USING SINGLE-CELL SORTING, FISH AND 13C-LABELING TO CULTIVATE AND ASSESS CARBON SUBSTRATE UTILIZATION OF ‘AIGARCHAEOTA’ AND OTHER NOVEL THERMOPHILES

Damon Kurtis Mosier
damon.mosier@csusb.edu

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USING SINGLE-CELL SORTING, FISH AND $^{13}\text{C}$-LABELING TO CULTIVATE AND ASSESS CARBON SUBSTRATE UTILIZATION OF ‘AIIGARCHAEOTA’ AND OTHER NOVEL THERMOPHILES

A Thesis
Presented to the
Faculty of
California State University,
San Bernardino

In Partial Fulfillment
of the Requirements for the Degree
Master of Science
in
Biology

by
Damon Kurtis Mosier
September 2019
USING SINGLE-CELL SORTING, FISH AND $^{13}$C-LABELING TO CULTIVATE AND ASSESS CARBON SUBSTRATE UTILIZATION OF ‘AIGARCHAEOTA’ AND OTHER NOVEL THERMOPHILES

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Approved by:

Dr. Jeremy Dodsworth, Committee Chair, Biology

Dr. Michael Chao, Committee Member

Dr. Laura Newcomb, Committee Member
ABSTRACT

‘Aigarchaeota’, a deeply branching lineage in the domain Archaea with no cultivated representatives, includes both thermophilic and hyperthermophilic microorganisms that reside in terrestrial and marine geothermal environments. The ‘Aigarchaeota’ consists of at least nine proposed genus-level groups that have been confirmed via 16S rRNA sequencing, with ‘Aigarchaeota’ Group 1 (AigG1) being the focus of this study. Based on cultivation-independent genomic data available from several AigG1 members in Great Boiling Spring (GBS), NV, and Yellowstone National Park, 22 different types of growth media were designed and tested for their ability to support growth of AigG1. One of these cultures, G1-10, was found to contain AigG1 at ~5% abundance, as well as other novel thermophilic microbial groups including a new species of the genus *Pyrobaculum*, members of the candidate phyla ‘Calescamentes’ and ‘Fervidibacteria’, and the novel archaeal lineage NAG1 (‘Geoarchaeota’). To attempt to obtain pure cultures of AigG1 and other novel thermophiles, a single-cell sorting system using an optical trap and a microfluidic device was constructed. The system was validated by sorting *E. coli* cells, which demonstrated that single, viable cells could be reliably obtained. Using this single cell sorting device on the G1-10 culture, a pure culture of a member of the genus *Pyrobaculum* was obtained, which was shown to represent a distinct species in this phylum by whole genome sequencing and in silico DNA-DNA hybridization. Additionally, a pure culture of the first representative of the candidate phylum
‘Fervidibacteria’ from an enrichment culture derived from G1-10. Additionally, to aid in morphology-based sorting of AigG1 and stable isotope labeling studies, fluorescence in situ hybridization (FISH) based on catalyzed reporter deposition (CARD-FISH) were developed and an AigG1-specific probe was tested. CARD-FISH was successfully used to detect AigG1 in both the G1-10 culture and in natural sediment samples from GBS. Stable isotope labeling incubations were performed with a variety of $^{13}$C-labeled substrates (bicarbonate, amino acids, sugars, and short chain fatty acids) on GBS sediments and G1-10 culture samples, and CARD-FISH was used to specifically detect AigG1 in the fixed samples. Nanometer-scale secondary-ion mass spectrometry (nano-SIMS) will then be used to determine whether AigG1 was capable of taking up the different carbon substrates tested. Overall, the results and accomplishments from this project and follow up nano-SIMS analysis will allow a better understanding of the metabolic potential of AigG1 and will aid future efforts to attempt to obtain pure cultures of this novel lineage.
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CHAPTER ONE

BACKGROUND

While more than 1.8 million eukaryotic species have been identified to date, a mere 15,626 species of prokaryotes, a combination of the domains Bacteria and Archaea, have been described since the invention of the petri dish by Julius Richard Petri over 100 years ago (Parte, A.C, 2018; http://www.bacterio.net/-number.html). Although there is a drastic difference in the number of described species, it is not reflective of the total abundance of prokaryotes; it is predicted, based on continued genomic studies, that the diversity of prokaryotes is likely vastly greater than that of eukaryotes, with estimates of the total number of species ranging from 246,841 to approximately 1 trillion (Locey and Lennon, 2016; Yarza et al., 2014). This large discrepancy in numbers is largely due to the lack of cultivated representatives. Lack of cultivation can be due to a variety of reasons, including the inability of some microbes to grow on solid media or their slow growth rates and low abundance in natural samples. Many of these organisms also come from extreme environments that require unique growth conditions or undergo obligatory interactions that prevent the attainment of pure cultures. The term “microbial dark matter” has been used to define this unknown microbial majority, with an overwhelming majority of microorganisms being uncultivated to date (Marcy et al. 2007; Alain and Querellou, 2009; Wu et al. 2009; Dodsworth et al. 2013; Rinke et al. 2013).
Studying microbial species in a laboratory setting allows for varied growth environments and medium to be used to perform phenotypic tests, a valuable option when paired with environmental studies. Because of difficulties associated with cultivation, the vast majority of microbes are known only in the form of 16S rRNA gene clones obtained from metagenomic environmental samples (Stott et al. 2008). Although this sequencing data is helpful for basic identification and taxonomic classification, it is of limited value in predicting the functions of microbes, especially those with no close relatives that have been isolated and studied. Obtaining pure cultures or defined co-cultures of these currently uncultivated microbes will allow for a better understanding of their metabolic capabilities and the interactions they likely undergo in their natural environment.

Cultivation Methods

A number of techniques have been (and are still) used in attempts to cultivate, with the most common being streaking for isolation on agar plates. This method requires growth on solid media and is ideal for microbes with relatively short doubling times - both characteristics that are uncommon for many of the uncultured majority (Puspita et al., 2012). Other methods of isolation include inoculating into/onto selective, differential, or enrichment media, or dilution-to-extinction (serial dilution) experiments. Inoculating into/onto selective, differential, or enrichment media includes introducing a mixed sample of microbes to either solid or liquid media that is preferential to the microbe of interest. This method requires prior knowledge of the microbe of interest's growth conditions, which is
uncommon when there are no currently cultivated representatives or close relatives. Dilution-to extinction (serial dilution) includes a step-by-step decrease in the concentration of microbes found in mixed culture, with the goal being to reach a concentration where only one species is present. While useful, this method can only be conducted successfully when the microbe of interest is relatively abundant in the mixed culture.

Technological advances have led to increased interest in uncultivated microbes in an effort to develop our current understanding of taxonomic diversity (Stott et. al 2008). Developments include the equipment and techniques necessary for single cell isolation, genome acquisition, transcriptomics, proteomics, and others (Hu, 2016). Such advances allow for a greater idea of overall microbial abundance and provide hope that further efforts will lead to successful discoveries.

Single cell isolation is an alternative method of microbial isolation that has the potential to overcome a number of common obstacles when attempting to cultivate, including low microbial concentrations, slow growth rates, undefined cultures, etc. There are a number of different single cell sorting methods, including fluorescence-activated cell sorting (FACS), magnetic-activated cell sorting (MACS), laser capture microdissection (LCM), manual cell picking (micromanipulation), and optofluidics (optical trapping within a microfluidic device), that are used to study heterogeneous cell mixtures (Hu, 2016). While all of these methods can be used for single cell isolation, each has both benefits
and downfalls. System throughput, specificity, skill level required, cell survival rates, and associated costs are all factors to take into account when comparing these isolation methods.

Fluorescence-activated cell sorting (FACS) is a type of flow cytometry that uses cell size, granularity, and fluorescence to identify different cell types. Most widely used for isolated cell populations, FACS offers high specificity and throughput and can be used for mixed culture samples. Magnetic-activated cell sorting (MACS) is a method that binds specific proteins to the target cells via magnetic beads. These beads activate when subjected to a magnetic field, thus polarizing any labeled cells. Although this method offers high specificity, throughput, and is cost effective, it is best utilized when a culture is purified >90%, a feature that is uncommon when studying novel microbes. Laser capture microdissection (LCM) is an isolation method with two current classes, infrared (IR LCM) and ultraviolet (UV LCM). Although accurate at detecting specific cell types, a requirement is that you are able to accurately identify the target cells, a luxury that is not available when concerning uncultivated microbes with no known close relatives. Manual cell picking (or micromanipulation) is a method that uses a microscope and mechanical stages with connected micropipettes. Much like LCM, the cells of interest must be identifiable in order for this method to be effective. Optofluidic single cell sorting, a method for viewing and separating live microbial cells via microscopy, is another technique that can be used to isolate cells for cultivation but is not often implemented due to the level of difficulty and
associated costs (Dodsworth et al., 2013; Landry et al., 2013). This method is most often used in single-cell genomics studies but can be adapted to be used to single cell isolation for cultivation. Adaptations include bypassing certain portions of the microfluidic device as well as including the use of different surfactants to prevent cell adhesion.

Cultivation-Independent Methods

In addition to these novel methods of cultivation, a number of cultivation-independent techniques exist to study microbes of interest. The most common is the use of 16S rRNA (ribosomal RNA) gene sequences (or gene sequence clones), known to contain both highly conserved and variable sequences in prokaryotic organisms. Often obtained from environmental samples, this sequencing data is helpful for basic identification and taxonomic classification of novel microbes. Methods such as PCR and qPCR utilize 16S rRNA in order to create standards, while genomic and metagenomic methods use 16S rRNA as a tool to expand the library of currently uncultivated microbes. Although a number of cultivation-independent methods for studying microorganisms exist, those that will impact the proposed work include fluorescence in situ hybridization (FISH), nanoscale secondary ion mass spectrometry (nano-SIMS), and stable isotope probing (SIP).

Fluorescence in situ hybridization (FISH) utilizes site-specific probes, often targeting 16S rRNA, to identify and enumerate specific microbial taxa within complex mixtures. Variations of this method include Clone-FISH and CARD-
FISH. Clone-FISH allows for construction of pseudo-positive controls for FISH probes targeting uncultivated microbes and involves heterologous expression of 16S rRNA genes of uncultivated microbes in an appropriate *E. coli* strain (Schramm et al, 2002). Catalyzed Reporter Deposition FISH (CARD-FISH) techniques are often used to increase fluorescent signal through the use of the enzyme Horseradish Peroxidase (HRP), which reacts to create a fluorescently-labeled tyramide radical intermediate, which will then bind to adjacent proteins. One issue with this method is achieving permeabilization of cells without completely degrading cell structure (Kubota, 2013). For Archaea this is often achieved by testing treatment with proteinase K (or other proteases) under a variety of different concentrations, incubation times/temperatures, to aid in permeabilization of archaeal proteinaceous, S-layer-based cell walls.

Nanoscale Secondary Ion Mass Spectrometry (nano-SIMS) can be used as a method to “investigate the dynamics of carbon and nitrogen assimilation activities in individual microbial cells” (Morono et al, 2011). Through the introduction of isotopically labelled compounds, substrate uptake can be analyzed for these microbes of interest. This introduction of substrates labelled with stable elements that are not naturally abundant (e.g. $^{13}\text{C}$, $^{15}\text{N}$, $^{18}\text{O}$) into essentially any given sample, including both laboratory cultures and natural samples, will allow for the assessment of specific utilization of these substrates and for further assessment of potentially ideal growth conditions. Upon yielding successful results from fluorescence in situ hybridization experiments, these
methods can be combined (FISH-nano-SIMS) where cells found to be positive (based on fluorescence) can be targeted when running nano-SIMS experiments.

Uncultivated Microbes and Candidate Phyla

Although less widely studied than Bacteria, Archaea are likely to prove to be of great evolutionary importance, with a number of recent studies proposing the possibility of Archaea being more closely related to the domain Eukarya than the domain Bacteria (Spang et al., 2015; Koonin and Yutin, 2014). Proposed to be the oldest of the three domains of life, establishing links to Eukarya could result in a greater understanding of the evolution of life on Earth and the derivation of eukaryotes. There were only two recognized phyla of Archaea for more than a decade leading into the early 2000’s, with any detected 16S rRNA sequences simply being placed under these phyla; recently, however, many additional archaeal phyla have been recognized (Spang et. al, 2017). Several of these Archaeal phyla have since been grouped together to form the “TACK” superphylum, named for its composition that consists of the ‘Thaumarchaeota’, ‘Aigarchaeota’, ‘Crenarchaeota’, and ‘Korarchaeota’ phyla (Guy and Ettema, 2011; Figure 1, top). Although this expansion of the archaeal phylogenetic tree expands our knowledge on the diversity of archaea, there is still much to be learned about these microbes.
Figure 1. Phylogenetic diversity of archaea, both cultured and uncultured (top). Eukaryotic signature proteins found in various archaeal taxa (bottom). Figures taken from Guy and Ettema (2011) and Spang et al. (2017) for top and bottom, respectively.
Genomic analyses of members of the “TACK” superphylum have identified the presence of a number of eukaryotic signature proteins (ESPs; Figure 1, bottom), leading to the possibility that they may represent a sister lineage to the progenitor of Eukaryotes (Guy and Ettema, 2011; Spang et. al, 2015). Although progress has been made using genomic methods, testing the functions of these ESP’s in Archaea will be greatly facilitated if we have these microbes in pure or mixed laboratory culture.

