BASICS OF ANIMAL CELL CULTURE

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Cell Culture

The maintenance of cells outside of the living animal (in vitro) for easier experimental manipulation and regulation of controls.

- **Pros**
  - Use of animals reduced
  - Cells from one cell line are homogenous and have same growth requirements, optimizing growing patterns.
  - *In vitro* models allow for control of the extracellular environment
  - Able to monitor various elements and secretions without interference from other biological molecules that occurs *in vivo*
Classification of Cell Cultures

- **Primary Culture**
  - Cells taken directly from a tissue to a dish

- **Secondary Culture**
  - Cells taken from a primary culture and passed or divided *in vitro*.
  - These cells have a limited number of divisions or passages. After the limit, they will undergo *apoptosis*.

- Apoptosis is programmed cell death
Making a Primary Culture

1. Remove tissue
2. Mince or chop
3. Digest with proteolytic enzymes
4. Place in culture

Enzymatic Dissociation
Types of cell culture

Primary culture
- Tissue or organ fragment
  - Transfer to culture
    - Primary culture
      - Subculture (passage)
        - Cell line
          - Continuous
          - Finite
          - Stem cell

Secondary culture
- Example: Embryonic Fibroblasts
- Example: HeLa
- Example: Stem Cells
Culture characteristics

**Suspension:**
- ATCC Number: CRL-13007
- Designation: IMM002.69.47.4

**Adherent:**
- ATCC Number: CCL-2
- Designation: HeLa

**Fibroblast-like cell**

**Epithelium-like cell**
Confluency Measure

At the time of seeding

After attachment

Complete Monolayer
SOURCING CELL LINES
Sources of cell lines

American Type Culture Collection ATCC
  – www.atcc.org

• NIH
  – Coriel Cell Repositories http://locus.umdnj.edu/ccr/

• European Collection of Cell Culture
  – www.ecacc.org.uk

• DSMZ (German Collection of Microorganisms and Cell Cultures)
  - www.dsmz.de (very good for plant cell lines)

- NCCS, Pune
PRE-REQUISITES FOR STARTING CELL CULTURE LAB
Essential Equipment

- Laminar flow hood
- (biological safety cabinet)
- CO2 incubator (for most cells)
- Inverted microscope
- Pipette aid
- Aspiration pump
- Centrifuge
- Water bath
- Cold storage (refrigerator)
- Cryopreservation equipment
Laminar flow hood
Horizontal Air Flow Hood

HEPA Filters
Filtered Air
Room Air

HEPA Filters
CO₂ incubator

- maintains CO₂ level (5-10%), humidity and temperature (37° C) to simulate in vivo conditions.
Water bath

- To warm media, TRED (Trypsin-EDTA) and PBS (phosphate buffered saline) before placing on cells
- Can harbor fungi and bacteria, spray all items with 70% ethanol before placing in the hood.
- Usually takes 10-15 minutes for media to warm, 5-10 for TRED to thaw
Vacuum pump

- For permanent aspiration of liquids (media, PBS and TRED).
- Use unplugged glass pasteur pipets, throw into sharps box when done.
Inverted Phase Microscope

- A phase contrast microscope with objectives *below* the specimen.
- A phase plate with an annulus will aid in exploiting differences in refractive indices in different areas of the cells and surrounding areas, creating contrast.
A comparison

Phase contrast microscopy
Can be used on living cells

Light microscopy
Requires stain, thus killing cells
Liquid Nitrogen containers

- Contamination by microorganisms and other cell lines
- Need for distribution to other users

Equipment for Cryopreservation

Liquid nitrogen
Liquid phase (-196°C) or vapor phase (-156°C)
Cell culture vessels

- Tissue culture flasks
- Multi well plates
- Roller bottle
- Spinner flask
- Bioreactor
Small scale cultivation

Culture Plate

Culture flask
Tissue culture Flasks

Filter Caps
Large scale cultivation

Culture bottle

Roller Bottle

Culture dish

Stack culture chamber
MEDIA AND IT’S COMPONENT
Basic Constituents

Cell culture medium

- Water: 68%
- Inorganic salts: 11%
- Amino acids: 2%
- Vitamins: 1%
- Sugars: 5%
- Other: 3%
- Serum: 10%

Image: Bottles containing cell culture medium.
• **Energy sources**- eg. Glucose, Galactose

• **Serum** – eg. FBS, FCS. Complex mixture of albumins, growth factors and growth inhibitors

• **Trace Elements**- eg. Co-factors (Zn, Cu) for biochemical pathways

• **Amino Acids**- eg. Glutamine, Cystine

• **Vitamins** - Metabolic co-enzymes for cell replication

• **Antibiotics** – eg. Penicillin, Streptomycin etc.

