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Review

Single-cell approaches in human microbiome research

Verónica Lloréns-Rico,[1,2,3] Joshua A. Simcock,[1,2,3] Geert R.B. Huys,[1,2] and Jeroen Raes[1,2,*]
[1]Laboratory of Molecular Bacteriology, Department of Microbiology and Immunology, Rega Institute, KU Leuven, Leuven, Belgium
[2]Center for Microbiology, VIB, 3000 Leuven, Belgium
[3]These authors contributed equally
*Correspondence: jeroen.raes@kuleuven.be
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SUMMARY

Microbial culturing and meta-omic profiling technologies have significantly advanced our understanding of the taxonomic and functional variation of the human microbiome and its impact on host processes. The next increase in resolution will come by understanding the role of low-abundant and less-prevalent bacteria and the study of individual cell behaviors that underlie the complexity of microbial ecosystems. To this aim, single-cell techniques are being rapidly developed to isolate, culture, and characterize the genomes and transcriptomes of individual microbes in complex communities. Here, we discuss how these single-cell technologies are providing unique insights into the biology and behavior of human microbiomes.

INTRODUCTION

Throughout the years, the human microbiome field advanced through technological innovations, gradually increasing in complexity, scale, and resolution while teasing apart the structure, diversity, and functionality of the host-associated microbiota. In the past decades, improved culturing approaches have resulted in culture collections harboring thousands of human-derived strains for genomic and metabolic characterization as well as for *in vitro* mechanistic studies on microbe-microbe and microbe-host interactions (Sommer, 2015; Vrancken et al., 2019). In parallel, culture-independent amplicon or shotgun metagenomic sequencing approaches have demonstrated their power in illuminating microbiome variation and its links to host physiology and health (Falony et al., 2016; Kurilshikov et al., 2021). In the slipstream of metagenomics, other "meta-omics" technologies such as metatranscriptomics, metaproteomics, or meta-metabolomics have been used to elucidate the functionality of the human microbiome (Fettweis et al., 2019; Lloyd-Price et al., 2019; Zhou et al., 2019b).

Although these technologies have become standard in contemporary human microbiome studies, they are not exempt from limitations and challenges. For instance, low-abundant taxa are difficult to isolate or culture under laboratory conditions, and their genomes are underrepresented in metagenomics samples. It has been estimated that over 70% of cataloged bacteria in the heavily studied human gut microbiome still lack a cultured representative (Almeida et al., 2021; Vrancken et al., 2019). Furthermore, "omics" technologies are unable to discriminate and validate the function of individual microorganisms. Even within a single strain, microbial cell populations can be highly heterogeneous, with clonal populations of bacteria displaying remarkable heterogeneity in their gene expression (Elowitz et al., 2002). This is crucial to strain survival in specific conditions, for instance, in bacterial persisters during antibiotic treatment (Windels et al., 2019). To study this type of microdiversity, bulk "meta-omics" approaches are clearly falling short. To overcome these challenges, a next wave of innovations is increasingly focusing on the single-cell level. In fact, at every step of today's microbiome research workflow (Figure 1), single-cell analyses have been introduced and are continuously being fine-tuned to address new biological questions.

In this review, we present the state-of-the-art single-cell approaches for isolation, culturing, and genomic and transcriptomic profiling that have contributed to distinct facets of microbiome research. Although we focus mainly on studies concerning the human microbiome, we also acknowledge works in other areas such as animal or environmental microbiomes, particularly in terms of method development. Finally, we also highlight the specific challenges of human microbiome samples that need to be overcome to facilitate single-cell techniques in this field.

Single-cell approaches for bacterial isolation, culturing, and screening

Compared with traditional approaches such as agar dilution plating, recent advances in single-cell-based technologies have revolutionized microbiome isolation processes in both taxonomic breadth and throughput. The physical dissection of a sample's microbial community into spatially separated cells overcomes one of the major issues in microbial isolation, i.e., the fact that differences in individual growth rates as well as nutritional and spatial competition inevitably promote overgrowth by fast-growing organisms. For this reason, single-cell approaches have the potential to recover a higher species richness beyond the usual suspects commonly isolated in bulk

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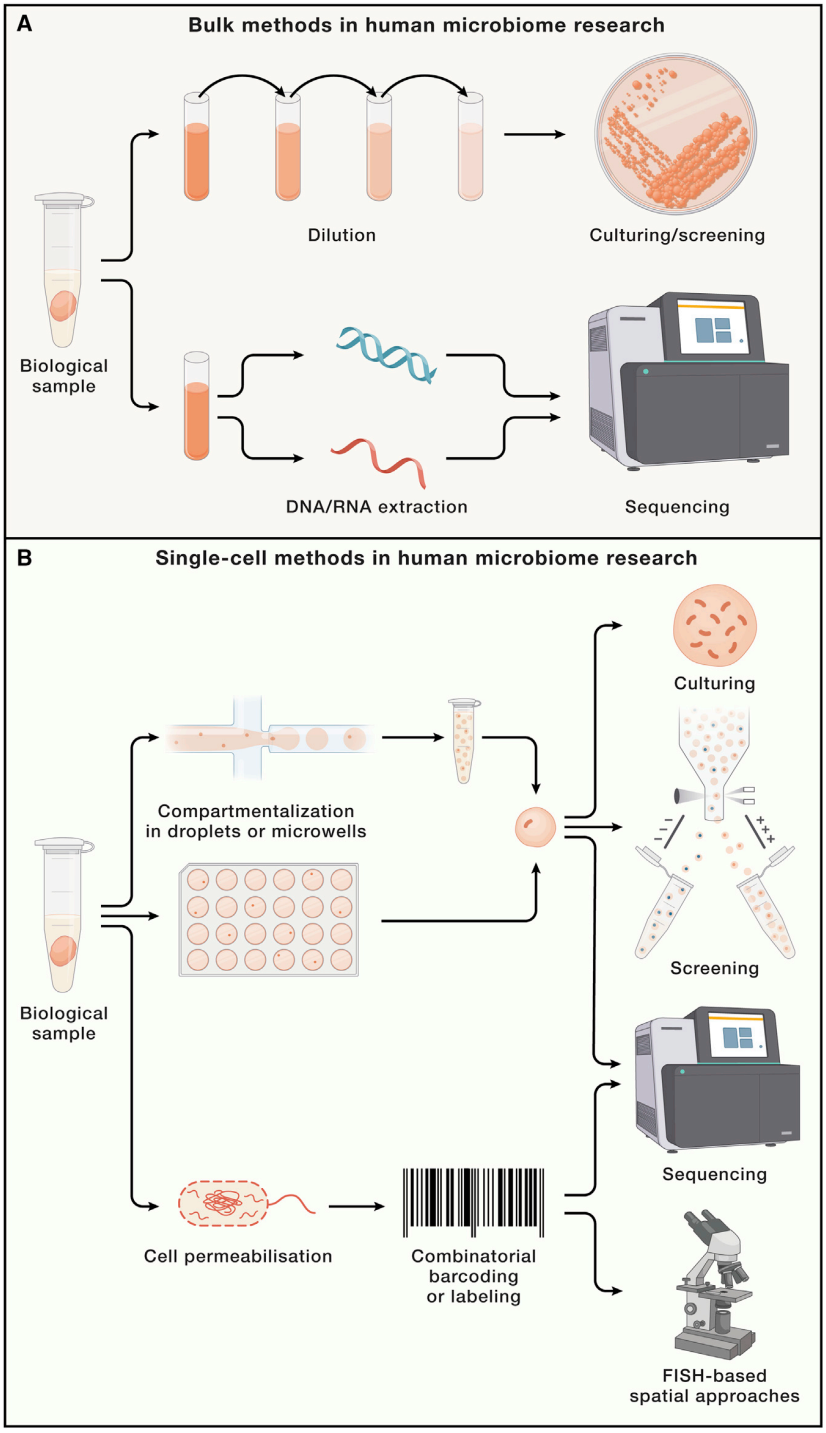

Figure 1. Bulk and single-cell methods in human microbiome research

(A) In bulk approaches, biological samples (such as feces, biopsies, swabs, etc.) are either subjected to dilution for culturing and isolation of its members or to bulk nucleic acid extraction for sequencing applications.

