Methods in Cell Biology

B. Balen

Diversity of the living world

~ 1 200 000 animal species

~ 360 000 plant species

~ 80 000 fungi species

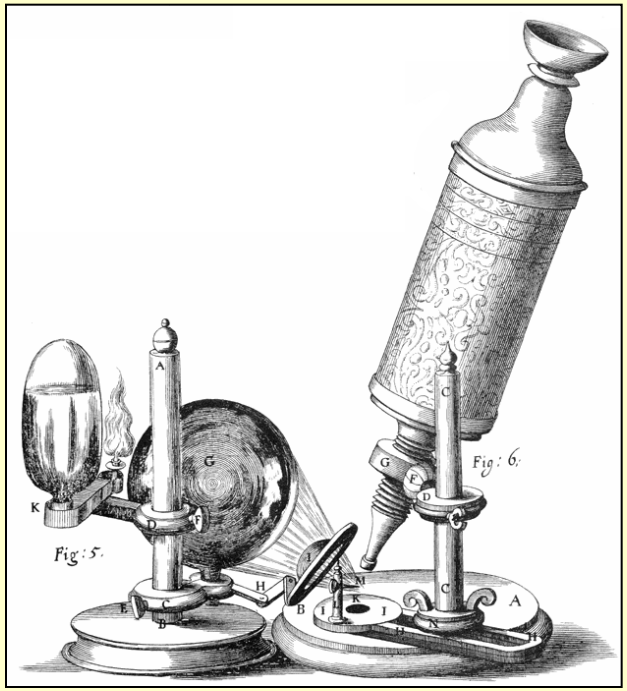
~ 3 000 bacterial species

All diversity of the living world can be resumed to the common cellular basis.

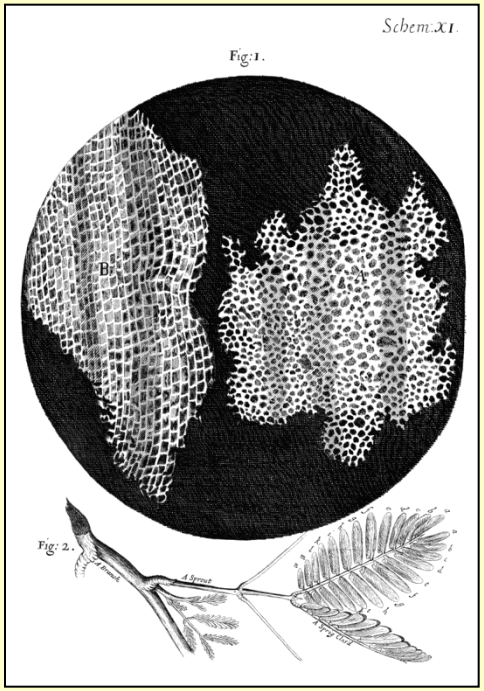
When were the first cells discovered?

➢Robert Hook, *Micrographia*, 1665.g.

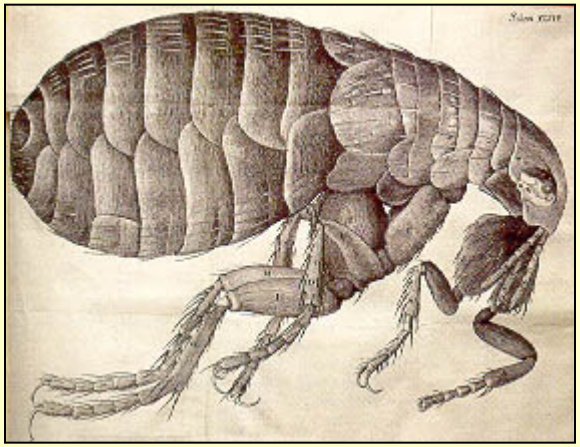
-introduced term the “cell” by observing cork pieces with his light microscope



Light microscope
(magnification 30 x)



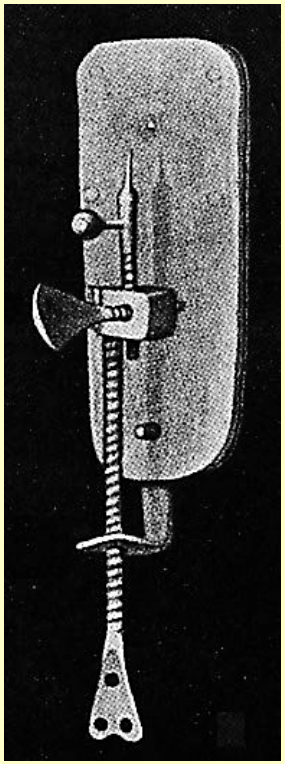
Drawing of the cork
from *Micrographia*



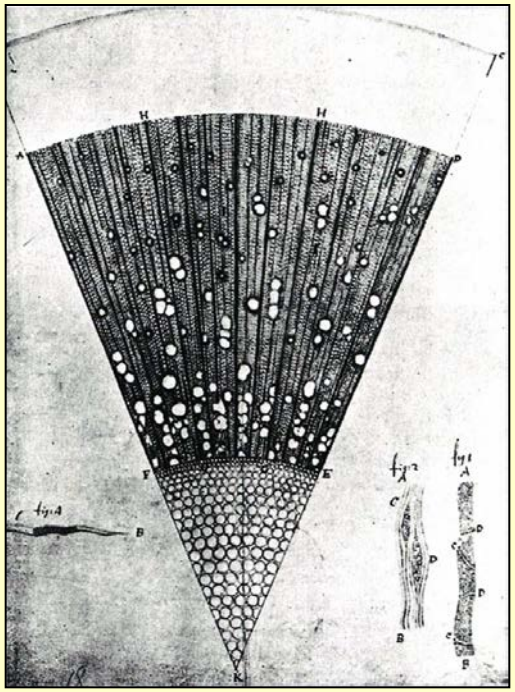
Drawing of the flee from
Micrographia

➢Antony van Leeuwenhoek, 1670. g.

- discovered the world of bacteria by watching the water droplet from the pond, blood cells and animal sperm cells

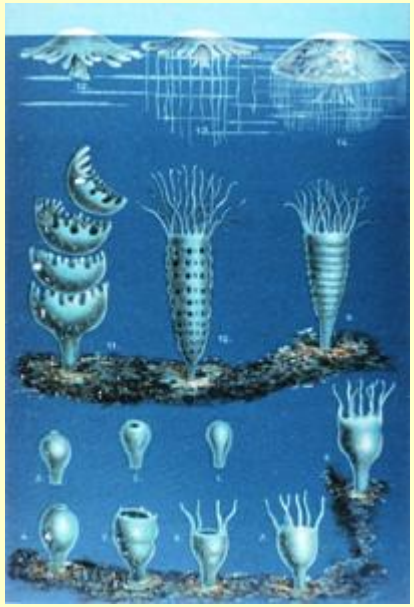


Light microscope
(magnification 300 x)



Drawing of the section of the ash tree

➢Matthias Schleidenn and Theodor Schwann, 1838. g.
-microscopic analyses of plant and animal tissues



Development of the jellyfish from the Schleiden book *Das Meer*

✓ discovered that all living organisms are built out of cells

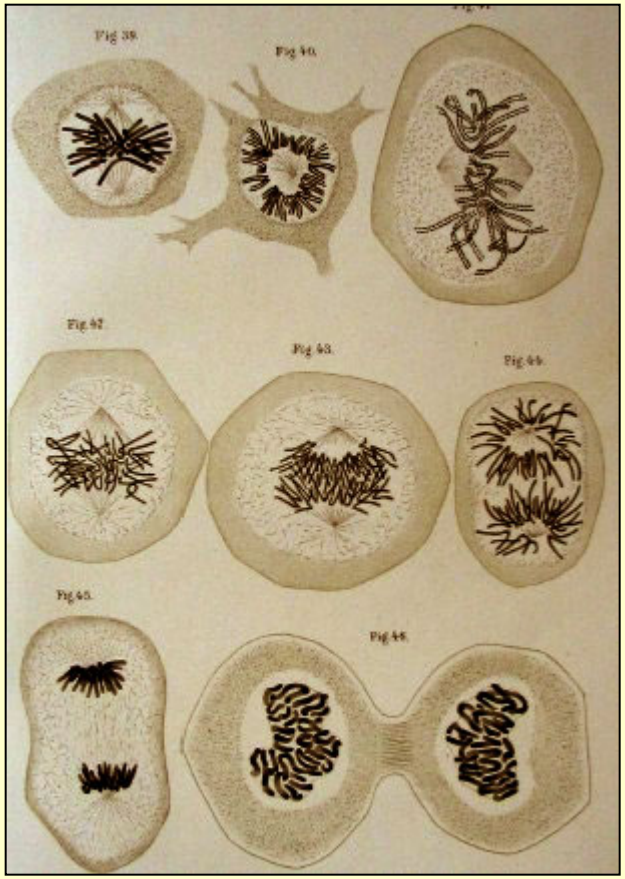
✓ cell – basic unit of life

✓ beginning of the Cell Biology Science

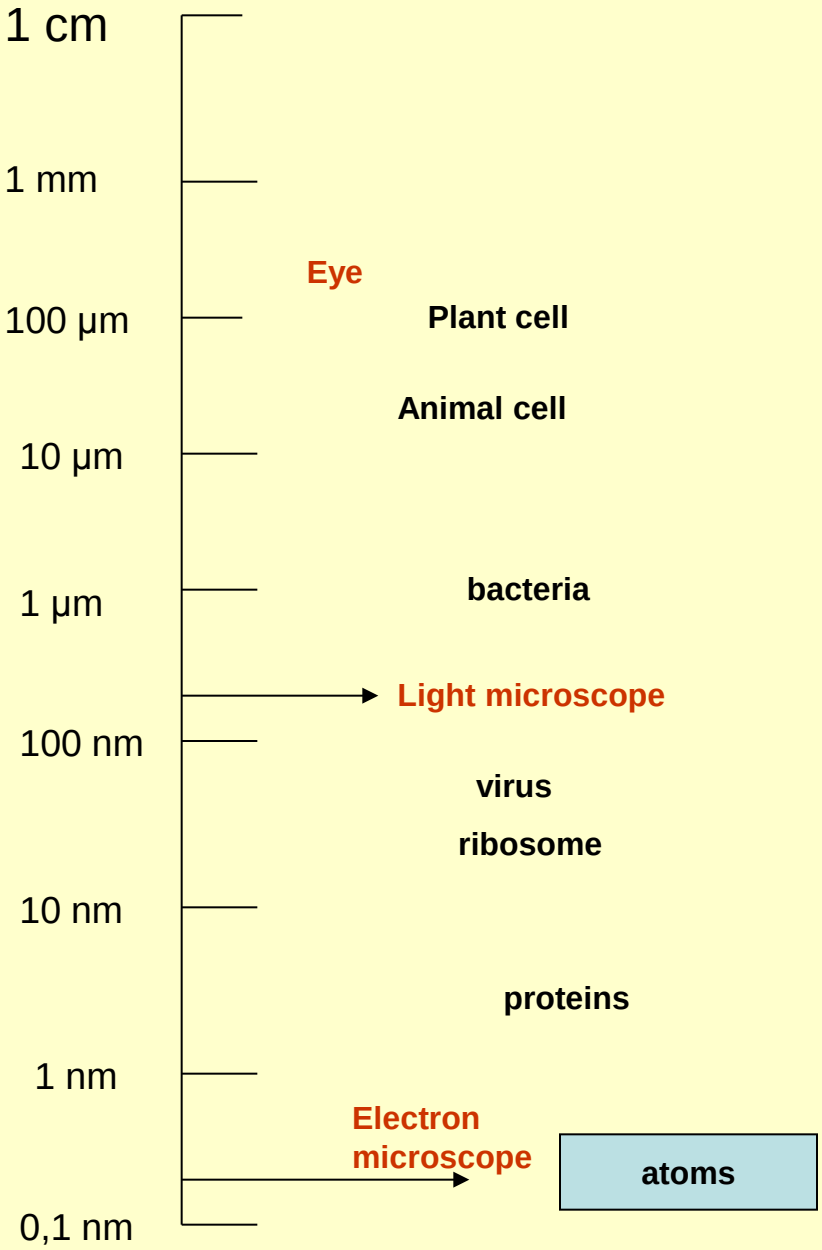
✓ proposed **cell theory or cell doctrine: all living organisms are built out of cells or at least one cell**

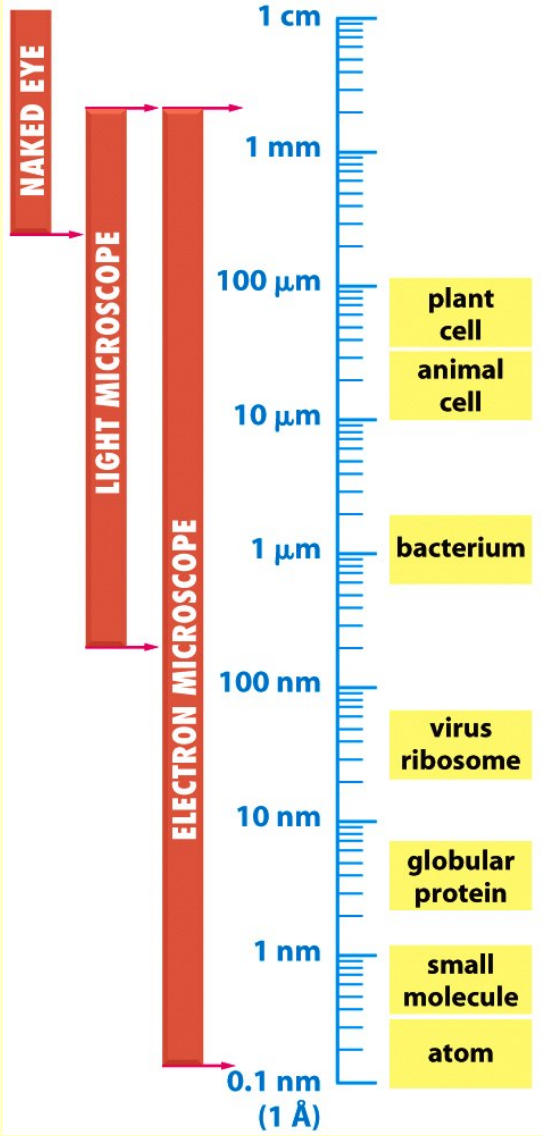
✓ **Formal birth of contemporary cell biology!**

➢Rudolf Virchow, 1885. g.
“*Omnis Cellula ex Cellula*”



