AMERICAN SOCIETY FOR MICROBIOLOGY | Applied and Environmental Microbiology

| Spotlight Selection | Environmental Microbiology | Minireview

Microbial single-cell applications under anoxic conditions

Ciara Keating,[1] Kerstin Fiege,[2] Martijn Diender,[3,4] Diana Z. Sousa,[3,4] Laura Villanueva[2,5]

AUTHOR AFFILIATIONS See affiliation list on p. 16.

ABSTRACT The field of microbiology traditionally focuses on studying microorganisms at the population level. Nevertheless, the application of single-cell level methods, including microfluidics and imaging techniques, has revealed heterogeneity within populations, making these methods essential to understand cellular activities and interactions at a higher resolution. Moreover, single-cell sorting has opened new avenues for isolating cells of interest from microbial populations or complex microbial communities. These isolated cells can be further interrogated in downstream single-cell "omics" analyses, providing physiological and functional information. However, applying these methods to study anaerobic microorganisms under *in situ* conditions remains challenging due to their sensitivity to oxygen. Here, we review the existing methodologies for the analysis of viable anaerobic microorganisms at the single-cell level, including live-imaging, cell sorting, and microfluidics (lab-on-chip) applications, and we address the challenges involved in their anoxic operation. Additionally, we discuss the development of non-destructive imaging techniques tailored for anaerobes, such as oxygen-independent fluorescent probes and alternative approaches.

KEYWORDS anoxic cultivation, single-cell, anaerobes

Some of the world's most fascinating ecosystems are characterized by anoxic environments, devoid of molecular oxygen. The absence of oxygen imposes energetic constraints on microorganisms in these environments, requiring the utilization of alternative electron acceptors with lower redox potentials (1, 2). Anoxic settings are found in soils, sediments, rice paddy fields, the gut, and the deep sea. Microorganisms living in anoxic environments can exhibit either anaerobic (conducted in the absence of measurable oxygen) or facultative anaerobic metabolism. The term "anaerobic" is commonly interchanged by anoxic, while anoxic is not linked to metabolisms and refers more generally to an environment or culture setting which is oxygen-free or with oxygen concentrations below the detection level. Environments can also be "hypoxic" referring to the presence of low or depleted oxygen concentration (<21% O_2). The terms "micro-oxic" or "micro-anoxic" are also occasionally used for similar environments. In contrast, oxic environments are those which contain oxygen (the term aerobic will be used throughout this manuscript).

Anaerobic metabolism relies on the presence of alternative electron acceptors to oxygen, while facultative anaerobes are more versatile and can also utilize oxygen when available. Some anaerobes can tolerate oxygen (aerotolerants), or require low levels of oxygen (microaerophiles), while others are extremely sensitive to its presence (strict anaerobes); for example, most methanogenic (methane producing) archaea are notoriously sensitive to oxygen concentrations (poisoned at >5 ppm O_2) (3).

Anaerobes play key roles in organic matter degradation, biogeochemical cycling, and even contribute or alleviate climate change by the production or consumption of greenhouse gases (4). They are also found in the human and animal digestive tract, where some aid in food digestion and nutrient delivery to the host, while others may be involved in pathogenicity or providing resistance to antimicrobials (5, 6). Anaerobes

Editor Jennifer B. Glass, Georgia Institute of Technology, Atlanta, Georgia, USA

Address correspondence to Ciara Keating, ciara.keating@durham.ac.uk, Kerstin Fiege, kerstin.fiege@nioz.nl, or Martijn Diender, martijn.diender@wur.nl.

Ciara Keating, Kerstin Fiege, and Martijn Diender contributed equally to this article. The authorship order was decided by alphabetical first names.

The authors declare no conflict of interest.

See the funding table on p. 16.

Published 30 September 2024

are also essential for biotechnological processes, such as anaerobic digestion, biomanufacturing commodity chemicals, and fuels or bioremediation (7–9). Therefore, anaerobes are of significant interest across environmental, and clinical microbiology disciplines.

In the last decades, there has been great progress on elucidating microbial diversity within anoxic environments, mainly due to the introduction of DNA-based sequencing methods. Yet, microbial cultivation and physiological studies remain critical to understanding the metabolism of anaerobic microorganisms and their response to biotic and abiotic factors. Thus, the development and integration of novel culturing methods and microscopic techniques under anoxic conditions, mimicking the conditions in which anaerobic microorganisms live, are essential.

Anaerobes are inherently challenging to study due to their slow growth and sensitivity to oxygen. Besides, method development for anaerobic cultivation has been slow or stagnant. The methods of Hungate (1950, 1969) (10, 11) and Balch (1976) (12) remain the cornerstones of current lab techniques despite being laborious and time-consuming. An overview of anoxic cultivation techniques has been extensively reviewed elsewhere (4, 13). One of the drawbacks of anoxic culturing is the reliance, in most cases, on batch growth conditions with a typical shift from high to depleted nutrient conditions, which are not reflective of true environmental conditions. Anoxic continuous culture or chemostat cultivation is possible although tedious and dependent on expensive equipment and lab infrastructure to keep anoxic conditions, e.g., an anaerobic glovebox or glove bag, also known and sold as anaerobic chambers (14). However, all traditional anoxic culturing approaches fail to provide insight into the individual cell responses, cell spatial organization, and cell-cell interactions (such as chemotaxis, changes in individual cell growth rates, and physiological cell changes) of anaerobic microorganisms. Research has, therefore, come to depend on time-lapse culture-independent omics and cell-destructive microscopy approaches to provide snapshots into these missing links. These snapshots give hints regarding cell-to-cell interactions, but ultimately, experimental validation is needed to find causal relationships. We, therefore, lack a mechanistic understanding of how key anaerobic taxa interact with their environment and each other to ultimately influence biotechnology, environmental or host ecosystems at the macroscale level (15, 16).

To achieve this mechanistic understanding, we need to radically redesign how we conduct our experiments. Previous studies have proposed, for example, sophisticated non-destructive single-cell physiological experimentation, which they termed "next-generation physiology" (17). This approach uses a combination of culturing, phenotyping, sorting, and processing of cells of interest to yield insight into how individual cells respond to environmental conditions (or other tested parameters). However, to apply such an approach to anaerobes while maintaining non-destructive experimentation (to maintain viable cells) is still incredibly challenging. In contrast, aerobic culturing has experienced a "cultural renaissance" (18), where innovative cultivation efforts and single-cell applications, such as high-throughput lab-on-chip and microscopy methods, combined with genetic engineering and genomics are quickly transforming our understanding of the dynamic and subtle interactions between members of microbial consortia in complex environments [e.g. soils (15)], or the gut (19). Single-cell technologies involve experimenting with or visualizing single cells from microbial populations. A search on PubMed (National Library of Medicine, NIH) shows that manuscripts using aerobic single-cell applications totaled over 3,000 up to December 2023, whereas anaerobic single-cell applications yielded fewer than 100 publications during the same time period (Fig. 1).

