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Targeted Cell Labeling and Sorting of Prokaryotes for Cultivation and Omics Approaches

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Abstract
To date, the vast majority of prokaryotic organisms escapes detailed characterization because they cannot be isolated in axenic cultures. These organisms are referred to as microbial dark matter. Targeted labelling and sorting of these microorganisms pave the way for single-cell, enrichment, or cultivation approaches. In this review, we describe an array of different methods ranging from labeling-free to specific labelling techniques. In addition, different cell sorting methods and their combinations with targeting strategies are summarized and downstream applications like sequencing and cultivation are reviewed. Recent advances, challenges, and limitations of the particular methods are discussed with respect to cell viability, genome integrity as well as throughput, in order to help researchers select the most suitable methods for their specific research questions.

Introduction

Microorganisms harbor a huge potential for biotechnological applications, e.g., the production of novel antibiotics, the conservation and storage of bioenergy, or the remediation of hazardous compounds [Mullis et al., 2019; Jeyavani et al., 2021; Wan et al., 2021]. Since the late 1980s, studies of prokaryotic communities based on 16S rRNA gene sequences have shown a vast diversity of microorganisms in the environment [Cho, 2021]. However, the majority of prokaryotic species has yet to be characterized and is therefore referred to as microbial dark matter (MDM) [Lok, 2015; Kaster and Sobol, 2020; Jiao et al., 2021]. Despite breakthroughs in cultivation techniques [Berdy et al., 2017; Zoheir et al., 2022], attempts to grow the majority of microorganisms and to generate sufficient biomass for further analysis have mostly failed (also known as the great plate count anomaly) [Staley and Konopka, 1985]. This is why researchers still primarily rely on omics methods, such as meta- and single-cell omics to study MDM [Kaster and Sobol, 2020; Jiao et al., 2021; Kumar et al., 2021; Münch et al., 2023].

Genomes derived from metagenomics data – so-called metagenome-assembled genomes (MAGs) – are useful for understanding microbial community compositions and metabolic potentials. Nevertheless, creating reference-quality genomes at the species level can be

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difficult and expensive with this approach. The quality of genome reconstruction is largely dependent on sequencing coverage and covariance-based binning [Amarasinghe et al., 2020; Dam et al., 2020; Kaster and Sobol, 2020], which renders the characterization of low-abundance microorganisms in a habitat a challenge, particularly in ecosystems with great diversity [Bickhart et al., 2022]. Despite advances in sequencing technologies (e.g., long-read sequencing) [Amarasinghe et al., 2020] and binning algorithms [Arisdakessian et al., 2021; Nissen et al., 2021; Zhang and Zhang, 2021], MAGs still are often consensus genomes derived from cells with high homologous regions. In addition, mobile genetic elements such as plasmids cannot be assigned to particular MAGs [Vollmers et al., 2022].

Single-cell genomics (SCG) on the other hand is the separation of single cells from a community prior to analysis, followed by sequencing and data analyses, which results in so-called single amplified genomes (SAGs) [Kaster and Sobol, 2020; Jiao et al., 2021]. However, obtaining SAGs can be difficult and expensive, too. This is especially true for minority community members that are statistically rarely sorted. In addition, cells might already lyse during the sorting process or do not lyse at all when applying standard lysis conditions [Rinke et al., 2014; Dam et al., 2020; Wiegand et al., 2021]. Moreover, SCG requires amplification of DNA since a single cell only contains femtograms of nucleic acids, which is too little to be directly used in sequencing. Multiple displacement amplification (MDA) is the most common method used; however, it suffers from random biases as well as biases against high GC regions and chimera formation, which often results in incomplete genomes [Parras-Moltó et al., 2018; Tu et al., 2021; Lawrence et al., 2022; Sobol and Kaster, 2023]. This is why standard SCG and metagenomics can become very expensive in regard to time and cost when one is interested in a specific organism with a certain taxonomy or metabolism, and especially when looking for minority members of microbial communities.

That is why other methods have been developed in the recent past. "Mini-metagenomics" uses small pools of typically 5–100 sorted cells from which the DNA is then amplified via MDA and sequenced [McLean et al., 2013; Alteio et al., 2020]. While the higher amount of cells is considered to reduce amplification bias, amplification is still necessary to obtain enough DNA for sequencing. To overcome the problem of low DNA input amounts for sequencing, "midi-metagenomics" was developed. In this approach, cells are fractionated into larger pools of several million cells in regard to different properties which can be distinguished by flow cytometers (e.g., different shape and size, metabolic, or physiological markers). From these pools, DNA can be directly isolated and sequenced resulting in assemblies that are suitable for co-variance-based binning approaches, yielding MAGs of significantly higher quality compared to conventional metagenomic concepts and avoiding additional costs for amplification [Vollmers et al., 2023].

Instead of sequencing nucleic acids of single cells, one can also attempt to cultivate from a single organism [Hu et al., 2020; Täuber et al., 2020; Anggraini et al., 2022]. This technique has the advantage of having a certain microorganism already separated from its community, hence avoiding competition for nutrients or the danger of being destroyed by competitors. Currently, there are different methods available for targeted cell detection and isolation. These methods can be either rather unspecific, label-free approaches, or aim at selectively targeting cells with a specific trait, taxonomy, or metabolism using radioactive or stable isotopes, or – in the majority of cases – fluorescent labels. However, since the mere detection of cells is not the ultimate goal, labelling methods have to be compatible with downstream processes for sequencing or cultivation. The same holds true for cell sorting techniques such as flow cytometry (FC), microfluidic devices, or cell printing. In this review, we aim to describe challenges as well as recent advances in prokaryotic cell labeling and sorting allowing downstream omics as well as live recovery of cells for cell cultivation.

Targeting of Prokaryotic Cells

Cultivation-dependent and cultivation-independent processes are the two approaches mainly applied for characterizing prokaryotic species. However, it has been demonstrated frequently that communities characterized using cultivation-independent methodologies often show greater complexity compared to traditional cultivation-based methods. In general, minorities in prokaryotic communities, but also anaerobes or cells with tough cell walls are usually neglected and understudied. This also holds true for the domain of Archaea, which is predominated by extremophiles [Rampelotto, 2013; Reed et al., 2013; Kaushik et al., 2021]. Low-abundance microbes in complex communities might, however, still play important roles in biogeochemical processes (e.g., due to high enzyme affinities to certain substrates) or might have biotechnological or environmental relevance [Frias-Lopez et al., 2008;

