Rare Cell Analysis and Sorting by Flow Cytometry

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Presentation Outline

• Introduction with list of rare cell applications
• Basic Flow Cytometry
• Points to Consider for Rare Cell Analysis
• Application Examples
• Conclusions
• Key Contributors
Rare Cell Examples
(<1% of cell sample)

• Epithelial tumor cells, circulating in blood (CTCs)
• Tumor stem cells
• Fetal cells in maternal blood
• Hematopoietic stem cells
• Antigen specific T-cells
• Leukocytes in depleted platelet or erythrocyte preps
• Basophils
• …
Limitations for Accurate and Precise Particle/Cell Counting

- Sampling
- Sample Preparation
- Counting statistics

Source: Desatoya LLC
Detection Methods for Rare Cells

- Immunohistochemistry and high speed imaging
- PCR
- Flow Cytometry

(rare cell pre-enrichment may be valuable for all approaches, flow cytometry offers an thresholding option, which acts like a pre-enrichment)
Flow Cytometry

Single cell analysis with

- High sensitivity (single molecule sensitivity by fluorescence)
- Wide dynamic range (100 to 10,000,000 cells mL\(^{-1}\))
- High analysis and sorting rates to \(~100,000\) particles sec\(^{-1}\)
- Light scatter for label-free particle analysis and counting
- Multi-color fluorescence, multi-parameter analysis
- Live/dead discrimination
- Recovery of viable cells possible (sorting)
- Ease-of-use for the analysis of cell/particle suspensions
History of Flow Cytometry

- 1968 1\textsuperscript{st} fluorescence-based flow cytometry device (ICP 11) by Prof. Göhde from the University of Münster, Germany, and first commercialized in 1968/69 by German developer and manufacturer Partec through Phywe AG in Göttingen.
- 1971 Cytofluorograph, Ortho
- 1973 PAS 8000, Partec
- 1974 1\textsuperscript{st} FACS instrument, BD
- 1977 Epics Instrument, Coulter
- 2002 Microfluidic Cytometer, Quake, Caltech
- 2003+ many microfluidic flow cytometers and sorters
Physical Parameters for Cytometry

- Light scatter
- Absorbance
- Fluorescence
- Phosphorescence
- Raman
- Electrical properties
- Mechanical properties
- Element mass

...
Flow Cytometer Schematic

Source: BD, San Jose CA
Flow Cytometer Fluidics

Cell Input

Hydrodynamic or acoustic focusing

Injector Tip

Sheath fluid

Fluorescence signals

Focused laser beam

Cells after analysis, available for culture

CD-ROM Vol 3 Purdue University Cytometry Laboratories

V. Kachel, H. Fellner-Feldegg & E. Menke - MLM Chapt. 3
Flow Cytometer Optical Systems

Source: BD, San Jose CA
“Droplet-based” Sorting

Source: BD, San Jose CA
### Basic Data Processing

#### Numbers in Memory

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<tr>
<th>Cell</th>
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<th>P3</th>
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#### Event histogram

```
0 10 20 30
#events
0 10 20 30
P3 intensity
```

#### Dotplot

```
0 10 20 30
P3
0 10 20 30
P4
```

*Source: BD, San Jose CA*
Why Single Cell Analysis

Cell by cell intensity analysis detects population heterogeneity.

Source: Desatoya LLC
Benefits of Subset Analysis

Intensity Histogram

Intensity Ratios

Subpopulation analysis detects changes better, especially for rare subpopulations.

Source: Desatoya LLC
Subset Analysis Example

profiling a lymphoma patient

Slide provided by Nikesh Kotecha, CytoBank Inc, Mountain View CA

Data: J. Irish, Stanford
Optimizing Flow Cytometry for Rare Cell Analysis and Sorting

• Counting statistics
• Specific Cell Markers
  – Brightness
  – Combinations
• Instrument System Background
  – Carryover
  – System noise
• Raw Data Analysis
• High yield, high purity sorting
  – Pre-enrichment e.g. enrichment sort 1st
• …
Counting Statistics

Ignoring Counting Statistics Can Lead to Erroneous Conclusions

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<th>Sample 1</th>
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<th>Sample 3</th>
<th>Sample 4</th>
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<td>6</td>
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| Mean     | 2.4      | 4        | 4.6      | 5.2      |
| St.Dev   | 2.2      | 1.9      | 2.1      | 2.2      |

Overall Mean: 4.1
Overall St.Dev: 2.2

Source: Desatoya LLC
Marker Brightness

Brighter markers resolve rare populations better
Marker Combinations

An optimized combination of markers, including an exclusion marker decreases background substantially.

