

What is Cell Culture?

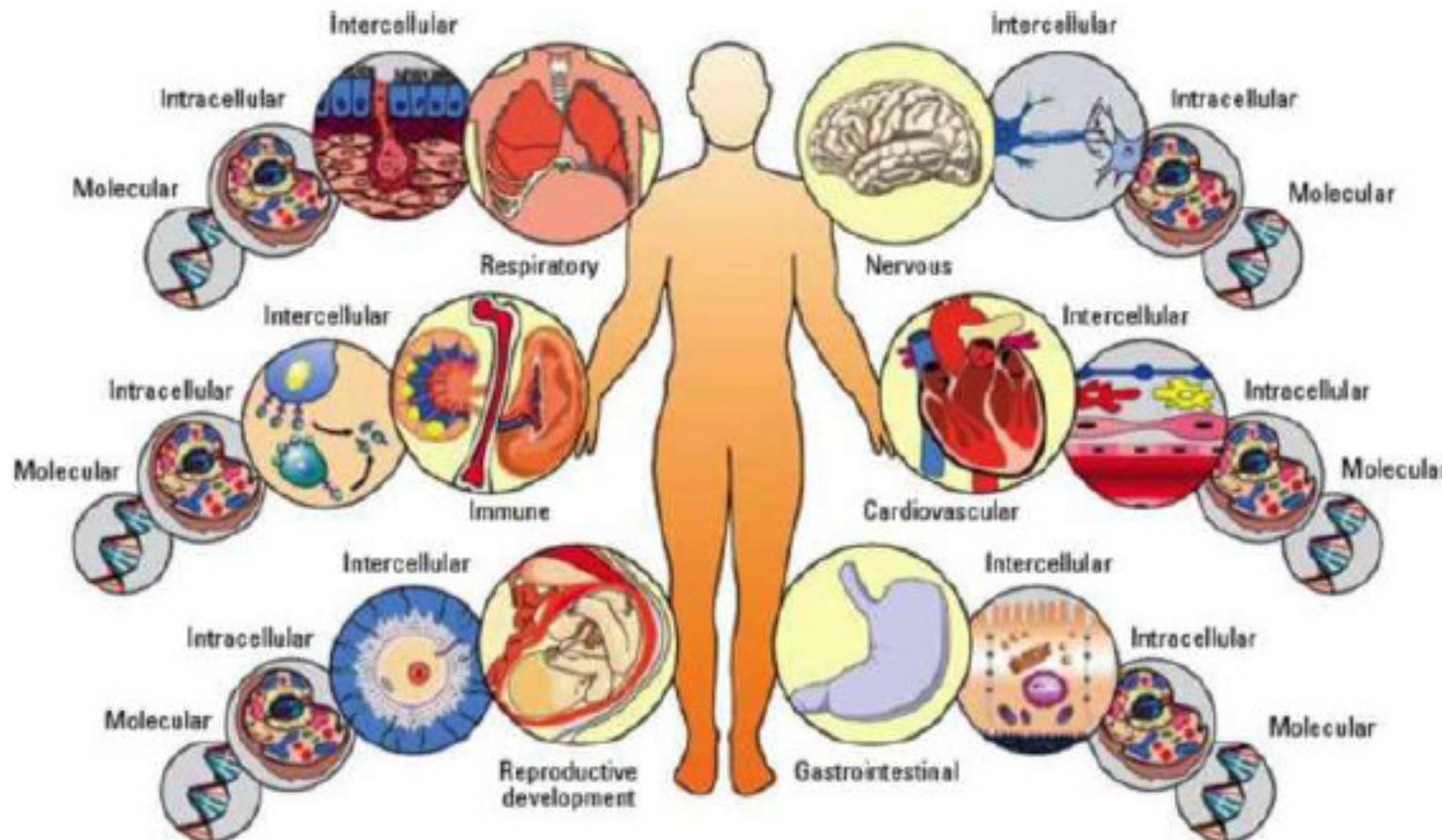
- The process by which prokaryotic, eukaryotic or plant cells are grown under controlled conditions

But in practice it refers to the **culturing of cells derived from animal cells/tissue.**

- *In vitro* culture (maintain and/or proliferate) of cells, tissues or organs
- Tissue from an explant is dispersed, mostly enzymatically, into a cell suspension which may then be cultured as a monolayer or suspension culture.

Why we need to culture animal cells?

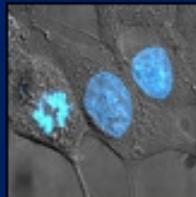
- Cell is the Basic Unit for the Life



- Understanding functions and roles of various cell are crucial approach for modern biology

HISTORY OF ANIMAL CELL CULTURE

- The primary impetus for the development of cell culture was to study, under the microscope, normal physiological events of cells.
- **Haberlandt** (1902) stated that the *in vitro*-culture techniques for plants primarily to facilitate basic physiological research.
- **Ross Granville Harrison** (1907) developed a culture to study the development of nerve fibers using Frog as a source.



HeLa (Henrietta Lacks) cells
one of the earliest human cell lines cultured
by **George Otto Gey** to create
an immortal cell line for medical research.



- **Alexis Carriel** (1912) used tissue and embryo extracts as culture media to keep the fragments of chick embryo heart alive.
- Mammalian cell cultures can be a suitable alternative for the use of whole animal tests to establish the potential toxicity of compounds.

History

- **1885:** Roux maintained embryonic chick cells alive in saline solution for short lengths of time
- **1912:** Alexis Carrel cultured connective tissue and showed heart muscle tissue contractility over 2-3 months
- **1943:** Earle et al. produced continuous rat cell line
- **1962:** Buonassisi et al. Published methods for maintaining differentiated cells (of tumour origin)
- **1970s:** Gordon Sato et al. published the specific growth factor and media requirements for many cell types
- **1979:** Bottenstein and Sato defined a serum free medium for neural cells
- **1980 to date:** Tissue culture becomes less of an experimental research field, and more of a widely accepted research tool

Major development's in cell culture technology

- 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.

Why do we need Cell culture?

1. Research

To overcome problems in studying cellular behavior such as:

- Confounding effects of the surrounding tissues
- Variations that might arise in animals under experimental stress
- Reduce animal use

2. Commercial or large-scale production

Production of cell material: vaccine, monoclonal antibodies (Mabs), hormone etc. which are impossible to produce synthetically.

Terminologies

- **Primary Cell Culture:** When cells are surgically removed from an organism and placed into a suitable culture environment they will attach, divide and grow.
- **Cell Line:** When the primary culture is subcultured and they show an ability to continuously propagate.
- **Anchorage dependency:** Cells grow as monolayers adhering to the substrate (glass/ plastic)
- **Passaging/ subculturing:** The process of splitting the cells.
- **Finite cells:** When the cells has finite life span.
- **Continuous cell lines:** When the cells can grow upto infinite lifespan.

Cell Culture Morphology

Morphologically cell cultures take one of two forms:

Anchorage independent cells (Suspension culture)

- Able to survive and proliferate without attachment to the culture vessel
- Cells from blood, spleen, bone marrow, etc.
- **Advantage:** large numbers, ease of harvesting

Anchorage dependent cells (Adherent Culture)

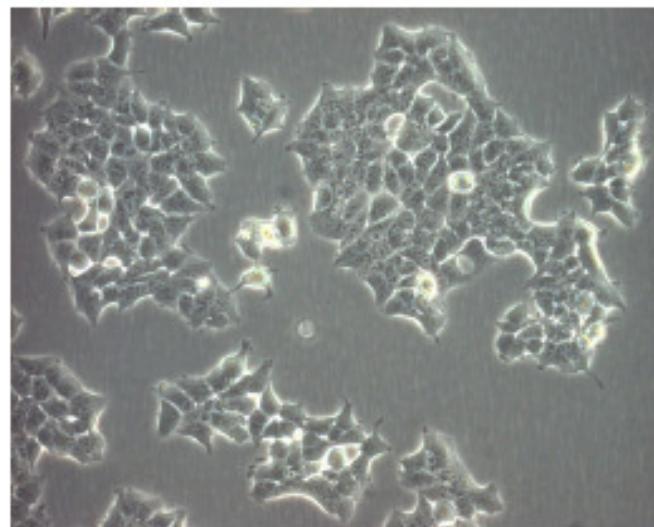
- Grow in monolayer, attached to the surfaces of the culture vessels
- All ectodermal or endodermal embryonic cells, e.g. fibroblasts, epithelial cells
- Various shapes but generally are flat (rounded in suspension)
- **Advantage:** spread on surfaces such as coverslips, easy for microscopy or other functional assays

