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Report on the Advanced Workshop on Fluorescent Probes for Marine Flow Cytometry: Use of Fluorescent Probes in the Study of Phytoplankton Physiology and Cellular Biochemistry

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There has been a recent explosion of techniques utilizing fluorescent indicators of cellular biochemistry and physiology in the field of biomedicine. Molecules have been synthesized that indicate intracellular pH, redox potential, mitochondrial membrane potential, esterase activity, calcium ion activity, and a variety of other biochemical properties of cells based on their fluorescent characteristics (Darzynkiewicz and Crissman 1990). These indicators can become fluorescent, or change the intensity or spectral quality of their fluorescence that can be readily measured by flow cytometry.

In October 1992 we had the opportunity to offer a workshop on the use of fluorescent probes with flow cytometry for the study of single-celled marine organisms. The workshop was sponsored by and held at the Individual Particle Analysis Facility at the Bigelow Laboratory for Ocean Sciences. It was attended by 12 students and scientists from around the U.S. and from several European countries. The probes we used and their fluorescence and biochemical indicator properties are summarized in Table 1. UV (360 nm) excited probes were analyzed on a Coulter EPICS V with an air cooled Argon laser. Blue (488nm) excited probes were analyzed on a BD FACScan with a 488nm laser. The data was compiled and analyzed using the self learning flow cytometric list mode data classification program, CLASSIF1 (Valet et al. 1992).

Prior to the workshop we screened a wide range of phytoplankton, protozoan and marine sponge cultures for their ability to take up the stains. We needed to be sure that the stains were able to penetrate the cell walls of healthy cells. These were first examined microscopically and then observations were confirmed by flow cytometry and instrument settings were established. All cultures tested were observed to take up all the stains.

The format of the workshop was for participants to set up simple experimental conditions that might be expected to change the intracellular physiology of the cells, which could then be measured using the fluorescent probes. This format produced results from a variety of single experiments designed to yield preliminary ideas about how these probes might be more fully exploited. Several of the experiments are worth mentioning.

1. Oxidative burst response of marine protozoa to natural prey. Dr. M. Sieracki (Bigelow Laboratory) and Karina Gin (M.I.T.).
2. Nutrient starvation of *Thalassiosira weissflogii* , Dr. Monica Orellana (University of Washington).
3. Effects of $CuCl_2$ on *Phaeodactylum tricornutum* - Angeles Cid (University of Coruna, Spain)
4. Effects of light levels on several clones (dinoflagellate, cryptomonad, prochlorophyte) - T. Cucci (Bigelow Laboratory).
5. Responses of marine sponge cells to chemical stimulation - Dr. S. Pomponi (Harbor Branch Oceanographic Institution)

Result from these preliminary experiments are being analyzed by the various participants and experiments will need to be replicated under carefully controlled conditions. Some results were expected and many were unexpected, but the potential application of these probes to indicate cellular physiology in marine organisms has been demonstrated. We are optimistic that these methods can be applied to marine phytoplankton and protozooplankton to yield new knowledge about their cellular responses to anthropogenic and natural variations in their environment.

Table 1. List of Probes Used in Workshop

Probe	Abbrev.	Excitation	Indicates	Reference
1,4-diacetoxy-2,3 - dicyano-benzene	ADB	UV (365nm)	Intracellular pH/esterase activity	Valet et al. 1981
INDO1 pentaaceto-oxymethyl-ester	INDO1/AM	UV	calcium ion	
ortho-phthal-dialdehyde	OPT	UV	free glutathion/free protein SH-groups	Treumer and Valet 1986
Hoechst H33258	--	UV	vital DNA stain	
Hoechst H33342	--	UV	vital DNA stain	
3,3-dihexyl-oxacarbocyanin	DIOC6	blue (488nm)	transmembrane potential	Hasmann, et al. 1989
rhodamine 123	R123	blue	mitochrondrial membrane potential	
2',7'-dichloro-fluorescein-diacetate	DCF	blue	H_2O_2 and peroxidase activity	Burow and Valet 1987
dihydrorhodamine 123	DHR	blue	sensitive H_2O_2 indicator system	Rothe, et al. 1988
hydroethidine	HE	blue	O_2 radical formation, general oxidation	
rhodamine 110 derivatives	R110	blue	protease substrates	Rothe, et al. 1992

Literature Cited

Burow, S. and G. Valet. 1987. Flow cytometric characterization of stimulation, free radical formation, peroxidase activity and phagocytosis of human granulocytes with 2,7-dichlorofluorescien (DCF). European J. Cell Biology 43:128-133.

Darzynkiewicz, Z. and H.A. Crissman. (eds.) 1990. Flow Cytometry. Methods in Cell Biology, Vol. 33. Academic Press, Inc. NY.

Hasmann, M., G.K. Valet, H. Tapiero, K. Trevorrow, T. Lampidis. 1989. Membrane potential differences between adriamycin-sensitive and -resistant cells as measured by flow cytometry. Biochemical Pharmacology. 38:305-312.

Rothe, G., A. Oser, G. Valet. 1988. Dihydrorhodamine 123: a new flow cytometric indicator for respiratory burst activity in neutrophil granulocytes. Naturwissenschaften 75:354-355.

