Chapter 1

WHOLE CELL IMMUNOMAGNETIC ENRICHMENT OF ENVIRONMENTAL MICROBIAL CONSORTIA USING RRNA-TARGETED MAGNETO-FISH

Abstract

Magneto-FISH, in combination with metagenomic techniques, explores the middle ground between single cell analysis and complex community characterization in bulk samples to better understand microbial partnerships and their roles in ecosystems. The Magneto-FISH method combines the selectivity of Catalyzed Reporter Deposition -Fluorescence In-Situ Hybridization (CARD-FISH) with immunomagnetic capture to provide targeted molecular and metagenomic analysis of co-associated microorganisms in the environment. This method was originally developed by Pernthaler et al. and Pernthaler & Orphan (2008; 2010). It led to the discovery of new bacterial groups associated with anaerobic methane-oxidizing (ANME-2) archaea in methane seeps as well as provided insight into their physiological potential using metagenomics. Here, we demonstrate the utility of this method for capturing aggregated methanotrophic consortia using a series of nested oligonucleotide probes of differing specificity designed to target either the ANME archaea or their *Deltaproteobacteria* partner, combined with 16S rRNA and *mcrA* analysis. This chapter outlines a modified Magneto-FISH protocol for large and small volume samples and evaluates the strengths and limitations of this method predominantly focusing on 1) the relationship between FISH probe specificity and sample selectivity, 2) means of improving DNA yield from paraformaldehyde-fixed samples, and 3) suggestions for adapting the Magneto-FISH method for other microbial systems, including potential for single cell recovery.

Introduction

As advancements in high-throughput sequencing technology allow deeper and more cost effective means of sequencing complex microbial assemblages, we are left with more data, but not necessarily more means to understand it. The development of microbiological techniques to isolate and visualize environmental microorganisms *a priori* can be used to meaningfully parse environmental samples before metagenomic processing, and thereby provide additional context for downstream bioinformatic data interpretation. There is also increasing awareness that microbe-environment and microbe-microbe interactions are important factors in assessing microbial systems, their metabolic potential, and how these relationships affect larger scale processes such as ecosystem nutrient cycling.

A range of *in situ* techniques are currently available for physical separation of microorganisms of interest from environmental samples. Methods involving selection from a complex microbial sample often involve a stage of phylogenetic identification, such as 16S rRNA-based fluorescence *in situ* hybridization (FISH), coupled to a means of physical separation such as flow sorting (Amann et al. 1990; Yilmaz et al. 2010) (also see chapters in this volume by Zehr and Haroon), optical trapping (Ashkin 1997), microfluidics (Melin & Quake 2007), or immunomagnetic beads (Šafařík & Šafaříková 1999). This is in contrast to separation methods where selection is based on a property other than identity, such as metabolic activity (Kalyuzhnaya et al. 2008), followed by downstream identification of the population exhibiting the property of interest. The majority of these methods have focused on single cell analysis, rather than examining intact multi-species microbial associations, with the exception of intracellular microbial interactions (Yoon et al. 2011).

The Magneto-FISH method was originally developed by Pernthaler et al. (2008) to enrich for and characterize microbial associations in the environment. This technique was specifically developed for studying inter-species partnerships between anaerobic methane-oxidizing (ANME) archaea and sulfate-reducing *Deltaproteobacteria* (SRB) in anoxic marine sediments (Boetius et al. 2000; Orphan et al. 2002). This method is based on 16S rRNA Catalyzed Reporter Deposition (CARD)-FISH (identity) (Pernthaler et al. 2002) and immunomagnetic bead capture (separation) (Pernthaler et al. 2008; Pernthaler & Orphan 2010). The Magneto-FISH method was shown to successfully

concentrate the population of interest and aid in microbial association hypothesis development that could be further supported with metagenomics, microscopy, and isotope-labeling techniques. This provides a means to study metabolic potential at a level that is not defined in separate units of species identity, but operational groups of organisms that have evolved to serve a function, such as the symbiotic consortia mediating methane oxidation coupled to sulfate reduction. Magneto-FISH is also compatible with the physical challenges of sediment associated ANME-SRB aggregates, namely their heterogeneous morphology, wide size range (~3-100 μm diameter), and frequent association with mineral and sediment particles.

In evaluating the application of Magneto-FISH to other environmental populations, it is important to consider sample input constraints such as microbe size and morphology, sample output requirements such as yield and purity, and of course time and expense. Autofluorescent sediment particles and diverse ANME/SRB consortia size complicated the successful application of flow sorting approaches to the AOM system. In other environments, FAC sorting has been shown to be an effective means of cell separation, but often requires DNA amplification (Rodrigue et al. 2009; Woyke et al. 2011). Yield and purity are also often opposing constraints. For example, FAC sorting can provide high sample purity, but may require significant instrument time for collecting sufficient material without including a post-amplification step (Woyke et al. 2011). Sample yield remains an issue with Magneto-FISH, as well. Initial application of Magneto-FISH required Multiple Displacement Amplification (MDA) before construction of metagenomic libraries for 454 pyrosequencing (Pernthaler et al. 2008). However, advances in library preparation (e.g. Nextera XT) have significantly lowered the minimum DNA concentrations required. Magneto-FISH can be completed in a day and does not require the use of any specialized equipment beyond an epifluorescent microscope. The main expense is reagents, which scales with amount of sample processed and diversity of FISH probes needed. Another advantage is the versatility of this method. It is compatible with a broad range of oligonucleotide probes incorporated into the same basic protocol; no instrument adjustment or recalibration is required between runs or with different microbial targets.

This chapter introduces three modifications to the Magneto-FISH protocol of Pernthaler et al. (2008) to improve DNA recovery and labor efficiency: 1) immuno-based attachment of magnetic beads for single cell capture, 2) magnetic separation in a standard magnetic holder, and 3) DNA

cross-link reversal incubation during extraction. Using this modified method, we evaluate the DNA recovery and microbial target specificity using a nested set of oligonucleotide probes and discuss 1) increasing target DNA yield for current template requirements amplification, 2) the relationship between sample purity and FISH probe specificity, 3) controls for association selectivity, and 4) DNA quality for metagenomic techniques.

Methods

Samples and controls used in Magneto-FISH capture experiments

Sediment samples were collected in September 2011 from methane seeps within the S. Hydrate Ridge area off the coast of Oregon at a depth of 775 m using the R.O.V *JASON* and the R/V *Atlantis*. Marine sediment was collected in a push core (PC-47) associated with a sulfide-oxidizing microbial mat adjacent to an actively bubbling methane vent. A sediment slurry from the upper 0-15 cm depth horizon was prepared with one volume N_2 sparged artificial seawater to one volume sediment, over-pressured with methane (3 bar) and incubated at 8ºC in a 1L Pyrex bottle sealed with a butyl rubber stopper. A 4 ml sample from the incubation was collected on November 19, 2012. Samples were immediately fixed in 0.5 ml sediment aliquots in 1.5% paraformaldehyde (PFA) for 1 hr at room temperature (fixation can alternatively be performed at 4°C overnight). Samples were washed in 50% 1x PBS: 50% EtOH, then 75% EtOH: 25% DI water, and resuspended in 2 volumes (1 ml) 100% ethanol. Samples were centrifuged at 1000 x*g* for 1 min between wash steps.

