Cell and Organelle Purification for Proteomics

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Tissue Proteomics, Identifying Disease-related Proteins

http://cgap-mf.nih.gov/BtoB/BtoBSampleAcquisitionAndProcessing.html
Proteomics: From Sample to Result

Blood → Tissue → Cell Culture → Suspension of Single Cells → Purified Single Cells → Cell Homogenate → Purified Cell Substructure Fractions → Genes/GFP → Transfect

Genome Analysis
Proteome Analysis
Analog Signals

FSC

SSC

FITC

APC

Laser delay

Many more...
Digitize in 16,384 Levels

- FSC
- SSC
- FITC
- APC

Laser delay

14 bits
Sample 10,000,000 per Second

10 MHz

- FSC
- SSC
- FITC
- APC

Laser delay
Digitization and Sampling

- Digitize in 14-bits
  - 16,384 levels
- Sample at 10Mhz
  - 10 million times/sec
- 16 channels
Flow Cytometer Data

<table>
<thead>
<tr>
<th>Cell</th>
<th>P1</th>
<th>P2</th>
<th>P3</th>
<th>P4</th>
<th>P5</th>
<th>Pop#</th>
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<tbody>
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<td>135</td>
<td>704</td>
<td>175</td>
<td>612</td>
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<td>2</td>
<td>146</td>
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<td>526</td>
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<td>6</td>
<td>269</td>
<td>169</td>
<td>2</td>
<td>659</td>
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<td>1</td>
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<tr>
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<td>171</td>
<td>679</td>
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<tr>
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<td>152</td>
<td>74</td>
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<td>828</td>
<td>532</td>
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<td>87</td>
<td>110</td>
<td>904</td>
<td>560</td>
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</tbody>
</table>

**Event histogram**

**"Dotplot"**
Six color example

- CD3    FITC
- CD56   PE
- CD8    PE-Texas Red
- CD19   PE-Cy7
- CD14   APC
- CD4    APC-Cy7
Six color example
Six color example
Six color example
Six color example
FACS
Pre-Sort

45 psi
64 kHz
20,000 events/s
## Purity

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Value 1</th>
<th>Value 2</th>
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<tbody>
<tr>
<td>FITC</td>
<td>8.2%</td>
<td>99.1%</td>
</tr>
<tr>
<td>PE</td>
<td>9.5%</td>
<td>99.2%</td>
</tr>
<tr>
<td>PerCP</td>
<td>8.0%</td>
<td>99.5%</td>
</tr>
<tr>
<td>APC</td>
<td>0.6%</td>
<td>99.4%</td>
</tr>
</tbody>
</table>

**BD Biosciences Immunocytometry Systems**
### Recovery, 60kHz, 6% population

<table>
<thead>
<tr>
<th>Event Rate</th>
<th>4k/sec</th>
<th>20k/sec</th>
<th>24k/sec</th>
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</thead>
<tbody>
<tr>
<td>DiVa sort counter</td>
<td>15k</td>
<td>46k</td>
<td>48k</td>
</tr>
<tr>
<td>Analog sort counter</td>
<td>14k</td>
<td>38k</td>
<td>39k</td>
</tr>
<tr>
<td><strong>More cells with DiVa</strong></td>
<td><strong>7%</strong></td>
<td><strong>23%</strong></td>
<td><strong>23%</strong></td>
</tr>
<tr>
<td>Theoretical recovery</td>
<td>90%</td>
<td>63%</td>
<td>58%</td>
</tr>
<tr>
<td>Diva recovery</td>
<td>88%</td>
<td>65%</td>
<td>57%</td>
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</table>
## Molecule Copy Numbers

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Per T-cell</th>
<th>fmoles/10^6 cells</th>
<th>LC-MS</th>
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</thead>
<tbody>
<tr>
<td>CD3</td>
<td>8.1 x 10^4</td>
<td>130</td>
<td>++</td>
</tr>
<tr>
<td>CD4</td>
<td>5.9 x 10^4</td>
<td>98</td>
<td>-</td>
</tr>
<tr>
<td>CD8</td>
<td>1.4 x 10^5</td>
<td>230</td>
<td>+</td>
</tr>
<tr>
<td>CD11a</td>
<td>2.7 x 10^4</td>
<td>45</td>
<td>+</td>
</tr>
<tr>
<td>CD16</td>
<td>7.9 x 10^4</td>
<td>130</td>
<td>+</td>
</tr>
<tr>
<td>CD18</td>
<td>3.1 x 10^4</td>
<td>52</td>
<td>+</td>
</tr>
<tr>
<td>CD45</td>
<td>1.9 x 10^5</td>
<td>320</td>
<td>-</td>
</tr>
</tbody>
</table>

Parameters for Selection of Cell Subsets

Analyse and Sort based on:

• light scatter
• immunofluorescence
• fluorescent in-situ hybridization
• DNA content
• transfection with fluorescent proteins
• protein content
• auto-fluorescence
• enzyme activity
• pH
• redox potential
• other components detectable by fluorescence
DNA Content as Tumor Marker

Advantages of FACS™ based sorting

- Yield large numbers of purified, primary cells (i.e., not cultured) from patients.
- Yield high quality biomolecules.
- May potentially provide molecular profiles very similar to cells in vivo due to their rapid processing after removal from the patient.
- Can be separated into specific subsets of cells based on molecular markers on the cell type of interest.

