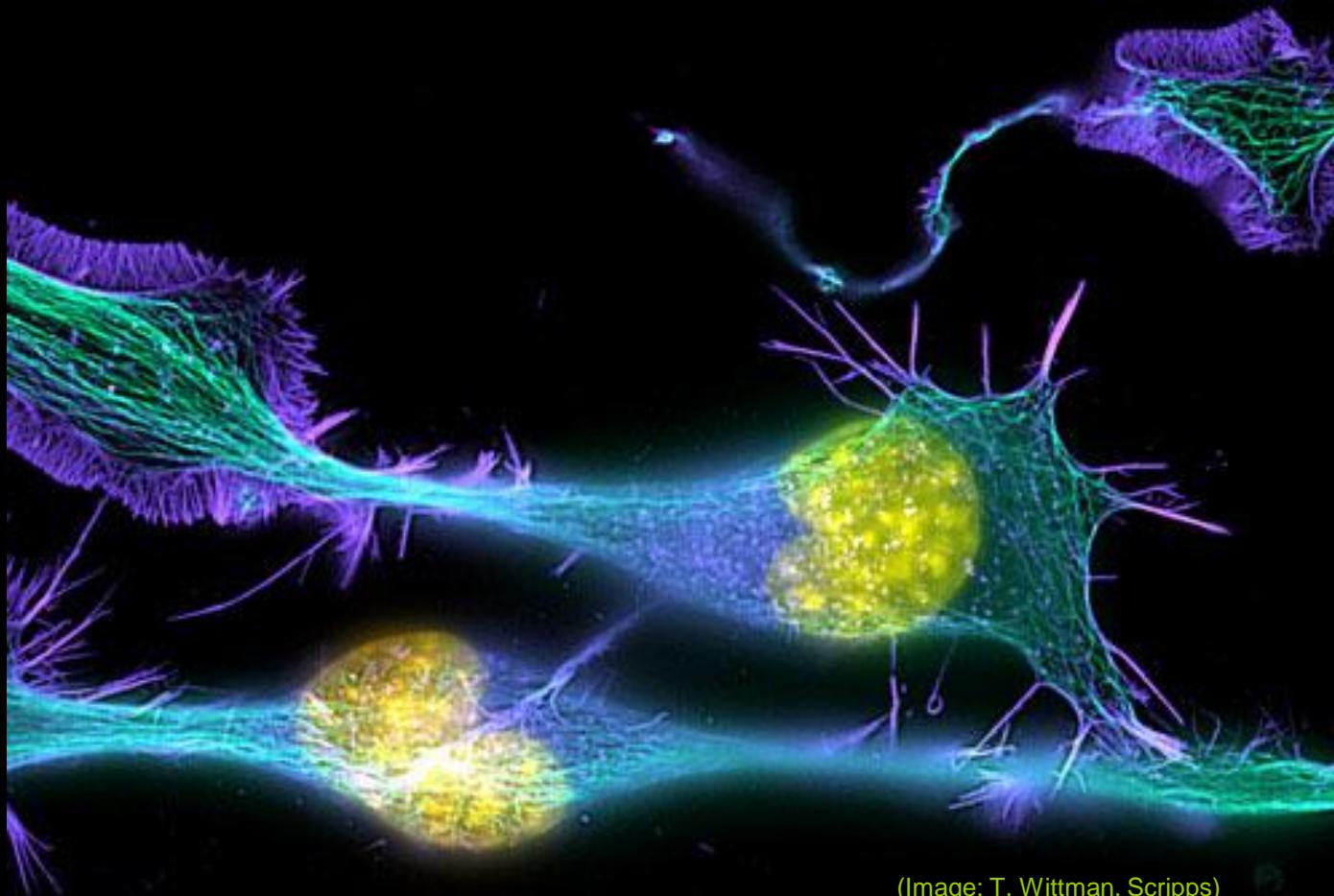


# Introduction to Light Microscopy



(Image: T. Wittman, Scripps)

# The Light Microscope

- Four centuries of history
- Vibrant current development
- One of the most widely used research tools



Fig 41.

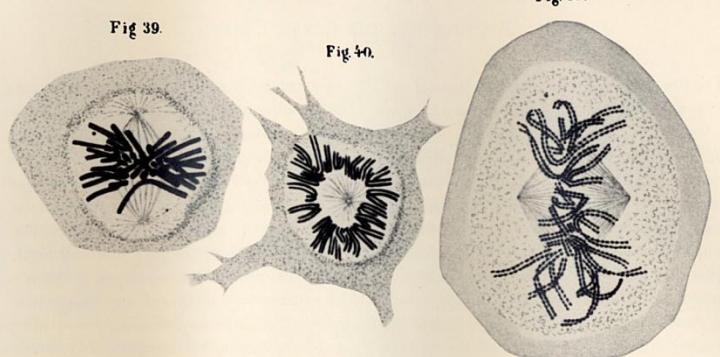


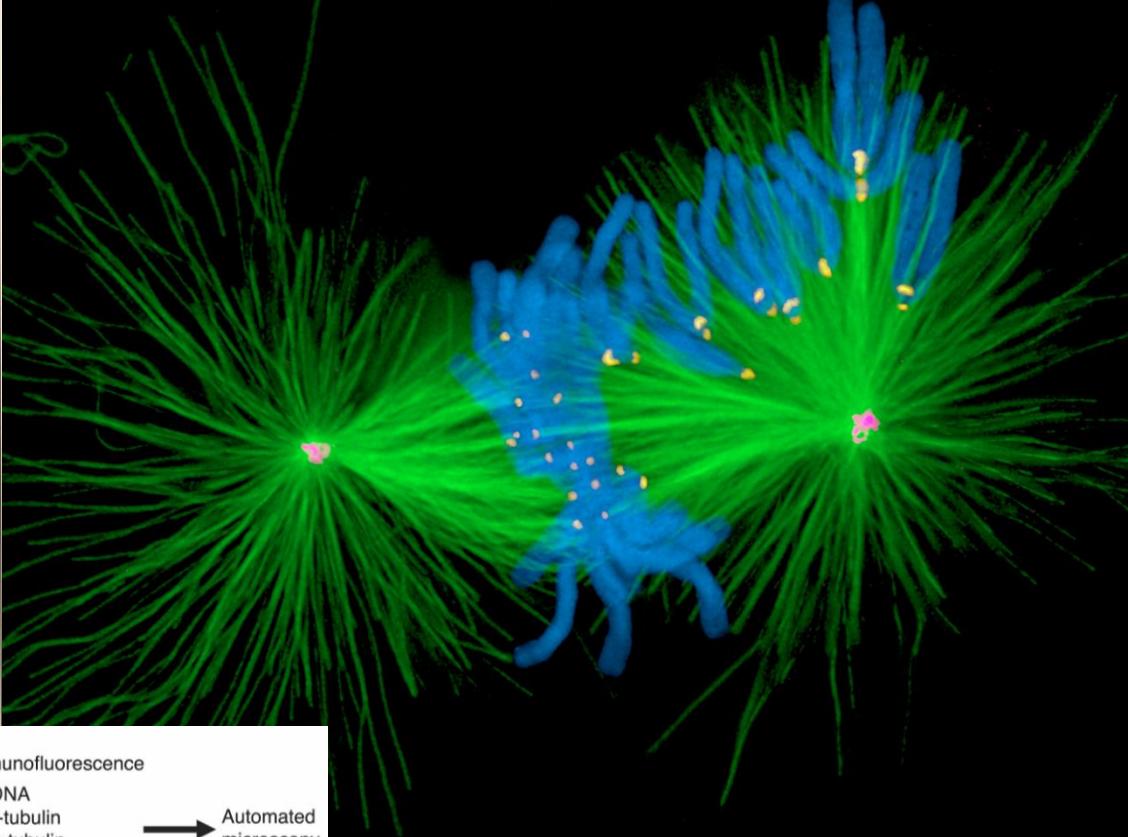
Fig 39.

Fig 40.

Fig 42.

Fig 43.

Fig 44.



a

14000 dsRNA  
(96-well plate x 146)



S2 cell  
+ Cdc27 dsRNA  
4 days

96-well, glass-bottom plate  
(ConA-coated)



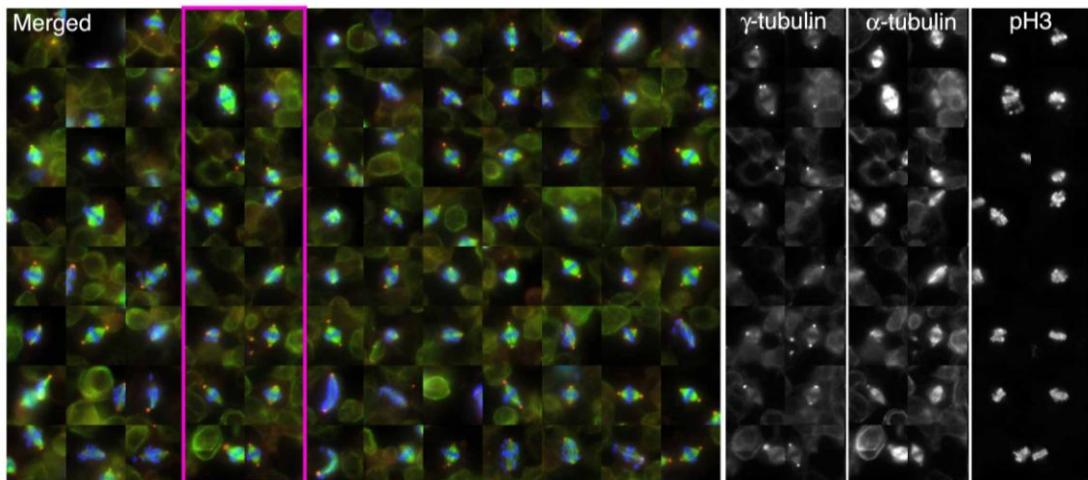
Immunofluorescence

DNA  
 $\gamma$ -tubulin  
 $\alpha$ -tubulin  
phospho-HistoneH3



Automated  
microscopy

b



# **Major Imaging Functions of the Microscope**

- Magnify
- Resolve features
- Generate Contrast
- Capture and Display Images

# An Upright Epifluorescence Microscope

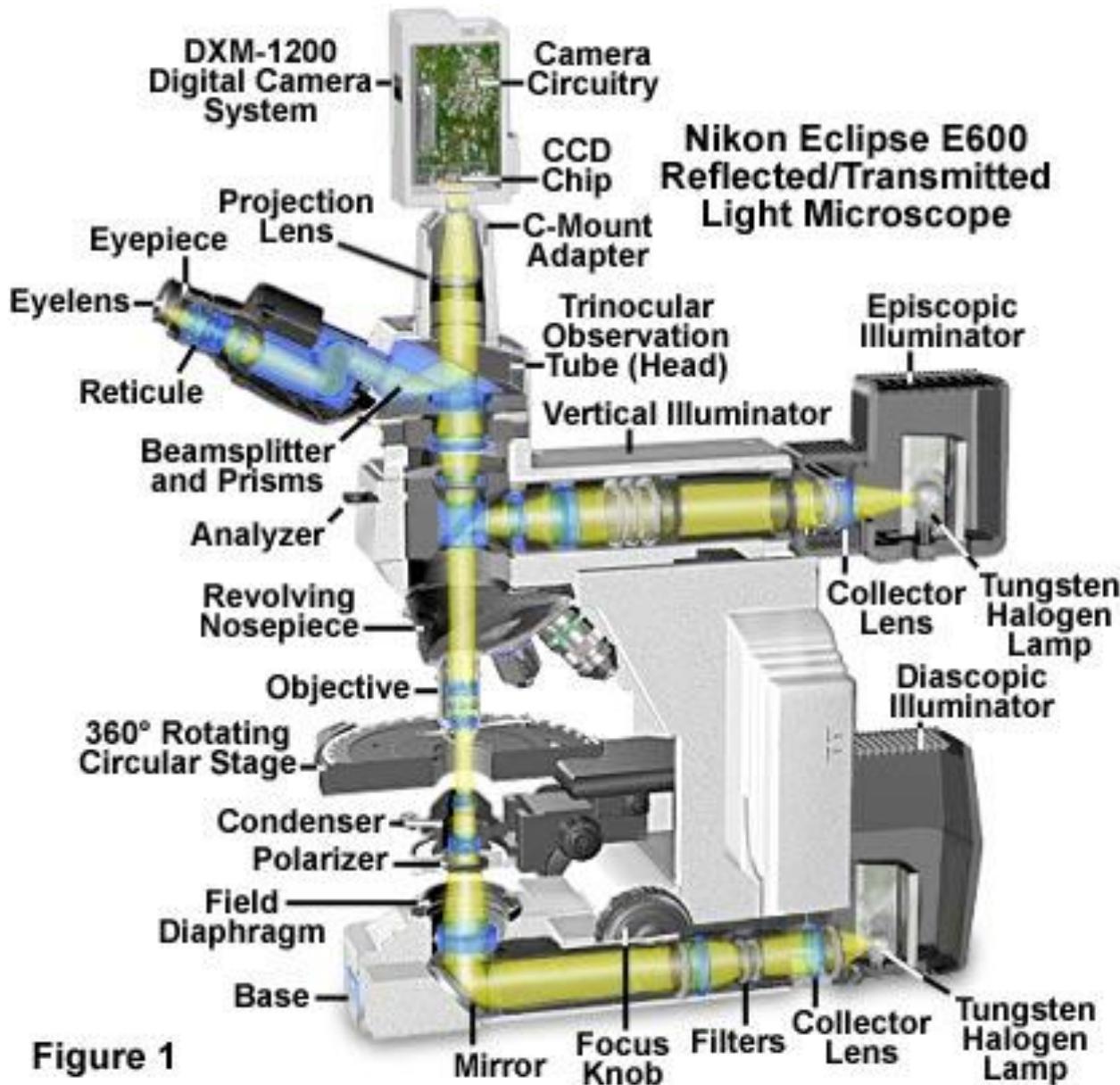
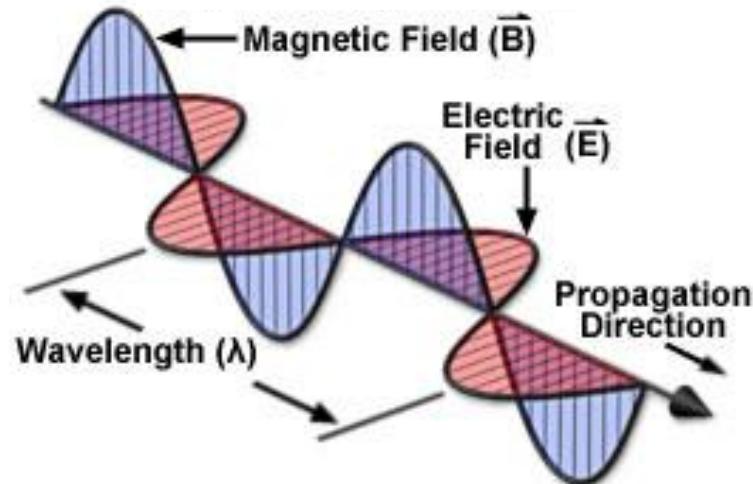


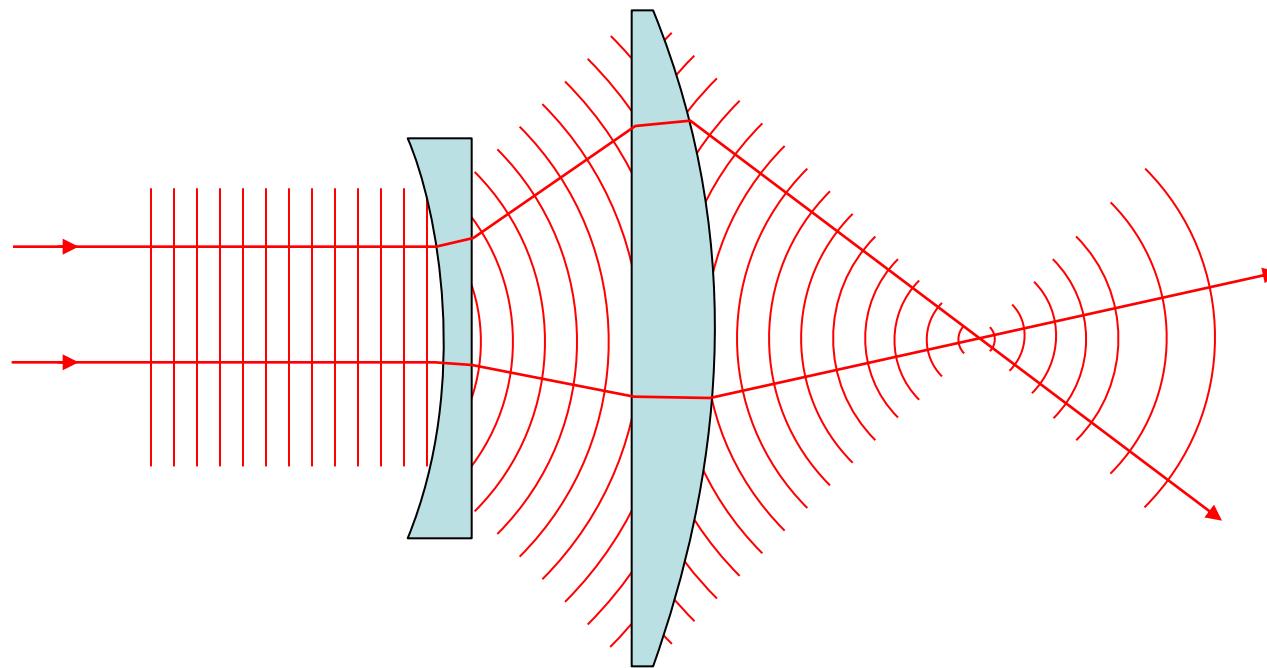
Figure 1

# Waves vs. Photons vs. Rays

- Quantum wave-particle duality
- Rays: photon trajectories
- Rays: propagation direction of waves



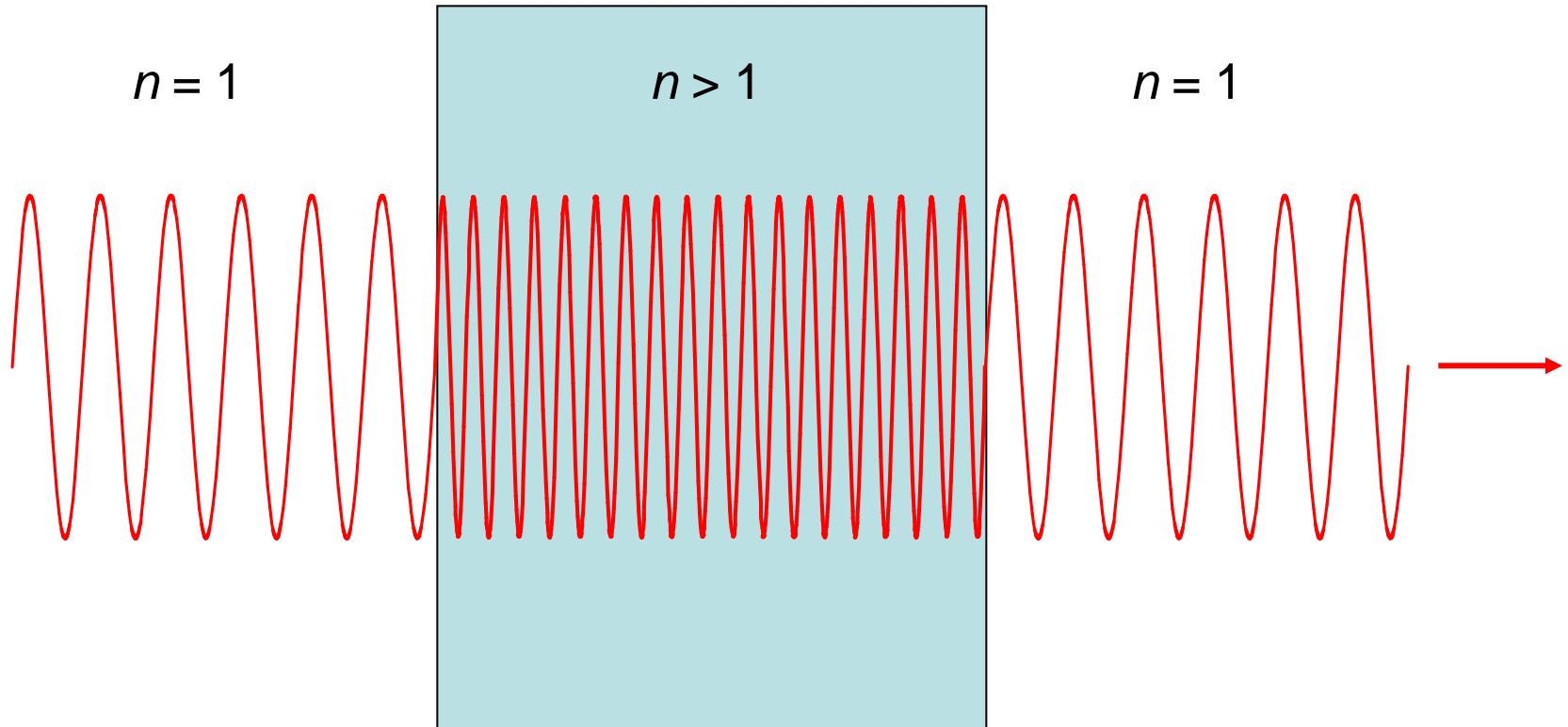
# Rays are perpendicular to wavefronts



# Light travels more slowly in matter

The speed ratio is the ***Index of Refraction, n***

$$v = c/n$$



# Refractive Index Examples

- Vacuum 1
- Air 1.0003
- Water 1.333
- Cytoplasm 1.35–1.38 ?
- Glycerol 1.475 (anhydrous)
- Immersion oil 1.515
- Fused silica 1.46
- Optical glasses 1.5–1.9
- Diamond 2.417

