Notación/convención de signos:

TABLE 5.2 Meanings Associated with the Signs of Various Thin Lens and Spherical Interface Parameters

Quantity	Sign +	Sign −
s_o	Real object	Virtual object
s_i	Real image	Virtual image
f	Converging lens	Diverging lens
y_o	Erect object	Inverted object
y_i	Erect image	Inverted image
M_T	Erect image	Inverted image

TABLE 5.3 Images of Real Objects Formed by Thin Lenses

Convex

Object	Image			
Location	Type	Location	Orientation	Relative Size
$\infty > s_o > 2f$	Real	$f < s_i < 2f$	Inverted	Minified
$s_o = 2f$	Real	$s_i = 2f$	Inverted	Same size
$f < s_o < 2f$	Real	$\infty > s_i > 2f$	Inverted	Magnified
$s_o = f$		$\pm\infty$		
$s_o < f$	Virtual	$\lvert s_i \rvert > s_o$	Erect	Magnified

Concave

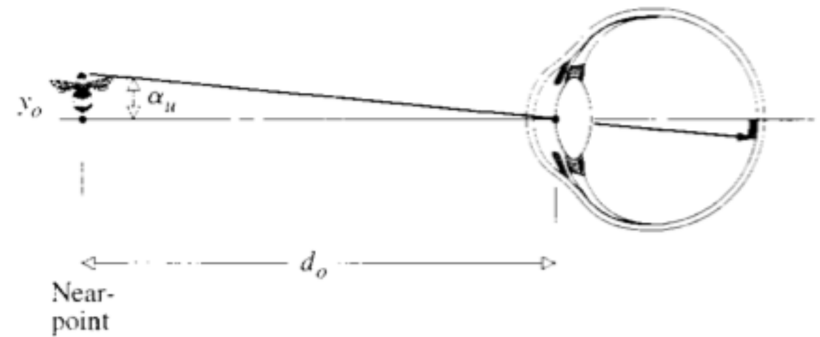
Object	Image			
Location	Type	Location	Orientation	Relative Size
Anywhere	Virtual	$\lvert s_i \rvert < \lvert f \rvert$, $s_o > \lvert s_i \rvert$	Erect	Minified

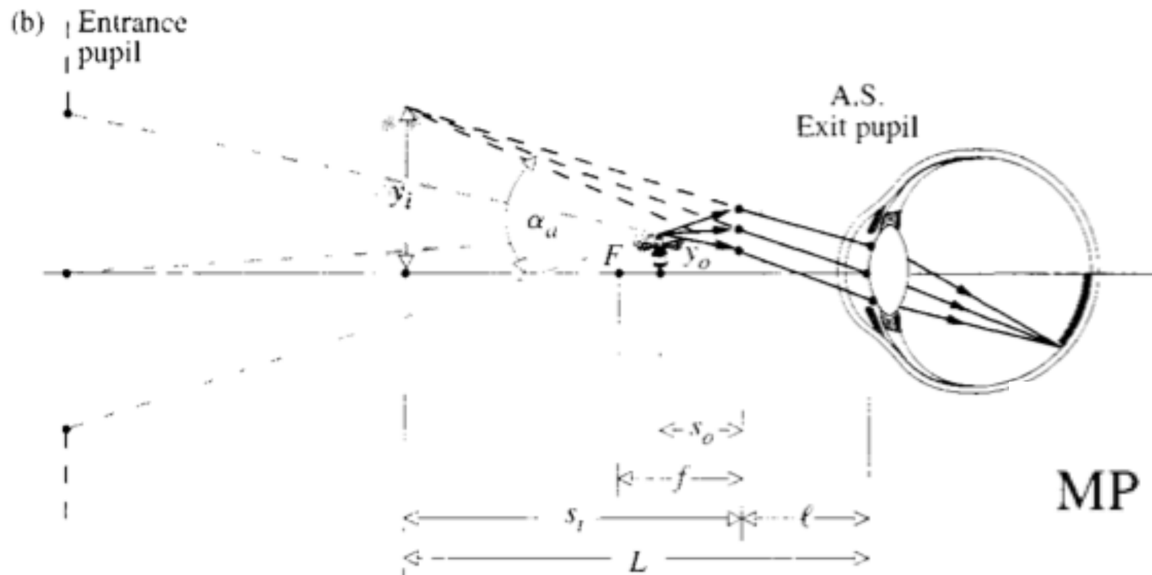
Sistemas Ópticos

Lupa. Del objeto a una imagen virtual de mayor tamaño

El aumento (magnificación angular) es el cociente de tamaños de la imagen en la retina cuando se ve al objeto con y sin la lupa al colocarlo (en el segundo caso) a la distancia óptima de visión distinta. Es equivalente al cociente de los ángulos

(a)





$$\mathrm{MP} = \frac{\alpha_a}{\alpha_u}$$

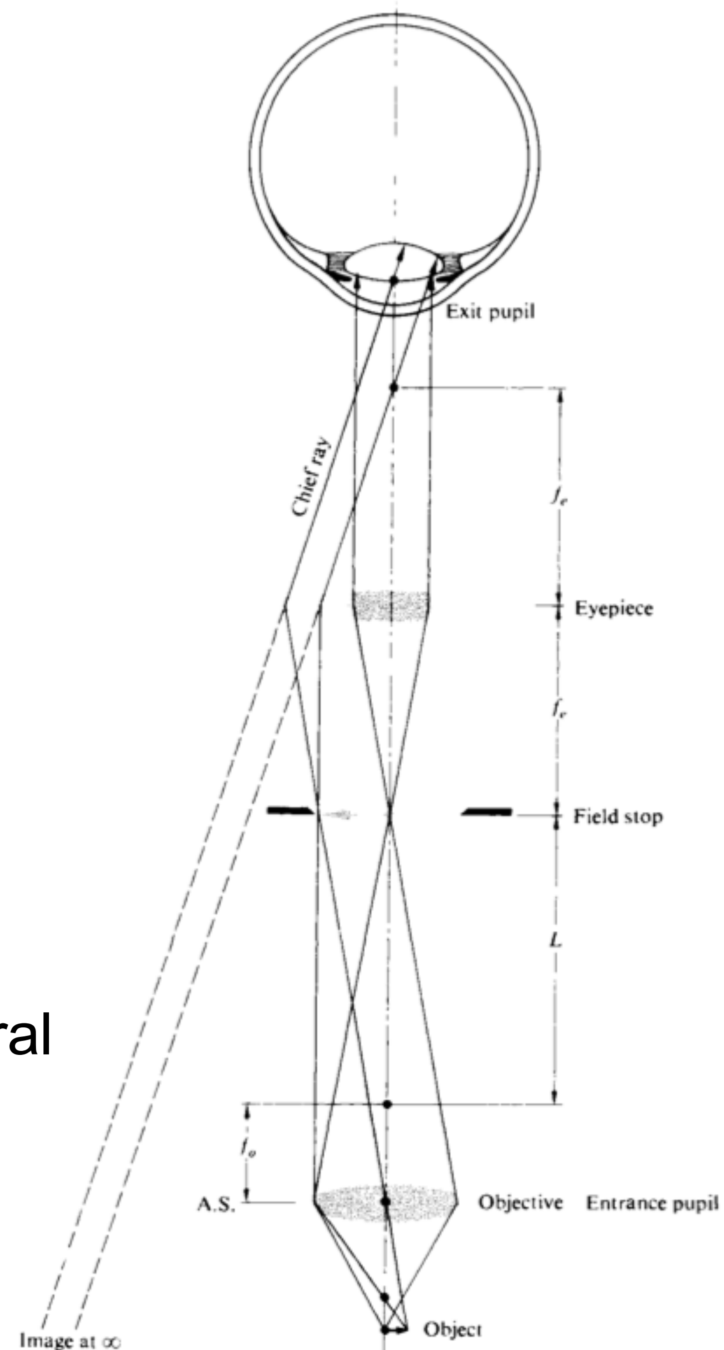
$$\mathrm{MP} = \frac{y_i d_o}{y_o L}$$

$$\mathrm{MP} = -\frac{s_i d_o}{s_o L} = \left(1 - \frac{s_i}{f}\right)\frac{d_o}{L}$$

Microscopio (compuesto). Usa una lente que se ubica cerca del objeto de modo de poder recolectar la mayor cantidad de luz (lente objetivo) que enfoca una imagen real del objeto en dentro del microscopio. Esa imagen es magnificada por una segunda lente o grupo de lentes (ocular) formando una imagen virtual invertida de mayor tamaño.

Acá el aumento es el producto del lateral del objetivo por el angular del ocular

$$\mathrm{MP} = M_{To} M_{Ae}$$



Telescopio. Similar al microscopio pero diferente la ubicación relativa de los focos de las dos lentes.

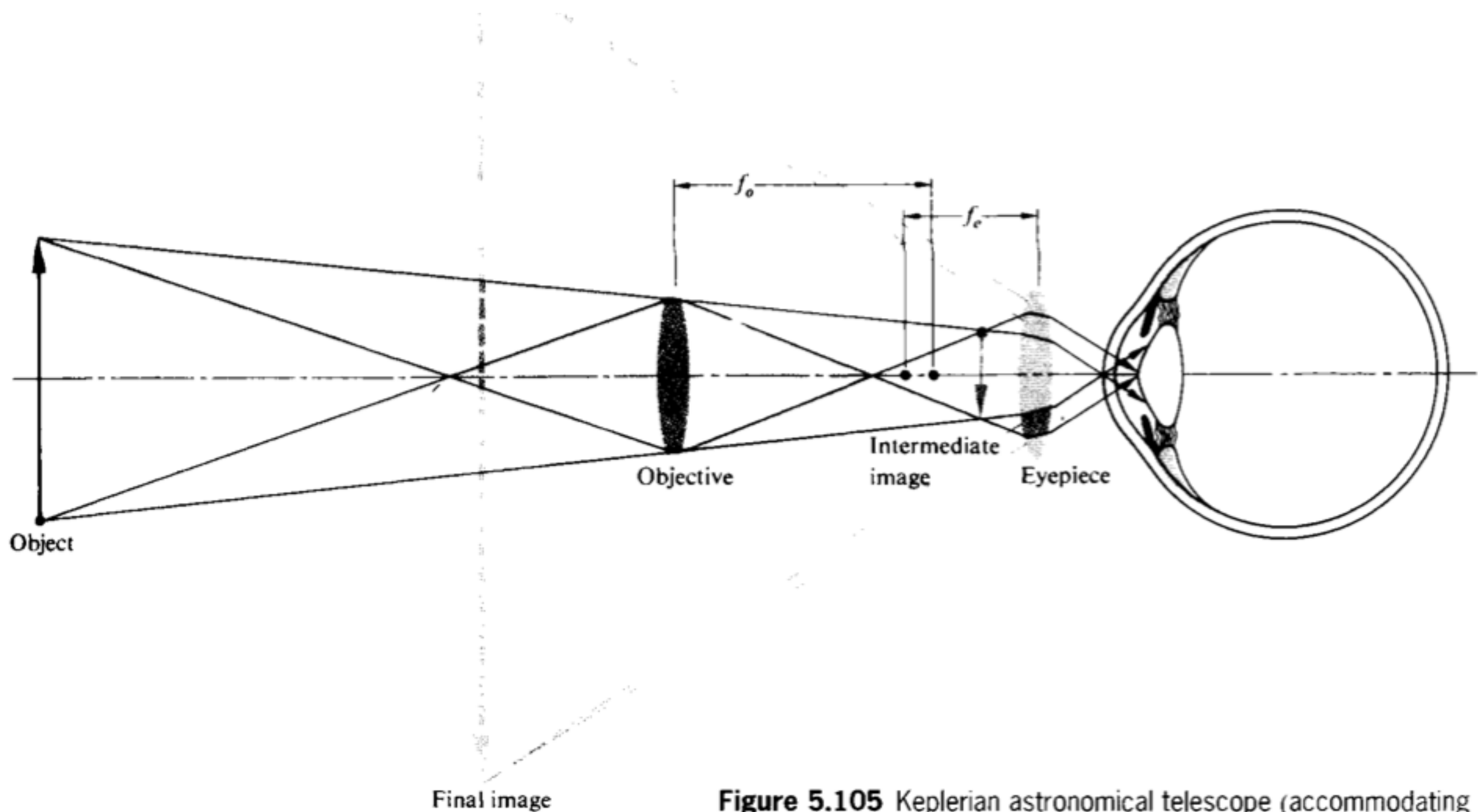


Figure 5.105 Keplerian astronomical telescope (accommodating eye).

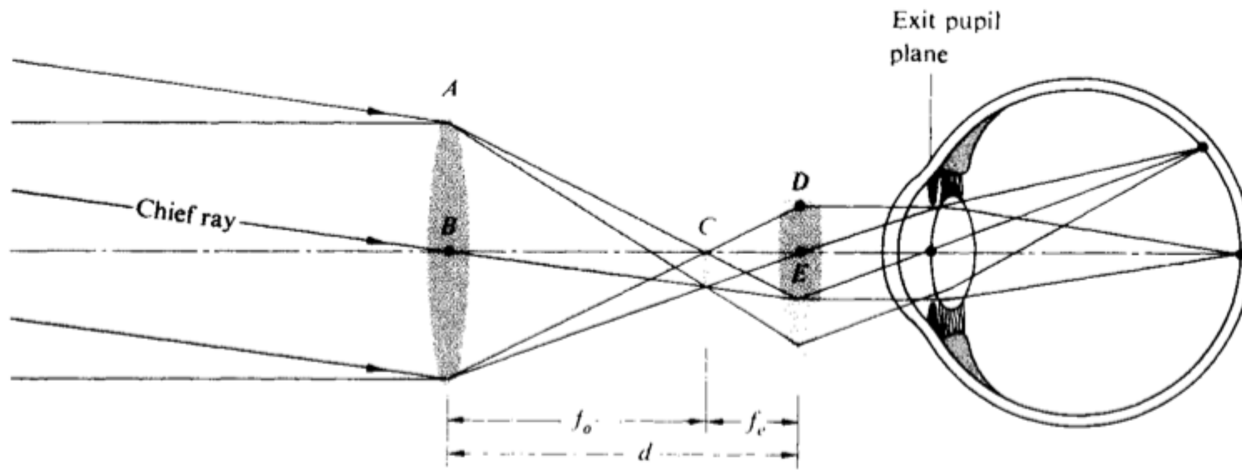
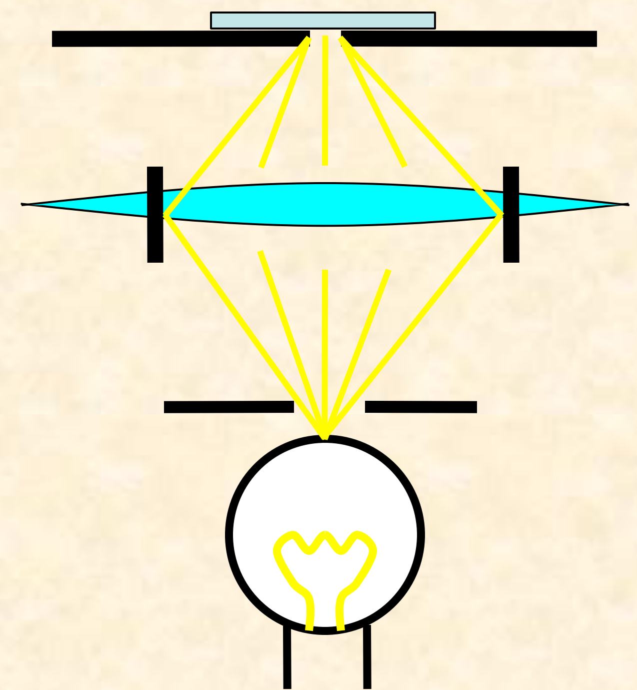


Figure 5.106 Astronomical telescope—infinite conjugates.

Microscopía óptica

Sistema de Iluminacion

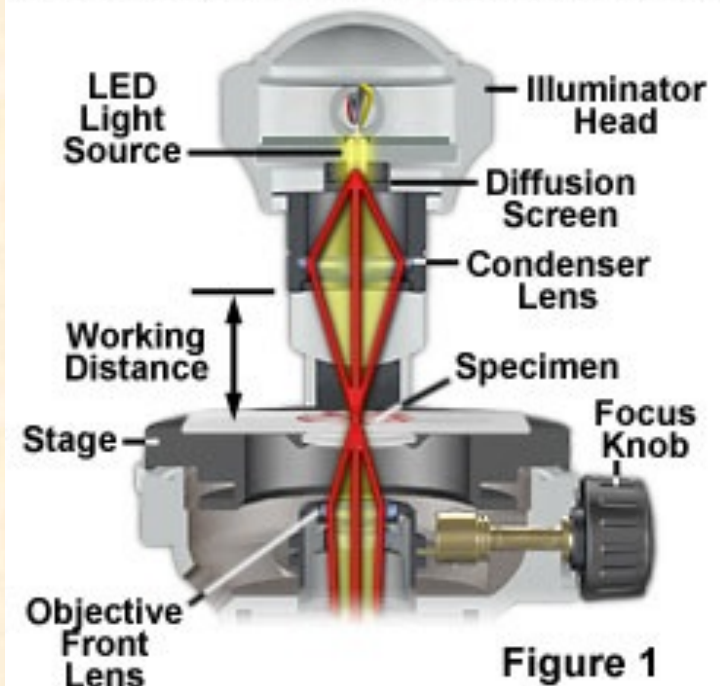
- Parts
  - Plano de la muestra
  - Condensador
  - Diafragma
  - Fuente de luz



Condiciones de Iluminacion:

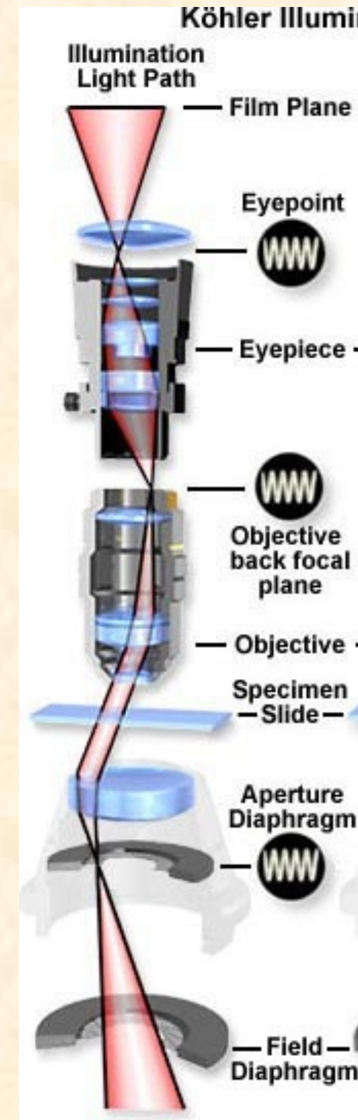
- Iluminacion critica
  - El condensador enfoca la luz en el plano del especimen.