As a control to test association specificity, 0.5 ml of sediment slurry was spiked with 10 µl of turbid *Paracoccus denitrificans*, strain ATCC 19367. After addition the sample was quickly vortexed, fixed, and washed as described above. 16S rRNA diversity surveys of the original sediment incubation sample supported the absence of *P. denitrificans* in the bulk sediment.

Magneto-FISH

A detailed protocol is provided in Table 1 and additional information and explanation of the major steps are provided below. When using Magneto-FISH with marine sediment samples, 100 µl of fixed sediment slurry (resuspended in 100% ethanol) is the recommended starting volume for the

recovery of PCR-amplifiable DNA. The method has been tested with sediment volumes ranging from 75-3000 µl. Smaller sample sizes have higher target purity, but lower DNA yield. For the purposes of this chapter, all reagent amounts are given for the 100 µl starting sample size (small scale prep), but can be scaled up as indicated for larger samples. There are two means to scale up these reactions: 1) using more of the starting sample with the same oligonucleotide probe, 2) or using more of starting sample, but with different probes. With option 1, all sample aliquots can be combined during wash steps as indicated. For option 2, sample aliquots can be combined during the initial permeabilization stages, but can no longer be combined after probes have been applied. All reagents should be sterilized by filtration (0.22µm) prior to use, and sterile sample containers should be used in subsequent steps. Additionally, after fluor addition samples should treated as light-sensitive.

Permeabilization and inhibition of endogenous peroxidases

The TE pH 9 heating step serves to permeabilize cells and loosen sediment particles. The hydrogen peroxide addition inhibits endogenous peroxidases prior to the CARD reaction. To remove ethanol, spin sediment-ethanol slurry at 16,000 x*g* for 1 min, remove supernatant, and resuspend in TE (pH 9). When performing multiple reactions with the same sediment, they can be combined during these steps (i.e. for 6 captures, add 600 µl (original volume) of sediment slurry to 100ml Tris-EDTA (TE), pH 9) after removing ethanol.

Liquid CARD-FISH

All oligonucleotide probes and corresponding formamide concentrations used are summarized in Table 2. When using a histological microwave for hybridization, formamide concentrations were lowered by 10% below the concentrations optimized for a conventional hybridization oven (Fike et al. 2008). *Note*: A hybridization oven can also be used for liquid CARD-FISH, but incubation time should be increased to at least 2 hrs or more.

If doing multiple reactions, evenly divide sediment pellet among all samples in each CARD hybridization buffer with the appropriate formamide concentration. For histological microwave use, orient the beaker and samples such that only water, and no samples, is in the path of the temperature probe. Inverting or vortexing samples a few times during this incubation can improve

mixing of probe and sample, since sediment will tend to settle out of suspension during the incubation. All samples with the same probe can be combined during wash steps, but different probe samples must be kept separate.

For the amplification reaction, samples must be evenly divided into their initial starting proportions (if started with 600 µl of slurry, then separate into 6 aliquots) for proper target to probe ratios, but like-samples can be recombined during subsequent wash steps. For a larger combined wash, samples can remain in a 50 ml tube with the appropriate amount of PBS and PPi after blocking reagent and washing steps. Hybridized samples can also be stored overnight at 4°C before proceeding with magnetic capture.

Table 1: Step-by-step detailed instructions for Magneto-FISH protocol. Additional information and suggestions are included in the text for each section. Recommended equipment list: hybridization microwave [BP-111-RS-IR, Microwave Research & Applications], centrifuge (microtubes and 50 ml tubes), sonicator with tapered microtip probe [Branson Sonifier W-150 ultrasonic cell disruptor], rotating or shaking incubator/hybridization oven, magnetic holder [Dynal MPC-1.5ml], waterbath, bead beating tubes with garnet sand [PowerSoil DNA Kit PowerBead Tubes, MO BIO] and bead beater [FastPrep FP120, Thermo Electron Corp.], cellulose spin columns [Microcon, Millipore], vortex [Vortex-Genie 2, MO BIO], 1.5 ml maximum recovery centrifuge tubes [Flex-Tubes 1.5ml, Eppendorf]. Special reagents: Linear Acrylamide, Dextran Sulfate, Blocking Reagent, HRP-probes, fluor-labeled tyramide(s), biotin tyramide, anti-fluor mouse monoclonal IgG antibody [Life Technologies], Dynabeads Pan Mouse IgG [Life Technologies].

Magneto-FISH

1. Permeabilization and inhibition of endogenous peroxidase
    a. Add 100µl sediment slurry to 100 ml TE pH 9 in a sterile 250 ml glass beaker (or other flat-bottomed vessel to maximize surface area).
    b. Microwave 2 min at 65°C in a hybridization microwave (100% power) [BP-111-RS-IR, Microwave Research & Applications].
    c. Transfer to two 50 ml Falcon tubes and spin at 5000 x*g* for 5 min at 4°C (all spin steps should be performed in this manner unless otherwise indicated).
    d. Decant supernatant taking care to retain the sediment pellet by pouring slowly and all in one motion.
    e. Resuspend in 50 ml 1x Phosphate Buffered Saline (PBS), 0.01M Sodium Pyrophosphate (PPi), 0.1% H_2O_2 and incubate at room temperature for 10 min, inverting tubes occasionally to keep sediment in suspension.
    f. Sonicate for three 10 s pulses on setting 3 (~6V(rms) output power) [Branson Sonifier W-150 ultrasonic cell disruptor] at room temperature with sterile remote tapered microtip probe [Branson] inserted into the liquid.
    g. Spin and decant.
2. Liquid CARD-FISH
    a. Resuspend sediment in 2 ml CARD buffer [0.9M NaCl, 20mM Tris-HCl

pH 7.5, 10% w/v Dextran Sulfate, 1% Blocking Reagent (in pH 7.5 maleic acid buffer), 0.02% w/v SDS] and transfer to a 2 ml Eppendorf tube.

