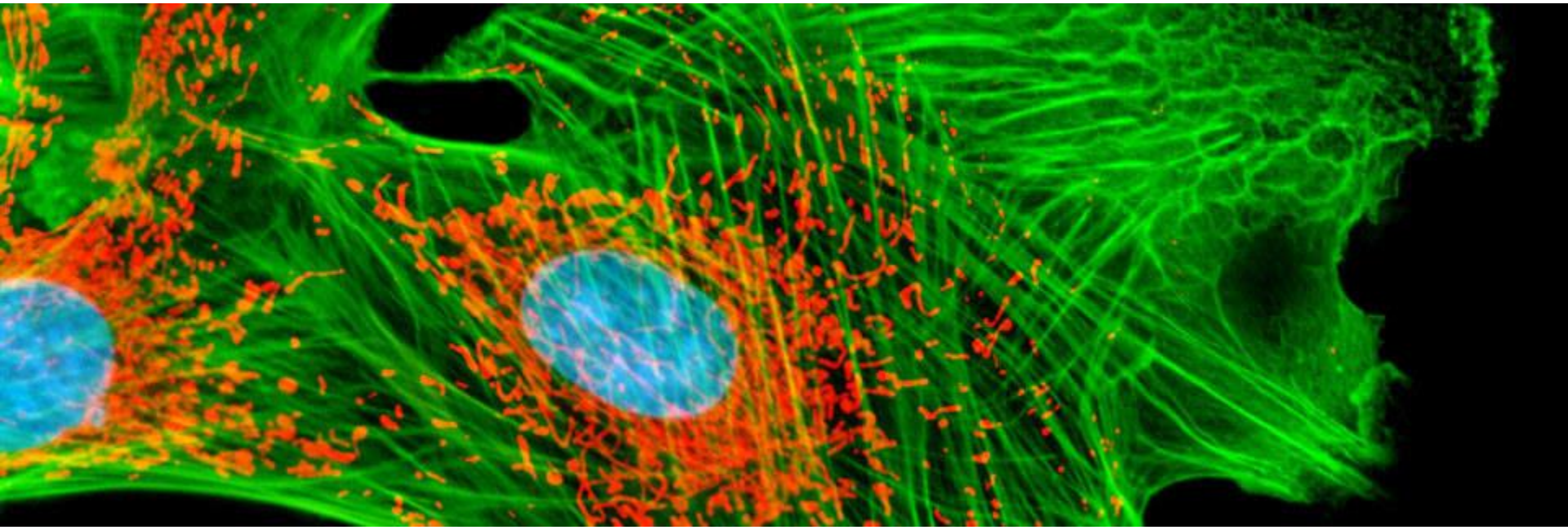
Basic Concepts of Microscopy



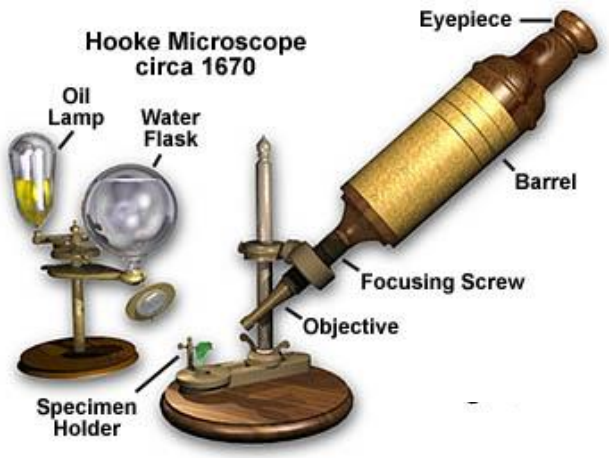
Valentina Russo (UniTe)-Angelo Balsamo (Nikon)

Optical microscope

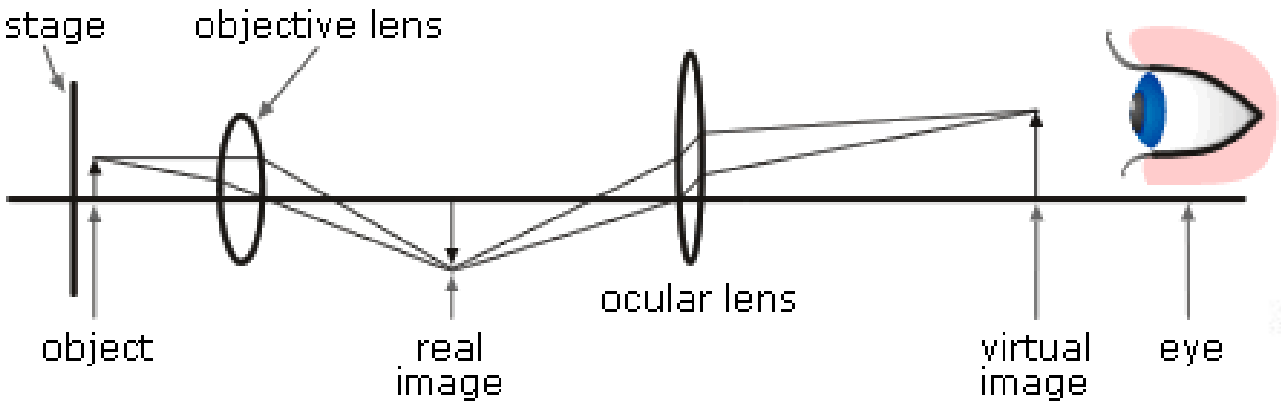
Microscopes are instruments designed to produce magnified visual or photographic images of small objects.

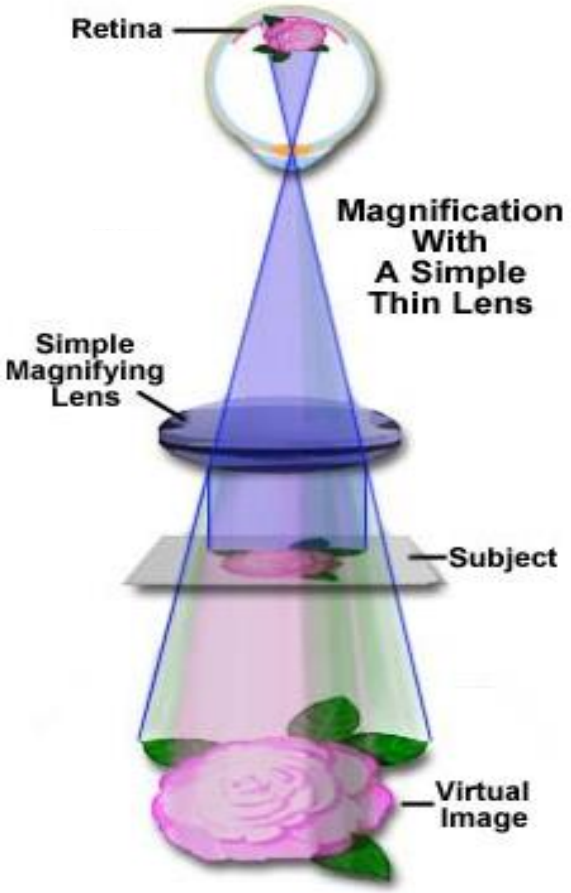
The microscope must accomplish three tasks:

- produce a magnified image of the specimen
- separate the details in the image
- render the details visible to the human eye or camera



Magnification





Magnification

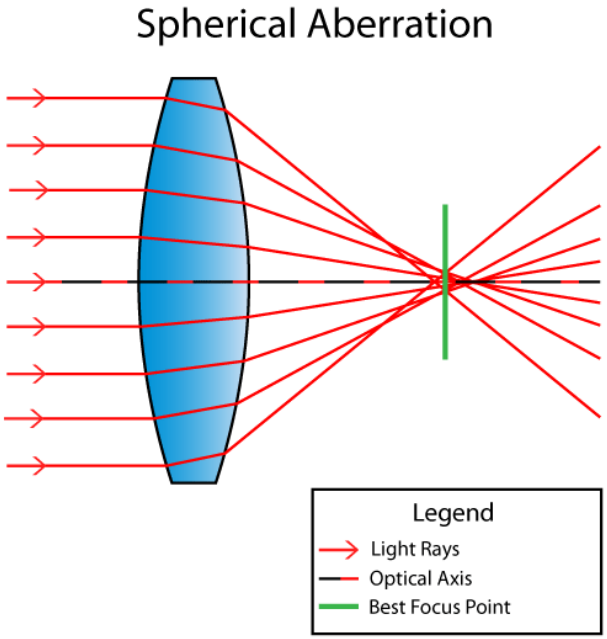
Objective Magnification	Eyepiece (Ocular lens)	Total Magnification
4x	10x	40x
10x	10x	100x
20x	10x	200x
40x	10x	400x
60x	10x	600x
100x	10x	1000x