Figure 2 illustrates a timeline parallel of developments in aerobic and anaerobic single-cell microbiological studies. The very first bacterial single-cell isolators were invented as far back as 1911 (20), such as the Barber's single-cell isolator (21) and the Chambers micro-manipulator (22). Barber adopted the single-cell isolator for anoxic conditions in 1920 (23). From this point on, several developments were made concerning aerobic single-cell culture, such as droplet isolation (24), and oil-chamber and

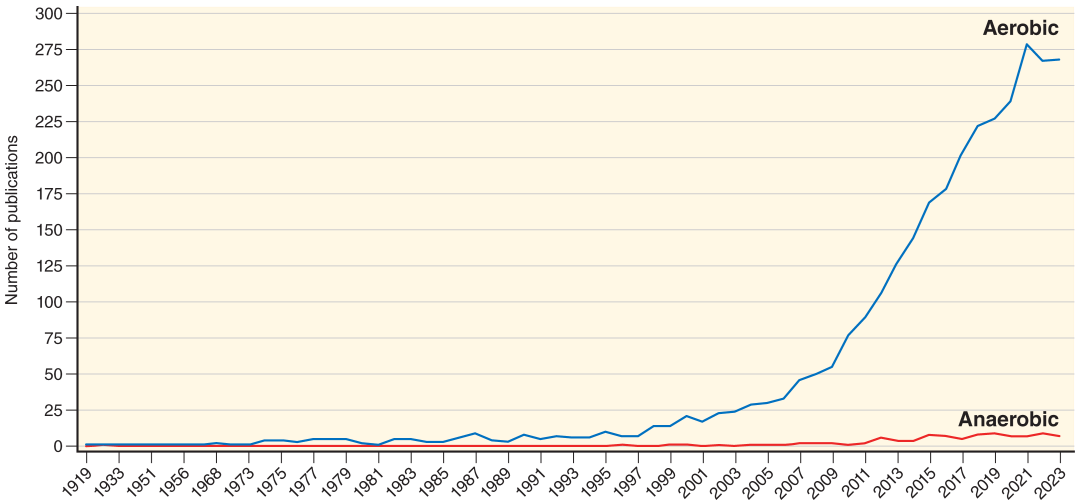

FIG 1 Results of a search from PubMed showing the results for searches for "Aerobic" single-cell bacterial studies ("Bacteria" AND "Single-cell"—3,086 papers) and "Anaerobic" single-cell studies ("Bacteria" AND "Single-cell" AND "Anaerobic"—98 papers).

continuous microscopy (25, 26). Although these techniques were first adapted for anaerobes in 1941 (27), they were not widely implemented. Only recently have droplet-based single cell-sorting techniques been revived for anaerobes (28, 29).

The next breakthrough in aerobic single-cell applications occurred with the invention of optical trapping of particles using lasers by Ashkin in 1978 (30), which was later applied to bacteria in 1987 (31). It took almost a decade for this methodology to be adapted to anaerobes by Huber et al. in 1995 (32). From the late 1990s, (aerobic) single-cell applications experienced exponential growth with the development of advanced microscopy facilities for timelapse (e.g., automated stages, autofocus capability, and digital imaging) and the innovation of BioMEMs (biomedical or biological microelectro-mechanical systems) or lab-on-chip applications, initially for mammalian cell-culture and other biological applications (33–35). These were rapidly applied to bacterial single cells for their culture and isolation (36, 37). Microfluidics for (aerobic) bacterial single-cell applications became more common from 2005 onward (38–40) and the incremental development of the mother machine device to monitor individual cell growth rates (41, 42). The next major advancement has been the development of highly sophisticated *in situ* environments for single-cell analysis such as the "organ-on-a-chip" (43). Adoption of lab-on-chip devices to anoxic single-cell applications has been slow and only carried out by a handful of researchers (Fig. 2), for example, the use of anoxic single-cell pure-culture in 2007 (44) and again in 2015 (45). However, developments in gut-on-a-chip applications have been substantial (19, 46).

The slow adoption of aerobic single-cell imaging techniques to anoxic applications is likely due to the multidisciplinary skillset needed to develop these experimental conditions (for example, nanofabrication and fluid handling) and the technical challenges of maintaining anoxic conditions. Handling anaerobic microorganisms presents unique challenges due to the requirement for low-oxygen or oxygen-free environments to maintain their viability and physiological properties. Some challenges associated with using single-cell imaging methods with anaerobic species include sample handling, imaging setup, live-imaging, fluorescent probes, and cell viability. Therefore, specialized equipment and techniques are needed, for instance, closed imaging systems to secure anoxic conditions, which often require customized setups, or new labeling strategies to visualize cells and their cellular processes.

In this mini-review, we provide a comprehensive overview of single-cell applications in anoxic conditions involving live-cell conditions. We present the key advances, from single-cell imaging, microfluidic experimentation, and cell sorting. We further highlight

