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

Roland Hatzenpichler ✉, Viola Krukenberg, Rachel L. Spietz and Zackary J. Jay

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.

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

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

Department of Chemistry and Biochemistry, Center for Biofilm Engineering, and Thermal Biology Institute, Montana State University, Bozeman, MT, USA.

✉*e-mail: roland.hatzenpichler@montana.edu*

https://doi.org/10.1038/s41579-020-0323-1

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 associate with virtually all multicellular lifeforms, including plants, animals and humans. The microbiome 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 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 in a given 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[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, 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 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 of a microorganism and the way it functions in its native habitat.

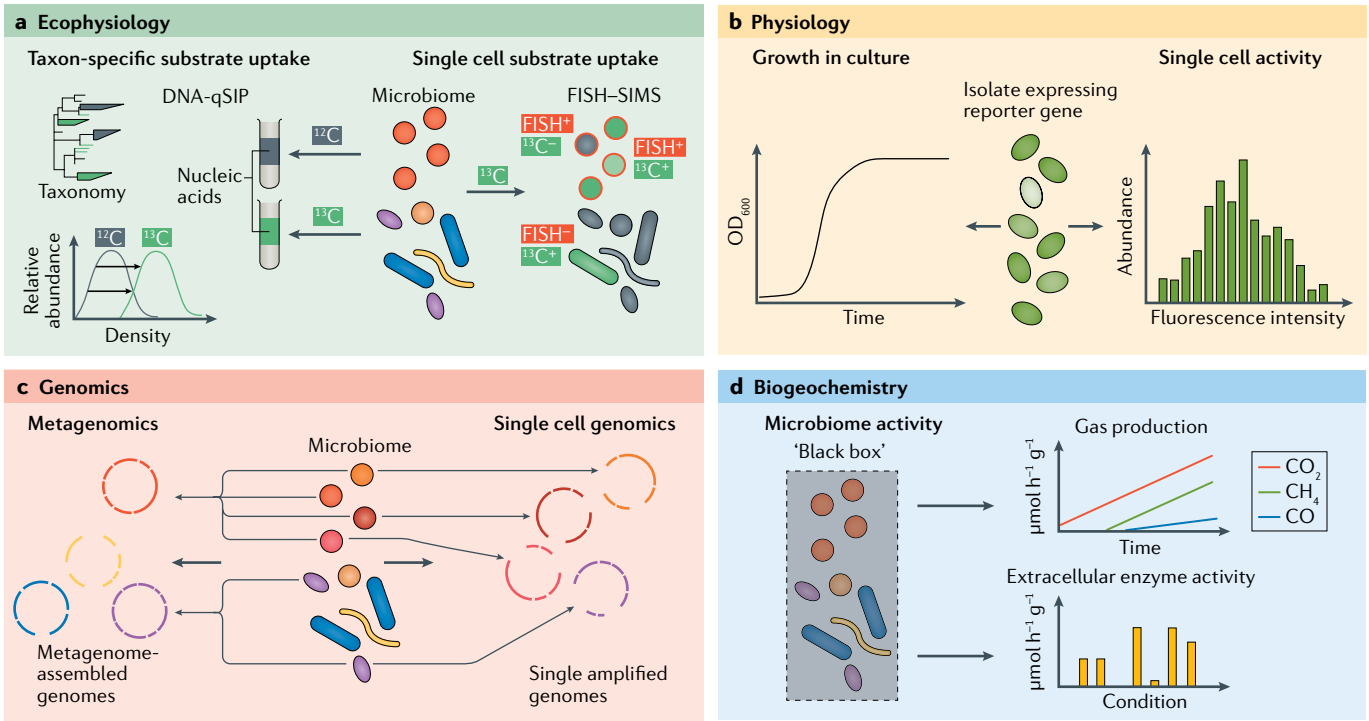


Fig. 1 | **Examples of traditional approaches to study microbial physiology.** Most physiology-targeted techniques in the microbiome field depend on the availability of genetically amenable pure cultures, rely on destructive analyses that cannot directly link genotype with phenotype or are unable to study functional activity at the level of single cells. **a** | Stable isotope probing 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 analysed by 16S ribosomal RNA gene sequencing or metagenomics. **b** | If genetically tractable microorganisms are available, they can be studied using reporter–gene constructs, which enable direct insights into variation of metabolic and anabolic activity between cells. **c** | The genetic make-up 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. **d** | Many biogeochemical approaches treat microbiome samples as an undefined 'black box' but provide highly sensitive and precise measurements of overall community activity. OD_{600}, optical density at 600 nm.

Ecophysiology experiments typically target phenotypes of populations or cells based on predictions of their metabolic potential from sequencing of enzyme marker genes, metagenomics or single cell genomics. 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) and nano-scale secondary ion mass spectrometry (nanoSIMS) 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 (SIP)[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 quantitative SIP 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 reproducible but currently cannot be applied at a scale relevant to microorganisms (micrometres to millimetres; with the notable exception of microsensors). Because these methods are destructive, are incompatible with correlative methods or have limited spatial resolution, one frequently has to first determine the genotype of a cell before subsequently characterizing the phenotype of a different cell in a separate experiment.

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. Next-generation physiology approaches are independent of the need for prior knowledge about the genetic make-up of a microbial community and focus on cellular function. They do not require laboratory cultivation and are non-destructive, thus

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.

Genotype
The sets of genes or the entire genome of an organism.

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
The random shotgun sequencing of DNA from a sample containing more than one genotype.

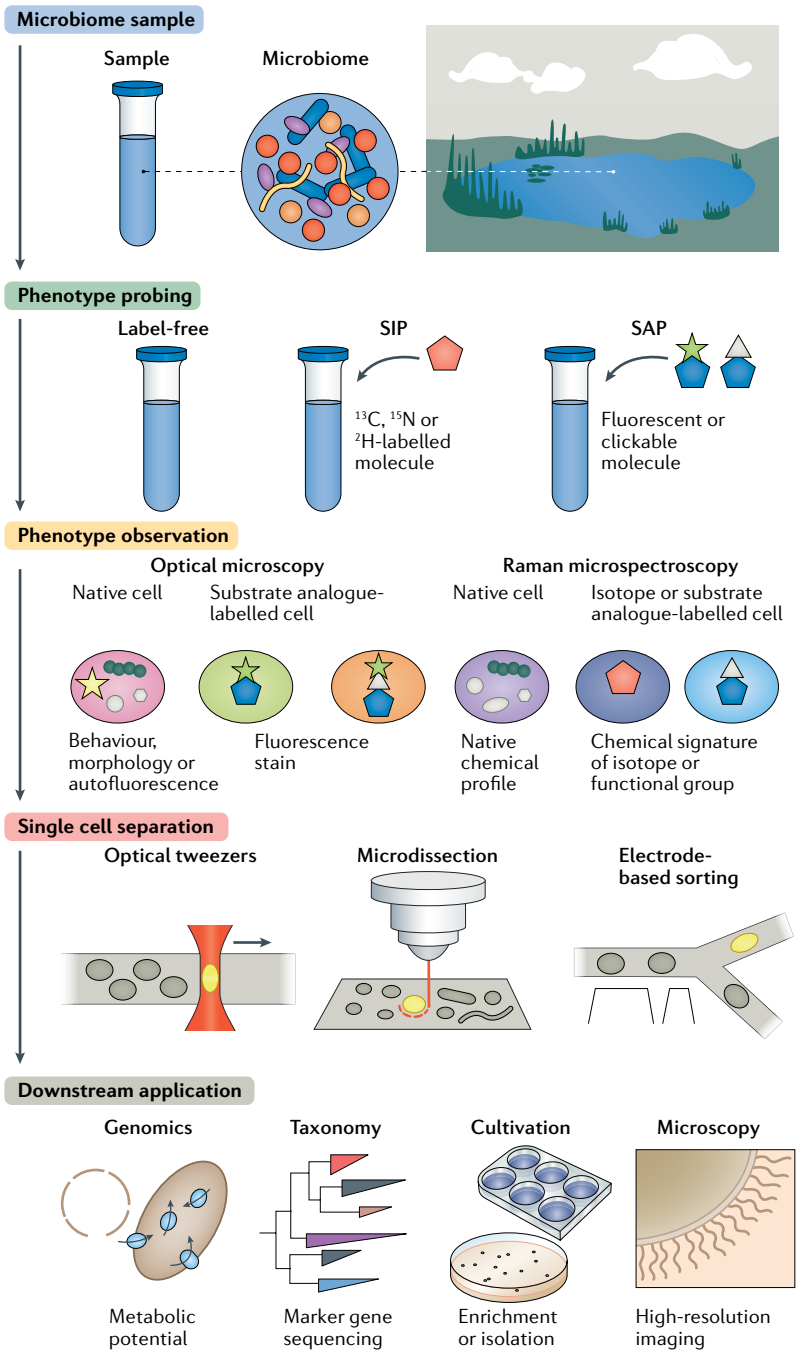


Fig. 2 | **Next-generation physiology workflow to study microorganisms.** A microbiome sample is obtained using minimally invasive protocols, and a phenotype of interest is detected using non-destructive methodology, for example by light or fluorescence microscopy or Raman microspectroscopy. Label-free approaches are directed at intrinsic properties of a cell, including chemotactic behaviour, 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 analogue probing (SAP) uses 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 FIG. 3.

enabling microbiologists to bridge the gap between historically separated fields in microbiome research (FIG. 1). Although 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 analyse 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: non-destructive phenotype observation; sorting of the observed cell based on its phenotype; and 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 s 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

Box 1 | **The chemistry underlying many next-generation 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 (E_0, excitation energy) and inelastically scattered (re-emitted) photons are analysed (E, emitted energy). 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 (see the figure, part **a**). In very rare cases (one in every 10^6–10^8 photons), the wavelength of a scattered photon is shifted compared with 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 with 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 with 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 analysing cells with high levels of autofluorescence. Several techniques are available for signal enhancement and faster acquisition times, including surface-enhanced or tip-enhanced Raman spectroscopy, stimulated Raman scattering, coherent anti-Stokes Raman spectroscopy and resonance Raman spectroscopy[67,150–155]. Although these advanced Raman microspectroscopy techniques have sporadically been applied to microbial isolates[151,152,156–159] 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.

