

SHORT COMMUNICATION

Fixation-free fluorescence *in situ* hybridization for targeted enrichment of microbial populations

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We modified the standard ribosomal RNA-targeted fluorescence *in situ* hybridization (FISH) protocol by removing the fixation steps to allow recovery of unmodified nucleic acids. Using this method, hybridized cells could be visualized in two bioreactor sludges and termite hindgut samples by epifluorescence microscopy. We then targeted one bacterial and one archaeal population in the sludge samples with group-specific oligonucleotide probes using in-solution fixation-free FISH and sorted hybridized populations using fluorescence-activated cell sorting (FACS). We could show that sorted populations were highly enriched for the target organisms based on 16S rRNA gene sequencing, thus confirming probe specificity using the modified FISH protocol. This approach should facilitate subsequent genomic sequencing and analysis of targeted populations as DNA is not compromised by crosslinking during fixation.

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Fluorescence *in situ* hybridization (FISH) is a powerful method for visualizing microbial populations in a community setting (Amann and Fuchs, 2008) and can be used as the basis for separating targeted populations from a multi-species background through fluorescence-activated cell sorting (FACS) (Kalyuzhnaya *et al.*, 2006; Podar *et al.*, 2007). Cell fixation is an accepted and reportedly integral part of the FISH protocol that serves to stabilize cell integrity and facilitate probe permeability for imaging (Amann *et al.*, 1995). For microorganisms, this is commonly achieved with paraformaldehyde that crosslinks proteins and nucleic acids (Hayat, 2000). However, for genomic or proteomic applications, unfixed biomass is desirable to minimize possible sequencing biases introduced by crosslinking and also to avoid possible cell lysis difficulties (Wallner *et al.*, 1997). Ribosomal RNA-targeted FISH using unfixed microbial samples has been reported for *Escherichia coli*, *Salmonella enterica* and *Pseudomonas putida* using specialized quenched autoligation oligonucleotide

probes (Silverman and Kool, 2005; Silverman *et al.*, 2008) that are not yet commercially available. In addition, unfixed mammalian tissue has been shown to produce better imaging results than paraformaldehyde-fixed tissue using *in situ* hybridization histochemistry (Dagerlind *et al.*, 1992). The aim of this study is to determine whether fixation-free FISH using standard probes works well enough on environmental samples to allow specific sorting of targeted populations for subsequent genomic applications.

We made a simple modification to the standard FISH protocol by removing the paraformaldehyde fixation and ethanol dehydration steps, and applied broad- and narrow-specificity FISH probes to two bioreactor sludges and a termite hindgut community (Table 1). The quality of the images was good enough to clearly delineate numerous distinct morphotypes regardless of probe, fluorochrome and sample combination, suggesting that the method may be applicable to a broad range of species (Figures 1c and d). Although hybridization specificity was not systematically addressed in this initial microscopy test, we noted that the group-specific probes highlighted only the expected morphotypes (Figures 1a and b) using stringency conditions optimized for standard FISH (Table 1). Paraformaldehyde and ethanol fixation also serves to preserve environmental samples, suggesting that fixation-free FISH may only be suitable for freshly

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Table 1 Details of environmental samples, oligonucleotide probes and hybridization conditions used for the fixation-free FISH method

Target population(s)	Environmental source	Sample storage	FISH probe-fluorochrome	Probe sequence (5'-3')	FA (%)	Probe specificity (reference)	Related figure
<i>Candidatus</i> Accumulibacter phosphatis type II ANME-2D-like archaea	EBPR sludge	-20 °C in 10% glycerol	AccII444-Alexa488	CCCGTGC AATT CTTCCCG	35	CAP type II (Flowers <i>et al.</i> , 2009)	1a and 2a
	DAMO sludge	Fresh	Arc915-FITC	GTGCTCCCGCCCA ATTCTT	40	Most archaea (Stahl and Amann, 1991)	1b and 2b
Bacteria	EBPR sludge	Fresh	Darch872-Cy3	GGCTCCACCGGTT GTAGT	20	Various Euryarchaeota including ANME groups (Raghoebarsing <i>et al.</i> , 2006)	1c
Bacteria	<i>Nasutitermes corniger</i> hindgut	-80 °C without cryoprotectant	EUB338mix-Alexa488	GCWGCCWCCCGTA GGWGT	20	Most bacteria (Daims <i>et al.</i> , 1999)	1d

Abbreviations: ANME, anaerobic methanotroph; Cy, cyanine; DAMO, denitrifying methane oxidizing; EBPR, enhanced biological phosphorus removing; FA, formamide; FISH, fluorescence *in situ* hybridization; FITC, fluorescein isothiocyanate.

collected material. However, we found that samples stored at -80 °C, even in the absence of cryoprotectants, produced satisfactory microscopy images (Figure 1d). Although these are promising preliminary results, rigorous testing of the method will be required to determine whether fixation-free FISH can broadly substitute for fixation-based FISH methods. This includes, but is not limited to, confirmation that (1) the method works with cells with low signal intensities and Gram-positive cells (and associated permeabilization protocols), (2) probes are specific by testing with perfect and single-mismatch target sites at different hybridization stringencies and (3) quantification of targeted populations is reproducible. Until these tests are carried out, we recommend using the method only for qualitative applications with independent verification of probe specificity, for example, by marker gene sequencing.

