Cell Biology
Laboratory Manual

University of Debrecen
Medical and Health Science Center

Department of Biophysics and Cell Biology

Zsolt Bacsó
Péter Nagy
Mózes Péter
Tamás Varga
György Vereb
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modifications by Zsolt Fazekas
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Color code to modifications:
-additional text is indicated by yellow colour
- ~~crossed~~ text in red colour indicates parts do not need to study or do

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note: due to the additional texts, most of page numbers gone wrong.

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Studying the physical and chemical processes leading to cell death (a.k.a. Testing cell viability)

Mechanism of cell death

A living cell is system which is in dynamic equilibrium with its environment. It can adapt to changes in the parameters of its surroundings. However, if the extent of changes exceeds a certain limit, cell damage ensues.

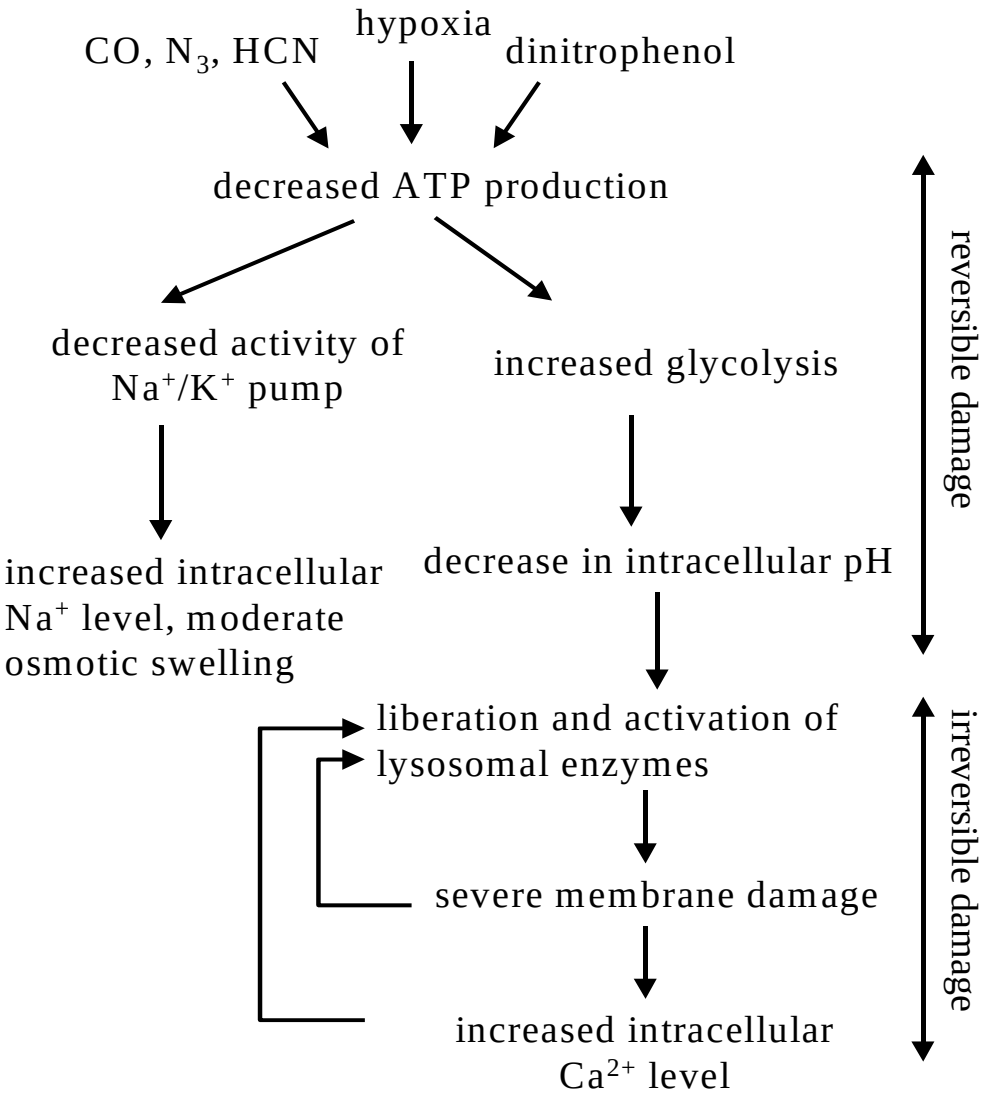


Fig.1. Simplified effect of agents destroying mitochondrial energy production

There are numerous things that give rise to cell damage, but secondary processes induced by primary ones are more schematic. Primary processes usually liberate different mediators (e.g. free radicals, as discussed later) and start secondary processes, that finally result in cell damage or death. Based on secondary processes we discuss two mechanisms: primary **damage in the metabolic activity** of cells and **destruction of the cell membrane**. However, as we will see later, these processes are often interrelated (e.g. membrane damage is also observed in cells with impaired metabolic activity). Several primary processes can give rise to impaired metabolic activity:

- hypoxia (decrease in the concentration of O_2, e.g. in the case of a heart attack in the area supplied by the occluded artery),
- agents impairing mitochondrial function (e.g. azide, cyanide and carbon-monoxide, that all destroy the mitochondrial electron transport system because they stabilize either the ferric (azide and cyanide) or ferro (carbon-monoxide) state of iron; dinitrophenol, which permeabilizes the inner mitochondrial membrane for protons thereby eliminating the proton electrochemical gradient and ATP production)
- long starvation of a cell.

Each of the above processes decreases the production of ATP. A cell tries to defend itself against the deleterious effects of low ATP concentration: if mitochondrial energy production shuts down (e.g. because of hypoxia, since oxygen is the final electron acceptor in the electron transport system), the cell tries to produce ATP in glycolysis. If low serum glucose level precludes glycolysis, fatty acids and proteins start to be degraded. If the factor responsible for the damage is permanent and robust, cellular ATP level considerably decreases in spite of these adaptive processes. As a result the activity of ATP-dependent ion pumps in the plasma membrane (at the beginning mainly Na^+/K^+ pump) decreases. This results in an increased sodium influx into the intracellular space. This process also occurs in healthy cells (as a result of the unspecific permeability of the cell membrane and is partly connected to certain cellular activities like, e.g. sodium influx occurs in neurons during action potentials), but it is counterbalanced by the activity of ion pumps in unimpaired cells. If sodium is not pumped out of the cell, intracellular sodium concentration increases. This results in osmotic swelling of the cell, since sodium is followed by water to maintain osmotic equilibrium. Alterations in the plasma membrane take place: membrane protrusions (blebs) appear. Water influx into the cell is made possible by the anomalosly high water permeability of the cell membrane. In some cells (e.g. red blood cells) a membrane protein, which forms water permeable pores in the membrane and is called CHIP (channel forming integral protein) or aquaporin, is responsible for this.

A cell tries to defend itself from osmotic damage. If a cell is osmotically swollen, certain ion channels are activated (e.g. chloride or potassium channels, but never sodium channels), the opening of which gives rise to outflux of

certain ions (e.g. chloride or potassium in the cases referred to above). This is accompanied by loss of water from the intracellular space. These ions are transported without ATP consumption down their electrochemical gradient. If, in other cases, a cell is placed in a hyperosmotic environment, sodium channels open, through which sodium, and as a result, water enters the cell.

As long as plasma membrane damage is not extensive, cell injury is reversible. If degradation of structural elements (lipids and proteins) continues and the intracellular ATP levels remains low for a long time, irreversible damage occurs. The most important factor in this process is the increased calcium permeability of the plasma membrane and intracellular membranes. As a result a lot of calcium enters the cytosol from the extracellular space and from intracellular stores. High intracellular calcium concentration activates calcium dependent proteases and lipases, that further deteriorates the barrier function of membranes. Membranes become permeable for proteins, that results in protein loss from the intracellular space on the one hand, and the liberation of lysosomal degrading enzymes on the other, that causes further digestion of the cytoplasm and the nucleus and finally complete loss of cellular integrity.

The target of another group of noxious factors is the plasma membrane and intracellular membranes. (Damage of membranes is present in the previous mechanism also, but not as a primary factor.) The function of proteins can be impaired (e.g. a ouabain is a specific blocker of Na^+/K^+ ATPase, that causes an increase in the intracellular sodium concentration and osmotic swelling; mercury containing compounds inhibit the function of sulfhydryl group containing proteins including ATP-dependent ion pumps of the plasma membrane). Factors belonging to this group can also attack membrane lipids. Detergents and free radicals are among these agents. Free radicals are neutral compounds that have an unpaired electron. These molecules are unstable and reactive. Free radicals attack double bonds of unsaturated fatty acids and form peroxides, that increase the aspecific permeability of membranes very much. Free radicals can also damage proteins and nucleic acids. Free radicals are generated in the following circumstances:

- exposure to ionizing radiation,
- as a by-product of oxidative metabolism (when an electron is not transported to its suitable acceptor),
- in white blood cells catalyzed by enzymes during the elimination of bacteria and fungi (certain enzymes, e.g. NADPH oxidase, are able to promote the formation of free radicals. These free radicals are able to damage bacteria and fungi. Since the process in under control, it does not greatly damage the host),
- in the case of reperfusion injury. This process occurs when oxygen-rich blood supply is restored to a previously hypoxic area. Oxygen free radicals are probably generated by white blood cells in the blood),

- in some chemical reactions catalyzed by metal ions (e.g. iron in Fenton reaction).

The above processes have three different outcomes:

- the cell quickly dies. This process is called necrosis (this process was described in detail above)
- in other cases another type of cell death occurs that is called apoptosis. The cell actively takes part in this type of cell death by executing a genetic program. The release of hydrolyzing enzymes is much less pronounced in apoptosis, since membranes are less severely affected in this type of cell death. Therefore damage of the surrounding tissue is more restricted.
- the cell is able to adapt to the most moderate injuries.

It is worth mentioning that several important features are shared between apoptosis and necrosis, e.g. the activation of some enzymes occurs in both of the cases. It is the type and strength of the injury (some factors induce apoptosis or necrosis in low or high dose, respectively) and the cell type (some cells easily succumb to injuries and undergo apoptosis, while others not) that determine which particular type of cell death occurs in a given case.

The above mentioned processes are mainly responsible for short-term effects. If the agent attacks DNA, it can have deleterious effects in the long run. Overwhelming damage of DNA can also result in necrotic cell death, but the development of cancer poses an equally important risk on multicellular organisms. A cell tries to defend itself against mutations:

- it tries to repair the mutated genes,
- if the repair of the affected gene is unsuccessful, the cell may undergo apoptosis to avoid the development of cancer. p53 protein plays an essential role in this process.

Recently some modifications have been introduced in the above mentioned nomenclature. Two different types of cell death are defined: apoptotic and oncotic. Oncotic cell death is equivalent to the first steps in necrosis described above. Both processes converge towards a final common pathway. According to the new designation, these final steps are called necrosis. The recognition of the common steps in apoptosis and necrosis (according to the old nomenclature) and the realization that all cell death types finally lead to the complete lysis of the cell led to the introduction of these modifications.

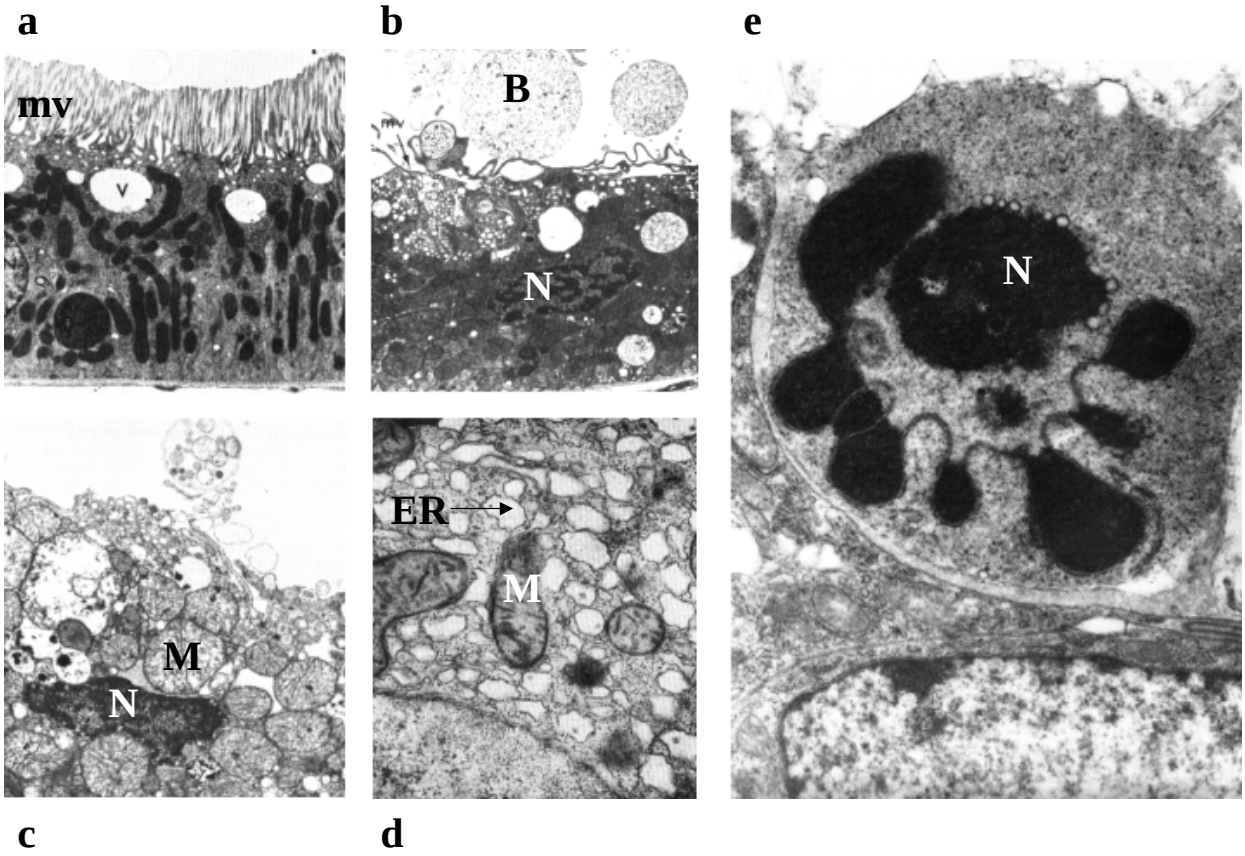


Fig.2.

a. *Normal epithelial cell from the proximal tubule of kidney (mv - microvillus)*
b. *Reversible damage caused by hypoxia in a proximal tubule cell. Microvilli disappeared, and blebs are clearly visible. (B – bleb, N –nucleus)*
c. *Irreversible damage caused by hypoxia in a proximal tubule cell. Large swollen mitochindria (M), severely disrupted plasma membrane, electrondense nucleus (N).*
d. *The effect of CCl_4 poisoning on a liver cell in rat. Endoplasmic reticulum (ER) is swollen. (M – mitocondrium)*
e. *An apoptotic cell in the breast. Nucleus (N) is condensed, cytoplasm and plasma membrane are almost unimpaired. Part of an intact cell can be seen in the bottom of the image.*

Investigation of the viability of cells

Determination of the percentage of dead cells is often an important prerequisite to any kind of experiment. Investigation of viability generally means giving this percentage. Results obtained using different experimental approaches only partly correspond with each other, because it is not straightforward how to define a dead cell, and the sensitivity of experimental approaches of viability is different.

