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Laboratoř buněčné biologie a genetiky

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Introduction

The laboratory of cell biology and genetics is designed as a series of individual projects. The main aim is to provide not only basic skills of work in the cell laboratory, but also a view into design and evaluation of individual experiments required to assess biological properties of materials. Students will therefore perform experiments with their own cell population they thaw and will work with that population for the rest of time. In a case of suspicion of contamination, they will evaluate microbiology tests and report the results. Students will perform the projects listed below and design them individually following this manual. The project is considered to be fulfilled if the protocol is accepted by the teacher. Within the individual lectures the following assays are expected to be done:

Lecture No. I.

- Design of cell culture laboratory, equipment and safety aspects.
- Working in laminar flow hood.
- Pipetting and transferring fluids.
- Counting of concentration of cells / cm² of tissue culture flasks.
- Preparation of time-schedule of experiments (based on the expected population doubling time).
- Thawing of cells.

Lecture No. II.

- Subculture of cell lines growing in monolayer.
- Determination of cell adhesion (microscopy).
- Preparation of cell proliferation assay – cell seeding.
- Preparation of cell migration assay – cell seeding.

Lecture No. III.

- Sterilization/disinfection of surfaces (glass slides, Tissue Culture Polysterene, Polystyrene).
- Determination of cell proliferation (MTT assay, ATP assay).
- Preparation of cell migration assay - scratch (microscopy).
- Preparation of cytotoxicity by direct contact assay – cell seeding.
- Preparation of cytotoxicity by extracts assay – cell seeding.

Lecture No. IV.

- Determination of cell migration assay - scratch (microscopy).
- Determination of cytotoxicity by direct contact assay – cell seeding.
- Determination of cytotoxicity by extracts assay – cell seeding.
- Preparation of DNA damage assay – cell seeding.

Lecture No. V.

- Determination of DNA damage - Comet assay.
- Preparation of ingrowth within the scaffolds.

Lecture No. VI.

- Determination of ingrowth within the scaffolds.

Project Report No. I.: Accuracy and reproducibility, sterilization efficiency

- Pipetting accuracy - statistical evaluation using mean and SD.
- Sterilization protocol – microbiological evaluation.

Project Report No. II.: Cell cultivation and growth

- Growth curve.
- Calculation of population doubling time.

Project Report No. III.: Cell adhesion on the surfaces

- Preparation of surfaces.
- Quantification of cell adhesion on individual surfaces.
- Determination of differences between cell adhesion on variable individual surfaces (e.g. t-test).

Project Report No. IV.: Cell viability and cytotoxicity.

- Preparation of tested substances and materials.
- Quantification of cell viability.
- Determination of differences between the cell viability (e.g. t-test).

Project Report No. V.: DNA damage

- Preparation of tested substances.
- Determination of DNA damage.

Project Report No. VI.: Cell proliferation and migration on the surfaces

- Preparation of surfaces.
- Quantification of cell proliferation and migration on individual surfaces.
- Determination of differences between the cell behaviour on individual surfaces (e.g. t-test).

Project Report No. VII.: Cell ingrowth within the scaffolds

- Preparation of materials.
- Determination of cell ingrowth.

1 Design of cell culture laboratory, equipment and safety aspects

Background and general information:

Working with cell cultures require, besides other, aseptic working area. Therefore, specific design of laboratory is essential. Several conditions ensure appropriate laboratory practice (Pedersen & Fant, 2018). One of the most important is to maintain cleanliness which includes good air condition and washable surfaces. Another important requirement is the organization of the laboratory. For example, aseptic area, wash-up area, storage and microscopy room should be separated. Regarding the maintenance, all devices, such as laminar box and incubators, should be regularly controlled, washed and disinfected. There are several pieces of equipment essential for cell culture laboratory (Mather & Roberts, 1998).

Laminar flow hood provides clean working area protecting the samples as well as the operator himself. This is ensured by the flow conditions and using HEPA (high efficiency particulate air) filters. Laminar flow hoods can be in class I, II and III depending on the level of protection. All equipment in the hood has to be sterile and the surface area has to be accessible for manipulating and washing (Figure 1).

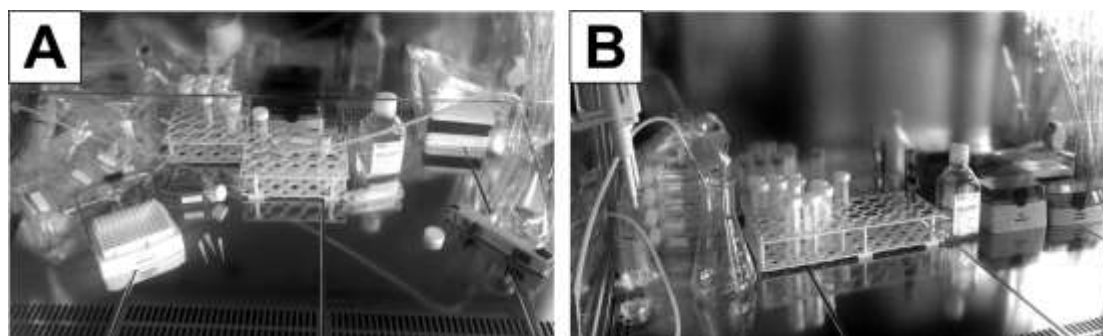


Figure 1 The working area of laminar low hood. A) Good practice; B) bad practice.

Incubator is one of the most important accessories in the cell culture laboratory as the cell cultures require controlled conditions for growth. The fundamental requirement is stable temperature, humidity and CO₂ level. Exact values of gas concentration are cell line dependent. Some of the cultures also need controlled O₂ level as well.

Before and during every work cells have to be examined visually using the **microscope**. To image the cells, microscope with a phase contrast is necessary. (Thorn, 2016). Phase contrast allows the phase shift which results in changes of

brightness. Moreover, inverted microscope should be used to visualise adherent cell line.

Other commonly used devices are autoclave, water bath, centrifuge, fridge, freezer, cryo storage container. Sterile consumables are also needed to maintain aseptic conditions. Nowadays, cell culture plastic ware, such as culture flasks, dishes, plates, tubes as well as pipettes are available in sterile single packaging.

Besides chemical risk, safety aspects in cell culture laboratory introduce also biological risk connected with a manipulation with human or animal cells. Moreover, many used chemicals are highly cytotoxic, carcinogenic or mutagenic. Therefore, it is more than necessary to keep the good laboratory practise and legislative rules. For more information see the Guidance document on good in vitro method practices in OECD series on Testing Assessment (Pedersen & Fant, 2018).

Output:

Students will familiarize the appropriate laboratory design and introduced legislative rules. Each student will sign a form to confirm that he/she knows the laboratory safety rules.

