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Sequencing Single-cell Genomes of Uncultivated Bacteria in the Human Oral Microbiome

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Abstract

The microbial world has the most diversity of all living organisms. The fact that the majority of microbial organisms remain unexplored holds enormous potential to discover new metabolic pathways that can be applied in biotechnological processes or used to develop new therapeutics. We present a workflow for exploring such microbial dark matter, based on single-cell isolation and genetic characterization. Single-cell dispensing with the B.SIGHT allows label-free isolation of microorganisms with image-based cell detection visualization. Subsequent single-cell whole genome amplification is performed using a multiple displacement amplification (MDA) protocol. Precise dispensing of single bacteria into PCR plates, combined with contact-free low-volume liquid handling enables cell lysis in only 350 nL and a miniaturization of the amplification reaction to 1/20 of the original volume and results in significant cost savings. Downstream sequencing of the 16S rRNA gene allows taxonomic classification of the isolated bacterial cells. Since all steps are performed in standard microwell plates, this workflow can easily be scaled up to analyze thousands of individual organisms to gain insight on the composition and heterogeneity of complex microbial samples without the need for cultivation.

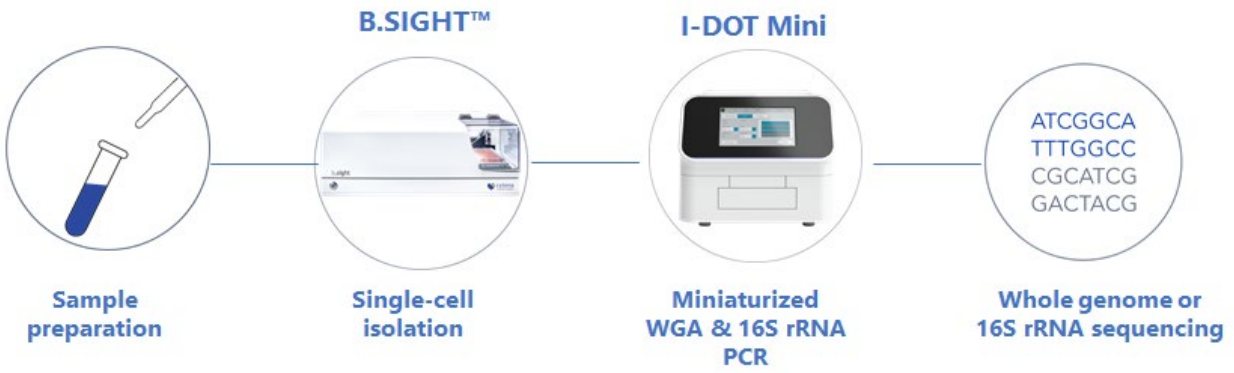

Figure 1. *Workflow for the separation and analysis of single microbial genomes from a complex sample.*

Introduction

The biosphere of microbial organisms has an estimated diversity between 10^5 and 10^{12} different species. While some prominent representatives are well studied and characterized, the vast majority of microbial organisms remain unknown. The characterization of unknown microbial species is of high scientific interest with a high potential for discovering new metabolic pathways or regulatory mechanisms that could result in new therapeutic approaches. It also contributes to a better understanding of the composition and potential interactions within a microbial consortium. Established methods for the isolation of pure microbial cultures from single cells are based on dilution-to-extinction or streaking on agar plates and quickly reach their limits in terms of throughput and automation capability. Furthermore, methods based on cultivation and subsequent characterization face the major challenge of finding suitable growth conditions for the complex metabolisms of individual microorganisms. Despite numerous efforts and great advances in improved cultivation methods,

estimates indicate that 99% of all microbial species remain uncultured, either because cultivation is too time consuming or proves impossible because of the high complexity of their metabolism. For genetic analyses of these unculturable species, independent cultivation methods have been developed. Shotgun sequencing and subsequent reconstruction of metagenome-assembled genomes, as well as single-cell genomic approaches, shed light on the recovery of the identity and genomes of these unculturable species. While shotgun sequencing is usually performed for a complex sample in bulk, rare organisms or subtle differences between related organisms might be overseen in the mass of highly abundant species (Woyke, 2017). The separated characterization of single cells prevents such a bias and is therefore the method of choice to reflect the original heterogeneity of a complex sample.

We introduce a high-throughput workflow for the label-free isolation of single microorganisms from a human sputum sample, followed by miniaturized reactions for whole genome amplification and the amplification of 16S rRNA gene sequences for taxonomic classification.

