Basic Techniques

R.Moldzio
University of Veterinary Medicine, Vienna (Vetmeduni Vienna)

Why to use Cell Cultures

- cell culture studies are mandatory for the development of new drugs
- studies for regulatory more and more evolve into an animal replacement direction

Why to use Cell Cultures

- the designs of animal studies are often unacceptable (lack of suitable controls, housing problems, inappropriate parameters measured)
- animal experiments are very expensive due to the abundant mandatory tests (hormon levels, clinical parameters, surgical interventions)
- ethical issues

Basic rules

- Keep doors and windows closed while working
- No eating, drinking or chewing gum
- Wear labcoats and other protective clothing if necessary
- Handling of fluides and aerosols with gloves and safety glasses
- After work, clean everything in the lab carefully, but also yours hands
- Avoid contaminations

Important rules

- lab safety is mandatory, there are rules about
  - decontamination
  - resistance against antibiotics
  - handling of animals
  - others

Important rules

Three important tools for standardization

- GLP „good laboratory practice“
- GCCP „good cell culture practice“
- GSP „good scientific practice“

Important rules

standardization

- control of materials (lot numbers etc.)
- cleanliness
- documentation (data management)
- quality management

Important rules

quality management

- SOP (standard operation procedure)
- using reference substances
- maintenance of equipment
- good instruction of employees
- use of blinded studies (cross studies)
- statistics

Work with genetically modified Organisms (GMOs)

classification of risk potential

- the Cartagena protocol

legal framework

- international, national and institutional

Implementation

- biosafety levels 1-4
- Laws, regulations, marking of rooms and work areas, training, courses
- Working under sterile conditions
- SOPs and handbooks

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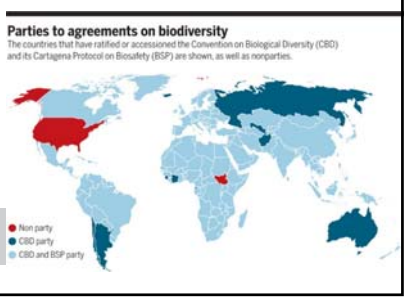
Work with genetically modified Organisms (GMOs)

The Cartagena protocol

- based on the idea that GMOs, especially in crop, can endanger biodiversity, the Convention on Biological Diversity (CBD) was established in 1992, and the Cartagena Protocols on biosafety (BSP) in 2003
- Protocols deal with biosafety, so they were expanded to human health, not only the environment
- Information are shared by the Biosafety Clearing House (BCH)

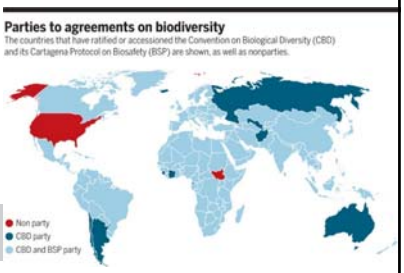


Work with genetically modified Organisms (GMOs)

the Cartagena protocol

- http://www.egbch-eg.com/En/
https://www.facebook.com/BiosafetyEgypt/

- Information are shared by the Biosafety Clearing House (BCH)



Work with Genetically modified Organisms (GMOs)

Nonetheless, in 2015

- Egypt takes a permissive approach to genetically modified organisms (GMOs), and its public policy does not oppose growing, importing, and exporting genetically modified crops. Egyptian activists have voiced their rejection to this policy. Egyptian laws do not contain restrictions on researching, producing, or marketing genetically modified crops and food products. The country also has no restrictions on releasing genetically modified organisms into the environment. A draft law on biosafety was not approved by the Egyptian Parliament.

https://www.loc.gov/law/help/restrictions-on-gmos/egypt.php 12/40

Work with Genetically modified Organisms (GMOs)

National Biosafety Systems in Egypt

- A policy on biotechnology, including biosafety
- Regulatory framework for biosafety
- Mechanism to handle requests for permits
- Enforcement and monitoring
- Public information and public participation

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Work with Genetically modified Organisms (GMOs)

History:

- 1994: Egypt ratifies the Biodiversity Convention (CBD)

- article 8: "In situ conservation of biodiversity"
- article 19: "Handling biotechnology and distribution of its benefits"

- 1995: Establishment of the National Biosafety Committee (NBC)
- 2006: Establishment of the National Competence Authority for the functions of the CPB

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The National Biosafety Committee

- Formats for Authorizations

- a summary of the request
- a summary of the procedure followed
- a summary of the risk assessment
- the final decision.

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National Profile on Occupational Safety and Health

2. LEGISLATIVE FRAMEWORK

General

2.1 References to OSH requirements in the constitution of the country
2.2 Major occupational safety and health laws and regulations
2.2.1 Law No. 12 (2003), Book V on OSH
2.2.2 Executive Ministerial decrees
MD 126/2003: Notification of accidents and diseases at the workplace
MD 211/2003: Safe working environment (TLVs etc.)
MD 134/2003: OSH committees
2.2.3 Infringements and Sanctions
2.2.4 Latest development on OSH legislation in Egypt
2.3 Other laws and regulations covering aspects related to OSH:
2.3.1 Mines
2.3.2 Ionizing radiation
2.3.3 Compensation: Health Insurance Legislation and Organizations
2.3.4 Protection of the Environment: Law No. 4 / 1994
2.4 Laws and regulations indirectly related to OSH
2.4.1 Related to health
2.4.2 Related to conditions of work
2.4.3 Related to women and children
2.4.4 Related to special categories of workers
2.5 Correspondence with ILO instruments

Occupational Safety and Health in EGYPT

A National Profile

Dr. Gehad A. Abo El Ata

Ms. Michèle Nahmias

January 2005

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National Profile on Occupational Safety and Health

Information e.g. on

- National Institute for Occupational Safety and Health
  (http://www.niosh.gov.eg/ar)

- International Labour Organization
  (https://www.ilo.org/dyn/legosh/en/f?p=14100:1100:0::NO::P1100_ISO_CODE3,P1100_YEAR:EGY,2013)

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Egyptian Laws and Regulation for Managing Chemicals