2 Aseptic techniques

Background and general information:

Crucial requirements for work in cell culture laboratory is to avoid harm of the operators as well as cells. Cells should be kept free from all microorganisms, such as bacteria, fungi or viruses. They should be prevented also from cross contamination with other cell lines. Aseptic techniques provide a barrier between the microorganisms (can be introduced by contaminated material or operator) and sterile cell cultures. Reduction of contamination consists of a set of procedures. The major components of aseptic techniques are a sterile working area, proper personal hygiene, sterile reagents and media, and sterile handling (Coecke et al., 2005).

Purpose of Procedure:

The main goal is to familiarize with: Working in flow box; Pipetting and transferring fluids; Preparing sterile culture medium; Washing and sterilizing glassware; Basic work with plastics.

Equipment and Materials:

1. Autoclave
2. Laminar flow hood
3. Micropipettes

2.1 Working in laminar flow hood

Background and general information:

Successful work with cell culture presumes to maintain cells without any contamination. Working in a laminar flow hood is the simplest way how to reduce possible contamination. The hood is equipped with HEPA-filters which protect the working environment from dust and other contaminants. There are three classes of laminar flow hood according to their safety level. Class I protects operators. However, this class does not protect cultures from contamination. Class II provides a good protection for operators as well as cell cultures. These cabinets are intended for biosafety levels (BSL) 1, 2 and 3. Hoods classified in class III are designed for work in BSL 4. These hoods provide the highest level of protection. Working place in a laminar flow hood should be maintained clean and sterile. Every item placed into the hood should be disinfected by spraying with 70% ethanol. Pipettor, pipettes and tips should be placed in a laminar flow hood, not outside the box. Operator should avoid moving any other materials in or out of the hood while work is in progress. Prior and after the experiment, working area has to be disinfected with 70% ethanol and sterilized with UV-lamp.

Purpose of Procedure:

Understand the principles of work in a laminar flow hood.

Standard Protocol:

1. Switch on the laminar flow hood.
2. Pull out the window to the correct position. Wait until the air conditions are at working values (see the indicator on the display).
3. Put on the gloves, disinfect the gloves with 70% ethanol.
4. Wipe the working area in the laminar cabinet with 70% ethanol.

Note: Every experiment with cells should be performed in the laminar flow hood!

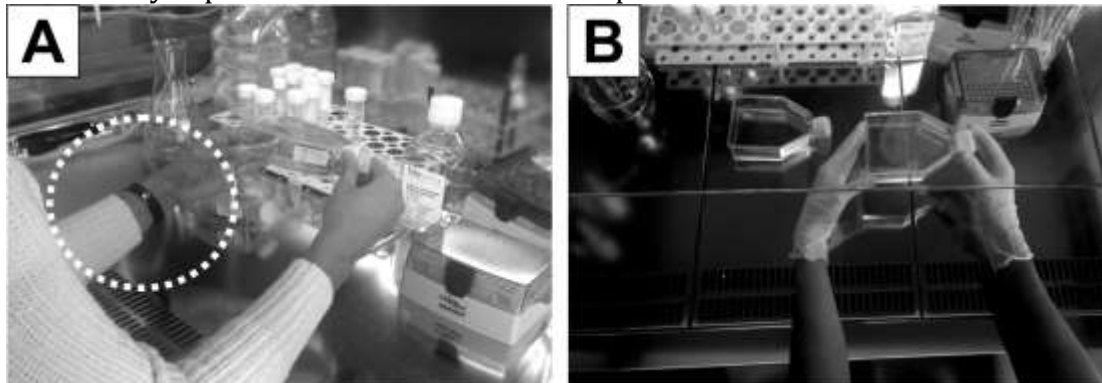


Figure 2 Working in laminar flow hood. A) Jewellery and watch should not be worn; B) use the gloves all the time

Output:

Be able to work with a laminar flow hood.

2.2 Pipetting and transferring fluids

Background and general information:

To obtain repeatable and accurate results it is crucial to manipulate with pipettes correctly. Without accurate pipetting, the experiment would not be reproducible. There are several rules which have to be followed for accurate pipetting. Be sure to use the correct type of pipette tips all the time. Never lay the pipette containing liquid horizontally. Hold the pipette vertically and return it to the pipette holder when you are not working with it. The pipette tip should be pre-wetted before the work. Use reverse pipetting when working with viscous liquids. Immerse the tip sufficiently below the surface of the liquid, shallow or too deep immersion could lead to pipetting inaccurate volume.

Purpose of Procedure:

Accurate pipetting. Safety liquid transfer.

Standard Protocol:

1. Set the volume on the pipette.
2. Depress the plunger to the 1st stop.
3. Immerse the tip under the surface of the liquid in vertical angle and let the plunger go to the rest position.
4. Place the pipette to the receiving chamber and slowly depress the plunger to 2nd stop.

5. Let the plunger go to the rest position.
6. Remove the tip.
7. Pipette 50 μl of non-viscous liquid for 5 times. Weight it each time separately.
8. Pipette 500 μl of viscous liquid for 5 times. Weight it each time separately.

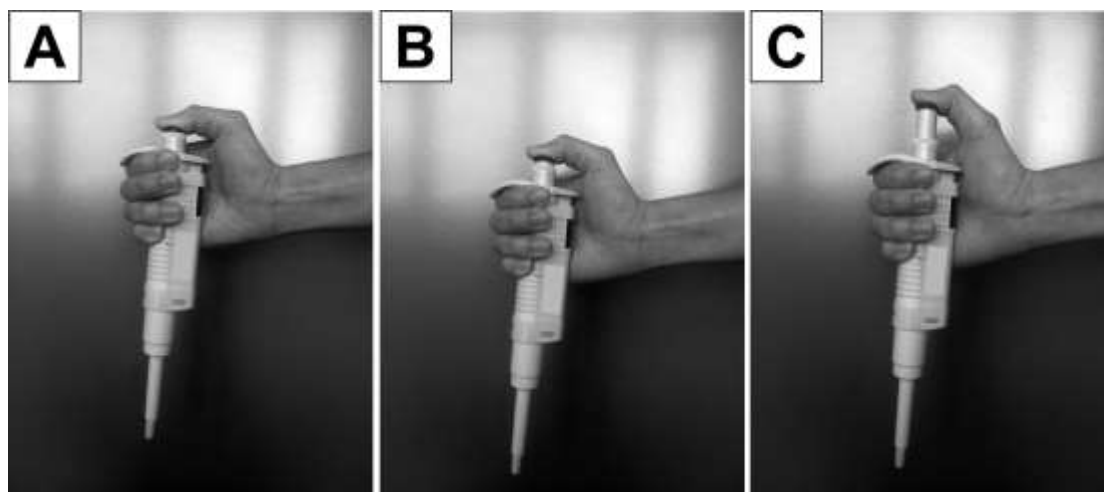


Figure 3 Plunger positions. A) 1st; B) 2nd; C) 3rd.

