Cell culturing

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Reading resources

Qiagen
https://www.qiagen.com/za/resources/molecular-biology-methods/animal-cell-culture/

Gibco cell culture basics- material, videos and virtual culturing
http://www.thermofisher.com/za/en/home/references/gibco-cell-culture-basics.html

Amazing, thank you Gibco, not only am I learning a lot before I start my Quantitive Biology course in March, even better, it is an amazing way to get young biologists, if not already familiar with lab protocols, to learn protocols before they enter a lab.

Gibco cell culture basics book
http://www.thermofisher.com/content/dam/LifeTech/global/life-sciences/pdfs/March2015_PG1315-PJ5831-CO012890-REPRINT-Gibco-Cell-Culture-Basics-Handbook-Americas-FLR.pdf

Learning Objectives

- After the session, students should be able to explain
  - the meaning of tissue culture and various types of cell cultures
  - the application of cell culture
  - the advantages and disadvantages of each type of cell culture
  - the significance of culture environment on cell culture
  - the basic procedure of cell culture
  - the safety considerations for cell culture work
  - Instrumentation and technologies used with cell culturing

What is tissue culture?

- In vitro culture (maintain and/or proliferate) of cells, tissues or organs
- Types of tissue culture
  - Organ culture
  - Tissue culture
  - Cell culture

Organ culture

- The entire embryos or organs are excised from the body and culture
- Advantages
  - Normal physiological functions are maintained.
  - Cells remain fully differentiated.
- Disadvantages
  - Scale-up is not recommended.
  - Growth is slow.
  - Fresh explantation is required for every experiment.

Tissue Culture

- Fragments of excised tissue are grown in culture media
- Advantages
  - Some normal functions may be maintained.
  - Better than organ culture for scale-up but not ideal.
- Disadvantages
  - Original organization of tissue is lost.

Cell Culture

- Tissue from an explant is dispersed, mostly enzymatically, into a cell suspension which may then be cultured as a monolayer or suspension culture.
- Advantages
  - Development of a cell line over several generations
  - Scale-up is possible
- Disadvantages
  - Cells may lose some differentiated characteristics.

What is cell culture used for?

Areas where cell culture technology is currently playing a major role.

- 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

Contd….

- Virology

  Cultivation of virus for vaccine production.

- 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

Advantages of Cell culture

- Advantages:
  - Absolute control of physical environment
  - Homogeneity of sample
  - Less compound needed than in animal models
- Disadvantages:
  - Hard to maintain
  - Only grow small amount of tissue at high cost
  - Dedifferentiation (loss of specialized function)
  - Instability, aneuploidy

Introduction

- Cell culture is 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.
- 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

Cell Culture *in vitro* - A brief history

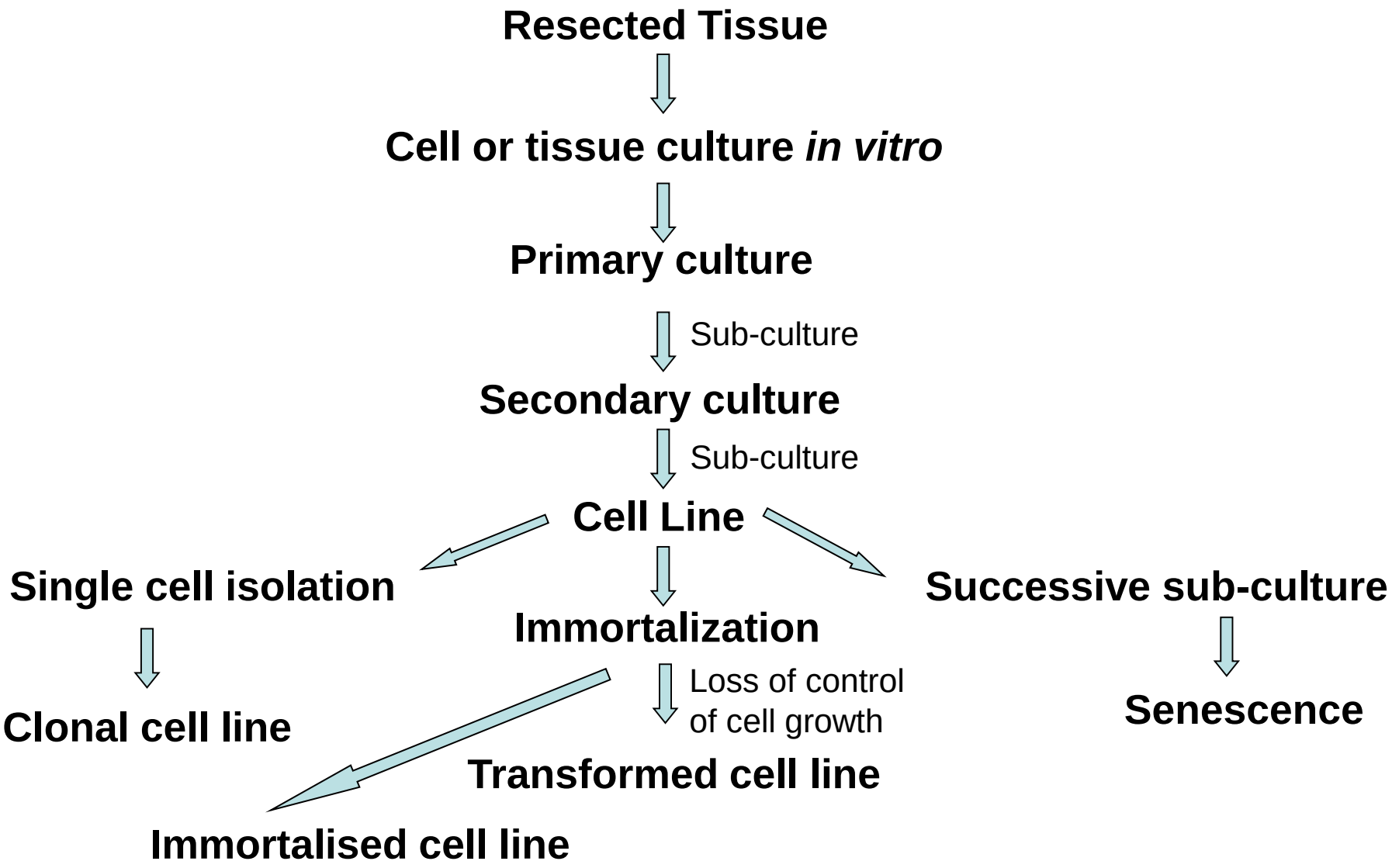
- **1885: Roux maintained embryonic chick cells alive in saline solution for short lengths of time**
- **1912: Alexis Carrel cultured connective tissue and showed heart muscle tissue contractility over 2-3 months**
- **1943: Earle et al. produced continuous rat cell line**
- **1962: Buonassisi *et al*. 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**

