



A method to analyze, sort, and retain viability of obligate anaerobic microorganisms from complex microbial communities



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ABSTRACT

A high speed flow cytometric cell sorter was modified to maintain a controlled anaerobic environment. This technology enabled coupling of the precise high-throughput analytical and cell separation capabilities of flow cytometry to the assessment of cell viability of evolved lineages of obligate anaerobic organisms from cocultures.

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Mapping functional interactions across microorganisms is essential to fully characterize microbial community structure and ecology, with important implications in human medicine, resource management and extraction, and environmental science. Anaerobic microorganisms are critical components of many important microbiomes, such as in anaerobic digesters (Fernández et al., 1999), anaerobic hydrocarbon deposits (Aitken et al., 2004), and anoxic waters and sediments (Musat et al., 2008). Notwithstanding major advances in laboratory microbiology techniques to study the physiology, systems biology, and microbial interactions of anaerobic microorganisms (Walker et al., 2009, 2012; Hillesland and Stahl, 2010; Hillesland et al., 2014; Yoon et al., 2011, 2013; Stolyar et al., 2007), a major challenge in characterizing anaerobic communities emerges from the technical difficulty of performing high throughput single cell analysis of live cells in complete absence of oxygen.

Flow cytometry is a useful technique for high-throughput analysis and isolation of live single cells for cultivation or experimentation (Chisholm et al., 1992; Davey and Kell, 1996; Wallner et al., 1997; Czechowska et al., 2008). Recently, flow cytometry has facilitated

single-cell analyses of genotypic and phenotypic variation that are particularly useful for understanding microbial population community structure and diversity. However, most applications have been limited to aerobic microbial systems because the sort stream and cell deposition areas of flow cytometers are open to the oxygenated atmosphere. Here, we present a new method that enables the separation of target cells from complex samples and preserves the viability of anaerobic cells for downstream experimentation by flow cytometric analysis, cell sorting, and single cell growth under anoxic conditions.

A BD Influx cell sorter (BD Biosciences) was equipped for anaerobic work by eliminating oxygen from the sort stream and cell deposition areas (Fig. 1). An anodized aluminum column was placed around the sort stream, seated in a groove on the cytometer frame, and sealed to the nozzle by a vinyl gasket, which spanned the gap between the aluminum column and upper aspect of the nozzle, creating a sealed interrogation chamber (Fig. 1B). Windows of 12.5 mm diameter MgF₂-coated glass (Edmund Optics, Inc., Barrington, NJ) were installed in the interrogation chamber at 180 and 90° angles to the incident laser line to allow forward scattered light (FSC), side scattered light (SSC), and fluorescence to reach the detectors. The internal seams of the cytometer frame were sealed with acrylic latex caulk (DAP Products Inc., Baltimore, MD). A glove box (12 × 14 × 9 in.) made of anodized

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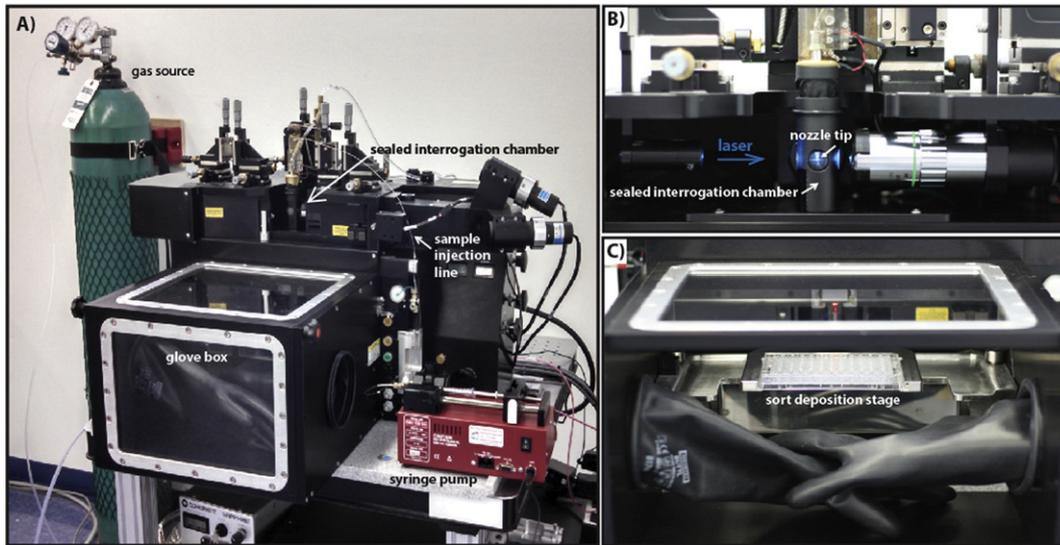


Fig. 1. A BD Influx cell sorter (BD Biosciences) (A) was equipped for anaerobic work by eliminating oxygen from the sort stream (B) and cell deposition area (C).

aluminum with clear polycarbonate thermoplastic windows and 4-inch diameter holes for placement of 16-mil chemical-resistant butyl gloves (Honeywell, Morris Township, New Jersey) was secured to the sort deposition chamber with aluminum bolts, in place of the standard deposition area door, forming a sealed sort chamber and workspace (Fig. 1 C). A port in the glove box was plumbed to a regulated (5–10 psi) tank of high purity nitrogen gas to create a positive-pressure oxygen-free environment during sorting. Oxygen concentration was measured with an

Oxygen Pen (Sper Scientific, Scottsdale, AZ) placed inside the sealed sort chamber and was maintained at less than 0.1% during sorting.

