BASICS OF ANIMAL CELL CULTURE

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Cell Culture

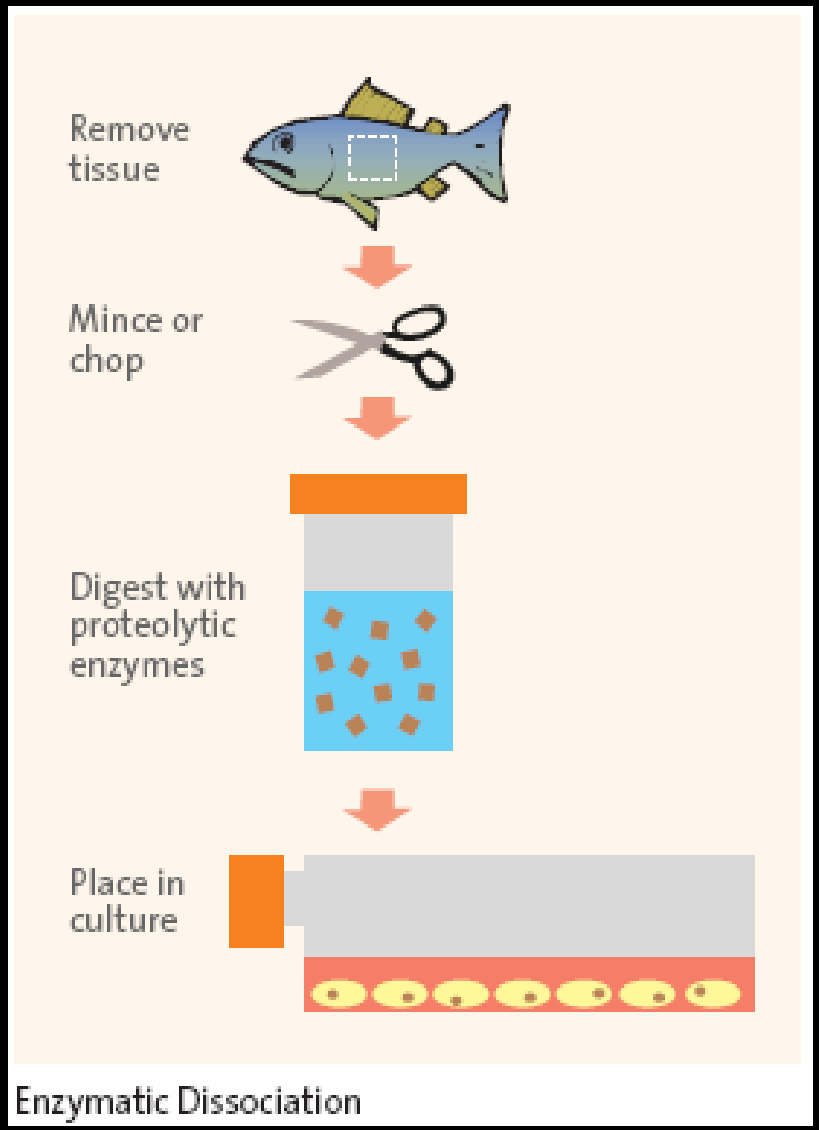
The maintenance of cells outside of the living animal (*in vitro*) for easier experimental manipulation and regulation of controls.

- **Pros**
- Use of animals reduced
- Cells from one cell line are homogenous and have same growth requirements, optimizing growing patterns.
- *In vitro* models allow for control of the extracellular environment
- Able to monitor various elements and secretions without interference from other biological molecules that occurs *in vivo*

Classification of Cell Cultures

- **Primary Culture**
  - Cells taken directly from a tissue to a dish
- **Secondary Culture**
  - Cells taken from a primary culture and passed or divided *in vitro.*
  - These cells have a limited number of divisions or passages. After the limit, they will undergo *apoptosis.*
    - Apoptosis is programmed cell death

Making a Primary Culture



Enzymatic Dissociation

Types of cell culture

Tissue or organ fragment

Transfer to culture

Primary culture

Subculture (passage)

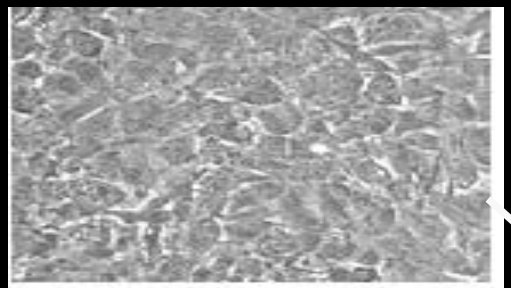
Cell line

Continuous

Finite

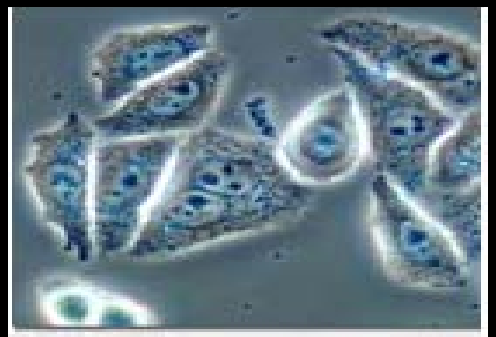
Stem cell

Primary culture



Example:
Embryonic Fibroblasts

Secondary culture

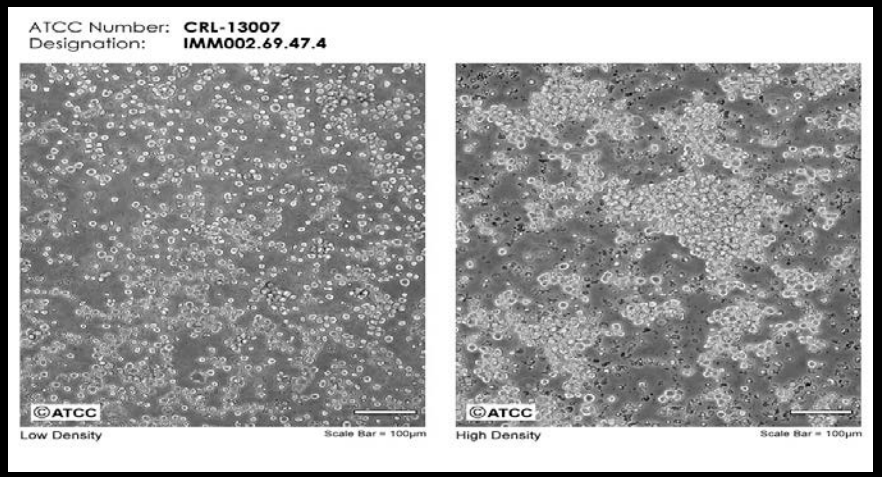


Example:
HeLa

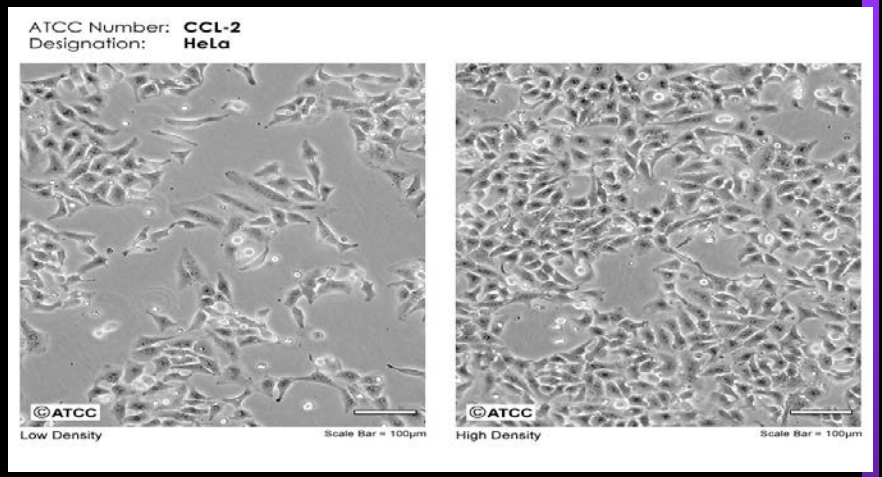
Example:
Stem Cells

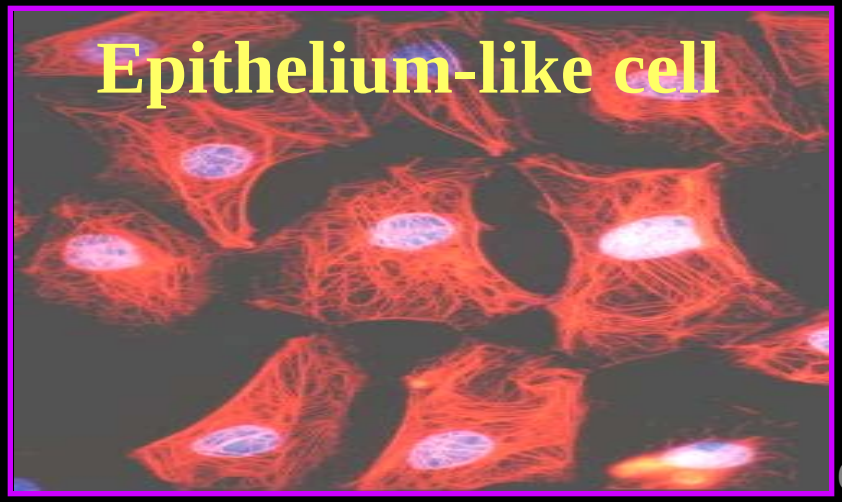
Culture characteristics

Suspension:

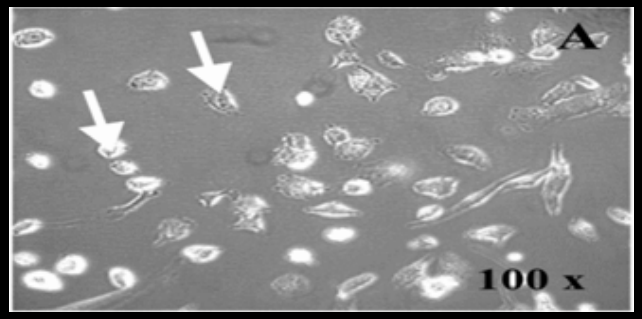


Adherent :

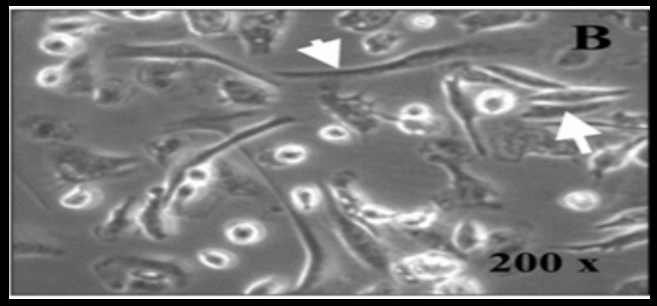




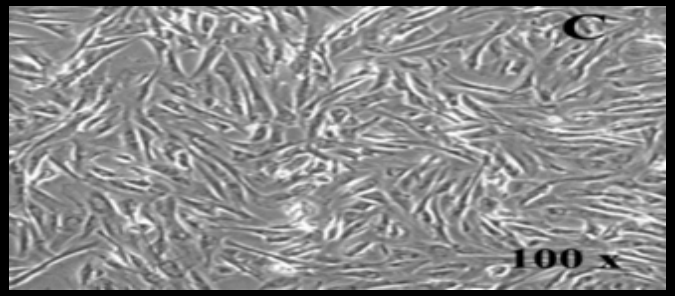
Confluency Measure



At the time of seeding



After attachment



Complete Monolayer

SOURCING CELL LINES

Sources of cell lines

American Type Culture Collection ATCC

– *www.atcc.org*

• NIH

– Coriel Cell Repositories http://locus.umdnj.edu/ccr/

• European Collection of Cell Culture

– *www.ecacc.org.uk*

• DSMZ (German Collection of Microorganisms and Cell Cultures)