Single-cell WGA per reaction	Full volume (µL)	1/20 volume (µL)
Lysis		
Buffer DLB	2.75	0.1375
DTT, 1 M	0.25	0.0125
PBS sc	4	0.2
Total	**7**	**0.35**
Neutralization		
Total	**3**	**0.15**
Whole genome amplification		
REPLI-g sc Reaction buffer	29	1.45
H_2O sc	9	0.45
Repli-g sc DNA Polymerase	2	0.1
Total	**40**	**2**

Table 1. *Dispensed volumes of reagents in the standard single-cell WGA reaction (50 µL) and 20-fold reduction (2.5 µL).*

Materials and methods

Sample preparation and single-cell isolation

Sputum samples were obtained from healthy individuals. The samples were diluted 10-fold with PBS and filtered through a 10 µm membrane. After filtration, the sample was centrifuged at 4000*g* for 10 minutes, the supernatant was discarded, and the pellet was resuspended in 40 µL filtered PBS before being loaded into a new sterile bagged dispensing cartridge. Single microbial cells were dispensed into 384-well PCR plates using a B.SIGHT equipped with a module for automated offset correction (AOC). The settings for sorting were selected for a size range between 0.5 and 10 µm and a roundness between 0.1 and 1 to sample the full morphologic spectrum of the complex mixture.

Lysis and whole genome amplification with the I-DOT

Cell lysis and whole genome amplification (WGA) reactions were performed using the REPLI-g Single Cell Kit (Qiagen), which allows isothermal amplification of genomic DNA based on multiple displacement amplification. Dispensing the reagents with the I-DOT or I-DOT Mini enabled the miniaturization of the reaction volume 20-fold compared to the original protocol (see **Table 1**). To avoid the amplification of potential DNA contaminations, all reagents were UV-radiated (Stratagene Stratalinker) for 60 minutes prior to dispensing (Woyke, 2011). The amplification was run for 12 hours at 30°C, then for 5 minutes at 65°C to inactivate the polymerase.

DNA concentration measurement

DNA concentrations were determined using the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen). The samples were diluted 1/20 and 1 µL was used in 10 µL of PicoGreen working solution. A standard curve consisting of lambda DNA was dispensed by the I-DOT or I-DOT Mini for quantification. Fluorescence intensities were measured using a Spark Multimode Microplate Reader (Tecan).

PCR amplification of the 16S rRNA gene and sequencing

Amplifications of the full length 16S rRNA genes were performed using the OneTaq 2x MasterMix with Standard Buffer (NEB) and the primer pair 27F (5'-AGAGTTTGATYMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3') in a total volume of 10 µL per well. DNA samples were diluted 1/20 after the WGA reaction and 1 µL of each reaction was used as a template for the PCR. After PCR amplification, the samples were loaded to an agarose gel, and samples showing a product with the correct length (~1550 bp) were sent for Sanger sequencing in 96-well format (Eurofins Genomics) using 27F as a sequencing primer.

Sequence quality control and trimming

Only sequences that passed sequencing quality criteria were further analyzed. Clipped sequences were chosen for further analysis, based on sequencing quality values.

Taxonomic classification and phylogenetic tree calculation

Sanger sequences were aligned with SINA against the SILVA RefNR SSU database using the online tool Silva-ARB (www.arb-silva.de). Phylogenetic trees were calculated with PhyML 3.1 (Guindon, 2003) using 100 bootstrap replicates for calculating branch support values. A custom python script was written to replace sequence IDs with genus or family names, to collapse taxa that occurred multiple times, and for drawing a phylogenetic distance tree. The latter was done with the iTOL visualization tool (Leitunic, 2019).

