The Abdus Salam
International Centre
for Theoretical Physics

Basic Concepts of Microscopy

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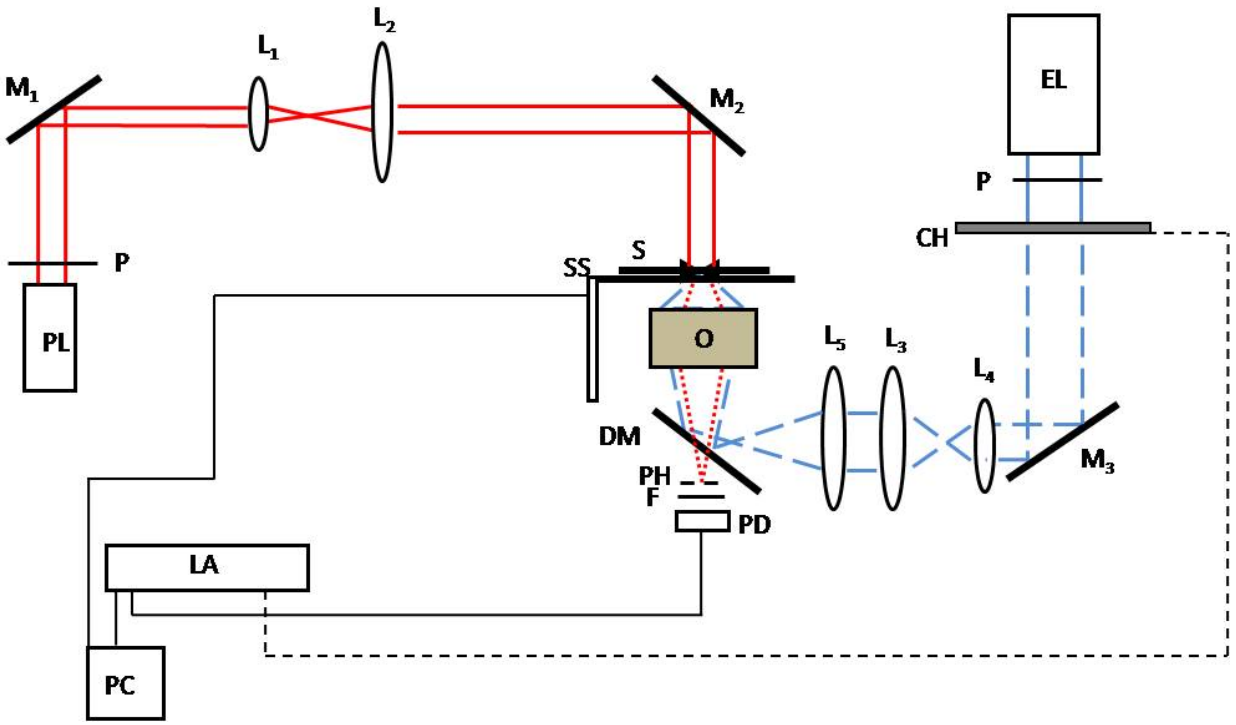
Light: a Bridge between Earth and Space: Preparatory School

- Introduction
- Lens formula, Image formation and Magnification
- Resolution and lens defects
- Basic components and their functions
- Collimators
- Specialized Microscopy Techniques
- Typical examples of applications

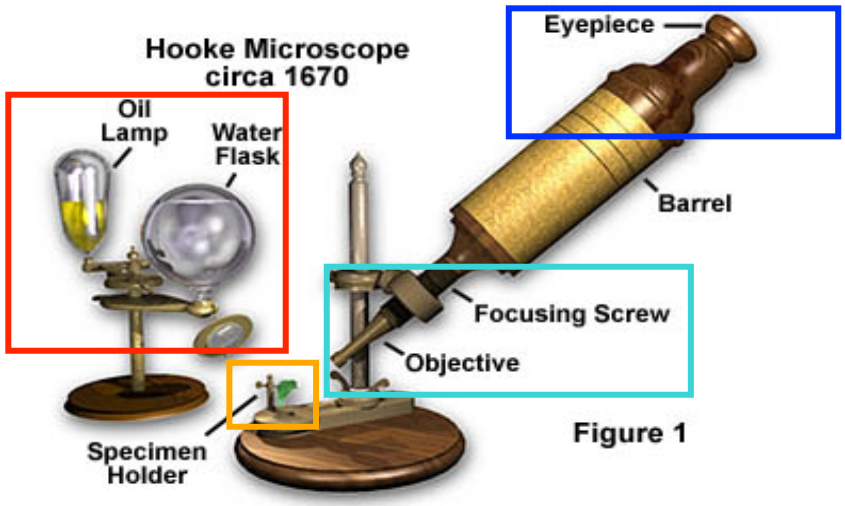


Schematic diagram of the TLM, S: sample; SS: sample stage 3-D control; M_1, M_2 and M_3: mirrors; CH: chopper; DM: dichroic mirror; P: linear polarizer; L_1, L_2, L_3; L_4 and L_5: lenses; O: focusing objective lens; PH: pinhole; F: interference filter at 632.8 nm; PD: photodiode; LA: lock-in amplifier; PC: personal computer; EL: excitation laser; PL: probe laser.

Similar to confocal optical (fluorescence, Raman) microscope, and optical tweezers

Microscope Components

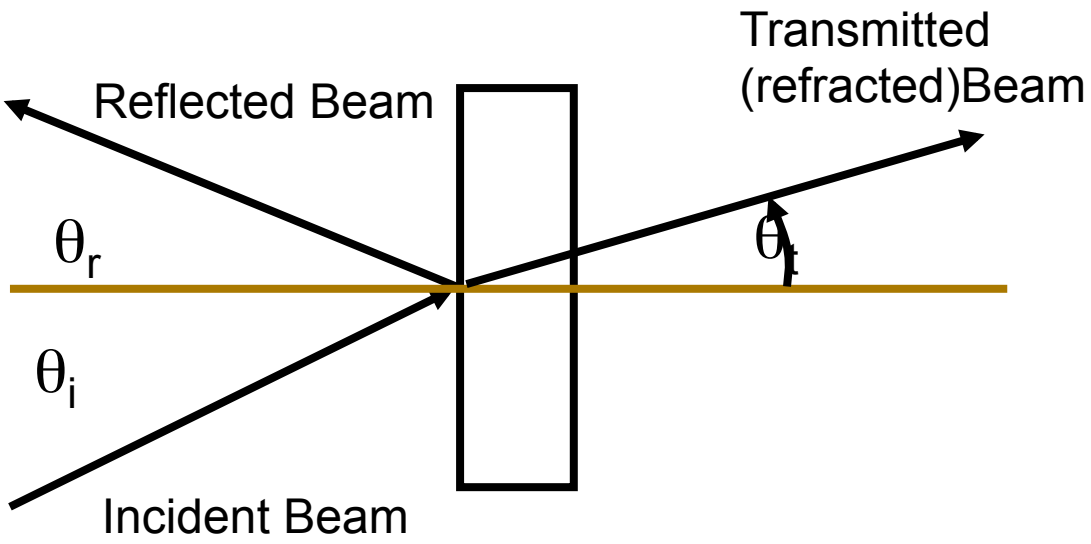
- Ocular
- Objectives
- Condenser
- Numerical Aperture
- Refractive Index
- Aberrations
- Optical Filters



Basic components and their functions

(1) **Eyepiece (ocular lens)**
(2) Revolving nose piece (to hold multiple objective lenses)
(3) **Objective lenses**
(4) And (5) **Focus knobs**
    (4) Coarse adjustment
    (5) Fine adjustment
(6) **Stage** (to hold the specimen)
(7) **Light source** (lamp)
(8) **Condenser lens** and diaphragm
(9) Mechanical stage (move the specimen on two horizontal axes for positioning the specimen)

Reflection and Refraction

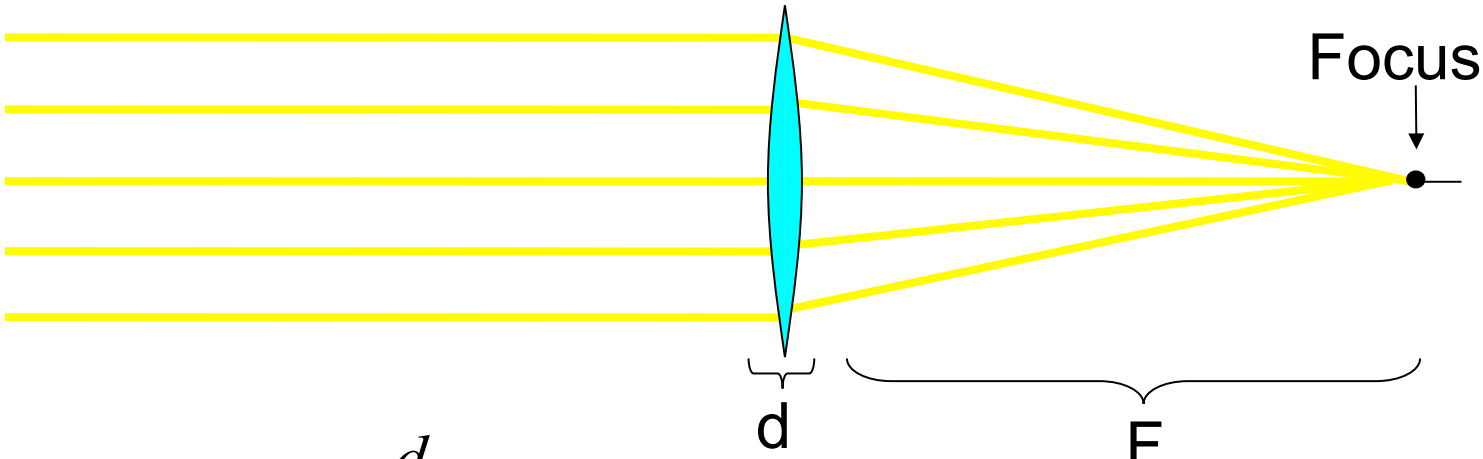


- **Snell's Law**: The angle of reflection ($Ø_r$) is equal to the angle of incidence ($Ø_i$) regardless of the surface material
- The angle of the transmitted beam ($Ø_t$) is dependent upon the composition of the material

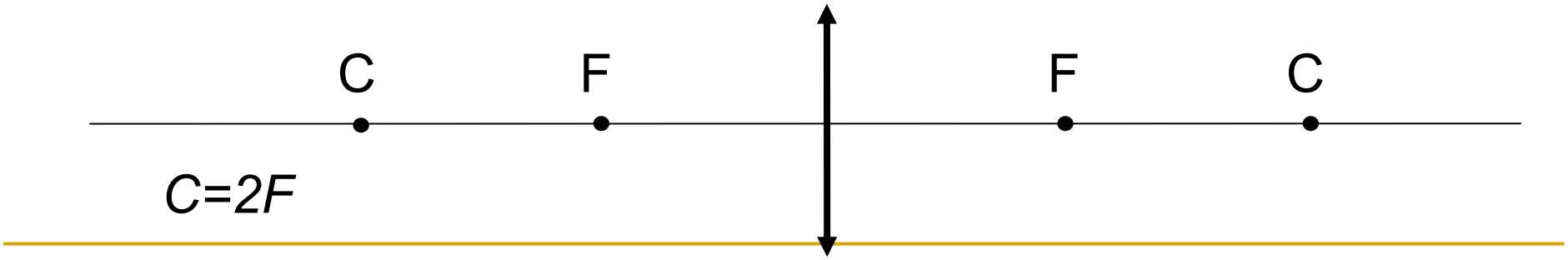
$$n_1 \sin Ø_i = n_2 \sin Ø_t$$

The velocity of light in a material of refractive index n is c/n

Optics of a thin lens (1)

