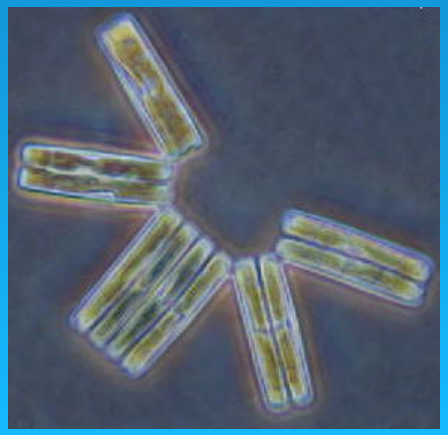
Principles of Flow and Imaging Cytometry for the Study of Ocean Microbes

Mike Sieracki
Nicole Poulton
J.J. MacIsaac Aquatic Cytometry Facility
Bigelow Laboratory for Ocean Sciences

June 2008



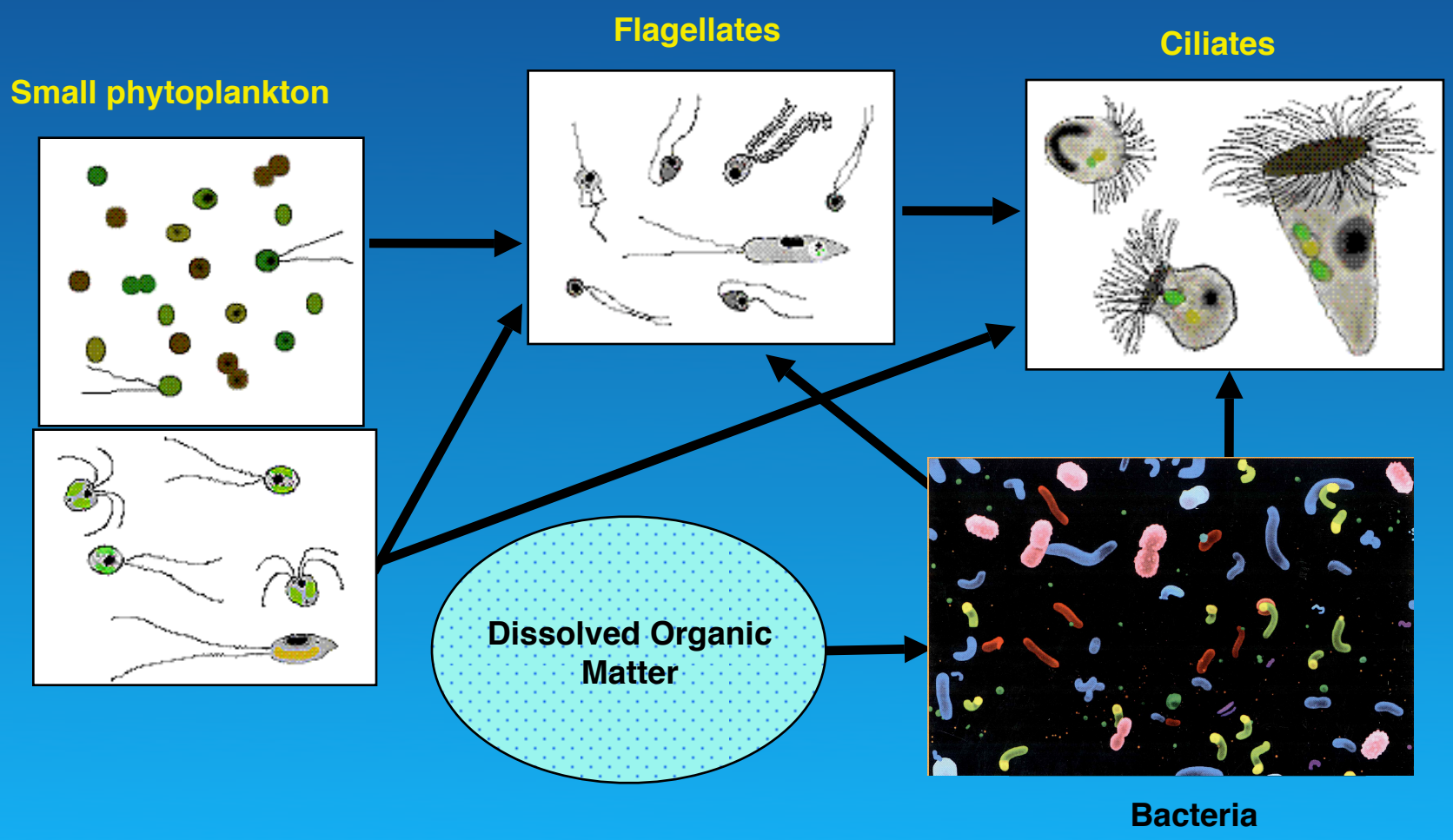
Lecture outline

- Principles of flow cytometry
- Imaging-in-flow (FlowCAM)
- In-situ instrument developments

What is where and how many?

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

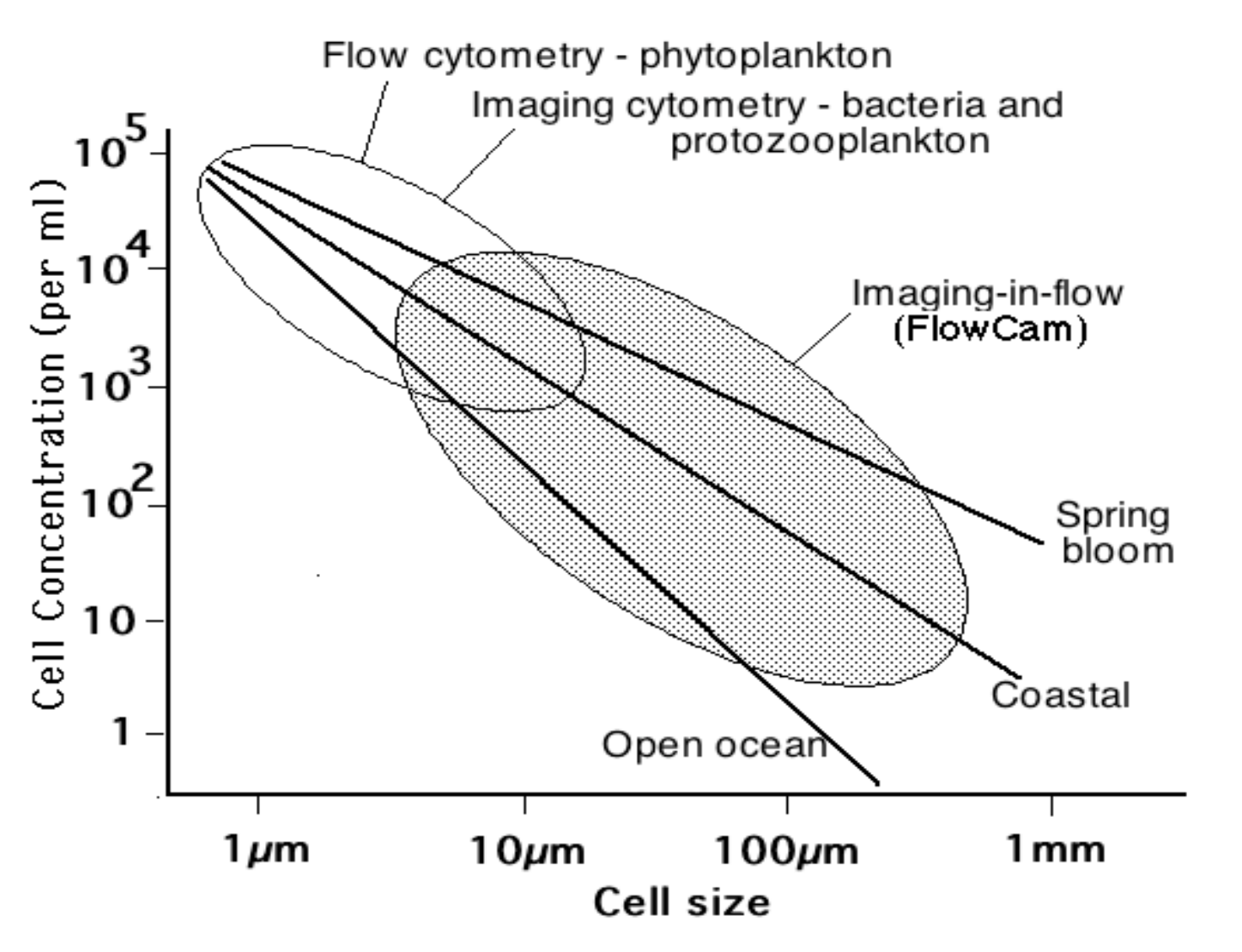
You can know precisely **HOW MANY** there are

BUT

You cannot know both

--> Can technology solve this dilemma??

Plankton size spectra

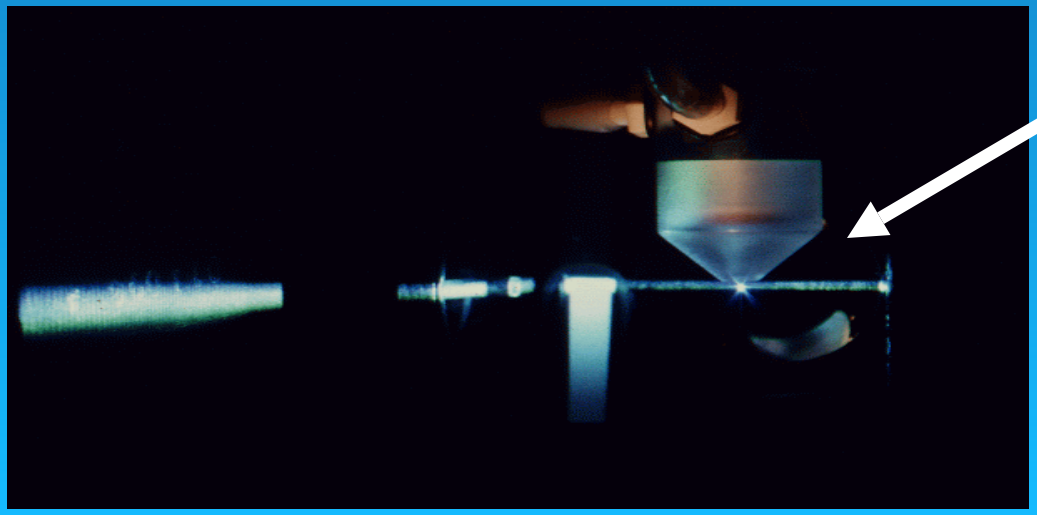


What is Flow Cytometry ?

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

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

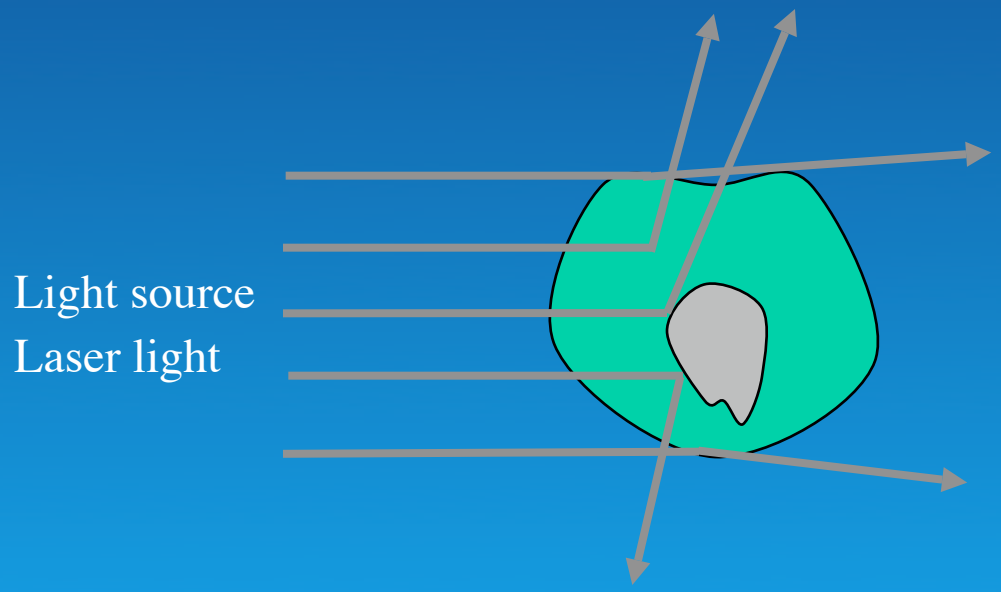
Flow cytometry - detecting cells single file within a fluid stream
Can measure several parameters for several thousand cells per second with high precision (up to ~10,000 cells/sec).



Flow Cell

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?

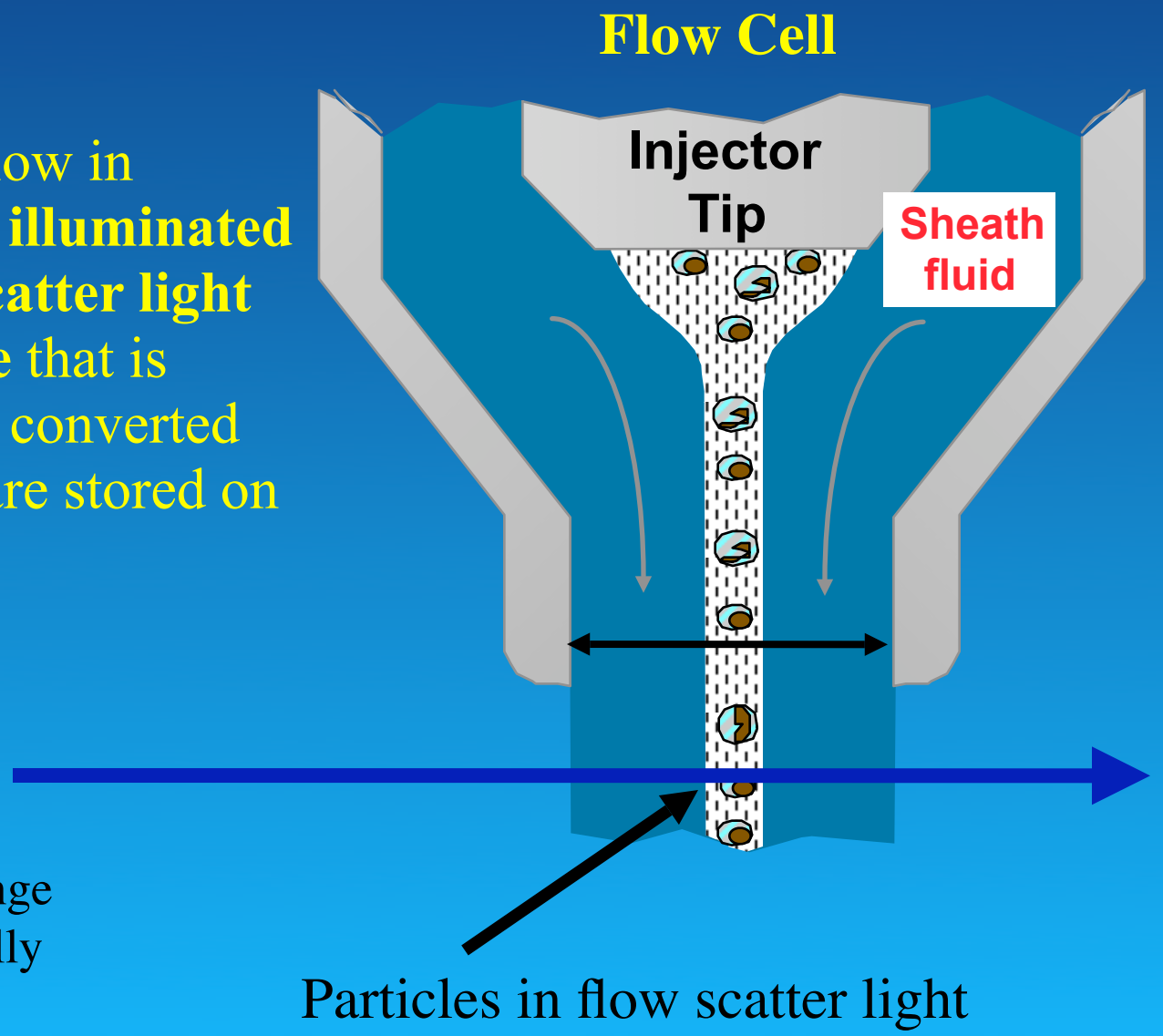
1. Counts and measures single cells/particles.
2. Rapid, quantitative measurements
3. Can physically sort sub-populations for cultivation or further analysis.

Sample requirements

- Cells/particles in liquid suspension
- Optimal concentration: $5x10^3$ to $5x10^6$ per mL

Basics of Flow Cytometry

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.