Depends on wavelength and temperature

# Refraction by an Interface

Refractive index  $n_1 = 1$

Speed =  $c$

Refractive index  $n_2$

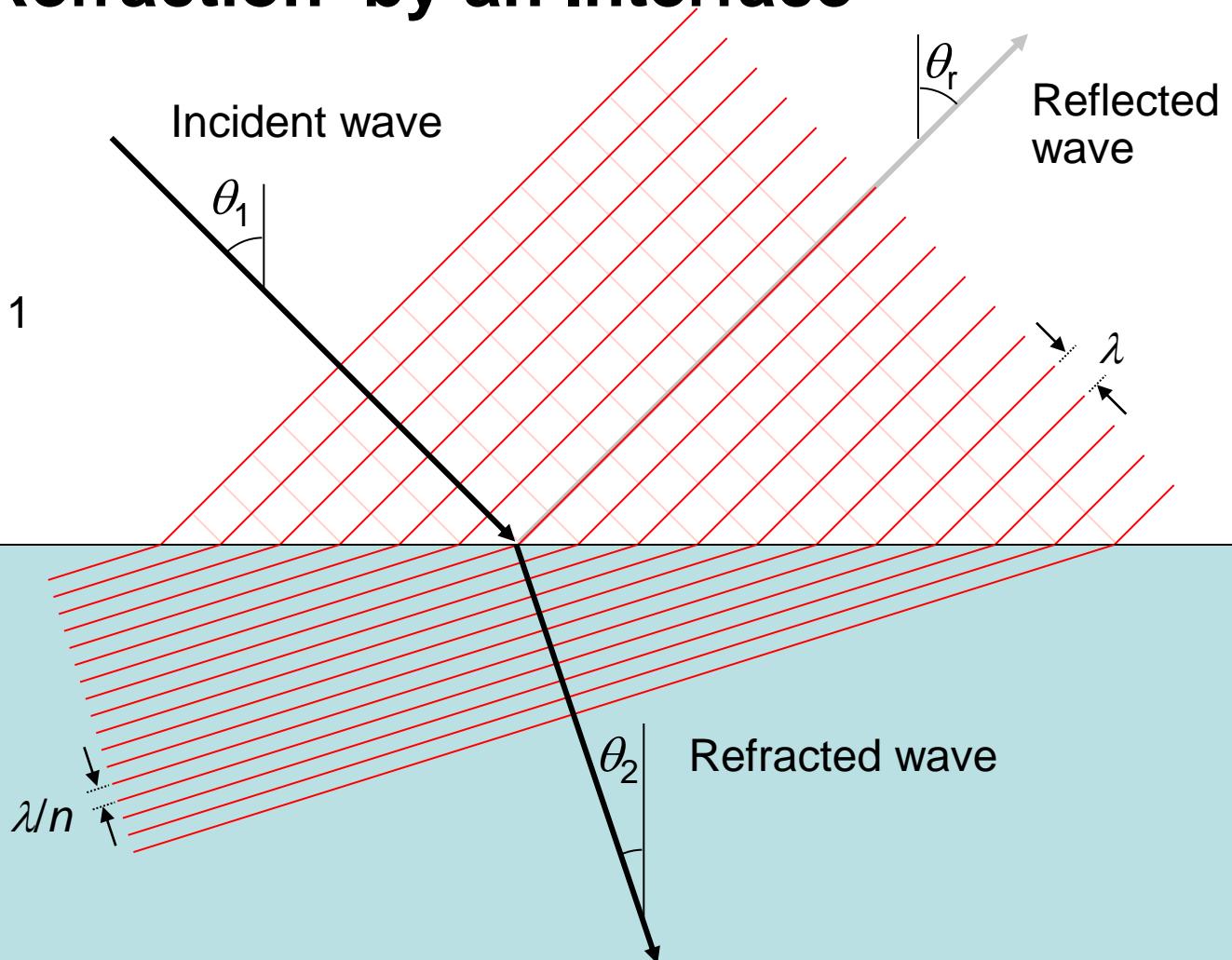
Speed =  $c/n$

⇒ Snell's law:

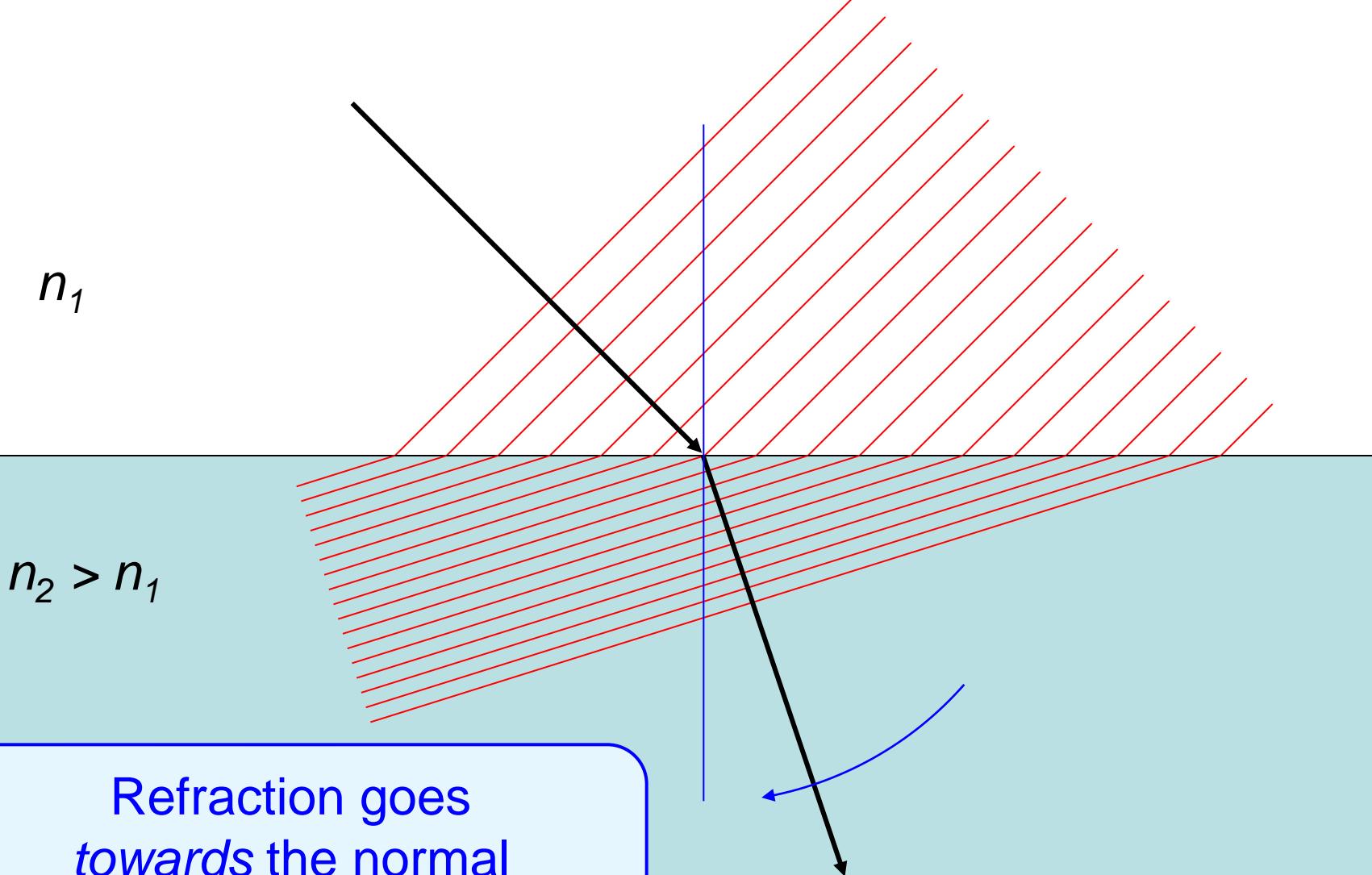
$$n_1 \sin(\theta_1) = n_2 \sin(\theta_2)$$

Mirror law:

$$\theta_r = \theta_1$$

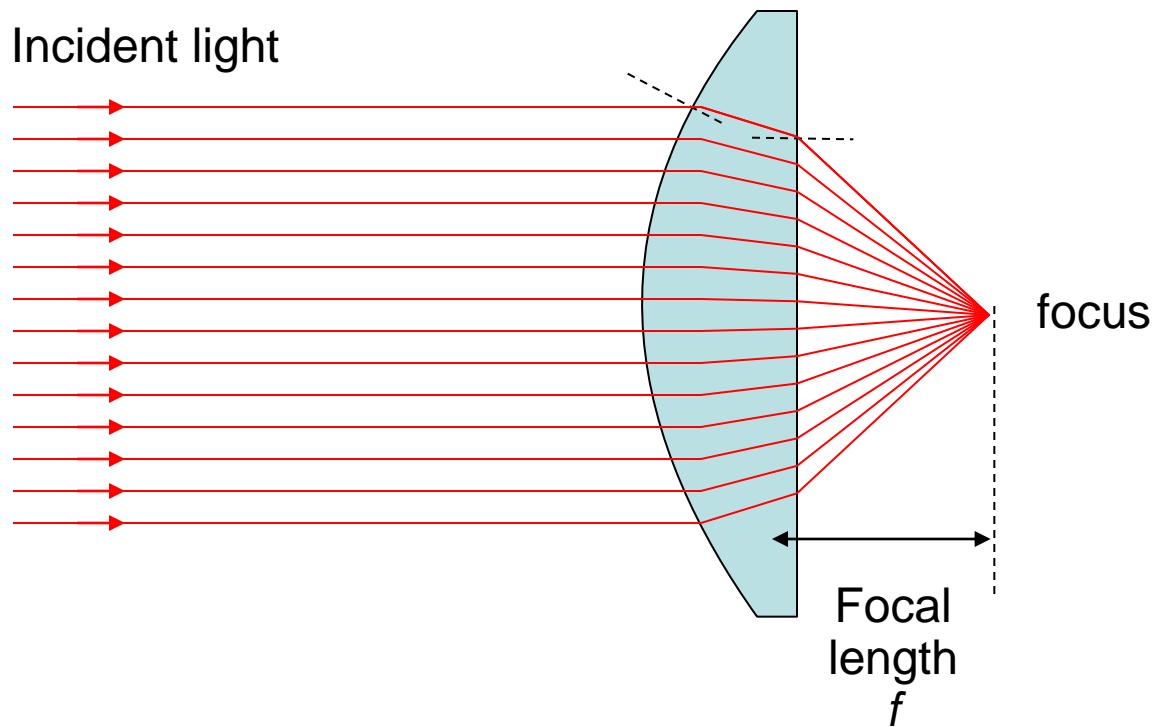


# Which Direction?



Refraction goes  
towards the normal  
in the *higher-index* medium

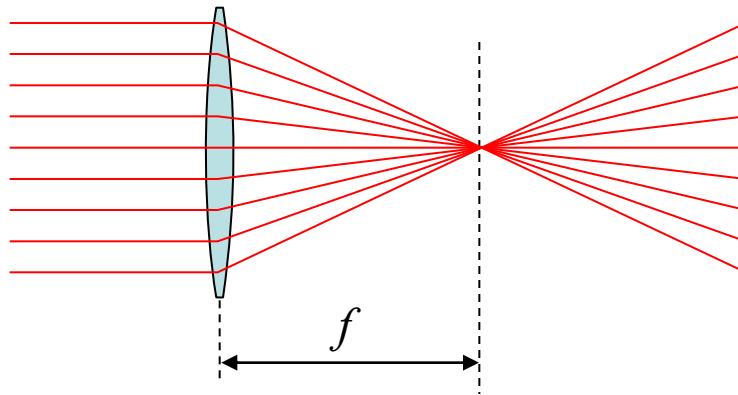
# Lenses work by refraction



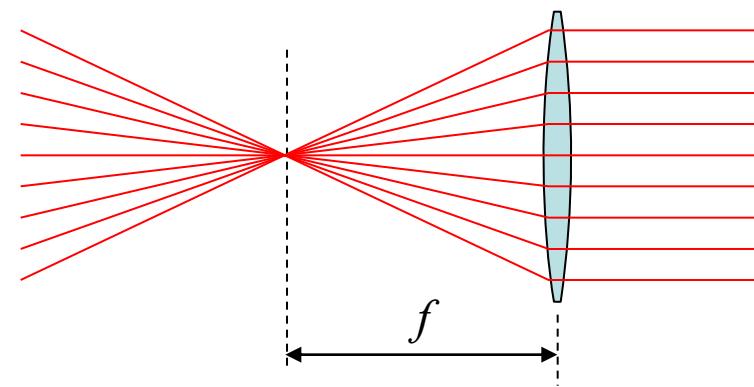
# Ray Tracing Rules of Thumb

(for thin ideal lenses)

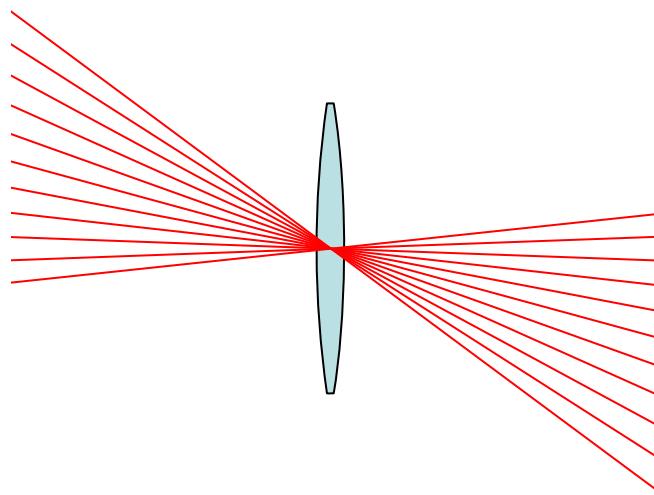
Parallel rays converge at the focal plane



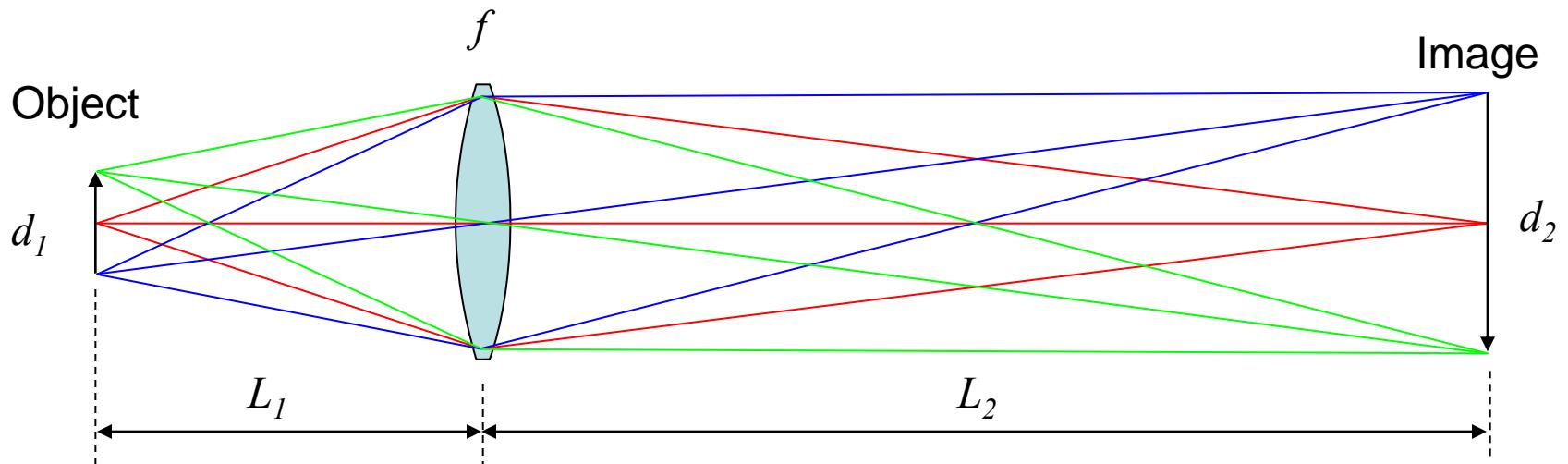
Rays that cross in the focal plane end up parallel



Rays through the lens center are unaffected



# Imaging



The lens law:

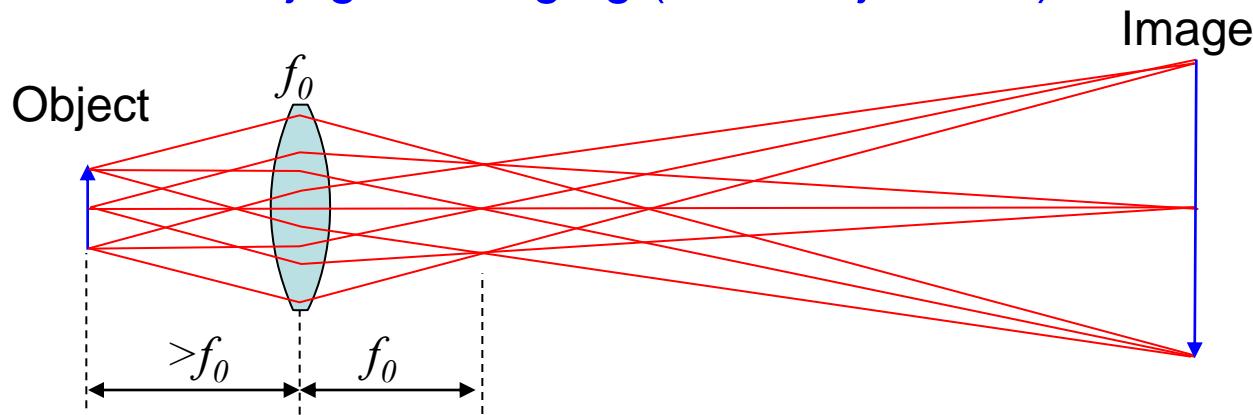
$$\frac{1}{L_1} + \frac{1}{L_2} = \frac{1}{f}$$

Magnification:

$$M = \frac{d_2}{d_1} = \frac{L_2}{L_1}$$

# Finite vs. Infinite Conjugate Imaging

- Finite conjugate imaging (older objectives)



- Infinite conjugate imaging (modern objectives).