Just for information

Major developments 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.

Isolation of cell lines for *in vitro* culture



Types of cell cultured *in vitro*

Primary cultures

- **Derived directly from animal tissue**
  embryo or adult? Normal or neoplastic?
- **Cultured either as tissue explants or single cells**
- **Finite life span *in vitro***
- **Retain differentiated phenotype**
- **Mainly anchorage dependant**
- **Exhibit contact inhibition**

Contact inhibition is a growth mechanism which functions to keep cells growing into a layer one cell thick (a monolayer)

Types of cell cultured *in vitro*

Secondary cultures

- Derived from a primary cell culture
- Isolated by selection or cloning
- Becoming a more homogeneous cell population
- Finite life span *in vitro*
- Retain differentiated phenotype
- Mainly anchorage dependant
- Exhibit contact inhibition

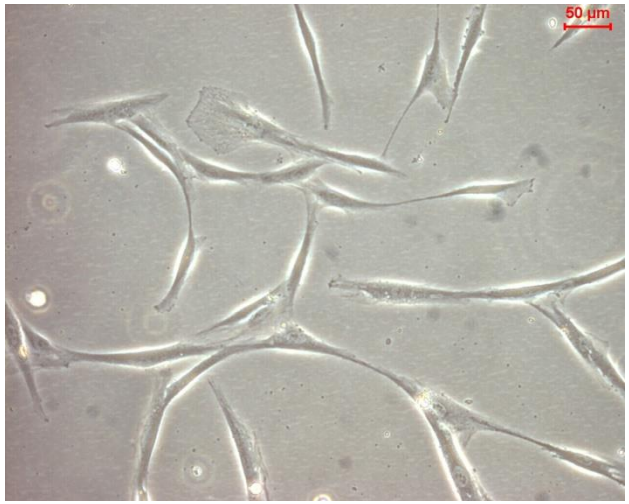
Types of cells

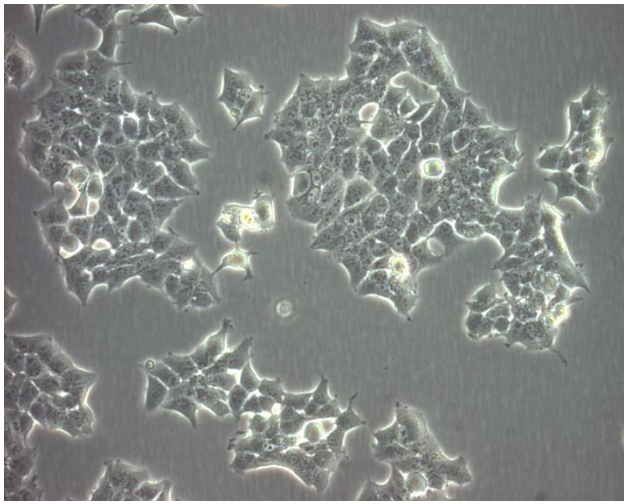
On the basis of morphology (shape & appearance) or on their functional characteristics. They are divided into three.

- 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 a substrate appear elongated and bipolar