b. Add 20 µl of 50 ng/µl CARD probe and vortex [Vortex-Genie 2, MO BIO] briefly to mix.
c. Wrap tubes in parafilm and tape to the sides of a beaker filled with DI water, such that tubes float in an approximately horizontal orientation.
d. Microwave for 30 min at 46°C, power setting of 100%.
e. Remove samples from the water bath and remove parafilm.
f. Spin tubes at 10,000 x*g* for 2 min.
g. Decant supernatant into formamide waste and resuspend hybridized sediment in 50 ml 1x PBS.
h. Incubate at room temperature for 10 min, shaking occasionally.
i. Centrifuge, decant supernatant, resuspend in fresh 1x PBS, centrifuge and decant again, leaving pellet.
j. Resuspend in 2 ml amplification buffer [1x PBS, 1% Blocking Reagent, 10% w/v Dextran Sulfate, 2M NaCl] in 2 ml Eppendorf tube.
k. Add 2 µl fluor-labeled tyramide (0.5 µg/ml), 2 µl biotin tyramide (0.5 µg/ml), and 5 µl 0.0015% H_2O_2.
l. Wrap tube(s) in foil to protect from light and incubate with gentle shaking or rotating at 37°C for 1.5 hrs.
m. Spin at 10,000 x*g* for 2 min.
n. Decant supernatant and resuspend in 50 ml 1x PBS in 50 ml centrifuge tube.
o. Incubate for 10 min at room temperature in the dark, shaking occasionally.
p. Spin, resuspend in 50 ml 1x PBS, and spin again.
q. Resuspend in 49.5 ml 1x PBS and 0.5 ml 10% blocking reagent in a 50 ml falcon tube.
r. Microwave [BP-111-RS-IR, Microwave Research & Applications] in a vessel large enough to submerge 50 ml tubes for 20 min at 40°C in DI water.
s. Centrifuge, decant, and resuspend in 50 ml 1x PBS, then centrifuge and decant again.
t. Resuspend each sample in 1 ml 1x PBS, 0.01M PPi in a 1.5 ml Eppendorf tube
u. Counterstain a sample aliquot with DAPI and verify hybridization by microscopy.

3. Magnetic Bead preparation and Magnetic Cell Capture
    a. Sonicate sample in 1.5 ml tube for 5 sec, setting 3 at room temperature to resuspend cells.
    b. Add 5 µl anti-fluor mouse monoclonal IgG antibody [Life Technologies] per 1 ml reaction volume and incubate at 4°C for 20 min rotating to keep sediment in suspension [Hybridization Oven, VWR].
    c. While the sample is incubating, prepare beads:
        i. Add 25 µl of Dynabeads Pan Mouse IgG [Life Technologies] per reaction to 1 ml of Buffer1 [1x PBS, 0.1% BSA] and place in magnetic holder [Dynal MPC-1.5ml].

ii. Invert holder and tube(s) multiple times to wash all beads down to magnet. Remove liquid with pipet and treat as azide waste. Remove tube from holder and resuspend washed beads in 30 µl of Buffer1.

d. After 20 min incubation, spin sample at 300 x*g* for 8 min at 4°C.
e. Decant supernatant, resuspend sediment pellet in Buffer1, and spin again as in step 3d. Decant supernatant.
f. Add 30 µl of washed beads and 1 ml Buffer1 per sample volume.
g. Incubate 1.5 hrs at 4°C in dark while rotating to keep sediment in suspension.
h. Place sample(s) into magnetic holder slots. Invert multiple times and let sit 1 min until sediment has settled to the bottom of the tube. Remove liquid including all sediment while trying not to disturb magnetic beads.
i. To wash beads and target cells, remove tube from magnetic holder and add 1 ml Buffer1 while aiming pipet tip at magnetic beads to resuspend them. If all beads are not resuspend when adding 1 ml, pipet up and down slowly to resuspend remaining beads from side of the tube. After a few washes, counterstain a sample aliquot with DAPI and verify bead attachment by microscopy. Repeat wash step at least 9 more times (10 total).
j. Save any sample necessary for further microscopy before proceeding to DNA extraction.
k. After final wash, resuspend washed beads and cells in 400 µl of TE buffer (pH 8).

DNA Processing

1. Cell lysis and reversing crosslinks in DNA
   a. Add lysis reagents (10 µl 5M NaCl and 25 µl 20% SDS) to 400 µl TE with beads from step 3k.
   b. Remove liquid from screw cap 2 ml bead beating tube with garnet sand (PowerSoil DNA Kit PowerBead Tubes, MO BIO].
   c. Add total volume of sample and lysis reagents (435 µl) to bead beating tube.
   d. Bead beat at setting 5.5 for 45 s [FastPrep FP120, Thermo Electron Corp.].
   e. 3 rounds of alternating Freeze/Thaw (-80°C and 65°C were used in this study).
   f. Incubate samples for at least 2 hrs, up to 48 hrs, in a 65°C water bath.
2. DNA Extraction
   a. Add 0.5 ml phenol [pH 8, 0.1% hydroxyquinoline] to bead beating tube.
   b. Vortex to mix, and spin for 2 min at 16,000 x*g*.
   c. Transfer supernatant to a new tube while avoiding particulates at TE/phenol interface.
   d. Add 250 µl phenol and 250 µl Chloroform:IAA (24:1).
   e. Vortex to mix, spin 1 min at 16,000 x*g*, and transfer supernatant to new tube.
   f. Add 500 µl Chloroform:IAA, vortex briefly, spin 2 min at 16,000 x*g*.

g. Add 200 µl TE to cellulose spin column [Microcon, Millipore], then add DNA supernatant.
h. Spin 8 min, 14,000 x*g*. Wash DNA on spin column 3x with 500 µl TE.
i. Elute into new tube at 1,000 x*g* for 3 min, as per manufacturer directions.

3. Concentration
   a. Transfer DNA from elution tube to 1.5 ml maximum recovery centrifuge tube [Flex-Tubes 1.5 ml, Eppendorf] and bring volume up to 37.5 µl with TE.
   b. Add 12.5 µl 10M Ammonium Acetate (2.5 M final concentration), 0.2 µl Linear Acrylamide, and 125 µl cold EtOH (2.5 volumes).
   c. Precipitate DNA overnight in wet ice (0°C).
   d. Spin 18,000 x*g* in a microfuge for 30 min at 4°C to pellet DNA.
   e. Decant supernatant, careful to retain pellet.
   f. Lay tube on its side with cap open on a heat block at 65°C to evaporate remaining liquid. Resuspend in 10 µl Tris-HCl (pH 8).

