The technique for morphology

Visualizing cells

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How can we “see” what a cell looks like and behaves?

Imaging

Sample Preparation

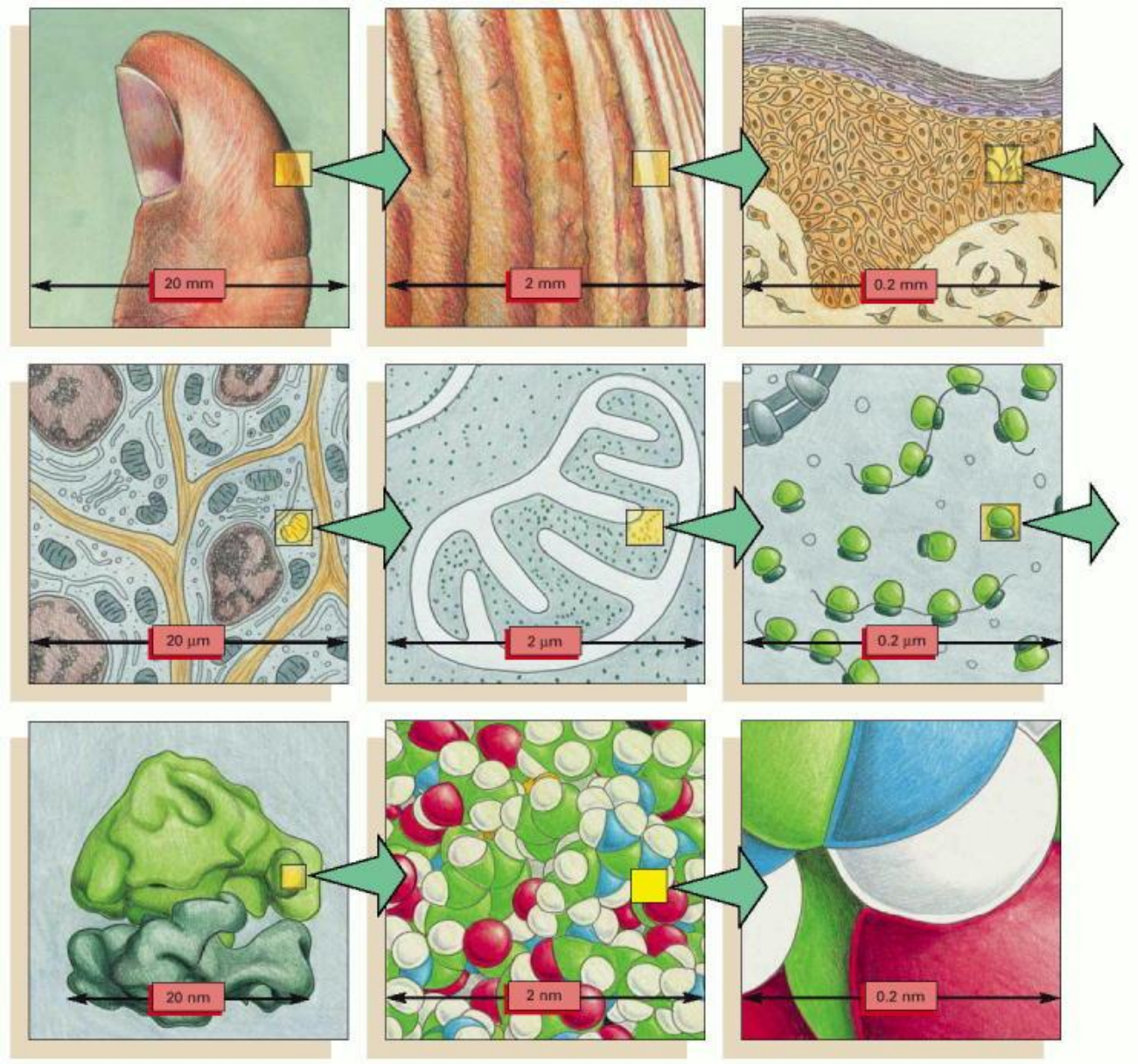
Types of Samples

Live		Fixed
Cultured Cells	*In-vitro*	Cultured Cells
Tissues or Tissue Slices	*Ex-vivo*	Tissues or Tissue Slices
Animals	*In-vivo*	Animals

Imaging

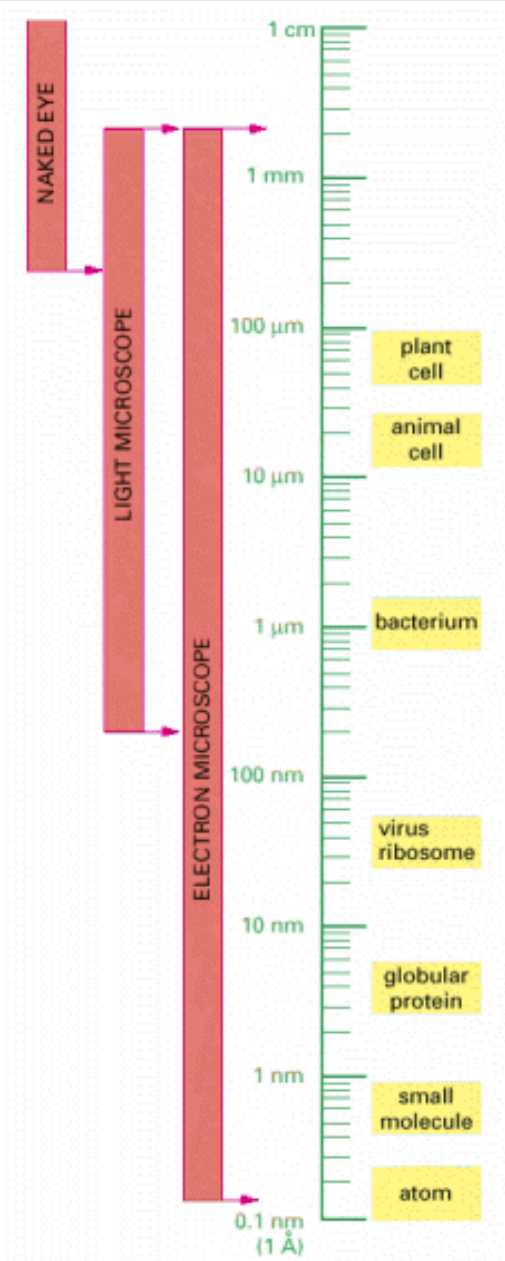
安东尼-列文虎克(荷兰人)
微生物开拓人
1632-1732

A sense of scale



From Molecular Biology of the Cell, 4th edtion

A sense of scale



Fluorescence Microscope (Dr. Xiang YU)

Scanning Electron Microscope (Dr. Shujia ZHU)

A sense of scale

Bruj K Halifa Tower (~828 meters)= 2岁孩子的平均身高*1000

Stereomicroscope (Dissecting microscope)

Features	Disadvantages	Use

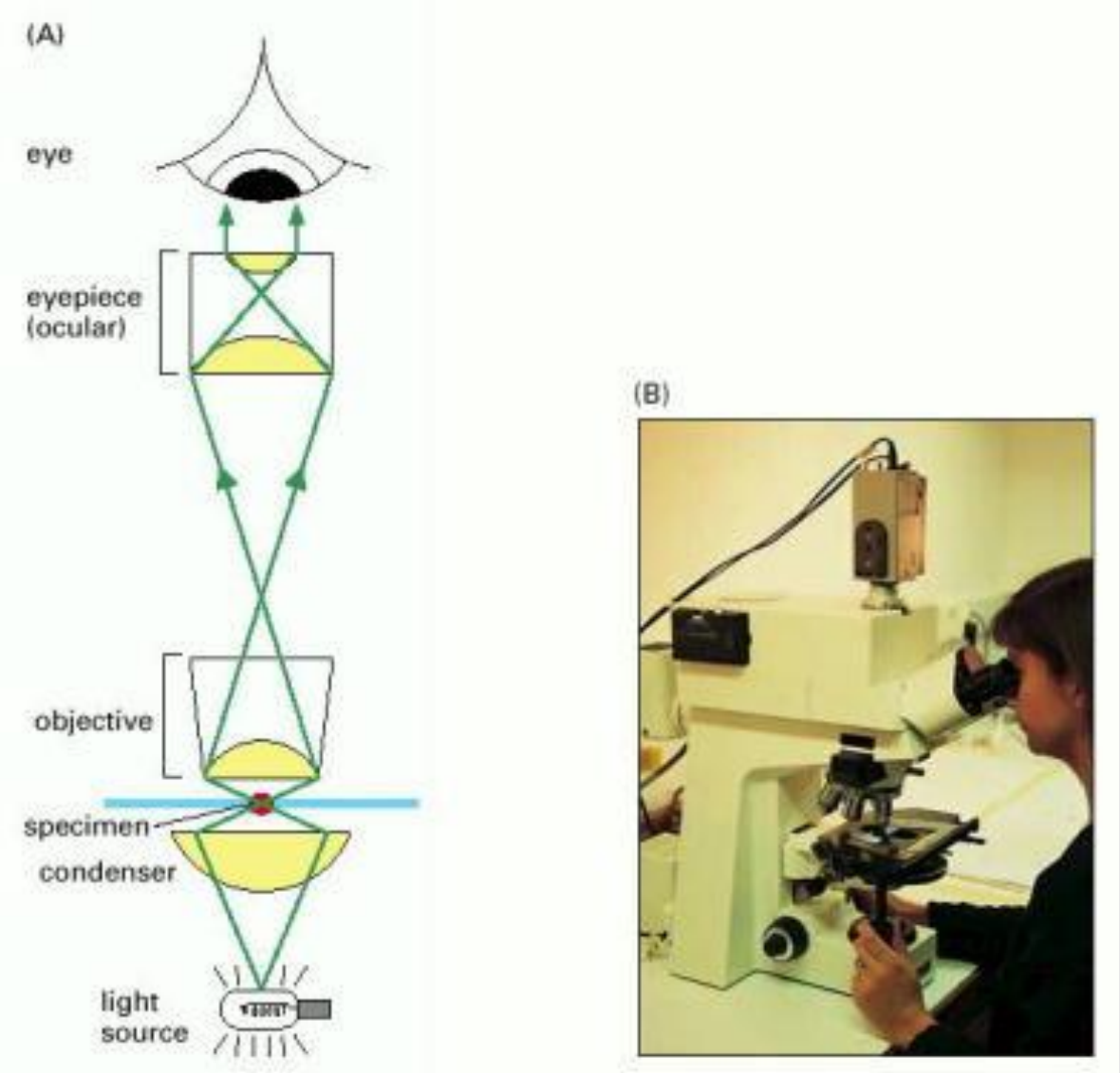
Stereomicroscope (Dissecting microscope)

