Human Dermal Fibroblast Viability and Proliferation \textit{in vitro}
OBJECTIVES

- Compare cell counting techniques
  - MTT Assay

- Develop relationships between cell density and physical properties (i.e. absorbance)
  - MTT Assay

- Relate percentage of Fetal Bovine Serum (FBS) to cell proliferation rate and doubling time
  - Anti-PCNA Staining
  - Cell Proliferation Assay
MTT Viability Assay Compares 2 Different Cell Counting Techniques

- **Spectrophotometer**
  - Cells dyed with MTT dye and incubated for 2 hours
  - Solubilization/Stop solution was added to cells for 45 minutes
  - Cells placed in cuvette and absorbance measured at 570nm in spectrophotometer

- **Coulter Counter**
  - Each well trypsinized for cell detachment
  - Cells added to Isoton in Coulter Counter vials
  - Cell number determined using Coulter Counter

Both techniques used cell dilutions ranging from 0 – 50,000 cells/mL
Absorbance is Linearly Related to Measured Cell Concentration

\[ R^2 = 0.9954 \]

Indicative of a good fit

*The 6\(^{th}\) point was determined to be an outlier and was therefore removed from this plot.*
MTT Viability Test Can Be Used To Detect Cell Concentration

- Absorbance related linearly to cell concentrations measured by Coulter Counter
  - MTT Assay may be used as reliable cell counting technique

- MTT Assay may be more accurate than Coulter Counter
  - Metabolic dye used in MTT Assay insures that dead cells and cell debris are not counted
Anti-PCNA Staining is Used to Highlight Cells in S-Phase

- Cells initially suspended at 20,000 cells/mL in 1%, 5% and 10% FBS and incubated for 2 days
- The following were added to the cells during this assay

<table>
<thead>
<tr>
<th>Added to Cells</th>
<th>Formalin</th>
<th>H$_2$O$_2$</th>
<th>Primary Antibody</th>
<th>Secondary Antibody</th>
<th>AEC Solution</th>
<th>Hematoxylin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Function</td>
<td>Fixes Cells</td>
<td>Eliminates Peroxidase Activity</td>
<td>Binds to PCNA in cell nucleus</td>
<td>Binds to primary antibody</td>
<td>Reacts with HRP Tag</td>
<td>Blue dye which dyes entire cell - nonspecific</td>
</tr>
</tbody>
</table>

- Cells in S-Phase synthesize PCNA
  - HRP (red fluorescent) tag bound to PCNA
- Cells observed under fluorescent microscope
Cells in S-Phase Increase with Increasing FBS Percentage

<table>
<thead>
<tr>
<th>FBS in Media</th>
<th>Confluency</th>
<th>Red Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>1%</td>
<td>20%</td>
<td>0%</td>
</tr>
<tr>
<td>5%</td>
<td>40%</td>
<td>30%</td>
</tr>
<tr>
<td>10%</td>
<td>60%</td>
<td>60%</td>
</tr>
</tbody>
</table>

- Increased FBS percentage leads to increase in cell concentration
- Higher cell concentration give rise to greater amounts of cells in S-Phase

* Data acquired from partner, confluency unknown *
Determination of Cell Proliferation With Respect to FBS Percentage

- 33 wells were seeded with 5,000 cells/mL each
- Cell concentrations measured after incubation
  - 4 hours, 2 days, 5 days & 7 days
  - Sets of wells are specified for each time point
  - Each set contains 9 wells
- Well subsets contain DMEM with 1%, 5% and 10% FBS
- Sets were trypsinized and counted using Coulter Counter at specified time points
- Media for remaining wells was replenished
Cellular Proliferation is Exponential

ANOVA: F = 418  F_{crit} = 3.402 on Day 7

All cell concentrations are significantly different
Doubling Time Decreases as FBS Percentage Increases

- Cell proliferation shows exponential behavior
- Doubling time inversely related to FBS percentage
  - $T_d = \frac{\ln(2)}{k}$ where $k$ is from exponential fit $y = ae^{kt}$

<table>
<thead>
<tr>
<th>FBS Amount</th>
<th>1%</th>
<th>5%</th>
<th>10%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doubling Time</td>
<td>5.12 days</td>
<td>1.90 days</td>
<td>1.54 days</td>
</tr>
</tbody>
</table>

- Cell concentrations for different amounts of FBS are statistically different
  - ANOVA
Anti-PCNA Results Can Be Related to Cell Proliferation Assay Results

- As FBS percentage increases in DMEM, cell proliferation increases
- More cells are in S-Phase with increasing FBS%
  - More cells are dividing at any given time
  - Cells are multiplying more rapidly
- Cell doubling time decreases with increased FBS%
  - Cell number is greater at any given time as FBS % increases
  - Cells increase exponentially because of doubling time
Various Techniques Allow For Analysis of Cell Viability and Proliferation *in vitro*

- Cell concentrations can be measured by MTT Viability Test
  - MTT Test will only measure cells in metabolic state
  - Test does not count cell debris or dead cells
- Higher FBS percentage in DMEM increases cell proliferation
  - Cells can be grown faster with high percentages of FBS% in media
  - This can provide experiments with an ongoing supply of cells