Basics of Cell Culture

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Introduction

- **Cell culture is the process by which prokaryotic, eukaryotic or plant cells are grown under controlled conditions**

Animal cell/tissue culture is the term used for "the process of growing cells artificially in the laboratory";

Animal cell/tissue culture produces clones, in which all product cells have the same genotype (unless affected by mutation during culture).

- **Cell culture was first successfully undertaken by Ross Harrison in 1907**
- **Roux in 1885 for the first time maintained embryonic chick cells in a cell culture**

Historic Perspective

•1665 - Hooke called "cells" to the small holes he saw in crossections of cork.

•1692 - Leeuwenhoek saw and drew the first protozoa and bacteria, "little animals" as he called them.

•1838/39 - Schleiden and Schwann formulated the so called "cell theory" theory which stated that all life is based on individual cells.

• Rudolf Virchow in 1855 formulated that all cells are derived from cells and do not form spontaneously.

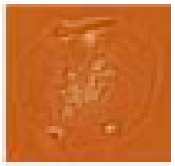
• Wilhelm Roux cultivated - 1885 - the first cells from chick embryos in salt solutions.

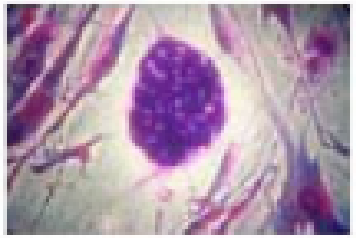
• Ross Harrison - 1907 - isolated pieces of frog embryonic tissue known to give rise to nerve fibres ("hanging drop method"). He was credited as the first to work successfully with "artificial" tissue culture.

• Another great step forward was the development of a protocol by Alan Parkes in 1949 to freeze cells using a cryoprotectant (*e.g.* glycerol).

• 1952 – Establishment of the most famous cultured cells - HeLa cells - because they came from Henrietta Lacks, a thirty-one-year-old Baltimore woman who died in 1951 of cervical cancer.

History of Cell Culture





1885 **Roux** cells can live outside the body

1907 **Harrison** nerve outgrowth

1913 **Carrel** aseptic conditions

1952 **Gey** Established HeLa cells

1955 **Eagle** "defined" medium

Late '50s and '60s **Stoker, Dulbecco, Green** viral transformation

1961 **Hayflick and Moorhead** Finite number of divisions

1964 **Littlefield** somatic cell hybrids

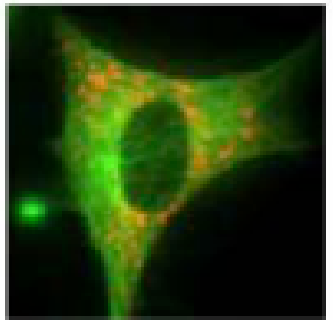
1965 **Ham** clonal growth of mammalian cells

1976 **Sato** hormones and growth factors required in SF medium

1977 **Wigler and Axel** transfections

1986 **Martin and Evans** Mouse ES cells

1998 **Thomson and Gearhart** human ES cells



Source: Domingos Henrique, *Cell and Tissue Engineering* Classes, IST, 2008-2009

Why animal cells?

- Over the years, tissue culture studies have move to medically relevant, warm-blooded animals (rather than plants, frogs…);
- Animal cells are normally part of an organ where they differentiate to perform specific functions;
- Animal cells are eukaryotic cells which vary in size (10-30 µm) and shape (spherical, ellipsoidal). They are bound together by intercellular material to form tissue.

Tissue Culture – Concepts

- **Primary culture:** cells obtained from animal tissue
- **Passing:** process by which cells are often diluted and replaced in new vessels as they divide
- **Cell line:** primary culture after the first passage (*i.e.* cells continue to grow for a limited number of generations)

Tissue Culture – Concepts (continued)

- **Immortalized cell line:** cell line able to grow continuously (*i.e.* over an extended time period); these arise from tumor cells or embryonic tissues. Therefore, **they have specific differences** in **gene expression** (*e.g.* telomerase) than primary cells.

Advantages:

✓Easier to maintain *in vitro* than primary cells or cell lines;

✓Potentially lead to highly reproducible results;

though:

× Altered characteristics as compared to their tissue of origin or following interaction with different cell environments (need for culture condition standardization, number of passages specified, …).

Note: Some primary cells can be transformed into immortalized cell lines by uptake of new genetic material, which is associated with **genetic instability** and can lead to **immortalization**, **aberrant growth control** and **malignancy**.

Major development's

- First development was the use of antibiotics which inhibits the growth of contaminants
- Second was the use of trypsin to remove adherent cells to subculture further from the culture vessel
- Third was the use of chemically defined culture medium

Select from these cell culture antibiotics

- **Actinomycin D**
- **Ampicillin**
- **Carbenicillin**
- **Cefotaxime**
- **Fosmidomycin**
- **Gentamicin**
- **Kanamycin**
- **Neomycin**
- **Penicillin Streptomycin (Pen Strep)**
- **Polymyxin B**
- **Streptomycin**

Keep your irreplaceable cultures contamination free

Trypsin

Trypsin is an endopeptidase produced by the gastro-intestines of mammals, and has an optimal operating pH of about 8 and an optimal operating temperature of about 37 °C. *I*

n vivo, trypsin is produced in the pancreas in the form of inactive zymogen, trypsinogen.