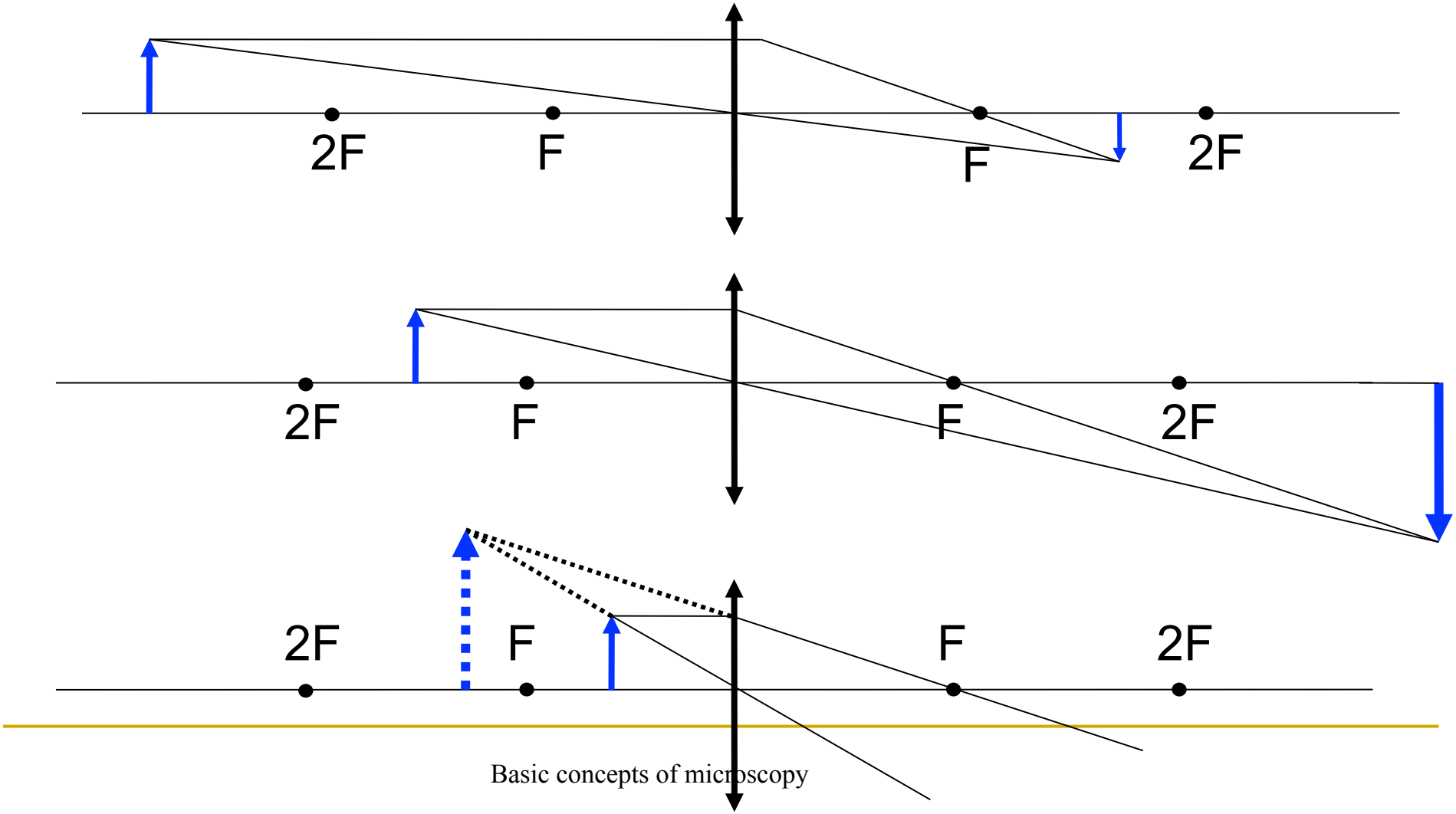


Thin Lens: $\frac{d}{F} \to 0$

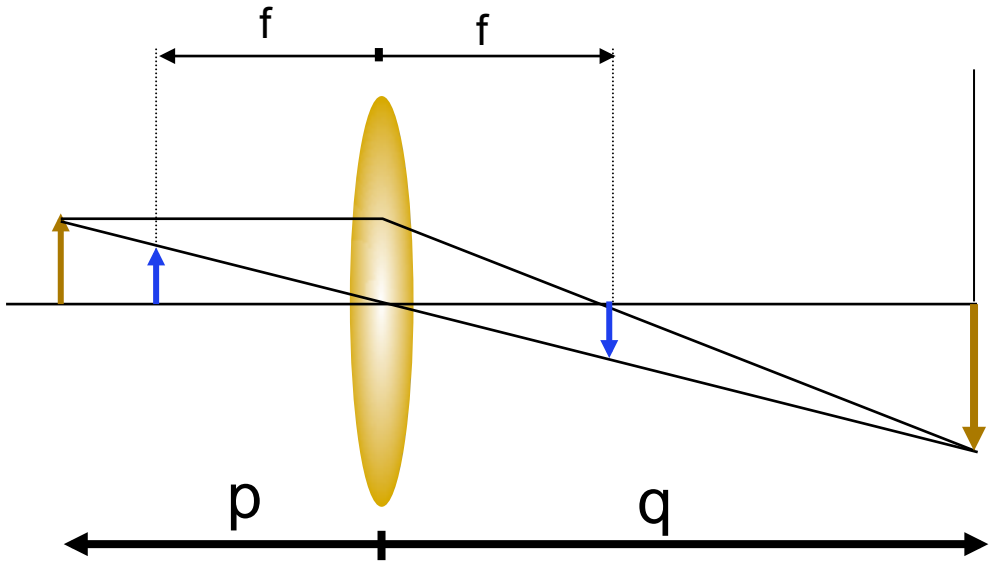


Optics of a thin lens (2)

- Three different scenarios:



Properties of thin Lenses



$$\frac{1}{p} + \frac{1}{q} = \frac{1}{f}$$

$$\text{Magnification} = \frac{q}{p}$$

The Concept of Magnification

Magnification of the Microscope

- M Microscope = M Objective X M Eyepiece X M Intermediate Factor

M = Magnification

- Example: Objective = 60 x

Eyepiece = 10 x

Intermediate Factor = 1 x

Overall M = 600 x

The characteristics of objectives

Figure 1

Objectives configurations

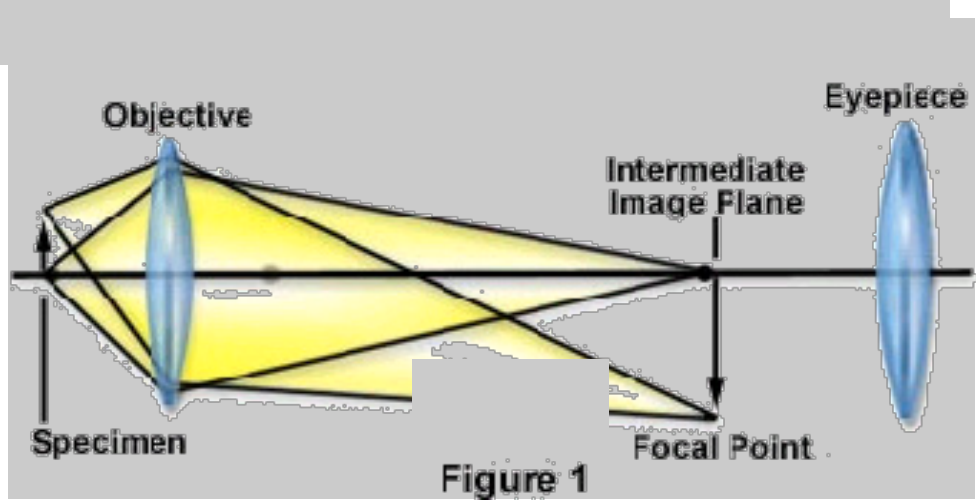


Figure 1

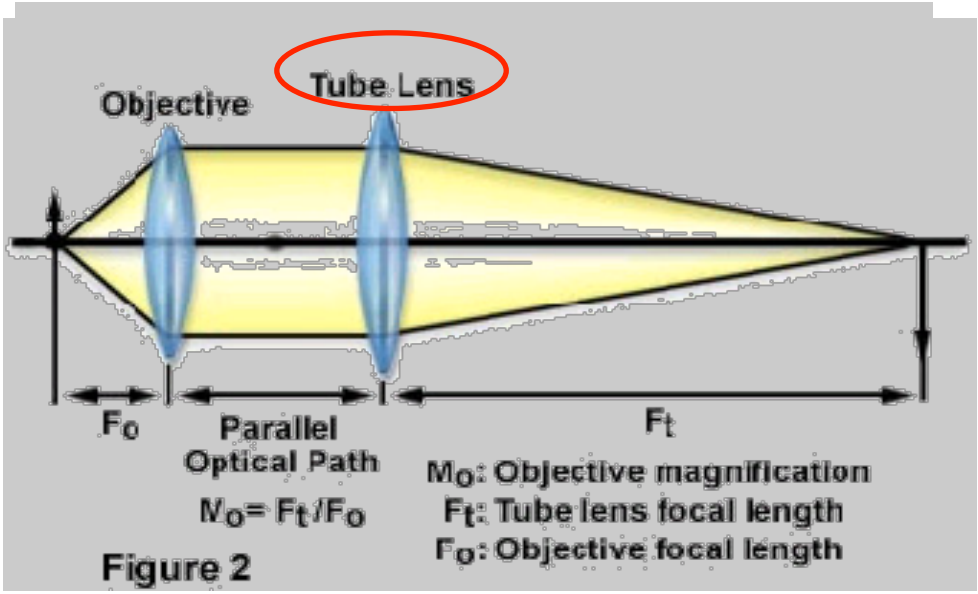
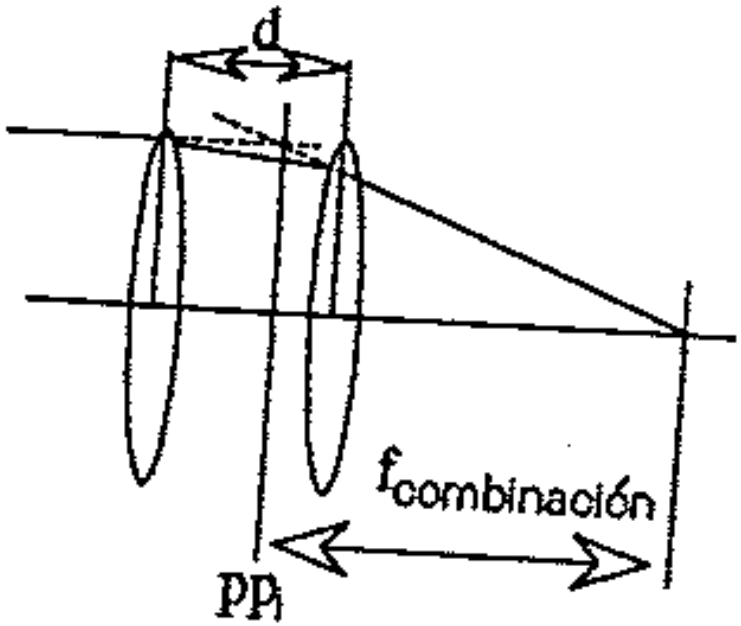


