Single Cell Analysis
Matching Technology with Application Needs

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### Flow Cytometry in the Context of Biological Systems and Other Technologies

<table>
<thead>
<tr>
<th>Organism</th>
<th>NMR</th>
<th>X-ray imaging</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ultrasound</td>
<td>2-photon imaging</td>
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<tr>
<td>Organ</td>
<td>In-vivo cytometry</td>
<td>Light microscopy</td>
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<tr>
<td>Tissue</td>
<td>Electron microscopy</td>
<td><strong>Flow cytometry</strong></td>
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<tr>
<td>Single Cell</td>
<td>Cell imaging</td>
<td></td>
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<tr>
<td>Organelle</td>
<td>NA sequencing</td>
<td>Mass spectrometry</td>
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<tr>
<td></td>
<td>TIRF microscopy</td>
<td>Electrophoresis</td>
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<tr>
<td>Macromolecule</td>
<td></td>
<td>Sample prep</td>
</tr>
<tr>
<td>Small molecules</td>
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</tbody>
</table>
Why Single Cell/Particle Analysis

Intensity Histogram for Single Particles

Cell by cell intensity analysis detects population heterogeneity.
Subpopulation analysis detects changes better, especially for rare subpopulations.
Single cell analysis reveals heterogeneity, which is masked by averaging, when analyzing groups of cells.
Points To Consider

- Understand the capabilities of your system
- Evaluate effects of specimen collection and sample preparation
- Know the quality of your reagents
- Controls are essential
- Validate data analysis
Particle Counting (abs. counts or percentages)

Counting Statistics

<table>
<thead>
<tr>
<th>Sample 1</th>
<th>Sample 2</th>
<th>Sample 3</th>
<th>Sample 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>2</td>
<td>6</td>
<td>8</td>
</tr>
<tr>
<td>3</td>
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</tr>
<tr>
<td>1</td>
<td>4</td>
<td>6</td>
<td>3</td>
</tr>
</tbody>
</table>

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

Applications:
- Cell Counting
- Molecule Counting
  - Digital PCR
  - Immunoassays

Ignoring Counting Statistics Can Lead to Erroneous Conclusions

Source: Desatoya LLC
Cytometry Worksteps

- Cell observations (photography including video)
- Cell culture monitoring
- Total Cell Counting
- Cell subset counting
- Multi-parameter Cell Analysis
  - Flow Cytometry
  - Imaging
- In-vivo single cell analysis
- Cell Sorting
- Single Cell Sequencing
- Data Analysis
- Sample Preparation
- Workflow Automation
Low Magnification Video and Photography

- Documentation of changes in cell/tissue culture by digital photography or video recording.
- RGB jpeg data have limited quantitative information.
- For subsequent quantitative analysis uncompressed data formats like tiff provide good data.
- Intensity depth is generally 8 bits/color, some consumer cameras provide 12 bits/color, scientific cameras 16 bits/color.

Link to an image analysis introduction
Microscopy

- Brightfield
- Darkfield
- Fluorescence
- Phosphorescence
- Phase contrast
- Interference contrast
- Polarization

Quantitative measurements

see link ImageJ for microscopy
(select cancel, when prompted for password)
Cell Growth Monitors/ Tissue Culture

- Low Magnification Microscopy/Imaging
- Impedance

Low-cost Cell Counters
(many include viability and single color subset analysis)

• Imaging
• Flow
• Impedance

http://www.nexcelom.com
Simple Cell-Subset Counters

- Fluorescence Detection by Imaging (left below)
- Fluorescence Detection by Flow (right on bottom)
- Multi-Frequency Impedance (on right)
Cell Cycle Analysis

- High-resolution Flow Cytometry
- Imaging
- BrDU

Kinetic Cell Measurements

- Imaging  
  (single cell kinetics)
- Flow Cytometry  
  (population kinetics)

Examples:
- Ca^{++} flux
- Enzyme kinetics
- Cell proliferation

Legend. The Indo-1 ratio was calculated from the violet (440 nm) and green (530 nm) emissions and plotted against the Time parameter. Jurkat T-cells were loaded with 1 μM Indo-1 for 45 min at 37°C. Ionomycin (10 μg/ml) was added after 30 seconds resulting in a rapid rise in Indo-1 Ratio against Time.

http://www.icms.qmul.ac.uk/flowcytometry/uses/functionalanalysis/calciumflux/ratiometric/index.html
Multiparameter Cell Analysis

- Imaging
  - Immunocytochemistry
  - Multi-color Immunofluorescence
  - Sequential Stain-Destain Immunofluorescence
  - Raman including SERS
- Flow Cytometry
  - Multi-color Immunofluorescence
    - Filter-based
    - Spectral Analysis
  - Raman including SERS
  - CyTOF
- Imaging Flow Cytometry (ImageStream)