Bright-field Microscope

Upright

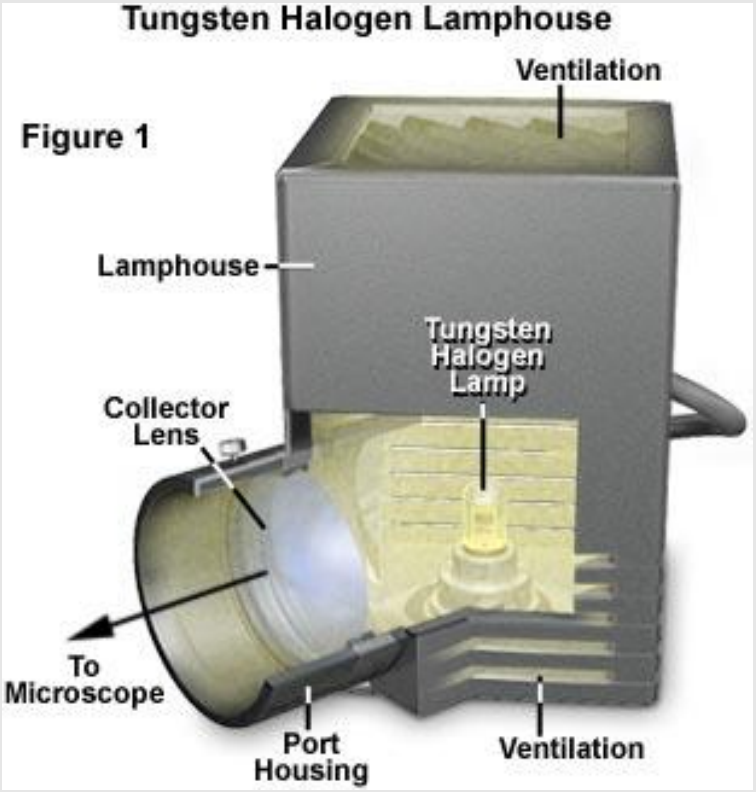
Inverted

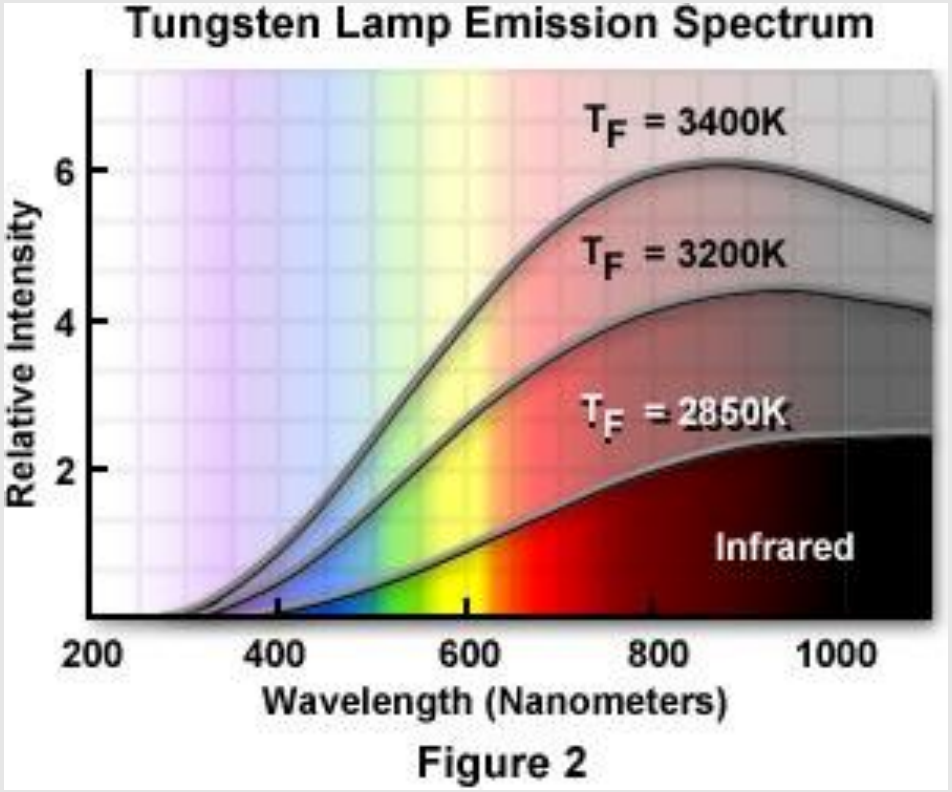
Light microscope



From Molecular Biology of the Cell, 4th edtion

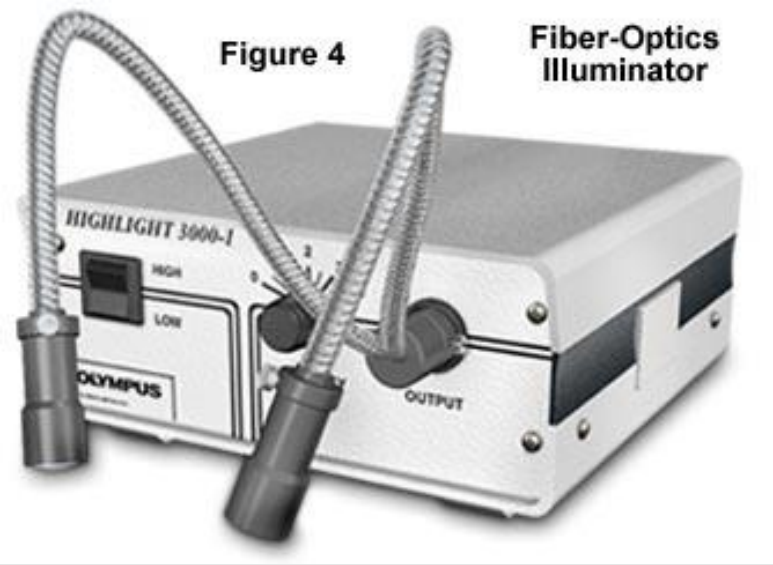
Light source



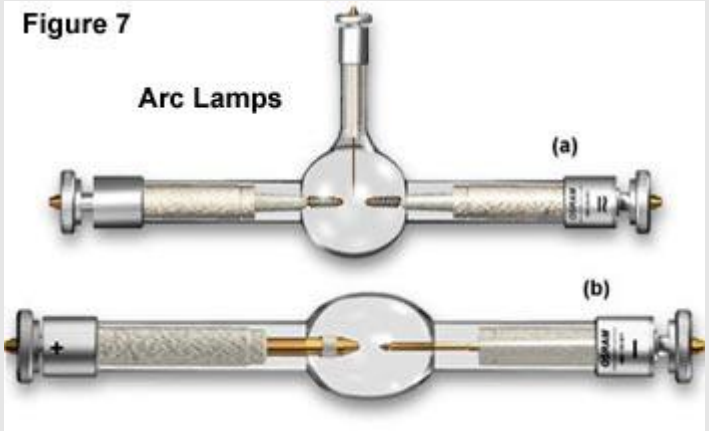


300-1400-nm light

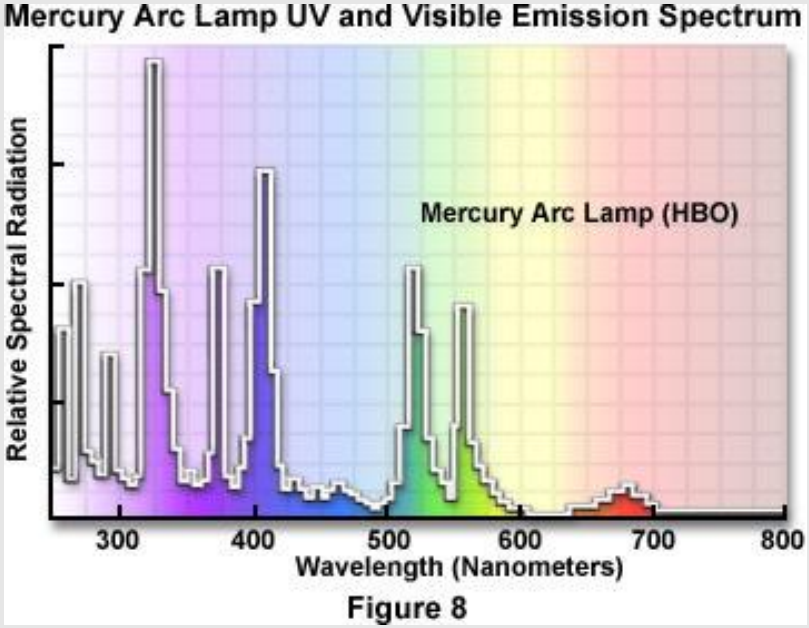
Light source



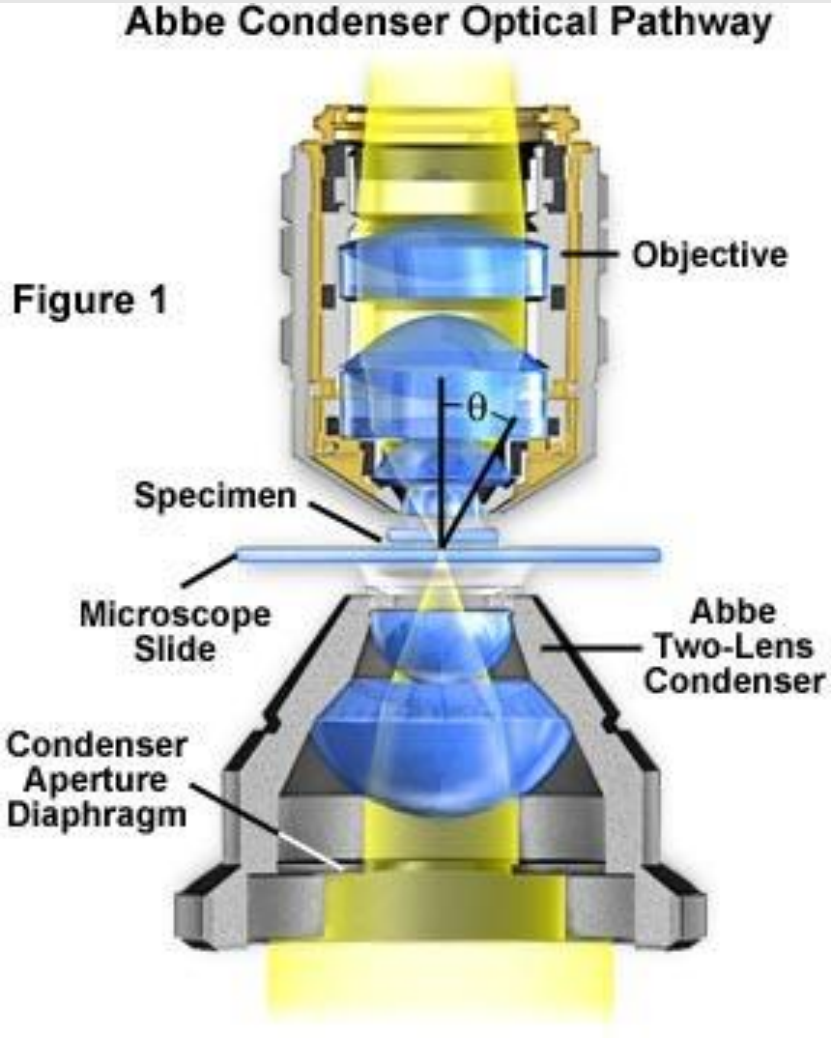
Light source



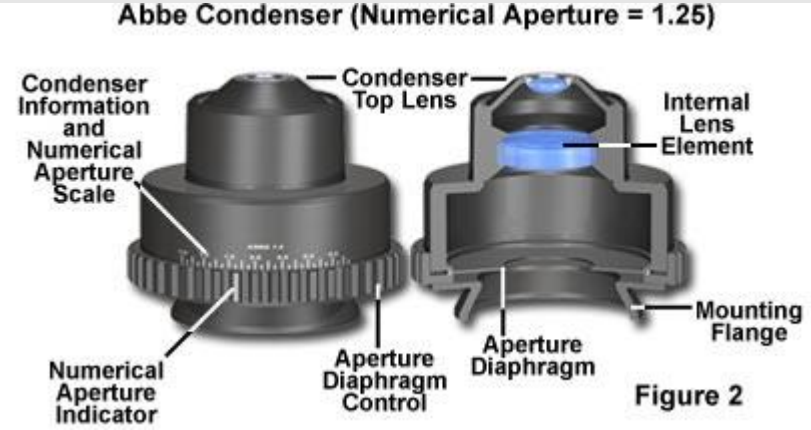
Mercury Vapor Lamp

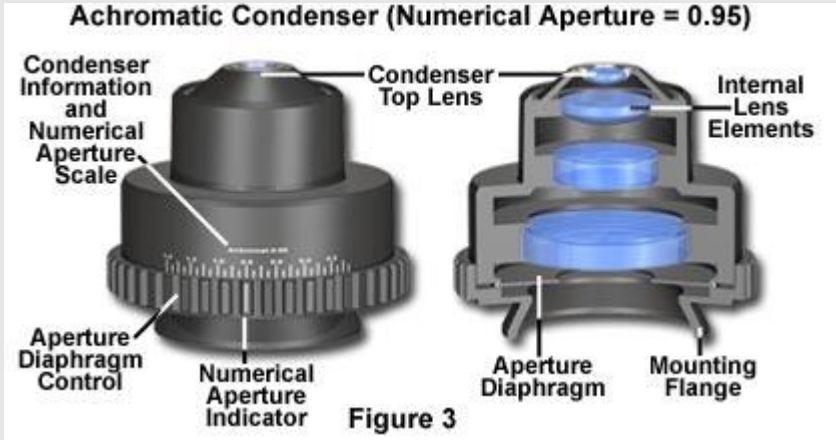


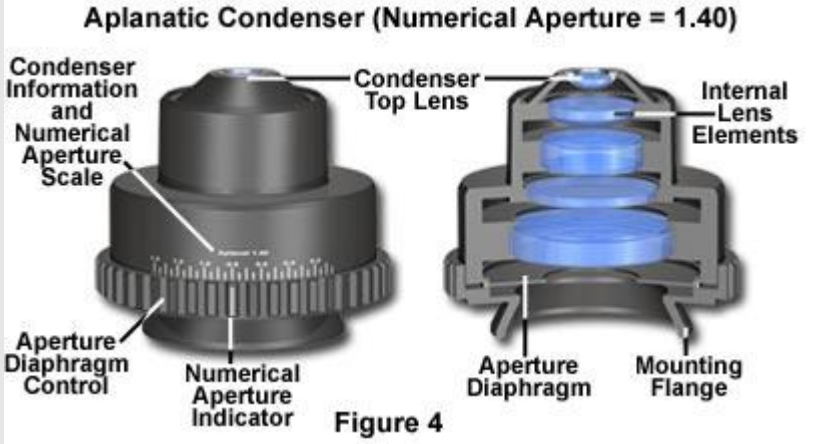
Condenser

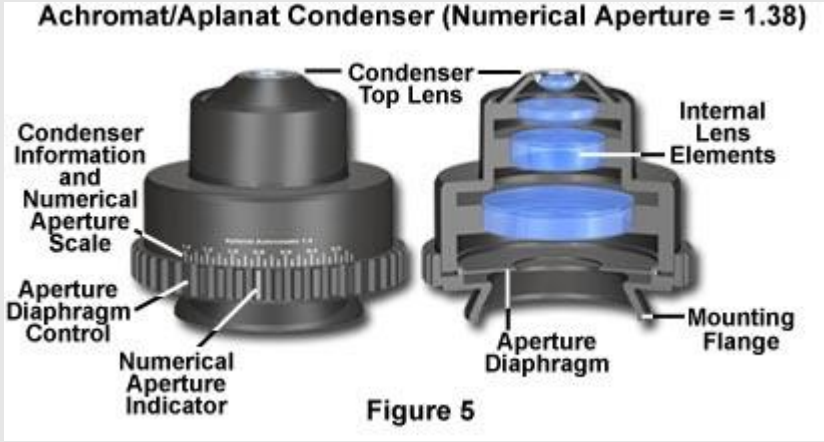

Abbe Condenser Optical Pathway
Figure 1
Objective
θ
Specimen
Microscope Slide
Abbe Two-Lens Condenser
Condenser Aperture Diaphragm