(B) In single-cell methodologies, microbial cells need to be compartmentalized either in droplets or in microwell plates. Alternatively, the cells themselves might be used as individual compartments by permeabilizing their cell walls to perform barcoding or labeling of nucleic acids inside the cells. Single cells can later be then subjected to culturing, screening procedures to select for specific features, and sequencing or microscopy-based spatial approaches.

approaches. This said, as some single cells might not be able to grow when separated from their supporting (e.g., cross-feeding) species, combination with bulk approaches remains recommended.

Most recent technological innovations in human microbiome single-cell-based isolation and culturing take one of two main approaches: (1) encapsulation of free cells from an aqueous phase in single or double water-oil emulsions through flow-based droplet microfluidics or (2) space-limited compartmentalization of individual cells in miniaturized arrays of physical microwells or microvalves.

Droplet-based microfluidics

Droplet-based microfluidics relies on two-phase microchannel fluidics to capture individual cells or molecules in aqueous monodisperse spherical particles that are circulated by a continuous phase of biocompatible oil (Kaminski et al., 2016). Triggered by successful implementations in molecular and cell biology labs, this technology is increasingly recognized as a key component of single-cell-based microbiome isolation and culturing workflows due to its potential in terms of throughput, parallelization, physicochemical standardization, and dynamic process control (Tan and Toh, 2020; Liu and Walther-Antonio, 2017). Given that most passive cell capture processes are dictated by Poisson statistics, only a proportion of the generated microdroplets will effectively contain a single cell against a background population of empty microdroplets and droplets containing two or more cells (Collins et al., 2015). Single-cell encapsulation rates are usually maximized by optimization of sample inlet dilutions, but more advanced modifications such as real-time imaging can be used for selective sorting of only single-cell droplets via machine learning algorithms (Sesen and Whyte, 2020).

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In anerobic ecosystems of the human body, such as gut, vaginal, or subgingival crevice microbiomes, droplet microfluidics for isolation, culturing, and assaying single microbial cells face the additional challenge that sample processing, growth monitoring, and droplet sorting need to be performed under oxygen-free conditions (e.g., 80% N_2/10% H_2/10% CO_2; Table 1). As many state-of-the-art microfluidic pipelines comprising a droplet generator coupled to a flow cytometric instrument and/or a sorting device are difficult to integrate into standard anerobic workstations without compromising operator or incubation space, alternative approaches for growth monitoring have been investigated. For instance, the microfluidic streak plate (MSP) technique essentially merges microfluidics with the standard plate-streaking principle in such a way that microchannel-confined nanoliter droplets are spaced by uniform oil plugs and thus can be streaked manually or through spiral plating on surface-modified petri dishes (>1,000/dish) (Jiang et al., 2016). The typical MSP workflow is compact enough to allow a full operational setup in anerobic workstations. MSP approaches enabled single-cell isolation of bacteria from a wood-feeding termite gut microbiome (*Reticulitermes chinensis*) at both aerobic and anerobic conditions (Zhou et al., 2019a). Additionally, MSP single-cell culturing was integrated in high-throughput functional screening workflows such as chemotactic selection of imidazolinone-degrading bacteria (Chen et al., 2019).

Another approach to this problem is offered by the MicDrop platform (Villa et al., 2020). Here, anerobic encapsulation and incubation of individual bacterial cells in single-emulsion (water-in-oil) picoliter-sized microfluidic droplets are combined with Illumina MiniSeq sequencing for indirect non-continuous growth monitoring. MicDrop's concept is built on the assumption that the resulting 16S rDNA sequence variants (SVs) can function as barcodes that are shared between droplets containing the same bacterial taxa. This way, growth of individual species can be tracked outside the anerobic workflow by fitting growth curves at SV-level using a combination of 16S rDNA-derived sequencing and qPCR data. The high culture throughput of the MicDrop platform allows users to perform functional screenings assessing growth dynamics, taxonomic identity, and abundance of single-cell droplets within human microbiome subpopulations anerobically enriched under selective medium conditions. One such functional assay investigated the diversification of dietary carbohydrate degradation capacities across fecal inocula from nine healthy human donors (Villa et al., 2020), which opens up new possibilities for personalized prebiotic-based gut modulation strategies.

In contrast to the MSP system, the MicDrop platform focuses on culturing and assaying but is not primarily designed for isolation purposes due to the destructive nature of longitudinal droplet sampling. To this end, Watterson et al. (2020) developed a droplet-based microfluidic workflow with an integrated microscopic detection that combines all three basic functionalities, i.e., isolation, culturing, and sorting. By defining optical-density-like threshold criteria, bacterial colonies developed from a single cell can be selectively deflected, harvested, and subcultured for further taxonomic and functional characterization. A multiplexed fecal isolation study using antibiotic selection showed that the taxonomic richness and rare taxa recovered by this workflow were far superior compared with conventional agar plating (Watterson et al., 2020).

Although anerobic incubation is essential for culturing gut or vaginal bacteria, this is less of a concern for other human microbial ecosystems such as superficial regions of the oral cavity and for human microbiota-derived metagenomic clone libraries. Aerobic working conditions also enable more complex and space-demanding microdroplet setups for in-depth functional screening purposes. One of the major developments in this context is the combination of biocompatible microfluidic double water-in-oil-in-water emulsion droplet technology (MDE) and fluorescence-activated cell sorting (FACS) to study microbial interactions at single-cell level between a fluorescently labeled target strain and potential effector strains (Terekhov et al., 2017). As a proof-of-concept of the MDE-FACS platform, it was demonstrated that co-encapsulation of *Staphylococcus aureus* cells expressing GFP with oral microbiota suspensions facilitated selection of *S. aureus* inhibitors. The screening process was further refined by liquid chromatography-mass spectrometry analysis of secretomes for identification of metabolites with potential bioactivity against *S. aureus*. The identified anti-*S. aureus* component amicoumacin A was then co-encapsulated with single cells from oral and fecal microbiota ((Terekhov et al., 2018)), demonstrating the downstream validation potential of MDE-FACS.

Integrated high-throughput screening can also be extended toward clone libraries. For instance, a library of 20,000 fosmid *Escherichia coli* clones covering 0.7 Gb of metagenomic DNA from a human ileal mucosa sample (Gloux et al., 2011) was screened by droplet microfluidics for β-N-acetylgalactosaminidase activity using a commercially available fluorogenic glycoside analog (Tauzin et al., 2020). Subsequent deep sequencing (100×) and functional profiling of selected clones allowed identification of pathways contributing to the degradation of human gangliosides and milk oligosaccharides, demonstrating single-cell-in-droplet microfluidics' promise for functional metagenomics projects.