-*www.dsmz.de* (very good for plant cell lines)

- NCCS, Pune

PRE-REQUISITES FOR STARTING CELL CULTURE LAB

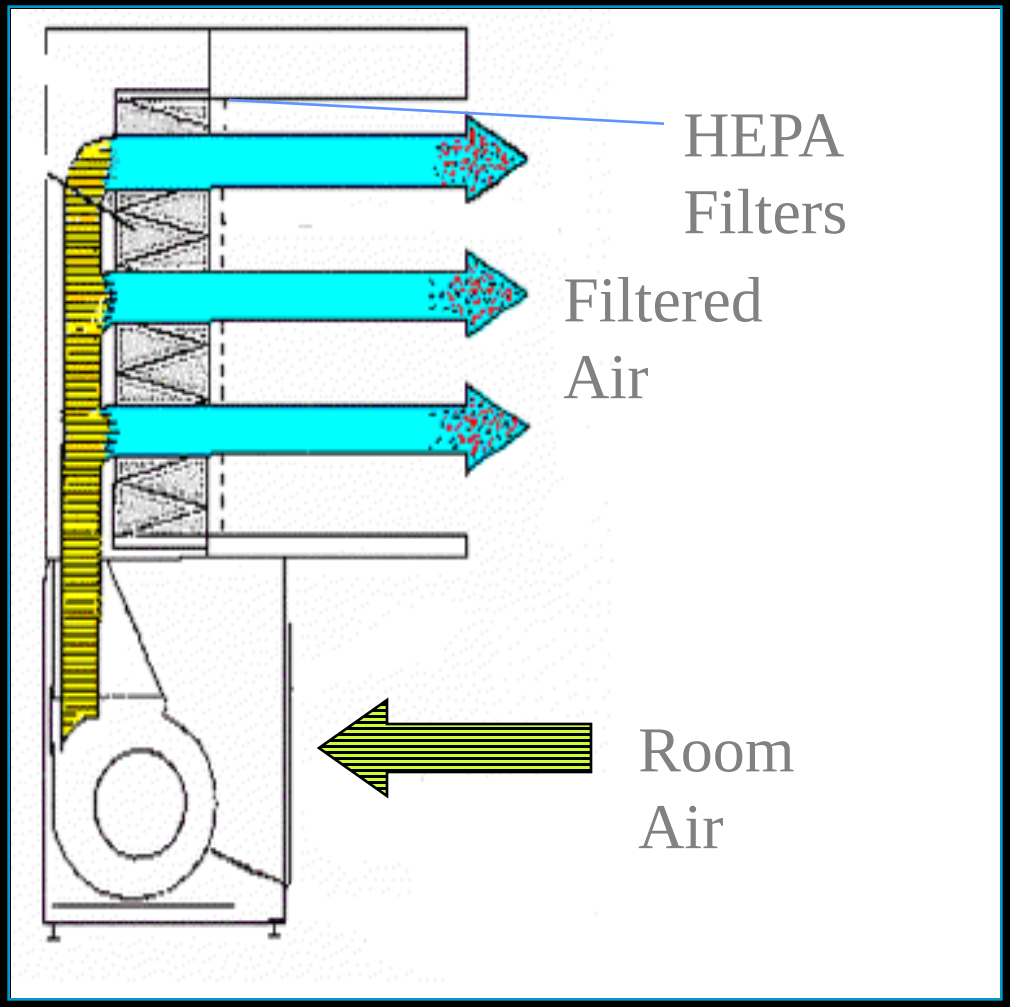
Essential Equipment

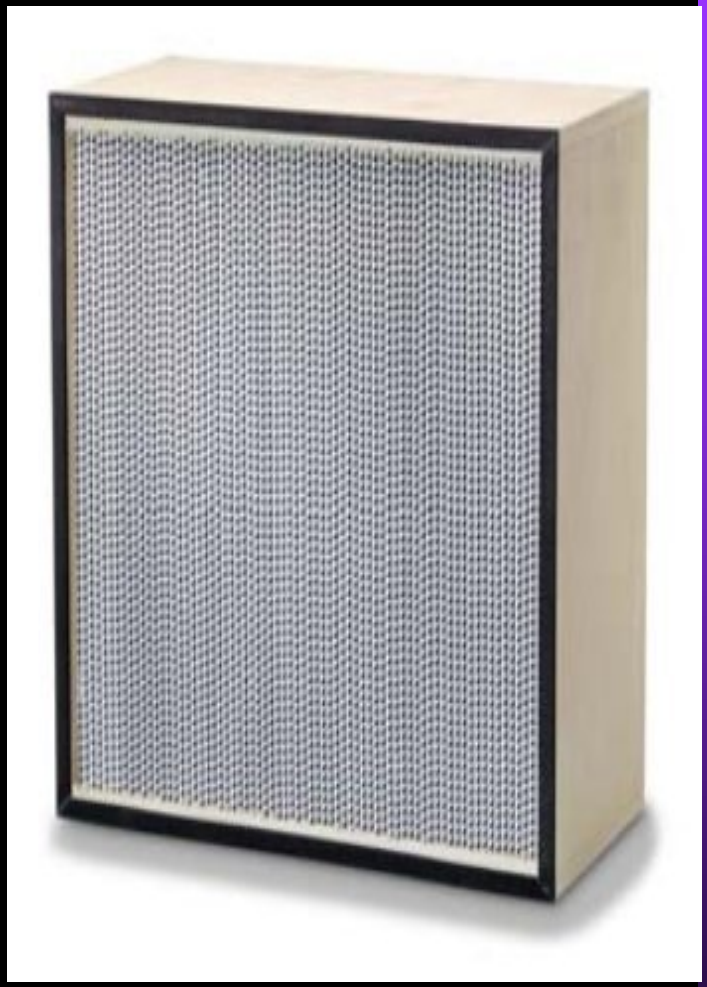
- Laminar flow hood
- (biological safety cabinet)
- CO2 incubator (for most cells)
- Inverted microscope
- Pipette aid
- Aspiration pump
- Centrifuge
- Water bath
- Cold storage (refrigerator)
- Cryopreservation equipment

Laminar flow hood


BH2000 Series
BIOLOGICAL SAFETY CABINET CLASS II – FOR PERSONNEL, ENVIRONMENT AND PRODUCT PROTECTION
NOT DESIGNED FOR USE WITH FLAMMABLE, EXPLOSIVE OR HIGHLY VOLATILE LIQUIDS OR CYTOTOXIC DRUGS
DANGER

Horizontal Air Flow Hood





HEPA Filters

CO_2 incubator

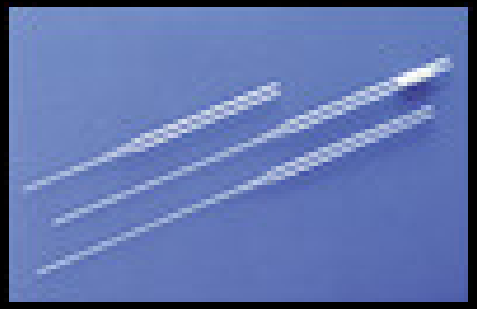
- maintains CO_2 level (5-10%), humidity and temperature (37^o C) to simulate in vivo conditions.

Water bath

- To warm media, TRED (Trypsin-EDTA) and PBS (phosphate buffered saline) before placing on cells
- Can harbor fungi and bacteria, spray all items with 70% ethanol before placing in the hood.
- Usually takes 10 -15 minutes for media to warm, 5-10 for TRED to thaw

Vacuum pump

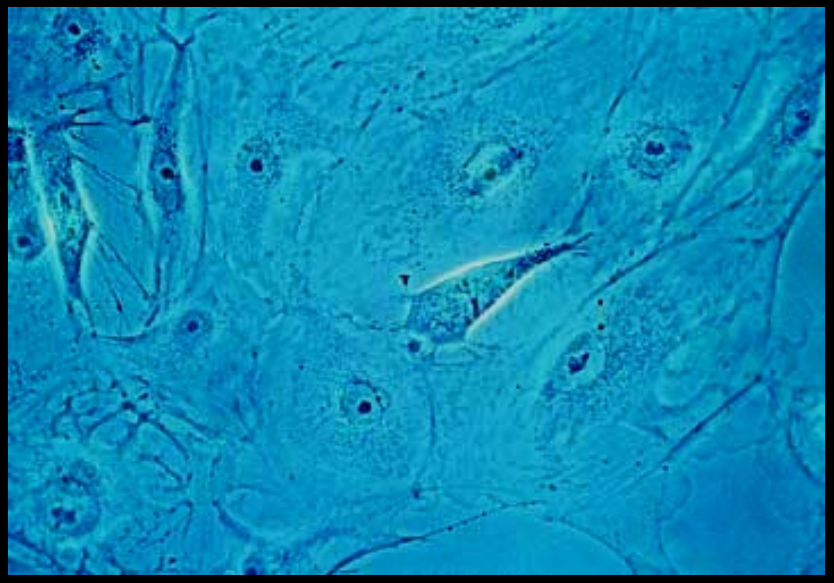
- For permanent aspiration of liquids (media, PBS and TRED).
- Use unplugged glass pasteur pipets, throw into sharps box when done.

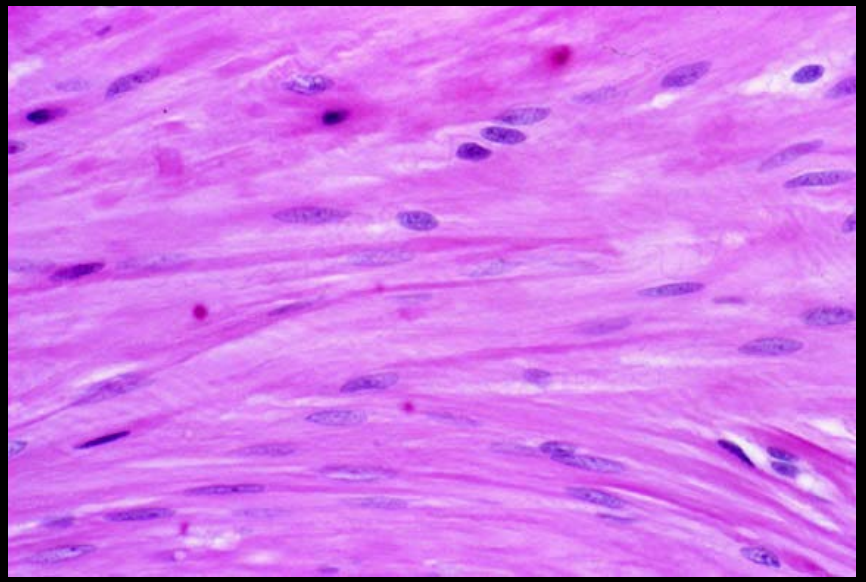


Inverted Phase Microscope

- A phase contrast microscope with objectives *below* the specimen.
- A phase plate with an annulus will aid in exploiting differences in refractive indices in different areas of the cells and surrounding areas, creating contrast

A comparison





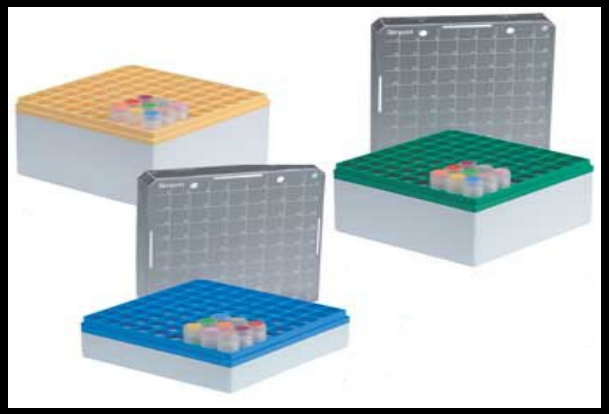
Phase contrast microscopy
Can be used on living cells

