Supplemental Methods

Biosafety considerations

Biosafety protocols were developed for sample handling, FACS analysis, aerosol containment testing, and instrument and workspace decontamination. For the current biosafety standards for high speed flow cytometric sorting of potentially infectious samples, and discussion of BSL level practices and risk assessment for specimens, see (Schmid et al. 2007). Nycodenz fractionation, and subsequent aliquoting and dilution of EtOH fixed cells from human derived samples, were performed using BSL2 procedures in a biosafety cabinet. EtOH fixed samples were considered “unfixed” for the purposes of this work and to potentially contain infectious agents, and BSL2 biosafety practices were used. In contrast, BSL1 practices are generally used for formaldehyde fixed human derived samples.

For high speed sorting of EtOH fixed single cells, BSL2+ practices (BSL2 procedures with some BSL3 practices) included locating the FACS Aria II instrument, fitted with an aerosol management system (BD), in an enclosed, dedicated room with positive air pressure, and for personal protection included use of personal protective gear including full body gowns, Moldex N95 ventilation masks, acrylic face shields, and gloves.

Sorting of single cells by flow cytometry

***Equipment*.** Single cell sorting was performed on a custom FACS Aria II equipped with a 100 mW 488 nm laser, forward scatter photomultiplier tube (FSC-PMT) 488 nm filtered, side scatter (SSC-PMT) 488 nm filtered, and green fluorescence (SYBR-PMT) 512 nm filtered detectors

(BD Biosciences, San Jose, CA). The Aria II was also equipped with a Whisper aerosol management system (AMS, BD Biosciences) as a biosafety feature for aerosol containment during sorting, and in the event of a clog. An Olympus IX70 inverted epifluorescent microscope was used to visualize bacterial cells and fluorescent beads.

Aerosol containment test. Prior to sorting EtOH fixed cells from specimens, a test of the AMS containment function was performed essentially as described by (Oberyszyn and Robertson 2001), but using 3 μm yellow-green fluorescent microspheres (Spherotech) diluted to a concentration of ~25,000 events/sec. Blank, cleaned glass microscope slides were mounted on the FACS instrument inside the sort chamber, on top of the sort chamber, and on top, left and right sides of the instrument. An aerosol event was generated by obstructing the waste stream inside the sort block while fluorescent beads were being detected. With the AMS vacuum powered to 20%, aerosols were generated for 2 min and slides were then examined manually by fluorescent microscopy for presence of beads. No beads were observed on slides positioned outside the sort chamber, and the slides inside the sort chamber, contained minimal amounts of beads, indicating that the AMS and sort block were effectively containing aerosol generated in the test.

Single cell sort targeting. Confirmation of single cell sorting accuracy in the 384-well format was determined by using 10 μm green fluorescent beads as proxy while sorting under the detection settings determined empirically to be most appropriate for a given sample. Single 10 μm beads were first sorted onto the top surface of 4 wells on each corner of a sealed 384-well PCR plate. Inverting the plate and imaging the location of the beads using fluorescence microscopy allowed for an initial targeting adjustment from within the FACS control software such that beads to be targeted to the center of the well. A second series of single beads were then targeted into 16 empty wells on each end of an unsealed 384-well plate. The plate was then inverted and beads were directly imaged at the bottom of the targeted empty wells. A second series of minor targeting adjustments were made in the FACS control allowing for precise targeting to the bottom of the well. After confirmation of accurate targeting of sorted beads, single cell sorting proceeded.

FACS detection and single cell sorting. After aersosol containment was established, FACS detection was performed on the Nycodenz fractionated bacteria. Filter sterilized (0.2 μm) phosphate buffered saline (PBS, 1X) was used as sheath fluid, and for sample dilution. Unstained and SYBR Green I (0.5X) stained material was 35 μm filtered and a 1:1000 dilution was assessed for event rate at low flow rate (<2,000 total events/sec) and adjusted if necessary. The low flow rate is critical to reduce the likelihood of sorting coincident events. Fluorescent events were collected using 549V FSC-PMT, 220V SSC, and 700V SYBR Green Channel, with thresholds of 200, 200, and 500 respectively, with no compensation. Appropriate voltage and threshold settings need to be determined empirically for different samples. Bi-exponential scatter plots were generated using FSC-PMT vs. SSC and FSC-PMT vs. SYBR Green (Supplemental Figure S1). Unstained material few fluorescent events. 1000 events from each gate were sorted at a lower purity setting onto glass slides for viewing with fluorescent microscopy on an Olympus IX70 inverted fluorescence microscope at 60X magnification to confirm the presence of cellular morphologies. A bead targeting strategy (see above) was employed to ensure only single cells were sorted from the sample into each well of a 384-well PCR plate (FrameStar). Single cell events were sorted into 4 μL of a low EDTA TE (10 mM Tris 0.1 mM EDTA, pH 8.0) and immediately frozen on dry ice and held there until transfer to -80°C for storage prior to processing.

