Journal of Microbiological Methods 124 (2016) 21–27

Contents lists available at ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

Phytoplankton IF-FISH: Species-specific labeling of cellular proteins by immunofluorescence (IF) with simultaneous species identification by fluorescence immunohybridization (FISH)

Megan E. Meek [a,b,*], Frances M. Van Dolah [a,b]

[a] Graduate Program in Marine Biology, Grice Marine Laboratory, College of Charleston, 205 Fort Johnson Rd., Charleston, SC 29412, United States
[b] NOAA Marine Biotoxins Program, Center for Coastal Environmental Health and Biomolecular Research, 219 Fort Johnson Rd., Charleston, SC 29412, United States

ARTICLE INFO

Article history:
Received 22 December 2015
Received in revised form 28 February 2016
Accepted 29 February 2016
Available online 4 March 2016

Keywords:
Immunofluorescence
In situ hybridization
Dinoflagellate
Karenia brevis
Phytoplankton
Flow cytometry

ABSTRACT

Phytoplankton rarely occur as unialgal populations. Therefore, to study species-specific protein expression, indicative of physiological status in natural populations, methods are needed that will both assay for a protein of interest and identify the species expressing it. Here we describe a protocol for IF-FISH, a dual labeling procedure using immunofluorescence (IF) labeling of a protein of interest followed by fluorescence in situ hybridization (FISH) to identify the species expressing that protein. The protocol was developed to monitor expression of the cell cycle marker proliferating cell nuclear antigen (PCNA) in the red tide dinoflagellate, *Karenia brevis*, using a large subunit (LSU) rRNA probe to identify *K. brevis* in a mixed population of morphologically similar *Karenia* species. We present this protocol as proof of concept that IF-FISH can be successfully applied to phytoplankton cells. This method is widely applicable for the analysis of single-cell protein expression of any protein of interest within phytoplankton communities.

1. Introduction

Phytoplankton play important roles as primary producers in marine and freshwater ecosystems, accounting for >50% of primary production worldwide. Yet some phytoplankton species disrupt ecosystem processes — either by producing toxins that sicken or kill other organisms (Landsburg, 2002; Van Dolah, 2005; Anderson et al., 2012) or by depleting dissolved oxygen in the water column (Sunda et al., 2006; Paerl et al., 2007). Understanding the processes that regulate the physiology, growth, and succession of phytoplankton populations has therefore received much interest. Although many physiological processes can be studied in laboratory cultures of phytoplankton, monitoring them in the field can be challenging because phytoplankton rarely exist as unialgal populations. Methods to selectively monitor the activity of a particular species within a mixed population are needed to study algal physiology in situ. Here we describe a flow cytometry method to identify algal cells dually labeled for specific protein expression using immunofluorescence (IF) and species using fluorescence in situ hybridization (FISH). Although developed for a cell cycle marker, proliferating cell nuclear antigen (PCNA), in the dinoflagellate *Karenia brevis*, the method is broadly applicable to other proteins and phytoplankton species of interest.

Karenia brevis is a neurotoxin-producing dinoflagellate which forms extensive blooms, or red tides, in the Gulf of Mexico that are responsible for fish kills, marine mammal mortalities, and human respiratory distress. Current bloom monitoring and forecasting practices rely on cell abundance measurements, chlorophyll fluorescence measurements obtained from satellites and in-water optical remote sensors, and hydrodynamic models of winds and currents (Heil and Steidinger, 2009). Improved insight into the cell composition, physiology and growth rates within bloom patches would further enhance our understanding of bloom dynamics. Gulf of Mexico red tides often include other species of *Karenia* besides *K. brevis*, including *Karenia mikimotoi*, *Karenia papilionacea*, and *Karenia selliformis*, and little is known about the interaction or succession among these species (Steidinger et al., 2008; Wolney et al., 2015). All have similar morphology, and can therefore be hard to distinguish by microscopy alone. Therefore, efforts have been made to develop molecular methods to assist in distinguishing these species in the field.