Microarray or compartmentalization-based technologies

Compared with droplet microfluidics, the main advantage of compartmentalization-based workflows is that they offer larger culture chambers for expansion of single cells to high-density microcolonies in bigger volumes. This may not only reduce the loss of isolates that fail to grow upon sorting but may also allow longer incubation times and increase sensitivity of biological readouts. In addition, downstream harvesting of selected microcolonies is generally more straightforward from discrete microcompartments compared with droplets. Although many of the early microcompartmentalization devices such as the micro-Petri dish (Ingham et al., 2007), the iChip (Berdy et al., 2017; Nichols et al., 2010), and the SlipChip (Ma et al., 2014a) could be easily manipulated in anerobic workstations, very few were validated on human-associated anerobic bacteria. Ma et al. (2014a) demonstrated that the SlipChip platform, a microfluidic chip consisting of two plates etched with microcompartment wells for single cells and ducts as fluid conduits, can be used for culturing gut anerobes either as pure cultures (i.e.,

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Table 1. Challenges of single-cell techniques within human microbiome research

Aerobic tolerance of human-associated microbes	A large majority of microbes residing in human-associated environments are obligate anerobes with next-to-zero oxygen tolerance. However, integration of state-of-the-art microfluidics-based single-cell isolation platforms into standard anerobic workstations is a spatial challenge. Current solutions for this include the spatially compact microfluidic streak plate (MSP) technique (Jiang et al., 2016), the comprehensive sequencing-based platform MicDrop (Villa et al., 2020), as well as the integrative isolation, culture and sorting platform devised by Watterson et al. (2020).
Bacterial cell aggregation and low bacterial biomass in specific ecosystems	Autoaggregation into multicellular clumps is common among bacteria (Trunk et al., 2018), posing a problem for the efficient isolation of single cells. Additionally, in some human ecosystems (e.g., in oral, nasopharyngeal, or vaginal samples), the expected bacterial biomass is low compared with the gut. Samples from these ecosystems, usually obtained via swabbing, require bacteria to be detached from both swabs and host cells. Potential solutions for this problem include sonication and physical disruption as well as filtering, although this may reduce overall recovery.
Diverse bacterial cell walls	The robust and structurally diverse nature of bacterial cell walls represents a major challenge for many classic single-cell approaches that require lysis of individual cells in picolitre volumes of reagents for genomic or transcriptomic sequencing. Alternative solutions involve the permeabilization of cell walls for combinatorial barcoding inside the cells (Blattman et al., 2020; Kuchina et al., 2021).
Lower amount and increased lability of bacterial mRNA	Prokaryotic mRNA half-lives are in the range of minutes as opposed to hours in eukaryotes. Prokaryotes also produce much lower amounts of mRNA overall; roughly, 100-fold lower copy numbers per transcript than is typically seen in eukaryotes. This poses a particular problem for scDual-seq techniques. One current solution for this issue is targeted enrichment of bacterial RNA in dual scRNA-seq samples (Betin et al., 2019).
Lack of poly-adenlylated mRNA	Many eukaryote-focusing scRNA-seq approaches rely on poly(T) primers to capture polyadenylated mRNAs. However, mRNA polyadenylation is absent in prokaryotic mRNAs, except in transcripts bound for degradation. One solution to this problem is to polyadenylate bacterial mRNAs prior to capture and reverse transcription (Kuchina et al., 2021; Wangsanuwat et al., 2020). Another alternative is the use of random hexamer primers, which are capable of binding native prokaryotic mRNA (Blattman et al., 2020). Both these solutions however lead to unwanted capture of the rRNA, which comprises approximately 95% of all prokaryotic RNA. Future optimisation of prokaryotic scRNA-seq approaches may therefore include steps to either block rRNA reverse transcription with blocking primers (Wangsanuwat et al., 2020) or remove rRNA cDNA via cas9 nuclease (Prezza et al., 2020).
Smaller size of bacterial cells	Classic FISH microscopy techniques employing barcoded mRNA indexing require that mRNA molecules be individually resolved. Due to the smaller size of bacterial cells, many mRNA molecules may overlap. This problem can be addressed by use of sequential FISH techniques combined with the measurement of fluorescence intensity per spot, a value linearly related to the number of mRNA molecules per spot (Dar et al., 2021).

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Bacteroides thetaiotaomicron) or from a complex community (i.e., a mucosal biopsy). In an accompanying paper (Ma et al., 2014b), a human caecal biopsy was used for the genetically targeted anerobic isolation of an unidentified *Ruminococcaceae* genus representative from the Human Microbiome Project's "Most Wanted" list for whole-genome sequencing (Fodor et al., 2012). To circumvent the destructive nature of most genetic assays, a key feature of the replica-SlipChip technology is that it allows to physically split microcolonies allowing one replica to be used for 16S rDNA PCR and one for preservation and upscaling of confirmed target organisms. A modified version of the device, the serial dilution SlipChip, incorporated a multistep process to generate serial dilution nanoliter arrays in high throughput through a series of sliding motions (Yu et al., 2019).

In recent years, single-cell workflows progressed from homegrown laboratory setups to more easy-to-use commercial benchtop devices. In this area, the Prospector system (General Automation Lab Technologies) is an automated array-based compartmentalized culturing platform that integrates optical growth monitoring, selection, and transfer of microcultures in a single instrument that fits in medium-sized anerobic cabinets. In an initial study (Liang et al, 2020), approximately half of the 45 species isolated with this system from six human fecal samples were considered low-abundant ($\leq$0.2%) taxa based on analysis of paired 16S rDNA metagenomic data, suggesting that this type of microarray technology has the potential to also recover the rarer members of gut microbiomes. On the other hand, a recent comparison between Prospector-guided isolations and more conventional agar plating and extinction methods to analyze the root endomicrobiome of lettuce (*Lactuca sativa* L.) revealed no differences in species richness, although cultured diversity varied across methods (Persyn et al., 2022). As is the case for most miniaturized isolation workflows, here also the actual throughput of pure culture recovery is much lower than the system's intrinsic capture capacity ($\sim$6,000 3 nL microwells) because it is not possible to ensure that (1) each individual culture volume (well or droplet) is loaded with a single cell and (2) cells are alive at the time of loading. It has been demonstrated that both issues can be simultaneously addressed by the integration of a FACS optical platform with a live/dead discrimination feature (e.g., using thiazole orange/propidium iodide dye staining) upstream of the cell loading stage (Liu et al., 2021a).

Beyond the need to separate and grow single cells lies the next-level challenge to capture the temporal evolution of individual cells over multiple generations, which would reveal essential information on their growth, physiological dynamics, and responses to abiotic parameters. One approach that specifically addressed this challenge is single-cell isolation following time-lapse imaging (SIFT), a workflow aimed at tracking spatially confined living cells by multigenerational time-lapse microscopy (Luro et al., 2020). In the two-layer SIFT microfluidic chip, cells are separated in a flow layer comprising 16,680 trenches of 25 × 1.5 μm, whereas a second layer integrates a series of microfluidic push-down valves, flow channels, and an optical trap for off-chip collection of individual cells. Although non-destructive multigenerational imaging and tracking of individual bacteria with SIFT followed by downstream isolation and upscaling of cells of interest has so far only been demonstrated for bacterial libraries, this technology might also have applications for high-throughput isolation and screening of dynamic single-cell phenotypes from (anerobic) human microbiomes.

