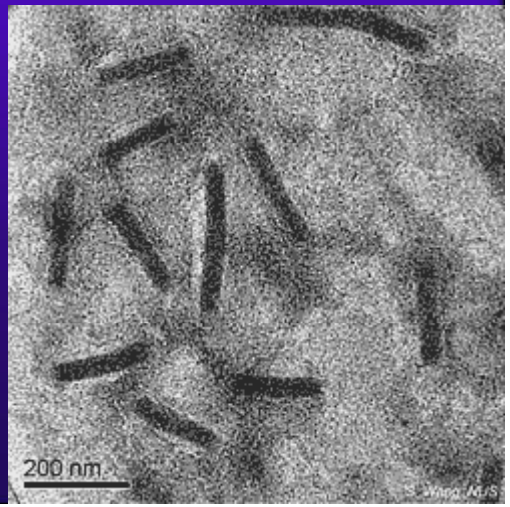
Cell Culture



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

Historical events in the development of cell culture

- 1878: Claude Bernard proposed that physiological systems of an organism can be maintained in a living system after the death of an organism.
- 1885: Roux maintained embryonic chick cells in a saline culture.
- 1897: Loeb demonstrated the survival of cells isolated from blood and connective tissue in serum and plasma.
- 1903: Jolly observed cell division of salamander leucocytes *in vitro.*
- 1907: Harrison cultivated frog nerve cells in a lymph clot held by the 'hanging drop' method and observed the growth of nerve fibers *in vitro* for several weeks.
- 1910: Burrows succeeded in long term cultivation of chicken embryo cell in plasma clots. He made detailed observation of mitosis.

Contd..

- 1911: Lewis and Lewis made the first liquid media consisted of sea water, serum, embryo extract, salts and peptones. They observed limited monolayer growth.
- 1913: Carrel introduced strict aseptic techniques so that cells could be cultured for long periods.
- 1916: Rous and Jones introduced proteolytic enzyme trypsin for the subculture of adherent cells.
- 1923: Carrel and Baker developed 'Carrel' or T-flask as the first specifically designed cell culture vessel. They employed microscopic evaluation of cells in culture.
- 1927: Carrel and Rivera produced the first viral vaccine - Vaccinia.
- 1933: Gey developed the roller tube technique

Contd..

- 1940s: The use of the antibiotics penicillin and streptomycin in culture medium decreased the problem of contamination in cell culture.
- 1948: Earle isolated mouse L fibroblasts which formed clones from single cells. Fischer developed a chemically defined medium.
- 1952: Gey established a continuous cell line from a human cervical carcinoma known as HeLa (Helen Lane) cells. Dulbecco developed plaque assay for animal viruses using confluent monolayers of cultured cells.
- 1954: Abercrombie observed contact inhibition: motility of diploid cells in monolayer culture ceases when contact is made with adjacent cells.
- 1955: Eagle studied the nutrient requirements of selected cells in culture and established the first widely used chemically defined medium.
- 1961: Hayflick and Moorhead isolated human fibroblasts (WI-38) and showed that they have a finite lifespan in culture.
- 1964: Littlefield introduced the HAT medium for cell selection.
- 1965: Ham introduced the first serum-free medium which was able to support the growth of some cells.

Contd..

- 1965: Harris and Watkins were able to fuse human and mouse cells by the use of a virus.
- 1975: Kohler and Milstein produced the first hybridoma capable of secreting a monoclonal antibody.
- 1978: Sato established the basis for the development of serum-free media from cocktails of hormones and growth factors.
- 1982: Human insulin became the first recombinant protein to be licensed as a therapeutic agent.
- 1985: Human growth hormone produced from recombinant bacteria was accepted for therapeutic use.
- 1986: Lymphoblastoid γIFN licensed.
- 1987: Tissue-type plasminogen activator (tPA) from recombinant animal cells became commercially available.
- 1989: Recombinant erythropoietin in trial.
- 1990: Recombinant products in clinical trial (HBs, factor VIII, HIVgp120, CD4, GM-CSF, EGF, mAbs, IL-2).

2007 Mario Capecchi, Oliver Smithies of USA and Martin Evans of Britain received Nobel Prize in Medicine for their work on "Gene Targeting" commonly known as gene "knockout". They genetically manipulated mouse embryonic stem cell leading to laboratory rodent that replicate human disease.

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 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, 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

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.

Tissue culture

1.Tissue culture: Is used as a generic term to include organ culture and cell culture .

2. Organ culture: Will always imply a three – dimensional culture of un disaggregated tissue retaining some or all of the histological features of the tissue in vivo.

3. Cell culture : refers to a culture derived from dispersed cells taken from original tissue , from a primary culture , or from a cell line or cell strain by enzymatic , mechanical , or chemical disaggregation .

Tissue culture

4.Histotypic culture: implies that cells have been reaggregated to recreate a three dimensional tissue-like structure ,e.g., by cultivation at high density in a filter well, perfusion and overgrowth of a monolayer in flask or dish , reaggregation in suspension over agar, or infiltration of a three- dimensional matrix such as collagen .

5. Organotypic culture: implies the same procedures but recombining cells of different lineages, e.g., epidermal keratinocytes in combined culture with dermal fibroblasts.

Tissue culture

Advantages of tissue culture:

1- Control of pH, temperature, osmolarity and dissolved gases.
2- Control of hormone and nutrient concentrations.
3- Regulation of matrix , cell – cell interactions.
4- Availability of selective media.
5- Cytology and immunostaining are easy performed.
6- Can be stored in Liquid nitrogen.
7- Origin, history and purity can be recorded.
8- quantitation is easy.
9-Reduced volumes, direct access and lower cost.
10-Ability to define dose , concentration and time.
11- Cytotoxicity and screening of pharmaceutics , cosmetics, etc

Essential equipment

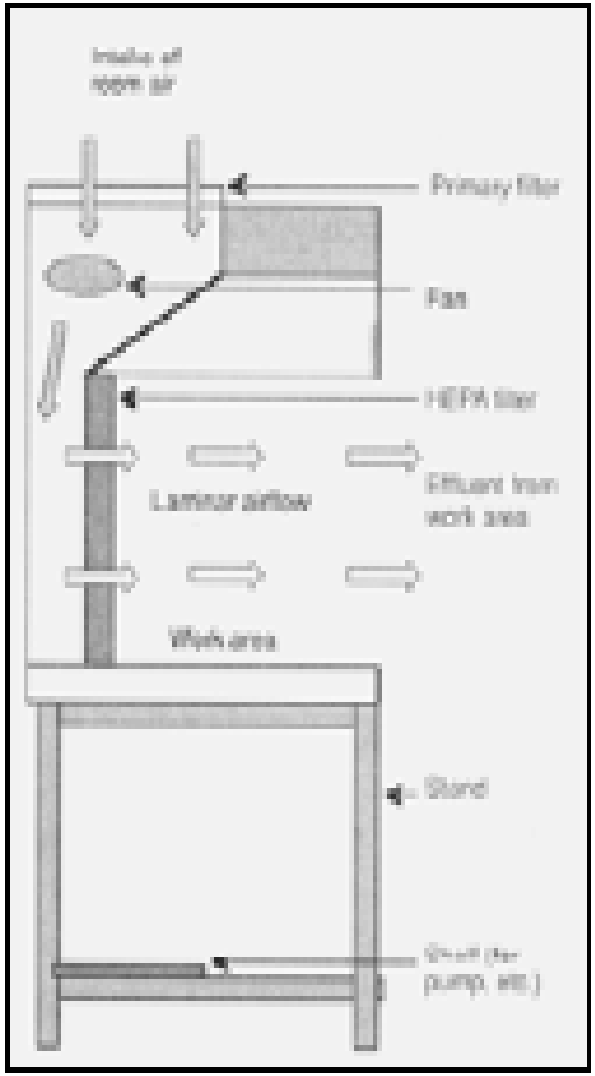
- Biological safety cabinet
- CO2 incubator
- Inverted microscope
- Pipette aid
- Aspiration pump
- Centrifuge, waterbath
- Refrigerator
- Cryopreservation equipment

Additional equipment

- Sterilization equipment
- Balances, Vortex
- Water purification – distiller, deioniser
- Magnetic stirrer, pH meter
- Micropipettes
- Cell counter
- Video camera and monitor

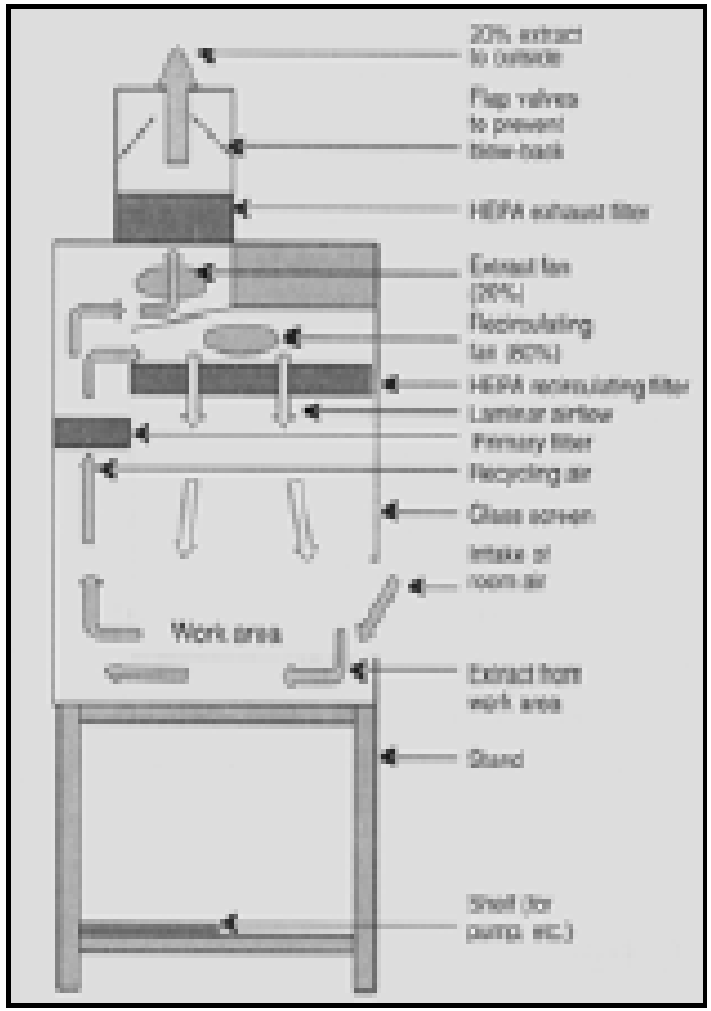
Horizontal vs. vertical hood

- Horizontal
  - Maintains sterile working area
  - Air blows directly at the investigator
  - Enough to protect your cultures but not you



Horizontal vs. vertical hood

- Vertical
  - Air is blown vertically in front of the investigator
  - Usually there is additional glass barrier in front of the investigator
  - Air is recycled and goes through HEPA filter before being returned to the room



Class I Microbiological Safety Cabinet

Class II Microbiological Safety Cabinet

Class III Microbiological Safety Cabinet

Incubator
Temp: 28 – 36 C
Humidity: 95% - 100%
CO2 level: 5 – 10%
Water jacket incubator or keep an open dish with distilled water on the bottom shelf

Incubator

Inverted microscope

- It is vital to look at the culture regularly
- A morphological change is often the first sign of deterioration in the culture
- Inverted because it is not good to open tissue culture dishes
- It should have phase contrast

- Most cells are not dense enough to be visible in regular light without staining

Fluorescence microscope

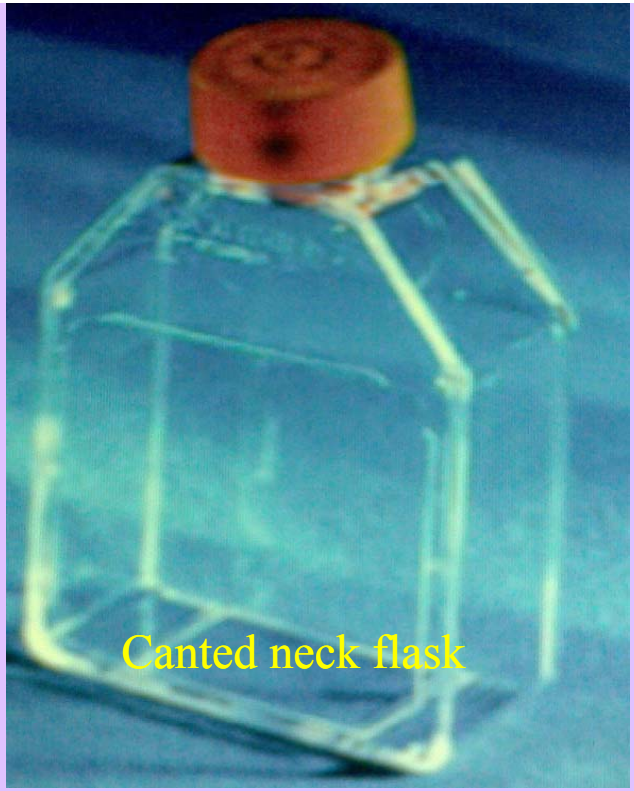
Equipment for cryopreservation

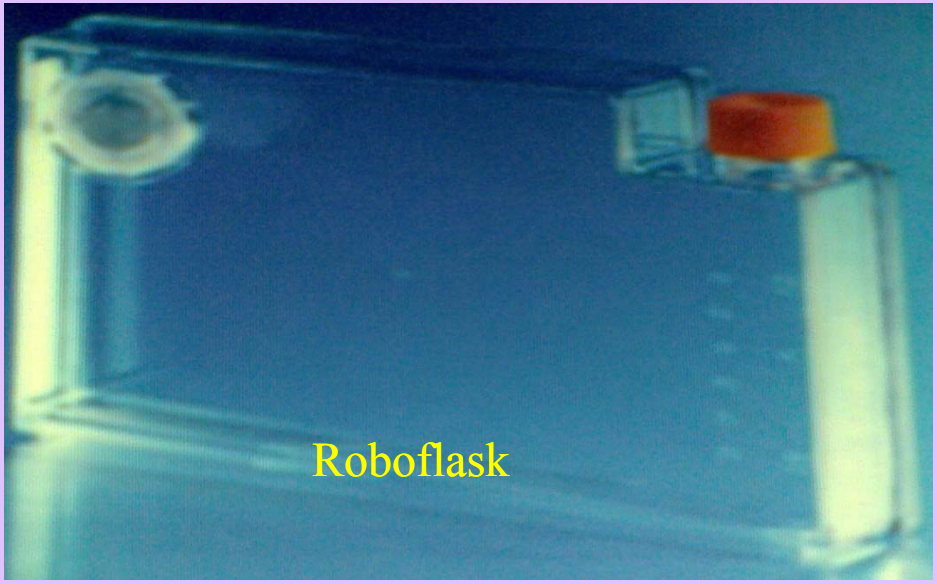
- Liquid nitrogen
  - Liquid phase (-196°C) or vapor phase (-156°C)
- Fancy freezing chambers to regulate the freezing rate
  - Styrofoam containers work too

Choice of culture container

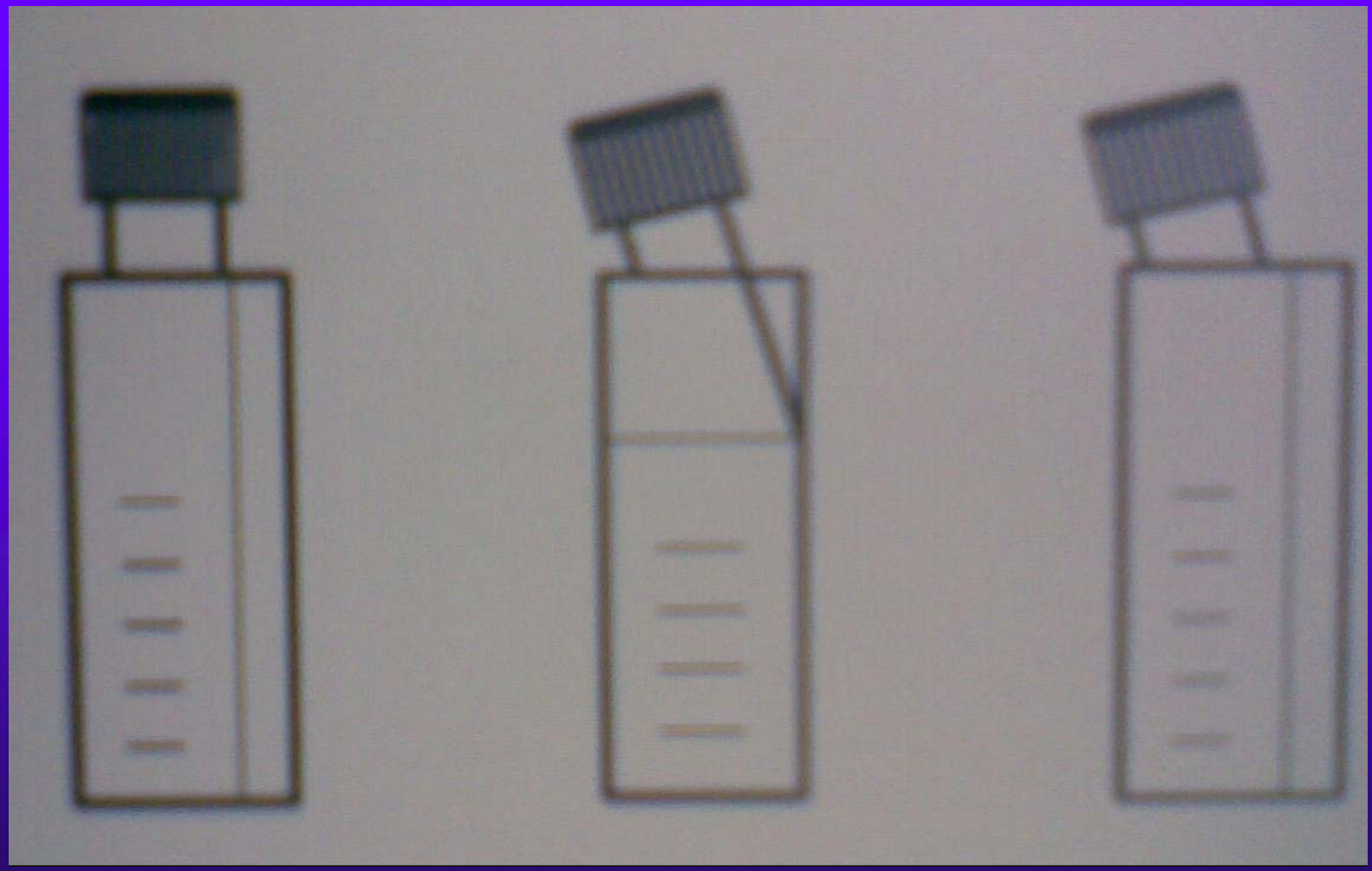
- Majority of vertebrate cells in vitro grow as monolayers on an artificial substrate
- Most cells need to spread out on a substrate in order to proliferate
- Overcrowding will inhibit proliferation
- But cells don't like to be lonely
- Cell yield is proportional to the available surface

Triangular Flask


Canted neck flask

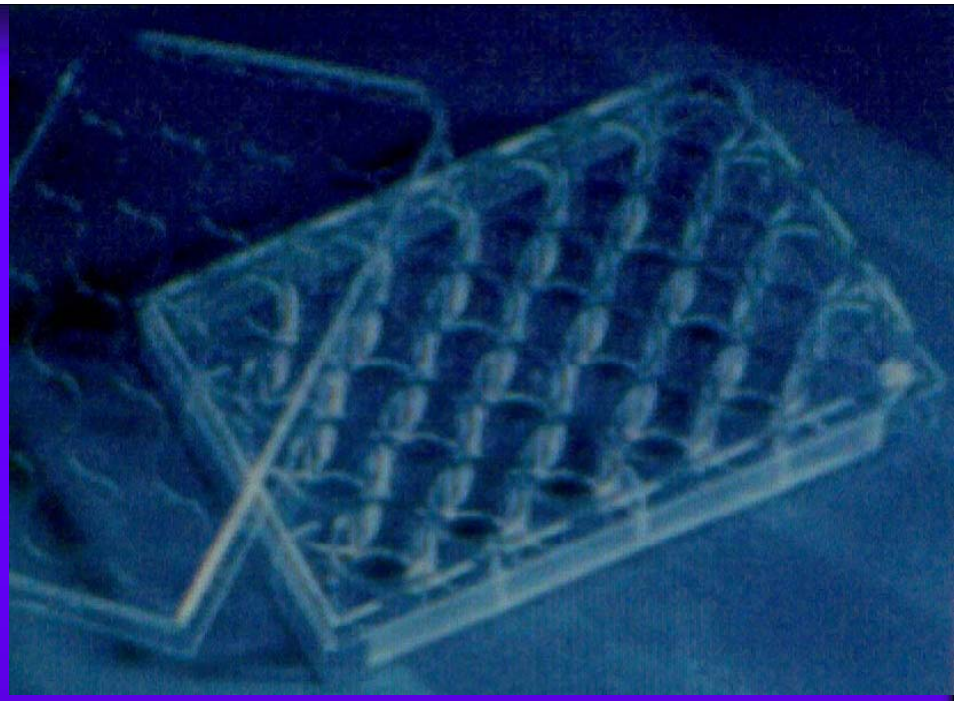

Roboflask

Flask neck styles

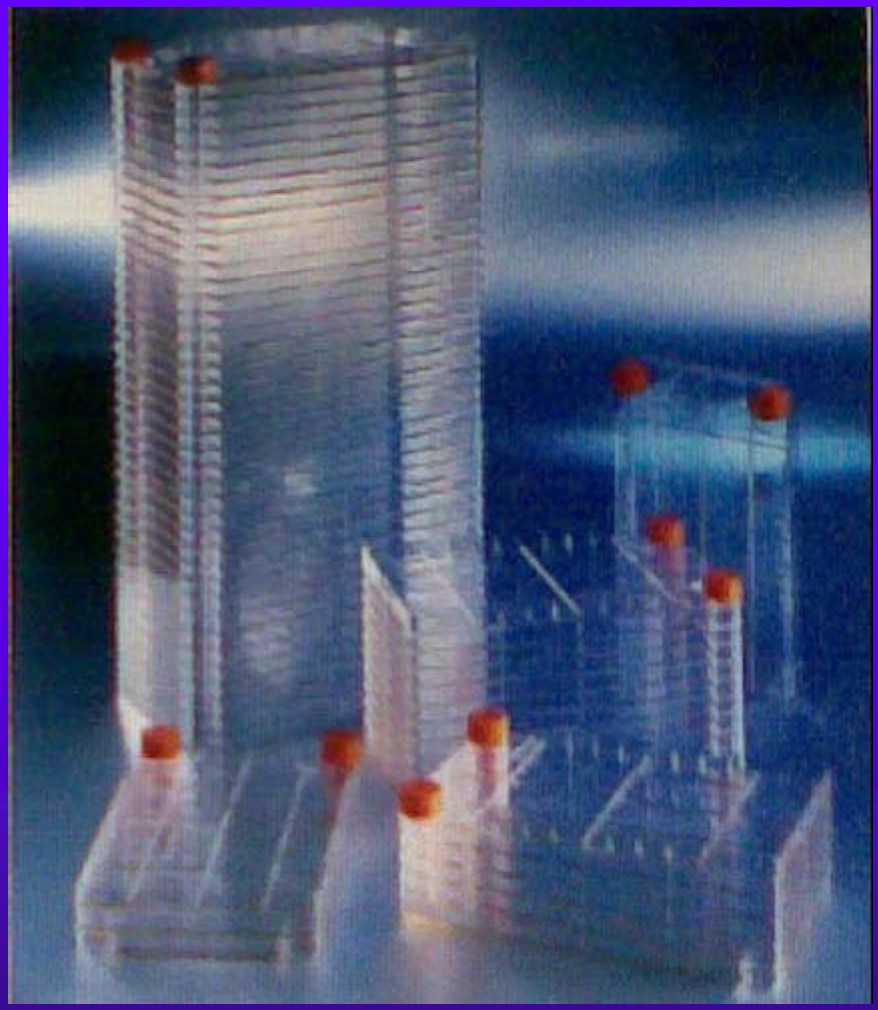


Straight neck Canted neck Angled neck

24-well plate



48-well plate



Cell STACK with 1 to 40 culture chambers

Cell scrapers handle L 28 cm, blade W 1.8 cm

Cell lifter designed for use with dishes and cell culture clusters.

blade L 1.9 cm, handle L 18 cm; sterility: sterile; y-irradiated

Cell Culture Protocols

- **The "Do's" and "Don'ts" of cell culture**

The Do's

- Use personal protective equipment (PPE)
- Wear dedicated PPE for tissue culture facility and keep separate from PPE in the general lab environment.
- Keep all work surfaces free of clutter.
- Correctly label reagents including flasks, medium.
- Only handle one cell line at a time.
- Clean the work surfaces with a suitable disinfectant (e.g. 70% ethanol).
- Wherever possible maintain separate bottles of media for each cell line.
- Examine cultures and media daily for evidence of gross bacterial or fungal contamination.
- Quality control all media and reagents prior to use.
- Ensure that incubators, cabinet, centrifuges and microsocpes are cleaned.
- Test cells for mycoplasma on a regular basis.

The Don’ts

- Do not continuously use antibiotics in culture medium.
- Don’t allow waste to accumulate particularly within the incubators and culture area.
- Don’t handle cells from unauthenticated sources in the main cell culture suite.
- Avoid keeping cell line continually in culture without returning to frozen stock.
- Avoid cell culture becoming fully confluent. Always sub-culture 70-80% confluency or as advised on European Collection of Cell Cultures ECACC’s cell culture data sheet.
- Don’t allow media to go out of date.
- Avoid water baths dirty.
- Don’t allow essential equipment to become out of calibration.

Technique and instrument

Laminar flow

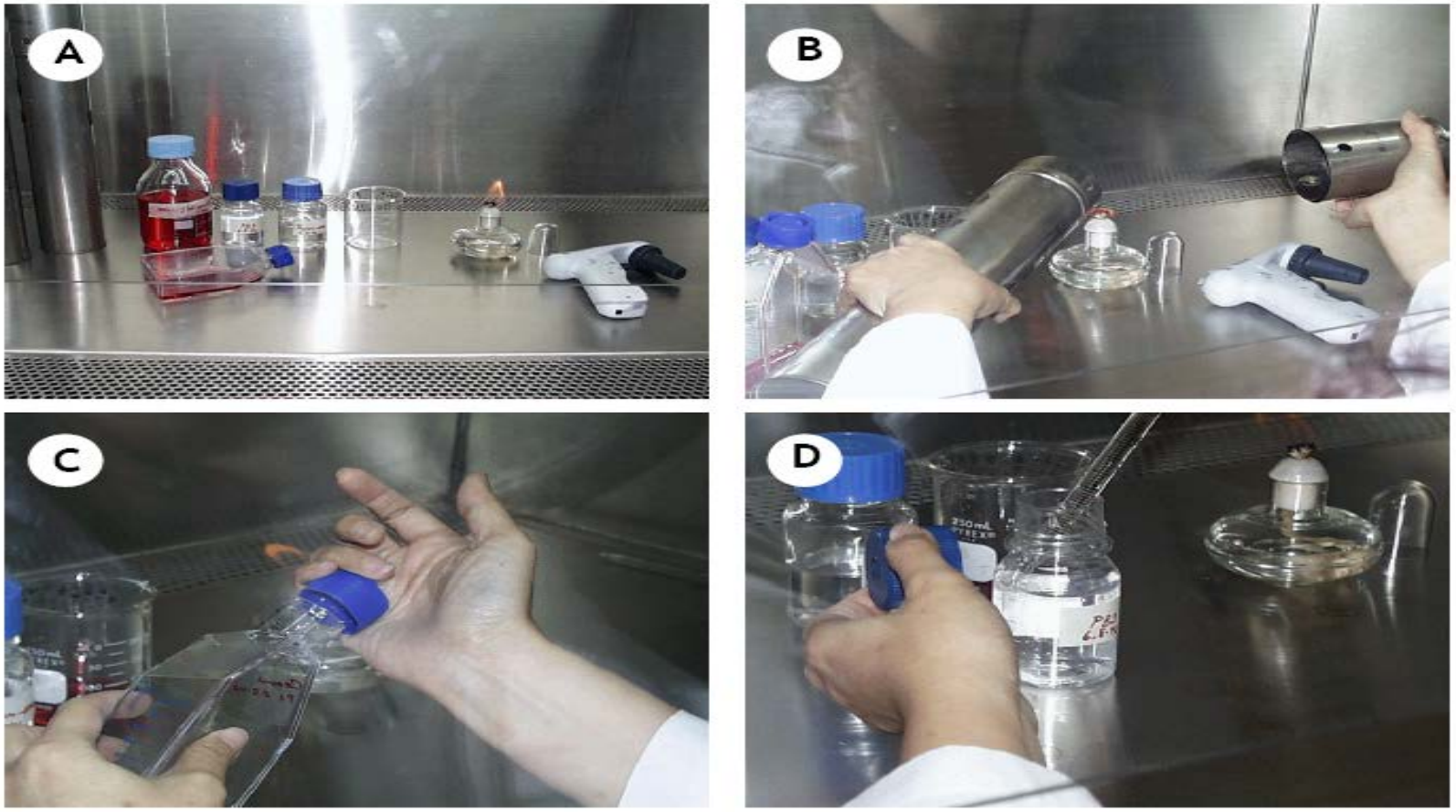


Figure 8. Basic aseptic techniques. A: Performing all maneuvers in a laminar flow hood. B: Using flames to fix microorganisms on container necks. C: Holding a bottle cap with the little finger. D: Avoid touching tops of open vessels while transferring their content.

Carbon dioxide incubator

Figure 1. A CO_2 controlled incubator.

Microscope

Figure 2. An inverted microscope equiped with bright field, Normaski, phase-contrast, and fluorescence optics. Attached CCD video camera connects microscopic visual fields to the TV monitor and computer.

Tissue culture Ware

Figure 3. The disposable plasticware used for culturing cells. A: Multi-well plate (6 wells). B: Flask (75 cm^2).

Culture Media Sterilization

Figure 4. Media sterilization through a 0.22 µm membrane filter assembled in a filter holder.

Cell counting

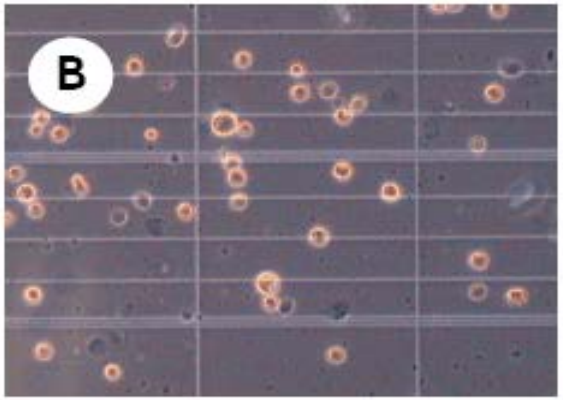


Figure 9. Quantitation of cell viability by trypan blue exclusion. A: Filling the counting chamber with a trypan blue-stained cell suspension. B: Counting viable and non-viable cells under a 100x microscopic field.

Changing Medium

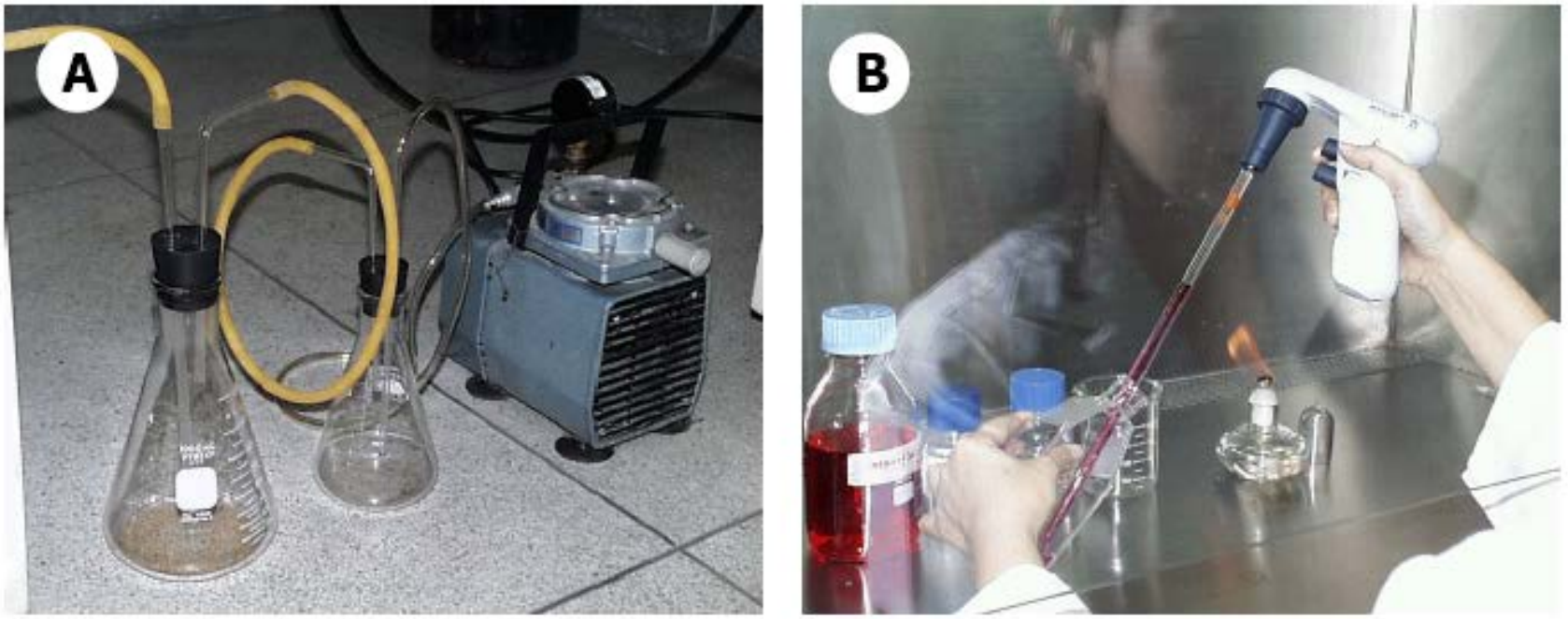
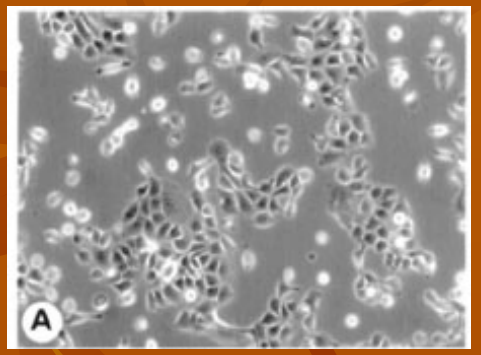
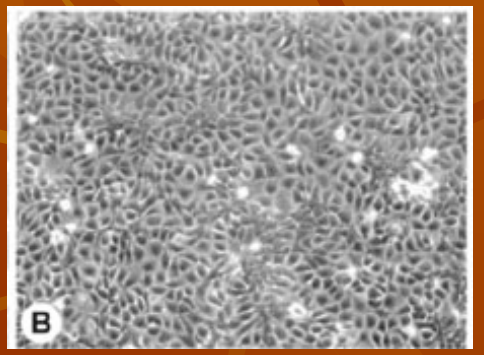


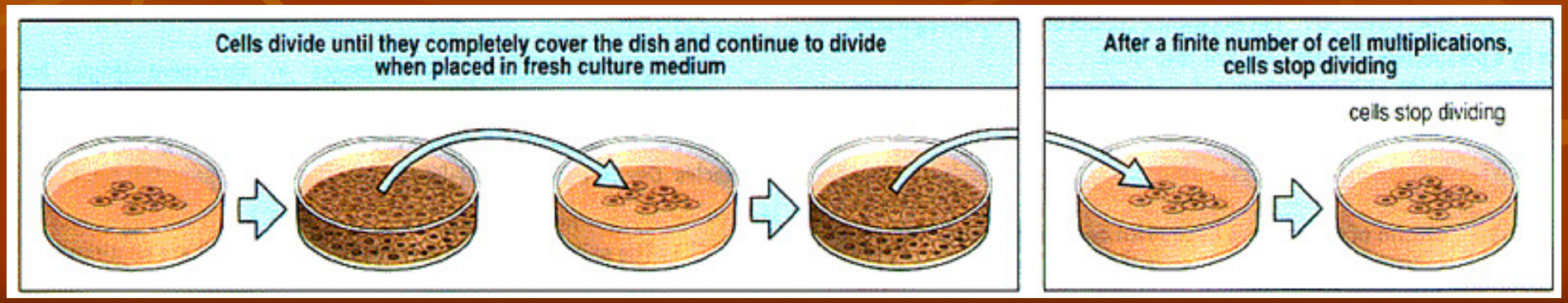
Figure 5: A: The vacuum pump with a 'trapping flask' protection. B: Glass or plastic pipettes with some form of suction aid are practical for transferring larger volumes.

Passaging cells (subculturing cells)

- Process of diluting cell number in order to keep cells actively growing
- For adherent cells, when they cover tissue culture dish, they need to passaged
  - Otherwise, the cells will become unhealthy and stop growing







Other miscellaneous Equipment

- Fridge Freezer for storing medium
- Liquid nitrogen Container for cryopreservation of cells
- Incubator for warming up of the medium
- Bench centrifuges to separate out cell pellet.

Sterile technique

- Procedures by which cultures are manipulated without infecting the worker or contaminating the cultures or the laboratory environment
- Important for the cell culture
  - You want to be sure you are growing only the cells you want to grow a single unwanted cell can ruin an experiment or a multimillion $ production run
- Important for the lab worker
  - Some cultured cells can pose health threats to workers if they are inhaled, ingested, or absorbed--sterile technique prevents exposure of the worker to cultured cells

Sterile technique: Bacteria

- Media is sterilized in an autoclave
  - Very high pressure and temperature kills
- Minimizing contamination through awareness
  - Inoculation loop, pipets, pipet tips, etc. should never touch contaminating surfaces
  - Containers holding media and other cell additives should be kept closed until needed, then opened briefly
- Tubes and flasks are “flamed” whenever they are opened
  - The purpose of flaming is not to sterilize, but to warm the tube and create warm air convection currents up and away from the opening. This "umbrella" of warm, rising air will help to prevent the entrance of dust particles carrying contaminating microbes

Sterile technique: Tissue culture

- Media is usually sterilized by filtration
  - Standard biological filters are 0.22 μm - 0.45 μm; these remove most microbes by trapping them in the filter
    - This does not remove all microbes (such as mycoplasm), and will not remove viruses
  - Unlike bacterial media, animal cell media cannot be autoclaved, because this would destroy many of the growth factors and other molecules needed for cell growth
- Working with cells in a laminar flow hood
  - HEPA High-Efficiency Particulate Air filter
- Disinfect
  - 70% Ethanol is sprayed in hood, onto bottles entering hood
- Minimizing contamination through awareness
  - Inoculation loop, pipets, pipet tips, etc. should never touch contaminating surfaces
  - Containers holding media and other cell additives should be kept closed until needed, then opened briefly

Sterilization methods

- Dry heat
  - Hot air oven at 160 °C for 75 min.
- Autoclave
  - Applies heat under high pressure; this increases the boiling point of water to 121ºC (normal boiling point of wat
  - 15-20 min. is sufficient to kill most microbes
- Filtration
  - Large volumes: suction filter
  - Small volumes: syringe filter 0.22 μm - 0.45 μm
- UV radiation
  - Causes mutations to form in the DNA of microbes, causing genetic damage and eventual death
  - Used to sterilize surfaces (such as the surface of laminar flow hoods)

- Antiseptics
  - Antibiotics
- Sterilization
  - Removing Microbes
    A. Filtration
    B. Centrifugation
  - Killing Microbes
    A. Heat …. Dry and moist
    B. Irradiation UV
    C. Chemicals (antibiotics)

What is in the media?

- Dulbecco' Modified Eagle's Media (DMEM)
  - Contains glucose, some proteins, and essential salts
  - Contains a pH indicator (**phenol red**) Media looks pink/red at pH 7.2
    - **Acidic** -yellow or orange (cell growth, bacterial growth)
    - **Basic** -purple (no cell growth, not enough CO_2)

Media preparation

- Process of combining and sterilizing ingredients of a particular medium
- Proper media prep is essential for cell culture systems!
  - If the media is lacking any components, the cells would either die or be unhealthy
  - If the media is not properly sterilized, cells will be contaminated with microorganisms
  - If glassware, pipettes, etc. used to prepare media are not properly cleaned, cell cultures can be contaminated with chemical residues
- As a result, the cells would not produce the desired product effectively

More media components

- Antibiotics (penicillin and streptomycin)
  - Prevent bacterial contamination
- Salts and buffers
  - To simulate *in vivo* environment
- Serum
  - Portion of blood after the cells and fibers have clotted
  - From cow, horse, sheep
  - added to media as a nutrient source for growing cells
- Lipids, proteins

Phosphate Buffered Saline

- Used to wash/remove excess serum that inhibits the function of TRED.
- Must be warmed in the water bath before use so cells are not shocked by cold liquid.

Trypsin EDTA

- An enzyme used to detach the cells from a culture dish.
- EDTA binds calcium ions in the media that would normally inhibit trypsin.