Minimizing contaminating DNAs. Special precautions are used to minimize the possibility of DNA contamination during single cell sorting, MDA, and PCR. As molecular biology reagents can contain bacterial contaminating DNAs that yield bacterial sequences following amplification, MDA and PCR reagents, including buffers, water, primers, hexamers, and dNTPs are subjected to quality control analysis prior to use so that reagent lots with confounding amounts of contaminating DNAs can be avoided.

Wherever possible, plastic ware used in MDA and PCR reagent preparation and reactions are single-use, sterile, RNase-, DNase-, and Pyrogen-free. Where glass is used in preparation of MDA lysis stocks, it is either autoclaved or treated with 10% bleach, followed by 70% EtOH, and rinsed in nuclease free water (Ambion). MDA and PCR reagents are prepared in a PCR prep hood equipped with dedicated pipetmen and a UV source light for DNA decontamination. DNA AWAY (Molecular BioProducts) and 70% EtOH are used to clean pipetmen and surfaces before and after use, including at the flow cytometry station, and in the Agilent BioCel robotic system (see below).

Plastic ware or glassware used to make reagents for the FACS instrument are either autoclaved or treated with 10% bleach, followed by 70% EtOH, and rinsed in nuclease free water (Ambion). Glassware, tubing, and the stainless steel sheath flow tank used for the FACS instrument are cleaned with 18.2 MΩ purified water (MilliQ) and Vesphene IIse detergent (Steris), followed by autoclaving for 30 min at 125 ^{o}C. The sheath flow tank and glassware are vented during autoclaving using a cotton filtered syringe attachment. The sealed and sterile tank is filled with filter sterilized PBS (prepared in a large glass carboy) using a peristaltic pump.

The FACS instrument fluidics are cleaned as follows. Prior to bead targeting and sorting on the FACS instrument, standard startup procedures are followed, with one exception: the sheath tank filter is typically stored in 70% ethanol upon shutdown and is washed with 1L of sheath fluid after startup. After startup, the standard aseptic sort setup routine is performed per manufacturer instructions, with the exception that the filter assembly is re-attached to the line instead of being replaced. Shutdown of the instrument is as per the manufacturer's instructions with one exception: the sheath filter assembly is connected to the ethanol tank and 70% ethanol is flushed through the sheath filter assembly before shutdown.

Free DNA in the sample is also a possible source of contaminating DNA. Reiterative sorting approaches were not used in this work, but can be used to reduce the likelihood of contamination originating from free DNA in the sample (Rodrigue et al. 2009).

PCR and analysis of 16S rRNA genes. 16S rRNA genes were amplified from diluted MDA product (1:20 into TE) using universal bacterial primers 27f and 1492r (Lane 1991). with the following reaction condition: 94 °C for 3 min, 35 cycles of 94 °C for 30 sec, 55 °C for 30 sec, 72 °C for 90 sec, and 72 °C for 10 min. PCR products were treated with Exo I and shrimp alkaline phosphatase (both from Fermentas) prior to direct cycle sequencing with 27f and 1492r primers at the Joint Technology Center (JTC, J. Craig Venter Institute, Rockville, MD). 16S tracefiles were analyzed and trimmed with the CLC Workbench software program (CLC bio, Muehltal, Germany). Read lengths of less than 200 bp were discarded. Only a minority of 16S read pairs could form a contig, and in some cases only the forward or reverse read was used to establish taxonomy. Chromatogram quality was assessed manually, and MDA products with both forward and reverse reads of poor quality were excluded from further analysis. MDA products with 16S rRNA gene taxonomy similar to those NTC reactions were excluded from further analysis

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