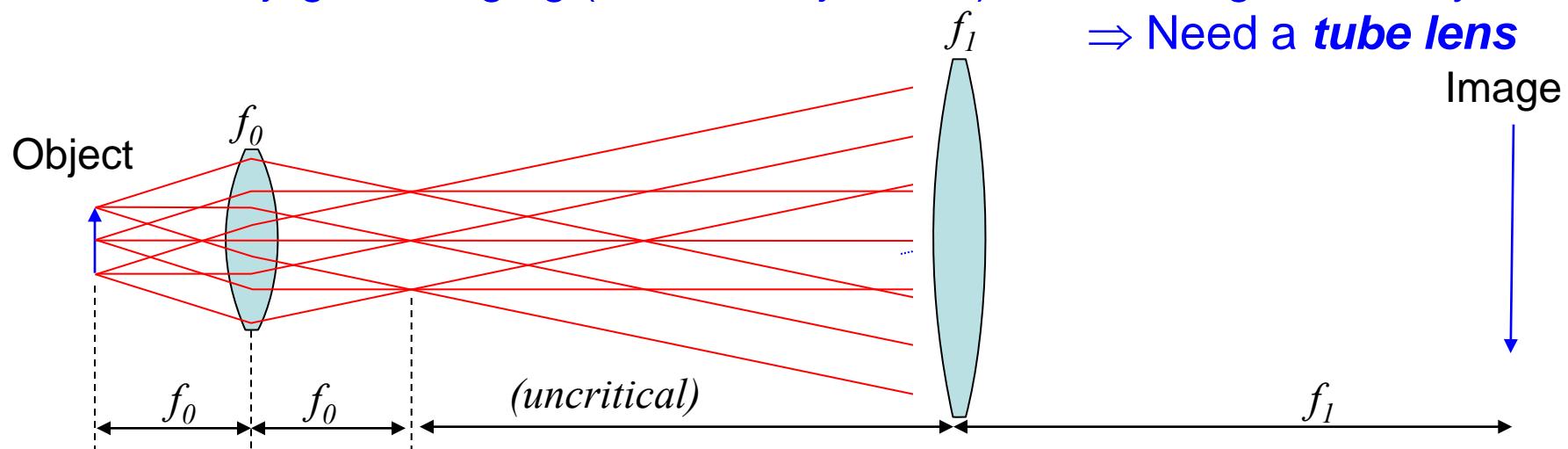
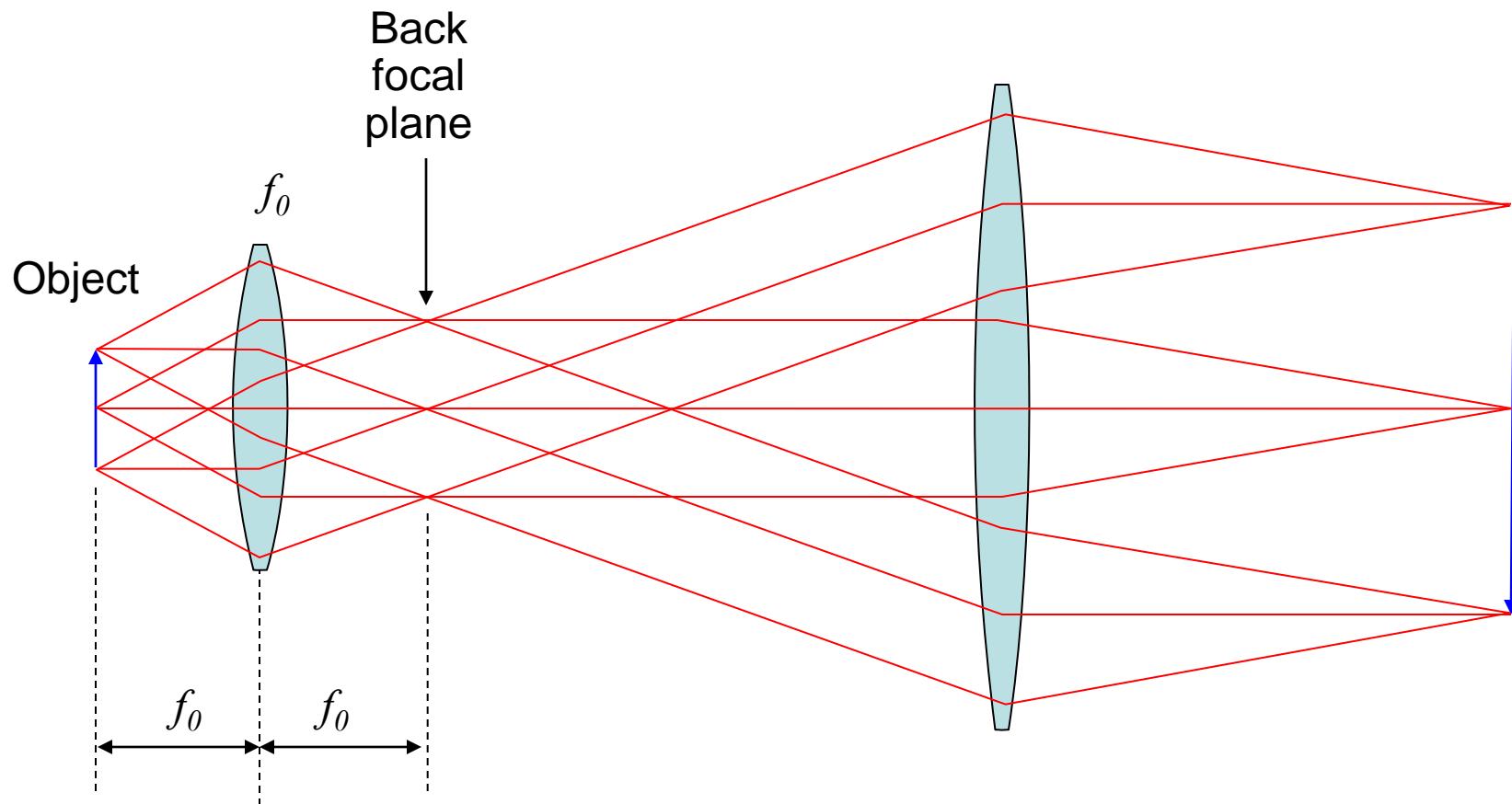


Image at infinity  
⇒ Need a **tube lens**

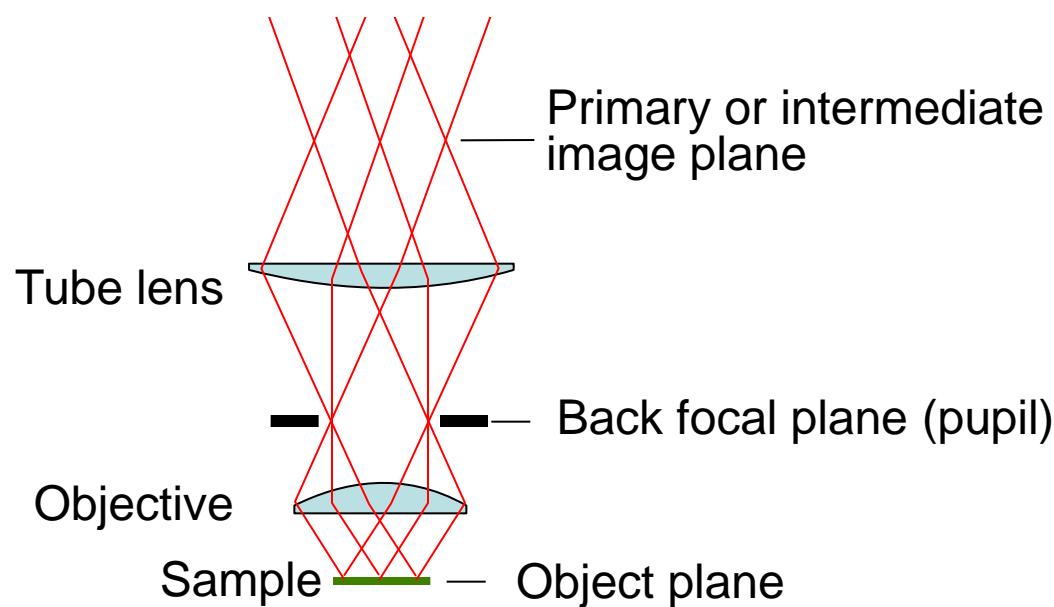
Magnification:  $M = \frac{f_1}{f_o}$

# Back focal plane

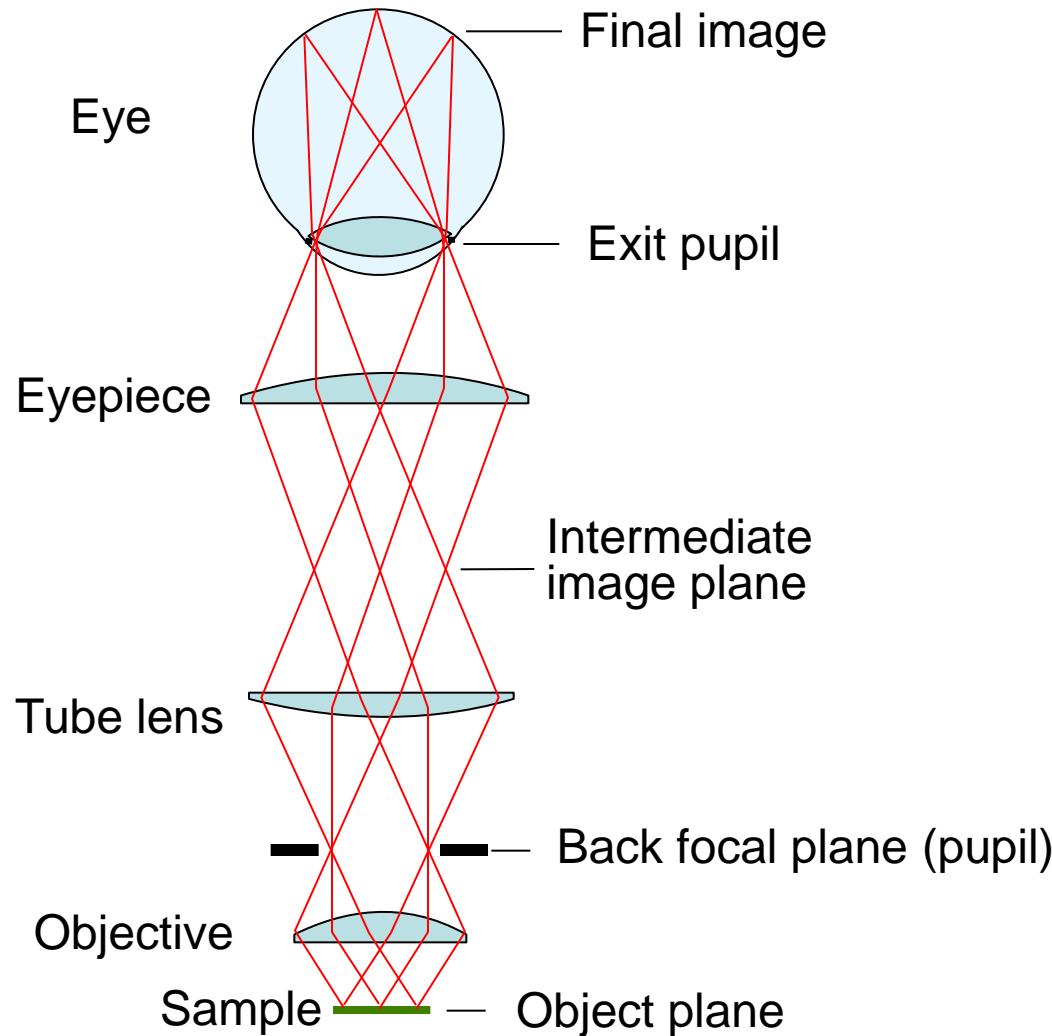


Rays that leave the object with the same angle  
meet in the objective's *back focal plane*

