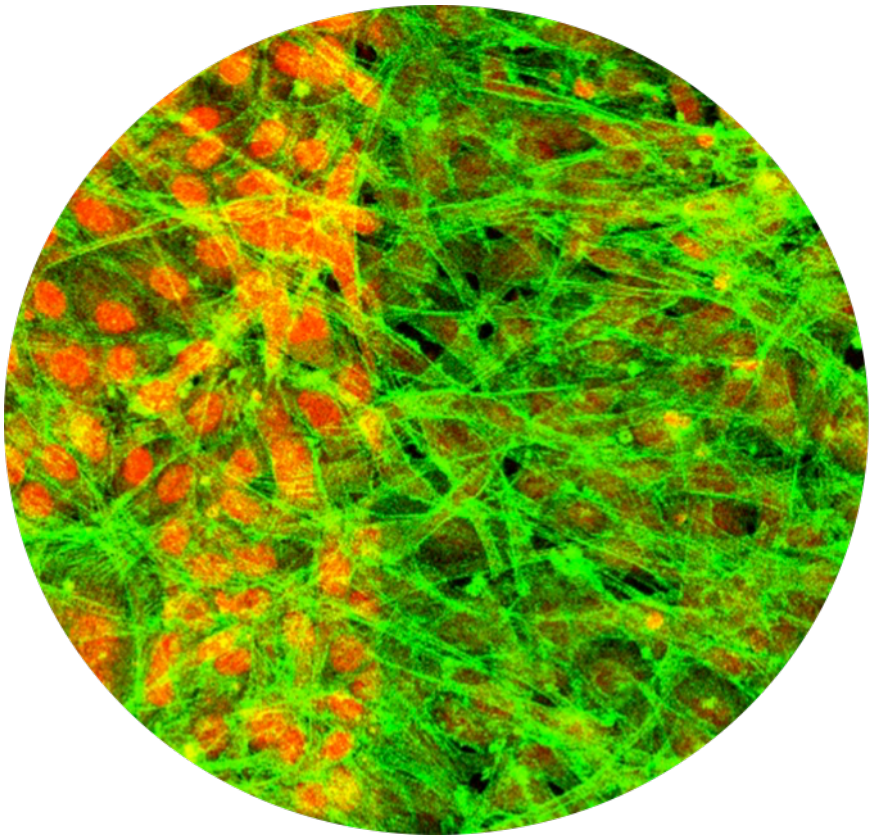
ITMO UNIVERSITY

**A.F. Fakhardo, S.A. Ulasevich,**
**A.Y. Prilepskii, V.V. Vinogradov**

# RESEARCH METHODS IN MOLECULAR AND CELL BIOLOGY



**Saint- Petersburg**
**2021**

MINISTRY OF EDUCATION AND SCIENCE OF THE RUSSIAN FEDERATION
ITMO UNIVERSITY

**A.F. Fakhardo, S.A. Ulasevich,
A.Y. Prilepskii, V.V. Vinogradov**

# RESEARCH METHODS IN MOLECULAR AND CELL BIOLOGY

STUDY GUIDE

RECOMMENDED FOR USE AT ITMO UNIVERSITY

in the field of training 19.04.01 Biotechnology
as a teaching aid for the implementation of basic professional educational programs of higher education magistracy

**Saint- Petersburg
2021**

Fakhardo A.F., Ulasevich S.A., Prilepskii A.Y., Vinogradov V.V. Research methods in molecular and cell biology – SPb: ITMO University, 2021. – 52 p.

Reviewers:
Elena Ivanovna Koshel, PhD of Biological Sciences, Associate Professor (qualification category "Ordinary Associate Professor") of the ChemBio Cluster, ITMO University.

The manual outlines the main methods of working with adherent animal cell cultures, provides examples of determining the cytotoxicity of nanoparticles using the assessment of mitochondrial respiratory activity (MTT test), staining with fluorescent and absorption dyes, describes the necessary laboratory equipment: laminar box, incubator, inverted microscope, cell counter. The manual is aimed at developing basic skills in working with cell cultures during toxicological experiments.

It is intended for graduate students of the ChemBio cluster of ITMO University enrolled in the Molecular Biology and Biotechnology program (disciplines "Methods in nanomedicine", "Nanotoxicology" and "Advanced Materials for Biomedical Applications"), and can also be recommended to students of natural sciences when they perform several works in specialized workshops.

ITMO UNIVERSITY

**ITMO University** – is a leading Russian university in the field of information and photonic technologies, one of the few Russian universities that received the status of a national research university in 2009. Since 2013, ITMO University has been a participant in the program to improve the competitiveness of Russian universities among the world's leading research and educational centers, known as the "5 in 100" project. The goal of ITMO University is to become a world-class research university, entrepreneurial in type, focused on the internationalization of all areas of activity.

**Table of contents**

# 1. GENERAL RULES OF WORK IN LABORATORIES

## *1.1. Being in the cell culture lab*

1. It is allowed to stay in the culture room only in a lab coat and changeable shoes, which are not used anywhere else. Long hair should be gathered and pulled behind the head.

2. Before starting any work, make sure that all the necessary reagents and tools are in a cell culture room. It is necessary not to go in and out during work.

3. Avoid using a mobile device in the cell culture room. In case of emergency, use the device with a hand without a glove.

4. No more than four people can be in the cell culture room for a long time at a time. Except for the moments of training, **it is forbidden to be in the room without doing work**.

5. It is forbidden to work with toxic volatile substances (paraformaldehyde, chloroform, phenol and trizol, methanol, 2-mercaptoethanol) and strong-smelling substances (dithiothreitol, isopropanol, isoamyl alcohol, fixation varnish) in the cell culture room.

6. After working with the microscope, make sure to turn off the microscope lamp.

7. The incubator should be opened only with gloves, pre-treated with alcohol. Keeping the incubator open more than required is unacceptable. It is advisable to pay attention to the water level in the sump; if it is not enough, it is necessary to fill it with deionized water.

8. Only sterile dishes used for cell cultivation (dishes, plates, vials) are allowed to be placed in the incubator. Cells in test tubes should be incubated in a water bath, not in an incubator.

9. All prepared aliquots, dilutions and weighed portions, and personally prepared media and dishes with cell cultures must be legibly signed. The signature of personal reagents and dishes with cell cultures must contain the name of the solution or cell line, an indication of the owner of the reagent or cell culture; for solutions and media, the date of preparation is also indicated. It helps to avoid confuses when working in the laboratory with many lab members.

10. It is necessary to be careful about reagents' storage conditions: for some of them, being at the wrong temperature can lead to a rapid loss of their original properties. Do not keep serum (FBS) aliquots, trypsin solution and culture media at room temperature longer than required. A new open bottle of serum must be aliquoted into 50 mL tubes, having previously completely defrosted in the refrigerator or at room temperature (not at 37°C). Do not leave a whole bottle of serum outside the refrigerator for longer than it takes to defrost. Aliquots of drugs and heat-sensitive dyes should also be placed in a refrigerator at the required temperature in their original place immediately after work.

## *1.2. Rules for working in sterile conditions (in a laminar flow hood)*

1. When planning work in a laminar flow hood, it is a good idea to create an electronic calendar for booking of the laminar flow hood as well as for all the other equipment if there are more than 3-4 workers in the laboratory to avoid the lines and to plan the work. Regardless of the order of recording, it is necessary that between work, the laminar flow hood is treated with UV irradiation for at least 20 min to avoid contamination.

2. The following accessories can be kept in the laminar flow hood permanently: automatic pipettes with a stand, serological pipettes, open packs of culture dishes, a box with 1000 µL tips, a box with 200 µL tips, trash box and permanent marker.

3. At the beginning of the working day, 15/50 mL universal racks can be placed in the laminar flow hood, after which they should be treated with UV irradiation for 20 min. At the end of the working day, they must be taken out from the laminar box's space (it is the duty of the last one recorded in the calendar).

4. Before working in the laminar flow hood, you should put in it the accessories necessary for your work: culture plates, the required number of tubes and serological pipettes, microtubes, tweezers. Check if each type of tip is sufficient for your work. Everything brought into the laminar

flow hood must be pre-treated with 70% ethanol. *Do not put unnecessary or excessive accessories in the laminar as they can disturb the laminar airflow.*

5. Before work, the laminar flow hood's space **must** be treated with UV irradiation for at least 20 min. After burning, turning on the laminar flow and calibrating (takes about 90 sec, when the red indicator on the right panel stops flashing — for Lamsystems hood), wipe the working surface of the laminar flow hood with a lint-free paper moistened with 70% ethanol.

6. In the process of work, without preliminary burning, sterile solutions necessary for cultivation, drugs (sterile, if possible) and cell cultures can be brought into the laminar flow hood. Everything, except for dishes with cell cultures, must be pre-treated with 70% ethanol.

7. When working in the laminar flow hood, if possible, you should follow the three-section division: on the left is a clean area (new cups, tips, pipettes), in the center of the working area (the pipette holder system, rack with tubes), on the right is a dirty area (trash box, aspirator). *Before starting any work, you should place everything in the box space in the most convenient way in such a manner for not to make unnecessary movements during work.*

8. If possible, you should start the work from the cleanest and then continue to the dirtiest: first prepare the medium and the necessary aliquots of sterile solutions, then work with untransformed cell lines, and work with cancer cell lines at the very end. *If it is necessary to work with the primary culture, it is highly desirable to do this at the end of the working day and then treat the laminar with UV irradiation for 1-2 hours.*

9. If possible, do not use the Bunsen burner in a laminar flow hood.

10. Sterile Eppendorf tubes may only be taken with metal tweezers.

11. To avoid cross-contamination, do not keep plates with different cell cultures in the laminar flow hood at the same time.

### *1.3. At the end of work (in a laminar flow hood)*

1. At the end of the work, you should ensure that all the solutions you used are tightly sealed and remove them to their proper places. *All instruments that should not be kept permanently in the laminar flow hood should also be moved to their original places outside the laminar flow hood.*

2. The trash box should be emptied, treated with 70% ethanol and returned to the laminar flow hood. If some glass was used to put the cell culture excess into it, the contents of the glass should be mixed with a disinfectant (detergents, chlorine water, etc.), emptied into the sink, and the glass itself is washed with distilled water, treated with 70% ethanol and returned to the laminar flow hood.

3. If there are less than two filled rows in one of the pipette tip racks, replace it with a new one from among the sterilized ones, fill the pipette tip racks, sterilize it, and put it back into the cell culture room.

4. The cone of the pipettes used during the work should be wiped with lint-free paper moistened with 70% ethanol.

5. The laminar flow hood's working surface and buttons should be wiped with lint-free paper moistened with 70% ethanol.

6. The laminar flow should be turned off before closing the laminar flow hood, then turn on the UV irradiation.

7. Wipe the outer surface of the laminar flow hood glass with a napkin moistened with 70% ethanol.

### *1.4. At the end of work (before leaving the room)*

1. If you used an aspirator in your work, you need to rinse it (first with 5-10 mL of distilled water, then with 5-10 mL of 70% ethanol). *If, during the rinsing process, water or ethanol in the corresponding test tubes has run out, you must refill them.*

2. If the Goryaev chamber was used during the work, it is necessary to wipe it with a lint-free paper moistened with 70% ethanol.

3. If no one plans to work immediately after you (check according to the calendar), then turn off the bath and the centrifuge, check the gas level on the $CO_2$ reducer (0.03 MPa on the blue pressure gauge, more than 1 MPa on the black pressure gauge), make sure that the incubator is closed tightly and the microscope is turned off. Turn on the UV cleaner-recirculator for 90 min.

4. Before leaving the pre-box, you should put the indoor shoes in the shoe racks.

### *1.5. Cultivation*

1. It is good to have some shared files (Tables) with the list of cell cultures and their storage scheme at –80°C freezer and liquid nitrogen. When defrosting a cell line from a –80°C refrigerator, you must first find the ampoule's exact location in the racks using the Table, then remove from the table the cell corresponding to the ampoule you plan to thaw. After working with the refrigerator at –80°C, check that the refrigerator lid is **tightly closed**.

2. When thawing a cell line, it is necessary to cultivate it for 1–1.5 weeks with the control of morphology and viability. Only after this period, the culture can be taken into experiments.

3. Cell lines should be frozen no earlier than one week after thawing and no later than three weeks after thawing, at the passage between the third and the eighth.

4. The cell line's name and the number of cells should be written on the vials to be frozen. Also, indicate the month and year of freezing, the name of the person who froze it.

5. The frozen cultures after the appropriate operations are placed in free wells of racks in a refrigerator at –80°C.

6. It is recommended to use the same antibiotic at the same concentration throughout the laboratory. For example, one of the most common is 50 μg/mL of gentamicin. Pharmacy-bought antibiotics that are not traditional for use in cell culture should not be used for cell lines. Deviations from the antibiotic protocol must be justified.

7. If contamination is found, it is necessary to inform other labmates indicating the contaminated line and the medium in which it was cultivated. The culture in which contamination is detected and the medium on which the culture was grown, and other solutions used when working with the culture (phosphate buffers, trypsin and versene solutions) should be discarded **immediately**. **Contaminated culture dishes must not be opened within the cell culture laboratory.**

### *1.6. Rules for working in a PCR and electrophoresis room*

1. Staying in the PCR room is allowed **only** in a lab coat and indoor shoes, different from those used in the cell culture room.

2. It is strictly **forbidden** to move pipettes and tips from electrophoresis to the PCR area and vice versa. In the PCR room, it is forbidden to take out pipettes and tips into the PCR laminar.

3. After finishing work, it is necessary to remove all used consumables and reagents to their original places and throw trash out. It is necessary to fill the racks with tips after work so that they are filled. In the PCR room, it is necessary to fill the tips using tweezers.

4. After working in the PCR laminar, it is necessary to wipe the surface with 70% ethanol. After filling all used racks, you must leave them open and after closing the PCR hood door, turn on the UV lamp.

5. It is necessary to pay attention to the storage regimes of reagents, for some of them being in an unsuitable temperature can very quickly lead to the loss of their original properties. It is forbidden to keep revertase, antibodies, RNAse A, protein and oligonucleotide ladder marker, and diluted primer stocks at room temperature longer than required. When working with the specified reagents, you can take them out of the refrigerator only before use. When working, keep them on ice or in a cryo-rack.

6. Before using the device in the PCR or electrophoresis room, it is necessary to book it in the corresponding Google calendar. **After you have finished using the devices, they must be turned off.**

### *1.7. Rules of using an automatic pipette*

1. Do not keep the pipettes in a horizontal position for a long time.
2. The tip should be fitted as tightly as possible on the pipette cone. Do not apply force to put on the tip. Do not put the sterile tip on the pipette using your hands.
3. The tip should be immersed in the sampled liquid no more than 10% – 20% of its length.
4. When immersed in the sampled liquid, the pipette should be kept **strictly vertical**.
5. It is not recommended to use pipettes for accurate titration in a spring position less than 35% of the maximum nominal volume.
6. The collection of liquid volumes larger than 20 µL is carried out in the following way: a) pressing the piston up to the soft stop before immersing the tip in the liquid; b) immersion of the tip into the liquid; c) smooth push-up of the piston to its original state; d) removal of the tip from the liquid; e) draining the sampled liquid by pressing the piston until it stops softly.
7. **It is inadmissible for a liquid to enter the pipette cone.** If a liquid is thrown inside, remove the filter (if any) from the pipette, let the pipette dry out, replace the filter with a new one, and check the pipette work's accuracy. In the case of autoclavable pipettes, sterilize them.
8. At the end of work with the pipette, wipe the cone with a napkin moistened with ethanol.
9. When working with a pipette filler, first open the package with a serological pipette of the required volume, insert the open pipette into the filler, hold the outer package, and immediately turn it towards you with the desired scale. After that, you can remove the packaging and work, picking up liquid according to pipette volume. **If a liquid occasionally gets into the filler, it is necessary to replace the filter inside. Continued use of the filter soaked in media may result in contamination for all laboratory members.**

# 2. ROUTINE PROCEDURES WITH CELL CULTURES

Special media are required to maintain any cell culture. Usually, cell lines are bought in the cell culture collections (ATCC, Biolot, Russian collection of cell cultures) and are supplemented with passports, in which you can find all the cultivation conditions. In the table below, the composition of media for some cell lines can be found.

