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CELLULAR AND MOLECULAR BIOLOGY PRACTICUM

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Lesson 1. Microscopy

MICROSCOPE

Microscopes that you are going to use in the practicum are of Zeiss brand. They are modern school microscopes with two eyepieces. Today there are more complex types of microscopes (ultramicroscope, phase contrast microscope, polarizing microscope, interference /Nomarski/, fluorescence, acoustic, confocal) used for research purposes or for cytological analysis, but basically, all light microscopes are working on the same microscope principle.

We distinguish between mechanical and optical parts of the microscope. The mechanical parts carry optical components of the microscope and are used for their alignment and adjustment of the sample with the object that we want to observe, whereas optical parts of the microscope are a set of lenses and mirrors needed to create an image of the observed object (Figure 1.1).

Mechanical parts: base, arm, tube, revolving nosepiece, stage, coarse focusing knob, fine focusing knob.

Optical parts: eyepiece/ocular, objectives, condenser, iris diaphragm, illumination.

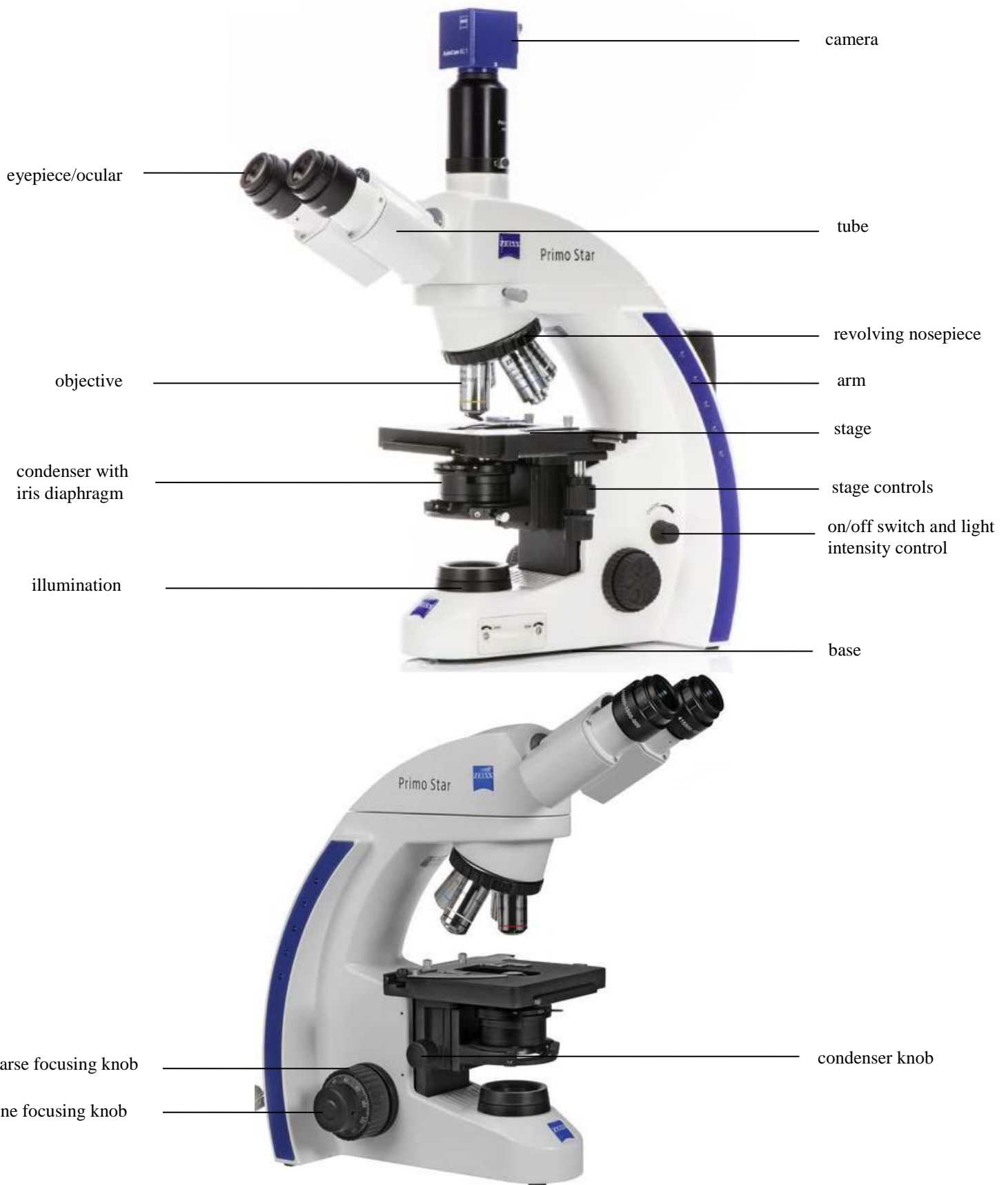


Figure 1.1. Light microscope of Zeiss brand used in the practicum. Figure prepared by P. Peharec Štefanić and D. Pavoković.

HOW ARE IMAGES CREATED IN THE MICROSCOPE?

A prerequisite for you to become a good microscopist is to understand how your instrument works. Be aware that light passes through the sample, and that the interactions between structures in the sample and light occur. Moreover, light directed to your eyes by the lenses of the microscope carries information that the brain processes and interprets. Be sure to repeat basic concepts of optics: refraction of light beams, the passage of light through the plane parallel plate, optical prism and lens, the refractive index of light, scattering or dispersion of light, diffraction of light on a narrow slit, interference. Use the physics textbook to study how images are formed in the microscope.

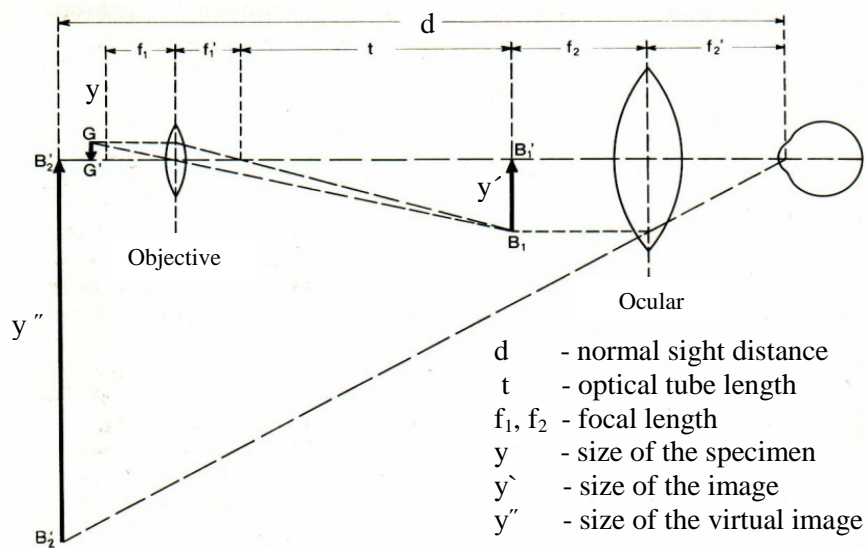


Figure 1.2. Image creation by the microscope (simplified schematic drawing).

The optical system of the microscope consists of two major systems of lenses: objectives and the eyepieces/oculars. The objective gives magnified, reverse and realistic image of the object, while an eyepiece additionally magnifies this realistic image. Therefore, the quality of the objective is crucial, because if in the real image, created by the objective, small details are not visible, i.e. resolved, the eyepiece will only magnify the image with details that are not visible. This is so called blank increase.

Total magnification (M_{total}) of the microscope is the magnification of the eyepiece or ocular lens (M_{ocular}) multiplied with the magnification of the objective lens ($M_{\text{objective}}$).

$$M_{\text{total}} = M_{\text{ocular}} \times M_{\text{objective}}$$

MICROSCOPING

1. Adjust the height of the chair to your body, so that you can approximate your eye to the eyepiece with no tension. The microscope may be right in front of you. (It is important to sit properly. Otherwise, the eye, neck and spine will hurt.)

Eyeglass wearers should use the microscope **without glasses**, unless you are very short-sighted (this type of microscope you can also use with eyeglasses).

2. Switch on the light using the knob on the right side of the microscope and **adjust the light intensity**.

The field of view must be uniformly illuminated!

You will notice that the field of view is not uniformly illuminated using the lowest-power objective. The central part is more illuminated than the border. If you are working with a microscope whose condenser can move with the knob, then while microscoping with the lower-power objective you will lower the condenser, and raise it when you are using stronger-power objectives. On the microscopes in this practicum **condensers cannot and must not be moved!** From Figure 1.3. you will understand that by lowering the condenser or using of prelens, we lower the focus of this lens system; therefore, the sample is illuminated by wider cone of divergent light beams.

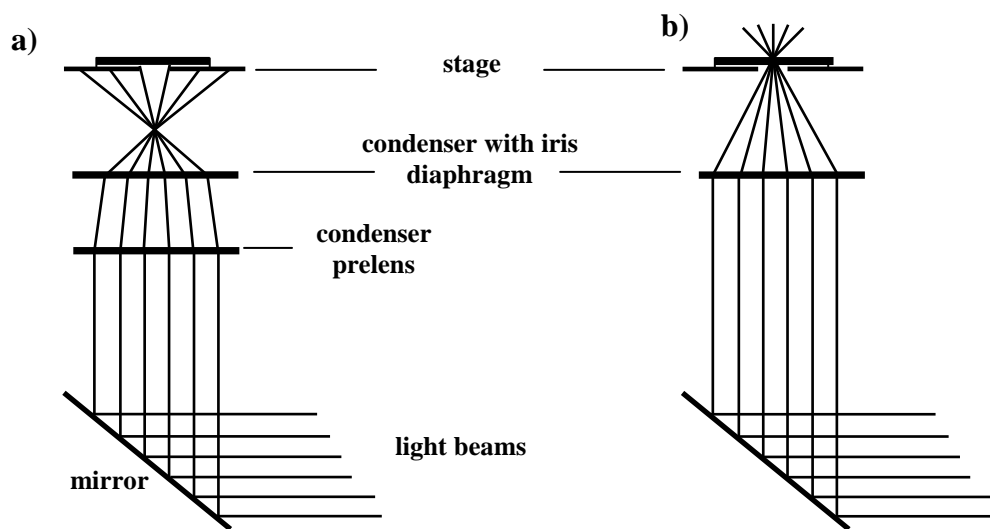


Figure 1.3. The role of the condenser and its prelens: a) when the condenser is in the top position, its focal point is in the plane of the sample; b) if we lower the condenser or place the prelens, focal point is lowered, thereby achieving a uniform illumination field of view when working with lower-power objectives. Image by M. Krsnik-Rasol and B. Balen.

Ensure that the image created by objective is magnified, real and inverted!

Draw a small arrow on the microscope slide with a thin felt pen!

Place the slide on the microscope stage and remember the direction of the arrow. Lift the stage with coarse focusing knob so that the distance of objective lens with weakest magnification from the sample (arrow on slide) is about 0.5 cm. Remove the eyepiece from the tube and put the plate of frosted glass on the opening of the tube. With one hand hold the plate, and with the other hand slowly lower the stage (coarse focus knob), while on plate you see an image of the arrow. (If the room is highly illuminated it will be difficult to notice the arrow.) Compare the size and the direction of the arrow on the slide with the size and direction of the arrow on the image you see on the glass plate. Would you be able to see the virtual or apparent image on the glass plate?

Now put back the eyepiece in the tube, take the frosted glass and move it away and the approach it to eyepiece lens. Catch sharp bright circle on the glass - it's **an ocular circle** in which all the beams coming from the microscope intersect and here is your eye when you

look in a microscope. Check direction of an arrow when you move away and approach the glass to eyepiece!

FOCUSING THE IMAGE IN THE MICROSCOPE

Focusing means to move optical systems of the microscope until a clear and sharp image appears.

Start to use the microscope with the lowest-power objective as it follows:

1. Place a slide on the microscope stage, turn on the lamp and adjust the light intensity.
2. Carefully lift the stage (coarse focusing knob) so that the objective lens with the weakest magnification is about 0.5 cm away from the sample. When raising the stage you should not look into the eyepiece/ocular in order not to strike and crash a microscope slide and damage the objective lens.
3. While you are looking in the eyepiece/ocular, lower the stage (coarse focusing knob) until in the field of view a sample image appears. With this type of microscope we always focus by increasing the distance! The distance between the frontal objective lens and sample, when its image is sharp, is the working distance of the lens. The working distance of the objective is smaller with higher-power objectives.
4. Focus the sample image with a fine focusing knob, if necessary. Move the sample, scan it carefully, and select the place you want to explore in more detail with higher-power objectives.
5. When the image is focused using the lower-power objective, it will be in the focus for higher-power objectives. Turn the revolving nosepiece and set a higher-power objective (40x, **but not the strongest one, i.e. immersion objective!**), and then focus only with the fine focusing knob.

ABSORPTION AND DIFFRACTION-REFRACTION NATURE OF THE IMAGE

Depending on the nature of the sample it is necessary to change the width of the cone of light that illuminates the specimen. This can be achieved by **iris diaphragm**. It is located near the bottom of the focal plane of the condenser. As a rule, we light up coloured samples with wider light beams, and discoloured with narrower light beams.

We notice details in the sample because they are either coloured (light absorption of certain wavelengths, the nature of colour) or they differentiate from the environment due to refractive index (diffraction-refraction image). Absorption image comes to the fore when the sample is illuminated with wide cone of light beams, and diffraction image comes to the fore when the cone of light is narrower. The image in the microscope is usually at the same time absorption and diffraction-refraction nature.

We observe the sample first with fully open iris diaphragm, and then we start to close the iris diaphragm until we achieve an optimum contrast.

If we narrow the aperture of iris diaphragm too much, due to optical phenomena diffraction colobars around the tiny structures will appear, although they are not really present in the sample.

When we use the lower-power objectives (dry objectives), iris diaphragm should be narrowed, but with higher-power objectives (immersion objective) it should be opened.

DETERMINING THE RELATIVE REFRACTIVE INDEX OF THE MICROSCOPE

The refractive index (n) is the ratio of the velocity of propagation of light through a vacuum and a medium (air, water, glass). The rays of light are propagating more slowly through the water ($n = 1.33$) than through the air ($n = 1.0$). In the cell they propagate faster through the cytoplasm than through the nucleus, but even more slowly they are propagating through the nucleolus. The velocity of propagation of light through some medium depends on its optical density. On the basis of the relative refractive index we can recognize some structures in the sample. For example, in the living cell of onion epidermis we will differentiate the mitochondria from sferosoma (fatty droplets), according to the difference in the refractive index.

Usually a student in the sample first perceives the air bubble, although it is not a part of the object for investigation, but is inadvertently embedded into the sample.

With the help of sample preparation in which air bubbles are embedded into the water or oil, you will learn to recognize spherical inclusions with smaller refractive index, while by observing the oil droplets in water, you will learn how to recognize structures with larger refractive index.

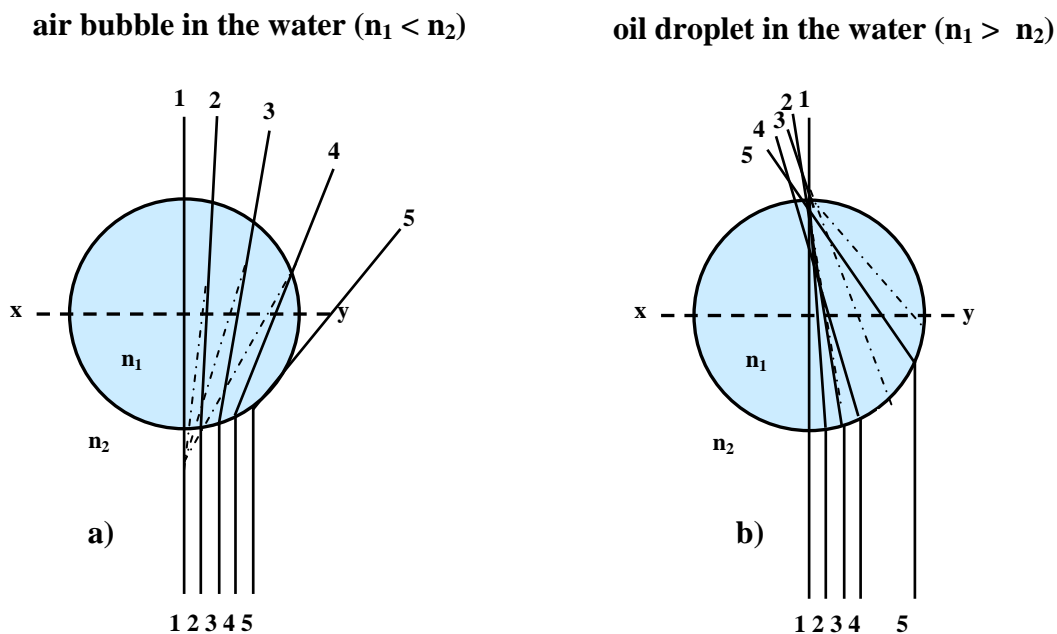


Figure 1.4. a) Air bubble in the water, b) oil droplet in the water. Figure prepared by M. Krsnik-Rasol and B. Balen.