Light microscopy
requires stain, thus killing cells

Liquid Nitrogen containers

- Contamination by microorganisms and other cell lines
- Need for distribution to other users

Equipment for Cryopreservation



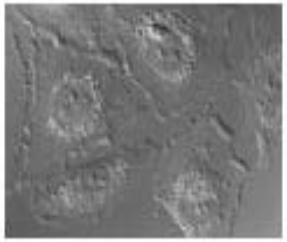
Liquid nitrogen

Liquid phase (-196ºC) or vapor phase (-156ºC)

Cell culture vessels

Tissue culture flasks

Multi well plates



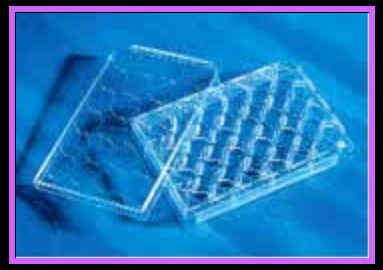
Roller bottle

Spinner flask

Bioreactor

Small scale cultivation



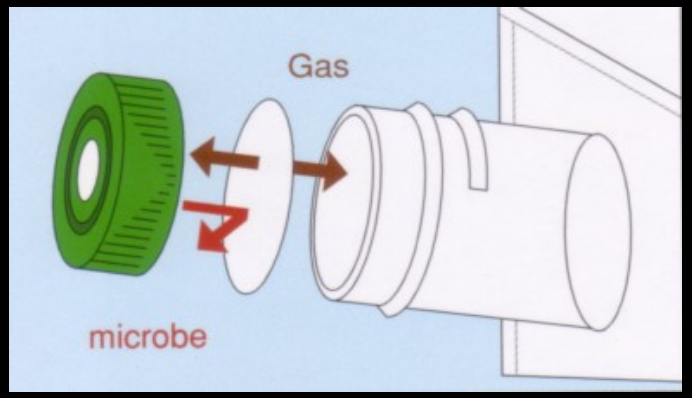
Culture Plate



Culture flask

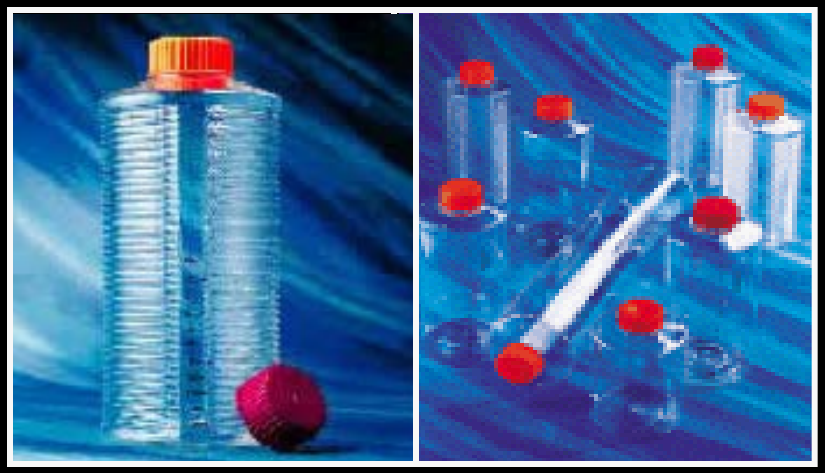
Tissue culture Flasks

Filter Caps

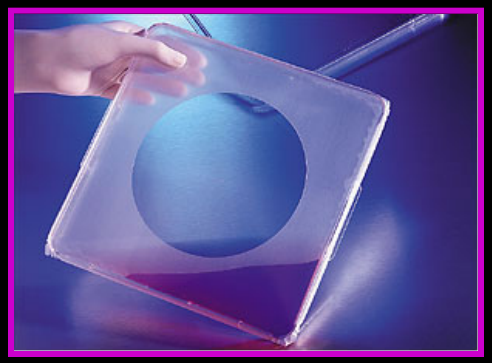


Large scale cultivation

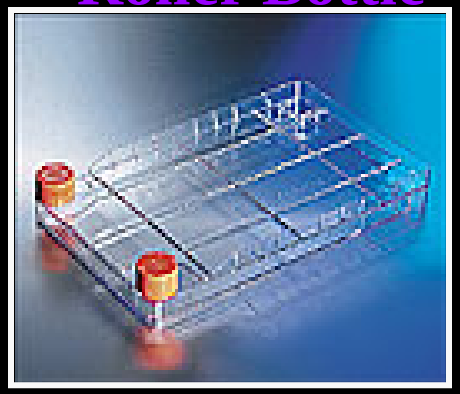
Culture bottle



Roller Bottle



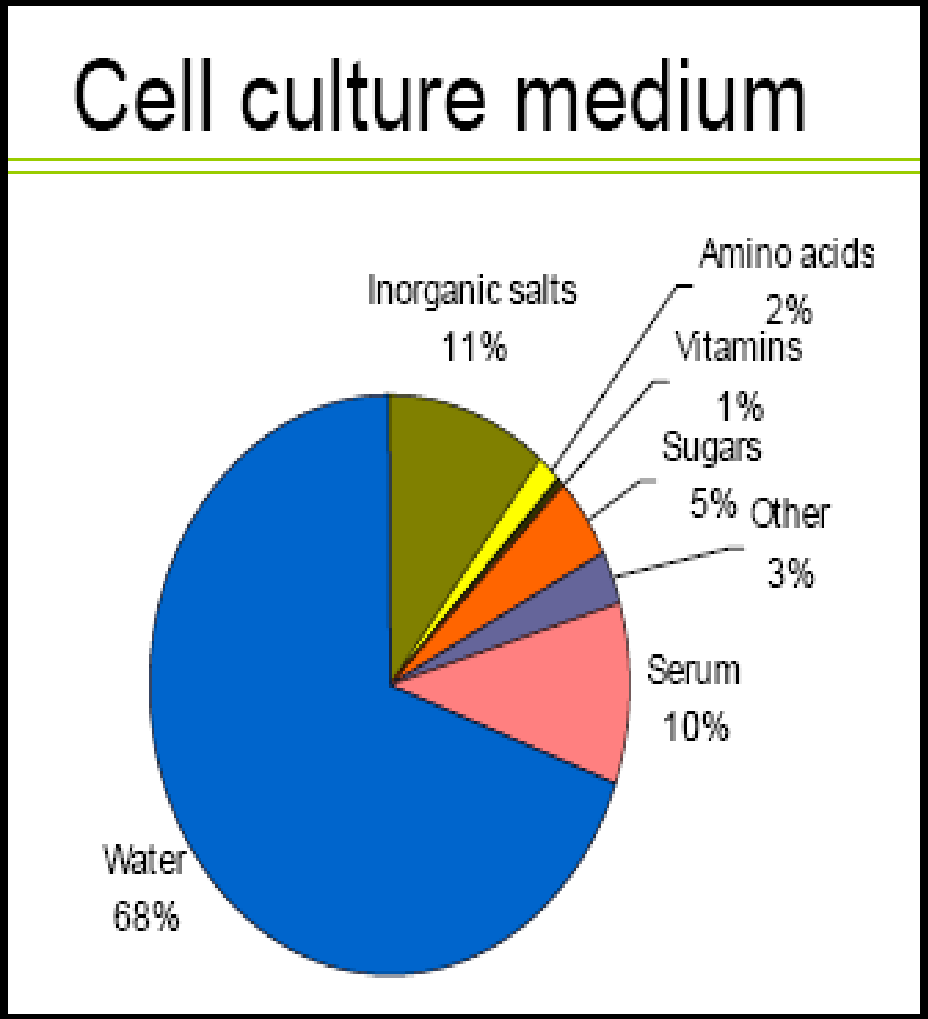
Culture dish



Stack culture chamber

MEDIA AND IT'S COMPONENT

Basic Constituents



- **Energy sources-** eg. Glucose, Galactose
- **Serum** – eg. FBS, FCS. Complex mixture of albumins , growth factors and growth inhibitors
- **Trace Elements-** eg. Co-factors (Zn, Cu) for biochemical pathways
- **Amino Acids-** eg. G*lutamine ,* Cystine
- **Vitamins** - Metabolic co-enzymes for cell replication
- **Antibiotics** – eg. Penicillin, Streptomycin etc.
- **Balanced salt solutions** ,Inorganic ions-Osmotic balance – cell volume

Ready made Media: RPMI (**Roswell Park Memorial Institute medium**), DMEM **(Dulbecco's Modified Eagle's *medium)*** , MEM (***Minimum Essential Medium* (*MEM*), developed by Harry Eagle**

Serum and its importance

- Complex mixture of albumins , growth factors and growth inhibitors.
- Increase the buffering capacity
- Protect against mechanical damage
- Source of growth factors
- Bind and neutralize toxins.

☒ risk of contamination- BVDV virus, mycoplasma.