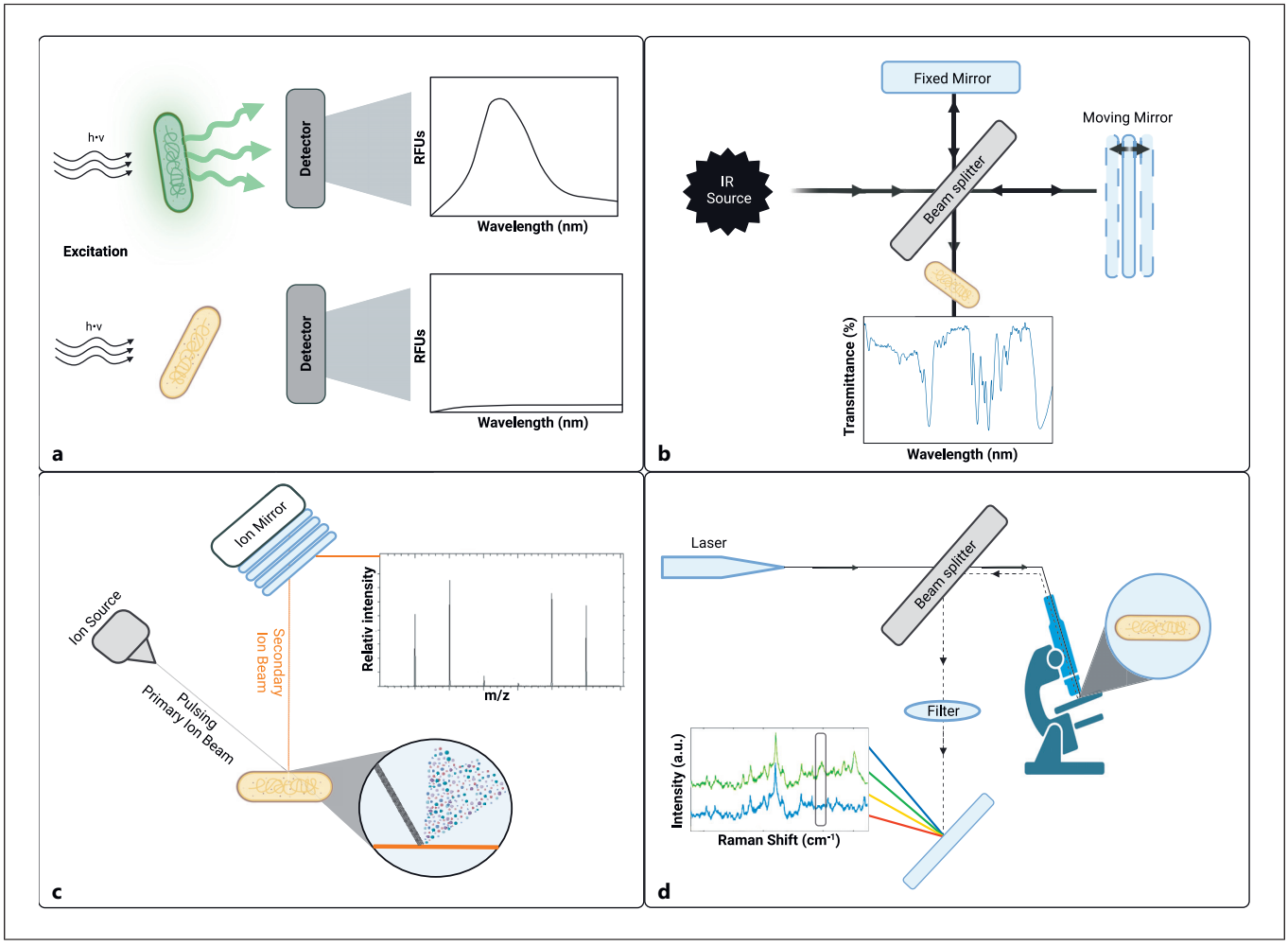

Fig. 1. Label-free detection methods. **a** Autofluorescence. Cells exhibiting autofluorescence are excited by light and fluorescence can be monitored via detectors or microscopy. **b** Fourier transform infrared spectroscopy (FT-IR). The split beam generates an interferogram that is transformed into an absorption spectrum. **c** Secondary ion mass spectrometry (SIMS). Bombardment of the sample with primary ions causes sputtering of secondary ions that are analyzed via mass spectrometry. **d** Raman spectroscopy (RS). The sample is excited via a laser and generated Raman-shifted photons are collected. For detailed explanations, we refer to the text. h, Planck's constant; ν, frequency; RFU, relative fluorescence units; IR, Infrared radiation; m/z, mass/charge ratio; a.u., absorption units. Created with Biorender.

Pratscher et al., 2018]. Consequently, targeted separation and sorting approaches are needed to overcome the limitations of conventional methods to allow the enrichment or isolation of formerly unidentified or underrepresented species.

Label-Free Approaches and Detection

Label-free approaches focus on natural and intrinsic cellular features that provide useful information about a cell's phenotype [He et al., 2019; Jayan et al., 2022]. Autofluorescence, e.g., caused by a variety of internal fluorescent biomolecules (e.g., cofactors) can be utilized to identify and categorize prokaryotic species [Ammor, 2007; Kang et al., 2020] (Fig. 1a). These compounds excite in the UV/VIS spectral range usually between 250 nm and 450 nm and emit fluorescence in the spectral range of 280 nm–540 nm [Ammor, 2007]. It was demonstrated that even different serotypes of species from reference genera (*Xanthomonas*, *Stenotrophomonas*, *Burkholderia*, and *Pseudomonas*) can show different autofluorescence signals [Tourkya et al., 2009]. However, this approach's efficacy is constrained for environmental samples since many different species might exhibit the same autofluorescence.

Fourier Transform Infrared Spectroscopy

An organsisms' biochemical fingerprint can be identified using Fourier Transform Infrared spectroscopy (FT-IR) since infrared radiation (IR) is absorbed by various biological components (e.g., nucleic acids, proteins, lipids, or carbohydrates) within the cell [Quintelas et al., 2018; Phelan et al., 2020; Novais and Peixe, 2021]. Here, a split IR radiation beam is used to determine a sample-specific interferogram that can be analyzed against reference databases to identify molecular configurations as well as whole cells (Fig. 1b). Only a few studies have examined single microbial cells in their native environments using FT-IR [Harrison and Berry, 2017], but the technique was used, e.g., to identify 14 different strains of *Enterobacter cloacae* from patients of a neonatal intensive care unit and the results were consistent with results from whole genome sequencing analyses [Vogt et al., 2019]. Despite its nondestructive nature, cost-effectiveness, and speed, the technology has a number of drawbacks, including a shortage of band frequencies for distinct chemical compounds, a lack of comprehensive commercial spectral libraries and water being a strong absorber of IR radiation. So far, these drawbacks have significantly limited successful application in identifying specific cells from natural habitats so far.

Secondary Ion Mass Spectrometry

Secondary ion mass spectrometry (SIMS) provides sufficient resolution to investigate individual microbial cells. In this approach, analyte ions (secondary ions) are formed by the bombardment of the sample surface with high-energy particles (primary ions). Secondary ions are then directed into a mass spectrometer for analysis [Watrous and Dorrestein, 2011] (Fig. 1c). Mass spectrometric imaging analyses such as SIMS are most commonly used on flat surfaces (depth resolution 1–2 nm). As a result, mass spectrometric imaging 3D mapping of complex microbiological forms (e.g., aggregates or biofilms) can be difficult. Additional measures are required for successful sample preparation, demanding further stabilization or freezing, which generally results in loss of live cells [Watrous and Dorrestein, 2011; Grujcic et al., 2022]. Moreover, SIMS imaging employs a harsh and destructive ionization technique leaving cells in a nonviable state after analysis [Watrous and Dorrestein, 2011]. Another issue with this method is its difficulty in precisely selecting a specific microorganism.

A further development is the so-called NanoSIMS, which has a greater lateral resolution [Gao et al., 2016; Nuñez et al., 2018] but requires expensive instrumentation. Briefly, this method enables the generation of nanoscale maps of elemental or isotopic distribution, and high mass resolution (10–50 times greater than conventional SIMS) [Kilburn and Wacey, 2014; Oehler and Cady, 2014]. This is achieved by using Cs^+ and O^- as primary ion beams with narrow focal planes (50–200 nm for Cs^+ primary ion beam) [Gates et al., 2018]. Additionally, a double-focusing design is used in NanoSIMS for secondary ion collection and detection. Most NanoSIMS analysis efforts utilize isotope and/or rare element labeling to enable the detection of processes or species of interest inside biological samples [Nuñez et al., 2018]. NanoSIMS is now frequently used in combination with other measures such as fluorescent in situ hybridization (FISH) or bioorthogonal noncanonical amino acid tagging (BONCAT) [Grujcic et al., 2022] (discussed below). Although the method is destructive and downstream applications such as cultivation and omics approaches are not possible, its application in a variety of studies shows a valuable information gain in the detection of MDM and description of prokaryotic communities on single-cell level (reviewed in, e.g., [Orphan and House, 2009; Gao et al., 2016; Musat et al., 2016; Nuñez et al., 2018]).

Raman Spectroscopy

Raman spectroscopy (RS) is a powerful tool to identify cellular components and/or individual cells based on their specific Raman spectrum. The Raman effect describes the inelastic scattering of photons interacting with molecules [Raman and Krishnan, 1928]. Inelastic scattering occurs on two energy levels: wavelengths of inelastically scattered photons can be shifted to either higher (stokes) or lower (anti-stokes) wavelengths. The Raman shift (difference of wavelengths between excitation and Raman signal) is specific for different molecular vibrational modes and can be used as "fingerprint" to identify certain components in a sample (Fig. 1d). Raman excitation is typically based on laser light, with 532 nm and 785 nm being the most frequent laser wavelengths for biological samples due to the lower fluorescence background [Tu and Chang, 2012; Wang et al., 2021]. RS is a point-based technique that only probes a distinct spatial region of the sample. In order to provide chemoselective images of larger biological structures (e.g., in μm scale), raster scanning can be performed [Lohumi et al., 2017]. Collecting spatially resolved Raman spectra with scanning devices results in Raman "maps" harboring information about the chemical fingerprints at distinct spots but are rather time-consuming depending on the signal strength [Le et al., 2010; Stewart et al., 2012]. RS has been

applied to heterogeneous prokaryotic cell populations. Kanno et al. [2021] recently classified three bacterial and three archaeal species from various phyla by using single-cell Raman microspectroscopy combined with machine learning algorithms [Kanno et al., 2021]. Song and colleagues were able to identify (and sort) different bacterial strains from the Red Sea according to their carotinoid content and moreover to classify the identified carotenoids into subgroups [Song et al., 2017]. Despite the potential of the method, the main issue remains the lack of spectral databases to identify phenotypic differences among different prokaryotes and their physiological states [Spitsyn et al., 2021]. In addition, energy acquired during signal acquisition (i.e., heat) has proved to be a major issue when aiming for genomics approaches [Su et al., 2020b]. Raman signals are also often masked by fluorescence, frequently emitted by biological molecules, especially when probed in the UV or short-wavelength visible spectral region [Le et al., 2010; Spitsyn et al., 2021; Tang et al., 2021].

