Genetics 319

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Tissue Culture Technology

Khaled Adly Mohamed Khaled

Genetics Department, Faculty of Agriculture,
Beni-Suef University

What is it tissue culture?

- Tissue culture is the term used for "the process of growing cells artificially in the laboratory" *(OSMS.otago.ac.nz/main/bursary)*
- Tissue culture involves both plant and animal cells
- Tissue culture produces clones, in which all product cells have the same genotype (unless affected by mutation during culture)

What is tissue culture?

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

What's the Background?

Haberlandt

Carrel

- Tissue culture had its origins at the beginning of the 20th century with the work of Gottleib Haberlandt (plants) and Alexis Carrel (animals)

- The first commercial use of plant clonal propagation on artificial media was in the germination and growth of orchid plants, in the 1920's

The Background

- In the 1950's and 60's there was a great deal of research, but it was only after the development of a reliable artificial medium (Murashige & Skoog, 1962) that plant tissue culture really 'took off' commercially
- A more recent advance is the use of plant and animal tissue culture along with genetic modification using viral and bacterial vectors and gene guns to create genetically engineered organisms

What is needed?

Tissue culture, both plant and animal has several critical requirements:

- Appropriate tissue (some tissues culture better than others)
- A suitable growth medium containing energy sources and inorganic salts to supply cell growth needs. This can be liquid or semisolid
- Aseptic (sterile) conditions, as microorganisms grow much more quickly than plant and animal tissue and can over run a culture

What is needed?

- Growth regulators - in plants, both auxins & cytokinins. In animals, this is not as well defined and the growth substances are provided in serum from the cell types of interest
- Frequent subculturing to ensure adequate nutrition and to avoid the build up of waste metabolites

Why do Plant Tissue Culture?

- A single explant can be multiplied into several thousand plants in less than a year - this allows fast commercial propagation of new cultivars
- Taking an explant does not usually destroy the mother plant, so rare and endangered plants can be cloned safely
- Once established, a plant tissue culture line can give a continuous supply of young plants throughout

Why do Plant Tissue Culture

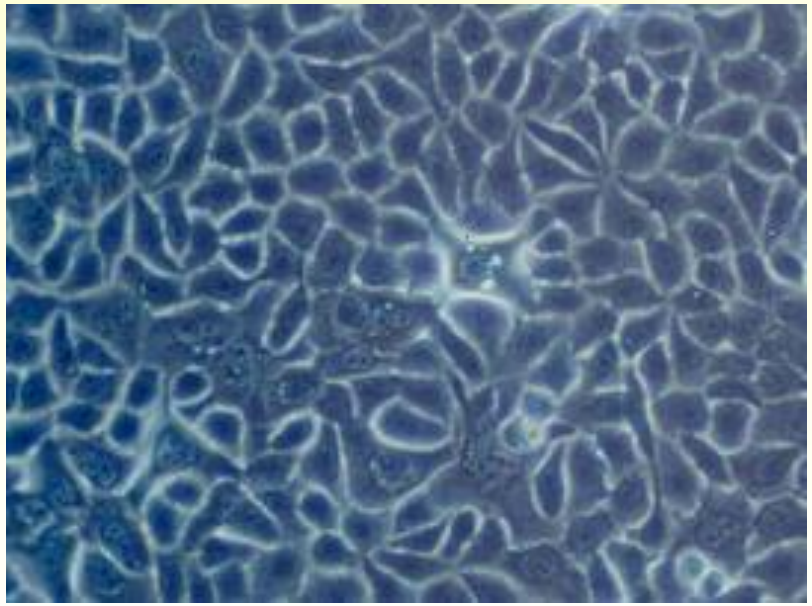
- In plants prone to virus diseases, virus free explants (new meristem tissue is usually virus free) can be cultivated to provide virus free plants
- Plant 'tissue banks' can be frozen, then regenerated through tissue culture
- Plant cultures in approved media are easier to export than are soil-grown plants, as they are pathogen free and take up little

Why do Plant Tissue Culture

- Tissue culture allows fast selection for crop improvement - explants are chosen from superior plants, then cloned

- Tissue culture clones are ‘true to type’ as compared with seedlings, which show greater variability

Uses of Animal Tissue Culture



- Growing viruses - these require living host cells
- Making monoclonal antibodies, used for diagnosis and research
- Studying basic cell processes
- Genetic modification & analysis

Uses of Animal Tissue Culture

- 'Knockout' technology - inactivating certain genes and tracing their effects
- Providing DNA for the Human Genome Project (and other species' genome projects)

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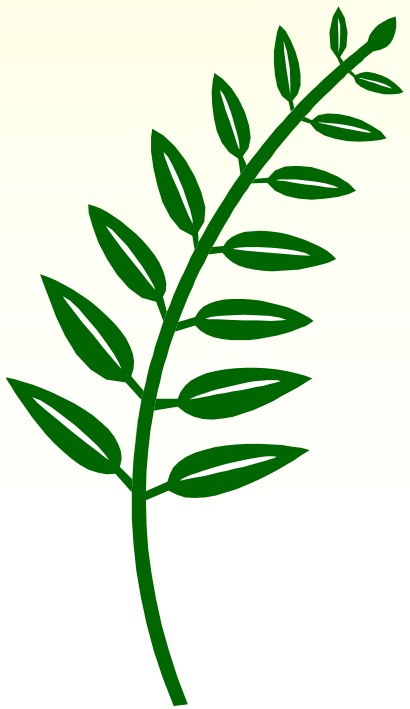
Plant Tissue Culture



Terminology used in tissue culture

- Acclimatization
- Androgenesis
- Basal medium
- Callus
- Clone
- Cryobiology
- Explant
- *In vitro*
- Inoculation
- Micropropagation (clone propogation)
- Organogenesis
- Protoplast
- Regeneration
- Shoot tip culture
- Somaclonal variation
- Somaclone
- Somatic embryogenesis
- Stock plant
- Sub-culture
- Suspension culture
- Totipotency
- Transplant stage

Basic need of tissue culture

1. nutrient medium
2. aseptic condition
3. aeration

nutrient medium

- "culture medium" or "Basal medium" or "growth medium"
- depends on their nutrient medium composition
- supplies energy and essential metabolites to growing tissues within
- state of culture medium solid = *"Callus culture"* or liquid = *"Suspension culture"*

Ingredients of nutrient medium

- carbon source (e.g. sucrose, glucose, starch which supplies energy)
- inorganic salts (major and minor elements),
- vitamins,
- hormones (auxin, cytokinin sometime GA).




Table 25.1 Composition of Two Growth Media

Chemical constituents	*M S medium mg/1.*	*Nitsch's medium mg/1*
$NH_4 NO_3$	1650	720
KNO_3	1900	950
$CaCl_2 2H_2O$	440	166
$MgSO_4 7H_2O$	370	185
$KH_2 PO_4$	170	68
Na_2 EDTA	37.3	37.3
$FeSO_4 7H_2O$	27.8	27.8
$MnSO_4 4H_2O$	22.3	25
$ZnSO_4 4H_2O$	8.6	10
$Na_2MOO_4 2H_2O$	0.25	0.25
$CuSO_4 5H_2O$	0.025	0.025
$CaCl_2 2H_2O$	0.025	–
KI	0.83	–
H_2BO_3	6.2	10
Glycine	2.0	2.0
Nicotinic acid	0.5	–
Pyridoxine HCl	0.5	0.25
Thiamine–HCl	0.1	0.5
Kinetin	0.04–10.0	–
IAA	1.0–3.0	–
Myo–inositol	100.0	100
Sucrose	30 gm/litre	20,000
pH	5.7	–
Niacin	–	5
Folic acid	–	0.5
Biotin	–	0.05

