LIMNOLOGY
and
OCEANOGRAPHY: METHODS

Limnol. Oceanogr.: Methods 6, 2008, 653–658

Lighting up phytoplankton cells with quantum dots

Karen M. Orcutt[1], Kjell Gundersen[1], Mark L. Wells[2], Nicole J. Poulton[3], Michael E. Sieracki[3], and G. Jason Smith[4]*
[1]University of Southern Mississippi, Department of Marine Science, Stennis Space Center, MS, USA
[2]University of Maine, School of Marine Sciences, Orono, ME, USA
[3]Bigelow Laboratory for Ocean Sciences, West Boothbay Harbor, ME, USA
[4]Moss Landing Marine Laboratories, Moss Landing, CA, USA

Abstract

Quantum dots (Qdots) are semiconducting nanocrystals composed of periodic elements with different intrinsic band-gap energies that yield unique fluorescent signatures. Unlike conventional organic fluorophores, Qdots are photo-chemically stable and have a wide absorption spectrum, but a narrow, tunable emission spectrum. Multiple colors can be imaged from a single excitation wavelength allowing for labeling of many different target sites (e.g., membrane proteins) simultaneously.

We conjugated Qdots to primary antibodies specific for the soluble enzyme nitrate reductase (NR) and a light-harvesting structural protein localized in the chloroplast, the fucoxanthin chlorophyll *a/c* protein (FCP), in the marine diatom *Skeletonema costatum.* By fluorescence microscopy, we successfully detected NR and FCP in single cells of *S. costatum* with a clarity and definition that was not obtainable with conventional organic fluorophores. Biotinylated cells labeled with Qdot-strepavidin conjugate and Qdot-FCP immuno-labeled cells were detected by flow cytometry. Qdot bioconjugates provide an alternative photostable probe for surface or intracellular protein immuno-localization in the study of marine bacteria and phytoplankton metabolism and physiology.

Introduction

Fluorescent probes are proven tools to identify intracellular and cell surface molecules that often are visualized by immunodetection coupled with a fluorescent signal. Fluorescent probes allow the assessment of changes in relative abundance of both cell surface and intracellular antigens under specific environmental conditions (Orellana and Perry 1992, 1995, Jochem et al. 2000, Kloepfer et al. 2003). As a consequence of this utility and sensitivity, fluorescence-based immunodection by flow cytometry has become a powerful tool in phytoplankton ecology (Peperzak et al. 2000). However, molecular and photochemical instabilities of organic fluorophores and their tendency for photobleaching under prolonged exposure both contribute to inefficiencies in detection of low abundance analytes. Moreover, the wide emission bands of organic fluorophores severely restricts the application of simultaneous labeling of different cellular components of interest. Overcoming these limitations would greatly increase the functionality of cellular probes in marine applications. Recently, novel and highly fluorescent inorganic particles (quantum dots) were introduced in medical and cell biology to counter these problems. However, the use of this nanotechnology for immuno-detection in pigment-rich phytoplankton cells remains untested.

Quantum dots (Qdots) are semiconducting nanocrystals composed of periodic groups of period II-VI, III-V, or IV-VI elements. Commonly used Qdots in biological and medical applications are comprised of a CdSe/ZnS (core/shell) crystal that form stable aqueous suspensions and are biocompatible (Medintz et al. 2005, Dubertret et al. 2002, Jaiswal et al. 2004). The unique optical properties of Qdots may provide new probes for cell imaging and flow cytometry. Unlike organic fluorophores, Qdots exhibit broad excitation absorption spectra, narrow emission spectra, and are resistant to photobleaching. In contrast, organic fluorophores characteristically have narrow excitation bands and broad red-tailing emission bands that limit their effectiveness in multiple color imaging as well as being prone to photobleaching. This broad emission also can be a significant challenge with phytoplankton due to the high natural autofluorescence of cellular pigments. Qdots have the potential to overcome many of these limitations due to their unique properties. CdSe/ZnS Qdots exhibit a size-dependant tunable bright photoluminescence that spans the visible spectrum. As a consequence, Qdots provide an ideal alternative for studies that require long-term and multicolor

*Corresponding author: E-mail: Karen.orcutt@usm.edu

Acknowledgments

This work was supported by a Small Grant for Exploratory Research (SGER) from NSF (grant no. 0530568) to KMO, KG, MLW, and MES.

imaging of cells such as detection of low abundance cellular antigens by fluorescence microscopy.