Investigate the Figure 1.4. and answer the following questions:

1. What happens to the rays of light when they are passing from a medium with higher refractive index to the medium of smaller refractive index? And what happens when they are passing from medium with smaller refractive index to the medium of high refractive index?
2. What is characteristic for the passage of ray **1** in Fig 1.4. through the spherical inclusions?
3. What happens to the rays **2** and **3** at the interface water - air, and what at the interface of water - oil? (We have not considered refraction of light rays at the border of medium of different optical density on the way out from the bubble due to the clarity of the drawings)
4. Will rays **4** and **5** enter the objective lens of the microscope after passing through an air bubble (Fig. 1.4.a) or a drop of oil (Fig. 1.4. b)?
5. If you compare spherical inclusions with optical lens, which inclusion acts as a spreader, and which like gatherer?

By investigating the sample ensure that this basic knowledge of optics can help biologist to recognize cell organelles or inclusions and to conclude something about their nature. For example, cell nucleus has a higher refractive index than the surrounding cytoplasm (acts like a gatherer lens); by raising the stage, the boundary line of nucleus travels inward, while by lowering the stage it travels outward.

MICROSCOPE AS A MEASURING INSTRUMENT

Measurement in the horizontal plane - determining the length and width of the object

With the microscope we can measure the lengths with the help of an **eyepiece for measurement** (ocular micrometre). In such an eyepiece a transparent plate with a scale on which 1 cm is divided into 100 parts is inserted. The sample should be adjusted in the way that the structure that we want to measure overlaps with the bars of the scale. To do that, move the slide and rotate the eyepiece until the scale is not in the desired position. Note how many bars cover the length and width of an investigated object. However, you still do not know the real dimensions of the measured structure because the size of the gap between the two bars on a scale of eyepiece measurement changes depending on the objective magnification. Therefore, for each objective the scale in the eyepiece should be calibrated, to determine its micrometre value (distance between the two bars in μm).

Micrometre slide (object micrometre) is required for calibration,. Micrometre slide is the slide on which the scale of 1 mm is divided into 100 parts (sometimes 2 mm divided into 200 parts). Thus, the distance between the two bars is 10 μm .

Approximate methods of measurement

In the initial observations of the images in the microscope, sizes of different parts of the sample are inadvertently compared. For example, the sizes of cells are compared as well as the ratios of the cell size and the nucleus size or nucleus and chloroplasts size etc. The goal is

to determine roughly the size of some cell by comparison with a structure whose size we know. In histological samples of animal tissues, erythrocytes serve as a good comparison.

Approximate measurements we can make in the following manner:

We estimate the size of any structure in the field of view (eg, cells, nuclei, starch grains) in millimetres and divide that value by multiplying with objective and eyepiece magnification i.e. with the total magnification of the microscope.

IMMERSION OBJECTIVE

The objective provides a realistic image of the object, which is additionally magnified by the eyepiece. The image quality depends primarily on the quality of the objective lens.

Between the sample and the frontal objective lens of low and medium magnification is air (dry systems). Immersion objective lens is immersed in a drop of immersion oil, which is placed on the coverslip. Immersion oil has a refractive index equal to the refractive index of glass ($n = 1.515$); thus, the space between the frontal objective lens and sample is homogenised in the optical sense, and the border between the medium with higher refractive index (coverslide) and a lower refractive index (the air, $n = 1$) disappears. Figure 1.5. (b) shows linear spreading of light beam through the optical homogeneous space, while the Figure 1.5. (a) shows refraction of light beam at the glass - air interface. In dry systems part of light beams (beams 3, 4 and 5) will not enter the objective lens due to refraction at the glass-air interface. In the optically homogeneous systems there is no refraction of the light beam, so more light beams are entering the objective lens.

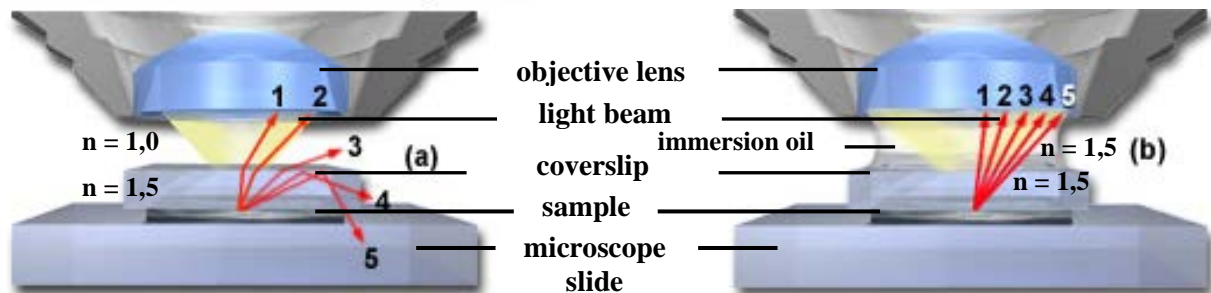


Figure 1.5. a) Due to the refraction of light upon the transfer from the sample (glass, $n = 1.5$) to the air ($n = 1.0$), beams 3, 4 and 5 will not enter the objective lens; **b)** In an optical homogeneous medium there is no refraction of light because the glass and immersion oil have the same refractive index, $n = 1.5$, so the beams 1, 2, 3, 4 and 5 enter the objective lens. Figure prepared by B. Balen.

Working with immersion objective

The immersion objective should be positioned very close to the sample, because its working distance (distance between the objective frontal lens and sample) is very small (0.13 to 1.15 mm). Therefore, while focusing we have to **handle it very carefully**. This is done as follows:

1. Switch on the lamp and adjust the light intensity.
2. Scan the sample using the objective with the lowest magnification. The structures you want to investigate further, place in the centre of the field of view.
3. By using the objective with higher magnification, examine the sample quality and adjust more precisely the selected part of the sample.
4. Using a coarse focusing knob move away the objective lens from the sample about 2 cm and then rotate the revolving nosepiece with objectives so that the above the sample is an empty space.
5. Place a drop of immersion oil on the cover slip, exactly where you see a circle of light coming from the light source (illumination) and the condenser.
6. Rotate the revolving nosepiece so that the immersion objective comes to the place of dry objective.
7. Tilt your head to see the frontal lens of the objective, and lift the stage with a coarse focusing knob until the objective lens touches a drop of immersion oil. At the moment of contact, immersion oil suddenly adheres to the lens, and you will notice a light flash.
8. Now look into the eyepiece and slowly lift the stage with a fine focusing knob; if the image does not appear slowly lower the stage until you get a sharp image. (Be careful not to squash or break the prepared sample or to damage the objective by impulsive and careless stage lifting!).

Note!

If the image is not clear and the sample is poorly prepared, immersion objective will not fix these preparation flaws! Make sure that the cover slip sits well on the sample, and that is not sidelong due to too big pieces of tissue or too thick sections.

If we are operating with objectives with stronger magnifications, we must constantly focus, i.e. to focus the image with fine focusing knob. We do this in order to avoid ambiguities, which are caused by curvature of the image plane, but also to explore the different planes of the sample. Be aware that the microscopic image of the cell is two-dimensional, while the cell itself is three-dimensional structure.

Practical work:

Note: while performing the exercises, fill in the Form 1.

Exercise 1. On a clean slide seep a drop of tap water and put inside the leaf of moss or pondweeds (*Elodea canadensis* L.), thin section of leaf or some other object that will be used in microscopy exercise. Follow the above explained instructions and investigate the sample using the low magnification (objective 10x) and then with a higher magnification (objective 40x).

Answer the questions:

1. Do you see the same part of the sample with a objective of the lower and objective of the higher magnification?
2. Does the whole sample fit in the field of view when using a) a low magnification objective (10x), b) a higher magnification objective (40x)?

Exercise 2. On the slide seep a drop of distilled water and transfer the starch grains in the water with the tip of the needle. (Another way to get the starch grains is to scratch the cut potato tuber or beans with a razor). Investigate the sample (objective 40x) and draw a few grains of starch as you see them with a completely open, too closed and optimally set iris diaphragm. Then add a drop of Lugol solution at the edge of coverslip and with filter paper, which you pressed alongside with coverslip on the opposite side, soak up some water to make a solution more quickly penetrate in the sample.

Answer the question:

1. What do you conclude about the nature of the image in the case of not stained and stained starch grains?

Exercise 3. Cut the onion (*Allium cepa* L.) bulb longitudinal into slices about 1cm thick. Separate the fleshy scales and on the concave side cut a epidermis (not separating it from the scale) into rectangles about 0.3 x 0.5 cm. Pieces of scales along with notched epidermis put into a flask with water and using a vacuum suction exhaust the air. If you do not have a vacuum suction, you can use the syringe as well; put the water in the syringe to half its volume, then put the tissue inside, squeeze out all the air with the plunger, and then plug the hole of the syringe with your finger and pull the syringe plunger and allow it to go back by itself. Repeat this several times. Separate the small piece of epidermis with tweezers and put it (with the wounded surface facing up) in a drop of water on a slide. Make sure that the epidermis does not bent or roll up! Place the coverslip to avoid embedding of air bubbles.

Answer the questions:

1. How will you distinguish between oil droplet in water from the air bubble in water?
2. What happens with light beam when it falls at an angle to the border between water (oil) and air, and that when it falls to the border between the water and oil?
3. If you compare the act of spherical inclusions with the action of the lens, which inclusion acts like a spreader lens, and which like gatherer lens?
4. Why do we need to suck air from the bulbs tissue?
5. What do you see by looking at the nucleus and cell walls when you do the fine movement of the stage with the fine focusing knob?

Lesson 2. Cellular Organization

COMPARISON OF EUKARYOTIC AND PROKARYOTIC CELL

Cells are the basic structural, functional and reproduction units of life; small machines that facilitate and sustain every process within a living organism. Regardless of their diversity, all cells can be classified into two basic cell types, **prokaryotic** and **eukaryotic** cell.

Prokaryotic cells are also unicellular organisms, among which we can distinguish archaea or archaebacteria and bacteria or eubacteria. All other organisms (protista, fungi, animals and plants) are eukaryotic and can be either unicellular or multicellular.

The term prokaryotic and eukaryotic reflects the difference in cellular organization between these two cell types, whereat prokaryotic cells are devoid of nuclear envelope and therefore lack nucleus (greek *karyon* = nucleus) in the form of the cell organelle. In them, genetic material is a circular DNA molecule located in the cytoplasm. That part of cytoplasm in which DNA is, functionally is equivalent to nucleus and is called nucleoid.

Among prokaryotes, the smallest and most simple organisms are mycoplasmas (size is around 0.5 μm). Mycoplasmas are parasites and they don't have cell wall like all other bacteria. Their DNA contains only 500-700 genes. Mycoplasmas are called minimal cells, as they have that minimum of size and organization needed for life. On the biochemical point of view mycoplasma are not so simple, and their molecular organization is very complex. The most complex prokaryotes are the cyanobacteria, which contain in their cytoplasm membranes - lamellae (thylakoids) that are very similar to photosynthetic membranes in the chloroplasts of plant cells. In these membranes the photosynthesis takes place in a manner corresponding to that in the plants, but not like in other bacterial cells.

The bacterial cells are running all important metabolic processes including the three main pathways for energy: glycolysis, respiration and photosynthesis. Simpler organization of prokaryotic cells is expressed in their small size, lack of intracellular membranes, smaller DNA and short generation time.

Eukaryotic cells are larger and more complex than prokaryotic. Their genome is located in the nucleus, which is clearly morphologically differentiated and separated from the cytoplasm with the nuclear envelope (consisting of two membranes). In the cytoplasm of eukaryotic cells are organelles enveloped by envelope (plastids, mitochondria) or only one membrane (endoplasmic reticulum, Golgi complex, lysosomes). Eukaryotic cell membranes divide them in different reaction spaces.

Table 2.1. Differences between prokaryotic and eukaryotic cells

Prokaryotic cells	Eukaryotic cells
Simple organisms	Simple but also complex multicellular organisms
Nucleoid (equal to nucleus)	Nucleus enveloped with envelope
DNA-circular molecule	DNA-organized with proteins in chromatin and chromosome
Size 0.1-10 μm	Size 10-100 μm and more
No compartment (organelles)	Compartment (cell organelles) are separated with membranes
No cytoskeleton	cytoskeleton
Binary division	Mitosis, meiosis

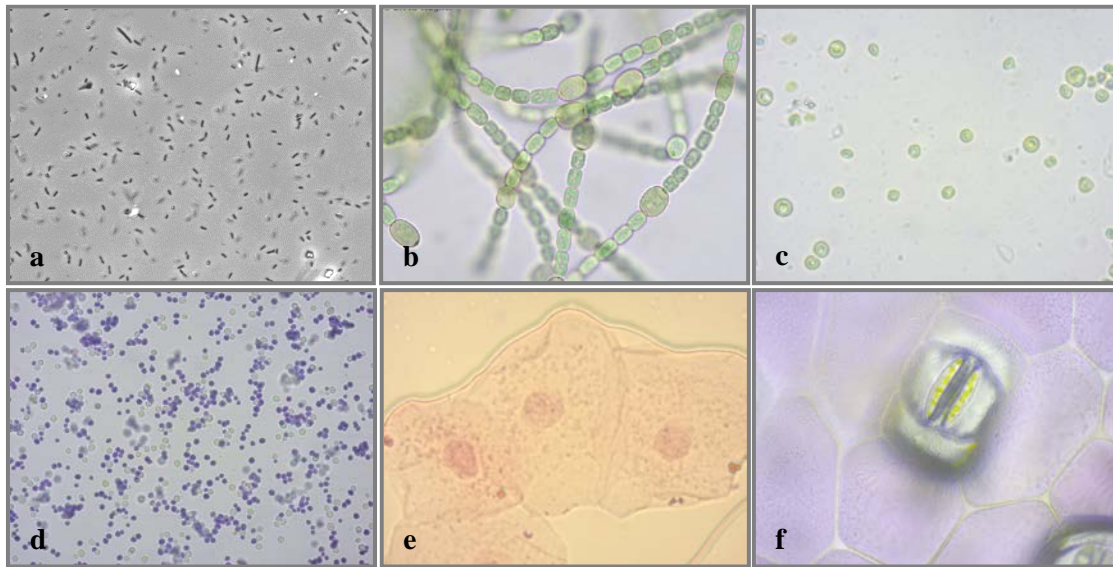


Figure 2.1. Diversity of cells. **a)** bacterium *E. coli* (objective 100x), **b)** cyanobacterium *Nostoc* (objective 100x), **c)** green algae *Chlorella* (objective 100x), **d)** yeast *Saccharomyces* sp. (objective 40x), **e)** epithelial cells from the human mouth (objective 100x) **f)** cells of lower epidermis of leaf of *Rhoeo discolor* with stomata (objective 100x). Prepared by P. Cvjetko and B. Balen.

PRACTICAL WORK

Why drawing at the beginning of the 21st century? Drawing plays an important role in microscopy and still cannot completely replace the photograph or digital display image. To be able to accurately draw the structure of the sample, it must be carefully monitored. You must observe the proportions of individual structures and their relationship and position. The cell you see only in the optical cross section is always as a two-dimensional structure. You will notice that cell image changes when focusing, because then become clearly visible different planes of your sample. Cooperating your hands (which raise and lower the tubus) and the brain (which integrates a variety of images of the planes), you can get an idea of the three-dimensional structure of the sample.

Anyone can learn how to draw. You need a simple accessory: a quality paper, several pencils of varying hardness, good eraser. The paper on which you draw put right by microscope (or left if you draw with your left hand). With a little practice anyone can learn!

Drawing can be a simple sketch. It contains no details, but it can clearly point the place that is interesting and you want someone else to show. Simplified drawing is more detailed, but still some structures are simplified (for example, plant cells are bounded by a simple line). A detailed drawing shows as faithfully as possible the structure of the sample. That detailed drawing can draw only one cell or part of the station. Figs. 2.2 and 2.3. give examples of drawing from microscopic slides.

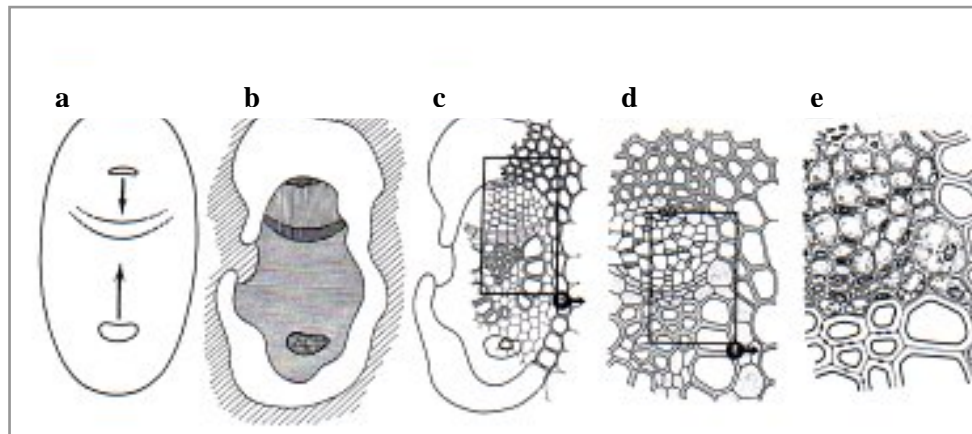


Figure 2.2. Drawing possibilities that show microscopic object. **A)** a sketch, **b)** schematic (different tissues are graphically differently marked), **c)** faithful picture of the cell with single contours, **d)** cut-out from **c)** drawn more detailed, **e)** cut-out from **d)**, cells are faithfully drawn with double contours (cell walls) and cellular content (Sample – *Ranunculus* sp., vascular tissue in cross section). Drawings by M. Krsnik-Rasol.

