Microscopy

Paul Herzmark
pherzmark@gmail.com

Ellen Robey lab
Molecular and Cell Biology

http://tinyurl.com/PaulHerzmark-microscopy

Microscopy

Background-Koehler illumination, Numerical Aperture

fluorescence

Optimizing imaging

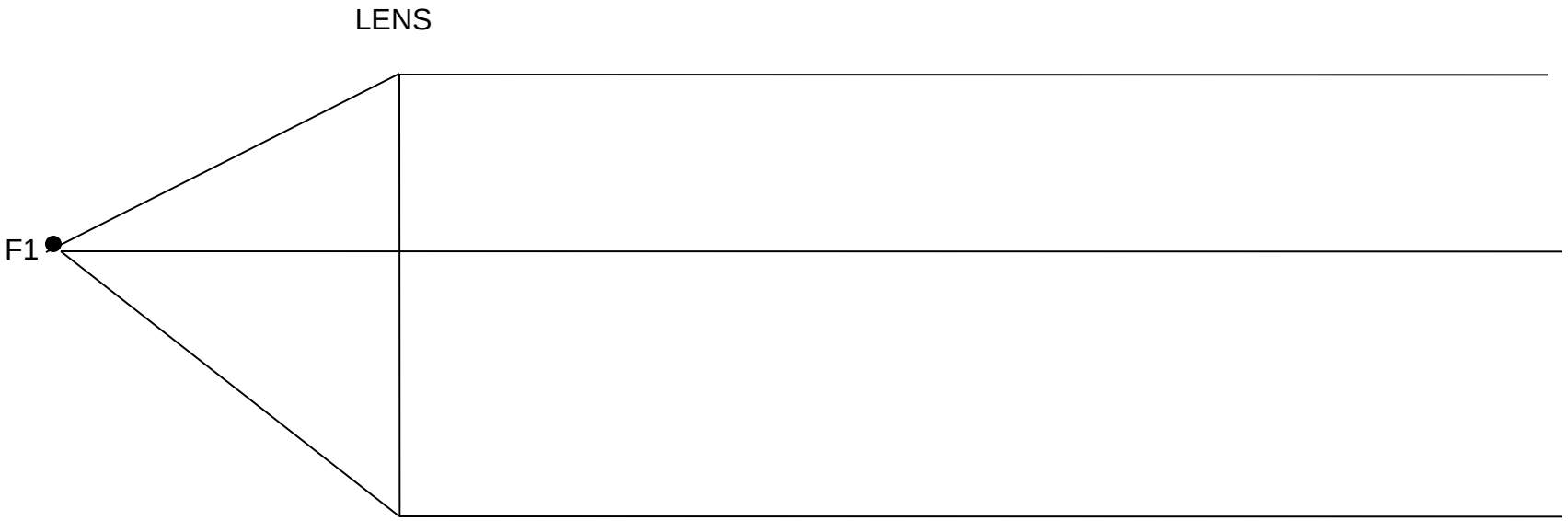
Comparing the different microscopes

Test:

- How can you make your fluorescent samples brighter?
- How can you keep them from bleaching and dying?

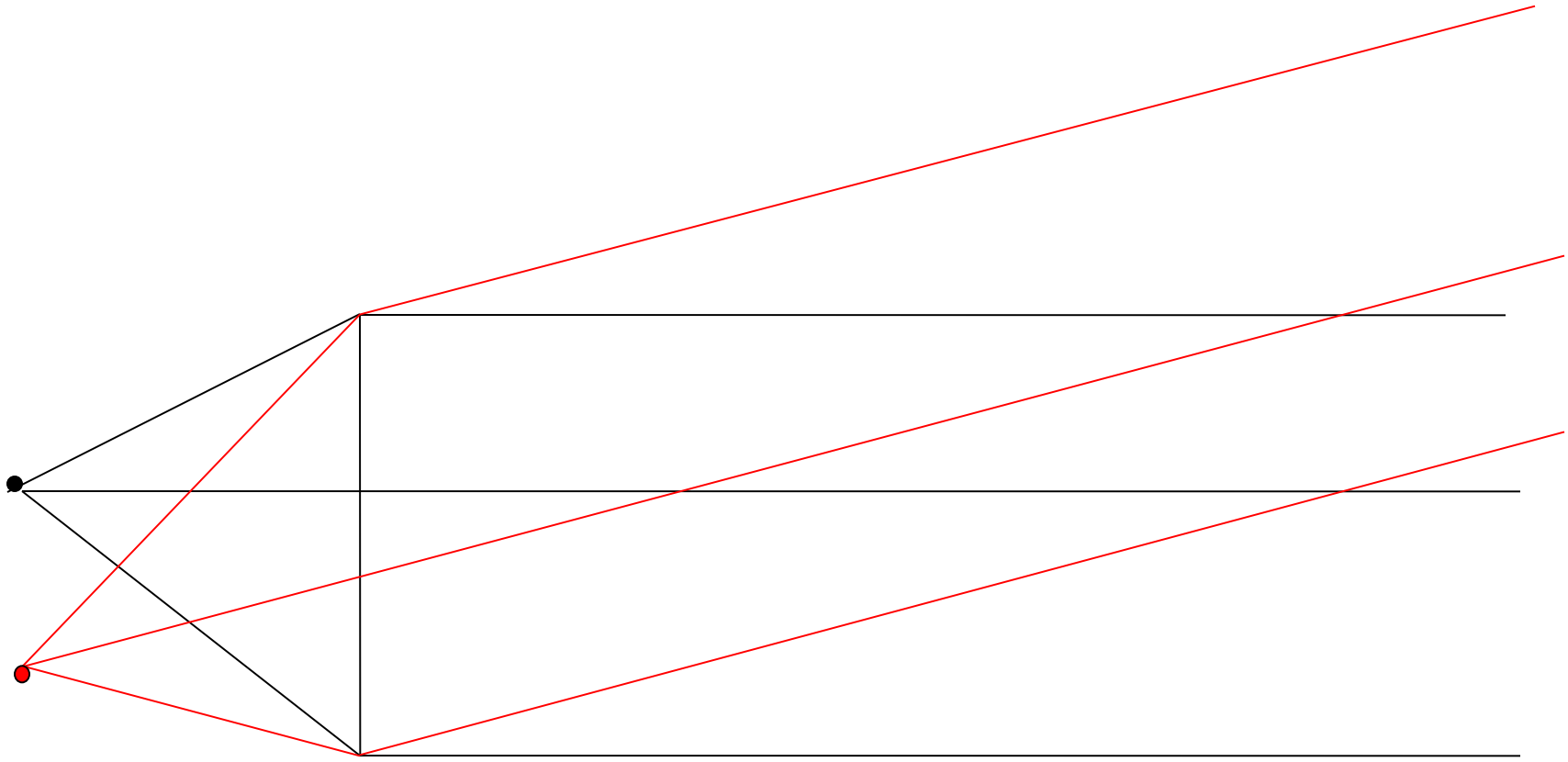
Lens and rays

Ray tracing



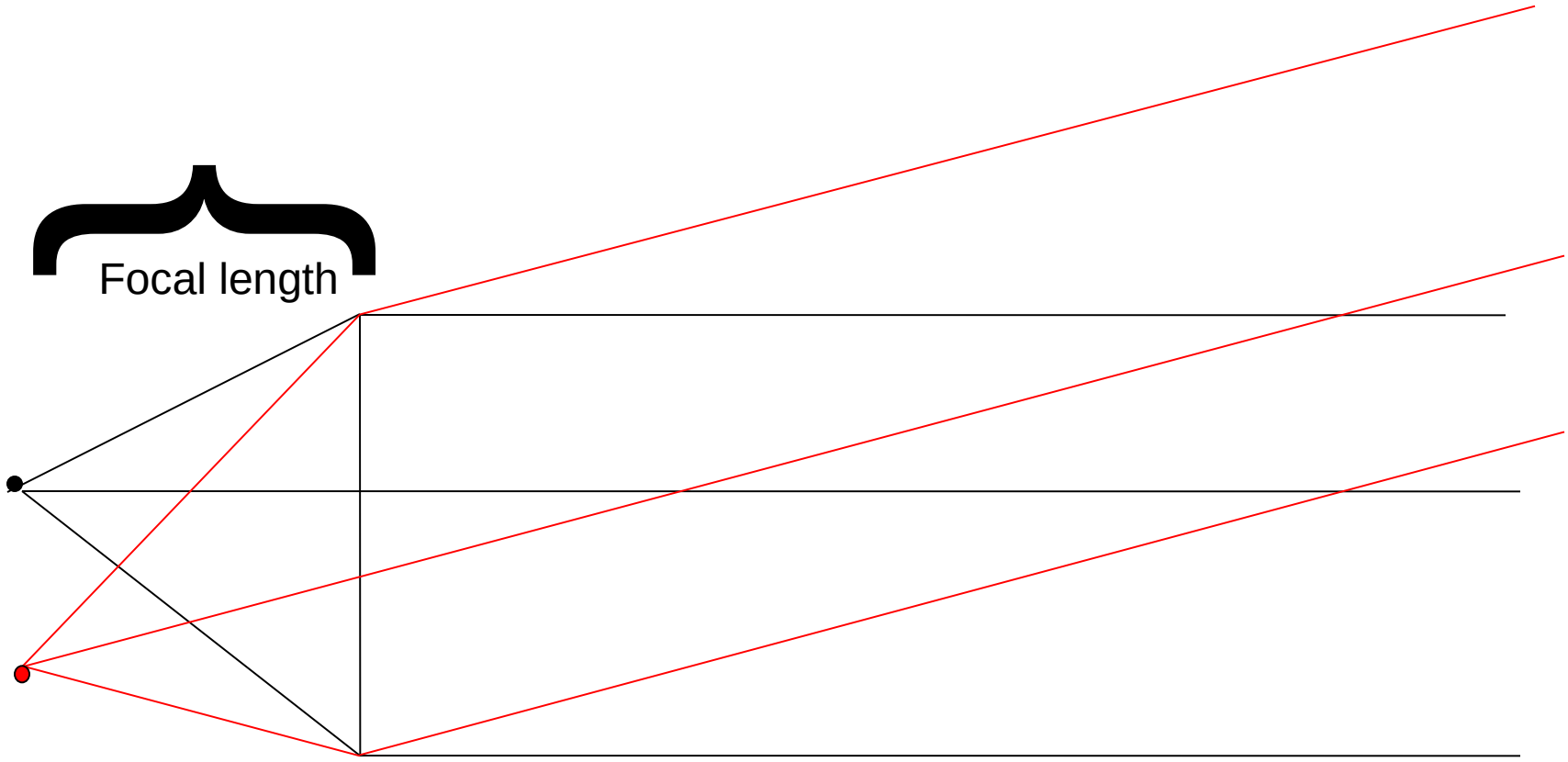
- At infinity lines are parallel
- Light through the center is unbent

Ray tracing



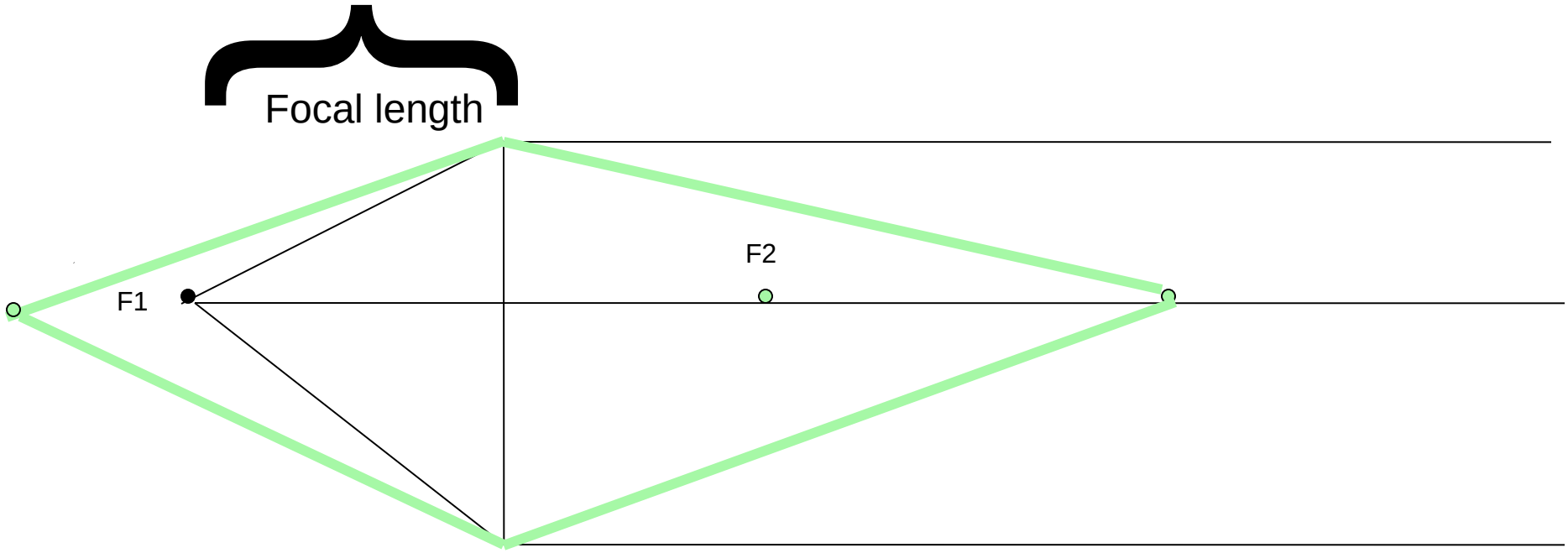
- At infinity lines are parallel
- Light through the center is unbent