• **Balanced salt solutions**, Inorganic ions-Osmotic balance – cell volume

**Ready made Media:** RPMI (Roswell Park Memorial Institute medium), DMEM (Dulbecco's Modified Eagle's medium), MEM (Minimum Essential Medium (MEM)), developed by Harry Eagle
Serum and its importance

- Complex mixture of albumins, growth factors and growth inhibitors.
- Increase the buffering capacity
- Protect against mechanical damage
- Source of growth factors
- Bind and neutralize toxins.

- Risk of contamination: BVDV virus, mycoplasma.
- Consistency in quality
PART - 2
CELL CULTURE TECHNIQUES
Media Preparation and Filtration

- Powder media
- Reconstitute in D/w
- Dissolve completely
- Adjust pH
- Filter
Sub culturing

Cells are harvested when the cells have reached a population density, which suppresses growth. Ideally, cells are harvested when they are in a semi-confluent state and are still in log phase.
Subculture – Adherent Cells

Routine maintenance of cell lines is called subculture.

<table>
<thead>
<tr>
<th>Flasks</th>
<th>PBS</th>
<th>Trypsin</th>
<th>Cell Seeding</th>
</tr>
</thead>
<tbody>
<tr>
<td>T25 cm²</td>
<td>5-7ml</td>
<td>0.5 ml</td>
<td>1-5 x 10⁵ cells</td>
</tr>
<tr>
<td>T75 cm²</td>
<td>10-15 ml</td>
<td>1.0 ml</td>
<td>9-20 x 10⁵ cells</td>
</tr>
<tr>
<td>T175 / T180 cm²</td>
<td>20-25 ml</td>
<td>4.0 - 5.0 ml</td>
<td>30-45 x 10⁵ cells</td>
</tr>
<tr>
<td>Roller Bottles</td>
<td>~50 mL</td>
<td>5.0 - 10.0 mL</td>
<td>~ 10 x 10⁶ cells</td>
</tr>
</tbody>
</table>

Caution: When adding (or replacing) medium, never touch the neck of the culture flasks with the bottle containing the medium or use the same pipette to transfer medium to more than one bottle. Ideally, aliquot the total amount of medium required for each batch of culture bottles being handled and store the remainder at 4–8°C. Dedicate separate medium for each cell line.
Sub culturing - adherent cell line

1. **Trypsin-EDTA addition:** incubate in RT/37 deg for 1-3 min.
   - if ~80-90% confluency
   - Give media change
   - Incubate back
   - Give PBS wash
   - Aspirate spent media
   - Observe under microscope - till ~80% cells appear rounded
   - Aspirate Trypsin-EDTA soln. completely
   - 1-3 sharp taps from the surface of flask
   - Addition of growth media dispersion of cells to single cell suspension – repeated pipetting

Good laboratory practice: Work with one cell line at a time.
Cell viability

- Cell viability is determined by staining the cells with trypan blue.
- As trypan blue dye is permeable to non-viable cells or death cells whereas it is impermeable to this dye.
- Stain the cells with trypan dye and load to haemocytometer and calculate % of viable cells.

\[
\text{% of viable cells} = \frac{\text{No. of unstained cells}}{\text{Total no. of cells}} \times 100
\]
Cell counting-
Manual

% of viable cells = \( \frac{\text{No. of unstained cells}}{\text{Total no. of cells}} \times 100 \)
Hemacytometer

- Specialized chamber with etched grid used to count the number of cells in a sample.
- Use of trypan blue allows differentiation between living and dead cells.
Looking at the grid under the phase contrast microscope
Count 10 squares
Any 10 will do but we will follow convention

Watch for stringy, reddish material—those aren’t cells!
Automated Cell Counter
Cryopreservation

The process of preservation of cells by freezing at extremely low temperatures using cryo-protective agents (DMSO), so as to maintain the existing form, structure and chemical composition of all the constituent elements of the specimens for future use.
General Tips:

• Do not use overgrown cells.
• Make sure that there are no clumps
• Preserve correct number of cells per vial (1 – 10 x 10^6 cells / ml).
• Do not leave cells in DMSO for long.
• Seal the vials tightly.
• Label the vials appropriately.
**Cell banking**

Collection of containers of uniform composition stored under defined conditions, each containing an aliquot of a single pool of cells.
Problem: Rapid pH shift in medium