**Table 1.** Composition of media for the cultivation of the most common cell lines

| Cell line | Medium (for 50 mL/25 mL) | Fetal bovine serum (FBS) | Gentamicin or penicillin/streptomycin |
|---|---|---|---|
| **Mesenchymal stem cells (MSC)** | 90% DMEM 1 g/L glucose (45 mL/22.5 mL) | 10% | 50 µg/mL, 100 EU/mL /100 µg/mL |
| **Human embryonic lung fibroblasts (HELF)** | 90% EMEM | 10% | 50 µg/mL, 100 EU/mL/100 µg/mL |
| **Human postnatal fibroblasts (immortalized)** | 95% EMEM | 5% | 50 µg/mL, 100 EU/mL/100 µg/mL |
| **HeLa cells** | 95% EMEM | 5% | 50 µg/mL, 100 EU/mL/100 µg/mL |

| | | | |
|---|---|---|---|
| **HEp-2 cells** | 95% EMEM | 5% | 50 µg/mL,<br>100 EU/mL/100 µg/mL |
| **K 562 cells** | 95% RPMI | 5% | 50 µg/mL,<br>100 EU/mL/100 µg/mL |
| **HCT 116 cells** | 95% DMEM | 5% | 50 µg/mL,<br>100 EU/mL/100 µg/mL |
| **HCT 116 -/- cells** | 95% DMEM | 5% | 50 µg/mL,<br>100 EU/mL/100 µg/mL |
| **C2C12** | 90% DMEM or α-MEM<br>(44.5/22.25 mL) | 10% | -,<br>50-100 EU/mL/50 µg/mL |
| **MC3T3-E1** | 90% alpha-MEM | 10% | -,<br>100µg/mL/<br>100 µg/mL |

## *2.1. Subculture of cells*

When the cells grow, they have several growth stages such as lag phase, exponential "log" phase and the plateau phase. At the first one, cells grow slowly; at the second one – rapidly; and finally slowly again. For the monolayer cell cultures, the cells reach the confluency at the plateau phase. At this stage, they need to be passaged to maintain a healthy state and viability. The protocol can be found below.

***Subculture of monolayer cells***

1. Discard the media using the aspirator.
2. Wash the cells two times with the sterile phosphate-buffered saline (PBS).
3. Add 1 mL of trypsin-versene solution (1:1). Incubate 2 to 4 min depending on the cell line.
4. When the cells begin to round up but have not yet detached, remove the trypsin-versene solution.
5. Resuspend cells in 2 mL of media. Depending on the speed of cell culture growth, subculture cells 1:2–1:10. This dilution means that either ½ or 1/10 (1 mL or 200 µL) from the initial number of cells should be transferred to the new Petri dish.
6. Add the media up to 5 mL (for 60 mm dish) or 10 mL (for 100 mm dish). Mix the media with cells with gentle movements of the dish.

***Subculture of suspension cells.***

1. Resuspend cells in the media using the pipette. Depending on the speed of cell culture growth, subculture cells 1:2–1:10. This dilution means that either ½ or $^{1}/_{10}$ (1 mL or 200 µL) from the initial number of cells should be transferred to the new Petri dish.
2. Add the media up to 5 mL (for 60 mm dish) or 10 mL (for 100 mm dish). Mix the media with cells with gentle movements of the dish.

## *2.2. Cell freezing protocols*

We usually do not cultivate cells for longer than 1.5 months as they accumulate mutations (when speaking about cancer cells). Alterations in cell gens can even lead to change in their morphology. After this period of cultivation, a new vial of the cells should be thawed. For the long-term storage of cells, we use cryopreservation at –80°C (for the period of half a year) or in

liquid nitrogen (for the long-term storage of the cell culture collection for many years). Below you can find the freezing protocol.

1. Prepare the freezing medium.

***For cancer and immortalized cells:*** 10% DMSO, 90% of media which is used to cultivate a specific cell line.

***For Human embryonic lung fibroblasts (HLEC), mesenchymal stem cells (MSC):*** 10% DMSO, 90% FBS.

2. Sign the tubes: cell line, date, number of cells, name of the worker.

Steps for ***suspension cell cultures.***

1. Resuspend cells in a Petri dish, transfer them to the 15 mL tube, resuspend once more. Take 10 μL of cell suspension, count in a cell counter.
2. Calculate the volume containing $2\times10^6$ cells, multiply it by the number of microtubes (Cryo Freeze Vials), which will be freezed, transfer this volume to the tube.
3. Centrifuge the cells in a tube (5 min at 300 g), remove the supernatant.
4. Resuspend cells in freezing medium with DMSO (see section 1). The volume of medium (mL) is calculated to be equal to the number of vials freezed.
5. Place 1 mL of cell suspension into each vial, which should be carefully pre-marked, tightly close, leave for 3 min at +4°C.
6. Place the cryovials into the Cryo Freeze filled with isopropyl alcohol (IPA), put it in the refrigerator at –80°C. Mind that after every 5 cycles of freezing, the IPA must be replaced.
7. After a minimum of 4 hours (usually the next day), transfer the cryovials from the Cryo Freeze to the cryo rack, put the row and cell identification with the name of the frozen cell culture into the shared table or Google drive document. If it is necessary to transfer cryovials to the liquid nitrogen, it should be done very fast not to avoid the rise of temperature, also **do it the next day after freezing.**

Steps for ***monolayer cell cultures.***

1. Cells should reach 80–90% of confluence in a Petri dish or a flask.
2. Remove medium from the dish, wash the cells with sterile PBS 2 times, add 1 mL of trypsin-versene solution (1:1). Incubate 2–4 min depending on the cell line.
3. When the cells begin to round up but have not yet detached the surface, remove the trypsin–versene solution. Resuspend cells in the freezing medium (see paragraph 1 $V_{medium}$ = 1mL×number of cryovials that will be frozen. Usually, the cells from one dish are freezed in 1 cryovial).
4. Place 1 mL of cell suspension into each vial, which should be carefully pre-marked.
5. Distribute 1 mL of cell suspension in freezing medium into cryovials, tightly close, leave for 3 min at +4°C.
6. Place the cryovials into the Cryo Freeze filled with isopropyl alcohol (IPA), put it in the refrigerator at –80°C. Mind that after every 5 cycles of freezing, the IPA must be replaced.
7. After a minimum of 4 hours (usually the next day), transfer the cryovials from the Cryo Freeze to the cryo rack, put the row and cell identification with the name of the frozen cell culture into the shared table or Google drive document. If it is necessary to transfer cryovials to the liquid nitrogen, it should be done very fast not to avoid the rise of temperature, also **do it the next day after freezing.**

## *2.3. Seeding cells for the experiments*

After planning the design (number of tested drugs/conditions, number of replicates), cells should be seeded in a certain number into the appropriate to experiment plate. For screening experiments such as viability screening in the MTT/sulforhodamine assay, there is no need for a large number of cells, and usually, several different doses are tested in such experiments. 96-well plate is the most suitable for this purpose. For flow cytometry, a moderate number of cells is

enough. As 10000 events are analyzed in most cases, so 24- or 12-well plate should be used. Finally, for analyzing DNA/RNA or proteins using PCR or Western blotting, a large number of cells is needed, so the cells should be plated into the 6-well plate or 35/60 mm Petri dishes. Some plate characteristics can be found in Table 2.

**Table 2.** Cell culture plates parameters

| **Number of wells per plate** | 6 | 12 | 24 | 48 | 96 |
|---|---|---|---|---|---|
| **Area of the well, $cm^2$** | 9.5 | 3.8 | 1.9 | 0.95 | 0.32 |
| **Optimal volume of culture medium, mL** | 3 | 1–1.5 | 0.5–1 | 0.3 | 0.1 |
| **Maximal volume*, mL** | 16.8 | 6.9 | 3.4 | 1.6 | 0.35 |
| **Number of cells**** | $10^6$ | $5\times10^5$ | $2.5\times10^5$ | $10^5$ | $5\times10^4$ |
| **Number of cells for 60% confluency** | $0.25\times10^6$ | $10^5$ | $0.5\times10^5$ | $0.25\times10^5$ | $10^4$ |

* For Corning plates

** Average linear size of cells 10 μm (A549, MDCK, HEK293, Vero)

## *2.4. Counting the cells using the cell counter TC10 (Bio-Rad) and trypan blue dye*

1. *For monolayer cells:* remove the medium from the Petri dish, wash the cells with sterile PBS 2 times, add 1 mL of trypsin-versene solution (1:1). Incubate 2–4 min depending on the cell line. *For suspension cells:* start from step 3.

2. When the cells begin to round up but have not yet detached, remove the trypsin-versene solution.

3. Resuspend cells in the Petri dish in media and transfer them to the 15 mL tube, resuspend once more. Then follow step 4 or step 5 depending on whether the measurement of the percentage of dead cells is necessary.

4. *If there is no need to count live/dead cells*. Take 10 μL of cell suspension and put it into the counting plate's chamber's outer hole.

5. *If you need to count live/dead cells*. Dilute 1 part of the trypan blue dye and 1 part of cell suspension in a tube. For example, mix 10 μL of cell suspension and 10 μL of trypan blue dye in the tube. Resuspend the cells, take 10 μL of cell suspension and put it into the counting plate's chamber's outer hole.

6. Insert the counting plate into the Cell counter. It will automatically initialize the counting process.

7. The counting results are displayed on the current count screen. For samples without trypan blue dye, the instrument displays the total number of cells per mL. For samples with trypan blue dye, the instrument displays the total number of cells per 1 mL, the number of live cells per 1 mL and the percentage of live cells per 1 mL. *If a sample with counted elements contains trypan blue dye, the instrument considers a 1:1 dilution of trypan blue dye and cells.*

# 3. METHODS OF WORKING WITH NUCLEIC ACIDS

## *3.1. Reverse transcription-polymerase chain reaction (RT-PCR)*

**RNA isolation**

1. Seed $10^6$ cells per 60 mm Petri dish for each probe.
2. After the end of incubation, transfer the supernatant to the 15 mL tube. Remove the cells with trypsin-versene and transfer them to the same test tube.
3. Centrifuge for 3 min at 300 g.
4. Discard the supernatant, add 0.8 mL of trizol or Extract RNA Evrogen reagent, carefully resuspend, leave for 3–4 min.
5. Add 160 μL of chloroform, shake for 15 s.
6. Take individual Eppendorf tubes, add 500 μL of isopropanol.
7. In the Eppendorf tubes with cells, the aqueous phase and the precipitate are separated for about 5-7 min.
8. Centrifuge for 10 min at 12000 rpm.
9. Transfer the water phase to the Eppendorf tubes with isopropanol using the pipette as much as possible. **It is important not to capture the precipitate.** Shake the mix.
10. Incubate for 10 min (room temperature), shake occasionally.
11. Centrifuge for 10 min, 12000 rpm.
12. Take isopropanol (supernatant) and discard. RNA will be visible as a dark point and a yellow spot at the bottom. Add 500 μL of 70% ethanol to the tube.
13. Centrifuge for 5 min at 12000 rpm.
14. You can leave the samples at –20°C for a week. *You can skip this stage.*
15. Cut the filter paper into triangles (pre-wipe the scissors with ethanol).
16. Discard ethanol, remove residues with triangles from filter paper, trying not to touch the precipitate, keep Eppendorf tubes on ice all the time.
17. Add 20 μL of nucleases-free water to Eppendorf tubes with RNA (you can use water for injections) and resuspend until the RNA dissolves. Transfer everything to the small Eppendorf tubes (500 μL). Perform this and the next steps on ice.
18. Measure the RNA solution's optical density (you can take 2 μL of RNA + 998 μL of water if you use a standard spectrophotometer). If you use the nanophotometer, it will estimate the RNA concentration automatically. There should be an absorption peak at 260 nm and two peaks with 1.5 times lower intensity at 230 and 280 nm. Calculate the required volume for the reaction based on the fact that there should be 2 μg of RNA/reaction.

**Reverse transcription**

19. Mix RNA volume containing 2 μg + 1 μL oligo dT (100 μM) or Thermo Scientific Random Hexamer primer (100 μM) (100 pM per reaction) + nucleases-free water (up to 9 μL of the total volume).
20. Incubate tubes for 5 min at 70°C (*secondary and tertiary structure of RNA degrades to primary*), then put on ice.
21. Prepare the reaction mixture (components are given for the Evrogen reagents, **Table 3**) for the appropriate number of samples in the Eppendorf tube. Add the last rivertase, keep only on ice.

**Table 3.** Components ratio for preparation of the reaction mixture

| Component | V, μL (per 1 probe) | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|---|
| $H_2O$ | 2.5 | 5 | 7.5 | 10 | 12.5 | 15 | 17.5 | 20 | 22.5 | 25 |
| ×5 buffer | 4 | 8 | 12 | 16 | 20 | 24 | 28 | 32 | 36 | 40 |
| dNTP 5 mM | 1.5 | 3 | 4.5 | 6 | 7.5 | 9 | 10.5 | 12 | 13.5 | 15 |
| DTT 20 mM | 2 | 4 | 6 | 8 | 10 | 12 | 14 | 16 | 18 | 20 |
| MMLV rivertase | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |

22. Add 11 μL of the reaction mixture to each Eppendorf tube with RNA.
23. Put in the thermocycler with the following parameters: 25°C – 10 min, 42°C – 50 min, 70°C – 10 min, 10°C - 10 s. As a result, cDNA is produced.
24. Put it in the freezer at –20°C. DNA is very stable and can be stored for a long time.

**PCR**

25. Prepare the PCR mixture according to **Table 4** (volumes and reagent names are given according to the Evrogen protocol).
26. Put 2 μL of cDNA into Eppendorf tubes (500 μL, if PCR will be performed in Terzik, or 200 μL if PCR is done in the BioRad thermocycler). Add 23 μL of the PCR mixture, described above, to them. *Mix everything carefully so that there are no bubbles.*
27. Make one more test tube without cDNA, only reagent mix for each primer pair with 2 μL of nuclease-free water as a negative control. In this probe, the PCR product should not appear. The total volume of the mixture is 25 μL.
28. Add 15 μL of mineral oil to the Eppendorf tubes to cover the PCR mixture's surface to avoid evaporation during PCR if the thermocycler does not have the heated lid (for example, Tercik). *If the Real-Time PCR Detection System is used (for example, CFX-96) — skip this step.*
29. Set up the thermocycler with the following cycles:
    Cycle 1 (1 repeat): 94°C – 3 min, 60°C – 40 s, 72°C – 40 s.
    Cycle 2 (28 repetitions): 94°C – 10 s, 60°C – 10 s, 72°C – 20 s.
    Cycle 3 (1 repeat): 72°C – 3 min.
    Cycle 4 (storage): –10°C.
30. Dilute the original primers from 100 μM to 10 μM, use 1-2 μL of this solution (10–20 pM/reaction).

**Table 4.** Reagent ratio for preparation of PCR mixture

| Reagent | Volume, µL | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| 5x qPCR mix | 5 | 10 | 15 | 20 | 25 | 30 | 35 | 40 | 45 | 50 |
| Forward primer, 10µM | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| Reverse primer, 10µM | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| Nuclease-free water | 16 | 30 | 45 | 60 | 75 | 90 | 105 | 120 | 135 | 150 |

**Gel electrophoresis**

1. If the thermocycler does not have a real-time detection system, use gel electrophoresis to detect the PCR products. Prepare TAE buffer with 0.5 µg/mL EtBr (can be used multiple times). *200 mL of the buffer will be enough for the Mini Sub-cell GT Cell (Biorad) or 600 mL for the Sub-cell GT Cell (Biorad).*

3. Prepare 1% agarose diluted with TAE buffer, containing EtBr. *50 mL is enough for the mini system, 200 mL – for the regular system.* Put the glass flask with the agarose solution into the microwave for 5–6 min until it becomes transparent. Wait for it to cool down (unless you can touch the flask with your hand). Insert the comb into the gel tray, **which should be installed strictly horizontal**, pour the agarose into it, and allows the gel with the inserted comb to harden. **Pull out the comb carefully.**

4. Mix 2 µL of 6x loading dye + 2 µL GeneRuler 100 bp DNA ladder 0.5 µg/mL (Thermo Scientific).

5. Mix 2 µL of 6x loading buffer + 10 µL of the sample (PCR product).

6. Put the agarose tray into the electrophoresis cell, pour out the TAE buffer. *Place the samples into the agarose wells carefully, without touching the bottom of the well with the tip.*

7. Run electrophoresis at 80 V for a small camera for about 30 min. The ladder marker should "run-up".

8. Evaluate the results on a transilluminator or ChemiDoc Imaging Systems.

## *3.2. Investigation of internucleosomal DNA degradation (DNA fragmentation)*

1. Cells are seeded in 60 mm Petri dishes in the logarithmic phase ($10^6$ cells in 5 mL of culture medium). Alternatively, use two 35 mm dishes for each concentration/time point (combine them during the experiment). *There are $5\times10^5$ cells in 3 mL of medium in a 35 mm dish*. Leave in a $CO_2$ incubator overnight.