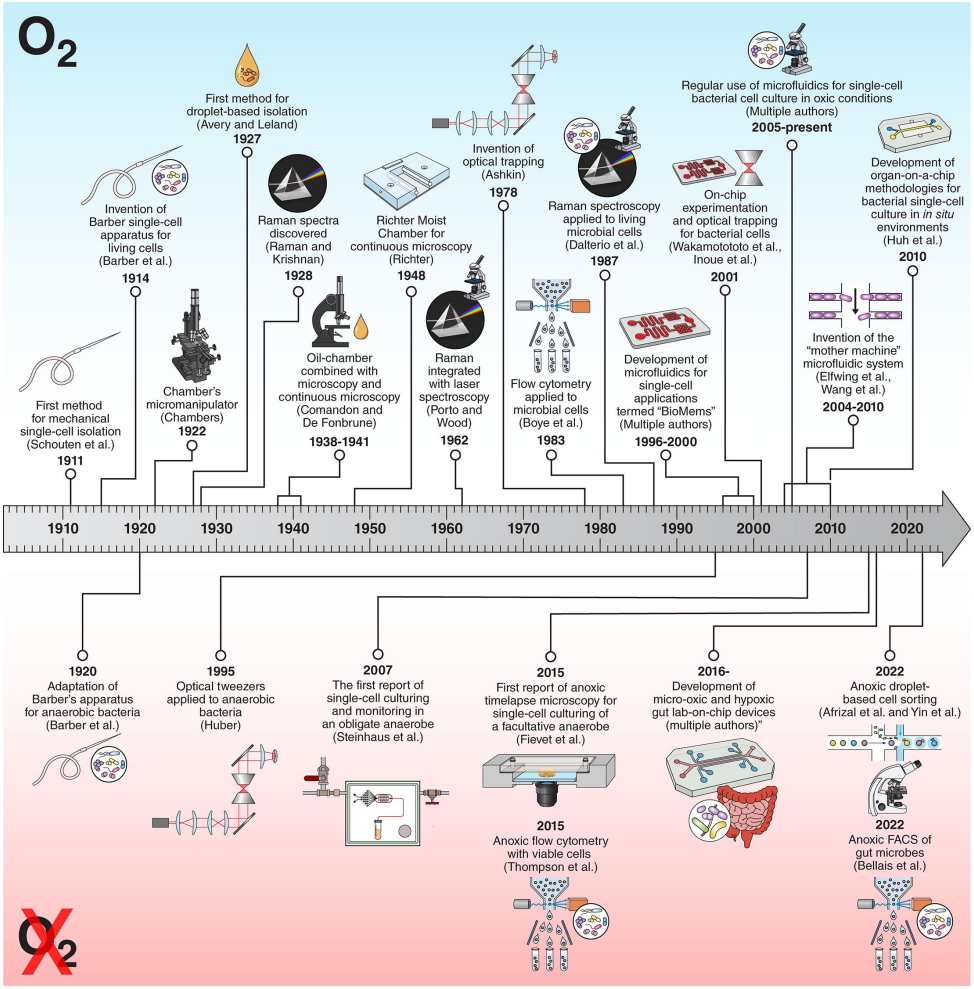

FIG 2 Timeline showing the developments in aerobic microbiology (shown on top) parallel to the developments in anaerobic microbiology (shown on the bottom, represented with crossed out O_2) in the context of single-cell analysis images and instrumental advances. The full list of references is provided in File S1.

the challenges and limitations of anoxic single-cell applications and pose exciting future research opportunities.

SINGLE-CELL IMAGING IN ANOXIC CONDITIONS

As an integral part of modern cell biology, single-cell imaging encompasses the exploration of living cells through fluorescence microscopy. It enables researchers to visualize biological processes and track and quantify molecules. Imaging of living anaerobic cells has two major requirements: first, labeling the cells while keeping them anoxic and viable and second, maintaining anoxic conditions during imaging. Visualization based on fluorescence proteins (FP), dyes, and other fluorophores is widely used under oxic conditions. Using genetically encoded FPs to specifically label molecules in living cells also circumvents the challenge of cell permeability of externally added dyes or molecules, e.g., antibodies, being then one of the most widely used tools in fluorescence microscopy. A broad range of FPs with different properties, such as color, pH, and temperature stability (47–50), photo-conversion (51), photo-activation (52, 53), or split variants for protein-protein interactions (54), are readily available. However, most of these conventional FPs, such as the blue-green fluorescent protein (GFP) (55) or red mCherry (56), are dependent on the presence of O_2 to develop a fluorescence signal (57, 58). In fact, GFP has been used as a reporter for hypoxia at O_2 concentrations of at

least 0.1%, while conditions below 0.02% O_2 lead to a significant loss of fluorescence signal (59), clearly showing the limitation of GFP in oxygen-limited experiments. FPs like GFP or mCherry have been used as reporter proteins for anaerobic organisms; however, O_2 was added at some point to the culture, during fixation or imaging to obtain the fluorescence signal (45, 60). It is expected that the necessary oxygen concentrations to obtain a fluorescence signal would lead to oxygen-stress in the cells during live-cell imaging with consequences in growth behavior or loss of viability (45), which greatly limits the application of FPs for live-cell imaging of anaerobic microorganisms.

Imaging methods based on genetically encoded labeling

In the past years, alternative fluorescence labeling methods have been established to compensate for the lack of oxygen-independent FPs (61–63). One of the first FPs demonstrated to function as fluorescence reporter genes in live anaerobic bacteria was Flavin mononucleotide (FMN)-based fluorescence proteins (FbFPs) (62). They are based on the bacterial blue-light sensitive and photoactive light-oxygen-voltage domain (LOV). Different variants of FbFPs such as iLOV, evoglow, and miniSOG are known to be functional as fluorescent markers in gut bacteria under different biological conditions (64–68) but also in eukaryotic cells under anoxic conditions (63, 69). The characteristics and applications of FbFPs have been reviewed before (70). The advantage of FbFPs is their small size and the availability of FMN in most cells. However, the fluorescence signal emitted by FbFPs remains dim in comparison to enhanced GFP (eGFP). In addition, the signal is in the blue spectral range and, therefore, overlaps with intrinsic cellular fluorescence making the differentiation of proteins labeled with FbFPs challenging. This can especially be a disadvantage when studying methanogenic archaea and some bacteria which possess a strong green blue autofluorescence attributed to the methanogenic redox cofactor F_{420} or other fluorescent molecules present in cells (71, 72). Several studies have been performed to generate FbFPs with shifted spectra by exchanging amino acids forming a stable FPs with moderate success (73).

Bioorthogonal labeling approaches for live imaging have gained traction in recent years (74, 75). There are a variety of bioorthogonal labeling methodologies. In this section, we will discuss those which require *a priori* knowledge of the genetic system and have been tested in anaerobic organisms. For example, self-labeling protein tags, such as HaloTag (Promega) (76), SNAP-tag (77), or CLIP-tag (78) (New England Biolabs), enable the attachment of ligands that provide high fluorescence signals and are more photostable than FPs. These tags are highly specific to their ligand forming an irreversible protein-ligand bond. Ligand libraries with a broad range of spectral behaviors are available. The HaloTag system has been shown to be functional for anoxic single-molecule imaging of *Bacteroides thetaiotaomicron* (79, 80). Furthermore, SNAP-tag, CLIP-tag, and HaloTag have been demonstrated to be suitable anoxic fluorescence reporters in some *Clostridium* species and *Porphyromonas gingivalis* (81–83). The organic ligands, however, can be cytotoxic, are often poorly cell permeable, and can require excessive washing to avoid background fluorescence. In anoxic applications, extensive wash steps are opportunities for oxygen intrusion. An alternative innovative approach is the use of fluorogenic proteins, including the bilin-binding UnaG, infrared FP IFP2.0, and near infra-red FPs, which rely on protein-ligand complex formation for fluorescence signal generation, thus mitigating the need for washing steps. UnaG has similar spectral characteristics as GFP and has been demonstrated to be functional under anoxic conditions during two-color imaging with IFP2.0 in *Bacteroides* species and in mammalian cells (84–87). IFP2.0 emits fluorescence in the far-red spectrum. The emergence of the fluorescence-activating absorption-shifting tag (FAST) has further expanded the anoxic imaging toolkit, with successful applications in diverse bacteria and archaea (88–92), offering reversible, quantitative, and bright fluorescence upon ligand binding. Nevertheless, challenges persist. Oxygen-independent fluorescence labels often require exogenous ligands, introducing limitations due to cellular permeability. So far, most fluorescence labels are either used for protein localization or tracking and gene

expression analysis. However, the development of split variants of SNAP-, SNAP/CLIP-, and FAST-tag for protein-protein interactions might lead to future studies of cellular processes in anaerobic organisms (93–95). Besides FPs and self-labeling protein tags, FlAsH, a biarsenical derivative of the dye fluorescein-5-isothiocyanate (FITC), has been used in *B. thetaiotaomicron* for live single-molecule fluorescence imaging of an outer membrane protein under anoxic conditions (80). FlAsH covalently binds to a tetracysteine motif introduced into the protein sequence.