After secretion into the duodenum, the enzyme enteropeptidase activates a small number of the enzymes into trypsin by proteolytic cleavage, followed by autocatalysis of trypsins to activate the whole secreted mass. Then, trypsin acts to hydrolyse pepsin-digested peptides by hydrolysis of peptide bonds.

The aspartate residue (Asp 189) located in the catalytic pocket (S1) of trypsins is responsible for attracting and stabilizing positively-charged lysine and/or arginine. Thus, trypsin predominantly cleaves proteins C-terminally of the amino acids lysine and arginine, except when either is followed by proline. Trypsins should be stored at very cold temperatures (between −20 °C and −80 °C) or at a pH of 3 in order to prevent autolysis.

Usage of Trypsin for Cell Passage

Preparation of a 10 % Trypsin stock solution

Solubilise 10 g Trypsin in 100 ml 10 x Trypsin buffer
Sterilize by filtration (0.2 µm) and store in aliquots (e.g. 10 ml each) at -20 °C or -80 °C. Durability approx. 1 year.

10 x Trypsin buffer

80 g NaCl (endotoxin-free Art. No. HN00)
3 g KCl (endotoxin-free Art. No. HN02)
0.73 g Na2HPO3 x 2 H2O (Art. No. 4984)
0.2 g KH2PO3 (Art. No. 3904)
20 g Glucose (endotoxin-free Art. No. HN06)
7 g EDTA (Art. No. 8043)
fill with water ad 1 Litre, pH 7.2 (NaOH, Art. No. K021)

Preparation of working solution

Dilute stock solution 1:10 with sterile distilled water. Store at +4 °C.
Durability approx. 1 week.
We recommend to use endotoxin-free water for preparation of stock- und working solution. Water made
by common distillers frequently is polluted with bacteria. Although these microorganisms are removed
by sterile filtration, the released endotoxins may affect cultured cells heavily. Endotoxin-free water can
be obtained under order number 3255.

Why is cell culture used for?

- **Model systems for**
  - -studying basic cell biology
  - -interactions between disease causing agents and cells
  - -effects of drugs on cells
  - -process and triggering of aging & nutritional studies

- **Toxicity testing**
  - Study the effects of new drugs

- **Cancer research**
  - Study the function of various chemicals, virus & radiation to convert normal cultured cells to cancerous cells

- **Virology**

  Cultivation of virus for vaccine production, also used to study there infectious cycle

- **Genetic Engineering**

  Production of commercial proteins, large scale production of viruses for use in vaccine production e.g. polio, rabies, chicken pox, hepatitis B & measles

- **Gene therapy**

  Cells having a functional gene can be replaced to cells which are having non-functional gene

Basic cell culture infrastructure

- **Laminar cabinet- Vertical are preferable**
- **Incubation facilities- Temperature of 25-30 C for insect & 37 C for mammalian cells, CO2 2-5% & 95% air at 99% relative humidity.**
- **To prevent cell death incubators set to cut out at approx. 38.5 C**
- **Refrigerators- Liquid media kept at 4 C, enzymes (e.g. trypsin) & media components (e.g. glutamine & serum) at -20 C**
- **Microscope- An inverted microscope with 10x to 100x magnification**
- **Tissue culture ware- Culture plastic ware treated by polystyrene**

Tissue culture

- In vitro cultivation of organs, tissues & cells at defined temperature using an incubator & supplemented with a medium containing cell nutrients & growth factors is collectively known as tissue culture

- Different types of cell grown in culture includes connective tissue elements such as fibroblasts, skeletal tissue, cardiac, epithelial tissue (liver, breast, skin, kidney) and many different types of tumor cells

How you start cell culture ?

Cultures can be initiated from

- **Tissue or organ fragments**
- **Single cell suspensions**

Choices to be made

- **Disaggregation techniques**
- **Media**
- **Culture conditions**
- **Selection procedures**

Classification of tissue cultures
(based on the origin of the cells)

- Primary culture (directly from animal or plant tissue)
- Extended culture (multipassage culture) – cell strain
- Established (transformed) cell lines

Classification

3 Types of Primary Culture:

- ***Cell Culture*:** the harvested tissue is mechanically or enzymatically dispersed into a cell suspension. This cell suspension can be cultured as:

  ✓ **adherent monolayer,** requiring a wettable solid surface to grow on– **adhesive substrate** - such as glass or plastic (ex. epithelial-like - flattened and polygonal in shape - and fibroblast-like cells - elongated and bipolar);