Red-Alkaline pH  Neutral pH  Acidic pH

Possible Causes:
1. Incorrect carbon dioxide (CO₂) tension
   Action: Increase or decrease percentage of CO₂ in the incubator based on concentration of sodium bicarbonate in medium.
2. Overly tight caps on tissue culture flasks – No penetration of CO₂
   Action: Loosen caps one-quarter turn.
3. Insufficient bicarbonate buffering
   Action: Add HEPES buffer.
4. Incorrect salts in medium
   Action: Check for the Media Composition.
5. Bacterial, yeast, or fungal contamination
   Action: Discard culture and medium or try to decontaminate culture.
Fungal Contamination

- Fungus-Molds
- Fungal - yeast

Come through:
Improper Handling, Gowning, Cleaning

Aseptic handling

**Antibiotics:** Amphotericin B, Mycostatin
Antibiotics: Penicillin, Streptomycin, Gentamycin

Bacterial contamination

Come through:
Improper Cleaning, handling, cross contamination

Antibiotics: Penicillin, Streptomycin, Gentamycin
Applications of Cell culture
Biopharmaceuticals

Are medical drugs produced using **biotechnology**. They are **proteins** (including **antibodies**), **nucleic acids** (DNA, RNA or **antisense oligonucleotides**) used for therapeutic or **in vivo** diagnostic purposes, and are produced by means other than direct extraction from a native (non-engineered) **biological** source.

Blood factors ([Factor VIII](#) and [Factor IX](#))
Thrombolytic agents ([tissue plasminogen activator](#))
**Hormones** ([insulin](#), glucagon, growth hormone, gonadotrophins)
Haematopoietic growth factors ([Erythropoietin](#), [colony stimulating factors](#))
**Interferons** ([Interferons-α, -β, -γ](#))
**Interleukin**-based products ([Interleukin-2](#))
**Vaccines** ([Hepatitis B](#) surface **antigen**)
**Monoclonal antibodies** ([Various](#))
Additional products ([tumour necrosis factor](#), therapeutic enzymes)
Antibodies Production:

**Examples:** Transplant rejection - Muronomab-CD3-CD28
Cardiovascular disease - Abciximab
Cancer - Rituximab
Infectious Diseases - Palivizumab
Inflammatory disease – Infliximab

**Cell line used:** CHO, NSO, SP20

Recombinant proteins:

**Example:** Tissue plasminogen activator (t-PA)

EPO
Blood clotting factors

**Cell lines used:** CHO-K1 cells, Baby Hamster Kidney Cells (BHK)
Vaccine Production

Example: Rabies vaccine
Polio vaccine

Cell lines used: Chick embryo, Vero (African green monkey kidney epithelial cell line)
Cell biology

1. Studies on intracellular activity, e.g. cell cycle and differentiation, metabolism, drug metabolism, transcription, translation, energy metabolism

2. Elucidations of intracellular flux, e.g. hormonal receptors, signal transduction, nutritional studies, membrane trafficking, metabolites
Stem cell Therapy

Definition:
A cell that has the ability to continuously divide and differentiate (develop) into various other kinds of cells/tissues

SO.....WHAT ARE STEM CELLS?

• CELLS THAT CAN MAKE MORE OF THEMSELVES

• CELLS THAT CAN BECOME ALMOST ANY CELL - MULTIPOTENT
Gene therapy
Definition: Genetic alteration of somatic cells to treat disease.

The insertion of genes into an individual's cells and tissues to treat a disease, such as a hereditary disease in which a deleterious mutant allele is replaced with a functional one.
Currently gene therapy is being developed to treat a variety of genetic diseases and disorders as well as vascular disease, immune deficiencies, neurodegenerative diseases, blood disorder and some cancers. Example: thalassaemia, cystic fibrosis, Sickle Cell Disease.
Bioassays

Modern scientific assessment of drug safety is increasingly using cell-based assays.

Cell based assays are used to assess drug behavior in the body (pharmacokinetics, drug metabolism), genotoxic liabilities, developmental toxicity (teratogenicity), cardiac toxicities, potential drug-drug interactions and other distinct toxicological mechanisms.

Cell lines used: WISH, M-NFS60, UT7, UMR106
Toxicity Studies

**Toxicology** is the study of the adverse effects of chemicals on living organisms.

*In vitro* toxicity testing is the scientific analysis of the effects of toxic chemical substances on cultured mammalian cells. *In vitro* testing methods are employed primarily to identify potentially hazardous chemicals and/or to confirm the lack of certain toxic properties in the early stages of the development such as therapeutic drugs, agricultural chemicals and direct food additives that may or may not taste good.

