Introduction

• An essential tool in the study of cell biology is the use of *in vitro* cell culture.

• Variables in cell culture include cell source, isolation techniques, growth conditions such as matrix proteins and soluble factors, and cell age.

• Basic laboratory practices are sometimes overlooked as a source of discrepancy in data; however the application of fastidious and reproducible technique can reduce cell culture as a source of data variation.

• In this presentation we will identify and discuss basic principles of *in vitro* mammalian cell culture that influence the quality of experimental results.
Why Culture Living Cells?

To Study:
- Intracellular activities
  - DNA replication
  - Transcription (RNA)
  - Translation (protein)
  - Signal Transduction
- Cell-environment interaction
  - Drug action, infection, cytotoxicity, receptor/ligand interaction
- Cell-cell interaction
- Genomics and proteomics

To Produce:
- Cells, cell products, and cell secreted products
  - Protein, DNA, RNA, antibody, transfected protein product
Why Standardize Techniques?

• Cultivation in a more physiological environment
  = increased value of experimental data

• Cultivation in a controlled environment
  = defined parameters that can be changed/measured

• Cultivation in an optimized environment
  = increased signal/noise ratio
Why Standardize Techniques?

As an example:

It is estimated that it takes ~12 to 15 years and ~$800 million - $1.7B to develop a new drug

  http://www.pharmainfo.net/reviews/drug-development-process-review

Failure of drug candidate is 10% when moving from ADME to Clinical Testing

– Chris Bode, PhD, Vice President, Corporate Development, Absorption Systems, L.P. (January 12, 2009) Going In Vitro. Drug Discovery & Development  

A 10% improvement in predicting compound failure before clinical trial could save up to 100 million $ in development costs per drug
Experimental Data is Influenced by:

1) Cell Source
2) Initial Growth Conditions
3) Medium Composition
4) Cell Counting Procedure
5) Physiological Parameters
6) Characterization
   • Age, Karyotype
7) Substrate, Matrix, or Not
   • Morphology
8) Dissociation Method
9) Contamination Control
   • Aseptic Technique
10) Cryopreservation
Cell Source

Primary Cells:
Cells isolated from an organism prior to the first subcultivation
- Tissue Source
- Isolation Technique
- Species
- Donor

Cell Line:
Propagated culture after the first subculture
- Certified Stock
- Not your neighbor

MCF-7 (breast cancer cell line) labeled with Hoechst (blue), Mitotracker green (green) and wheat germ agglutinin (red)

BD Pathway™ 855 Bioimager

Cross-Contamination of Cell Cultures: A Call for Vigilance and Authentication
—Lucille A. Ouellette and Roland M. Nardone
ASCB NEWSLETTER JULY 2007
Cell Source: Tissue Source

Example: Endothelial Cells

- EC tube
- EC tube PCs BM
- EC tube IEL SMCs BM EEL

Legend:
- Endothelial cell (EC)
- Pericyte (PC)
- Smooth muscle cell (SMC)
- Fibroblast (FB)
- Internal elastic lamina (IEL)
- External elastic lamina (EEL)
- Basement membrane (BM)
- Lymphatic endothelial cell
- Extracellular matrix (EM)
Cell Source: Isolation Techniques

Example: Isolation of Rat Hepatocytes

Collagenase Perfusion activates immediate Early Growth Response Genes (c-jun, jun-b, c-myc)

FIG. 1. (A) Activation of IEG during perfusion of the liver with collagenase. Northern blot analysis of total RNA (25 µg per lane) isolated from liver or hepatocytes at the indicated stages of the cell isolation procedure, as outlined in Materials and Methods, is shown.

(Basabi Rana, et. al. MOLECULAR AND CELLULAR BIOLOGY, Sept. 1994, p. 5858-5869)
Cell Source: Species

- Know what you want in experimental data
- Example: *In Vitro* Testing of Drug Compound Bioavailability
  - MDCK Cells, Dog Kidney
  - MDCK2 Cells, Dog Kidney transfected with MDR-1
  - Caco2 Cells, Human intestinal epithelial

