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

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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.

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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	–

2.5. FISH

After the washes were complete, samples were fixed again in 2% PFA in D-PBS (w/v) for 10 min on ice and centrifuged (4 °C) at 1900 ×*g*. After fixation and a subsequent 5 min wash in D-PBS, cells underwent one transition wash in 50% D-PBS 50% hybridization buffer [5× SET (0.75 M NaCl, 5 mM EDTA, 0.1 M Tris pH 7.8), 0.1% IGEPAL-CA630 (Sigma, St. Louis, MO), 25 μg ml^{-1} poly A (Sigma)] for 5 min, followed by a 5 min wash in 100% hybridization buffer. Cell samples were hybridized with 5 ng ml^{-1} of Kbprobe7 in 100% hybridization buffer at 50 °C in a Hb-1D Hybridise hybridization oven (TECHNE by Bibby Scientific Limited, Staffordshire, UK) for 1.5 h, and centrifuged (1650 ×*g*) at room temperature. In parallel, separate samples from the same cultures were hybridized with 5 ng ml^{-1} of either a universal eukaryote SSU-targeted oligonucleotide probe with a 5′-fluorescein label as a positive control or a 5′-fluorescein labeled oligonucleotide probe, the sequence of which is the reverse complement of the positive control probe sequence, as a negative control (Table 1). The samples were washed with preheated 5X SET buffer for 10 min at 52 °C, centrifuged again at 1650 ×*g* at room temperature, and finally resuspended in D-PBS. Samples were immediately analyzed on the Beckman Coulter Epics XL-MCL flow cytometer.

2.6. Flow cytometry

The FISH probes were analyzed on the FL1 detector (525/50 nm band pass filter set), while the antibody, linked to the PE-Cy5.5 conjugate, was analyzed on the FL4 detector (675/45 nm band pass filter set). A 5% compensation was applied to the FITC detector (FL1-5% FL4) to account for overlap of the PE-Cy5.5 fluorophore signal into the FL1 channel.

Fluorescence minus one (FMO) controls and negative controls were used to set gates around positive and negative cell populations for each parameter. FMO controls include all fluorochromes in a multicolor panel except for one, ensuring that any incidental spread of fluorescence into the other channels is properly identified and accounted for when setting the gates. For the two-color panel used in dual labeling there are two FMO controls, one which omits the PE-Cy5.5 secondary antibody and one that uses the negative control FISH probe. Additionally the negative control removes both signals by using secondary antibody only and negative control FISH probe to assess for residual autofluorescence.

2.7. PCR and sequencing of the D1–D3 LSU rDNA region of K. selliformis

To confirm the specificity of KbProbe-7, the D1–D3 rRNA gene sequences of the four *Karenia* species were analyzed for nucleotide differences at the site of the probe. Of the four species, only *K. selliformis* lacked a published sequence for the D3 region of LSU rRNA. To obtain this sequence, DNA was extracted from triplicate mid-log phase cultures of *K. selliformis* using a modified protocol of Scholin et al. (1994). Briefly, cells were harvested by centrifugation (600 ×*g* for 10 min) and resuspended in nuclease-free water, to which a final concentration of each of the following was added to each sample in this order: 1% SDS, 10 mM EDTA, 10 mM Tris–HCL (pH 7.5), and 0.7 M NaCl. Next, 10% CTAB in 0.7 M NaCl was added, mixed, and the samples were incubated for 10 min at 65 °C (final volume: 450 μl). Sequential extractions with an equal volume of 24:1 chloroform:isoamyl alcohol (CI) and 25:24:1 phenol:chloroform:isoamyl alcohol were performed to purify the DNA, followed by an additional extraction in CI. The purified DNA was precipitated with ice-cold isopropanol overnight at −20 °C, and rinsed with ice-cold 70% ethanol. Finally, DNA was dissolved in Tris-EDTA buffer (TE, pH 7.5) overnight at 4 °C. DNA was quantified using a NanoDrop 2000 spectrophotometer (Thermo Scientific).