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Introduction	1
Executive Summary of the National Profile	3
Chapter 1: National Background Information	7
Chapter 2: Chemical Production, Import, Export and Use	19
Chapter 3: Priority Concerns Related to Chemical Production Import, Export, and Use	21
Chapter 4: Legal Instruments and Non-Regulatory Mechanisms for Managing Chemicals	25
Chapter 5: Ministries, Agencies and Other Institutes Managing Chemicals	35
Chapter 6: Relevant Activities of Industry, Public Interest Groups & Research Sector	39
Chapter 7: International Linkages	45
Chapter 8: Awareness Understanding of Workers and the Public	48

NATIONAL PROFILE

FOR

THE MANAGEMENT OF CHEMICALS IN EGYPT

JANUARY 1999

CONTENTS

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Egyptian Laws and Regulation for Managing Chemicals

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NATIONAL PROFILE

FOR

THE MANAGEMENT OF CHEMICALS IN EGYPT

JANUARY 1999

CONTENTS

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Egyptian Laws and Regulation for Managing Chemicals

The National Profile for
the Management Of Chemicals in Egypt

An Executive Summary

April 2002

Legal Instrument(Type, Reference, Year)	*Responsible Ministries or Bodies*	*Chemical Use Categories Covered*	*Objectives of Legislation*	*Enforcement Ranking[4]*
Law No.4 of 1994	MSEA	Industrial chemicals, Agricultural chemicals (pesticides – fertilizers), Petroleum products Consumer chemicals and Chemical waste.	Environmental Protection and Pollution Control in Egypt	(2)

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Egyptian Laws and Regulation for Managing Chemicals

Drugs and narcotic compounds

Egyptian Drug Authority (http://www.eda.mohp.gov.eg)

Law

- Pharmacy Law No. 127/1955
- Suppression Deception And Fraud Law [No. 48/1941]
  - First Modification of Suppression Deception And Fraud Law [No. 127/1955]
  - Second Modification of Suppression Deception And Fraud Law [No. 127/1955]
- Law [No. 120/1982]

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Work with Biological Material

Classification of Risk Potential

- grading of risk exposure
- determination of biological safety level
- work with GMOs must be done in certified Labs
- violation is punishable

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Biosafety Levels

Biosafety Level 1 (BSL-1)

BSL-1 is the basic level of protection common to most research and clinical laboratories, and is appropriate for agents that are not known to cause disease in normal, healthy humans.

Biosafety Level 2 (BSL-2)

BSL-2 is appropriate for moderate-risk agents known to cause human disease of varying severity by ingestion or through percutaneous or mucous membrane exposure. Most cell culture labs should be at least BSL-2, but the exact requirements depend upon the cell line used and the type of work conducted

Biosafety Level 3 (BSL-3)

BSL-3 is appropriate for indigenous or exotic agents with a known potential for aerosol transmission, and for agents that may cause serious and potentially lethal infections.

Biosafety Level 4 (BSL-4)

BSL-4 is appropriate for exotic agents that pose a high individual risk of life-threatening disease by infectious aerosols and for which no treatment is available. These agents are restricted to high containment laboratories.

Biosafety Level 1

- **genetic engeneering without risk for humans and environment, according to the current state of research**
- **specific rules for the use of biological agents, e.g., plasma of SPF animals, pathogen-free tissue specimen, most of cell cultures (including non-immortalized cell lines)**

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Guidelines Required by Law

S1 labs

- specified rooms (obligation to notify)
- protective clothing is mandatory (lab coat)
- gloves and aerosol protection recommend, but not mandatory
- appropriate waste disposal / inactivation (like unspecified medical waste)
- identifiable trash bags (waste should be burnt, not dumped)
- sterilization by autoclav, desinfection of surfaces with 70% EtOH, incidur, or other disinfectants

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Guidelines Required by Law

S1 labs

- specified rooms (obligation to notify)
- protective clothing is mandatory (lab coat)

- do not wear the S1 lab coat in other rooms-

- appropriate waste disposal / inactivation (like unspecified medical waste)

- do not mix S1 and other waste

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Biosafety Level 2

- genetic engeneering with „minor“ risk for humans and environment, according to the current state of research
- specific rules for the use of biological agents, e.g., plasma of humans or animals without human pathogens, tissue specimen, cell cultures (including viral /retroviral immortalized cell lines)

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S2 - Guidelines Required by Law

- compulsory report to the authorities
- S2 rooms must be indicated and have to contain washbasin, eye shower and a first-aid kit
- entrance for personell only after specific annual training
- protective clothing (distinguishable from other lab clothing), aerosol protection, gloves and mask are mandatory
- doors have to be closed while work, otherwise locked
- manuals, SOPs, emergency and hygiene plans must be displayed
- biological material must be tranported in a closed shatter-proofed box
- record requirement: (project, experimenter and organisms)

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S2 - Guidelines Required by Law

and of course you must keep a detailed lab journal

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Biosafety Levels

project leader is responsible and ensures

- the compliance of legal regulations and official requirements and orders
- the compliance of operating instructions and adequate qualification and regular instruction of employees
- initiating occupational health check-ups and recording accidents

biological safety officers are responsible for

- supervision of the facility
- for composing an annual written assessment for the institution

Biosafety Level 2

the institutional biological safety committee provides information about significant circumstances, injuries and incidents

- display of important emergency contacts
- rules for accident and emergency procedures
- general safety regulations and code of conduct
- working in the hood (biosafety cabinet)
- virus waste handling
- S2 lab spill handling protocol
- general hygiene rules
- skin safety plan
- emergency plan

Biosafety Level 2

Use SOPs and write a handbook

Handbook for the Biosafety Level 2 (S2) Lab in the Institute for Medical Biochemistry
Room HA06G47
University of Veterinary Medicine Vienna
Veterinärplatz 1, A-1210 Vienna, Austria