1. The basis of the most generally applied technique is the detection of the increased aspecific permeability of the plasma membrane in dead cells.
   - This is the basis of *the propidium iodide (PI) and ethidium bromide (EBr)* method. The membrane of living cells is impermeable for these indicators. However, they are able to go through the impaired plasma membrane of dying or dead cells. Once inside the cell, they are free to diffuse to the nucleus, where they bind to DNA. This interaction considerably increases their fluorescence quantum efficiency. Based on the above reasoning, only dead cells display fluorescence. Propidium iodide and ethidium bromide are mainly used in flow cytometry. Since this method is based on the fact that live cells exclude these dyes, this approach is often called propidium iodide or ethidium bromide exclusion test. Ethidium bromide is inferior to propidium iodide since it can slowly go through the membrane of live cells, too, due to its lower molecular weight, so live-dead discrimination is compromised.
   - Staining with *trypan blue* (trypan blue exclusion method) has a similar foundation to the above mentioned. This indicator is also only taken up by dead cells with increased aspecific membrane permeability. Trypan blue adsorbs to proteins in the intracellular space. This dye is not fluorescent, but its blue color can be seen in a conventional light microscope. The major drawback of this approach is that trypan blue is able to get into live cells after a longer incubation time.
   - Live cells are able to take up *fluorescein-diacetate (FDA)* and aspecific esterases hydrolize it in the intracellular space. In this reaction a fluorescent dye, fluorescein is generated from non-fluorescent FDA. Due to its hydrophil character fluorescein cannot go thorough the plasma membrane and is retained in the cytosol. Dead cells are not able to hydrolize FDA, since they have already lost their intracellular enzymes, but even if they hydrolyze it, fluorescein diffuses out of the cytosol. Using this approach live cells display an intense green fluorescence. The FDA method is mainly used in flow cytometry and fluorescence microscopy. FDA and PI or EBr can be used simultaneously. In this case live and dead cells display green and red fluorescence, respectively.
   - Measurement of *radioactive chrome (^{51}Cr) release* is basically similar to the FDA method. In this case cells are labeled with radioactive chrome in advance and the percental release of chrome is measured after a certain

treatment. Radioactive chrome is released from cells with increased aspecific plasma membrane permeability. From the percental release the ratio of cells damaged by the treatment can be found out. Recently *europium* is used instead of radioactive chrome. Europium is detected with its luminescence, so the use of radioactivity is obviated. Other methods based on the release of intracellular enzymes (e.g. lactate dehydrogenase, creatin kinase) are very similar to the above mentioned. It is worth mentioning that such assays are used in medicine in the diagnosis of heart attack. In this case the activity of enzymes released from damaged cells is measured.

- The maintenance of membrane potential is indirectly related to the integrity of the membrane. The potential of the plasma membrane and the inner mitochondrial membrane can be separately measured using special indicators.

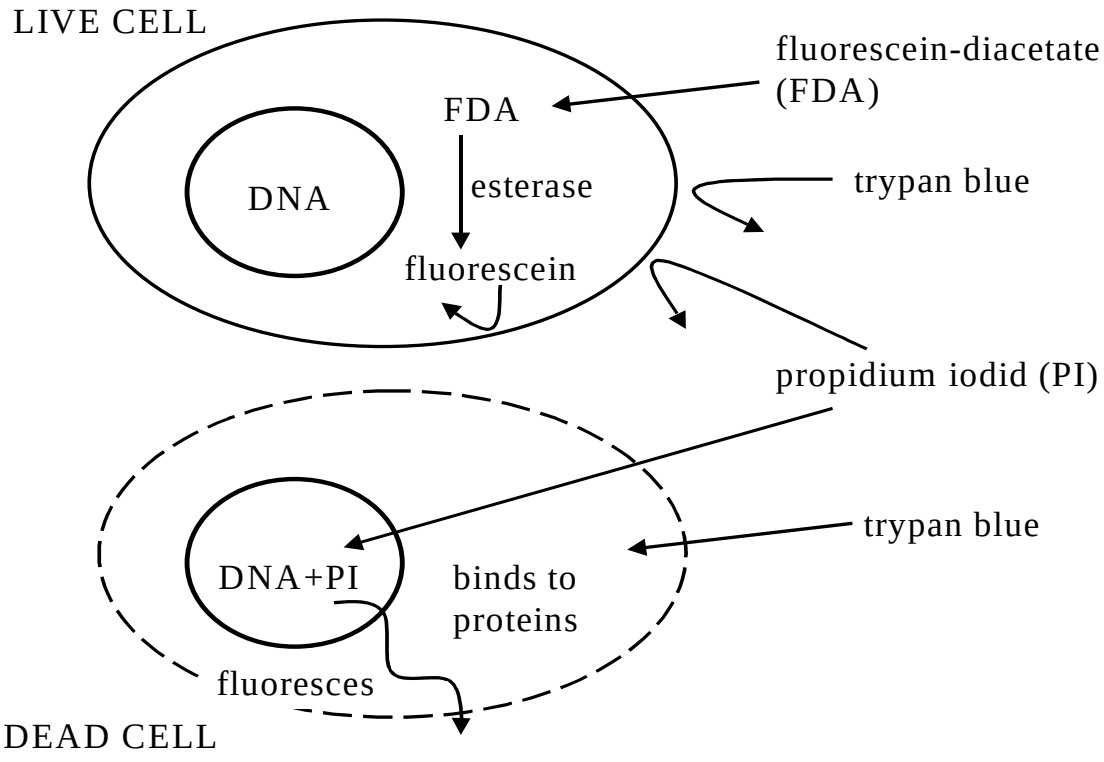


Fig.3. Determination of cell viability using membrane a permeability assay

2. In some experiments cell viability is measured by the assessment of *mitochondrial energy production.* The mitochondrial inner membrane and the mitochondrial matrix is the site of energy production which is accompanied by electron transport and oxidative processes. Therefore if cells are treated with a water-solvable tetrazolium salt (e.g. 3-(4,5-di**m**ethyl-**t**hiazol-2-yl)- 2,5-diphenyl**t**etrazolium bromide, *MTT*), mitochondrial

dehydrogenases reduce them to insolvable, blue formazan, so live cells display blue color. Since mitochondrial electron transport and energy production are needed for cell viability, this method offers a suitable alternative to membrane permeability based techniques. Microculture tetrazolium assays (*MTA*) are used to test the effect of drugs on the viability of cells in culture. MTA is very useful since live-dead discrimination is reliable and only a conventional light microscope is needed.

A related method relies on the measurement of intracellular ATP content to assess cell viability.

3. In experiments where the division of cells is under investigation, viability does not simply mean the integrity of membranes, but it is the cells' ability to respond to a stimulus by division. DNA synthesizing cells incorporate nucleotides. If a cell is incubated in the presence of *radioactive thymidine*, it takes up this nucleoside and synthesizes thymidine-triphosphate from it, which is a substrate of DNA synthesis. Since RNA does not contain timin base, cells not synthesizing DNA do not incorporate thymidine. Measurement of the uptake and *incorporation of brome-deoxyuridine (BrdU)* has the same principle. Brome-deoxyuridine is used instead of timidine in DNA synthesis. This is detected by fluorescent anti-BrdU antibodies.

Tasks to be preformed

Stock solutions

- 0.4 % trypan blue solution in PBS (**p**hosphate **b**uffered **s**aline)
- 0.5 mg/ml fluorescein-diacetate dissolved in acetone (keep refrigerated). Prepare a 20-fold dilution to obtain a 25 µg/ml solution used for staining (prepare freshly each day)
- 1 mg/ml propidium iodide dissolved in PBS (keep refrigerated). Prepare a 5-fold dilution to get a 200 µg/ml solution for staining (prepare freshly each day)

Protocol

1. The viability of HL60 cells (a leukaemic cell line) will be examined on the practice. Suspend the cells, centrifuge them at 400 g for 10 minutes. Then resuspend them in 10 ml Hank's buffer, count the cells. The description of the cell counting can be found with the "Separation of lymphocytes and granulocytes …" experiment, on page 16. Please study that for this experiment, too. Centrifuge them again at 400 g for 10 minutes and resuspend them at a concentration of 10^7/ml in Hank's buffer. Put 80-80 µl of this suspension to four numbered Eppendorf tubes (Sample 1-4).

- Sample 1 will be the **control**, the viability of treated samples will be compared to this sample. Incubate it in a water bath at 37 ^{o}C for 40 minutes.
- Sample 2: Add **0.5 % Na-azide** to this tube at the beginning of the incubation, then incubate it in a water bath at 37 ^{o}C for 40 minutes.
- Sample 3: Put this sample into a **freezer** for 20 minutes, then put it back into the water bath for 40 minutes.
- Sample 4: Add 40 µl distilled water to this sample at the beginning of the experiment, then incubate it in a water bath at 37 ^{o}C for 40 minutes.

At the end of the experiment put the samples on ice. In this way no more change in the viability of cells will occur (or the speed of these processes slows down considerably compared to that at room temperature or 37 ^{o}C).

2. Staining with trypan blue. Investigation with a light microscope
   Put 30 µl 0.4 % trypan blue solution to numbered Eppendorf tubes. Add 30 µl cell suspension to each of these tubes. Incubate them at room temperature for 3 minutes. Drip 10 µl of each solution on slides and cover them with coverslips. Count 50 cells on each slide using a light microscope and a 100x immersion objective. Give the percentage of trypan blue positive (dead) and negative (viable) cells. The color of trypan blue positive cells is very faint.

3. Labeling of cells with fluorescein-diacetate and propidium iodide. Investigation with a fluorescent microscope
   Put 30 µl 25 µg/ml fluorescein-diacetate and 30 µl 200 µg/ml propidium iodide solution into numbered Eppendorf tubes. Add 30 µl cell suspension to each tube. Incubate them at room temperature for 5 minutes. Drip 10 µl of these suspensions on slides and cover them with coverslips. Determine the ratio of propidium iodide positive and fluorescein positive cells. (A cell is usually either propidium iodide positive (completely red) or fluorescein positive (completely green). In some cases cells, that are about to die, still retain their fluorescein content, but are already permeabilized for propidium iodide. These cells appear in orange color. Do not count these cells.)
4. Plot the ratio of dead cells obtained using the trypan blue exclusion and the propidium iodide-fluorescein diacetate method in a bar chart. What results did you get with the two methods?

	Treatment	trypan blue		PI+FDA	
		positive	negative	PI positive	fluorescein positive
1	Control				
2	0.5 % azide				
3	freezing-thawing				
4	200 mOsm				

Separation of lymphocytes and granulocytes, Feulgen and May-Grünwald-Giemsa stain (a.k.a. Separation of blood)

The main constituents of blood

Blood consists of plasma (approx. 56%) and blood cells (approx. 44%). There are three main types of blood cells: red blood cells (erythrocytes), white blood cells (leukocytes) and thrombocytes (platelets). Red blood cells do not have a nucleus, they are disk (doughnut) shaped. Their diameter is 7.2 µm, their thickness is 2 µm at the edges; in the middle it is less. Being flexible they are able to pass through capillaries. In hypoosmotic medium they can easily be lysed. Their concentration in blood is 4.5-5.5 million/µl. White blood cells (leukocytes) possess nuclei, their concentration in the blood is 5000-8000 /µl. There are three types of white blood cells: granulocytes, lymphocytes and monocytes. Granulocytes have multilobed nuclei (that is why they are called polymorphonuclear cells) and granules in their plasma. Three subgroups can be distinguished on the basis of the staining properties of their granules: neutrophil, eosinophil and basophil granules. Lymphocytes make up 20-40 % of the total number of cells in the blood. With the help of a light microscope the small and the large lymphocytes can be distinguished. 92 % of the lymphocytes are small. Lymphocytes are spherical, their nucleus is surrounded by a small layer of plasma. Monocytes make up 3-8 % of white blood cells.

Separation of lymphocytes and granulocytes

Boyum in 1968 published a technique suitable for separating mononuclear cells (lymphocytes, monocytes) from peripheral blood and bone marrow. This method is based on the different densities of the blood cells. Since then separation of other blood cells has become also possible by developing the method. By introducing new techniques it is possible to separate the sub-fractions of different cell types. Separation of blood cells is often used in clinical diagnostics and in research. The densities of blood cells are different: the density of lymphocytes and monocytes is the smallest, the density of granulocytes is higher and the density of red blood cells is the highest.

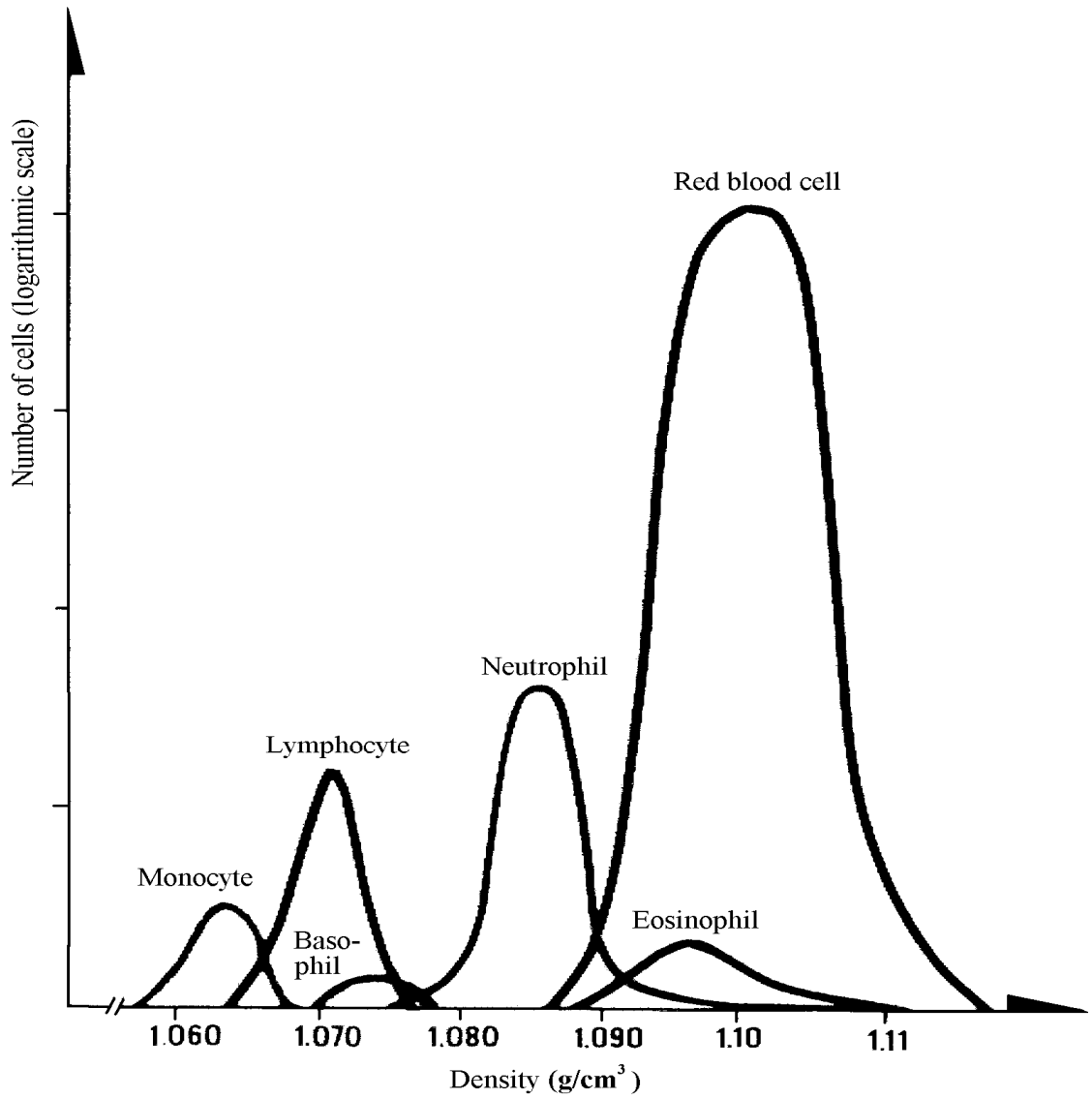


Figure 1: Density distribution of blood cells

Layering diluted blood onto the top of a separation fluid, whose density is higher than that of the mononuclear cells but smaller than the density of the granulocytes, and applying high gravity by centrifugation the mononuclear cells can be separated. Mononuclear cells stay on the top of the separation fluid, but the other cells sediment to the bottom of the tube. The density of separation fluid applied at the practice is 1.077 g/ml. At the end of the centrifugation the plasma is situated on the top, under it the thin opaque layer on the top of the separation fluid contains the mononuclear cells. The bottom layer contains the erythrocytes and the granulocytes. Mononuclear cells can be harvested from the opaque layer. Granulocytes can be separated form erythrocytes dextran sedimentation of the bottom layer (pellet).

Feulgen-staining

The principle of this method is the following: Acid hydrolysis (HCl) removes purin bases from the DNA, thereby unmasking free aldehyde groups. The aldehyde groups then react with Schiff's reagent, which results in the purple staining. RNA is not hydrolyzed by the HCl treatment and, thus, the reaction is DNA-specific.

Tasks to be performed - practical instructions

I. Necessary calculations

Determine the rotor length of the centrifuge provided and calculate the number of revolutions per minute (rpm) to achieve 400g and 250g relative centrifugal acceleration. Use the following formula: $a=0.011\times N^2\times r$, where a is the centrifugal acceleration, N is the rpm and r is the rotor length (measured from the center of rotation).