Single-cell genome analysis in the human microbiome

Single-cell techniques for genomic profiling of complex microbiome samples can provide further insights into their phylogeny, ecology, and evolution. These technologies have been used not only to expand and improve the catalog of sequenced human microbes but also to resolve microbial interactions at the single-cell level and to map the spatial localization of individual bacteria in their natural ecosystem.

Single-cell genomic sequencing expands the microbial tree of life

The first wave of methods for obtaining single amplified genomes (SAGs) from individual microbial cells appeared before shotgun metagenomic sequencing became widely used. These methods were primarily developed to help shed light on the "microbial dark matter," i.e., the large fraction of microbial taxa so far only identified through 16S rDNA-based surveys but lacking cultured representatives or high-quality reference genomes. The first human-derived SAG targeted a member of the candidate phylum TM7 (later renamed to Saccharibacteria), isolated from the oral cavity (Marcy et al., 2007). In this work, authors used a dedicated microfluidic device where the different steps of the process—isolation, lysis, and genome amplification—occurred in separate chambers.

With the expansion of shotgun metagenomics technologies and the development of computational assembly methods, the number and quality of metagenomic-assembled genomes (MAGs) obtained from the human microbiome has increased dramatically in the past few years (Almeida et al., 2021; Saheb Kashaf et al., 2022; Zhu et al., 2021). Even with the increasing quality of MAGs recovered from shotgun metagenomics, single-cell genome sequencing still expands the microbial catalog, improves assemblies, and provides "real" biological validation and/or backbones of complete genome sequences from individual cells. As an example, several studies have combined single-cell genome sequencing with shotgun metagenomics for the purpose of comparing both techniques (Bowers et al., 2021) or to integrate them for better assemblies (Alneberg et al., 2018; Arikawa et al., 2021). In the human microbiome, this dual approach led to the assembly of two genomes from different *Staphylococcus hominis* skin strains, whereas only one could be assembled using a shotgun metagenomics approach alone (Arikawa et al., 2021). These two strains differed in their associated plasmids, which could point to differences in their antibiotic resistance profiles or virulence factors.

One of the advantages of single-cell genomics derives from the possibility of targeting specific taxa with certain metabolic capabilities or rare microbiome members. Using flow cytometry sorting of individual cells on microwell plates, genome amplification, and selection of specific 16S rDNA gene sequences (Figure 2A), early studies recovered human-associated bacterial SAGs from the oral cavity (Campbell et al., 2014, 2013b) or the gut (Brito et al., 2016). Alternatively, specific bacteria can be enriched in the samples

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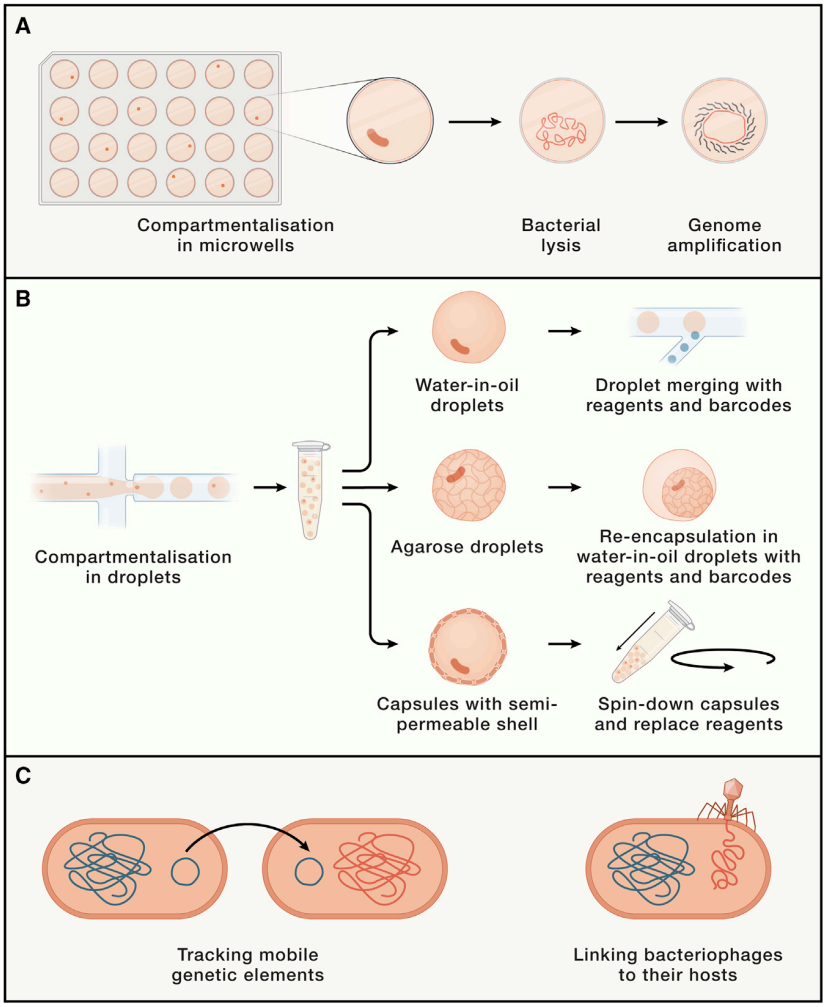

Figure 2. Single-cell genomic approaches
(A) Microbial compartmentalization in microwells and processing steps prior to sequencing.
(B) Different possibilities of microbial single-cell compartmentalization in microfluidic droplets: water-in-oil droplets (top), molten agarose droplets (middle), or liquid capsules with a semi-permeable shell (bottom). For each of these options, there are different options to manipulate the droplets or capsules to achieve the steps depicted in (A): water-in-oil droplets require droplet merging with reagents via microfluidic manipulation; agarose droplets can be re-encapsulated in water-in-oil droplets containing necessary reagents; and the semipermeable shell capsules can be washed by spinning-down the tubes to pellet the capsules and replacing the liquid reagents.
(C) Examples of applications of single-cell genomic approaches: tracking mobile genetic elements such as plasmids or linking bacteriophages to their specific hosts.

prior to isolation. In the human microbiome field, Deltaproteobacteria-specific fluorescence *in situ* hybridization (FISH) probes were used to target and enrich single *Desulfobulbus* and *Desulfovibrio* cells from the oral cavity via FACS for genome sequencing (Campbell et al., 2013a). Other enrichment strategies have used IgG antibodies (e.g., for oral Saccharibacteria; Cross et al., 2019). More recently, the development of droplet microfluidics has streamlined the process of screening and targeting specific gut microbiome members (Pryszlak et al., 2022). Although this method has not yet been applied to the generation of SAGs, isolation of individual cells after the screening process could be applied to generate single-cell genomes.

Improvements in genome amplification methods and sequencing throughput have also maximized the parallel sequencing capacity of large numbers of single cells in untargeted studies. For instance, these improvements have been implemented in single-cell genomic sequencing of the mouse gut microbiome, resulting in 298 high-coverage microbial assemblies (Lyalina et al., 2022).

