Workshop part I:

Additional Lectures

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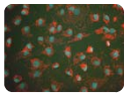
Cell Culture Workshop

Lecture:



Basic Techniques

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Basic Techniques I

Fundamental Techniques

- Design and Equipment for the Cell Culture Laboratory
- Cell Culture Medium
- Sterile Techniques and Good Cell Culture Practice
- Avoidance of Contamination

Design and Equipment for the Cell Culture Laboratory

Design of Laboratory

- quarantine area
- main cell and tissue culture area

Fundamental Equipment for Cell Culture Laboratory

Laminar Air Flows

- Laminar airflow - Horizontal
- Laminar airflow - Vertical

UV-Light

It will provide a clean working enviroment.

Fundamental Equipment for Cell Culture Laboratory

Incubators

- Possibility to keep temperature constant.
- For buffering the culture medium, a carbonite buffer is used. So, the CO_2 must be around 5% in the incubator
- The humidity must be just below 100% to avoid evaporation of the medium

Fundamental Equipment for Cell Culture Laboratory

Inverted (fluorescent) microscope

- Cell must be controlled regarding
  - contamination
  - growths rates
  - differenciation/maturation/aging

Fundamental Equipment for Cell Culture Laboratory

Autoclaves

+98°C: solutions (PBS,..)

+121°C: plasticware

+134°C: glassware

stainless instruments

Fundamental Equipment for Cell Culture Laboratory

Centrifuges

- Indicate rotational speed never in RPM, but RCF (relative centrifugal force), which is

$$RCF = r\omega 2 / g$$

R = earth's gravitational acceleration

ω = rotational radius

g = angular velocity in radians per unit time

Fundamental Equipment for Cell Culture Laboratory

Centrifuges

- Indicate rotational speed never in RPM, but RCF (relative centrifugal force), which is

$$RCR = 1.118 \times 10^{-6} r N^2$$

r = rotational radius (mm)

N = rotational speed (RPM)

Fundamental Equipment for Cell Culture Laboratory

Laboratory Refrigerators and Freezers

Refrigerator: +4°C

Freezer: -20 to -40°C

Freezer: -80°C

Fundamental Equipment for Cell Culture Laboratory

Water bath

- To warm up medium to +37°C
- For disolving different reagents (gelatine,..)
- Thawing cells

Fundamental Equipment for Cell Culture Laboratory

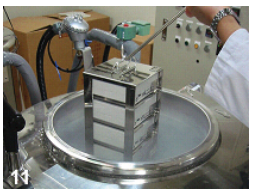
Water destiller

Deionised water should be autoclaved before use ! The use of high purity , toxin-free water is essential for successful cell culture!

Fundamental Equipment for Cell Culture Laboratory

Liquid nitrogen tank

- Freezing cells for long storage period



Fundamental Equipment for Cell Culture Laboratory

Others:

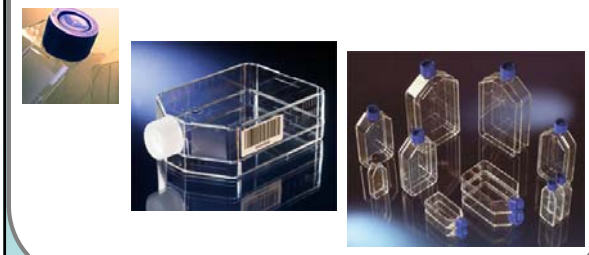
- **ph meter**
- **vacuum pump**
- .....

Fundamental Equipment for Cell Culture Laboratory

Plasticware and Consumables

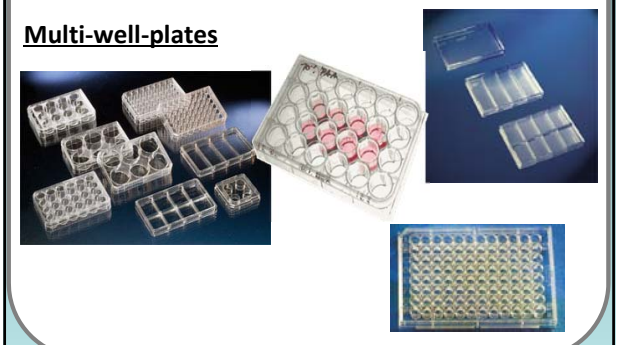
Fundamental Equipment for Cell Culture Laboratory

Cell Culture Flask



Fundamental Equipment for Cell Culture Laboratory

Multi-well-plates



Fundamental Equipment for Cell Culture Laboratory

Pipettes

- Electric pipette "Pipet boy"
- Mechanical pipettes and pipet tips

Cell Culture Media

Cell culture media

- for proliferation,
- growth enviroment
- differentiation and cell functions
- Neutralisation of waste products

Cell Culture Media

Content

- Amino acid
- Salts
- Vitamins
- Carbohydrates (glucose,glycerol…)
- Growth factors, hormones, blood serum,…

Cell Culture Media

Different types of media

- Undefined Media
  Basic or standard medium contains glucose, water and various salts
- Defined Media
  - Minimal media
    all chemicals used are known
  - Complete Media
    include serum and growth factors

Cell Culture Media

Defined media

DMEM (Dulbecco's Modified Eagle Medium)
MEM (Minimum Essential Medium)
MEM alpha (Minimum Essential Medium alpha)
RPMI 1640 (Roswell Park Memorial Institute)
Developed by Moore et al for the culture of human normal and neoplastic leukocytes

Cell Culture Media

Advantages of minimal media

- Exact composition is known
- exactly adapted to each kind of micro-organisms
- Accounting possible for individual components

Cell Culture Media

Disadvantages of minimal media

- Elaborate production
- Separate sterilization of various components required
- Heat-sensitive components must be sterilized by filtration
- Preliminary tests required for media optimization

Cell Culture Media

Types of Sera

- Fetal Calf Serum
- Newborn Calf Serum
- Calf Serum
- Horse Serum

Cell Culture Media

Fetal Calf Serum

- from blood of bovine fetuses between 3rd and about 7th gestation after slaughter
- After coagulation of blood is centrifuged
- Blood clotting is a natural way as a growth factor is released from platelets in serum
- Serum is sterile filtered through cartridge filters

Cell Culture Media

Newborn Calf Serum

Serum of newborn calves (NCP):

- from blood at 1-10 days old calves, more immunoglobulins than in FCS
- cheaper than FCS
- for some cell lines, less growth -

Cell Culture Media

Calf Serum

- can be used for short-term cultivation of relatively "unassuming" cells
- contains high levels of antibodies (gamma - globulin)

Cell Culture Media

Horse Serum (HS)

- blood obtained at slaughter
- Donor-horses = blood, which is taken again and again → ensures serum with less contamination

Cell Culture Media

Principles in the serum using

- Serum should be stored at -20 ° C, storage without quality loss of 1 years
- slow thawing to prevent precipitation of lipoproteins
- Repeated freezing and thawing reduces the growth-promoting properties
- Add serum in concentrations 1% to 25%

Cell Culture Media

Disadvantages of using serum-containing media

- Serum is not a physiological fluid
- Serum constituents can vary widely from batch to batch
- may contain inhibitors
- may contain substances that may forming cytotoxic substances with the secretions of the cells
- Disturbance of the use of cell cultures or their metabolic products
- Microorganisms can enter the cell cultures

Cell Culture Media

Reasons for Using Serum Free Media

- Avoidance of qualitative and quantitative variations of culture media components
- Working under defined and controlled conditions
- Prevention of microbial contamination
- Cell products can be more easily isolated

Steril Techniques and Good Cell Culture Practice

- **Aim**

To ensure all cell culture procedures are performed to a standard that will prevent contamination from bacteria, fungi and mycoplasma and cross contamination with other cell lines.