Recent Systems for Multi-parameter Cytometry

- **Element-Label Flow Cytometry**
  (CyTOF, addresses fluorescence spectral overlap issue by using elements as labels, Anal. Chem., 2009, 81 (16), pp 6813–6822)

- **SERS-Label and Spectral Flow Cytometry**
  (uses spectral (fine)-structure to distinguish labels, Cytometry, 2008, 73A(2), pp 119-128, SONY cytometer)

- **Sequential Stain De-stain Cytometry**
  (Cytometry, 2009, 75A(4), pp 362-370)

- **Highly fluorescent polymer dyes (Brilliant violet, …)**
  (Cytometry, 2012, 81A(6), pp 456-466,
  http://www.sirigen.com/sirigen_technology.html
CyTOF Cytometer

From single cells to deep phenotypes in cancer
Sean C Bendall & Garry P Nolan
Imaging Flow Cytometer

Images of Cells/Particles are captured in a fluid stream and stored individually.

In-vivo Single Cell Analysis

- Intra-vital Imaging
- In-vivo Flow Cytometry

Cell Sorting

- Bulk Sorting
  - Magnetic
  - Gravity
  - Acoustic
  - ...
- FACS
- Tyto/OWL
- DEP sorter
- Other sorters
New Cell Sorting Technologies

[Diagram of cell sorting process]

http://www.siliconbiosystems.com/deparrey-technology
Single Cell Sequencing

Gene expression analysis of single cells from blood or tissues

Source: http://www.nanostring.com

http://www.genome.duke.edu/cores/microarray/services/fluiddigm/
Novel Affinity Reagents

Antibodies

- Antibodies from different species (e.g. Llama 15 kDalton fragments with 10^{-9}M Kd and high stability, potential for intracellular use)
- Recombinant antibody fragments
- ...

Synthetic affinity reagents

- Aptamers
- Protein scaffolds
- Molecular Imprinted Polymers
- ...

Fodey T et al; Trends in Anal. Chem. 30(2011) 254ff

Fc-receptor binding: CD158a-PE on PBMC

Dr. Christian Dose, Miltenyi Biotec
Gene Expression Analysis
RNA Flow Cytometry

RNA Probes:
• Detection of specific RNA sequences after hybridization and amplification
• Single copy limit of detection

Label Selection

- Brightness
- Spectral Overlap
- Application (surface vs. internal)
- Detection System
- ...

Data Analysis

- Manual Gating

- Automated Analysis

Enrico Lugli, Mario Roederer, Andrea Cossarizza (2010) Cytometry 77A:705ff
Automated Sample preparation

Presently available
• Fully automated sample to result e.g. BC Acquios CL, MACSQuant
• xyz-fluid handling robots e.g. BD SPA or BC Biomek
• Washing by special centrifugation e.g. BD LWA
• Membrane Filtration e.g. MSP FlowCytoPrep

Promising future technologies
• Deterministic lateral displacement
• Acoustic focusing
• Inertial flow
Droplet-based Integrated Bio-Assay System Technology

Heterogeneous Tumor Cells
Disaggregate & label
Generate single cell droplets
Sort by phenotype
Lyse
Merge Amplification reagents
Amplify
Sort & dispense into individual wells

Raindance Technologies
http://raindancetech.com/

Single cell microarray analysis
Targeted enrichment (RDT1000)
Single cell sequencing

Generate Co-Flow Merge Split Mix Timing Thermal Zones Detect Sort Collect
New Detector-Label Combinations

- New photodetectors extend the available spectrum
  (Si avalanche photodiodes extend detection into the far infrared, Xitogen system)

- New dyes add excitation in the UV, some detection in the IR
  (Fluorescent polymers, bacteriochlorins, …)
New Detection Technologies

- High spatial resolution and multi-parameter capability with X-ray / synchrotron radiation fluorescence
  (super-high resolution with element labels or direct element imaging)
- Medium resolution, multi-parameter mass spectrometric imaging
  (CyTOF like element labels, direct metabolite or structural component detection)
- Label-free imaging with Raman
  (measuring cellular components by their Raman spectra)
- Label-free high resolution NMR imaging
  (direct chemical detection)
Conclusions

• For optimal results use an adequate technology
  (flow cytometry has enormous capabilities, but is not always the technology to use e.g. single cell kinetics)

• Understand the limitations of the system
  (limits of detection, non-specific binding of reagents, …)

• Validate results with appropriate controls
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- Holden Maecker, Stanford
- Bob Hoffman
- Ming Yan
- Maria Jaimes
- Hrair Kirakossian
  ...

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