

LIGHT MICROSCOPY – SCANNING ELECTRON MICROSCOPY:

Limitation and advantages in diagnostic procedure.

Maris Gonidi, cytopathologist,

President of Medical Corfu Association, member of Board of Panhellenic Scientific Research Institute.

Since 1595, when Zacharias Jansen created the first compound light microscope that produced magnification up to 9x, microscopes have come a long way. Today's strongest compound light microscopes have magnifying power of 1000x to 2000x. By 1900 the theoretical principles of microscopy were well understood. The development of technology and photography techniques have led to the modern microscope as an essential tool for professional scientists all over the world. [Fig 1]

In 1665 the first published work, based upon the use of light microscope by the English scientist Robert Hooke entitled "Micrografia" or "Some physiological descriptions of minute bodies made by magnifying glasses with observations and inquiries thereupon". The above text contains description and illustrations of parts of plants and animals, using for first time the term "cell" in biological context. A few years later Leeuwenhoek, an amateur scientist using a single glass lens in a simple microscope, with his observations on blood cells, bacteria, plants and minerals gave a boost to our understanding of microscopy.

By about 1830, the understanding of distortion such as the spherical and chromatic aberration related to the shape and type of glass used in lens production, led to the light compound microscope with relatively high magnification and good resolution. Ernst Abbe, around 1870, formulated his famous theoretical resolution of the light microscope in the following equation: $Resolution = 0.61\lambda / nsin\theta$. Where λ = the wave length for light used, n = the refractive index of the medium separating the specimen from objective and condenser lenses, θ = half the angular width of the cone of rays collected by the objective lens from a typical point in the specimen. $nsin\theta$ is called the numerical aperture of the lens.

Abbe's theory demonstrates the importance of the numerical aperture of the lenses used and the wavelength of light. According to Abbe's theory, the higher the numerical aperture (NA) and the shorter the wavelength, the better the resolving power is. NA of the lens is its function of light collecting ability.

In light microscopy we take advantage of waveform properties of light. The waves when produced at the particular source vibrate at right angles to the line of propagation. Wave length varies with the color and intensity of the source.

Magnification and resolution are two basic terms to microscopy. **Magnification:** is the degree by which dimensions in an image are, or appear to be, enlarged with respect to the corresponding dimensions in the object. The total magnification when viewing an image with compound light microscope is the power of the objective lens (4x,10x,40x) multiply by the power of the eyepiece (typically10x) **Resolution:** is the act or result of displaying fine detail in an image. Resolution depends on both objective and condenser lens (width of the cone of illumination). Magnification without resolution would be meaningless. [Fig 2]

Understanding how light microscope works is critical of how electronic microscope works, as both of them share similarities in optical principles. Most modern light microscopes are divided into two categories:

a) The brightfield microscope, "normal microscopy", in which direct transmitted light pass through the objective lens and illuminates the background against which the image can be seen. Brightfield microscopy uses specimens that contain inherent contrast color or as for cells and tissues special staining techniques. Using stained prepared slides can be seen cellular smears, chromosomes, organelles, bacteria and thin tissue sections. Using unstained mounts living protists or metazoans and plant cells can be seen. Generally brightfield microscope is used for the study of detailed gross internal structures. [Fig 3]

b) Fluorescence microscopy uses special stains (fluorophores), which are molecules that absorb one wave length of light and emit a second longer wavelength, targeted to the molecule of interest. Fluorescence microscopy is an essential tool for the study of cell biology and physiology. [Fig 4]

The Scanning Electron Microscope (SEM)

The German physicist Ernst Ruska and the electrical engineer Max Knoll constructed the prototype Electron Microscope in 1931 capable of four hundred power magnification. [Fig 5]

The scanning electron microscope (SEM), uses beams of electrons for image formation. SEM produces excellent images of the surfaces of cells and small organisms, providing information for studying surface morphology. Samples are scanned in vacuum conditions and must be prepared undergoing dehydration and conductive coating with Au, Cr etc. There is a wide variety of interactions which may occur between the beam of electrons and the specimen. The exact interactions that occur are dependent on the sample material and beam conditions. [Fig 6]

The typical magnification of SEM is approximately x30,000 in general terms can provide magnifications of at least 100 greater than a typical light microscope. Comparing the resolution of SEM with that of a light microscope, SEM can display and detect around 400x as much detail as a light microscope. [Fig 7]

Back Scattered Electrons contain information relating to the composition of the material, Scattered Electrons provide surface information and the high resolution gives to the human eye the subjective impression of a higher depth of field, allowing a stereoscopic image observation. Offering control over the magnification, SEM is used by the researchers in the physical, medical and biological sciences to examine a huge range of specimens.

Microscopy offers a large and very active research field. Different types of microscopes offer different information that can meet the needs of the researcher, according to his planning experiment.

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 -Dean KM, Palmer AE (2014). Advances in fluorescence labeling strategies for dynamic cellular imaging. *Nat Chem Biol*. 10: 512-523
 -Murphy DB, Davidson MW (2012) *Fundamentals of light Microscopes and Electronic Imaging* John Wiley& Sons, Hoboken, NJ
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Fig. 1. Robert Hooke 1665 "Micrographia"

Fig. 2. Compound Light Microscope



Fig. 3. Columnar epithelium (H&E, 100x)

Fig.4. glioblastoma (actin-red, transglutaminase-green, nuclei-blue)

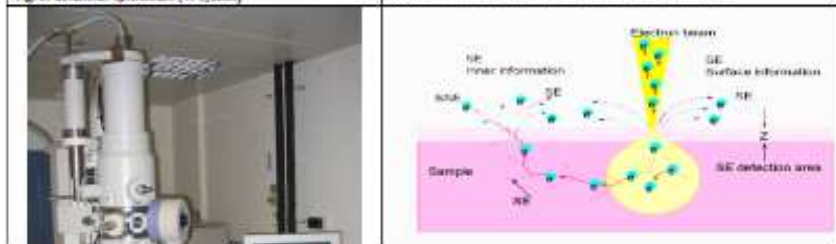


Fig.5 Beam – Sample interaction (SE: secondary electrons, BSE: back scattered electrons)



Fig.6 Scanning Electron Microscope (SEM)

Fig.7 Fibroblast on collagen SEM (30,000 x)

**LIGHT MICROSCOPY – SCANNING
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LIMITATION AND ADVANTAGES IN
DIAGNOSTIC PROCEDURE.**

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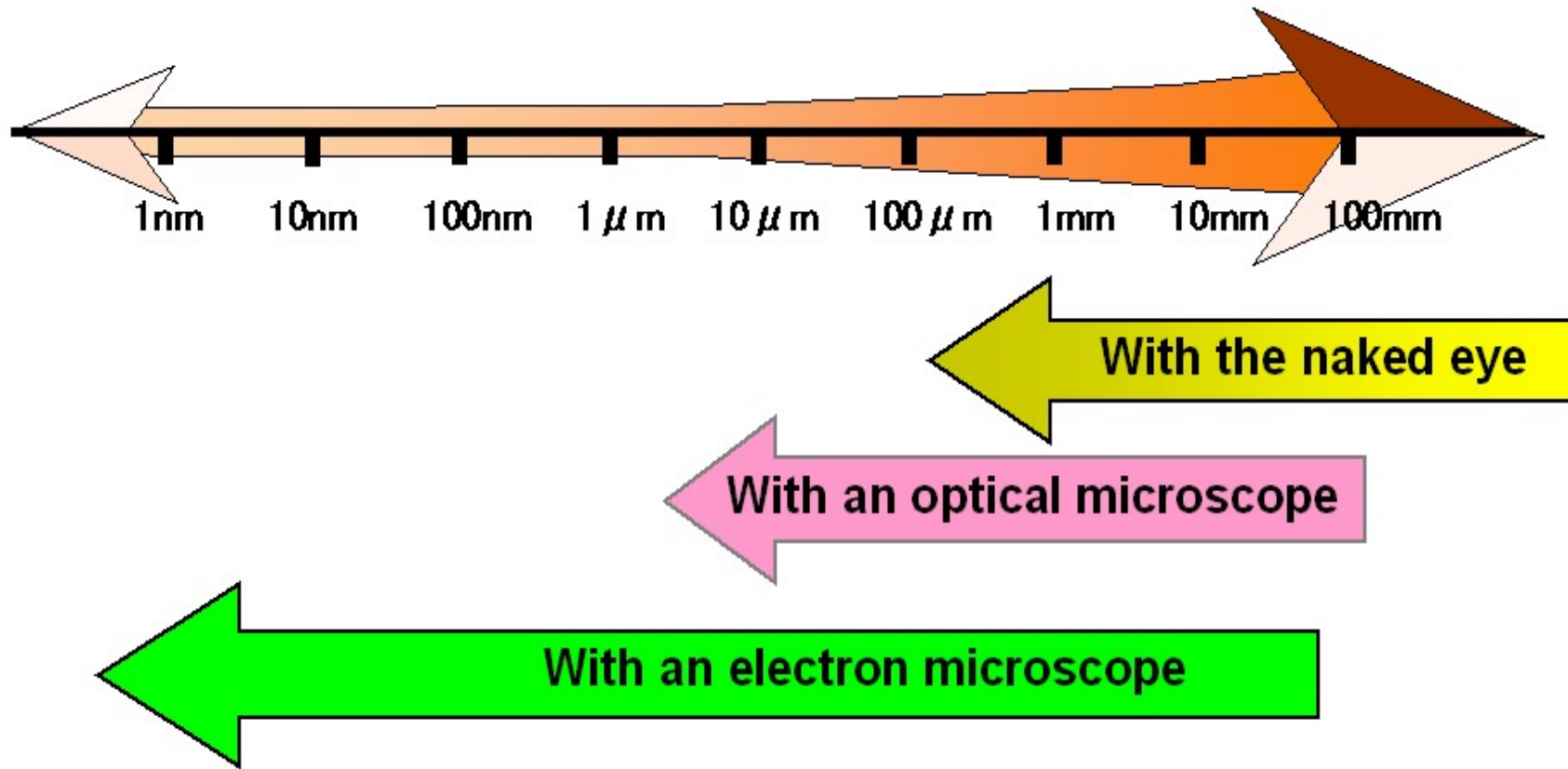
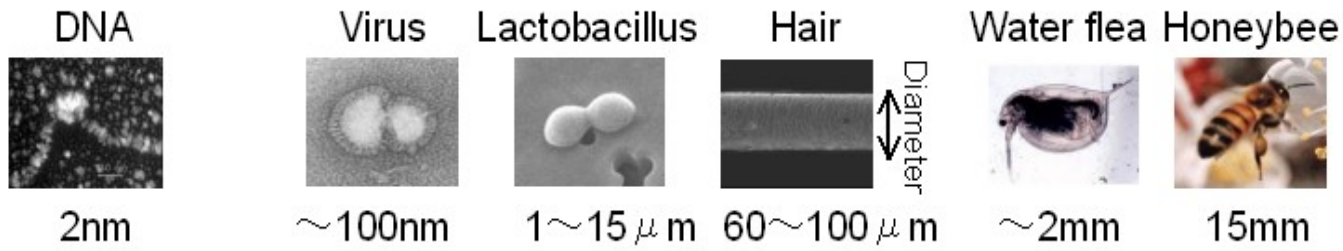
General Philosophy

Human beings use
two means to gauge
objectives

- ✓ *Seeing* : through eyes
(light)
optical microscope
electronic microscope
- ✓ *Touching*



Microscopes – What for ?



Historic Figures in Microscopy



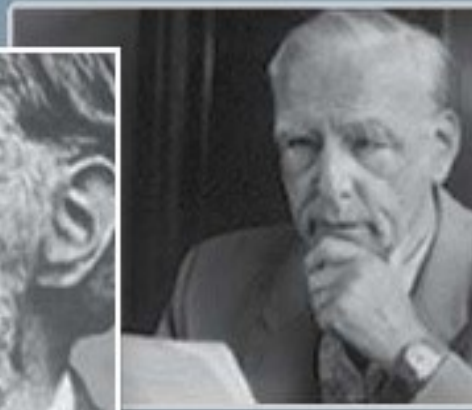
Antony van Leeuwenhoek
(1632-1723)



Robert Hooke
(1635-1703)



Ernst Abbe
(1840-1905)



Ernst Ruska
(1906-1988)



Richard Feynman
(1918-1988)

Development of microscope takes us back almost 400 years ago

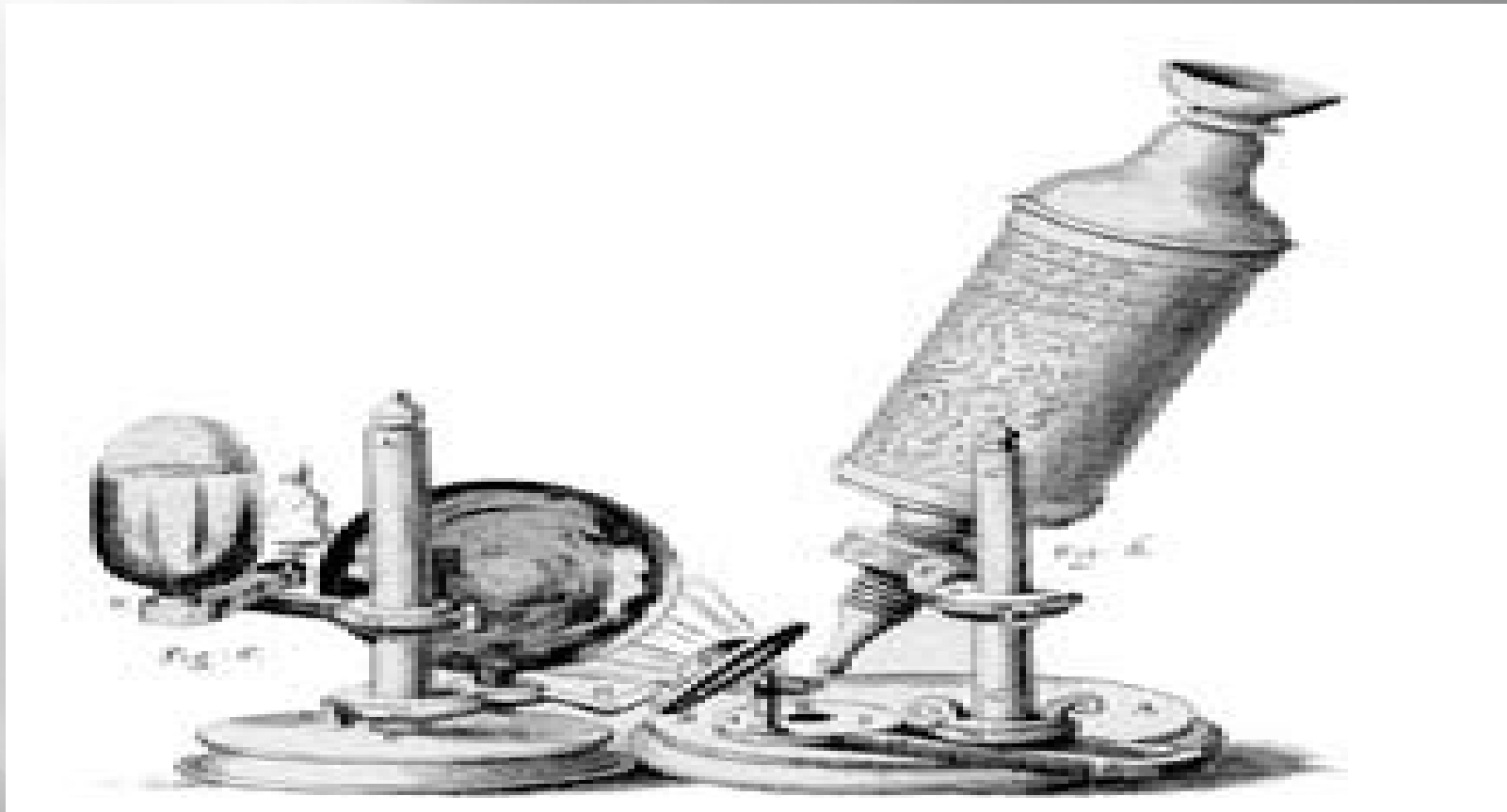


image of Hooke's compound microscope 1665

Robert Hooke 1665

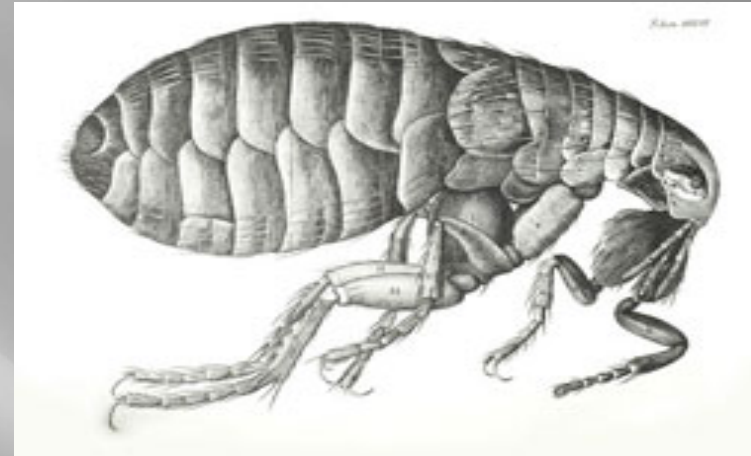
“Micrografia”

- ▣ *“ Micrografia” or “ Some physiological descriptions of minute bodies made by magnifying glasses with observations and inquiries thereupon “.*
- ▣ In 1665 the first published work , based upon the use of light microscope by the English scientist Robert Hooke.
- ▣ Hooke used a compound microscope, having two lenses



The English scientist, Robert Hooke (1635-1703)

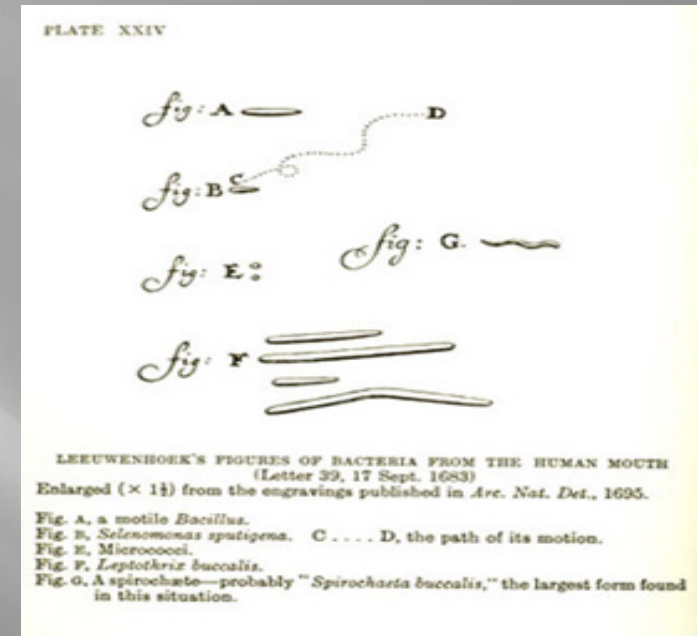
- ▣ *"Micrographia"*
contains description
and illustrations of
parts of plants and
animals , using for
first time the term
"cell" in biological
context



From "Micrographia", by Robert Hooke, 1665: plate showing the drawing of a flea

Antoni van Leeuwenhoek, a Dutch cloth merchant and amateur scientist

- ▣ Leeuwenhoek made observations on bacteria, plants, blood cells and minerals and is generally considered to have made a greater contribution to our understanding of the microscopical world than Hooke.