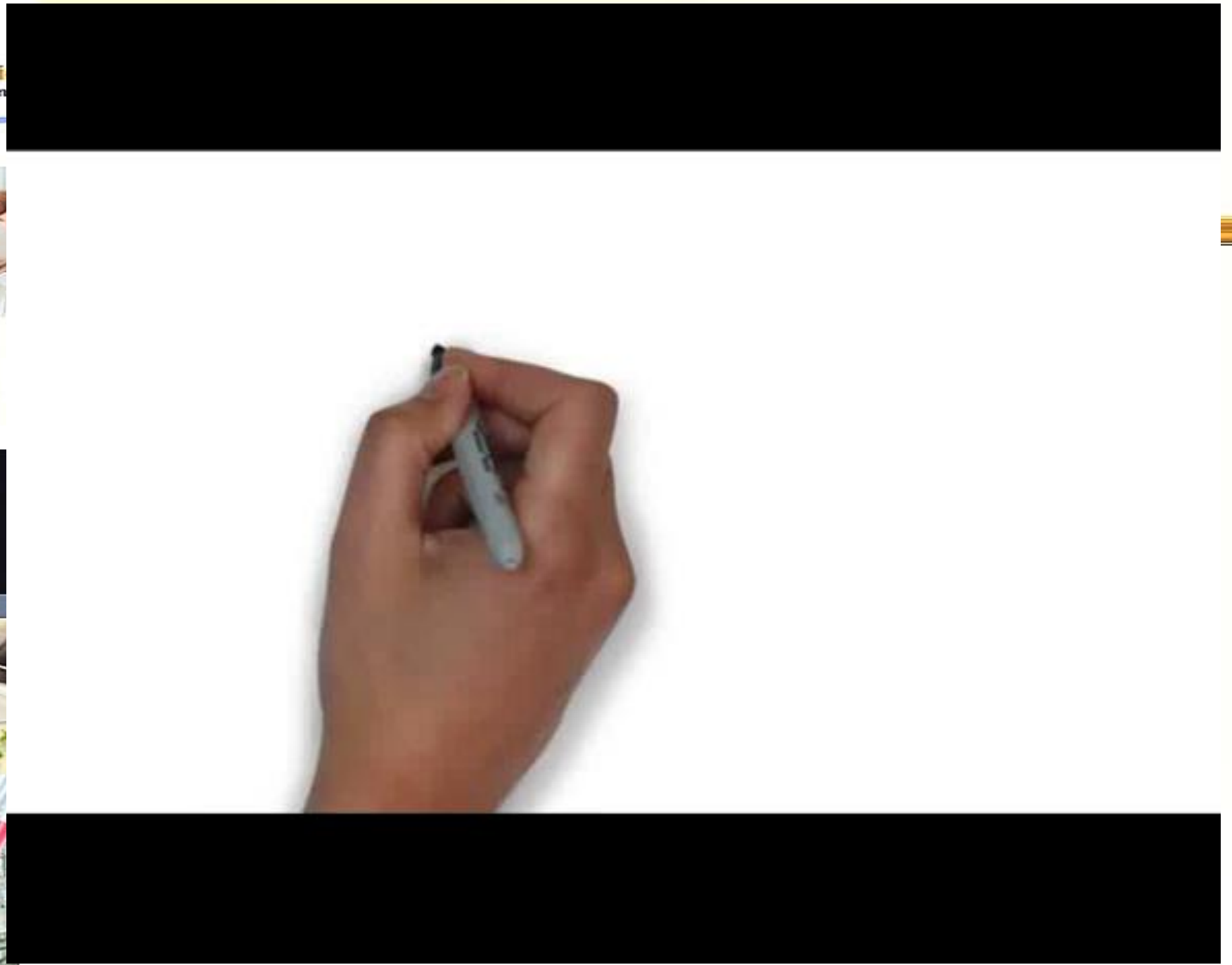
M S medium-Murashige and Skoog medium.

MS media

- Murashige and Skoog

plant tissue culture laboratory equipment's


ANIMAL CELL CULTURE



aseptic condition

- Utensils, glass ware, nutrient medium, culture room, inoculation chamber must be in aseptic condition.
- microbes compete with growing tissue and finally kill it
- maintain aseptic condition in and around the culture equipments in tissue culture experiments.

- There are three sources of contamination:

(i) through' utensils and culture medium

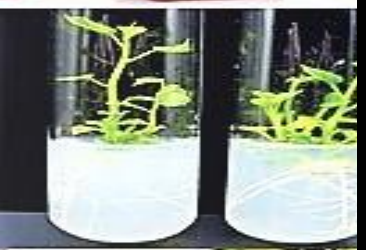
(ii) through explant material

(iii) through inoculation (transfer of explant to culture medium)

- avoid contamination through utensils - surface sterilized with
  - sodium hypochlorite (bleaching powder) or
  - calcium hypochlorite or
  - chlorine water or
  - $HgCl_2$ (0.1 %) and
- autoclaved at 121°C for 30 minutes for complete sterilization.
- Microorganisms get killed at this temperature by autoclaving.
- Explant is thoroughly washed with chlorine water or calcium hypochlorite and then with distilled water.

- To prevent the entry of microbes at the time of inoculation, laminar air flow cabinet (chamber used for culture) is sterilized with ultra violet light or filter sterilized air.
- The principal advantage of working in these cabinets is that the flow of air does not hamper the use of spirit lamp.
- Under tropical and subtropical areas, where dust particles are very high, it must be kept in double door fitted culture room in order to prolong the effective life of the filter.


ANIMAL CELL CULTURE



aeration

- If the plant part is grown on the surface of a semi solid medium it acquires sufficient aeration without a special device.
- But if liquid medium is used, submerged plant part requires some special device for aeration.
- One such device is a "filter paper bridge" whose two legs remain dipped in the medium and the horizontal part carrying the plant part is raised above the level of the medium.
- Common method - shaking the cultural flasks/tubes on an automatic shaker.

- The culture vials containing medium are plugged with non-absorbing cotton wrapped in cheese cloth; such a closure allows the exchange of gases but does not permit the entry of micro-organisms into the culture vials.

Steps of tissue culture

- Inoculation and callus formation
- Multiplication or sub-culturing callus
- Development and differentiation of sub-culture
- Acclimatization

Inoculation and callus formation

- explant - vegetative parts or reproductive part, young or old.
- Old parts have accumulation of phenols, terpenoids, coumarins, steroids (secondary metabolites) - pre-soaked in an anti-oxidant solution to remove the substances
- desirable to take explant from young plant especially from meristem and cortical parenchyma

- Explant is extracted with a sharp scalpels - tissues remain undamaged.
- Damaged tissues release various compounds which are air oxidized.
- explant surface is sterilized with chlorine water or HgClz (0.1 %),
- then it is washed with distilled water to remove adherent chemical particles.
- explant is transferred to sterilised culture medium kept in test tubes or flasks in culture chamber.
- This chamber is also pre-sterilized with ultra violet ray emitting tubes.

- all inoculated tubes/flasks are kept at 25 ± 2°C temperature in culture chamber with light exposure intensity of 2000 lux for 16 hours
- light is not essential for callus formation in tissue culture experiment.
- It is essential for plant regeneration only.
- Callus formation is observed after two weeks of inoculation, i.e. incubation period is of two weeks.

Multiplication

- Callus produced in above stage is taken out
- cut into pieces and
- each piece is transferred to fresh culture medium in separate tubes or flasks.
- After some time these pieces develop into big masses of callus.
- This is called *sub-culture* or *multiplication stage* as callus get multiplied here

Development and differentiation of sub-culture

- The differentiation of organs (root and shoot) occurs from callus.
- This differentiation is dependent upon auxins/cytokinins ratio.
- High ratio favours root formation and low ratio favours shoot formation, while appropriate proportion of the two favours root and shoot formation together.
- In this phase, entire plant is obtained from callus, it is also called *regeneration phase.*
- Embryoids may also develop in this phase if medium concentration is modified.
- Embryo is an independent structure devoid of vascular supply - it cannot be regarded as an organ.

Acclimatization

- Cultured plants are taken out of test tubes/flasks
- thoroughly washed under running tap water to remove adhered agar molecules.
- Plants are kept at low minimal salt medium for 24 - 48 hours
- transferred to pots containing autoclaved sterilized mixture of clay and core soil leaf moulds in equal proportions.
- Plantlets of the pots are covered with transparent polythene to maintain humidity for 15-30 days.
- Plantlets then are transferred to green house for a week and then to field.
- In the field agricultural techniques are applied like other crops/ plants.

Types of cultures:

- Whole plant culture
- Plant organ culture
- Protoplast culture




- 1. Whole plant culture
  - Seed culture
  - Embryo culture
- 2. Plant organ culture
  - Androgenesis
    - Anther culture
    - Isolated pollen culture
  - Ovary culture
  - Nucellus culture
  - Ovule culture
  - Vegetative organ culture
    - Shoot culture
    - Root culture
    - Leaf culture
- 3. Protoplast culture
  - *Synkaryon* (Both nuclei fuse)
  - *Heterokaryon* (Both nuclei exist)
  - *Cybrid* (one nucleus disappears)

Whole plant culture

- natural condition - Seed embryo normally develops into whole plant
- also be developed into plantlet through tissue culture technique.
- Embryo culture is useful in such plants which have abortive embryo.
- The function of endosperm is to feed embryo but due to incompatibility between endosperm and embryo this relationship is not maintained.
- If such embryo is excised from seed and supplied exogenously by tissue culture technique, plantlet is regenerated.

- Orchids - have very small seeds without food reserves,
- can be regenerated through tissue culture technique even in the absence of fungal association (which is essential for their normal germination).
- Fungus *Rhizoctonia* has symbiotic relationship with orchids seeds.

