Introduction to animal cell culture
CELL CULTURE

Why do it?

- Research proteins of interest
- Antibody production
- Gene Therapy
- Embryo culture
- Stem cells
- Primary Human Cell culture
Cell Culture: why do it?

- Tool for the study of animal cell biology *in vitro* model of cell growth
- Mimic of *in vivo* cell behaviour
- Model for optimising treatments (for further *in vivo* studies)
- Artificial (some cell types are thus difficult to culture)
- Highly selective 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 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 cell line*
Yaffe and Saxel (1977)
Myoblasts cultured from the thigh muscle of C3H mice 70 h after a crush injury
Cells were shown to be capable of differentiation
Model to study factors controlling differentiation, fusion and maturation into skeletal muscle cells
Passaging or sub-culture

Cell dissociated from flask

Split 1 in 2
Contact inhibition
Initiation, establishment and propagation of cell cultures
Cultures can be initiated from
- tissue or organ fragments
- single cell suspensions

Choices to be made
- 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 (e.g., fibroblasts vs myoblasts).
- Some cells are prone to spontaneous transformation.
- Limited life span of some cultures.
(1) Dispersal of tissues

- Mechanical
  - Mincing, shearing, sieves

- Chemical

- Enzymatic (proteases)
  - Trypsin, pronase, collagenase, dispase

- Can be a combination
The cell culture environment
Factors affecting cell behaviour *in vivo*

- The local micro-environment
- Cell-cell interactions
- Tissue architecture
- Tissue matrix
- Tissue metabolites
- Locally released growth factor and hormones
(2) Culture Surface

- Most adherent cells require attachment to proliferate
- Change charge of the surface: different kinds of plastic (originally used glass)
  - 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
# DMEM
(Dulbecco’s Modified Essential Medium)

<table>
<thead>
<tr>
<th>COMPONENTS</th>
<th>Molecular Weight</th>
<th>Concentration (mg/L)</th>
<th>Molarity (mM)</th>
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<tr>
<td>Amino Acids</td>
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<td></td>
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<tr>
<td>Glycine</td>
<td>75</td>
<td>30</td>
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<tr>
<td>L-Alanyl-Glutamine</td>
<td>217</td>
<td>962</td>
<td>3.97</td>
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<td>L-Arginine hydrochloride</td>
<td>211</td>
<td>94</td>
<td>0.398</td>
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<td>L-Cystine</td>
<td>31.3</td>
<td>48</td>
<td>0.153</td>
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<td>L-Histidine hydrochloride·H2O</td>
<td>210</td>
<td>42</td>
<td>0.200</td>
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<tr>
<td>L-Isoleucine</td>
<td>131</td>
<td>105</td>
<td>0.802</td>
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<td>L-Leucine</td>
<td>131</td>
<td>105</td>
<td>0.802</td>
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<tr>
<td>L-Lysine hydrochloride</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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<tr>
<td>L-Phenylalanine</td>
<td>165</td>
<td>66</td>
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<td>L-Serine</td>
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<td>L-Threonine</td>
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<td>L-Tryptophan</td>
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<td>L-Tyrosine dihydrochloride</td>
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<td>L-Valine</td>
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<td>Vitamins</td>
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<td>Choline chloride</td>
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<td>D-Calcium pantothenate</td>
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<td>Folic Acid</td>
<td>44</td>
<td>4</td>
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<td>i-Inositol</td>
<td>180</td>
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<td>Niacinamide</td>
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<td>Riboflavin</td>
<td>376</td>
<td>0.4</td>
<td>0.00106</td>
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<td>Thiamine hydrochloride</td>
<td>337</td>
<td>4</td>
<td>0.0019</td>
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<td>Inorganic Salts</td>
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<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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<td>Magnesium Sulphate (MgSO₄) (anhyd.)</td>
<td>120</td>
<td>97.67</td>
<td>0.014</td>
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<td>Potassium Chloride (KCl)</td>
<td>75</td>
<td>400</td>
<td>5.33</td>
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<tr>
<td>Sodium Bicarbonate (NH₄CO₃)</td>
<td>64</td>
<td>3700</td>
<td>44.05</td>
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<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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<td>Other Components</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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*Note*
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

- Basic nutrients
- Hormones and growth factors
- Attachment and spreading factors
- Binding proteins (albumin, transferring) carrying hormones, vitamins, minerals, lipids
- Protease inhibitors
- pH buffer

Use rich foetal calf serum (FCS) or chick embryo extract (CEE) for best growth/proliferation of cells.