Figure 2

Lens systems and collimators (telescopes)



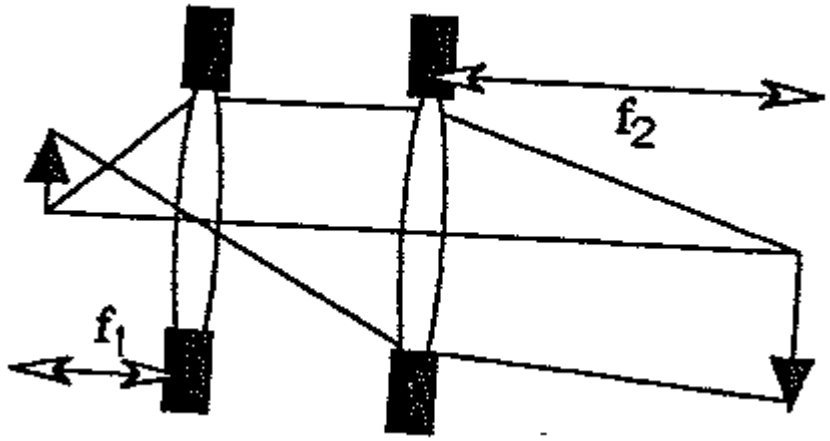
$$f_{comb} = \frac{1}{\frac{1}{f_1} + \frac{1}{f_2} - \frac{d}{f_1 f_2}}$$

2 thins lens separated by distance d

If d tends to zero

$$\frac{1}{f_{comb}} = \frac{1}{f_1} + \frac{1}{f_2}$$

Exampe, if d=3 cm, then f=1.5 cm for the combined system



Transporting system

$$M_{\text{transportador}} = \frac{h_{\text{img}}}{h_{\text{obj}}} = -\frac{f_2}{f_1}$$

Afocal telescopes or collimators

If d=f1+f2, then fcomb is indefined therefore the afocal telescopes can not be represented as a single lens.

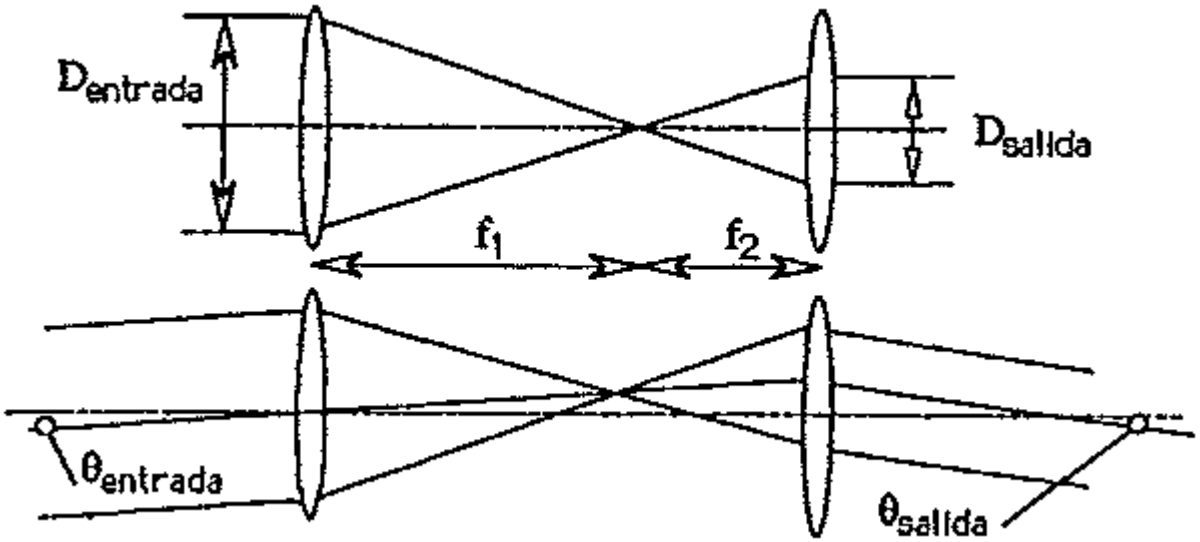
There is no single lens with this behavior.

$$f_{comb} = \frac{1}{\frac{1}{f_1} + \frac{1}{f_2} - \frac{d}{f_1 f_2}}$$

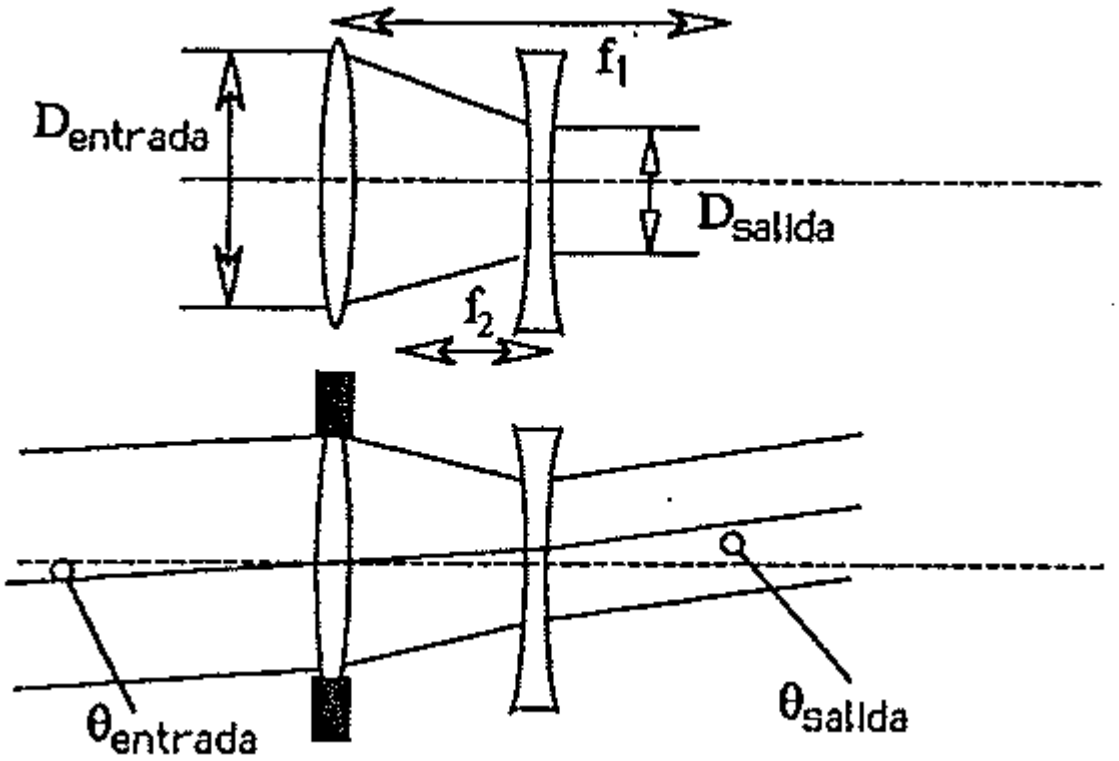
$$\mathcal{M}_{angular} = \frac{\theta_{salida}}{\theta_{entrada}} = -\frac{f_2}{f_1}$$

$$\left|\mathcal{M}_{angular}\right| = \frac{D_{entrada}}{D_{salida}} = \left|\frac{f_2}{f_1}\right|$$

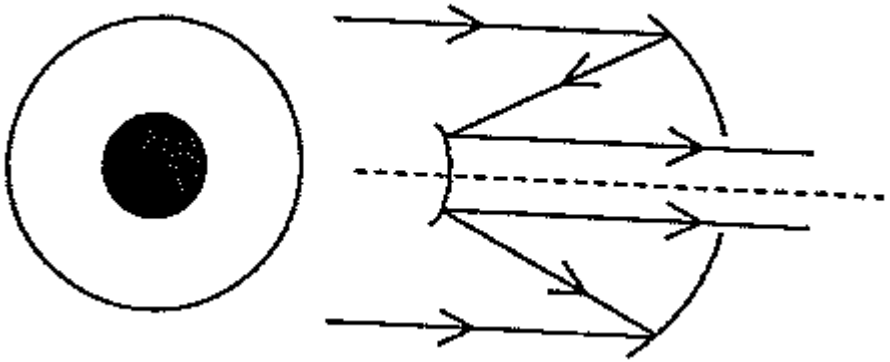
Kepler Telescope



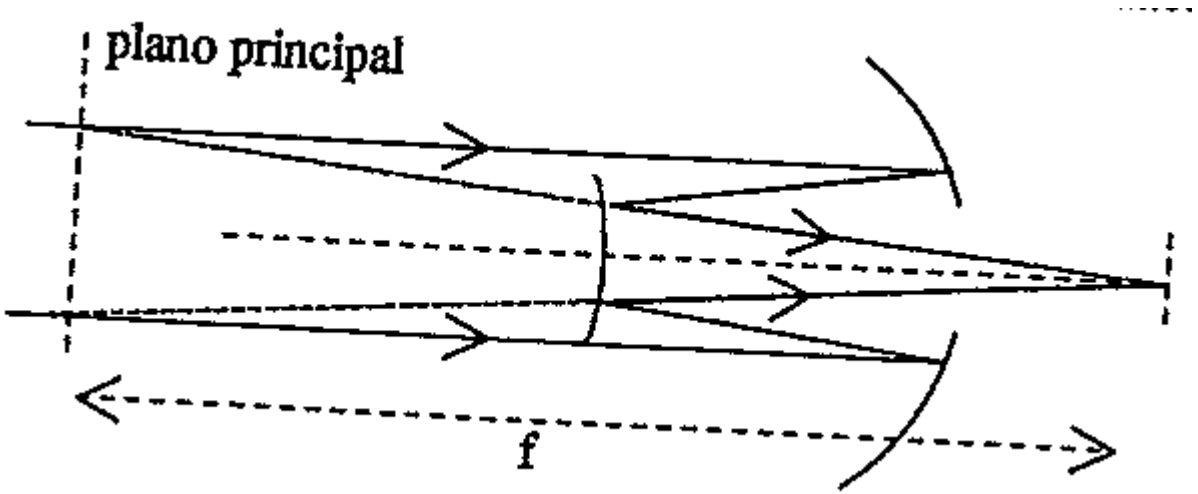
Galileo Telescope



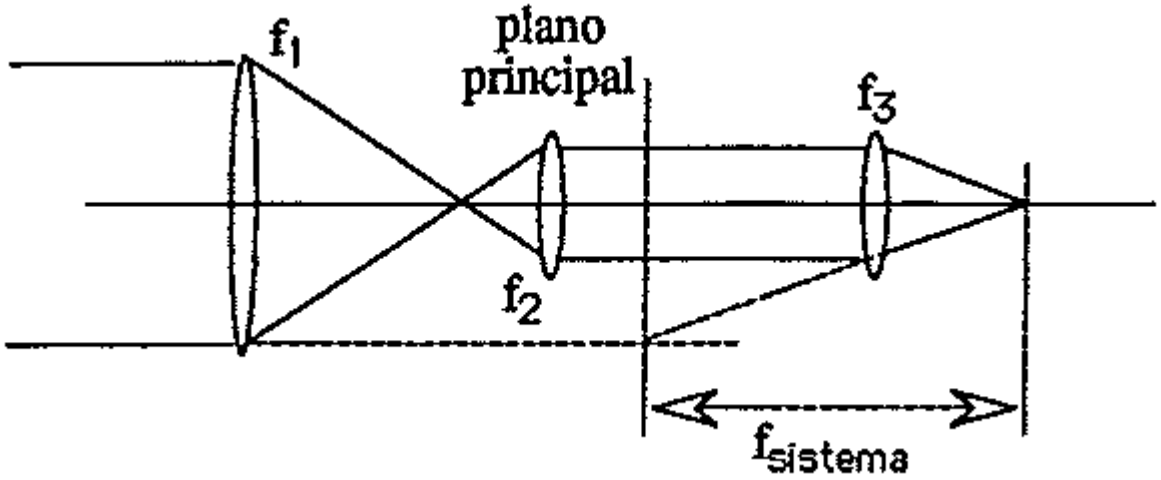
Reflective Galileo Telescope



Cassegrain telescope



T are used to modify the eye field



$$f_{comb} = \frac{f_1 \times f_3}{f_2} = \left|\mathcal{M}_{angular}\right| \times f_3$$

$$f_{comb} = \frac{f_1 \times f_3}{f_2} = f_1 \times \left|\mathcal{M}_{transportador}\right|$$

The characteristics of objectives

Figure 1

Numerical Aperture (N.A.)