Scale of size of different biological structures





Methods in Cell Biology

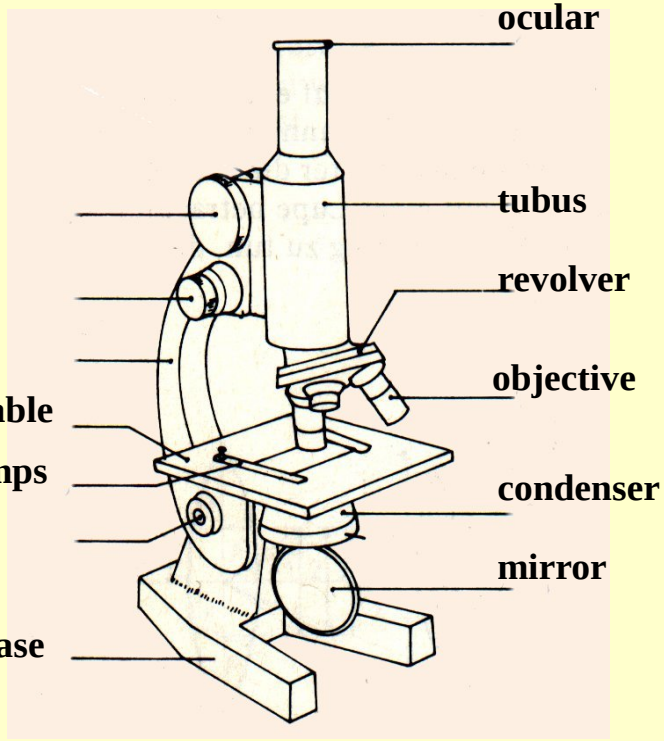
1. Microscopy

1.1. Light microscopy

1.2. Electron microscopy

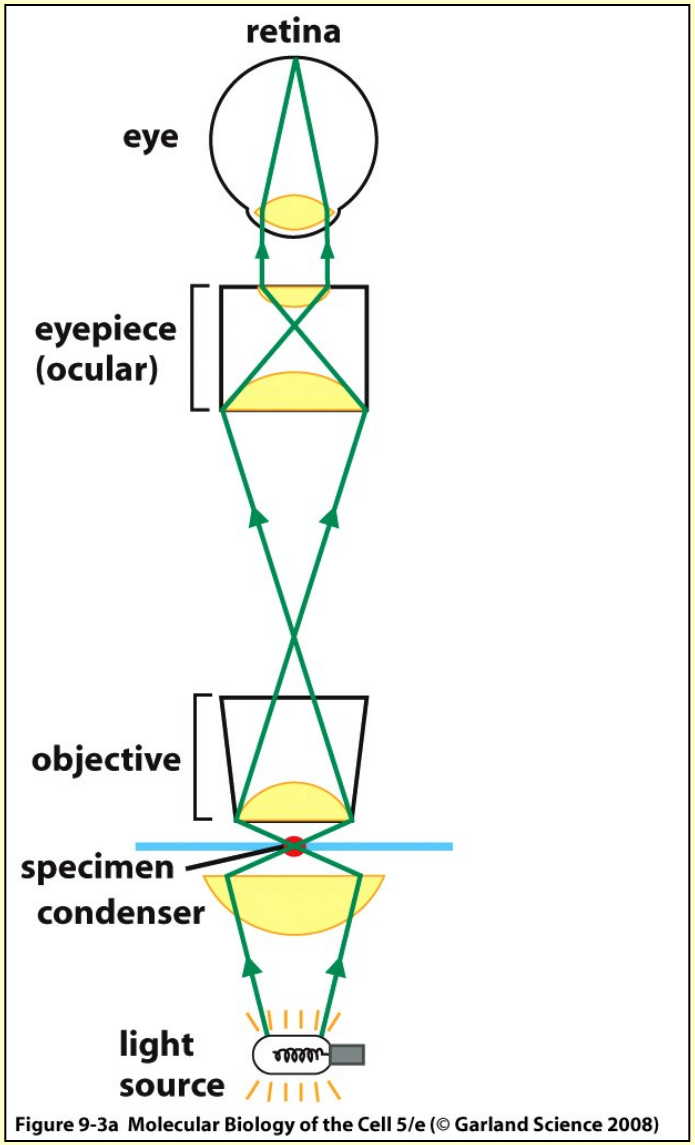
1.1. Light microscopy

1.1.1. Ordinary light microscope



Primo Star, Zeiss

How does the light microscope function?



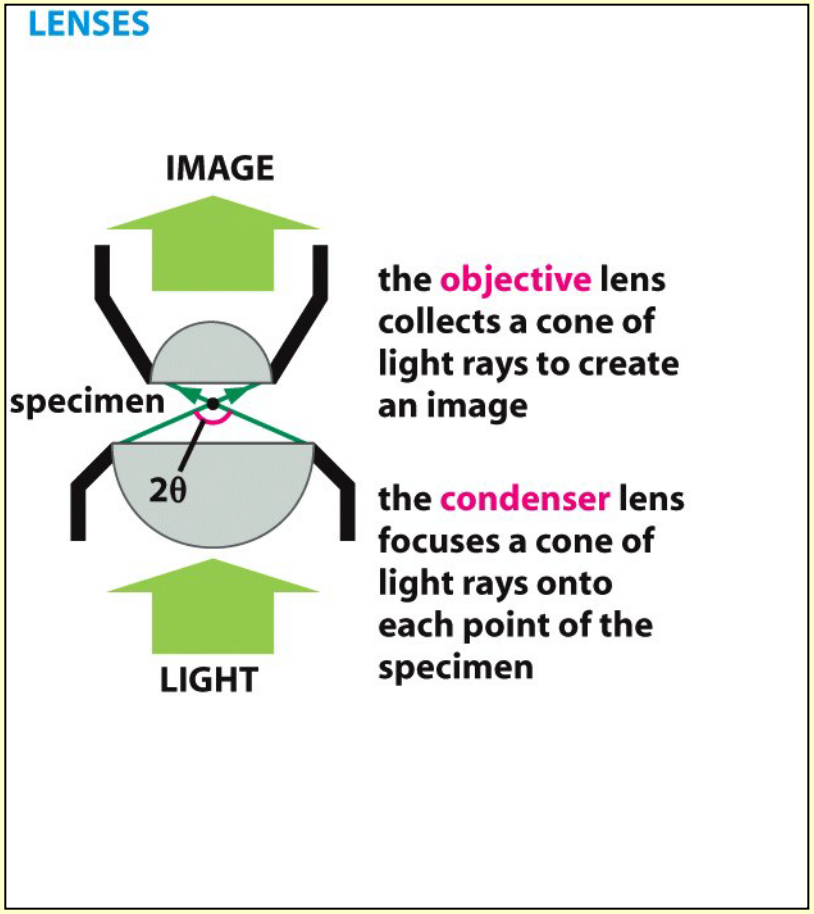


Figure 9-6 *Molecular Biology of the Cell* (© Garland Science 2008)

Modern light microscopes

- magnification up to 1000x

- disadvantage – limit of resolution **(max. resolution 0.2 µm)**

d_{min} =0.2 µm

Figure 9-3b Molecular Biology of the Cell 5/e (© Garland Science 2008)

Microscope resolution

1. What is resolution of the microscope?

Its ability to resolve very tiny structures in the specimen

2. It is dependent on:

(**a**) Wavelength (**λ**) of the visible light (0,4 – 0,7 μm)

(**b**) Ability of the microscope lenses to collect light (**numerical aperture)**

Resolution (d_{min}) = 0.61 λ / NA

λ (white light) = 0,55 μm

NA = numerical aperture

0.61 = correction factor

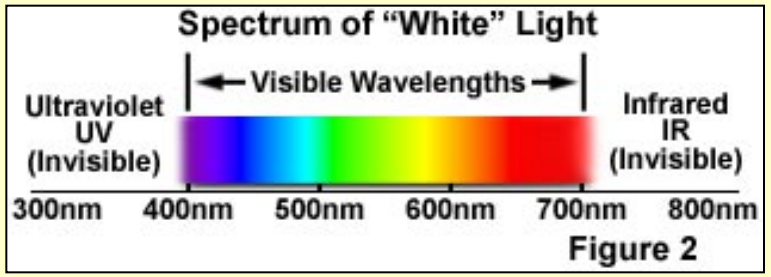
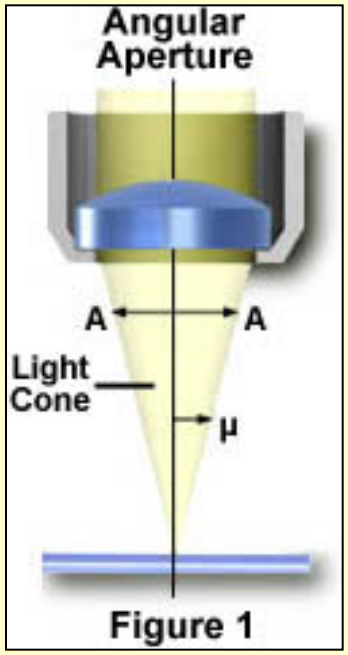


Figure 2

Numerical aperture

→ it is a function of the light-collecting ability of the microscope

$$NA = n \times \sin \mu$$



http://micro.magnet.fsu.edu

n – refractive index of the medium which separates the specimen from the objective and condenser lenses ($\mathbf{n_{air} = 1.0}$)

μ – half the angular width of the cone of the rays collected by the objective lens (max. μ=90°)

• <u>maximal NA</u>:

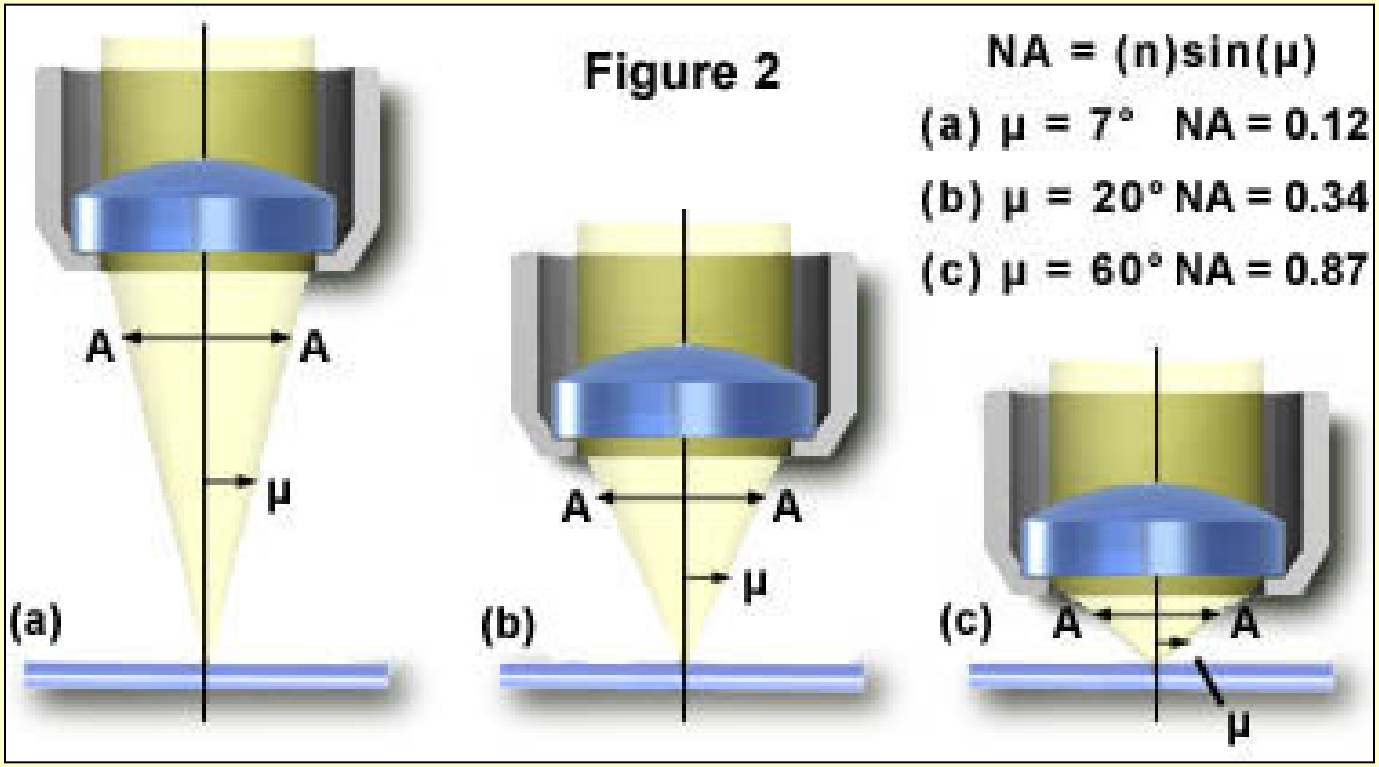
$\mathbf{n_{imersion\ oil} = 1.4}$

sin 90° = 1,0 → $\mathbf{NA_{max} = 1,4 \times 1,0 = 1,4}$

• <u>max. resolution of the light microscope</u>:

$$\mathbf{d_{min} = 0,61 \times 0,55\ \mu m / 1,4 = 0,22\ \mu m}$$

Different objectives – different numerical apertures

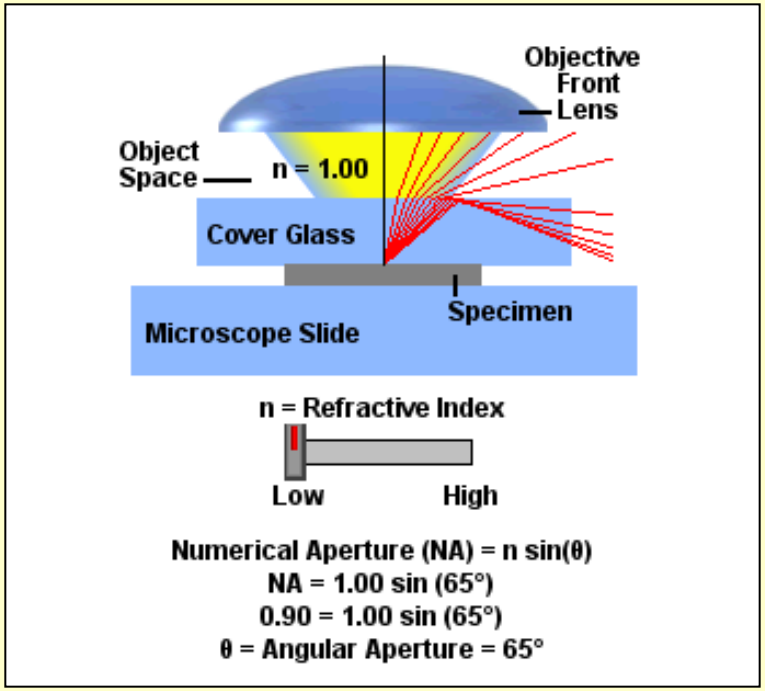


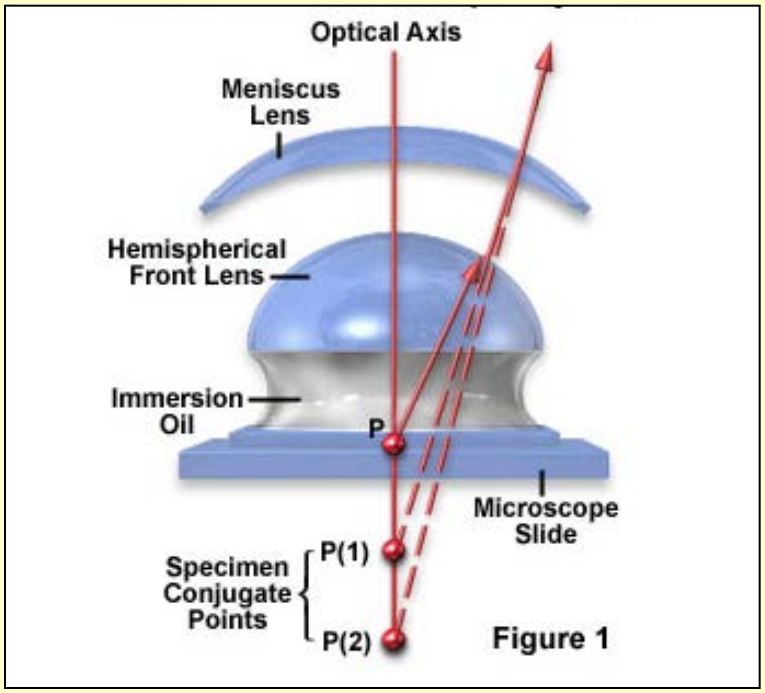
http://micro.magnet.fsu.edu

⇨ the wider the microscope opens its eye, the more sharply it can see!

How to increase the resolution of the light microscope?