‘Aigarchaeota’

The candidate phylum ‘Aigarchaeota’ is a deeply branching lineage in the Domain Archaea found in terrestrial and marine geothermal environments worldwide (Guy and Ettema, 2011; Beam et al., 2015; Hedlund et al., 2015). No members of the ‘Aigarchaeota’ have been isolated, and their maintenance in mixed laboratory culture has not yet been reported. This phylum-level lineage is thought to contain at least nine proposed genus-level groups (Figure 2) based upon analysis of 16S rRNA gene sequences (Hedlund et al., 2015).
While metagenomic studies have shown a global distribution of ‘Aigarchaeota’, there are sites of particular interest that may answer the questions that we have about these novel microbes including terrestrial, marine, and subsurface thermal environments (Hedlund et al., 2015). One such site, Great Boiling Spring (GBS), is found in Gerlach, Nevada in the northwestern part of the United States Great Basin (Costa et al., 2009). Great Boiling Spring is a circumneutral geothermal hot spring that hosts both thermophilic (exhibit optimal growth at temperatures between 45-80 °C) and hyperthermophilic (optimally grow at temperatures over 80 °C) bacteria and archaea (Cole et al., 2013).

Great Boiling Spring (GBS) serves as the main research and sampling site for this study and offers temperature variation and distinct sediment and water microbial communities, providing an ideal environment with high output potential. Metagenomic studies on these communities have shown GBS to contain a high proportion of novel microbial lineages (Cole et al., 2013; Hedlund et al., 2015). Among these lineages is ‘Aigarchaeota’ Group 1 (AigG1), one of the nine
previously mentioned genus-level groups, which is abundant in sediments of hot springs in the GBS thermal area (Figure 2; Costa et al., 2009). Based on genomes reconstructed from metagenomic data from Octopus Spring, Yellowstone National Park (YNP), as well as from hot springs in Tengchong, China, AigG1 is predicted to be an aerobic, chemoorganoheterotroph that utilizes oxygen as the primary electron acceptor (Figure 3; Beam et al. 2015) and is capable of fixing carbon (Hua et al. 2018).

![Figure 3. Predicted metabolism of ‘Aigarchaeota’ lineages. Figure taken from Beam et al, 2015.](image-url)
Laboratory Cultures

Attempts to cultivate AigG1 have been made in the Dodsworth lab at CSUSB, yielding a stable laboratory culture containing AigG1. Methods to establish and maintain this culture were determined by the Dodsworth lab as described below. Based on metabolic predictions and cultivation-independent genomic data available from several AigG1 members in Great Boiling Spring (GBS), NV, and Yellowstone National Park, 22 different types of growth media were designed and tested for their ability to support growth of AigG1 (Figure 4). These media were inoculated in the field with freshly collected GBS sediment, incubated at 80°C in the laboratory, and transferred (1/100 vol.) after 3-4 weeks of growth.

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Figure 4. Media conditions (top) and optimal growth times verified via qPCR (bottom) of ‘Aigarchaeota’ Group 1.

Of the 22 growth conditions, only two were found to support significant growth of AigG1 based on 16S rRNA quantitative PCR (Figure 4) and gene tag sequencing (Figure 5). These growth conditions included hot spring mat extract with 2% oxygen and casamino acids with 10% oxygen. AigG1 ranged from 1-20% of the total population of microorganisms, reaching densities up to $10^7$ 16S rRNA gene copies/mL. These cultures (named AigG1-10) are now maintained in lab and represent the first laboratory cultures containing AigG1. Interestingly, these cultures also contain several other novel microbes, including members of the candidate bacterial phyla ‘Fervidibacteria’ and Calescamantes, uncultivated archaeal lineage “Geoarchaeota”, and novel species- to family-level groups of
Archaea in the phylum Crenarchaeota. This laboratory culture offers an opportunity to further enrich for and potentially isolate AigG1 and other novel lineages, and to study these lineages in mixed culture under defined laboratory conditions.

Figure 5. Growth rates of ‘Aigarchaeota’ and other novel lineages in media conditions listed in Figure 4 above.

Specific Project Goals

Attempts to cultivate AigG1 have been made in the Dodsworth lab at CSUSB, yielding a stable laboratory culture containing AigG1. With this culture being the basis of much of the proposed work, the following goals for this thesis project have been established in an effort to further studies on and cultivate ‘Aigarchaeota’ Group 1 and/or other novel microbes:
- Goal 1: Set up, validate, and use an optofluidic single cell sorting system to obtain pure or more simplified cultures of AigG1 and other novel thermophiles
- Goal 2: Develop and use FISH methods coupled with nano-SIMS to track the uptake of $^{13}$C-labeled compounds by AigG1

Figure 6. Major steps in accomplishing Goals 1 and 2, showing potential use of FISH methods (Goal 2) for targeting AigG1 cells for sorting (Goal 1).
CHAPTER TWO

METHODS

Cultivation and Maintenance of G1-10 Cultures

As mentioned previously, stable laboratory cultures (named G1-10) that contain Aig-G1 have been established. Continued growth of AigG1 is maintained with a medium consisting of a 1:1 mixture of GBS salts medium (Dodsworth et al., 2014) and filtered GBS spring water. This medium is sparged with nitrogen gas to remove oxygen, and 50 mL aliquots are added to 150 mL bottles that are sealed with butyl rubber stoppers and aluminum crimps in an anaerobic chamber. The headspace of the bottles is exchanged with nitrogen by three cycles of vacuuming and filling with nitrogen at 12 psi, and bottles are subsequently autoclaved. The medium is supplemented with additional components from sterile, anaerobic stocks to the following final concentrations: 2 mM HEPES pH 7.5, 0.2 mM bicarbonate, 5 mL/L of a trace elements solution (Dodsworth et al., 2014), a nutrient mixture (added to final concentrations of 2 mL/L vitamin mixture, 0.002% yeast extract, and 0.1 mM sodium phosphate pH 7), and 0.02% casamino acids. 50 mL of air is then added via a syringe and 0.2 µm filter, yielding ~10% oxygen. The media is typically inoculated with 0.5 mL of growth from a previous culture, incubated at 80°C, and transferred every 3 weeks.
qPCR for Assessing Abundance of AigG1 in Laboratory Cultures

In an effort to assess abundance of Aig-G1 in the G1-10 cultures, quantitative PCR (qPCR) is done regularly. This not only provides us with a relative abundance of AigG1 and other novel microbes in the culture but allows for confirmation that they are persisting over time.

Following the three week incubation period and the transfer of 0.5 mL into a new incubation bottle, G1-10 cultures are chilled on ice, vortexed, and centrifuged in 50 mL conical tubes at 9500 rpm for 10 minutes. All but 1 mL of supernatant is discarded, the pellet is resuspended in leftover supernatant, transferred to a 1.5 mL microcentrifuge tube, and centrifugation is done at 14,000 rpm for 5 minutes. Supernatant is discarded and the pellet is stored in -80°C until DNA extraction.

DNA extraction is done using the steps and materials from the FastDNA SPIN Kit for Soil available from MP Biomedicals (Irvine, CA), essentially as described in Costa et al (2009) except that samples were processed in a bead beater twice for 30 seconds at setting 4.5 m/s. This is a DNA extraction kit designed for extraction of DNA from soil samples. It is relatively robust, and often used for unknown samples in the case the cells are difficult to lyse. DNA was eluted in 100 µL of DES solution, and 1/10 dilutions were made in 10 mM Tris buffer pH 8. Extracts were stored at -20°C until further experiments.

Following extraction of DNA, qPCR is done in a 96 well plate using the ThermoScientific ABsolute Blue qPCR reagent mix, along with our own specific
primer sets and template. The ThermoScientific ABsolute Blue qPCR reagent mix comes as a 2x stock, and it contains everything necessary for the reaction except template and primers, i.e. it has buffer, MgCl₂, dNTPs, and polymerase. It also contains two dyes: SYBR Green, which binds to double-stranded DNA; and a small amount of the fluorescent dye ROX, which is used as a standard. Primers used for specific detection of AigG1 16S rRNA genes are AigG1for (5’ TGCcGGCTAGCTGTCGGGG) and AigG1rev (5’ TcGTCAGGTAGGGTCGTcA) with an annealing temperature of 64°C. Primers for determining total bacterial and archaeal gene copies are 515F (5’ GTGYCAGCMGCCGCGGTAA) and 806R (5’ GGACTACTCHVGGGTWTCTTAAT), adapted from Kozich et al (2013), with an annealing temperature of 55°C. Together these primer sets can be used to determine the absolute abundance (AigG1-specific) of AigG1 in mixed samples, while relative abundance (AigG1-specific/universal) will be determined with the following: bacterial forward primer 9bF (5’ GRGTTTGATCCTGGCTCAG), archaeal forward primer 8aF (5’ YCYGGTTGATCCTGCC), and universal reverse primer 1512uR (5’ ACGGHTACCTTGTTACGACTT). Template used is obtained from the DNA extractions from G1-10 cultures described above (done in triplicate), while the standard curve consists of duplicate reactions of 10⁴-10⁸ copy/µL dilutions of the plasmid G04b_L4_A09, which contains a near-full length AigG1 16S rRNA gene clone (Costa et al., 2009). Most often, the 1/10 dilutions of template DNA are used for qPCR, although in some cases undiluted template or further diluted template may be used. To perform qPCR, a “master mix”
consisting of 2x qPCR buffer/mix, 400 nM forward primer, 400 nM reverse primer, and PCR-grade water is made, and 20 µL aliquots of this master mix are added to wells in a 96 well plate. Template (5 µL) is then added to each well, with 5 µL of water used in negative control reactions. Following template addition, the 96 well plate is sealed with optically clear sealing tape, vortexed, and centrifuged briefly. Plate is inserted into an Applied Biosystems StepOne real time PCR machine and the run is started using the following cycling parameters: 15 minutes at 95°C; 40 cycles of 15 sec at 95°C, 30 sec at 64°C (AigG1) or 55°C (Bacteria/Archaea), and 45 seconds at 72°C. Fluorescence is detected during the 72°C step of each cycle. This is followed by a melt curve from 60-95°C at 0.3°C steps (5 seconds each). StepOne software calculates a threshold fluorescence based on the standards, and Ct values are subsequently calculated from the resulting standard curve for each of the tested experimental templates.

Setup of Optical Trap (Laser Tweezers)

Based on a previous design used for single-cell genomics (Landry et al., 2013), a Nikon Ti-U Eclipse microscope was adapted for single-cell isolation using optical trapping (laser tweezers). A 2-watt, 980 nm infrared laser is used to prevent toxicity to live cells, while the shutter in the beam path allows for controlled exposure to the Polydimethylsiloxane (PDMS) optofluidic device and any cells of interest. For optimal trapping of particles (cells), the 2-watt, 980 nm infrared laser (DL980-3T0-O, Crystalaser, Reno, Nevada) is connected by a fiber optic cable to an initial lens (PAF-X-18-PC-B, ThorLabs, Newton, New Jersey),
directed by broadband mirrors (BB1-E03, ThorLabs) through a Galilean beam expander consisting of two lenses (LB1374-B and LB1596-B, ThorLabs) and a periscope assembly (RS99, ThorLabs) into the Nikon microscope, where the beam is reflected into the back of a 100x phase contrast objective lens (DRD31905, Nikon, Melville, NY) using a dichroic mirror (DMSP805R, ThorLabs) installed in a filter cube (MDFM-TE2000, ThorLabs). Reflection of the laser to the microscope camera is prevented using a shortpass filter (FESH0750, ThorLabs) installed in the filter cube. With this end goal of the laser aligning perfectly with the objective lens, all contents were placed in general areas and carefully moved accordingly with the help of kinematic mounts for precision. Since the 980 nm wavelength is not within the visible light spectrum, a special card (VRC4 ThorLabs) that allowed for viewing was used to approximate location of the beam. The schematic (Figure 7) and setup were based on work by Paul Blainey and collaborators at Stanford University (Landry et al, 2013).
Figure 7. Photograph (a) and schematic (b) of an optical trap for single-cell sorting, set up on a Nikon Ti-U Eclipse microscope.

Optofluidic Cell Sorting

An optofluidic technique involving optical trapping within a microfluidic device has been applied in single-cell genomic studies (Landry et al., 2013), where it was successfully used to isolate rare members of the candidate phylum OP9 based on morphology (Dodsworth et al., 2013). Utilizing this experience and the Nikon Ti-U Eclipse microscope equipped with optical trapping (laser tweezers) for cell sorting and a microfluidic device made from polydimethylsiloxane (PDMS), a cell sorting protocol was developed (Figure 8).
Figure 8. Single-cell sorting for cultivation. (a) Schematic of a 48 chambered PDMS microfluidic device for optofluidic sorting. (b) Photograph of the microfluidic device on an inverted microscope stage. (c) Major steps in preparing the microfluidic device, sorting single cells by laser tweezers, and harvesting from the device.

Basic steps for sorting include: 1) treatment of the microfluidic chip with a sorting solution (varies by experiment, but often contains surfactants to prevent cell adhesion); 2) Pressurization of the control layer and the addition of cell
sample through the proper port; 3) Trapping and sorting of desired cell into the cell holding chamber; 4) Closing off the cell in the holding chamber and flushing the remaining cell sample from the chip out of the waste port; 5) Harvest the cell into a pipet tip and inoculate media appropriately. Step-by-step instructions for cell sorting using this setup can be found in Appendix 1, while variations by experiment and cell sample type are explained below.