PCR reactions were performed on the purified DNA using the D1R and D3Ca primers developed by Scholin et al. (1994) to span the hypervariable regions D1–D3 of LSU rDNA (Lenaers et al., 1989). Reactions consisted of 20 mM Tris–HCl (pH 8.4), 50 mM KCl, 3 mM $MgCl_2$, 2.5 units of Taq DNA polymerase (Life Technologies), 200 μM dNTPs, 0.5 μM of each primer, and 20 ng of DNA template (final volume: 100 μl). Following a 3 min denaturation at 94 °C, 31 cycles of denaturation (45 s at 94 °C), annealing (1 min at 45 °C), and extension (45 s at 72 °C) were run on an iCycler (Bio-Rad, Hercules, CA), followed by a final extension at 72 °C for 7 min. Product sizes were visualized using a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA) DNA 12000 Kit, and quantified with a NanoDrop spectrophotometer. PCR products were prepared for sequencing using the ChargeSwitch®-Pro PCR Clean-Up Kit (Invitrogen). Samples were sent for Sanger sequencing to GE Healthcare SeqWright Genomic Services (Houston, TX) with the internal forward primers D2Ra and modified D3R (Mikulski et al., 2005) and the internal reverse primers D1C and D2C (Scholin et al., 1994) in addition to the PCR primers to ensure sequencing in both directions of the entire amplified fragments. The obtained sequences were aligned using MAFFT Version 7 multiple alignment software (Katoh and Standley, 2013), following which the alignment was imported into BioEdit Sequence Alignment Editor Version 7.0.0 (Hall et al., 1990) in order to obtain a consensus sequence. The resulting sequence for *K. selliformis* regions D1–D3 LSU rRNA gene was aligned using ClustalW (Thompson et al., 1994) in BioEdit to LSU rRNA genes of the other three *Karenia* species for sequence comparisons at the site to which the KbProbe-7 FISH probe was originally designed.

3. Results and discussion

3.1. Dual fluorescence labeling of cellular proteins and rRNA: IF-FISH

The IF-FISH method described in this paper successfully separated *K. brevis* from other closely related species, *K. mikimotoi and K. papilionacea*, enabling the assessment of PCNA fluorescence intensity in *K. brevis* cells within artificially mixed populations. To quantify species-specific PCNA fluorescence, flow cytometry gate positions were first determined based on the "fluorescence minus one" (FMO) samples, in which one fluorescence label is iteratively left out (Fig. 1). In column A, all species, labeled with the negative FISH probe and secondary antibody only, appear in quadrant 3, with low autofluorescence in both FL1 (FITC) and FL3 (Cy5.5) channels. All *Karenia* species cross-reacted with the α-PCNA antibody (column B), so all cells are found in quadrant 1 when labeled with α-PCNA and the negative FISH probe. When incubated with the universal FISH probe (column C), all species were found in quadrant 4 and exhibited approximately 100-fold higher fluorescence intensity than with the negative probe (column A). When incubated with KbProbe-7 and α-PCNA, *K. brevis* cells were found in quadrant 2, whereas *K. mikimotoi* and *K. papilionacea* were found in quadrant 1 (KbProbe-7 negative, α-PCNA positive). *K. selliformis* was not distinguishable from *K. brevis* for reasons discussed below. Additionally, single-species controls were used to validate the position of the PCNA antibody and universal FISH probe fluorescence intensities for each species (not shown).

To further focus the analysis on the species of interest (in this case, *K. brevis*) an alternative gating strategy can be used (Fig. 2). Here *K. brevis* cells, labeled with the KbProbe-7, are separated from *K. mikimotoi and K. papilionacea* based on FITC fluorescence intensity. An amorphous gate was set around the *K. brevis* population (column A) and the fluorescence intensity of α-PCNA staining was then measured on the cells within this gate (column B). The position of the linear gate (D) on the PE Cy5.5 axis (column B) was based on the same FMO controls as seen in Fig. 1. When the same populations were hybridized with the negative FISH probe (column C), all cells, including *K. brevis*, had low intensity fluorescence in the FITC channel (FL1) due to autofluorescence. The absence of cells in the amorphous *K. brevis* gate resulted in no cells to measure the fluorescence of in the Cy5.5 channel 9 (FL4, column D). As in Fig. 1, *K. selliformis* was not distinguishable from *K. brevis*. This gating strategy would allow for species-specific analysis of