II. Separation of lymphocytes and granulocytes

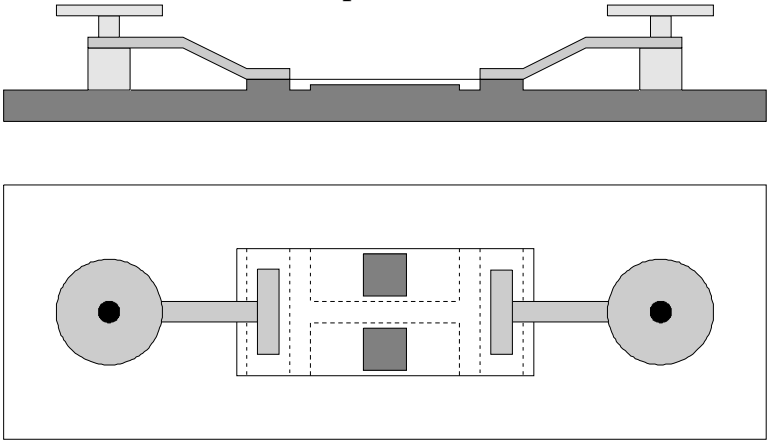
1. Dilute 1 part blood with 1 part PBS (phosphate buffered saline).
2. Carefully layer 2 parts diluted blood onto one part separation solution in at least two centrifuge tubes. It helps to tilt the tube and carefully pipette the diluted blood on the wall of the tube.
3. Centrifuge at 400g for exactly 30 minutes at room temperature, gradually (over 1-2 minutes) accelerating the centrifuge. Make sure the break is off. During this process **prepare the smears from whole blood**.
4. After centrifugation carefully aspirate (with pipette), the layer of the opaque interfaces containing mononuclear cells. Discard the upper layers. (Removing the upper layer is not necessary if you use a Pasteur pipette for transferring the opaque layers to the other centrifuge tube.) Carefully transfer the opaque layers into a clean centrifuge tube. Wash the cells twice. After the second one add 0.3 ml 10% FCS/PBS and mix gently; **prepare smears** using this sample.
5. Remove the separation solution from the layer containing the erythrocytes and the granulocytes. Resuspend the cells in PBS and fill up the tube with PBS so that the total volume will be about 10 ml. Split cell suspension to 5-5 ml portions in two tubes. Add dextran solution (6% dextran 2000 in 0.9% NaCl) to one of the tubes so that the final dextran concentration will be **2-3%**. Compare the sedimentation of red blood cells in the dextran treated and not treated tubes following a ½-1 hour sedimentation. After sufficient

sedimentation in the presence of dextran the upper layer will contain the granulocytes while erythrocytes will be accumulated in the pellet

6. **Procedure for washing:** Fill up the centrifuge tube with PBS and centrifuge at 250 g for 10 minutes. Decant supernatant and resuspend the cells.

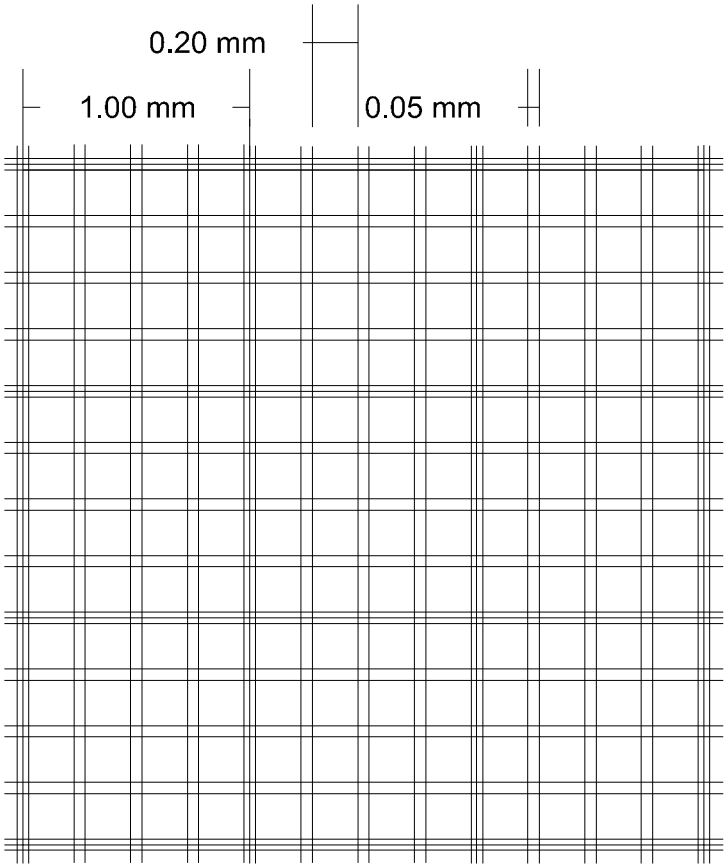
III. Determining the cell concentration:

The haemocytometer (Buerker chamber) was designed for determining cell concentration is supension.



Haemocytometer side view (above) and top view (below)

It consists of a thick glass base and a special coverslip. On the top of the haemocytometer's base two grid areas can be seen. If the special coverslip is placed correctly onto the base then the gap between the base and the covers is exactly 0.1 mm. Place 10 µl of suspension onto the base next to the side of the coverslip close to one of the grid areas. The space between the platform and the slip should be almost completely filled. Since we know the depth of the chamber (0.1 mm) and the distances between the grid lines we can determine the volume of the block defined by any of the squares and the depth of the chamber.



The grid area of the haemocytometer

You do not need to count blood cells. Cell counting will be done during the "Studying the physical and chemical processes leading to cell death" experiment. However, the principles of haemocytometer usage must be studied and will be asked here.

~~Determine the cell concentration of the granulocyte and lymphocyte suspensions using the haemocytometer.~~ Dilute a small volume (10 µl) of the cell suspension in another tube so that the appropriate concentration of the cells will be 10-100 cells/ml. Determine the number of cells inside a 1 mm x 1 mm square. The volume of such a block is 0.1 mm^3. Count the cells above at least 3 squares and calculate the average. Determine the total number of cells. Calculate the number of cells separated from 1 ml ~~blood. Organize your data in the table below:~~

	~~Lymphocyte~~	~~Granulocyte~~
~~1.~~		
~~2.~~		
~~3.~~		
~~4.~~		
~~5.~~		
~~Average~~		
~~Total number of cells~~		
~~Total/ml~~		

IV. Smear preparation and staining

Smear preparation:

1. Degrease the slide with acetone and wait until it becomes dry.
2. Drop the sample onto the slide and streak the drop with another slide.
3. Wait until the smear becomes dry.
4. Fix with methanol for 25 minutes.
5. Dry the smear.

Feulgen stain:

1. Hydrolyze the smears **in 1 N HCl for 10 minutes at +60 °C** (or in 5 N HCl for 40 minutes at room temperature).
2. Rinse in distilled water.
3. Stain for 10 minutes in Schiff reagent.
4. Rinse in 0.05 M $Na_2S_2O_3$.
5. Wash in tap water.

Duplicate/ triplicate smears should be made from whole blood and the lymphocyte layer.

May-Grünwald-Giemsa staining:

This is the commonly used staining of blood smears. Similarly, to other methods in histology it is based on the electrostatic interaction between dye and target molecules. The staining solutions contain methylene blue (a basic dye), related azures (also basic dyes) and eosin (an acid dye). The basic dyes carry net positive charges, consequently they stain nuclei (because of the negative charges of phosphate groups of DNA and RNA molecules), granules of basophil granulocytes and RNA molecules of the cytoplasm of white blood cells. The

eosin carries net negative charge and stains red blood cells and granules of eosinophil granulocytes. It was originally thought that the granules of neutrophil granulocytes were stained by a „neutral dye" that formed when the above-mentioned dyes were combined, but the correct mechanism is not clear.

The nuclei of white blood cells and the granules of basophil granulocytes appear in blue (staining with basic dyes), while red blood cells and eosinophil granules in red (because of red color of eosin). The cytoplasm of white blood cells are light blue, because of the low concentration of RNA molecules.

Solutions:

1. May-Grünwald stain diluted with an equal volume of distilled water
2. Giemsa stain diluted with 9 volume of distilled water

Procedure of staining:

1. Stain the fixed smears for 5 minutes in May-Grünwald stain diluted with an equal volume of distilled water!
2. Put the smears without washing for 30 minutes into Giemsa stain diluted with 9 volume of distilled water!
3. Wash the smears in distilled water and let them dry!
4. Observe the stained blood cells in light microscope!

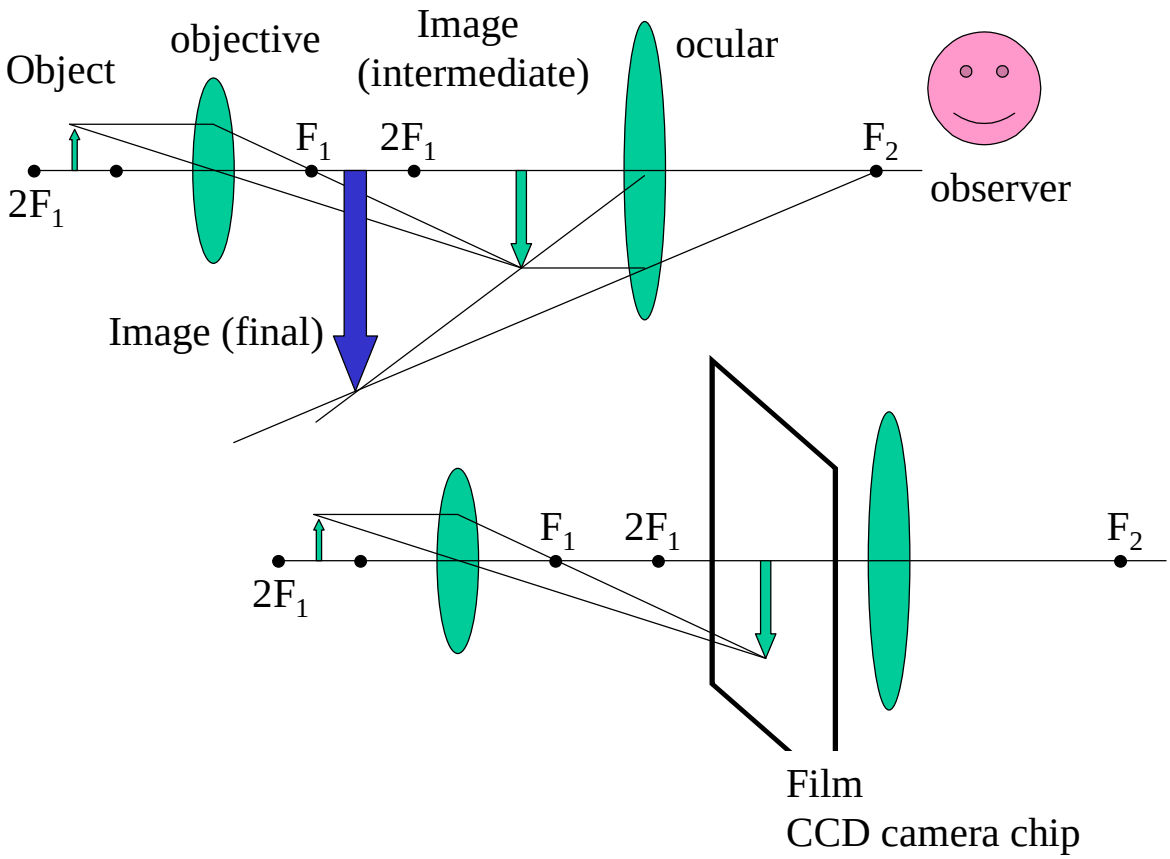
Results of the staining:

The nuclei of white blood cells appear in blue, whereas the cytoplasm in light blue color. The cytoplasm of granulocytes contain some granules staining according to the type of the cell. The red blood cells appear in red. Look for different types of leucocytes! Draw them into the lab book!

Luminescent labelling and microscopic detection of cellular components (a.k.a. Fluorescent microscopy)

Basic principles of microscopy

Microscopes are compound optical systems that are used to magnify the image of small objects. They are made of an objective facing the object of observation and an ocular, or eye-piece, facing the observer. To refresh memories of high school physics, the object has to be located in between the focus (F) and twice the focus (2F) of the objective, and its image, magnified, real and inverted, is on the other side of the objective is outside the 2F distance. The eye-piece is placed so that this real image is inside the focus of the eye-piece. Looking into the ocular we see the virtual, upright, magnified image of this first image. In the end, we see the doubly magnified, inverted, virtual image of the object. If we want to make a picture in the microscope, we normally take the first real image created by the objective, by projecting it onto photographic film, or a the chip of a digital camera.



The parts of the microscopes can be classified into (1) optical parts serving illumination and magnification, and (2) mechanical parts that hold and move the former. The optical parts of the traditional light microscope are comprised of the

condenser (for illumination), the objective and the ocular. For looking at the image with both eyes, a binocular tube is employed, which doubles the first image by means of a prism onto two separate oculars.

Magnification, resolution and visibility

The **magnification** of the microscope is defined by the product of the magnifications of its optical elements. These are the following: the objective (typically between 10x és 100x), ocular (typically between 5x és 20x), and sometimes the prism in the binocular tube (generally 1,5x). The magnification of individual optical elements is the ration of the image distance to the object distance.

The **resolution limit**, that is, the *smallest* distance at the end of which two points are still conceived as two separate points is given by the Abbe formula

$$D = 2/\pi \cdot \lambda / n \sin\varphi = 0.61 \cdot \lambda / NA$$

where $NA = n \cdot \sin\varphi$ is the numeric aperture of the objective, n is the diffraction index of the medium between the objective and the sample, and φ is the angular aperture (1/2 of the angle of light rays the lens can take in when focussed). Thus, using green light of 543 nm wavelength, an objective of NA=1.25 the resolution limit is 265 nm. **Resolving power** is defined as the reciprocal of the resolution limit, R=1/D.
When the object is illuminated from the outside, the NA of illumination is also to taken into consideration as it limits the resolving power. The NA in the Abbe formula then must be estimated as the average of numerical apertures for the illuminating and detecting optics. Thus, using green light of 543 nm wavelength, an objective of NA=1.25 and a condenser of NA=0.9, the resolution limit is 308 nm.

The resolving power often limits the usefulness of magnification. If, for example, we use a magnification of 1000x (e.g., objective 100x and ocular 10x), the smallest resolved distance in the above case (i.e. 265 nm) will be magnified to 0.265 mm. Our eye is capable of resolving 10 separate points on a 1 mm line, so in this magnified 0.265 mm line we will be able to distinguish 2 points, and hence will clearly see the two points separated by the 0.265 mm line. Now suppose we increase our magnification 3-fold by using a 20x ocular and a 1.5x binocular prism instead of the 10x ocular and the 1x binocular prism. The images of the best-resolved two points will be separated by 0.795 mm, in which we could in principle distinguish roughly 8 individual points, but the resolving power of the objective only allows us to see 2 points. Thus we are wasting

magnification power. The rule of thumb is not use a magnification exceeding 1000x the NA of your objective.

As it can be deduced from the above, the resolving power can be improved in the following ways (see the Abbe formula):
- decrease the wavelength
    e.g. UV microscope, or, as an extreme example the electron and the X-ray microscope
- increase the NA
    e.g. by increasing the angular aperture (better design of objective, 2 or three objectives)
    or by increasing the refractive index (use of immersion oil with n=1.5, rather than n=1.0 for air. Immersion oil should be used only with the appropriate objectives marked with a black ring around their body, or labelled “HI” or “oil”.

One should not confuse resolving power with **visibility**. The latter is not related to the separability of two points, but rather to a single point far away from all others, and states the minimum conditions of size, brightness and contrast against background under which this point is still visible. Seeing this object does not mean we know anything about its size or shape.

Aberrations

Objectives and lenses therein have aberrations that impair image quality. Regarding the theoretical background, we refer to the Biophysics lecture notes provided by the Department. The main aberrations are:

1. Spherical aberrations occur because light rays passing through the edge of the lens are refracted more than those through the centre. Thus the image of a point will in itself be blurred. Overall this also results in an image that is either in focus at the centre or at the edge of the field, but never both at the same time. Correction for this results in a “flat” image, at the price of some loss in resolving power. Corrected objectives bear the forename “*plan-*”.
2. Chromatic aberrations are caused by the wavelength dependence of the refractive index of any material. Henceforth a single lens will have different focal lengths for different colours. This can be corrected for by making a compound lens of single lenses from diverse materials that compensate for each others chromatic aberrations. Objectives corrected for two colours are called *achromat*, those corrected for 3 are called *apochromat.* Apochromats are further corrected for the fourth colour of the visible spectrum (the 4 being blue, green, yellow and red) by a special compensating ocular.