Cell morphologies vary depending on cell type

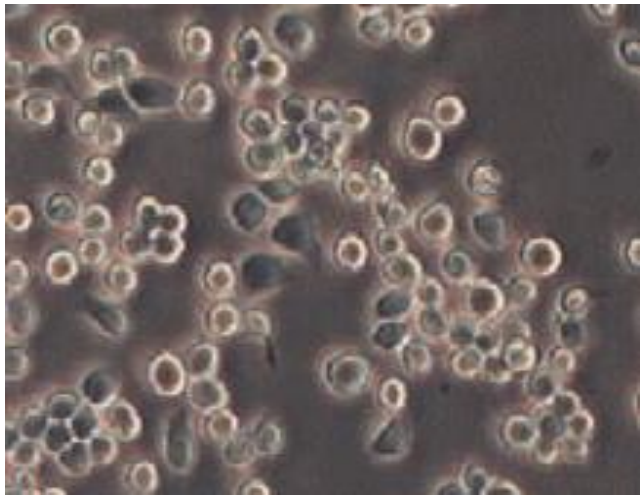
Fibroblastic

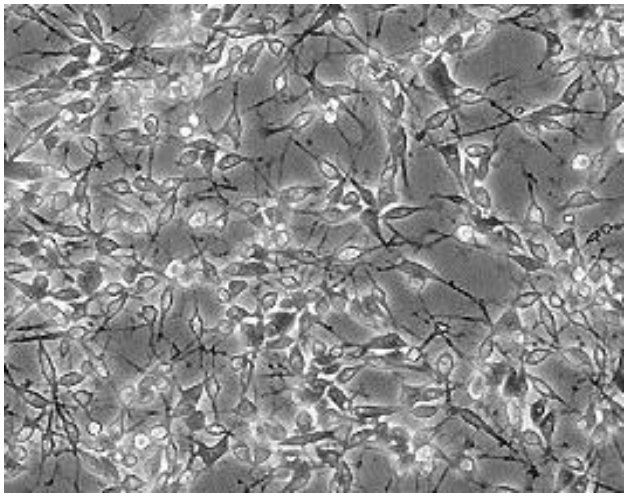




Epithelial

Lymphoblastic

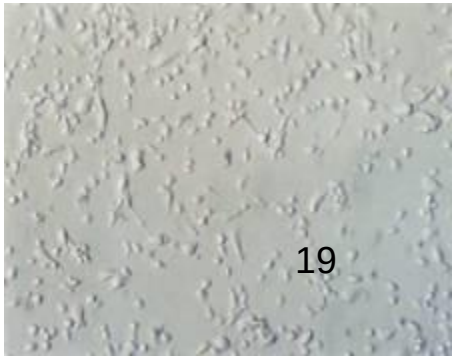




Neuronal

Culturing of cells

- Cells are cultured as anchorage dependent or independent
- Cell lines derived from normal tissues are considered as anchorage-dependent grows only on a suitable substrate e.g. tissue cells
- Suspension cells are anchorage-independent e.g. blood cells
- Transformed cell lines either grows as monolayer or as suspension



How do we culture cells in the laboratory?

Revive frozen cell population
Isolate from tissue

⇩

Maintain in culture (aseptic technique)

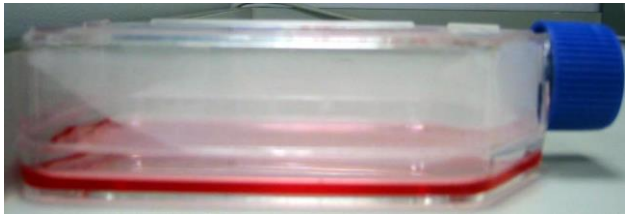
⇩

Sub-culture (passaging)

⇩ **Count cells**

Cryopreservation

Containment level 2 cell culture laboratory



Typical cell culture flask

'Mr Frosty' Used to freeze cells

Passaging Cells

Check confluency of cells

⇩

Remove spent medium

⇩

Wash with PBS

⇩

Incubate with trypsin/EDTA

⇩

Resuspend in <u>serum</u> containing media

⇩

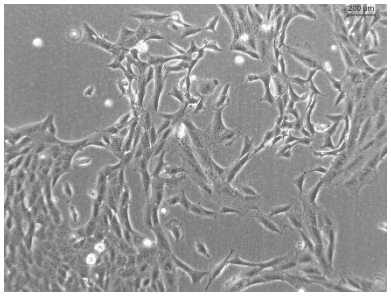
Transfer to culture flask

Why passage cells?

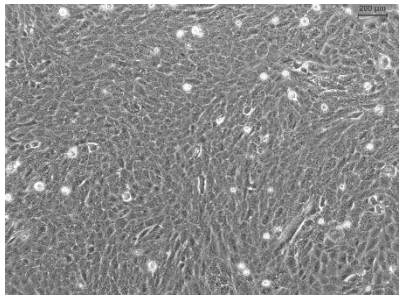
- To maintain cells in culture (i.e. don't overgrow)
- To increase cell number for experiments/storage

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 media (inactivates trypsin)
- Transfer dilute cell suspension to new flask (fresh media)
- Most cell lines will adhere in approx. 3-4 hours



70-80% confluence

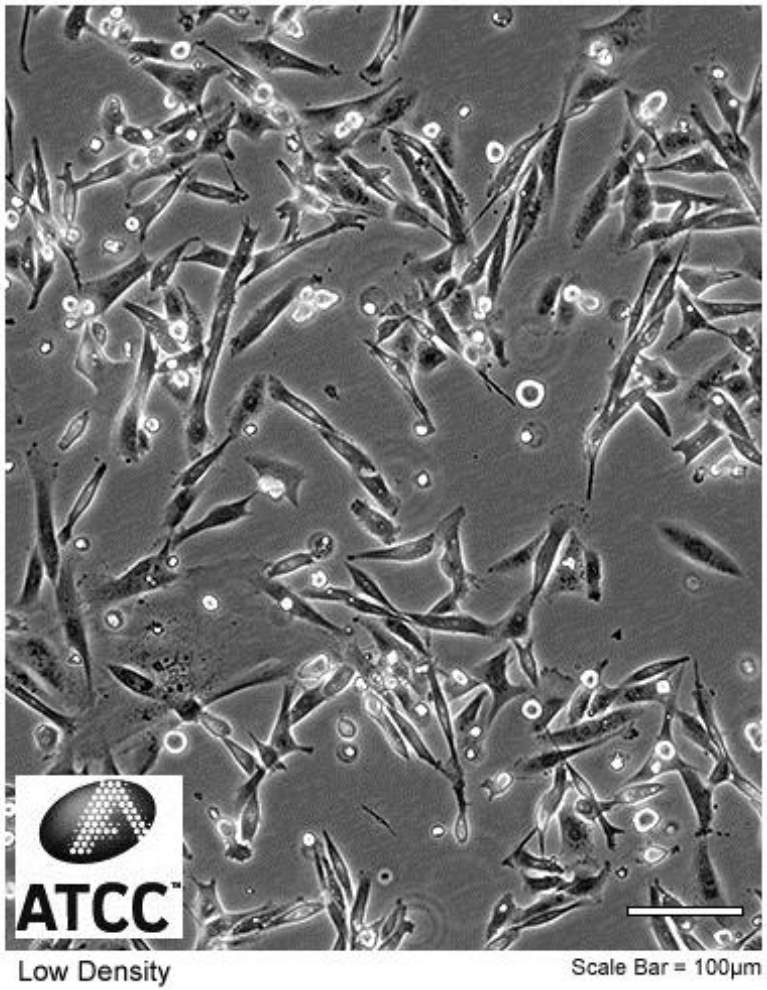


100% confluence

Adherent cells

- Cells which are anchorage dependent
- 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 minutes
- 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