Data and their analysis:

Calculate the mean weight of liquid in each flask. Calculate the standard deviations of the repeats.

2.3 Sterilization/disinfection

Background and general information:

Contamination is a serious issue to consider in every cell culture laboratory. Contamination may lead to adverse effects on cultures, loss of cells and samples, biased results as well as personal embarrassment and loss of time. Therefore, appropriate knowledge and practice of sterilization and disinfection procedures are necessary for operators. Sterilization/disinfection can be achieved by physical, chemical or physicochemical methods, such as dry heat, moist heat, radiation, filtration (membrane filters), using liquids or gases (e.g. ethylene oxide). The proper method depends on the nature of agent/material which is going to be sterilized.

Among the most common methods belongs (Unchern, 1999):

- Glasses, laboratory equipment - hot air oven, 160°C for 1 hour;
- Tips, micropipettes, plastics, waste disposal – autoclave, 121°C for 20 minutes;
- Surfaces, flat samples (e.g. thin films) – UV-light for 30 minutes;
- Culture media, sera – membrane filters with a pore size of 0,22 μm ;
- Surfaces, materials which cannot be exposed to heat – 70% ethanol.

Purpose of Procedure:

Familiarize correct procedures used for sterilization of individual materials.

Standard Protocol:

1. Inoculate 100 μL of bacterial suspension to the agar plate. Prepare it in quadruplicates.
2. Spread the inoculum homogenously on the agar surface.
3. Place two plates to autoclave at 121°C for 20 minutes.
4. Let all plates incubate for 48 hours at 35°C .
5. Check the number of colonies on agar plates.

Data and their analysis:

Count the number of colonies. Calculate the mean and standard deviations of the repeats.

3 Introduction to culture of cells

Cell culture is a population of cells derived from living tissues and consequently cultured in an artificial environment with controlled conditions. Actual conditions depend on individual cell type. Generally, these conditions include suitable culture medium, essential nutrients, growth factors, hormones, temperature, and the accurate concentration of gases. Appropriate surface for the growth of adherent cells is also very important. There are two types of cell lines in terms of growth, adherent and suspension cell lines. Adherent cell lines grow in a form of monolayer attached onto a suitable surface while suspension cell lines do not attach onto the surface, they float freely in a culture medium. Adherent cell lines will be applied within all experiments.

Cells derived directly from excised tissue are known as primary cultures. These cultures are initially heterogeneous and can be divided *in vitro* only for a few populations before losing their ability to proliferate, which is a genetically determined matter known as senescence; these cell lines are finite. Continuous lines have undergone a transformation when finite cell line acquires the ability to divide indefinitely. This transformation may occur spontaneously (e.g. cancerous lines) or can be chemically or virally induced (e.g. immortalized lines) (Gad, 2000). There exist even further types of cell lines e.g. embryonic stem cells and induced pluripotent stem cells.

Definitions of basic terms are also very important to know:

- **Subculturing/passaging** is a detachment and subsequent transferring of all or some of the cells to a new culture medium.
- **Passage number** is generally the number of times the cells have been subcultured into a new vessel.
- **Confluency** expresses the surface area covered by cells. Usually, confluency is given as percentage of covered surface. Fully confluent cells refer to entirely covered surface. If half of the surface is covered by the cells, we consider 50% confluence.

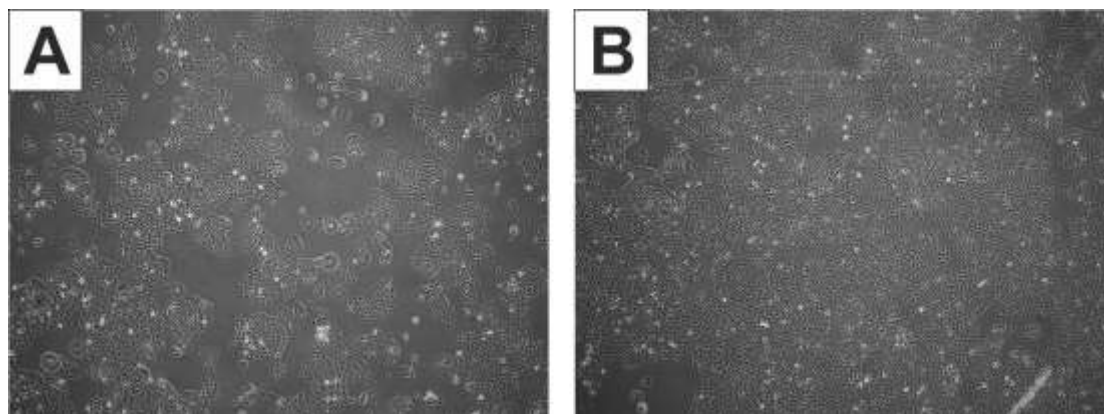


Figure 4 HaCaT cell line. A) semiconfluent; B) confluent.

Equipment and Materials:

1. Centrifuge
2. Cryo freezing container
3. Freezer (-80°C)
4. Incubator
5. Inverted microscope
6. Laminar flow hood
7. Micropipettes
8. Syringe filters
9. Water bath

3.1 Preparation of complete culture medium

Background and general information:

Culture medium is a liquid containing nutrients needed for cell cultures. It contains amino acids, glucose, salts and vitamins. The exact composition depends on the type of the cell line. One of the most common culture media in cell biology is DMEM (Dulbecco's Modified Eagle's Medium), MEM (Minimum Essential Medium), Ham's F-10, McCoy's 5A and RPMI-1640. Culture medium is generally supplemented with serum, antibiotics and growth factors (Yao & Asayama, 2017). Specific requirements of individual cell lines can be found within the protocol defined by author of cell lines or cell culture collector.

Purpose of Procedure:

To prepare a complete cultivation medium.

Standard Protocol:

1. Add DMEM powder to 900 mL of ultrapure water.
2. Add 3,7 g of NaHCO_3 to the solution of DMEM.
3. Add ultrapure water to 1 L.
4. Dissolve the DMEM and NaHCO_3 in ultrapure water.
5. Filter the prepared medium using filter with pores of 0,22 μm to sterile flasks.

6. Add 10% of sterile calf serum.
7. Add 1% of penicillin/streptomycin.
8. Mark the flask with the type of medium, serum, ATB and date.

3.2 Thawing

Background and general information:

Cell lines can be ordered from culture collection. It is highly recommended to order only from reputable and trustworthy specialists, such as European collection of authenticated cell cultures (ECACC) or American type culture collection (ATCC). These companies also provide information about the origin of the cell line as well as the recommendation how to culture the cell line. Cell lines are usually supplied frozen in cryovials at dry ice; therefore, the first step is to thaw it correctly.