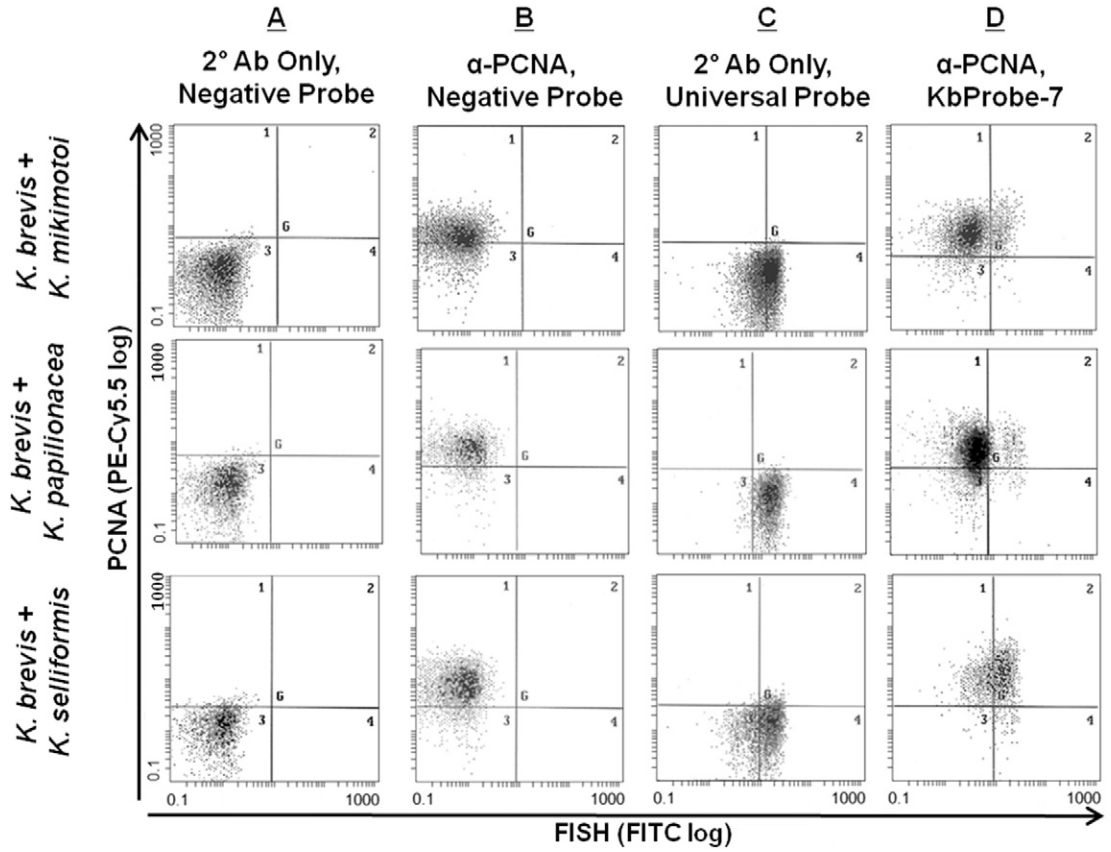

Fig. 1. Dual labeling of rRNA with FISH and PCNA with IF in artificially mixed *Karenia* species populations. In all scatter plots, FISH is represented by FITC fluorescence on the X-axis and PCNA is represented by PE-Cy5.5 fluorescence on the Y-axis. First column, the negative control (secondary antibody only, negative FISH probe). Second column, FMO control with the negative FISH probe and PCNA. Third column, FMO control with the universal FISH probe and secondary antibody only. Control single-species samples of non-targeted *K. mikimotoi* and *K. papilionacea* and targeted *K. brevis* were also analyzed to validate the fluorescence intensity positions of each signal in each species (not shown).

two or more proteins of interest stained with various fluorescent markers measured in different channels on a flow cytometer equipped with multiple lasers.

3.2. Specificity of KbProbe-7

An unexpected result of this study was that *K. selliformis* appeared to positively hybridize with KbProbe-7. As this probe was designed against a sequence in the D3 hypervariable region of the LSU rDNA, and to date *K. selliformis* LSU rDNA has only been sequenced in the D1–D2 domain (GenBank Accession #U92250; Haywood et al., 2004), the D1–D3 domain of *K. selliformis* was sequenced to assess the potential for KbProbe-7 to hybridize in *K. selliformis*.

Amplification of *K. selliformis* DNA with the D1R/D3Ca primer pair yielded a PCR product of the expected size (985 bp). Six sequencing primers were used to obtain overlapping regions within the D1–D3 region, including the D2 region of the LSU rRNA gene. The top hits from BLASTn analysis of a consensus sequence created from these amplicons were all LSU rRNA gene sequences of various *Karenia* species. The D1–D2 region had 100% identity to the existing *K. selliformis* D1–D2 domain LSU rRNA gene sequence (U92250.1) on Genbank. To compare the *K. selliformis* D3 sequence with the KbProbe7, an alignment of the reverse complemented *K. selliformis* D3 domain with other reverse complemented *Karenia* spp. was performed (Fig. 3). One nucleotide difference occurred between *K. papilionacea* and *K. brevis*, and two base-pair differences occurred between *K. mikimotoi* and *K. brevis*, but there were no differences between *K. selliformis* and *K. brevis* at the position of KbProbe-7. The latter explains the positive cross-reactivity of *K. selliformis* to KbProbe-7. The sequence for *K. selliformis* D1–D3 region of the LSU rRNA gene has been submitted to GenBank (accession number KT020848).

