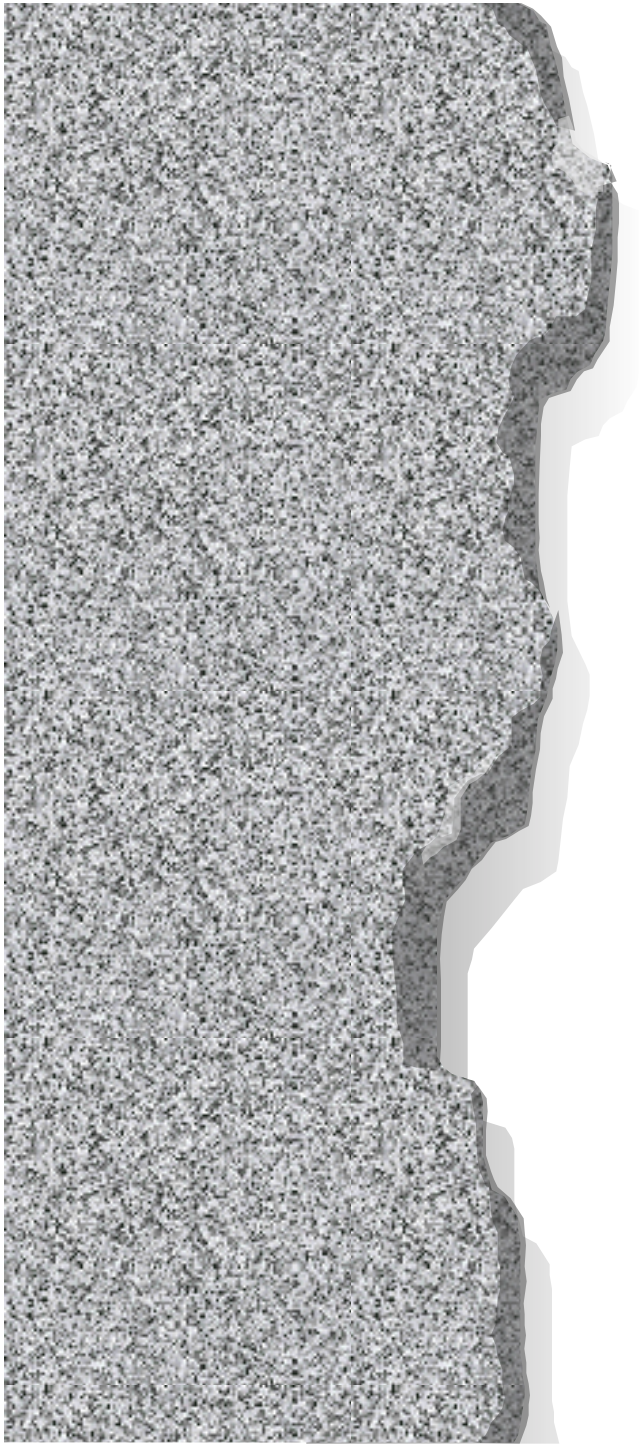


Counting Cells and Microscopic Particles: Introduction to Flow Cytometry, EpiFluorescence Microscopy, and Coulter Counters

Karen Selph
SOEST Flow Cytometry Facility
Department of Oceanography
University of Hawaii
selph@hawaii.edu
www.soest.hawaii.edu/sfcf

The Microbial World

- Particles to chemists, small critters to biologists…
- Size range (excluding viruses):
  ~0.4 μm to a few 100 μm's in diameter
- Includes all bacteria, phytoplankton, & most primary planktonic consumers, as well as a range of abiotic particles (clay to fine sand)
- Ubiquitous, highly diverse functionally & taxonomically, variable activities
- Responsible for most of the transformations of organic matter in the ocean, as well as much of the gas transfers (O_2, CO_2, etc.)

How do we study them?

- Microscopic, so need methods that will resolve small particles.
- Today, I'll introduce you to 3 instruments that can resolve such small particles and give us useful information about them:
  - Epifluorescence Microscope
  - Flow Cytometer
  - Coulter Counter

Why use an epifluorescence microscope?

- *Quantitative* detection and enumeration of microbes, including viruses
- *Ability to concentrate particles* to see rarer populations
- *Estimate microbial biomass*, using biovolume estimates.
- *Separate classes*, e.g., autotroph from heterotroph, prokaryote from eukaryote, even by species for some organisms

Why use a flow cytometer?

- ✓ *Rapid* counting of microbes (minutes)
- ✓ *Enumerate several populations* in one sample, if their scatter or fluorescence signatures are distinct
- ✓ *Enumerate dimmer cells* or cells that bleach out (fade) quickly
- ✓ *Sort live cells* from bulk solution

Why use a Coulter Counter?

- *To count particles* (~1 to ~200 μm)
- To estimate the size of particles
- *Main limitation*: will detect all particles in solution, and can not discriminate between them except by size

Fluorescence

- Both the flow cytometer and the epi-fluorescence microscope rely on the property of fluorescence
- The Coulter Counter does not (measures changes in resistance)

Fluorescence: What is it?

One of several pathways of the de-excitation process by which an excited state electron gives up energy and returns to the ground state.

- photon absorbed by electron: ~10^{-15} s
- energy released by fluorescence: ~10^{-8} s

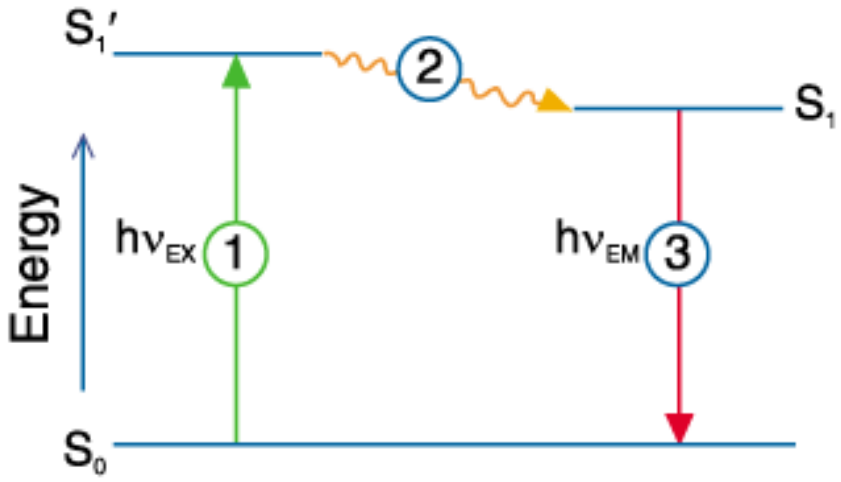


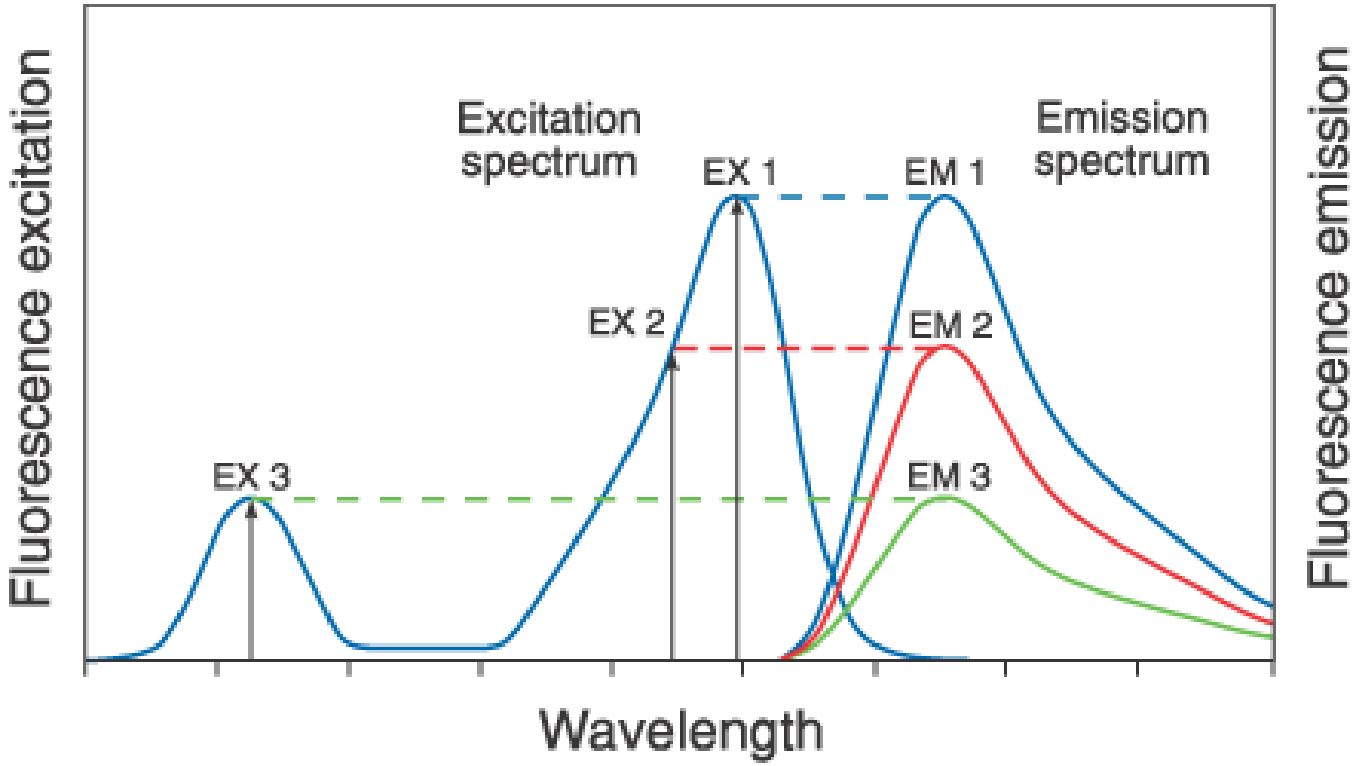
figure courtesy of Molecular Probes, www.probes.com

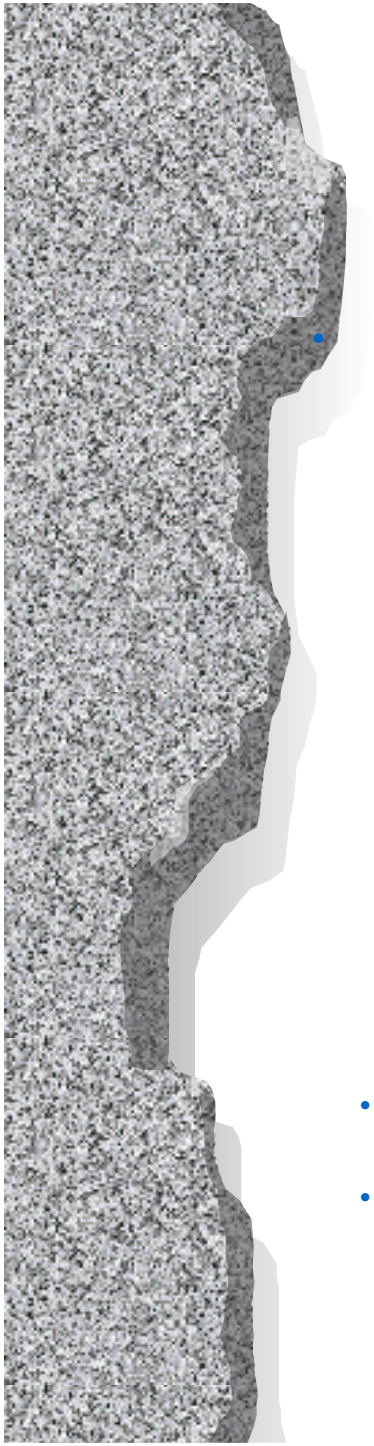
Stokes Shift

- The energy loss between excitation and emission, resulting in longer emission wavelengths relative to absorbance/ excitation wavelengths.
- Fundamental property, allowing detection of emitted photons against a low background, isolated from excitation photons.

Emission Profile

Excitation of a fluorophore at three different wavelengths (EX 1, EX 2, EX 3) does not change the emission profile but does produce variations in fluorescence emission intensity (EM 1, EM 2, EM 3) that correspond to the amplitude of the excitation spectrum.





Autofluorescence

Natural fluorescence of molecules in cells.