☒Consistency in quality

PART – 2

CELL CULTURE TECHNIQUES

Media Preparation and Filtration

Powder media

↓

Reconstitute in D/w

↓

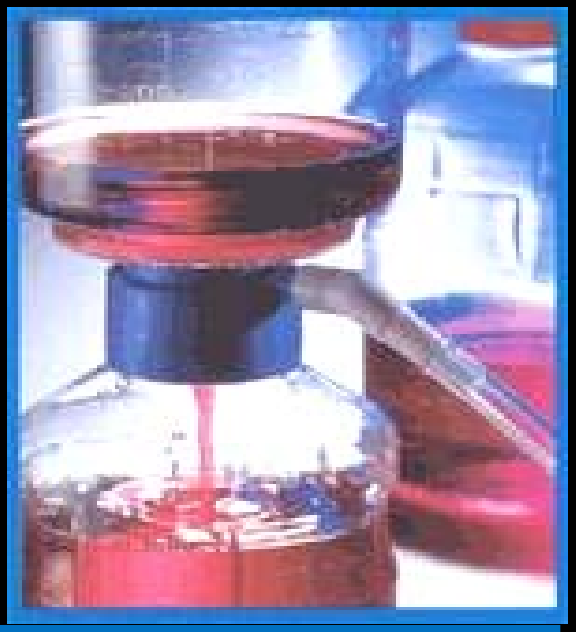
Dissolve completely

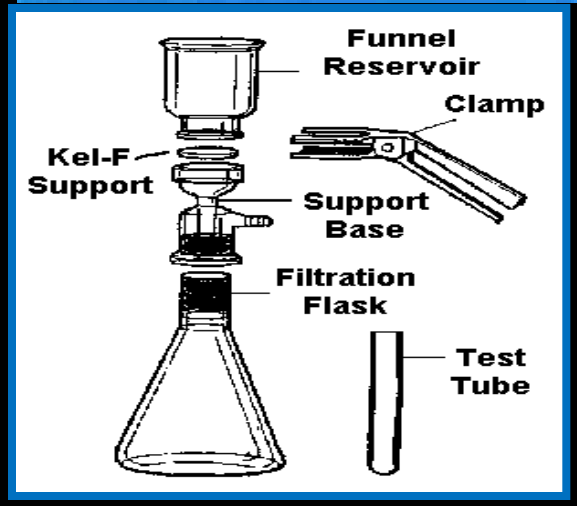
↓

Adjust pH

↓

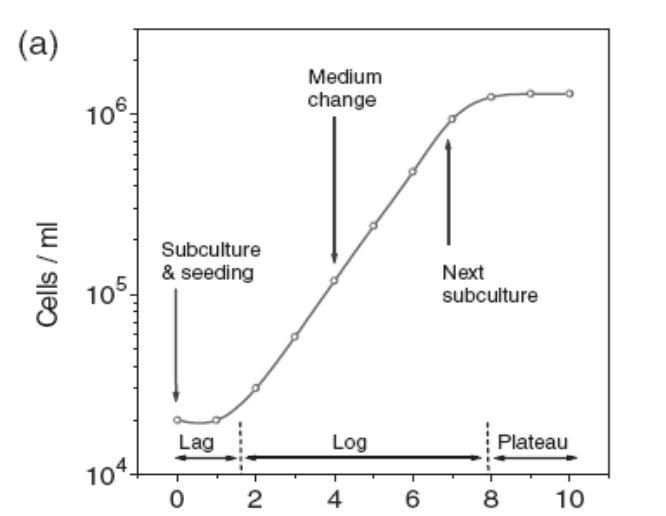
Filter

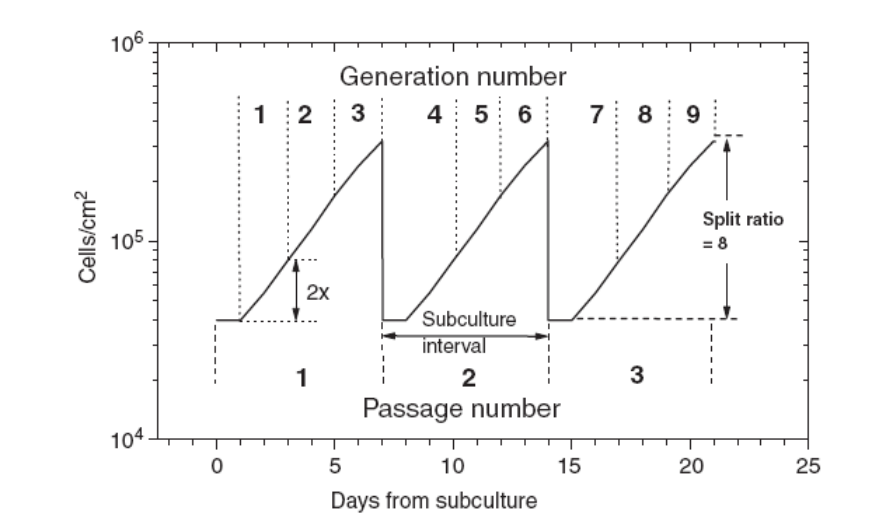




Sub culturing

Cells are harvested when the cells have reached a population density, which suppresses growth. Ideally, cells are harvested when they are in a semi-confluent state and are still in log phase.





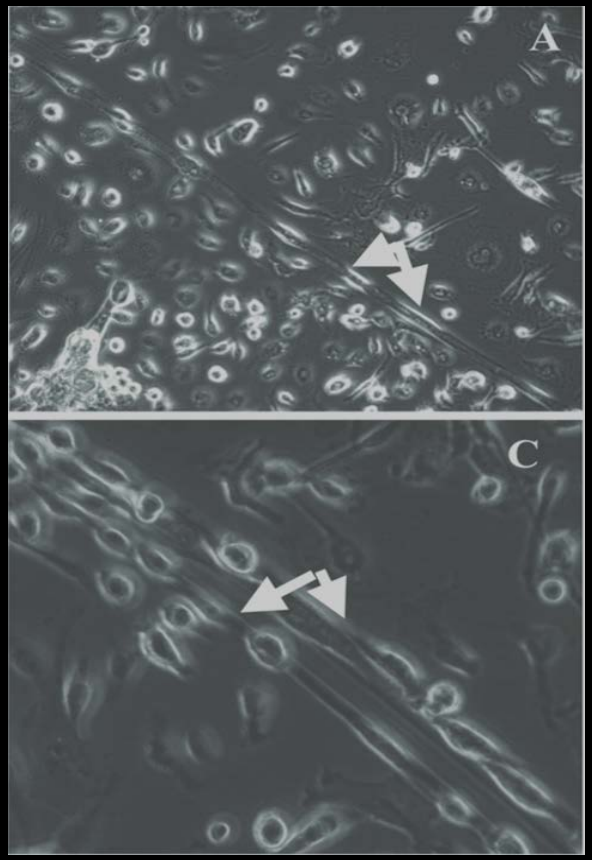
Subculture – Adherent Cells

Routine maintenance of cell lines is called subculture.

Flasks	PBS	Trypsin	Cell Seeding
T25 cm^2	5-7ml	0.5 ml	1-5 x 10^5 cells
T75 cm^2	10-15 ml	1.0 ml	9-20 x 10^5 cells
T175 / T180 cm^2	20-25 ml	4.0 - 5.0 ml	30-45 x 10^5 cells
Roller Bottles	~50 mL	5.0 - 10.0 mL	~ 10 x 10^6 cells

Caution: When adding (or replacing) medium, never touch the neck of the culture flasks with the bottle containing the medium or use the same pipette to transfer medium to more than one bottle. Ideally, aliquot the total amount of medium required for each batch of culture bottles being handled and store the remainder at 4–8°C. Dedicate separate medium for each cell line.

Sub culturing- adherent cell line



Observation of cells

if ~80-90% confluency

Give media change Incubate back

Aspirate spent media

Avoid liquid transfer by pouring.

Give PBS wash

Trypsin-EDTA addition incubate in RT/ 37 deg for 1-3 min.

Observe under microscope- till ~80 % cells appear rounded

Aspirate Trypsin-EDTA soln. completely 1-3 sharp raps from the surface of flask.

Addition of growth media dispersion of cells to single cell suspension –repeated pipetting

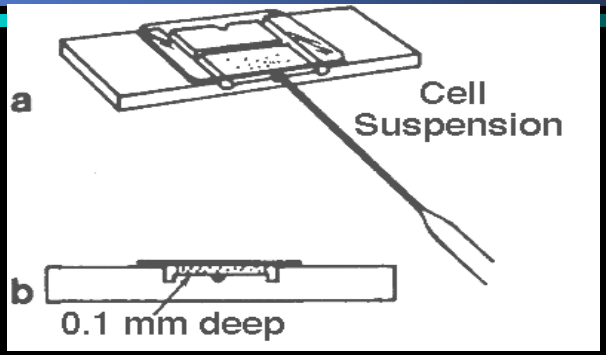
Good laboratory practice: Work with one cell line at a time.

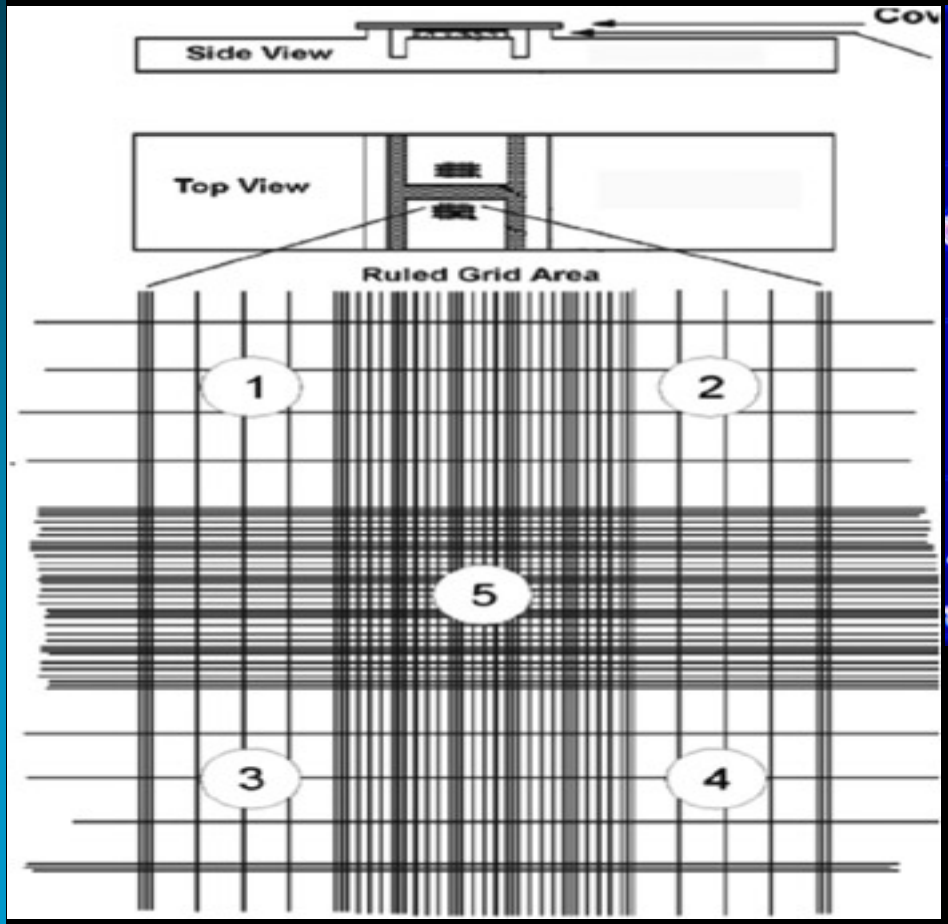
Cell viability

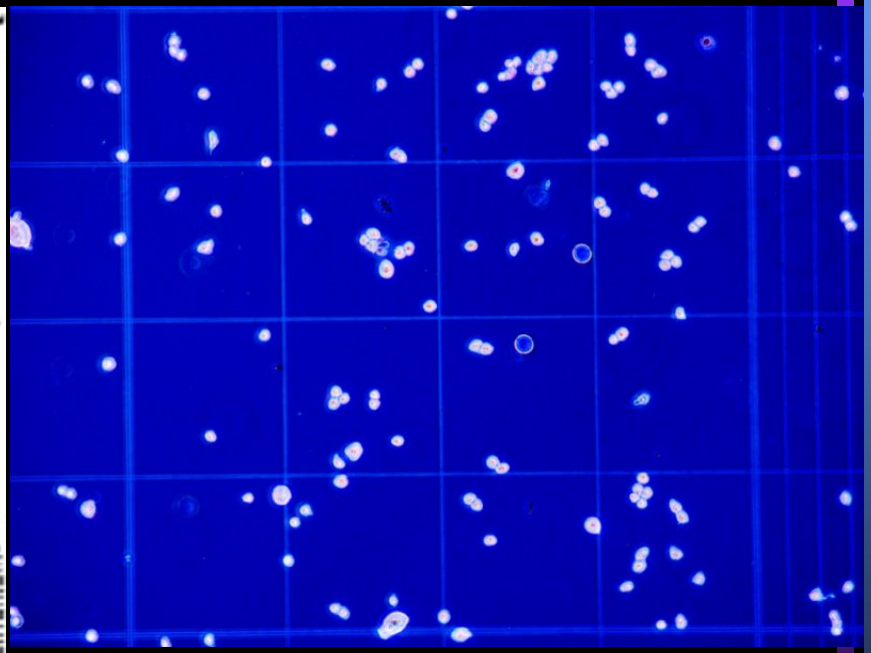
- Cell viability is determined by staining the cells with trypan blue
- As trypan blue dye is permeable to non-viable cells or death cells whereas it is impermeable to this dye
- Stain the cells with trypan dye and load to haemocytometer and calculate % of viable cells

$$\% \text{ of viable cells} = \frac{\text{No. of unstained cells}}{\text{Total no. of cells}} \times 100$$