Condenser

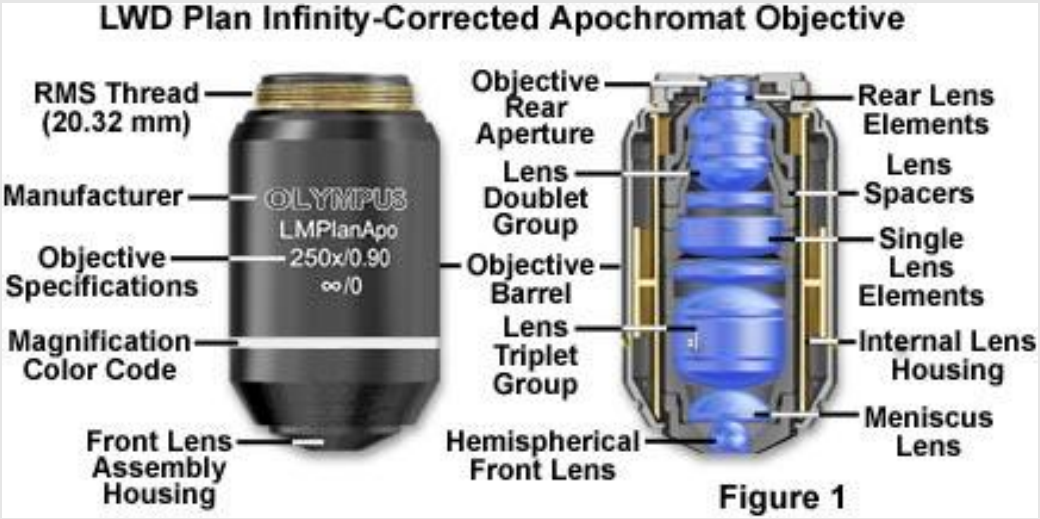

Abbe Condenser (Numerical Aperture = 1.25)
Condenser Information and Numerical Aperture Scale
Condenser Top Lens
Internal Lens Element
Mounting Flange
Numerical Aperture Indicator
Aperture Diaphragm Control
Aperture Diaphragm
Figure 2


Achromatic Condenser (Numerical Aperture = 0.95)
Condenser Information and Numerical Aperture Scale
Condenser Top Lens
Internal Lens Elements
Aperture Diaphragm Control
Numerical Aperture Indicator
Aperture Diaphragm
Mounting Flange
Figure 3


Aplanatic Condenser (Numerical Aperture = 1.40)
Condenser Information and Numerical Aperture Scale
Condenser Top Lens
Internal Lens Elements
Aperture Diaphragm Control
Numerical Aperture Indicator
Aperture Diaphragm
Mounting Flange
Figure 4


Achromat/Aplanat Condenser (Numerical Aperture = 1.38)
Condenser Top Lens
Condenser Information and Numerical Aperture Scale
Internal Lens Elements
Aperture Diaphragm Control
Numerical Aperture Indicator
Aperture Diaphragm
Mounting Flange
Figure 5

Objectives



Amplification: 10x; 20x; 25x; 30x; 40x; 60x; 100x

Resolution : $R = \lambda / 2n(\sin(\theta))$

NA

Objectives

Types of Objectives

air (NA<1.0)
Water (NA: up to 1.2)
Oil (NA: up to 1.4)

Working Distance (WD)

Eyepieces and Camera

Figure 1

Eyepieces and Camera

Viewfield Diameter = (FN) / (M(O) × M(T))

where **FN** is the field number in millimeters, **M(O)** is the objective magnification, and **M(T)** is the tube lens magnification factor (if any)

Magnification (x)	Viewfield Diameter (mm)
1	21.2
2	10.6
4	5.3
10	2.12
20	1.06
40	0.53
50	0.42
60	0.35
100	0.21

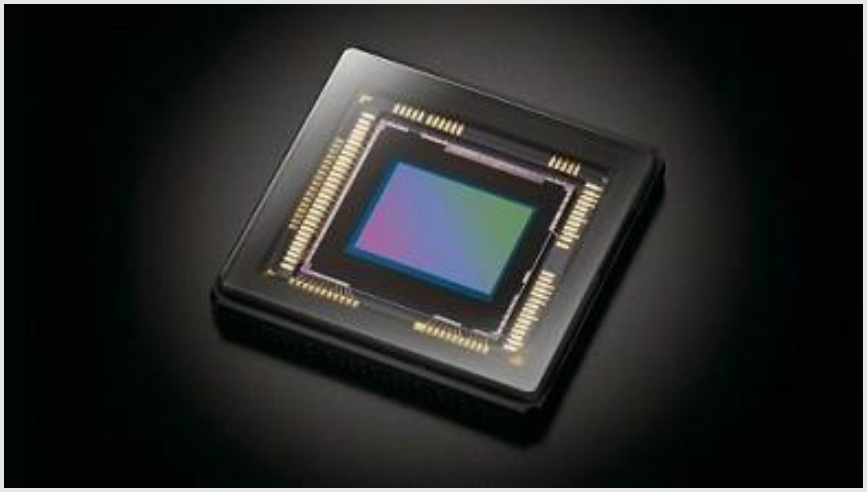
Eyepieces and Camera

500 NA to 1000 NA

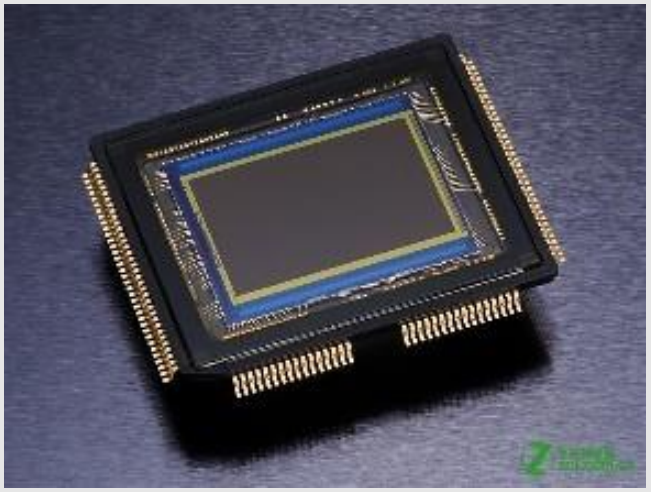
NA	10x	12.5x	15x	20x	25x
2.5x (0.08)	√	√	√	x	x
4x (0.12)	√	√	x	x	x
10x (0.35)	v	x	x	x	x
25x (0.55)	x	x	x	x	√
40x (0.70)	x	x	x	√	√
60x (0.95)	x	x	x	√	√
100x (1.42)	x	x	√	√	√

Eyepieces and Camera

Charge-coupled Device (CCD)



Complementary metal oxide semiconductor (cMOS)