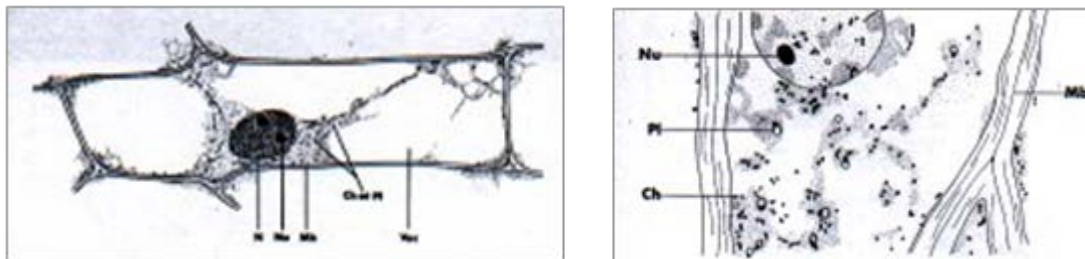


Figure 2.3. The drawing of the whole cell (left) and depiction of the part of the cell (right).

Practical work:

Note: while performing the exercises, fill in the Form 2.

Exercise 1. Bacterial cells.

On the slide you put a drop of water and use a glass needle to pick bacteria that grow on nutritive media and transfer them into the water. The site of applying the bacteria in water will be too thick, but this place can facilitate finding images at low magnification to a rookie. When you have focused in low magnification, inspect the sample at higher objective (40x) and then find a picture using the immersive oil objective (100x). Draw a picture of bacteria which gives the immersive oil objective.

Exercise 2. Eucaryotic cells

a) Yeast cells. On the clean slide drip a suspension of yeast. Some cells are living and some dead. They differ in that the dead cells are stained with methylene blue, a living are discolored. Inspect the sample in the usual way and draw a picture that is visible with the immersion objective.

b) Algae cell *Chlorella* sp. In the drop of water over the glass put green alga *Chlorella* sp. using glass needle. Algae are grown in culture on nutritive media. Observe the algae with the largest objective and draw it.

c) Cells of the leaf from species *Zebrina pendula* or *Rhoeo discolor*.

On the clean slide drip a drop of water in it and lay a thin cut section of the list. Cuts look at first in low and medium increase, and then draw a picture that gives the immersion objective.

d) Epithelial cells from the human mouth. Wash your hands thoroughly. On clean slide put a drop of water. Pull index finger on the mucosa inside the cheek and transfer hulled cells in a drop of water. Use coverslips and inspect the sample. Draw a picture of epithelial cells, using available on immersion objective. Use aceto-orcein or aceto-carmin to stain cells (drip the paint along the edge of coverslips and put filter-paper on the opposite side to pull out the water and to replace it with color).

Answer the questions:

1. Is *Chlorella* prokaryotic or eukaryotic type of cell?
2. Is *Chlorella* simple or multicellular organism?
3. On which one easily visible characteristic you can classify the cell *Chlorella* in one of two basic types of cell?
4. Which ones are easily visible characteristics of plant cell?
5. What was marked in non-stained epithelial cells?
6. Which part of epithelial cells is more intensively stained?
7. Conclude about size of prokaryotic or eukaryotic type of cells? Estimate the size of prokaryotic and eukaryotic cells you saw during practical work!

Lesson 3. Biomembranes – indirect visualization

The plasma membrane (also called the cell membrane) encloses the cell and determines its area and as a selectively permeable barrier maintains important differences between cells and their environment. All biological membranes are built on the same principle, and they are built by lipids, proteins and glycolipids and glycoproteins.

Lipid bilayer forms a structural matrix of the membrane. Membrane lipids are amphipathic molecules (one end is hydrophilic and the other end is hydrophobic) which are each spontaneously oriented, depending on whether they are in an aqueous medium or in a nonpolar solvent, or the border of the two phases. In the aqueous medium they can be spontaneously assembled into a double layer. Such a double layer acts as a two-dimensional liquid in which molecules can diffuse laterally. (Liquid mosaic model, Singer and Nicolson, 1972, Fig. 3.1)

Membrane proteins are mainly responsible for membrane function. They act as specific receptors, enzymes, carriers or make water channels for the passage of hydrophilic substances through the membrane. Proteins can be immersed in the lipid bilayer - the integral proteins, anchored therein or connecting to membrane as peripheral surface proteins.

Sugars not come alone but are covalently bonded to proteins or membrane lipids. They are located on the outside of the plasma membrane, and if they are in the interior of eukaryotic cells, they are turned away from the cytoplasm (the interior of the organelle membranes, for example lysosome). Their roles are multiple and they are responsible for the mutual recognition of cells.

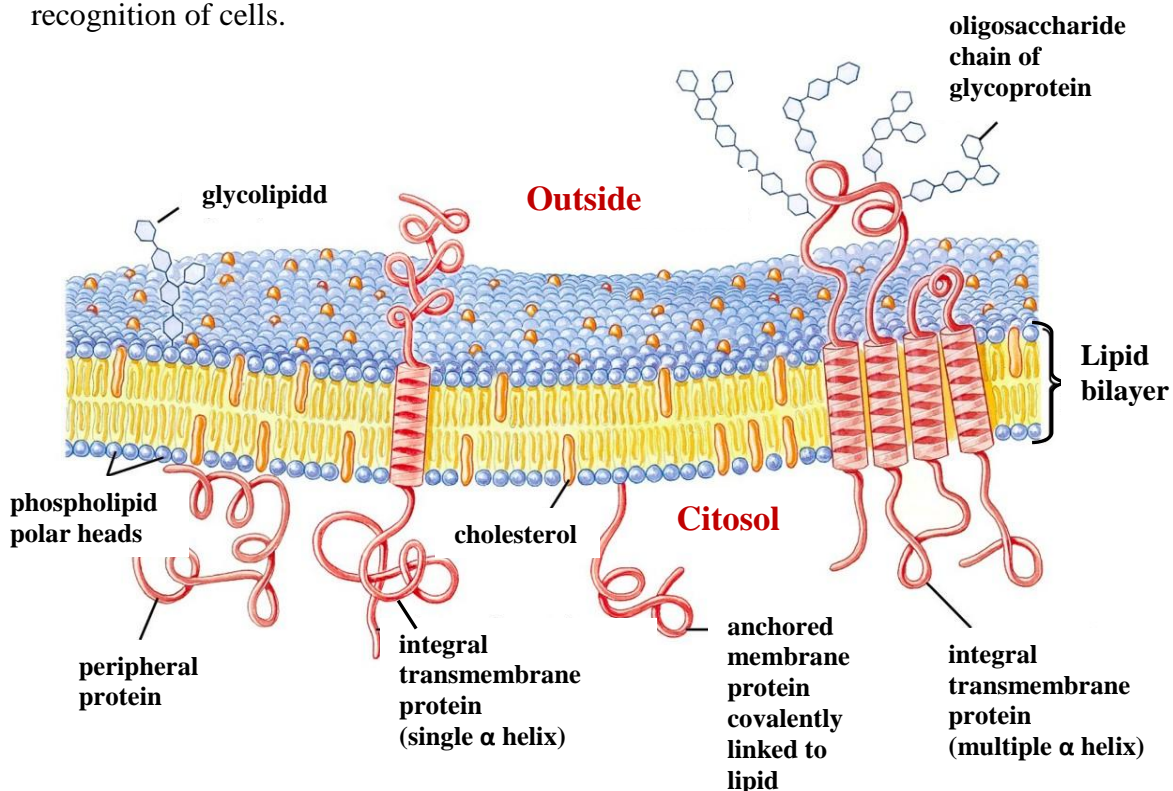


Figure 3.1. Membrane proteins may be extended across the lipid bilayer as integral proteins, be anchored to one of the monolayers or connected as peripheral proteins. Prepared by B. Balen.

INDIRECT PROOFS OF EXSISTANCE OF BIOMEMBRANES – PLASMOLYSIS

The term plasmolysis means separation of cell membrane and cell protoplast of the cell wall when the plant cells are found in hypertonic solution (Figure 3.2.). The phenomenon is caused by osmosis. Experimentally it can be induced in living plant cells that are placed in a sugar or salt solution that has a higher concentration than the cytoplasm (vacuole content). The concentration of cell interior is hypotonic in relation to the sugar solution; therefore, water is coming out of the vacuole and the protoplasmic layer and the cellular liquid exits the cell. Vacuole decreases until it equals the concentration of its content with concentration of external solution (a solution which induced plasmolysis).

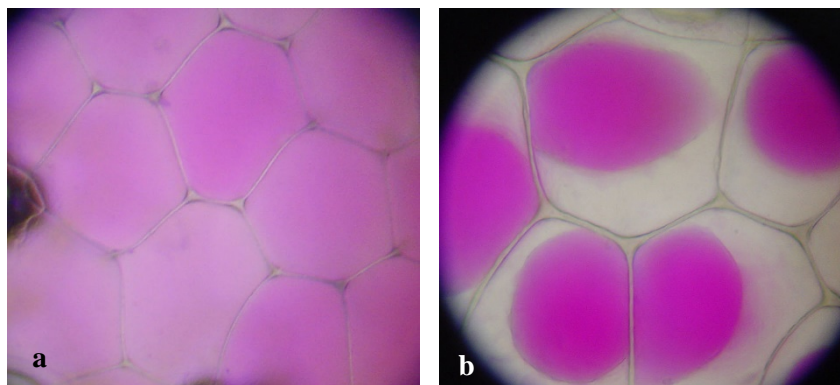


Figure 3.2. Plasmolysis in the cells of the lower epidermis of leaf *Rhoeo discolor*. **A)** turgescient cells in isotonic solution; **b)** cells in hypertonic solution. Preparation and imaging by B. Balen.

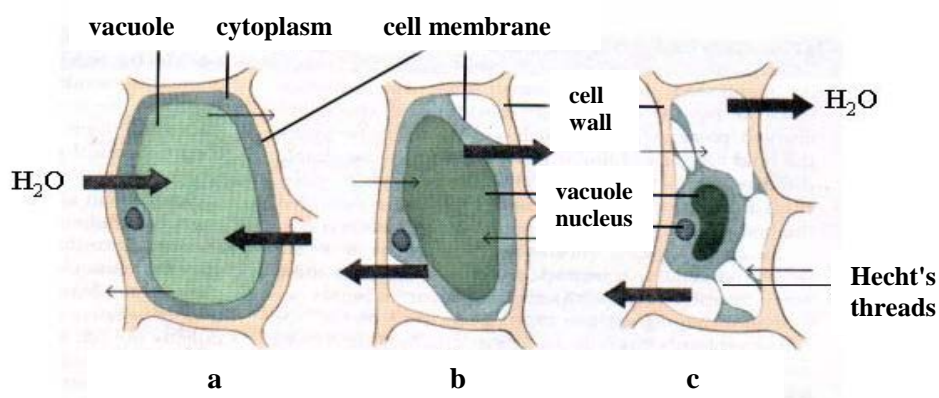


Figure 3.3. Plasmolysis in the epidermal cells of leaves. **A)** normal isotonic conditions - turgescient cells; **b)** hypertonic solution - plasmolysis starts; **c)** more concentrated hypertonic solution - plasmolysed cells. Figure prepared by M. Krsnik-Rasol.

CELL AS AN OSMOMETER

Practical work:

Note: while performing the exercises, fill in the Form 3.

1. Create a 100 mL stock solution of sucrose concentration of 1.0 M. (relative molecular mass of sucrose: 342.3).
2. Diluting stock solution suit a range of solution concentration of 0.2 M to 1.0 M (prior to the work, complete the Table 3.1 of Form 3).
3. Using sharp razor cut on the underside of the leaf *Rhoeo discolor* thin tangential sections (also work well a leaf *Tradescantia* sp., onion epidermis, beet root, etc.) and immerse them in the small bottles you have prepared a series of dilutions of sucrose. After ten minutes prepare slides for microscopy.
4. On the slide you put a drop of water (from the first vessel in the series) and in it several thin cross section (from the same bowl). Inspect the product at low and medium increase. Select the cells that are purple-colored due to anthocyanins in the vacuole and draw such turgescient cells.
5. Inspect all sections in solutions of different concentrations of sucrose. Draw two cells: one in which plasmolysis just begun and another in which it progressed (obj. 40: 1).
6. At weak objective increase inspect every sample and evaluate for most cells the level of plasmolysis. At which sucrose concentration plasmolysis begun?

Exercises

Exercise 1. Draw one turgescient cell, in which plasmolysis just started and the cell with the ultimate degree of plasmolysis.

Exercise 2. Using the cells as a small osmometric measure, based on experience from previous exercises determine the approximate concentration of the given solution of sucrose.

Answer the questions:

1. What is happening with vacuole during the plasmolysis?
2. What defines the osmolality of the cells?
3. Must two solutions which are isosmotic with the cytoplasm also be isotonic? Explain the answer!

Lesson 4. Plastids

Plastids are organelles characteristic for plant cells. Some fungi and highly specialized plant cells are devoid of plastids. One plant cell always contains only one plastid type, while on the whole plant level different plastid types can be found. Plastids are semiautonomous organelles and contain genetic information in *plastid DNA* (ptDNA), which, however, is not sufficient for independent life of plastid outside of the cell. Plastids are surrounded by two membranes, which form *plastid envelope*. Outer and inner plastid membranes are different and actually the inner membrane represents the actual barrier towards the cytoplasm. The space between two membranes is called intermembrane space. The inner membrane wraps the plastid *stroma* in which ribosomes, circular ptDNA (present in more than one copy), starch grains, plastoglobules and the third membrane system, *thylakoids*, are located.

It is known that some plastid types can be reversibly transformed from one type to another. Which plastid type will be present in the cell is dependent on the cell's developmental stage, its location within the plant and physiological conditions, among which light is the key factor.

Proplastids are poorly differentiated, colourless plastids of meristem cells (Fig. 4.1.a). They contain a few inner structures, called *prothylakoids*. They divide and develop into a certain plastid type, which depends on the differentiation direction of the cell that they are placed in.

Chloroplasts are photosynthetically active plastids (Fig. 4.1.b). They contain chlorophyll (bound to membrane proteins), carotenoids and other membrane-bound components of the photosynthetic system in *thylakoid membranes*. Individual thylakoids are named *stroma thylakoids*, while multiple thylakoid layers are called *grana thylakoids*. Enzymes for CO₂ fixation and related synthetic pathways are located in the *chloroplast stroma*. Beside photosynthesis, numerous other activities are taking place in chloroplasts, such as synthesis of lipids, fatty acids and starch.

Etioplasts are colourless plastids found in the cells of plants grown in darkness (Fig. 4.1.c). Some reactions in the construction of the photosynthetic apparatus and thylakoid membranes are dependent on light. Etioplasts contain paracrystalline structures called *prolamellar body* and a few membranes – *prothylakoids*, which contain green magnesium-containing pigment *protochlorophyll*. During exposure to sunlight, prolamellar body is degraded and functional thylakoids are formed. *Protochlorophyll reductase*, a light-dependent enzyme, converts protochlorophyll to chlorophyll by reduction under the influence of light.

Chromoplasts are yellow, orange or red coloured plastids of flowers and fruits. They contain carotenoids and a lot of lipids in the form of lipid globules (Fig. 4.1.e and f). Beside globular type, chloroplasts with tubule-like membranes and chloroplasts with carotenoid crystals can be found.

Leucoplasts are colourless plastids found in non-photosynthetic tissues of plants, such as roots, bulbs and seeds. They may be specialized for bulk storage of starch, lipid or protein and are then known as amyloplasts (Fig. 4.1.d), elaioplasts, or proteinoplasts (also called aleuroplasts) respectively. However, in many cell types, leucoplasts do not have a major storage function and are present to provide a wide range of essential biosynthetic functions, including the synthesis of fatty acids, many amino acids, and tetrapyrrole compounds such as heme. In general, leucoplasts are much smaller than chloroplasts and have a variable morphology, often described as amoeboid.

A **gerontoplast** is a plastid found in formerly green tissues that are currently senescing (yellow leaves). The formation of gerontoplast from chloroplast during senescence involves extensive structural modifications of thylakoid membrane and degradation of chlorophyll with the concomitant formation of a large number of plastoglobuli with lipophilic materials.