- NADH, riboflavin and flavin coenzymes excited at 350 - 500 nm
- proteins excited at 250 - 280 nm (because of tryptophan, tyrosine, and phenylalanine)
- phytoplankton pigments, especially chlorophyll and phycoerythrin

Fluorescent Dyes and Stains

- bind to specific molecules or organelles
- allow detection of cellular components or whole cells

Fluorescence measurements: Microscopy

- Sample is illuminated, and light that is transmitted through or scattered, reflected, or emitted from specimen (depending on type of microscope) is sent to the eye.
- The eye obtains a real image of the specimen, and the sample can be moved manually.

- Response time for human observer:

  milli-seconds

- Main disadvantage: slow sample processing time

Hg arc lamp: common light source for epifluorescence microscopy

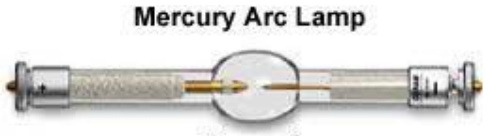


Figure 1

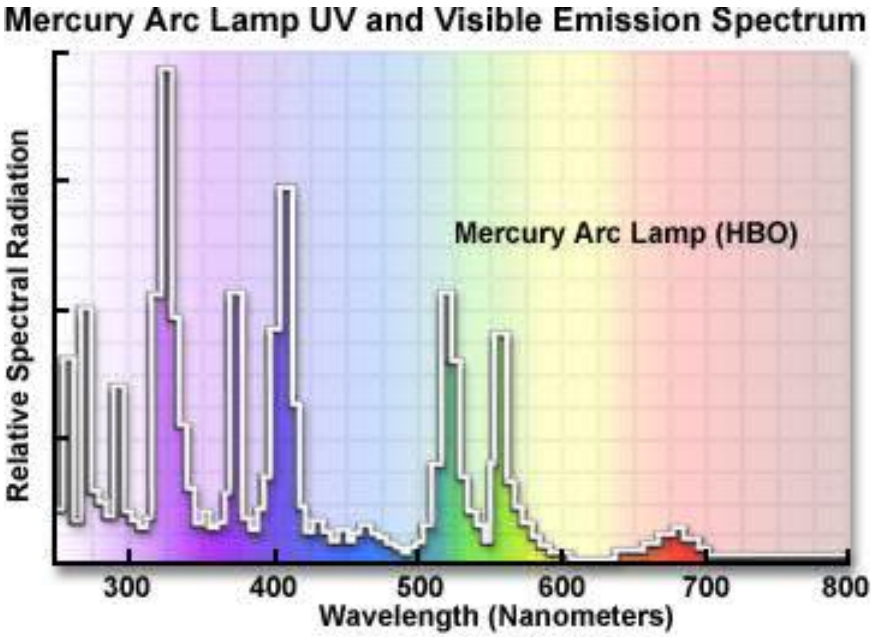


Figure 2

http://www.olympusmicro.com

Filters

- Excitation filters: allows only certain wavelengths to pass
- Dichroic filters: short-pass or long-pass, e.g., 488 DCLP will block and deflect wavelengths shorter than 488 nm and pass wavelengths longer than 488 nm.
- Emission filters: allow only certain wavelengths to get to detector

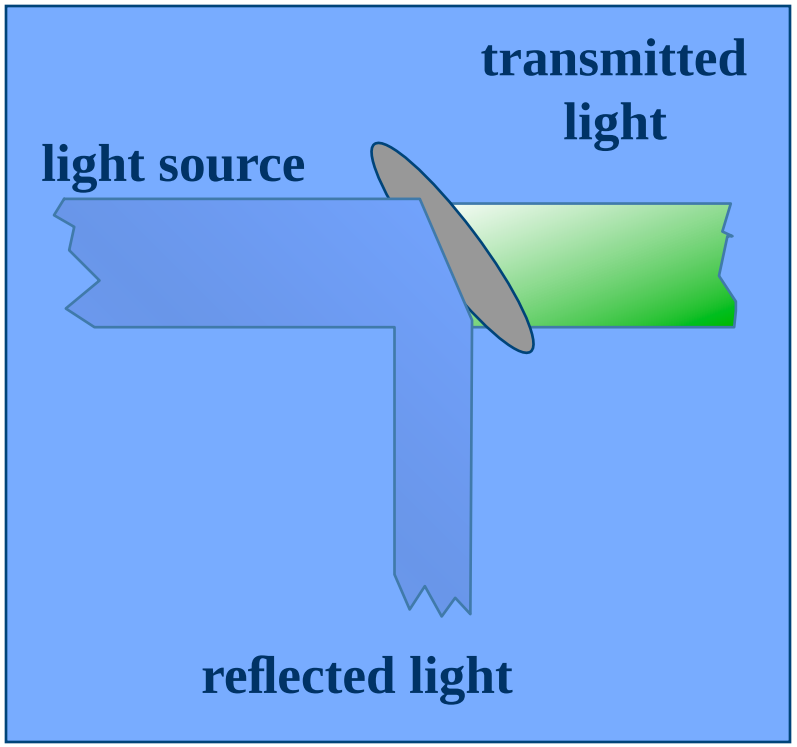
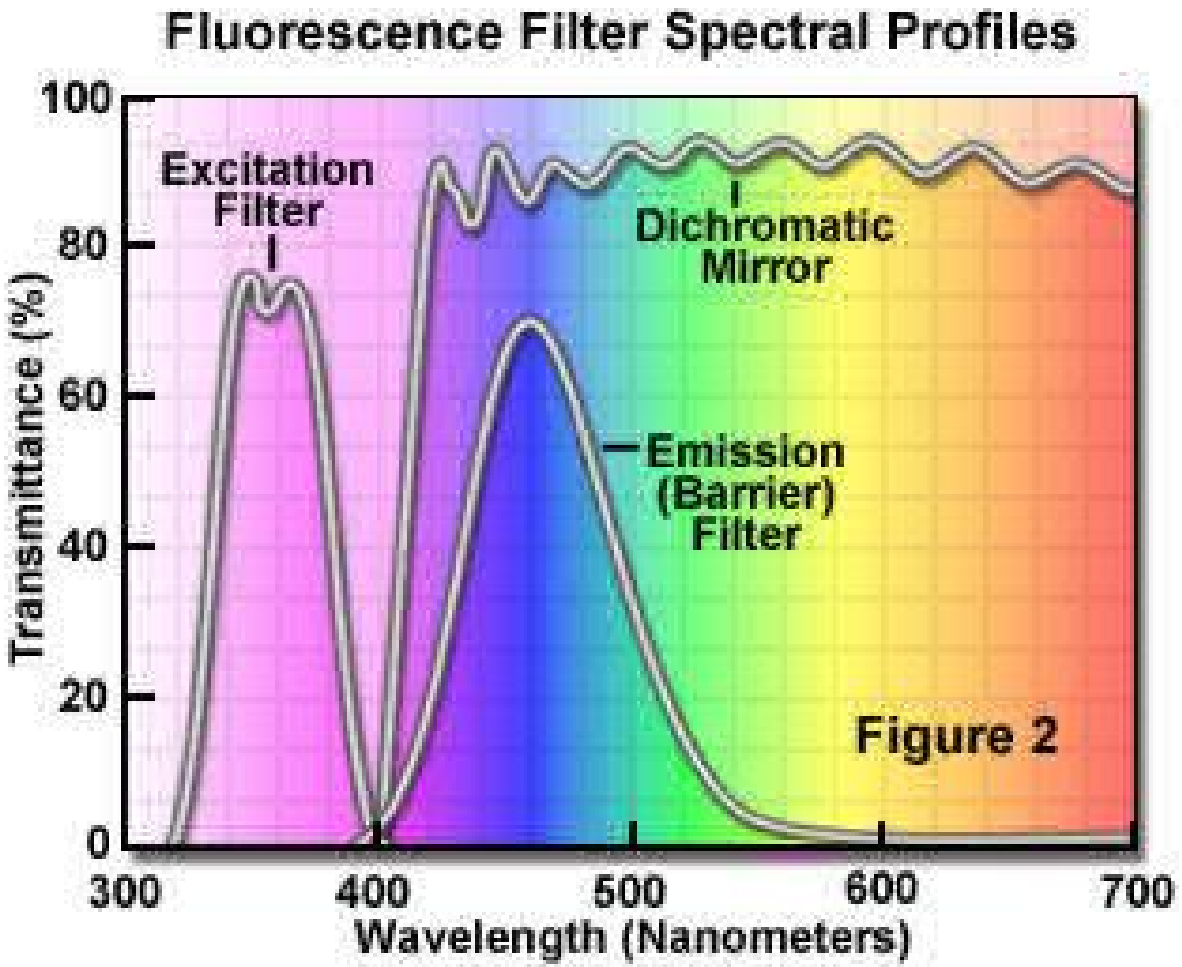


figure modified from
J. Paul Robinson, Purdue

Omega Optical (www.omegafilters.com)

Example of Ex/DC/Em Filters



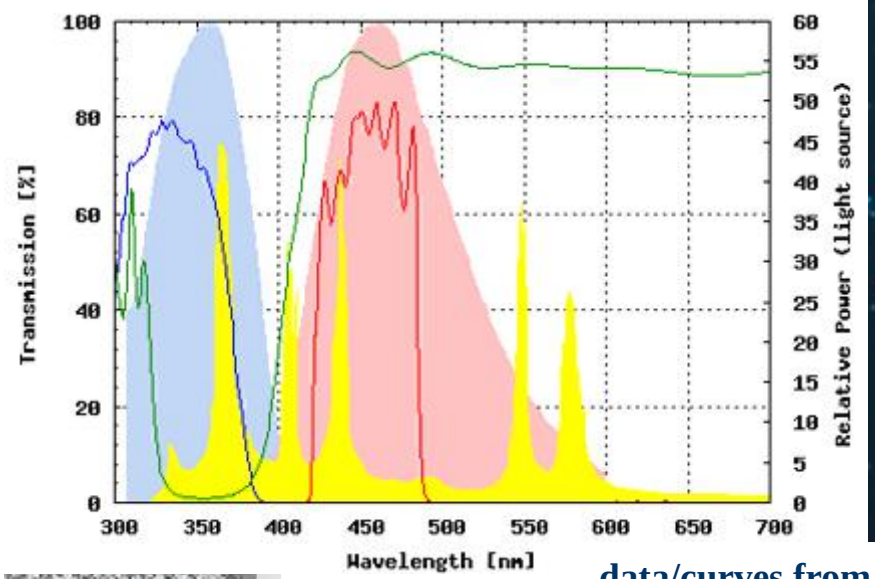
Typical microscope image of seawater

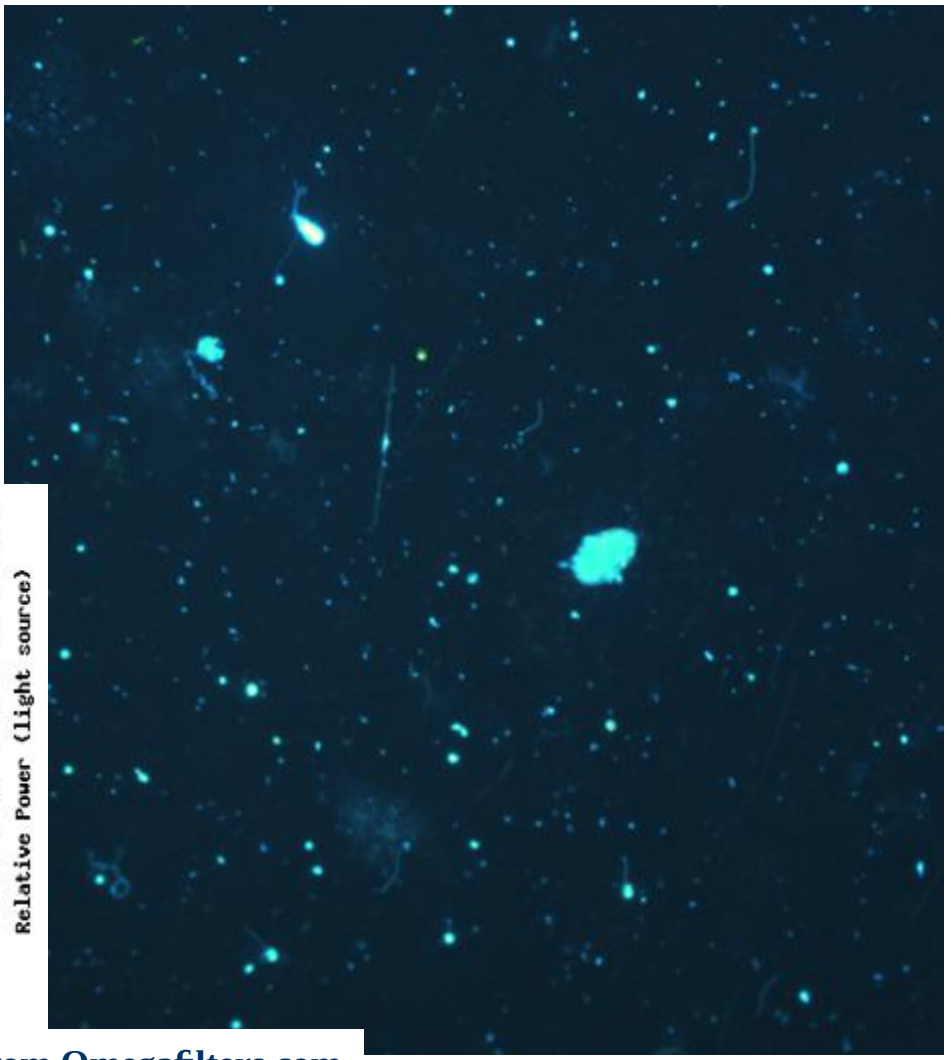
(sample (50 ml) stained with DAPI and filtered onto 1 µm filter and excited with UV light)