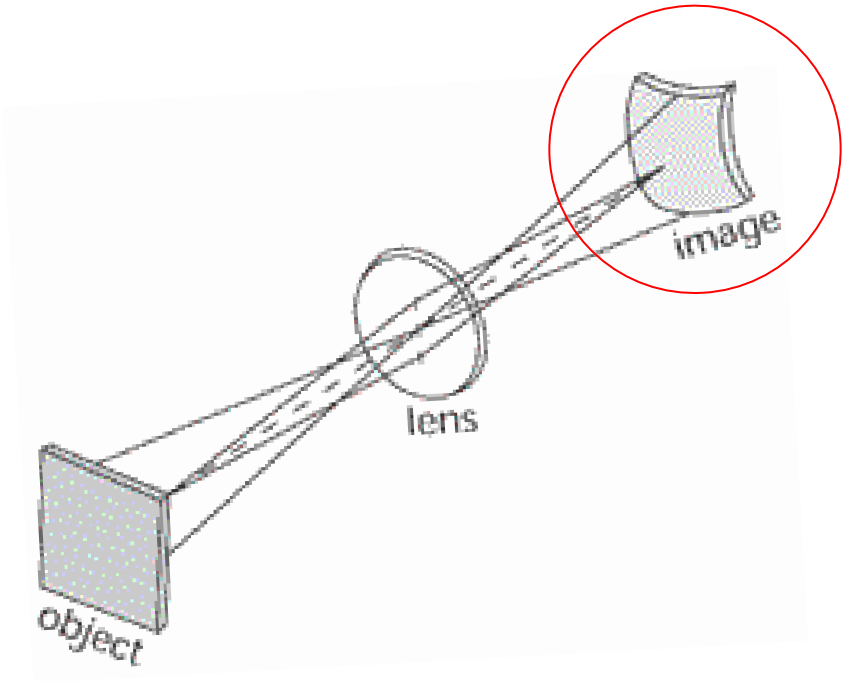
Figure 1

X

Spherical aberration

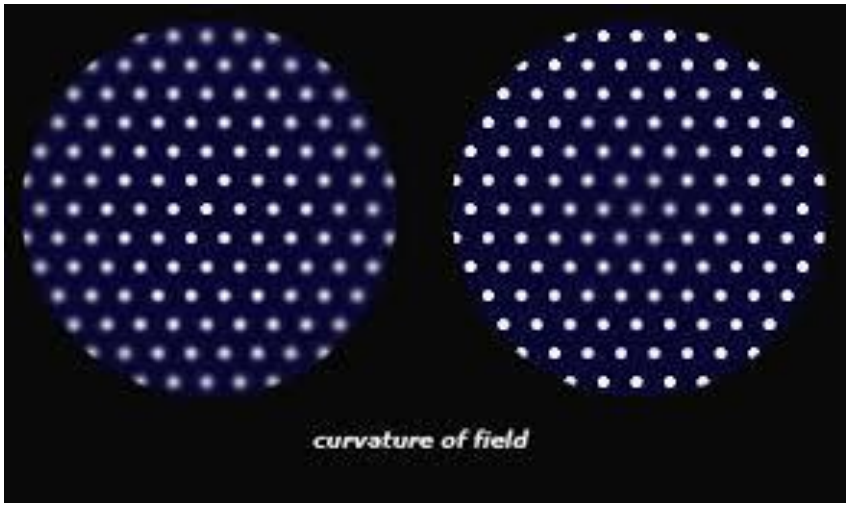
Spherical aberration causes beams parallel to but away from the lens axis to be focussed in a slightly different place than beams close to the axis. This manifests itself as a blurring of the image.





Geometrical Aberrations

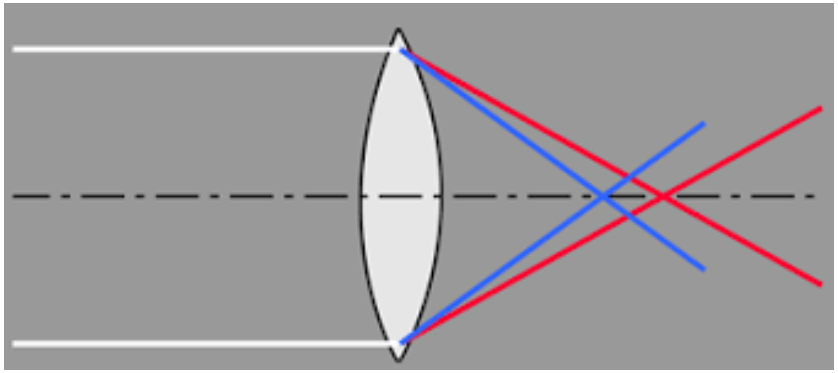
Spherical aberration



Cromatic Aberrations

Chromatic aberration is caused by a lens.

In a simple optical system the different wavelengths will be focused on different positions in the focal plane.
Chromatic aberration is seen as fringes of colour around the image.



Objectives

Microscope objectives are perhaps the most important components of an optical microscope because they are responsible for primary image formation and play a central role in determining the quality of images that the microscope is capable of producing.

In each objective you will find all the functional information as:

- Lateral Magnification
- Flat and Aberration Correction
- Numerical Aperture
- Working Distance
- Type: dry or oil immersion
- Cover Glass Specification

Flat and Aberration Correction

Objective types

Achromatic

- Good colour correction – exactly for two wavelengths.
- No good geometrical corrections.

Plan-Achromatic

- Good colour correction – exactly for two wavelengths.
- Good geometrical corrections

Semi Plan-Apochromatic

- Excellent colour correction for at least three wavelengths.
- Excellent geometrical corrections

Plan-Apochromatic

- Perfect colour correction (correction for four wavelengths!).
- Excellent geometrical corrections

Numerical Aperture (N.A.)

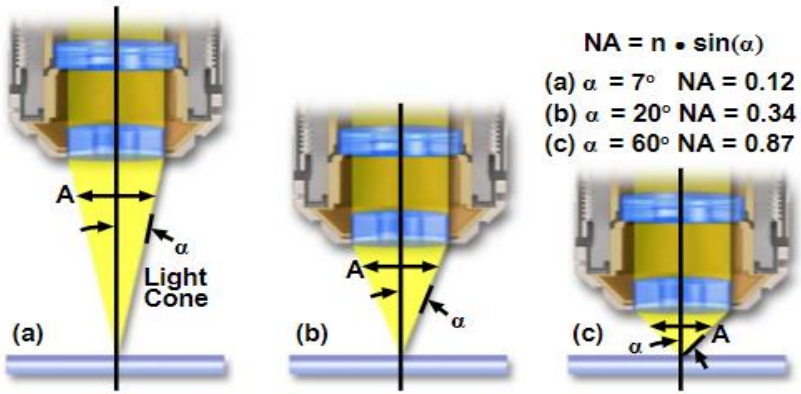
Numerical Aperture = N.A. = n · sin α

α is half the opening angle of the objective.

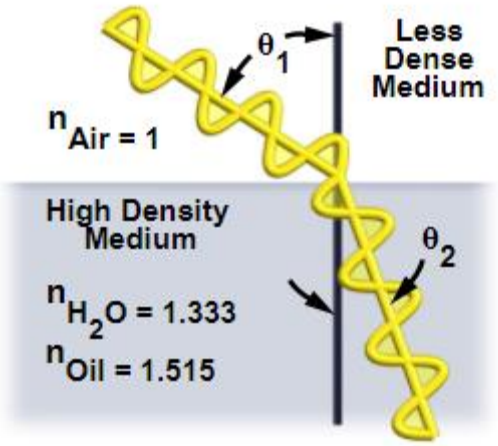
n is the refractive index of the immersion medium used between the objective and the object.

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

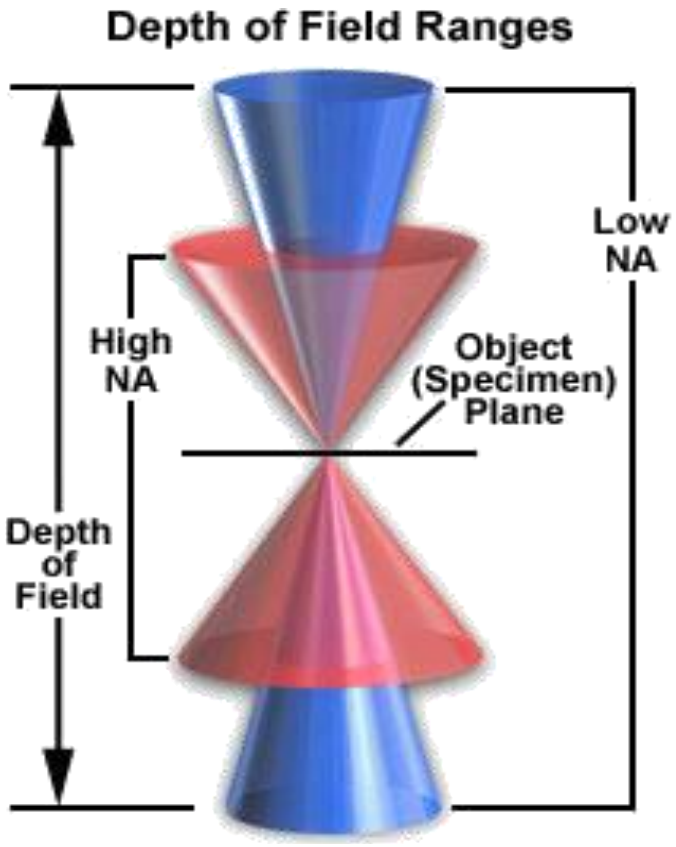
Numerical Aperture



Refraction of Light



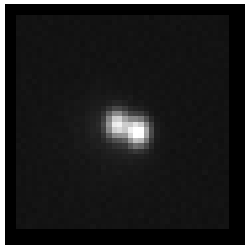
Numerical Aperture (N.A.)

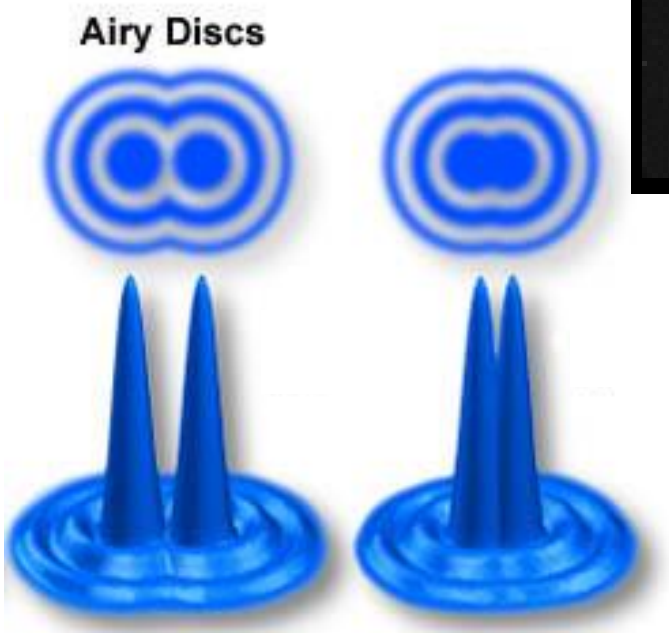


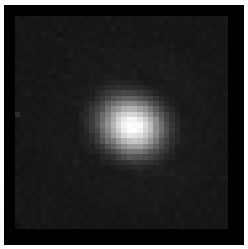
Magnification	Numerical Aperture	Depth of Field (μm)
4x	0.10	50
10x	0.25	7.7
20x	0.40	2.9
40x	0.65	0.9
60x	0.85	0.36
100x	0.95	0.17

Resolution

Resolution describes the minimal distance of two points that can be distinguished.