2. The next day, add the test compound to the cultures, incubate for 24–72 hours at 37°C, 5% $CO_2$. Leave one Petri dish (or a couple of dishes) untreated (as control).

3. After each period of 24 h, the cells are detached from the dishes with a versene solution and centrifuged (1000 rpm, 15 min). For suspension cultures: resuspend in a dish, transfer to Eppendorf tubes, centrifuge for 5 min at 4000 rpm.

4. Lyse the cell pellet in 0.5 mL of buffer containing 0.35 M NaCl, 20 mM Tris-HCl (pH7.4), 2 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 0.5% NP-40 (*does not lyse mitochondria and can be replaced with 0.3% sodium dodecyl sulfate (SDS), which also destroys mitochondria and nuclei membranes*), and distilled water (Table 5). Stay on ice for 30 min.

5. Centrifuge the supernatant taken from tubes with cells for 10 min at 12000 rpm. DNA strands will be visible in the supernatant.

6. Remove the supernatant, dissolve the pellet in 100 μL of lysis buffer and add this solution to the main cell pellet. At this stage, it is possible to freeze pellets at -20°C for indefinitely long storage. Do the same with time points 48 hours and 72 hours.

**Table 5.** Reagent for preparation of 10 mL of lysis buffer

| Reagent | Stock solution concentration | Volume, μL |
|---|---|---|
| NaCl | 5 M | 700 |
| Tris HCl | 1 M | 200 |
| $MgCl_2$ | 25 мМ | 800 |
| DTT | 100 мМ | 100 |
| SDS | 10% | 300 (0.3% of the total volume) |
| $H_2O$ | | 7700 |

7. Add 600 μL of buffered (neutral) phenol and ⅕ of total volume (150 μL) chloroform to the lysates. *In fact, add chloroform to the top.* Centrifuge it at 12000 rpm for 10 min. *You should observe two phases separated by a fluffy white bloom. The upper phase is water, with DNA in it.*

*8.* The bottom one is organic; discard it. Transfer the aqueous phase (600–700 μL) to new tubes, add 60 μL of 3 M sodium acetate (pH 5.2; salting out) and 1.5 volumes (almost to the top) of 96% ethanol (to remove water). Place at –20°C for at least 2 hours (or overnight). *You can add phenol-chloroform a second time to isolate DNA with better quality.*

9. Remove lysates from the freezer. Centrifuge the tubes at 12000 rpm for 20 min to precipitate DNA. Remove the supernatant, add 0.5 mL of 70% aqueous ethanol to the sediment, centrifuge at 12000 rpm for 10 min.

10. Prepare a buffer for phoresis: 40 mL of 50x TAE stock solution + 1960 mL $H_2O$ + 100 μL EtBr (10 mg/mL). Prepare the agarose gel: dissolve 3 g of agarose in 200 mL of TAE buffer, place in the microwave oven to heat. Then cool slightly, pour in a tray with a comb, and wait until it hardens (20-30 min).

11. Remove supernatant from Eppendorf tubes. Dry the precipitates by opening the Eppendorf tubes, then dissolve in the mixture:

- 6x loading buffer (3.5 μL per sample);
- 1x TAE, 15.4 μL per sample;
- RNAse A 10 mg/mL (2.1 μL per sample, concentration in the sample 10 μg/mL);
- marker, DNA ladder 100 bp (3.5 μL per sample + 3.5 μL of 6x loading buffer);

Dispense 21 μL of sample mixture into the tubes.

12. Incubate the samples in a thermostat for 30 min at 37°C. *If the mixture is viscous, you can put it at 60°C for 2 min (or boil 95°C for 5 min).*

13. Perform electrophoresis of DNA in 1.5% agarose gel (*for better visualization, you can make a 2% gel*), visualize in ultraviolet light after staining with EtBr (0.5 μg/mL). Electrophoresis conditions: 70–100 V, at least 5 cm of a run.

# 4. METHODS OF WORKING WITH PROTEINS

## *4.1. Determination of the protein amount by the Bradford method*

For some experiments, protein concentration can be a critical factor, for example, western blotting. The concentration of a specific protein of interest will be proportional to the total protein amount, so the initial differences in the control and experimental group will result in a false interpretation of the results. One of the most common and reliable methods for the determination of protein concentration is the Bradford method.

To estimate the protein amount, you will need to plot a calibration curve. The vertical axis is optical density. The horizontal axis is the amount of protein per sample (0.5, 1, 2, 4, 6, 10, 20, and 50 μg of protein per sample).

1. Prepare the bovine serum albumin (BSA) solution with the different concentrations. Use the two-fold dilutions to prepare the solutions with precise concentrations. For instance, there is a BSA solution in water with a concentration of 10 mg/mL (or 10 μg/μL) — solution A. Make the intermediate solution B — dilute solution A 10 times (90 μL water + 10 μL stock A), final concentration 1 μg/μL. Dilute solution B 10 times (90 μL of water + 10 μL of stock B) to obtain solution C, with a concentration of 0.1 μg/μL.

2. Prepare Bradford solution. Dissolve 200 mg of Coomassie Blue G-250 (Serva Blue g) in a bit of $H_3PO_4$ in a clean, dry glass, stir with a glass rod, gradually pouring in the acid so that there is no sediment. The total volume of added acid is 200 mL. Then add 100 mL of 96% ethanol, stir and put in a dark place overnight (or more). Transfer the mixture to the cylinder, add deionized water (Milli-Q) up to 2 L, filter through the 0.22 μm membrane. **The solution should have a light brown color. The method is very sensitive even to very low concentrations of proteins. If the Bradford reagent has a blue color, it cannot be used for the experiment.**

3. Put the BSA solutions with the lysis buffer into the Eppendorf tubes, add water to 100 μL, then add 900 μL of Bradford solution.

***Control (blanc probe):***

- 900 μL Bradford solution;
- 98 μL distilled water;
- 2 μL lysis buffer with inhibitors.

***Calibration probes:***

- 900 μL Bradford solution;
- The correct amount of a standard protein solution (BSA or ovalbumin) in distilled water, which will provide a certain final concentration;
- 2 μL lysis buffer with inhibitors.

4. Incubate samples for 15 min at room temperature, then transfer 600 μL of the sample to the cuvette. Measure the absorbance on the wavelength λ = 595 nm using the spectrophotometer. *At first, the blanc sample, which does not contain any protein, should be measured, then the BSA samples.*

## *4.2. Western blotting*

1. Seed the $5\times10^5$ cells per 60 mm Petri dish overnight (for monolayer cultures).
2. Add the drug for 24–72 hours.
3. Transfer the medium into 15 mL tubes, remove the cells with versine solution, transfer to the same tubes. Centrifuge at 5000 rpm, 5 min.
4. Prepare RIPA cell lysis buffer. You can prepare 50 mL of the buffer; it can be stored in the refrigerator for a long time. For 1 mL RIPA, add 20 μL of Protease Inhibitor Cocktail (PIC, Sigma-Aldrich, P8340) and 20 μL of Phenylmethanesulfonyl fluoride (PMSF , Sigma-Aldrich, P7626 ) – proteinase inhibitors, so that proteins do not break down.

**Table 6.** Reagent ratio for preparation of 50 mL of RIPA solution.

| Reagent | Final concentration | Required volume for 50 mL of RIPA, mL |
|---|---|---|
| NaCl | 150 mM | 1.5 mL 5 M NaCl |

| | | |
|---|---|---|
| NP-40 | 1% | 0.5 mL stock solution |
| SDS | 0.1% | 0.5 mL 10% SDS |
| Tris pH=8.0 | 50 mM | 2.5 mL 1 M Tris |
| $H_2O$ | | 45 mL |

5. Remove the supernatant from the cells after centrifugation. Add 100 µL of RIPA, resuspend cells, lyse on ice for 30 min.

6. Prepare gels for the electrophoresis. Separating gel equal to 8%, but if the proteins to be determined have little difference in molecular weight, you can prepare up to 12%. Add TEMED immediately before pouring the gel. Assemble glasses, check if they are leaking with water. Pour water, dry with filter paper.

7. Pour 5 mL of separating gel into glasses. Pour 200 µL of isopropanol on top to solidify the gel.

8. Check if the gel hardens (about 15 min). If only isopropanol is leaking, then it is hardened. Discard the isopropanol, rinse with distilled water.

9. Pour the concentrating gel quickly up to the edge of the glass, insert the comb.

10. The gel can be frozen for a day: wrap the glasses with a wet filter paper, place them in a zip bag and store at –20°C.

11. Treat the samples with the ultrasound 3 times for 4 s with a pause of 4 s. In the beginning, the DNA should be attached to the tip like a thread. After the ultrasound treatment, it should not be detected by the eye. This step is optional.

12. Centrifuge the samples at 11000 rpm, +4°C, 15 min.

13. Transfer the supernatant (proteins are dissolved in it) to separate Eppendorf tubes.

14. Prepare BSA calibration solutions for the Bradford protein calibration. Use 7 concentrations, $D_{max}$ = 1 mg/mL, then two-fold dilutions, $8^{th}$ sample – pure water.

15. Build the calibration curve in Excel or other statistical programs, determine the amount of protein in each sample (mg). Right-click on the graph allows you to see the trend line and show the equation on the diagram.

16. Calculate the volume of cell lysate, which should be taken into the experiment to have 35 µg of protein in each well. Select the maximum required volume, bring the rest of the samples to the same volume with water. Calculate the loading buffer volume for each sample (dilute the 5x loading buffer 5 times with the sample).

17. Mix samples and protein loading buffer, incubate 5 min at 99°C.

*5X Loading Buffer:*

- 0.313 M Tris HCl (pH 6.8 at 25°C);
- 10% (w/v) SDS;
- 0.05% (w/v) bromophenol blue;
- 50% (v/v) glycerol.

18. Place the glasses into the vertical phoresis chamber, fill it with the running buffer:
    - 25 mM Tris
    - 190 mM glycine
    - 0.1% SDS

You can prepare a ×10 buffer from Tris and glycine, then dilute it immediately before the experiment.

*Composition of 10x Tris-glycine buffer:*

- 30 g Tris;
- 144 g glycine;
- Add water till 1 L.

Then, to prepare the running buffer, you will need:

- 1 g/L SDS (0.1%);
- 10x Tris-glycine buffer (10%);
- Water (89.9%).

19. Transfer the samples to the wells. Place the protein ruler in the first well (1 µL). Adjust the samples up to 25 µL volume.
20. Run electrophoresis at 120 V for about 1.5 hours (until the blue bar almost reaches the bottom).
21. Prepare the blotting cassettes, put them in a tray with a buffer, black side down. Lay out a sponge, thick paper, gel, nitrocellulose membrane. Carefully remove the bubbles with a spatula. Then put 2 layers of thin paper, again remove the bubbles, then put a sponge. Close the cassettes and place them in the phoresis chamber with the black side of the cassette against the black wall. **Put refrigerant on the side!** Otherwise, the buffer may boil.
22. To prepare 1 L of the transferring buffer (to transfer the proteins from the gel to the membrane), mix the following components in **a specific order; otherwise, a precipitate will form.**

*Transfer buffer (wet):*

- 25 mM Tris;
- 190 mM glycine;
- 20% methanol;
- Check the pH and adjust to 8.3.

*Or*

- 100 mL 10x Tris-glycine buffer (10%);
- 700 mL water (70%);
- 200 mL methanol (20%).

23. Run electrophoresis at 250 mA for 1 hour.
24. Remove the glasses from the chamber, separate the membrane, place them in a bath with distilled water. It is important to remember where the "face" is ¾ the side that adjoined the gel – there are proteins on it. Discard the water, pour the Ponceau solution for the development of proteins.

*Ponceau S Staining Solution (0.1% (w/v) Ponceau S in 5% (v/v) acetic acid):*

- 1g Ponceau S;
- 50 mL acetic acid;
- Add distilled water to 1L;
- Store at 4°C**. Do not freeze**.

25. Rinse the membrane with distilled water. Cut the membrane edges.
26. Pour 5% solution of skimmed milk powder in TBST (mixture of tris-buffered saline (TBS) and Polysorbate 20 (also known as Tween 20). Put on a shaker for 30 min.
27. Prepare the antibody solutions. Make 1% BSA in TBST. It is essential to stain each membrane with actin or tubulin antibodies – these proteins are in all cells, and their amount is

rarely affected by the compounds. Therefore, these proteins' bands should be the same in all samples, which indicates that the total amount of protein was initially the same in all probes.

28. If you want to analyze several proteins with different molecular weights from the same probe, you can cut the membrane along the protein ladder according to the molecular weights of the proteins of interest.

29. Stick the parafilm slices onto the Petri dishes, drop 500 µL of primary antibodies and place the membrane sections "face down" on them. Put wet filter paper on the dishes' walls to maintain the humidity level inside and protect the antibodies from drying out. Close the upper cup and incubate at +4°C overnight (if you need to save the time ¾ incubate for 2 hours at room temperature).

30. Remove the membranes, wash in TBST 3 times for 5 min on a shaker, replace the TBST after each washing procedure.

31. Dilute the second antibodies in a 5% solution of skimmed milk powder in TBST (usually 1:1000, but see each antibody's instruction).

32. Put 500 µL of the second antibodies onto new dishes with parafilm, put the membrane on them, close the dish, incubate for 1 hour at room temperature (for some antibodies at +4°C).

33. Remove the membranes, wash in TBST 3 times for 5 min on a shaker, replace TBST after each washing procedure.

34. Prepare the enhanced chemiluminescence (ECL) solution: 6 mL ECL + 3 µL 30% hydrogen peroxide. *If you can take hydrogen peroxide from the pharmacy (3%), just take the volume 10 times more.* Moisten membranes in this mixture, put on a tray "face-up". Second antibodies are conjugated with horseradish peroxidase; therefore, when interacting with peroxide, a glow occurs. Analise the chemiluminescence on a ChemiDoc imaging system.

*ECL working solution:*

- Distilled water – 71.4 mL;
- Luminol (5-amino-2,3-dihydro-1,4-phtalazidenion) – 400 µL;
- Coumaric acid – 176 µL;
- Tris 1 M pH = 8.5 – 8 mL.

**General notes:**

- Nitrocellulose membranes 0.4 are suitable for high molecular weight proteins 0.2–200 kDa. 0.2 – for proteins 0.1–200 kDa. A membrane of 0.2 kDa can be used in almost all cases.
- Choosing the percentage of the gel: the smaller the protein' molecular weight, the greater should be the percentage of gel.
- Approximately 5 mL of gel is needed per glass. One gel is concentrating, and the other is separating. For 4 glasses, 10 mL concentrating, 25 mL separating gel will be needed (depending on the glasses' size). ABCAM Guide can be used for searching the composition of buffers.

# 5. SPECTROPHOTOMETRIC METHODS FOR MTT ASSAY

There are many cases when you need to estimate the drug cytotoxicity. For this purpose usually, relatively simple and cheap screening methods are used. One of the most popular ones is the MTT assay. This method is based on the reduction of yellow dye Thiazolyl Blue Tetrazolium Bromide (MTT) with mitochondrial NAD(P)H-dependent cellular oxidoreductase enzymes into the formazan, which has a purple color. The intensity of the purple staining is proportional to the cells' enzyme activity which helps to estimate the number of viable cells indirectly.

