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Next-generation physiology approaches to study microbiome function at the single cell level

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Abstract

The function of cells in their native habitat often cannot be reliably predicted from genomic data or from physiology studies of isolates. Traditional experimental approaches to study the function of taxonomically and metabolically diverse microbiomes are limited by their destructive nature, low spatial resolution, or low throughput. Recently developed technologies can offer new insights into cellular function in natural and human-made systems and how microorganisms interact with and shape the environments that they inhabit. In this Review, we provide an overview of these next-generation physiology approaches and discuss how the non-destructive analysis of cellular phenotypes, in combination with the separation of the target cells for downstream analyses, provide powerful new, complementary ways to study microbiome function. We anticipate that the widespread application of next-generation physiology approaches will transform the field of microbial ecology and dramatically improve our understanding of how microorganisms function in their native environment.

ToC blurb

In this Review, Hatzenpichler et al. introduce next-generation physiology, which is a suite of new techniques that enable to investigate the phenotypes of individual cells in a non-destructive manner. Next-generation physiology complements genomics and culturing and provides new insights into microbiome function.

Introduction

Microorganisms dominate every ecosystem on our planet. They are the main drivers of global biogeochemical cycling, control the levels of many climate-active gases, and

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associate with virtually all multicellular lifeforms, including plants, animals, and humans. The microbiome **[G]** of each human is estimated to contain 10^{13}-10^{15} microbial cells from 10^{3}-10^{4} bacterial, archaeal and fungal species[1] and recent predictions suggest a total number of more than 10^{30} microbial cells and 10^{31} viruses in the biosphere[2]. High-throughput sequencing technologies have revolutionized microbial community studies and led to a more complete view of the diversity of life on Earth[3–5]. However, in order to understand how microorganisms function and interact with their biotic and abiotic environment, experiments targeting the phenotype **[G]** of cells in their native habitat must complement cultivation-based and sequencing-based work. Physiology, the functioning of a cell at a given time and set of physiochemical conditions, is an emergent property that cannot be reliably predicted from genomic data or metabolic reconstructions alone. Rather, these approaches formulate valuable hypotheses that require experimental testing before definitive conclusions can be drawn about the physiology of a specific microorganism.

The realization that heterogeneity of gene expression and as a result changes in cellular phenotype are observed in synchronized, clonal cultures[6,7] led microbiologists to study physiology at the level of the individual cell (Fig. 1). In natural systems, the need to work at such high resolution is more pronounced; most DNA-sequencing and bioinformatic methods cannot differentiate between strains of the same species, and microorganisms sometimes have dramatically different genotypes **[G]** [8] and *in situ* phenotypes[9] despite indistinguishable or near-identical 16S ribosomal RNA (rRNA) gene sequences (the most commonly used taxonomic marker gene for bacteria and archaea). Although many powerful approaches exist to study microbial physiology, most of these techniques are only applicable to genetically amenable model archaea and bacteria that can be grown in pure culture. Commonly, these techniques depend on genetically encoded fluorescent reporters **[G]**, the creation of deletion mutants to causally link genotype and phenotype, and/or the ability to reliably grow microorganisms under tightly controlled conditions, such as in bioreactors or microfluidic devices[10–14]. Outside of laboratory-based experiments, however, microorganisms live as members of spatially structured, taxonomically diverse, and metabolically interdependent communities, which are exposed to varying physicochemical conditions. These complexities are an important reason why most taxa have so far proven recalcitrant to cultivation[15,16]. Even if representatives of environmentally and medically relevant taxa can be isolated, it is sometimes unclear to what extent laboratory findings can inform us about the ecophysiology **[G]** of a microorganism and the way it functions in its native habitat.

Ecophysiology experiments typically target phenotypes of populations or cells based on predictions of their metabolic potential from sequencing of enzyme marker genes, metagenomics **[G]**, or single cell genomics **[G]**. All of these methods require the destruction of the original sample (through cell lysis), thereby preventing subsequent analyses. Metabolic predictions are tested using experimental approaches that also destroy cells. For example, microautoradiography (MAR) **[G]** and nano-scale secondary ion mass spectrometry (nanoSIMS) **[G]** are arguably the most successfully applied ecophysiology techniques capable of single cell resolution[9,17–20] but are incompatible with downstream applications such as cultivation or genome sequencing. Quantitative stable isotope probing

(qSIP) **[G]** [21] provides a complementary and more high-throughput approach to study microbial physiology and can provide a direct link between cell taxonomy and substrate uptake. Although qSIP has led to fascinating discoveries in microbial ecology and is particularly powerful when combined with meta-omics[22–24], it cannot distinguish between individual cells. Similarly, many biogeochemistry-targeted approaches, such as extracellular enzyme assays, gas production measurements, or metabolome profiling are sensitive and easily replicable but currently cannot be applied at a scale relevant to microorganisms (μm to mm; with the notable exception of microsensors). Because these methods are either destructive, incompatible with correlative methods or have limited spatial resolution, one frequently has to determine the genotype of a cell first before subsequently characterizing the phenotype of a different cell.

In the past 15 years several new techniques have been developed in the fields of microbial ecology, chemical engineering, and analytical chemistry that radically break from the above approach. They enable studying the function of cells informing about, for example, their role in biogeochemical cycling, biotechnological potential, or medical relevance, irrespective of cell identity or genotype[25,26]. To distinguish these novel approaches from traditional methodologies, we introduce the term next-generation physiology **[G]**. Next-generation physiology approaches are independent of the need for prior knowledge about the genetic makeup of a microbial community and focus on cellular function. They do not require laboratory cultivation and are non-destructive, thus enabling microbiologists to bridge the gap between historically separated fields in microbiome research (Fig. 1). While cultivation, omics, and traditional physiology techniques are central components of microbiology research, next-generation physiology approaches provide a novel, complementary, and highly resolved view into the lives of microorganisms.

In this Review, we first discuss the general concept of next-generation physiology approaches before describing in detail the currently available techniques for studying cellular phenotypes without destruction of studied cells. We discuss how these approaches can be combined with cell sorting techniques and a suite of powerful downstream applications, including genetic characterization and cultivation-based experimentation.

Concept of next-generation physiology

We define a next-generation physiology approach as any combination of techniques that analyses the phenotype of an individual cell in a microbiome in a non-destructive way, which enables the physical separation of this cell based solely on its phenotype for subsequent, downstream applications. Ideally, these approaches can be applied in high-throughput (10^3–10^7 cells per hour).

Next-generation physiology approaches can be either label-free or label-dependent. Label-free approaches target native and inherent cellular properties and provide valuable information about the phenotype of a cell under non-invasive conditions. Label-based approaches introduce a chemical reporter into the cell that can provide a more comprehensive or complementary view of dynamic cellular processes. Before we discuss label-free and label-dependent approaches in detail, we provide an outline of the three steps

of every next-generation physiology approach: (i) non-destructive phenotype observation, (ii) sorting of the observed cell based on its phenotype, and (iii) downstream analyses (Fig. 2).

Non-destructive phenotype observation

The phenotype of an organism is defined by its observable characteristics in a given environment. Microscopy-based imaging is essential for studying the phenotype of individual microbial cells and is ideally coupled with molecular analysis to obtain taxonomic information. Microscopy uses transmitted light to visualize morphological features and optical properties or detects fluorescence characteristics upon excitation with light of specific wavelengths. Coupling microscopy with spectral analysis by Raman spectroscopy (Raman microspectroscopy) provides high-resolution (submicron spatial scale) spectral information. Raman spectroscopy measures the vibrational energy of molecular bonds after excitation with monochromatic light, which is informative of the molecular and, to some extent, isotopic composition of a cell (Box 1). The Raman spectrum of a cell typically consists of over 1,000 Raman bands (data points), each representing specific biochemical properties. Measurements are rapid (0.1–10 seconds per measurement) and can be non-destructive, thus enabling monitoring of living cells over time. There are reports of detrimental effects of laser irradiation on microorganisms and cell exposure to laser beams can have a range of outcomes from no observable effects to physical disintegration of the cell. However, negative effects are typically only observed after long-term exposure to intense laser light[27].

The Raman spectrum of a cell is a unique fingerprint of its chemical composition and contains information on its taxonomic identity and physiological state[28–30]. Label-based phenotype studies use introduced reporters (that is, stable isotopes, functional groups, or fluorophores) to detect unique chemical signatures or fluorescence properties using Raman microspectroscopy or fluorescence microscopy, respectively.