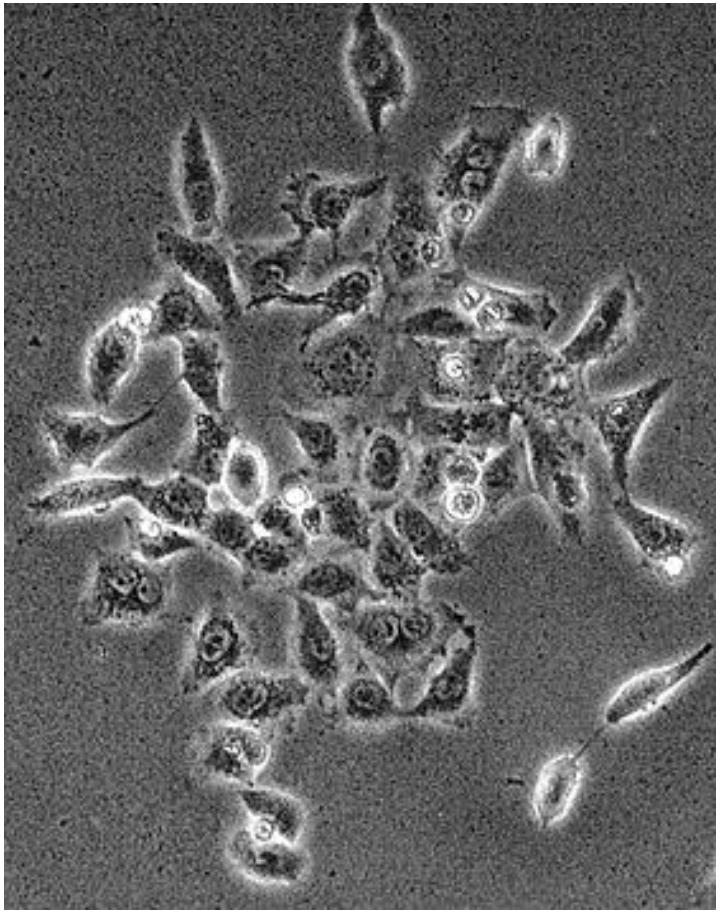
MDA-MB-231 (Human breast adenocarcinoma)



- Elongated with spindle-like projections
- Metastatic (derived from breast tissue)
- Isolated from pleural effusion (metastasised to lung tissue)

Adapted from: http://www.atcc.org/products/all/HTB-26.aspx

HEK (Human embryonic kidney cells)



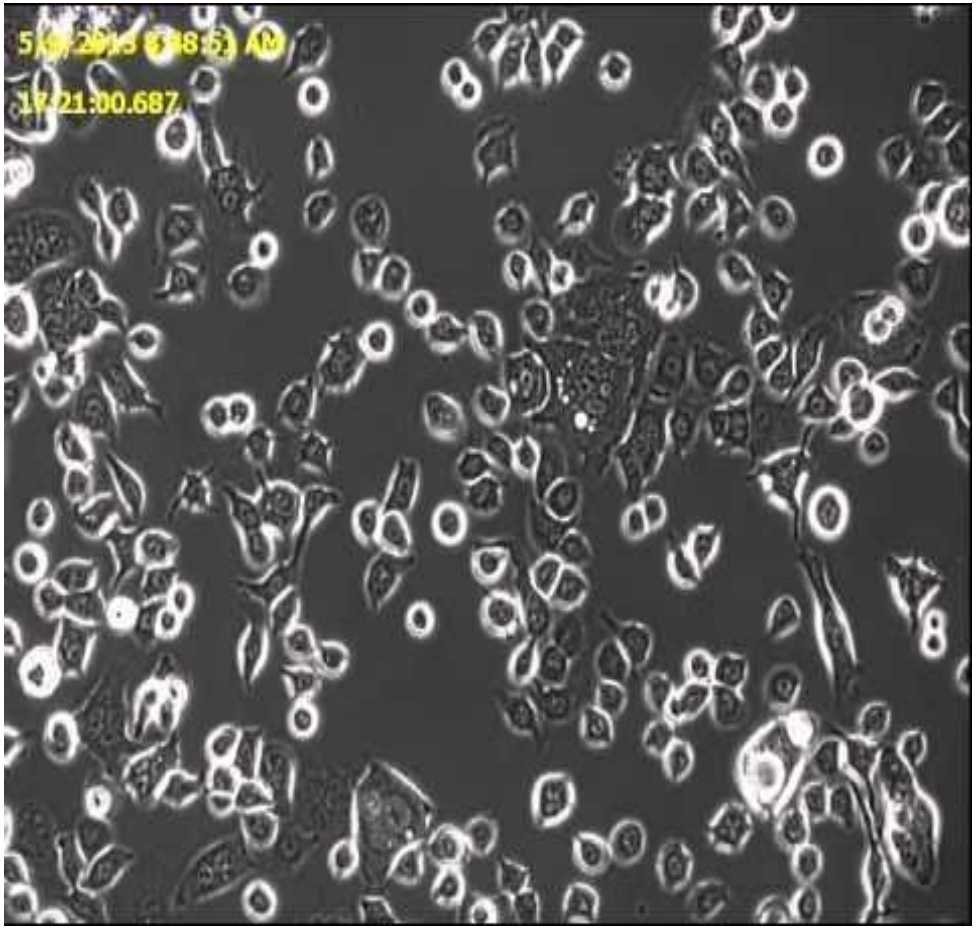
http://en.wikipedia.org/wiki/HEK_293_cells