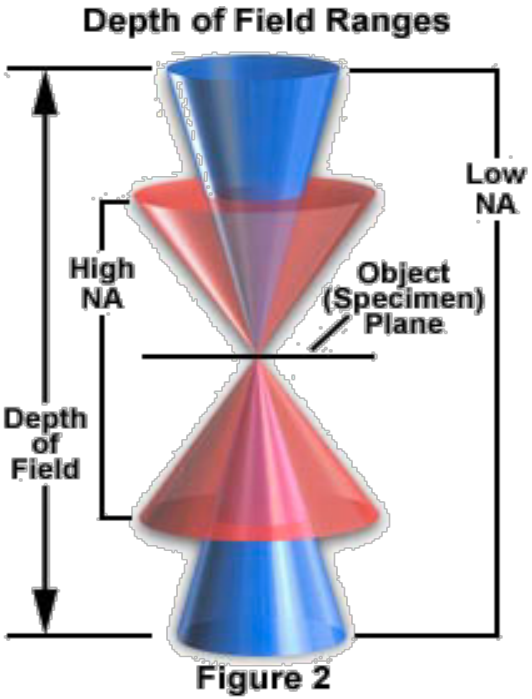


Figure 2

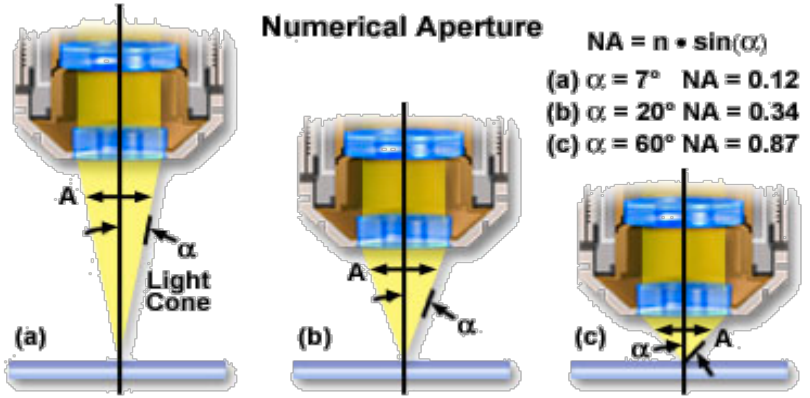


Figure 1

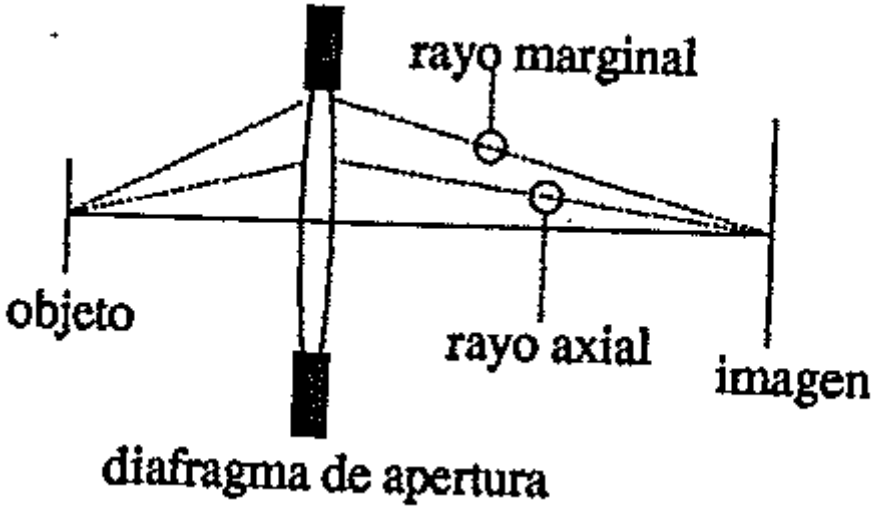
Numerical Aperture = N.A. = n · sin α

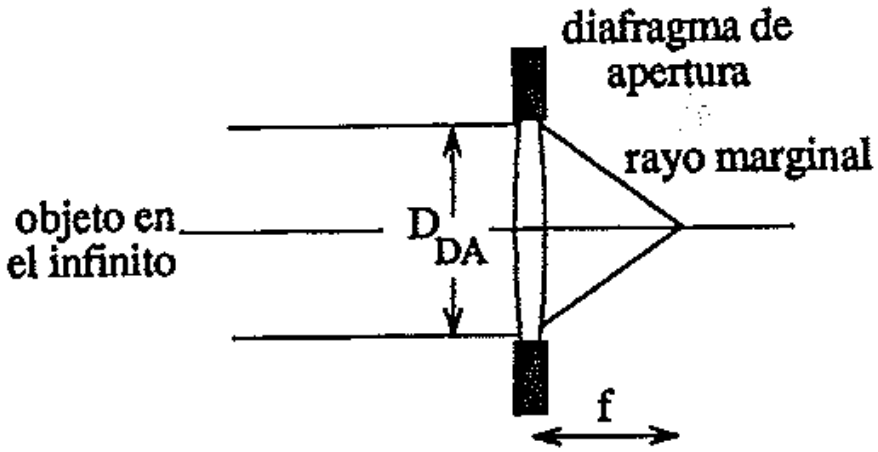
α is half the opening angle of the objective.

n is the refractive index of the immersion medium used between the objective and the object.

(n = 1 for air; n = 1.51 for oil or glass)

Aperture diaphragm (stop) and number of diaphragm

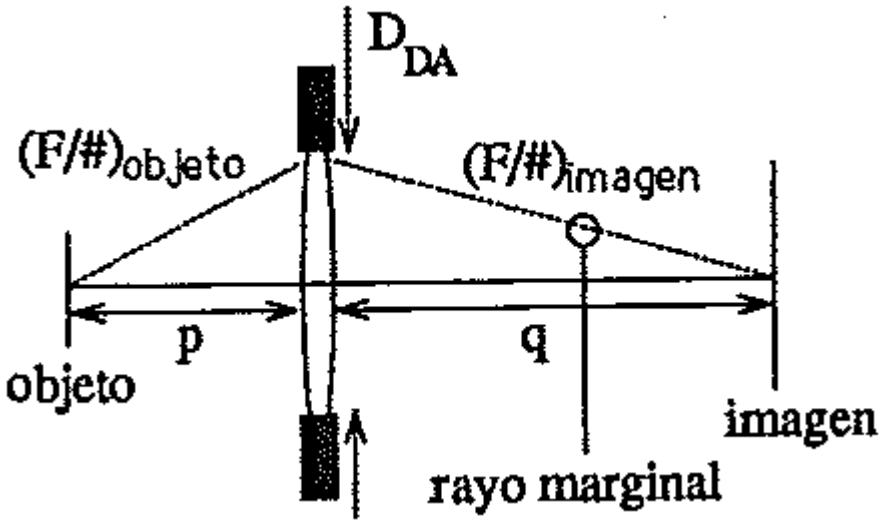




$$(F/\#)_{\text{imagen}} = \frac{f}{D_{DA}}$$

Number of diaphragm defined in image space by the margin ray

And for conjugate points in object and image space

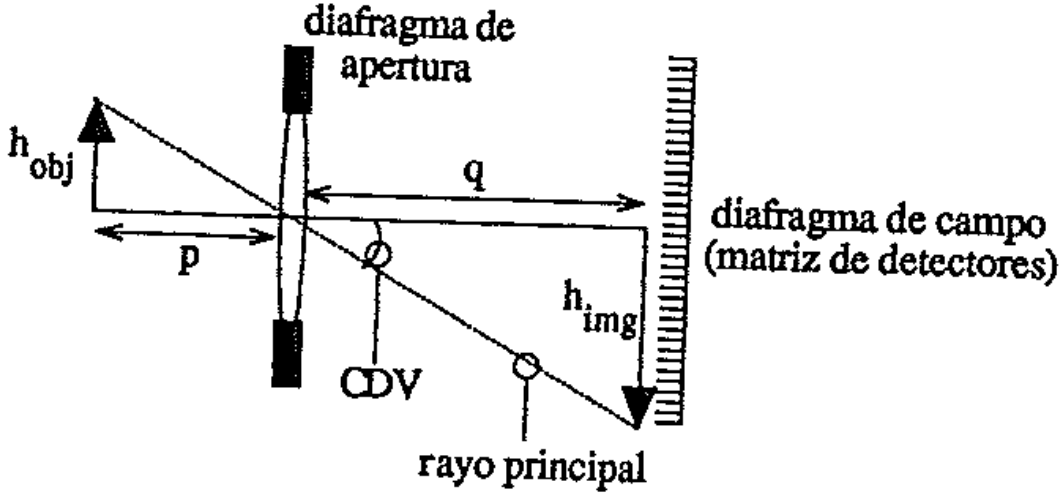


$$(F/\#)_{objeto} = \frac{p}{D_{DA}}$$

$$(F/\#)_{imagen} = \frac{q}{D_{DA}}$$

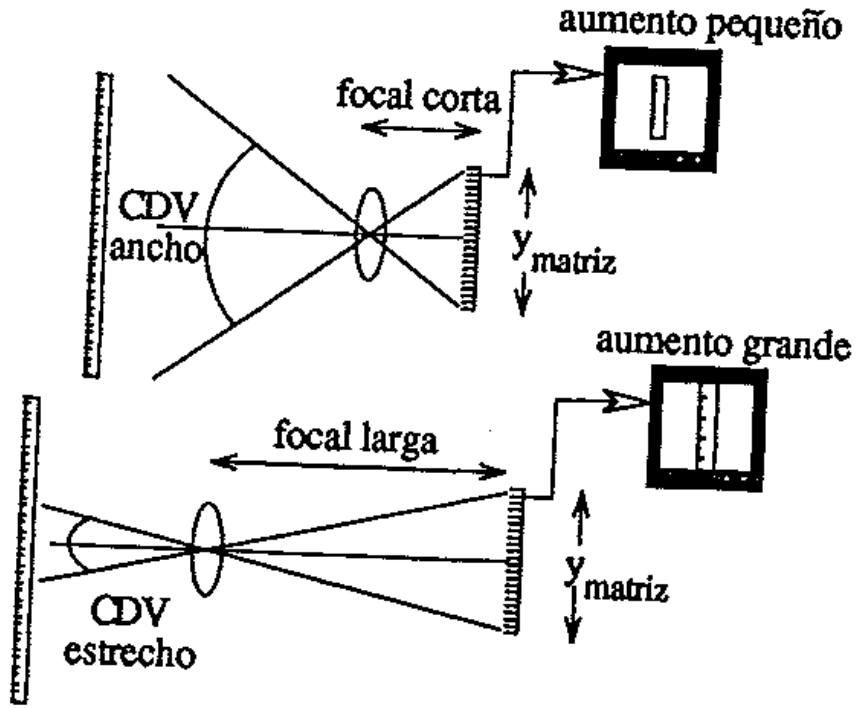
The number of diaphragm (ND) is inverse to the diameter of the aperture diaphragm. Then increasing ND is an slow system which need more exposure time.