Exciter: 330WB80

Dichroic: 400DCLP

Emitter: 450DF65

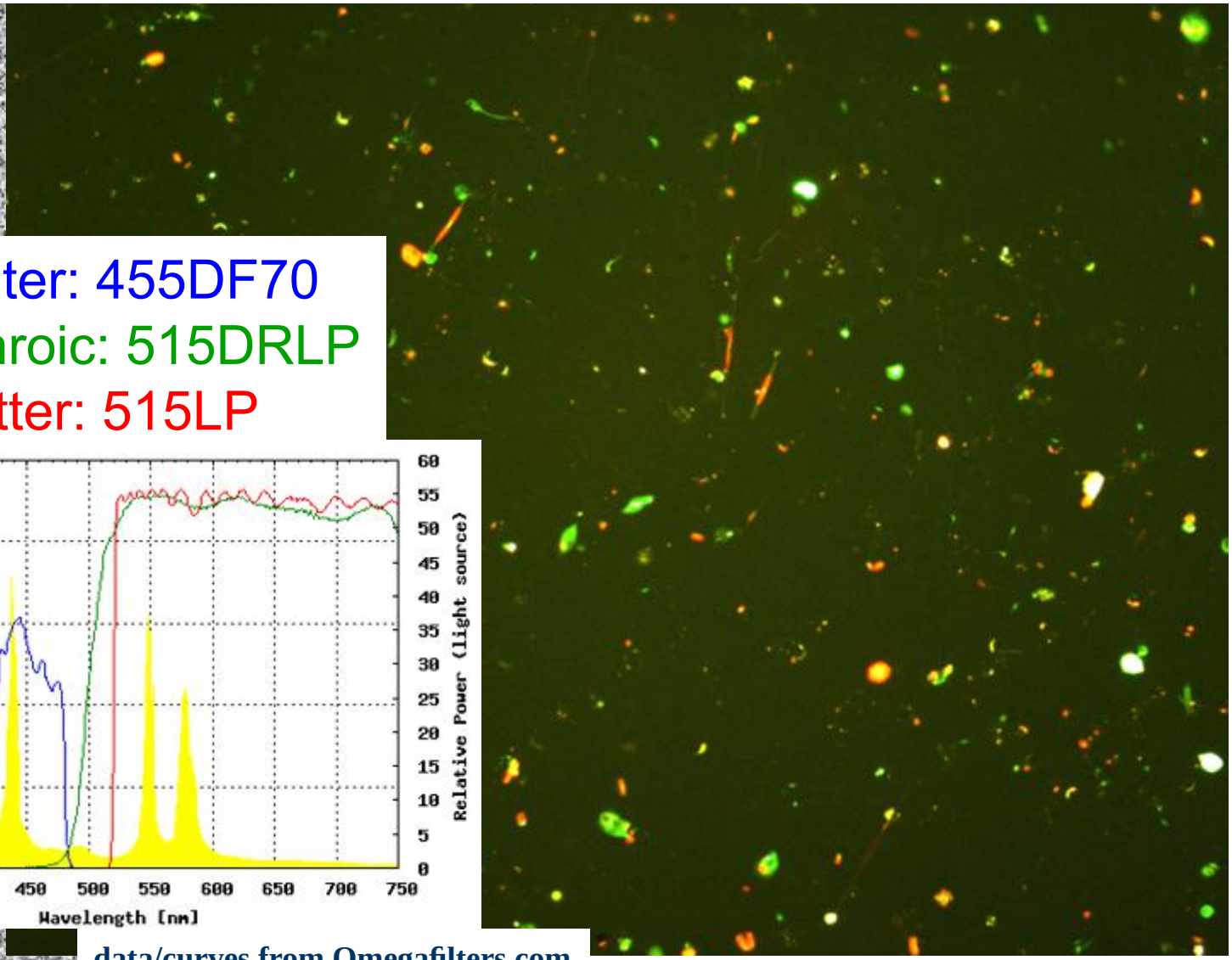




data/curves from Omegafilters.com

Typical image of phytoplankton and heterotrophs (protists)

(sample (50 ml) stained with proflavin and DAPI and filtered onto a 1 µm filter, excited with blue light)



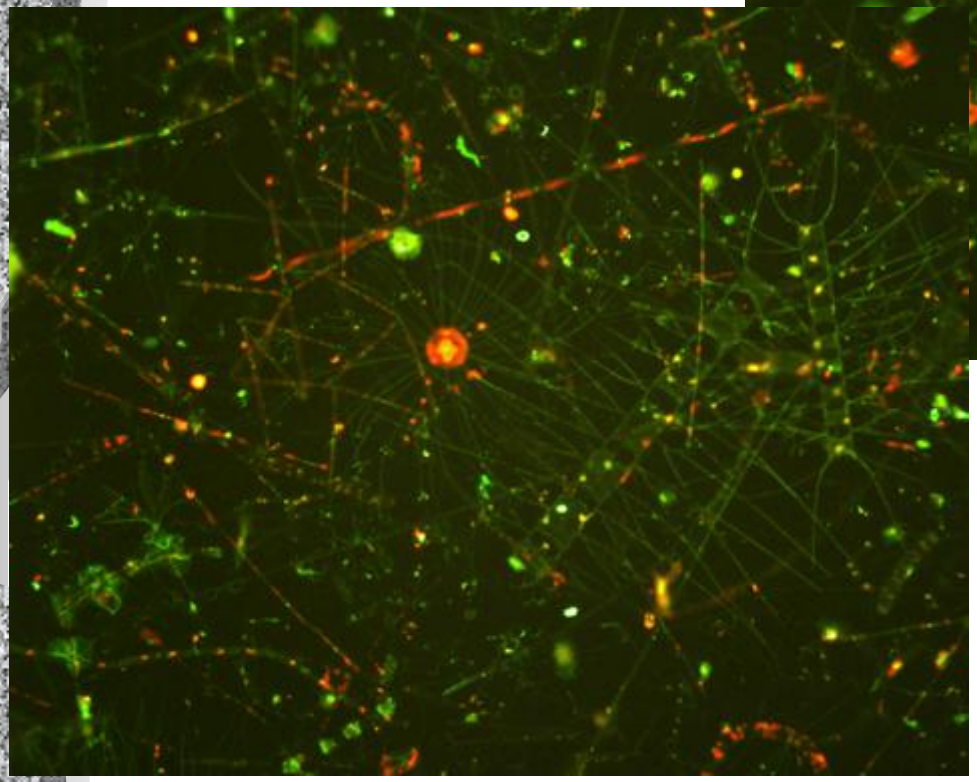
Exciter: 455DF70

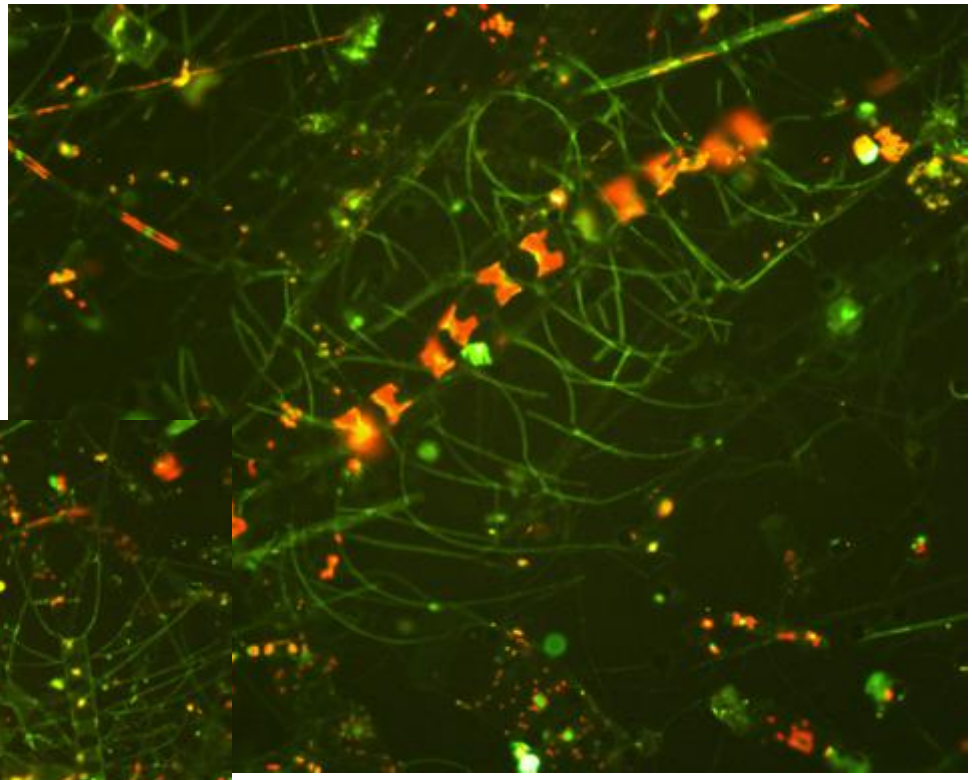
Dichroic: 515DRLP

Emitter: 515LP

data/curves from Omegafilters.com

Spring diatom bloom in the Kuroshio Current (off Japan), May 2002





Centric diatoms, mainly Chaetoceros spp.

XL Benchtop (sea-going) Flow Cytometer

Altra Sorting Flow Cytometer POST 20

Influx (BD) Cytometer

What is a Flow Cytometer?

- *It characterizes particles in suspension*, based on their light scatter properties and their auto- and induced fluorescence properties

- *It consists of*
  - laser light source(s)
  - fluid controls for sample and sheath streams
  - light scatter and fluorescence detectors
  - software to analyze particle signatures

Fluorescence measurements: Flow Cytometry

- Sample is illuminated and light is collected by detectors selectively (i.e., certain wavelengths), which then produce electronic signals. These signals are quantified in various ways. The sample moves in a fluid stream, past the detectors.
- Response time: <10 μseconds.
- Main disadvantage: limited resolution -- can only classify plankton on basis of optical characteristics, not ultrastructure details.

Light sources: microscopy vs. flow cytometry

- Epifluorescence microscope: usually a Hg-arc lamp.
- Flow cytometer: usually a laser or short-arc lamp with much more powerful light output (~1000 times brighter than Hg-arc lamp) to collect the same signal as the microscope. This is because the sample is flowing past the detectors very rapidly -- can not integrate the fluorescence signal like the eye does with a stationary sample.