### TABLE 3
Number of transcripts per microgram of total RNA

<table>
<thead>
<tr>
<th>Transcript $^{a}$</th>
<th>Jejunum $^{b}$</th>
<th>Caco-2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± S.D.</td>
<td>Minimum–Maximum</td>
</tr>
<tr>
<td>MDR1/10$^{6}$</td>
<td>7.9 ± 1.4</td>
<td>5.3–10.3</td>
</tr>
<tr>
<td>MDR3/10$^{8}$</td>
<td>5.2 ± 3.0</td>
<td>2.5–12.9</td>
</tr>
<tr>
<td>MRP1/10$^{6}$</td>
<td>1.8 ± 0.5</td>
<td>1.1–2.5</td>
</tr>
<tr>
<td>MRP2/10$^{6}$</td>
<td>2.4 ± 0.6</td>
<td>1.7–3.3</td>
</tr>
<tr>
<td>MRP3/10$^{6}$</td>
<td>4.6 ± 1.3</td>
<td>2.5–6.9</td>
</tr>
<tr>
<td>MRP4/10$^{4}$</td>
<td>6.5 ± 1.3</td>
<td>5.1–9.4</td>
</tr>
<tr>
<td>MRP5/10$^{5}$</td>
<td>2.1 ± 0.5</td>
<td>1.2–3.0</td>
</tr>
<tr>
<td>MRP6/10$^{5}$</td>
<td>2.5 ± 0.7</td>
<td>1.4–3.7</td>
</tr>
<tr>
<td>BCRP/10$^{6}$</td>
<td>2.7 ± 0.6</td>
<td>2.1–3.7</td>
</tr>
<tr>
<td>LRP/10$^{6}$</td>
<td>2.7 ± 0.8</td>
<td>1.8–4.5</td>
</tr>
<tr>
<td>CYP3A4/10$^{7}$</td>
<td>1.4 ± 0.3</td>
<td>0.8–1.8</td>
</tr>
</tbody>
</table>

$^{a}$ The /10$^{n}$ in the Transcript column is the multiplier for the mean ± S.D. column.

$^{b}$ Results are based on 13 jejunal biopsies and presented as mean ± S.D. The maximum and minimum values for each transcript are also given.

(Taipalensuu, J. et.al. JPET 299:164-170, 2001)
Cell Source: Donor Variability

Comparison of VEGF-response in HUVEC cells from different sources

*The data in this graph is representative of three independent endothelial cell migration experiments.
Initial Growth Conditions

Example: Caco-2 Cell Culture

Collagen I  TC-treated Polystyrene
# Initial Growth Conditions

## Example: Caco-2 Cell Culture

**Digoxin Permeability Comparison**

<table>
<thead>
<tr>
<th>Caco-2 Line</th>
<th>Pore Size</th>
<th>Papp A-B</th>
<th>Papp B-A</th>
<th>Papp Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>10% FBS</td>
<td>1 uM</td>
<td>mean: 2.9</td>
<td>9.7</td>
<td>3.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SD: 0.8</td>
<td>1.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>% CV: 27</td>
<td>19</td>
<td>(n=4)</td>
</tr>
<tr>
<td>20% FBS</td>
<td>1 uM</td>
<td>mean: 1.3</td>
<td>17.3</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SD: 0.24</td>
<td>2.50</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>% CV: 19</td>
<td>14</td>
<td>(n=8)</td>
</tr>
</tbody>
</table>
Medium Composition

Must supply proper nutrients, including essential amino acids, pH balance, proper osmolarity, proper surface tension and viscosity, and buffering for gas exchange

• Serum supplies a variety of required components
  – Growth factors and hormones
  – Proteins, including those needed for cell adherence
  – Trace minerals
  – Inhibitors of cell proliferation
    • Cell proliferation inhibitors can enhance differentiation
Media Watch-outs

- Control for water source and reagent preparation
- FBS
  - Lot to lot (uncontrolled) variation in hormone and growth factor levels
  - Test FBS or use serum free
- Antibiotics or Not
- Feed cells every 2 days, warm media to 37°C
- Store media at 4°C, in the dark, observe expiration dates
Fluorescent Lighting

HEPES, Riboflavin, Tryptophane

\[ \text{Hydrogen Peroxide and Free radicals} \]

\[ = \]

Toxic to Cultured Cells
Cell Counts

- Standardized Technique
- Cell Preparation
  - Single Cell Suspension
  - Dilutions
- Sampling technique
  - Well mixed
  - Accurate pipetting
- Filling of Chamber
  - Over/under filling
  - Bubbles
- Clean chamber, even distribution
- Coverslip, heavier than normal
  - to maintain chamber height
Physiological Parameters