To address these issues, Surface Enhanced Raman spectroscopy (SERS) has been developed [Fleischmann et al., 1974; Jeanmaire and Duyne, 1977]. Here, Raman signals can be increased by SERS-active substrates such as roughened noble-metal surfaces or noble-metal nanoparticles. In this approach, the Raman signal intensity increases by orders of magnitude when microorganisms are brought in close vicinity of the substrate [Cui et al., 2019; Weiss et al., 2019]. However, since only cell components in close proximity to the SERS-active substrate contribute to the Raman spectrum, the spatial resolution is decreased. While a "conventional" Raman spectrum represents a cross section profile of all Raman-active molecules of a particular cell, a SERS spectrum represents just a subfraction of components that are closely oriented to the surface (e.g., membrane components). Since the spatial orientation of the sample on the surface has a great impact on the obtained Raman spectrum, one has to make sure that cells are evenly adhered to the surface following the surface selection rules [Le Ru et al., 2011; Xu et al., 2011]. Studies have been performed on the analysis of pathogenic and non-pathogenic microorganisms using label-free SERS methods [Bodelón et al., 2018; Akanny et al., 2020; Liu et al., 2021]. It is noteworthy that SERS signals still strongly rely on the operational conditions related to sample preparation (i.e., nanoparticle morphology, chemical composition, concentration as well as the chemical nature of the SERS substrate) [Witkowska et al., 2019; Sportelli et al., 2022]. In addition, some metals, especially silver, show antibacterial effects and might affect microorganisms' viability. By conducting a comparative study on nontoxic gold with toxic silver nanoparticles, the effects of nanoparticle concentration and incubation time on *Lactobacillus fermentum* and *Escherichia coli* SERS spectral variance were investigated. The results demonstrated both nanoparticle concentration and incubation time to be closely related to the toxicity of silver particles [Cui et al., 2015]. A fundamental understanding of the origin of the Raman signal and the correlation between the SERS signals from single cells and their metabolic activity is essential for reliable analysis [Sportelli et al., 2022]. An analysis of six different prokaryotic species (one archaeon, two Gram-positive bacteria, and three Gram-negative bacteria) demonstrated that the metabolic activity of the studied cells has a significant impact on the corresponding SERS signal at the single-cell level [Weiss et al., 2019]. Therefore, a large database and standardized analysis methods are required to have broader applications for this method [Cui et al., 2019; Matanfack et al., 2020].

Coherent Anti-Stokes Raman scattering (CARS) is an additional method for amplifying Raman scattered light [Volkmer et al., 2002; Camp and Cicerone, 2015]. CARS imaging was frequently used for identification of mammalian brain tissue, tumors, and lipids in vivo [Cui et al., 2022] but was also applied for imaging of *Bacillus subtilis* spores in water, illustrating that limitations of conventional Raman signal acquisition with respect to signal strength could be overcome using CARS [Petrov et al., 2005]. For all Raman methods, photodamage of cells is, however, a major problem, limiting the applications for downstream processing [Fu et al., 2006; Tripathi et al., 2008; Pilo-Pais et al., 2014]. For detailed information about Raman-based techniques as well as profound background information, we refer to the excellent review written by Cialla-May and colleagues [Cialla-May et al., 2022].

Labeling Approaches

The abovementioned techniques use chemico-physical properties of certain molecules of cells or cell fractions/compounds to classify cells. However, target molecules (e.g., carotenoids or cofactors) might be expressed by different strains and show the same (or similar) detection behavior. This might lead to a collection of cells or strains showing the same signals but still belong to different clades of prokaryotes with different metabolic properties. A simple example is the well known live/dead staining, where dyes staining nucleic acids (e.g., propidium-iodide, SYBR® or SYTO® dyes) allow for discrimination of living from dead cells according to the cellular viability

(i.e., membrane integrity). However, numerous research questions require more specific labeling of cells and high-resolution selection which can be accomplished using sophisticated labeling techniques. Here, subfractions of a given community are targeted according to different (characteristic) properties that allow discrimination. The following section introduces a set of methods to specifically label cells and discusses their possible downstream analyses.

Isotope-Based Labeling

Stable Isotope Probing. Stable isotope probing (SIP) can identify metabolic fluxes and functional microorganisms using different stable isotopes (^{13}C, ^{15}N, ^{2}H, or ^{18}O) [Coyotzi et al., 2016; Berry and Loy, 2018; Jayan et al., 2022] (Fig. 2a). Here, samples are incubated with a substrate labeled with a heavy stable isotope, and the labeled molecules, such as proteins, DNA, or RNA, are traced [Campana et al., 2021]. Using isopycnic centrifugation, the heavy-labeled molecules form different fractions in a cesium chloride density gradient matrix. Molecules with higher incorporation rates of the heavy isotopes migrate deeper in the applied gravity field and can then be isolated and sequenced. Thus, the identity of the organism refers to the uptake of certain substrates or metabolic processes [Campana et al., 2021]. SIP has been extensively used in microbial ecology, e.g., applying ^{13}C SIP revealed that alternate carbon sources, such as acetate, play a substantial role in the metabolism of potential atmospheric methane oxidants in highland soils [Pratscher et al., 2011]. DNA-SIP combined with metagenomics (metagenomic-SIP) could link specific functions to distinct genomes of complex communities, as well as help assemble target genomes [Wilhelm et al., 2019; Barnett and Buckley, 2020]. Nine MAGs belonging to *Proteobacteria*, *Gemmatimonadetes*, and *Chloroflexota* were retrieved from SIP-^{13}C-labeled DNA in a project investigating active bacteria involved in methane metabolism in high Arctic cryosols and the authors were able to identify non-culturable active methane oxidizers [Altshuler et al., 2022]. Starr et al. traced the carbon flux from plants to a microbial community of the rhizosphere via quantitative SIP and were able to identify different fractions of SIP-labeled prokaryotes [Starr et al., 2021]. However, since the resolution of SIP is low, detection of stable isotope incorporation on single-cell level is not possible to date.

The combination of SIP with NanoSIMS (NanoSIP) can be employed to identify and target species of interest in a community of microorganisms. One important advantage of NanoSIP is the capacity to image multiple isotopes at the same time [Nuñez et al., 2018]. Many microbiological studies involving NanoSIP focus on microbial metabolism, where primarily ^{13}C and ^{15}N are used to follow interspecies transfer of metabolites [McGlynn et al., 2015; Berry and Loy, 2018; He et al., 2021]. In addition, catabolic degradation and subsequent uptake of extracellular DNA in anoxic marine sediments could be monitored using NanoSIP [Wasmund et al., 2021]. Furthermore, specific carbon assimilation rates from glucose in *Pseudomonas putida* as well as the cellular density of certain elements like nitrogen, phosphor, and oxygen could be determined on the single-cell level [Stryhanyuk et al., 2018]. In a recent approach, Kitzinger and colleagues [2019] were able to determine nitrite production from urea and cyanate by Thaumarchaeota on single-cell level [Kitzinger et al., 2019]. Using a combination of $^{15}N_2$-based NanoSIP, CARD-FISH (see below), 16S rRNA and *nifH* gene sequencing, Woebken and colleagues were able to correlate metabolic function (i.e., N_2-incorporation) to a distinct Chloroflexota species of a complex microbial consortium from a photosynthetic microbial mat derived from Monterey Bay (CA, USA) [Woebken et al., 2012]. In a similar approach, sulfate-reducing bacteria could be excluded as potential diazotrophs, whereas *Lyngbia* sp. could be clearly identified as major N_2-fixing organisms in microbial mats from Laguna Ojo de Liebre (Mexico) [Woebken et al., 2015]. However, as mentioned above, the underlying mechanism of SIMS is highly destructive and cells cannot be subjected to further growth experiments or to sequencing approaches. Still, as a supplementary tool combined with other techniques, NanoSIP has shown to be useful in community description, albeit it requires expensive instrumentation.

SIP can also be combined with Raman microscopy and other imaging and spectroscopic techniques to explore the phenotypic characteristics and activities of microbial communities at single-cell resolution [Matanfack et al., 2020; Lee et al., 2021; Jayan et al., 2022]. Muhamadali and co-workers used a simultaneous combination of Raman and FT-IR for distinguishing *E. coli* cells grown under different carbon and nitrogen isotope concentrations. Based on the different isotope ratios, the combined methods allowed a distinct discrimination of the cells [Muhamadali et al., 2015]. Deuterium in heavy water (D_2O) could also be used to identify metabolically active cells via its incorporation into newly synthesized biomolecules. It was shown that incorporation rates highly depend on complexity of the substrate metabolized by the cells. Rather low complex substrates like sugars, short-chain fatty acids, and aromatics yielded higher deuterium