CELL MORPHOLOGIES VARY DEPENDING ON CELL TYPE

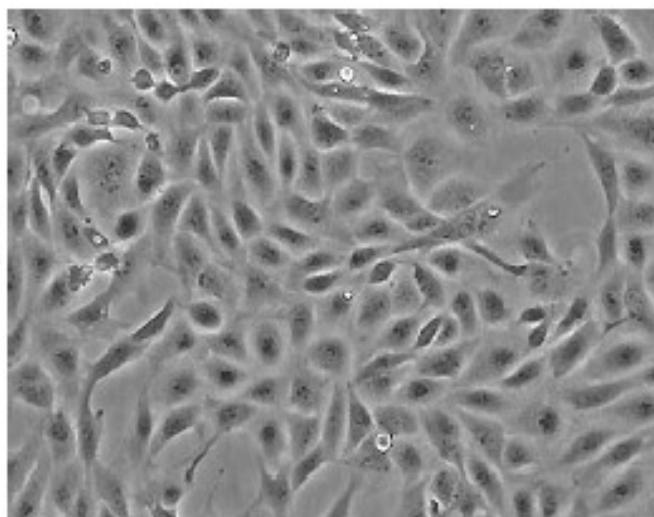
Fibroblastic



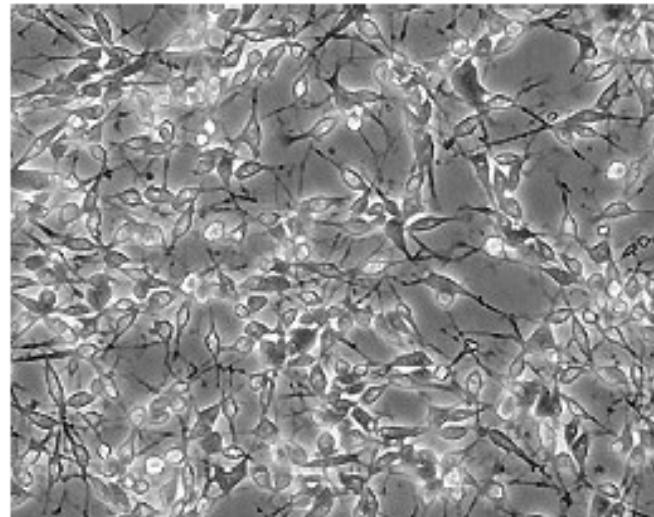
Epithelial



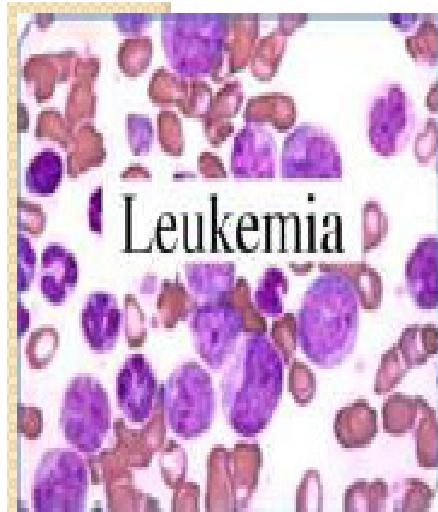
Endothelial



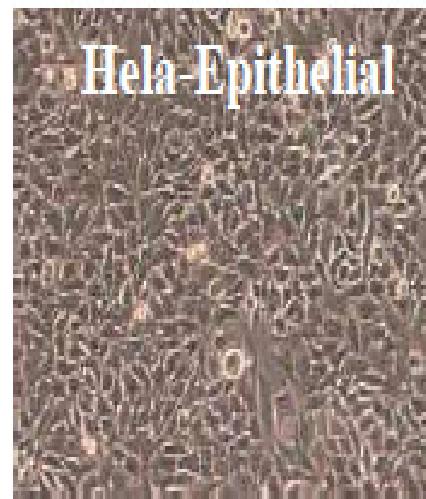
Neuronal



Cell Culture Morphology



Leukemia



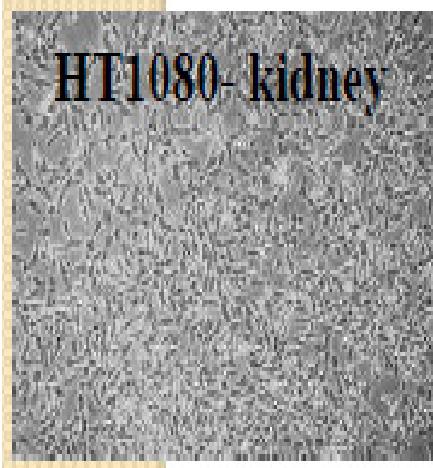
HeLa-Epithelial



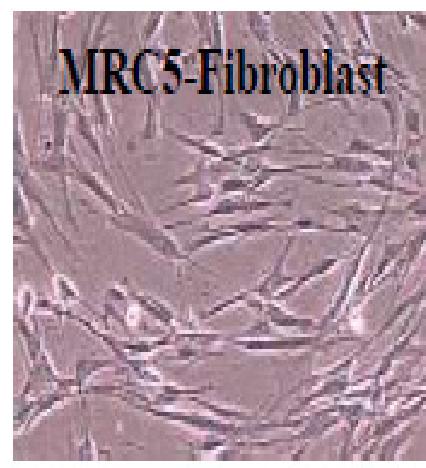
BAE1-Endothelial



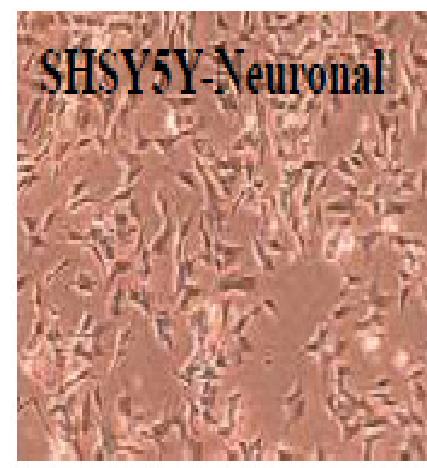
MCF-7 breast



HT1080-kidney



MRC5-Fibroblast



SHSY5Y-Neuronal



3LL - lungs

Types of Animal Cell culture

Primary Cultures

- Derived directly from excised tissue and cultured either as
- ❖ Outgrowth of excised tissue in culture
- ❖ Dissociation into single cells (by enzymatic digestion or mechanical dispersion)
- Retain differentiated phenotype.
- Mainly anchorage dependant.
- Exhibit contact inhibition.