**Background Levels**

- 
++ no exc $10^{-4}$
+ exc $10^{-6}$
++ exc $10^{-8}$

**CTC Gate**

FSC & SSC cell size
Fl1 & Fl2 CTC specific
Fl3 exclusion markers

*Data: Gross HJ et al, Cytometry*
# Particle Carry-Over

## Carry-over specifications

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<th>Instrument</th>
<th>Carry-over (%)</th>
<th>Loader Carry-over (%)</th>
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<td>Beckman Coulter FC500</td>
<td>&lt;1%</td>
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<tr>
<td>Beckman Coulter</td>
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<tr>
<td>BD FACSCanto II</td>
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<td>Life Technologies Attune</td>
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<tr>
<td>Miltenyi MACSQuant</td>
<td>&lt;0.01%</td>
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</tr>
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</table>

Sources: Desatoya LLC

Eliminating fluidic system particle carry-over is vital for reliable rare cell analysis.
System Noise

Eliminating system noise from fluidics perturbations and other sources improves the limit of detection for rare cell analysis.

Gross HJ et al, Cytometry
Raw Data Analysis

Computer algorithms allow better raw data extraction for multi-parameter rare cell analysis.

A “genetic algorithm” finds more rare cells than manual analysis by an expert (30% vs. 10% yield at low frequencies).

*Data: Gross HJ et al, PNAS*
High yield, high purity sorting

Rare CTC sorting from peripheral blood leukocytes

<table>
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<th>Target fraction</th>
<th>Yield</th>
<th>Time [min]</th>
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<td>Enrichment</td>
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<tr>
<td>Purification</td>
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Source: Desatoya LLC calculation

Useful sorting Guidelines:
http://hcc.musc.edu/research/resources/flowcytometry/FACSaria%20Guidelines%20070208.pdf

Pre-enrichment helps sample throughput, for sorting.
Example Data for Rare Cell Applications

- Leukocyte-subset analysis without erythrocyte lysis
- Counting of hematopoietic progenitor cells
- Measuring Basophil Activation
- CTC isolation for gene expression analysis
Counting of hematopoietic progenitor cells

![Graph showing comparison between Original Fraction and CD34+ Fraction with percentage differences.] 

Aaron B. Kantor, Ian Gibbons, Stefan Miltenyi, and Jürgen Schmitz Magnetic Cell Sorting with Colloidal Superparamagnetic Particles. in: Diether Recktenwald, and Andreas Radbruch eds.; Cell Separation Methods and Applications; Marcel Dekker Inc., New York, 1997
CTC isolation for genomic analysis

1. Cancer patient blood 5 – 20 mL
2. Immunomagnetic enrichment of epithelial cells
3. Flow cytometric analysis and sorting, based on nuclear stain, epithelial marker, and leukocyte exclusion marker
4. Genomic analysis on gene arrays after whole genome amplification

Result: Genomic alterations in CTCs from prostate cancer were detected. Removal of non-CTC cells is essential for the analysis.

Leukocyte-subset analysis without erythrocyte lysis

- increase of analysis rate with a fluorescence threshold
- good correlation between lysis and no-lyse method

Source: BD, San Jose CA
Basophils are activated by stimulating with Anti-IgE or fMLP which cross-link the IgE receptors on the basophil cell membranes.

CD63 is an activation marker in basophils which is upregulated upon stimulation by IgE. This causes the granules within the basophil to move to the cell membrane and degranulate, releasing several inflammatory chemical mediators including Histamine & IL-4.

Source: IntelliCyt Corp.
An Integrated Solution

iQue™ Screening System
- High Throughput
- High Content
- Multiplexed
- Cells and Beads

Integrated Application Solution

ForeCyt® and iDM® Software
Rapidly transforms massive data sets into actionable results

Assay Reagent Kits
Robust, plug & play application specific kits

Automated high sample through-put flow cytometry system capable of rare cell analysis.

Source: IntelliCyt Corp
Conclusions

• With a high analysis rate of up to 100,000 cells/sec, flow cytometry performs robust rare cell analysis and isolation.

• Thresholding allows focusing at a cell subset for higher analysis rates.

• Optimized systems are commercially available.

• New technology developments will further help to enhance the use of automated single cell analysis.
Key contributors

- Hans-Joachim Gross, David Houck (BD) rare cell analysis by flow concept and data
- Ben Verwer (BD) algorithms
- Chia-Huei Chen (BD) un-lysed blood analysis
- Janette Phi (AmCell) CD34 counting
- Hrair Kirakossian, Liping Yu (BD) CTC analysis and sorting
- Nikesh Kotecha (CytoBank) data analysis
- ...

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