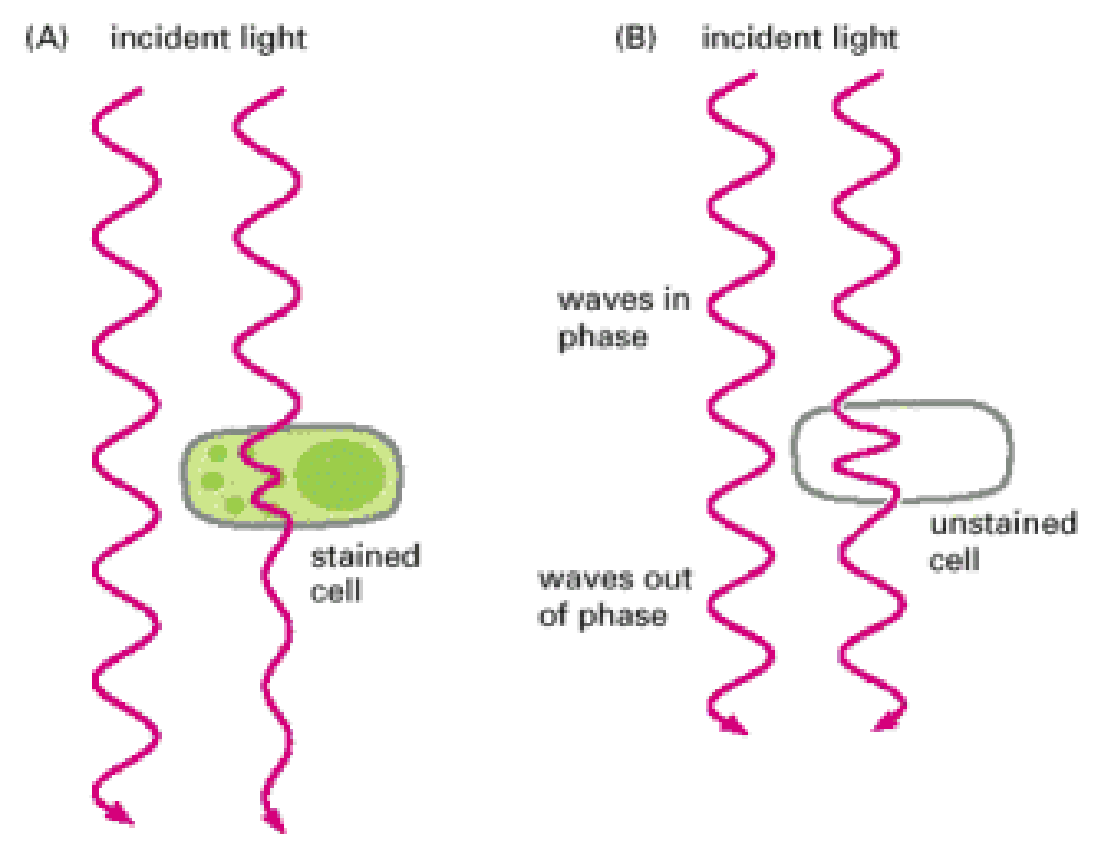
Exposure time
Resolution
Binning
Gain

Eyepieces and Camera

CCD and cMOS 区别

	CCD	cMOS
成像过程	统一输出	每个传感器单独输出
集成性	工艺高	工艺简单，成本低
速度	速度慢	速度快 (>500 fps)
噪音	低	较高

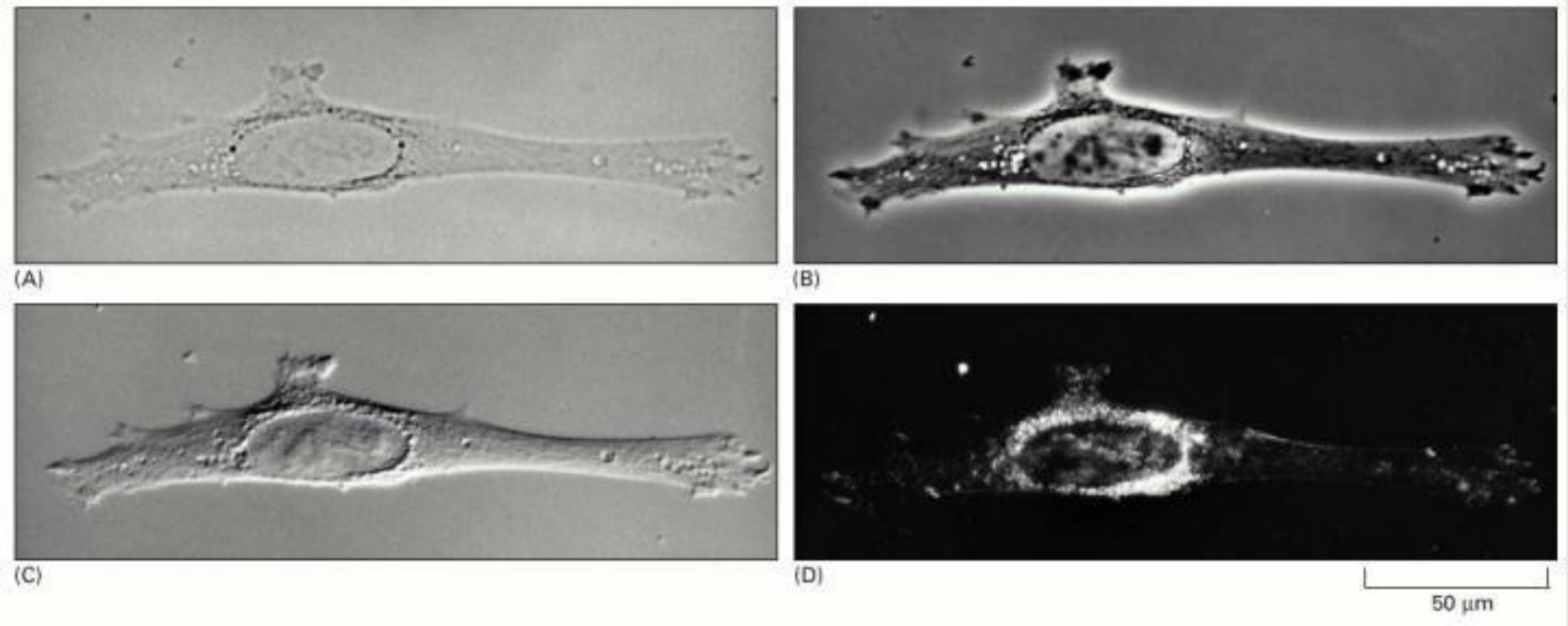
Imaging Principles



A stained tissue section

From Molecular Biology of the Cell, 4th edtion

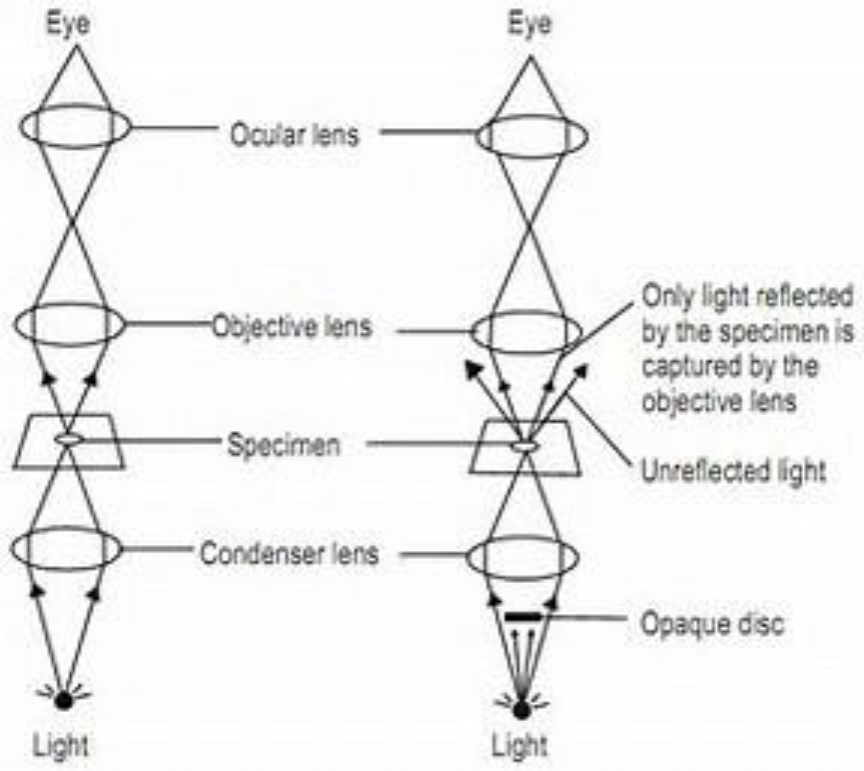
Four types of specialized imaging



(A) Bright-field microscopy.
(B) Phase-contrast microscopy.
(C) Nomarski differential-interference-contrast microscopy.
(D) Dark-field microscopy

From Molecular Biology of the Cell, 4th edtion

Bright-field microscopy



Advantages

1. Simple setup
2. Live cells can be visualized by bright-field microscopes

Bright-field microscopy

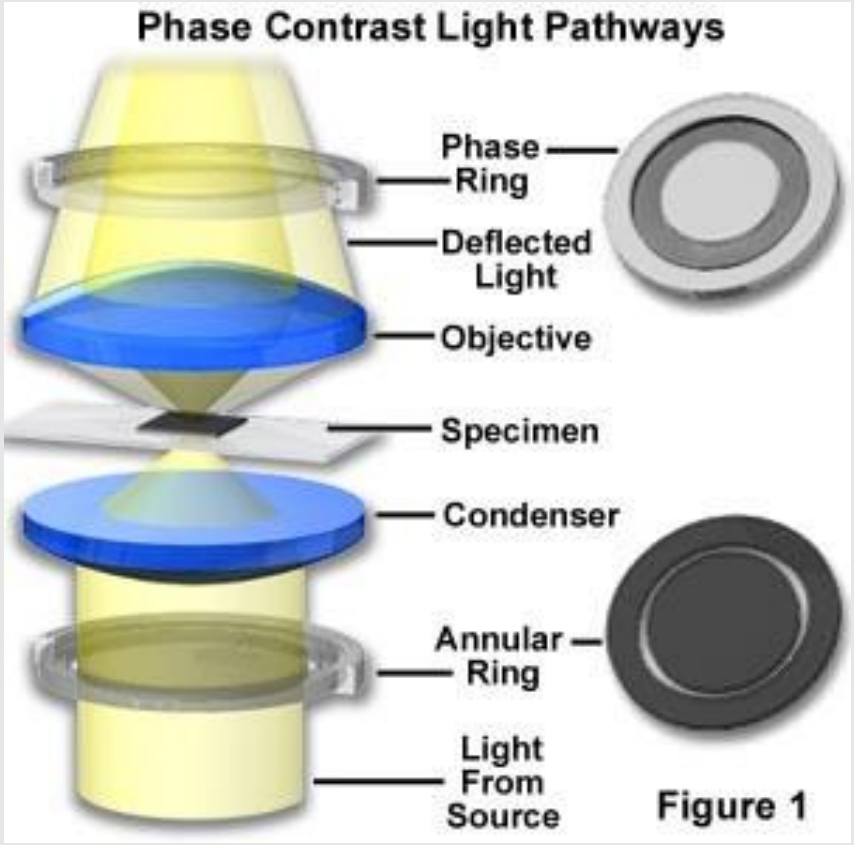
Limitations

1. Low contrast
2. Maximal magnification is about 1300 x (otherwise, resolution drops)
3. Low resolution due to the blur of out-of-focus material
4. Colorless and transparent samples are not applicable

Enhancement

1. Change the amount of the light source
2. Use of an oil-immersion objective
3. Staining: methylene blue, safranin, crystal violet
4. Use of a colored or polarized filter on the light source to highlight features

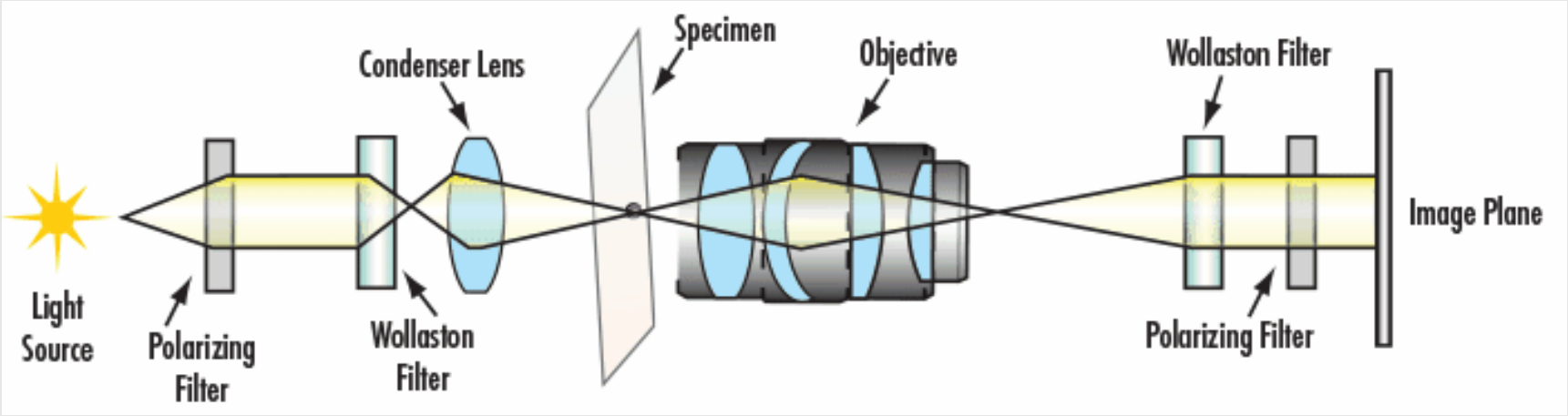
Phase contrast microscopy



针对于未染色的标本

环形光阑
相位板
合轴调节望远镜
绿色滤光片

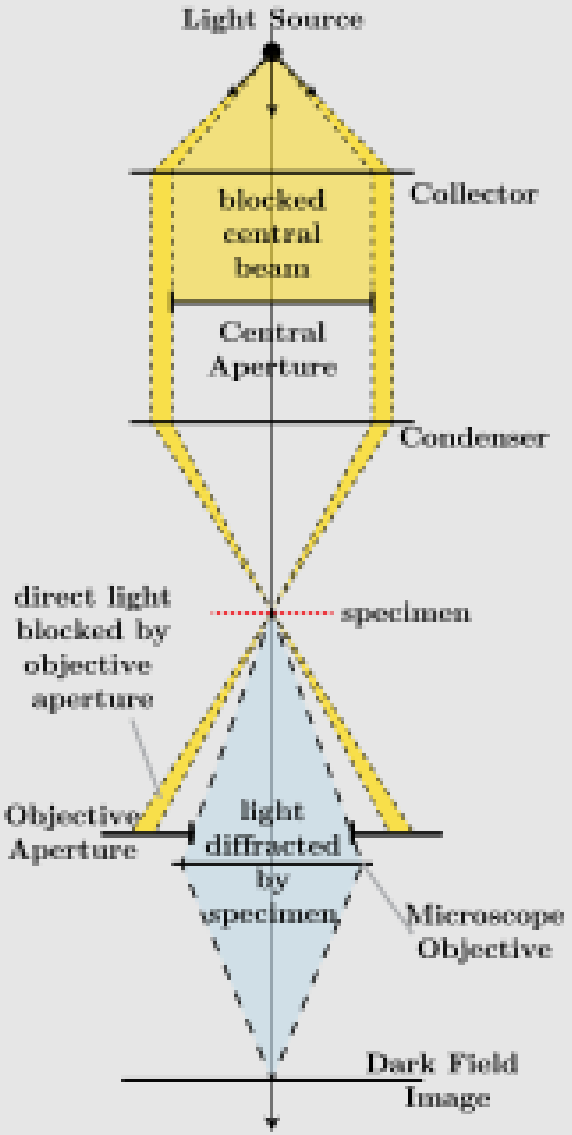
Nomarski differential-interference-contrast microscopy (DIC)



偏振器 (Polarizer)
DIC棱镜
DIC滑行器
检偏器 (Analyzer)

优点：针对细胞结构，核，线粒体等
能显示结构的三维立体投影影像
标本可略厚一点，折射率相差更大

Dark-field microscopy



“丁达尔效应”

Imaging

Sample Preparation

Sample Preparation

1. Sectioning
2. Fixation
3. Staining

Sectioning

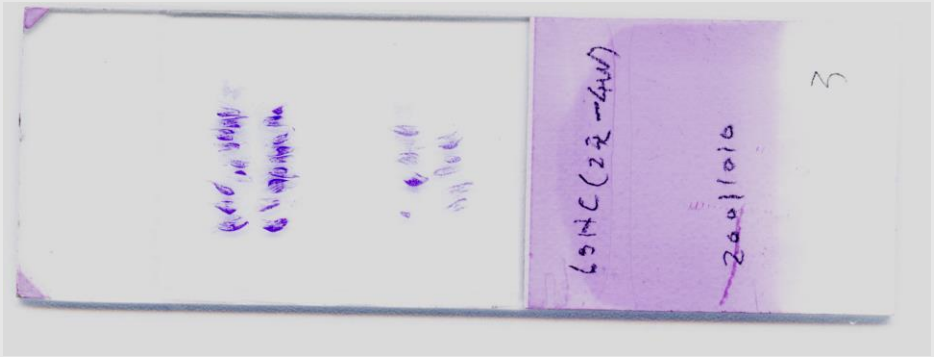
石蜡切片　　冰冻切片　　震动切片

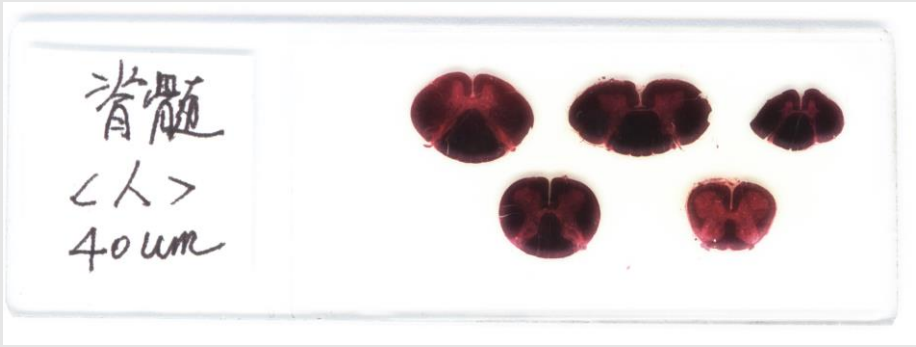
cryostat

Microtome（石蜡切片5μm）

Vibratome

Section





明胶硫酸铬钾　（免疫组化浸载片用）

2　g　　gelatin

0.2 g　$KCr(SO_4)_2 \cdot 12H_2O$

400 ml H_2O

加热溶化，但不要超过50°C；

过滤；

浸片子1-2 min;

片子干后，可重复浸泡1-2 min一次，干后即可。

(室温下48 h 内溶液有效，也可在4度冰箱内保存较长时间)

Sample Preparation

Fixation

Autolysis

Fixative (prevention of autolysis; limit microbial growth)

Fixative (4% paraformaldehyde)