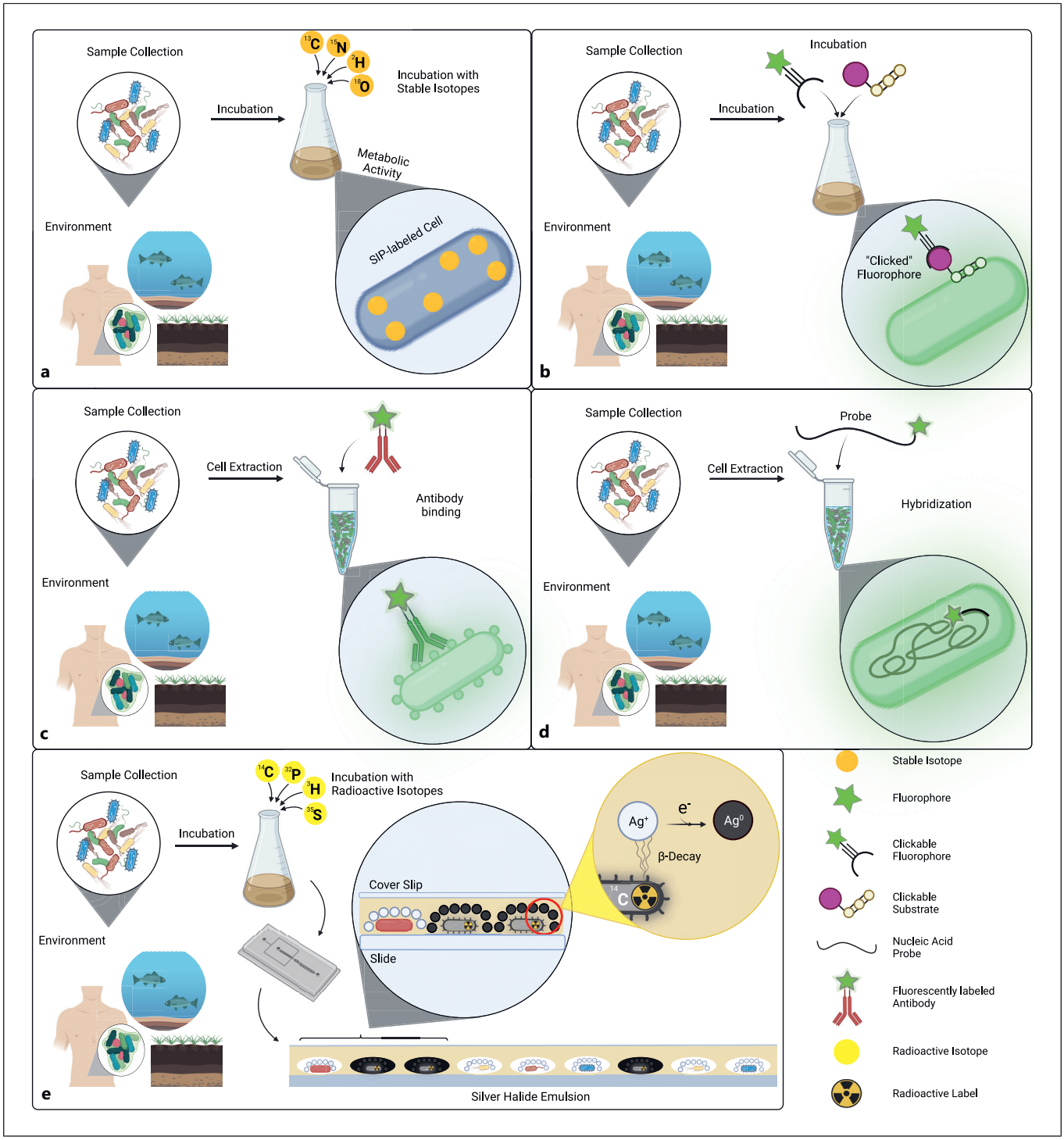

Fig. 2. Labeling methods for prokaryotic cells. **a** Stable isotope probing (SIP). Samples are incubated with stable isotope-labeled substrates. Substrate metabolization results in uptake of stable isotopes. **b** Click chemistry. Samples are incubated with a synthetic substrate suitable for click chemistry followed by incubation with a clickable fluorophore. Click reaction results in fluorescent labeling of cells that metabolized clickable substrates. **c** Antibody labeling. After sample preparation, samples are incubated with a fluorescently labeled antibody. After recognition of the target epitope by the antibody , cells exhibit fluorescence. **d** Fluorescence in situ hybridization (FISH). Samples are incubated with a fluorescent probe. After successful probe hybridization cells exhibit fluorescence (for details, see Fig. 3). **e** Microautoradiography (MAR). Like SIP, the radioactively labeled substrate is metabolized by the cells. β-decay causes black amorphous silver-precipitation by reduction of silver ions which can then be monitored. See text for further details. Created with Biorender.

incorporation rates compared to peptides or complex medium. Although Raman-based signal identification of deuterium incorporation is comparably easy to detect (due to the signal occurrence in the Raman silent region, 2,040–2,300 cm^{-1}), organism-specific metabolism as well as substrate incorporation have to be taken into account [Matanfack et al., 2020]. Raman-SIP is also substantially less sensitive compared to NanoSIP [Wang et al., 2016].

Single-Cell Capturing via Microautoradiography and Genome Sequencing

Microautoradiography (MAR) uses radioactively labeled compounds such as, e.g.,^{14}C aromatics to label prokaryotic cells based on their metabolic activity. Based on ^{14}C uptake, radioactively labeled cells trigger the conversion of silver cations in radiation-sensitive silver halide emulsion to amorphous, black silver atoms (Fig. 2e) [Brock and Brock, 1966; Nielsen and Nielsen, 2005], which can then be identified as black spots. A major drawback is that MAR can only detect metabolic activity and does not provide any species information. To overcome this issue, MAR-FISH (FISH is discussed below) was established [Lee et al., 1999; Ouverney and Fuhrman, 1999; Wagner et al., 2006]. However, since FISH requires prior knowledge about the sequences to label, this combination excludes unknown taxa from further analysis. Recently, a new technology named single-cell capturing via microautoradiography and genome sequencing was established to close this knowledge gap (Lo et al. under review). Here, radioactively labeled single cells are encapsulated in photoemulsion-hydrogels using microfluidics technology. After conversion of silver ions to black silver atoms, the microcapsules turn black. The black emulsion microcapsules can then be easily detected and sorted via FC. The sorted capsules can be dissolved and the DNA of the cell can be subjected to sequencing. Lo and colleagues isolated and sequenced single benzene-degrading *Pseudomonas veronii* cells from a mock community. Although application to environmental samples is needed, this technique holds a lot of promise for determining the identity of microorganisms metabolizing certain compounds in a variety of habitats. In contrast to the aforementioned destructive NanoSIP, this technique is highly compatible with downstream applications like genome sequencing on the single-cell level.

Fluorescent Labeling

Due to its good visualization properties, fluorescent labeling is the most common labeling technique in microbiology where a fluorophore is coupled to a targeting molecule (e.g., oligonucleotide, antibody). Fluorescein-derivates (fluorescein–isothiocyanate, 5-[-6-] carboxyfluorescein-N-hydroxysuccinimide-ester) and rhodamine-derivates (tetramethyl-rhodamine-isothiocyanate [TRITC], Texas Red) are popular dyes [Moter and Göbel, 2000]. Cyanine dyes such as Cy3 or Cy5 enhance fluorescence intensity since they show improved resistance to photobleaching. Alexa Fluor® 488, 555, 594, and 647 coumarin dyes are also widely used and have, e.g., enabled the simultaneous localization of numerous sequence-specific RNA species [Frickmann et al., 2017].

Substrate Analog Probing

An alternative to the SIP approach is the so-called substrate analog probing. Here, synthetic substrate analogs are used to identify specific cells based on metabolic characteristics. Artificial substrates are either labeled with a fluorophore or are suitable for azide-alkyne click chemistry (see below). Once taken up by the cells these molecules can be tracked [Hatzenpichler et al., 2020]. As opposed to most SIP and some label-free techniques, substrate analog probing has the benefit of using infrastructure that is more easily available, such as fluorescence microscopes and fluorescence-activated cell sorting (FACS) devices. For example, Doud and colleagues were able to identify a new cellulose degrader from the rare biosphere using this function-driven approach [Doud et al., 2020].

Azide–Alkyne Click Chemistry

"Click chemistry" refers to chemical reactions that produce stable heteroatom bonds that can be used for specific identification of particular compounds [Kolb et al., 2001; Moses and Moorhouse, 2007; Hatzenpichler et al., 2020].). Here, a specific cellular molecule is combined with a molecule that is easy to detect in a simple and fast reaction. The tagged cells can then be subjected to identification and sorting methods (Fig. 2b). There are various reactions in nature forming heteroatom bonds. However, click reactions must be modular, stereospecific, broad in scope, provide large yields, and produce only non-toxic byproducts [Kolb et al., 2001]. In more detail, click reaction refers to two types of labeling reactions yielding triazole conjugates: a Cu(I)-catalyzed and a metal-free version. Copper-free click chemistry has been widely used in a variety of biological projects [Baskin et al., 2007; Fugier et al., 2015; Cañeque et al., 2018; Zhang et al., 2021]. Synthetic substrates used in click chemistry are commercially available in several different forms: synthetic substitutes of L-methionine (L-azidohomoalanine [AHA] and L-homopropargylglycine [HPG]),

modifiable N-acetylmuramic acid derivatives (MurNAc, NAM [DeMeester et al., 2018; DeMeester et al., 2019]), lipopolysaccharide component analog 8-azido-8-deoxy-Kdo (8AzKdo [Wang et al., 2017a]) and thymidine analogs (5-bromo-2′-deoxyuridine [BrdU] and 5-ethynyl-2′-deoxyuridine [EdU] [Borneman, 1999; Tada and Grossart, 2014; Taniguchi and Eguchi, 2020]) are some examples. Natural substances, such as D-glutamic acid and D-alanine [Liang et al., 2017], as well as numerous forms of glycans [Li et al., 2020; Han et al., 2021], have also been used as labels due to their uniqueness in bacteria. The click reaction offers several benefits, the most notable of which is that it is an enzyme-free reaction that is straightforward to use. In context of targeted labeling of cells, click chemistry reactions are often visualized using fluorescent labels [Ishizuka et al., 2016; Cañeque et al., 2018; Hatzenpichler et al., 2020] (Fig. 2b). New generations of clickable fluorophores, such as picolyl dyes and fluorogenic "turn-on" azide probes (that only react with a fluorescent alkene), are especially well-suited to a wide range of complex samples [Leizeaga et al., 2017; Müller et al., 2019; Hatzenpichler et al., 2020]. Alkyne-conjugated Alexa Fluor®488 in combination with HPG was used to assess the activity of marine bacterial communities in both fast- and slow-growing cells [Samo et al., 2014]. It was possible to discriminate single *Alteromonas* cells actively synthesizing protein from the majority of resting cells, which remained uncovered by MAR approaches. Still, most clickable substrates have to be incorporated actively by the cells (e.g., during cell wall synthesis and protein biosynthesis). Therefore, click chemistry is rather unsuitable for identification of resting cells or discrimination of slowly growing cells from cells with similar metabolisms with respect to substrate incorporation.