1. Seed $5\times10^3$ cells/100 μL in each well of the 96-well plate without feeling the outer rows to avoid the edge effects. Leave the cells to adhere overnight.
2. On the following day, add the drug in different concentrations. A series of successive two-fold dilutions in the media are prepared from the stock solution (often 5–10 mM stock in DMSO). Prepare the tested substance at concentrations two times higher than you need to test in 100 μL volume (number of replicates +1). Then add the drug solution to 100 μL of media in the wells so that the final concentration will be decreased two times.
3. Incubate the plate for 24–72 hours in the $CO_2$ incubator at 37°C. For each concentration, 3–4 replicates are usually used.
4. Add 20 μL of 10x MTT solution (5 mg/mL) in sterile PBS into each well. *It takes some time to dissolve the MTT in PBS, so it is better to prepare the solution in advance. Use a vortex when dissolving the powder. Prepared 10x stock can be used for several weeks if stored at +4°C.* Mix the plate gently with round movements on the table. Incubate for 1.5 hours at 37°C in a $CO_2$ incubator.
5. Remove the MTT solution from the wells using the aspirator. Dissolve the formazan crystals by adding 200 μL of DMSO into each well. Add the DMSO in a well A1 – it will be the blanc well.
6. Incubate the plate on a shaker for 15 min at 300–350 rpm.
7. Measure the optical density on an Infinite F50 (Tecan) (or Tecan Spark microplate reader). The values are obtained using the Magellan50 program. Select the start of measurements → MTT flat transparent → check the plate type and the wavelength range of 570 nm → detect → export the obtained data to Excel.

# 6. FLOW CYTOMETRY METHODS

Flow cytometry is a technique providing an incredible amount of different applications for monitoring processes inside each cell. It gives exceptional results because of huge data collection (typically 10000–20000 of events), providing good statistics. Flow cytometry has such advantages compared to fluorescence/confocal microscopy, like a quantitative estimation of relative fluorescence intensity, which is very useful when you need not only the "yes/no" answer but also wants to compare the parameter level in different probes. A description of several flow cytometry protocols which can be helpful in cytology and cancer research is given below.

## *6.1. Sample preparation protocol for Annexin-PI staining*

When testing the cytotoxic efficiency of anticancer drugs, the next question after estimating its half-maximal inhibitory concentration ($IC_{50}$) is evaluating the mechanism of action. For this purpose, the understanding of the type of cell death will be a reasonable beginning. Typical ways of cell death caused by cytotoxic drugs are apoptosis and necrosis. At the beginning of the apoptosis process, phosphatidylserine flips down from the inner to the cell membrane's outer side. Annexin V, conjugated with the fluorophore (FITC), binds with the phosphatidylserine, so the FITC-positive cells are considered apoptotic ones. At the same time, propidium iodide (PI) penetrates only the damaged membranes, which is a necrosis sign. Thus, the double staining helps to define the live cells, apoptotic and necrotic ones.

For correct analysis, it is also necessary to prepare controls:

- negative control for adjusting the device (unstained intact cells);
- cells, treated with the apoptotic inducer, stained with Annexin V only;
- cells, treated with the induced necrosis (for example, incubated 10 min at 90°C), stained with PI only.

**1.** Transfer all cells together with the media in which they were incubated with the drug (it contains the dead cells, which are the object of interest) to the 15 mL tubes, centrifuge at 3000 rpm, 5 min. Discard the supernatant. **Make all the further procedures on ice.**

2. Wash the cells by adding 0.5 mL of PBS, centrifuge at 3000 rpm, 5 min. Discard the PBS.

3. Wash the cells by adding 0.5 mL of 1x binding buffer, centrifuge at 3000 rpm, 5 min. Discard the binding buffer.

4. Prepare Annexin V in 1x binding buffer: 5 µL of Annexin V per 100 µL of 1x buffer — for one sample.

5. Resuspend cells in 100 µL Annexin V in 1x buffer.

6. Incubate 10–15 min in the dark at room temperature.

7. Wash the cells by adding 0.5 mL of 1x binding buffer to the samples with Annexin V, centrifuge at 3000 rpm, 5 min. Discard the binding buffer.

8. Wash once more with 0.5 mL of 1x binding buffer, centrifuge at 3000 rpm, 5 min. Discard the binding buffer.

9. Prepare PI in 1x binding buffer: 5 µL PI per 200 µL 1x buffer — per sample.

10. Resuspend cells in 200 µl of PI in 1x buffer.

11. Incubate 5 min in the dark on ice (you can cover samples with foil to protect them from light).

1. Samples should be stored no longer than 4 hours, but it is better to analyze them immediately.

12. Samples should be analyzed using the flow cytometer. Use the blue laser (488 nm) to excite both FITC and PI. FITC is detected in the 525/40 nm channel, while PI is detected in the 585/42

nm channel. Compensation should be made and applied to all the samples as the two fluorophores spectrums are crossed.

## *6.2. Cell cycle flow cytometry*

Another application of flow cytometry for investigating anticancer drugs' mechanisms is the cell cycle measurement. Blocking of cells in a specific cell cycle stage is one of the possible options. The method is based on the ability of propidium iodide (PI) to intercalate between the DNA strands, so the fluorescence level will be proportional to the amount of DNA, which changes during the cell cycle stages.

1. Seed $3\times10^5$–$5\times10^5$ of cells per 60 mm Petri dish overnight.
2. Add the drug for 24–72 hours on the next day.
3. After the incubation, transfer the medium into 15 mL tubes, remove the cells with versene, transfer to the same tubes. If the culture is in suspension, simply resuspend and transfer to tubes. Centrifuge at 5000 rpm for 5 min (or 1000 g for 3 min).
4. Discard the supernatant. Add 0.4 mL of lysis buffer, pipette thoroughly, vortex, transfer to the Eppendorf tubes, incubate for 30 min in the dark.

**Table 7.** Reagent content and ratio for Lysis buffer composition:

| Reagent | Final concentration | Stock solution concentration | The volume of stock solution for 5 mL of buffer |
|---|---|---|---|
| Sodium citrate | 0.1% | 10% | 50 µL |
| NP40 | 0.3% | 100% | 15 µL |
| RNAse A | 100 µg/mL | 10 mg/mL | 50 µL |
| Propidium iodide (PI) | 50 µg/mL | 10 mg/mL<br>or 2 mg/mL* | 25 µL<br>125 µL |
| $H_2O$ | | | Up to 5 mL |

* It is better to make a PI stock 2 mg/mL

*5.* Analyse results on a flow cytometer using the 488 nm blue laser at the PI channel (585/42 nm). Samples can be preserved at +4°C for up to 5 days, wrapped in foil to protect PI from degradation. *For some cell lines, fixing the cell pellet in the 70% ethanol with 30% PBS at –20°C for 20 min helps, after which the fixed cells are centrifuged, and lysis buffer is added. It helps to improve the pattern of cell distribution by DNA ploidy.*

## *6.3. Transfection with lipofectamine*

1. Take 50 µL of medium + 2 µL of lipofectamine. Mix and wait 20 min.
2. Then add 50 µL of the medium with DNA/siRNA amount needed for the experiment. Mix and wait 15 min. The result is a mixture of 2×50 µL for two wells of a 24-well plate.
3. Add the resulting 50 µL to 250–500 µL of a medium in the well with attached cells.

**Table 8.** Preparation of Lipofectamine complexes with DNA

| Procedure details | | | |
|---|---|---|---|
| Component | 96-well | 24-well | 6-well |
| Adherent cells | $1–4\times10^4$ | $0.5–2\times10^5$ | $0.25–1\times10^6$ |
| Opti-MEM® Medium | 25 µL × 4 | 50 µL × 4 | 150 µL × 4 |
| Lipofectamine® 2000 Reagent | 1, 1.5, 2, 2.5 µL | 2, 3, 4, 5 µL | 6, 9, 12, 15 µL |
| Opti-MEM® Medium | 125 µL | 250 µL | 700 µL |
| DNA (0.5–5 µg/µL) | 2.5 µg | 5 µg | 14 µg |
| Diluted DNA Total | 25 µL | 50 µL | 150 µL |
| Diluted Lipofectamine® 2000 Reagent | 25 µL | 50 µL | 150 µL |
| Incubate for 5 min at room temperature | | | |
| Component | 96-well | 24-well | 6-well |
| DNA-lipid complex per well | 10 µL | 50 µL | 250 µL |
| Final DNA used per well | 100 ng | 500 ng | 2500 ng |
| Final Lipofectamine® 2000 Reagent used per well | 0.2–0.5 µL | 1.0–2.5 µL | 5.0–12.5 µL |
| Incubate cells for 1–3 days at 37°C. Then analyze transfected cells | | | |

### *6.4. Colony-forming assay*

1. Discard the media after the end of incubation of cells with the drug of interest.
2. Fix cells with 100% ice-cold methanol for 10 min at +4°C (5 mL of methanol per 10 cm Petri dish).
3. Discard the methanol and add crystal violet. Stock 1% solution of crystal violet in prepared in distilled water. Working solution of crystal violet is prepared from the stock solution by dilution 10 times in 25% methanol in distilled water. To stain a 10 cm dish, 5 mL of staining solution is sufficient.
4. Incubate at room temperature for 10 min.
5. Wash each cup with distilled water 3 times.
6. Take a picture of the dishes, count the number of colonies.

### *6.5. Protocol for the generation and detection of ROS + their neutralization with N-acetylcysteine*

*For K-562 (for adhesive, we simply change the medium instead of washes).*

1) Select the required number of cells (usually $10^5$ cells per Eppendorf tube).

2) Centrifuge and replace the media with the new one, 500 µL (without antibiotic). Leave for 15 min.

*3)* Add NAC to the probe, which will be a control for oxidative stress neutralisation (N-acetylcysteine, the stock is stored in a PBS solution at –20°C, 500mM) 5mM (for 500 mL of

medium – 5µL of NAC). The medium turns yellow (because NAC is an acid), bring the pH back with NaOH. *If you use 1 mM NaOH, then take 2 times less volume than the NAC volume, which is 2.5 µL.*

4) Incubate for 10 min at room temperature.

5) Add DCFDA 5 µM (the stock 1 and 10 mM is stored in the freezer, defrosts very quickly, it must be stirred with a pipette before adding), the Eppendorf tube with NAC does not need to be centrifuged – add DCFDA directly into media with NAC.

6) Incubate for another 5 min.

7) Centrifuge and resuspend in a new media.

8) Add 5mM $H_2O_2$ (for 3% $H_2O_2$ add 882 mM).

9) Incubate 30 min (while the incubation is in progress, you can turn on the cytometer).

10) Centrifuge, resuspend in a new medium and analyze on a flow cytometer (measure right in the medium, then the result is better, but you need to rinse the machine well after yourself).

## *6.6. Carboxyfluorescein succinimidyl ester (CFSE) staining*

The CFSE is a cell-permeable fluorescent dye that can be used for estimation of the proliferation rate. Live cells are all stained, but when cell division occurs, fluorescence intensity decreases, so the number of peaks at day X is equal to the number of cell divisions at the specific time after the first measurement.

1. Resuspend cells in a dish, take 500 µL into Eppendorf tube and centrifuge for 5 min, 500 g.
2. Prepare 1 µM CFSE in sterile PBS (5 mL:1 µL CFSE stock (5 mM) + 5 mL sterile PBS).
3. Discard the cell supernatant, resuspend the cells in 2 mL of the abovementioned solution.
4. Incubate 10 min at room temperature.
5. Centrifuge for 5 min, 500 g. Discard the supernatant, resuspend cells in 3 mL of medium.
6. Transfer cells to the plate, take 200 µL into the flow tube immediately. Analyze using the flow cytometer at blue laser 488 nm in the FITC channel (517 nm).
7. Gently resuspend the cells every day, take 200 µL and analyze. When working with monolayer cells, place them initially into several different wells of the 12-well plate and take one well for analyses every day.

# 7. CELL STAINING FOR MICROSCOPY

## *7.1. Protocol for immunofluorescence staining of cells for gamma H2AX*

1. Place the cover slides in ethanol, burn quickly over a fire, then put in PBS and next into a plate. Add 500 μL of medium to the wells while counting cells.

2. Seed cells into a plate, $5–10\times10^4$ cells per well of a 24-well plate, $10^5$ per well of a 12-well plate, leave overnight for adhesion.

3. The next day, discard the medium and add 800–1000 μL of the medium with the drug (control, $IC_{50}$ and a relatively non-toxic dose, at least 2 replicates for each sample).

4. After the incubation, discard the medium with the drug, wash with PBS (1 mL of PBS per well, pour on the wall, immediately discard).

5. Dilute a 4% paraformaldehyde solution: 600 mg of paraformaldehyde + 15 mL of PBS, heat in a water bath for 75°C, 20 min until the solution becomes transparent.

6. Add 1 mL of 4% paraformaldehyde solution (to fix the cells) per well **in a fume hood!**, incubate for 15 min.

7. Wash with PBS 3 times for 5 min. *After this step, you can leave cells in the refrigerator for a week.* After each washing, turn the plate upside down on filter paper, knock the plate to remove the PBS residues.

8. Prepare Triton X-100 solution: 1 mL of 100% Triton X-100 + 9 mL of PBS (10% intermediate solution), 1 mL of 10% solution + 49 mL of PBS (0.2% final working solution).

9. Add 0.2% Triton at 1 mL/well for membrane permeabilization, incubate for 20 min.

10. Discard Triton X-100 solution, wash with PBS 3 times for 5 min.

11. Prepare PBST solution. Firstly, prepare 0.1% Tween 20 solution. 5 mL of Tween 20 + 45 mL of PBS (PBST stock with 10% Tween 20). Next, 0.5 mL of 10% stock + 49.5 mL of PBS (PBST with 0.1% Tween 20). Finally, prepare PBST: 99.9% of PBS + 0.1% of Tween 20.

12. Add 3% (1% to 5%, for antibodies with high affinity – 3%, for low affinity – 1%) BSA solution in PBST, incubate 45–60 min at room temperature to block the proteins.

13. Transfer the coverslips to a Petri dish with a parafilm piece, then do all the washings on it, 500 μL of PBST each time.

14. Dilute the first antibodies (https://www.cellsignal.com/products/primary-antibodies/phospho-histone-h2a-x-ser139-antibody/2577) 1: 350 (formally it is necessary to dilute 1:300; if they bind poorly, up to 1:50) in BSA/PBST **using sterile tips** (100 μL of diluted antibodies per coverslip). For 10 coverslips: 2.9 μL of antibodies + 997.1 μL of 3% BSA/PBST. Incubate overnight at +4°C.

15. Add 100 μL of 3% BSA/PBST without antibodies to the negative control well. It is necessary to do negative control without the first antibodies since they have a threshold level of luminescence, which must be considered when working with a confocal microscope.

16. The next day washes the coverslips 3 times for 5 min in PBST.

17. Dilute secondary antibodies (https://www.thermofisher.com/antibody/product/Goat-anti-Rabbit-IgG-HL-Cross-Adsorbed-Secondary-Antibody-Polyclonal/A-11008) 1:1000 in BSA/PBST. *If the protocol does not work with this dilution, dissolve according to instruction (1:500).* **Work only under weak light to protect the fluorophores from quenching!** Add 100 μL of second antibodies, incubate for 1 hour in the dark at room temperature.

18. Wash 3 times for 5 min with PBST.

19. Add 10 μL of the antifade mountant (ProLong ™ Gold Antifade Mountant with 4′,6-Diamidine-2′-phenylindole dihydrochloride (DAPI)) onto the glass slides, cover them with prepared coverslips (turn them upside down, cells down). Seal with transparent nail polish, being careful not to climb onto the preparation to fix the glass.

## *7.2. Senescence associated β-galactosidase staining*

Cell senescence is a process when cell division stops due to several reasons. Senescent cells are characterized by a permanent cell-cycle arrest. One of the most popular markers is β-galactosidase which can be detected using the chromogenic substrate X-Gal. The stock solutions should be prepared to perform the protocol (Table 9).