- Temperature: 37-38.5°C
- pH: 7.2-7.4
- CO₂ @ 5% in 95% air
- Oxygen Tension: 1-10%
- Osmotically Balanced Medium
- Humidification: saturated
  - limit evaporation to maintain osmolarity
Physiological Parameters: pH

- Most Mammalian Cells grow well at pH 7.4.
- Cells produce waste products that tend to be acidic.
- Thus, generally require a buffered medium.
- Sodium bicarbonate is usually the buffering agent.
- The pH is governed by the reaction
  - $\text{H}_2\text{O} + \text{CO}_2 \leftrightarrow \text{H}_2\text{CO}_3 \leftrightarrow \text{H}^+ + \text{HCO}_3^-$
- Cultures must be incubated in a CO$_2$ environment in equilibrium with the H$_2$CO$_3$ in the medium.
Physiological Parameters: Humidity

- 96-well plate evaporation study
- Plate map of volumetric changes over time

Vibration

- Non-random adherence and growth of cell suspension due to vibration or resonance in the incubator
Cell Age

- Cell Characteristics can vary over time
- Use Standardized Protocols to control and understand the age of your cells

### Population Doublings, Generation Number:

<table>
<thead>
<tr>
<th>1</th>
<th>2</th>
<th>4</th>
<th>8</th>
<th>16</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
</tbody>
</table>

...n, number of generations

\[ n = 3.32(\log N - \log X) \]

N= final population, and X=initial population

### Passage Number:
- Number of times a culture has been subcultured or split
- Does not reflect population age unless there is a consistent time between splits and a unvarying split ratio

(Kruse and Patterson, Tissue Culture Methods and Applications, 1973)
Vertebrate cells have negative surface charges, and can be cultured on either positive or negative charged surfaces

- Glass and Tissue culture treated polystyrene plastic carry a negative charge
- BD Primaria™ cultureware carries both positive and negative charges
- Poly-lysine surfaces have positive charges

Extracellular matrix components (2D or 3D)

- Cells spread using the ECM they lay down and the serum or media derived attachment factors
- Fibronectin is key attachment factor derived from serum
- BD BioCoat™ cultureware

Microporous membranes (cell culture inserts)
Native polystyrene is rendered hydrophilic by a process that adds a variety of negatively charged functional groups to the surface.

**BD Falcon™ cultureware is vacuum-gas plasma treated** under specific conditions of pressure and temperature...

**Substrates: Polystyrene**

**Untreated polystyrene**

**Vacuum-gas plasma treatment- oxygen**
Substrates: Polystyrene

Other Modifications to Polystyrene

Vacuum-gas plasma treatment- oxygen + ammonia

Some BD BioCoat Extracellular proteins

BD Primaria cultureware
HEK-293 cells were cultured under serum-free conditions on TC-treated or BD BioCoat PDL 384-well black/clear plates. Samples were washed using a Skatron Washer (Molecular Devices).
Human Umbilical Vein Endothelial Cells (HUVEC) Attach Better on BD BioCoat Collagen I

TC Plastic

BD BioCoat Collagen I
Human Microvascular Endothelial Cells Form Tubules on BD Matrigel Matrix

Collagen I (2D)

BD Matrigel™ Matrix (3D)
BD BioCoat Collagen I

Amorphous (2D)  Fibrillar (3D)
Primary Hepatocytes Exhibit Differentiated Morphology on 3D Growth Substrates

Morphology: Phenotype

Col I (2D thin coat)  Col I (3D gel)  BD Matrigel™ Matrix (3D)

(Technical Bulletin # 420)
Morphology: Phenotype

Aortic Smooth Muscle Cells

Collagen I  GFR BD Matrigel™ Matrix
What is BD PureCoat Cultureware?

The Next Generation of Advanced Cell Culture Surfaces

- Chemically defined, animal-free surfaces
- Manufactured by a proprietary thin-film coating technology
- BD PureCoat™ Carboxyl
  - Negatively charged surface
- BD PureCoat Amine
  - Positively charged surface

Webinar scheduled on October 22, 2009

Application Note No. 466
Routine Maintenance

- Medium changes
- Monitor cell morphology
- Stay on a strict schedule
- Subculture at 80% confluence

Representative Cell Growth Curve
Dissociation Techniques

Mechanical
- Cell scraper, trituration
- Colony division, scalpel

Enzymatic
- Trypsin, collagenase, dispase, pronase
- Rinse with Balanced Salt Solution without Ca\(^{++}\) Mg\(^{++}\)
- Enzymes should be aliquoted and frozen at -20°C