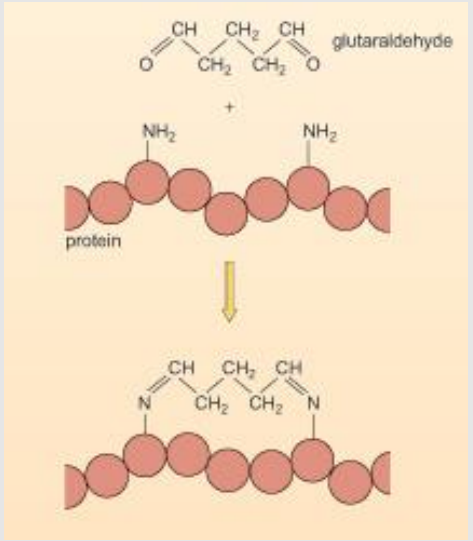
Cross-linking (paraformaldehyde, glutaraldehyde)

Precipitant fixatives (alcohol, methanol, acetone)

pH (acidic fixative, neutral fixative)

Procedure (perfusion, immersion, post-fixation)

Sucrose (10%、20%、30%)



Sample Preparation

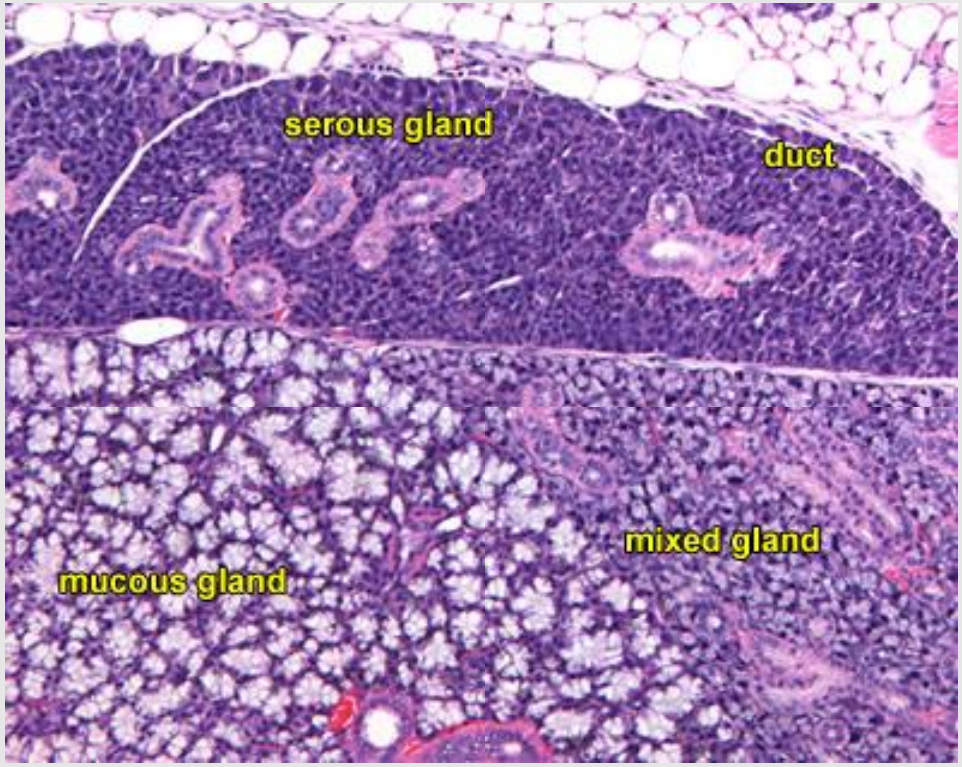
Staining

Routine staining of tissue sections

Hematoxylin-and-eosin, or H&E staining, is used for routine staining of tissue sections.

Hematoxylin, a basic dye, binds to acidic components of a tissue, which are thus said to be "basophilic." The color of the stained structures depends on the mordant used to make the hematoxylin dye bind to the molecules of the tissue. Potassium alum, the most common mordant, gives the dye a blue to purple color. Basophilic nuclei, bacteria, calcium, and so on are stained "blue" with hematoxylin.

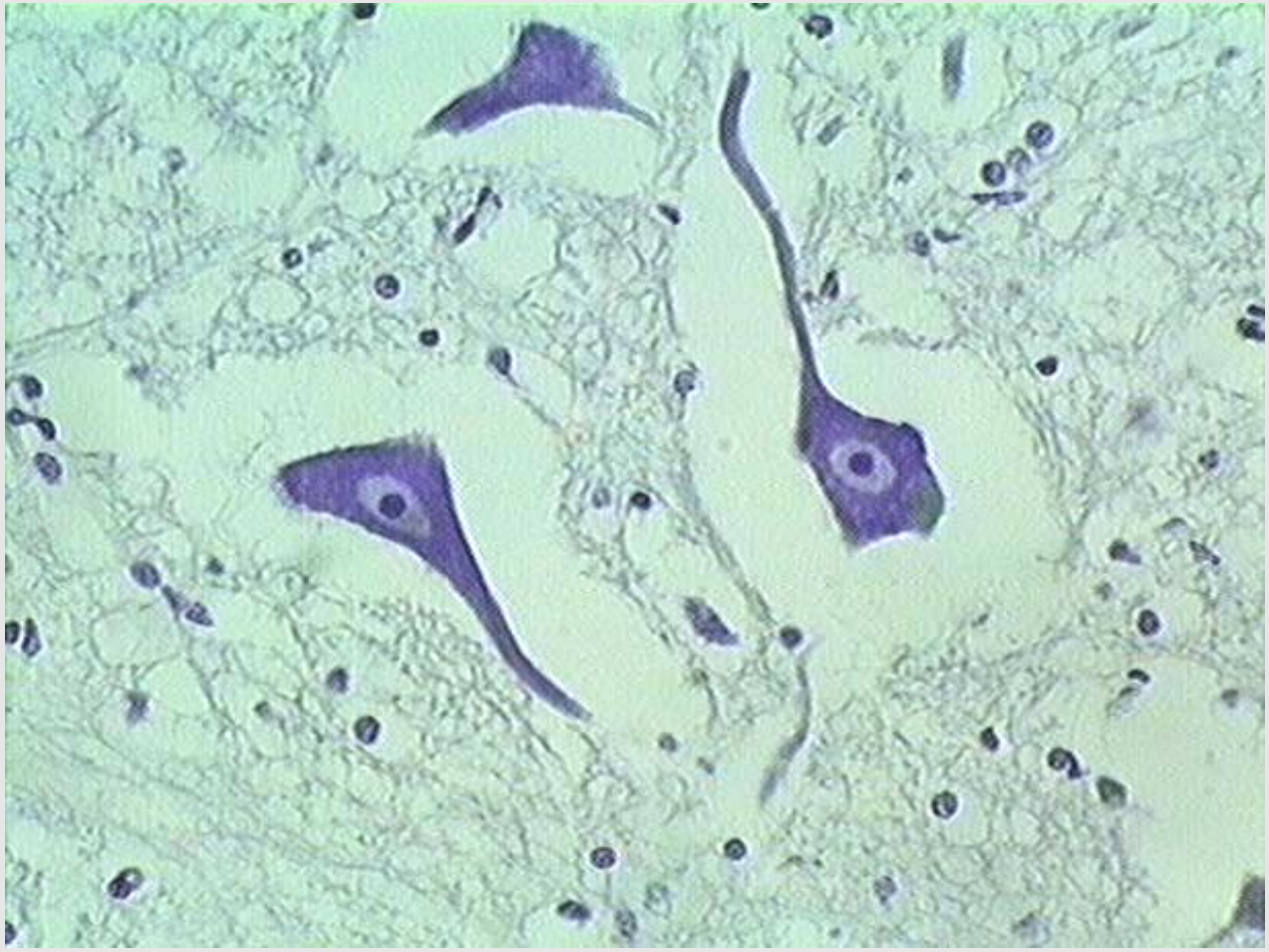
Eosin, an acidic dye, binds to basic components of a tissue, which are thus said to be "acidophilic." The structures stained by eosin are typically colored pink to red. Eosinophilic cytoplasm, connective, and all other tissues are counterstained "red" with eosin.



H&E-staining of mouse salivary glands fixed in formalin

Cresyl Violet (Nissl stain)

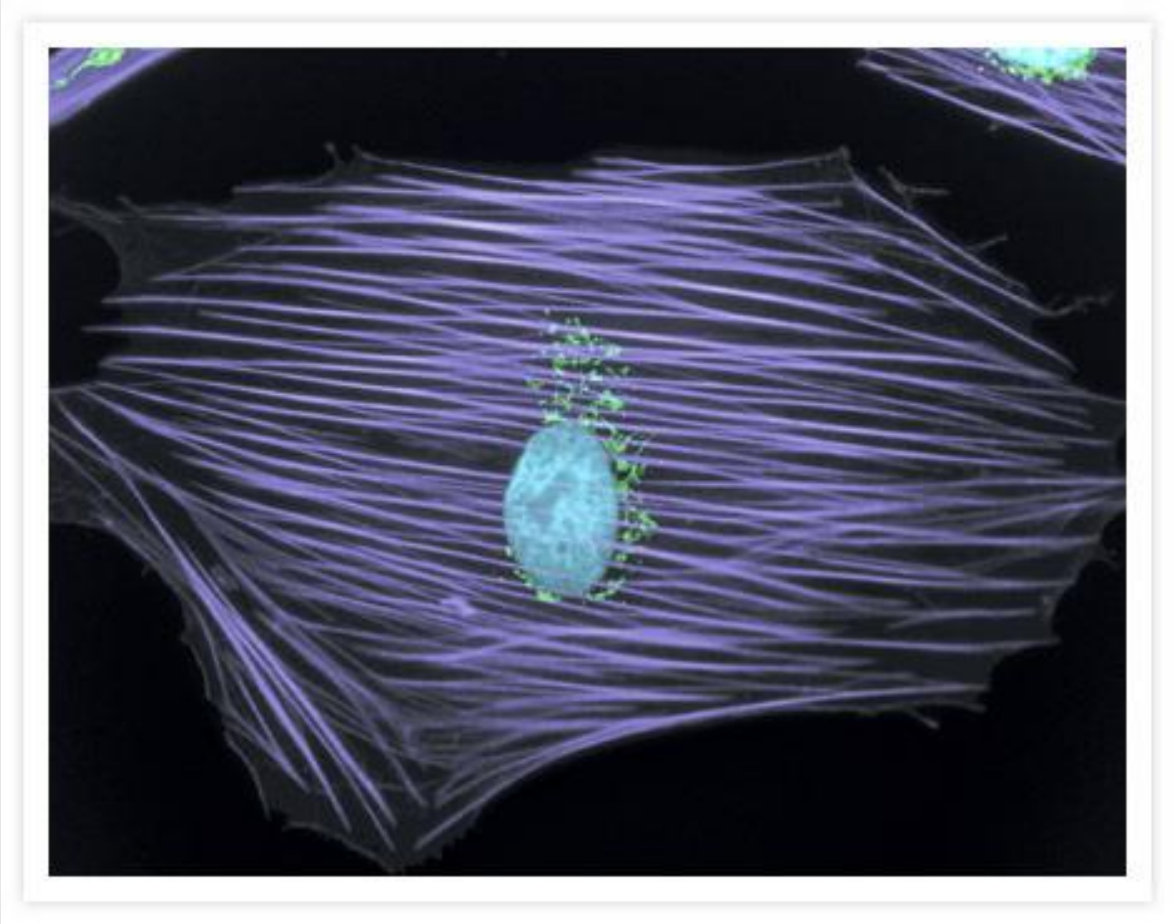
Nissl stain stains RNA and DNA purple. The so-called Nissl substance in the cytoplasm of nerve cells has been identified as ribosomes and the rough endoplasmic reticulum. This stain gives the Nissl substance a purple color.



This section from the spinal cord has been stained with cresyl violet, known as the Nissl stain, which stains RNA.