Illumination in the microscope

Once we have a high quality microscope and a good sample, the most common errors that hinder image quality are problems with adjusting the illumination. The following generic points should be considered.

1. The intensity of illumination should provide and optimal balance of brightness and contrast.
2. The light ray should be symmetric, centred at the optical axis.
3. The field of vision should be illuminated evenly, and no light should fall on points outside the field, as photons scattered from there ruin image quality.
4. The NA of illumination should match that of the objective. Thus, for high NA objectives, the condenser should be high and its diaphragm open, for low NA objectives vice versa.

Some important types of microscopes

Bright field

This microscopy applies white light to illuminate the object that is required to be studied. Both unstained and stained cells can be visualized with this instrument. In the case of the former ones the different parts of cells are distinguished from protoplasm as darkened objects as the result of the light absorption and the scattering away from the objective lens. The latter ones are stained with various dyes (red, blue, green etc.), which bind to a certain organelle or to molecules in the cell (e.g., the nucleus). These dyes absorb all wavelengths except that region of the spectrum that corresponds to their colour. Reducing the intensity of light illuminating the specimen can improve contrast and depth of field. However, this will result in the decrease of effective numerical aperture and resolving power.

Koehler illumination

To see sharp images, the microscope must be equipped with Koehler illumination. In the case of microscopy using external light source this operation provides more intensive and even illumination without decreasing the resolution. It is achieved by focussing the light source onto the sub-stage (condenser) iris diaphragm, which is at the same time the rear focal plane of the condenser lens. From every point of the image of the light source formed on the iris diaphragm, a parallel ray is projected towards the sample. This results a nearly uniform

bundle of parallel rays impinging on the specimen and hence a more precise and detailed image with less artefacts.

Dark field

Dark field illumination involves the technique of blocking out the direct light completely and viewing an object by means of diffracted light. A special condenser prevents the direct light from reaching the objective but light from rays that are scattered on objects will still enter the objective. This technique enables us to study sudden changes in the refracting properties at the edges or discontinuities in an object, subtle colouration of objects. This method can also reveal the presence of objects below the resolving power of the same lens system because of the sensitivity of human eye to the intensity difference. (Dark background - bright object)

Phase contrast

It was developed in 1932 by F. Zernike (Nobel Prize in 1953). The phase contrast microscope enhances the contrast of the unfixed and unstained cells. Most cell components are transparent to light and have a high refractive index. Light transmitted through these structures is withheld a bit, resulting approximately 1/4 wavelength ”out-of phase“ shift relative to the light passing through the clear medium. The phase microscope transforms this phase difference to a change in brightness in the following way: separating the diffracted from the non-diffracted light, it retards one or the other by an additional 1/4 wavelength and afterwards the two beams are recombined in the final image. The interference of the “in-phase” (not diffracted) or the “out of phase” (diffracted) beams produces exaggerated contrasts of objects. The beam separation is achieved at a phase plate in the objective’s rear focal plane according to the following method: (1) The direct light is made into a narrow cone with a help of a sub-stage transparent annulus (normally built into the condenser). (2) The image of the annulus is adjusted to coincide with a ring in the phase plate of the objective. (3) The remainder of the phase plate is illuminated with the diffracted beam. There are two strategies of phase contrast microscopy:

Positive (dark) phase: It is applied when the more refracting object is desired to look darker than its surroundings. The diffracted light is retarded by an additional 1/4 wavelength on the phase plate, i.e. by using a magnesium fluoride layer on the phase plate except the ring. Eventually, this light beam becomes ”out of phase” with direct rays so their interference results in a dark spot at the eyepiece.

Negative (bright) phase: The direct light is retarded by a layer at the phase plate ring and as a result the diffracted and the direct beam come into phase again, yielding a brighter image in contrast to the background.

To enhance the contrast effect, the direct beam must be attenuated by means of an absorbing layer at the phase ring. This absorption has efficiency of 75-95%, therefore only a little fraction of the original intensity can be observed. If this causes a problem, it can be circumvented either by using stronger illumination, or by longer exposure (in photomicrography). A practical problem in phase microscopy is the wavelength-dependence of refractive index: to avoid this, frequently yellow-green filters are used with maximum transmission around 550 nm. This wavelength range is a good choice as the human eye is very sensitive to this region of spectrum.

Fluorescence microscopy

In contrast to the previously mentioned microscopic techniques where the specimen is illuminated externally, fluorescence microscopy makes use of the self-luminescence of objects. In this type of microscopy, samples (e.g. cells) are stained (e.g. study of viability) or labelled (e.g. various antigens) with fluorescent dyes and are illuminated with a wavelength of light that corresponds to the excitation (absorption) maximum of the dye. For light sources, mercury or xenon lamps are used to ensure wide excitation spectrum. With application of proper filters, the desired wavelength of light can be selected. The emitted fluorescence light at longer wavelength is detected. To observe only the fluorescent light, filters and dichroic mirrors are placed between the objective and the eyepiece. (The dichroic mirrors are transparent to a certain wavelength and the remainder of the spectrum is reflected.) The emission filters absorb the scattered photons from the exciting beam, so only the emitted light rays reach the ocular. The fluorescent objects appear as bright spots (with colour coming from their emission spectrum) in the dark background. It is thus easier to study fluorescent images in a darkened room. By applying different types of dyes, many parameters of the cell can be studied: vitality, membrane potential, ligand-receptor bindings, conformational changes of proteins etc..

Microscopic methods in the investigation of cellular and molecular systems – an outline

I. Investigation of biological effects of various substances on cells

1. From biochemical material isolated before and after treatment

a. On the molecular level

i. Physico-chemical
ii. Fluorescence spectroscopy
iii. Fluorescence quenching
iv. Raman / IR spectroscopy
v. Correlation spectroscopy
vi. Surface plasmon resonance

b. Biochemical

i. Inhibition / enhancement of enzyme activity
ii. Substrate specificity / effectiveness
iii. Affinity

2. Detection of *in situ / in vivo* effetcs

⇨ Microscopies

II. Classification of Microscopies

1. **Optical (Formation of an image is based on some light-related property of the material examined)**
   a. Classical optical microscopies (diffraction limit!)
      i. Transmission
         - phase contrast
         - differential interference contrast
         - dark field
         - polarisation
      ii. Fluorescence
   b. Near field scanning optical microscopy = NSOM
2. Non-optical
   a. Electron microscopy (transmission and scanning)
   b. acoustic
   c. X-ray
   d. Scanning probe microscopies
      i. atomic force
      ii. shear force (e.g. NSOM, which forms two types of images, optical *and* shear force)
      iii. tunneling

III. Major parts of the compund microscope (in order of light path)

1. **Transmission**
   Light source, condenser, stage, objective, tube, eyepiece (or camera)
2. **Epifluorescent**
   Light source, collector, excitation filter, dichroic mirror, objective, stage, objective, dichroic mirror, emission filter, tube, eyepiece (or camera)

IV. Image formation by the compound microscope

1. **Objective (compound object lens)**
   Inverted, magnified, real image
   Can be projected onto a screen (film, camera chip)
2. **Eyepiece (compound eye lens)**
   Its object is the image formed by the objective
   Upright, magnified, virtual image, seen by the observer in the direction of the stage

V. Modalities of image formation in classical microscopies

1. **Full field**
   a. Illumination: full field (with Köhler optics)
   b. Detection
      i. by eye
      ii. camera (film)
      iii. digital camera
2. **Scanning**
   a. Illumination: point (laser light source focussed by objective)
   b. Detection: irrespective of the localization of the incoming photon, e.g., photo multiplier tube (PMT), avalanche photodiode
   c. Scanning mechanism: stage (motorised) or laser (deflected by moving mirrors)

VI. Comparison of Fluorescence and transmission (absorption) image formation

1. Absorption of living samples is relatively low, contrast of organelles is small
2. Possibilities for improvement: dark field (ultramicroscope), Phasecontrast, polarisation
3. Observing fluorescence *in vivo* can provide high sensitivity and good contrast, since
   a. Relative to a dark background every photon means a 100% improvement in background

b. Fluorescent labels can be attached to the molecule or organelle of our choice
c. Most fluorescent labels cause little or no harm to living cells

4. The most widely applied technique for observing fluorescence in the microscope is epifluorescence

VII. Selected application of fluorescence microscopy

1. Morphological, quantitative characterisation

a. Observation of organelles (e.g. DNA in the nucleus with propidium iodide (PI) staining. Uptake of PI occurs through membranes with increased permeability caused by involuntary or deliberate damage)
b. Demonstration of cell surface and intracellular proteins (antigens). Most often via binding a fluorescent dye (e.g. FITC, TRITC, Cy3) to specific monoclonal antibodies or substrates. Distribution of labelled proteins can be indicative of structure. (e.g. actin filaments of the cytoskeleton labelled with FITC-phalloidin).

2. Investigation of dynamic properties

a. *In situ* observation of proteins (see 1/b)
b. Determining the extent of DNA synthesis (Br-deoxiuridine is incorporated into DNA during synthesis, this can be qualitatively detected with fluorescently labelled anti-BrDU antibody)
c. Measurement of transmembrane potential. Distribution probes: bis–oxonol (negative charge), carbocyanines (positive charge). Charge shift probes: e.g. di-4-ANEPPS.
d. Measurement of intracellular free calcium concentration. The advantage of ratio imaging is its independence of the amount of indicator in the cell. Frequently applied fluorescent calcium chelator dyes: Indo-1 (emission ratio is measured) and Fura-2 (excitation ration measured).
e. Measurement of other ion concentrations: Na – SBFI, K – PBFI, H (pH) – BCECF
f. Investigation of protein-protein interactions: energy transfer microscopies

VIII. Resolving power

1. The limit of resolution in the microscope

$d_{min} \sim 0.61\ \lambda / n \sin\phi$, where d_{min} is the smallest distance of two points that still are observed as two separate points (that is, d_{min} is the reciprocal of resolving power), λ is the wavelength of light used for observation, and $n \sin\phi = NA$, the numeric aperture of the objective, (n is the refractive index of the medium between the objective and the

observed object, and ϕ is the half angle of observation through the objective).

2. Increasing the resolving power

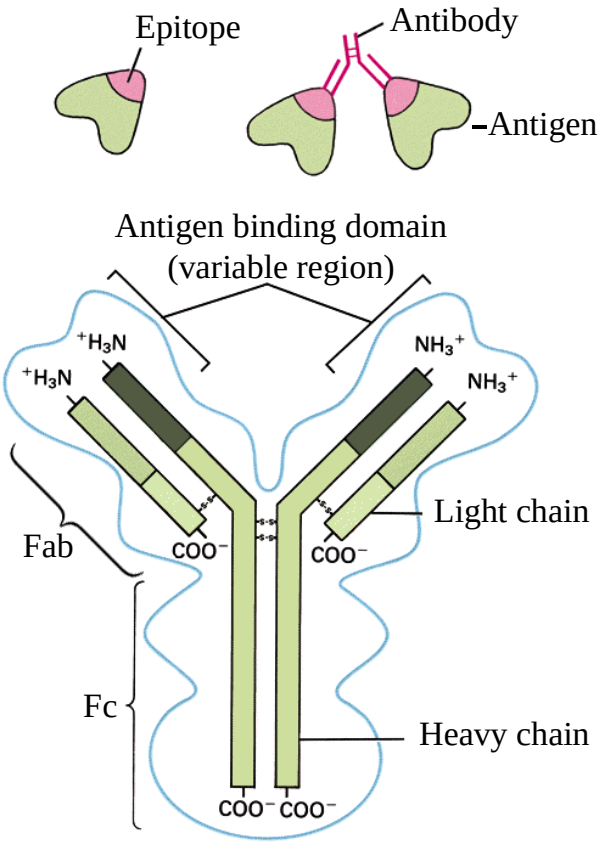
a. Decreasing the observation wavelength: UV microscope, X-ray microscope, electron microscope (see de Broglie's principle of material particles as a wave in Biophysics)
b. Increasing NA: the upper limit of n is 1.5, $y=\sin\phi$ reaches its maximum at y=1.0, thus even the best objectives are below NA = 1.5. When applying more illuminating and/or observing objectives, numeric apertures are added (4π microscopy).
c. Imaging is not done in the full field, but on a point by point basis (scanning principle). In this case it is the size of the volume element observed that matters, and not the distance of the two points to be seen separately (provided that the scanning is done in sufficiently high-resolution steps.) One possibility is the application of the confocal principle. The pinhole applied in the focal plane (and point) decreases the detected volume both along the z axis (causing the optical slicing of the sample) and along the x-y axes (improving lateral resolution) The detected volume element is a rotated ellipsoid with its long axis along z.
d. In *Theta microscopy* there is a second objective detecting perpendicular to the excitation, and hence the effectively detected volume element is the intersection of the two perpendicular ellipsoids, one formed by the excitation objective and the other by the emission objective and the pinhole.
e. In 2-photon microscopy, two photons arriving co-temporarily are used to excite an energy transition equivalent to the sum of the two photon's energies. The probability of absorbing 2 photons is proportional to the intensity squared at a given point in space, which is a narrower function than the distribution of laser beam intensity across the diameter of the illuminated spot. Hence most photons will be absorbed at the centre of the illumination spot.
f. In scanning near-field microscopy (SNOM), the sample is approached to a few nm by the probe, so that photons can cover a path of only a fraction of their wavelengths, and therefore interference, which is the inherent cause of the diffraction limit, cannot arise. Resolution then is only determined by the spot size, which can be down to a few 10 nm when using a pulled-out, metal-coated optical fiber tip as the illumination source.
g. Other scanning probe microscopies do not use light, but rather physically map the surface of the sample at high resolution, using precise piezo-scanners and fine, elastically suspended tips of

molecular dimensions at their end. Besides the 3D structure, specific markers can be located, e.g. by using colloidal gold-conjugated antibodies.

Labelling techniques in fluorescence microscopy

The absorption of light by living samples is very small, hence contrast in the microscope based on the absorption of light is very poor. Methods utilising fluorescence provide a far better contrast as they are based on the detection of photons originating from specific places of the sample against a dark background. In addition, fluorescent labelling is of relatively low impact even in living systems as opposed to various methods applied to specifically enhance contrast in absorption based microscopy.

Fuorescent labelling of targets in the cell - proteins and nucleic acids - is most often done using specific tags, molecules that bind specifically and also fluoresce. The fluorescence can come from the specifically binding molecule, or it can be a label conjugated to this molecule before adding it to the target. These two options belong to the category of direct labelling. We can also apply indirect labelling, where the fluorophor is conjugated to a molecule that will bind to the specific tag attached to the target.



Labelling with antibodies

Proteins and other molecules with antigenicity can be labelled using antibodies. The antigen-binding region (Fab) of antibodies shows a great diversity, various plasma cells produce different, but highly specific and strongly binding antibodies. The protein epitope bound by the antibody is called the antigen.

For immunofluorescence we prefer to use monoclonal antibodies, which are produced by plasma cells of the same clone, and are therefore structurally identical. If we want to visualise epitopes of low density, use of polyclonal antibodies, that bind to many epitopes on the same molecule can be more efficient.

As whole antibodies are bivalent (see figure), identical

epitopes can be cross-linked by whole antibodies, that can lead to the change of spatial localisation of examined proteins, and, in some cases, also to activation of otherwise inactive functions. In these cases it is useful to employ the Fab fragments of the antibody in question, which can be derived from the whole antibody by papain digestion.
Along with the 2 Fab fragments, and Fc fragment is formed, which is of constant aminoacid sequence for a given species and given type of antibody, hence the index *c* (constant). This Fc part of the antibody, being constant, can be used as a target of secondary antibodies in indirect labelling.

onoclonal antibodies are most often produced by the hybridoma method: antibody producing plasma cells are fused to cells of a myeloma cell line, forming - after selection - cells that not only produce antibodies but also proliferate continuously, a property inherited from the myeloma, which in fact is a tumour originating from white blood cells.