**E. coli Control Sorting Experiments**

To validate that single, viable cells could reliably be obtained using the optofluidic device, control sorting experiments were performed using *E. coli*. The cell sorting protocol was carried out as mentioned above, with the sorting solution including the use of surfactants tween 20 and F127 pluronic in low concentrations of 0.02% and 0.04% respectively to prevent cell adhesion. Putative single-cell suspensions harvested from the device were pipetted into 500 µL of LB growth medium, aliquoted equally five times in 100 µL portions into a 96 well plate, incubated for 2 days at 37°C, and scored for growth by turbidity following the 2 day period. Some of the putative single-cell suspensions were taken from wells that were not sorted into, while other wells in the 96 well plate were simply aliquoted with sterile LB growth medium, generating possibilities for a false positive (contamination). The positive control channel of the chip was also utilized to test for growth conditions in the 96 well plate.
G1-10 and Other Sorting Experiments

In an attempt to obtain single viable AigG1 cells, single cell sorting is performed on the G1-10 lab cultures. Cultures are sampled at times when AigG1 proves to be most abundant (after 3 weeks of incubation), with the cell sorting protocol being carried out as mentioned above. The sorting solution will include some variety of surfactants (likely tween 20 and/or F127 pluronic) in low concentrations until desired sorting and growth conditions are found. Putative single-cell suspensions harvested from the device are pipetted into 500 µL of G1-10 culture medium, aliquoted into 10 mL bottles using a sterile needle and syringe, incubated long-term at 80°C, and scored for growth by turbidity every 3...
days. The positive control channel of the chip is also utilized to test for growth conditions, necessary surfactant concentrations, and in attempts to achieve a simpler mixed culture. Successful sorting experiments (cultures that exhibit growth) will undergo cell harvest and DNA extraction, followed by PCR with general bacterial (9bF - GRGTTTGATCCTGGCTCAG) or archaeal (8aF - YCYGGTTGATCCTGCC) forward primers and a universal reverse primer (1512uR - ACGGHTACCTTGTTACGACTT) at an annealing temp of 55°C to identify cell types as described in Costa et al (2009). Products obtained are identified by Sanger sequencing using the appropriate forward primer at Retrogen (San Diego, USA; www.retrogen.com), and resulting sequences are identified by BLASTn using the NCBI non redundant nucleotide (nr/nt) database (Altschul et al., 1990; Morgulis et al., 2008; Zhang et al., 2000). Those containing pure cultures of AigG1 or other taxa of interest will be characterized for growth at different temperatures and on various substrates, and whole genome sequencing, in preparation for formal description as new taxa.
Follow-up Experiments

The DNA extraction was done essentially as described in the "JGI Bacterial DNA isolation CTAB-2012" protocol (which can be found at the website jgi.doe.gov/user-programs/pmo-overview/protocols-sample-preparation-information), with libraries being made using the Nextera DNA Flex Library Prep kit (#20018704, Illumina), and 2x150 bp sequencing performed using the Illumina iSeq instrument according to the manufacturer’s instructions. Sequence read processing was done using Trimmomatic (Bolger et al 2014), assembly using SPAdes (Bankevich et al 2012) as implemented in Unicycler (Wick et al 2017) was done on the online platform Galaxy (Afgan et al 2018).

In addition to genome sequencing, dilution-to-extinction (serial dilution) and streak plating are attempted on any potential isolates. Dilution-to-extinction
will be performed based on cell counts prior to transfer, while streak plating will be done using a standard approach of making the liquid medium regularly used for growth and adding a solidifying agent at a concentration of 1.2%. Samples will then be incubated at 80°C and observed for growth on a weekly basis. Single-cell sorts that exhibit growth but are suspected to be the same as a previously grown isolate will undergo Restriction Fragment Length Polymorphism (RFLP) analysis using the Msp I restriction enzyme. The process includes combining 5 µL of the PCR product with 1 µL of the Msp I restriction enzyme, incubating at 80°C for 1 hour then holding at 4°C to stop activation. Following the incubation period, this was run out on a gel next to a 100bp DNA ladder using previous successful Pyrobaculum sorts as positive controls. This will allow for analysis of band sizes in an effort to identify duplicate sorts and eliminate unnecessary sequencing costs.

FISH

In preparation for FISH, cell fixation and washing must be done. Throughout all fixation and washing steps, cells are kept on ice. All steps following fixation are done using filtered pipet tips to provide an RNase free environment to prevent degradation of the rRNA. The fixation protocol used includes the following steps: 1) Pellet cells at 4°C in cooled centrifuge at 10,000 x g for 5 minutes, then discard supernatant (for large volumes, this can first be done in a 50 mL conical tube and then stepped down to a 1.5 mL tube); 2) Wash once with 500 µL of 1x PBS, resuspend, and centrifuge at 10,000 x g for 5
minutes. Discard supernatant; 3) Resuspend cell pellet in 0.5 mL 1% ice-cold paraformaldehyde (solution contains 13.5 mL high purity water, 0.15 g paraformaldehyde (PFA), 5 µL 5 M NaOH, 5 µL 5 M HCl, and 1.5 mL 10x PBS passed through a sterile 0.2 µm filter, with the HCl and 10x PBS being added after PFA is dissolved into solution); 4) Incubate on ice for 1 hour to fix; 5) Pellet cells by centrifugation at 10,000 x g for 5 minutes, then remove supernatant and discard in the appropriate waste container located under a fume hood; 6) Wash cells three times in 500 µL 1x PBS by centrifuging at 10,000 x g for 5 minutes and removing supernatant between each wash; 7) Resuspend remaining pellet in 100 µL DEPC water and add an equal volume of 100% ethanol, then mix well and store at -20°C.

Once fixed, washed, and stored at -20°C, cell samples can then be used to perform FISH experiments. FISH preparation begins by adding 2 µL of the fixed cell sample to a well of a gelatin-coated slide, drying in the fume hood, and dehydrating with 80%, then 95% ethanol by dipping in the solution then allowing to air dry. Based on predetermined formamide concentrations (usually optimized when performing clone-FISH), a hybridization solution is prepared for AigG1 (the optimal formamide percentage was found to be 40%) with the solution containing 360 µL 4.5 M NaCl, 36 µL 1 M Tris pH 8, 720 µL Formamide, 682.2 µL DEPC treated water, and 1.8 µL 10% SDS (following the table provided below).
Table 1. Volumes of hybridization solution additions used for varying formamide concentrations.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>0%</th>
<th>10%</th>
<th>20%</th>
<th>30%</th>
<th>40%</th>
<th>50%</th>
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<tr>
<td>4.5 M NaCl</td>
<td>360</td>
<td>360</td>
<td>360</td>
<td>360</td>
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<td>36</td>
<td>36</td>
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<tr>
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<td>720</td>
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<tr>
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<td>1042.2</td>
<td>862.2</td>
<td>682.2</td>
<td>502.2</td>
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<tr>
<td>10% SDS</td>
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Following preparation of the hybridization solution, the desired fluorescent oligonucleotide probe is added at 0.25 µL of 200 ng/µL probe stock per well. For example, for 60 µL of hyb solution (enough for 6 wells), add 1.5 µL of 200 ng/µL probe stock. Mix well, then transfer 10 µL of hybridization solution/probe mixture to each well that is to be hybridized. Per slide, one hybridization chamber is prepared by pipetting the remaining ~1.7 mL hybridization solution (without probe) onto a folded kimwipe (4x folded, cut at 2/3 length) inserted onto the bottom of a 50 mL centrifuge tube. The hybridization chamber is then placed horizontally (liquid side up) in a test tube rack covered in foil and allowed to incubate overnight in a hybridization oven at 46°C.

The next day, about an hour before hybridization is concluded, 50 mL of wash solution is prepared per slide. The wash solution mixture is dependent upon the formamide concentration selected for hybridization - for AigG1 (40% formamide) the wash solution consists of 1 mL 1 M Tris-HCl pH 8, 625 µL 4.5 M
NaCl, 500 µL 0.5 M EDTA, 47.7 mL sterile H₂O, and 50 µL 10% SDS. The volume of 1 M Tris-HCl pH 8, 0.5 M EDTA, and 10% SDS are constant across the commonly used formamide percentages, while 4.5 M NaCl and sterile H₂O volumes vary based on the table below. This 50 mL wash solution is brought to 48°C prior to moving on to the next step (usually takes approximately 30-45 minutes).

Table 2. Volumes of wash solution additions used for varying formamide concentrations.

<table>
<thead>
<tr>
<th>% Formamide (hyb. Buffer)</th>
<th>NaCl (mM)</th>
<th>NaCl (µl of 4.5 M NaCl) in wash buffer</th>
<th>0.5M EDTA (µl) in Wash buffer</th>
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<tr>
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<td>10000</td>
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<tr>
<td>50</td>
<td>28</td>
<td>312.5</td>
<td>500</td>
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</tbody>
</table>

Once the wash solution reaches 48°C, the slide is carefully removed from the hybridization chamber, immersed in fresh DEPC treated water for three seconds, and incubated in the wash solution in a 48°C water bath for 20 minutes without shaking. The slide is immersed in fresh DEPC treated water for three seconds, and quickly dried in the flow hood with minimal light exposure on a test tube rack covered with aluminum foil. Once dry, the slide is immersed into 1 µg/mL DAPI solution for 5 seconds (making sure that all hybridized wells are submerged), immersed in DEPC treated water for 5-10 seconds, and allowed to
air dry under the fume hood with minimal light exposure. After the slide has dried, 3 µL 1:4 Vectashield/Citifluor mixture is pipetted onto each well and a coverslip is gently placed over the wells without any air bubble formation. The slide is then ready to view under a fluorescence microscope or can be stored at 4°C for later viewing.

Clone-FISH

With no known positive controls for the currently uncultivated ‘Aigarchaeota’, pseudo-positive controls must be generated to test probes and to identify optimum formamide concentrations during hybridization. Generation of a vector-insert construct in an appropriate *E. coli* host strain (JM109(DE3)) provides heterologous expression of AigG1 16S rRNA, thus allowing for testing of Group 1 ‘Aigarchaeota’-specific oligonucleotide probes.

Generation of a vector-insert construct is performed by: 1) amplification of the near full length AigG1 16S rRNA gene from clone G04B_L4_A09 (Costa et al., 2009) by PCR with primers 8aF and 1406uR; 2) ligation into pGEM-T expression vector; 3) transformation, selection, isolation, and confirmation of construct by Sanger sequencing; 4) transformation into host strain *E. coli* JM109(DE3) with IPTG-inducible T7 RNA polymerase; and 5) expression of AigG1 16S rRNA by treatment with IPTG and chloramphenicol. Following successful induction and thus expression of the AigG1 16S rRNA in the host cells, samples are fixed, washed, and stored at -20°C in preparation for FISH.
Figure 11. Methods for construction of a pseudo-positive control for testing FISH probes targeting 16S rRNA of uncultivated AigG1 using the clone-FISH technique developed by Schramm et al. (2002). Probes AigG1-180 and AigG1-1012 specific for AigG1 were designed based on manual inspection of alignments of AigG1 and other ‘Aigarchaeota’ genus-level groups and verified using the Ribosomal Database Project (RDP) Probe Match utility (Cole et al., 2014).

Although a probe was previously published to be effective in detecting presence of AigG1, we designed two probes in order to compare intensities. Our probes were designed based on manual inspection of alignments of AigG1 and other ‘Aigarchaeota’ genus-level groups and verified using the Ribosomal Database Project (RDP) Probe Match utility (Cole et al., 2014). The previously published probe (AigG1-800 [Cy3] GGCCCGTAGCCGCCCCGA; Beam et al., 2015) as well as our designed probes (AigG1-180 [Cy3] ACCGGGACTTTCCGCGACC) and (AigG1-1012 [Cy3]...
AGGTAGGGTCGTCAGCCCGA) were tested for specificity using the methods listed above. In addition, formamide concentrations were optimized by testing 0-50% formamide in 10% increments. Negative controls were then tested at the formamide concentrations found to be optimal to ensure probe specificity.

CARD-FISH

Similarly to FISH (or mono-FISH as it is often referred), once cell samples are fixed, washed, and stored at -20°C CARD-FISH preparation begins by adding 2 µL of the fixed cell sample to a well of a gelatin-coated slide and is allowed to dry in the fume hood. After drying, cells are permeabilized in a humidity chamber (consisting of 1 mL DEPC water on a Kimwipe in a 50 mL centrifuge tube) via treatment with a fresh proteinase K (for Archaea) or lysozyme (for Bacteria) solution for 1 hour at 37°C. The proteinase K solution contains 100 µL EDTA, 100 µL Tris, 800 µL DEPC water, and 10 mg of proteinase K. The lysozyme solution contains 100 µL EDTA, 100 µL Tris, 800 µL DEPC water, and 10 mg of lysozyme. Proteinase K is used when working with archaea, such as AigG1, due to their cell wall structure that lack peptidoglycan but instead have a protein component (e.g. an S-layer). Following the incubation period, the slide is immersed in fresh DEPC water for washing. Next, to inactivate any endogenous peroxidases that may be present, the slide is incubated in 0.01 M HCl (4 µL of 2.5 M HCl in 996 µL DEPC water) for 15 minutes at room temperature, immersed in fresh DEPC water three times for 10 seconds to wash, immersed in 95% ethanol for 1 minute, then allowed to air dry.
Table 3. Volumes of hybridization solution additions with varying formamide concentrations.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Formamide concentration in hybridization solution</th>
<th>0%</th>
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<th>20%</th>
<th>30%</th>
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</tbody>
</table>

Using the above table, the hybridization solution is made ahead of time and can be stored at -20°C for up to three months. Per slide, 1 µL of 50 ng/µL AigG1-specific horseradish peroxidase (hrp) labeled probe stock is added to a 200 µL aliquot of the 40% formamide hybridization solution. A hybridization chamber (consisting of 1.5 mL mixture of 40% formamide and DEPC water added to a folded kimwipe and inserted onto the bottom of a 50 mL centrifuge tube) is prepared, 10 µL of the probe/hybridization mixture is added to each well to be hybridized, the slide is placed in the chamber liquid side up, the chamber is placed horizontally in a test tube rack covered with foil, and incubation in a 46°C hybridization oven occurs overnight.
The next day, about an hour before hybridization is concluded, 50 mL of wash solution is prepared per slide. The wash solution mixture is dependent upon the formamide concentration selected for hybridization - for AigG1 (40% formamide) the wash solution consists of 1 mL 1 M Tris-HCl pH 8, 625 µL 4.5 M NaCl, 500 µL 0.5 M EDTA, 47.83 mL sterile DEPC water, and 50 µL 10% SDS. The volume of 1 M Tris-HCl pH 8, 0.5 M EDTA, and 10% SDS are constant across the commonly used formamide percentages, while 4.5 M NaCl and sterile DEPC water volumes can be found in the table below. This 50 mL wash solution is brought to 48°C prior to moving on to the next step (usually takes approximately 30-45 minutes).