Anther Culture/Androgenesis

- culture of plantlet from pollen grains either by placing anthers or *isolated* pollen grains in culture medium

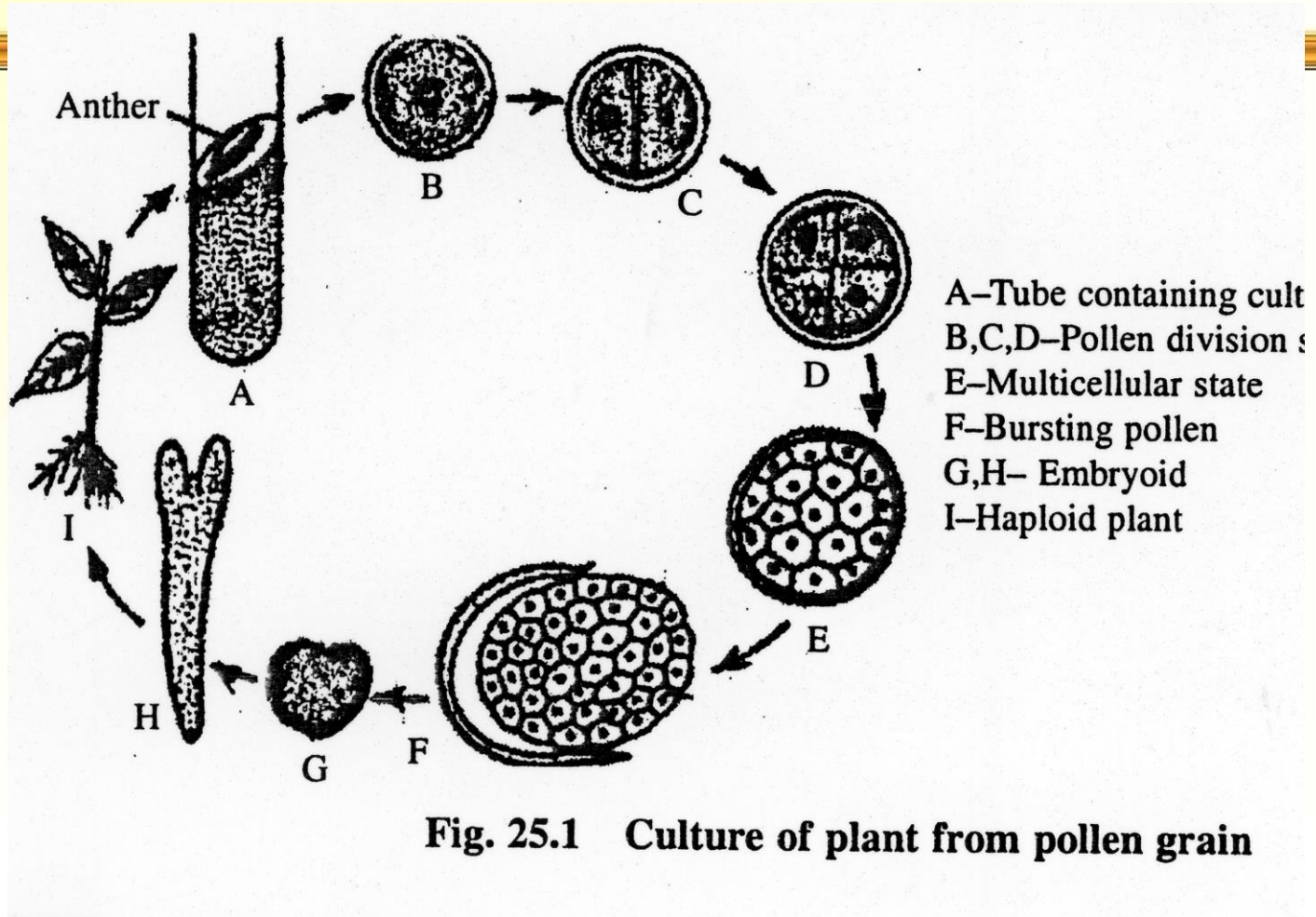


Fig. 25.1 Culture of plant from pollen grain

- Embryoids (non-zygotic embryo) are produced *in vitro* condition from pollen grains.
- Embryoids later on develop into plantlet.
- Plantlets produced through anther culture may be haploid or mixploid.
- If it is produced by connective tissues (sterile cells), it is mixploid but if it is produced from pollen grain, it is haploid as pollen grains are haploids.

- Technique - extraction of explant (pollen grains of intact anther/isolated pollen grains) from surface sterilized buds and inoculation of the same in nutrient medium.
- Depending on the nature of cultured species and composition of nutrient medium, the pollens may develop either directly into embryoids or into callus.
- Callus later grows into embryoids.
- Anther culture process is influenced by age of the plant (at the time of taking anther) and temperature.

- Intact anthers were taken from a single flower bud
- put horizontally on the surface of a semisolid nutrient medium.
- A filter paper disc was placed over these anthers.
- suspension of pollen from anthers of another bud was prepared separately in liquid medium (10 pollen/O.5 ml medium).
- Ten pollen grains were transferred to each filter paper disc.
- Numerous clones were formed in four weeks

Ovary Culture

- seedless tomato fruits from unpollinated ovaries' culture on a medium supplemented with 2, 4. D.
- addition of IBA (Indole butaric acid) to the nutrient medium resulted into development of parthenocarpic fruits in Althea rosea
- The ovaries of *Cucumis* and *Lycopersicon* excised from pollinated flowers developed into mature fruits containing viable seeds (Fig. 25.3).

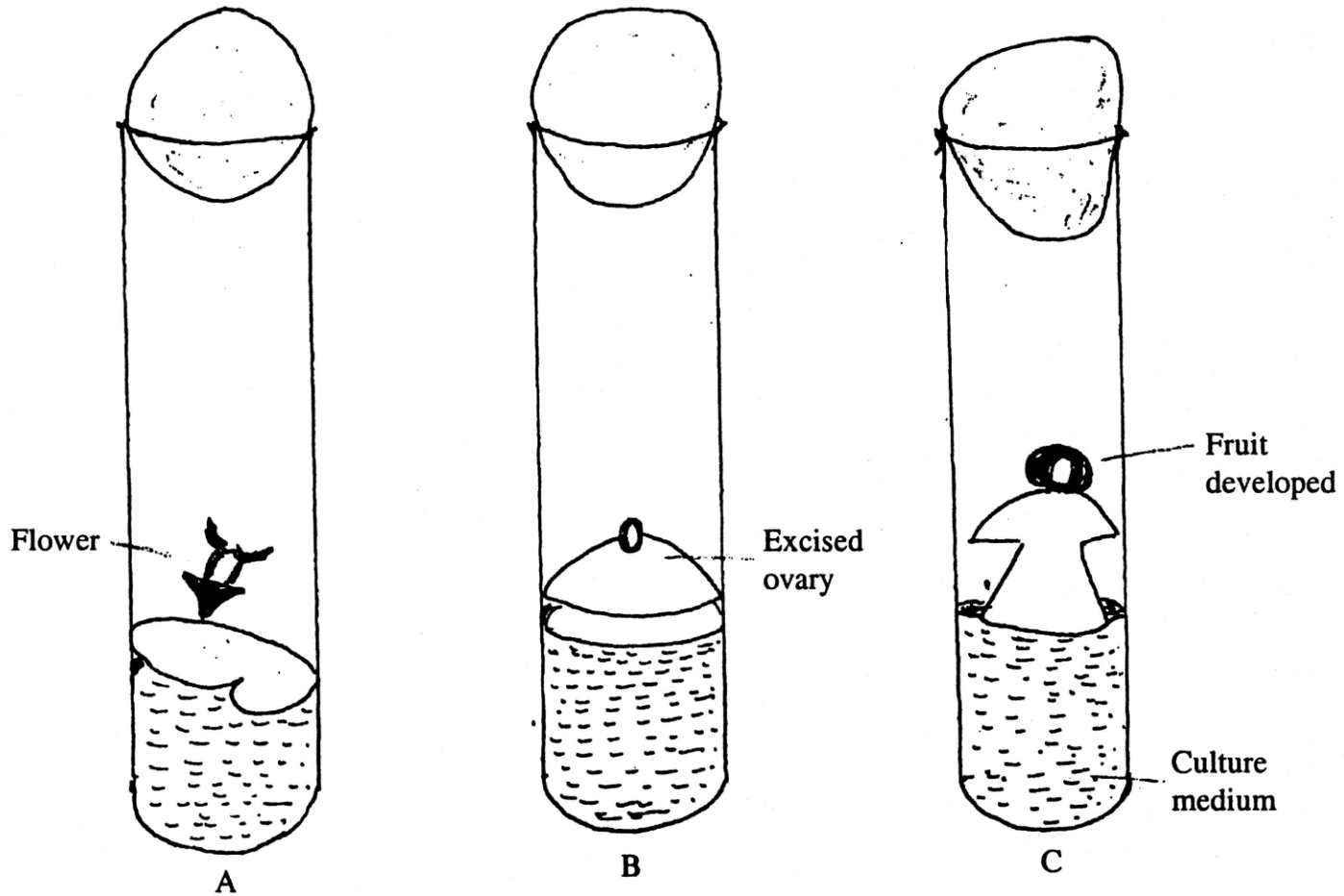


Fig. 25.3 Culture of ovary

- In vitro culture of ovary has revealed that the perianth lobes, especially sepals, do not give only protection to the essential parts but also have a positive role in fruit development.
- Culture of ovaries after calyx removal results into poor development of fruits.