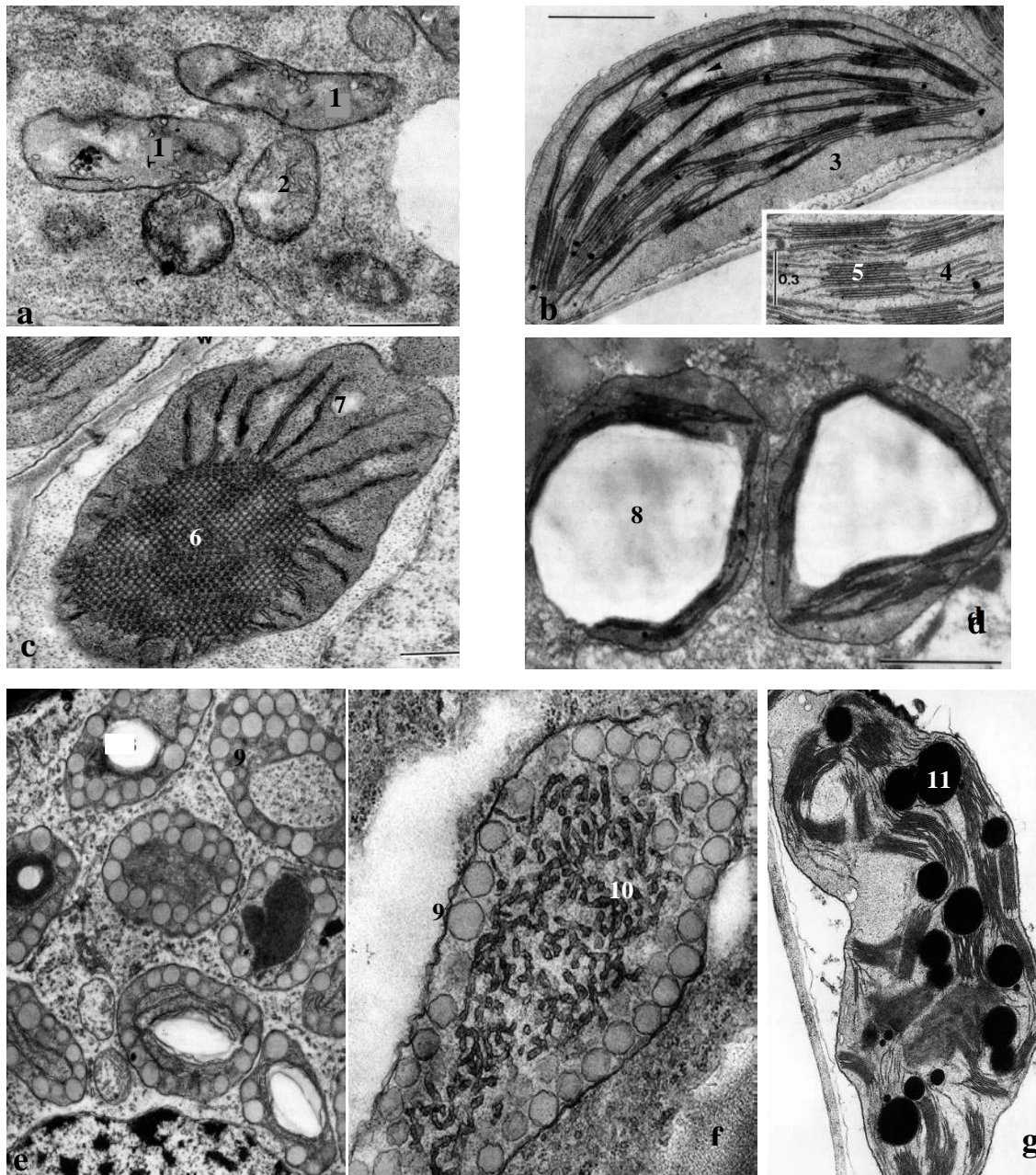


Figure 4.1. Plastids – organelles specific for plant cells ; **a)** proplastids, **b)** chloroplast, **c)** etioplast, **d)** amyloplasts (starch containing leucoplasts), **e)** and **f)** chromoplasts, **g)** gerontoplast.

1 – prothylakoids (rudiment of thylakoid membranes), **2** – mitochondrion, **3** – stroma, **4** – stroma thylakoids, **5** – grana thylakoids (granum), **6** – prolamellar body, **7** – prothylakoids, **8** – starch grain in leucoplast, **9** – chromoplast globules, **10** – chromoplast tubules, **11** – plastoglobules. Preparation and imaging by M. Krsnik-Rasol.

Exercises

Note: while performing the exercises, fill in the Form 4.

Exercise 1. Isolate apical meristem of *Elodea canadensis* Rich.

The apical tip has to remain intact and cells need to stay alive. Do it in a following way: Use the tweezers to remove the leaflets and when you observe a white lump (apical meristem covered with only a few leaflets), place it in a drop of water on the microscope slide. Use the razor to cut the tip (above the leaflet insertion spot). Use the needle to remove the leaflets, but you can leave them in the water drop since they will hold the cover slide and protect apical meristem from squeezing.

Study the apical meristem anatomy using the small magnification. Using the immersion objective observe the nuclei and proplastids in apical cells cytoplasm.

Exercise 2. Prepare thin longitudinal leaf sections. Place them immediately in the water and use water pump or medical syringe to remove the air from intercellular space. Prepare microscopic sample and define the plastid type present in: a) epidermal cells, b) stomata guard cells, c) stomata accessory cells, d) leaf parenchyma cells (mesophyll). Thicker leaves of plant species like *Kalanchoe daigremontiana*, *Tradescantia sp.*, *Zebrina pendula*, *Pulmonaria officinalis* and *Elodea canadensis* are appropriate for this type of exercise.

Draw the detail of the cell which you can observe using the immersion objective. Compare the light microscope micrograph with the one obtained by electron microscope. Draw the detail of the ultrastructure which can be observed on the electron micrograph (envelope, thylakoids, plastoglobules etc.).

Exercise 3. Define the origin of the yellow or orange colour of some flowers. Prepare thin sections of heartsease (*Viola tricolor*) or iris (*Iris germanica*) yellow and blue coloured petals.

Exercise 4. Define the origin of the yellow or orange colour of some fruits. Prepare thin sections of yellow or red coloured paprika fruit of carrot root.

Answer the questions:

1. Which plastids are present in the cells of apical meristem?
2. Which plastid type is present in a) epidermal cells, b) stomata guard cells, c) stomata accessory cells, d) leaf parenchyma cells (mesophyll)?
3. What is the origin of the yellow, red or orange colour of some flowers and fruits?
4. Which type of chromoplasts is present in carrot root cells?

Lesson 5. Nucleus - Mitosis

All eukaryotic cells possess a nucleus, while in prokaryotic cells function of the nucleus is conducted by nucleoid as equivalent of the eukaryotic nucleus.

The nucleus is composed of following structures:

- Nuclear envelope
- Nuclear lamina
- nucleoplasm
- nuclear matrix
- chromatin/chromosomes
- nucleolus

CELL CYCLE

For better understanding of cell growth and division, scientists have divided the processes involved in the cell cycle in several phases. The cell cycle is divided in two major phases, interphase and mitosis. Interphase is divided in three phases: **G₁, S and G₂** (G means gap and S means synthesis, Fig. 5.1), and mitosis in five phases: **prophase, prometaphase, metaphase, anaphase and telophase**. Division of the nucleus is followed by the division of the cell (**cytokinesis**). In the non-dividing cells, nucleus is metabolically active and morphological changes characteristic for dividing cells are not present. Therefore, such nuclei are in resting phase. However, more suitable name for non-dividing nucleus could be “working nucleus” (what do you think why?). “Working nucleus” is morphologically similar to interphase nucleus but their activity is different.

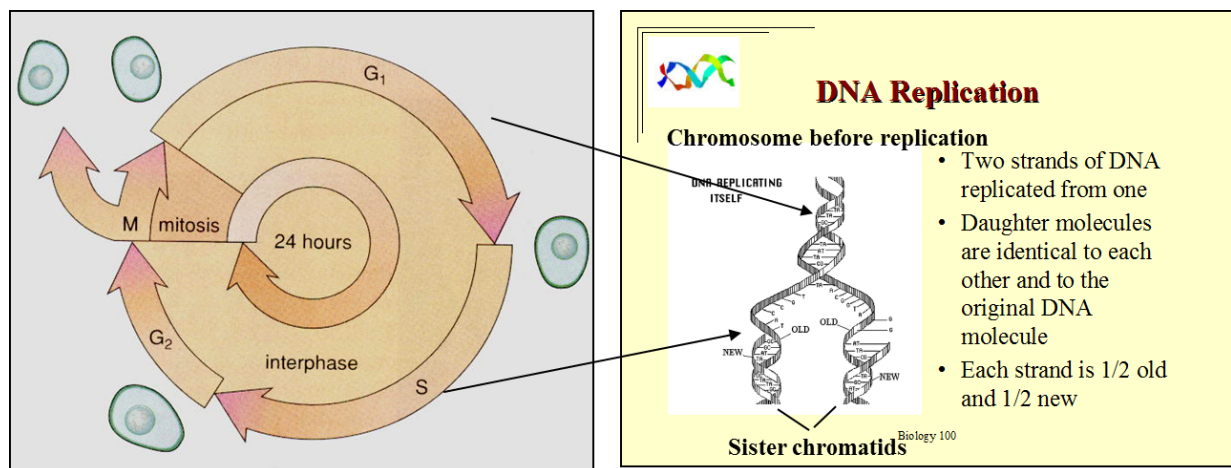


Figure 5.1. Cell cycle (cell division) in the most eukaryotic cells is divided in four phases: G₁, S, G₂ and M. Figure preparation by V. Besendorfer.

INTERPHASE

Interphase is a part of the cell cycle between two subsequent divisions and occupies 90% of cell cycle. Crucial event in interphase is doubling (reduplication) of the genetic material, i.e. reduplication of the DNA. Reduplication occurs in semiconservative way which means that each of two newly synthesised DNA molecules consist of one old (original) and one new polynucleotide strand (Fig. 5.1). After doubling, genetic material will be distributed into the daughter nuclei during mitotic division (mitosis). In that way both daughter cells that occur after cell division (cytokinesis) will contain identical genetic information and the same chromosome number like the mother cell. The biological meaning of mitosis is the maintenance of the genetic material during cell division.

Under the light microscope, the interphase nucleus stained with aceto-carmine, aceto-orceine or as a result of Feulgen reaction become visible as dense granular structure called **chromatin** (complex of protein and DNA) (Fig. 5.4.a.). In some regions, chromatin is more compact, intensively stained and looks like dark spots. These structures are called **chromocenters** and correspond to highly condensed chromatin. Therefore, chromatin represents stained part of the nucleus content, which corresponds to decondensed chromosomes which become visible during mitosis.

MITOSIS

Mitosis represents the division of the nucleus that occurs after doubling (reduplication) of the genetic material (DNA) in the S phase.

Mitosis starts with **prophase**. Chromosomes look like decondensed fibres folded in a ball structure (Fig. 5.4.a). During prophase chromosomes become more condensed and thicker, and at the end of the prophase two chromatids – sister chromatids - could be seen under the microscope. Sister chromatids are wrapped around each other forming a structure called relational spiral (coil). They are tightly connected in the **centromeric** region – the region composed of DNA sequences specific for centromeres. Centromeres represent location on chromosomes essential for binding of specific protein complex called the **kinetochore**. (Figs. 5.2. and 5.3). Formation of the **mitotic spindle** starts at the end of prophase. Mitotic spindle is a bipolar fibre structure composed of microtubules (Fig. 5.2). Nucleolus is degraded (vanish).

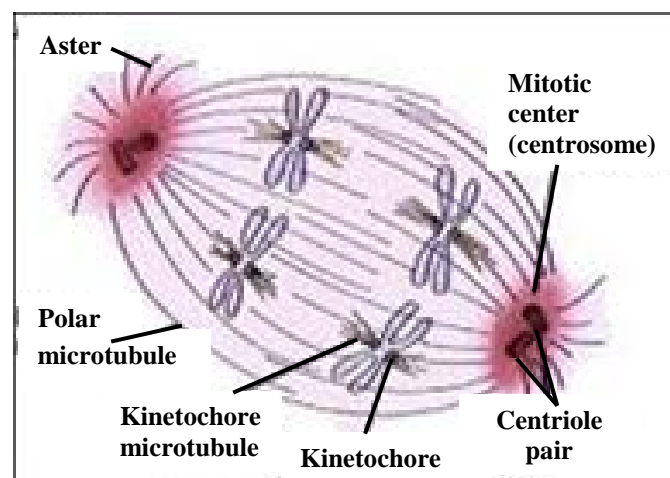


Figure 5.2. Mitotic spindle includes three types of microtubules – aster, kinetochore and polar microtubules. Plant cells do not have aster microtubules. (<http://ridge.icu.ac.jp>)

Prometaphase started with degradation of nuclear envelope into the membrane vesicles. On each side of the centromere, kinetochore apparatus is forming and binds to specific group of microtubules called **kinetochore microtubules**. Other microtubules of the mitotic spindle are **polar microtubules and aster microtubules** (Fig. 5.2). In prometaphase, chromosomes are condensed, shorter than those in the prophase and visible as separate structures if they are not overlapped (Fig. 5.4.a). They are dispersed in the cell, and sister chromatids as well as centromeres are could be easily recognized (Fig. 5.4.a).

In metaphase, chromosomes are located in the middle part of the cell. By the activity of the kinetochore microtubules chromosomes are moved in the middle part of the cell with centromeres positioned in the equatorial plate (vertical to the direction of the microtubule axis), while chromosome arms are turned toward spindle poles (Figs. 5.3. and 5.4.c).

Anaphase started with segregation of the sister chromatids in the centromeric region, continues with their segregation along chromosome arms and by their movement toward opposite spindle poles. Mitotic spindle composed of **polar** and **kinetochore** microtubules are not visible under the light microscope (except under the phase-contrasting microscope and application of immuno-fluorescence techniques). Movement of sister chromatids toward poles is a consequence of two independent processes. First, kinetochore microtubules shorten and second, polar microtubules elongate what lead to the additional separation (moving apart) of mitotic poles. When **sister chromatids** are completely separated they represent **chromosomes** (Figs. 5.4.d and 5.4.e). These chromosomes are single chromosomes containing only one DNA molecule.

In **telophase**, chromosomes reach mitotic poles and kinetochore microtubules vanish due to the microtubule depolymerisation. Polar microtubules continue with elongation to ensure chromosome separation on the cell poles. Telophase is characterized by chromosomes decondensation (chromosomes again look like long thin fibres), mitotic spindle depolymerisation/degradation and nuclear envelope formation (Fig. 5.4.f). Nucleoli, which were degraded and vanish in prophase, are assembled again.

Mitosis ends with **cytokinesis**. In the middle part of the animal cell, perpendicular to the mitotic spindle axis and between two daughter nuclei, the **cleavage furrow** is formed. Furrow invagination continues as long as division of the cell is ended with the appearance of two daughter cells. Plant cells, because of the cell wall cannot form a cleavage furrow. Instead they form a **cell plate** (phragmoplast) – barrel-like structure composed of numerous Golgi vesicles used for building of new cell wall.

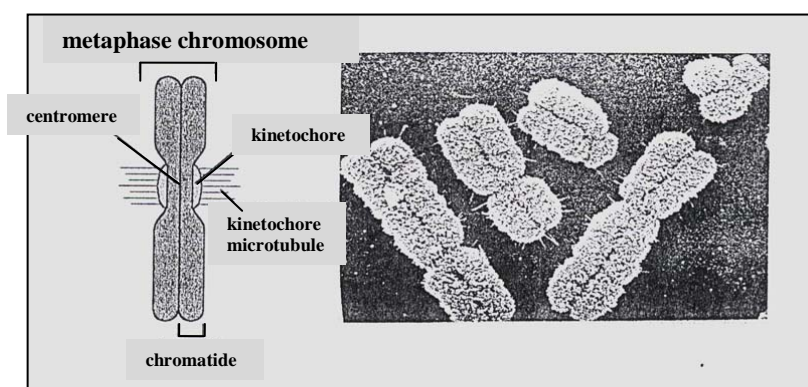


Figure 5.3. Drawing and SEM photo of metaphase chromosomes.