Advantages:

- ❖ usually retain many of the differentiated characteristics of the cell *in vivo*

Disadvantages:

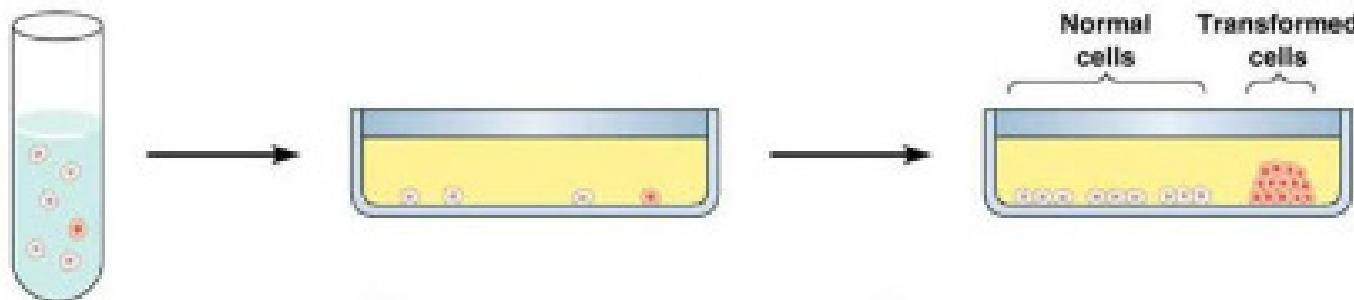
- ❖ Initially heterogeneous but later become dominated by fibroblasts
- ❖ Preparation is labour intensive
- ❖ Limited life span

Continuous Cultures

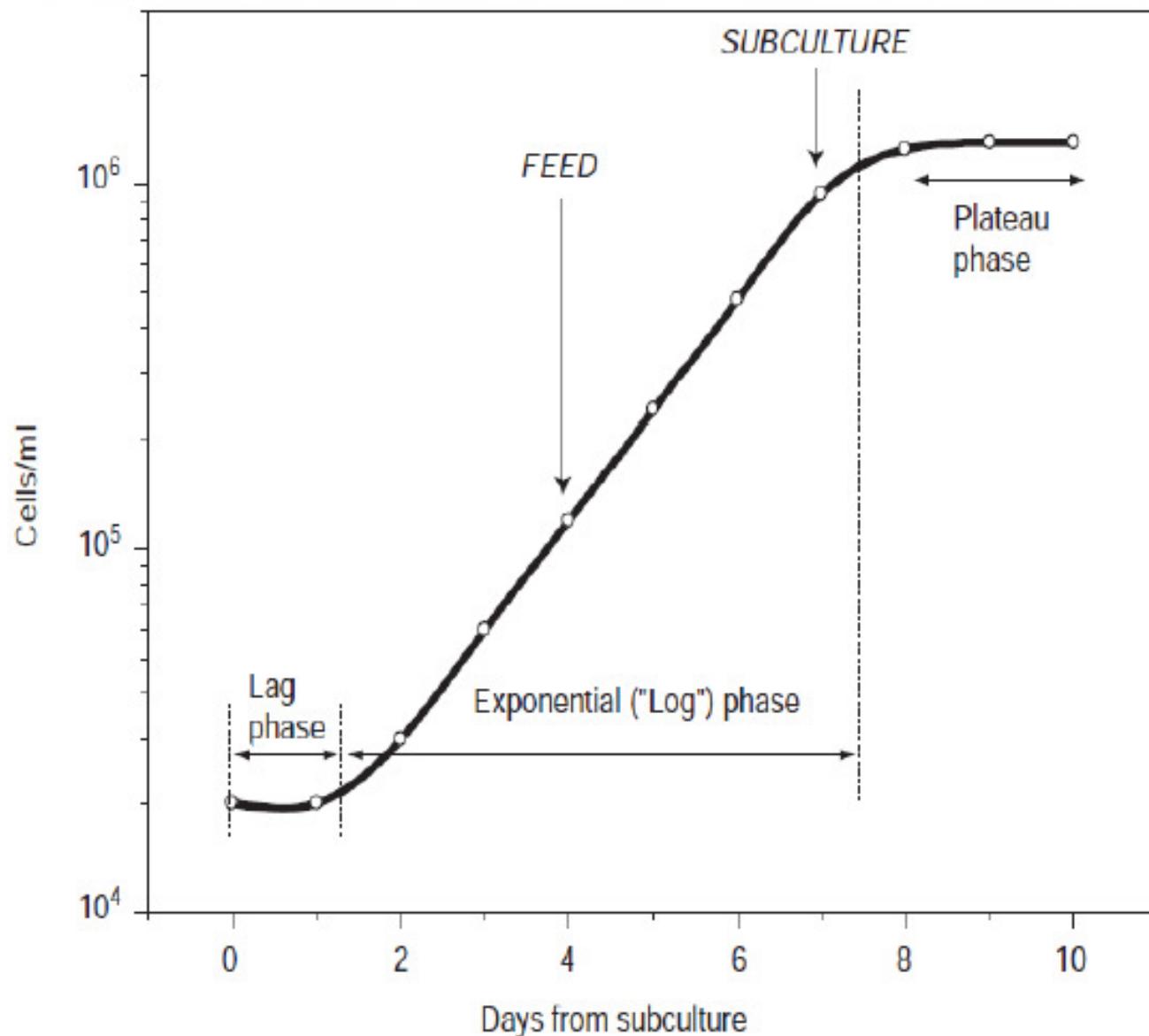
- Derived from subculture (or passage, or transfer) of primary culture

Subculture: the process of dispersion and re-culture the cells after they have increased to occupy all of the available substrate in the culture

- Usually comprised of a single cell type
- Can be serially propagated in culture for several passages

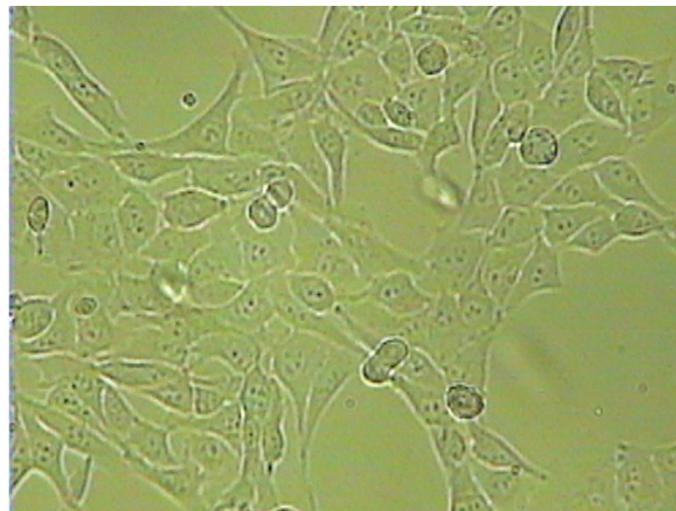


Subculture



Confluency

- ❖ Once the available substrate surface is covered by cells (a confluent culture) growth slows & ceases.
- ❖ Cells to be kept in healthy & in growing state have to be subcultured or passaged , It's the passage of cells when they reach to 80-90% confluence in flask/dishes/plates
- ❖ Enzyme such as trypsin, collagenase in combination with EDTA breaks the cellular glue that attached the cells to the surface



70 % confluent culture



100 % confluent culture

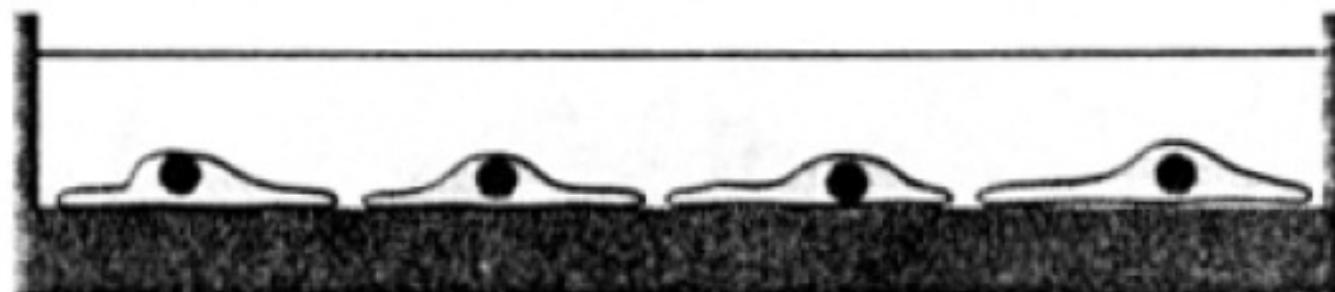
Why passage cells?

- To maintain cells in culture (i.e. don't overgrow)
- To increase cell number for experiments/storage

How?