Culture samples were loaded into the cytometer by a New Era Pump Systems (Farmingdale, NY) syringe pump. Each sample was run for 5 min at 20 μL per minute before sorting to ensure removal of residual oxygen from the sample line. Cells were interrogated with a 200 mW 488-nm laser (Coherent, Santa Clara, CA) and SSC and FSC were collected for each cell, with FSC as the data collection trigger. Sort gates

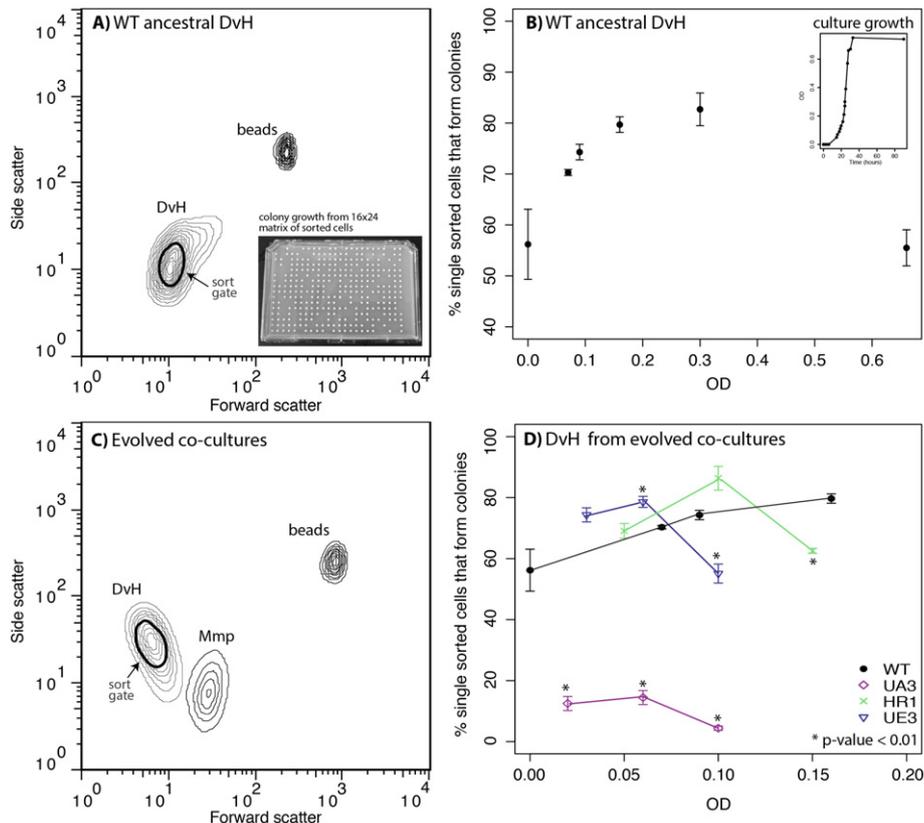


Fig. 2. Comparison of DvH single cell recovery rates from an ancestral monoculture (A, B) and three 1000-generation evolved co-culture lineages with *Methanococcus maripaludis* (UA3, HR1, and UE3) (C, D) in log phase and early stationary phase growth (WT only). Note that the characteristics of the DvH populations varied between wild type (A) and coculture (C) relative to standard beads (1 μm diameter). Inset in (A) displays a typical matrix of colonies growing for the 16 \times 24 sort matrix. In (D), p-values are calculated to compare each evolved line to the WT at similar OD. Inset in (B) displays growth data from the DvH WT culture from which single cells were sampled to test single cell growth efficiency plotted in (B).

were drawn on SSC and FSC parameters (Fig. 2 A and C) and 384 single cells were sorted into 16 × 24 matrices onto agar medium prepared in Nunc OmniTrays (Thermo Scientific, Waltham, MA). After sorting, within the sealed sort chamber, plates were placed in a gas-tight anaerobic transport box with AnaeroPack-Anaero (Mitsubishi Gas Chemical Co., Japan) as an oxygen-absorption agent. The anaerobic transport box containing the plates was then removed from the anaerobic sort chamber and transferred immediately to an anaerobic hood (98.5% N₂, 1.5% H₂) for growth at 37 °C for 3 days. Single standard reference beads spiked into the samples were sorted as negative controls and experiments were repeated under oxygenated conditions as an additional negative control, for which no growth was observed.