Bleach

- Used to destroy any remaining cells in dishes and tubes before they are tossed in the trash can.
- Add enough to change media to clear,
  - wait 5 minutes,
  - rinse solution down sink
  - throw away the dish/flask/plate in the trash can.

Potential sources of contamination in cell culture

- Equipment
  - Glassware, instruments, incubators.
- Solutions
  - Media or reagents added into media
- Room air
- Work surfaces
- Operators
  - Hands, hair, clothing, breath, etc.
- Incoming cells

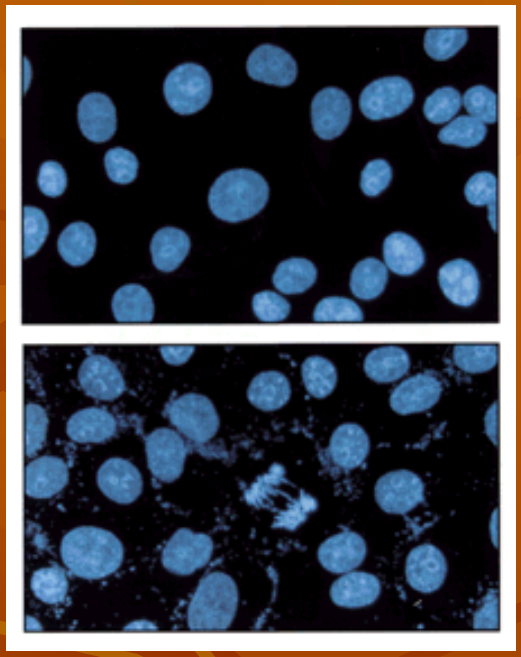
Types of contamination in animal cell culture systems

- Biological
  - Bacteria
  - Fungi
  - Cross-contamination by other cell cultures
- Chemical
  - Residues left from detergents or disinfectants on glassware, pipettes, instruments, etc.
  - Metal ions, other impurities in water
  - Endotoxin: highly bioreactive part of the cell wall of some types of bacteria (endotoxin molecules are shed from bacteria and are left behind even after bacteria die)

Characteristics of microbial contamination in cell cultures

- pH
  - Sudden change in pH is often a strong indicator of contamination
- Turbidity
  - Media looks cloudy
- Microscopic evaluation
  - Can see individual microorganisms, often because their motion can be seen easily under the microscope

Further detection of contamination in cell cultures



- Mycoplasma
  - Smallest free-living prokaryotes
  - Not killed easily by many antibiotics
  - Contamination can't be seen by evaluation
  - Mycoplasma testing should be done routinely (several tests are available)
  - Long-term effects of mycoplasma contamination include reduced growth rate, changes in cell shape and metabolism, and chromosome abnormalities

- Endotoxin
  - LAL test: an extract from the blood of horseshoe crabs is used to test for endotoxin (horseshoe crab blood contains a protein that binds endotoxin & can be detected) **Limulus amebocyte lysate (LAL)**

Minimizing contamination

- Very difficult to prevent entirely, but good lab practices can keep contamination incidents to a minimum

- Proper cleaning and sterilization of glassware, pipettes, and other lab instruments.
- Practicing sterile technique when working with cell cultures

Substrates for Cell Growth

Ghassan Mohammad Sulaiman , Ph.D.

Assist. Prof.
School of biotechnology
Department of applied science
University of Technology

- Glass
- Plastic
- Metals
- Palladium
- Modification of Substrate Surface

Glass

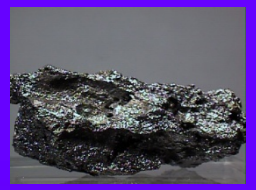
- most commonly used substrate
- easily washed and readily sterilized
- optically clear to allow microscopic observation of cultures
- Glass made of alum-borosilicate (e.g. Pyrex) is preferred to soda-lime glass as soda-lime glass releases alkali into the medium. Soda-lime glass can be detoxified by boiling in weak acid, before use
- Slides, test tubes and flasks, used as substrates must be washed with non-toxic detergent

Plastic

- nonautoclavable.
- Sterile substrates available are meant for single use.
- most commonly used plastic
  - polystyrene.; Polyethylene,;
  - polycarbonate,; perspex;
  - polyvinyl chloride; teflon;
  - Cellophane; cellulose acetate, etc.

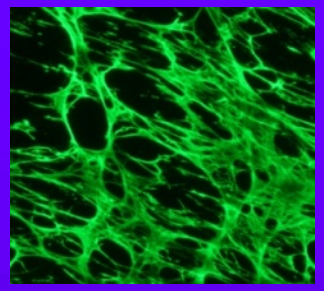
- Thin teflon films are available as petri dishes or as membranes to be used as rafts. These films are permeable to O2 and CO2 and can be sectioned for microscopy.

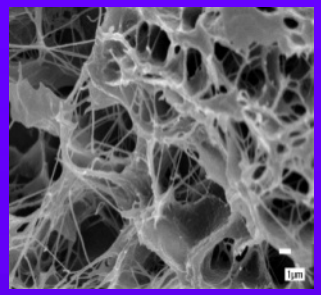
Metals

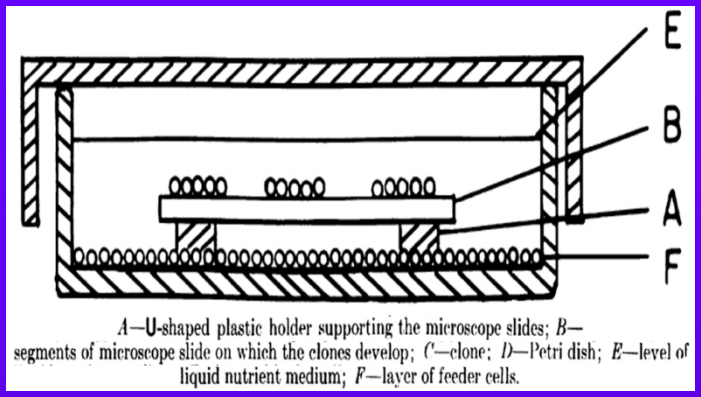


- Stainless steel and titanium, being relatively chemically inert and negatively charged, are suitable for use as substrates.
- If the stainless steel is not of proper grade, it will release toxic ions.
- Stainless steel should be acid washed, to remove surface impurities.
- Palladium deposited on agarose is used as a substrate for growth of fibroblasts and glia.

Modification of Substrate Surface







A—U-shaped plastic holder supporting the microscope slides; *B*—segments of microscope slide on which the clones develop; *C*—clone; *D*—Petri dish; *E*—level of liquid nutrient medium; *F*—layer of feeder cells.

The efficiency of the cultured cells through increased cell attachment can be enhanced by treating the surface of the substrate with purified fibronectin or collagen.

- The chemical is poured onto the surface of the dish, excess chemical drained, and then dried and sterilized using UV light.

- The surface of the substrate is also treated with a monolayer of special types of cells. This layer is called **feeder layer**, since it feeds the growing cells.
- The cells used as feeder layer are glial cells, normal foetal intestine, mouse embryo, fibroblasts, etc. The feeder layer is used for the growth of neurons, epithelium of breast and coelom, and production of transgenic animals.

CELL CULTURE MEDIA AND GROWTH FACTORS

Ghassan Mohammad Sulaiman , Ph.D.

Assist. Prof.
School of biotechnology
Department of applied science
University of Technology

Introduction

- ***Animal cell culture*** basically involves the in vitro maintenance and propagation of animal cells in a suitable nutrient media.
- Culturing is a process of growing cells artificially
- selection of an appropriate growth medium for the in vitro cultivation of cells is the important and essential step.
- the choice of the medium mostly depends on the type of the cells to be cultured, and the purpose of the culture (growth, differentiation, production of desired products).

Culture Media …

- Classified based on
  - Source – Natural/ Artificial
  - Composition – Defined/ Undefined
  - Consistency – Solid, Semi Solid, Liquid
  - Serum content – Serum and Serum-free media
  - Special Need

- Animal cells need either a completely natural medium, or an artificial medium supplemented with some natural products.

Natural media

Artificial media

Natural media

- In the early years, natural media obtained from various biological sources were used.
- Body fluid: plasma, serum, lymph, amniotic fluid, etc. These fluids are tested for sterility and toxicity before their utility.
- Tissue extract: among the tissue extract chick embryo extract was most commonly employed. The extract of liver, spleen, bone marrow and leucocytes also used as culture media.
- Plasma clots have been in use for a long time and are now available commercially.
- Bovine embryo extract prepared from bovine embryos of different age (up to 10 days).

Artificial media

- The artificial media containing partly defined components. The minimal criteria needed for choosing a media for animal cell culture are listed below:

- The medium should provide all the nutrients to the cell.
- Maintain the physiological pH around 7 with adequate buffering.
- The medium must be sterile, and isotonic to the cells.

- The basis for the cell culture media was the balanced salt solution -used to create a physiological pH and osmolarity required to maintain the cell in vitro.

- For promoting growth and proliferation of cells, various constituents were added and several media developed. Eg: serum containing media and serum-free media.

Artificial medium

Physio-Chemical Properties of Culture Media:

- The culture media is expected to possess certain physiochemical properties (pH, Oxygen, carbon dioxide, buffering, osmolarity, viscosity, temp., etc«) to support good growth and proliferation of cultured cells.

- **pH:** Normally most cells can grow at pH in the range 7-7.4, although there are slight variations depending on the type of cells. The indicator phenol red commonly used for visible detection of pH in the media.

Carbon dioxide, Bicarbonate and Buffering:

- Carbon dioxide in the medium is in a dissolved state and the concentration depends on the atmospheric carbon dioxide tension and temperature.

- Carbon dioxide in the medium exists as carbonic acid and HCO3 and H+ ions.

$CO_2 + H_2O \rightarrow H_2CO_3 \leftrightarrow H^+ + HCO_3^-$

- Concentration of carbon dioxide, bicarbonate and pH are inter related.
- By increasing the atmospheric carbon dioxide, the pH will be reduced making the medium acidic.

Carbon dioxide, Bicarbonate and Buffering:

- Addition of sodium bicarbonate (as the component of bicarbonate buffer) neutralize bicarbonate ions.

$NaHCO_3 + H_2O \rightarrow Na + HCO_3^- + H_2O \rightarrow$
$Na + H_2CO_3 + OH^- \leftrightarrow Na + OH^- + H_2O + CO_2$

- The presence of pyruvate in the medium results in the increased endogenous production of carbon dioxide by the cells.

- This is advantageous since the dependence of oxogenous supply of carbon dioxide and bicarbonate will be less. In such case, the buffering can achieved by high concentration. HEPES : N-2 hydroxyethylpiprazine-N-2ethane sulphonic acid

Oxygen:

- The scale up of animal cell culture is very dependent up on the ability to supply sufficient oxygen without causing cell damage.
- Oxygen is only sparingly soluble in culture media.
- Cultured cells mostly rely on the dissolved O2 in the medium which may be toxic at high concentration due to the generation of free radicals.
- adequate quantities of O2 must be supplied so that the cellular requirements are met avoiding toxic affects.

Temperature:

- Animal cells are sensitive to temperature higher than37c [Culture from birds-38.5c,Cool blooded animals -15-25c]
- Aqueous solution has low thermal conductivity and with a low co reaction rate in animal cell culture due to gentle mixing, high temperature gradient may occur with heating.
- Low gradient heating systems mostly based on the water jacked principle and with a high surface area are favorable.
- In a compact loop bioreactor this can be achieved by using a thermo stated draught tube.
- Besides directly influencing growth of cells, temperature also affects the solubility of O2 [Higher temperature enhances solubility]

Osmolality:

- Osmolality for most of the cultured cells is in the range of 260-320mosm/kg, comparable to the osmolality of human plasma(290mosm/kg).

- Once an osmolality is selected for a medium it must be maintained at that level [+/-10mosm/kg].

- When ever there is an addition of acids, bases ,drugs etc .to the medium osmolality alters and is measured using Osmometer

Point Osmometer:

SERUM MEDIA

Although natural media are very useful and convenient for a wide range of cases, they suffer from the disadvantages of poor reproductability due to lack of knowledge of exact composition.

Reasons 4 using synthetic media:

*Media for Immediate survival.

*Media for Prolonged survival.

*Media for Indefinite growth.

*Media for Specialized functions.

- For the immediate survival, a ³balanced diet´ solution with defined osmotic pressure and pH is added. [Combination of inorganic salts and glucose].

- For long survival, serum may be used or the balanced salt solution may be supplemented with amino acids, O2,vitamins, and serum proteins.

- MEM-Minimum Essential Medium [Eagles 1955]-used for mammalian cell culture.

- Role of serum in culture is extremely complex and contains mixture of many large and small bio molecules [growth promoting and growth inhibitory factors].

Type of Serum

- Commonly used : bovine calf, fetal bovine, adult horse and human serum.
- Calf (CS) and fetal bovine (FBS) – used for cell lines and cloning.
- Human serum – used for human cell lines.
- Horse serum is consistent from batch to batch- Less polyamines.

Fetal Bovine Serum

- By-product of meat industry
- Produced from blood collected bovine fetus at the abattoir (Slaughter houses).
- Collected after the pregnant uterus has been removed from the slaughtered cow.

Fetal Bovine Serum

- Serum is collected as early as 5 minutes after slaughter or as long as 25–40 minutes after, at which point the fetuses may still be alive.
- Invasive procedures, involving insertion of a 12–16 gauge needle between the 4th and 5th rib of the fetal heart are often employed.
- Most widely used serum for cell culture

Fetal Bovine Serum

- Low in antibodies
- Contains more growth factors
- The globular protein, bovine serum albumin (BSA) is a major component of fetal bovine serum.
- The rich variety of proteins in fetal bovine serum maintains cultured cells in a medium in which they can survive, grow, and divide.

Foetal Calf/Bovine Serum (FCS & FBS)

Heat Inactivation (56ºC for 30 mins) – why?

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

Care! Overdoing it can damage growth factors, hormones & vitamins and affect cell growth

Function of serum:

- Basic nutrient in solution and bound to proteins.
- Hormones and growth factors stimulating cell growth and specialized functions.
- Attachment and spreading factor.
- Binding proteins (albumin) carrying hormones vitamins minerals lipids etc.
- Non specific protection factors against mechanical damage and viscosity.
- pH buffer

SERUM FREE MEDIA

Due to some disadvantages associated with the use of serum in media ,Serum Free media have been developed.

Disadvantages of Serum in Media:

- *Variable Composition*: there is no uniformity in the composition of serum (highly variable).

- *Quality Control* : to maintain uniform quality of the serum special testes need to be performed with each batch of serum before its use.

- Contamination: very difficult to get serum from virus contamination.

- *Presence of Growth Inhibitors* -

in some cases growth inhibitors like TGF- β may dominate and inhibits cell proliferation.

- *Availability and cost* -

availability is restricted due to its dependence on cattle.

- *Down stream processing* –

presence of serum in the culture medium interferes with the isolation and purification of cell culture products.

For this reason additional steps are needed for the isolation

SERUM FREE MEDIA

Advantage and Disadvantage of Serum Free Media

ADVANTAGE :

* *Selection of media with defined composition-*

Main advantage of serum free media is to control growth of the cells as desired with a well defined medium.

**Regulation of differentiation –*

It is possible to use a set of factors to achieve differentiation of cells with the desired and specialized functions

DISADVANTAGE:

*Slow cell proliferation

:

* Need for Multiple media

:

*Purity of reagents

:

*Availability and cost

:

[Costly than serum media as they use pure chemicals which are expensive]

Protein

- **Albumin** – important carrier of lipids, minerals and globulins
- **Fibronectin** – promote cell attachment
- **Fetutin** – enhance cell attachment
- **Transferrin** – binds iron
- Increases viscosity of medium, reducing shear stress during pipetting and stirring and adds to medium's buffering capacity

Growth Factors

- Platelet-derived growth factor (PDGF)
- Fibroblast growth factor (FGFs)
- Epidermal growth factor (EGF)
- Insulin-like growth factors IGF-I and IGF-II
- **Help in Mitogenic activity and stability**

Hormones

- **Hydrocortisone** – present in fetal bovine serum – can promote cell attachment and proliferation or cell differentiation

- **Insulin** – uptake of glucose and amino acids – binds to IGF receptors

Testing serum

- Plating efficiency: check growth of cells in Cloning, count them
- Stain and count colonies
- **Plating efficiency (survival) and colony size (cell proliferation)**
- PE(%)= (# Cells Day 1 /# Cells Plated Day 0) x 100
- PE(%)=(Colonies Counted / Cells Inoculated) x 100

Testing serum

- **Growth curve**: lag period, doubling time and saturation density
- **Lag period –** culture has to adapt to serum
- **Saturation density** – more cells will grow in a given amount of serum
- **Preservation of cell culture characteristics**
- **Sterility**

Cell culture - types

Ghassan Mohammad Sulaiman , Ph.D.

Assist. Prof.
School of biotechnology
Department of applied science
University of Technology

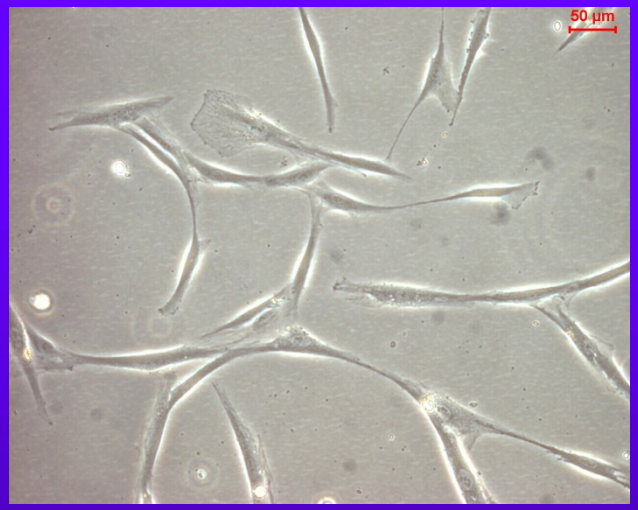
Types of cells

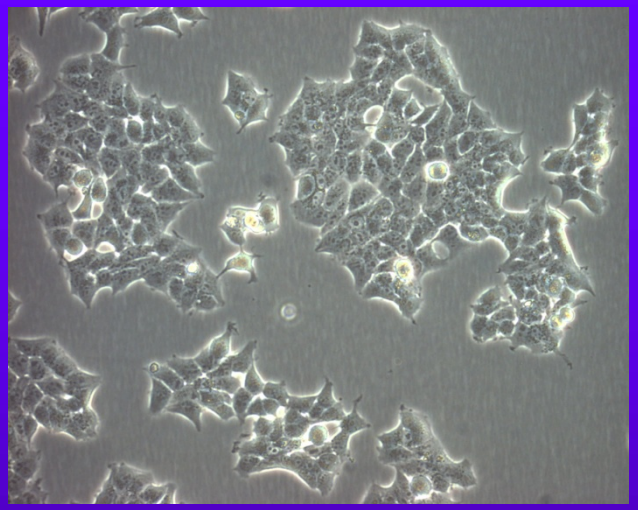
Three types based on morphology or functional characteristics.

- 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; appears elongated and bipolar

Cell morphologies vary depending on cell type

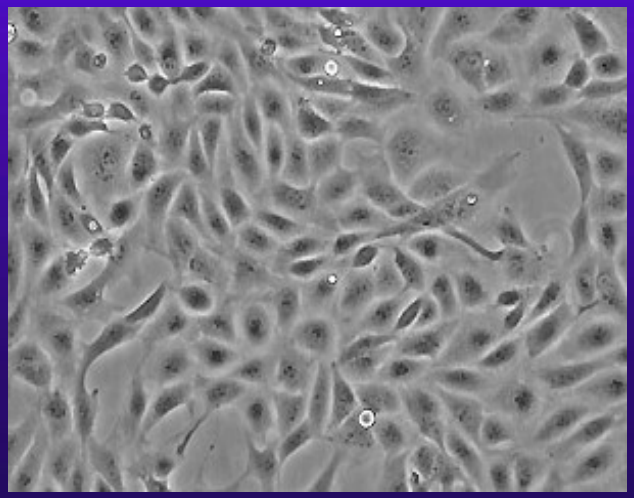
Fibroblastic

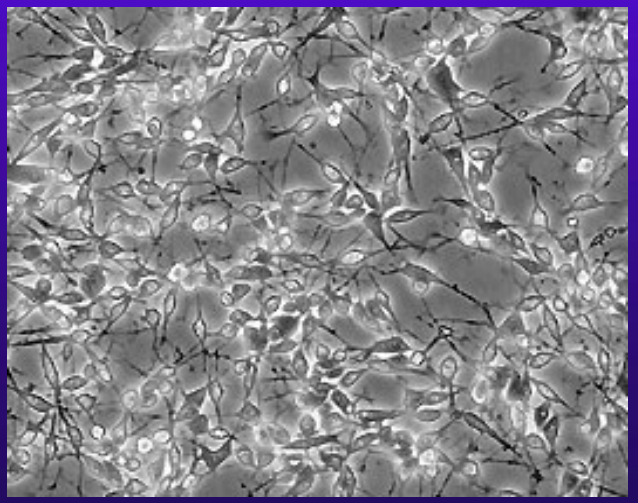




Epithelial

Endothelial





Neuronal

Terms associated with cell culture

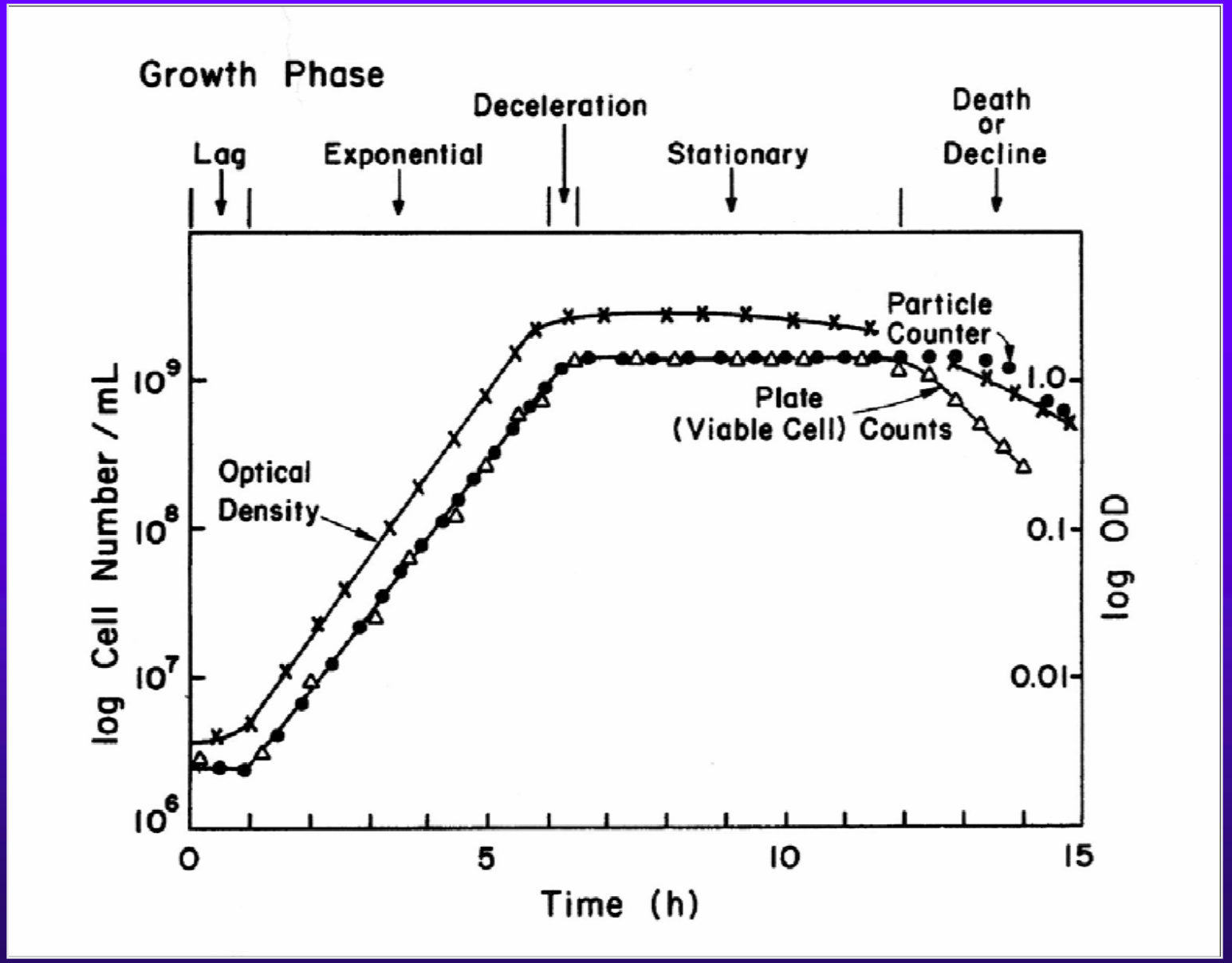
- **Split ratio-** divisor of the dilution ratio of a cell culture at subculture
- **Passage number-** number of times that the culture has been cultured
- **Generation number-** number of doublings that a cell population has undergone

Growth Kinetics

- Growth patterns and kinetics in batch culture
- growth phases

In batch culture:

- lag phase
- logrithmic or exponential growth phase
- deceleration phase
- stationary phase
- death phase


Growth Phase
Lag
Exponential
Deceleration
Stationary
Death or Decline
Particle Counter
Plate (Viable Cell) Counts
Optical Density
log Cell Number / mL
log OD
Time (h)
10^9
10^8
10^7
10^6
1.0
0.1
0.01
0
5
10
15

Cell Lines

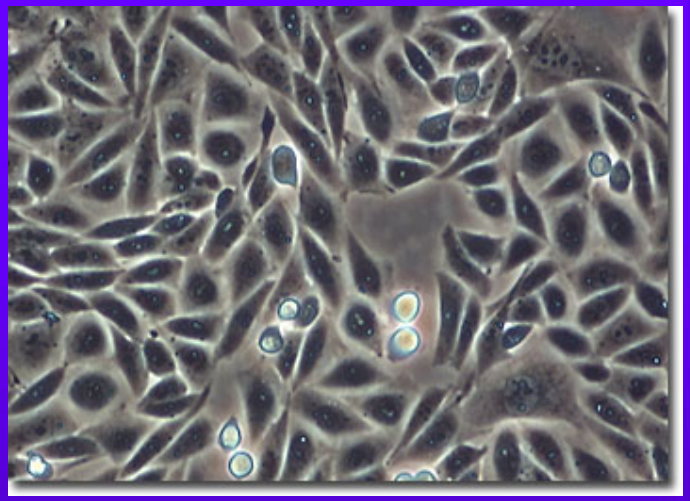
- **Cell Line**
  - Cells that have undergone a mutation and becomes immortal (won't undergo apoptosis after a limited number of passages) i.e. Attain indefinite growth.
- **Transformed cell line**
  - A cell line that has been transformed by a tumor inducing virus or chemical that has the ability to cause tumors if injected into animal.
- **Hybrid cell line (hybridoma)**
  - Fused cells with two different characteristics

Classification of Cell Cultures

- Primary cell cultures
- Diploid (Euploid) cell cultures (cell strains) (Semi-continuous cell cultures)
- Continuous cell cultures (cell lines) (Aneuploid / heteroploid cell lines)

Adherent vs Suspension cells for tissue culture

- **Adherent cells**: cells grow in a single layer (called a monolayer) attached to the tissue culture dish
  - Cell growth is limited by available surface area on which cells can grow
  - To passage adherent cells, the cells must be released from the dish (done either enzymatically, chemically, or mechanically)



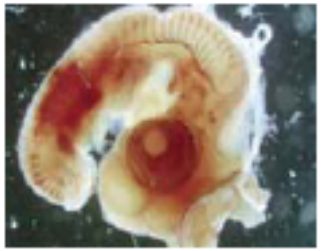
- **Suspension cells** : cells are suspended in liquid as single cells or as free-floating clumps of a few cells
  - To passage suspension cell cultures, a proportion of the cells in culture are diluted into a larger volume of medium

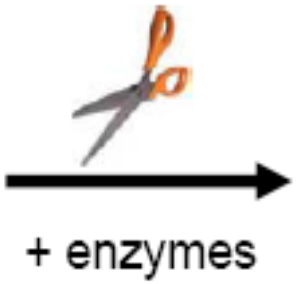
- Primary
  - Heterogeneous – many cell types
  - Closest to animal
  - Technical hassle
- Diploid cell strain
  - Relatively homogeneous – fewer cell types
  - Further from animal
  - Technically less hassle
- Continuous cell line
  - Immortal
  - Most homogeneous
  - Genetically weird – furthest from animal
  - Hassle free
  - Suspension or monolayer

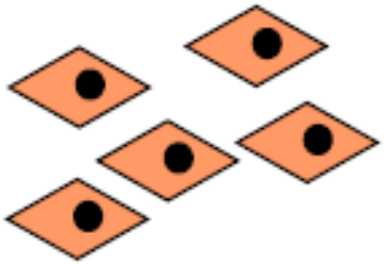
Primary culture

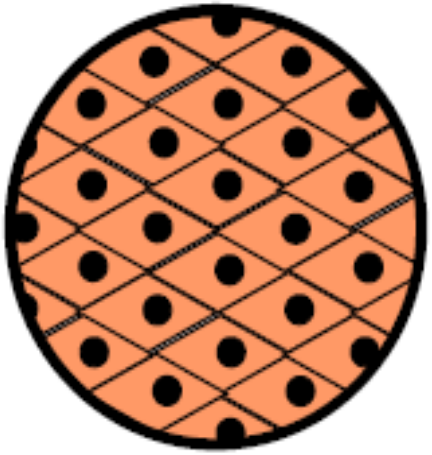
- Cells when surgically or enzymatically removed from an organism and placed in suitable culture environment will attach and grow.
- Primary cells have a finite life span.
- Primary culture contains a very heterogeneous population of cells.
- Sub culturing of primary cells leads to the generation of cell lines.
- Cell lines have limited life span, they passage several times before they become senescent.
- 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.

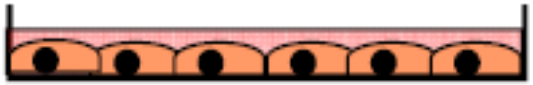
Primary cell culture



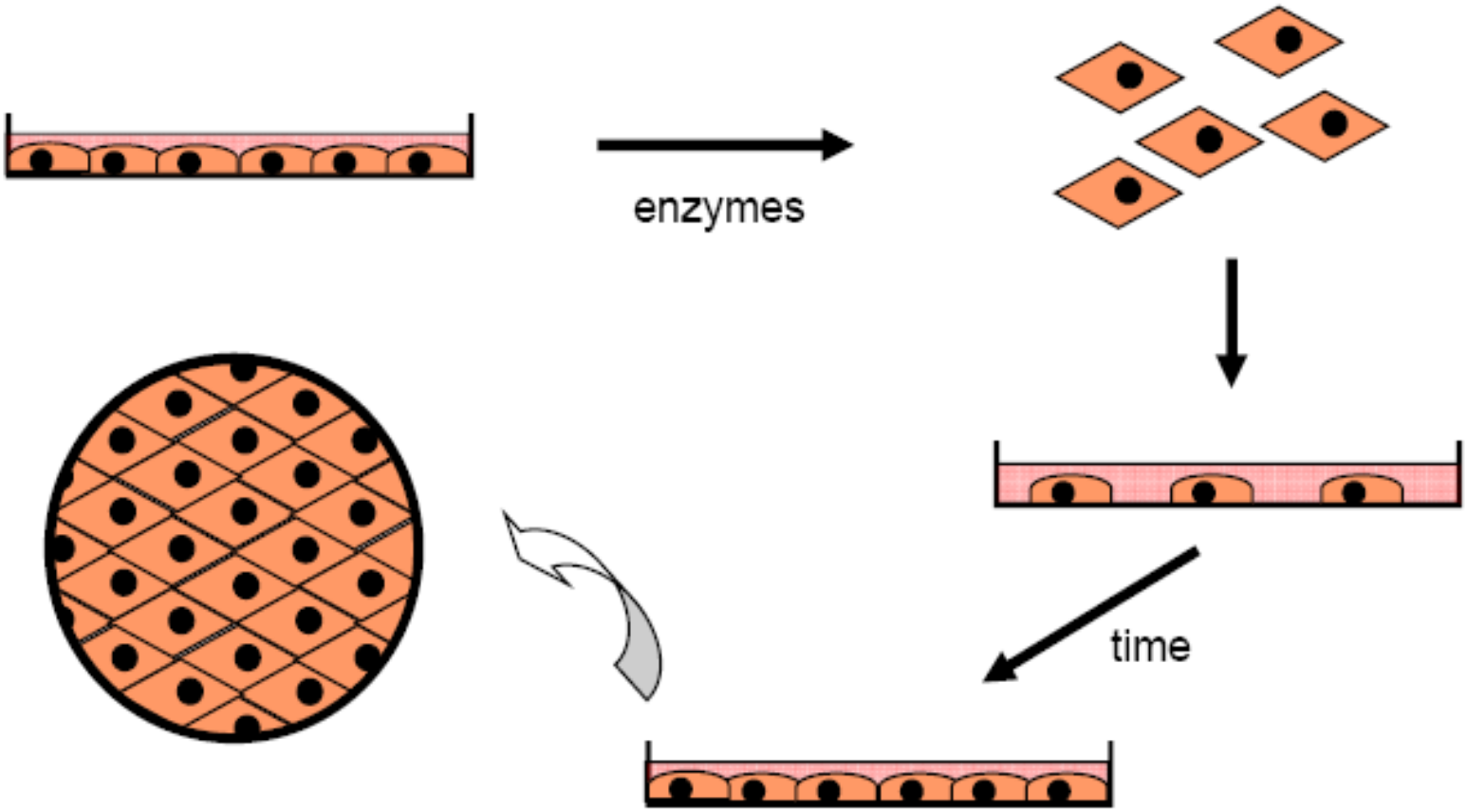








Subculture



Continuous cell lines

- By transformation (spontaneous or engineered) of cell strains or from tumours.
- Loss of contact inhibition
- Indefinite subculture
- Chromosome No. not exact multiple of haploid No.
- High plating efficiency
- Faster growth rate; subculture twice weekly
- Frequent pH adjustment necessary

Continuous cell lines

- Characteristics of continuous 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 up to higher cell density

-different in phenotypes from donor tissue

-stop expressing tissue specific genes

Disaggregation of cells

Ghassan Mohammad Sulaiman , Ph.D.

Assist. Prof.
School of biotechnology
Department of applied science
University of Technology

What is Primary Cell Culture?

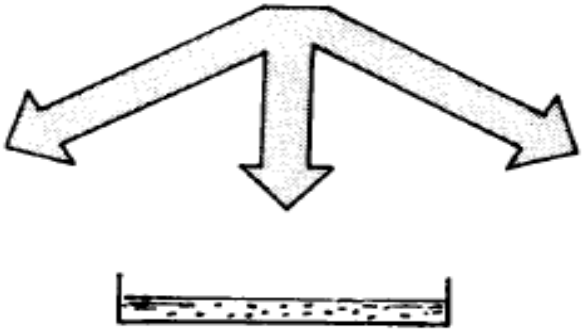
- That stage of culture obtained after isolation of cells but before first subculture

How can you obtain Primary Cell Culture?

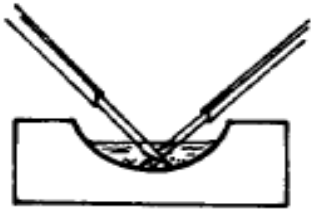
- **Stages**: 1. Acquisition of sample
- 2. Isolation of tissue
- 3. Dissection and/or disaggregation
- 4. Culture after seeding into culture vessel

Primary cell culture

ENZYME DIGESTION

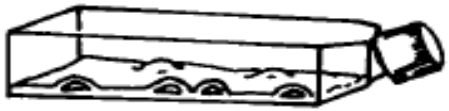


FINELY CHOPPED

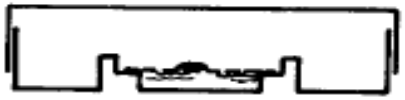


FURTHER DISSECTION IF NECESSARY

CELL CULTURE



PRIMARY EXPLANTS



ORGAN CULTURE

Disaggregation of cells

- Cells can be allowed to migrate out from an explant
- Mechanical dissociation (mincing)
- Enzymatic dissociation
- Exception – hematopoietic cells do not need to be
- disaggregated, they already are

Enzymatic Disaggregation

- The two important enzymes used in tissue disaggregation are collagenase and trypsin.
- **Collagenase**It is used for disaggregation of embryonic, normal and malignant tissues, by digesting the collagen of intracellular matrix
- **Trypsin**Use of trypsin for disaggregation of tissue is called trypsinization. It may be of two types:
  1. Warm trypsinization and
  2. Cold trypsinization.

What are the different enzymes used for tissue disaggregation?

- **Trypsin**, **Collagenase**, Elastase, Pronase, Dispase, DNAase and Hyaluronidase or in various combinations (**Mammalian enzymes**)

- Trypzean, TrypLE, Accutase and Accumax (**Nonmammalian enzymes**)

Effectiveness of different enzymes

- Trypsin and Pronase give complete disaggregation
- Trypsin and Pronase may damage the cells
- Collagenase and dispase give incomplete disaggregation
- Collagenase and dispase are less harmful

Hyaluronidase + Collagenase digests intracellular matrix

DNase – disperses DNA released from lysed cells

What are the set of conditions required commonly by most of the cultures?

- **Fat and necrotic tissue** are removed during dissection
- **Chopping** should be **fine** with sharp instruments
- **Enzymes** used for **disaggregation** should be removed
- **Concentration of cells – primary > subculture**
- Choice of **Rich medium**
- Choice of **Embryonic tissue**

Rules to follow before working with human or animal tissues

- Medical ethical rules or current legislation on experimentation with animals
- In UK, **the use of embryos or fetuses beyond 50% gestation or incubation** – regulated under Animal Experiments act of 1986
- Work with **human biopsies or fetal material** – require consent from local ethical committee or relatives

What are the different techniques to get primary culture?