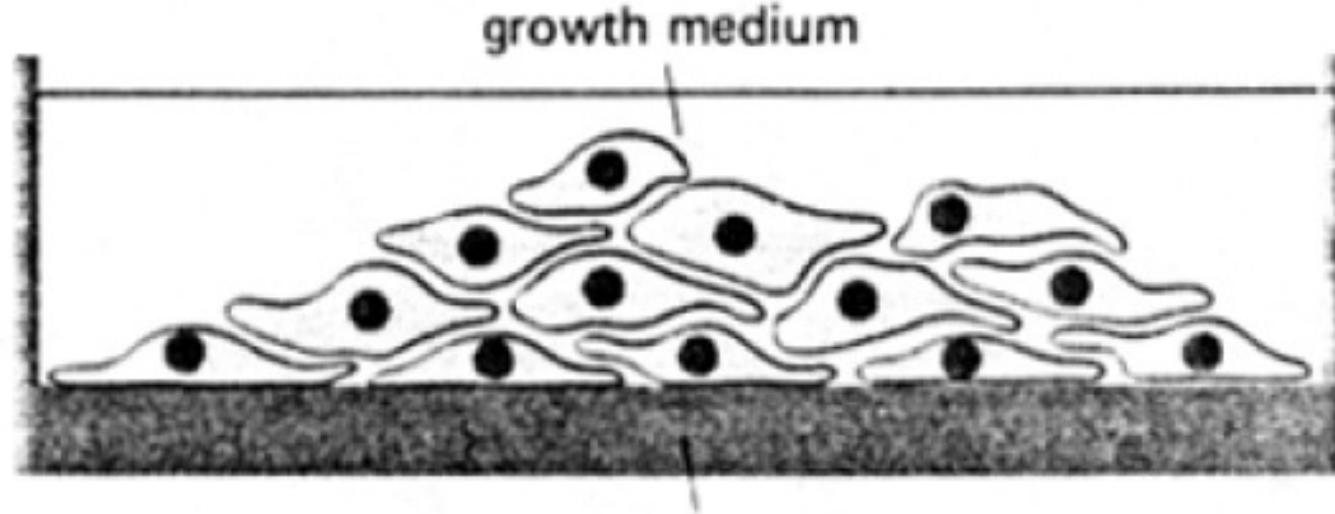
- 70-80% confluency
- Wash in PBS to remove dead cells and serum
- Trypsin digests protein-surface interaction to release cells (collagenase also useful)
- EDTA enhances trypsin activity
- Resuspend in serum (inactivates trypsin)
- Transfer dilute cell suspension to new flask (fresh media)
- Most cell lines will adhere in approx. 3-4 hours

Contact inhibition



contact-inhibited
monolayer of
normal cells

Therefore need
to split them to
maintain growth



multilayer of
uninhibited
cancer cells

plastic tissue culture dish

PASSAGING CELLS

Check confluence of cells



Remove spent medium



Wash with PBS



Incubate with
trypsin/EDTA

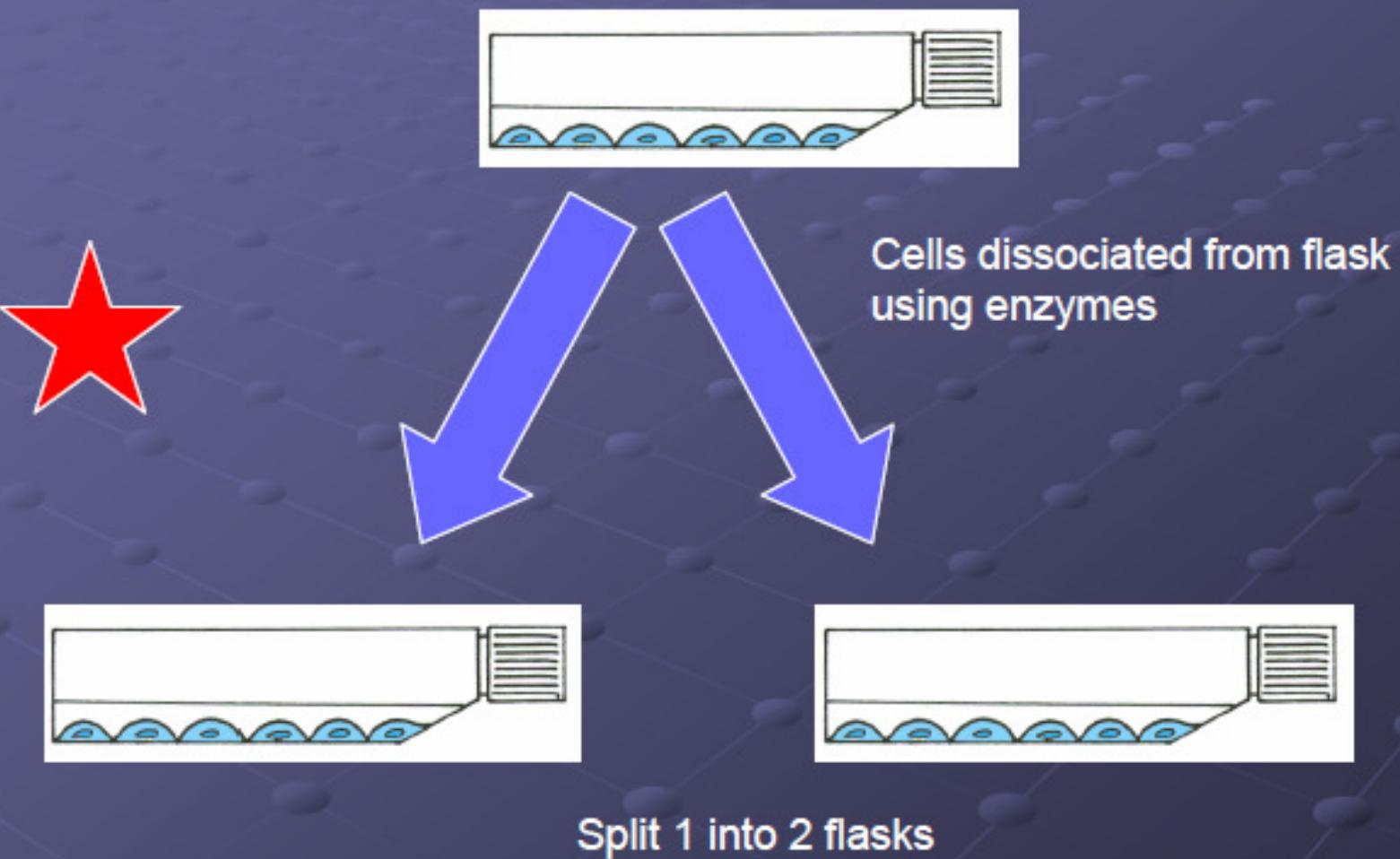


Resuspend in serum
containing media



Transfer to culture flask

Passaging or sub-culture



Types of continuous cultures

- ❖ Cell lines
- ❖ Continuous cell lines

Types of continuous culture

Cell lines

- ❖ Finite life, senesce after approximately thirty cycles of division
- ❖ Retain differentiated phenotype (Usually diploid)
- ❖ Essential to establish a system of Master and Working banks in order to maintain such lines for long periods
- ❖ Isolated by selection or cloning.
- ❖ Becoming a more homogeneous cell population.
- ❖ Finite life span in vitro.
- ❖ Mainly anchorage dependant.
- ❖ Exhibit contact inhibition.

Types of continuous culture

Continuous cell lines (immortalized cell line)

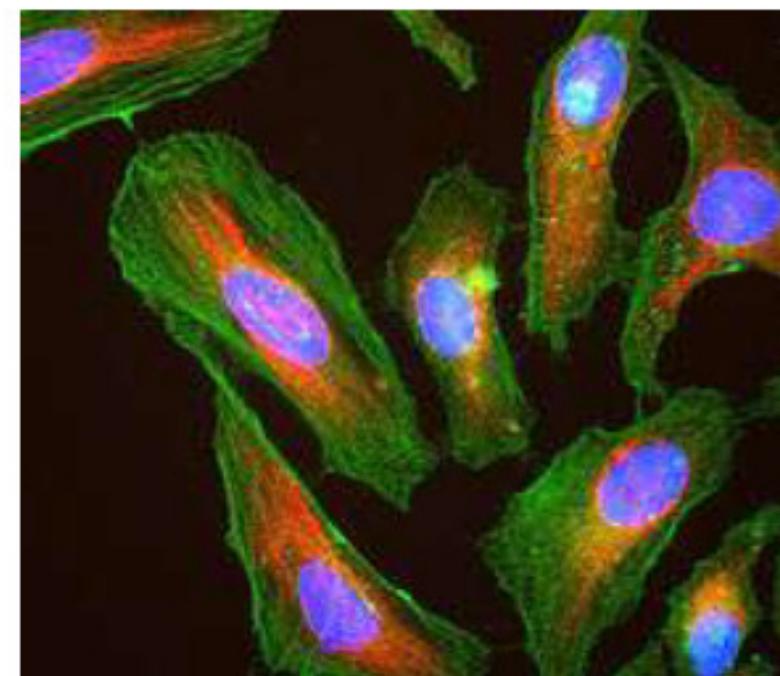
- ❖ Derived from a primary or secondary culture
- ❖ **Immortalised:**
 - Spontaneously (e.g.: spontaneous genetic mutation)
 - By transformation vectors (e.g.: viruses &/or plasmids)
- ❖ Serially propagated in culture showing an increased growth rate
- ❖ Homogeneous cell population
- ❖ Loss of anchorage dependency and contact inhibition
- ❖ Infinite life span in vitro
- ❖ **Disadvantages:** Retain very little of the original *in vivo* characteristics
Fast growth and have aneuploid chromosome number