Argon Ion Gas Laser

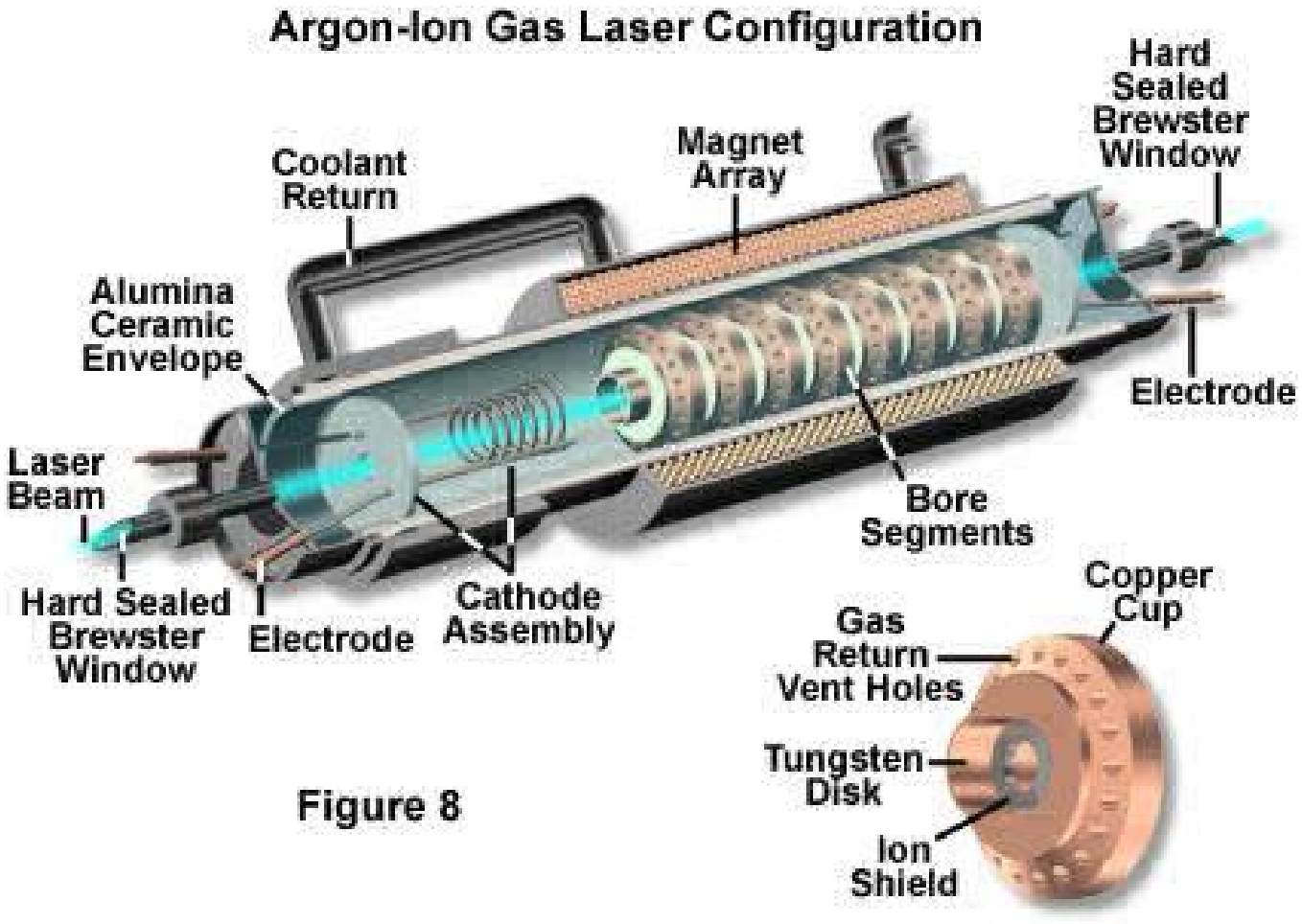


Figure 8

L.A.S.E.R. = Light Amplification by the Stimulated Emission of Radiation

Solid State Lasers

-Use a solid crystalline material (ruby, garnet, sapphire crystals in rod form) as the "lasing" medium.
-These cylindrical rods are mounted in an optical cavity which forms the bulk of the laser.
- Certain atoms found inside the crystalline host "excited" by external light: producing laser light.

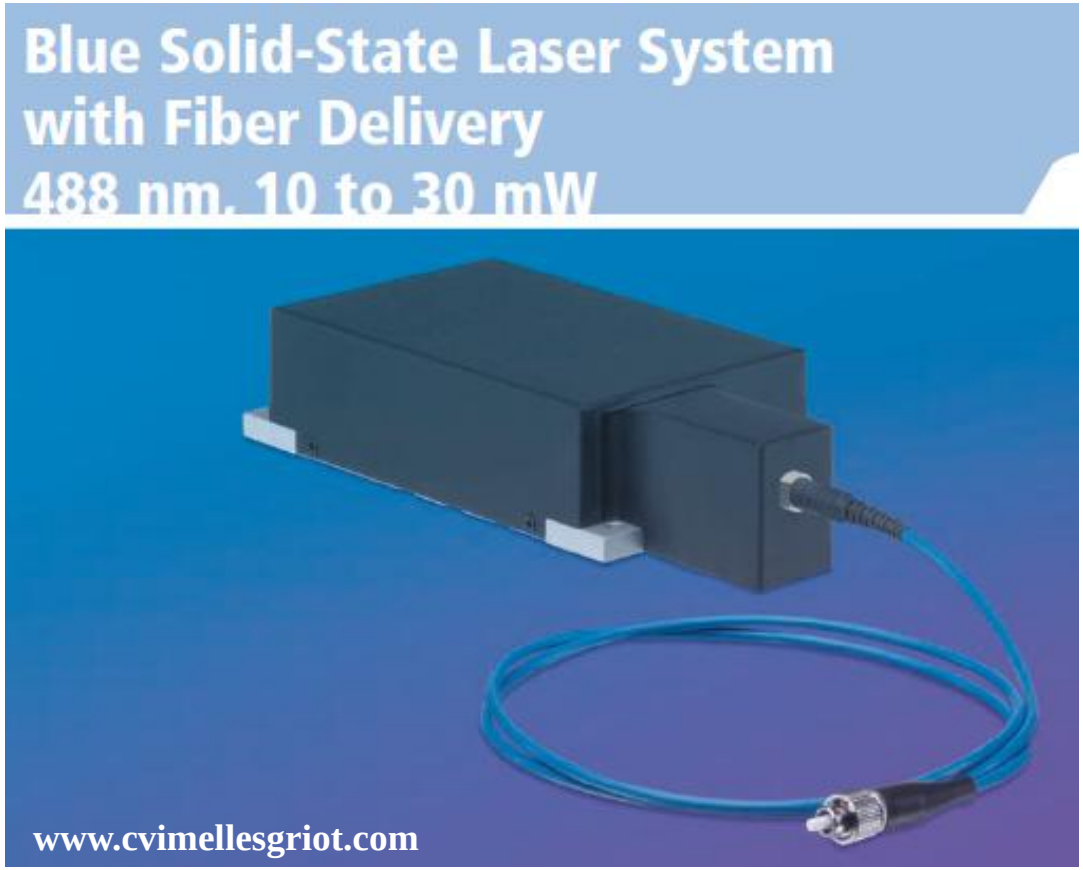
Have the following components:

1) A solid, crystalline lasing medium that can be "pumped" to a higher state of energy

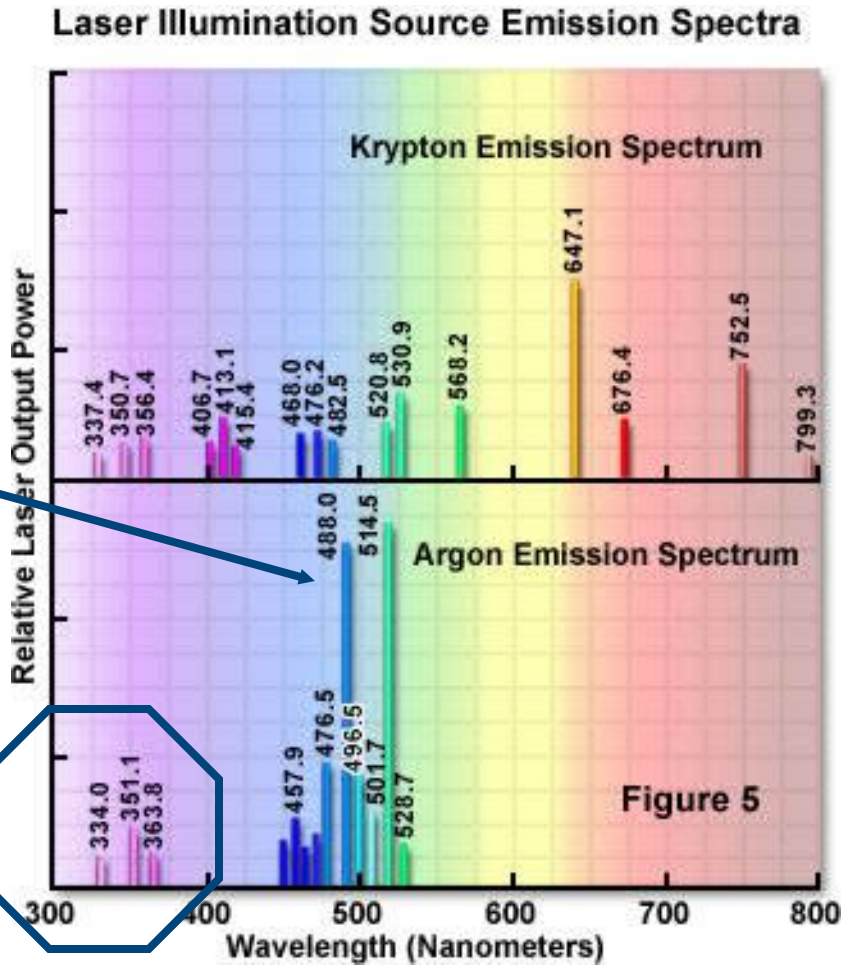
2) A pumping system to pump energy into the lasing medium (usually an optical pumping system)

3) A resonator (usually a pair of mirrors mounted at each end of the laser) to bounce stimulated light through the lasing medium

More efficient delivery of light, more flexibility in laser location



Laser light sources



most commonly used

"UV" excitation

Also common, He-Ne lasers at 546 nm and 633 nm

Schematic of Flow Cytometer's fluidics system

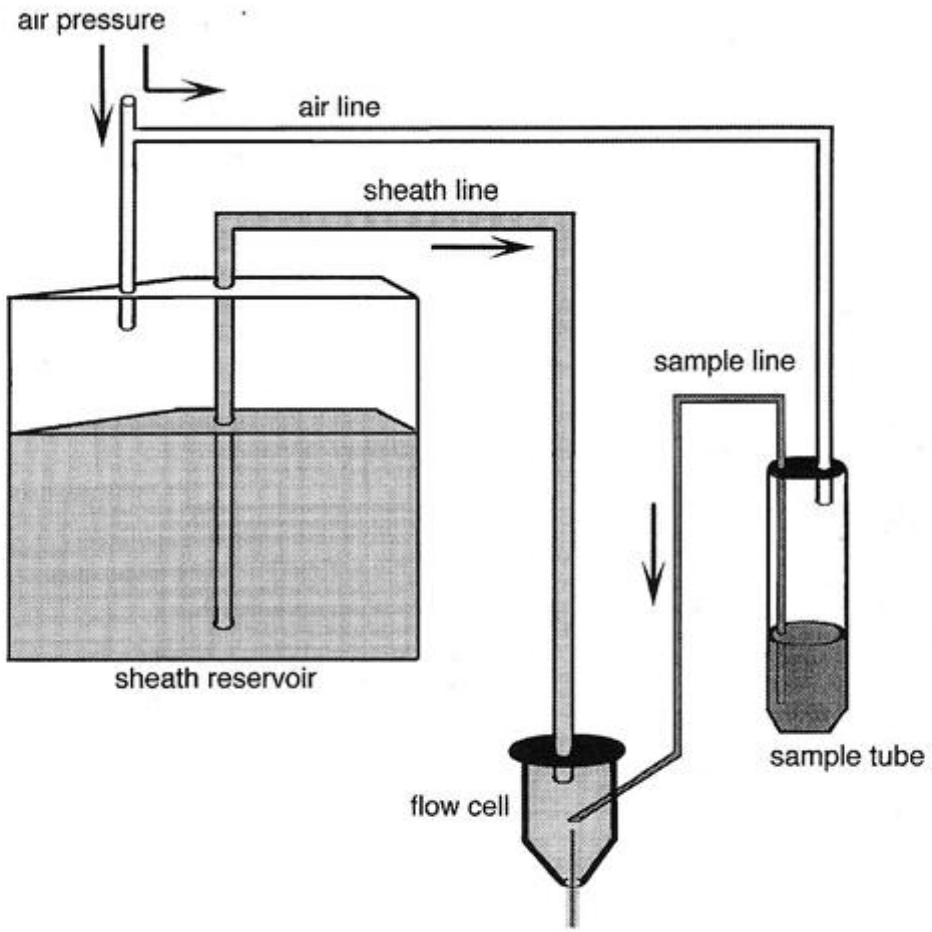


Fig. 3.2. The fluidics system, with air pressure pushing both the sample (with suspended cells) and the sheath fluid into the flow cell.