Dyes

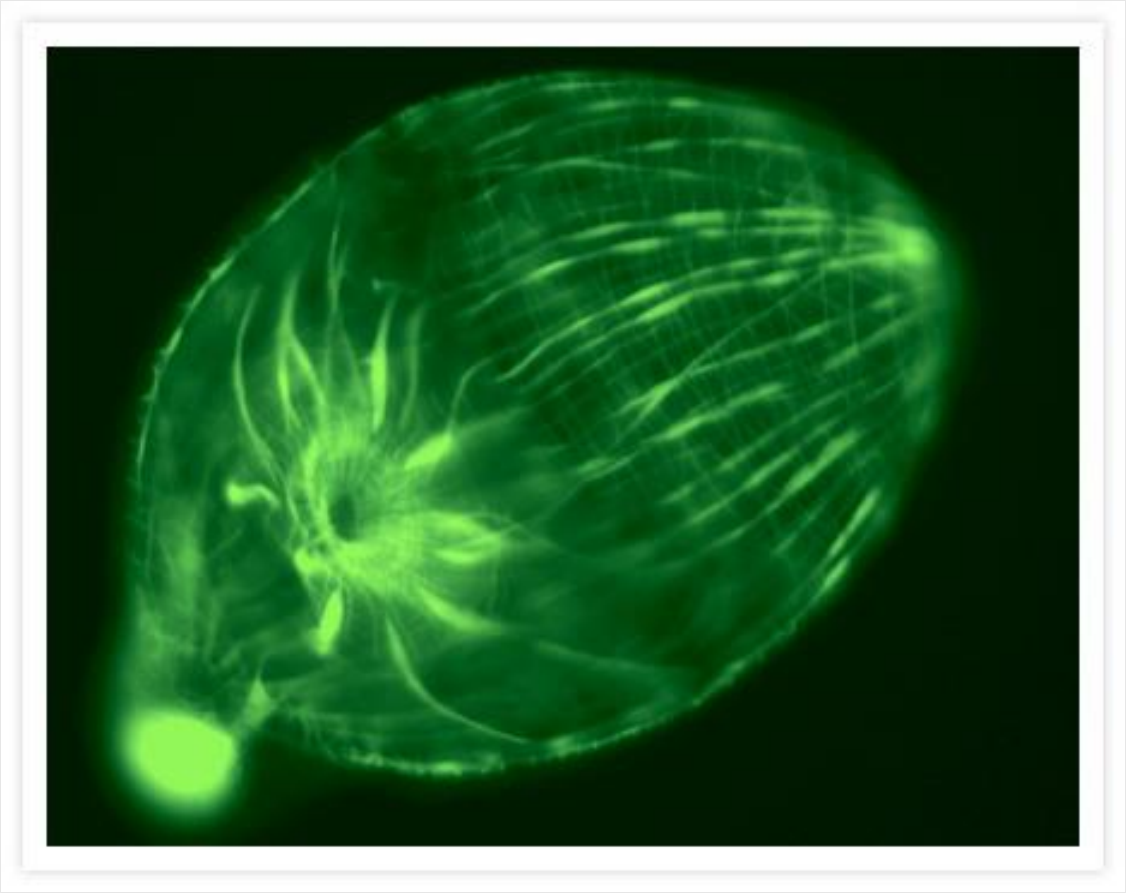
- Nucleic acid detection
- Actin and tubulin probes
- Apoptosis probes
- Calcium indicator
- Organelle probes: golgi apparatus, endoplasmic reticulum, lysosomes, vacuoles and other acidic organelles, mitochondria

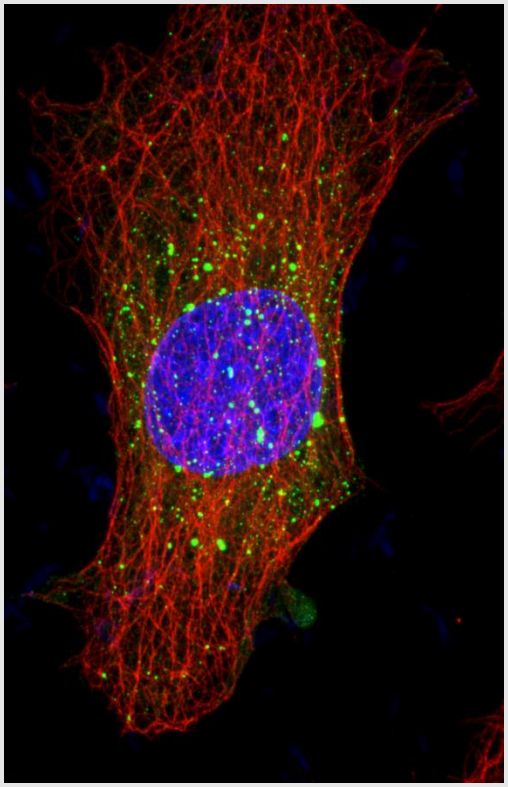


Golgi bodies were labeled with anti–Golgin-97 antibody
Filamentous actin was labeled with Alexa Fluor 680 phalloidin (pseudocolored purple).
Nuclei were stained with blue-fluorescent DAPI.

From Molecular Probes

Actin and tublin probes

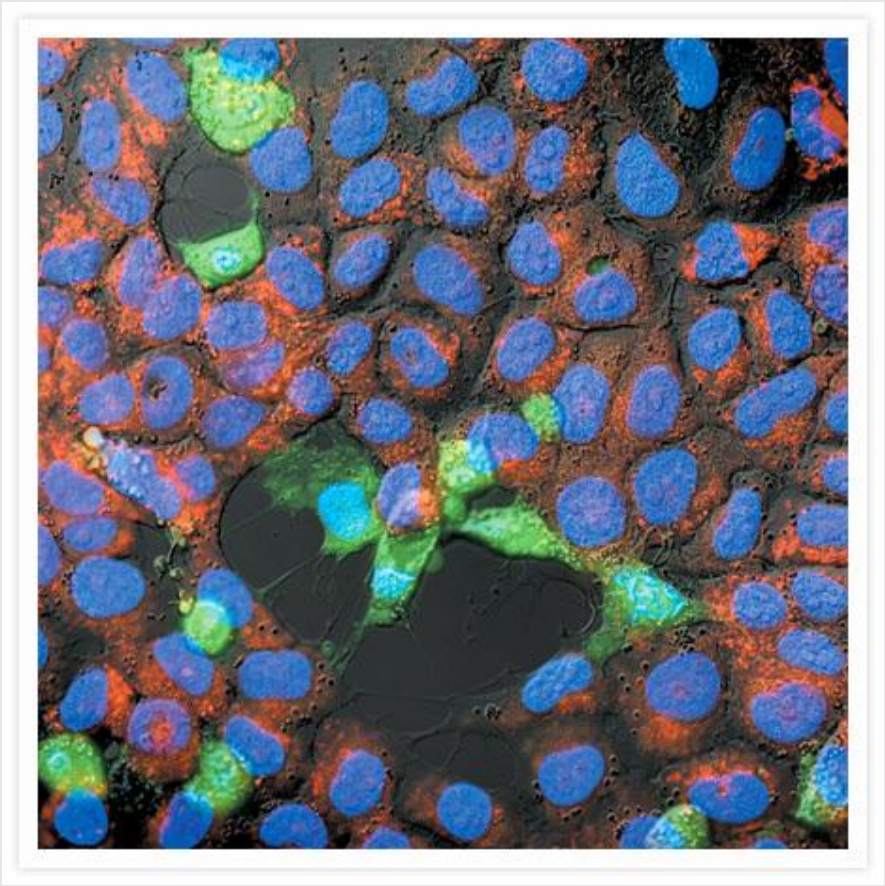




Provided by Wei Bian

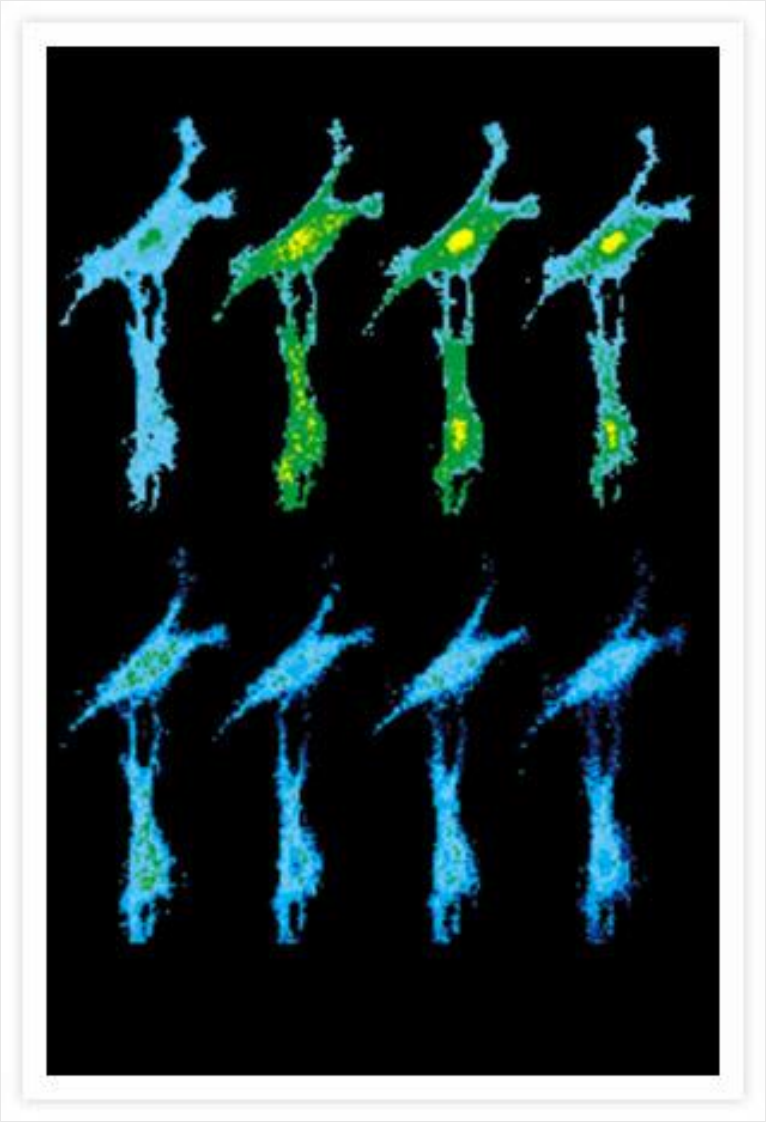
Actin filaments of the turbellarian flatworm *Archimonotresis* sp. stained with Alexa Fluor 488 phalloidin to reveal a meshwork of longitudinal, circular and diagonal muscles.

From Molecular Probes



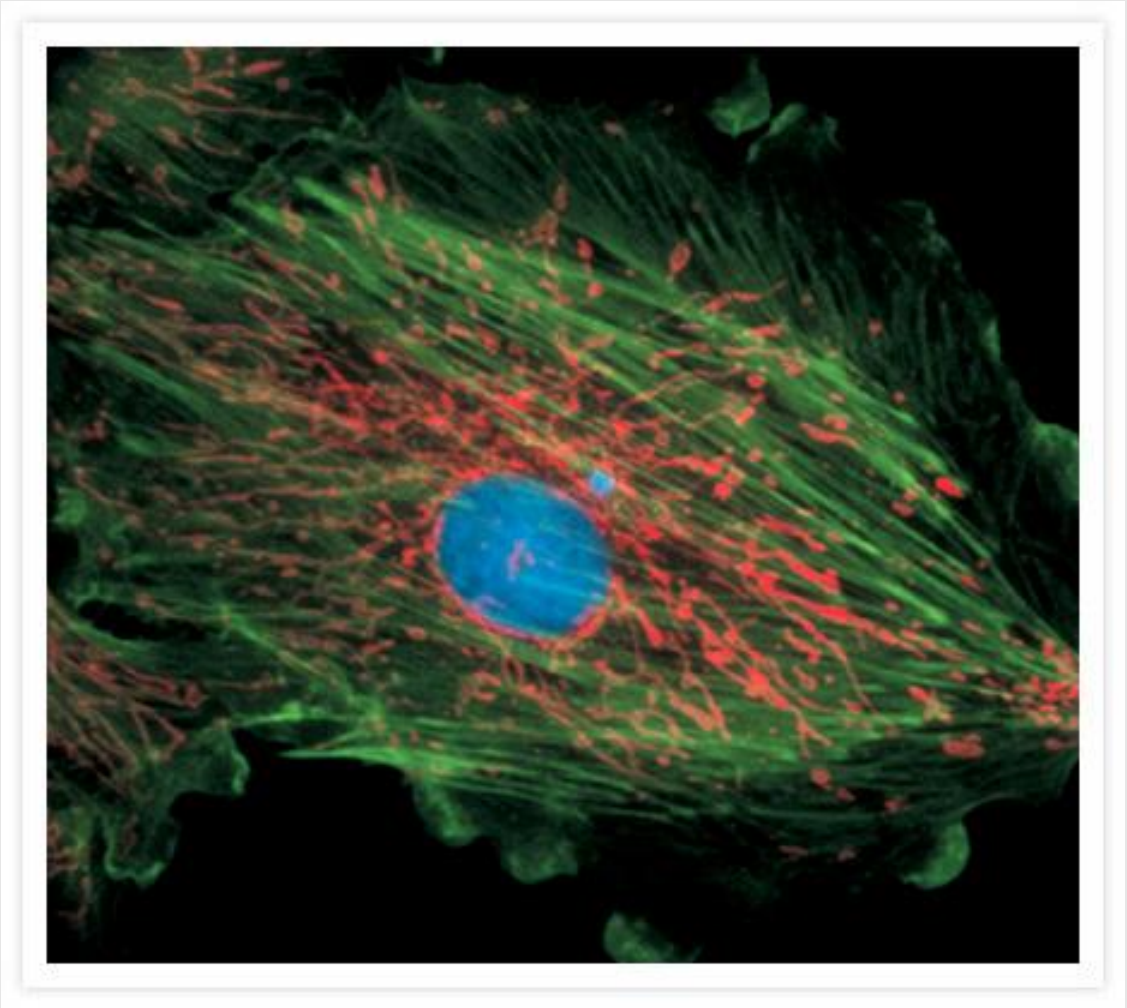
Detection of apoptosis in SK-N-MC neuroblastoma cells
Hoechst 33342 (blue),
Tetramethylrhodamine ethyl ester (TMRE, red), mitochondria marker
Rhodamine 110, bis-L-aspartic acid amide (green), apoptosis marker