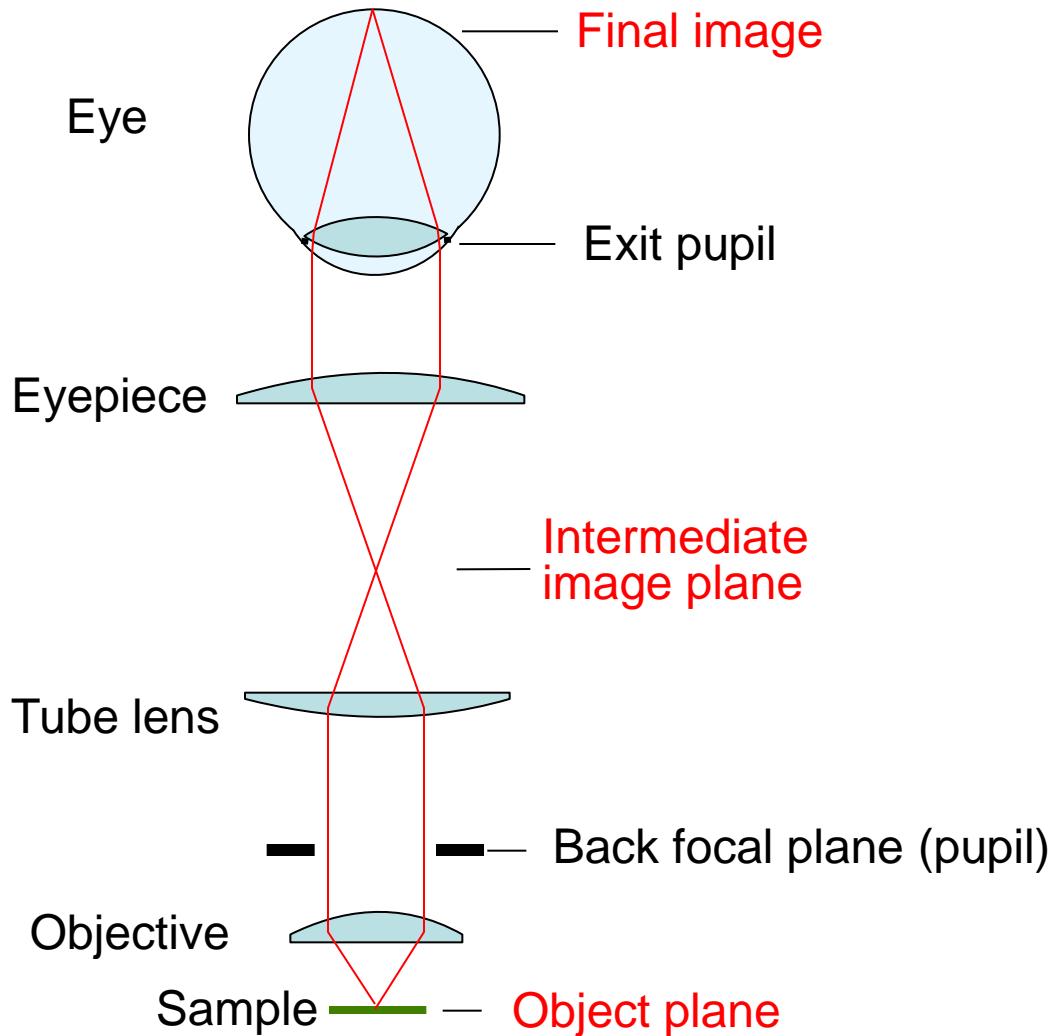
# The Compound Microscope



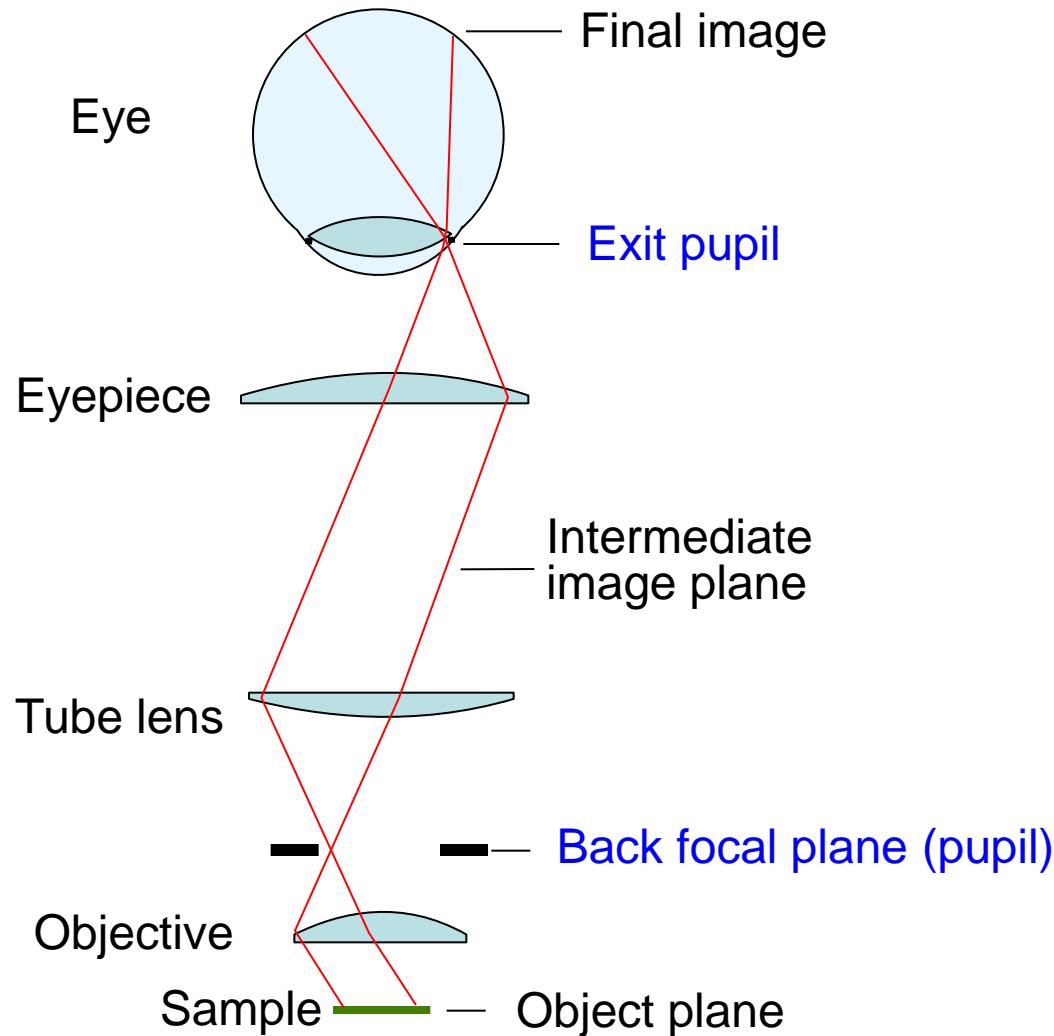
# The Compound Microscope



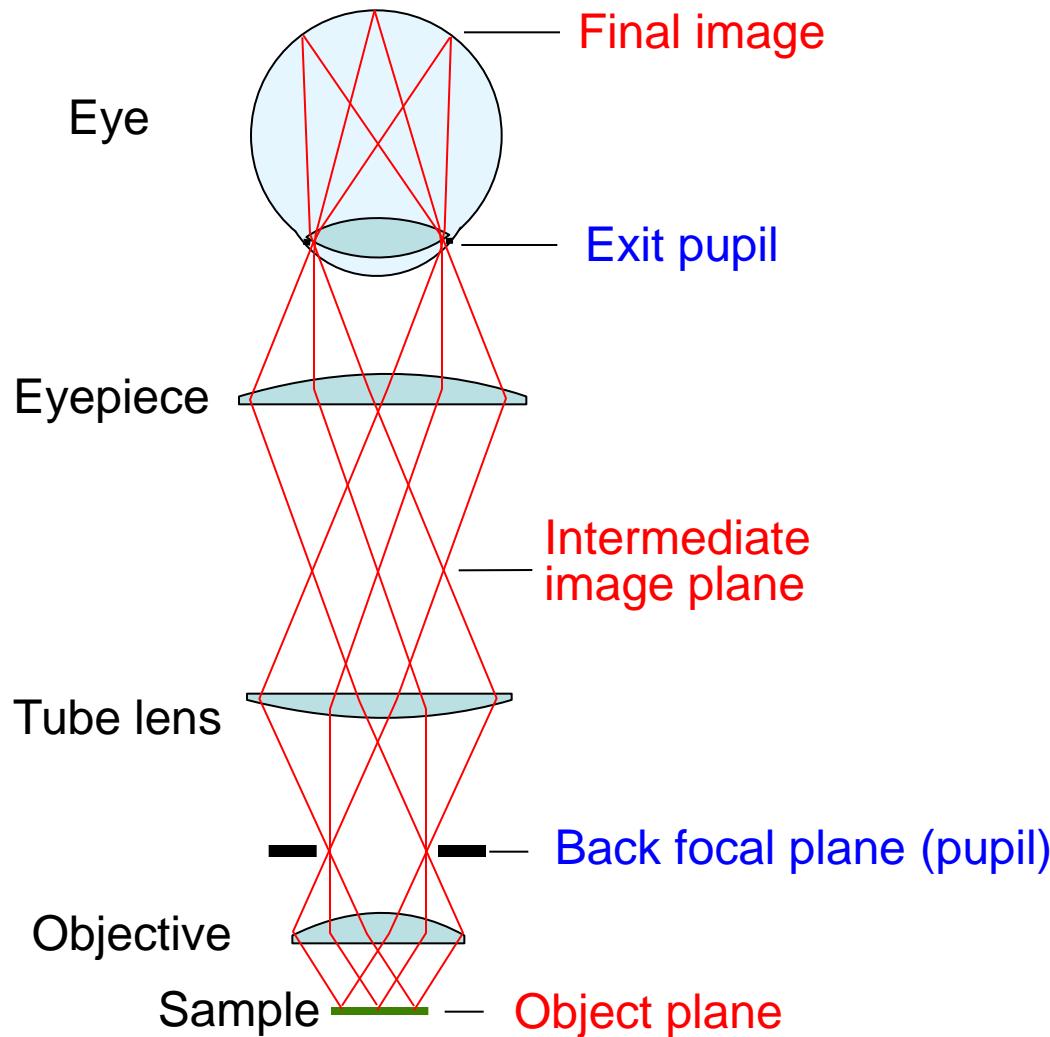
# The Compound Microscope



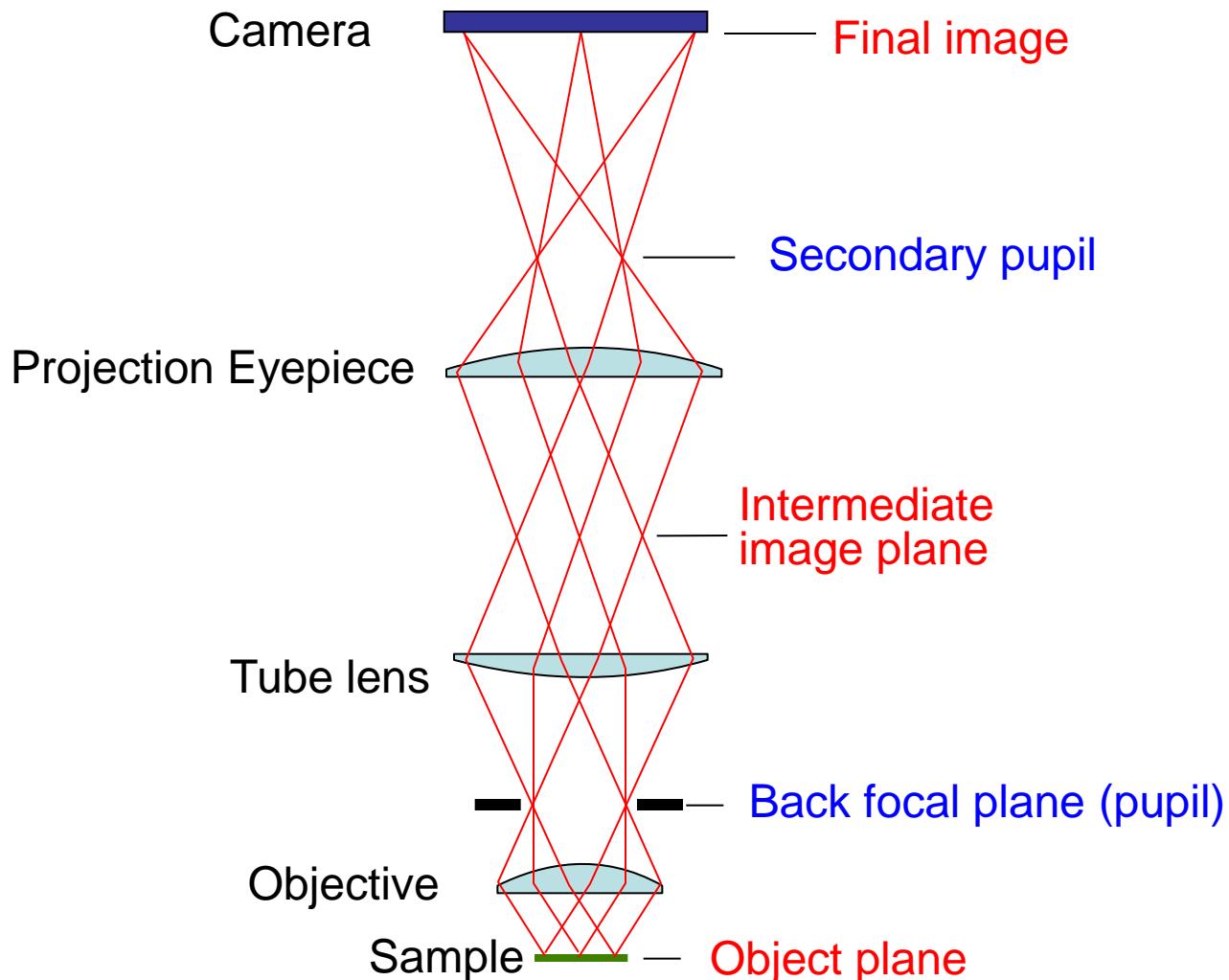
# The Compound Microscope



# The Compound Microscope



# The Compound Microscope



# Eyepieces (Oculars)

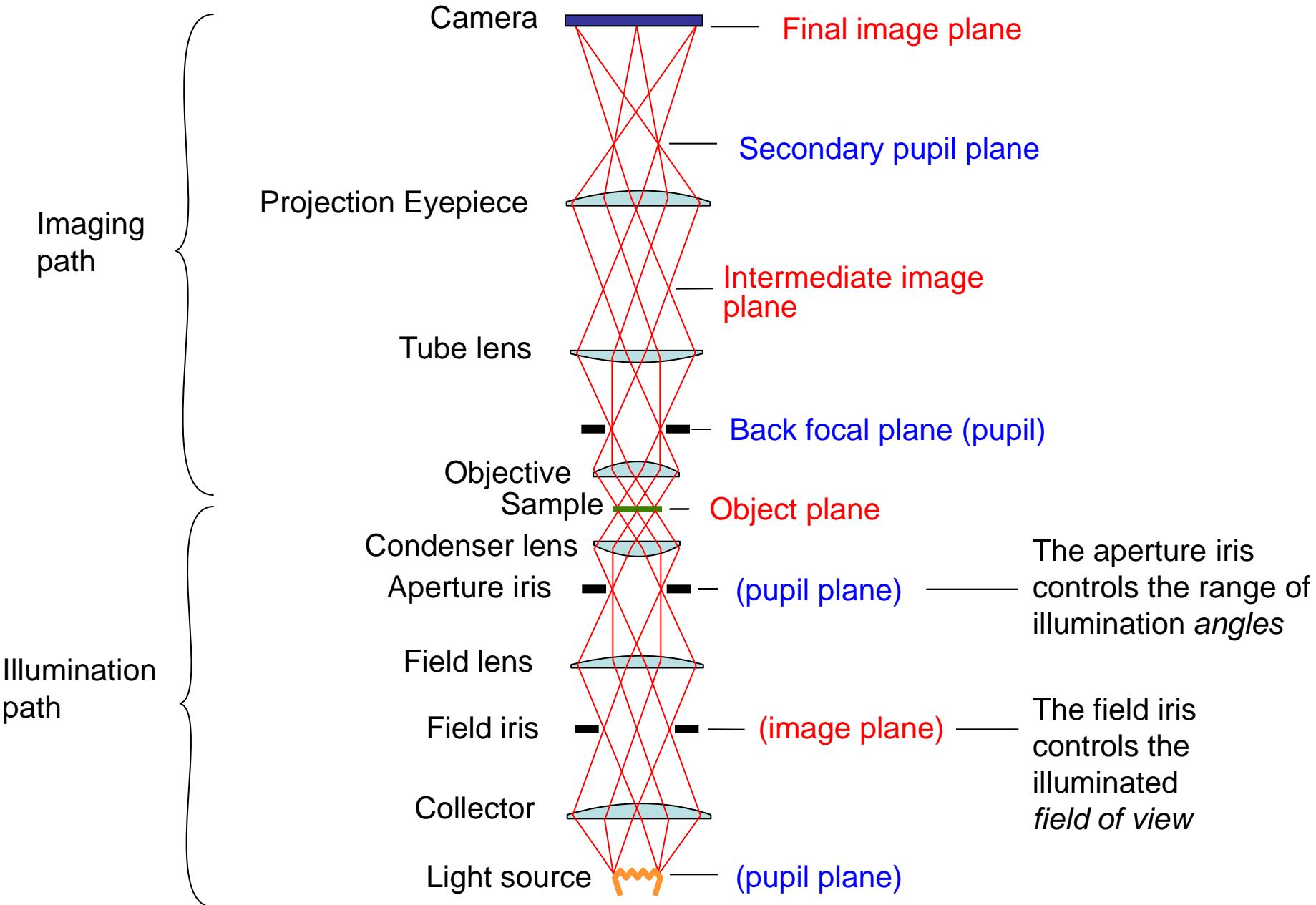
Aberration-Free 10x Eyepiece With Diopter Adjustment



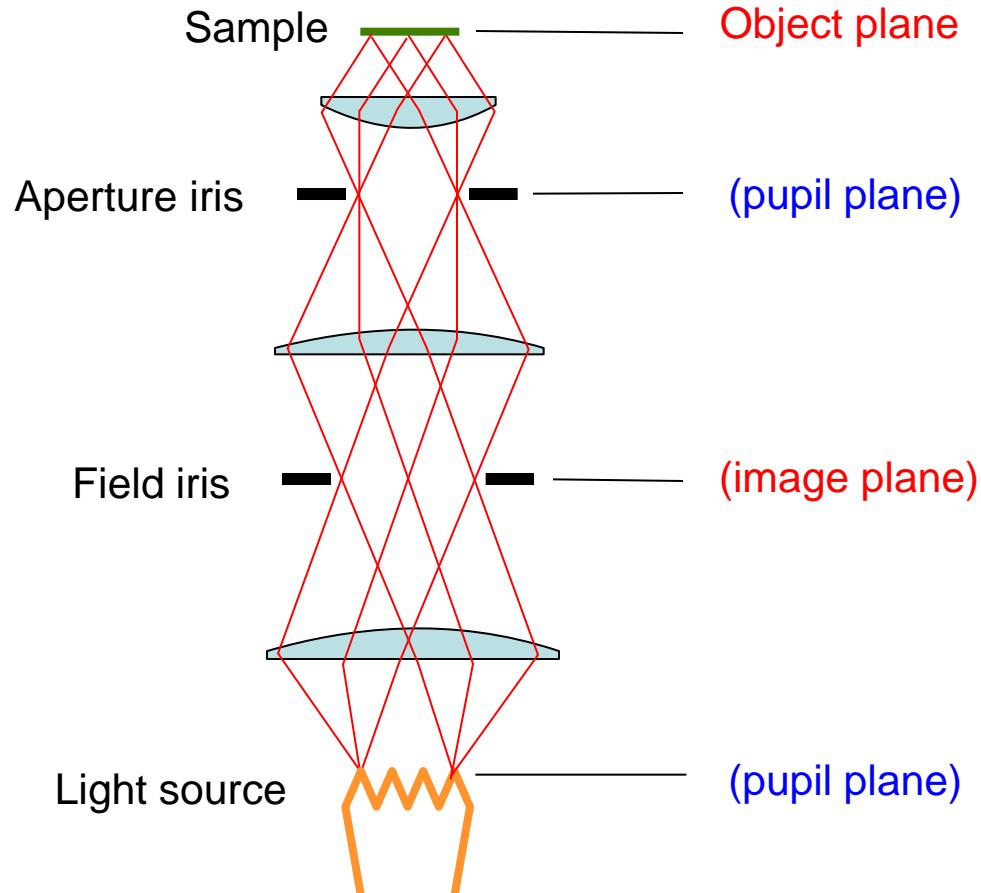
## Features

- Magnification (10x typical)
- “High eye point” (exit pupil high enough to allow eyeglasses)
- Diopter adjust (at least one must have this)
- Reticle or fitting for one
- Eye cups

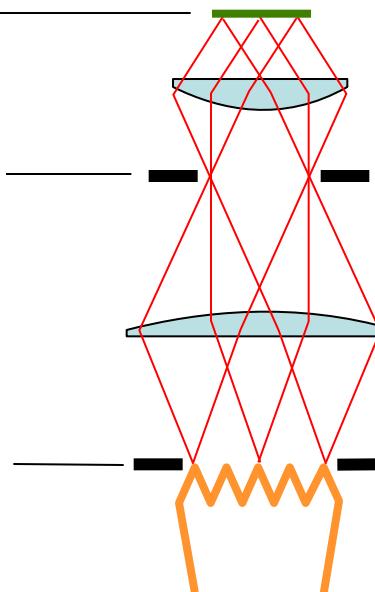
# Trans-illumination Microscope



# Köhler Illumination

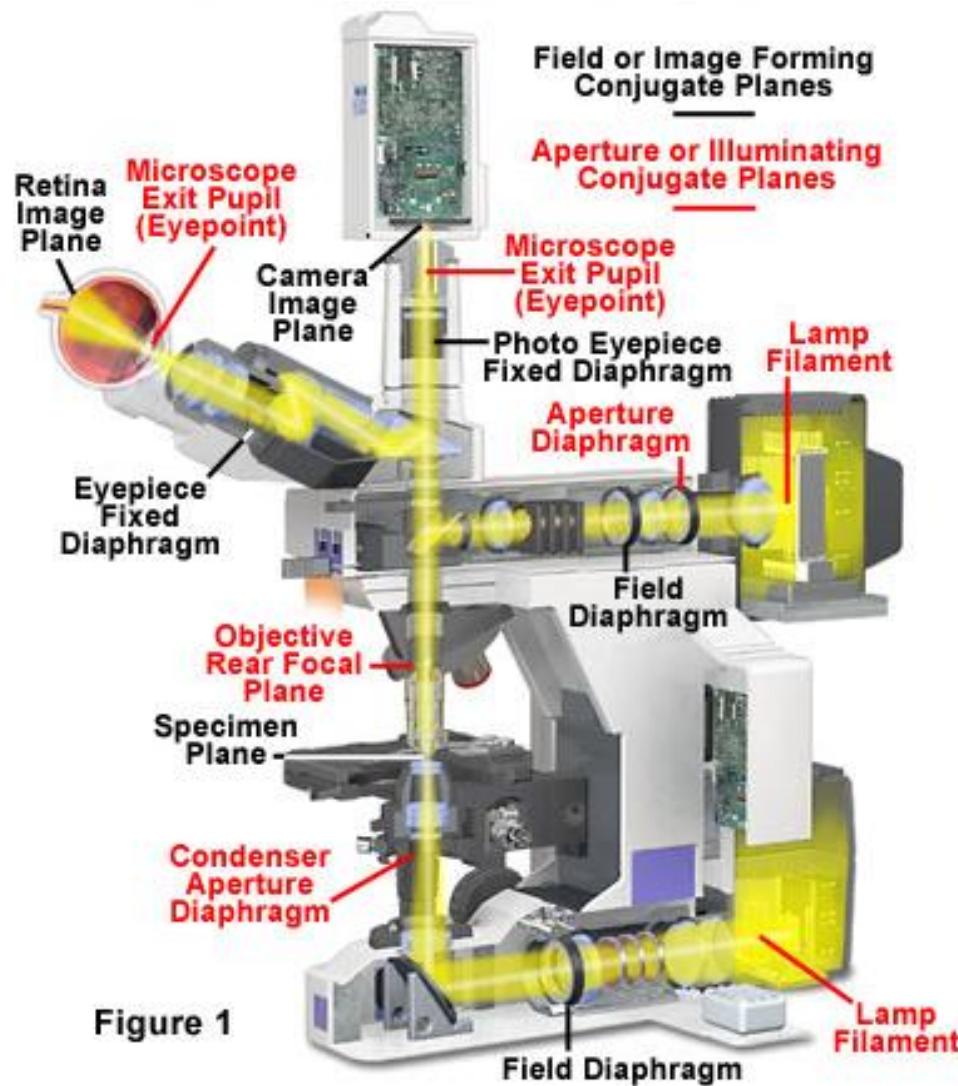


# Critical Illumination



- Each light source point produces a parallel beam of light at the sample
- Uniform light intensity at the sample even if the light source is “ugly” (e.g. a filament)
- The source is imaged onto the sample
- Usable only if the light source is perfectly uniform

# Conjugate Planes in A Research Microscope

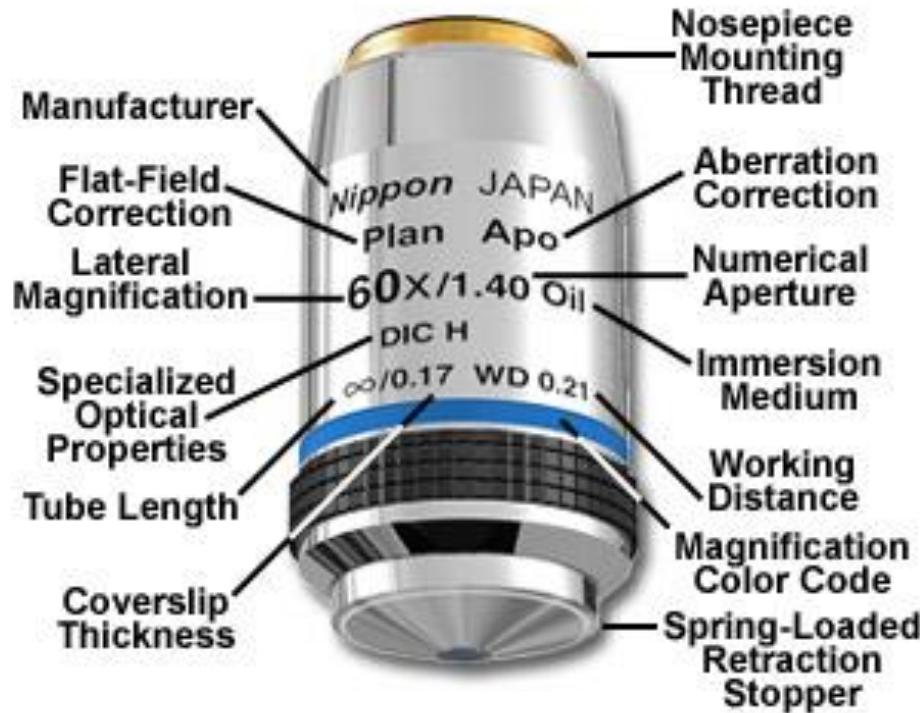


# **How view the pupil planes?**

**Two ways:**

- “Eyepiece telescope”
- “Bertrand lens”

By far the most important part:  
***the Objective Lens***



Each major manufacturer sells 20-30 different **categories** of objectives.  
What are the important distinctions?