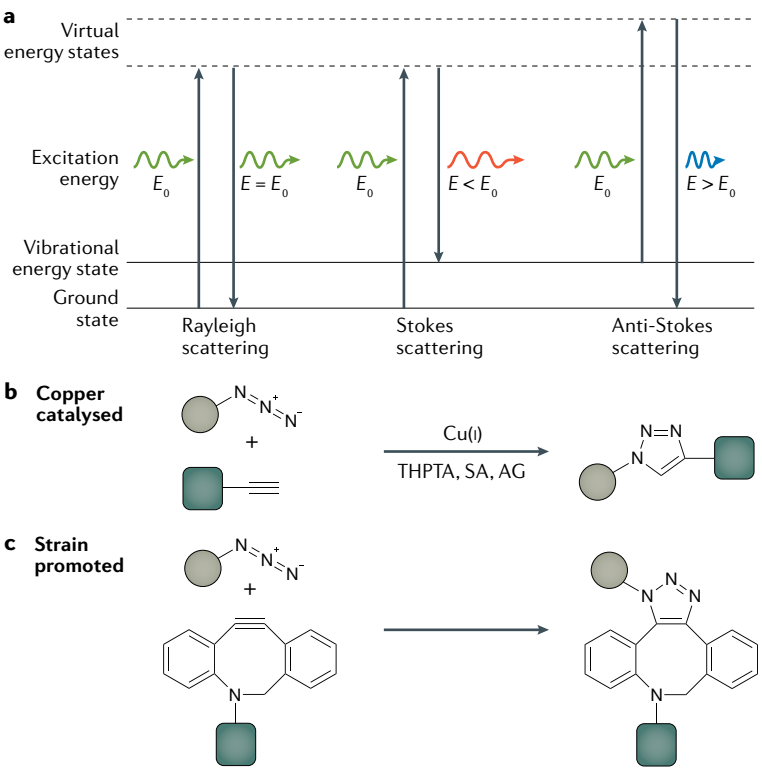
Azide–alkyne click chemistry

Click chemistry refers to any reaction that creates heteroatom links and that is modular and easy to perform and features fast kinetics, high chemoselectivity and stereoselectivity, as well as very high yields[160]. Although many reaction types fulfil these criteria[81,83,161], 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 labelling reactions yield triazole conjugates through azide–alkyne click chemistry: a Cu(I)-catalysed version that ligates an azide with a terminal alkyne (see the figure, part b); and a metal-free, strain-promoted reaction that links a highly reactive (strained) cyclooctyne-containing molecule (for example, dibenzocyclooctyne) with a reporter azide[81,83,162,163] (see the figure, part c).

In Cu(I)-catalysed click reactions, chelating ligands for copper (such as Tris[(1-hydroxypropyl-1*H*-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 (SA) maintains copper in the catalytically active Cu(I) state. To avoid protein cross linking by by-products of ascorbate oxidation, aminoguanidine (AG) is added to the reaction mix. Fluorescent dyes containing copper-chelating picolyl motifs raise the effective concentration of Cu(I) at the reaction site[164], 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)-catalysed click reactions. However, strain-promoted click chemistry can be accompanied by non-specific 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 non-specific labelling, 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 (totalling ~$500 for the clickable substrate analogue and dye as well as all necessary reagents[117]) and a small number of working steps. Labelling and washing protocols are well established and can be completed in 1 h (copper-catalysed click) to 3 h (strain-promoted click)[117]. 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[164] and fluorogenic 'turn-on' azide probes[165], 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[42,46,111–114,117], ethanol-fixed[46] or intact, not chemically fixed[42,46,111] cells.



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 labelled substrates into cells through formation of silver grains after exposure to a photographic emulsion. MAR is limited in its widespread 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 analyses them by time-of-flight mass spectrometry. nanoSIMS provides unrivalled sensitivity and spatial resolution but has very low throughput and destroys the sample.

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

Next-generation physiology
Any approach enabling study into 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.

Click chemistry
A 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.

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 owing to isotope incorporation; FIG. 2) can be separated by Raman-activated cell sorting (RACS). 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 s per spectrum currently limit the throughput of RACS compared with 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 is 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 optimization for each sample type and thorough testing by comparing the in situ community composition with 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), 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 (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. Multiple displacement amplification can lead to uneven genome coverage, genome rearrangements including chimaera 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 culture of cells separated from a sample based on their phenotype has not yet been achieved. 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 behaviour 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 also provides insights into dynamic cellular processes. Phenotypic observations by optical microscopy include the formation of spores, storage compounds, cellular segmentation, the behavioural responses of cells to external stimuli (for example, aerotaxis, chemotaxis, magnetotaxis or phototaxis) 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 autofluorescent 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 presents 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 cells, which requires the incorporation of chemical reporters to be tracked on a single cell level.

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			
Behavioural reaction to external stimuli	Aerotaxis, chemotaxis, magnetotaxis or phototaxis	NA	Magnetic enrichment and single cell genomics of magnetotactic bacteria[179]
Cofactors	Cofactor F_{420} in methanogenic pure and enrichment cultures[54]	Cofactor F_{420} in an ammonia-oxidizing archaeon[180]	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	Endospore detection by differential interference contrast microscopy	*Bacillus cereus* spores[181]	–[a]
Extracellular polymeric substance	Stains for extracellular DNA, proteins or polysaccharides[144,145]	Proteins and polysaccharides in biofilm matrix[59,60]	–[a]
Carbon storage	NA	Glycogen, polyhydroxyalkanoate and polyhydroxybutyrate in waste-water sludge bacteria[182,183]	–[a]
Cytochromes	NA	Cytochrome *c* in nitrifiers[183], anammox bacteria[183] and *Beggiatoa* spp.[184]; cytochrome redox state[185]	Automated RACS of cytochrome *c*-rich cells from a marine enrichment culture[33]
Magnetosomes	NA	Magnetotactic bacteria containing magnetite and greigite[186]	–[a]
Phosphate storage	NA	Orthophosphate and polyphosphate in cultured[186] and environmental[182,183,186] bacteria	–[a]
Sulfur inclusions	NA	Polysulfides in sulfur-oxidizing *Beggiatoa* spp.[184]; cyclo-octasulfur in pure cultures[186] and bacterial symbionts of flatworms[187]	–[a]
Stable isotope probing (with substrate or heavy water)			
^{2}H	NA	Naphthalene and glucose degradation by *Pseudomonas* spp. and *Escherichia coli*[57]	–[a]
^{13}C	NA	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	NA	$^{15}N_2$ fixers in soil[63]; ammonia, nitrite and N_2 assimilation in freshwater bacteria[156]	–[a]
$^{2}H_2O$	NA	Mucin degraders in mouse gut microbiome[33]; cellulose degraders[128]; detection of antibiotic-resistant bacteria in freshwater[36]; degraders of organic matter in groundwater[188]	Manual sorting using optical tweezers, followed by 16S rRNA gene sequencing[35]; automated sorting using optical tweezers on a microfluidic platform, followed by metagenomics[33]
$H_2{}^{18}O$	NA	–[a]	–[a]
Substrate analogue probing			
Fluorescent analogues	Glucose uptake in rumen[76]; xylan and lamarin uptake by bacterioplankton[77]; fluorescent amino acids[75]; fluorescent cellulose[78]	–[a]	FACS and 16S rRNA gene sequencing and single cell genomics of cells taking up fluorescent glucose[76] or polysaccharides[77], respectively
Non-canonical substrates	Clickable nucleosides[129,130,137], L-amino acids[46,111–114,118], D-amino acids[80], sugars[116,136] and fatty acids[131,132]	Alkyne-containing amino acids, nucleosides, sugars and fatty acids visualized by SRS[67,68,88]	FACS followed by 16S rRNA gene sequencing[42,46]
Activity-based and affinity-based protein profiling	Ammonia monooxygenases, antibiotic-reactive proteins, ATPases, ATP-binding proteins, cellulases, cytochromes, fatty acid synthases, glycoside hydrolases, lipases, redox-reactive proteins and vitamin transporters[138,140,141]	–[a]	FACS separation and 16S rRNA gene sequencing of β-glucuronidase active cells[143]

Anammox, anaerobic ammonium oxidation; FACS, fluorescence-activated cell sorting; NA, not applicable; RACS, Raman-activated cell sorting; rRNA, ribosomal RNA; SRS, stimulated Raman scattering. [a]Application feasible but not yet demonstrated.

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 (ribosomal RNA 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 (for example, DNA, RNA, proteins and lipids).

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

Isotope probing

Isotope probing approaches involve the incubation of a microbial sample with an isotopically labelled reactant (substrate or water) and track its incorporation into cellular components, identifying anabolically active microorganisms. Incubation with an isotopically labelled 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 labelling 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 labelled cells are identified by characteristic peak shifts in their Raman spectrum owing 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} owing 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 labelled with heavy isotopes can be used to identify all members of a microbial community that can specifically assimilate the substrate. In addition, isotopically labelled 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 labelled compound, which could alter natural substrate concentrations and change the composition of the incubated communities.

Detection of ^{13}C and ^{15}N-labelled cells has been achieved by Raman microspectroscopy in multispecies communities and has been successfully combined with FISH[59,60], genome sequencing[37,60] or cultivation[60]. An initial study detected labelled cells after incubation with ^{13}C-glucose based on peak shifts in the Raman spectrum owing to incorporation of the heavy isotope[61]. Subsequently, substrate SIP–Raman microspectroscopy was used to investigate the niche differentiation of naphthalene degraders in groundwater 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-labelled 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-labelled cells from complex samples[37,39], demonstrating the importance of this approach to ecophysiology research (TABLE 1).