Se ve la imagen del filamento de luz

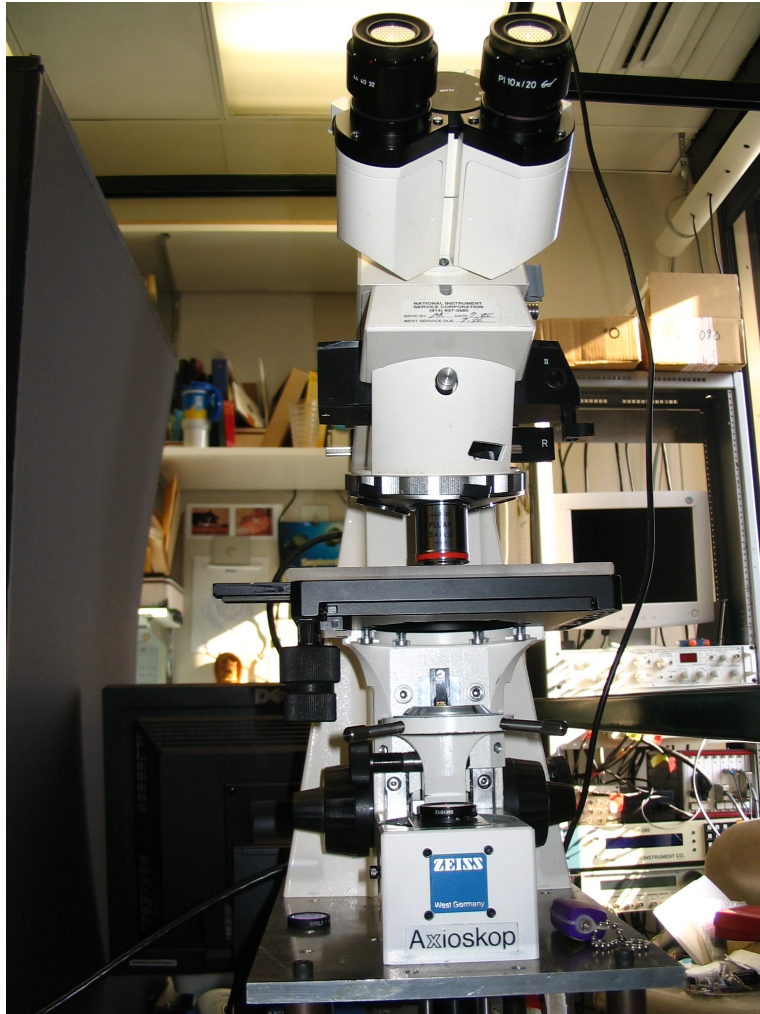


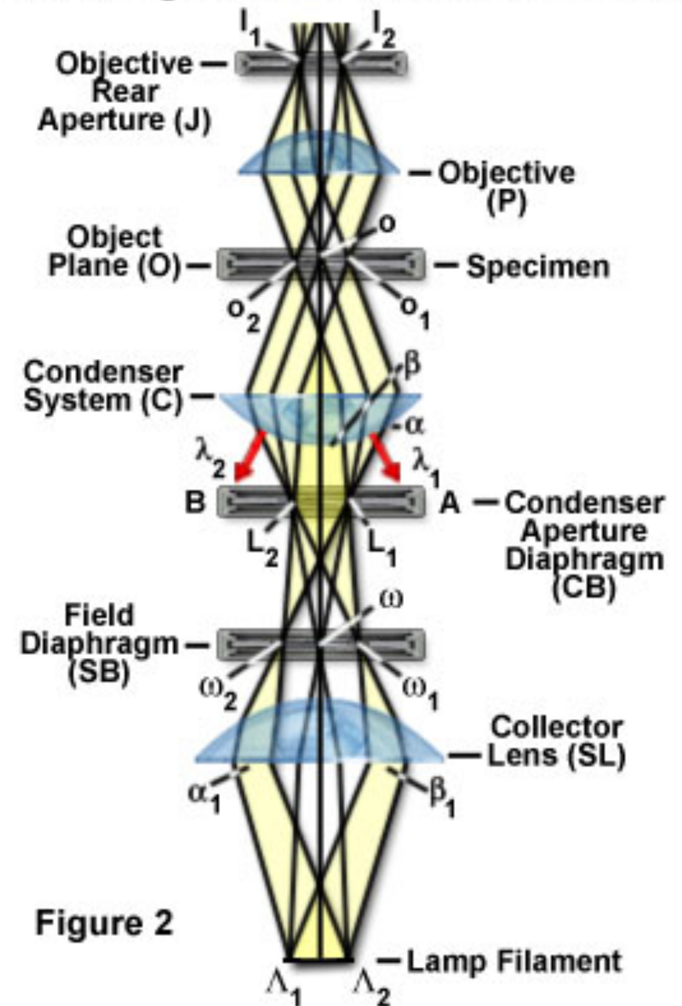
Köhler illumination

- La iluminación del especimen es uniforme
- Las imágenes del especimen y de la lámpara están en distintos planos.



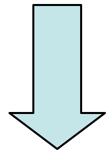
Iluminación Köhler

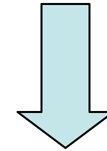


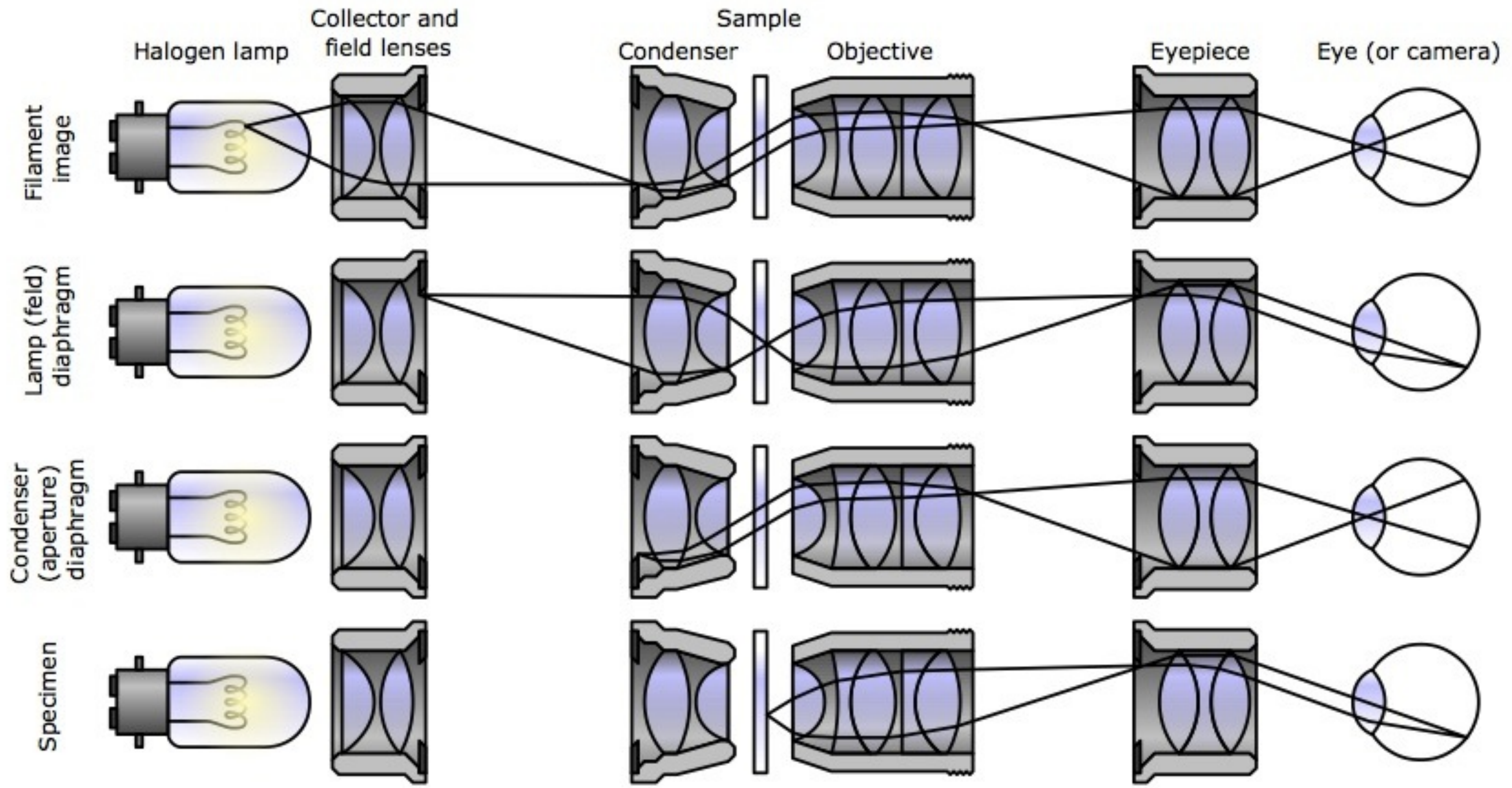


El propósito de la iluminación Köhler es generar una iluminación muy pareja de la muestra asegurándose de que la fuente (por ejemplo, el filamento de una lámpara) no sea visible en la imagen adquirida. Para eso hace que la imagen de la fuente esté totalmente desenfocada en el plano de la muestra y sus planos conjugados.

Planos conjugados

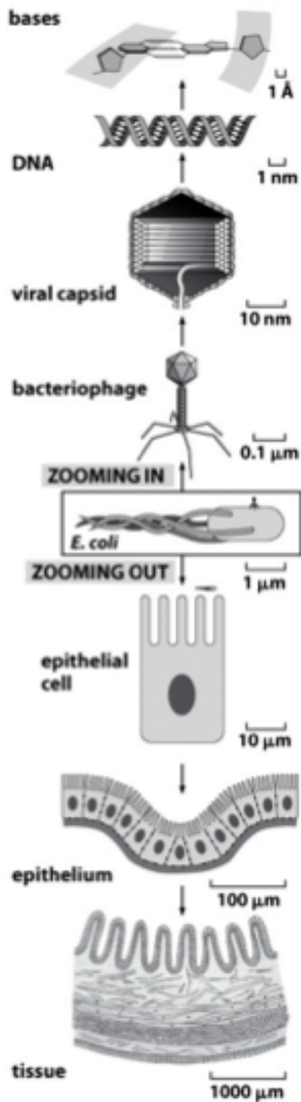


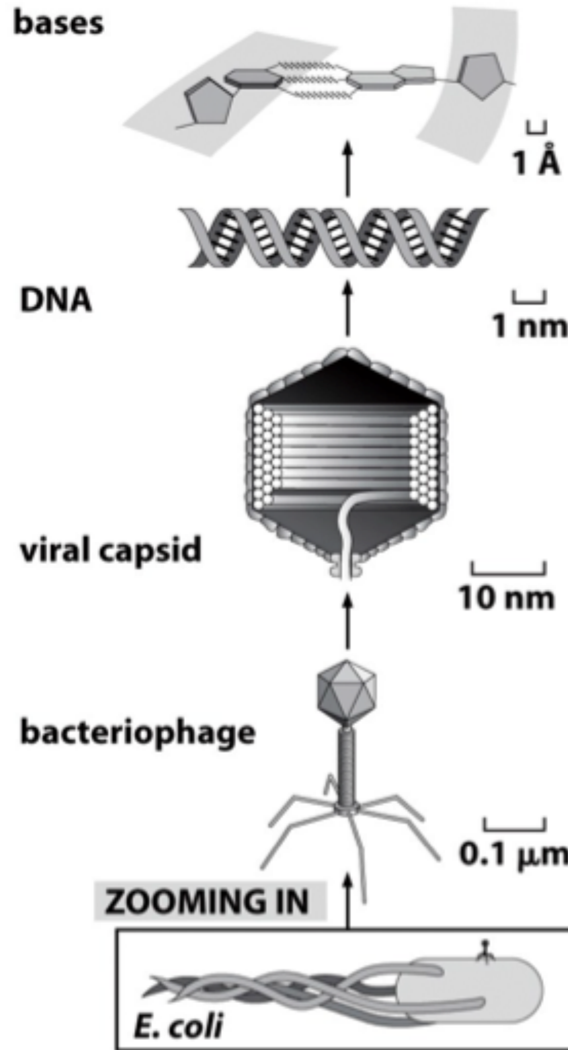


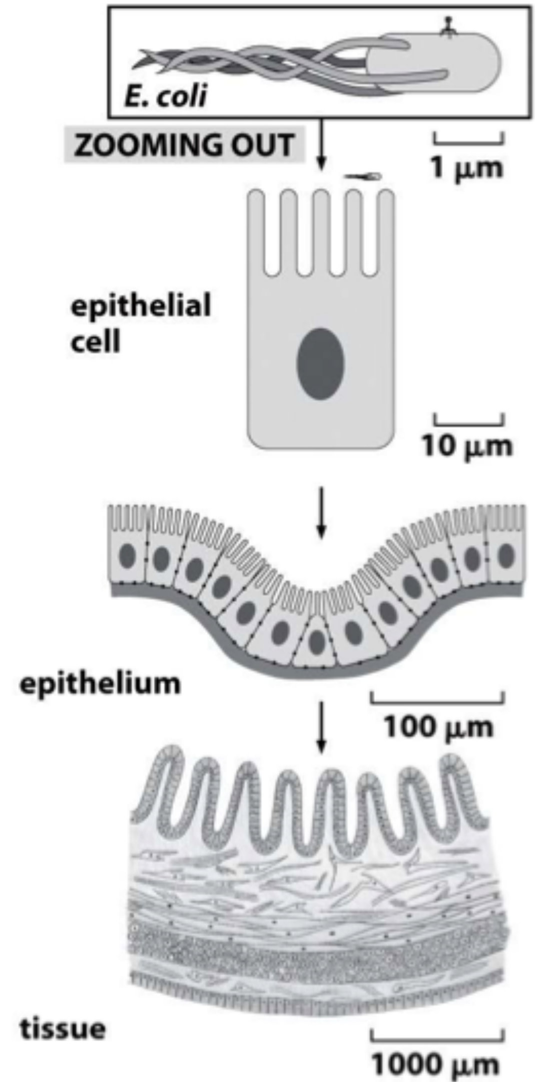


De Wikipedia

Biological Scale







http://learn.genetics.utah.edu/content/begin/cells/scale/

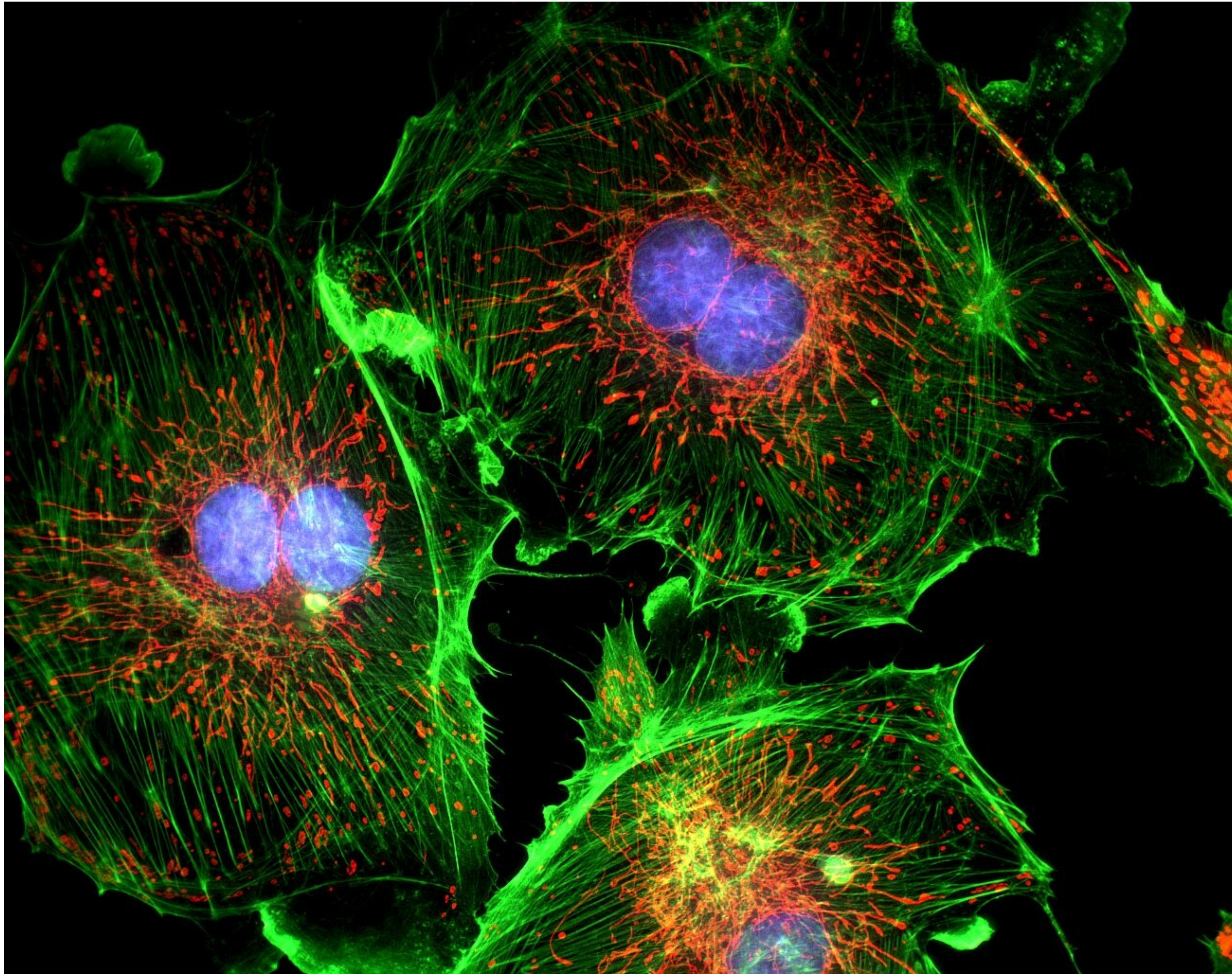
Hay especímenes que no absorben luz. Existen distintas opciones para observarlos (microscopía de contraste de fase, scattering, etc).

Otra solución para poder ver con microscopios (ópticos) células, tejidos u organismos:

Marcarlos con alguna sustancia fluorescente

Pero para entender esto necesitamos saber algo más sobre la interacción entre la luz y la materia

Microscopía de Fluorescencia lifefaculty.biu.ac.il/



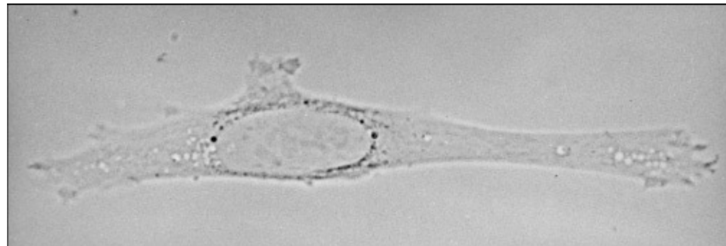
Distintas técnicas ópticas

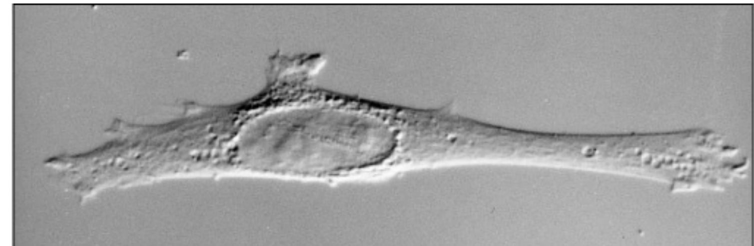
- Brightfield - absorption
- Darkfield - scattering
- Phase Contrast - phase interference
- Polarization Contrast - polarization
- Differential Interference Contrast (DIC) - polarization + phase interference
- Fluorescence Contrast - fluorescence

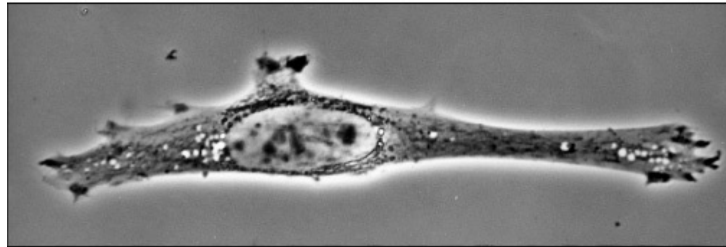
Algunas imágenes

Bright-field

DIC


(A)


(C)


(B)

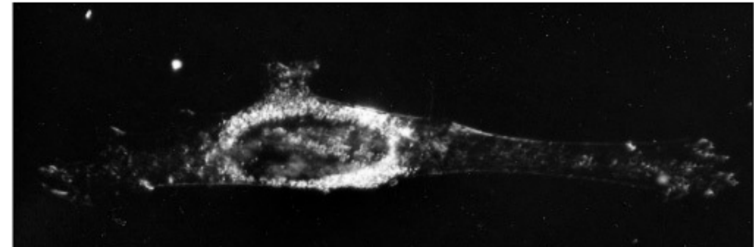

(D)

Figure 9–8 part 1 of 2. Molecular Biology of the Cell, 4th Edition.

Figure 9–8 part 2 of 2. Molecular Biology of the Cell, 4th Edition.