Cell sorting

Cells can be separated from complex samples based on morphological, optical, fluorescence, or Raman spectral properties. Optical microscopy and cell separation via optical tweezers or laser microdissection are manual and often tedious processes with limited throughput (10–100 cells per hour). By contrast, fluorescence activated cell sorting (FACS) automates separation and can sort 10^3-10^4 cells per second by combining fluorescence detection of individual cells with flow cytometry or microfluidics-based separation. Furthermore, cells with unique chemical signatures in their Raman spectrum (for example, compound-specific bands or peak shifts due to isotope incorporation; Fig. 2) can be separated by Raman-activated cell sorting (RACS) **[G]**. RACS techniques (reviewed in [31]) combine single cell Raman spectral acquisition with cell separation via optical tweezers[32], microfluidic sorting[33–36] or cell ejection[36–39]. Although a recently developed automated RACS platform that combined optical tweezers, microfluidics, and Raman spectral acquisition provided improved sorting efficiency (200–500 cells per hour[33]), Raman signal acquisition times of 0.1–10 seconds per spectrum currently limit the throughput of RACS compared to FACS. Future modifications of Raman microspectroscopy signal enhancement (Box 1) could

theoretically achieve spectral acquisition rates over 100 times faster than classical Raman microspectroscopy.

A potential bias associated with all cell sorting is that the initial separation of cells from the sample matrix depends on the specific sample and can lead to preferential cell recovery. Proper cell extraction particularly important for samples with high structural complexity or high numbers of particle-attached or otherwise immobilized cells. To achieve maximal cellular yields at minimal risk of preferential recovery, cell extraction protocols typically require optimisation for each sample type and thorough testing by comparing the *in situ* community composition to the extracted cell fraction[25,40,41]. Although no single protocol works for all sample types, a combination of washes with mild detergents, sonication, and density gradient centrifugation with or without filtration has been reported to yield the best results for complex samples, including sediments and soils[41–46]. Finding the appropriate cell extraction protocol often is the most time-consuming step in any next-generation physiology workflow.

Downstream analyses

After separation and sorting of individual cells with a desired phenotype they can be used for subsequent investigation. The main applications in microbiome research identify taxa through rRNA-targeted fluorescence *in situ* hybridization (FISH) **[G]**, taxonomic marker gene sequencing, genotype characterization through single cell or metagenome sequencing, or further phenotypic characterization with different microscopy techniques (for example, electron microscopy or atomic force microscopy[47]). Because chemical fixation can dramatically decrease DNA quality (for example, formaldehyde cross-links proteins and DNA), intact cells **[G]** (cells that have not been chemically fixed) are desired for DNA-targeted downstream applications[40,48]. Genome amplification from ultra-low biomass samples, including single cells, is commonly achieved by multiple displacement amplification (MDA). MDA can lead to uneven genome coverage, genome rearrangements including chimera formation, or erroneous nucleotide incorporation. Most of these biases, however, can be overcome through long mate-pair libraries, high sequence coverage, and post-sequencing normalization[40,41,48–50].

Alternatively, intact, sorted cells can be used as inoculum for cultivation, which enables in-depth culture-dependent physiology, biochemistry, and systems biology studies[51,52]. These downstream investigations complement initial phenotype characterization and lead to a more comprehensive understanding of the ecophysiology of a microorganism. To the best of our knowledge, high-throughput axenic cultures of cells separated from a sample based on their phenotype has not been achieved yet. However, a study demonstrated that cells separated from lake sediment by FACS based on their activity response to methane addition, could be regrown in enrichment media[52].

Label-free approaches

Non-invasive optical microscopy and Raman microspectroscopy observe the behavior and native chemical composition of individual cells. This is mostly informative of the presence of transient traits, but in the case of time-resolved analyses of living cells, it also provides

insights into dynamic cellular processes. Phenotypic observations by optical microscopy include the formation of spores, storage compounds, cellular segmentation, the behavioral responses of cells to external stimuli (for example, aero-, chemo-, magneto, or photo-taxis), or the occurrence of intrinsic autofluorescence from cofactors, pigments, or vitamins. Similarly, compounds with known Raman bands can be identified in the Raman profile of a cell based on database comparisons. Cells with specific characteristics can be separated based on their optical properties[53] (for example, cell volume or refractory index) or their chemical composition, such as the presence of auto-fluorescent compounds[54] or compound-specific Raman bands[31]. For example, RACS of a functional guild was elegantly demonstrated in a recent study[38], which separated uncultured bacteria from the Red Sea based on distinctive Raman bands of their carotenoid pigments. RACS-separated cells were further characterized by single cell genomics, revealing novel insights into carotenoid biosynthesis and previously unknown phototrophs[38]. Table 1 provides an extensive list of reporters available for label-free imaging and sorting of individual microbial cells.

As these label-free approaches to phenotypic characterization detect inherent cellular properties, they have limited application in studying metabolically active **[G]** cells, which requires the incorporation of chemical reporters to be tracked on a single cell level.

Isotope probing

Isotope probing approaches involve the incubation of a microbial sample with an isotopically labeled reactant (substrate or water) and track its incorporation into cellular components, identifying anabolically active **[G]** microorganisms. Incubation with an isotopically labeled substrate (for example, ^{13}C-glucose or $^{15}NH_4^+$) enables substrate incorporation into biomass and tracking of the flow of intermediates within a system. Alternatively, incubation with heavy water (^2H_2O or $H_2{}^{18}O$) provides a labeling strategy in which all anabolically active cells are detected independently of assimilatory capacities[36,55–57].

Single cell resolved isotope probing, such as MAR or nanoSIMS combined with FISH, has seen wide application in microbial ecophysiology studies[9,17–20]. However, MAR and nanoSIMS destroy cells and thus preclude subsequent downstream analysis. Raman microspectroscopy is a non-destructive analysis strategy. Isotopically labeled cells are identified by characteristic peak shifts in their Raman spectrum due to the replacement of a light isotope by a heavy isotope, which changes the vibrational energy of a molecule through the increased molecular mass introduced by the heavy isotope (for example, shift of the C-H peak from 2,935 cm^{-1} to 2,178 cm^{-1} due to ^{2}H incorporation[58]; Fig. 3). The intensity of this shift towards smaller wavenumbers correlates with the amount of assimilated heavy isotope[55,59]. Detection limits of isotope uptake depend on the specific capabilities of the Raman microspectroscopy system but typically are ~10% ^{13}C, ~10% ^{15}N, and ~0.2% ^{2}H replacement of cellular carbon, nitrogen, and hydrogen, respectively[55,58,59].

Substrate stable isotope probing

Substrates labeled with heavy isotopes can be used to identify all members of a microbial community that can specifically assimilate the substrate. In addition, isotopically labeled

metabolic intermediates (degradation products of the initially added substrate) can reveal cross-feeding within a community and metabolic interactions between cells. However, some isotope-containing compounds, in particular those of high structural or compositional complexity (such as cellulose, lignin, or mucin) often are prohibitively expensive or commercially unavailable. In addition, substrate SIP requires amendment of a microbial sample with an isotopically labeled compound, which could alter natural substrate concentrations and change the composition of the incubated communities [.

Detection of ^{13}C and ^{15}N labeled cells has been achieved by Raman microspectroscopy in multi-species communities and has been successfully combined with FISH[59,60], genome sequencing[37,60] or cultivation[60]. An initial study detected labeled cells after incubation with ^{13}C-glucose based on peak shifts in the Raman spectrum due to incorporation of the heavy isotope[61]. Subsequently, substrate SIP-Raman microspectroscopy was used to investigate the niche differentiation of naphthalene degraders in ground water communities[59,60], uptake of phenylalanine in an amoeba-*Chlamydiae* symbiont system[62], and assimilation of different carbon and nitrogen sources in environmental communities[60,63–66]. Since the first successful separation of individual isotope labeled cells from a cell mixture into sterile capillaries using optical tweezers[32], different types of RACS have been used to sort ^{13}C or ^{15}N labeled cells from complex samples[37,39], demonstrating the importance of this approach to ecophysiology research (Tab. 1).

Using stimulated Raman scattering (SRS) microscopy, ^{2}H-containing nucleosides, amino acids, and fatty acids can be used to visualize DNA-, RNA-, protein-, and membrane-synthesizing cells[67,68]; however, this application has, to the best of our knowledge, not yet been demonstrated for microbial samples.

