Introduction to animal cell culture
CELL CULTURE
Why do it?

- Make proteins: commercial scale
- Stem and cancer cells
- Primary Human and animal Cell culture
- Antibody production: monoclonals
- Gene Therapy

Embryo culture
Cell Culture: why do it?

- Tool for the study of animal cell biology using convenient *in vitro* model of cell growth

- Mimic of *in vivo* cell behaviour (e.g. cancer cells)

- Artificial (some cell types are thus difficult to culture)

- Highly selective and defined environment which is easily manipulated (used to optimise cell signalling pathways)
Cell Culture is a *Fussy Discipline*

In the tissue culture laboratory:

- bench tops should be kept clear and clean
- wearing a long sleeve lab coat: minimises contamination from street clothing (hair, etc)
- wearing gloves while doing tissue culture work: minimises contamination from skin organisms
- Surfaces, gloves, solutions and plasticware sprayed with 70% alcohol before placed into the biological hood
- solutions, reagents and glassware used in tissue culture work should not be shared with non-tissue culture work
Primary application of animal cell culture in the investigation of:

- Mechanisms of cell cycle control
- Characteristics of cancer cells
- Detection, production and function of:
  - growth factors
  - hormones
  - viruses
- The study of:
  - differentiation processes
  - specialised cell function
  - cell-cell and cell-matrix interactions
Primary culture vs Cell line

**Primary culture** freshly isolated from tissue source

**Cell line**
- Finite cell line: dies after several sub-cultures
- Continuous cell line: transformed ‘immortal’

*In our lab: C2C12 immortalised skeletal muscle cell line*

Myoblasts were extracted from the thigh muscle of C3H mice 70 h after a crush injury and cultured. They became immortalised. (Yaffe and Saxel, 1977). Very useful tool to study effects of various factors on myoblast proliferation and differentiation and myotube formation.

*Note: mouse cells readily immortalise whereas human cells do NOT. This is due to telomerase* (discussed later under ageing).

- Can STORE cells, cryopreserved in liquid nitrogen for years
Passaging or sub-culture

Cells dissociated from flask using enzymes

Split 1 into 2 flasks
Contact inhibition

Therefore need to split them to maintain growth
Initiation, establishment and propagation of cell cultures
Cultures can be initiated from tissue or organ fragments, single cell suspensions, and other sources.

Choices to be made include:
- Disaggregation techniques
- Media
- Culture conditions
- Selection procedures
Considerations

- Sensitivity to mechanical dispersal or enzymes; cell-cell contact may be required for proliferation
- Dispersed cells in culture are vulnerable
- Most primary cells require satisfactory adherence
- Some cells are not normally adherent in vivo and can be grown in liquid suspension
- In a mixed primary culture differences in growth rate may mean a loss of the cell type of interest – selection techniques
- Some cells are prone to spontaneous transformation
- Limited life span of some cultures
(1) Dispersal of tissues

- **Mechanical**
  - Mincing, shearing, sieves

- **Chemical**

- **Enzymatic (proteases that affect ECM)**
  - Trypsin, pronase, collagenase, dispase

- Can be a combination
The cell culture environment
8 well culture dish. Allows comparison of 8 samples: can have different stains or are fixed at different times. THEN- remove wells and gasket. Leaves ONE slide with 8 separate samples for easy microscopic analysis of (stained) cells

96 well plate Allows comparison of many culture conditions. Samples often in triplicate.
Factors affecting cell behaviour in the complex *in vivo* environment

- The local micro-environment: metabolites, local growth factors, ECM, architecture
- Cell-cell interactions
- Circulating proteins, cytokines, hormones

How to best mimic this in vitro?
(2) Culture Surface

Most adherent cells require attachment to proliferate

Change charge of the surface
  - Poly-L-lysine

Coating with matrix proteins
  - Collagen, laminin, gelatin, fibronectin
(3) Media formulation

- Initial studies used body fluids
  - Plasma, lymph, serum, tissue extracts

- Early basal media
  - Salts, amino acids, sugars, vitamins supplemented with serum