Purpose of Procedure:

To be able to accurately thaw the cell lines.

Standard Protocol:

1. Preheat the culture medium at 37°C, prepare culture flasks. Fill the culture flask with the appropriate volume of the culture medium.
2. Thaw the vial from the freezer at water bath at 37°C. Thawing should be rapid, it takes approximately 2 minutes.
3. Decontaminate the vial with cells by spraying with 70% alcohol. Transfer the cells to 15 mL centrifuge tube, add culture medium to 10 mL. Centrifuge the cells at 1100 rpm for 5 minutes.
4. Remove the supernatant with the cryoprotectant. Resuspend the cells in 1 mL of culture medium and transfer the cells to the culture flask.
5. Transfer the culture flask with cells to the incubator.
6. Check the cell adhesion after 5, 20 and 60 minutes and take microphotographs.
7. Check the cell growth after 1 week.

Data and their analysis:

Evaluate the cell adhesion after thawing using the microphotographs.

3.3 Subculture of cell lines growing in monolayer

Background and general information:

To keep the cells in the good condition, adherent cell lines should be subcultured regularly. The exact procedure as well as split ratios (from 1:2 to 1:8) or the degree of confluence (usually around 80-90%) are cell line dependent. Generally, as first cells have to be placed into suspension. In most cases, proteases (trypsin, collagenase) are used to release intercellular and intracellular bonds between the

cells and cells and surface. In some cases, the exposure of cells to proteases is harmful; therefore, the cell scrapers with a small amount of medium are used.

Purpose of Procedure:

To keep the cells in good conditions and to be able to detach them for future experiments.

Standard Protocol:

1. Preheat the culture medium, trypsin-EDTA solution and PBS in water bath at 37°C.
2. View cell cultures using an inverted microscope to assess the degree of confluency and confirm the absence of bacterial and fungal contaminants – take a microphotograph.
3. Estimate the approximate number of cells according to confluency (Table 1).
4. Trypsinize the cells.
 - a. Discard the culture medium from the flask.
 - b. Rinse the cell monolayer with PBS (0.2 mL cm⁻²) and subsequently remove PBS.
 - c. Add trypsin-EDTA solution (0.1 mL cm⁻²), incubate until cells are detached (max. 20 min). Check the cell dissociation using a microscope. During this process take microphotographs every minute.
 - d. Add growth medium in the same amount as trypsin to the cells to inactivate trypsin.
 - e. Transfer the cell suspension to centrifuge tube. Centrifuge cell suspension for 3-5 min at 1100 rpm and 37°C.
 - f. Remove supernatant and resuspend cell pellet in the culture medium.
5. Divide the cells in a split ratio (1:5) and transfer them to new tissue culture flasks. Culture flasks have to be marked with the type of cell line and passage number.
6. Place the flasks to the incubator. Change the medium as needed.
7. Check the cell adhesion after 5, 20 and 60 minutes and take the microphotographs.
8. Check the cell viability after 1 week.

| Culture vessel | Working volume (mL) | Area (cm ²) | Approximate cell yield (HeLa) |
|-------------------|---------------------|-------------------------|-------------------------------|
| T25 flask | 5 | 25 | 5×10^6 |
| T75 flask | 25 | 75 | 2×10^7 |
| 96 well plate | 0.1 | 0.3 | 1×10^5 |
| 12 well plate | 1 | 2 | 7.5×10^5 |
| 24 well plate | 1 | 2 | 5×10^5 |
| 3.5 cm petri dish | 2 | 8 | 2×10^6 |
| 6 cm petri dish | 5 | 21 | 5×10^6 |

Table 1 Characteristics of selected culture vessel (Freshney, 2005).

Data and their analysis:

Evaluate if the subculturing was performed correctly by measuring the cell viability after one week. Describe the cell morphology using the microphotographs.

3.4 Freezing

Background and general information:

To ensure long-term supply of cells, cryopreservation is necessary. The main advantage is that there is no need to subculture the cell lines when you are not working with them. Cell lines can be stored in freezers at -80°C or in liquid nitrogen. The main key to successful cryopreservation is slow freezing. In general, cells should be cooled by -1°C per a minute. It is very important to use cryoprotectant which prevents from formations of ice crystals leading to cells rupture (Phelan & May, 2015).

Purpose of Procedure:

Familiarize the principle of long term storage of cells in liquid nitrogen or -80°C Lab Freezers.

Standard Protocol:

1. Check the cells. The confluency should be around 90%.
2. Trypsinize the cells.
3. Label cryotubes with the cell line, passage number and date.
4. Resuspend cells to a concentration 1×10^6 per mL in culture medium.
5. Add the cryoprotectant dimethyl sulfoxide (DMSO) to a final concentration of 5%. Work quickly after adding the DMSO!
6. Dispense the cell suspension into prelabeled cryotubes.
7. Put the cryotubes to the cryo freezing container (Figure 4). If required, fill the container with isopropyl alcohol.
8. Displace the container to the freezer at -80°C.



Figure 5 Examples of cryo freezing containers.

3.5 Microscopy:

Background and general information:

Microscopy plays a very important role in cell culture laboratory. Cell lines have to be examined regularly not only for their morphology but also to check their health conditions. Before the start of any experiment, the cell culture should be examined by microscope.

To observe adherent cell culture an inverted microscope is essential. An inverted microscope has the condenser and light source on the top while the objectives and turret are placed on the bottom. What is more, phase contrast is an essential feature to display cells clearly. Phase contrast is a technique which converts phase shifts into light passing through a transparent sample resulting in brightness changes in the displayed image. Cells compartments are not visible without a phase contrast.

Purpose of Procedure:

Observe the cell by phase contrast microscope; Take the microphotographs within magnifications of 4x, 10x and 20x.; Check the cells using correct phase contrast as well as the incorrect one.

Standard Protocol:

1. Check the cells visually using a microscope.
2. Trypsinize the cells.
3. Dilute the cells to the concentration of 1×10^6 per mL.
4. Seed the cells onto tissue polystyrene and glass samples in the volume of 1 mL. Let them incubate for 1 hour.
5. Discard the medium. Wash the cells with PBS. Add the required amount of PBS.
6. Add $5 \mu\text{g mL}^{-1}$ of Hoechst 33258 and let it in the dark for 20 minutes.
7. Examine the cell adhesion and take microphotographs within magnifications of 4x, 10x and 20x.

Data and their analysis:

Qualitative data: Micrographs with scales within different magnifications.

3.6 Growth curve**Background and general information:**

Growth curve defines growth characteristics of each cell line, such a doubling time or the best time for subculturing. Growth curve consists of three phases: 1) lag phase; 2) exponential phase; 3) stationary phase. The lag phase is the time needed for the cells recovery after trypsinization. In the next exponential phase, the cell population doubles. The time in which cells are doubled is known as doubling time and can take from a few hours up to 48 hours depending on the type of the cell line. After this exponential growth, when the cells are confluent, the nutrients are metabolized which results to a very slow growing and cells enter a stationary phase (Assanga & Lujan, 2013).