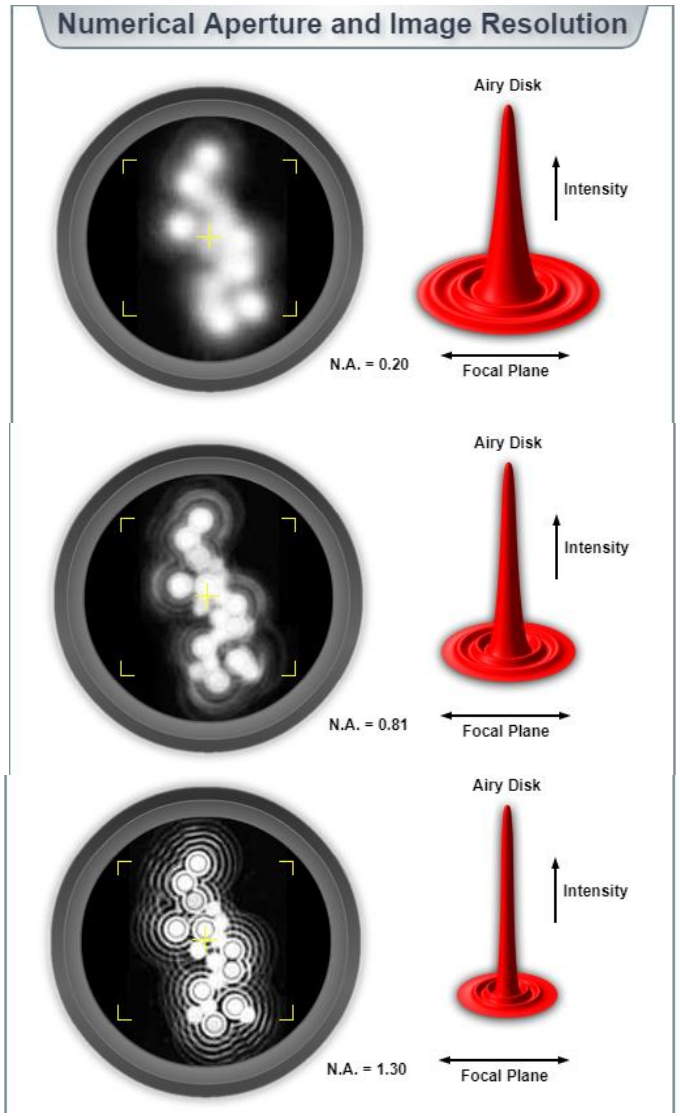






Resolution (r) = λ/(2NA)

Resolution

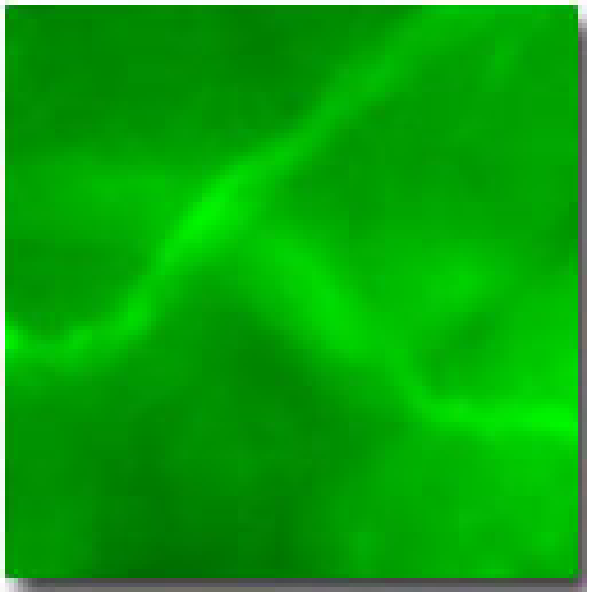


	Objective Type					
	Plan Achromat		Plan Fluorite		Plan Apochromat	
Magnification	N.A.	Resolution (µm)	N.A.	Resolution (µm)	N.A.	Resolution (µm)
4x	0.10	2.75	0.13	2.12	0.20	1.375
10x	0.25	1.10	0.30	0.92	0.45	0.61
20x	0.40	0.69	0.50	0.55	0.75	0.37
40x	0.65	0.42	0.75	0.37	0.95	0.29
60x	0.75	0.37	0.85	0.32	0.95	0.29
100x	1.25	0.22	1.30	0.21	1.40	0.20

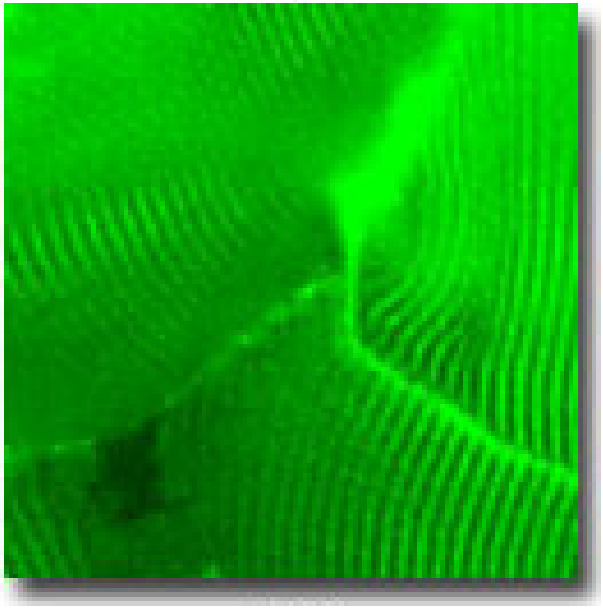
N.A. = Numerical Aperture

Resolution describes the minimal distance of two points that can be distinguished!