Emergency Plan, Code of Conduct, Hygiene Plan

- **General hygiene rules**

Subject	Required Action	Use	When	Those Responsible
Hands	Wash Disinfect Moisturise	Baktolin® sensitive Sterillium® classic pure Baktolan® lotion pure	As required and after work (EH72.3 Roth)	Everyone
Lab Coats	Autoclave	double-bagged waste	Regularly or when contaminated	Everyone
Work Surfaces	Disinfect and clean	Meliseptol Rapid	After each use or if contaminated	Everyone
Equipment	Disinfect and clean	2% Virkon® or Meliseptol Rapid	After each use	Everyone
Solid Waste	Autoclave	Autoclave	As required	Trained Personnel (names documented)
Contaminated Floors	Disinfection	Meliseptol®	Once per month or if contaminated	Everyone
Floors	Cleaning	Cleaning Products	Weekly	Trained Support Staff (names

Emergency Plan, Code of Conduct, Hygiene Plan

- **S2 lab spill handling protocol**

Clean-up requires:

Masks	Gloves	overshoes
Disposable lab coats	Disposable forceps	
Absorbent paper	Virkon powder	
Surface Disinfectant	Autoclave bags	

Infectious Hazard Clean-up:

1. Place the hazardous spill warning sign at the door, to make sure no one may enter accidentally.
2. Be sure to wear a mouth protecting mask and two pairs of gloves.
3. Assemble equipment for clean-up from the “Spill kit”.
4. Remove solid materials first. Use forceps to pick up contaminated items.
5. Absorb fluids with Virkon powder, wait at least 3 minutes before mopping up with absorbent paper and putting in autoclave bag.

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Emergency Plan, Code of Conduct, Hygiene Plan

- **accident and emergency procedures**
  - In case of accident, injury or exposure immediately inform the project leader (and/or the BBS if not available). If there is an urgent need for treatment or a risk of deferred health issues, Greiner, Claudia, Dr.med. (25077-1515) should be seen **immediately**, as the incident might need to be reported as an occupational accident.
  - Contaminated skin should be washed with copious amounts of clinical soap and water.
  - For a sharps injury, bite or scratch - following ANY exposure, immediately, but gently encourage free bleeding. Then wash liberally with clinical soap and running water but without scrubbing. Wounds should not be sucked.
  - Exposed mucous membranes, including conjunctivae, should be irrigated copiously with water, before and after removing any contact lenses.
  - Blood or bodily fluids in eyes or mouth - irrigate with copious quantities of cold water.

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What to do in Case of an Emergency?

- **initiation of emercency measures**
- **information of project leader and biosafety officers**

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Steril Technique 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 Technique 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

Steril Technique and Good Cell Culture Practice

Procedure

- clean the cabinet using 70% ethanol before work
- clean 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 speakingtalking, sneezing and coughing is allowed

Hygiene

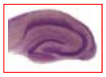
- Always wear appropriate personal protective equipment. Change gloves when contaminated, and dispose used gloves with other contaminated laboratory waste.
- Wash your hands after working with potentially hazardous materials and before leaving the laboratory.

Hygiene

Skin, face, mouth

- skin
  - wear an lab coat and appropriate shoewear
- hands
  - wash with antimicrobial soaps follwowed by use of disinfactants
  - lab gloves
- face
  - Do not talk if possible
  - wear masks
  - use hair bobbles or hairgrips if necessary

Hygiene



- Do not eat, drink, smoke, handle contact lenses, apply cosmetics, or store food for human consumption in the laboratory.
- Follow the institutional policies regarding safe handling of sharps (i.e., needles, scalpels, pipettes, and broken glassware).

Hygiene

- Take care to minimize the creation of aerosols and/or splashes.
- Decontaminate all work surfaces before and after your experiments, and immediately after any spill or splash of potentially infectious material with an appropriate disinfectant. Clean laboratory equipment routinely, even if it is not contaminated.

Hygiene

- Decontaminate all potentially infectious materials before disposal.
- Report any incidents that may result in exposure to infectious materials to appropriate personnel (e.g., laboratory supervisor, safety officer).

Lab for Sterile Working

- separated room, only for cell culture purposes
- economic equipment (only a few devices)
- closed doors and windows
- laminar flow
- incubator
- sterilizer
- autoclave

General requirements

three defined working areas

- cleaning area
- prearrangement area
- sterile area

General requirements

three defined working areas

- cleaning area

- lab dish washer
- dryer
- autoclave
- storage of lab ware

General requirements

three defined working areas

- cleaning area
- prearrangement area
- **sterile area**
  - **flow**
  - **incubator**
  - **water bath**
  - **centrifuge**
  - **microscope**
  - **storage cabinet**

Design and Equipment for the Cell Culture Laboratory

Design of the Laboratory

- **quarantine area**
- **main cell and tissue culture area**

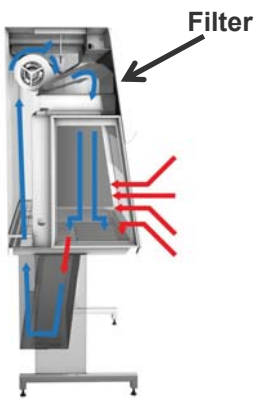
Basic Equipment for Cell Culture Laboratories

Laminar Air Flows

- Laminar airflow - Horizontal
- Laminar airflow - Vertical

UV-Light

The laminar flow - biosafety cabinet



- Running 15 min beforé and
- after use

Basic Equipment for Cell Culture Laboratories

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

Basic Equipment for Cell Culture Laboratories

Inverted (fluorescent) microscope

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

Basic Equipment for Cell Culture Laboratories

Autoclaves

- +98°C: solutions (PBS,..)
- +121°C: plasticware
- +134°C: glassware
  stainless instruments

Basic Equipment for Cell Culture Laboratories

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

Basic Equipment for Cell Culture Laboratories

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)

Basic Equipment for Cell Culture Laboratories

Laboratory Refrigerators and Freezers

- Refrigerator: +4°C
- Freezer: -20 to -40°C
- deep Freezer: -80°C

Basic Equipment for Cell Culture Laboratories

Water bath

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

Basic Equipment for Cell Culture Laboratories

Water destiller

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

Basic Equipment for Cell Culture Laboratories

Liquid nitrogen tank

- Freezing cells for long storage period

Basic Equipment for Cell Culture Laboratories

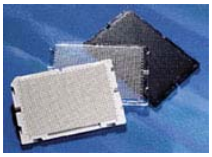
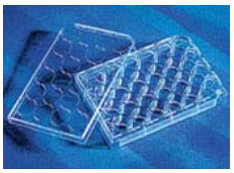
Others

- pH-meter
- vacuum pump
- …..