Droplet microfluidics technologies have further been instrumental in increasing the throughput in microbial single-cell genomic sequencing (Figure 2B). Single-cell genomic sequencing (SiC-seq), the first droplet-based method for this purpose, was used to characterize a marine microbiome sample (Lan et al., 2017). In this method, individual cells are encapsulated in molten agarose microdroplets, which upon polymerization provide a semipermeable matrix that immobilizes the bacterial cells but allows reagents to reach their contents. These microgels are then processed in microfluidics devices to generate single-cell genomic libraries for sequencing (Lan et al., 2017; Figure 2B). Later, this method was simplified by sorting the agarose droplets in microwell plates, therefore reducing the microfluidic processing steps. This adaptation, named SAG-gel, was applied to study the gut microbiome of mice, where a total of 346 SAGs was generated from two independent samples (Chijiiwa et al., 2020). Recent studies have employed alternatives to agarose microgels as matrices to capture single cells. Microbe-seq encapsulates single bacteria in water-in-oil droplets containing lysis reagents. Droplets then undergo a series of merging steps with additional reagents in a microfluidic device to perform genome amplification followed by barcoding. Microbe-seq has been used to profile human stool samples, yielding thousands of SAGs per sample (Zheng et al., 2022). A different method is based on the generation of microcapsules, in which a semipermeable hydrogel shell contains an aqueous core in which single bacteria are isolated (Leonaviciene et al., 2020). These microcapsules can be processed without the need for sorting in microwell plates nor the use of complex microfluidic devices, simply by washing the microcapsules and exchanging buffers. This approach was used in a proof-of-concept study in which high-

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quality SAGs were obtained from a mock community formed by 15 human gut microbiome strains (Aoki et al., 2021; preprint).

Despite the many improvements in whole-genome amplification protocols, a common pitfall of SAGs is that they tend to be highly fragmented and incomplete. The incorporation of long-read technologies into SAG generation could potentially help to address these issues, provided that sufficient intact DNA is available. Recently, a study using an Oxford Nanopore MINion device has shown that it is possible to reduce the amount of starting DNA to that of a single bacterial cell (~10 fg) by using microfluidic devices to perform the whole-genome amplification and library preparation steps (Liu et al., 2021b). Thus, this work opens the door for further explorations of incorporating long-read technologies into the available workflows for single-cell microbial genomic sequencing.

Microbial genome profiling at single-cell resolution provides insights into human microbial ecology and evolution

Single-cell microbial genomic sequencing can also provide insights into the ecology and evolution of the human microbiome, such as the spread of mobile genetic elements. Brito and coworkers identified over 20,000 mobile genes in 180 single-cell fecal bacterial genomes. The majority of identified mobile genes (62.4%) were detected in individuals of multiple human populations, but diet greatly influenced their abundance (Brito et al., 2016). A more recent study of within-person horizontal gene transfer (HGT) showed that the majority of observed HGT events occurred between pairs of gut bacteria from the same phylum. Additionally, strain-specific HGTs could be mapped in strains of *Faecalicatena faecis* and *Agathobacter faecis* (Zheng et al., 2022). Finally, using a targeted single-cell approach, individual plasmids were linked to their specific host cells (Diebold et al., 2021). This method was used to identify specific bacteria carrying beta-lactamase genes in the stool of two patients in which these antibiotic resistance genes had been previously identified by shotgun metagenomic sequencing (Diebold et al., 2021).

Single-cell genomics has also been used to study phage-host interactions. A targeted strategy of "viral tagging" combined with single-cell genome sequencing was developed to find viral-host pairs in gut species (Džunková et al., 2019). Viral tagging consists of the addition of fluorescently labeled viral particles to potential host bacteria, after which specific host cells can be enriched via FACS (Deng et al., 2012). By adapting the original method to single-cell isolation and sequencing, 363 unique host-phage pairings were discovered. Additionally, by swapping the bacterial and viral fractions from different individuals, researchers identified substantial phage-host cross-reactivity between subjects, suggesting that for the majority of viruses, host specificity is defined at the species level instead of the strain level (Džunková et al., 2019).

Recently, new avenues are being explored with single-cell genomic approaches. In the area of spatial genomics, mapping the microbial biogeography of human-associated ecosystems at high resolution is an emerging priority. FISH-based approaches now enable *in situ* microbial identification at the single-cell level. Early approaches include combinatorial labeling and spectral imaging FISH (CLASI-FISH) (Valm et al., 2011), using 16S probes. Using combinatorial probes to detect 15 common oral genera, this study distinguished individual cells and explored cross-genus physical associations in the dental plaque. By pushing this concept of combinatorial labeling, high-phylogenetic-resolution microbiome mapping by FISH (HiPR-FISH) (Shi et al., 2020) discriminated up to 1,023 different taxa via 16S labeling, using only 10 different fluorophores in a single step. HiPR-FISH showed the antibiotics-based disruption of bacterial spatial networks in the murine gut and identified spatial multispecies consortia in dental plaque. Although targeting of 16S rRNA sequences limits taxonomic resolution, these techniques may be adapted for strain-level resolution in the future. Another limitation is the need to design specific FISH probes for the taxa of interest, limiting this method to known taxa.

Illuminating functional heterogeneity with single-cell transcriptomics

The techniques described above advance our ability to identify and genomically catalog human microbiome members. Nonetheless, additional approaches are required to achieve full functional characterization of host-associated microbes, as functional heterogeneity among populations of isogenic bacteria is well known (Windels et al., 2019). The advent of single-cell transcriptomics has greatly advanced our ability to characterize the mechanisms and patterns of such heterogeneity in eukaryotic cell populations, and a decade of rapid evolution of these approaches is finally beginning to be harnessed, revealing individual patterns of gene expression in prokaryotes.

Measuring intrinsic functional heterogeneity with single-cell RNA-seq

Recent advances in *in situ* combinatorial indexing techniques resolve some of the challenges of techniques requiring individually compartmentalizing bacteria within picoliter volumes (Table 1). Specifically, the ability to add molecular barcodes to cDNA constructs before cell lysis abrogates the need for physical compartmentalization in droplets or wells, as the cells themselves act as the individual compartments, facilitating multistep processing of samples (Cao et al., 2017; Rosenberg et al., 2018). Cells containing uniquely barcoded transcripts can then be pooled and lysed in bulk. Two techniques, prokaryotic expression profiling by tagging RNA *in situ* and sequencing (PETRI-seq) and microbial split-pool ligation transcriptomics (Micro-SPLiT), have recently leveraged this technology for the study of bacterial cells (Blattman et al., 2020; Kuchina et al., 2021; Figure 3A). Building on the existing SPLiT-seq protocol (Rosenberg et al., 2018), these approaches are capable of comprehensively profiling gene expression across tens of thousands of cells and identifying rare or novel subpopulations. PETRI-seq applied to *S. aureus* revealed a rare subpopulation of cells undergoing prophage induction (Blattman et al., 2020). Micro-SPLiT, on the other hand, helped create an atlas of changes in metabolism and lifestyle of *Bacillus subtilis* cells at various growth stages (Kuchina et al., 2021). It also identified novel and unexpected gene expression states, including a subpopulation of cells displaying a rare stress response mechanism inducing competence. This response could help this subset of cells evolve faster by incorporating foreign DNA into their genome. With minimal differences in methodology,