From "Anthony Van Leeuwenhoek and his 'little animals'", edited by Clifford Dobel: plate showing the drawings of bacteria from the human mouth

Ernst Abbe (around 1870) formulated his famous theoretical resolution of the light microscope

- ▣ the understanding of *distortion* such as the spherical and chromatic aberration *related to the shape and type of glass used in lens production*, led to the light compound microscope with relatively high magnification and good resolution

Historic Figures in Microscopy



Antony van Leeuwenhoek
(1632-1723)



Robert Hooke
(1635-1703)



Ernst Abbe
(1840-1905)



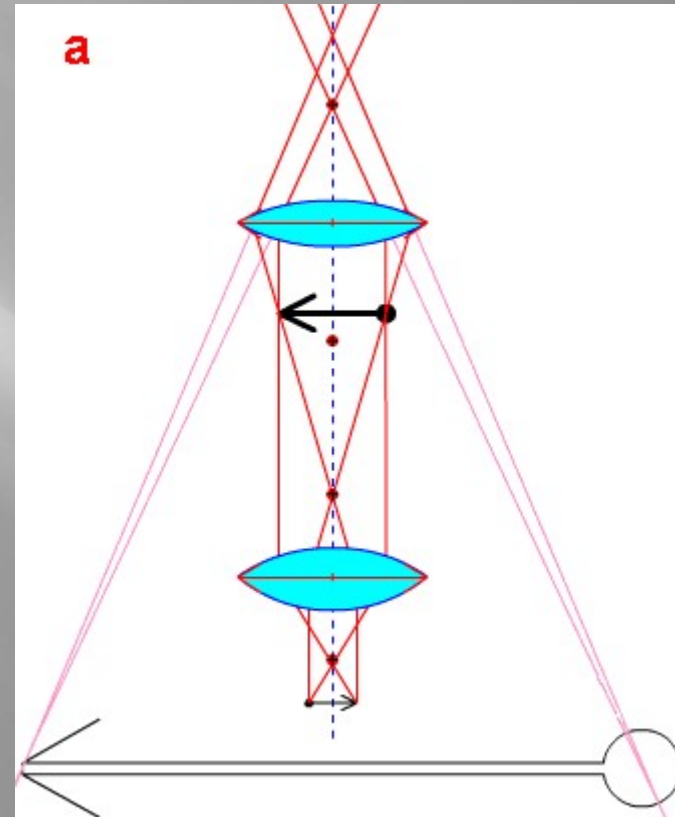
Ernst Ruska
(1906-1988)



Richard Feynman
(1918-1988)

waveform properties of light

- ✓ Sample is imaged by its effect on the light passing through it as the sample absorbs, scatters, or deflects the light.
- ✓ In light microscopy we take advantage of *waveform* properties of light. The waves when produced at the particular source vibrate at right angles to the line of propagation.
- ✓ Wave length varies with the color and intensity of the source



Magnification and resolution

Magnification and *resolution* are two basic terms to microscopy:

- ✓ *Magnification*: is the degree by which dimensions in an image are, or appear to be, enlarged with respect to the corresponding dimensions in the object. The total magnification when viewing an image with compound light microscope is the power of the objective lens (4x.10x.40x) multiply by the power of the eyepiece (typically10x)

Magnification and resolution

Magnification

- Magnification is the **enlargement of the image**
- The magnification of a microscope is given by-

$$M_{\text{microscope}} = M_{\text{ocular}} \times M_{\text{eyepiece}}$$

- Generally used class microscope has following magnification-

	Magnification	Ocular lens	Total Magnification
Scanning	4x	10x	40x
Low Power	10x	10x	100x
High Power	40x	10x	400x

Magnification and resolution

- ✓ ***Resolution:*** is the act or result of displaying fine detail in an image. Resolution depends on both objective and condenser lens (width of the cone of illumination).
- ✓ ***Magnification without resolution would be meaningless.***

Ernst Abbes' equation

$$\text{Resolution} = 0.61\lambda / n\sin\theta$$

Where λ = the wave length for light used

n = the refractive index of the medium separating the specimen from objective and condenser lenses

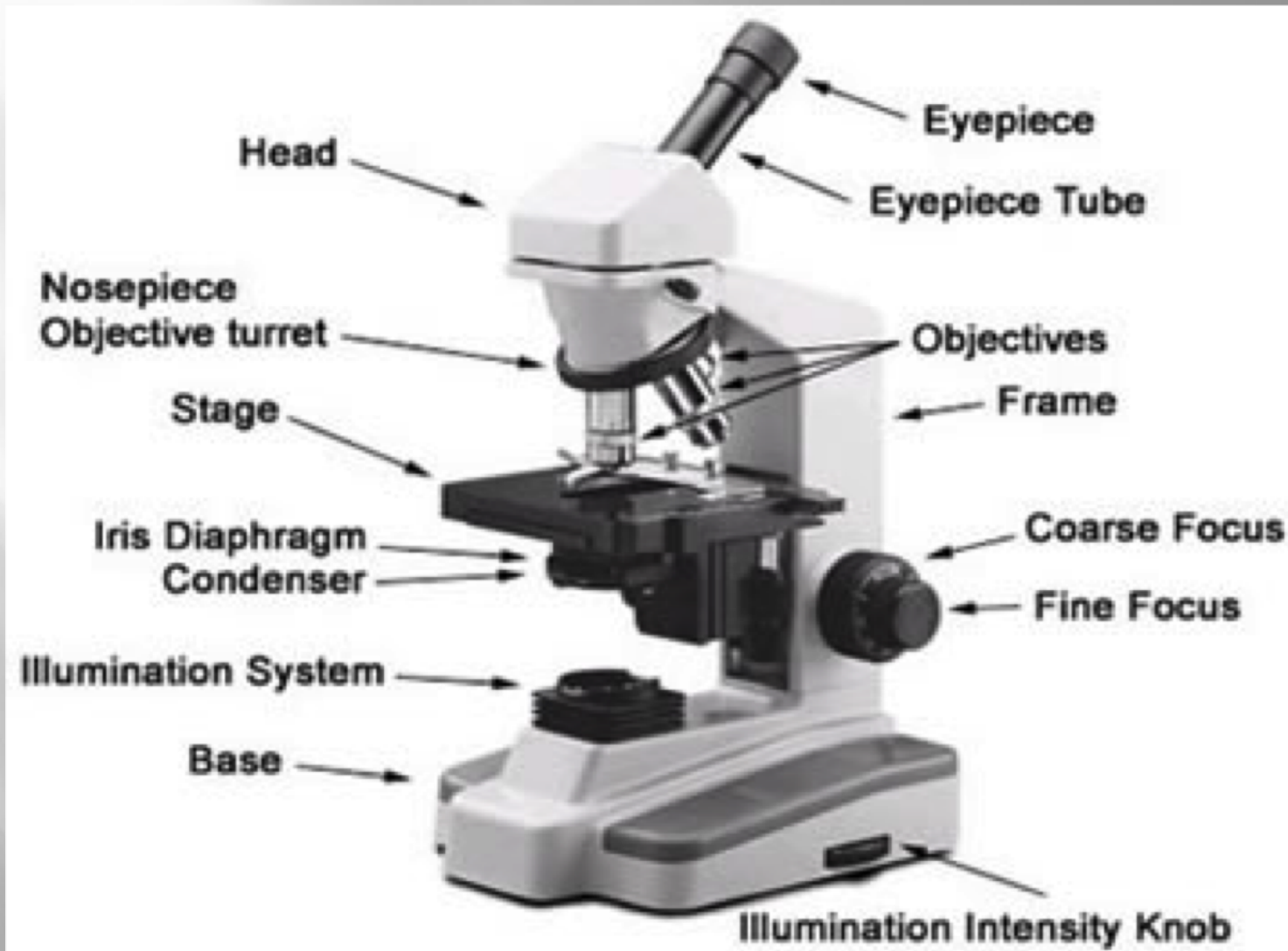
θ = half the angular width of the cone of rays collected by the objective lens from a typical point in the specimen.

$n\sin\theta$ is called the numerical aperture (NA) of the lens.

Abbe's theory, demonstrates the importance of the numerical aperture of the lenses used and the wavelength of light. According to Abbe's theory, the *higher the numerical aperture (NA) and the shorter the wavelength, the better the resolving power.*

NA of the lens is its function of light collecting ability.

Compound Light Microscope

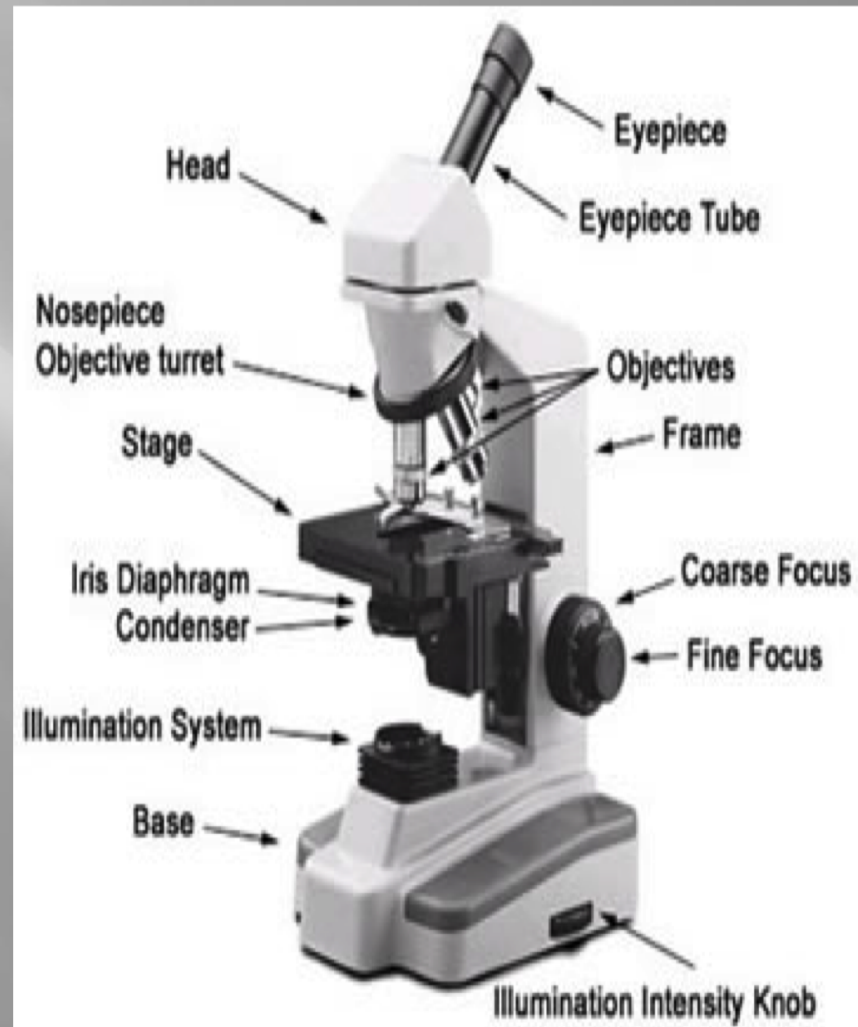


Types of Microscope

- Types of microscope.
 - Light microscope
 - Bright field microscope
 - The Dark-Field Microscope
 - The Phase-Contrast Microscope
 - The Fluorescence Microscope
 - Scanning and electron microscope

Transmitted Light Microscopy

- Transmitted light microscopy is the general term used for any type of microscopy where **the light is transmitted from a source on the opposite side of the specimen to the objective lens**. Usually, the light is passed through a condenser to focus it on the specimen to get maximum illumination. After the light passes through the specimen it goes through the objective lens to magnify the image of the sample and then to the oculars, where the enlarged image is viewed.



Transmitted Light Microscopy

- ▣ The *Objective Lens* is the first part of the imaging system; the objective lens forms a primary, enlarged image of the object. Very fine details are distinguished with the objective lens. The *eyepiece* sometimes called the ocular lens, is the second lens, which forms a secondary, further enlarged image. By multiplying the magnifying power of the objective lens and the magnifying power of the ocular the final magnification is found. A *Substage Condenser lens* is the third optical component. It is placed on a platform beneath the object.

Transmitted Light Microscopy

- ▣ Light is directed through the substage condenser and converges to a point at the position of the specimen. The light rays diverge as they pass through the specimen and form an inverted cone, whose base is just large enough to fill the aperture of the objective. The size of the light beam is controlled by a diaphragm beneath the condenser called the aperture diaphragm

Transmitted Light Microscopy

- ✓ In order to get a usable image in the microscope, the specimen must be properly illuminated. The light path of the microscope must be correctly set up for each optical method and the components used for image generation. *The first and perhaps the most important element are the lenses.* The condenser was invented to concentrate the light on the specimen in order to obtain a bright enough image to be useful.

Transmitted Light Microscopy

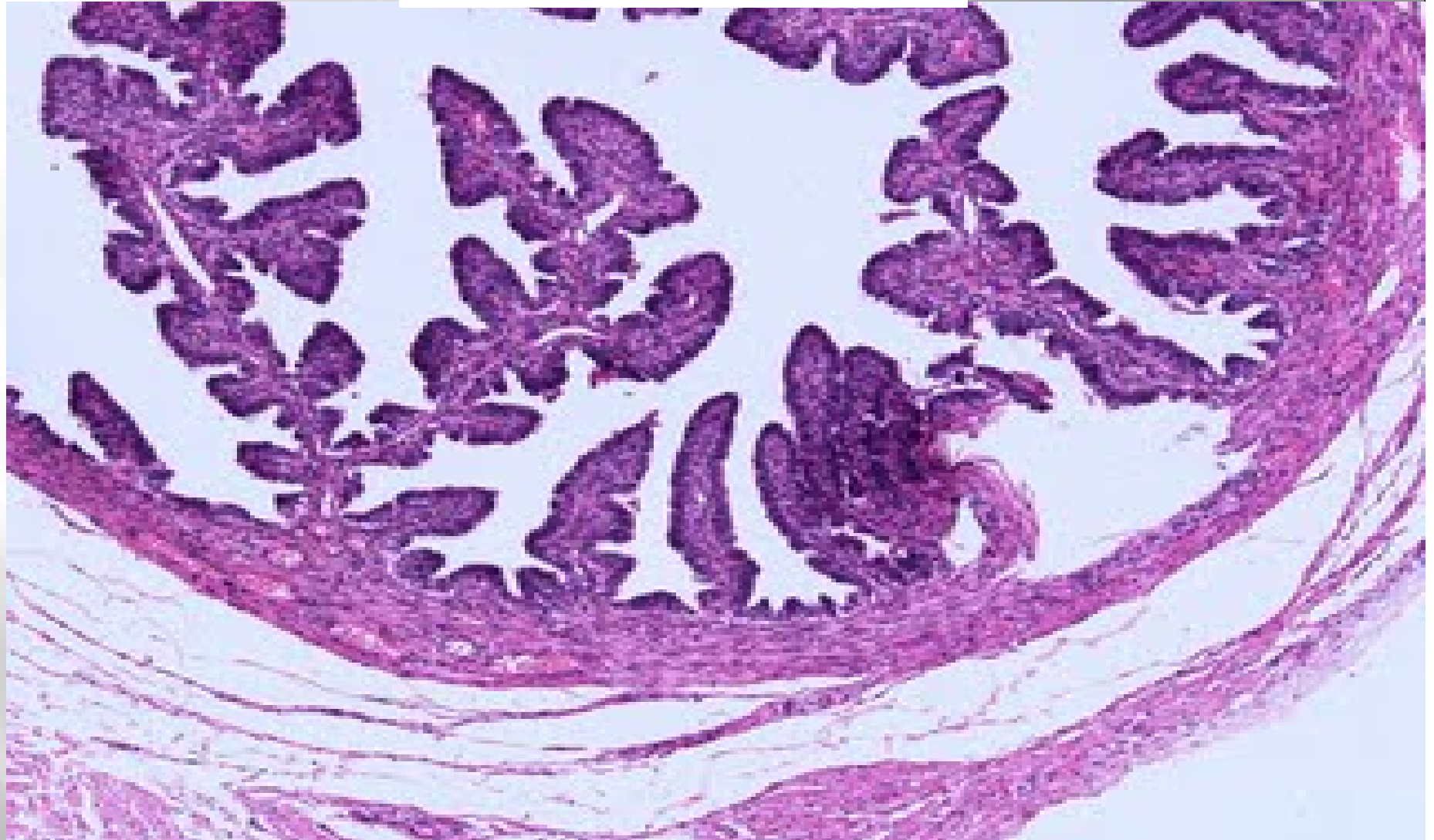
- ✓ The microscope techniques requiring a transmitted light path include bright field, dark field, phase contrast, polarisation and differential interference contrast optics
- ✓ The condenser is used to focus parallel rays of light on the specimen, as if coming from infinity, thereby giving you the advantages of an evenly illuminated field, a bright image without glare and minimum heating of the specimen. **As most cells and tissues have insufficient contrast in themselves, staining techniques are generally used. .**

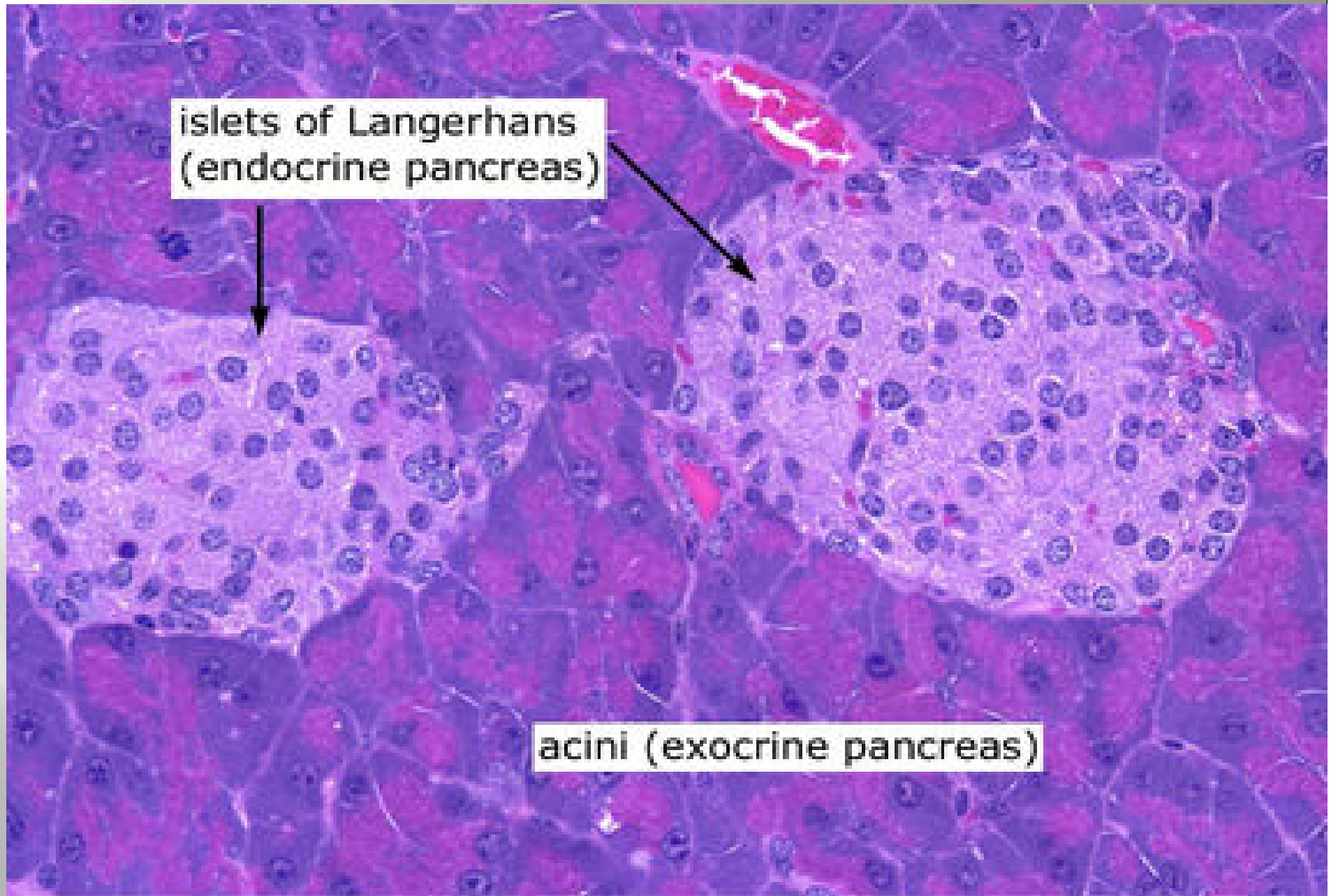
✓ Common stains include Papanicolaou stain, which is used for cervical smears, H-E stain used for semi-thin sections of all tissue types, MGGiensa for lymphocytes and most of FNA bopsies

✓ Generally, brightfield microscope is used for the study of detailed gross internal cellular structures.

✓ With the development of monoclonal or polyclonal antibodies to specific cell components further insights into cell derivation and function can be achieved by immunocytochemistry

Columnar epithelium biopsy, intestine(H-E,100x)