Table 4. Volumes of wash solution additions used for varying formamide concentrations.

<table>
<thead>
<tr>
<th>% Formamide</th>
<th>NaCl (mM)</th>
<th>(X) 4.5 M NaCl (µl)</th>
<th>(Y) 0.5M EDTA (µl)</th>
<th>(Z) sterile DEPC water (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>900</td>
<td>10000</td>
<td>500</td>
<td>38.45</td>
</tr>
<tr>
<td>10</td>
<td>450</td>
<td>5000</td>
<td>500</td>
<td>43.45</td>
</tr>
<tr>
<td>20</td>
<td>225</td>
<td>2500</td>
<td>500</td>
<td>45.95</td>
</tr>
<tr>
<td>30</td>
<td>112.5</td>
<td>1250</td>
<td>500</td>
<td>47.20</td>
</tr>
<tr>
<td>40</td>
<td>56.25</td>
<td>625</td>
<td>500</td>
<td>47.83</td>
</tr>
<tr>
<td>50</td>
<td>28</td>
<td>312.5</td>
<td>500</td>
<td>48.14</td>
</tr>
</tbody>
</table>

Once the wash solution reaches 48°C, the slide is carefully removed from the hybridization chamber and incubated in the wash solution in a 48°C water bath for 15 minutes without shaking. Following washing, the slide is immersed in fresh DEPC treated water for three seconds and incubated in a 50 mL conical
tube with 1x PBS for 15 minutes at room temperature. During this 15 minute incubation period, CARD solutions are generated in preparation for the next step.

For the CARD step, a chamber (a tissue inserted onto the bottom of a 50 mL tube and soaked with ~1.5 mL DEPC water), fresh 0.15% H$_2$O$_2$ (2 µL in 398 µL PBS), and amplification buffer (for 40 mL - 2 mL of 20x PBS pH 7.6, 0.4 ml Roche Blocking Reagent (10%), 16 ml 5 M NaCl, add sterile dH$_2$O to a final volume of 40 ml, add 4 g of dextran sulfate and heat in 48°C water bath until dissolved) must be prepared. Once prepared, mix amplification buffer with 0.15% H$_2$O$_2$ solution in a ratio of 100:1 (final concentration of 0.0015%), then add fluorescently labeled tyramide at a rate of 0.1 µL per well, mix well, and keep in the dark. Once the 15 minute incubation mentioned above is complete, remove excess liquid by tapping on a Kimwipe then add 10 µL of amplification mix to each hybridized well and incubate at 46°C in a foil-covered 50 mL conical tube for 45 minutes in the dark. Incubate in 1 x PBS for 5 minutes at room temperature in the dark, wash slide twice in fresh DEPC for 1 minute at room temperature in the dark and immerse in 95% ethanol for 1-2 minutes then allow to air dry in the dark. Once dry, the slide is immersed into 1 µg/mL DAPI solution for 5 seconds (making sure that all hybridized wells are submerged), immersed in DEPC treated water for 5-10 seconds, and allowed to air dry under the fume hood with minimal light exposure. After the slide has dried, 3 µL 1:4 Vectashield/Citifluor mixture is pipetted onto each well and a coverslip is gently
placed over the wells without any air bubble formation. The slide is then ready to
view under a fluorescence microscope or can be stored at 4°C for later viewing.

Stable Isotope Probing and nano-SIMS

For inference on what AigG1 are metabolizing, stable isotope probing was
conducted both in a natural setting at GBS in Nevada and in a lab setting on the
G1-10 cultures followed by sample fixation. CARD-FISH was then performed on
samples spotted on poly-lysine-coated slides (used specifically for nano-SIMS),
and samples were sent off for nano-SIMS analysis at Lawrence Livermore
National Lab (LLNL). While it is unknown exactly what substrates AigG1 is
capable of metabolizing, predictions allow for educated guesses (Beam et al.,
2015; Hua et al., 2018). For this reason, a variety of isotopically-labeled
substrates were added to both lab and natural samples (Tables 5 and 6). While
the $^{15}$N and $^{18}$O labels are used essentially as positive controls for detecting
activity, the substrates labelled with $^{13}$C are those that will allow for an analysis of
the metabolic capabilities of AigG1.
Table 5. Isotope-labeling experiments on natural samples

<table>
<thead>
<tr>
<th>Sample name</th>
<th>Incubation Condition</th>
<th>Incubation time (hrs.)</th>
<th>$^{13}$C label$^1$</th>
<th>$^{15}$N-NH$_4$Cl (50 µM)</th>
<th>$^{18}$O –H$_2$O (10%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sed 1-0</td>
<td>Aerobic</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Sed 3-4</td>
<td>Aerobic</td>
<td>4</td>
<td>Bicarb (0.5 µM)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Sed 5-4</td>
<td>Aerobic</td>
<td>4</td>
<td>BPA (0.5 µM)</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Sed 7-4</td>
<td>Aerobic</td>
<td>4</td>
<td>Algal AA (0.005%)</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Sed 9-4</td>
<td>Aerobic</td>
<td>4</td>
<td>Starch (0.005%)</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Sed 11-24</td>
<td>Aerobic</td>
<td>24</td>
<td>Bicarb (0.5 µM)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Sed 13-24</td>
<td>Aerobic</td>
<td>24</td>
<td>BPA (0.5 µM)</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Sed 15-24</td>
<td>Aerobic</td>
<td>24</td>
<td>Algal AA (0.005%)</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Sed 17-24</td>
<td>Aerobic</td>
<td>24</td>
<td>Starch (0.005%)</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Sed 22-0</td>
<td>Anaerobic</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Sed 23-4</td>
<td>Anaerobic</td>
<td>4</td>
<td>Bicarb (0.5 µM)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Sed 25-4</td>
<td>Anaerobic</td>
<td>4</td>
<td>BPA (0.5 µM)</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Sed 27-4</td>
<td>Anaerobic</td>
<td>4</td>
<td>Algal AA (0.005%)</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Sed 29-4</td>
<td>Anaerobic</td>
<td>4</td>
<td>Starch (0.005%)</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Sed 31-24</td>
<td>Anaerobic</td>
<td>24</td>
<td>Bicarb (0.5 µM)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Sed 33-24</td>
<td>Anaerobic</td>
<td>24</td>
<td>BPA (0.5 µM)</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Sed 35-24</td>
<td>Anaerobic</td>
<td>24</td>
<td>Algal AA (0.005%)</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Sed 37-24</td>
<td>Anaerobic</td>
<td>24</td>
<td>Starch (0.005%)</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

$^1$Abbreviations: Bicarb, Bicarbonate; BPA, Butyrate Propionate & Acetate; AA, Amino Acids
Table 6. Isotope-labeling experiments on laboratory cultures

<table>
<thead>
<tr>
<th>Sample name</th>
<th>Incubation time (hrs.)</th>
<th>$^{13}$C label$^1$</th>
<th>$^{15}$N-NH$_4$Cl (50 µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lab 1-6</td>
<td>6</td>
<td>Bicarb (0.5 µM)</td>
<td>+</td>
</tr>
<tr>
<td>Lab 2-6</td>
<td>6</td>
<td>Formate (0.5 µM)</td>
<td>+</td>
</tr>
<tr>
<td>Lab 3-6</td>
<td>6</td>
<td>Acetate (0.5 µM)</td>
<td>+</td>
</tr>
<tr>
<td>Lab 4-6</td>
<td>6</td>
<td>Propionate (0.5 µM)</td>
<td>+</td>
</tr>
<tr>
<td>Lab 5-6</td>
<td>6</td>
<td>Butyrate (0.5 µM)</td>
<td>+</td>
</tr>
<tr>
<td>Lab 6-6</td>
<td>6</td>
<td>Algal AA (0.005%)</td>
<td>+</td>
</tr>
<tr>
<td>Lab 7-6</td>
<td>6</td>
<td>Glucose (0.005%)</td>
<td>+</td>
</tr>
<tr>
<td>Lab 8-6</td>
<td>6</td>
<td>Xylose (0.005%)</td>
<td>+</td>
</tr>
<tr>
<td>Lab 9-6</td>
<td>6</td>
<td>Ribose (0.005%)</td>
<td>+</td>
</tr>
<tr>
<td>Lab 10-6</td>
<td>6</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Lab 1-24</td>
<td>24</td>
<td>Bicarb (0.5 µM)</td>
<td>+</td>
</tr>
<tr>
<td>Lab 2-24</td>
<td>24</td>
<td>Formate (0.5 µM)</td>
<td>+</td>
</tr>
<tr>
<td>Lab 3-24</td>
<td>24</td>
<td>Acetate (0.5 µM)</td>
<td>+</td>
</tr>
<tr>
<td>Lab 4-24</td>
<td>24</td>
<td>Propionate (0.5 µM)</td>
<td>+</td>
</tr>
<tr>
<td>Lab 5-24</td>
<td>24</td>
<td>Butyrate (0.5 µM)</td>
<td>+</td>
</tr>
<tr>
<td>Lab 6-24</td>
<td>24</td>
<td>Algal AA (0.005%)</td>
<td>+</td>
</tr>
<tr>
<td>Lab 7-24</td>
<td>24</td>
<td>Glucose (0.005%)</td>
<td>+</td>
</tr>
<tr>
<td>Lab 8-24</td>
<td>24</td>
<td>Xylose (0.005%)</td>
<td>+</td>
</tr>
<tr>
<td>Lab 9-24</td>
<td>24</td>
<td>Ribose (0.005%)</td>
<td>+</td>
</tr>
<tr>
<td>Lab 10-24</td>
<td>24</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

$^1$Abbreviations: Bicarb, Bicarbonate; AA, Amino Acids

For natural samples sediment and water slurry was collected from the “C site” at GBS in Nevada (defined by Cole et al., 2013), a site exhibiting temperature and pH ranges of 77.5-79.4 °C and 7.54-7.59 respectively and dosed with the noted amounts of labeled substrates (see above). The samples were then held at an incubation temperature of 75 °C and harvested at 4- and 24-hour time points, with multiple experiments spanning a three day time period from July 18-20, 2017. Post-incubation, cells are fixed in preparation for FISH
and nano-SIMS. Due to sensitivity issues with the nano-SIMS machine, natural samples containing sediment must be treated with nycodenz to remove larger particles from the fixed samples. Steps for nycodenz treatment include 200 µL of fixed samples are added gently on top of 200 µL of 80% nycodenz in a 1.5 mL microcentrifuge tube then centrifuge at 13k rpm for 10 minutes. Then harvest the upper sample layer and the sample/nycodenz interface (approximately 230 µL) and transfer to a new 1.5 mL tube, add DEPC water to a total volume of 500 µL, mix well and centrifuge at 13k rpm for 5 minutes. Remove and discard the supernatant without disturbing the pellet and wash in 500 µL 1x PBS and centrifuge at 13k rpm for 5 minutes (do this twice). Following the third wash, resuspend the pellet in 50 µL of 50% ethanol in water, and store at -20°C for later use in CARD-FISH and nano-SIMS experiments.
Figure 12. Basic steps for performing stable isotope probing (SIP) experiments on natural samples from GBS using $^{13}$C, $^{15}$N, and $^{18}$O-labelled substrates.

Laboratory samples were derived from the already existing G1-10 cultures. Following the regular 3-week incubation period, labeled substrates were added and incubation continues for 24 more hours. Cultures were harvested at 6- and 24-hour time points, and cells are fixed in preparation for FISH and nano-SIMS.
Figure 13. Basic steps for performing stable isotope probing (SIP) experiments on laboratory cultures using $^{13}$C and $^{15}$N-labelled substrates.

The combination of FISH with nano-scale Secondary Ion Mass Spectrometry (FISH-nano-SIMS) allows for studying the metabolic uptake of individual cells in the absence of pure cultures. FISH-nano-SIMS is a tool that, if successful, could allow for a better understanding of the interactions within mixed laboratory cultures such as the G1-10. The method has been successfully used, but to our knowledge has never been applied to high-temperature terrestrial geothermal systems. CARD-FISH was performed at CSUSB, nano-SIMS will be done at LLNL, and subsequent analysis of nano-SIMS images will be done at CSUSB using L’image software (http://limagesoftware.net/). This software allows for comparison of nano-SIMS and CARD-FISH images, and individual cells positive for AigG1 probes by CARD-FISH are indicated as “regions of interest” (ROI) by manually drawing borders around the cells. The $^{13}$C content is then
calculated for each ROI by the L’image program and expressed as average percent enrichment (APE) of $^{13}\text{C}$ above natural abundance (Woebken et al, 2015).
CHAPTER THREE

RESULTS

Goal 1: Set up, Validate, and Use an Optofluidic Single Cell Sorting System to Obtain Pure or More Simplified Cultures of AigG1 and Other Novel Thermophiles

_E. coli_ Control Sorting Experiments

To test the sorting protocol and validate that single, viable cells could reliably be obtained, control sorting experiments were performed using _E. coli_. Putative single-cell suspensions harvested from the device were split between five different aliquots of LB growth medium and incubated for 2 days in a 96-well plate at 37 °C. In all cases, only one of the five aliquots yielded growth, and between 40-90% of sorted cells yielded growth in three separate experiments (Figure 14). To prevent individual cells from sticking to the inner walls of the microfluidic chip during sorting, Tween 20 and F127 pluronic were used in low concentrations of 0.02% and 0.04%, respectively, in initial experiments. These results confirm that the optofluidic cell sorting device, originally designed for single-cell genomics techniques, can be reliably applied for isolation of individual, viable microbes.
Figure 14. Putative single-cell suspension harvested from the device were split between five different aliquots of LB growth medium and incubated for 48 hours in a 96 well plate. In all cases, only one of the five aliquots yielded growth, and between 40-90% of sorted cells yielded growth in three separate experiments. + = sorted cells; - = no cell sorted (negative control); green = expected outcome; red = unexpected outcome. For the positive control, cells were loaded directly into the cell holding chamber (channel 48 of chip).