Nucellus Culture

- When nucellar tissue excised from post pollinated carpels (when the adventitious embryos are already in torpedo shaped stage) in polyembryonate species of *Citrus* is grown in White's medium supplemented with casin hydrolysate, it forms callus, which later differentiates into numerous pseudobulbils and then into embryoids.

- Embryoids germinate to form seedlings.

- *Rangan et al* (1968) cultured nucellus by taking it from post fertilized ovule of monoembryonate species of Citrus and observed embryoid formation without callusing/pseudobulbil-like structure.

- The embryoids later developed into plantlets.

Root Culture

- Surface sterilized root tips are taken as explant from seedling roots of 20 mm length, developed from sowing of sterile seeds.
- Each tip was allowed to grow on culture medium in test tubes or Erlenmeyer flask.
- Roots grew in length and the laterals emerge from the main axis.
- Roots were cut into pieces bearing four laterals. The pieces were transferred to new culture medium separately, where laterals grew in length to produce new laterals.
- *Datta Bordoloi and Sarma* (1997) took explant from roots of sweet potato and cultured it in MS medium. They observed callus development after four weeks of incubation. Sub-culturing of callus produced shoot buds.

Shoot Tip Culture

- Cultivation of axillary or apical meristems is called *"Meristem culture".*
- Meristem culture involves development of already existing shoot meristem.
- Technique is used for quick vegetative propagation of a large number of plant species in a short period.
- Shoot tip culture produces virus free callus and eventually plantlet.





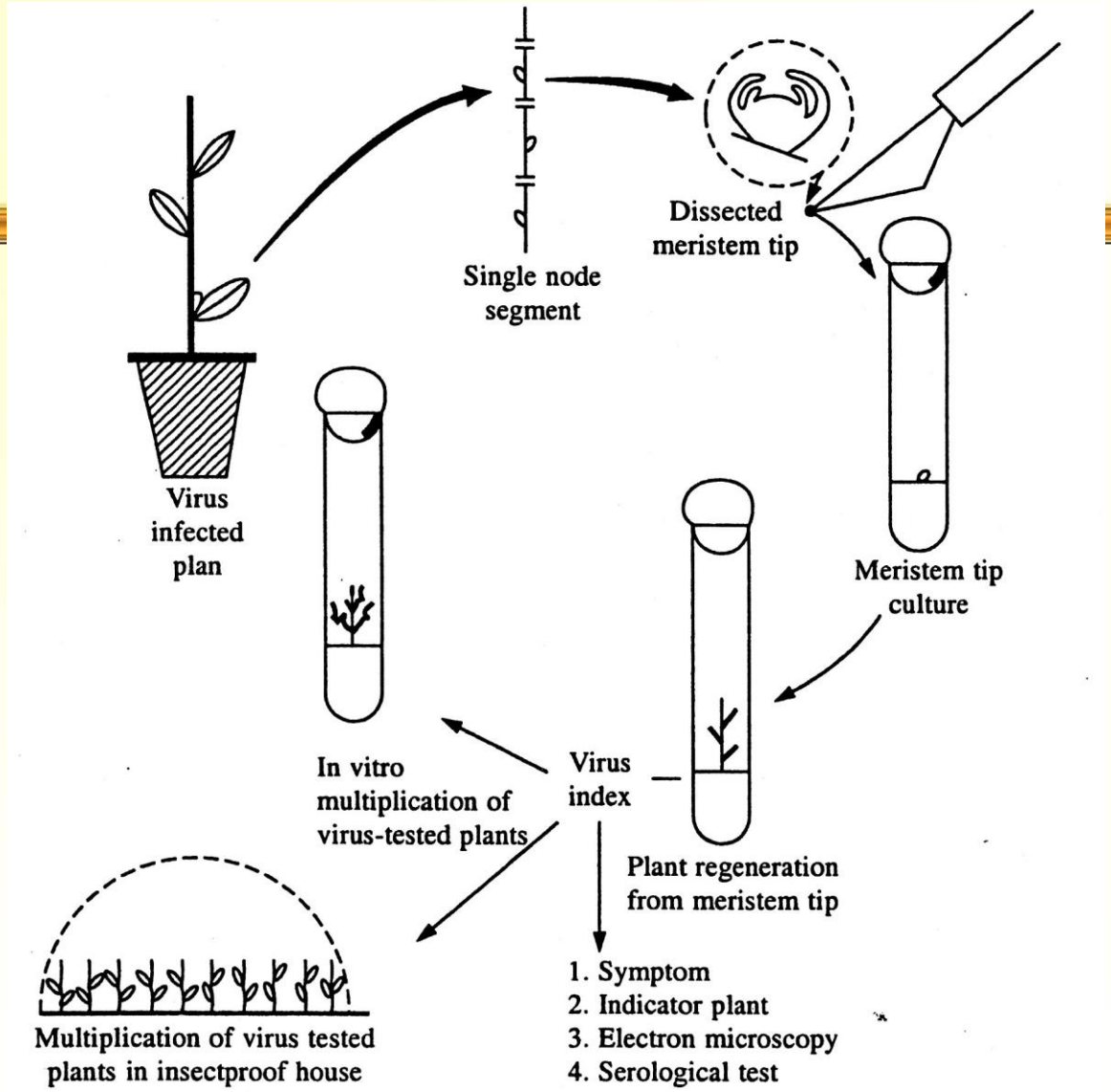


Fig. 25.4 Meristem culture

Leaf Culture

- Leaf culture is more simple, common and widely employed technique in tissue culture.
- it produces callus/plantlet very rapidly.
- explant were taken from different leaf portions, viz-apical, middle, basal region with midrib.
- Explants were grown in MS medium.
- After a week long incubation callus formation began.
- Whole explant turned into callus after 2 months.
- Subculturing of callus yielded plantlets.

Somatic Hybridization

- Production of hybrid-plants through the fusion of somatic cell protoplasts of two different plant species/varieties is called *"Somatic hybridization".*
- It is one of the methods of parasexual hybridization.
- It is an alternative mode of sexual hybridization.
- During the process neither meiosis nor fusion of gamete takes place.
- Such hybrids are called *somatic hybrids.*
- *Deng et al* (1992) sucessfully obtained inter generic somatic hybrids between *Fortunella crassifolia* and *Citrus sinensis.*

Somatic and sexual hybrids differ as follows:

1. Somatic hybrids are tetraploid, not diploid, as fusion occurs between two diploid somatic cells
2. This process does not involve meiosis
3. Nuclear gene of hybrid may be uniparental (cybrid) or biparental (hybrid)
4. Cytoplasmic contribution in the hybrid is equal from both parents.