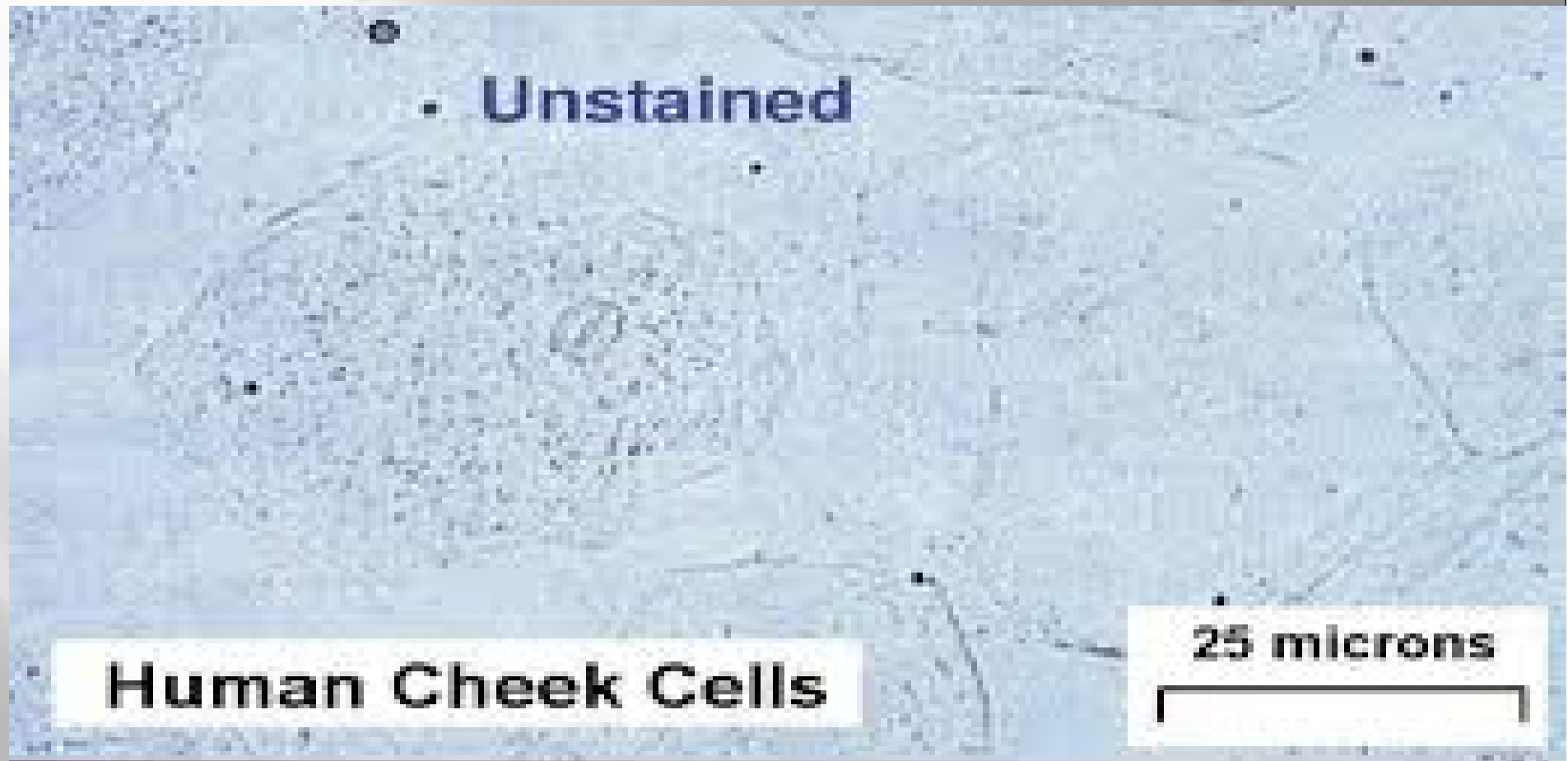


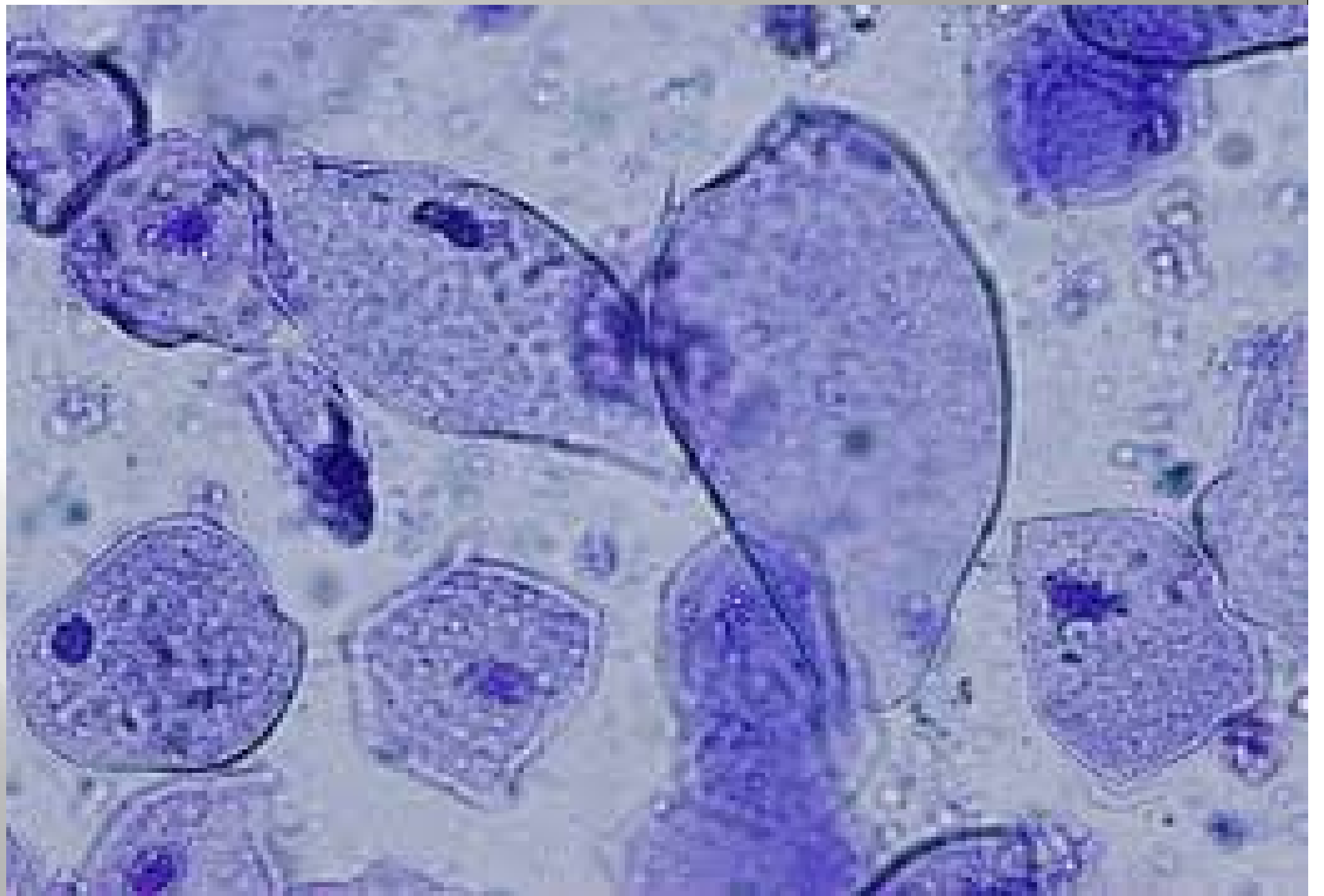


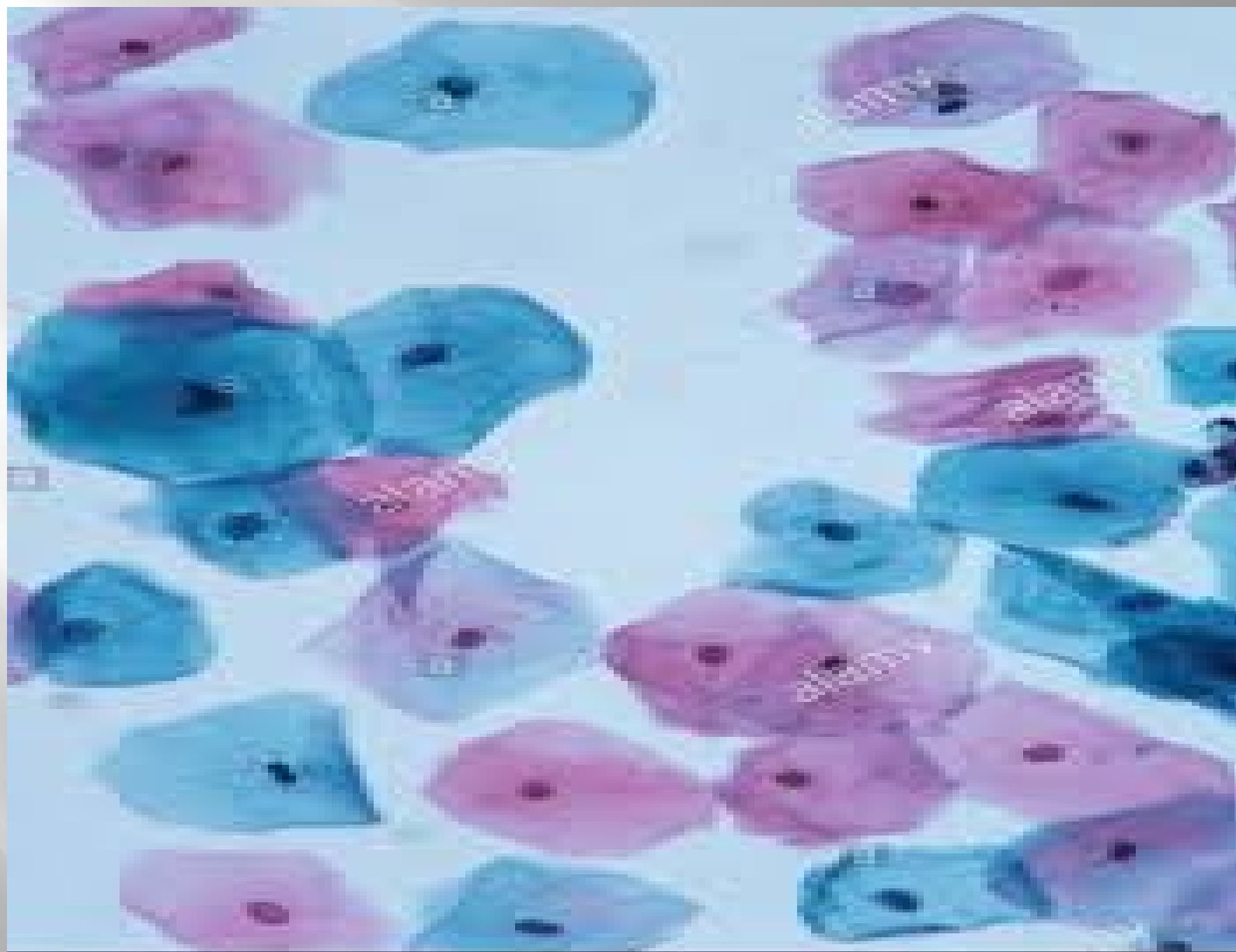
islets of Langerhans
(endocrine pancreas)

acini (exocrine pancreas)

Most cells are thin and transparent:
they do not absorb much light

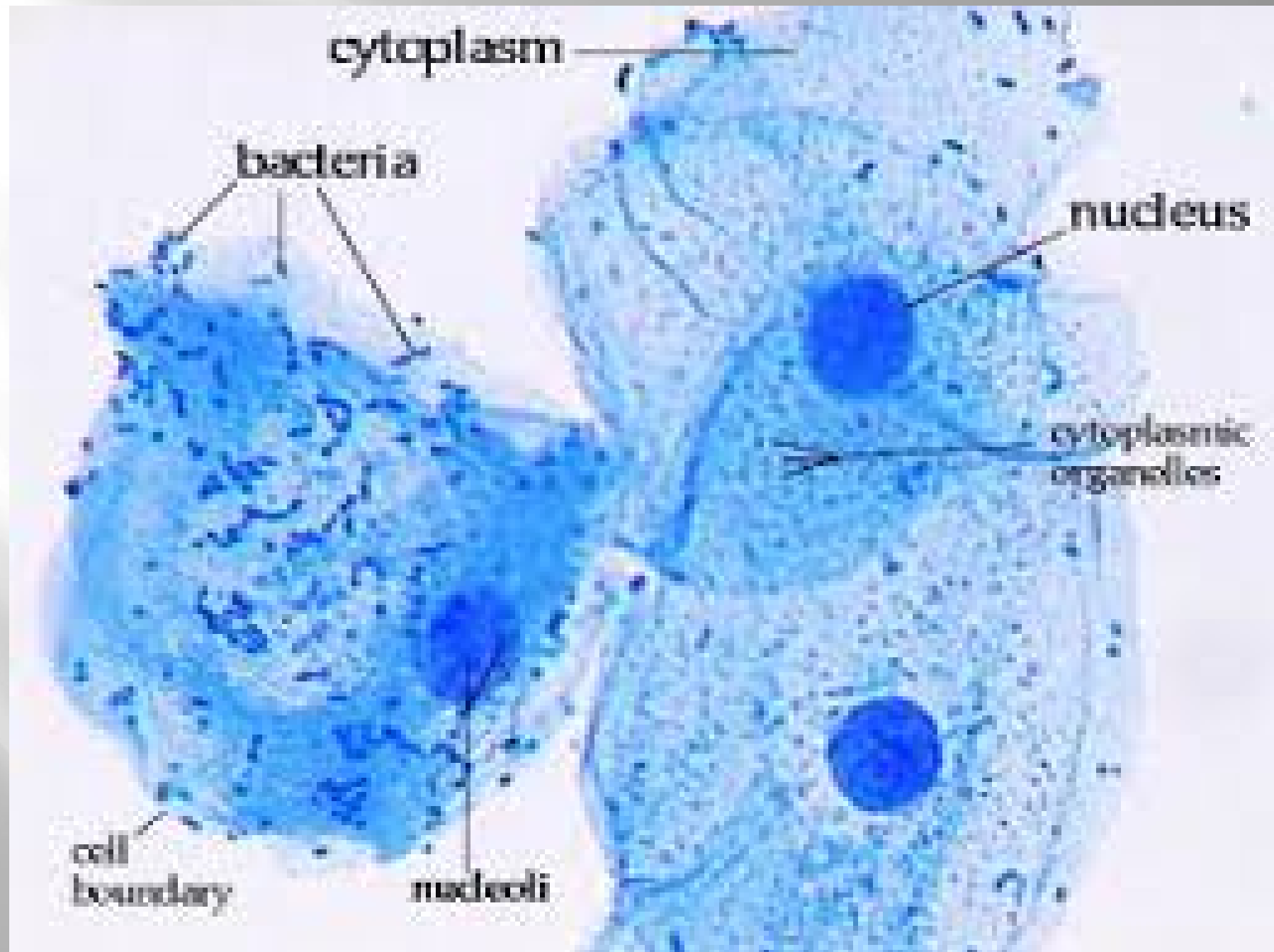




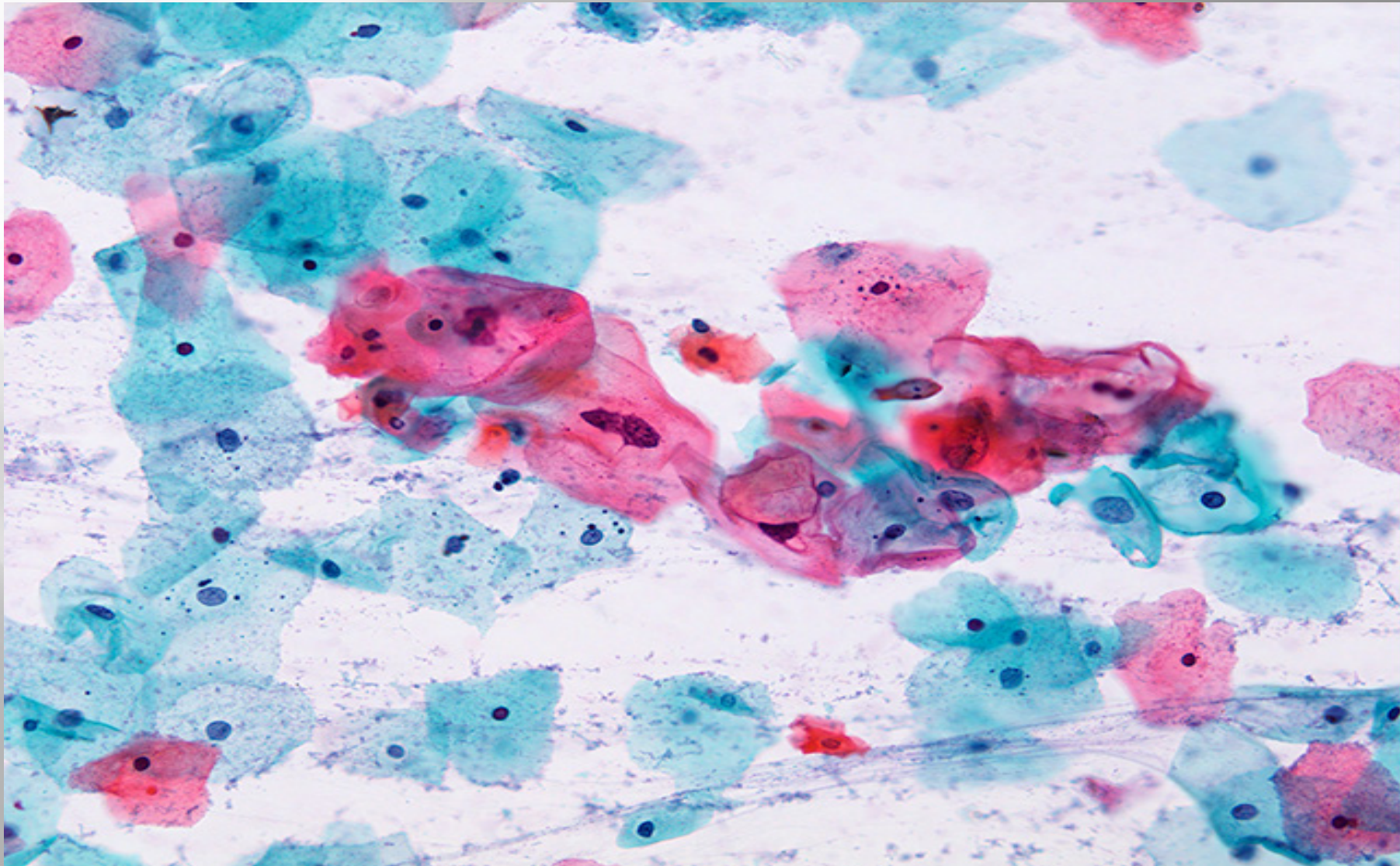


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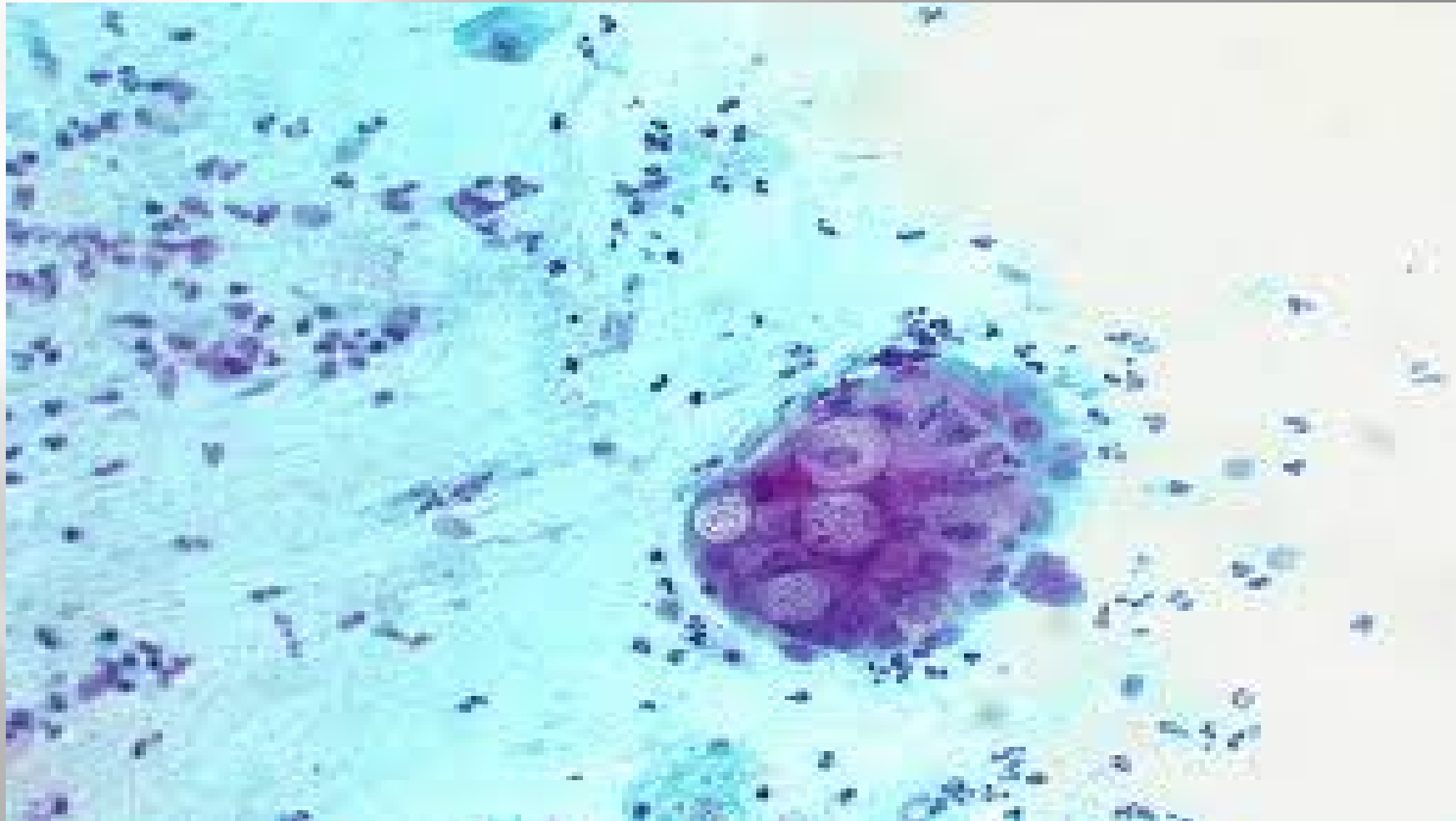
Cervical smears- Papanicolaou stain Ls1l