Chelating Agents
- Citrate and Ethylene-diamine-tetra-acetic acid (EDTA, Versene)
- Calcium and Magnesium are important regulators of cell to cell junctional complexes

*Standardized Routine Techniques When and How*
Cryo-preservation

Cell Response to Cryo-preservation:
- Reduced Metabolic Activity
- Ionic balance and Osmotic balance are disrupted
- Free radicals accumulate
- Ice forms around the cell
- Molecular stress responses are initiated

Solutions:
- Tissue culture media
- Serum (10-90%)
- Proteins such as albumin
- Cryo-protective agents
  - Dimethylsulphoxide (DMSO)
  - Glycerol
- Vitrification

(Baust, JG, Baust, JM, Advances in Biopreservation, 2007)
Contamination

Contamination Sources:

- Chemical: endotoxins, plasticizers, disinfectants, fluorescent lighting
- Biological: Bacterial, viral, fungal, mycoplasmal, cellular
Contamination: Chemical

- **Endotoxins**: lipopolysaccharide containing by-product of gram-negative bacteria
  - Water, sera, culture additives
- **Plasticizers**: in storage containers, tubing
- **Reused storage containers**
- **Disinfectants**: deposits left from washing glassware
- **Germicides**: used to disinfect Incubators
- **CO₂**: Use medical grade
Contamination: Biological

• **Bacteria, Mold, Yeast**
  – Easily detected: pH, turbidity, cell morphology, better to avoid antibiotics

• **Viral, Mycoplasma**
  – Alter host cell function, hard to detect

• **Cell Cross-contamination**
  – Technique, don’t borrow cells

Cross-Contamination of Cell Cultures: A Call for Vigilance and Authentication

—Lucille A. Ouellette and Roland M. Nardone

ASCB NEWSLETTER JULY 2007
Yeast

- Does not cause early pH changes
- Medium becomes cloudy
- Later, yeast can cause media to become basic (purple media)
- Distinctive bread-like odor

(www.biochemweb.org)
Bacterial
Causes changes in pH of Medium
– Medium becomes acidic,
– Cloudy and bright yellow
– Often produce toxins that destroy cells
Mold

Does not cause immediate pH changes

- Often is not cytotoxic
- Easy to observe under a low power microscope and can even be seen without magnification in advanced stages of contamination.
- Tough to catch early
- Appears whiteish, yellowish, or black in culture
Contamination: Biological

Mycoplasma

Cannot be seen under normal magnification
  • No overt effects in culture, only subtle ones
  • The only way to confirm mycoplasma contamination is by routine testing

Mycoplasma can cause changes in:
  • Cell growth characteristics
  • Cell metabolism
  • Disruption of nucleic acid synthesis
  • Chromosomal aberrations
  • Changes in cell membrane antigenicity
  • Can alter transfection rates and virus susceptibility.
Contamination: Control

Control

- Certified seed stock
- Aseptic technique
- Periodic testing
- Antibiotic-free media
- Sterile equipment, glassware and media
- Quality reagents
- Use frozen cell stock

Most Dangerous:

- Undetected contaminants
Aseptic Technique

- Gown appropriately
- Frequently disinfect gloved hands with 70% ethanol
- Disinfect your work surface before you begin
- Laminar Flow hoods provide a physical barrier to contamination
  - HEPA filters trap airborne contaminants
  - Blowers move filtered air at specified velocities in a non-mixing (laminar) stream across a work surface
  - Not 100% sterile and are not replacement for good microbiological aseptic technique
  - Do not overcrowd work space
  - View window should be lowered to the proper operating height of 8 in.
  - Before use - allow blower to run 10 min to filter the cabinet air of any particulates
  - Recertify / Replace HEPA filters at 1000 hours
  - Periodically disinfect the catch basin
  - Environmental Bioburden Testing
  - Use UV light before and after work
Experimental data is influenced by:

- Cell Source
- Initial Growth Conditions
- Medium Composition
- Cell Counting Procedure
- Physiological Parameters
- Characterization
  - Age, Karyotype
- Substrate, Matrix, or Not
  - Morphology
- Dissociation Method
- Contamination Control
  - Aseptic Technique
- Cryo-preservation

Standardization is really just a good laboratory technique.
Questions?

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