Purpose of Procedure:

To understand rapidity of cell growth and their doubling.

Standard Protocol:

1. Check the cells visually using a microscope.
2. Trypsinize the cells.
3. Dilute the cells to the concentration of 1×10^4 per mL.
4. Seed the cells on two 6-well plates. Put the plates into the incubator.
5. Count the duplicates every 24 hours for 4 days.
6. Plot the results on a log-linearscale.
7. Calculate the cell doubling time (DT) using the equation (Korzyńska & Zychowicz, 2008):

$$DT = \frac{\Delta t}{\log_2\left(\frac{\Delta N}{N_0 + 1}\right)}$$

Where N_0 is the initial concentration of cells, ΔN is an increase of cell concentration during the cultivation time Δt .

Data and their analysis:

Prepare a growth curve and calculate doubling time.

4 Cell viability

Background and general information:

To know whether cells are able to maintain alive after the exposition to different agents it is important to quantify the cell viability. Cell viability refers to a number of healthy cells related to the reference (untreated cells). There are several assays that can be used for this purpose. These tests can be based on different parameters, such as the integrity of cell membranes, metabolic activity or redox potential of the cell population. One of the commonly used assays is a measurement of metabolic activity using MTT assay and ATP detection (ATP assay). These assays offer a quick overview of the potential cytotoxic effect of any compound.

Purpose of Procedure:

Familiarize determination of cell viability using their quantification.

Equipment and Materials:

1. Centrifuge
2. Incubator
3. Inverted microscope
4. Laminar flow hood
5. Luminometer
6. Micropipettes
7. Spectrophotometer
8. Water bath

Standard Protocol:

Cell preparation:

1. Check the cells visually using a microscope.
2. Trypsinize the cells.
3. Transfer the cells onto the 96-well plate.
 - a. Prepare one plate with the cell concentration of 1×10^5 cells per mL.
 - b. Prepare one plate with different concentrations of cells. Use concentrations of 1×10^6 ; 5×10^5 ; 1×10^5 ; 5×10^4 and 1×10^4 cells per mL. Each concentration will be prepared in quadruplicates.

Exposition to cytotoxic agent:

1. Take the 96-well plate with the cell concentration of 1×10^5 cells per mL and check the cells by microscopy. Take the microphotograph.

2. Discard the old medium.
3. Add the liquid cytotoxic agent in the concentrations of 50; 25; 10; 5, 1 and 0.5%. Dilute the agent in the culture medium.
4. Place the plates into the incubator.
5. Continue with MTT and ATP assay.

4.1 MTT assay

MTT is a widely used method to determine cell viability. This assay is dependent on cellular metabolic activity. It is based on the enzymatic reduction of yellow MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to purple MTT-formazan (Figure 5). This reduction is catalysed by mitochondrial dehydrogenase. Formed formazan is proportional to the number of living cells. The amount of formazan is measured by recording changes in absorbance. Hence, the MTT assay is colorimetric reaction that can be analysed spectrophotometrically. As formazan is water-insoluble salt, it has to be solubilized before the measurement of absorbance. Widely used solvents are DMSO, isopropanol, or SDS (Stockert, Horobin, Colombo, & Blázquez-Castro, 2018).

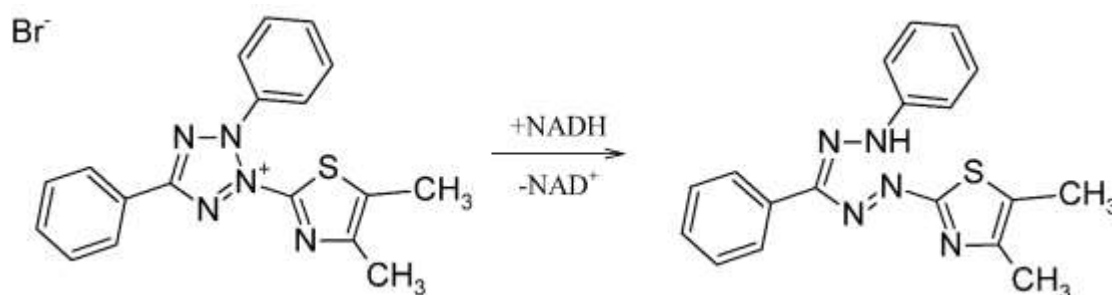


Figure 6 Structure of MTT and formed formazan.

Standard Protocol:

1. Prepare the solution of MTT in ultrapure water in a concentration of 5 mg mL⁻¹.
2. Discard the old medium and add the preheated fresh medium.
3. Add MTT solution to the final concentration of 0.5 mg mL⁻¹.
4. Place the plate into the incubator for 2 hours.
5. Remove the medium carefully. Dissolve the crystals of formazan by adding DMSO. Let it dissolve for 15 minutes by gently shaking.
6. Read the absorbance at 570nm, set the reference wavelength at 690nm.

Data and their analysis:

- Calculate means and standard deviations for each repetition.
- The percentage of viable cells calculate using the following equation:

$$\% \text{ viability} = \frac{\text{absorbance}_{\text{sample}}}{\text{absorbance}_{\text{reference}}} \times 100$$

4.2 ATP assay

Background and general information:

MTT assay has a lower sensitivity than fluorescent or luminescent assays. Moreover, redox active compounds can affect the reduction of MTT and lead to falsely positive results. Therefore, using different method is much more appropriate for such substances. ATP-bioluminescence assay is one of the most sensitive and fastest viability assays. In contrast with MTT assay, the luminescent signal reaches a steady state within a few minutes after the addition of reagents; therefore, no incubation period with reagent is needed. However, ATP assay is much more sensitive for accurate manipulation, especially precise pipetting. This method is based on the conversion of luciferin to oxyluciferin achieved by addition of the enzyme luciferase. This reaction is dependent on the present amount of ATP and accompanied by the production of light (Figure 6). Hence, the generated light is proportioned to the level of ATP. The light emission is measured using luminometer (Morciano et al., 2017).