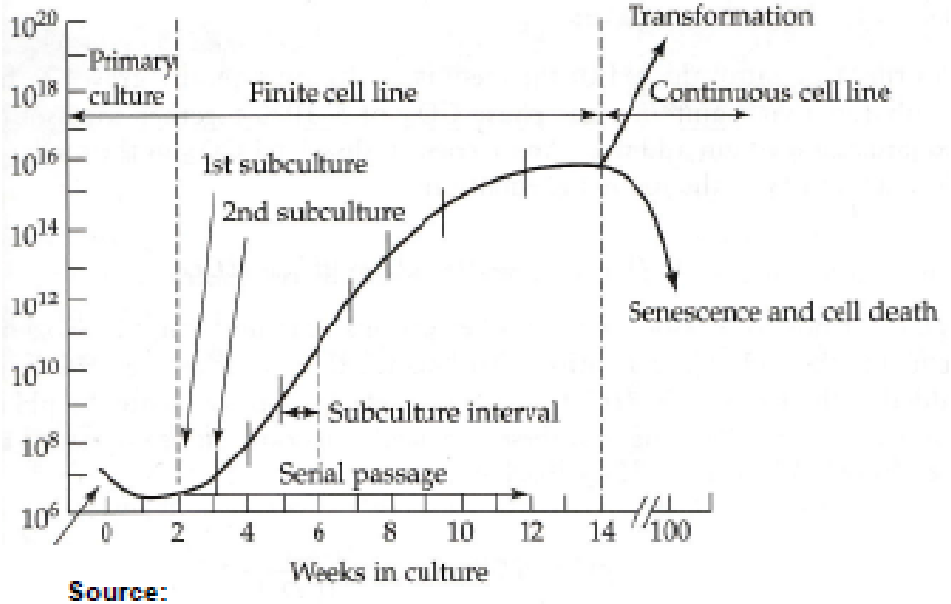
  ✓ **nonadherent suspension** in liquid medium (ex. hematopoietic cells).

- ***Primary Explant Culture***: a fragment of tissue is placed at a liquid-solid interface where, upon attachment, outgrowth and migration of cells occurs in the plane of the substrate;

- ***Organ Culture*:** the tissue explant retains, at least partially, its architectural characteristics; it is placed in a culture environment that favors retention of this 3-D shape.

Evolution of a primary cell culture into a cell line:

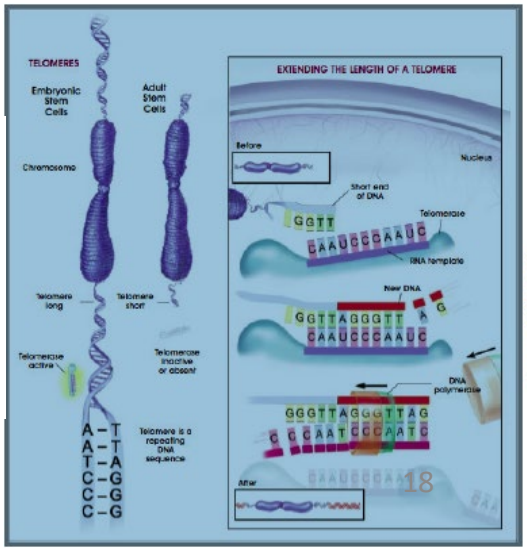
1) Growth is relatively slow in the primary culture;
2) Growth enters an exponential growth phase;
3) Cells reach their lifetime limit and undergo senescence and death.



Source:
Palsson, B.Ø. and Bhatia, S.N., Tissue Engineering, Pearson Prentice Hall Bioengineering, 2004

➢ Classically, the lifetime of a cell line is referred to as the **Hayflick limit** and is equivalent to approximately **50 generations**;

➢ The molecular mechanisms for the **Hayflick limit** depend not only on **telomere length**, but also on **telomerase activity**, **DNA repair machinery**, and other factors as variables that contribute to **senescence**.



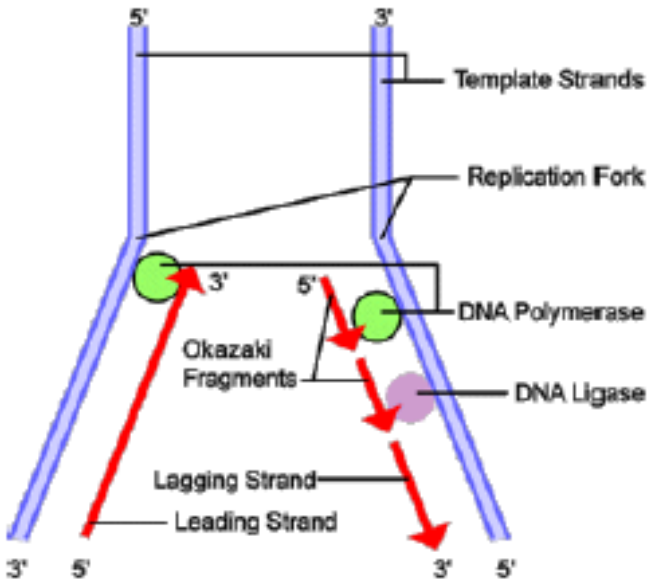
Source: NIH, 2001

Hayflick Limit

- Leonard Hayflick discovered that most human cell lines, unless they are tumor lines, begin to die off after about 40-60 cell divisions (1965).