**Table 9.** Preparation of solutions

| Stock solution | Preparation | Storage conditions |
|---|---|---|
| Citric acid (0.5M) | 21.0 g of citric acid (monohydrate)<br>Bring volume to 100 mL with $dH_2O$ | RT |
| Citric acid/$Na_2HPO_4$ (0.5M, pH 6.0) | 7.1 g $Na_2HPO_4$<br>~75 mL $dH_2O$<br>**Adjust pH to 6.0 with 0.5 M citric acid**<br>Bring volume to 100 mL | RT |
| $K_4Fe(CN)_6$ (50mM) | 2.11 g $K_4Fe(CN)_6$<br>Bring volume to 100 mL with $dH_2O$ | 4°C in dark |
| $K_3Fe(CN)_6$ (50mM) | 1.65 g $K_3Fe(CN)_6$<br>Bring volume to 100 mL with $dH_2O$ | 4°C in dark |
| NaCl (5M) | 29.2 g NaCl<br>Bring volume to 100 mL with $dH_2O$ | RT |
| $MgCl_2$-$6H_2O$ (1M) | 20.3 g $MgCl_2 \times 6H_2O$<br>Bring volume to 100 mL with $dH_2O$ | RT |
| X-Gal (20 mg/mL) | 100 mg X-gal<br>5 mL dimethylformamide | **–20°C in dark** |
| Fix Solution (PBS/2% Formaldehyde/0.2% Glutaraldehyde) | 5.4 mL 37% Formaldehyde<br>0.4 mL 50% Gluteraldehyde<br>94 mL $dH_2O$ | **Make fresh fix solution each time** |
| Staining Solution (10 mL) | 6.3 mL $dH_2O$<br>0.8 mL citric acid/$Na_2HPO_4$<br>1.0 mL $K_3Fe(CN)_6$ (50mM)<br>1.0 mL $K_4Fe(CN)_6$ (50mM)<br>0.3 mL NaCl (5M)<br>0.02 mL $MgCl_2 \times 6H_2O$ (1M)<br>0.5 mL X-gal<br>**Prepare and filter through a 0.45 µm filter** | **Make fresh each time** |

1. Wash cells 2–3 times with PBS.
2. Cover with Fix Solution for 5–10 min at RT.
3. Wash 2–3 times with PBS.
4. Add Staining Solution and incubate at 37°C for 24–48 hr (or longer if control stays blank).
5. Wash 2–3 times with PBS.

6. If crystals have formed on your plate during the incubation, add a small amount of DMSO to dissolve, then wash again with PBS.

7. Cover with 50% glycerol in $H_2O$ and store at 4°C.

### *7.3. Protocol for actin cytoskeleton staining using Alexa Fluor® 488 phalloidin and TO-PRO®-3 iodide (tissue in scaffolds)*

This protocol is used for staining the individual cells to estimate their viability. The protocol is suitable for staining the cells on hard substrates.

1) Discard the cell medium.

2) Add 2 mL of 4% paraformaldehyde (PFA) solution and put in a fridge at 4°C for 15 min to fix the tissue (in scaffolds).

3) Wash 3 times (fast) in PBS solution.

4) Permeabilise the cells with 0.1% Triton X-100 (2 mL) for 10–15 min at room temperature.

5) Wash three times in PBS (fast).

6) Stain the scaffolds with Alexa Fluor® 488 phalloidin (Invitrogen, Eugene, Oregon, USA) 1:20 dilution for 60–90 min in the dark at +4°C.

- Dilute 100 μL of Alexa Fluor® 488 phalloidin in 2 mL of PBS solution.
- Pour 100 μL of AlexaFluor solution on the sample.
- Put the samples in a box and put them into the fridge for 60 min.

7) Wash in PBS twice briefly and then three times for 3 min each.

8) Stain the nuclei with TO-PRO®-3 iodide (Invitrogen, Eugene, Oregon, USA) 1:300 dilution for 5 min.

- Dilute 5 μL of TO-PRO®-3 iodide in 1.5 mL of PBS.
- Pour 100 μL of TO-PRO®-3 iodide solution on the sample.

9) Wash briefly the samples three times in PBS.

10) Drop the Vectashield mounting medium on the sample surface and mount the sample in an inverted position on a thin coverslip to observe under a confocal microscope.

### *7.4. Immunofluorescent staining on solid, smooth and porous surface (glass, metal, titania) for focal adhesions and the actin cytoskeleton*

This protocol is used for staining the individual cell focal contacts to estimate their adhesion on the substrate surface.

1) Discard the cell medium.

2) Add 2 mL of 4% PFA and put in a fridge at +4°C for 10 min to fix the tissue (in scaffolds).

3) Wash 3 times (fast) in PBS solution.

4) Quench for 5 min in 50 mM $NH_4Cl$.

5) Wash three times in PBS (fast).

6) Add 2 mL of 4% PFA and put it in a fridge at 4°C for 15 min to fix the tissue (in scaffolds).

7) Wash 3 times (fast) in PBS solution.

8) Block for 1 hour in 3% BSA in PBS solution.

9) Incubate **overnight** with anti-vinculin antibody (AB) (Sigma *V9131*).

- Prepare 3% BSA-PBS solution
- Dilute 10 μL of anti-vinculin antibody in 3 mL of 3% BSA-PBS solution.

**Next day:**

1) Wash 3 times (fast) in PBS solution.
2) Incubate with secondary AB, goat anti-mouse Alexa 594 (Invitrogen) for 1 hour.

- Dilute 10 µL of goat anti-mouse Alexa 594 in 3 mL of PBS.

3) Wash 3 times (fast) in PBS solution.
4) Incubate with Alexa Fluor® 488 phalloidin for 20 min in the dark.

- Dilute 10 µL of Alexa Fluor® 488 phalloidin in 3 mL of PBS.

5) Wash 3 times (fast) in PBS solution.

6) Stain the scaffolds with DAPI, 1:2000 dilution for 5 min in the dark at 4°C.

- Prepare 1µg/µL of DAPI solution in PBS.
- Add 10 µL of DAPI solution on the sample surface.
- Stain the samples for 5 min in the dark at +4°C.

7) Wash 3 times (fast) in PBS solution.
8) Mount glass slides with fluoromount.

## *7.5. Protocol of nuclei staining with DAPI*

1. Place coverslips in alcohol, then in PBS and finally into a plate. Add 500 µL of medium to the wells while counting cells.

There are life hacks for providing better adhesion of cells to coverslips. For example, you can leave them in a well plate with an open lid under a UV lamp in a laminar for 10 min. Alternatively, cover them with a serum medium for 30–60 min to ensure protein adhesion or put the coverslips in a small Petri dish and add 0.5 mL of pure serum on the glass.

2. Seed 5–10×$10^4$ cells per well of a 24-well plate, leave to attach overnight.
3. The next day, discard the medium and add 800–1000 µL of the medium with the drug ($IC_{50}$ and a relatively non-toxic dose, at least 2 replicates for each sample). Leave one well as a control.

Usually, a standard medium with serum is used for growing cells on coverslips. To slow down the cells' proliferation, you can dilute drugs in a serum-free medium (for cancer cells). If the cells are very sensitive (normal cells), then you can reduce the percentage of serum to 5%.

4. After the end of incubation, discard the medium, wash the wells with PBS **once!** (1 mL of PBS per well, **immediately discard**). *Pour PBS on the well's walls, not on the glass, to avoid washing of cells.*

5. Dilute a 4% PFA solution: 600 mg of PFA in 15 mL of PBS, heat in a water bath at 75°C for 20 min. **Do not store the solution for more than 3 weeks!**

6. Add 1 mL (if you lack the solution, 250 µl will be enough) of 4% PFA solution in **fume hood** into each well to fix the cells, incubate for 15 min.

7. Wash with PBS 3 times.

8. Add 1 mL per well of Triton X-100 (0.1%) for membrane permeabilization, incubate for 5 min.

Triton X-100 is a very viscous liquid. To prepare a 0.1% solution, you need to prepare the right consumables. Firstly, cut a pipette tip to make a larger hole. This will ease the filling process. Then take the Triton X-100 several times to moisten the tip from the inside. Then take 0.1 mL and dilute it in 0.9 mL of PBS. Shake properly to dissolve.

9. Wash the samples 3 times with PBS.

10. Dilute DAPI dye. The stock solution is 5 mg/mL (add 2 mL of deionized water to the 10 mg in the tube). Make aliquots 50–100 µL each and store them at –20°C. For a working solution, dilute the stock solution immediately before the staining 40000 times: 5 µL of stock + 195 µL of PBS. Then take 5 µL from this solution + 4995 µL of PBS.

11. Add 300 µL of DAPI working solution per well, incubate for 3–5 min.

12. Discard the DAPI solution, wash the wells with PBS one time.

13. Proceed as described in the general procedure of preparation of glasses for microscopy from step 10.

### *7.6. MitoTracker™ Red CMXRos Staining*

1. Dilute 1 mM of dry dye in DMSO ($M_r$ = 531.5). It is better to dilute immediately a 50 µg supplied in the tube in 94 µL of DMSO, make aliquots 20 µL each and store at –20°C.

2. For convenience, immediately before the experiment, you can prepare an intermediate stock solution with a concentration of 10–100 µM in a buffer or medium without serum. *You can store this solution at –20*°C *for a short time if the experiments are carried out one after another).*

3. Staining proceeds without fixation. Depending on the density of cells and their type, use the range of final concentrations of 25–500 nM (if the glasses need to be fixed for long-term storage, then use at least 100 nM); the lower the concentration, the better for preserving living cells. Time for staining varies in the range of 15-45 min. For example, to stain $8 \cdot 10^5$ THP-1, HCT-116, or MCF-7 on 35 mm coverslips use 500 nM for 30 min.

4. If you plan to stain cells for 30 min, then add the dye 30 min before the end of incubation with the studied drug. The dye from the intermediate stock is added directly to the medium. When adding, use the dispenser tip to distribute dye over the entire volume of the well at once, mix thoroughly by shaking the plate. Incubate at 37°C.

5. After incubation, wash the cells from dye excess two or three times in PBS (if you will detach or fix cells, it is better to do it twice). If necessary, cells can be fixed using standard protocols.

6. Optimal wavelength parameters for measuring fluorescence are at 579/599 nm. Results can be analyzed both with the flow cytometry (use the APC configuration) and the RHOD cube of the microscope.

**Figure 1** shows the HCT-116 cells stained with 500 nM MitoTracker™ Red CMXRos and 250 ng/mL DAPI, fixed in paraformaldehyde. **Figure 2** shows the THP-116 cells after the same sample preparation procedure but analyzed using flow cytometry.

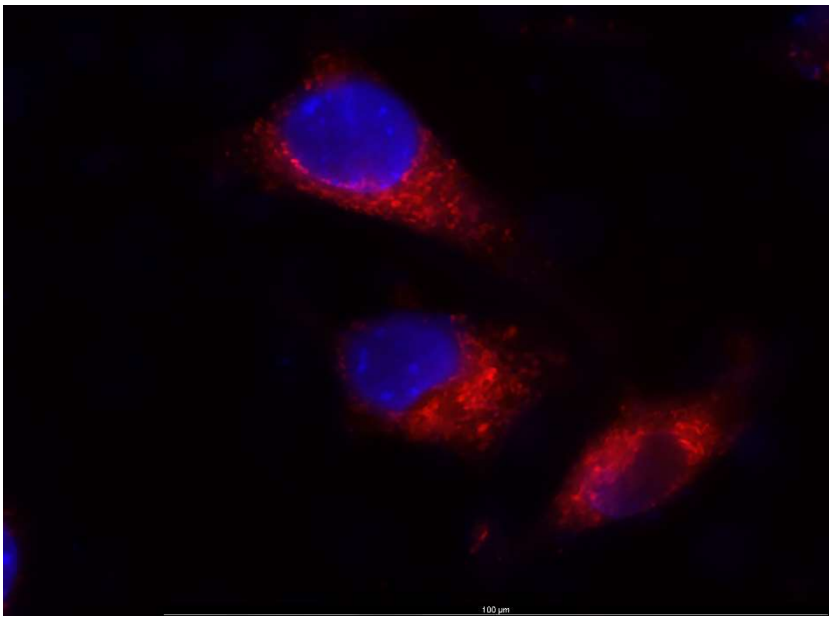


**Figure 1.** HCT-116 cells stained with 500 nM of MitoTracker™ Red CMXRos and 250 ng/mL of DAPI, fixed in PFA

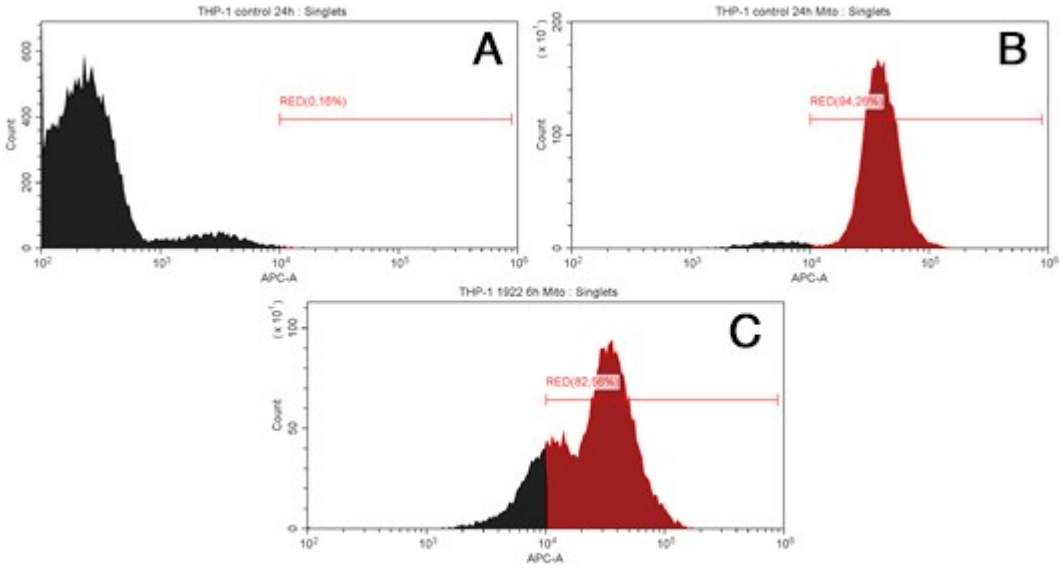



**Figure 2.** THP-1 cells, 500 nM MitoTracker™ Red CMXRos. A: unstained. B: stained. C: stained after treatment with mitochondrial poison

## *7.7. Genotoxicity study by DNA comet method*

1. Seed the cells for the experiment. Add the test compound for the required time. As a positive control, add for 24 **hours! (Take into account the experiment duration. Otherwise, the cells damage will be too significant)** hydrogen peroxide at 500 μM concentration (Sigma 19.8% wt.)

2. Degrease the glasses by immersion in 96% ethanol for 30 min. Dry and immerse them in 1% agarose solution, immediately pull out, place on foil to dry.

3. Prepare lysis buffer: 2.5 M NaCl, 0.1 M EDTA, 10 mM Tris pH 10, 10% DMSO, 1% Triton X-100. Store it at +4°C.

4. The pH of the buffer should be 10. Adjust pH with 5M NaOH at 100 µL step. If pH was adjusted to more than 10, you could add concentrated hydrochloric acid to drop it down.

Store the lysis buffer for no more than 2 weeks **without Triton X-100!** Add Triton X-100 only on the experiment's day, refrigerate buffer in the refrigerator before the experiment. **Prepare the buffer in plastic!** Do not adjust the volume to a liter immediately; **check the pH first!**

5. Prepare 0.5% solution of low-melting agarose. Dissolve 200 mg of low-melting agarose in 40 mL of PBS, put in a microwave, heat, but not boil, control the dissolution. It can be done in a 50 mL plastic tube. Put in a water bath at 37°C, so that when added to the cells, they are not damaged by high temperature.