figure from Givan 2001

Core within a core: Hydrodynamic focusing of particles in the fluid stream

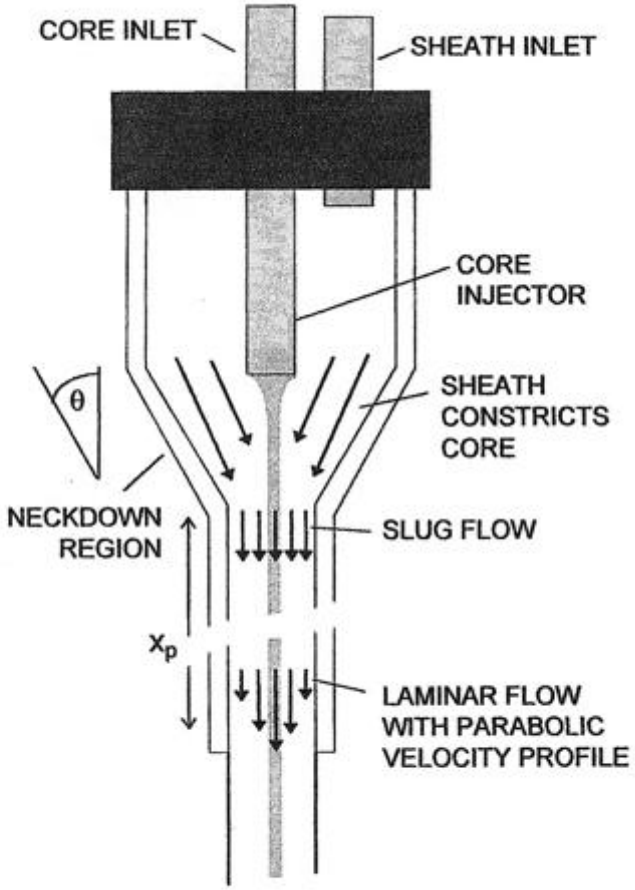


Figure 4-28. Fluid flow in a flow cytometer.

Shapiro 1995

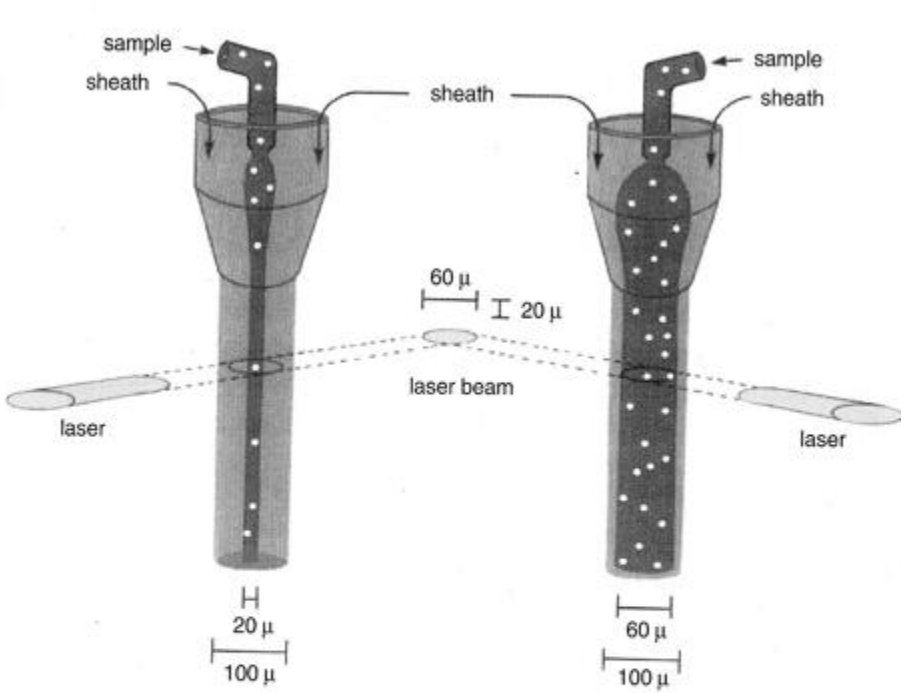
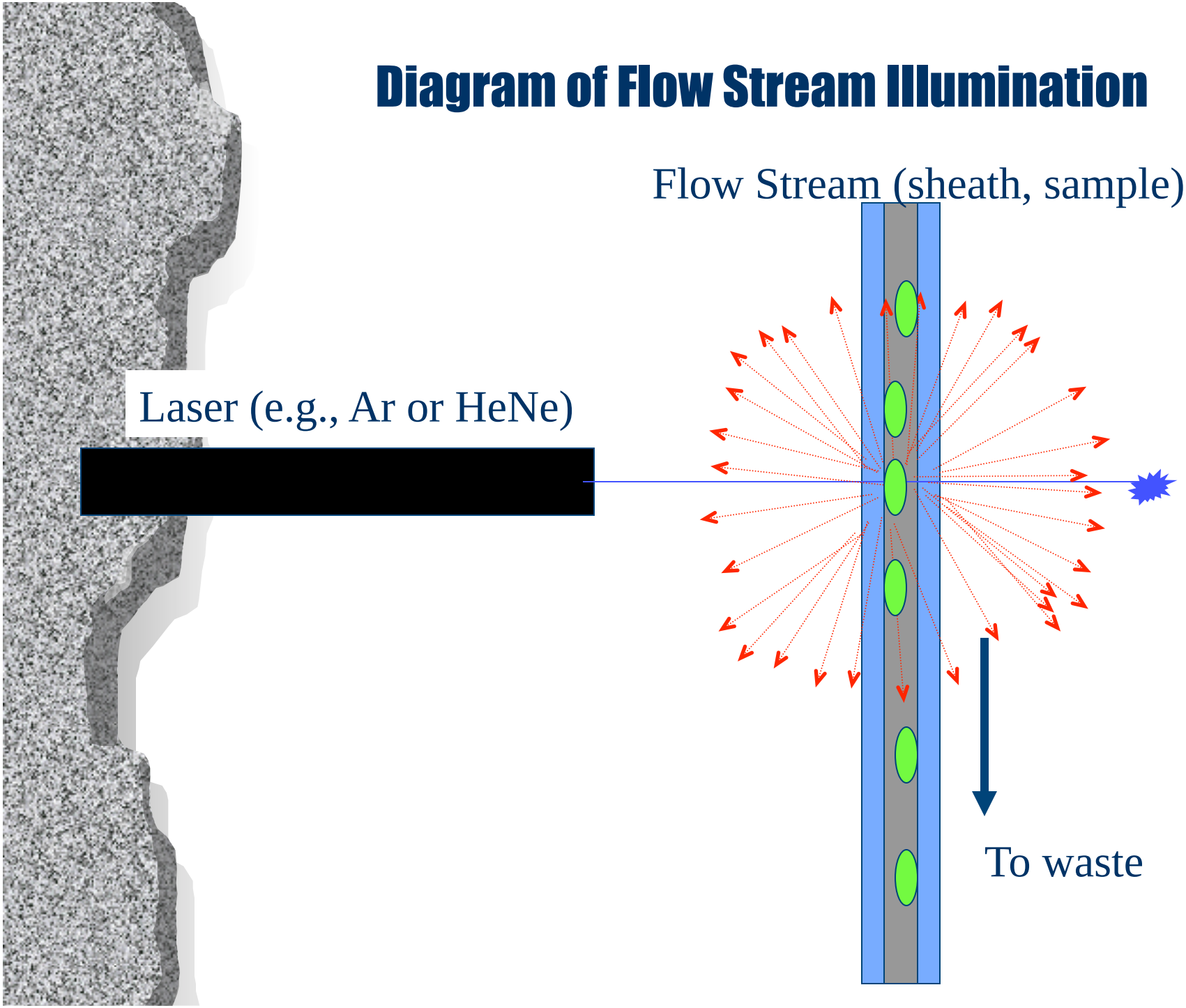


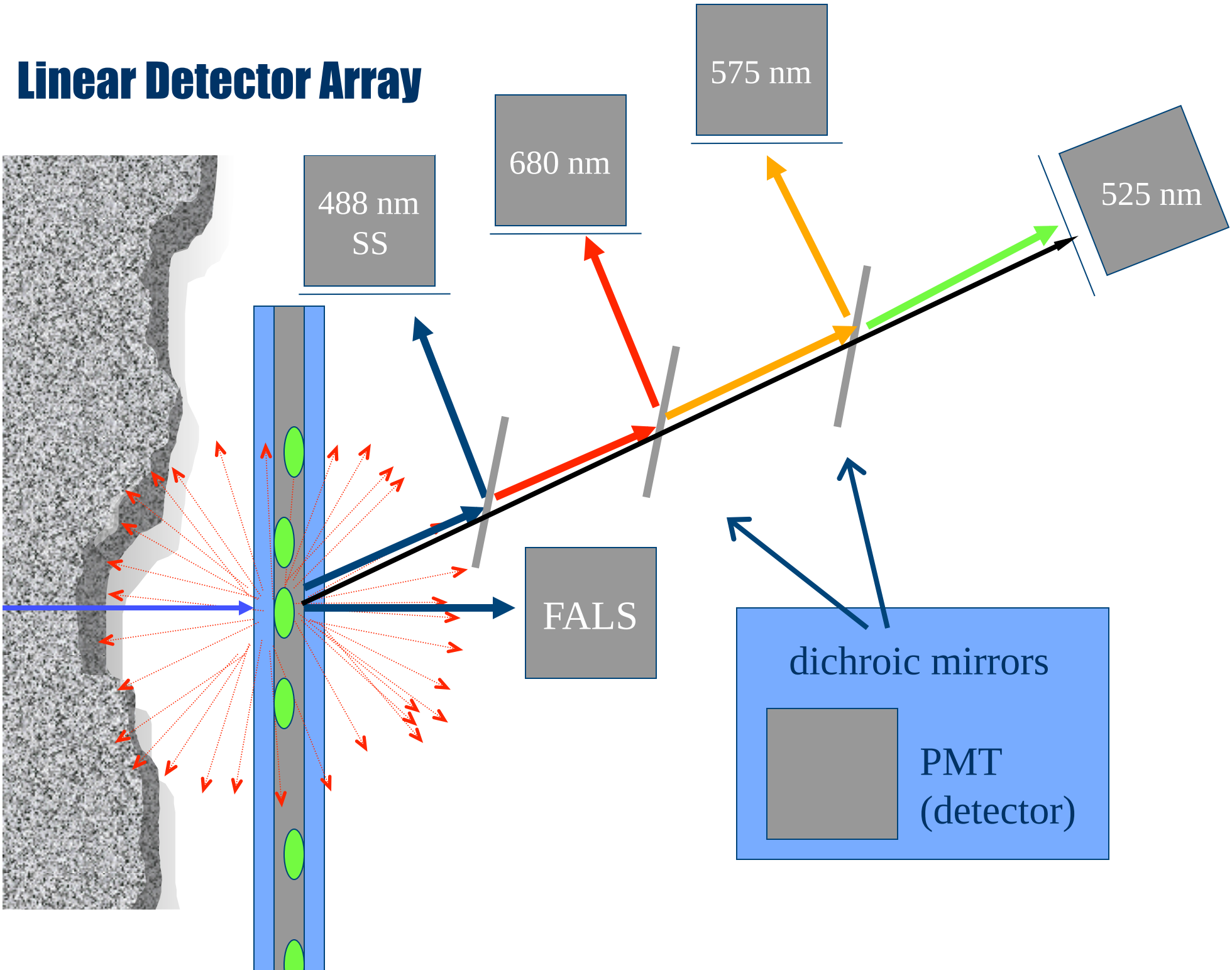
Fig. 3.5. The flow of cells within the core of sheath fluid through the analysis point in the illuminating beam. When the sample is injected slowly (left), the core is narrow and the cells flow one at a time through the center of the laser beam. When the sample is injected too rapidly (right), the core is wide (somewhat exaggerated in this drawing); the cells may be illuminated erratically because they can stray from the center of the beam. In addition, more than one cell may be illuminated at the same time.