G1-10 Sorting Experiments

While cell survival rates were not an issue when testing with *E. coli*, initial results on mixed thermophilic cultures indicate that the amount of surfactants Tween 20 and F127 pluronic used to prevent cell binding to the PDMS chip surface severely decreased viability. In the absence of these surfactants, however, cell adhesion becomes an issue and prevents successful sorting. To determine suitable conditions that allows for sorting of viable, single cells of
targeted thermophiles, including ‘Aigarchaeota’, various concentrations of surfactants were added to cell suspensions and sorting was attempted to determine if cell binding to PDMS occurred. In cases where binding was not observed, several single cells as well as a positive control channel (loaded with approximately 0.3 nL of the cell suspension used for sorting, corresponding to about 100 cells) were sorted, harvested, and inoculated into media used for growth and maintenance of the mixed culture containing ‘Aigarchaeota’ (casamino acids as a carbon source, at pH 7.5, with 50% air headspace, at 80°C); dilution to extinction was also performed on the harvested cell suspension and on cell suspension not loaded onto the chip. Growth at different dilutions was compared to theoretical cell numbers (based on direct counts using a Petrof-Hausser counting chamber) to determine the percentage of viable cells. These experiments were performed in triplicate (most probable number technique). In addition, qPCR was performed to ensure presence of AigG1 in mixed culture following treatment with surfactants.

Initial experiments were conducted with varying amounts of the two surfactants (Tween 20 and Pluronic) introduced to newly-transferred cultures, and it was expected that some combination of surfactants would allow for effective sorting (preventing adherence to the PDMS) while retaining viability during the lengthy growth period (Table 7). All cultures that contained Tween 20, even in very low (0.02%) concentrations, did not yield growth while the addition of Pluronic at low concentrations did not seem to inhibit cell growth. Results from
these experiments suggest that exclusion of Tween 20 and lower (0.01%) concentrations of Pluronic may yield results deemed satisfactory, based on the growth of positive controls and the ability to sort with limited cell adhesion, therefore such conditions were used for future single cell sorting experiments.

Table 7. AigG1 growth and sorting conditions under various surfactant concentrations

<table>
<thead>
<tr>
<th>Surfactant</th>
<th>Concentration</th>
<th>AigG1 Growth?</th>
<th>Sorting Conditions (based on cell adhesion)</th>
</tr>
</thead>
<tbody>
<tr>
<td>none</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>tween 20 and F127 pluronic</td>
<td>0.02% and 0.04%</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>tween 20 and F127 pluronic</td>
<td>0.002% and 0.004%</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>tween 20</td>
<td>0.02%</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>F127 pluronic</td>
<td>0.04%</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>F127 pluronic</td>
<td>0.004%</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>F127 pluronic</td>
<td>0.002%</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Once sorting conditions suitable for the G1-10 cultures were found, a number of attempts were made at obtaining pure (or even more simplified) cultures. Over the course of approximately three months, 7 sorting sessions
yielded a total of 42 sorted individual cells, 10 sorted cell pairs or clusters, and both a positive and negative control from each session to ensure proper growth conditions were provided and contamination-free results, respectively. As presented below (Table 8) the number of sorts that exhibited growth varied widely, ranging from 0.0 - 71.4% on separate sort dates. Cultures “G1-10 Sort 25” and “G1-10 Sort 32” are those derived from previous successful sorts, and were re-sorted in an attempt to further isolate, if possible, due to unclear sequencing results. One of the sorting dates (190516) exhibited less-than-ideal conditions, where the internal pressure of the sorting device being uneven and cells freely moving past reagent gates. Results from that day are thus not to be trusted.

Table 8. G1-10 sorting experiments summary table

<table>
<thead>
<tr>
<th>Date</th>
<th>Culture (Age)</th>
<th>Total Sorted</th>
<th>Exhibited Growth</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>190205</td>
<td>G1-10 (3 wk)</td>
<td>9</td>
<td>0</td>
<td>0.0%</td>
</tr>
<tr>
<td>190227</td>
<td>G1-10 (3 wk)</td>
<td>9</td>
<td>1</td>
<td>11.1%</td>
</tr>
<tr>
<td>190301</td>
<td>G1-10 (3 wk)</td>
<td>7</td>
<td>5</td>
<td>71.4%</td>
</tr>
<tr>
<td>190326</td>
<td>G1-10 Sort 25 (5 days)</td>
<td>5</td>
<td>1</td>
<td>20.0%</td>
</tr>
<tr>
<td>190326</td>
<td>G1-10 Sort 32 (5 days)</td>
<td>5</td>
<td>0</td>
<td>0.0%</td>
</tr>
<tr>
<td>190513</td>
<td>G1-10 (2 wk)</td>
<td>9</td>
<td>5</td>
<td>55.6%</td>
</tr>
<tr>
<td>190516</td>
<td>G1-10 (2 wk)</td>
<td>8</td>
<td>1</td>
<td>12.5%*</td>
</tr>
</tbody>
</table>

A total of 13 sorts exhibited growth and were processed in preparation for eventual identification and characterization, should they be a novel lineage. Following observed growth, microbes were identified by DNA extraction, 16S
rRNA gene PCR, and sequencing. Table 9 below shows the individual successful cell sorts that were derived from G1-10 cultures, including the morphology at the time of sorting, date of observed growth, and the closest hit identified by BLASTn using the NCBI non redundant nucleotide (nr/nt) database (Altschul et al., 1990; Morgulis et al., 2008; Zhang et al., 2000).

As seen in the table all successful results from G1-10 cultures, including those that were secondarily sorted from previously grown sorts (G1-10 Sort 25), had similar hits in the BLASTn database. Estimated to be a new species of the genus *Pyrobaculum*, which currently contains 7 isolated and published species in addition to a few unpublished candidates. Whole genome analysis of this proposed new species can be found later in this chapter.
Table 9. G1-10 successful sorts

<table>
<thead>
<tr>
<th>Name</th>
<th>Culture (Age)</th>
<th>Morphology</th>
<th>Observed Growth Date</th>
<th>Organism Identity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>19027_25</td>
<td>G1-10 (3 wk)</td>
<td>medium rod (2 cells)</td>
<td>190302</td>
<td>Pyrobaculum spp. (98.86%)</td>
</tr>
<tr>
<td>190301_25</td>
<td>G1-10 (3 wk)</td>
<td>medium rod</td>
<td>190305</td>
<td>Pyrobaculum spp. (98.85%)</td>
</tr>
<tr>
<td>190301_26</td>
<td>G1-10 (3 wk)</td>
<td>medium rod</td>
<td>190305</td>
<td>Pyrobaculum (RFLP)</td>
</tr>
<tr>
<td>190301_28</td>
<td>G1-10 (3 wk)</td>
<td>long rod</td>
<td>190305</td>
<td>Pyrobaculum spp. (98.87%)</td>
</tr>
<tr>
<td>190301_30</td>
<td>G1-10 (3 wk)</td>
<td>medium rod</td>
<td>190320</td>
<td>Pyrobaculum spp. (98.87%)</td>
</tr>
<tr>
<td>190301_32</td>
<td>G1-10 (3 wk)</td>
<td>medium rod</td>
<td>190305</td>
<td>Pyrobaculum (RFLP)</td>
</tr>
<tr>
<td>190326_28</td>
<td>G1-10 Sort 25 (5 days)</td>
<td>medium rod</td>
<td>190402</td>
<td>Pyrobaculum spp. (97.80%)</td>
</tr>
<tr>
<td>190513_25</td>
<td>G1-10 (2 wk)</td>
<td>discus</td>
<td>190527</td>
<td>Pyrobaculum (RFLP)</td>
</tr>
<tr>
<td>190513_26</td>
<td>G1-10 (2 wk)</td>
<td>filamentous rod</td>
<td>190521</td>
<td>Pyrobaculum spp. (95.16%)</td>
</tr>
<tr>
<td>190513_27</td>
<td>G1-10 (2 wk)</td>
<td>cell pair (attached ends)</td>
<td>190521</td>
<td>Pyrobaculum spp. (98.24%)</td>
</tr>
<tr>
<td>190513_28</td>
<td>G1-10 (2 wk)</td>
<td>medium rod (dark ends)</td>
<td>190521</td>
<td>Pyrobaculum spp. (98.22%)</td>
</tr>
<tr>
<td>190513_30</td>
<td>G1-10 (2 wk)</td>
<td>discus</td>
<td>190521</td>
<td>Pyrobaculum spp. (98.24%)</td>
</tr>
<tr>
<td>190516_27</td>
<td>G1-10 (2 wk)</td>
<td>cell cluster</td>
<td>190528</td>
<td>Pyrobaculum (RFLP)</td>
</tr>
</tbody>
</table>

Other Sorting Experiments

While cell survival rates were not an issue when testing with *E. coli*, initial results on mixed thermophilic cultures indicate that the amount of surfactants in addition to the G1-10 cultures, other laboratory cultures known to contain novel
thermophilic microbes were used for single cell sorting. Following the same procedures used for the G1-10 cultures, 4 sorting sessions yielded a total of 26 sorted individual cells and both a positive and negative control from each session to ensure proper growth conditions were provided and contamination-free results, respectively. As presented below (Table 10), the number of sorts that exhibited growth ranged from 0.0 - 57.1% on separate sort dates. Sorted cultures include laboratory cultures derived from GBS in Gerlach, NV known to host Kryptonia (culture names: G29-3 and 3NO₃/N₂O) and laboratory cultures from our collaborators at UNLV known to contain a member of the candidate phylum ‘Fervidibacteria’ in what is potentially a pure culture (FV XG UNLV and FV LBG UNLV). The G29-3 culture is growing under similar media conditions to that of G1-10, with the addition of 0.02% starch as an additional substrate. The 3NO₃/N₂O culture is growing under similar media conditions to that of both G29-3 and G1-10, with the addition of 0.02% starch and 200 µM nitrite as additional substrates and 10% N₂O air headspace. Both the “FV XG UNLV” and “FV LBG UNLV” cultures were derived from the G1-10 culture but were further enriched for ‘Fervidibacteria’ using serial dilution methods at UNLV, with xyloglucan and locust bean gum used as primary growth substrates. While it was thought to be a pure culture, the inability to grow on solid media led to doubts. Successful sorts from this culture would eliminate any such doubts about the purity.
Table 10. Other sorting experiments summary table

<table>
<thead>
<tr>
<th>Date</th>
<th>Culture (Age)</th>
<th>Total Sorted</th>
<th>Exhibited Growth</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>190227</td>
<td>G29-3 (5 days)</td>
<td>9</td>
<td>2</td>
<td>22.2%</td>
</tr>
<tr>
<td>190313</td>
<td>3 NO3/N2O (5 days)</td>
<td>7</td>
<td>4</td>
<td>57.1%</td>
</tr>
<tr>
<td>190417</td>
<td>FV XG UNLV (4 days)</td>
<td>5</td>
<td>1</td>
<td>20.0%</td>
</tr>
<tr>
<td>190417</td>
<td>FV LBG UNLV (4 days)</td>
<td>5</td>
<td>0</td>
<td>0.0%</td>
</tr>
</tbody>
</table>

A total of 7 sorts exhibited growth and were processed in preparation for eventual identification and characterization, should they be a novel lineage. Following observed growth, microbes were identified by DNA extraction, 16S rRNA gene PCR, and sequencing. Table 11 below shows the individual successful cell sorts that were derived from these cultures, including the morphology at the time of sorting, date of observed growth, and the closest hit identified by BLASTn using the NCBI non redundant nucleotide (nr/nt) database (Altschul et al., 1990; Morgulis et al., 2008; Zhang et al., 2000).

Although the sequencing results for the ‘Fervidibacteria’ were not as expected (the closest BLASTn hit was not an uncultivated ‘Fervidibacteria’), the successful sort showed up positive with ‘Fervidibacteria’-specific PCR primers and gave promising results when a digestion was done with a restriction enzyme. While sorts from both the G29-3 and 3NO3/N2O cultures yielded successful results, all were found to be already published species.
Table 11. Successful sorts deriving from other cultures

<table>
<thead>
<tr>
<th>Name</th>
<th>Culture (Age)</th>
<th>Morphology</th>
<th>Observed Growth Date</th>
<th>Organism Identity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>190227_39</td>
<td>G29 (5 days)</td>
<td>medium, fat rod</td>
<td>190302</td>
<td>*Thermus oshimai JL-2 (98.31%)</td>
</tr>
<tr>
<td>190227_43</td>
<td>G29 (5 days)</td>
<td>medium/long rod (pair)</td>
<td>190302</td>
<td>*Thermus oshimai JL-2 (96.86%)</td>
</tr>
<tr>
<td>190313_25</td>
<td>3 NO3/N2O (5 days)</td>
<td>short rod</td>
<td>190409</td>
<td>*Not yet sequenced</td>
</tr>
<tr>
<td>190313_30</td>
<td>3 NO3/N2O (5 days)</td>
<td>short, fat rod</td>
<td>190318</td>
<td>*Thermus oshimai JL-2 (98.10%)</td>
</tr>
<tr>
<td>190313_32</td>
<td>3 NO3/N2O (5 days)</td>
<td>medium rod</td>
<td>190409</td>
<td>*Not yet sequenced</td>
</tr>
<tr>
<td>190313_37</td>
<td>3 NO3/N2O (5 days)</td>
<td>short rod (bouncy)</td>
<td>190329</td>
<td>*Not yet sequenced</td>
</tr>
<tr>
<td>190417_26</td>
<td>FV XG UNLV (4 days)</td>
<td>cell pair (attached ends)</td>
<td>190421</td>
<td>*Amplified with FV-specific 9bF primers</td>
</tr>
</tbody>
</table>

**New Pyrobaculum spp.**

As mentioned, sorting from G1-10 cultures yielded what is likely to be a new species of the *Pyrobaculum* genus. Upon observed growth, microbes were identified by DNA extraction, 16S rRNA gene PCR, and sequencing. After running through BLASTn and recognizing the potential for a new species, the culture was transferred and grown to optimal cell counts. A second round of sorting on the grown up single cell culture (named G1-10 Sort 25) was then done, followed by dilution-to-extinction (serial dilutions) and plating to ensure purity of the culture. Dilution-to-extinction yielded growth up to the $10^{-9}$ dilution (as expected based on cell counts of $2.37 \times 10^8$/mL from the transferred culture). Plating did not yield successful growth, neither when incubated at 73 °C in
polystyrene plates or at 80 °C using glass plates. Whole genome sequencing was done by students in the Genomics (Bio 490) course on campus taught by Dr. Dodsworth during the Spring 2019 term. Rast assembly stats for the isolated *Pyrobaculum* spp. are as follows: 2,430,315 bp, 57.0% GC content, 585 contigs, 33,569 bp N50 value, 3,464 protein coding genes, and 41 RNA’s.