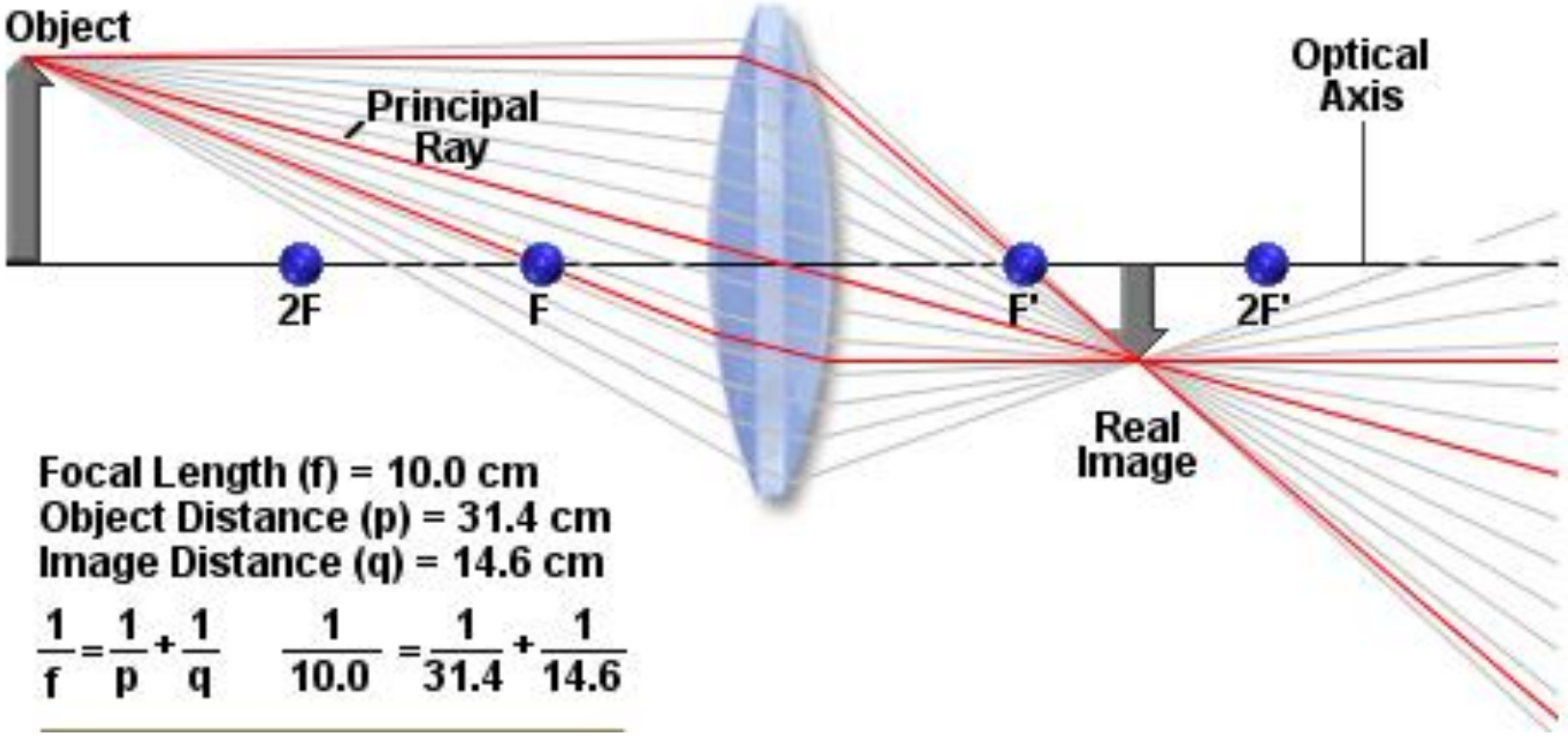
Ray tracing



- At infinity lines are parallel
- Light through the center is unbent

Ray tracing

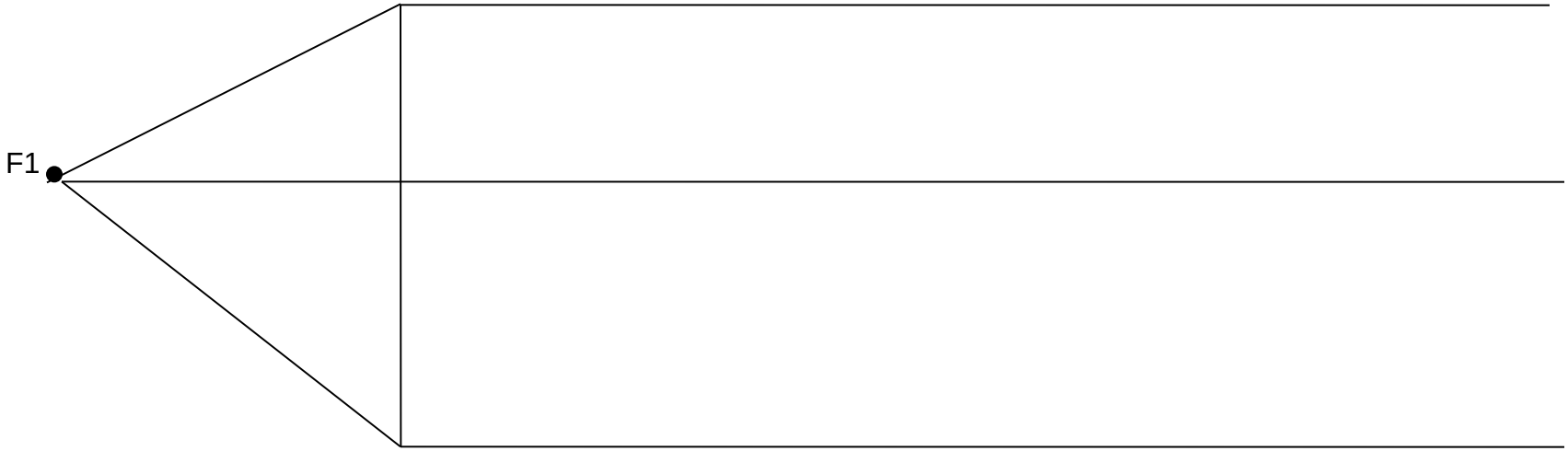


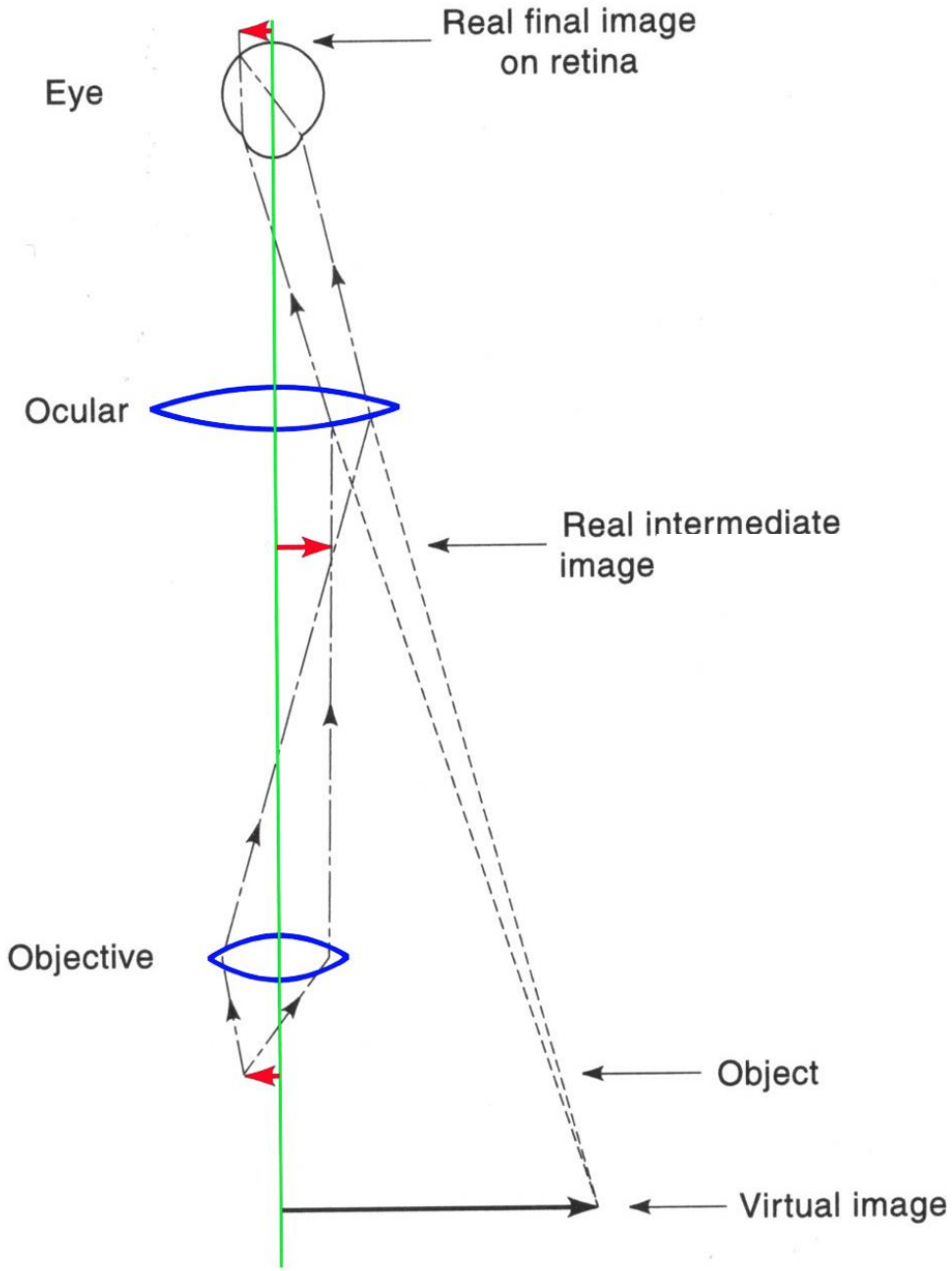


Focal Length (f) = 10.0 cm
Object Distance (p) = 31.4 cm
Image Distance (q) = 14.6 cm

$$\frac{1}{f}=\frac{1}{p}+\frac{1}{q} \qquad \frac{1}{10.0}=\frac{1}{31.4}+\frac{1}{14.6}$$

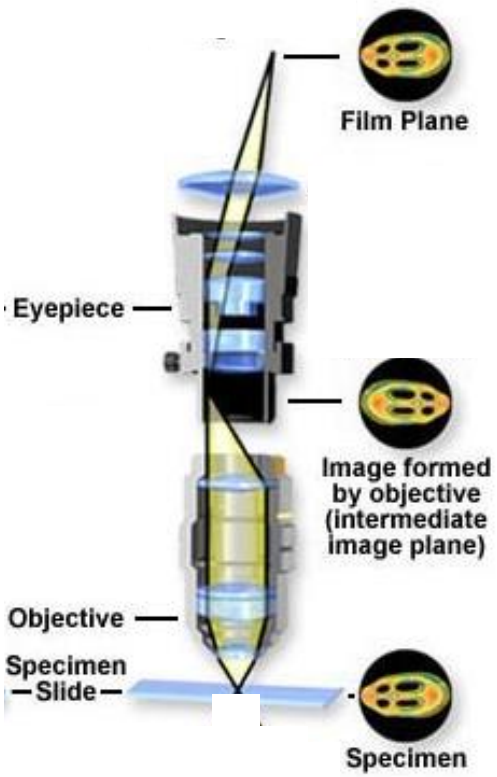
Determine focal length of a lens



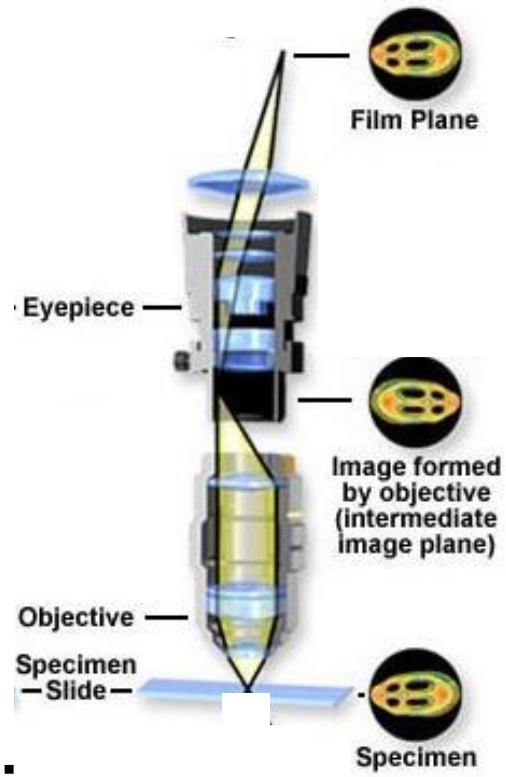

Real final image
on retina
Eye
Ocular
Real intermediate
image
Objective
Object
Virtual image

Koehler illumination

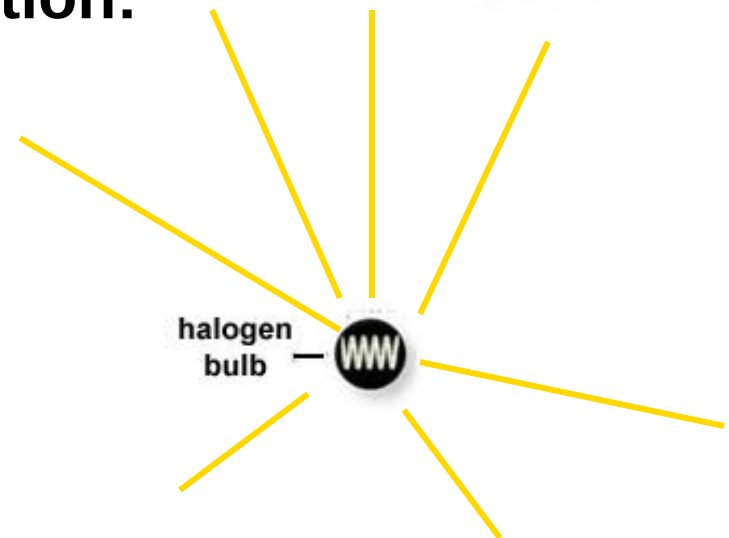
The nuts and bolts
(But why Paul?)



Now make the specimen bright.



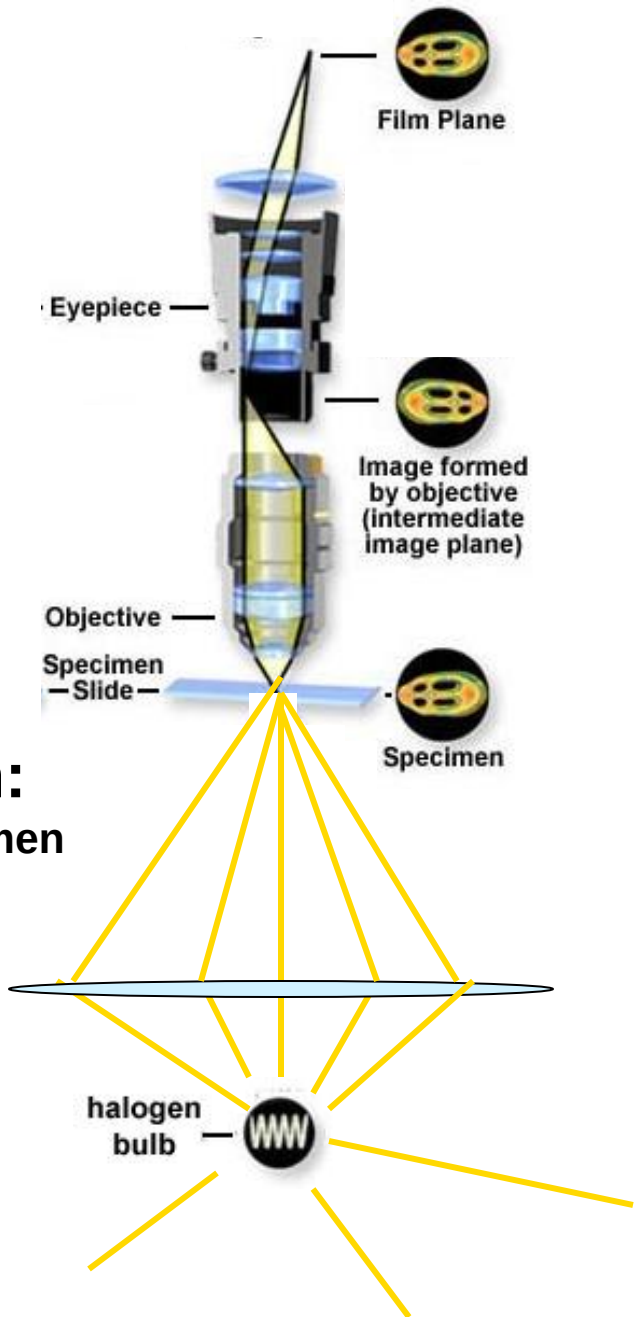
Light bulb illumination: even, dim



Critical illumination:

Focus the light onto the specimen

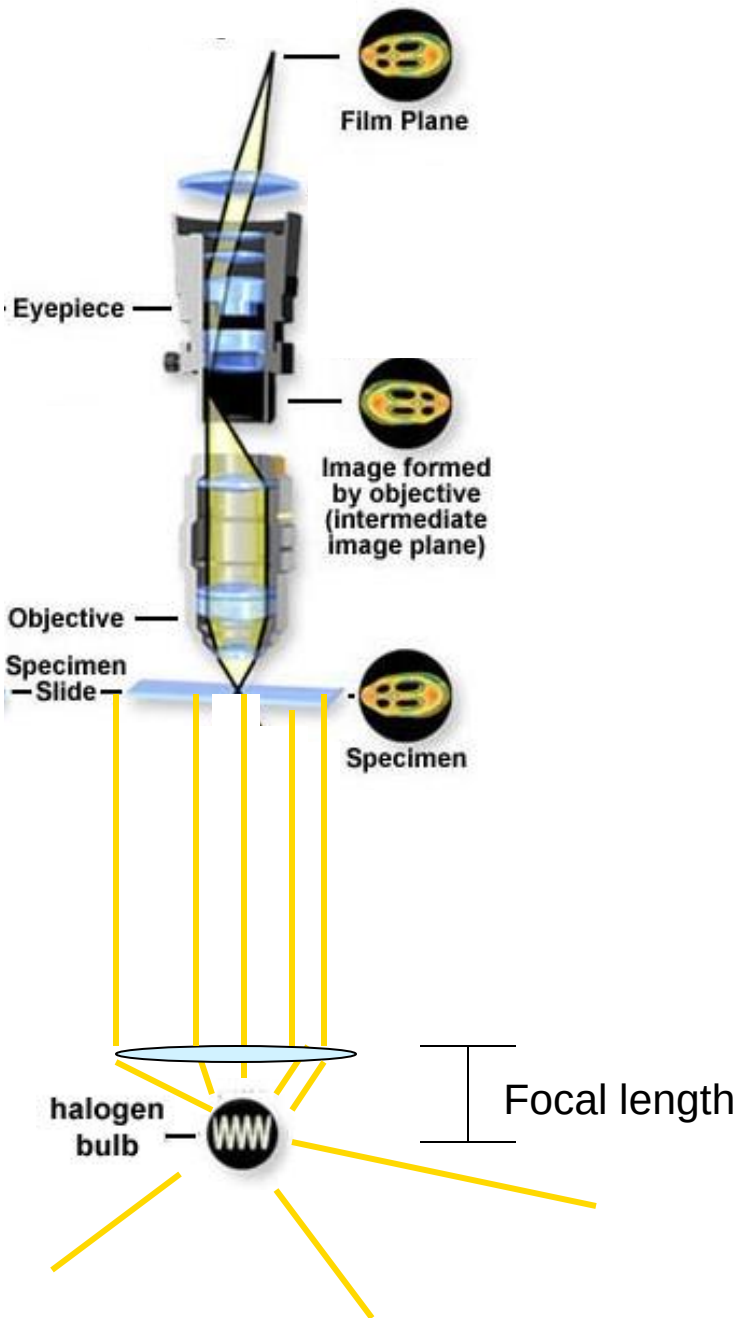
bright but uneven



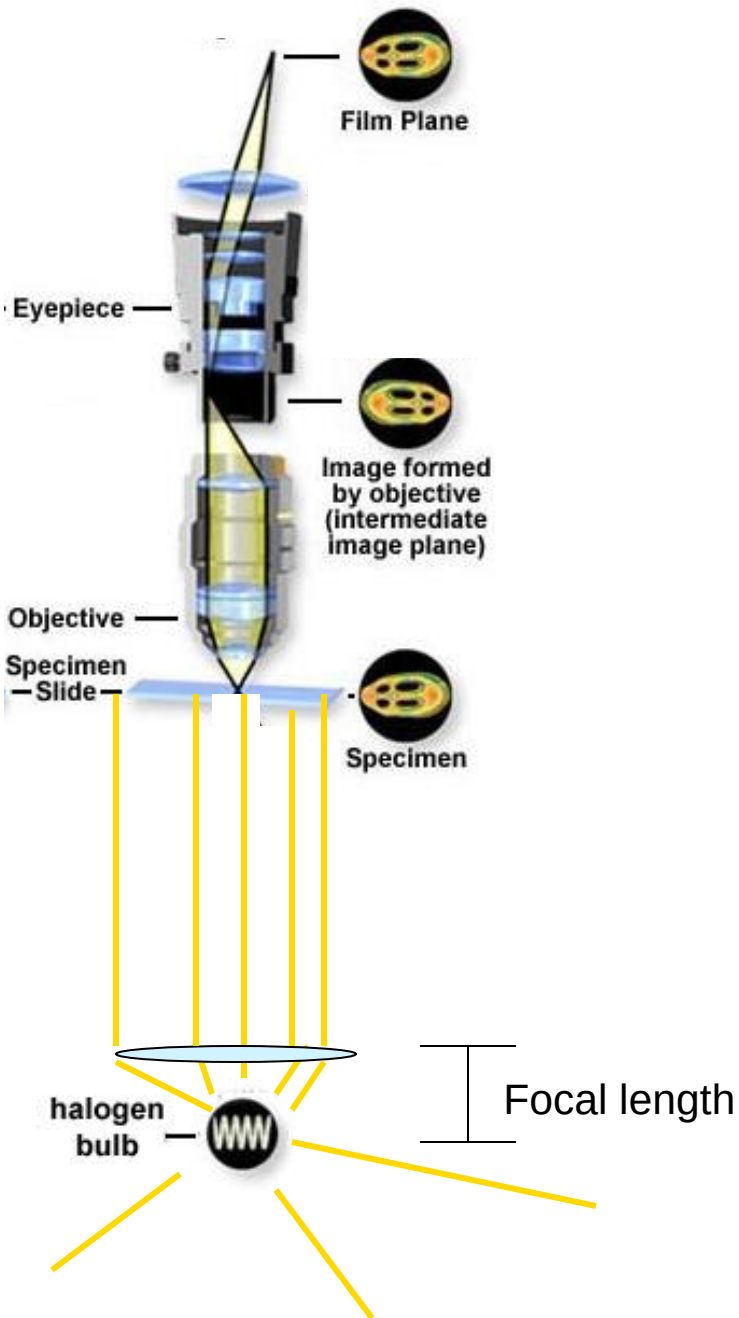
Kohler illumination:

Infinity focused light

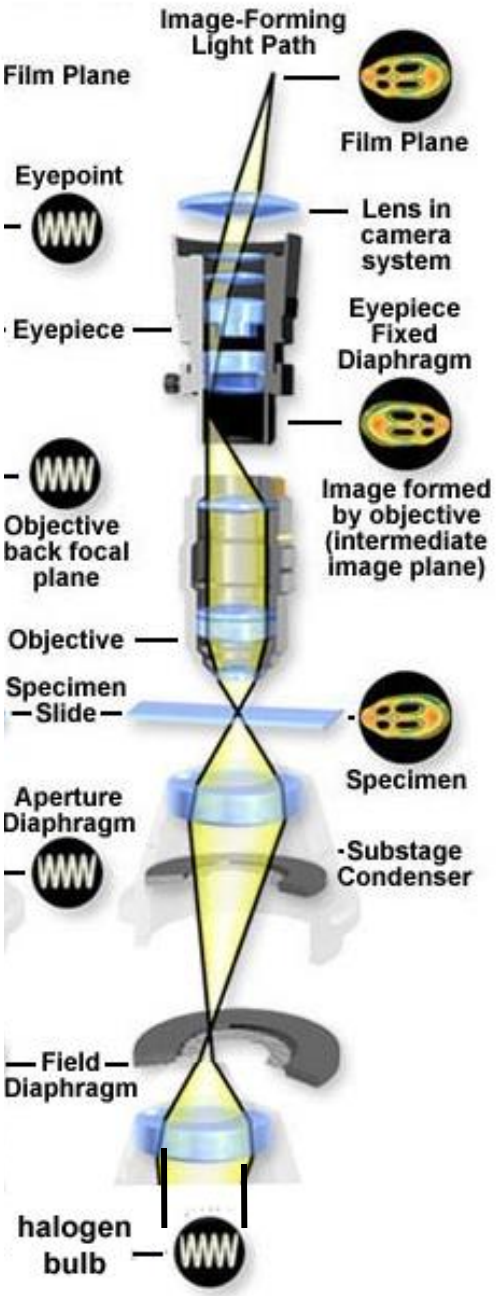
even, bright



The light bulb is out of focus. What is in focus?

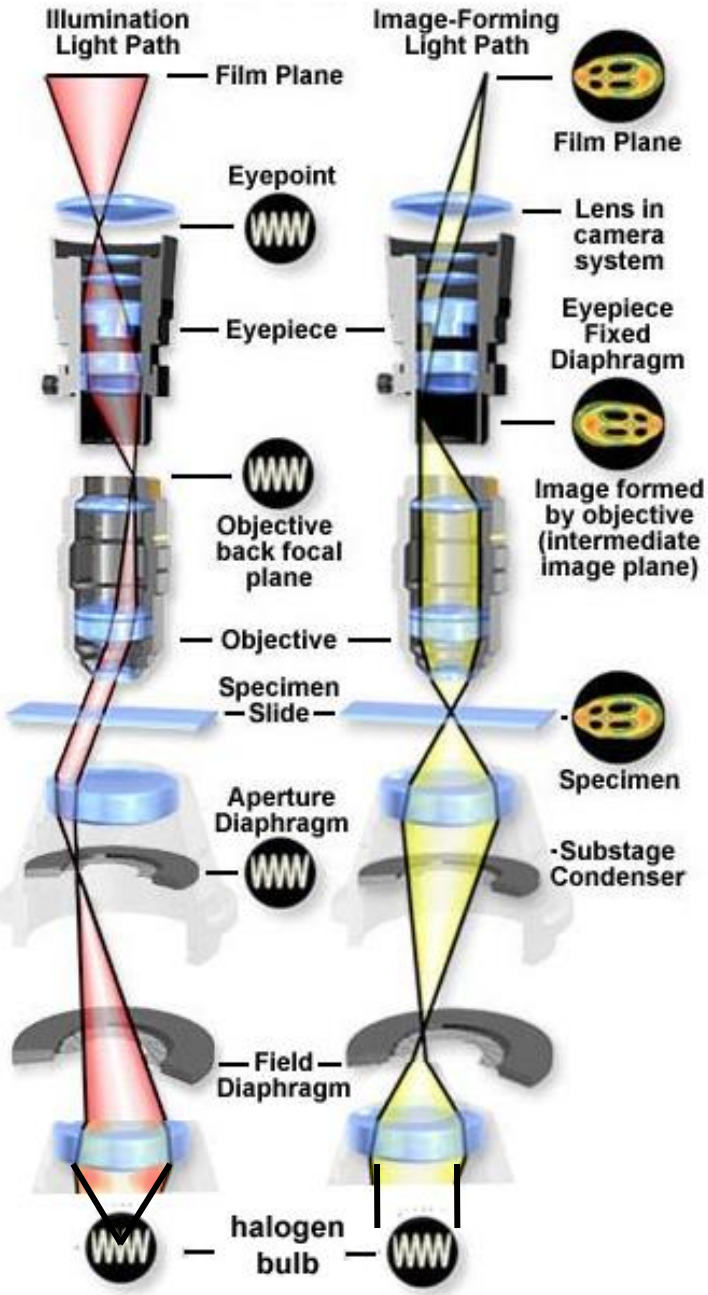


Köhler Illumination

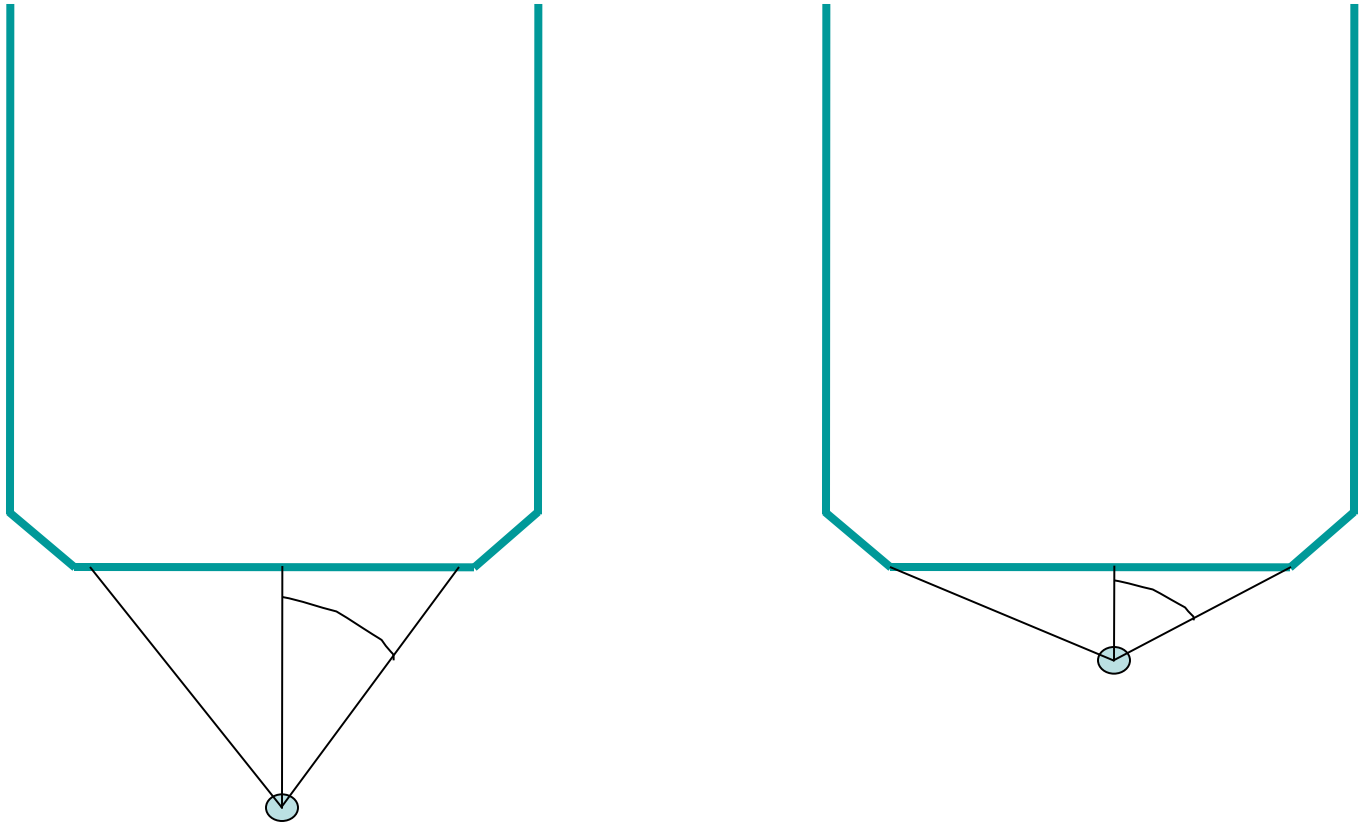


The light bulb is out of focus. What is in focus?