The use of Qdots for cellular detection has developed significantly in the biological sciences (Bruchez et al. 1998, Alivisatos 2004, Michelet et al. 2005, Medintz et al. 2005). Biological conjugates of Qdot nanocrystals have been used to label bacterial cells (Kloepfer et al. 2003) and Qdot-conjugated antibodies have been used to label the parasitic *Giardia* and *Cryptosporidium* (Zhu et al. 2004). More recently, Qdot bioconjugates have been applied in studies using plant cells (Ravindran et al. 2005, Müller et al. 2006). Although Alvisatos (2004) speculated that Qdot applications in flow cytometry might be limited because of their radiative rate (emission lifetimes of tens of nanoseconds), Gao and Nie (2004) used quantum dot–encoded mesoporous beads and obtained flow cytometry count rates up to 1000 beads/s. They concluded that the multiple excited state lifetimes and relaxation pathways of Qdots do not limit their detection in flow cytometry. Qdots have increased the fluorochromes available for flow cytometry (Bruchez 2003) enabling expansion of flow cytometric detection capabilities beyond the prior 12 color system by increasing the color capability of the violet laser (407 nm) from 2 to 8 parameters (Perfetto et al. 2004). However, the potential of Qdots has yet to be applied and tested in environmental science in general, and biological oceanography in particular.

In this paper, we describe the first application of Qdots to study marine phytoplankton. We conjugated Qdots to primary antibodies generated against soluble nitrate reductase (NR) from the diatom *Skeletonema costatum* (Gao et al. 1993) and to the fucoxanthin chl *a/c* protein (FCP) family from the diatom *Phaedactylum tricornutum* (Friedman and Alberte 1984) in the marine diatom *S. costatum*. By using Qdot bioconjugates, we detected NR and FCP by fluorescent microscopy in single cells of *S. costatum.* We also demonstrate compatibility of Qdots for flow cytometric analysis of phytoplankton (*S. costatum)* by the detection of both a Qdot-strepavidin conjugate of surface biotinylated cells and cells labeled internally with a Qdot-FCP bioconjugate.

Methods

Qdot bioconjugation—Qdots (Invitrogen) were conjugated to enriched primary antibody fractions using a thiol-maleimide coupling described in the manufacturer's antibody conjugation kit protocol. The IgG primary antibodies were purified using a Melon Gel IgG purification kit (Pierce), and the antibody concentration determined using the Pierce Bicinchoninic Acid (BCA) protein assay. Briefly, the Qdots are activated by incubation in (maleimidomethyl)-1-cyclohexanecarboxylic acid N-hydroxysuccinimide ester (SMCC) at room temperature for 1 h. Excess SMCC was then removed by size exclusion chromatography. IgGs were purified from immune sera and dispersed in 300 μL phosphate buffered saline (PBS) (10 mM phosphate, 138 mM NaCl, 2.7 mM KCl, pH 7.4) at a concentration of 1 mg mL^{-1}. The antibody was reduced by adding 6.1 μL of 1M Dithiothreitol (DTT) at room temperature for 30 min, after which the excess DTT was removed by size exclusion chromatography. The activated Qdots were covalently coupled to the reduced antibody for 1 h at room temperature, and the conjugation reaction quenched by adding β-mercaptoethanol to a final concentration of 100 μM for 30 min. The Qdot conjugate was purified and concentrated as described in the protocol provided (Invitrogen). Sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE) was used to qualitatively assess the labeling of IgG batches by visualization of the unfixed gels on a long-wave UV (366 nm) transilluminator.