# Working Distance

## Objective Working and Parfocal Distance



In general, high NA lenses have short working distances

However, extra-long working distance objectives do exist

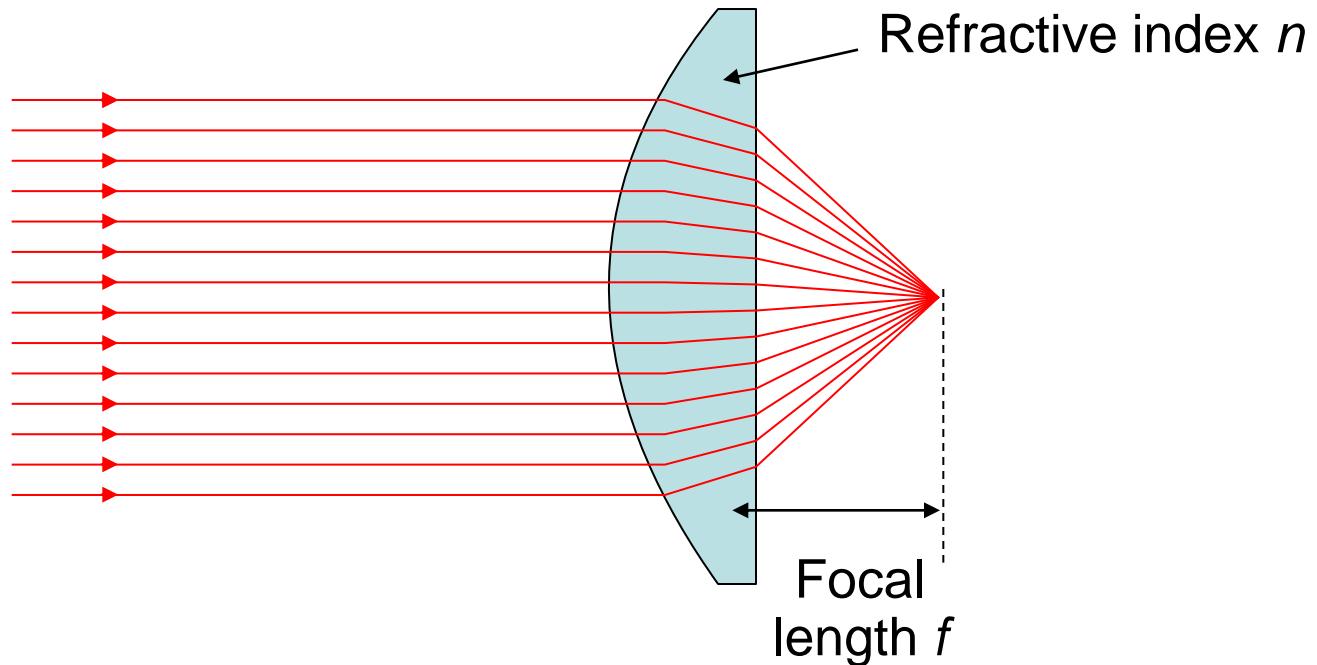
Some examples:

10x/0.3 WD = 15.2mm

20x/0.75 WD = 1.0mm

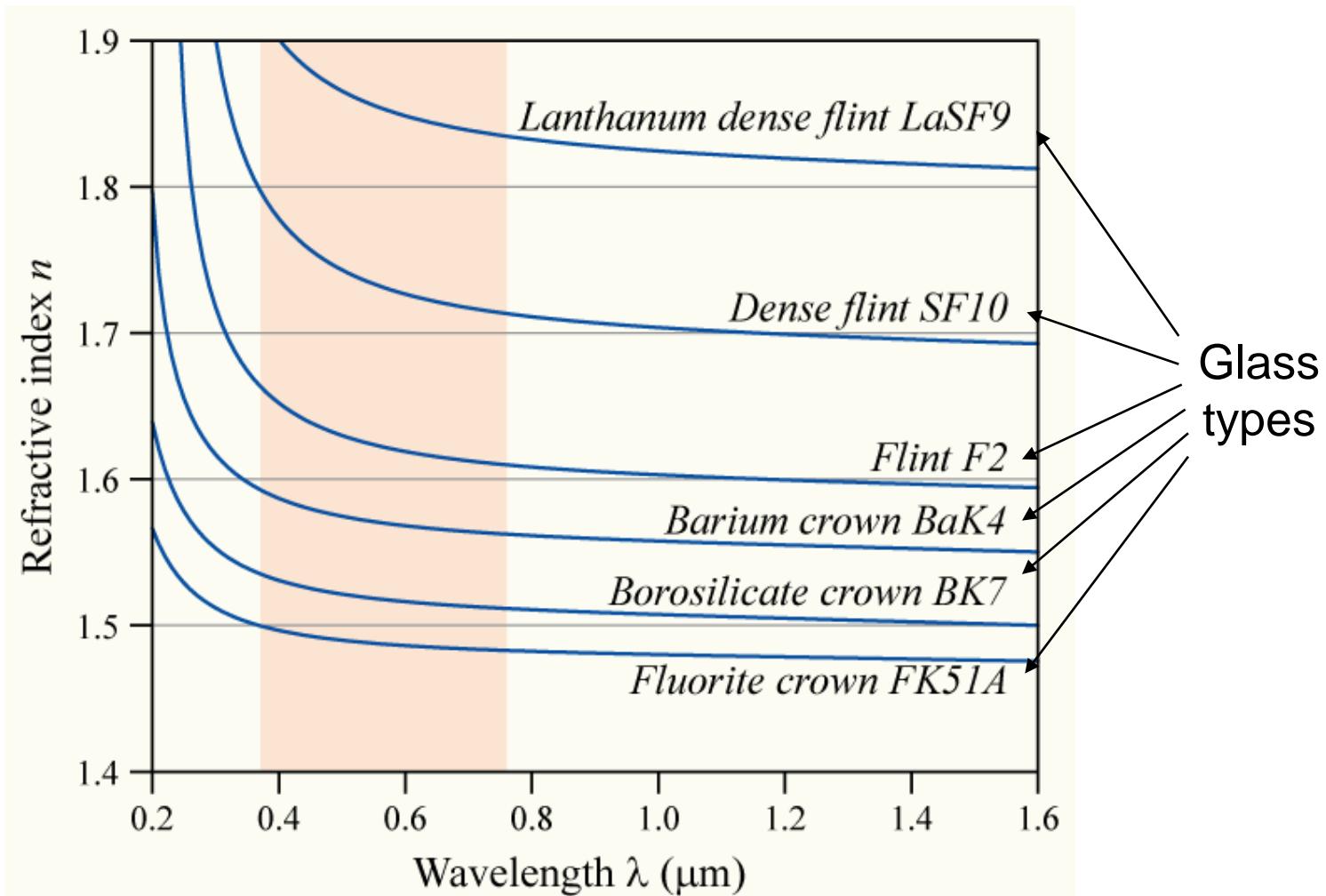
100x/1.4 WD = 0.13mm

# The focal length of a lens depends on the refractive index...



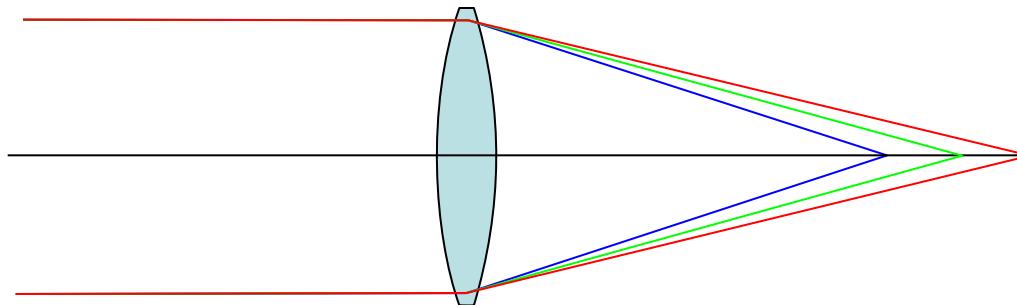
$$f \propto 1/(n-1)$$

... and the refractive index depends on the wavelength (“dispersion”)

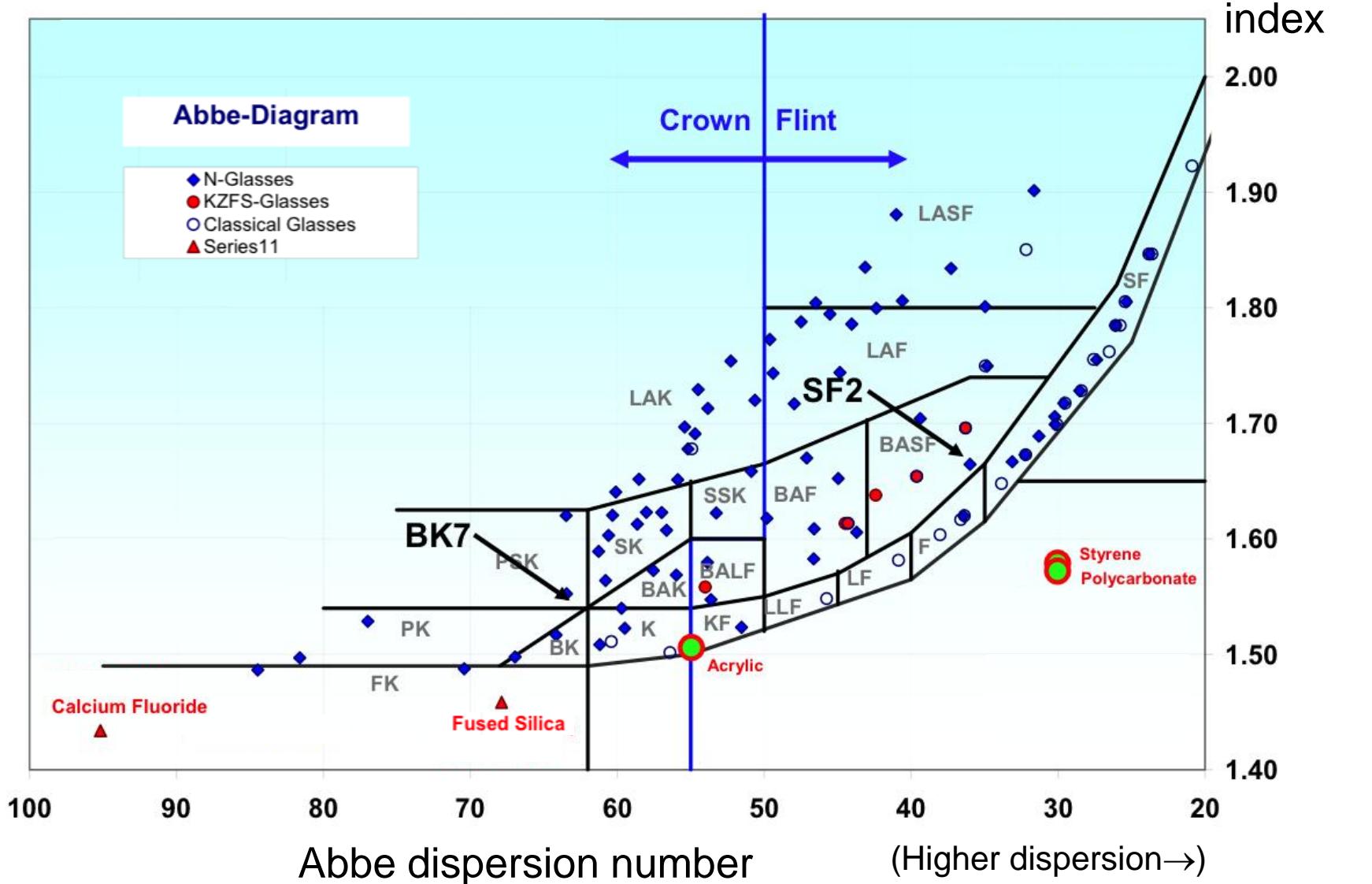


## ⇒ Chromatic aberration

- Different colors get focused to different planes
- Not good...

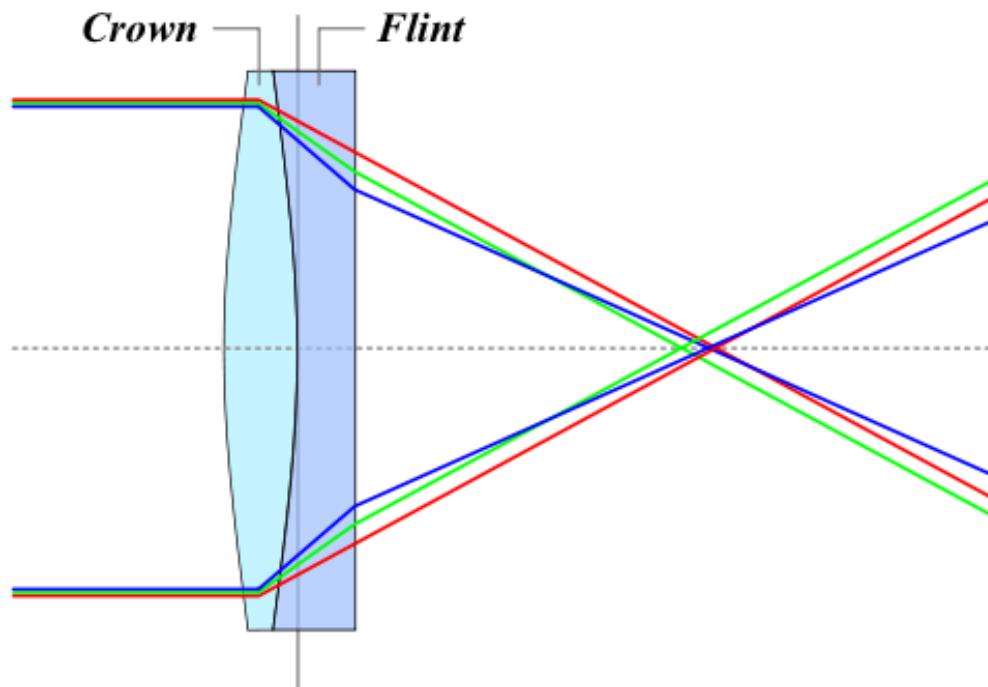


# Dispersion vs. refractive index of different glass types

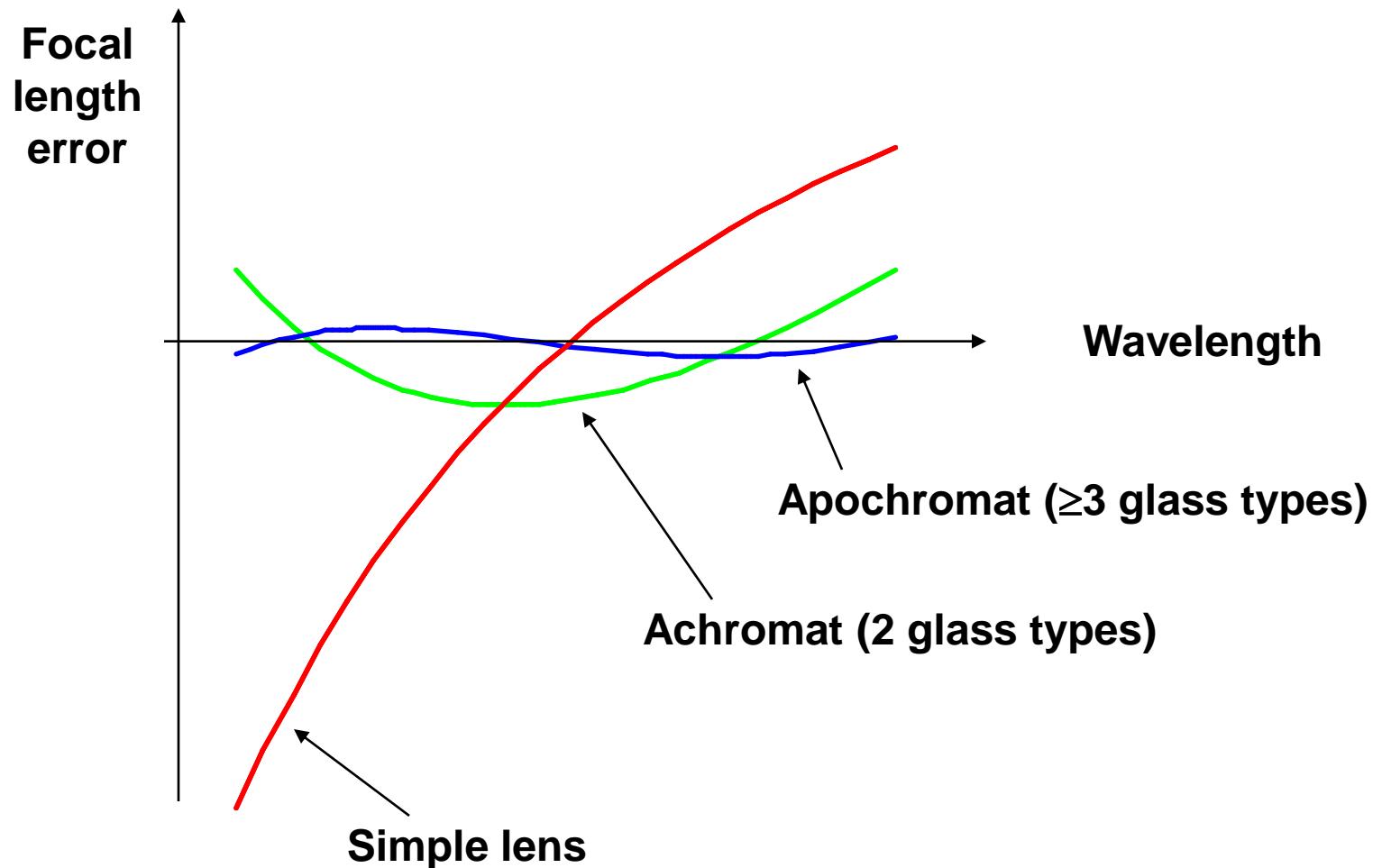


# Achromatic Lenses

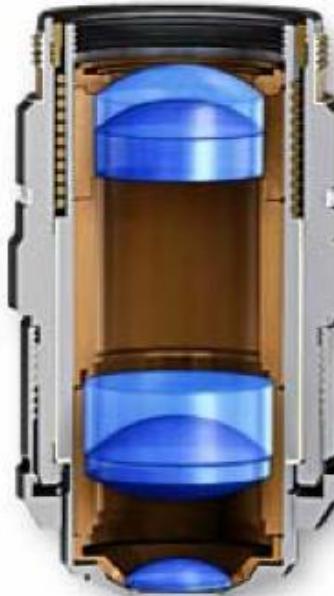
- Use a weak negative flint glass element to compensate the dispersion of a positive crown glass element



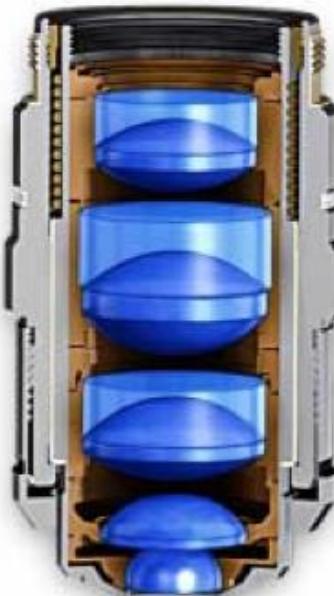
# Achromats and Apochromats



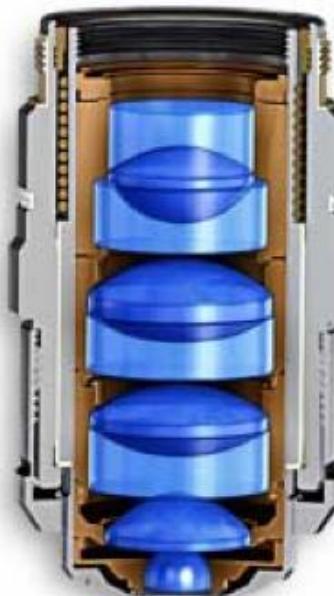
# Correction classes of objectives



**Achromat**  
(cheap)



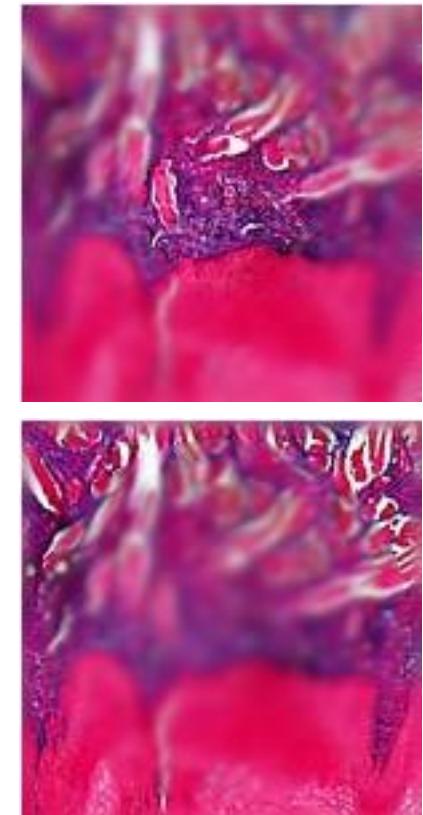
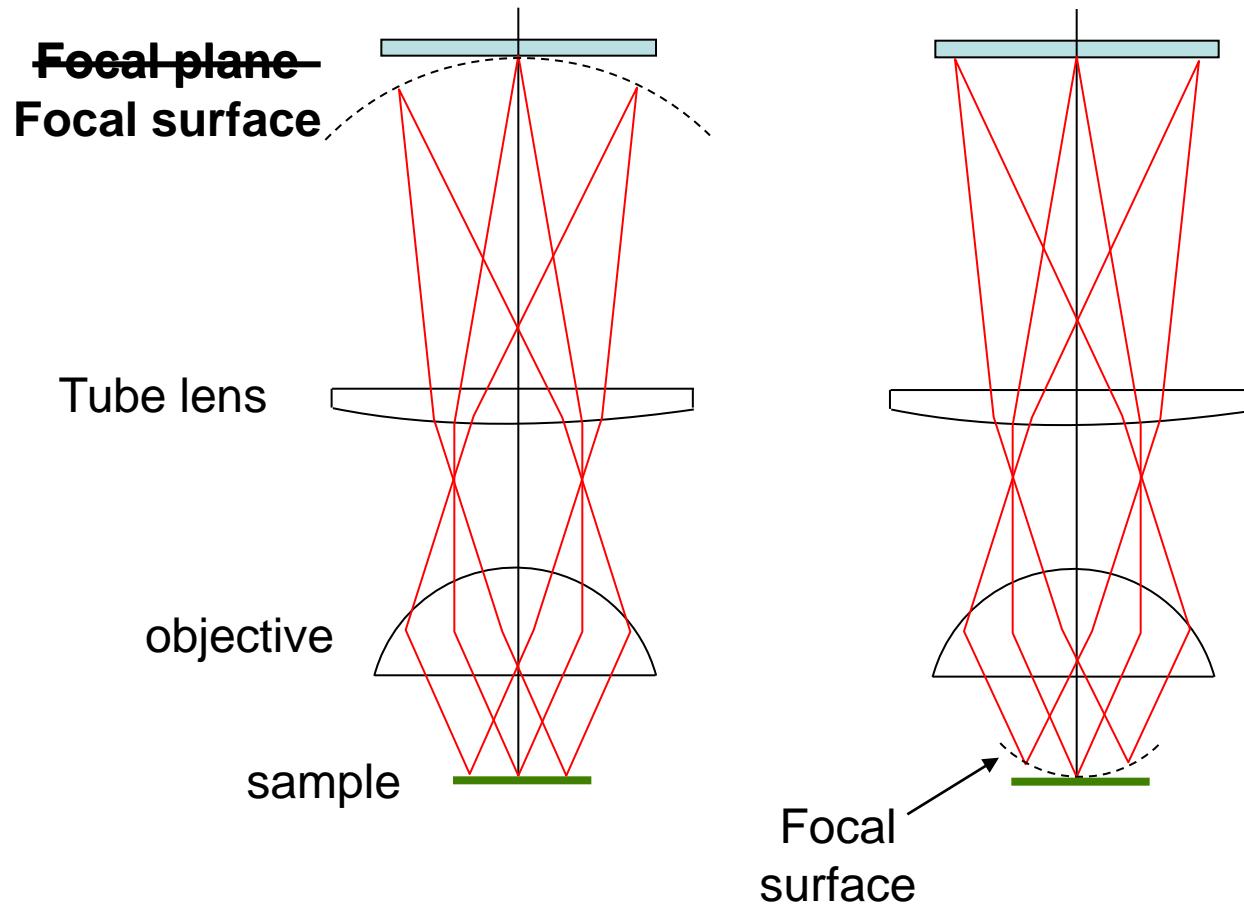
**Fluor**  
“semi-apo”  
(good correction,  
high UV  
transmission)