"*We showed that when normal human embryonic cells are grown under the most favorable conditions, aging and death is the inevitable consequence after about fifty population doublings*"

Hayflick Limit:
The end replication Problems



- On the leading strand, DNA polymerase can make a complementary DNA strand without any difficulty because it goes from 5' to 3 ';
- In the lagging strand, however, DNA polymerase's synthesis is based on a series of fragments, called Okazaki, each requiring an RNA primer;
- The DNA polymerase can start replication at that point and go to the end of the initiation site;
- More RNA primers attach further on the DNA strand and DNA polymerase comes along and continues to make a new DNA strand;
- the last RNA primer attaches, and DNA polymerase, RNA nuclease and DNA ligase come along to convert the RNA (of the primers) to DNA, and seal the gaps in between the Okazaki fragments;
- But in order to change RNA to DNA, there must be another DNA strand in front of the RNA primer;
- This happens at all the sites of the lagging strand, but it doesn't happen at the end where the last RNA primer is attached. Ultimately, that RNA is destroyed by enzymes that degrade RNA left on the DNA;
- The result is the "end-replication problem" in which a sequence is lost at each round of DNA replication.

Cell lines

A. Continuous cell line: transformed 'immortal'
B. Finite cell line (primary culture): dies after several sub-cultures

A. Continuous cell line

- Most cell lines grow for a limited number of generations after which they ceases
- Cell lines which either occur spontaneously or induced virally or chemically transformed into Continous cell lines

Characteristics of continous cell lines

-smaller, more rounded, less adherent with a higher nucleus /cytoplasm ratio

-fast growth and have aneuploid chromosome number

-reduced serum and anchorage dependence and grow more in suspension conditions

-ability to grow upto higher cell density

-different in phenotypes from donar tissue

-stop expressing tissue specific genes

Can STORE cells, cryopreserved in liquid nitrogen for years

Primary culture

B. Primary culture : **Primary culture freshly isolated from tissue source**

- **Cells when surgically or enzymatically removed from an organism and placed in suitable culture environment will attach and grow are called as primary culture**
- **Limited growth potential**
- **Cell lines have limited life span, they passage several times before they become senescent**
- **Primary culture contains a very heterogeneous population of cells**
- **Sub culturing of primary cells leads to the generation of cell lines**
- **Cells such as macrophages and neurons do not divide in vitro so can be used as primary cultures**
- **Lineage of cells originating from the primary culture is called a cell strain**

Types of cells

On the basis of morphology (shape & appearance) or on their functional characteristics, they are divided into three types:

- Epithelial like- attached to a substrate and appears flattened and polygonal in shape
- Lymphoblast like- cells do not attach remain in suspension with a spherical shape
- Fibroblast like- cells attached to an substrate appears elongated and bipolar