- By applying the immersion oil





Dry objectives – air between the specimen and the lens

$n_{glass}(1,4) > n_{air}(1,0)$

➢**greater diffraction**

Immersion objectives – immersion oil between the specimen and the lens

$n_{glass}(1,4) = n_{imersion\ oil}(1,4)$

➢**less diffraction**

Absorption and diffraction of light waves

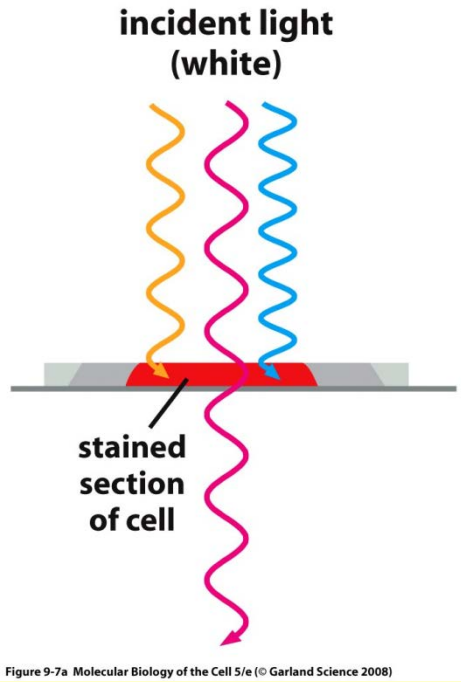


Figure 9-7a Molecular Biology of the Cell 5/e (© Garland Science 2008)

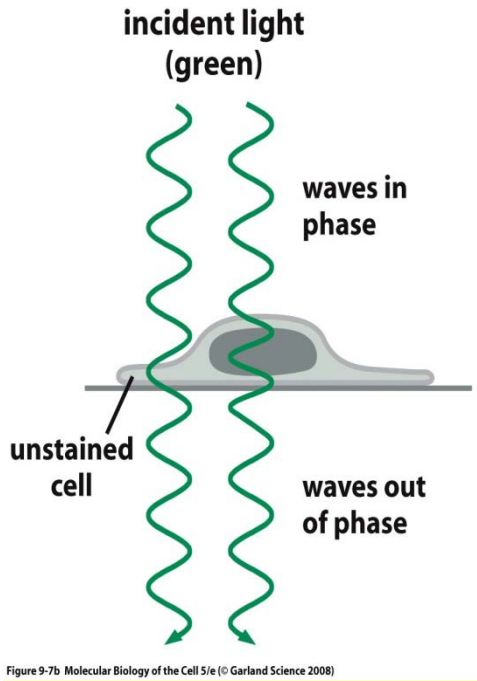


Figure 9-7b Molecular Biology of the Cell 5/e (© Garland Science 2008)

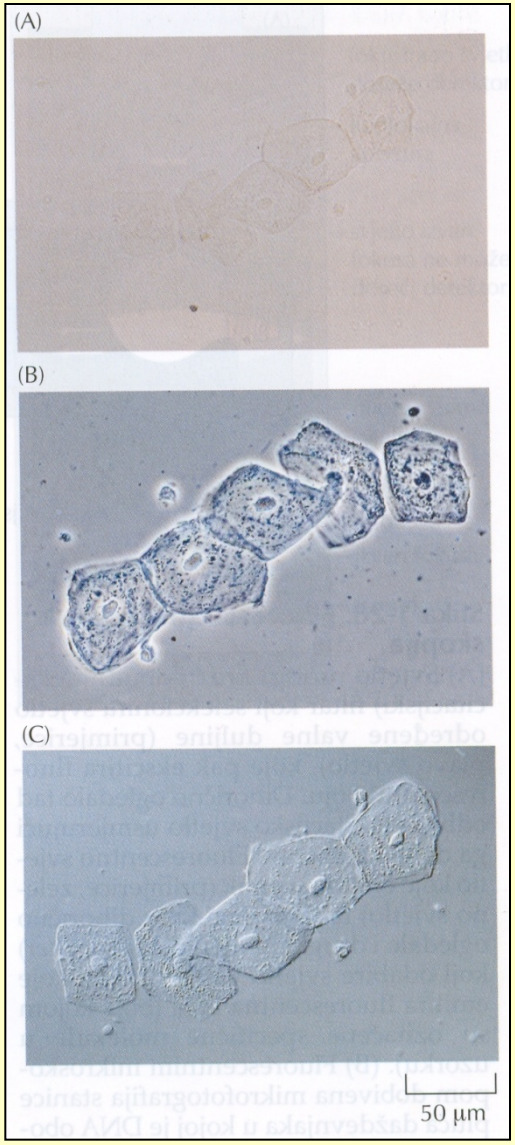
Small details in the specimen are visible because of:

- The color – absorption of the certain wavelengths
- The difference in the refractive index causes the change in phase of light wave - colorless and translucent cells (**difraction and refraction**)

Image - usually the combination!

Phase-Contrast Microscope
Differential-Interference-Contrast Microscope
Dark-Field Microscope

Light passing through thick or dense part of the cell (nucleus) is retarded



Microphotographs of cells obtained by :

A) Regular Light Microscopy

B) Phase-Contrast Microscopy

C) Differential-Interference-Contrast Mmicroscopy

2004 Cooper and Hausmann

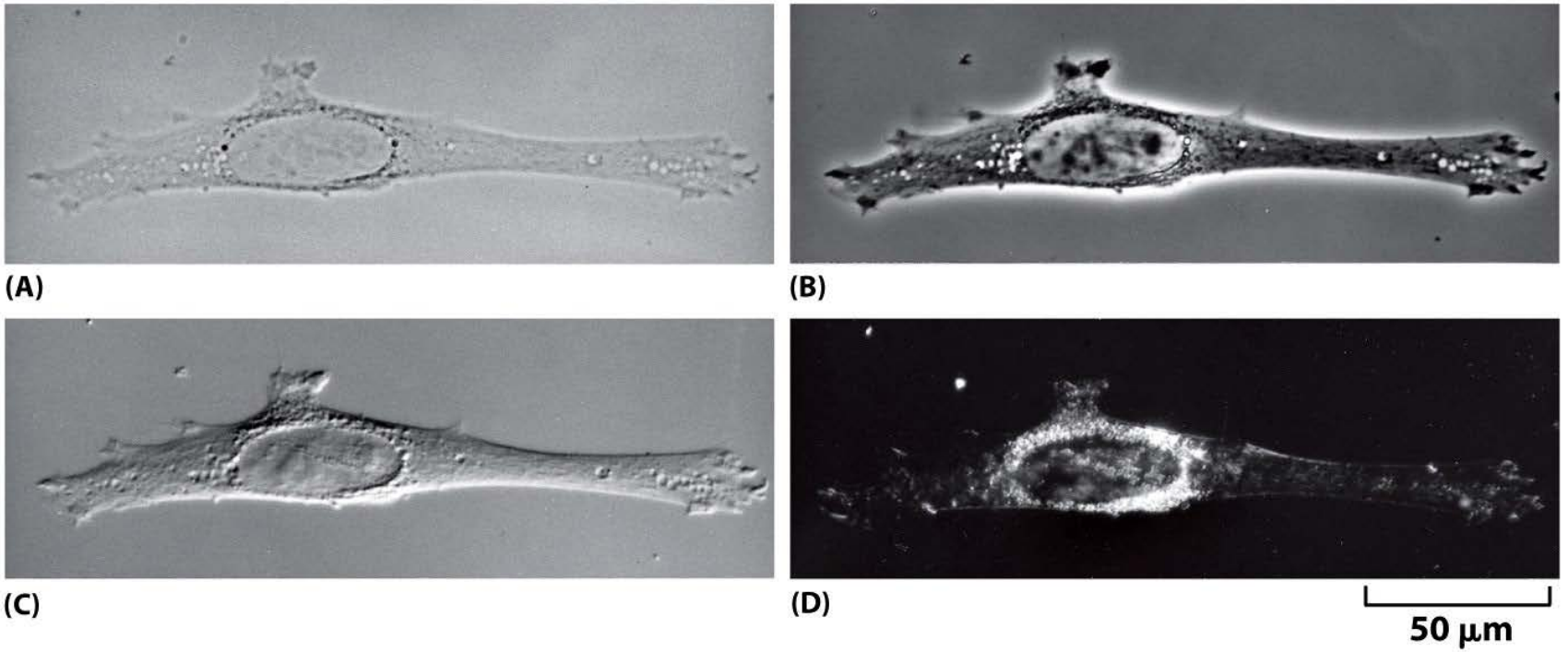


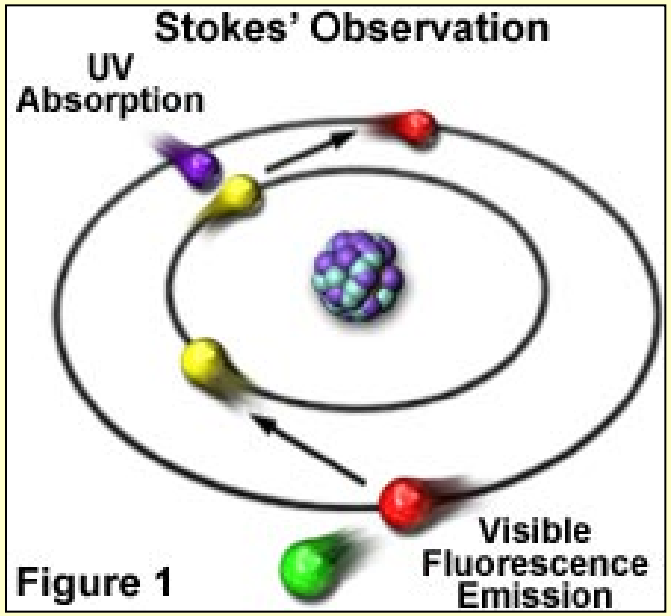
Figure 9-8 Molecular Biology of the Cell 5/e (© Garland Science 2008)

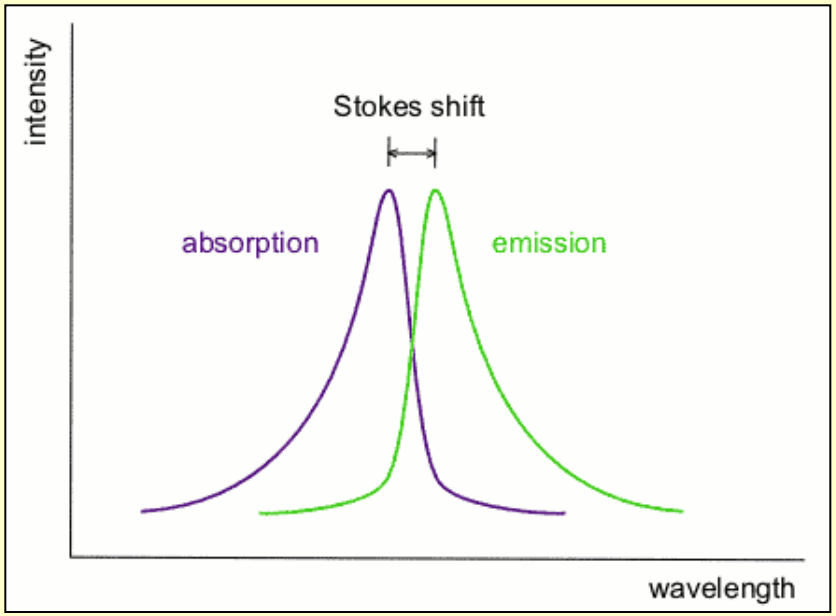
Four types of light microscopy. Four photographs of the same fibroblast cell

(**A**) Regular light microscopy, (**B**) Phase-contrast microscopy , (**C**) Nomarski differential-interference-contrast microscopy, (**D**) Dark-field microscopy.

1.1.3. Fluorescence microscope

- fluorescent dies for labeling certain molecule inside **fixed** or **living** cells
- very sensitive method for analyzing the intracellular distribution of molecules





Sir George G. Stokes - **Stokes shift**.

http://micro.magnet.fsu.edu/primer/lightandcolor/fluorointroduction.html

How does the fluorescence microscope work?

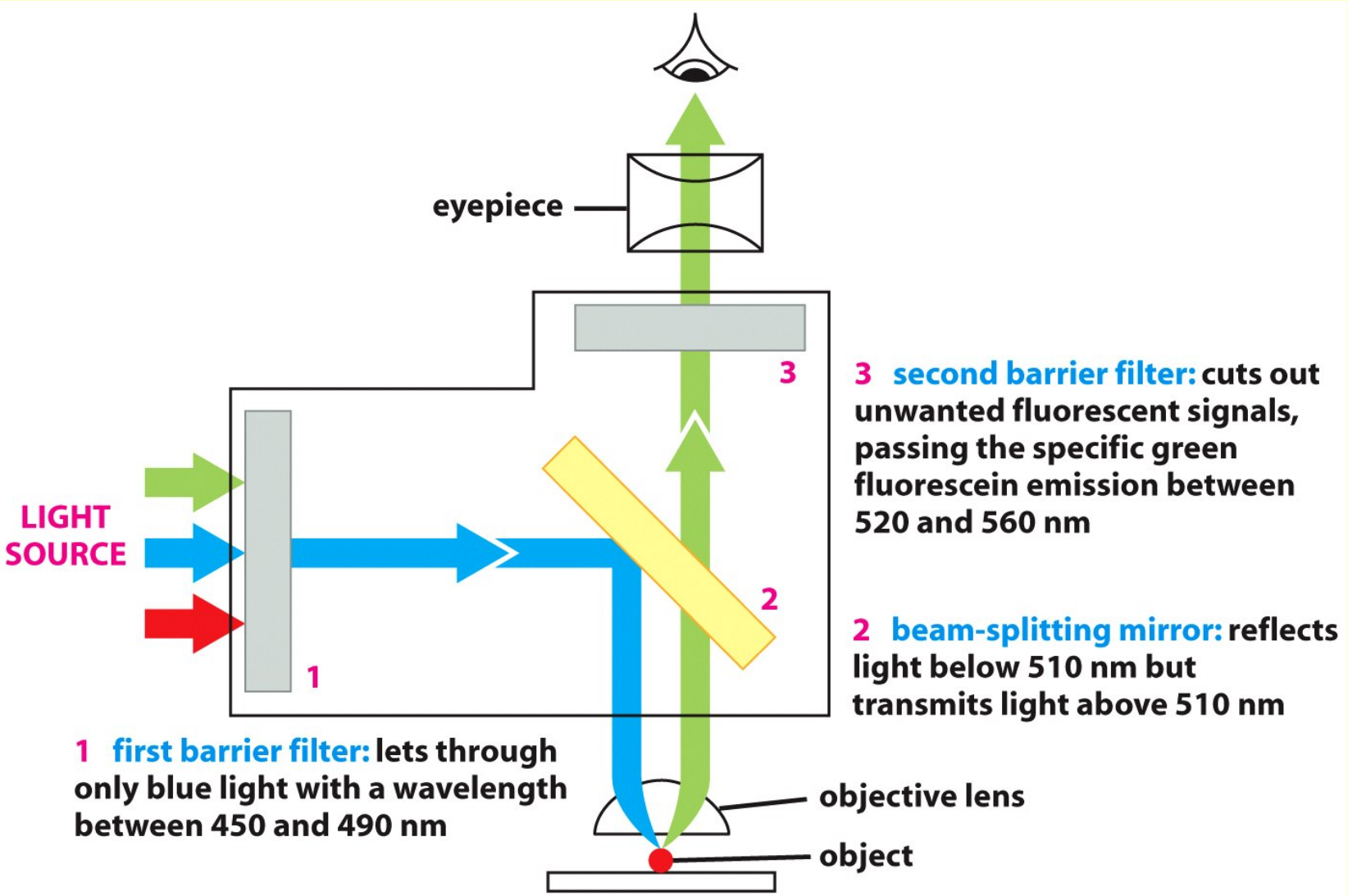
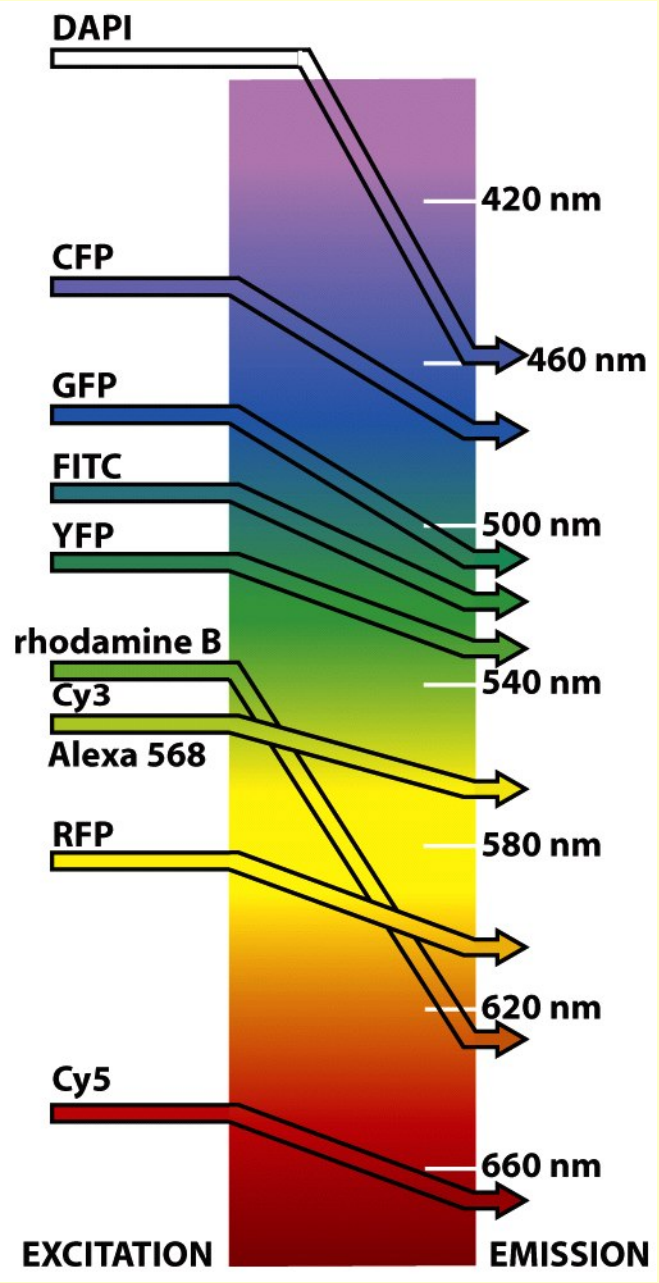
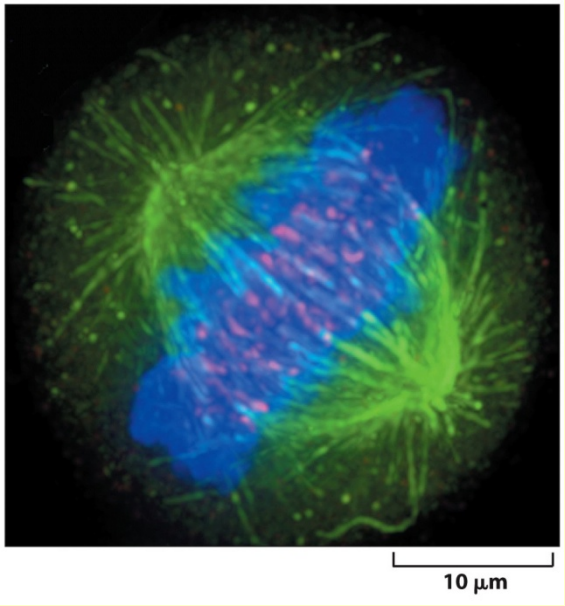
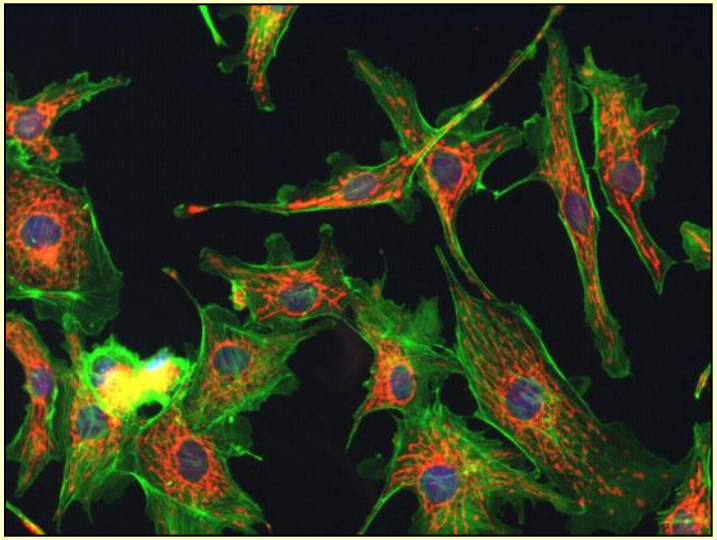