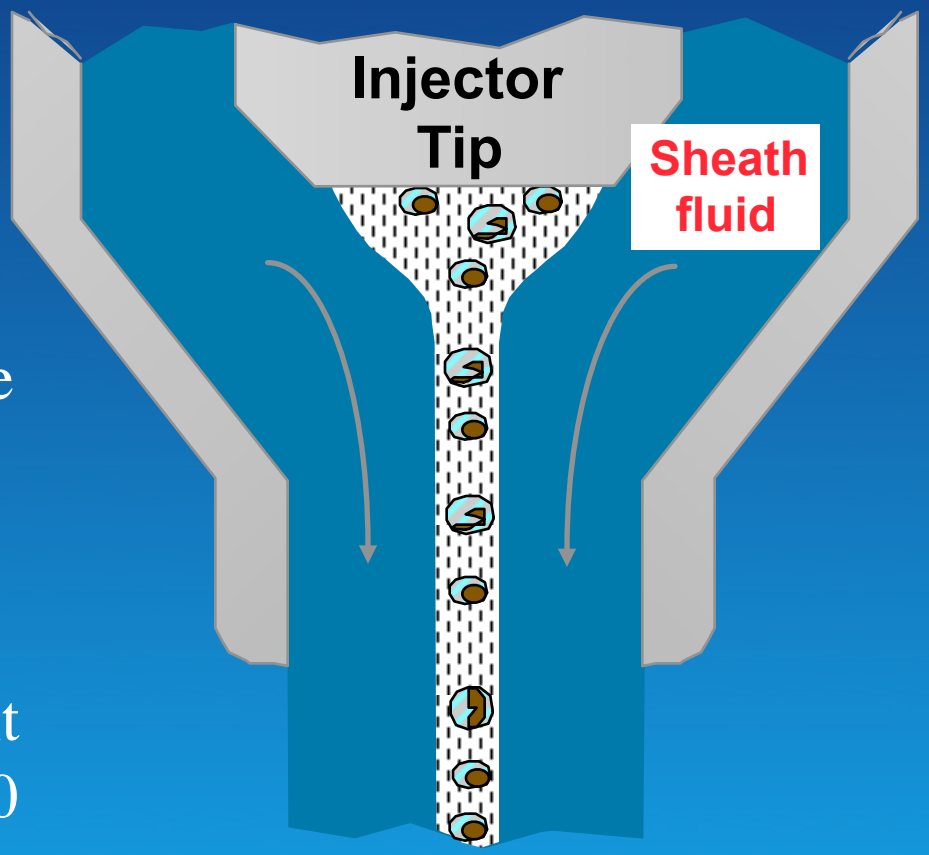


Flow cell orifice can range from 5 to 300 μm, usually 70 - 200 μm.

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

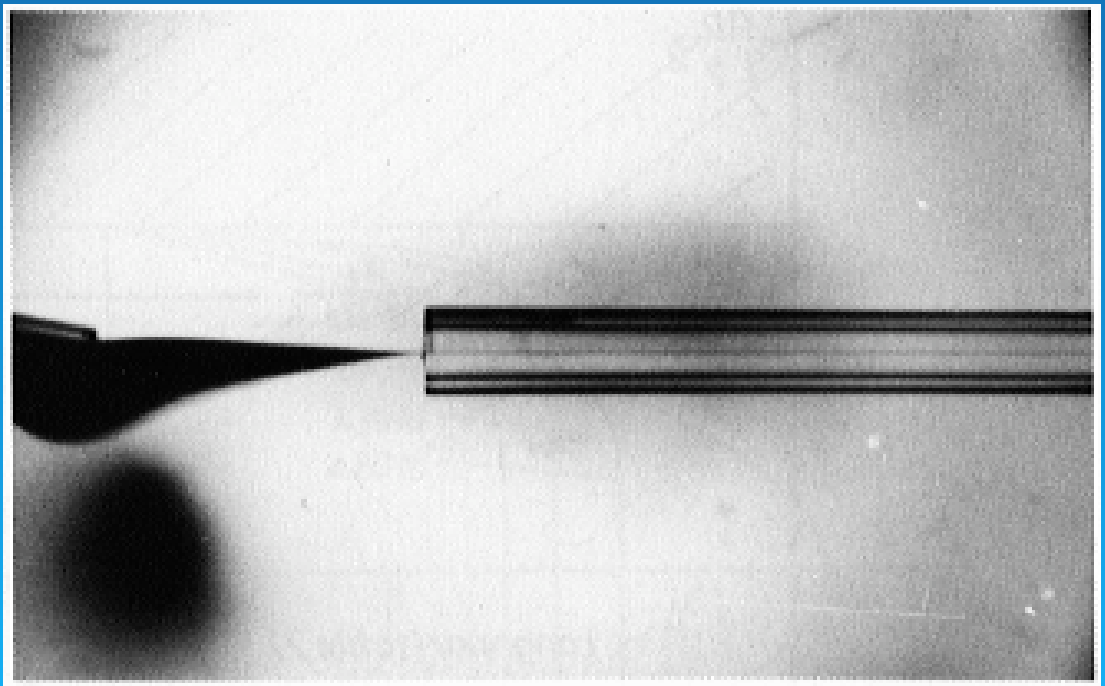
In most instruments, accomplished by injecting sample into a **sheath fluid** as it passes through a small (50-400 μm) **orifice**

Sample fluid flows in a central core that does not mix with the sheath fluid, i.e. l**aminar flow.**

Fluidics

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**

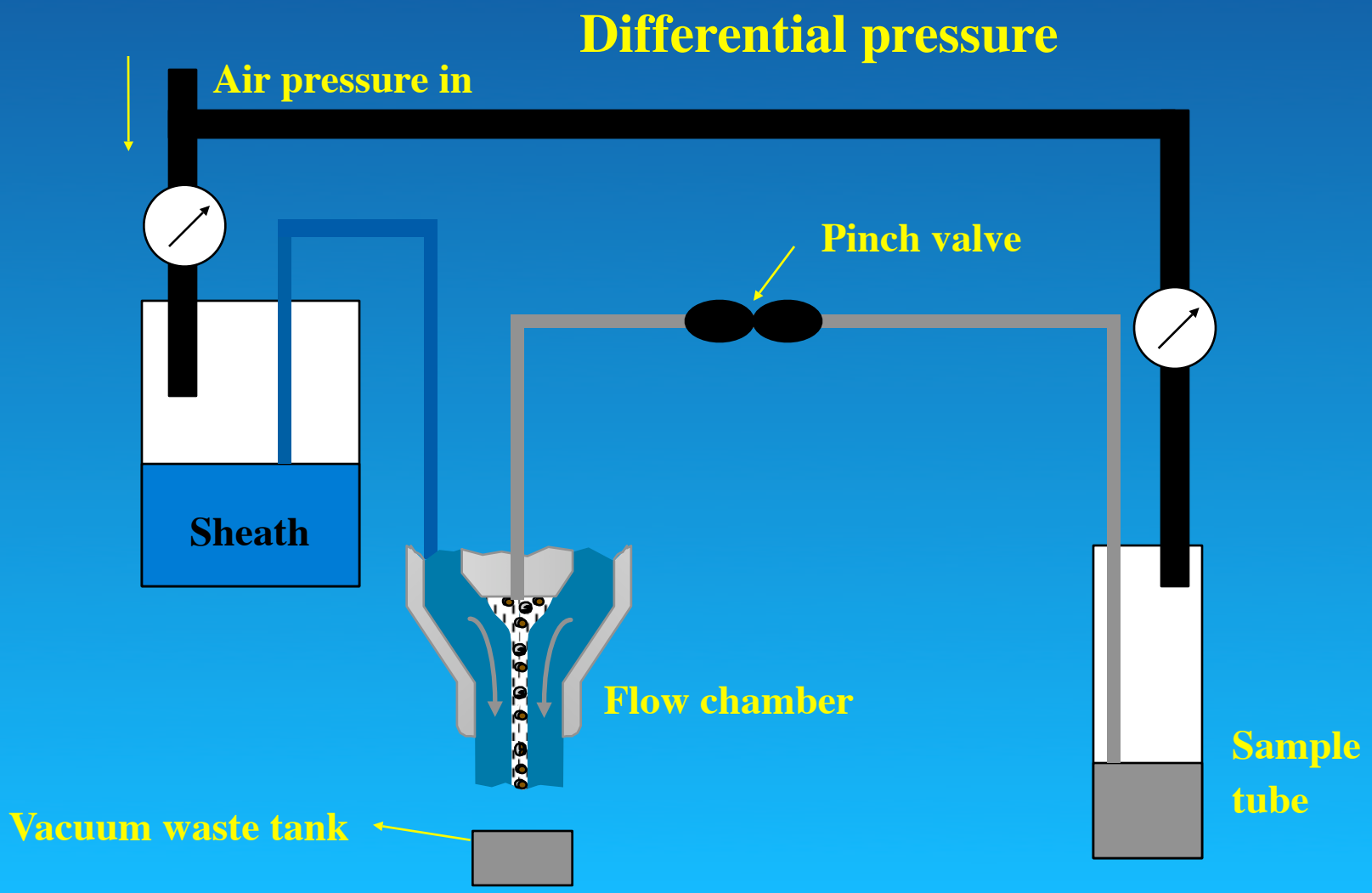
As particles are hydrodynamically focused, they experience different shear stresses that cause them to orient along their axis.



Hydrodynamic focusing during laminar flow

Fluidics

How do we accomplish sample injection and regulate sample flow rate?



Fluidics - differential pressure

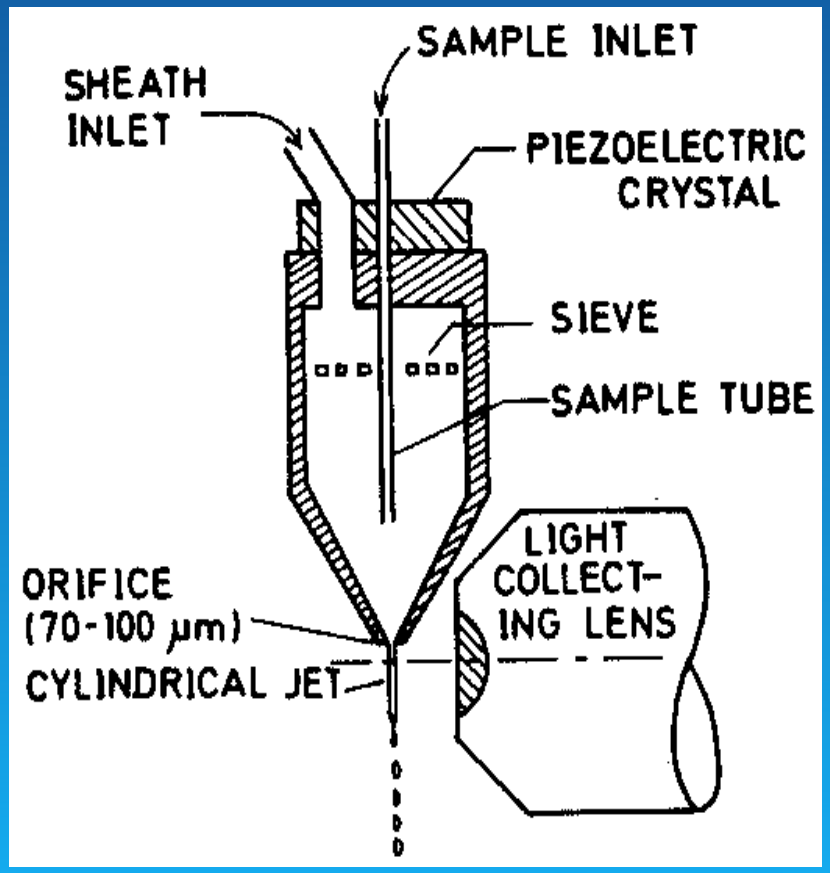
1. Sheath pressure will set the **sheath volume flow rate** (assuming sample flow is negligible)

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

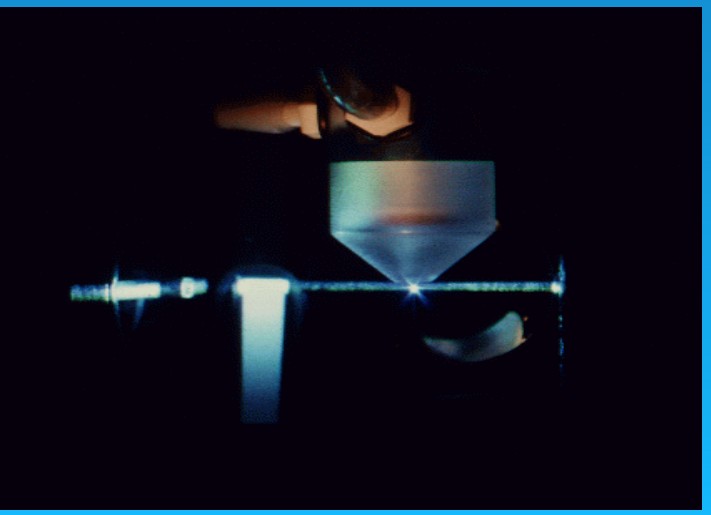
3. Control is not absolute - changes in friction cause changes in sample volume flow rate.

