Principles of Flow and Imaging Cytometry for the Study of Ocean Microbes

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Lecture outline

• Principles of flow cytometry
• Imaging-in-flow (FlowCAM)
• In-situ instrument developments
A fundamental tenet of ecology is to understand and explain the distributions of organisms.
Microbial Food Web

All forms produce dissolved organic matter
“Ecological Uncertainty Principle”

You can know precisely **WHO** the organisms are

OR

You can know precisely **HOW MANY** there are

BUT

You cannot know both

--> Can technology solve this dilemma??
Plankton size spectra

- Flow cytometry - phytoplankton
- Imaging cytometry - bacteria and protozooplankton
- Imaging-in-flow (FlowCam)
- Spring bloom
- Coastal

Cell Concentration (per ml)

Cell size

- Open ocean
- Coastal
- Spring bloom

Log scale:
- $10^5$
- $10^4$
- $10^3$
- $10^2$
- $10^1$
- $10^0$
- $10^{-1}$

Log scale:
- $1\mu m$
- $10\mu m$
- $100\mu m$
- $1mm$
What is Flow Cytometry?

**Cytometry** - rapid simultaneous measurements of individual particles (cells).

**Measurements:**
- light scatter
- fluorescence (autofluorescence or stain-induced)

**Flow cytometry** - detecting cells single file within a fluid stream.
Can measure several parameters for several thousand cells per second with high precision (up to ~10,000 cells/sec).
What can a flow cytometer tell us about a cell?

1. Size.

2. Relative granularity or internal complexity.

3. Relative fluorescence intensity.
Why use flow cytometry?

1. Counts and measures single cells/particles.
2. Rapid, quantitative measurements
3. Can physically sort sub-populations for cultivation or further analysis.

Sample requirements

- Cells/particles in liquid suspension
- Optimal concentration: $5 \times 10^3$ to $5 \times 10^6$ per mL
Cells in **suspension** flow in **single-file** through an **illuminated volume** where they **scatter light** and emit **fluorescence** that is collected, filtered and converted to digital values that are stored on a computer.

**Flow cell orifice can range from 5 to 300 µm, usually 70 - 200 µm.**
Systems of a Flow Cytometer

1. **FLUIDICS** - to introduce and align the cells/particles for interrogation.

2. **OPTICS** - An excitation source and collection optics to generate and collect the optical signals.

3. **ELECTRONICS** - To convert the optical signals to proportionate electronic signals and digitize them for computer analysis.

4. **SORTING** - physical separation of specific cells/particles
Fluidics

Need to have cells in suspension flow in single file through an illuminated volume.

In most instruments, accomplished by injecting sample into a sheath fluid as it passes through a small (50-400 µm) orifice.

Sample fluid flows in a central core that does not mix with the sheath fluid, i.e. laminar flow.
The introduction of a large volume into a small volume in such a way that it becomes “focused” along an axis is called **Hydrodynamic Focusing**.

As particles are hydrodynamically focused, they experience different shear stresses that cause them to orient along their axis.
How do we accomplish sample injection and regulate sample flow rate?

**Fluidics**

- Air pressure in
- Sheath
- Flow chamber
- Pinch valve
- Sample tube
- Vacuum waste tank

**Differential pressure**
Fluidics - differential pressure

1. Sheath pressure will set the sheath volume flow rate (assuming sample flow is negligible).

2. Difference in pressure between sample and sheath will control the sample volume flow rate.

3. Control is not absolute - changes in friction cause changes in sample volume flow rate.
Fluidics - Flow Chambers

**Jet-in-air**
best for sorting,
inferior optical properties

**MoFlo cytometer**
Flow-through cuvette
excellent optical properties
not as good for sorting

FACScan flow cytometer
Optics - Excitation

1. Need to have a light source focused at the point that cells have been focused (the illumination volume)

Two types of light sources:

- **Lasers** *(MoFlo and FACScan)*
- **Arc-lamps** *(NPE instrumentation)*

2. Need lenses to shape and focus the laser beam (or illumination source)
Optics - Light Sources

Lasers

1. Provides a single wavelength of light (a laser line) or (more rarely) a mixture of wavelengths
2. Provides from milli-watts to watts of illumination power
3. Lower power air-cooled lasers and laser diodes (less expensive)
4. High-power solid-state and water-cooled lasers (more expensive)

Laser lines available from an argon-ion laser

Ultraviolet - 351, 363 nm
Visible - 457, 476, 488, 496, 514, 528 nm (632 nm)
Optics - Collection and detection

Collection optics consists of a system of filters and dichroic mirrors that route specified wavelengths to designated detectors.

Two types of optical detectors
- Photomultiplier tubes (PMTs)
- Photodiodes
Scatter

- the amount of light scattered in the forward direction (along the same axis that the laser light is traveling) is detected in the forward scatter channel
- the amount of light scattered to the side (perpendicular to the axis that the laser light is traveling) is detected in the side or 90° scatter channel

Forward scatter is primarily effected by particle size.
Side scatter is effected by particle size, shape, and optical homogeneity.
The fluorescence emitted by each fluorochrome (pigment or stain) is detected in a unique **fluorescence channel** at 90 degrees from laser angle.