Köhler Illumination

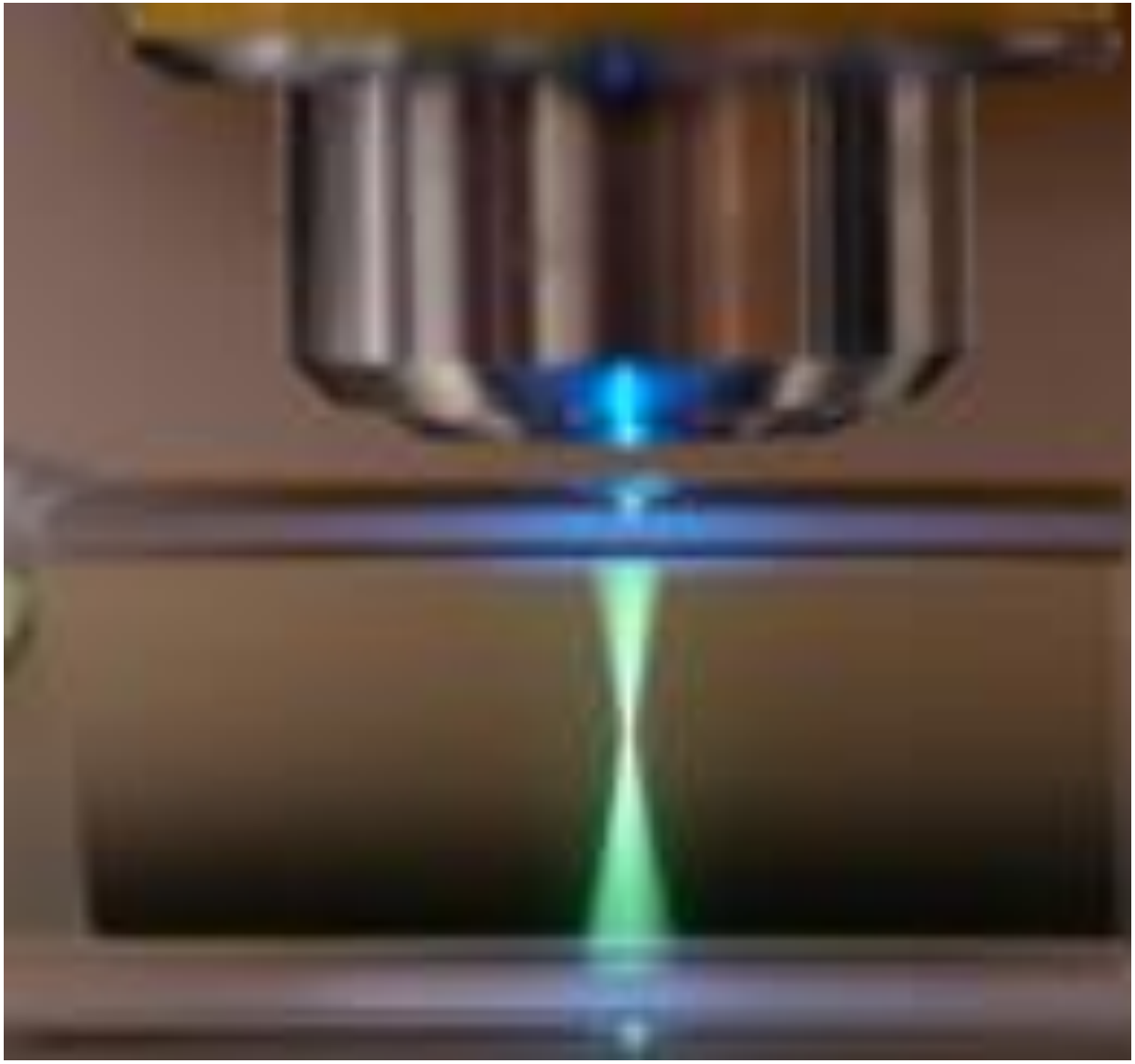


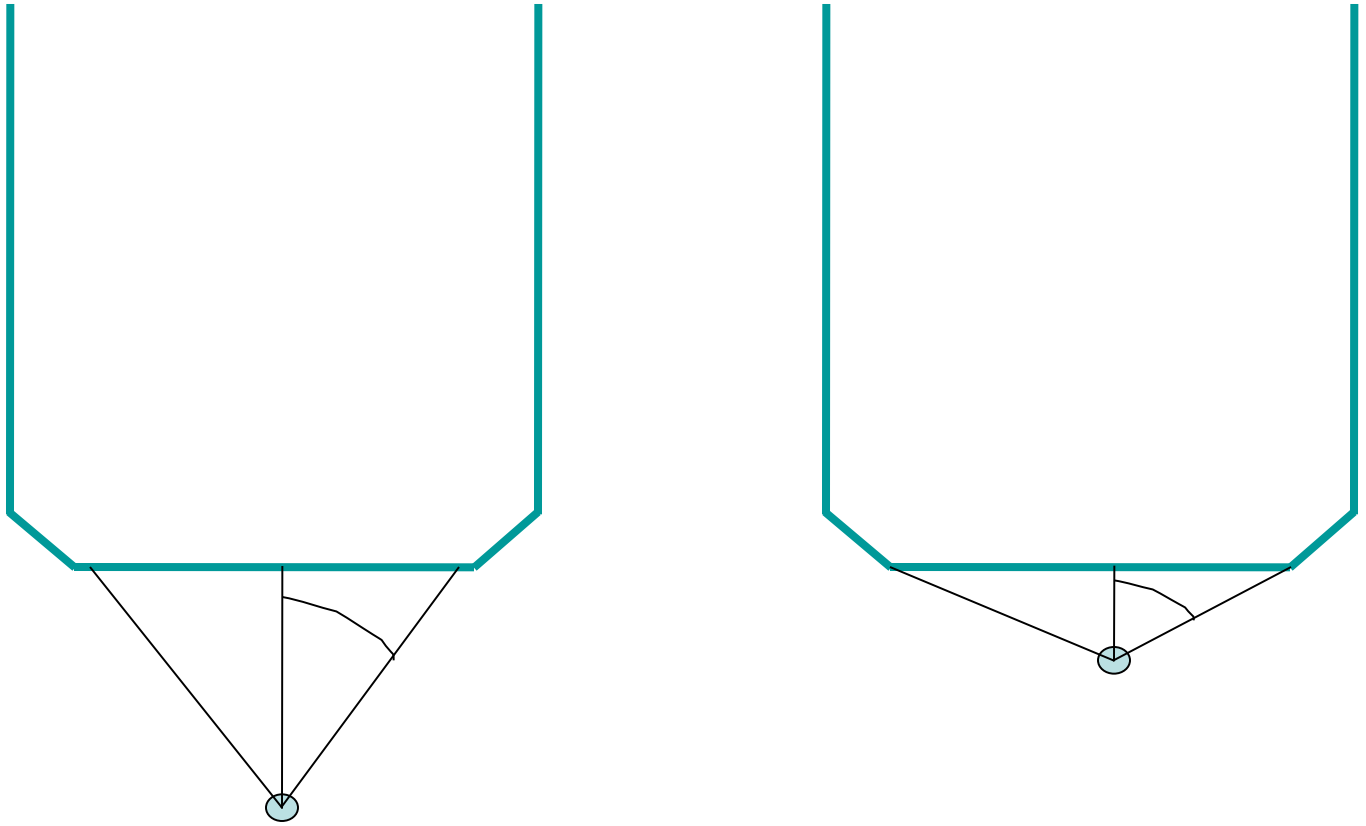
Numerical Aperture (NA)



NA=(sine of half angle) X (refractive index of immersion medium)

Air=1 oil =1.51

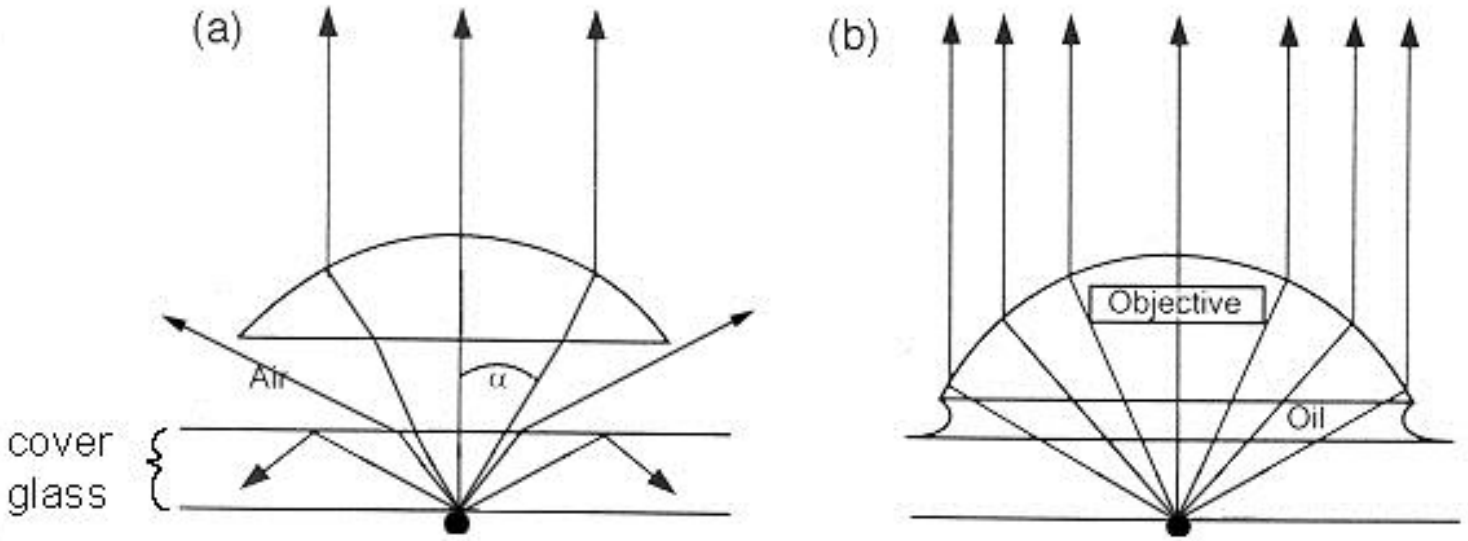




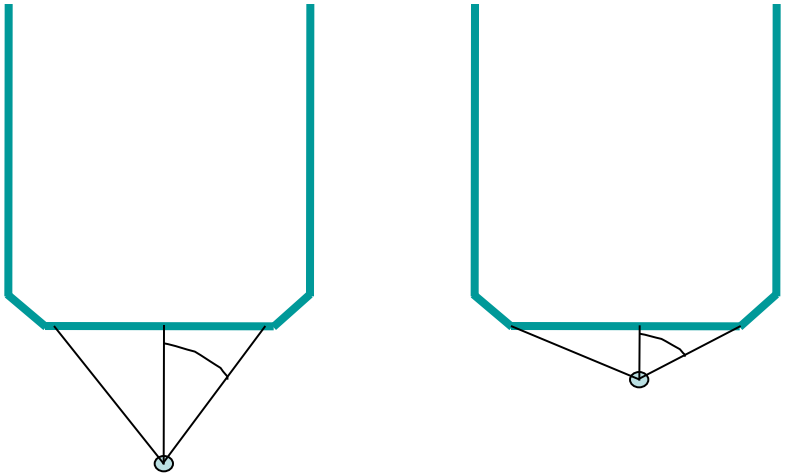
NA=(sine of half angle) X (refractive index of immersion medium)

High NA = high light gathering

High NA = high resolution



Influence of the refractive index of the immersion medium [(a) air; (b) oil] on the theoretical maximum of the numerical aperture.



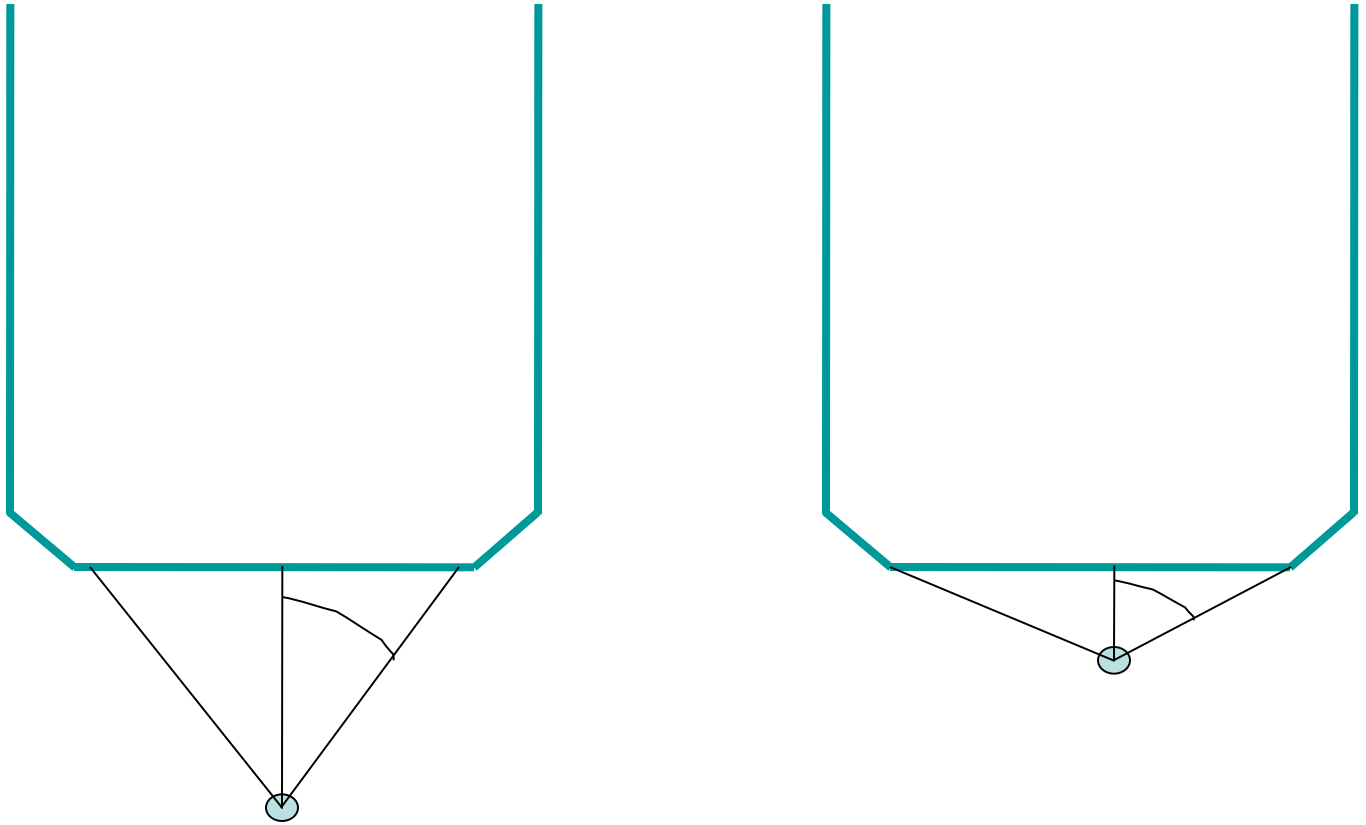
High NA = high resolution

XY resolution = 0.61 wavelength / NA

Z resolution = 2 X wavelength X refractive index medium / NA^2

resolution means separation

This is different from see or detect.



High NA = high resolution

demonstration

Resolution means separation

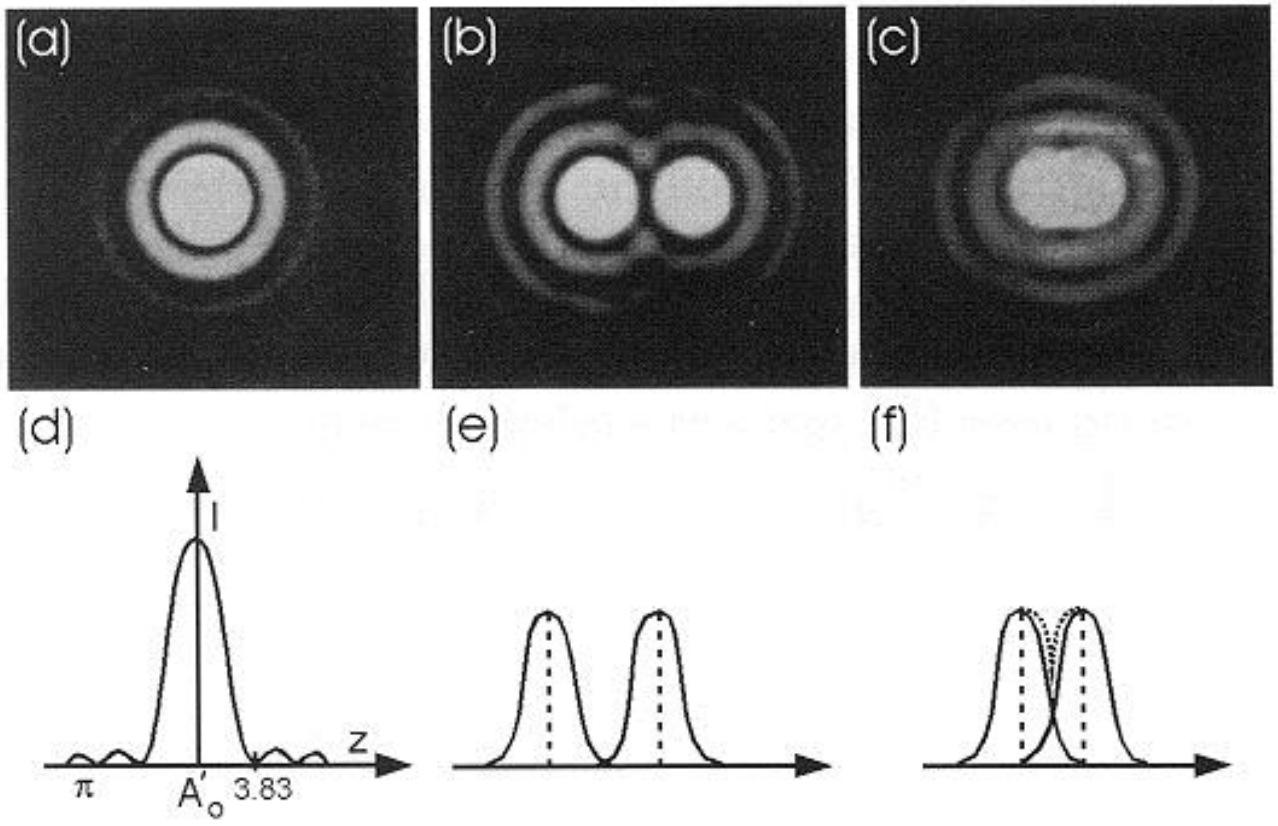


Figure 2.3. Airy disc and intensity distribution in the diffraction pattern. (a, d) Airy disc and intensity distribution. (b, e) Two Airy discs and their intensity distributions. The separation of the two Airy discs is greater than their radii, and they are resolvable. (c, f) Two Airy discs and their intensity distributions under conditions where the centre-to-centre distance equals the radius (Rayleigh criterion). Adapted with permission of Plenum Press, New York, from Inoué, S. (1986) *Video Microscopy*.