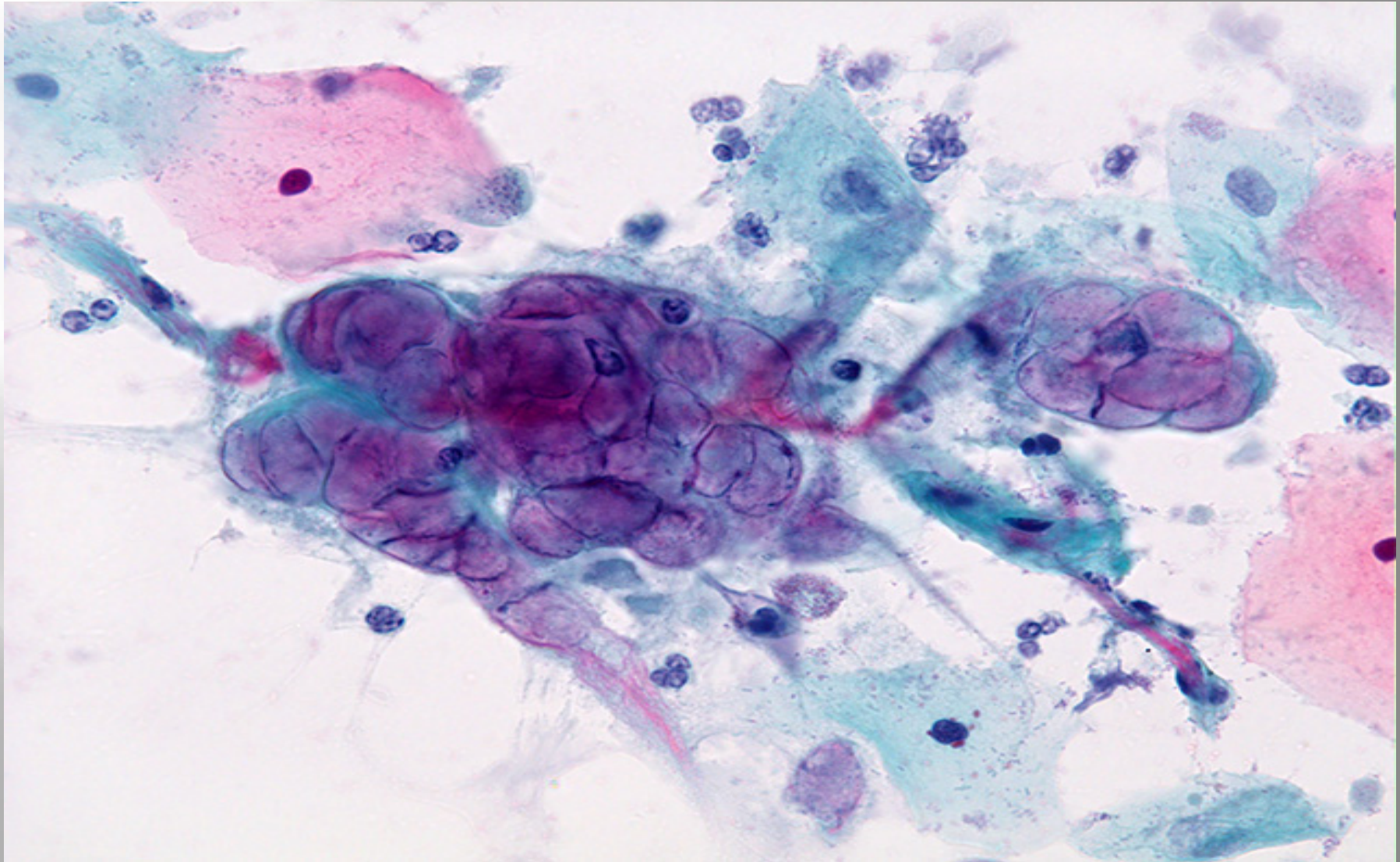
Cervical smear- HPV infection



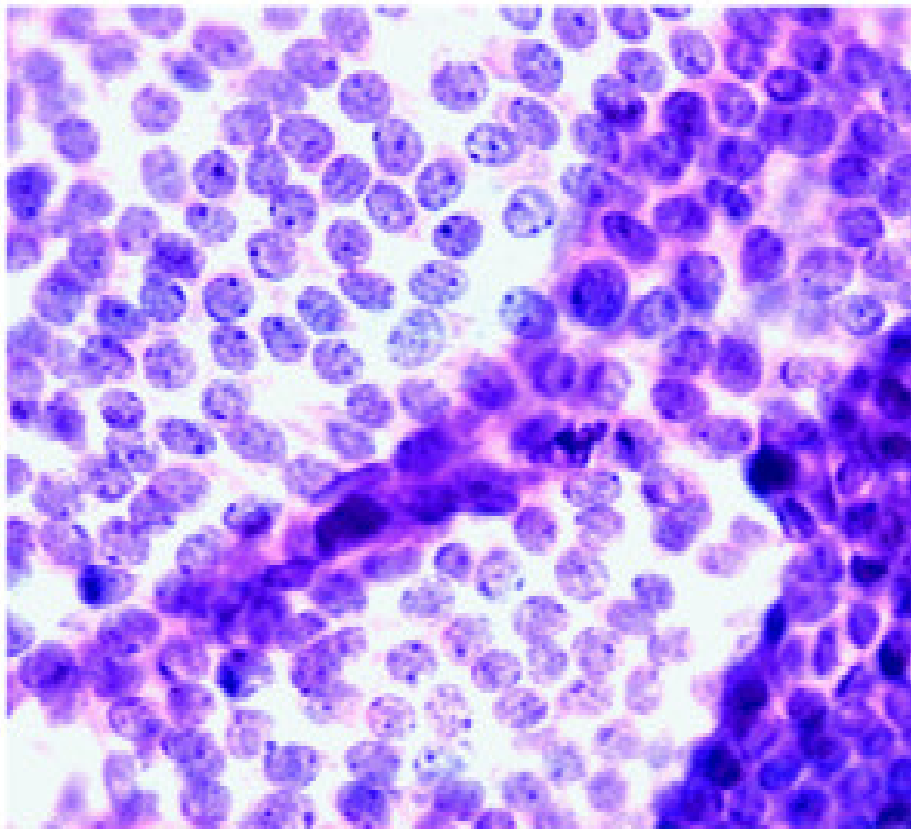
Cervical smear- chlamydia infection



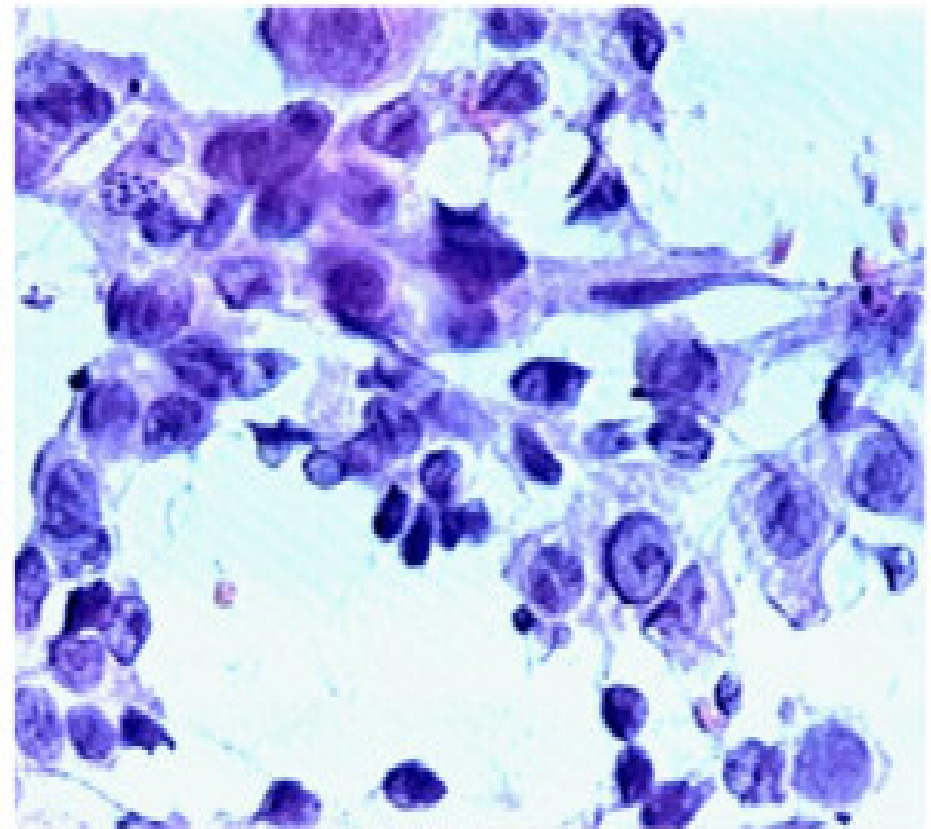
Cervical smear Herpes virous



FNA breast - Giemsa

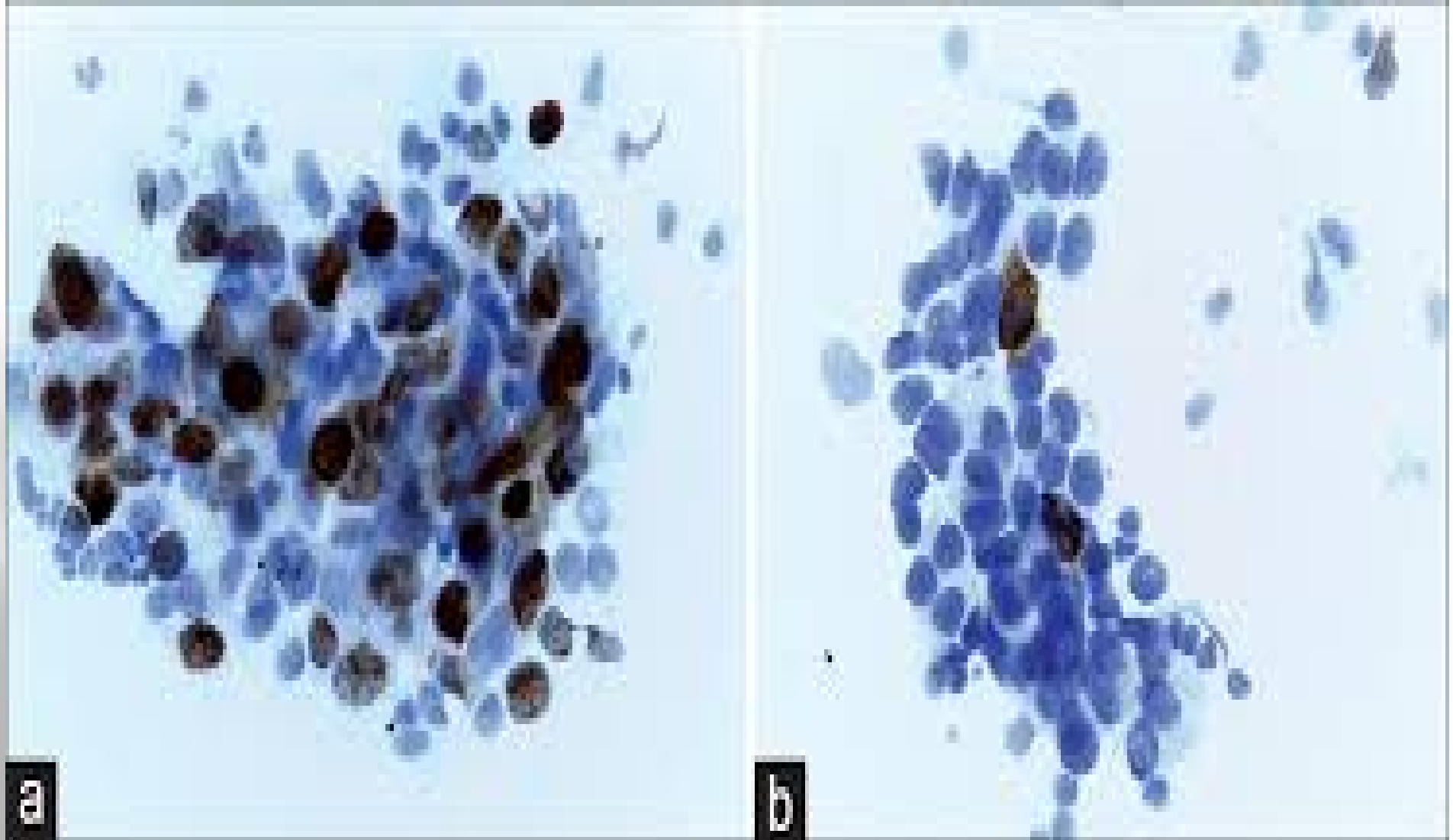


Smear with BENIGN diagnosis – uniform nucleus of cells, symmetrical, homogeneous, with areas within normal size

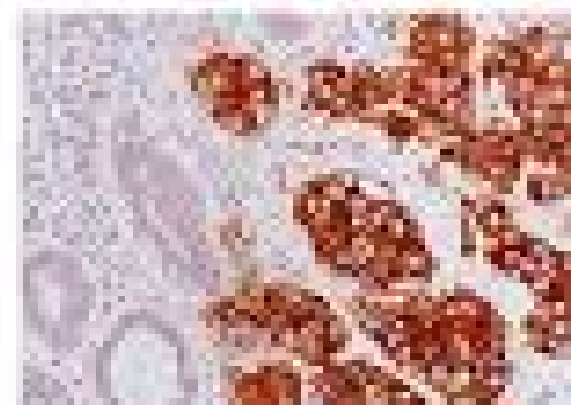
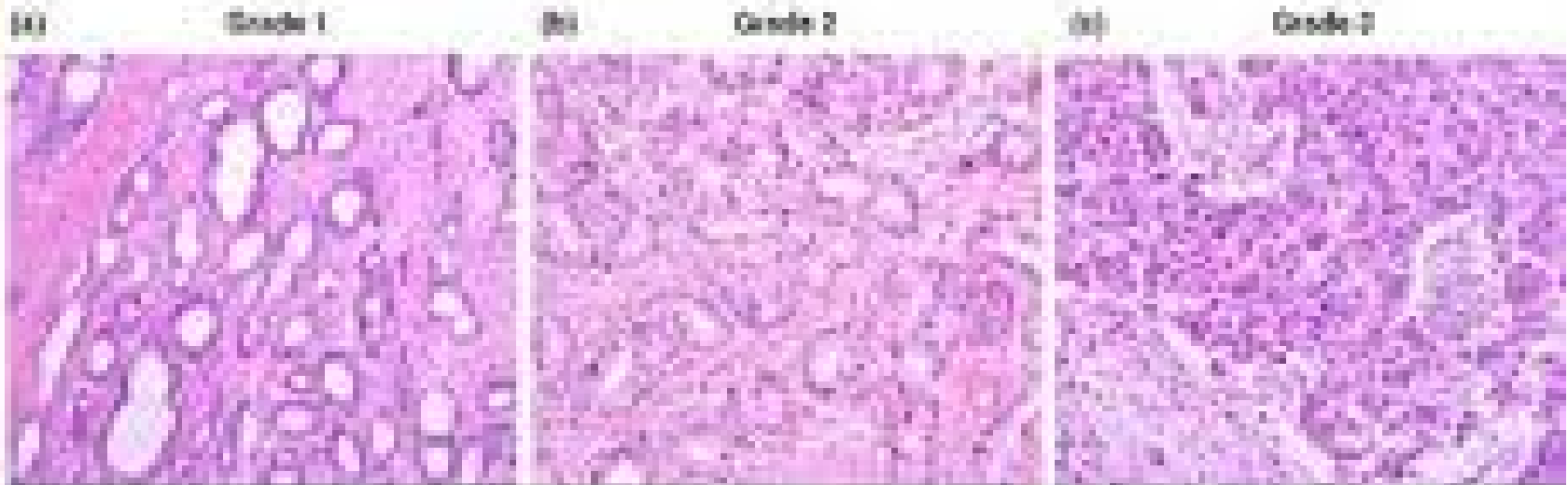


Smear with MALIGNANT diagnosis – nucleus of cells without uniformity, asymmetrical, not homogeneous (multiple sizes) and with areas above normal size

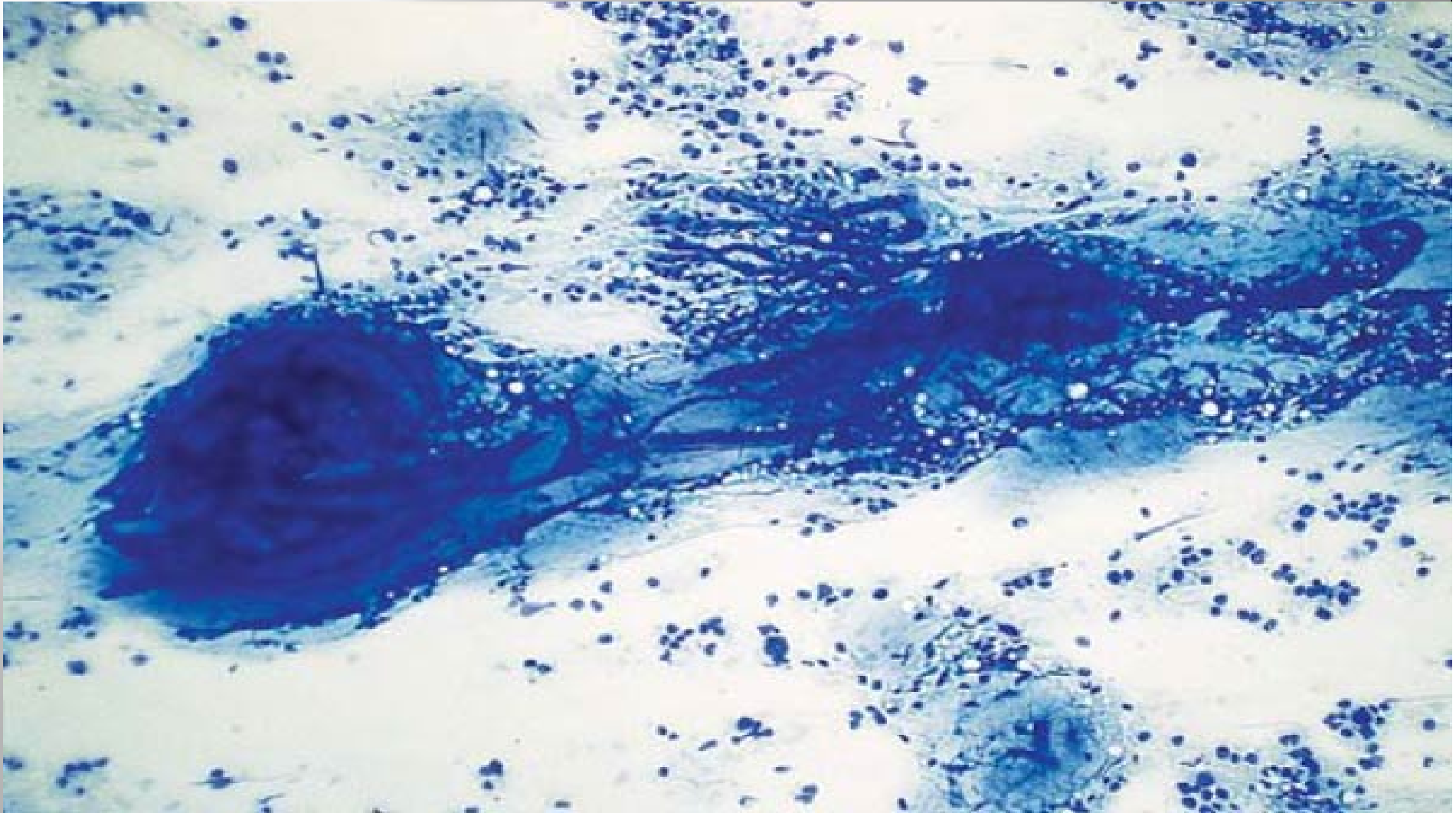
ER ,PR nuclear expression. Breast cancer cells



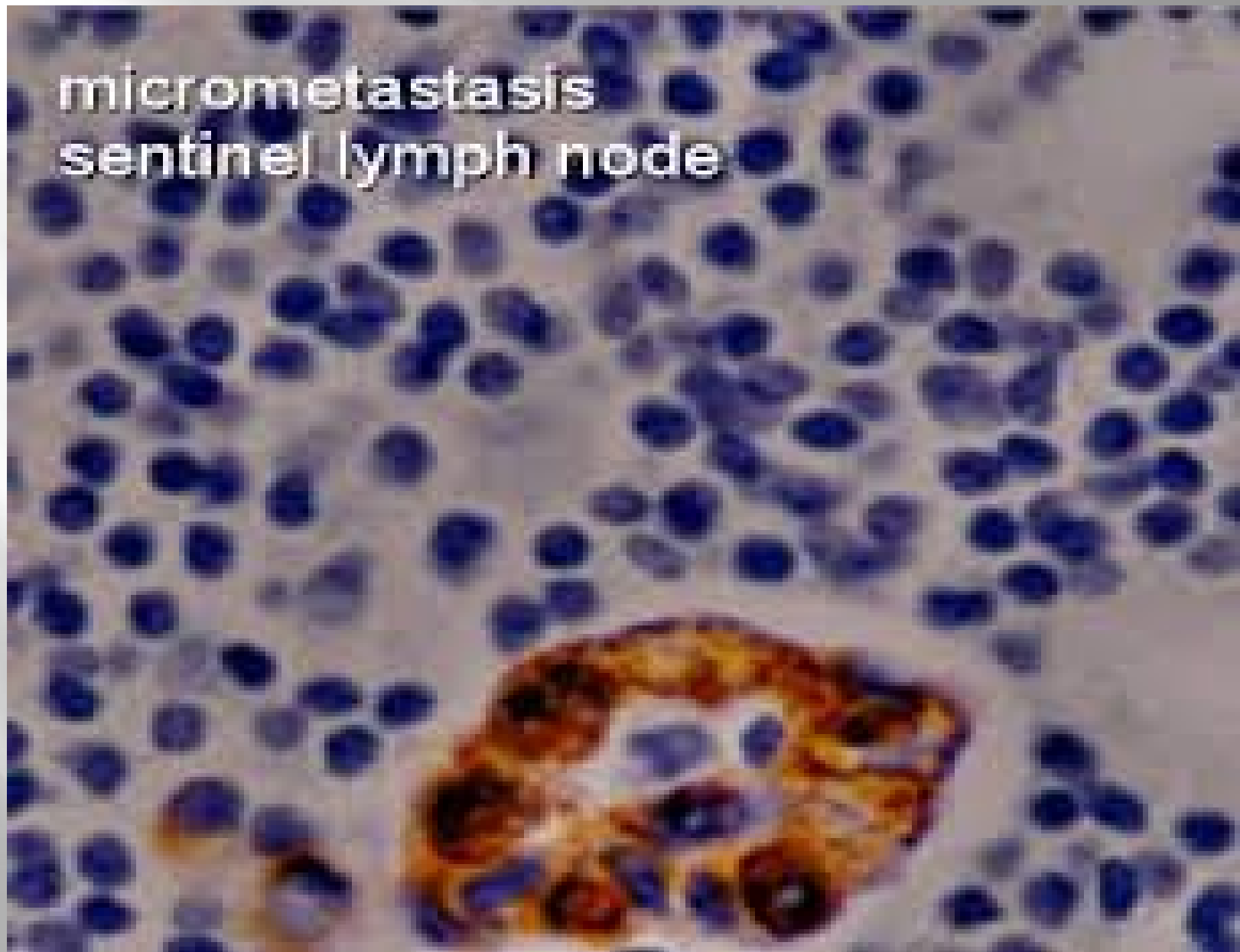
Grading and ER/PR/HER2 testing of Breast Cancers