6. Prepare 0.8% solution of agarose (not low-melting, used for routine gel electrophoresis). Dissolve 0.64 g of agarose in 80 mL of PBS in **glass** dishes, put in the microwave. *Do not let it boil; stop if you see the bubbles, stir and start again until dissolved.* The dish can be left at room temperature while you prepare the cells.

7. Put the Eppendorf tubes in a water bath to warm up to 37°C.

8. Count the number of cells in all dishes for the experiment (detach the cells with trypsin-versene solution, discard it, resuspend the cells in 1 mL of versene, count).

9. 6 glasses are required per sample. For each glass, 75 µL of low-melting agarose + volume of cell suspension in versene contains 25 000 cells.

10. Next, you need to calculate how much agarose and cells are needed for 7 glasses (6 are required, one for the reserve). The volume of the cell suspension should not exceed 10 µL to avoid agarose dilution. If more than 10 µL due to the low concentration of cells, the cells must be centrifuged and resuspended in such a versene volume to provide 25000 cells in 10 µL.

11. Add cells to the low-melting agarose in a dry incubator in the Eppendorf tubes at 37°C (for example, 7×75 µL of agarose + 7×10 µL of cell suspension).

12. Then drop 80 µL of a heated mixture of cells with low-melting agarose on each coverslip 24×50 mm and cover with another coverslip (square, 24×24 mm) **immediately!**

13. Wait for the slides to dry for about 15 min and remove the coverslips **carefully, without tearing.**

14. Melt the usual agarose again, cool it to about 37°C so that the cells do not blow up.

15. Pour150 µL of agarose onto the coverslip (for an average room temperature of 20–22°C, the 100 µL of agarose solution is enough) and immediately cover it with a coverslip, wait for it to dry and remove carefully. **All glasses should be removed "sideways", not ripped up.**

16. Put the glasses in flat trays and fill them with lysis buffer. **The procedure should be performed without light.** *Pour the buffer on the walls, not on the glasses, to cover the glasses with about 5 mm layer!* **Refrigerate at +4°C overnight!** (can be left from 2 hours to a week).

17. The next day, wash the glasses with distilled water (discard the lysis solution and pour distilled water, **not** on the glasses! Wash three times (put coverslips in boxes when washing).

18. Prepare approximately 800 mL of phoresis buffer (0.3 M NaOH, 1 mM $Na_2EDTA$, pH 13.6).

**Calculation of portions:**

Mr (NaOH) = 40

Mr (EDTA) = 372.24

$M_{sample}$ = molarity × Mr × required volume, ie.

M (NaOH) = 0.3 ×40 × 0.8 = 9.6 g

Mr (EDTA) = 0.001 × 372.24 × 0.8 = 0.298 g

19. Take out the glasses, put them in the phoresis chamber, in the direction from the electrode to electrode. You can overlay places with labels of samples on top of each other.

20. Perform phoresis.

Doing phoresis for the first time, you need to adjust the time: check 20, 25, 30 min and observe where there will be no “tails” in the control cells. Control should have a maximum of 1 tail per 10 nuclei. The voltage is equal to the length between the electrodes in the chamber. For a medium camera, voltage is 15 V. The current should be in the range of approximately 300–350 mA. If the current is too high, you need to add more buffer. Initially, the buffer should cover the glass with a 5 mm layer.

21. Neutralize the phoresis buffer with 0.4 M Tris HCl. Transfer the slides from the phoresis chamber to the trays, fill with Tris HCl for 5 min, discard.

22. Transfer the glasses to filter paper. You can moisten it and put it in the refrigerator overnight.

23. The next day, add 50 - 100 μL green fluorescent nucleic acid staining solution ( alternatively, EtBr can be used) to the slides, stain for 15 min at room temperature in the dark. Rinse the slides briefly in $dH_2O$ and dry completely at 37 °C in the dark. Cover with coverslips and analyze immediately.

24. Take an image of about 100 cells, process them in the Comet score software, carry out statistical analysis.

# 8. MICROSCOPY METHODS

## *8.1. Capturing and processing images on fluorescent microscope Leica DMi8*

This section describes the process of obtaining images using the inverted fluorescent microscope Leica DMi 8. The main control elements of the microscope are shown in Figure 3 and 4. Touch screens are shown in Figure 5. Image capturing is carried out by the LAS X program. The main control elements of the program are shown in Figure 6.

a b

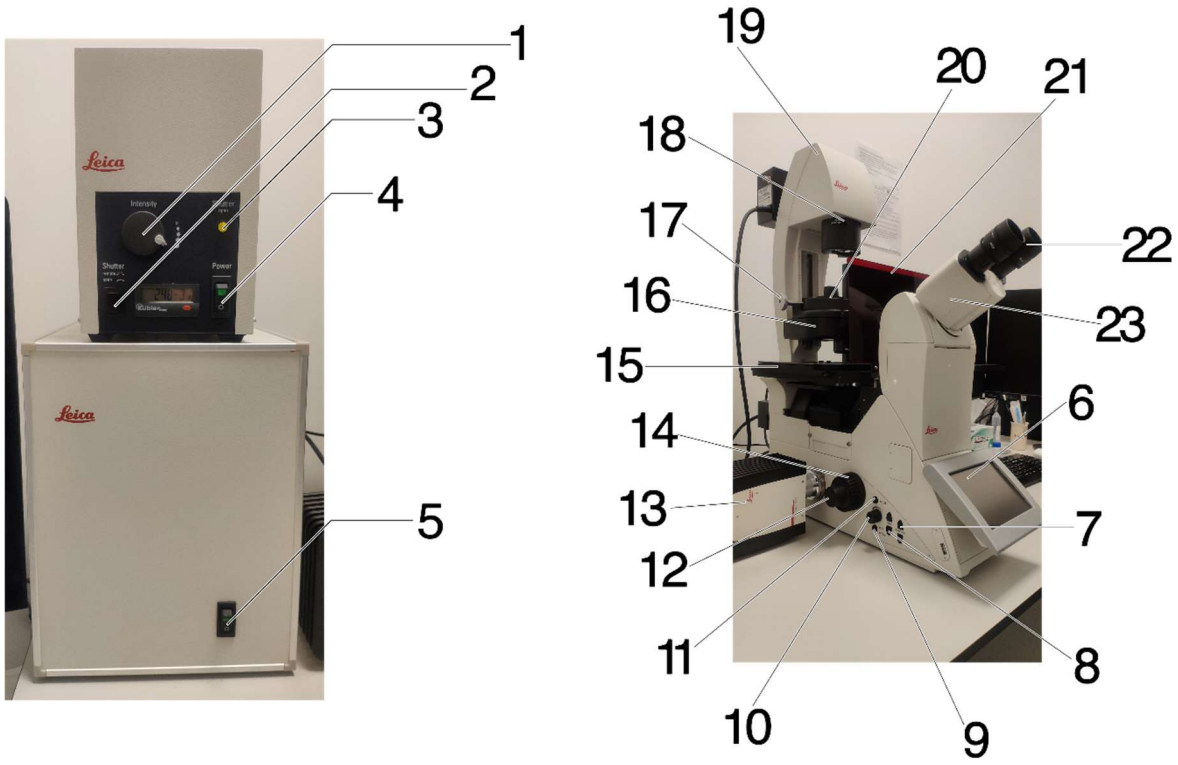



**Figure 3.** Main microscope controls (left side): 1 – Fluorescence intensity controller, 2 – luorescence axis shutter button, 3 – fluorescence axis shutter indicator, **4 – fluorescence lluminator main power switch, 5 – the main power switch,** 6 – touch screen, 7 – aperture djustment, 8 – field diaphragm switching, 9 – transmitted light/fluorescence switch, 10 – ntensity controller, 11 – shutter switch, 12 – fine focus wheel, 13 – camera, 14 – coarse focus vheel, 15 – table, 16 – condenser block, 17 – condenser block z-axis adjustment, 18 – transmitted ight diaphragm, 19 – transmitted light tilted column, 20 – polarization analyser, 21 – protective creen, 22 – oculars, 23 – adjustable oculars head.

To get access to the microscope, you need to pass an exam. Contact the responsible staff to do so. Before going to the microscope, you need to sign in to e-calendar via the link you get from responsible staff. *Remember, that priority in using the microscope is for the one who is recorded in the e-calendar.* It would be best if you prepare your sample **before** going to the microscope.

1. Remove protective cover from the microscope. Be sure nothing is left on the microscope table, and everything looks fine (no spilled liquid, broken parts, etc.). ***If anything strange happens, do not try to repair it by yourself, contact responsible staff.***
2. Sign in a journal near the microscope with the actual time of work start. Turn on the microscope with button **5**. If you need fluorescence, you can turn on the fluorescent illuminator with button **4**. *All buttons must be switched fast, do not hold them in the middle position.*
3. Wait for the microscope to calibrate, do not place anything on table **15** before calibration. After calibration ends, start the LAS X application.
4. Choose the appropriate sample holder (for Petri dishes or multiwell plate). To insert the sample holder into the table, place *red dots* on the holder and table close to each other, push the holder to the marked edge, hold it in place and then push down the opposite side of the holder.

a b

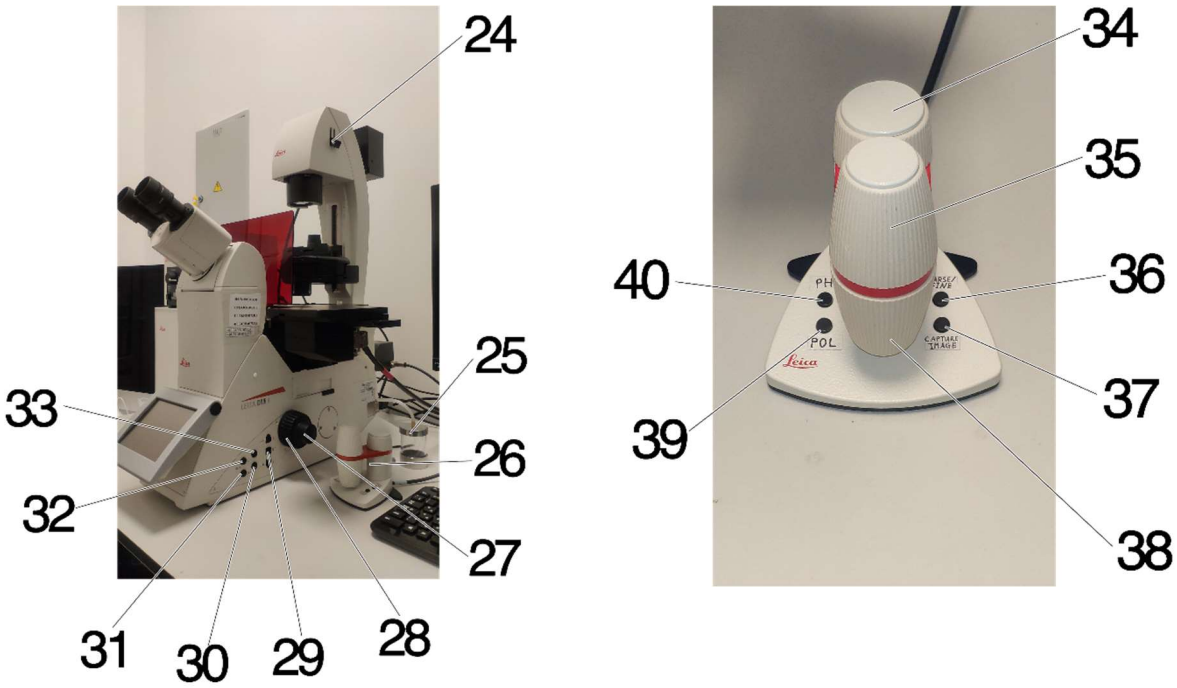



**Figure 4.** Main microscope controls (right side): 24 – transmitted light axis shutter, 25 – spilled liquid tank, 26 – smart stick, 27 – fine focus wheel, 28 – coarse focus wheel, 29 – z-axis fixed positions, 30 – filter cube anticlockwise button, 31 – filter cube clockwise button, 32 – objectives clockwise button, 33 – objectives anticlockwise button, 34 – focus wheel, 35 – y-axis movement wheel, 36 – coarse/fine movement switch button, 37 – capture image switch button, 38 – x-axis movement wheel, 39 – polarization mode switch button, 40 – phase-contrast mode switch button.

5. Place your sample into the holder. *On an inverted microscope, you should place your samples upside down (coverslip down).*

6. Check what axis is turned on (green lamp near button **9**). Press button **9** to switch to transmitted light if necessary. Open shutter with button **11.**

7. Choose objective x5 either with buttons **31** or **32** or via **screen 3**. **Remember that you must switch to ×5 objective when you end working!** In this case, you will always start from ×5. Switch to phase-contrast mode on **screen 2**.

8. Adjust oculars **22** and ocular head **23** to suit your eyes. Then adjust the light intensity with regulator **10. Be careful, on ×5 objective and with high light intensity, you can be blinded. Reduce light intensity in advance.**

9. Find a focus with focus wheels **12**/**14** or **27**/**28** or smart stick wheel **34**. Focus wheels are doubled on both sides of the microscope. Coarse and fine wheels are moving together. To switch between coarse/fine movements, press button **36**. *The difference between these coarse/fine movements is especially noticeable at low magnification.*

10. After finding the focus on the ×5 objective, you can increase magnification with buttons **31** or **32** or via screen **3**. *It is better to increase magnification gradually so as not to lose the object from the field of view.*

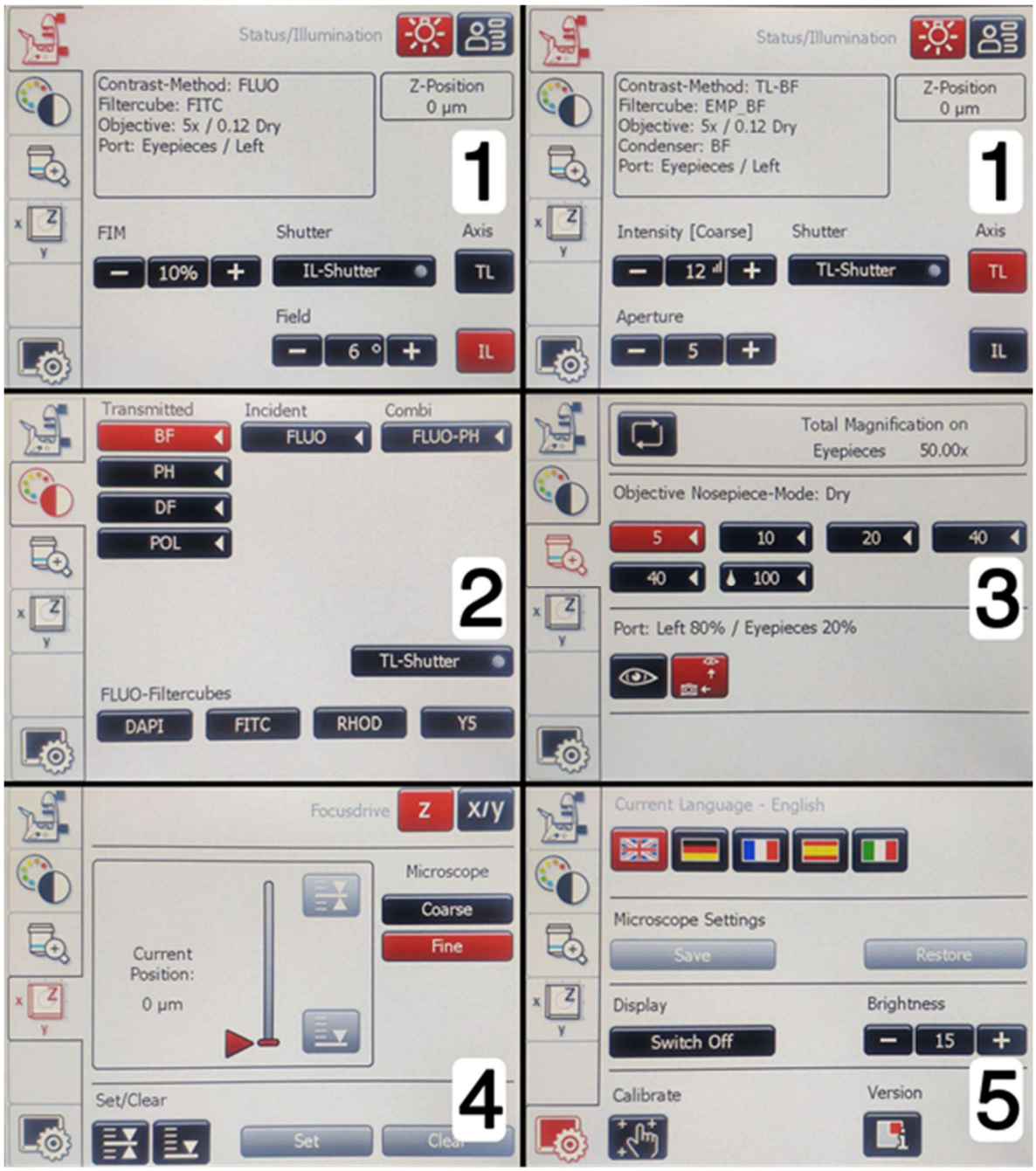



**Figure 5.** Microscope touch screens: 1 – first screen in transmittance and fluorescence modes, 2 – second screen for choosing modes and filter cubes, 3 – third screen for choosing objectives, 4 – fourth screen for choosing fixed z-axis positions and switching from coarse to fine movement mode, 5 – fifth screen with options.