- More defined media
  - Cell specific extremely complex

- PLUS SERUM
# DMEM

<table>
<thead>
<tr>
<th>COMPONENTS</th>
<th>Molecular Weight</th>
<th>Concentration (mg/L)</th>
<th>Molarity (mM)</th>
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<tbody>
<tr>
<td><strong>Amino Acids</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Glycine</td>
<td>75</td>
<td>30</td>
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<tr>
<td>L-Arginine</td>
<td>217</td>
<td>962</td>
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<td>L-Ornithine</td>
<td>211</td>
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<td>L-Histidine</td>
<td>210</td>
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<td>L-Idiocline</td>
<td>131</td>
<td>105</td>
<td>0.602</td>
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<tr>
<td>L-Leucine</td>
<td>131</td>
<td>105</td>
<td>0.602</td>
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<tr>
<td>L-Lysine</td>
<td>193</td>
<td>146</td>
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<td>L-Methionine</td>
<td>149</td>
<td>30</td>
<td>0.201</td>
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<td>L-Phenylalanine</td>
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<td>L-Serine</td>
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<td>L-Threonine</td>
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<tr>
<td>L-Tryptophan</td>
<td>214</td>
<td>16</td>
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<td>L-Tyrosine</td>
<td>251</td>
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<td>0.559</td>
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<td>L-Valine</td>
<td>117</td>
<td>94</td>
<td>0.809</td>
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<td><strong>Vitamins</strong></td>
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<td>Choline chloride</td>
<td>140</td>
<td>4</td>
<td>0.0296</td>
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<td>D-Calcium pantothenate</td>
<td>477</td>
<td>4</td>
<td>0.00839</td>
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<td>Folic acid</td>
<td>441</td>
<td>4</td>
<td>0.00967</td>
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<td>i-Insitol</td>
<td>180</td>
<td>72</td>
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<td>Nicotinamide</td>
<td>122</td>
<td>4</td>
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<td>Pyridoxal</td>
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<td>4</td>
<td>0.1956</td>
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<td>Riboflavin</td>
<td>276</td>
<td>64</td>
<td>0.00106</td>
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<tr>
<td>Thiamine</td>
<td>337</td>
<td>4</td>
<td>0.0119</td>
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<td><strong>Inorganic Salts</strong></td>
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<td></td>
<td></td>
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<tr>
<td>Calcium chloride (CaCl2) (anhyd.)</td>
<td>111</td>
<td>200</td>
<td>1.80</td>
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<tr>
<td>Ferric Nitrate (Fe(NO3)3*9H2O)</td>
<td>404</td>
<td>0.1</td>
<td>0.000248</td>
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<tr>
<td>Magnesium Sulfate (MgSO4) (anhyd.)</td>
<td>120</td>
<td>97.67</td>
<td>0.014</td>
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<tr>
<td>Potassium Chloride (KCl)</td>
<td>75</td>
<td>400</td>
<td>5.33</td>
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<tr>
<td>Sodium Bicarbonate (NaHCO3)</td>
<td>84</td>
<td>3700</td>
<td>44.05</td>
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<tr>
<td>Sodium Chloride (NaCl)</td>
<td>58</td>
<td>6400</td>
<td>110.34</td>
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<tr>
<td>Sodium Phosphate monobasic (NaH2PO4-H2O)</td>
<td>138</td>
<td>125</td>
<td>0.906</td>
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<tr>
<td><strong>Other Components</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>D-Glucose (Dextrose)</td>
<td>180</td>
<td>4500</td>
<td>25.00</td>
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<tr>
<td>Phenol Red</td>
<td>376.4</td>
<td>15</td>
<td>0.0099</td>
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</tbody>
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Media Formulation

- Inorganic ions
  - Osmotic balance – cell volume

- Trace Elements
  - Co-factors for biochemical pathways (Zn, Cu)

- Amino Acids
  - Protein synthesis
  - Glutamine required at high concentrations

- Vitamins
  - Metabolic co-enzymes for cell replication

- Energy sources
  - glucose
Serum provides the following:

[Horse serum, foetal calf serum, chick embryo extract: all not fully defined]

- Basic nutrients
- Hormones and growth factors
- Attachment and spreading factors
- Binding proteins (albumin, vitronectin, transferrin), hormones, vitamins, minerals, lipids
- Protease inhibitors
- pH buffer
Table 1. Major serum components and profile of fetal calf serum (Lindl and Bauer, ref. 12)