Two current molecular methods for differentiating *Karenia* species use the hypervariable regions of large subunit (LSU) ribosomal RNA (rRNA) genes. The first method, a sandwich hybridization assay, developed for all four species targeting the D1–D2 domains of this gene, requires cell lysis but not purification of nucleic acids (Haywood et al., 2007). The second method leaves cells intact as a fluorescent oligonucleotide probe is

Abbreviations: PCNA, proliferating cell nuclear antigen; IF, immunofluorescence; FISH, fluorescent in situ hybridization; LSU, large subunit; rRNA, ribosomal RNA; PFA, paraformaldehyde; D-PBS, DEPC-treated PBS; D-PBST, DEPC-treated PBS 0.5% Tween 20; FMO, fluorescence minus one.

* Corresponding author.
E-mail address: meekme@g.cofc.edu (M.E. Meek).

http://dx.doi.org/10.1016/j.mimet.2016.02.017
0167-7012/

hybridized to the D3 domain in situ (FISH). Cell abundance is quantified by analysis of fluorescence using microscopy or flow cytometry (Mikulski et al., 2005). Both of these methods have been tested in the field and showed promising results for distinguishing *Karenia* species (Haywood et al., 2007; Mikulski et al., 2005).

In addition to knowing what species are present in a bloom patch, information on the growth rates within that bloom would be useful for forecasting its growth or demise. Within a bloom, cellular growth occurs by vegetative cell division, which follows the typical eukaryotic cell cycle with characteristic G1, S, G2 and mitotic phases. In *Karenia*, progression through the cell cycle is regulated by a circadian rhythm, such that the different cell cycle phases occur at predictable times of the day. Taking advantage of this, flow cytometric cell cycle analysis has been successfully used to measure in situ growth rates of *K. brevis* blooms (Van Dolah et al., 2008); however, this method requires sampling from the same bloom patch repeatedly in a 24 h period. An alternative approach is to calculate growth by measuring 14C uptake over 24 h; however, this method is also not species-specific and cannot be done without a large research vessel. A cell cycle marker combined with species-specific probes would therefore be useful in determining species-specific bloom growth rates.

PCNA is a cell cycle dependent protein that is critical to DNA replication in S-phase. PCNA was first detected in marine phytoplankton by Lin et al. (1994). Subsequent studies have tested its use as a growth rate marker in the green microalgae *Dunaliella tertiolecta* and *Dunaliella salina*, the coccolithophorid microalga *Pleurochrysis cartarae*, the diatom *Ethmodiscus rex*, and the dinoflagellates *Alexandrium catenella* and *Prorocentrum donghaiense*, all with promising results (Lin et al., 1995; Lin and Carpenter, 1995; Lin and Corstjens, 2002; Liu et al., 2005; Huang et al., 2010). PCNA was characterized in *K. brevis* by Brunelle and Van Dolah (2011), and was shown by immunolocalization to translocate to the nucleus from the cytosol during S-phase and, by Western blotting, to concurrently increase in abundance and undergo an increase in molecular weight. The relationship between S-phase of the cell cycle and PCNA makes it a potential candidate as a marker of growth in *Karenia* species.

The goal of this research was to develop a species-specific assay for PCNA expression by combining FISH, using rRNA gene targeting oligonucleotide probes, and intracellular staining of PCNA in a flow cytometry format. Each technique was first optimized separately, and then combined using the guidance of recent studies that successfully combined RNA and protein detection in *Drosophila* (Toledano et al., 2012; Zimmerman et al., 2013). This dual labeling technique has not to our knowledge previously been applied to phytoplankton, and would be broadly applicable to monitoring protein expression in mixed populations to provide insight into mechanisms regulating algal bloom physiology.

2. Methods

2.1. Cultures and culturing conditions

Cultures of *K. brevis* (NOAA-1, isolated by Steve Morton, from the Florida Gulf Coast), *K. mikimotoi* (NOAA-2, isolated by Steve Morton from the Florida east coast), *K. selliformis* (CAWD79, isolated by Lincoln MacKenzie from Fouveaux Strait, N.Z.), and *K. papilionacea* (Kpap PA, isolated by Carmelo Tomas from Port Aransas, Texas) were used in this study. All cultures were cultivated individually in sterile filtered seawater from the seawater system at the Florida Institute of Technology field station, Vero Beach, Florida, at 36‰ salinity. The seawater was enriched with f/2 media (Guillard, 1975) modified with ferric sequestrene in the place of ethylenediaminetetraacetic acid (EDTA)·Na_2 and $FeCl_3 \cdot 6H_2O$ and the addition of 0.01 μM selenous acid. Cultures were maintained in 1-l bottles or 250 ml flasks at 22 °C ± 1 °C with a 16:8 light:dark photoperiod with light provided by cool white light bulbs at 60–70 μmol photons m^{-2} s^{-1}, as measured by a LI-COR LI-250 Quantum light meter (Lincoln, NE). All species were acclimated to 22 °C over 3 serial transfers performed at late log phase of growth.