In comparing the full D1–D3 domain of LSU rRNA genes of *Karenia* species, it is difficult to find a region where there was an at least one nucleotide difference between all four species. In fact, *K. brevis*-specific probes developed for regions with differences were previously tested with no success other than KbProbe-7 (Mikulski et al., 2005). Therefore further work is needed in order to determine the best probe for distinguishing *K. brevis* from *K. selliformis*. Improved species-specificity might also be achievable using internal transcribed spacer regions of the rRNA genes or protein coding genes such as rubisco or cytochrome oxidase (Medlin and Kooistra, 2010).

3.3. Special considerations for combining IF and FISH

Because the antibody labeling and the FISH labeling require very different conditions (e.g., 4 °C vs. 50 °C) consideration of the requirements for each must be made. Based on the precedence found in dual labeling protocols used for mammalian tissue slices, it was decided that the antibody labeling must be done first, followed by FISH. Antibody labeling was performed essentially as described above for the flow cytometry application, with the exception that the secondary antibody was cross-linked by a second fixation in PFA. Once the secondary antibody and fluorescent tag were found to be stable through the rigorous conditions used by the FISH labeling step, the FISH protocol was optimized.

For FISH, it is critical to protect the rRNA from degradation during the immunolabeling steps. To do this, several measures were taken. First, the percentage of PFA used was increased from the original 0.2% originally used in the separate protocols to ensure rigorous fixation of the whole cells, and to successfully hold the cells together during the