1. Sexual hybrids are diploid as fusion occurs between two haploid gametes.
2. This process involves meiosis
3. Nuclear gene of the hybrid is essentially biparental. Each parent contributes equally and shows biparental inheritance
4. Cytoplasmic contribution in hybrids is unequal. It is mainly contributed by female parent. Male parent contributes only in the form of minute pollen grain. It shows maternal inheritance

- Somatic hybrids may be synkaryon or cybrid.
- In synkaryon, cytoplasm and nucleus of the two cells fuse
- In cybrid, nucleus of two cells does not fuse. Only cytoplasm of two cells fuse. Nucleus of only one cell remains. Other is removed before fusion.
- Literal meaning of cybrid is cytoplasm hybrid

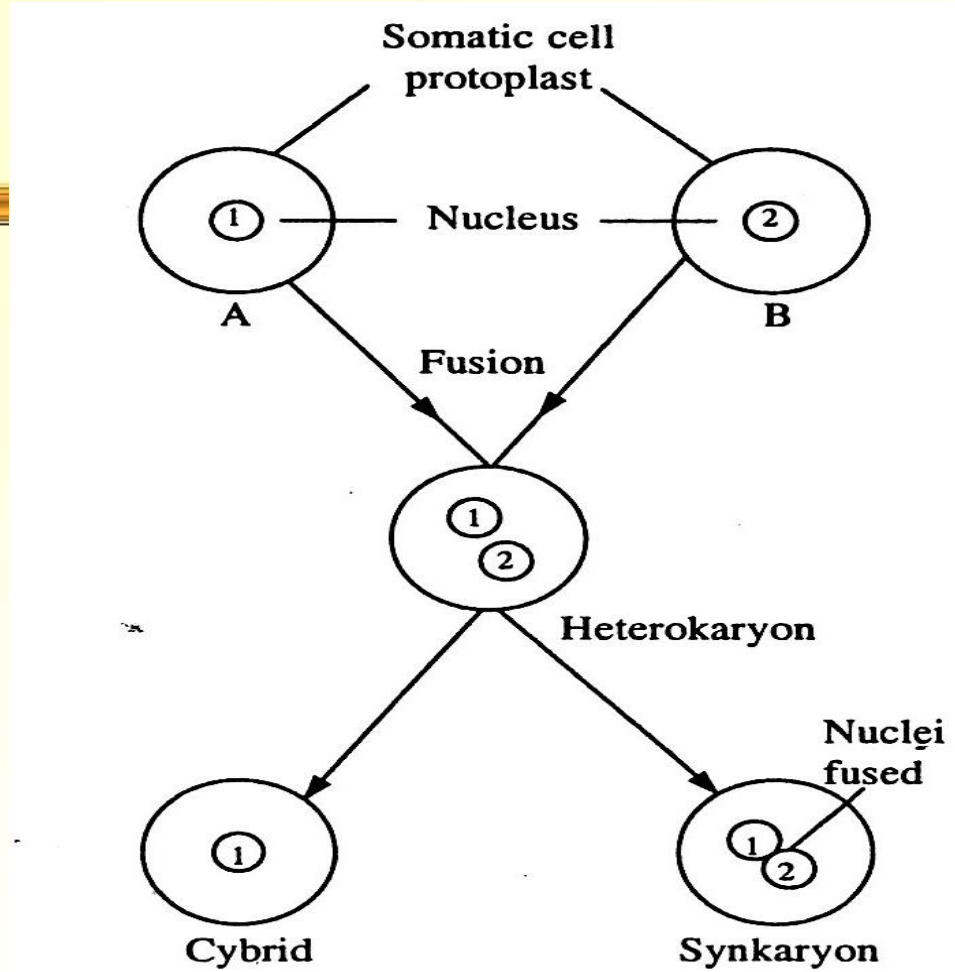


Fig. 25.5 Somatic hybrids

Steps of Somatic Hybridization

There are three steps of somatic hybridization:

(i) Isolation of protoplasts

(ii) Fusion of protoplasts of desired species

(iii) Culture of hybrid protoplast to produce whole plants.

to be cont….

Tutorial

Application of tissue culture

- Crop improvement
- Horticulture
- Synthetic seeds production
- Forestry
- Propagation of rare plants
- Production of secondary metabolite
- Shortening of breeding cycle
- Production of disease-free plants

Organ culture

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

Tissue Culture

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

Cell Culture

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

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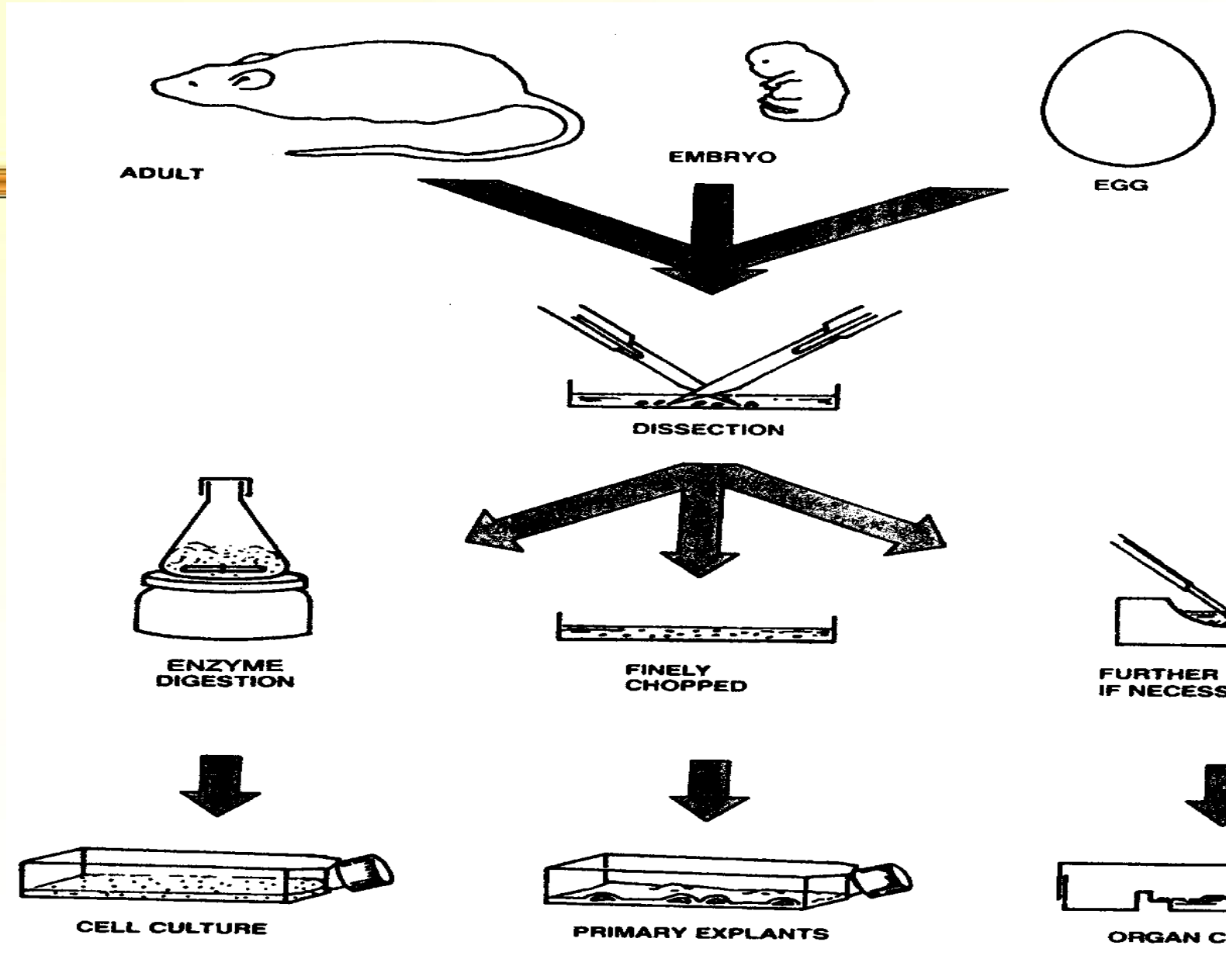
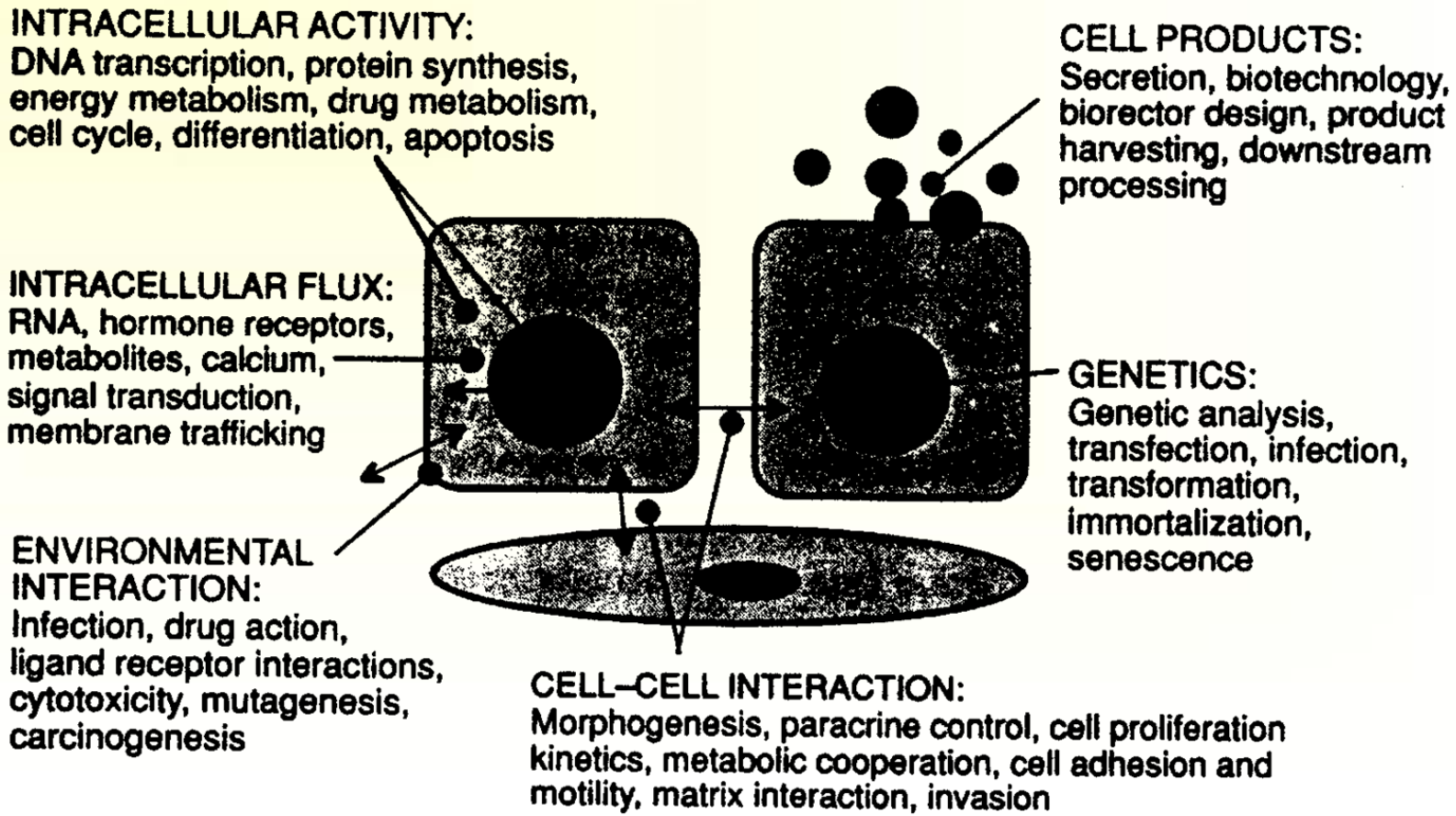