Cell counting- Manual



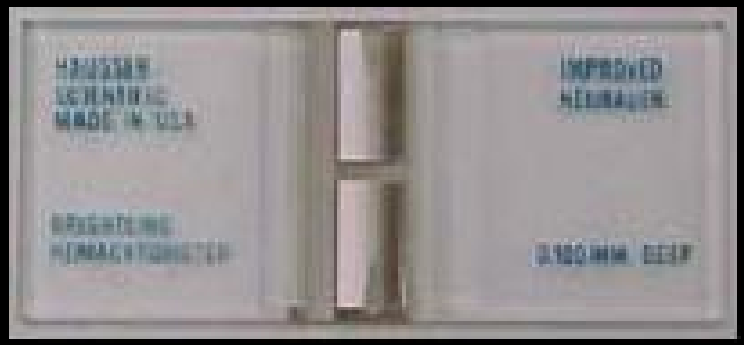


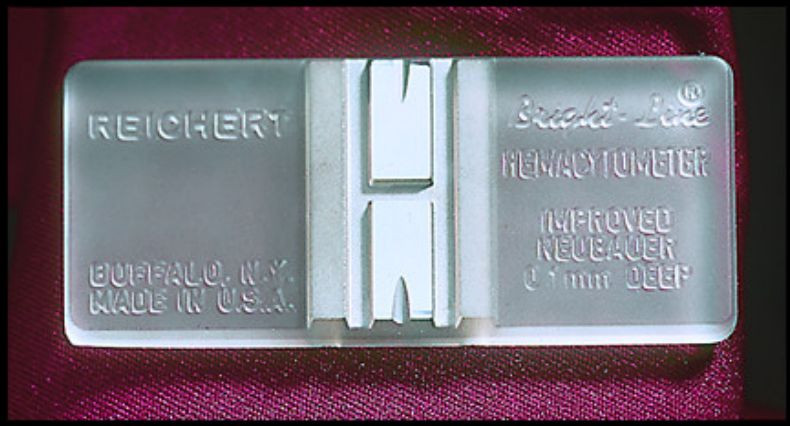


% of viable cells= No. of unstained cells x 100
Total no. of cells

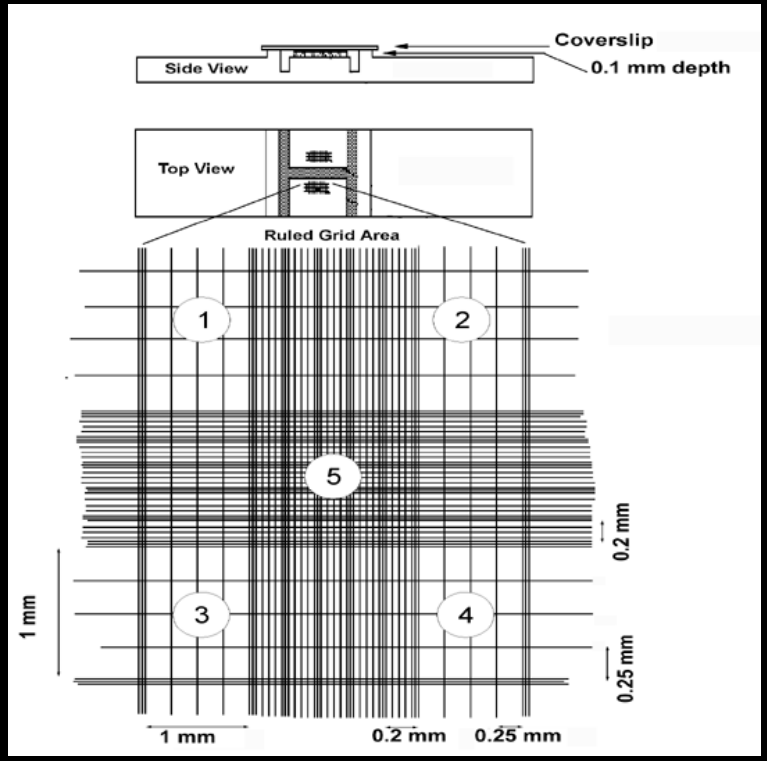
Hemacytometer

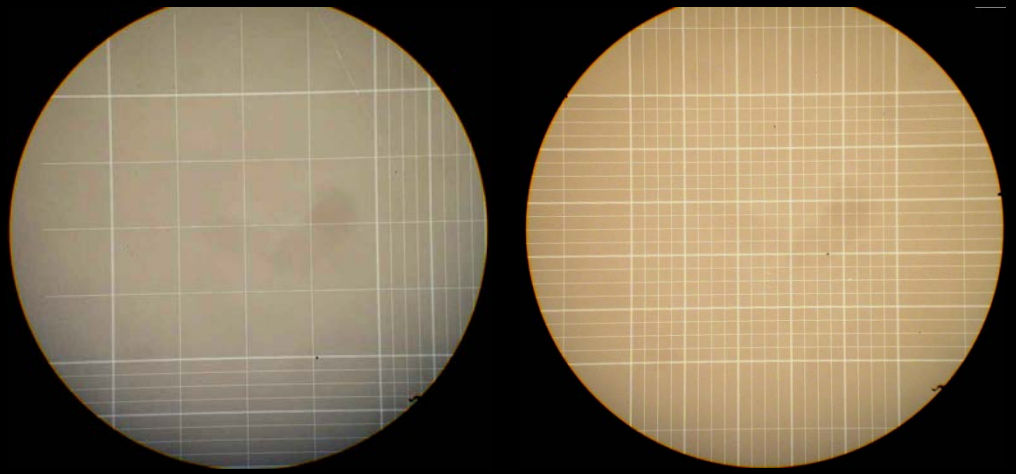
- Specialized chamber with etched grid used to count the number of cells in a sample.
- use of trypan blue allows differentiation between living and dead cells





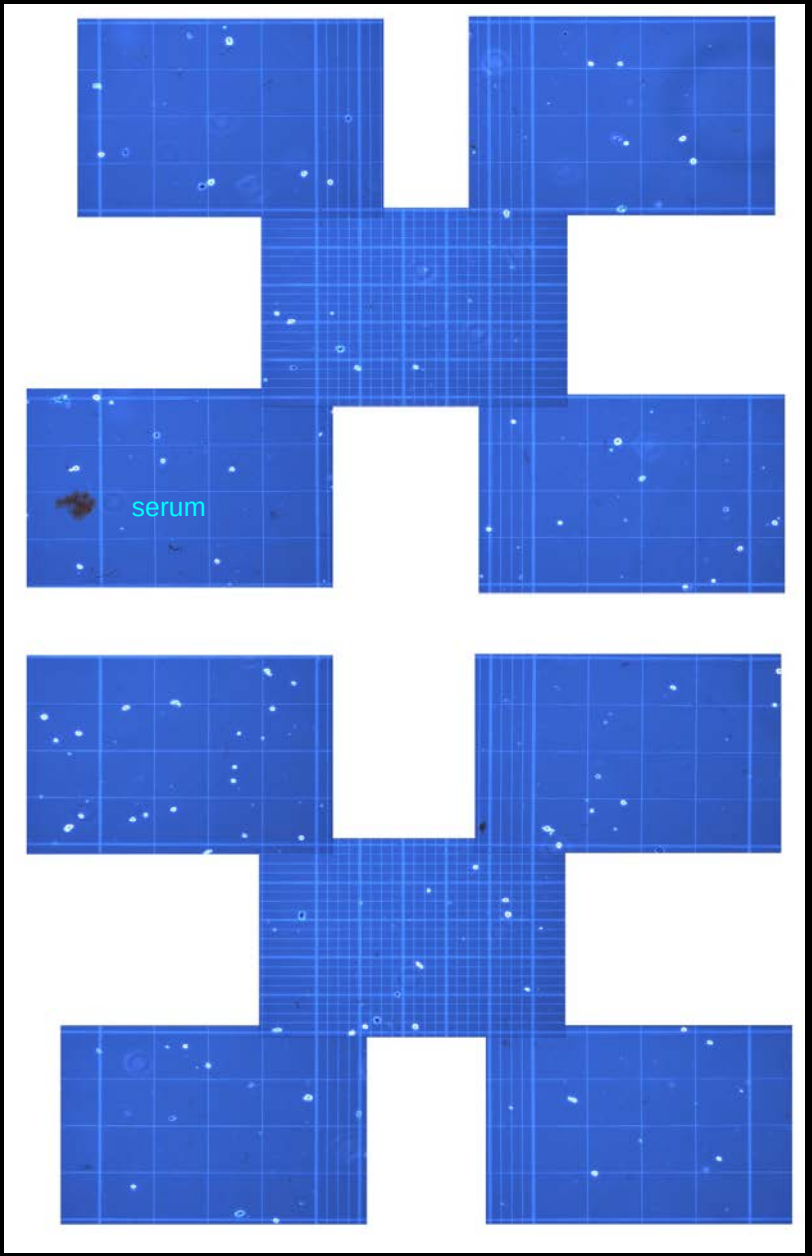
Looking at the grid under the phase contrast microscope



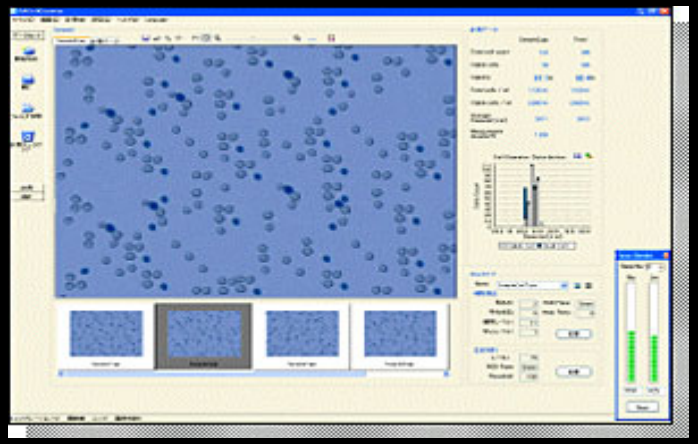


Count 10 squares
Any 10 will do but we will follow convention

Watch for stringy, reddish material—those aren't cells!

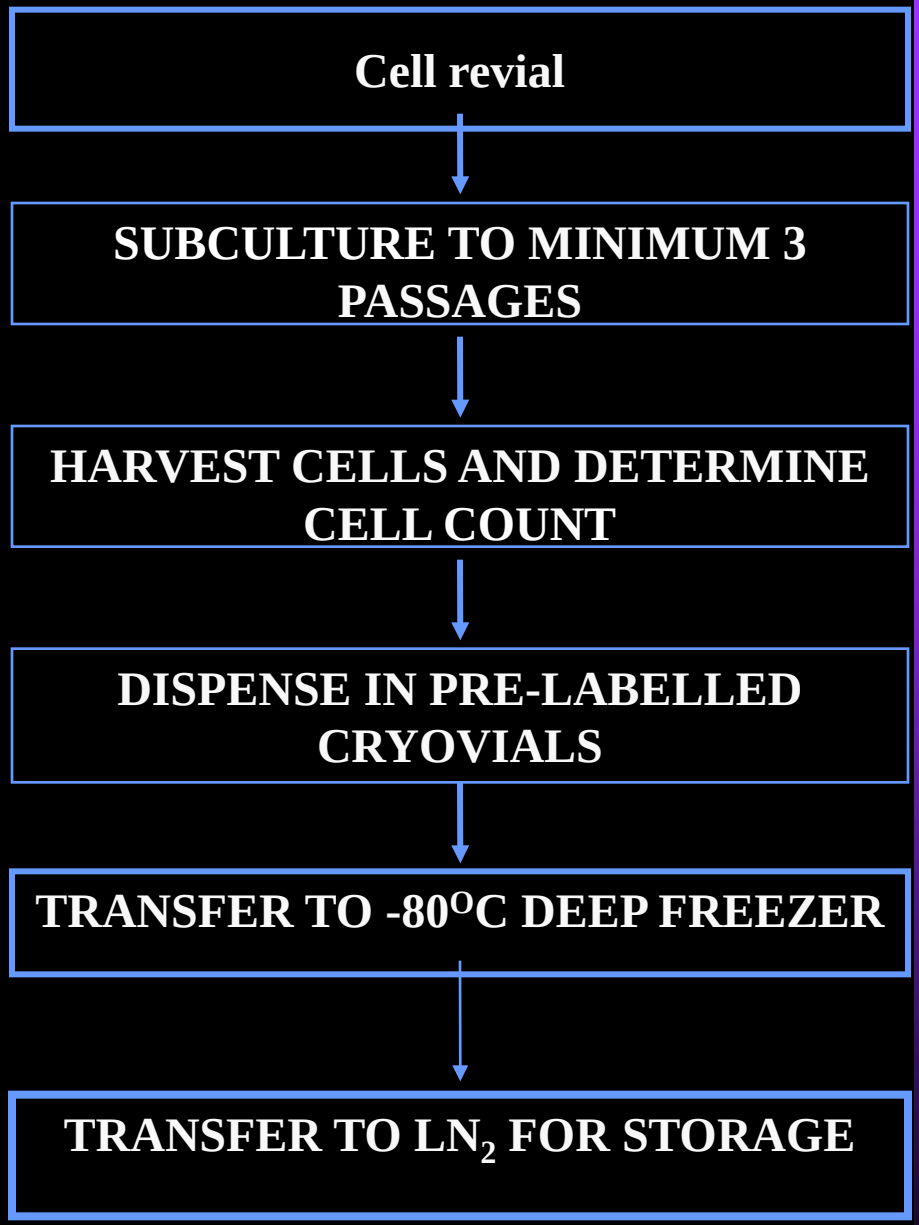


Automated Cell Counter



Cryopreservation

The process of preservation of cells by freezing at extremely low temperatures using cryo-protective agents (DMSO), so as to maintain the existing form, structure and chemical composition of all the constituent elements of the specimens for future use.