Phase-contrast

Dark-field

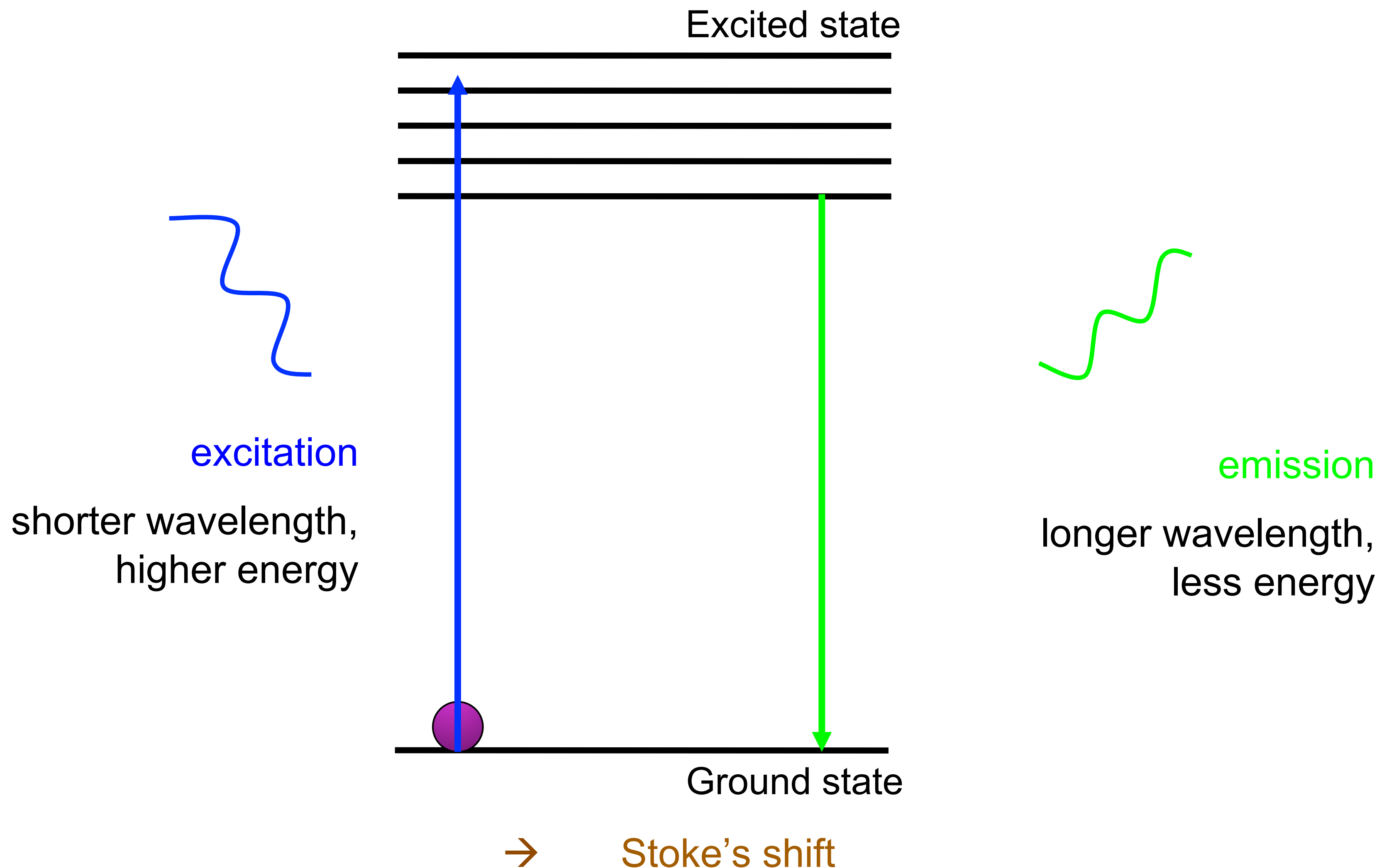
Técnicas de microscopia de fluorescencia

- Standard techniques: wide-field
  confocal
  2-photon

- Special applications: FRET
  FLIM
  FRAP
  Photoactivation
  TIRF

Fluorescencia

Es la emisión de una molécula en una longitud de onda al ser excitada con luz de otra longitud de onda



Fluorescencia

Fundamental Concepts Underpinning Fluorescence Microscopy

Jablonski Energy Diagram

Excitation (Absorption) 10^{-15} Seconds

Internal Conversion and Vibrational Relaxation (10^{-14} - 10^{-11} Sec)

Fluorescence (10^{-9} - 10^{-7} Sec)

Intersystem Crossing

Quenching

Non-Radiative Relaxation

Excited Singlet States

S_2

S_1

S_0

Vibrational Energy States

Internal Conversion

Delayed Fluorescence

Excited Triplet State (T_1)

Intersystem Crossing

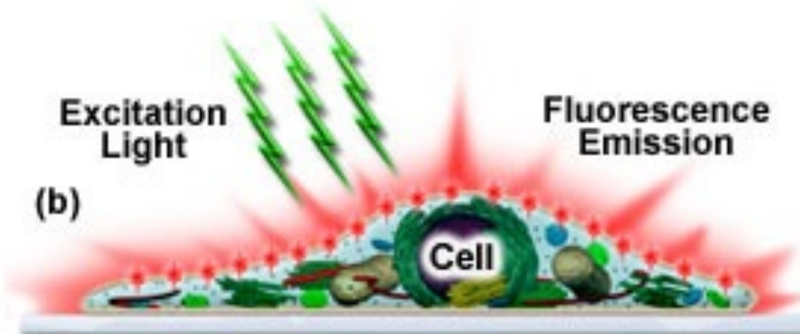
Non-Radiative Relaxation (Triplet)

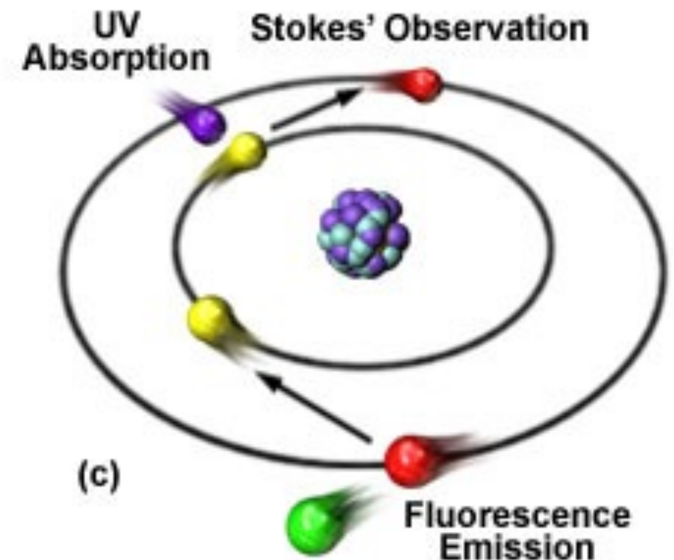
Phosphorescence (10^{-3} - 10^{2} Sec)

(a)

Ground State

Figure 1





Algunas moléculas son naturalmente fluorescentes

Proteínas:

Heme proteins (hemoglobin, myoglobin, cytochrome C, hemocyanin)

Redox & photoproteins (phytochromes, phycoerythrin, phycocyanin, green fluorescent protein (http://www.ucmb.ulb.ac.be/.../ GFP/Fluorophore.gif; http://www-bioc.rice.edu/.../Phillips/ Papers/gfpbio.html; dwb.unl.edu/.../projects/ gmocz/gfp.htm), phycobiliproteins, rhodopsin, ferredoxins)

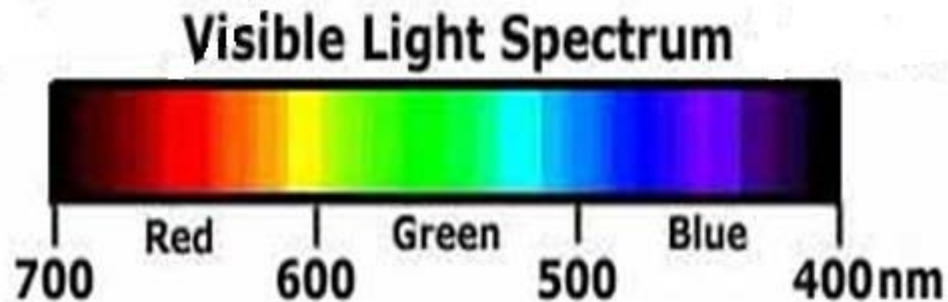
Pigmentos:

flavins, stilbenes, tryptophan, tyrosine, purines..

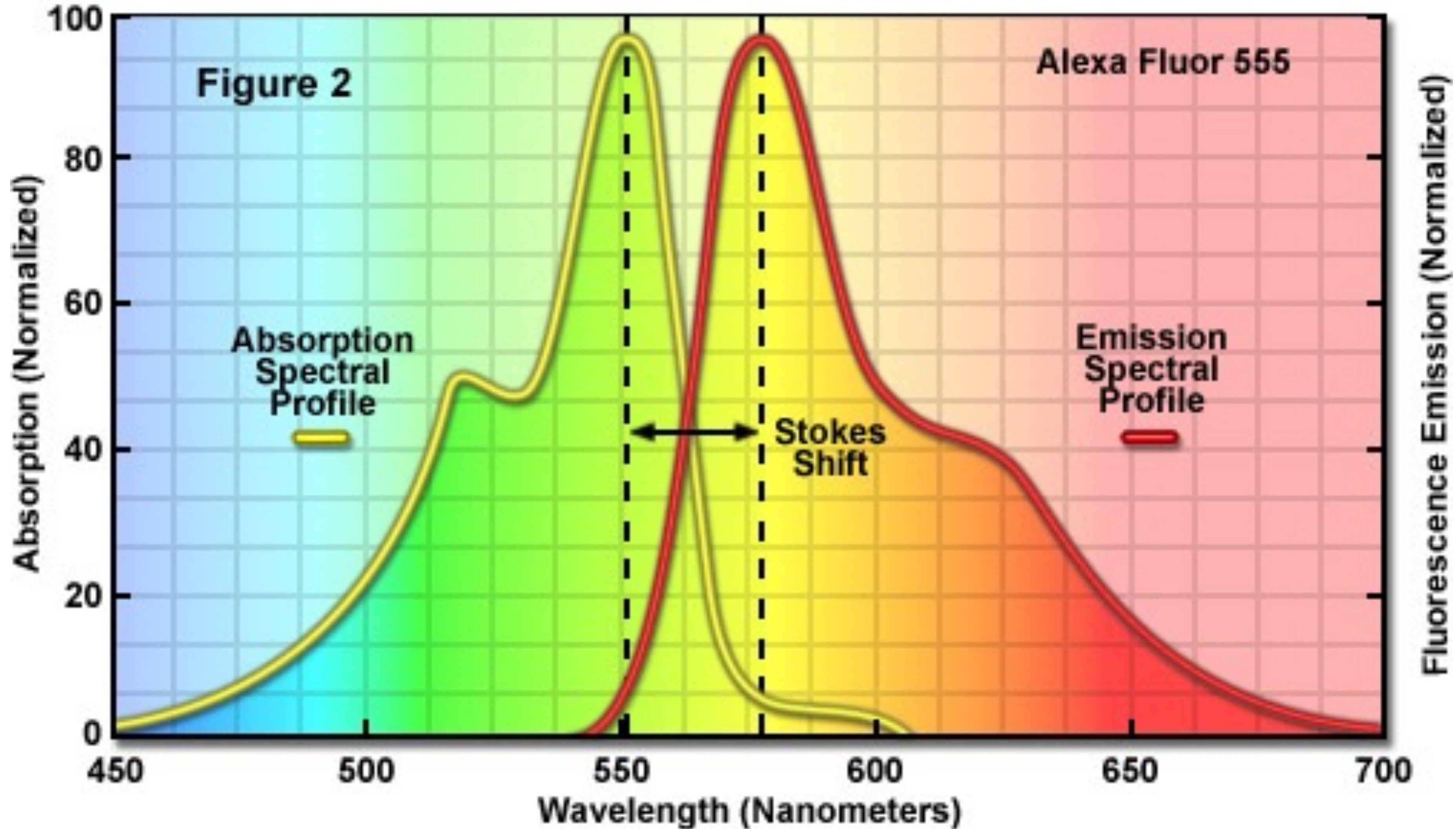
También se pueden sintetizar moléculas fluorescentes (ya veremos cómo usarlas)

Fluoróforos (Fluorochromes, chromophores)

- Tienen una estructura particular
- Están caracterizados por un espectro de excitación y uno de emisión

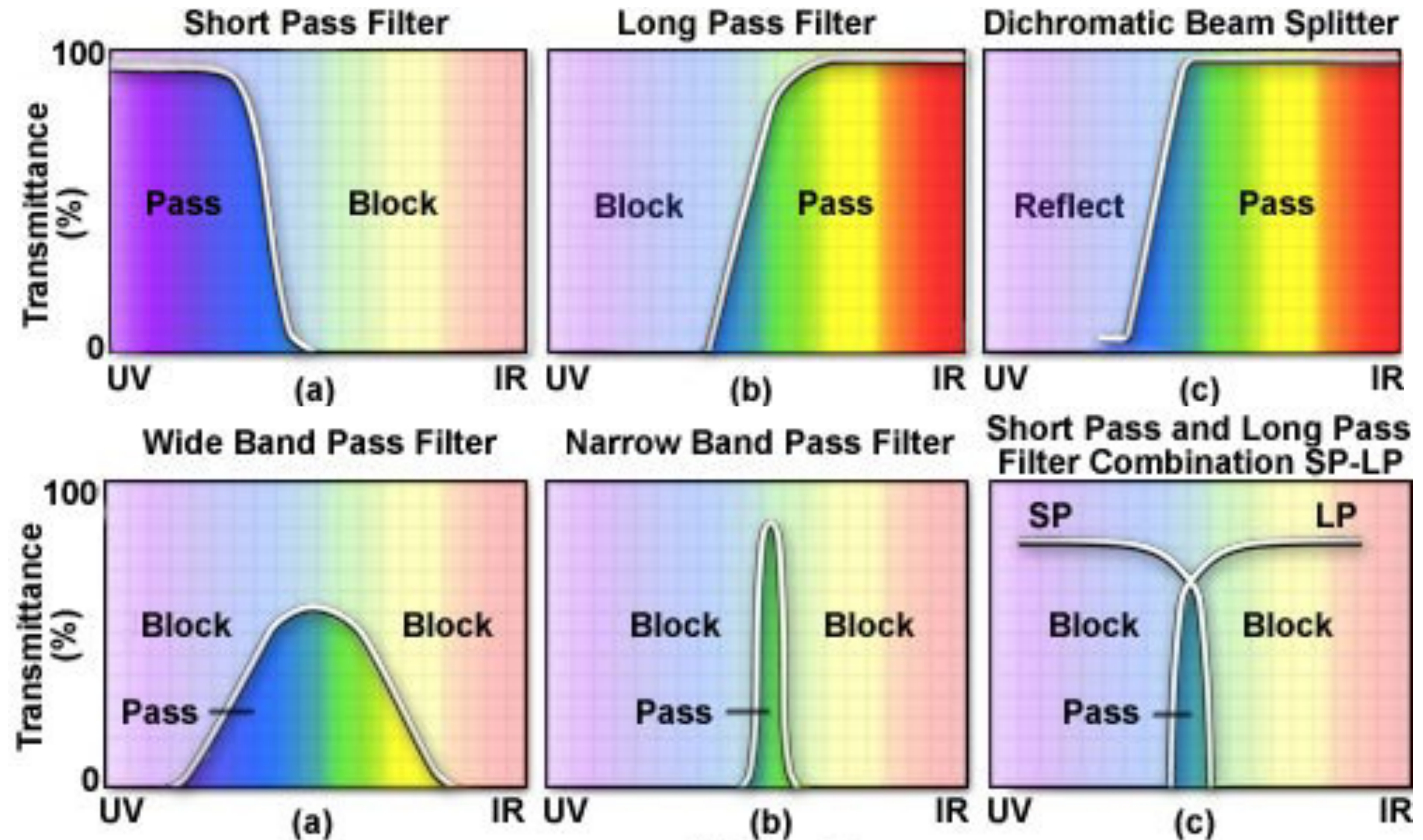


Fluoróforos



Filtros

Se usan para separar la luz dentro de un rango de longitudes de onda

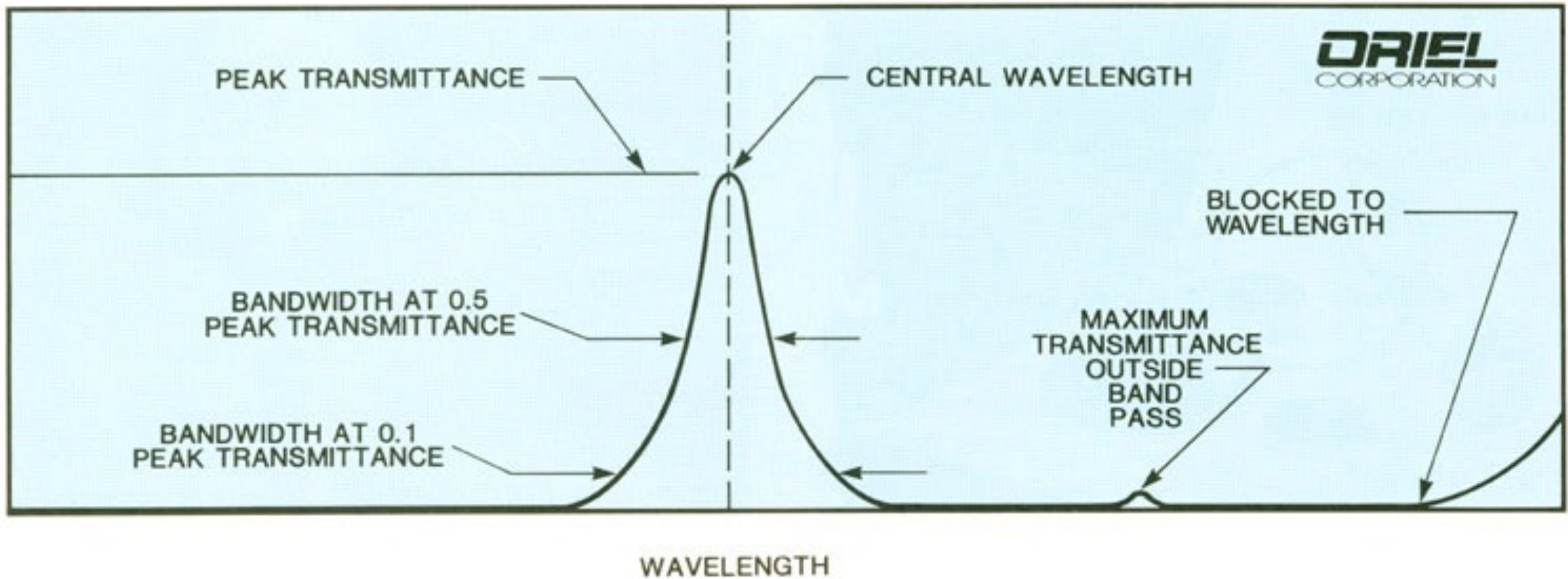


Filtros, nomenclatura

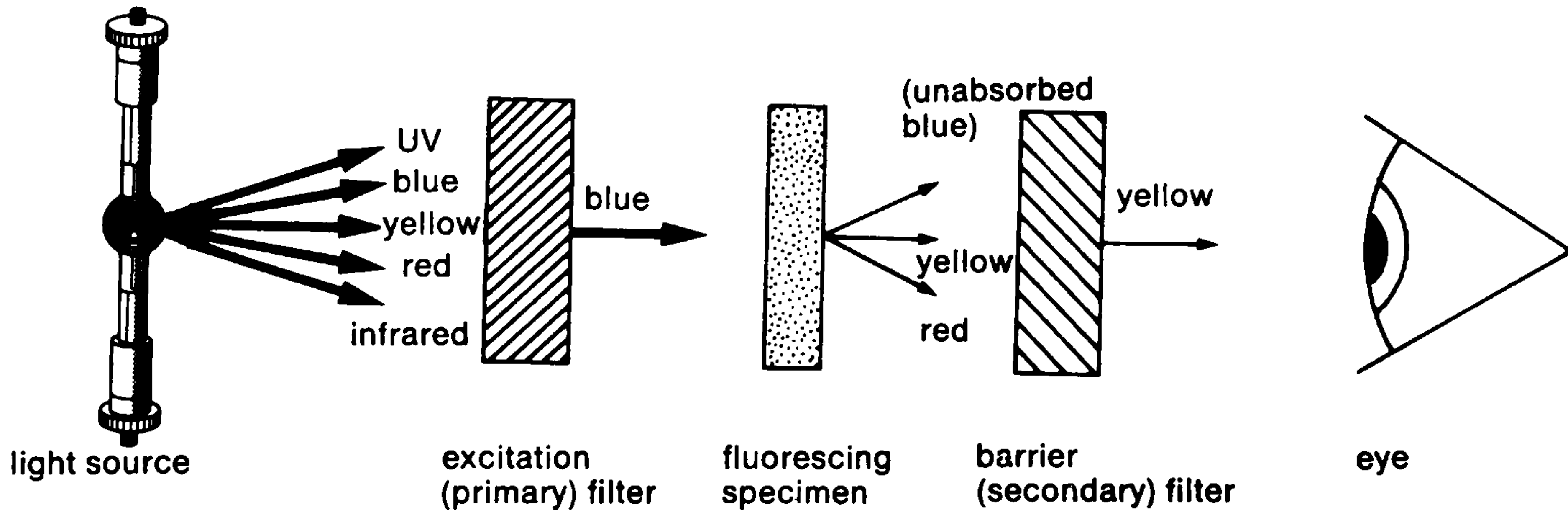
- Filtros de excitación: x
- Filtros de emisión: m
- Beamsplitter (espejo dicroico): bs, dc, FT