From Molecular Probes



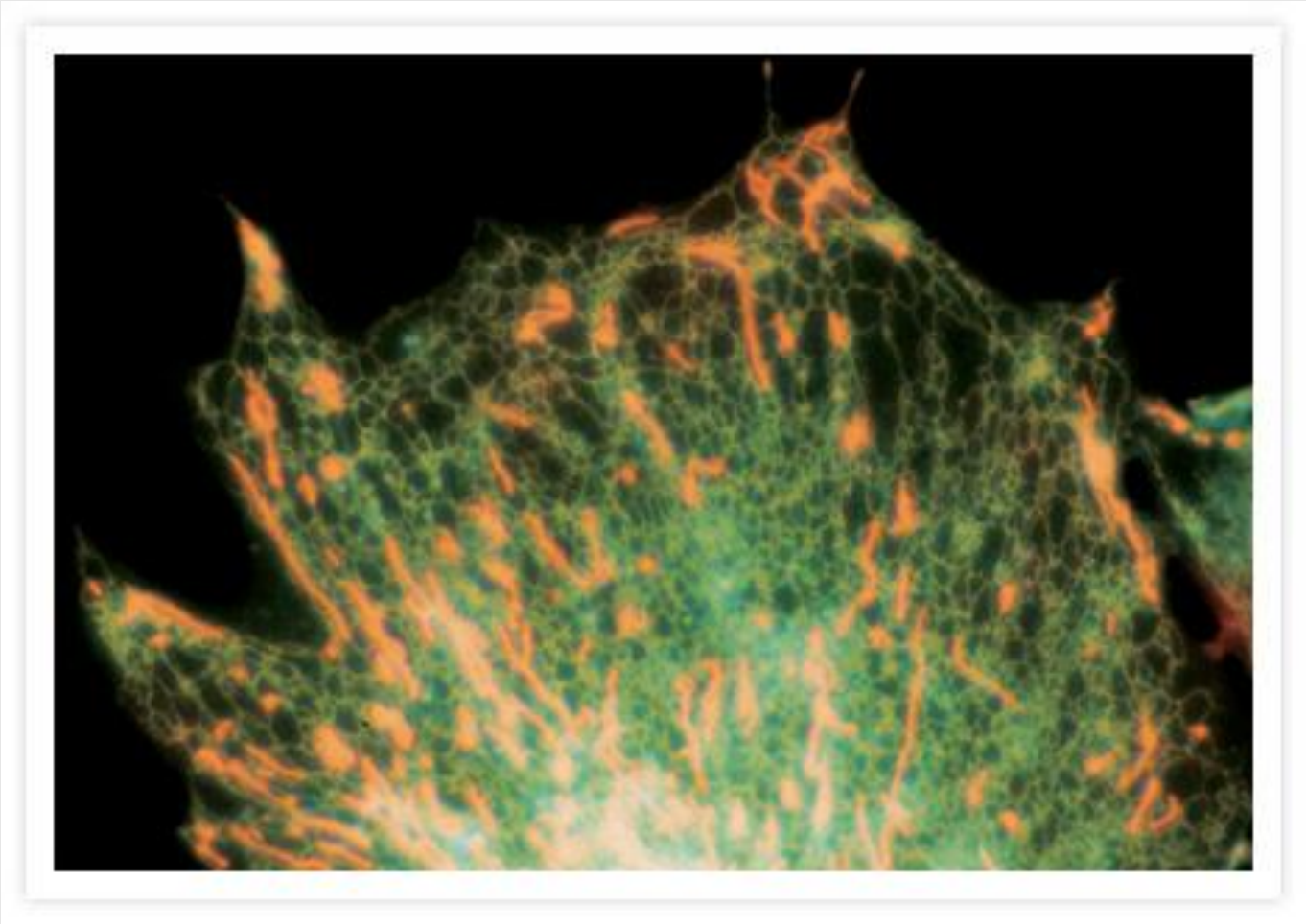
REF-52 fibroblasts loaded with fura-2 AM following microinjection of Cyclic AMP Fluorosensor (FlCRhR)

From Molecular Probes



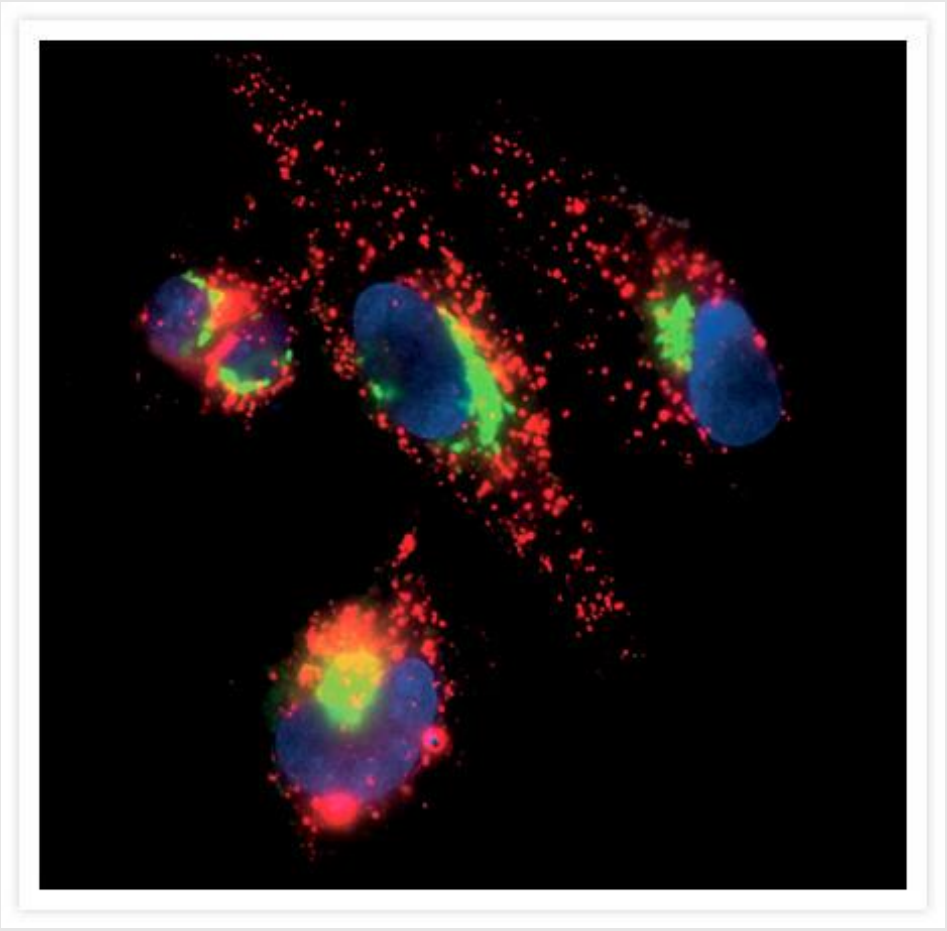
Bovine pulmonary artery endothelial cells incubated with MitoTracker Red CMXRos to label the mitochondria. After fixation and permeabilization, the cells were stained with BODIPY FL phallacidin to label the F-actin filaments and finally counterstained with DAPI to label the nucleus.

From Molecular Probes



ER-Tracker: Blue-White DPX; MitoTracker: Red CM-H2XRos

From Molecular Probes



Viable Madin-Darby canine kidney (MDCK) cells sequentially stained with BODIPY FL C_5-ceramide, LysoTracker Red DND-99 and Hoechst 33258.

From Molecular Probes

Immunohistochemistry (IH)

Immunocytochemistry (IC)

Definition: The use of labeled antibodies as specific reagents for localization of tissue/cell constituents (antigens) *in situ*.