Steril Technique and Good Cell Culture Practice

Procedures

Chemical	Mechanical	Physical
Ethanol	**Microfiltration**	**Humidity**
Oxidant	**Ultrafiltration**	**Dry heat**
Aldehyde...		**UV- radiation**

Steril Techniques and Good Cell Culture Practice

Important rules for working in cell culture

- 70% ethanol in water
- 1% formaldehyde based disinfectant
- Personal protective equipment (sterile gloves, laboratory coat, safety visor)
- Do not eat, drink or smoke
- Do not pipette by mouth

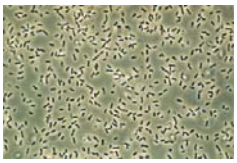
Steril Technique and Good Cell Culture Practice

Procedure:

- Sanitize the cabinet using 70% ethanol before work.
- Sanitize gloves by washing them in 70% ethanol.
- Put all materials and equipment into the cabinet prior to start working.
- Equipment in the cabinet or that which will be taken into the cabinet during cell culture procedures should be wiped with 70% ethanol prior to use.
- No Speech, sneezing and coughing.

Avoidance of Contamination

- **Microbial contamination**
- **Contamination source, identification, effects and treatment** of:
  - -bacteria
  - -mycoplasma
  - -fungi and yeast
  - -virus
- **crosscontamination**

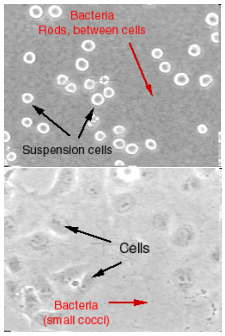


Avoidance of Contamination

Microbial contamination

three principle forms of bacteria:
- •round-shaped (cocci),
- •rod-shaped (bacilli),
- •spiral-shaped (spirilla)

Bacteria produce toxins that disrupt cell function and destroy cell cultures!



Avoidance of Contamination

Antibiotics

The most commonly used antibiotics in cell culture are penicillin/streptomycin (pen/strep)

Typically, pen/strep are used together and gentamicin and kanamycin can be used together.

as much as possible!

as much as is necessary!

Avoidance of Contamination

Mycoplasma

- a class (Mollicutes) of bacteria that lack cell walls and may cause disease, esp. of the joints and lungs, in humans and domestic animals, or may be pathogenic for plants

Avoidance of Contamination

Contamination source
- Serum
- Trypsinsolution

Effects
- Inhibition of PBS
- Decrease of cell division
- Cell necrosis
- Facilitation of virus increase

Avoidance of Contamination

Identification
- Alteration of proliferation rate
- Cavities on cell monolayers
- Agglutination of cells

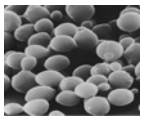
Detection
- Fluorescence (DAPI-Test)
- Electron microscopy
- PCR

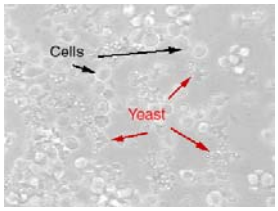
Treatment
- Autoclaving
- Mycoplasma desinfectant

Avoidance of Contamination

Fungi and yeast

- member of a large group of eukaryotic organisms that includes microorganisms such as yeasts and molds
- lack of chlorophyll
- live as parasites

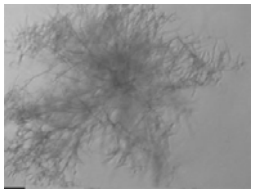




Avoidance of Contamination

Identification

- Optically easy to identyfy



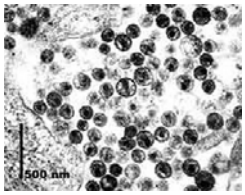
Treatment

- Antifungal agents
  - Amphotericin B
  - Nystatin

Avoidance of Contamination

Virus

Any of a large group of submicroscopic agents that act as parasites and consist of a segment of DNA or RNA surrounded by a coat of protein.



Avoidance of Contamination

Identification

- Infected cell lines
- Serum
- Trypsin

Effects

- Changes of cell morphology and cell physiology

Avoidance of Contamination

Decontamination of Cultures with Antibiotics

- When a cell culture becomes contaminated, attempt to eliminate or control the contamination.
- First, determine if the contamination is bacteria, fungus, mycoplasma, or yeast.
- Isolate the contaminated culture from other cell lines.
- Clean incubators and laminar air flows with a laboratory disinfectant, and check HEPA filters.

Avoidance of Contamination

Crosscontamination

- Mixture of cell lines
  of different donors or spezies
- Cells, which grow faster will eliminate slow growing cells

Avoidance of Contamination

- Turn on the UV lamp for 20 minutes to sterilize the surface.
- The laminar air flow should be turned on about 10-20 min before being used.
- Wipe down all surfaces with 70% ethanol before and after each use.

Basic Techniques II

Routine methods

- Media change of
  - Adherent Cells
  - Suspension Cells
- Subculture of
  - Adherent Cells
  - Suspension Cells
- Cell Quantification
- Storage, Cryopreservation of Cells

Media Change

Why?

"Feeding"

Creating optimum growing conditions for the cells

Components of the medium are metabolized or broken

→ acidification of the medium

-visible color change

Media Change of Adherent Cells

Before and after each medium change:

View cultures using an inverted microscope

2 options:

Change of complete medium or

Part of the medium

Removal of the old medium, either by tilting or aspiration

Media Change of Adherent Cells

Procedure

1. To change media, warm up fresh culture media at 37°C in water bath or incubator for at least 30 min.
2. Carefully pour of the media from the flask into a waste pot containing some disinfectant.
3. Immediately replace the media with 50 ml of fresh pre-warmed culture media and return to CO2 37°C incubator.

Media Change of Adherent Cells

Frequency

- Dependent from cell line metabolism, growth rate
- usually every 2-3 days
- also depends on the experimental design

Media Change of Suspension Cells

Rather unusual

If necessary:
Add new medium
Aspirate part of the suspension cells

→ better to subculture suspension cells

Subculture of Adherent Cells

Adherent cells must be detached from the bottom of the flask!

