

b.sight™ | High-throughput Cultivation Workflow for Isolation of Anaerobic Gut Bacteria

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Introduction

The gut microbiota refers to very diverse communities of microorganisms that reside in the gastrointestinal tract and play an important role in regulating the health of the host. As shifts in the structure and functions of this microbial ecosystem have been associated with the development of chronic disorders such as metabolic diseases¹, inflammatory bowel disease², and colorectal cancer³, studying the genetic diversity of the microbiota is of major interest. The advancement of modern sequencing technologies has revealed the existence of many yet unknown species and highlighted the tremendous complexity of gut microbiota⁴. Nevertheless, cultivation-based studies are essential because they facilitate proper taxonomic description of novel microbes and open avenues for downstream functional studies^{5,6}.

A critical step in the process of isolating and describing microbes from complex communities is efficient separation of single community members for the generation of axenic cultures. This is complicated

substantially by the uneven distribution of taxa in native communities such as those in human feces. Most common approaches of obtaining pure microbe cultures rely on colony streaking on agar plates or “dilution-to-extinction” procedures⁷. In the latter case, samples are serially diluted in microwell plates until no bacterial growth is observed. While this method can produce pure cultures, it is quite laborious and low throughput since only a small percentage of wells give rise to clonal cultures. Hence, improving the isolation of single cells from complex matrices is crucial for enhancing the efficiency of cultivation approaches.

Here we introduce a simple workflow for rapid generation of axenic cultures of gut bacteria. We bypassed the low efficiency “dilution-to-extinction” step by using the b.sight™, an instrument designed to rapidly sort single microbial cells and enable direct generation of clonal cultures. The b.sight can readily isolate and deposit a single cell per well in liquid culture media or directly onto solid agar. The device is benchtop-sized and can be accommodated in anaerobic workstations commonly used for handling strict anaerobes in

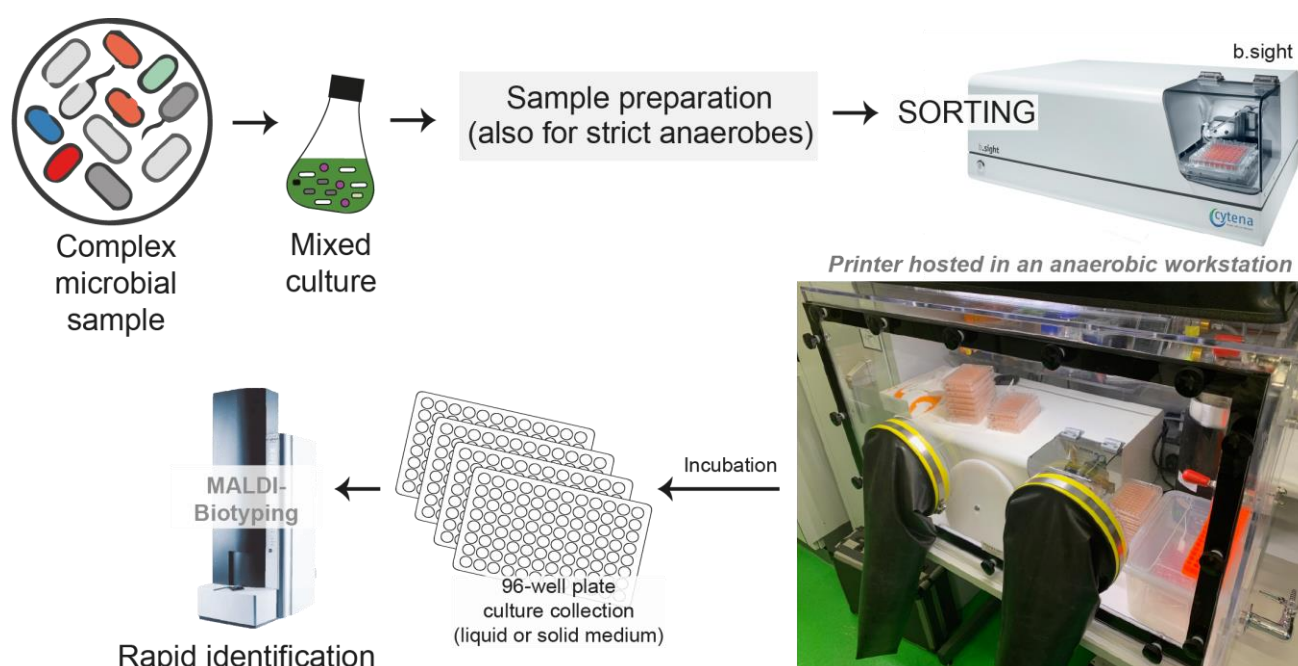


Figure 1. Schematic overview of the new bacterial isolation workflow using the b.sight.