Basic Equipment for Cell Culture Laboratories

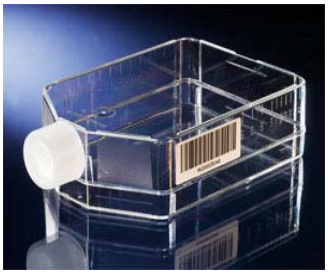
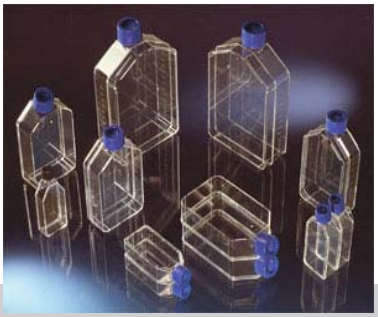
Plasticware and Consumables




Basic Equipment for Cell Culture Laboratories

Cell Culture Flask

Material of culture dishes/plates

- very important → influence on healthiness of the culture
- cell surface has negativ charges (Glycocalyx)
- cell culture dishes should have a specific charge density
- cell-dish electrostatic interaction is based on Van-der-Waals-Force

Plastic

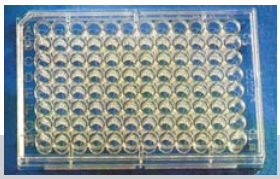
All forms constist of

- polystyrene, polycarbonate, polytetrafluoroetylene ,…
- on the inner walls they have a hydorphobic surface
- *Polymers can also be used as microcarrier* (polyacrylamide, cellulose, dextran polymers)

Basic Equipment for Cell Culture Laboratories

Multi-well-plates

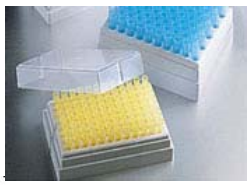


Basic Equipment for Cell Culture Laboratories

Pipettes

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


Cell Culture Media

Support of

- proliferation
- growth environment
- differentiation and cell functions
- neutralisation of waste products

Cell Culture Media

Content

- amino acid
- salts
- vitamins
- carbohydrates
- growth factors, hormones, blood, serum,…

Cell Culture Media

different types of media

- undefined media
- basic or standard medium containg glucose, water and various salts

- defined media
- minimal media (all compounds are known)
- complete media (include serum and growth factors

Cell Culture Media

defined media

- DMEM (Dulbecco's Modified Eagle Medium)

- MEM (Minimum Essential Medium)

- 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

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 month
- after coagulation, blood is centrifuged
- blood clotting is a natural way as growth factors are released from platelets into the serum
- serum is sterile filtered through cartridge filters

Cell Culture Media

newborn calf serum

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

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

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

Cell Culture Media

principles of serum use

- 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 form 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

Why to use Cell Cultures

Before you start, you have to decide, what kind of cell culture you want to use

Types of Cell Cultures

adherend	non-adherend cells
mono- or multilayer attached to the dish derived from tissues where they are not tranlocating coherent cell layers density-dependent inhibition of proliferation	grow in suspension do not stick to the polymers of the dish cells of the immune system oder their precursors
primary	**transformed cells**
isolated from tissues limited live expectancy => finite cultures	infinite proliferation span in vitro either tumor cells or immortalized cells

Types of Cell Cultures

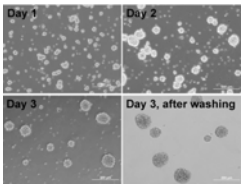
primary culture

tissue taken directly from an animal

Primary Cell Cultures

reaggregate culture

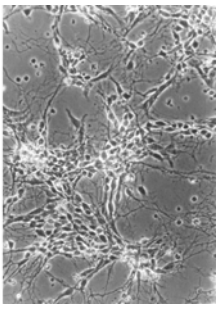
- dissociated cells kept in suspension rather than allowed to settle on and attach to solid substrate
- cells tend to reaggregate into small balls
- culture permits cells to develop in three dimensions



Primary Cell Cultures

primary dissociated culture

obtained when taken tissue is dissociated, mechanically or enzymatically, into single cells which could be plated on a coated surface



neural cell culture

Primary Cell Cultures

primary dissociated culture

cultured cells are derived either by

- outgrowth of migrating cells from a fragment of tissue (Only cells with capacity to migrate from the explants survive the disaggregating techniques and adhere to the substrate or survive in suspension will form the basis of the primary culture)
- mechanical or Enzymatic dissociation of the tissue to single cells

primary mesencephalic culture

Primary Cell Cultures

organ culture

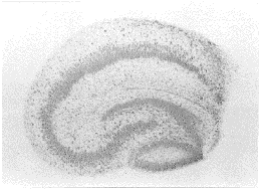
maintenance of a piece of tissue, a part of organ or a whole organ in vitro

organ culture

Primary Cell Cultures

organotypic (slice) culture

- small pieces of tissue of interest attach to an appropriate substrate and are cultured in enriched media
- preserve some aspects of the tissue's architectures



hippocampal slice culture

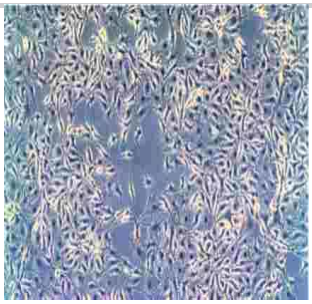
Types of Cell Cultures

cell Lines

immortal/immortalized cells

Cell Lines

- primary cell cultures can be passaged a finite number of passages before reaching a crisis (senscence)
- passages before crisis are referred to as a cell strains
- cells that survive the crisis and continue to grow are referred to as a cell line



B16-F10 Melanoma Cell Line

Types of Cell Lines

- continuous cell line:

- population of cells that can be passaged indefinitely and express reasonable stable phenotype

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- cell lines obtained from tumor cells