To apply fixation-free FISH to FACS, we adopted an in-solution FISH protocol (Wallner *et al.*, 1993; Kalyuzhnaya *et al.*, 2006) that does not require the solid support (filter or slide) of the standard protocol (Manz *et al.*, 1992; Glockner *et al.*, 1996) or resuspension of cells removed from a solid support (Sekar *et al.*, 2004). Two specific populations were targeted in the bioreactor communities, the bacterium *Candidatus* Accumulibacter phosphatis (CAP) type II (Flowers *et al.*, 2009) and methanotrophic archaea closely related to ANME-2D (Mills *et al.*, 2004) (Table 1). After in-solution fixation-free FISH, these populations were homogenized by sonication and flow sorted, using two-stage sorts, from their respective communities by gating cells based on their enhanced fluorescence and light scattering properties (Figure 2). We noted that unfixed cells homogenized more easily than fixed cells, perhaps because of the absence of crosslinking that may act on extracellular polymeric substances holding the cells together (Garcia Martin *et al.*, 2006). After two sorts, the CAP-II population was enriched from 2.8% of the starting community to 97.6% (35-fold enrichment) and the ANME-2D population was enriched from 1.8% to 93.6% (52-fold enrichment; Figure 2). Multiple subsets of the second-sort hybridized populations (presumptively >93% pure), ranging in size from 10² to 10⁵ cells, were sorted into different microtiter plate wells to confirm hybridization specificity (Supplementary Table S1). Sorted populations were alkaline lysed and their genomic DNAs amplified using multiple displacement amplification (MDA; Supplementary Figure S1). Primers broadly targeting either bacteria or archaea were then used to PCR amplify the 16S rRNA genes from the whole genome-amplified subset populations. The putative CAP-II populations produced PCR amplicons only with the bacterial primers, and the putative ANME-2D populations produced PCR amplicons only with the archaeal primers (Supplementary Figure S2). The resulting amplicons were directly Sanger sequenced

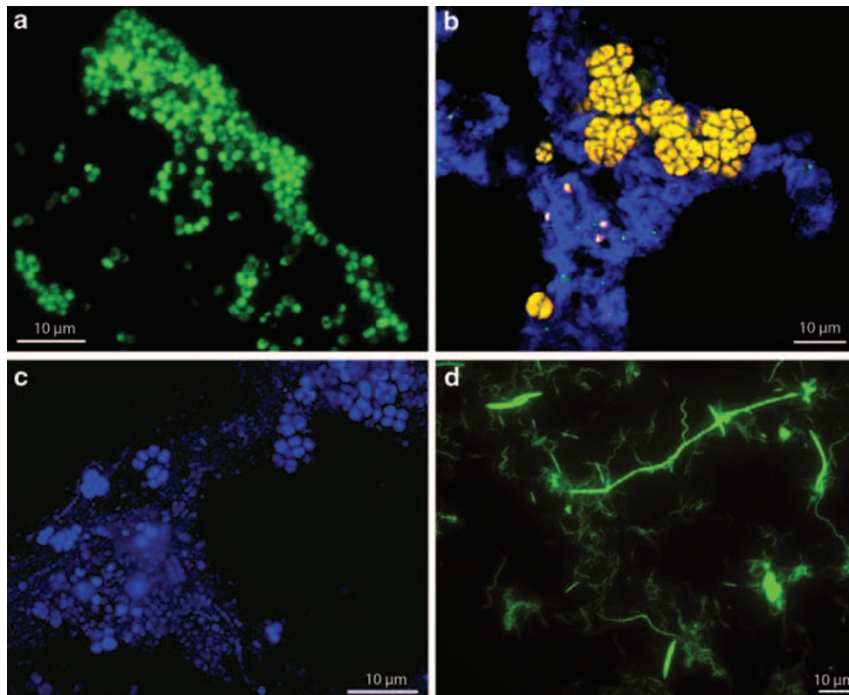


Figure 1 Epifluorescence microscopy images of fixation-free FISH applied to (a) sorted enhanced biological phosphorus removing (EBPR) sludge hybridized with a CAP II-specific probe highlighting the characteristic donut shape of CAP bacteria, (b) denitrifying anaerobic methane oxidizing (DAMO) sludge hybridized with archaeal-specific probes highlighting the distinctive tetrad clusters formed by ANME-2D archaea (the only archaeal members of the DAMO sludge community); the blue background is due to a concanavalin A counterstain for saccharides, (c) EBPR sludge hybridized with a broad-specificity bacterial probe highlighting several morphotypes and (d) *Nasutitermes corniger* hindgut community hybridized with a broad-specificity bacterial probe highlighting several morphotypes. See Table 1 for further details of the probes and hybridization conditions used.