Fluidics - Flow Chambers

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



MoFlo cytometer

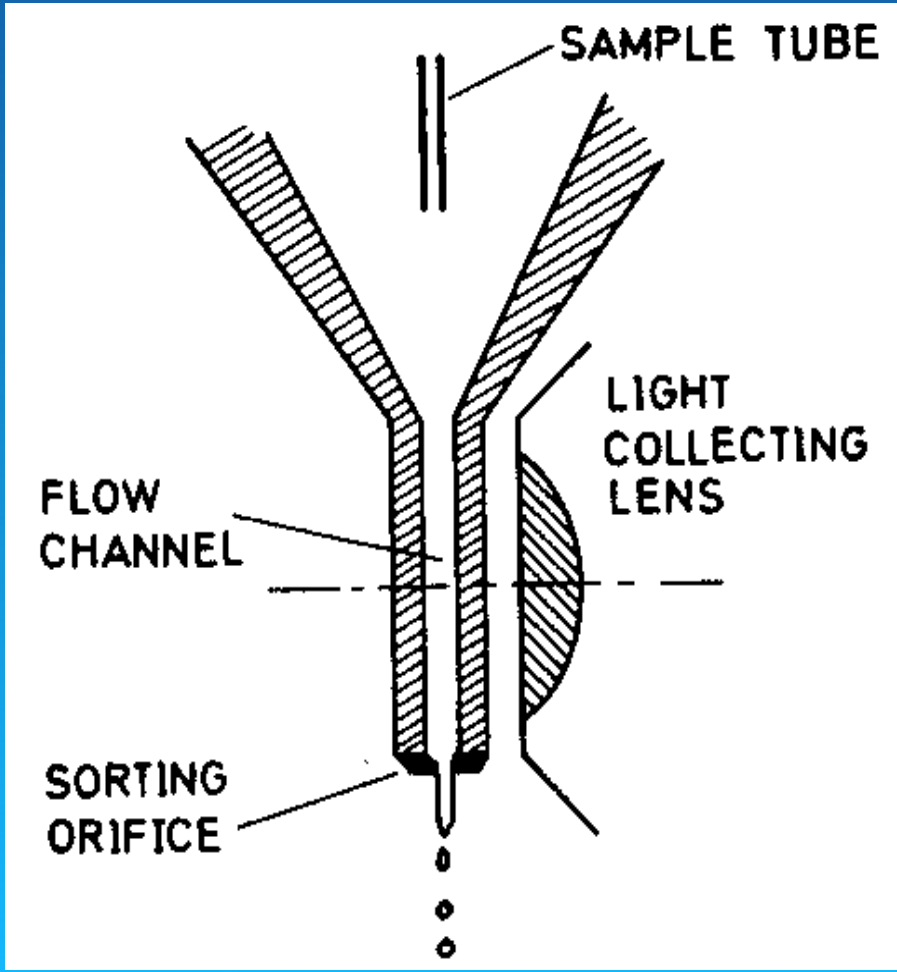


Fluidics - Flow Chambers

- defines the axis and dimensions of sheath and sample flow

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

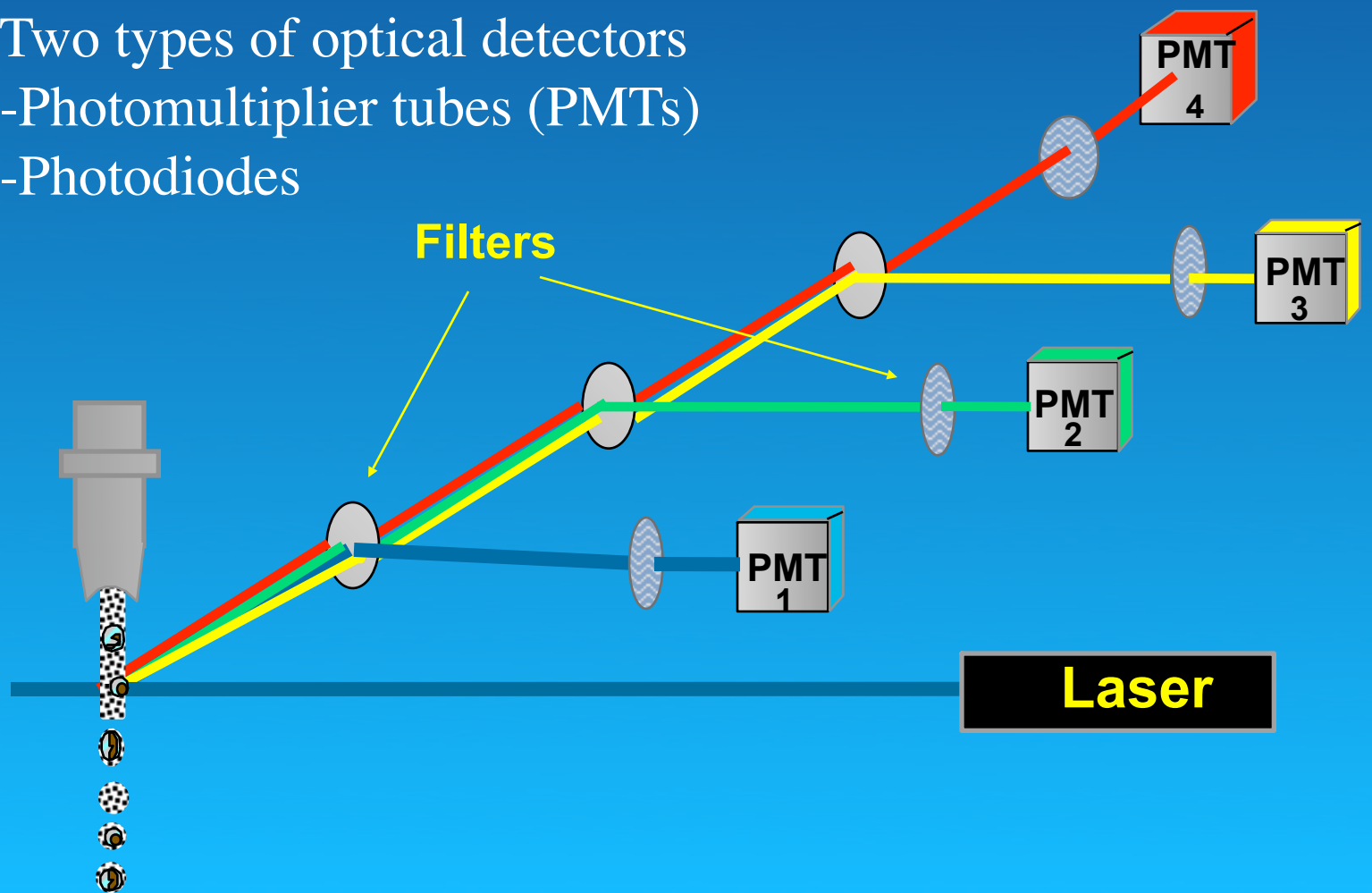
Ultraviolet - 351, 363 nm

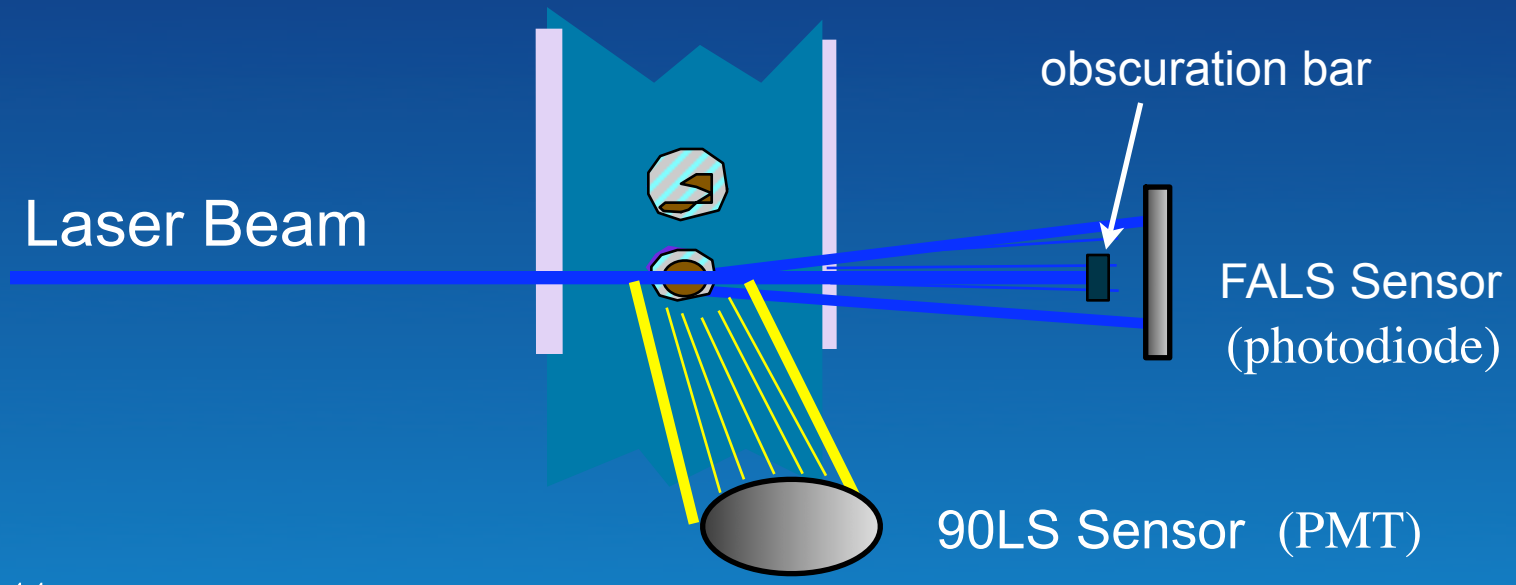
Visible - 457, 476, 488, 496, 514, 528 nm (632 nm)

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

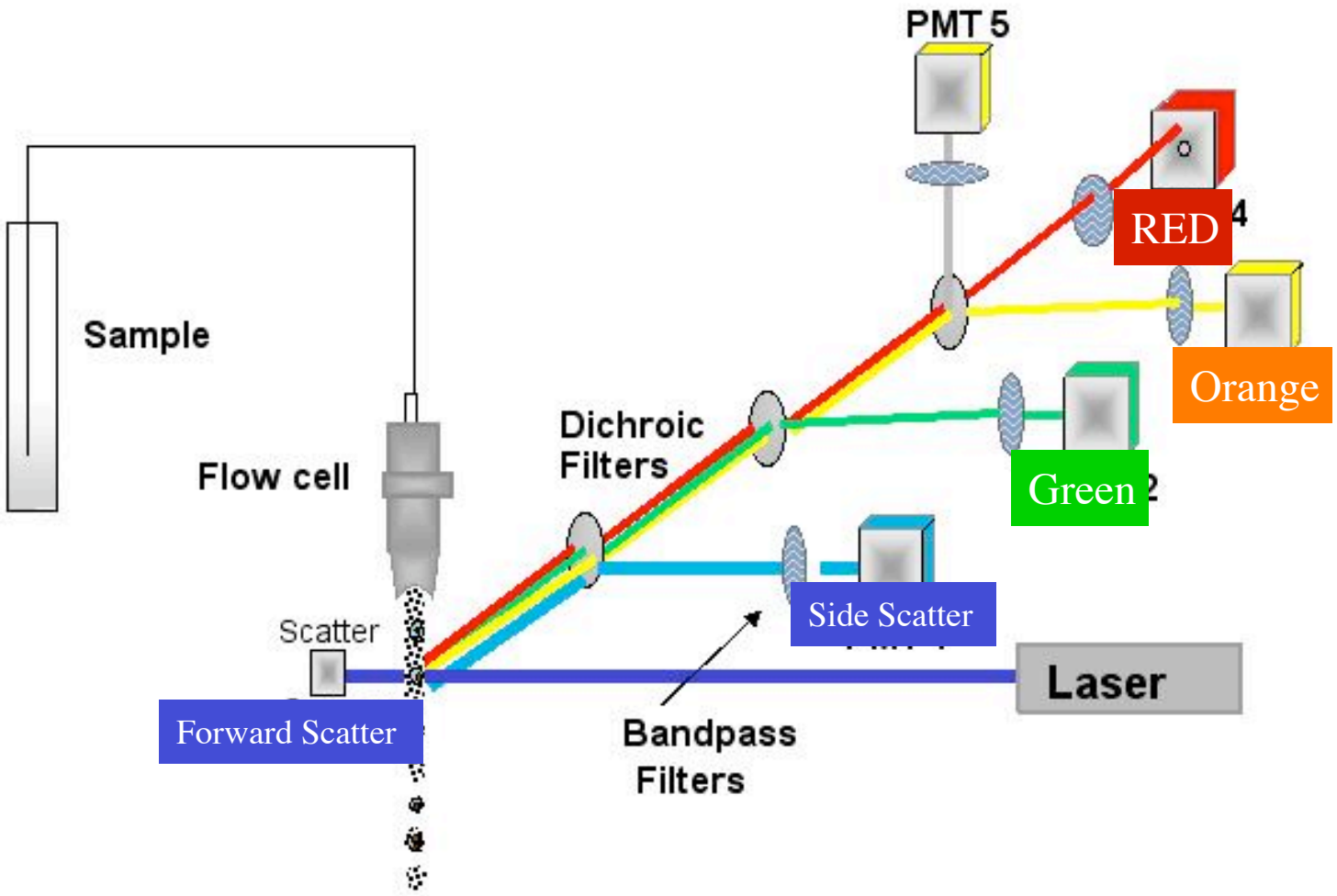
- the amount of light scattered in the forward direction (along the same axis that the laser light is traveling) is detected in the **forward scatter channel**
- the amount of light scattered to the side (perpendicular to the axis that the laser light is traveling) is detected in the **side or 90° scatter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

Forward scatter is primarily effected by particle **size**.
Side scatter is effected by particle **size**, **shape**, and optical **homogeneity**.

Optics - Fluorescence Channels

- 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



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.

Optics - Filters - Basic Characteristics

1. Filters must have very sharp cut - ons 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

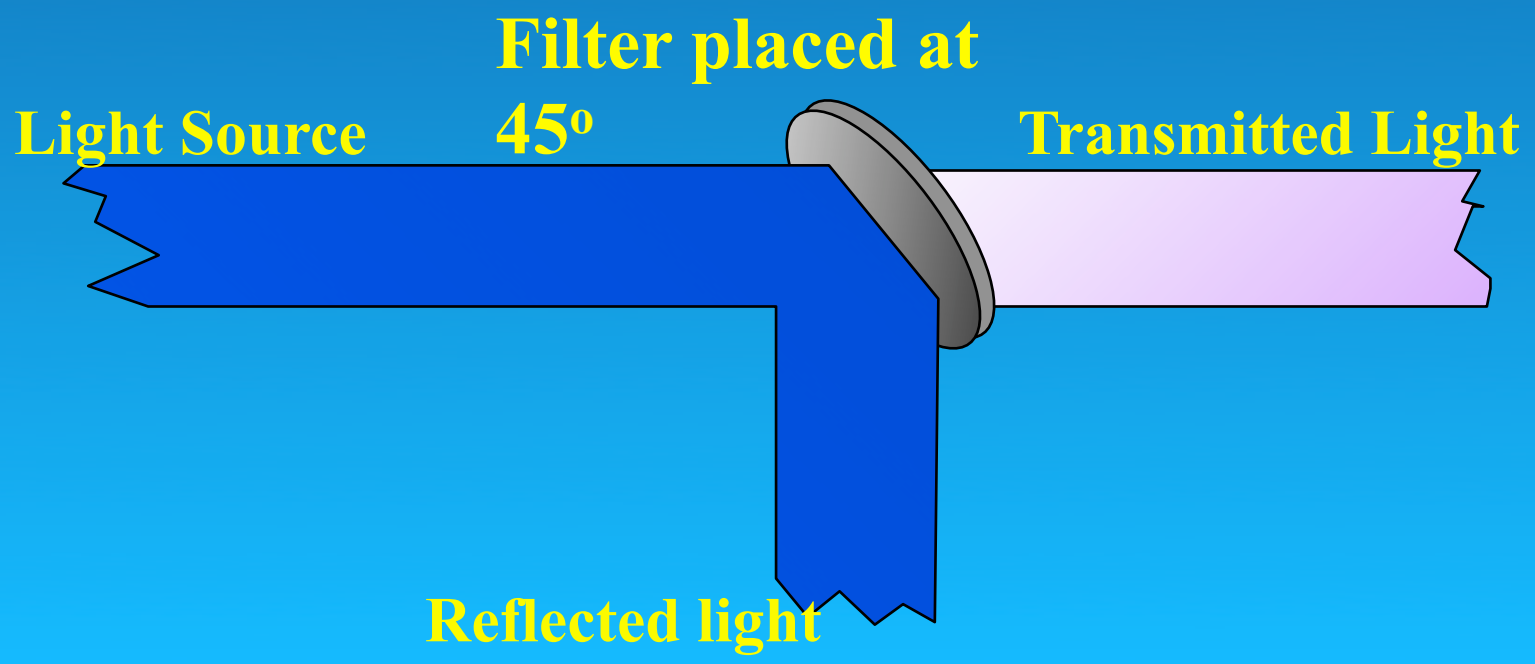
Long pass filters transmit wavelengths ABOVE a **cut-on** wavelength.

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

Optics - Filter Properties - Dichroic Filters

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).



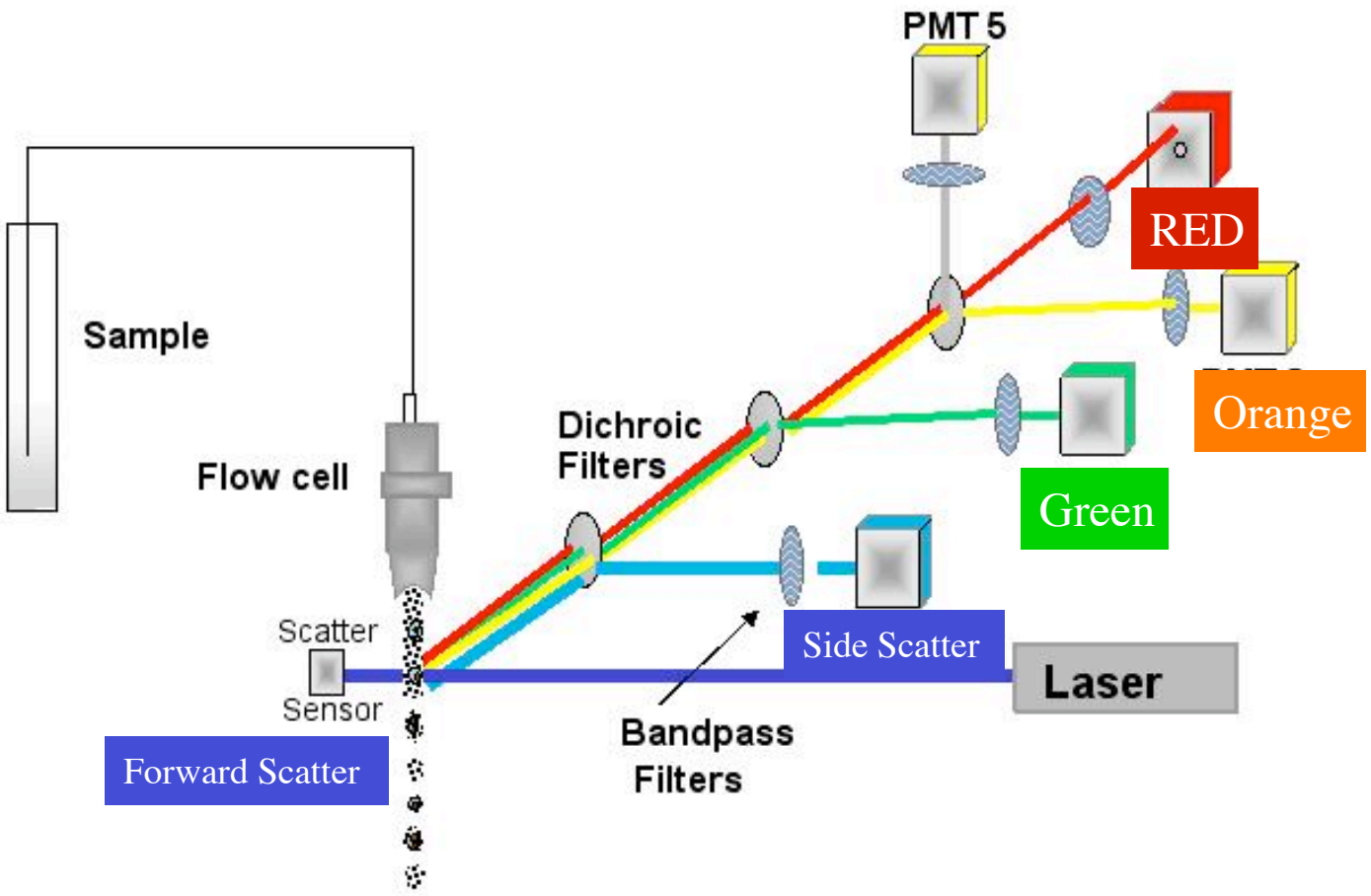
Optics - Filter Layout

To simultaneously measure more than one scatter or fluorescence from each cell, we typically use multiple channels (multiple detectors - PMTs)