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- transformed cell line:

- cell lines obtained from tumor cells

- infinite life span (immortality)
- divide rapidly
- do not require attachment for growth
- form tumors when introduced into animals

Types of Cell Lines

- continuous cell line:

- population of cells that can be passaged indefinitely and express reasonable stable phenotype

- transformed cell line:

- cell lines obtained from tumor cells

- clonal cell line:

- cells could be cloned in continuous cell lines to obtain genetically homogenous population

Types of Cell Lines

1 continuous cell line:

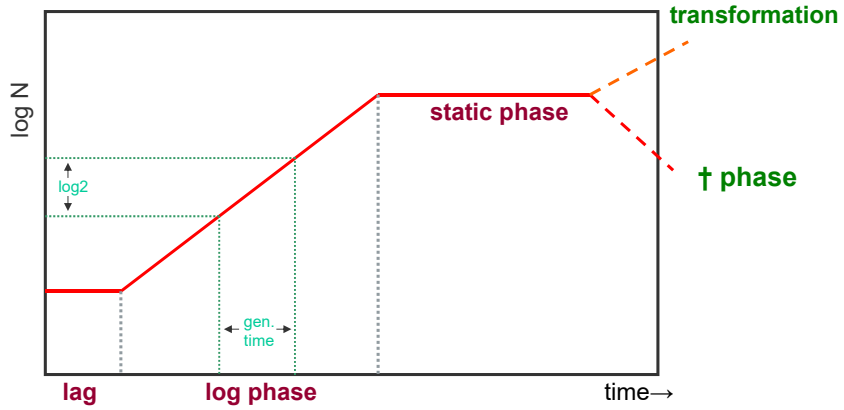
Population of cells that can be passaged indefinitely and express reasonable stable phenotype

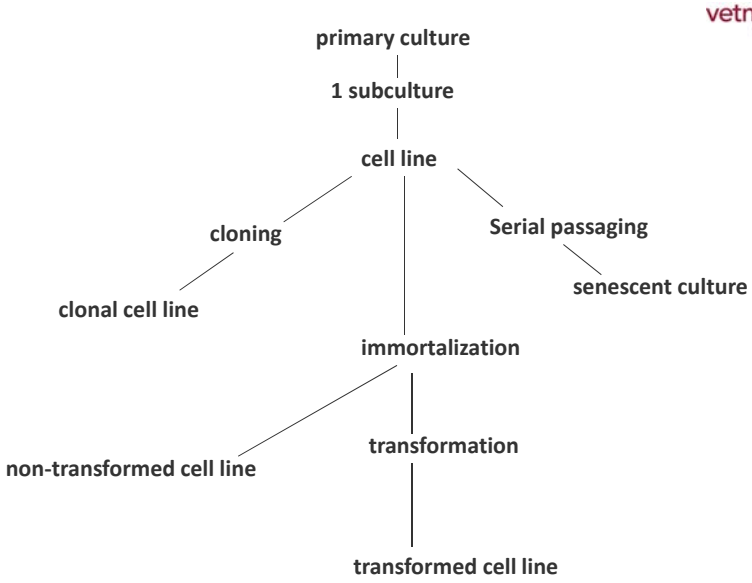
2 transformed cell line:

Cell lines obtained from tumor cells

- infinite life span (immortality)
- divide rapidly
- do not require attachment for growth
- form tumors when introduced into animals

Growth of Cell Lines





What kind of culture shall I use?

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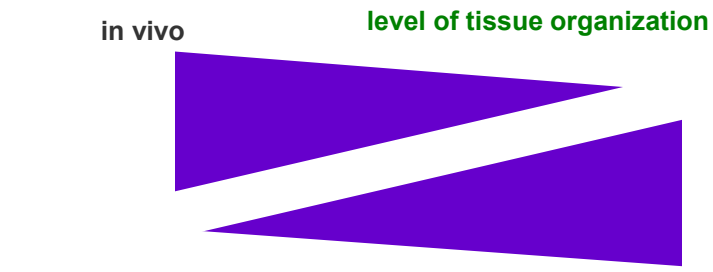
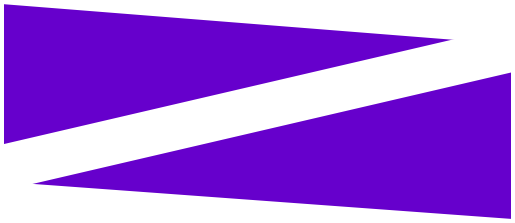


What kind of culture shall I use?

lines and clones

in vivo

level of tissue organization



accessibility to experimental manipulations

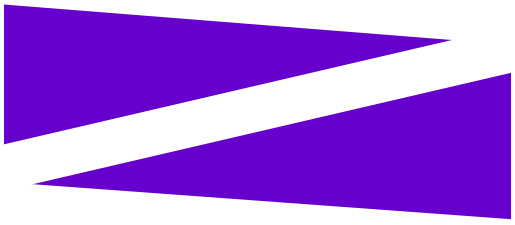
What kind of culture shall I use?

lines and clones

purified homogenous

in vivo

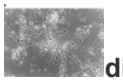
level of tissue organization



accessibility to experimental manipulations

What kind of culture shall I use?

lines and clones

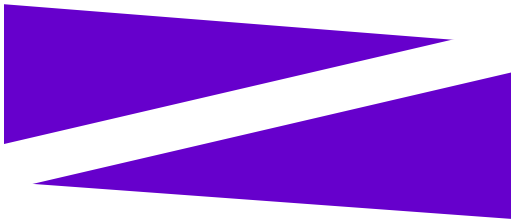


purified homogenous

dissociated

in vivo

level of tissue organization



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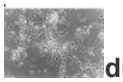