Heavy water SIP

Isotope labeling approaches with heavy water ($^{2}H_2O$ or $H_2{}^{18}O$) aim to identify all members of a community that are anabolically active[36,55–57], rather than those members involved in specific transformations. Heavy water SIP enables testing cellular activity under either close to *in situ* conditions or a specific physicochemical condition or substrate amendment. Heavy water has gained increasing interest in environmental microbiology as it generally can be used without prior knowledge of the growth substrate(s) of a microorganism, it does not interfere with the natural substrate pool, and it is inexpensive. SIP with heavy water commonly requires an experimental setup in which a portion of regular water (ideally 30–50%[55]) is replaced with heavy water to achieve high enough labeling percentages for reliable detection, a feat that can be challenging to achieve in certain sample types (for example, soil and aqueous samples). In addition, the effect of heavy water on the growth rates of physiologically diverse and taxonomically distinct cells has not been evaluated and the molecular underpinnings of this effect are not yet understood, demanding further investigation[55,69,70].

For Raman microspectroscopy analyses, labeling of cells with $^{2}H_2O$ is superior to $H_2{}^{18}O$ labeling because H from water readily exchanges with the NADPH pool of the cells, the main source of H for lipid synthesis[71–73]. The introduction of ^{2}H-C bonds is easily detectable in the Raman spectrum of a cell by a characteristic peak shift of the abundant C-H

peak into the silent region **[G]** of the cellular chemical profile (Fig. 3). $^{2}H_2O$ SIP-Raman microspectroscopy has been combined with FISH to detect targeted taxa and with RACS to select functionally active cells for 16S rRNA gene or whole-genome sequencing[33,36,55] (Tab. 1).

Substrate analog probing

An alternative approach to SIP is to incubate a sample with a synthetic compound that is a structural and/or functional analog of a naturally occurring molecule. Such experiments are either directed towards anabolic processes, such as non-canonical substrate labeling, or towards metabolic reactions catalyzed by specific enzymes or enzyme families, such as fluorescent substrate analog labeling or activity- and affinity-based protein profiling. To contrast these approaches from SIP, we here introduce the term substrate analog probing (SAP). An important advantage of SAP compared to most SIP and some label-free approaches is that SAP uses infrastructure that is readily available to most laboratories, that is, standard fluorescence microscopes and FACS instruments. Therefore, many SAP approaches, in particular those that use azide-alkyne click chemistry **[G]**, are comparatively easy to set up.

Fluorescent SAP

Fluorophore-tagged derivatives of natural compounds can be used to track the uptake of molecules on a cellular level. This provides a powerful approach for determining specific substrate uptake capabilities of individual microorganisms in multi-species communities. Examples of fluorescent SAP include the use of fluorescent cobalamin analogs to demonstrate the uptake of this vitamin into bacteria, worms, and plants[74], or the use of fluorescently labeled *D*-amino acids to visualize regions of active peptidoglycan-synthesis in cell walls of different bacterial pure cultures[75]. Furthermore, the combination of fluorescent substrate analog probing with FACS and subsequent marker gene and whole genome sequencing enabled the identification of diverse but low abundance degraders of glucose in the rumen[76],of xylan and laminarian in bacterioplankton[77], and cellulose degraders in a geothermal spring[78].

Fluorescent SAP specifically detects cells that take up the fluorescent substrate under the assumption that there is no transfer of the fluorescent group to other metabolites. The broader implementation of fluorescent SAP is limited by the development of fluorescent labeling techniques that target different molecule classes. Furthermore, the addition of a fluorescent tag directly to the substrate might interfere with enzyme-substrate binding and recognition. Newer, click chemistry-based approaches, such as non-canonical substrate labeling and activity- and affinity-based protein profiling, overcome these problems by making the detection of these molecules (for example, by dye staining) independent of the labeling chemistry by using substrate analogs. Examples of this are the use of clickable **[G]** vitamin B12[79] or *D*-amino acids[80] rather than fluorescently labeled vitamins or *D*-amino acids.

Non-canonical SAP

Non-canonical molecules are synthetic structural analogs of biological molecules that are incorporated into biomass due to enzyme promiscuity. Many non-canonical molecules contain a reporter group that can be specifically traced within the complex environment of the cell through a bioorthogonal reaction **[G]**. These reactions are chemical transformations that do not interact with functional groups present in naturally occurring molecules, have no or only minimal byproducts, and do not interfere with cellular processes[81–83]. Azides and terminal alkynes are particularly attractive reporter groups because they rarely occur in biology, are biocompatible, and can be fluorescently detected by azide-alkyne click chemistry conjugation reactions (Box 1). To our knowledge, only one natural azide-containing molecule (a secondary metabolite produced by a dinoflagellate) has been identified[84]. Terminal alkynes, as functional groups of amino acids and fatty acids, are more common but still restricted to only a few lineages[85–87]. An alternative to detecting azides or alkynes through a bioorthogonal fluorescence labeling reaction is to use SRS to trace them inside the cell[68,88].

Biorthogonal labeling approaches are well established in the study of bacterial[89–93] and eukaryotic[94–97] model organisms. In multi-species systems, however, they have mainly been used to study *de novo* protein synthesis. Indeed, proteins are the most promising target for *in situ* studies because they constitute the largest proportion of cellular dry weight (50–65%)[98–101]. This results in a higher sensitivity for proteins than other molecules, as the cellular dry weights of DNA (1–3%), RNA (10–20%), and lipids (10–25%) are much lower (Fig. 3).

Bioorthogonal non-canonical amino acid tagging—Labeling of newly translated proteins with synthetic amino acids can be accomplished through bioorthogonal non-canonical amino acid tagging (BONCAT)[102–104]. BONCAT achieves the co-translational labeling of proteins by exploiting the substrate promiscuity of amino-acyl tRNA synthetases, which are enzymes responsible for catalyzing the esterification of amino acids with their cognate tRNAs. Only two clickable amino acids, *L*-azidohomoalanine (AHA) and *L*-homopropargylglycine (HPG), which both replace *L*-methionine (Met) during translation (Fig. 3), can be incorporated without genetic modification[104–106]. Since its inception[103], BONCAT has been used to study protein synthesis in a range of microbial pathogens[89,107–111] and was recently applied in several complex samples, including marine and freshwater sediments[46,112], surface[113,114] and deep[115] seawater, soil[42], and an oral biofilm[112]. In these studies, BONCAT was applicable to cultured and uncultured members of at least 20 archaeal and bacterial phyla[42,46,112–118] as well as bacteriophages[119] and eukaryotic viruses[119,120]. Because of their structural similarity to Met and their low activation rate by methionyl-tRNA synthetase[104], HPG and AHA have only small effects on rates of protein synthesis and degradation in *E. coli*[121] and mammalian cells[95,103,121], as well as on protein tertiary structure[122]. BONCAT correlates well with other independent proxies of growth, such as the incorporation of $^{15}NH_3$ into single cells visualized by nanoSIMS[112], ^{35}S-Met uptake as measured by MAR[113], or incorporation of ^{3}H-leucine into bulk biomass measured by scintillation counting[115]. In a study on deep-sea methane seeps, no measurable effect on either microbial community composition or rates of sulfide

production and methane oxidation was observed when sediment samples were incubated with HPG[46]. When AHA or HPG are used at levels that resemble the intracellular concentration of Met (~100 μM)[123] or over more than two generations, growth rates of some bacterial cultures are negatively affected[112]. Therefore, low concentrations of AHA or HPG (nM to μM range) and no-addition (blank) controls are required to compare and minimize effects on growth rates as well as unwanted reactions with naturally occurring azides or terminal alkynes. Incubation times should also be optimized (ideally to less than one to a few cell generations[46,114,118]) to avoid excessive substitution of Met, which could lead to nonfunctional proteins. It is still unknown how non-canonical amino acids enter the cell and interact with the translational machinery, which currently limits the ability to directly quantify, on a single cell level, newly made proteins in complex communities (Box 2). It is also unknown whether AHA or HPG are misrecognized for Met by enzymes other than Met-tRNA-synthase; if so, the azide and alkyne functional groups could be transferred to other molecules.