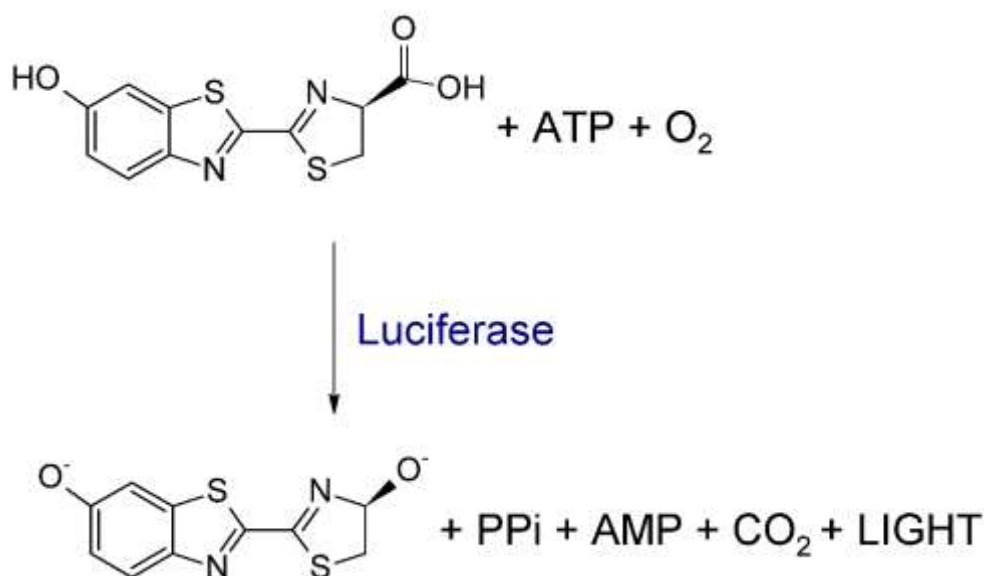


Figure 7 Conversion of luciferin to oxyluciferin.

Standard Protocol:

1. Prepare the reaction solution by mixing following chemicals: 89 vol.% distilled H₂O, 5 vol.% 20X reaction buffer, 1 vol.% 0.1 M DTT, 5 vol.% 10 mM D-luciferin and 0.025 vol.% 5 mg mL⁻¹ of firefly luciferase
2. Prepare data to obtain standard curve:
 - a. Add the reaction solution to the well plate.
 - b. Add ATP solution in quadruplicates in the concentrations of 1 nM, 10 nM, 50 nM, 100 nM, 500 nM, 1000 nM.
 - c. Measure the luminescence.

3. Discard the medium from the cells.
4. Add the reaction solution. Do not add ATP solution!
5. Read the luminescence.

Data and their analysis:

Protocol with the results of MTT and ATP assays. Results should be in a form of a column graph with the mean values and standard deviations (Figure 7). Moreover, calculate statistical differences using T-test.

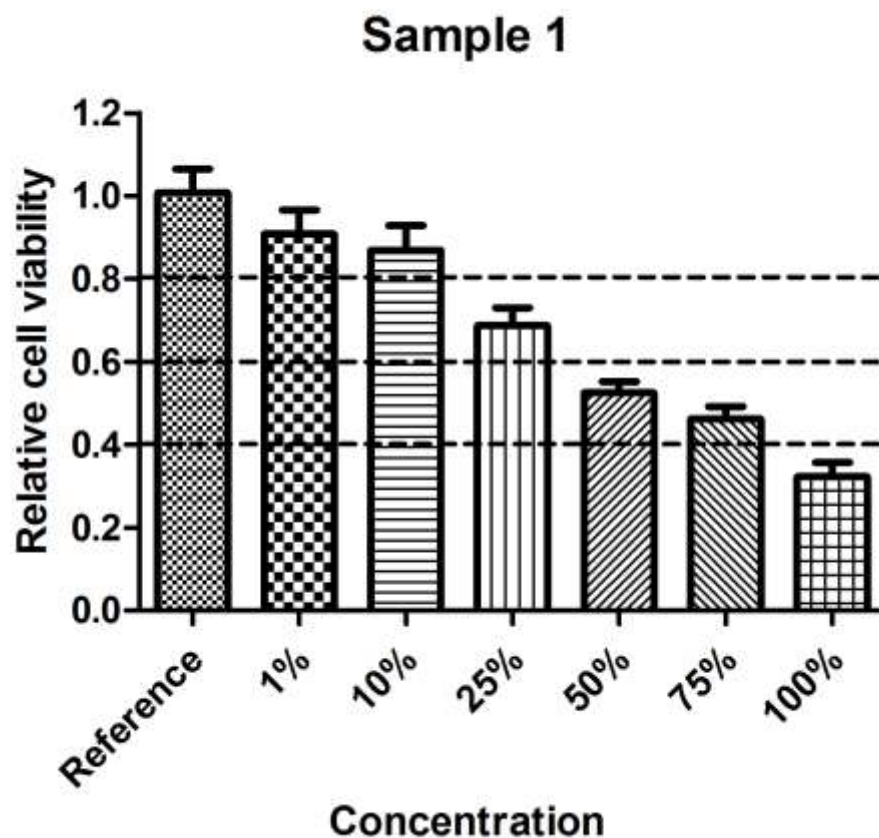


Figure 8 Example of data processed using MTT assay.

5 Determination of DNA damage - Comet assay

Background and general information:

Comet assay is a sensitive technique to identify whether any agent causes DNA damage and to what extent at the level of an individual cells. Comet assay, also known as SCGE (single cell gel electrophoresis), is based on the charge applied on the gel with cells resulting in separating damaged DNA from the nucleus. The name comet assay originates from the appearance of DNA of individual cells. As they migrate through gel, damaged DNA forms a tail resembling comets (Figure 8). These comet tails are visualized by fluorescence staining. The brightness and length of the tail correspond to the number of DNA breaks (Olive & Banáth, 2006).

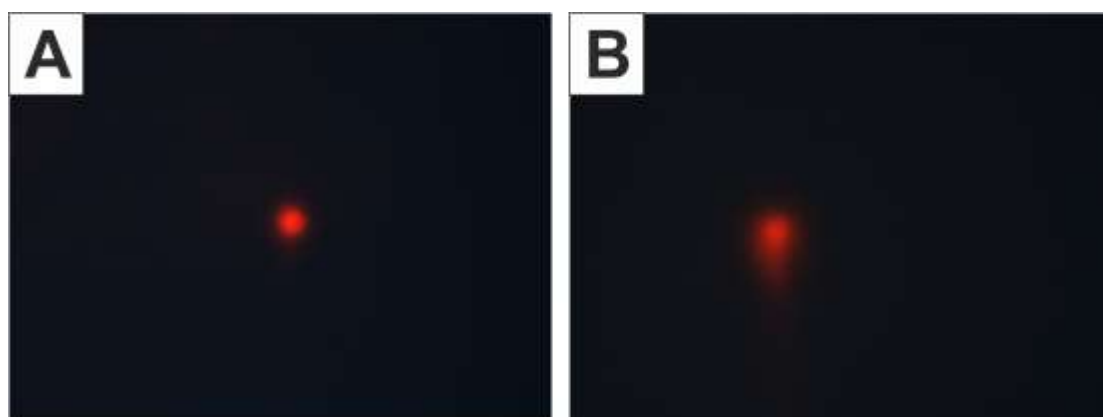


Figure 9 Evaluation of DNA damage. A) DNA without damage; B) damaged DNA with the tail.