Figure 9-13 Molecular Biology of the Cell 5/e (© Garland Science 2008)

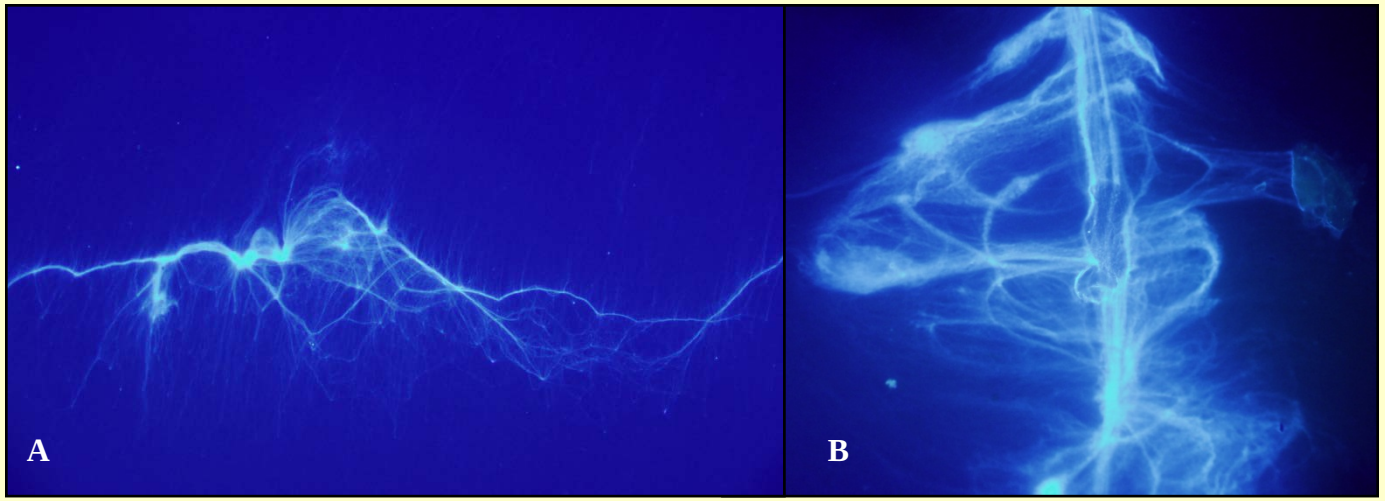






Figures 9-14; 9-15 *Molecular Biology of the Cell* (© Garland Science 2008)

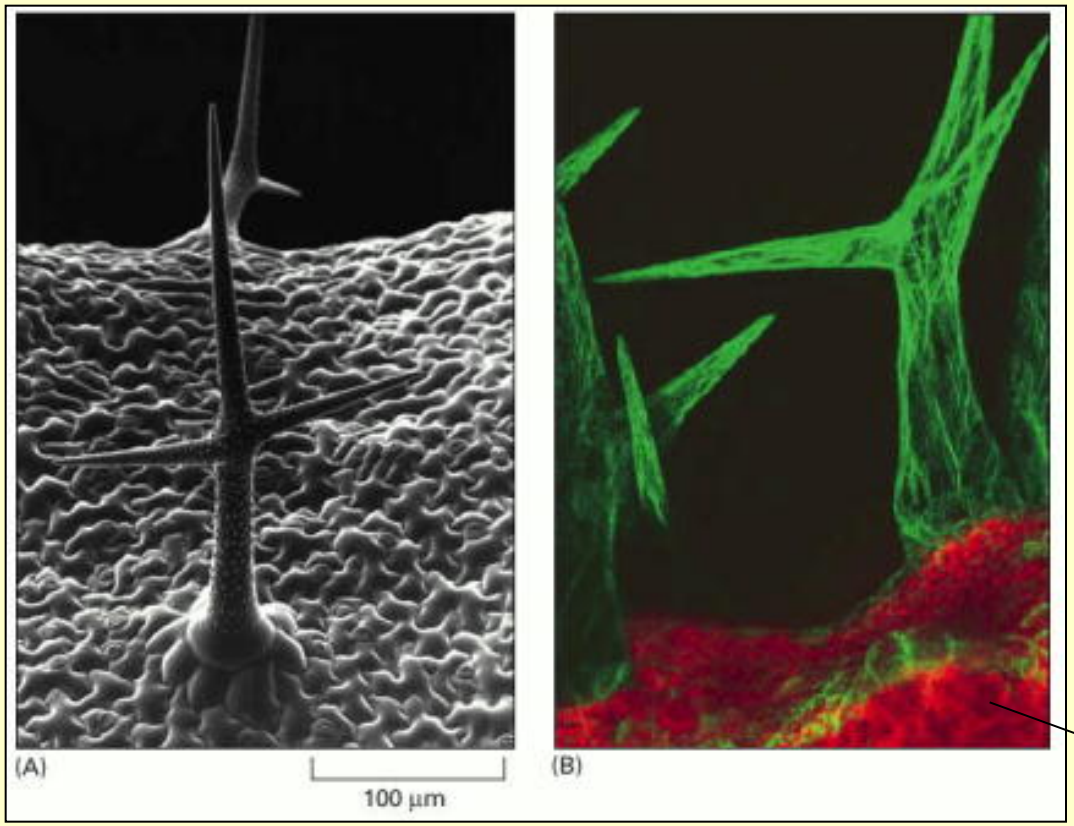
➢**DAPI** → DNA visualisation (Practicum!)



DNA fibers from *Allium cepa* labeled with DAPI: **A** – image magnification 200x, **B** – image magnification 400x.

2006 B. Balen and P. Peharec

➢**GFP**→ protein visualisation



Practicum!

Hairs at the surface of the Arabidopsis leaf

➢green fluorescent protein (GFP) incorporated in cytoskeleton proteins (**green**)

➢chlorophyll natural fluorescence (**red**)

2002 Bruce Alberts, et al.

Fluorescent microscopes at the Division of Molecular Biology

Confocal microscope

✓ fluorescence microscope that uses laser as a light source

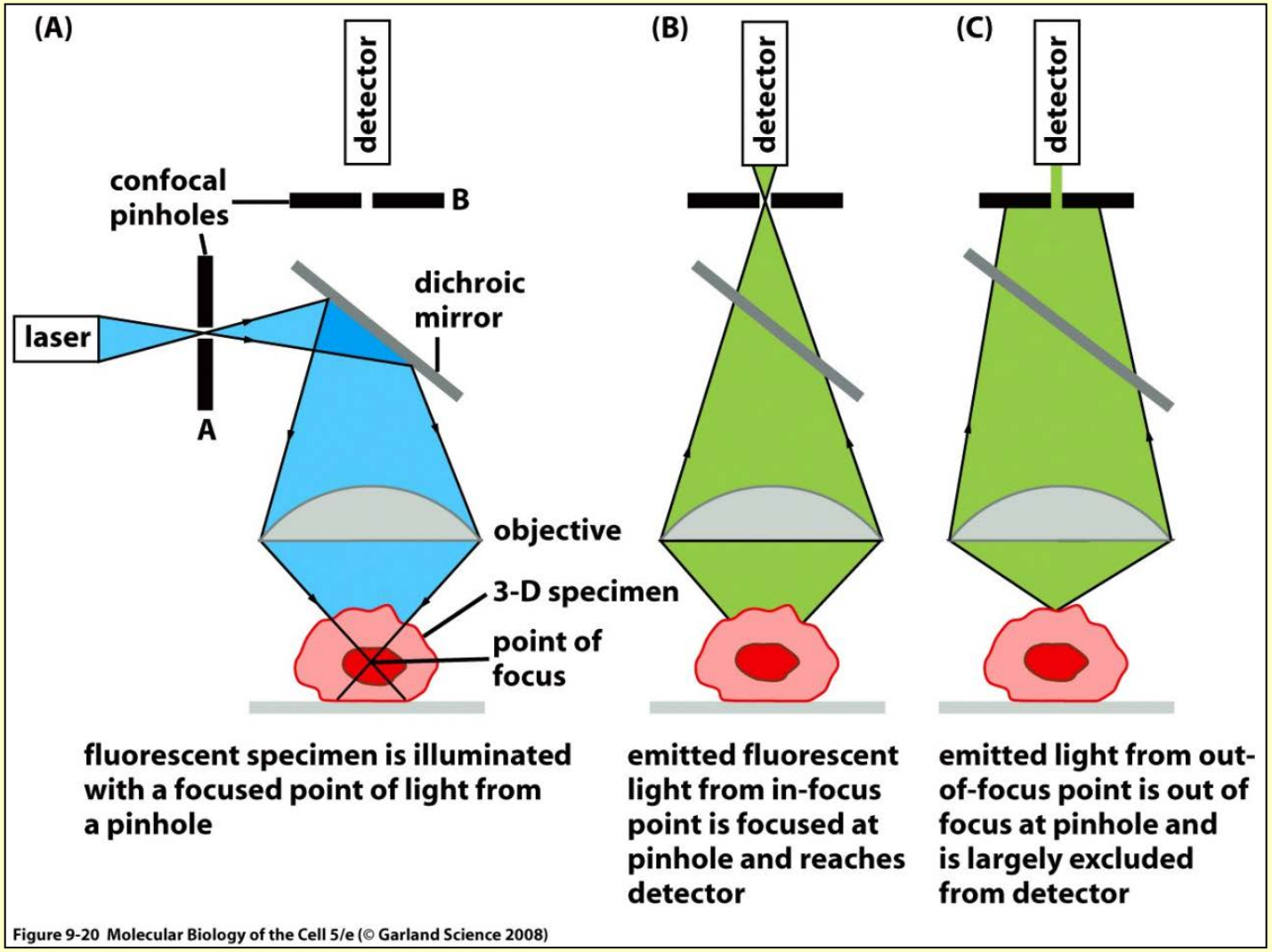


Figure 9-20 Molecular Biology of the Cell 5/e (© Garland Science 2008)

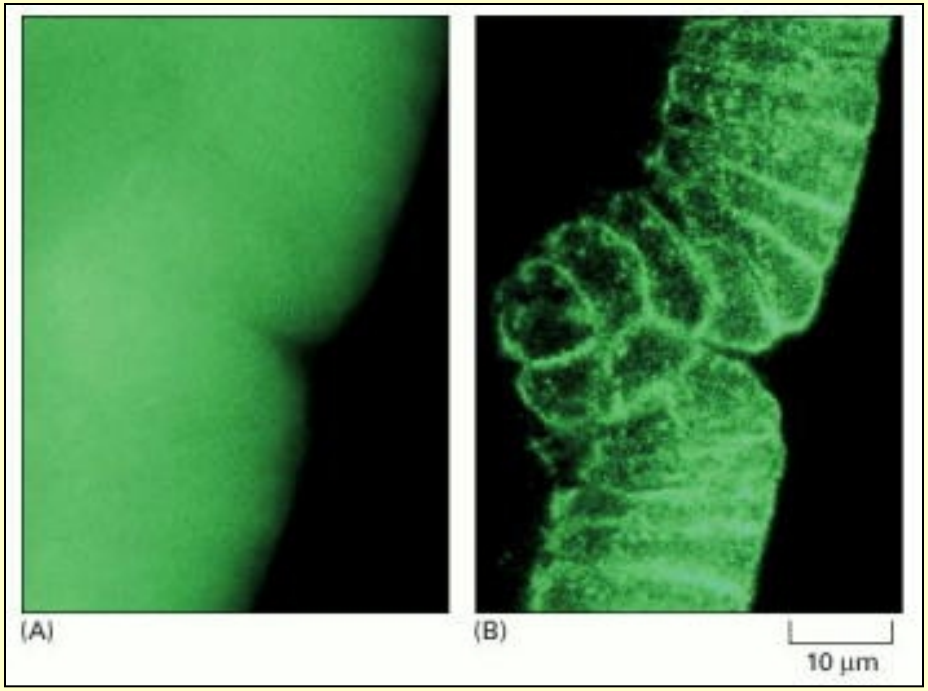


Figure 9-21. Conventional and confocal fluorescence microscope.

Fruit fly embryo in gastrula phase stained with fluorescent dye which binds to actin.