In Barrett's esophagus, flow cytometry can be used to identify patients who are at low- or high-risk for progression to high grade dysplasia or cancer.

(www.barrettsinfo.com/content/6a1_use_of_flow_cytometry)

http://cgap-mf.nih.gov/BtoB/BtoBSampleAcquisitionAndProcessing.html
Analysis of Sorted Populations

CD8

96.1%
5 x 10^6 cells

CD4

23.4%
40.9%

1.3%
1.2 x 10^7 cells

0.07%
99.5%
## Cell-surface Proteins Identified On Sorted T-cells

### CD4+ Cells
- T-cell receptor beta chain VJ region (*CD3*)
- Integrin alpha L precursor; antigen *CD11A* (p180),
- Integrin alpha 9 protein
- Integrin alpha-7B
- Low affinity IgG FC region receptor III-A precurso (*CD16-A*)
- Complement receptor type I (C3B/C4B receptor) (*CD35* antigen)
- T-cell surface glycoprotein E2 (*CD99*)
- Mast/stem cell growth factor receptor precursor (C-KIT) (*CD117* antigen)
- Interleukin-1 receptor, type II precursor (Antigen *CDW121B*)

### CD8+ Cells
- T-cell antigen receptor alpha chain (*CDR3*)
- T-cell surface glycoprotein *CD8* beta chain isoform
- Fc-gamma receptor III-2 (*CD 16*)
- Leukocyte adhesion protein beta chain (*CD18*) precursor
- Leukocyte surface antigen *CD47* precursor
- Integrin, alpha-2 (*CD49B*; alpha-2 subunit of VLA-2 receptor)
- Integrin, alpha V (vitronectin receptor, antigen *CD51*)
- Interleukin-22 receptor
- Transferrin receptor (p90, *CD71*)
- Putative. B7,3 molecule of *CD80-CD86* family
- Leukocyte differentiation antigen *CD84*
- Cell-surface antigen heavy chain (4F2HC) (*CD98 ANTIGEN*)
- T-cell surface glycoprotein E2 (*CD99*)
- Tumor necrosis factor receptor 2 precursor (TBPII) (P80) (*CD120B*)
- Protocadherin beta 9 precursor (*CDB9*)
Cell Isolation and Purification from Tissues for Proteomics

- Mechanical or Enzymatic Tissue Disruption
- FACS™ or immunomagnetic sorting for the purification of cell subsets
- Protein extraction
- Analysis by LC-MS, 2D gels or protein micro-arrays

(MS analysis by ThermoFinnigan demonstrated, that enough low abundant protein can be obtained for global MS Id.)
Tissue Proteomics, Liver Model

- Collagenase perfusate of human liver (BD Gentest, Woburn, MA)
- Anti-CD45-APC / propidium iodide Sort (viability, CD45 expression, scatter) LC-MS/MS analysis

Starting cell suspension

CD45^+ Hepatocytes

99.3%

2.1%

0.6%

97.9%
Preliminary Results - Hepatocyte Fraction

- 35 proteins identified with high confidence from 54 unique peptides
- 8 mitochondrial-specific proteins and precursor proteins
- Hepatic arginase
- Large proportion of cytoplasmic proteins
Identification of Specific Organelles for FACS™

- Specific Enzymes with Fluorogenic Substrates
- Component-Specific Dyes
- Autofluorescence
- Organelle Targeted Vectors for Expression of Fluorescent Proteins

Figure 9. Organelles targeted by Living Colors™ Subcellular Localization Vectors.
Organelle Purification for Proteomics

- Cell disruption to release sub-cellular structures (organelles)
- Purification with anti-body coated Imag magnetic particles and cell sorting based on antibody reactivity or other specific properties.

From the website of the Catholic University of Nijmegen

Cell breakage  FACS organelle sorting  Protein extraction

BD Biosciences Immunocytometry Systems
Mitochondria

Homogenate from mouse liver cells

\[ \downarrow \]

Antibodies against metaxin or Tim23 (BD Biosciences Pharmingen)

\[ \downarrow \]

Anti-Kappa-RPE

\[ \downarrow \]

Analysis & sorting

BD Biosciences Immunocytometry Systems
Organelle/Cell/Tissue Proteomics

Summary

William D. Gleason, University of Minnesota
gleason@maroon.tc.umn.edu
O'Reilly Bioinformatics Technology Conference
Tucson, AZ

Organelle, Cell, Tissue, Organism

Alternative extraction protocols
Preconcentration, removal of major contaminants

Heterogeneous Protein Sample

Narrow pH IPG
ICAT, other Cys-peptide techniques
2D microbore chromatography
Database leveraging

Chromatographic Separation
2D-gel or...

Proteolysis, Chemical cleavage

FACS
Organelle Capillary electrophoresis
Laser capture microdissection
Organelle MS
2D Gel Databases
2D in a day
Alternative gel formulations
Protein Microarrays

Protein Identification of Individual Constituent Proteins Using Mass Spectral Data or...

Ion tree analysis
Conclusions

- Flow cytometric and immuno-magnetic particle separation methods yield sufficient highly purified preparations for proteomic analysis of low abundance proteins.

- Flow cytometry offers the best flexibility in parameter selection for cell and organelle separations, while immunomagnetic techniques provide higher throughput.