RNA-based fluorescent biosensors like Spinach2 have been shown to detect c-di-GMP in *E. coli* under anoxic conditions (96). The Spinach2 aptamer is a short single-stranded RNA molecule which binds an external chromophore. Fused to a second detection aptamer, the fluorescence signal is dependent on the presence of a ligand. It provides high fluorescence, fast activation, and specific binding to study c-di-GMP signaling in live bacteria under oxygen-free conditions. In addition, Wang et al. (96) have proposed that related aptamer-dye pairs could also be used for other small molecules or proteins and for imaging of gene expression levels. Another oxygen-independent fluorescence labeling is expanding the genetic code by incorporating unnatural amino acids (UAAs) into proteins. This method enables fluorescence live-cell imaging and bypasses the disadvantage of bulky fluorescence labels and, therefore, compatible with cell viability. Genetically encoded UAAs provide target-specific labeling (97). With a minimal distance between probe and target, UAAs have been successfully used for super-resolution microscopy. Several fluorescent amino acids to be incorporated at amber codons are available (98, 99). An overview of anoxic fluorescence microscopy labeling methods is shown in Fig. 3.

Alternative labeling strategies for microbial species without genetic systems

The FP overviewed above rely on the use of expression vectors, and thus, they require the availability of genetic systems and expression vectors for the target microorganism. Unfortunately, genetic systems are only available for a limited number of microbial species. This excludes the use of expression vector-coded FPs for yet-uncultured microorganisms or those with a non-yet established genetic system. To circumvent this problem, an alternative is the use of fluorescence labeling strategies with non-genetically encoded labels, like fluorescence *in situ* hybridization (FISH) methods.

Conventional FISH-based methods are typically destructive for anaerobes, requiring long and complicated protocols (up to 30 h) including fixation, permeabilization, and washing steps. Fixation-free FISH methods omit the fixation step but still need long incubation times for hybridization and washing (100, 101). These steps can compromise cell integrity, leading to a loss of cell viability. While these methods can be used for downstream applications like proteomics or metagenomics, a shorter protocol reducing oxygen exposure and free of chemicals that could damage the cells would be preferred for continuous imaging of viable living cells. FISH of transcript-annealing molecular beacon (FISH-TAMB) requires only a short incubation time (which can be performed in an anaerobic chamber), and it is fixation-free, both minimizing the risk of oxygen contamination and of cell damage considerably (102). FISH-TAMB has been used for imaging of the strict anaerobic archaeon *Methanosarcina barkeri* and anaerobic methanotrophic archaea, leading to the detection of non-culturable taxa, active gene expression, and different transcript expression levels. Harris et al. have shown that FISH-TAMB-treated cells show little impact on cell viability (102). As it is a quite new method, not many studies using FISH-TAMB are published, and it likely needs further improvements to be used for other species.

Alternatives to FISH-TAMB could be, for example, small cell permeant fluorescence dyes, e.g., MitoTracker, CellMask, or SYBRSafe dyes (Invitrogen), which are independent of both molecular oxygen and of an available genetic system. These stains have been shown to be non-cytotoxic and lead to efficient fluorescence staining under different extreme physiological conditions, e.g., high-salt, high-temperature, and low pH (103, 104). However, the dye permeability and functionality are strongly dependent on the

Fluorescence label	Advantages	Disadvantages	Used application	Organism	Reference
FMN-based FPs					
LOV	- no external ligand necessary in FMN producing species	- only blue emission • overlaps with intrinsic fluorescence • no multicolor imaging	Fluorescent marker	*Rhodobacter capsulatus*	(62)
iLOV (phiLOV/CreiLOV)			Gene expression reporter	*Clostridium* species	(68)
Evoglow®-Bs1, -Bs2, -Pp1			Fluorescent marker	*Clostridium* species, Lactic acid bacteria, *Enterococcus faecium, Bifidobacterium breve*	(64-66)
CaFbFP			Fluorescent marker	*Candida albicans Saccharomyces cerevisiae*	(69)
Fluorogenic proteins (reversible)					
UnaG	- UnaG: only protein/ligand complex fluorescent, photoswitchable - FAST-tag: split-variant available for interactions studies	- external ligand necessary for most species • Not always cell permeable	Fluorescent marker	*Bacteroides* species, mammalian cells	(84, 85-87)
IFP2.0			Fluorescent marker	*Bacteroides* species	(84)
FAST-tag			Fluorescent marker	*Clostridium* species, *Methanococcus maripaludis, Eubacterium limosum*	(88, 89, 92)
Self-labeling protein-tag (irreversible)					
HaloTag®	- stable covalent ligand bond - enhanced brightness - high specificity - broad variety of ligands for different applications - HaloTag®: exchangeable ligands - SNAP-tag®: split-variant for interactions studies	- External ligand necessary • Not always cell permeable • Can require extensive washing - ligand itself fluorescent - most tag/ligand binding irreversible	Single-molecule imaging, Fluorescent marker	*Bacteroides thetaiotaomicron, Clostridium* species	(80, 81, 83)
SNAP-tag®			Fluorescent marker	*Clostridium* species, *Porphyromonas gingivalis*	(81, 82)
CLIP-tag™			Fluorescent marker	*Clostridium* species	(83)
Biarsenical-tetracysteine tag					
TC-FlAsH™	- only protein/ligand complex fluorescent	- non-specific binding	Single-molecule imaging	*Bacteroides thetaiotaomicron*	(80)
FISH-based					
FISH-TAMB	- no genetic system necessary - no fixation - only probe/mRNA complex fluorescent	- permeability dependent on species	Species identification, mRNA labeling	*Methanosarcina barkeri, ANME consortium*	(102)
Aptamers					
Spinach2, Ct, Dp, PIA, PI-B	- fluorescence only in presence of ligand	- external chromophore	c-di-GMP biosensor	*Escherichia coli*	(96)
Bioorthogonal chemistry with linked dyes					
Alexa fluor dyes linked via azide-modified sugars	- no external ligand required for fluorescence - enables labeling of metabolically active cells	- labels all metabolically active cells	Active cell labeling & live imaging	Anaerobic gut bacteria	(111)
Reaction with azide-modified sugars and labeling with fluorescent alkyne or dibenzocyclooctyne (DIBO) via copper-independent reaction				Anaerobic gut bacteria (*Bacteroidetes fragilis*)	(112)

FIG 3 Overview of fluorescence labeling methods used under anoxic conditions.

investigated organism and its physiological characteristics. Nanobodies (tiny, recombinantly produced domains of heavy-chain only antibodies) and chromobodies (small intracellular functional antibody genetically fused to an FP) represent further options to broaden the spectrum of available live-cell imaging methods for anaerobic organisms (105) as their functionality is independent of oxygen. However, their applicability in anaerobic microorganisms has yet to be demonstrated.