Labelling with toxins and other specific molecules

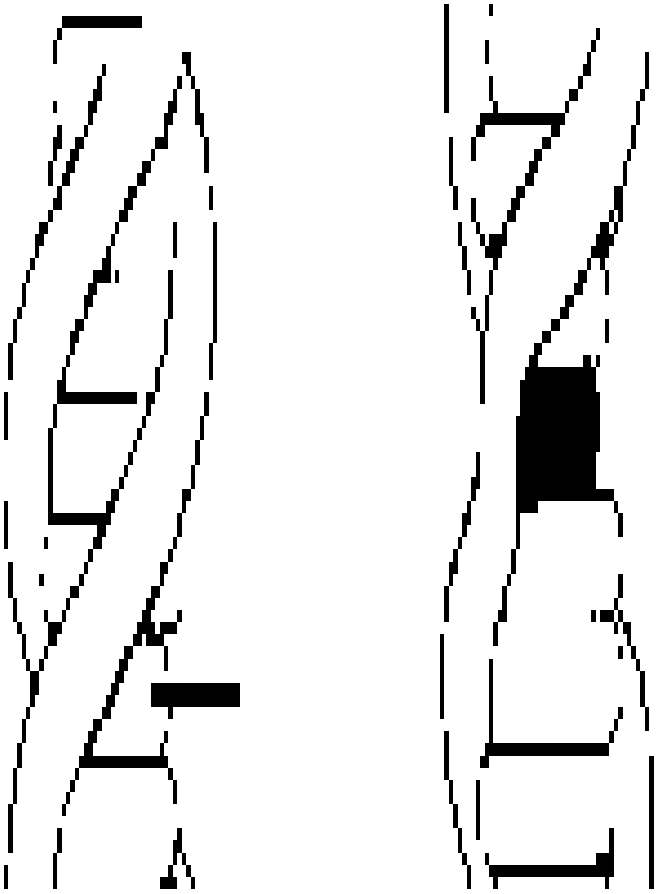
The requirements of small size and specific binding are fulfilled by other molecules in addition to antibodies and Fab fragments. These are usually toxins and other biologically active molecules (metabolites, alkaloids, lectins). While binding is strong and specific, so the Kd is very small, we need to observe that these molecules are biologically active. The table illustrates a few possibilities of labelling with toxins, etc.

labelled macromolecule	**Specific probe**
F-actin	Phalloidin
Voltage gated K^+ channel	β-scorpion toxin
Voltage gated Na^+ channel	tetrodotoxin, saxitoxin
N type calcium channel	ω-conotoxin
L type calcium channel	dihydropiridins, phenylalkylamins
intracellular (IP_3) calcium channel	Ryanodin
Na^+/K^+ ATP-ase	Ouabain
nicotinic acetylcholin receptor	α-bungarotoxin
μ-opioid receptor	naloxone
α-adrenergic receptor	prasosine

Labelling with substrate and ligand

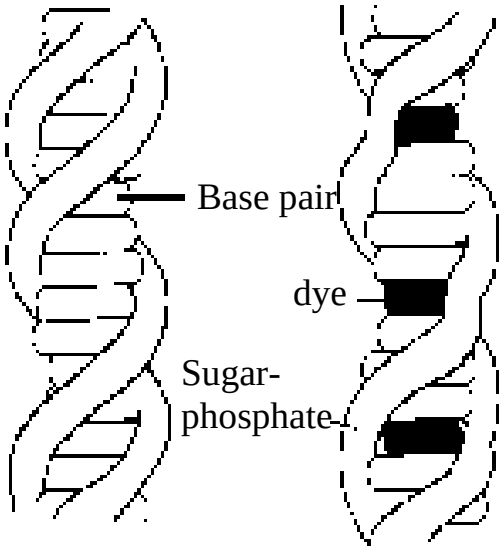
Proteins with enzyme activity can be labelled with their substrates, or modified forms thereof (called pseudo-substrates), that are luminescent and fit into the active site, but often bind to it irreversibly. Ligand of receptors can also be fluorescently labelled and used to tag the receptors. Care has to be taken that the luminescent label does not alter the structure, and hence the binding, of the ligand. One needs to note that the formation of the ligand – receptor complex can start signalling processes, which can influence the system we intend to examine. Thus we often deliberately use a fluorescent ligand to start a signalling system and follow the activated receptor or the path of the ligand (e.g. internalisation) at the same time. We can slow this process down by cooling the system to 4 degrees C, but this only stops lateral diffusion in the membrane, activation that requires the change od protein conformation exclusively is only slowed down, but not prevented.

Dyes of nucleic acids (DNA and RNA dyes)



Many fluorescent dyes are planar molecules with aromatic rings that have a tendency to form aggregates with each other or molecules of similar structure. Some of them bind to DNA: the binding can be (a.) from the outside, (b.) into minor groove (e.g. daunomycin and acridin orange), or (c) of the intercalating type (major groove binding, e.g. ethidium bromide, proflavin, and propidium iodide); some examples of the chemical structure of these dyes and the mechanism of intercalation is shown in the figures.

Major and minor groove binding dyes can be used to measure the DNA content of cells, to determine the single or double stranded state of nucleic acids, and some are even appropriate to preferentially label DNA sequences rich in certain base pairs, e.g., Hoechst

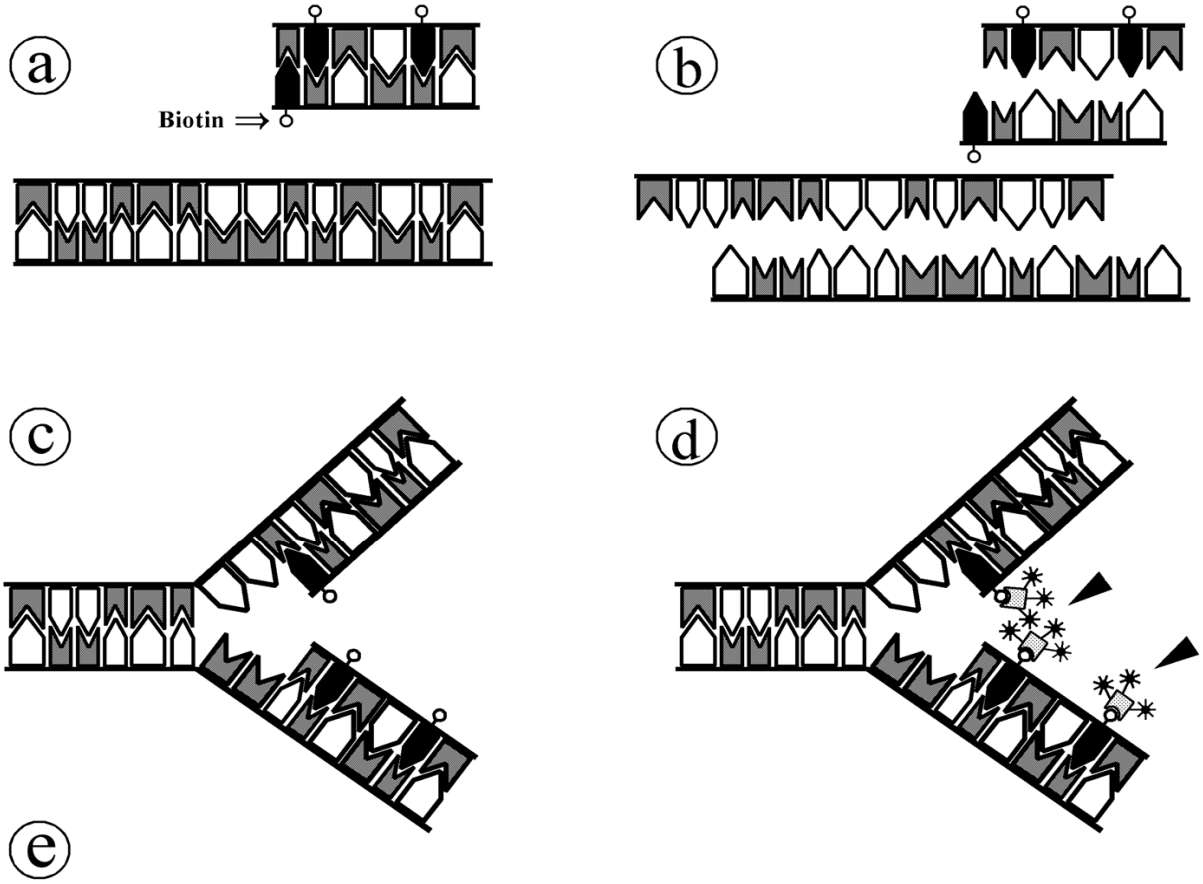


dyes and DAPI bind to regions rich in AT pairs. This serves as the basis of banding metaphase chromosomes as well.

Since these dyes interfere with DNA, they can severely influence DNA replication and transcription and are thus considered toxic and carcinogenic. The quantum yield of these dyes increases 10 - 100-fold upon binding to DNA because the dye molecules are protected from collisional quenching by the solvent.

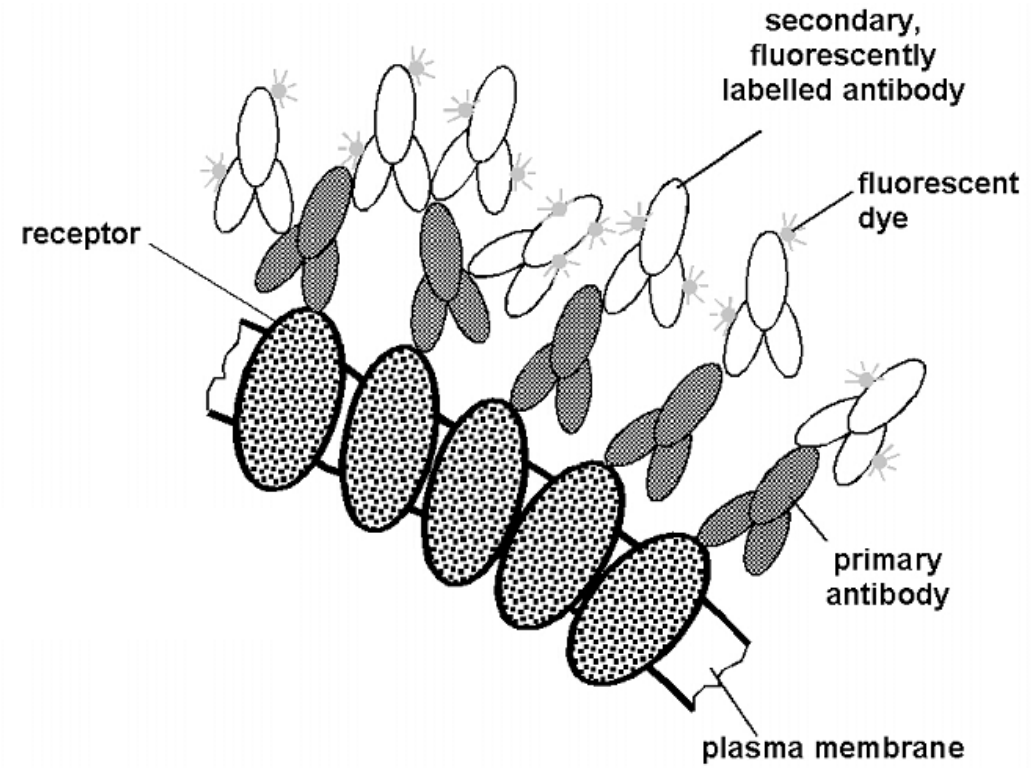
Sequence-specific nucleic acid probes

It is often not morphology (localisation of the nucleus) or quantity of nucleic acids (haploid or diploid set of chromosomes) that we are investigating, but rather, the occurrence of a given base pair sequence in a set of DNA molecules (chromatin). Furthermore, we often wish to know the number of the sequences (genes), their position in the genome, or transcription to RNA. In these cases we create a synthetic sequence that can be labelled specifically and visualized later in the experiment. The synthetic DNA is then added to the cellular DNA (a, see figure) and both are melted (the double strands are separated by heat and salt) (b). Upon cooling, the strands pair up again (c), and because the synthetic probe is in great excess, it will hybridise to the complementary target sequence. The excess of probe DNA can also cause a multiple labelling of the target, with each probe molecule pairing up with only a part of the complementary target sequence. This multiple binding is responsible for a reasonably strong signal. The probe DNA contains a specific marker (eg biotin or digoxigenin), which can then be tagged with a fluorescent label (eg. avidin-FITC) (d). Part e in the figure shows such a hybridisation tartgeting the pericentromeric region of chromosome 1. As the chromosomes are labelled as they are found in the interphase cell, the process is calles *in situ* hybridisation. If a fluorescent label is use, it is termed fluorescent in situ hybridisation, or, for short “FISH”. The cells in the figure are malignant tumour cells with 6 copies of chromosome 1 instead of the usual 2, and also the sizes of the pericentromeric region are irregular. The specific nucleic acid probes can also be used in comparative genomic hybridisation (CGH), or, in the case of antisense and nonsense oligonucleotides, for inhibiting gene expression.



Indirect labelling

As already indicated, we sometimes use indirect labelling of targets. This can help increase the limited number of fluorophores that can bind to the target, but is also an applicable method when the primary label is unsuitable for accepting fluorescent tags (eg. it is inactivated by conjugating a fluorophore to it). A prominent form of indirect labelling is the use of two antibodies, one monoclonal binding to the target, and a fluorophore-conjugated second antibody (often polyclonal) second antibody, directed against the first antibody. An example of this approach is demonstrated in the experiment that you will perform in the lab - see later in this text.



To make a very small number of molecules visible, often the so-called sandwich methods are applied. These utilise a set of molecules, e.g. the specifically binding pair **biotin** and **avidin**, and a primary label conjugated to one of these, to build larger molecular complexes that carry many luminescent molecules.

Targeting the probes to the labelled molecular entities

Probes that cannot penetrate the cell membrane but are directed against intracellular molecules, furthermore the quest to label molecules *ex vivo* and replenishing them into the cell have brought along methods that permit the introduction of these molecules into the cell without interfering severely with the life processes of the cell. These methods include microinjection techniques, closing the probes into lipid vesicles that fuse with the cell membrane, and scrape loading (in the case of adherent cells, the membrane opens up transiently upon scraping the cell with a needle and labelled probes from the buffer enter the cytoplasm before the membrane closes up again). The application of acetoxi-methyl groups for masking polar carboxyl groups of probes is a noteworthy idea. These masking groups are cleaved in most cells by esterases after the probe has trespassed the membrane and the probe is consequentially trapped in the cytosol. Ion indicator dyes such as Fura-2 or Indo-1 are often loaded into cells in this manner.

If keeping the viability and integrity of the cells is not a must, we can permeabilize the cells after, or along with, the fixation process. One option is to

fix in formaldehyde (or paraformaldehyde) and than permeabilize with a weak detergent, like Triton X-100. The other frequent approach is the utilisation of some organic solvent, like acetone, or a 1:1 mixture of acetone and methanol, which fixes and permeabilizes at the same time.