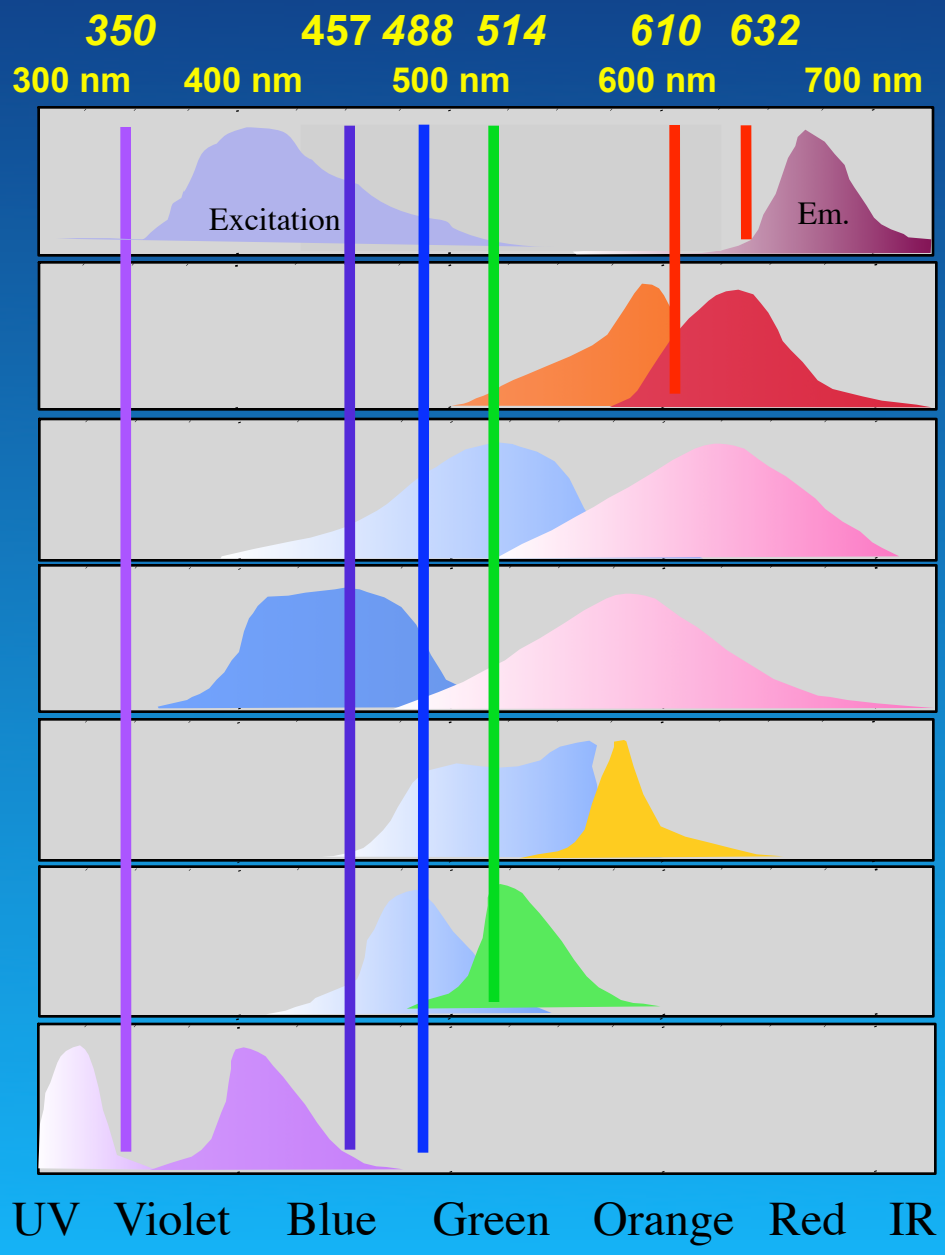
Design of multiple channel layout must consider

- **spectral excitation and emission** of fluorochromes
- **proper sequence and position** of filters and mirrors

Fluorescence Detectors - 90 degrees



Common Laser Lines



Chlorophyll

Texas Red

Propidium iodide

Ethidium bromide

Phycoerythrin

Fluoroscein (FITC)

cis-Parinaric acid

Electronics

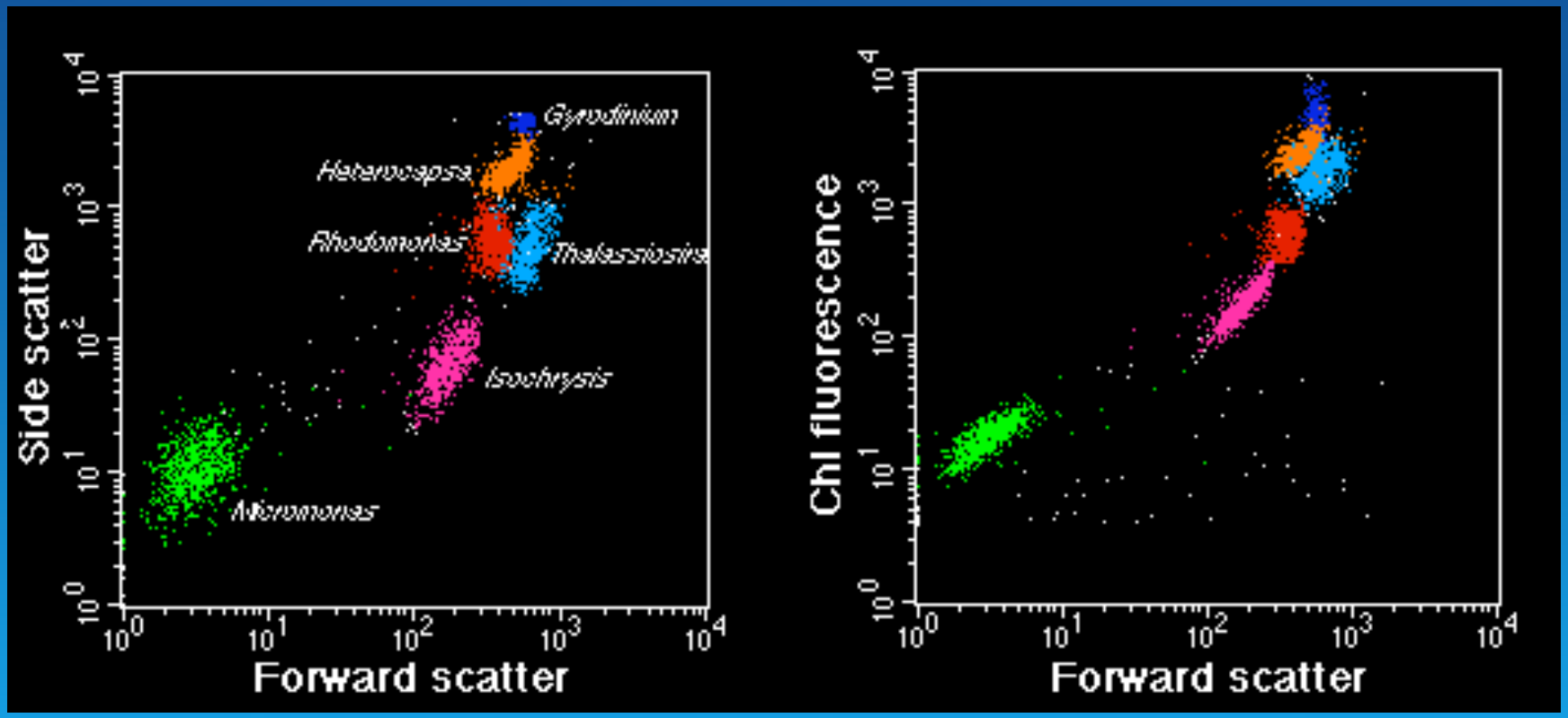
- Voltage received from the PMT is directly proportional to the number of photons that strike the photocathode.

- 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



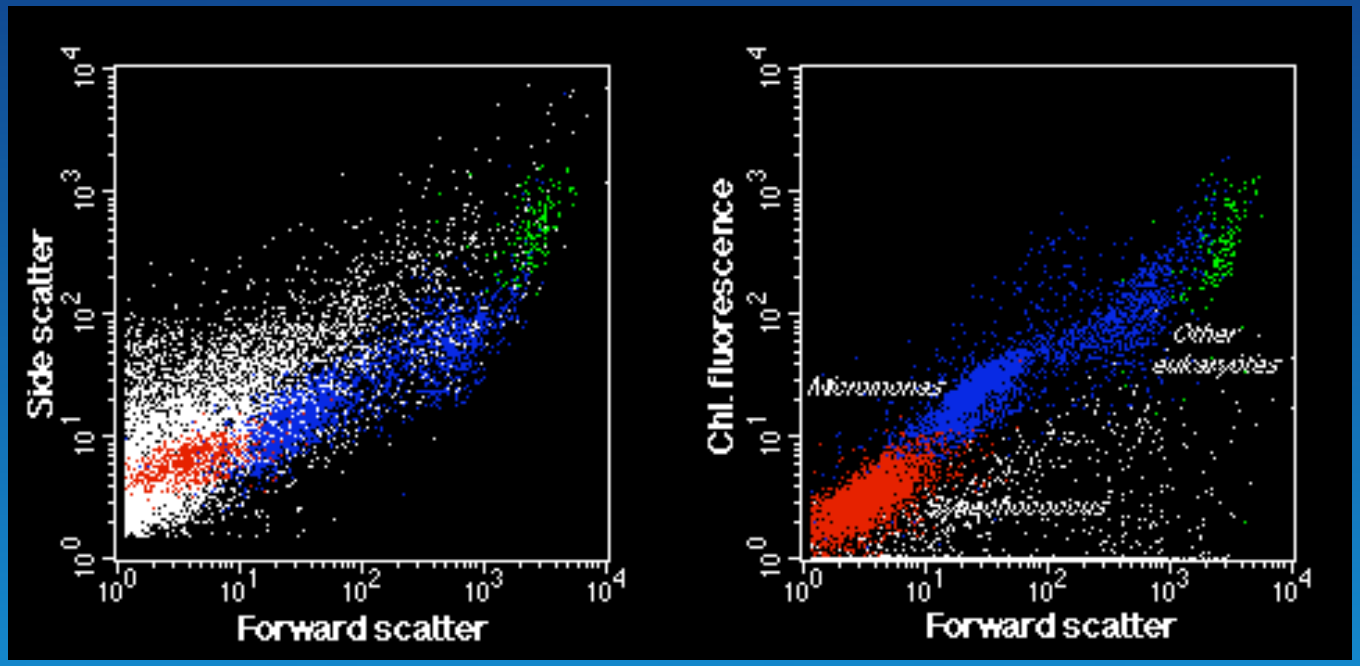
2-D dotplots

can also use contour plots, 3D plots, 1-D histograms

Natural Marine Samples

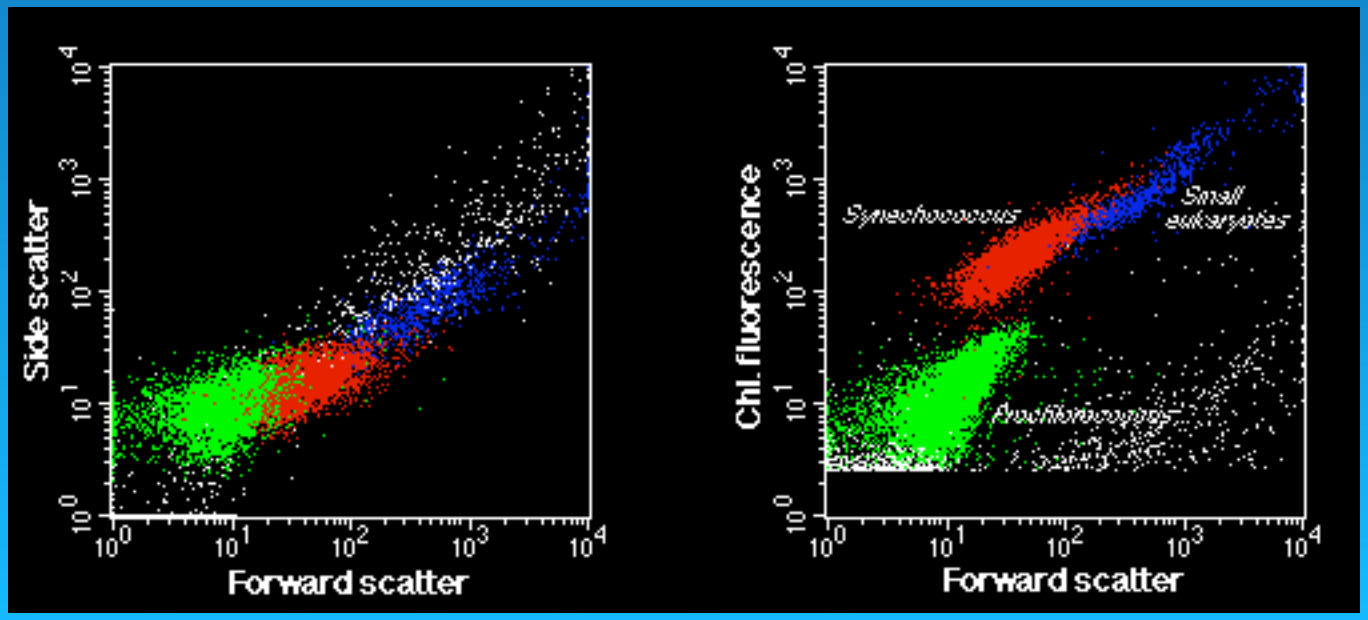
Continental Shelf

Georges Bank

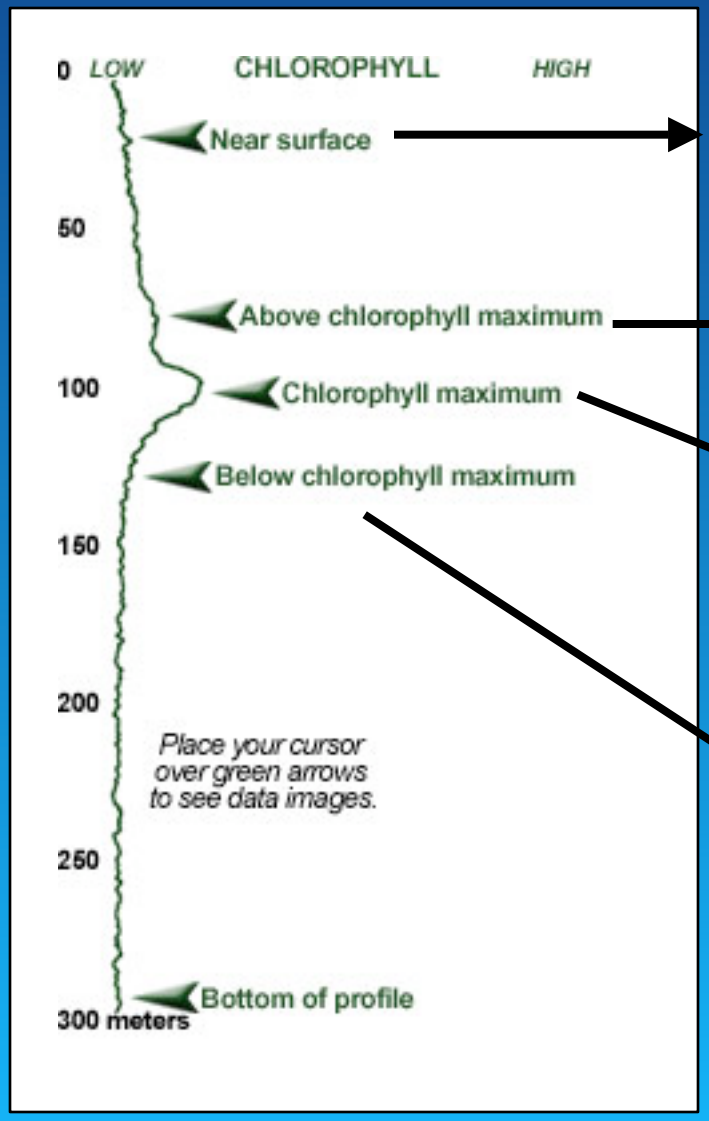


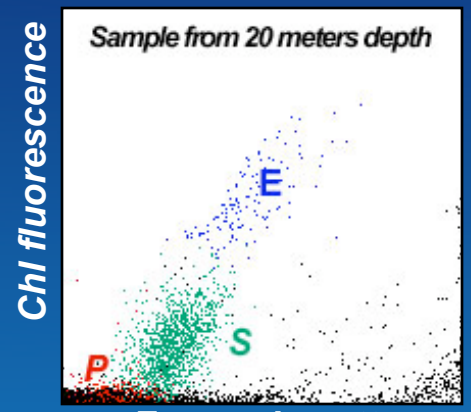
Open Ocean

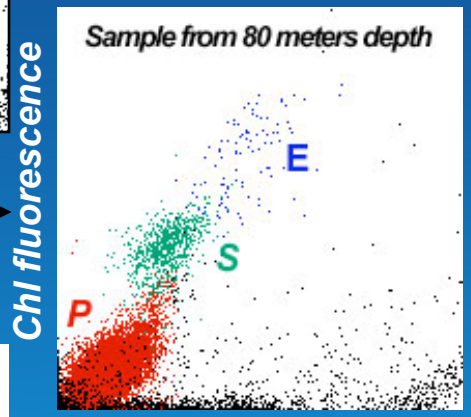
Sargasso Sea

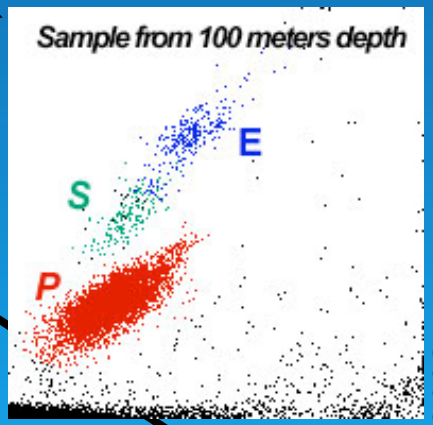


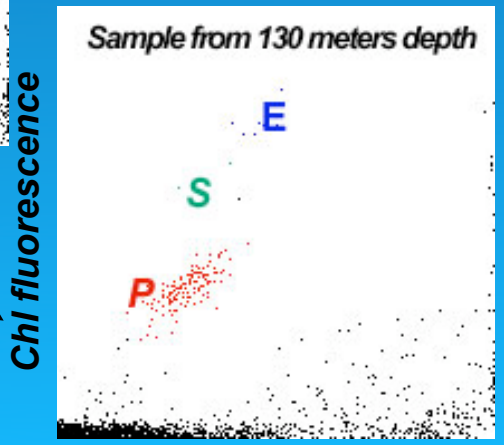
Flow cytometric signatures from the open ocean