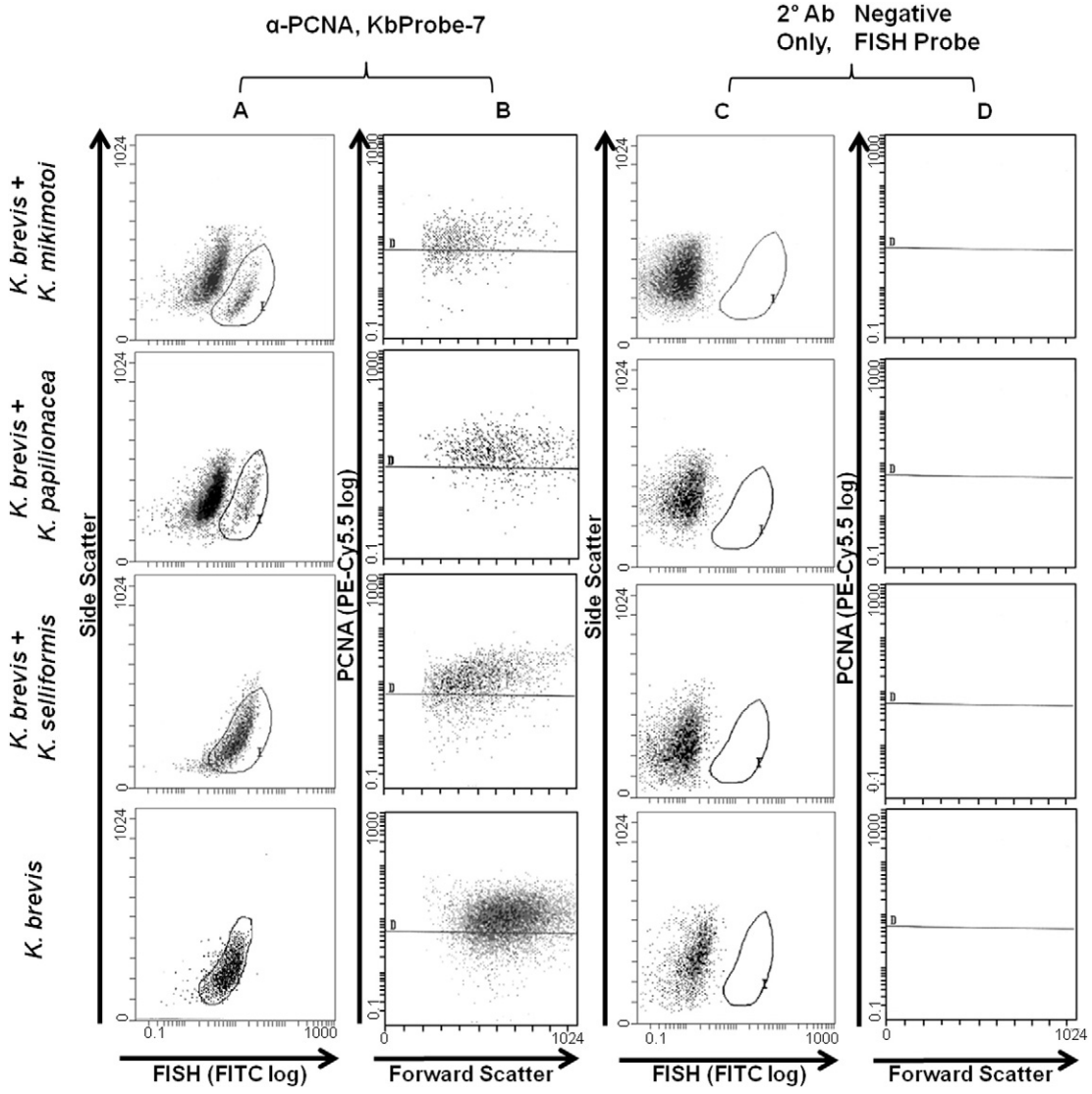

Fig. 2. An alternate gating strategy for dual labeling of rRNA with FISH and PCNA with IF in artificially mixed *Karenia* species populations. Scatter plots of FITC versus side scatter (SS) were used to gate cells positive for KbProbe-7 (columns A and C), and then PCNA was analyzed on a plot of forward scatter (FS) versus PE-Cy5.5 (columns B and D), which was gated on the amorphous gate in columns A and C so that only cells positive for KbProbe-7 were analyzed. As in Fig. 1, control single-species samples of non-targeted *K. mikimotoi* and *K. papilionacea* and targeted *K. brevis* were also analyzed to validate the fluorescence intensity positions of each signal in each species (not shown).

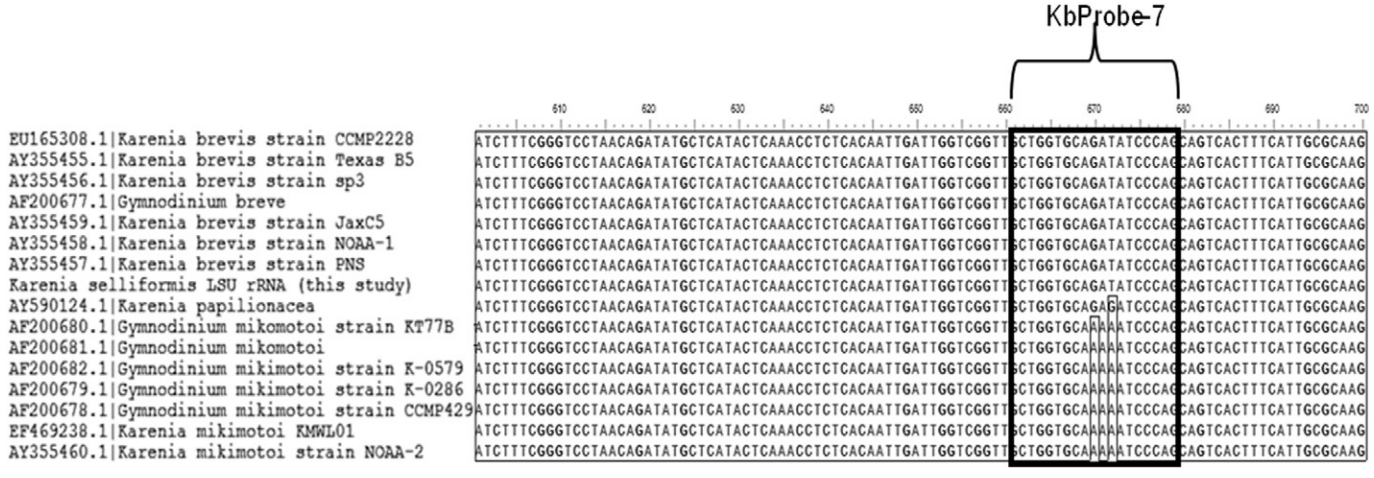

Fig. 3. Alignment of LSU rRNA gene sequences encompassing the D3 domain for all four *Karenia* species present in the Genbank database (accession numbers are listed first). Sequences are modified to the reverse complement in order to determine the position of KbProbe-7. *K. mikimotoi* exhibits 2 base-pair differences from Kbprobe-7, and *K. papilionacea* exhibits 1 nucleotide difference. Both *K. brevis* and *K. selliformis* (this study) match KbProbe-7 at every position, so this probe is specific for both species.