- Fine dissection to obtain explant
- Mechanical disaggregation involves dissection with or without maceration
- Enzymatic disaggregation

Enzymatic disaggregation

- Enzymes used for disaggregation – trypsin + EDTA or only trypsin
- **Warm trypsin -** @ 37°C + removed by centrifugation + neutralized with serum and medium

- **Cold trypsin** – Soak tissue in trypsin at 4°C for 6 – 18 hrs followed by incubation at 37°C for 20-30 minutes for disaggregation

Differences between warm and cold trypsin treatment

- **Warm trypsin**
- Shorter time period
- Lesser yield
- Centrifugation is required

- **Cold trypsin**
- Gives higher yield of viable cells with improved survival after 24 h culture
- No stirring or centrifugation is required
- Incubation at 4 C can be done overnight
- Embryonic organs

Treatment with Collagenase enzyme

- Used for embryonic, adult, normal and malignant
- Human tumor, mouse kidney, human adult and fetal brain and liver
- **Used for too fibrous or too sensitive**
- Requires no mechanical agitation or special equipment
- **More than 1 gram tissue is used - collagenase can be expensive**

Mechanical disaggregation

- Collecting cells that spill out when tissue is sliced – **Scraping/Spillage**
- Pressing the dissected tissue through series of sieves - **Sieving**
- Forcing the tissue fragments through a **Syringe**
- **Pipetting** repeatedly
- **Soft tissues** – Spleen, embryonic liver, embryonic and adult brain and some human and animal soft tumors

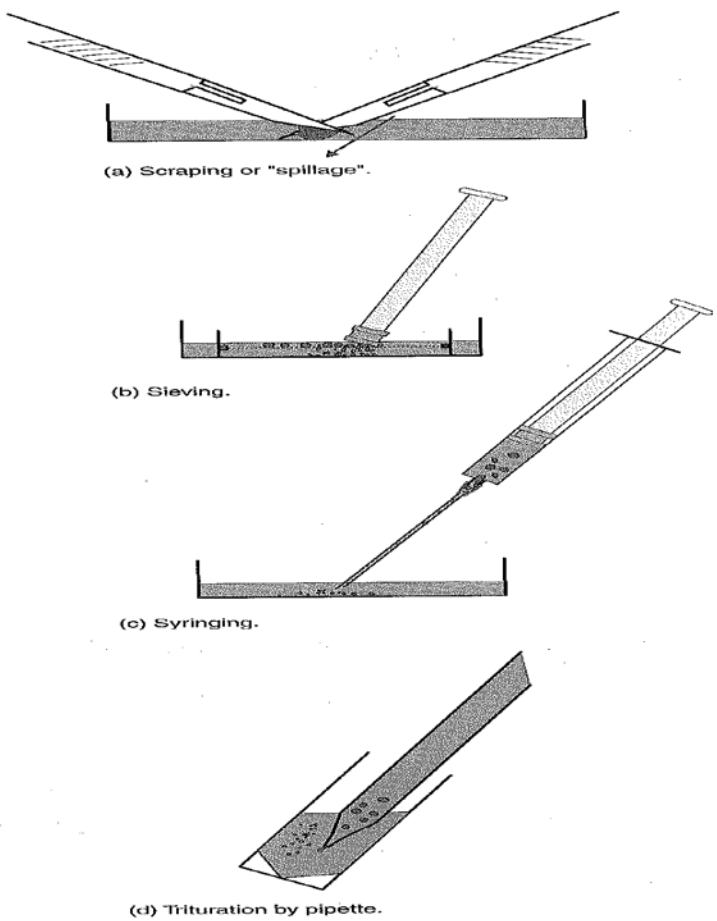
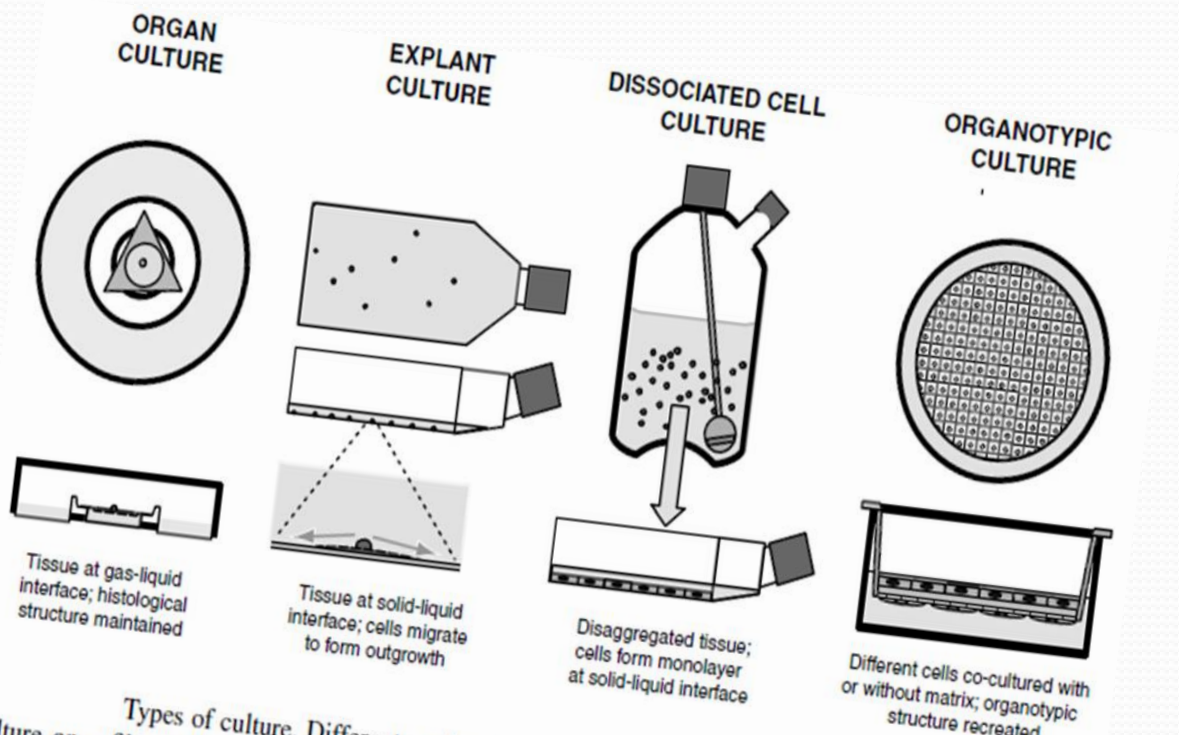


Fig. 12.13. Mechanical Disaggregation. (a) Scraping or "spillage". Cutting action, or abrasion of cut surface, releases cells. (b) Sieving. Forcing tissue through sieve with syringe piston. (Falcon Cell Strainer can be used; *see* Fig. 12.8.) (c) Syringing. Drawing tissue into syringe through wide bore needle or canula and expressing. (d) Trituration by pipette. Pippetting tissue fragments up and down through wide bore pipette.

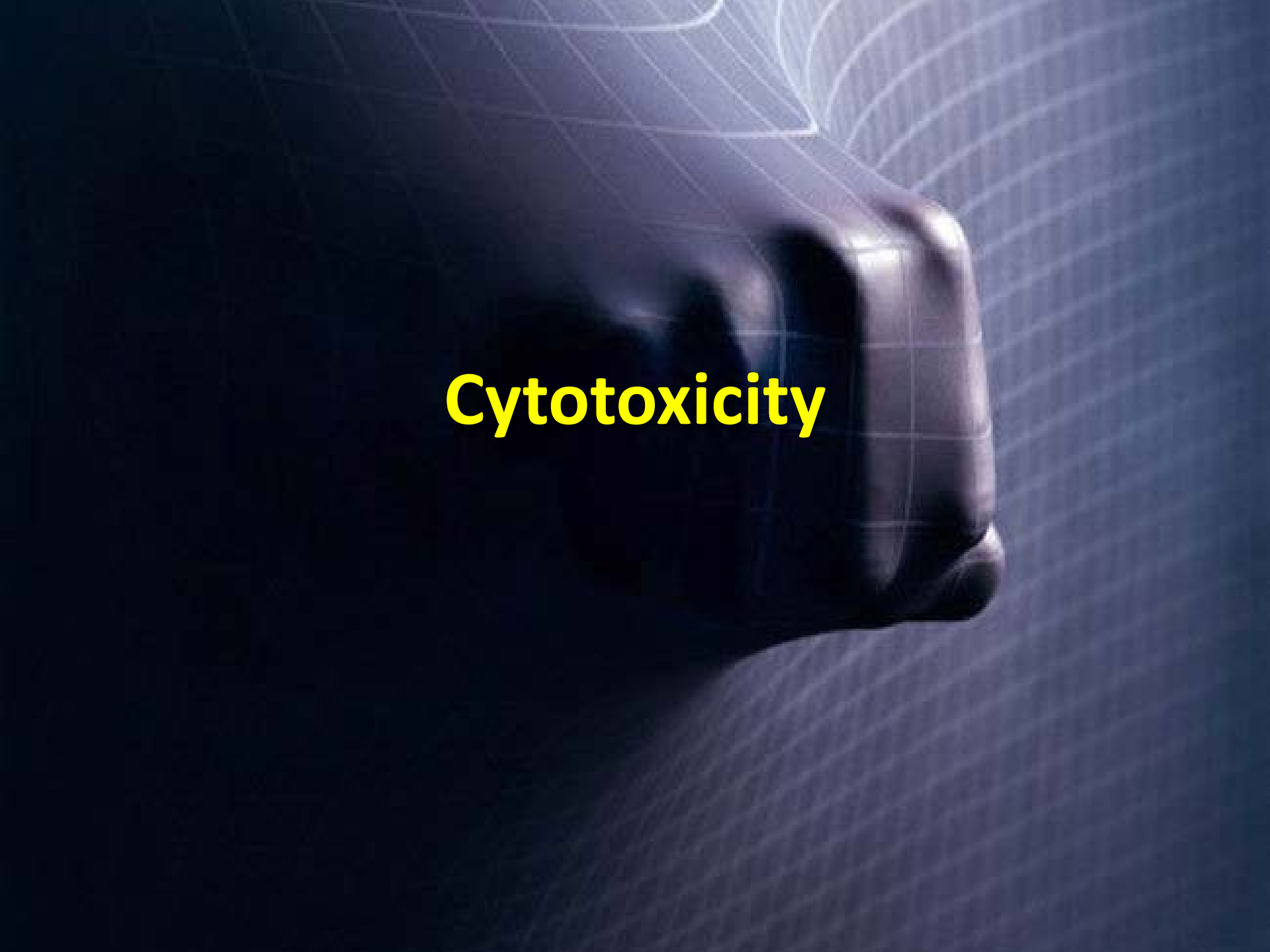
TYPES OF CULTURE

From Freshney, 2005



Types of culture. Different modes of culture are represented from left to right. First, an organ culture on a filter disk on a triangular stainless steel grid over a well of medium, seen in section in the lower diagram. Second, explant cultures in a flask, with section below and with an enlarged detail in section in the lowest diagram, showing the explant and radial outgrowth under the arrows. Third, a stirred vessel with an enzymatic disaggregation generating a cell suspension seeded as a monolayer in the lower diagram. Fourth, a filter well showing an array of cells, seen in section in the lower diagram, combined with matrix and stromal cells.

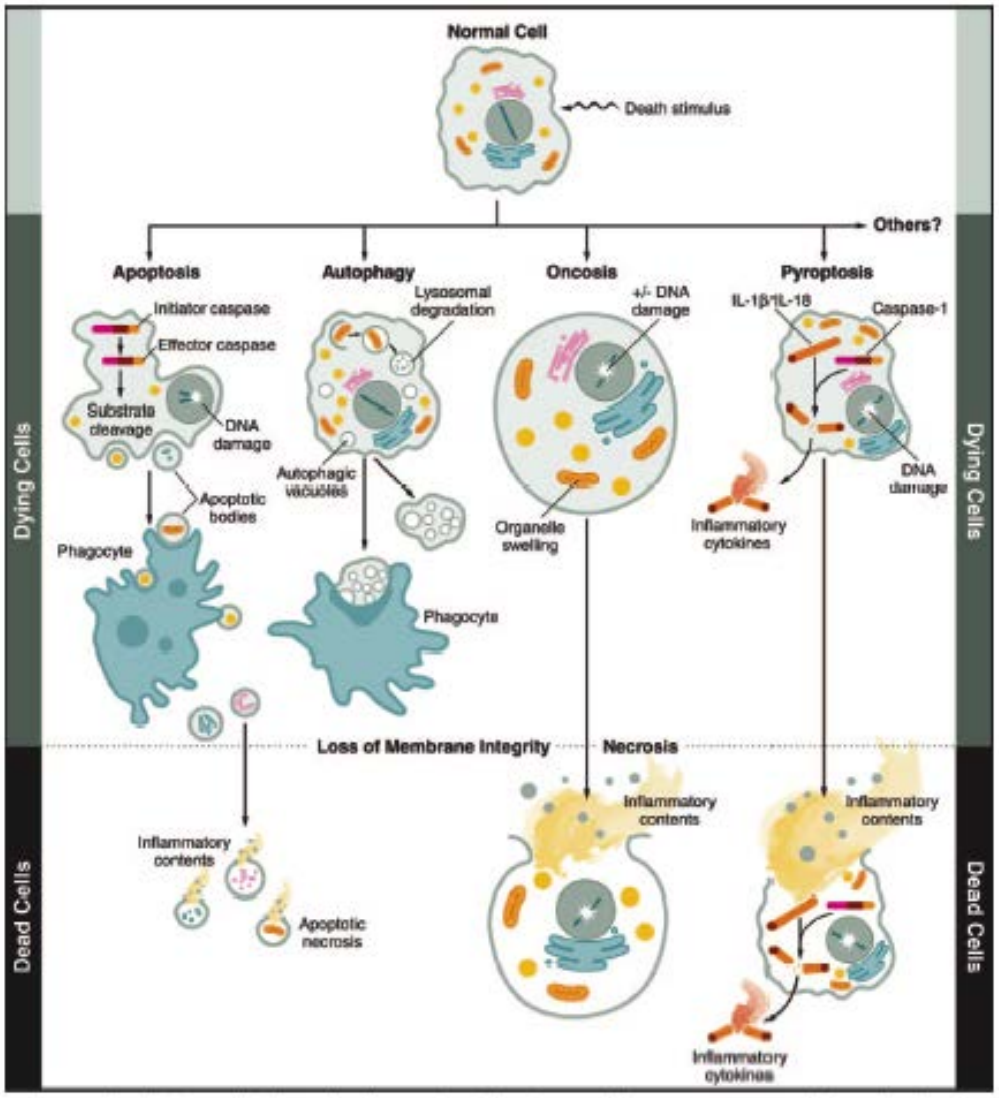
CATEGORY	ORGAN CULTURE	EXPLANT	CELL CULTURE
Source	Embryonic organs Adult tissue fragments	Tissue fragments	Disaggregated tissue, primary culture, propagated cell line
Effort	High	Moderate	Low
Characterization	Easy, histology	Cytology Markers	Biochemical, molecular, immunological, cytological assay
Histology	Informative	Difficult	Not applicable
Biochemical differentiation	Possible	Heterogenous	Loss, but may be reduced
Propogation	Not possible	Possible from outgrowth	Standsrd procedure
Replicate samppling, reproduciblity, homogeneity	High intersample variation	High intersample variation	Low intersample variation
Quantitation	Difficult	Difficult	Easy, many techniques available


Cytotoxicity

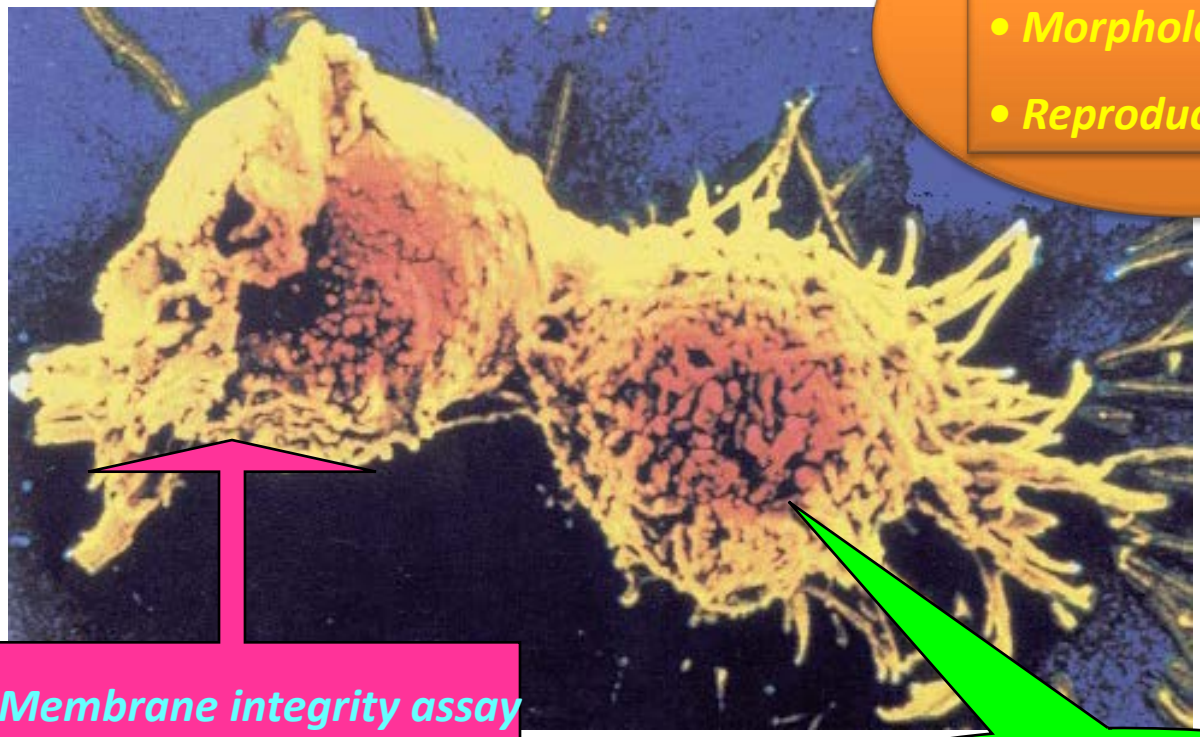
What is cancer

- *Neoplasia* is any new or continued cell growth not needed for normal development or replacement of dead and damaged tissues.
- Tumor & Cancer : In medical language, a neoplasm is often referred as tumor.
- Malignant
- Benign
- Metastasis

There are many ways to die…..



Cell Viability



- DNA labeling assay
- Morphological assay
- Reproductive assay
- Membrane integrity assay
- Functional assay

The major criteria employed in viability assay

Category of viability assay	Assays	Principles
Membrane integrity assay	-Exclusion dyes -Fluorescent dyes -LDH leakage	The determination of membrane integrity via dye exclusion from live cells
Functional assay	-MTT, XTT assay -Crystal violet/ Acid phosphatase(AP) assay -Alamar Blue oxidation-reduction assay - Neutral red assay -[3H]-thymidin/ BrdU incorporation	Examining metabolic components that are necessary for cell growth
DNA labeling assay	-Fluorescent conjugates	Simultaneous cell selection and viability assay
Morphological assay	-Microscopic observation	Determination of morphological change
Reproductive assay	-Colony formation assay	Determination of growth rate

Cell counting

Measurement	Characteristics	Methods
Direct	- Distinguish viable and non-viable cell - Simple, quick, cheap - A small fraction of the total cells from a cell suspension	- Microscopic counting(Hemocytometer) : Trypan blue, Crystal violet - Electronic counters : Coulter counter
Indirect	- Monolayer - Immobilized in matrix	- Lactate dehydrogenase activity - Functional assay - DNA labeling assay

- ***Membrane integrity assay***

  Hemocytometer Trypan blue assay / Automatic trypan blue method
  LDH(lactate dehydrogenase) leakage
  Fluorescent dyes

- ***Functional Assay***

  MTT / XTT assay
  Crystal violet / Acid phosphatase(AP) assay
  Alamar Blue oxidation-reduction assay / Neutral red assay
  [3H]-thymidin and BrdU incorporation

- ***DNA Assay***

  Enzymatic DNA labeling / DNA-binding dye labeling

- ***Morphological assay***

- ***Reproductive Assay***

  Colony-forming efficiency

- ***Laser scanning confocal microscopy***

- ***Nuclear magnetic resonance methods***

Membrane integrity assays

Dye exclusion methods

- Viability assays measure the percentage of a cell suspension that is viable.
- This is generally accomplished by a dye exclusion stain, where cells with an intact membrane are able to exclude the dye while cells without an intact membrane take up the coloring agent.
- The dye used for exclusion stain is usually trypan blue but erythrosin and naphthalene black have also been used.
- A dye uptake stain can be used to measure viability as well. In this case, the dye is normally taken up by viable cells but not by the non-viable cells. Diacetyl fluorescein is an example of a dye used for dye uptake assays.

Trypan blue

- A stain which will only enter across the membranes of dead/non-viable cells.

- Cause cancer in lab. animals

- Appropriate precaution should be taken when handling trypan blue (use of extraction hood and gloves)

- Dilution by trypan blue

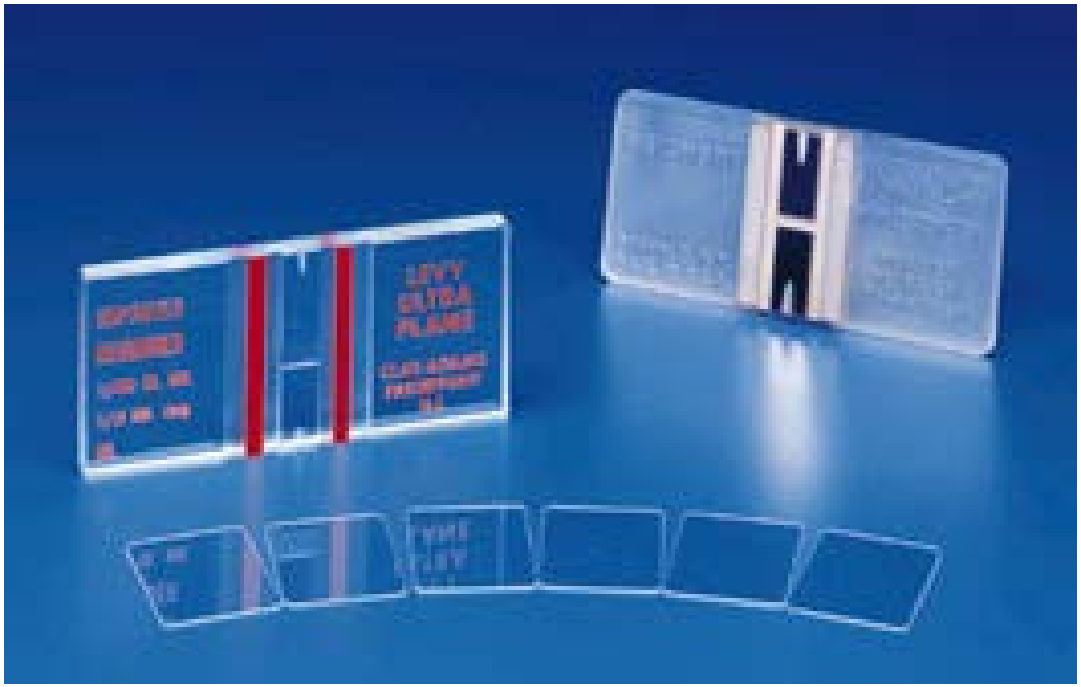
- Viable cells : small, round and refractive

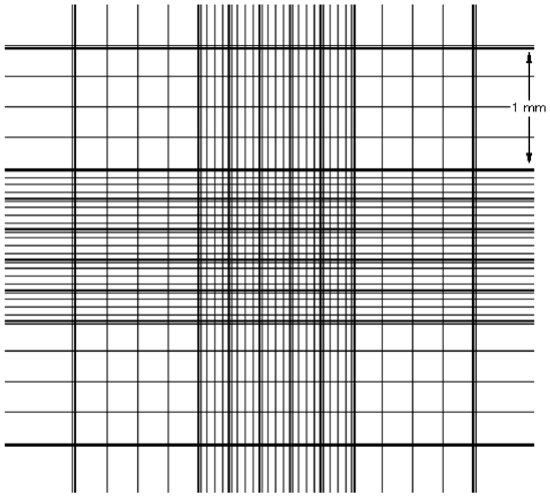
- Non-viable cells : swollen, larger, dark blue

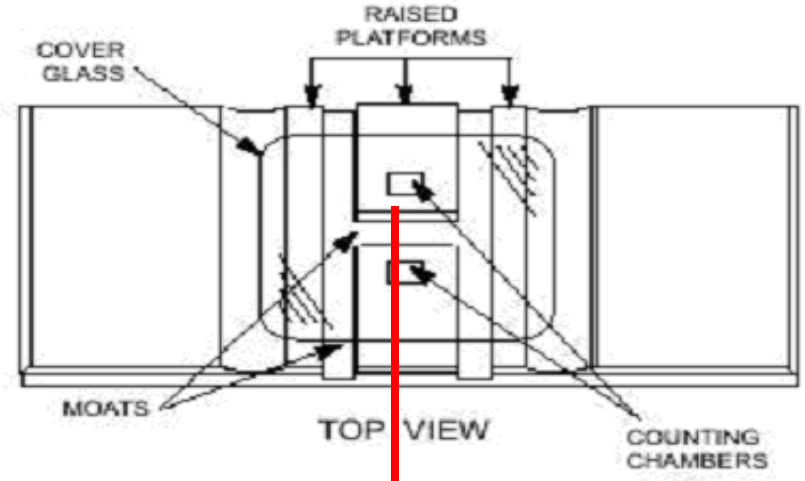
Hemocytometer Cell Counts

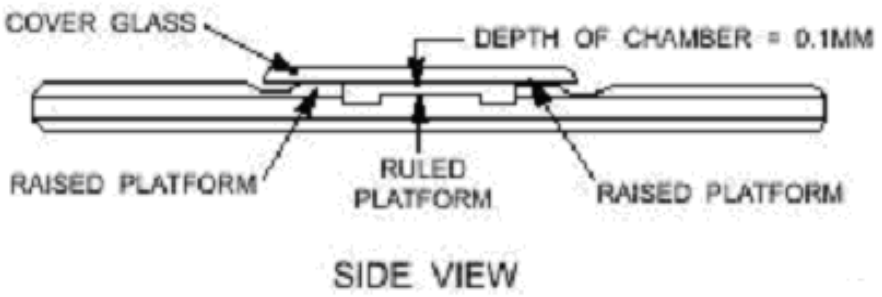
❖ Hemocytometer

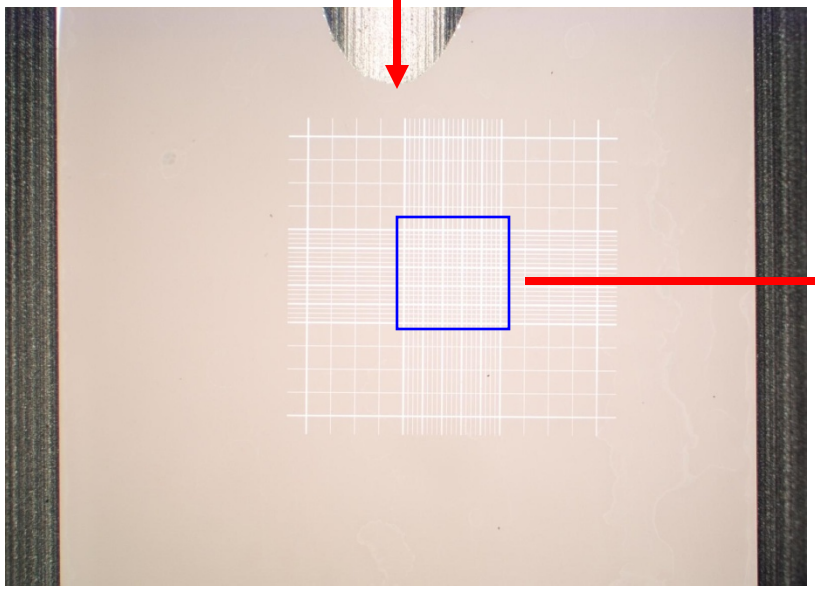
The most common routine method for cell counting which is efficient and accurate is with the use of a hemocytometer.

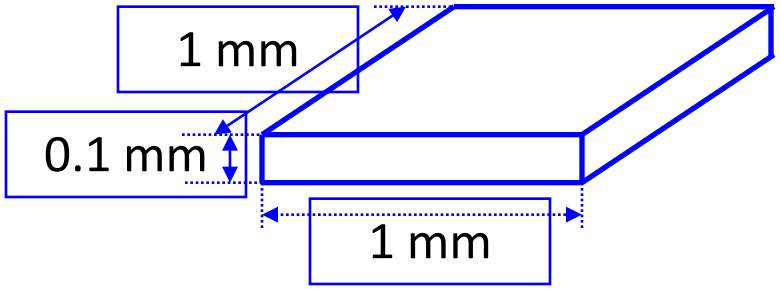






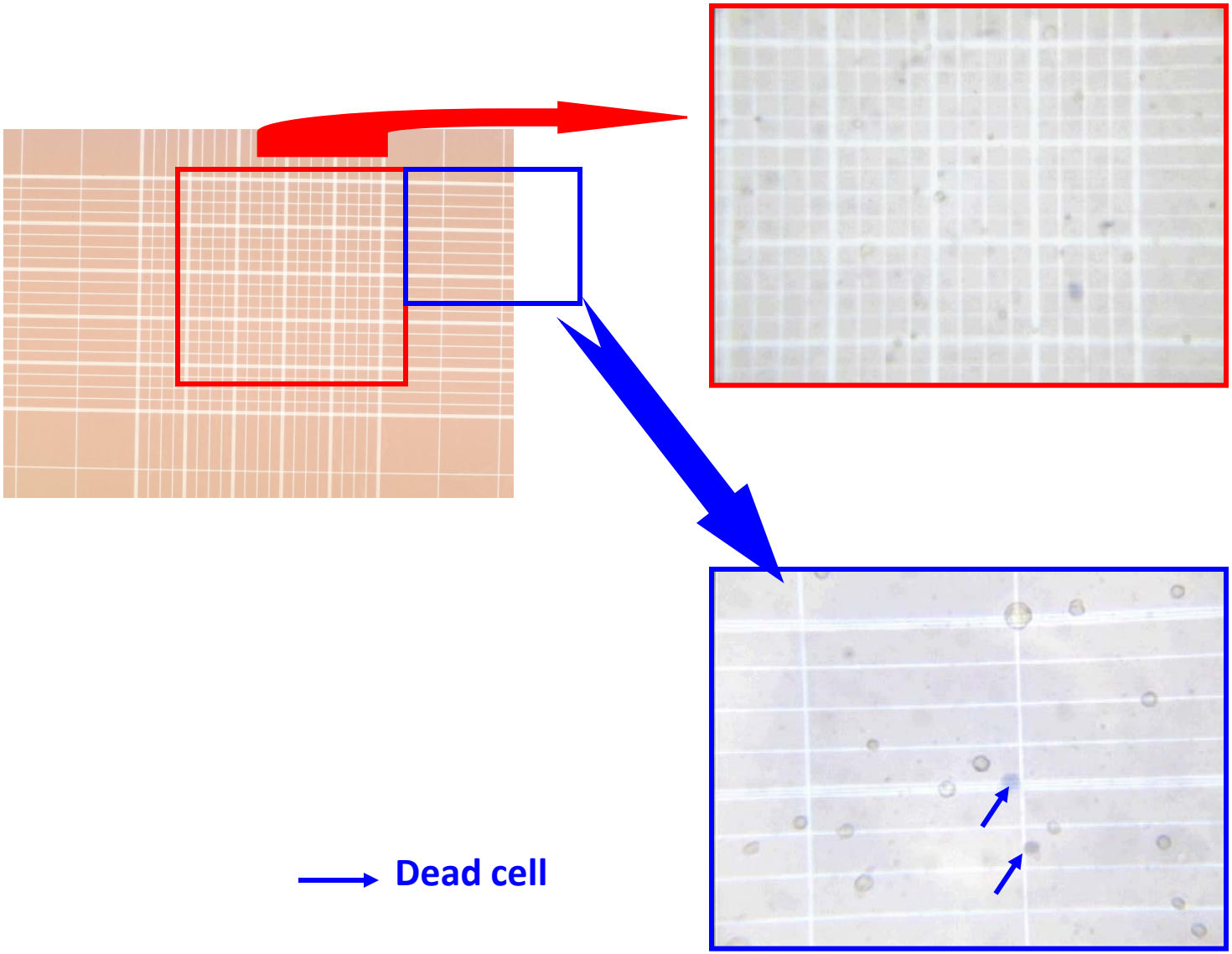






Volume : 0.1mm^3

1 ml = 1 cm^3 = 1000 mm^3


Dead cell

Materials and Equipment

- Trypan blue (0.4 g trypan blue in 100 ml physiological saline) → pass through a 0.22 μm filter
- Hemocytometer with coverslip
- Hand-held counter
- Microscope

Methods

1) Clean hemocytometer & coverslip and wipe with 70% alcohol before use
2) Place coverslip on hemocytometer
3) Mix the cell suspension gently
4) Aliquot 0.1 ml cell suspensions
5) Add 0.1 ml (2-fold dilution), 0.3 ml (4-fold dilution) or 0.9 ml (10-fold dilution) trypan blue : appropriate range of cells to be counted
6) Draw a sample into a Pasteur pipette after mixing
7) Draw the cell suspension in to fill the chamber
8) Using a light microscope at low power, count the number of cells
9) Count the viable & non-viable cells in both halves of the chamber

10) Calculations

A = Vol. Of cell solution (ml)

B = Dilution factor in trypan blue

C = Mean number of unstained cells

D = Mean number of dead/stained cells

10^4 = Conversion of 0.1 mm^3 to ml

(1) Total number of viable cells

$A \times B \times C \times 10^4$

(2) Total dead cell count

$A \times B \times D \times 10^4$

(3) To give a total cell count

Viable cell count + dead cell count

(4) % viability

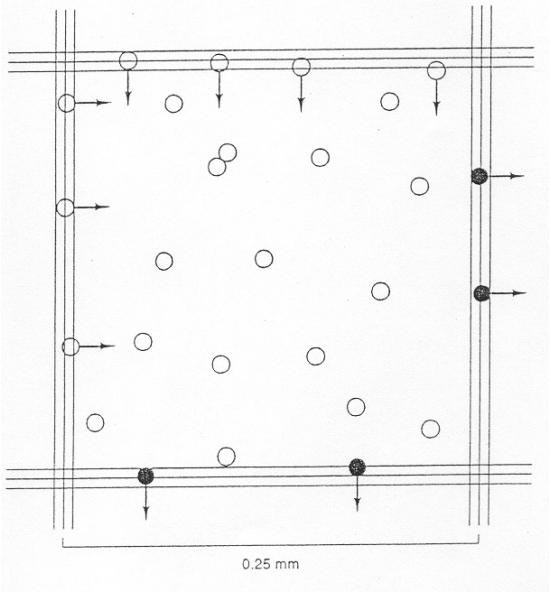
(Viable cell count/Total cell count) $\times$ 100

Example

1) Vol. : Volume
2) CS : Cell Solution
3) TB : Trypan blue

○ Count

● Uncount



Dilution factor	Vol. of CS	Cell count	Total viable cells
0.1 ml CS + 0.1 ml TB (2)	20 ml	23	$20 \times 2 \times 23 \times 10^4 = 9.2 \times 10^6$ cells
0.1 ml CS + 0.3 ml TB (4)	15 ml	//	$15 \times 4 \times 23 \times 10^4 = 1.38 \times 10^7$ cells
0.1 ml CS + 0.9 ml TB (10)	10 ml	//	$10 \times 10 \times 23 \times 10^4 = 2.3 \times 10^7$ cells

Automated trypan blue method for optimal cell viability determination

www.innovatis.com

Introduction

- **The trypan blue dye exclusion assay is the most commonly used and accepted method for the measurement of cell viability**
- **It relies on the alteration in membrane integrity as determined by the uptake of dye by dead cells, thereby giving a direct measure of cell viability**
- **Based on optimal image analysis, the technology allow precise cell-viability and cell-density determination**
- **The system performs automatic and reproducible measurements of human or animal suspension cell densities as well as standardized differentiation between viable and dead cells, based on the trypan blue dye exclusion method**
- **The direct and automated optical analysis by means of modern pattern recognition methods allows cell identification and a standardized differentiation between viable and dead cells and also cell debris**

- *The system consist of three functional part: the liquid handling unit, image capture hard ware, and a data processing system, including the user interface*

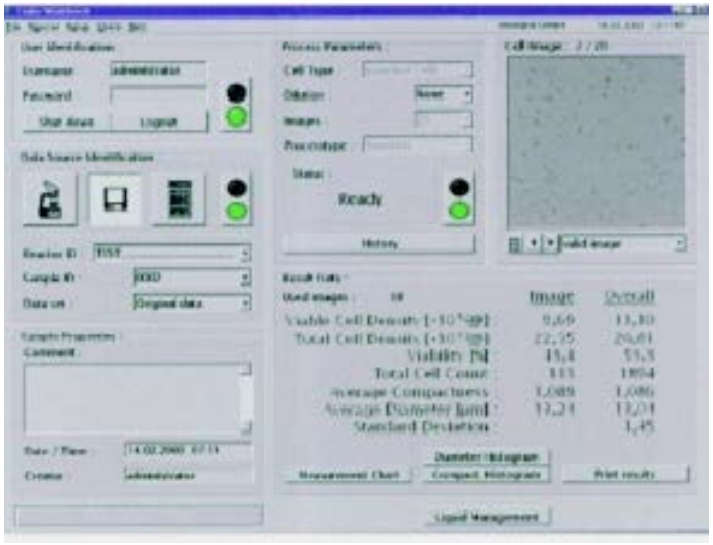
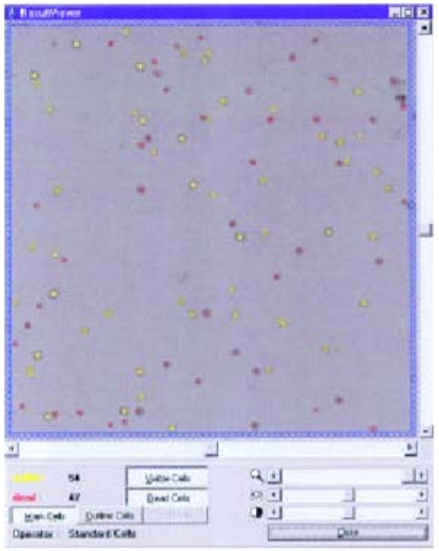
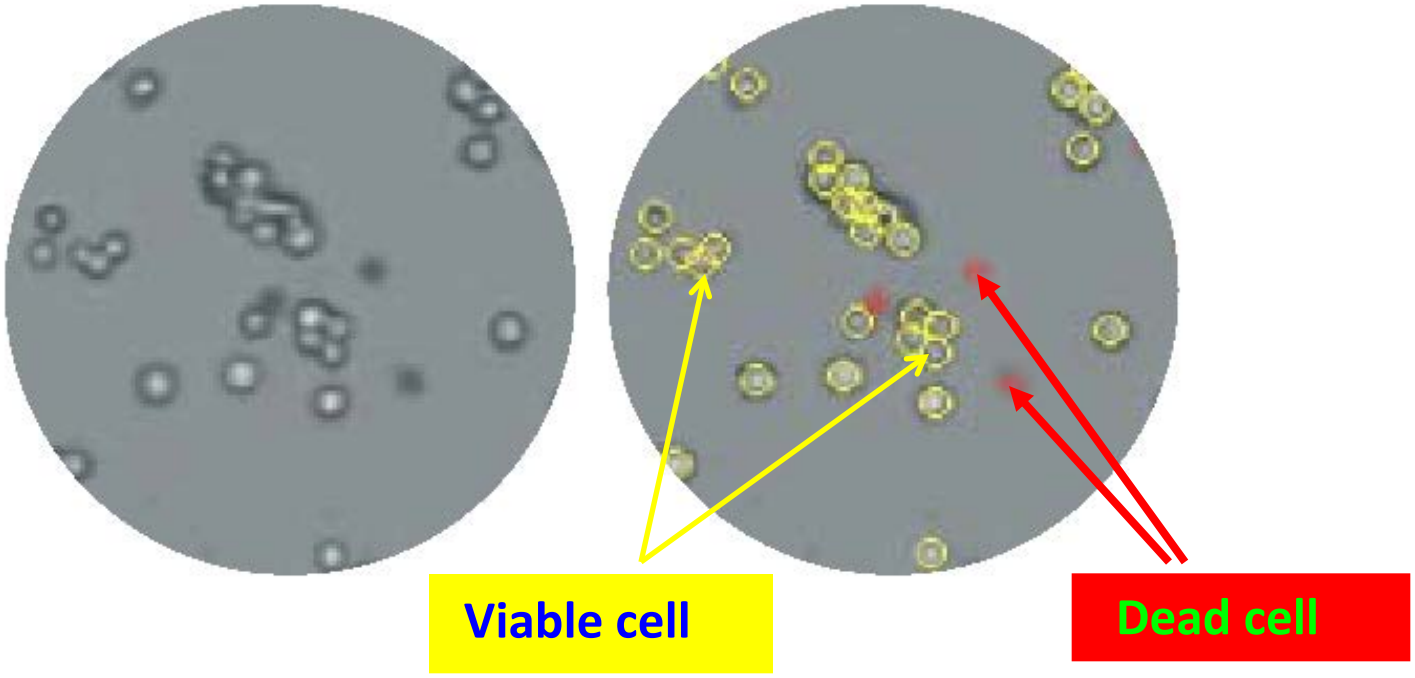


Fig. *Cedex workbench*

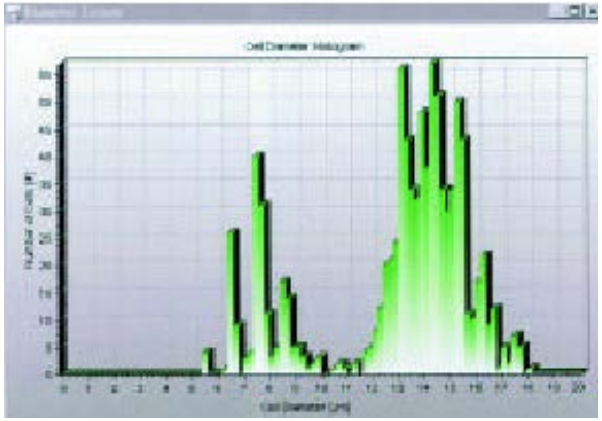


The IP Result viewer enables the user to control whether the Cedex system recognizes the cells correctly, and whether it reliably differentiates and between viable and dead cells