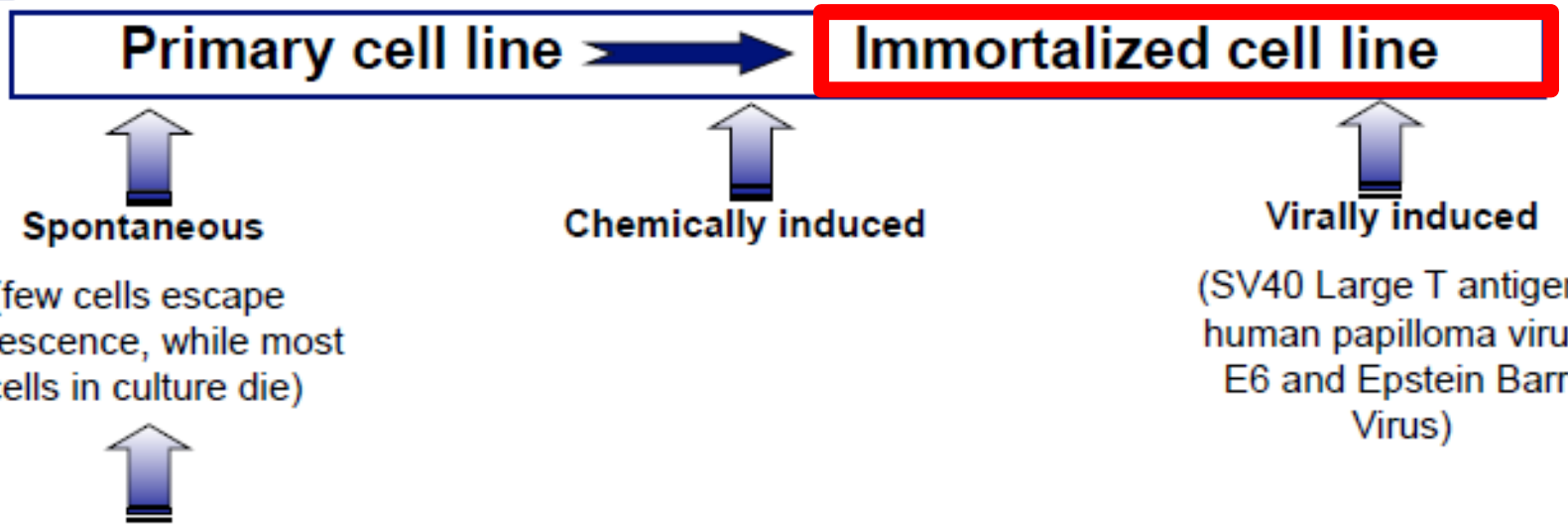
Adherent cells

- **Cells which are anchorage dependent** (characterize cells requiring a solid substratum for growth)
- **Cells are washed with PBS (free of Ca & Mg) solution**
- **Add enough trypsin/EDTA to cover the monolayer**
- **Incubate the plate at 37 C for 1-2 mts**
- **Tap the vessel from the sides to dislodge the cells**
- **Add complete medium to dissociate and dislodge the cells with the help of pipette which are remained to be adherent**
- **Add complete medium depends on the subculture requirement either to 75 cm or 175 cm flask**

Suspension cells

- Easier to passage as no need to detach them
- As the suspension cells reach to confluency
- Asceptically remove 1/3rd of medium
- Replaced with the same amount of pre-warmed medium

Immortalized Cell lines



Genetic Instability: loss of functional p53 or Rb (tumor suppressors, arresting cell-cycle progression in the setting of DNA damage), overexpression of telomerase or mutations in a group of "senescence genes".

✓ While immortalized cells **are easier** to maintain in culture,

They are used as imperfect models for primary cells, displaying:

× Aberrant growth control and chromossal numer (*i.e.* aneuploidy);
× Potential loss of anchorage dependence and contact inhibition;
× Reduced need for serum growth factors;
× Potential to undergo further transformation into tumorigenic cells.

Examples of Cell lines

HeLa (epithelial human cells)

Hybridomas cells (resulting from the fusion of antibody-producing B lymphocytes with tumor cells): production of monoclonal antibodies and glycosylated proteins.

Myeloma cells: production of recombinant proteins, in particular recombinant antibodies.

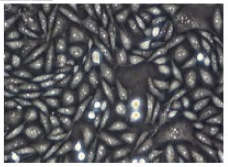
CHO (fibroblasts from chinese hamster ovary) cells are the favored industrial cells for production of recombinant proteins (monoclonal antibodies).

BHK (fibroblasts from baby hamster kidney) cells have been used for vaccine production, *e.g.* the veterinarian vaccine for rabies.

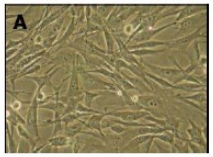
Stem Cells: Embryonic (ESC), Hematopoietic (HSC), Neural (NSC),… for cell therapy.

HeLa (epithelial human cells)

CHO (fibroblasts from chinese hamster ovary)



BHK (fibroblasts from baby hamster kidney)



Animal Cell Culture media

Eagle's medium and derivatives thereof:

BME (Basal Medium Eagle's)

EMEM (Eagle's Minimal Essential Medium)

DMEM (Dulbecco's Modified Eagle's Medium)

GMEM (Glasgow's Modified Eagle's Medium)

Media designed for use with serum:

Liebovitz

Trowell

Williams

Media from Roswell Park Memorial Institute (RPMI):

RPMI 1630

RPMI 1640

Media designed for a specific cell line for use without serum:

CMRL 1060

Ham's F10 and F12

TC 199

IMDM (Iscove's Modified Dulbecco's Medium)

Choice of media depends on the type of cell being cultured

Culture media

- The nutritional requirements of animal cells are more stringent than those of microorganisms because animal cells do not metabolize inorganic nitrogen.

Cell culture medium: Liquid in which cells are maintained and propagated *in vitro*, supplying nutrients and inorganic salts and providing a physiological pH and osmolality.