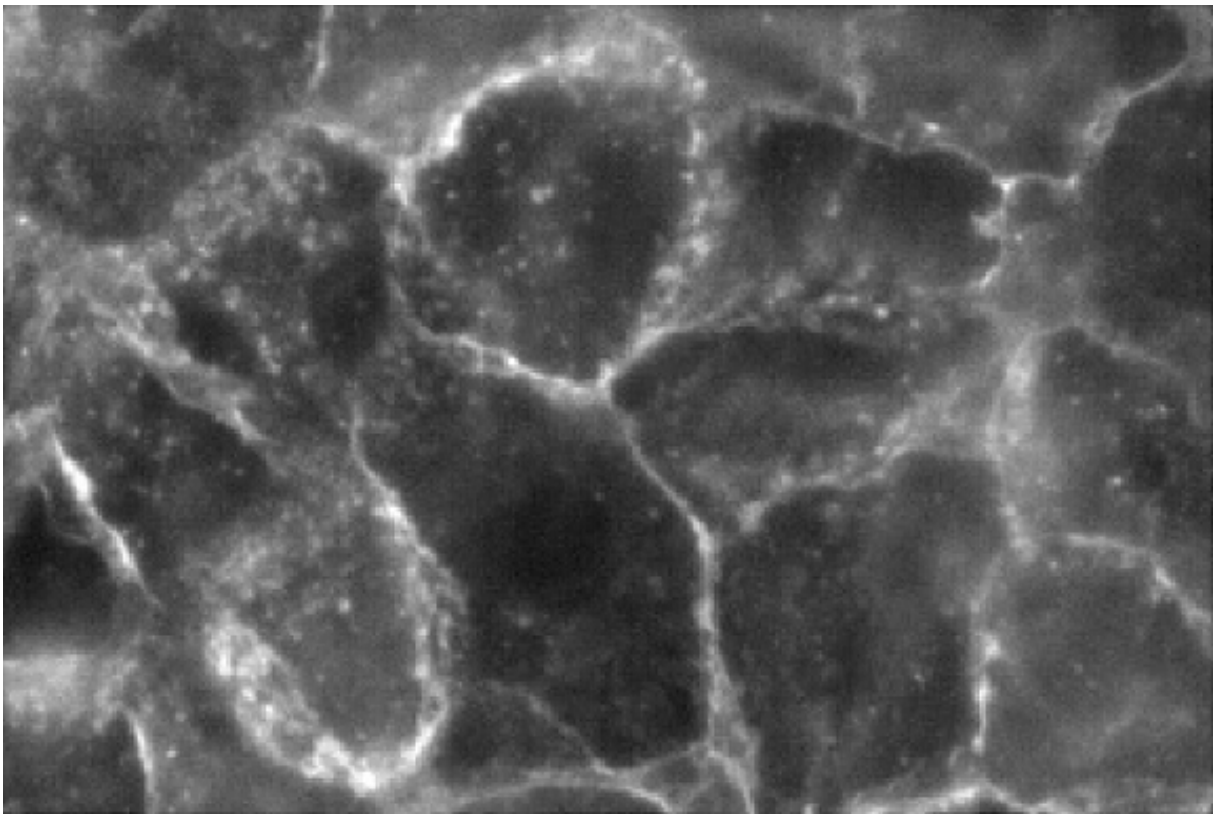
Tasks to be performed and specific background: Fluorescent labelling of A431 epidermoid carcinoma cells

Targets to be visualised are:

- EGF receptors in the plasma membrane
- Filamentous actin, a constituent of the cell cytoskeleton
- DNA (nucleus)

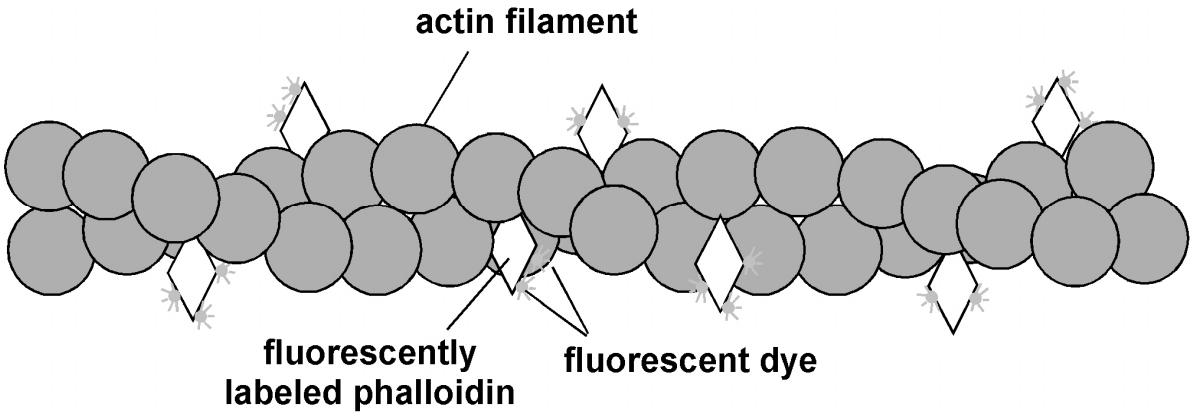
Labelling EGF receptors in the plasma membrane

A431 carcinoma cells are of human origin. They are adherent cells that grow in a monolayer. Their plasma membrane contains ~10^6-10^7 EGF (epidermal growth factor) receptors per cell. The EGF receptor belongs to the tyrosine kinase family of membrane receptors. We shall label these receptors using indirect immunofluorescence. The primary antibody is a monoclonal termed "528" produced by a hybridoma of mouse origin. To visualise these primary antibodies that we have bound to the receptors, fluorescein conjugated secondary antibodies are used. These are polyclonal antibodies produced in goat against the Fc fragment of mouse immunoglobulins. Since both the primary and the secondary antibodies are bivalent, the labelled EGF receptors can be cross-linked during the labelling process. This, similarly to the binding of EGF, can initiate the tyrosine-phosphorylation of EGF receptors, which is termed autophosphorylation. As a matter of fact, the cross-linked or dimerised receptors phosphorylate their nearby neighbours, and not themselves. This is followed by the internalisation of activated (phosphorylated) receptors. In this practical, we aim to visualise the receptors in the plasma membrane, and will inevitably see those receptors in the cytosol that were cross-linked, activated and internalised. To avoid the excessive internalisation of the receptor, we shall do the labelling on ice: the low temperature rigidifies the membrane and slows down (but does not stop) the internalisation. We also fix the cells in formaldehyde after the labelling to prevent further internalisation and also to avoid the detachment of cells from the coverslips on which they were grown and labelled.



Labelling filamentous actin, a constituent of the cell cytoskeleton

Adherent cells are characterised by cytoplasmic bundles of filamentous actin (F-actin), the so-called stress filaments. This probably plays a role in maintaining the attached, spread form of the cells. F-actin can be specifically



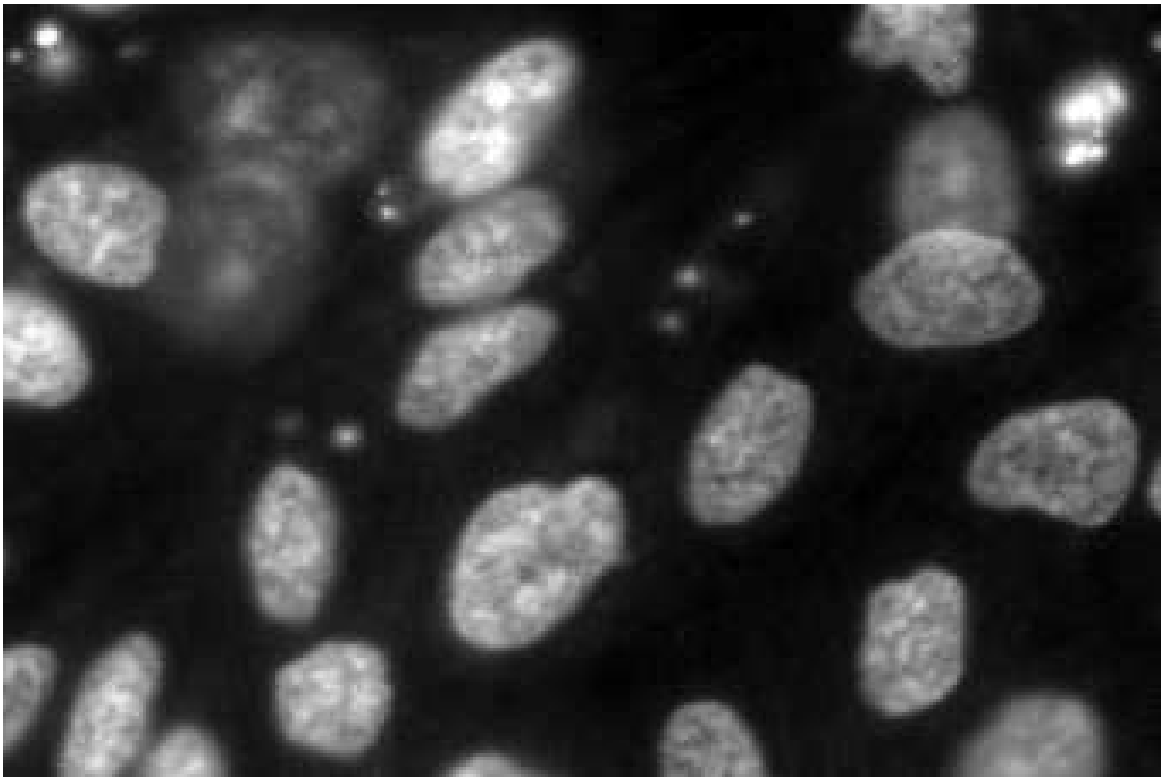
labelled by antibodies, and even better by phallatoxins (phalloidin and phallacidin). These latter are bicyclic peptides isolated from the poison mushroom *Amanita phalloides* and bind selectively to the polymerised actin but not the globular monomer. Binding is stochiometric and shifts the equilibrium *F-actin* $\leftrightarrow$ *G-actin* towards polymerisation. At the same time, because of their small size (12-15 Å) phallatoxins binding to F-actin do not inhibit its function in cell motility, its binding to myosin and tropomyosin and contraction. In our experiment, we shall label actin using phalloidin that was conjugated covalently

to a fluorescent dye (fluorescein). Caution! Phalloidin is toxic! The quantities used in the experiment are orders of magnitude smaller than the toxic dose, nonetheless the same extreme caution is required of the experimenter. The labelled phalloidin will be handed out in an Eppendorf tube diluted to the nanomolar concentration used for labelling. Please discard the pipette tip used for measuring the labelling volume into the beaker allocated exclusively for this purpose.

Labelling DNA content (esp. the nucleus)

To label the nucleus, a fluorescent dye, propidium iodide (PI), intercalating with DNA will be used. PI is not a selective label of nuclear DNA, it also binds to mitochondrial DNA and double stranded RNA. Under the conditions we use for observation (suboptimal excitation and detection) we can only expect a well-defined signal from the nucleus (see fig. below). Since PI intercalates into DNA, it is mutagenic and therefore great care must be exercised upon its handling. The diluted (20 μg/ml) dye directly used for labelling will be handed out.

The intact cell membrane is impermeable to both phalloidin and PI. Hence these intracellular components can only be labelled after permeabilising the plasma membrane. We shall apply acetone fixation as the first step of this labelling which is an easy, well controllable procedure to achieve the preservation of cellular structure, morphology, and permeabilisation the membrane.



Tasks to be performed in the lab

N.B. While coverslip I is incubated, start doing coverslip II. While this one is incubated, you can look at the results of the first one.

Procedure **Cells will be handed out in medium, grown on 12-mm coverslips**	Time (min)
Coverslip I – Direct labelling of F-actin with FITC-phalloidin and of the nuclear DNA with PI ***(All steps are done at room temperature. Start washing immediately after starting the first 30 min. incubation of coverslip I.***	**60+20**
Wash 3x 3 minutes in 500 µl room temp PBS.	**15**
Fixation in acetone. After removing the PBS, hold the coverslip with the tweezers and dip for 2 min into acetone. Don't let the coverslip go, and keep track of the side with the cells! After 2 min, submerge the coverslip into a beaker with ~10 ml room temp PBS. Keep there for 2 min and then place into the wet chamber, cells up. Makes sure the lining of the chamber is wet enough.	**5**
Wash 2x 2 minutes in 500 µl room temp PBS. Pipette carefully 500 µl PBS onto the edge of the coverslip. Surface tension will take care for properly distributing the buffer above the coverslip. Use the pipette to remove the buffer after 3 minutes. Change the tip and repeat the process 2 more times. Be quick after removing the liquid so that the cells do not dry out. After removing the third wash, instantly proceed to the next step.	**5**
Incubate at room temp in wet chamber with FITC-phalloidin and PI. The solution provided contains 1 µg/ml FITC-phalloidin and 20 µg/ml PI in PBS. Carefully pipette 30 µl of it onto the coverslip (keep the pipette tip at the edge!) and incubate for 20 min.	**20**
Wash 3x 3 minutes in 500 µl room temp PBS	**10**
After removing the PBS, pipette about 15 µl antifade (e.g. Mowiol, or whatever is provided at the lab – this will prevent free radicals from damaging excited state fluorophores) onto the coverslip and carefully turn the coverslip over (cells down) onto a cleaned glass slide. Use the tweezers and touch one edge of the coverslip to the slide. Slowly let the whole coverslip down flat, so that no bubbles are formed. Carefully Drain the excess fluid on the edges with paper tissue. *Make sure not to move the coverslip!* Wait for drying for about 20 minutes (keep the sample covered to prevent bleaching) and then place 3 small droplets of nail polish at the edge of the coverslip to prevent it from moving. Wait for the nail polish to dry perfectly! (~10 min). Examine the sample in the fluorescence microscope. Excitation and emission maximum of PI is	**5+25**

at 535 and 617 nm, respectively, however, the filter used for FITC excitation in the blue range of the spectrum still provides an acceptable excitation of PI as well, while observing all emitted light above 520 nm allows the detection of both green and red (orange) fluorescence. Thus FITC-phalloidin and PI can be observed co-temporarily, visualising in green the Fluoresceine molecules fluorescing on the actin stress filaments and in orange the propidium iodide fluorescing in the nuclei. Observe the mutual orientation of the labelled entities and their relation to cell shape. Draw your observations in colour.	
Coverslip II. – Indirect Immunofluorescent labelling of membrane EGF receptors ***(Up to the fixation step, all processes are done on ice and with chilled solutions, and thereafter at room temperature)***	**120+20**
Check whether the paper in the wet chamber is wet enough. Place the wet chamber on ice. Use the tweezers to take a coverslip from the medium and place onto the parafilm on the bottom of the chamber (Cells upwards!). The parafilm will be wrapped around a piece of metal to keep the cells ice-cold more easily. Wash 3x in ice cold PBS (phosphate buffered saline. After removing the third wash, instantly add the first antibody (see next step.)	**15**
Incubation on ice, in wet chamber, with "528" antibodies to label EGF receptors. "528" is a monoclonal antibody produced by a mouse hybridoma which. One could use other specific antibodies (e.g. 2E9, which is specific to the low affinity type human EGF receptor). 20 μg/ml antibody is provided in PBS. Pipette 30 μl carefully onto the edge of the coverslip.	**30**
Wash 3x 3 minutes in ice cold PBS (as in the first step)	**10**
Incubate in the wet chamber, on ice, with the second, FITC- or Cy3-conjugated antibody. The second antibody is a GAMIG (goat anti mouse immunoglobulin), a polyclonal antibody raised in goat against the constant (Fc) region of the mouse IgG. Measure 30 μl of the pre-diluted antibody onto the coverslip.	**30**
Wash 3x 3 minutes in ice cold PBS (as in the first step)	**10**
Fix with 3.8 % PFA (paraformaldehyde), or 2% formaldehyde. After the last wash in PBS, pipette 100 μl ice cold PFA or formaldehyde onto the coverslip, keep on ice for 5 min and than take the chamber out onto the bench and let it warm to room temp. (another 5 min.)	**10**
Wash 3x 3 minutes in room temp (!) PBS.	**10**
After removing the PBS, carefully turn the coverslip over onto a cleaned glass slide (cells down!). Follow the instructions given for coverslip I. Examine the sample in a fluorescence microscope.	**5+20**

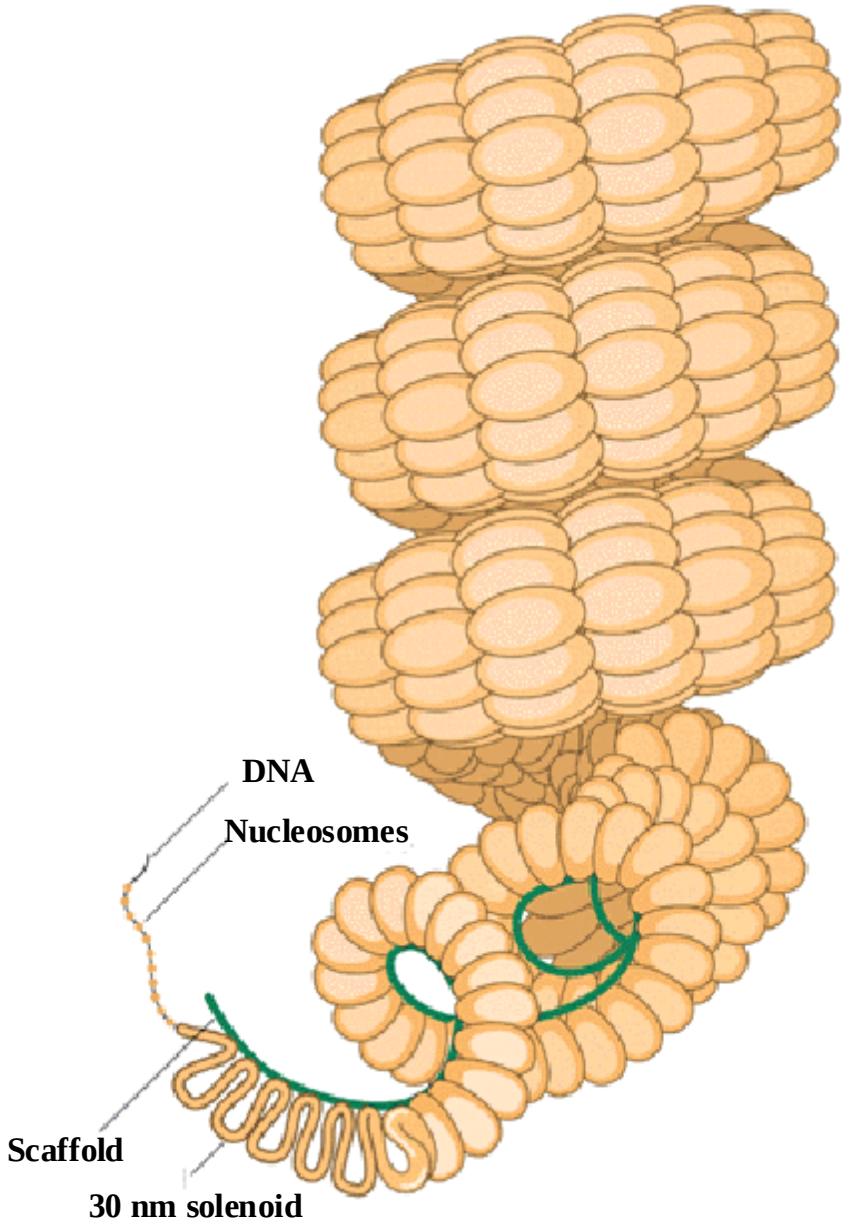
Fluorescein can be excited with blue light (excitation maximum is at 494 nm), and detected in the green (above 520 nm). Cy3 is excited in the green and emits in the red. (maxima at 554 and 568 nm). Observe plasma membrane fluorescence highlighting cell boundaries, and internalised receptors. Draw your observations in colour.	