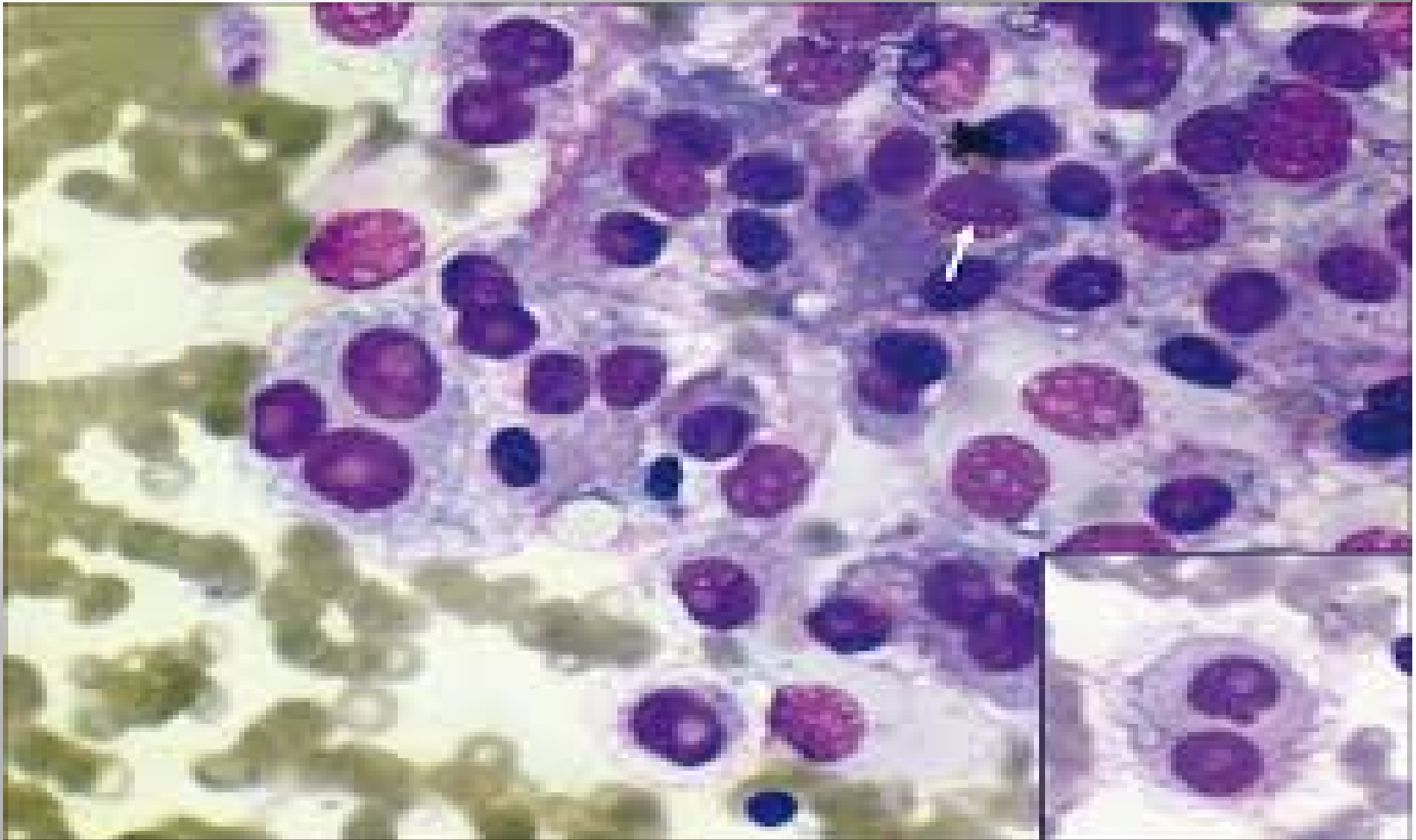
Mucoid breast cancer



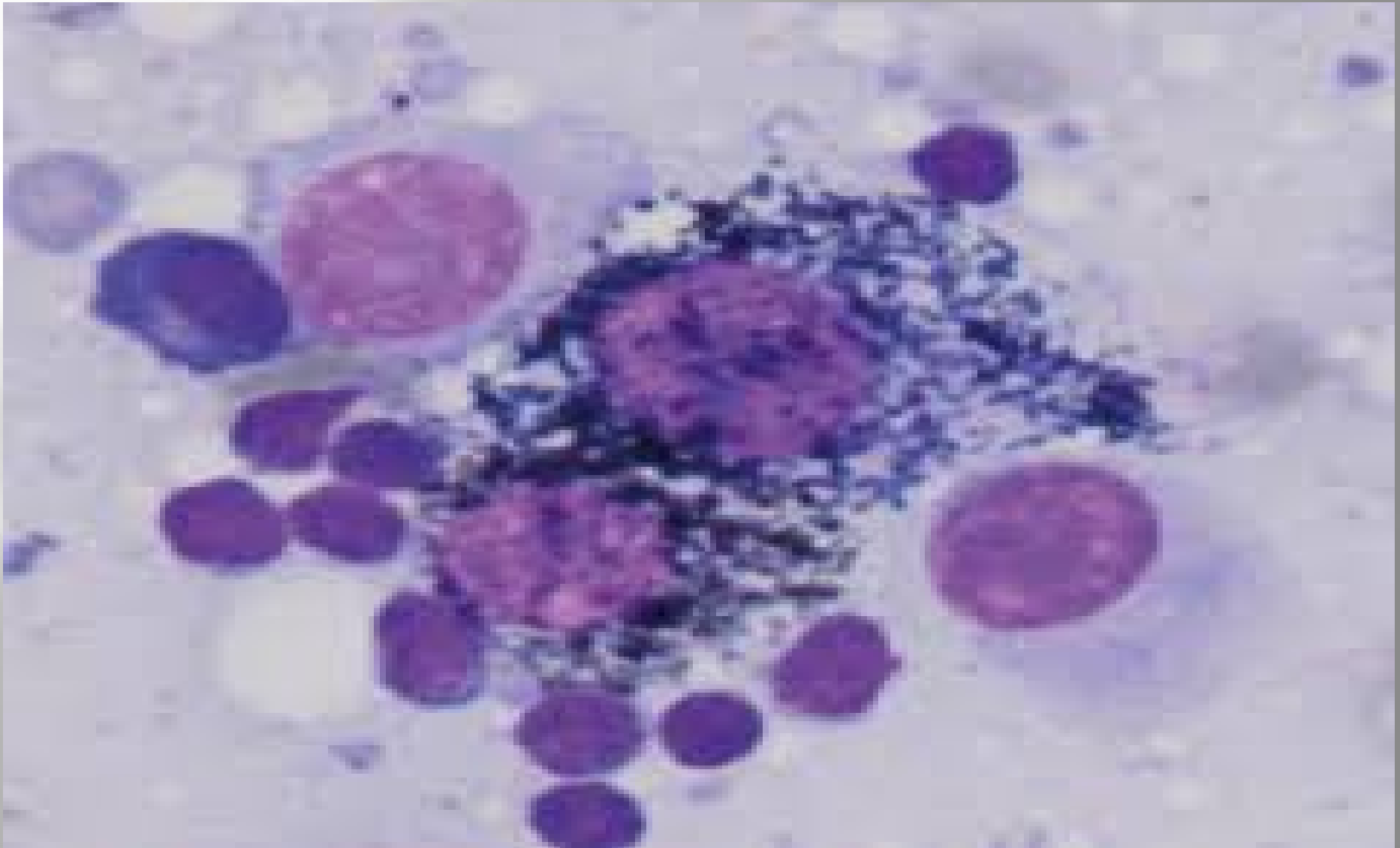
micrometastasis
sentinel lymph node



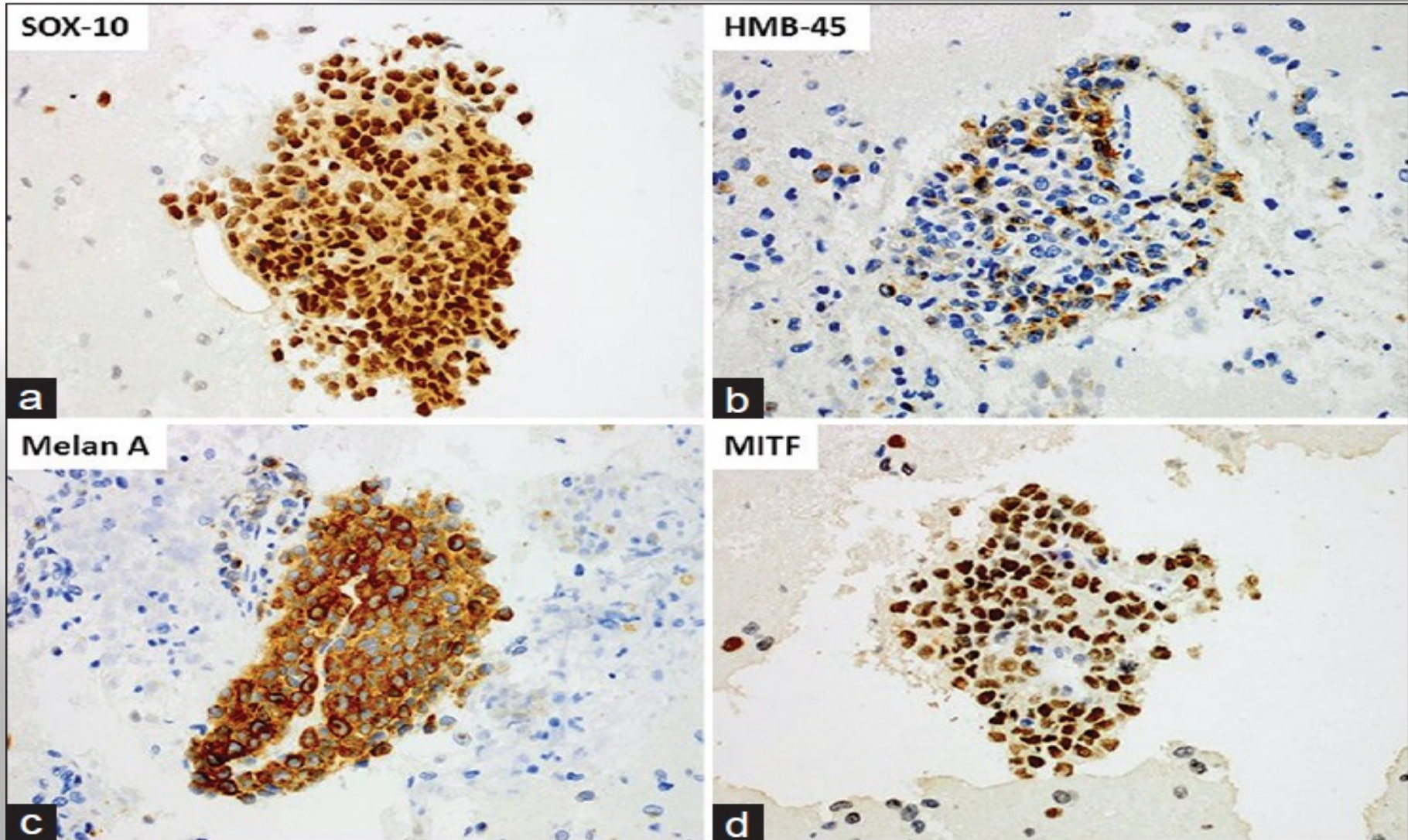
Melanoma -fna



Melanoma, melanocyte



Amelanotic Melanoma immunocytochemistry



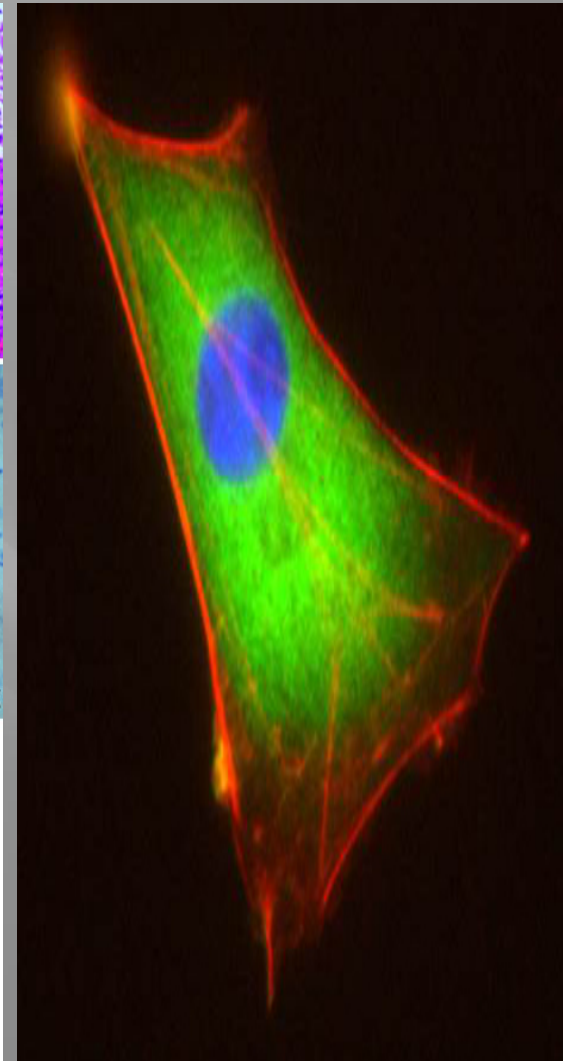
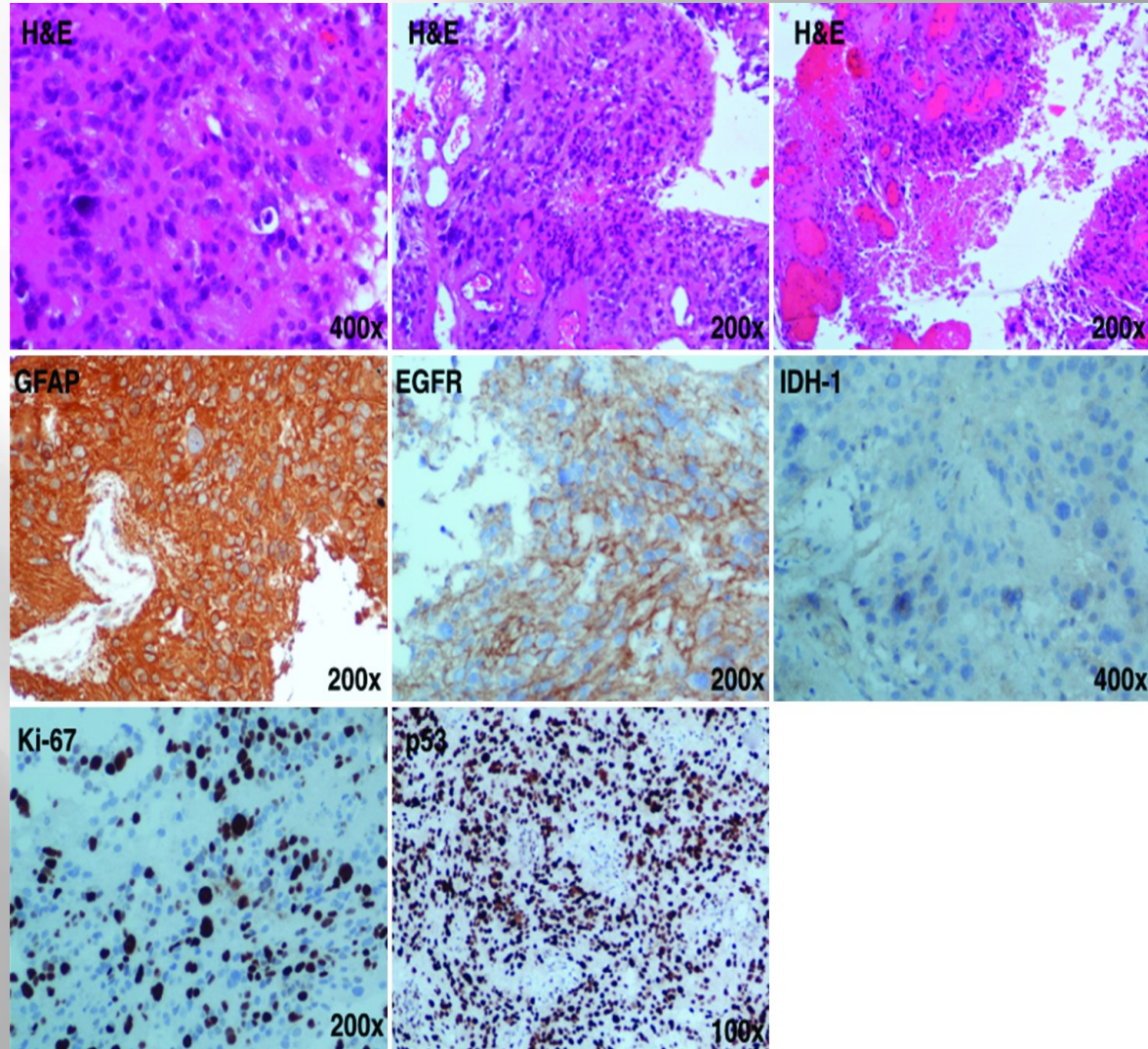
FLUORESCENCE MICROSCOPY

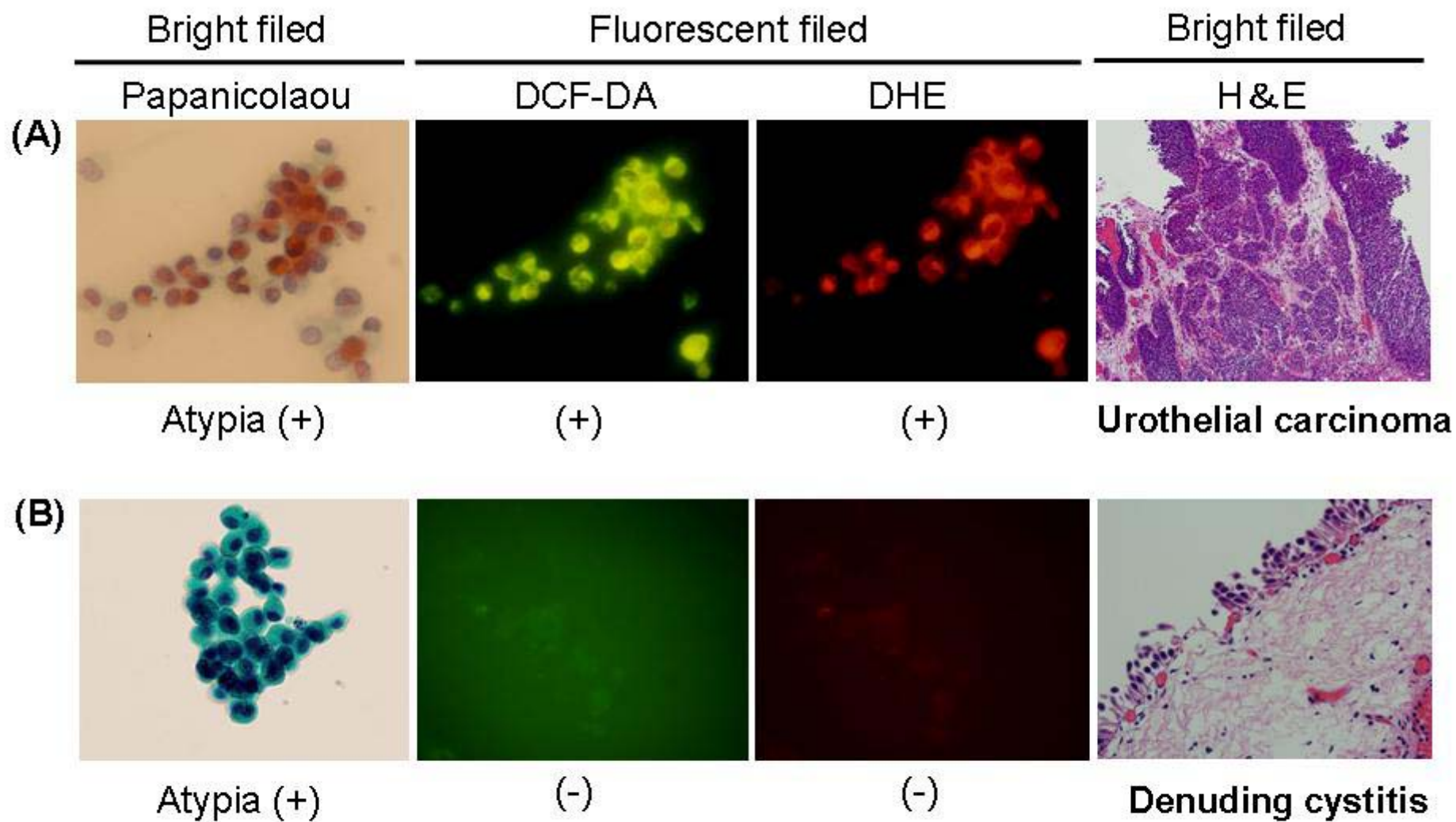
- ✓ *Essential tool for the study of cell biology and physiology*
- ✓ Fluorescence microscopy uses fluorescent dyes (fluorophores), which are molecules that absorb one wavelength of light (the excitation wavelength) and emit a second, longer wavelength of light (the emission wavelength). Most molecules in the cell are not very fluorescent, so fluorescent labels to be imaged are typically introduced by the experimenter.

FLUORESCENCE MICROSCOPY

- ✓ This allows the labels to be targeted to the molecule(s) of interest, either by genetically encoding a fluorescent protein or by binding a fluorescently labeled antibody. *Multiple different fluorescent molecules can be distinguished simultaneously and can be detected at very low abundance (single molecules can be imaged), making this a very powerful technique*

Glioblastoma





Limitations of fluorescence Microscopy

- ✓ Fluorophores used might interfere with metabolic pathway studied
- ✓ Excitation light might damage live tissue
- ✓ Excited fluorophore might react with oxygen and generate free radicals toxic to cell
- ✓ photobleaching

Inverted epifluorescence microscopy

- ▣ **Live cells** are most commonly imaged on an *inverted epifluorescence microscope*. In such a microscope, the objective images the sample from below. Inverted microscopes are popular for cell biological imaging because they allow imaging through a glass coverslip to *see cells grown above*. The detection camera cannot distinguish this out-of-focus light from the light emitted by the focal plane of the sample. Hence the in-focus information that we seek to image is obscured by blurred images of the out-of-focus regions of the sample for thick, densely stained samples or in cases in which we wish to achieve well-resolved 3D images, this *out-of-focus light can obscure valuable information*.

Confocal microscopy

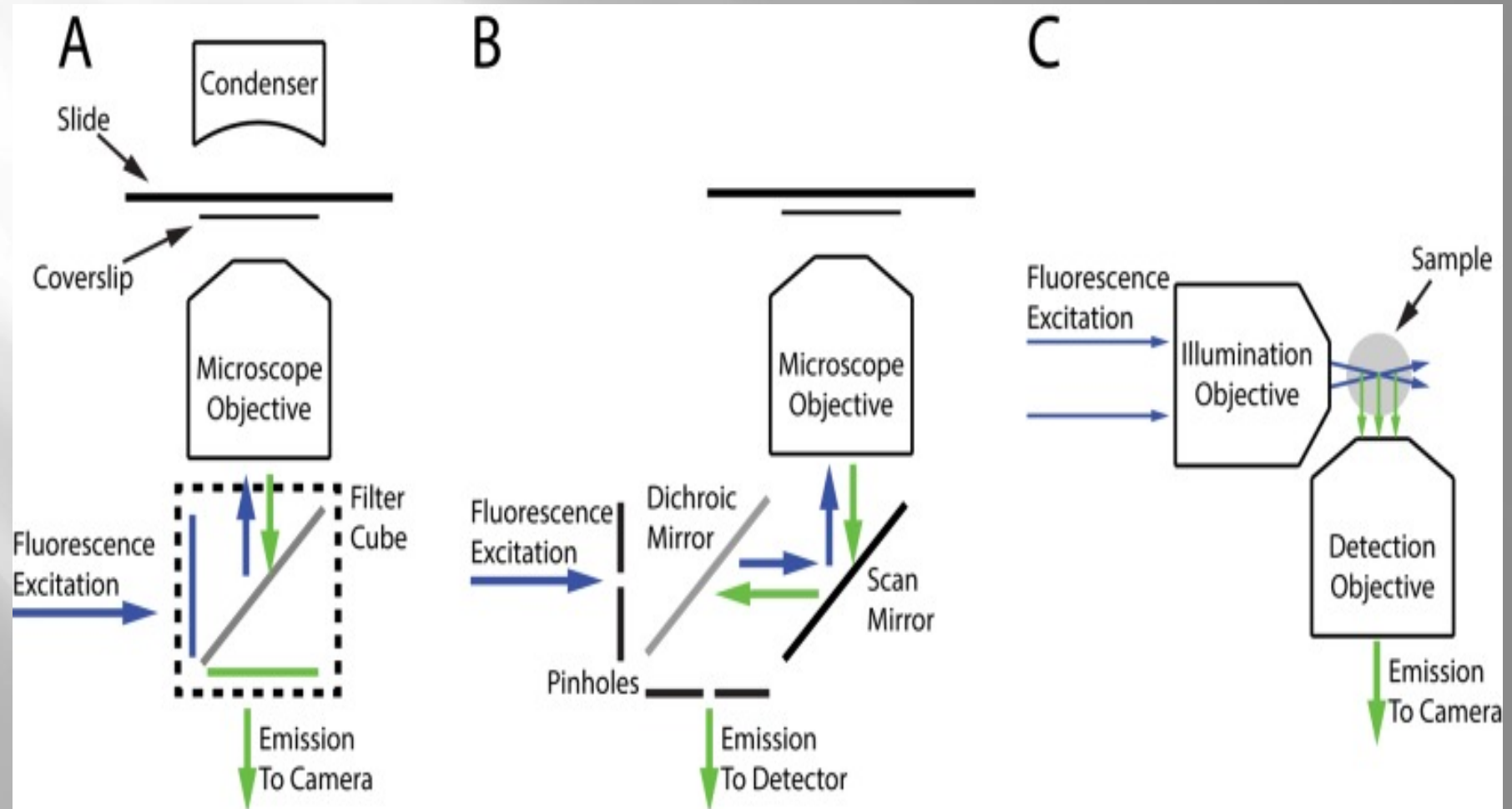
- ✓ Many techniques have been developed to **eliminate this out-of-focus light**. The most commonly used is *confocal microscopy*, in which **the sample is illuminated by a focused laser beam at a single point in the sample focal plane**
- ✓ Light from this point is detected after passage through a pinhole, such that only light emitted from the focal plane makes it through the pinhole and is recorded on the detector. Light from out-of-focus planes is blocked by the pinhole, and so the confocal only records light from the focal plane of the sample.
- ✓ confocal microscopes combine ease of use, high speed (up to hundreds of frames per second), and **high sensitivity**, so they have become widely used in cell biology Spinning-disk confocal microscopy is widely used for imaging **protein and organelle dynamics in single cells** – for example, imaging mitochondrial inheritance in yeast (or imaging microtubule dynamics in mammalian cells)

LIGHT SHEET MICROSCOPY

- ✓ These are microscopes that illuminate the sample from a plane orthogonal to the imaging plane. This *eliminates the problem of out-of-focus light* because only light from the focal plane or very close to it is excited.
- ✓ This selective illumination also reduces the total light exposure of the sample, which in turn reduces photobleaching and phototoxicity
- ✓ Light sheet microscopes are not yet widely available
- ✓ **a revolution in 3D microscopy of live cells**

Schematic drawings of microscopy techniques.