Qdot immuno-labeling of whole cells and protein extracts—The Qdot bioconjugates were tested for immunodetection of proteins in whole cells and protein extracts. Intact phytoplankton cells were concentrated by centrifugation at 15°C (10 min, 2000 *g*) and stored at –20°C in 95% ethanol for a minimum of 24 h. Prior to staining, the cells were reconcentrated by centrifugation and rinsed in 1 mL of 3% bovine serum albumin (BSA) diluted in phosphate buffer saline (100 mM PBS 32, pH 6.8). The cells were again concentrated by centrifugation and blocked with 1 mL of 3% BSA-PBS 32 for 1 h at 4°C. Dimethyl sulphoxide (DMSO, 2% final conc.) was added to the blocking solution containing the cells and allowed to react (0.5 h) at 4°C. The DMSO treatment was terminated by centrifugation and 2 rinses (with intermittent concentration by centrifugation) of the cells in 1 mL BSA-PBS 32. Finally, the cells were concentrated by centrifugation and stained with the antibody conjugate (Qdot565-NR or Qdot565-FCP or Qdot605-FCP diluted 1:100 in a 3% BSA solution) for 1 h at 20°C. Staining was terminated by concentrating the cells by centrifugation, followed by 2 rinses with Tween-20 (0.05% diluted in PBS 32). The stained cells were dispensed in 1 mL of PBS 32, treated with fresh phenylmethylsulfonlyfluoride for 10 minutes (PMSF, 1 μM final conc.), and preserved in paraformaldehyde for 30 min (PFA, 1 % final conc.) at 20°C. The preserved cells were stored at 4°C until microscope or flow cytometer analysis (usually within 1-2 d).

Protein extracts were subjected to SDS-PAGE and immunodetection was performed directly by an in-gel technique (Odyssey, LI-COR Biosciences) using one of the primary Qdot bioconjugates (Qdot605-FCP). Qdot immunolabeled *S. costatum* cells were detected by flow cytometry as described below and by fluorescent microscopy using an Olympus BX51 fluorescent microscope equipped with a DP71 12-bit color digital camera and software.

We note that the Qdot products should never be frozen and care must be taken when ordering these products in winter regions with outside temperatures below freezing. It is recommended by the manufacturer that the final Qdot conjugate is stored at 4°C, and this limits the shelf life after synthesis to approximately 6 mo. We attempted to conjugate IgY antibodies to Qdots without success, and it is also pointed out by the

manufacturer that the conjugation kit has not been tested for these antibodies. The IgY we used had a very low antibody titer. The supplier recommends using IgG antibodies for their Qdot conjugation kit.

Qdot detection by flow cytometry—Live *S. costatum* cells were biotinylated using FluoReporter Cell-Surface Biotinylation Kit (Invitrogen). Strepavidin conjugated Qdot565 (Invitrogen) were applied to visualize the biotinylation as described in the manual provided by the manufacturer. Live Qdot labeled *S. costatum* cells were then detected by flow cytometry as described below.

Samples were analyzed using a dual-beam Dako Cytomation MoFlo flow cytometer equipped with a 100-mW 488-nm air-cooled argon-ion laser and a 90-mW 365-nm water-cooled laser. Natural red fluorescence emissions (indicative of chlorophyll) were collected using a 660 long pass (LP) filter. All natural fluorescent pigments were excited using the 488-nm laser. A UV (365 nm) excitation source was used to determine the presence of 2 types of Qdots (Qdot565, Qdot605). The emissions from the UV lasers were split using a 585 dichroic mirror (Chroma Technology Co.). The emissions from the Qdot565 and the Qdot605 conjugates were measured using a 565/20 bp filter and a 605/20 bp filter (Chroma Technology Co.), respectively. Side scatter (SSC) was used to monitor presence of all particles/organisms within each sample (approximating a size range of 0.5 to 50 □m). Flow cytometric measurements were collected in logarithmic mode and analyzed using Summit software (DakoCytomation). The volume of the sample run on the flow cytometer was determined gravimetrically. Regions of interest (ROI) were used to define the presence of different Qdot labeled populations based on flow cytometric signatures of preliminary sample runs. Numerical abundance of these populations were determined by the ROI-specific particle count and the total sample volume analyzed.