Objectives ×5, ×10, and second ×40 can be used to observe samples on glass slides, in Petri dishes or multiwell plates. Objectives ×20 and first ×40 can be used only for coverslips (they have a very short focus distance). Objective ×100 is for oil immersion and can work only with coverslips.

11. When you are sure that the observed object is what you are looking for, you can switch to other capturing modes via screen **2: bright-field, phase-contrast, dark-field, polarization,**

**fluorescence.** *The shutter position **11** for each mode is remembered, so you need to open the shutter manually after switching to another mode.*

**Bright-field (BF)** is best for observing colored objects like stained bacteria (Gram staining). To ensure the best possible resolution in BF, you must keep the aperture on screen **1** as close as possible (1 is best, 24 is worst). Increase light intensity with regulator **10** to do so.

**Phase-contrast (PH)** is best for observing transparent objects (with the same refractive index as glass or water) like cells. In this mode, objects will look a bit bluish.

**Dark-field (DF)** is best for observing objects smaller than the Abbe limit (micro- and nanoparticles). In DF mode, such objects will scatter light, and the observed picture would look like a dark field with small gold-colored bright dots.

**Polarization (POL)** is best to observe optically-active objects which can polarize the light, like cellulose nanocrystals.

**Fluorescent mode (FLUO)** with different filter cubes is used to observe pre-stained biological objects or objects with intrinsic fluorescence. The wavelength of excitation and emission of each cube can be found in LAS X. Hold the mouse over the cube's image (color circle) for a couple of seconds).

12. To capture an image in LAS X, you need to press the Live button in the left bottom corner. For images in transmitted light, you need to adjust the image's brightness with regulator **10** and sliders Exposure and Gain.


**Figure 6.** LAS X main controls. The exposure slider can be moved from 1 ms to 100 s on a logarithmic scale. In most cases, there is no need to accumulate images longer than 2 s. Gain can be adjusted within the 1 – 10 range. FIM (Fluorescence Intensity Manager) have fixed values 10%, 17%, 55% and 100%. In transmitted light, intensity can be adjusted between 1 and 255.

For PH images, Gain and Exposure may be kept at the lowest value for better quality. Remember that Gain adds noise to the image, thus reducing its quality. Also, if your object is moving, try to reduce Exposure to prevent blurry images. In the case of moving objects increase Gain or light intensity with regulator **10.**

13. To capture fluorescent images, for best results, you can follow the following protocol.

**Note: When working with a fluorescence, a protective screen 21 must be permanently installed. Do not look behind the protective screen during operation!**

- Turn on the Live image.
- While in PH mode, find and place your object in focus, switch to the required objective.
- Switch to a proper filter cube.
- Shortly switch to FLUO by opening the shutter and ensuring that your object is fluorescent, so the staining was done correctly. Close shutter to prevent bleaching. Adjust fluorescence intensity.

In FLUO, image intensity can be adjusted in four different ways: with regulator **1**, with regulator **10** (same as FIM intensity in LAS X or screen **1**), with Exposure (LAS X), and with Gain (LAS X). **Regulator 1 must always be switched to the max!** In most cases, FIM intensity is set to 100%. At these parameters, Exposure is usually 300–1000 ms, and Gain can be adjusted to 2–5. *In case your fluorescence is fading very fast, first reduce FIM to a lower value and increase Exposure and Gain.*

- Open shutter and capture image.

14. To save your images, you need to go to the Open Projects section in LAS X. When you open a program, a blank Project is created. You can save it under the required name by pressing the diskette button near its name.

15. To export images, you can right-click either on the image name or on the Project name and choose export options. In the opened dialog, you need to choose your folder, check the “Separate folder” and Scale bar options.

16. After work, you need to switch to the transmitted light axis, objective x5, remove your sample and clean all spilled liquids. Do not forget to save your project or export images. Check in the journal with the end time.

17. First, close the LAS X, switch off fluorescence with button 4, then control block with button 5.

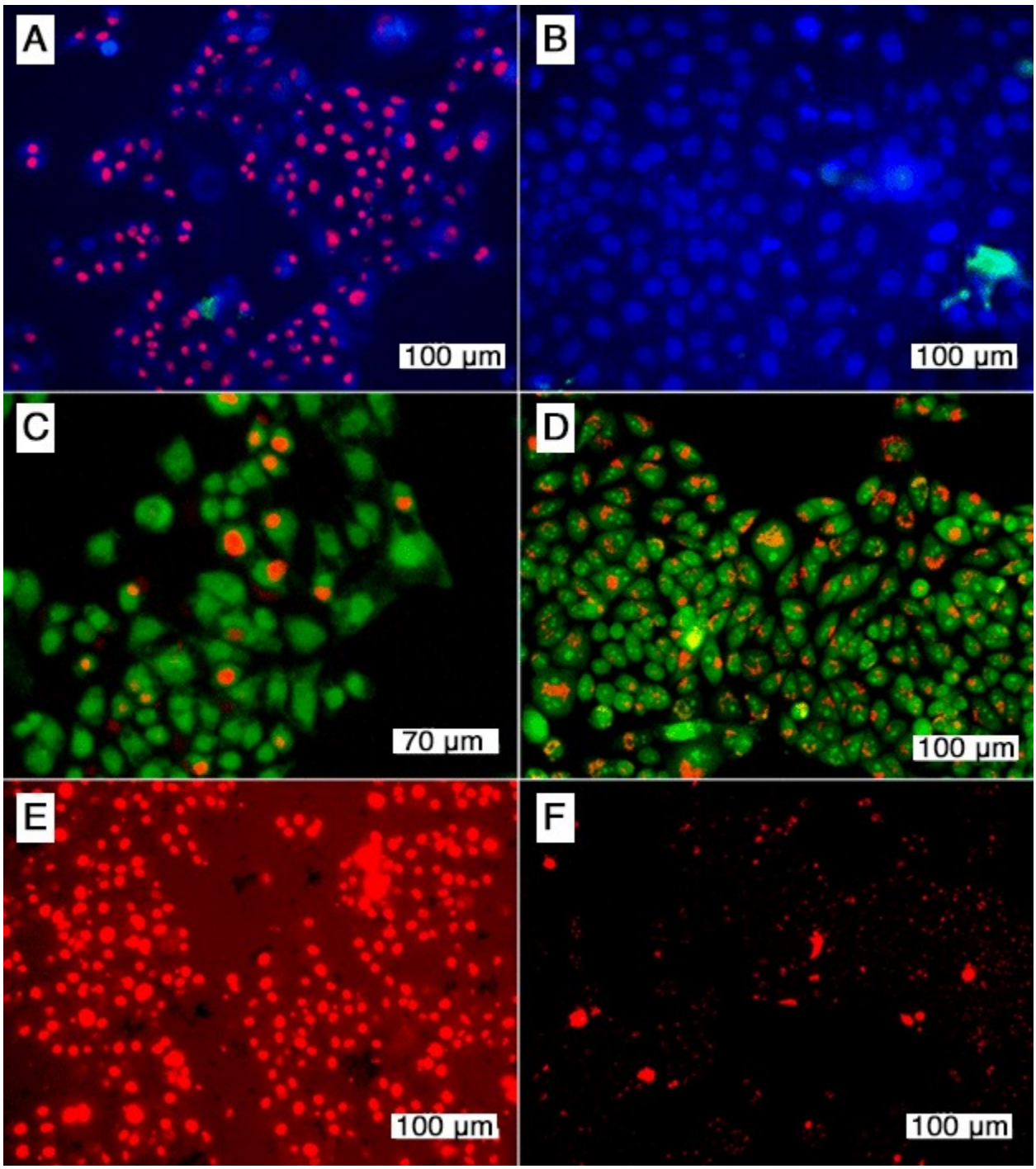



**Figure 7.** Fluorescent image of SPEV-2 cells stained with various dyes. A, C, D – after exposure to toxicants, B, D, E – control. A, B – staining with DAPI and PI. C, D – staining with acridine orange (AO) and PI. D, E – staining with PI. In image D, the tiny red dots located around the nucleus are ribosomes. In the rest of the images, the red dots are nuclei stained with PI

## *8.2. General procedure for preparing samples for microscopy*

1. For best results, all samples for microscopy should be prepared on glass slides and covered with coverslips.

2. All glasses should be checked for dust and other impurities, even if they were just taken from the box. To clean glasses from dust, you can use lint-free wipes (e.g. Kimtech). You should

always take glasses by the edges using tweezers, wrap in wipes and press on both sides while cleaning. Take care when cleaning coverslips since they can be easily broken.

3. The sample for microscopy should be as thin as possible. Cells grown on glass are the best option. To grow cells on coverslips, you need to place a sterile coverslip (pretreated in a vial with ethanol) in a 6-well culture plate. As another option, you can use special cell culture round coverslips, which can be placed in either 12- or 24- well plate, depending on their diameter. Then you need to add culture media and the required amount of cells. Cells will grow on the glass surface.

4. It is better to use sharp-edged tweezers to remove coverslips from the well. Be careful not to press too strongly to prevent the glass from cracking. In case you broke the glass, you still can use pieces, but the final sample will be not that beautiful.

5. All other microscopy samples should be cut into thin pieces with a razor blade or scalpel (or microtome, if available).

6. It is better to fix samples in formaldehyde or glutaraldehyde. Typical concentrations of these fixative solutions are 4% (in PBS or other buffer solutions). **Always work in fume hoods during fixation because all fixative solutions can be toxic to the lungs and epithelium.**

7. To fix samples, you need to immerse them in fixative solution entirely for at least 30 mins. Coverslips with cells should be placed as they were in culture well.

8. After fixation, you need to rinse your samples with PBS a couple of times. You can do so by immersing them in PBS or carefully pour on them while holding them in tweezers.

9. If you want to stain your sample, follow the required procedure. Typically, you should immerse your sample in PBS and add dye solution in a required amount. After staining, do not forget to rinse your sample with PBS to avoid nonspecific fluorescence.

10. Then you need to place your sample on a glass slide. For typical 18×18 mm coverslips, you can place up to 3 samples on a standard glass slide. For coverslips with grown cells, you need to turn your glass upside down.

11. All samples must be covered with a clearing solution that helps decrease the difference in refractive indices of the sample, glass and air. The simplest clearing solution is glycerol, but you can also use commercially available ones like ProLong™ Gold Antifade.

12. In the case of cells grown on coverslips, you need to place a small drop (about 10 μL) of glycerol on a glass slide, then turn your coverslip upside down, and put it on the drop. Wait for glycerol to spread out to coverslip edges. You can gently tap on the coverslip using the tweezers to remove air bubbles.

13. In the case of another sample, you can first put your sample and then pour glycerol on top of it. Remember that you must keep the glycerol amount as small as possible. Large amounts will lead to problems with the sealing of the glass and sample visualization.

14. Then you need to seal your sample to prevent glycerol from leaking and, most importantly, from moving your sample under the action of convection flows of glycerol.

15. Transparent nail polish is usually used for sealing purposes. You should carefully seal all the edges of the coverslip. Avoid leaking of the glycerol because if you mess the coverslip with it, it would be almost impossible to obtain good pictures.

However, there is still an option to clean your coverslip. To do so, you need to seal it completely, wait for nail polish to harden. Then you need to try to wash glycerol with dish soap like Fairey. It is better to soak a sponge with a soap solution and then wipe the glycerol completely. Rinse the glass in water and dry with wipes. Check the result under a microscope, and, if required, repeat.

16. After sealing your sample, be sure that nail polish completely hardens. After that, you need to remove PBS left after rinsing on top of the coverslip. It is better to wait until it dries up and remove the salt crystals with a wipe.

17. Then place your sample upside down (coverslip down) in a microscope sample holder and enjoy!

18. Properly fixed and sealed samples can be stored in the dark for a long time. For better results, place them in the fridge (+4°C).

# 9. STATISTICAL ANALYSIS

An important part of our research and performing biological experiments is the statistical analysis of obtained results. The variance analysis is most often used in statistical calculations, aimed at finding dependencies in experimental data by examining the significance of differences in mean values.

This method could compare samples based on their means and depict how different these samples are from one another. Besides, the analysis of variance (ANOVA test) can be used to describe complex relations among variables. The ANOVA was suggested by the statistician Ronald Fisher. The law of total variance is the main in ANOVA basis. The observed variance in a particular variable is partitioned into components attributable to different sources of variation. The simplest variant of the ANOVA test provides a statistical test of whether two or more population means are equal. Thus, ANOVA generalizes the t-test (Student's test) beyond two means.

At first, let us see the t-test on the example of obtained results.

## *9.1. Student's t-test*

The final stage in data processing (both images or numerical data) is analysing the data obtained. This is necessary to say with confidence that the obtained data are statistically reliable and can be trusted. We will use the most common method called Student's t-test.

Let us consider an example of data analysis obtained in the MTT test (Table 9).

**Table 10.** The optical density of the samples obtained during the MTT test

| Row | Well 1 | Well 2 | Well 3 | Well 4 | Well 5 | Well 6 | Control |
|---|---|---|---|---|---|---|---|
| 1 | 0.097 | 0.166 | 0.14 | 0.158 | 0.239 | 0.258 | 0.32 |
| 2 | 0.186 | 0.211 | 0.229 | 0.275 | 0.225 | 0.263 | 0.369 |
| 3 | 0.164 | 0.143 | 0.208 | 0.231 | 0.182 | 0.252 | 0.359 |

Table 10 shows the example of data obtained by measuring the optical density in wells of a 96-well plate. The wells 1–6 correspond to the two-fold dilutions of the toxicant; in well 1 the concentration is maximal, and in well 6 it is minimal. The rows are triplicated. We need to find the average for each well. To do this, you can use the standard AVERAGE function in Microsoft Excel or calculate manually (Table 11).

**Table 11.** Average values of optical density based on Table 10

| Sample | Well 1 | Well 2 | Well 3 | Well 4 | Well 5 | Well 6 | Control |
|---|---|---|---|---|---|---|---|
| Average value | 0.149 | 0.173 | 0.192 | 0.221 | 0.215 | 0.257 | 0.349 |

Each MTT test must be repeated several times. Usually, 3–5 repetitions are sufficient. The result is the following dataset shown in Table 12.

**Table 12.** Average values of optical density obtained from three repetitions of the experiment.

| # | **Well 1** | **Well 2** | **Well 3** | **Well 4** | **Well 5** | **Well 6** | **Control** |
|---|---|---|---|---|---|---|---|
| 1-st exp | 0.149 | 0.173 | 0.192 | 0.221 | 0.215 | 0.257 | 0.349 |
| 2-nd exp | 0.134 | 0.181 | 0.198 | 0.191 | 0.225 | 0.277 | 0.370 |
| 3-rd exp | 0.159 | 0.175 | 0.189 | 0.211 | 0.200 | 0.259 | 0.365 |

There is no need to plot the graph to analyze the data statistically. Our task is to compare the obtained data in pairs. We need to establish whether the optical density values differ significantly between experimental and control wells, as well as between wells 1 and 2, 2 and 3, etc. If the values differ significantly, it means that substance concentrations in wells, for example, 1 and 2, have a different effect on the cells.