Table 12 below shows a pairwise whole genome comparison of the newly isolated *Pyrobaculum* spp. from GBS and related species, with values representing DDH (DNA-DNA hybridization) calculated by the DSMZ GGDC program (Meier-Kolthoff et al., 2013). All available genome sequences associated with isolates in the genus *Pyrobaculum* available in the Genbank database, including all described species as well as isolates that have not been formally described, were used for comparison. These analyses show that the newly isolated *Pyrobaculum* spp. from GBS is likely to be a new species.
Table 12. Genomic comparison of isolated *Pyrobaculum* and related species

<table>
<thead>
<tr>
<th>Isolate</th>
<th>100</th>
<th>17.2</th>
<th>17.1</th>
<th>18.3</th>
<th>18.2</th>
<th>18.3</th>
<th>17.1</th>
<th>17.2</th>
<th>22.1</th>
<th>30.7</th>
<th>32.8</th>
<th>33.0</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. calidiformis</em></td>
<td>17.2</td>
<td>100</td>
<td>17.9</td>
<td>18.1</td>
<td>19.3</td>
<td>20.4</td>
<td>17.2</td>
<td>17.3</td>
<td>17.9</td>
<td>17.6</td>
<td>18.3</td>
<td>17.5</td>
</tr>
<tr>
<td><em>P. islandicum</em></td>
<td>17.1</td>
<td>17.9</td>
<td>100</td>
<td>17.8</td>
<td>17.8</td>
<td>17.0</td>
<td>25.2</td>
<td>18.5</td>
<td>16.4</td>
<td>17.1</td>
<td>18.1</td>
<td>18.0</td>
</tr>
<tr>
<td><em>P. arsenaticum</em></td>
<td>18.3</td>
<td>18.1</td>
<td>17.8</td>
<td>100</td>
<td>63.8</td>
<td>18.9</td>
<td>17.5</td>
<td>18.3</td>
<td>20.2</td>
<td>18.9</td>
<td>24.6</td>
<td>18.6</td>
</tr>
<tr>
<td><em>P. oguniense</em></td>
<td>18.2</td>
<td>19.3</td>
<td>17.8</td>
<td>63.8</td>
<td>100</td>
<td>20.0</td>
<td>18.7</td>
<td>18.1</td>
<td>19.3</td>
<td>19.1</td>
<td>19.8</td>
<td>19.2</td>
</tr>
<tr>
<td><em>P. aerophilum</em></td>
<td>18.5</td>
<td>20.4</td>
<td>17.0</td>
<td>18.9</td>
<td>20.0</td>
<td>100</td>
<td>16.8</td>
<td>17.7</td>
<td>19.5</td>
<td>16.6</td>
<td>17.4</td>
<td>16.5</td>
</tr>
<tr>
<td><em>P. neutrophilum</em></td>
<td>17.1</td>
<td>17.2</td>
<td>25.2</td>
<td>17.5</td>
<td>18.7</td>
<td>16.8</td>
<td>100</td>
<td>17.8</td>
<td>18.3</td>
<td>17.7</td>
<td>18.4</td>
<td>18.6</td>
</tr>
<tr>
<td><em>P. ferrireducens</em></td>
<td>17.2</td>
<td>17.3</td>
<td>18.5</td>
<td>18.3</td>
<td>18.1</td>
<td>17.7</td>
<td>17.8</td>
<td>100</td>
<td>20.3</td>
<td>17.9</td>
<td>17.9</td>
<td>17.3</td>
</tr>
<tr>
<td><em>P. ignelimii</em></td>
<td>22.1</td>
<td>17.9</td>
<td>16.4</td>
<td>20.2</td>
<td>19.3</td>
<td>19.5</td>
<td>18.3</td>
<td>20.3</td>
<td>100</td>
<td>20.3</td>
<td>21.1</td>
<td>20.7</td>
</tr>
<tr>
<td>Strain WP30</td>
<td>30.7</td>
<td>17.6</td>
<td>17.1</td>
<td>18.9</td>
<td>19.1</td>
<td>16.6</td>
<td>17.7</td>
<td>17.9</td>
<td>20.3</td>
<td>100</td>
<td>55.8</td>
<td>64.9</td>
</tr>
<tr>
<td>Strain OCT_11</td>
<td>32.8</td>
<td>18.3</td>
<td>18.1</td>
<td>24.6</td>
<td>19.8</td>
<td>17.4</td>
<td>18.4</td>
<td>17.9</td>
<td>21.1</td>
<td>55.8</td>
<td>100</td>
<td>56.8</td>
</tr>
<tr>
<td>Strain JCHS_4</td>
<td>33.0</td>
<td>17.5</td>
<td>18.0</td>
<td>18.6</td>
<td>19.2</td>
<td>16.5</td>
<td>18.6</td>
<td>17.3</td>
<td>20.7</td>
<td>64.9</td>
<td>56.8</td>
<td>100</td>
</tr>
</tbody>
</table>

Goal 2: Develop and Use FISH Methods Coupled with Nano-SIMS to Track the Uptake of $^{13}$C-Labeled Compounds by AigG1

To better detect and quantify AigG1 in natural samples and enrichment cultures, Clone-FISH (fluorescence in situ hybridization) techniques were used to test possible oligonucleotide probes and to optimize hybridization conditions for FISH. A near-full length 16S rRNA gene obtained from AigG1 was cloned into the plasmid pGEM-T. This construct was transformed into *E. coli* JM109(DE3) and the AigG1 16S rRNA was subsequently induced by treatment with isopropyl beta-D-1-thiogalactopyranoside (IPTG) and chloramphenicol. FISH was then conducted with probes targeting the AigG1 16S rRNA. Fluorescence signal intensities were observed using an epifluorescence microscope for induced and
uninduced samples, as well as for a number of negative controls, at various formamide concentrations with fluorescently-labeled bacterial-specific (positive control for hybridization), archaeal-specific, and ‘Aigarchaeota’-specific oligonucleotide probes. Induced, but not uninduced, cells were positive for the Archaea-specific probe, indicating successful induction of the AigG1 16S rRNA gene transcript (Figure 15).

Figure 15. Fluorescence in situ hybridization (FISH) using DAPI counterstain (blue), FAM-labeled Bacteria-specific probe (Bact927; green), and Cy3-labeled Archaea-specific probe (Arch344; red) comparing IPTG-induced and chloramphenicol treated cells (A-C) and uninduced cells (D-F). Scale bar is 20 µm.

The previously published AigG1-specific probe (AigG1-800) showed specificity but had a relatively low signal intensity in comparison to the archaeal probe at all formamide concentrations, suggesting poor binding of this probe. Two other newly developed ‘Aigarchaeota’ (G1)-specific probes were tested and
compared at six different formamide concentrations, one of which (AigG1 180 Cy3, data not shown) was found to have low intensity and the other (AigG1 1012 Cy3) that was about 4-fold brighter than the previously published probe, with apparent optimal hybridization conditions at 30-40% formamide (Figure 16).

Figure 16. Fluorescence in situ hybridization (FISH) using DAPI counterstain (blue) and Cy3-labeled AigG1-specific probe (red) comparing the proposed AigG1-800 probe (Beam et al, 2015) to the AigG1-1012 probe at formamide percentages ranging from 0-50% in 10% increments, seeking optimization. Scale bar is 20 µm.

To test for specificity of the AigG1-1012 probe, FISH was performed on the generated pseudo-positive control against the uninduced sample and two other negative controls, *Staphylococcus aureus* and *Bacillus subtilis* (Figure 17). Additional negative controls relevant to thermal environments (not pictured) were
tested, confirming that the probe will potentially be useful for detection of AigG1 in natural and laboratory samples. All future FISH experiments would thus use the AigG1-1012 probe.

**Figure 17.** Fluorescence in situ hybridization (FISH) using DAPI counterstain (blue) and Cy3-labeled AigG1-specific AigG1-1012 probe (red) on IPTG-induced and chloramphenicol treated cells, uninduced cells, *Staphylococcus aureus* (negative control), and *Bacillus subtilis* (negative control) to test for probe specificity. Scale bar is 20 µm.

**CARD-FISH and nano-SIMS**

Many attempts were made at performing FISH on both natural and laboratory samples, each time yielding unsuccessful results with little to no detectable fluorescence signal. Based on recommendations from collaborators, the focus moved to CARD-FISH.

Catalyzed Reporter Deposition (CARD-FISH) techniques were used to determine the morphology of AigG1 cells to help enable morphology-based
sorting of members of this lineage. One critical issue with CARD-FISH is achieving permeabilization of cells without completely degrading cell structure (Kubota, 2013). For Archaea this is often achieved by testing treatment with proteinase K (or other proteases) under a variety of different concentrations, incubation times/temperatures, to aid in permeabilization of archaeal proteinaceous, S-layer-based cell walls. Using our designed AigG1-1012 probe, CARD-FISH was performed on fixed samples obtained from GBS (Figure 18) and from G1-10-derived laboratory samples (Figure 19). As seen in the figures, cells positive for Cy3 (red) signal show up with both DAPI and Cy3 and only fluoresce when an HRP-labeled probe is added. These results are positive and allowed for us to proceed with the FISH-nano-SIMS experiments.

Figure 18. Fluorescence in situ hybridization (FISH) using DAPI counterstain (blue) and HRP-labelled AigG1-specific probe (red, detection with Alexa Fluor 555 tyramide) on GBS natural samples using 40% formamide. No fluorescence was observed in the no probe control when observing a number of fields per well. Scale bar is 20 µm.
With successful detection of AigG1 by CARD-FISH, stable isotope labeling experiments coupled with CARD-FISH and nano-SIMS were able to proceed. Results for these experiments are not yet available, but experiments will be conducted at Lawrence Livermore National Lab (LLNL) by myself and collaborators in the coming months.
CHAPTER FOUR
DISCUSSION AND FUTURE DIRECTIONS

Goal 1: Set Up, Validate, and Use an Optofluidic Single Cell Sorting System to Obtain Pure or More Simplified Cultures of AigG1 and Other Novel Thermophiles

With successful trapping and sorting using the optofluidic system, the sorting system has been demonstrated to allow for isolation and cultivation of individual cells, and sorting buffer conditions (appropriate F127 pluronic and Tween 20 concentrations) compatible with AigG1 viability have been determined. The system was used in attempts to isolate novel microbes from both the G1-10 cultures as well as other laboratory cultures. While it is unknown why such a great variance occurred in exhibited growth percentage among sorted cells it is not thought to be due to contamination or inhabitable media conditions, confirmed by consistent growth in positive controls and lack of growth in negative controls. Sorting on G1-10 cultures will persist, targeting specific morphologies thought to be AigG1 or other novel microbes present in the culture. While conditions for sorting were optimized, it is noteworthy that using these amounts of surfactants when trying a new chip resulted in cell adhesion. It is thought that pretreatment followed by an autoclaving cycle may be necessary. Initial experiments testing this hypothesis confirm that one treatment with the sorting solution followed by an autoclaving cycle allows for sorting conditions with limited cell adhesion.
While results were unsuccessful in that single cell isolates of AigG1 were not obtained, there were minor successes along the way. The common rod-shaped morphology of AigG1 and the similarity to that of *Pyrobaculum* is likely what led to a large number of sorts yielding duplicative results. In addition, the sorted cultures that led to isolated *Pyrobaculum* exhibited a number of different cell sizes ranging from small, stumpy cells to medium rods, suggesting that this microbe may be somewhat pleomorphic. The isolated *Pyrobaculum* is likely to be a distinct species based on the DDH value comparison to already isolated related species from the genus. The next step for this isolate is to fully characterize by testing growth at a variety of different temperatures, pH, and with a number of different substrates. Following characterization, the isolate will be sent to a culture collection and a paper will be written describing it as a new taxon by future lab members.

Also resulting from the single cell sorting was an isolate from the phylum Fervidibacter, which has no currently cultivated members. Following isolation via single cell sorting the sequencing results for the “Fervidibacteria“were not as expected, with the closest BLASTn hit not being an uncultivated Fervidibacter. However, the successful sort showed up positive with Fervidibacter-specific PCR primers and gave promising results when a digestion was done with a restriction enzyme. This is promising and suggests that the unexpected sequencing results were potentially due to short sequences or poor sequencing results. These experiments are likely to be included in a future publication describing the
isolation and characterization of this new phylum, one which I would be included on.