Using stimulated Raman scattering microscopy, ^{2}H-containing nucleosides, amino acids and fatty acids can be used to visualize DNA-synthesizing, RNA-synthesizing, protein-synthesizing 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

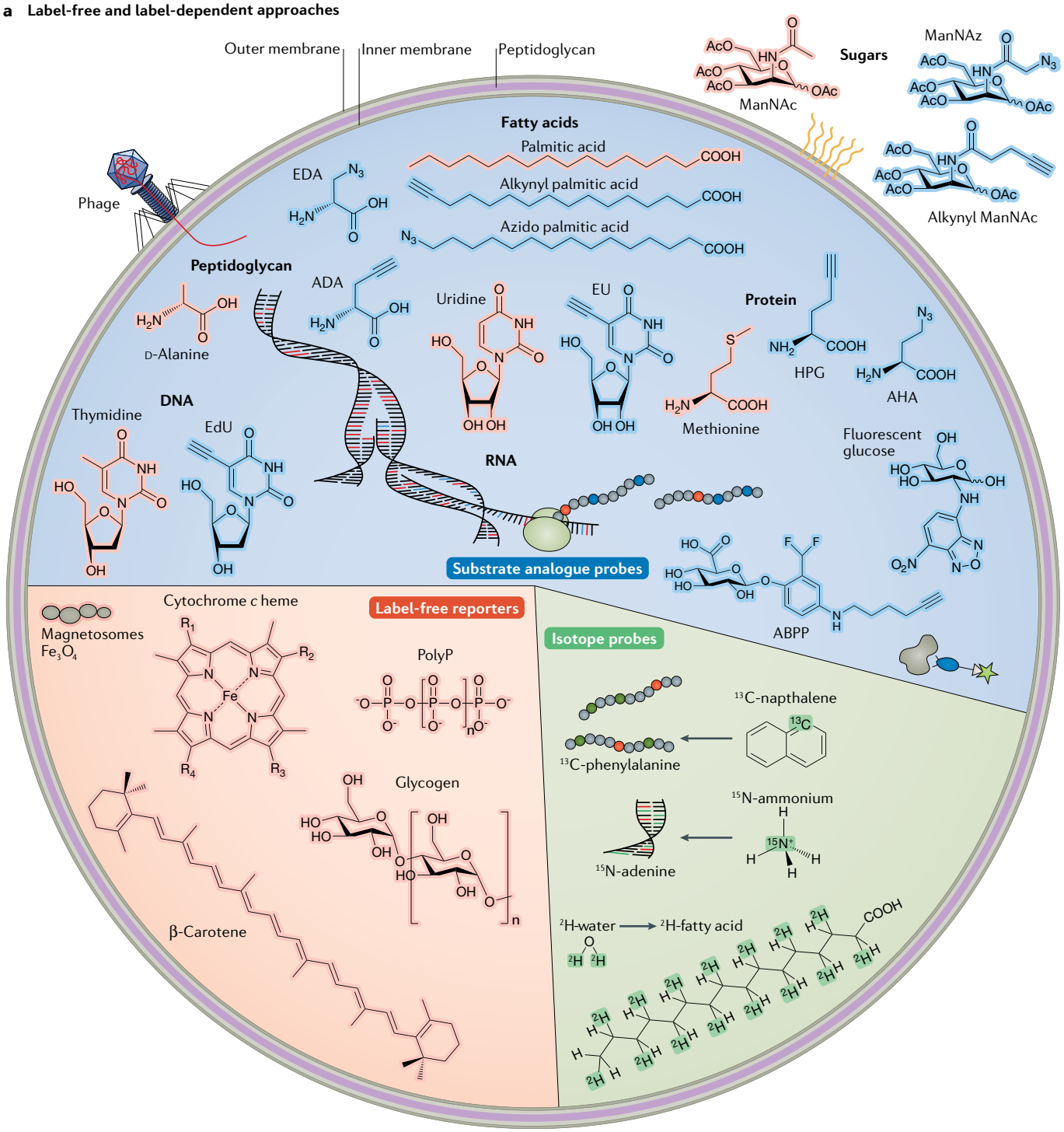
Isotope-labelling approaches with heavy water (^2H_2O or $H_2{}^{18}O$) aim to identify all members of a community that are anabolically active[36,55–57], rather than those members involved in specific transformations. Heavy water SIP enables testing of cellular activity under either close to in situ 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 substrates of a microorganism, it does not interfere with the natural substrate pool and it is inexpensive. SIP with heavy water commonly requires an experimental set up in which a portion of regular water (ideally 30–50% (REF.[55])) is replaced with heavy water to achieve high-enough labelling 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, labelling of cells with ^2H_2O is superior to $H_2{}^{18}O$ labelling because hydrogen from water readily exchanges with the NADPH pool of the cells, the main source of hydrogen 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 of the cellular chemical profile (FIG. 3). ^2H_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] (TABLE 1).

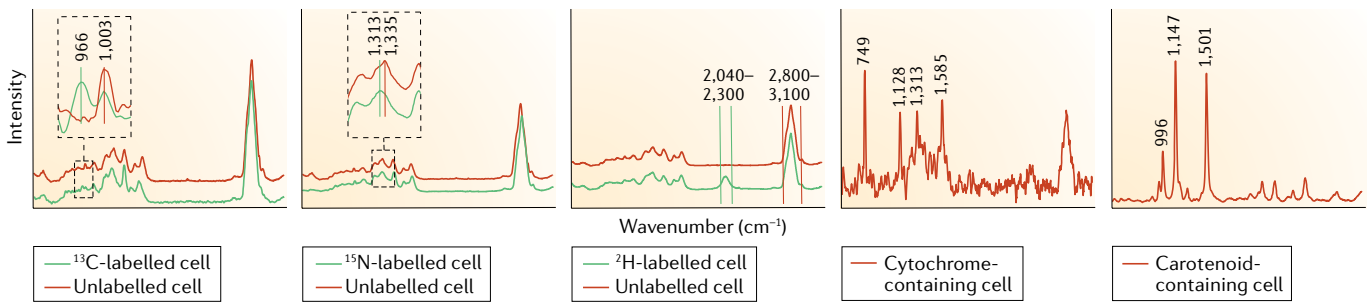
Substrate analogue probing

An alternative approach to SIP is to incubate a sample with a synthetic compound that is a structural and/or functional analogue of a naturally occurring molecule. Such experiments are either directed towards anabolic processes, such as non-canonical substrate labelling, or towards metabolic reactions catalysed by specific enzymes or enzyme families, such as fluorescent substrate analogue labelling or activity-based protein profiling (ABPP) and affinity-based protein profiling. To contrast these approaches with SIP, we here introduce the term substrate analogue probing (SAP). An important

a Label-free and label-dependent approaches



b Raman spectra of cells



◀ Fig. 3 | **Reporters and their associated Raman spectral fingerprints in microbial next-generation physiology. a** | Label-free reporters are produced by the cell and do not require addition by the researcher. Substrate analogue probes (blue) are traceable compounds that are amenable to bioorthogonal labelling and that are incorporated instead of their native counterparts (red) into biomass by the cell of interest after addition to the microbiome sample. Many substrate analogue probes contain azide or terminal alkyne groups, which can be conjugated to a fluorescent dye for detection by click chemistry. Finally, isotope probes (green) can be added to a sample to trace the uptake and incorporation of isotopically labelled compounds. **b** | Incorporation of stable isotopes into biomass leads to spectral shifts towards lower wavenumbers in the spectrum of labelled cells compared with unlabelled 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 left to right, 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-labelled substrate analogues and some label-free compounds, for example cytochromes and carotenoids, have distinct Raman spectral fingerprints that also can be used for phenotype detection[38,68,88,178]. TABLE 1 includes detailed information on the application of all depicted reporters and references to the primary literature. ABPP, activity-based protein profiling; ADA, azido-D-alanine; AHA, L-azidohomoalanine; EDA, ethynyl-D-alanine; EdU, 5-ethynyl-2′-deoxyuridine; EU, 5-ethynyl-uridine; HPG, l-homopropargylglycine; ManNAc, *N*-acetylmannosamine; ManNaz, *N*-azidoacetylmannosamine. Part **b** courtesy of Anthony Kohtz, Montana State University.

advantage of SAP compared with 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, are comparatively easy to perform.

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 multispecies communities. Examples of fluorescent SAP include the use of fluorescent cobalamin analogues to demonstrate the uptake of this vitamin into bacteria, worms and plants[74], or the use of fluorescently labelled D-amino acids to visualize regions of active peptidoglycan synthesis in cell walls of different bacterial pure cultures[75]. Furthermore, the combination of fluorescent SAP 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] and 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 labelling 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 labelling, ABPP and affinity-based protein profiling, overcome these problems by making the detection of these molecules (for example, by dye staining) independent of the labelling chemistry by using substrate analogues. Examples of this are the use of clickable vitamin B12 (REF.[79]) or D-amino acids[80] rather than fluorescently labelled vitamins or D-amino acids.

Non-canonical SAP

Non-canonical molecules are synthetic structural analogues 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. These reactions are chemical transformations that do not interact with functional groups present in naturally occurring molecules, have no or only minimal by-products 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 labelling reaction is to use stimulated Raman scattering to trace them inside the cell[68,88].

Bioorthogonal labelling approaches are well established in the study of bacterial[89–93] and eukaryotic[94–97] model organisms. In multispecies 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%)[98–100]. 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. Labelling of newly translated proteins with synthetic amino acids can be accomplished through bioorthogonal non-canonical amino acid tagging (BONCAT)[101–103]. BONCAT achieves the co-translational labelling of proteins by exploiting the substrate promiscuity of aminoacyl-tRNA synthetases, which are enzymes responsible for catalysing 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[103–105]. Since its inception[102], BONCAT has been used to study protein synthesis in a range of microbial pathogens[89,106–110] and was recently applied in several complex samples, including marine and freshwater sediments[46,111], surface[112,113] and deep[114] seawater, soil[42] and an oral biofilm[111]. In these studies, BONCAT was applicable to cultured and uncultured members of at least 20 archaeal and bacterial phyla[42,46,111–117] as well as bacteriophages[118] and eukaryotic viruses[118,119]. Because of their structural similarity

Clickable
A molecule carrying a functional group that is amenable to azide–alkyne click chemistry.