X-Y resolution (theoretical!)

Rayleigh d = 0.61wavelength (in air) / NA

Delta Vision

magnification	NA	Camera Pixel size (nm)	λ	calculated resolution (nm)	2 pixels equal (nm)
60 X	1.4	170	550 nm	240	340
40 X	1.3	260	550 nm	258	520
20 X	0.75	520	550 nm	447	1040
10 X	0.3	1010	550 nm	1118	2020

(Bin 1, no Optivar)

Z resolution

d = 2 (wavelength)(refractive index medium) / NA ^{2}

magnification	NA	λ	calculated resolution (nm)
60 X	1.4	550 nm	847
40 X	1.3	550 nm	983
20 X	0.75	550 nm	2,953
10 X	0.3	550 nm	18,456
4 X	0.13	550 nm	98,284

Electron microscope

A 50,000 volt electron has a wavelength of 0.0055nm.

The numerical aperture calculation:
1 degree aperture
Sine of 1^{o} = 0.017
Index of refraction (vacuum) = 1

Rayleigh d = 0.61wavelength (in air) / NA

Resolution is 0.20nm.

NA and magnification effects on image brightness

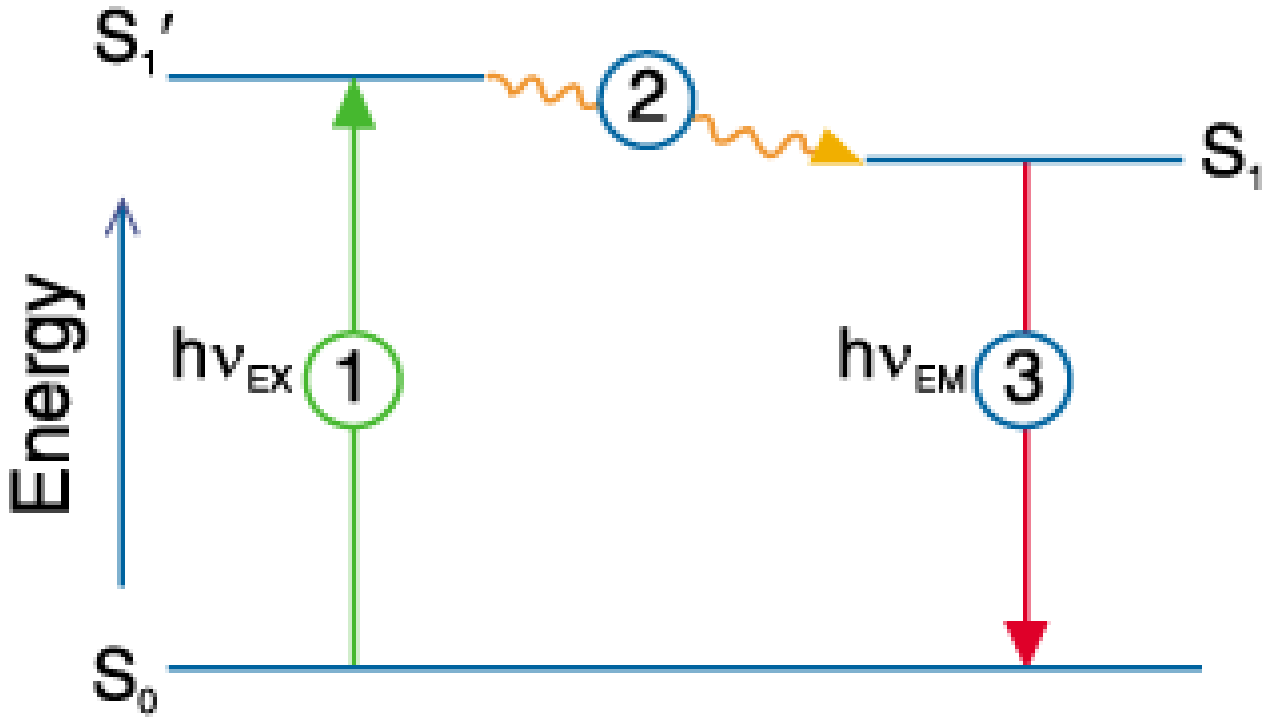
Brightness increases $(NA)^4$ (epifluorescence)

Brightness decreases $1/magnification^2$

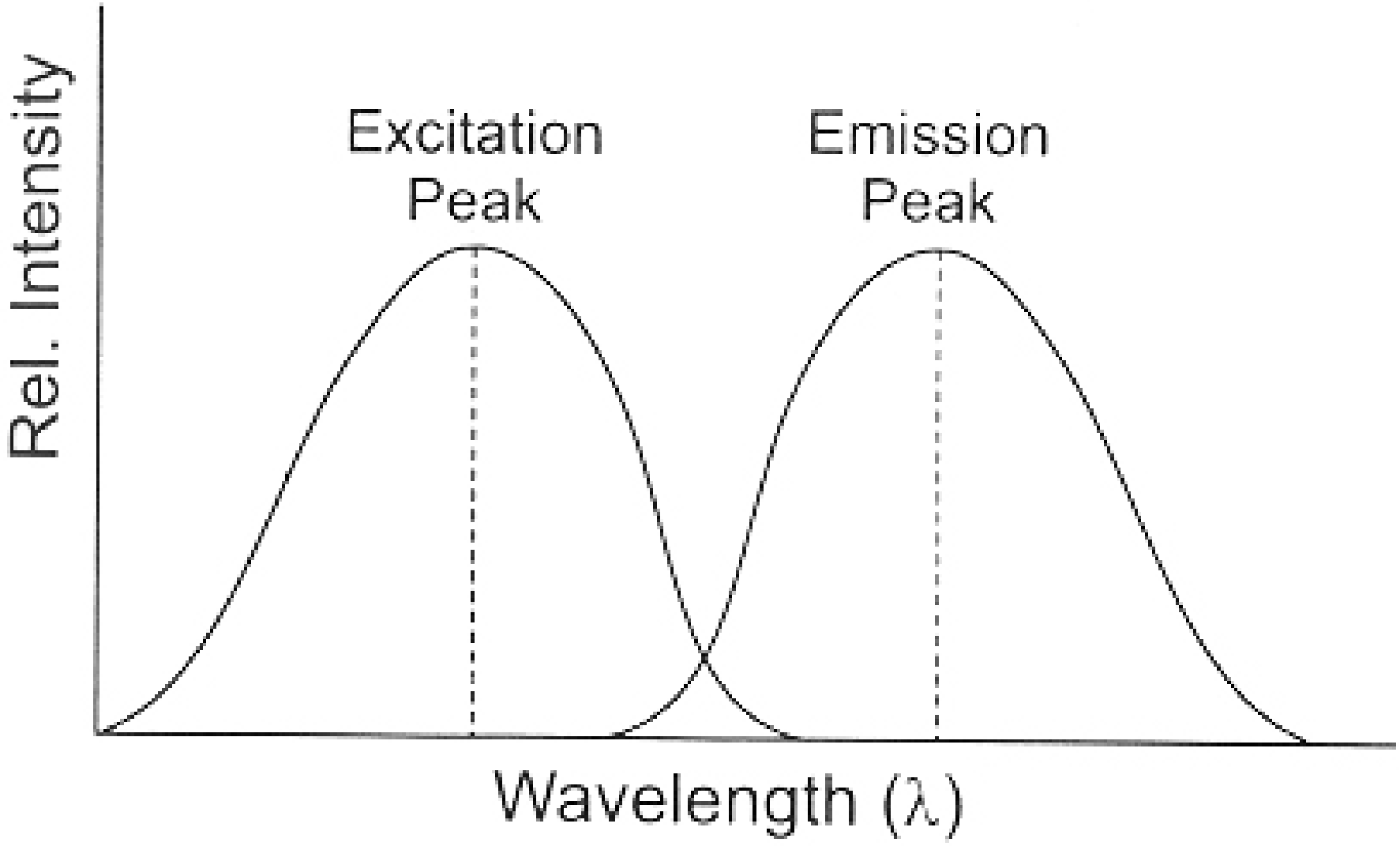
		relative brightness
60 X	**1.4**	**1.0**
40 X	**1.3**	**1.7**
20 X	**0.75**	**0.7**
10 X	**0.3**	**0.1**

Fluorescence microscopy

Fluorescence
demo

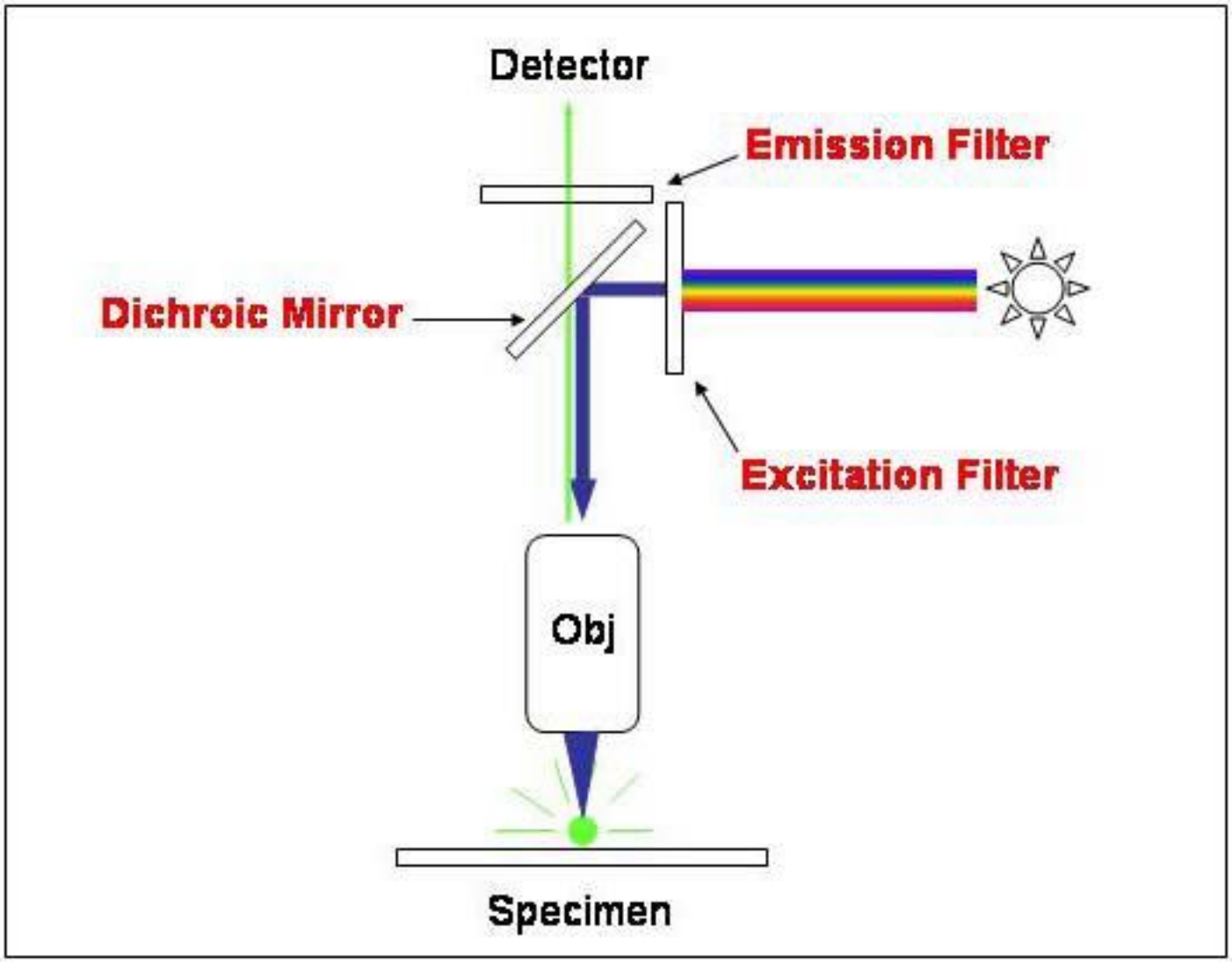


Jablonski diagram


Rel. Intensity
Excitation Peak
Emission Peak
Wavelength (λ)