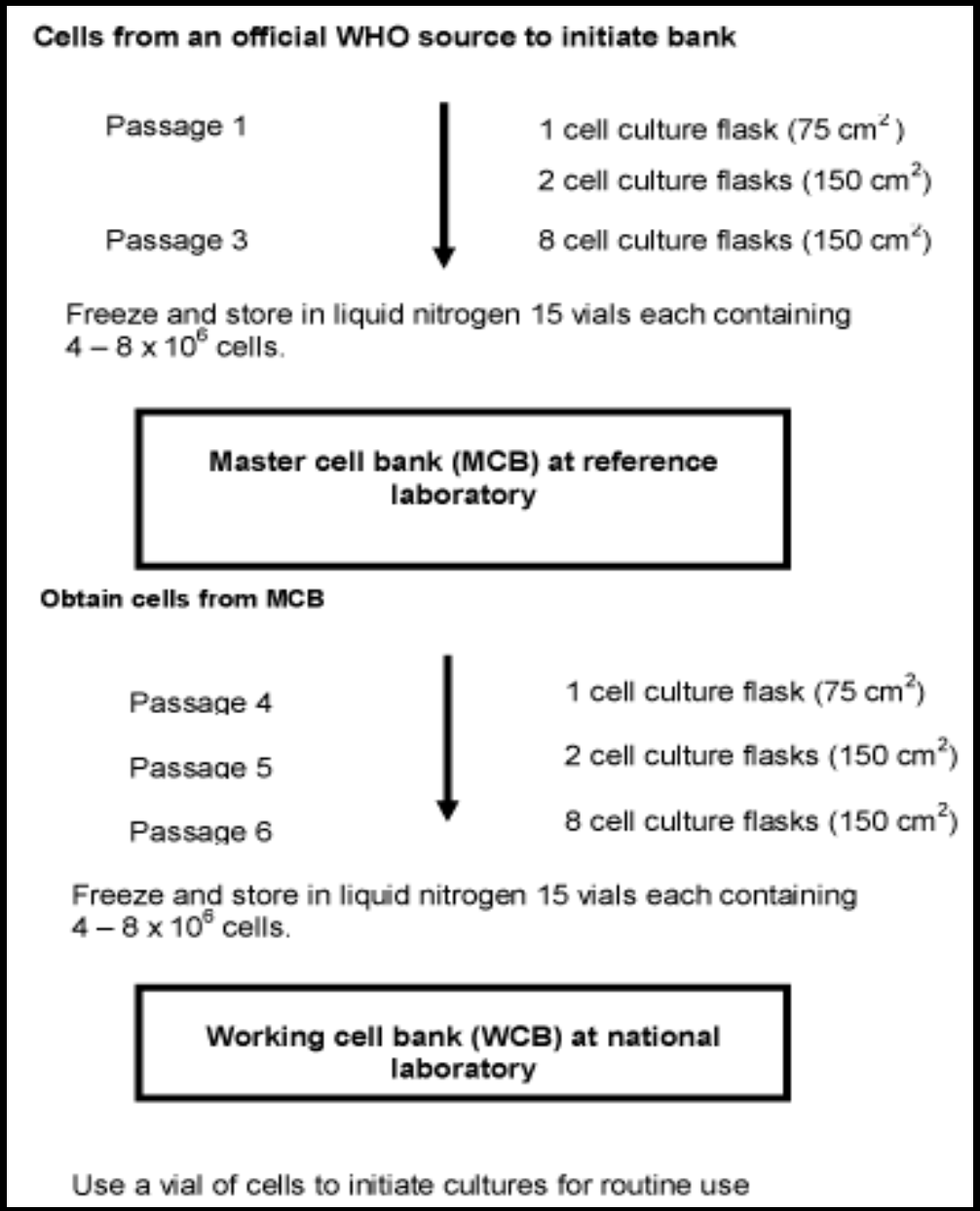


General Tips:

- Do not use overgrown cells.
- Make sure that there are no clumps
- Preserve correct number of cells per vial ($1 – 10 \times 10^6$ cells / ml).
- Do not leave cells in DMSO for long.
- Seal the vials tightly.
- Label the vials appropriately

Cell banking

Collection of containers of uniform composition stored under defined conditions, each containing an aliquot of a single pool of cells.



Problem: Rapid pH shift in medium

Red-Alkaline pH  Neutral pH

Acidic pH 

Possible Causes:

1. Incorrect carbon dioxide (CO_2) tension

Action: Increase or decrease percentage of CO_2 in the incubator based on concentration of sodium bicarbonate in medium.

2. Overly tight caps on tissue culture flasks – No penetration of CO_2

Action: Loosen caps one-quarter turn.

3. Insufficient bicarbonate buffering

Action: Add HEPES buffer.

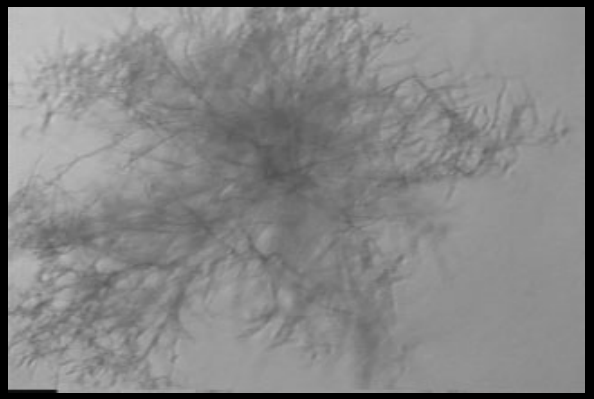
4. Incorrect salts in medium

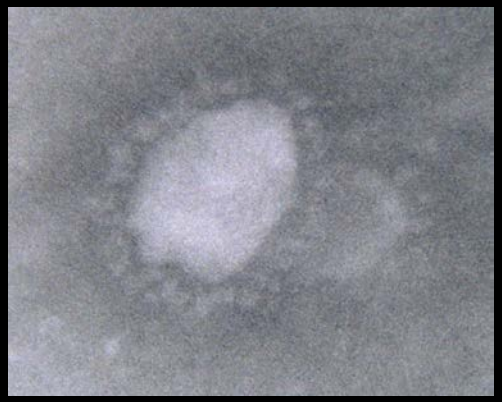
Action: Check for the Media Composition.

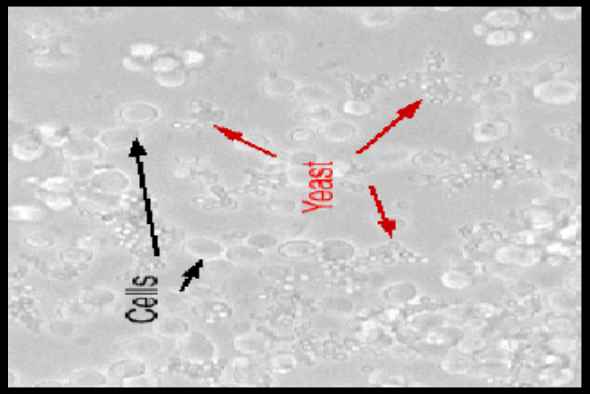
5. Bacterial, yeast, or fungal contamination

Action: Discard culture and medium or try to decontaminate culture.

Fungal Contamination







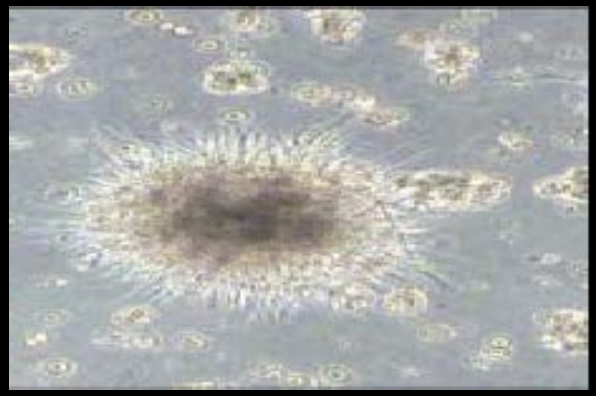
- Fungus-Molds
- Fungal - yeast

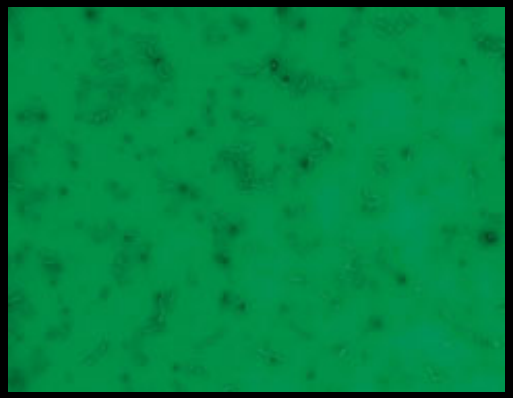
Come through:

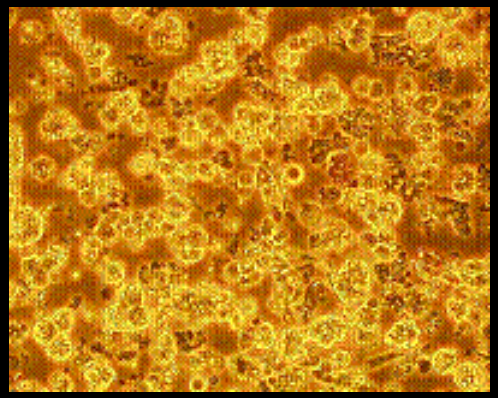
Improper Handling, Gowning, Cleaning

Aseptic handling
Antibiotics: Amphotericin B, Mycostatin

Bacterial contamination





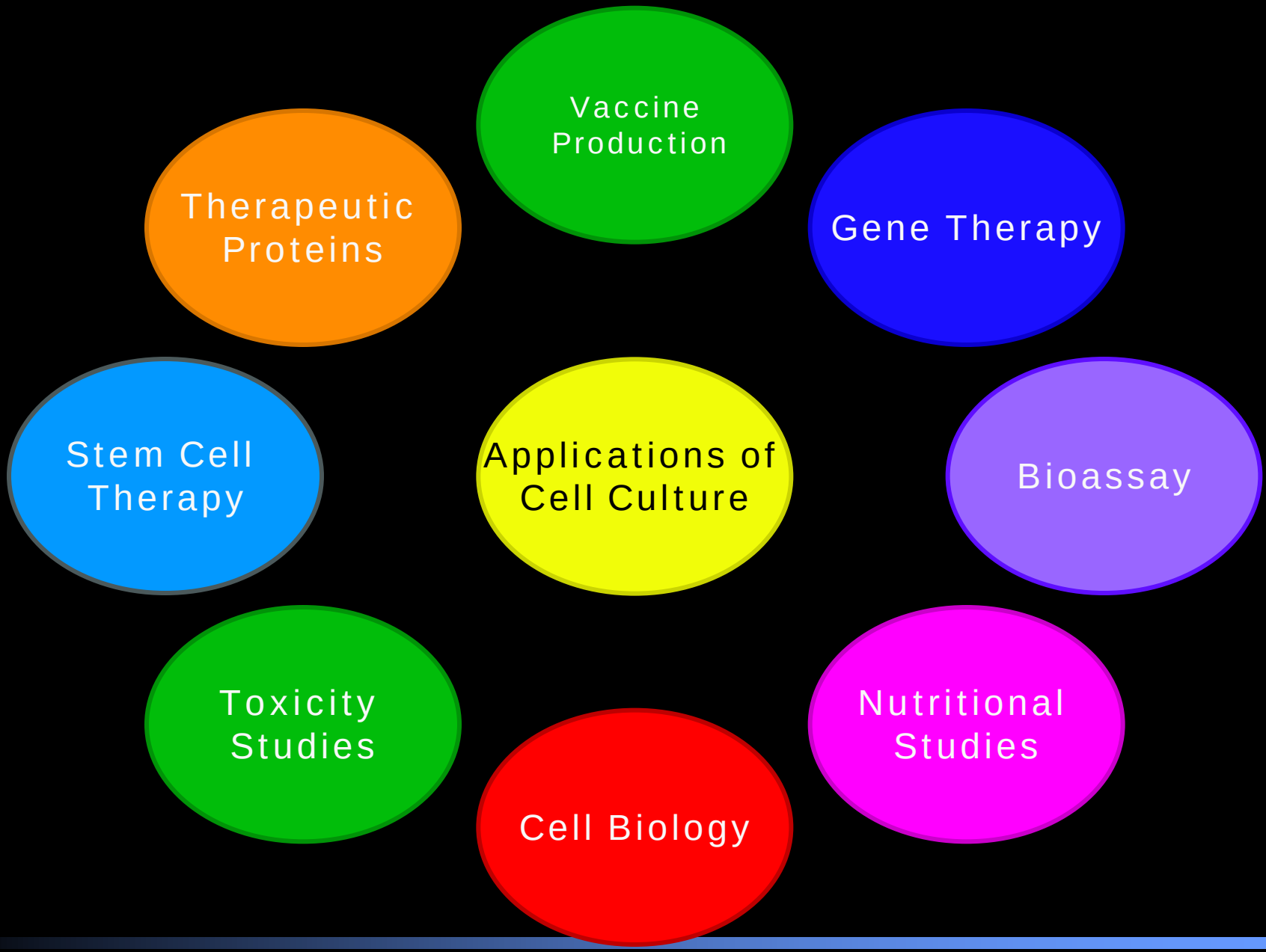


Come through:

Improper Cleaning, handling, cross contamination

Antibiotics: Penicillin, Streptomycin, Gentamycin

Applications of Cell culture

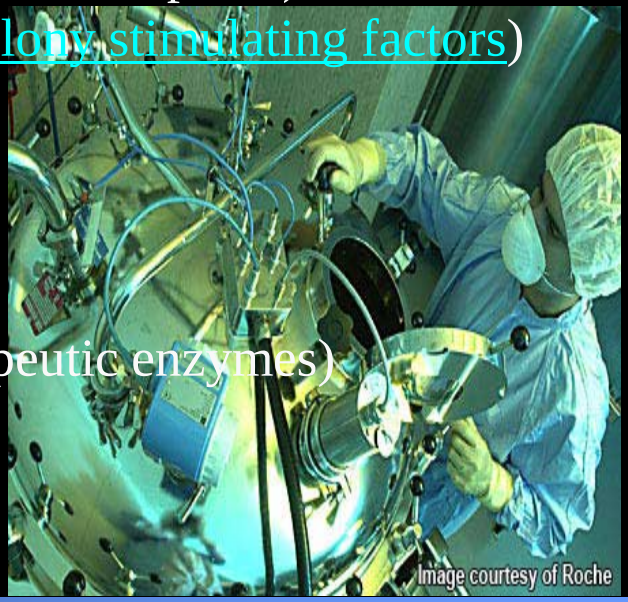

Vaccine Production
Therapeutic Proteins
Gene Therapy
Stem Cell Therapy
Applications of Cell Culture
Bioassay
Toxicity Studies
Nutritional Studies
Cell Biology

Biopharmaceuticals

Biopharmaceuticals

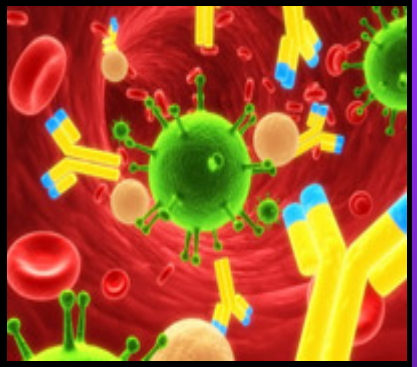
Are medical drugs produced using biotechnology. They are proteins (including antibodies), nucleic acids (DNA, RNA or antisense oligonucleotides) used for therapeutic or *in vivo* diagnostic purposes, and are produced by means other than direct extraction from a native (non-engineered) biological source.

Blood factors (Factor VIII and Factor IX)
Thrombolytic agents (tissue plasminogen activator)
Hormones (insulin, glucagon, growth hormone, gonadotrophins)
Haematopoietic growth factors (Erythropoietin, colony stimulating factors)
Interferons (Interferons-α, -β, -γ)
Interleukin-based products (Interleukin-2)
Vaccines (Hepatitis B surface antigen)
Monoclonal antibodies (Various)
Additional products (tumour necrosis factor, therapeutic enzymes)

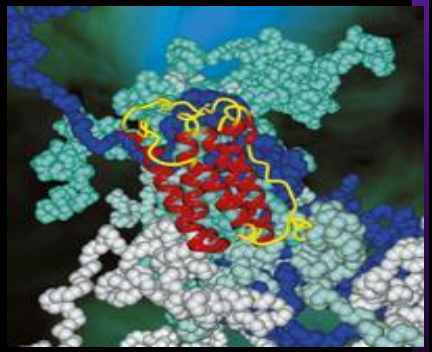


Antibodies Production:

Examples: Transplant rejection - Muronomab-CD3-
Cardiovascular disease - Abciximab
Cancer - Rituximab
Infectious Diseases - Palivizumab
Inflammatory disease – Infliximab
Cell line used: CHO, NSO, SP20



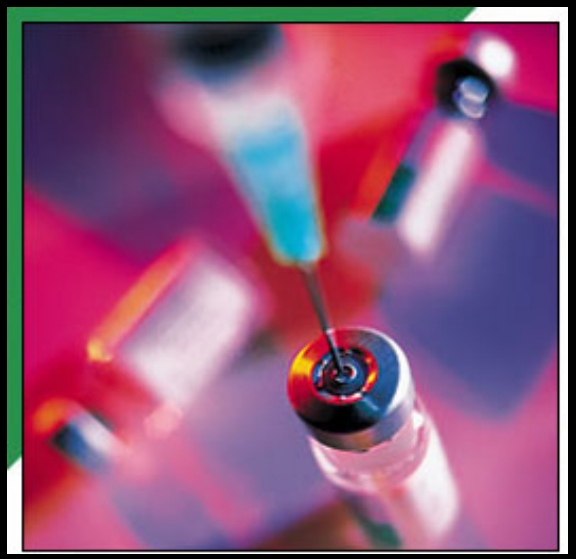
Recombinant proteins:
Example: Tissue plasminogen activator (t-PA)
EPO
Blood clotting factors
Cell lines used: CHO-K1 cells, Baby Hamster Kidney Cells (BHK)



Vaccine Production

Example: Rabies vaccine
Polio vaccine

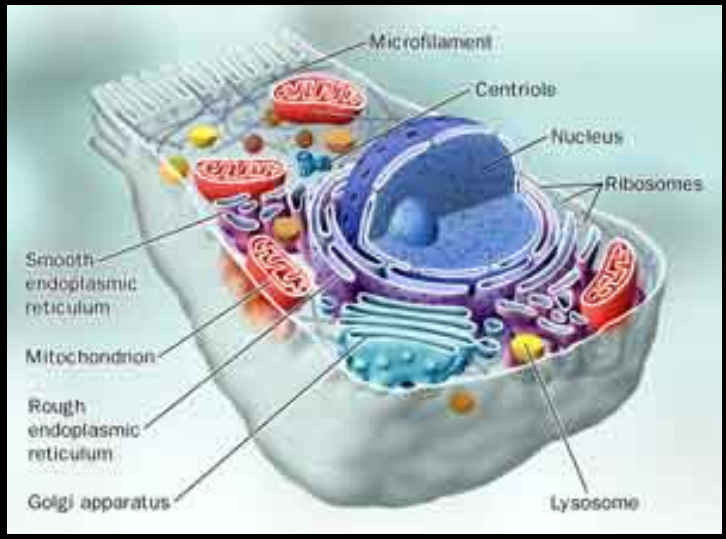
Cell lines used: Chick embryo, Vero (African green monkey kidney epithelial cell line)



Cell biology

1.Studies on intracellular activity,
e.g. cell cycle and differentiation
metabolism, drug metabolism
transcription, translation energy metabolism

2. Elucidations of intracellular flux,
e.g. hormonal receptors,
signal transduction, nutritional studies
membrane trafficking, metabolites



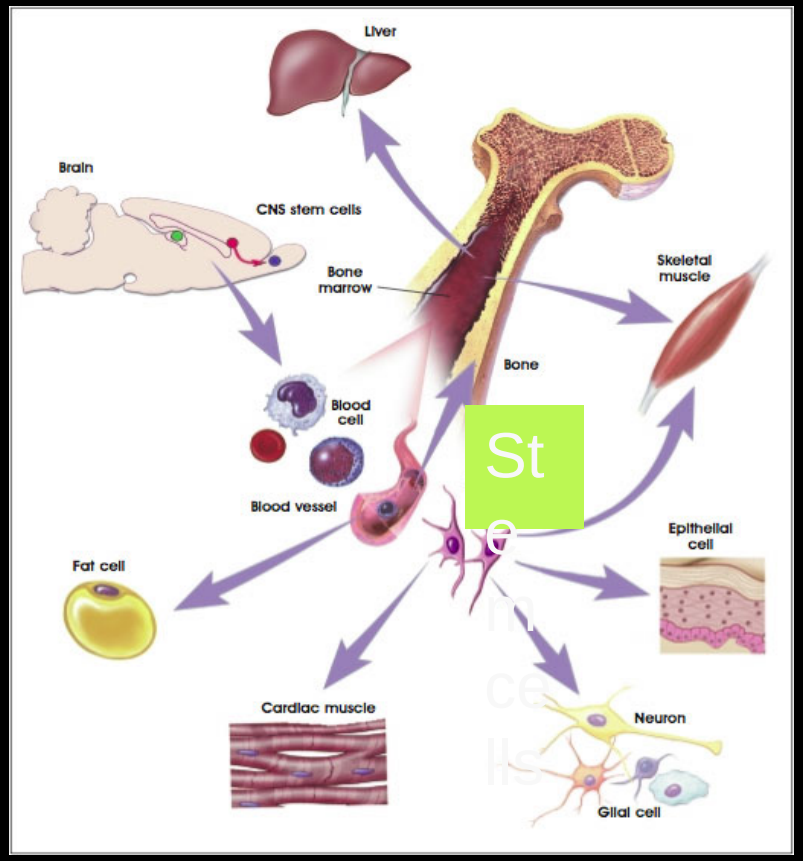
Stem cell Therapy

Definition:

A cell that has the ability to continuously divide and differentiate (develop) into various other kinds of cells/tissues

SO…..WHAT ARE STEM CELLS?