- 480/30 = the center wavelength is at 480nm; full bandwidth is 30 [= +/- 15]
- BP = bandpass, light within the given range of wavelengths passes through (BP 450-490)
- LP = indicates a longpass filter which transmits wavelengths longer than the shown number and blocks shorter wavelengths (LP 500)
- SP = indicates a shortpass filter which transmits wavelengths shorter than the shown number, and blocks longer wavelengths

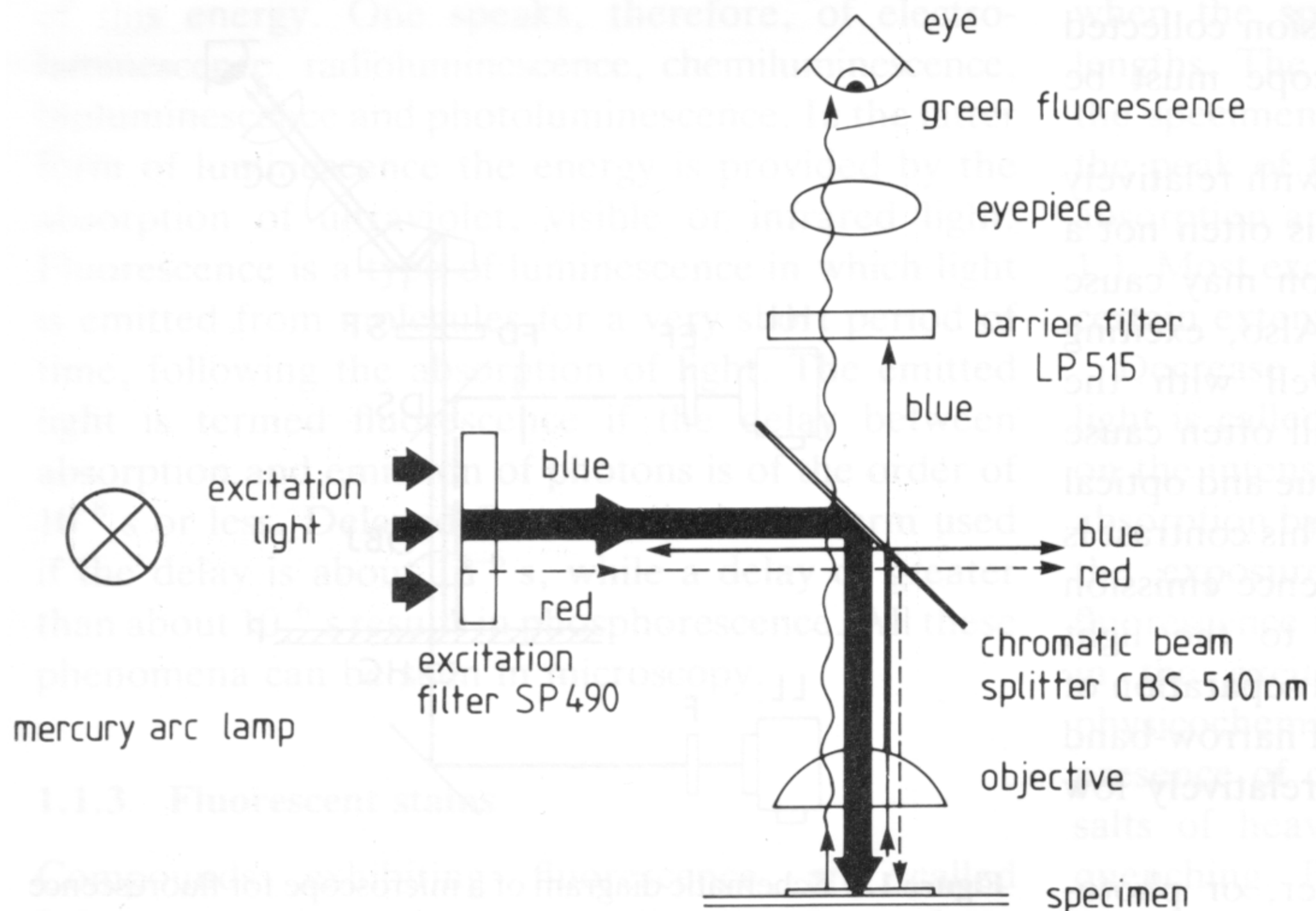
Filtros, nomenclatura



Idea básica

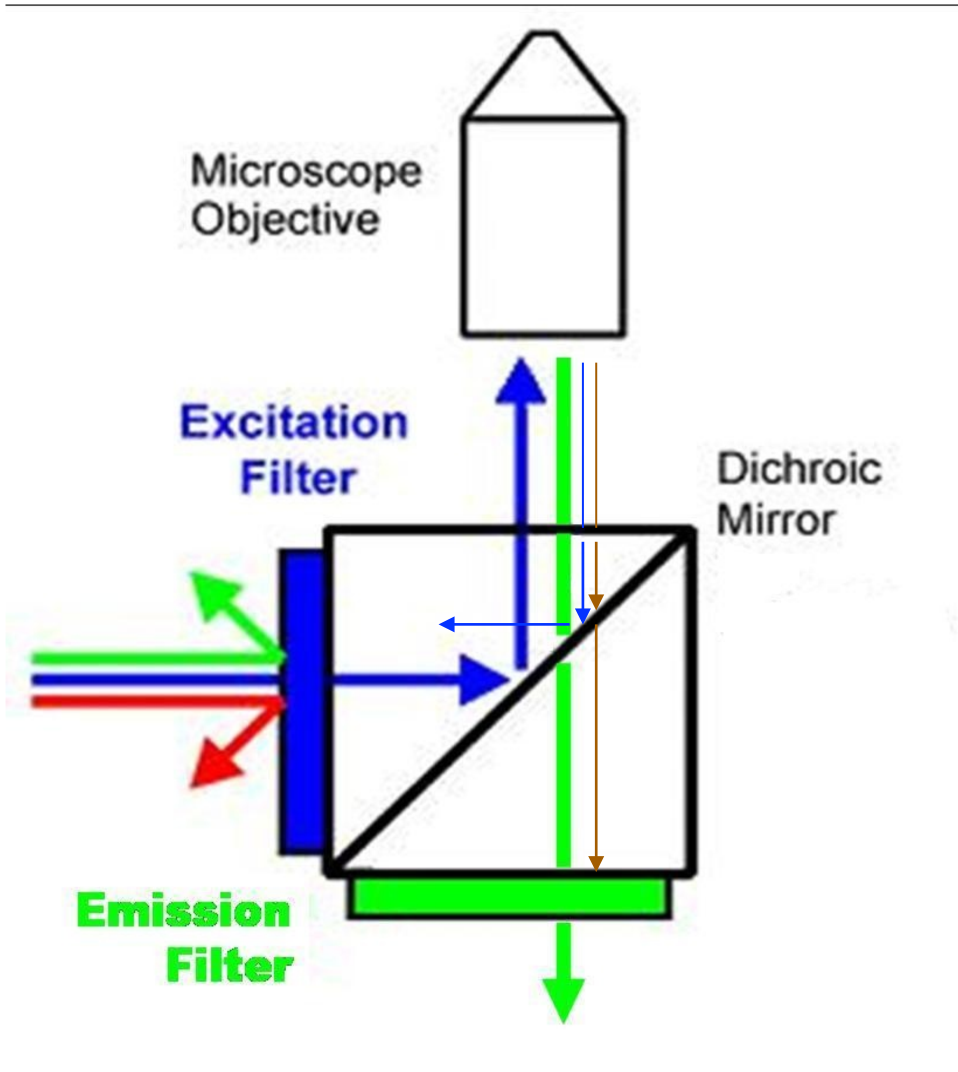


Diseño básico para epi-fluorescencia



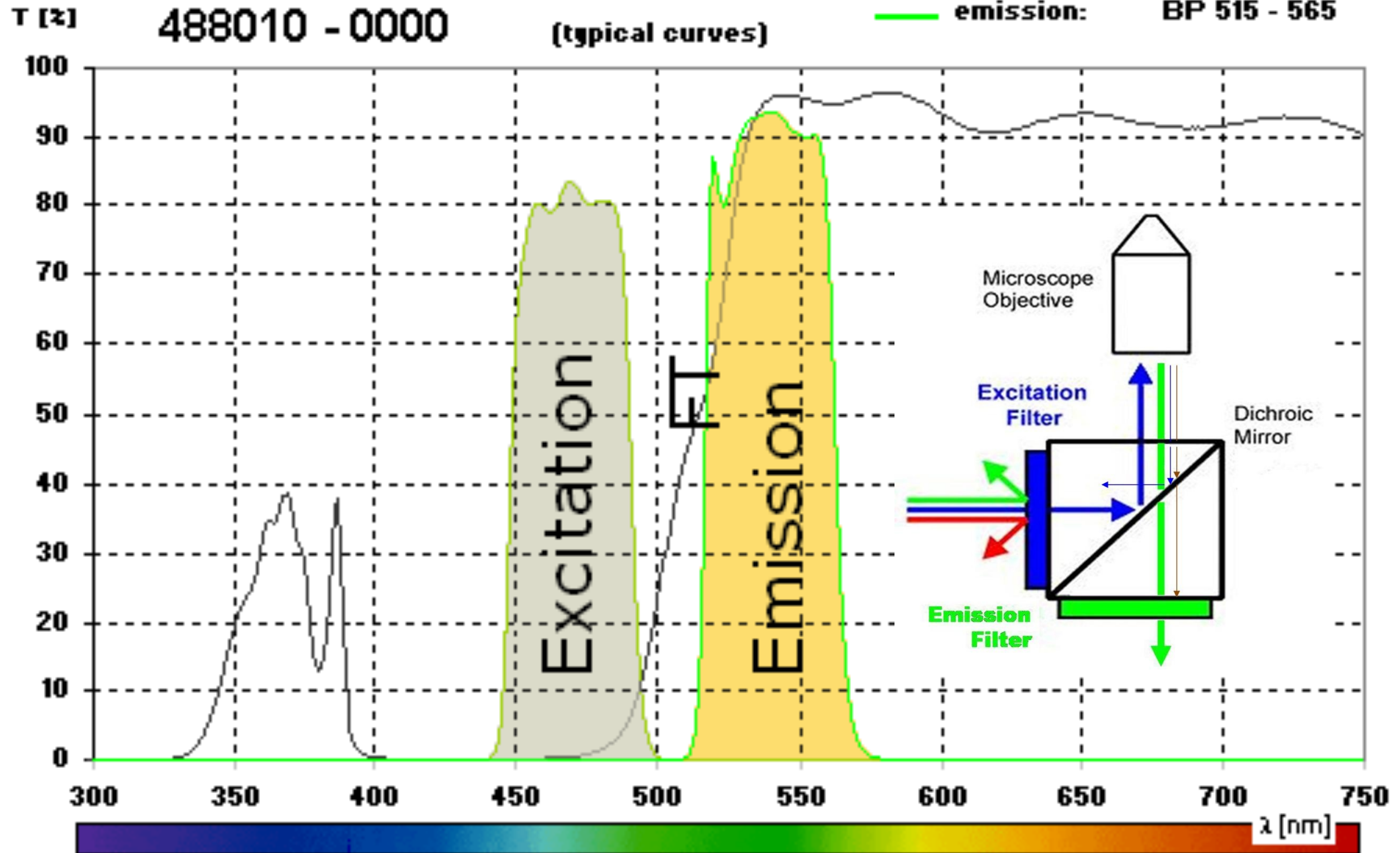
El objetivo actúa como condensador; la luz de la excitación es reflejada para que no llegue al ojo

El cubo

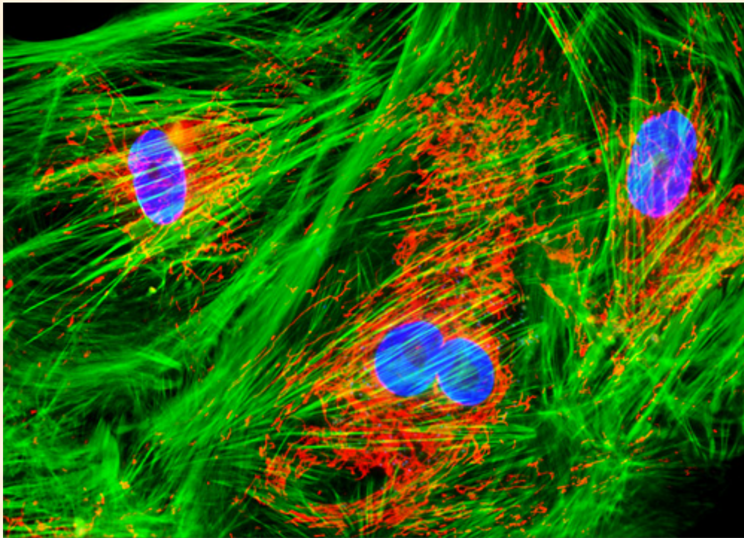


a) Filtro de excitación
b) Espejo dicroico
c) Filtro de emisión

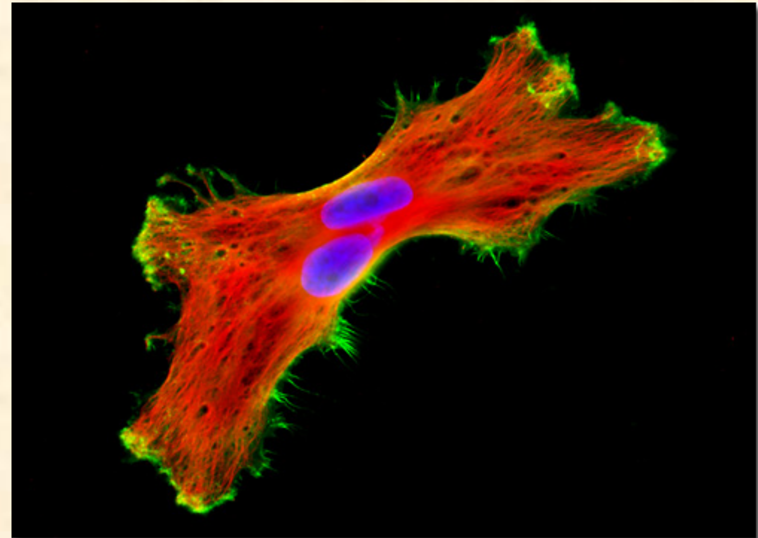
El cubo



Fluorescent Microscopic images (1)

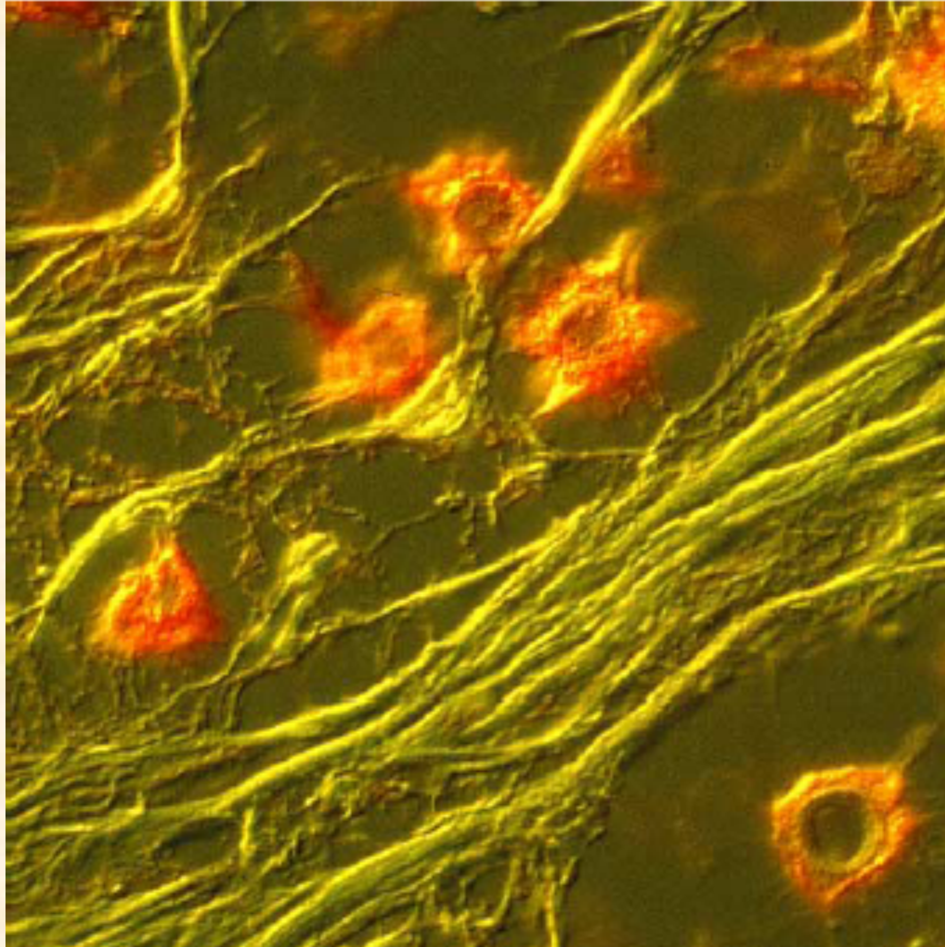


Human cortical neurons



Human brain glioma cells

Fluorescent Microscopic images



Fluorescence/DIC combination, cat brain tissue infected with Cryptococcus

Photobleaching

- **<u>Photobleaching ("blicheo")</u> - Cuando un fluoróforo pierde su propiedad fluorescente debido al daño producido por la propia luz de excitación.**