HeLa

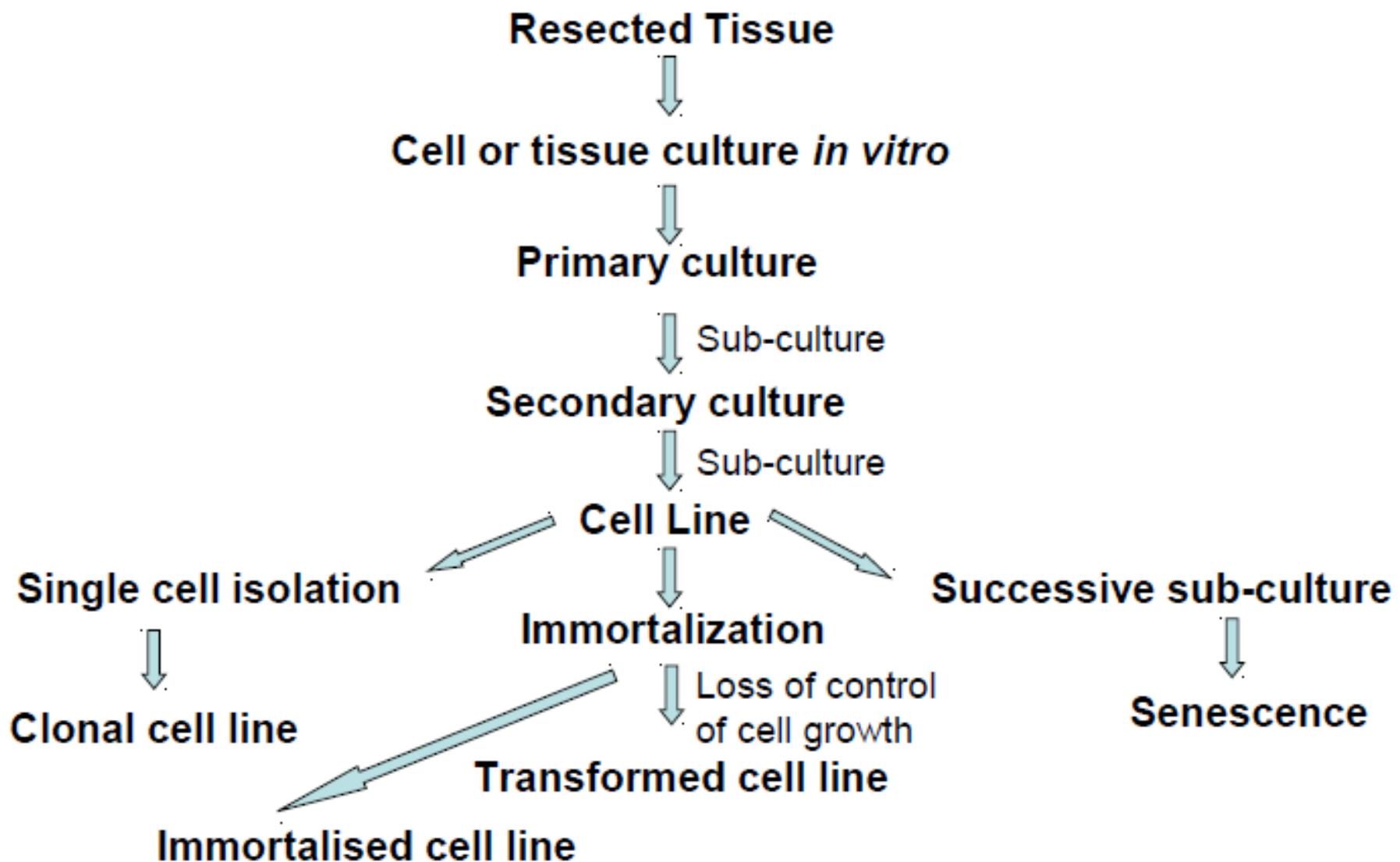
- The oldest and most commonly used human cell line
- 1951 : Derived from cervical cancer cell taken from **Henrietta Lacks** (1920-1951)



Henrietta Lacks (circa 1945)



ISOLATION OF CELL LINES FOR *IN-VITRO* CULTURE



EQUIPMENTS USED ROUTEINLY IN A TYPICAL CELL CULTURE LABORATORY



- Laminar Air Flow
- Vacuum Oven
- CO₂ Incubators
- Centrifuge
- Microscopes
- Culture Dishes
- Culture Media
- Autoclaves
- Freezers
- Dewar Flasks
- Multi-mode Reader
- Real-time PCR
- FACS/MACS
- Electroporator

etc...

Laminar air flow (biosafety cabinet)

- * The working environment is protected from dust and contamination by a constant, stable flow of filtered air
- Two types:
 - Horizontal**, airflow blow from the side facing you, parallel to the work surface, and is not circulating;
 - Vertical**, air blows down from the top of the cabinet onto the work surface and is drawn through the work surface and recalculated

Vertical LAF are preferable. They are fitted with HEPA filters and UV.

Laminar air flow (biosafety cabinet)