- Rounded in shape with spindle-like edges
- Derived from human embryonic kidney cells grown in tissue culture
- Epithelial-like morphology

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

AsPC-1 pancreatic cancer cells



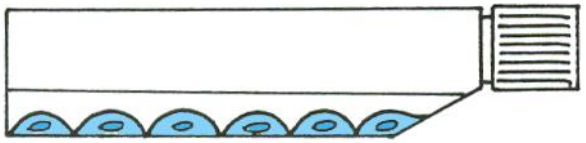
- Spherical with spindle-like protrusions/ endings
- Rapid and aggressive growth
- Metastatic
- Derived from the ascites of a patient with pancreatic cancer

https://www.google.com/search?q=aspc+1+cells&source=lnms&tbm=isch&sa=X&ei=WEn4VKHpNsavU-yhhNgO&ved=0CAcQ_AUoAQ&biw=1240&bih=890#imgdii=_&imgrc=bdFPKhnW-jbAGM%253A%3BwL6rCSSpvQDzHM%3Bhttp%253A%252F%252Fi1.ytimg.com%252Fvi%252F4GNactizyhg%252F0.jpg%3Bhttp%253A%252F%252Farticle.wn.com%252Fview%252F2014%252F04%252F29%252FChina_acts_on_dumping_of_TBHQ_imports%252F%3B480%3B360

Why sub culturing.?

- 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 sub-cultured or passaged
- It’s the passage of cells when they reach to 80-90% confluency in flask/dishes/plates
- Enzyme such as trypsin, dipase, collagenase in combination with EDTA breaks the cellular glue that attached the cells to the surface

The cell culture environment



Factors affecting cell behaviour *in vivo*

- The local micro-environment
- Cell-cell interactions
- Tissue architecture
- Tissue matrix
- Tissue metabolites
- Locally released growth factor and hormones

Cell culture environment (*in vitro*)

What do cells need to grow?

- **Substrate or liquid (cell culture flask or scaffold material)**
  - **chemically modified plastic or coated with ECM proteins**
  - **suspension culture**
- **Nutrients (culture media)**
- **Environment (CO_2, temperature 37^{o}C, humidity)**
  - **Oxygen tension maintained at atmospheric but can be varied**
- **Sterility (aseptic technique, antibiotics and antimycotics)**
  - **Mycoplasma tested**

pH Control

- Physiological pH 7
- pH can affect
  - Cell metabolism
  - Growth rate
  - Protein synthesis
  - Availability of nutrients
- CO_2 acts as a buffering agent in combination with sodium bicarbonate in the media

(-pKa of sodium bicarbonate is 6.3 at 37°C which results in suboptimal buffering throughout the physiological pH range.
-carbon dioxide is released in the atmosphere with a resulting increase in alkalinity)

Temperature and Humidity

- Normal body temperature 37^{o}C

- Humidity must be maintained at saturating levels as evaporation can lead to changes in
  - Osmolarity
  - Volume of media and additives

Introduction of Cell Culture Lab (Equipment)

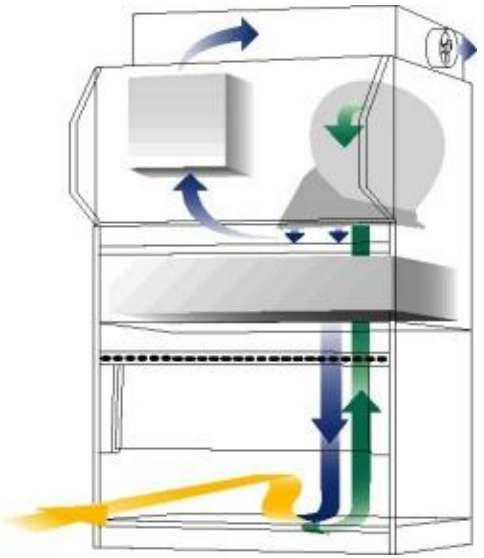
- CO_2-thermostats
- Airflow
- Solutions
- Dishes
- Freezers
- Liquid nitrogen
- Centrifuges
- Autoclave
- Vacuum ovens
- Cryotubes
- Microscopes
- ELISA-readers

CO_2 Incubators

- Water Jacketed CO_2 incubator
- 3 Gas/CO_2 Incubator with RH Control
  - Precise control of Oxygen levels combined with CO_2, and RH ensure accurate conditions for applications such as, hypoxic cell studies and cancer research.

Laminar Flow Box

- HEPA filter rated at 99.99% efficient for 0.3 micron particulates. The HEPA filtered air is then directed vertically across the work surface.



Dishes

- Dishes
- Multiwell plates
- Flasks

Freezers

Centrifuges

Vacuum Ovens

Microscopes

ELISA readers

Contamination

Minimise the risk

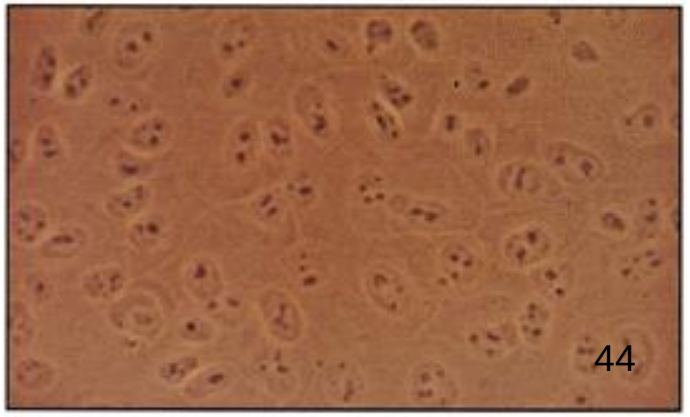
Cell culture- general

- Cell cultivation has to be carried out in a sterile environment to prevent contamination of the cultivated cells with bacteria or viruses.
- For this reason, cell cultivation is normally done in a separate lab, which is fitted especially for this purpose. The most important equipment is a sterile working place.