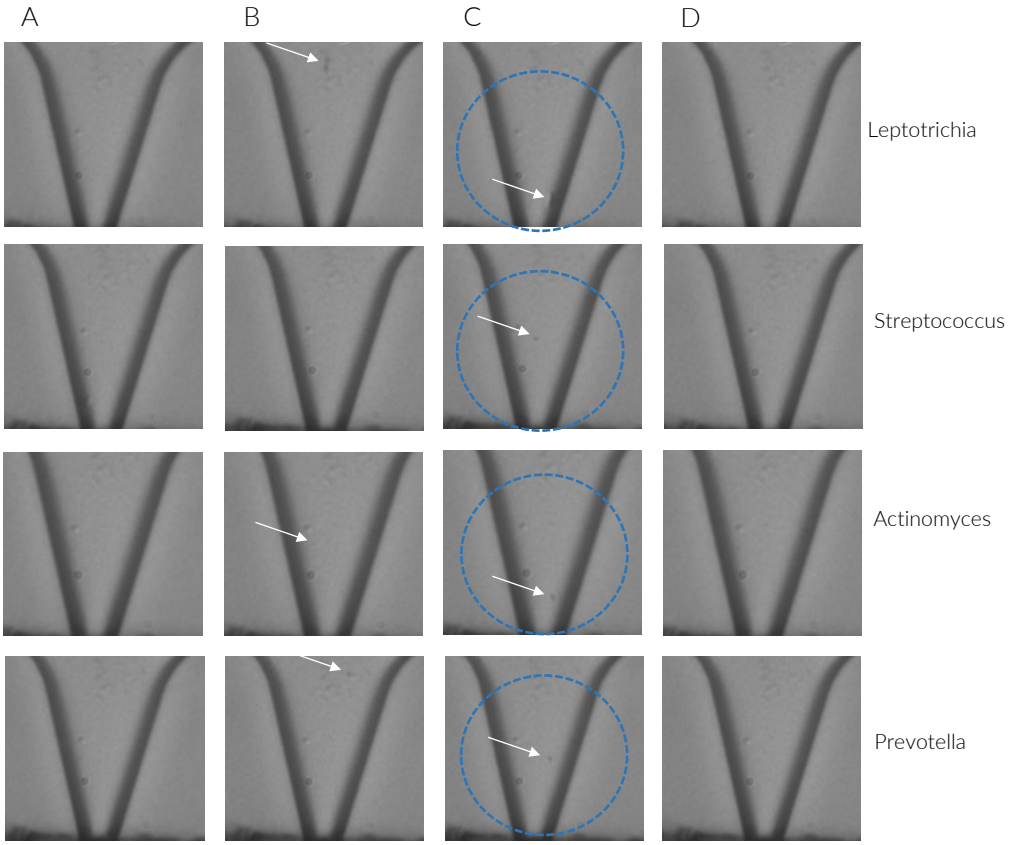

*Figure 2. B.SIGHT nozzle images showing the isolation of a single microbial cell. The cell approaches the nozzle (**A**, **B**). The cell is detected in the nozzle by the automatic image processing algorithm (**C**). Image **D** shows the empty nozzle after the droplet with the single cell was dispensed.*

Results and discussion

Dispensing single microbial cells to a precise plate position

A total number of 344 single cells were dispensed. In 19 of the dispensed wells, two cells were dispensed unintentionally as determined by nozzle images. These wells were not further processed in the following workflow. A selection of typical nozzle images obtained by the B.SIGHT are displayed in **Figure 2**.

The deposition of the single microbial cell into the center of the conically shaped well of the PCR plate is crucial when working in low assay volumes (**Figure 3**). The B.SIGHT is equipped with a module for automated offset correction (AOC). The AOC module automatically measures the droplet impact position to ensure that droplets with single cells are dispensed into the center of the wells without the need for manual alignment procedures. In addition, a deionizer removes electrostatic charges from the microwell plate to prevent deflection of the droplet by electrostatic forces. The gentle dispensing by the B.SIGHT reduces the risk of pressure-induced cell lysis that might cause DNA degradation.

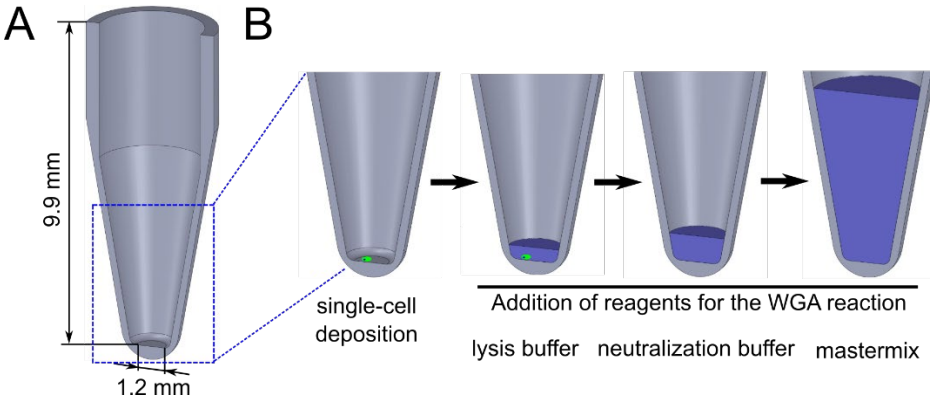

Figure 3. *Precise dispensing of a single cell into conical PCR plates. Plate deionization to remove electrostatic charges and the automated offset correction (AOC) module enable the deposition of a single cell (green sphere) onto the bottom of the well (**A**) as a prerequisite for the lysis and WGA in miniaturized volumes (**B**).*