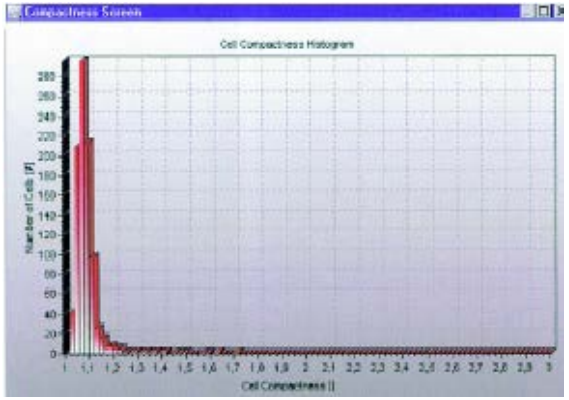
Result view of the image processing



Marked viable and dead cells



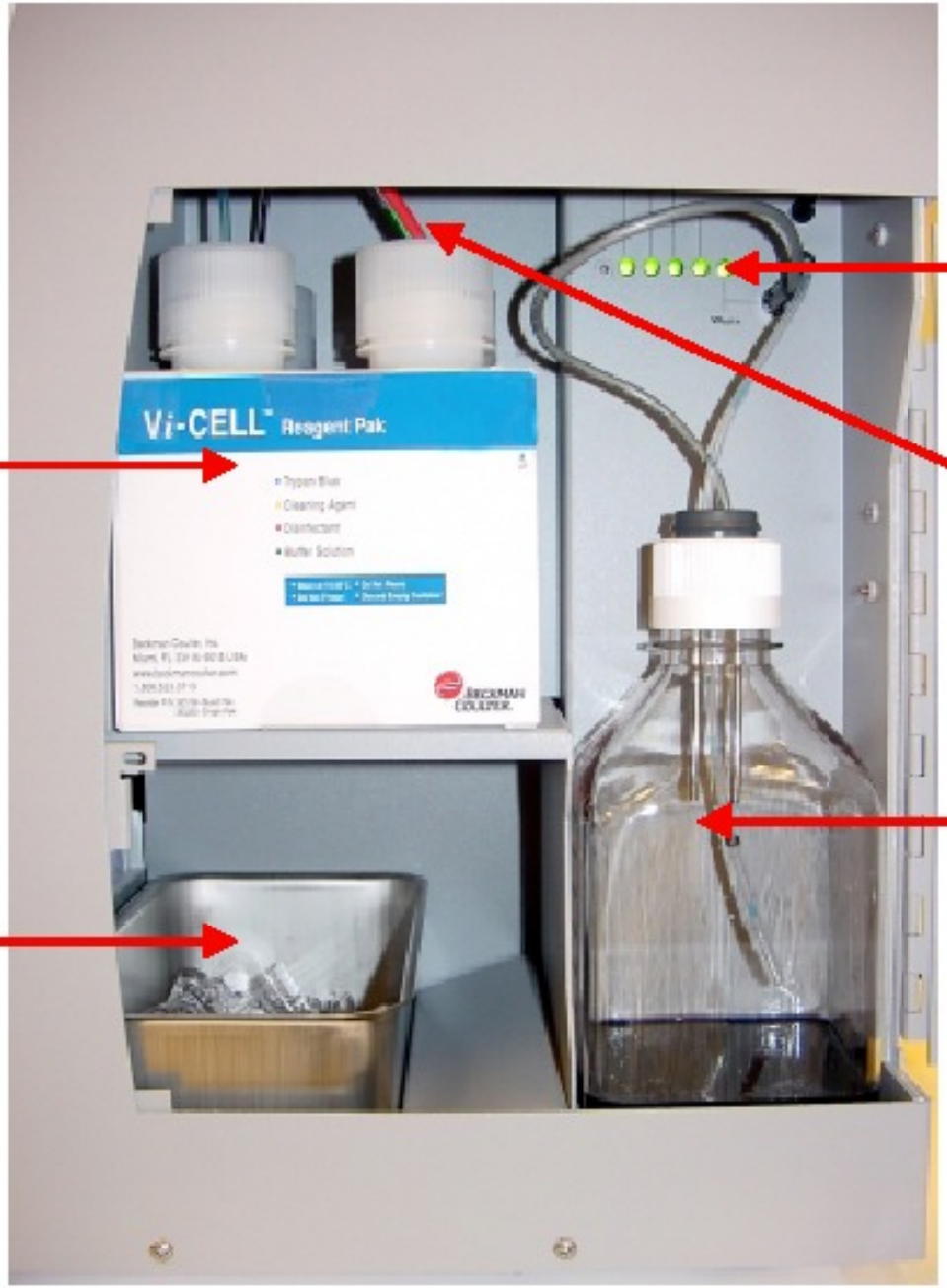
(a)



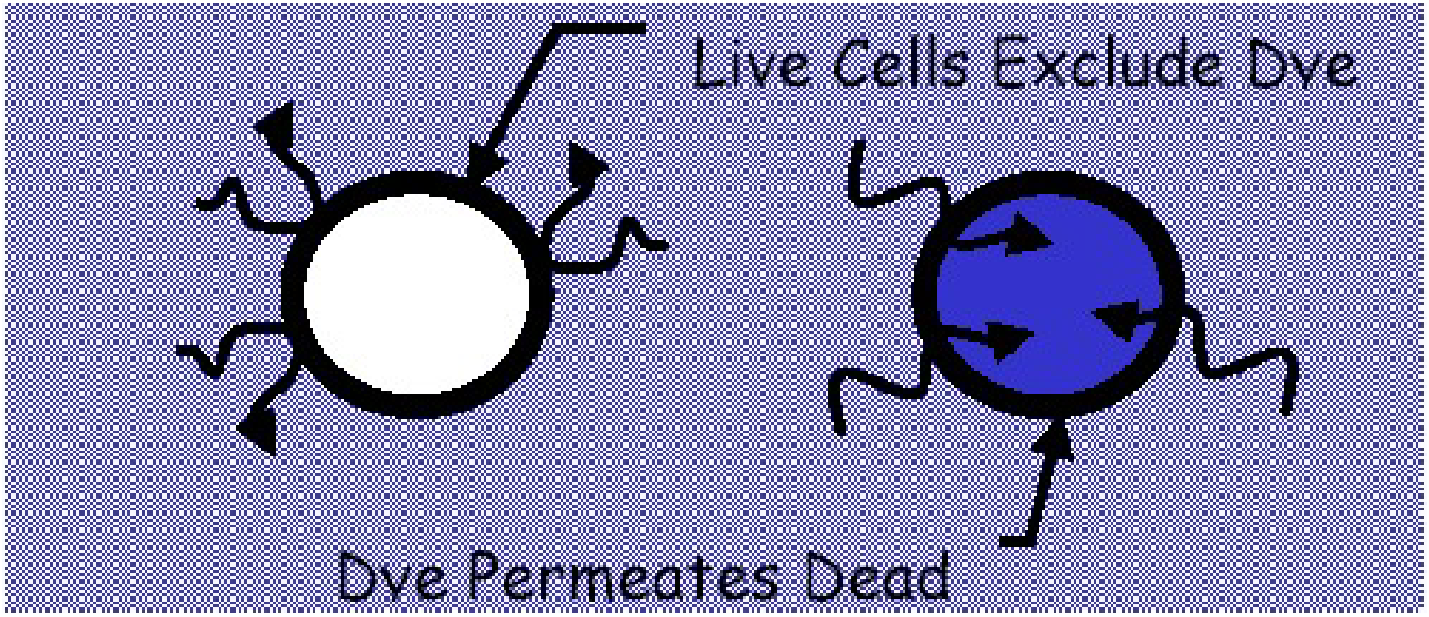
(b)

Fig. a) Distribution histogram of the viable cell diameter for a human leukemia cell sample. b) Histogram of compactness for a human leukemia cell sample. The abscissa represents the ratio of cell circumstance to cell area normalized to the value of 1 for an ideal sphere

Vi-CELL ™ CELL Viability Analyzer

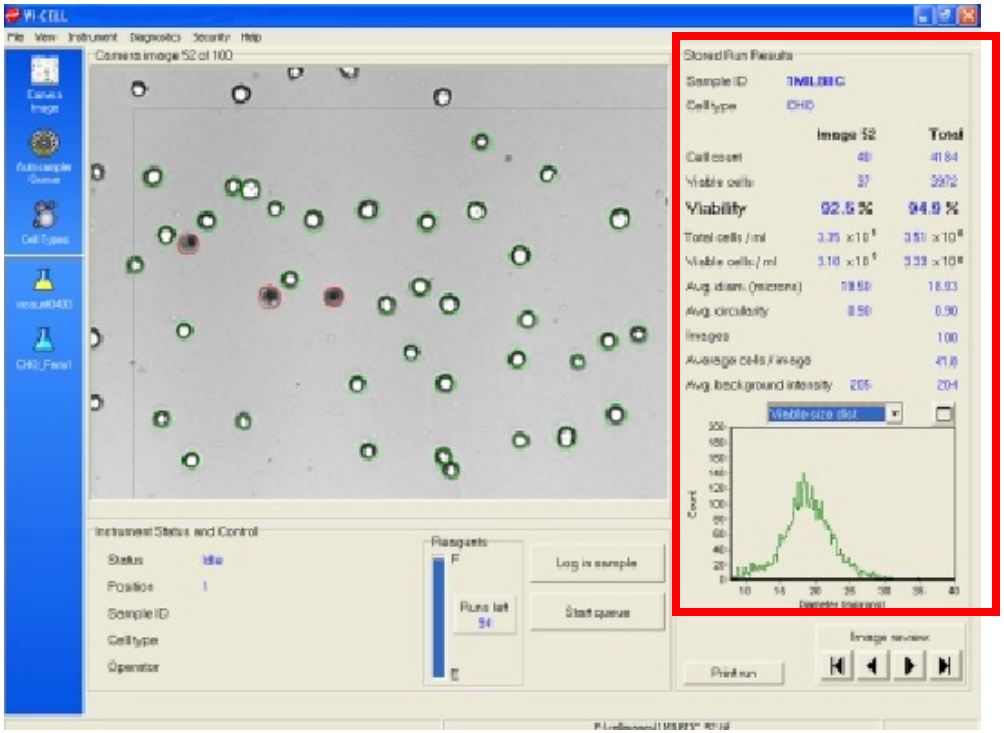

Vi-CELL™ Reagent Pak
Reagent Pack
Used Cups Receptacle
Reagent Detection LED's
Color Coded Reagent Tubing
Waste Container

Principle



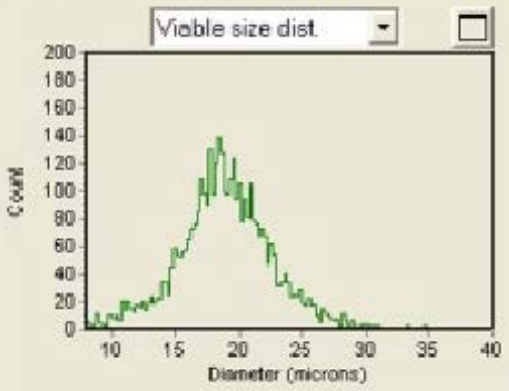
Trypan Blue dye Exclusion Methods

Run results



Stored Run Results

Sample ID	1MILBIIC	
Cell type	MILBIIC	
	Image 52	Total
Cell count	40	4181
Viable cells	37	3966
Viability	92.5 %	94.9 %
Total cells / ml	2.83 x10^6	2.96 x10^6
Viable cells / ml	2.62 x10^6	2.80 x10^6
Avg. diam. (microns)	19.50	19.00
Avg. circularity	0.94	0.93
Images		100
Average cells / image		41.8
Avg. background intensity	205	204



LDH Release

- One parameter of cell death is the integrity of the cell membrane.
- It can be measured by cytoplasmic enzyme activity released by damaged cells.
- Lactate dehydrogenase is a stable cytoplasmic enzyme present in all the cells.
- The LDH is rapidly released to the culture supernatant upon damage of the plasma membrane.

LDH (lactate dehydrogenase) Leakage

- Quantitative value for the loss of cell viability
- The activity of LDH can be measured as the reduction of pyruvate to lactate.
- The reduction is coupled to the oxidation of NADH to NAD^+, which is followed spectrophotometrically at 340nm

$$\text{Pyruvate} + \text{NADH} + H^+ \xrightleftharpoons{\text{LDH}} NAD^+ + \text{lactate}$$

- As NADH has a high absorbance at 340nm compared to NAD^+, the reaction is measured as the rate of decrease in absorbance at 340nm.

Requirement of LDH assay

Greater process productivity (e.g. High-cell-density entrapped-culture systems) : difficulty of cell isolation

→ Metabolic parameters(glucose uptake) : compromised because uptake/production rates can alter as a result of the cell switching carbon source.

→ Analysis of the release of intracellular enzymes can be used enzyme in cell culture studies is LDH.

❖ Assumptions of LDH assay

: Intracellular enzymes are only released after damage to the cell membrane

: Rapidly released from damaged cells.

Pitfalls of LDH assay

- The release of LDH activity can be related to the total No. of dead & lysed cells.
- The stability of LDH can vary considerably, ranging from the loss of a few percent per day to a half-life of 12h depending upon the cell type.
- Assumed that the release of LDH occurs rapidly after damage to the cell membrane. This assumption is not necessarily correct.
- The release of LDH can be complete in cells which are considered dead by dye exclusion methods.
- Complete release may only occur upon cell lysis.
- This point is further complicated because dye exclusion methods do not measure lysed cells.

Fluorescent dyes

- Ethidium bromide (EtBr) and propidium iodide (PI)
- PI is impermeable to intact plasma membrane.
- PI binds to nucleic acids upon membrane damage : flow cytometric techniques depend on fluorescence, PI is ideally suitable for the rapid evaluation of the permeability properties of large numbers of cells while maintaining good statistical accuracy.
- Intercalates with DNA or RNA $\Rightarrow$ red

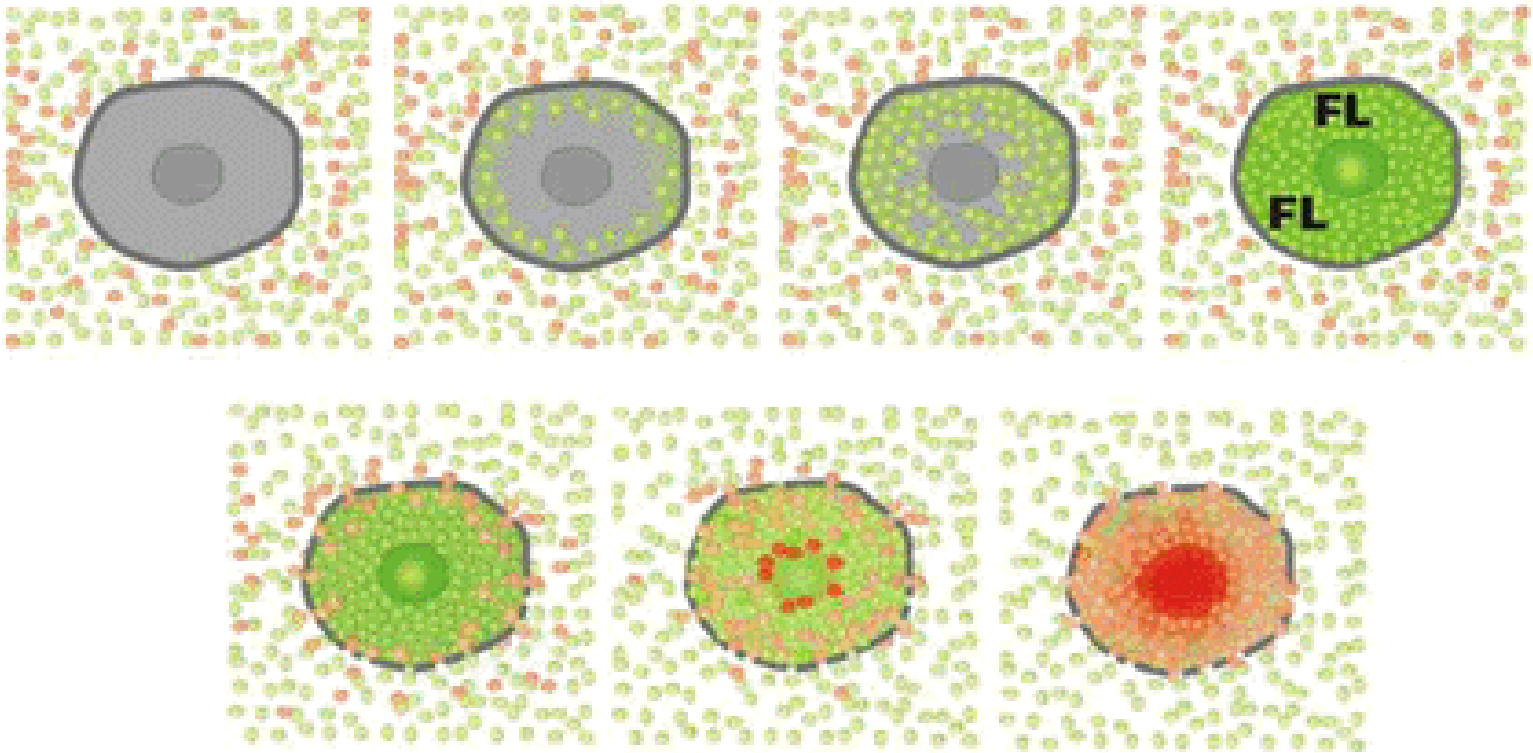
- Fluorescein diacetate (FDA) is a nonpolar ester which passes through plasma membranes and is hydrolyzed by intracellular esterases to produce free fluorescein, the polar fluorescein is confined within cells which have an intact plasma membrane and can be observed under appropriate excitation conditions.
- Undamaged cell : highly fluorescent fluorescein dye
- Damaged cell : fluoresce only weakly
- greenish-yellow at 450-480 nm

Schematic illustration of the principle of PI/FDA cell viability assay

● FDA (Fluorescein diacetate)
● PI (Propidium iodide)

Intact cell –
PI and FDA is added

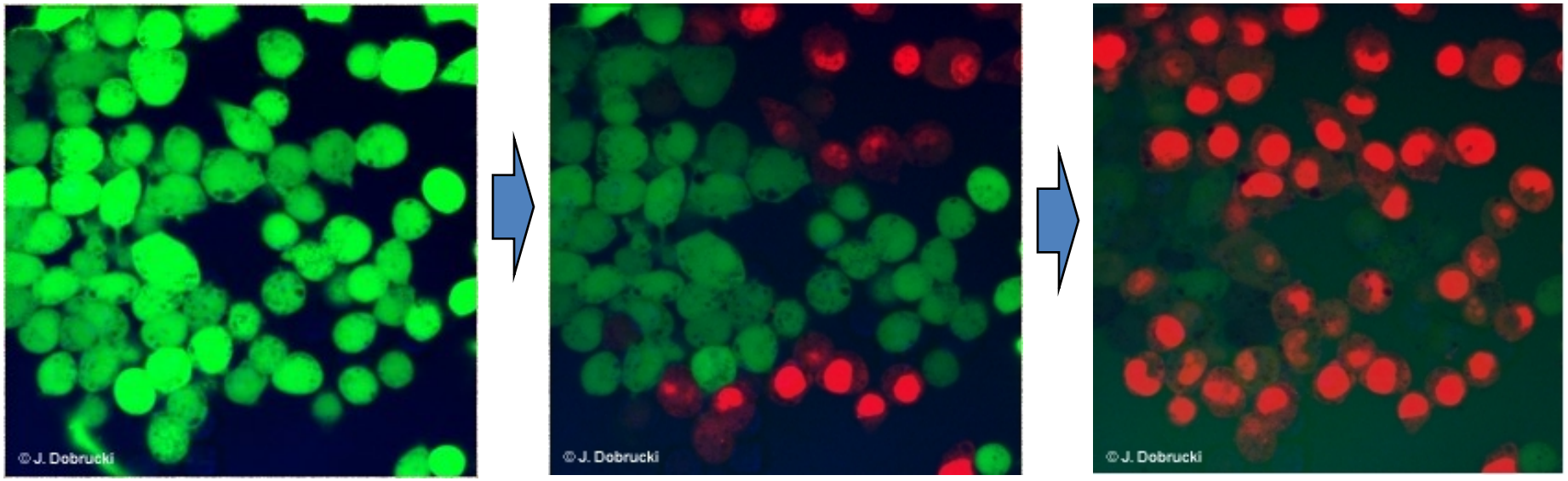
Fluorescein in intact cells



Plasma membrane is damaged ; fluorescein leaks out

PI enters and strains nucleic acids

Example 1 ; Observation of cell death



A group of hepatoma cells exposed to a diffusing wave of digitonin. Intact cells (green) are damaged by digitonin, loose the green fluorescence and acquire red fluorescence of PI.

Functional assays

Evaluate viability by examining the metabolic components that are necessary for cell growth, on the premise that cellular damage will inevitably result in the loss of ability to maintain and provide energy for metabolic function and growth.

Colorimetric assay

- Rapid and accurate assessment of viable cell number
- Miniaturized into 96-well plates
- Measure using an Microplate reader
- Permit many sample to be analyzed rapidly
- Reduce medium and plastics costs

- These assay are read at 570 nm (except for the Acid Phosphatase (A.P) assay-wavelength is 405 nm) on a ELISA plate reader, using a 620 nm filter as reference wavelength.
- It is important to remove any bubbles from the well before absorbance readings.

Examples are

MTT / XTT assay

Crystal violet dye elution (CVDE)

Acid phosphatase (AP) assay

Alamar blue oxidation-reduction assay

Neutral Red (NR) assay

[^{3}H]-thymidine and BrdU incorporation

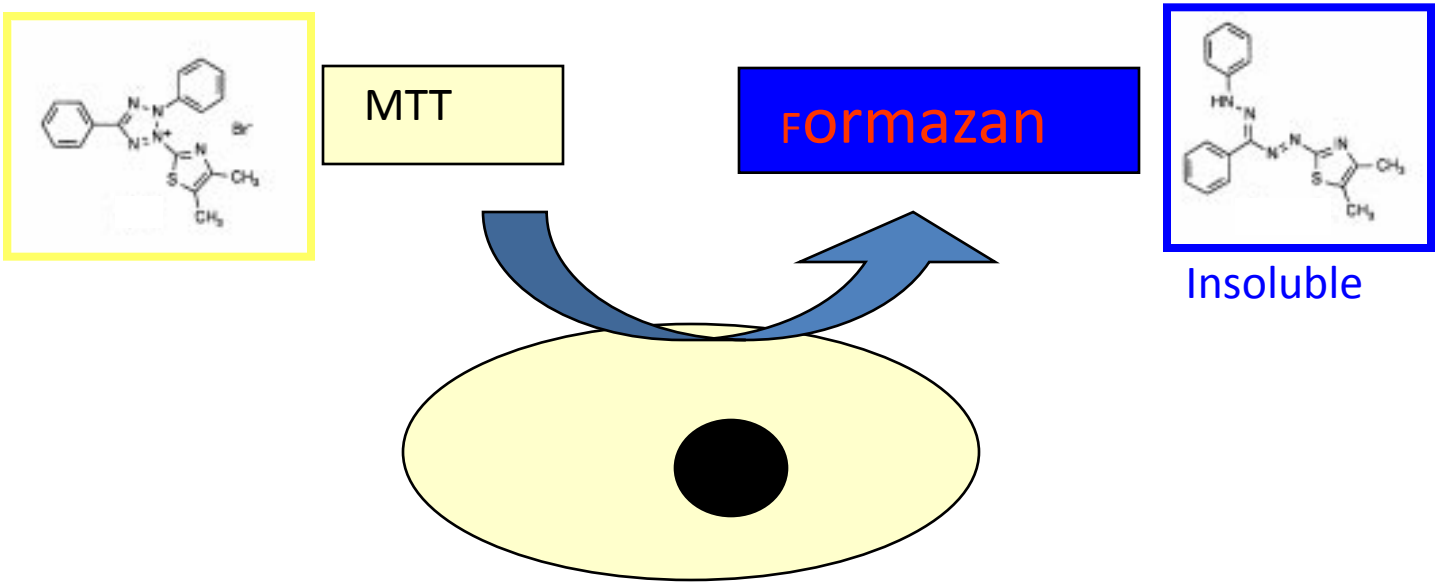
MTT Assay

Introduction

- This assay is a sensitive, quantitative and reliable colorimetric assay that measures viability, proliferation and activation of cells.

- The assay is based on the capacity of mitochondrial dehydrogenase enzymes in living cells to convert the yellow water-soluble substrate 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) into a dark blue formazan product which is insoluble in water.

- The amount of formazan produced is directly proportional to the cell number in range of cell lines.



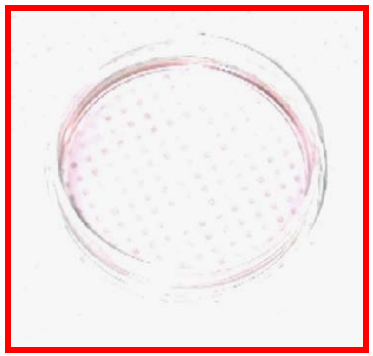
Advantages of MTT assay

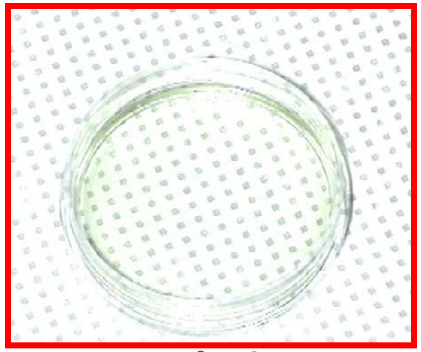
- Considered a major advance; used the most prevalent in vitro assay
- Rapid, versatile, quantitative and highly reproducible
- Adaptable to large-scale screening; relevant for most cells
- MTT reduction correlates to indices of cellular protein and earlier cell number
- More sensitive and earlier predictor of toxicity than classical LDH or neutral red measurements

Disadvantage of MTT assay

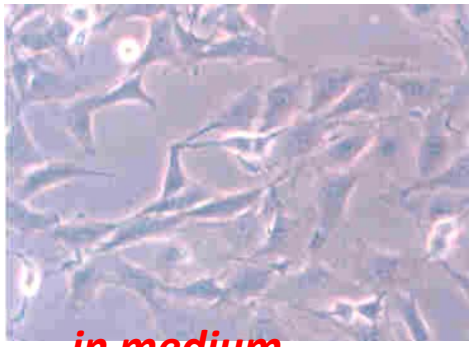
1. Production of the MTT product is dependent on the MTT concentration in the medium. The kinetics and degree of saturation are dependent on cell type.
2. Assay is less effective in the absence of cell proliferation.
3. MTT cannot distingulish between cytostatic and cytocidal effect.
4. Individual cell numbers are not quantitated and results are expressed as a percentage of control absorbance.
5. Test is less effective if cells have been cultured in the same media that has supported growth for a few day, which leads to underestimation of control and untreated samples.

Example; NIH J2 3T3 cell

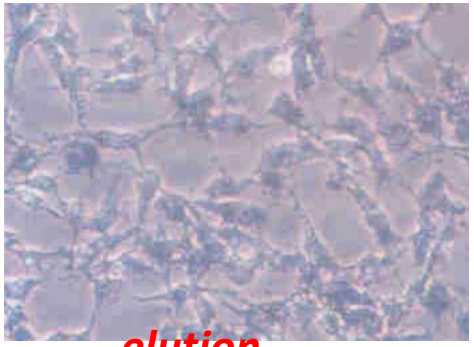




MTT solution



in medium



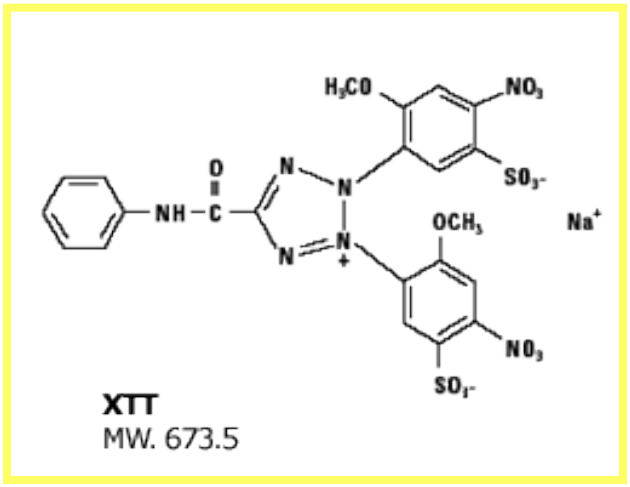
elution

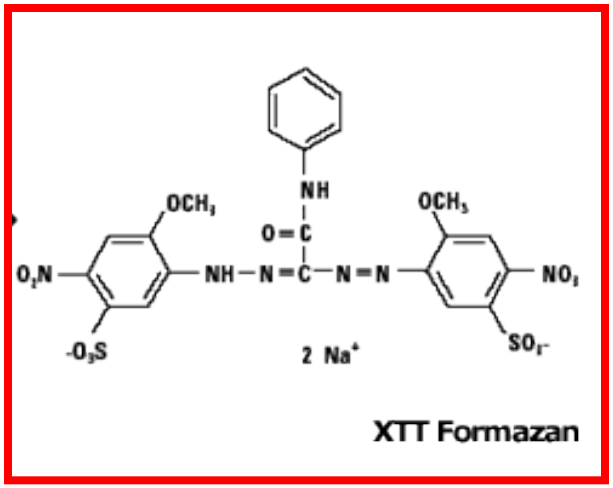
Absorbance at 540 nm

XTT assay

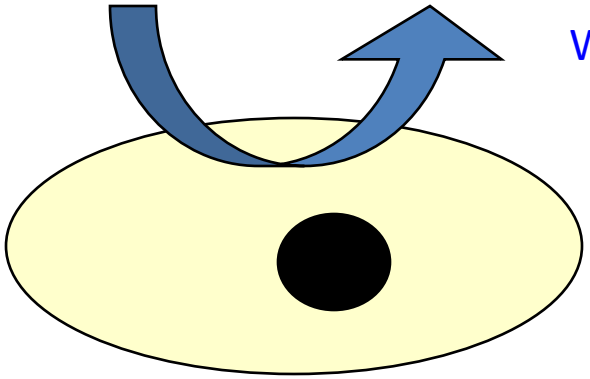
Introduction

- The assay is based on the cleavage of the yellow tetrazolium salt XTT to form an orange formazan dye metabolic active cells.
- This conversion only occurs in viable cells.
- The formazan dye formed is soluble in aqueus solution and is directly quantified using Microplate reader.
- Both MTT and XTT work by being to a formazan dye only by metabolic active cells.



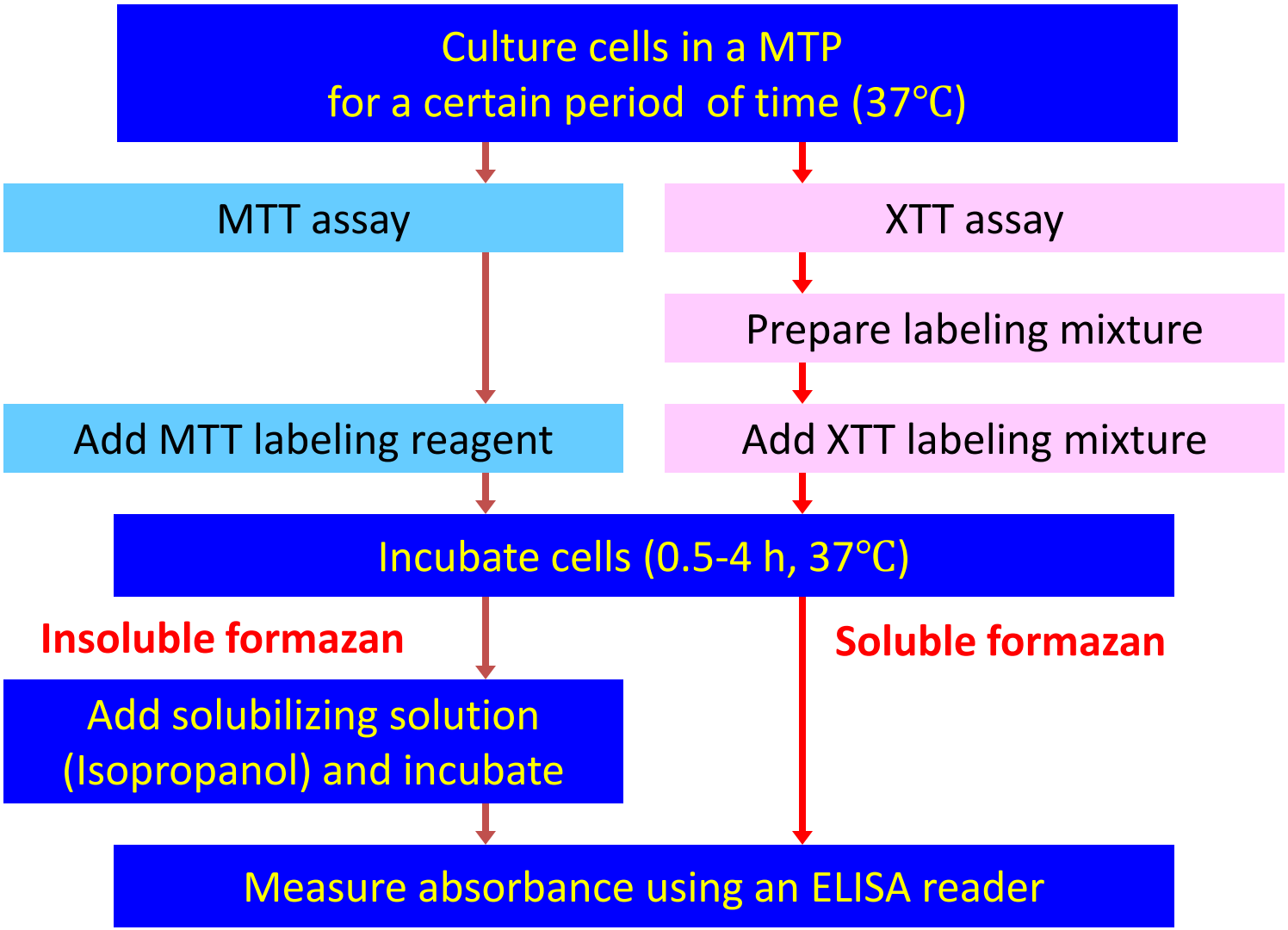


Water soluble

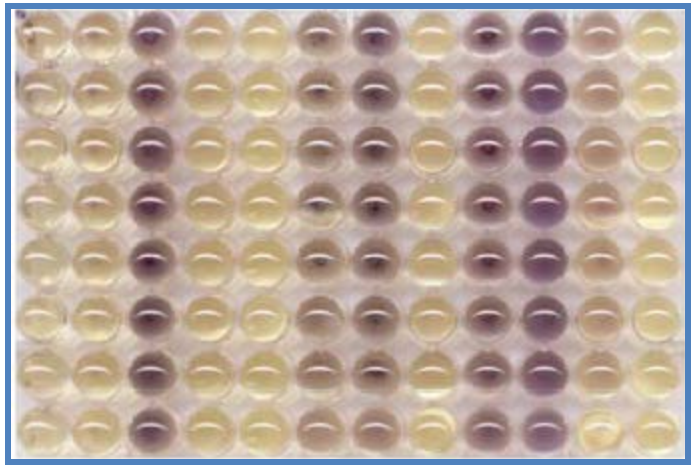


metabolically active cell

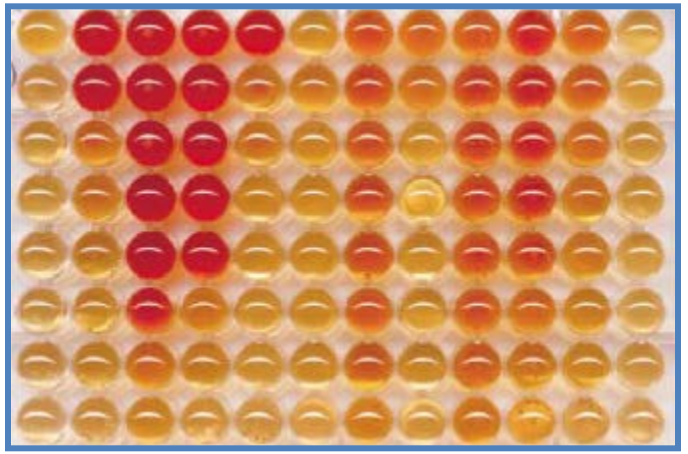
Compare with MTT assay and XTT assay



Example: MTT and XTT



MTT



XTT

Jenny G., Mark H., Anna J., Inger K., Douglas Mc., Roland M., 2002.
Evaluation of redox indicators and the use of digital scanners and spectrophotormeter for quantification of microbial growth in microplates. J. Micro. Methods. 50:63-73

Crystal violet

Principle

- Dye elution:

Cell up-taken dye was measured colorimetric method after acetic acid dye elution.

- Nuclei counting

Incubation of cell samples in a mixture of citric acid and crystal violet causes cells to lyse and the released nuclei to stain purple.

Procedure

❖ Dye elution

① After removal of medium, rinse 96 well plates with 100 μℓ/well of PBS and stain with 100 μℓ 0.25% (g/10ml) aqueous crystal violet for 10 min.

② Rinse plats four times in tap water.

③ Dry the outsides of the plates with paper to help avoid water stains, and then dry the plates at 37℃. When dry, add 100 μℓ per well of 33% glacial acetic acid (33 ml/100ml) and mix the contents of each well before reading at 570 nm.

❖ Nuclei counting

① Allow microcarriers from a culture sample (1ml) to settle to the bottom of a centrifuge tube.

② Removed clear supernatant by aspiration.

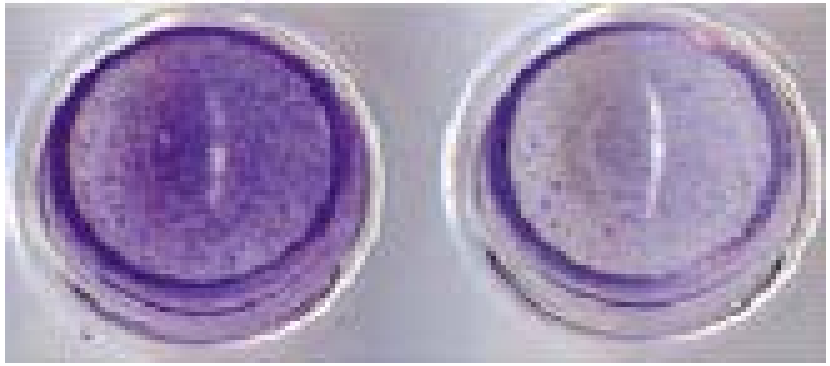
③ Add 1ml of crystal violet reagent.

④ Incubate at 37℃ at least 1 h.

⑤ Introduce a sample into the hemocytometer chamber and count the purple-stained nuclei as for whole cells.

Example; Monolayer culture

Crystal Violet



Acid phosphatase (AP) assay

Introduction

- The action of this enzyme in many of tissue is to cleave a waste product called pyrophosphate and effectively convert it to a useable phosphate.

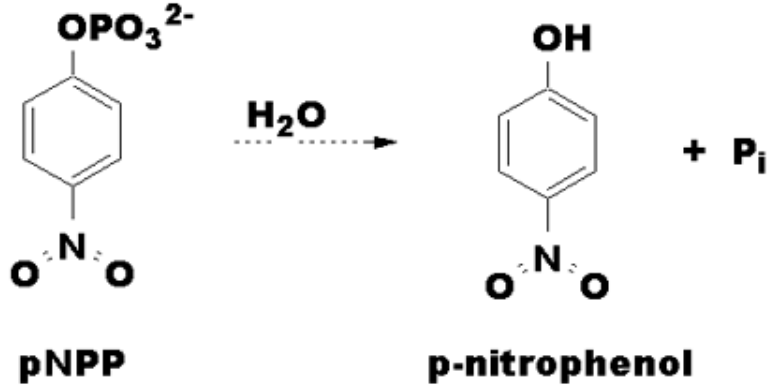
- P-nitrophenyl phosphate will be the substrate and nitrophenol is the product of this reaction.

- Nitrophenol is colorless but when the pH of the reaction solution is alkaline, it is appears yellow. The pH of the reaction solution will be changed by the addition of NaOH.

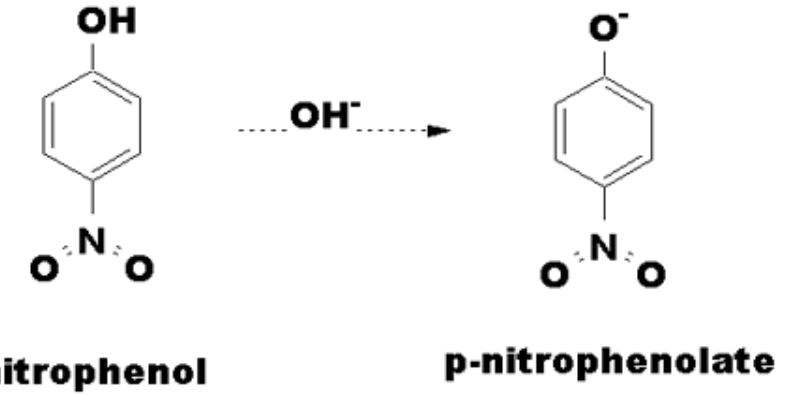
P-nitrophenyl phosphate + Acid phosphatase

→ Nitrophenol + HPO_4^{-2}

1. Phosphatase Catalyzed Reaction



2. Color Reaction (add NaOH)



Alamar Blue oxidation-reduction assay

- Principle

In the presence of cellular metabolism the color of Alamar Blue (ALB) changes from a fully oxidized, non fluorescent blue to a fully reduced, fluorescent red. ALB will be reduced by a variety of enzymes and small molecules, including the cytochrome system, FMN, FAD, NAD, and NADP.

- Advantages

Simple, rapid, inexpensive, required no lysis, extraction or washing of sample

- Disadvantages

Unstable during storage (absorbance of oxidized ALB-3day, reduced ALB-increase from one day to the next)

- Characteristics

- Sensitivity :

Propidium iodide (PI), Sulforhodamine B (SRB) > ALB=MTT

- The ALB assay is faster, simpler, and less artefact prone than the MTT assay.

Neutral Red assay
(3-amino-7dimethyl-2-methyphenazine hydrochloride)

❖ Principle

- The incorporaton of NR into the lysosomes of viable cells after their incubation with test agents.

❖ Use

- Industrial, pharmaceutical, environmental and other testing laboratories concerned with acute toxicity testing.