Contaminants 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



Sources of Contamination

- Bacteria
- Fungi
- Mould
- Yeast
- Mycoplasma
- Other cell types

- Free organisms, dust particles or aerosols
- Surfaces or equipment

Class II Biological Safety Cabinet

Protection of
- **personnel**
- **environment**
- **product**

Class 1 Cabinets protect the product only

Cultivation of mammalian cells

Laminar flow box (Safety level 2)

•the constant air flow prevents particles from the room air coming near the working bench.
•The experiments as well as the experimenter are thus protected from being contaminated.

Cultivation of cells in incubators

- Cells should be cultivated under conditions as close to *in-vivo*- conditions. Therefore, special cell incubators are used. They provide constant temperature (37°C), in a humidified 5% CO_2 atmosphere (to provide constant pH via the CO_2/HCO_3-buffer system).

Mammalian cell Incubators --- provide optimal growth conditions

- CO_2 – incubators provide a humidified 5% CO_2 atmosphere at 37°C, which is optimal for cell growth.
- Growth media contain all necessary growth factors for the cells. Growth factors like amino acids or salts can be added through media additives.
- Generation time of mammalian cells: approx. 24 h

Safety….

Use of Cell Culture areas

- The cell culture area, as any other laboratory is a working area

- Do not bring your friends in with you
- Do not eat, drink or smoke in these areas
- Do not use a mobile phone

- Do wear a lab coat at all times whether in a cell culture area or a laboratory
- Do wear disposable gloves, but make sure that you dispose of them in the correct way before you leave the area

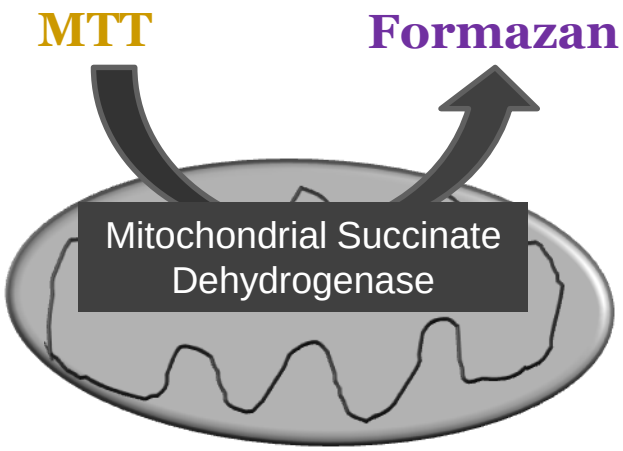
- Do not wear disposable gloves in the corridors or write-up areas

Techniques

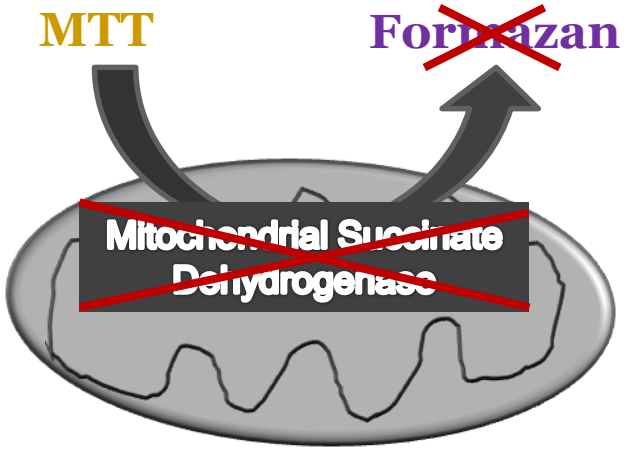
- Metabolic activity (MTT)- cell viability
- Detection of Apoptosis and Necrosis
- Western blot from cells
- Transfection
- Flow Cytometry Methods
- Microscopy

Cell Viability (MTT Assay)

Viable



Non Viable

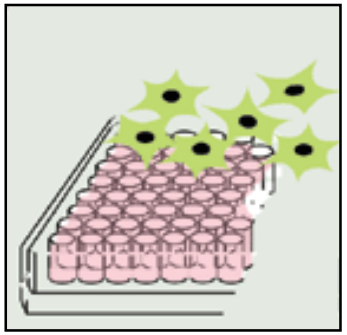


The MTT (3-(4, 5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide) assay is used to determine percentage cell survival in response to a drug, antibody, etc. MTT is a yellow tetrazolium salt that becomes converted into purple formazan crystals by the enzyme mitochondrial succinate dehydrogenase which is present in the mitochondria of viable cells. The absorbance of the formazan crystals is directly proportional to cell viability and is read at 570nm.
Since the formazan crystals are only produced in viable cells, the higher the absorbance, the higher the number of viable cells in the solution. PCA (protocatechuic acid) will be used to induce cell death.