Alternatively, BONCAT can be used, a method of tracking newly synthesized peptides/proteins by the use of amino acid surrogates (e.g., AHA and HPG) [Dieterich et al., 2006]. Since proteins account for the majority of cellular dry weight (50–65%) [Beck et al., 2018], incorporation of amino acid surrogates into newly translated proteins can be used to trace metabolic activity of certain cells in microbial communities [Kiick et al., 2002; Dieterich et al., 2006]. It was corroborated that incorporation of HPG and AHA has only minor impacts on the activity or community structure of bacteria [Hatzenpichler et al., 2016; Steward et al., 2020], as well as on protein tertiary structure [Lehner et al., 2017]. Researchers also used BONCAT to tag the active fraction of anammox microbiota [Chen et al., 2021], discover process-specific microbial features [Du and Behrens, 2021], investigate their in situ activity [Lindivat et al., 2020], and monitor the metabolic response of microbial community members to changing environmental conditions [Reichart et al., 2020]. It was also demonstrated that BONCAT-based labeling can be comparably very fast using AHA (down to 2% of the respective doubling time in *E. coli*). When BONCAT is combined with rRNA-targeted FISH or CARD-FISH (described below), active cells can be monitored and identified [Hatzenpichler et al., 2016; Pereira et al., 2022]. Furthermore, this technique was applied for the isolation of new strains [Han et al., 2021], classification of bacteria based on their structure (such as Gram-negatives and Gram-positives) [Wang et al., 2017b], studying the composition of the actively growing bacteria at the taxon level [Tada and Grossart, 2014], and examination of extracellular DNA from bacteria [Alhede et al., 2020]. In summary, this technique harbors the potential to label, isolate, and cultivate prokaryotic cells in a rather ambient way for further sequencing as well as cultivation approaches. However, it harbors similar drawbacks like the aforementioned click reactions of only targeting active cells due to the need for active substrate import. Additionally, stability of the substrates was reported to be an issue, especially during long-term exposures in alkaline and/or sulfide-rich environments [Hatzenpichler et al., 2014].

Antibody Labeling

Bio-recognition elements like antibodies and aptamers are excellent tools for specifically labeling particular cellular characteristics. Due to their three-dimensional recognition quality, it is possible to take advantage of structural components of cells in situ as binding motifs to specifically target cells (e.g., cell wall components, outer membrane proteins) (Fig. 2c). Antibodies can be coupled to a variety of reporters (e.g., enzymes, metals, and fluorophores) and be used for labelling also in combination with other methods such as FISH (discussed below) and SERS (see above) [Pal et al., 2017; Lee et al., 2019b; Wang et al., 2021]. Since binding of antibodies happens on the outside of the cell this technique circumvents drawbacks like fixation and permeabilization steps coming along with, e.g., FISH-based labeling methods (described below). Using polyclonal antibodies designed against reference strains, Bellais et al. were able to isolate the gut bacterium *Faecalibacterium prausnitzii* from human fecal samples. The approach was also successfully applied for isolating the rarely present *Christensenella minuta* from the same samples using FC [Bellais et al., 2022]. A promising approach to target cells from MDM where no isolates or closely related reference strains are available is to apply reverse genetics. A proof of principle was recently shown

by Cross and colleagues. Based on SAG sequence information for protein domain prediction extracellularly exposed peptide fragments (epitopes) were synthesized and served as antigens. Human oral samples labeled with these antibodies showed 25–100% recovery of the target organism *Saccharibacterium* sp. TM7 based on 16S rRNA sequencing [Cross et al., 2019]. Although the underlying genetic information was SAG-derived, this approach might also be useful if only MAG information is available when targeting specific (outer) membrane components. Although antibodies harbor a great potential for specifically labeling distinct organisms due to unique epitopes they, however, usually exhibit a certain cross-reactivity resulting in reduced selectivity and resolution [Kramer et al., 1997; Holm et al., 2012; Landegren et al., 2012].

Fluorescent in situ Hybridization

Fluorescently labeled oligonucleotides complementary to certain DNA or RNA sequences are widely used for the targeted labelling of cells (Fig. 2d, 3) [Antón et al., 1999; Pernthaler et al., 2001; Wagner et al., 2003a; Sekar et al., 2004; Fazi et al., 2007]. In general, short oligonucleotide probes (15–25 nucleotides) attach to defined sequence motifs of nucleic acids (i.e., genes, mRNA, lncRNA). FISH methods usually comprise permeabilization of the cell wall allowing the probe to enter the cell, binding of the labeled probe to the target sequence, and fluorescence-based detection of the bound probe either via microscopy or other fluorescence-based detection methods (e.g., FC, see below). However, in many cases the fluorescence signal from bound probes is too faint for proper detection for manifold reasons. For example, low cellular activity can be an issue when probes are designed for mRNA or rRNA targets and low genomic copy numbers cause weak signals when probes are designed to match specific marker genes causing signal strengths close to or even below the detection limit [Albertson et al., 1995; Wagner et al., 2003b].

To increase the fluorescence signal several improved methods have been developed, such as double-labeled oligonucleotide probes FISH (5′ and 3′ ends, Fig. 3a) or multi-labeled oligonucleotide probes FISH (Fig. 3b) which rely on the use of fluorescently labeled probes that have been labeled multiple times [Stoecker et al., 2010; Schimak et al., 2016]. In addition one or more types of fluorescent dyes can be used [Behnam et al., 2012]. Using the double-labeled oligonucleotide probes FISH technology, a novel species within the Beijerinckiaceae, closely related to *Methylocapsa*, was discovered in a study to detect atmospheric methane-oxidizing bacteria in the USCα clade from forest soil [Pratscher et al., 2018]. The first finding of an archaeal symbiont-host relationship was made by using multi-labeled oligonucleotide probes FISH [Schwank et al., 2019]. An alternative strategy for increasing the fluorescence signal is to label an organism with two or more probes targeting different genes [Azimi et al., 2022] which is called combinatorial labeling and spectral imaging-FISH (Fig. 3c) [Valm et al., 2011]. For example, a study revealed the microbial complexity in the human tongue using this technique [Wilbert et al., 2020].

Fluorescent signals can also be amplified based on an isothermal amplification technology. In situ DNA-hybridization chain reaction (DNA-HCR) uses an initiator probe partially hybridizing against the target sequence. The unbound part serves as a target to trigger the polymerization of two fluorescently labeled amplifier probes [Yamaguchi et al., 2015; Jia et al., 2021] (Fig. 3d). DNA-HCR is described to be a reasonable labeling technique for environmental samples reaching the same or even higher labeling efficiency compared to CARD-FISH (described below) and is suitable for downstream applications like SIP, FC, and next-generation sequencing [Yamaguchi et al., 2015]. DNA-HCR in combination with FACS was used for subsequent whole-genome sequencing and retrieval of MAGs for genomic annotation and characterization of uncultured phytoplankton bloom-associated flavobacterial clade Vis6 [Grieb et al., 2020].

Catalyzed reporter deposition FISH (CARD-FISH) is a FISH variation approach that can boost the fluorescence signal up to 41 times compared to standard FISH [Pereira et al., 2022]. Here, peroxidase activity leads to the deposition of a considerable number of labeled tyramine molecules [Eickhorst and Tippkötter, 2008]. Tyramines are phenolic substances that can be dimerized by the enzyme horseradish peroxidase (HRP). Many fluorescent molecules can be incorporated at the hybridization site in situ if fluorochrome-labeled tyramides are used [Pernthaler et al., 2002]. Here, HRP is used to label the probes. The signal from fluorescently labeled tyramides is then amplified by catalyzed reporter deposition (CARD) [Kubota, 2013] (Fig. 3e). CARD-FISH was first applied to environmental microbes in 1997 in attempts to identify prokaryotes from natural environments which feature a low ribosome content compared to cultured microbes [Lebaron et al., 1997; Schönhuber et al., 1997]. The first visual evidence for Heimdallarchaeota was observed using specifically tailored CARD-FISH probes [Salcher et al., 2020]. The same study revealed a different cell shape of Lokiarchaeota in environmental samples compared to cultured strains and indicated a condensed DNA