Bioorthogonal fluorescence labeling approaches that do not require prior knowledge of the cell's genetic system are an alternative solution for studying *in situ* interactions in anaerobic microbial communities (106). These methods enable protein tagging, cell labeling, and tracking physiological activity in microbial communities in a broad range of conditions by using labels that minimally interfere with the biology of the cell (107). In terms of anaerobic applications, methods like bioorthogonal noncanonical amino acid tagging (BONCAT) have been applied to label the translationally active members of microbial communities. BONCAT-labeled cells can be further visualized and identified using FISH as shown in an anaerobic methane-oxidizing (ANME) community (108) and various bacterial and archaeal isolates (109). However, the FISH step in these cases requires genetic information to design specific probes. Alternatively, BONCAT can be combined with click chemistry for imaging using oxygen-independent fluorophores, as shown in studies where BONCAT with TAMRA-alkyne was used to investigate protein expression during anaerobic survival in *Pseudomonas aeruginosa* (110). A limitation of these techniques, including the previously mentioned methodologies for fluorescence microscopy, is the limited number of applications under anoxic conditions in live-imaging experiments and, therefore, limited information on cell viability (Fig. 3). Thus, it remains often unclear how the cells are affected in their viability by factors such as label and reagent toxicity, and efficiency of label uptake. Live-imaging conducted in the gut environment suggests that copper-free BONCAT reactions improve cell viability as tested in the anaerobic bacterium *Bacteroidetes fragilis* (111, 112). Increased use of bioorthogonal methods in anoxic conditions will improve the methodology and expand the toolbox that can be used to study anaerobes and their role in communities.

Imaging methods using microfluidic experimentation

Microfluidic (or lab-on-chip) devices can confine cells into a controlled space, allowing their visualization and tracking using high-resolution microscopy. Chemical conditions in these devices can be tightly controlled to mimic *in situ* conditions, and depending on the device design, the placement of microbial cells can also be configured. Single-cell microfluidic platforms can show heterogeneity in individual cell growth rates and other cell responses that would otherwise be masked by batch culture (113). For a review of single-cell microbial cultivation using microfluidics, see Anngraini et al. (114). Despite this field experiencing exponential growth from the early 2000s, applications in anoxic conditions remain relatively few. We will discuss the key advances in single-cell imaging using microfluidics under anoxic conditions below.

One of the first instances of anoxic (anaerobic), single-cell imaging using microfluidics was performed by Steinhaus et al. (44) using the methanogen *Methanosaeta concilli*, arguably one of the strictest anaerobes known (44). This sophisticated system contained a sealed portable chamber containing either a shear flow or concentration gradient microfluidic device (Fig. 4A). Hydrogen sulfide was used to scavenge oxygen from the chamber. Additionally, the system was placed in an anaerobic glovebox and was removed once per day for imaging purposes. During imaging, the system was sparged with N_2 to minimize oxygen intrusion. Using this setup, the authors determined the optimum shear rates for maximum biomass production, as well the pH and ammonium concentrations required for optimal growth of *M. concilli*. It was over a decade later that single-cell imaging in anoxic conditions was again applied to an environmental organism (115). In this application, *Desulfovibrio vulgaris* was cultured in a microfluidic device combining a cover slip flow chamber with an agar pad, sealed within a gasket chamber [(45); Figure 4B]. The device was continuously monitored under the microscope. Anoxic

conditions, however, were not stringent as the authors tested the response of *D. vulgaris* to oxygen stress. Real-time visualization with high-resolution microscopy was conducted, with cells visualized via fluorescence using GFP labeling (requiring oxygen exposure). The study provided valuable insight into cell division in *D. vulgaris* and showed that cell division was halted by oxygen exposure. However, the agar pad methodology used in the study has disadvantages such as the squeezing of cells, challenges in tracking cells, and difficulty in maintaining a homogenous environment during experimentation (116).

In clinical research, there has been a surge in organ-on-chip devices to study host systems. One such area is the interface between host cells and anaerobic microbial populations (for example, the intestinal system), which has been extensively reviewed elsewhere (117–119). A review by Bossink et al. (120) paid particular attention to anoxic systems, covering progress, and considerations such as oxygen sensing and device fabrication (120). Some examples of anoxic applications include Shah et al. (19), Greenhalgh et al. (121), Jalili-Firoozinezhad et al. (46), Wang et al. (122), and Shin et al. (123), achieving variable oxygen concentrations from 0.1% to 0.9% oxygen. These authors all used some variation of a microfluidic device to monitor the interaction between an endothelial cell layer and anaerobic bacteria. Most included oxygen-sensing capacity in the devices. However, given the reported variation in sensor capacity, oxygen monitoring, and reported concentrations (often in percentages rather than in parts per million, ppm), therefore, these studies may be considered to be performed in micro-oxic or hypoxic conditions). While these examples highlight the technical challenge of hypoxic to micro-oxic cultivation in complex *in situ* environments with the capacity for single-cell imaging, the focus was geared toward culturing and other downstream

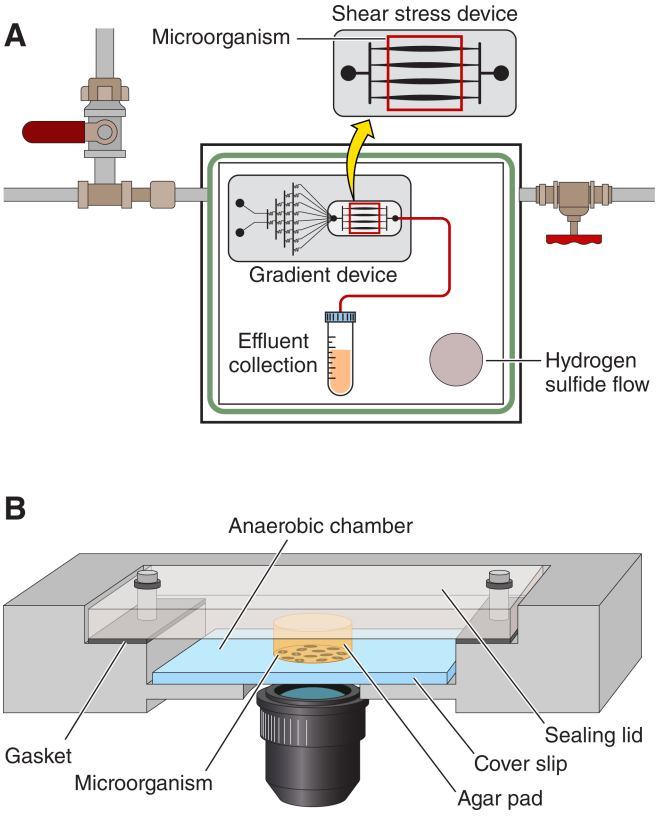

FIG 4 Schematic of two anoxic single-cell applications using microfluidic experimentation. (A) shows the system developed by Steinhaus et al. (44) which uses microfluidic devices encased in an anoxic environment sparged with N_2 gas. The device was placed in an anaerobic glovebox and imaged on a microscope once per day. (B) shows the system employed by Fievet et al. (45) and adapted from Ducret et al. (115) which employs a simple coverslip and agar pad which was closed via plates and gaskets and continuously monitored on a high-throughput microscope.

applications, and thus, they lack insight into the physiology and activity of individual anaerobic microorganisms.