Several possibilities:

- Trypsin, Trypsin/EDTA
- Shake off
- Other enzymes
- Scraping

Subculture of Adherent Cells

Procedure

1. View cultures using an inverted microscope to assess the degree of confluency and confirm the absence of bacterial and fungal contaminants.
2. Remove old medium.
3. Wash the cell monolayer with PBS without Ca^{2+}/Mg^{2+}.
4. Pipette trypsin/EDTA onto the washed cell monolayer using 1ml per 25cm2 of surface area.
5. Rotate flask to cover the monolayer with trypsin.

Subculture of Adherent Cells

6. Examine the cells to ensure that all the cells are detached and floating.
7. Resuspend the cells in a small volume of fresh serum-containing medium to inactivate the trypsin.
8. Transfer the required number of cells to a new labeled flask containing pre-warmed medium.
9. Incubate as appropriate for the cell line.
10. Repeat this process as demanded by the growth characteristics of the cell line.

Subculture of Adherent Cells

Shake off - cells are brought by tapping and rinsing in suspension

1. Beat vigorously with your fingers on the underside of the culture flask or rinse well
2. Transfer cell suspension to new culture flasks (cell number!) and fill with medium or
3. Centrifuge the cell suspension and resuspend the pellet with new medium to spread to new culture flasks

Subculture of Adherent Cells

Other enzymes

Collagenase, Dispase, pronase

Gentle subculture of particularly sensitive cells

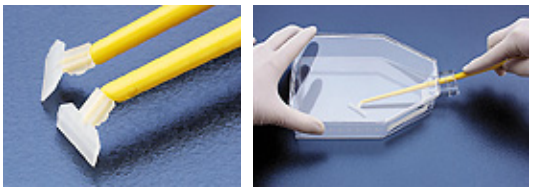
! Addition of serum does not stop activity!
→ enzymes must be separated by centrifugation

Subculture of Adherent Cells

Scraping - mechanical dissociation

1. Remove old medium
2. wash with PBS
3. gently scrape with a rubber scraper
4. Mix to dissolve cell clumps
5. Transfer cell suspension to new culture flasks (cell number!) and fill with medium or
6. After centrifugation add new medium to spread to new culture flasks

Subculture of Adherent Cells



Subculture of Suspension Cells

Procedure

1. View cultures using an inverted phase contrast microscope. Cells growing in exponential growth phase should be bright, round and refractile.
2. Do not centrifuge to subculture unless the pH of the medium is acidic (phenol red = yellow) which indicates the cells have overgrown and may not recover. If this is so, centrifuge at 1280rpm for 6 minutes, re-seed at a slightly higher cell density.

Subculture of Suspension Cells

3. Take a small sample of the cells from the cell suspension.
4. Calculate cells/ml and re-seed the desired number of cells into freshly prepared flasks without centrifugation just by diluting the cells. The data sheet will give the recommended seeding densities.
5. Repeat this every 2-3 days.

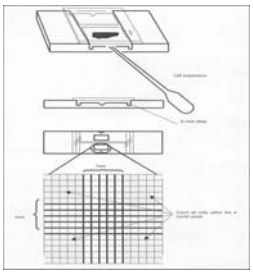
Cell Quantification

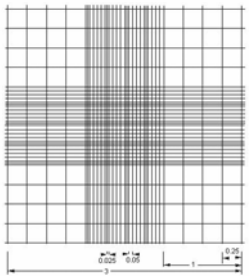
Cell number determination

- Counting chamber (hemocytometer)
- Electronic cell counter

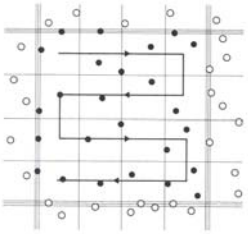
Cell Quantification

Cell Quantification





Cell Quantification



Cell Quantification

Procedure

1. Bring adherent cells into suspension using trypsin/EDTA as above and resuspend in a volume of fresh medium. For cells that grow in clumps centrifuge and resuspend in a small volume and gently pipette to break up clumps.
2. Under sterile conditions remove 10-20µL of cell suspension.

Cell Quantification

4. Add an equal volume of Trypan Blue (dilution factor =2) and mix by gentle pipetting.
5. Slide the cover-slip over the chamber back and forth using slight pressure until Newton's refraction rings appear.
6. Fill both sides of the chamber (approx. 10µL) with cell suspension and view under a light microscope using x20 magnification.

Cell Quantification

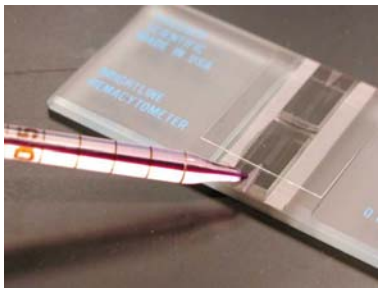
6. Count the number of viable (seen as bright cells) and non-viable cells (stained blue) - Ideally >100 cells should be counted in order to increase the accuracy of the cell count. Note the number of squares counted to obtain your count of >100.
7. Calculate the concentration of viable and non-viable cells and the percentage of viable cells.

Cell Quantification

Trypan blue staining

- Trypan blue is a vital stain used to selectively colour dead cells blue.
- Trypan blue dye is an acid whose anion binds to cellular proteins.
- It penetrates through broken cell membranes of dead cells into the cytosol and turns these cells blue
- Living cells appear bright light

Cell Quantification



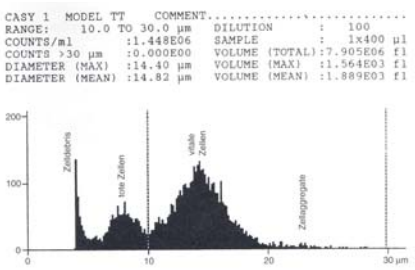
Cell Quantification

Electronic Cell Counter

Measurement of:

- cell number
- cell size
- cell aggregation
- viability of the cells

Cell Quantification



Storage

- For a short time at -80 ° C
- For years, at -196 ° C in liquid nitrogen
  ! Protective substances: glycerol and DMSO
  → prevent crystal formation inside and outside the cell, and dehydration of the cytoplasm
  ! Controlled freezing and rapid thawing is important

Resuscitation of Frozen Cells

Procedure

1. Thaw vial quickly in a 37°C water bath.
2. Immediately transfer cell suspension to a sterile, 15 ml centrifuge tube and dilute with appropriate media.
3. Spin cells down at ~1200 rpm for 10 mins, 4°C.

Resuscitation of Frozen Cells

4. Resuspend cells in 10 mls of media and count viability.
5. Spin cells down: ~1200 rpm, 10 mins, 4°C.
6. Resuspend pellet in appropriate volume of media.
7. Seed cell suspension into flasks.

Cryopreservation of Cells

Procedure

1. View cultures using an inverted microscope to assess the degree of cell density and confirm the absence of bacterial and fungal contaminants.
2. Bring adherent cells into suspension using trypsin/EDTA as above and re-suspend in a volume of fresh medium at least equivalent to the volume of trypsin. Suspension cell lines can be used directly.