This cell sorting technique proves to be a low-cost option when high-throughput is not necessary and/or sorting rare cells based on morphological characteristics is desired, especially when an appropriate microscope is already available. In addition to the successful results above, it was found that the microfluidic devices remained fully functional after multiple autoclaving cycles when the control and flow channels were filled with water to prevent collapsing. This allows for microfluidic devices to be sterilized and reused, eliminating the high cost and lengthy production time of obtaining new ones. The PDMS microfluidic chips currently in use cost approximately $300 each, so the ability to autoclave devices for multiple reuses will significantly decrease the cost of single-cell sorting using this technique. This device and the associated sorting protocol will allow for future members of the Dodsworth lab to obtain isolated single cells in attempts to cultivate novel microbes. Other future plans include the development of antibodies for AigG1 and other ‘Aigarchaeota’ lineages, which could be used for fluorescent tagging of live cells during sorting.
Goal 2: Develop and Use FISH Methods Coupled with nano-SIMS to Track the Uptake of $^{13}$C-Labeled Compounds by AigG1

While the original plan was to use the simpler methods for Fluorescence *in situ* hybridization (FISH) to study AigG1, continued unsuccessful results and recommendations from collaborators led to CARD-FISH methods being utilized. Following optimization and minor tweaks to the protocol, successful results were achieved for both laboratory samples and nycodenz-treated natural samples from GBS. These results allow for CARD-FISH to be paired with nano-SIMS to analyze potential substrate utilization. In addition, we found that AigG1 exhibits a different morphology in the laboratory cultures vs. the natural samples from GBS sediments. The shorter, fatter rod morphology commonly exhibited in laboratory cultures will allow for targeting a more specific morphology when doing single-cell sorting on these cultures.

Future plans include sending these samples to Lawrence Livermore National Lab (LLNL) for nano-SIMS analysis. I will visit LLNL to complete these experiments, and the resulting nano-SIMS image data will be downloaded at CSUSB and the L’image software will be used for calculating average percent enrichment of $^{13}$C in AigG1 cells under various incubation conditions. Follow-up experiments will be performed with any substrates that were taken up by AigG1. These follow-up experiments will be done at a more detailed time course (2 hours, 4 hours, 8 hours, and 24 hours) to potentially calculate uptake rates and to assess whether AigG1 directly take up the substrate in question (uptake at early timepoints, increasing $^{13}$C over time) or whether AigG1 may be cross-feeding on
products produced by other microbes in the culture (uptake only at later time points). The latter might also be the case if uptake by AigG1 is not occurring under the initial culture conditions (growing on casamino acids) but might be induced by the addition of the substrate in question. These follow up experiments are likely to be included in a future publication.
APPENDIX

SINGLE CELL SORTING PROTOCOL
Some things to remember…

- **MOST IMPORTANT**: Laser safety! THE IR LASER IS INVISIBLE TO THE EYE AND EXTREMELY DANGEROUS. ENSURE THE LASER BEAMPATH IS BLOCKED IN TWO PLACES when the microscope is not shielded (small black cardboard box over the chip/holder, and black cloth over the scope). **Close or (depower) the ThorLabs shutter** AND turn the upper filter turret so that the filter is not in place (laser is not reflected up into objective). Alternatively, key off the laser at the laser power supply (however, please minimize the power cycling of the laser, e.g. do not key on/off the laser more than two times per day).

- **Always try to handle the chip by the PDMS**, rather than the cover slip, if possible. This will prevent damage/breaking of the coverslip. If the coverslip breaks/cracks beneath the chip, it will likely render the chip non-functional (control lines will be compromised).

- Prior to first use, chips should be mounted, and the coverslip should be gently cleaned of excess PDMS using a kimwipe moistened with isopropanol. The chip should be inspected at 2.5x mag objective for: 1) Collapsed flow lines or valves, 2) Lines or punches cut by edge of chip, 3) Improperly located or missing punches.

- To prevent damage to objective lenses, make sure that the objective turret is FULLY LOWERED (using focus knob) before changing stages. ALSO, do not install the 100x trapping objective until the motorized stage is installed, and remove the 100x trapping objective before taking off the motorized stage.

- **Before detaching any control line**, always make sure that the line is depressurized. Otherwise the water in the control line will quickly flow out.

- **Before detaching any tubing connected to the flow layer**, first disconnect the ‘Aigarchaeota’ stub from the microfluidic controller manifold and lower the ‘Aigarchaeota’ stub to near the level of the chip/stage. Otherwise the liquid will flow out of the tubing.

- When “filling” tubing with reagent, do not fill the tubing all the way; leave about 3-5 cm of air between the solution and the ‘Aigarchaeota’ stub.

- **When filling the tubing with cells, it is only necessary to fill to a few cm of tubing** (not much!)

- When connecting a “filled” tubing to the manifold…

  First: Fill by drawing up fluid with a 1 mL syringe connected to the ‘Aigarchaeota’ stub.

  Second: Connect the tubing to the desired port (using clean forceps), taking care to not introduce too much air into the tubing (don’t raise it too much beyond the height it was filled relative to the syringe) and to not lose too much sample/reagent (don’t lower it too much beyond the height it was filled relative to the syringe).

  Third: AFTER the tubing is connected to the chip, disconnect the syringe and attach the ‘Aigarchaeota’ stub to the flow line manifold at the top of the microfluidic valve controller.

  Fourth: Adjust the valves on the manifold so that the tubing is pressurized.

I. **Prepare and sterilize the chip and tygon tubing (should be done ~2 days in advance!!!)**

1. Prepare a Tygon tubing set:
   a. Four (4) or more ~50 cm tubings with leur stubs and 2 cm stainless pins (for adding reagents/cells),
   b. Two (2) ~8 cm tubings with 2 cm stainless pins only (for sample waste ports)
c. One (1) or more 125 cm tubing with a stub and a pin (for product recovery)

NOTE: Previously used sets can be used, as long as they are flushed with water immediately after use.

2. Place each tygon tubing in a separate glass petri dish with cover (the two waste tubings can be put in along with one of the 50 cm tubings).

3. Take a previously inspected and functional microfluidic chip and, using the microfluidic controller, fill all flow layer lines with clean, 0.2 µm-filtered water. This should help prevent collapsing of the flow lines during autoclaving. This can be done using the microfluidic valve controllers (described below), or carefully using a syringe connected to a tubing and carefully/gently injecting 0.2 um-filtered water into the reagent port until water is observed coming out of the cell harvest ports. However, for a chip that has not been used recently, it is better to do it with the valve controller so that the control lines fill as well. For doing it with the valve controller:
   a. If not already done so, mount the chip in the motorized stage holder, being careful to not overtighten the thumb screws. Connect the control lines to their respective ports. When all are connected, pressurize the control lines (~23 psi) and allow all of them to fill.
   b. Fill a 50 mm tubing with 0.2 um filtered water, insert pin into the diluent or reagent port, and connect to the upper 'Aigarchaeota' port. Pressurize to ~6 psi.
   c. After water is clearly coming out the product ports, close the reagent outlet (valve 21) so that flow will be concentrated at other outlets. Water should now be coming out of the reagent purge and the diluent port. When it does, close these ports (valves 22 and 3) and allow water to come out of the cell waste ports, sample ports, and control ports (these might take a while).
   d. Inspect the chip to make sure that all control and flow layer lines are filled (non-filled portions should be easier to see because of greater contrast with the PDMS). If some are not filled, additional valves may need to be closed to concentrate flow in certain areas.
   e. Depressurize the tubing containing the water, disconnect from the manifold, and remove from the reagent port. Gently wipe off excess water from the top of the chip using a kimwipe.
   f. Open/depressurize all control valves.
   g. Disconnect all the control lines and insert them into a dummy chip (or other chip).
   h. Remove the chip from the holder and place into a glass petri dish. Proceed to the following steps (covering ports with PCR tape and autoclaving) ASAP, so that the water that was added doesn't evaporate.

4. Take the chip into the laminar flow hood (PCR hood in BI-322) and place appropriately cut strips of PCR plate sealing tape over the following ports using clean forceps:
   a. harvest ports for channels 1-24 (right side) and 25-48 (left side); one strip for each side
      a. Diluent and cell waste ports (can use a single piece of tape for these)
      b. Reagent port
      c. Each of the two cell sample ports
   b. Carefully place the chip in a glass petri plate and gently tape down one end of the coverslip with lab tape (with a tab on it, to make it easy to remove).
   c. Autoclave the petri plates with the chip and tubings on liquid cycle for 20 minutes. After autoclaving, allow to dry for at least 2 days (until all haziness in chip and tubings clears).

II. Prepare the microscope and chip for sorting - Set up stage and microfluidic controller. Mount chip connect control lines, rinse chip, align microscope condenser ring.

1. Turn on UV lights in cell sorting cart UV chamber for 30 minutes (can be done well prior to preparation, as long as the chamber is not used subsequently).
2. If not, the motorized stage is not already mounted, remove the oil stage, and move the objective turret to its lowest position (using focus knob). Mount the motorized stage on the microscope, being careful not to contact/scratch objective lenses. Secure the stage with hex wrench screws. If not already attached, attach the X and Y encoder cables to the ProScanIII box, and attach...
the multi-pin cable (which should already be plugged into the ProScanIII box) to the right side of the stage.

3. Power on the ProScanIII box (swich on back), light should turn red, then green. Green lights should appear on the joystick as well. The control box should already be connected to the computer via USB and serial cables (necessary for adjusting joystick settings and computer control of the stage…not required, but likely will be desired).

4. Attach the Z focus sleeve to the Nikon microscope focus knob on the left side of the scope: slide on sleeve fitting, loosely tighten the three hex screws on the main body, then push firmly on the back and tighten the larger silver thumbscrew, and fully tighten the small hex screws. Focus can now be controlled with the knob on the side of the joystick. NOTE that both the focus and XY joystick movements are in the default "coarse" setting when initially powered on...you will likely want to switch to "extra fine" when working at 100x.

5. **After installing the stage**, carefully install the 100x trapping objective lens (should be in the cell sorting cabinet).

6. Make sure that the microfluidic controller is secured to the optical table (on the wooden stand), securing the wooden stand to the table with thumb nuts and the controller to the stand with wing nuts.

7. If not already connected, plug in the compressed air source (blue plastic tubing) to the push-to-connect fitting at the back of the controller chassis. Also connect the controller DC power source and USB plug (these should already be plugged into the AC outlet and the computer, respectively). Prior to turning on the air source (valve near the vacuum and gas, on the wall in the middle of the room), make sure that all control tubings are connected to something (e.g. a chip already in a holder) or are otherwise plugged. Turn on the house air source (should be at about 30-35 psi, set by regulator between air source and the controller).

8. Turn on the microscope camera (button on end of camera) and white light source (black switch on front of the small white box...do not switch on the fluorescence source, which is the larger tower-like box).

9. Turn on computer. Open Nikon Elements application (not the analysis one) and press the "play" button in the upper tool bar to begin imaging. Select brightfield and set exposure time to 20 ms. When using phase contrast, you may have to increase the hardware gain somewhat (from 1 to 3 or 4) to get a bright image, but this will probably not be necessary. Have the Nikon window open in the lower monitor?

10. Ensure that the controller is connected to the power source and the computer by USB (done in step above). Open the Microfluidic Controller folder on the desktop and start the LabView vi program for the "full" 48x chip. There is a button on the Windows task bar for this, but you will need to right click on it and select the top option, otherwise it will just start the labview application and not the specific app for the controller. To start the Labview microfluidic controller vi, click on the white arrow button in the top left of the tool bar in the labview window. After a bit of a delay, the controller solenoid valves should be able to be opened/closed by clicking on the dark/green buttons corresponding to different control lines. Have this window open in the upper monitor.

11. Start the program for the Prior ProScanIII stage controller with the button on the task bar and have this window open in the upper monitor as well. You can use this to control the stage if desired. You can also use this to reverse the x- and y-axis on the joystick (may be useful) and can decrease the speed on the joystick to make it less sensitive to small movements.

12. Prepare solutions for sorting (may vary depending on the type of cells/sample that is being sorted)
   a. In the cell sorting cart UV chamber, prepare a 1 mL aliquot of 1x PBS in a sterile 1.5 mL tube.
   b. Add 2 uL 10% Tween 20 (to 0.02% total) and 4 uL 10% F127 pluronic (to 0.04% total).
   c. Label this solution “PBS PL Tw”

13. Carefully remove the autoclaved chip from the glass petri dish and mount the chip on the plastic stage holder. Gently clean excess PDMS and other debris off the bottom of chip’s cover slide
using an isopropanol-moistened kimwipe. Mount the chip/holder on the stage, removing the “dummy” chip/holder.

14. Make sure that all control lines are depressurized (all buttons should be “green” on the labview vi, and all lights on the solenoid valves should be red).

15. One by one, move control lines from the “dummy” PDMS chip to the new, autoclaved chip. If any are empty or nearly empty, fill with clean 0.2 um filtered water using a syringe and tubing connected to the pin on the control line. Also, make sure that there are no or minimal air bubbles in the control line tubings before connecting to the new chip.

16. When all control lines have been connected to the new chip, fill the chip control lines by setting the control pressure to 23 psi (it should already be at this setting, but check to make sure) and closing all valves. If some control lines are slow to fill, ‘open’ the valve and re-fill control tubing (slow filling is likely due to air bubbles in the control line tubing).

17. Check for proper chip function of each of the control lines by finding the given control line at 2.5x objective and opening/closing the valve.

18. Remove PCR film from ‘diluent’ and ‘reagent’ ports. Fill a new tubing with PBS/PL/Tw. Insert tubing into the ‘diluent’ port and connect it to the control line manifold panel (top of the microfluidic controller). Open the valve and pressurize the tubing at 7 psi using the regulator at the top of the controller (marked in green tape). If desired, use a sharpie to mark the level of the liquid in the tubing.