<table>
<thead>
<tr>
<th>Component</th>
<th>Average concentration per litre</th>
</tr>
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<tbody>
<tr>
<td>Na⁺</td>
<td>137 meq</td>
</tr>
<tr>
<td>K⁺</td>
<td>11 meq</td>
</tr>
<tr>
<td>Cl⁻</td>
<td>103 meq</td>
</tr>
<tr>
<td>Fe²⁺, Zn²⁺, Cu²⁺, Mn²⁺, Co²⁺, VO₄²⁻, Mo₇O₂₄⁶⁻, SeO₃³⁻</td>
<td>μg to ng</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>136 mg</td>
</tr>
<tr>
<td>Inorganic phosphorous</td>
<td>100 mg</td>
</tr>
<tr>
<td>Glucose</td>
<td>1250 mg</td>
</tr>
<tr>
<td>Nitrogen (urea)</td>
<td>160 mg</td>
</tr>
<tr>
<td>Total protein</td>
<td>38 g</td>
</tr>
<tr>
<td>Albumin</td>
<td>23 g</td>
</tr>
<tr>
<td>α₂-Macroglobulin</td>
<td>3 g</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>35 mg</td>
</tr>
<tr>
<td>Uric acid</td>
<td>29 mg</td>
</tr>
<tr>
<td>Creatinine</td>
<td>31 mg</td>
</tr>
<tr>
<td>Haemoglobin</td>
<td>113 mg</td>
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<tr>
<td>Bilirubin (total)</td>
<td>4 mg</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>255 U</td>
</tr>
<tr>
<td>Lactate dehydrogenase</td>
<td>860 U</td>
</tr>
<tr>
<td>Insulin</td>
<td>0.4 μg</td>
</tr>
<tr>
<td>TSH (thyroid stim. hormone)</td>
<td>1.2 μg</td>
</tr>
<tr>
<td>FSH (follicle stim. hormone)</td>
<td>9.5 μg</td>
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<tr>
<td>Bovine growth hormone</td>
<td>39 μg</td>
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<tr>
<td>Prolactin</td>
<td>17 μg</td>
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<tr>
<td>T₃ (trilodothyronine)</td>
<td>1.2 μg</td>
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<tr>
<td>Cholesterol</td>
<td>310 μg</td>
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<tr>
<td>Cortisone</td>
<td>0.5 μg</td>
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<tr>
<td>Testosterone</td>
<td>0.4 μg</td>
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<tr>
<td>Progesterone</td>
<td>80 ng</td>
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<tr>
<td>Prostaglandin E</td>
<td>6 μg</td>
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<tr>
<td>Prostaglandin F</td>
<td>12 μg</td>
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<tr>
<td>Vitamin A</td>
<td>90 μg</td>
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<tr>
<td>Vitamin E</td>
<td>1 mg</td>
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<tr>
<td>Endotoxin</td>
<td>0.35 μg</td>
</tr>
</tbody>
</table>

Table 2. Further serum components essential for cell survival and growth in vitro

Proteins
- Fibronectin
- α₂-Macroglobulin
- Fetuin
- Transferrin

Growth factors
- Insulin-like growth factors I and II (IGF)
- Somatomedin A and C
- Multiplication stimulating activity
- Platelet-derived growth factor (PDGF)
- Epidermal growth factor (EGF)
- Fibroblast growth factor (FGF)
- Endothelial cell growth factor (ECGF)

Amines
- Amino acids
- Polyamines (spermine, spermidine)

Peptide
- Glutathione

Lipids
- Linoleic acid
- Phospholipids

(4) The gas phase

**Oxygen**
- Aerobic metabolism
- Atmospheric 20%
- Tissue levels between 1-7%

**Carbon dioxide**
- Buffering
CO₂ Incubator

- Controlled CO₂
- Humidified
- 37°C
(5) pH Control

Physiological pH 7

pH can affect
- Cell metabolism
- Growth rate
- Protein synthesis
- Availability of nutrients

CO$_2$ acts as a buffering agent in combination with sodium bicarbonate in the media
(6) Temperature and Humidity

- Normal body temperature 37°C

- Humidity must be maintained at saturating levels as evaporation can lead to changes in
  - Osmolarity
  - Volume of media and additives
You have the ingredients
Now let us look at the procedures.