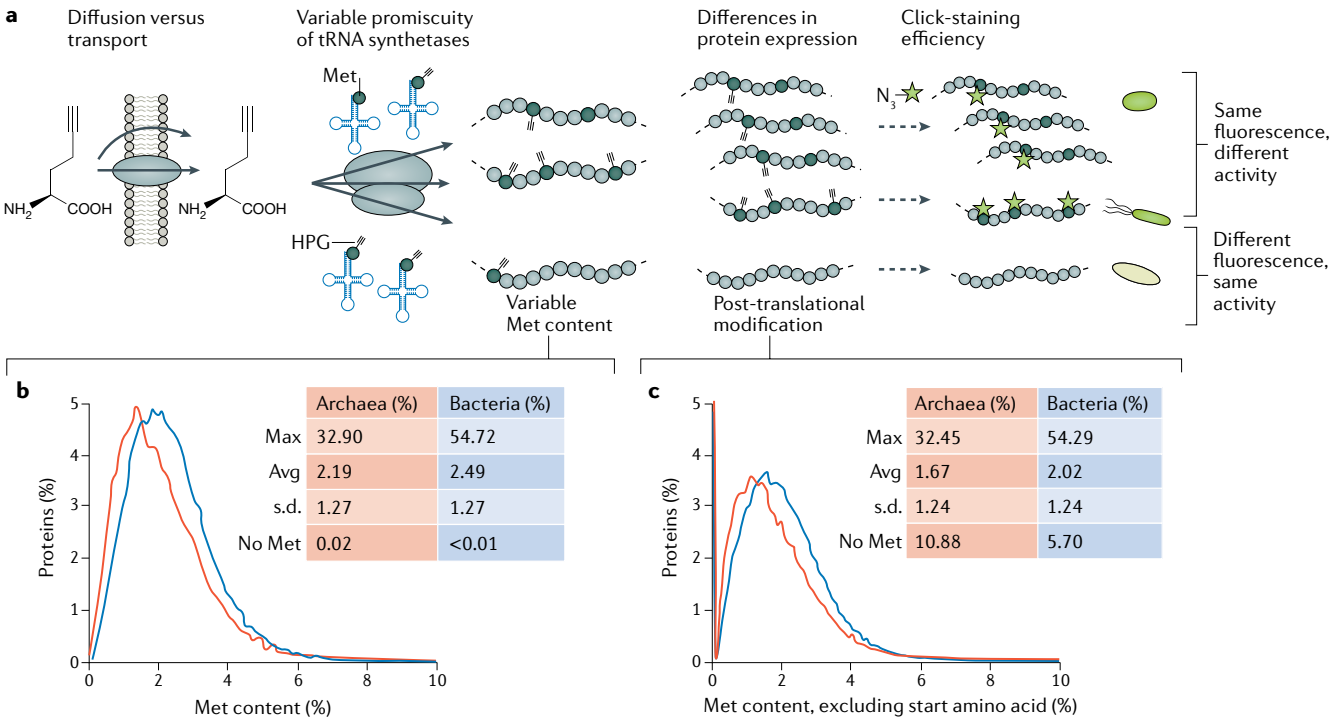
Bioorthogonal reaction
A reaction that does not interfere with biological processes; it can be used to label a cell or molecule with a reporter.

to Met and their low activation rate by methionyl-tRNA synthetase[103], HPG and AHA have only small effects on rates of protein synthesis and degradation in *Escherichia coli*[120] and mammalian cells[95,102,120], as well as on protein tertiary structure[121]. BONCAT correlates well with other independent proxies of growth, such as the incorporation of $^{15}NH_4^+$ into single cells visualized by nanoSIMS[111], ^{35}S-Met uptake as measured by MAR[112] or incorporation of ^{3}H-leucine into bulk biomass measured by scintillation counting[114]. 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)[122] or over more than two generations, growth rates of some bacterial cultures are negatively affected[111]. Therefore, low concentrations of AHA or HPG (nanomolar to micromolar 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[46,113,117]) to avoid excessive substitution of Met, which could lead to non-functional 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

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 (see the figure, part **a**). 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. 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 L-methionine (Met):HPG and 1:390 for Met:AHA[103]), 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 labelling intensity. Furthermore, variations in the Met content of proteins and 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 multispecies samples[6,7,166]. Lastly, 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-labelling intensity. Similar limitations probably exist for other non-canonical substrate analogue probing approaches capable of labelling DNA, lipids or peptidoglycan but are currently untested.

Analysis of genomes deposited in the National Center for Biotechnology Information (NCBI) Reference Sequence (RefSeq) database reveals a range in the use of Met in proteins (see 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. More than 99.9% of these proteins contain Met, suggesting that virtually all proteins are in principle amenable to labelling 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 (see the figure, part **c**). For these calculations, only one genome from each species was analysed, 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. Avg, average; Max, maximum; s.d., standard deviation.



	Archaea (%)	Bacteria (%)
Max	32.90	54.72
Avg	2.19	2.49
s.d.	1.27	1.27
No Met	0.02	<0.01

	Archaea (%)	Bacteria (%)
Max	32.45	54.29
Avg	1.67	2.02
s.d.	1.24	1.24
No Met	10.88	5.70

HPG are misrecognized for Met by enzymes other than methionyl-tRNA synthetase; 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 catalysed reporter deposition FISH (BONCAT–FISH[111] or BONCAT–CARD–FISH[46]), active cells can be identified, thus revealing taxonomy–function relationships and co-localization patterns of taxonomically identified active cells[46,111,113,114]. 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 labelled with BONCAT[42]. This result was in stark contrast to previous, more labour-intensive studies, such as DNA-SIP[123] or labelling with the thymidine surrogate 5-bromo-2′-deoxyuridine (BrdU)[124], which suggested that up to 95% of cells in soil are inactive at a given time. Recent studies that employed quantitative SIP methodology are consistent with findings by BONCAT–FACS[125]. This discrepancy can be explained by the inherent biases associated with BrdU labelling[124,126,127].

The ability to combine bioorthogonal labelling 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[46,111]. BONCAT is particularly useful for studying non-assimilatory pathways or if isotope-labelled 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[46,111]. 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-labelled cells by RACS[33,36,55,128]. Neither BONCAT nor ^2H_2O-Raman microspectroscopy can disentangle whether cell labelling is owed to direct substrate uptake or metabolic cross feeding, but measuring multiple samples over the course of an incubation may help reconstruct metabolic interactions and population dynamics within communities.

Targeting non-proteinaceous cell components and viruses. BONCAT is arguably the most sensitive non-canonical substrate labelling approach owing 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 straightforward 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[129]. Click chemistry-based detection of EdU can be performed in 1 h and yields cell labelling rates comparable with the more biased and experimentally more complex BrdU-labelling approach[124,126,127]. By contrast, the alkyne-carrying uridine analogue 5-ethynyl-uridine is incorporated into RNA due the promiscuity of RNA polymerase[130] but has not yet been used on complex samples.

Other bioorthogonal labelling approaches use azide-modified or alkyne-modified fatty acids[131,132], D-amino acids[80,133,134] or sugars[116,135,136] to label the lipid membrane, peptidoglycan layer or cell surface polysaccharides, respectively (FIG. 3; TABLE 1). Because capacities for lipid and cell wall biosynthesis as well as the use of peptidoglycan and cell wall modifications, differ widely across the tree of life, these approaches lack the general applicability of protein labelling via BONCAT. Although some of these substrate analogues 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 labelling to cultured virus–host models of pathogenic[119] and environmental relevance are also very promising[118,137]. 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[137]. Furthermore, BONCAT was recently used to quantify in situ marine viral production rates by fluorescence staining[118]. 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 and of the viral impacts on elemental cycling through the release of nutrients from lysed cells, and might help to identify new virus–host relationships[118,137].

Activity-based protein profiling

A complementary set of SAP techniques targets catabolic rather than anabolic functions of the cell. 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[138–141] (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[142], 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 analogues 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 analogue to the enzyme, photoactivatable groups can be used[140,141].

ABPP 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[143]. 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[143]. The study 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 ABPP and affinity-based protein profiling lies in the challenge to design a substrate analogue that reacts and binds to only one particular enzyme or enzyme class; however, substrate analogues are already available for a wide variety of enzyme classes[138,140,141] (TABLE 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 matrices 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[144,145]. Furthermore, most commercially available stains of metabolic activity have been shown to be inapplicable to complex samples for various 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

Box 3 | **Alternative cell-staining approaches**

‘Vitality’ and ‘viability’ dyes

Advertised as ‘vitality’ and ‘viability’ stains (see the 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 of these stains have some limitations that restrict their use in many complex samples, resulting in rough estimates of vital or viable microorganisms at best[167,168]. 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[52,169]. 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 the phylum Saccharibacteria (formerly known as TM7) and the candidate phylum ‘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)	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[170,171]; counts of CTC^+ cells were 2–100 times lower than microautoradiography counts[172–174]	Practically unsuitable for structurally complex sample types (such as sediments, soils or biofilms) because cell extraction reduces cell activity; general applicability to physiologically and 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 with independent measures of activity or cell growth other than the formation of colony-forming units
Live–dead stains (for example, LIVE/DEAD™ BacLight™, SYTOX Red Dead, FUN®-1 or ReadyProbes)	Mixture of a cell-permeable (for example, SYTO TM9) and membrane-impermeable DNA stain (for example, propidium iodide)	Not useful or yield inaccurate results for cells with hard to permeate cell walls or membranes (for example, spores; Gram-positive versus Gram-negative bacteria)[167,175,176]	Background fluorescence, bleaching, fluorescence resonance energy transfer between dyes, double staining and a decrease in vitality during staining[167,175,177]	

complement more established methodologies including cultivation, enzyme characterization and meta-omics. Once appropriate instrumentation becomes more widely available and experimental protocols are 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 microorganisms at a hitherto unprecedented speed[33,42,46,55]. These and other single cell-targeted approaches will be aided by the anticipated progress of microfluidics for culture-independent assays. Most currently available microfluidics approaches still depend on the ability to grow microorganisms on-chip, use genetically encoded fluorescence reporters or target the genotype rather than the phenotype[10,13,146–149].

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 laboratory-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.