(A) Conventional fluorescence microscopy – blurry image due to light out of focus

(B) Confocal fluorescence microscopy – crispy image – light out of focus is excluded from the detector

Tridimensional reconstruction of the pollen grains by confocal microscope

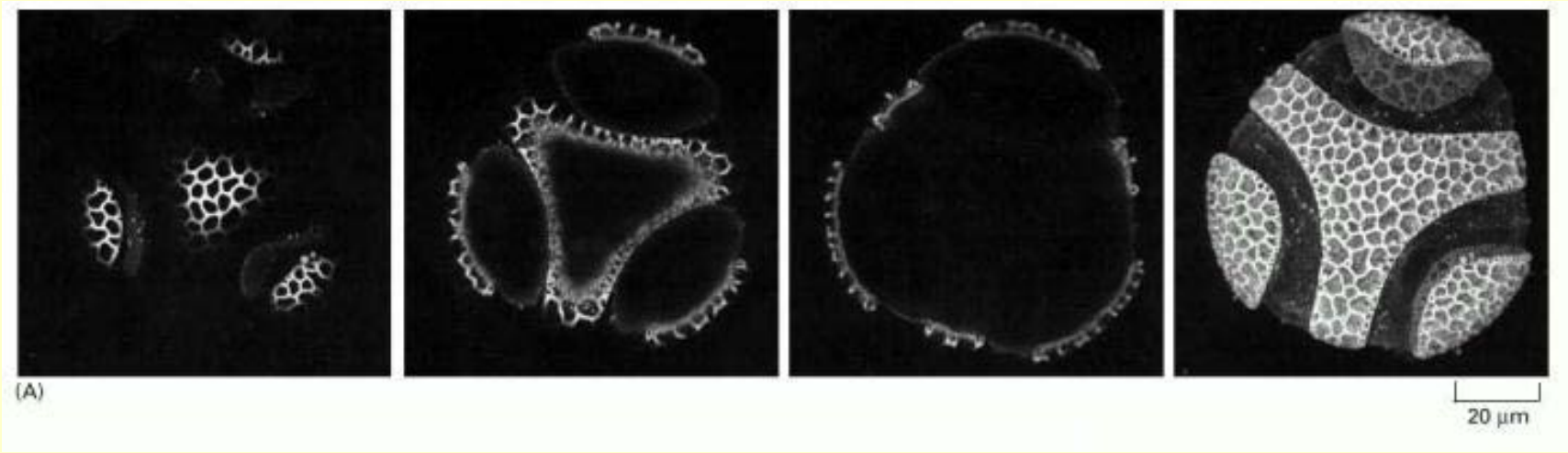


Figure 9-22.

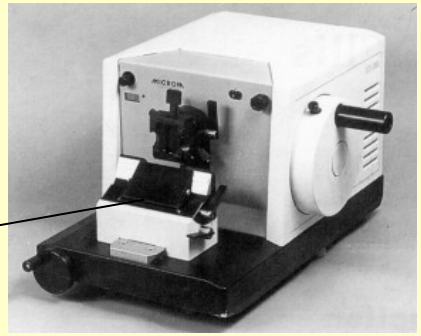
How to prepare the specimen?

1. **Fixing** – formaldehyde and glutaraldehyide (to immobilize, kill and preserve cells)
2. **Embedment** – wax and resin – form solid block (tissue soft and fragile)

Can be replaced by rapid freezing

3. **Cutting** – microtome (sections thickness 1 - 10 μm)
4. **Staining** – toluidin blue; malachite green, sudan black (specific affinity for particular subcellular components)

-20° C



microtome

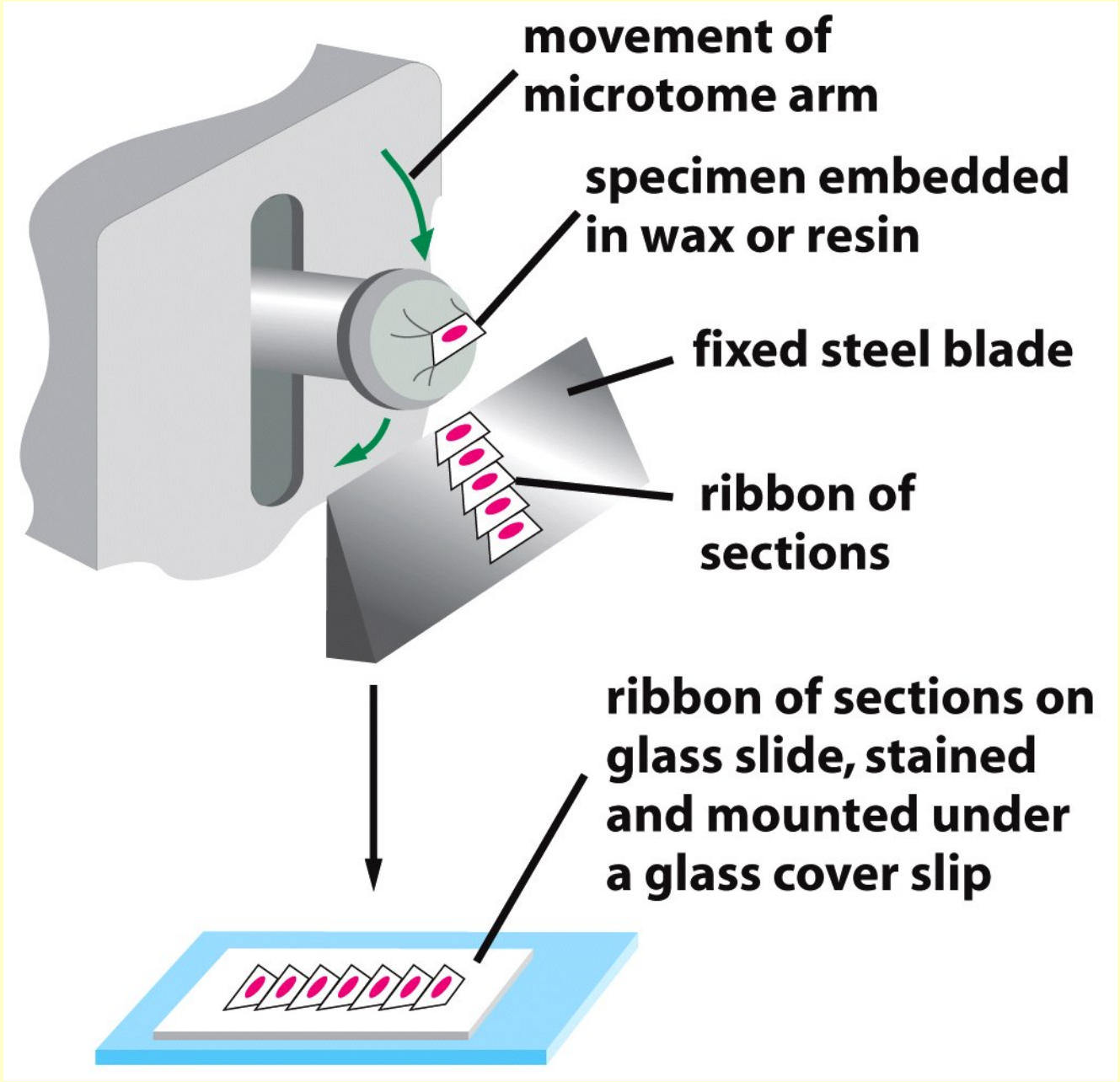
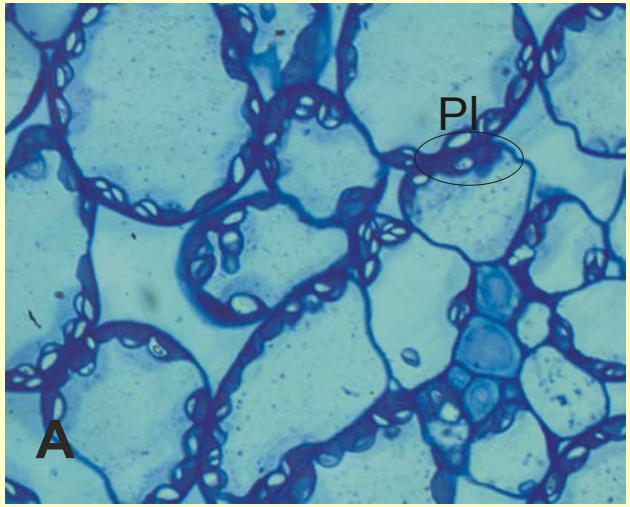
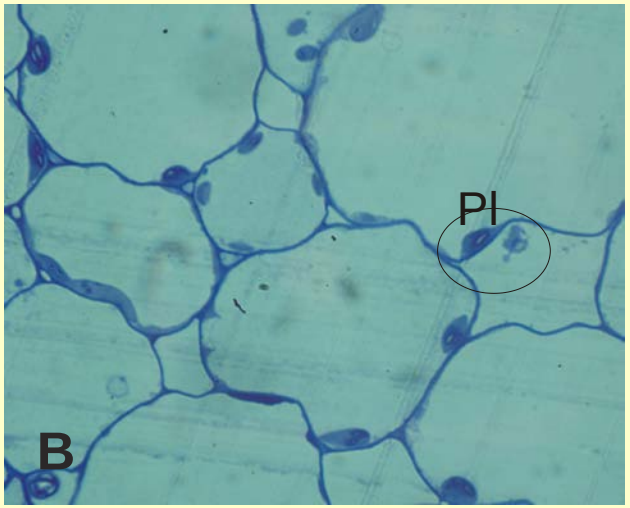
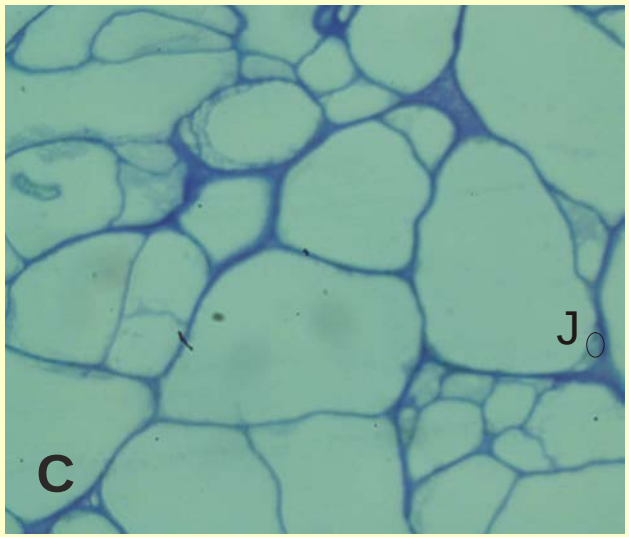


Figure 9-10 *Molecular Biology of the Cell* (© Garland Science 2008)

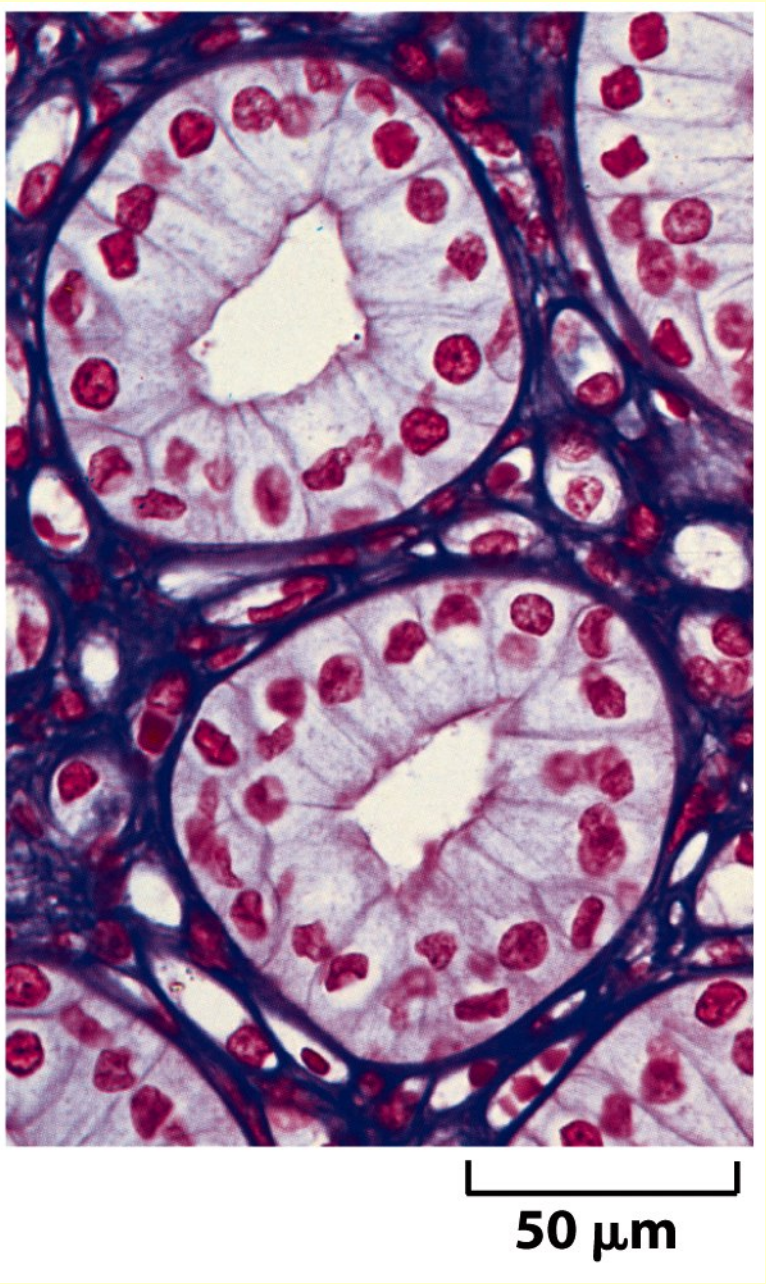






Light microscopy of semi-thin sections. Dye - **toluidin blue**, immersion objective magnification 100:1. A – horseradish leaf, B – teratom, C – tumor.
Pl – plastid, **J** – nucleus

2006 P. Peharec



Section of cells in urine-collecting ducts of kidney.

Dyes - hematoxylin and eosin

Nuclei - **red**
Extracellular matrix - **purple**

Figure 9-11a *Molecular Biology of the Cell* (© Garland Science 2008)

1.2. Electron microscopy

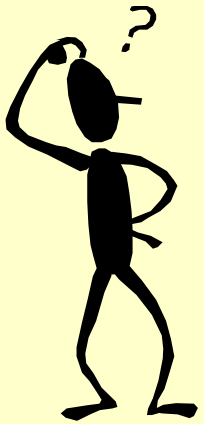
1924. De Broglie - Nobel prize → discovery of the wave nature of fast e^-

$$\lambda = h / m v$$

h – Plank constant

m – mass

v – speed



Problem:
How to increase the resolution of the microscope?

Solution:
Shorter wavelength ⇨ better resolution!!

1932. **Ernst Ruska and Max Knoll** – prototype of the electron microscope

1939. **Siemens** – commercial production of EM

1940 and 1950 **Claude, Porter and Palade** – application of EM in analyses of biological samples

EM resolution

➢**λ (electrons) = 0.004 nm in EM** ⟶ **theoretical d_{min} = 0.002 nm**

(**λ** (white light) = **0.55 μm**)

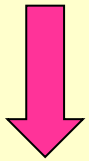
but!

➢**NA (electron lens) = around 0.01** ⟶ **d_{min} = 0.2 nm**

(NA (glass lens) = **1.4**)

but!

➢**The lack of contrast in biological samples!**



The resolution power of EM is 2 nm for biological samples (around 100 x higher than light microscope!!!).