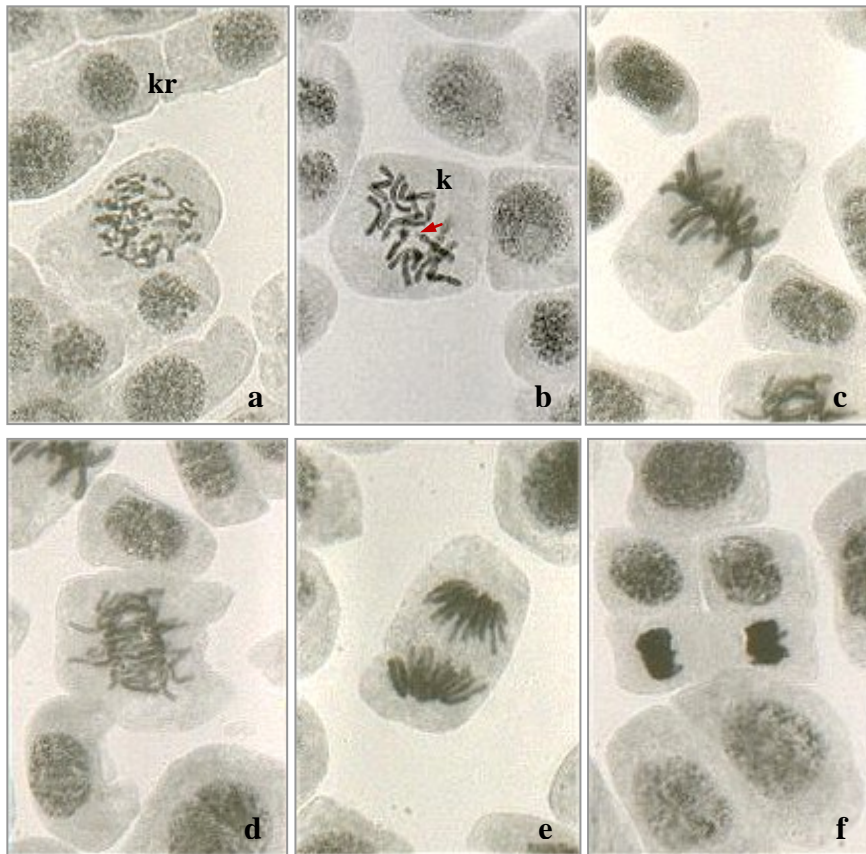


Figure 5.4. Light microscope images of mitosis in the meristematic cells of the onion root tip (*Allium cepa* L). Cells are stained with aceto-carmin, and photos are taken under immersion objective (100x). **a)** Three meristematic cells in **prophase**. **b)** **Prometaphase**, visible individual chromosomes. One satellite chromosome is marked with arrow. **c)** **Metaphase**, chromosomes in equatorial plate. **d), e)** **Anaphase**, movement of single chromosomes (one chromosome = one DNA molecule) to the opposite pole of the mitotic spindle. **f)** **Telophase**, decondensation of chromosomes and formation of new nuclei. kr – chromatin, k – chromosome. Slides and images prepared by M. Krsnik-Rasol.

Practical work

Note: while performing the exercises, fill in the Form 5.

Preparation of slides for studying mitosis in root tip meristematic cells (onion, *Allium cepa*, or some other plant species).

1. Select healthy onion bulbs and remove old roots by cutting the bottom of the bulb with sharp blade.
2. Put the bulbs (cut site) in the vessel with tap water.
3. After 24 h new roots will appear. Leave bulbs in the water for a few days (change the water each day). After few days roots will be 3-5 cm long and ready for use.
4. Put the dye (1-2% aceto-carmin or aceto-orceine in 45% acetic acid) in the glass tube. Cut the tip of the root (1 cm) and put it in the tube.
5. Heat slowly by pulling the glass tube through the flame until first bubbles appear. Move from the flame immediately. Be careful with this step!
6. Transfer roots with dye in the Petri dish and cover.
7. On clean glass slide put a drop of dye and one root with the tip of the root immersed in the dye. Detach meristematic part of the root tip and leave it in the dye. Remove the rest of the root from the slide.
8. With blunt end of the pencil or blunt end of the histological needle macerate the meristematic tissue in small pieces.
9. Carefully, avoiding bubbles cover the macerated tissue with cover slip.
10. Put 2-3 layers of the filter paper over the cover slip and strongly squash with finger. On that way the meristematic cells will spread around the slide and extra stain will be removed. This technique of slide preparation is called *squash technique*.

Exercise 1. Prepare 2 slides for studying mitosis and each step of slide preparation represent schematically.

Exercise 2. Study each mitotic phase and interphase, and count how many dividing cells in particular mitotic phase is present on hundred counted cells in your slide. The counting should be done independently on both slides. Fill out the table.

Exercise 3. Count the mitotic index for both slides. Mitotic index is ratio between dividing cells and total number of cell.

Exercise 4. Draw metaphase and anaphase using the immersion objective of the light microscope. Annotate all structures (key words: cell, nucleus, chromatin, chromosome, chromosome arms, chromatids, centromere, secondary constriction, satellite).

Answer the question:

1. Are all interphase cells of equal size? If not, explain why?

Lesson 6. Endomitosis. Polytene chromosomes and C-mitosis

ENDOMITOSIS, POLYTENY AND GIANT CHROMOSOMES

In some somatic cells the quantitative changes of the genome occur as a consequence of **endomitosis**. Endomitosis is the process of DNA replication without mitosis. The polyploid (endopolyploid) nuclei appear through repeated endocycles (amplification of the whole genome). These nuclei are bigger than diploid one, and according to prevailing ratio of nucleus and cell size, these polyploid cells are also bigger.

Specific example of endopolyploid nuclei are those with **polytene or giant chromosomes**. Polytene chromosomes are characteristic for salivary gland cells in diptera (Fig. 6.1), but also it could be found in plant cells, for example in some legumes. Characteristic of polytene chromosomes are decondensed chromatids which stay connected during repeated replication cycles and look like thick chromosome bundles 10 μm in diameter. Polytene chromosomes are up to 100 times longer in comparison to metaphase chromosome in other somatic cell of the same species. They are present in the cell as haploid chromosome set because of homolog chromosome pairing (somatic pairing of homologs). E. G. Balbiany has described polytene chromosomes in 1881. Banding pattern of polytene chromosomes could be seen after chromosome staining, i.e. bright and dark bands alternatively appeared across polytene chromosomes. Dark bands are named chromomeres and represent part of the condensed chromatin. Bands also reflect linear order of the genes on chromosomes. Gene activation, which could be induced by hormones (for example hormone ecdysone) is cytologically visible as decondensed region in the position of light bands that looks like a puff. The biggest puffs are named Balbiany's ring according to Balbiany. Microautoradiography ($^3\text{H-U}$) clearly shows that puffs represent the place of intensive transcription, e.i. they represent morphological manifestation of gene activity.

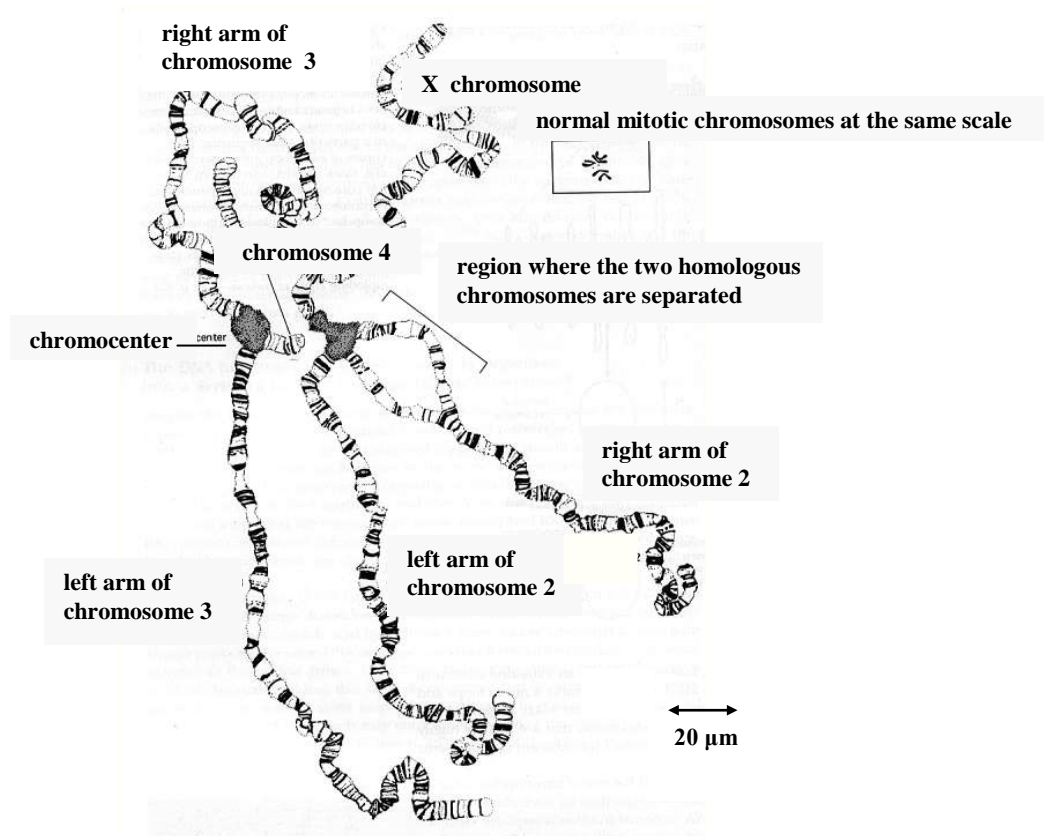


Figure 6.1. Polytene chromosomes in salivary gland cell of fruit fly (*Drosophilla melanogaster*). Image prepared by V. Besendorfer.

C-MITOSIS, KARYOTYPE AND KARYOGRAM

The treatment with cytostatics such as colchicine could induce endomitosis called **C- mitosis**. Cytostatics inhibit formation of the mitotic spindle and chromosomes will not move to the equatorial plate. Instead, they will be spread throughout the cell. Condensed and well spread metaphase chromosomes are suitable for chromosome counting and analysis of their morphology. Morphology of each chromosome is defined by the position of the centromere (primary constriction). **Centromere** is located between two **chromosome arms**, therefore its position determines the length of chromosome arms. If the centromere is in the central (median) position of the chromosome then both chromosome arms are of equal size. Such chromosome is designated as **metacentric** chromosome. When centromere is located a bit far from the centre (submedian position) one chromosome arm will be shorter. Such chromosome is designated as **submetacentric**. **Akrocentric** chromosome possess centromere located near the end of the chromosome (subtelomeric position), while in **telocentric** chromosome the centromere is located at chromosome end and such chromosome has only one chromosome arm. Some chromosomes possess secondary constriction and short chromosome fragment called satellite. Because of the satellite such chromosomes are called satellite chromosomes. Onion (*A. cepa*) possesses one pair of satellite chromosomes.



Figure 6.2. Karyotype of *Anemone hortensis*. Slides and image prepared by V. Besendorfer and J. Mlinarec Novosel.

A **karyotype** is the number and appearance of chromosomes in the nucleus of a eukaryotic cell (Fig. 6.2). Since karyotype in all cells of one organism is the same, we can say the karyotype is characteristic for individual organism or species. Karyotypes of species could be very different. **Karyogram** represent systematic presentation of the chromosomes in the karyotype (Fig. 6.3). Karyogram could be made by cutting the chromosomes from the micrograph and their arrangement in homologous pairs in order from the largest to the smallest. Schematic chromosome representation by which more detail differences could be shown between chromosomes is called **idiogram**. With the help of the computer programs karyotype assembly is easier today. Chromosome analyses are important not only for the study of species-specific karyotypes and karyotype evolution but also, in the case of human genome, for identification of changes in chromosome number and structure which are connected with various diseases including cancer.



Figure 6.3. Karyogram of *Anemone hortensis*. Slides and image prepared by V. Besendorfer and J. Mlinarec Novosel.

CYTOGENETIC TECHNIQUES – SLIDE PREPARATION FOR CHROMOSOME ANALYSIS

Among all cytogenetic techniques the most widely used is the squash technique. The method for chromosome preparation consists of several steps:

1. pre-treatment
2. fixation
3. storage of material
4. staining
5. slide preparation

Depending on the purpose some steps could be omitted. For example, some dyes could be used for fixation and chromosome staining in the same time.

1. PRE-TREATMENT

Depending of the aim of the study, leaving cells could be pre-treated before fixation. For example, if the goal of the study is determination of the chromosome number and morphology characteristic for plant or animal species, the chromosomes must be well spread throughout the cell. Condensed metaphase chromosomes are the most suitable for study chromosome morphology. If the metaphase chromosomes are not pre-treated they will be overlapped and placed in the equatorial plate. Squash techniques will be not sufficient to solve this problem. To prepare good chromosome spreads the cells should be treated with chemicals or ice water in order to prevent formation of the mitotic spindle. Such chemicals are colchicine, α -monobromnaphthalene, 8-hydroksyquinoline etc. (Table 8.1.). Thus, pre-treatment with colchicine (alkaloid from meadow saffron *Colchicum autumnale* L.) will block the mitotic spindle formation, and with the help of squashing, condensed metaphase chromosomes will be well spread throughout the cell. The concentration of colchicine working solution is 0.05 – 1.0%. Duration of the pre-treatments depends upon colchicine concentration. Pre-treatment with 0.05% colchicine lasts 3-4 hours in uncovered bottles to ensure aeration. After pre-treatment the cell division must be stopped by fixation in fixative. COATION! Cytostatics are dangerous chemicals for your health!!

Table 6.1. Chemicals widely used for pre-treatments of roots for the study of somatic chromosomes (Sharma and Sharma 1972).

CYTOSTATIC	CONCENTRATION	PRE-TREATMENT	TEMPERATURE
Ice water	-	24 h	4 °C
colchicine	0.5 - 1 % 0.05%	30 min. - 1h 3-4 h	Room temperature (RT)
α -monobrom-naphthalene	Saturated solution	10 min. - 4 h	10 -16 °C
8-hidroksikinoline	0.002 M	3 - 4 h	12 -16 °C

2. FIXATION

The purpose of fixation is to fix, or stop the cell at the desire stage of the cell division without distortion, swelling, or shrinking of the chromosomes. The most widely used fixatives are solutions of glacial acetic acid and ethanol as listed in Table 6.2.

Table 6.2. Widely used fixative for the study of nucleus and chromatin/chromosome.

FIXATIVE	ETHANOL (96%)	Acetic acid (99,5%)	Chlorophorm
CARNOY I	3 volume portion	1 volume portion	-
CARNOY II	6 volume portion	1 volume portion	3 volume portion

3. STORAGE OF MATERIAL

The material is sometimes not available all the time and thus it should be stored. Both animal and plant material could be stored either in fixative or alcohol (70% ethanol or methanol).

4. STAINING (staining with aceto-dyes)

Mostly used aceto-dyes are carmine and orceine in concentration of 1-2% prepared in 45% acetic acid.

The basis of **Feulgen staining** is cytochemical reaction that enables qualitative and quantitative evidence of DNA in the cell. Hydrolization of the DNA remove purine bases in DNA molecule what result in formation of aldehyde groups that react with Schiff's reagent giving compound a red to violet colour.

Preparation of dye solutions:

Aceto-orceine (working solution: 1% prepared in 45% acetic acid):

Add 2.2 g orceine in 100 ml of glacial acetic acid, mix well and boil gently for about 10 min with frequent stirring. Cool, dilute and filtrate the solution.

Aceto-carmine (working solution: 0.5% prepared in 45% acetic acid)

Mix 45 ml glacial acetic acid and 55 ml distilled water (deH₂O), and add 0.5 g carmine. Boil gently 5 min, mix well, cool and filtrate. Duration of boiling depends on concentration of solution: duration of boiling increases with solution concentration.

Leuco-basic fuchsin (Schiff's reagent):

Add 200 ml boiling deH₂O on 1 g of basic fuchsin. Mix well, cool and filtrate solution. Add 30 mL 1N HCl and add 3 g of potassium metabisulphite (K₂S₂O₅). Solution must be stored 24 h in the darks in container close with stopper, sealed with parafilm and wrapped with aluminium foil. If solution shows faint straw colour, add 0.5 g activated charcoal powder, shake thoroughly and keep overnight on 4 °C.

5. CHROMOSOME PREPARATION (Squash technique)

Practical work

Note: while performing the exercises, fill in the Form 6.

Exercise 1. Make preparation of polytene chromosomes from salivary glands of fruit fly (*Drosophilla melanogaster*). Draw the polytene chromosomes under lower magnification of your microscope and then under the immersion objective draw polytene chromosome with puffs.

Instructions for polytene chromosome preparation:

1. Under the stereo-microscope, in a drop of physiological solution, remove a head of the larva with the histological needle. Salivary glands are connected with pharynx and could be easily recognized according to large, glossy cells.
2. Add drop of aceto-carmin or aceto-orcein on the cells.
3. Cover slide with cover-slip and macerate tissue by rapping with needle (*squash technique*). By this procedure the nuclei will be released from the cell and chromosomes will spread.

Exercise 2. Make few slides of C- mitosis, and find cells with wellspread metaphase chromosomes. Count the chromosomes and determine their morphology. Draw one cell with well-spread metaphase chromosomes.

Instructions for slide preparation:

1. Put the root tips in the colchicine solution (0.05%) or α -monobromnaphthalene (saturated solution – a few drops of α -monobromnaphthalene in 5-10 ml deH₂O, mix thoroughly), 3-4 h, at room temperature (RT).
2. Gently wash the roots in water (**deH₂O) and transfer them in the fixative (fixation must not be shorter than 1 h).
3. Wash roots with **deH₂O (1-2 x) and transfer in 1 M HCl, previously warmed to 60 °C and **hydrolyse** 6-8 min. (Optimal duration of the hydrolysis is defined experimentally). During hydrolysis root tips become soft (cell maceration), purines are removed and aldehyde groups are formed. Aldehyde groups will react with Schiff's reagent.
4. After washing** transfer roots in small bottle with 1-2 ml of Schiff's reagent (see exercise 8), close with stopper and put in the dark at least 30 min or better 1-2 h.
5. If the cytochemical reaction is good the root tips will become violet. Intensity of coloration is weak in the root elongation zone (Why?)
6. Put drop of 45% acetic acid on clean glass slide and prepare cell spreads using squash technique as in previous exercises. In the case of faint staining with Feulgen, the aceto-carmin could be used instead of acetic acid.
7. For preparation of permanent slides, coat cover slip with mixture of glycerol and albumen in equal volumes. Dry cover slip by passing it through the flame. After squashing the cells will adhere to the cover slip.

