Seawater sample collection:

Raw seawater- Raw seawater is defined as water sampled from the ocean which has not be processed or manipulated in any fashion. Seawater samples such as this are collected in 1 to 10L increments in a translucent to semi-translucent autoclaved containers. Sample to be used for flow cytometer are subsampled from this volume directly into cytometry sample tubes in 1 to 3 mL incriments.

Concentrated seawater samples- An effective strategy for collecting biomass from rare or low concentration organisms in seawater can be to concentrate seawater atop filters via low pressure filtration. Caution should be taken, as filtration can be very detrimental to cells larger than 3 μm in diameter.

Seawater samples are collected in 1 to 10L increments in a translucent to semi-translucent autoclaved containers. Samples are concentrated approximately 100-fold by low-pressure vacuum filteration (<5 psi) atop a 45 mm diameter, 0.22 μm Type GV Durapore filters. Cells captured atop the filter are resuspended by vortex in 10 mL of the seawater filtrate with a 15mL Falcon centrifuge tube. Concentrated cells are analyzed on the flow cytometer immediately or stored in 15 mL centrifuge tubes for a period no longer than 24 hours at 4°C.

Seawater samples collected at sea are concentrated by the same method and then flash frozen in freeze resistant tubes (15mL Falcon centrifuge tubes work well) using liquid nitrogen. Tubes may then be stored at -80°C until analyzed.

Preserved Seawater samples- Samples intended for cell counts are preserved using two potential fixatives:

1. Paraformaldehyde (PFA): Natural seawater samples may be preserved with paraformaldehyde (0.5% to 1% final concentration). We purchase a 10% solution from a manufacturer (Electron Microscopy Sciences Cat# 15712-S, Electron Microscopy Grade). Seawater samples are transferred to cryovials and 10% PFA solution is added directly to the samples. Once capped, cryovials are mixed thoroughly by inverting the vials several times and frozen immediately in liquid nitrogen. Sample may be stored at -80°C for long-term preservation.

2. Glutaraldehyde: Natural seawater samples may be preserved with Glutaraldehyde (0.25% final concentration). We purchase a 25% solution from a manufacturer (Ted Pella Cat# 18426, Electron Microscopy Grade, 25%). Seawater samples are transferred to cryovials and 25% PFA solution is added directly to the samples creating a 1:100 dilution. Once capped, cryovials are mixed thoroughly by inverting the vials several times and frozen

immediately in liquid nitrogen. Sample may be stored at -80°C for long-term preservation.

Seawater intended for FACS to molecular biology protocol:

1. DNA: Organisms to be sorted from seawater for DNA analysis require no special preservation additive. Cell sorts maybe obtained from raw seawater, seawater that has been frozen previously or seawater that has been concentrated before or after freezing. As mentioned previously, some cells are altered greatly by freezing suggesting caution be taken when sorting from frozen samples.
2. RNA: Seawater to be used to obtain cells for FACS assisted molecular biology analysis is preserved first in RNA Later (Qiagen Cat# 76104, recipes are available online for bulk production) by mixing RNA Later in a 50:50 mix with seawater. Immediately, this mixture is filtered atop 45 mm diameter, 0.22 µm Type GV Durapore filters. Cells from atop the filter may then be re-suspended in filtered seawater for immediate FACS or the filters may be frozen dry (-80°C) for later use.

Seawater Analysis:

Scatter detectors-

1. FSC- forward light scatter can be a limited proxy for cell size on a relative scale.
2. SSC- side light scatter (orthogonal scatter) can offer some insight as to the complexity or granularity of the cytoplasm of cells

Fluorescence detectors-

1. 531/40- Green filter (FITC- commonly used as a label on Antibodies and nucleic acid probes, SYBR stain detection- a common DNA stain)
2. 572/27- Yellow filter (detects the presence of Phycoerythrin, a light collection protein common to phototrophic bacteria)
3. 692/20- Red filter (detects the presence of Chlorophyll, common to all phototrophic organisms)

Preparations and protocols for FACS to molecular biology workflow

Flow cytometer:

1. Sheath Fluid- We use a preservative-free, 8X concentrate sheath fluid (Biosure, inc, Cat# 1020) prepared fresh prior to sorting for cells to be incorporated into a FACS to molecular biology work flow using nano-purified, distilled water to dilute to a 1X working solution.
2. The BD Influx reservoir is through cleaned and autoclaved.
3. The sheath and sample line plumbing are disinfected with a 5% bleach solution (500mL) then flushed with 15L of nano-purified, distilled water.
4. Expose the internal sort chamber of the Influx cytometer to UV light for 5

minutes.

Sample tubes:

1. FACS product can be sorted into a variety of different tubes.
    a. Large sorts (>5,000 cells; 1.5mL micro-centrifuge tubes in 20 to 100 μL 5kD filtered, nano-purified, distilled water or TE buffer)
    b. Moderate sorts (100 to 5,000 cells; 0.2mL reaction tubes (individual or strips of 8) in 10 to 20 μL 5kD filtered, nano-purified, distilled water)
    c. Small sorts (1-100 cells; 0.2mL reaction tubes (strips of 8, or 96 and 384 well plates) in 5 to 10 μL 5kD filtered, nano-purified, distilled water)
2. Reaction tubes (both PCR and optical tubes for qPCR) are purchased sterile and, DNase and RNase free.
3. All reaction tubes are exposed to 10 minutes of UV light prior to the addition of water or TE buffer.
4. Reaction tubes are stored capped until ready for sorting

Sort strategy:

Single Cell Sorts-

Cells from a candidate population or a rare event region can be sorted into 8 optical tube strips or 96 well optical plates (Applied Biosystems, Foster City, CA) for real time PCR or into a conventional containing 96-well PCR plates containing 10 μL of 5 kD filtered distilled water in each well. Cells are then sorted at 1 cell per well into the top 72 wells contained in row A through row F. Wells in row G and H were left empty for Real-Time PCR nifH DNA standards in duplicate and no template controls. Cells are immediately frozen upon the completion of sorting and stored at -80°C until assayed. Prior to the assay the sorted cell mixture is thawed and the remaining volume of Universal PCR Master Mix (Applied Biosystems, Foster City, CA) including the primer probe mixture is added to each well to a final volume of 25 μL.

Culture sorts-

Cells may be sorted as a means of isolating organisms for culture or purifying a contaminated culture. To ensure a sort free of contaminating cells I try to keep the source population fairly dilute limiting the sort rate to 100s of cells per sec or lower and sorting on a stringent sort parameter of 1 Drop Pure. Cells are sorted into media at a concentration of at least 10,000 cells per mL. Recovery can be slow. Sorting results in dramatic cell damage or death followed by a length recovery time (single digit weeks).

List of stuff to add to web site…

1. Cytometry setup and Troubleshooting
2. Sample collection of seawater (raw; concentrated; preserved; unpreserved; for molecular biology)
3. Rudimentary seawater sample analysis
4. Bacterial and eukaryote cell counts
5. Special precautions for the preparation of the flow cytometer and cell sorter for sorting for cell culture and sorting for downstream molecular biology protocols
6. Sorting options and software instructions for multiple sorting parameters
7. Whole Cell Molecular Biology Assay Protocols