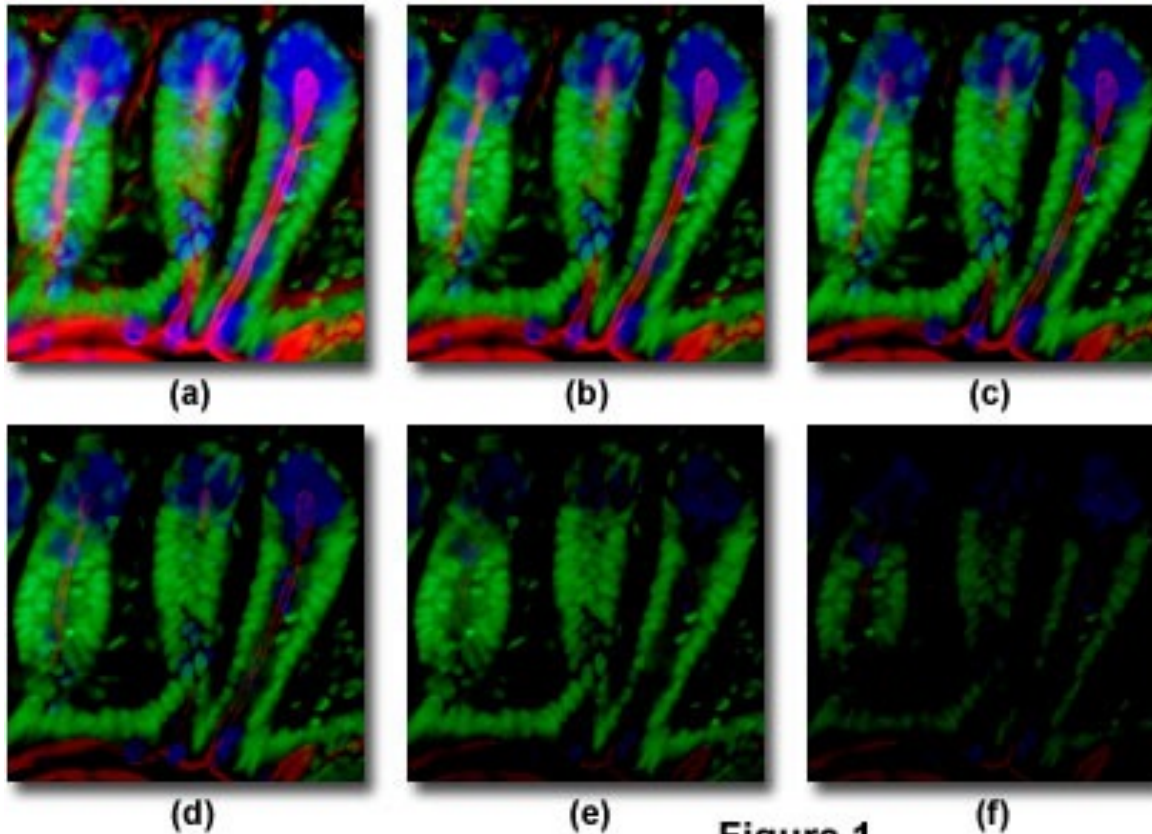
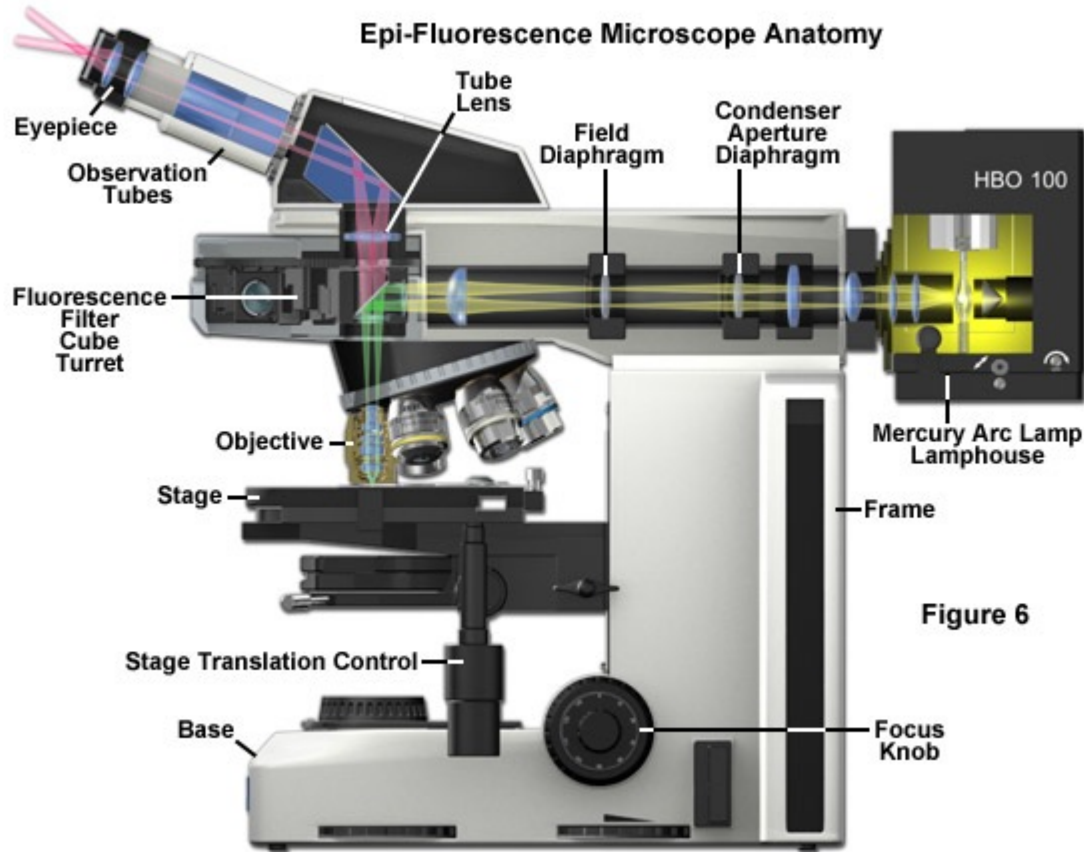


Figure 1

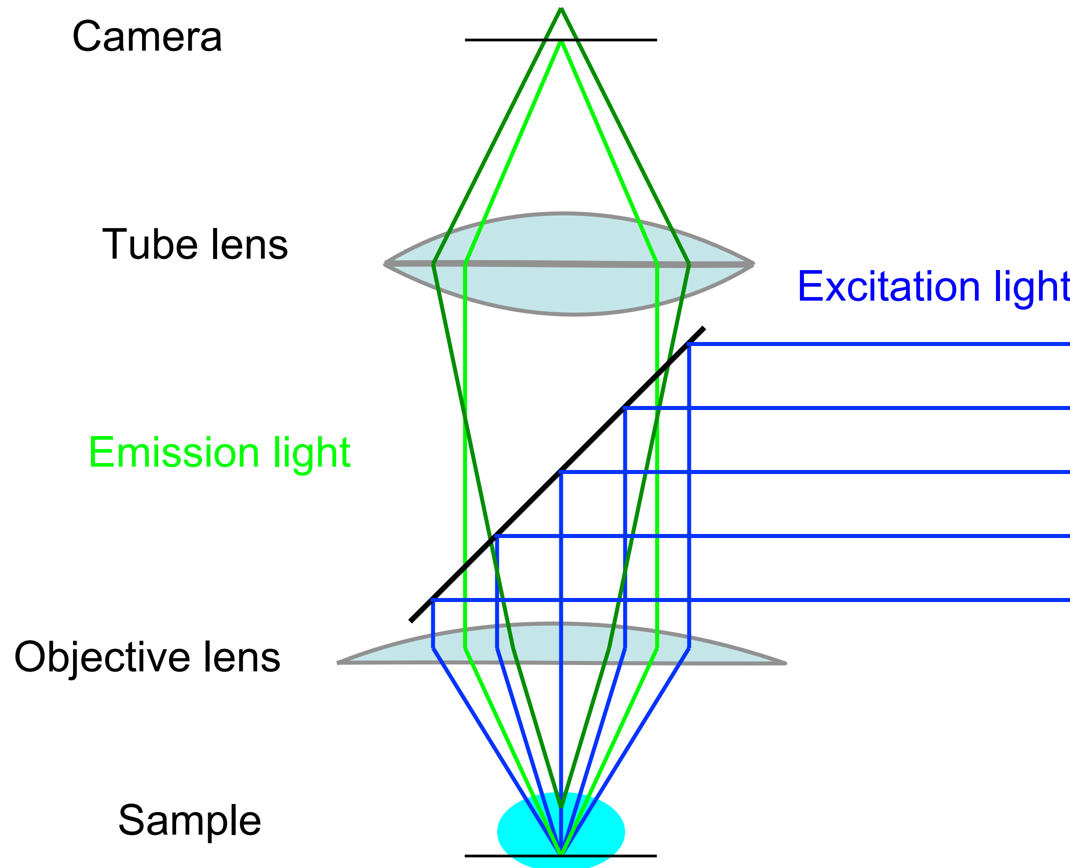
Técnicas standard

- wide-field
- Confocal
- Spinning disk confocal
- 2-photon

Wide-field fluorescence



- reflected light method
- Multiple wavelength source (polychromatic, i.e. mercury lamp)
- Illumination of whole sample



Problema – la fluorescencia es emitida a lo largo de todo el cono de iluminación.

Widefield vs iluminación de un punto

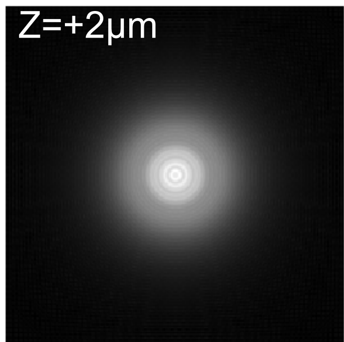
Pero, ¿cómo iluminamos toda la muestra con un punto? Escaneo

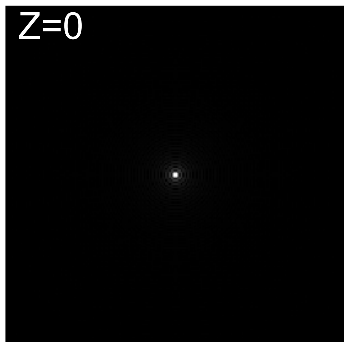
De todos modos, debido a la naturaleza ondulatoria de la luz la

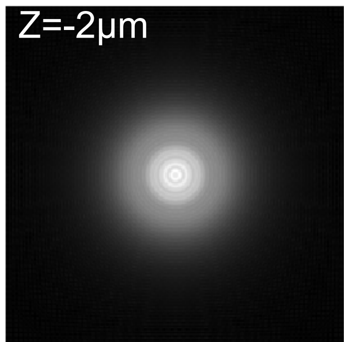
The Point Spread Function (PSF)

2D PSF
for different defocus

Imagen de un objeto puntual

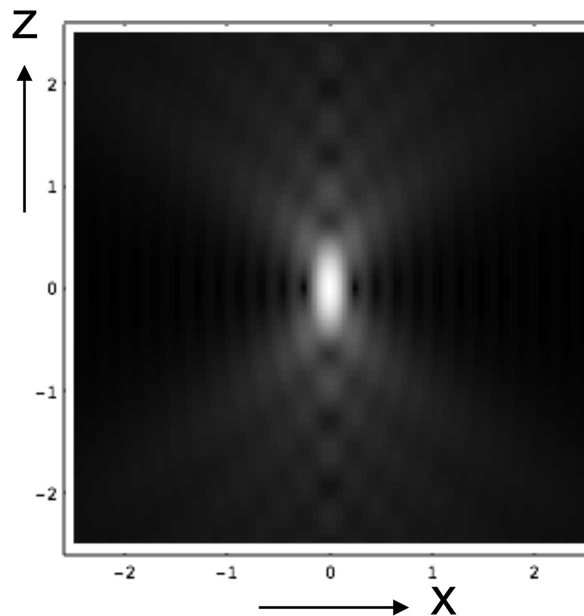




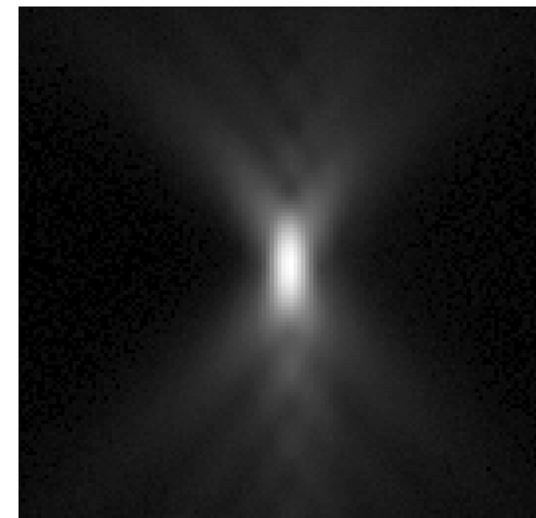


3D PSF

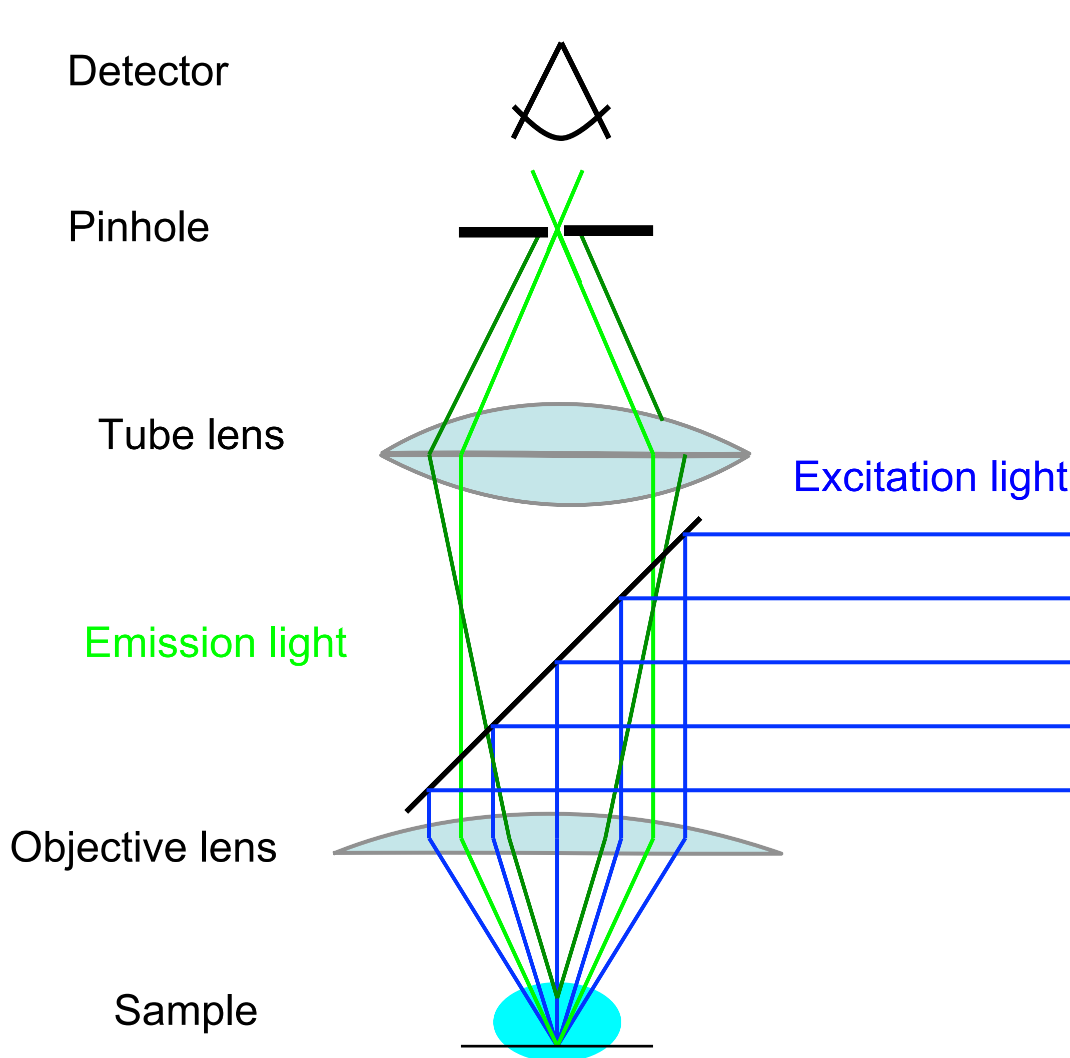
Calculated



Measured



The confocal microscope



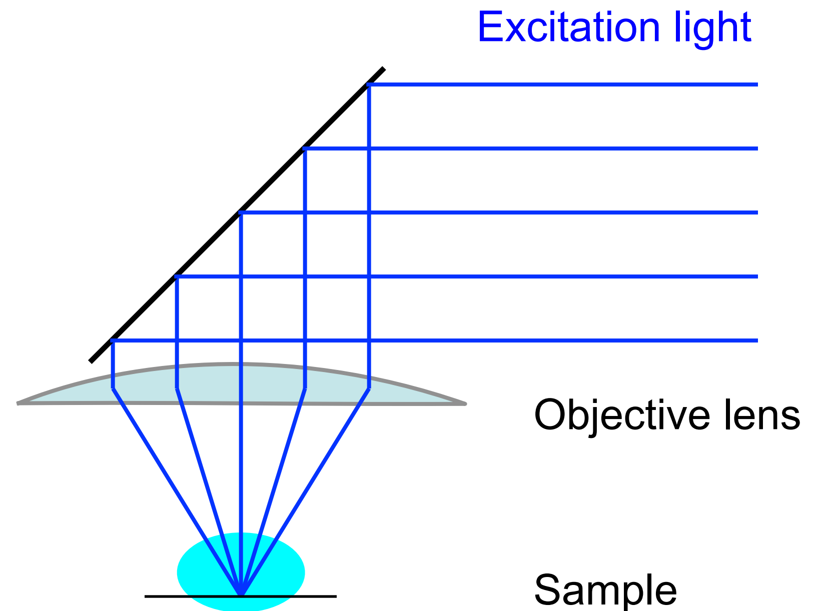
Scan excitation spot point-by-point to build up image

Fuentes de luz

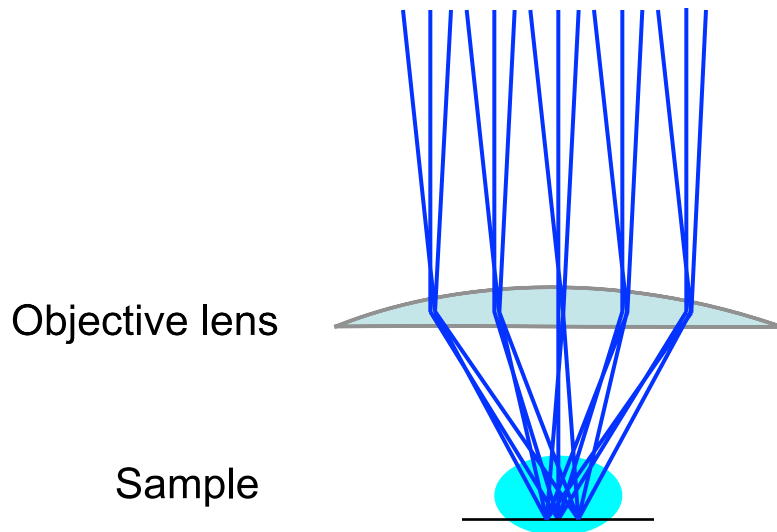
Excitation light must be focused to a diffraction limited spot