Whole genome amplification

Dispensing of all WGA reagents was performed using the I-DOT (Immediate Drop-on-demand Technology). The I-DOT allows fast and fully automated reagent dosage in the sub-microliter range. The high precision and low-volume dispensing of the I-DOT enables the miniaturizing of cell lysis and whole genome amplification reaction steps to 1/20 of the original volume, saving both costs and time. The reduced volumes in comparison to the original protocol are summarized in **Table 1**. Since the WGA is based on unspecific priming and amplification, it is critical to avoid any nucleic acid contamination. The contact-free dispensing evades any cross contaminations in these highly sensitive DNA amplification steps. Negative control reactions yielded no detectable DNA in the PicoGreen assay, accounting for a contamination-free amplification reaction.

PCR amplification and sequencing of 16S rRNA genes

For a phylogenetic classification and identification of the isolated prokaryotes, the genes coding for 16S rRNA were amplified. 157 out of 344 samples showed a visible band around 1550 bp on an agarose gel, which corresponds to the average size of the 16S rRNA gene. In wells where no 16S rRNA gene could be amplified, although a single cell was detected in the nozzle, the amplification might have failed because of insufficient cell lysis or the fragmented nature of the WGA DNA obtained from the MDA. Here, the targeted 16S rRNA sequence is 1465 bp and therefore relatively long. In case this region is fragmented, amplification will not take place. Purification and Sanger sequencing of the PCR products was performed in a batch of 95 samples at Eurofins Genomics.

Taxonomic classification and calculation of phylogenetic distances

The obtained 16S rRNA gene sequences were aligned against the SILVA RefNR SSU reference database for a taxonomic classification. **Figure 4** shows the distribution of bacteria in the sample at the phylum level. A total of 77 sequences met the sequencing quality criteria and were included in the alignment. The most abundant phyla were *Firmicutes* (35%) and *Bacteroidetes* (35%), followed by *Proteobacteria* (17%) and *Actinobacteria* (16%). A phylogenetic tree of the sample is presented in **Figure 4**. Alignment against the human

oral microbiome database (Chen, 2010) identified seven sequences as closely related to uncultured bacteria, among those two sequences related to *bacterium HMT 352* with 99.9% and 99.6% identity, *Campylobacter sp. HMT 044* (99.7%), *Ruminococcaceae [G-1] bacterium HMT 075* (99.5%), two sequences for *Ruminococcaceae [G-2] bacterium HMT 085* (99.7% and 99.8%) and *Alloprevotella sp. HMT 913* (98.9%).