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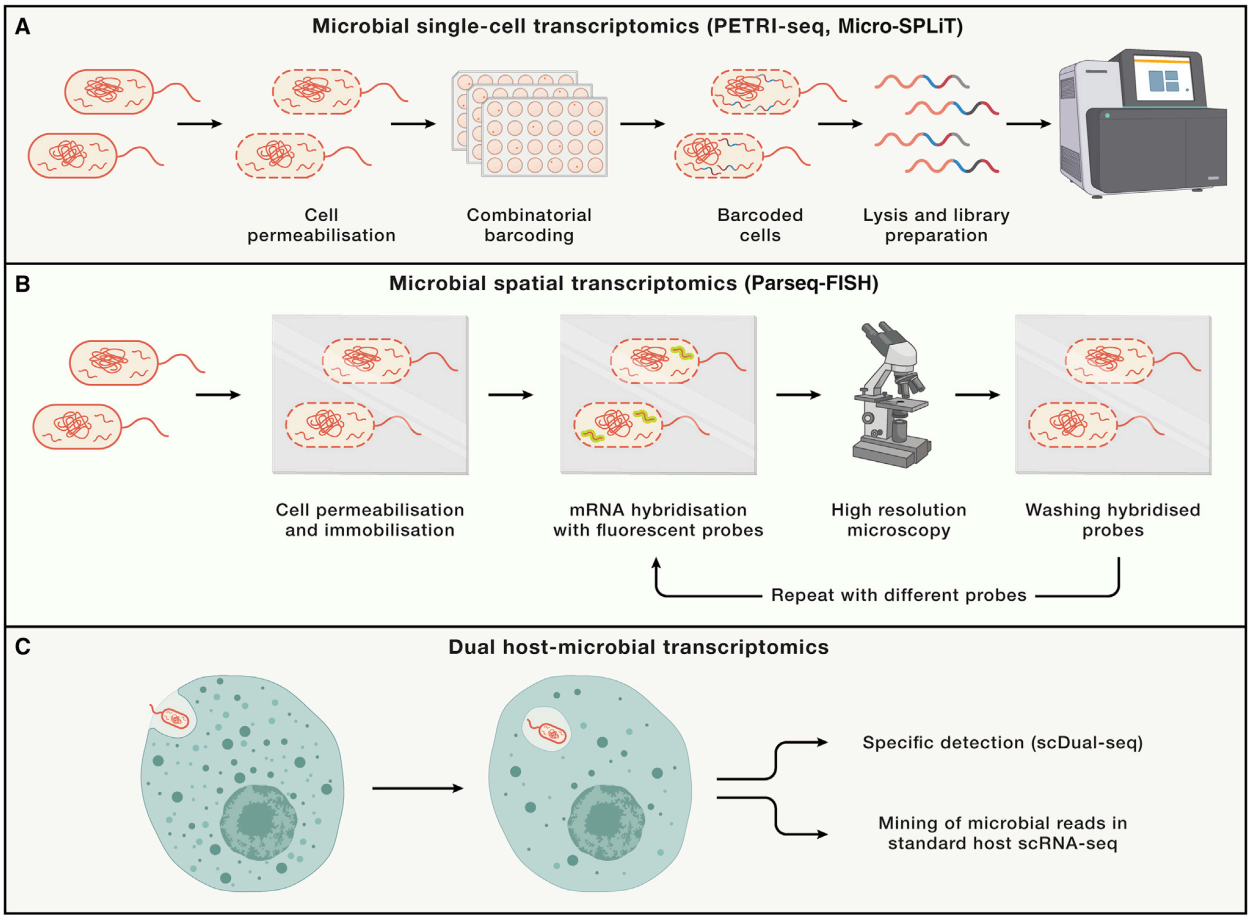

Figure 3. Microbial single-cell transcriptomics applied to microbiome

(A) Steps required for single-cell transcriptomics based on combinatorial barcoding inside the cells.

(B) Major steps in spatial transcriptomics approaches.

(C) Methods on dual host-microbe single-cell RNA-seq. A host cell internalizes one bacterium (left), and host-microbial interactions can be detected following two different approaches (right): specific detection approaches (with lower throughput, such as scDual-seq) or mining microbial reads in host scRNA-seq datasets (less specific but having higher throughput; see Figure 4).

it remains to be seen which technique proves to be the most widely successful. One notable difference is that PETRI-seq was tested on Gram-positive and Gram-negative isolates separately, employing varying enzymes in each case, whereas Micro-SPLiT was tested on a mixed population of bacteria, where it showed a bias toward the mRNA capture of Gram-negative cells. Both techniques may therefore require further optimization for a wider applicability in the context of complex microbiome samples. Nonetheless, the adaptation of combinatorial barcoding scRNA-seq for prokaryote targets promises to be an efficient and cost-effective method for illuminating inherent transcriptional heterogeneity in human microbiome populations.

Although such combinatorial barcoding strategies eliminate the need for single-cell isolation, leveraging the power of existing commercial platforms developed for droplet-based isolation and scRNA-seq of eukaryotic cells has also proved possible. 10X Genomics' Chromium system is a widely used platform comprising commercially available kits and benchtop microfluidics technology, capable of scRNA-seq profiling up to 10,000 individual eukaryotic cells per run (10X Genomics, Pleasanton, CA; Zheng et al., 2017). After being adapted to yeast cells (Jariani et al., 2020), this platform has recently been adapted for scRNA-seq of bacterial cells (McNulty et al., 2021; preprint), using a large oligonucleotide probe library complementary to all protein-coding sequences within a specific target bacterial genome. Probes include a 3′ poly(A) tail that enables prokaryotic mRNA capture using the 10X Chromium technology. Applied to *E. coli* and *Bacillus subtilis* pure cultures, this approach identified multiple cell subpopulations including previously unknown ones with different metabolic, chemotaxis, and motility expression profiles. Of note was the differential expression of arginine biosynthesis genes across different subpopulations, suggesting a potential spatial separation of chemically incompatible metabolic processes, a tactic widely employed in multicellular organisms.

Illuminating such spatial patterns of gene expression, however, is currently not possible with these technologies and may

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best be achieved by more specialized spatially resolved approaches. Furthermore, it is important to note that the reliance on genome-specific probes is an important limitation when applying this approach to complex human microbiome samples, as the number of taxa that may be detected in a single experiment may be limited by the number of probe sets that can be designed. Additionally, this technique can currently only be used on well-characterized taxa, with sequenced and annotated genomes.

FISH-based approaches resolve spatial functional heterogeneity in bacterial cell populations

Despite recent efforts to map taxonomic biogeography at the single-cell level (Shi et al., 2020), resolution of the spatial heterogeneity of gene expression in microbiomes is currently lacking. Although traditional microscopy work incorporating FISH techniques is capable of recording the spatial heterogeneity of gene expression at a single-cell resolution, it is limited to a small number of target genes. However, similarly to scRNA-seq, advances in combinatorial indexing have also been recently expanded to FISH-based techniques, increasing the number of potential gene targets to hundreds and thousands.