Intact or chemically fixed cells identified by BONCAT can be stained with clickable fluorophores (Box 1) that serve as reporter groups in fluorescence microscopy studies. When coupled to rRNA-targeted FISH or catalyzed reporter deposition FISH (BONCAT-FISH[112], BONCAT-CARD-FISH[46]), active cells can be identified, thus revealing taxonomy-function relationships and co-localization patterns of taxonomically identified active cells[46,112,114,115]. BONCAT-FISH has been used to visualize the cell organization of protein-synthesizing methane-oxidizing archaeal-bacterial consortia in deep-sea sediments[46]. In the same study, BONCAT was, for the first time, combined with FACS of both ethanol-fixed and intact (chemically unaltered) cells (BONCAT-FACS) for subsequent whole genome amplification and gene sequencing. Recently, the same approach was used to study the active cell fraction in soil[42], an ecosystem that is notoriously difficult to investigate due to its structural complexity and high microbial diversity. The study revealed that a large fraction (20–70%) of soil-extractable cells was translationally active and that a high diversity of bacterial taxa was labeled with BONCAT[42]. This result was in stark contrast to previous, more labor-intensive studies, such as DNA-SIP[124] or labeling with the thymidine surrogate 5-bromo-2’-deoxyuridine (BrdU)[125], which suggested that up to 95% of cells in soil are inactive at a given time. Recent studies that employed qSIP-methodology are consistent with findings by BONCAT-FACS[126]. This discrepancy can be explained by the inherent biases associated with BrdU-labeling[125,127,128].

The ability to combine bioorthogonal labeling incubations with other compounds enables designing experiments to screen for physicochemical factors (such as temperature, pH, or O_2 levels in the headspace) or growth substrates that drive cellular, population, or community activity[46,112]. BONCAT is particularly useful for studying non-assimilatory pathways or if isotope labeled substrates are not available. Accordingly, BONCAT-FISH and BONCAT-FACS combined with marker gene or whole-genome sequencing can be used to monitor microbial community dynamics or identify specific taxa with changing activity after substrate changes[46,112]. This approach is conceptually similar to tracking the growth response of cells to substrate addition in the presence of heavy water and separating ^2H_2O-labeled cells by RACS[33,36,55,129]. Neither BONCAT nor ^2H_2O-Raman microspectroscopy can disentangle whether cell labeling is due to direct substrate uptake or metabolic cross-

feeding, but measuring multiple samples over the course of an incubation may help reconstructing metabolic interactions and population dynamics within communities.

Targeting non-proteinaceous cell components and viruses—BONCAT is arguably the most sensitive non-canonical substrate labeling approach due to the large contribution of proteins to cellular biomass; however, many other biomolecules can be targeted, including nucleic acids, lipids, and polysaccharides (Fig. 3). The introduction of (deoxy)ribonucleoside surrogates amenable to click chemistry, for example, provides a straight-forward approach for detecting cells that synthesize RNA and DNA. A recent proof-of-concept study demonstrated the applicability of the alkyne-carrying thymidine surrogate 5-ethynyl-2'-deoxyuridine (EdU) to studying DNA synthesis in individual marine microorganisms by azide-dye staining[130]. Click chemistry-based detection of EdU can be performed in an hour and yields cell labeling rates comparable to the more biased and experimentally more complex BrdU-labeling approach[125,127,128]. By contrast, the alkyne-carrying uridine analog 5-ethynyl-uridine (EU) is incorporated into RNA due the promiscuity of RNA polymerase[131] but has not yet been used on complex samples.

Other bioorthogonal labeling approaches use azide- or alkyne-modified fatty acids[132,133], *D*-amino acids [80,134,135], or sugars[117,136,137] to label the lipid membrane, peptidoglycan layer, or cell surface polysaccharides, respectively (Fig. 3; Tab. 1). Because pathways for lipid and cell wall biosynthesis, the use of peptidoglycan, and cell wall modifications differ widely across the tree of life, these approaches lack the general applicability of protein labeling via BONCAT. Although some of these substrate analogs have been used in studies targeting specific microorganisms, they have yet to be tested on taxonomically and physiologically diverse pure cultures and their effect on cellular activity remains unclear. Thus, researchers interested in applying these activity proxies in their research should proceed carefully before applying them to diverse samples.

Recent successful application of bioorthogonal labeling to cultured virus-host models of pathogenic[120] and environmental relevance are also very promising[119,138]. In 2012, a study demonstrated that EdU-modified T4 phages can infect *E. coli* and that T4-containing cells stained with a clickable dye can be separated by FACS from an artificial waste water community[138]. Furthermore, BONCAT was recently used to quantify *in situ* marine viral production rates by fluorescence staining[119]. These pioneering studies demonstrated that non-canonical SAP approaches have strong potential to increase our understanding of the turnover rates of viruses in single cells as well as microbial communities, the viral impacts on elemental cycling through the release of nutrients from lysed cells, and might help to identify new virus-host relationships[119,138].

Activity- and affinity-based protein profiling

A complementary set of SAP techniques targets catabolic rather than anabolic functions of the cell. Activity-based protein profiling (ABPP) is arguably the most broadly applicable catabolism-targeted approach that identifies active enzymes. Most importantly, in contrast to all other methods discussed in this Review, ABPP enables researchers to reveal the function of open reading frames in microbial genomes lacking functional prediction. ABPP achieves

this objective with catalytic mechanism-based, electrophilic reactive groups (‘warheads’) that covalently label the active site of specific enzymes or enzyme classes[139–142] (Fig. 3). The bound enzyme is later detected by a functionalizable reporter attached to the warhead by a spacer group. Although other reporter groups are available[143], terminal azides and alkynes are the most commonly used and adaptable reporter tags owing to their biocompatibility and small molecular size, which guarantees minimal interference with substrate binding and reactivity and improves cell permeability.

Affinity-based substrate analogs are similar to their counterparts used in ABPP but rather than relying on enzymatic activity, the analogous substrates interact with proteins based on structural mimicry of the substrate rather than by bond creation with the active site of the enzyme. Thus, affinity-based protein profiling cannot resolve catalytically active from inactive enzymes. To achieve irreversible covalent linking of the affinity-based substrate analog to the enzyme, photoactivatable groups can be used[141,142].

Activity- and affinity-based protein profiling are well-established approaches for identifying new enzymes in cultured microorganisms but, to our knowledge, have only once been applied to complex microbiomes[144]. Their potential importance for single cell ecophysiology studies, however, cannot be overstated. In an approach called ABP-FACS, a recent study used activity-based probes (ABP) to fluorescently detect, separate by FACS, and taxonomically identify β-glucuronidase active members of the mouse gut microbiome[144]. They also demonstrated that treating mice with vancomycin drastically affects glucuronidase activity and leads to strong shifts in the taxonomic composition of glucuronidase-active cells separated by ABP-FACS.

The limitation of activity- and affinity-based protein profiling lies in the challenge to design a substrate analog that reacts and binds to only one particular enzyme or enzyme class; however, substrate analogs are already available for a wide variety of enzyme classes[139,141,142] (Tab. 1). In the future, microbiologists will need to more frequently and effectively collaborate with analytical chemists, chemical engineers, protein biochemists, and synthetic organic chemists to identify the most promising targets for functional studies and develop specific reporters for probing the activities of specific enzymes as well as intact cells.

Although other fluorescence-based tracers of enzyme activity, cell integrity, or cell structure are in use, most of them suffer from limitations that currently restrict their widespread application in microbial ecology. Many stains used for staining extracellular matrixes or cell internal structures are class-specific (for example, DNA, polysaccharides, or protein) but not compound-specific, and their specificity has not been validated using independent methods[145,146]. Furthermore, most commercially available stains of metabolic activity have been shown to be inapplicable to complex samples for a variety of reasons (Box 3).

Outlook

The non-destructive nature of next-generation physiology approaches enables crucial downstream analyses of individual cells that express a phenotype of interest. These unique,

phenotype-targeted approaches complement more established methodologies including cultivation, enzyme characterization, and meta-omics.. Once appropriate instrumentation becomes more widely available and experimental protocols more broadly adapted by the research community, the concepts we have described will enable highly parallelized characterization of microbiome function. For example, we expect that BONCAT-FACS and $^{2}H_{2}O$-RACS will soon be widely applied to study the activity response of microbial communities to substrate addition or environmental changes, thus allowing physiological characterization of uncultured microbes at a hitherto unprecedented speed[33,42,46,55]. These and other single cell targeted approaches will be aided by the anticipated expansion of droplet microfluidics to employ culture-independent assays. Most currently available microfluidics approaches still depend on the ability to grow microorganisms on-chip, exploit genetically encoded fluorescence reporters, or are targeted at the genotype rather than the phenotype[10,13,147–150].