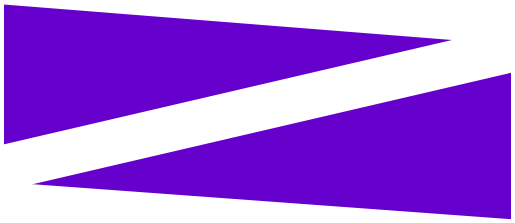
purified homogenous

dissociated

reaggregating

in vivo

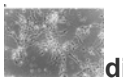
level of tissue organization



accessibility to experimental manipulations

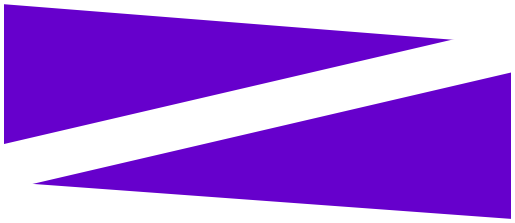
What kind of culture shall I use?


lines and clones


purified homogenous
dissociated
reaggregating
explant

in vivo

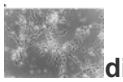
level of tissue organization



accessibility to experimental manipulations

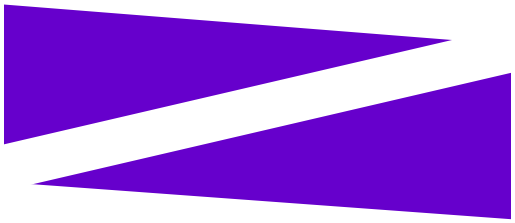
What kind of culture shall I use?


lines and clones


purified homogenous
dissociated
reaggregating
explant


organotypic
in vivo

level of tissue organization



accessibility to experimental manipulations

Pitfalls

- it is well known that cell lines lose some characteristics after passaging more than allowed. So, some parameters are to be studied to confirm the suitability
- primary cell cultures do reflect the in vivo situation properly, but they tend to show accelerated aging
- primary cells as well lose some characteristics during cultivation
- in cultures, an increased cell degeneration occurs that influences the outcome of the study

Three Dimensional Cultures

3D cell cultures, microtissues, and organoids are increasingly being used to bridge the gap between 2D cell cultures and *in vivo* animal models. 3D models are more physiologically relevant than biochemical assays and 2D cell cultures, as they more closely represent the microenvironments, cell-to-cell interactions, and biological processes that occur *in vivo.* They show a higher degree of morphological and functional differentiation – again, similar to *in vivo* cell characteristics.

http://www.perkinelmer.de/category/3d-cell-culture-analysis?gclid=EAIaIQobChMIgKKIkMbS1wIVihbTCh0nmwViEAAYASAAEgLehPD_BwE

Three Dimensional Cultures

advantages

- you can investigate easily cell migration
(e.g. neuronal development, stratification)
- you can investigate tumor development
(e.g. angiogenesis)
- you can investigate cell-matrix interaction
- you can investigate the pharmacodynamic and -kinetics of a drug

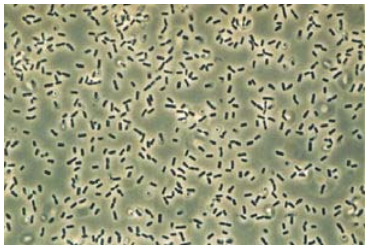
Three Dimensional Cultures

often hydrogels are used to mimic the extracellular matrix

- hydrogels are composed of interconnected pores with high water retention, which enables efficient transport of e.g. nutrients and gases
- there are different types of hydrogels, e.g. polymer, peptide or protein hydrogels
- also other 3D systems are in use (hanging drop, rotating cultures, bioreactors etc.)

Avoidance of Contamination

- microbial contamination
- contamination source, identification, effects and treatment of:
  - -bacteria
  - -mycoplasma
  - -fungi and yeast
  - -viruses
- cross contamination

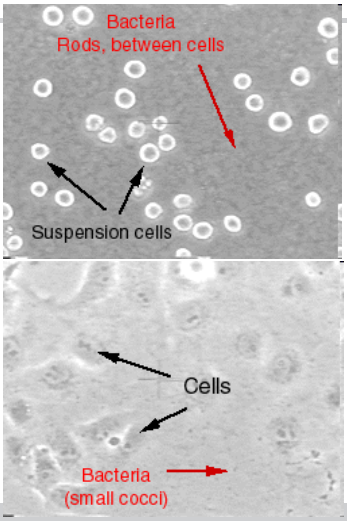


Avoidance of Contamination

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

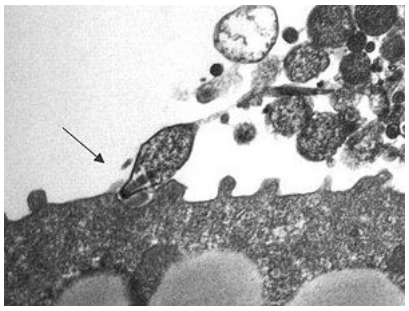
- the most commonly used antibiotics in cell culture are penicillin/streptomycin (pen/strep)
- typically, pen/strep are used together and gentamicin and kanamycin can be used together.

as little as possible, as much as 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



Mycoplasma pneumoniae

Avoidance of Contamination

contamination source

- serum
- trypsin solution

effects

- inhibition of PBS
- decrease of cell division
- cell necrosis
- facilitation of virus infection

Avoidance of Contamination

identification

alteration of proliferation rate; cavities on cell monolayers; agglutination of cells

detection

fluorescence (DAPI); electron microscopy, PCR

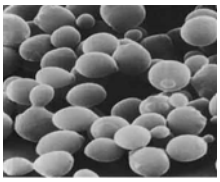
treatment

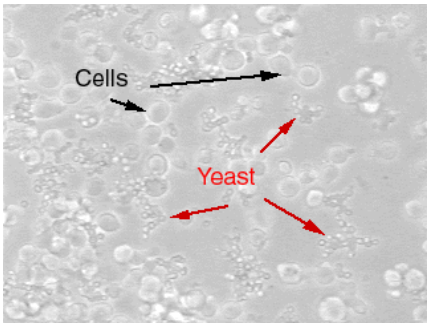
autoclaving; mycoplasma desinfectant

Avoidance of Contamination

fungi and yeast

member of a large group of
eukaryotic organisms
that includes microorganisms
such as yeasts and molds