Medium Components	Amount	Purpose
Sodium chloride	6-8 g/L	Adjust osmotic pressure
Inorganic salts	0.8-1 g/L	Electrolyte balance (≅blood)
Sodium bicarbonate	2-3 g/L	Buffering capacity (pH=7.4)
Glucose	1 g/L	Source of energy, carbon
Aminoacids	1 g/L	Source of nitrogen
Vitamins	0.01 g/L	Cofactors
Growth factors and hormones	1-10 μg/L	Growth stimulators
Antibiotics	1-50 μg/L	Prevent contamination by m.o.
Phenol red	0.01 g/L	Visual pH indicator
Animal serum	1-20% v/v	Enhances growth, attachment…

Adapted from: Palsson, B.Ø. and Bhatia, S.N., Tissue Engineering, Pearson Prentice Hall Bioengineering, 2004

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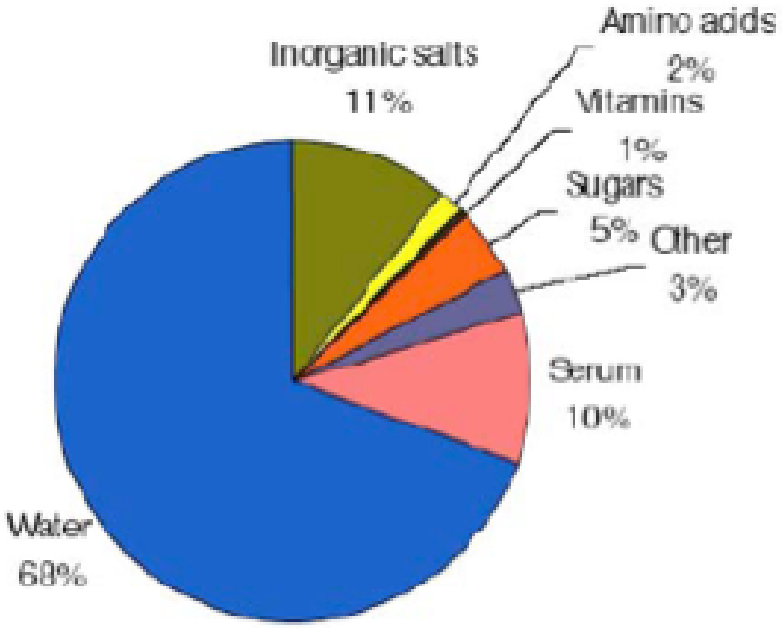

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Animal Serum

- **Serum:** Liquid that remains after plasma (noncellular fraction of blood) is allowed to clot.
  - ➢ It is typically added to culture medium in a proportion of approximately **1** to **20%** by volume;
  - ➢ Examples: **calf** (bovine), **fetal bovine**, **horse** and **human**.

MAJOR CONSTITUENTS OF SERUM	
Serum Components	**Effect on cells**
Albumin, globulins, transferrin	Carriers for fatty acids, iron, etc.
Fibronectin	Attachment factors
α2-macroglobulin	Inhibits proteases
Growth factors (*e.g.* PDGF)	Promotes cell proliferation
Hormones (*e.g.* insulin)	Stimulate uptake of glucose and aminoacids; alter cell growth rate
Carbon and nitrogen sources	Nutrients
Mineral and trace elements	Essential for the activity of metalloenzymes
Inhibitors (*e.g.* endotoxins)	Inhibit cell proliferation

Adapted from: Palsson, B.Ø. and Bhatia, S.N., Tissue Engineering, Pearson Prentice Hall Bioengineering, 2004

Animal Serum: Disadvantages

✓ Serum contains important growth factors, adhesion factors, minerals, lipids and hormones that can be **key to successful tissue culture**;

BUT present some **disadvantages** such as:

× High cost;

× Batch-to-batch variability (need for tight controls of the lots of sera);

× Risk of contaminations (mycoplasma and viral);

× Potential regulatory hurdles, envisaging the clinical application of cultured cells;

× Difficulties in downstream separation processes (*i.e.* interference with detection of cell products);

× Inflicts animal suffering during its collection (ethical concern).

Development of serum-free media allowing more reproducible results

Serum free media: Disadvantages

- **Multiplicity of media**

  cell lines of different origins require different media

- **Selectivity**

  cells at different stages of development may require different formulations

- **Requires higher reagent purity**

  removal of serum also removes the protective, detoxifying action that some serum proteins may have

- **Reduced cell proliferation**

- **Limited availability**

Physiochemical Properties of Media

Oxygen

- Oxygen consumption can be a critical variable for certain cell types (ex. **hepatocytes** consume 5- to 10-fold more oxygen than other cells, while **BM stem cells** reside in a physiological hypoxia);
- Oxygen delivery in culture is different from *in vivo* because of the lack of red blood cells (with hemoglobin) as oxygen carriers;
- *In vitro*, oxygen delivery is **limited** by:
  - its low solubility at 37ºC (0.22 mM @ 1 atm);
  - its transport from gas phase to cell surface.
- The correct oxygen tension should allow a compromise between fulfilling the respiratory requirements and avoiding toxicity (*e.g.* oxygen radicals);

Osmolality

Osmolalities between 320 and 340 mOsm/kg are quite acceptable to most animal cells.

Physiochemical Properties of Media

- **Temperature**

Recommended value for most human and warm-blooded animal cell lines is 37 ºC.