To reach these goals, microbiologists are encouraged to work hand-in-hand with researchers outside the microbiome sciences, including analytical chemists, synthetic organic chemists, and biological and chemical engineers. Tremendous opportunities exist for non-microbiologists who are willing to go outside their comfort zone and break into the realm of living systems. Examples for their potential impact on microbiome sciences include the synthesis of new probes to interrogate cellular and enzyme function under non-invasive conditions, the adaptation of lab-on-the-chip designs to characterize uncultured microbial cells extracted from complex samples, or the development of new high-speed phenotype-based cell-sorting devices. Whereas fluorescence microscopes and FACS instruments are already widely available to most microbiome researchers, university core facilities are now beginning to incorporate advanced microscopy techniques (such as Raman microspectroscopy and cryo-electron tomography), microfluidics, and nanofabrication equipment.

We predict that once broadly applied, next-generation physiology approaches will greatly help with the transition of microbiome research from correlative studies to a causal understanding of microbial activity and function.

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Glossary

Microbiome

Synonymous with microbial community; all microscopic organisms, including archaea, bacteria, unicellular eukaryotes, and their viruses, within a sample

Phenotype
An observable characteristic of an organism that is manifested on molecular, cellular, or population level. A phenotype of a cell varies over time and with changing physicochemical conditions.

Genotypes
A genotype is the set of genes or the entire genome of an organism

Reporters
Molecules or chemical motifs that can be specifically traced within the cell; ideally, the reporter group is entirely absent from the target cell under natural conditions

Ecophysiology
The functioning of a cell in its native habitat under a given set of conditions, including interactions with other cells and the abiotic environment

Metagenomics
Random shotgun sequencing of DNA from a sample containing more than one genotype

Single cell genomics
An individual cell is separated from a microbiome and its genome is amplified and sequenced

Microautoradiography (MAR)
A method that detects uptake of radioactively labeled substrates into cells through formation of silver grains after exposure to a photographic emulsion. MAR is limited in its wide-spread application because of its dependency on isotopes with a suitable half-life, its low throughput, and its destructive nature.

Nano-scale secondary ion mass spectroscopy (nanoSIMS)
A technique that expels secondary ions from a sample surface through a focused ion beam in high vacuum, extracts them by an electric field, and analyzes them by time of flight mass spectrometry. nanoSIMS provides unrivaled sensitivity and spatial resolution but has very low throughput and destroys the sample

Quantitative SIP (qSIP)
A technique that separates isotopically heavy biomolecules (for example, ^{13}C-containing DNA) from unlabeled molecules by buoyant density centrifugation. By collecting multiple density fractions and determining their taxonomic and genetic makeup, taxon-specific isotope enrichments can be calculated.

Next-generation physiology
Any approach enabling to study the physiology of an individual cell in a microbiome in a non-destructive way, thus enabling physical separation of this cell based on its phenotype for further downstream applications

Raman activated cell sorting (RACS)
A set of techniques that combines Raman spectral acquisition with single cell separation

Fluorescence in situ hybridization (FISH)
A technique that uses single-stranded DNA probes and fluorescence microscopy to visualize cells based on their taxonomic identity (rRNA FISH) or gene expression (mRNA FISH)

Intact cells
cells that have not been exposed to a chemical fixative (such as formaldehyde or ethanol) that might interfere with downstream analyses (such as cultivation or DNA-sequencing)

Metabolically active
A cell carrying out specific metabolic function (such as redox activity or activity of a specific enzyme); this term is agnostic about whether this activity leads to the build-up of new biomass (that is, anabolic activity)

Anabolically active
Performing de novo synthesis of specific macromolecules (e.g. DNA, RNA, proteins, and lipids)

Silent region
Area in the Raman spectrum of a cell that is free of background interference from cellular vibrations (~1,800–2,700 cm^{-1})

Click chemistry
Summary term for a range of reactions with a high thermodynamic driving force and extremely high yields and reaction efficiencies. The term is often used synonymously for azide-alkyne cycloaddition reactions, which are the most commonly used type of click chemistry reactions in biology

Clickable
Used here to characterize a molecule carrying a functional group that is amenable to azide-alkyne click chemistry

Bioorthogonal reaction
A reaction that does not interfere with biological processes; can be used to label a cell or molecule with a reporter

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Box 1.

The chemistry underlying many nextgeneration physiology approaches

Raman microspectroscopy

Raman spectroscopy is a classical technique in analytical chemistry that measures the vibrational energy of molecular bonds. In Raman microspectroscopy analyses, the molecules in a sample are excited with monochromatic light and inelastically scattered (re-emitted) photons are analyzed. Following excitation, most molecules return to their ground vibrational state and emit photons with the same wavelength as the incident light, an effect referred to as Rayleigh scattering (figure part a). In very rare cases (one in every 10^6-10^8 photons) the wavelength of a scattered photon is shifted compared to the incident light by either Stokes or anti-Stokes inelastic scattering. Stokes scattering, the more common form, occurs when an excited molecule returns to a state of elevated vibrational energy compared to the ground state, resulting in increased vibrational energy and emission of photons with lower energy. Alternatively, a molecule that is already in an excited state can be further excited and return to its vibrational ground state, emitting a photon with higher energy compared to the incoming light (anti-Stokes scattering). The detection of these scattered photons can be used to study the chemical composition of a sample.

Spontaneous Raman scattering, the most commonly used Raman microspectroscopy method, is limited by inherently low signal intensities. Low signal intensity can be problematic when analyzing cells with high levels of autofluorescence. Several techniques are available for signal enhancement and faster acquisition times, including surface- or tip-enhanced Raman spectroscopy (SERS or TERS), stimulated Raman scattering (SRS), coherent anti-Stokes Raman spectroscopy (CARS), and resonance Raman spectroscopy[67,163–168]. Although these advanced Raman microspectroscopy techniques have sporadically been applied to microbial isolates[161,164,165,169–171] and hold great promise for microbial ecophysiology, they are currently absent from the microbiome literature. The acquisition of a Raman spectrum is relatively fast and easy, although Raman spectra can be very complex and their interpretation requires robust data analysis and reliable reference databases.

Azide-alkyne click chemistry

Click chemistry refers to any reaction that creates heteroatom links and that is modular and easy to perform, features fast kinetics, high chemo- and stereo-selectivity, as well as very high yields[172]. Although many reaction types fulfill these criteria[81,83,173], the widely used azide-alkyne [3+2] cycloaddition reaction yielding a triazole conjugate has become the gold standard and is often simply referred to as the ‘click reaction’. Two types of labeling reactions yield triazole conjugates through azide-alkyne click chemistry (figure part b): a Cu(I)-catalyzed version that ligates an azide with a terminal alkyne, and a metal-free, strain-promoted reaction that links a highly reactive (strained) cyclooctyne-containing molecule (for example, dibenzocyclooctyne) with a reporter azide[81,83,174,175].

In Cu(I)-catalyzed click reactions, chelating ligands for copper (such as tris[(1-hydroxypropyl-1H-1,2,3-triazol-4-yl)methyl]amine, THPTA) improve reaction kinetics and protect the cell from oxidative damage, whereas addition of the reductant sodium ascorbate maintains copper in the catalytically active Cu(I) state. To avoid protein crosslinking by byproducts of ascorbate oxidation, aminoguanidine is added to the reaction mix. Fluorescent dyes containing Cu-chelating picolyl motifs raise the effective concentration of Cu(I) at the reaction site[176], which permits the use of lower metal concentrations and thus lowers the risk of copper cytotoxicity for downstream analyses that require viable cells, such as cultivation attempts.

Exploiting the reactivity of cyclooctyne-containing molecules with azides provides a metal-free alternative to Cu(I)-catalyzed click reactions. However, strain-promoted click chemistry can be accompanied by nonspecific reactions with free thiols (for example, the thiol group of reduced cysteine). Hence, free thiols must be blocked prior to the click reaction to avoid nonspecific labeling, which is typically achieved by incubation with a haloacetamide (for example, 2-chloroacetamide).

Azide-alkyne click chemistry reactions to fluorescently label cells are simple to perform because they involve cheap reagents (totaling ~$500 for the clickable substrate analog and dye as well as all necessary reagents [118]) and a small number of working steps. Labeling and washing protocols are well established and can be completed in one (copper-catalyzed click) to three hours (strain-promoted click)[118]. Both types of click reactions are solvent- and pH-independent and are not affected by the presence of complex organic or inorganic matrices (for example, the extracellular polymeric substance of a biofilm, sediment particles or minerals), ensuring a low level of background noise when applied to microbiome samples. New generations of clickable fluorophores, including picolyl dyes[176] and fluorogenic ‘turn-on’ azide probes[177], which only become fluorescent upon reaction with an alkyne, are particularly well suited for complex sample types. The low molecular weight of all reagents (<1 kDa) makes it possible to click-stain cells without the ethanol-dehydration or permeabilization steps (such as treatment with lysozyme or proteinase K) required for successful fluorescence *in situ* hybridization. Click chemistry-mediated fluorescence-staining can be achieved on formaldehyde-fixed[42,46,112–115,118], ethanol-fixed[46], or intact, not chemically fixed[42,46,112] cells.