Givan 2001

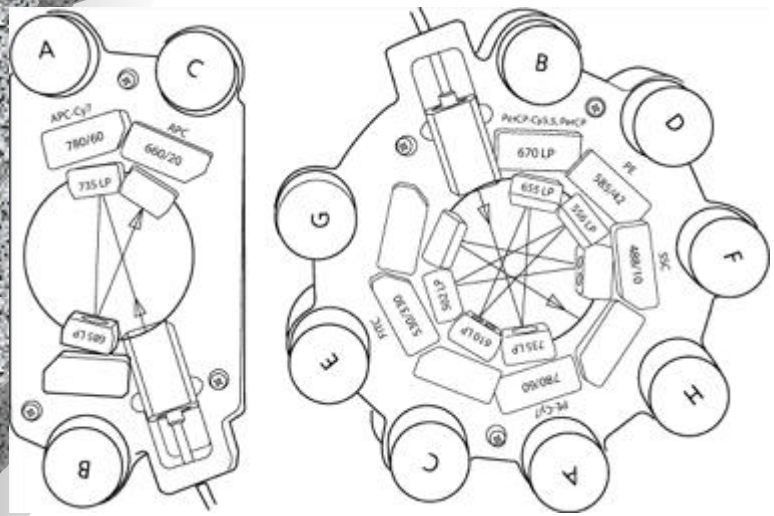
Diagram of Flow Stream Illumination



Linear Detector Array



Other Detector Configurations: serial reflective design



BD Trigon and Octagon Detectors

BD FACSVerse Heptagon Detectors

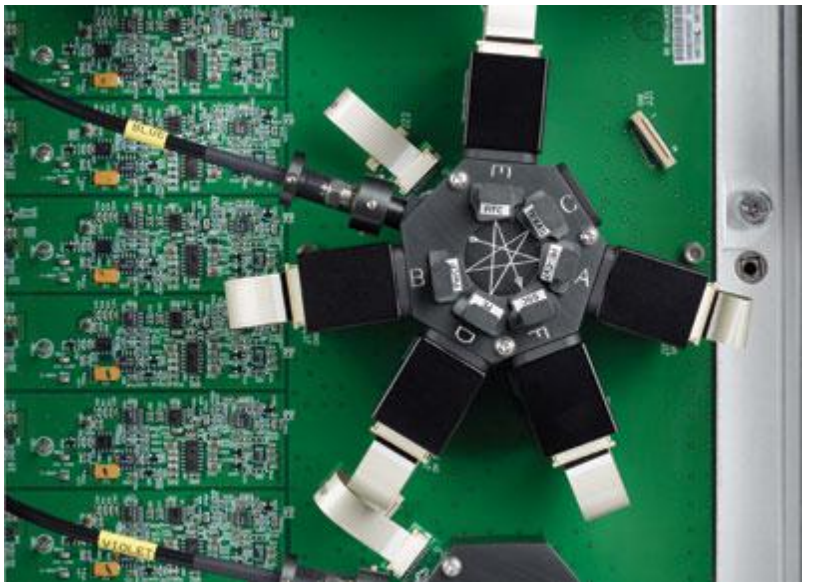


Diagram of sorting

Shapiro 1995

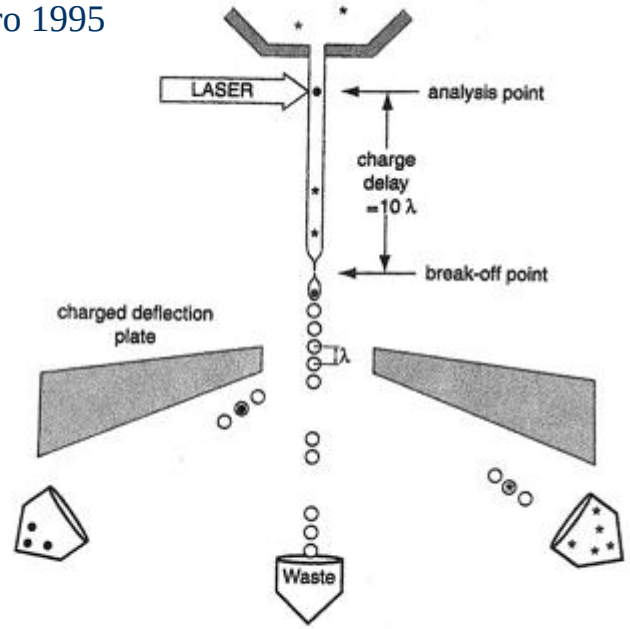
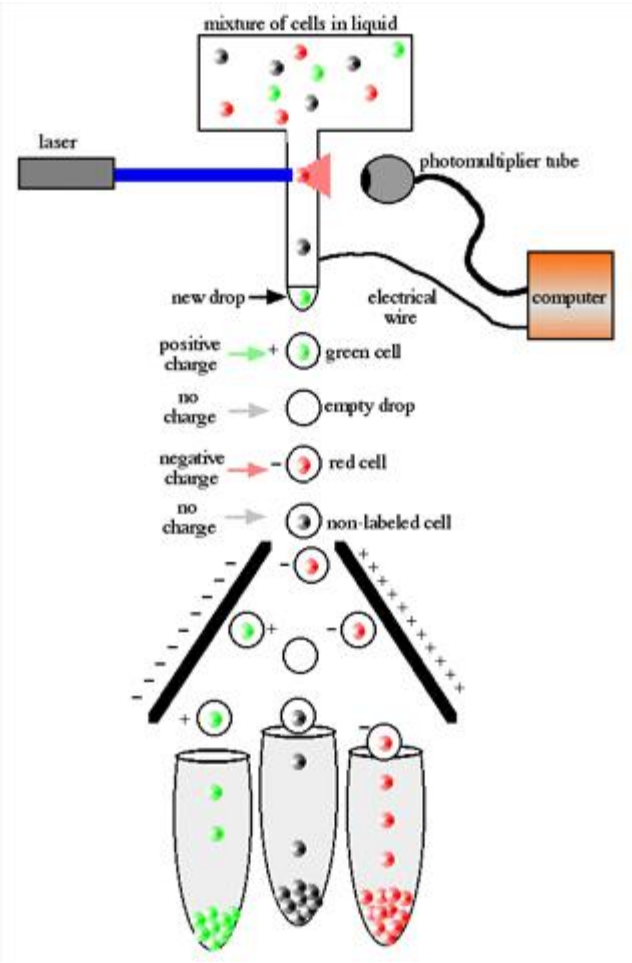


Fig. 9.3. Droplet formation for sorting. A time delay is required between analysis of a particle and the charging of the stream so that only the drop (or three drops) surrounding the desired particle will be charged and then deflected.



www.bio.davidson.edu/genomics/method/FACS.html

Microbial Oceanography: Common Measurement Parameters

Cell Size Proxies:

FALS: Forward angle light scatter

RALS: Right angle light scatter (90°light scatter)

DNA Proxy:

Blue Fluorescence: UV-excitation of Hoechst-bound DNA emits in blue (~450 nm)

Pigment Autofluorescence:

Orange fluorescence: 488 nm excitation of phycoerythrin, emits in orange (575-630 nm): cyanobacteria

Red fluorescence: 488 nm excitation of chlorophyll, emits in red (~680 nm).

Prochlorococcus spp.

The most abundant phytoplankton (~0.6 µm diameter) in the oceans, discovered with the use of flow cytometry in 1988.

Chisholm et al. (1988) A novel free-living prochlorophyte abundant in the oceanic euphotic zone.
Nature 334:340-343

Chisholm et al. (1992) Prochlorococcus marinus nov. gen. Nov.sp.: An oxyphototrophic marine prokaryote containing divinyl chlorophyll a and b.
Archives of Microbiology 157:297-300.

Synechococcus spp.

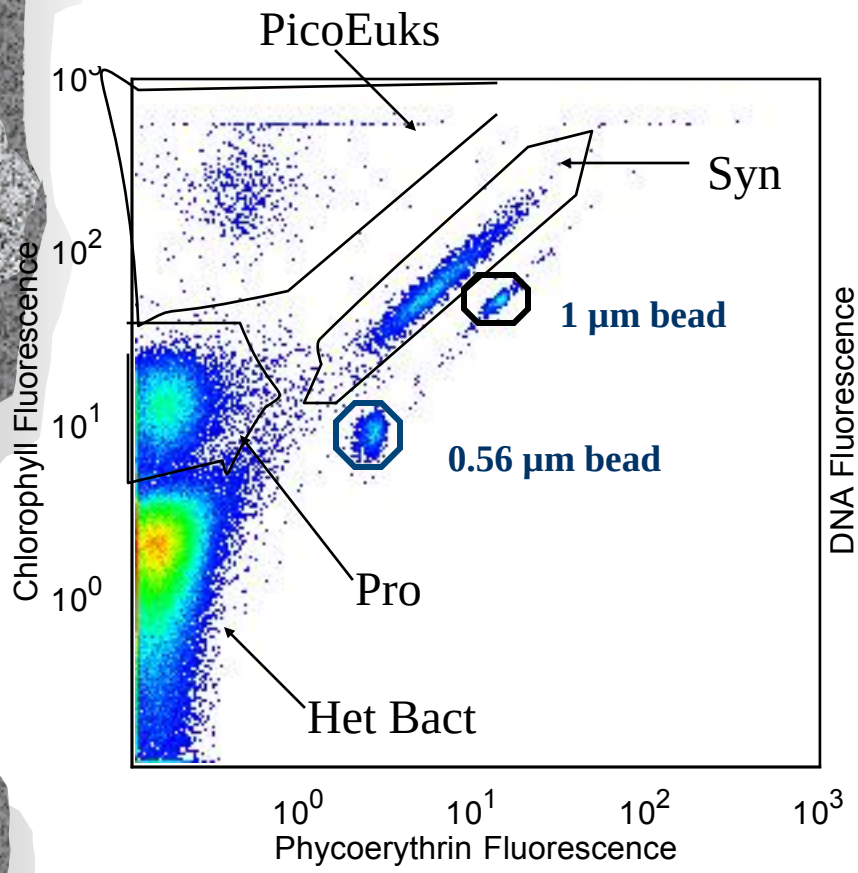
- Another prokaryotic phytoplankton, ~0.9 µm diameter (somewhat larger than *Prochlorococcus)*.
- Found near the coast and in the open ocean, often co-occurs with *Prochlorococcus*.
- Distinctive flow cytometric signature: chlorophyll and phycoerythrin

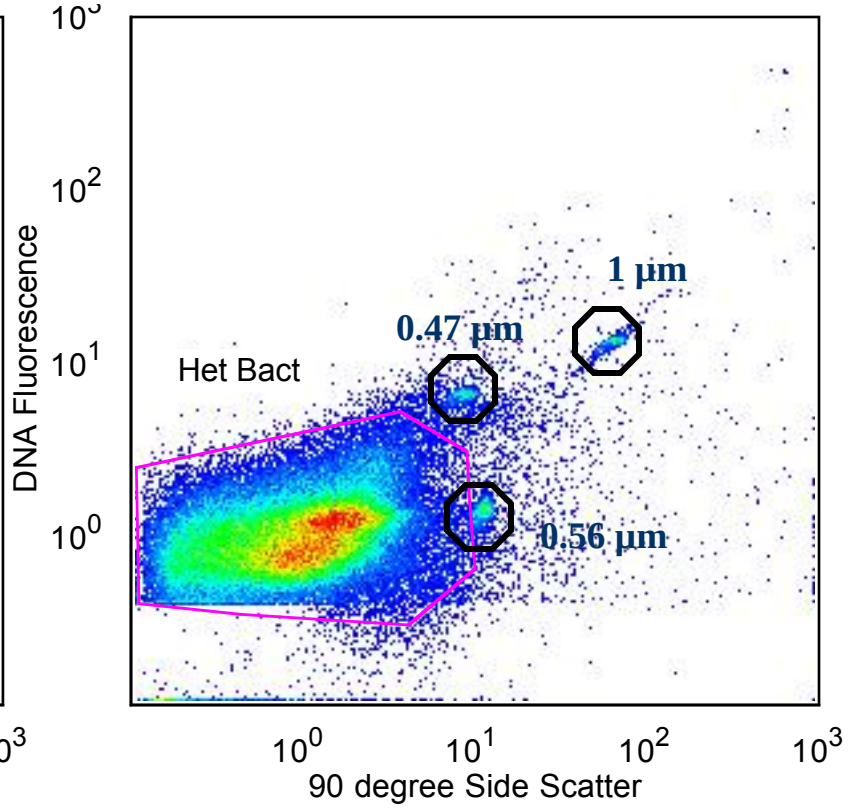
Non-pigmented Bacteria

- Commonly enumerated using flow cytometry (faster and better counting statistics than microscopy)
- Abundances of 10^5 - 10^6 cells per ml of seawater
- Usually stained with a DNA stain for visualization (Hoechst, Sybr-stains, etc.)