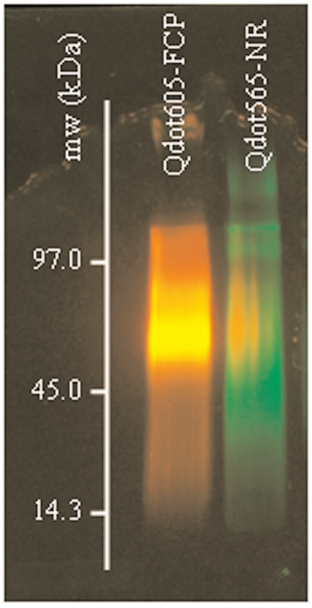

Fig. 1. Detection of Qdot bioconjugates (Qdot605-FCP and Qdot565-NR) on SDS-PAGE.

Assessment

Qdots were reliably conjugated to primary fractions enriched from crude rabbit immune sera raised against the protein antigens FCP and NR. These antibodies were previously isolated from the marine diatoms *P. tricornutum* and *S. costatum* (Freidman **Q2** and Alberte 1984, Gao et al. 1993). The Qdot bioconjugates were verified by SDS-PAGE, which exhibited major product bands near the predicted molecular weight of ~50 kDa expected for IgG peptides (Fig. 1). The associated smearing in fluorescence signal was attributed to multiple labeling events and influence of Qdot size/mass on migration rate through this gel system.

Immunofluorescence assays of *S. costatum* cells preserved from cultures grown in nitrate as the only N-source showed Qdot565-NR labeling predominantly in the cytoplasm closely aligned to the cell frustules. In contrast, Qdot565-FCP labeling was more intense and confined to the cells chloroplast consistent with its known cellular localization and abundance (Fig. 2A,C). Compared with traditional indirect immunodetection methodology,

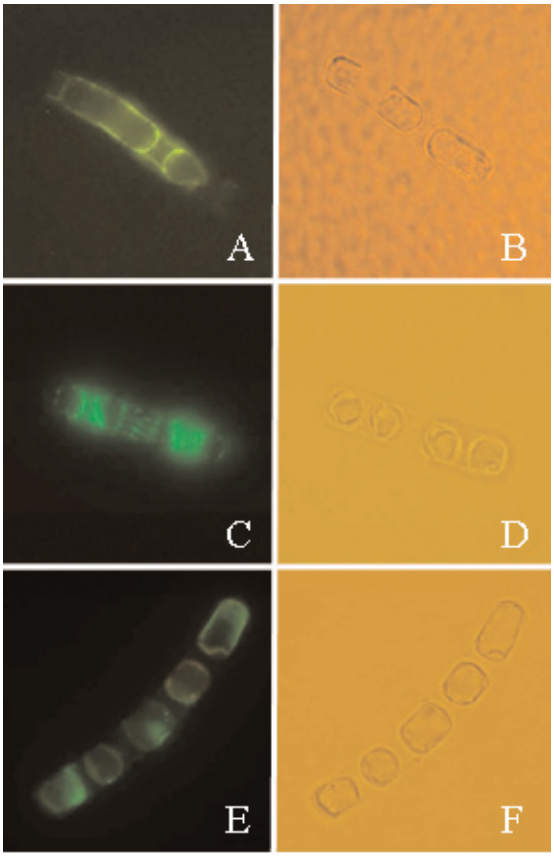

Fig. 2. Epifluorescence (A, C, E) and light microscope images (B, D, F) of *S. costatum* stained with Qdot565-NR conjugate (A, B), Qdot565-FCP conjugate (C, D), and a combination of FCP (primary) and FITC (secondary) antibodies (E, F).