Magnetic Capture

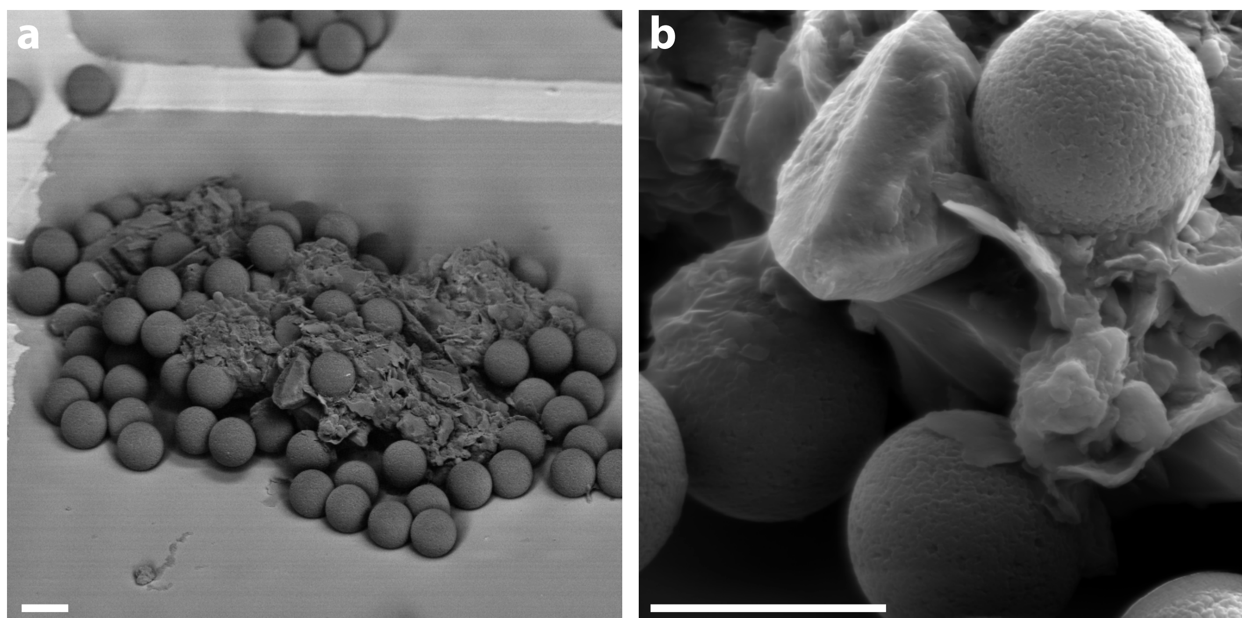


Figure 1. a. Electron backscatter image and b. close-up secondary electron image of Magneto-FISH aggregate. Bar is 5 µm.

The magnetic capture consists of three main steps: 1) Incubation of anti-fluor antibodies with fluor labeled cells (Figure 1), followed by two centrifugation wash steps to remove any unassociated anti-fluor. Increasing the centrifugation speed/force does not appear detrimental and could be

optimal for other systems in order to retain more material (for example, non-sediment associated microbes, single cells, and smaller aggregates). We recommended saving supernatants from the washing steps until satisfied with magneto capture, in the event that steps need to be repeated or re-optimized during bead attachment. 2) Incubation of magnetic beads with anti-fluor attached cells. 3) Removing remaining sediment and cells that did not attach to beads using a magnetic tube holder. It is also recommended to retain the first two sediment washes until satisfied with magnetic capture and to evaluate efficiency (number of captured cells/cells remaining in wash). Bead resuspension between washes should be done as gently as possible to reduce the strain on bead-cell association. When performing larger reactions, multiply number of reactions by 1 ml Buffer1 to calculate volume of wash to use. Larger magnetic holders for 15 ml or 50 ml tubes may also be necessary. To reduce larger volumes down to 400 µl for extraction, adding additional washes in increasingly smaller volumes before final suspension in TE may be helpful. After the final resuspension, it is easier to work with low retention tips as beads can stick to tips and tubes when in TE.

In Pernthaler et al. (2008) the magnetic beads and anti-fluor antibodies were incubated together before application to the sediment. Here, anti-fluor and magnetic beads are added in separate, successive reactions. We have found that addition of the anti-fluor antibodies independently, followed by subsequent addition of magnetic beads, resulted in higher recoveries, likely a result of improved antibody-cell hybridization, which may avoid steric hindrance caused by bulky magnetic beads during the attachment stage (*R.S. Poretsky and V.J. Orphan*, unpublished). Pernthaler et al. (2008) also developed a separatory funnel apparatus outfitted with a neodymium ring magnet to allow large volumes of buffer to continually wash the magnetic beads and attached cells (Pernthaler & Orphan 2010). To simplify this procedure, and increase the recovery of cells after magnetic capture, a conventional magnetic tube holder for 1.5 ml and 50 ml falcon tubes (Dynal) was used in combination with multiple washes to remove residual sediment particles and collect the bead-attached cells. We found that these modifications achieved a similar level of target cell enrichment with small samples.

DNA Processing

Lysis and reversing paraformaldehyde crosslinks

Higher DNA yields have been reported after 48 hrs cross-link reversal incubation with no degradation of sample (Gilbert et al. 2007), but may not be necessary if fixation duration and time since fixation are short, or a different fixative is used. Gilbert et al. (2007) also provide a review of other published amendments to DNA extraction methods for PFA fixed DNA that may provide further insight for optimizing this method for different sample types or downstream goals.

Extraction and Concentration

DNA extraction and concentration methods are based on Sambrook and Russell (2001) and Crouse and Amorese (1987). Bead beating can be replaced by vortexing at maximum speed for 10 min. Freeze/thaw cycles can be performed at a range of freezing and thawing temperatures. -80°C and 65°C were chosen based on equipment available and for rapid cycling between states.

Quantification

The extremely low DNA concentrations from magneto-FISH samples requires the highest possible sensitivity for detection, reduction of sample loss during quantification, and minimization of contamination during processing or from reagents (Woyke et al. 2011). For DNA quantification prior to metagenomic library construction, the use of a Qubit fluorometer and HS dsDNA Assay kit (Life Technologies) is recommended, though it may require as much as half of the final DNA extract for the small-scale preparation (5 µl) to obtain a reading above detection.

PCR and Cloning

Archaeal *16S rRNA* Primers, annealing 54°C:

- Arc23F (DeLong 1992; Waldron et al. 2007) – TCC GGT TGA TCC YGC C
- U1492R (Lane 1991) – GGY TAC CTT GTT ACG ACT T

mcrA Primers, annealing 52°C:

- ME1 (Hales et al. 1996) – GCM ATG CAR ATH GGW ATG TC
- ME2 (Hales et al. 1996) – TCA TKG CRT AGT TDG GRT AGT

Paracoccus denitrificans, annealing 50°C:

- Bac27F (Lane 1991) – AGA GTT TGA TYM TGG CTC
- PAR1244R (Neef et al. 1996) – GGA TTA ACC CAC TGT CAC

Hot start Taq DNA polymerases, such as HotMaster (5 PRIME), are recommended for PFA fixed samples, especially when trying to amplify larger (>1000 bp) fragments such as full length 16S

rRNA (Imyanitov et al. 2006). All Magneto-FISH PCR reactions were 12.5 µl total volume containing 1 µl DNA template. The following thermocycler conditions were used: 95°C initial denaturation of 2 min, followed by 40 cycles of 94°C for 20 s, annealing for 20 s at temperatures listed above for primers, 1-1.5 min extension at 72°C, and a final extension of 10 min at 72°C. PCR reagents were used at the following concentrations: 1x HotMaster buffer with 25 mM Mg^{2+}, 0.22 mM dNTPs, 0.2 µM forward and reverse primer, 0.2 U HotMaster Taq per µl reaction. Prior to cloning, an additional reconditioning PCR step of 5 to 8 cycles was performed in 25 µl, using 5 µl of template from the original PCR reaction (Thompson et al. 2002). Reconditioned PCRs were quantified by gel electrophoresis (1% gel, SYBR safe stain), filtered (MultiScreen PCR Filter Plate #MSNU03010, Millipore) to remove primers, and concentrated in 10µl Tris-HCl (pH 8). Approximately 4 µl of PCR product was used per reaction according to guidelines for TOPO TA Cloning Kit for Sequencing with pCR4-TOPO Vector and One Shot Top 10 chemically competent *E. coli* (Life Technologies). An ABI Prism 3730 DNA sequencer was used for all sequencing.