The specificity of detection is controlled by the wavelength selectivity of optical filters and mirrors.
Fluorescence Detectors - 90 degrees

- Forward Scatter
- Bandpass Filters
- Side Scatter
- Dichroic Filters
- Flow cell
- Sample
- Laser
- PMT 5
- RED
- Orange
- Green
Optics - Filters - Basic Characteristics

- Optical filters are constructed from materials that absorb or reflect certain wavelengths and are most often characterized by their transmittance spectra.

- Transitions between absorbance and transmission are not perfect; the sharpness can be specified during filter design.
1. Filters must have very sharp cut-offs and cut-offs since there will be many orders of magnitude more scattered laser light than fluorescence.

2. Can specify wavelengths that filter must reject to a certain tolerance.

*(e.g., reject 488 nm light at 10^{-6} level: only 0.0001% of incident light at 488 nm gets through)*
Optics - Filter Properties

**Long pass filters** transmit wavelengths ABOVE a cut-on wavelength.

**Short pass filters** transmit wavelengths BELOW a cut-off wavelength.

**Band pass filters** transmit wavelengths in a narrow range around a specified wavelength. **Band width** can be specified.
When a dichroic filter is placed at a 45° angle to the light source, shorter wavelengths are transmitted and longer wavelengths are reflected (at a 90° angle).
To simultaneously measure more than one scatter or fluorescence from each cell, we typically use multiple channels (multiple detectors - PMTs)

Design of multiple channel layout must consider

- spectral excitation and emission of fluorochromes
- proper sequence and position of filters and mirrors
Fluorescence Detectors - 90 degrees
Common Laser Lines

300 nm 400 nm 500 nm 600 nm 700 nm

UV  Violet  Blue  Green  Orange  Red  IR

Excitation

Em.

Chlorophyll

Texas Red

Propidium iodide

Ethidium bromide

Phycoerythrin

Fluorescein (FITC)

cis-Parinaric acid
Electronics

• Voltage received from the PMT is directly proportional to the number of photons that strike the photocathode.

• Amplitude of the “voltage pulse” is dependent on:
  1. Number of photons detected
  2. PMT voltage
  3. Amplifier Gain

• Upon analysis of the pulse height the voltages (0-10.24V) are converted to a digital number representing 0-1024 channels (Analog to Digital Conversion - ADC)

• Linear and Logarithmic collection
Flow Cytometric Signatures of Phytoplankton Cultures

2-D dotplots

can also use contour plots, 3D plots, 1-D histograms
Natural Marine Samples

Continental Shelf
Georges Bank

Open Ocean
Sargasso Sea
Flow cytometric signatures from the open ocean

Chlorophyll maximum

Depth

Forward scatter

Chl fluorescence

Sample from 80 meters depth

Forward scatter

Sample from 100 meters depth

Forward scatter

Sample from 130 meters depth

Forward scatter

Sample from 20 meters depth

Forward scatter
Cytomation MoFlo high speed cell sorter
Basics of Flow Sorting

1. **Droplet formation**
   As liquid is ejected into air, it will form droplets. By vibrating the nozzle at a defined frequency, the size of these droplets and the position along the stream where they form can be controlled with great precision.

2. **Timing**

3. **Purity and Efficiency**
- Hydrodynamic focusing of sample
- Interrogation using a light source.
- Cells/particles of interest are identified
- Droplet formation and charge applied.
- Droplet deflection and collection.
Sorting takes place following analysis in the Illumination Chamber.

- Droplets are formed by vibrating the nozzle at very high frequency.
- The sorting function involves many facets of the MoFlo that require very precise timing and calibration.
- The more stable the droplet break-off and charge translates directly to the purity of your sort.

IMPORTANT - Air-free fluidics.
The Sort (MoFlo®)

- A decision is made at the interrogation point whether to sort a cell or abort a cell.

- If a cell is to be sorted, the electronics waits for the “drop delay” time until the split second when the cell will be contained in the “last attached drop”.

- The electronics sends a charge throughout the sheath/sample stream.

- The last attached drop breaks off carrying a charge that can be positive or negative depending on the direction to be sorted.

- As the charged droplet falls between the sort deflection plates, it is deflected into the proper tube for collection.
## Sort Modes (MoFlo®)

<table>
<thead>
<tr>
<th>Mode</th>
<th>Characteristic</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enrich</td>
<td>Tries to capture all positive events regardless of the presence of negative events.</td>
<td>High recovery/yield</td>
</tr>
<tr>
<td>Purify</td>
<td>Sorts positives only in the absence of a negative event.</td>
<td>High purity</td>
</tr>
<tr>
<td>Single</td>
<td>Same as Purify mode except will only accept one positive event per sort decision.</td>
<td>Single Cell Deposition</td>
</tr>
<tr>
<td>Mixed Mode</td>
<td>Sorts Purify Mode left and all the aborts to the right.</td>
<td>High Purity one direction while recovering all aborted positive events the other direction.</td>
</tr>
</tbody>
</table>
Flow cell nozzle orifice of 100 µm
Sample pressure was about 25 psi (172 kPa)
Total drop volume was 2.785 nanoliters
Equivalent spherical diameter (ESD) of 177 µm.