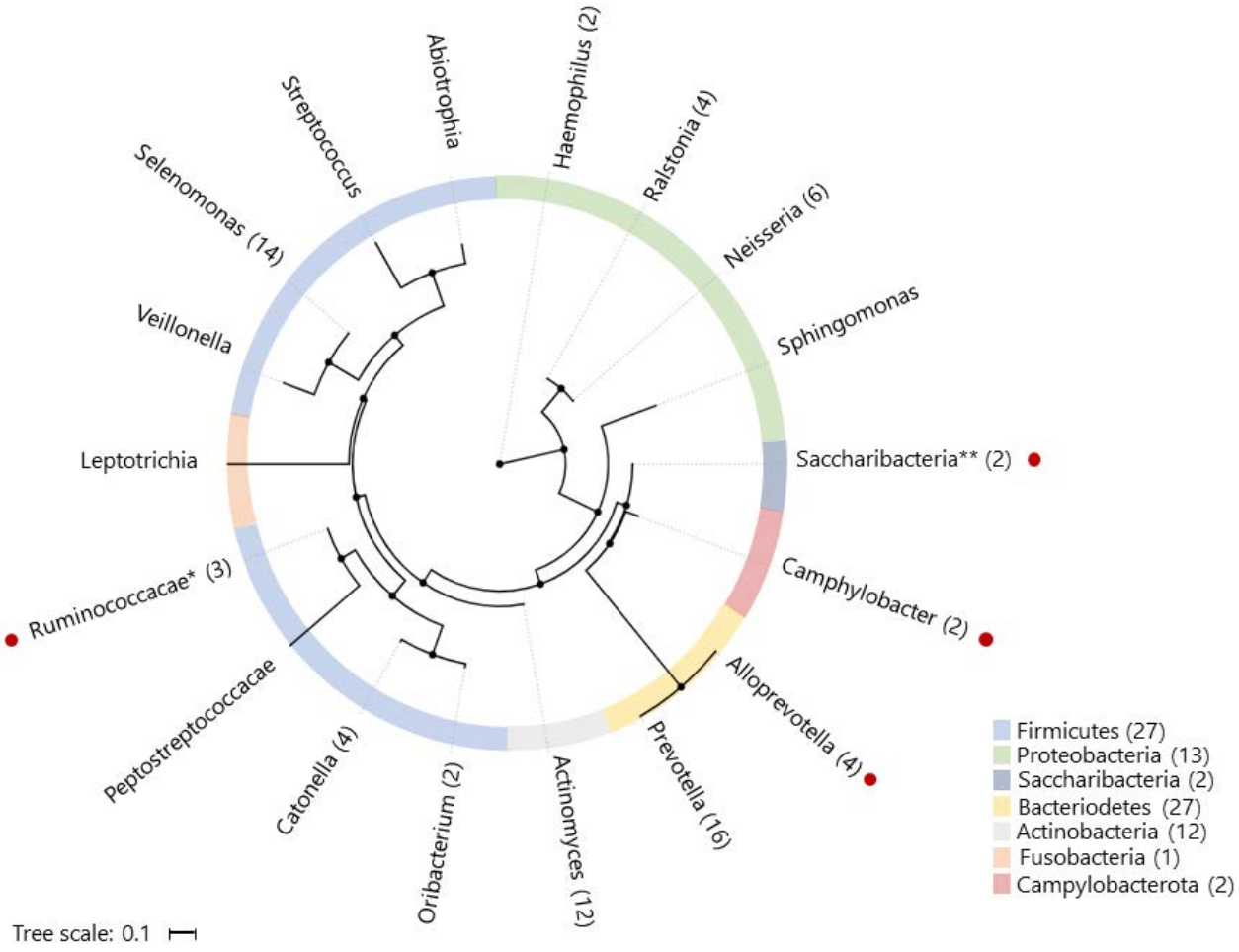

Figure 4. *Phylogenetic tree showing the identified cells in the sputum sample. If multiple single cells belong to the same group, the nodes were collapsed and their number is displayed in brackets. Leaf names indicate identification down to genus, family (*) or phylum level (**). A red dot indicates that an uncultured representative was found within this group.*

Conclusions

- By combining the B.SIGHT with the I-DOT/I-DOT Mini, we established an automated high-throughput workflow for single-cell whole genome sequencing.
- Single-cell dispensing allows for label-free isolation of individual bacteria from complex microbial samples.
- Precise deposition of single cells using AOC and plate deionization allows for single-cell lysis in 350 nL lysis buffer using standard 384-well plates.
- Using I-DOT technology, we scaled down the volumes of the WGA reactions by 20x and significantly reduced the cost for such analysis.
- The small footprint of the instrument allows users to perform the entire workflow in a flow bench, reducing the risk of contamination.
- The B.SIGHT can be optionally equipped with a laser for fluorescent sorting of bacteria, and the entire workflow can be easily integrated into laminar flow or anaerobic cabinets.

References

1. Woyke T, Doud DFR, Schulz F. The trajectory of microbial single-cell sequencing. *Nature Methods*. 2017; 14(11): 1045–1054. DOI:10.1038/nmeth.4469.
2. Woyke T, Sczyrba A, Lee J, et al. Decontamination of MDA reagents for single cell whole genome amplification. *PLoS ONE*. 2011; 6(10): e26161. DOI:10.1371/journal.pone.0026161.
3. Guindon S, Gascuel O. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Systematic Biology*. 2003; 52(5): 696–704. DOI:10.1080/10635150390235520.
4. Letunic I, Bork P. Interactive Tree Of Life (iTOL) v4: Recent updates and new developments. *Nucleic Acids Research*. 2019; 47(W1): W256–W259. DOI:10.1093/nar/gkz239.
5. Riba J, Gleichmann T, Zimmermann S, et al. Label-free isolation and deposition of single bacterial cells from heterogeneous samples for clonal culturing. *Scientific Reports*. 2016; 6(1): 32837. DOI:10.1038/srep32837.
6. Chen T, Yu WH, Izard J, et al. Human Oral Microbiome Database: A web accessible resource for investigating oral microbe taxonomic and genomic information. *Database: The Journal of Biological Databases and Curation*. 2010; 2010: baq013. DOI:10.1093/database/baq013.

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