Use serum from adult (non-growing) animals, e.g. horse serum (HS), for cell maintenance.

Fully defined, serum–free media are available: expensive but more reproducible. May be essential for clinical transplantation of cells into humans (to avoid issues with animal products).
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>
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<tr>
<td>Na⁺</td>
<td>137 meq</td>
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<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⁺</td>
<td></td>
</tr>
<tr>
<td>VO₃⁻, Mo₇O₂₄⁶⁻</td>
<td>μg to ng</td>
</tr>
<tr>
<td>SeO₂³⁻</td>
<td>26 μg</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>136 mg</td>
</tr>
<tr>
<td>Inorganic phosphorus</td>
<td>100 mg</td>
</tr>
<tr>
<td>Glucose</td>
<td>1250 mg</td>
</tr>
<tr>
<td>Nitrogen (urea)</td>
<td>160 mg</td>
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<tr>
<td>Total protein</td>
<td>38 g</td>
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<tr>
<td>Albumin</td>
<td>23 g</td>
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<tr>
<td>α-2-Macroglobulin</td>
<td>3 g</td>
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<tr>
<td>Fibronectin</td>
<td>35 mg</td>
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<tr>
<td>Uric acid</td>
<td>29 mg</td>
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<tr>
<td>Creatinine</td>
<td>31 mg</td>
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<tr>
<td>Haemoglobin</td>
<td>113 mg</td>
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<tr>
<td>Bilirubin (total)</td>
<td>4 mg</td>
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<tr>
<td>Alkaline phosphatase</td>
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<td>Lactate dehydrogenase</td>
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<tr>
<td>Insulin</td>
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<tr>
<td>TSH (thyroid stim. hormone)</td>
<td>1.2 μg</td>
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<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 (triiodothyronine)</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>
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<tr>
<td>Progesterone</td>
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<tr>
<td>Prostaglandin E</td>
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<tr>
<td>Prostaglandin F</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>
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</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 (4%)**
  - Buffering
(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
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)
C2C12 skeletal muscle culture stained with desmin (green) – to identify myotubes and Hoescht (blue) to identify cell nuclei.
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
Laminar Flow Hood
Class 2 Cabinets:  
Protection of personnel, environment and product  
Laminar Flow Hood
Class II Biological Safety Cabinet

HEPA filters
Laminar flow
NATA certified

Exhaust Fan
Exhaust HEPA Filter
Laminar Flow Fan
Laminar HEPA Filter
Air Barrier
Vertical Laminar Airflow
“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 $15 \times 10^6$ to $30 \times 10^6$ particles per minute into the cleanroom environment.’
First have a 5-10 minute break and stretch.
Aseptic Technique 1

- Controlled environment
  - Traffic, air flow
- Sterile media and reagents
- Avoids aerial contamination of solutions
- Avoids manual contamination of equipment
Aseptic Technique 2

- Minimise traffic
- Clear work area
- 70% ethanol swab
- Minimise work area (field of vision)
- Keep work area clean
- Do not lean over open vessels
- UV irradiation before and after
- Only use disposable equipment once
Aseptic Technique 3

- Minimise exposure to air
- Flame bottles if on open bench
- Avoid repeated opening of bottles
- Avoid liquid accumulation around necks and lips of bottles
- Avoid excessive agitation
- Only one cell type at a time
- Do not open contaminated solutions
- No burner in hood
Establishing a Primary Culture

Skeletal Muscle
Muscle regeneration in vivo - likeness to formation of myotubes in culture.

Haematoxylin and Eosin stained paraffin embedded section of mouse muscle 12 days after injury (viewed by bright field microscopy)

Myotubes formed by myoblasts grown in culture for 7 days.