appropriate conditions. Using this workflow, we generated 1,181 clonal cultures of *Escherichia coli* and *Bacteroides vulgatus* and 1,666 clonal cultures of mouse gut bacteria. In the ultimate cultures, MALDI-TOF-MS identified up to 500 isolates, revealing the presence of potentially new species.

Materials and Methods

Samples and culture conditions

Strains of *E. coli* and *B. vulgatus* were obtained from in-house collections of bacteria. Complex microbial communities from the intestine of a mouse were obtained from previously generated mixed cultures; no mice were sacrificed for the purpose of the present work. Strains and samples were grown in appropriate culture media in anaerobic conditions (89.3% N₂, 4.7% H₂, 6% CO₂) in a 999-AC-118 Chamber (Plas-Labs Inc., MI, USA). Cells were dispensed in different media (BHI, Brain Heart Infusion; WCA, Wilkins-Chalgren Anaerobe; GMM, Gut Microbiota Medium). All materials and media were brought into the anaerobic workstation at least 24 hours before starting work.

Working principle of b.sight's single-cell dispensing

The b.sight is an automated laboratory instrument that enables microbial cell isolation, as previously published⁸. A dispenser chip rapidly generates small droplets on-demand when the silicon membrane is deflected by a piezo-actuated piston. The dispenser

chip's nozzle region is imaged continuously during operation. A cell detection algorithm analyzes each passing particle until it predicts that the next droplet is a single cell with correct parameters and allows the cell to dispense into a well of the 96- or 384-well plate below. Unwanted droplets (i.e., void droplets or droplets with multiple cells) are removed by vacuum prior to dispensing. A series of images of the nozzle region is automatically stored for each deposited droplet, and single-cell ejection can be verified by reviewing the image series.

Workflow for microbial single-cell isolation

A brief overview of the workflow is shown in **Fig. 1**. Prior to cell isolation, single bacterial strains or mixed communities were cultured for 24 hours in liquid medium under anaerobic conditions to generate suspensions free of debris from intestinal content and of sufficient density. These cultures were serially diluted in filter-sterilized (0.02 µm) phosphate buffer saline (PBS) supplemented with 0.5% (w/v) peptone and reducing agents. The final dilution was filtered through a 10 µm membrane and dispensed (50-70 µl) into a sterile dispensing cartridge with a 20 µm nozzle and mounted onto the b.sight for single-cell dispensing. Preparation of the b.sight instrument typically takes 5-10 minutes. Single cells were dispensed into 96-well plates filled either with liquid or agar media. Typical dispenser conditions were a downstroke velocity of 49-61 µm/s and a stroke length of 3 µm. Post-dispensing,