❖ Advantages

- Simplicity, speed, economy, and sensitivity

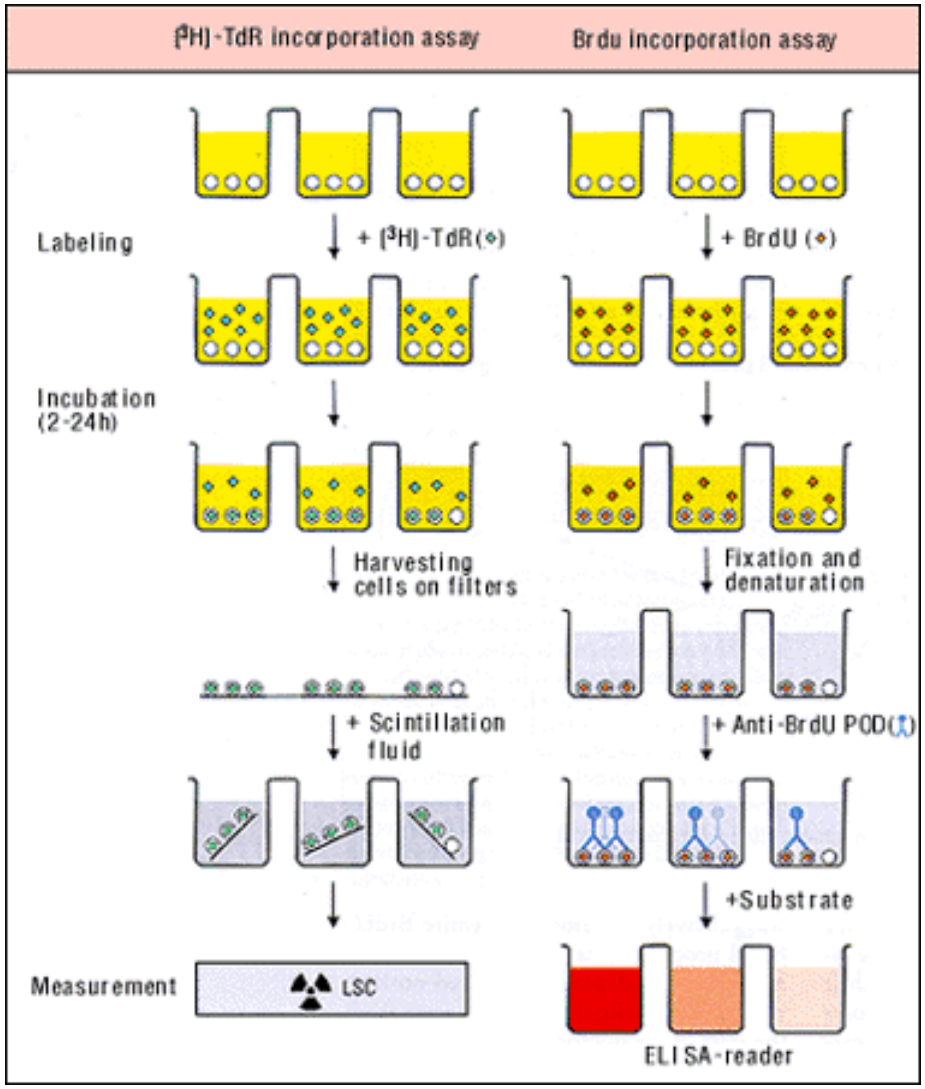
[^{3}H]-thymidine and BrdU incorporation

(DNA synthesis measurement)

Principle

The rate of DNA synthesis is a reflection of proliferation under many condition.To measure the proliferative rates by [^{3}H]-thymidine uptake, cells are cultured in microtitre wells, thymidine is added, and the uptake by DNA is measured , after lysing and washing on, by scintillation counting. Bromodeoxyuridine(BrdU) can be incorporated instead of [^{3}H]-thymidine and the incorporation can be assayed with antibodies to BrdU in a non-radioactive assay.

Schematic diagram of [^{3}H]-TdR and BrdU



Labeling index with [^{3}H]-thymidine

① Set up the culture at $2x10^4$ cells/ml~ $5x10^4$ cells/ml in 24 well plates containing cover-slips. Grow to the desired cell density.

② Add [^{3}H]-thymidine to the medium . 100KBq/ml(~5μCi/ml)and incubate for the cultures 30 min.

③ Remove the labeled medium, and discard it into a designed container for radioactive waste.

④ Wash the cover-slips three times with PBSA.

⑤ Add 1:1 PBSA: acetic methanol, 1ml per well, and remove it immediately

⑥ Add 1ml of acetic methanol at 4 to each well, and leave the cultures for 10min.

⑦ remove the cover-slips, and dry them with a fan

⑧ mount the cover-slip on a microscope slide with the cells uppermost.

⑨ Leave the mountant to dry overnight.

DNA synthesis by [^{3}H]-thymidine

① Grow the culture to the desired density.

② [^{3}H]-TdR, 40 KBq /ml(~1.0μCi), 2 MBq/mol(~50 μCi/mol) in HBSS.

※1Ci =3.7*10^{10} 호/ s = 3.7*10^{10} Bq , 1Bq =1호/s

③ Incubate the cell for 1-24 h.

④ Remove the radioactive medium carefully.

⑤ Wash the cell carefully with 2 ml of HBSS, PBSA, and 2 ml ice-cold 0.6 M TCA for 10 min.

⑥ Wash the cell with TCA twice 5 min each time.

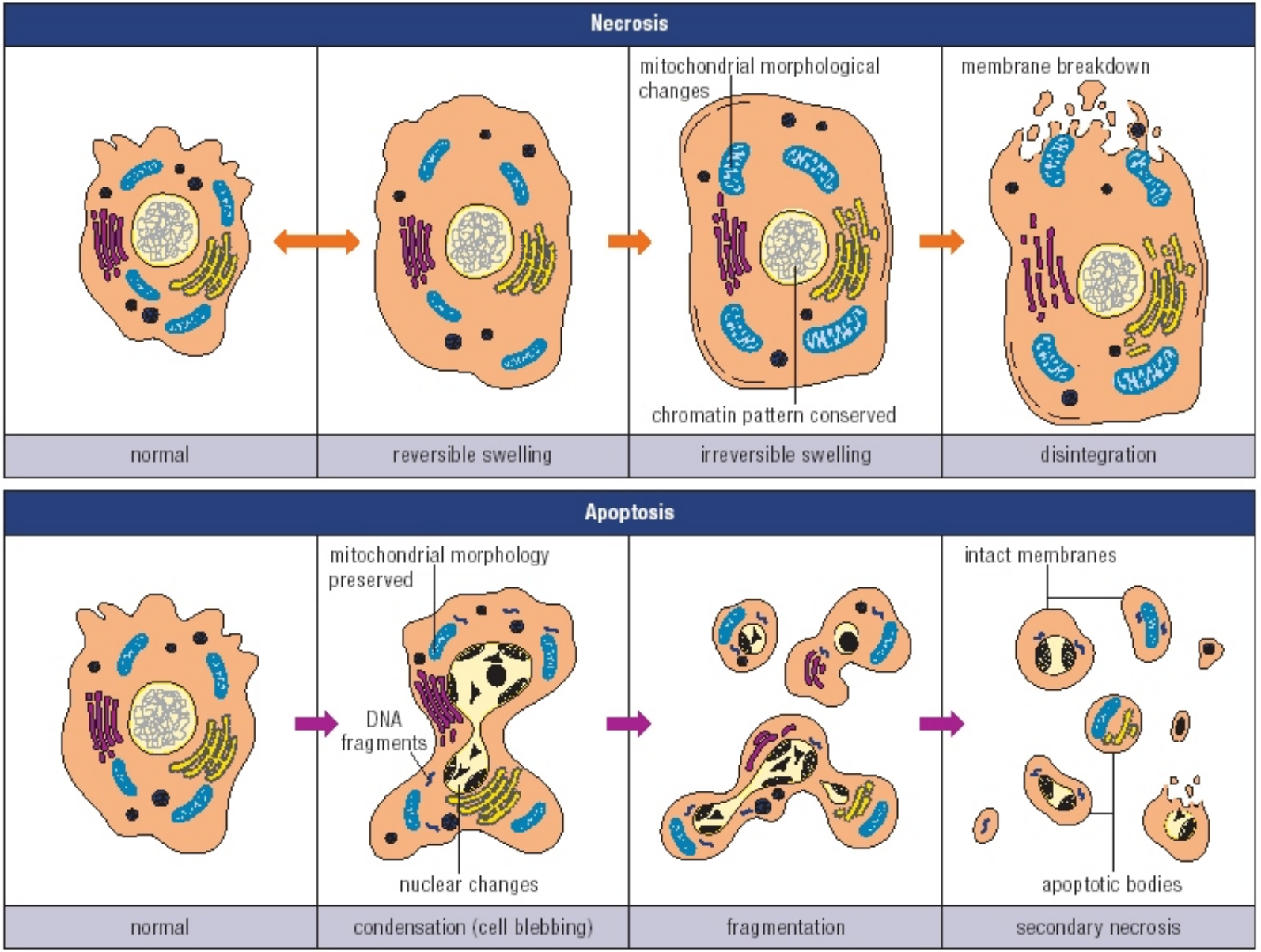
⑦ 0.5 ml of 2 M perchloric acid, a hot plate at 60℃ for 30min and allow the solution to cool.

⑧ Add 0.5ml SLS in NaOH incubate the solution at 37℃ for 30 min or overnight at room temperature.

⑨ Collect the solubilized pellet and determine the radioactivity.

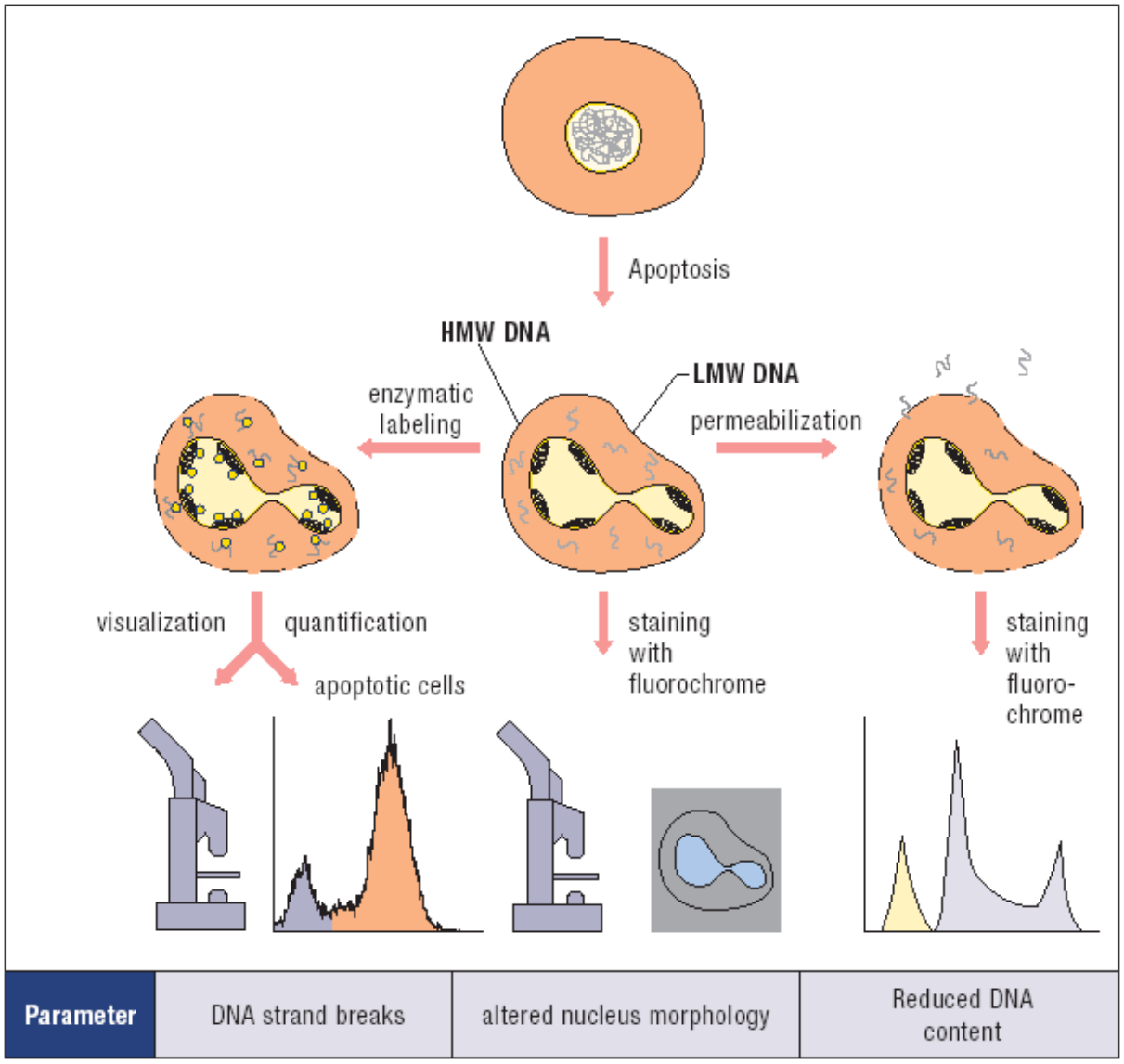
DNA labeling assay
(using fluorescent probes assay)

Two types of nonviable cells

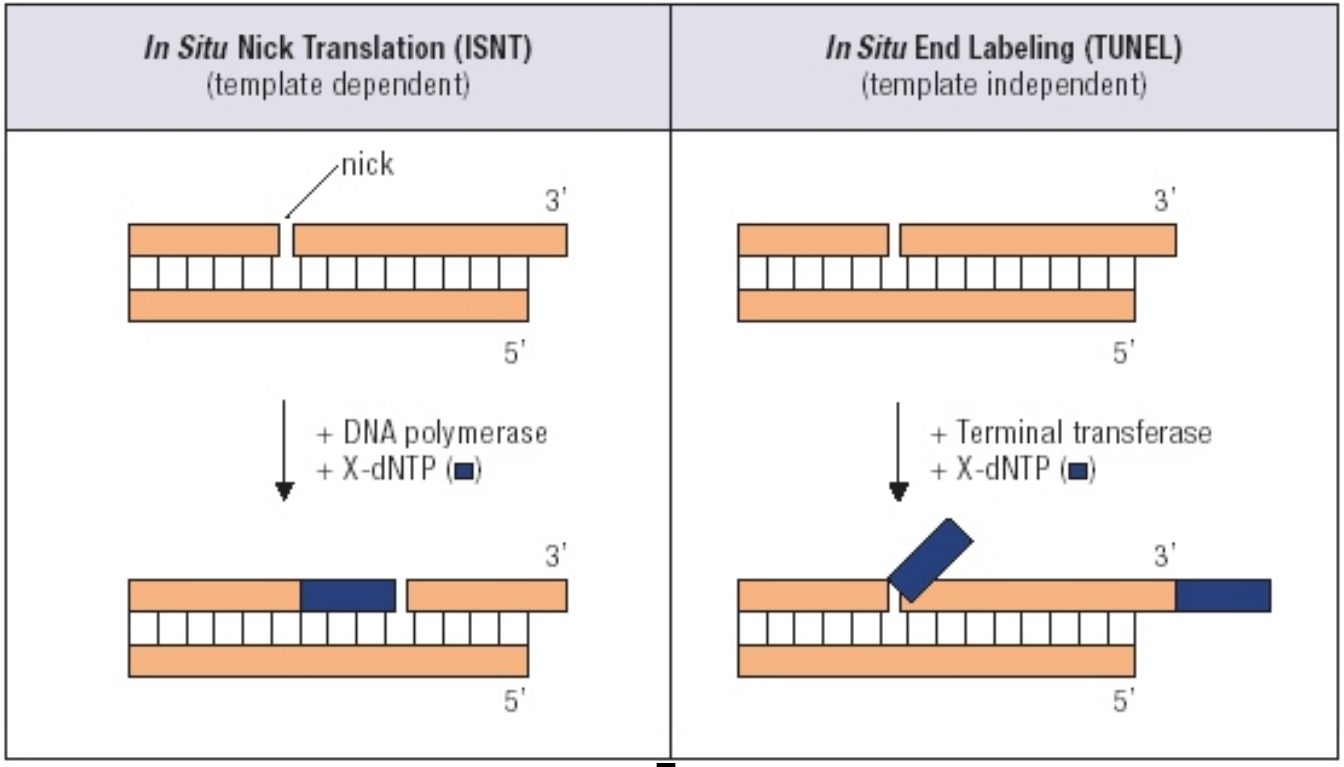


Two methods:

Enzymatic DNA labeling and DNA-binding dye labeling



I . Enzymatic DNA labeling



dNTP	dUTP		
	Direct	Indirect	
X	Fluoresein, FITC, PE etc	Biotin	DIG
		Avidin conjugated with fluoresein, AP, POD	Anti-DIG antibody conjugated with fluoresein, AP, POD

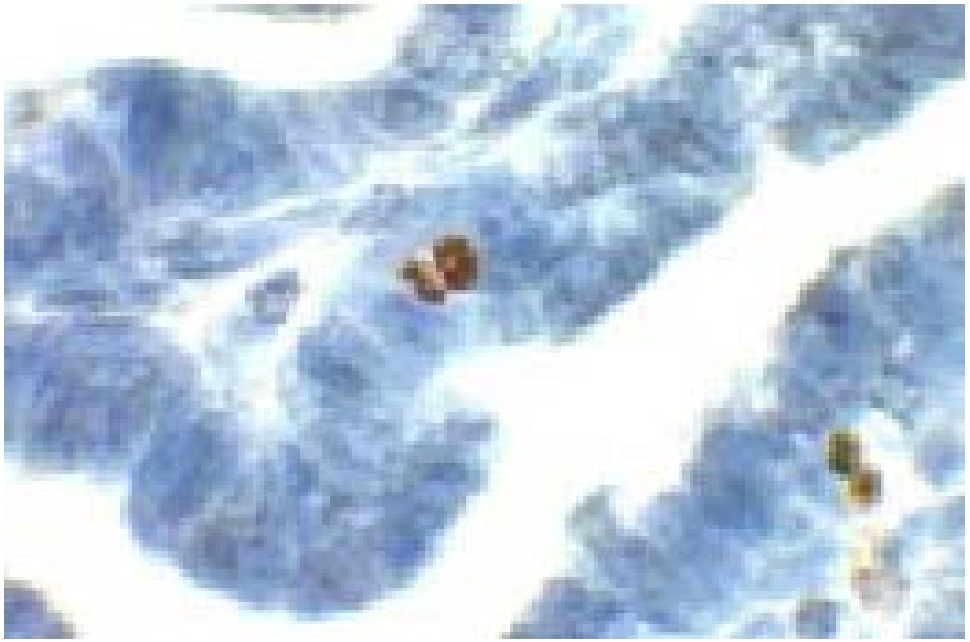


Fig. Immunostaining of apoptotic cells (dark brown) by TUNEL
Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) and peroxidase staining in rabbit endometrium

Ⅱ. DNA-binding dye labeling

DNA-binding dyes: fluorochrome

Dye	Permeability via intact membrane	Staining	
		DNA	RNA
Acridine orange	Yes	Green	Red-orange[1]
Hoechst 33342	Yes	Blue	No
Hoechst 33258	Yes	Blue	No
DAPI (4,6-diamidino-2-phenylindole)	Yes	Bright blue	No
EtBr (Ethidium bromide)	No	Orange	Slightly red[1]
PI (Propidium iodide)	No	Red	No[2]

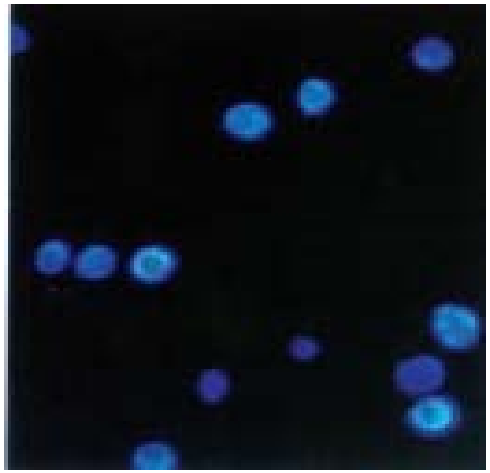
[1] RNase treatment is required

[2] RNase treatment is required because PI could stain double strand RNA

Pattern of dye staining according to color and chromatin morphology

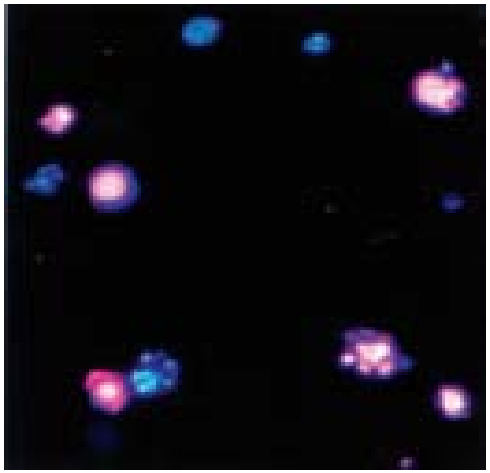
Dye	Apoptosis		Necrosis
	Early apoptosis	Late apoptosis*	
Acridine orange	Green Condensed	Green Fragmented	Green Diffuse, Intact
Hoechst 33342	Blue Condensed	Blue Fragmented	Blue Diffuse, Intact
Hoechst 33258	Blue Condensed	Blue Fragmented	Blue Diffuse, Intact
DAPI	Blue Condensed	Blue Fragmented	Blue Diffuse, Intact
Ethidium bromide	No (Orange, Condensed if permeabilized)	Orange Fragmented	Orange Diffuse, Intact
Propidium iodide	No (Red, Condensed if permeabilized)	Red Fragmented	Red Diffuse, Intact

*** late apoptosis is regarded as the stage of membrane fragmentation and secondary necrosis**



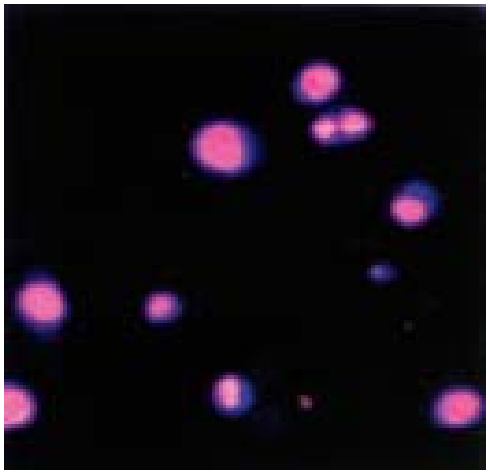
Apoptotic(0%)

Necrotic(10.5%)



Apoptotic(85.2%)

Necrotic(11.2%)



Apoptotic(1.2%)

Necrotic(92.5%)

Fig. In Hoechst 33258 / PI double staining, cells with blue intact nuclei were viable cells, whereas those with blue fragmented nuclei were early apoptotic cells. Cells with pink intact nuclei were necrotic cells, whereas cells with pink fragmented nuclei were late apoptotic cells. (blue against Hoechst33258, red against PI)

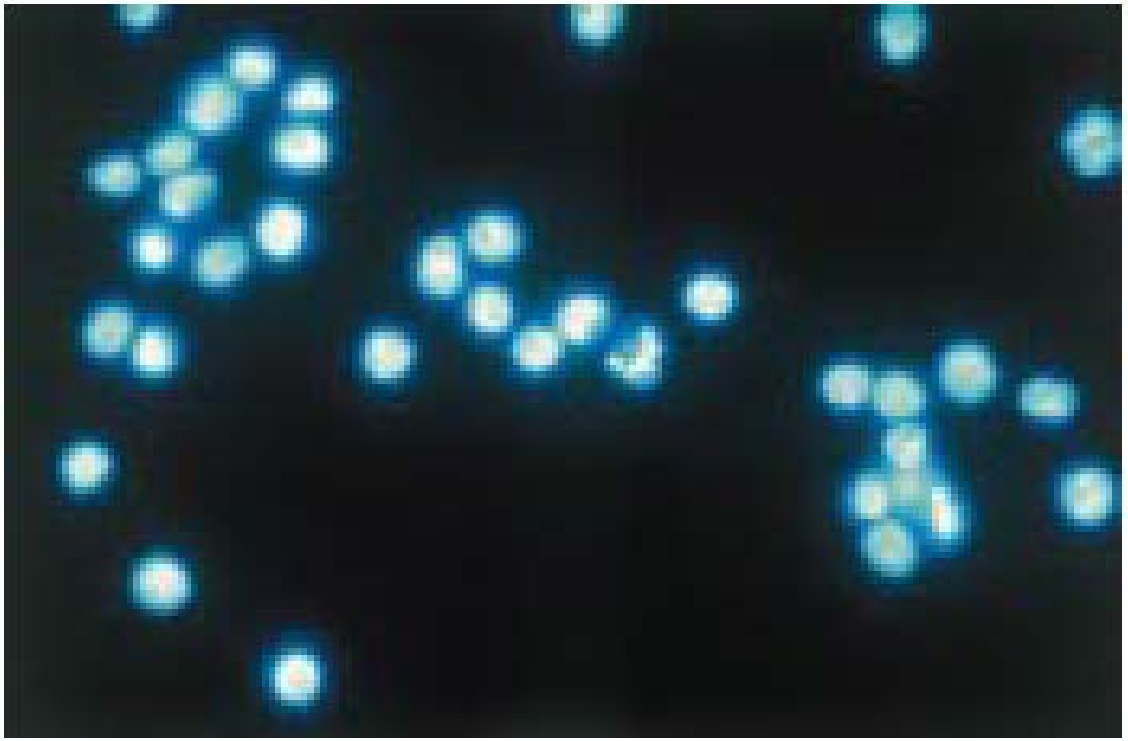
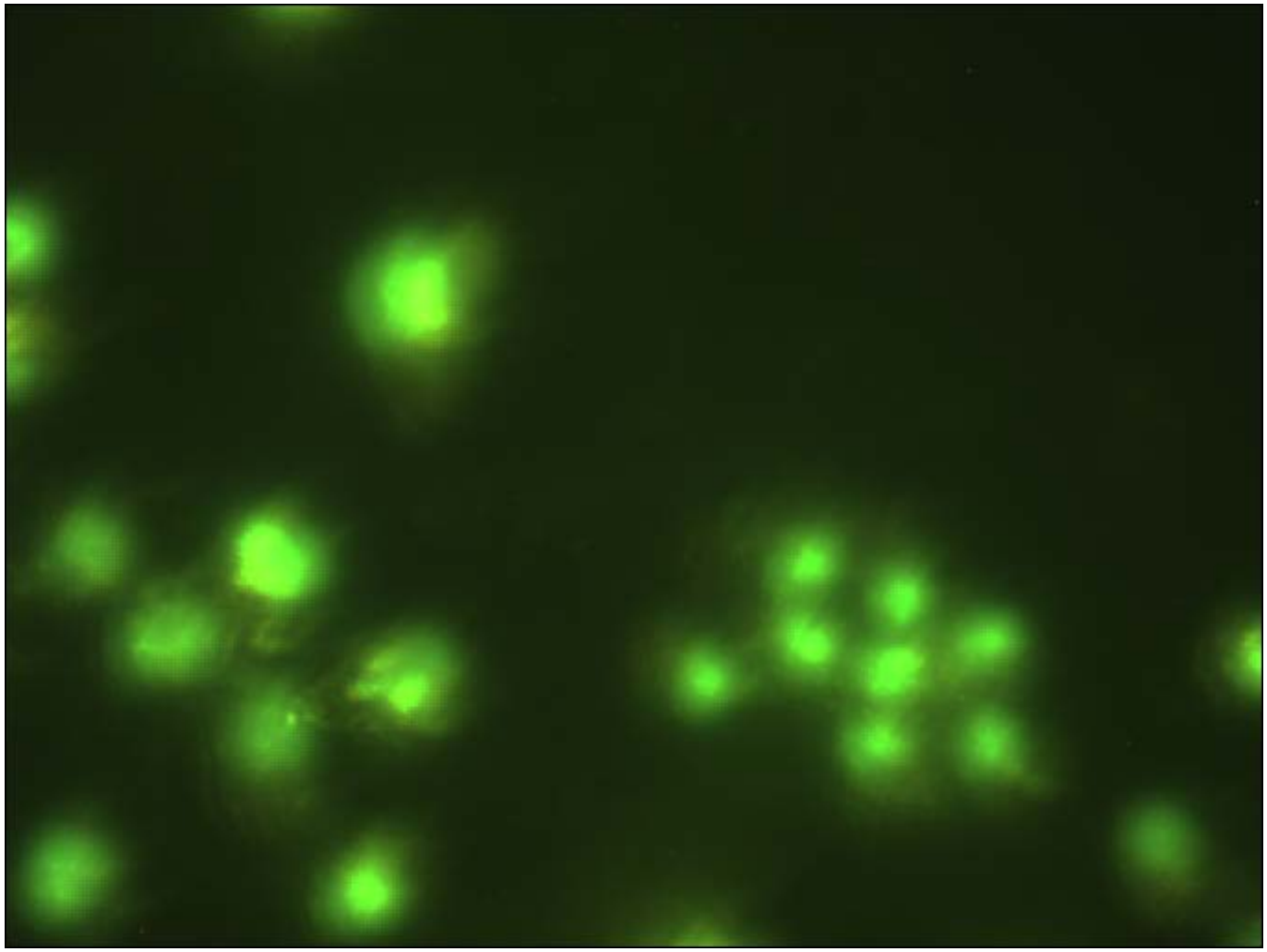


Fig. DAPI staining of condensed nuclei of apoptotic cells

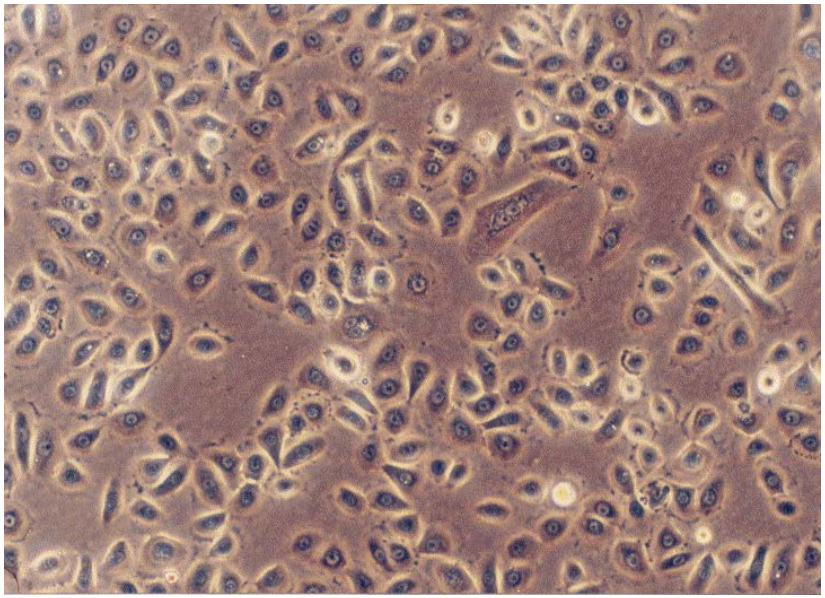
Acridine orange



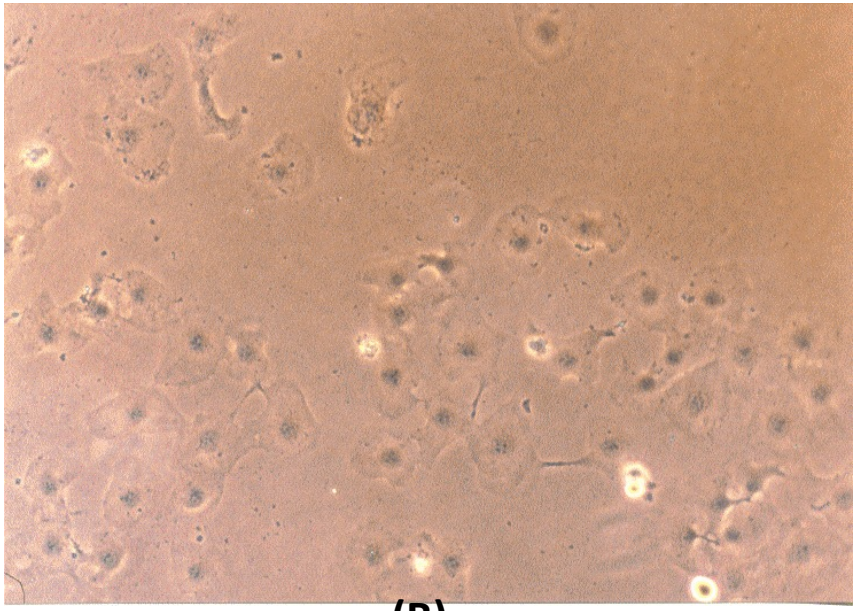
Morphological assay

- Large-scale, morphological changes that occur at the cell surface, or in the cytoskeleton, can be followed and related to cell viability.
- Damage can be identified by large decreases in volume secondary to losses in protein and intracellular ions of due to altered permeability to sodium or potassium.
- Necrotic cells: nuclear swelling, chromatin flocculation, loss of nuclear basophilia
- Apoptotic cells: cell shrinkage, nuclear condansation, nuclear fragmentation

Example; Morphological feature (Human skin keratinocyte)



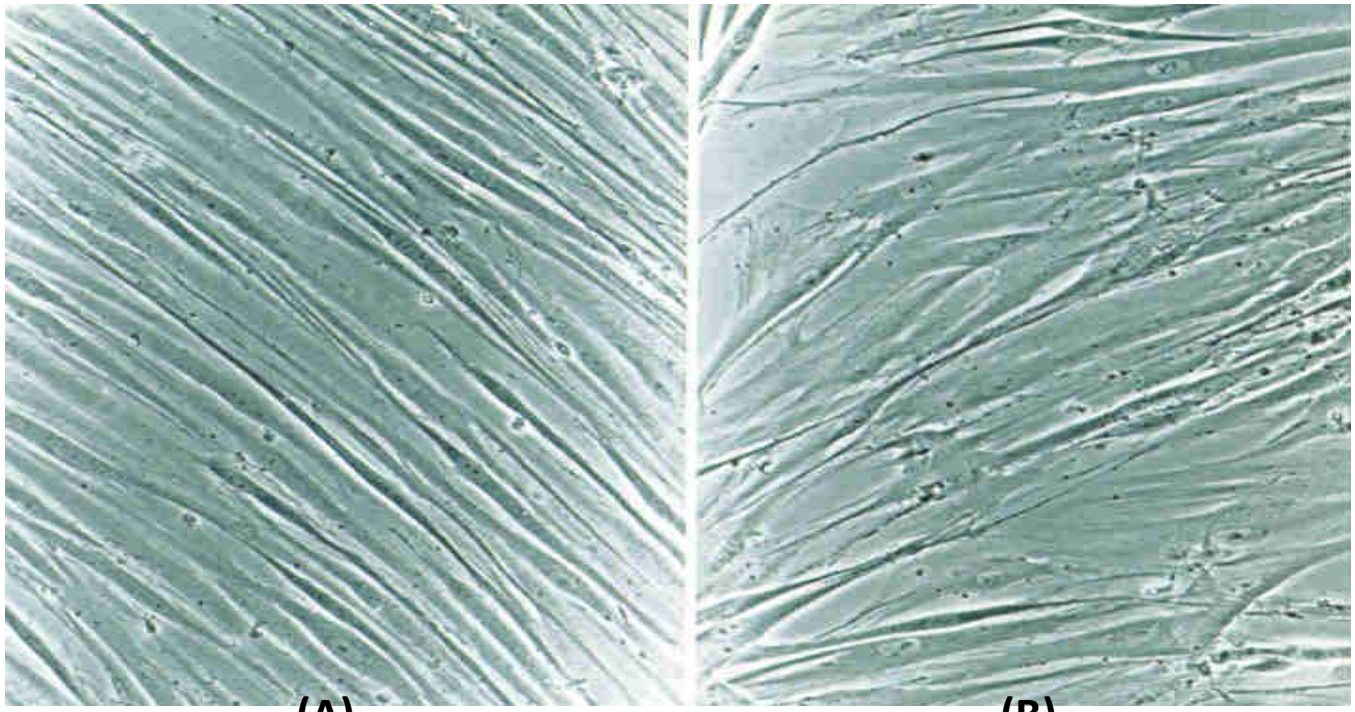
(A)



(B)

Fig. Morphological feature of (A) normal human skin keratinocyte, and differentiated human skin keratinocyte(B).

Example; Morphological feature (Human skin fibroblasts)



(A) **(B)**

Fig. Morphological feature of (A) normal human skin fibroblasts, and aging human skin fibroblasts(B).