<table>
<thead>
<tr>
<th>Pressure differential</th>
<th>Ratio of $V_s$ to $V_d$ (R)</th>
<th>Sample stream diameter (µm)</th>
<th>Sample droplet volume, $V_s$ (pL)</th>
<th>ESD of $V_s$ (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2</td>
<td>$3.13 \times 10^{-3}$</td>
<td>5.6</td>
<td>8.7</td>
<td>25.9</td>
</tr>
<tr>
<td>0.4</td>
<td>$4.98 \times 10^{-3}$</td>
<td>7.0</td>
<td>13.9</td>
<td>30.3</td>
</tr>
<tr>
<td>0.6</td>
<td>$7.93 \times 10^{-3}$</td>
<td>8.9</td>
<td>22.1</td>
<td>35.3</td>
</tr>
<tr>
<td>0.8</td>
<td>$9.71 \times 10^{-3}$</td>
<td>9.8</td>
<td>27.0</td>
<td>37.8</td>
</tr>
</tbody>
</table>

If $10^6 \cdot$ mL$^{-1}$ bacteria cells were present in the sample, then there would be one bacterium every 40 to 110 drops. (Sieracki et al. 2005)
Sorting Applications

- cell isolation and cultivation
- culture purification
- single cell isolation for DNA amplification
- sorting of phytoplankton, heterotrophic protists, and bacteria
- sorting select populations using DNA or antibody stains
Sorting for Identification and/or Isolation

Establishing cultures or identifying groups by sorting on different populations

Post-sorting microscope identification

Sort regions

Fibrocapsa japonica

Leptocylindrus danicus

Thalassiosira minima
Sorting for purification or isolation

• sorting success from bacterized cultures (Sensen et al. 1993)
  • *Cyanophora*
  • *Haematococcus*
  • *Monomastix*
  • *Scherffelia* and *Spermatozopsis*
  • *Prochlorococcus* (Moore et al. 1998)

• sorting natural samples in Wadden Sea - clonal isolates
  • *Synechococcus* (Reckermann and Colijn, 2000)
  • *Microcystis*
  • *Hemiselmis*
  • *Teleaulax*
  • *Rhodomonas*, and *Thalassiosira*
Sorting for Isolation or Cultivation

- Number of Cells to Sort
- Physical Setup
  - Nozzle Tip Size
  - Pressure and differential
  - Sheath (and fluidics preparation)
  - Laser Power
- Sort Trigger and Criteria (sort modes)
- Tubes vs. Plates
- Culture Media
- Culture or Field Sample Preparation
- Screening

Volume of a drop
Isolation from Cultures

Cell Sorting

- for culture purification
- clonal and axenic
- establishing new culture from field isolations

96 vs. 24 well plates

Positive Control Wells

- 1 cell/well
- 10 cell/well
- 100 cell/well
- 1000 cell/well
Sorting for PicoEukaryotes in Gulf of Maine

- 3um filter screen and incubation
- Excluded cyanobacteria from sort criteria
  - Sort single one drop - 1 and 10 cell sorts
Sampling Pico-Algal Diversity from Gulf of Maine

N.J. Poulton, R.A. Andersen, M.E. Sieracki
Fluorescence of Marine Protists
FlowCAM - Imaging in Flow

Benchtop

Portable
FlowCAM

Fluorescence (or light scatter)-triggered imaging in flow system
FlowCAM Imaging

- No sheath fluid
- Fluorescence-triggered
- Cell subimages extracted in real-time
- Digital images stored
FlowCAM: Interactive Scattergram
Color FlowCAM Images

Diatoms

Tetraselmis
FlowCAM deployed on floating docks
In-situ Flow Cytobot Deployment

CytoSense, CytoBuoy, CytoSub

- Pulse processing
- Wide dynamic range
- Designed for biological oceanography
Protozoan Isolation using LysoTracker

Detection of Flagellates and Ciliates

- LysoTracker is a fluorescent acidotropic probe that labels acidic organelles in live cells (e.g., vacuoles and lysosomes).
- Specific for eukaryotes (must have internal organelles).
- Allows for the detection & isolation of small heterotrophic protists that are not mixotrophs (both photosynthetic and heterotrophic).

![Graph showing the use of LysoTracker for protist isolation.](image)
LysoTracker Green - Identification of Heterotrophic Protists

Negative control (preserved)

Positive Control (live)
Microbial Food Web

All forms produce dissolved organic matter