Cell and tissue culture work

o BASIC RULES

Before you start working in a tissue culture lab you must realize where you‘re going to work. The basic and the most important rule is to keep your **work STERILE** and **GERM-FREE**. Just that will save quite a lot of cells and experiments – it's not only matter of infections, but also involuntary distorted results. That's something you really should realise when you work with tissue cultures.

1. **Always keep your hands and everything you work with clean –** before opening an incubator, touching plates with cells, before you start working in a flow-box, spray your hands and forearms with **70% ethanol** (it’s usually in a wash bottle, if it's empty, you should mix it – take the brown bottle with 2 yellow marks on it - fill with **denatured ethanol** from the stock till the first mark and **add destilled water** till the second mark). Wearing lab gloves is also a good idea, it can often save your health, so take care of you...

2. Everything you want to take inside the flow box has to be washed with **70% ethanol** as well.

3. Your clothes shouldn’t be inside the flow box - if you have jumper, roll up the sleeves. And you have to wear softie lab shoes! **No outside shoes in the tissue culture lab.**

4. If you take the last aliquot, bottle or tube... of anything, you are obliged to get new ones. That means - take a new bottle of **serum from -80°C or trypsin, P/S, L-G from -20°C,** let it thaw, aliquot it and store properly (-20°C freezer in the tissue culture lab). It's important to label the reagents - mark the contents and date of preparation. This rule is valid in all the lab! You should learn to think in advance and never forget that you are not the only one person working in the lab. Try to watch how much reagents we use (mediums, serum, trypsin, enzymes, antibodies, acrylamide, protein markers...). It's a big problem if we run out of some basic reagent, it may take up to 3 weeks before we get a new supply from the company. So if you finish or almost finish our supply of some chemicals, let somebody know about it!!! Tell your supervisor - there might be some stocks or aliquots you don't know about - or ask directly Paja or James to order it. Remember it all takes time and many people's work depend on you. Paja and James unfortunately cannot notice everything. So check regularly the chemicals you work with, it will save us a lot of trouble :-)

5. **Work inside the flowbox (more info follows on next pages)** - learn to work sterile, learn to organise you work... that's something you should learn as a first thing from your supervisor. Just basic rules - flow-boxes are cleaned properly once a month, meanwhile you should keep it clean and sterile. That means - always wash the working surface with 70% ethanol after you finish your work, clean the sucking pipette with ethanol, throw away all contaminated pipettes. The sucking pipette should be turned off when you don´t need it, and in time of use try to not throttle it so the engine stays fine longer. If you reuse pipettes for medium or PBS..., label them with date and your name. Close the incubators if nobody's going to work after you. Don't let it run all the day, it get's damn hot in the lab. That's not comfortable for you and it might also influence your cells! If the bottle of the sucking machine is ful, add some SAVO in the liquid, throw the waste in the sink. Then add some SAVO to the empty bottle and put it back.

If you finish a bottle of PBS, sterile MQ water, gelatine or box of pipette tips, wash it in the sink (not the box for pipette tips :-D) and let dry next to the sink. James or Hanka will gather it for autoclaving, but it's not their duty to wash every bottle after everyone! You should wash everythnig you finish yourself! Extra boxes with tips, sterile eppendorfs, culturing plates or solutions are stored in the room

next to the tissue culture lab. Only 15cm plates are always in a paper box in the hall (just outside the tissue lab).

6. If you have some **cells you want to discard,** kill them first with SAVO and then discard in sink (pour a lot of watter). **NEVER** discard liquids in flask, dishes or tubes in waste bin!!! When you have an empty bottle of medium, wash it properly and store for collection of dirty pipette tips (above JIPA's flow-box). Wash clean also the used tubes with screw cap and put in the box under centrifuge (not sterile - for spiders). If you need wrapping plastic bags, you'll find them in the drawer under the „bryjalab“ incubator.

7. **In case of infection**, kill all the cells with SAVO **OUTSIDE the cultivation room.** Always inform your supervizor so that we prevent mass infection.