Cryopreservation of Cells

3. Remove a small aliquot of cells (100-200uL) and perform a cell count. Ideally the cell viability should be in excess of 90% in order to achieve a good recovery after freezing.
4. Centrifuge the remaining culture at 1280 rpm for 6 minutes.
5. Re-suspend cells at a concentration of 2-4x106 cells per ml in freeze medium.
6. Pipette 1ml aliquots of cells into cyroprotective ampules that have been labeled with the cell line name, passage number, cell concentration and date.

Cryopreservation of Cells

7. Place ampules inside a passive freezer e.g. Nalgene Mr. Frosty.
8. Fill freezer with isopropyl alcohol and place at –80ºC overnight.
9. Frozen ampules should be transferred to the vapor phase of a liquid nitrogen storage vessel and the locations recorded.

Cell Culture Workshop

Lecture:

Testing of Toxic Effects in Cell Lines

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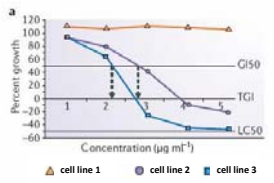
Tissue Cultures in Screening for Tocicity or putative drugs

Screening results of compounds includes three parameters

- **GI50 =**
50% growth inhibition (usually use -log(GI50))

- **TGI =**
total growth Inhibition

- **LC50 =**
50% lethal concentration



Tissue Cultures in Screening for Tocicity or putative drugs

Screening results of drugs or antagonists includes two parameters

- **EC50** = half maximal effective concentration

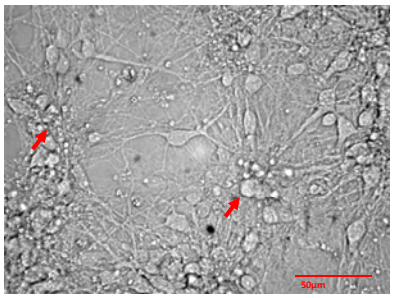
concentration of a drug, antibody or toxicant which induces a response halfway between the baseline and maximum after some specified exposure time. It is commonly used as a measure of drug's potency

- **IC50** = half maximal inhibitory concentration (IC50)

The is a measure of the effectiveness of a compound in inhibiting biological or biochemical function. This quantitative measure indicates how much of a particular drug or other substance (inhibitor) is needed to inhibit a given biological process (or component of a process, i.e. an enzyme, cell, cell receptor or microorganism) by half. It is commonly used as a measure of antagonist drug potency in pharmacological research

Markers for Cell Degeneration

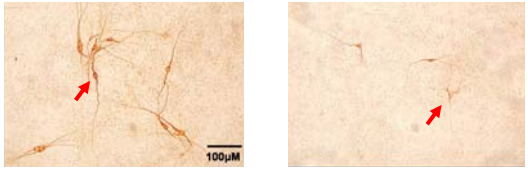
Morphology



Mesencephalic culture

Markers for Cell Degeneration

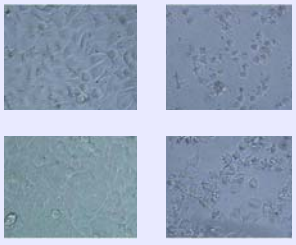
Morphology



Dopaminergic Neurons

Markers for Cell Degeneration

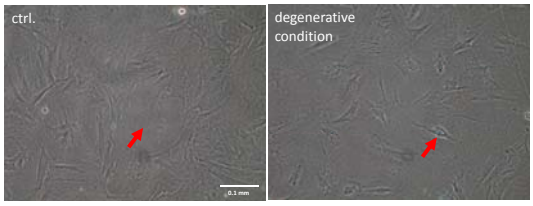
Morphology



HeLa Cells

Markers for Cell Degeneration

Morphology



Fibroblast Culture on 12DIV

Markers for Cell Degeneration

Cell Count

- hemocytometer

cell lines

Markers for Cell Degeneration

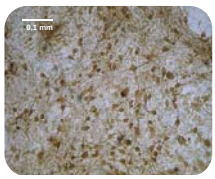
Cell Count

- hemocytometer

cell lines

- inside wells

adherent cells using light microscope (phase contrast and grid, cell counter, …)



Markers for Cell Degeneration

Cell Count

- hemocytometer

cell lines

- inside wells

adherent cells using light microscope (phase contrast and grid, cell counter, …)

- densitometrically

adherent cells using fluorescence microscope (live/dead kit, MTT assay, crystal violet for DNA,…)

- others

dyes for cell nuclei, FACS, etc.

Markers for Cell Degeneration

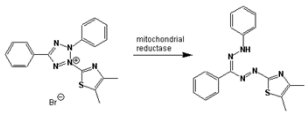
MTT Assay

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

- standard colorimetric assay

- measuring the activity of enzymes that reduce MTT + phenazine methosulfate to formazan (purple colour)

- a solubilization solution (DMSO) is added to dissolve the insoluble purple formazan product into a colored solution



Markers for Cell Degeneration

MTT Assay

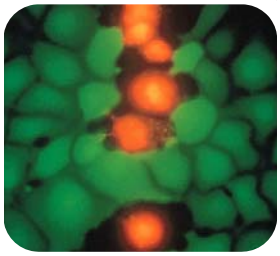
3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

- absorbance can be quantified by spectrophotometry (500 and 600 nm depending on the solvant)

Markers for Cell Degeneration

Live / Dead® Kit (Invitrogen)

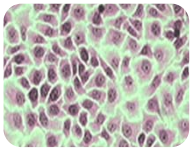
cells stained with ethidium homodimer-1 and an esterase substrate calcein AM



Kangaroo rat (PtK2) cells

Markers for Cell Degeneration

What the Nucleus tells us about Cell Condition

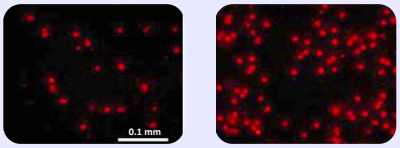


hematoxylin staining

Markers for Cell Degeneration

What the Nucleus tells us about Cell Condition

propidium iodide staining (DNA)

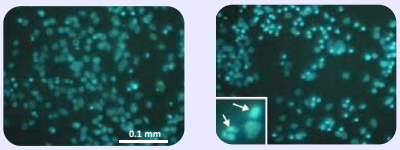


PI intercalates into DNA of cells with damaged membranes (necrotic cells)

Markers for Cell Degeneration

What the Nucleus tells us about Cell Condition

Hoechst33342 staining (DNA)



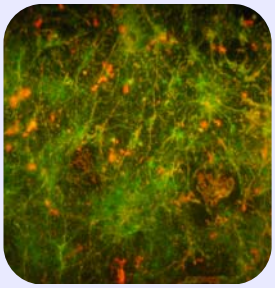
PI intercalates into DNA of living cells

(detects apoptotic bodies)

Markers for Cell Degeneration

Mitochondrial Marker JC-1

- cationic dye for studies on mitochondrial membrane potential
- exhibits potential-dependent accumulation in mitochondria (fluorescence emission shift from green (~529 nm) to red (~590 nm))
- mitochondrial depolarization is indicated by a decrease in the red/green fluorescence intensity ratio



Markers for Cell Degeneration

Mitochondrial Marker JC-1

- cationic dye for studies on mitochondrial membrane potential
- exhibits potential-dependent accumulation in mitochondria (fluorescence emission shift from green (~529 nm) to red (~590 nm))
- mitochondrial depolarization is indicated by a decrease in the red/green fluorescence intensity ratio
- potential-sensitive color shift is due to concentration dependent formation of red fluorescent J-aggregates
- JC-1 is more specific for mitochondrial versus plasma membrane potential

Markers for Cell Degeneration

Markers for Reactive Oxygen and Nitrogen Species

There are many fluorescent dyes indicating the formation of free radical, e.g.