Therefore the higher the absorbance of formazan
The higher the number of viable cells

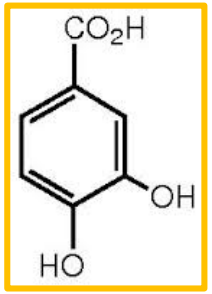
MTT Assay

0h



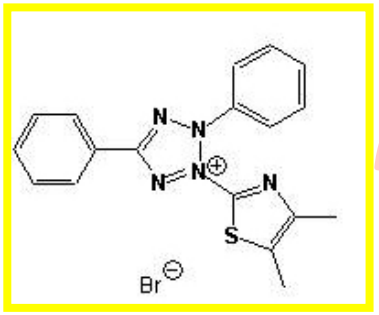
1) Seed Cells ($1x10^5$ cells per well)

24h



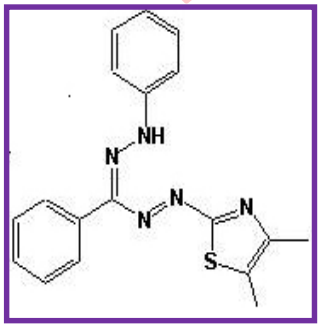
2) Add Protochatechuic Acid (PCA)/H2O2 to 3 wells –
serves as a positive control as it induces apoptosis

48-72h



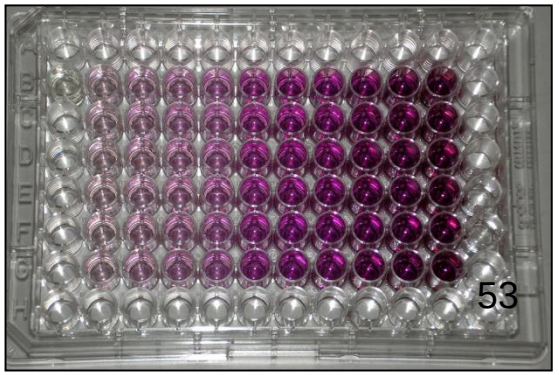
3) Add 1mg/ml MTT
Allow cells to metabolize for at least 2 h

Mitochondrial succinate dehydrogenase



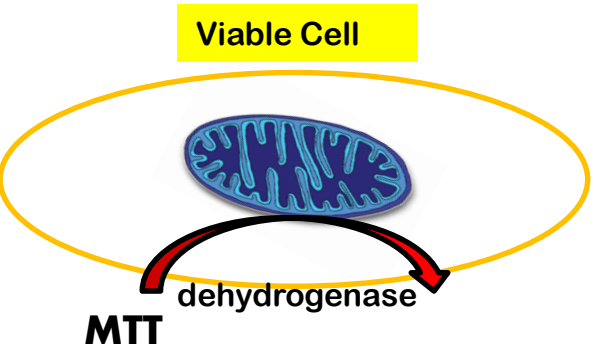
4) Formation of purple Formazan crystals