19. Insert 10 cm tubings into the sample waste ports.

20. Add the PBS/PL/Tw to the chip by opening the reagent inlet (1), reagent gates (8), diluent (11), and all A gates (4, 5, 10, 16), i.e. open valves 1, 8, 11, and 4, 5, 10, 16, to fill chip.

21. Flush at least 10 uL buffer (3 cm level change in Tygon tubing) out ‘reagent port,’ wiping dry with clean kimwipe. Close valve 1 (‘reagent inlet’) to purge flow lines of air bubbles.

22. With valve 1 closed, open valves 3, 6, 9 and 17 (‘B gates’) to fill the cell holding chambers. Then fill the first, second, and third lysis chambers and the “reaction” chamber by opening valves 7, 18, 19, and 20. Make sure ALL the air has escaped the template, lysis and reaction chambers (air will be pushed out of the PDMS by dead-end filling). After all of these are completely filled, close all the valves. Then re-open the valves for the A and B gates (open valves 3, 4, 5, 6, 9, 10, 16, 17) …having these open a little while (2-3 minutes wait) will help “treat” the PDMS surface in the “gates” and prevent cell adhesion.

23. Turn pressure off PBS/PL/Tw solution at manifold, move to the tubing to the ‘reagent’ port (first disconnect the ‘Aigarchaeota’ stub from the manifold, then use forceps to move tubing…then reconnect ‘Aigarchaeota’ stub to manifold), and pressurize at ~2-3 psi.

24. Set up condenser alignment and phase. Move the stage so that you are viewing the middle of the channel 1 sorting intersection (just to the left of the “A” gate on channel 1). Because the condenser alignment is extremely sensitive at 100x, do rough alignment with the 4x, 10x, and 40x air objectives before attempting fine alignment at 100x. Reduce field diaphragm size to lowest size, center condenser ring with screws, focus on the flow layer, and align condenser with silver knob to make edge of field diaphragm image sharp.

25. When ready to go to 100x, carefully remove the chip/holder from the stage. Bring the 100x trapping objective into position. Apply a drop of immersion oil to the 100x lens, and also apply some along the middle top-bottom axis of the chip, where the cell sorting will take place. Carefully re-mount the chip/holder in the stage. Make sure the lamp illumination is bright enough, you may have to increase the gain on the camera with the Nikon Elements software.

26. Align condenser for optimal phase contrast at 100x (reduce field diaphragm, misalign it by slightly turning one of the small silver screws to visualize the “edge”, align condenser with silver knob to make edge of field diaphragm image sharp, re-center field diaphragm with hex screw, and re-open the field diaphragm).

27. Confirm that all valves are closed (i.e. close the A, B, lysis, and reagent inlet gates if they are still open).
III. Cell Preparation and Sorting

Dilute sample, set sorting intersection coordinates, and sort cells.

28. Dilute cell sample using PBS/PL/Tw solution, or other appropriate solution. For dense cultures (e.g. E. coli), dilute 1/50 in PBS/PL/Tw. For less dense cultures, dilute 1/5 or 1/2. You will want to see a decent number of cells per field (~10-20) at 100x, but not so many that it is difficult to sort individual cells. If your cell suspension is too concentrated or too dilute, you can always make a different dilution and add it to the chip, flushing the original cell suspension out.

29. Transfer cell solution into a tubing (as little as 1 uL) using the sterile syringe. Insert tubing into the “sample 1” port (for sorting in channels 1-24, on the right side of the chip), connect the ‘Aigarchaeota’ stub to the flow layer manifold (separate port from where the PBS/PL/Tw tubing is connected) and pressurize tubing to ~4 psi. To load cells, open the cell sample inlet and 1-24 waste gates (open valves 2 and 15), raising the pressure as high as 5 psi if necessary, to drive flow.

30. Purge air from the sample line by closing the waste for the appropriate sample (Waste 1-24, valve 15 for samples loaded into the right side of the chip; Waste 25-48 valve for the left side) while valve 2 (sample inlet) is open and the sample is pressurized at 4-5 psi. When the sample flow stops, all air has been purged. Set pressure to 2-3 psi, close valve 2 (‘sample inlet’), and open the waste for the appropriate sample (valve 7 or 10) when finished.

31. Make sure that the upper filter wheel has the IR mirror/filter in place. Also make sure that the lower filter wheel is in a position where no filter is in place.

32. Make sure laser enclosure is closed/secured (it should be normally) and microscope illuminator is in forward position with shields properly in place. The shields should include 1) the small, cut, black cardboard box in position over the chip/holder, and 2) black cloth over the microscope (but not over the brightfield illumination lamp).

33. MOST IMPORTANT: THE LASER IS INVISIBLE TO THE EYE AND EXTREMELY DANGEROUS. ENSURE THE LASER BEAMPATH IS BLOCKED IN TWO PLACES when the microscope is not shielded (small black cardboard box over the chip/holder, and black cloth over the scope). Close or (depower) the ThorLabs shutter AND turn the upper filter turret so that the filter is not in place (laser is not reflected up into objective). Alternatively, key off the laser at the laser power supply (however, please minimize the power cycling of the laser, e.g. do not key on/off the laser more than two times per day).

34. Turn on ThorLabs shutter controller (small red switch on the front of the box), turn key to unlock, and make sure the shutter is closed (should be default setting).

35. With cardboard box and black cloth shields in place, turn on the laser power source (switch on the back), and key on the laser. Set the power to 1.00 watt to start. If necessary, power can be increased.

36. Examine template chambers and sort path; make a note of the particle background between the ‘A’ and ‘B’ gates and in the template chamber & reagent lines.

37. Close all valves. Adjust manifold (connected to PBS/PL/Tw in reagent port, and sample tubing in sample inlet port) to about 2-3 psi.

38. To equalize pressure, open valves 1, 8, and ‘B gates’ (3, 6, 9, 17) for 10 seconds. Then close 1, 8, 20, and B gate of first quadrant to be sorted. For improved surface treatment, leave B gates on unsorted quadrants open (must close before sorting these quadrants!).

39. Before opening the A gate for each sort, open valves 1 and 3. This will counter drying between the A and B gates, preventing an influx of sample solution.

40. When moving to a new quadrant, equalize the pressure as in the previous step, and also open the relevant B gate to additionally counter drying past the B gate and ensure high B gate performance.

41. Search for cells to sort. If necessary, flush more sample by quickly double-clicking sample waste valve 15 (or 12) with the sample inlet (valve 2) pressurized.
42. Once you have found a cell, briefly open and close valve 8, with valve 2 open and valve 15 (or 12) closed (this will equilibrate pressure on both sides of the A gate). Open A gate (e.g. valve 4 for channels 1-12), confirm that cell is trapped by the laser, and move the cell past the A gate. Close the A gate, untrap the cell (close shutter) and check to see you just have one cell. If you have more than one, wait for them to separate. (There is no need to move the unwanted cell back across the A gate.) Re-trap the desired cell and move it towards the B gate (to the right on the right half of the chip, channels 1-24; to the left on channels 25-48 on left half of chip).

43. Move the cell to the B gate. Then back away from the B gate so it is just no longer in view. Open the B gate (e.g. valve 3 for channels 1-12). The liquid displacement from opening the gate will likely untrap the cell and move it closer to, but not quite into, the B gate. Find and retrap the cell and move it through the B gate. Close the B gate after the cell has passed through it. Retrap the cell and move it to the far side of the holding chamber, within view of the “lysis 1” gate. Untrap the cell.

44. After the sort, take a short movie of the cell using the BSR screen recorder program. Once launched, set the data directory to a folder named with the date in following format YYMMDD. Check that the recording region is appropriately sized for the cells being sorted (recording at 5 – 10 fps, no compression: ‘uncompressed’ setting). F6 starts recording and F7 stops recording. Save videos with the date and chamber number, e.g. YYMMDD_chXX, in a folder labeled with the sorting date.

45. IF YOU CAN'T TRAP: check the following: laser power supply on, laser power supply key at on position, upper filter wheel is in appropriate position (look for pink tape with “IR”), shutter is powered on and keyed on, shutter is open (can get stuck closed...toggle a few times if necessary). MAKE SURE TO LEAVE THE SHIELDING IN PLACE WHILE TROUBLESHOOTING MICROSCOPE SETTINGS, OR TO BLOCK LASER APPROPRIATELY IF REMOVING SHEIDING.

46. Sort any and all chambers except 24 and 48 (to be used for positive controls). Some chambers can be left blank as negative controls. For sorting on the left side of the chip (channels 25-48), close the shutter, move the upper turret so that the IR filter is not in place, then remove shielding. Either load a new sample into a new tubing and insert into the Cell sample 2 inlet (removing the tubing from sample 1 inlet first), or simply move the tubing from sample 1 inlet to sample 2 inlet if you want to keep sorting the same sample.

IV. Wash out unsorted cells and harvest sorted cells

47. When done sorting, make sure all valves are closed.

48. Close the shutter, key off shutter, key off laser, and turn laser off.

49. Remove shielding from microscope.

50. Carefully remove the chip/holder from the stage, and gently wipe off immersion oil from the coverslip using a kimwipe. Follow up with a kimwipe moistened with isopropanol.

51. Using lens paper (NOT kimwipes!), wipe oil off of the 100x sorting objective, using multiple lens papers if necessary. At this point, the 100x objective can be removed and placed back in its holder. Make sure to place the cap back on the empty port on the turret where the lens was.

52. Move the 2.5x objective into place and remount the chip/holder on the stage.

53. Detach the cell sample tubing from the manifold, remove the cell sample tubing from the chip and, using a syringe, expel the remainder of the cell suspension onto a kimwipe.

54. Replace the empty cell sample tubing into the cell sample port, connect to manifold, and pressurize to ~4-5 psi. Flush cells out of the sorting area by opening the sample inlet and sample waste valves (open valves 2 and either 15 or 12). You should see air eventually flush through the cell sample channel, indicating that the cells have been flushed out. Repeat for the other side of the chip, if it was used. When finished, remove the cell sample tubing from the chip (disconnect from manifold first) and set aside.

55. Remove the reagent tubing from the ‘reagent’ port (disconnect from manifold first) and connect to the diluent port. Do not reconnect the ‘Aigarchaeota’ stub to the manifold, just leave it
level on the stage…it will be used to collect additional fluid used to flush out cells between the A and B gates in the steps below (preventing it from coming out onto the surface of the chip).

56. Fill the 125 cm tubing nearly full (∼5 cm from ‘Aigarchaeota’ plug) with solution that the cells will be harvested in, e.g. the PBS/Tw/PI solution that may have been used for sorting and chip loading. Insert in reagent port and pressurize at the left panel to 7 psi.

57. Purge the reagent manifold of air by opening valves 1 and then 22 (reagent purge), closing 22 when no air remains.

58. Rinse one quadrant of the chip at a time by opening valves 1, 8, 11, and one A gate at a time (4, 5, 10, 16) to flush individual quadrants of the chip. Flush 1 cm of buffer thorough each quadrant, repeating until 12 cm of buffer has been consumed. Flush an additional 4 cm through with all four A gates open. Close all valves when finished.

59. Spray some bleach solution on your gloves and wipe off with a kimwipe. Repeat with isopropanol. Remove PCR film from outlets. Place filter-tip gel loading (20 uL) pipette tips in the chip collection ports, taking care to not touch the lower part of the pipet tip.

60. To harvest from all 48 channels, open valves 1, 8, all the B gates (3, 6, 9, 17), and the 1st/2nd/3rd lysis chambers and reaction chamber inlet/outlet (7, 18, 19, 20, 21). To harvest from only certain quadrants of the chip, only use the appropriate B gates (i.e. to harvest from the right side of the chip, channels 1-24, have B valves 3 and 17 open, but 6 and 9 shut).

61. Monitor the chip and tubing closely, checking that the tips are filling with buffer evenly and that the buffer in the tubing does not run out. Continue harvest until ~5 µL has been collected in each tip. Close all valves. **IF THE BUFFER RUNS OUT, THE SAMPLE WILL BE LOST INTO THE PIPETTE TIP FILTERS!!!**

62. Add the solution from each pipette tip to the corresponding collection tube, making sure to not contact any portion of the tip that will enter the collection tube with anything other than a sterile (bleach- and alcohol-treated is OK) forceps. To do so, handle the tip by the top only. DO NOT insert the tip into a pipetman until the tip is in the collection tube. When the end of the tip is in a collection tube, insert pipetman and expel the fluid into the collection tube.

63. Use cells to inoculate media as appropriate.

64. Rinse chip with clean filtered water. If the chip will not be autoclaved immediately, just leave it hooked up to the controller.

65. Rinse used tubings with clean water. If the tubing will not be reused, remove the ‘Aigarchaeota’ stubs and pins from the tubing, and discard tubing in biohazard waste…the ‘Aigarchaeota’ stubs and pins can be saved for reuse. If tubing will be reused, leave stubs and pins connected.

66. Soft-stop the vi, by clicking the ‘stop vi’ button. Shut off Prior stage/joystick controller box, camera. Turn off the air supply to the valve controller.

67. Replacing oil stage…make sure to completely lower the filter turret (using focus knob) before doing so!!!
1: Reagent inlet
2: Cell sample inlet
3: Ch 1-12 B gate
4: Ch 1-12 A gate
5: Ch 25-36 A gate
6: Ch 25-36 B gate
7: 1st lysis chamber
8: Reagent gates
9: Ch 37-48 B gate
10: Ch 37-48 A gate
11: Diluent
12: Waste Ch 25-48
13: Ch 48 control sol'n
14: Ch 24 control sol'n
15: Waste Ch 1-24
16: Ch 13-24 A gate
17: Ch 13-24 B gate
18: 2nd lysis chamber
19: 3rd lysis chamber
20: Rxn chamber inlet
21: Rxn chamber outlet
22: Reagent purge
23: <not used>
24: <not used>
REFERENCES