8. To remove cover slip, put microscope slide with cover slip upside down in 40% ethanol. Put a few glass sticks on the bottom of the dish that cover slip could fall down on the bottom.
9. To dehydrate the cell, transfer the cover slip by forceps in the dish with 80% ethanol for 2 min and then 2 times in 96 % ethanol also for 2 min.
10. Put the drop of synthetic resin "EUPARAL" on the clean microscope slide and carefully put down cover slip with your cells.
11. Dry slides few days at 60 °C (in the oven, on the radiator or some other warm place). Such prepared slides could be used for several years.
12. In laboratory conditions, dry ice method (freezing with CO₂) is used for dehydration of the slides. Cover slip is removed after freezing and microscope slide with cell spreads is used for preparing of permanent slides after overnight drying at room temperature.

** In routine work washing of roots could be omitted

Lesson 7. Meiosis

Two major events characterize sexually reproductive organisms: fertilization (union of haploid gametes) and **meiosis**. Sexual reproduction requires the alternation of haploid and diploid generations and the period of meiosis occurrence during the life cycle is different in eukaryotic species.

1. In multicellular animals, majority of protozoa and some evolutionary lower plants meiosis occur before differentiation of gametes (gametal or final meiosis).
2. In some alga, fungi and protozoa meiosis occur immediately after fertilization. On other words vegetative haploid cells occurred by meiosis of zygote (initial meiosis).
3. In plants, the occurrence of haploid spores through meiosis is followed by mitotic events that result in formation of haploid gametophyte that possess egg and sperm cells (intermediate meiosis).

In diploid cell each chromosome is present in two copies (chromosome pairs). One chromosome of the chromosome pair originates from father and other from mother. Thus, chromosome pairs represent **homologous chromosomes**. During mitosis, after chromosome doubling (DNA reduplication) in interphase, sister chromatids segregate (anaphase) and each daughter cell possess both copies of homologous chromosomes. After meiosis, gametes or spores in plants possess only one of homologous chromosome (one chromosome of the chromosome pair) as a result of the homologous chromosome pairing in metaphase I (**first meiotic division**) and their segregation in anaphase I. Homologous pairing of father and mother chromosomes is characteristic of meiosis (Fig. 7.1). Two paired homologous chromosomes represent structure called **bivalent** (Fig. 7.2), and because each chromosome of the bivalent possess two chromatids, bivalent is composed out of four chromatids (chromatid tetrads). After segregation of homologous chromosomes two nuclei with haploid chromosome numbers occurred. Each chromosome in haploid cell possess two sister chromatids. In **second meiotic division** sister chromatids segregate and four haploid cells occurred. Each haploid cell possesses chromosomes with one chromatid (Fig. 7.3).

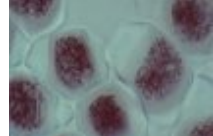

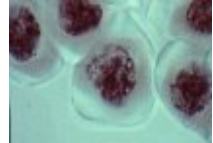





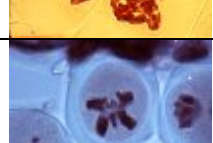

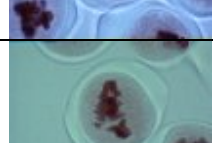

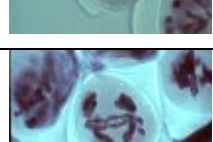

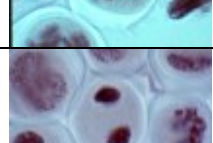

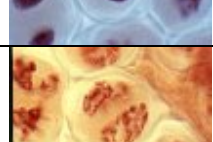





		Prophase I, leptotene – decondensed chromosomes with two sister chromatids folded in a ball structure. Chromomere – heavily staining masses of coiled chromatin
		Prophase I, zygotene – pairing of homologous chromosomes started, the chromosome pairing is precise that means each chromomere of the homologous chromosome precisely coincides. (Synaptonemal complex is not visible under the light microscope).
		Prophase I, pachytene – chromosomes are completely paired and bivalents are formed. (In this phase genetic recombination events, crossing over, occurred. It is an exchange of the genetic material between non-sister chromatids.
		Prophase I, diplotene – homologous chromosomes in bivalent become more condensed and chiasma, a cytological phenomenon of the crossing over, is visible.
		Prophase I, diakinesis – bivalents with chiasmata that moved toward the chromosome ends are clearly visible (chiasma terminalisation). The bivalents are spread all over the cell.
		Metaphase I – kinetochores of homologous chromosomes attach to microtubules from the opposite spindle poles, positioning the bivalents with chiasmata in equator of the spindle apparatus, while centromeres are oriented toward spindle poles.
		Anaphase I – segregation of the homologous chromosomes toward opposite spindle poles (reduction of chromosome number). Each chromosome possess two chromatids.
		Telophase I – chromosome despiralization and formation of two nuclei.
		Prophase II – after interkinesis (without DNA reduplication) the condensation of chromosomes started.
		Metaphase II – chromosomes are positioning in equator of the spindle apparatus like in mitosis with centromeres in the centre of the mitotic spindle. The chromatids are not wrapped around each other.
		Anaphase II i telophase II – segregation of the chromatids, and chromatids become chromosomes. Chromosome despiralization and formation of new nuclei which are genetically different.

Figure 7.1. Meiosis in pollen mother cells of the *Gasteria* sp. ($2n = 14$) floral buds. Left – photos of the meiotic phases (objectiv 100x, ocular 10x); middle – drawing of the meiotic phases (drawn according to microscope slides but not identical to the photos on the left); right – name and description of the phase. Slides and photos prepared by M. Kršnik-Rasol.

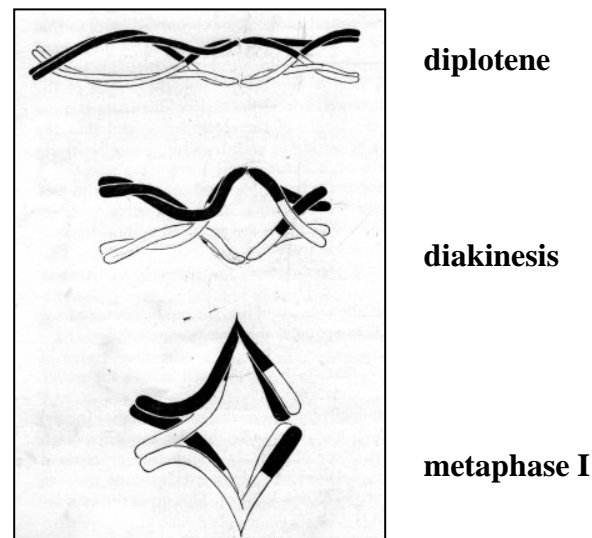


Figure 7.2. Model of the bivalents with two chiasmata. The changes from diplotene through diakinesis to the metaphase I are presented. The condensation of the chromosomes in bivalents is followed by chiasma terminalisation. In diakinesis and metaphase I the position of chiasma is not overlapped with crossing over.

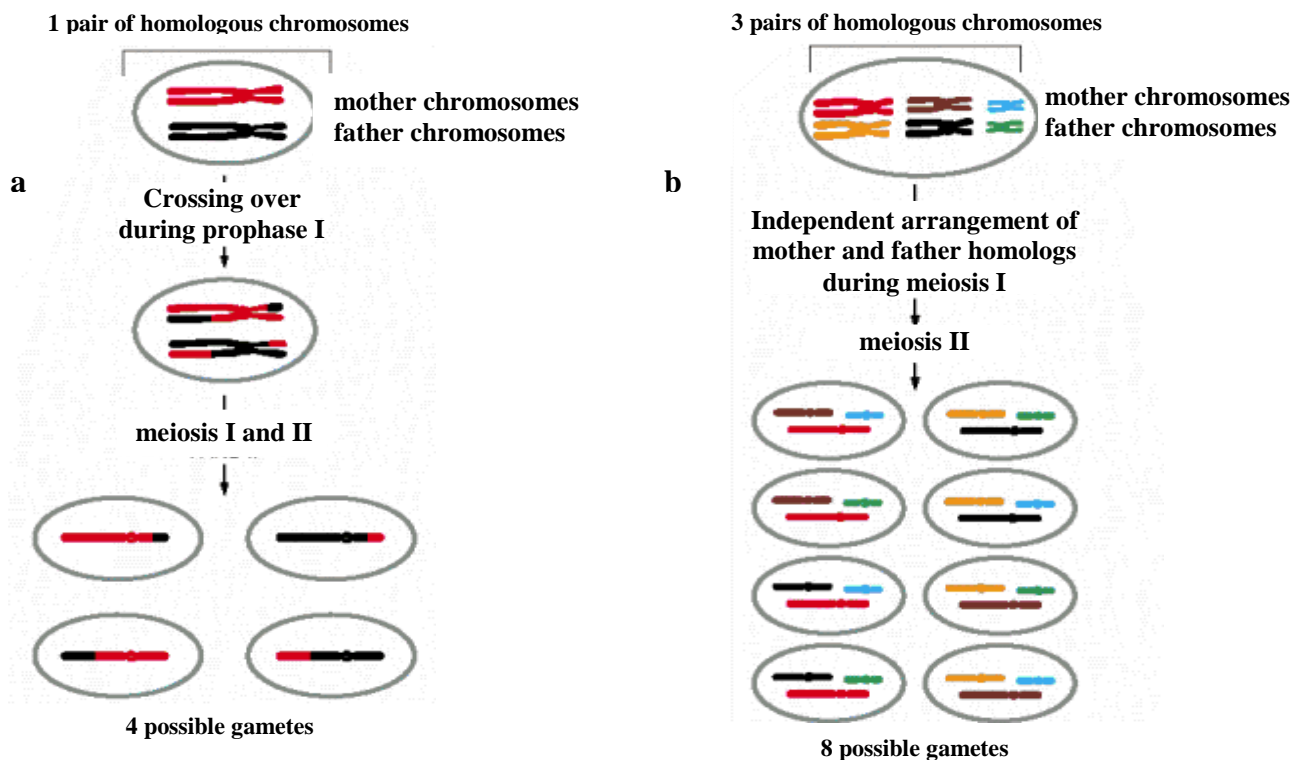


Figure 7.3. Two major contributions to the arrangement of the genetic material that happened in meiosis during gamete formation: **a)** genetic consequences of the crossing over – parts of the homologous chromosomes are exchanged during prophase I and **b)** independent arrangement of mother and father homologs during meiosis I produce 2^n different haploid gametes in an organism with chromosome number n . In this particular case chromosome number $n = 3$ and therefore 8 different gametes could be produced. Both mechanisms increase genetic variability of an organism. Figure prepared by B. Balen.

Exercise

Note: while performing the exercises, fill in the Form 7.

The meiosis will be analysed in the pollen mother cells from the floral buds of plant species: *Gasteria* sp., *Aloe* sp. ($2n = 14$), *Lilium martagon* (or some other lily species), *Allium ursinum*, *Vicia faba*.

Instruction for slide preparation:

1. Take the floral buds from, for example, *Gasteria* sp. or *Aloe* sp., whose floral buds are green and closed.
2. Break away bud and open it with the razor blade and needle open to release the anthers. Put a few anthers in a drop of a dye (aceto-carmin or aceto-orcein), which you previously have placed on the clean glass slide.
3. By squeezing the anthers, they will burst and pollen mother cells will be released from the anther. The dye is used as a fixative and for chromosome staining. Remove anther tissue.
4. Cover cells with cover-slip. After 1-2 min remove surplus dye with filter paper. Avoid formation of air bubbles. For better staining of the cells warm the slides under the flame.

If you choose an appropriate floral bud, the round shaped cells in various phases of meiosis could be seen under the lower microscope magnification. If you cannot find dividing cells than you should repeat the whole procedure with younger or older flower buds. To find all meiotic phases in one slide you should prepare a slide from buds of different maturity (size).

Exercise 1. Check the slide and with the help of Fig. 7.1. and other available photos of meiosis define meiotic phases. Using the immersion objective on your microscope draw cells in diplotene, diakinesis, metaphase I, anaphase I, metaphase II and anaphase II. Annotate all drawn structures.

Exercise 2. By simple schematic drawing display the procedure of meiotic slide preparation.

Exercise 3. Answer the questions dealing with crossing over and genetic recombination in meiosis. (The questions will be provided by the assistant).

Lesson 8. Isolation of nuclei and DNA fibres

In eukaryotic cells, DNA organization is much more complex than in prokaryotic cells. Eukaryotic genome is composed out of several linear DNA molecules that are organized into structures called chromosomes. In interphase nucleus, chromosomes are visible in the form of thick granular structures called **chromatin**. Chromatin is a complex of proteins and DNA molecule. Protein part of the chromatin is built out of histones, small proteins with high content of basic amino acids (arginine and lysine), which facilitate the binding of the negatively charged DNA molecule. Histone core, around which the DNA molecule is coiled, is built out of four histone types, H2A, H2B, H3 and H4, which are present in the form of protein dimers (two molecules out of each histone type). This structure is called **nucleosome** and it represents basic chromatin structure. Hence, the first level of the DNA packaging is formation of nucleosome, which is with the help of the fifth H1 histone packed more tightly, and additionally condensed by folding into fibres (thickness 30 nm), called **chromatin fibres**.

Chromosome organization in the form of chromatin fibres (30 nm) is visible not only by electron microscope, but also by light microscope by application of the technology of the elongated DNA fibres (Figure 8.1). This technology is based on the release of the chromatin fibres from the interphase nucleus on the microscope slide. Elongated fibres released from the proteins are decondensed, and the level of the condensation is approaching the length of the DNA molecule (Figures 8.2 i 8.3).

The procedure has two phases:

1. Isolation of interphase nuclei and fixation on the microscope slide
2. Nucleus lysis and protein extraction by which the chromatin fibres are released from the interphase nuclei.

Disruption of nucleus and chromatin structure is obtained by the usage of detergents and EDTA, which brings to stretching of DNA fibres along the microscope slide. Degree of fibre stretching is dependent on the DNA origin (tissue type, plant or animal cell) and the extraction conditions.

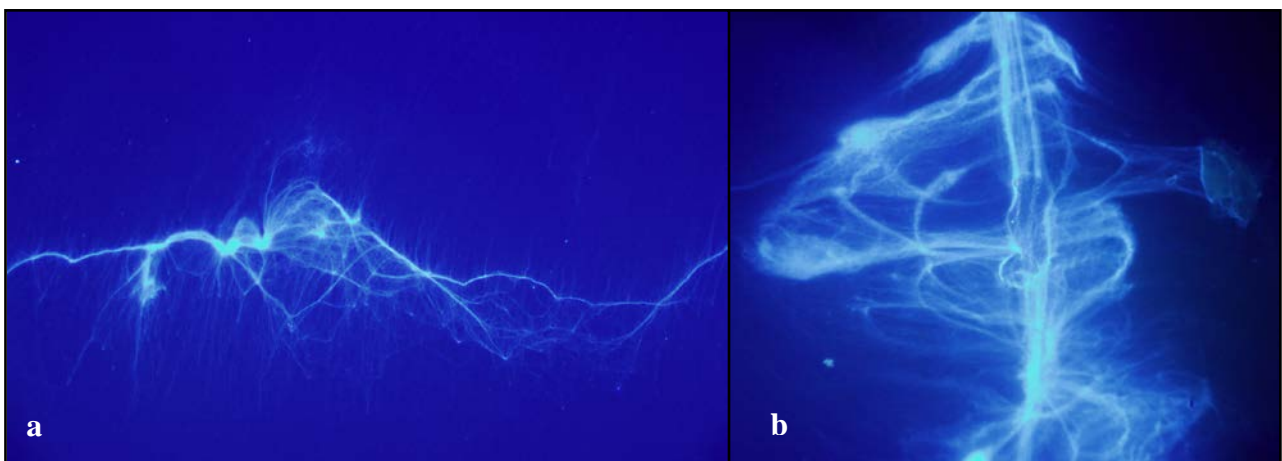


Figure 8.1. DNA fibres of *Allium cepa* stained by fluorescent dye DAPI: **a)** image at total magnification of 200x, **b)** image at total magnification of 400x. Preparation and imaging by P. Peharec Štefanić and B. Balen.