5) Crystals are dissolved in DMSO



6) Read absorbance at 570nm

Principle

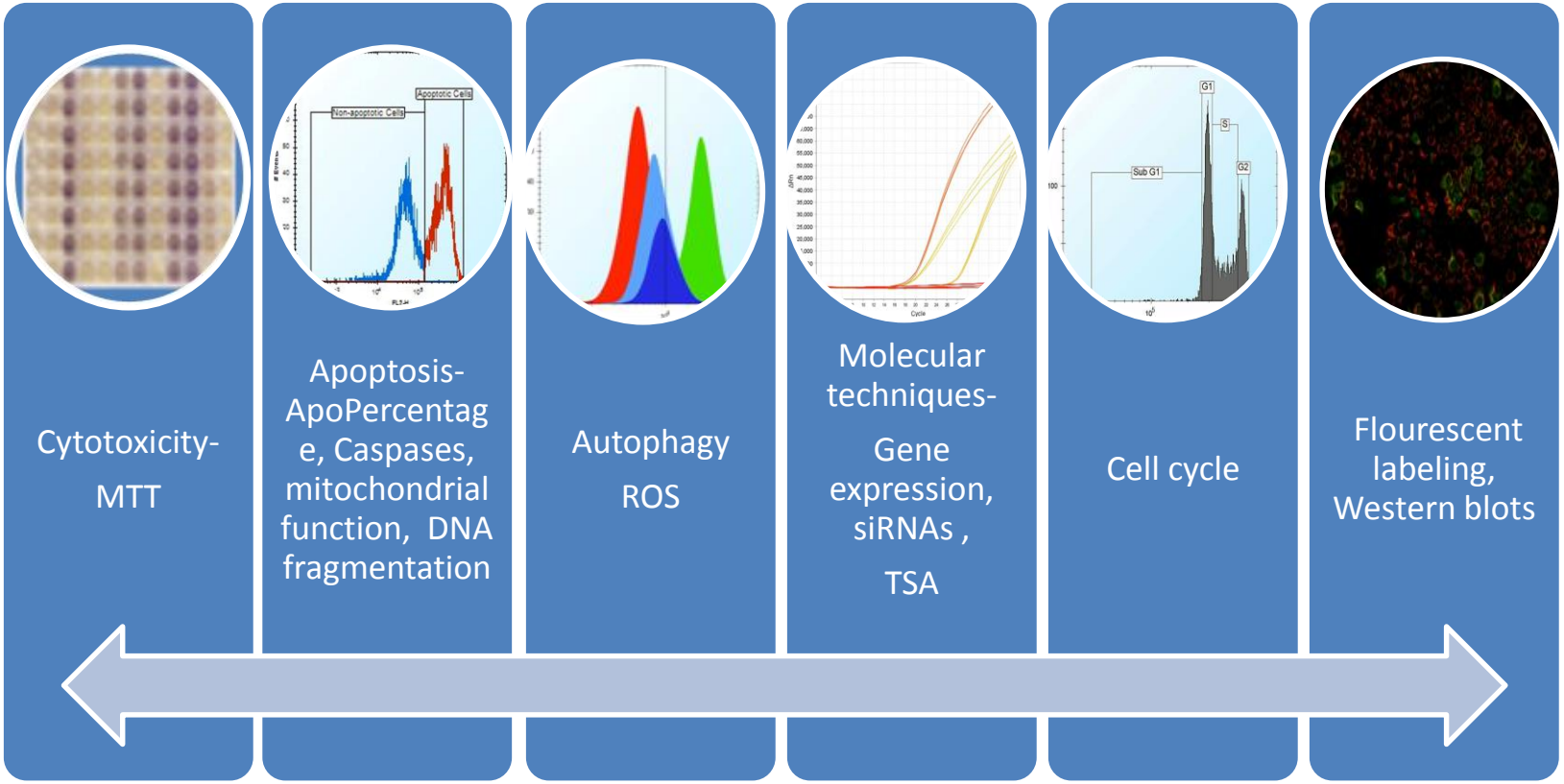


MTT Formazan

Applications of cell culturing

Highthroughput Screening

Cell-based Throughput Screening



'Add and analyze' protocols

Anticancer and can be adapted for Neuroprotection and other diseases

Goal: *To explore natural resources for novel bioactive molecules*

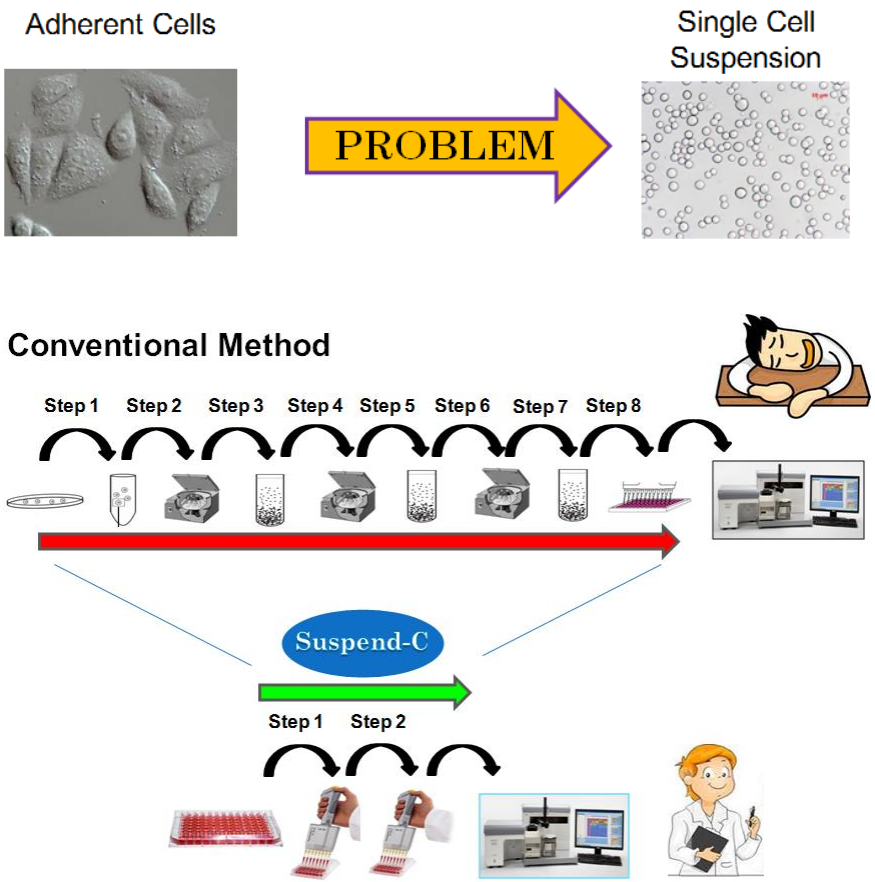
Applications of cell culturing

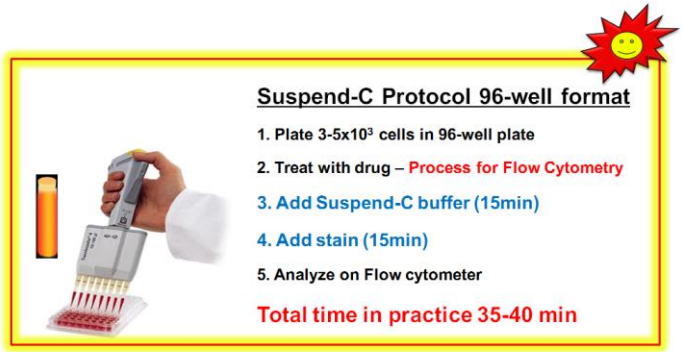
Assay Development

Assay Development

To develop new assays for use in cancer research

http://www.youtube.com/watch?v=rd-l0aN9syE&feature=youtu.ber.





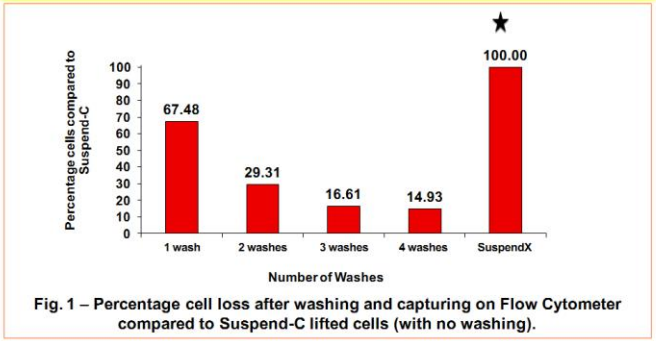


Fig. 1 – Percentage cell loss after washing and capturing on Flow Cytometer compared to Suspend-C lifted cells (with no washing).

Kaur and Esau. Two-step protocol for preparing adherent cells for high-throughput flow cytometry. Biotechniques. 2015 Sep 1;59(3):119-26.

More resources..

- Flow cytometry virtual

http://www.unsolvedmysteries.oregonstate.edu/flow_06

- Flow cytometry applied in tissue culture

http://cdn.intechopen.com/pdfs-wm/40183.pdf

- Microscopy in cell biology

https://cellbiology.med.unsw.edu.au/cellbiology/index.php/2014_Lab_8#Fluorescence_Microscopy