Resolution



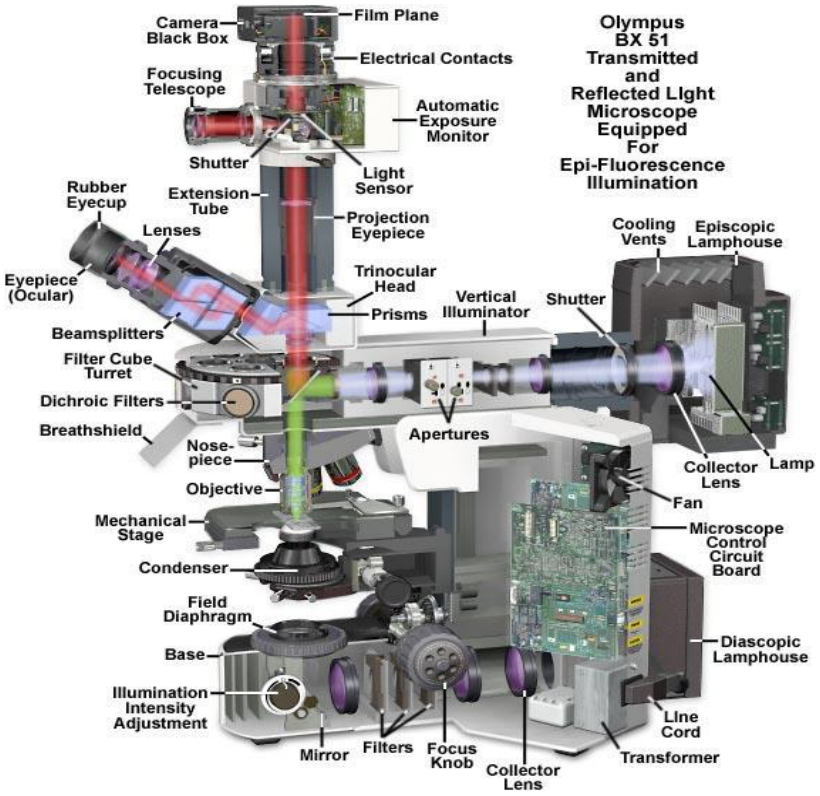
Low NA



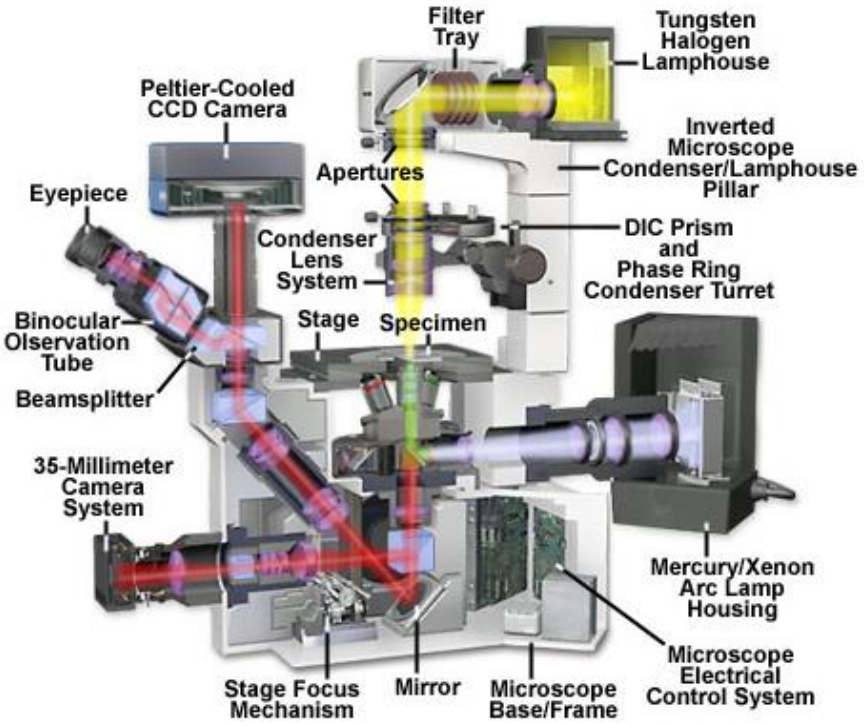
High NA

Microscope Optical Components

Upright Microscope



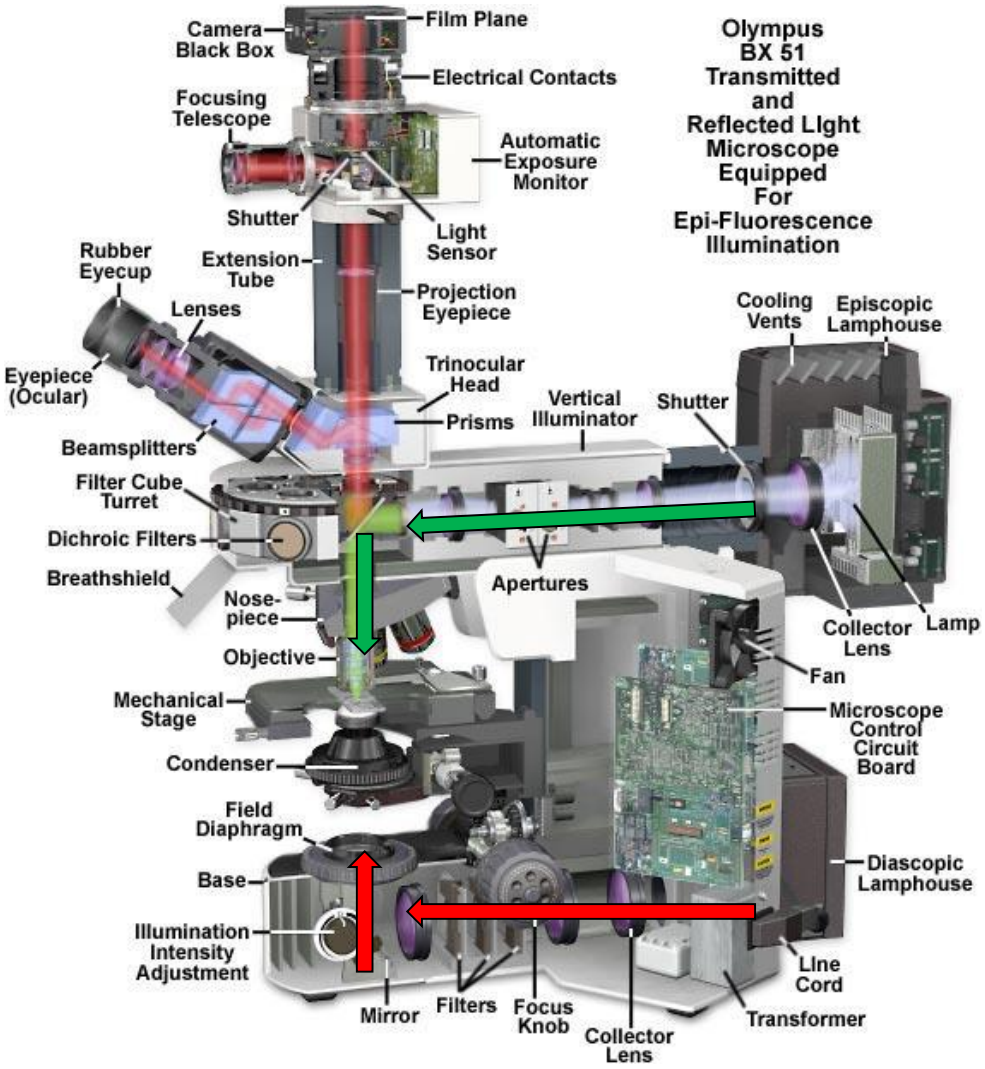
Inverted Microscope



Microscope Optical Components

Types of observation:

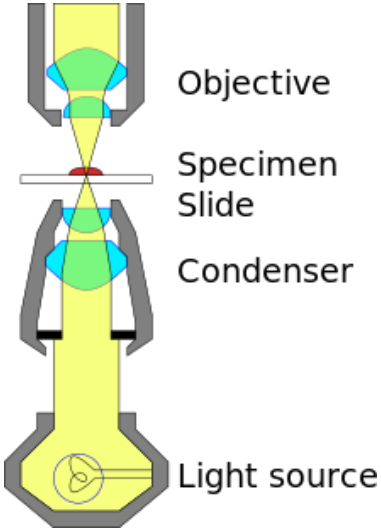
- **Diascopic (Bright Field)**
- **Episcopic (Fluorescence)**

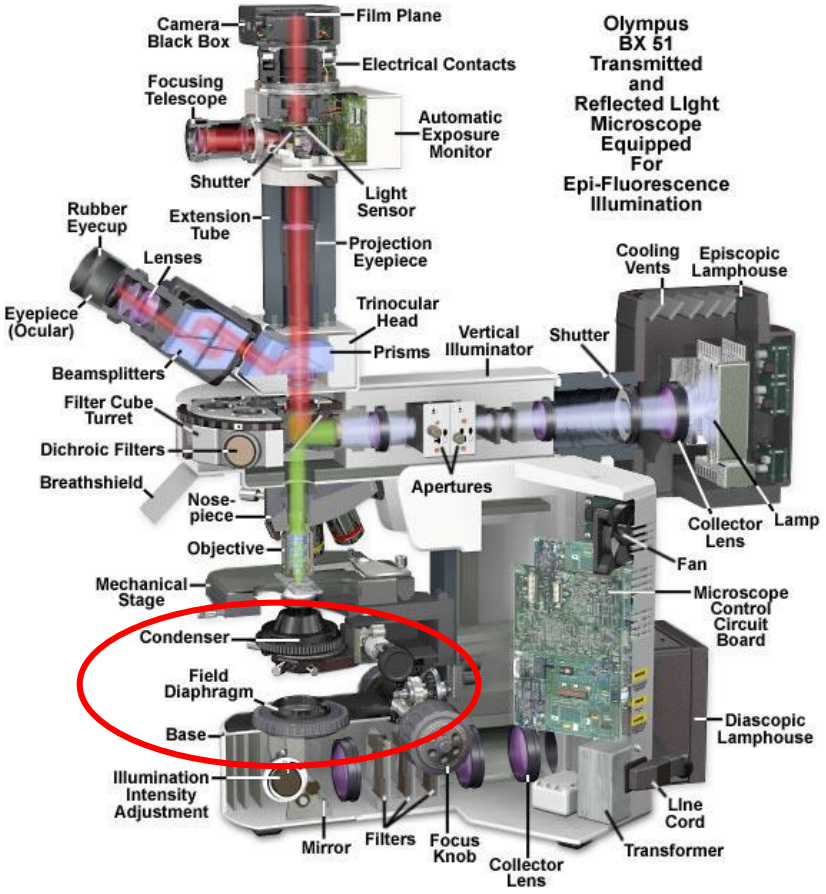


Microscope Optical Components

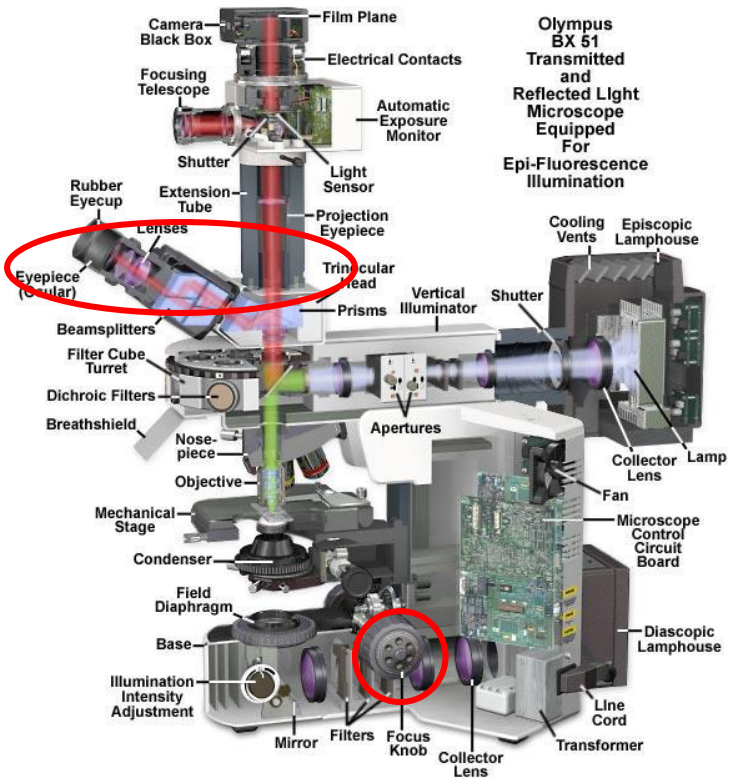
Condenser

is a part of the Diascopic illumination system. Converges the light beam to illuminate an object, it requires a setting such as Köhler illumination by the Field Diaphram





Microscope Optical Components



Binocular or Trinocular tube:
The hollow tube through which light passes. It holds the lenses apart.

Eyepiece:
The lens you look through that magnifies the specimen.

Course Focus:
Raises or lowers the tube to focus

Fine Focus:
Raises and lowers the tube and used to bring objects into focus

Observation Techniques

The differences in intensity and/or color create image contrast.

Use Staining method or particular Lighting Equipment to increase the image quality.

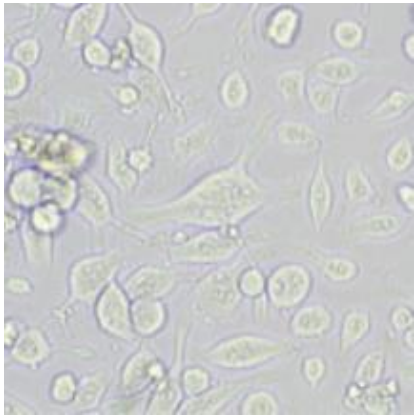
Observation Techniques	Stainig metod	Lighting Equipment
Bright Field	X	
Fluorescence	X	
Phase Contrast		X
DIC (Differential Interference Contrast)		X
Fluorescence/DIC	X	X

Microscope Optical Components

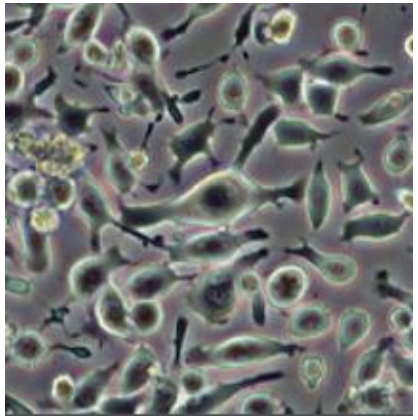
Phase Contrast

Increase the image contrast
of specimen not stained
by Phase Rings

w/o Ph. Contrast



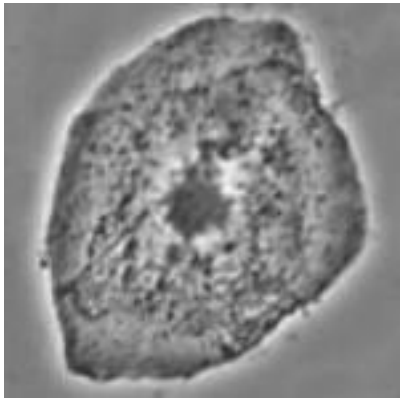
Ph. Contrast



D.I.C.

Increase the image contrast
of specimen not stained
by bias retardation

Ph. Contrast

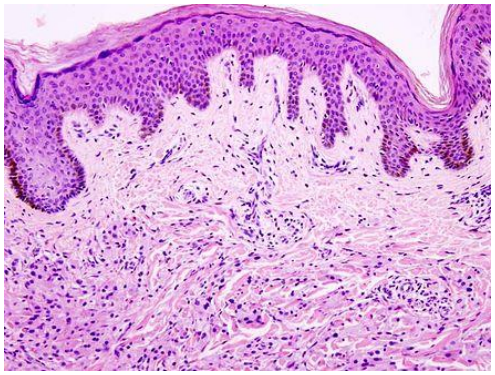


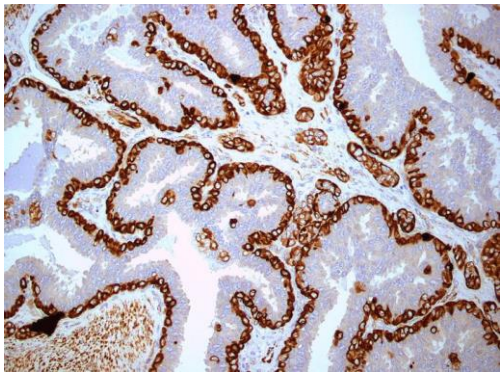
D.I.C.