Current sequential FISH (seqFISH)-based approaches (Chen et al., 2015; Eng et al., 2019) involve two hybridization steps. First, specific mRNA sequences are hybridized by primary non-fluorescent probes flanked by short gene-specific sequences. Then, transcripts can be detected via secondary hybridization of a fluorescently labeled readout probe targeting the primary probes. Readout probes can be quickly stripped, and new probes hybridized in successive rounds, with a handful of genes targeted per round. This technique has been recently adapted for bacteria with the parallel sequential FISH (ParseqFISH) approach (Dar et al., 2021; Figure 3B). ParseqFISH was applied to the clinically important pathogen *Pseudomonas aeruginosa* and allowed identification and spatial resolution of heterogeneity in numerous metabolic and virulence-related transcriptional states that emerged dynamically throughout planktonic growth. Of clinical relevance, ParseqFISH was also capable of identifying distinct physiological states in biofilms, spatially resolving this heterogeneity at a micrometer scale. The potential power of this approach to illuminate heterogeneity within commensal microenvironments is an exciting prospect. For instance, spatial approaches could help profile expression of specific bacterial cells in the context of tumor microenvironments, helping to understand bacteria-host interactions with potential implications for therapy. However, again, the need for genome-specific probes hampers its direct application to complex host-associated communities.

Dual scRNA-seq reveals mechanisms of host-microbe interactions

Dual scRNA-seq techniques, which leverage existing eukaryote approaches to capture and sequence the mRNA of host and microbes in bacterially exposed host cells, have demonstrated great potential in illuminating host-microbe interactions. In single-cell Dual sequencing (scDual-seq), the first method to simultaneously sequence both host and bacterial RNA at a single-cell scale, Avital and colleagues replaced poly(A)-based primers with random hexamers to capture both host and bacterial mRNA (Avital et al., 2017; Table 1). Subsequent processing and sequencing were achieved with a modified version of an existing eukaryote-based scRNA-seq technique, cell expression by linear amplification and sequencing 2 (CEL-seq2; Hashimshony et al., 2016; Figure 3C). Resulting reads could then be mapped to host and bacterial genomes. Initial testing with murine macrophages exposed to *Salmonella, in vitro*, allowed distinction of three subpopulations of macrophages as well as characterizing a linear cellular progression through them. Importantly, two subpopulations of *Salmonella* were also identified, displaying clear patterns of transcriptional heterogeneity that correlated strongly with specific macrophage subpopulations. Although promising in this specific setup, this approach is limited in its ability to capture bacterial mRNA by the many challenges posed by bacterial physiology (Table 1), with >10 bacterial cells needed per host cell to achieve suitable sequencing depth of bacterial transcriptomes (Avital et al., 2017).

To address the inherent limitations of dual scRNA-seq, some methods have added specific steps to enrich microbial mRNA. The pathogen hybrid capture (PatH-Cap) method, for example, enables the capture of increased numbers of bacterial transcripts per gene, and sequencing of samples before and after enrichment can achieve remarkably efficient sequencing of host and pathogen mRNA, respectively (Betin et al., 2019). This technique was applied to study intracellular infection of host cells by *P. aeruginosa*, typically involving less than three bacterial cells per host cell. scDual-seq combined with PatH-Cap was able to demonstrate rapid gene expression adaptations in intracellular bacteria as well as revealing the necessity of NF-κB host-cell signaling for bacterial survival inside bladder epithelial cells (Penaranda et al., 2021). This combined approach was also able to identify a dynamic host-pathogen relationship between *Mycobacterium tuberculosis* and murine macrophages. Although infected macrophages appear to reduce intracellular iron availability, intracellular *M. tuberculosis* cells increase expression of genes associated with iron scavenging (Betin et al., 2019). Although PatH-Cap is limited by its need for well-annotated target transcriptomes, its ability to enrich lowly abundant targets may provide promising applications in the study of complex human microbiome samples. The ability to identify such dynamic and specific host-microbe interactions may be incredibly beneficial to the study of both intracellular pathogens as well as host-cell interactions with a wide range of microbes during normal immune regulation of microbiome communities.

Mining human scRNA-seq data for microbial RNA uncovers hidden information

Although many studies have focused on efficiently capturing both host and microbial mRNA, recent evidence has suggested that there may be a wealth of microbial transcriptional information hiding among existing host scRNA-seq datasets. Several groups have recently developed approaches to mine host scRNA-seq data for such information in the form of inadvertently captured microbial mRNA. Although the presence of microbial RNA among such datasets has historically been considered unwanted contamination, several groups are now demonstrating

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the power of inadvertently captured microbial sequences to illuminate a range of host-microbiome interactions.

The first of these approaches, termed Viral-Track, allowed simultaneous profiling of infected host cells as well as taxonomic profiling of the responsible viruses (Bost et al., 2020). Viral-Track was able to successfully differentiate hepatitis B infected cells in human clinical samples, as well as identify specific host factors associated with viral replication. The authors also applied the approach to bronchoalveolar lavage (BAL) samples from COVID-19 patients. They were able to develop a cellular and viral atlas of immune responses across a range of patients, in particular revealing differential effects of viral infection on immune cells of patients with mild and severe disease. Viral-Track was also able to implicate co-infection with human Metapneumovirus in dampening the immune response to COVID-19.

Host scRNA-seq mining approaches are now also being applied beyond specific pathogens to target all microbial reads (Figure 3C). The first example of this comes from a recent study also investigating COVID-19 (Lloréns-Rico et al., 2021). Here, host scRNA-seq data were mined for microbial reads to investigate the potential role of the lung microbiome in disease progression. The authors applied an in-house pipeline to scRNA-seq data from BAL samples of patients with COVID-19 and non-COVID-19 pneumonia controls. Similar to Viral-Track, this pipeline was designed for the discovery of host cell-associated microbial transcripts from host scRNA-seq data as well as taxonomic profiling of uncovered sequences and characterization of differential microbial abundances across host cell types (Figure 4). This approach identified a subset of bacteria associated with host immune cells, in particular neutrophils, monocytes, and macrophages. Notably, it was found that associations with monocytes and macrophages were unique to COVID-19 patients and that bacteria-associated host cells displayed distinct proinflammatory phenotypes. This expression profile in the immune cells suggests that the bacteria found in these samples could be opportunistic pathogens exacerbating inflammation in these severe COVID-19 patients. However, this study could not elucidate whether these associations were due to COVID-19 or to mechanical ventilation, as most COVID-19 patients were ventilated, whereas controls were not. Overall, this study clearly demonstrates the power of scRNA-seq mining approaches to reveal and illuminate complex and potentially clinically significant relationships between the human microbiome and host cells.

Another recent addition to the scRNA-seq mining approach, identification of cell-type-specific intracellular microbes (CSI-Microbes; Robinson et al., 2021; preprint), was initially tested on two scRNA-seq datasets of human immune cells exposed to *Salmonella*, showing a difference in resolution linked to the sequencing depth achieved by the two platforms used (Smart-seq2 and 10X). Application of CSI-Microbes on Smart-seq2-generated data from lung cancer samples led to identification of sixteen species previously associated with lung cancer. The algorithm was also able to identify differential abundances of bacteria associated with tumor, immune, and stromal cells. Subsequent analysis of host-cell gene expression patterns also identified downregulation of antigen processing and presentation in infected tumor cells, suggestive of a mutualistic relationship between intracellular bacteria and tumor cells.

A common limitation of retroactive scRNA-seq data mining approaches is that they are limited by both the quality produced by the original scRNA-seq approach as well as the chosen approach's ability to capture microbial RNA. Additionally, the risk of detecting common contaminants or sequencing artefacts requires the application of strict controls and/or filtering strategies, either experimentally or bioinformatically. Nevertheless, with appropriate methods, these scRNA-seq mining approaches can provide significant first insights and hypotheses into host-microbe interactions. Although the low level of microbial RNA captured in host scRNA-seq data as well as biases introduced through polyA based capturing approaches are hampering full, quantitative functional characterization, these recent studies prove that microbial taxonomic identification is achievable. Such promising initial results suggest a wealth of insights may remain untapped within existing publicly available scRNA-seq datasets. These approaches therefore present a remarkably quick way to mine the vast amount of existing data and generate testable hypotheses for follow-up studies.