Cytomation MoFlo high speed cell sorter

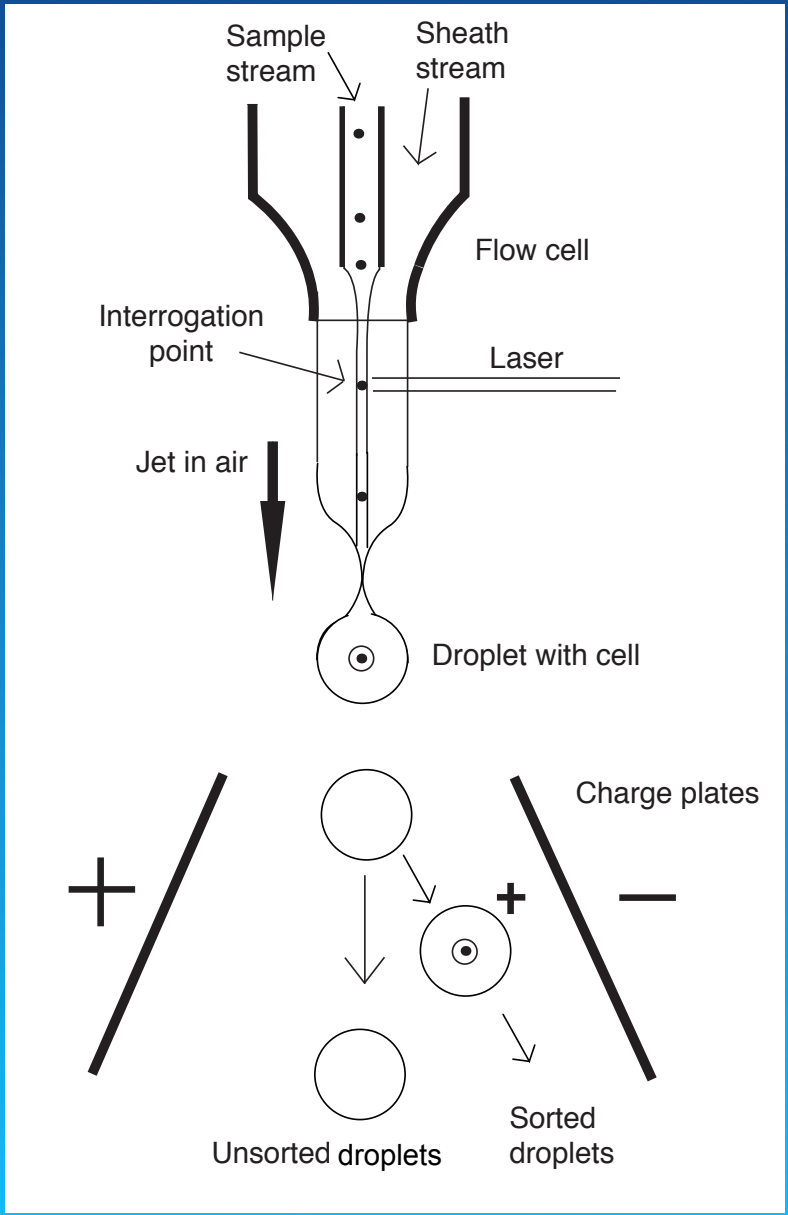
Basics of Flow Sorting

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.

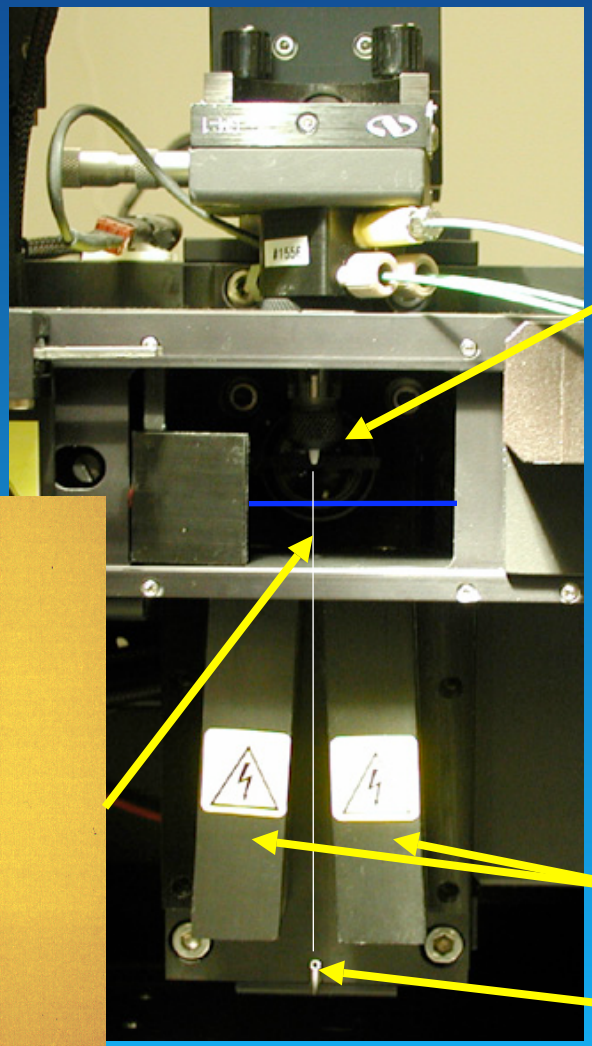
2. Timing
3. Purity and Efficiency

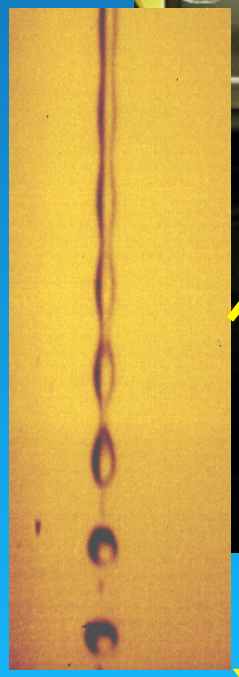
Sorting - Overview - Setup and Hardware



- Hydrodynamic focusing of sample
- Interrogation using a light source.
- Cells/particles of interest are identified
- Droplet formation and charge applied.
- Droplet deflection and collection.

Sort Setup Using MoFlo®





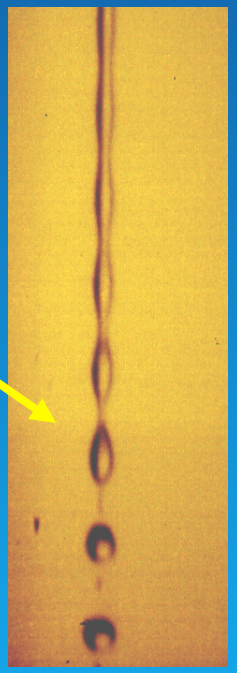
Magnified view of droplet formation

- 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®)

Mode	Characteristic	Application
Enrich	Tries to capture all positive events regardless of the presence of negative events.	High recovery/**yield**
Purify	Sorts positives only in the absence of a negative event.	High **purity**
Single	Same as Purify mode except will only accept one positive event per sort decision.	Single Cell Deposition
Mixed Mode	Sorts Purify Mode left and all the aborts to the right.	High Purity one direction while recovering all aborted positive events the other direction.

Volume of a sort drop

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.

Pressure differential	Ratio of V_s to V_d (R)	Sample stream diameter (µm)	Sample droplet volume, V_s (pL)	ESD of V_s (µm)
0.2	3.13×10^{-3}	5.6	8.7	25.9
0.4	4.98×10^{-3}	7.0	13.9	30.3
0.6	7.93×10^{-3}	8.9	22.1	35.3
0.8	9.71×10^{-3}	9.8	27.0	37.8

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

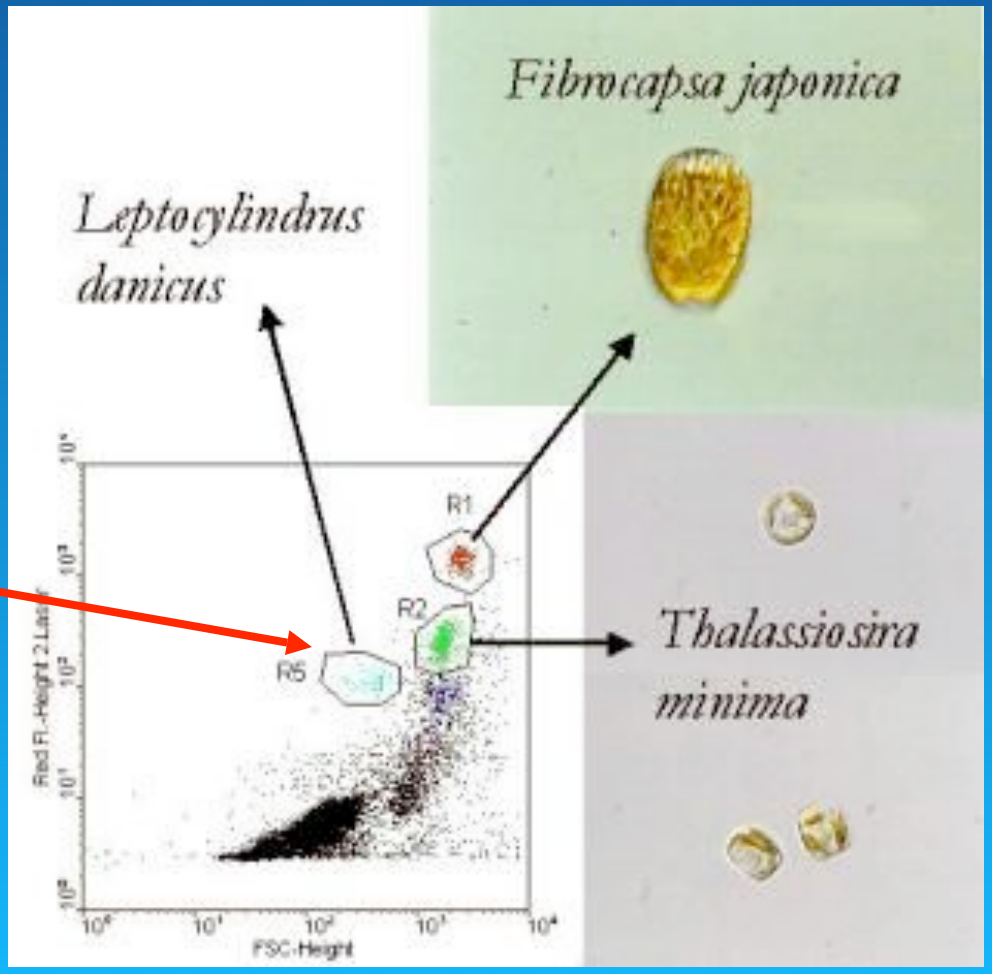
- cell isolation and cultivation
- culture purification
- single cell isolation for DNA amplification
- sorting of phytoplankton, heterotrophic protists, and bacteria
- sorting select populations using DNA or antibody stains

Sorting for Identification and/or Isolation

Establishing cultures or identifying groups by sorting on different populations

Post-sorting microscope identification

Sort regions



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*

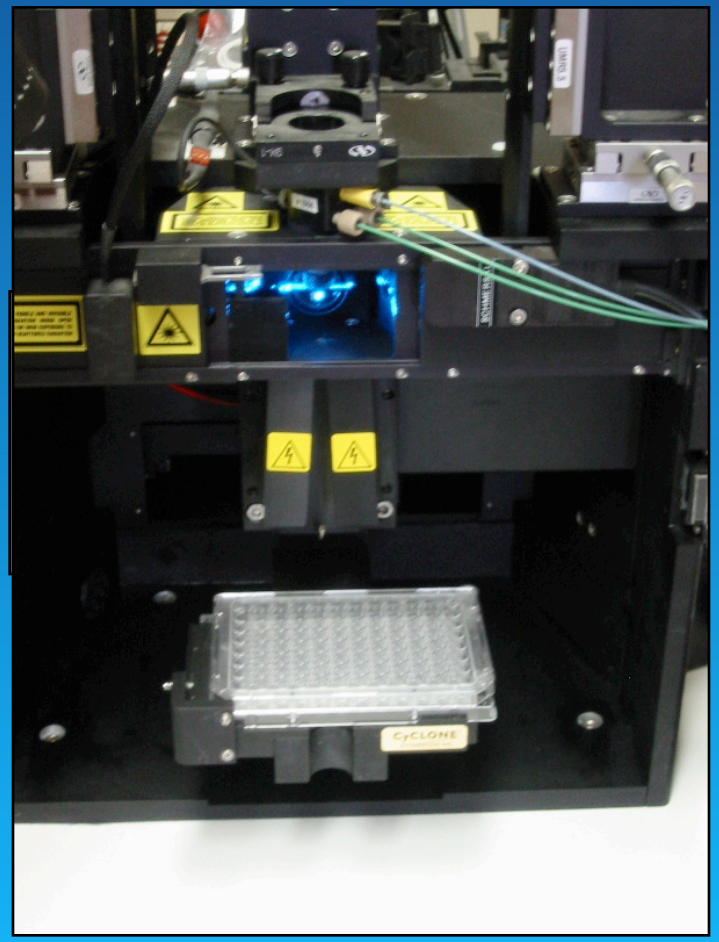
Sorting for Isolation or Cultivation

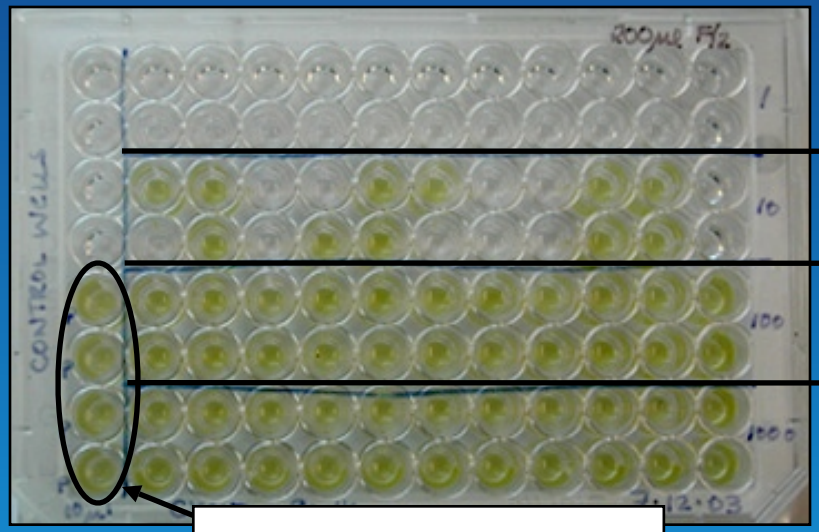
- Number of Cells to Sort
- Physical Setup
  - Nozzle Tip Size
  - Pressure and differential
  - Sheath (and fluidics preparation)
  - Laser Power
- Sort Trigger and Criteria (sort modes)
- Tubes vs. Plates
- Culture Media
- Culture or Field Sample Preparation
- Screening