Could be done with an arc lamp and pinhole – but very inefficient

Enter the laser:
Perfectly collimated and high power



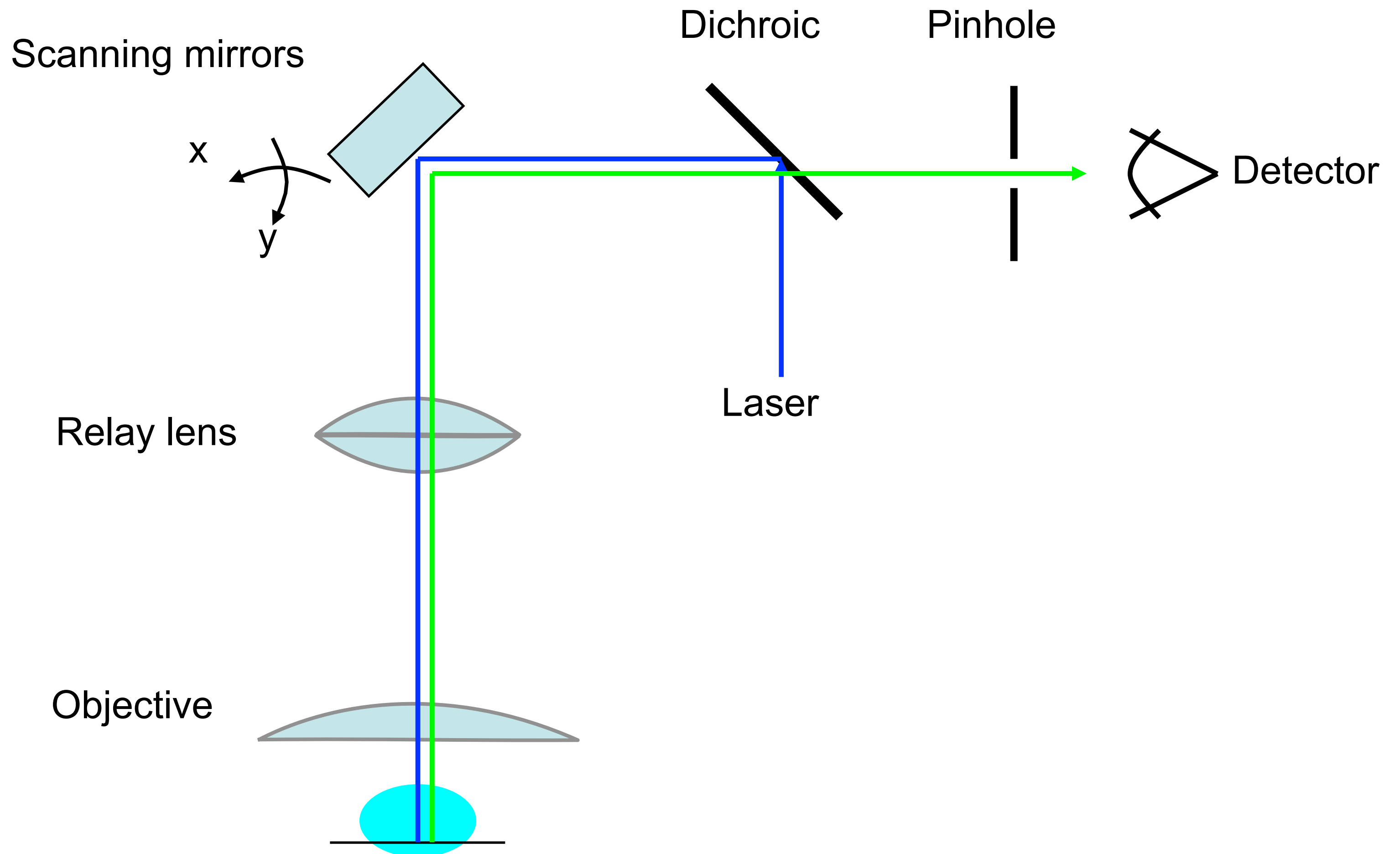
Scanning



Changing entrance angle of illumination moves illumination spot on sample

The emission spot moves, so we have to make sure pinhole is coincident with it

Confocal optical path

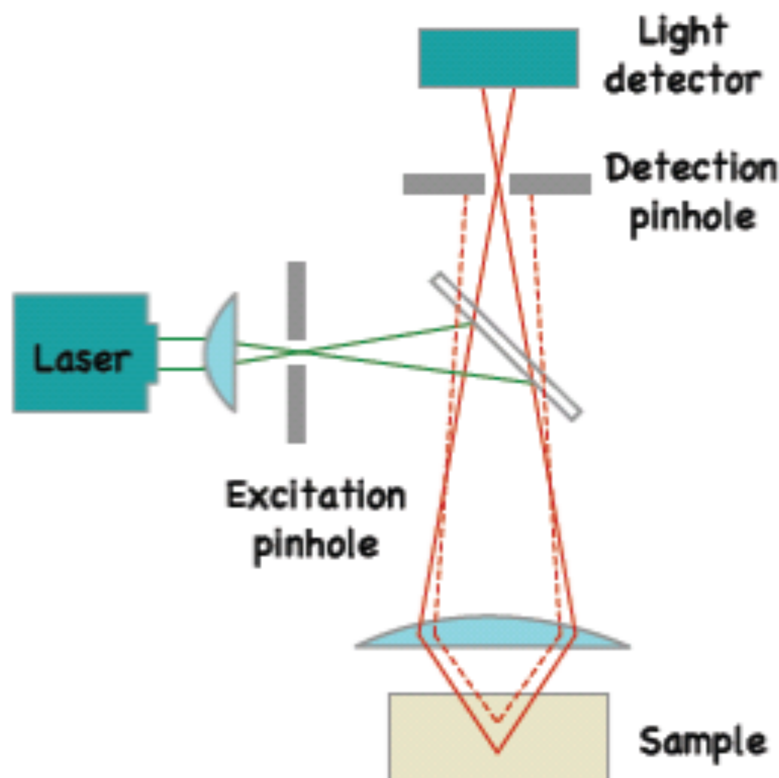


3D fluorescence microscopy

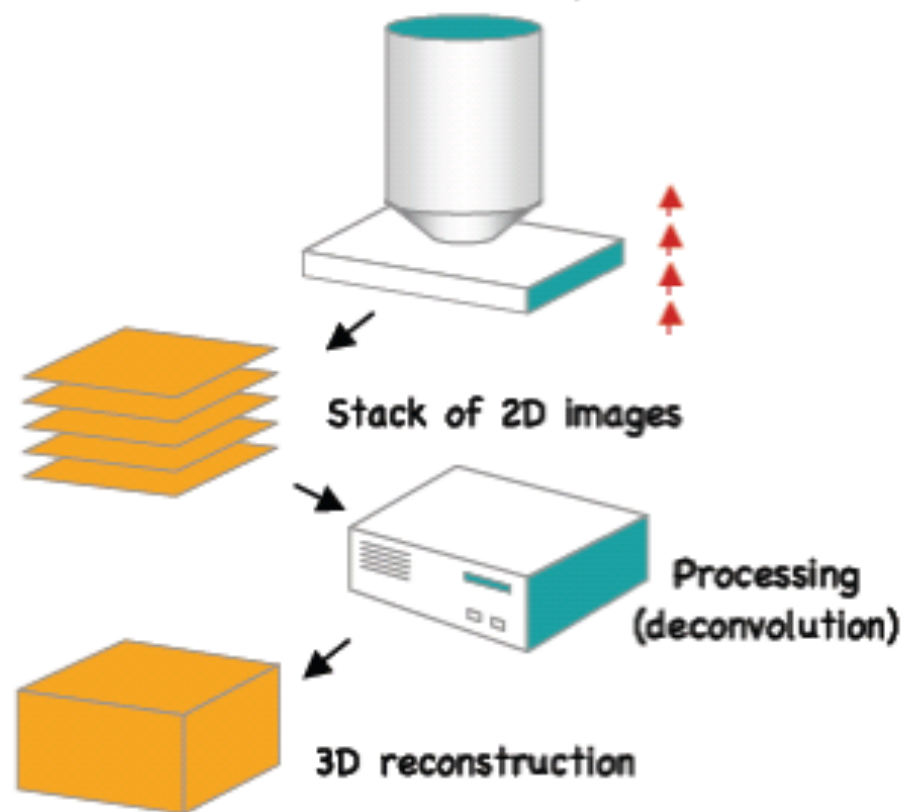
Acquire a "focal series" (stack) of images

Problem:
Each image contains out-of-focus blur from other focal planes

<u>Approach 1</u>
Physically exclude the blur
Example: <u>confocal</u> microscopy



<u>Approach 2</u>
Remove the blur computationally



How well can this be done?

Light source for fluorescence microscopy

Arc lamps

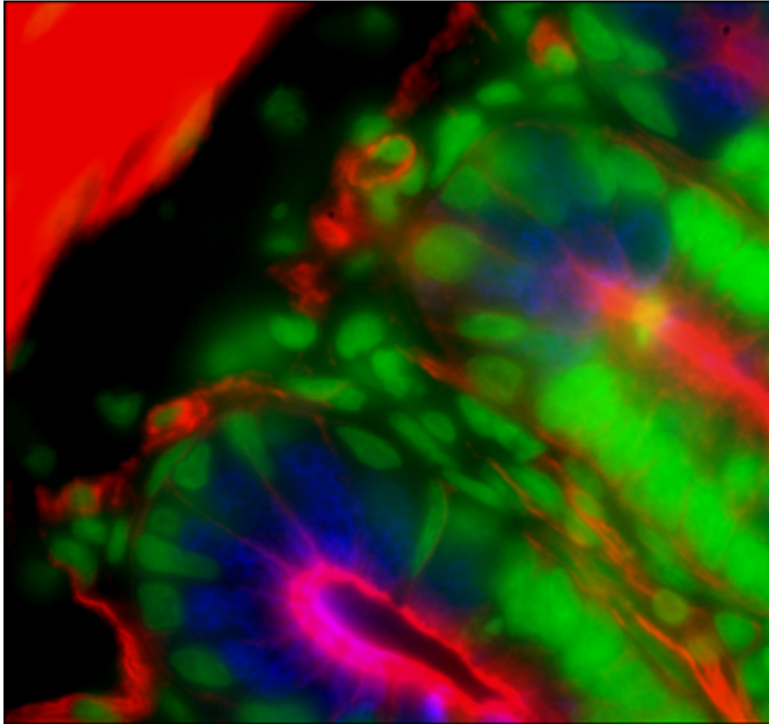
Xenon
Mercury

Laser types

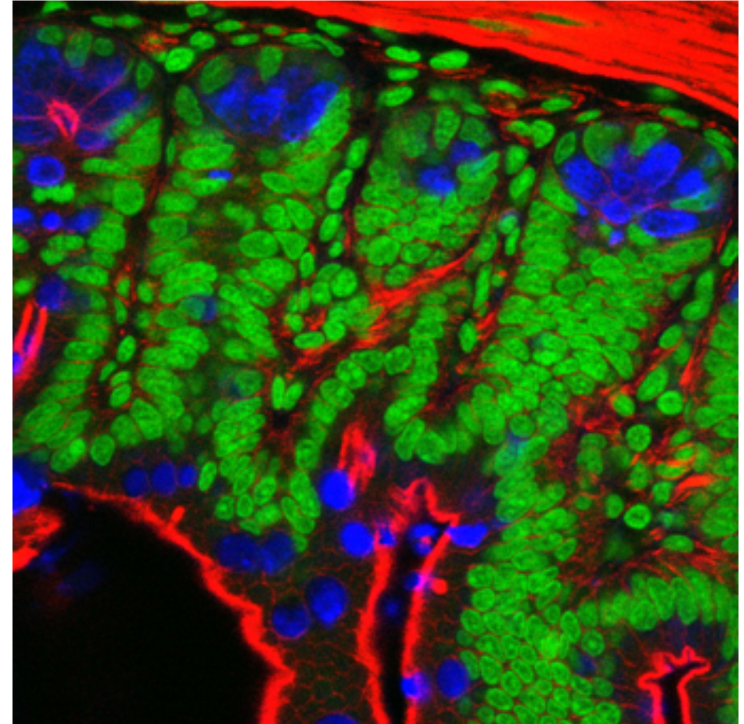
UV — IR

Laser	Wavelengths (nm)
Argon	351 364 457 477 488 514
Blue diode	405 440
Helium-Cadmium	354 442
Krypton-Argon	488 569 647
Green Helium-Neon	543
Yellow Helium-Neon	594
Orange Helium-Neon	612
Red Helium-Neon	633
Red diode	635 650
Ti:Sapphire	720-980