Published online 13 February 2020

1. Gilbert, J. A. et al. Current understanding of the human microbiome. *Nat. Med.* **24**, 392–400 (2018).
2. Flemming, H. C. & Wuertz, S. Bacteria and archaea on Earth and their abundance in biofilms. *Nat. Rev. Microbiol.* **17**, 247–260 (2019).
3. Castelle, C. J. & Banfield, J. F. Major new microbial groups expand diversity and alter our understanding of the tree of life. *Cell* **172**, 1181–1197 (2018).
4. Karst, S. M. et al. Retrieval of a million high-quality, full-length microbial 16S and 18S rRNA gene sequences without primer bias. *Nat. Biotechnol.* **36**, 190–195 (2018).
5. Hug, L. A. et al. A new view of the tree of life. *Nat. Microbiol.* **1**, 16048 (2016).
6. Ackermann, M. A functional perspective on phenotypic heterogeneity in microorganisms. *Nat. Rev. Microbiol.* **13**, 497–508 (2015).
7. Lidstrom, M. E. & Konopka, M. C. The role of physiological heterogeneity in microbial population behavior. *Nat. Chem. Biol.* **6**, 705–712 (2010).
8. Ansorge, R. et al. Functional diversity enables multiple symbiont strains to coexist in deep-sea mussels. *Nat. Microbiol.* **4**, 2487–2497 (2019).
9. Gruber-Dorninger, C. et al. Functionally relevant diversity of closely related nitrospira in activated sludge. *ISME J.* **9**, 643–655 (2015).
10. Vasdekis, A. E. & Stephanopoulos, G. Review of methods to probe single cell metabolism and bioenergetics. *Metab. Eng.* **27**, 115–135 (2015).
11. Rosenthal, K., Oehling, V., Dusny, C. & Schmid, A. Beyond the bulk: disclosing the life of single microbial cells. *FEMS Microbiol. Rev.* **41**, 751–780 (2017).
12. Nai, C. & Meyer, V. From axenic to mixed cultures: technological advances accelerating a paradigm shift in microbiology. *Trends Microbiol.* **26**, 538–554 (2018).
13. Oomen, P. E., Aref, M. A., Kaya, I., Phan, N. T. N. & Ewing, A. G. Chemical analysis of single cells. *Anal. Chem.* **91**, 588–621 (2019).
14. Taheri-Araghi, S., Brown, S. D., Sauls, J. T., McIntosh, D. B. & Jun, S. Single-cell physiology. *Annu. Rev. Biophys.* **44**, 123–142 (2015).
15. Lloyd, K. G., Steen, A. D., Ladau, J., Yin, J. & Crosby, L. Phylogenetically novel uncultured microbial cells dominate earth microbiomes. *mSystems* **3**, e00055–18 (2018).
16. Steen, A. D. et al. High proportions of bacteria and archaea across most biomes remain uncultured. *ISME J.* **13**, 3126–3130 (2019).
17. Nielsen, J. L., Christensen, D., Kloppenborg, M. & Nielsen, P. H. Quantification of cell-specific substrate uptake by probe-defined bacteria under in situ conditions by microautoradiography and fluorescence in situ hybridization. *Env. Microbiol.* **5**, 202–211 (2003).
18. Lee, N. et al. Combination of fluorescent in situ hybridization and microautoradiography — a new tool for structure–function analyses in microbial ecology. *Appl. Env. Microbiol.* **65**, 1289–1297 (1999).
19. McGlynn, S. E., Chadwick, G. L., Kempes, C. P. & Orphan, V. J. Single cell activity reveals direct electron transfer in methanotrophic consortia. *Nature* **526**, 531–535 (2015).
20. Kopf, S. H. et al. Heavy water and ^{15}N labelling with nanoSIMS analysis reveals growth rate-dependent metabolic heterogeneity in chemostats. *Env. Microbiol.* **17**, 2542–2556 (2015).
21. Hungate, B. A. et al. Quantitative microbial ecology through stable isotope probing. *Appl. Env. Microbiol.* **81**, 7570–7581 (2015).
22. Ziels, R. M., Sousa, D. Z., Stensel, H. D. & Beck, D. A. C. DNA-SIP based genome-centric metagenomics identifies key long-chain fatty acid-degrading populations in anaerobic digesters with different feeding frequencies. *ISME J.* **12**, 112–123 (2018).
23. Eyice, O. et al. SIP metagenomics identifies uncultivated methylophilaceae as dimethylsulphide degrading bacteria in soil and lake sediment. *ISME J.* **9**, 2336–2348 (2015).
24. Fortunato, C. S. & Huber, J. A. Coupled RNA-SIP and metatranscriptomics of active chemolithoautotrophic communities at a deep-sea hydrothermal vent. *ISME J.* **10**, 1925–1938 (2016).
25. Doud, D. F. R. & Woyke, T. Novel approaches in function-driven single-cell genomics. *FEMS Microbiol. Rev.* **41**, 538–548 (2017).
26. Singer, E., Wagner, M. & Woyke, T. Capturing the genetic makeup of the active microbiome in situ. *ISME J.* **11**, 1949–1963 (2017).
27. Yuan, X. et al. Effect of laser irradiation on cell function and its implications in Raman spectroscopy. *Appl. Env. Microbiol.* **84**, e02508–17 (2018).
28. He, Y., Wang, X., Ma, B. & Xu, J. Ramanome technology platform for label-free screening and sorting of microbial cell factories at single-cell resolution. *Biotechnol. Adv.* **37**, 107388 (2019).
29. Harrison, J. P. & Berry, D. Vibrational spectroscopy for imaging single microbial cells in complex biological samples. *Front. Microbiol.* **8**, 675 (2017).
30. Lorenz, B., Wichmann, C., Stockel, S., Rosch, P. & Popp, J. Cultivation-free Raman spectroscopic investigations of bacteria. *Trends Microbiol.* **25**, 413–424 (2017).
31. Zhang, Q. et al. Towards high-throughput microfluidic Raman-activated cell sorting. *Analyst* **140**, 6163–6174 (2015).
32. Huang, W. E., Ward, A. D. & Whiteley, A. S. Raman tweezers sorting of single microbial cells. *Env. Microbiol. Rep.* **1**, 44–49 (2009).
33. Lee, K. S. et al. An automated Raman-based platform for the sorting of live cells by functional properties. *Nat. Microbiol.* **4**, 1035–1048 (2019).
This study describes the development and application of the first microfluidic platform for automated Raman-activated sorting of isotope-labelled microorganisms. SIP–RACS and metagenomics are used to characterize mucin-degrading bacteria from a mouse colon.
34. McIlvenna, D. et al. Continuous cell sorting in a flow based on single cell resonance Raman spectra. *Lab Chip* **16**, 1420–1429 (2016).
35. Wang, X. et al. Raman-activated droplet sorting (RADS) for label-free high-throughput screening of microalgal single-cells. *Anal. Chem.* **89**, 12569–12577 (2017).
36. Song, Y. et al. Raman-deuterium isotope probing for in-situ identification of antimicrobial resistant bacteria in Thames river. *Sci. Rep.* **7**, 16648 (2017).
37. Jing, X. et al. Raman-activated cell sorting and metagenomic sequencing revealing carbon-fixing bacteria in the ocean. *Env. Microbiol.* **20**, 2241–2255 (2018).
38. Song, Y. et al. Single-cell genomics based on Raman sorting reveals novel carotenoid-containing bacteria in the Red Sea. *Microb. Biotechnol.* **10**, 125–137 (2016).
This study uses label-free RACS and single cell genomics to characterize as yet uncultured carotenoid-containing microorganisms.
39. Wang, Y. et al. Raman activated cell ejection for isolation of single cells. *Anal. Chem.* **85**, 10697–10701 (2013).
40. Rinke, C. et al. Obtaining genomes from uncultivated environmental microorganisms using FACS-based single-cell genomics. *Nat. Protoc.* **9**, 1038–1048 (2014).
41. Rinke, C. et al. Insights into the phylogeny and coding potential of microbial dark matter. *Nature* **499**, 431–437 (2013).
42. Couradeau, E. et al. Probing the active fraction of soil microbiomes using BONCAT–FACS. *Nat. Commun.* **10**, 2770 (2019).
43. Morono, Y., Terada, T., Kallmeyer, J. & Inagaki, F. An improved cell separation technique for marine subsurface sediments: applications for high-throughput analysis using flow cytometry and cell sorting. *Env. Microbiol.* **15**, 2841–2849 (2013).
44. Eichorst, S. A. et al. Advancements in the application of nanoSIMS and Raman microspectroscopy to investigate the activity of microbial cells in soils. *FEMS Microbiol. Ecol.* **91**, fiv106 (2015).