Box 2.

Limitations of single cell BONCAT studies

Several unknowns currently limit our ability to absolutely quantify protein synthesis rates in individual cells, which challenge the use of bioorthogonal non-canonical amino acid tagging (BONCAT) in quantifying activity rates of single cells (figure part a). (i) The routes by which non-canonical amino acids enter a cell are unknown, and the roles of facilitated diffusion and/or transporters could differ between species. (ii) In addition, although the catalytic efficiency of methionyl-tRNA synthetase of *Escherichia coli* for *L*-homopropargylglycine (HPG) and *L*-azidohomoalanine (AHA) is known (1:500 for Met:HPG and 1:390 for Met:AHA[104]), the extent of this substrate promiscuity might differ between organisms. Varying promiscuity would lead to differences in the substitution rate of Met in new proteins and ultimately labeling intensity. Furthermore, (iii) variations in the Met content of proteins and (iv) the rate at which proteins are expressed might compound interpretations. Heterogeneity in gene expression rates is observed even in clonal cultures and is likely amplified in multi-species samples[6,7,178]. Lastly, (vi) variability in click staining efficiency as a result of differences in the rate of dye entry into the cell could also lead to differences in cell labeling intensity. Similar limitations probably exist for other non-canonical substrate analog probing approaches capable of labeling DNA, lipids, or peptidoglycan but are currently untested.

Analysis of genomes deposited in NCBI RefSeq reveals a range in the use of Met in proteins (figure part b; $N_{archaea}$=1,561,087 proteins; $N_{bacteria}$=14,597,681 proteins). On average, predicted bacterial and archaeal proteins have a Met content of 2.49% and 2.19%, respectively. >99.9% of these proteins contain Met, suggesting that virtually all proteins are in principle amenable to labeling by AHA or HPG. However, possible modifications to the start Met (for example, N-formyl-Met, which uses a separate tRNA) could render some proteins unamenable to replacement by AHA and HPG, which depends on the promiscuity of methionyl-tRNA synthetase. If the starting amino acid is ignored, 5.70% and 10.88% of predicted bacterial and archaeal proteins do not contain Met (figure part c). For these calculations, only one genome from each species was analyzed and only complete genomes were considered for bacteria. Average values for archaea and bacteria are shown in each plot. The number of archaeal and bacterial bins for drawing plots were 410 and 270 in part b and 550 and 350 in part c, respectively.

Box 3.

Alternative cell-staining approaches

‘Vitality’ and ‘viability’ dyes

Advertised as ‘vitality’ and ‘viability’ stains (table), commercially available redox stains and mixes of membrane-permeable and impermeable dyes have lately seen use in microbiome studies to identify supposedly ‘living’ or ‘active’ cells. However, all these stains have some limitations that restrict their use in many complex samples resulting in rough estimates of vital or viable microorganisms at best[179,180]. Nevertheless, these stains can be useful in mixed-species samples, but only after extensive testing, including with pure cultures relevant to the specific study system. RedoxSensor™ Green has been successfully applied in combination with substrate stimulation and fluorescence-activated cell sorting (FACS) to investigate metabolically active methane oxidizers in Lake Washington[52,181]. Although such targeted applications are possible, researchers should apply caution when using these dyes.

Genome-inferred antibody engineering

An exciting new approach at the interface of phenotypic and taxonomy-based cell separation is ‘reverse genomics’[51]. In this workflow, antibodies are raised against proteins predicted to be located in the outer membrane or cell wall and FACS is used to sort fluorescent antibody-stained cells from a sample for subsequent single cell cultivation. The power of this approach was elegantly demonstrated by a study that used it to culture individual cells of ‘Saccharibacteria’ (formerly known as TM7) and the candidate phyla ‘Absconditabacteria’ (SR1) from human saliva[51]. Genome-inferred antibody engineering depends on the availability of genomes from cells of interest and cannot differentiate between metabolically active and inactive cells. However, if suitable cell surface antigens can be identified and specific antibodies targeting them can be developed, reverse genomics could be a promising tool to bring new microorganisms into culture.

Type of stain	Working principle	Method-specific limitations	Dye-specific limitations	General limitations of all ‘viability’ and ‘vitality’ dyes
Redox stains (for example, 5-cyano-2,3-ditolyltetrazolium chloride (CTC) or RedoxSensor™ Green (RSG))	Redox dyes that depend on activity of electron transport chain	Not useful for tracking activity of microorganisms that lack an electron transport chain (for example, strict fermenters)	CTC suppresses cellular activity[182,183]; counts of CTC positive cells were 2–100 times lower than microautoradiography counts[184–186]	Practically unsuitable for structurally complex sample types (such as sediments, soils, or biofilms) because cell extraction reduces cell activity; general applicability to physiologically and
Live-dead stains (for example, LIVE/DEAD™ BacLight™, SYTOX Red Dead, FUN®−1, ReadyProbes)	Mixture of a cell-permeable (for example, SYTO TM9) and	Not useful or yield inaccurate results for cells with hard to permeate cell walls or membranes (for	Background fluorescence, bleaching, fluorescence resonance energy transfer between dyes, double staining,	

Type of stain	Working principle	Method-specific limitations	Dye-specific limitations	General limitations of all 'viability' and 'vitality' dyes
	membrane-impermeable DNA-stain (for example, propidium iodide)	example, spores; Gram positive versus Gram negative bacteria)[179,187,188]	and decrease in vitality during staining[179,187,189]	taxonomically diverse communities is unknown; dyes are typically tested only on a small subset of clinically relevant, easy to culture, heterotrophic bacteria adapted to high nutrient conditions; rarely compared to independent measures of activity or cell growth other than the formation of colony-forming units

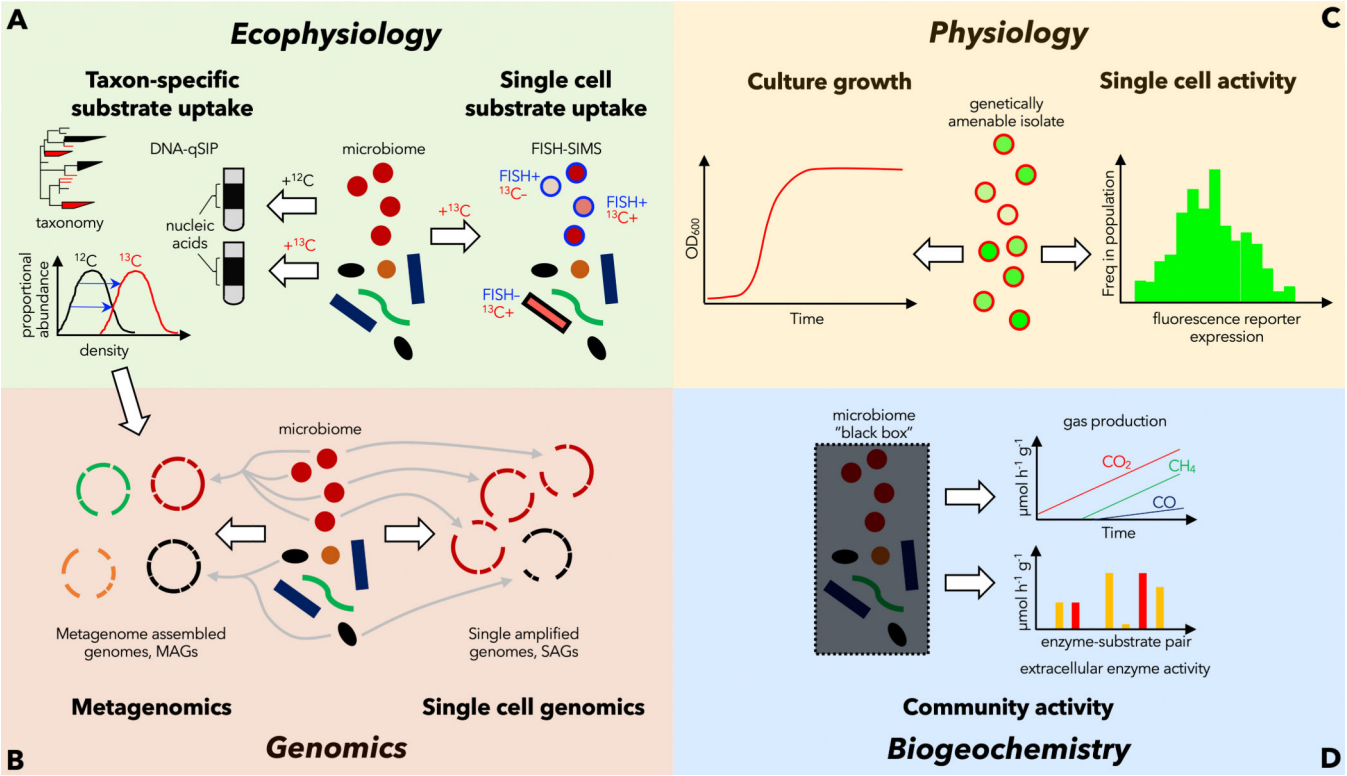

Figure 1. Examples of traditional approaches to study microbial physiology.