A: inverted epifluorescence microscope,
B: Confocal microscope, C: Light sheet microscope



Advantages and disadvantages of optical microscope

Advantages

- ✓ **Color imaging**
- ✓ Easily prepared sample material
- ✓ Easy to be integrated with digital camera systems for data storage and analysis
- ✓ Possible to observe living or non living specimens and observe movement

Disadvantages

- ✓ Low resolution , mainly due to the light diffraction limit

Scanning Electron Microscope

- ✓ The scanning electron microscope (SEM), uses beams of electrons for image formation. SEM produces excellent images of the surfaces of cells and small organisms, providing information for studying surface morphology and composition.



Electronic Microscope for higher resolution

- ✓ Resolution limit of LMs is due to light diffraction ; roughly optical resolution can be estimated as $\lambda/2NA$ (NA is Numerical Aperture of lens usually $\sim 1,0$) for white light wavelength is around 500nm. The best resolution thus is a few hundreds nm
- ✓ Decreasing the wavelength is the way to improve resolution , though nobody would deal with UV light.
- ✓ *Electron wave is a unique medium that can be used in imaging. By accelerating the electrons into high energy beam, the wavelength thus created is far shorter than white light*
- ✓ *theoretically, it can be used to image a species as small as 0.3 Å. Most atoms are in size of 2-3 Å.*

What is SEM ??

- ▣ In scanning electron microscopy (SEM) an electron beam is focused into a small probe and is rastered across the surface of a specimen.
- ▣ • Several interactions with the sample that result in the emission of electrons or photons occur as the electrons penetrate the surface.
- ▣ • These emitted particles can be collected with the appropriate detector to yield valuable information about the material.
- ▣ • The most immediate result of observation in the scanning electron microscope is that it displays the shape of the sample.
- ▣ • The resolution is determined by beam diameter.

ADVANTAGES & DISADVANTAGES OF SEM

Advantages

- It gives detailed 3D and topographical imaging and the versatile information garnered from different detectors.
- This instrument works very fast.
- Modern SEMs allow for the generation of data in digital form.
- Most SEM samples require minimal preparation actions.

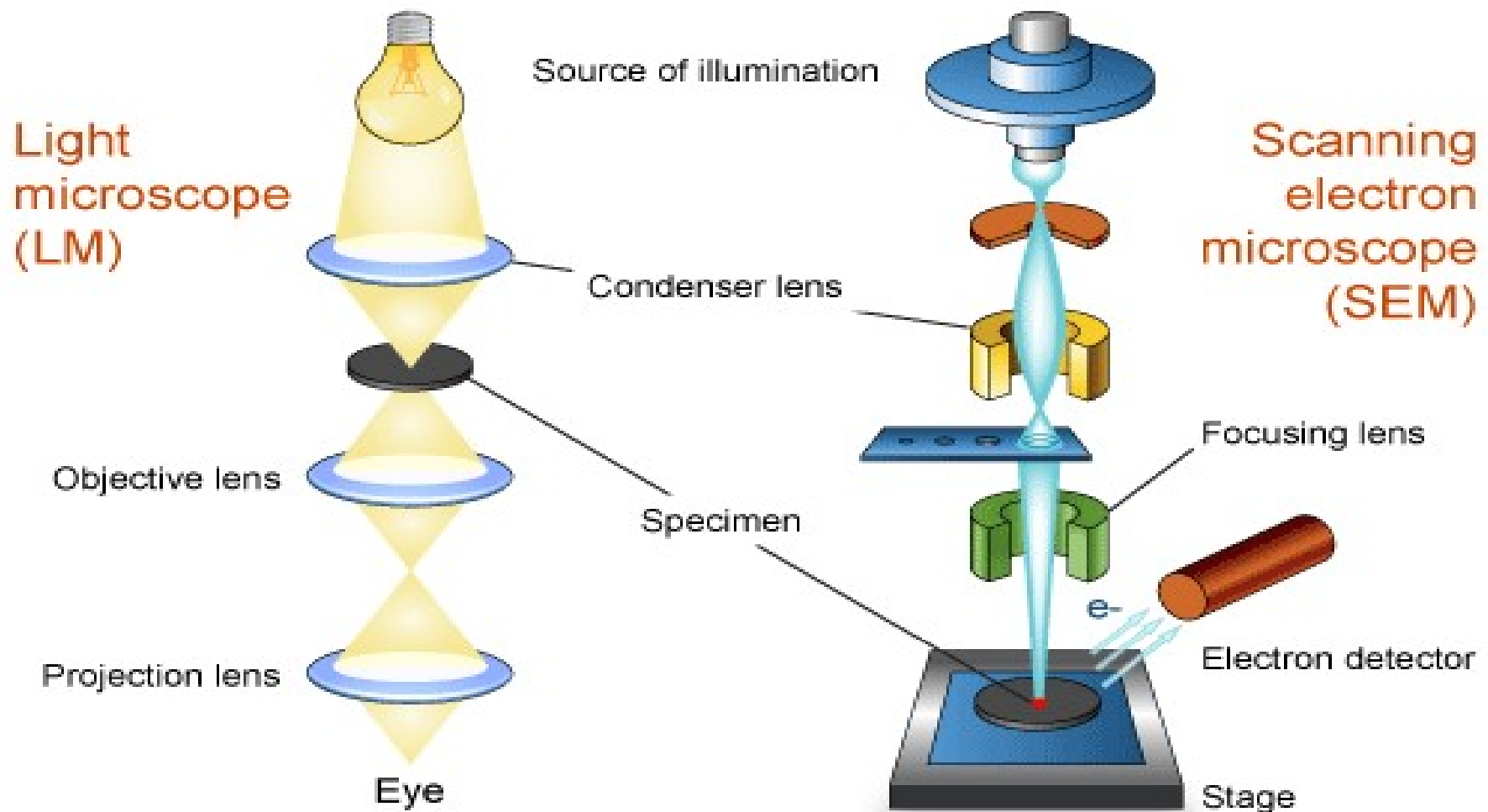
Disadvantages

- SEMs are expensive and large.
- Special training is required to operate an SEM.
- The preparation of samples can result in artifacts.
- SEMs are limited to solid samples.
- SEMs carry a small risk of radiation exposure associated with the electrons that scatter from beneath the sample surface.

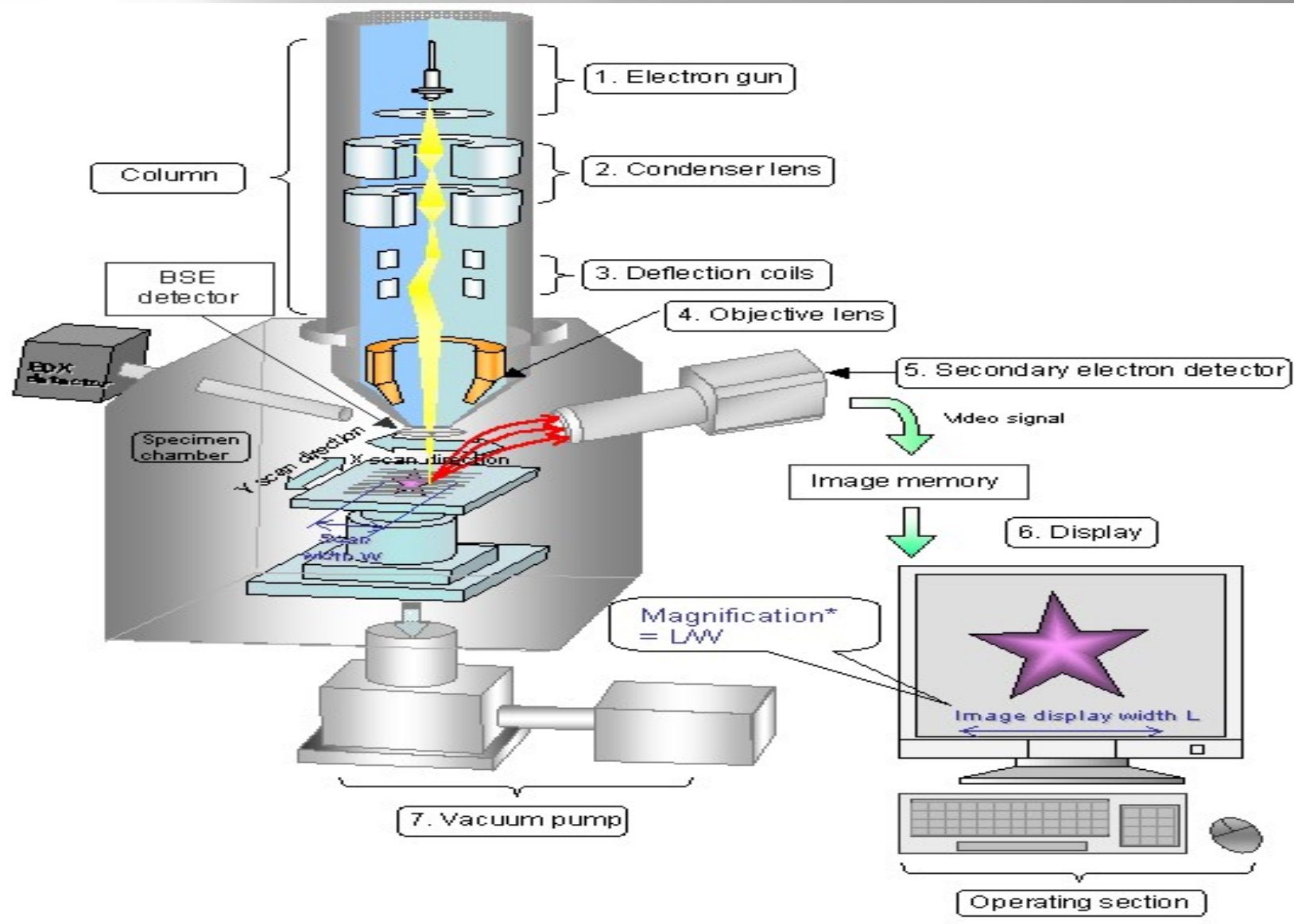
DIFFERENCES BETWEEN OM AND EM

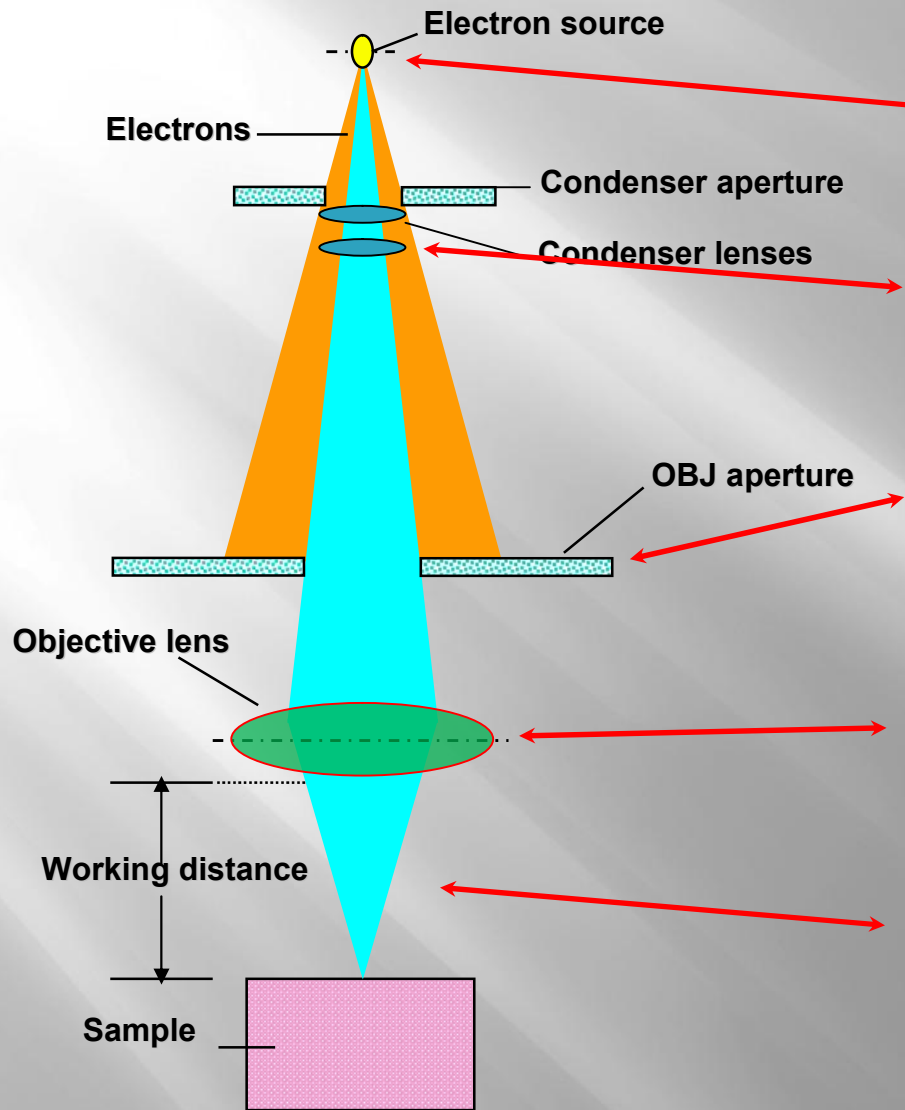
OPTICAL MICROSCOPE	ELECTRON MICROSCOPE
<ol style="list-style-type: none">1. The source of light.2. The specimen.3. The lenses that makes the specimen seem bigger.4. The magnified image of the specimen that you see.	<ol style="list-style-type: none">1. The light source is replaced by a beam of very fast moving electrons.2. The specimen usually has to be specially prepared and held inside a vacuum chamber from which the air has been pumped out (because electrons do not travel very far in air).3. The lenses are replaced by a series of coil-shaped electromagnets through which the electron beam travels.4. The image is formed as a photograph (called an electron micrograph) or as an image on a TV screen.

Light microscope - Scanning electron microscope



Principal and Structure of the SEM



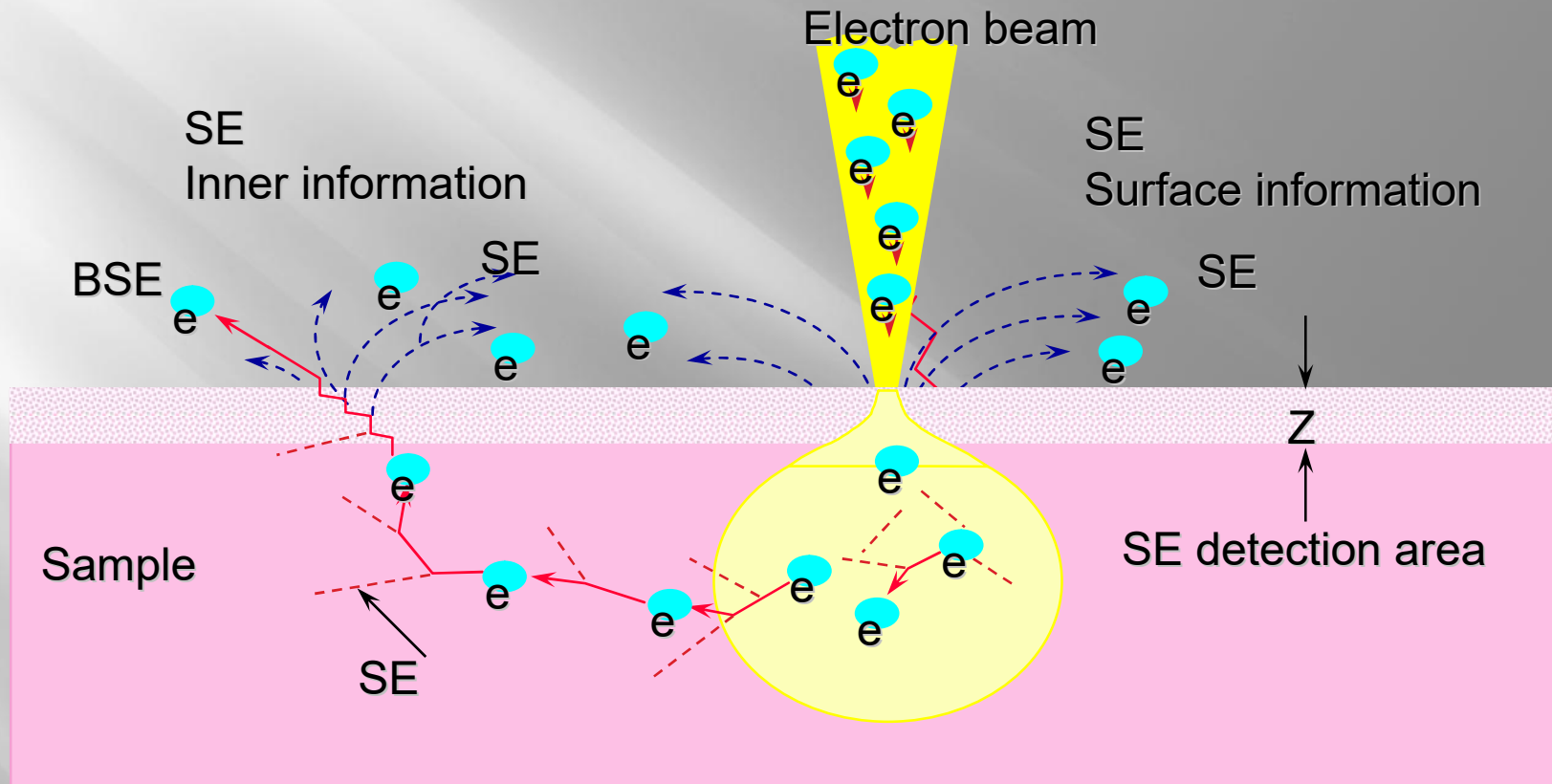


1. **Electron Source/Filament**
The filament is where we produce electrons from a tungsten filament that eventually form a continuous beam.
2. **Condenser Lenses**
The lenses “condition” the beam and allows us to “control” it better
3. **Condenser & Objective Apertures**
The apertures remove unwanted electrons in the the beam and forms a more monochromatic (electrons of equal energy) beam
4. **Objective Lens**
The Objective lens finally focuses the electron beam on the surface of the sample
5. **Working Distance W.D. or D**
The Working Distance is the distance between the Objective lens and the surface of the sample.

Beam \leftrightarrow Sample Interaction

- There is a wide variety of interactions which may occur between the beam electrons and the specimen.
- The exact interactions which occur are dependent on the *sample material* and *beam conditions*
- Secondary electrons = **SE**
- Backscattered electrons = **BSE**

Beam - Sample interaction (SE: secondary electrons, BSE: back scattered electrons)



Beam \leftrightarrow Sample Interaction

Secondary Electrons = SE

Yield (how many electrons are released) has very little relationship to the material composition.

S.E. yield is essentially a function of topography.
(Surface Shape & Roughness)

Backscattered Electrons = BSE

yield is related to the atomic number of the sample.