Why do we need Cell culture?

- <u>Research</u>
  - To overcome problems in studying cellular behavior such as:
    - confounding effects of the surrounding tissues
    - variations that might arise in animals under experimental stress
  - Reduce animal use
- <u>Commercial or large-scale production</u>
  - Production of cell material: vaccine, antibody, hormone

Cell culture application



Advantages of Cell culture

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

Types of Cell culture

1. **Primary Cultures**
   - Derived directly from excised tissue and cultured either as
     - Outgrowth of excised tissue in culture
     - Dissociation into single cells (by enzymatic digestion or mechanical dispersion)
   - Advantages:
     - usually retain many of the differentiated characteristics of the cell *in vivo*
   - Disadvantages:
     - initially heterogeneous but later become dominated by fibroblasts.
     - the preparation of primary cultures is labor intensive
     - can be maintained *in vitro* only for a limited period of time.

Types of Cell culture

2. Continuous Cultures

- derived from subculture (or passage, or transfer) of primary culture
  - Subculture = the process of dispersion and re-culture the cells after they have increased to occupy all of the available substrate in the culture
- usually comprised of a single cell type
- can be serially propagated in culture for several passages
- There are two types of continuous cultures
  - Cell lines
  - Continuous cell lines

Types of continuous culture

1) <u>Cell lines</u>
   - finite life, senesce after approximately thirty cycles of division
   - usually diploid and maintain some degree of differentiation.
   - it is essential to establish a system of Master and Working banks in order to maintain such lines for long periods

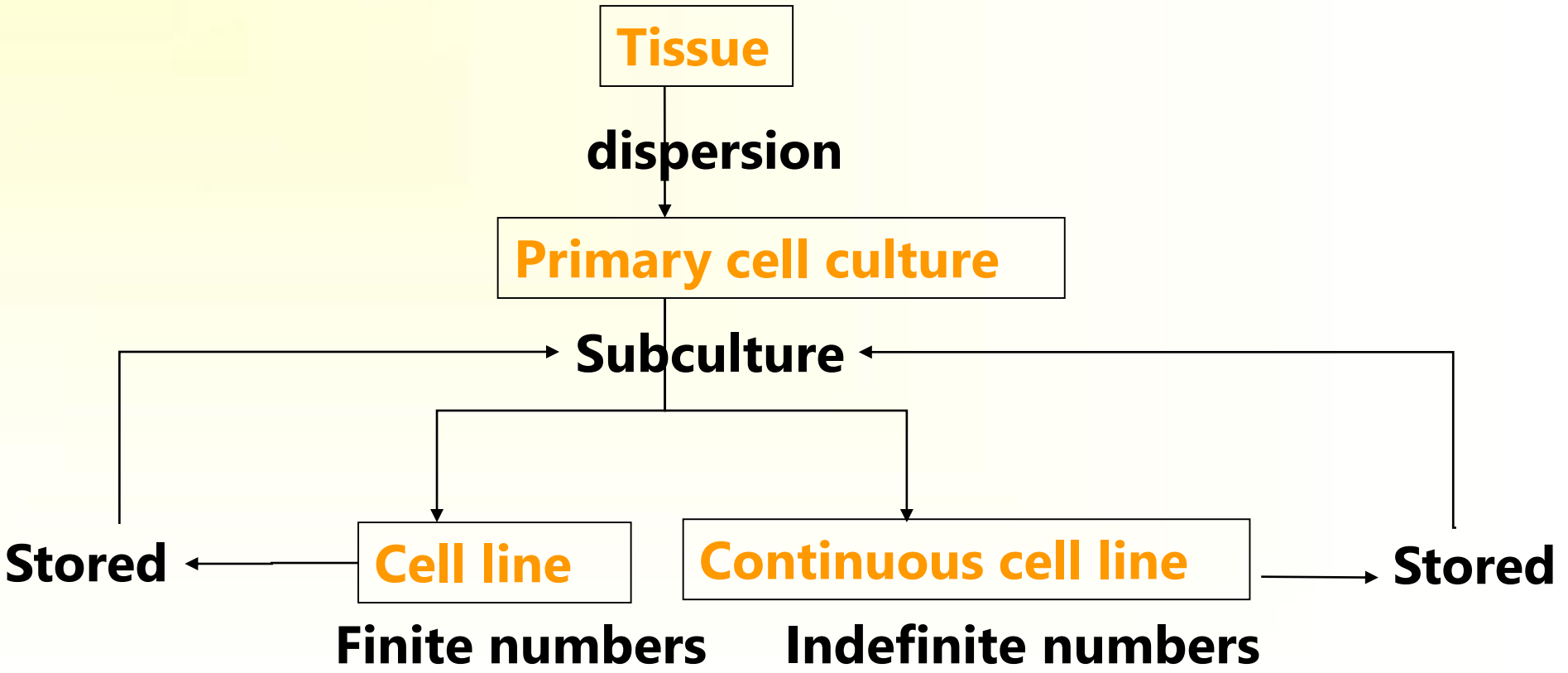
Types of continuous culture

2) <u>Continuous cell lines</u>
   - can be propagated indefinitely
   - generally have this ability because they have been transformed
     - tumor cells.
     - viral oncogenes
     - chemical treatments.
   - the disadvantage of having retained very little of the original *in vivo* characteristics

Transformation VS Transfection

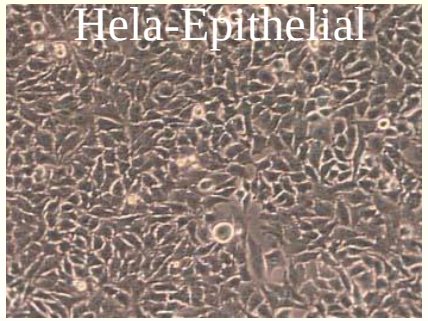
- Transformation
  - Spontaneous or induced permanent phenotypic changes resulting from change in DNA and gene expression
    - growth rate
    - mode of growth (loss of contact inhibition)
    - specialized product formation
    - longevity
    - loss of need for adhesion
- Transfection
  - Introduction of DNA into a cell (like viral DNA)