1.2.1. Transmission electron microscope (TEM)

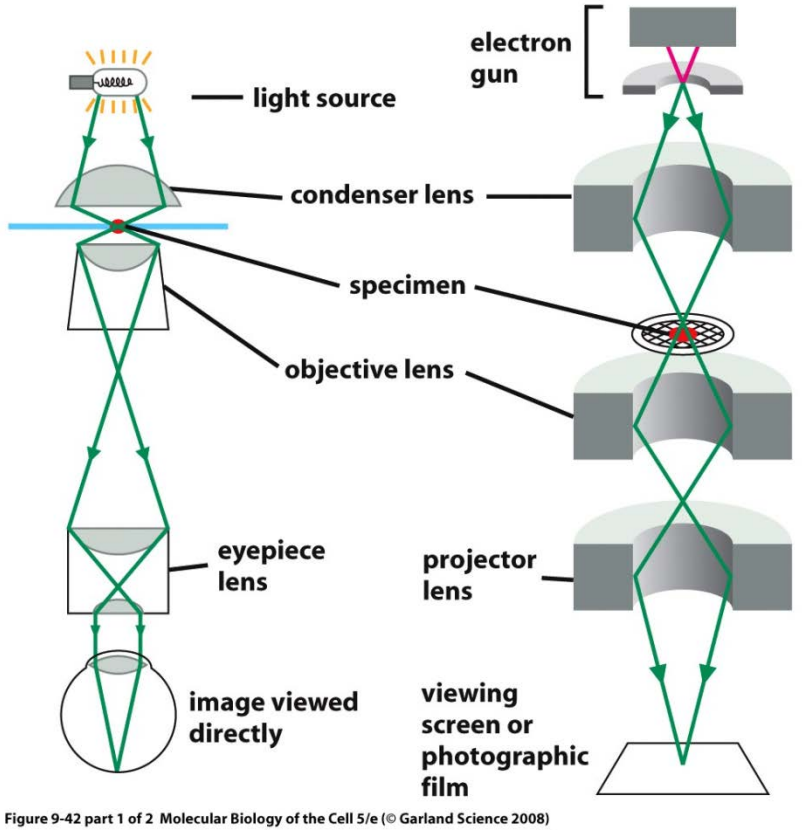


Figure 9-42 part 1 of 2 Molecular Biology of the Cell 5/e (© Garland Science 2008)


Figure 9-42 part 2 of 2 Molecular Biology of the Cell 5/e (© Garland Science 2008)

Light microscope **TEM**

TEM ⇨ similar design with light microscope but larger and upside-down

e^- scattered by collision with air molecules ⇨ air must be pumped out (vacuum!!!)

e^- that have passed through the sample

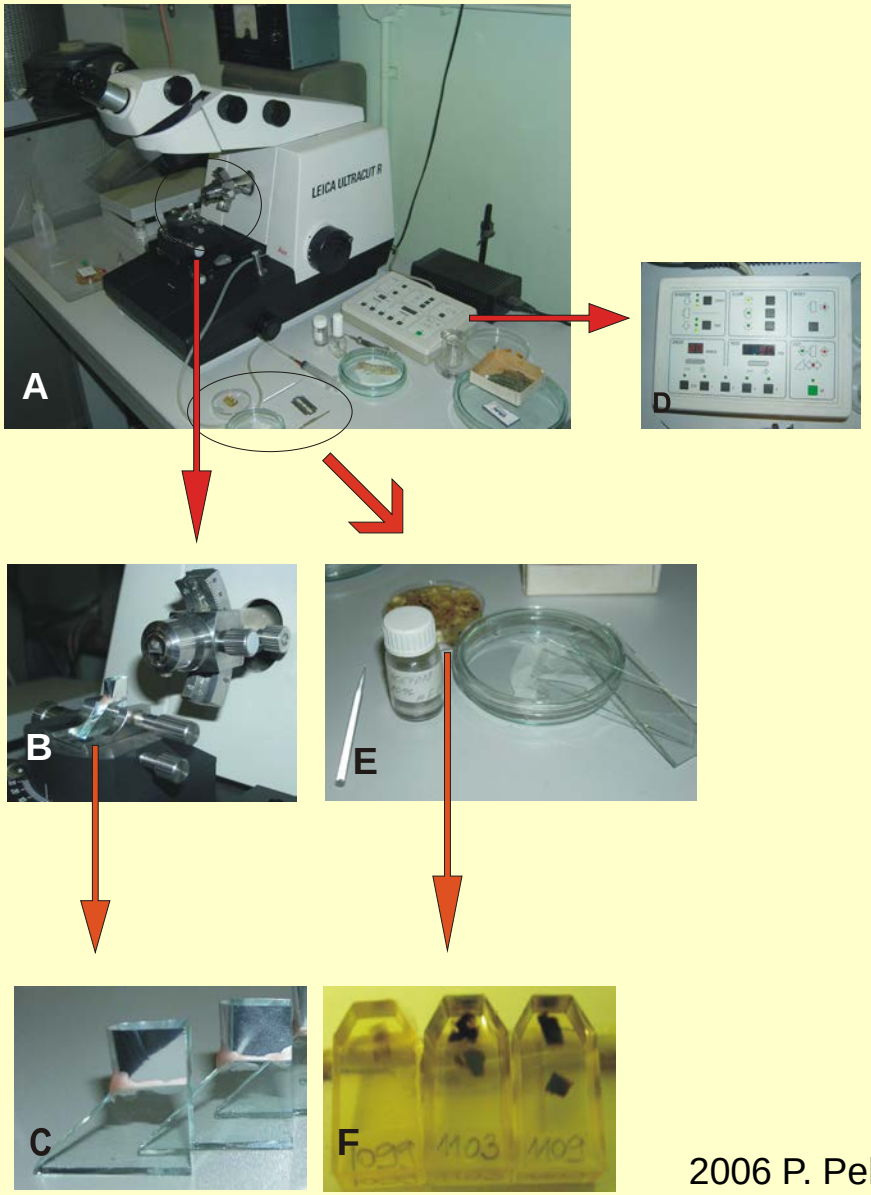
2002 Bruce Alberts, et al.

Preparation of the samples:

⇨ **it is not possible to analyze living cells! (vacuum)**

1. Fixation – glutaraldehyde osmium tetroxide
2. Dehydration – series of ethanol, ethanol and acetone, acetone
3. Embedment – epoxy resin or wax
4. Cutting – ultramicrotome (thickness 50-70 nm)

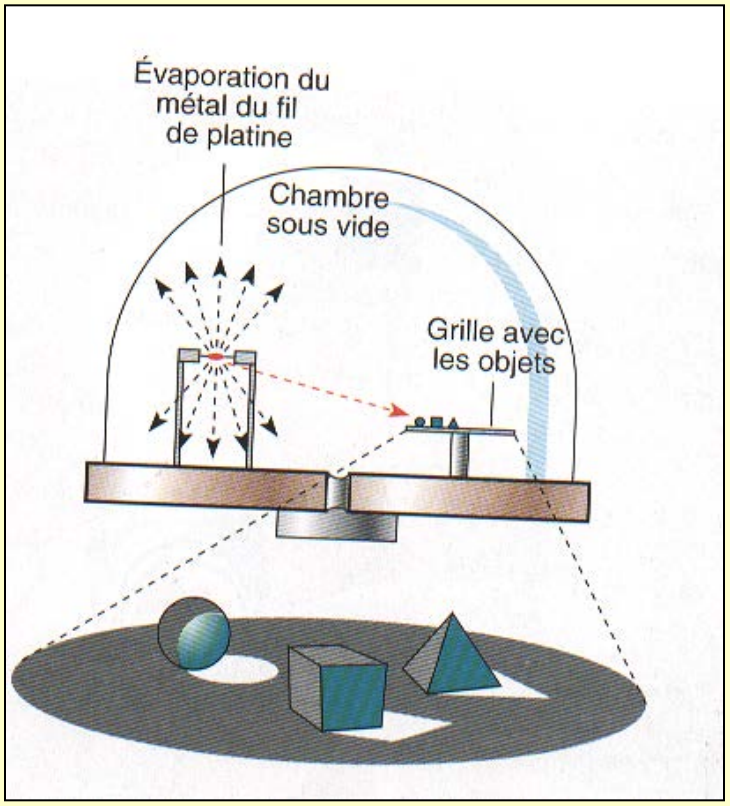
⇨ **e^- have very limited penetrating power**



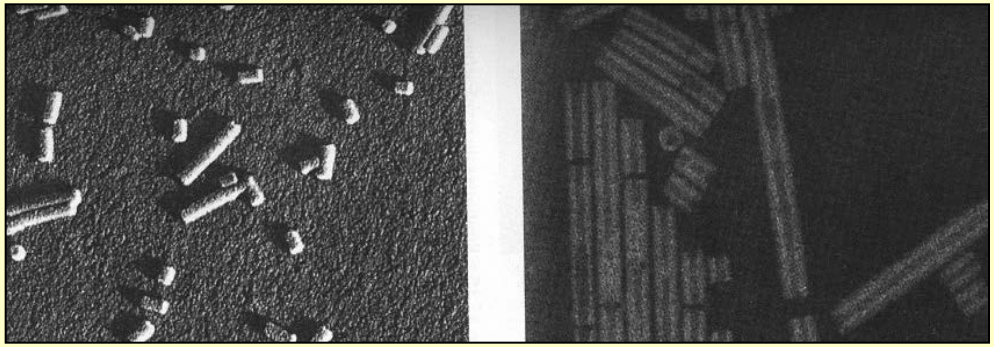
5. Constrating – contrast depends on atomic number!

Biological samples composed of atoms of very low atomic number – C, H, O, N!

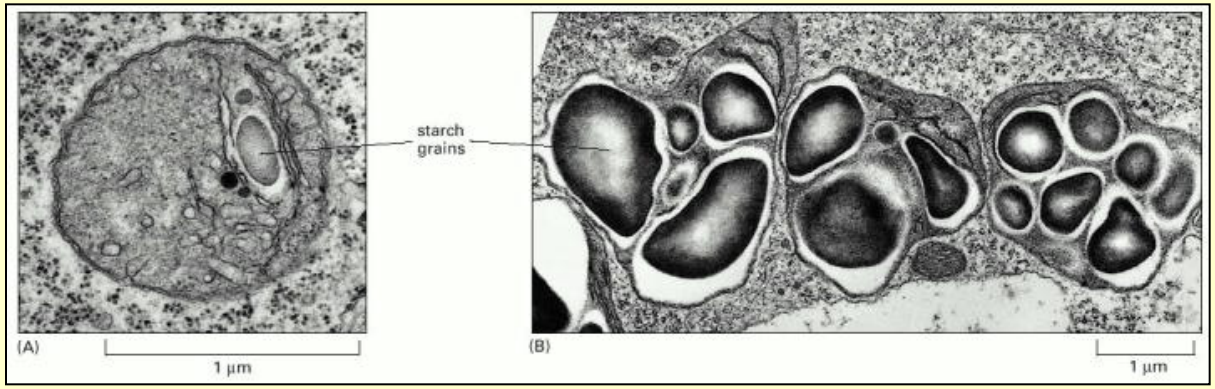
- **Positive contrasting** (OsO_4)
- **Negative contrasting** (phosphor-volfram acid)
- **Metal shadowing** (gold, paladium, platinum)



Platinum shadowing

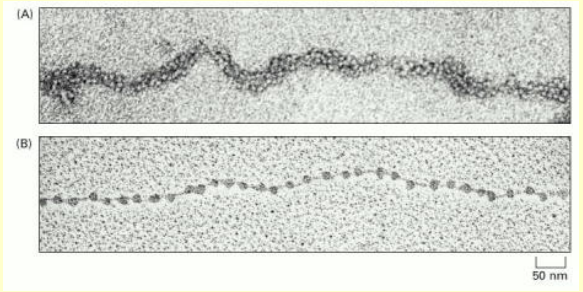


A specimen prepared by **negative contrasting** and **shadowing** (G. Karp pp.736)

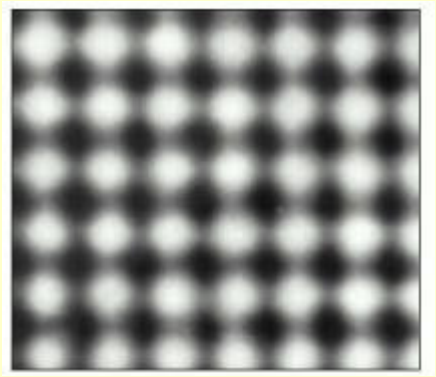


A specimen prepared by positive contrasting and shadowing

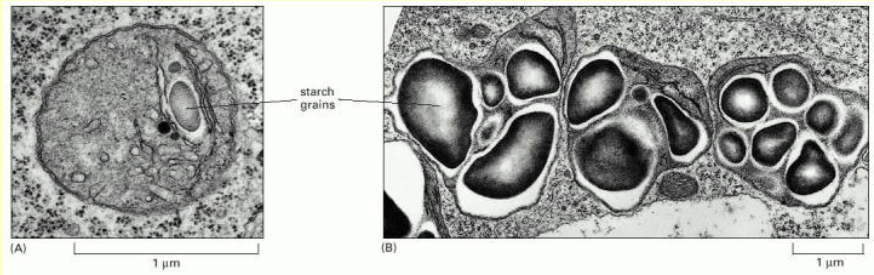
The power of resolution of TEM.



Nucleosome in EM



Thin golden plate – distance between the atoms is 0.2 nm



(A) Proplastid (B) Amiloplast

2002 Bruce Alberts, et al.

1.2.2. Scanning electron microscope (SEM)

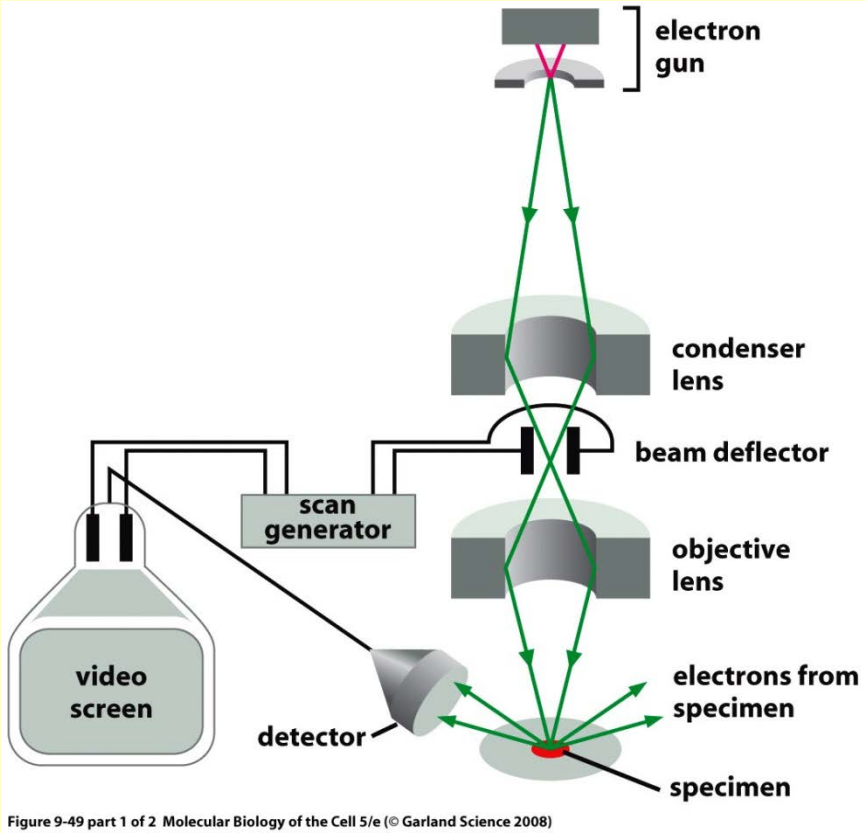


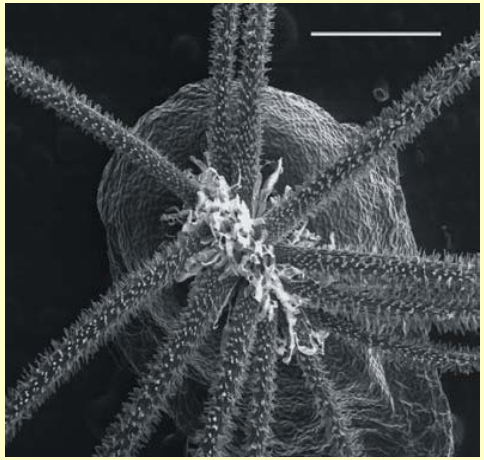
Figure 9-49 part 1 of 2 Molecular Biology of the Cell 5/e (© Garland Science 2008)

- smaller, cheaper and simpler
- el ⁻ which are scattered or emitted from the surface
- resolution – not very high (about 10 nm)
- studying whole cells and tissues

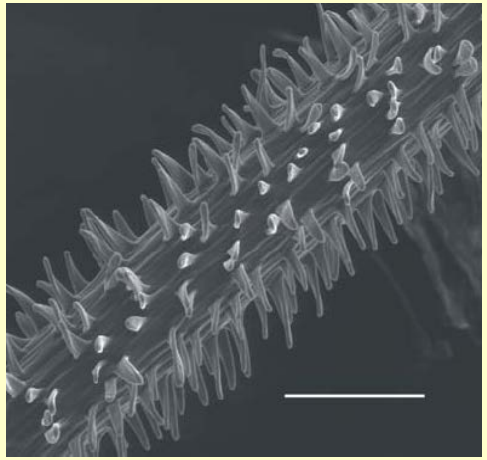
2002 Bruce Alberts, et al.