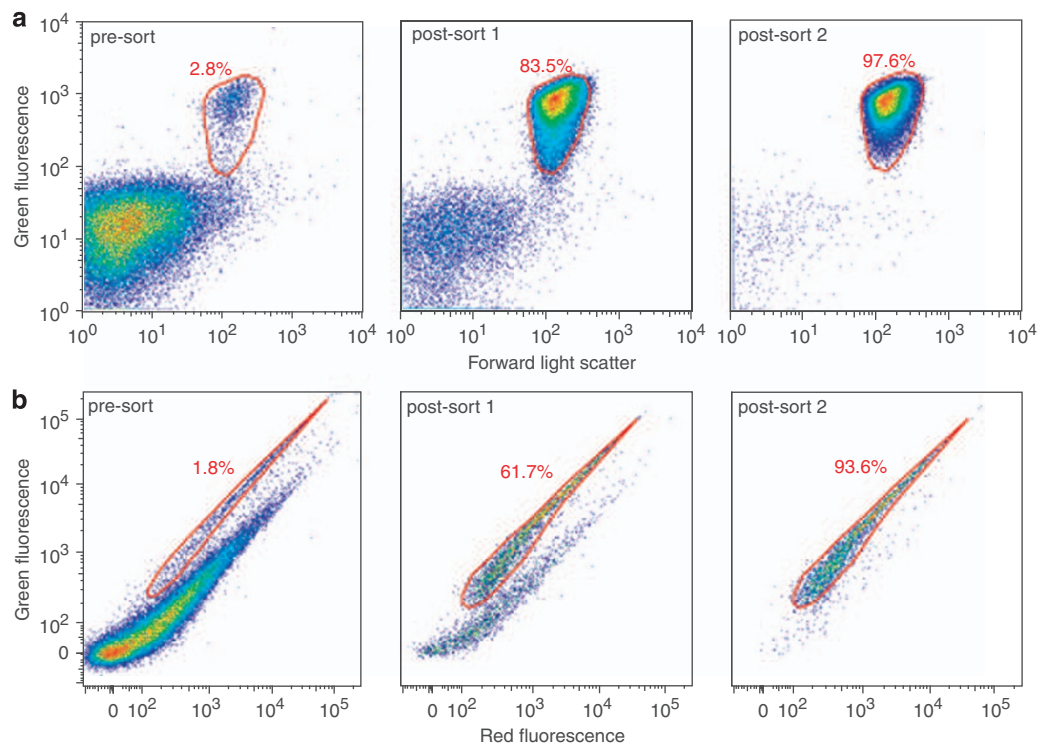


Figure 2 Flow cytometric analysis of pre- and post-sorts of (a) enhanced biological phosphorus removing (EBPR) sludge hybridized with AccII-444-Alexa488 and (b) denitrifying anaerobic methane oxidizing (DAMO) sludge hybridized with Arc915-fluorescein isothiocyanate (FITC) and Darch872-cyanine 3 (Cy3). The regions with a red outline indicate the gating used for the subsequent round of sorting (panel to right) and the values shown indicate the percentage of gated (fluorescent) cells relative to total cells.

without cloning. We have previously found that mixed templates will produce sequence chromatograms with secondary peaks using broad-specificity primers (data not shown). Comparative analysis of the amplicon sequences confirmed hybridization specificity due to the presence of the FISH probe target sites (Table 1, Supplementary Figure S3) and the expected identity of the sorted populations (Supplementary Table S1). In the majority of cases the sequencing chromatograms showed negligible background signal, indicating that the populations were highly enriched for the target organisms by the two-stage sorts (Supplementary Figure S3). However, it is not feasible to sort more than $\sim 10^6$ cells because of sorting volume and time constraints (Eek *et al.*, 2007), which limits the total amount of recoverable DNA to <5 ng, and much less for smaller population sizes. With this caveat in mind, we anticipate that in-solution fixation-free FISH will allow enrichment of a range of targeted bacterial and archaeal populations for recovery of unmodified nucleic acids. This will facilitate downstream genomic applications and possibly applications requiring live material.