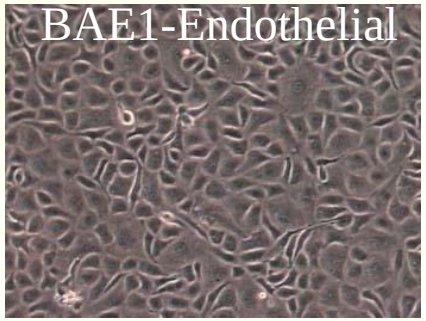
Initiation of culture

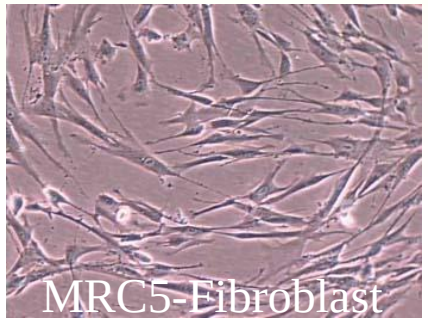


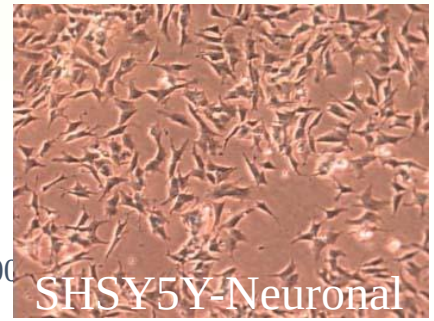
Cell Culture Morphology

- Morphologically cell cultures take one of two forms:
  - growing in suspension (as single cells or small free-floating clumps)
    - cell lines derived from blood (leukaemia, lymphoma)
  - growing as a monolayer that is attached to the tissue culture flask.
    - cells derived from solid tissue (lungs, kidney), endothelial, epithelial, neuronal, fibroblasts









Special types of Cell culture

Cells in the culture can be grown to adopt *in vivo* characteristic

- Histotypic culture
  - Single cell lineage
- Organotypic culture
  - Multiple cell lineages

Biology of Culture cells

- Cell growth and differentiation in the culture depends on:
  - The nature of cells
  - The culture environment
    - the nature of the substrate on which cell grow
    - the physicochemical and physiological constitution of culture medium
    - the constitution of gas phase
    - the incubation temperature
    - the cell-cell and cell-matrix interaction

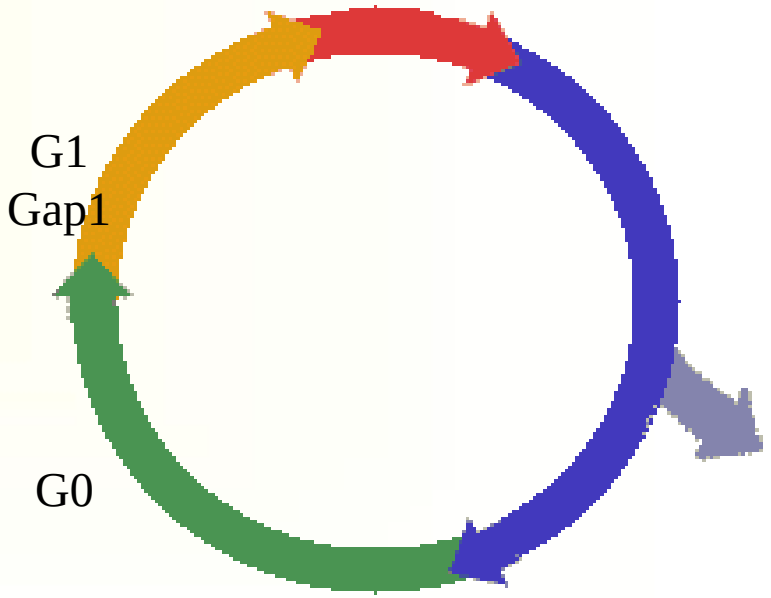
Cell cycle

G2 check point
- DNA replicated
- cell big
- environment suitable

G2
Gap2

M
Mitosis

Metaphase check point
- chromosome align on spindle



S
Synthesis

G1 check point
- cell big
- environment suitable

Cell cycle

- **Interphase:**
  - **generally lasts at least 12 to 24 hours in mammalian tissue**
  - **the cell is constantly synthesizing RNA, producing protein and growing in size**
    - **Gap 0 (G0):** cell will leave the cycle and quit dividing temporary or more permanent
    - **Gap 1 (G1):** Cells increase in size, RNA and protein synthesis, there is a G1 Checkpoint
    - **S Phase:** The DNA replication occurs
    - **Gap 2 (G2):** The cell will continue to grow and produce new proteins. There is a G2 Checkpoint
- **Mitosis or M Phase:**
  - **Cell growth and protein production stop**
  - **the cell cycle divides into two similar daughter cell**
  - **Mitosis last perhaps only one to two hours**
  - **there is a Checkpoint in the middle of mitosis (Metaphase Checkpoint) that ensures the cell is ready to complete cell division.**

Factors affecting cell proliferation

- Promotion of cell proliferation
  - low cell density (leaves the cell with free edge)
  - signals from environment: Growth factors
- Inhibition of cell proliferation
  - Density limitation: high cell density
  - Contact inhibition: cell contact
  - signals from environment: p53 gene product

Factors affecting cell diferentiation

- Cell differentiation is important for normal cell functions
- Factors promoting cell differentiations
  - high cell density
  - cell-cell and cell-matrix interaction
  - inducers: hydrocortisone, retinoid, matrix

Factors affecting cell adhesion

- Cell adhesion is important for cell proliferation and differentiation (signaling through cytoskeleton)
- Cell adhesion molecule
  - Cell-cell interaction: CAMs, cadherins
  - Cell-matrix interaction: integrin, transmembrame proteoglycan
- Tight junctional complex in epithelial cells for cell-cell interaction

Factors affecting cell adhesion

- Enzymatic disaggregation digests the adhesion molecule and extracellular matrix
- Most cells from solid tissues grow as adherent monolayer
- Matrix-coated surface promotes cell proliferation and differentiation

Factors affect cell culture success

- Appropriate cells
- Suitable environment
  - Solid phase
    - substrate or phase on which the cell grow eg. glass, plastic, collagen, agar
  - Liquid phase
    - physicochemical and physiological constitution of the medium
  - Gaseous phase
  - Temperature
  - Aseptic environment

Solid phase

- Anchorage dependent cells require a nontoxic, biologically inert to attach and allow movement for growth
- The most convenient vessels are polystyrene plastic
- other growth surface such as glass, filter wells
- The surface can be treated by
  - coated with matrix substrate eg. Collagen, poly-l-lysine, matrigel
  - Feeder layers: monolayer of supporting cells, perhaps promote cell growth and differentiation by cell contact and substance secreted
    - Neurons on glial cell feeder layers

Liquid phase

- Components of culture media
  - **Inorganic Salts**
    - retain the osmotic balance of the cells
    - regulate membrane potential by provision of sodium, potassium and calcium ions.
    - are required in the cell matrix for cell attachment and as enzyme cofactors.
  - **Carbohydrates**
    - Most media contain 4-20 mM glucose
    - main source of energy from glycolysis

Liquid phase

- **Proteins and Peptides**
  - are used to replace those normally present in serum eg. transferrin, fibronectin
- **Amino acids**
  - important for cell proliferation and differentiation
  - glutamine can enter Kreb's cycle
- **Fatty Acids and Lipids**
  - important in serum free media e.g. cholesterol and steroids essential for specialized cells.

Liquid phase

- **Vitamins**
  - vitamins B are necessary for cell growth and proliferation
  - precursors for numerous co-factors
  - The vitamins commonly used in media include thiamine, riboflavin and biotin
- **Trace Elements**
  - zinc, copper, selenium and tricarboxylic acid intermediates.
  - Selenium is a detoxifier and helps remove oxygen free radicals.

Liquid phase

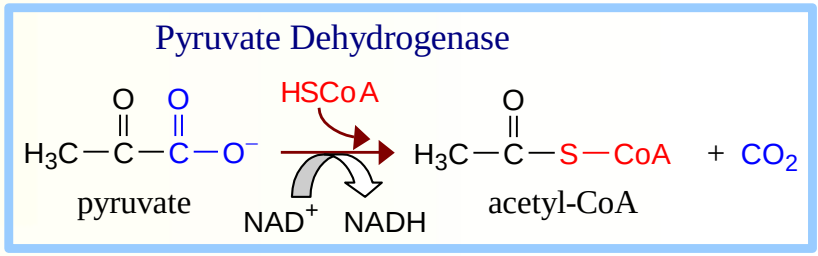
- **Buffering Systems**
  - most cells need optimal pH conditions in the range 7.2 - 7.4
  - close control of pH is essential for optimum culture conditions
    - bicarbonate/CO_2 buffering systems
    - Chemical buffering: HEPES
  - Most commercial culture media include phenol red as a pH indicator
    - yellow (acid) or purple (alkali)
- **Osmolarity**
  - similar to plasma osmolarity 290 mOsm