Most physiology-targeted techniques in the microbiome field depend on the availability of genetically amenable pure cultures, rely on destructive analyses that cannot directly link genotype with phenotype, or are unable to study functional activity at the level of single cells. (A) Stable isotope probing (SIP) can be coupled to secondary ion mass spectrometry (SIMS) and fluorescence *in situ* hybridization (FISH) to link cell function and identity.Isotopically heavy DNA can be separated from light DNA via buoyant density centrifugation. In quantitative stable isotope probing (qSIP), multiple density fractions are collected and analyzed by 16S rRNA gene sequencing or metagenomics. (B) The genetic makeup of entire microbial communities or individual cells can be studied by metagenomics or single cell genomics. Whereas single cell genomics typically captures only the most abundant members of a microbial community, metagenomics integrates the genomic information obtained from many individual cells into population genomes, that is, metagenome-assembled genomes (MAGs). (C) If genetically tractable microorganisms are available, they can be studied using reporter-gene constructs, which enables direct insights into variation of metabolic and anabolic activity between cells. (D) Many biogeochemical approaches treat microbiome samples as an undefined ‘black box’ but provide highly sensitive and precise measurements of overall community activity.

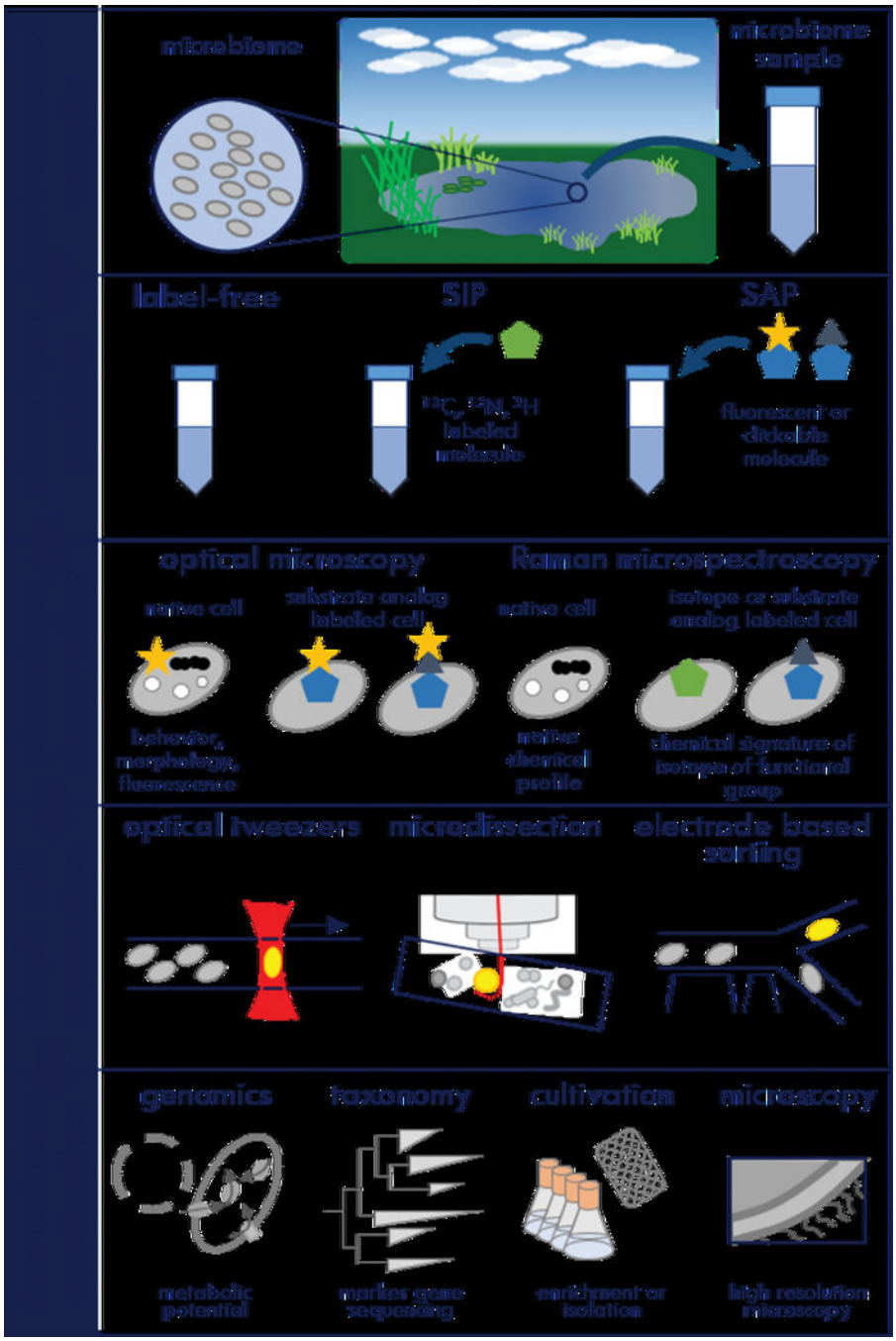

Figure 2. Next-generation physiology workflow to study microorganisms.

A microbiome sample is obtained using minimally invasive protocols and a phenotype of interest is detected using non-destructive methodology, for example via light or fluorescence microscopy or Raman microspectroscopy. Label-free approaches are directed at intrinsic properties of a cell, including chemotactic behavior, the expression of cofactors or pigments, or the presence of storage compounds. Label-based approaches introduce a chemical reporter into the cell that provides information about dynamic processes. Stable isotope probing (SIP) in combination with Raman microspectroscopy reveals substrate assimilation.

Substrate analog probing (SAP) employs molecules that carry either a fluorescence tag or a side-group amenable to azide-alkyne click chemistry to obtain information on the overall biosynthetic activity or specific enzymatic function of the cell. After identifying a cell expressing the phenotype of interest that same cell is separated from the sample using, for example, optical tweezers, laser microdissection, or electrostatic deflection. The unaltered, sorted cell is then committed to downstream applications, which could include whole-genome sequencing, targeted cultivation, or complementary microscopic analyses. Different reporters used in next-generation physiology are described in table 1 and figure 3.