Rothe, G., S. Klingel, I. Assfalg-Machleidt, W. Machleidt, C. Zirkelbach, R. B. Banati, W.F. Mangel, G. Valet. 1992. Flow cytometric analysis of protease activities in vital cells. Biol. Chem. Hoppe Seyler.

Treumer, J. and G. Valet. 1986. Flow-cytometric determination of glutathione alterations in vital cells by o-phthaldialdehyde (OPT) staining. Exper. Cell Res. 163:518-524.

Valet, G., L. Raffael, E. Moroder, E. Wuensch, G. Ruhenstroth-Bauer. 1981. Fast intracellular pH determination in single cells by flow cytometry. Naturwissenschaften. 68:265-266.

Valet, G., M. Valet, D. Tschope, G. Rothe, W. Kellermann, H. Kahle. 1992. White cell and thrombocyte disorders: standardized, self learning flow cytometric list mode data classification with the Classif1 program system. Ann. N.Y. Acad. Sci.

A Note on Losses of *Prochlorococcus* Cells Due to Preservation

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Flow cytometry is currently the only method reported in the literature for quantifying *Prochlorococcus* populations from natural samples. These cells were first identified and isolated by automated flow cytometry and cell sorting of fresh samples aboard ship (Chisholm, et al. 1988, Olson, et al. 1990). Vaulot, et al. (1989) first reported a method to preserve samples for flow cytometric analysis using 1% glutaraldehyde followed by rapid freezing in liquid nitrogen. A good method for preserving these and other picoplankton cells is critical since it relieves the necessity of shipboard flow cytometry. We report here a review of cell loss using this method from papers in the literature and our own results. We would like to encourage further discussion of this issue and especially solicit comments/results from others who may have experiences with preservation methods for *Prochlorococcus* or other picoplankton for flow cytometric analysis.

Methods

Natural populations of *Prochlorococcus* were analyzed from a vertical set of samples from the Sargasso Sea collected in November 1992 near the Bermuda time series station. Replicate samples were analyzed fresh and preserved by method #2 below. The preserved samples were analyzed in the lab after 32 days of storage.

We fixed 5 replicates from a culture of *Prochlorococcus* using the following three methods:

1. 0.5% glutaraldehyde, liquid N_2, stored in liq. N_2.
2. 0.5% glut., liq. N_2, stored in a freezer (-20°C).
3. 0.5% glut., stored in a refrigerator (4°C).

All samples were analyzed on a Becton-Dickinson FACScan instrument fresh, immediately after glutaraldehyde, and then after 7 days of storage. Cell loss was calculated as the difference from the fresh samples. Counts were determined by adding a known concentration of standard microbeads to each sample before analysis. The mean cell loss due to glutaraldehyde alone (no freezing) was 15%.

Results and Discussion

Preservation efficiency of natural *Prochlorococcus* populations from the two reports in the literature averaged 71 and 81% (Table 1). Our own attempts yielded only a 46% preservation efficiency. Our method differed from the published method in 2 respects: 1) we used a lower glutaraldehyde concentration (0.5 vs. 1%) and 2) after freezing the samples in N_2, we stored them in the freezer rather than in liquid N_2. Follow-up studies on cultured *Prochlorococcus* yielded cell losses of 20 and 26% for samples stored in the freezer and liquid N_2, respectively. This suggests that the storage temperature may affect cell loss less than the preservation itself, at least for a storage period of 1 week.

Preservation and storage increased forward light scatter by 2 to 3 times but did not significantly change side scatter or chlorophyll fluorescence (data not shown).

The differences in preservation efficiency may be due to minor differences in technique or could represent variable sensitivity of the natural populations to preservation. It could be argued that cultured cells might be more hardy than natural populations and could represent the "best case" for preservation. If this is so, then 80% may represent the highest preservation efficiency attainable by this method, and a better preservation method is needed to yield accurate measurements of these cells in nature.

Table 1. Comparison of cell loss due to preservation of *Prochlorococcus* from natural samples (Sargasso Sea verticals) and from a culture.

	Sample	% Glut	Method	N samples	N reps per sample	Mean loss, % of cells
Vaulot et al. 1989	Sargasso Sea vertical	1	N_2, stored in N_2	6	1	19
Olson et al. 1990	Sargasso Sea vertical	1	N_2, stored in N_2	10	1	29
Sieracki & Cucci (unpubl.)	Sargasso Sea vertical	0.5	N_2, stored 32d in freezer	6	1	54
Sieracki & Cucci (unpubl.)	culture	0.5	N_2, stored in N_2	1	5	26
Sieracki & Cucci (unpubl.)	culture	0.5	N_2, stored in freezer	1	5	20
Sieracki & Cucci (unpubl.)	culture	0.5	refrigerated only	1	5	36

Literature Cited

Chisholm, S. W., R. J. Olson, E. R. Zettler, R. Goericke, J. B. Waterbury and N. A. Welschmeyer. 1988. A novel free-living prochlorophyte abundant in the oceanic euphotic zone. Nature 334:340-343.

Olson, R., S. W. Chisholm, E. R. Zettler, M. A. Altabet and J. A. Dusenberry. 1990. Spatial and temporal distributions of prochlorophyte picoplankton in the North Atlantic Ocean. Deep-Sea Research 37:1033-1051.

Vaulot, D., C. Courties and F. Partensky. 1989. A simple method to preserve oceanic phytoplankton for flow cytometric analyses. Cytometry 10:629-635.