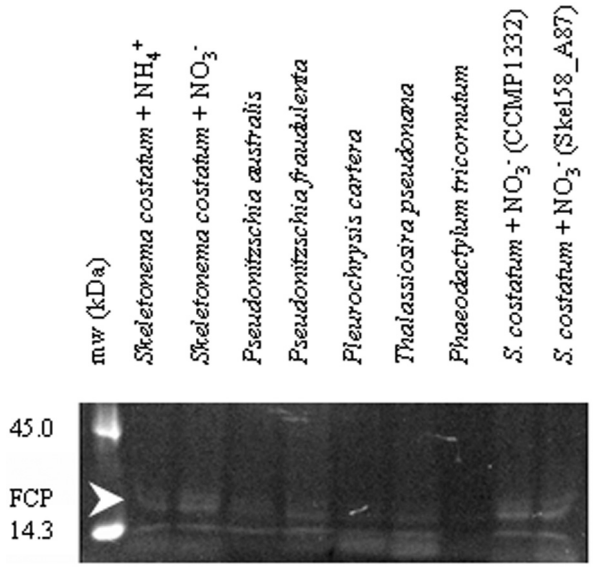

Fig. 3. Phytoplankton monocultures, solubilized, and subjected to 10% SDS-PAGE. The gel was stained with the Qdot565-FCP conjugate (titer 1:500), and the region of the two FCP sub-units (white arrow) is shown. The pigment front is visible at the 14.3 kDa mark.

using a FITC-labeled goat-antirabbit secondary antibody (Fig. 2E), the direct labeling of the primary antibody with Qdot reporters yielded a stronger signal, longer lasting, and more defined localization of the FCP antigen in these cells (Figs. 2C and 2E). The efficacy of direct labeling with Qdot probes was also demonstrated by immunofluorescent detection of FCP directly on SDS-PAGE gels which cross reacted with diatoms *Pseudonitzchia australis*, *Pseudonitzchia fraudulenta*, *Pleurochrysis cartera*, *Thalasiosira psuedonana*, and *Phaeodactylum tricornutum* (Fig. 3).

S. costatum cells were detected by flow cytometry using a Qdot565-strepavidin conjugate that was bound to live biotinylated *S. costatum* cells (Fig. 4). The Qdot labeling and detection efficiency was nearly 100% with this exterior label. Cells of *S. costatum* immunolabeled with Qdot605-FCP were also detected by flow cytometry and could be seen as an increase in the 605 emission (Fig. 5). The internal protein target FCP labeling efficiency was calculated to be 62% of all cells of *S. costatum*. Detection of FCP-labeled cells by flow cytometry was more successful than with NR-labeled cells, most likely due to higher protein abundance of FCP. The NR-labeled cells showed some detection by flow cytometry but did not produce the robust signal seen with FCP-labeled cells. Initially, we used flow cytometer filters with a broader emission spectrum but these readings were marred by background pigment inference. Background fluorescence was greatly reduced by using filters with a specific wavelength (565 and 605 nm) and a narrow band pass (20 nm).

Discussion

Qdots provide a unique fluorescent reporter technology for the study of phytoplankton ecology and physiology. Our

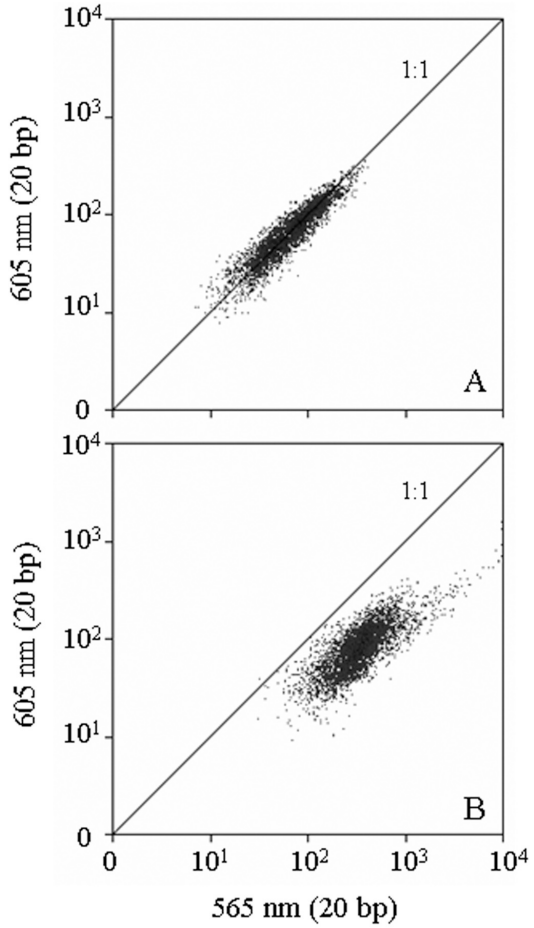

Fig. 4. Single cells, chains, and clusters of *S. costatum* without biotinylation in the presence of Qdot565-streptavidin conjugate (A) and biotinylated cells stained with a Qdot565-streptavidin conjugate (B). The diatom cells were detected using emission filters at 565 and 605 nm wavelength (20 nm band pass).