CONCLUSIONS AND PERSPECTIVE

The vast heterogeneity of human-associated microbial communities has long been recognized, but only recently, technical developments in the field of single-cell biology allow revealing the individual variability in these ecosystems. However, we currently sit at different developmental stages regarding the different applications of these single-cell techniques. Although single-cell bacterial isolation and culturing and single-cell DNA sequencing have been successfully applied to both environmental and human-associated microbiomes, and even some commercial platforms exist, efforts in microbial single-cell transcriptomics are still largely focused on cultured strains due to the diverse challenges that remain to be addressed, such as diversity of cell wall structures in complex communities, low mRNA abundances, or poor mRNA stability (Table 1). Despite these limitations, it has been proven that to a certain extent, bacterial sequences can be captured by single-cell methods even in host-associated tissues. This possibility will open the door to the development of joint host-microbiome single-cell genomics and transcriptomics methods that will provide the technological basis needed to disentangle complex host-microbiota interactions at the individual cell level.

The development of these microbiome single-cell technologies should not be the end goal, and we should see these as tools to improve our current understanding of the human microbiome: to identify and characterize low-abundant members and to explore the intrinsic functional variability of microbiomes, even within strains, to assess how they impact the ecosystem-level behavior. For instance, it has recently been shown that diet directly impacts the mutational profile of *Bacteroides thetaiotaomicron*, a prevalent member of the gut microbiota (Dapa et al., 2022). Single-cell techniques could potentially be used to determine how these specific mutations induced by alterations in the diet led to changes in gene expression in a subset of these bacteria, leading to a higher fitness and thus eventual outgrowth of these variants. Additionally, these techniques need to be combined with bulk approaches in metagenomics and

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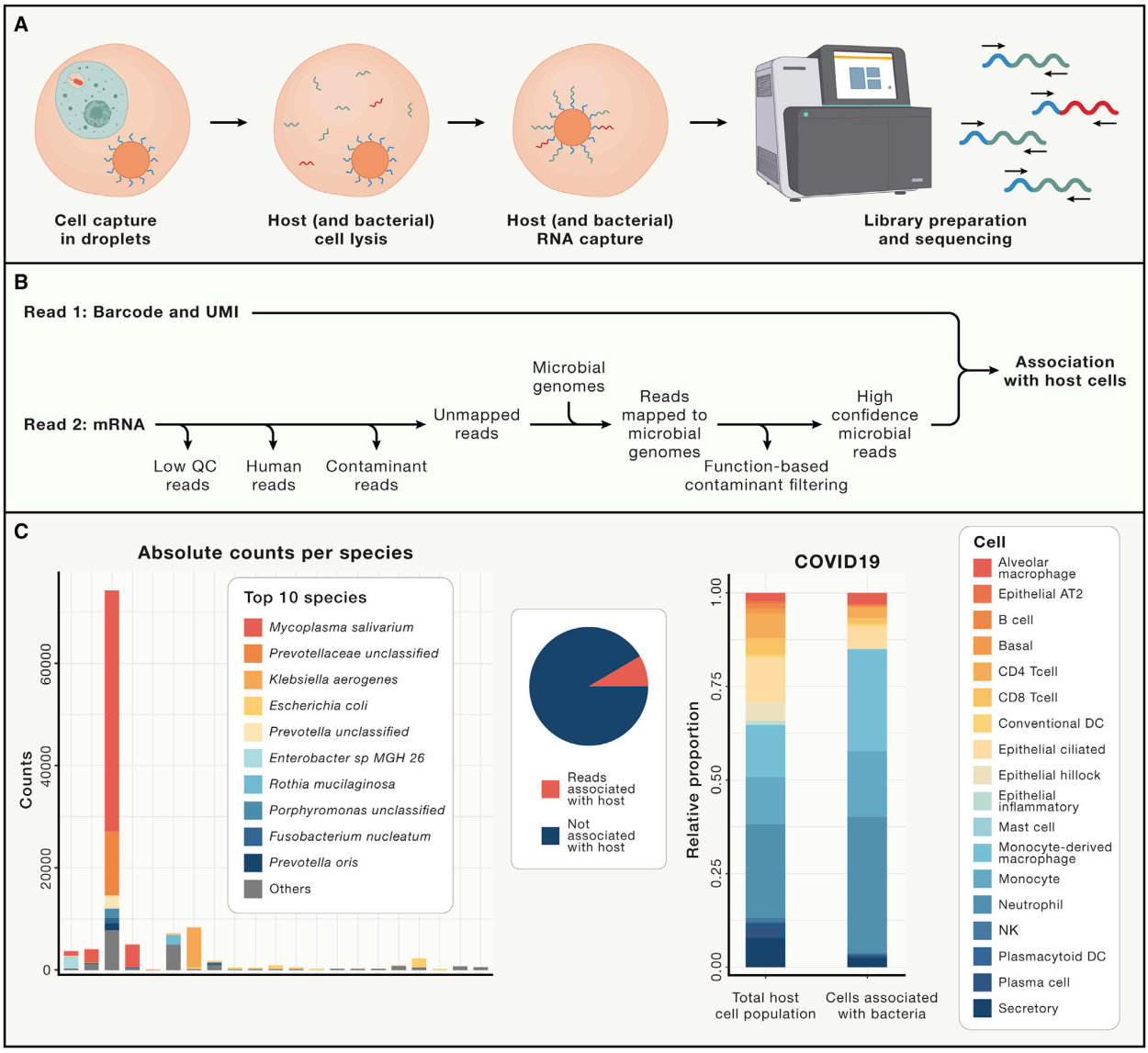

Figure 4. Mining microbial reads in host single-cell RNA-seq datasets

(A) Major steps in host single-cell RNA-seq protocols, showing how bacteria-containing host cells could lead to microbial transcripts being captured by these approaches.

(B) Major steps in microbial detection pipelines in scRNA-seq datasets. The most important steps involve decontamination and removal of potential sequencing artefacts or contaminants introduced during sample preparation.

(C) Microbial reads detected in a COVID-19 patient cohort, using scRNA-seq datasets from bronchoalveolar lavage data. Number of total microbial reads detected for each sample is shown on the left bar plots. Inlet pie chart shows the percentage of microbial reads whose barcodes are assigned to a host cell. On the right, the percentages of each host cell type in the whole cohort are compared with the percentages of host cells with associated bacterial reads, showing a higher fraction of monocytes, macrophages, and neutrophils with associated microbes.

metatranscriptomics, but also in other techniques such as metaproteomics or metabolomics, to achieve an integrative understanding of the human microbiome. For example, gene expression or even growth of individual bacteria may depend on supporting species via cross-feeding, and therefore, combination with bulk approaches is required for the isolation and study of these microbiota members.

To this end, we should aim our efforts at making these tools broadly available to microbiome researchers, by standardizing current techniques and making them affordable and parallelizable to attain large sample sizes. Only then will we be able to explore the large diversity of microbial strains and functions within the human microbiome and the mechanisms by which they interact with the host, impacting health and well-being, one cell at a time.

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DECLARATION OF INTERESTS

The authors declare no competing interests.

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