Phylogenetic analysis of 16S rRNA and metabolic genes (mcrA)

Translated methyl-coenzyme reductase alpha subunit (*mcrA*) nucleotide sequences were added to an *mcrA* database and aligned in ARB utilizing the ARB alignment features (Ludwig et al. 2004). 16S rRNA sequences were aligned using Silva online aligner (Quast et al. 2013) and then imported into ARB to verify alignment. Representative sequences were selected from the alignments and cropped to a common region containing no primers: 451 nucleotide containing positions for *mcrA* and 901 nucleotide containing positions for 16S rRNA. Sequences were then exported from ARB and phylogenies were computed using MrBayes (Ronquist et al. 2012). Convergence was determined by an average standard deviation of split frequencies <0.01. Both phylogenies were computed by nucleotide. Inverse gamma rates and default recommendations from Hall (2004) were used for all other MrBayes parameters.

Results and Discussion

Evaluating the quantification and specificity of captured targets using general and species-specific FISH probes

In the initial Magneto-FISH publication by Pernthaler et al. (2008), a clade specific probe targeting the archaeal subgroup ANME-2c (Knittel et al. 2005) was used to successfully enrich this group and physically associated bacteria from Eel River Basin methane seep sediments, increasing the percentage of recovered ANME-2c from 26% in the original sediments to 92% of the Magneto-FISH captured archaeal diversity. Here we expand upon this work, specifically evaluating how FISH probe selectivity affects Magneto-FISH microbial target selectivity. Five different CARD-FISH probes, including Domain-level and group-specific probes targeting major methane seep archaeal and sulfate-reducing bacterial groups were evaluated (Figure 2 and Table 2). The three archaeal probes used were ANME-2c_760, Eel-MSMX_932 (general ANME; Boetius et al. 2000) and Arc_915 (general archaea; Stahl & Amann 1991). Two bacterial probes, Seep-1a_1441 (Schreiber et al. 2010) and Delta_495a (Loy et al. 2002), were also used to target *Deltaproteobacteria* that commonly associate with ANME archaea. Seep-1a_1441 is a probe designed to hit a specific subgroup of the *Desulfococcus/Desulfosarcina* (DSS), shown to be a dominant partner of ANME-2c archaea in methane seeps (Schreiber et al. 2010). However, greater diversity of SRB and other bacteria exist in association with ANME in seeps (Holler et al. 2011; Knittel et al. 2003; Löesekann et al. 2007; Niemann et al. 2006; Orphan et al. 2002). Delta_495a targets a broader range of SRB, and is expected to recover additional diversity if present in the sample. This allows investigation of both the effectiveness of target species enrichment, as well as providing information on the breadth of associated ANME partners.

Total DNA recoveries from each Magneto-FISH capture ranged from below detection to 1.2 ng, depending on the specificity of the FISH capture probe (Table 2). The total DNA extracted for each sample was consistent with the predicted yield based on oligonucleotide probe specificity, where clade specific probes (ANME-2c_760 and Seep-1a_1441) yielded lower DNA recoveries relative to Magneto-FISH captures with more general probes (Eel-MSMX_932, Delta_495a, and Arc_915). The DNA recovered from the group-specific Seep-1a_1441 probe is reported as not detected in the table, however, only 1µl (10%) of the total DNA extract was quantified to preserve sample material. Typically 5 µl (50%) was necessary for detection of other Magneto-FISH captures. Based on PCR amplification, the Seep-1a_1441 Magneto-FISH capture most likely recovered a DNA concentration similar to that observed with ANME-2c_760.

In an attempt to quantify the level of confidence in Magneto-FISH microbial associations, we spiked a bulk sediment sample with a known volume of an alien cultured organism, *Paracoccus denitrificans*. This pure culture has a diagnostic morphology and was not detected in any of our bulk sediment analyses. After confirming with FISH and microscopy that the introduced *P. denitrificans* cells were present in the sediment sample after fixation and at an environmentally relevant concentration (visible in each field of view, but not a dominant species), this spiked sample was used for Magneto-FISH with the Eel-MSMX_932 probe. Using a primer specific to *P. denitrificans* (Neef et al. 1996), DNA recovered from the capture did not reveal *P. denitrificans* contamination after 40 cycles of PCR. There was also positive PCR amplification of *P. denitrificans* from the spiked bulk sediment DNA extraction. Universal bacterial 16S rRNA primers were used to confirm that the Eel-MSMX_932+*P. denitrificans* sample did not have amplification inhibition. This suggests that microorganisms associated with target Magneto-FISH samples are unlikely present due to non-specific attachment during the Magneto-FISH or sample preservation protocol.