Volume of a drop

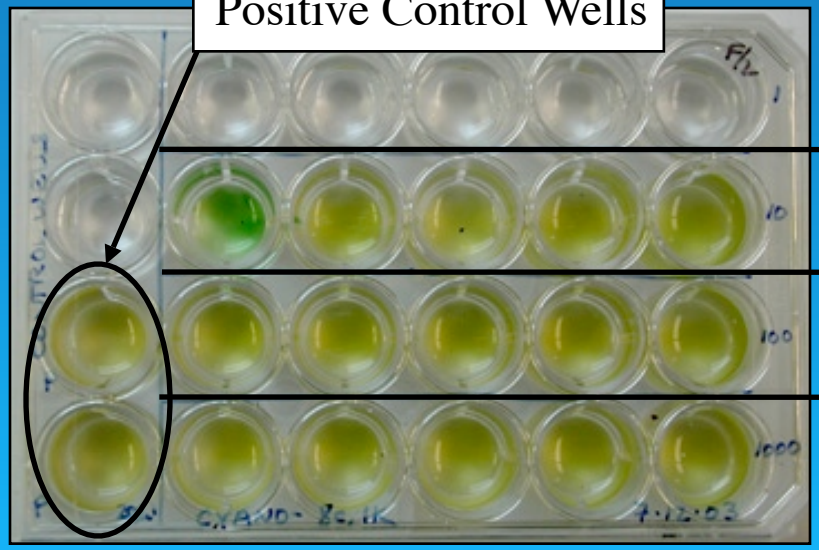
Isolation from Cultures

Cell Sorting



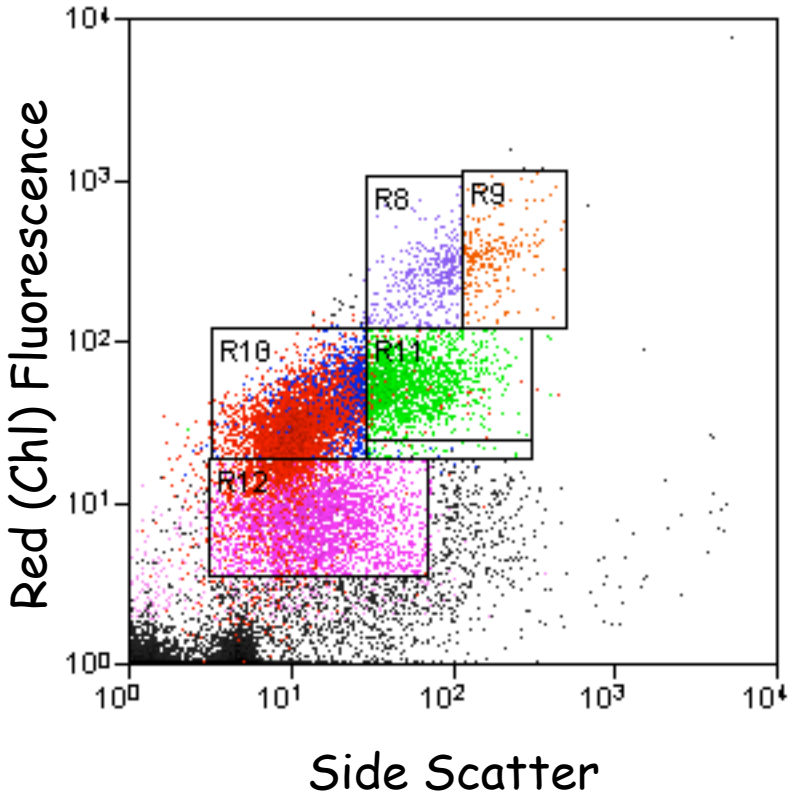


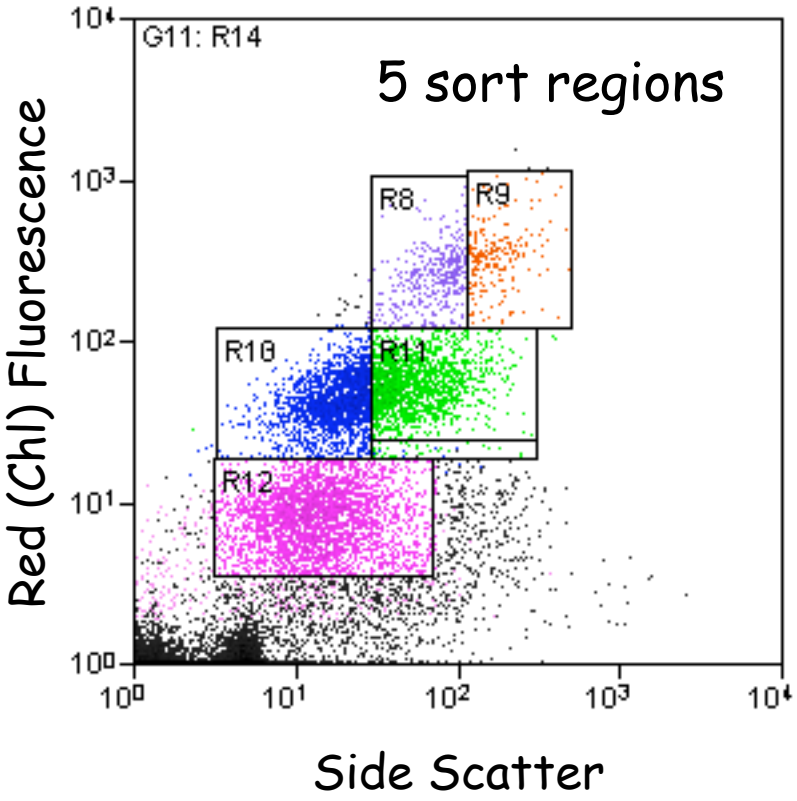
Positive Control Wells



96 vs. 24 well plates

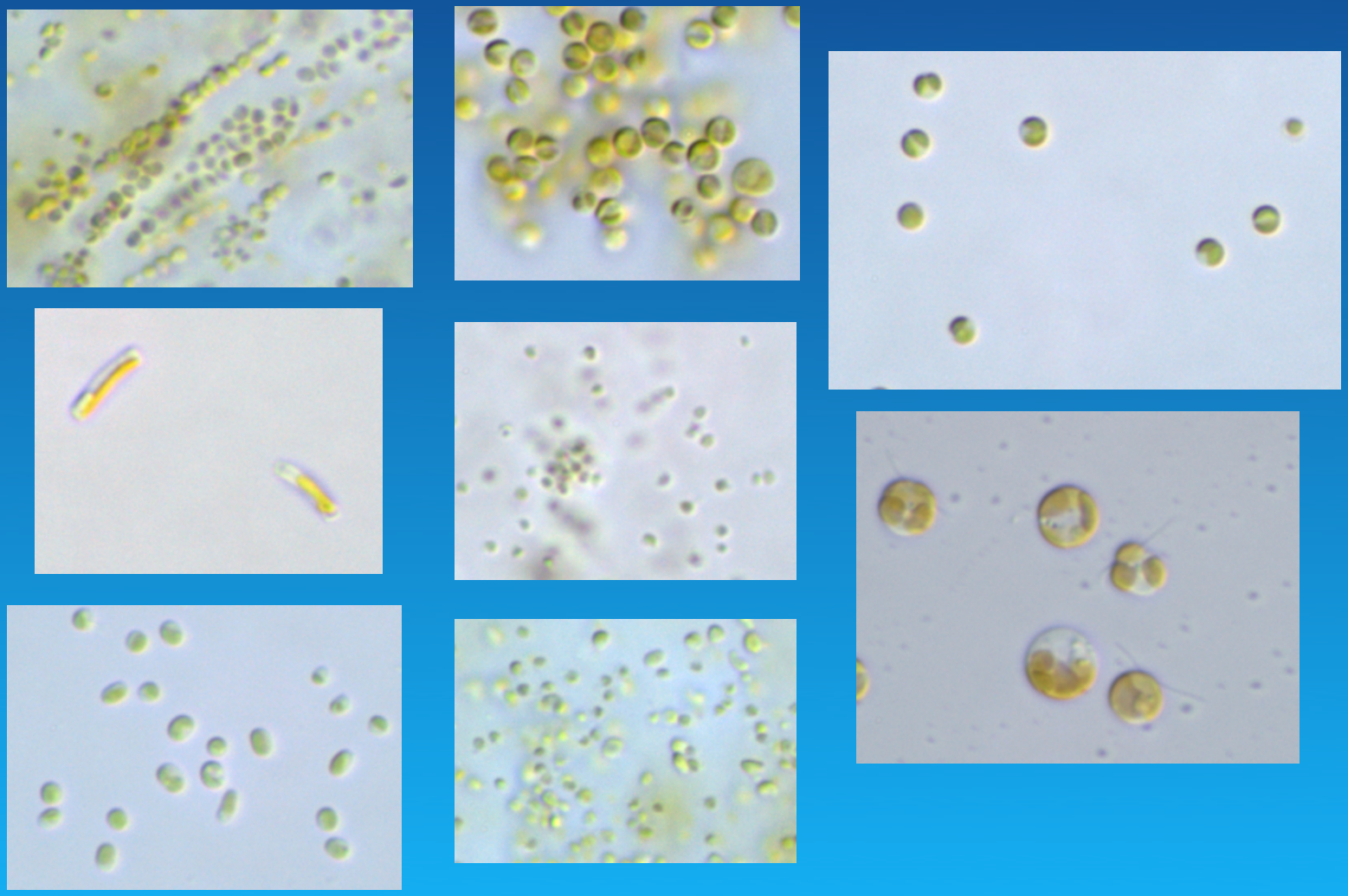
Sorting for PicoEukaryotes in Gulf of Maine





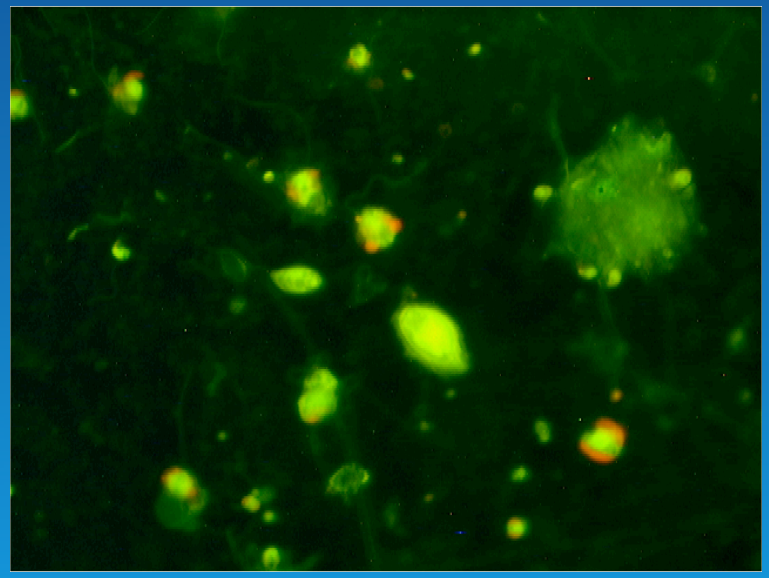
- 3um filter screen and incubation
- Excluded cyanobacteria from sort criteria
  - Sort single one drop - 1 and 10 cell sorts

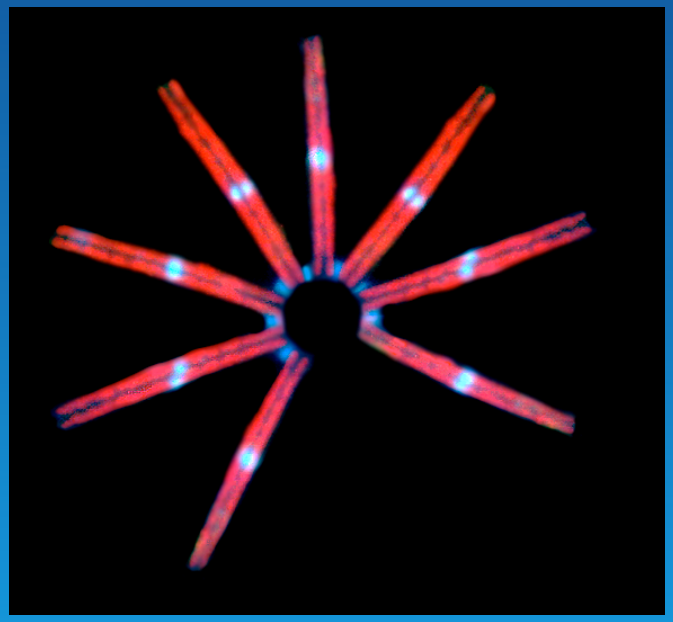
Sampling Pico-Algal Diversity from Gulf of Maine

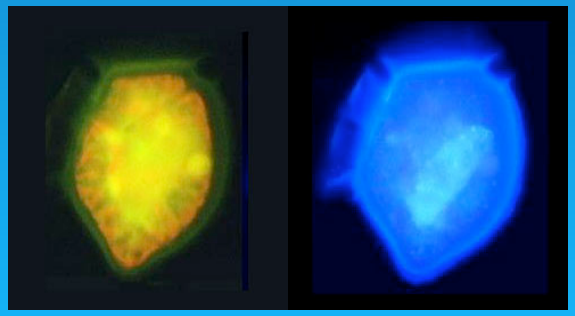


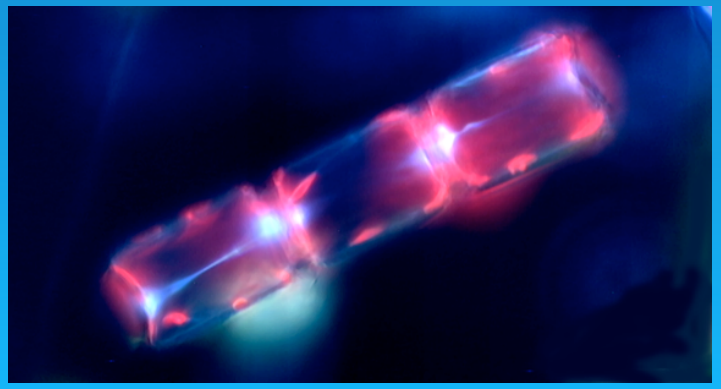
N.J. Poulton, R.A. Andersen, M.E. Sieracki