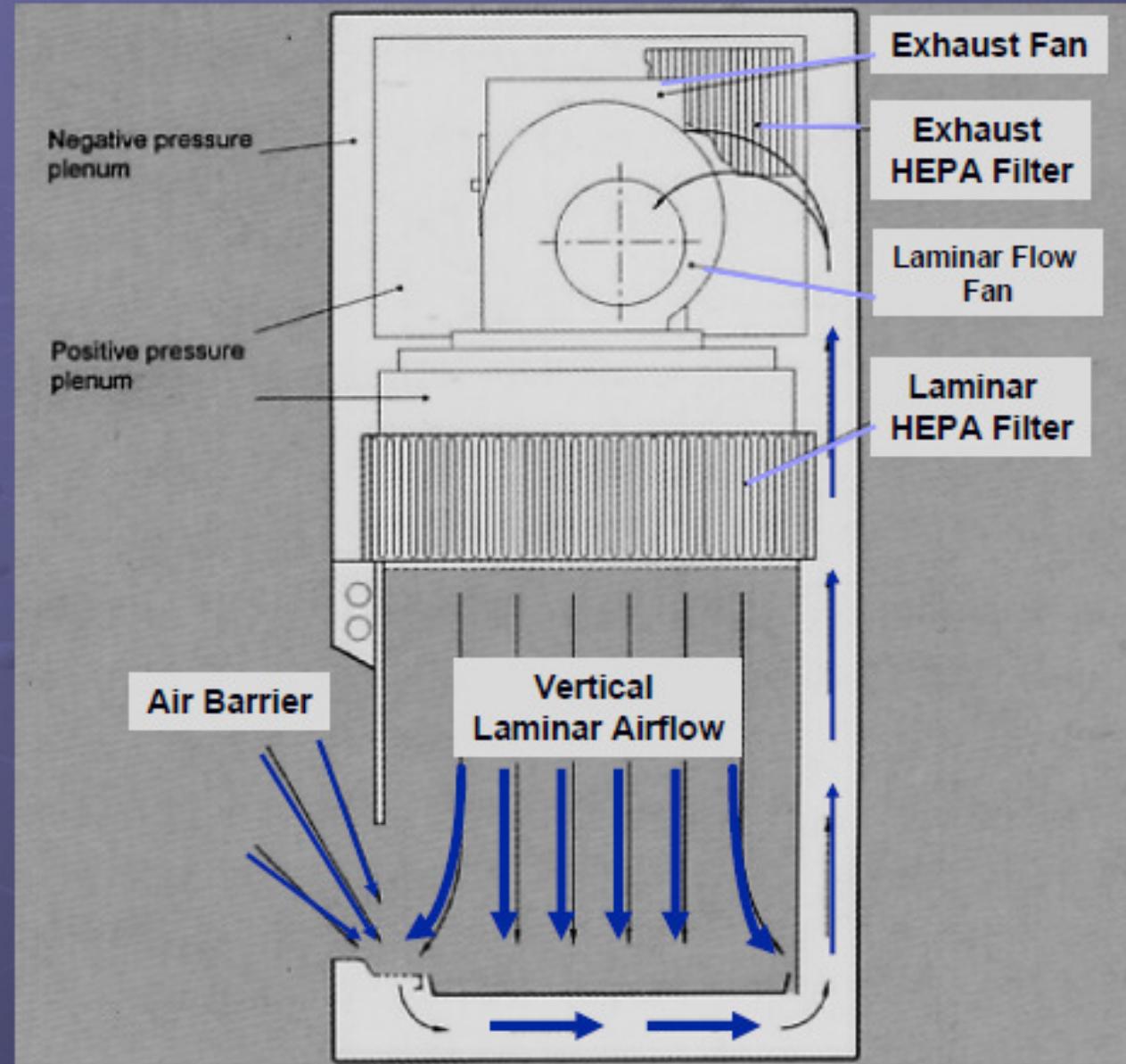
- It is used to provide sterile environment and protect the laboratory worker from exposure to aerosols from cell culture.
- Air is filtered through a **HEPA** (high efficiency particulate air) filter before exiting the cabinet. They are classified at levels I, II, and III.
- **Class I** cabinets are the simplest and easiest to maintain but offer least sterile protection to the cell culture.
- **Class II** cabinets are the most widely used for cell culture work and offer good protection to both the operator and cell cultures since air passing over the working area is HEPA filtered.
- **Class III** cabinets are completely sealed units and are used for more hazardous types of work.

Class 2 Cabinets: Protection of personnel, environment and product Laminar Flow Hood



Class 2 Biological Safety Cabinet

HEPA filters
Laminar flow



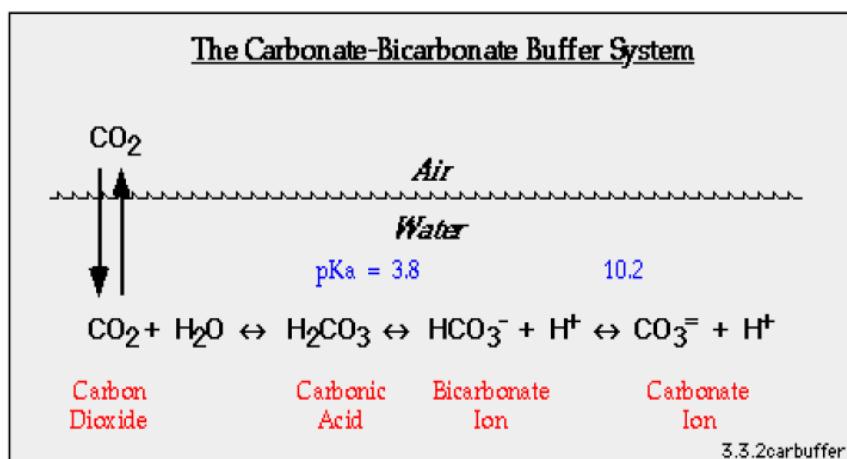
CO₂ incubator

Incubation facilities –

- ❖ Temperature of 37°C,
- ❖ CO₂ - 5%
- ❖ 95% air at 99% relative humidity.



Buffering in Cell Culture



- In a natural buffering system, gaseous CO₂ balances with the CO₃²⁻/HCO₃⁻ content of the culture medium.
- Cultures with a natural buffering system need to be maintained in an air atmosphere with 5-10% CO₂, usually maintained by an CO₂ incubator
- Natural buffering system is low cost and non-toxic

Centrifuge and Inverted microscope



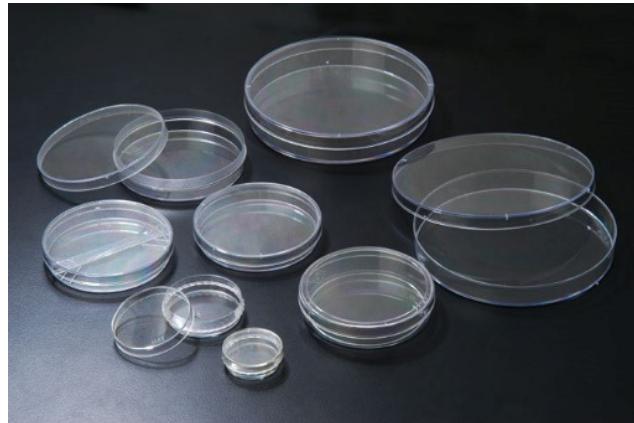
For separation of cells



For visualization of cells

Cell culture vessels (dishes, flasks, plates)

- Flasks and plates are treated with Poly-L-lysine, collagen etc.
(For anchorage dependent cells)



Filters



Other equipment

Refrigerators and deep freezers



Water bath



Liquid nitrogen cans



-20 °C deep
freezer

-80 °C deep
freezer

Cell culture environment (*in vitro*)

Requirements

Substrate: Substrate or liquid (cell culture flask or scaffold material) chemically modified plastic or coated with ECM proteins.

Nutrients (culture media)

Environment (CO₂, temperature 37°C, 100% humidity), Oxygen tension maintained at atmospheric but can be varied.

Sterility (aseptic technique, antibiotics and antimycotics), Mycoplasma tested.

- **Penicillin (100 U/ml) for bacteria,**
- **Streptomycin (100 mg/ml) for bacteria,**
- Gentamycin (50mg/ ml) for bacteria,
- Nystatin (50mg/ml) for fungi and yeast
- Amphotericin B for Fungi

Types of Cell culture media

	Media Type	Examples
Natural media	Biological Fluids	plasma, serum, lymph, human placental cord serum, amniotic fluid
	Tissue Extracts	Extract of liver, spleen, tumors, leucocytes and bone marrow, extract of bovine embryo and chick embryo
	Clots	coagulants or plasma clots
Artificial media	Balanced salt solutions	Eagle's BS, Hank's BS,
	Basal media	MEM DMEM
	Complex media	RPMI-1640, IMDM

Basic Components of Culture Media

- Culture media (as a powder or as a liquid) contains:

- amino acids
- Glucose
- Salts
- Vitamins
- Other nutrients

The requirements for these components vary among cell lines, and these differences are partly responsible for the extensive number of medium formulations .

Cell culture media

Culture medium is a liquid designed to support the growth of cells

Basal Media

- Maintain pH and osmolarity (260-320 mOsm/L).
- Provide nutrients and energy source.