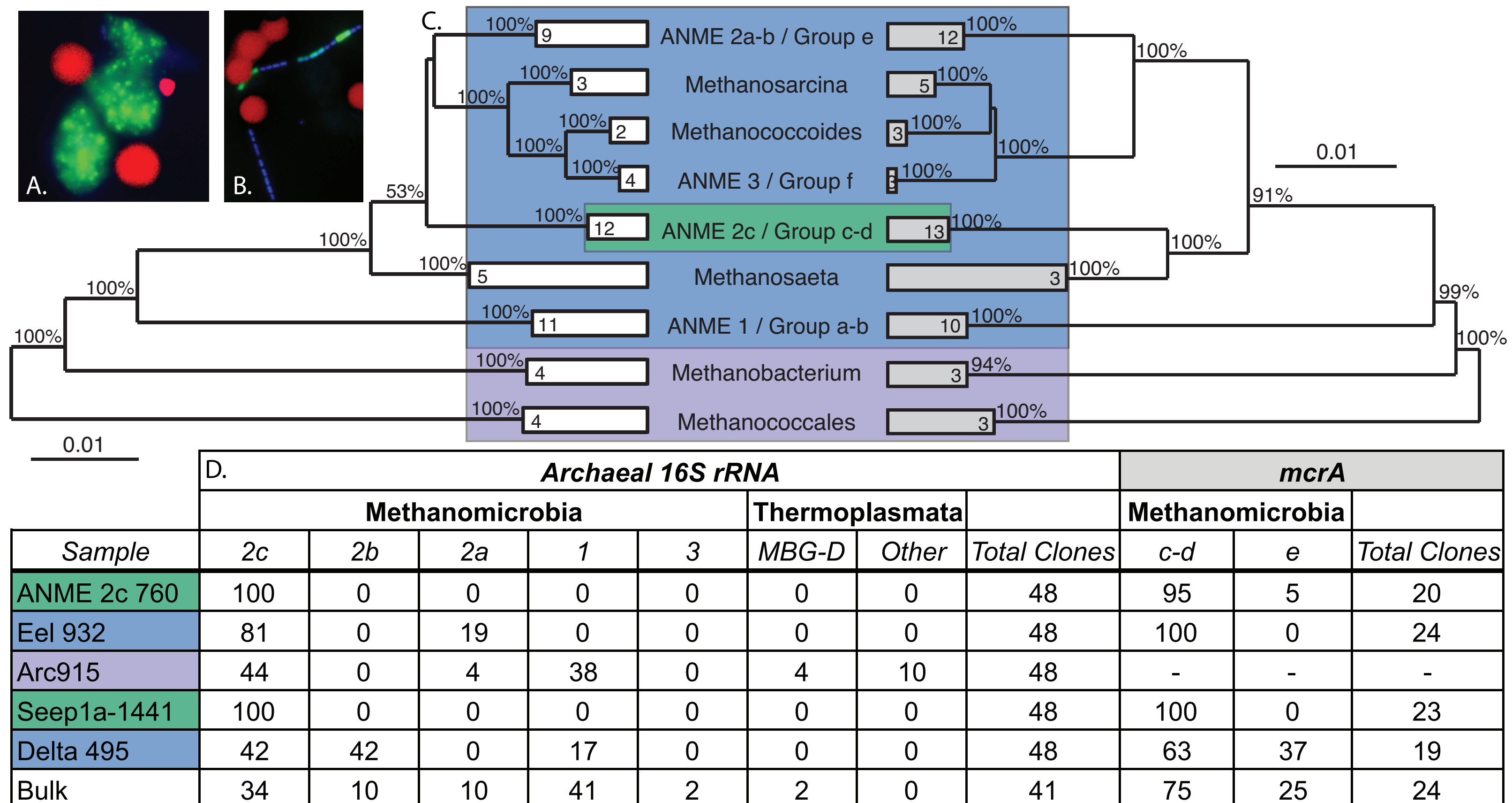
Table 2: CARD-FISH probes, microbial target organisms (Ar, archaea; Ba, bacteria) and associated formamide concentrations (FA %) used in this study with corresponding total DNA yield in nanograms quantified on a Qubit fluorometer from small scale (100 µl) Magneto-FISH captures. The percent bulk yield is calculated by dividing the total DNA recovered for each Magneto-FISH capture by the total bulk DNA recovered from a same volume of paraformaldehyde fixed sediment. Both loss during processing and selectivity of FISH probes used in Magneto-FISH contribute to the estimated percent bulk yield. Seep-1a_1441 DNA concentration was below detection (BD), but only 10% of sample was analyzed due to sample volume constraints. Probe references: Seep-1a_1441 (Schreiber et al. 2010); ANME-2c_760 (Knittel et al. 2005); Eel-MSMX_932 (Boetius et al. 2000); Arc_915 (Stahl & Amann 1991); Delta_495a (Loy et al. 2002).

Sample	*Target Organism(s)*	*FA %*	*Total DNA (ng)*	*Bulk Yield (%)*
ANME-2c_760	Ar, ANME subgroup 2c	50	0.4	3
Seep-1a_1441	Ba, Desulfobacteraceae subgroup Seep-SRB1a	40	BD	-
Eel-MSMX_932	Ar, General ANME	35	0.9	8
Delta_495a	Ba, General Deltaproteobacteria	25	0.8	7
Arc_915	Ar, General Domain-level Archaea	25	1.2	11
Bulk sediment	-	-	11.0	-

To evaluate Magneto-FISH enrichment of target species, clone libraries for both archaeal 16S rRNA and methyl-coenzyme M reductase alpha subunit (*mcrA*) were constructed (Figure 2). *mcrA*

encodes for an enzymatic step common to methanogenic and methanotrophic archaea (Hallam et al. 2003; Luton et al. 2002). Conserved regions can be used as a measure of archaeal diversity in methane seeps, with similar tree topology to archaeal 16S rRNA (Hallam et al. 2003; Hallam et al. 2011; Luton et al. 2002). Parallel analysis of 16S rRNA and metabolic gene diversity in Magneto-FISH capture experiments using complementary (or nested; Amann et al. 1995) suites of FISH probes with differing specificities can assist in evaluating the affiliation of specific metabolic genes with a 16S rRNA phylotype. Results from five independent Magneto-FISH capture experiments, using different probes on the same starting material, recovered the predicted level of archaeal diversity, based on the specificity of the capture probe. For example, archaeal 16S rRNA diversity recovered from ANME-2c_760 and Seep-1a_1441 Magneto-FISH experiments were 100% affiliated with the ANME-2c group, with parallel *mcrA* analysis recovering 95% and 100% of *mcrA* groups c/d, respectively. The *DSS*-affiliated Seep-SRB1a group has been shown in environmental FISH surveys to predominately pair with ANME-2c (Schreiber et al. 2010). The abundance of ANME-2c in both the archaeal 16S rRNA and *mcrA* gene surveys from the SRB targeted Seep-1a_1441 capture is consistent with these findings. These experiments support the results from Pernthaler et al. (2008), demonstrating that high specificity can be achieved with Magneto-FISH. These data also demonstrate the ability to corroborate a microbial association hypothesis, such as ANME-2c/ Seep-SRB1a (Schreiber et al. 2010), with complementary Magneto-FISH experiments.

Figure 2: A. CARD-FISH epifluorescent image of an ANME-2c (FITC) aggregate counterstained with DAPI. B. CARD-FISH epifluorescent image of an ANME-1 (FITC) rod chain counterstained with DAPI. In both images cy3 was over-exposed to show beads (beads are 5 µm for scale). C. Consensus trees of Archaeal 16S rRNA (white boxes) and *mcrA* (gray boxes) genes with ANME clade (16S rRNA) and Group (*mcrA*) names separated by a slash. All other group names apply to both trees. The target range of CARD-FISH probes ANME-2c_760 and Seep1a_1441 (green), Eel_932 and Delta_495 (blue), and Arc_915 (purple) are indicated in the trees and table. D. Table includes percent of total archaeal clones from each Magneto-FISH capture for each archaeal group. *Thermoplasmata* was not included in the trees but is shown in the table to demonstrate the full diversity recovered. No *mcrA* group a-b or f were recovered from Magneto-FISH or Bulk samples, and are not included in the table. *mcrA* clone libraries were not constructed for the Arc_915 Magneto-FISH capture.