45. Lunau, M., Lemke, A., Walther, K., Martens-Habbena, W. & Simon, M. An improved method for counting bacteria from sediments and turbid environments by epifluorescence microscopy. *Env. Microbiol.* **7**, 961–968 (2005).
46. Hatzenpichler, R. et al. Visualizing in situ translational activity for identifying and sorting slow-growing archaeal–bacterial consortia. *Proc. Natl Acad. Sci. USA* **113**, E4069–E4078 (2016).
This study uses BONCAT–FISH and BONCAT–FACS in combination with 16S rRNA gene sequencing to characterize translationally active methane-oxidizing microbial consortia in deep-sea sediments.
47. Hao, L. et al. Novel prosthecate bacteria from the candidate phylum acetothermia. *ISME J.* **12**, 2225–2237 (2018).
48. Clingenpeel, S., Schwientek, P., Hugenholtz, P. & Woyke, T. Effects of sample treatments on genome recovery via single-cell genomics. *ISME J.* **8**, 2546–2549 (2014).
49. Clingenpeel, S., Clum, A., Schwientek, P., Rinke, C. & Woyke, T. Reconstructing each cell's genome within complex microbial communities—dream or reality? *Front. Microbiol.* **5**, 771 (2014).
50. Bowers, R. M. et al. Impact of library preparation protocols and template quantity on the metagenomic reconstruction of a mock microbial community. *BMC Genomics* **16**, 856 (2015).
51. Cross, K. L. et al. Targeted isolation and cultivation of uncultivated bacteria by reverse genomics. *Nat. Biotechnol.* **37**, 1314–1321 (2019).
This study uses genome-informed antibody engineering to sort individual TM7 and SR1 cells from oral microbiome samples and regrow them in cultivation media.
52. Kalyuzhnaya, M. G., Lidstrom, M. E. & Chistoserdova, L. Real-time detection of actively metabolizing microbes by redox sensing as applied to methylotroph populations in Lake Washington. *ISME J.* **2**, 696–706 (2008).
This study demonstrates that, if carefully applied, redox sensing dyes can be used to sort metabolically active methylotrophic bacteria by FACS and bring sorted cells into enrichment culture.
53. Koch, C., Gunther, S., Desta, A. F., Hubschmann, T. & Muller, S. Cytometric fingerprinting for analyzing microbial intracommunity structure variation and identifying subcommunity function. *Nat. Protoc.* **8**, 190–202 (2013).
54. Lambrecht, J. et al. Flow cytometric quantification, sorting and sequencing of methanogenic archaea based on F420 autofluorescence. *Microb. Cell Fact.* **16**, 180 (2017).
55. Berry, D. et al. Tracking heavy water (D_2O) incorporation for identifying and sorting active microbial cells. *Proc. Natl Acad. Sci. USA* **112**, E194–E203 (2015).
This study for the first time combines heavy water labelling, RACS and 16S rRNA gene sequencing and uses this workflow to identify glucosamine-degrading and mucin-degrading bacteria from mouse caecal samples.
56. Kopf, S. H. et al. Trace incorporation of heavy water reveals slow and heterogeneous pathogen growth rates in cystic fibrosis sputum. *Proc. Natl Acad. Sci. USA* **113**, E110–E116 (2016).
57. Xu, J. et al. Raman deuterium isotope probing reveals microbial metabolism at the single-cell level. *Anal. Chem.* **89**, 13305–13312 (2017).
58. Wang, Y., Huang, W. E., Cui, L. & Wagner, M. Single cell stable isotope probing in microbiology using Raman microspectroscopy. *Curr. Opin. Biotechnol.* **41**, 34–42 (2016).
This review provides an excellent overview of the principle and potential applications of single cell-targeted SIP–Raman studies.
59. Huang, W. E. et al. Raman–FISH: combining stable-isotope Raman spectroscopy and fluorescence in situ hybridization for the single cell analysis of identity and function. *Env. Microbiol.* **9**, 1878–1889 (2007).
This study combines Raman microspectroscopy and FISH for the first time and uses it to identify ^{13}C-naphthalene degraders in groundwater and quantifies isotope incorporation into individual cells.
60. Huang, W. E. et al. Resolving genetic functions within microbial populations: in situ analyses using rRNA and mRNA stable isotope probing coupled with single-cell Raman–fluorescence in situ hybridization. *Appl. Env. Microbiol.* **75**, 234–241 (2009).
61. Huang, W. E., Griffiths, R. I., Thompson, I. P., Bailey, M. J. & Whiteley, A. S. Raman microscopic analysis of single microbial cells. *Anal. Chem.* **76**, 4452–4458 (2004).
62. Haider, S. et al. Raman microspectroscopy reveals long-term extracellular activity of Chlamydiae. *Mol. Microbiol.* **77**, 687–700 (2010).
63. Angel, R. et al. Application of stable-isotope labelling techniques for the detection of active diazotrophs. *Env. Microbiol.* **20**, 44–61 (2018).
64. Li, M. et al. Rapid resonance Raman microspectroscopy to probe carbon dioxide fixation by single cells in microbial communities. *ISME J.* **6**, 875–885 (2012).
65. Muller, A. L. et al. Bacterial interactions during sequential degradation of cyanobacterial necromass in a sulfidic arctic marine sediment. *Env. Microbiol.* **20**, 2927–2940 (2018).
66. Taylor, G. T. et al. Single-cell growth rates in photoautotrophic populations measured by stable isotope probing and resonance Raman microspectrometry. *Front. Microbiol.* **8**, 1449 (2017).
67. Hu, F., Shi, L. & Min, W. Biological imaging of chemical bonds by stimulated Raman scattering microscopy. *Nat. Methods* **16**, 830–842 (2019).
68. Wei, L. et al. Live-cell bioorthogonal chemical imaging: stimulated Raman scattering microscopy of vibrational probes. *Acc. Chem. Res.* **49**, 1494–1502 (2016).
69. Crespi, H. L., Conard, S. M., Uphaus, R. A. & Katz, J. J. Cultivation of microorganisms in heavy water. *Ann. N. Y. Acad. Sci.* **84**, 648–666 (1960).
70. Kselikova, V., Vitova, M. & Bisova, K. Deuterium and its impact on living organisms. *Folia Microbiol.* **64**, 673–681 (2019).
71. Zhang, X., Gillespie, A. L. & Sessions, A. L. Large D/H variations in bacterial lipids reflect central metabolic pathways. *Proc. Natl Acad. Sci. USA* **106**, 12580–12586 (2009).
72. Valentine, D. L., Sessions, A. L., Tyler, S. C. & Chidthaisong, A. Hydrogen isotope fractionation during H_2/CO_2 acetogenesis: hydrogen utilization efficiency and the origin of lipid-bound hydrogen. *Geobiology* **2**, 179–188 (2004).
73. Sessions, A. L., Jahnke, L. L., Schimmelmann, A. & Hayes, J. M. Hydrogen isotope fractionation in lipids of the methane-oxidizing bacterium *Methylococcus capsulatus*. *Geochim. Cosmochim. Acta* **66**, 3955–3969 (2002).
74. Lawrence, A. D. et al. Construction of fluorescent analogs to follow the uptake and distribution of cobalamin (vitamin B12) in bacteria, worms, and plants. *Cell Chem. Biol.* **25**, 941–951.e6 (2018).
75. Kuru, E. et al. In situ probing of newly synthesized peptidoglycan in live bacteria with fluorescent d-amino acids. *Angew. Chem. Int. Ed. Engl.* **51**, 12519–12523 (2012).
76. Tao, J. et al. Use of a fluorescent analog of glucose (2-NBDG) to identify uncultured rumen bacteria that take up glucose. *Appl. Env. Microbiol.* **85**, e03018–18 (2019).
77. Martinez-Garcia, M. et al. Capturing single cell genomes of active polysaccharide degraders: an unexpected contribution of verrucomicrobia. *PLoS One* **7**, e35314 (2012).
78. Doud, D. F. R. et al. Function-driven single-cell genomics uncovers cellulose-degrading bacteria from the rare biosphere. *ISME J.* https://doi.org/10.1038/s41396-019-0557-y (2019).
This study uses fluorescent substrate analogue probing, FACS of active cells and mini-metagenomics to identify thermophilic cellulose degraders.
79. Rosnow, J. J. et al. A cobalamin activity-based probe enables microbial cell growth and finds new cobalamin–protein interactions across domains. *Appl. Environ. Microbiol.* **84**, e00955–18 (2018).
80. Liechti, G. W. et al. A new metabolic cell-wall labelling method reveals peptidoglycan in *Chlamydia trachomatis*. *Nature* **506**, 507–510 (2014).
81. Best, M. D. Click chemistry and bioorthogonal reactions: unprecedented selectivity in the labeling of biological molecules. *Biochemistry* **48**, 6571–6584 (2009).
82. Devaraj, N. K. The future of bioorthogonal chemistry. *ACS Cent. Sci.* **4**, 952–959 (2018).
83. Sletten, E. M. & Bertozzi, C. R. Bioorthogonal chemistry: fishing for selectivity in a sea of functionality. *Angew. Chem. Int. Ed. Engl.* **48**, 6974–6998 (2009).
84. Griffin, R. J. The medicinal chemistry of the azido group. *Prog. Med. Chem.* **31**, 121–232 (1994).
85. Marchand, J. A. et al. Discovery of a pathway for terminal-alkyne amino acid biosynthesis. *Nature* **567**, 420–424 (2019).
86. Zhu, X., Liu, J. & Zhang, W. De novo biosynthesis of terminal alkyne-labeled natural products. *Nat. Chem. Biol.* **11**, 115–120 (2015).
87. Zhu, X. & Zhang, W. Terminal alkyne biosynthesis in marine microbes. *Methods Enzymol.* **604**, 89–112 (2018).
88. Wei, L. et al. Live-cell imaging of alkyne-tagged small biomolecules by stimulated Raman scattering. *Nat. Methods* **11**, 410–412 (2014).
This study combines non-canonical substrate analogue probing and Raman microspectroscopy to visualize alkyne-containing nucleoside, amino acid and fatty acid analogues in various eukaryotic cells.
89. Sinai, L., Rosenberg, A., Smith, Y., Segev, E. & Ben-Yehuda, S. The molecular timeline of a reviving bacterial spore. *Mol. Cell* **57**, 695–707 (2015).
90. Shieh, P., Siegrist, M. S., Cullen, A. J. & Bertozzi, C. R. Imaging bacterial peptidoglycan with near-infrared fluorogenic azide probes. *Proc. Natl Acad. Sci. USA* **111**, 5456–5461 (2014).
91. Bagert, J. D. et al. Time-resolved proteomic analysis of quorum sensing in *Vibrio harveyi*. *Chem. Sci.* **7**, 1797–1806 (2016).
92. Babin, B. M. et al. SutA is a bacterial transcription factor expressed during slow growth in *Pseudomonas aeruginosa*. *Proc. Natl Acad. Sci. USA* **113**, E597–E605 (2016).
93. Mahdavi, A. et al. Engineered aminoacyl-tRNA synthetase for cell-selective analysis of mammalian protein synthesis. *J. Am. Chem. Soc.* **138**, 4278–4281 (2016).
94. Glenn, W. S. et al. BONCAT enables time-resolved analysis of protein synthesis in native plant tissue. *Plant. Physiol.* **173**, 1543–1553 (2017).
95. Calve, S., Witten, A. J., Ocken, A. R. & Kinzer-Ursem, T. L. Incorporation of non-canonical amino acids into the developing murine proteome. *Sci. Rep.* **6**, 32377 (2016).
96. Yuet, K. P. et al. Cell-specific proteomic analysis in caenorhabditis elegans. *Proc. Natl Acad. Sci. USA* **112**, 2705–2710 (2015).
97. Dieterich, D. C. et al. In situ visualization and dynamics of newly synthesized proteins in rat hippocampal neurons. *Nat. Neurosci.* **13**, 897–905 (2010).
98. Taymaz-Nikerel, H., Borujeni, A. E., Verheijen, P. J., Heijnen, J. J. & van Gulik, W. M. Genome-derived minimal metabolic models for *Escherichia coli* MG1655 with estimated in vivo respiratory ATP stoichiometry. *Biotechnol. Bioeng.* **107**, 369–381 (2010).
99. Beck, A. E., Hunt, K. A. & Carlson, R. P. Measuring cellular biomass composition for computational biology applications. *Processes* **6**, 38 (2018).
100. Zavrel, T. et al. Quantitative insights into the cyanobacterial cell economy. *eLife* **8**, e42508 (2019).
101. Beatty, K. E. et al. Fluorescence visualization of newly synthesized proteins in mammalian cells. *Angew. Chem. Int. Ed. Engl.* **45**, 7364–7367 (2006).
102. Dieterich, D. C., Link, A. J., Graumann, J., Tirrell, D. A. & Schuman, E. M. Selective identification of newly synthesized proteins in mammalian cells using bioorthogonal noncanonical amino acid tagging (BONCAT). *Proc. Natl Acad. Sci. USA* **103**, 9482–9487 (2006).
103. Kiick, K. L., Saxon, E., Tirrell, D. A. & Bertozzi, C. R. Incorporation of azides into recombinant proteins for chemoselective modification by the Staudinger ligation. *Proc. Natl Acad. Sci. USA* **99**, 19–24 (2002).
104. Lang, K. & Chin, J. W. Cellular incorporation of unnatural amino acids and bioorthogonal labeling of proteins. *Chem. Rev.* **114**, 4764–4806 (2014).
105. Ngo, J. T. & Tirrell, D. A. Noncanonical amino acids in the interrogation of cellular protein synthesis. *Acc. Chem. Res.* **44**, 677–685 (2011).
106. Chakrabarti, S., Liehl, P., Buchon, N. & Lemaitre, B. Infection-induced host translational blockage inhibits immune responses and epithelial renewal in the drosophila gut. *Cell Host Microbe* **12**, 60–70 (2012).
107. Sherratt, A. R. et al. Rapid screening and identification of living pathogenic organisms via optimized bioorthogonal non-canonical amino acid tagging. *Cell Chem. Biol.* **24**, 1048–1055.e3 (2017).
108. Mahdavi, A. et al. Identification of secreted bacterial proteins by noncanonical amino acid tagging. *Proc. Natl Acad. Sci. USA* **111**, 433–438 (2014).
109. Ouellette, S. P., Dorsey, F. C., Moshiach, S., Cleveland, J. L. & Carabeo, R. A. Chlamydia species-dependent differences in the growth requirement for lysosomes. *PLoS One* **6**, e16783 (2011).
110. Siegrist, M. S. et al. d-Amino acid chemical reporters reveal peptidoglycan dynamics of an intracellular pathogen. *ACS Chem. Biol.* **8**, 500–505 (2013).