In order to demonstrate possible applications for this method, we compared the recovery rate of single sorted cells from an ancestral wild-type monoculture of *Desulfovibrio vulgaris* Hildenborough (DvH) and from three distinct lines (UA3, HR1, and UE3) that had evolved for 1000 generations in co-culture with *Methanococcus maripaludis* (Mmp) under syntrophic growth conditions without added sulfate (Hillesland and Stahl, 2010). Whole genome sequencing showed that DvH from each evolved lineage had accumulated distinct mutations in sulfate respiration pathway genes at different frequencies (Table 1) (Hillesland et al., 2014), leading us to investigate the consequence of the mutations for DvH sulfate reduction capability when separated from Mmp and the linkage of the mutations in individual DvH genomes. Growth conditions and culture handling techniques were performed as previously reported by our group for wild type ancestral DvH monocultures (lactate–sulfate medium LS4D) (Mukhopadhyay et al., 2006) and evolved DvH and Mmp coculture lineages in the same medium without sulfate (coculture medium A CCMA) (Stolyar et al., 2007; Hillesland and Stahl, 2010).

First, we examined single cell recovery rates across different stages of growth in batch cultures of the wild-type ancestral DvH strain (Fig. 2A and B). Specifically, for each time point that corresponded to a different cell density and growth phase (reported as optical density at 600 nm or OD) we counted the fraction of 384 cells, sorted in three replicate sets of 384 single cells from one culture, that were able to form colonies from single cells on lactate–sulfate medium agar plates (Fig. 2A, inset). There was a positive relationship between OD and percent of sorted single cells that formed colonies until entry into stationary phase growth when recovery of colony-forming cells decreased (Fig. 2B). This pattern demonstrates that there is a direct relationship between growth stage and physiological state for sulfate reduction, with maximum recovery observed in late log phase growth. To our knowledge this is the first systematic assessment of single cell recovery rates of an anaerobic microbe that was enabled by the live anaerobic sorting capability. While other studies have estimated the percent of viable cells in a culture based on inoculation volume and cell concentration (Jones et al., 1983), our method enables precise deposition of cells in an anaerobic environment, eliminating pipetting error and ensuring equal nutrient availability for each deposited cell. This result underscored the importance of testing the growth efficiency of single cells over

different phases of growth, or ODs, in order to perform statistically significant and biologically meaningful comparisons between strains.

Next, to demonstrate the capability of anaerobic cell sorting to separate a target cell population from a mixture, we tested the viability of single DvH cells from three evolved co-cultures with Mmp by sorting and testing for viability on lactate–sulfate medium at three different ODs during log phase growth (Fig. 2C and D). In each lineage of evolved coculture, the two cell populations were easily distinguished by SSC and FSC signals and the identity of each population was confirmed as Mmp or DvH using PCR targeting unique genes in each genome (PCR data not shown). As described above for WT DvH, single cells were sorted onto agar plates to test for single cell growth efficiency. We found lowest recovery rates for evolved strain UA3, suggesting that mutations in at least one of three sulfate respiration genes (Table 1) prohibit growth independent of Mmp on lactate–sulfate medium. In contrast, single cell recovery rates of evolved strains HR1 and UE3 were similar to WT at equivalent ODs (Fig. 2D). This result suggested that the *dsrC* mutation in UE3 (in 99% of the population) is not a loss-of-function mutation. This result allows us to proceed with studies on how mutations accumulate in the evolved lines with a better understanding of the capability of DvH cells within each coculture to grow independently.

These insights into the independent growth physiology of DvH cells that had accumulated mutations in coculture with Mmp would not have been possible without the anaerobic cell sorter-enabled separation of DvH from a mixed assemblage in high throughput and at single cell level. Future work will involve resequencing of the viable colonies from evolved lines to determine how the mutations are linked in individual genomes.

The anaerobic cell sorter offers a powerful technique to separate and test the viability of live anaerobic cells towards characterizing the dynamics of changes in biodiversity and structure of an anaerobic microbial community from the single cell to population level. We have shown the utility of the method for separating target populations from co-cultures, however the method can easily be expanded to the isolation, genotyping, and cultivation of anaerobic microorganisms sorted from complex natural communities.

Competing financial interests

The authors MJC, BW, and TWP are employees of BD Biosciences, the company that makes the BD Influx cell sorter used in this study.

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Table 1

Frequency of sulfate reduction pathway mutations in three lineages of DvH evolved to grow syntrophically with Mmp and tested for their ability to grow independently on lactate sulfate medium. Data from Hillesland et al. (2014).

Line	Gene	Mutation frequency	Mutation effect
UA3	<i>dsrC</i>	0.93	Non-synonymous
	<i>sat</i>	0.95	Frame shift
	<i>apsB</i>	0.97	Frame shift
HR1	<i>sat</i>	0.34	Frame shift
	<i>apsB</i>	0.35	Stop
UE3	<i>dsrC</i>	0.99	Non-synonymous
	<i>sat</i>	0.33	Non-synonymous
	<i>apsA</i>	0.48	Stop

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