	Archaeal 16S rRNA								mcrA		
	Methanomicrobia					Thermoplasmata			Methanomicrobia		
Sample	2c	2b	2a	1	3	MBG-D	Other	Total Clones	c-d	e	Total Clones
ANME 2c 760	100	0	0	0	0	0	0	48	95	5	20
Eel 932	81	0	19	0	0	0	0	48	100	0	24
Arc915	44	0	4	38	0	4	10	48	-	-	-
Seep1a-1441	100	0	0	0	0	0	0	48	100	0	23
Delta 495	42	42	0	17	0	0	0	48	63	37	19
Bulk	34	10	10	41	2	2	0	41	75	25	24

The interesting pattern of ANME-2 diversity in the more general Eel-MSMX_932 and Delta_495a Magneto-FISH samples is another example of the more nuanced information that can be recovered by this technique. Note that the simplified trees in Figure 2 do not resolve the distinction between ANME-2a and 2b; ANME-2b sequences form a coherent clade related to the ANME-2a group (see Figure 2 in Orphan et al. 2001). While ANME-2a and ANME-2b were equally represented in the bulk sediment diversity survey, these closely related archaeal groups showed differential distribution in the Eel-MSMX_932 and Delta_495a Magneto-FISH captures. While these two probes are expected to have similar levels of target group specificity in this system, ANME-2a was not detected in Eel-MSMX_932 samples and conversely ANME-2b was absent in the Delta_495a capture. The Eel-MSMX_932 probe was designed to target all Eel River Basin clones affiliated with the order *Methanosarcinales* (Boetius et al. 2000), but archaeal 16S rRNA ANME-2 diversity contained ANME-2c (81%) and ANME-2a (19%) sequences and no 2b. The Delta_495a Magneto-FISH capture, selecting for general *Deltaproteobacteria*, recovered an equal number of ANME-2c and ANME-2b clones (42%), as well as 17% affiliated with ANME-1, but no 2a. This would suggest that, in this sample, ANME-2c and ANME-2b might be more likely to form associations with *Deltaproteobacteria* than ANME-2a. These hypotheses can be tested with independent FISH hybridization experiments with the original sediment sample (see Pernthaler et al. 2008).

Magneto-FISH can also aid in correlating diagnostic metabolic genes (e.g. *mcrA, dsrAB, aprA, nifH,* etc.) to 16S rRNA identity. Since 42% of the clones in the Delta_495a capture were ANME-2b, *mcrA* sequences that are distinct from the previously described ANME-1 group a-b or ANME-2c affiliated group c-d, may be associated with ANME-2b, a currently undefined *mcrA* group designated here as e'. The bulk sediment distribution within ANME-2 archaeal 16S rRNA sequences alone is 64% - 2c, 18% - 2b, 18% - 2a. The Delta_495a ANME-2 archaeal 16S rRNA distribution is 50% - 2c and 50% - 2b. The bulk sediment distribution of ANME-2 *mcrA* sequences is 75% c-d (2c), 4% e' (2b), 21% e (2a). The Delta_495a distribution of ANME-2 *mcrA* sequences is 63% c-d (2c), 37% e' (2b), 0% e (2a). Since all three ANME-2 groups are found in both archaeal 16S rRNA and *mcrA* clone libraries for bulk sediment, but only 2c (c-d) and 2b (e') are found in Delta_495a there are multiple lines of evidence to support the hypothesis of group e' *mcrA*. It should also be noted that the *mcrA* primers are not complementary to the majority of

ANME-1 sequences, so investigation of ANME-1 correlations between archaeal 16S rRNA and *mcrA* was not possible.

Optimization for Metagenomics

Advances in library preparation and high throughput sequencing protocols have significantly lowered the required amount of DNA for metagenomics (as low as 1 ng DNA with the Nextera XT). However, our small-scale Magneto-FISH captures yield DNA in amounts that are still below current thresholds without including a post DNA amplification (e.g. Multiple displacement amplification, MDA), similar to that used in single cell genomics ((Woyke et al. 2011) and other chapters in this volume) and used in the Magneto-FISH ANME-2c metagenome (Pernthaler et al. 2008).

To determine where the protocol could be optimized to increase recovery and DNA yield, we evaluated the losses associated with the different steps of the Magneto-FISH protocol. The Magneto-FISH cell retention efficiency was assessed by extracting DNA from wash step supernatants during a large-scale Magneto-FISH ANME-2c_760 capture (Table 3). The DNA concentration of the supernatants was then compared to the amount of DNA extracted from PFA fixed bulk sediment of the same initial volume (3 ml slurry). We estimate that ~6% total DNA is lost during the initial liquid CARD-FISH hybridization. An additional 28% is lost after the antibody (IgG) incubation, which can be improved by increasing the speed during centrifugation (discussed in methods). The sample remaining in the post-capture wash is due to both intended (selectivity from magnetic capture) and unintended (poor hybridization and/or unsuccessful magnetic capture) losses. The DNA yield from Magneto-FISH before the magnetic capture step can be estimated by adding the DNA recovered from the post Magneto-FISH wash (430 ng) to the yield from Magneto-FISH sample (145 ng) for a total of 575 ng. Using the specific ANME-2c_760 capture probe, the DNA yield from ANME2c-760 Magneto-FISH is 25% of this estimated total yield. As 33% of the recovered bulk sediment clones are ANME-2c, this is close to the expected level of selectivity. Assuming ANME archaea are the dominant archaea and about 1/3 of the total microbial assemblage based on the ANME:SRB ratio of 1:3 from other Hydrate Ridge studies (Nauhaus et al. 2007; Orphan et al. 2009), and 1/3 of those archaea are ANME-2c (bulk clone library results, Figure 2D), then 1/6 of the bulk sediment extracted DNA would result in a

theoretical yield of 166 ng. The experimental ANME-2c DNA yield (144.6 ng) is 87% of this theoretical yield.

Table 3: DNA recovered from different stages of a large-scale ANME 2c-760 Magneto-FISH sample to examine losses and selectivity. Initial sample was from 3 ml of PFA fixed slurry in EtOH. The percent bulk yield is calculated by dividing the total DNA recovered at each Magneto-FISH step (accounting wash volume differences) by the total bulk DNA recovered from a same volume of paraformaldehyde fixed sediment. *ANME2c-760, post capture sample* is target cells attached to beads at the end of the protocol. *ANME2c-760, post liquid CARD-FISH* is a 50 ml 1x PBS wash supernatant. *ANME-2c_760, post IgG* is the supernatant after 300 x*g* spin to remove remaining anti-body. *ANME-2c_760, post capture wash* is the sediment and Buffer1 removed after the first wash when the sample is in the magnetic holder (remaining non-target cells).