111. Hatzenpichler, R. et al. In situ visualization of newly synthesized proteins in environmental microbes using amino acid tagging and click chemistry. *Env. Microbiol.* **16**, 2568–2590 (2014).
This study for the first time uses bioorthogonal labelling and click chemistry on complex, multispecies samples and demonstrates that BONCAT–FISH can be used to link the identify and in situ function of uncultured microorganisms.
112. Samo, T. J., Smriga, S., Malfatti, F., Sherwood, B. P. & Azam, F. Broad distribution and high proportion of protein synthesis active marine bacteria revealed by click chemistry at the single cell level. *Front. Mar. Sci.* **1**, 1–18 (2014).
113. Leizeaga, A., Estrany, M., Forn, I. & Sebastian, M. Using click-chemistry for visualizing in situ changes of translational activity in planktonic marine bacteria. *Front. Microbiol.* **8**, 2360 (2017).
114. Sebastian, M. et al. High growth potential of long-term starved deep ocean opportunistic heterotrophic bacteria. *Front. Microbiol.* **10**, 760 (2019).
115. Kjeldsen, K. U. et al. On the evolution and physiology of cable bacteria. *Proc. Natl Acad. Sci. USA* **116**, 19116–19125 (2019).
116. Geva-Zatorsky, N. et al. In vivo imaging and tracking of host–microbiota interactions via metabolic labeling of gut anaerobic bacteria. *Nat. Med.* **21**, 1091–1100 (2015).
117. Hatzenpichler, R. & Orphan, V. J. in Hydrocarbon and Lipid Microbiology Protocols Vol. 7: Single-cell and single-molecule methods (ed T. J. McGenity) 145–157 (Springer, 2015).
118. Pasulka, A. L. et al. Interrogating marine virus–host interactions and elemental transfer with BONCAT and nanoSIMS-based methods. *Env. Microbiol.* **20**, 671–692 (2018).
This study applies BONCAT to microscopically quantify viral production rates in model systems and seawater and uses nanoSIMS to quantify carbon and nitrogen transfer rates between viruses and their microbial hosts.
119. Muller, T. G., Sakin, V. & Muller, B. A spotlight on viruses — application of click chemistry to visualize virus–cell interactions. *Molecules* **24**, 1–30 (2019).
120. Bagert, J. D. et al. Quantitative, time-resolved proteomic analysis by combining bioorthogonal noncanonical amino acid tagging and pulsed stable isotope labeling by amino acids in cell culture. *Mol. Cell Proteom.* **13**, 1352–1358 (2014).
121. Lehner, F. et al. The impact of azidohomoalanine incorporation on protein structure and ligand binding. *Chembiochem* **18**, 2340–2350 (2017).
122. Bennett, B. D. et al. Absolute metabolite concentrations and implied enzyme active site occupancy in *Escherichia coli*. *Nat. Chem. Biol.* **5**, 593–599 (2009).
123. Neufeld, J. D. et al. DNA stable-isotope probing. *Nat. Protoc.* **2**, 860–866 (2007).
124. Urbach, E., Vergin, K. L. & Giovannoni, S. J. Immunochemical detection and isolation of DNA from metabolically active bacteria. *Appl. Env. Microbiol.* **65**, 1207–1213 (1999).
125. Papp, K. et al. Quantitative stable isotope probing with $H_2^{18}O$ reveals that most bacterial taxa in soil synthesize new ribosomal RNA. *ISME J.* **12**, 3043–3045 (2018).
126. Pernthaler, A., Pernthaler, J., Schattenhofer, M. & Amann, R. Identification of DNA-synthesizing bacterial cells in coastal North Sea plankton. *Appl. Env. Microbiol.* **68**, 5728–5736 (2002).
127. Hamasaki, K. Comparison of bromodeoxyuridine immunoassay with tritiated thymidine radioassay for measuring bacterial productivity in oceanic waters. *J. Oceanography* **62**, 793–799 (2006).
128. Olaniyi, O. O., Yang, K., Zhu, Y. G. & Cui, L. Heavy water-labeled Raman spectroscopy reveals carboxymethylcellulose-degrading bacteria and degradation activity at the single-cell level. *Appl. Microbiol. Biotechnol.* **103**, 1455–1464 (2019).
129. Smriga, S., Samo, T. J., Malfatti, F., Villareal, J. & Azam, F. Individual cell DNA synthesis within natural marine bacterial assemblages as detected by 'click' chemistry. *Aquat. Microb. Ecol.* **72**, 269–280 (2014).
130. Jao, C. Y. & Salic, A. Exploring RNA ranscription and turnover in vivo by using click chemistry. *Proc. Natl Acad. Sci. USA* **105**, 15779–15784 (2008).
131. Kho, Y. et al. A tagging-via-substrate technology for detection and proteomics of farnesylated proteins. *Proc. Natl Acad. Sci. USA* **101**, 12479–12484 (2004).
132. Neef, A. B. & Schultz, C. Selective fluorescence labeling of lipids in living cells. *Angew. Chem. Int. Ed. Engl.* **48**, 1498–1500 (2009).
133. Garcia-Heredia, A. et al. Peptidoglycan precursor synthesis along the sidewall of pole-growing mycobacteria. *eLife* **7**, e37243 (2018).
134. Bublitz, D. C. et al. Peptidoglycan production by an insect-bacterial mosaic. *Cell* **179**, 703–712.e7 (2019).
135. Saxon, E. et al. Investigating cellular metabolism of synthetic azidosugars with the Staudinger ligation. *J. Am. Chem. Soc.* **124**, 14893–14902 (2002).
136. Siegrist, M. S., Swarts, B. M., Fox, D. M., Lim, S. A. & Bertozzi, C. R. Illumination of growth, division and secretion by metabolic labeling of the bacterial cell surface. *FEMS Microbiol. Rev.* **39**, 184–202 (2015).
137. Ohno, S. et al. A method for evaluating the host range of bacteriophages using phages fluorescently labeled with 5-ethynyl-2′-deoxyuridine (EdU). *Appl. Microbiol. Biotechnol.* **95**, 777–788 (2012).
138. Liu, Y. et al. Advancing understanding of microbial bioenergy conversion processes by activity-based protein profiling. *Biotechnol. Biofuels* **8**, 156 (2015).
139. Jehmlich, N., Vogt, C., Lunsmann, V., Richnow, H. H. & von Bergen, M. Protein–SIP in environmental studies. *Curr. Opin. Biotechnol.* **41**, 26–33 (2016).
140. Sadler, N. C. & Wright, A. T. Activity-based protein profiling of microbes. *Curr. Opin. Chem. Biol.* **24**, 139–144 (2015).
141. Whidbey, C. & Wright, A. T. Activity-based protein profiling-enabling multimodal functional studies of microbial communities. *Curr. Top. Microbiol. Immunol.* **420**, 1–21 (2019).
142. Willems, L. I., Overkleeft, H. S. & van Kasteren, S. I. Current developments in activity-based protein profiling. *Bioconjug. Chem.* **25**, 1181–1191 (2014).
143. Whidbey, C. et al. A probe-enabled approach for the selective isolation and characterization of functionally active subpopulations in the gut microbiome. *J. Am. Chem. Soc.* **141**, 42–47 (2019).
This study demonstrates the high potential of ABPP for single cell physiology studies. ABPP is combined with FACS and 16S rRNA gene sequencing to identify β-glucuronidase active members of the mouse gut microbiome.
144. Flemming, H. C. & Wingender, J. The biofilm matrix. *Nat. Rev. Microbiol.* **8**, 623–633 (2010).
145. Schlafer, S. & Meyer, R. L. Confocal microscopy imaging of the biofilm matrix. *J. Microbiol. Methods* **138**, 50–59 (2017).
146. Kehe, J. et al. Massively parallel screening of synthetic microbial communities. *Proc. Natl Acad. Sci. USA* **116**, 12804–12809 (2019).
147. Lan, F., Demaree, B., Ahmed, N. & Abate, A. R. Single-cell genome sequencing at ultra-high-throughput with microfluidic droplet barcoding. *Nat. Biotechnol.* **35**, 640–646 (2017).
148. Spencer, S. J. et al. Massively parallel sequencing of single cells by epicPCR links functional genes with phylogenetic markers. *ISME J.* **10**, 427–436 (2016).
149. Terekhov, S. S. et al. Ultrahigh-throughput functional profiling of microbiota communities. *Proc. Natl Acad. Sci. USA* **115**, 9551–9556 (2018).
150. Ando, J., Palonpon, A. F., Sodeoka, M. & Fujita, K. High-speed Raman imaging of cellular processes. *Curr. Opin. Chem. Biol.* **33**, 16–24 (2016).
151. Chisanga, M., Muhamadali, H., Ellis, D. I. & Goodacre, R. Surface-enhanced Raman scattering (SERS) in microbiology: illumination and enhancement of the microbial world. *Appl. Spectrosc.* **72**, 987–1000 (2018).
152. Ivleva, N. P., Kubryk, P. & Niessner, R. Raman microspectroscopy, surface-enhanced Raman scattering microspectroscopy, and stable-isotope Raman microspectroscopy for biofilm characterization. *Anal. Bioanal. Chem.* **409**, 4353–4375 (2017).
This review discusses the potential of advanced Raman microspectroscopy application, with a focus on the characterization of the extracellular polymeric substances and cells in microbial biofilms.
153. Cicerone, M. Molecular imaging with CARS micro-spectroscopy. *Curr. Opin. Chem. Biol.* **33**, 179–185 (2016).
154. Camp, C. H. & Cicerone, M. T. Chemically sensitive bioimaging with coherent Raman scattering. *Nat. Photonics* **9**, 295–305 (2015).
155. Opilik, L., Schmid, T. & Zenobi, R. Modern Raman imaging: vibrational spectroscopy on the micrometer and nanometer scales. *Annu. Rev. Anal. Chem.* **6**, 379–398 (2013).
156. Cui, L., Yang, K., Zhou, G., Huang, W. E. & Zhu, Y. G. Surface-enhanced Raman spectroscopy combined with stable isotope probing to monitor nitrogen assimilation at both bulk and single-cell level. *Anal. Chem.* **89**, 5793–5800 (2017).
157. Ivleva, N. P., Wagner, M., Horn, H., Niessner, R. & Haisch, C. Raman microscopy and surface-enhanced Raman scattering (SERS) for in situ analysis of biofilms. *J. Biophotonics* **3**, 548–556 (2010).
158. Kubryk, P. et al. Exploring the potential of stable isotope (resonance) Raman microspectroscopy and surface-enhanced Raman scattering for the analysis of microorganisms at single cell level. *Anal. Chem.* **87**, 6622–6630 (2015).
159. Weiss, R. et al. Surface-enhanced Raman spectroscopy of microorganisms: limitations and applicability on the single-cell level. *Analyst* **144**, 943–953 (2019).
160. Kolb, H. C., Finn, M. G. & Sharpless, K. B. Click chemistry: diverse chemical function from a few good reactions. *Angew. Chem. Int. Ed. Engl.* **40**, 2004–2021 (2001).
161. Moses, J. E. & Moorhouse, A. D. The growing applications of click chemistry. *Chem. Soc. Rev.* **36**, 1249–1262 (2007).
162. Codelli, J. A., Baskin, J. M., Agard, N. J. & Bertozzi, C. R. Second-generation difluorinated cyclooctynes for copper-free click chemistry. *J. Am. Chem. Soc.* **130**, 11486–11493 (2008).
163. Agard, N. J., Prescher, J. A. & Bertozzi, C. R. A strain-promoted [3+2] azide–alkyne cycloaddition for covalent modification of biomolecules in living systems. *J. Am. Chem. Soc.* **126**, 15046–15047 (2004).
164. Uttamapinant, C. et al. Fast, cell-compatible click chemistry with copper-chelating azides for biomolecular labeling. *Angew. Chem. Int. Ed. Engl.* **51**, 5852–5856 (2012).
165. Shieh, P. et al. CalFluors: a universal motif for fluorogenic azide probes across the visible spectrum. *J. Am. Chem. Soc.* **137**, 7145–7151 (2015).
166. Zimmermann, M. et al. Phenotypic heterogeneity in metabolic traits among single cells of a rare bacterial species in its natural environment quantified with a combination of flow cell sorting and nanoSIMS. *Front. Microbiol.* **6**, 243 (2015).
167. Netuschil, L., Auschill, T. M., Sculean, A. & Arweiler, N. B. Confusion over live/dead stainings for the detection of vital microorganisms in oral biofilms — which stain is suitable? *BMC Oral Health* **14**, 1–12 (2014).
This review provides an in-depth discussion of the shortcomings of viability and vitality stains.
168. Emerson, J. B. et al. Schrodinger's microbes: tools for distinguishing the living from the dead in microbial ecosystems. *Microbiome* **5**, 86 (2017).
169. Konopka, M. C. et al. Respiration response imaging for real-time detection of microbial function at the single-cell level. *Appl. Env. Microbiol.* **77**, 67–72 (2011).
170. Ullrich, S., Karrasch, B., Hoppe, H.-G., Jeskulke, K. & Mehrens, M. Toxic effects on bacterial metabolism of the redox dye 5-cyano-2,3-ditolyl tetrazolium chloride. *Appl. Env. Microbiol.* **62**, 4587–4593 (1996).
171. Hatzinger, P. B., Palmer, P., Smith, R. L., Penarrieta, C. T. & Yoshinari, T. Applicability of tetrazolium salts for the measurement of respiratory activity and viability of groundwater bacteria. *J. Microbiol. Methods* **52**, 47–58 (2003).
172. Karner, M. & Fuhrman, J. A. Determination of active marine bacterioplankton: a comparison of universal 16s rRNA probes, autoradiography, and nucleoid staining. *Appl. Env. Microbiol.* **63**, 1208–1213 (1997).
173. Servais, P., Agogue, H., Courties, C., Joux, F. & Lebaron, P. Are the actively respiring cells (CTC^+) those responsible for bacterial production in aquatic environments? *FEMS Microbiol. Ecol.* **35**, 171–179 (2001).
174. Nielsen, J. L., Aquino de Muro, M. & Nielsen, P. H. Evaluation of the redox dye 5-cyano-2,3-tolyl-tetrazolium chloride for activity studies by simultaneous use of microautoradiography and fluorescence in situ hybridization. *Appl. Env. Microbiol.* **69**, 641–643 (2003).
175. Stiefel, P., Schmidt-Emrich, S., Maniura-Weber, K. & Ren, Q. Critical aspects of using bacterial cell viability assays with the fluorophores SYTO9 and propidium iodide. *BMC Microbiol.* **15**, 1–9 (2015).
176. Berney, M., Hammes, F., Bosshard, F., Weilenmann, H. U. & Egli, T. Assessment and interpretation of bacterial viability by using the LIVE/DEAD BacLight Kit in combination with flow cytometry. *Appl. Env. Microbiol.* **73**, 3283–3290 (2007).
177. Nocker, A., Cheung, C. Y. & Camper, A. K. Comparison of propidium monoazide with ethidium monoazide