Antigen-antibody reaction

Methods: Direct/Indirect

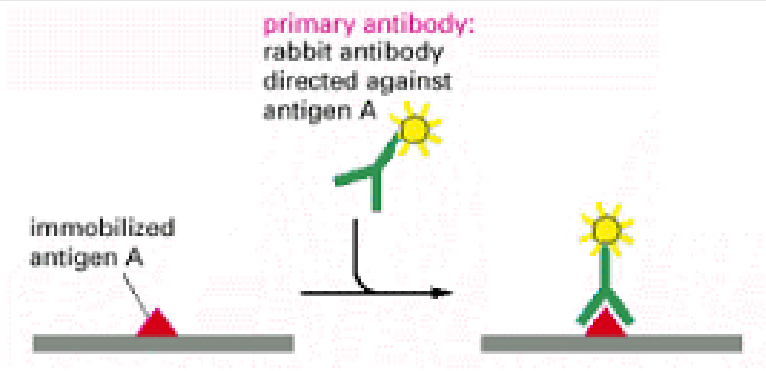
Immunofluorescence

single-, double-, triple- labeling

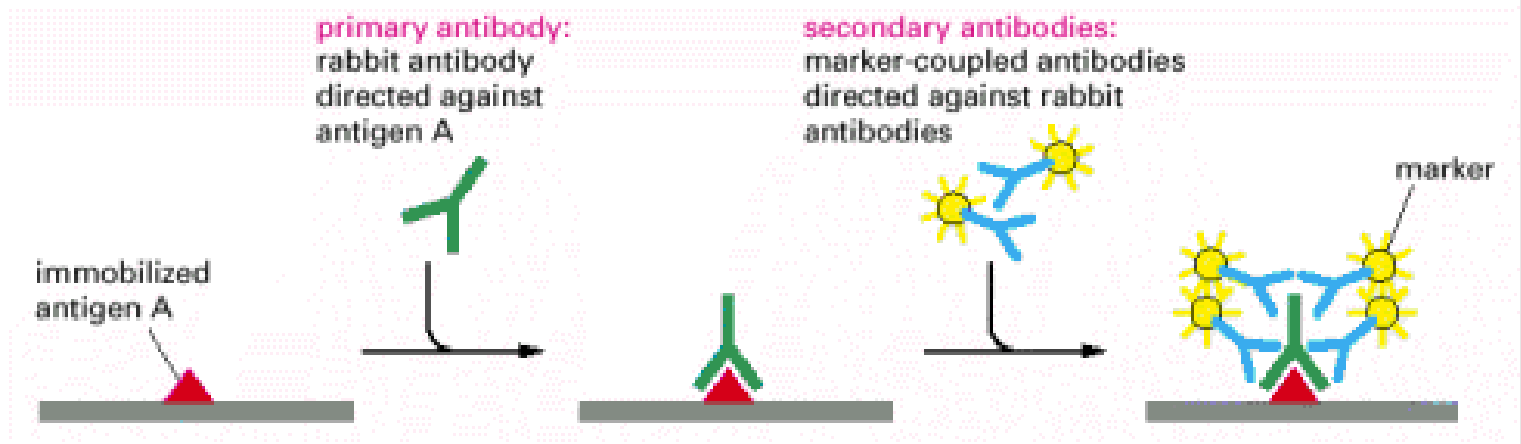
Immunoperoxidase

Immunofluorescence labeling

Direct immunostaining

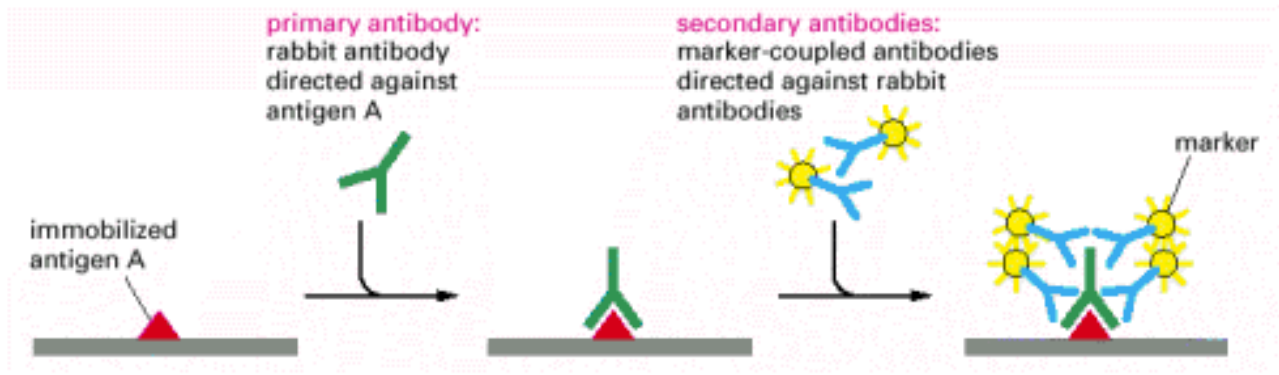


Indirect immunostaining

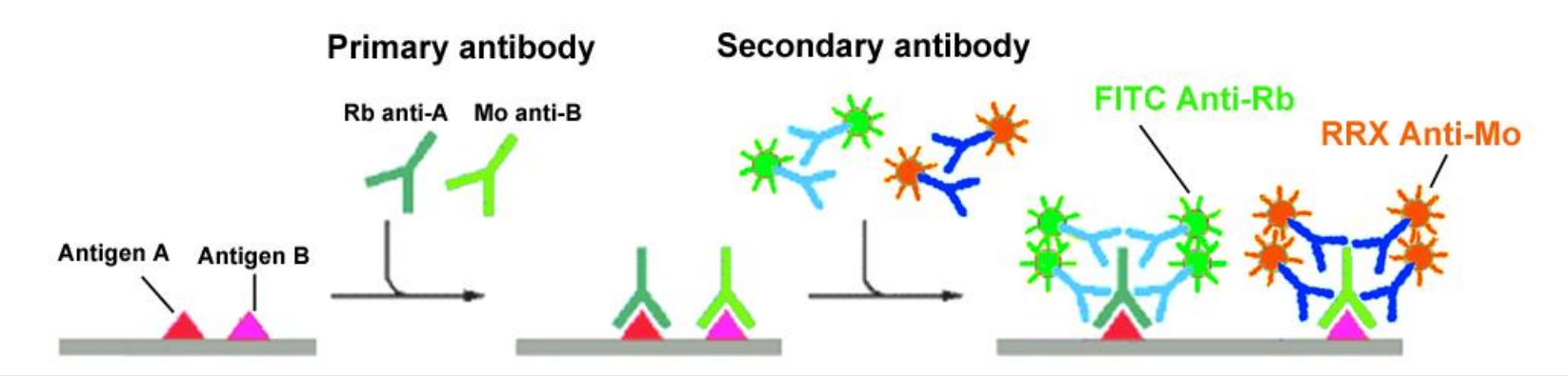


Immunofluorescence labeling

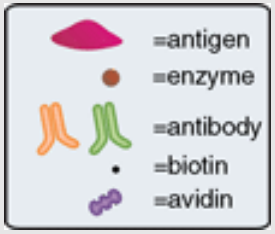
Single immunostaining



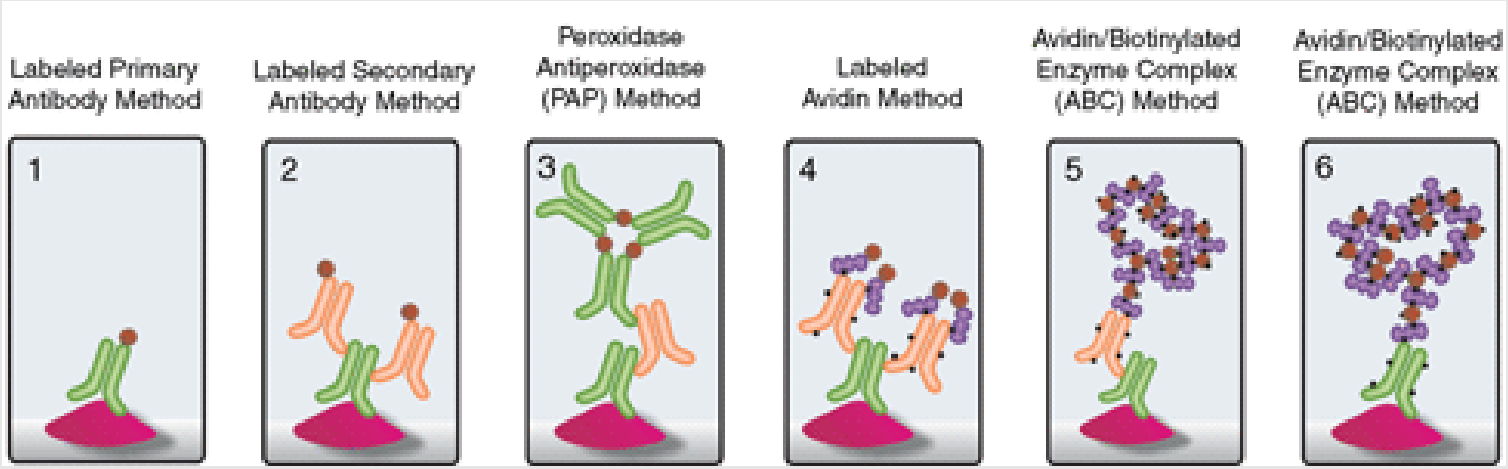
Double immunostaining



Immunoperoxidase labeling



Avidin-Biotin-Peroxidase



From Vector Laboratories

Essential conditions for immunocytochemistry:

1. Preservation of the antigen in tissue context (fixation)
2. Well characterized antibodies
3. Specific and sensitive staining with absence of non-specific staining
4. Efficient detection

Preparation and storage of specimens:

1. Frozen sections on adhesive-coated glass slides
2. Cell smears and cytocentrifuged cells
3. Fixative-fixed, paraffin-embedded sections on adhesive-coated glass slides.

Primary antibody:

Host (Mo, Rb, Go, GP, Ch)

Application (IB, IH, IP, IC)

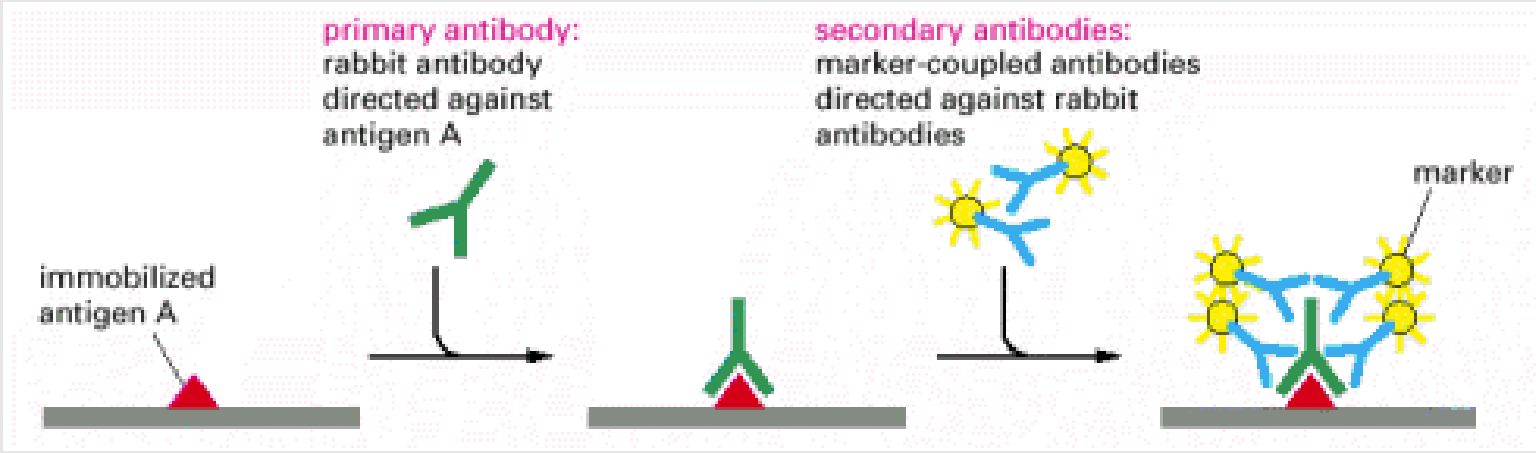
Cross reaction (Rt, Mo, Hu, Rb)

Storage (4℃, -20℃, -70℃)

Characteristics of a 'good' antibody:

high affinity, high avidity (stickiness),

high titre or concentration

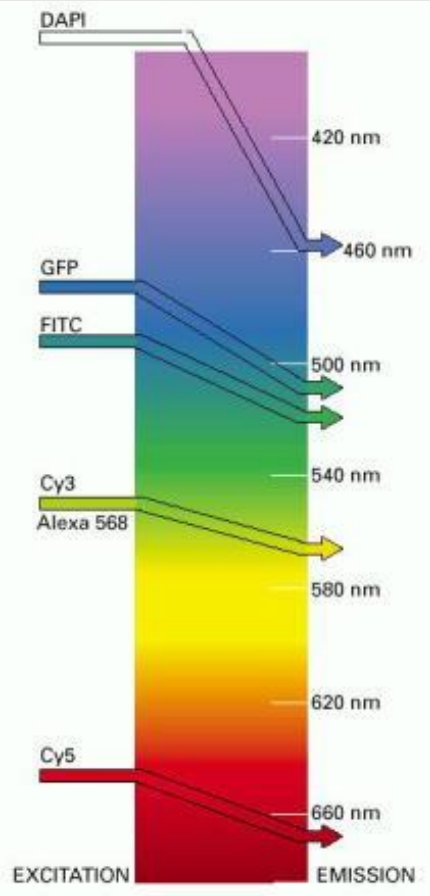


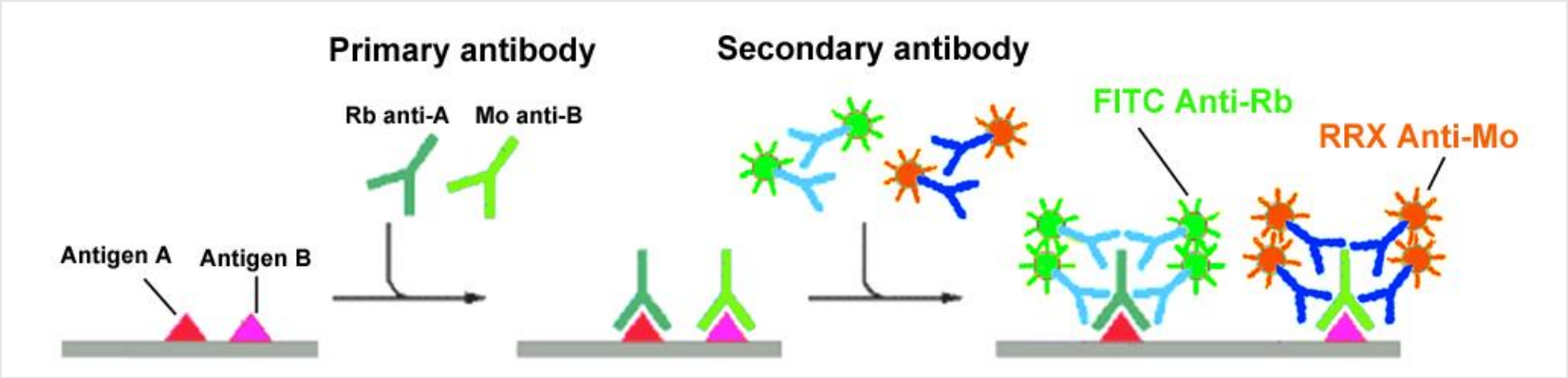
Secondary antibody:

Specificity (Mo, Rb, Go, GP, Ch)

Fluorescence (FITC, Rhodamine, Cy3, Cy5)

Specificity (minx Rt, Mo, Hu)





Immunohistochemistry Controls

Positive Staining Control

Antibody Positive Control: Stain tissues known to contain the antigen under investigation.

Negative Staining Controls

Null Control: Omit primary antibody and incubate tissues with buffer.

Absorption: Incubate antigen with antibody for 24 hours, and then incubate the tissue with this buffer.（使用过量的抗原结合抗体后再进行组化，检验非特异性结合的情况）

Problems and remedies

High level of background staining

1. Dilute the primary antibody further.
2. Check negative control with second primary antibody.
   Absorption with tissue powder (acetone-dried liver)
3. If background is still present, it could be due to:

(a) Tissue factors (non—specific binding sites, Fc receptors, basic proteins)

Remedy: (i) Increase concentration of blocking protein/serum.
(ii) Add detergent to buffer rinse (0.05% Tween 20).
(iii) Raise NaCl content of antibody diluent to 2.5%.
(iv) Raise pH of buffer to 9.0.
(v) Use F(ab) portions of antibodies.
(vi) Add 2 mg poly-L-lysine (a basic protein to each ml of diluted antibody.

(b) Cross-reaction between anti-species immunoglobulin and host-tissue immunoglobulins

Remedy: (i) Absorb cross-reacting antibody with 1% of host-tissue species normal serum or immunoglobulin.
(ii) Use species-specific antibodies.

Problems and Remedies

(c) Aldehyde groups in tissue left from fixative
Remedy: (I) Wash tissue well before processing and embedding.
(ii) Treat preparation with freshly made 0.02-1% sodium or potassium borohydride in 0.1 M phosphate buffer or water for 2-30 min at RT.
(iii) Add 10-100 mM NH_4Cl to the blocking serum.

Immunostaining weak or absent

1. Method sensitivity is insufficient for small quantity of antigen present

Remedy: Increase sensitivity.

2. Antigen is hidden (over-fixed).

Remedy: (i) Antigen retrieval by protease treatment
(ii) Heat-mediated antigen retrieval

3. Antibody deterioration
4. Wrong antibody sequence applied in error

Blocking of unwanted non-specific staining:

Source of unwanted staining, besides poor knowledge of the antibody reactivity and malice, is due to:

- Endogenous enzymes or fluorochromes.
- Endogenous biotin.
- Endogenous antibody binding activity (Fc receptors).
- Crossreactivity of the secondary reagents with endogenous proteins.

Blocking of endogenous enzymes
Endogenous enzymes such as AlkPhos, AcPhos and esterases are destroyed by boiling, even a short time at 100 C. Peroxidase is not.
Blocking of endogenous peroxidase is done by preincubating the slides in 3% H_2O_2 .

Blocking of endogenous fluorochromes.
Blocking of endogenous fluorochromes is impossible. One may choose fluorochromes emitting in the UV range of spectrum, where endogenous autofluorescence of tissue is minimal.

Blocking endogenous biotin

Can be done with commercially available kits or by buying the isolated components of the kits, free biotin and free avidin.

Blocking of endogenous Fc blocking.

Specimens not paraffin embeddded may have significant Fc binding activity by macrophages, B cells, T cells and other cell types.

By exploiting the preferential avidity of Fc receptor for human > mouse Ig> rabbit > swine > goat immunoglubulins, one may use a blocking of the receptors with a reagent which will not interfere with the secondary reagents and with which the secondary antibodies can be absorbed (1% serum added).

Blocking of crossreactive antigens in the tissue.
Typical example is staining mouse monoclonals in mouse tissue, where endogenous immunoglobulins will be specifically detected by the antibody aimed at the exogenous antibody used.

Thanks for your attentions !