Microscope Optical Components

Bright Field

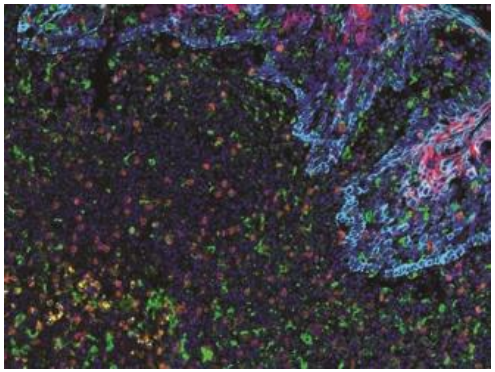
- Tissue and Cells Staining
- Immunohistochemistry

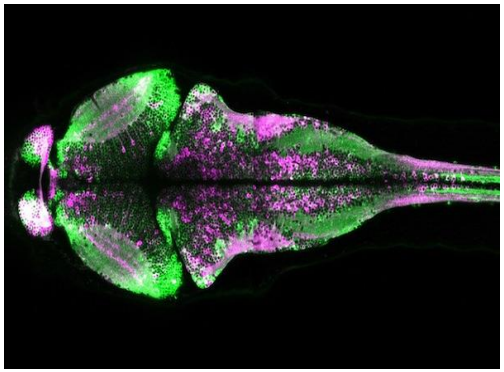




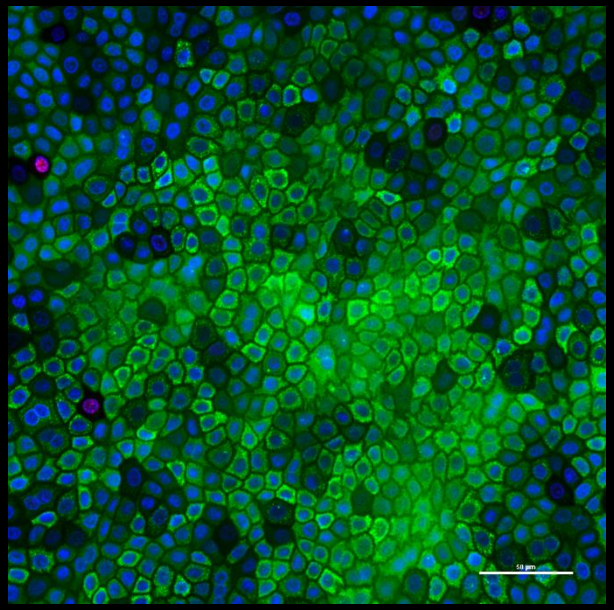
Fluorescence

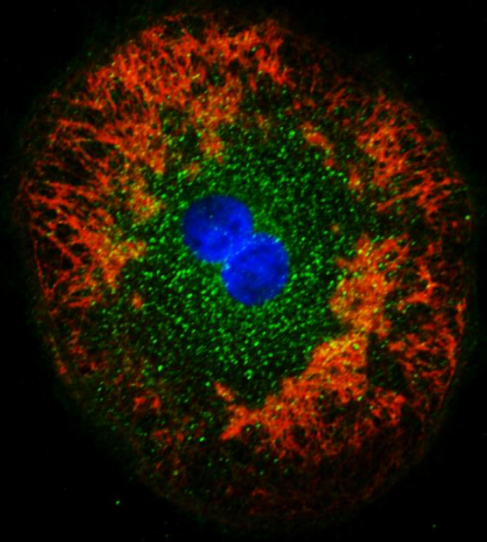
- Tissue and Cells Staining
- Immunohistochemistry
- GFP
- Zebra Fish

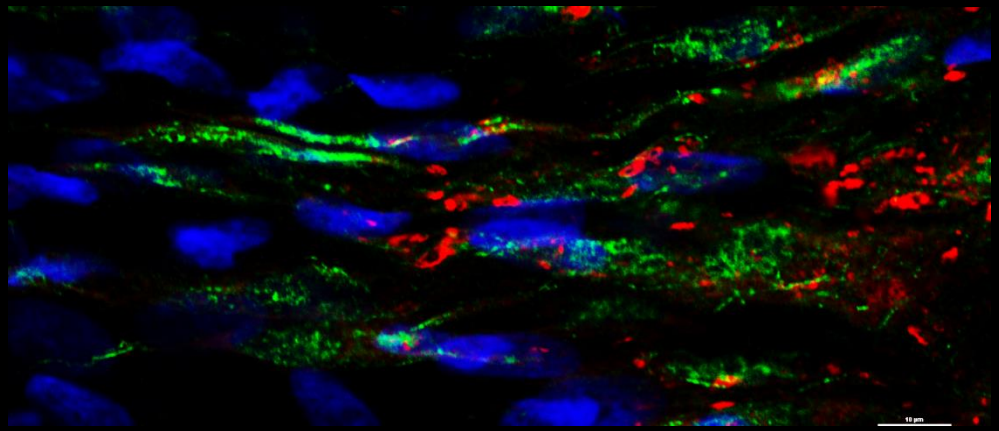


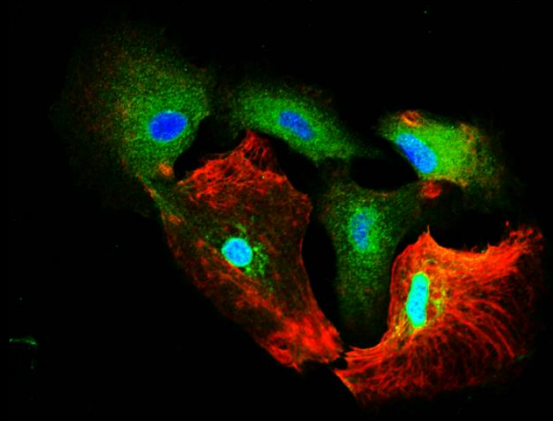


ImmunoHistoChemistry







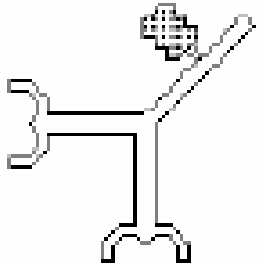


ImmunoHistoChemistry

- Immunohistochemistry (IHC) identifies specific tissue components by means of a specific antigen/antibody reaction tagged with a visible label.
- IHC makes it possible to visualize the distribution and localization of specific cellular components within a cell or tissue.
- IHC utilizes labeled antibodies to localize specific cell and tissue antigens, and is among the most sensitive and specific histochemical techniques.

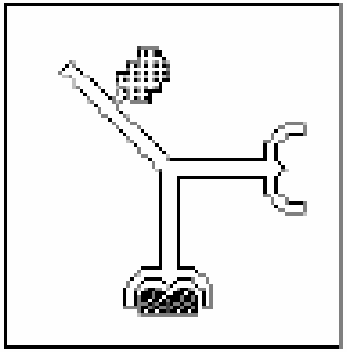
The immunological reaction

Direct method



Primary antibody that binds to a specific antigen conjugated with a marker

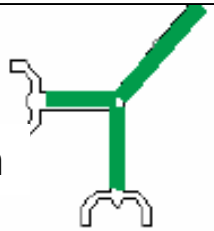
Antigen



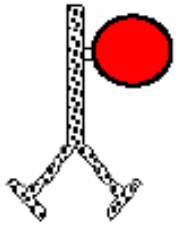
The primary antibody specifically binds to the antigen and it is revealed with a visible label

Indirect method

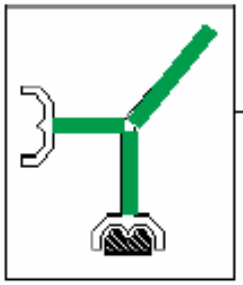
Antigen



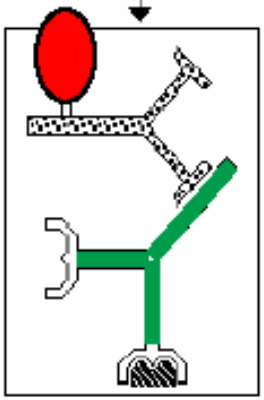
Primary antibody that binds to a specific antigen



Secondary antibody conjugated with a marker



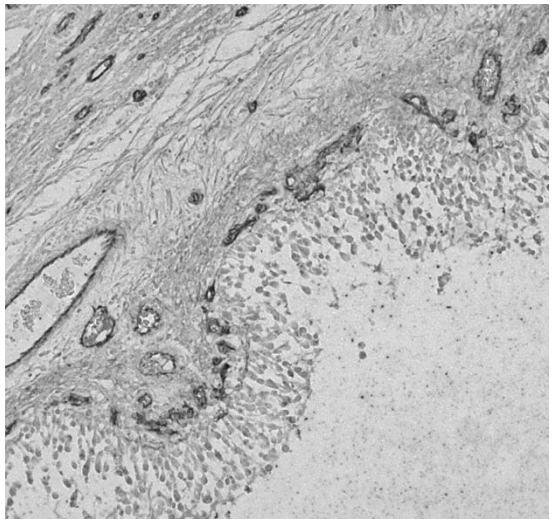
The primary antibody binds to the antigen



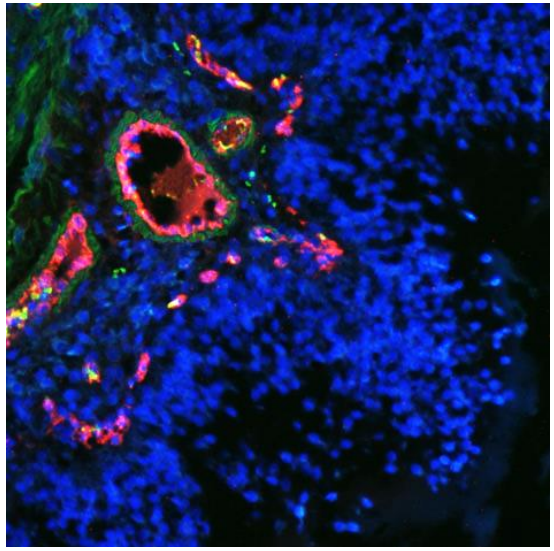
The labelled secondary antibody binds to the primary antibody and it is revealed with a visible label

IHC Staining

Enzymatic and chromogen detection system (peroxidase + DAB)