results demonstrate that direct labeling of primary antibodies to diverse intracellular targets not only enables clear localization of antigen distributions, but also provides enhanced signal detection over the more laborious and costly use of fluorescent secondary antibodies for signal amplification. While ideally suited for epifluorescence microscopy assays of phytoplankton and other marine microbes, the application of Qdot technologies in flow cytometric assays of these organisms is less straightforward, but our results demonstrate that the fluorophore is tractable. One possibility for the problems with detection in flow cytometers is that Qdots have a relatively slow radiative rate compared to organic fluorophores (Alivisatos 2004), and this may place constraints on flow stream rate to generation of robust fluorescent signals. This signal limitation will impair detection of low abundance targets such as NR, which was reliably detected by flow cytometry of traditional FITC-secondary antibody labeled cultures (Jochem et al. 2000). Our experiments also indicate that cell surface antigens are more readily labeled than intracellular targets by

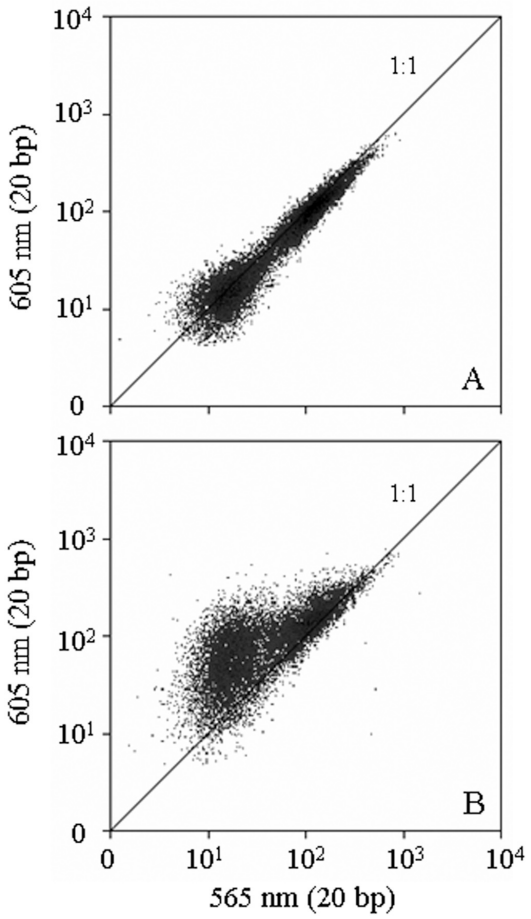

Fig. 5. Natural fluorescence of *S. costatum* (A) and cells stained with Qdot605-FCP conjugate (B). The diatom cells were detected using emission filters at 565 and 605 nm wavelength (20 nm band pass).

Qdot conjugates, possibly indicating permeabilization-based diffusion limitation of the quantum moieties. The assessment of cell surface proteins may be better than intracellular proteins in flow cytometry due to problems and interference from intracellular pigments and the overall quantity of the protein within a cell. Nonetheless, the application of Qdot bioconjugates in flow cytometry is a promising technique when used with surface cellular proteins in high abundance. The flow cytometric results obtained using Qdot-strepavidin biotinylated cells show a clear increase in the signal-to-noise ratio supporting this hypothesis (Fig. 4). Qdot-strepavidin biotinylated cells were clearly detected by flow cytometry and demonstrate that radiative rate or, cycling from excited state to ground level state, is not a major limitation in flow cytometric detection of high abundance cell surface proteins.

In summary, we have successfully conjugated Qdots to primary antibodies and applied these probes for cellular detection using epifluorescence microscopy and flow cytometry. Qdot bioconjugates make ideal probes for surface or intracellular protein immuno-localization using epifluorescence microscopy because they do not photobleach. Qdot bioconjugate probes show a great potential for both long-term labeling as well as in simultaneous use of multiple probes in marine microplankton studies of metabolism and physiology. However, more work is needed in labeling efficiencies of intracellular targets, particularly low abundance proteins, before enhanced signal detection by flow cytometry can be realized as anticipated by Peperzak et al. (2000).