At first, you need to calculate three values: the arithmetic mean *M*, standard deviation σ, and the number of experiments performed *n* (in our case, n = 3).

The arithmetic mean is the sum of values divided by their number:

$$M_x = \frac{\sum x_i}{n}$$

In our case, we have to sum up three values for well 1 and three for well 2 and divide each resulting number by 3. For well 1 $M_1 = 0.147$, for well 2 – $M_2 = 0.176$.

Now, let us calculate the standard deviation. The standard deviation is the estimation of a random variable $x_i$ relative to its mathematical expectation $M_x$ based on an unbiased estimate of its variance $D_x$:

$$\sigma_x = \sqrt{D_x} = \sqrt{\frac{\sum (x_i - M_x)^2}{n-1}}$$

For example, for well 1, it is:

$$\sigma_1 = \sqrt{\frac{(0{,}149 - 0{,}147)^2 + (0{,}134 - 0{,}147)^2 + (0{,}159 - 0{,}147)^2}{3-1}} = 0.0125$$

For well 2, the $\sigma_2 = 0.0041$.

The formula for calculating Student's t-criterion:

$$t_e = \frac{|M_1 - M_2|}{\sqrt{\frac{\sigma_1^2}{N_1} + \frac{\sigma_2^2}{N_2}}}$$

$$t = \frac{|0.147 - 0.176|}{\sqrt{\frac{0.0125^2}{3} + \frac{0.0041^2}{3}}} = \frac{0.029}{0.0076} = 3.81$$

Now we need to calculate the degrees of freedom *df*. The degrees of freedom *df* is the number of values that can freely change after the statistic value has been calculated. To understand what the degrees of freedom is, here is an example.

One can pick a set of numbers that have a mean (average) of 10. These sets can be: 9, 10, 11 or 8, 10, 12 or 5, 10, 15. However, once you have chosen the first two numbers in the set, the third is fixed. In other words, you cannot choose the third item in the set. The only numbers that are free to vary are the first two. You can pick 9 + 10 or 5 + 15, but once you have decided, you must choose a particular number that will give you the mean you are looking for. Thus, the degrees of freedom for a set of three numbers is two.

In our case, we have two data sets with three values in each, so degrees of freedom well be:

$$df = (3-1) + (3-1) = 4$$

Now it is necessary to refer to the table of the critical values of the Student's test (t-test), which can be found on the Internet or in the literature. For a degree of freedom equal to 4, we find that our value of 3.81 is greater than 2.776, so the significance level is less than 0.05.

This means that there is a statistical difference between the values, and the concentrations of toxicants in wells 1 and 2 have different effects on the cells. Likewise, it is necessary to calculate the criterion for each pair of wells sequentially. ***The lower the level of significance, the better.***

When analyzing the number of living and dead cells using dyes, which stain specifically either live or dead cells, you need to proceed in the same way. Suppose there are wells with different concentrations of toxicants. We get three images from each well and find the mean. The data obtained correspond to the data in Table 13.

The subsequent analysis is not difficult and is carried out by analogy.

**Table 13.** Results of cells counting

| Row | Number of the alive cells | Number of the dead cells | Total number of the cells | Live/dead ratio,% |
|---|---|---|---|---|
| 1 | 723 | 381 | 1104 | 65% |
| 2 | 685 | 360 | 1045 | 65% |
| 3 | 751 | 330 | 1081 | 69% |

Based on the data obtained, different conclusions can be drawn. Let us look at an example. According to the metabolic activity graph, based on Table 12 values, we found that it crosses the 50% metabolic activity at OD = 0.192 in well 3. However, the value in the next well 4 OD = 0.221 does not differ significantly from the value in well 3 (Figure 7).

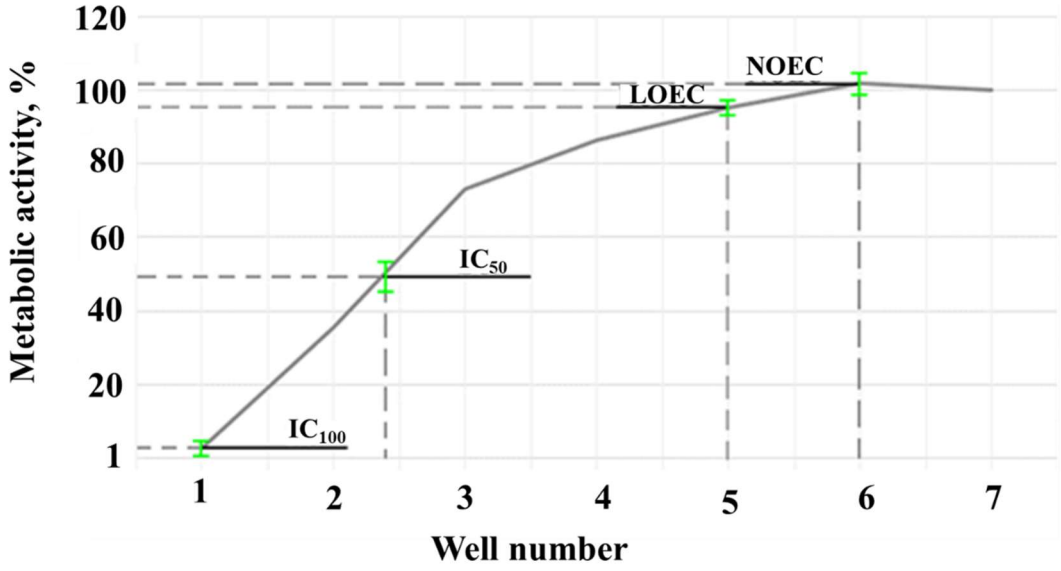



**Figure 8.** Example of the MTT assay results. The graph shows the main toxicometric characteristics. $IC_{100}$ – concentration of toxicant, which leads to a 100% death of cells. $IC_{50}$ – concentration of toxicant, which leads to a 50% death of cells. LOEC – lowest observed effect concentration; concentration of the smallest toxic effect. NOEC – no observed effect concentration; smallest concentration which causes no toxic effect. The control is in well 7. **The green color bars indicate 3σ**

Thus, we cannot say in which of these wells we reached $IC_{50}$. Sometimes it happens that the values, for example, wells 1 and 2, and 2 and 3 do not differ significantly from each other. This does not mean, however, that well 1 is no different from well 3; in this case, it is necessary to carry out an additional calculation and compare wells 1 and 3.

### *9.2. The Three Sigma Rule*

It is also worth noting another way to assess the differences between the two concentrations of toxicants. This method is associated with calculating the standard deviation, denoted by the letter σ (sigma), and is called the three sigma rule. It states that almost all values of the normally distributed random variable $x_i$ lie in the interval $(x_i - 3\sigma; x_i + 3\sigma)$. If we want to describe it more strictly, with approximately a probability of 0.9973, we define that the value of the normally distributed random variable $x_i$ lies in the indicated interval.

In Figure 8, the sections $(x_i-3\sigma; x_i + 3\sigma)$ for the values of all the main toxicometric characteristics are indicated as green bars. If the 3σ values for two adjacent measured values do not overlap, this means that the values differ significantly. If the 3σ overlap, then it cannot be said with certainty that the concentrations of toxicants have different effects on cells. To establish the slight differences between the effects of various concentrations of toxicants, it is possible to increase the number of experiments to decrease the value of σ (i.e., to clarify the value of metabolic activity at a given concentration of the toxicant) or to increase the incubation time to make the effect of the toxicant more pronounced.

### *9.3. One Way ANOVA*

Considering our above example of analysing MTT results, we can assume that the toxicants will affect the cell viability or will not. These statements are called Hypotheses. The ANOVA uses a Null hypothesis and an Alternate hypothesis. The Null hypothesis in ANOVA is valid when all

the sample means are equal, not significantly different. Thus, they can be considered as a part of a larger set of the same results.

On the other hand, the alternate hypothesis is valid when at least one of the sample means is different from the rest of the sample means. So, the alternate hypothesis states that at least one of the sample means different from another. However, we still cannot tell which one specifically. For that, we need to use other methods.

Let us return to the example with MTT-test mentioned above. For example, we assume that the toxicants' maximal concentration will kill more cells than minimal (well 1 and well 6, respectively). At the same time, the control demonstrates no influence of the toxicants on cells growth. But what if the chosen substances are not toxic?

To figure this out, we decided to implement it on a smaller group of randomly selected optical density values from three different wells (Table 14). Now, we will calculate the means and the Grand mean.

**Table 14.** The optical density of the samples obtained during the MTT test

| | 1st exp | 2nd exp | 3rd exp | **Mean** |
|---|---|---|---|---|
| **Control** | 0.32 | 0.369 | 0.359 | 0.349 |
| **Well 1** | 0.097 | 0.186 | 0.164 | 0.149 |
| **Well 6** | 0.258 | 0.263 | 0.252 | 0.257 |
| | | | **Grand mean** | 0.252 |

Looking at the above table, we might assume that the mean score of cells from the control well is definitely greater than the other two wells, so the treatment produces the effect. Maybe it is true, but there is also a slight chance that we have selected the best results from the control well, which resulted in bigger cell density values (remember, the selection was made randomly). This method does not take into account the following questions:

- How do we decide that these three results performed differently because of the different situations and not merely by chance?
- In a statistical sense, how different are these three samples from each other?
- What is the probability of cells from the control well having cell viability actually different from the other two wells?

To answer all these questions, first, we will calculate the f-statistic, which can be expressed as the ratio of Between Group variability and Within Group Variability.

A one-way ANOVA tells us that at least two groups are different from each other. However, it does not show which groups are different. If our test returns a significant f-statistic, we may need to run a post-hoc test to tell us exactly which groups have a difference in means.

## *9.4. Steps to perform one-way ANOVA with post hoc test in Excel 2013*

**1.** Put your data into columns or rows in Excel. For example, if three wells with cells are tested, spread the data into three columns (Table 15).

**Table 15.** An example of results table plot for ANOVA analysis

| **Repetition** | **Well 1** | **Well 2** | **Well 3** | **Well 4** | **Well 5** | **Well 6** | **Control** |
|---|---|---|---|---|---|---|---|
| 1-st exp | 0.149 | 0.173 | 0.192 | 0.221 | 0.215 | 0.257 | 0.349 |
| 2-nd exp | 0.134 | 0.181 | 0.198 | 0.191 | 0.225 | 0.277 | 0.370 |
| 3-rd exp | 0.159 | 0.175 | 0.189 | 0.211 | 0.200 | 0.259 | 0.365 |

**2.** Click the “Data” tab and then click “Data Analysis.” If you do not see Data Analysis, load the “Data Analysis Toolpak” add-in.

**3.** Click “ANOVA Single Factor” and then click “OK.”

**4.** Type an input range into the Input Range box. For example, if the data is in cells A1 to C10, type “A1:C10” into the box. Check the “Labels in first row” if we have column headers, and select the Rows radio button if the data is in rows.

**5.** Select an output range. For example, click the “New Worksheet” radio button.

**6.** Choose an alpha level. For most hypothesis tests, 0.05 is standard.

**7.** Click “OK.” The results from ANOVA will appear in the worksheet.

Results for our example look like this (Figure 9):

| | A | B | C | D | E | F | G |
|---|---|---|---|---|---|---|---|
| 1 | ANOVA: Single Factor | | | | | | |
| 2 | | | | | | | |
| 3 | Results | | | | | | |
| 4 | *Groups* | *Count* | *Sum* | *Average* | *Variance* | | |
| 5 | Well 1 | 3 | 0,442 | 0,1473333 | 0,000158333 | | |
| 6 | Well 2 | 3 | 0,529 | 0,1763333 | 1,73333E-05 | | |
| 7 | Well 3 | 3 | 0,579 | 0,193 | 0,000021 | | |
| 8 | Well 4 | 3 | 0,623 | 0,2076667 | 0,000233333 | | |
| 9 | Well 5 | 3 | 0,64 | 0,2133333 | 0,000158333 | | |
| 10 | Well 6 | 3 | 0,793 | 0,2643333 | 0,000121333 | | |
| 11 | Control | 3 | 1,084 | 0,3613333 | 0,000120333 | | |
| 12 | | | | | | | |
| 13 | | | | | | | |
| 14 | ANOVA | | | | | | |
| 15 | *Source of variation* | *SS* | *df* | *MS* | *F* | *P-Value* | *F crit* |
| 16 | Between groups | 0,089926667 | 6 | 0,0149878 | 126,4029451 | 2,24052E-11 | 2,847725996 |
| 17 | Inside groups | 0,00166 | 14 | 0,0001186 | | | |
| 18 | | | | | | | |
| 19 | Total | 0,091586667 | 20 | | | | |

**Figure 9.** ANOVA results

Here, we can see that the F-value is greater than the F-critical value for the alpha level selected (0.05). Therefore, we have evidence to reject the null hypothesis and say that at least one of the three wells has significantly different means and thus belongs to an entirely different cell density.

# 8. References

1. Скибо, Ю.В. Методы исследования программируемой клеточной гибели: учеб.-метод. пособие для магистров по курсу «Теория апоптоза» / Ю.В. Скибо, З.И. Абрамова. – Казань: Изд-во ФГАОУ ВПО КФУ, 2011. – 61 с.; ил.

2. Klionsky, J.D. Guidelines for the use and interpretation of assays for monitoring autophagy / D. J. Klionsky et al. // Autophagy. – 2012. – Vol. 8, No 4. – P. 445–544.

3. Johnston, H.J. A review of the in vivo and in vitro toxicity of silver and gold particulates: particle attributes and biological mechanisms responsible for the observed toxicity / H.J. Johnston, G. Hutchison, F.M. Christensen, S. Peters, S. Hankin, V. Stone // Crit. Rev. Toxicol. – 2010. – Vol. 40, No 4. – P. 328–346.

4. URL: http://rosmedbio.ru/.

5. URL: http://www.biolot.ru/catalogue/Cell_lines/.

6. URL: http://www.cytspb.rssi.ru/.

7. URL: http://www.ronc.ru/node/1084.

8. Niks, M. Towards an optimized MTT-assay / M. Niks, M. Otto // J. Immunol. Meth. – 1990. – Vol. 130, No 1. – P. 149-151.

9. URL: https://www.thermofisher.com/order/catalog/product/C7025.

10. URL: http://www.sigmaaldrich.com/catalog/product/sigma/p4083?lang=en&region=RU.

11. Fabrikanos, V.A. Stabiler Hydrosole von. Gold 22 und Silber durch Reduktion mit Äthylendiamintetraessigsaure / V. A. Fabrikanos, S. Athanassio, K. H. Darstellung // Z. Naturforschg. – 1963. – Vol. 18b. – P. 612–617.

12. Austin, L.A. Cytotoxic effects of cytoplasmic-targeted and nuclear-targeted gold and silver nanoparticles in HSC-3 cells – a mechanistic study / L. A. Austin, S. Ahmad, B. Kang, K. R. Rommel, M. Mahmoud, M. E. Peek, M. A. El-Sayed // Toxicol. In Vitro. – 2015. – Vol. 29, No 4. – P. 694-705.

13. URL: http://bitesizebio.com/8970/how-to-make-your-own-ecl/ – Date: 13.03.2021.

14. URL: https://www.analyticsvidhya.com/blog/2018/01/anova-analysis-of-variance/. – Date: 13.03.2021.

14. Montgomery, Douglas C., and George C. Runger. Applied statistics and probability for engineers. John Wiley & Sons, 2010.

Fakhardo Anna Fabiovna
Ulasevich Svetlana Alexandrovna
Prilepskii Artur Yur'evich
Vinogradov Vladimir Valentinovich

# Research methods in molecular and cell biology

**Study guide**

In the author's edition
Editorial and Publishing Department of ITMO University
RIO director N.F. Gusarova
Signed to print
Order no.
Circulation
Printed on a risograph

**Editorial and publishing department**
**ITMO University**
197101, St. Petersburg, Kronverksky pr., 49, letter A