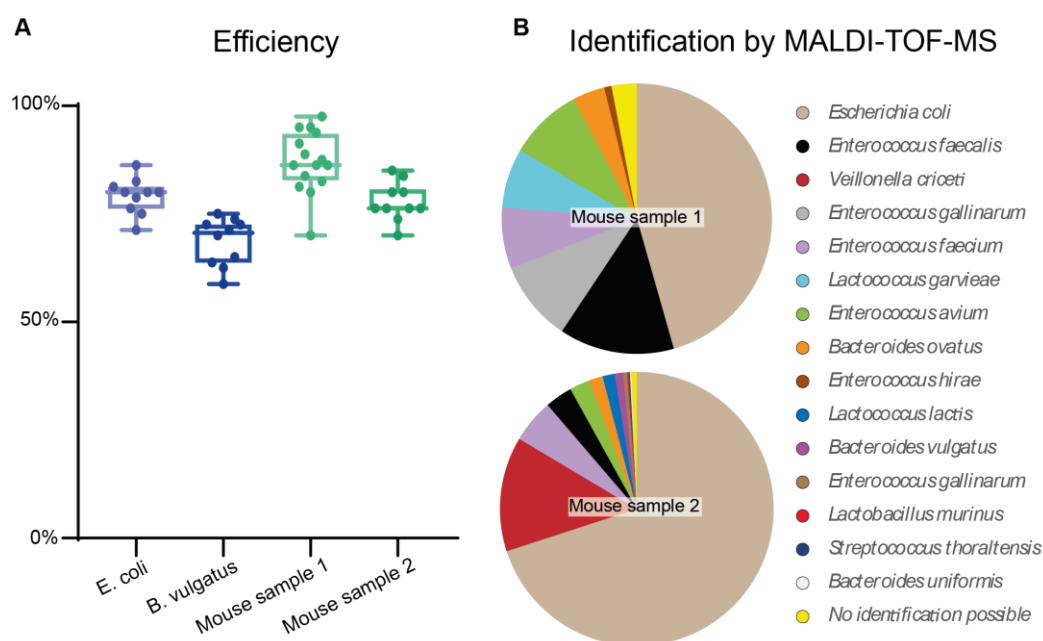


Figure 2. Outputs of single-cell dispensing experiments. **A.** Rounds of sorting were performed using cell suspensions of single strains and two mixed cultures generated with mouse gut content. Efficiency is defined as the percentage of wells addressed by single-cell dispensing giving rise to bacterial growth after OD measurement. **B.** The pie charts in the right panel show the proportions of isolates belonging to the various listed taxa ($n = 465$ and 620 for sample 1 and 2, respectively).

the samples were incubated at 37 °C for 1 to 7 days. Cultures in liquid media were stamped onto agar for subsequent downstream analysis.

Bacteria identification by MALDI-TOF-MS

Cell biomass from each of the bacterial colonies to be identified was applied directly onto the MALDI-target. Each spot was overlaid with 1 µl of α -Cyano-4-hydroxycinnamic acid (HCCA) matrix solution and allowed to dry at room temperature. MALDI-TOF measurement was performed using a Microflex LT mass spectrometer (Bruker Daltonics, Germany) according to manufacturer's instruction.

Results and Discussion

Clonal cultures from single strains

The first experiments were performed with single strains to evaluate the percentage of wells (each theoretically containing a single cell) giving rise to growth. An anaerobic species, *B. vulgatus*, was used in addition to the facultative anaerobic species *E. coli* to demonstrate feasibility of the approach for strict anaerobes. For each strain, ten 96-well plates were processed, resulting in 800 wells with single cells (two rows per plate being reserved for negative controls; one row each for empty droplets and culture medium). **Fig. 2a** shows the percentage of wells that exhibited growth as determined by OD₆₀₀ absorbance measurement: $79.1 \pm 4.0\%$ and $68.5 \pm 5.3\%$ of the wells were positive for *E. coli* and *B. vulgatus*, respectively.

Identification of single bacteria isolated from complex mouse gut samples

Complex communities from the intestine of a mouse were processed to evaluate printed bacterial diversity. For sample 1, single cells were dispensed onto solid agar media (six plates) or into liquid media (nine plates), resulting in 1,200 wells characterized by a cloning efficiency (percentage of wells with growth) of $87 \pm 6.9\%$ (**Fig. 2a**). Values for sample 2 (seven agar and three liquid plates with a total of 800 wells) were $77 \pm 5.8\%$ (efficiency).

After incubation, the identity of cultures was determined using a MALDI-biolyser. For sample 1, a total of 465 isolates were processed, revealing at least eight different species (**Fig. 2b**). For sample 2, the 620 isolates belonged to at least twelve species (**Fig. 2b**). Cultures with no match to the MALDI database were found in both samples, potentially representing unknown species within the mouse gut microbiota.

Conclusion

The high-throughput cultivation workflow presented here allows the generation of a high number of pure

cultures for downstream identification steps. The b.sight is flexible in its ability to deposit single cells, with image confirmation, both into liquid culture and onto solid agar. Additionally, its small footprint is compatible with anaerobic chambers and laminar flow hoods. Using this workflow, over 2,800 pure cultures with high cloning efficiency were generated. While the focus here was on the gut microbiota, this method is readily applicable to other types of microbial environments with appropriate adjustments of culture conditions and is a fast and efficient method to study the cultured fraction of complex microbial populations.

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