Materials and methods

Samples

Fresh samples from denitrifying anaerobic methane oxidizing bioreactor communities were collected and pelleted by centrifugation and washed twice with phosphate-buffered saline before immediate processing. Samples from enhanced biological phosphorus removing sludge were collected from a sequencing batch reactor at the end of the aerobic phase. Note that biomass was collected from both reactors when normal bioreactor function had ceased to ensure that the target populations were low ($<5\%$). Cells were pelleted by centrifugation and washed twice with phosphate-buffered saline and stored in 10% glycerol at -20°C until further processing. Worker termites stored at -80°C were prepared as previously described for hindgut segment P3 lumen fluid extraction (Warnecke *et al.*, 2007). Enhanced biological phosphorus removing sludge and termite samples were thawed on ice before hybridization.

In-solution fixation-free FISH

Samples were washed twice with phosphate-buffered saline and resuspended in $100\ \mu\text{l}$ of standard FISH hybridization buffer (Pernthaler *et al.*, 2001). Oligonucleotide FISH probes used for the different samples and associated formamide stringencies are listed in Table 1. Cells were incubated at 46°C for 2 to 3 h. After hybridization, cells were centrifuged for 3 min at $5000 \times g$, and washed twice with $100\ \mu\text{l}$ standard wash buffer (Pernthaler *et al.*, 2001) prewarmed to 48°C . Cell pellets were resuspended

in $100\ \mu\text{l}$ prewarmed wash buffer and incubated at 48°C for 20 min. Cells were concentrated by centrifugation, resuspended in 1 ml Tris-EDTA (pH 8) buffer or phosphate-buffered saline and homogenized by sonication at an output power of 165 W for 30 s with 5 s pulse using a Sonic Dismembrator (Fisher Scientific, Pittsburgh, PA, USA; Model 550: output power 550 W, frequency 20 kHz) or Branson Sonifier 250 (Branson Ultrasonics Corp., Danbury, CT, USA).

Fluorescence-activated cell sorting

Flow sorting of the CAP-II population from enhanced biological phosphorus removing sludge was performed using an Influx (Cytocpeia Inc., Seattle, WA, USA) cell sorter in two stages to reduce final sort volume. A 488-nm laser was used as the excitation source for light scattering and fluorescence. Homogenized unfixed samples were diluted to 10^6 – 10^7 cells ml^{-1} to prevent nozzle clogging, and hybridized cells with enhanced green fluorescence were detected at 531 ± 20 nm and sorted first at high speed ($25\ 000$ cells s^{-1}) into 50 ml centrifuge tubes on ice. Cells from the initial sort were then resorted at lower speed ($10\ 000$ cells s^{-1}) into PCR tubes (10^2 – 10^5 cells per tube). Flow sorting of ANME-2D archaea from denitrifying anaerobic methane oxidizing sludge was performed using a BD FACSAria (BD, Franklin Lakes, NJ, USA). A 488-nm laser was used as the excitation source for light scattering and fluorescence. Hybridized cells were sorted at a speed of $20\ 000$ cells s^{-1} into 2 ml Eppendorf tubes (10^4 cells per tube) based on forward scatter and fluorescence intensity. The effectiveness of population enrichment was verified by viewing aliquots of sorted cells using epifluorescence microscopy.

Whole genome amplification and 16S rRNA-based molecular identification

Genomic DNAs were released from sorted cells using alkaline lysis and amplified using the REPLI-g Ultrafast Mini kit (for the CAP-II population) (QIAGEN Inc., Valencia, CA, USA) or the Illustra GenomiPhiV2 DNA amplification kit (for the ANME-2D population) (GE Healthcare, Piscataway, NJ, USA) in a $20\ \mu\text{l}$ volume for 2 h (GenomiPhi) or 12 h (REPLI-g) at 30°C . The reaction was terminated by heating at 65°C for 3 min before cooling to 4°C . 16S rRNA genes were PCR-amplified from MDA products using broad-specificity primers: 27F (5'-A GAGTTTGATCMTGGCTCAG-3') and 1392R (5'-A CGGGCGGTGTGTRC-3') for the bacterial CAP-II population or Arc8f (5'-TCCGGTTGATCCTGCC-3') and 1492R (5'-GGTTACCTTGTACGACTT-3') for the archaeal ANME-2D population. Bacterial amplicons were directly Sanger sequenced, without cloning, using a 27F and/or 530F (5'-GTGCCAGCAGCC GCG-3') primer and archaeal amplicons sequenced using a 1391R (5'-GACGGGCGGTGWGTRCA-3')

primer. Amplicon sequences were compared against the publicly available databases to confirm population identity.

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