Moreover, the complex intestinal microfluidic systems described above tend to be used to culture facultative anaerobes and aerobes in layers and have the advantage that the system itself can become oxygen scavenging as oxygen-tolerant bacteria use oxygen in the system thereby increasing the anoxic conditions over time (123, 124). Interestingly, a study by Grant et al. (125) further advanced the organ-on-chip and assessed various means of oxygen control (respiration of aerobic cells, microfluidic device coatings, and fluid flow) to establish hypoxia (125). Nonetheless, it is anticipated that pure culture strict anoxic single-cell studies will be more challenging. However, these advances showcase the potential to adapt such technologies to the field of environmental anaerobic microbiology to reveal, for example, phenotypic heterogeneity (e.g., individual cell growth rates) in biotechnologically relevant organisms, the mechanisms underpinning syntrophic interactions, and, indeed, cellular mechanisms (e.g., cell division) in anaerobic microbes.

CELL SORTING UNDER ANOXIC CONDITIONS

The first attempts to perform cell isolation for anaerobes were done over a hundred years ago using an instrument specifically designed for anaerobic single-cell isolation (23). However, despite several successful isolation attempts using this method, it was considered challenging and was even described to be "wasteful of time, material, eyesight and nervous energy" (126). Currently, single-cell sorting of anaerobes is no less challenging, but with the development of automated systems and modernization of lab infrastructure, new attempts can be made to separate microbes from their communities (127, 128). Thompson et al. (129) used a cell sorter equipped with a sealed chamber fluxed with N_2 to perform the selection of the cells and the sorting, and an anaerobic glovebox around the stage where the cells were deposited after sorting, enabling sorting of cells in anaerobic fashion. This enabled the isolation of individual members of a co-culture community and allowed the assessment of the cell viability of differently evolved cell lines, showing the feasibility of this approach (129). Similarly, using fluorescence-activated cells via antibodies, Bellais and co-workers enabled cell sorting using flow-cytometry equipped with an anaerobic glovebox and obtained viable strict anaerobic pure cultures from fecal samples (130). Droplet-based cell sorting could similarly be used to obtain a large spectrum of viable anaerobic isolates without the use of labels (28, 29). This droplet-based approach was performed in an anaerobic glovebox and required a shorter experimental timeframe compared to the traditional plating approach and resulted in fast isolation of viable anaerobic pure cultures from fecal samples. A different approach to isolate individual cells from complex communities is via optical tweezers, where a strong light bundle is used to trap individual cells and move them to a separate environment. This method was applied to achieve viable pure cultures of anaerobic archaea (32). Briefly, the target cell is identified and fixed via a laser beam. The microscope stage is used to maneuver a single cell from the mixed sample inside a glass capillary connected to a micro-syringe. The glass capillary is then cut to separate the single cell from the mixed cells at the end of the capillary. The syringe is used to transfer the cell directly and swiftly into fresh anaerobic culture medium, enabling subsequent cultivation (Fig. 5). With a similar approach, this method has also been applied to co-cultures of the anaerobic archaea *Nanoarchaeum equitans* and *Ignicoccus hospitalis*, to study their interaction (131). While the optical tweezer approach in these studies is not described as being performed in anoxic fashion (e.g., in an anaerobic glovebox), still viable cells of anaerobic archaea could be obtained. It remains a question if more strict anaerobes, such as methanogenic archaea, would survive such a procedure.

The methods described above all rely on some form of selection, via either labeling or morphological identification (e.g., cell shape), and therefore, rely on prior knowledge about the target. In order to label and sort relevant microbes from undefined (and often

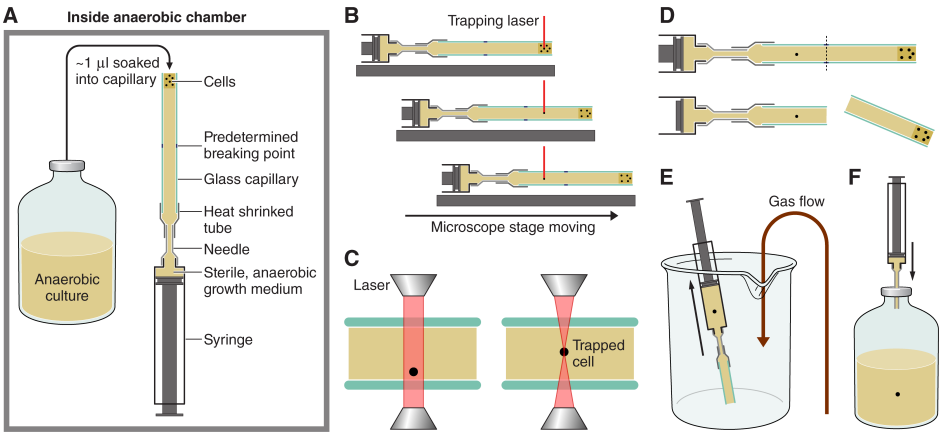

FIG 5 Isolation of a single cell via Optical Tweezer. To avoid exposure to oxygen, an anaerobic glovebox should be located next to the microscope. (A) A 1-mL syringe with a needle is connected via a tube to a rectangle glass capillary (inside 0.1 × 1 mm, length 10 cm) with a predetermined breaking point. The connection tube is heat-shrinked. The needle and capillary are filled with sterile, anaerobic growth medium up to 90% of the capillary volume. About 1 µL of an anaerobic culture is soaked into the open end of the capillary. (B) The capillary with the syringe is fixed on a microscope stage of an inverted microscope (Axiovert IM35, Zeiss, Oberkochen, Germany). For imaging, an oil immersion objective (100/1.3) and a Nd-YAG laser are used (wavelength 1,064 nm; maximum power 1 W, ADLAS, Lübeck, Germany). The cell suspension is imaged under 1,000×-fold magnification. Cell separation happens by moving the microscope stage and (C) optically trapping a single cell in the laser beam. (D) After separation, the capillary is taken out of the microscope and cut into two pieces at a predetermined breaking point. (D) Immediately, the syringe is put into a beaker filled with anoxic gas, and the cell is pulled "anoxically" into the syringe and (E) flushed into fresh anoxic growth medium for incubation [modified after reference (32)].

poorly understood) anaerobic communities, bioorthogonal chemistry approaches as described previously are an effective alternative (106). BONCAT can be followed up by FISH or fluorescence-activated cell sorting (FACS) and sequencing, allowing the user to sort and identify the active members of the community, as demonstrated in an anaerobic methane-oxidizing (ANME) community (108), anammox (132), and wastewater communities (133). If the cell sorting technique requires the anaerobes to remain alive, all reactions should be able to proceed in the absence of oxygen and, thus, also require fluorophores that work in such conditions. Further development of these bioorthogonal chemistry approaches could open up research opportunities related to fundamental physiology, spatial organization, host-microbe interactions, and growth rate assessments of strict anaerobic communities.