**Apochromat**  
(best correction)

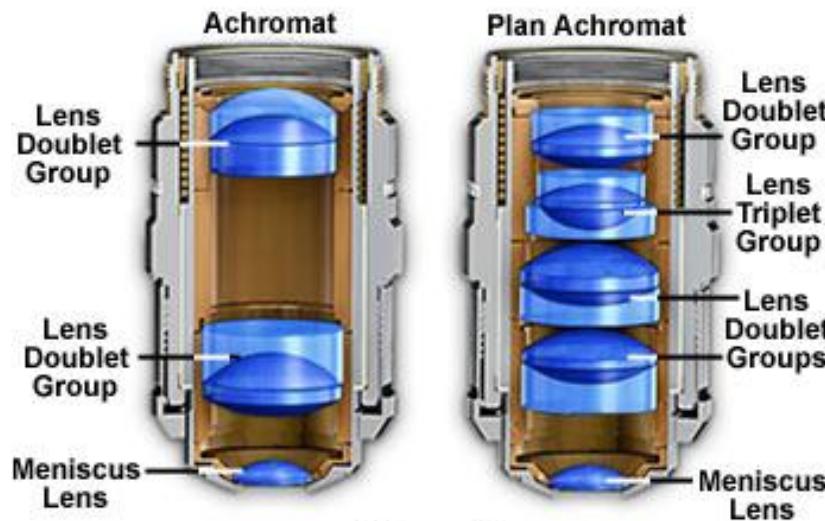
Correction for other (i.e. monochromatic) aberrations  
also improves in the same order →

# Curvature of Field



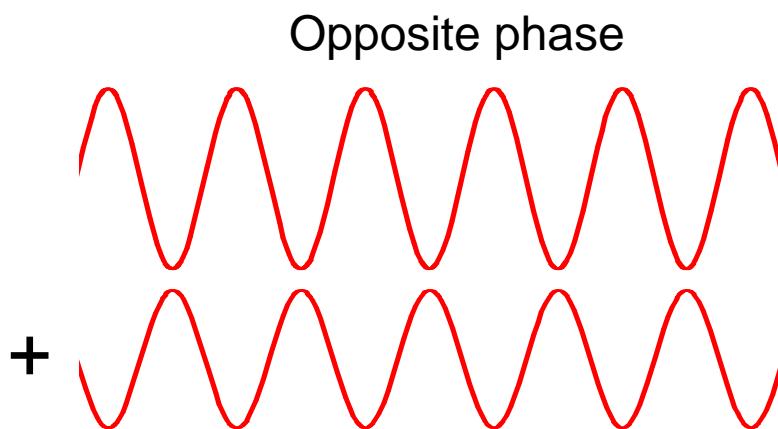
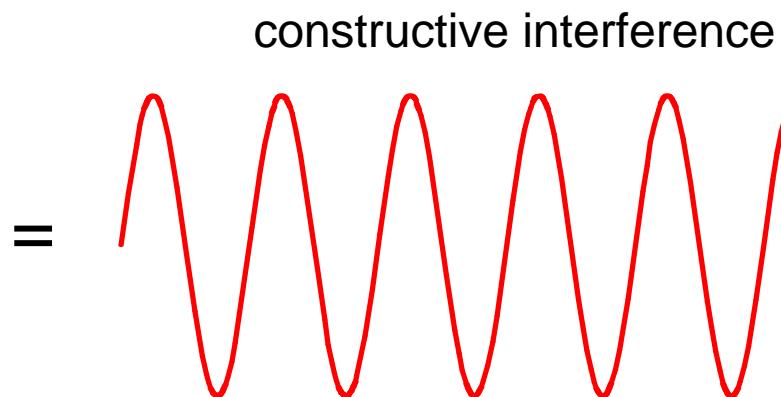
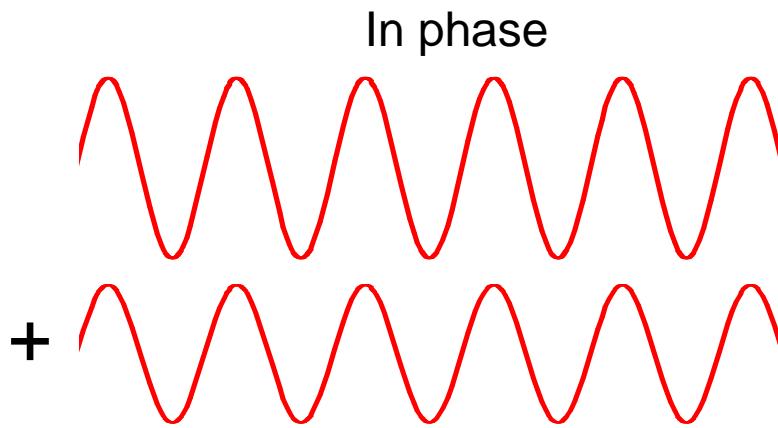
# Plan objectives

- Corrected for field curvature
- More complex design
- Needed for most photomicrography

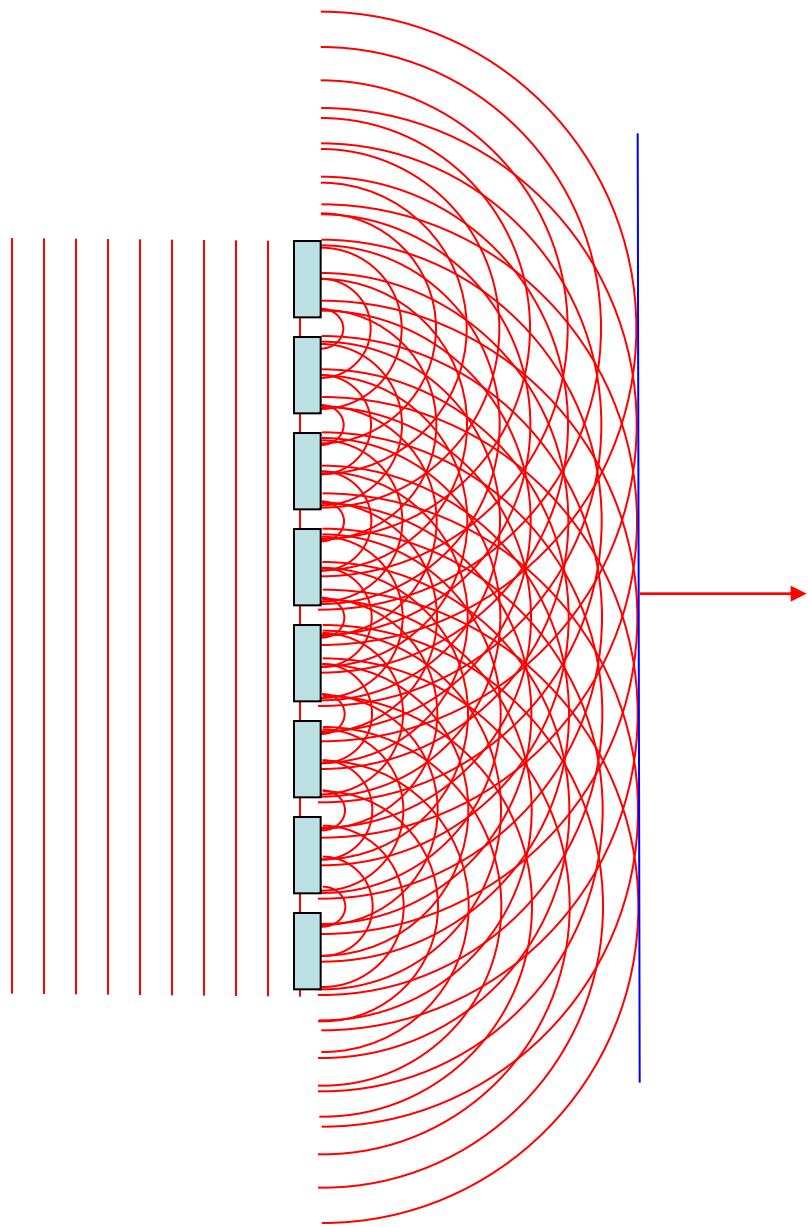


- **Plan-APOCHROMATS** have the highest performance  
(and highest complexity and price)

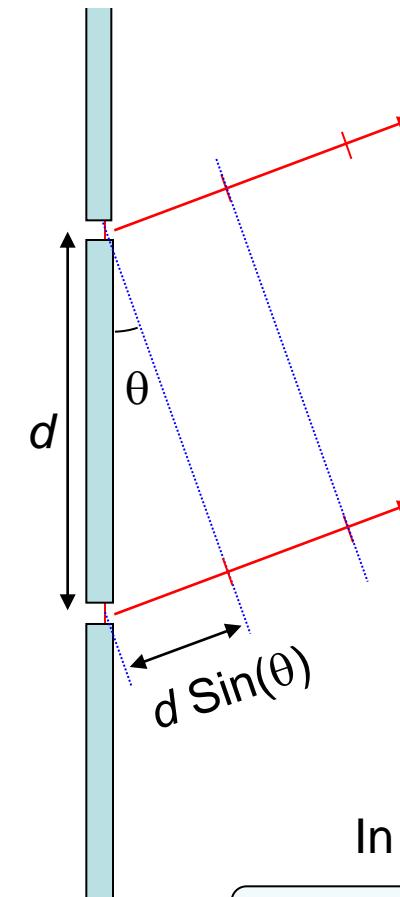
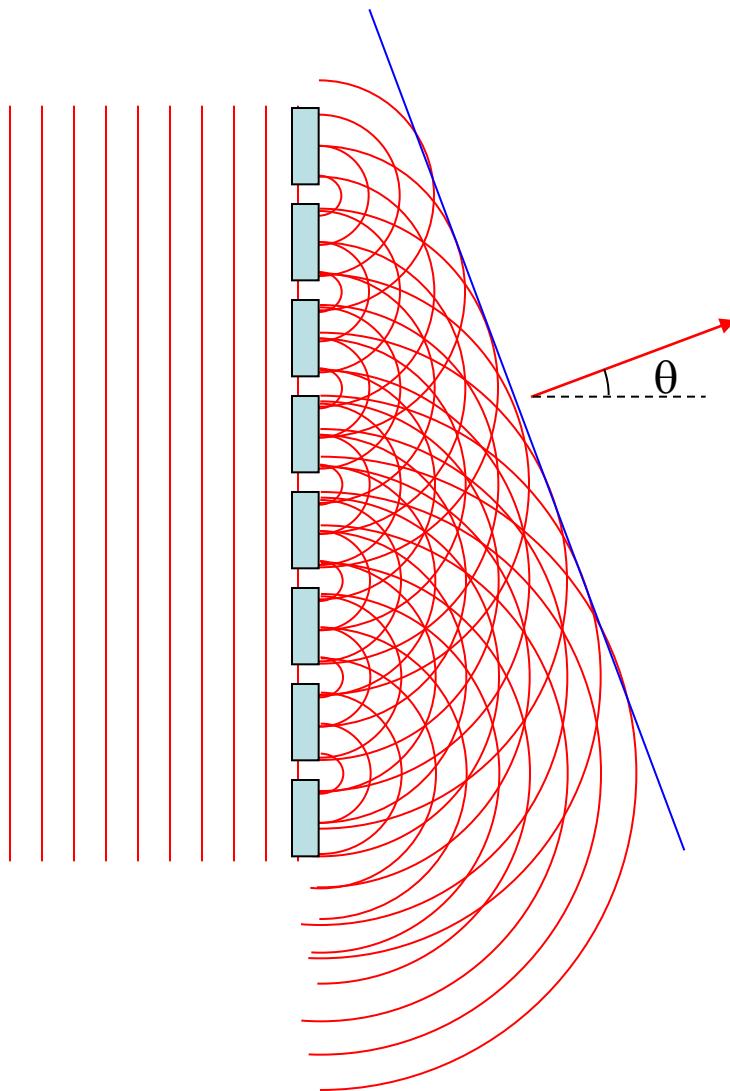
# Interference



# Diffraction by a periodic structure (grating)



# Diffraction by a periodic structure (grating)



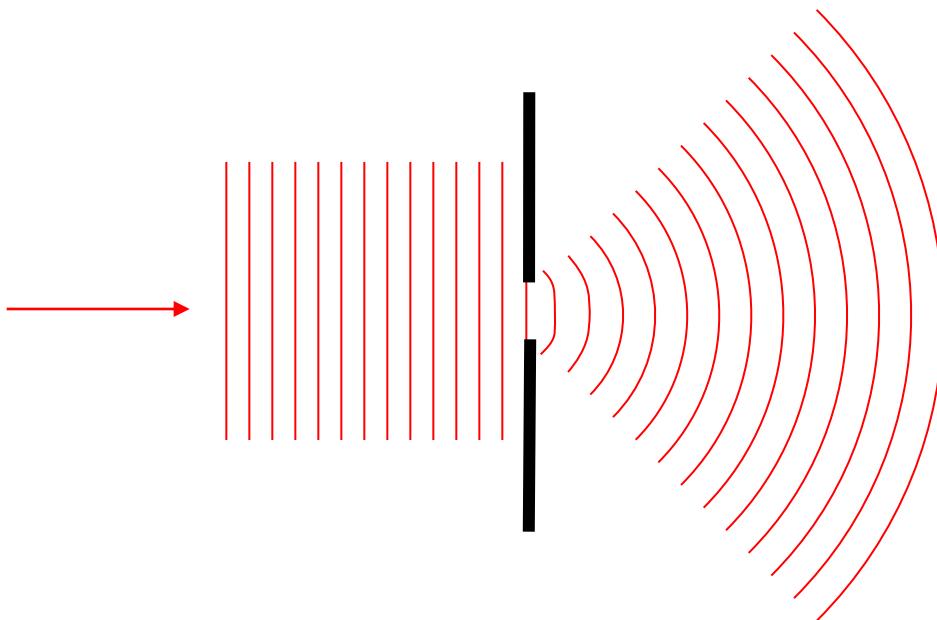
In phase if:

$$d \sin(\theta) = m \lambda$$

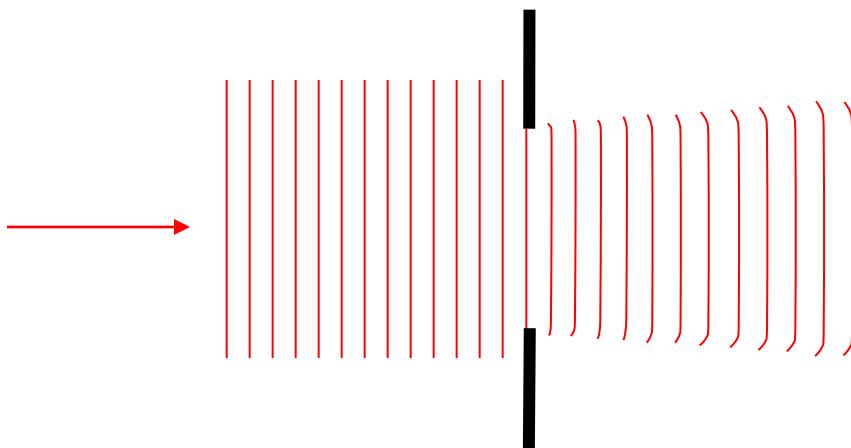
for some integer  $m$

# Diffraction by an aperture

drawn as waves



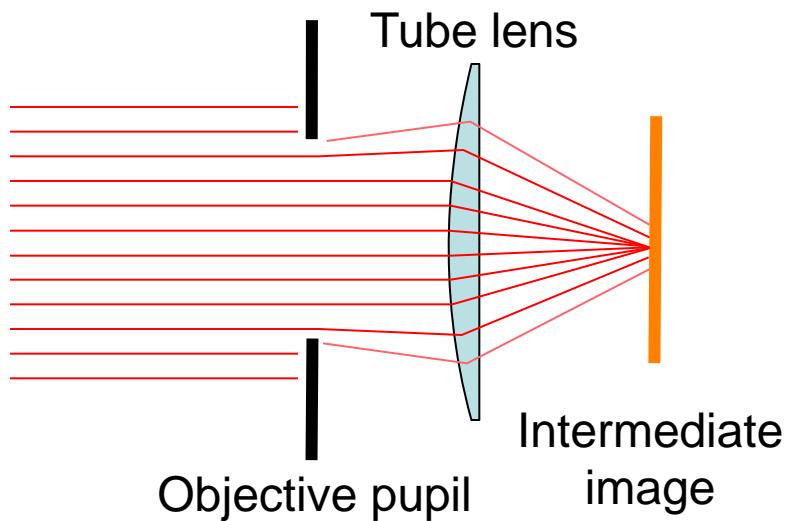
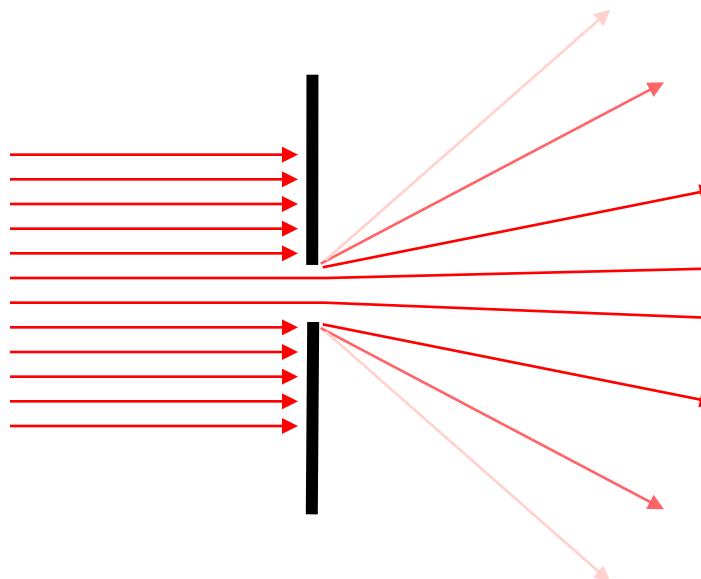
Light spreads to new angles



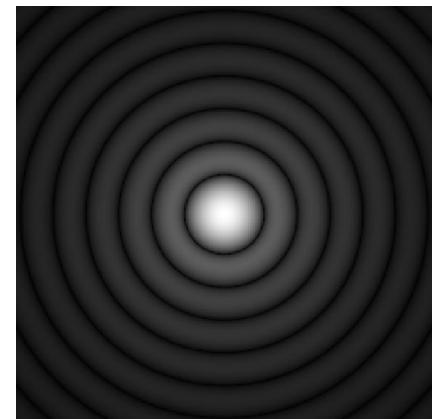
Larger aperture  
↔  
weaker diffraction

# Diffraction by an aperture

drawn as rays



The pure, “far-field”  
diffraction pattern  
is formed at  $\infty$  distance...