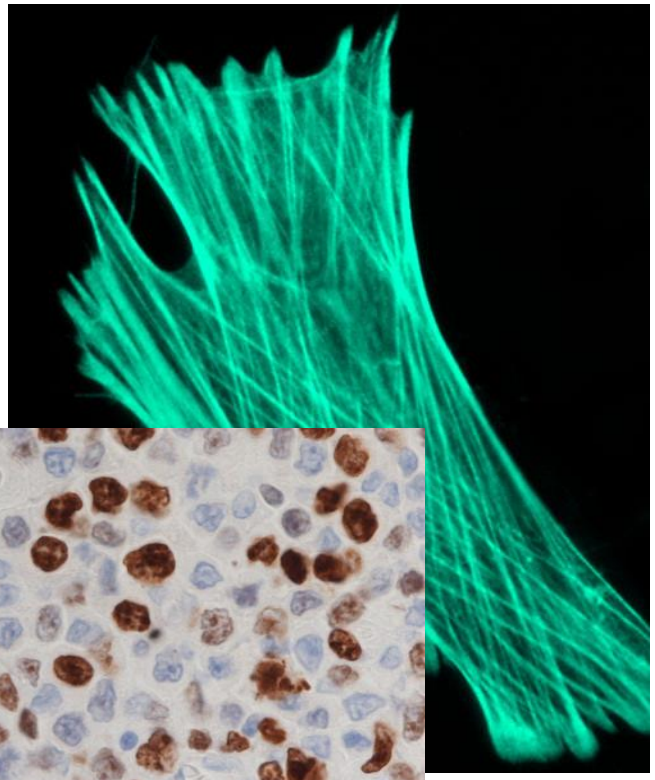
Fluorescent label detection system (FITC + CY3 + DAPI)

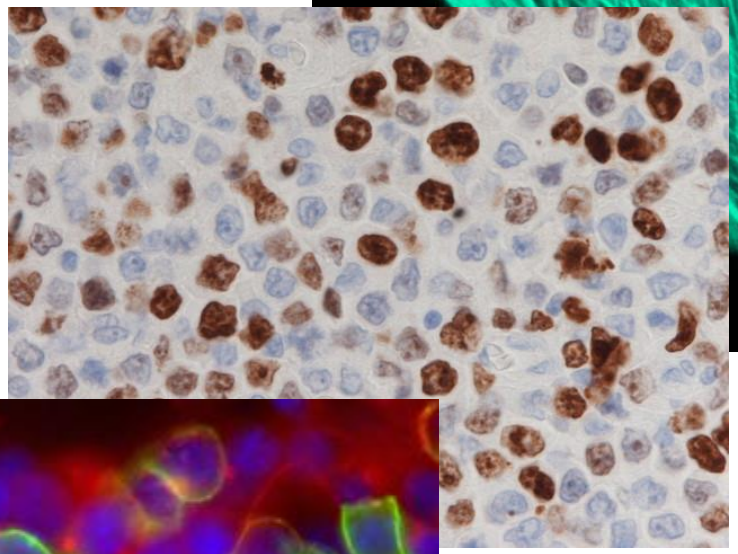


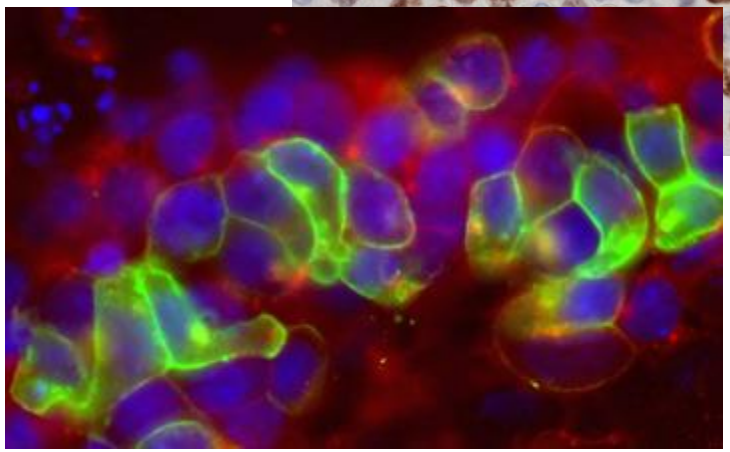
ImmunoHistoChemistry

What antigens can we target?

- Cytoplasmic
- Nuclear
- Cell membrane
- Extracellular matrix







Immunohistochemistry – what's good about it?

Antibodies bind to antigen in **specific manner**

Gives you a *spatial location*

Can be used to locate particular cells and proteins

Can be used to identify cellular events – e.g.apoptosis

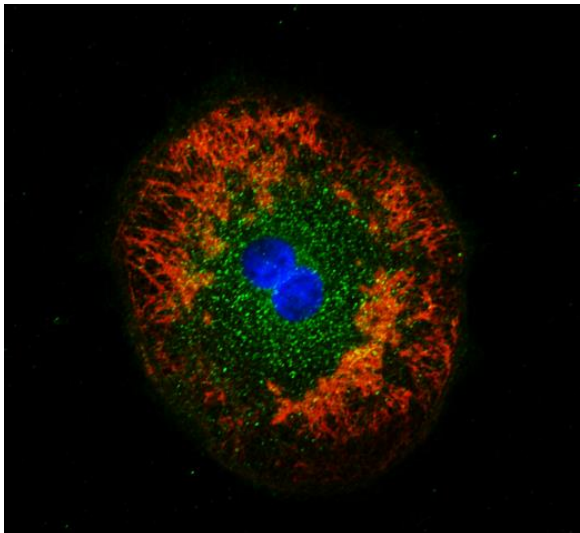
ImmunoFluorescence

WHY FLUORESCENCE MICROSCOPY?

• In all types of microscopes, cell constituents are not distinguishable.

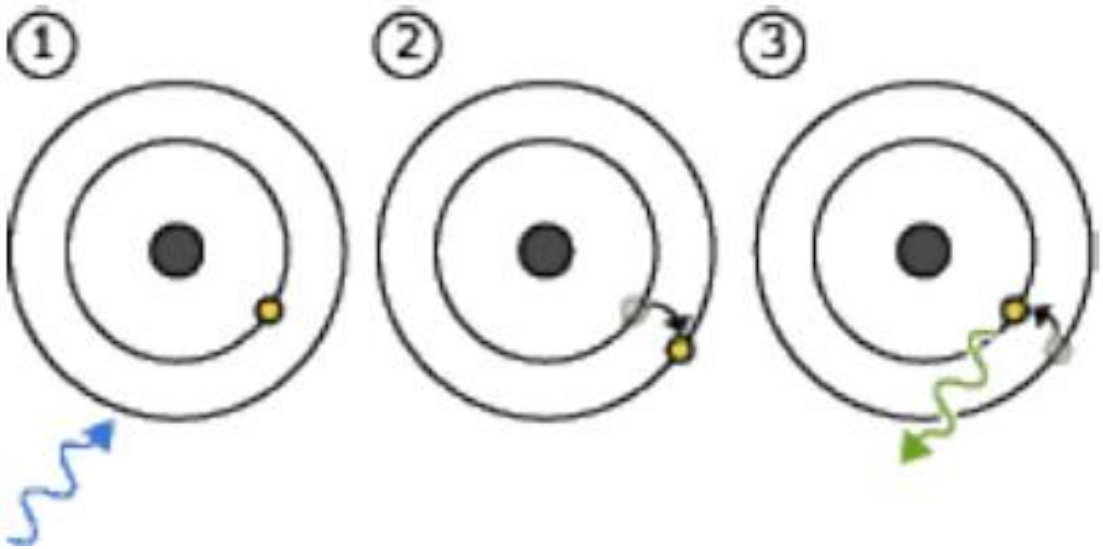
• In fluorescent microscopy, various fluorescent dyes are used which gives property of fluorescence to only specific part of the cell and hence it can be focused.

• Fluorescent microscopy depends upon illumination of a substance with a specific wavelength which then emits light at a longer wavelength .



Fluorescence Principle

When certain compounds are illuminated with high energy light, they then emit light of a different, lower frequency. This effect is known as **fluorescence**.

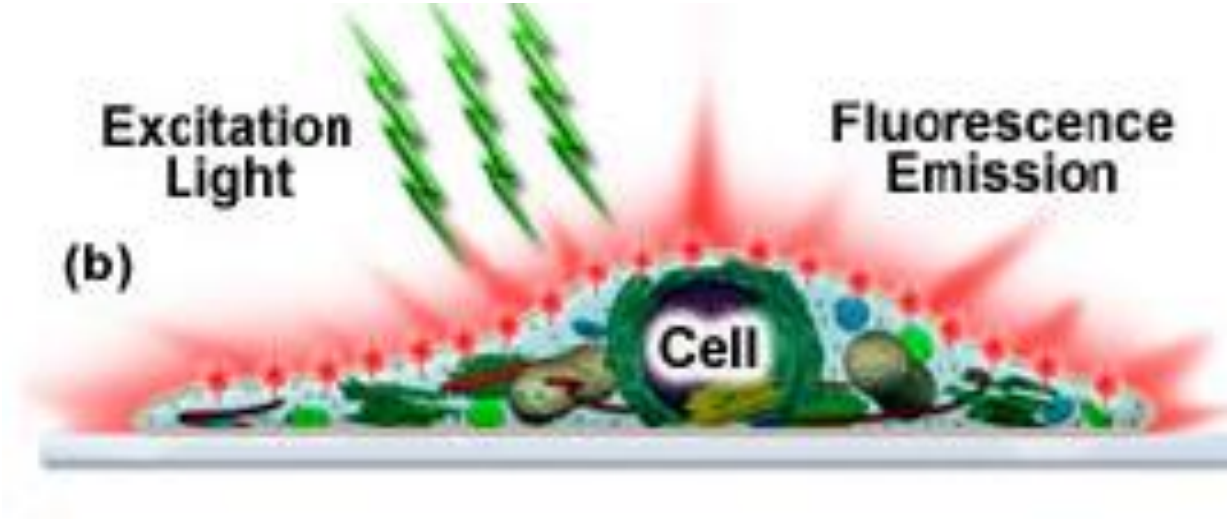


The radiation collides with the atoms **(1)**
In the specimen and electrons are excited to a higher energy level **(2)**.
When they relax to a lower level, they emit light **(3)**.