Contamination

Minimise the risk
Sources of Contamination

- Bacteria
- Fungi
- Mould
- Yeast
- Mycoplasma
- Other cell types
- Free organisms, dust particles or aerosols
- Surfaces or equipment
Class 1 Cabinets:
Preparation of primary cultures (removing muscle from mice) protect the product only
Class 2 Cabinets:
Protection of personnel, environment and product
Laminar Flow Hood
Class 2 Biological Safety Cabinet

HEPA filters
Laminar flow
Humans shed particles of skin, bacteria, fungi etc all the time

“Sitting or standing with no movement, wearing cleanroom garments, an individual will shed approximately 100,000 particles of 0.3um and larger per minute. The same person with only simple arm movement will emit 500,000 particles. Average arm and body movements with some slight leg movement will produce over 1,000,000 particles per minute; average walking pace 7,500,000 particles per minute; and walking fast 10,000,000 particles per minutes. Boisterous activity can result in the release of as many as 15x10^6 to 30x10^6 particles per minute into the cleanroom environment.’
Aseptic Techniques

- Controlled environment
  - Traffic, air flow
- Sterile media and reagents
- Avoid aerial contamination of solutions
- Avoid manual contamination of equipment
- Avoid repeated opening of bottles
- 70% ethanol swab
- UV irradiation before and after
- Only use disposable equipment once
Aseptic Technique

- Lab coat
- Gloves
- Tip does not touch the tube
- Holding of the tube
“Pipette-Aid”
- power or battery operated

Motorised intake and expelling of fluids transferred from one sterile container to another.

In line air filter.

“Transfer Pipette”
Disposable Enclosed Plastic

Packaged as Sterile so their contained air is sterile.
Clarification

Microfiltration

Ultrafiltration

Reverse Osmosis

SIZE

80
40
20
10
5
2
.8
.4
.2
.1
.05
.02
.008
.004
.002
.001
.0003

S I Z E

- Human Hair Diameter
- Smallest Visible Particle

- Erythrocyte

- Bacteria
- Mycoplasma

- Polio Virus

Micron = 10^{-6} m

0.8 \mu \text{ pre-filter}
0.22 \mu \text{ end filter}
0.1 \mu
Tissue culture medium cannot be autoclaved. It is filtered through 0.2µ membrane filters.

There are different filter membrane types for sterilizing gases, solvents and aqueous solutions.

NOW many items are purchased sterile (expensive)
In the LAB you will be studying **Myogenesis**: skeletal muscle formation

- **Myoblast**
- **Proliferation**
- **Differentiation**
- **Fusion**
- **Myotube maturation**

![Diagram of muscle cell development](image-url)

- Dividing myoblasts
- Cell alignment
- Cell fusion
- Multinucleate myotubes formed
- Cell division ceases
- Spontaneous contractions begin

**0 hours**

**+2 hours**

**52 hours**

**66 hours**
**IN VIVO**

Muscle regeneration - likeness to formation of myotubes in culture.

Haematoxylin and Eosin stained paraffin embedded section of mouse muscle 12 days after injury.

**IN VITRO**

Myotubes formed by myoblasts grown in culture for 7 days.

Culture viewed on an inverted phase microscope.

(phase microscopy)
Inverted Microscope
C2C12 Mouse Skeletal Muscle Cultured Cell Line

**Proliferation**
Scattered myoblasts 24h after subculture.
High Serum Media (20% FCS DMEM)

**Differentiation and fusion**
Myotubes formed at 7 days in fusion medium (2% HS DMEM)
Cell Counting - haemocytometer

Count cells on top and left touching middle line (○). Do not count cells touching middle line at bottom and right (⊙).
Media Formulation

Proliferation/maintenance
- Hams F10 nutrient mix (for primary cell culture)
- 20% FCS (foetal calf serum)
- 5ng/ml bFGF

Differentiation and fusion
- DMEM
- 2% horse serum (Note: change in serum type)
- Insulin
- Linoleic acid
Fusion

- Media changed from nutrient rich to nutrient poor
  - Induces withdrawal from the cell cycle giving the cells 3 choices
    - Die (apoptosis)
    - Senesce (become quiescent)
    - Differentiate – leading to fusion and myotubes
Analysis
Adult Mouse Skeletal Muscle - Primary culture

*Note: this also contains fibroblasts.*

cultured (on fibronectin) in 8 well slide,
fixed and stained for desmin
Mouse Skeletal Muscle Cell line (H-2Kb) cultured (on poly-D-lysine) in 35mm dish, fixed and stained for desmin.
C2C12 skeletal muscle cell line stained with desmin (green) – to identify myotubes and Hoescht (blue) to identify cell nuclei

Bjanka Gebski