Wide-field vs confocal

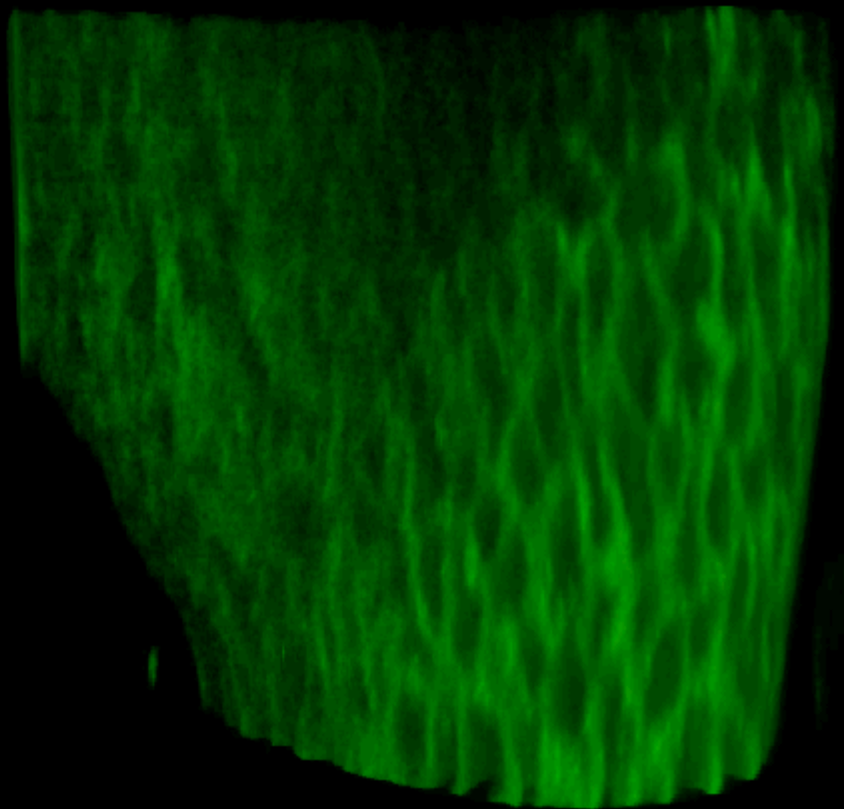


Wide-field image



confocal image

Molecular probes test slide Nr 4, mouse intestine

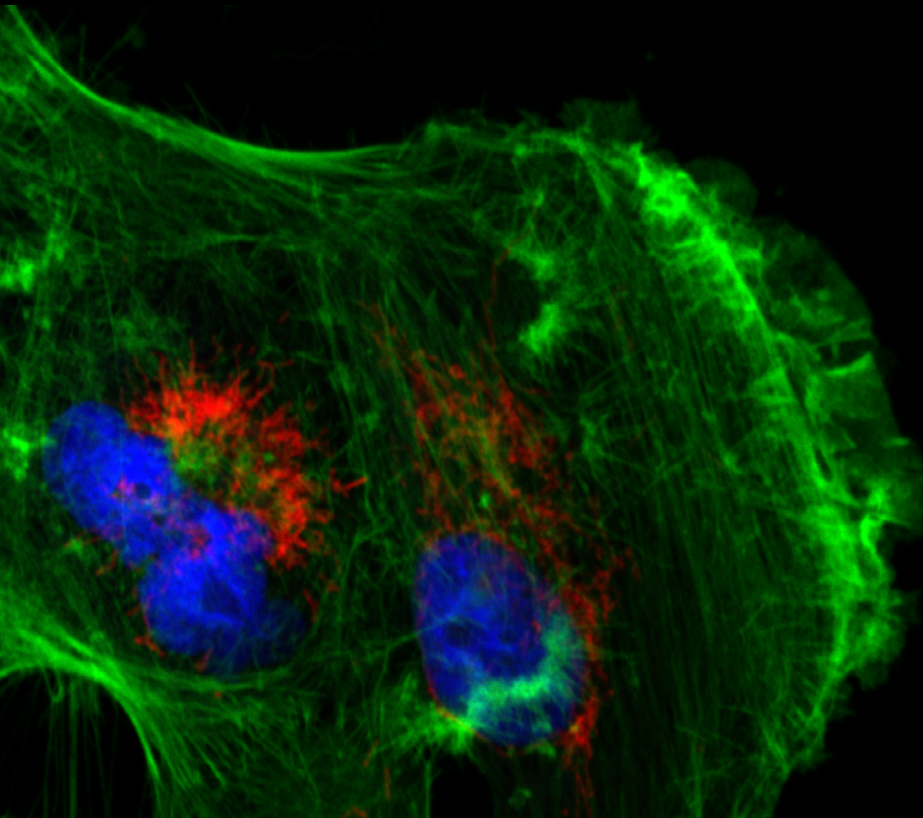


Laser-Scanning Confocal

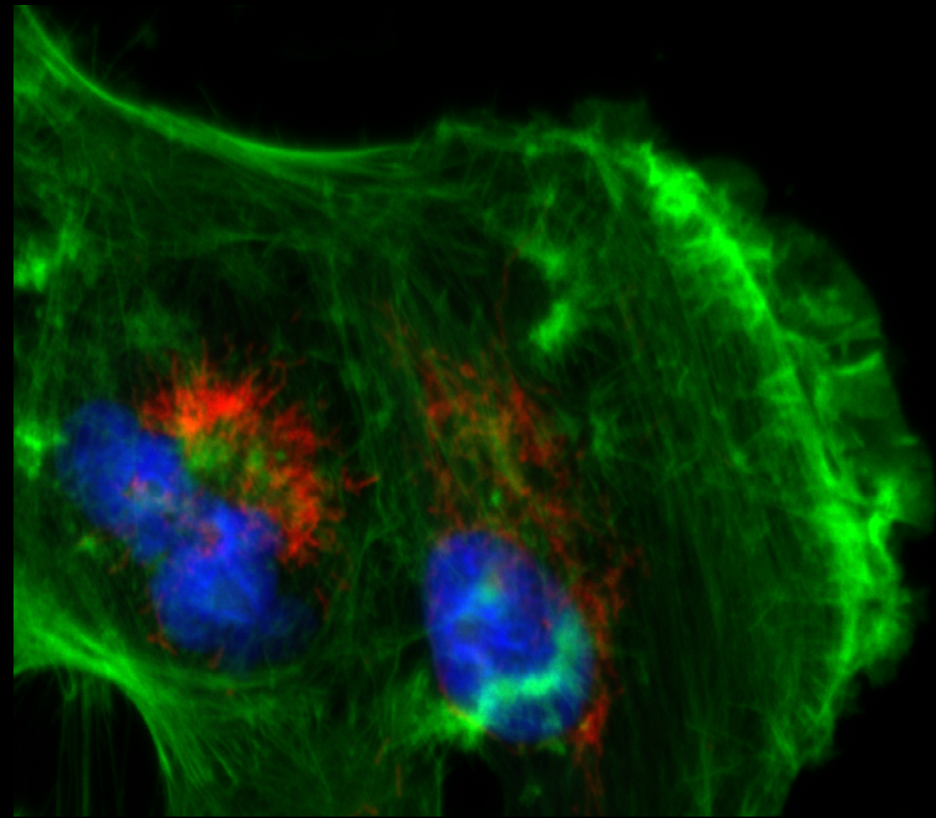
E11.5 mouse lung stained for E-cadherin

Nan Tang, Martin lab

Confocal vs. Widefield



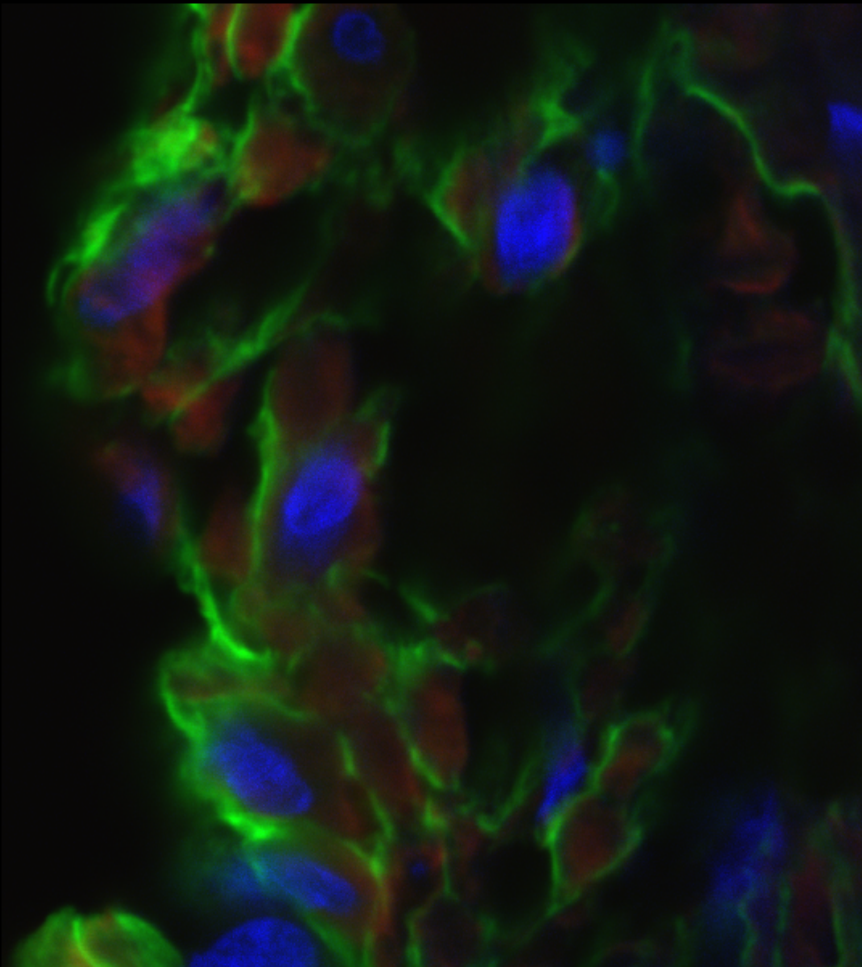
Confocal



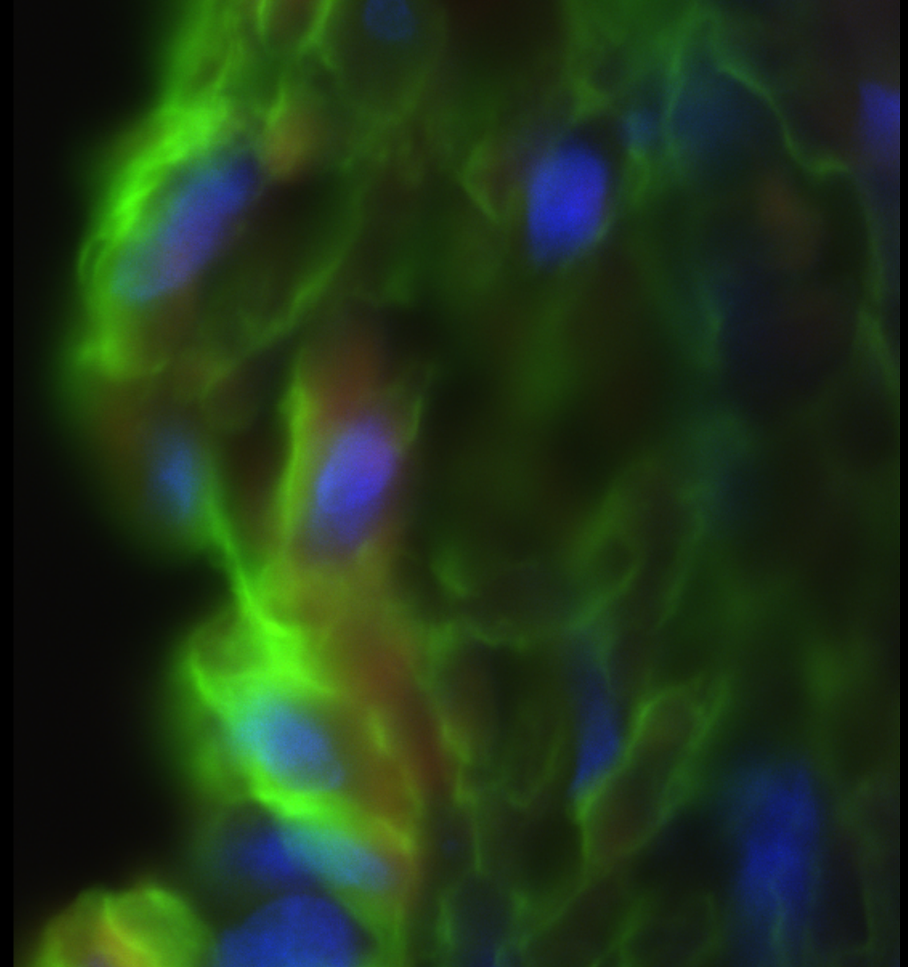
Widefield

Tissue culture cell with 60x / 1.4NA objective

Confocal vs. Widefield



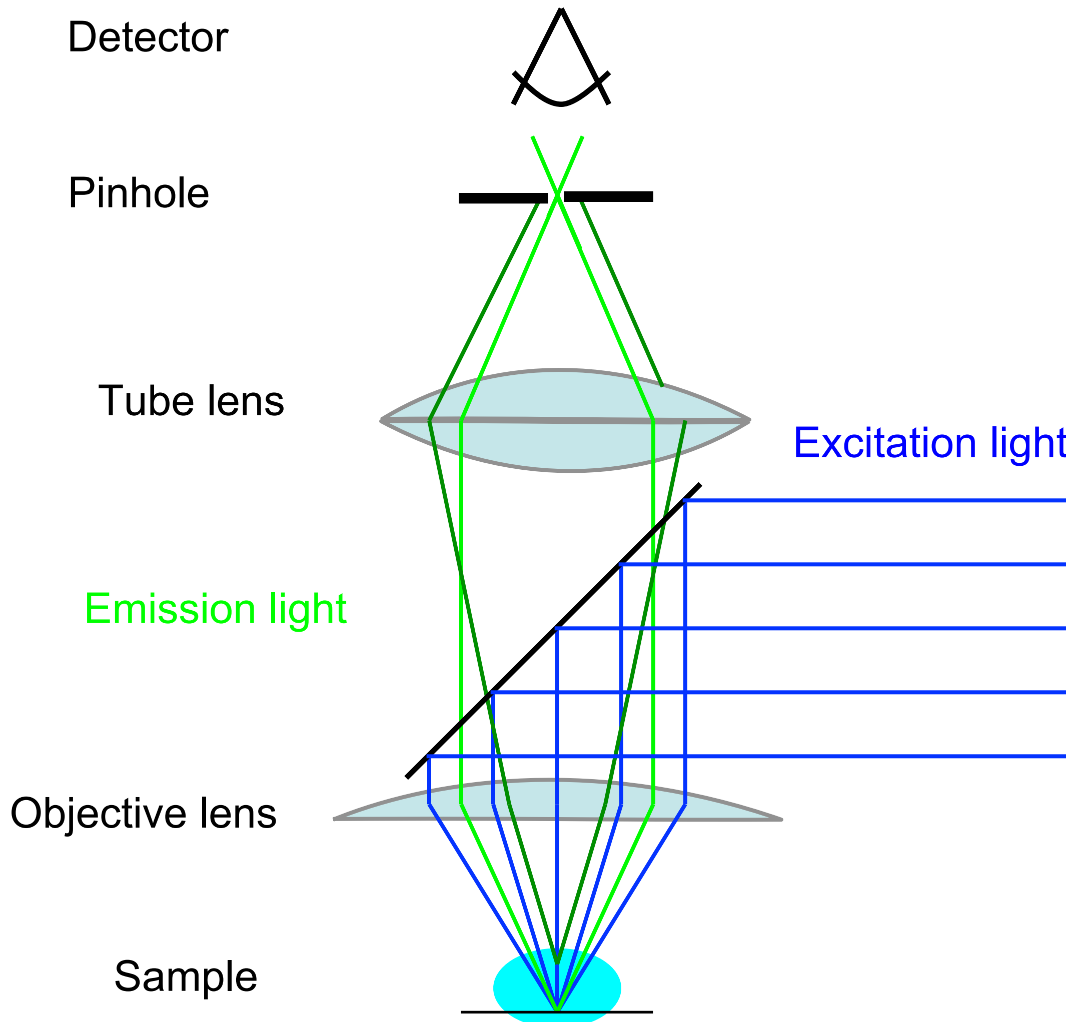
Confocal



Widefield

20 μm rat intestine section recorded with 60x / 1.4NA objective

The confocal microscope

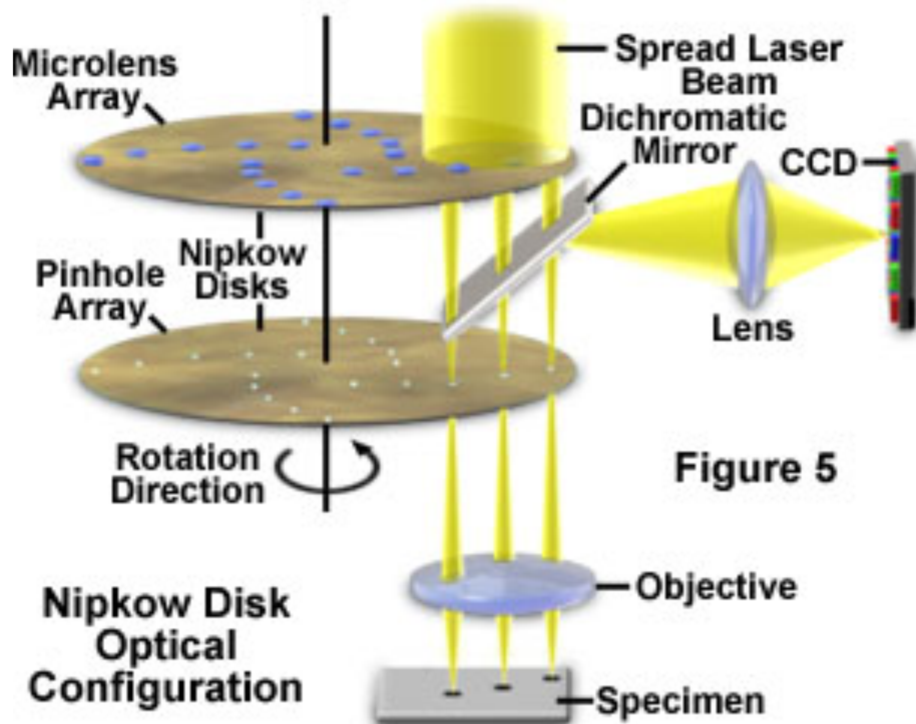


Scan excitation spot point-by-point to build up image

Problems:
Slow (~1 sec to acquire an image)
Low light efficiency (due to use of PMT as detector)

Solution:
Use multiple pinholes and a camera

A Solution: Spinning Disk Confocal



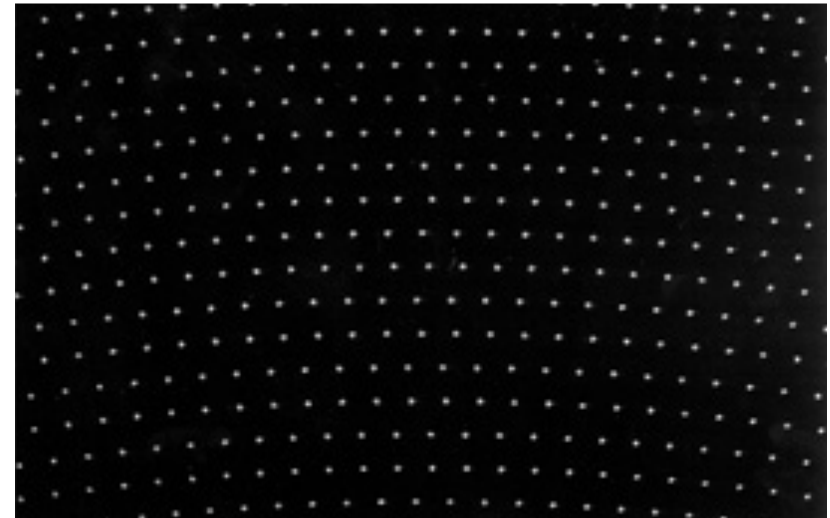


Image with many pinholes at once, so fast

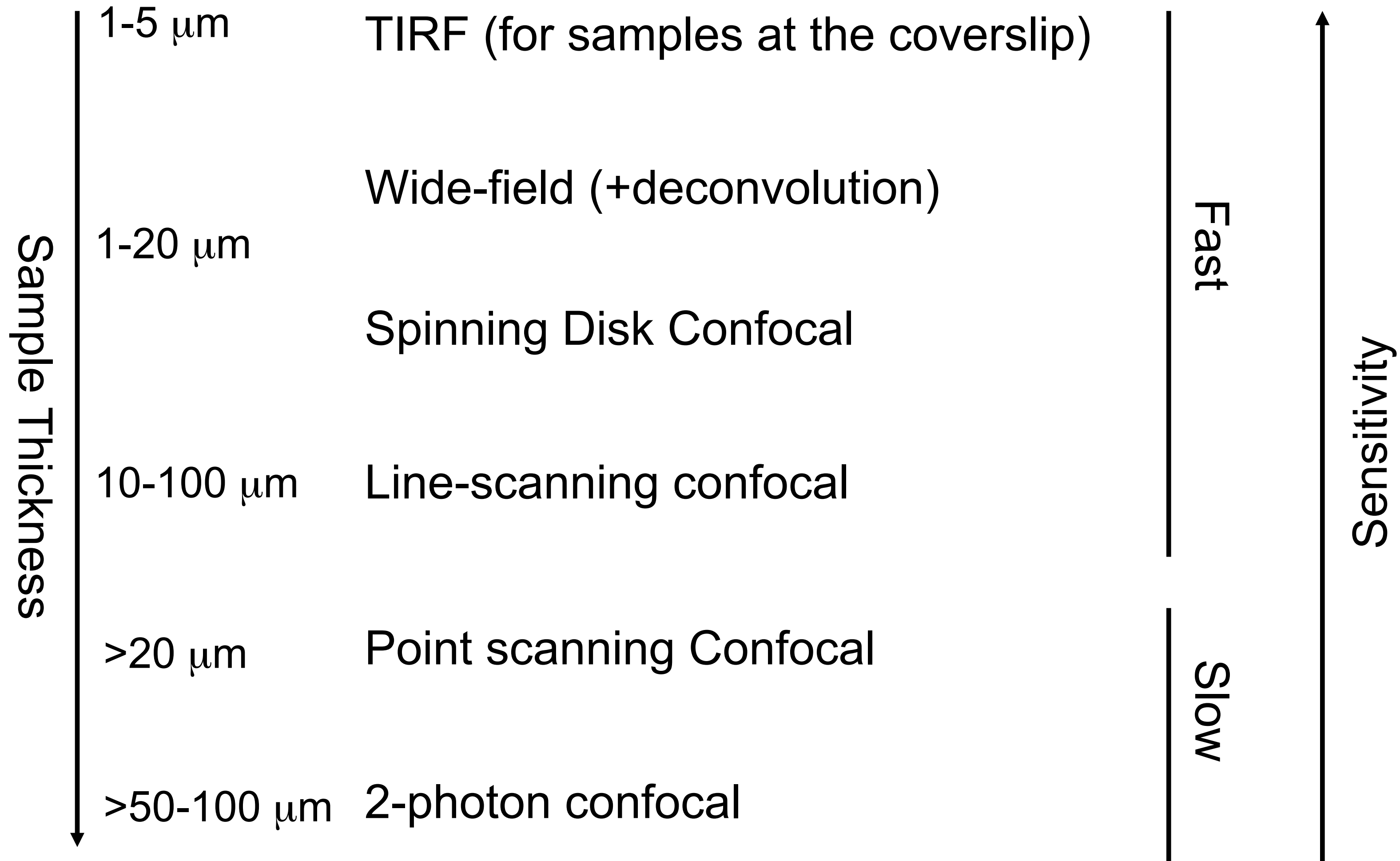
Use CCD as detector, so much higher QE

Pros/Cons of spinning disk

- Fast – multiple points are illuminated at once
- Photon efficient – high QE of CCD
- Gentler on live samples – usually lower laser power

- Fixed pinhole – except in swept-field
- Small field of view (usually)
- Crosstalk through adjacent pinholes limits sample thickness

Which imaging technique should I use?



Slides can be downloaded from:

http://nic.ucsf.edu/dokuwiki/doku.php?id=presentations

Resources

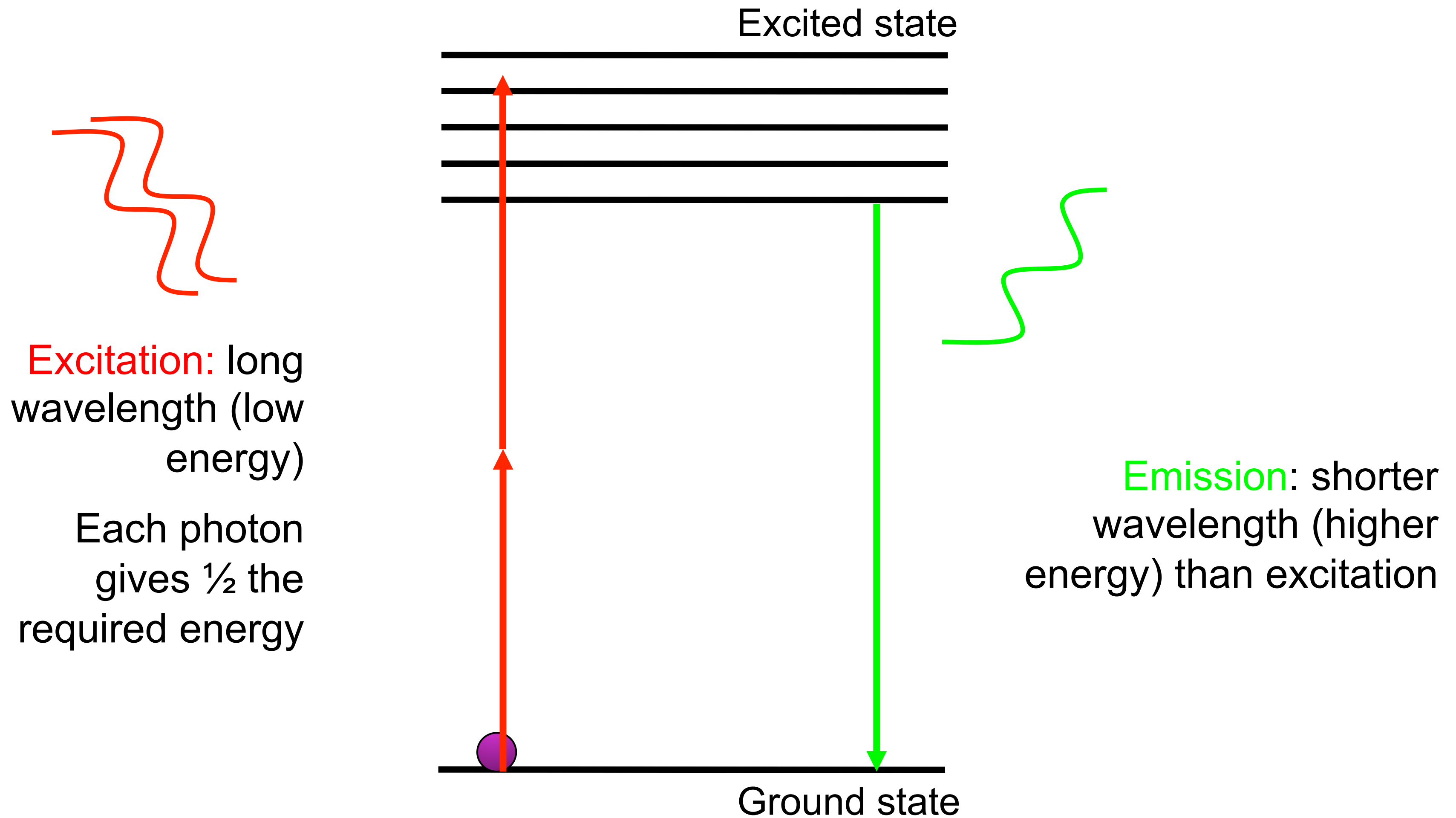
http://www.microscopyu.com

http://micro.magnet.fsu.edu

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

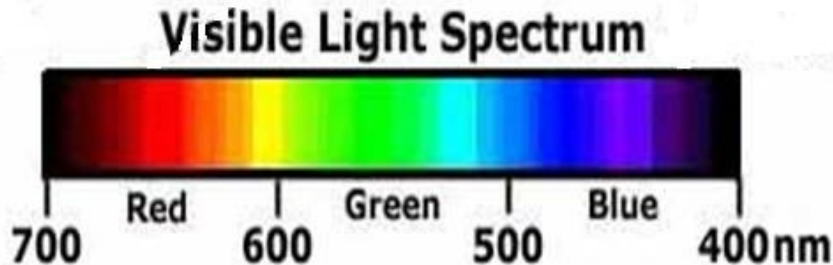
Murray JM et al., J. Microsc. 2007, 228: p.390-405

Microscopía de 2 fotones



2-fotones

➔ Use of lower energy light to excite the sample (higher wavelength)



1-photon: 488nm

2-photon: 843nm

Advantages:

❖IR light penetrates deeper into the tissue than shorter wavelength

❖2-photon excitation only occurs at the focal plane → less bleaching above and below the section