8. Especially in the summer try to reduce the heat production by turning off the flowboxes, sucking engines, watter baths always when nobody uses them.

o FACILITIES and APPARATUSES

1. **Flow box (laminar box) -** That's place for sterile work. It's endowed with filtres (0,3 μM pores) and vertical air flow which goes through the pores –the air's filtered. Laminar box must be sterilized daily by UV lamp for 30 minutes. Optimal is every morning or in the end of the day.

- ***Working in flow box:*** After putting gloves on and hands-washing, spray with ethanol and clean also everything that you want to take inside the flow-box.

  : Don't forget to keep working sterile. That means change a tip or glass/plastic pipette always if you touch box surface or another unwanted surface.

  : Don't take pipettes out of the flow box.

  : You can use same tip for same type of cells but don't use same pipette or tip for different types of cells. Cells can stay inside or outside on surface and you can carry them over to a wrong cell culture. Keep in mind, just one bad cell can infect your culture. Your experiments depend on work with single samples, so first think about that.

  : If you dip the tip/pipette which you've used for work with cells (or a different stock) to a bottle with PBS or medium, throw the contents away immediately in the sink and take new ones. Remember that other people will work in the same flow-box and you might cause them infection/contamination and ruin their experiments Basicly change the tips always if you work with a different sample/plasmid/reagent...

  : To suck liquids (medium, PBS...) use special suction flash and tidy tip (one for the same sample or the same type of cells). If the suction bottle is full, take off the lid, empty the bottle to the basin, wash it by normal water and add a little bit SAVO (strong desinfection) into the bottle. Then put it back...

  : Don't make mess in flow box. Cell plates and plastic pipettes are in drawers under the laminar box. Open a pack of plates inside the flow box, close it by adhesive tape and return it back to drawers. There should be opened packs with tubes as well. New packs of plates, tubes and pipettes are in a different room (now next to the tissue culture room). Ask a lab assistant or anybody when you cannot find them.

  : After you finish your work in the flow box, get everything back on the right places

(pipettes to the frame, tube-frames on the left side...) and clean the surface with ethanol and a paper cloth. If you know that nobody is going to work there, turn it off and in the end of the day turn on the UV lamp for 30min.
: There is a UV lamp with a timer which is turned on during night to make sure the room is also sterile. Keep it in mind if you plan some night treatments...

2. **Incubator (with 5% CO_2) –** Optimal conditions for cultivating mammalian cells are: 37°C, 5 % CO_2 and 95 % H_2O. You can see complete information on a display. If you catch something wrong or missing, tell person who takes care of the incubator (James, Hanka, JIPA, Jirka?).

3. **Inverse light microscope –** Usually used with phase contrast. You can watch morphology, contamination, colonies of cells and also take photos of them (the camera is usually in the room next to the tissue culture in a drawer). Don't switch the microscope off after your watching, just turn the light down. The microscope should be switched off in the end of day.

4. **Fluorescent microscope** - Always ask someone to show you how to work with it if it's your first time (how to open and close the shutter, how to take pictures...). Always let run at least 15min, not shorter, but also make sure it's not on too long. Don't forget to turn it off, the lamp could get burnt... If you work with it, make note about it in the notebook.

5. **Coulter counter –** Apparatus for counting and sizing particles and cells. A typical Coulter counter has one or more microchannels that separate two chambers containing electrolyte solutions. When a particle flows through one of the microchannels, it results in the electrical resistance change of the liquid filled microchannel. This resistance change can be recorded as electric current or voltage pulses, which can be correlated to size, mobility, surface charge and concentration of particles. After your work clean the coulter counter by hemasol.

Can be easily replaced by Accuri flow-cytometer! :-) ask Jirka for instruction.

6. **Fridge and freezer -** +4°C fridge for prepared mediums, using stocks, trypsin, antibiotics, plazmids. Freezers: -20°C for aliqots, -80°C for storage stocks (sometimes cells) and plasmid bank.

7. **Centrifuge –** for centrifuging cells (mainly). Optimum for cells is: 200 g, 5 min., room temperature.