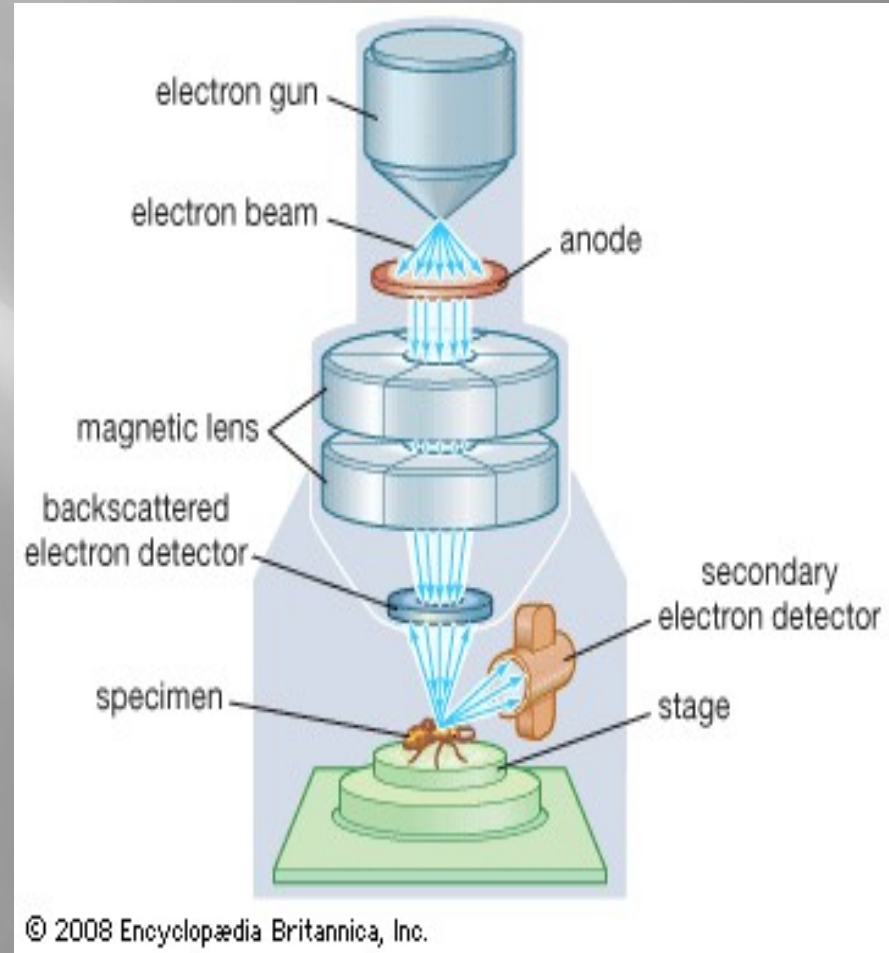
- High Atomic No. = High Yield
- Low Atomic No. = Low Yield

Secondary emission by Backscattered electrons

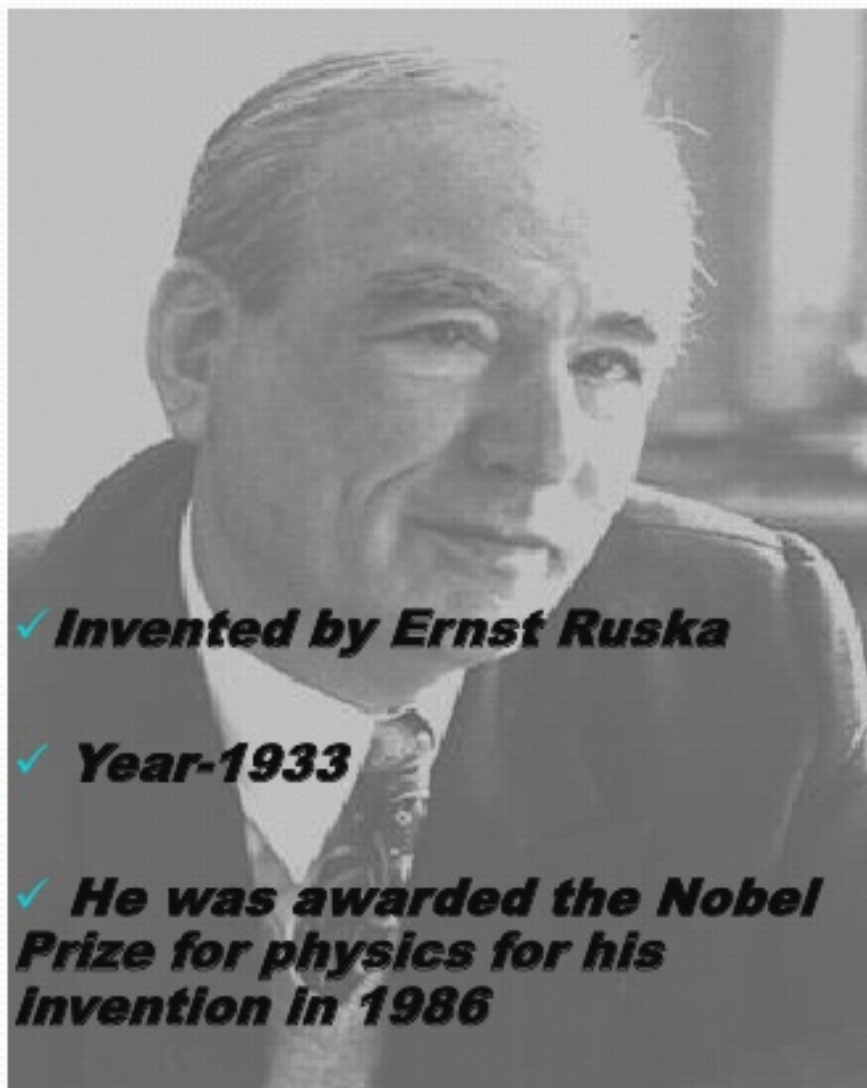
- This can be 4 times larger than the incident beam

SEM

- Electron microscope follows the same ideas of optical microscope, but uses electrons instead of light;
- •“Lens” here are not the optical materials (like glass), but electrical field.



First Electron Microscope



✓ ***Invented by Ernst Ruska***

✓ ***Year-1933***

✓ ***He was awarded the Nobel Prize for physics for his invention in 1986***



History of electron diffraction

1927 Davisson and Germer USA,
Thompson and Reid in Scotland, report
electron scattering by crystals giving
interference patterns. Share Nobel prize in
Physics 1937.



Lester Germer (right) with Clinton Joseph
Davisson (left) 1927
George Paget
Thompson



Ernst Ruska

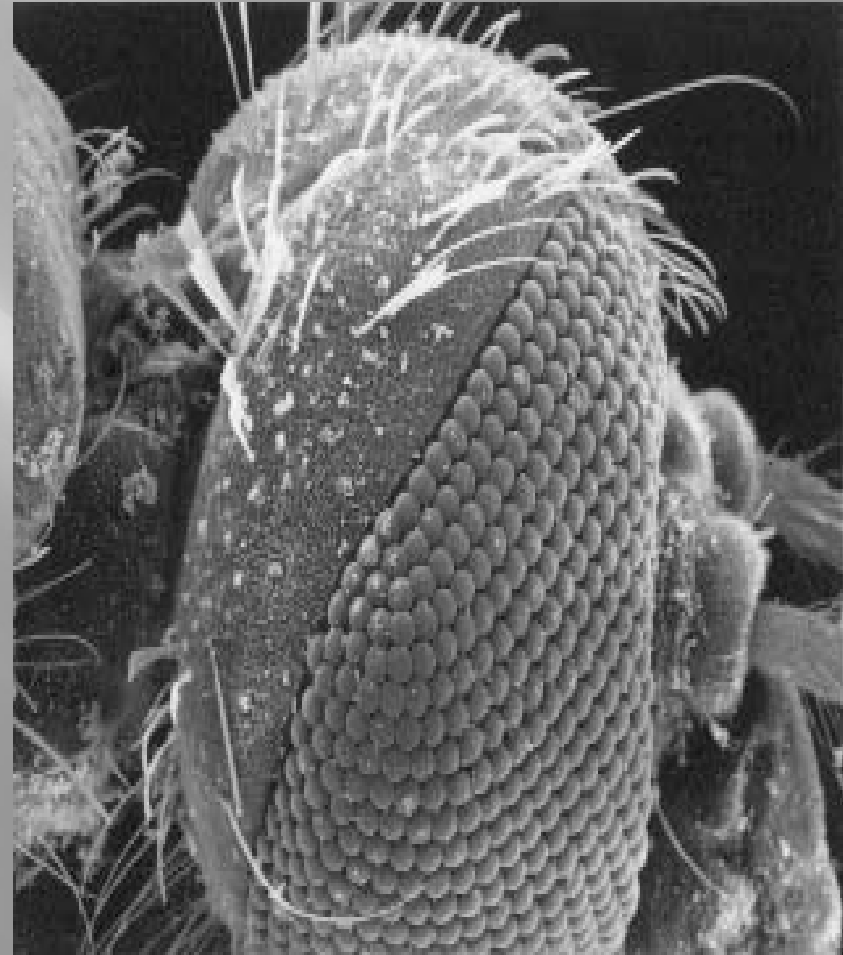
Ruska's
microscope

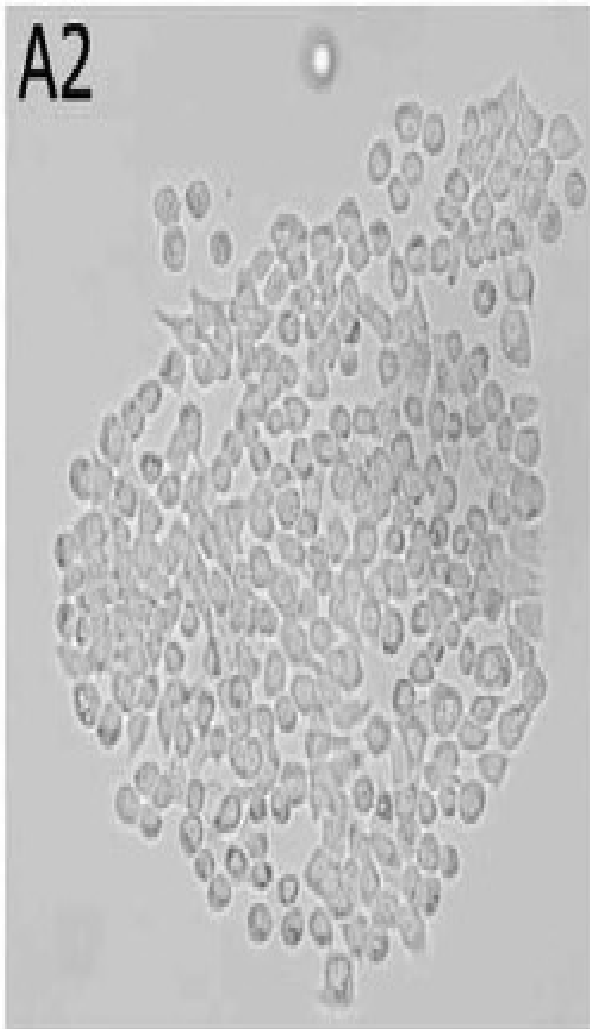
1932 Knoll and Ruska use the
term electron microscope and
present first electron images.
Nobel prize in Physics for Ruska
1986.

1936-39 First commercial TEMs
produced.

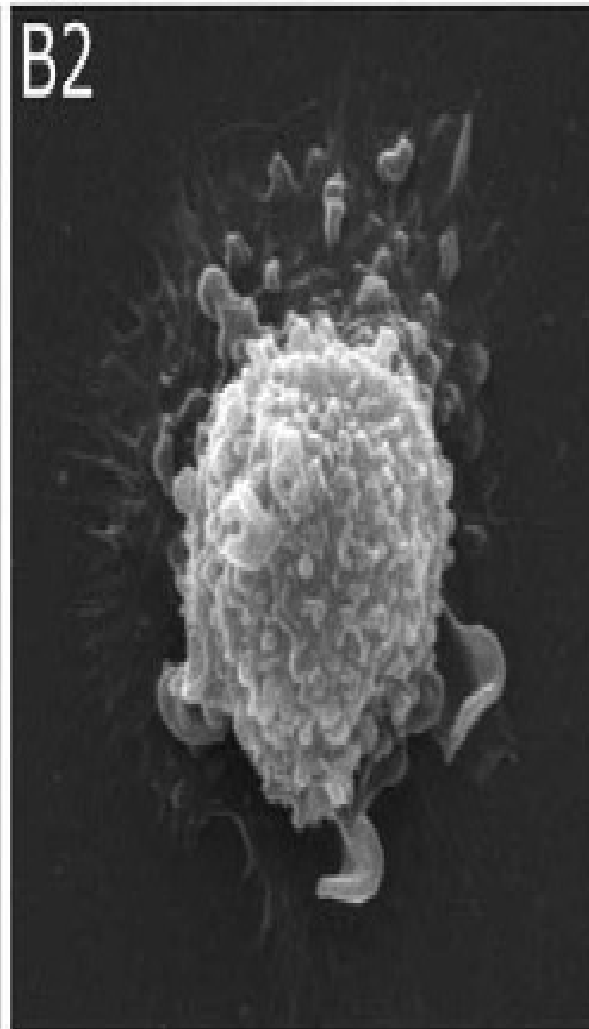
SEM images of bio-species

- ▣ The head of a mosquito is mostly eye. The eyes are compound eyes, made up of many tiny lenses.

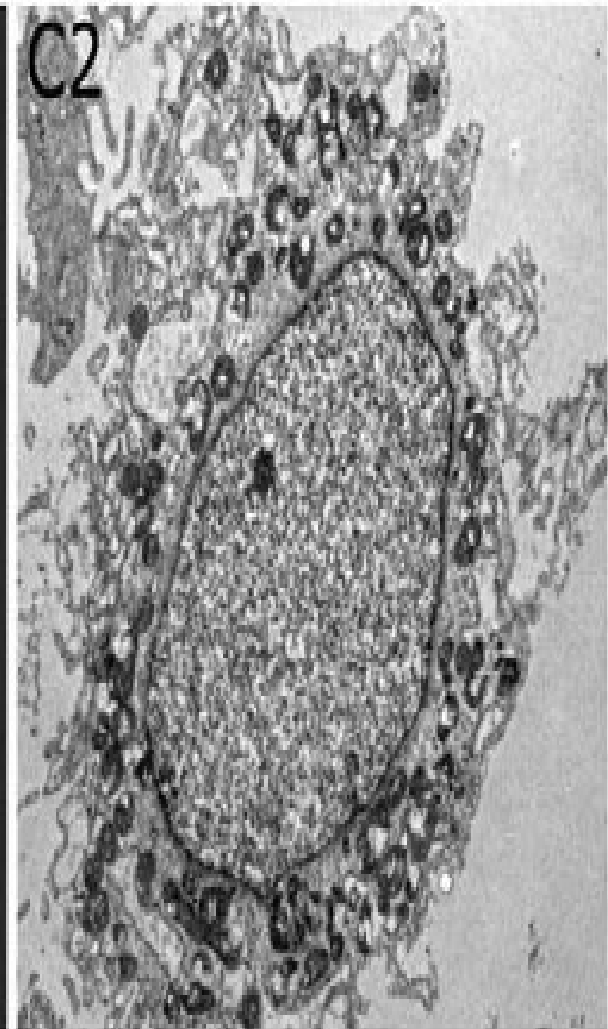




light microscope

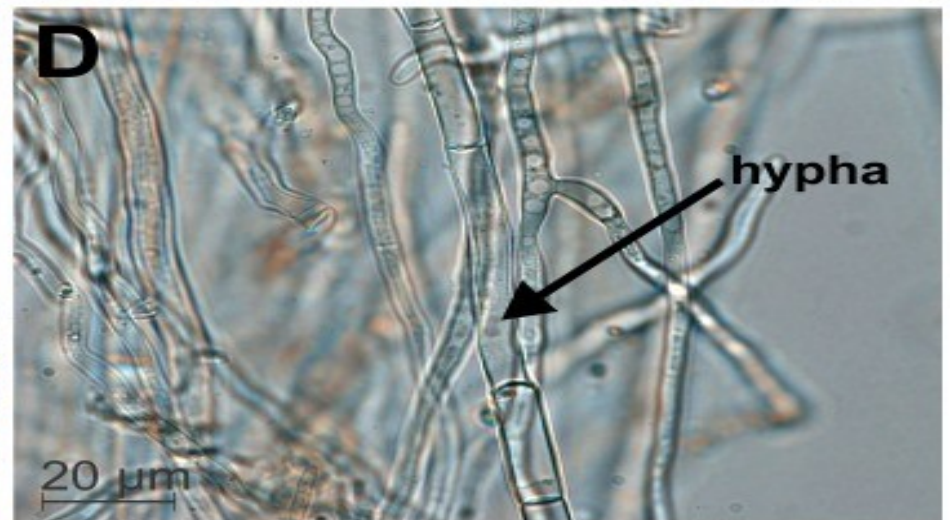
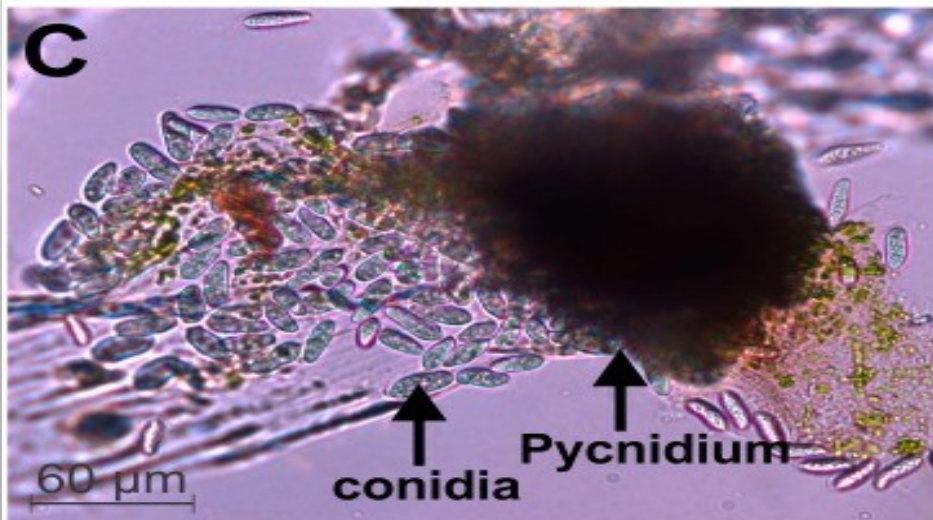
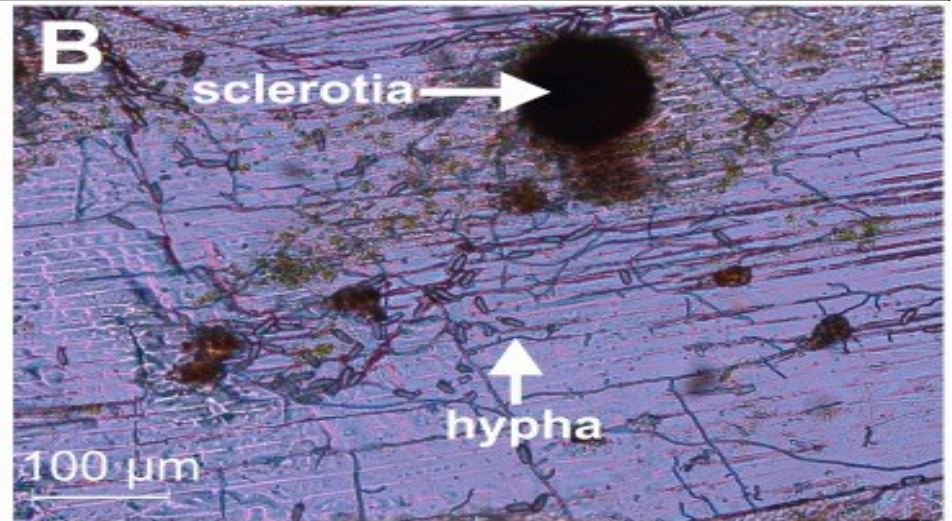
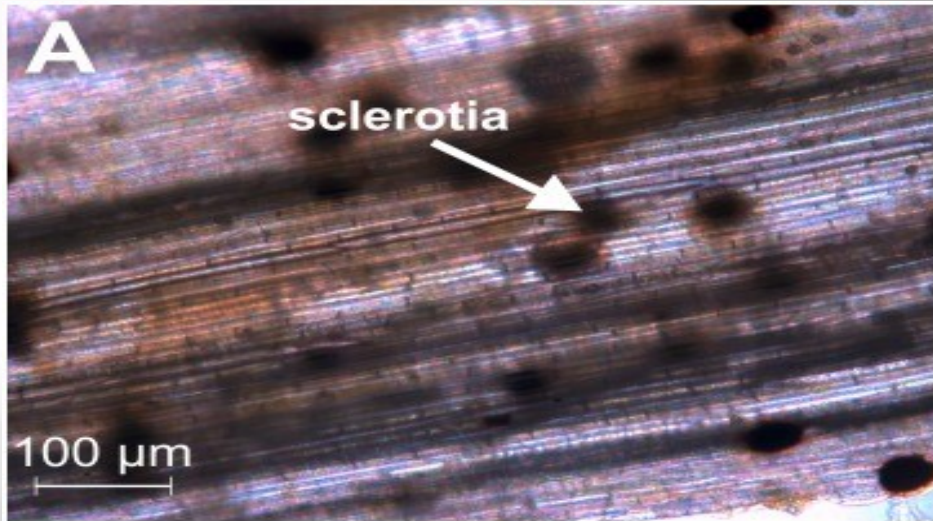


scanning electron
microscope

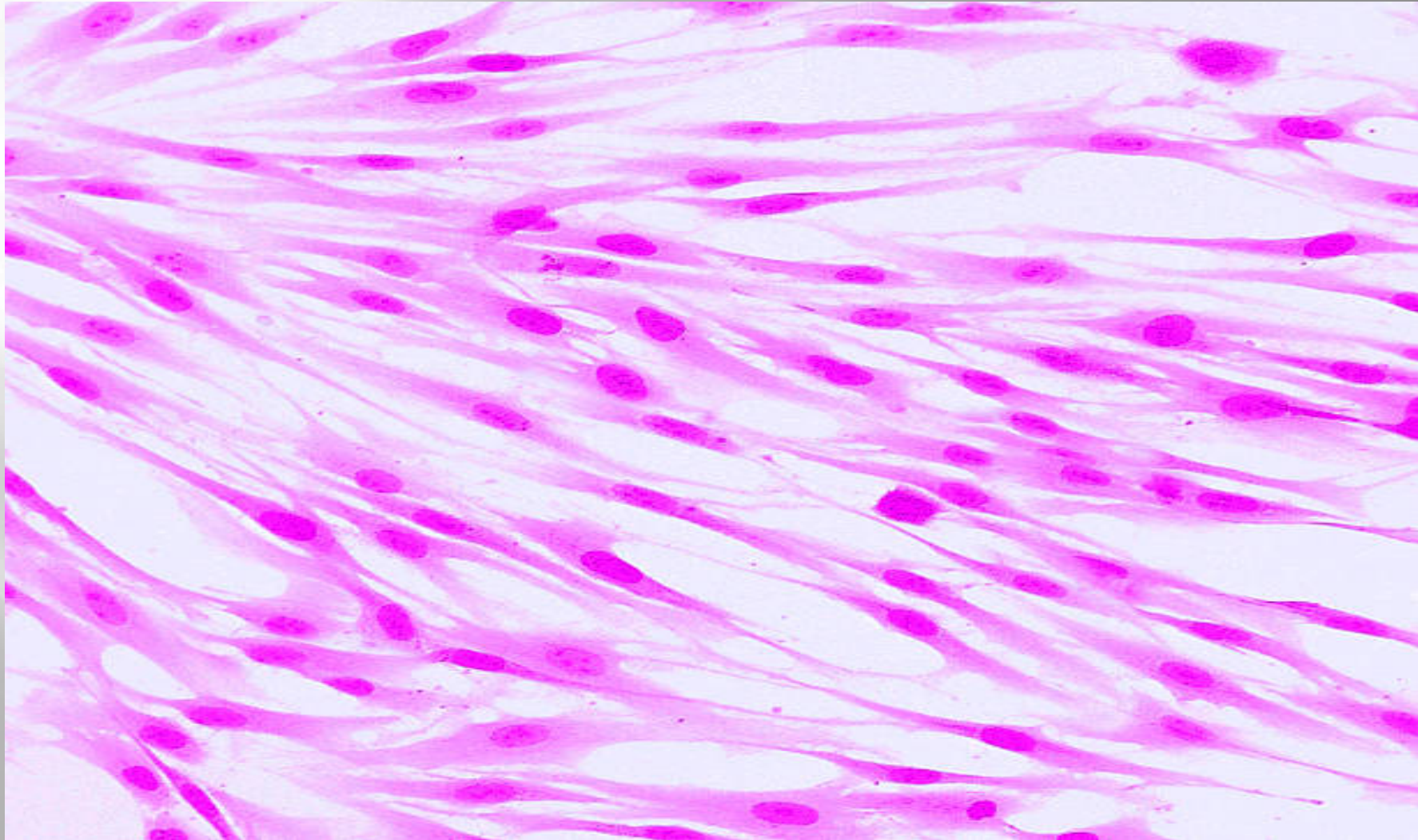


transmission electron
microscope

Light microscopy (A,B) SEM (C,D)