Field diaphragm and field of view (FV)



$$CDV_{\text{semiangular}} = \left|\tan^{-1}\left(\frac{h_{\text{obj}}}{p}\right)\right| = \left|\tan^{-1}\left(\frac{h_{\text{img}}}{q}\right)\right|$$

The maximal size of the object and the image is determined by the FV. Without FV there will be an extended infinite region outside in the object plane forming image in image plane.



If the object is in infinity we can relate the FV with the magnification $M = -q/p,\ q \approx f$, then larger focal lens five higher magnification.

Small ND and high FV give good flux of light but low quality image due to aberrations and the contrary high ND and low FV give quality images with low brigthness

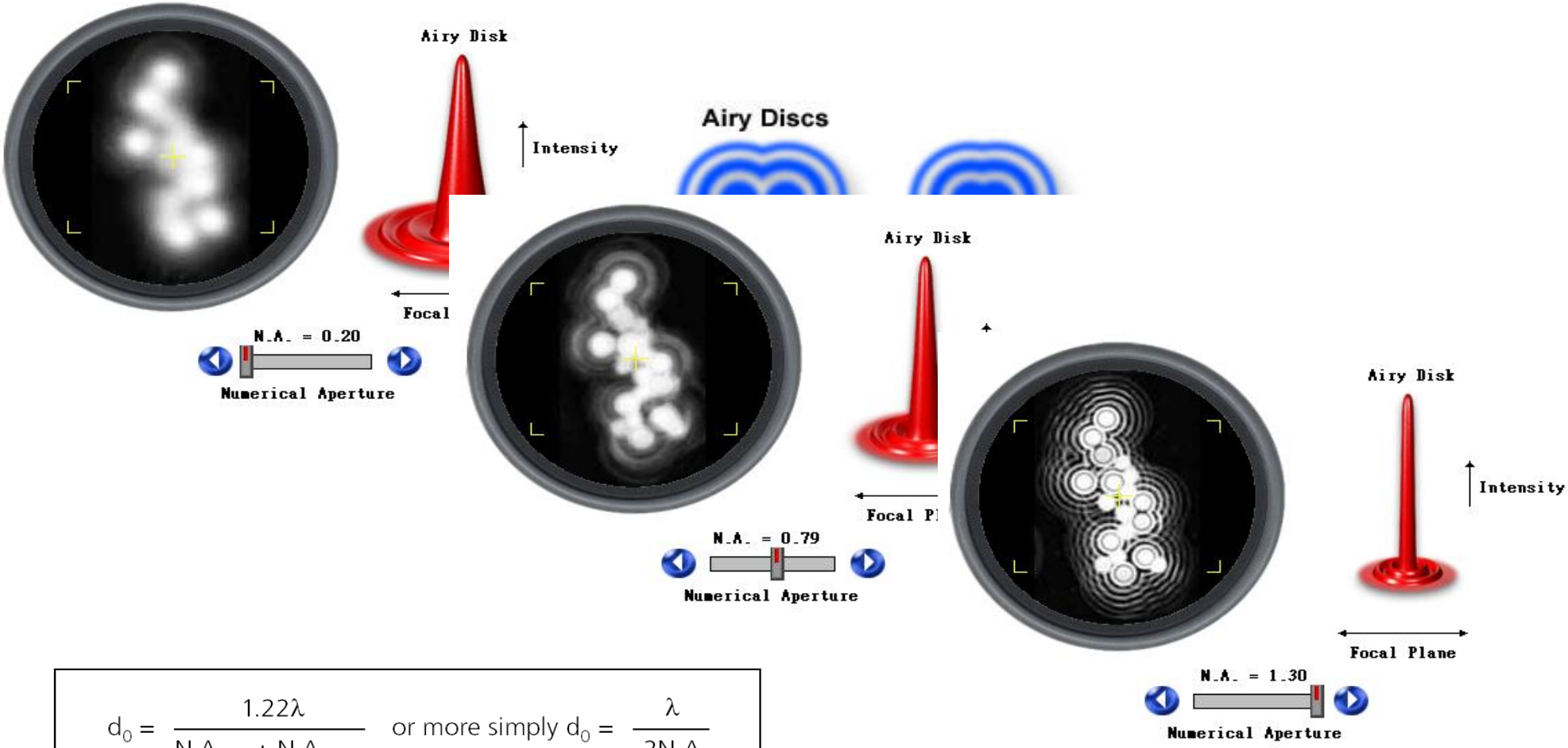
Depth of Focus

- We also need to consider the depth of focus (vertical resolution). This is the ability to produce a sharp image from a non-flat surface.

$$DOF \approx \frac{\lambda}{N.A.}$$

- Depth of Focus is increased by inserting the objective aperture (just an iris that cuts down on light entering the objective lens). However, this decreases resolution.

Resolution



$$d_0 = \frac{1.22\lambda}{N.A_{obj.} + N.A._{Cond}} \quad \text{or more simply } d_0 = \frac{\lambda}{2N.A}$$

λ = wavelength of light, e.g. 550 nm (green)

- **Resolving power, the limit up to which two small objects are still seen separately.**

Factors Affecting Resolution

- **Resolution (d_{min}) improves (smaller d_{min}) if $\lambda\downarrow$ or $n\uparrow$ or $\alpha\uparrow$**
- **Assuming that $\sin\alpha = 0.95$ ($\alpha = 71.8°$)**

Wavelength		Air (n= 1)	Oil (n = 1.515)
Red	650 nm	0.42 μm	0.28 μm
Yellow	600 nm	0.39 μm	0.25 μm
Green	550 nm	0.35 μm	0.23 μm
Blue	475 nm	0.31 μm	0.20 μm
Violet	400 nm	0.27 μm	0.17 μm
		Resolution $_{air}$	**Resolution $_{oil}$**

- **(The eye is more sensitive to blue than violet)**

Two sets of conjugate planes in the light microscope

Understanding the reciprocal relationship between the two sets of conjugate planes is crucial for properly understanding:

- Image formation
- Image resolution
- How phase-contrast and DIC work

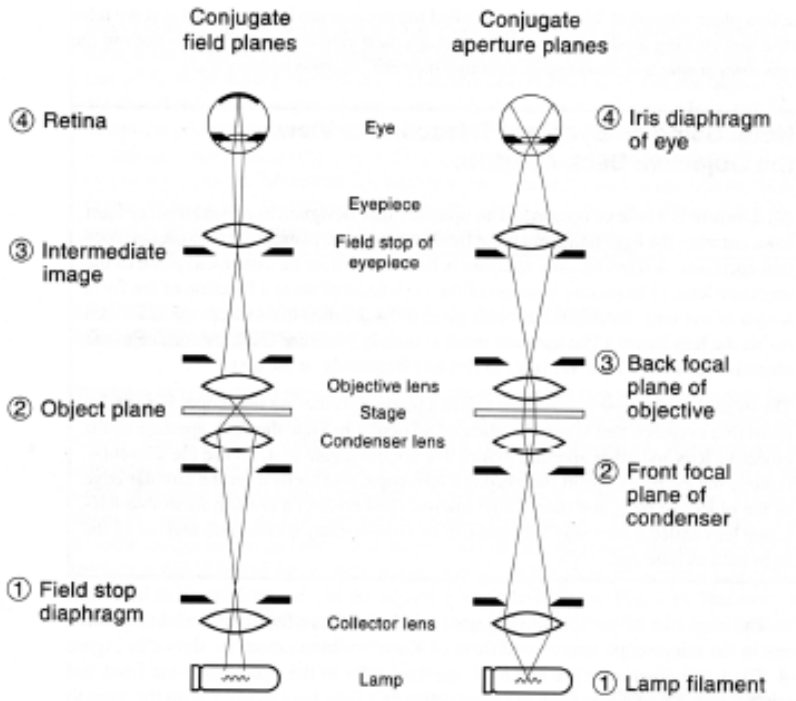


Figure 1-4

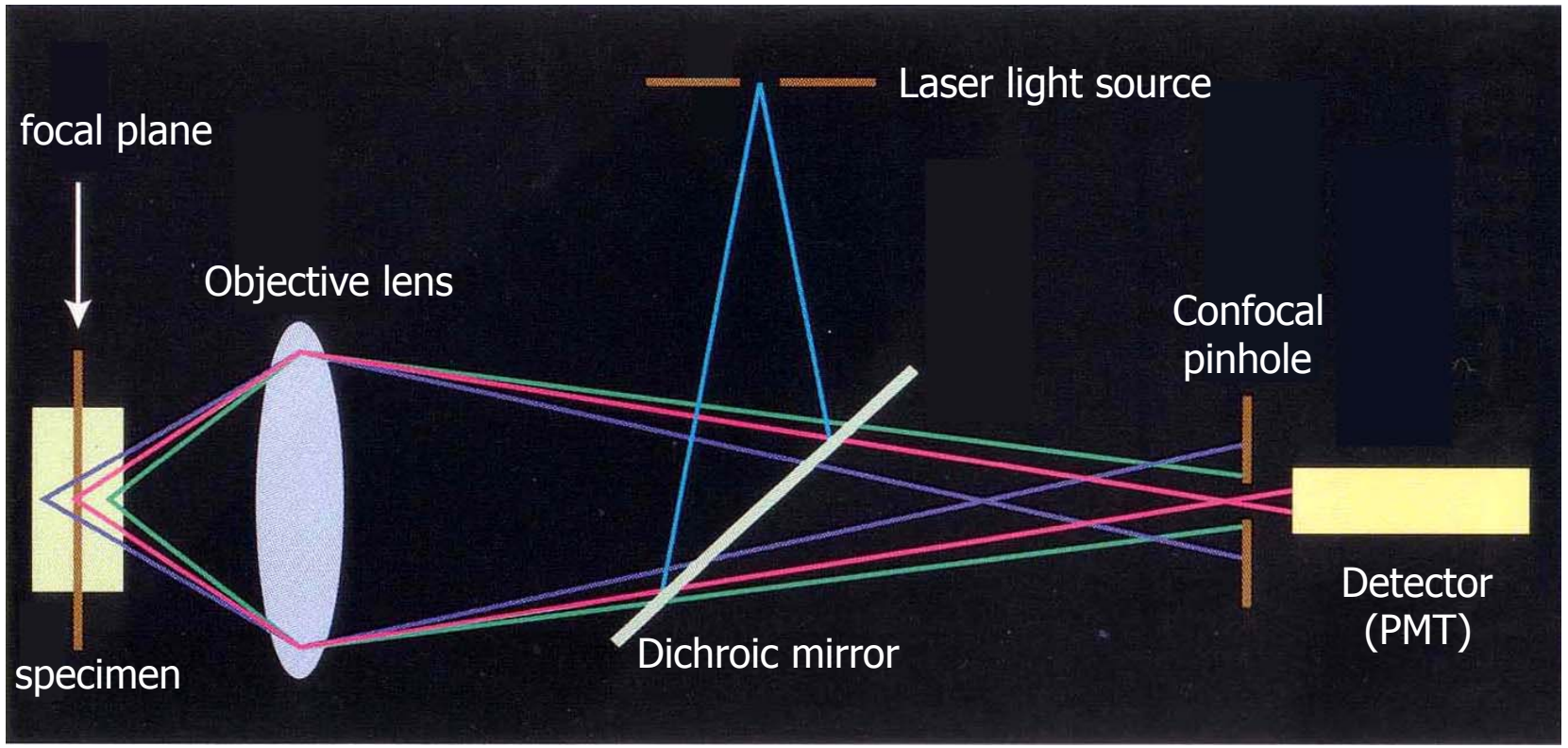
The locations of conjugate focal planes in a light microscope adjusted for Koehler illumination. Note the locations of four conjugate field planes (left) and four conjugate aperture planes (right) indicated by the crossover points of rays in the diagrams. The left-hand diagram shows that the specimen or object plane is conjugate with the real intermediate image plane in the eyepiece, the retina of the eye, and the field stop diaphragm between the lamp and the condenser. The right-hand drawing shows that the lamp filament is conjugate with aperture planes at the front focal plane of the condenser, the back focal plane of the objective, and the pupil of the eye.