Components of Basal Media

Inorganic Salts

- Maintain osmolarity
- Regulate membrane potential (Na^+ , K^+ , Ca^{2+})
- Ions for cell attachment and enzyme cofactors

pH Indicator – Phenol Red

- Optimum cell growth approx. pH 7.4

- **Buffers** (Bicarbonate, phosphates and HEPES)
- Bicarbonate buffered media requires CO_2 atmosphere
- HEPES Strong chemical buffer range pH 7.2 –7.6 (does not require CO_2)

Glucose

Energy Source

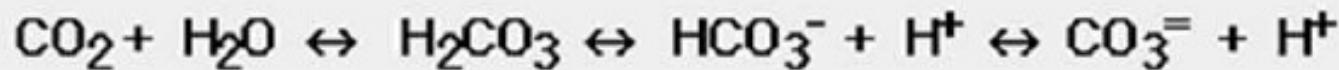


Properties and Special Requirements of Media

- pH: Optimum pH between 7.2 to 7.4 is generally needed for mammalian cells. Phenol red is used as an internal indicator.

pH	Colour of the medium
7.8	Pink / purple

The Carbonate-Bicarbonate Buffer System



Carbon
Dioxide

Carbonic
Acid

Bicarbonate
Ion

Carbonate
Ion

Components of Basal Media

Keto acids (oxalacetate and pyruvate)

- Intermediate in Glycolysis/Krebs cycle
- Keto acids added to the media as additional energy source
- Maintain maximum cell metabolism

Carbohydrates

- Energy source
- Glucose and galactose
- Low (1 g/L) and high (4.5 g/L) concentrations of sugars in basal media

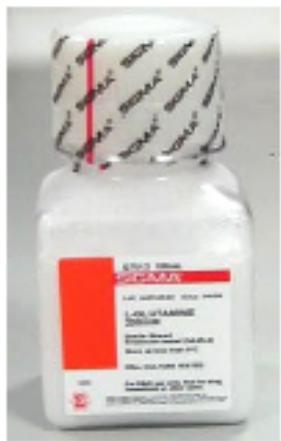
Vitamins

- Precursors for numerous co-factors
- B group vitamins necessary for cell growth and proliferation
- Common vitamins found in basal media is riboflavin, thiamine and biotin

Trace Elements

- Zinc, copper, selenium and tricarboxylic acid intermediates

Supplements



L-glutamine

- Essential amino acid (not synthesised by the cell)
- Energy source (citric acid cycle), used in protein synthesis
- Unstable in liquid media - added as a supplement

Non-essential amino acids (NEAA)

- Usually added to basic media compositions
- Energy source, used in protein synthesis
- May reduce metabolic burden on cells

Media supplements

Growth Factors and Hormones (e.g.: insulin)

- Stimulate glucose transport and utilisation
- Uptake of amino acids
- Maintenance of differentiation

Antibiotics and Antimycotics

- Penicillin, streptomycin, gentamicin, amphotericin B
- Reduce the risk of bacterial and fungal contamination
- Cells can become antibiotic resistant – changing phenotype
- Preferably avoided in long term culture

Foetal Calf/Bovine Serum (FCS & FBS)

- Growth factors and hormones
 - Aids cell attachment
 - Binds and neutralise toxins
 - Long history of use
-
- Infectious agents (prions)
 - Variable composition
 - Expensive
 - Regulatory issues (to minimise risk)



Heat Inactivation (56°C for 30 mins) – why?

- Destruction of complement and immunoglobulins
- Destruction of some viruses (also gamma irradiated serum).

Common Cell Culture Media

- Eagle's Minimum Essential Medium (EMEM)
- Dulbecco's Modified Eagle's Medium (DMEM)
 - Low glucose
 - High glucose
- RPMI-1640
- Ham's Nutrient Mixtures
- DMEM/F₁₂
- Iscove's Modified Dulbecco's Medium (IMDM)

Criteria for Selecting Media

- Immediate survival
- Prolonged survival
- Indefinite growth
- Specialized functions

it's always good to start with MEM for adherent cells and RPMI-1640 for suspension cells

Common media and their applications

Media	Tissue or cell line
IMDM	Bone marrow, hematopoietic progenitor cells, human lymphoblastoid leukemia cell lines
MEM	Chick embryofibroblast, CHO cells, embryonic nerve cells, alveolar type cells, endothelium, epidermis, fibroblast, glia, glioma, human tumors, melanoma
DMEM	Mesenchymal stem cell, chondrocyte, fibroblast, Endothelium, fetal alveolar epithelial type II cells, cervix epithelium, gastrointestinal cells, mouse neuroblastoma, porcine cells from thyroid glands, ovarian carcinoma cell lines, skeleton muscle cells, sertoli cells, Syrian hamster fibroblast
RPMI-1640	T cells and thymocytes, hematopoietic stem cells, human tumors, human myeloid leukemia cell lines, human lymphoblastoid leukemia cell lines, mouse myeloma, mouse leukemia, mouse erythroleukemia, mouse hybridoma, rat liver cells
Nutrient mixture F-10 and F-12	Chick embryo pigmented retina, bone, cartilage, adipose tissue, embryonic lung cells, skeletal muscle cells

Cell Line	Morphology	Species	Medium	Applications
HeLa B	Epithelial	Human	MEM + 2mM Glutamine + 10% FBS + 1% Non Essential Amino Acids (NEAA)	Tumourigenicity and virus studies
HL60	Lymphoblast	Human	RPMI 1640 + 2mM Glutamine + 10-20% FBS	Differentiation studies
3T3 clone A31	Fibroblast	Mouse	DMEM + 2mM Glutamine + 5% New Born Calf Serum (NBCS) + 5% FBS	Tumourigenicity and virus studies
COS-7	Fibroblast	Monkey	DMEM+ 2mM Glutamine + 10% FBS	Gene expression and virus replication studies
CHO	Epithelial	Hamster	Ham's F12 + 2mM Glutamine + 10% FBS	Nutritional and gene expression studies
HEK 293	Epithelial	Human	EMEM (EBSS) + 2mM Glutamine + 1% Non Essential Amino Acids (NEAA) + 10% FBS	Transformation studies
HUVEC	Endothelial	Human	F-12 K + 10% FBS + 100 µg/ml Heparin	Angiogenesis studies
Jurkat	Lymphoblast	Human	RPMI-1640 + 10% FBS	Signaling studies

Cryopreservation

- The preservation of cell stocks at temperatures below -130°C has allowed the long-term storage of cells for periods of at least 2–3 decades.
- Several features are important for optimizing the viability of cells.
- The use of cryoprotective agents that prevent ice crystals forming and the fragmenting of membranes is essential.
- The most commonly used cryoprotective agent is **dimethylsulfoxide (DMSO)**, but glycerol is an alternative.
- The rates of freezing and thawing also influence viability, and a freezing rate of approximately $1^{\circ}\text{C}/\text{min}$ is considered optimal.
- In contrast, thawing should be rapid and this is most easily achieved by placing ampules in a water bath at 37°C .
- Cells are generally stored in liquid nitrogen at -196°C , but can remain viable for short periods of time at -80°C .

- **Why cryopreserve cells?**
- Reduced risk of microbial contamination.
- Reduced risk of cross contamination with other cell lines.
- Reduced risk of genetic drift and morphological changes.
- Research conducted using cells at consistent low passage.

- **How?**

- Log phase of growth and >90% viability
- Passage cells & pellet for media exchange
- Cryopreservant (DMSO) – precise mechanism unknown but prevents ice crystal formation
- Freeze at -80°C – rapid yet ‘slow’ freezing
- Liquid nitrogen -196°C

CRYOPRESERVATION OF CELLS



HOW DO WE CULTURE CELLS IN THE LABORATORY?

Revive frozen cell population
Isolate from tissue



Maintain in culture (aseptic technique)



Sub-culture (passaging)

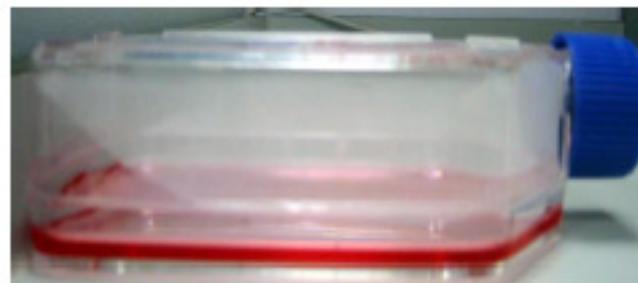


Count cells

Cryopreservation



Containment level 2
cell culture laboratory



Typical
cell culture flask



'Mr Frosty'
Used to freeze cells

Aseptic conditions

- 1. Switch on the laminar flow cabinet 20 mts prior to start working**
- 2. Swab all bottle tops & necks with 70% ethanol**
- 3. If working on the bench use a Bunsen flame**
- 4. Flame all bottle necks & pipette by passing very quickly through the hottest part of the flame**
- 5. Avoiding placing caps & pipettes down on the bench; practice holding bottle tops with the little finger**
- 6. Work either left to right or vice versa, so that all material goes to one side, once finished**