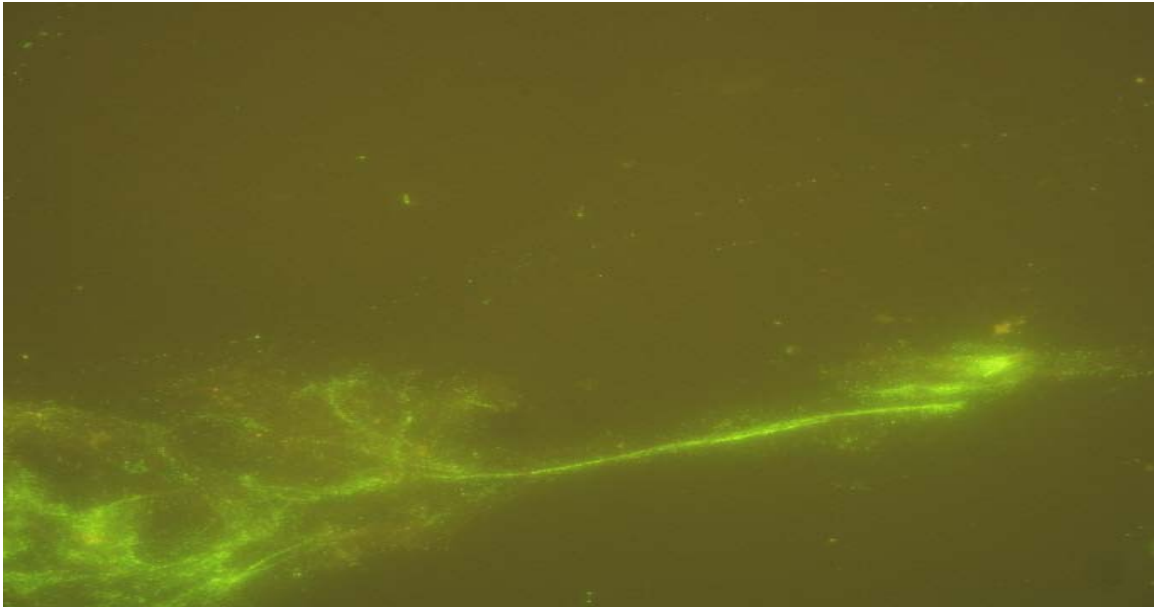


Figure 8.2. DNA fibres of *Anemone hortensis* – chromosome terminus. Fluorescent hybridisation *in situ* (FISH). Green labelled probe depicts telomere repetitive sequence (TTTAGGG) of DNA molecule. Preparation and imaging by V. Besendorfer and J. Mlinarec Novosel.

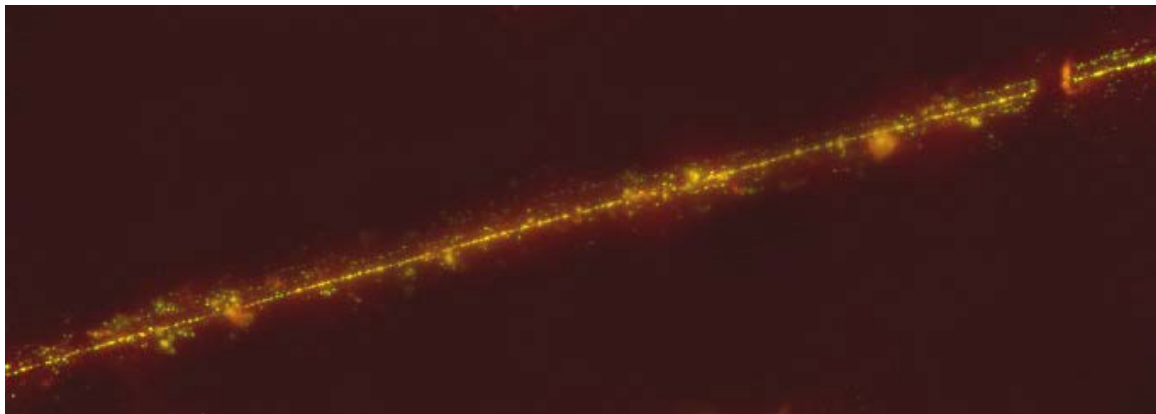


Figure 8.3. DNA fibres of *Anemone hortensis* – subtelomer ends of chromosomes. Fluorescent hybridisation *in situ* (FISH). Red labelled probe depicts repetitive sequence of DNA molecule. Overlap of green and red signal results with yellow coloration. Preparation and imaging by V. Besendorfer and J. Mlinarec Novosel.

ISOLATION OF NUCLEI FROM *Allium cepa* LEAVES**Practical work**

Note: while performing the exercises, fill in the Form 8.

IMPORTANT! The whole procedure should be conducted at 0-4 °C temperature

1. In a glass container seep 800 µl of 400 mM Tris-HCl buffer, pH=7 in which onion or sugar beet leaves (approximately 2-3 leaves) should be chopped by razor blade.
2. Leave 30 min on ice.
3. Centrifuge few seconds at 2000 x g through the grid.
4. Check efficacy of nuclei isolation under microscope (thick nuclei suspension).

(If you do not perform lysis of nuclei instantly, nuclei should be stored at -20 °C in a mixture of buffer and 100% glycerol, 1:1 v/v.)

Microscope sample preparation:

1. Seep 2-3 µl of nuclei suspension on the edge of microscope slide, stretch and wait for 4 min.
2. Seep 10 µl of STE buffer for nuclei lysis on nuclei and incubate for 4 min.
3. Put the microscope slide transversely and stretch fibres by dragging the cover slide downwards.
4. Leave the sample to dry at room temperature.
5. Fix the sample for 2 min in the mixture of acetone and alcohol (1:3 v/v) at room temperature.
6. Dry the sample at room temperature (approximately 15 min).

Sample staining with fluorescent dye DAPI:

1. Incubate the sample in McIlvain buffer, pH=7.0 for 15 min.
2. Drain the sample.
3. Seep 100 µl of DAPI stain solution (conc. 2.0 µg/ml) and cover that part with inner side of folium. Cover all together with cover plate and incubate for 10 min.
4. Wash the sample with deH₂O.
5. Embed the sample in the solution of McIlvain buffer and glycerol (1:1 v/v). Seep the small droplet of the solution (10-20 µL) on the sample and slowly place the cover slip on the top of the droplet.

Place the slide on the stage and view it with fluorescence microscope using the appropriate wavelength (360 nm).

STE buffer for nuclei lysis

0,5% SDS
5 mM EDTA
100 mM Tris*
Adjust pH=7 with addition of 1 M or 5 M HCl

Buffer should be autoclaved.

*stock solution: 1 M Tris-HCl - 60.57 g Tris base dissolve in 500 mL deH₂O and adjust pH at 7.5-7.8 with addition of concentrated HCl

McIlvain buffer

A) Dissolve 0.4208 g of citric acid in 20 mL deH₂O
B) Dissolve 7.1632 g of Na₂HPO₄ x 12 H₂O in 100 mL deH₂O or
Dissolve 2.8200 g of Na₂HPO₄ in 100 mL deH₂O

Mix 18 mL of solution A and 82 mL of solution B and fill up to 200 mL with deH₂O.

Exercise 1. Depict the scheme of the nuclei isolation procedure.

Exercise 2. Draw isolated nuclei stained with either aceto-orcein or aceto-carmin dye. Seep one droplet of the suspension on the microscope slide and then add one droplet of the stain solution. Cover with cover slide. Draw the image obtained with 40X magnification objective.

Exercise 3. Examine the DNA fibres with fluorescence microscope.

Lesson 9. Isolation of genomic DNA from plant material with „do it yourself“ method

As you already know, deoxyribonucleic acid (DNA) is the genetic/hereditary material present in the cells of all organisms, from bacteria to humans. It consists of two antiparallel strands of sugar (deoxyribose) and phosphate (PO_4^{3-}), connected with hydrogen bonds between the nitrogenous bases adenine and thymine, and guanine and cytosine. Phosphates (PO_4^{3-}) give DNA molecule a negative charge, making it water-soluble. The process of isolation of very pure DNA (that will be eligible for subsequent manipulations such as cutting with restriction endonucleases, radioactive or non-radioactive labelling, ligation, etc.) requires a relatively complex procedure. It almost always involves the use of special detergents, alkalis, organic solvents and/or enzymes. However, DNA can be isolated (and be seen with the naked eye!) by doing a pretty simple procedure which, in terms of material, does not require anything more than what you can find in your own kitchen.

Briefly, the DNA isolation procedure consists of three basic steps:

1. mechanically breaking the tissue and opening the cells
2. release of the cellular content into the solution
3. precipitation/sedimentation of DNA from the solution

Your task during this exercise will be the isolation of DNA in these three steps. There will be only “kitchen equipment” at your disposal: a mixer-cutter, coffee filters, table salt and detergent. Source of DNA will be different for each of the four groups (3-4 students per group). Each group will find the following isolation “tools” on their table: graduated cylinder, glass/plastic funnel, beaker, Erlenmeyer flask, glass rod, a wooden stick, a micropipette and a small plastic tube (colloquially: Eppendorf tube) of 1.5 mL. One of the critical moments in isolation of the DNA as pure as possible is its separation from the proteins with which it forms a complex. This is achieved in laboratory practice by using organic solvents phenol and chloroform (trichloromethane) or by adding some of the purified proteolytic enzymes (synonyms: proteases, proteinases, peptidases) that break peptide bonds between amino acids of a protein. In your case, the separation of DNA and protein will be improvised by using pineapple juice (it contains a proteolytic enzyme bromelain).

Exercise

Note: During the exercise fill in the Form 9.

Starting material:

- kiwi (2 pieces per isolation)
- broccoli (about 200 g per isolation)
- banana (1 piece per isolation)
- cucumber (0.5-1 piece per isolation)

(In all cases it will be necessary to add a smaller volume of water in the mixer-cutter for better homogenization of the material.)

“Extraction solution” (prepare around 150 mL)

- water : detergent = 9:1
- 2% table salt (w/v)

Procedure/protocol:

1. Shred your plant material in the mixer-cutter.
2. Add around 50 mL of each homogenate (shredded plant material) in a beaker and fill it with double the volume of the “extraction buffer”, incubate the suspension at 60 °C for 15 minutes. Stir occasionally with a glass rod.
3. Filter the suspension through a coffee filter (using a funnel) and collect the filtrate in a glass tube (you won't need more than 4-5 mL of filtrate); throw the rest of the homogenate in a garbage bin.
4. Cool the filtrate shortly on ice and, using a micropipette, add 0.75 mL of pineapple juice (bromelain). Incubate 10 minutes at 36 °C (use the closest natural thermostat).
5. Now carefully add the same volume of ice-cold 96% ethanol to the filtrate (slowly down the side of the tube; liquid phases should not be mixed!)
6. Set the glass tube on ice to enhance precipitation of DNA.
7. Try to wrap the precipitated DNA around a wooden stick or collect it using a micropipette with the blue plastic tip and transfer it in a small plastic tube (“Eppendorf”) of 1.5 mL.
8. You will try to precipitate the DNA to the bottom of the tube using centrifuge (max speed, 5 minutes), remove the excess ethanol, dry it in fume hood and then dissolve it in 50 µL of TE buffer (10 mM Tris-HCl, pH 7,5; 1 mM EDTA; Tris = Tris (hydroxymethyl) aminomethane buffer; EDTA = ethylenediaminetetraacetic acid, a chelator of metal ions).
9. You will store DNA at -20 °C.

Lesson 10. Isolation of genomic DNA from transgenic plant *Arabidopsis thaliana* and plasmid DNA from *Escherichia coli* laboratory strain

Now that you have learned how to isolate DNA from fruits and vegetables in a very simple way in the previous exercise, your assignment during this exercise will be to repeat the same thing using more complex laboratory methods on organisms *Escherichia coli* and *Arabidopsis thaliana* (thale cress). Also, we will define several new concepts: transgenic plant, transgene, plasmid, transformation, vector.

A strain of *E. coli*, which you will receive in the form of overnight liquid culture, contains plasmid. The task of each of the 4 groups will be to isolate the plasmid. What is plasmid? The plasmid is autonomous (independent), replicating (which is dividing), extrachromosomal (outside of the chromosome), circular DNA molecule that is located in the cytoplasm of bacterial cells separated from the bacterial genome (which is also circular DNA molecule, but much larger). Plasmids, as well as bacterial DNA (bacterial chromosomes) contain genes. Genes on plasmid have no effect on growth and survival of bacteria in normal conditions. However, under conditions of selective pressure plasmids allow survival of bacteria containing them. Thus, for example, plasmids may carry genes conferring resistance to heavy metals and /or antibiotics, genes responsible for bacterial virulence, genes that enable degradation of various complex compounds or genes that allow conjugation. By modification of natural plasmids, “artificial” plasmids were obtained, and they are used in recombinant DNA technology (“cloning”). Those plasmids are usually referred to as vectors. Vectors contain a sequence (sequence of nucleotides in DNA) that is of interest for us, usually it is gen for a particular trait. In our case, *E. coli* bacteria are used as a replicator/”factory” of vectors because during the each bacterial cell division plasmids/vectors are also divided and distributed to daughter cells. By isolating the plasmid we get a possibility for manipulation (eg. cutting it with restriction enzymes) and transfer to other organisms: eg. plants. Now we are getting to your second task: the isolation of genomic DNA from model plant *A. thaliana* (thale cress). One plant line of thale cress contains the same gene located on the plasmid in its genome, as the gene that you isolated from *E. coli*. In the plant it is built into plant DNA and does not exist as an independent extrachromosomal element. That gene (as well as any other foreign gene that we introduce into an organism) is called a transgene, and the process of its introduction into the plant is called transformation. The plant that carries such transgene is called a transgenic plant or a genetically modified plant (GMO – genetically modified organism). Two groups will isolate the DNA from transgenic line of *A. thaliana* (containing a part of plasmid/vector DNA), while the other two groups will isolate DNA from a wild type of *A. thaliana* (natural, nonmodified line). In the following exercise you will try to show that transgenic line of *A. thaliana* contains the same DNA sequence as the plasmid you isolated from *E. coli* (thereby, you can mark the plant as GMO), in contrast to the natural line/wild type which should not contain that sequence (as the plant was not transformed).

Exercise

Note: While doing the exercises at the same time fill in the Form 10.

1. Working solutions / buffers (for isolation from *E. coli*):

- GTE buffer (50 mM glucose; 25 mM Tris-Cl, pH 8; 10 mM EDTA, pH 8)
- cell lysis solution (0.2 M NaOH, 1% SDS - sodium dodecylsulfate, an ionic detergent)
- 3 M potassium acetate (CH₃COOK), pH 5.2
- isopropanol
- 70% ethanol
- TE buffer, pH 7.5 (see exercise 9)

The procedure/protocol for the isolation from *E. coli* (so-called mini prep):

1. Pour 1.5 mL of overnight *E. coli* culture in a plastic tube and centrifuge for 3 minutes at 10 000 rpm.
2. Pour off all the supernatant (SN) in an Erlenmeyer flask (never do it in a spout/sink; bacteria you are working with are transgenic organisms/GMO), add 0.2 mL of GTE buffer to the cell precipitate and dissolve it using a micropipette.
3. Prepare 0.4 mL of cell lysis solution (you have available: 0.5 M NaOH and 10% SDS). Add this 0.4 mL to your sample and stir gently by turning the plastic tube slowly several times for 180°.
4. Incubate the sample on ice for 4 minutes and then add 0.3 mL of 3 M potassium acetate.
5. Now gently mix the content of the plastic tube by turning it slowly several times for 180°.
6. Incubate the plastic tube on ice for 5 minutes, then centrifuge for 5 minutes at 13 000 rpm.
7. Transfer the supernatant (SN, around 0.8 mL) in a new plastic tube and throw the old plastic tube (to a garbage bin in your workplace).
8. Add 0.6 mL of isopropanol to the SN from the previous step and stir it as it was explained before.
9. Incubate the sample at -20 °C for 5-10 minutes and then centrifuge it for 5 minutes at 13 000 rpm.
10. Remove and throw out the SN (in Erlenmeyer flask).
11. Add 0.5 mL of 70% ethanol to your precipitate and centrifuge for 3 minutes at 13 000 rpm.
12. Remove all the ethanol and dry the precipitate in the fume hood.
13. Dissolve the precipitate in 0.05 mL of TE buffer, incubate at 37 °C for 10 minutes.
14. Mark and store your samples at -20 °C for the next exercise.

IMPORTANT: After centrifuge of bacteria pour off the SN into Erlenmeyer flasks. SN contains remains of bacterial cells that should not spread into the environment! You can also pour off SN from the other steps into the same Erlenmeyer flask, even though these steps do not involve living cells. While working with plant transgenic lines there is no need for caution because you are not working with plants that have already created the mature seed.

2. Starting materials and equipment:

- 2 lines of the model plant *Arabidopsis thaliana* (transgenic line and wild type)
- overnight culture of *E. coli* (cells contain plasmid with transgene)
- microcentrifuge
- mortar and pestle
- Eppendorf tubes (1.5 mL)
- a set of buffers and solutions
- micropipettes and plastic tips
- Erlenmeyer flask
- ice

Working solutions / buffers (for isolation from *A. thaliana*):

- 0.5 M NaOH
- 1 M Tris-HCl (or Tris-Cl), pH 7.4 (buffer)
- isopropanol (synonyms: 2-propanol, isopropyl alcohol)
- 70% ethanol
- TE buffer, pH 7.5 (buffer)

The procedure/protocol (for isolation from *A. thaliana*):

1. Add 300 μ L of 0.5 M NaOH to the bottom of the mortar using a micropipette.
2. Take 2 bigger or 3-4 smaller plantlets from the ground using a tweezer, remove the stem and put them in the mortar.
3. Using circular motions of the pestle finely chop up the plant material.
4. Take 250 μ L of the resulting green suspension with a micropipette and transfer it to an Eppendorf tube of 1.5 mL.
5. Put the same volume of 1 M Tris-HCl into the tube containing your suspension.
6. Stir and centrifuge for 3 minutes at 13 000 rpm.
7. Transfer the SN to a new Eppendorf tube and dispose precipitate and the old tube.
8. Add 0.5 mL of isopropanol to your SN and stir.
9. Incubate your sample on room temperature for 10 minutes.
10. Centrifuge the sample for 5 minutes at 13 000 rpm and remove SN.
11. Wash the precipitate in 0.5 mL of 70% ethanol and centrifuge for 3 minutes at 13 000 rpm.
12. Remove all ethanol and dry the precipitate in a fume hood.
13. Dissolve the precipitate in 0.05 mL of TE buffer, incubate at 65 °C for 10 minutes.
14. Mark and store your samples at -20 °C for the next exercise.