Culture viewed on an inverted phase microscope. (phase microscopy)
Considerations

- Highly structured tissue
  - disaggregation
- Heterogeneous
  - purification
- Multi-cellular versus single cell
  - media
- Contamination
  - Sterile technique
- Proliferation versus differentiation
  - Media
  - substrate
Collection

- **Sterility**
  - Wash mouse skin in 70% ethanol
  - Gentamicin
  - Penicillin/Streptomycin
  - (fungizone)

- **Speed**
  - 45 minutes

- **Tissue collected/clean**
  - Removal of tendon, fat, nerve
Primary Cell Culture Isolation

From a *Solid Tissue* - need to separate cells of interest from connective tissue and extra cellular matrix
- explant culture
- mechanical and enzymic dissociation
- washed in medium with serum
- filtered and plated in final medium

• ALL this done in a Class 1 lamina flow hood

From a *cell suspension tissue* - blood or body fluid (ascites)
- isolation by centrifugation (differential)
- grown in large volume of tissue culture medium
Disaggregation

- Mechanical mincing
  - scissors
- Collagenase
- Dispase
- Trypsin
- Washing
Laminar Flow Hood
Filtering

100 micron

- Removal of undigested material
- Lets through single cells
- Can count cells using haemocytometer
to plate at a particular cell density
Clarification

Microfiltration

Ultrafiltration

Reverse Osmosis

- Human Hair Diameter
- Smallest Visible Particle
- Erythrocyte
- Bacteria
- Mycoplasma
- Polio Virus

Micron = 10^{-6} m

0.8 µ pre-filter
0.22 µ end filter
0.1 µ

Micron = 10^{-6} m
Tissue culture medium cannot be autoclaved. It is filtered through 0.2µm membrane filters.

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

NOW many items are purchased sterile (expensive)
Aseptic Technique

- Lab coat
- Gloves
- tip does not touch the tube
- holding of the tube
**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.
Pipettes (glass or disposable) are plugged to minimise aerosol contamination, when solutions are expelled from them.
“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.
Cell Counting - haemocytometer

Count cells on top and left touching middle line (○). Do not count cells touching middle line at bottom and right (Ø).
Filtering and Pre-plate

100 micron
- Removal of undigested material
- Lets through single cells
- Can count cells to plate at a particular density

OR

Pre-plate
- 60 mins
- Differential attachment to culture plastic
Pre-plating — to remove other cells, e.g. fibroblasts

Supernatant Transferred from flask to new flask after given time

Putative Muscle Derived Stem Cell (MDSC)
Media Formulation

GROWTH MEDIUM
- Proliferation/maintenance
  - Hams F10 nutrient mix
  - 20% FCS (foetal calf serum)
  - 5ng/ml bFGF

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

Growth medium replaced by Fusion medium at Time 0

0 hours
- Dividing myoblasts

+2 hours
- Cell alignment

52 hours
- Cell fusion
  - Appearance of CPK, myosin, and actin

66 hours
- Multinucleate myotubes formed
  - Spontaneous contractions begin
Fusion

Media changed from nutrient rich to nutrient poor

- Induces withdrawal from the cell cycle giving the cells 3 choices
  - Die (apoptosis)
  - Senesce (age/stop)
  - Differentiate

[Note: in vivo differentiation may result from contact inhibition rather than decreased GF]
CO₂ Incubator

- Controlled CO₂
- Humidified
- 37°C
Inverted Microscope
Mouse Skeletal Muscle Cell Line C2C12

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)
Analysis
Primary muscle culture stained with desmin (green) – to identify myoblasts and Hoescht (blue) to identify cell nuclei
Adult Mouse Skeletal Muscle - Primary culture 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
CONTINUE TO GROW / MAINTAIN CELLS

- Proliferation media
- Trypsinisation and splitting/passaging of cultures
  - Contact inhibition
- 1% gelatin coated dishes

STORAGE

- Cryopreservation in liquid nitrogen
  - 10% FCS and 10% DMSO