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Go with the flow or solitary confinement: a look inside the single-cell toolbox for isolation of rare and uncultured microbes

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With the vast majority of the microbial world still considered unculturable or undiscovered, microbiologists not only require more fundamental insights concerning microbial growth requirements but also need to implement miniaturized, versatile and high-throughput technologies to upscale current microbial isolation strategies. In this respect, single-cell-based approaches are increasingly finding their way to the microbiology lab. A number of recent studies have demonstrated that analysis and separation of free microbial cells by flow-based sorting as well as physical stochastic confinement of individual cells in microenvironment compartments can facilitate the isolation of previously uncultured species and the discovery of novel microbial taxa. Still, while most of these methods give immediate access to downstream whole genome sequencing, upscaling to higher cell densities as required for metabolic readouts and preservation purposes can remain challenging. Provided that these and other technological challenges are addressed in future innovation rounds, integration of single-cell tools in commercially available benchtop instruments and service platforms is expected to trigger more targeted explorations in the microbial dark matter at a depth comparable to metagenomics.

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Introduction

The birth of metagenomics, the now standard approach to study microbial communities using high-throughput DNA sequencing technology, was essentially triggered by the intrinsic limitations of conventional culturing approaches. Concurrently, the common observation that the number of microbial colony forming units growing on an artificial isolation medium under laboratory conditions is vastly outnumbered by the microscopic cell count of the same sample, historically referred to as the 'great plate count anomaly', also fuelled intensive explorations into the phenomenon of unculturability. Whereas many efforts to bring 'unculturable' microbes into culture have focussed on relatively simple adaptations of conventional growth-stimulating and cultivation-stimulating strategies [1••], one largely underexplored challenge that still needs to be addressed in order to reach the depth and scale of metagenomics concerns the isolation process itself. Basically, microbial isolation is defined by the physical separation of individual cells from a community with a high microbial load and complexity. Although widely regarded as the most critical step in the discovery of novel species, it is striking that most isolation protocols still adhere to Robert Koch's landmark method [2] based on physical spreading of cells on solid, transparent culture media. The inability of spread cells to produce a colony upon incubation (e.g. because an essential growth factor is missing or growth is inhibited) or failure to detect such colonies (e.g. if their morphology only allows microscopic visualization) are two common scenarios in Koch's approach causing the target organism to escape cultivation and hence be considered 'unculturable' under the given isolation conditions.

A paradigm shift was achieved when the sequence of Koch's isolation steps was reversed. By isolating single cells before they are actually grown, limiting factors such as initial cell number and intercellular resource competition are expected to have far less impact on subsequent culture recovery [3]. In addition, only single-cell isolation techniques provide the absolute guarantee for the purity of a microbial culture, being an essential prerequisite for downstream phenotypic and genomic characterization of individual organisms. Such approaches also come with the necessary technological innovations; single-cell isolation methods move away from manual handling of plates and tubes to more complex microscale devices and (semi-) automated liquid handling platforms enabling flow-based cell sorting or stochastic confinement in small (e.g. microtiter plate wells) or ultra-small (e.g. microdroplets or microfabricated and nanofabricated chip compartments) volumes. At this scale, single-cell isolation technologies can also be combined or integrated with other analytical

tools which significantly extends their downstream application range, for example, to monitor cellular growth and metabolic responses upon perturbation, to screen for novel bioactive compounds such as enzymes and antibiotics, or to obtain whole genome sequences [4,5••].

Using single-cell technologies, a comparable level of throughput and miniaturization as achieved in metagenomic pipelines may also come within reach of culture-based approaches that specifically target members of the microbial dark matter. In the past few years, a number of excellent reviews have comprehensively covered the state-of-the art on available tools [6,7••] and physiological concepts [8••] for microbial analyses at the single-cell level. This review specifically deals with recent contributions of the single-cell toolbox for the isolation of previously uncultured species and the discovery of novel microbial taxa in the rare biosphere. Largely based on literature from the past three years, key studies and proof-of-concept examples were selected to critically assess the technological advances and application potential across a range of environmental and human microbiome ecosystems. We particularly focus on the two single-cell approaches that have been most intensively explored in a microbiological context, that is, analysis and separation of free cells by flow-based sorting and space-limited confinement of individual cells in microenvironment compartments (Figure 1).