Reproductive Assay

Colony-forming Efficiency

Clonogenic Cell:

- Defined as a cell with the capacity for sustained proliferation
- Have undergone a minimum of 5-6 doublings to give rise to colonies containing at least 50 cells

Colony-forming Efficiency (CFE)

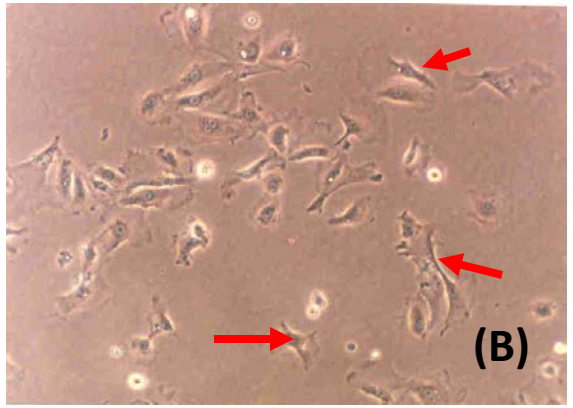
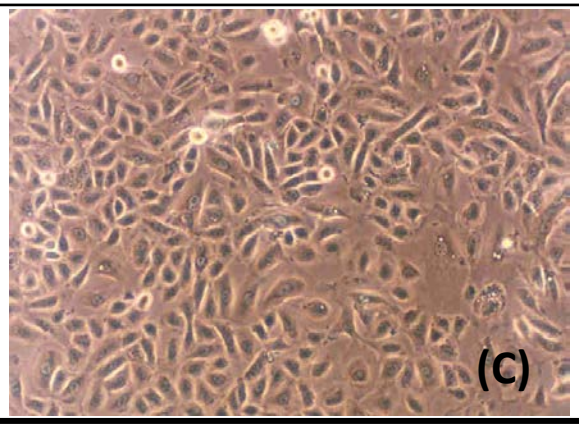
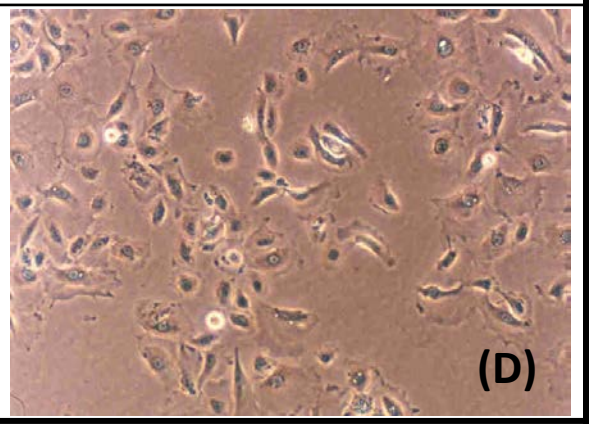
- The ability to form colonies is used as a measure of reproductive integrity
- It is often referred to as plating efficiency (PE)

$$CFE = \frac{Number\ of\ colonies\ formed}{Number\ of\ cell\ plates} \times 100\%$$

Clonogenic assays

- Be used to reflect stem cell content
- The basis of assays for determining the lethal effects of cytotoxic agents

Example; Rat keratinocytes

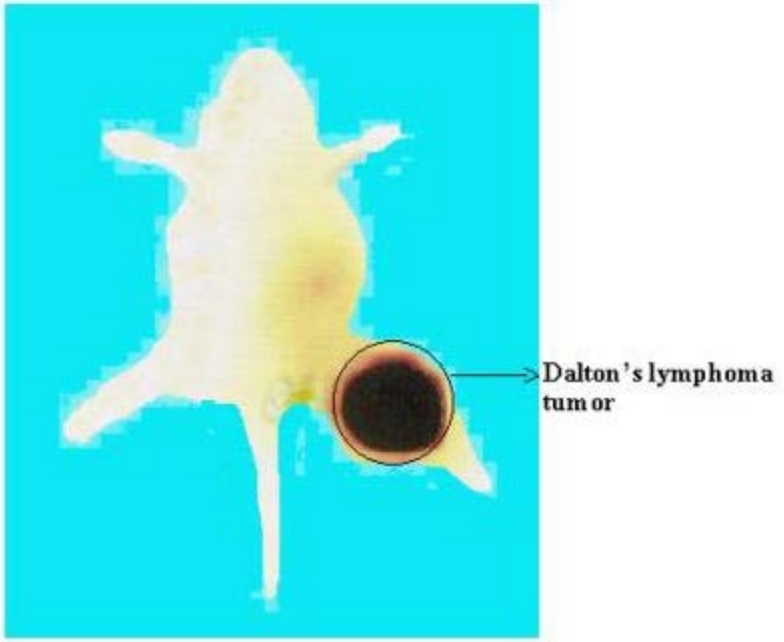
	Colony forming	*Non-colony forming*
48 hr after subculture		
6 days after subculture		

: colony , : Single cells

In-vivo models

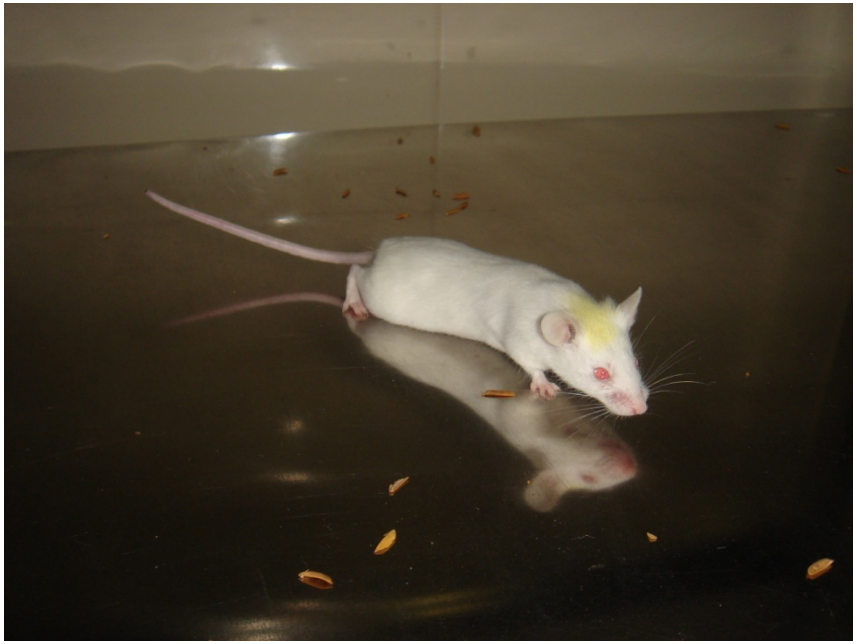
Models of transplantable tumour

- Murine solid tumour models with Daltons lymphomatic cells

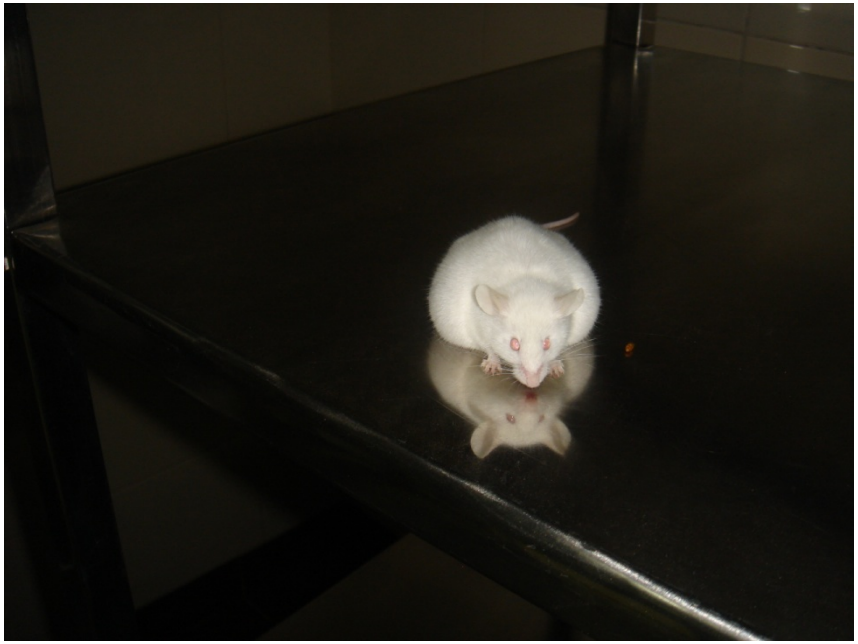


Ascites tumor model

Normal mouse



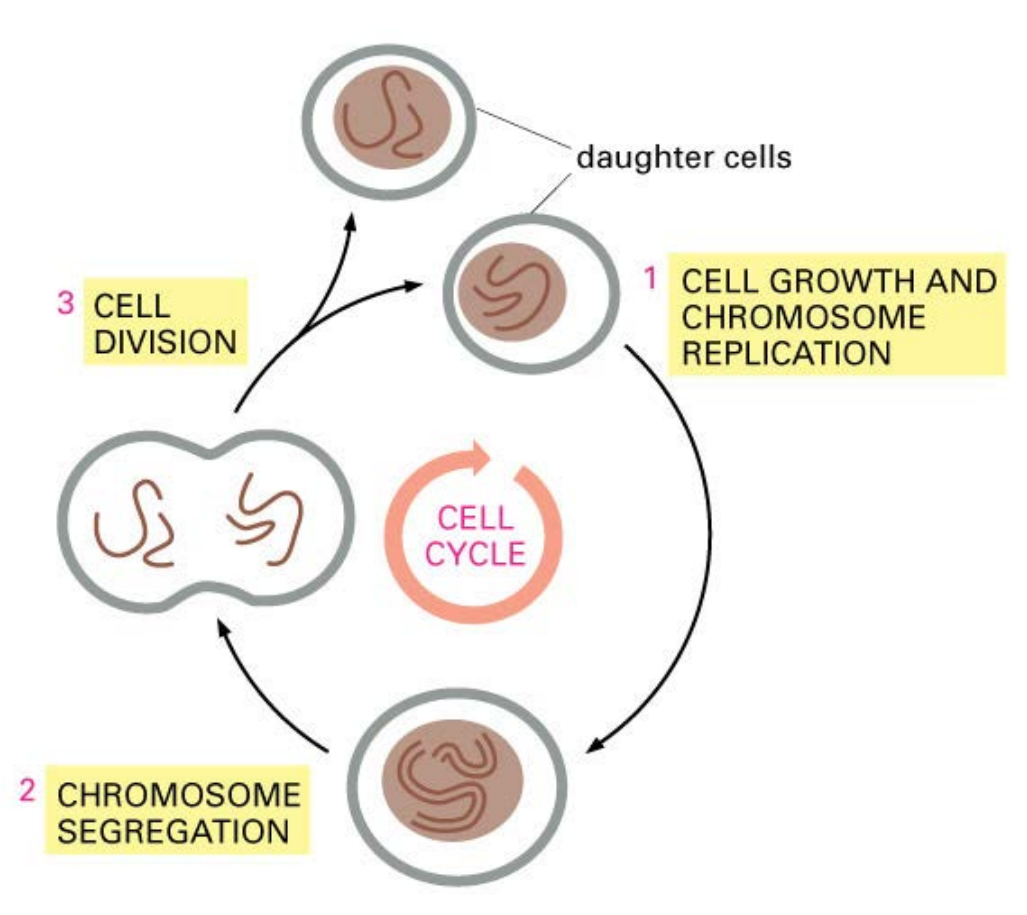
Tumor bearing mouse



Cell Cycle

Ghassan Mohammad Sulaiman , Ph.D.

Assist. Prof.
School of biotechnology
Department of applied science
University of Technology


daughter cells
1 CELL GROWTH AND CHROMOSOME REPLICATION
2 CHROMOSOME SEGREGATION
3 CELL DIVISION
CELL CYCLE

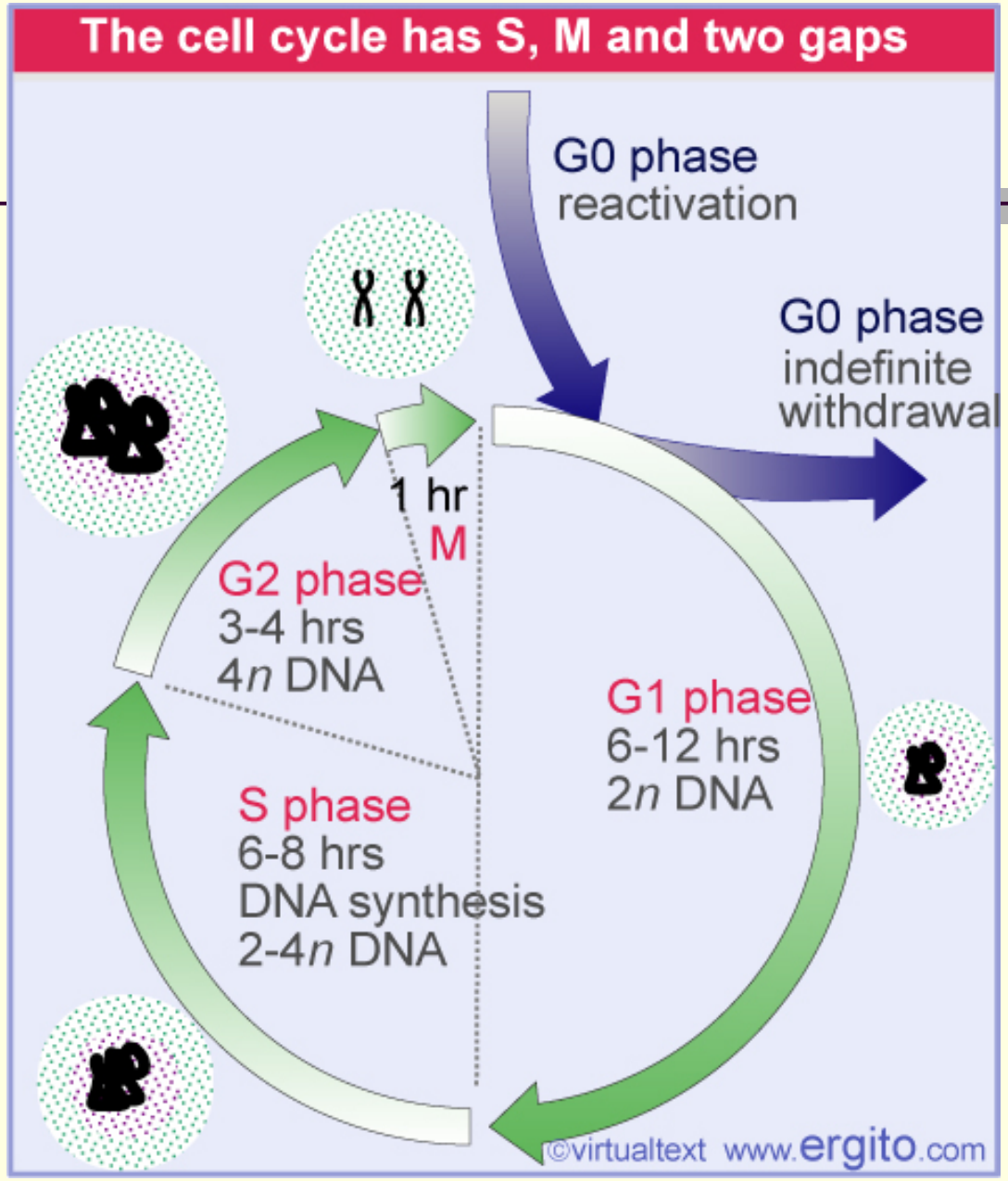
Cell Cycle

The eukaryotic cell cycle has 5 main phases:

1. G_1 (gap phase 1)
2. S (synthesis)
3. G_2 (gap phase 2)
4. M (mitosis)
5. C (cytokinesis)

} interphase

The length of a complete cell cycle varies greatly among cell types.


The cell cycle has S, M and two gaps
G0 phase
reactivation
G0 phase
indefinite
withdrawal
1 hr
M
G2 phase
3-4 hrs
4n DNA
G1 phase
6-12 hrs
2n DNA
S phase
6-8 hrs
DNA synthesis
2-4n DNA
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Interphase

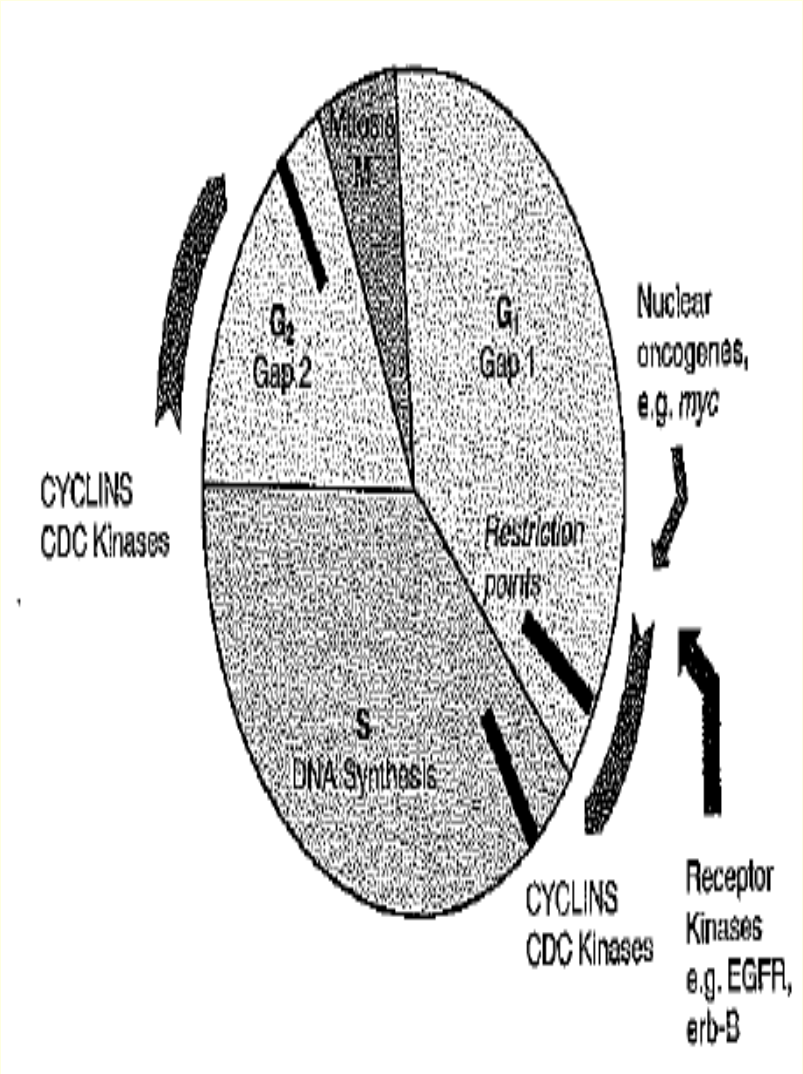
Interphase is composed of:

G_1 (gap phase 1) – time of cell growth

S phase – synthesis of DNA (DNA replication)

- 2 sister chromatids are produced

G_2 (gap phase 2) – chromosomes condense



G1 phase – major portion of the cell cycle period, is the gap between cytokinesis and DNA synthesis.

S phase- replica of genome is synthesized

G2 phase – Prepares cell for mitosis, fills gap between DNA synthesis and beginning of mitosis, mitochondria and other organelles replicate, chromosomes begin to condense and microtubules begin to assemble at spindle

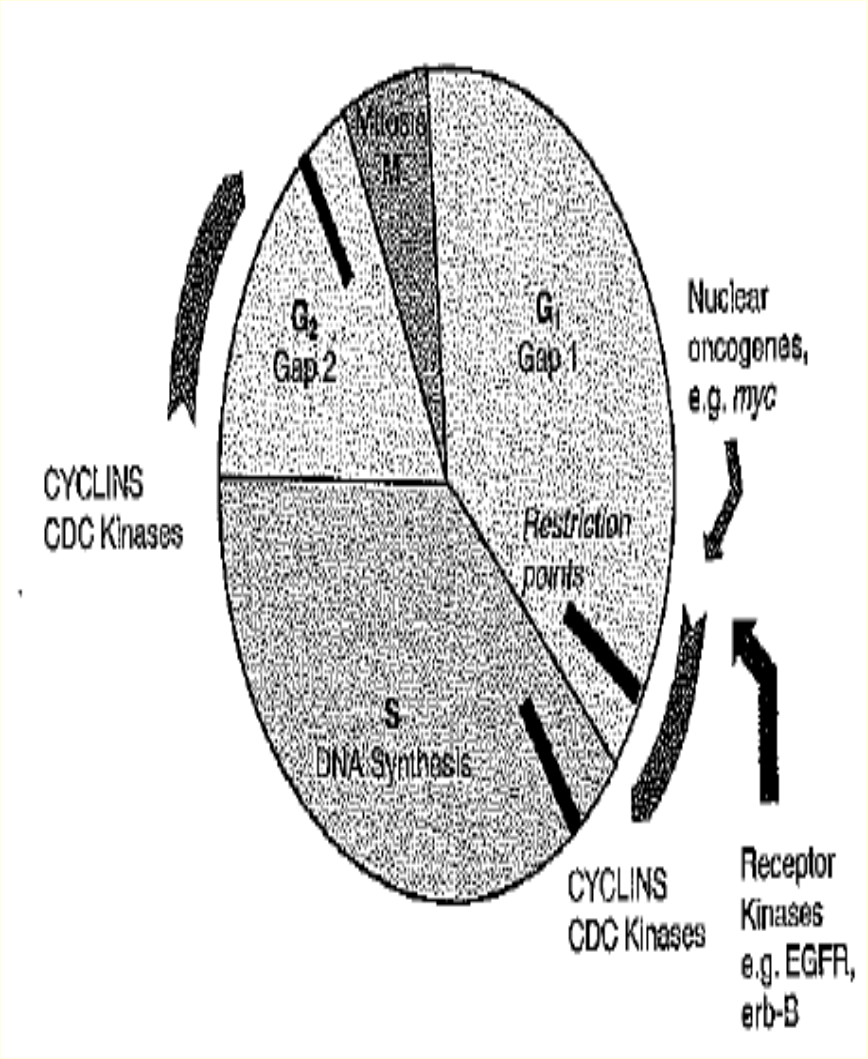
G0 phase-Cells often pause in G1 before DNA replication and enter a resting stage G0. ex: muscle and nerve cells remain there permanently others such as liver cells can resume G1 phase in response to factors release during injury.

Control of the cell cycle

The cell cycle is controlled at three checkpoints:

1. G_1/S checkpoint
   -the cell "decides" to divide

2. S/G2 checkpoint
   - the cell "decides" DNA repair or apoptosis



G1/S checkpoint –acts by deciding whether the cell should divide or not based on the availability of growth factors, the nutritional condition of the cell (whether starving or not). Once a cell has crossed this checkpoint then it has made a commitment to divide and any damage to DNA can result in halting cell cycle at this point.

G2/M checkpoint- The Cdks identified at this point are known as M phase-promoting factor. Passing this phase means the cell has made commitment to mitosis based on proper DNA replication. Without successful DNA replication the cycle can stop at this point.

Activation and inactivation at each checkpoint happens because of phosphorylation (but for activation cdk should bind to appropriate cyclins)

Spindle checkpoint – Arrangement of chromosomes on the metaphase.

G1 checkpoints

Rb (Retinoblastoma) prevents cell moving into S phase by binding to a transcription factor

When Rb is phoshporylated it cannot bind so cell can move into S phase

p53 (tumor suppressor protein) prevents damaged from dividing (by inhibiting Rb pathway)

Abnormalities in both genes are associated with hereditary forms of cancer

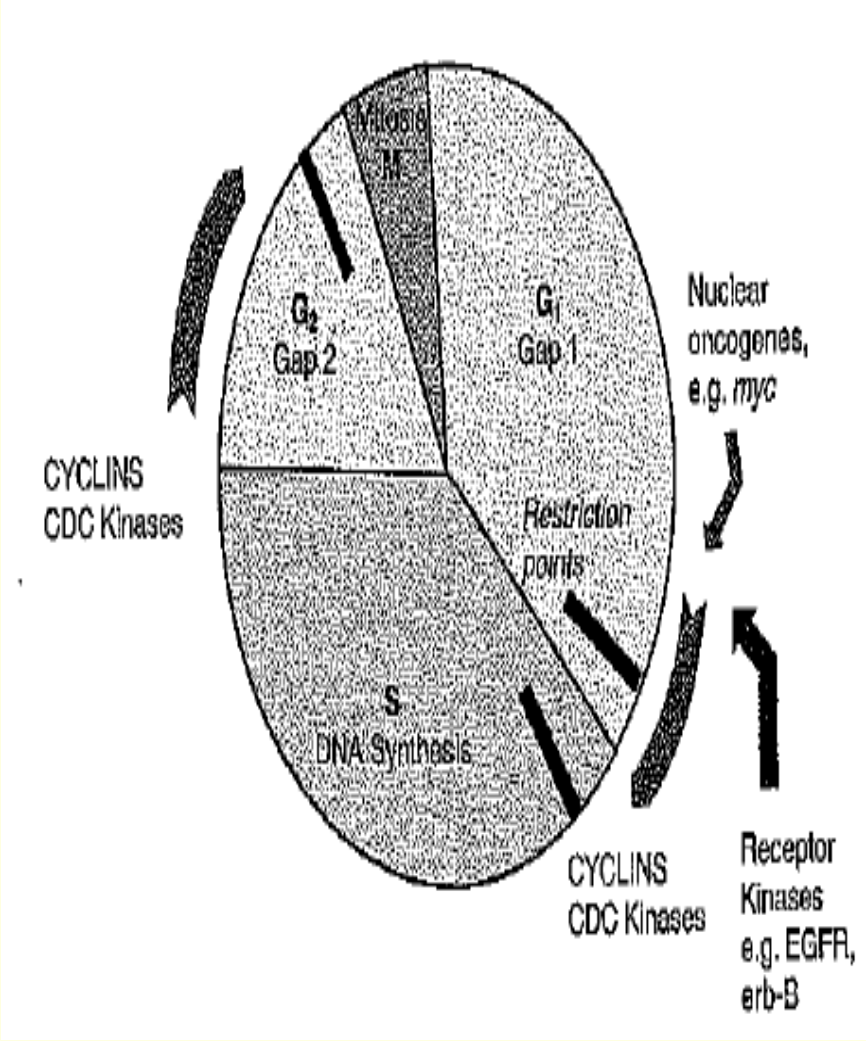
Control of the cell cycle

3. G_2/M checkpoint

   -the cell makes a commitment to mitosis

4. late metaphase (spindle) checkpoint

   -the cell ensures that all chromosomes are attached to the spindle



Enzymes Drive the Cell Cycle

- The cell cycle is driven by proteins called ***C***yclin-***d***ependent ***k***inases, or **Cdk's**

- Cdk's are active only when they bind to other proteins called **cyclins**

Quiescent cell-not going through cell cycle/division

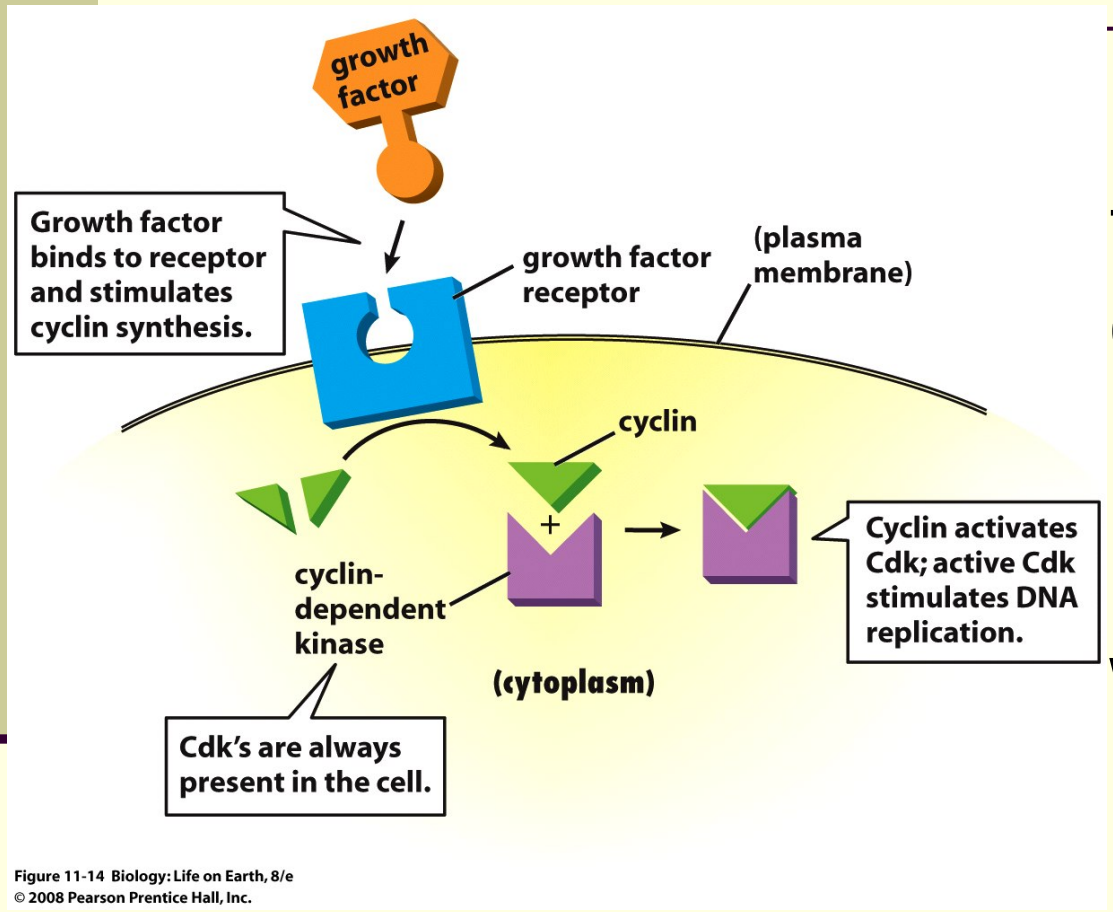


Figure 11-14 Biology: Life on Earth, 8/e
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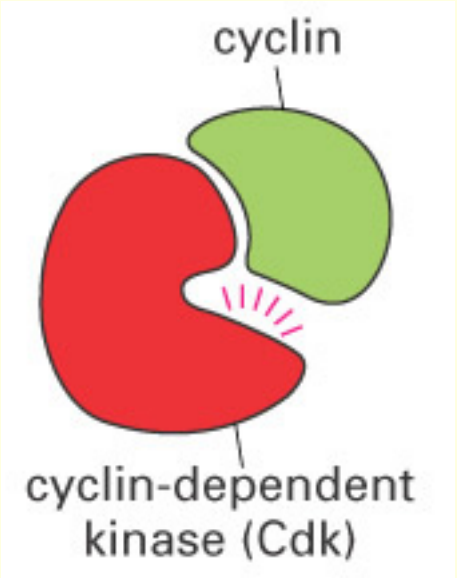
The cell cycle is driven by these **Cdk proteins**

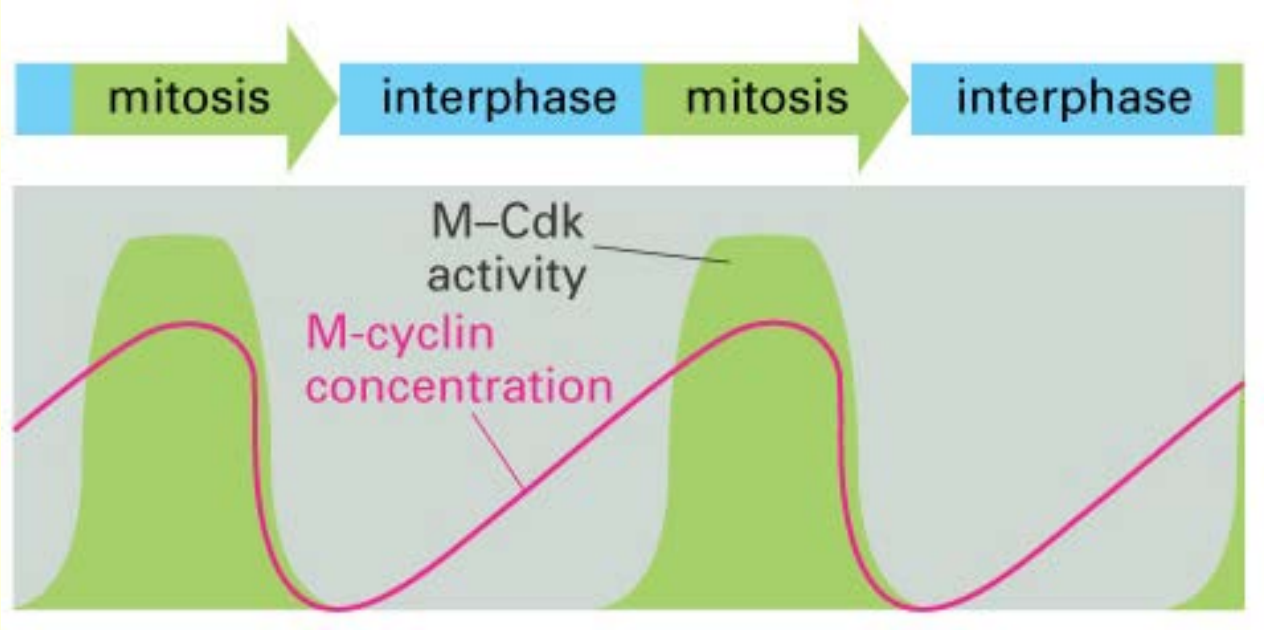
Which stimulate DNA replication

Cyclin-dependent protein kinases drive progression through the cell cycle

- Cyclin-dependent kinases (Cdks) are inactive unless bound to cyclins
- Active complex phosphorylates downstream targets
- Cyclin helps to direct Cdks to the target proteins

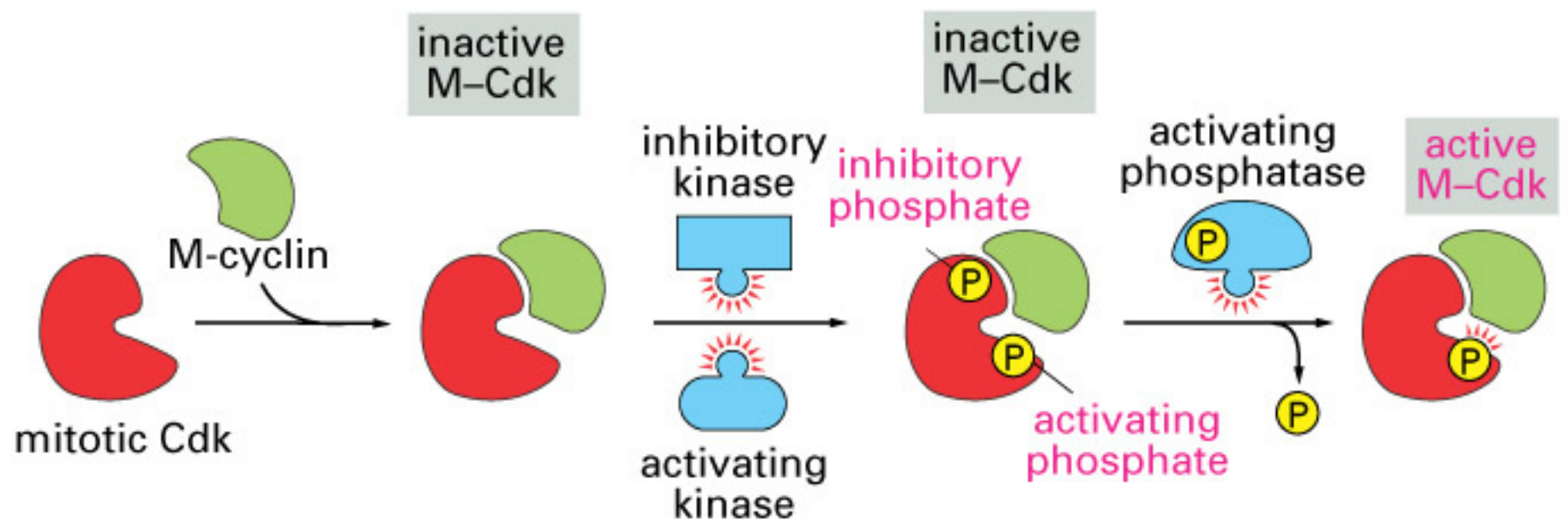


Cellular levels of (mitotic) M-cyclin rises and falls during the cell cycle

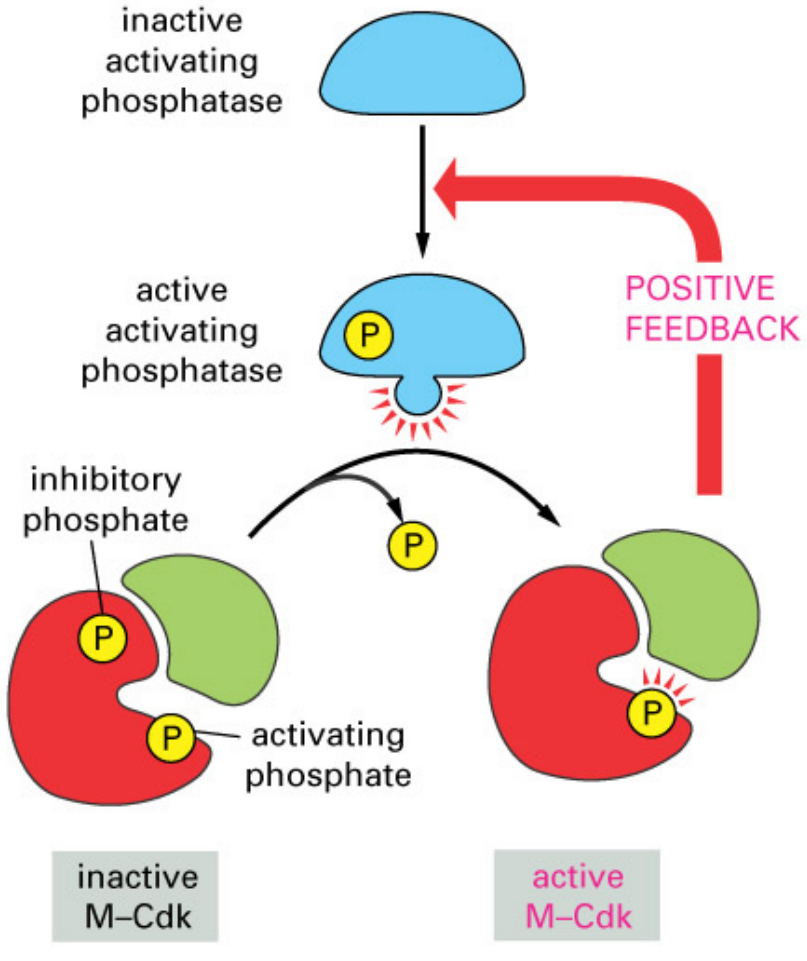


- M-cyclin levels are low during interphase but gradually increases to a peak level during mitosis
- M-cdk activity is, likewise, low in interphase but increases in mitosis

Cdks are also regulated by cycles of phosphorylation and dephosphorylation

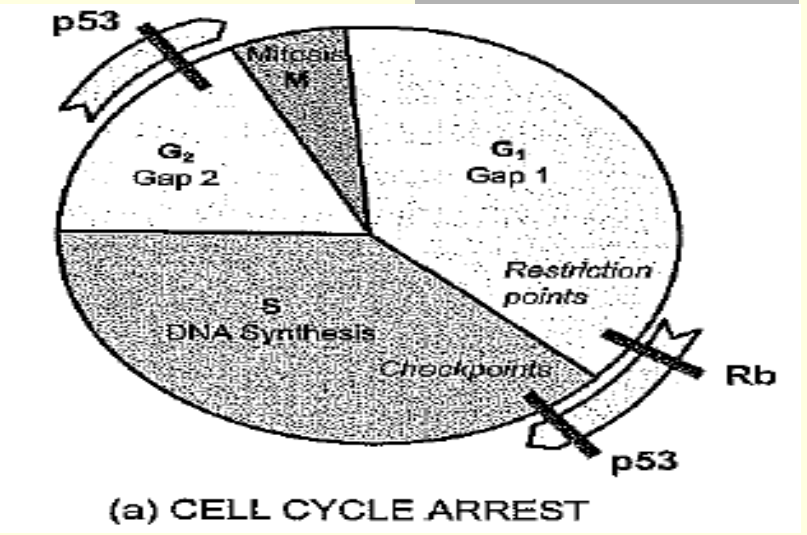


Cdk activates itself indirectly via a positive feedback loop

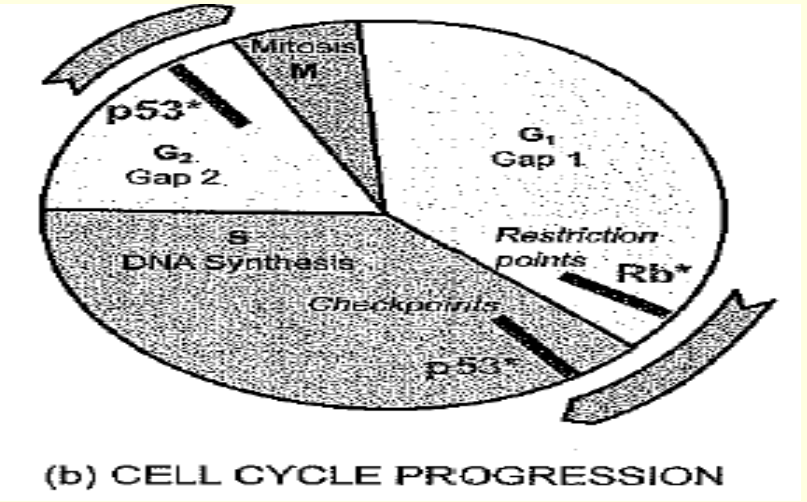


How cell cycle inhibitors control cell proliferation?

- Rb gene product, p53, and p16
- Block cell cycle progression/arrest
- Damage leads to cancerous cells
- Inactivation by phosphorylation-cell cycle progression



(a) CELL CYCLE ARREST



(b) CELL CYCLE PROGRESSION

Which Protein Is Activated by Cdk-Cyclin Complex?

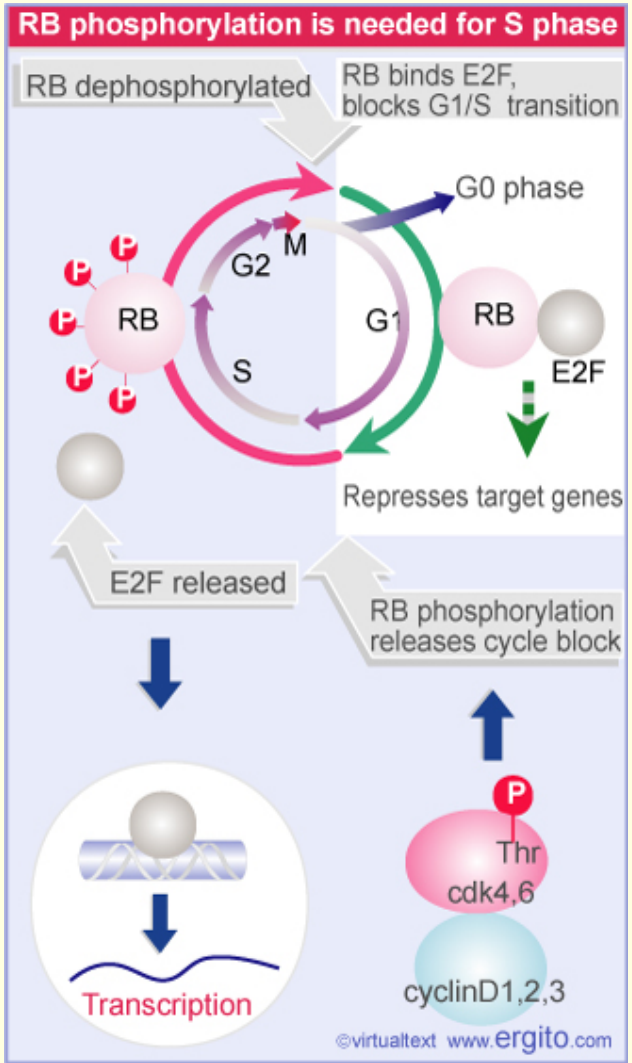
RB:

- Is a target of cdk-G1 cyclin (D) complexes
- Is a tumor suppressor

RB-E2F → **transcription inhibited**

RB-p E2F → **transcription activated**

Cdk-cyclin complex phosphorylates RB



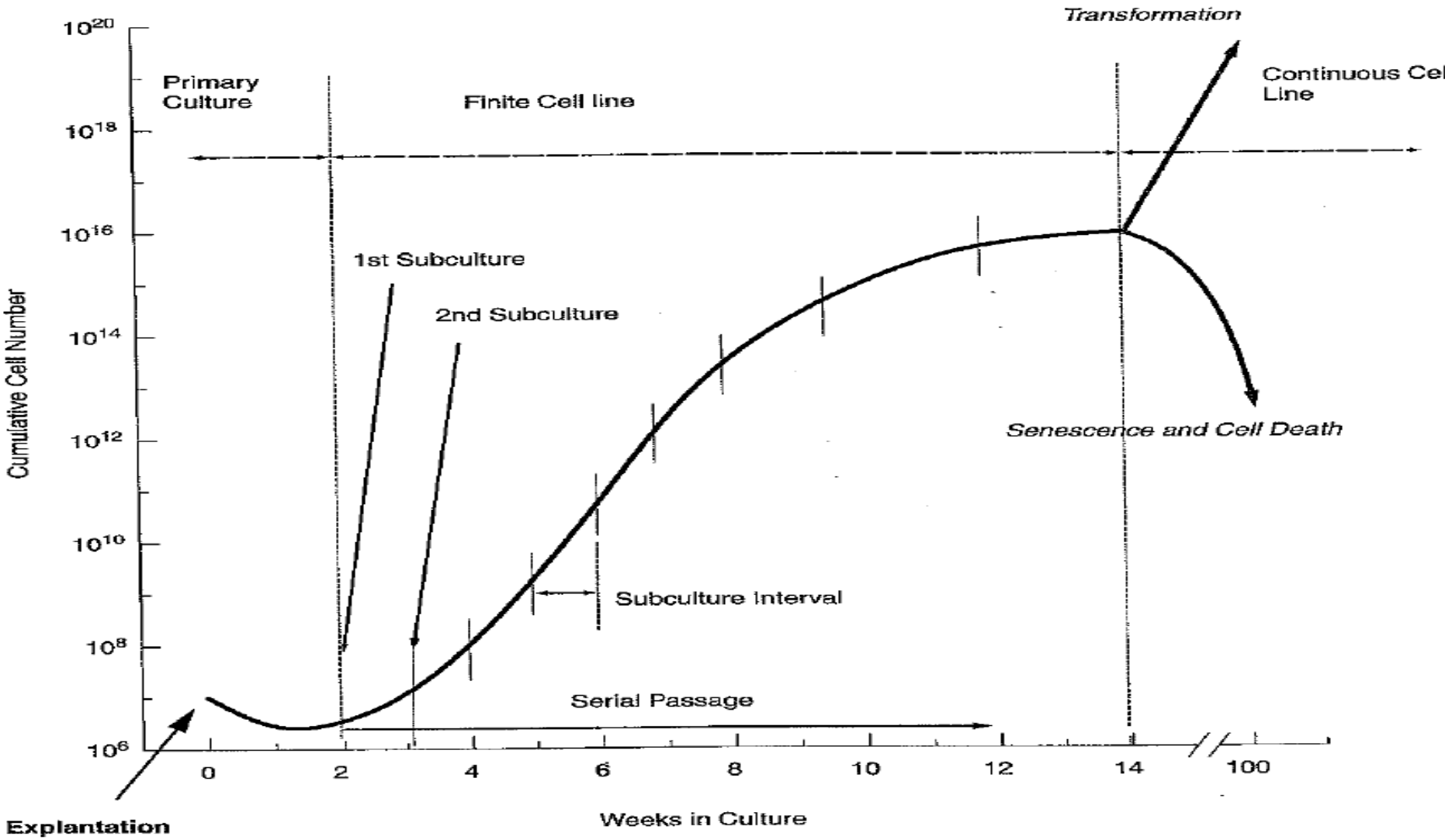


Fig. 3.8. Evolution of a Cell Line. The vertical (*Y*) axis represents total cell growth (assuming no reduction at passage) for a hypothetical cell culture. Total cell number (cell yield) is represented on this axis on a log scale, and the time in culture is shown on the *X*–axis on a linear scale. Although a continuous cell line is depicted as arising at 14 weeks, with different cells it could arise at any time. Likewise, senescence may occur at any time, but for human diploid fibroblasts it is most likely to occur between 30 and 60 cell doublings, or 10 to 20 weeks, depending on the doubling time. Terms and definitions used are as in the glossary. (After Hayflick and Moorhead [1961].)