for differentiation of live vs. dead bacteria by selective removal of DNA from dead cells. *J. Microbiol. Methods* **67**, 310–320 (2006).
178. Pätzold, R. et al. In situ mapping of nitrifiers and anammox bacteria in microbial aggregates by means of confocal resonance Raman microscopy. *J. Microbiol. Methods* **72**, 241–248 (2008).
179. Kolinko, S. et al. Single-cell analysis reveals a novel uncultivated magnetotactic bacterium within the candidate division OP3. *Env. Microbiol.* **14**, 1709–1721 (2012).
180. Spang, A. et al. The genome of the ammonia-oxidizing Candidatus Nitrososphaera gargensis: insights into metabolic versatility and environmental adaptations. *Env. Microbiol.* **14**, 3122–3145 (2012).
181. Chan, J. W. et al. Reagentless identification of single bacterial spores in aqueous solution by confocal laser tweezers Raman spectroscopy. *Anal. Chem.* **76**, 599–603 (2004).
182. Fernando, E. Y. et al. Resolving the individual contribution of key microbial populations to enhanced biological phosphorus removal with Raman–FISH. *ISME J.* **13**, 1933–1946 (2019).
183. Majed, N., Chernenko, T., Diem, M. & Gu, A. Z. Identification of functionally relevant populations in enhanced biological phosphorus removal processes based on intracellular polymers profiles and insights into the metabolic diversity and heterogeneity. *Env. Sci. Technol.* **46**, 5010–5017 (2012).
184. Berg, J. S., Schwedt, A., Kreutzmann, A.-C., Kuypers, M. M. M. & Milucka, J. Polysulfides as intermediates in the oxidation of sulfide to sulfate by *Beggiatoa* spp. *Appl. Env. Microbiol.* **80**, 629–636 (2014).
185. Bjerg, J. T. et al. Long-distance electron transport in individual, living cable bacteria. *Proc. Natl Acad. Sci. USA* **115**, 5786–5791 (2018).
This study uses Raman microspectroscopy to visualize gradients in cytochrome redox states along living cable bacteria.
186. Eder, S. H., Gigler, A. M., Hanzlik, M. & Winklhofer, M. Sub-micrometer-scale mapping of magnetite crystals and sulfur globules in magnetotactic bacteria using confocal Raman micro-spectrometry. *PLoS One* **9**, e107356 (2014).
187. Gruber-Vodicka, H. R. et al. Paracatenula, an ancient symbiosis between thiotrophic alphaproteobacteria and catenulid flatworms. *Proc. Natl Acad. Sci. USA* **108**, 12078–12083 (2011).
188. Taubert, M. et al. Tracking active groundwater microbes with D_2O labelling to understand their ecosystem function. *Env. Microbiol.* **20**, 369–384 (2018).

Acknowledgements
The authors are grateful to A. Kohtz for generating the Raman data depicted in Fig. 3. They thank R. Gerlach and H. Smith (Montana State University), J. Hemp (University of Utah), members of the Hatzenpichler laboratory – A. Kohtz, M. Lynes and N. Reichart – and the three reviewers for critical comments that improved the manuscript. Next-generation physiology research in the Hatzenpichler laboratory is supported through grants by the Gordon and Betty Moore Foundation (GBMF5999) and the National Science Foundation (MCB award 1817428 and RII Track-2 FEC award 1736255), as well as an Early Career Fellowship by the National Aeronautics and Space Administration to R.H. (80NSSC19K0449). Montana State University's Confocal Raman microscope was acquired with support by the National Science Foundation (DBI-1726561) and the M.J. Murdock Charitable Trust (SR-2017331).

Author contributions
R.H. designed the concept for this Review. All authors wrote the manuscript.

Competing interests
The authors declare no competing interests.

Peer review information
Nature Reviews Microbiology thanks Wei Huang, Aaron Wright and the other, anonymous, reviewer(s) for their contribution to the peer review of this work.

Publisher's note
Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.