Cactus spines – magnification glass and SEM (2007. Balen and Peharec)

Cactus shoot with spines

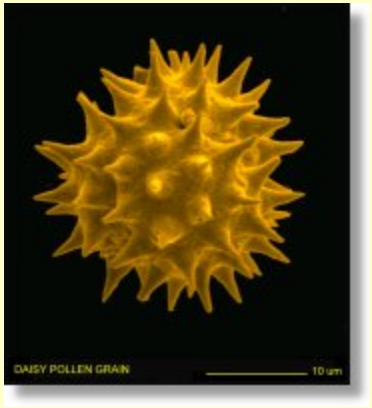


SEM - areole with spines



SEM – a part of the spine

An ant holding a microchip (Science Museum)



pollen grain

2. Cell fractionation and centrifugation

✓ EM allowes a detailed analysis of cellular structures

✓ Cell/organell isolation - in order to analyze their biochemical composition and physiology

✓Organelles have to remain intact and physiologically active!

2.1. Homogenization

✓ Ultrasound – ultrasonic vibration

✓ Mechanical

- mortar and pestle
- mechanical homogenizers and mixers (blenders)

✓ Lysis in hypotonic solution (osmotic shock)

✓ Enzymes

- Celullase and pectinase – plant cell walls
- Lysosyme – bacterial cell walls

1. Mortar and pestle

2. Mixer (Omni-mixer Sorval)

3. Homogenizer (Retsch MM 200)

Things in common:

- ✓ disruption of cell and ER membrane
- ✓ mild, quick and cold

- ✓ Keep the conditions like in the cell:

- **pH** (buffers)
- **isotonicity** (sucrose, sorbitol, mannitol 0,2-0,6 mol/L)
- **ion composition and concentration**
- **antioxidants** (mercaptoethanol, dithiotreithol, serum albumin, ascorbic acid) → oxidative protection
- **stabilization chemicals** (PVP)

2.2. Centrifugation

→ separation of compounds:

2.2.1. Differential centrifugation

2.2.2. Centrifugation in concentration gradient

A) velocity

B) equilibrium

2.2.1. Differential centrifugation

→ sedimentation of compounds at increasing speed in a certain time

→ important:

- **rotation speed** → (*g*)
- **time** (*min* or *h*)
- **temperature** (*°C*)

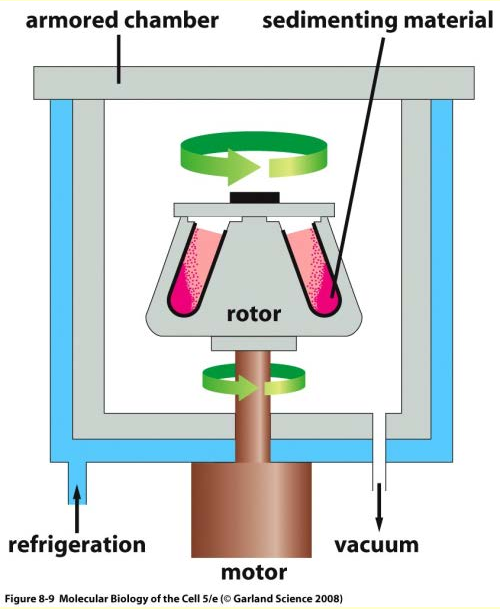
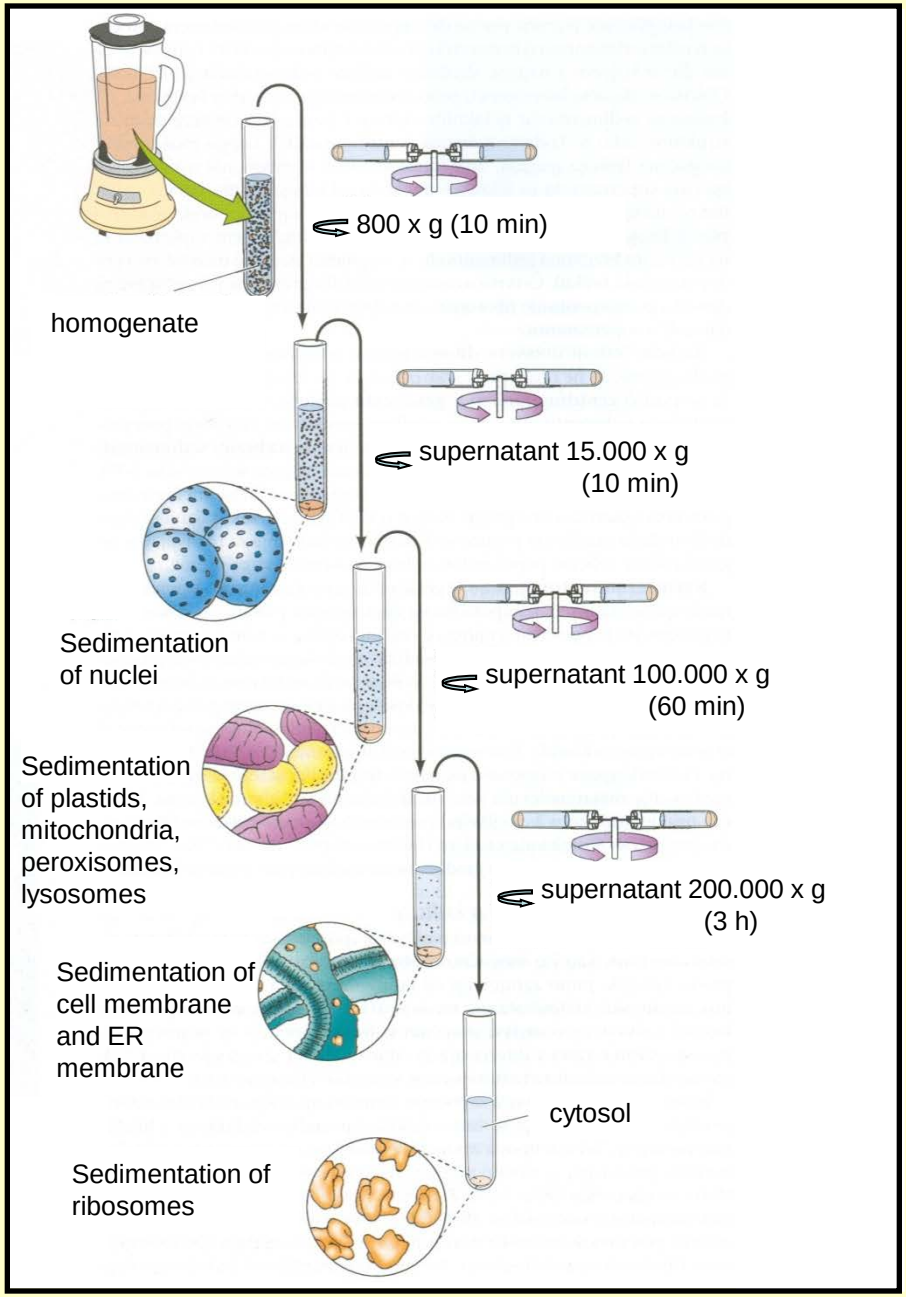
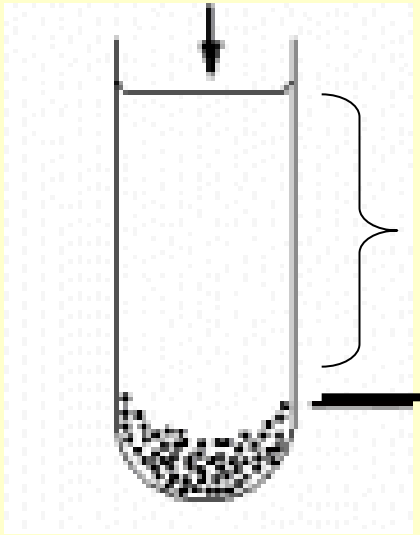


Figure 8-9. Ultracentrifuge

2002 Alberts, et al.





✓Sedimentation coefficient:

– unit **Svedberg (S)**

– $1S = 10^{-13}$ s

2004. Cooper and Hausmann (Fig. 1-38)

2.2.2. Centrifugation in concentration gradient

→ organelles are separated by sedimentation through density gradient

A) Velocity centrifugation

✓ shallow sucrose gradient (5 - 20%)

✓ separation according to size and shape

✓ molecules of the different size sediment by different speed → zones of the molecules of the same size

B) Equilibrium centrifugation

✓ steep sucrose gradient → **linear** or **gradient**

✓ separation according to density

✓ molecules travel through the gradient and stop where their density is equal to the gradient density

❖ very sensitive method→ it is possible to separate macromolecules which have incorporated heavy isotopes ^{13}C or ^{15}N from macromolecules with normal isotopes (^{12}C or ^{14}N)

Centrifugation in concentration gradient

according to size and shape

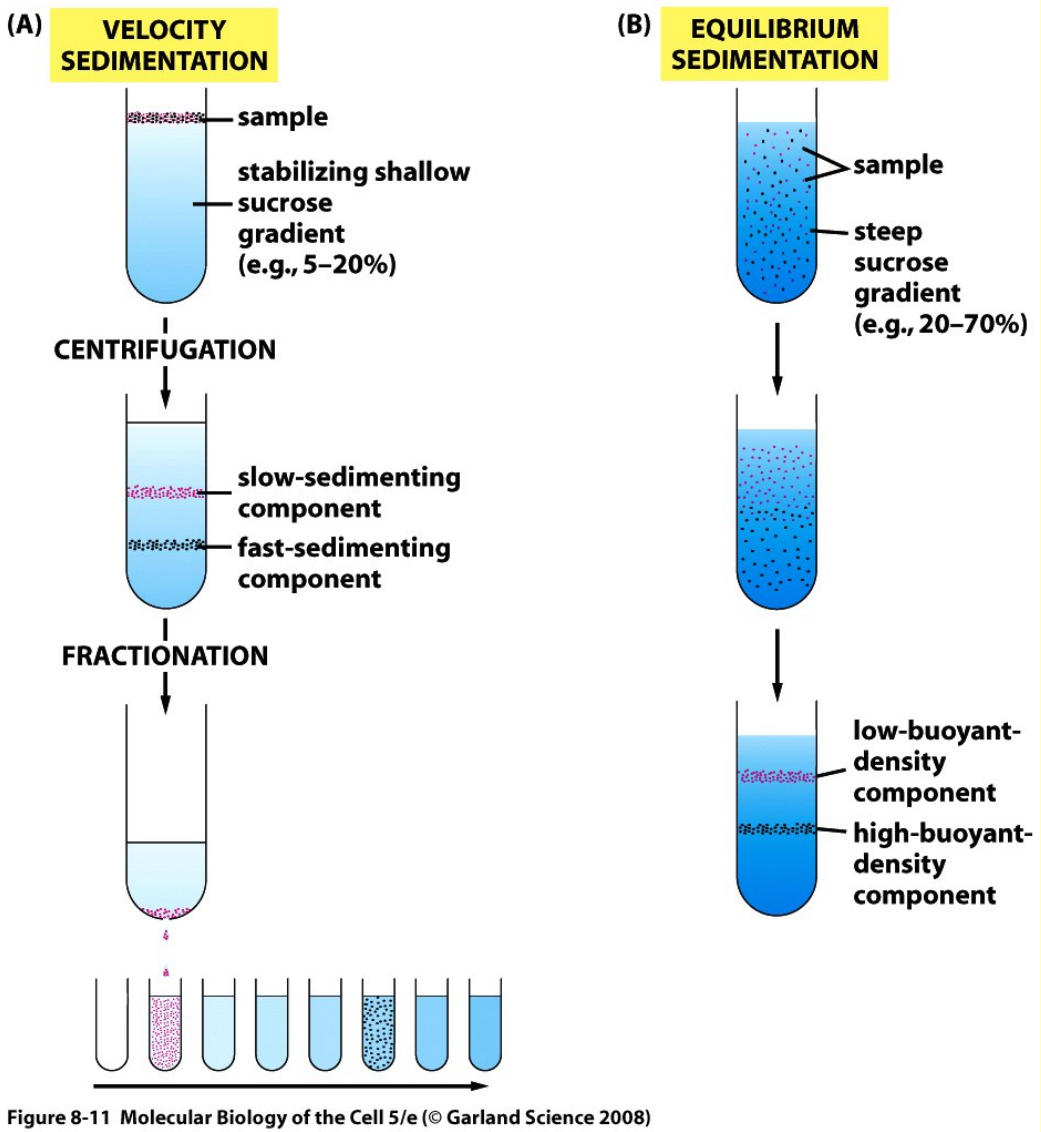


Figure 8-11 Molecular Biology of the Cell 5/e (© Garland Science 2008)

according to density

2008 Alberts, et al. (sl. 8-11)

3. Cell culture

✓great amount of material and large number of cells required for biochemical analyses

✓ sample – tissue part ⇨ heterogeneous cell population

✓ it is necessary to dissociate cells from tissues and to separate different cell types

✓ the aim: homogenous cell population which can be cultured *in vitro*

✓ animal as well as plant cells

3.1. Disruption of extracellular matrix

- ✓ Proteases (trypsin, collagenase) – degradation of extracellular matrix proteins
- ✓ Reagents (EDTA) bind Ca^{2+} - important for cell-cell adhesion

3.2. Separation of different cell types

- ✓ Centrifugation (large from small cells, dense from light ones etc.)
- ✓ Growing them on the plastic or glass surface (different adhesion strength)
- ✓ Antibodies labeled with fluorescent dies → *fluorescence-activated cell sorter*
- ✓ From thin tissues section→ *laser capture microdissection*

Mov2.mpg

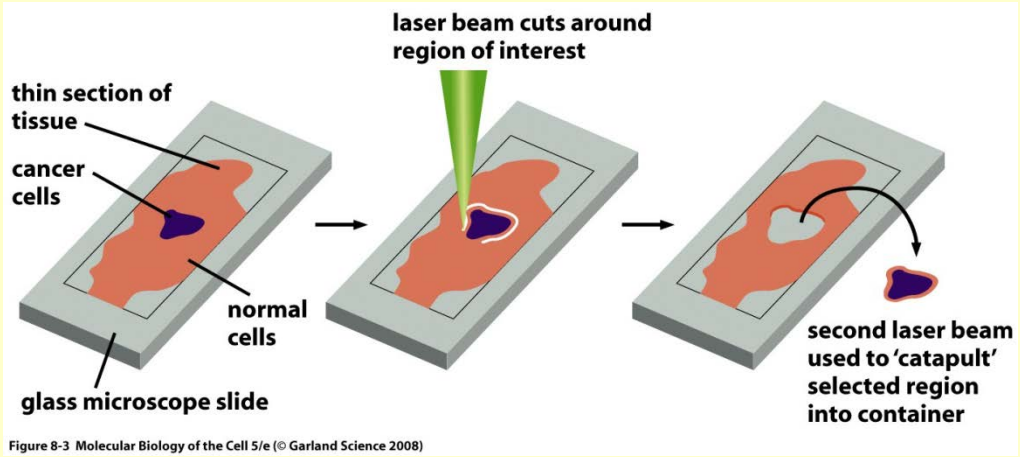


Figure 8-3 Molecular Biology of the Cell 5/e (© Garland Science 2008)

3.3. Cells can be grown in a culture dish

✓ majority of the animal and plant cells can live, multiply and even express differentiated properties in culture cell vessel

✓ different analyses can be performed:

• microscopy

• biochemical analyses

• monitoring of influence of different compounds (hormones, growth regulators, biotic or abiotic stressors)

• mixing of two different cell types

✓ experiments on cell in culture→ "*in vitro*"

✓ experiments on living organisms → "*in vivo*"