Conjugate planes are "parfocal" with each other

When something is in focus in one set of conjugate planes, it is "maximally out-of-focus" in the other set of planes

These two sets are often called "reciprocal" or "transform" planes (with respect to each other)

Laser Scanning Microscope
(Confocal System)



Light emitted from the focal plane

Light emitted from the out-of-focus region

Confocal Aperture

Decreasing the pinhole size rejects more out of focus light, therefore improving contrast and effective z resolution.

Decreasing the pinhole will increase x,y resolution (1.3x wide field)

Decreasing pinhole size decreases the amount of the Airy disk that reaches the detector. This results in less light from each point being collected

Generally, collecting the diameter of 1 Airy disk is considered optimal. This collects about 85% of light from a sub-resolution point.

Limits:

Open pinhole: nearly wide field resolution (still some confocality)

Closed: no image

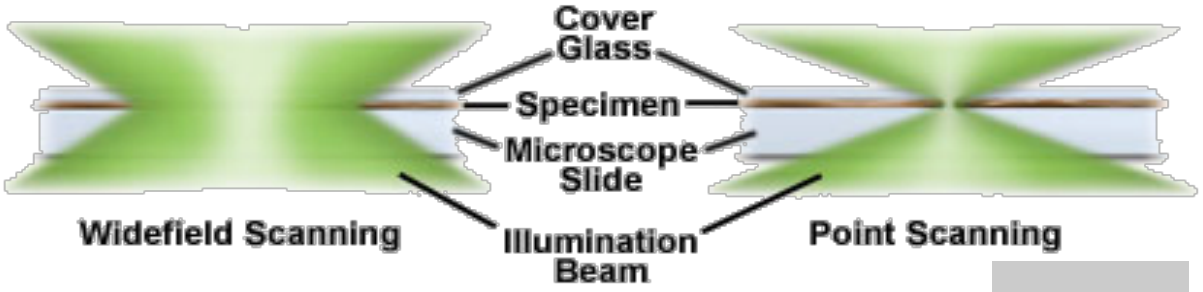
Confocal Aperture

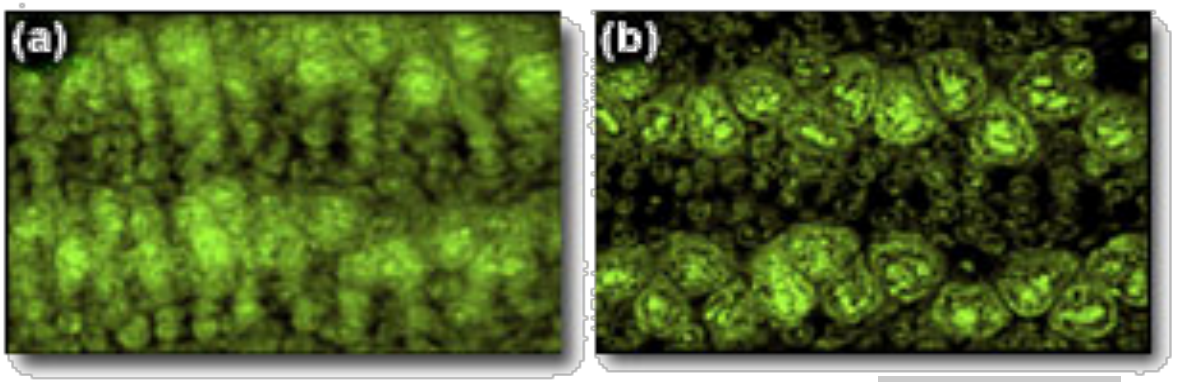
ALIGNMENT OF APERTURES IS CRITICAL

X, Y alignment: Different wavelengths focus at different lateral position. Lateral color aberrations can be important for multi-color imaging (multiple dyes with multiple lasers)

Z alignment: Different wavelengths focus at different depths in image plane. Chromatic aberrations can be important. Need well-corrected lenses

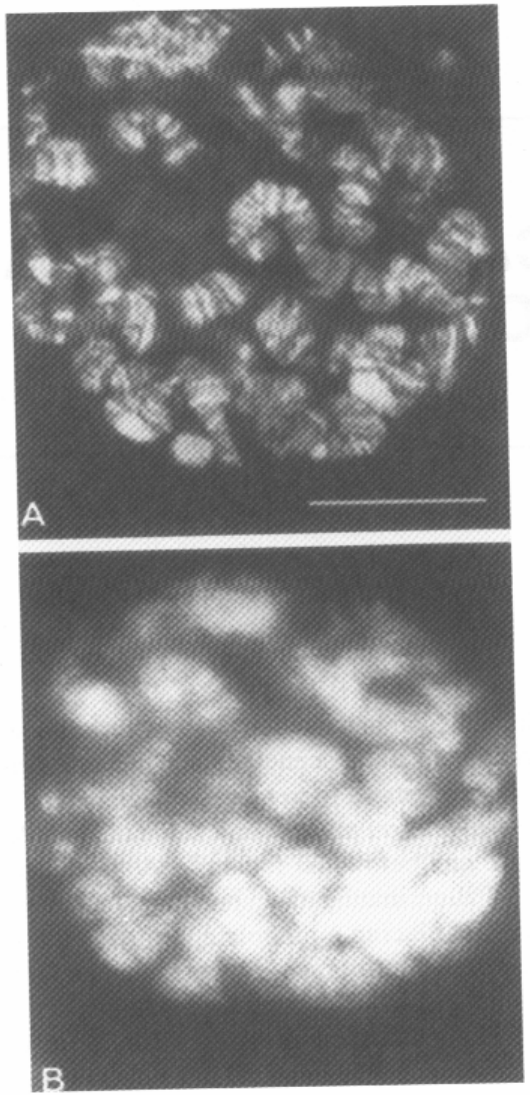
Wide field versus confocal scanning





Wide Field **Confocal**

WF vs C - Fluorescence Imaging



Confocal

Greatly reduces
Out of focus blur

Wide-field

Brighter but
No sectioning

More examples

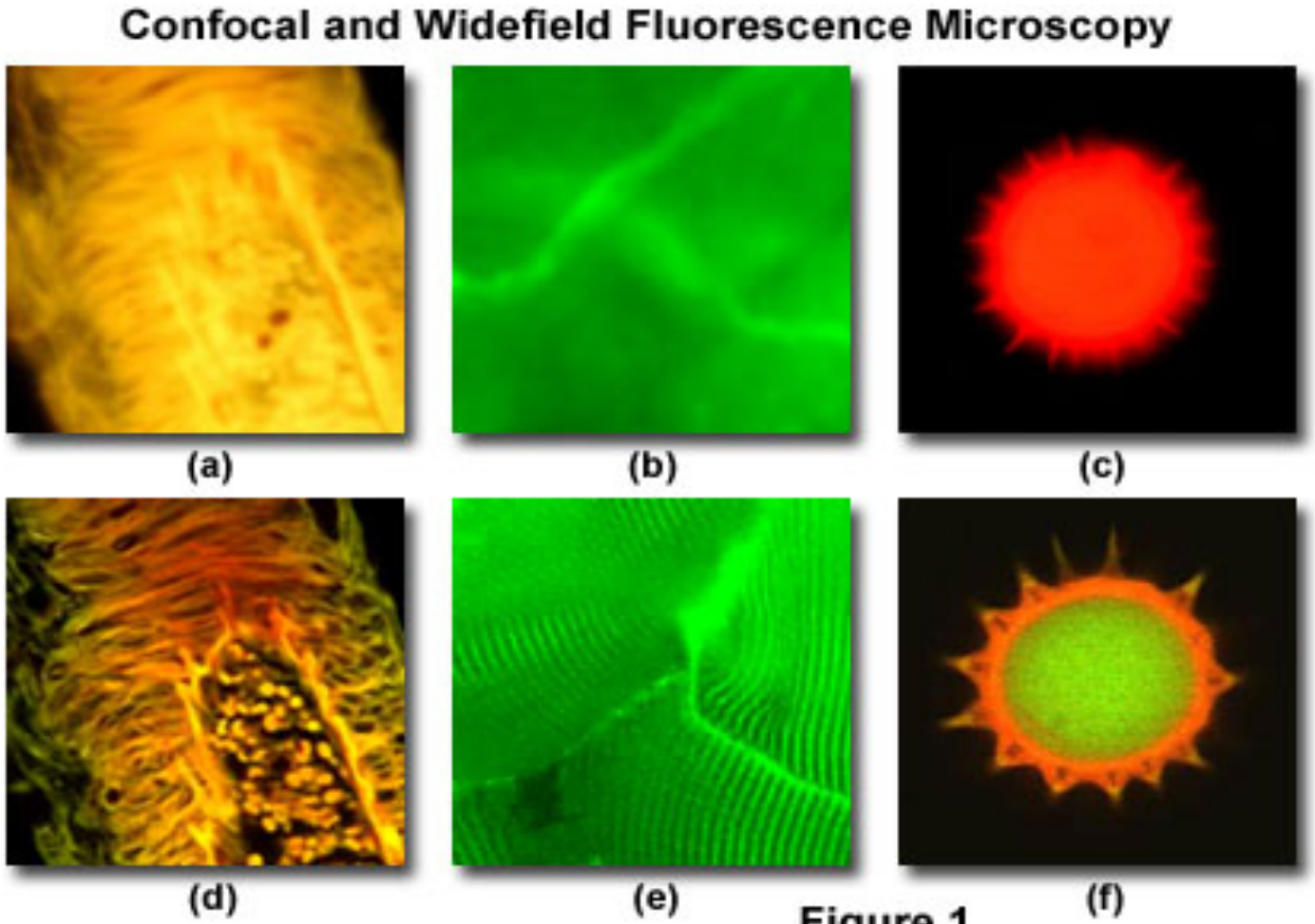
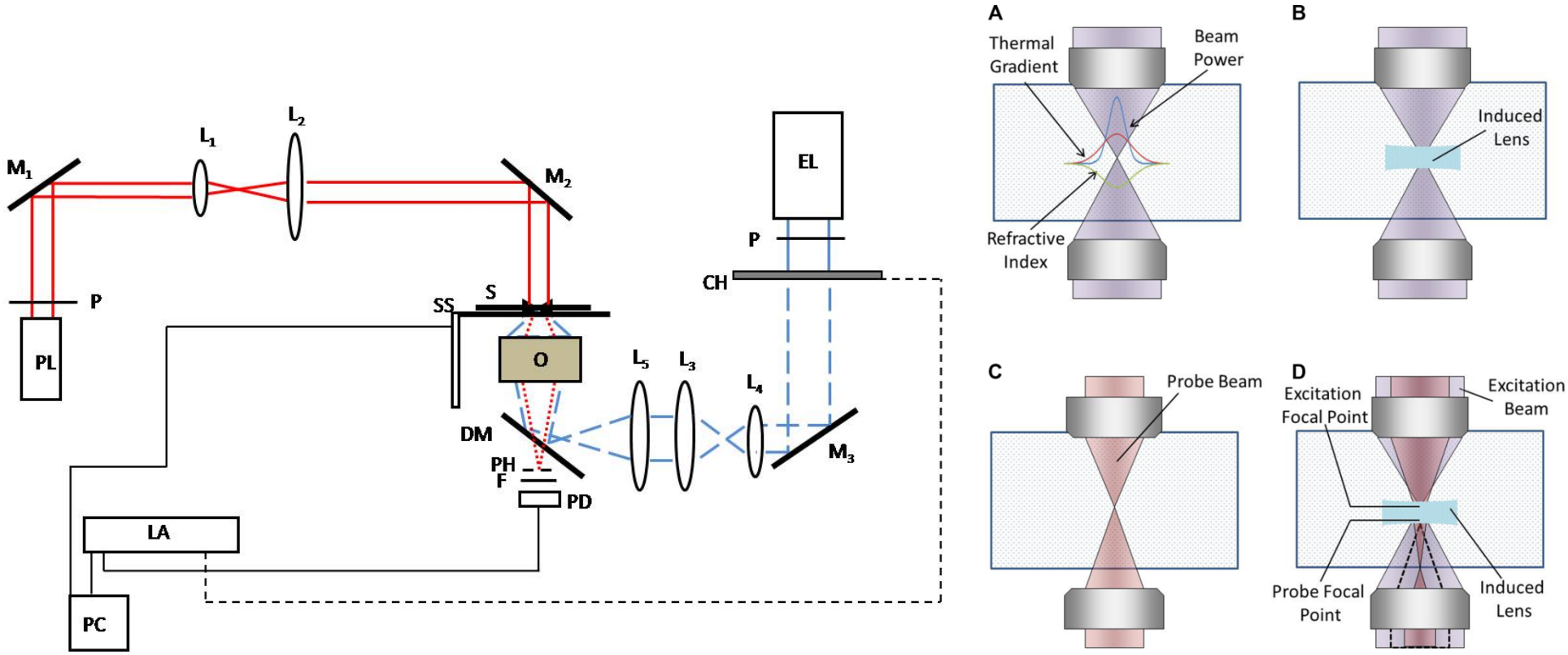