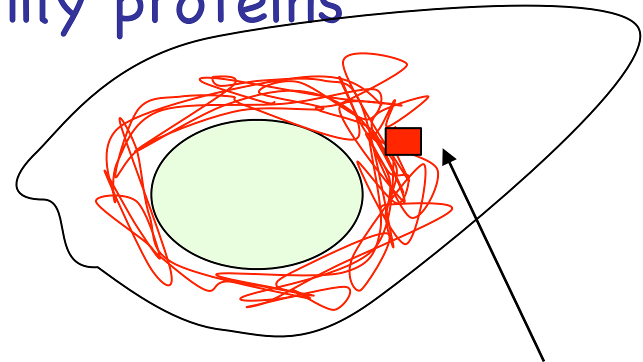
→ Use for deep tissue imaging

Photoactivation

(Better?) FRAP/FLIP alternative

Some fluorophores can be activated by light

- Photo-uncagable dyes
  - GFP-family proteins

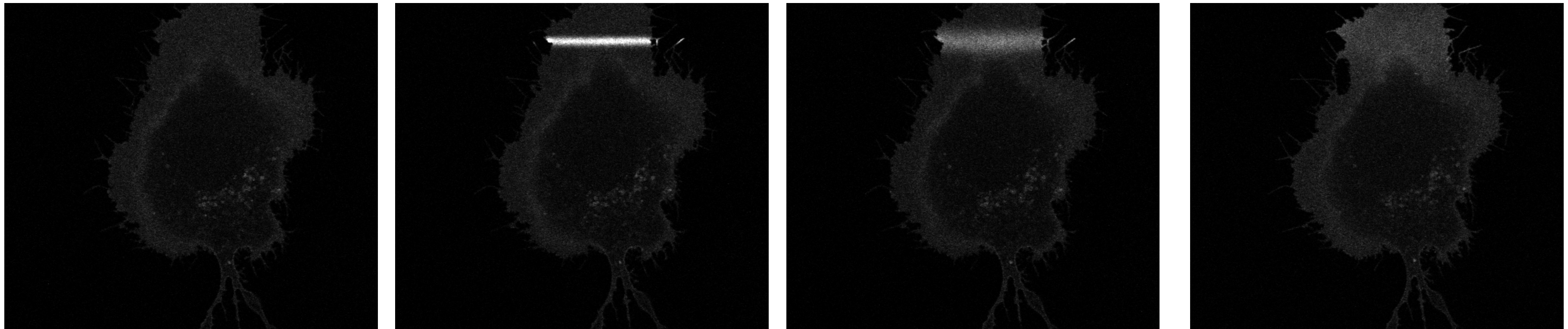


Look for weak light
against dark background
Instead of slight dimming
of bright background

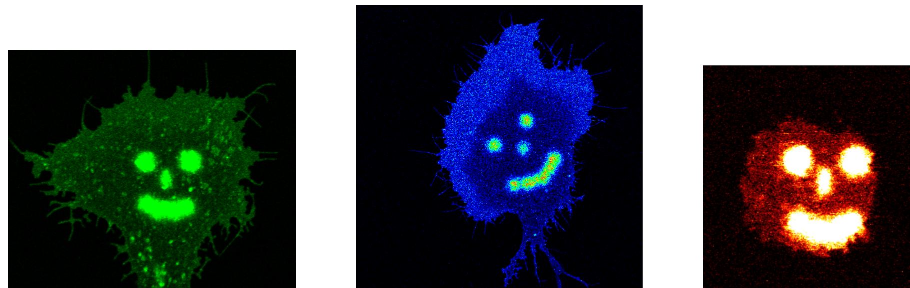
Activate a small area
Watch fluorescence spread

photoactivation

- Fluorophore only becomes active (= fluorescent) if excited (e.g. with 405 laser) due to structural change

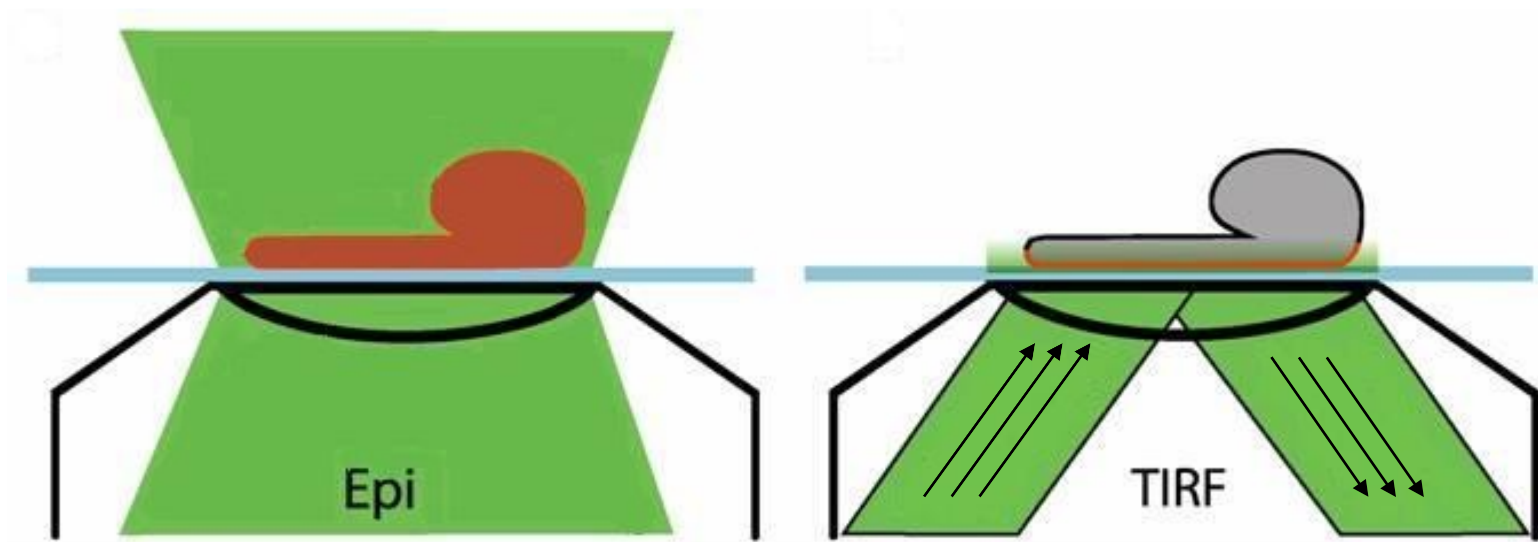


Pictures taken from a activation movie: activation of a line trough the lamellipodia of the cell, activated GFP_F diffuses quickly



TIRF

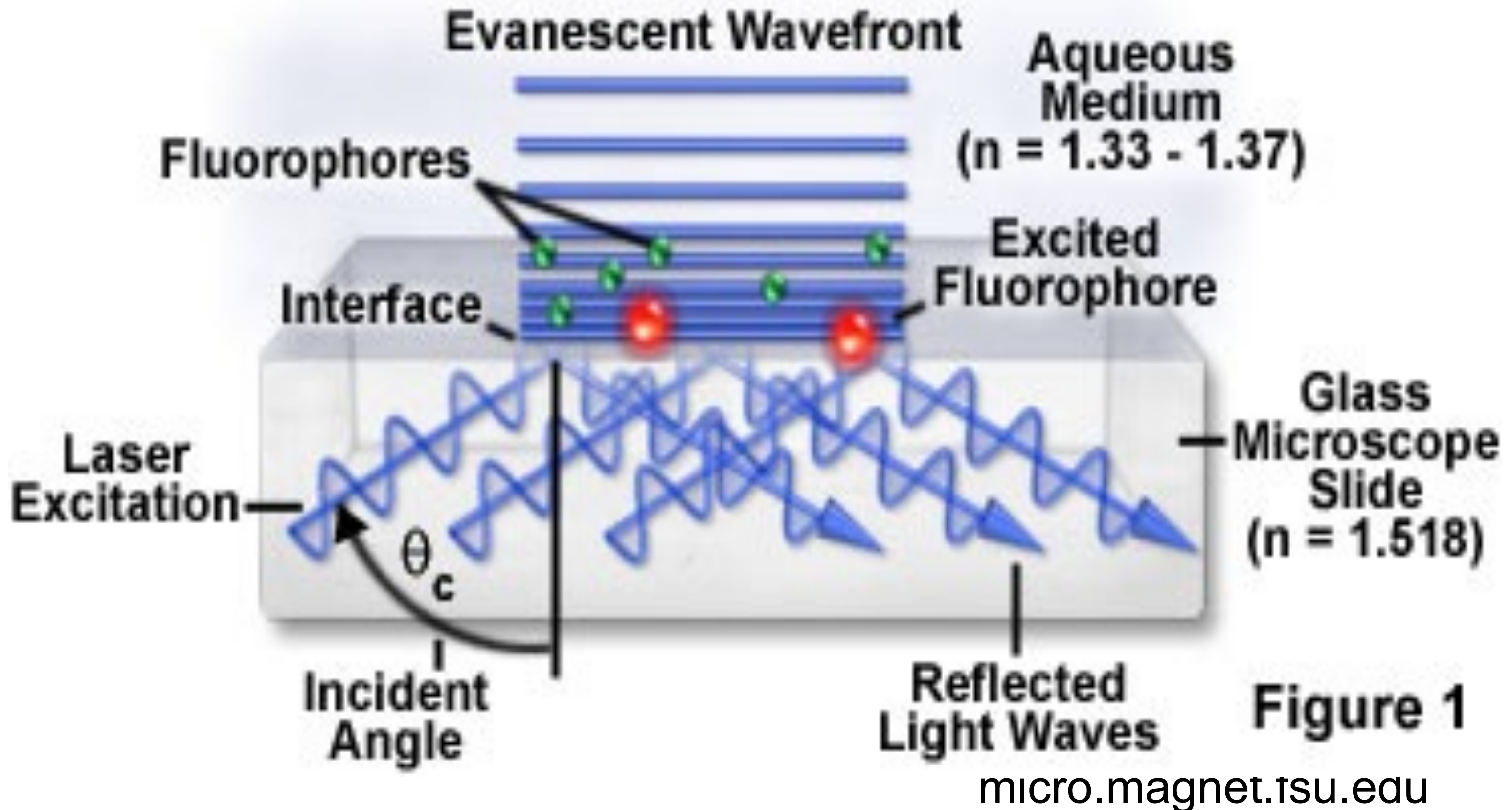
(Total Internal Reflection Fluorescence)



You need:

- TIRF objectives with high NA
- TIRF condensor, where you are able to change the angle of illumination
- Glass coverslips

TIRF



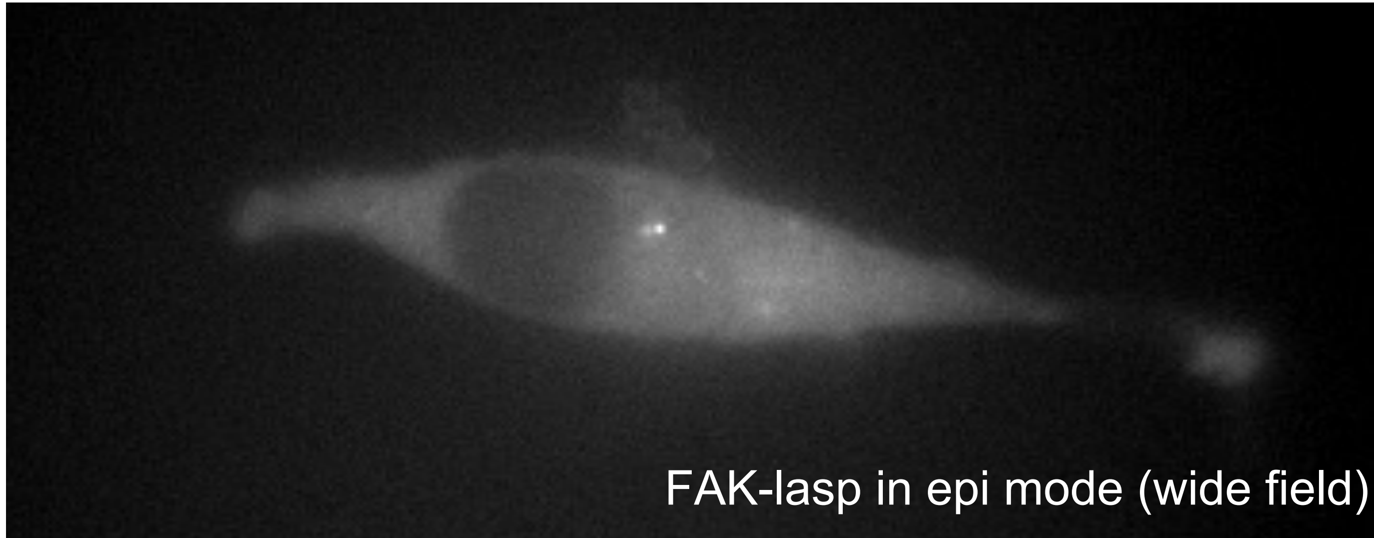
micro.magnet.fsu.edu

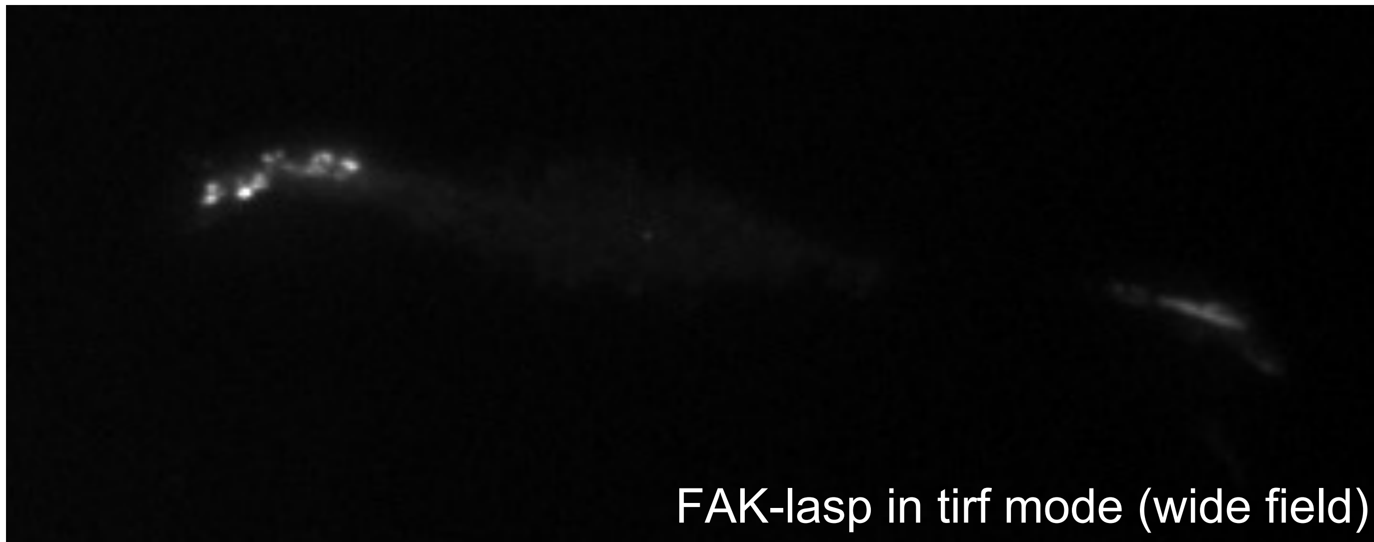
Result: very thin section at the bottom of the sample → 150-200nm

Use: to study membrane dynamics (endocytosis, focal adhesions, receptor binding)

→ *Nikon TE 2000*

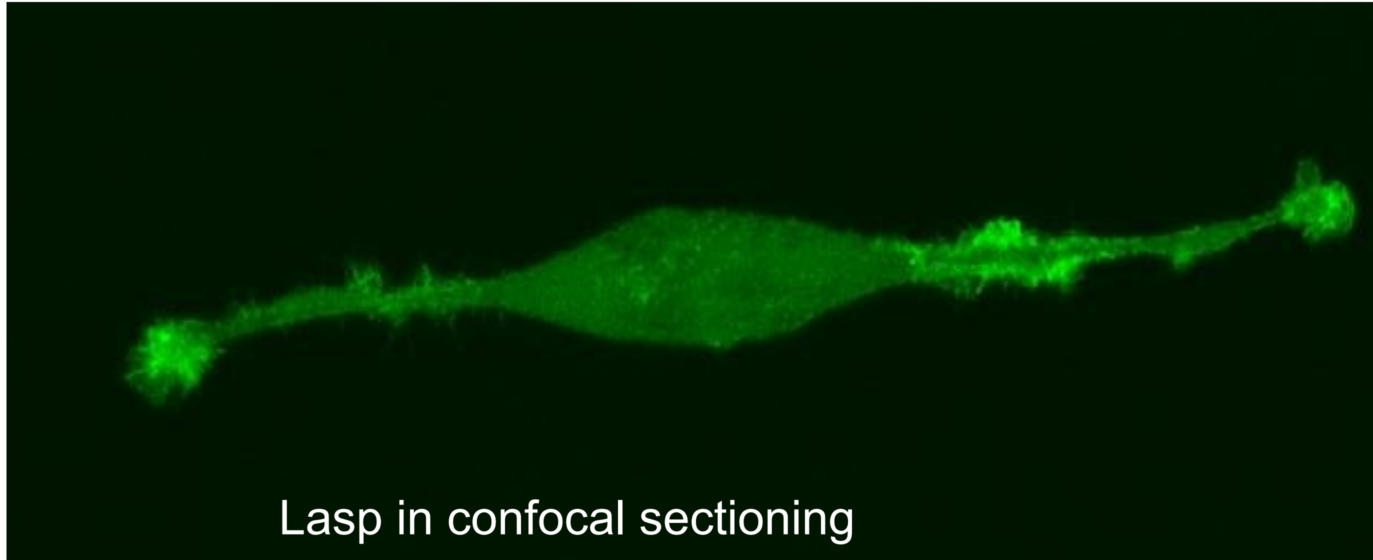
TIRF vs epi

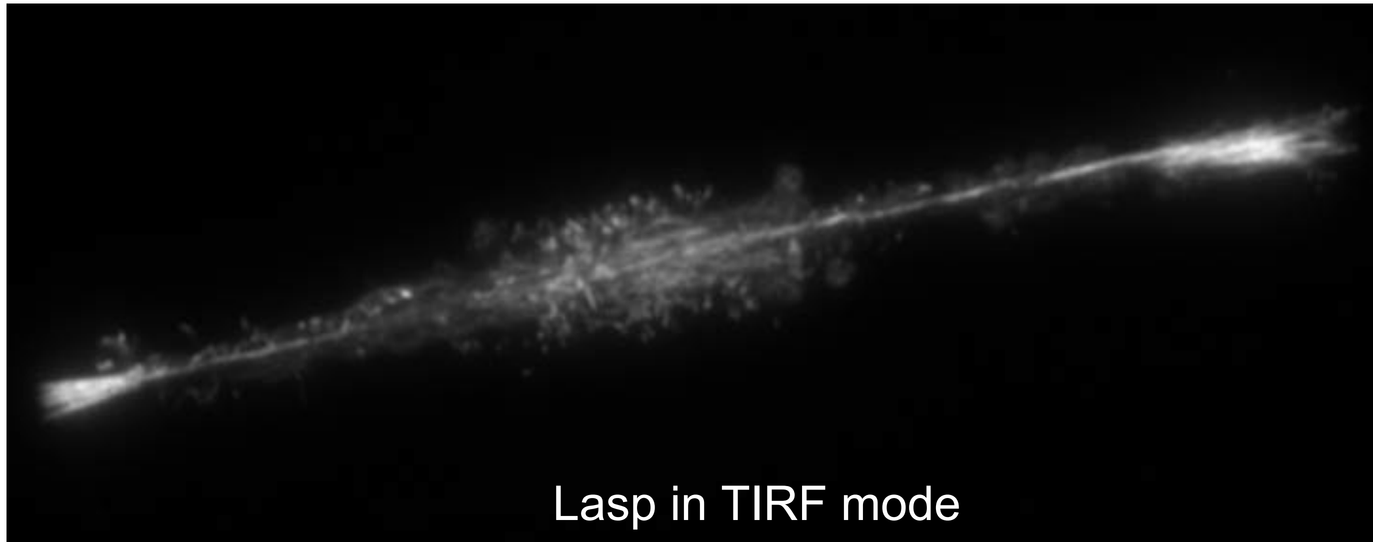




Heather Spence, R10

TIRF vs epi





Heather Spence, R10

Summary/comparison

method	excitation	detection	sectioning	use
Wide field	Whole sample	Whole sample	No sectioning	Simple fluorescence samples
confocal	Whole sample	One z-plane	350-500nm	High contrast images, optical sectioning
2-Photon	One z-plane	One z-plane	500-700nm	Deep tissue imaging, optical sectioning
TIRF	Only bottom plane	Only bottom plane	150-200nm	Membrane dynamics