Liquid phase

- **Serum**
  - Undefined factors: complex mix of albumins, growth factors and growth inhibitors
  - increase the buffering capacity of cultures
  - able to bind and neutralize toxins
  - can be important for slow growing cells or where the seeding density is low
  - Subject to batch to batch variation
  - Heat inactivation of serum (incubation at 56ºC for 30 minutes) can help to reduce the risk of contamination

Gaseous phase

- Carbondioxide
  - important for buffering system
    - 5-10% CO_2
    - Endogenous production: pyruvate



- Oxygen
  - most cells in culture require low oxygen tension
  - anaerobic glycolysis
  - high oxygen can produce toxic free radical

Temperature

- The optimum temperature depends on
  - the body temperature of animals from which the cells were obtained
  - anatomical variation of temperature (skin temperature may be lower than the rest of the body)




Aseptic techniques

- Microorganism remains a major problem in cell culture
- prevention of contamination
  - Antibiotics
  - improvement of laboratory condition
  - Aseptic techniques
    - Clean and tidy work surface
    - Personal hygiene
      - hand washing
      - caps, gowns, face mask
    - Reagents and media
    - Culture vessels

Cryopreservation of Cell Lines

- The aim of cryopreservation is to enable stocks of cells to be stored to prevent the need to have all cell lines in culture at all times
- Reduced risk of microbial contamination
- Reduced risk of cross contamination with other cell lines
- Reduced risk of genetic drift and morphological changes
- Work conducted using cells at a consistent passage number
- Reduced costs (consumables and staff time)

Cryopreservation of Cell Lines

Method	Advantages	Disadvantages
Electric (-135ºC) Freezer	• Ease of maintenance • Steady temperature • Low running costs	• Requires liquid nitrogen back-up • Mechanically complex • Storage temperatures high relative to liquid nitrogen
Liquid Phase Nitrogen	• Steady ultra-low (-196ºC) temperature • Simplicity and mechanical reliability	• Requires regular supply of liquid nitrogen • High running costs • Risk of cross-contamination via the liquid nitrogen • - 196ºC
Vapor Phase Nitrogen	• No risk of cross-contamination from liquid nitrogen • Low temperatures achieved • Simplicity and reliability	• Requires regular supply of liquid nitrogen • High running costs • Temperature fluctuations between - 135ºC and - 190ºC

Risk Assessment

Risks depend on:

- Source of material
- the nature of operation being carried out

Assesment:

- Pathogenicity
- Route of transmission
- Agent stability
- Infectious dose
- Concentration
- Availability of data from animal studies
- Availability of an effective prophylaxis
- Medical surveillance
- Experience and skill level of at-risk personnel

Risk groups for animal cell culture

- The level of risk depends on the cell line to be used and is based on whether the cell line is likely to cause harm to humans.
- **Low risk**
  - Non human/non primate continuous cell lines and some well characterized human diploid lines of finite lifespan
- **Medium risk**
  - Poorly characterized mammalian cell lines.
- **High risk**
  - Cell lines derived from human/primate tissue or blood.
  - Cell lines with endogenous pathogens (the precise categorization is dependent upon the pathogen)
  - Cell lines used following experimental infection where the categorization is dependent upon the infecting agent

Safety aspects of cell culture

- **SAFETY CONSIDERATIONS**
- Assume all cultures are hazardous since they may harbor latent viruses or other organisms
- The following safety precautions should also be observed:
  - pipetting: use pipette aids to prevent ingestion
  - keep aerosols down to a minimum
  - no eating, drinking, or smoking
  - wash hands after handling cultures and before leaving the lab
  - decontaminate work surfaces with disinfectant (before and after)
  - autoclave all waste
  - use biological safety cabinet (laminar flow hood)
  - use aseptic technique
  - dispose of all liquid waste after each experiment and treat with bleach

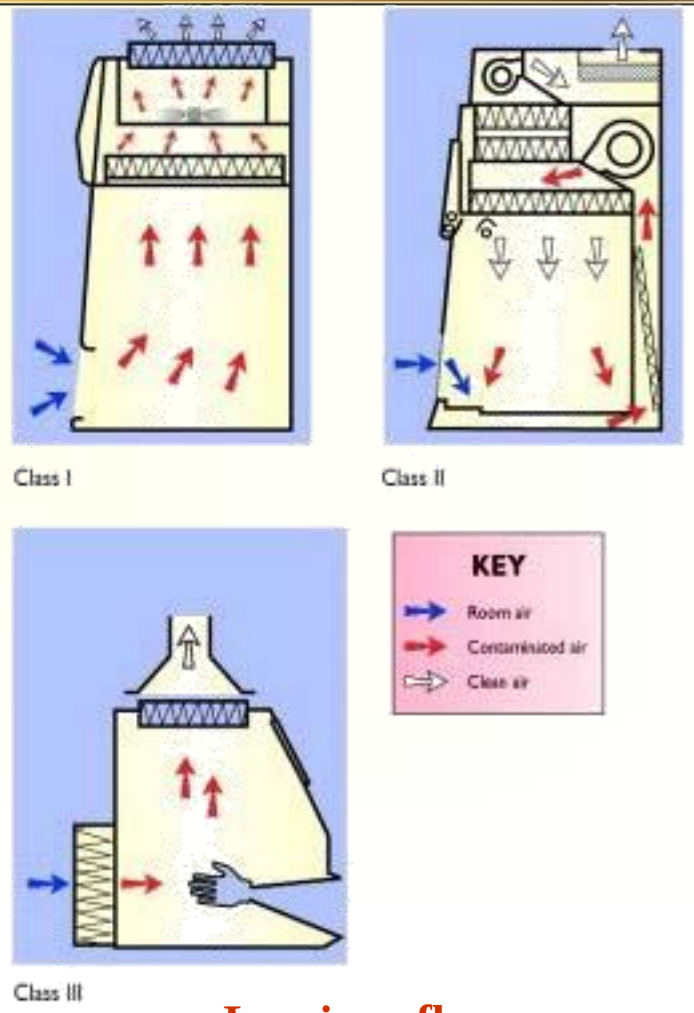
Risk Group (RG)

Classification is based on the potential effect of biological agent on healthy human adult

- RG1-agents are not associated with disease
- RG2-agents are associated with human disease which is rarely serious and for which preventive or therapeutic interventions are ***often*** available
- RG3-agents are associated with serious or lethal human disease for which preventive or therapeutic interventions ***may be*** available
- RG4-agents are likely to cause serious or lethal human disease for which preventive or therapeutic interventions are ***not usually*** available

Biosafety cabinets

- The Class I BSC provides personnel and environmental protection, but no product protection.
- The Class I BSC is hard-ducted to the building exhaust system, thimble-connected, or recirculated back into the room depending on use.
- The Class II (Types A, B1, B2, and B3)24 biological safety cabinets provide personnel, environmental and product protection.



Laminar flow

Recommended Biosafety Levels for Infectious Agents

BSL	Agents	Practices	Safety Equipment (Primary Barriers)	Facilities (Secondary Barriers)
1	Not known to consistently cause disease in healthy adults	Standard Microbiological Practices	None required	Open bench top sink required
2	Associated with human disease, hazard = percutaneous injury, ingestion, mucous membrane exposure	BSL-1 practice plus: • Limited access • Biohazard warning signs • "Sharps" precautions • Biosafety manual defining any needed waste decontamination or medical surveillance policies	Primary barriers = Class I or II BSCs or other physical containment devices used for all manipulations of agents that cause splashes or aerosols of infectious materials; PPEs: laboratory coats; gloves; face protection as needed	BSL-1 plus: Autoclave available
3	Indigenous or exotic agents with potential for aerosol transmission; disease may have serious or lethal consequences	BSL-2 practice plus: • Controlled access • Decontamination of all waste • Decontamination of lab clothing before laundering • Baseline serum	Primary barriers = Class I or II BCSs or other physical containment devices used for all open manipulations of agents; PPEs: protective lab clothing; gloves; respiratory protection as needed	BSL-2 plus: • Physical separation from access corridors • Self-closing, double-door access • Exhausted air not recirculated • Negative airflow into laboratory
4	Dangerous/exotic agents which pose high risk of life-threatening disease, aerosol-transmitted lab infections; or related agents with unknown risk of transmission	BSL-3 practices plus: • Clothing change before entering • Shower on exit • All material decontaminated on exit from facility	Primary barriers = All procedures conducted in Class III BSCs or Class I or II BSCs in combination with full-body, air-supplied, positive pressure personnel suit	BSL-3 plus: • Separate building or isolated zone • Dedicated supply and exhaust, vacuum, and decon systems • Other requirements outlined in the text

References

- R. Ian Freshney. Culture of Animal cells *a manual of basic technique*. 4th edition. Wiley-Liss, New York. 2000.

Tissue culture

P2 Room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ANIMAL CELL CULTURE


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