Fluorescence Principle

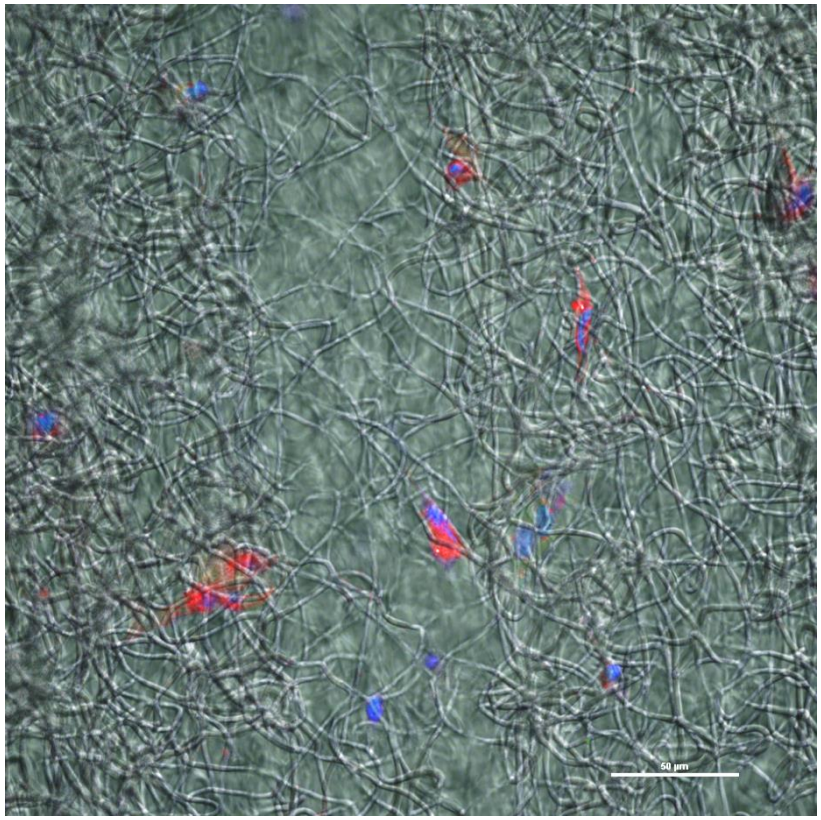
A component of interest in the specimen is specifically labeled with a fluorescent molecule called a **fluorophore.**

The specimen is illuminated with light of a specific wavelength which is absorbed by the fluorophores, causing them to emit longer wavelengths of light (of a different color than the absorbed light).

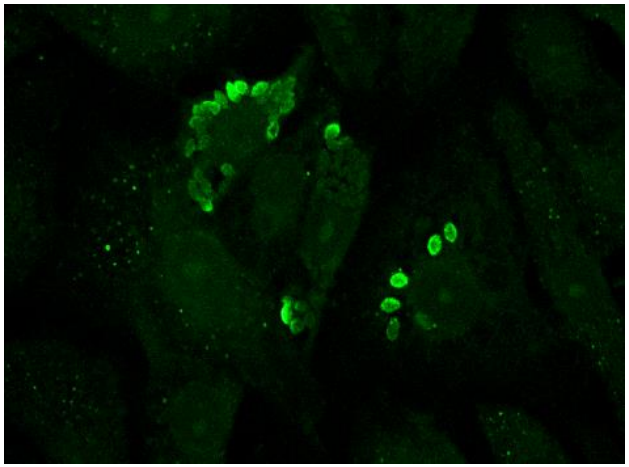


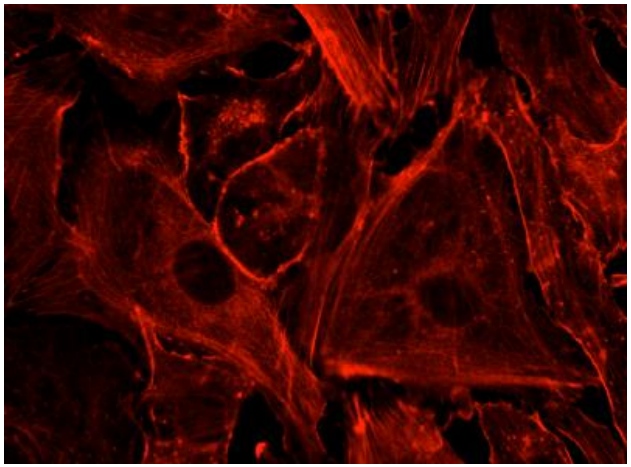
Fluorescence Principle

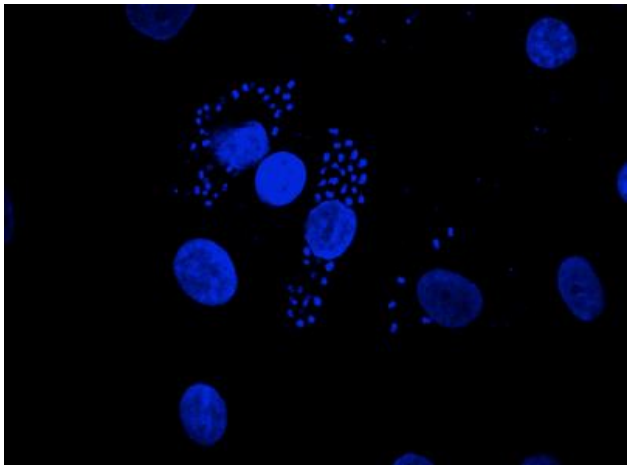
The key feature of fluorescence microscopy is that it employs **reflected** rather than transmitted light, which means transmitted light techniques such as phase contrast and DIC can be combined with fluorescence microscopy.

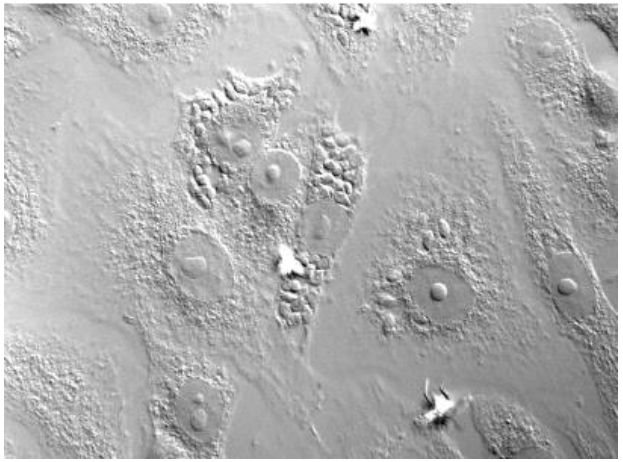


The Fluorescence Microscope





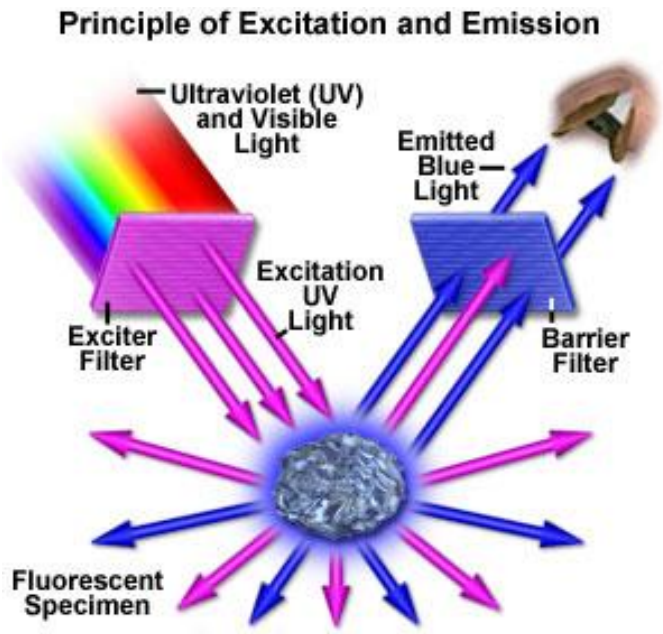


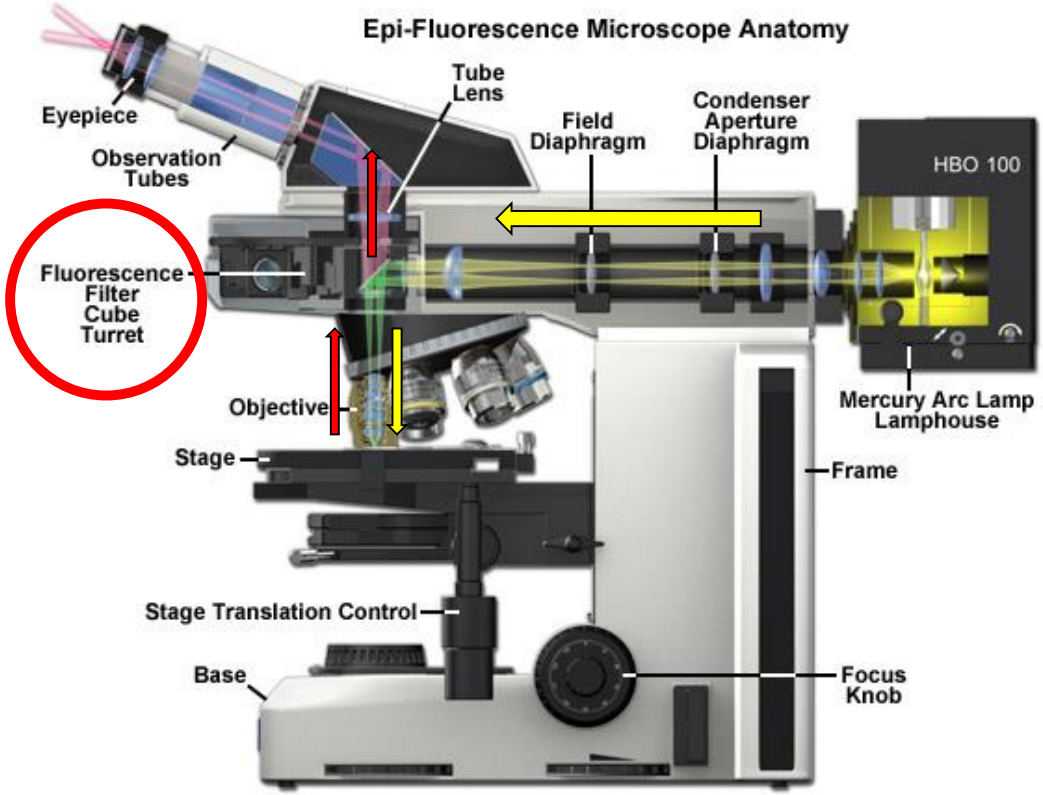


Actin - Rhodamine-phalloidin
Antibody to *T.cruzi* - FITC
DNA - Dapi

The Fluorescence Microscope

The basic function of a fluorescence microscope is to irradiate the specimen with a desired and specific band of wavelengths, and then to separate the much weaker emitted fluorescence from the excitation light.