Cell-sorting applications open a world of possibilities for anaerobes when integrated with downstream pipelines (Fig. 6). For example, single-cell RNA sequencing and single-cell genomics or strain-resolved genomics, while still technically challenging, can already be applied to some microbes (134). This gives insights into the transcripts, function, and taxonomy of individual cells. These types of methods do not require maintenance of anoxic conditions after the cell harvesting procedure, allowing it to follow the original protocols. Other applications, however, like live-cell-imaging or follow-up cultivation, do require maintenance of anoxic conditions after sorting; then the further downstream pipelines need to be specifically designed to maintain anoxic conditions (Fig. 6).

FUTURE RESEARCH OPPORTUNITIES

Techniques showing promise for future anoxic single-cell studies are highlighted below as well as the technical challenges to overcome and examples of lessons learned from aerobic studies.

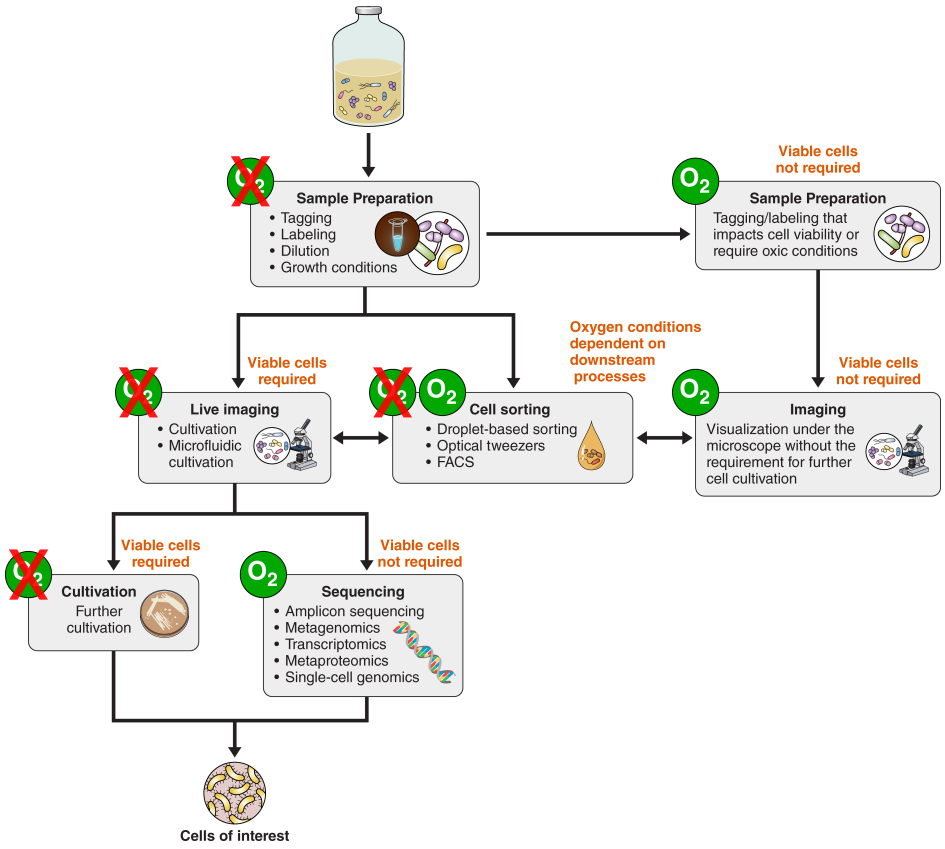

FIG 6 Overview of a sample experimental anoxic single-cell cultivation workflow and downstream processes, outlining where anoxic conditions may be required. Oxic/aerobic is represented with O_2, and anoxic/anaerobic as crossed out O_2. Note: Raman spectroscopy could be considered under the "imaging" or "live-imaging" heading depending on the approach used and the requirement for viable cells.

Parallels to aerobic cultivation

In this review, we have focused on the advancements made in anoxic single-cell studies. Many of these studies focus on method development, and this is warranted given the lack of tools available for anoxic single-cell cultivation. To highlight the value in these method developments, we will briefly describe interesting scientific discoveries that have been derived from aerobic single-cell cultivation studies. Microfluidics cell sorting and isolation have been used to overcome the problem of the uncultured majority, thus, identifying novel species (135). Samlali et al. (136) used droplet-based cell sorting to sort fungal cultures with a particular trait of interest—cell-wall-degrading enzymes to rapidly develop a population with the maximum desired activity (136). Microfluidics cultivation has also shed light on the phenotypic heterogeneity of clonal bacterial populations (137). Cell growth rates and population evolutionary history can also be determined using the Mother machine, which consists of trapping the mother or parent cell in a microchannel's dead-ends of a microfluidic device and letting the population grow (42, 138, 139). Microfluidics have also been used to elucidate microbial cross-feeding, cellular communication, and response to resource variations (e.g., (15). Similar advancements could be made in the field of anaerobic microbiology if these innovative methods were adapted accordingly (Fig. 7).

Raman spectroscopy

One technique which shows a great deal of promise for future anoxic live microbial single-cell applications is Raman spectroscopy. Raman has traditionally been used in

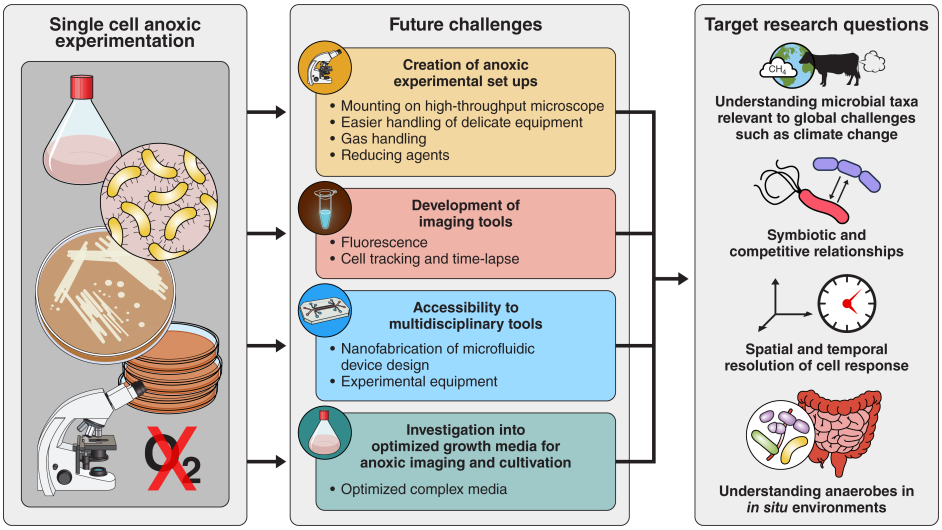

FIG 7 Overview summary describing the future challenges to be solved and potential research questions advances that single-cell anoxic applications could solve. Anoxic/anaerobic is represented with crossed-out O_2.

chemistry, material science, and the pharmaceutical industry but, in the past 15 years, has seen increased use in varying subfields of microbiology (140). In Raman spectroscopy, a laser light source is applied to a sample and the scattering of this light can be used to determine information on the molecular bonds and chemical structure of the sample. It is a non-destructive and rapid technique that can provide metabolic and physiological information from single cells. Lee et al. (2021a) provide an extensive overview of the potential of different Raman methodologies for microbial cells (140). For living cells, the technique can be used to analyze communities *in situ* in liquid culture. Regarding anaerobic microorganisms, these could be analyzed *in situ* as long as anoxic conditions are met, and potentially, they would be viable after the analysis to proceed with anoxic downstream analyses (Fig. 6).