Quantum Yield

$$Q = \frac{\text{Photons emitted}}{\text{Photons absorbed}}$$


Detector
Emission Filter
Dichroic Mirror
Excitation Filter
Obj
Specimen

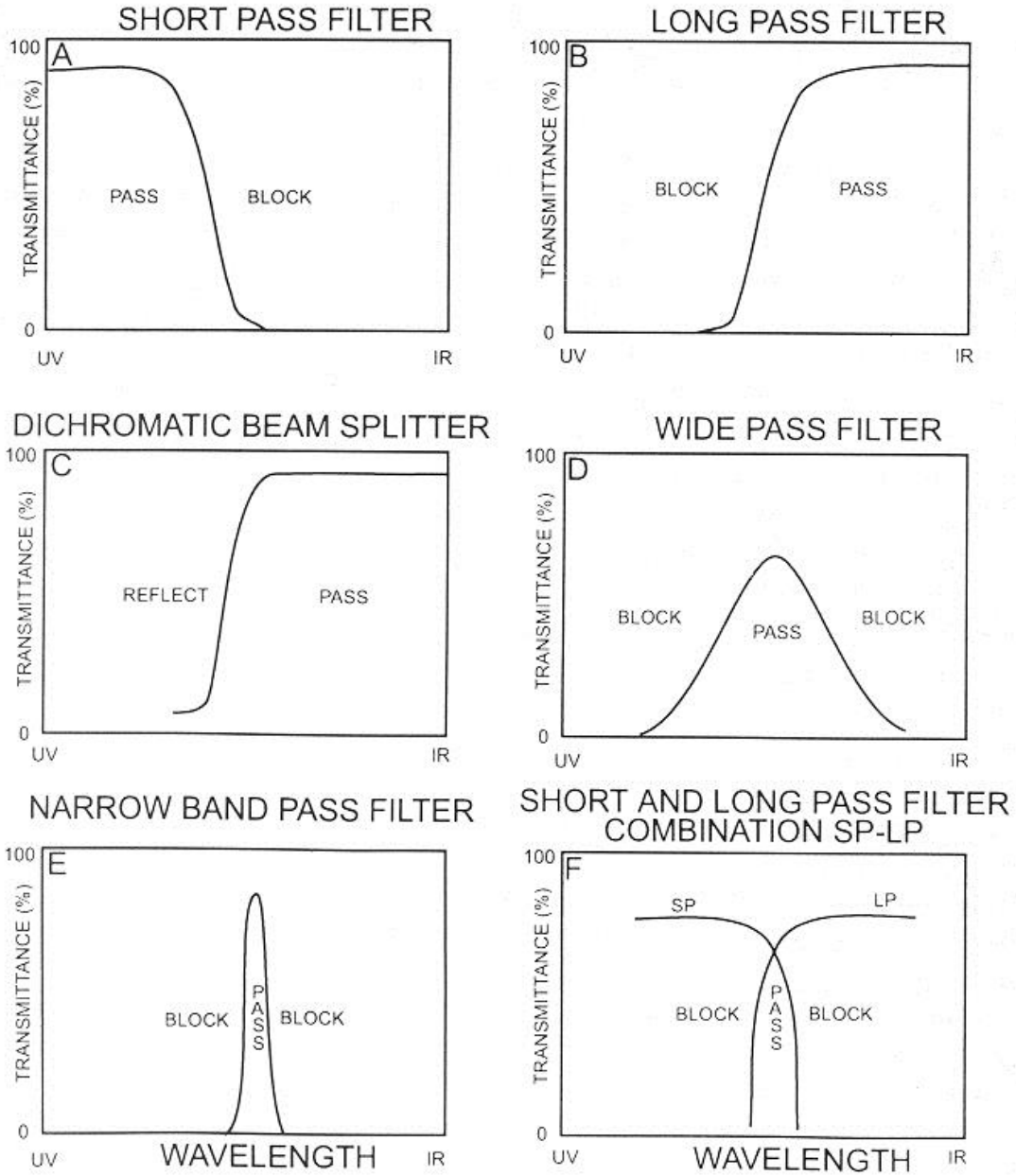


Figure 2.8. Various types of filters used in fluorescence microscopy. See text for details. Adapted with permission of Olympus-America, New York, from Abramowitz, M. (1993) *Fluorescence Microscopy: The Essentials.*

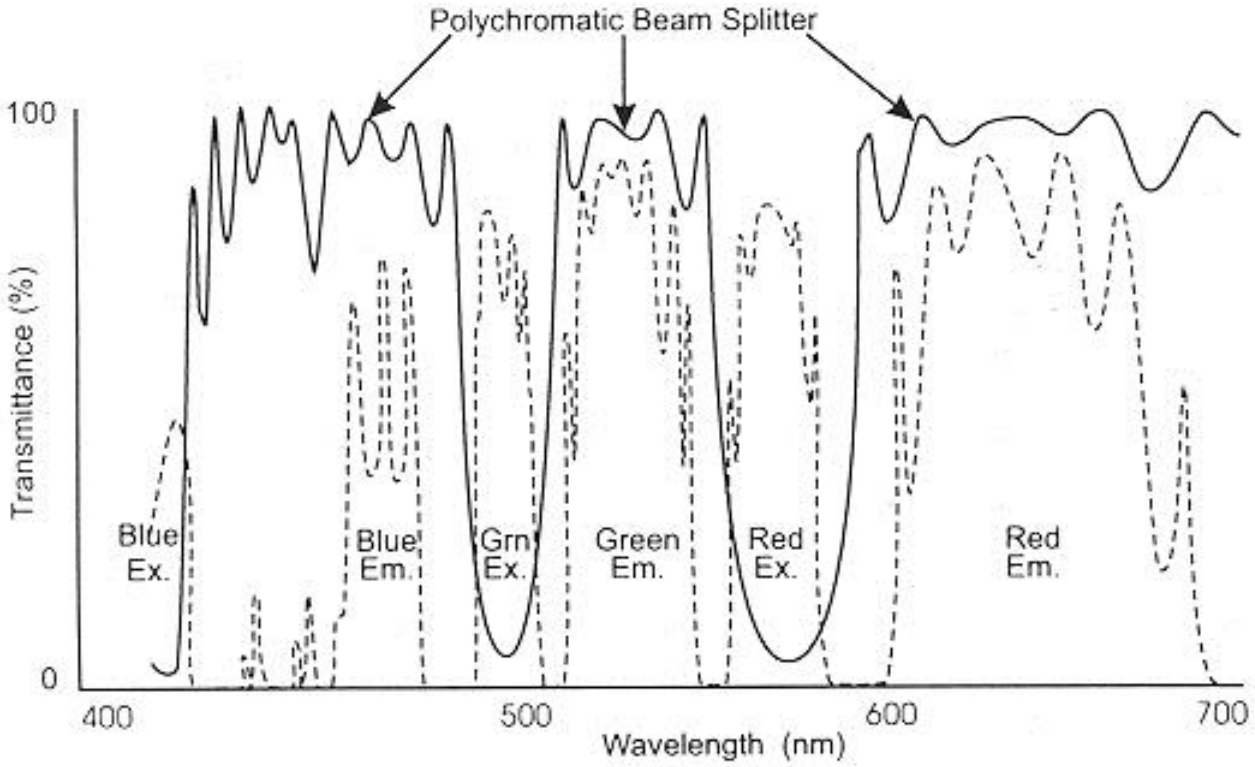
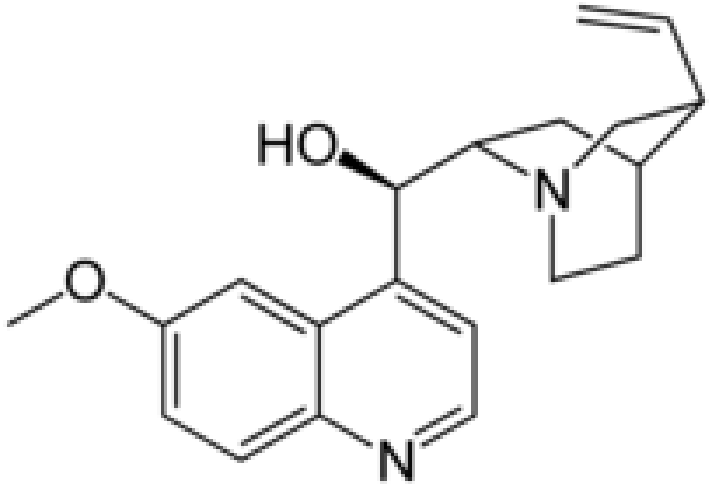


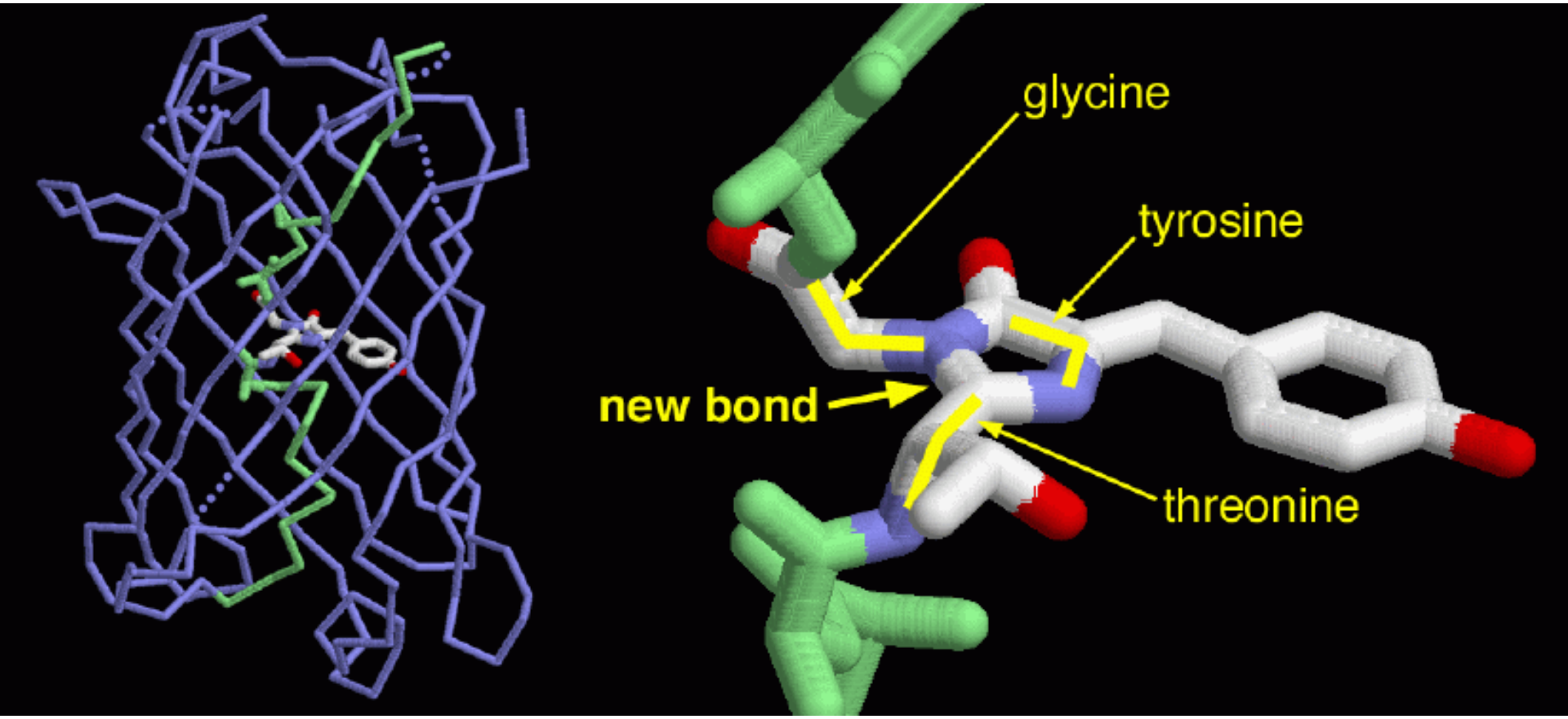
Figure 2.10. Spectra of a triple-band filter set designed for the dyes DAPI, FITC and Texas red. Adapted with permission of Chroma Technology Corp. from Reichman, J. *Handbook of Optical Filters for Fluorescence Microscopy*, P/N 61002 (1994).

Fluorescent structures



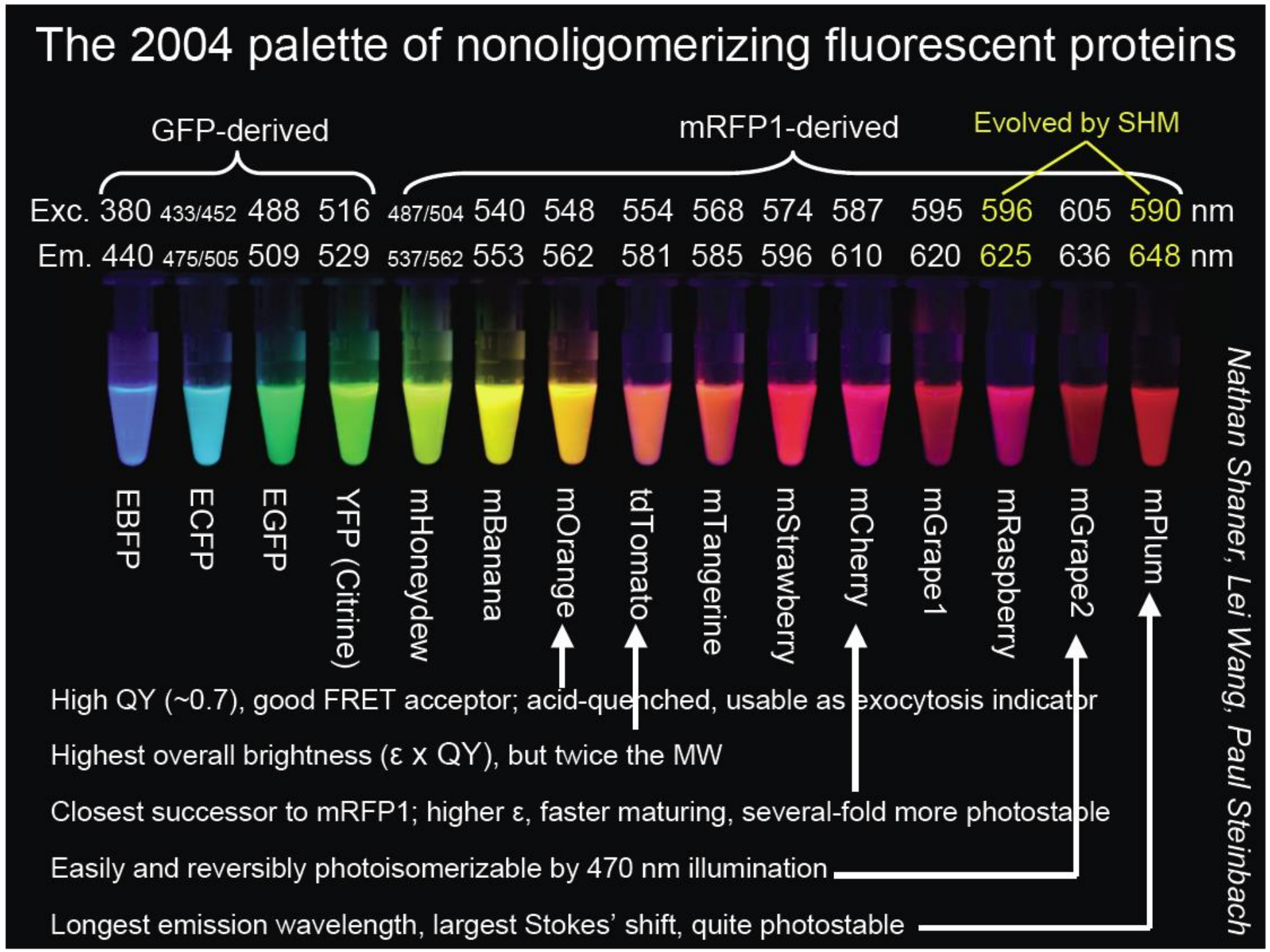
Quinine

- Anti malaria
- Anti fever
- tasty
- And fluorescent!