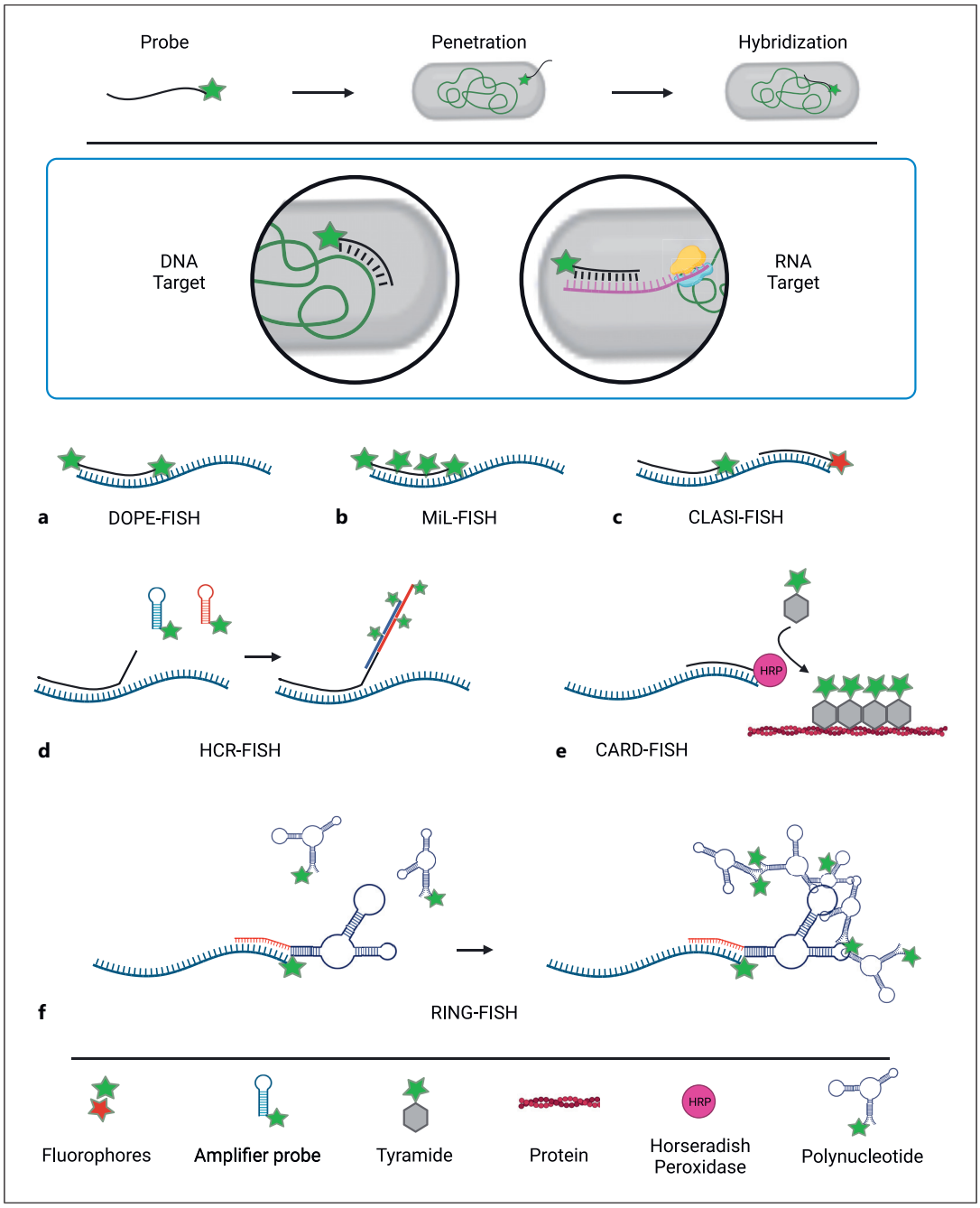

Fig. 3. Fluorescence in situ hybridization (FISH). The basic principle is probe penetration into the cell followed by hybridization against a target sequence. Hybridization is carried out with either DNA or RNA as target (blue box). Important subclasses of FISH techniques are shown. **a** DOPE-FISH uses double-labeled probes for higher signal strength. **b** MiL-FISH increases signal intensity by adding more fluorophores to the particular probe. **c** CLASI-FISH uses combinations of different fluorescently labeled probes. **d** HCR-FISH takes advantage of fluorescently labeled amplifier probes that hybridize against a special nonbinding part of the target-specific initiator probe. **e** CARD-FISH is conducted via a horseradish peroxidase (HRP) coupled to the probe. Tyramides attached to the fluorophore are converted to a radical state that causes immediate binding to aromatic compounds in the close vicinity (Trp, Phe) thereby increasing the signal intensity. **f** RING-FISH, recognition of individual genes is carried out using very long polynucleotides that form secondary structures. These molecules bind partially to each other thereby increasing fluorescence signal intensity. Created with Biorender. DOPE-FISH, double-labeled oligonucleotide probe FISH; MiL-FISH, multi-labeled oligonucleotide probe FISH; CLASI-FISH, combinatorial labeling and spectral imaging-FISH; HCR-FISH, hybridization chain reaction-FISH; CARD-FISH, catalyzed reporter deposition-FISH.

localization atypical for prokaryotes. Still, when it comes to FC-based sorting signal intensities might be too low for proper detection of very small cells with low ribosome content (e.g., ultramicrobacteria). To solve this problem, a second signal amplification step can be performed, which is mediated by HRP-labeled antibodies targeted to the fluorophores already deposited by CARD-FISH. This further development is called double CARD-FISH (2C-FISH), where two rounds of signal amplification are conducted [Neuenschwander et al., 2015]. The first round couples HRP-attached anti-fluorophore antibodies to the hybridized fluorophore, and the second is used for signal amplification with fluorophore-labeled tyramides. This technique was successfully used to sort LD12 ultramicrobacteria by FC from samples obtained from an oligo-mesotrophic lake. In a proof of principle study the authors were also able to show successful amplification of the 16S rRNA gene from whole-genome amplification (WGA) products (from multiple cells) after 2C-FISH treatment using ethanol as well as formaldehyde as initial fixative [Neuenschwander et al., 2015].

While traditional FISH methods target the 16S rRNA gene and its product, recognition of individual genes (RING)-FISH is used to target metabolic marker genes. The polynucleotide probe is labeled with a reporter molecule during probe synthesis via in vitro transcription. During hybridization, the probe subsequently anchors further probes due to secondary structures formed by incomplete binding (secondary structure formation) of the large probes, thereby forming a network around the cell periphery resulting in a halo-shaped fluorescent signal [Zwirglmaier et al., 2004; Pereira et al., 2022] (Fig. 3f). RING-FISH was, e.g., used to identify nonredundant type III secretion systems (T3SS) in *Vibrio parahaemolyticus* strains isolated from the Gulf of Mexico [Noriea et al., 2010].

A combination of different approaches can also be an effective way to improve cell labelling efficiency and increasing targeting specificity and thereby enhancing downstream sorting selectivity. The simultaneous application of FISH and BONCAT (see above) is a successful example of combining techniques. This approach links the cells' metabolic/translational active state (BONCAT) of the cell with species-specific markers (FISH), thus enabling the discrimination of metabolically active cells of a certain type from a whole community in situ. In a mock community of bacteria, the successful combination of FISH and BONCAT was able to detect Methylococcaceae sp. WF1 [Hatzenpichler et al., 2014], however, no sequencing or cultivation was performed.

Although FISH-based techniques have many advantages such as specificity, adaptability, and low costs, they also harbor some drawbacks, especially when it comes to sensitivity. Depending on the probe's target, the organism's physiological state and cell wall status as well as the detection method the intracellular fluorophore concentration might not exceed the detection limit. One major problem is the low penetration of the probe into the cell due to insufficient permeabilization of the cell wall. Common fixatives such as paraformaldehyde (for Gram-negatives) and ethanol (for Gram-positives) help permeabilizing the cell wall; however, they also inevitably destabilize the cell wall and damage the DNA thereby limiting downstream research possibilities such as sorting and/or single-cell sequencing approaches [Douglas and Rogers, 1998; Clingenpeel et al., 2014; Doud and Woyke, 2017; Dam et al., 2020]. There have been several studies published in the past years to overcome this problem, e.g., by eliminating the fixation steps from the conventional FISH protocols [Yilmaz et al., 2010; Haroon et al., 2013]. However, removing these chemical fixation substances also significantly reduces the probe's penetration rate and results in weak or no signals. To enhance the probe's penetration into the cell ethanol dehydration was shown to result in almost the same hybridization efficiency (*E. coli*) or an even slightly higher (*Bacillus megaterium*) when using the in-solution protocol omitting paraformaldehyde fixation [Haroon et al., 2013]. The live-FISH protocol developed by Batani and colleagues (based on the aforementioned protocol by Haroon et al. [2013]) was able to increase the outcome of viable cells by a factor of 10, resulting in >1% viable cells at the end of the protocol by replacing ethanol dehydration series with PBS washing and introducing an additional heat shock. The live-FISH protocol was shown to keep cells alive allowing cultivation even after undergoing a FACS-based sorting process [Batani et al., 2019]. Dam et al. simply used higher probe concentrations and longer hybridization times to gain sufficient fluorescent intensities in environmental samples. Following phylogenetic labeling of target cells and sorting, so far unknown Chloroflexota species from wastewater treatment plants could be isolated and subjected to genome sequencing [Dam et al., 2020].

Recently, a new fixation-free labeling approach for archaea and bacteria has been developed called FISH of transcript-annealing molecular beacons (FISH-TAMB) [Harris et al., 2022]. Here, a molecular beacon (MB) is designed to target the mRNA of bacteria and archaea. The MB comprises an oligonucleotide forming a hairpin structure connected to a fluorophore and a

fluorescence quencher at the opposite ends of the hairpin structure so that fluorescence is quenched in the unbound state. The hairpin structure is dissolved in the presence of the target sequence thereby releasing the quencher from the fluorophore's proximity and resulting in a fluorescence signal. MBs are transferred into the cells by cell-penetrating peptides which in experiments did not affect cell viability. FISH-TAMB is a promising approach to specifically target metabolically active cells since it targets mRNA and can potentially also discriminate between different levels of transcription. It has to be mentioned that so far only model organisms (*E. coli* and *Methanosarcina barkeri*) were tested under laboratory conditions and that FISH-TAMB still has to prove its value when it comes to environmental samples and in situ conditions.

Sorting of Cells

The opportunity to selectively identify or target cells from a community facilitates enrichment of certain subfractions of populations or even isolation at the single cell level. The downstream goal of the specific research interest ultimately specifies which sorting techniques are reasonable. The following section summarizes prominent techniques with respect to accuracy, throughput and downstream cell viability.