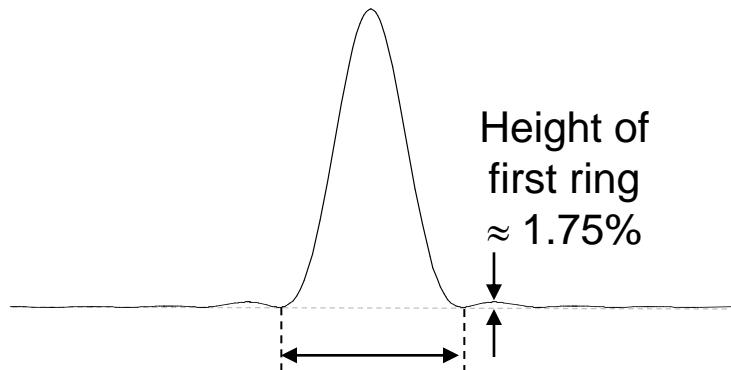
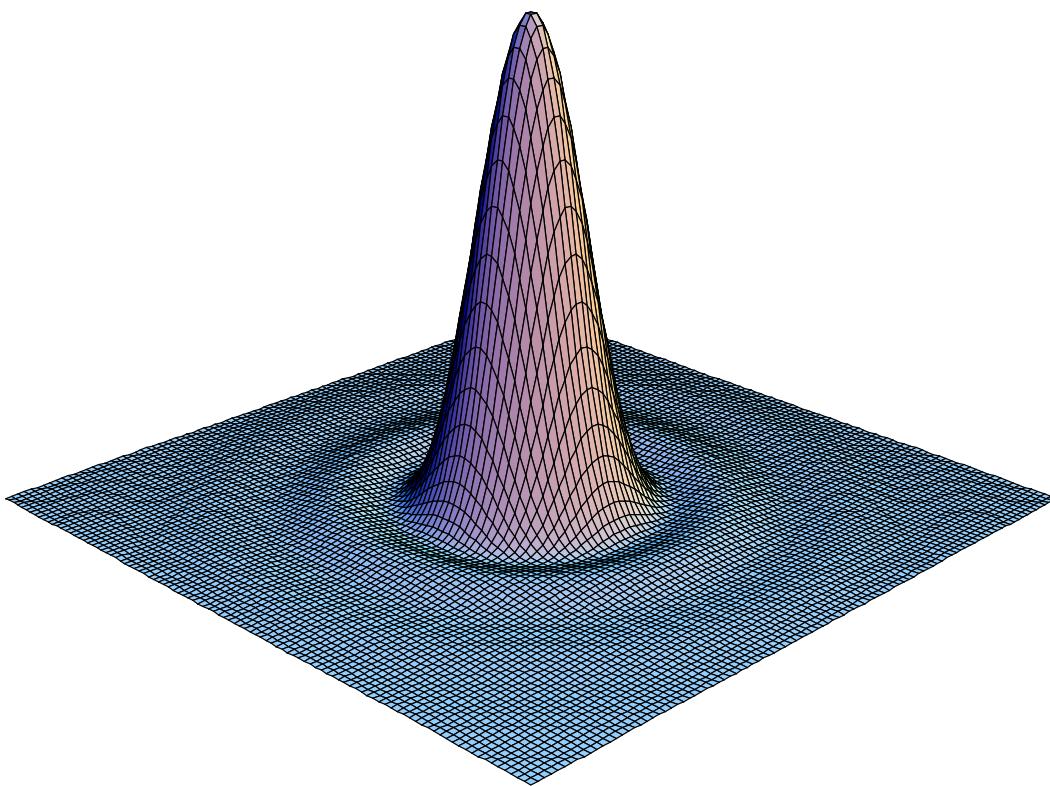


...or can be formed  
at a finite distance  
by a lens...

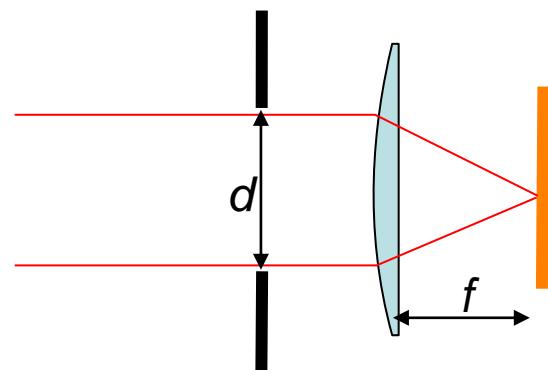
...as happens in a microscope

# The Airy Pattern

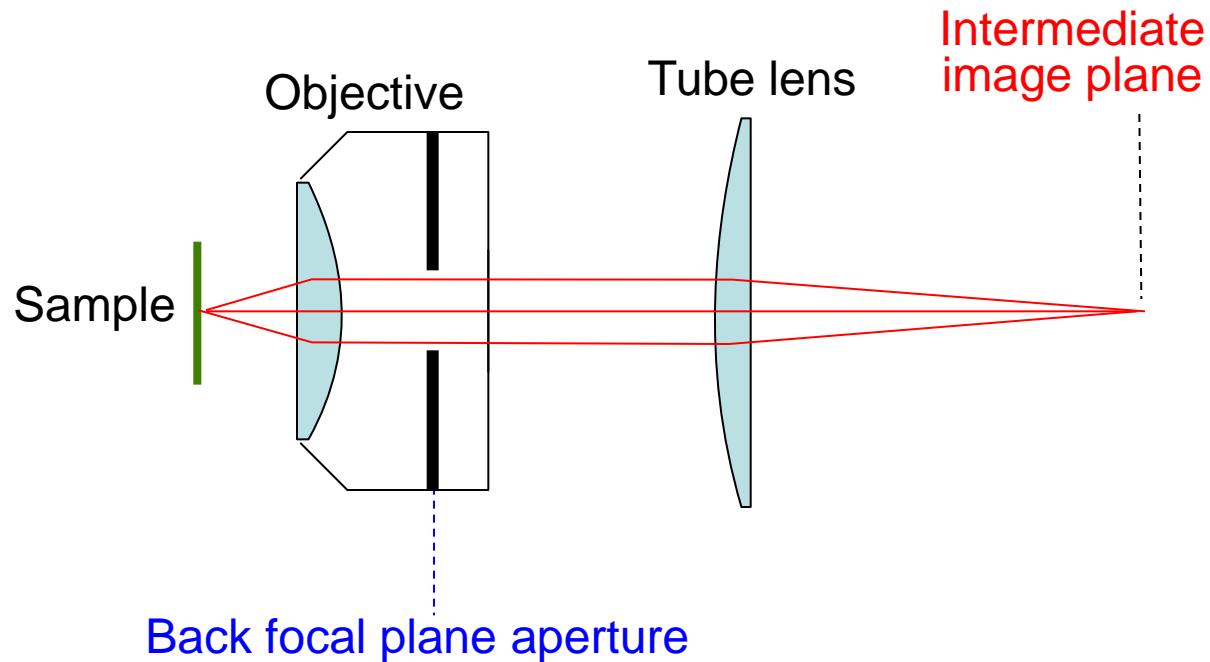
= the far-field diffraction pattern from a round aperture



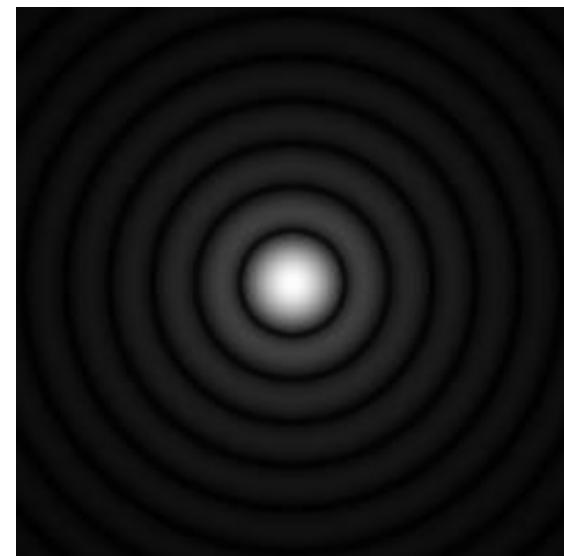
"Airy disk" diameter  
 $d = 2.44 \lambda f/d$   
(for small angles  $d/f$ )



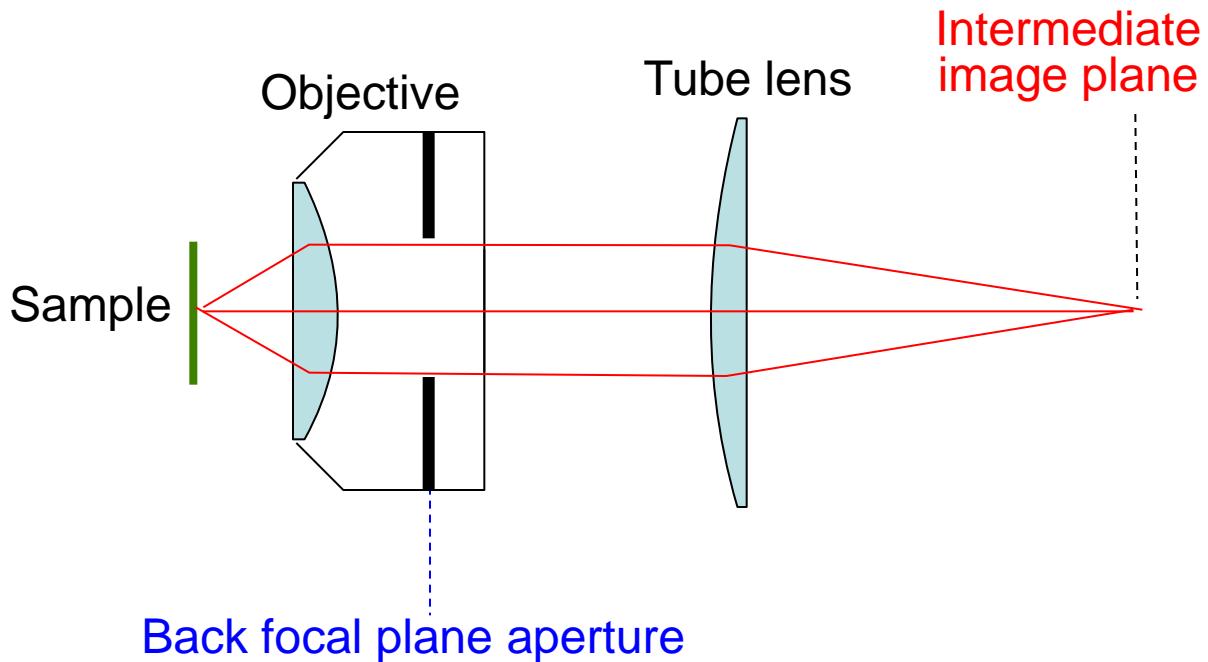
# Aperture and Resolution



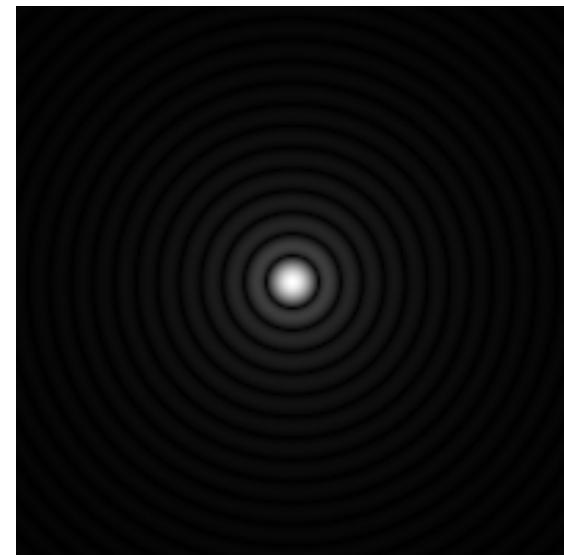
Diffraction spot  
on image plane  
= *Point Spread Function*



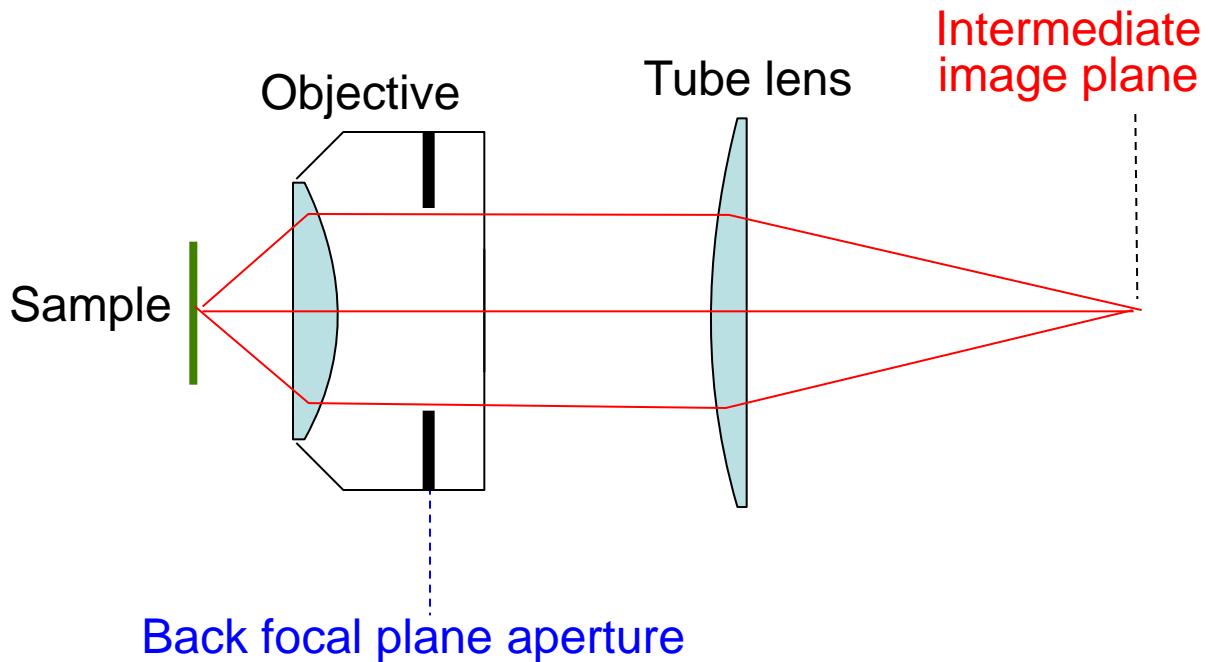
# Aperture and Resolution



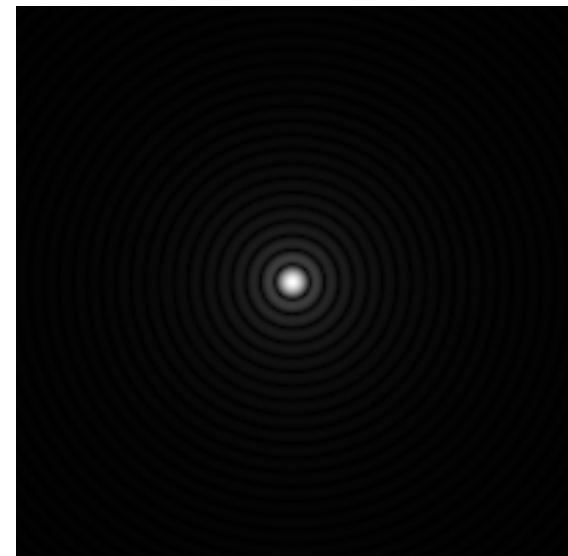
Diffraction spot  
on image plane  
= *Point Spread Function*



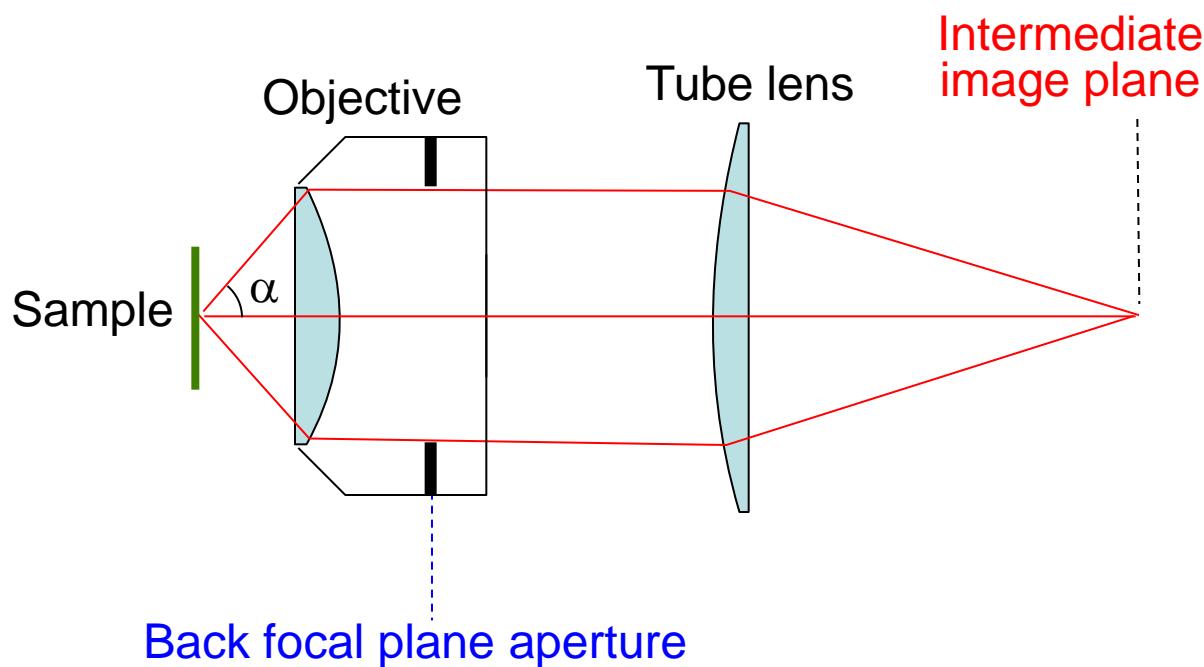
# Aperture and Resolution



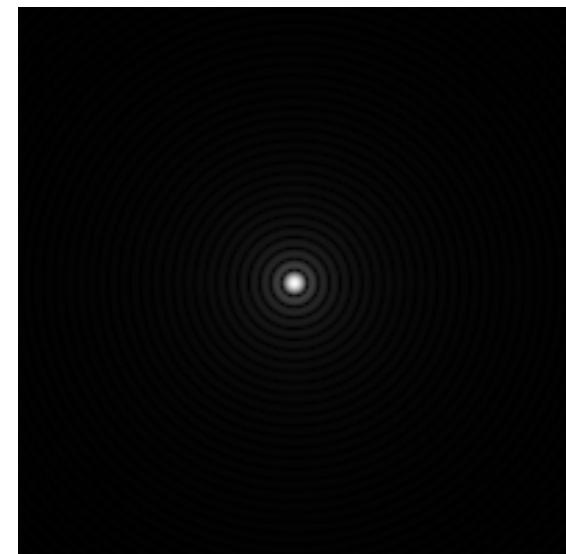
Diffraction spot  
on image plane  
= *Point Spread Function*



# Aperture and Resolution



Diffration spot  
on image plane  
(resolution)