Purpose of Procedure:

Familiarize the process of DNA damage and electrophoresis assay.

Equipment and Materials:

1. Centrifuge
2. Electrophoresis
3. Fluorescence microscope
4. Incubator
5. Laminar flow hood
6. Micropipettes

Standard Protocol:

1. Prepare the cells by standard protocol.
2. Add the cytotoxic agent to adherent cells. Let it affect the cells for one hour.
3. Prepare precoated agarose slides.
 - a. Prepare 1% low-gelling-temperature agarose solution.
 - b. Immerse a clean microscope slide to the agarose solution. Wipe one side of the slide to remove excess agarose.
 - c. Dry the slides at room temperature.
4. Prepare the cell samples. Cells have to be in a single-cell solution.
 - a. Detach the cells by standard protocol.

- b. Resuspend the cells.
 - c. Take 0.4 mL of cell solution, add 1.2 mL of 1% low-gelling-temperature agarose. Mix the solution carefully by gentle pipetting and transfer it immediately to the surface of precoated slide.
 - d. Let agarose rest to become gel for a few minutes.
5. Lyse the cells and run electrophoresis.
 - a. Gently place the slides to a lysis solution.
 - b. Let the cells to lyse for 1 hour in the dark (for standard protocol overnight, 18-20h).
 - c. Remove the slides and wash them with a rinse solution for 5 minutes for three times.
 - d. Transfer the slides into electrophoresis solution in electrophoresis chamber. The solution should be 2 mm above the top of the gel.
 - e. Proceed to electrophoresis at voltage of 0.6 V cm^{-1} for 25 minutes.
6. Stain the slides.
 - a. Remove slides from the chamber and wash them with distilled water twice.
 - b. Transfer the slides to propidium staining ($2.5 \mu\text{g mL}^{-1}$) solution for 20 minutes.
 - c. Wash the slides with distilled water twice.
7. Observe the comets using a microscope.

Data and their analysis:

Micrographs; length of comets – means and SD.

6 Cell adhesion, proliferation and migration

Background and general information:

Ability of cells to adhere and proliferate on the material is crucial to utilize material in tissue engineering. Cell adhesion is affected by three main classes of proteins 1) the cell adhesion molecules, 2) the extracellular matrix proteins, 3) the cytoplasmic membrane proteins. Regarding the material, cell adhesion and proliferation can be influenced by chemical composition, mechanical or physical properties. If material comes in a contact with cells, cell adhesion is the first interaction between them. If cells are able to adhere on the material, another important property is whether cells are able to divide and grow on it. The morphology of proliferating cells is checked by fluorescence microscopy. To visualize the cells, actin filaments and nucleus are stained.

Moreover, cell migration is important to mimic *in vivo* conditions of wound healing. Cell migration is studied using scratch assay. This method serves to observe cell-cell and cell-extracellular matrix interactions. Scratch assay can be performed only within the samples which allow cell growth as the scratch is created on a confluent cell monolayer. Cell migration is determined by microscopy as live cell imaging (Liang, Park & Guan, 2007).

Purpose of Procedure:

Fluorescence staining of cells to visualize their compartments.

Equipment and Materials:

1. Confocal microscope
2. Fluorescence inverted microscope
3. Incubator
4. Laminar flow hood
5. Water bath

6.1 Cell proliferation

Standard Protocol:

1. Check the cells visually using a microscope.
2. Trypsinize the cells.
3. Dilute the cells to the concentration of 1×10^5 per mL.
4. Seed the cells onto samples in the volume of 2 mL.
5. Place the samples into the incubator and let them incubate for 24 hours.
6. Fix and permeabilize cells using formaldehyde and Triton X-100.
 - a. Discard the medium from the cells.
 - b. Add 4% formaldehyde and leave for 15 minutes.
 - c. Discard the formaldehyde.
 - d. Wash the cells with PBS.
 - e. Add 0.5% Triton X-100 for 5 minutes.

- f. Wash the cells with PBS for three times.
- g. Add required amount of PBS.
7. Add $5\mu\text{g mL}^{-1}$ of Hoechst 33258 and two drops per 1 mL of ActinRed™ 555. Let it rest in the dark for 30 minutes.
8. Discard PBS with stains. Wash cells with PBS. Add required amount of PBS.
9. Take microphotographs within different magnifications.

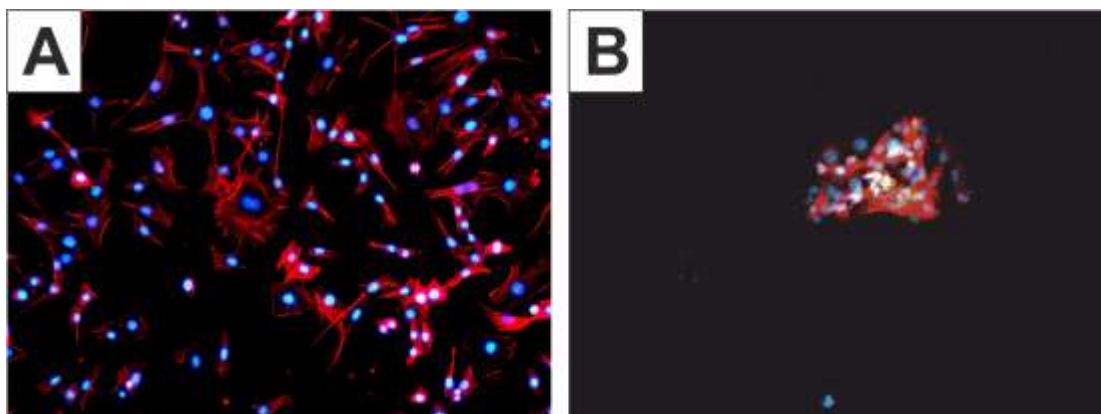


Figure 10 Cell proliferation. A) Good proliferation; B) bad proliferation.

6.2 Cell migration (scratch assay)

Standard Protocol:

1. Prepare marks with the permanent marker from the bottom of the sample to check the cell in the same place for all the times.
2. Check the cells visually using a microscope.
3. Trypsinize the cells.
4. Dilute the cells to the concentration of 1×10^5 per mL.
5. Seed the cells onto samples in the volume of 2 mL.
6. Place the samples into the incubator and let them incubate until they reach confluency.
7. Take the micropipette tip ($10\mu\text{L}$) and make the straight line (scratch) through the cell monolayer.
8. Remove the debris and wash the cells once with PBS.
9. Add the culture medium and put the samples with cells into the incubator.
10. Check the scratch after 1, 6, 12 and 24 hours. Take microphotographs.
11. Measure the width of the scratch.