Further tasks (also for times of incubation): Observation of various fluorescent labels using a computer database. Recognition of cellular components based on morphology and/or label specificity. Studying the calcium signal evoked by ligand-receptor interaction using digital ratio-imaging technique: playback of a time-resolved measurement.

Examination of DNA damage

Structure of the Chromatin

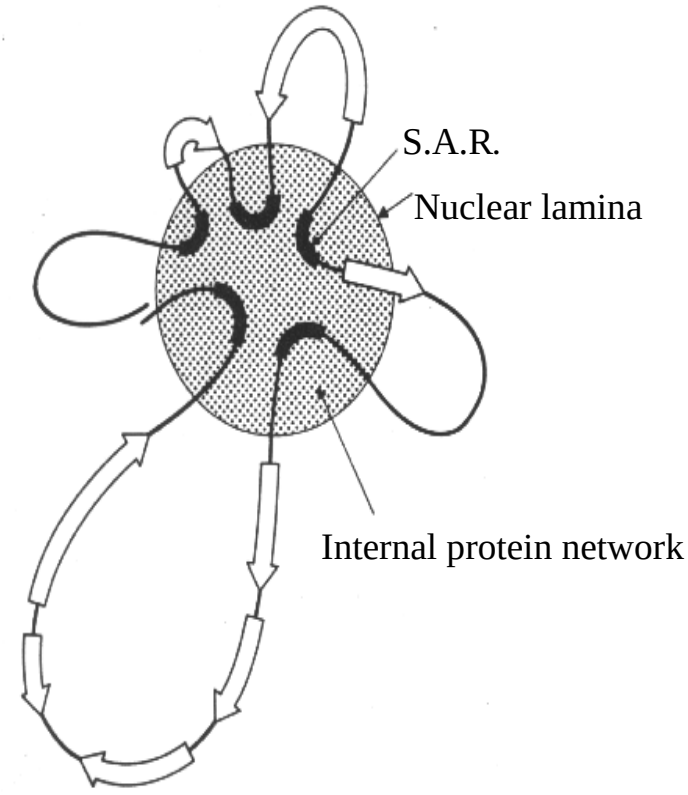
Diploid human genome consists of 6×10^9 base pairs of DNA. The length of the naked DNA in a single cell would be approximately 1 meter, and it is packaged into a nucleus with a diameter of 5-10 μm. This enormous condensation of DNA can be achieved through packaging steps that allow DNA and associated proteins to form chromatin.



The basic structural unit of chromatin is an assemblage called the nucleosome, composed of five types of histones (designated H1, H2A, H2B, H3 and H4) and DNA. A nucleosome consists of approximately 166 base pairs of DNA wound around a core particle of histone proteins. The core particle is a roughly heart-shaped octamer formed of 4 types of histones (two from each of H2A, H2B, H3 and H4). Approximately 146 base pairs are tightly bound to the core particle while the remaining 20 base pairs are associated with the H1 histon.

The DNA between two nucleosomes is called the linker segment. This linker segment gives the unfolded chromatin a so-called *beads-on-a-string* appearance (the nucleosomes are the beads). The length of the linker segments is

variable. If chromatin is isolated from cells under conditions similar to those expected in a cellular environment, chromatin does not have this appearance. Instead, the majority of chromatin threads appears to be composed of relatively smooth fibers about 25-35 nm in diameter. The folding mechanism that generates the *solenoid* structure is uncertain, and so is the mechanism of the further folding steps. However, it has been suggested that chromatin fibers (with the help of associated proteins) form loops containing 10-90 kb DNA. The DNA segments positioned at the bases of the loops are thought to be connected in some way to the *scaffold* or *matrix*, the putative protein skeleton of the interphase chromosomes. The other levels of packaging that exist, similarly to the nature of the associations that maintain the higher-level chromatin structure, are still largely unknown.



The Nuclear Halo

When permeabilized cells are dipped into salt solutions of increasing ionic strength (up to 2 M NaCl), the nuclei become depleted of soluble proteins and histones. In such samples, proteins ensuring condensation and chromatin-packaging are released from DNA wich tends to bulge out forming a "*halo*" surrounding the residual nuclear skeleton. These structures, comprising of a halo of DNA anchored to the nuclear matrix can be visualized with ethidium-bromide staining using a fluorescence microscope.

The superhelical structure of DNA

In addition to the normal helical configuration typical of all DNA molecules, circular DNA can be twisted upon itself to form a new, higher order helix, a supercoiled molecule. DNA without supercoils is said to be in its relaxed state.

To understand the mechanism of supercoiling, suppose that we cut both strands of a circular DNA molecule. If the molecule was supercoiled before the cut, it becomes relaxed immediately. Joining the ends of the molecule back together again does not result in any changes, the DNA is still in its relaxed state. However, if we first give the DNA a right-handed twist (i.e. a twist in the direction in which the strands are already entwined around each other) before sealing the ends, the DNA double helix becomes overwound and thrown into a positive supercoil. Conversely, if the DNA is given a left-handed twist before sealing (i.e. twisted in the direction opposite to which the double helix is wound), it becomes underwound and is thrown into a negative supercoil. There is a strong relationship between the number of turns of the Watson-Crick helix and the number of turns of the superhelix. Decreasing the number of Watson-Crick turns elicits overwinding while an increase results in underwinding. If DNA molecules are stained with ethidium-bromide, the number of Watson-Crick turns will decrease, therefore staining with this intercalating dye will introduce positive supercoiling into the double helix.

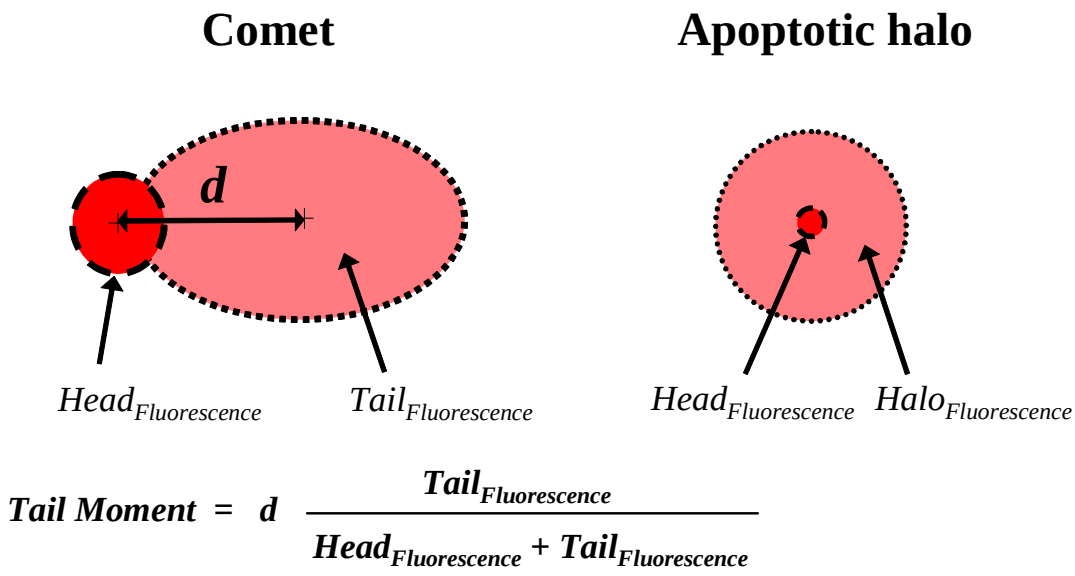
Supercoiling is not limited to circular DNA molecules, it also occurs in linear eukaryotic DNA, provided that the ends of the molecules are not free to rotate.

Molecules that differ only in their state of supercoiling are called topological isomers. Correspondingly, the enzymes that carry out the interconversions of relaxed and supercoiled forms of DNA (also *in vivo*) are called topoisomerases. Supercoiling markedly alters the overall form of DNA. A supercoiled DNA molecule is more compact than a relaxed DNA molecule of the same length.

DNA strand breaks

DNA is recognized to be the primary target for cell inactivation by ionizing radiation. Cell death induced by radiation is most likely the result of lack of repair or misrepair of complex lesions in DNA. *Ionization radiation is thought to produce about 1000 single-strand breaks (SSBs) and 25-40 double-strand breaks (DSBs) per diploid cell per Gray* (G) regardless of the cell type. (There are a few important exceptions, such as hypoxic cells, or cells deficient in glutathione). However, the *response to these lesions can differ widely for different cell types*. Variation in "intrinsic" radiosensitivity is often considered to be a result of differences in the ability of cells to repair DNA damage accurately. Clearly, the cellular components and efficiency of the repair enzymes are critical for DNA repair. DNA injury is generated in cells not only by *ionization radiation*, but DNA strand breaks is the primary cause of the cell inactivation by several anticancer agents, as well. Such damage is produced by many common *chemotherapeutic drugs* used in cancer therapy, including most alkylating agents and inhibitors of topoisomerases I (like camptothecin) or II

(such as etoposide). *Pesticides and herbicides* widely used in the modern agriculture form a very heterogeneous class of chemicals, may also play a role in cancer etiology, since they can also *generate DNA damages*.



$$\textit{Tail Moment} = d \frac{Tail_{Fluorescence}}{Head_{Fluorescence} + Tail_{Fluorescence}}$$

Figure 1. Quantitation of DNA damage in single cells.

Comet assay

There are several methods to measure DNA strand breaks. Conventional and pulsed field agarose gel electrophoresis, as well as filter elution detects DNA strand breaks in bulk cell populations. The *comet assay*, also called the single cell gel electrophoresis (SCGE) and microgel electrophoresis was introduced as a microelectrophoretic *technique for the direct visualization of DNA damage in*

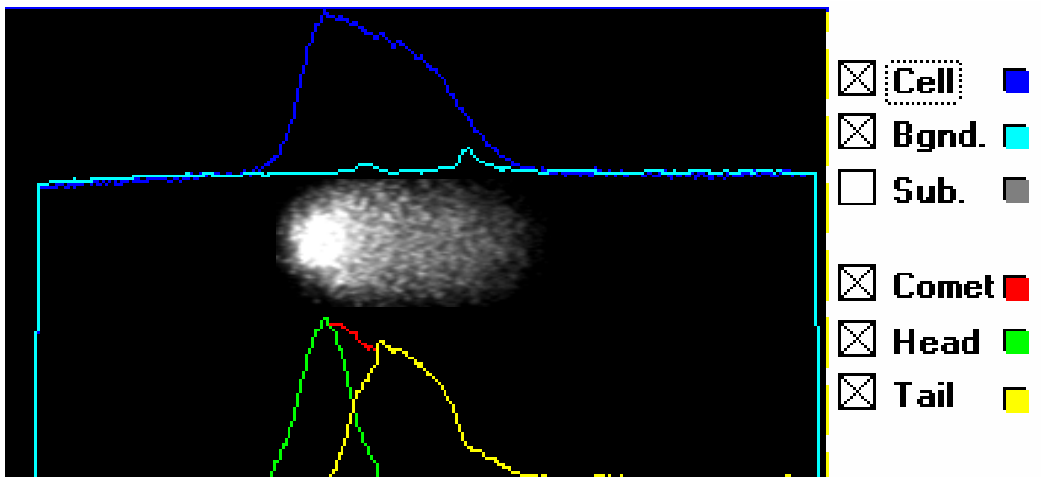


Figure 2. Image analysis for comet assay.

individual cells. A small number of irradiated cells suspended in thin agarose gel on a microscope slide are lysed, electrophoresed, and stained with a fluorescent DNA binding dye. The electric field pulls out the charged DNA from the nucleus so that relaxed and broken DNA fragments migrate further. The resulting images, which are named for their appearance as "comets", are measured to determine the extent of DNA damage. The extent of DNA liberated from the head of the comet during electrophoresis is a function of the dose of irradiation. It is possible, even, to follow DNA damage in cells recovered from biopsy samples of patients having received radiation therapy. Using this technique a heterogeneous response of cells to anticancer agents such bleomycin, was demonstrated. Because the unique design of the comet assay provides direct determination of the extent of DNA damage in individual cells, it is possible to determine whether all cells within a population demonstrate the

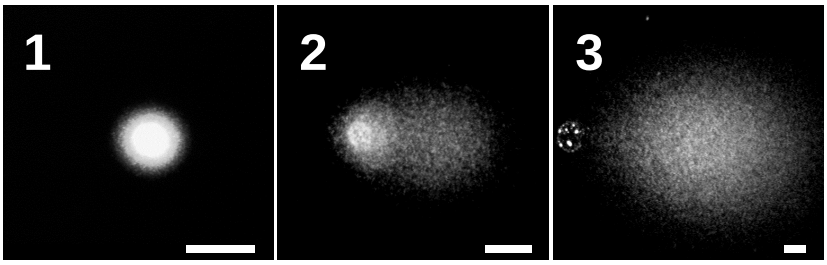


Figure 3. DNA damage resolved by comet assay.

Panel 1: undamaged cell (tail moment < 1). Panel 2: damaged cell (tail moment 6-60). Panel 3: highly damaged cell (tail moment > 60). Bars indicate 20 um.

same degree of damage. Heterogeneous response by cells during treatment can aid in the prediction of tumor response, to detect small subpopulations of cells that may be resistant. Ultimately, this technique allows predicting tumor response to radio- and chemotherapy regimens. The single cell gel assay has also been used to examine DNA damage and repair under a variety of experimental conditions. This technique may prove to be valuable in the dissections of the mechanisms of genotoxicity and DNA repair.

Procedures for measuring DNA strand breaks are generally based upon the principle that strand-breaking agents reduce the size of the large duplex DNA molecule. In addition, DNA single- and double-strand breaks can have dramatic effects on higher-order chromatin structure, by changing its supercoiling and tight packaging within the nucleus. Assays that measure DNA SSBs generally require unwinding of the double stranded DNA molecule for sensitive detection. High pH (>12.3, using NaOH) is generally utilized to

facilitate denaturation, unwinding, and expression SSBs as well as DNA breaks that only become apparent after exposure to alkali (so-called alkali-labile lesions).

Comet forms as broken ends and fragments of the negatively charged, deproteinised DNA molecules become free to migrate in the electric field towards the anode. Two main principles are believed to determine the pattern of comet formation. The ability of DNA to migrate is the function of both the size of the DNA and the number of broken "ends" which may be attached to larger pieces of DNA but which can still migrate a short distance from the comet head. Tail length initially increases with damage but it can reach a maximum that is primarily defined by the electrophoresis conditions, and not only by the size of the fragments. At low levels of damage the DNA loops may be cleaved only at a single site. In this case the free ends "stretch" out of the nucleus in the electric field, producing a short comet tail. At low number of broken ends, stretching of attached strands of DNA, rather than migration of individual pieces, is likely to occur (Figure 2). With increasing number of breaks, DNA pieces migrate freely into the tail of the comet, and at extreme (e.g. in the apoptotic cell, where the DNA is cut into very small molecular weight, oligo-nucleosome size fragments) the head and tail is well separated (Figure 3, panel 3). The intensity of the fluorescence in the tail relative to the head provides information about the number of strand breaks. These two concepts, stretching and migration of separated strands, are generally accepted to explain the DNA migration patterns observed in the comet assay.