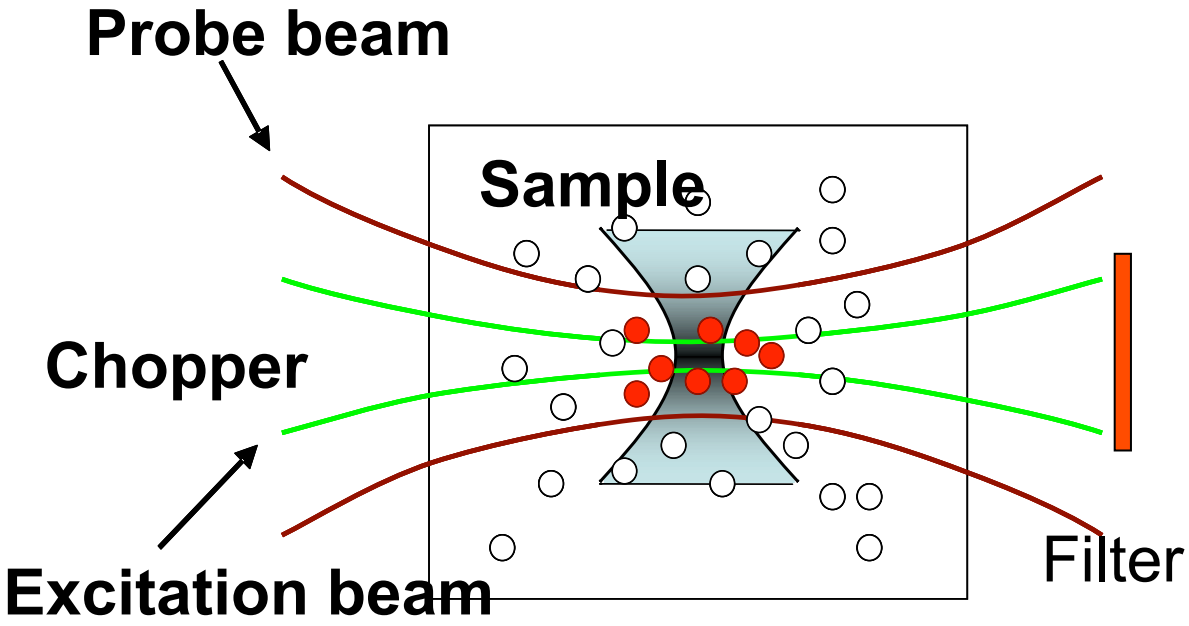
Figure 1

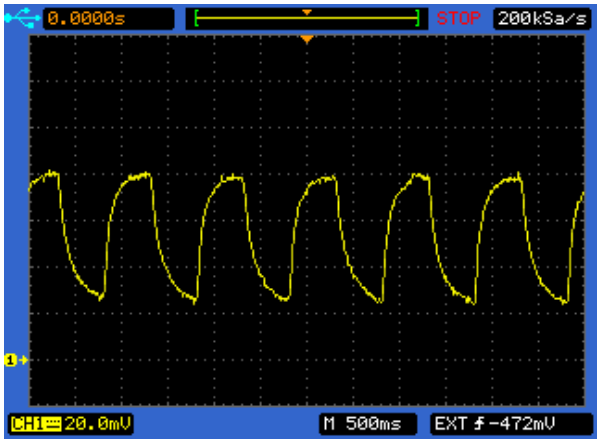
Thermal lens microscopy set up



Schematic diagram of the TLM, S: sample; SS: sample stage 3-D control; M_1, M_2 and M_3: mirrors; CH: chopper; DM: dichroic mirror; P: linear polarizer; L_1, L_2, L_3; L_4 and L_5: lenses; O: focusing objective lens; PH: pinhole; F: interference filter at 632.8 nm; PD: photodiode; LA: lock-in amplifier; PC: personal computer; EL: excitation laser; PL: probe laser.

Thermal lens effect and signal

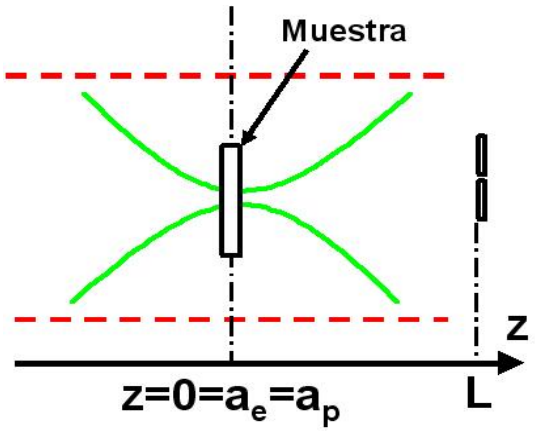




$$I(r) = \frac{2P_e}{\pi w_e^2} e^{-2r^2/w_e^2}$$

Physical mathematical model

$$z_p >> L >> z_e \;\; ; \;\; t \to \infty \;\; ; \;\; z_p \to \infty \;\; ; \;\; z = 0$$



$$S(z,t) = \Phi_0 \arctan\left\{ \frac{4m(z)\upsilon(z)t / t_c(z)}{\upsilon^2(z) + [1+2m(z)]^2 + [1+2m(z)+\upsilon^2(z)]2t / t_c(z)} \right\}$$