- DAF-FM

marker for NO radicals

- DHE

marker for superoxyde radicals

Markers for Cell Degeneration

Markers for Reactive Oxygen and Nitrogen Species

There are many fluorescent dyes indicating the formation of free radical, e.g.

- DAF-FM

marker for NO radicals

4-amino-5-methylamino-2',7'-difluorofluorescein

quantitating low concentrations of nitric oxide in solution. This compound is virtually nonfluorescent until it reacts with NO to form a fluorescent benzotrizole

Markers for Cell Degeneration

Markers for Reactive Oxygen and Nitrogen Species

There are many fluorescent dyes indicating the formation of free radical, e.g.

- DHE

marker for superoxyde radicals

dihydroethidium (hydroethidine)

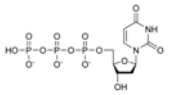
blue fluorescent in the cytosol before oxidation and intercalation into the DNA

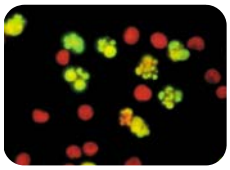
Markers for Cell Degeneration

Markers for Apoptosis

- Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)

method for detecting DNA fragmentation by labeling the terminal end of nucleic acids that are damaged in apoptosis by endonucleases





Markers for Cell Degeneration

Markers for Apoptosis

Apoptotic cell nuclei were labeled with YO-PRO®-1 dye (green)

Necrotic cells were detected with propidium iodide (red)

Markers for Cell Degeneration

Markers for Apoptosis

- Caspase 3 assay kits

protease

important role in apoptosis

kit is based on spectrophotometric detection of a chromophore after cleavage from the labeled substrate DEVD (Asp-Glu-Val-Asp) - chromophore

The *chromophore* can be quantified using a spectrophotometer or a microtiter plate reader

- Annexin V assay kits

most phosphatidylserines (PS) in cell membrane phospholipids translocate from the inner surface to the outer surface during early stage of apoptosis

Once the PS are on the outer surface, they can be detected easily by staining with an enhanced green fluorescent protein (EGFP) fused with annexin V, a protein that has strong natural affinity with PS.

Markers for Cell Degeneration

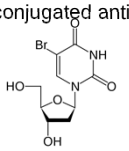
- **Cell Proliferation Assays**
  - BrdU
  - AlamarBlue™
- **PCR**
  - Bax / Bcl ratio
- **FACS**
- ...

Markers for Cell Proliferation

BrdU Assay

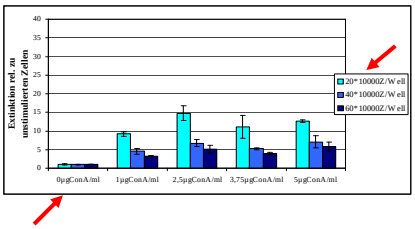
Test principle

- 5-bromo-2`-deoxyuridin (BrdU) is a pyrimidine analogue
- BrdU is inserted instead of thymidine in DNA of proliferating cells
- staining of BrdU with fluorochrome conjugated anti BrdU antibodies
- ELISA evaluation
- direct correlation with the amount of new formed DNA
  (number of proliferating cells)



Markers for Cell Proliferation

BrdU Assay

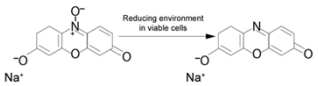


Markers for Cell Proliferation & Degeneration

Alamar Blue™ Assay

(Resazurin)

- **Resazurin is a redox dye commonly used as an indicator of chemical cytotoxicity in cultured cells**
- **It can readily be applied to both adherent and non-adherent cell cultures**



Resazurin sodium salt weakly fluorescent

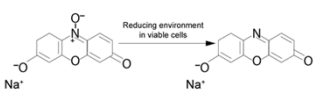
Resorufin sodium salt strongly fluorescent

Markers for Cell Proliferation & Degeneration

Alamar Blue™ Assay

(Resazurin)

- **The assay is based on the ability of viable, metabolically active cells to reduce resazurin to resorufin and dihydroresorufin**
- **This conversion is intracellular, facilitated by mitochondrial, microsomal and cytosolic oxidoreductases**



Resazurin sodium salt weakly fluorescent

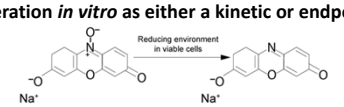
Resorufin sodium salt strongly fluorescent

Markers for Cell Proliferation & Degeneration

Alamar Blue™ Assay

(Resazurin)

- **Resorufin produced as a result of resazurin bioreduction is measured colorimetrically or fluorometrically**
- **Resazurin is non-toxic to cells and stable in culture medium, allowing continuous measurement of cell proliferation *in vitro* as either a kinetic or endpoint assay**



Resazurin sodium salt weakly fluorescent

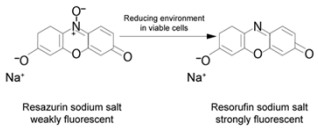
Resorufin sodium salt strongly fluorescent

Markers for Cell Proliferation & Degeneration

Alamar Blue™ Assay

(Resazurin)

- Toxic insult that impairs cell viability and proliferation also affects the capacity of cultures to reduce resazurin, and the rate of dye reduction is directly proportional to the number of viable cells present



Resazurin sodium salt weakly fluorescent

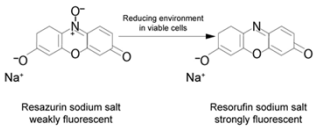
Resorufin sodium salt strongly fluorescent

Markers for Cell Proliferation & Degeneration

Alamar Blue™ Assay

(Resazurin)

- Therefore, as a direct measure of the metabolic competence of cell cultures, resazurin reduction may provide a convenient index of cell proliferation following irradiation



Resazurin sodium salt weakly fluorescent

Resorufin sodium salt strongly fluorescent

Markers for Cell Proliferation & Degeneration

Alamar Blue™ Assay

(Resazurin)

- redox potential of resazurin at 25° C and pH 7: 380 mV
- able to get electrons from NADPH and NADH (Eo=-320), FADH (Eo=-220), FMNH (Eo=-210) and cytochromes (Eo=80 - 290)

Advantage

- incubation of non-toxic resazurin together with your compound of interest
- measurements on different points in time