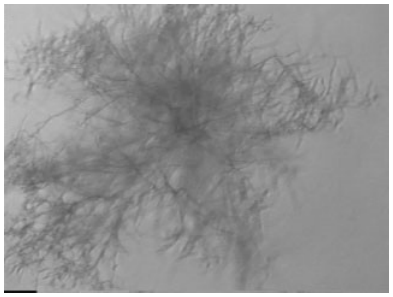
Avoidance of Contamination

identification

- optically easy to identify

treatment

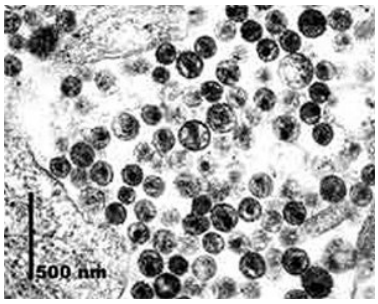
- antifungal agents
(amphotericin B, nystatin)



Avoidance of Contamination

viruses

Any of a large group of submicroscopic agents that 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

cross contamination

mixture of cell lines of different donors or spezies

Avoidance of Contamination

the laminar air flow

- turn on the UV lamp for 20 min 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

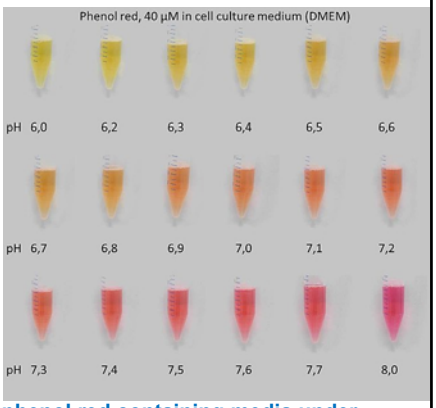
routine methods

- media change of adherent cells and cell suspensions
- subculture of adherent cells and cell suspensions
- cell quantification
- storage, cryopreservation of cells

Media Change

Why?

- "feeding" of cells for creating optimum growing conditions
- components of the medium are metabolized or broken, which leads to acidification of the medium
- visible colour change of phenol red medium



phenol red containing media under different pH values

Media Change

- before (occasionally also after) medium change check cultures using an inverted microscope

  you have 2 options:
  - change the complete medium or
  - only a part of the medium

- remove the old medium either by tilting or aspiration

Media Change of Adherend Cells

Procedure

- to change media, warm up fresh culture media at 37°C in water bath or incubator for at least 30 min
- carefully pour of the media from the flask into a waste pot containing some disinfectant
- immediately replace the media with fresh pre-warmed culture media and return to CO_2 incubator

Media Change of Adherend Cells

Frequency

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

Subculture of Adherend Cells

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

several possibilities

- **trypsin, trypsin/EDTA**
- **shake off**
- **other enzymes**
- **scraping**

Subculture of Adherend Cells

Procedure

- **view cultures using an inverted microscope to assess the degree of confluency and confirm the absence of bacterial and fungal contaminants**
- **remove old medium**
- **wash the cell monolayer with PBS without Ca^{2+}/Mg^{2+}**
- **pipette trypsin/EDTA onto the washed cell monolayer using 1 ml per 25 cm^2 of surface area**
- **move flask to cover the monolayer with trypsin.**

Subculture of Adherend Cells

- examine the cells to ensure that all the cells are detached and floating
- resuspend the cells in a small volume of fresh serum-containing medium to inactivate the trypsin
- transfer the required number of cells to a new labeled flask containing pre-warmed medium
- incubate as appropriate for the cell line
- repeat this process as demanded by the growth characteristics of the cell line

Subculture of Adherend Cells

shakeoff-cells are brought in suspension by tapping and rinsing

- beat vigorously with your fingers on the underside of the culture flask or rinse well
- transfer cell suspension to new culture flasks (cell number!) and fill with medium or
- centrifuge the cell suspension and resuspend the pellet with new medium to spread to new culture flasks

Subculture of Adherend Cells

other enzymes

- collagenase
- dispase
- pronase

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

Subculture of Adherend Cells

scraping - mechanical dissociation

- remove old medium
- wash with PBS
- gently scrape with a rubber scraper
- mix to dissolve cell aggregates
- transfer cell suspension to new culture flasks (cell number!) and fill with medium or
- after centrifugation add new medium to spread to new culture flasks

Subculture of Adherend Cells

cell scraper

Subculture of Suspension Cells

Procedure

- control cell morphology by an inverted phase contrast microscope:
  - Cells growing in exponential growth phase should be bright, round and refractile
- take a small sample of the cells from the cell suspension
- calculate cells/ml and re-seed the desired number of cells into freshly prepared flasks without centrifugation just by diluting the cells
- repeat this every 2-3 days

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

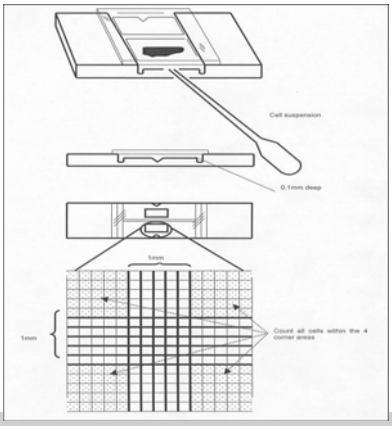
Cell Quantification

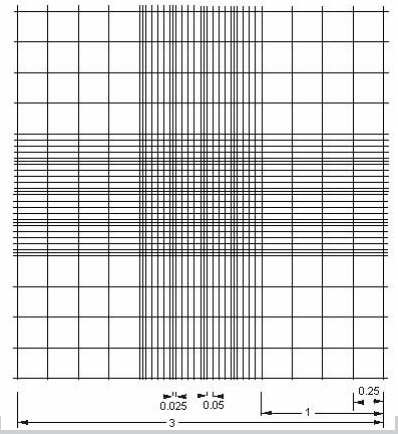
Cell Quantification

Trypan blue staining

- Trypan blue is a vital stain used to selectively detect dead cells
- 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