Flow Cytometry

FC is a technique for assessing large heterogeneous cell populations that uses a laser to detect light scattered or emitted by the cells [Givan, 2011; McKinnon, 2018]. FC coupled with specific fluorescence sensors allows for subclass cell sorting as well as sorting of single cells [Moor et al., 2016; Hiramatsu et al., 2019; Luo et al., 2020; Pereira et al., 2022]. FACS is frequently used to sort prokaryotic cells and sorters are commercially available from many companies in different price classes.

Fluorescence-Activated Cell Sorting

FACS technology relies on the detection of fluorescence signals caused by the cells themselves (autofluorescence), by fluorescently labeled antibodies or ligands that bind to certain cell-associated molecules. Besides fluorescence, simple scattering of light can also be detected and used for characterization of cells in terms of size and complexity/granularity as detected by forward scatter (FSC) and side scatter (SSC) [Veal et al., 2000; Luo et al., 2020; Vollmers et al., 2023]. Using a droplet deflection technique, FACS sorters are capable of sorting tens of thousands of cells per second enabling high throughput analyses to detect, count as well as sort subfractions of a complex heterogenous sample [Veal et al., 2000] (Fig. 4a). For example, Tada and Grossart identified a shift in community composition of actively growing bacteria in response to *N*-acetyl-glucosamine feeding at the taxon level by using a combination of BrdU-labeling and FACS-based cell sorting [Tada and Grossart, 2014]. Although FACS is a well-established, easy-to-use, fast, and precise option to sort cells, it has some limitations. The high pressure applied to cells during the sorting process might harm fragile cells. If researchers aim to cultivate from single-cells, these sorting conditions can be too harsh for some cell types [Pereira et al., 2022]. Low stability of the used fluorescence probes, bleaching and especially intrinsic background fluorescence from cells can lead to low sensitivity and "cross-talk" resulting in decreased purity and/or sorting efficiency. A major drawback is that obligately anaerobic microorganisms require strict anoxic conditions as long as cells should be kept alive after sorting. Due to large buffer volumes, these conditions are difficult to maintain, although a successful attempt has been reported [Thompson et al., 2015].

Microfluidic Technologies

Microfluidic technologies offer solutions for sample preparation, isolation, and identification of microorganisms. These techniques use very distinct configurations of microchannels embedded in a biocompatible matrix (Fig. 4b). Many studies use polydimethylsiloxane (PDMS) since it is cheap, inert, easy to handle, and allows gas penetration [Merkel et al., 2000; Lee et al., 2019a]. Other polymer-based materials are chosen primarily for their simplicity of surface modification and superior biocompatibility. Meanwhile, new materials for quick microbial detection, such as hydrogels and paper, have been incorporated into microfluidic circuits [Zhou et al., 2019]. Antibodies [Abafogi et al., 2020], antimicrobial peptides (AMPs) [Dao et al., 2018], aptamers [Su et al., 2020a], bacteriophages [Hussain et al., 2021], and molecularly imprinted polymers (MIPs) [Buensuceso et al., 2022] have all been exploited as functional coatings on the surface of microchannels.

Raman-Activated Cell Sorting

Raman-activated cell sorting spans the boundary between FC and microfluidics. Although the throughput of the RACS systems described below is higher than conventional microfluidic devices, a microfluidic chip or at

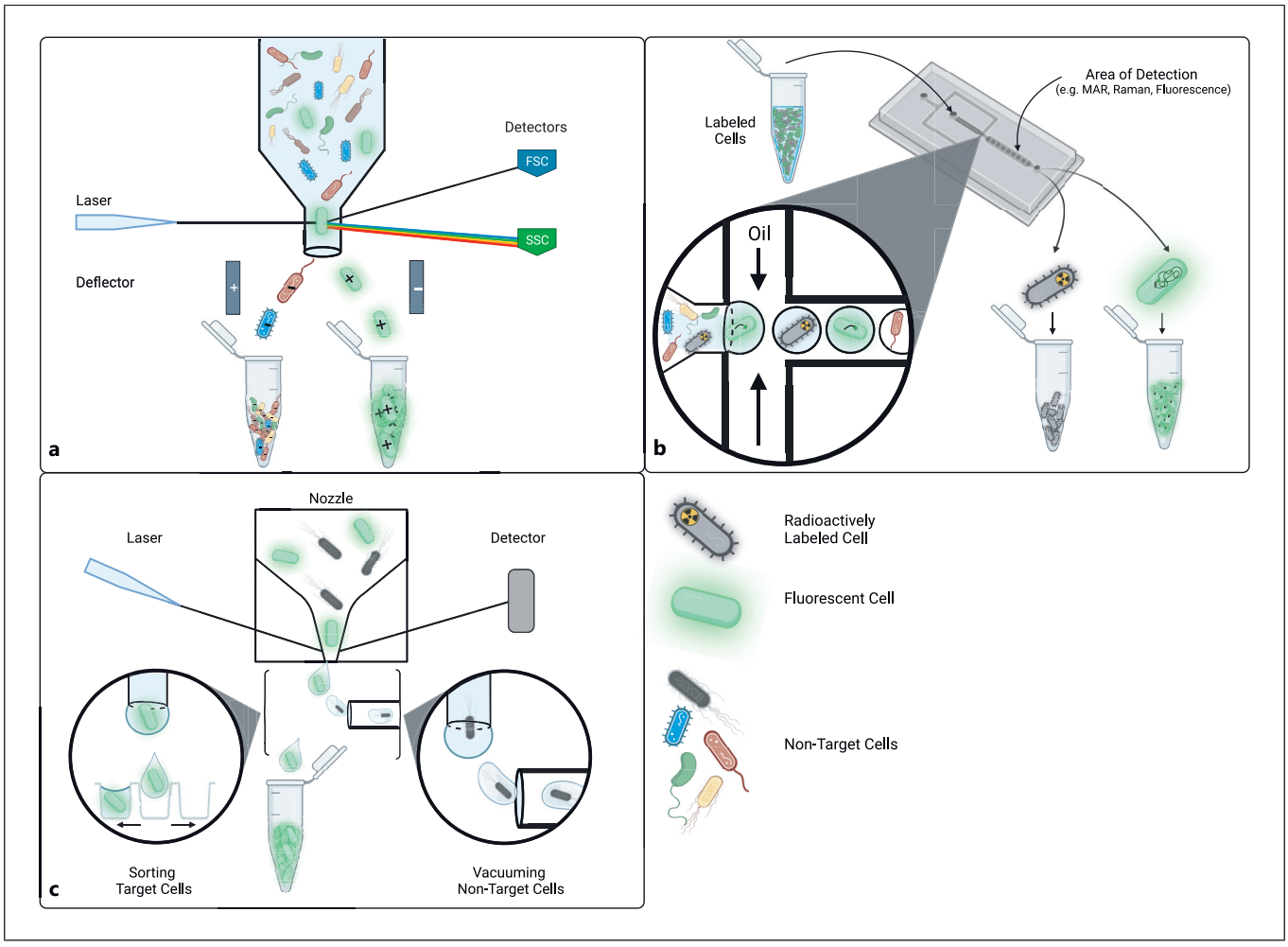

Fig. 4. Sorting techniques for prokaryotic cells. Cells can be subjected to labelling procedures prior to analysis or analyzed directly based on intrinsic sorting factors. **a** Flow-cytometry. Cells are diluted to a concentration, where single-cell droplets statistically occur. Cells in droplets are analyzed based on size, granularity, and/or fluorescence. Target cells can be chosen from the scattergrams based on the respective parameters and droplets are being charged accordingly. Deflectors guide the charged droplets to corresponding collection vessels (e.g., Eppendorf tubes or well plates). **b** Microfluidics platform. Cells are loaded onto the device in aqueous phase. Following a laminar flow droplet formation is accomplished by orthogonal oil application resulting in distinct droplets containing a single cell. Between droplet formation and ultimate sorting decision droplets can be analyzed, e.g., via fluorescence detectors, photodiodes, or Raman detectors (not shown). **c** Cell printer. Cells are diluted to a concentration, at which single-cell events occur at the tip of the nozzle where the droplet is formed by a piezo-actuated deflection of a silicon membrane. A fluorescence detector decides the fate of the droplet. Non-fluorescent droplets are eliminated from collection by a vacuum tube. Target cells are collected in batch (Eppendorf tube) or as single cells by use of microtiter plates (left inset). FSC, forward scatter; SSC, side scatter; MAR, microautoradiography. Created with Biorender.

least channel is the core component for separation of cells. Obtaining a full single-cell Raman spectrum (SCRS) is quite time-consuming (~1 s). Using coherent Raman scattering and coherent anti-Stokes Raman scattering, the enhanced signal reduces acquisition time down to ms or even μs [Hiramatsu et al., 2019, 2020; Suzuki et al., 2019]. Still, even when using SERS or CARS the throughput remains orders of magnitude lower compared to FACS [Hiramatsu et al., 2019; Gala De Pablo et al., 2021]. Although attempts were made to sort microalgae, yeast, and bacteria, the reported throughput and efficiency were rather low compared to "classical" FACS sorting [Camp et al., 2011; Huang et al., 2017; Hiramatsu et al., 2019]. However, RACS-based sorting is a useful tool in cases where label-free characterization is essential or the application of specific labels is ambiguous or not feasible.