Markers for Cell Proliferation & Degeneration

Alamar Blue™ Assay

Test principle

- absorption maximum oxidized form: 600 nm (blue)
- absorption maximum reduced form: 570 nm (purple)

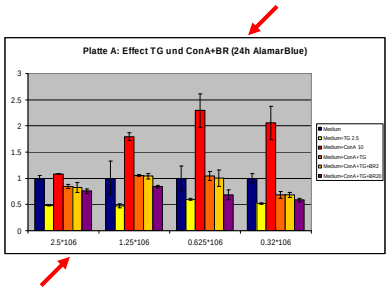
measurement of both wavelength with an ELISA reader

$$X = \frac{117{,}216 * A(\lambda 570) - 80{,}586 * A(\lambda 600)}{155{,}677 * A'(\lambda 600) - 14{,}652 * A'(\lambda 570)} * 100$$

A= absorption of test well
A´= absorption of reference well (medium alone)

Markers for Cell Proliferation & Degeneration

Alamar Blue™ Assay



Workshop part II:

Primary Cell Culture Protocols

Barbara Kranner
Christopher Krewenka

vetmeduni vienna

Nov 26-28 2017

Phosphate Buffer Saline (2 l)

NaCl	16.3 g
$Na_2HPO_4x\ 2H_2O$	3.56 g
$NaH_2PO_4x\ 1H_2O$	3.26 g
KH_2PO_4	0.82 g
adjust to pH=7,4	

Cell Culture Media

Endothelial Cells or Rat Aorta

DMEM	90 ml
Calf serum	10 ml
Glutamine	292 µg/ml
Penicillin	10 U/ml
Streptomycin	10 µg/ml

Murine Mesencephalic Basic Medium (BM)

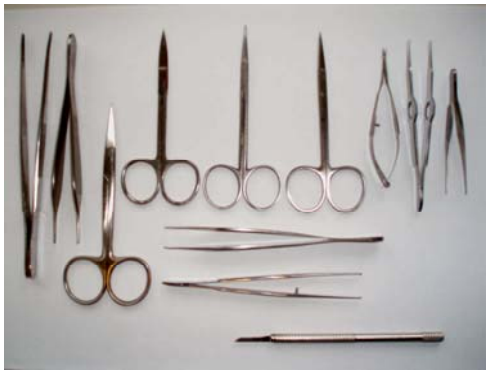
DMEM	90 ml
Calf serum	10 ml
Glutamine	292 µg/ml
Glucose	9.7 mM
Penicillin	10 U/ml
Streptomycin	10 µg/ml

Murin Mesencephalic Treatment Medium (N4)

DMEM	90 ml
B27 supplement	1 ml
Glutamine	292 µg/ml
Glucose	9.7 mM

Preparation of Mesencephalic Primary Cultures of Mouse

sterile instruments:



2 straight scissors
2 forceps
1 curved scissors
2 forceps with teeth
1 small scissors
2 small forceps
1 scalpel

1 turn on the water bath (37°C)

2 switch on lamina flow, clean worktop with alcohol (70%) 30´ before preparation

3 coat plates (in the incubator for 45´; 0.3 ml/well)

preparation of:

4 basic medium (BM)

5 trypsin solution (1 ml stock + 9 ml D-PBS)

6 trypsin inhibitor solution (2.5 mg trypsin-inhibitor + 10 ml D-PBS, sterile filter solution)

7 HBSS + DNAse (2.5 ml HBSS + 50 µl DNAse)

8 Petri dishes:

3 x diameter: 92 mm (one for the tools, 2 with 10 ml D-PBS)
4 x diameter: 50 mm with 5 ml D-PBS
1 x diameter: 25 mm with 1 drop of D-PBS

9 wash plates (point 3) with D-PBS, replace PBS with DMEM, and put them into the incubator

preparation:

1 sacrifice mouse with CO_2

2 use forceps and straight scissor to open the mouse's belly

3 remove the whole uterus and put it in one big Petri dish

starting from now, all work has to be done under the laminar flow

4 open uterus with curved scissors and forceps with teeth, put each embryo with placenta and embryonic membrane in a second big Petri dish

5 dissect placenta and embryonic membrane, put every embryo in the first medium sized Petri dish, lay each embryo on its left hand side

6 cut embryos with curved scissors and toothed forceps in half and put the upper half into the second medium Petri dish

7 put this Petri dish under the dissecting microscope (use surgery mask) and open the laminar flow

8 open the crania with small scissors and small forceps, remove the brains (cerebrum, midbrain, medulla oblongata) and put them into third medium Petri dish

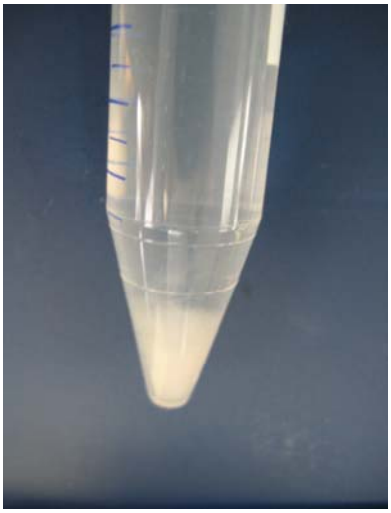
9 cut off the cerebellum and medulla oblongata with a scalpel and small forceps and transfer the midbrain into the fourth medium Petri dish

10 after removal of meninges with 2 small forceps, transfer midbrains in a small Petri dish

11 chop midbrains several times with the scalpel

12 transfer midbrain particles with 2 ml of trypsin solution in a `blue-cap-centrifuge tube´, add 2ml of HBSS + DNAse and incubate in a waterbath (37°C) for 7´

(in the meantime you may wash instruments and remove the dead mouse)



13 under the laminar flow, add 2 ml of Trypsin inhibitor

14 centrifuge at 100 RCF for 4´

15 under the laminar flow, aspirate supernatant and replace it with 3 ml of basic medium (BM) and 60 µl of DNAse

16 triturate with fire polished pasteur pipettes (about 7 times up and down)

17 allow suspension to stand for 10´

<u>in the meantime prepare a flask with 5ml BM and put it into the incubator</u>

18 **transfer the supernatant cell suspension (3 ml) in the flask with BM and add again 3 ml BM and 60 µl DNAse to the tube**

19 **repeat the trituration, wait another 10 min, transfer the supernatant in a flask and repeat step 17 and 18 twice**

20 **pipette a small volume (e.g. 20 µl) of cell suspension into an Eppendorf tube and add the same amount of trypan blue-solution (1+1 dilution)**

21 **count cells with a hemocytometer under a microscope**

22 **calculate the dilution needed for a final concentration of 750.000 cells per ml and dilute cell suspension, under the laminar flow, with BM**

23 **suck off the DMEM from the 4-well-plates and give 750 µl of cell-suspension into each well (shake the suspension gently after filling of 2-3 plates)**

24 **write the date of preparation onto the plates and put them into the incubator**