What is Senescence? Why and how does it happen?

- Normal cell lines will die after fixed number of population doublings – **Senescence**
- **Inability of terminal sequences of DNA in telomeres to replicate at each cell division**
- **Progressive shortening of telomeres – cell cannot divide**

Why Senescence does not take place in all cells?

- **Germ cells, stem cells, transformed cells, tumor lines** etc
- Express enzyme **telomerase** which is capable of **replicating** the terminal sequences of DNA in telomeres
- **Extends life span of cells**

What are Continuous Cell Lines?

- Ability of cell lines to grow indefinitely *in vitro*
- Alteration of a culture – **TRANSFORMATION and** giving rise to continuous cell line – **IMMORTALIZATION**
- Shows capacity for genetic variation/instability
- **p53 is mutated or deleted (if tumor cell line)**

Transformation and Immortalization

Ghassan Mohammad Sulaiman , Ph.D.

Assist. Prof.
School of biotechnology
Department of applied science
University of Technology

Role in cell line characterization/How will you characterize a cell line?

- **Transformation** is an event or series of events – depends on and promotes **genetic instability**
- Alters cell line properties – **growth rate, mode of growth, specialized product formation, longevity and tumorigenicity**
- Whether culturing cells from **neoplastic cells** and not normal blood vessel cells or inflammatory cells.

What is Transformation?

- Implies a **spontaneous or induced permanent phenotypic change** resulting from a heritable change in **DNA and gene expression**

- Artificial introduction of DNA or DNA transfer into mammalian cells – **Transfection**

- **Transformation – result of infection with virus or transfection with mutant *ras* genes or spontaneous exposure to ionizing radiation or chemical carcinogens**

What are the three major classes of phenotypic change?

- **Immortalization** – the acquisition of an infinite life span
- **Aberrant growth control** – the loss of contact inhibition of cell motility, density, limitation of cell proliferation and anchorage dependence
- **Malignancy** – Growth of invasive tumors *in vivo*

Genetic Instability

- Human finite cell lines are stable
- Mouse cell lines are genetically unstable and transform easily
- Continuous cell lines (tumorigenic) of all species are unstable
- **Two causes of genetic heterogeneity**
  - Spontaneous mutation is higher in vitro
  - Mutant cells cannot be eliminated without impairment in their growth capacity

Chromosomal Aberrations

- Genetic rearrangement – seen in **chromosome counts and karyotype analysis**
- Also determined by **sister chromatid exchange assay**
- Variations in **ploidy**, frequency of individual chromosomal **aberrations** and variations in chromosome **number** – tumorigenic cell lines

Immortalization

- Rodent cells are karyotypically normal at isolation and undergo changes after **12 th generation**
- If maintained at a low cell density and **not allowed to remain at confluence for any length of time**, they remain sensitive to contact inhibition and density limitation of growth
- If allowed to **remain at confluence for extended periods** – reduced contact inhibition- cells pile up – overgrowth- will be seen in subsequent subcultures - tumorigenic

Control of Senescence

- Senescence genes **in finite cells** negatively control cell cycle progression
- Regulates expression of telomerase – **synthesizes telomeric DNA**
- **Deletions and/or mutations** within senescence genes or **overexpression or mutation** of one or more oncogenes allows **reexpression of telomerase**

Control of Senescence

- Immortalization involves both inactivation of **senescence** and cell cycle **regulatory genes such as Rb and p53**
- Product of **SV40 LT gene** – T antigen binds to Rb and p53
- Allows **extended proliferative life span and restricts DNA surveillance activity of genes**
- Increases **genomic instability**
- Increases **chances of mutations**

Control of Senescence

- Immortalization – does not imply development of **aberrant growth control and malignancy**

- **Retains contact inhibition of cell motility, density limitation of cell proliferation and anchorage dependence and are non-tumorigenic**

- Some aspects of growth control are **abnormal** and likely increase of genomic instability

Immortalization with Viral genes

- **SV 40 virus** – immortalize cells with their large T **(LT) gene**
- Adenovirus E 1a, human papilloma virus (HPV) E6 and E7 and Epstein-Barr virus (EBV)
- Inhibits activity of genes such as CIP-1/WAF-1/p21, Rb, p53 and p16

Immortalization with Viral genes

- Mammalian cells transfected or retrovirally infected with **immortalizing gene** before they **enter senescence**

- Extends proliferative life span for **20-30 population doublings**
- Cells cease proliferation and enter **crisis**

- Up to **several months** in crisis **– subset of immortal cells overgrows**

- **Fraction** of immortal cells obtained

Telomerase-Induced Immortalization

- **Telomerase or terminal transferase –** composed of two subunits **– RNA component (hTR)** and a **protein catalytic subunit (hTERT**)
- hTR is expressed in normal and malignant tissues
- hTERT is expressed in tumors, germ cell lines and activated lymphocytes
- **Transfecting cells with hTERT extends life span of cell line**

- Some cells become immortal but not malignantly transformed

Aberrant growth control

- Tumorigenic cell lines exhibit
- **Lower serum or growth factor dependence**
- Form **clones** with higher efficiency
- Acquire **autonomous growth** control
- **Autocrine growth** control – secrete mitogens or receptors

Aberrant growth control by anchorage independence

- Transformation due to cell surface modifications in **cell surface glycoproteins and adhesion molecules**
- Leads to loss of cell-cell recognition – to disorganized growth pattern, loss of contact inhibition of cell motility and density limitation of cell proliferation
- In vitro exp. – **stirred suspension culture or semisolid media** as agar or methocel

Aberrant growth control by serum dependence

- Lower serum dependence due to **secretion of growth factors** by tumor cells
- Allows them to enter into **mitotic phases**
- Cause nontransformed cells to adopt **transformed phenotype and grow in suspension**
- Produce **interleukins 1,2 and 3** along with **colony stimulating factors (CSF)**
- **Hormones** released

Aberrant growth control by Oncogenes

- Overexpression of **oncogenes**
- Modified receptors **erb-B2 oncogene** product, modified **G protein** and overexpression of genes regulating stages **in signal transduction (*src* kinase) or transcriptional control (*myc*, *fos* and *jun*)**
- It is permanently active and cannot be easily regulated

Tumorigenicity by malignancy

- Transformation culminates into **neoplastic cells**
- **Malignant tumors** – increased growth rate, reduced anchorage dependence, more pronounced aneuploidy and immortalization
- All cell lineages present **within a tumor need not have same transformed properties**
- **Same properties cannot be expressed in every tumor**

Tumorigenicity by malignancy

- Cells have developed the capacity to generate invasive tumors if implanted **in vivo into an isologous host or if transplanted as a xenograft** into an immune-deprived animal.

Tumorigenicity by angiogenesis

- Tumor cells release factors – **VEGF, EGF-2 and angiogenin – capable of neovascularization**

- Tumor cells produce proteolytic enzymes – **plasminogen activator** – helps the tumor cells in not attaching to any surface – **help them invading other cells**

Stem Cells

Importance of Stem Cell Research

"Science has presented us with a hope called stem-cell research, which may provide our scientists with answers that have so long been beyond our grasp."

Nancy Reagan

"If the potential of stem cell research is realized, it would mean an end to the suffering of millions of people. If stem cell research succeeds, there isn't a person in the country who won't benefit, or know somebody who will."

Michael J. Fox

Stem Cell History

1998 - Researchers first extract stem cells from human embryos

1999 - First Successful human transplant of insulin-making cells from cadavers

2001 - President Bush restricts federal funding for embryonic stem-cell research

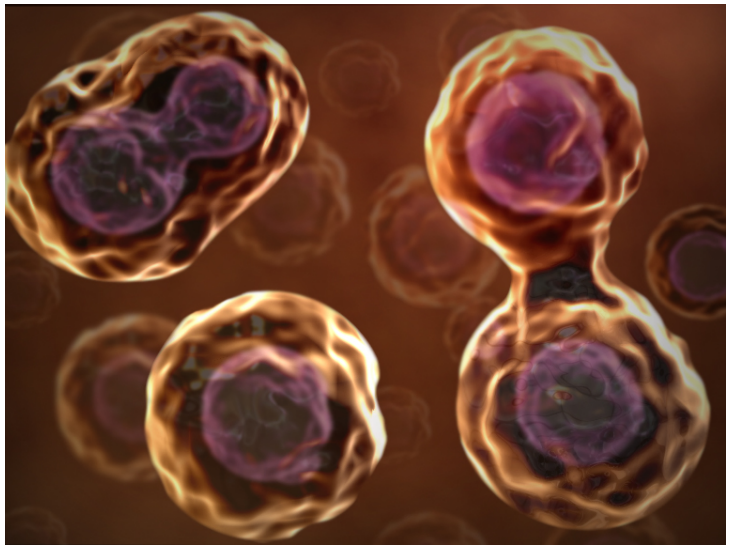
2002 - Juvenile Diabetes Research Foundation International creates $20 million fund-raising effort to support stem-cell research

2002 - California ok stem cell research

2004 - Harvard researchers grow stem cells from embryos using private funding

2004 - Ballot measure for $3 Billion bond for stem cells

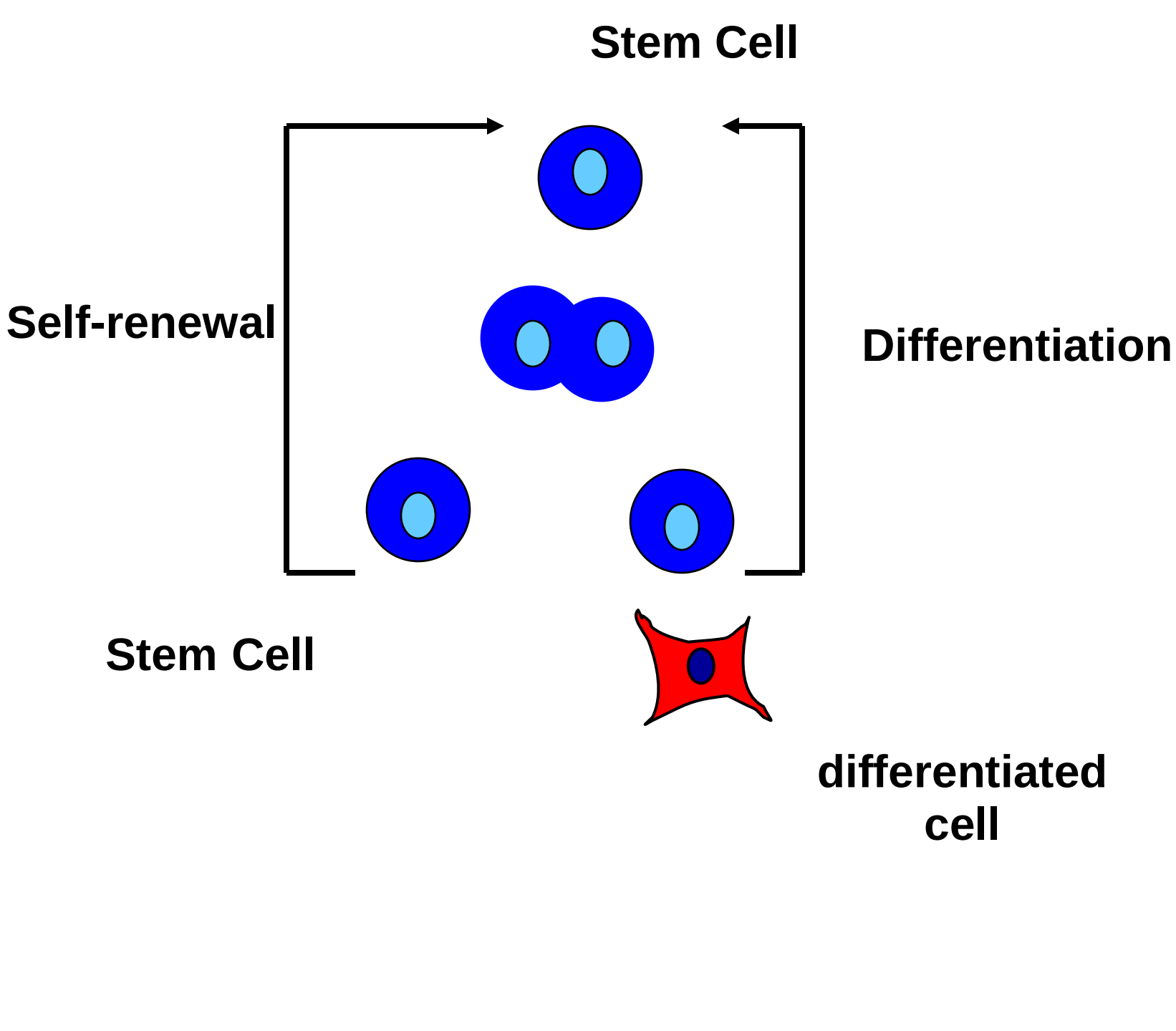
STEM CELL – DEFINITION



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

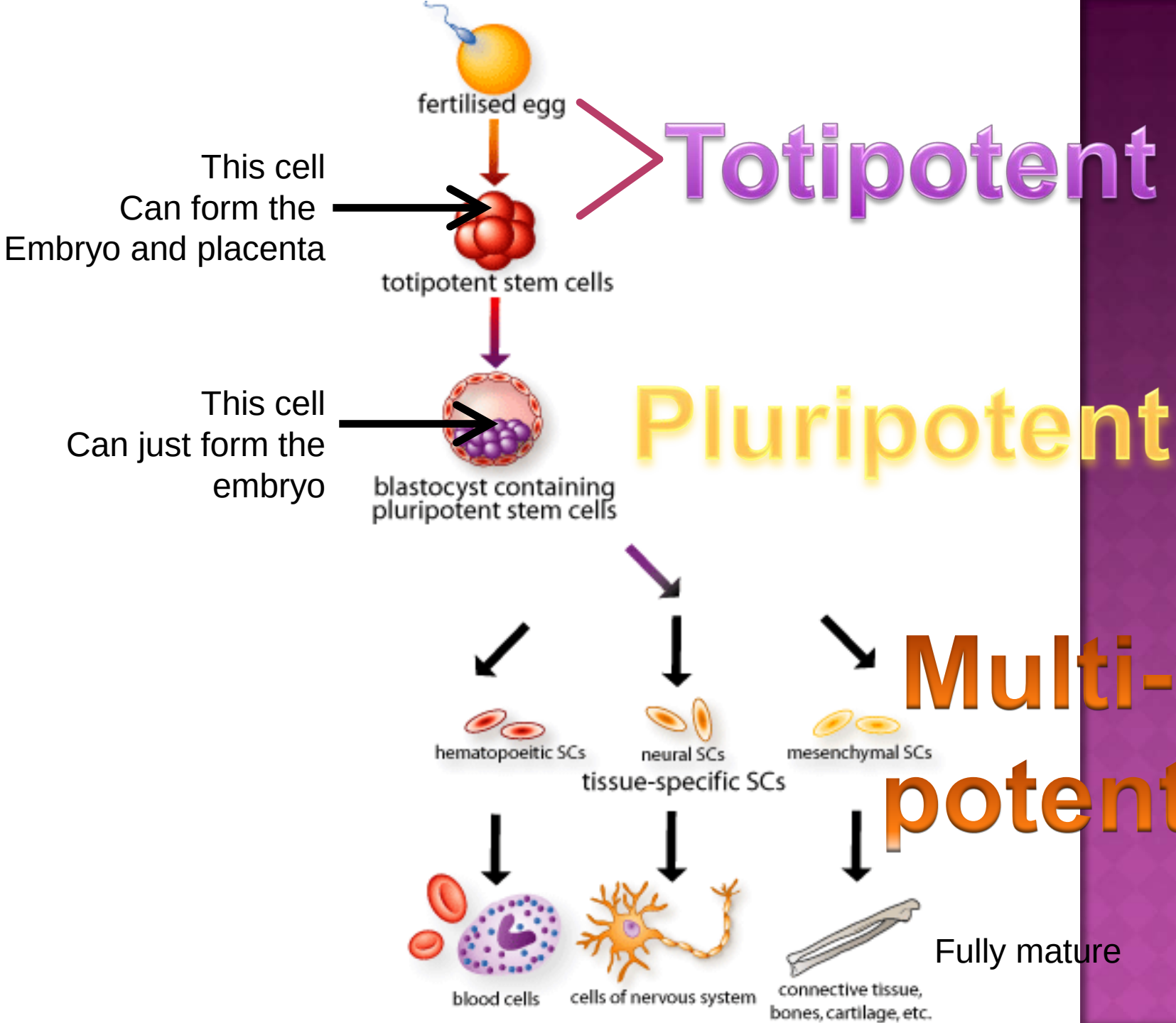
STEM CELL CHARACTERISTICS

- Plasticity: A stem cell is plastic, meaning it can develop into another type of cell.
- Differentiation: The process where a stem cell specializes or develops into another type of cell.
- Self-Renewal: a stem cell can divide (renew itself) *indefinitely* (go through mitosis) and without always developing into another cell.


Stem Cell
Self-renewal
Differentiation
Stem Cell
differentiated cell

KINDS OF STEM CELLS

Stem cell type	Description	Examples
Totipotent	Each cell can develop into a new individual	Cells from early (1-3 days) embryos
Pluripotent	Cells can form any (over 200) cell types	Some cells of blastocyst (5 to 14 days)
Multipotent	Cells differentiated, but can form a number of other tissues	Fetal tissue, cord blood, and adult stem cells


fertilised egg
This cell
Can form the
Embryo and placenta
totipotent stem cells
Totipotent
This cell
Can just form the
embryo
blastocyst containing
pluripotent stem cells
Pluripotent
hematopoeitic SCs
neural SCs
mesenchymal SCs
tissue-specific SCs
Multi-
potent
blood cells
cells of nervous system
connective tissue,
bones, cartilage, etc.
Fully mature

Kinds of Stem Cells

Embryonic stem cells

- five to six-day-old embryo

Embryonic germ cells

- derived from the part of a human embryo or fetus that will ultimately produce eggs or sperm (gametes).

Adult stem cells

- undifferentiated cells found among specialized or differentiated cells in a tissue or organ after birth
- appear to have a more restricted ability to produce different cell types and to self-renew.

Major Types of Stem Cells

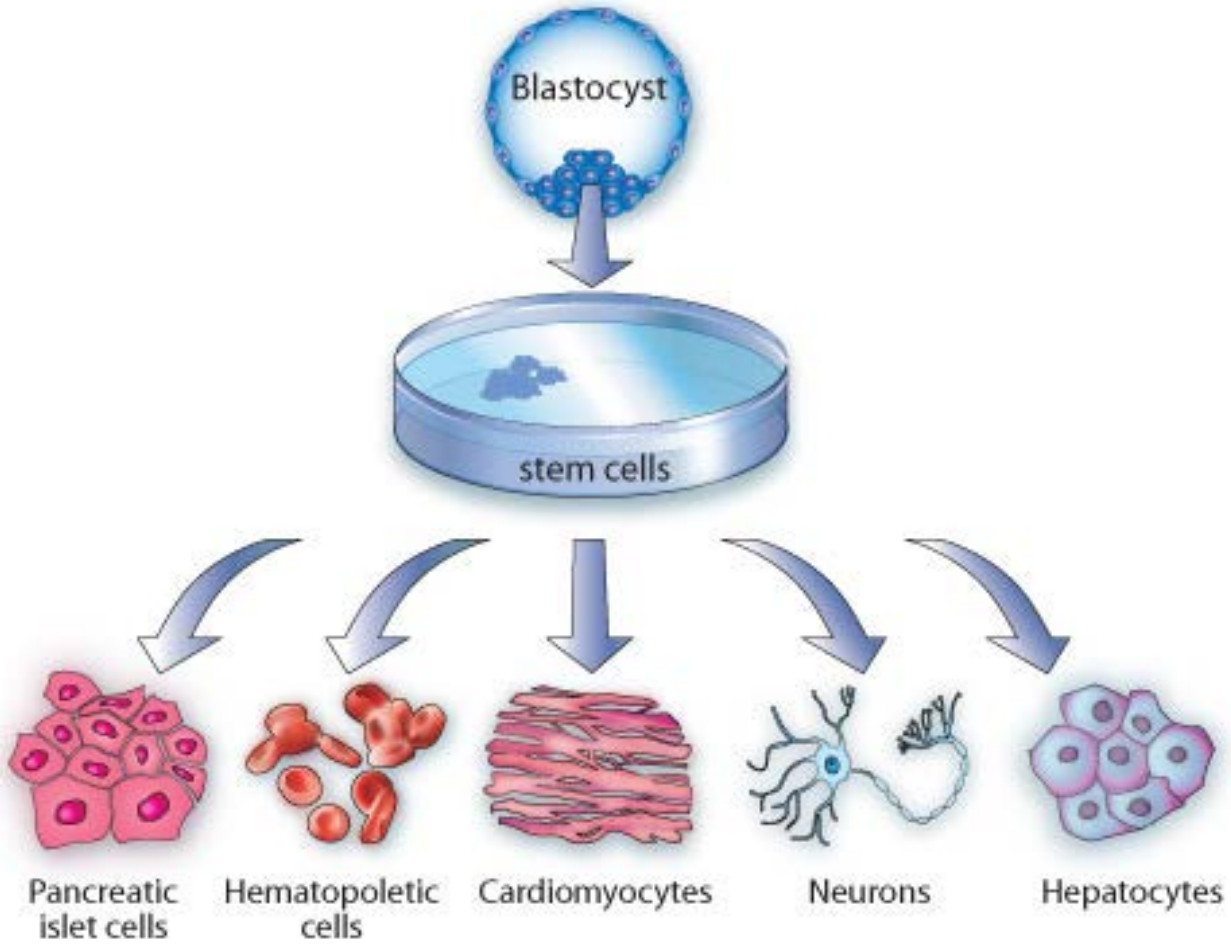
Embryonic Stem Cells

- Totipotent : cell can develop into all cell types
- "Immortal": can self-renew indefinitely
- Plentiful

Adult Stem Cells

- Multipotent: cell can develop into a few cell types but not all
- Located in few organs or may be unidentified
- Hard to find

PLURIPOTENT STEM CELLS – MORE POTENTIAL TO BECOME ANY TYPE OF CELL



MULTIPOTENT STEM CELLS

- Multipotent stem cells – limited in what the cells can become

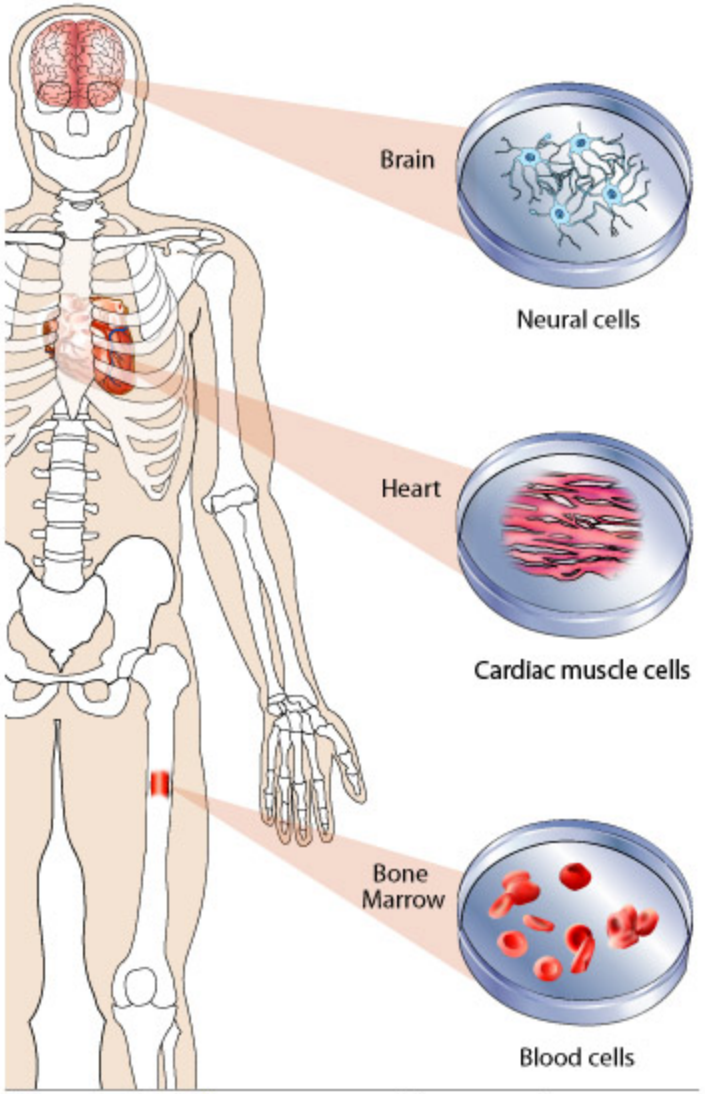
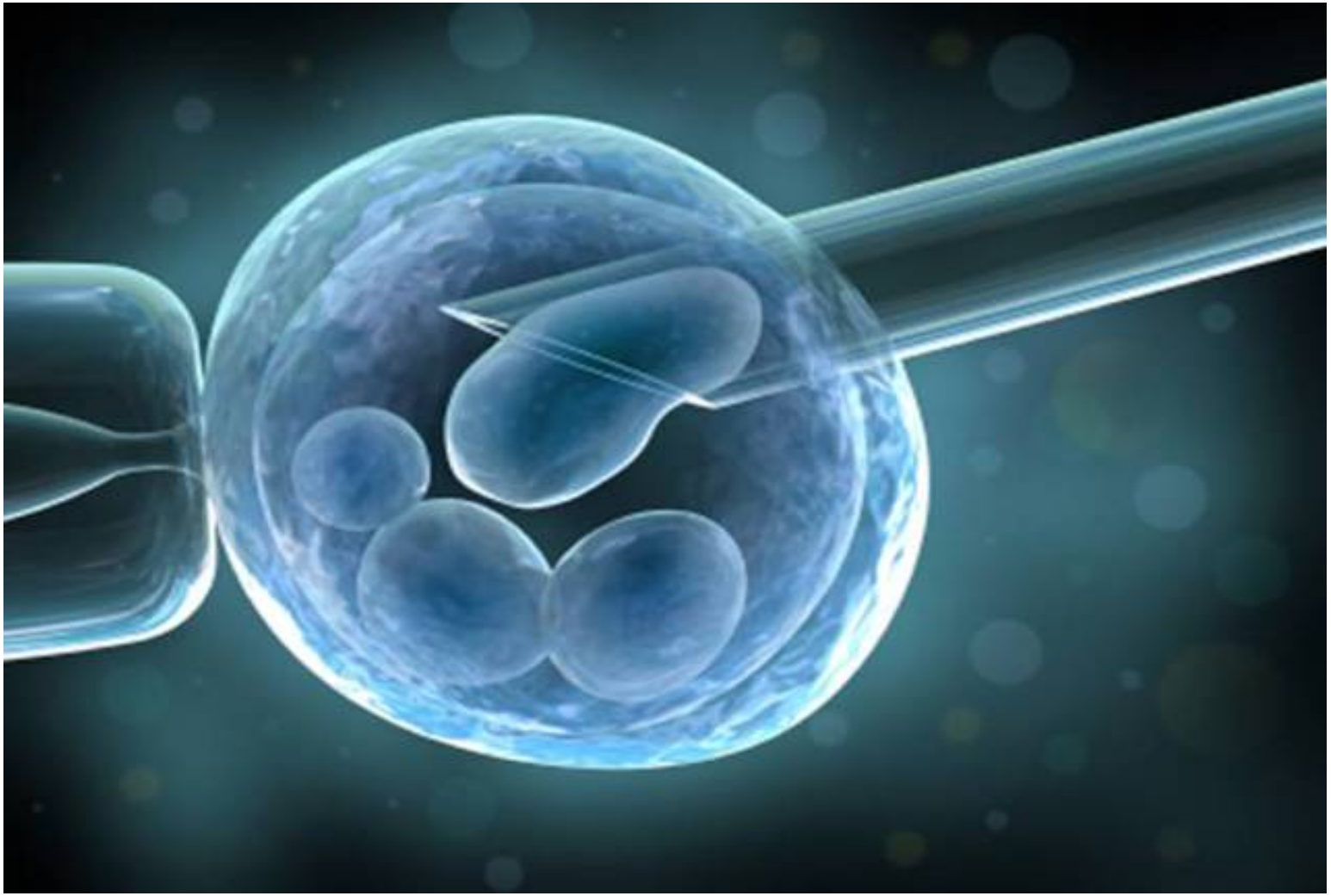


Illustration by Cell Imaging Core of the Center for Reproductive Sciences.

Embryonic Stem Cells

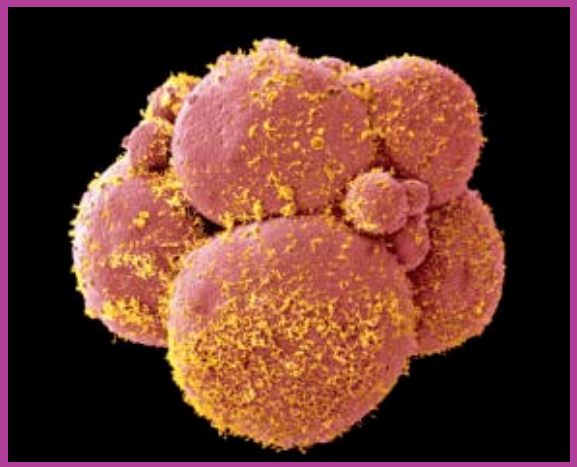


Mainly from IVF

SEXUAL REPRODUCTION

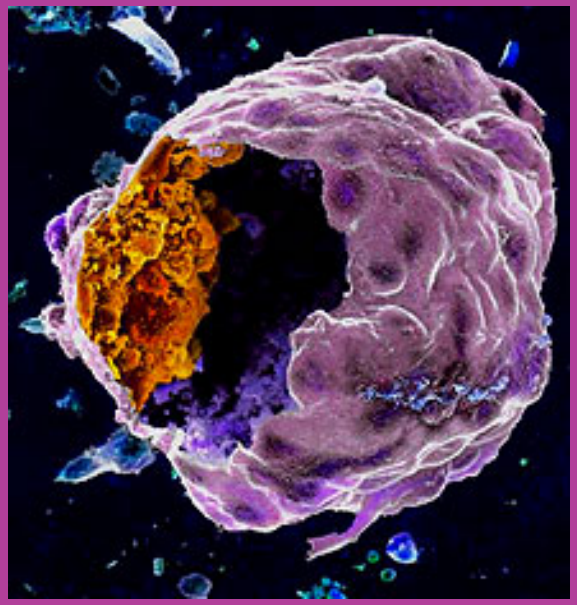
STAGES OF EMBRYOGENESIS

cleavage



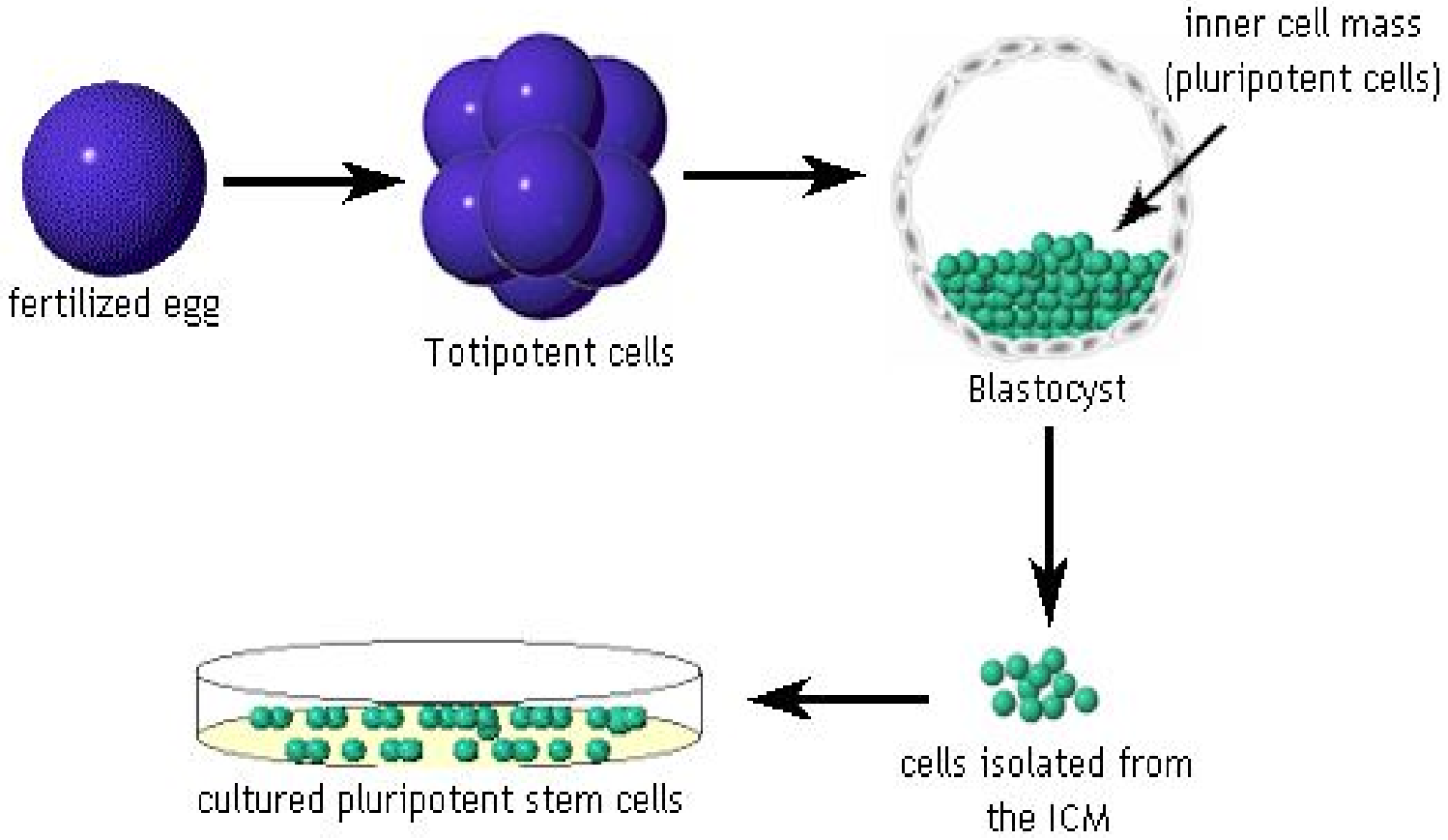
8-cell stage

blastocyst



Blastocyst inner mass cells

BLASTOCYST DIAGRAM

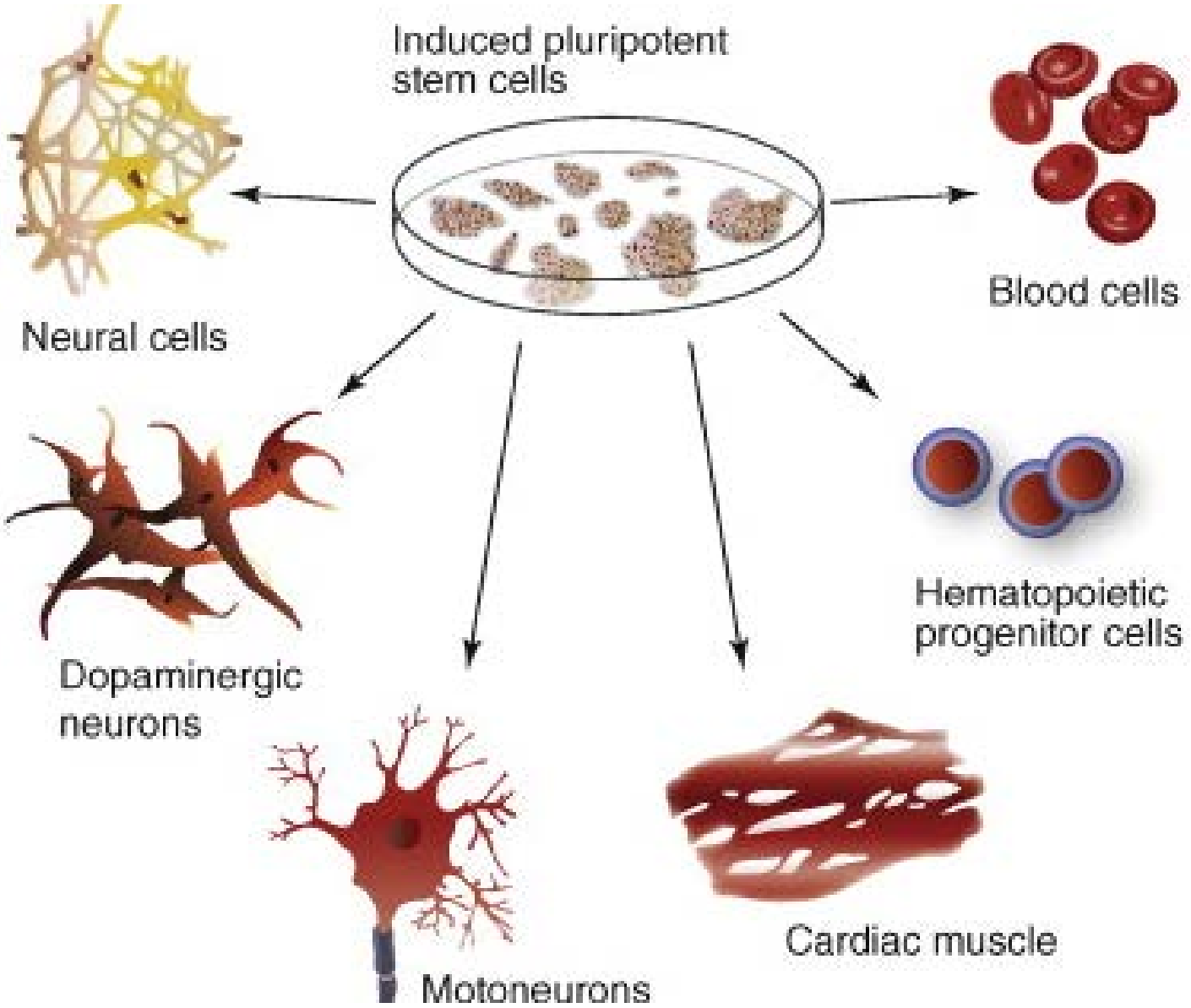


Adult Stem Cells

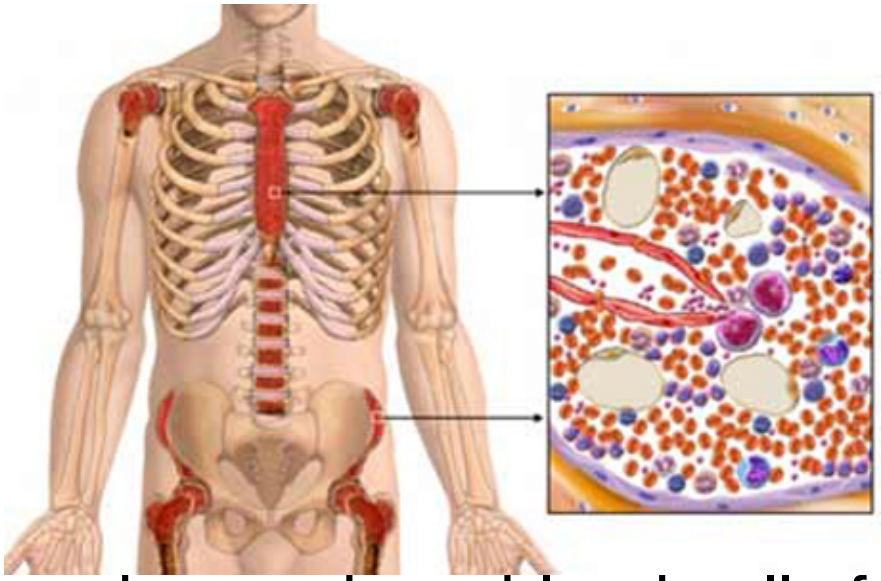
An undifferentiated cells found among specialized or differentiated cells in a tissue or organ after birth