2.2. Reagents

A PCNA antibody (EZBiolab, Carmel, IN) was designed against a *K. brevis* PCNA peptide sequence (DRIADFDLKLMQIESEH) located in the exposed area of the native folded protein (Brunelle and Van Dolah, 2011). This antibody was shown to cross react with PCNA of the other *Karenia* species (Meek, 2015). All FISH probes used are identified in Table 1: a 5′-fluorescein labeled oligonucleotide probe, Kbprobe-7, specific to the *K. brevis* LSU rRNA D1–D3 regions (Mikulski et al., 2005), a fluorescein labeled universal SSU rRNA probe used as a positive control, and its fluorescein-labeled reverse complement used as negative control.

2.3. Fixation

Samples containing cells from either a single species or two or more species (i.e. *K. brevis* alone or equal volumes of *K. brevis* and *K. mikimotoi*) were harvested in mid-log phase by centrifugation (600 ×*g* for 10 min). Pellets were resuspended in seawater containing 2% paraformaldehyde (PFA; w/v, prepared by heating to approximately 70 °C with stirring; Sigma, St. Louis, MO) for 10 min at room temperature. For each subsequent step, tubes were kept on ice or in a 4 °C refrigerator to prevent rRNA degradation, unless otherwise noted. All subsequent centrifugations were at 4 °C and 1650 ×*g* for 5 min, unless otherwise noted, and the supernatant aspirated.

2.4. Immunofluorescence

Following fixation, the cells were washed in diethyl pyrocarbonate (DEPC)-treated phosphate buffered saline (D-PBS, 137 mM NaCl, 2.7 mM KCl, 1.8 mM KH_2PO_4, 10 mM Na_2HPO_4, pH 7.4; treated with 1% DEPC for 1 h followed by autoclaving) and incubated in ice-cold 100% methanol for 1 h at 4 °C to permeablize them and remove pigments that might interfere with the fluorescence signal of the secondary antibody. Cells were washed in DEPC-treated PBS containing 0.5% Tween 20 (D-PBST) and incubated for 45 min at 4 °C with rotation (Mini Labroller; Labnet International; Edison, NJ) in D-PBST containing 1% UltraPure™ BSA (Life Technologies, Grand Island, NY) as a blocking agent. Samples were then incubated overnight at 4 °C with rotation in D-PBST containing the primary polyclonal rabbit anti-*K. brevis* PCNA antibody (1:600) in the presence of 1 U μl^{-1} RiboGuard™ RNase inhibitor (Epicenter, Madison, WI). Following primary antibody incubation, samples were washed twice in D-PBST with rotation for 5 min, and were incubated on ice for 1 h in the dark with the secondary antibody, goat anti-rabbit PE-Cy5.5 conjugate (Life Technologies) in the presence of 1 U μl^{-1} RiboGuard™ RNAse inhibitor. Samples were washed once in D-PBST and once in D-PBS for 10 min each with rotation.

Table 1
FISH probes used in this study, adapted from Mikulski et al. (2005). Aligned position refers to an alignment of *K. brevis* isolates against *Prorocentrum micans* LSU rRNA (GenBank no. AF260377; Mikulski et al., 2005). The positive probe is a universal SSU rRNA sequence, and the negative probe is the reverse complement of the positive (Miller and Scholin, 2000). Degenerate nucleotides: W = A or T; K = G or T; M = A or C.

Probe	Sequence (5′-3′)	Aligned position
KbProbe-7	GCTGGTGCAGATATCCCAG	877–896
Positive (universal)	GWATTACCGCGGCKGCTG	–
Negative	CAGCMGCCGCGGUAAUWC	–