Lesson 11. PCR, electrophoresis and DNA restriction analysis

In the previous exercise you isolated plasmid/vector from bacteria *Escherichia coli*. A part of that plasmid was embedded in the plant *Arabidopsis thaliana* genome using bacteria (*Agrobacterium tumefaciens*). You also isolated genomic DNA from a transgenic plant and a wild type of thale cress. To determine whether the part of the plasmid from bacteria *E. coli* was really embedded in the plant genome of the transgenic line you will apply today very widespread technique of PCR (PCR = Polymerase Chain Reaction). PCR concept was invented by a (bio)chemist Kary Mullis in the 70s. The idea was technically realized in the mid-80s and it was a real revolution in molecular biology. Kary Mullis won the Nobel Prize for Chemistry in 1993 because of his idea. What is “revolutionary” about PCR? The fact that we can get millions of identical copies of DNA in several hours using only a small number of initial DNA molecules. PCR requires: (1) DNA you want to multiply (so-called mold), (2) two so-called primers/initials (two oligonucleotides, each of which is complementary to one strand of the DNA template), (3) four deoxyribonucleotides (dATP, dCTP, dTTP, dGTP) as the building blocks of DNA and (4) DNA polymerase that catalyzes the formation of covalent (phosphodiester) bond between phosphate and deoxyribose, that is, connects nucleotides in a continuous chain. The PCR reactions consist of about 30 identical cycles. At the end of each cycle of multiplication the number of DNA molecules is doubled compared to the previous cycle (exponential multiplication, fig. 11.4). Each cycle consists of three consecutive steps in which the incubation temperature changes for the above mentioned components: (a) **denaturation** – at 95 °C denaturation of DNA template occurs (complementary strands are separated), (b) **annealing** – incubation temperature is lowered to 50 - 60 °C which leads to binding of primers (oligonucleotides) to the DNA template (each primer binds only to one of the two strands), (c) **new strands synthesis** – at 72 °C thermostable DNA polymerase (usually from a thermophilic bacteria *Thermus aquaticus*) synthesizes the new DNA strand starting from the binding sites of each primer. The synthesis at 72 °C is once again followed with the step of denaturation (a) and the process repeats for desired number of cycles (usually 30-35).

After PCR, you will apply the multiplied DNA to agarose gel and in a buffer expose it to electric field (Fig. 11.1.a). This process is called electrophoresis (Fig. 11.1.b). Since the DNA molecule is negatively charged (due to phosphates) it travels in this electric field towards the positive electrode (anode). During that travel DNA molecules of different sizes go at different speeds and in the end of electrophoresis can be seen as separate bands/fragments in the gel. Agarose that is used for gel production is a natural linear polysaccharide polymer (based on galactose) derived from some species of algae. It comes in the form of a white powder which melts by heating in water. Cooling leads to mutual connection of polymer chains with hydrogen bonds and formation of a porous gel. The pore size depends on the concentration of agarose which is usually 0.1 – 3% (w/v). Gels with high agarose concentration are better in separating small/short DNA molecules, whereas gels with low agarose concentration separate large/long DNA molecules better. Electrophoresis is usually done in TAE buffer (Tris-Acetate-EDTA), which is a good electrolyte. In the two pictures below you can see the apparatus for gel electrophoresis (Fig. 11.1.a) and bands/DNA fragments of different sizes separated after electrophoresis in agarose gel (Fig. 11.1.b).

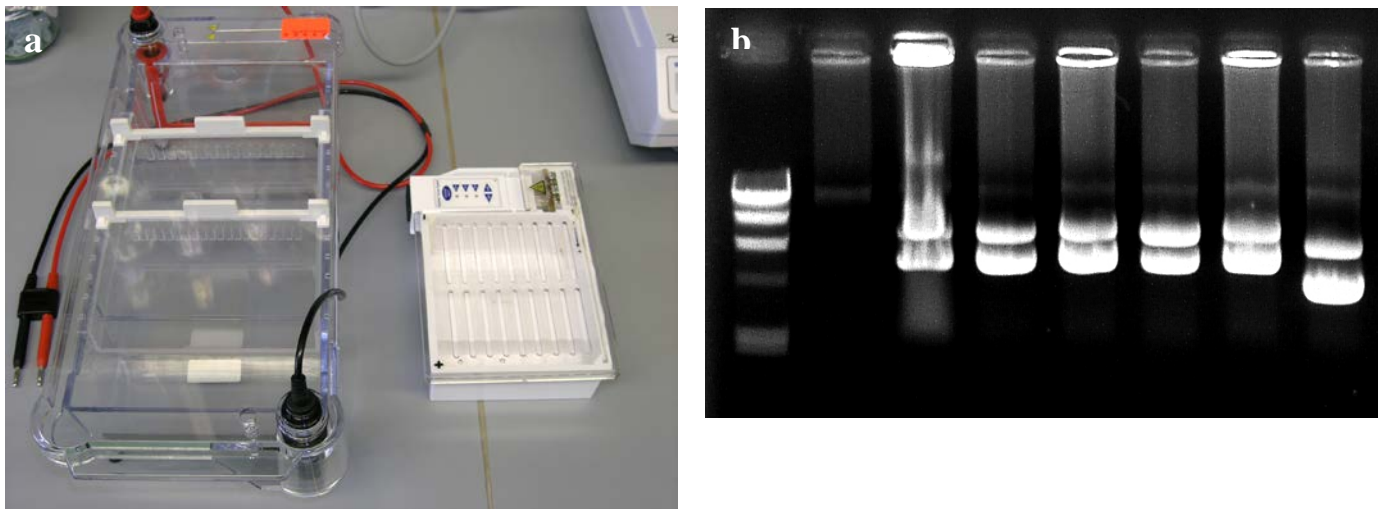


Figure 11.1. a) Two models of tubs for electrophoresis; b) agarose gel after the electrophoresis. DNA molecular marker can be seen in the far left lane. Two bands/DNA fragments can be seen in each lane. Prepared and recorded by N. Malenica.

Your task in this exercise is to check whether the transgenic plant from the previous exercise contains the same DNA sequence in its genome as the plasmid you isolated from *E. coli*. Also, you have to check if the wild type plant really is wild type, ie. if it contains the transgene. Furthermore, you need to cut the fragment of the plasmid that contains the same transgene embedded into the genome of transgenic plant *A. thaliana* using restriction endonuclease EcoRI.

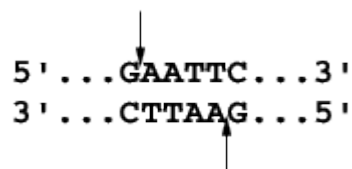
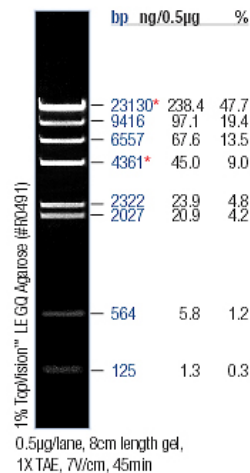


Figure 11.2. The sequence that is recognized and cut by endonuclease EcoRI. Note that cutting leaves protruding 5' ends. Sequences recognized by restriction enzymes are usually palindromic, ie. they are identical on both strands if they are read in the same direction. Image by N. Malenica.

Finally, you need to prepare a gel, apply all the DNA samples on it (three PCR reactions and cut plasmid with EcoRI), separate the fragments using gel electrophoresis and visualize DNA (make it visible). Visualization is done using ethidium bromide which embeds between the base pairs of the DNA and fluoresces in orange-red under UV light. Ethidium bromide does not fluoresce when not bound to the DNA. In addition to the samples, you will put so-called DNA markers on the gel. It is a mixture of DNA fragments of known sizes that travel in gel at the same time as your samples. After electrophoresis those fragments are used as a standard for the fragment size assessment in your sample (Fig. 11.3).

Figure 11.3. DNA marker. Fragment size in base pairs (bp) is marked on the right side. Below the gel photo are listed conditions in which electrophoresis was performed (1% agarose, TAE buffer, voltage 7V/cm, duration 45 min). (<http://www.fermentas.com>)



Important: Ethidium bromide is mutagenic and carcinogenic! Mandatory work with gloves!

Note: While doing the exercises at the same time fill in the Form 11.

1. The starting materials and equipment for PCR:

- PCR buffer (10x concentrated)
- 25 mM MgCl₂
- 2 primers (specific /complementary to the transgene)
- 2 mM dNTP solution (dATP, dGTP, dCTP, dTTP)
- Taq (*Termus aquaticus*) DNA polymerase
- DNA template (plasmid, genomic DNA from transgenic and wild type plant)
- dH₂O (distilled water)
- PCR device
- three eppendorf tubes for PCR

The procedure / protocol for preparing the PCR reaction solution:

1. the total volume of each of your PCR reactions will be 50 µL
2. you will make 3 PCR reactions; each with one of the DNA templates (plasmid, DNA of the transgenic plant and DNA of the wild type plant)

In each reaction add:

- the necessary amount of PCR buffer (dilute from 10x to 1x)
- MgCl₂ to a final concentration of 2 mM
- 1 µL of each primer
- 2 mM dNTP to a final concentration of 0.2 mM
- 1 µL Taq (*Termus aquaticus*) DNA polymerase
- 5 µL template
- dH₂O to 50 µL

- place the samples in the PCR device

2. Starting material and equipment for the restriction analysis of plasmid:

- enzyme/restriction endonuclease EcoRI
- buffer for EcoR I (concentrated 10x)
- plasmid DNA
- dH₂O
- thermostat

The procedure /protocol for restriction analysis of the plasmid:

- total volume of your reaction mixture will be 50 μ L

In each reaction add:

- buffer for EcoR I (dilute from 10x to 1x)
- plasmid DNA (5 μ L)
- 1 μ L enzyme EcoR I
- dH₂O to 50 μ L

- Place the reaction in a thermostat at 37 °C for 2-3 hours

3. Starting material and equipment for electrophoresis:

- agarose
- TAE buffer
- Erlenmeyer flask
- mold for pouring the gel with a "comb"
- adhesive tape for closing the mold for pouring the gel on all sides
- tub for electrophoresis
- power-supply

The procedure/protocol for restriction analysis of the plasmid:

1. Melt the agarose (1% w/v) in 50 mL of TAE buffer (in a microwave)
2. When the gel is cooled to about 60 °C, add ethidium bromide and pour into a mold with a "comb".
3. Wait 20 minutes for agarose to polymerize.
4. Apply PCR samples and restriction analysis of the plasmid in the gel.
5. Start electrophoresis (50-80 V).
6. Observe DNA on the transilluminator (source of UV light).

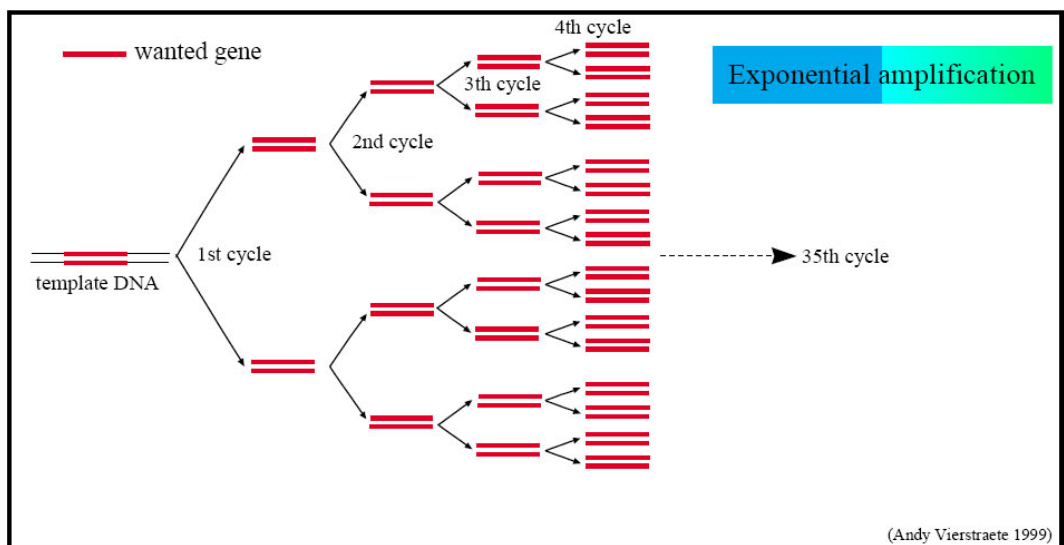
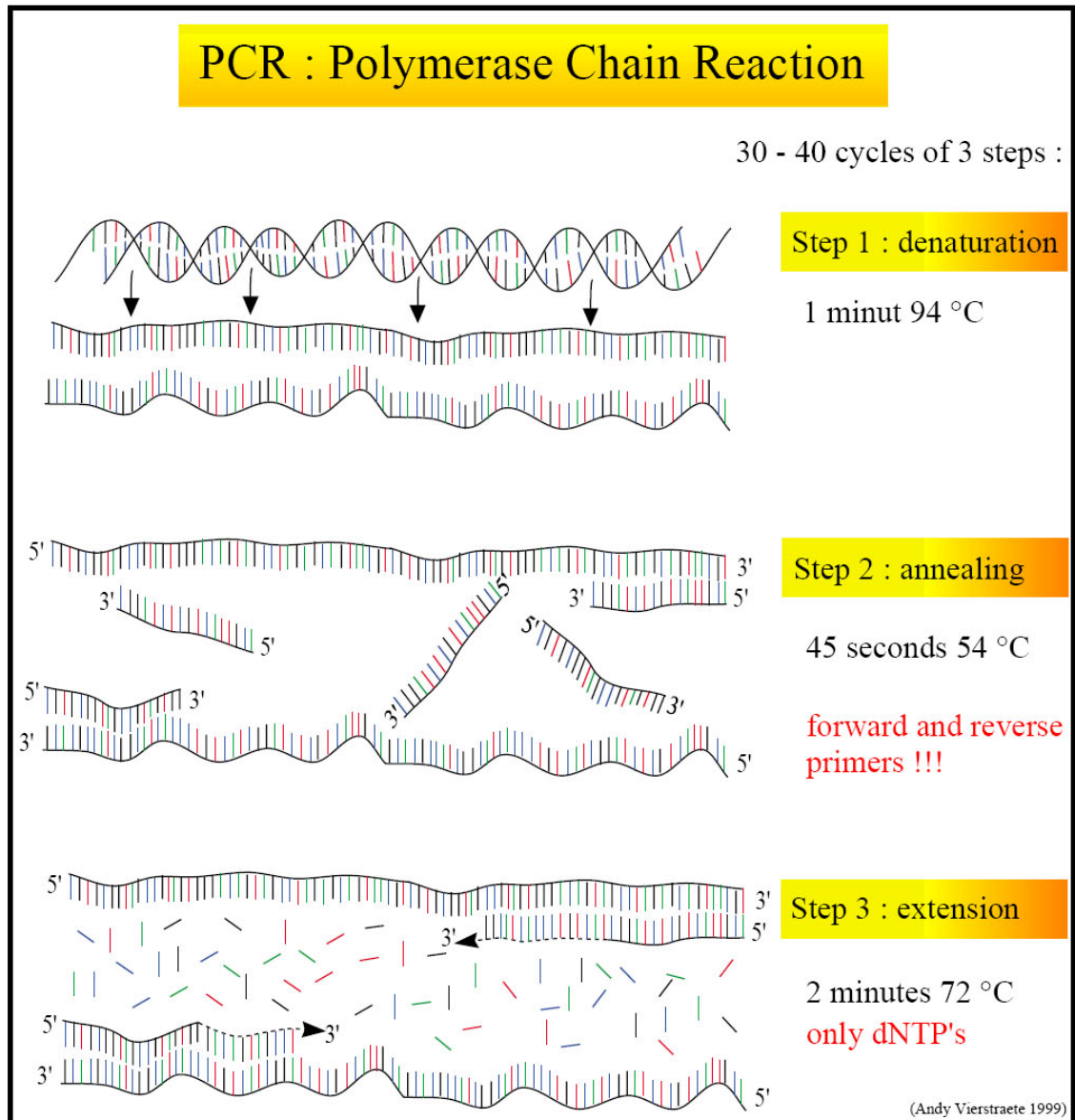


Figure 11.4. Schematic representation of PCR (Andy Vierstraete 1999, customized).

Literature

1. Molecular Biology of the Cell. 5th ed. (2008) Alberts et al. New York: Garland Publishing
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3. The Cell - A Molecular Approach. 6th ed. (2013) Cooper and Hausman, Sinauer Associates
4. Molecular Cell Biology. 4th ed. (2000) Lodish et al. New York: W. H. Freeman & Co.