GFP

The 2004 palette of nonoligomerizing fluorescent proteins



Fluorescent Protein Properties

Protein (Acronym)	Excitation Maximum (nm)	Emission Maximum (nm)	Molar Extinction Coefficient	Quantum Yield	*in vivo* Structure	Relative Brightness (% of EGFP)
GFP (wt)	395/475	509	21,000	0.77	Monomer*	48
Green Fluorescent Proteins						
EGFP	484	507	56,000	0.60	Monomer*	100
Emerald	487	509	57,500	0.68	Monomer*	116
Superfolder GFP	485	510	83,300	0.65	Monomer*	160
Azami Green	492	505	55,000	0.74	Monomer	121
mWasabi	493	509	70,000	0.80	Monomer	167
TagGFP	482	505	58,200	0.59	Monomer*	110
TurboGFP	482	502	70,000	0.53	Dimer	102
AcGFP	480	505	50,000	0.55	Monomer*	82
ZsGreen	493	505	43,000	0.91	Tetramer	117
T-Sapphire	399	511	44,000	0.60	Monomer*	79
Blue Fluorescent Proteins						
EBFP	383	445	29,000	0.31	Monomer*	27
EBFP2	383	448	32,000	0.56	Monomer*	53
Azurite	384	450	26,200	0.55	Monomer*	43
mTagBFP	399	456	52,000	0.63	Monomer	98
Cyan Fluorescent Proteins						
ECFP	439	476	32,500	0.40	Monomer*	39
mECFP	433	475	32,500	0.40	Monomer	39
Cerulean	433	475	43,000	0.62	Monomer*	79
CyPet	435	477	35,000	0.51	Monomer*	53
AmCyan1	458	489	44,000	0.24	Tetramer	31
Midori-Ishi Cyan	472	495	27,300	0.90	Dimer	73
TagCFP	458	480	37,000	0.57	Monomer	63
mTFP1 (Teal)	462	492	64,000	0.85	Monomer	162

Yellow Fluorescent Proteins						
EYFP	514	527	83,400	0.61	Monomer*	151
Topaz	514	527	94,500	0.60	Monomer*	169
Venus	515	528	92,200	0.57	Monomer*	156
mCitrine	516	529	77,000	0.76	Monomer	174
YPet	517	530	104,000	0.77	Monomer*	238
TagYFP	508	524	64,000	0.60	Monomer	118
PhiYFP	525	537	124,000	0.39	Monomer*	144
ZsYellow1	529	539	20,200	0.42	Tetramer	25
mBanana	540	553	6,000	0.7	Monomer	13
Orange Fluorescent Proteins						
Kusabira Orange	548	559	51,600	0.60	Monomer	92
Kusabira Orange2	551	565	63,800	0.62	Monomer	118
mOrange	548	562	71,000	0.69	Monomer	146
mOrange2	549	565	58,000	0.60	Monomer	104
dTomato	554	581	69,000	0.69	Dimer	142
dTomato-Tandem	554	581	138,000	0.69	Monomer	283
TagRFP	555	584	100,000	0.48	Monomer	142
TagRFP-T	555	584	81,000	0.41	Monomer	99
DsRed	558	583	75,000	0.79	Tetramer	176
DsRed2	563	582	43,800	0.55	Tetramer	72
DsRed-Express (T1)	555	584	38,000	0.51	Tetramer	58
DsRed-Monomer	556	586	35,000	0.10	Monomer	10
mTangerine	568	585	38,000	0.30	Monomer	34

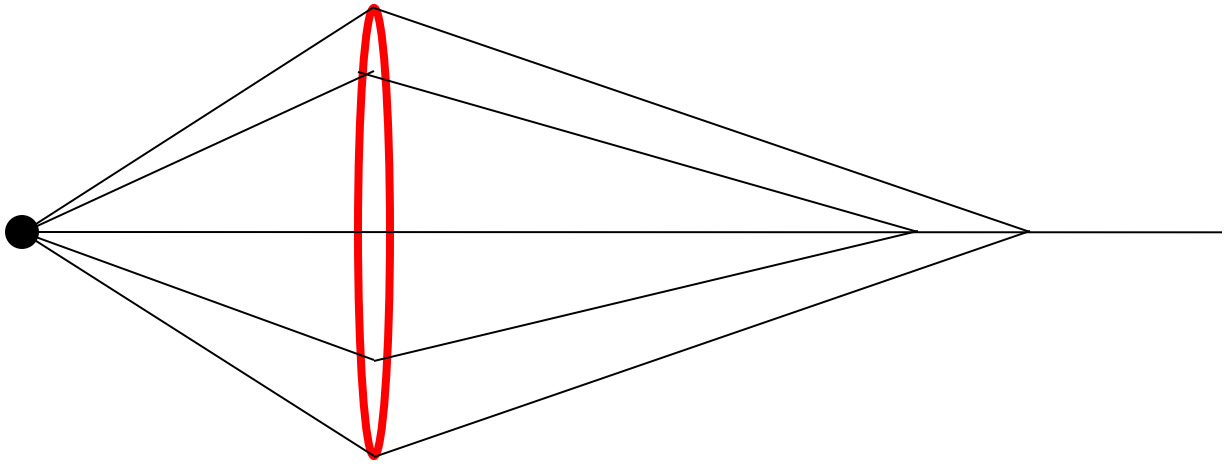
Red Fluorescent Proteins						
mRuby	558	605	112,000	0.35	Monomer	117
mApple	568	592	75,000	0.49	Monomer	109
mStrawberry	574	596	90,000	0.29	Monomer	78
AsRed2	576	592	56,200	0.05	Tetramer	8
mRFP1	584	607	50,000	0.25	Monomer	37
JRed	584	610	44,000	0.20	Dimer	26
mCherry	587	610	72,000	0.22	Monomer	47
HcRed1	588	618	20,000	0.015	Dimer	1
mRaspberry	598	625	86,000	0.15	Monomer	38
dKeima-Tandem	440	620	28,800	0.24	Monomer	21
HcRed-Tandem	590	637	160,000	0.04	Monomer	19
mPlum	590	649	41,000	0.10	Monomer	12
AQ143	595	655	90,000	0.04	Tetramer	11

* Weak Dimer

A Comparison of methods of microscopy

But first a digression

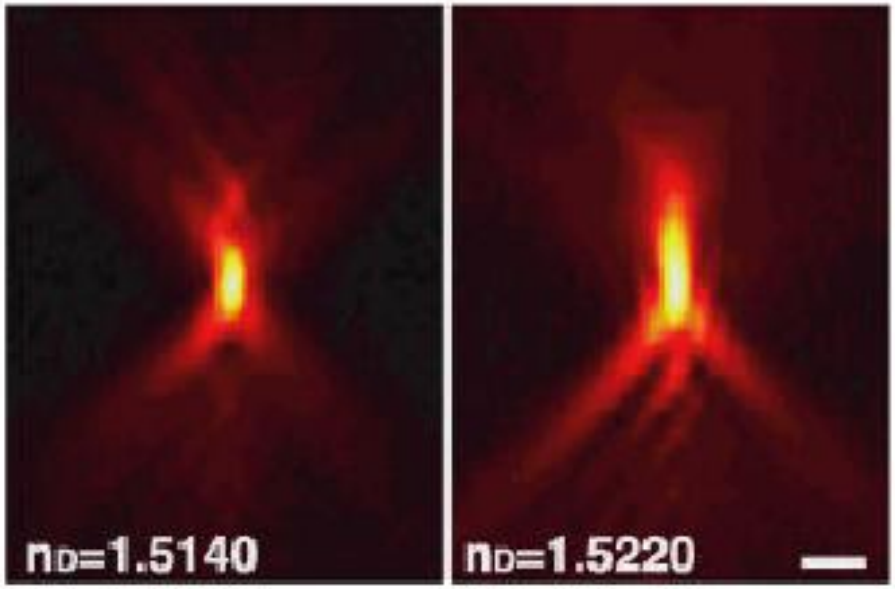
Spherical Aberration



Light going near the edges of the lens focuses at

A different plane than light going near the center

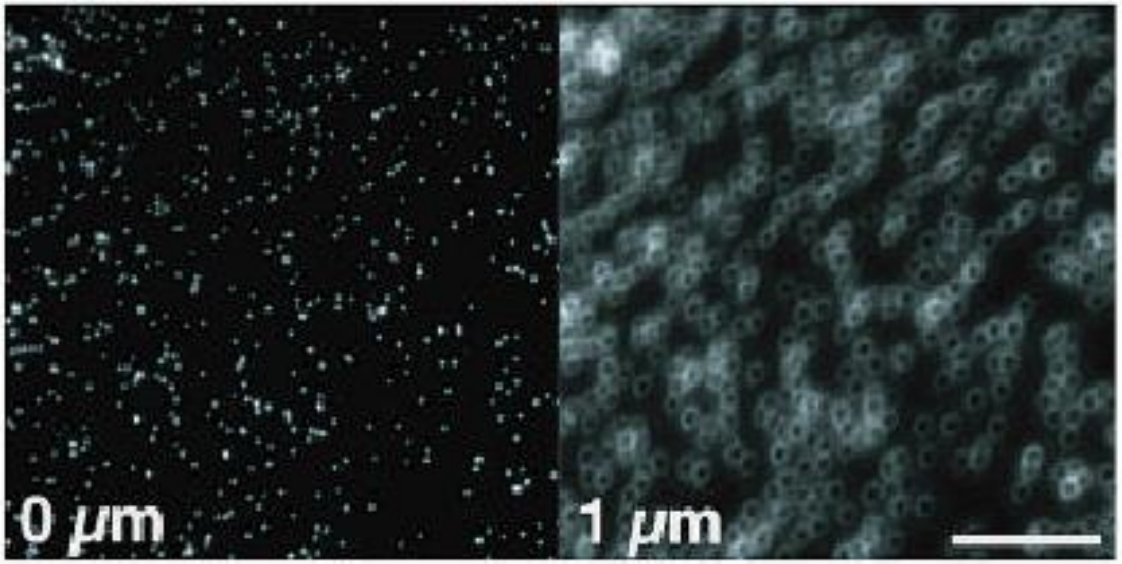
A.



Rings on one side
Blur on the other.

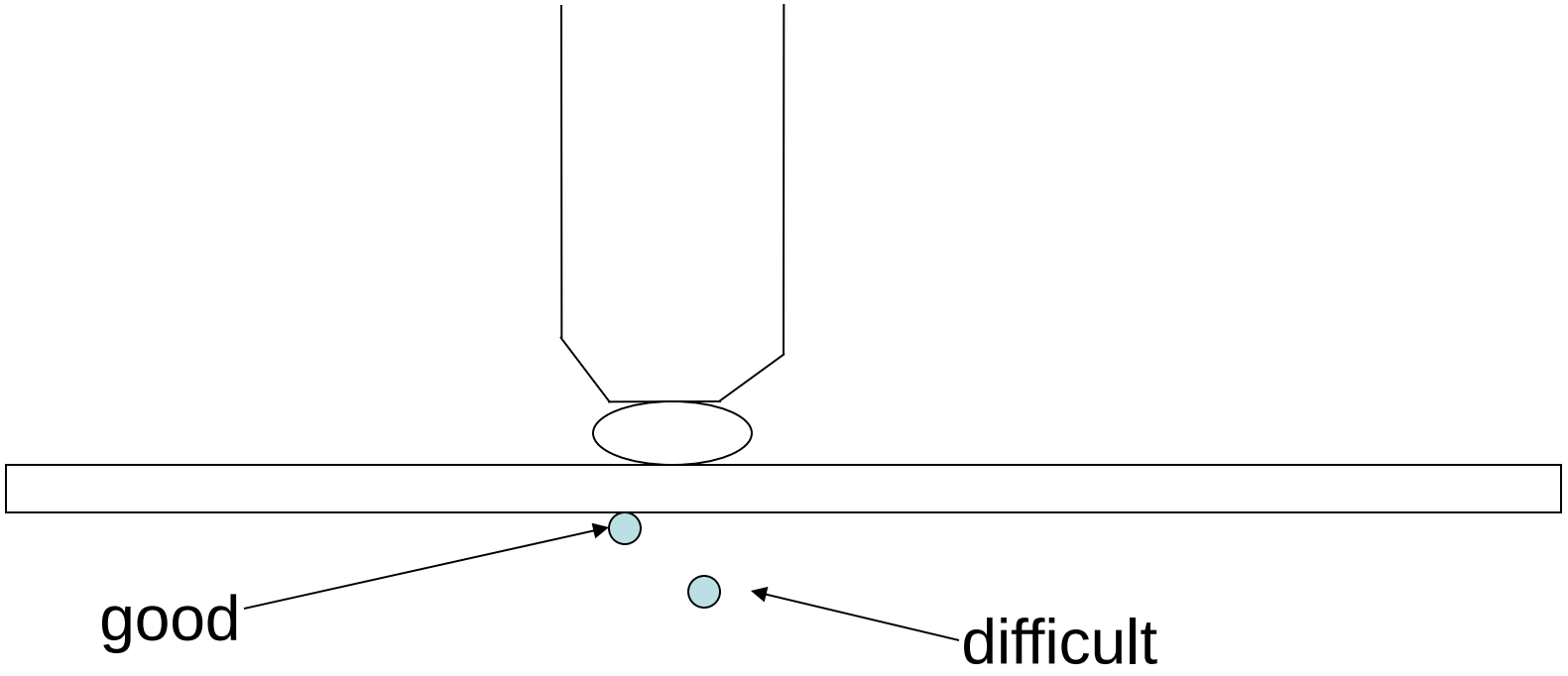
Spherical Aberration

B.



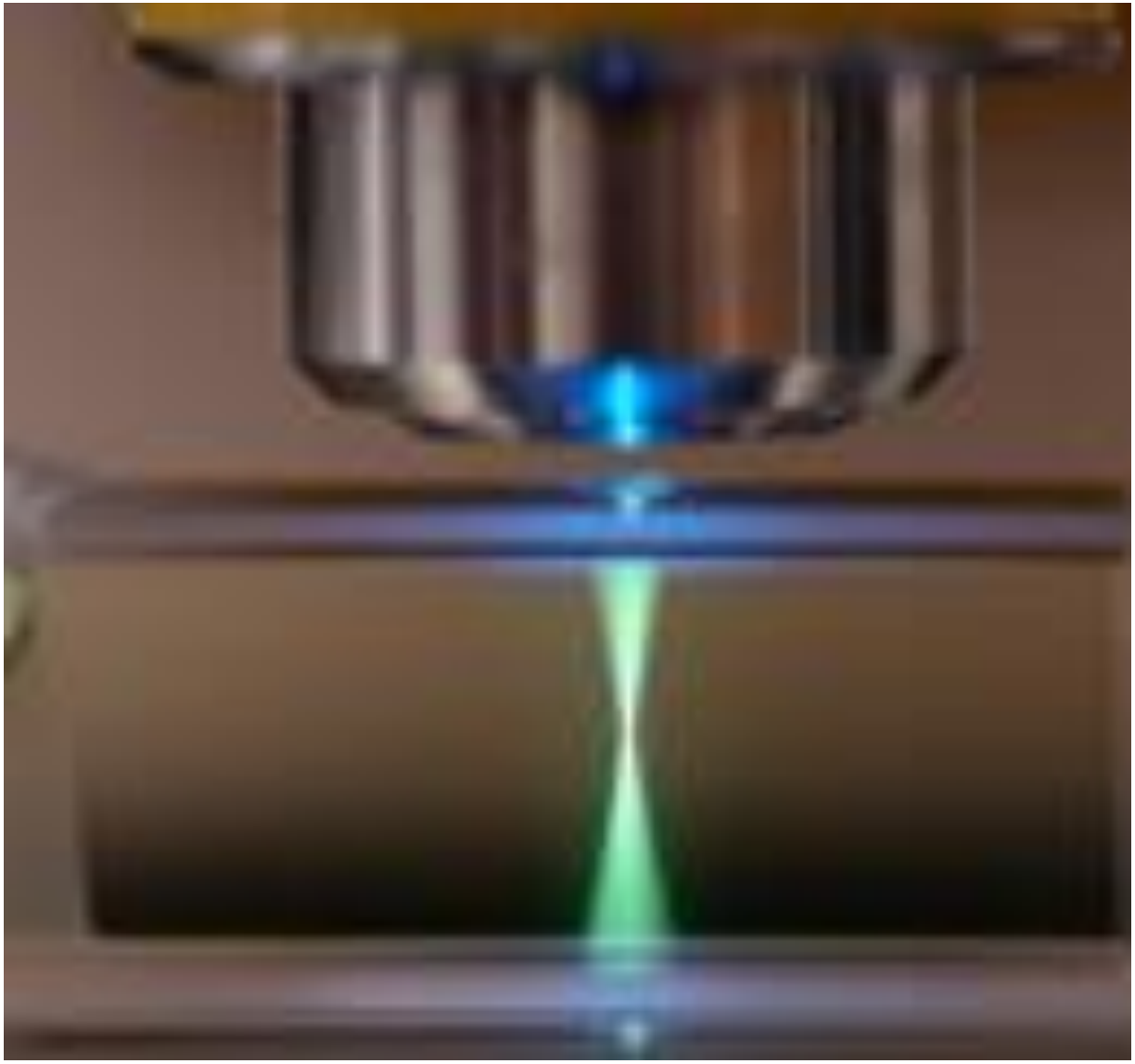
You can see this easily.