3.3.1. Animal cell culture

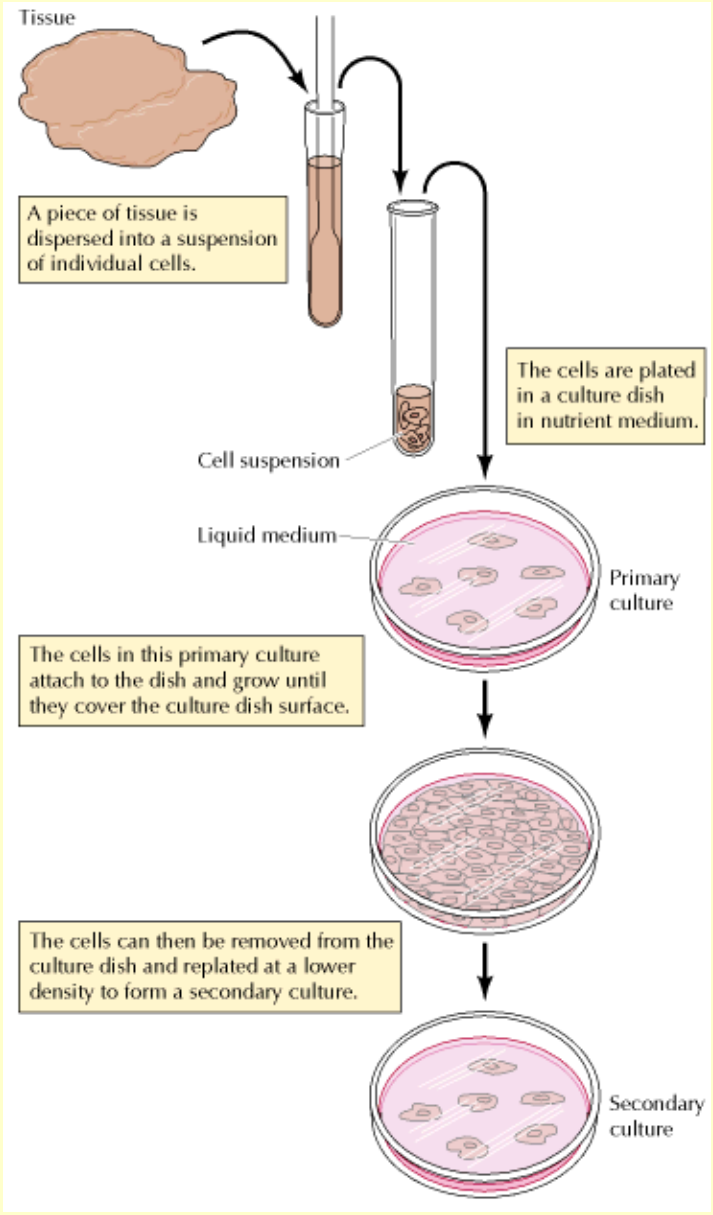
✓ First culture of animal cells - 1907. Harrison

Controversy in neurobiology:

Is nerve fiber an outgrowth of a single nerve cell or a product of the fusion of many cells?

✓ answer → one cell

✓ established foundation for cell culture development



2000 Geoffrey M. Cooper

Requirements for cell culture

✓ 1955. Harry Eagle → described the first medium for animal cell culture

✓ Requirements:

- growth vessel (Petri dish) - most cells are not adapted to live in suspension
- ingredients of extracellular matrix (collagen, laminin)
- nutrient medium (salts, glucose, vitamins, growth regulators, amino acids)

✓ Subcultivation every few weeks

✓ Maintain the characteristics of differentiated cells :

- fibroblasts→ secrete collagen
- neural cells → form axons and make synapses
- embryonic muscle cells → produce muscle fibers; spontaneous contractions

Cell culture types

Animal cell cultures can be divided in two groups:

✓ Embryonic and tumor cells ⇨ immortal cell lines

✓ Differentiated cells ⇨ restricted time of division in culture

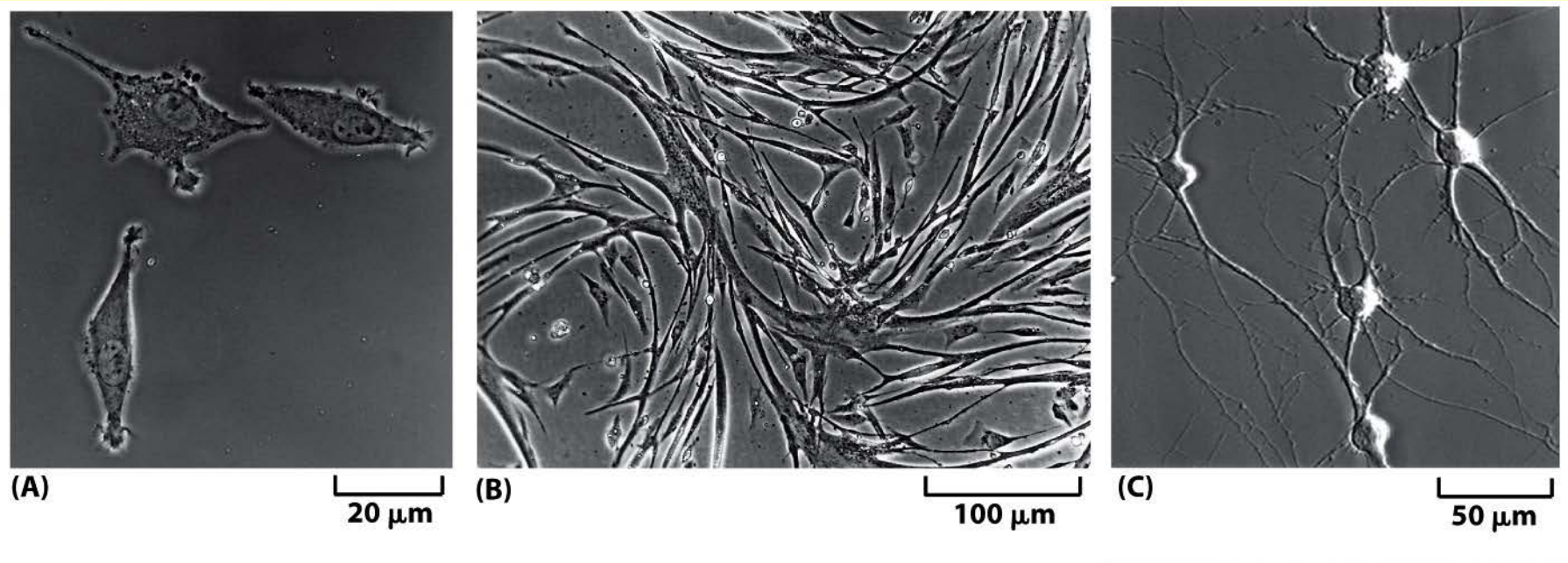
→ limited proliferation capacity

→ fibroblasts 25-40 divisions before they stop

→ reason: progressive shortening of **telomeres** due to turning off the **telomerase** (enzyme that maintains telomeres)

→ it is possible to incorporate the gen encoding the telomerase catalytical subunit – immortalized cell line

Animal cells in culture

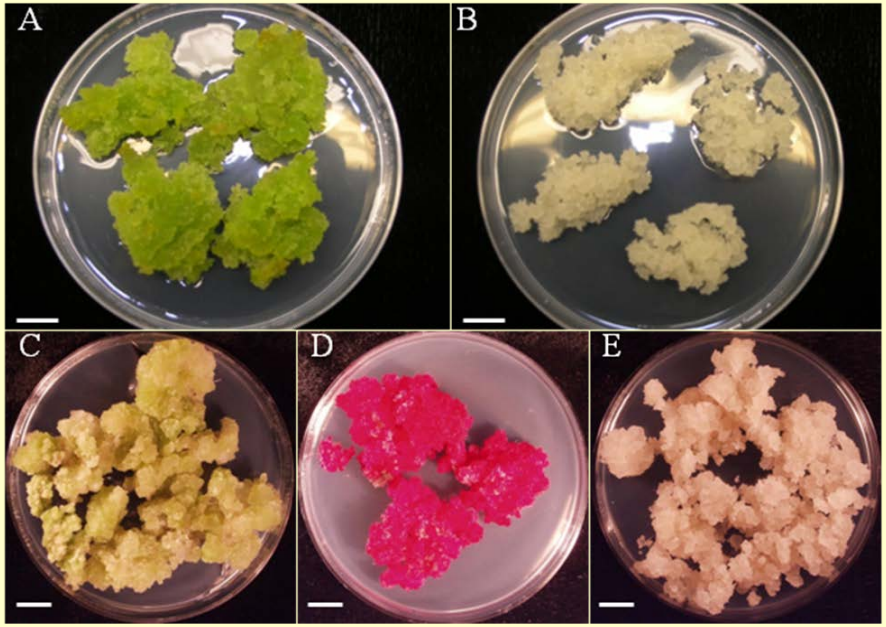


A – phase-contrast micrograph of fibroblasts in culture
B – myoblasts in culture – cells fusing to form multinucleate muscle cell
C – oligodendrocyte precursor cells

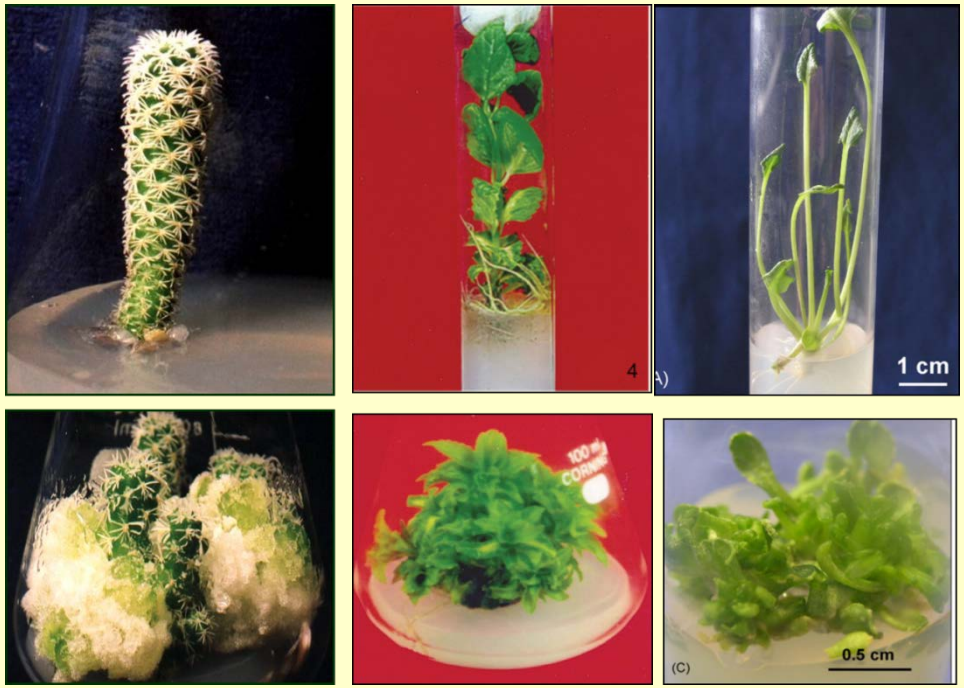
Figure 8-4 *Molecular Biology of the Cell* (© Garland Science 2008)

3.3.2. Plant cell culture

- ✓ unlimited cell growth on appropriate nutrient medium
- ✓ regeneration, cloning
- ✓ plant cell transformation
- ✓ production of genetically altered plants
- ✓ synthesis of secondary metabolites



Sugar beet cell lines. A) normal callus N; B) habituated line HNO; C) tumor line Tz; D) tumor line Tc; E) tumor line Tb. (Prepared by D. Pavoković). White line = 1cm.



Mammillaria gracilis *Coleus blumei* *Armoracia lapathifolia*

Production of genetically altered plants

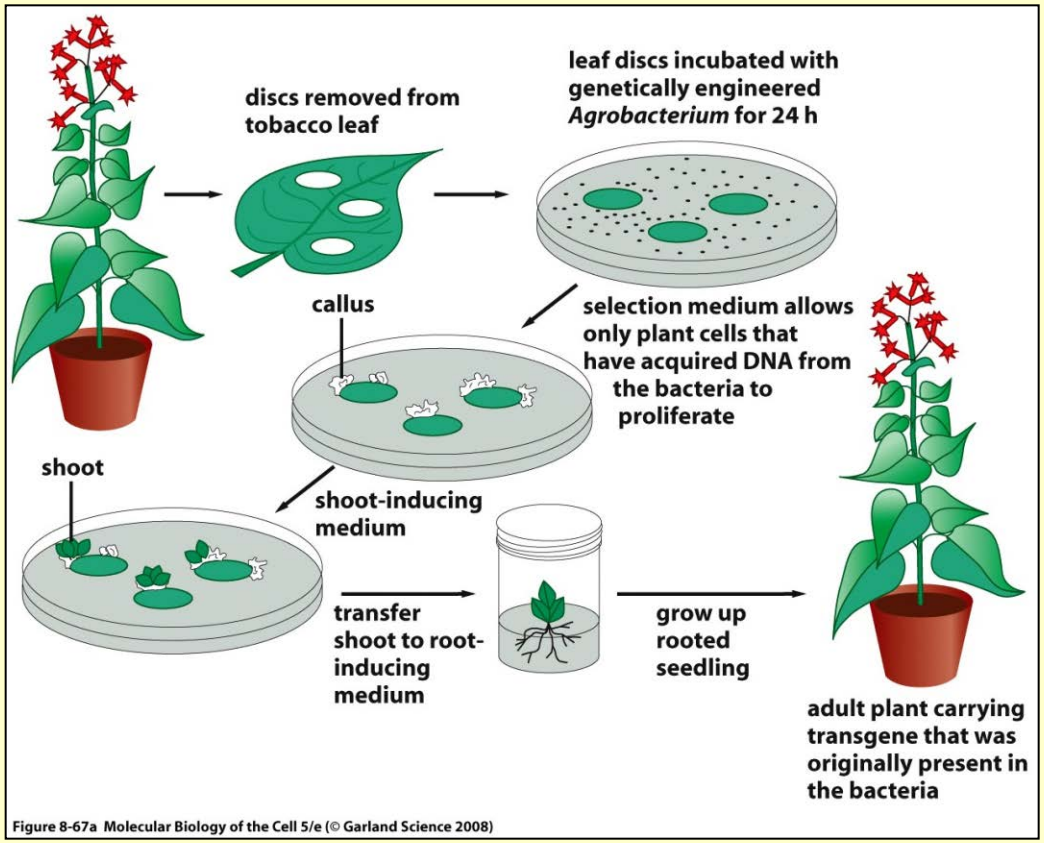


Figure 8-67a Molecular Biology of the Cell 5/e (© Garland Science 2008)

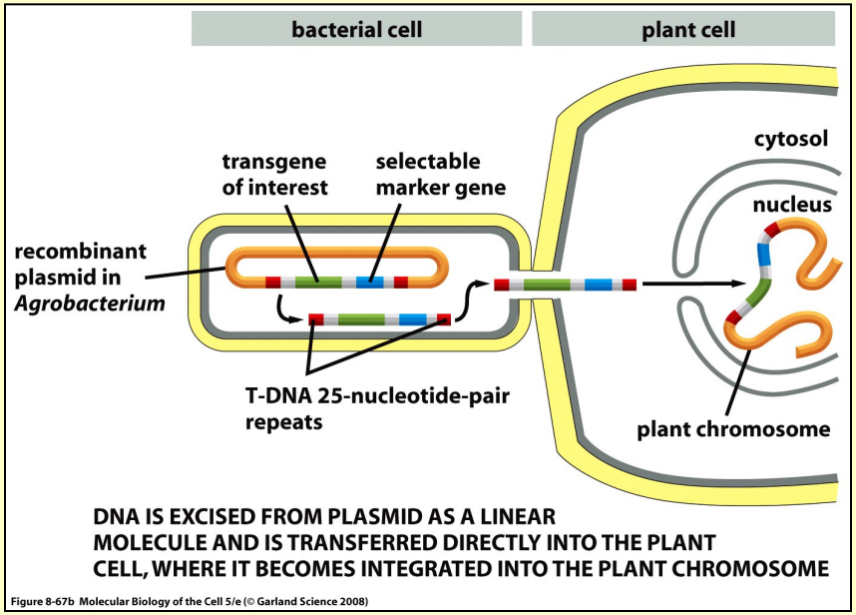


Figure 8-67b Molecular Biology of the Cell 5/e (© Garland Science 2008)