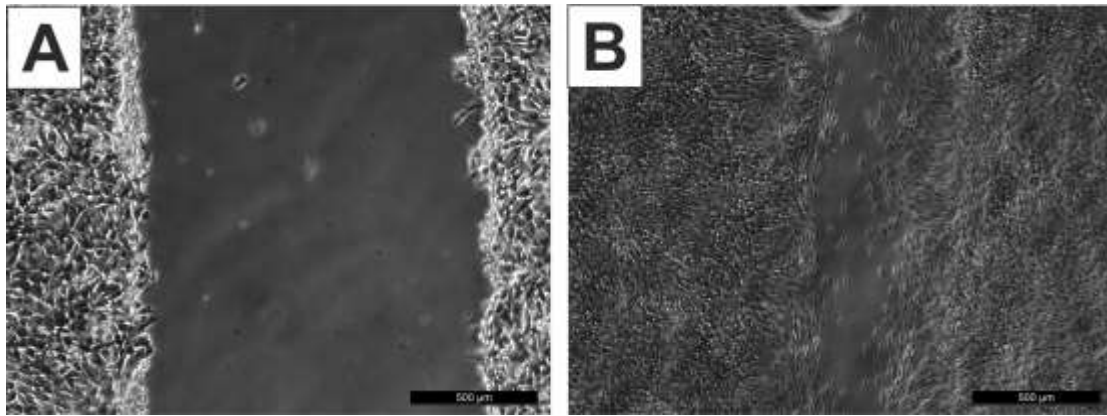


Figure 11 Scratch assay. A) Starting point; B) migration after 12 hours.

Data and their analysis:

Protocol containing micrographs of cell proliferation and migration on the samples and on tissue polystyrene. Describe the changes in cell morphology and migration.

7 Cell cultivation within the scaffolds

Background and general information:

Culturing in 2D systems plays an important role in the research; however, this model is not possible to use to mimic and simulate real conditions and structural organization in living systems accurately. In natural tissues cells occur in complicated interactions in 3D environment. Therefore, there is a considerable effort for development of 3D cultivation conditions. In 3D systems cells have more possibilities for mechanical inputs, they can exhibit also different morphology, speed of proliferation or gene expression (Edmondson, Broglie, Adcock & Yang, 2014).

One of the tools allowing the cell growth in 3D conditions is the cultivation within the scaffolds supported by cultivation in bioreactors. There are several aspects which affect the cell growth within the scaffolds. Naturally, scaffolds have to be biocompatible without any cytotoxic or immune response. Another requirement (in some applications) is the biodegradability of scaffolds leading to replace scaffold material and form extracellular matrix by cells. This introduces another condition by-products have to be biocompatible as well. The pore distribution, their size and structure are also very important properties of scaffolds. The pores should be interconnected for good ingrowth of cells. What is more, interconnected pores provide proper diffusion of either nutrients or metabolic waste, and degradation of by-products from scaffolds. Mechanical properties depend on the future utilization of scaffolds (should be similar to targeted tissue). As was mentioned before, the cultivation is supported by bioreactor. Bioreactors can mimic the real environment present in *in-vivo* systems. They can simulate the physiological conditions as the flow of body fluids, pressure or mechanical stress (Loh & Choong, 2013).

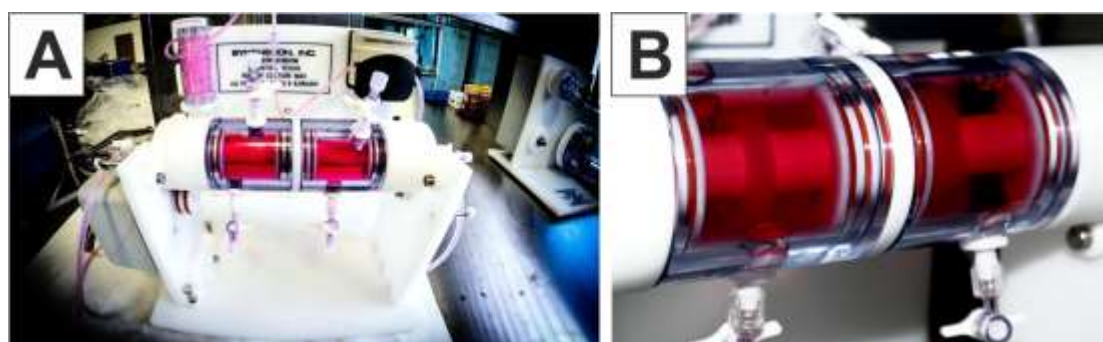


Figure 12 Bioreactor. A) Perfusion bioreactor; B) detail of the chamber.

Purpose of Procedure:

Understanding complicated *in vivo* conditions via mimicking them using cultivation in bioreactors.

Equipment and Materials:

1. Bioreactor
2. Centrifuge
3. Confocal microscope
4. Incubator
5. Laminar flow hood
6. Microtome
7. Water bath

Standard Protocol:

1. Prepare thin slides of samples by cutting them using microtome.
2. Check the cells visually by microscope.
3. Trypsinize the cells.
4. Dilute the cells to the concentration of 1×10^6 per mL.
5. Seed the cells into the sterile scaffold and let them adhere for two hours.
6. After adhesion, add the culture medium to the final concentration of 2 mL and let the cells proliferate.
7. Before working with bioreactor it is needed to sterilize chambers.
 - a. Release the screws.
 - b. Plastic screws wrap to aluminium foil.
 - c. Sterilize it in autoclave at 121°C for 20 minutes.
 - d. All components inappropriate for autoclaving have to be poured into 70% ethanol.
8. Place the sample with the cells into the appropriate chamber.
9. Fill the chamber with the culture medium, avoid bubbles. Place the bioreactor into the incubator, switch it on. Let the samples rotate for 2 weeks. Change the medium every 3 days.
10. After the incubation period, remove the samples from the bioreactors.
11. Fixed the cells on the sample by standard procedure. Stain it with fluorescence dyes and take microphotographs.
12. Wash the chamber with 70% ethanol and place them into the ultrapure water.

Data and their analysis:

Evaluate the cell morphology and growth in the sample.

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List of Abbreviations

| | |
|-------|--|
| ATB | antibiotic |
| ATCC | American type culture collection |
| ATP | adenosine triphosphate |
| BSL | biosafety level |
| DMEM | Dulbecco's modified Eagle's medium |
| DMSO | dimethyl sulfoxide |
| ECACC | European collection of authenticated cell cultures |
| EDTA | ethylenediaminetetraacetic acid |
| HEPA | high efficiency particulate arrestance |
| MEM | minimum essential medium |
| MTT | 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide |
| PBS | phosphate buffered saline |
| SCGE | single cell gel electrophoresis |

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