Fluorescence of Marine Protists

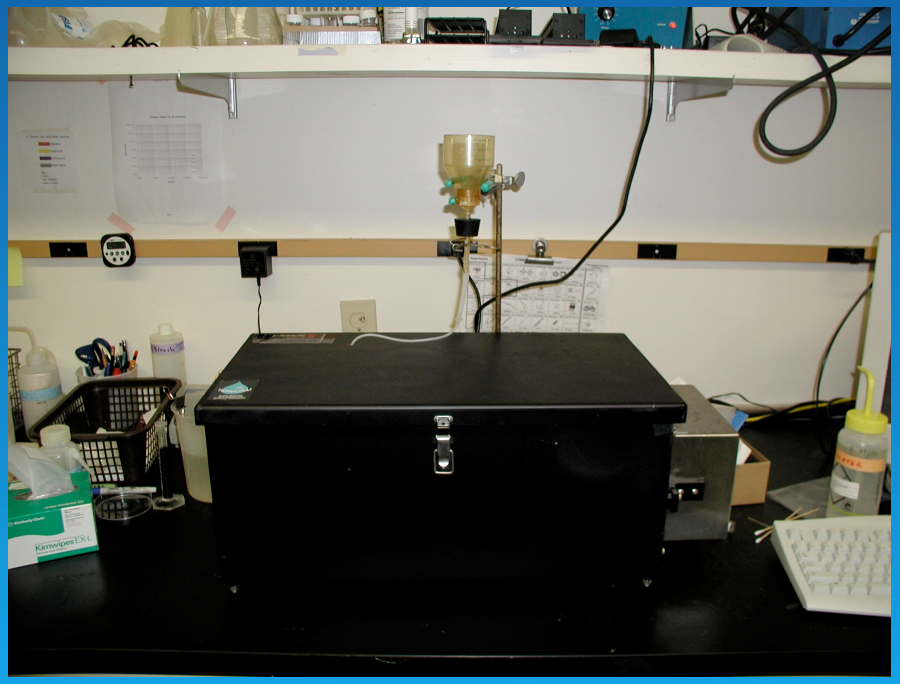








FlowCAM - Imaging in Flow

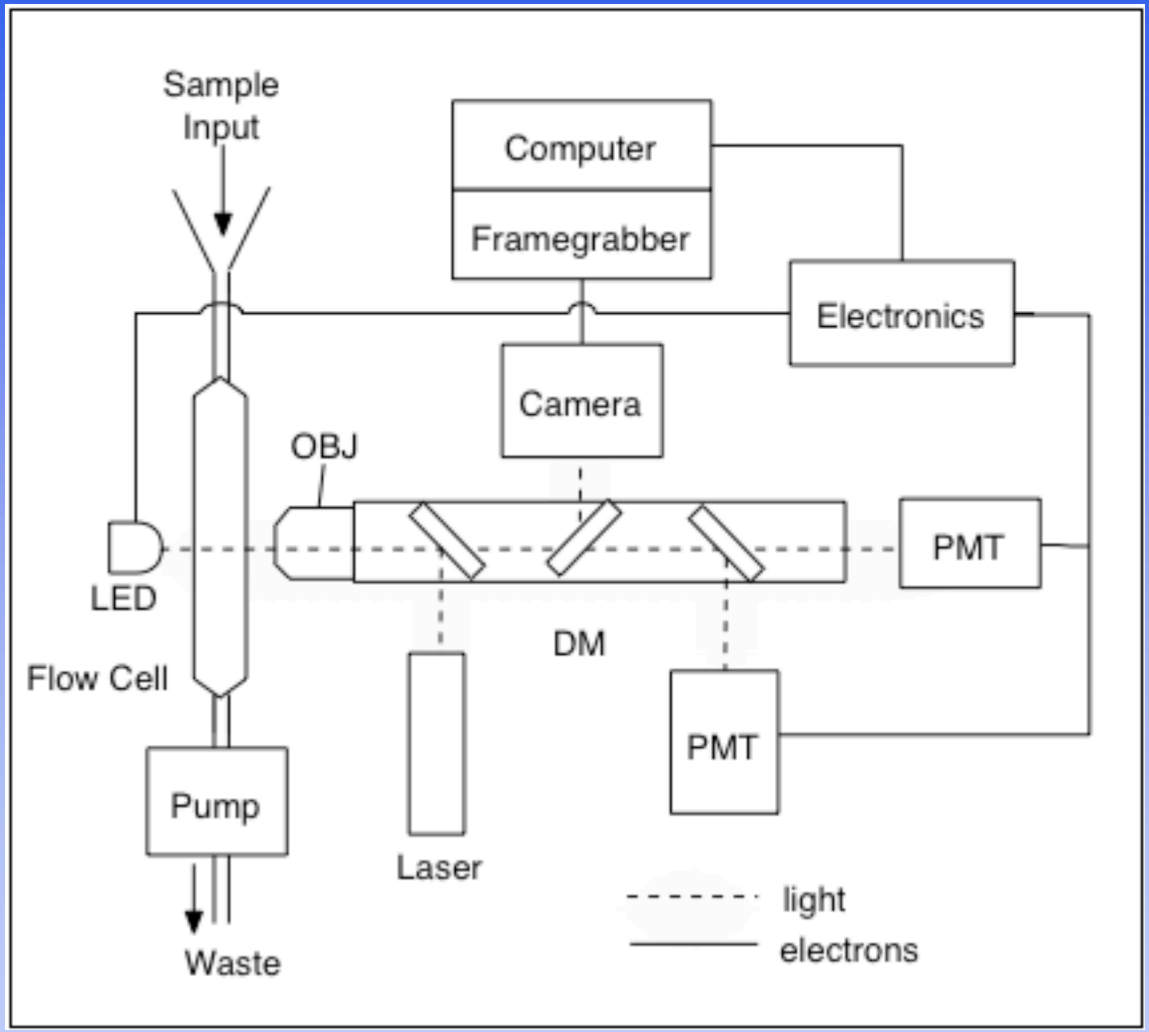


Benchtop

Portable

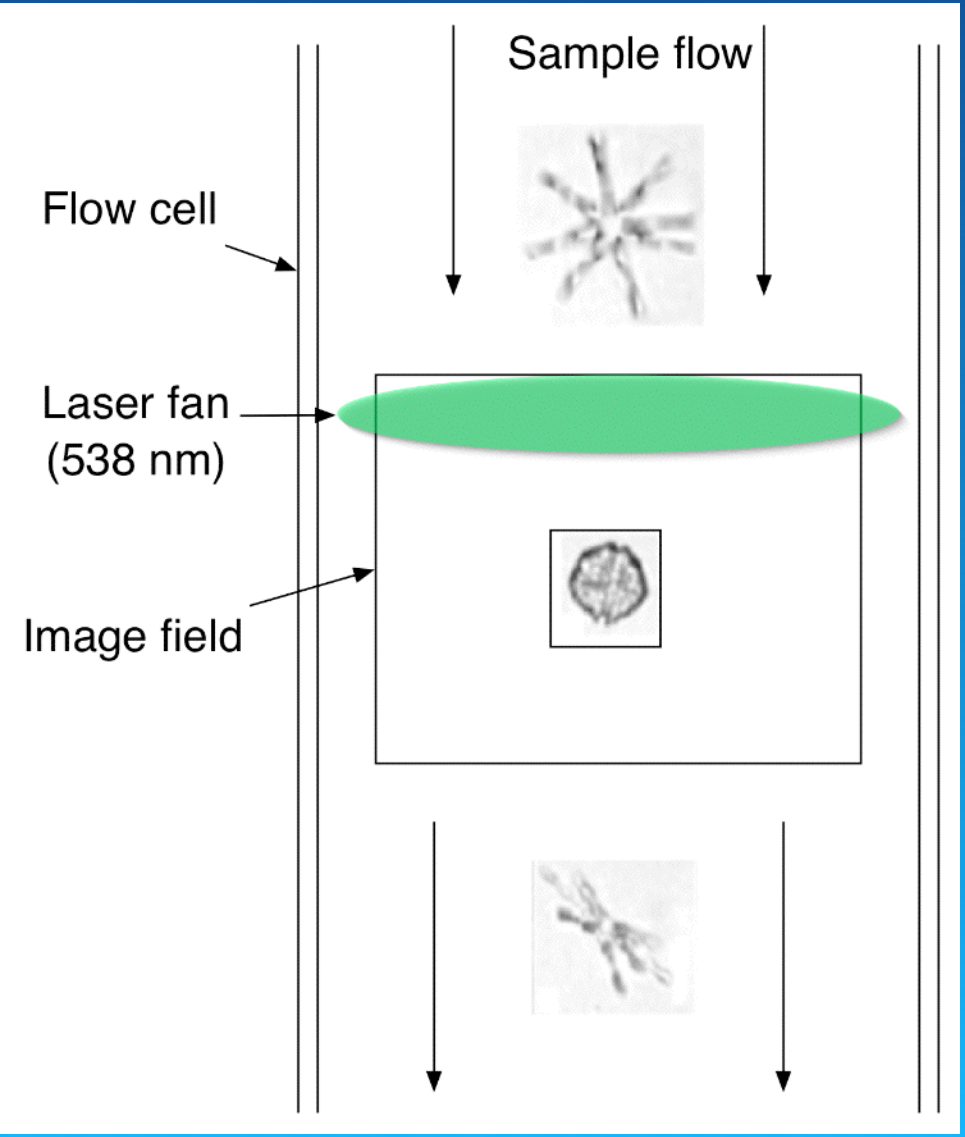
FlowCAM

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

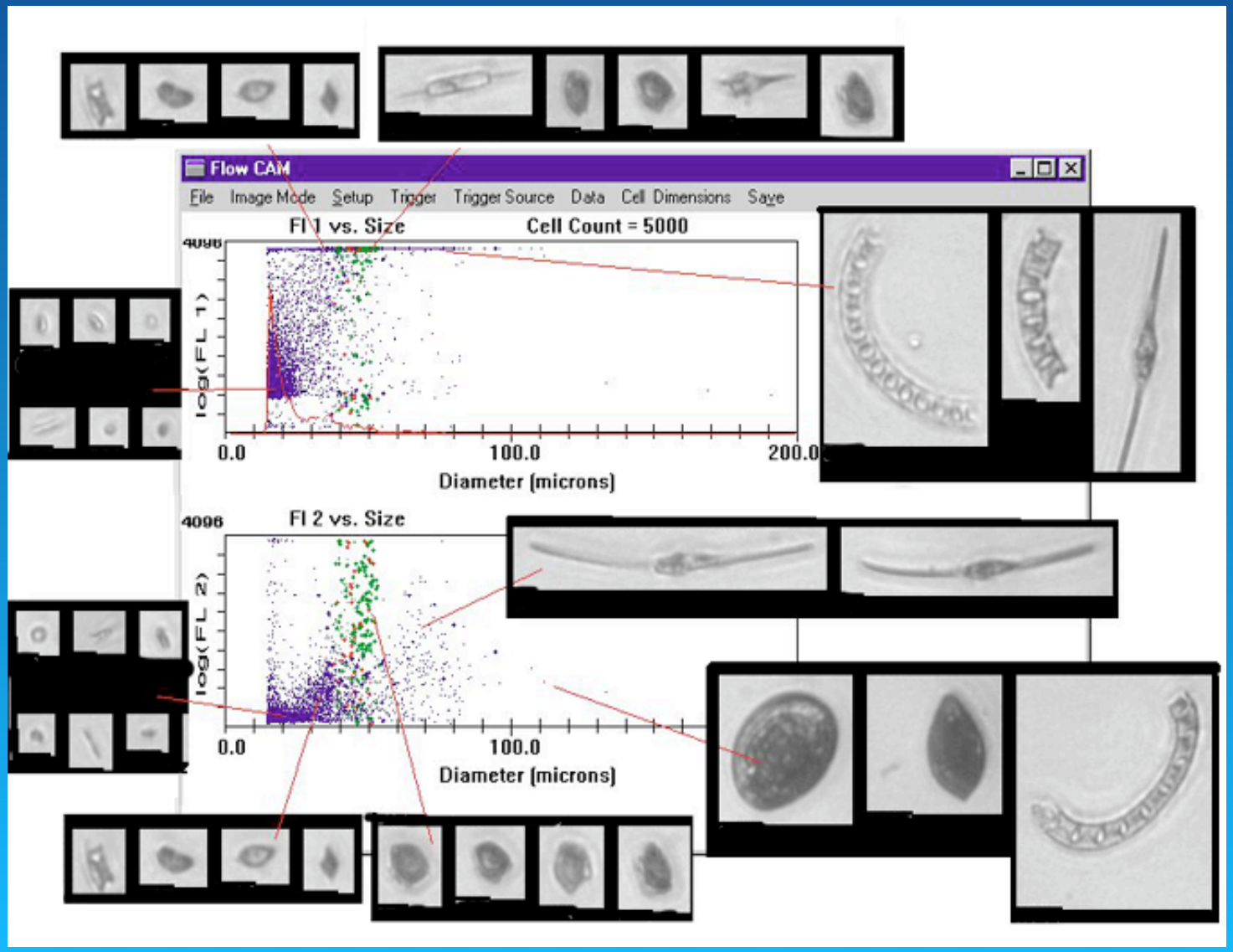


FlowCAM Imaging

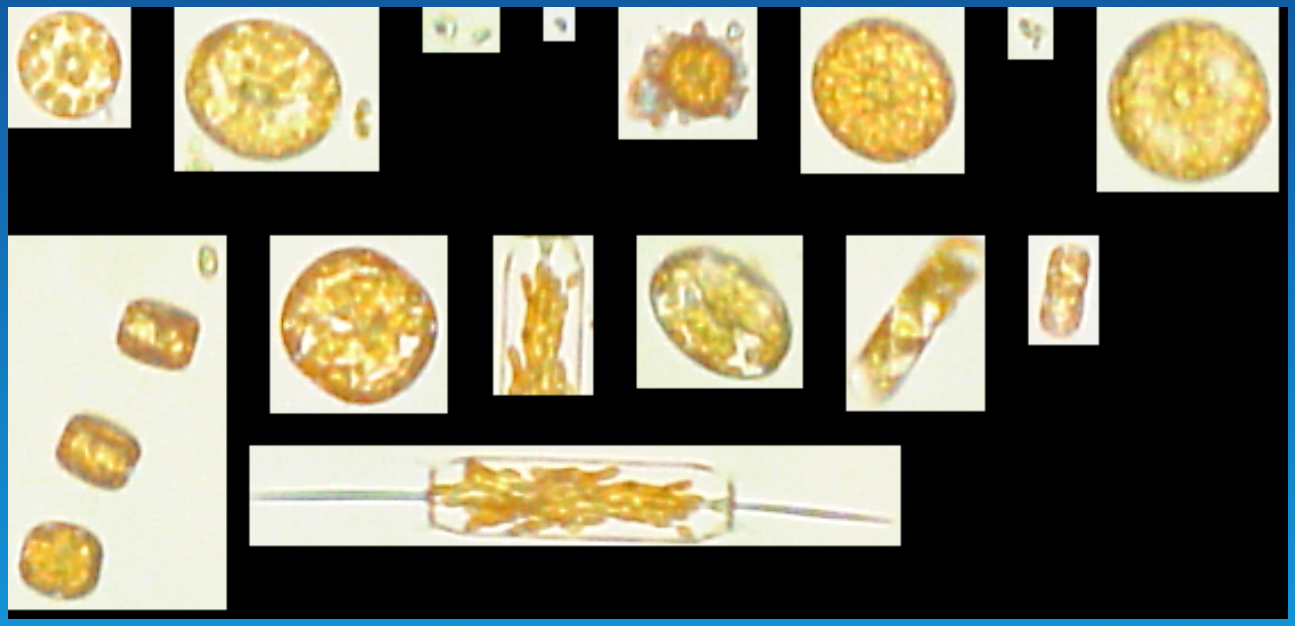
- No sheath fluid
- Fluorescence-triggered
- Cell subimages extracted in real-time
- Digital images stored