Aseptic conditions

- 7. Clean up spills immediately & always leave the work place neat & tidy**
- 8. Never use the same media bottle for different cell lines.**
- 9. If caps are dropped or bottles touched unconditionally touched, replace them with new ones**
- 10. Necks of glass bottles prefer heat at least for 60 secs at a temperature of 200 C**
- 11. Never use stock of materials during handling of cells.**

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
 - % of viable cells= $\frac{\text{Nu. of unstained cells}}{\text{total nu. of cells}} \times 100$

MANUAL CELL COUNT (HEMOCYTOMETER)

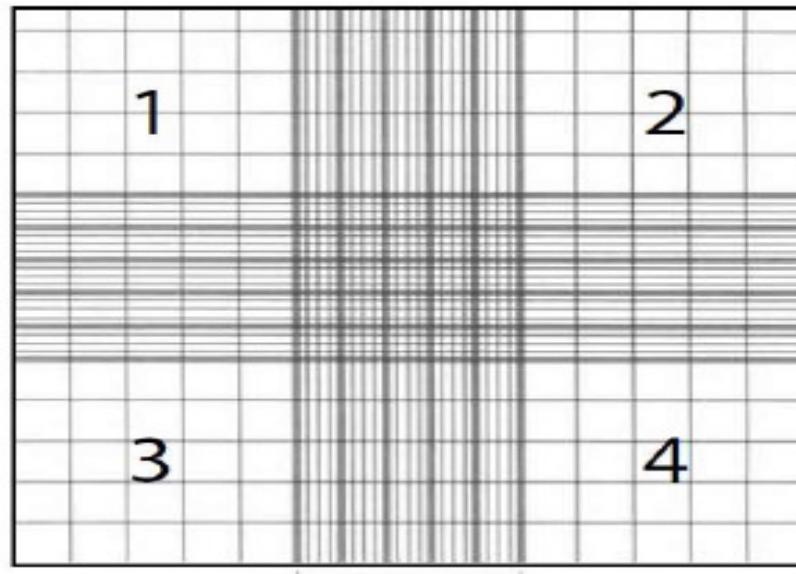
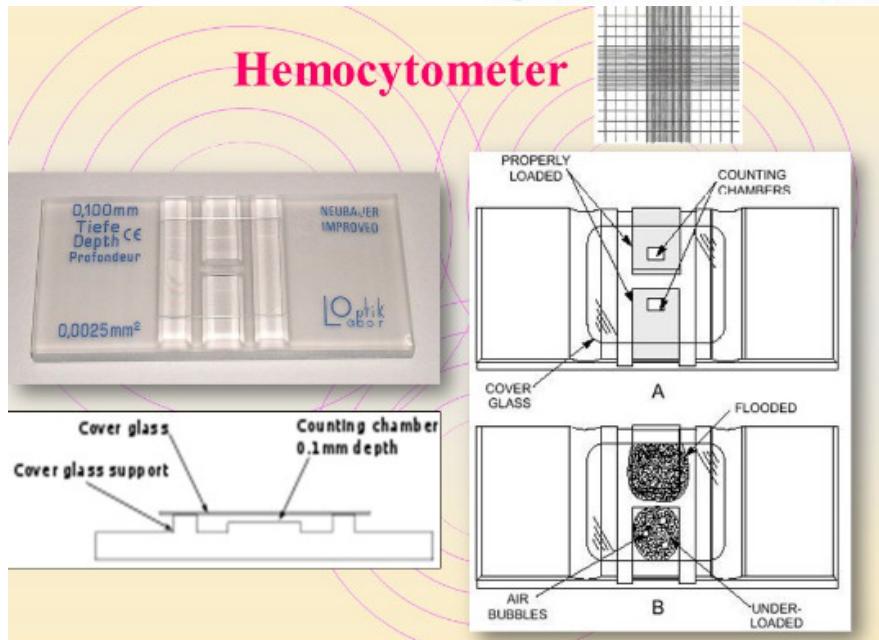


Diagram represent cell count using hemocytometer.

cell count

= average of cell number

× dilution factor of cell suspension × 10 000

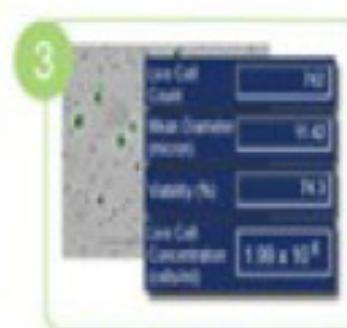
AUTOMATED CELL COUNT



20µL Sample



Insert Slide



Get Data

Celldometer lets you:

- View cell morphology, for visual confirmation after cell counting
- Take advantage of 300+ cell types and easy, wizard-based parameter set-up
- Save sample images with results securely on your computer, plus autosave results on the network for added convenience and data protection

CONTAMINATION

A cell culture contaminant can be defined as some element in the culture system that is undesirable because of its possible adverse effects on either the system or its use.

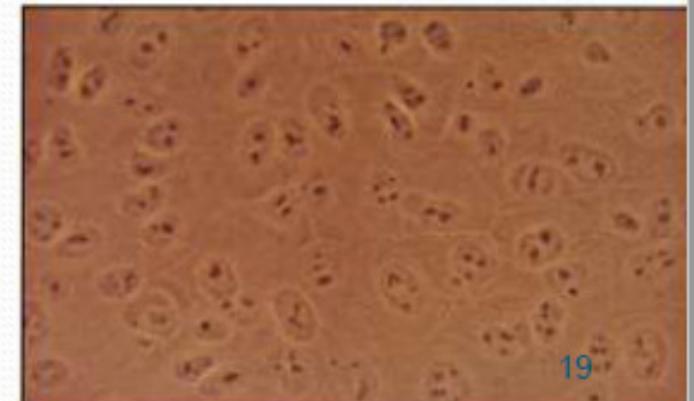
**1-Chemical
Contamination
Media
Incubator
Serum
water**

**2-Biological
Contamination
Bacteria and yeast
Viruses
Mycoplasmas
Cross-contamination by
other cell culture**

Contaminant's of cell culture

Cell culture contaminants of two types

- **Chemical-difficult to detect caused by endotoxins, plasticizers, metal ions or traces of disinfectants that are invisible**
- **Biological-cause** visible effects on the culture they are mycoplasma, yeast, bacteria or fungus or also from cross-contamination of cells from other cell lines



Effects of Biological Contamination's

- They competes for nutrients with host cells
- Secreted acidic or alkaline by-products ceases the growth of the host cells
- Degraded arginine & purine inhibits the synthesis of histone and nucleic acid
- They also produces H₂O₂ which is directly toxic to cells

Detection of contaminants

- In general: turbid culture media, change in growth rates, abnormally high pH, poor attachment, multi-nucleated cells, graining cellular appearance, vacuolization, inclusion bodies and cell lysis
- Yeast, bacteria & fungi usually shows visible effect on the culture (changes in medium turbidity or pH)
- Mycoplasma detected by direct DNA staining with intercalating fluorescent substances e.g. Hoechst 33258
- Mycoplasma also detected by enzyme immunoassay by specific antisera or monoclonal abs or by PCR amplification of mycoplasmal RNA
- The best and the oldest way to eliminate contamination is to discard the infected cell lines directly

Characteristic features of microbial contamination

Characteristic	Bacteria	Yeast	Fungi
Change in pH	pH drop with most infections	pH change with Heavy infections	pH changes sometimes
Cloudy medium: Under microscope (100–400x)	Shimmering in spaces between cells; rods or cocci may be observed	Round or ovoid particles that bud off smaller particles	Thin filamentous mycelia; sometimes clumps of spores

Applications of cell culture

**Cancer
Research**

**Vaccine
manufacture**

Gene therapy

**Recombinant
Protein**

**Drug selection
and
improvement**

**Stem Cell
Biology**

IVF Technology

Applications of cell culture

Model systems

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.

Virology

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

Cancer research

Study the function of various chemicals, virus & radiation to convert normal cultured cells to cancerous cells.

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

Applications of cell culture

Gene therapy

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

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

In vitro disease models and disease prognosis

Osteoarthritis, diabetes, Skin diseases etc.

Stem cell biology and research

Therapeutic effect of stem cells in various diseases and stem cells differentiation to different lineages