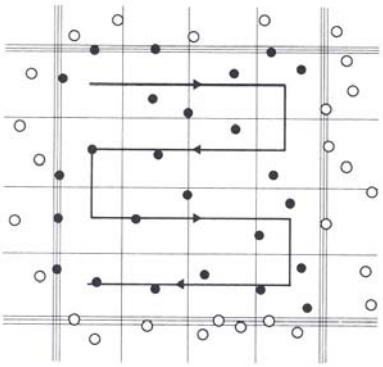
Procedure

- **bring adherent cells into suspension using trypsin/EDTA and resuspend them in a volume of fresh medium. For cells that grow in clumps, centrifuge and resuspend them in a small volume and gently pipette for disaggregation**
- **under sterile conditions, remove 10-20µL of cell suspension**
- **add an equal volume of Trypan Blue (dilution factor =2) and mix by gentle pipetting.**

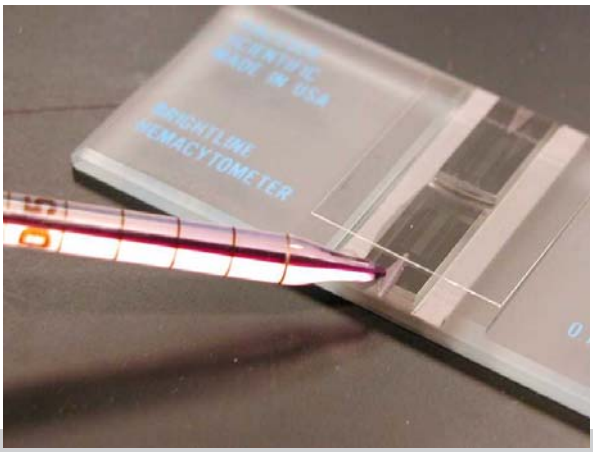
Cell Quantification

- slide the cover-slip over the chamber back and forth using slight pressure until Newton's refraction rings appear
- fill both sides of the chamber (approx. 10µL) with cell suspension and view under a light microscope
- 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. Calculate the concentration of viable and non-viable cells and the percentage of viable cells

Cell Quantification



Cell Quantification



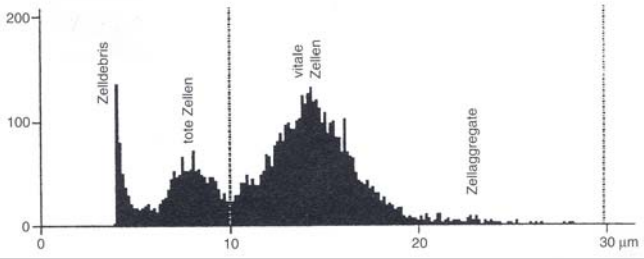
Cell Quantification

electronic cell counter; measurement of:

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

Cell Quantification

```
CASY 1  MODEL TT    COMMENT.....................
RANGE:    10.0 TO 30.0 µm   DILUTION       :   100
COUNTS/ml        :1.448E06   SAMPLE         :   1x400 µl
COUNTS >30 µm    :0.000E00   VOLUME (TOTAL):7.905E06 fl
DIAMETER (MAX)   :14.40 µm   VOLUME (MAX)   :1.564E03 fl
DIAMETER (MEAN)  :14.82 µm   VOLUME (MEAN)  :1.889E03 fl
```



Passaging

- number of generations
  - Total number of doublings of cells after start of cultivation
- doubling time
- contact inhibition
  - growth of monolayer cells until cell-cell contact

Passaging

- confluency:

densest neighbouring of cells in a monolayer

- passage:

Transfer of cells from one culture dish to another

- vital staining:

e.g., with Trypan blue

Storage

- For a short time at -80 ° C

- For years, at -196 ° C in liquid nitrogen

- Protective substances: glycerol and DMSO
  → prevention of crystal formation inside and outside the cell, and dehydration of the cytoplasm
- controlled freezing and rapid thawing is important

Resuscitation of Frozen Cells

Procedure

- thaw vial quickly in a 37°C water bath
- immediately transfer cell suspension to a sterile, 15 ml centrifuge tube and dilute with appropriate media; spin cells down at 4°C
- resuspend cells in 10 mls of media and count the viability; spin cells down at 4°C
- resuspend pellet in appropriate volume of medium and seed the cell suspension into flasks

Cryopreservation of Cells

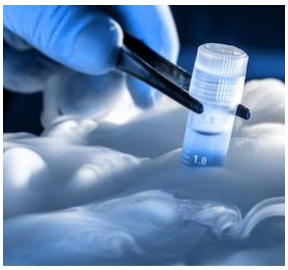
Procedure

- check cultures using an inverted microscope to assess the degree of cell density and confirm the absence of contaminations
- bring adherent cells into suspension using trypsin/EDTA as above and resuspend them in a volume of fresh medium at least equivalent to the volume of trypsin. Suspension cell lines can be used directly
- 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

Cryopreservation of Cells

- resuspend cells at a concentration of 2-4 x 10^6 cells / ml in freezing medium
- pipette 1ml aliquots of cells into cyroprotective ampules (labeled with the cell line name, passage number, cell concentration and date)
- Place ampules inside a passive freezer e.g. Nalgene Mr. Frosty
- Fill freezer with isopropyl alcohol and place at –80ºC overnight
- Frozen ampules should be transferred to the vapor phase of a liquid nitrogen storage vessel and the locations recorded

Handling of Probes in Fluid Nitrogen



https://www.sigmaaldrich.com/technical-documents/protocols/biology/cryopreservation-storage-cells.html

Before you start

- design a study
- including all groups and controls
- Incubation times (e.g., acute or chronic administration of drugs)
- write a schedule
- use at least duplicates and all necessary controls
- randomize your probes

Controls

for colorimetric assays (ELISA etc.)
- medium control without cells
- medium control without cells with vehicle

for all tests
- control with cells without vehicle
- control with cells with vehicle

Start with pretty simple tests

- reduced metabolism/ growth rate
  resazurin, BrdU
- breakdown of membrane permeability
  JC-1, PI
- tissue specificity of response
  comparison of different cell lines
- ability to metabolize toxic compounds
  quantification of compound in cell suspension and supernatant
- genetic effects/mutagenicity
  molecular biology

Cell Culture Application