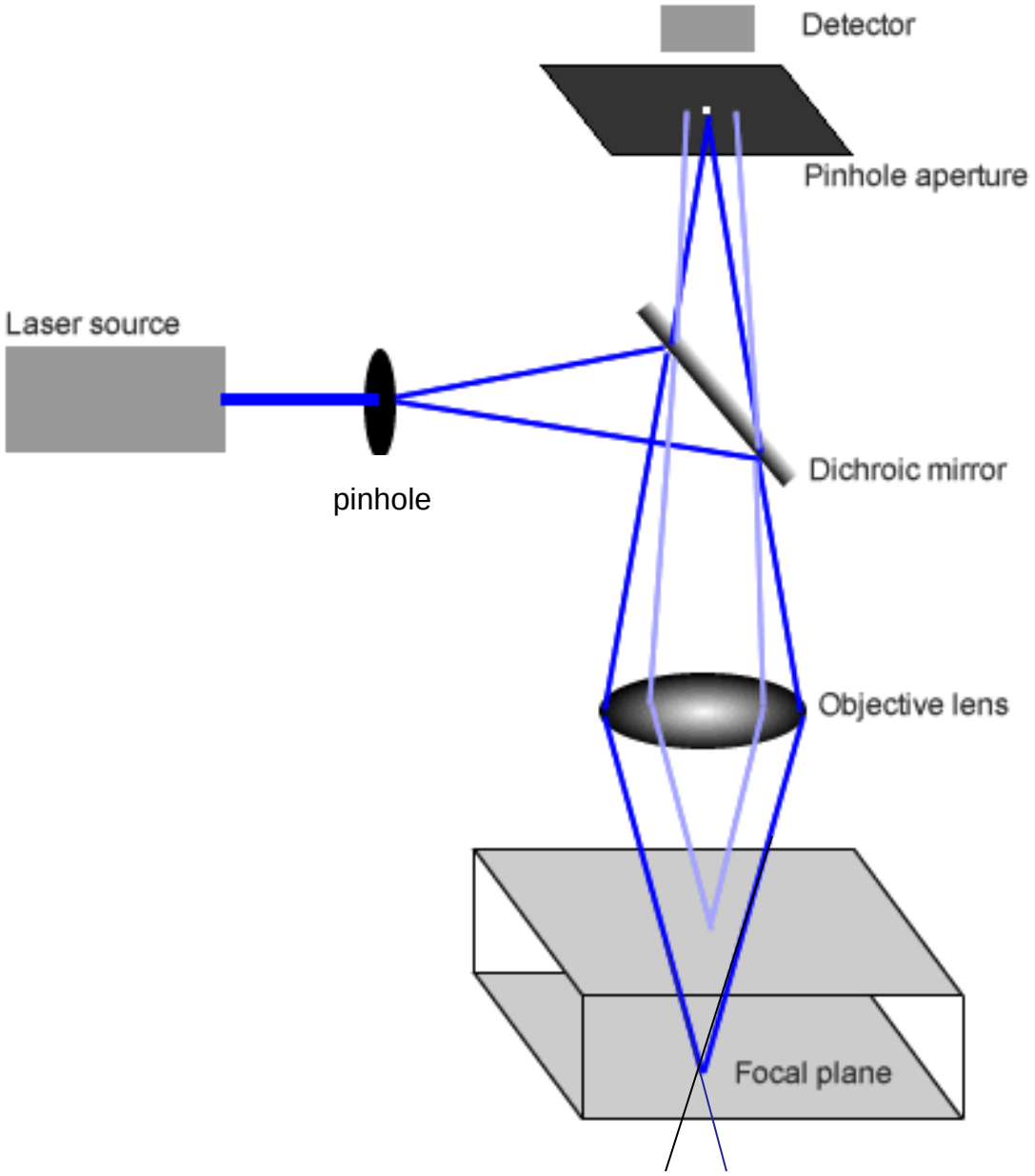
What causes Spherical Aberration?



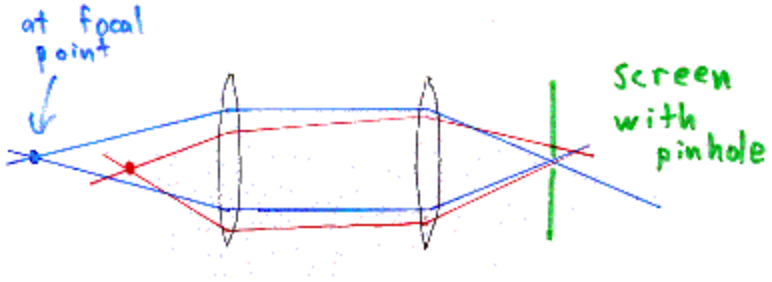
Incorrect refractive index

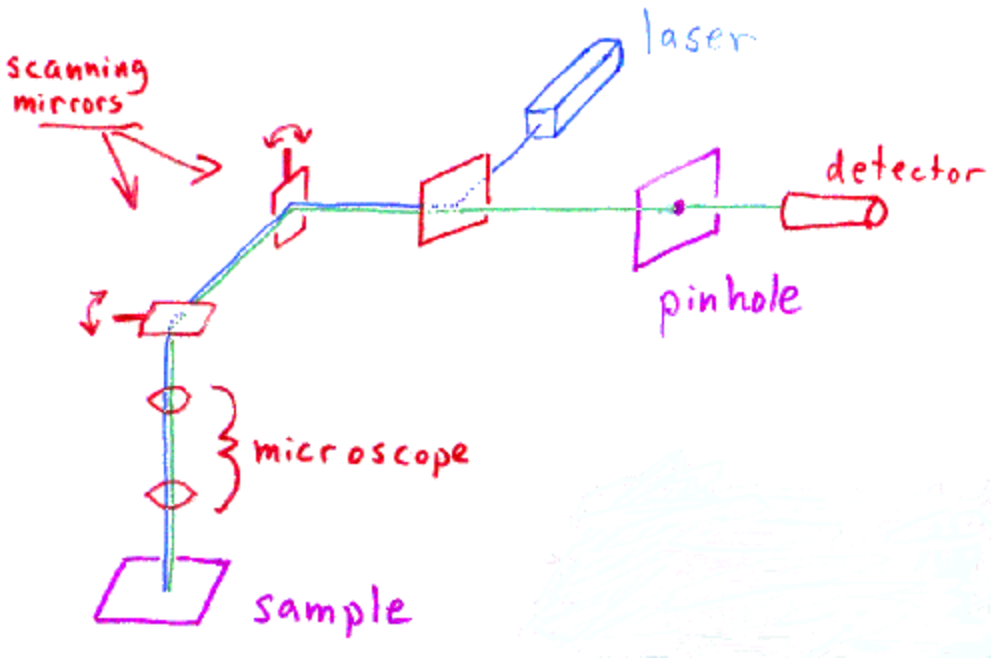
Confocal microscopy





A confocal microscope

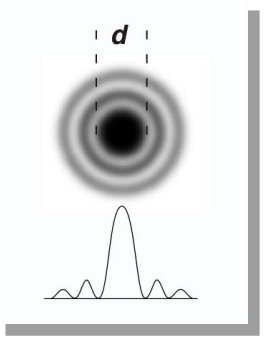


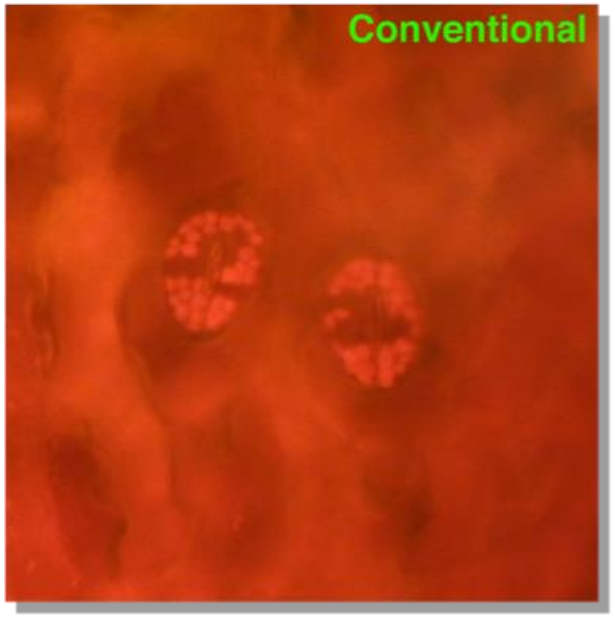


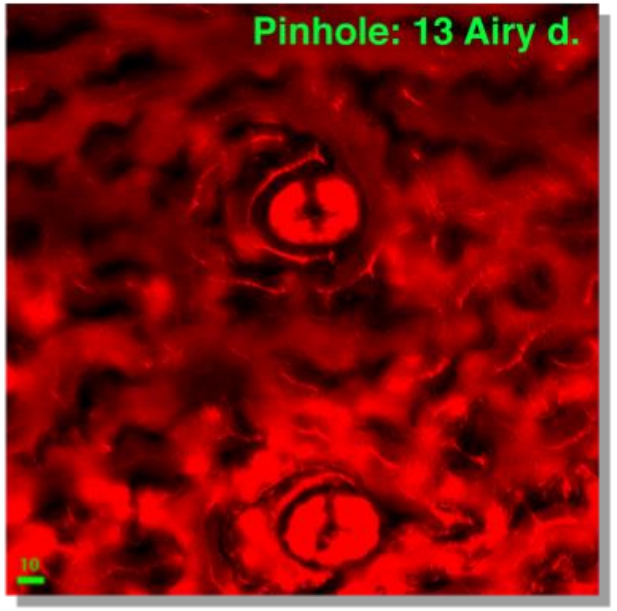
A confocal microscope

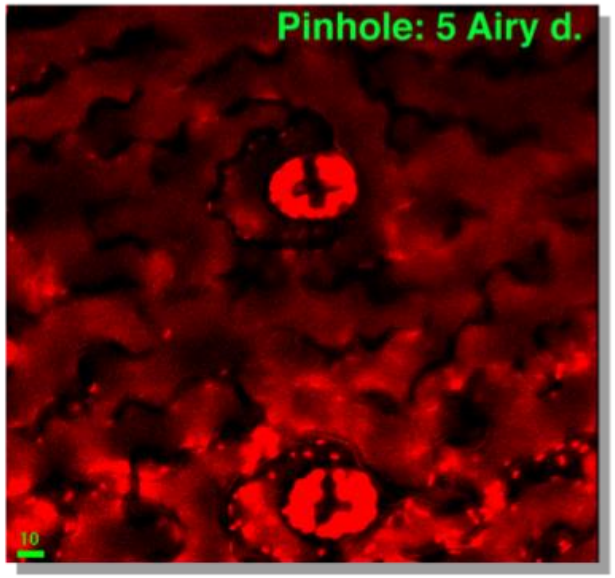
What happens when you open or close the pinhole?

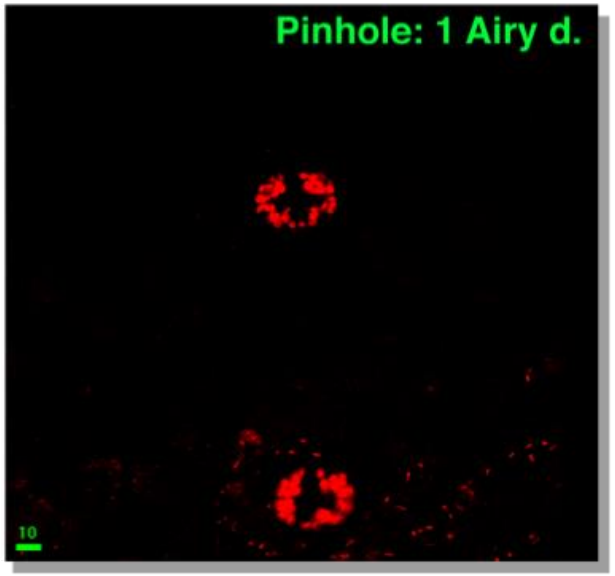
Pinhole Diameter and Confocal slice thickness











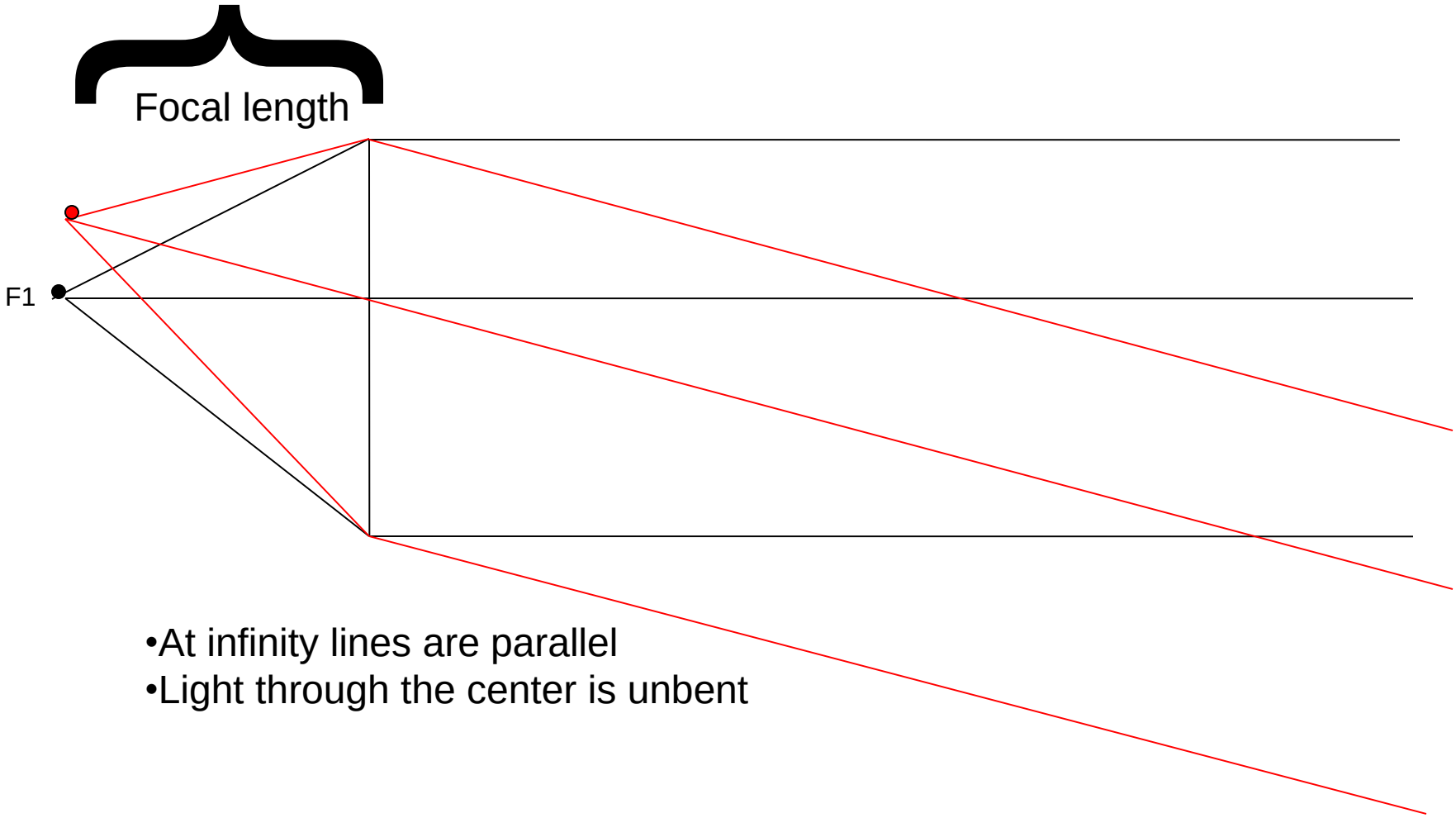
Test:
Your fluorescence is too dim, what can you do?

Test:
Your sample is bleaching, what can you do?

Optional quiz question:

When should you use a confocal microscope?

Ray tracing



- At infinity lines are parallel
- Light through the center is unbent