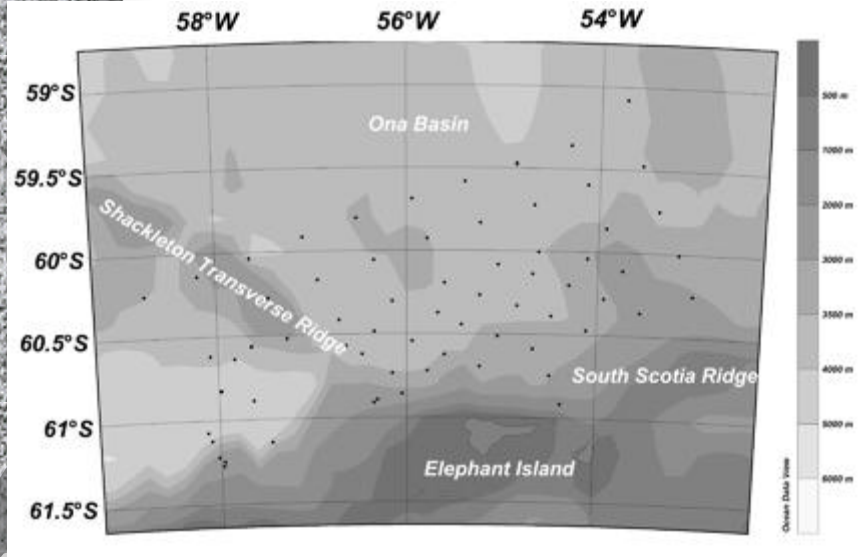
Examples of Flow Cytometry Data, showing picoplankton populations

(East China Sea, courtesy of J. Noh, KORDI)



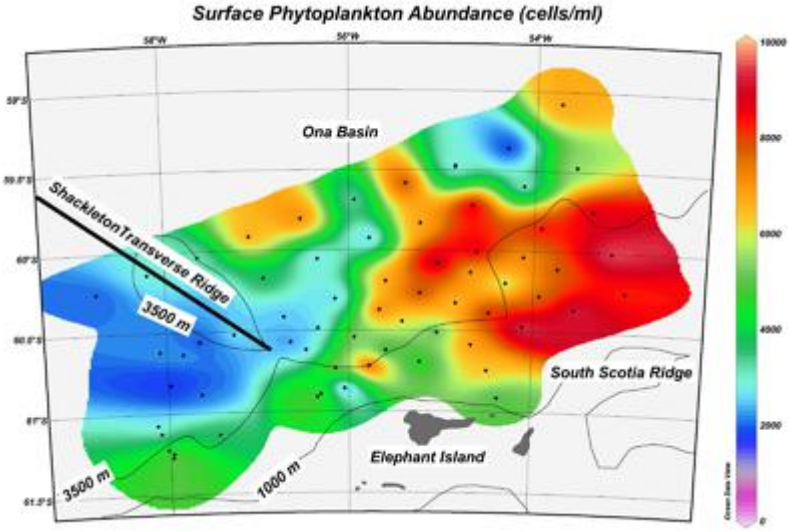


Phytoplankton in the Drake Passage, Antarctica



grid of ~70 stations collected water for analysis of many parameters, including phytoplankton abundance

flow cytometry analyses on-board ship of live samples (run within 6 hours of collection)



Some Other Applications of Flow Cytometry (not a complete list)

- ✓ Cell cycle analyses
- ✓ Grazing rate estimates
- ✓ Ploidy assessment
- ✓ Microbial physiology
- ✓ Antibody-based probes
- ✓ Nucleic acid probes
- ✓ Sorting for subsequent culturing/isolation

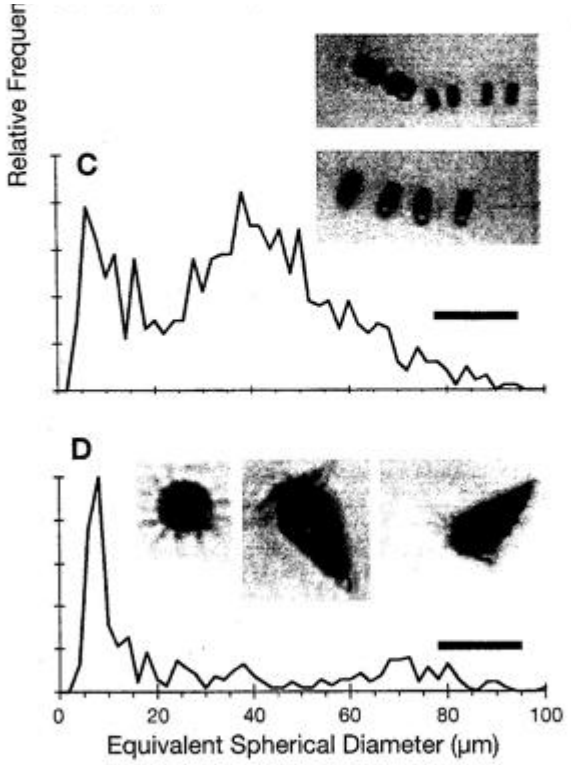
Custom-built flow cytometers for marine studies

Sieracki et al. (1998) An imaging-in-flow system for automated analysis of marine microplankton. Marine Ecology Progress Series 168:285-296.

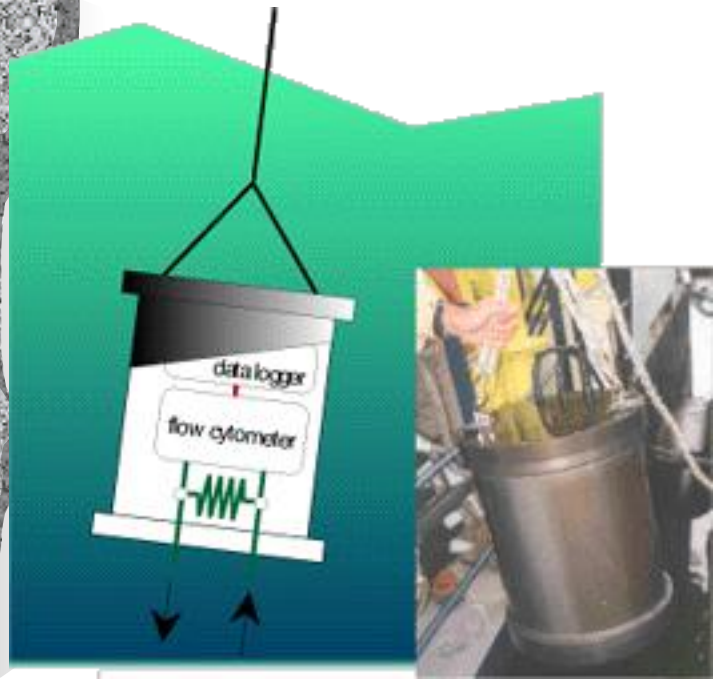
The “Flow CAM” combines an imaging epifluorescence microscope and a simple flow cytometer using a 200 W mercury arc lamp or blue laser for particle excitation.

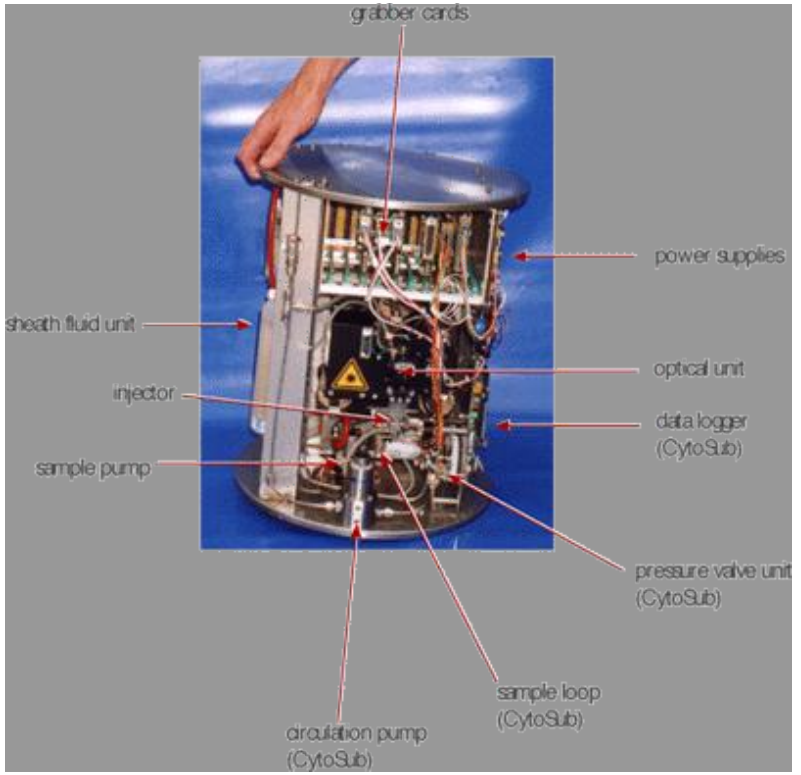
Can detect particles as small as ~2 µm in diameter and captures microscope images simultaneously with scatter and fluorescence data.

Has portable, submersible, flow-through configurations

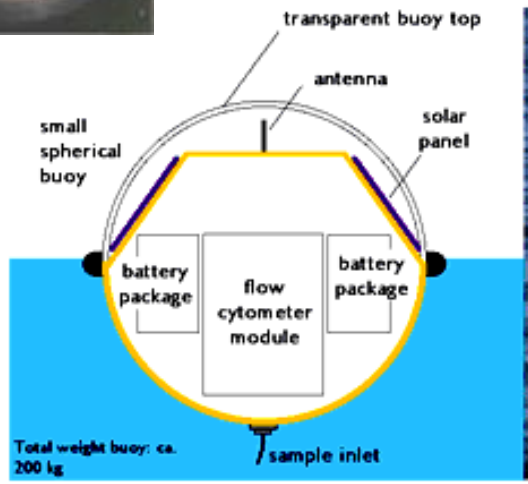


CytoBuoy





Benchtop, submarine, buoy styles:
possible to have 2 lasers and a camera, as options



Images from www.cytobuoy.com

FlowCytoBot

R. Olson, A. Shalapyonok, H. Sosik, 2003, An automated submersible flow cytometer for analyzing pico- and nano phytoplankton: Flow Cytobot

- **in-situ, unattended since 2003**
- **Coastal mooring off New Jersey (powered & cabled)**

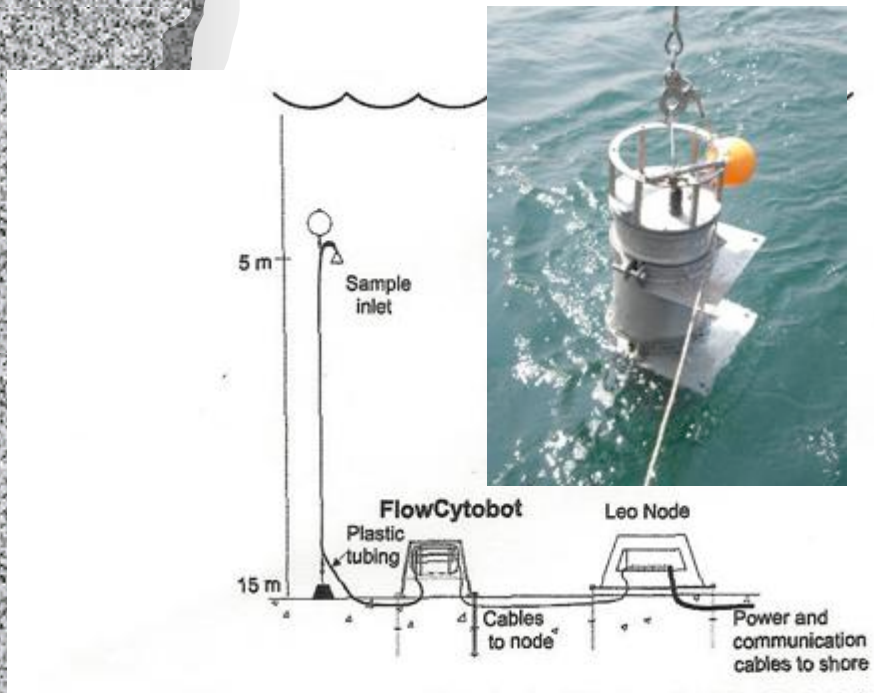


Fig. 5. Configuration of FlowCytobot at the LEO-15 mooring site off New Jersey. Inset: FlowCytobot in its frame, after testing off the WHOI dock.