References

Alivisatos, P. 2004. The use of nanocrystals in biological detection. Nature Biotechnol. 22:47-52.

Bruchez Jr, M., M. Moronne, P. Ginn, S. Weiss, and A. P. Alivisatos. 1998. Semiconductor nanocrystals as fluorescent biological labels. Science 281:2013-2015.

———. 2003. Qdot™ 655 streptavidin detection in flow cytometry. Quantum Dot Vision, Technical Note, Qdot Corporation.

Dubertret, B., P. Skourides, D.J. Norris, V. Noireaux, A. H. Brivanlou, and A. Libchaber. 2002. In vivo imaging of quantum dots encapsulated in phospholipids micelles. Science 298:1759-1762.

Friedman, A. L., and R. S. Alberte. 1984. A diatom light-harvesting pigment-protein complex. Plant Physiol. 76:483-489.

Gao, X., and S. Nie. 2004. Quantum dot-encoded mesoporous beads with high brightness and uniformity: rapid readout using flow cytometry. Anal. Chem. 76:2406-2410.

Gao, Y., G. J. Smith, and R. S. Alberte. 1993. Nitrate reductase from the marine diatom Skeletonema costatum. Plant Physiol. 103:1437-1445.

Jaiswal, J. K, E. R. Goldman, H. Matoussi, and S. M. Simon. 2004. Use of quantum dots for live cell imaging. Nature Methods 1:73-78.

Jochem, F. J., G. J. Smith, Y. Gao, R. C. Zimmerman, A. Cabello-Pasini, D. G. Kohrs, and R. S. Alberte. 2000. Cytometric quantification of nitrate reductase by immunolabeling in the marine diatom *Skeletonema costatum*. Cytometry 39:173-178.

Kloepfer, J. A., R. E. Mielke, M. S. Wong, K. H. Nealson, G. Stucky, and J. L. Nadeau. 2003. Quantum dots as strain- and metabolic-specific microbial labels. Appl. Environ. Microbiol. 69:4205-4213.

Medintz, I. L., H. T. Uyeda, E. R. Goldman, and H. Mattoussi. 2005. Quantum dot bioconjugates for imaging, labeling and sensing. Nature Materials 4:438-446.

Michelet, X., and others. 2005. Quantum dots for live cells, in vivo imaging, and diagnostics. Science 307:538-544.

Müller, F., A. Houben, P. E. Barker, Y. Xiao, J. A. Käs, and M. Melzer. 2006. Quantum dots – a versatile tool in plant science? J. Nanotechnol. 4:1-5.

Orellana, M. V., and M. J. Perry. 1992. An immunoprobe to measure Rubisco concentrations and maximal photosynthetic rates of individual phytoplankton cells. Limnol. Oceanogr. 37:478-490.

——— and ———. 1995. Optimization of an immunofluorescent assay of the internal enzyme ribulose-1,5-bisphosphate carboxylse (Rubisco) in single phytoplankton cells. J. Phycol. 31:785-794.

Peperzak, L., E. G. Vrieling, B. Sandee, and T. Rutten. 2000. Immuno flow cytometry in marine phytoplankton research. Scientia Marina 64:165-181.

Perfetto, S. P., P. K. Chattopadhyay, and M. Roederer. 2004. Seventeen-colour flow cytometry: unraveling the immune system. Nature 4:648-655.

Ravindran, S., S. Kim, R. Martin, E. M. Lord, and C. S. Ozkan. 2005. Quantum dots as bio-labels for the localization of a small plant adhesion protein. Nanotechnology 16:1-4.

Zhu, L., S. Ang, and W.-T. Liu. 2004. Quantum dots as a novel immunofluorescent detection system for *Cryptosporidium parvum* and *Giardia lamblia*. Appl. Environ. Microbiol. 70:597-598.

Submitted 8 July 2008
Revised 1 October 2008
Accepted 27 October 2008