Cell viability (cytotoxicity) assays used in *In-vitro* toxicology

Ex-NFS 60
**Tissue Culture Application (II)**

1. Production of antiviral vaccines

2. Understanding of neoplasia (cancer research)

3. Transfer of DNA to the cultured cells (or siRNA)

4. **Monoclonal antibody production** (immunology)

5. Production of human growth hormone, insulin, interferon

6. Stem cell culture differentiate into neurons

7. Implanting normal fetal neurons into patients with Parkinson diseases

8. Homografting and reconstructive surgery using individual’s own cells (tissue engineering)

9. In vitro fertilization (embryo culture)
Table 1-2 **Advantages of Tissue Culture**

<table>
<thead>
<tr>
<th>Category</th>
<th>Advantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Physico-chemical environment</td>
<td>Control of pH, temperature, osmolarity, dissolved gases (O₂ &amp; CO₂)</td>
</tr>
<tr>
<td>Physiological conditions</td>
<td>Control of hormone and nutrient concentrations</td>
</tr>
<tr>
<td>Microenvironment</td>
<td>Regulation of matrix, cell-cell interaction, gaseous diffusion</td>
</tr>
<tr>
<td>Cell line homogeneity</td>
<td>Availability of selective media, cloning</td>
</tr>
<tr>
<td>Characterization</td>
<td>Cytology and immunostaining are easily performed</td>
</tr>
<tr>
<td>Preservation</td>
<td>Can be stored in liquid nitrogen</td>
</tr>
<tr>
<td>Validation &amp; accreditation</td>
<td>Origin, history, purity can be recorded</td>
</tr>
<tr>
<td>Replicates and variability</td>
<td>Quantitation is easy</td>
</tr>
<tr>
<td>Reagent saving</td>
<td>Reduced volumes, direct access, lower cost</td>
</tr>
<tr>
<td>Control of C × T</td>
<td>Ability to define dose, concentration, and time</td>
</tr>
<tr>
<td>Mechanization</td>
<td>Available with microtitration and robotics</td>
</tr>
<tr>
<td>Reduction of animal use</td>
<td>Cytotoxicity and screening of pharmaceutics, cosmetics, etc.</td>
</tr>
</tbody>
</table>

Poorly defined materials: serum, supplementations, matrix,...
Cell line homogeneity

“After one or two passages, cultured cell lines assume a homogeneous constitution,
as the cells are randomly mixed at each transfer and the selective pressure of the
culture condition tends to produce a homogeneous culture of
the most vigorous cell type”

? ?
"Less reagent is required than for injection in vivo, where 90% is lost by excretion and distribution to tissues other than the interested cells under study."

**In Vitro Dosage v.s. In Vivo Dosage**

??
Reduction of animal use

“In vitro modeling of in vivo conditions”
## Table 1-3
**Limitations of Tissue Culture**

<table>
<thead>
<tr>
<th>Category</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Necessary expertise</td>
<td>Handling, Chemical contamination, Microbial contamination, Cross contamination</td>
</tr>
<tr>
<td>Environmental control</td>
<td>Workplace, Incubation, pH control, Containment and disposal of biohazards</td>
</tr>
<tr>
<td>Quantity and cost</td>
<td>Capital equipment, Consumables, Medium, serum, plastics, Heterogeneity, variability, Dedifferentiation</td>
</tr>
<tr>
<td>Genetic instability</td>
<td>Adaptation, Selection</td>
</tr>
<tr>
<td>Phenotypic instability</td>
<td>Expression of markers, Histology, cytology, Geometry and microenvironment</td>
</tr>
<tr>
<td>Identification of cell type</td>
<td></td>
</tr>
</tbody>
</table>
Microbial Contamination

“Animal cells grow much less rapidly than contaminants (bacteria, molds, yeasts)”

Cross-Contamination

“Many cell lines in common use are not what they are claimed to be, but have been cross-contaminated with HeLa or other growing cell line”
Cross-Contaminated cell lines

”HeLa cell”

George Otto Gey

Henrietta Lacks

”HeLa cell”

Cervical Cancer

http://en.wikipedia.org/wiki/Henrietta_Lacks
Cost

“The cost of producing cells in culture is about 10 times that of using animal tissue”

“Semimicro- or Micro-Assays”: reduced manipulation time (Quicker!!)
Genetic and Phenotypic Instability

“Dedifferentiation: a process assumed to be the reversal of differentiation”

“overgrowth of undifferentiated cells”

“Loss of the phenotypic characteristics typical of the tissue”

Possible Reasons:

(1) Specific cell interactions characteristics of the histology of the tissue are lost;

(2) The culture environment lacks systemically homeostatic regulation systems (nervous and endocrine system)
Cell Culture (II)

7. The derivation of continuous cell line or cell strain usually implies a phenotypic change, or transformation.

8. Cultured cell lines are more representative of precursor cells [most differentiated cells do not divide].

9. Cultured cells lack the potential for cell-cell and cell-matrix interaction.