Sample	Total DNA (ng)	Bulk Yield (%)
ANME-2c_760, post capture sample	144.6	14
ANME-2c_760, post liquid CARD-FISH	63.4	6
ANME-2c_760, post IgG	276.3	28
ANME-2c_760, post capture wash	430.5	43
Bulk	1000.0	-

We also examined DNA extraction efficiency by testing a range of methods to improve cell lysis, removal of formalin crosslinks, and losses during DNA precipitation. As discussed in the methods, implementation of an extended heating step was found to reduce PFA crosslinking issues and yielded the greatest improvement to DNA extraction efficiency. The use of conventional organic extraction with phenol:choloroform resulted in higher yields than tested kit protocols (PowerSoil DNA Isolation kit, MO BIO). Recovery of DNA after ethanol precipitation was enhanced by the use of ammonium acetate and linear acrylamide at 0°C (Crouse & Amorese 1987). The theoretical yield of bulk sediment DNA per ml slurry is 10^{-6} g per ml. This is based on 10^{7} aggregates per ml sediment slurry (calculated for this study) and estimates of 10^{2} cells per aggregate (Nauhaus et al. 2007) and 10^{-15} DNA per cell (Button & Robertson 2001; Simon & Azam 1989). Although this calculation does not account for single cells that also contribute to the bulk DNA, single cells are estimated to be 10% or less of the total biomass at Hydrate Ridge (Nauhaus et al. 2007). This theoretical yield is the same order of magnitude as the bulk sediment DNA (experimental) yield of 1000 ng per ml sediment slurry, indicating efficient DNA extraction.

The age of the fixed sample (time since fixation) can also impact the success of the Magneto-FISH capture and DNA recovery. Freshly fixed samples are recommended, when possible. We also evaluated ethanol as an alternative fixative to reduce cross-linking issues during DNA recovery. While CARD-FISH signals were not as bright, bead association was successful and expected clone diversity was recovered. Fixative choice and strength are recommended optimization areas for application of Magneto-FISH to other systems.

We also evaluated the ability of the Magneto-FISH procedure to meet metagenomic library preparation DNA concentration requirements without MDA amplification, by scaling up starting sample volume (large scale Magneto-FISH prep). This large-scale prep is similar to the procedure originally reported in Pernthaler et al. (2008) and outlined in Schattenhofer and Wendeberg (Schattenhofer & Wendeberg 2011) with a few modifications to the magnetic capture and washing steps (described in methods). In the large-scale Magneto-FISH prep, 3 ml of sediment slurry was used instead of 0.1 ml. From this volume of slurry, 48.2 ng DNA per ml slurry was obtained using the ANME-2c_760 specific probe. This is almost 14 times more DNA than a small scale ANME-2c_760 capture, and enough DNA for library preparation using the Nextera XT kit (minimum 1 ng) for Illumina miseq or highseq sequencing. However, the gain in total DNA yield also corresponded with a decrease in specificity. Only 53% of the 16S rRNA phylotypes associated with ANME-2c, compared with 100% in small scale Magneto-FISH captures. The scaled up protocol is still useful for enrichment of the target population, with 33% of the archaeal diversity associated with the ANME-2c target relative to 20% in the bulk sediment. For the larger volume Magneto-FISH protocols, the incorporation of more extensive washing procedures using a separatory funnel apparatus may aid in the removal of contaminating particles and enhance enrichment of the microbial target, as described in Pernthaler et al. (2008) and Schattenhofer & Wendeberg (2011).

Sample specificity and DNA yield should therefore be optimized for downstream needs; if high specificity is required then pooling many small-scale reactions is recommended, otherwise one large-scale reaction may be sufficient. It is also recommended that any samples that need be compared are run together with the same conditions and reagents to reduce any methodological variation.

Optimization for Other Environmental Systems

This Magneto-FISH protocol was developed and optimized for sediment-associated aggregated microorganisms, so optimal application to other systems likely requires adjustments to the liquid CARD-FISH protocol and washing steps for optimal cell recovery. Schattenhofer & Wendeberg (2011) reported enrichment of single SRB cells from hydrocarbon contaminated sediment using a Magneto-FISH protocol similar to Pernthaler et al. (2008). Schattenhofer & Wendeberg (2011) incubated cells with magnetic beads already labeled with antibodies, which may reduce single cell loss during antibody wash steps in the method described here.

To evaluate the method presented here for single cell Magneto-FISH, we focused on ANME-1. At Hydrate Ridge, ANME-1 are found predominately as single cells or chains of single cells rather than in association with SRB and have a distinctive rod-shaped morphology (Knittel et al. 2005). When using the general Arc_915 probe to target all archaea in small-scale Magneto-FISH experiments, we were able to recover ANME-1 phylotypes at bulk sediment clone abundance. We also observed single cells and chains attached to beads indicating the potential to enrich for non-aggregated cell types using this Magneto-FISH method. We then tried Magneto-FISH with an ANME-1 specific probe to select for a single cell population. We used ANME-1_350 (Boetius et al. 2000) with 30% formamide. We confirmed single cells and chains attached to beads by microscopy (Figure 2B). However, we did not recover quantifiable amounts of DNA and clone abundances were below bulk sediment ratios. Since ANME-1 represented 44% of the recovered archaeal bulk sediment diversity, this should not be due to issues with targeting too small a population.

A possible explanation is that more specific probes are more successful if they work at a higher stringency. When testing Magneto-FISH with without adding probe or adding non-sense probes at 5-10% formamide, it is possible to collect non-specifically bound aggregates. Non-specific capture was confirmed by microscopy (beads attached to aggregates without any CARD signal) and DNA extraction yields. DNA yields from these samples was below or near the limit of detection, but similar to the DNA concentration of ANME-2c_760 and Seep-1a_1441 samples. However, ANME-2c and Seep-1a captures return only the expected single species and do not show signs of non-specific binding. ANME-2c_760 (50%) and Seep-1a_1441 (40%) probes had higher

formamide concentrations than ANME-1_350 (30%). Only less specific probes such as Arc_915 and Delta_495a (25% formamide) returned the expected population at lower formamide concentrations. Optimization of Magneto-FISH for other systems and/or non-aggregate forming populations may be more successful when utilizing probes with targeted, high specificities.

Summary

Magneto-FISH provides a method to target microbial associations from environmental samples for metagenomic and other molecular analyses with high specificity. It is adaptable to a range of target populations within a system, working from the vast array of already vetted FISH probes or developing new ones. It is also an affordable technique since it does not require any special training or equipment beyond the contents of a normal microbiology laboratory. While the method was designed for ANME-2 aggregates and associated bacteria, it can be applied to and optimized for a range of microbial systems utilizing the recommendations described herein. By enriching for associations prior to metagenomic analysis, the genetic information obtained is for a working partnership that may otherwise be lost in a bulk environmental analysis. This middle ground will be invaluable in the effort to better understand all levels at which microbes function in an environment, and in particular in understanding how microbial associations on small scales reflect larger scale chemical and nutrient cycling.

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