Preparation of `Ring Cultures´ of Rat Aorta

one day before preparation:

1 sterilize cover glass in an oven at 180°C for min. 30´

2 sterilize tools

day of preparation:

1 cover polystyrene block with aluminium foil

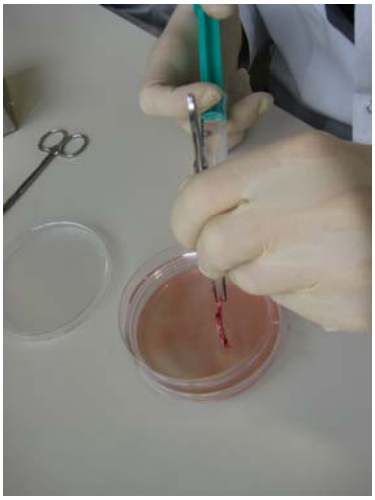
2 arrange one box of sterilized tools outside the laminar flow:

1) four needles for fixation
2) artery forceps
3) syringe (PBS) with needle
4) three Petri dishes (diameter: 92 mm) with PBS
5) two scissors
6) curved scissors
7) large forceps
8) small forceps

3 arrange another set of sterile tools inside the flow:

1) small scissors
2) three small forceps
3) scissors
4) four Petri dishes (diameter: 92 mm) with PBS
5) dissecting microscope
6) cover glasses (sterilized)
7) some Petri dishes (diameter: 25 mm)

preparation:



1 sacrifice rat (6-8 weeks old)

2 withdraw aorta

3 transfer aorta in a Petri dish containing PBS

4 rinse aortic tube with PBS by using the syringe

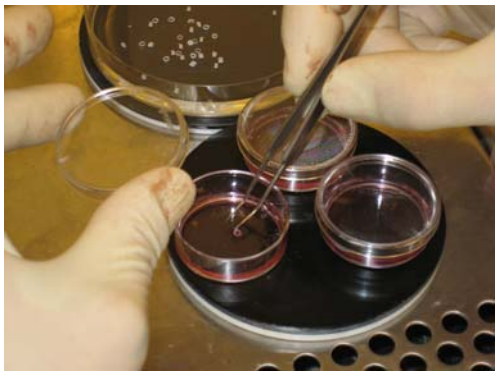
starting from now, all work has to be done under the laminar flow

5 transfer the aorta to another Petri dish filled with PBS

6 under the dissecting microscope, clean the aorta carefully by removing muscles and the adventitia

7 transfer the aorta to another Petri dish filled with PBS

8 cut rings of the size of about 1 mm without squeezing the aorta too much



9 put one cover glass per small Petri dish and fill with 1ml of medium

10 place one aortic ring on each cover glass and give Petri dishes into the incu-bator

Tyrosine Hydroxylase ICC

first day:

1 **remove medium**

2 **PFA (4%, 0.3 ml per well) 15´ at 4°C**

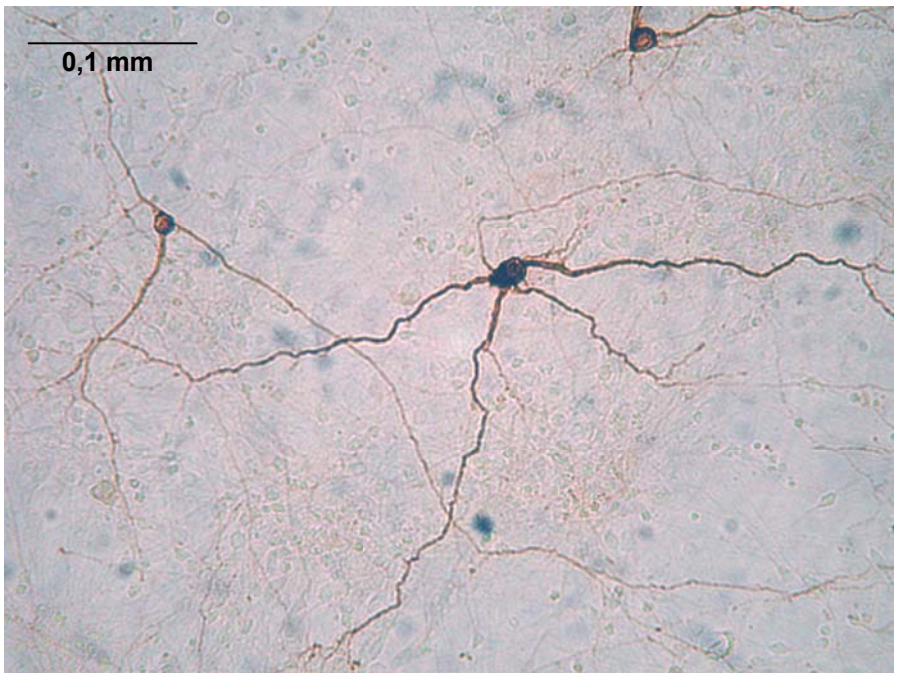
3 **wash 1x 2´ with PBS**

4 **0.4% Triton-X (1:10 dilution of 4% stock solution, 0.3 ml per well) at RT for 30´**

5 **wash 3x 2´ with PBS**

6 **horse serum solution (0.4 ml + 20 ml PBS; 0.3 ml per well) for 1.5 h at RT**

7 **mouse anti-tyrosine hydroxylase AB (primary antibody) (20 µl + 5 ml horse serum mixture (f.c. 80 ng/ml; dilution depends on the AB); 0.3 ml per well) overnight at 4°C**



the following day:

1 wash 3x 5´ with PBS

2 horse anti mouse biotinylated (secondary) antibody (20 µl to 10 ml PBS (f.c. 3 µg/ml; dilution depends on the AB); 0.3 ml per well) for 1.5 h at RT

prepare ABC-Kit Mouse (Vectastain):

1 1 drop A + 1 drop B + 5 ml PBS, shake well and incubate at RT for at least 30´

3 wash 3x 5´ with PBS

4 AB-solution for 1.5 h at RT (0.3 ml per well)

5 wash 3x 5´ with PBS

prepare DAB-solution:

solution I: 0.5 ml PBS + 20 µl H_2O_2
solution II: 5 mg DAB+ 5ml PBS + 100 µl solution I

6 incubate with DAB solution under optical control (microscope for 5-10´) (0.3 ml per well)

7 wash 3x 2´ with PBS

8 mount with Kaiser´s glycerol gelatine

GFAP ICC

first day:

1 remove medium

2 PFA (4%, 0.3 ml per well) 15´ at 4°C

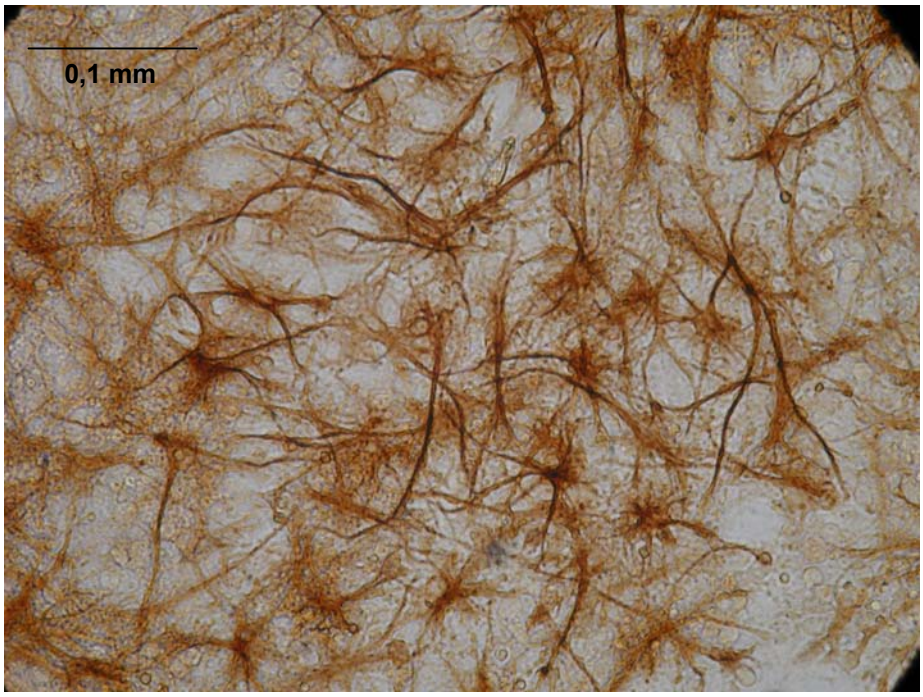
3 wash 1x 2´ with PBS

4 0.4% Triton-X (1:10 dilution of 4% stock solution, 0.3 ml per well) at RT for 30´

5 wash 3x 2´ with PBS

6 incubate with goat serum solution (0.4 ml + 20 ml PBS; 0.3 ml per well) for 1.5 h at RT

7 rabbit anti-GFAP AB (primary antibody) (20 µl + 5 ml horse serum mixture (f.c. 2 µg/ml, dilution depends on the AB); 0.3 ml per well) overnight at 4°C



the following day:

1 wash 3x 5´ with PBS

attention: Dim room when working with fluorescent dyes ! (otherwise use ABC-Kit Rabbit (Vectastain) for a DAB staining)

2 anti rabbit Alexa Fluor 488 (10 µl to 2.5 ml PBS (f.c.8 µg/ml; dilution depends on the AB) 0.3 ml per well) for 2h at 37°C

3 wash 2x 5´ with PBS

4 2 µM PI solution (prepared from 1 mM aqueous stock solution) for 5´

5 wash 2x 5´ with PBS

6 mount specimen

Von Willebrand factor ICC

first day:

1 **remove medium**

2 **PFA (4%) 15´ at 4°C**

3 **wash 1x 2´ with PBS**

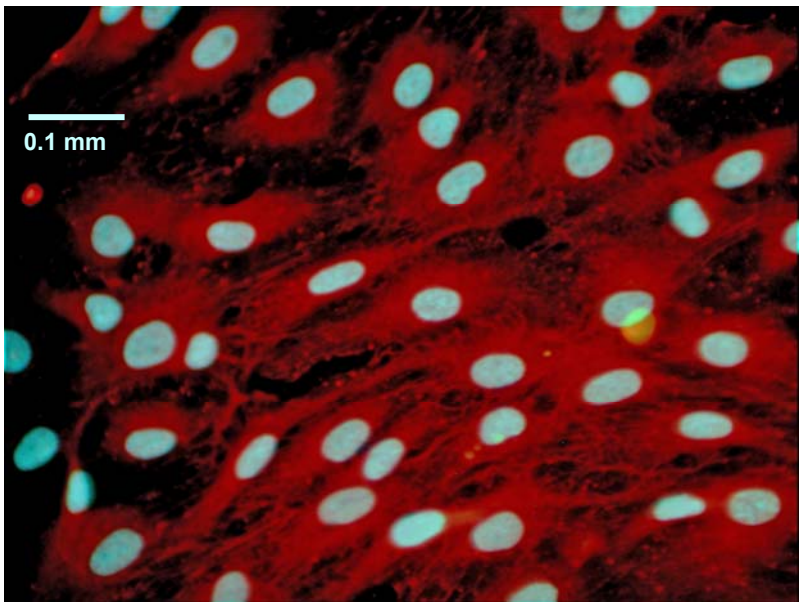
4 **0.4% Triton-X (1:10 dilution of 4% stock solution) at RT for 30´**

5 **wash 3x 2´ with PBS**

6 **goat serum solution (0.4 ml + 20 ml PBS) for 1.5 h at RT**

7 **wipe off carefully the solution around the cells and draw a circle with a Dako Pen**

8 **rabbit anti human von Willebrand factor AB (50 µl + 5 ml horse serum mixture (f.c.8µg/ml; dilution depends on the AB); 20µl per circle) overnight at 4°C**



the following day:

1 wash 3x 5´ with PBS

attention: Dim room when working with fluorescent dyes ! (otherwise use ABC-Kit Rabbit (Vectastain) for a DAB staining)

2 anti rabbit Alexa Fluor 568 (10 µl to 2.5 ml PBS (f.c.8 µg/ml) 0.3 ml per well) for 2 h at 37°C

3 wash 2x 5´ with PBS

4 2 µM DAPI solution (prepared from 1 mM aqueous stock solution; 0.3 ml/well) for 5´

5 wash 2x 5´ with PBS

6 mount specimen

Cell number determination

A with a hemacytometer

1 clean surface of hemacytometer and coverslip

2 mix cell suspension well, but carefully

3 take 10 µl of cell suspension and transfer to the edge of hemacytometer counting chamber

4 suspension will be drawn under coverslip by capillary action

5 fill second chamber

6 view slide on microscope with 100x magnification (10x ocular with a 10x objective)

7 count cells of the four corner squares

8 determine cells per ml by the following calculations:

cells/ml = average count per square x dilution factor x 10^4

B Cell viability determination with trypan blue stain

1 mix 1 part of 0.4% trypan blue and 1 part of cell suspension

2 load 10 µl to the counting chamber

3 count total number of cells and total number of viable (unstained) cells

4 calculate percent viable cells as follows:

$$\% \text{ viable cells} = \frac{\text{number of unstained cells}}{\text{total number of cells}} \times 100$$

Freezing procedure for adherent cells

1 remove and discard culture medium

2 wash with PBS

3 trypsinize cells (if necessary, with a minimum of amount)

4 add fresh medium (with serum) to stop effect of trypsin

5 count cells

6 centrifuge (e.g. for 6 min at 300-350 x g, RT)

7 remove supernatant

8 dilute pellet with 4°C freezing medium to a final concentration of 10^6 to 10^7 cells/ml and place on ice

12 pipet 1ml aliquots of cell suspension into labeled 2ml cryovials in cryobox

13 place vials overnight in a -70°C freezer

14 transfer to liquid nitrogen storage freezer