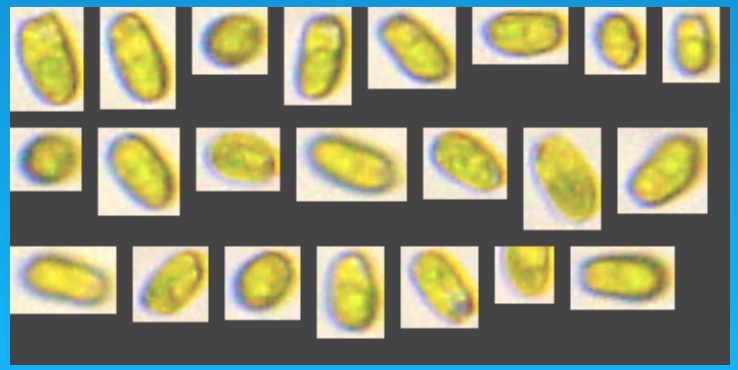
FlowCAM: Interactive Scattergram



Color FlowCAM Images

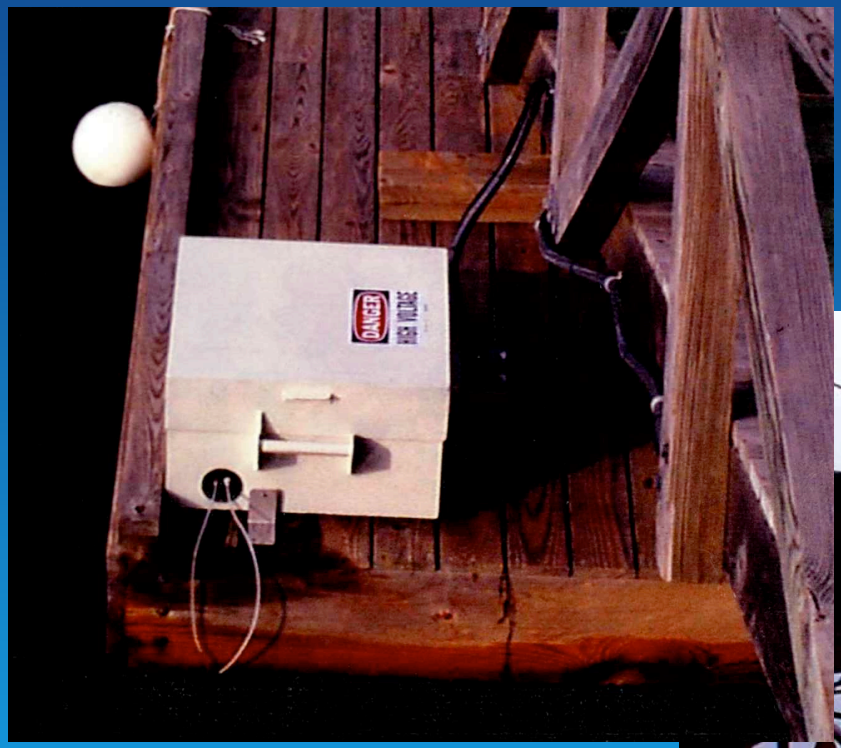


Diatoms

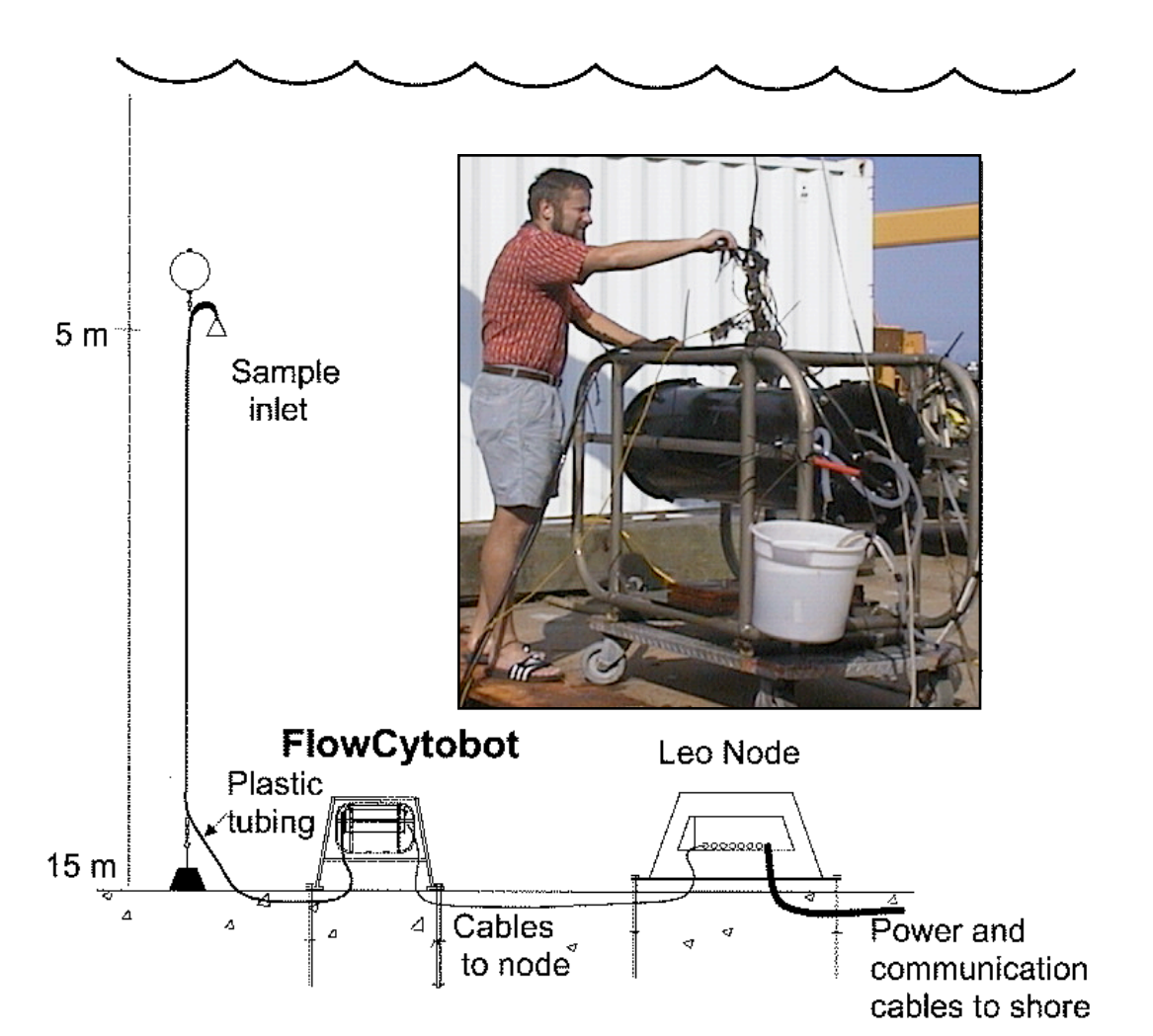


Tetraselmis

FlowCAM deployed on floating docks

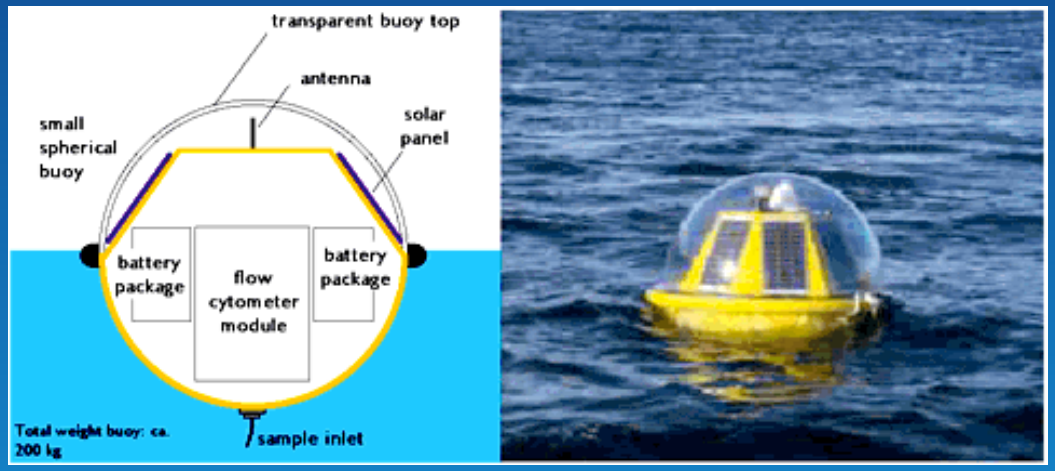


In-situ Flow Cytobot Deployment

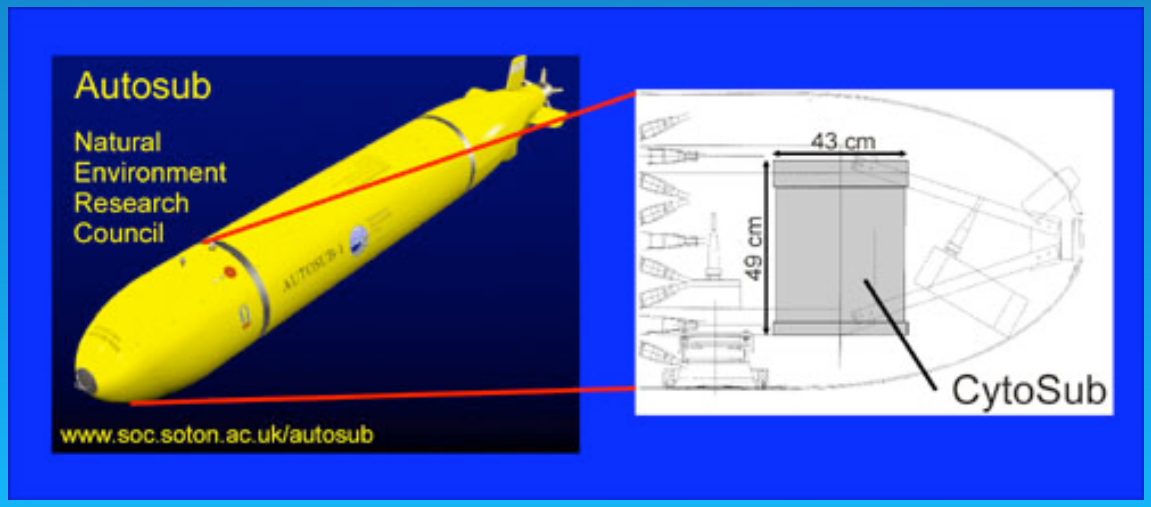


OLSON, R. J.,et al.. 2003.

CytoSense, CytoBuoy, CytoSub

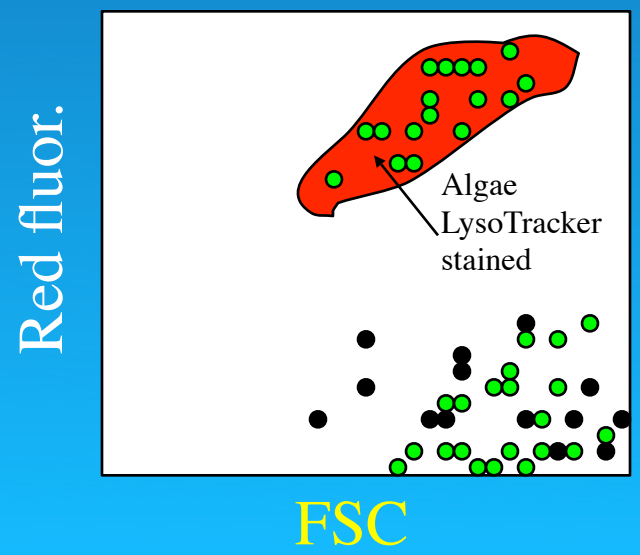


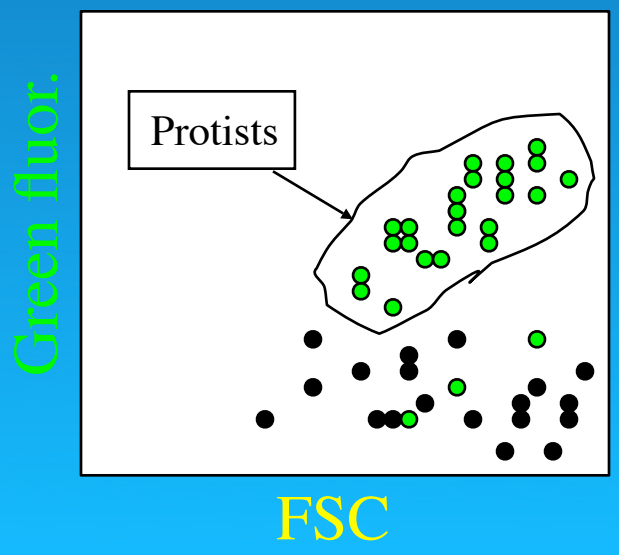
- Pulse processing
- Wide dynamic range
- Designed for biological oceanography



Protozoan Isolation using LysoTracker Detection of Flagellates and Ciliates

- LysoTracker is a fluorescent acidotropic probe that labels acidic organelles in live cells (eg. 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).



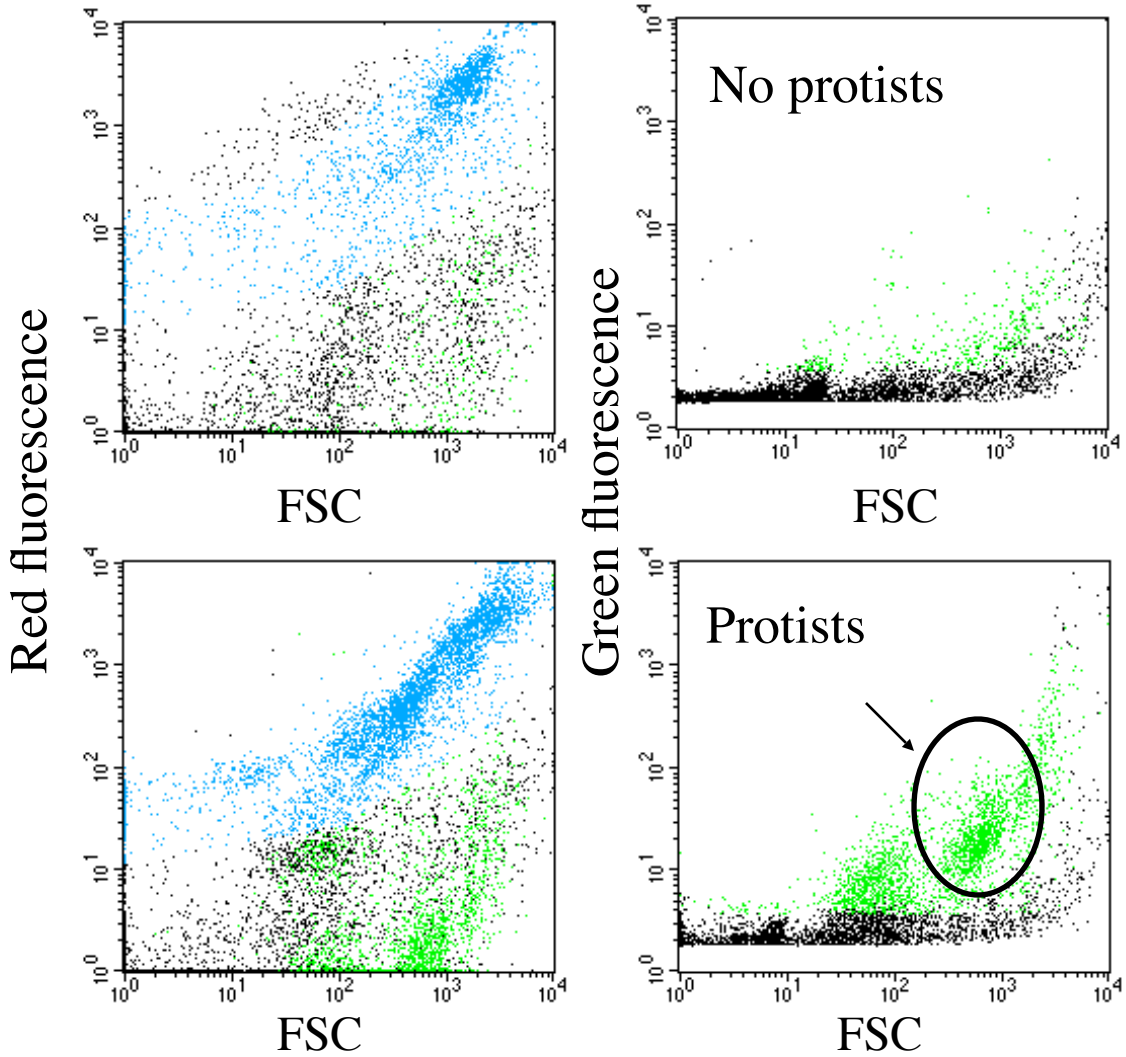


Green flu. trigger

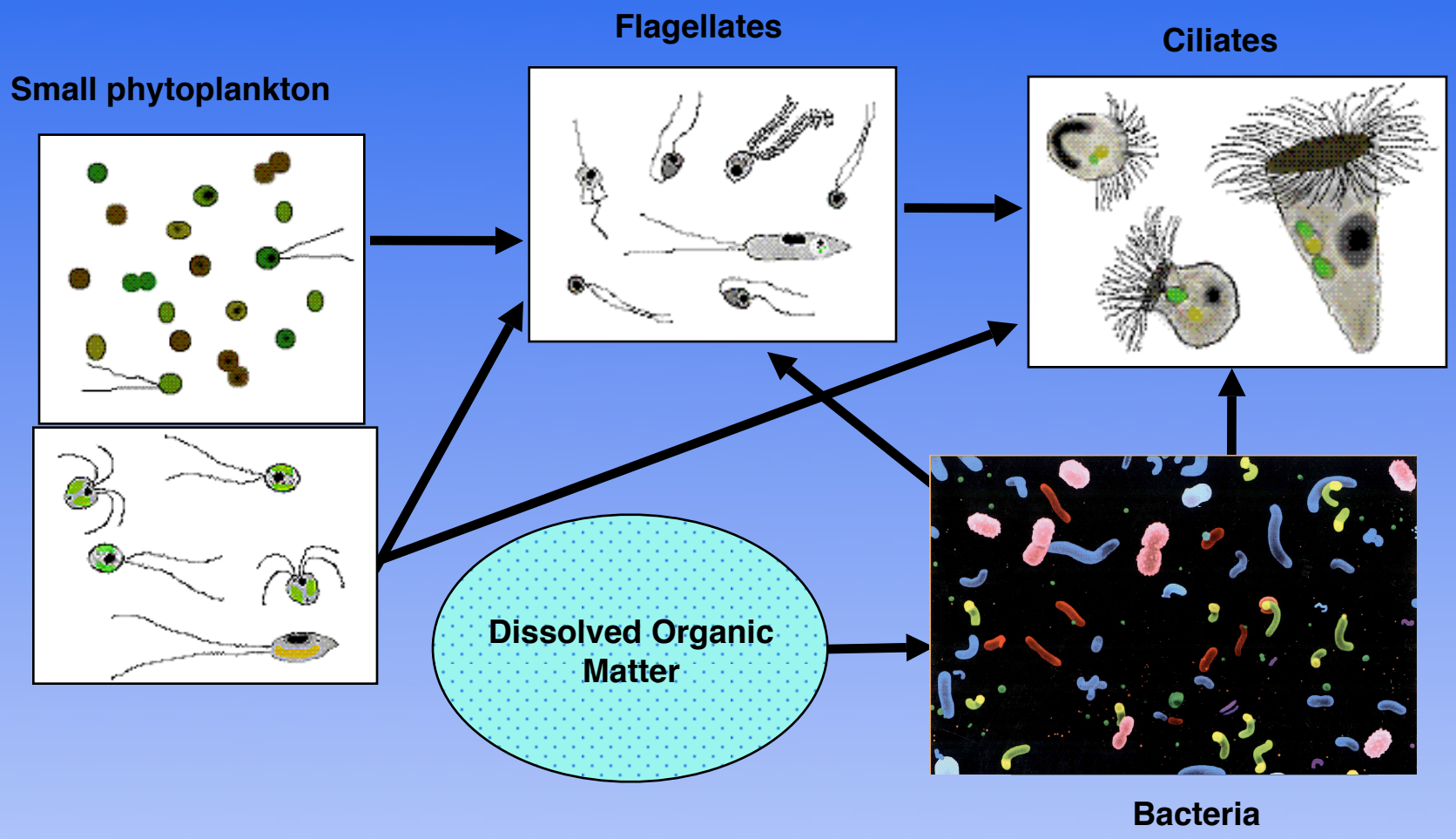
LysoTracker Green - Identification of Heterotrophic Protists

Negative control
(preserved)

Positive Control
(live)



Microbial Food Web



All forms produce dissolved organic matter