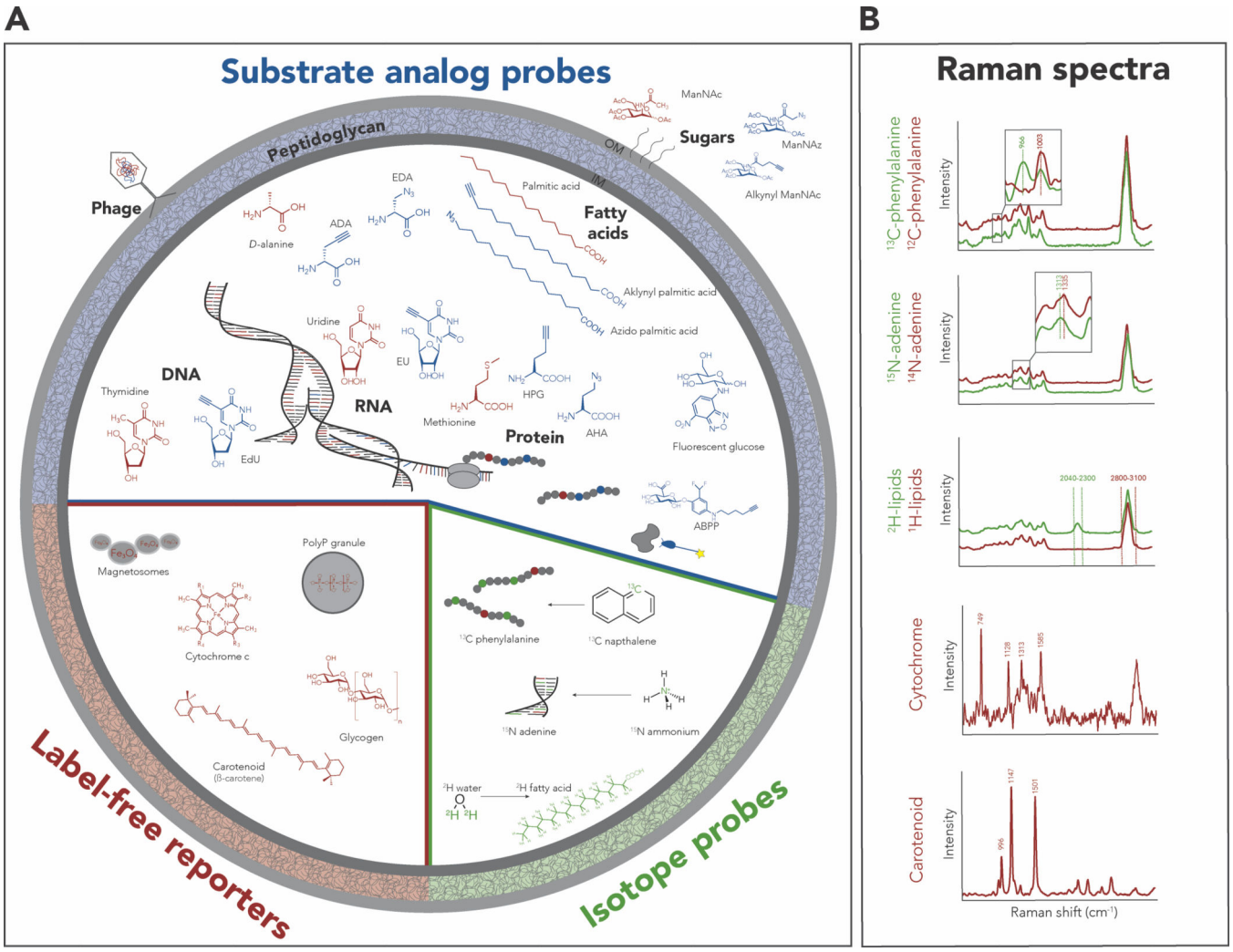

Figure 3. Reporters and their associated Raman spectral fingerprints in microbial next-generation physiology.

(A) Label-free reporters (red) are produced by the cell and do not require addition by the researcher. Substrate analog probes (blue) are traceable compounds that are amenable to biorthogonal labelling and that are incorporated into biomass by the cell of interest after addition to the microbiome sample. Finally, isotope probes (green) can be added to a sample to trace the uptake and incorporation of isotopically labeled compounds. Many substrate analog probes contain azide or terminal alkyne groups, which can be conjugated to a fluorescent dye for detection by click chemistry. **(B)** Incorporation of stable isotopes into biomass leads to spectral shifts towards lower wavenumbers in the spectrum of labeled cell compared to unlabeled cells and some of these shifts are pronounced enough to be detectable by Raman microspectroscopy[58]. The figure shows examples for the most commonly used indicator peak shifts used to trace isotope incorporation into single cells, including from top down, the symmetric ring breathing effect by phenylalanine (^{13}C), C–H stretching of adenine (^{15}N), and C–H stretching of lipids and proteins (^{2}H). Other reporters, including alkyne-labeled substrate analogs and some label-free compounds, have distinct Raman spectral fingerprints that also can be used for phenotype detection[38,68,88]. Table 1

includes detailed information on the application of all depicted reporters and references to the primary literature.

Table 1.

Next-generation physiology approaches to study microorganisms.

Reporter	Phenotype of single cells characterized by light or fluorescence microscopy	Phenotype of single cells characterized by Raman microspectroscopy	Phenotype-based cell separation and downstream application
Label-free approaches			
Behavioral reaction to external stimuli	Aerotaxis, chemotaxis, magnetotaxis, or phototaxis	N.A.	Magnetic enrichment and single cell genomics of magnetotactic bacteria[151]
Cofactors	Cofactor F_{420} in methanogenic pure and enrichment cultures[54]	Cofactor F_{420} in an ammonia-oxidizing archaeon[152]	FACS based on cofactor F_{420} autofluorescence and sequencing of marker gene of methanogens[54]
Pigments	Chlorophyll	Carotenoid-containing bacteria[38]	RACS and single-cell genomics of carotenoid-containing bacteria[38]
Spores	Endospores detection by differential interference contrast microscopy	*Bacillus cereus* spores[153]	*
Extracellular polymeric substance	Stains for extracellular DNA, proteins, or polysaccharides[145,146]	Proteins and polysaccharides in biofilm matrix[59,60]	*
Carbon storage	N.A.	Glycogen, poly-hydroxyalkanoate, and poly-hydroxybutanoate in wastewater sludge bacteria[154,155]	*
Cytochromes	N.A.	Cytochrome C in nitrifiers[156], anammox bacteria[156] and *Beggiatoa* spp.[157]; cytochrome redox potential[158]	automated RACS of cytochrome C rich cells from a marine enrichment culture[33]
Magnetosomes	N.A.	Magnetotactic bacteria containing magnetite and greigite[159]	*
Phosphate storage	N.A.	Orthophosphate and poly-phosphate in cultured[159] and environmental[154,155,159] bacteria	*
Sulfur inclusions	N.A.	Polysulfides in sulfur-oxidizing *Beggiatoa* spp.[157]; *cyclo*-octasulfur in pure cultures[159] and bacterial symbionts of flatworms[160]	*
Stable isotope probing (with substrate or heavy water)			
^{2}H	N.A.	Naphthalene and glucose degradation by *Pseudomonas* sp. and *Escherichia coli*[57]	*
^{13}C	N.A.	Naphthalene-degraders in groundwater[59,60]; phenylalanine uptake by extracellular *Chlamydiae*[62]; marine autotrophs[37]; degraders of cyanobacterial necromass[65]	Raman-activated cell ejection and single cell genomics of marine autotrophs[37]
^{15}N	N.A.	$^{15}N_2$ fixers in soil[63]; ammonia, nitrite and N_2 assimilation in freshwater bacteria[161]	*
$^{2}H_2O$	N.A.	Mucin degraders in mouse gut microbiome[33]; cellulose degraders[129], detection of antibiotic-resistant bacteria in freshwater[36]; degraders of organic matter in groundwater[162]	Manual sorting using optical tweezers, followed by 16S rRNA gene sequencing[55]; automated sorting using optical tweezers on a microfluidic platform, followed by metagenomics[33]
$H_2{}^{18}O$	N.A.	*	*
Substrate analog probing			
Fluorescent analogs	Uptake of glucose in rumen[76]; xylan and lamarin uptake by	*	FACS and 16S rRNA gene sequencing and single cell

Reporter	Phenotype of single cells characterized by light or fluorescence microscopy	Phenotype of single cells characterized by Raman microspectroscopy	Phenotype-based cell separation and downstream application
	bacterioplankton[77]; fluorescent amino acids[75]		genomics of cells taking up fluorescent glucose[76] or polysaccharides[77], respectively
Non-canonical substrates	Clickable nucleosides[130,131,138], *L*-amino acids [46,112–115,119], *D*-amino acids[80], sugars[117,137], fatty acids[132,133]	Alkyne containing amino acids, nucleosides, sugars, and fatty acids visualized by SERS[67,68,88]	FACS followed by 16S rRNA gene sequencing[42,46]
Activity- and affinity-based protein profiling	Ammonia monooxygenases, antibiotic-reactive proteins, ATP-ases, ATP-binding proteins, cellulases, cytochromes, fatty acid synthases, glycoside hydrolases, lipases, redox-reactive proteins, vitamin transporters[139,141,142]	*	FACS separation and 16S rRNA gene sequencing of β-glucuronidase active cells[144]

*application feasible but not yet demonstrated; anammox, anaerobic ammonium oxidation; FACS, fluorescence-activated cell sorting; N.A., not applicable; RACS, Raman-activated cell sorting; rRNA, ribosomal RNA; SERS, surface-enhanced Raman scattering.