- Skin
- Fat Cells
- Bone marrow
- Brain
- Many other organs & tissues

Induced Pluripotent Stem Cells

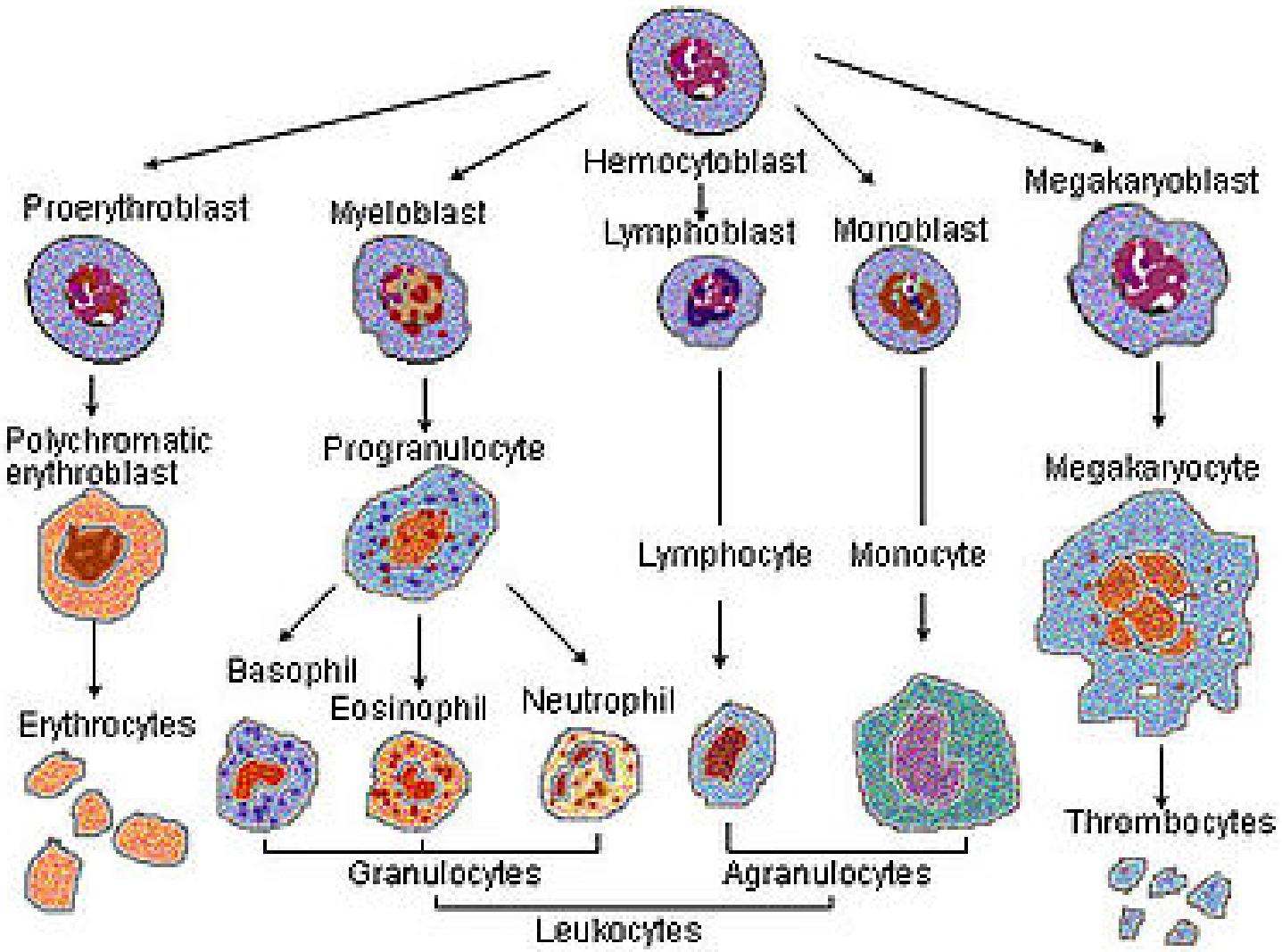


BONE MARROW



- Found in spongy bone where blood cells form
- Used to replace damaged or destroyed bone marrow with healthy bone marrow stem cells.
- treat patients diagnosed with leukemia, aplastic anemia, and lymphomas
- Need a greater histological immunocompatibility

Blood Cell Formation



UMBILICAL CORD STEM CELLS

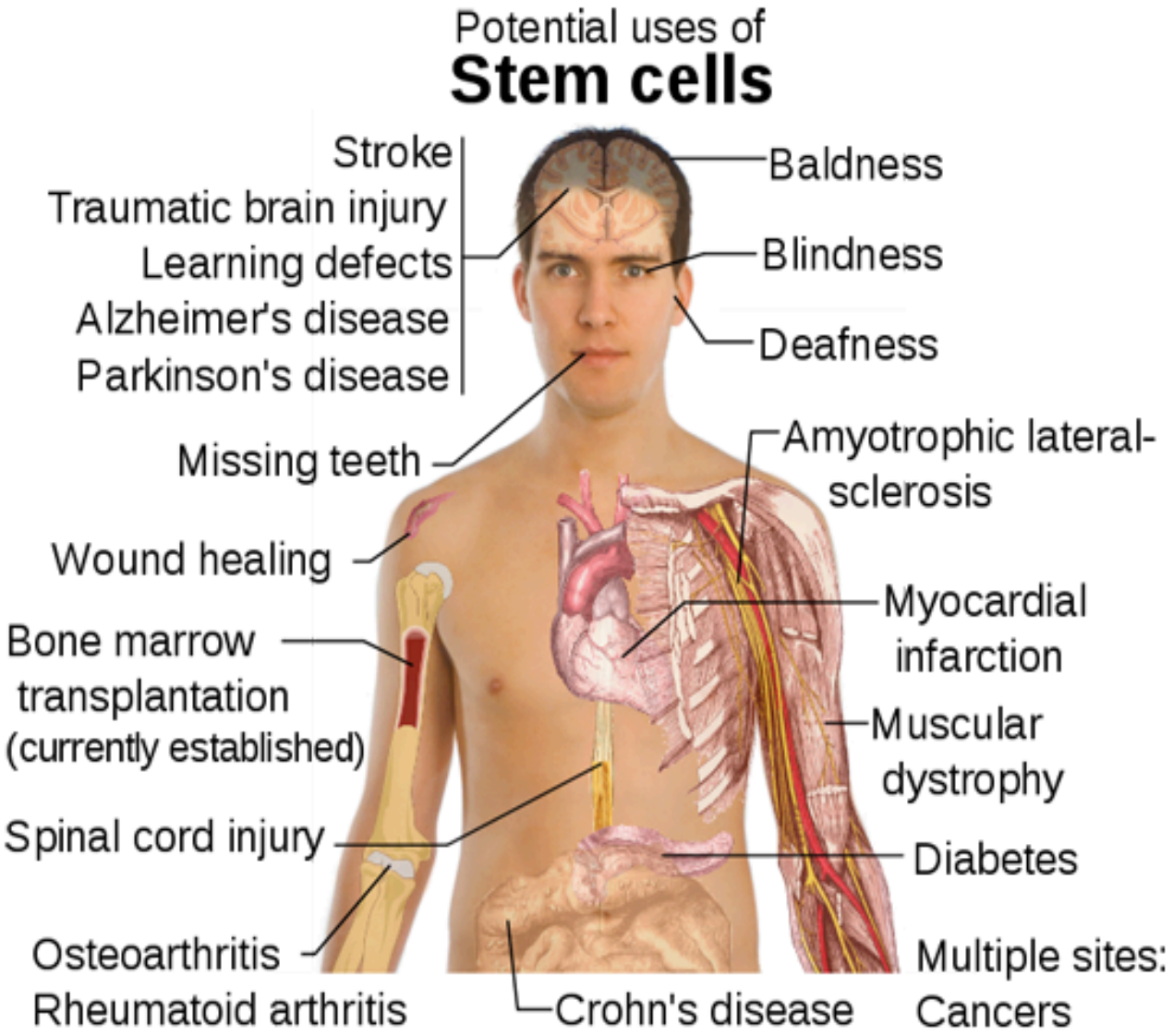
- Also Known as Wharton's Jelly
- Adult stem cells of infant origin
- Less invasive than bone marrow
- Greater compatibility
- Less expensive

UMBILICAL CORD STEM CELLS

Three important functions:

1. **Plasticity**: Potential to change into other cell types like nerve cells
2. **Homing:** To travel to the site of tissue damage
3. **Engraftment:** To unite with other tissues

Stem Cell Applications

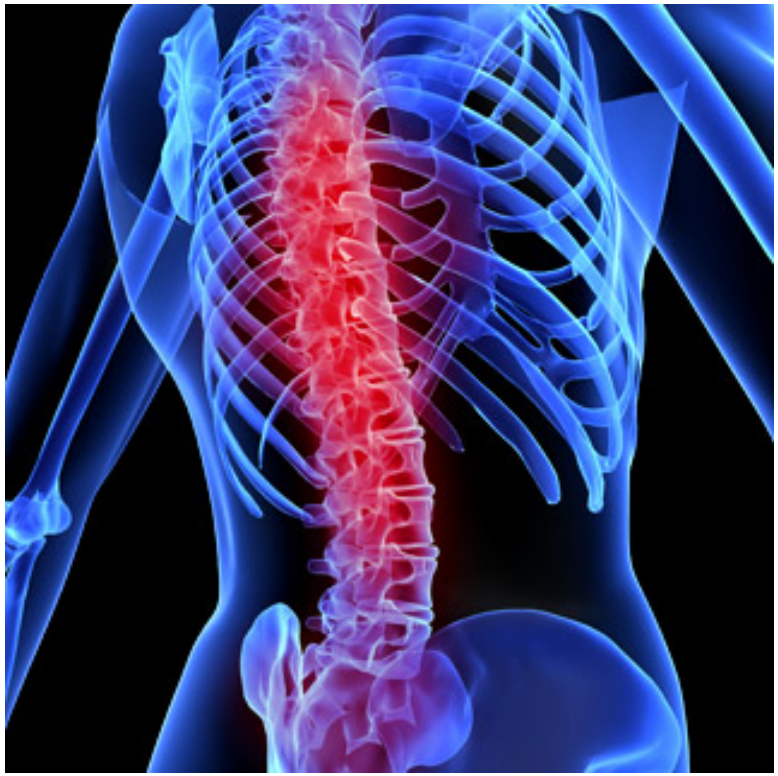


Stem Cell Applications

- Tissue repair
  - nerve, heart, muscle, organ, skin
- Cancers
- Autoimmune diseases
  - diabetes, rheumatoid arthritis.

Tissue Repair

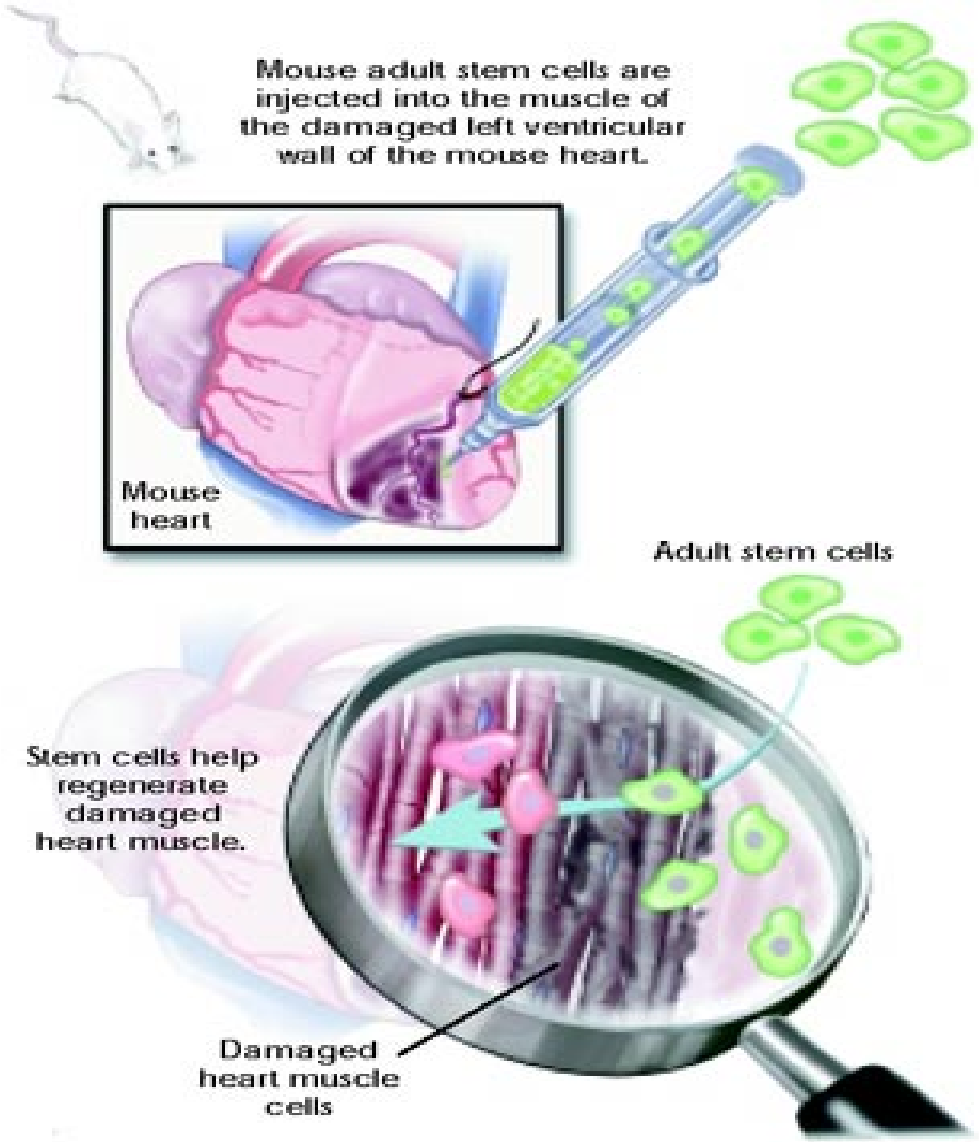
- Regenerate spinal cord, heart tissue or any other major tissue in the body.

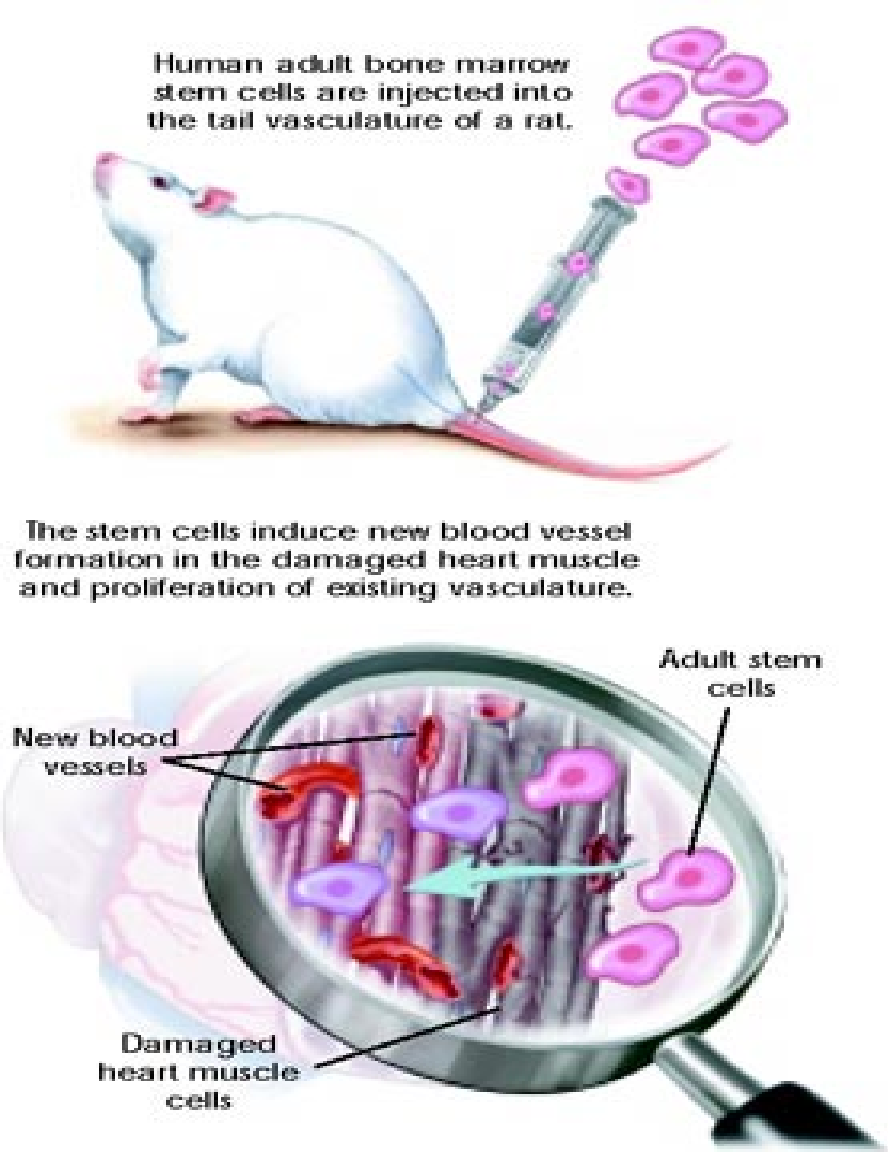


Replace Skin

Heart Disease

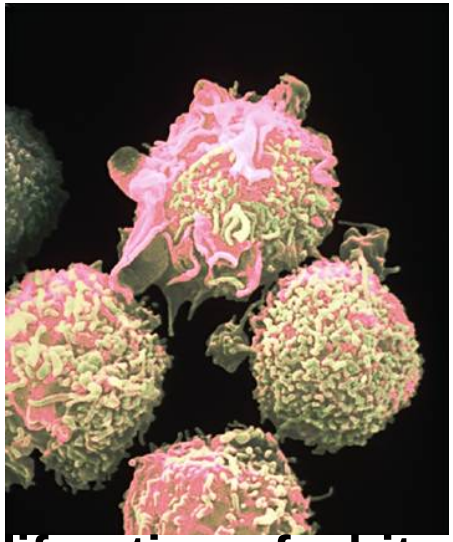
- Adult bone marrow stem cells injected into the hearts are believed to improve cardiac function in victims of heart failure or heart attack


Mouse adult stem cells are injected into the muscle of the damaged left ventricular wall of the mouse heart.
Mouse heart
Adult stem cells
Stem cells help regenerate damaged heart muscle.
Damaged heart muscle cells


Human adult bone marrow stem cells are injected into the tail vasculature of a rat.
The stem cells induce new blood vessel formation in the damaged heart muscle and proliferation of existing vasculature.
Adult stem cells
New blood vessels
Damaged heart muscle cells

Leukemia and Cancer

- Studies show leukemia patients treated with stem cells emerge free of disease.
- Injections of stem cells have also reduces pancreatic cancers in some patients.



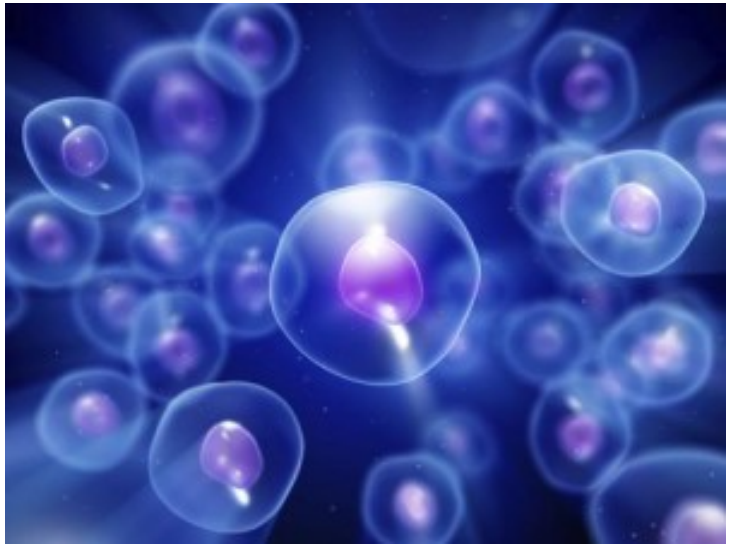
Proliferation of white cells

Rheumatoid Arthritis

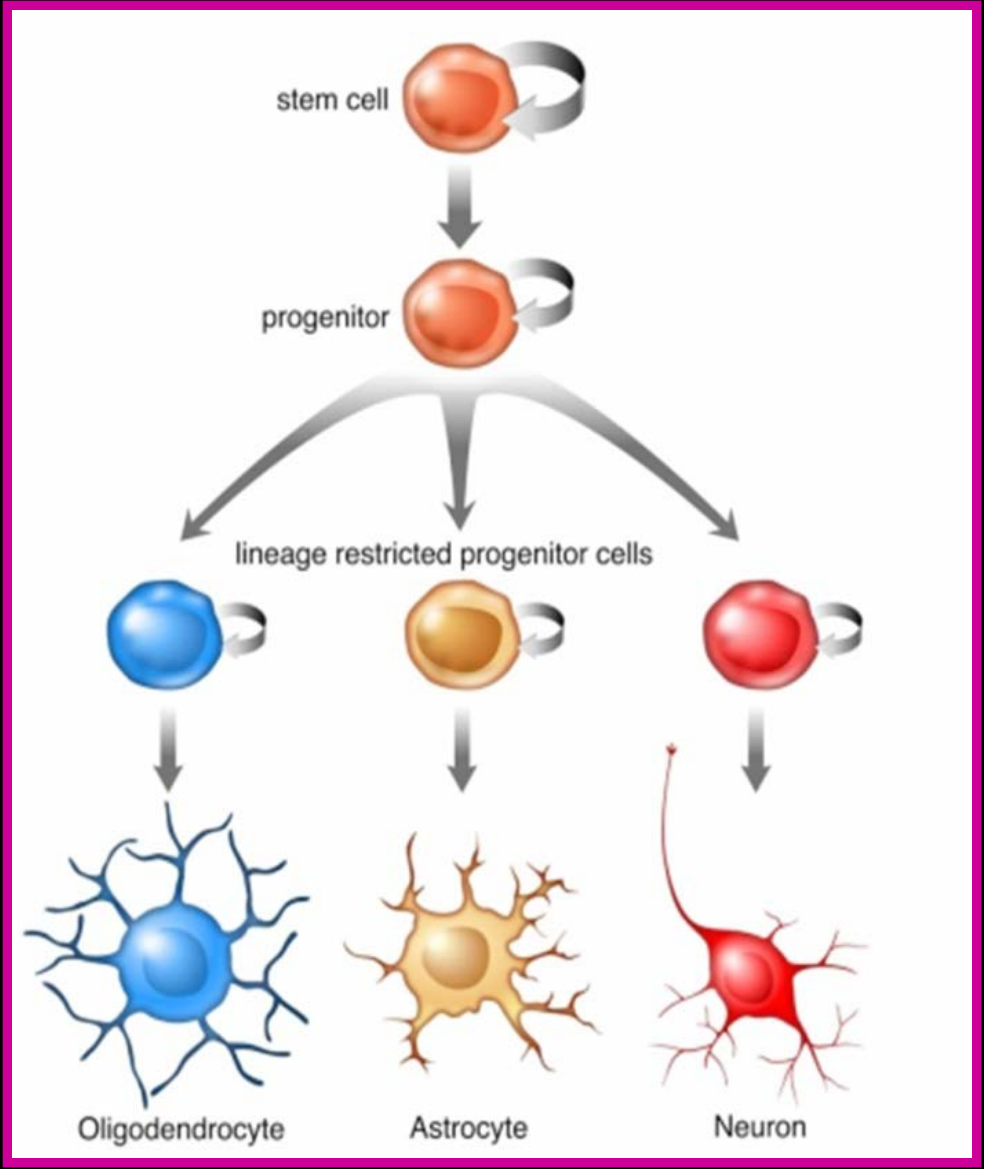
- Adult Stem Cells may be helpful in jumpstarting repair of eroded cartilage.

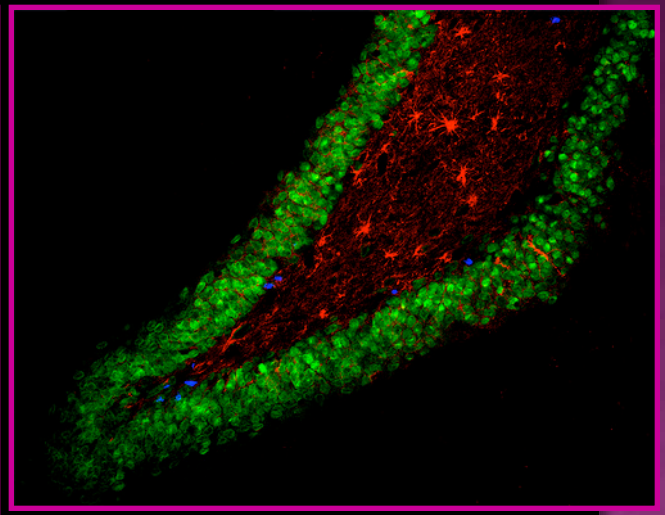
Type I Diabetes

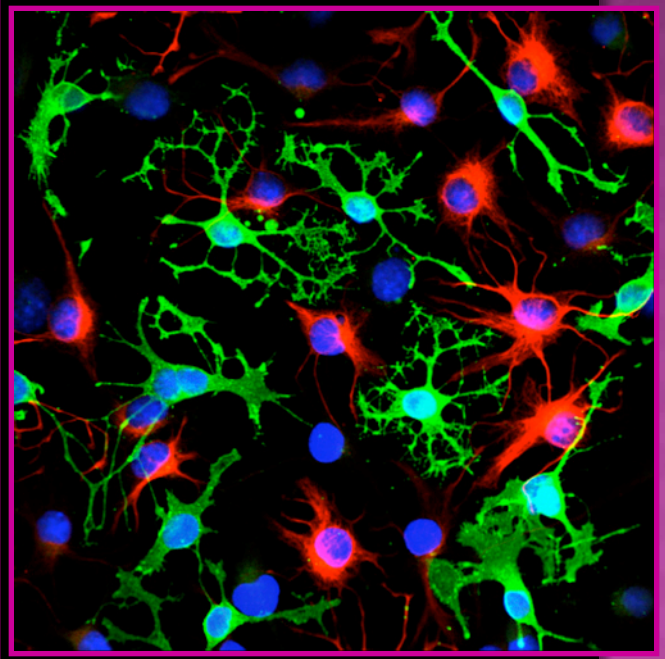
- Pancreatic cells do not produce insulin
- Embryonic Stems Cells might be trained to become pancreatic islets cells needed to secrete insulin.



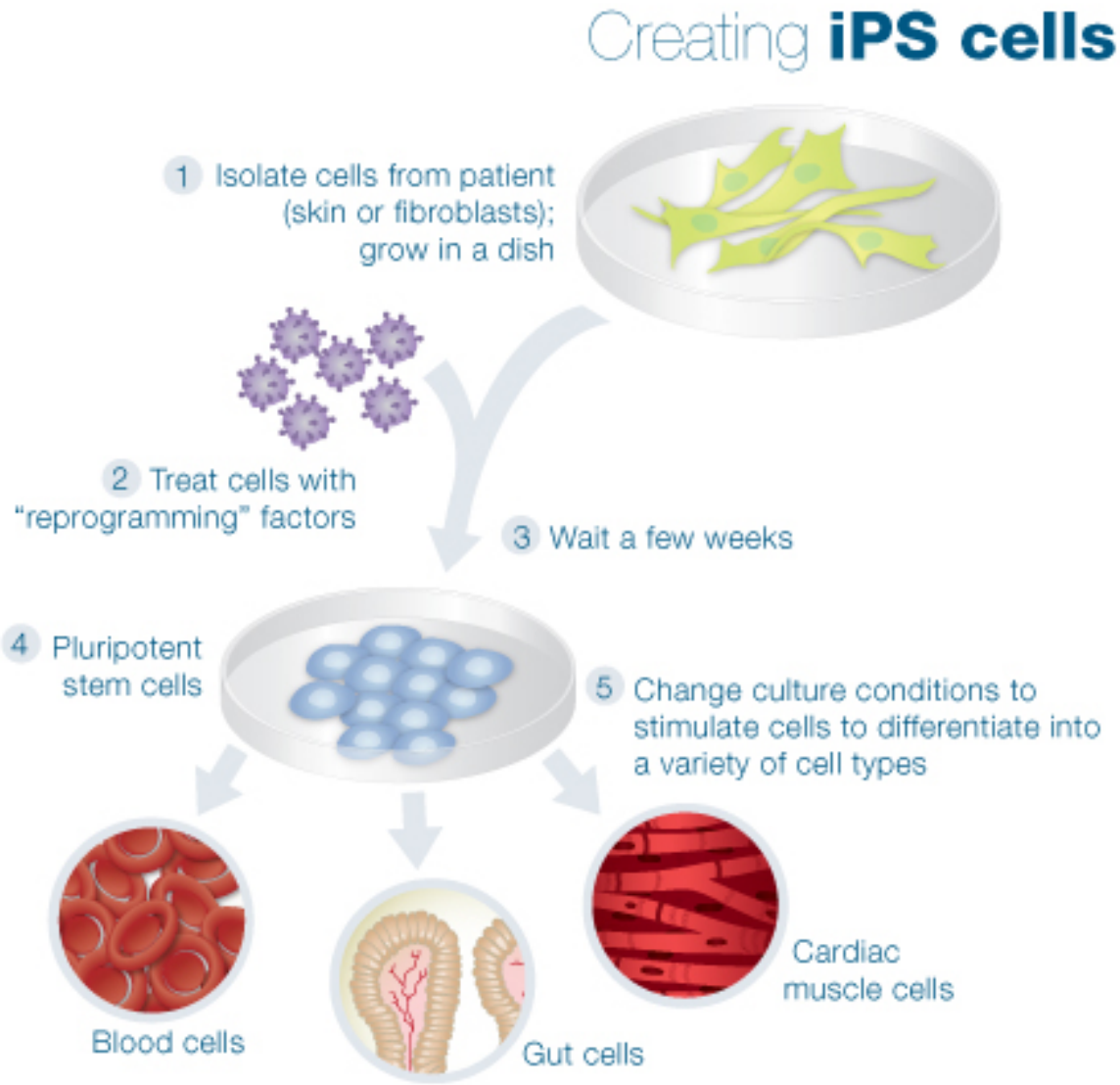
STEM CELLS IN THE ADULT BRAIN:







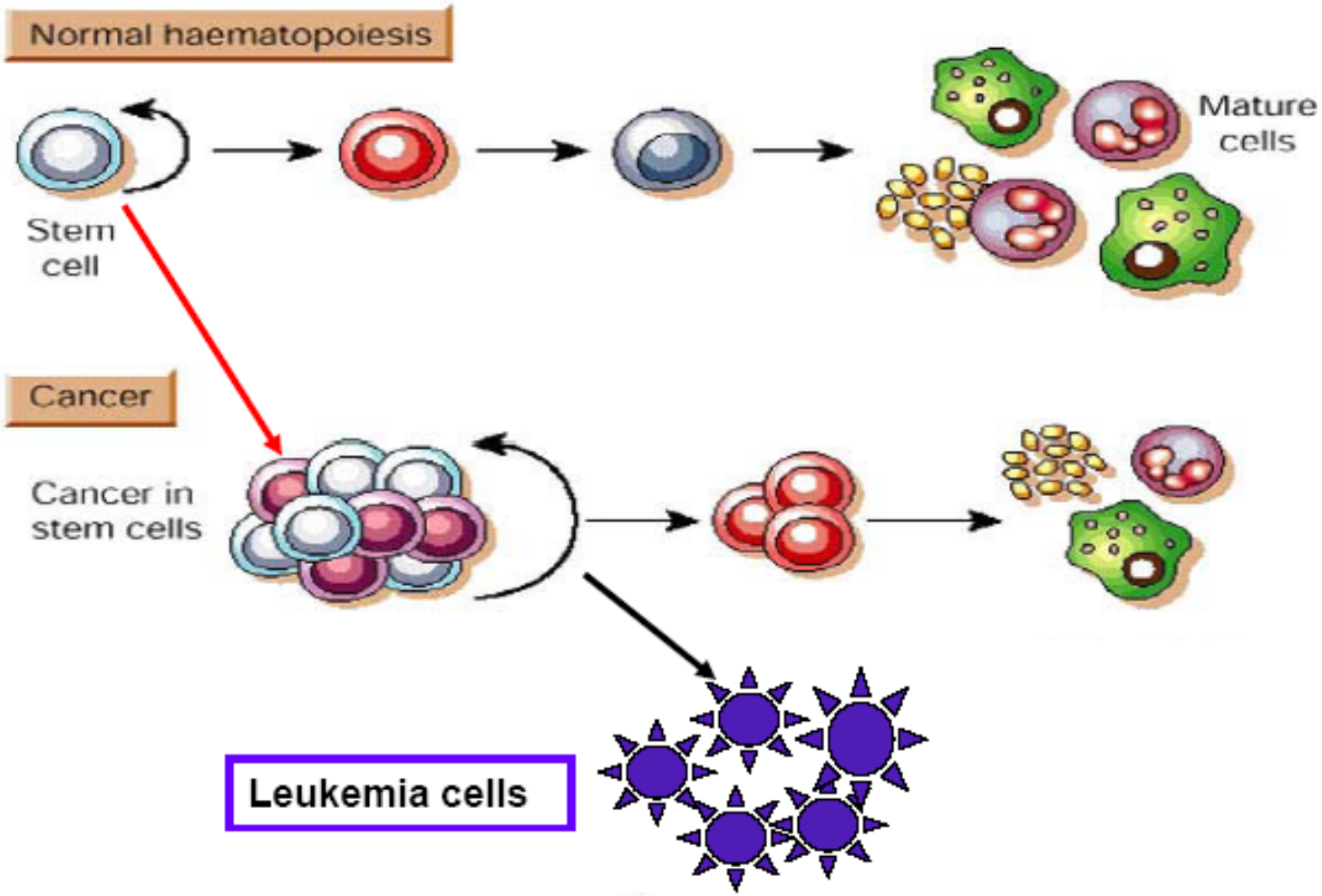
NEW RESEARCH – REPROGRAMMING CELLS



TECHNICAL CHALLENGES

- Source - Cell lines may have mutations.
- Delivery to target areas
- Prevention of rejection
- Suppressing tumors

Problems with Adult Stem Cells



Mutations can lead to leukemia

WHY IS STEM CELL RESEARCH SO IMPORTANT TO ALL OF US?

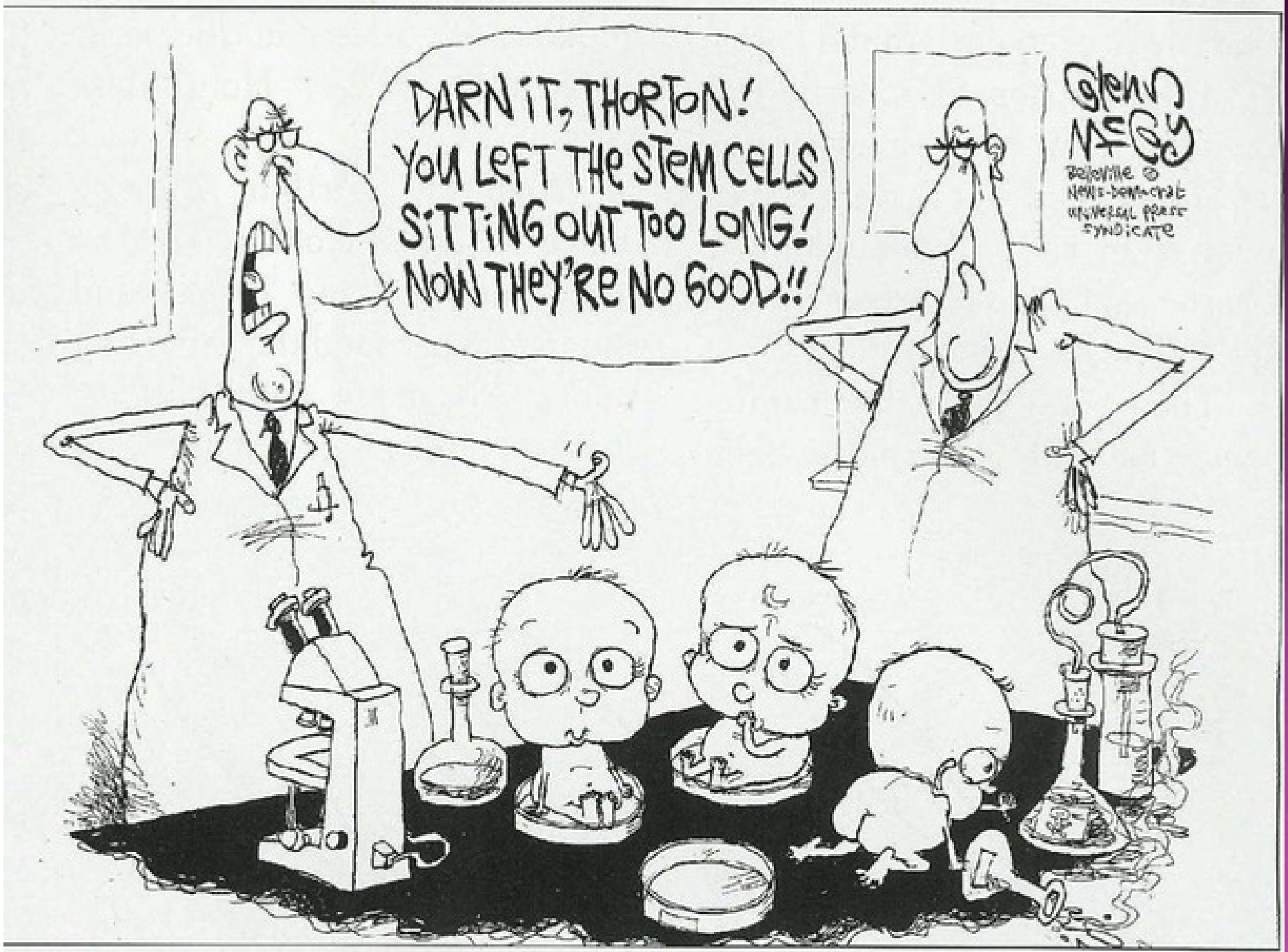
- Stem cells can replace diseased or damaged cells
- Stem cells allow us to study development and genetics
- Stem cells can be used to test different substances (drugs and chemicals)

WHY THE CONTROVERSY OVER STEM CELLS?

- Embryonic Stem cells are derived from extra blastocysts that would otherwise be discarded following IVF.
- Extracting stem cells destroys the developing blastocyst (embryo).

-Questions for Consideration-

- Is an embryo a person?
- Is it morally acceptable to use embryos for research?
- When do we become “human beings?”


DARN IT, THORTON! YOU LEFT THE STEM CELLS SITTING OUT TOO LONG! NOW THEY'RE NO GOOD!!
Glenn McCoy
Belleville News-Democrat
Universal Press Syndicate

Stem Cell Debate

Some people **oppose** stem cell research because they believe that the 4-5 day old ball of cells is a **living human being**. What some do not understand is that **unused embryos** are trashed regardless. Many people, religious and non-religious, believe it is better to use these embryos for research on how to cure human diseases rather than to just trash them without **purpose**. The underlying issue is **the personal belief when life actually begins**.

Misconceptions

- *Pregnancy, fetuses or babies are aborted or harmed in stem cell research.*

Fact: Fertilized Blastocysts donated from IVF labs are used and no pregnancy is aborted.

- *The fertilized embryos are removed from women's bodies and used for research*.

Fact: The embryos are left over from IVF and are frozen and are not implanted for pregnancy.

Misconceptions

- *A clone is grown in a lab without an embryo or born from a mother and is the same age and personality as you, and has no belly button*.

Fact: a clone would need a fertilized embryo and would have to be implanted in a woman's uterus. If you were cloned at 14, the clone would be 14 years *younger* than you (a baby you) and would have to have a mother to be born, so **yes**, it would have a belly button.

Misconceptions

- *If the embryos were not used in stem cell research they would have been used to make children*.

Fact: The embryos left over from IVF are discarded.

- *Men and women who donate their embryos do not know that their embryos are used for research.*

Fact: The embryos used in scientific research are donated with written and informed consent (permission) by the donors.