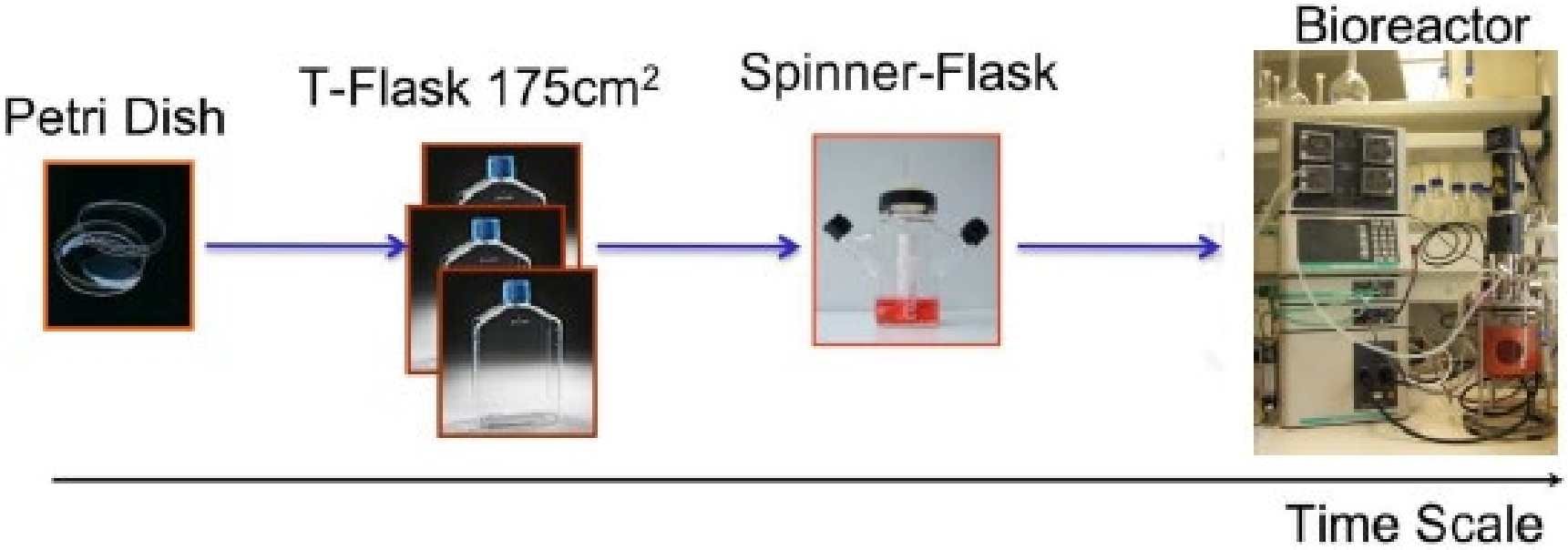
- **Viscosity**

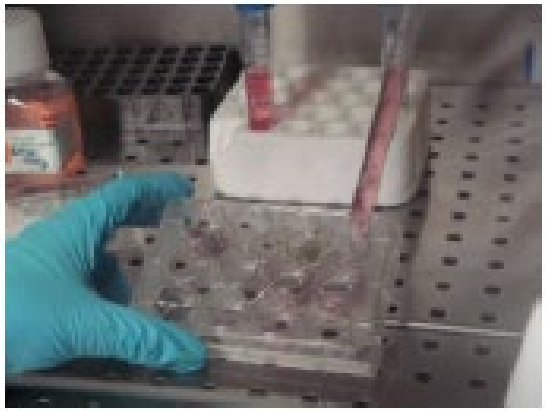
Is influenced by serum content and becomes important whenever a cell suspension is agitated (*e.g.* scale-up in stirred tank bioreactors).

- **Surface Tension and Foaming**

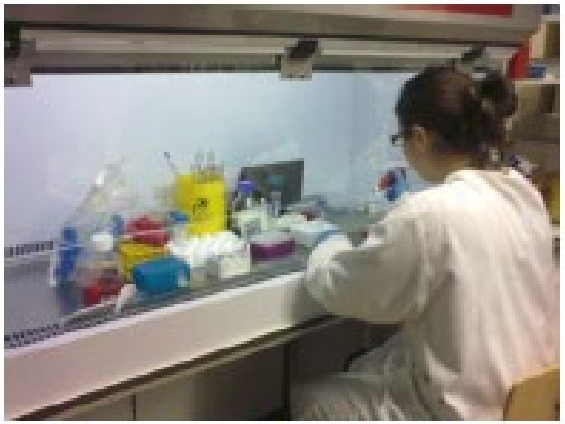
Foaming increases the risk of contamination and limits gaseous diffusion.