Quantitation of DNA damage in single cells

A computerized imaging system makes precise quantitation of DNA damage possible (Figures 1 and 2). For the purposes of quantitative analysis a special parameter the "tail moment" has been introduced. *Tail moment is defined as the product of the fraction of DNA in the tail and the distance (d) between centers of mass of the head and tail distributions* (Figures 1 and 3). Tail moment incorporates a measure of both the smallest detectable size of migrating DNA (reflected in the comet tail length) and the number of relaxed /broken pieces (represented by the intensity of DNA in the tail). With comet image analysis systems that calculate tail moment, it is also possible to determine the total comet fluorescence, which is proportional to the DNA content of the cell and thus with cell cycle position. Bivariate analysis of DNA damage and DNA content accommodates the application of more elaborate statistical methods which aid in further defining subtle changes in response to various treatments in cells within the cell cycle (Figure 4). Detecting such small differences was not possible before the introduction of these techniques.

Effect of etoposide and apoptosis on DNA

In the laboratory experiment, we will examine DNA damaging effect of *etoposide*, a widely used anticancer chemotherapeutic agent. Etoposide is a topoisomerase II poison that fixes DSBs generated transiently by topoisomerase II upon its binding to DNA (Figure 4).
Programmed cell death is a cellular process, which is triggered by a variety of environmental stimuli, e.g. ionization radiation or chemicals mentioned at the beginning of this section, and it results in a controlled end of the cell cycle, called *apoptosis*. The cellular program of apoptosis ultimately activates endogenous nucleases to cleave chromatin. This action generates a series of DNA fragments which are integer multiples of 180-200 base pairs. The comet assay efficiently resolves apoptotic cells with a very characteristic pattern formation (Figure 5, left panel).
The low molecular weight fragments generated during the apoptotic process

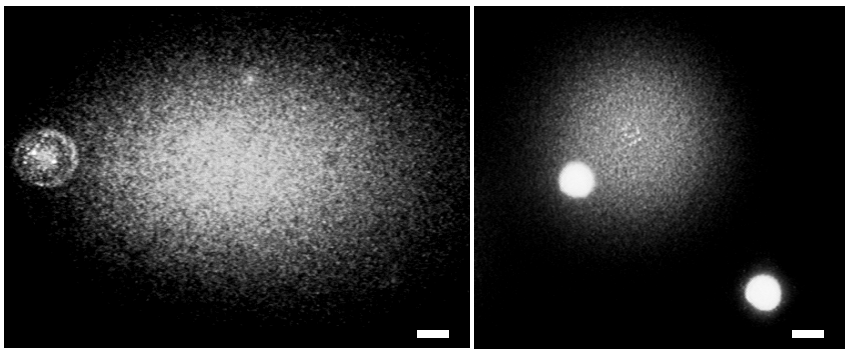


Figure 5. Apoptotic cells resolved by comet and halo assays.

In the left panel there is an apoptotic cell examined by comet assay. In the right panel there are two normal and an apoptotic cell observed by halo assay.

diffuse rapidly even without the electrophoresis. This characteristic is utilized in the *apoptotic halo assay*, where apoptotic cells are distinguished from the normal ones based on a large halo around the less intense nucleus (Figure 5, right panel).

Tasks to be performed in the lab

The aim of this experiment is to visualize single-strand and double-strand DNA breaks generated by etoposide treatment in HL-60 cells using the comet assay.

This assay is a cell biological method designed to quantitate DNA damage in single cells. It is also called single cell gel electrophoresis (SCGE).
The brief protocol of the comet assay is the following. Cells are embedded into agarose on the surface of a slide. The slide covered with agarose containing the cells is immersed into a lysis buffer, which deteriorates the cell and nuclear membranes and denatures the proteins and the DNA. The slide is put into electrophoretic buffer in an electrophoretic tank. After brief unwinding of DNA the cells are electrophoresed. Small molecular weight damaged DNA moves to the direction of the positive pole, while the genomic size undamaged DNA remains at the site of the cell nucleus. This procedure generates comet looking structures as observed in a microscope after staining the DNA with a DNA binding dye (Figure 1and 2). The head of the comet is the undamaged, genomic size DNA, while the damaged DNA moves out into the tail. The amount of DNA damage is proportional for a particular cell or nucleus to the length of the tail and to the amount of DNA in the tail. This comet pattern can be quantitated with an appropriate image analyzing software. The halo assay is a modified comet assay to visualize higher degree DNA damages. It is especially useful to visualize apoptotic DNA damage on the single cell level (Figure 2).

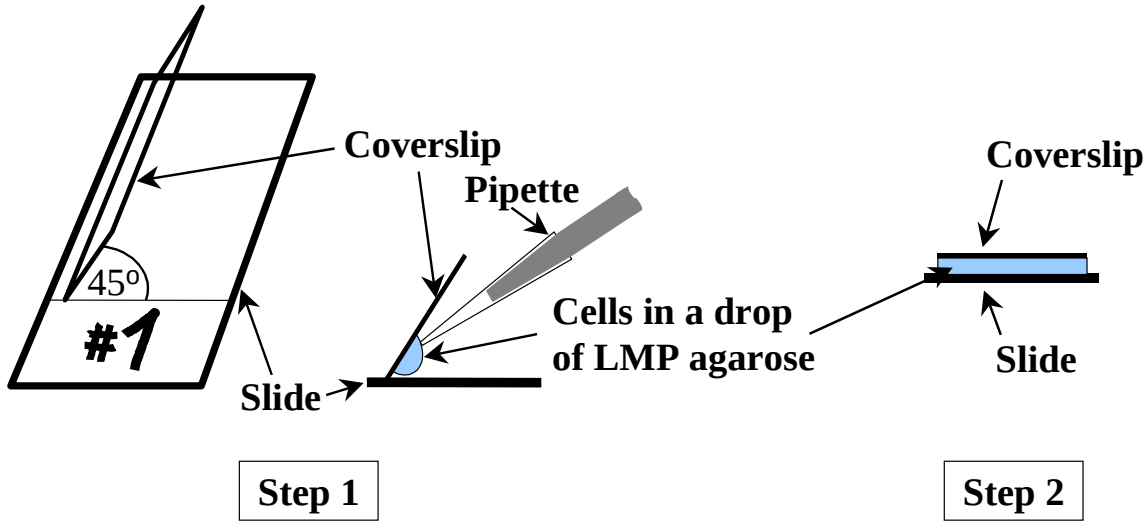


Figure 6. Layering cells on slide.

Preparation of cells

A day before the practice HL-60 cells transferred into fresh medium with 2.5x105/ml cell concentration (10ml/T25 flask - 10% FCS in RPMI). On the day of practice, 5 ml cultured HL-60 cells are prepared in 15-ml centrifuge tube.

Please use the "Short DNA damage protocol" (see below) instead of this!

Step by step protocol

Time (min.)	total time	Steps
5	5	Loosen up the cover of the 1% LMP (Low Melting Point) agarose containing tube and put it into the microwave oven for 3 seconds!!! (Count from 1 to 3 during this 3 seconds and stop the microwave.)
		Be careful, it is hot!!! If you leave it for longer time the agarose will be boiling out!!! If you do not loosen up the cover of the tube it will explode!!!
		Close firmly the cover of the 1% LMP agarose containing tube, shake it and put it into a 37 °C water bath for later usage.
10	15	Centrifuge at least 3 ml cultured cells in a 15ml centrifuge tube at 1200 rpm for 5 minutes, pour away the supernatant and keep the pellet.
		Resuspend cells in 3 ml PBS, count cells. During the time of counting centrifuge cells at 1200 rpm for 5 minutes.
15	30	Set cell concentration to $2x10^6$/ml in PBS: pour away supernatant, keep pellet and add appropriate amount of PBS to cells to resuspend them.
5	35	Treat cells with etoposide (272 µM) and control cells with vehicle:
		Label 4 Eppendorf tubes 1 to 4. Add cells, etoposide and ethanol into tubes according to Table 1.
30	65	Incubate cells for 30 minutes in a 37 °C water bath.
		Slide preparation:
3	68	Label 4 agarose-precoated slides 1 to 4. Prewarm slides and 4 coverslips by putting them on metal holder in the 37 °C water bath for 3 minutes or longer.
3	71	Label 4 new Eppendorf tubes I. to IV. and warm them up to 37 °C for 3 minutes or longer.
3	74	Mix 50 µl cells and 150 ul 1% 37 °C LMP agarose in prewarmed Eppendorf tubes labeled in accordance with the samples.
5	79	Apply 80 µl from the mixture on the 60x24-mm coverslip as shown in Figure 6.

		Release the coverslip, leaving the agarose to be layered between the two glass surfaces.
2	81	Let agarose solidify- laying slides on an ice-cold metal surface for 2 minutes.
2	83	Remove coverslips carefully by sliding them off on the sides.
3	86	Overlay a 2nd layer 37 °C 1% LMP agarose in PBS on top of the cells.
2	88	Let agarose solidify- laying slides on an ice-cold metal surface for 2 minutes.
2	90	Remove coverslips carefully by sliding them off on the sides.
2	92	* Turn the empty sides of slides #1 and #2 to each other and submerge them into 35-ml Alkaliner lysis buffer in a 50-ml centrifuge tube.
2	94	Repeat the previous step for slides #3 and #4 in another 50-ml tube.
60	154	Lysis for 1 hour in Alkaline lysis buffer at room temperature.
2	156	Take out slides from the lysis buffer and submerge them slowly and carefully into the Running buffer in the electrophoretic tank.
15	171	Orient slides according to Figure 7 and incubate them for DNA unwinding in Running buffer for 15 minutes.
		Take out slides #3 and #4 from the tank and slowly and carefully put them into Neutralization solution on the same way as in step *. Leave them and continue with the next step.
25	196	Electrophores slides #1 and #2: set power supply to constant current and adjust current to 200 mA. Run for 20 minutes.
		Stop electrophoresis: turn off the power supply. (Touching slides in the Running buffer without turning off electricity is dangerous!!!)
5	201	Take out slides #1 and #2 from the tank and put them for 5 minutes in Neutralization solution with replacing slides #3 and #4.
5	206	Stain all slides with 50 µl 20 µg/ml propidium iodine using a coverslip as it is indicated in Figure 6.
10	216	Observe comets, halos and undamaged nuclei in the fluorescence microscope at green excitation and red emission and make images.
20	236	Evaluate results: make notes and draw results into your laboratory logbook.

Total 3 hours and 56 minutes

Short DNA damage protocol. Please follow this!

1. Centrifuge HL-60 cells at 1200 rpm for 5 minutes, pour away the supernatant, and resuspend cells in 1 ml PBS buffer.
2. Treat cells with 272 mM etoposide according to the table at 37 ^{o}C for 15 minutes. During the incubation, preheat 4 prelabeled Eppendorf tubes in the 37 ^{o}C water bath. Measure 150 µl prewarmed 1% liquid LMP agarose into the prelabeled warm tubes.

	Label	Sample	Cells	Alcohol	Etoposide
Comet-assay	1	Etoposide	250 µl	–	2 µl
	2	Control	250 µl	2 µl	–
Halo-assay	3	Etoposide	250 µl	–	2 µl
	4	Control	250 µl	2 µl	–

3. Measure 50 µl cell suspension from the treated cell samples to the liquid agarose according to the labels. Mix samples with pipeting. Keep tubes in the water bath during the previous procedures.
4. Stratify 80µl from each mixture to the prewarmed slides according to the label, than put the slides to the metal surface in the ice for 1 minute in order to solidify agarose, than remove coverslips carefully by sliding them off on the slides.
5. Overlay a second layer from the liquid agarose to each slide and place the slides to the ice-cold surface and than remove the coverslips. Layer the liquid agarose quickly to extend uniformly before solidifying.
6. Submerge slides #1 and #2 as well as #3 and #4 by turning the empty sides to each other into 35 ml alkaline lysis buffer in a 50 ml centrifuge tube. Incubate the slides for 25 minutes at room temperature.
7. Place the slides into the electrophoresis tank and incubate them in the electrophoresis buffer for 15 minutes.
8. Take out slides #3 and #4 from running buffer and submerge them into neutralisation solution for 5 minutes, then stain them with 50-µl 20 µg/ml propidium iodine.
9. Adjust current to 200 mA and electrophores slides #1 and #2 for 20 minutes, then incubate them in neutralization solution for 5 minutes ,then stain them with 50 µl propidium iodine.
10. Examine nuclei with fluorescence microscope at green excitation.
11. Make intensity profile from the configurations with ScionImage program.
12. Make notes and draw results.

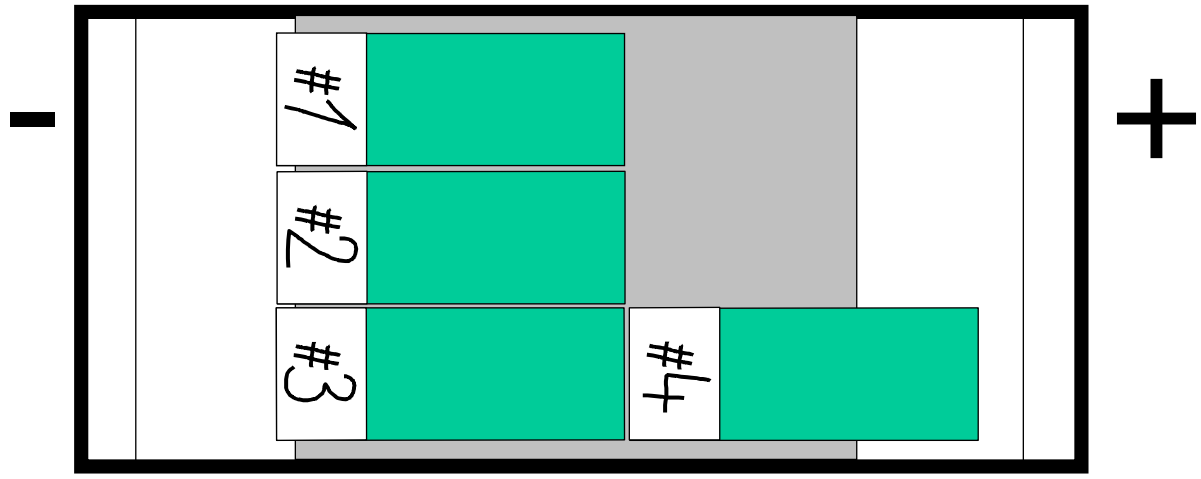


Figure 7. Electrophoretic tank.

Equipment used

Electrophoretic tank
Power supply with cables
37 oC water bath
Centrifuge
Microwave oven
Fluorescence microscope
Ice in container
Flat metal surface laying on the top of ice
Flat metal slide holder in the 37 oC water bath
Flat metal stand for slide preaparation
Pipettes: 200-µl and 10-µl
Sterile 200 µl pipette tips
1 x 15-ml centrifuge tube and container
8 x Sterile 1.5-ml Eppendorf tubes
6 x labeled 50-ml centrifuge tubes, stand alone or with container
4 x Agarose precoated slides: dip Superfrost+ Slides into 90-100oC 1% agarose in H2O and let them dry. Store in a container at room temperature indefinitely.
4 x 60x24-mm coverslips
Gloves
Forceps

Solutions used

Alkaline lysis buffer ***2 x 35 ml used/practice***

1% 1% N-Lauroylsarcosine sodium salt	10 g	(L-5125, SIGMA)
2.5 M NaCl	146.1 g	(S-3014, SIGMA)
10 mM Tris (base)	1.21 g	(Trisma Base T-1503, SIGMA)
NaOH	6 g	(S-0899, SIGMA)
100 mM EDTA	37.22 g	(E-5134, SIGMA, EDTA contains $2xH_2O$)

Set pH to 10

Fill to 1 L and mix till next day. Store indefinitely at room temperature closed.

On day of use add:
100 ml DMSO (10%, 1.28 M)
10 ml Triton-x-100 (1%, 16 mM)

Neutralization solution ***35 ml used/practice***

0.4M Trisma base	48.4g	(Trisma Base T-1503, SIGMA)

Fill to 1L H_2O

pH 7.5

Running buffer ***500 ml used/practice***

300 mM NaOH	24 g	(S-0899, SIGMA)
1 mM EDTA	0,372g	(E-5134, SIGMA, EDTA contains $2xH_2O$)

Fill to 1L H_2O

Etoposide (MW: 588.6) ***4 µl used/practice***

34 mM etoposide in ethanol
(from the original 20-mg/ml ampoule of Vepeside, Sandoz, Rueil Malmaison, France)

Propidium Iodine ***2 x 50 µl used/practice***

20 µg/ml in H2O (SIGMA)

Low Melting Point agarose ***920 µl used/practice***

1% LMP agarose in PBS (SIGMA)

PBS ***6 ml used/practice***

Ethanol ***4 µl used/practice***

Programs and program menu points used for image analysis