$$I(r) = \frac{2P_e}{\pi w_e^{\,2}} e^{-2r^2/w_e^2}$$

$$D = \kappa / \rho C_p$$

$$t_c(z) = \omega_e^{\,2}(z) / 4D$$

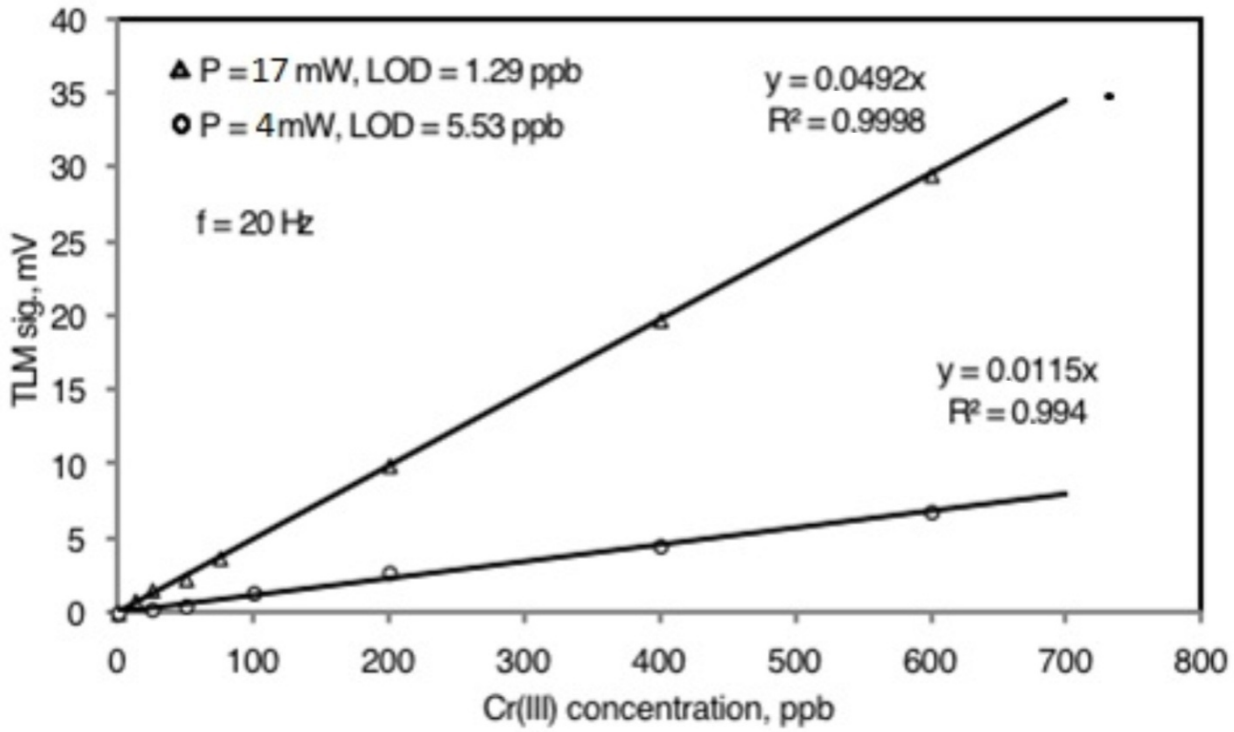
$$S_{max} = \pi \Phi_o / 2$$

$$\Phi_0 = \frac{P_e \alpha l}{\lambda_p k} \frac{dn}{dT}$$

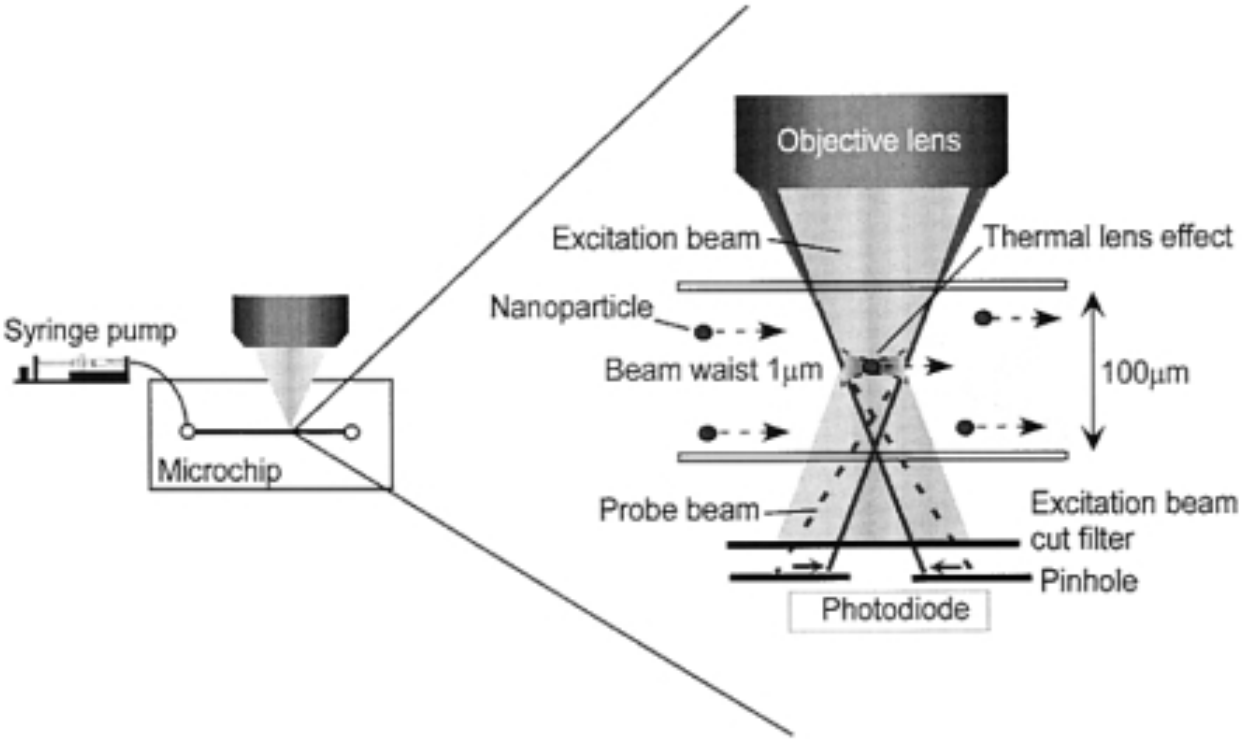
H Cabrera, J. Opt. Soc. Am. B, 23, 1408 (2006).

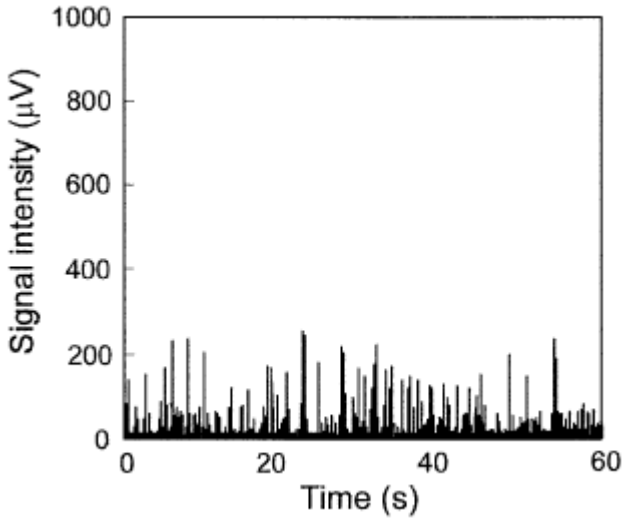
H. Cabrera, Appl. Phys. Lett. **94** 051103, (2009).

Applications



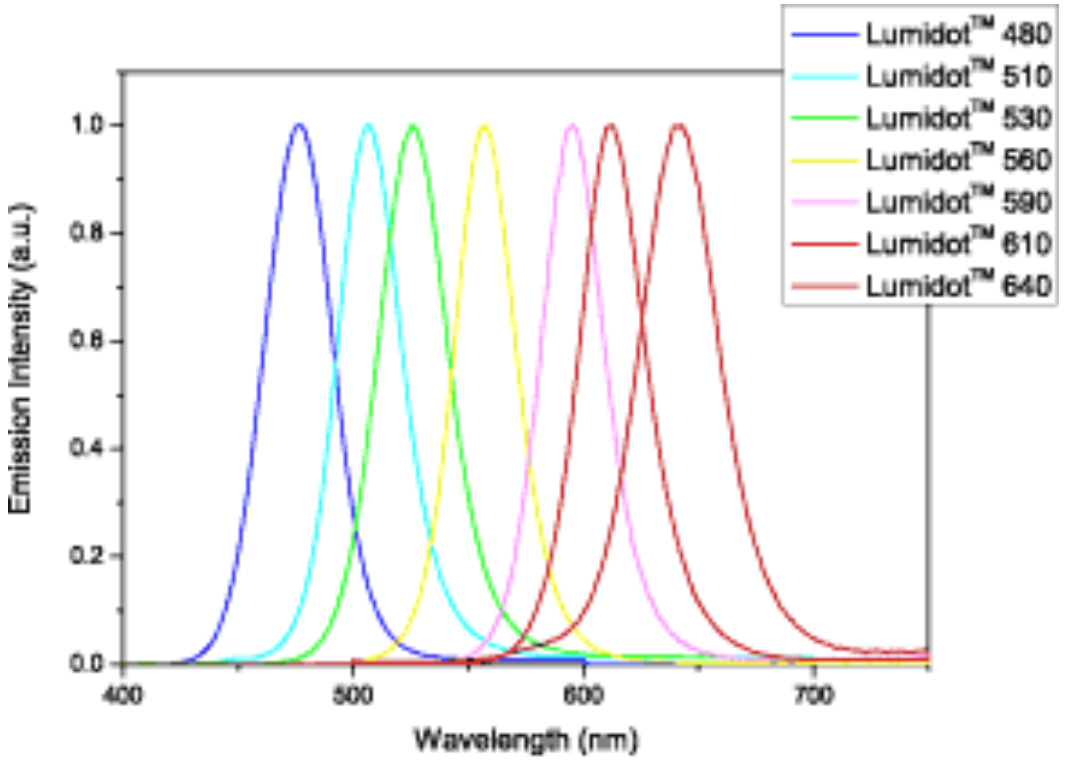
Calibration curves for Cr(III) solutions in 80% water with the addition of 20% of acetonitrile in 0.5 mm cell at 407 nm for 4 and 17 mW of excitation powers .


Objective lens
Excitation beam
Thermal lens effect
Nanoparticle
Beam waist 1μm
100μm
Syringe pump
Microchip
Probe beam
Excitation beam
cut filter
Pinhole
Photodiode

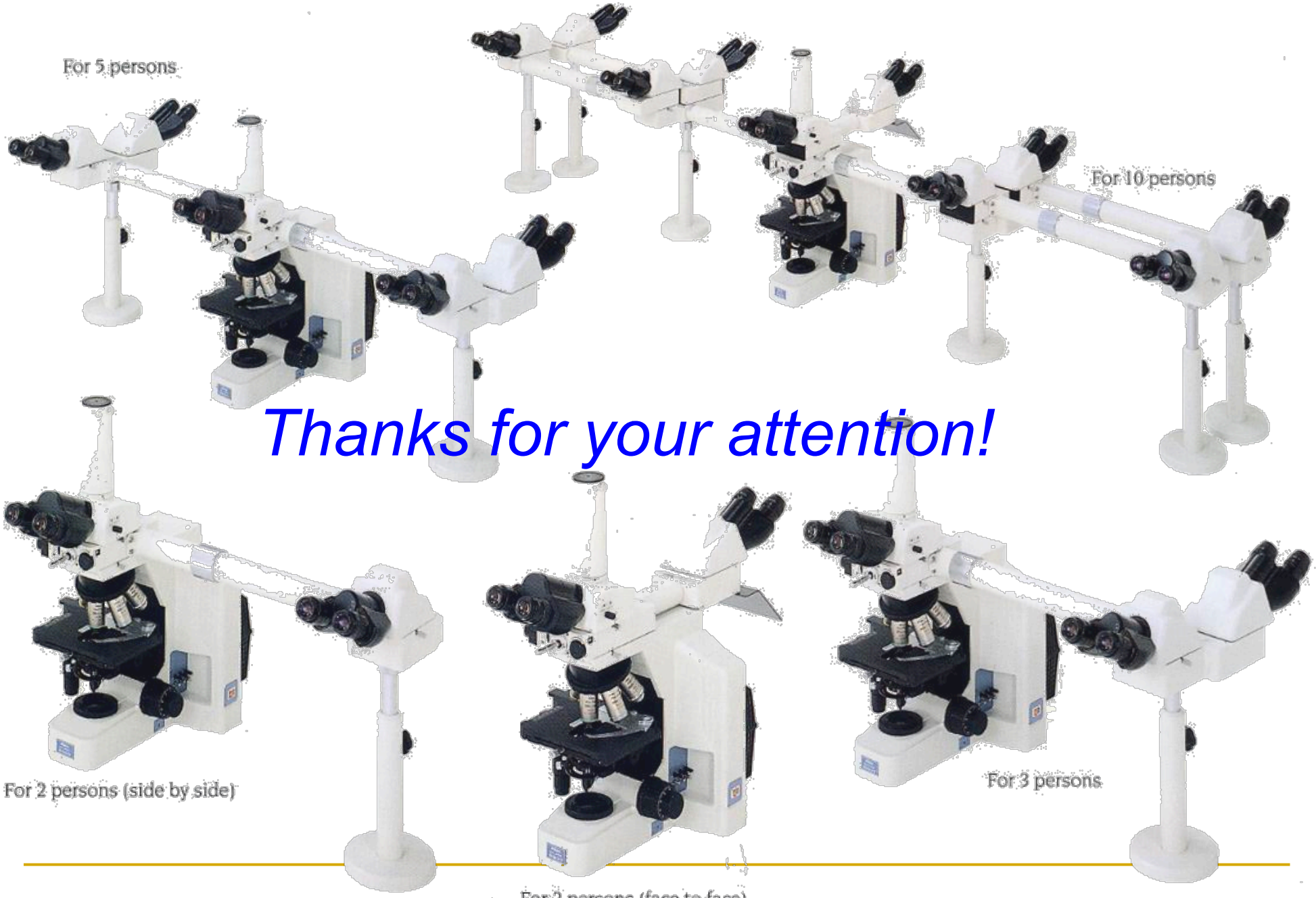

Signal intensity (μV)
1000
800
600
400
200
0
0
20
40
60
Time (s)

Lumidots: Quantum Dot Nanocrystals

CdSe/ZnS quantum dot nanocrystals



Emission spectra of Lumidot™ CdSe/ZnS nanocrystals



Thanks for your attention!