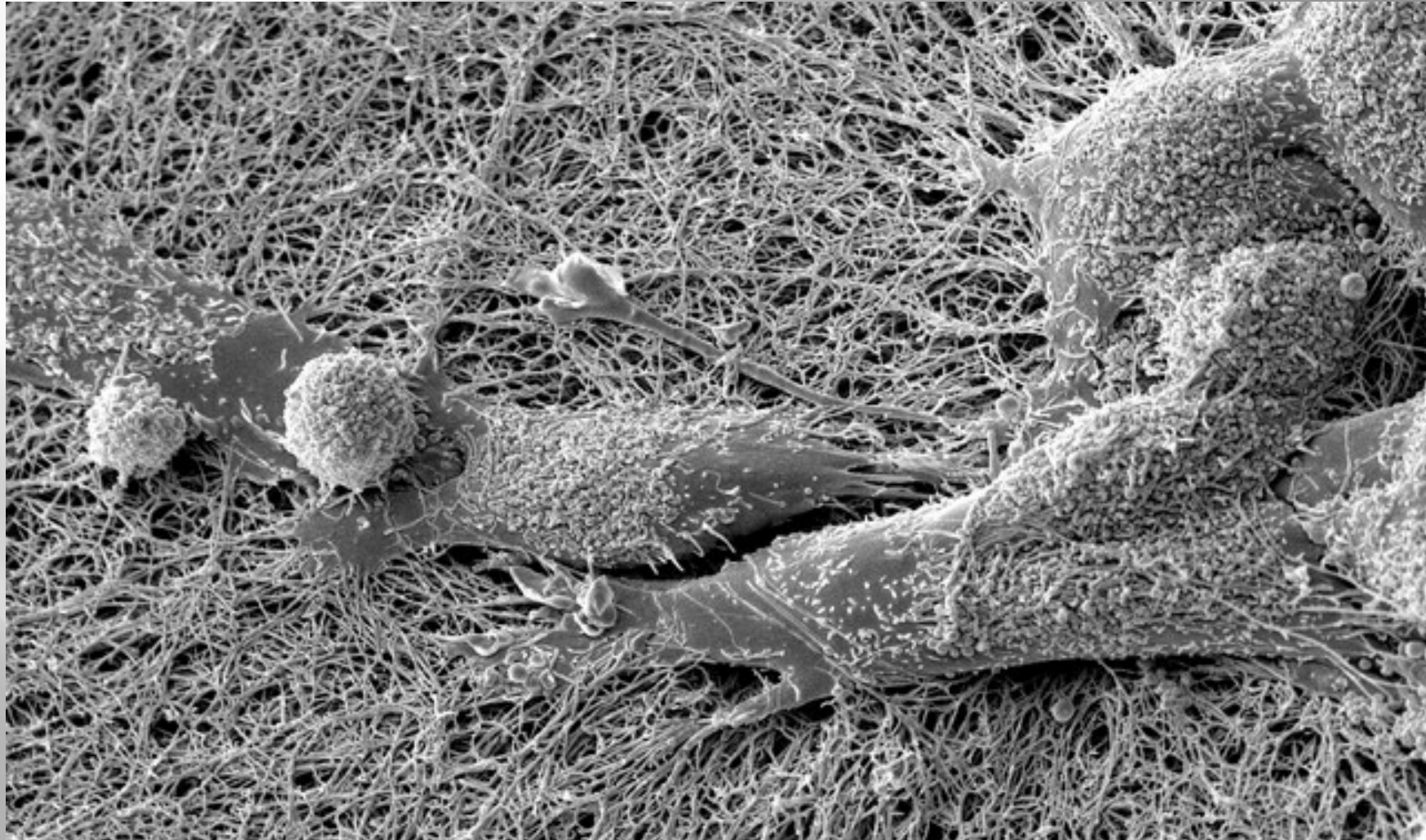
Fibroblast- MG Giemsa- Light Microscope



Fibroblast- fluoresceine



Fibroblast- SEM

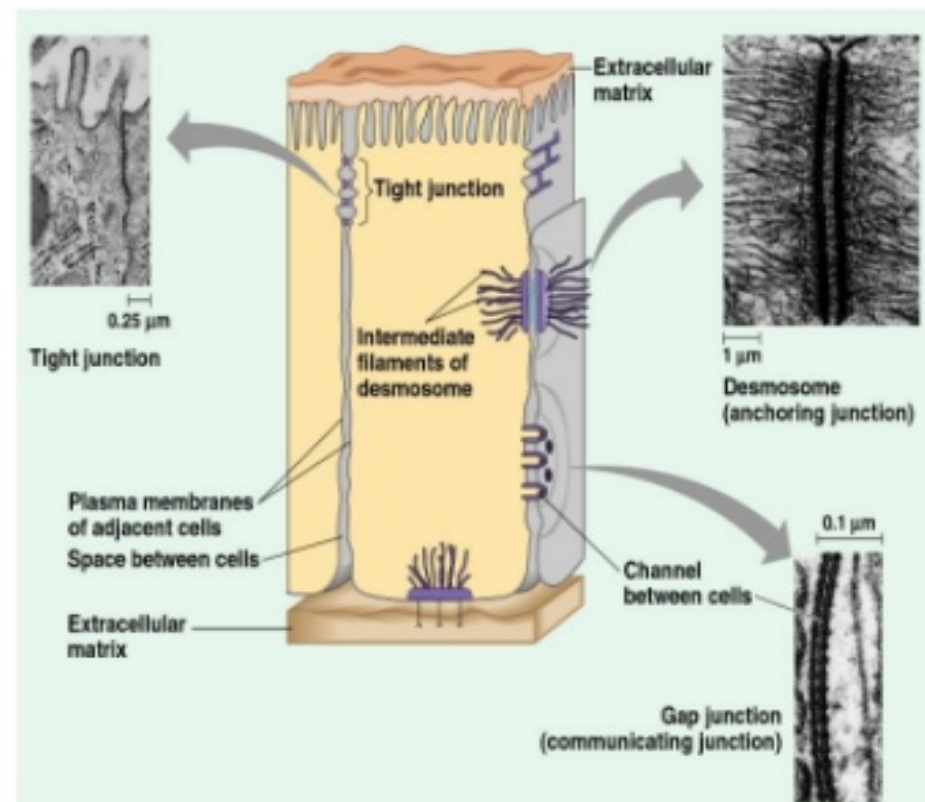


Adhesion junctions

● ● ● | Intercellular Junctions

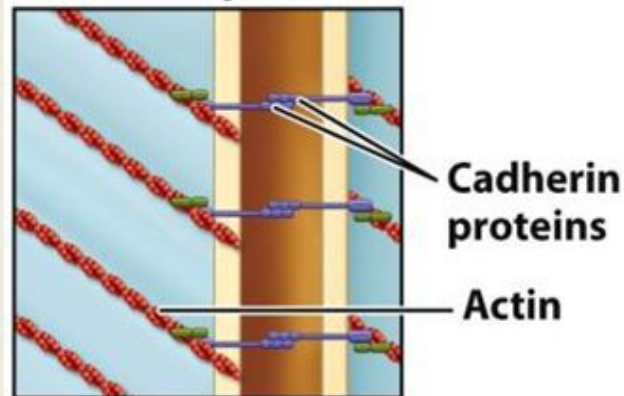
○ Gap junctions

- Communicating junctions
- Cytoplasmic channels between adjacent cells
- Salts, sugars, AAs, etc. can pass through



ADHERENS JUNCTIONS AND DESMOSOMES

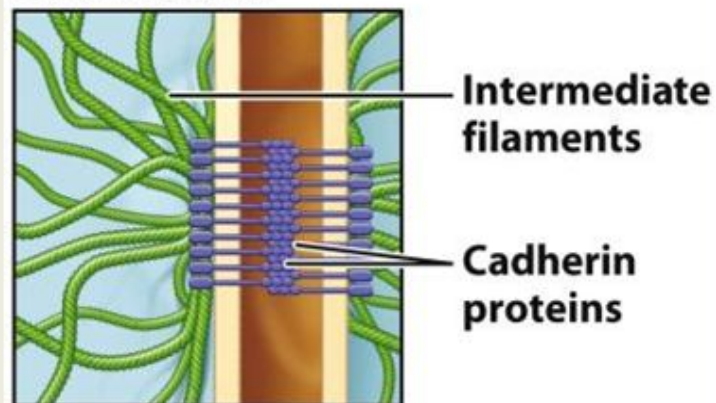
Adherens junction



Adherens junction:

Anchor cells to each other
Belt-like complex of cadherins
Reinforced by actin microfilaments

Desmosome

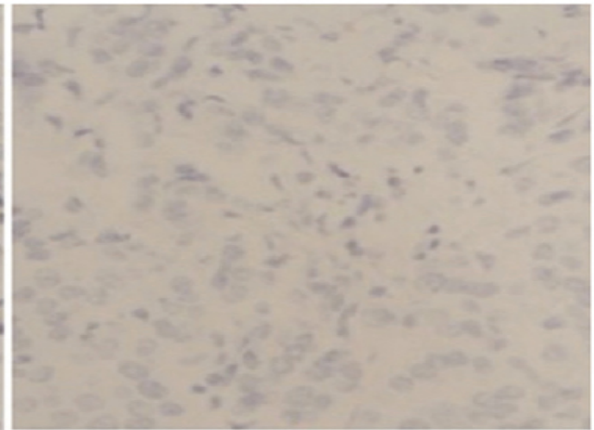
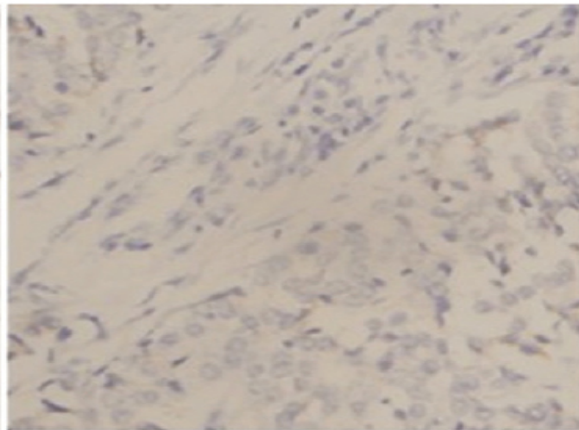
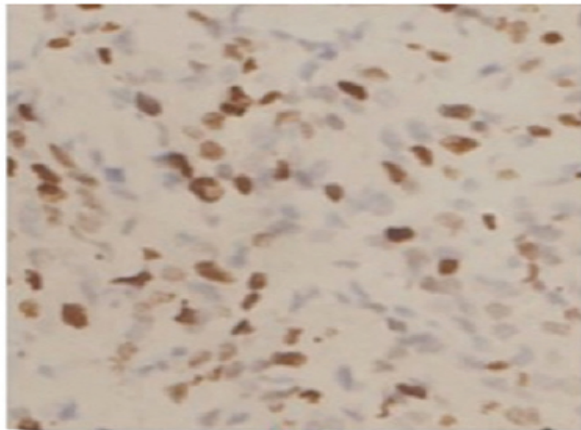


Desmosomes:

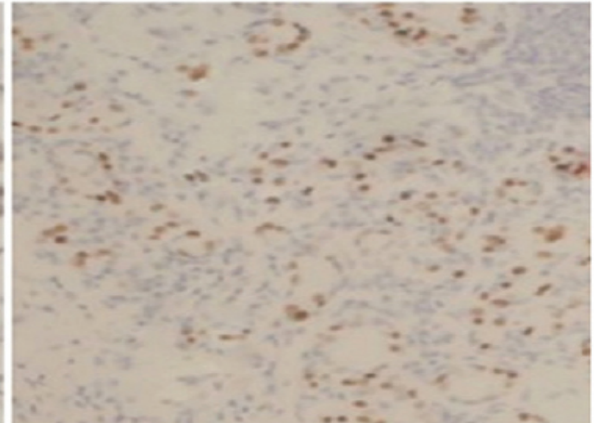
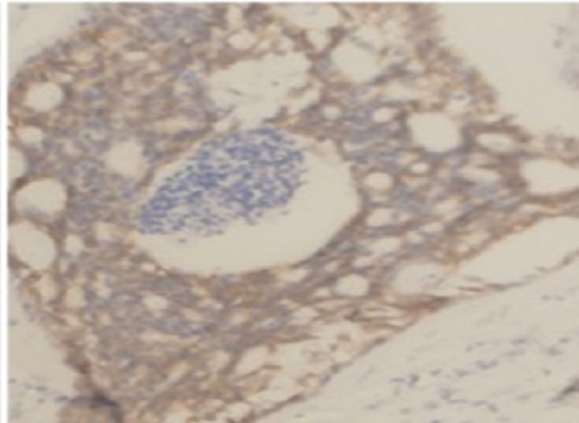
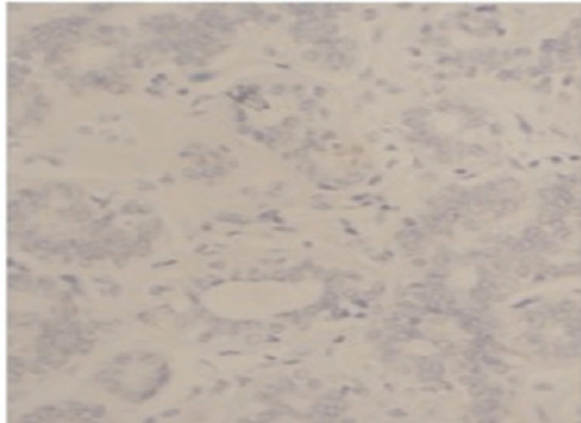
Button-like complex of cadherins
Reinforced by intermediate filaments

Breast cancer -immunocytochemistry cell adhesion molecule (CAM) E-Cadherine

Cancer tissues



Adjacent tissues



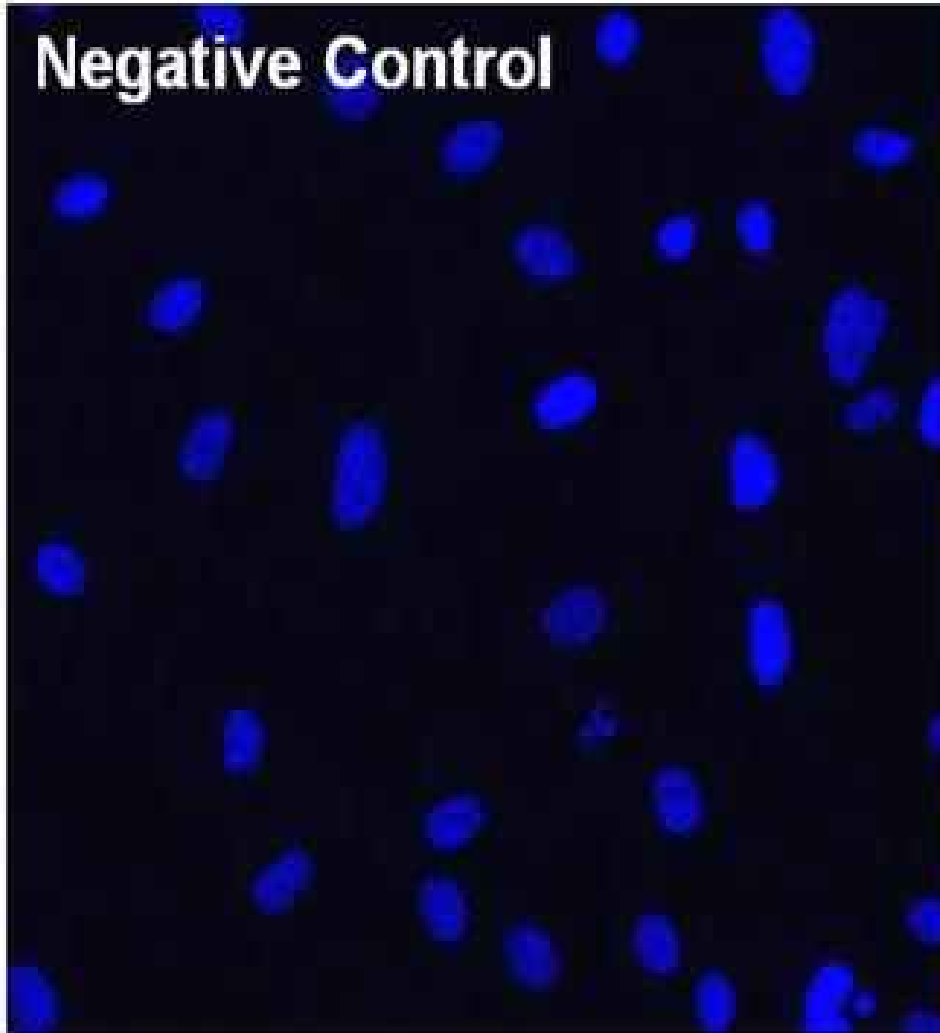
Twist

E-cadherin

ER

fluoresceine

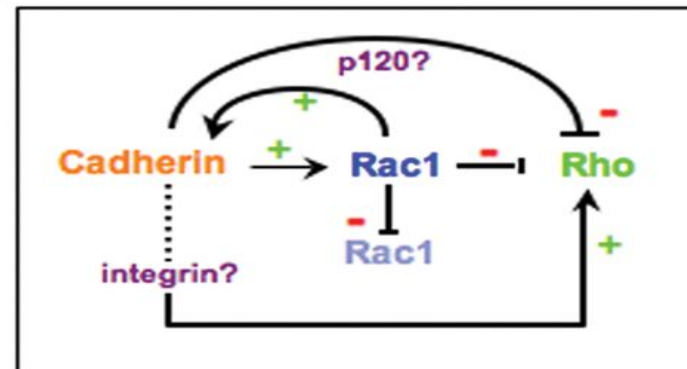
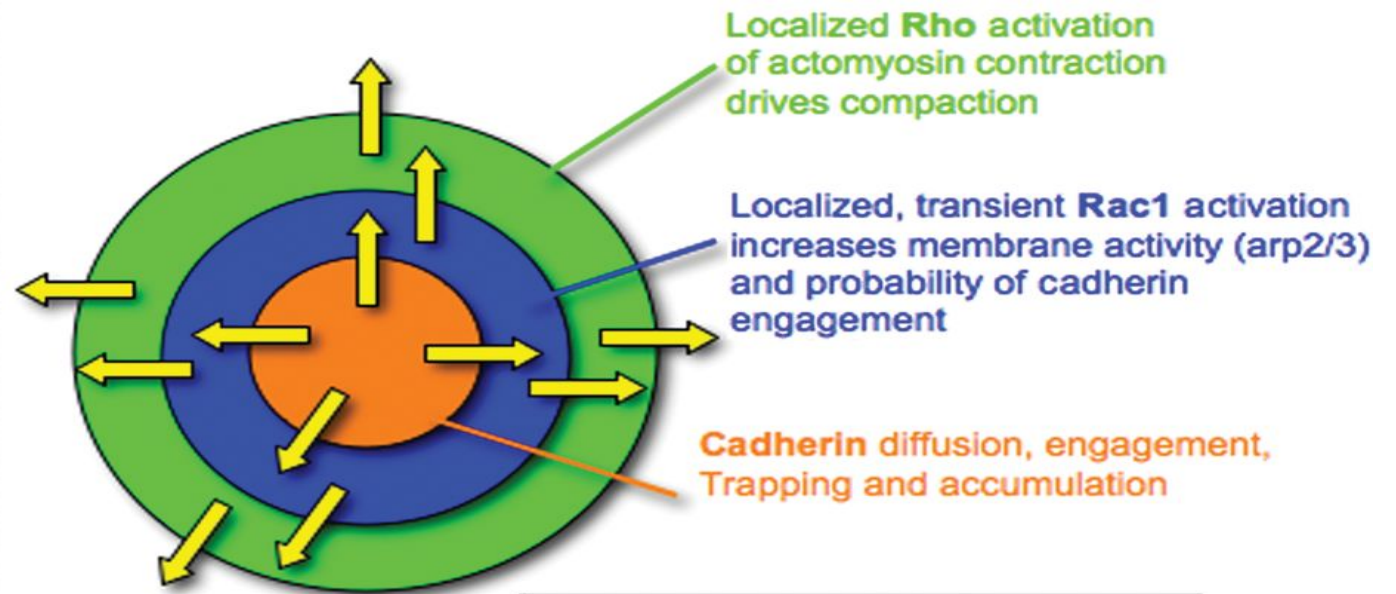
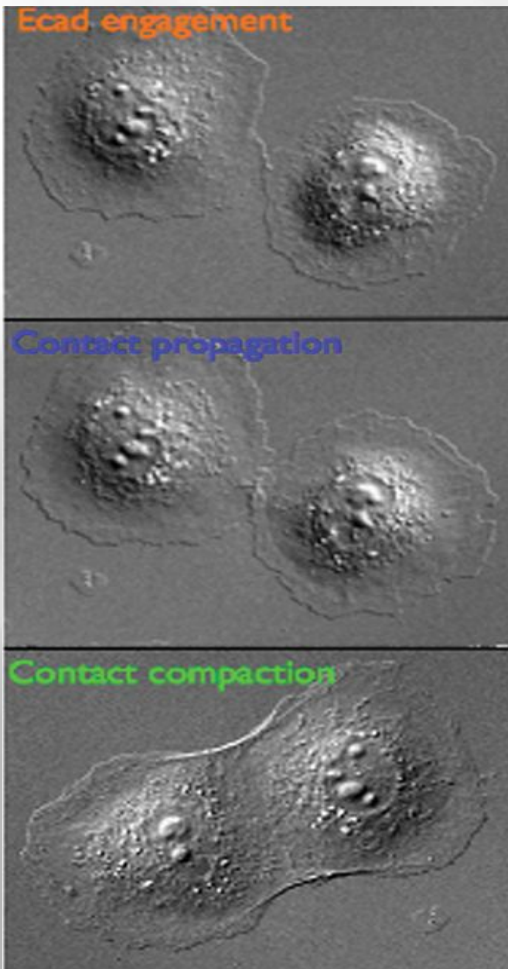
Negative Control



VE-Cadherin



SEM



Different types of microscopes offer different information that can meet the needs of the researcher, according to his planning experiment.