many agitation and centrifugation steps of the protocol. The PBS was DEPC treated to remove any RNases that may have been present, and molecular-grade products either certified RNase free or labeled as "no RNase detected" were used, including the Tween-20 and the BSA. The 1° antibody and the 2° antibody which did not specifically address RNase activity were treated with 1 U μl^{-1} RNase inhibitor as a precaution. Finally, all steps after fixation and up to hybridization were either performed on ice or in a 4 °C refrigerator.

UltraPure BSA was used as a blocking agent rather than the typical normal goat serum as the result of a series of trials that each left out one component of the assay (methanol, normal goat serum, 1° antibody, or 2° antibody). This study determined that the normal goat serum was degrading the rRNA, even when it was treated with RNase inhibitor. Increased concentrations of RNAse inhibitor were tested with inconsistent results, so the UltraPure BSA was tested and chosen as an alternative blocking agent.

The combination of IF and FISH has only recently been applied to *Drosophila* tissue sections and formalin-fixed paraffin embedded mouse retina (Toledano et al., 2012; Zimmerman et al., 2013; Stempel et al., 2014). These studies used FISH to detect mRNA rather than rRNA, but the overall principle is the same. The *Drosophila* studies determined that performing immunofluorescence first was necessary to protect the protein antigenicity before exposing the sample to the high temperatures needed for FISH. A second fixation step was also implemented before FISH in order to cross-link the antibody and further protect it (Zimmerman et al., 2013). Utilizing those modifications, the IF-FISH method for *K. brevis* was successful and flow cytometry could be used to analyze PCNA in *K. brevis* once it was separated from the other species by FISH. To our knowledge, this is the first application of this dual labeling approach to phytoplankton.

This approach to assessing PCNA expression in situ could be used to calculate an estimated growth rate for the population. Unfortunately there were no significant differences observed in this study between PCNA fluorescence intensity in early G1 phase and mid-S phase of the cell cycle due to substantial cytosolic staining by the existing PCNA antibody that overwhelmed differences in nuclear cell-cycle dependent staining when assessed by flow cytometry (data not shown). However PCNA has been studied in other phytoplankton species as a marker of cell proliferation and a growth rate estimator. Using the nuclear PCNA-stained phase as the 'terminal event' in the model originally developed by McDuff and Chisholm (1982) and Lin et al. (1995) estimated the growth rate of *D. tertiolecta*, a chlorophyte, and found that PCNA was a more accurate estimator of actual growth rate than the mitotic index. Qualitative relationships between PCNA abundance and growth rate have been additionally demonstrated for other species (Liu et al., 2005). Development of a more nuclear-specific PCNA antibody is needed to effectively use the dual labeling technique as an estimator of growth rate in *Karenia* species.

3.4. Conclusion

The IF-FISH protocol described here was developed to dually label the cell cycle protein, PCNA, and rRNA using a species-specific FISH probe for *K. brevis*, in order to attribute PCNA fluorescence to the species of interest among morphologically similar species known to co-occur in blooms. Our labeling strategy successfully preserved both the antibody-epitope complex and RNA integrity under substantially different hybridization requirements for the first time in a dinoflagellate species. Our objective to utilize PCNA florescence as a proxy for growth rate was not successful, due to the cytosolic staining overwhelming any differences in nuclear staining as previously observed by Brunelle and Van Dolah (2011). This may be improved by further antibody development and screening. However the dual labeling protocol described in this paper is broadly applicable to other phytoplankton research where the species-specific analysis of protein is warranted. For example, differential expression of nutrient assimilation proteins may be informative of iron, nitrogen or phosphorus limitation (La Roche et al., 1993; Dyhrman and Palenik, 2001). Dual labeling with a marker for stress or cell death (Johnson and Van Dolah, 2014) paired with FISH may be useful for identifying the imminent demise of a bloom or the succession of one species to another. Dual labeling may also use FISH to target messenger RNA (mRNA) rather than rRNA in a single species, as previously applied in *Drosophila* and mice (Toledano et al., 2012; Stempel et al., 2014) to look at both transcriptional and translational responses in the same cell. The method developed here is the first application of IF-FISH to phytoplankton, and serves as a proof of concept for its application to understanding biological processes that underlie algal bloom dynamics.

Notes

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