RACS has been shown to be capable of in situ single-cell detection, identification, and sorting of active cells in complex environments and was successfully used to sort carotenoid-containing cells (*Pantoea* spp., *Legionella* spp., *Massilia* spp., *Pseudomonas* spp., and *Pedobacter* spp.) from mock bacterial communities [Jing et al., 2021; Li et al., 2022]. Although Raman-based characterization performs well in the absence of markers, combining it with SIP by the preceding addition of, e.g., D_2O substantially expands the technique's potential. In a study by Jing and co-workers, metabolically active cells (*Corynebacterium* spp., *Clostridium* spp., *Moraxella* spp., *Pantoea* spp., and *Pseudomonas* spp.) were sorted by RACS after the bacterium was D_2O-marked [Jing et al., 2021]. Metabolic activity of single bacteria from mouse cecal microbiomes could be followed combining D_2O-labelling and downstream RACS sorting followed by MDA and 16S rRNA-based identification [Berry et al., 2015].

There are several combinations of Raman-based signal acquisition and sorting mechanisms described resulting in a multitude of RACS subclasses where Raman-activated microfluidic sorting (RAMS), Raman-activated droplet sorting (RADS), and Raman-activated cell ejection (RACE) are the most relevant [Yan et al., 2021]. RACE is a RACS variant allowing sorting of single cells. Here, Raman signals are used to characterize cells mounted on a coated slide attached to a collection vessel (e.g., PDMS chip containing cavities). Collection of single cells is conducted by selectively releasing selected cells from the mounting slide to the collection vessel by, e.g., using laser-induced forward transfer [Hopp et al., 2005]. Here, the coating material in the proximity of the cell is evaporated by a laser, thereby pushing or releasing the cell into the collection vessel underneath the mounting slide. RACE was also performed to classify five oral bacteria (*Streptococcus sanguinis*, *S. mutans*, *Porphyromonas gingivalis*, *Enterococcus faecalis*, *Actinomyces viscosus*). Although it was possible to obtain and amplify DNA from single cells, the isolated cells were quickly destroyed as a result of the procedure [Wang et al., 2013]. In a study conducted by Wang et al. [2013], D_2O-marked single antibiotic-resistant bacteria (ARBs) were characterized via SCRS, sorted using RACE, and the sorted cells were subjected to single-cell genome amplification downstream [Wang et al., 2020]. Although Raman-based signal acquisition is basically noninvasive, nondestructive and leaves the cells in a viable state after sorting, results of genome amplification after RACE are rather limited. Su and colleagues were able to show that laser energy utilized for Raman signal acquisition has a dramatic impact on downstream sequencing or WGA efficiency, most probably due to heat-generation caused by energy consumption during signal acquisition. The overall impact was determined as a reduction from 80.5% to 36.3% sequence coverage by acquiring SCRS before the ejection step. They were also able to determine the impact of laser energy absorbed by the cells where they showed that applying 3 mW for 10 s is a good compromise between a clear enough SCRS (signal-to-noise ratio above 3) and sufficient DNA quality for downstream WGA attempts, whereas signal acquisition at 3 mW for 20 s as well as 60 s decreased downstream genome amplification down to 33% and 0% success rate, respectively [Su et al., 2020b]. Of note, obtained WGA results based on MDA were carried out using at least 5 sorted cells per reaction since all single cell attempts failed at the level of WGA.

Zhang et al. [2015] developed a RAMS system that was able to discriminate carotenoid-producing yeast cells from non-producing strains with a subsecond event rate and an accuracy of around 73% [Zhang et al., 2015]. The combination of applying optical tweezers into a RAMS platform enabled automatization of the process. In this setup, cells enter a microfluidic device and are ordered via two sheath flows to form a row of cells. Optical tweezers capture a single cell and move it to the detection area where the SCRS is acquired. If the SCRS matches sorting criteria (e.g., presence of carotenes, incorporation of isotopes) the tweezers release the cell to the collection port, whereas they will move the cells back into waste stream if cells do not match sorting criteria [Lee et al., 2019a]. Using this technique combined with SIP, it was possible to analyze and collect up to 8 cells per minute. Since laser energies applied in this approach are low due to the short Raman signal acquisition (300 ms at 532 nm) cells sorted with this approach are generally viable and can be subjected to genomic analyses as well as cultivation attempts [Lee et al., 2021]. Studies aiming at sorting of specific cells also utilize oil to form small droplets wherein cells were trapped. Since droplet formation prior to Raman signal acquisition causes perturbations, droplets in RADS attempts were generated after spectral profiling and subsequently subjected to downstream applications. Using droplet-based microfluidic technologies Wang and colleagues were able to drastically increase screening rate of RAMS systems to 260 cells per minute [Wang et al., 2017b]. Moreover, the RADS setup used preserved sorted cells in a viable state with a proportion similar to untreated cells (>90%). In this setup, all investigated cells are incorporated into oil droplets. Depending on the experimental criteria droplets of cells considered positive are separated from the rest via an electric field. In addition, application of an electric field could replace optical

tweezers in the setup, thereby decreasing chances of photodamage. Besides throughput, the major drawback of RACS compared to FACS is that there is just one system commercially available. All setups described here are custom-build.

Cell Printing

Labeling and cell sorting techniques, as previously indicated, can exert stress on cells and can cause cell rupture. Comparatively mild conditions can be maintained by so-called cell printers. While there are different direct and indirect printing attempts described (starting from modified inkjet printers, reviewed in [Gross et al., 2013; Zhou et al., 2022]), we focus on the recent advances in contact-free cell dispensers enabling WGA, cultivation an automatization. The basic principle behind this printing technique is generation of very small (pico liter scale) droplets harboring a single cells that are then deposited to a micro-well or agar plates by means of a dispenser chip [Gross et al., 2015] (Fig. 4c). Droplets are generated via piezo-actuated deflection of a silicon-membrane and formation is monitored via a bright-field video microscope, allowing automatic single-cell sorting of different cell sizes. However, the sensitivity is considerably reduced compared to, e.g., FACS, making it challenging when sorting is based on weak signal strengths. Further improvements facilitate smaller droplet volumes (down to 35 pL) as well as higher resolution (>1 μm) to reliably identify and print single bacteria with efficiencies >90% with a collection rate of 96 wells (i.e., cells) in approximately 10 min. However, it should be noted that this rate is significantly lower if only a rare portion of the cells in a community are to be sorted since the appearance of targeted cells within the nozzle happens randomly. A now commercially available setup enabled the shape-based sorting (drop-on-demand) of a mixture of *E. coli* (rods) and *E. faecalis* (cocci) followed by successful cultivation of the sorted single cells [Riba et al., 2016]. Successful label-free single-cell printing was also conducted to isolate cells from a complex wastewater treatment plant sample. Subsequent WGA and sequencing revealed 80% success rate for genome amplification and finally resulted in 27 SAGs from novel members of Patescibacteria, indicating that the gentle sorting conditions are beneficial for downstream WGA-based applications [Wiegand et al., 2021]. Although throughput of this technique is rather low compared to microfluidics and FC, this technique has the benefit of requiring only very small sample volumes ranging from 5 to 100 μL [Gross et al., 2015]. Moreover, one of its benefits is that the whole setup can be easily placed in an anaerobic glove box facilitating anoxic conditions throughout the sorting process. A recent study showed the successful isolation of 21 taxa of the human gut microbiome using anoxic single-cell dispensing. This approach shows the applicability of cell printing with respect to cultivation attempts since the (untargeted) cells were printed directly onto agar plates and colony formation from single-cell growth was followed [Afrizal et al., 2022]. Moreover, it shows that single-cell sorting is possible also under anoxic conditions, thus allowing sorting of strictly anaerobic microorganisms that are often excluded using "classic" sorting methods like FACS as mentioned above. Unfortunately, the design of the instrument prevents high sensitivity in the detection of fluorescence signals at the moment, which is why weakly fluorescing cells cannot be reliably detected. Since the microscopical observation of the cells is maintained via mirrors it seems feasible to couple cell printers to fluorescence and also Raman detectors in the future, thereby extending applications to targeted sorting, resulting in a compromise of throughput and viable cells.

Conclusion

Workflows for targeted labelling and sorting of prokaryotic cells are still lagging behind methods for eukaryotic research. The obvious reason is the predicament of size and – for sequencing attempts – the reduced amount and stability of nucleic acids. Reduced cell size results in comparatively low signal strength and technical issues when it comes to sorting attempts. In addition, the manifold cell wall structures and complexities of prokaryotic organisms impede a precise prediction of cell lysis conditions – some cells might lyse during the sorting process while others do not lyse at all when it would be needed, even when applying lysis treatment after sorting. In addition, many of the techniques and their respective combinations (identification and sorting) described in this review are custom laboratory setups tailor-made for a particular research focus and only used in the specific laboratories that published the respective research. The considerable advances in the last years regarding prokaryotic cell labelling and sorting raise hope for further commercially available devices in the future. Finally, one has to individually decide which techniques are suitable for a certain research question and must carefully take all the pros and cons into account before selecting a suitable method. Researchers not aiming at cultivation might select rather invasive identification methods (e.g., NanoSIMS). When higher cell viability or integrity is needed the

smoother the targeting or identification techniques have to be. The same holds true for the particular question being addressed – methods aiming at specific metabolic processes significantly differ from methods targeting specific cell components. Likewise, complexity and origin of the sample have to be taken into account. The multitude of factors that must be considered when selecting a specific method makes targeted cell sorting an exciting and versatile tool for answering a wide variety of research questions.

Conflict of Interest Statement

The authors declare that they have no conflict of interest.

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Author Contributions

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