The Fluorescence Microscope

How do we separate the Excitation Light from Emission Light?

Fluorescence Filter Cube

- **Excitaction Filter**

  Select the Excitation light

- **Dicroic Mirror**

  Reflects Excitation Light

  Trasmitted Emission Light

- **Emission Filter**

  Select the Emission light

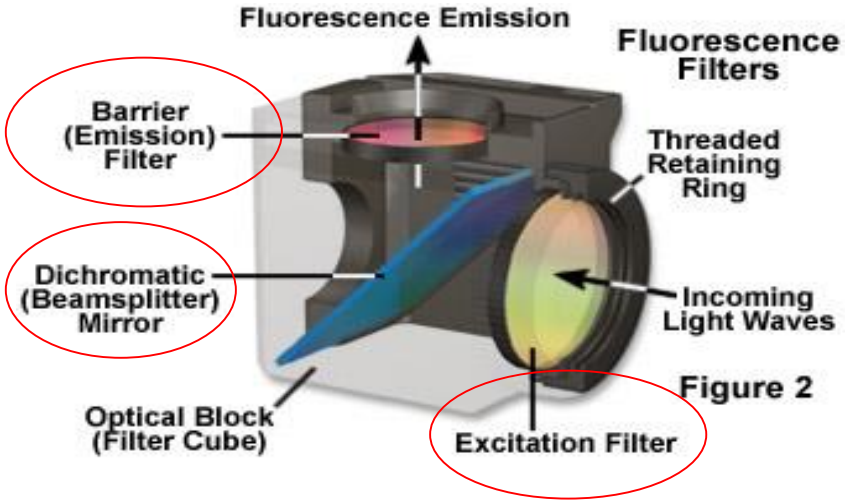
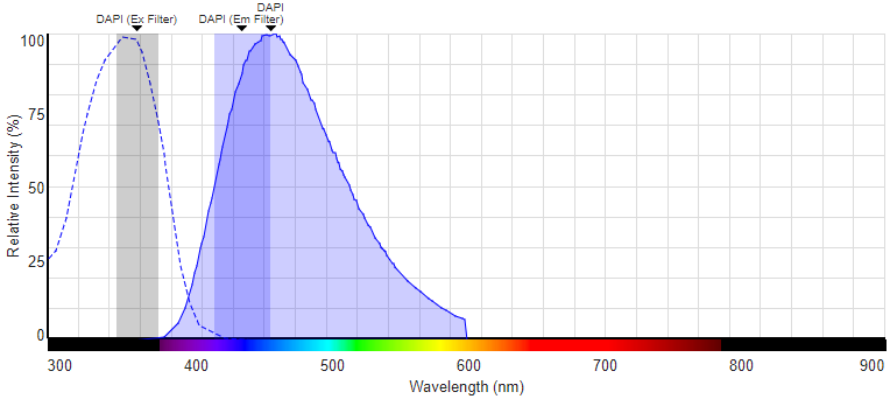
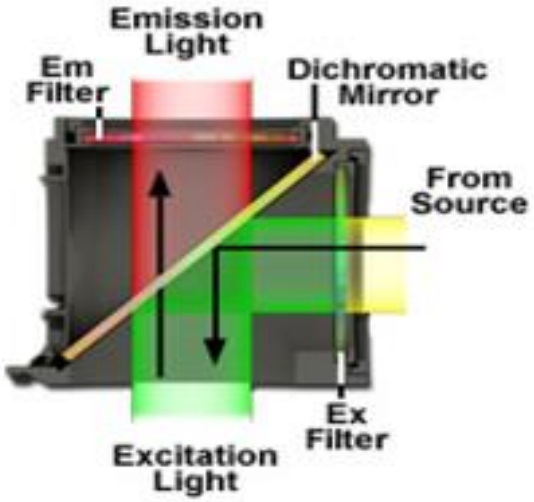


Figure 2

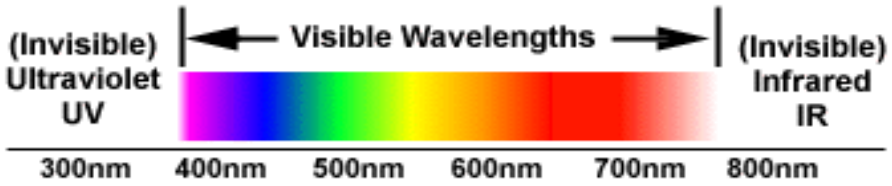




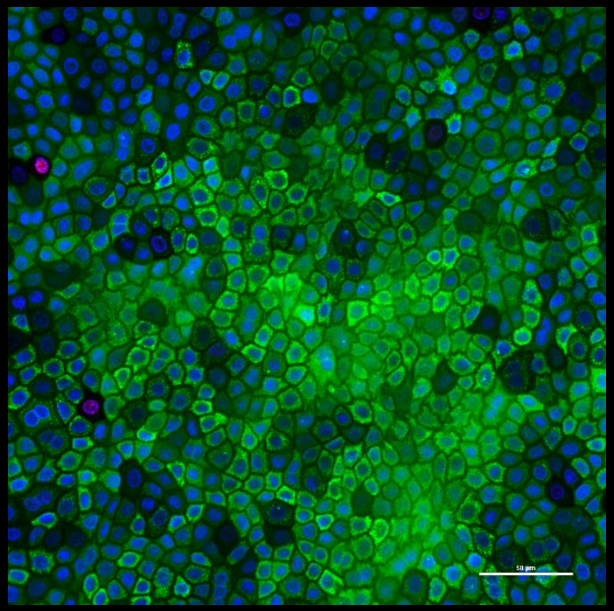
The principal fluorochromes

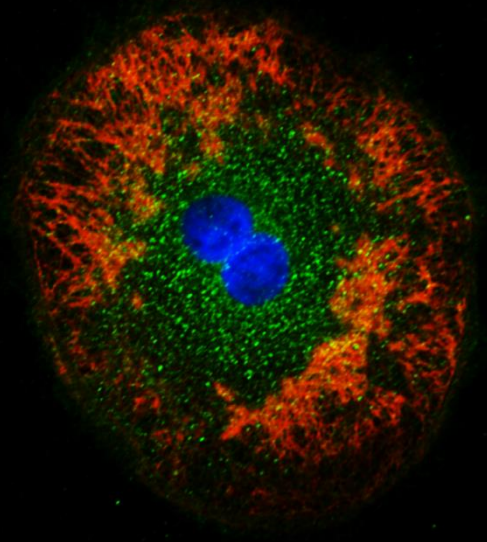
Dye Name	Excitation Max, nm	Emission Max, nm	Color
Alexa 350	346	442	Blue
Pacific Blue	416	451	
Marina Blue	362	459	Blue-Green
Acridine	362	462	
Edans	336	468	Green
Coumarin	432	472	
BODIPY 493/503	493	503	
Cy2	489	506	
BODIPY FL-X	504	510	Yellow-Green
DANSYL	335	518	
Alexa 488	495	519	
FAM	495	520	
Oregon Green	500	520	
Rhodamine Green-X	503	528	
NBD-X	466	535	
TET	521	536	
Alexa 430	434	541	
BODIPY R6G-X	529	547	Yellow
JOE	520	548	
Yakima Yellow	531	549	
Alexa 532	532	554	
VIC	538	554	
HEX	535	556	
R6G	524	557	

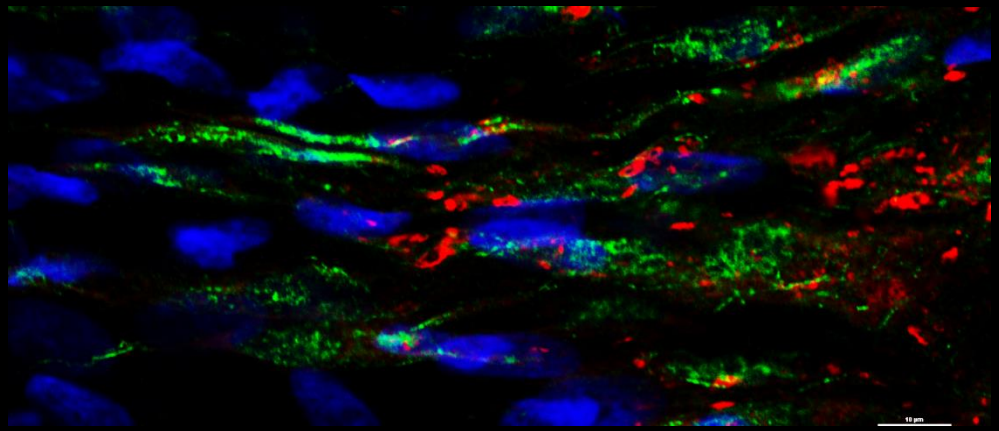
Dye Name	Excitation Max, nm	Emission Max, nm	Color
Alexa 555	555	565	Yellow-Orange
BODIPY 564/570	563	569	
BODIPY TMR-X	544	570	
Cy3	550	570	
Alexa 546	556	573	
TAMRA	555	576	
Rhodamine Red-X	560	580	
BODIPY 581/591	581	591	
Redmond Red	579	595	
Cy3.5	581	596	
ROX	575	602	Orange
Alexa 568	578	603	
Cal Red	583	603	
BODIPY TR-X	588	616	Orange-Red
Alexa 594	590	617	
BODIPY 630/650-X	625	640	Red
LC Red 640	625	640	
Alexa 633	632	647	
BODIPY 650/665-X	646	660	
Alexa 647	650	665	
Cy5	649	670	
Alexa 660	663	690	Far Red
Cy5.5	675	694	
Alexa 680	679	702	
LC Red 705	689	705	
Alexa 700	702	723	
Alexa 750	749	775	

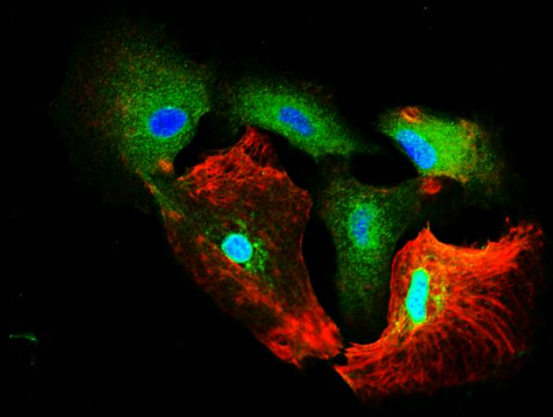


Multi-Wavelength Immunofluorescence Microscopy









Microscopy Tutorial

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https://www.microscopyu.com

Magnification