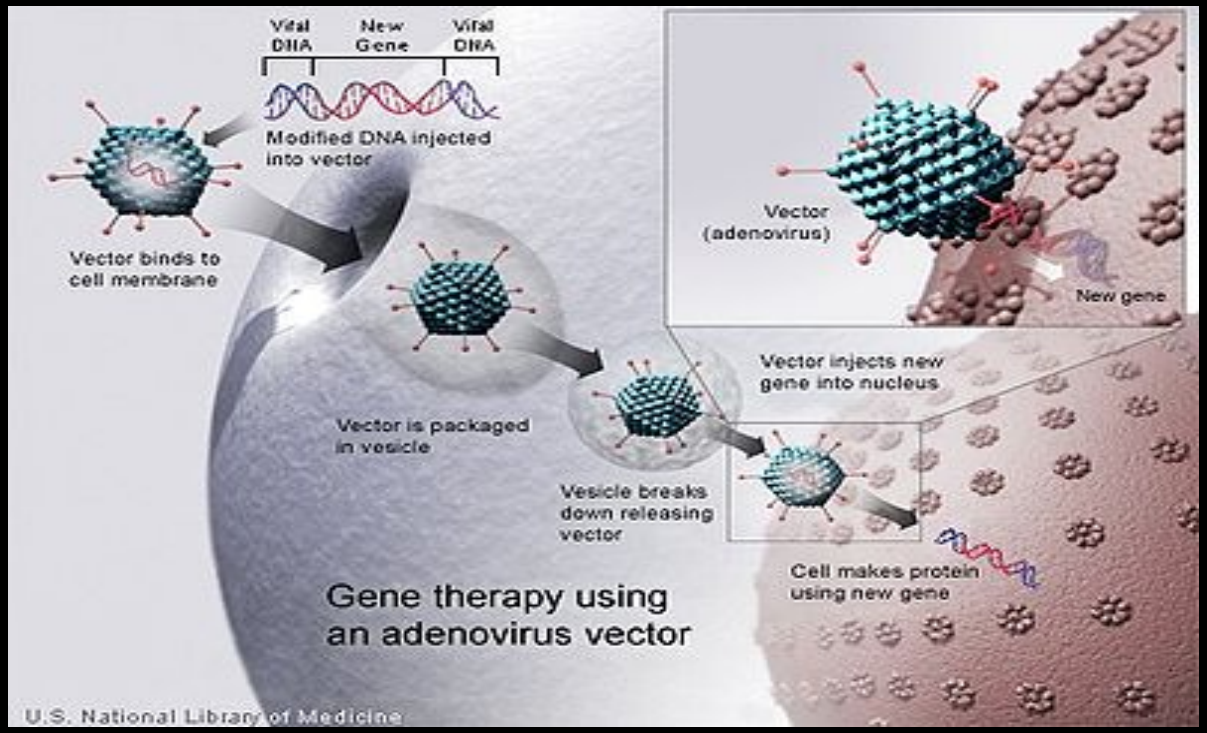
- CELLS THAT CAN MAKE MORE OF THEMSELVES
- CELLS THAT CAN BECOME ALMOST ANY CELL - MULTIPOTENT



Gene therapy

Definition: Genetic alteration of somatic cells to treat disease.

The insertion of genes into an individual's cells and tissues to treat a disease, such as a hereditary disease in which a deleterious mutant allele is replaced with a functional one.



Cont..

Examples of Vectors in gene therapy

Viruses

Retroviruses

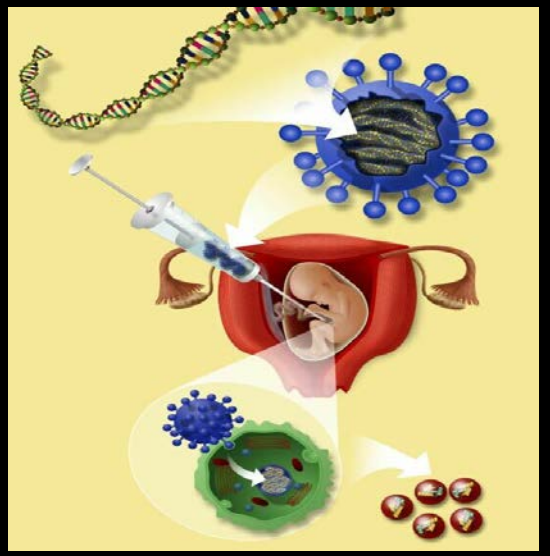
Adenoviruses

Adeno-associated viruses

Envelope protein pseudo typing of viral vectors

Currently gene therapy is being developed to treat a variety of genetic diseases and disorders as well as vascular disease, immune deficiencies, neurodegenerative diseases, blood disorder and some cancers

Example: thalassaemia, cystic fibrosis, *Sickle Cell Disease*



Bioassays

Modern scientific assessment of drug safety is increasingly using cell-based assays.

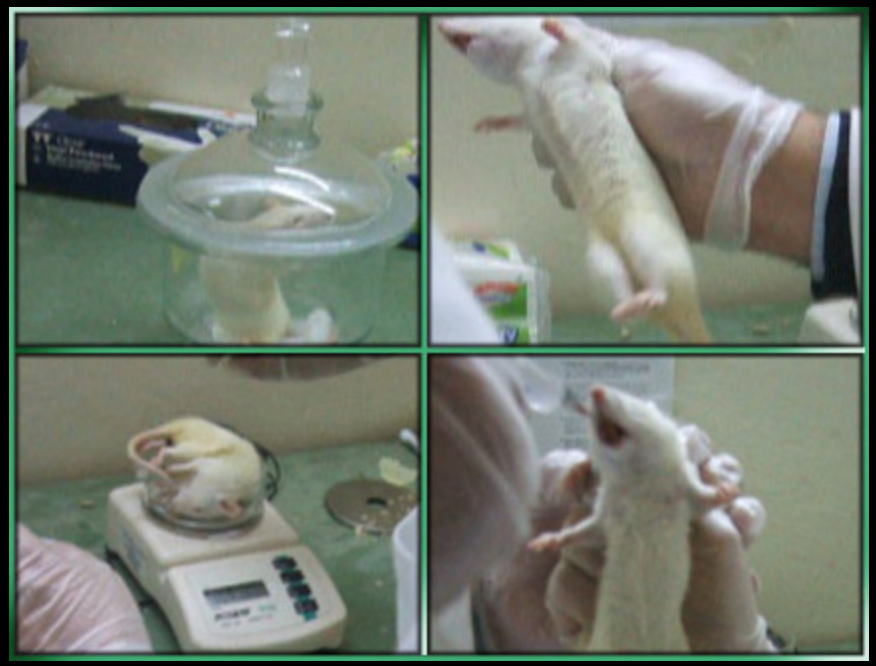
Cell based assays are used to assess drug behavior in the body (pharmacokinetics, drug metabolism), genotoxic liabilities, developmental toxicity (teratogenicity), cardiac toxicities, potential drug-drug interactions and other distinct toxicological mechanisms

Cell lines used : WISH, M-NFS60, UT7, UMR106

Toxicity Studies

Toxicology is the study of the adverse effects of chemicals on living organisms.

In vitro **toxicity testing** is the scientific analysis of the effects of toxic chemical substances on cultured mammalian cells.
In vitro testing methods are employed primarily to identify potentially hazardous chemicals and/or to confirm the lack of certain toxic properties in the early stages of the development
such as therapeutic drugs, agricultural chemicals and direct food additives that may or may not taste good.



Cell viability (cytotoxicity) assays used in *In-vitro* toxicology
Ex-NFS 60

Tissue Culture Application (II)

1. Production of antiviral vaccines
2. Understanding of neoplasia (caner research)
3. Transfer of DNA to the cultured cells (or siRNA)
4. Monoclonal antibody production (immunology)
5. Production of human growth hormone, insulin, interferon
6. Stem cell culture differentiate into neurons
7. Implanting normal fetal neurons into patients with Parkinson diseases
8. Homografting and reconstructive surgery using individual's own cells (tissue engineering)
9. In vitro fertilization (embryo culture)

Table 1-2 Advantages of Tissue Culture

Category	Advantages
(1) Physico-chemical environment	Control of pH, temperature, osmolarity, dissolved gases (O_2 & CO_2)
(2) Physiological conditions	Control of hormone and nutrient concentrations
Microenvironment	Regulation of matrix, cell–cell interaction, gaseous diffusion
Cell line homogeneity	Availability of selective media, cloning
Characterization	Cytology and immunostaining are easily performed
Preservation	Can be stored in liquid nitrogen
Validation & accreditation	Origin, history, purity can be recorded
Replicates and variability	Quantitation is easy
Reagent saving	Reduced volumes, direct access, lower cost
Control of C × T	Ability to define dose, concentration, and time
Mechanization	Available with microtitration and robotics
Reduction of animal use	Cytotoxicity and screening of pharmaceutics, cosmetics, etc.

Poorly defined materials: serum, supplementations, matrix,…

Cell line homogeneity

"After one or two passages, cultured cell lines assume a homogeneous constitution,

as the cells are randomly mixed at each transfer and the selective pressure of the

culture condition tends to produce a homogeneous culture of

the most vigorous cell type"

? ?

Reagent saving

"Less reagent is required than for injection in vivo,

where 90% is lost by excretion and distribution to tissues other than

the interested cells under study"

In Vitro Dosage v.s. *In Vivo* Dosage

? ?

Reduction of animal use

“In vitro modeling of in vivo conditions”

Table 1-3 Limitations of Tissue Culture

Category	Examples
Necessary expertise	Handling
	Chemical contamination
	Microbial contamination
	Cross contamination (Table 13-2)
Environmental control	Workplace
	Incubation, pH control
	Containment and disposal of biohazards
Quantity and cost	Capital equipment
	Consumables
	Medium, serum, plastics
Genetic instability	Heterogeneity, variability
Phenotypic instability	Dedifferentiation
	Adaptation
	Selection
Identification of cell type	Expression of markers
	Histology, cytology
	Geometry and microenvironment

Microbial Contamination

"Animal cells grow much less rapidly than contaminants

(bacteria, molds, yeasts)"

Cross-Contamination

"Many cell lines in common use are not what they are claimed to be,

but have been cross-contaminated with HeLa or other growing cell line"

Cross-Contaminated cell lines

”HeLa cell”

George Otto Gey

Henrietta Lacks

Cervical Cancer

”HeLa cell”

http://en.wikipedia.org/wiki/Henrietta_Lacks

Cost

“The cost of producing cells in culture is about 10 times that of using animal tissue”

“Semimicro- or Micro- Assays”: reduced manipulation time (Quicker !!)

Genetic and Phenotypic Instability

"**Dedifferentitation**: a process assumed to be the reversal of differentiation"

"overgrowth of undifferentiated cells"

" Loss of the phenotypic characteristics typical of the tissue"

Possible Reasons:

(1) Specific cell interactions characteristics of the histology of the tissue are lost;

(2) The culture environment lacks systemically homeostatic regulation systems
(nervous and endocrine system)

Cell Culture (II)

7. The derivation of continuous cell line or cell strain usually implies a phenotypic change, or transformation

8. Cultured cell lines are more representative of precursor cells [most differentiated cells do not divide]

9. Cultured cells lack the potential for cell-cell and cell-matrix interaction