8. **H_2O basin –** for warming up stocks and mediums when you work with cells. Optimal is 37°C. Distilled water within has to be changed every two weeks. It's necessary to add stock of pentahydrate copper(II) sulfate ("modrá skalice").

9. **Useful facilities:**

- The cells bank (liquid nitrogen, -196°C; -80°C freezer)
- Normal basin to wash glass and so on

- Sterilizer room - sterilizator, autoclave there - ask James, Hanka or Paja if you need something sterile... with a new autoclave it will be much easier and basicly anyone will be able to autoclave their things if necessary.

- CELL WORK

1. Introduction

In these days we know lots of cell lines and various types of their cultivations. We can work with primo-cultures or permanent (normal, cancerous) cultures. They both can be adherent or in suspension. In our lab, cells are cultivated on plastic plates (opened system). Plates are distinguished according to size of breadth (see *tab 1* below). Generally best conditions for cultivation of mammalian cells are:

37°C, 95 % H_2O, 5 % CO_2.

- MEDIUM

Cells are cultivated in medium. For different types of cells you may need differnt media (just have a look at some online protocols on web). Anyway, each medium has to contain: **water**, **dissolved inorganic salts** (salts are source of ions and ensure suitable pH (7.2 - 7.4); basic ions present in medium: **Na^+, K^+, Mg^{2+}, Ca^{2+}, Cl^-, SO_4^{2-}, PO_4^{3-}, HCO_3^-**), **saccharides** (mainly glucose, the source of energy**), amino acids**, **vitamins** and **trace elements**. That is called "minimal medium addition". Most of mammalian cells also need **insulin**, **selen** and **transferin**.

The other complements in medium can be lipids, hormones, peptides, cytokines, proteins, ECM, nucleosides, serum proteins etc. Majority of them is supplied by adding serum to medium (various sources: **fetal bovine/calf serum**, goatish, murine, embryonal….).

- POSSIBLE INFECTIONS

Quite usual is addition of antibiotics to medium. That is because of danger of heterogenous infections, including mycoplasmas (endogenous bacteria). In our lab basic useful antibiotics are **penicillin/streptomycin** and **sparfloxacin** (against mycoplasmas). If you find infection in your culture, the best solution is to throw the whole plate away. If you have a rare cell line, you can try to treat them with higher dose of antibiotics. Injurious infection in cell culture usually means: bacteria, yeasts or fungi.

2. Cultivation of cells –

We usually cultivate proliferating cells (that isn't related to postmitotic or terminal differentiated cells). Proliferating cells need to be splitted on regular base, because you have limited area for growing populations in plates. Defection of free place can be reason for **arrest of proliferation and increased apoptosis**. If the whole area of the plate is covered by cells, we say that the culture has 100% of confluence. In this point the culture needs to be splitted necessarily. **Splitting means periodical "dilution" of cells connected to change of medium**. If cells don't grow, it's good to change medium every third day because of nutritions (if medium gets to change color from red/pink to yellow, do it surely – phenol red is a marker of pH in DMEM medium and yellow shows acidity). Note each splitting of your culture and write total number on plates. After 20-30 splitings culture starts to change their charachteristics and obtain new mutations. **Therefore, freeze your cells till 20th splitting, that will be your own source of cells.** Always work with cells in a flow-box.

a) Adherent cells – mechanical or enzymatic splitting of cell-cell and cell-substrate attachments

- Enzymes: **trypsin** (it's inactivated by serum), colagenase

b) Suspension culture – single dilution

- **!!!** Remember, always work with cells **quickly** and **softly**, cells can be **VERY sensitive**, and brutal manipulation can kill them or change their abilities to grow. Leave plates with cells out of the incubator only for a necessary time. Simply, don't forget that you work with **live dynamic system** – everything can induce unknow (and unwanted) activities and changes in your cell and tissue cultures *in vitro* **!!!**

PROTOCOLS

3. Preparation of medium (in general) – add 50 ml bovine serum (FBS, 10%), 5 ml penicilin-streptomycin (P/S, 5%) and 5 ml glutamin (L-glutamin, 5%) to 500 ml DMEM/RPMI. DMEM is for adherent cells and RPMI for cell suspension culture.