Imaging FlowCytoBot

Multisizer III

The Coulter Principle

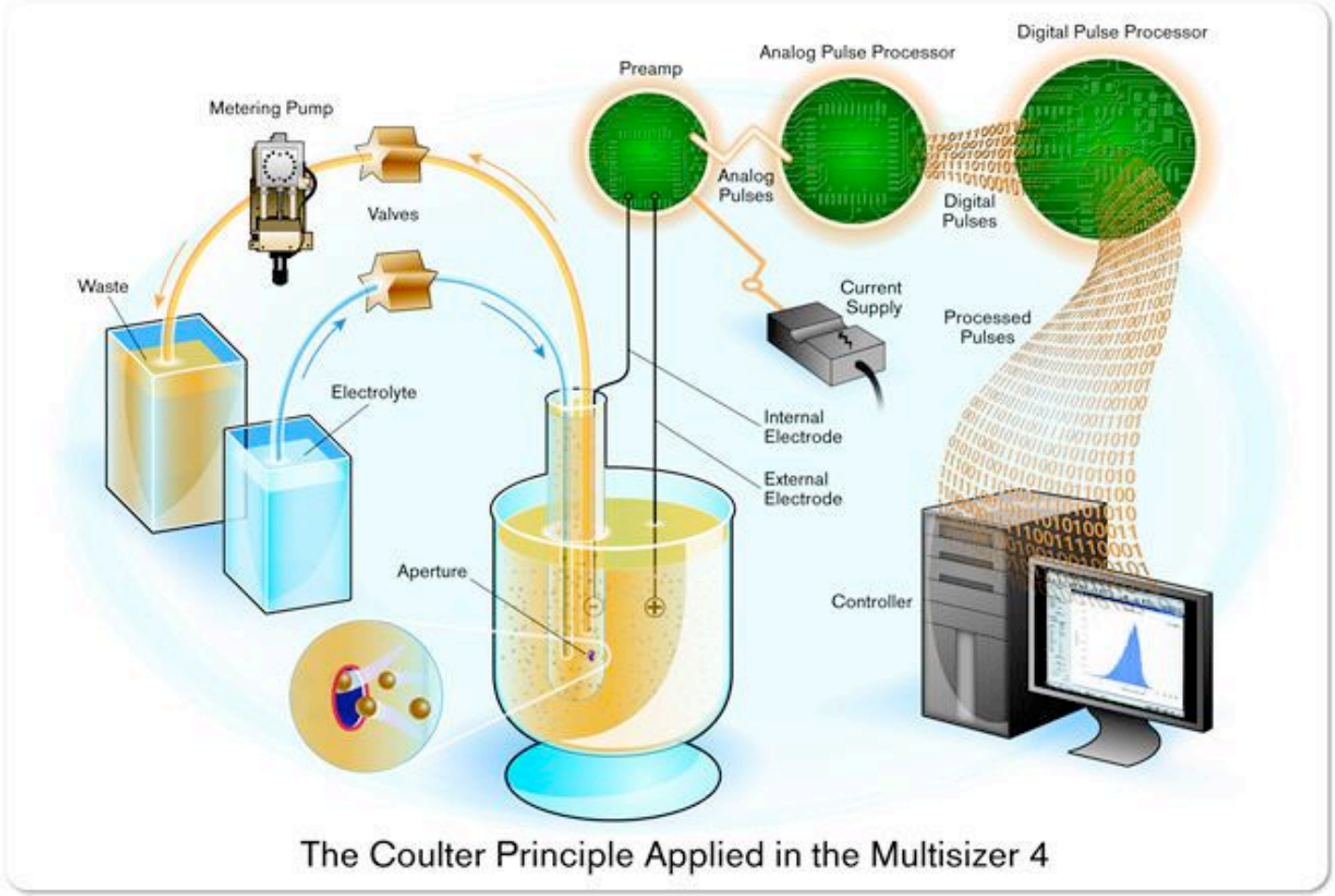
Based on measured changes in electrical resistance produced by nonconductive particles suspended in an electrolyte.

- A small opening (aperture) between electrodes is the sensing zone through which suspended particles pass.
- In the sensing zone each particle displaces its own volume of electrolyte.
- Volume displaced is measured as a voltage pulse; the height of each pulse being proportional to the volume of the particle.

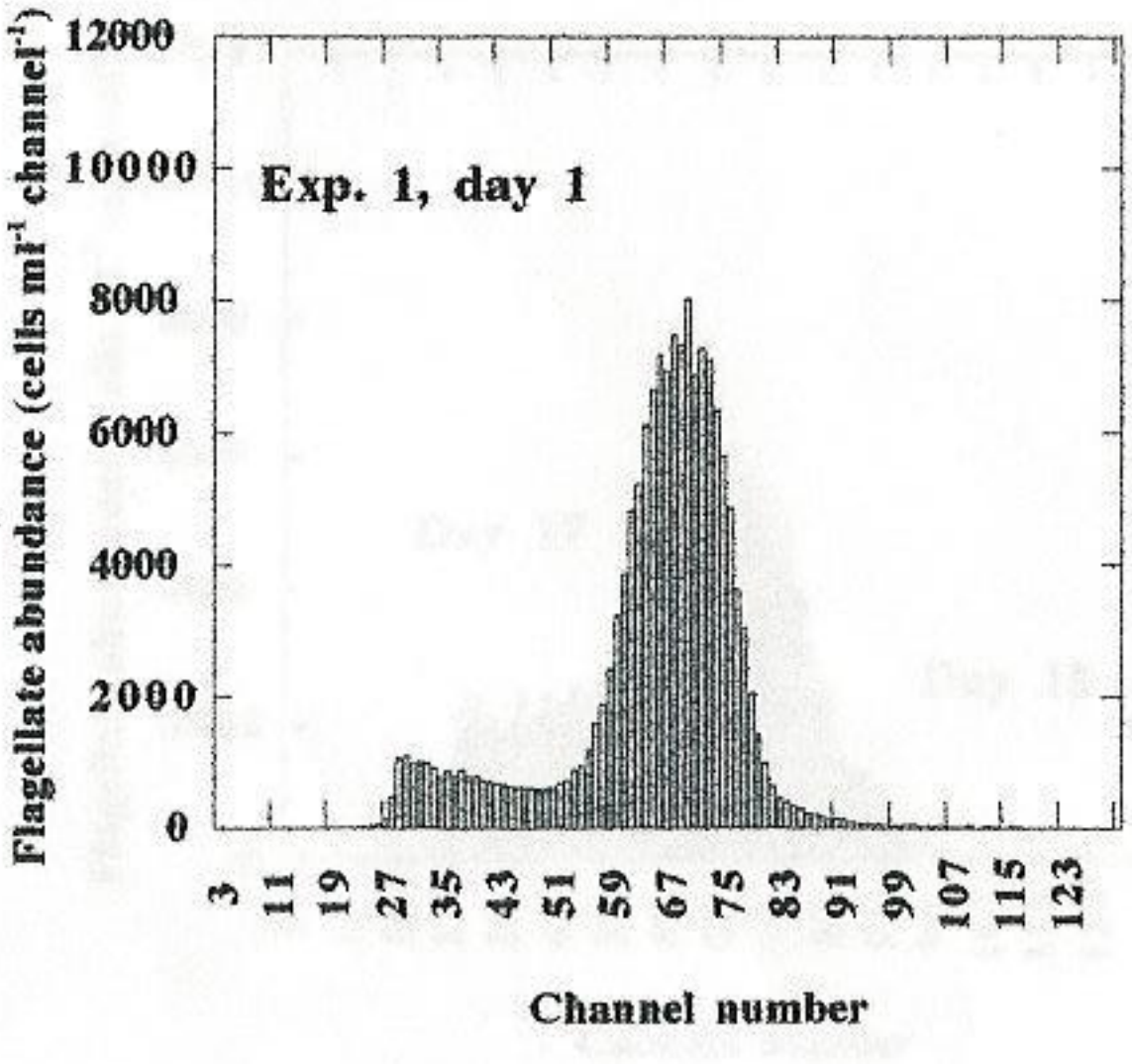
-- Volumetric quantity

-- Several thousand particles per second counted

Diagram of Coulter Counter



Example of Data Output



Comparison of Methods

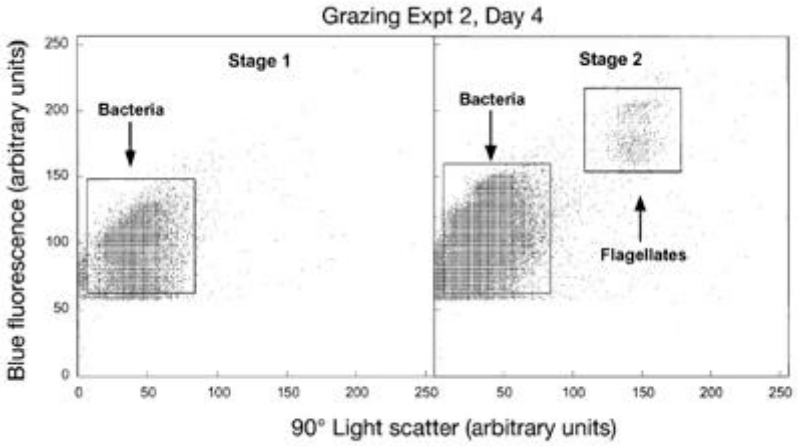
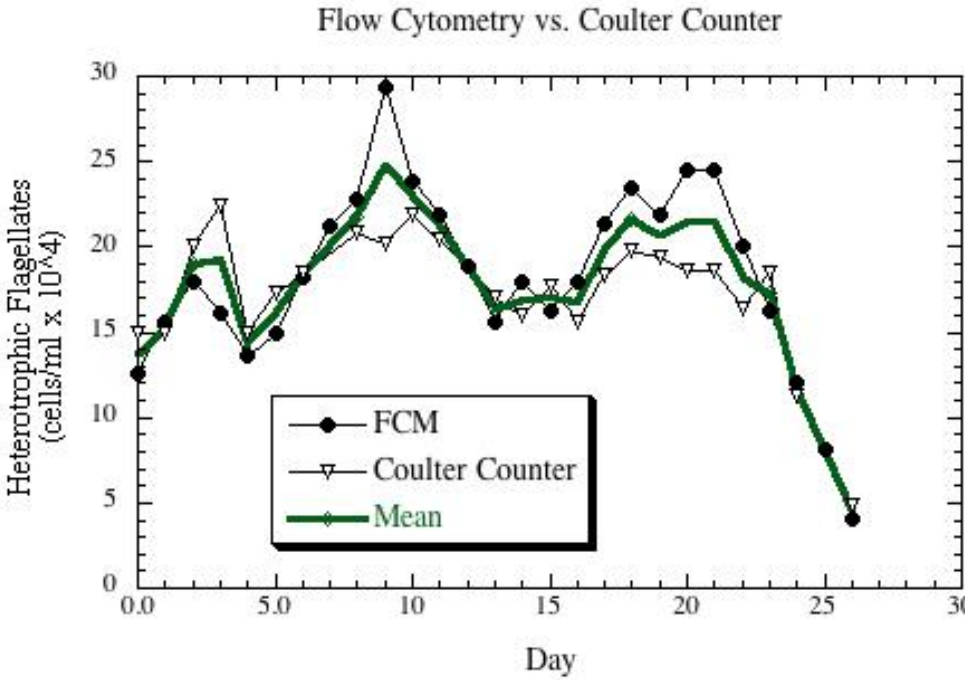


Fig. 1. Example cytograms of flow cytometry data from grazing Expt 2, Day 4. Plotted is blue fluorescence (a proxy for cellular DNA content) as a function of 90° light scatter (related to cell size and granularity). The Stage 1 sample from the chemostat (left) shows the heterotrophic bacteria in the sample, and the Stage 2 sample (right) shows the heterotrophic bacteria and the flagellate populations. These data are based on a 100 µl sample, diluted 1/100 (Stage 1) or 1/20 (Stage 2) in filtered seawater

Selph et al. 2003

Samples run on flow cytometer and Coulter counter

Data from Selph 1999



Great Resources for Flow Cytometry and Fluorescence-based applications in general

www.cyto.purdue.edu

This is the premier site for flow cytometry information.

www.probes.invitrogen. com

This is Molecular Probes web site: a supplier of fluorescent probes of all types, that has detailed explanations of all aspects of using dyes for just about any kind of biological research.

Practical reference for epifluorescence microscopy:

Handbook of methods in Aquatic Microbial Ecology, P.F. Kemp, B.F. Sherr, E.B. Sherr, and J.J. Cole (Eds), Lewis Pubs., 1993.