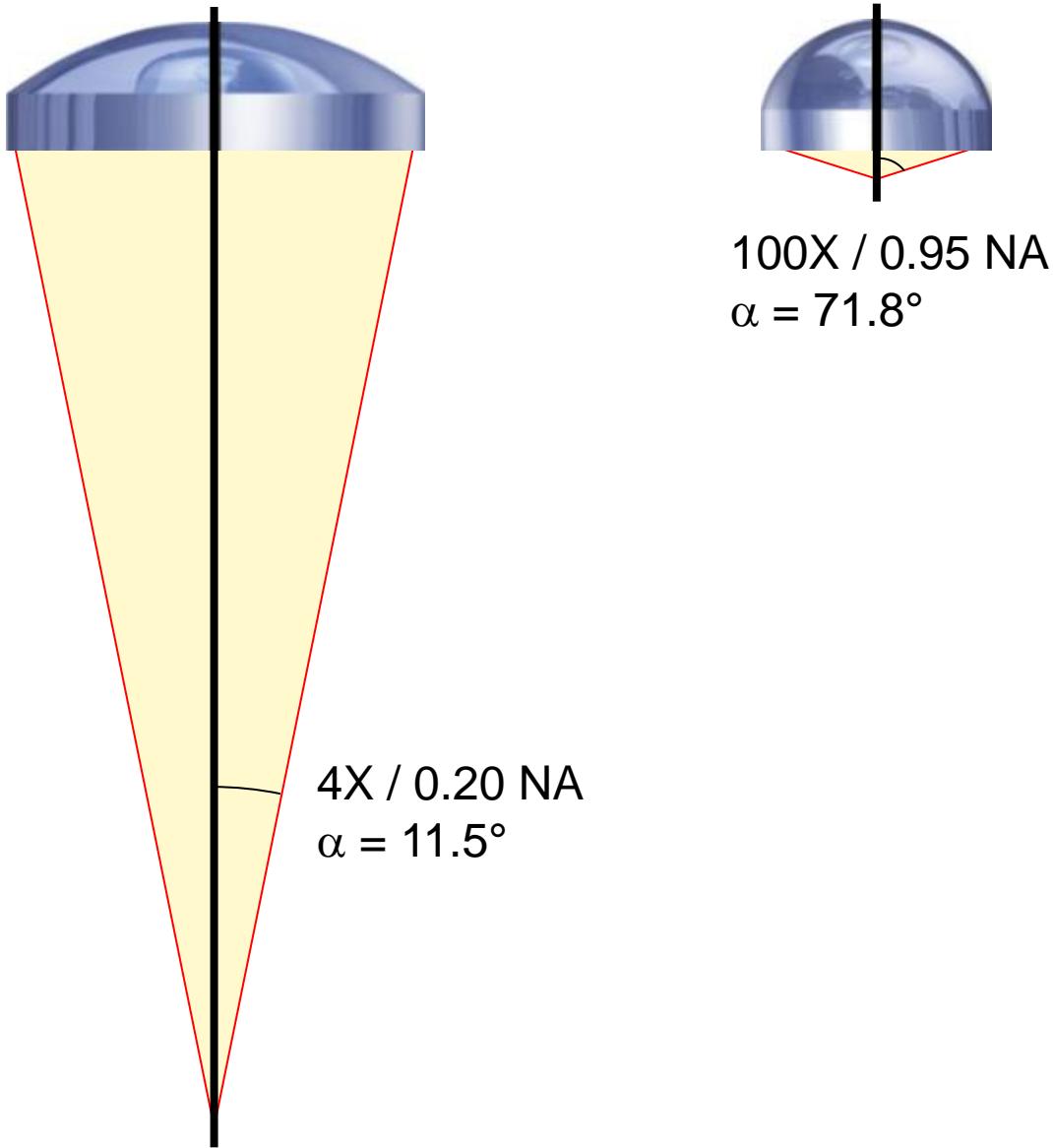
- Image resolution improves with ~~aperture size~~ Numerical Aperture (NA)

$$NA = n \sin(\alpha)$$

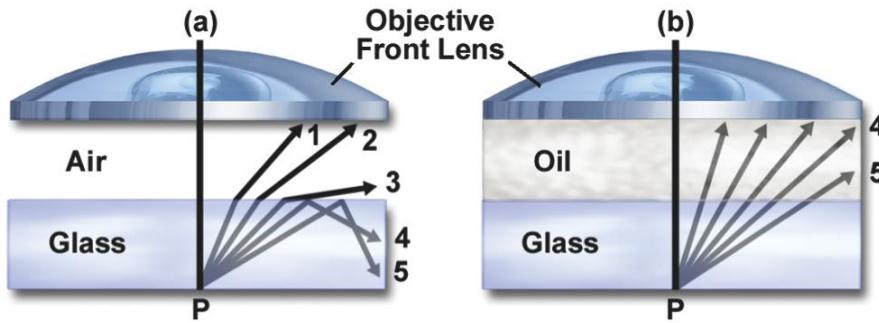
where:

$\alpha$  = light gathering angle  
 $n$  = refractive index of sample

# Numerical Aperture



# Immersion Objectives



- ⇒ NA cannot exceed the *lowest n* between the sample and the objective lens
- ⇒ NA > 1 requires ***fluid immersion***

NA can approach the index of the immersion fluid

Oil immersion:

$$n \approx 1.515$$

max NA  $\approx 1.4$  (1.45–1.49 for TIRF)

Glycerol immersion:

$$n \approx 1.45 \text{ (85%)}$$

max NA  $\approx 1.35$  (Leica)

Water immersion:

$$n \approx 1.33$$

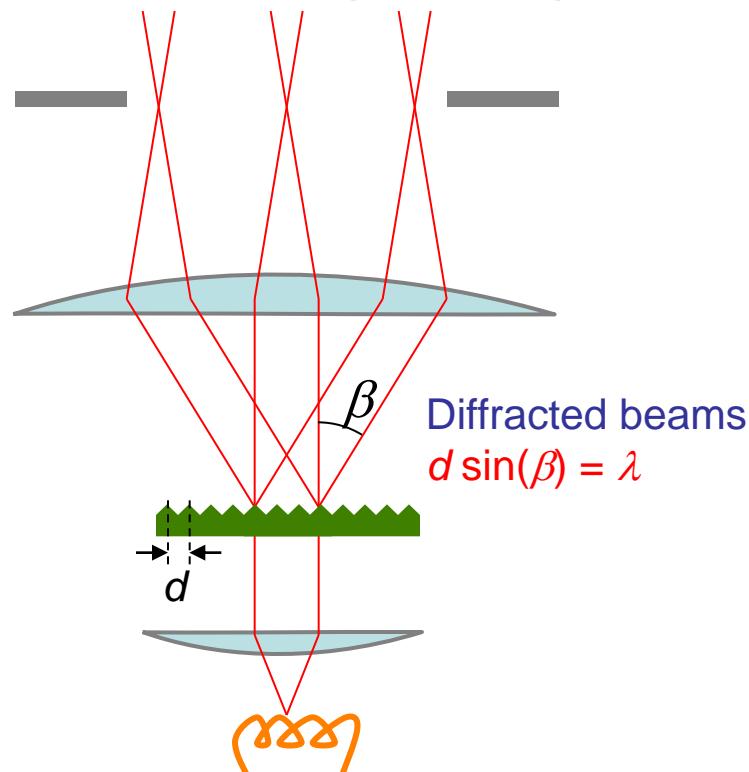
max NA  $\approx 1.2$

# Resolution

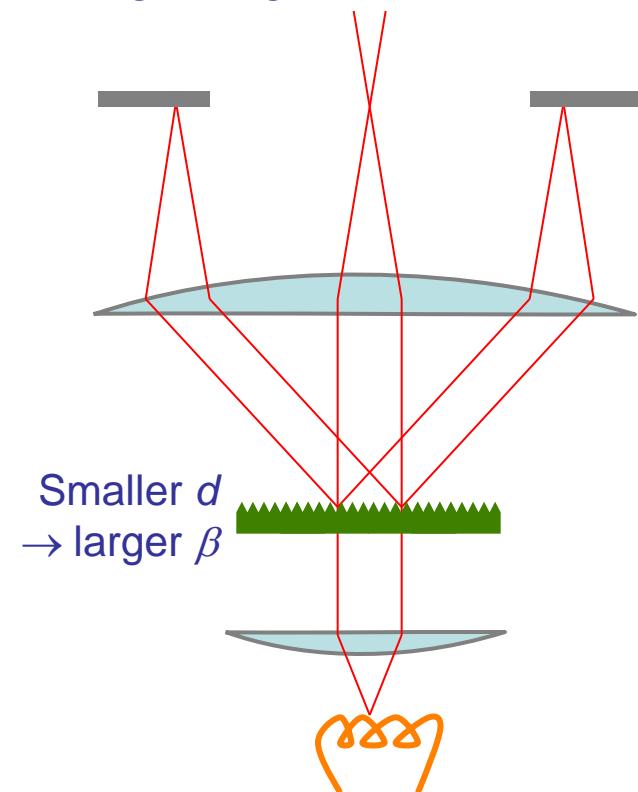
## Ernst Abbe's argument (1873)

Consider a striped sample  $\approx$  a diffraction grating

Back focal plane  
Objective lens  
Sample  
Condenser  
Light source



Consider first  
a point light source

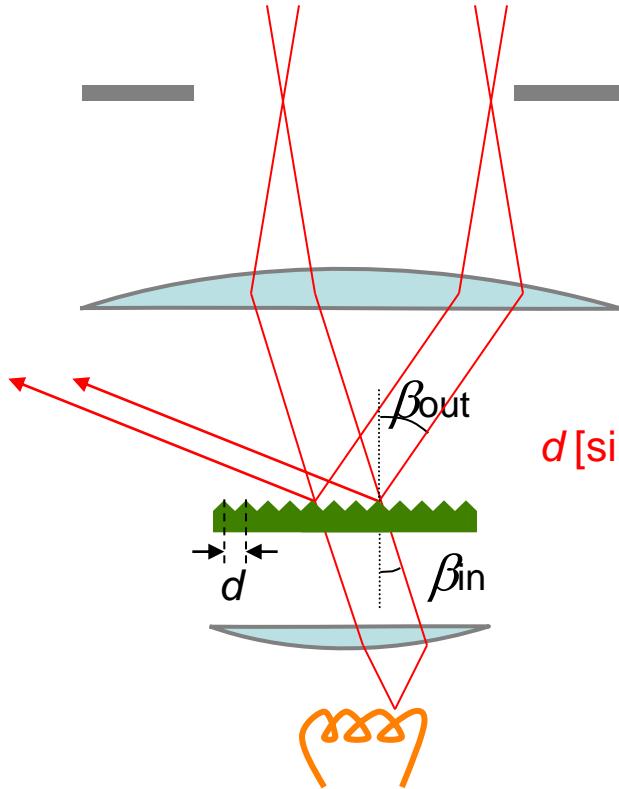


If  $\beta > \alpha$ , only one spot makes it through  
⇒ no interference ⇒ no image formed

Resolution (smallest resolvable  $d$ ):  
 $d_{\min} = \lambda_{\text{sample}} / \sin(\alpha) = \lambda / n \sin(\alpha) = \lambda / NA$

## (Abbe's argument, continued)

Now consider oblique illumination  
(an off-axis source point):



One spot hopelessly lost,  
but **two** spots get through  
→ interference → image formed!

$$d [\sin(\beta_{in}) + \sin(\beta_{out})] = \lambda$$

Two spots get through if  
 $\beta_{out} < \alpha$  and  $\beta_{in} < \alpha$ .

Resolution (smallest resolvable  $d$ )  
with incoherent illumination (all possible illumination directions):

$$d_{\min} = \lambda / (NA_{\text{obj}} + NA_{\text{condenser}})$$

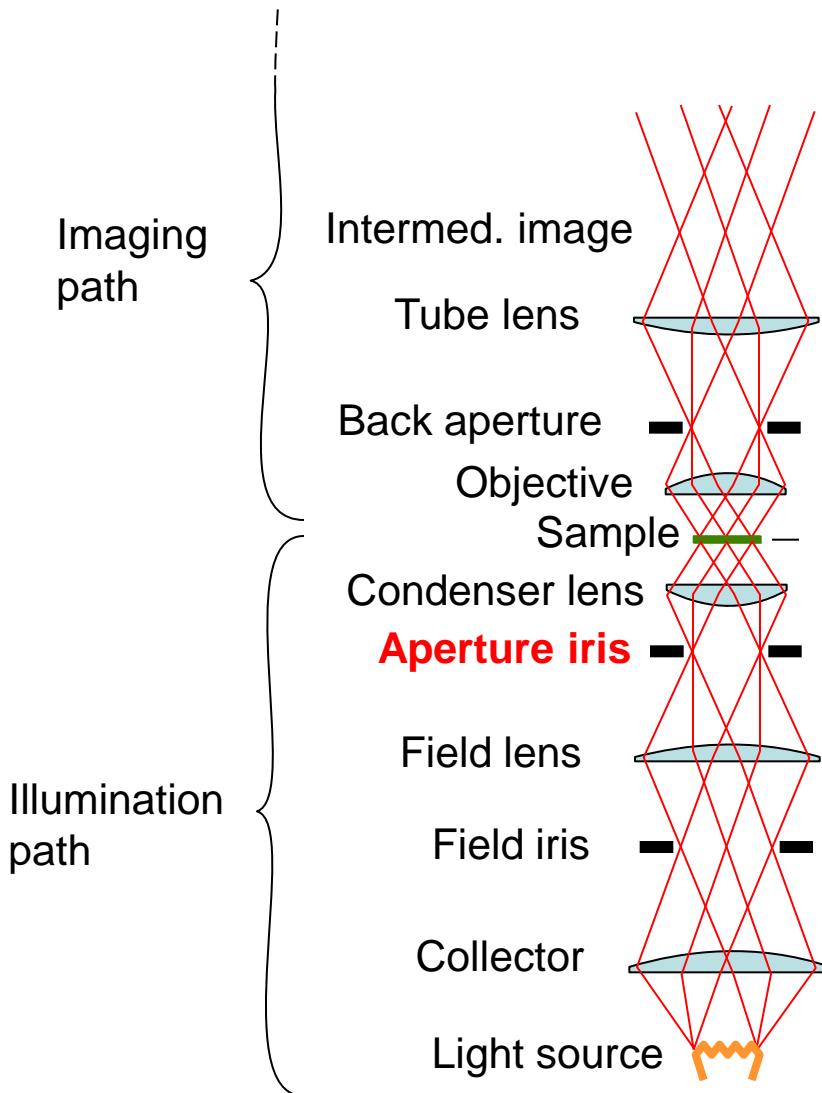


$$\lambda/2 \text{ } NA$$

if  $NA_{\text{condenser}} \geq NA_{\text{obj}}$  ("Filling the back focal plane")

# Aperture, Resolution & Contrast

Can adjust the condenser NA with the **aperture iris**

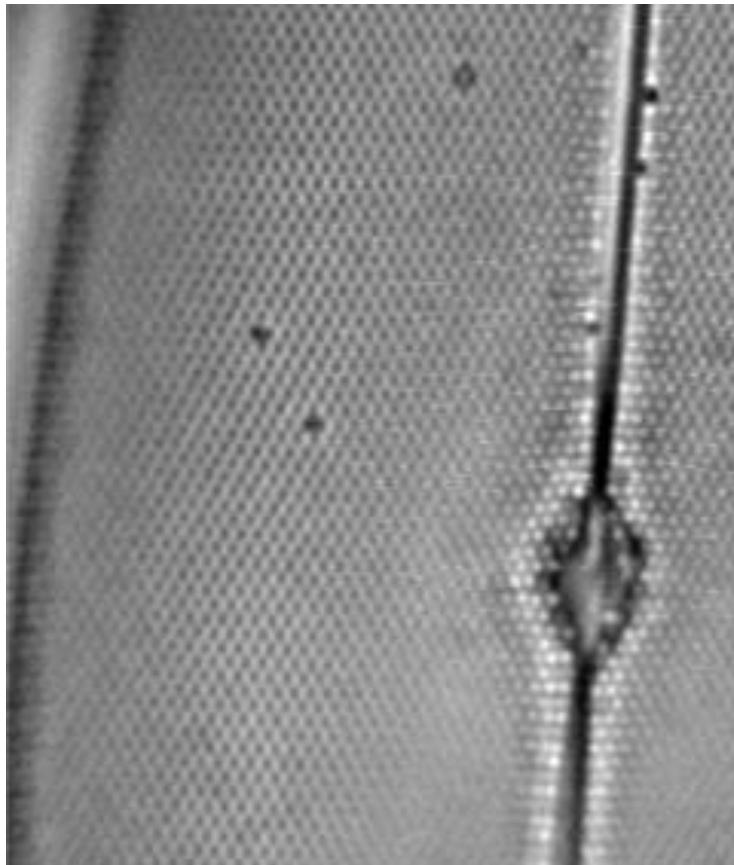


Q: Don't we always want it full open??

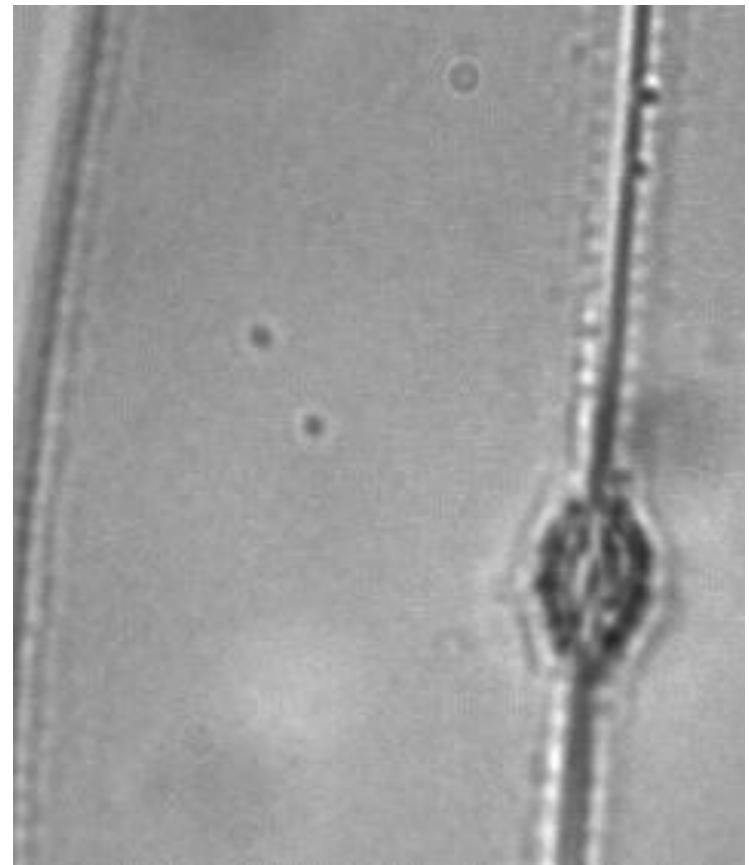
A: No

Why? Tradeoff:  
resolution vs. **contrast**

# NA and Resolution



High NA Objective



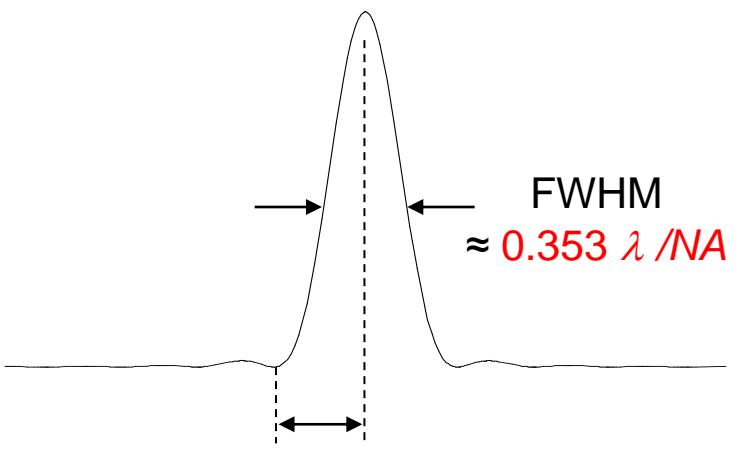
Low NA Objective

# Alternate Definitions of Resolution

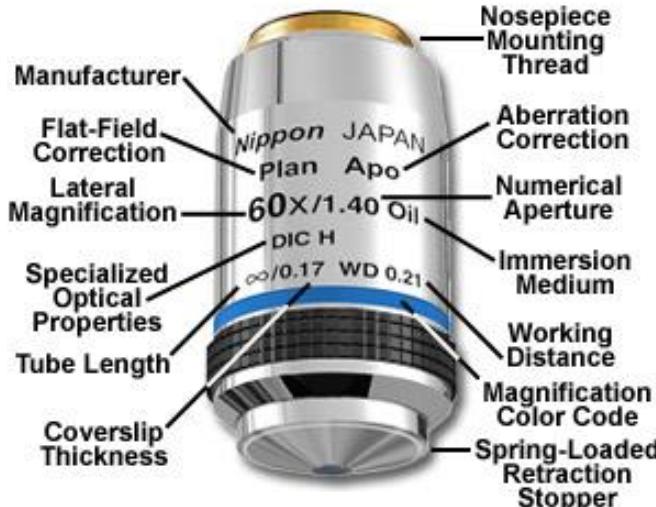
As the Full Width at Half Max  
(FWHM) of the PSF

As the diameter of the Airy disk  
(first dark ring of the PSF)  
= “Rayleigh criterion”

(Probably most common definition)



Airy disk radius  
≈  $0.61 \lambda /NA$



# Objective Types

## Basic properties

- Magnification
- Numerical Aperture (NA)
- Infinite or finite conjugate
- Cover slip thickness if any
- Immersion fluid if any

## Correction class

- Achromat
- Fluor
- Apochromat

## Field flatness

- Plan or not

## Phase rings for phase contrast

- Positive or negative
- Diameter of ring (number)

## Special Properties

- Strain free for Polarization or DIC

## Features

- Correction collar for spherical aberration
- Iris
- Spring-loaded front end
- Lockable front end

# Further reading

[www.microscopyu.com](http://www.microscopyu.com)

[micro.magnet.fsu.edu](http://micro.magnet.fsu.edu)

Douglas B. Murphy “Fundamentals of Light Microscopy and Electronic Imaging”

James Pawley, Ed. “Handbook of Biological Confocal Microscopy, 3rd ed.”

## Acknowledgements

Ron Vale / Mats Gustafsson