- These mediums are used for many types of cells, but not eg. for stem cells. So always ask somebody in the lab or check optimal frame of your wanted medium on web.

- To limit mycoplasma infection add sparfloxacin to plates with cells from time to time. Its dilution should be 1000x, that means add 10 µl 1000x sparfloxacin to 10 ml of medium. Don't add sparfloxacin just after thawing cells, wait for first splitting.

4. Splitting of cells – Keep the rules which were defined previously (sterile working in the flow-box, warming the medium and trypsin up....). Get plates into the flow-box.

- Drain medium by a tip placed on suction bottle. Wash the cells by PBS, drain PBS, add trypsin. The volume of PBS is like volume of medium (e.g. if you have cells on the 10 cm plates, you will use 10 ml of PBS). The recommended **volumes of medium/PBS** and **trypsin** are showed in the ***table 1***.

- Wait approximately 5 min (it depends on cells,– cells can't be affixed, you need to have suspension of cells and trypsin, you should check it by the microscope).

- Recline the plate and wash its surface with the mixture cells + trypsin. Resuspend cells carefully. If you need to split cells to next cultivations in the same volume, pipette the requested amount to the new plate with warm medium (take usually 1/3 of cells). If you need all volume of splitted cells, prepare falcon with medium, shift trypsined cells, wash the surface of plate by medium, add this to the falcon. Resuspend compound by pipetting 10 times. Separate compound to plates/wells.

- Remember, if you prepare cells for transfection, final confluence in plates/wells should be minimally 50%.

5. Freezing of cells – Trypsinize cells as described above.. Pipette cell suspension with trypsin to the falcon tube with media. To inactivate 1 ml of trypsin add 1 ml of FBS. Centrifuge falcons (1200 rpm(200 g)/5 min). During splitting/before (it can be stored in the fridge) prepare medium for freezing: for 10 ml - 7 ml DMEM/RPMI (70%), 2 ml FBS (20%) and 1 ml of DMSO (10%). DMSO acts as the antifreezer agent. Drain supernatant, if you used 1 ml of trypsin, use 1 ml of

freezing medium. Resuspend it. Add 500 µl to each frozen vial. It has to be written on vial: date, name of cells, passage, who frozen the cells. It is suggested to freeze cells with 80% confluence. Put the vials into Mr. Frosty or a polystyrene box and get it first to -80°C and the second day transfer them to liquid nitrogen (or you can move them into your own box in -80°C).

	growth surface per unit	growth surface	suggested TRYPSIN volume	suggested MEDIUM volume	minimal volume for MEDIUM
plates					
15 cm	147.8 cm^2	147.8 cm^2	3 ml	15 ml	
10 cm	60.1 cm^2	60.1 cm^2	1 ml	10 ml	4 ml
6 cm	22.1 cm^2	22.1 cm^2	0.5 ml	5 ml	2 ml
4 cm	9.2 cm^2	9.2 cm^2	0.4 ml	3 ml	1 ml
96 well	0.31 cm^2	29,76	50 µl	150 µl	
24 well	1.91 cm^2	45,84 cm^2	100 µl	0.5 ml	200 µl
12 well	3.66 cm^2	43,92 cm^2	200 µl	1 ml	400 µl
6 well	9.03 cm^2	54,18 cm^2	0.3 ml	3 ml	1 ml
culture flaks					
25 cm^2	25 cm^2	25 cm^2	0.5 ml	5 ml	
75 cm^2	75 cm^2	75 cm^2	2 ml	20 ml	

6. Thawing of cells – cells are placed in liquid nitrogen or in -80 °C. Prepare the plate with hot (37 °C) needed medium (it depends on the type of cells), take out from liquid nitrogen or -80 °C cells and quickly put freezing tube to the thermostat and let thaw, pipette the cells to the ready plate. Keep plate in incubator approximately 2 hours, change medium (use hot medium, it is necessary to smooth away DMSO that is present in freezing medium. DMSO is toxic for cells.