Free cells

Early attempts to physically separate single microbial cells, most of which relied on manual cell handling under a microscope with a micromanipulator device, all fell short in offering the throughput required for large scale microbiological isolations [9]. This was drastically improved when the concept of hydrodynamic focusing of cell populations in a narrow fluid stream led to the development of flow-based cell sorting and subsequent application of several types of externally applied forces (e.g. optical, magnetic, electrical) for single-cell separation [10]. In this section, we focus on recent achievements of free cell sorting for microbial isolation purposes based on optic manipulation.

Label-free flow cytometric cell sorting

Although flow cytometry and fluorescence-activated cell sorting (FACS) are probably the most common approaches for high-throughput separation and isolation of free cells from complex environments [[11•]], their application potential for downstream cultivation purposes has so far been fairly limited. As most bacterial cells from natural samples do not contain fluorescent pigments or molecules, additional labelling with DNA-binding or protein-binding fluorescent markers is usually required for laser-based detection but at the same time hampers subsequent cultivation of the sorted cells. In a number of recent studies, however, it was shown that label-free detection and isolation of previously uncultured bacteria is possible in a number of specific cases where target cells display distinct morphological features. For instance, morphology-specific light scattering signatures could be defined for FACS-based sorting in single-cell mode of ammonia-oxidizing bacteria from activated sludge environments that typically appear as microcolonies of aggregated cells. Physical enrichment of single-species microcolonies could be verified with fluorescence *in situ* hybridization (FISH) and resulted in the isolation of previously uncultured *Accumulibacter* and *Nitrospira* spp. [11•] and novel *Nitrosomonas* spp. [12]. This label-free cell enrichment approach thus further extends the application range of flow cytometric methods towards the potential isolation, genomic analysis, and physiological investigation of yet unculturable or unknown bacteria with atypical morphology.

Raman-activated cell sorting (RACS)

By offering an intrinsically richer spectrum for differentiation of non-confined single cells without requirement for external labelling, Raman-activated cell sorting (RACS) is currently one of the most promising alternatives to FACS for sorting and isolation of rare or unknown microbial species. The principle of Raman microspectroscopy is based on the use of a monochromatic laser and a confocal microscope objective lens to acquire cellular information on virtually all of its components (nucleic acids, protein, carbohydrates and lipids), resulting in a biochemical profile that allows to differentiate single cell types both at structural and metabolic levels [13]. The same laser can also be used to create a single beam optical gradient trap in a sample solution, termed Raman tweezers, which allows to separate and sort intact cells of interest in a non-destructive way. In recent years, various RACS optimization efforts have not only substantially improved the quality and reproducibility of single cell Raman spectra (SCRS), but have also expanded the technological options for the collection of targeted cells towards isolation systems operated in microfluidic flows (optofluidics) and on surfaces of coated slides, chips or microtubes [14]. However, the most important innovation was achieved by the direct combination of RACS and DNA, RNA, or protein-based stable isotope probing (SIP), with ^{15}N, ^{13}C and ^{2}H replacing their primordial isotopes (^{14}N, ^{12}C and ^{1}H). Single-cell Raman SIP not only proved a powerful approach to gain functional information from analysis of SCRS band shifts caused by nutritional or environmental fluxes [15••], but also shows great potential for taxonomic differentiation of microbial species in complex communities and the isolation of novel taxa.

Following the proof-of-principle study of Wang and co-workers [16] showing the ability of $^{13}C/^{15}N$-incorporated SCRS profiling to differentiate between bacterial species typical of the oral cavity, a number of recent studies have reported the isolation of new species from various

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