Culture Vessels

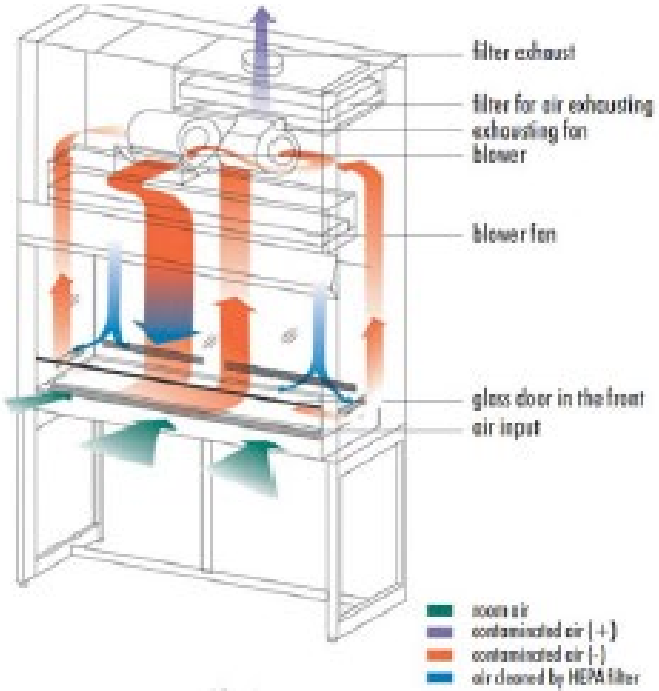




Lab



Laminar flow hood



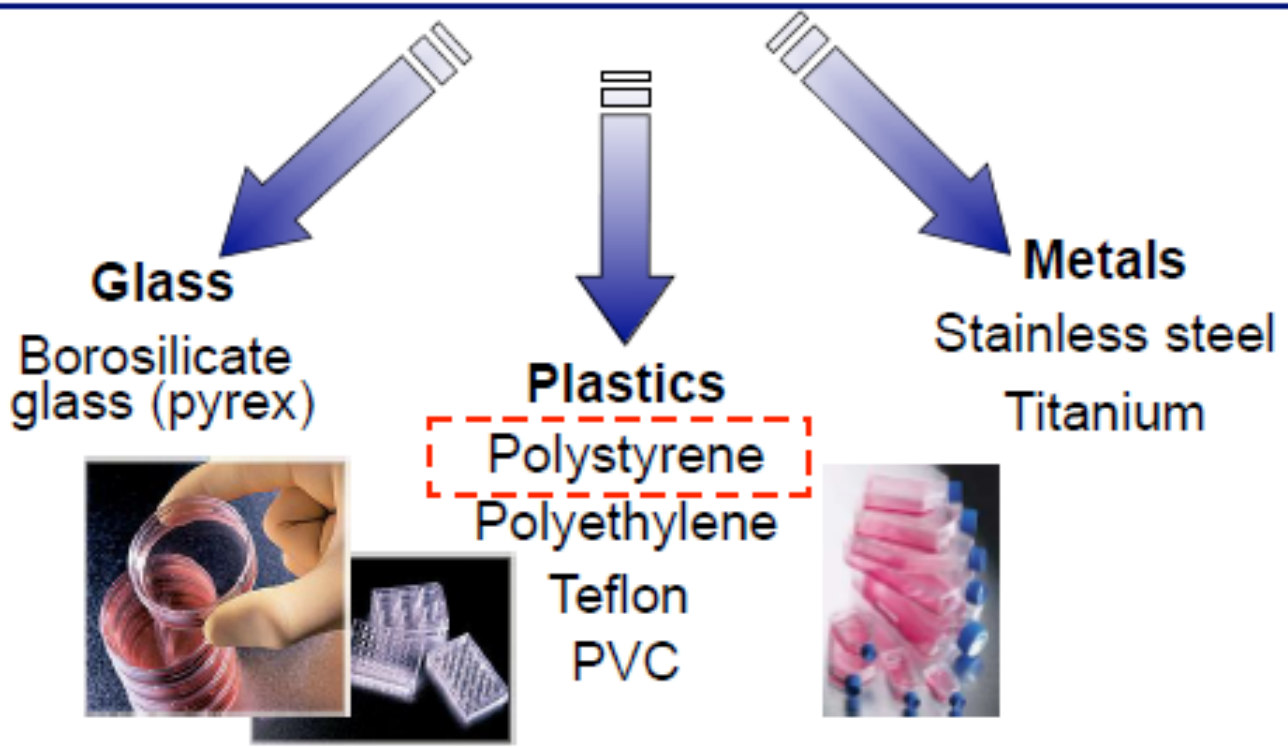
Incubator

- CO_2
- Water Jacked
- Humidity

Optical microscope

Tissue culture surfaces

Tissue culture surfaces are complex physicochemical substrates that have definite effects on cell morphology and growth



Pictures from: http://www.bdbiosciences.com/features/products/

- Clear polystyrene is generally exposed to ionized gas to introduce –OH groups on the surface and increase its hydrophylicity;
- Available in a variety of shapes and sizes affecting parameters such as surface area/ volume ratios, depth of media per unit volume, etc.

Please Follow
Basics of Cell Culture II