Specifically, in the application of Raman microspectroscopy, an integrated microscope can be used to select the area of interest in the sample. The sample can be held within a microfluidic device for highly controlled fluid flow (141, 142). Labeling approaches (see above sections on imaging labeling with and without a known genetic system) are advised as the application of Raman techniques to mixed and even pure cultures would generate a lot of noise due to variations in cellular composition and environmental conditions in the liquid medium (143, 144). Raman microspectroscopy has been also used in combination with stable isotope probing (141, 142) to label cells with the desired metabolic phenotypes of interest. FISH has also been applied together with Raman microspectroscopy to better pinpoint the diversity of the targeted microorganism(s) (145). Note that to maintain viable labeled cells during Raman, non-toxic labeling approaches may be more appropriate here (e.g., genetic labeling, FAST-tag, FISH-TAMB, or other approaches as outlined in Fig. 3). However, these labeling approaches are currently untested in combination with Raman microspectroscopy. Cell sorting via optical tweezers can then be used to sort the cells for downstream applications (142). The study by Lee et al. (142) highlights the development of a fully automated optofluidic platform for Raman-activated microbial cell sorting (RACS), and the authors provide a detailed protocol (146). To adapt these procedures to anoxic cultivation experiments would require placing the entire experimental set-up inside an anaerobic glovebox, which may be challenging due to the space requirement and additional risk assessments required for working with lasers and hydrogen mixed gas. The more feasible option would be to create an anoxic cultivation platform that could be embedded on the microscope or around the sample stage. For example, an oxic/hypoxic microfluidic system for mammalian muscle cell culture has been integrated with Raman

spectroscopy previously although the exact oxygen concentrations were not reported (147). Other considerations would include anoxic medium development compatible with this approach as Raman requires a non-photoluminescent media not masking cellular signals. In microfluidic systems, additional considerations include the device material and gas flow which may interfere with the Raman signal (142, 147).

Technical challenges for future anoxic single-cell applications

While, in recent years, significant developments have taken place, anoxic single-cell techniques remain underdeveloped compared to oxic methods. The requirement for the absence of oxygen results in physical challenges of equipment placement in anaerobic gloveboxes, resulting in more complex handling conditions and more expensive set-ups. For techniques operated on a relatively short timescale (e.g., cell sorting, short term-imaging), anoxic requirements are less strict and can be viable as long as oxygen exposure remains limited. Significant challenges arise, however, when it comes to prolonged experiments looking at population dynamics or extended growth curves, which are the common questions tackled with live-cell imaging methods. Due to the relatively slow growth of anaerobes, they suffer from environmental changes (e.g., substrate depletion, waste accumulation) that need to be controlled anoxically for prolonged periods of time. In addition, cell tracking for these prolonged periods can be challenging due to the small size of prokaryotes in general. Future research, therefore, requires the development of tools specifically for anaerobic high-throughput cultivation and imaging purposes. These could include, for example, the development of custom-made anoxic incubators that could be mounted on high-throughput microscopes, together with pipetting robots operating in an oxygen-free atmosphere, the development of anaerobic gloveboxes with greater flexibility for embedding large equipment, and further research on oxygen-independent fluorescent labels. An additional challenge is to further develop culture media for imaging purposes which does not interfere with the visualization due to cloudiness and precipitation of substrates. We have summarized the challenges in anoxic live-imaging and offered potential solutions in Fig. 8.

CONCLUSIONS

Overcoming the technical and methodological challenges described above would open up new horizons and advance our understanding of anaerobic microorganisms and microbial functions in anoxic environments (Fig. 7). For example, with better anoxic imaging and microfluidic experimentation, we could unravel phenotypic heterogeneity (i.e., differences in phenotype within a clonal population), phenotypic plasticity (i.e., differences in phenotype in response to variation in micro-climate), mutation rates, cellular function, and fitness rates of anaerobic populations which, at this point, are largely unknown. Moreover, these techniques could be integrated in further downflow processes (which may or may not require anoxic cultivation; Figure 6). For example, single-cell RNA sequencing and single-cell genomics or strain-resolved genomics, while still technically challenging, can already be applied to some microbes (134). This gives insights into the transcripts, function, and taxonomy of individual cells. Overall, we hope that this mini-review serves as inspiration for the use of single-cell applications under anoxic conditions. As a community of microbiologists, we need significant investment in anoxic methodological advances. Anaerobic microbiology can be an elitist field as considerable investment is needed to accommodate expensive and bulky equipment. Moreover, experiments are often slower and the reward slower in the current highly competitive academic landscape. This challenge is potentially the reason for the slower developments in innovative single-cell methodologies or why innovative methods are not widely or rapidly adopted (as shown in Fig. 2). To overcome this one avenue is for funding agencies to fund more projects focused on method development although this does not overcome the slower development time.

For individual labs and researchers, our general recommendation to implement single-cell application under anoxic conditions is to give priority to ensure anoxic

conditions in their set-up (either cell-sorting or visualization purposes). As it is challenging to get access to commercial anaerobic glove bags due to the high prices, an alternative would be to collaborate with industry to make custom-made boxes, and/or microfluidics devices, as well as to rely on collaborations with labs where these methods are well established. In this way, the resources available are more optimized and it promotes further collaborations with other research groups. Other more long-term solutions could include a global centralized state-of-the-art facility, akin to the diamond labs synchrotron facilities (https://www.diamond.ac.uk/Home.html) where researchers could obtain access through competitive proposals and conduct high-throughput multi-disciplinary anoxic experiments. However, the most tangible option is for microbiologists to come together as an organized community of researchers to reduce the research silos from different disciplines and different expertise areas (e.g.,

	Anaerobic live imaging challenges		Potential solutions	
Technical challenges (anoxic conditions)	**Complex handling conditions** including working anaerobically either inside or outside an anaerobic glovebox			
	Inside glovebox	*Outside glovebox*	*Inside glovebox*	*Outside glovebox*
	• Restrictive environment • Handling delicate equipment with large gloves (e.g. microfluidics) • Placing equipment inside the glovebox	• Maintenance of anoxic conditions • Provision of environmental conditions including gases and temperature	• Work inside glovebox with temperature control • Improve the design of anaerobic gloveboxes and reduce costs • Working outside of the glovebox	• Stage top incubators for temperature and gas control mounted on a microscope • Glass devices for less permeability to oxygen
	Precipitation of anaerobic medium Complex anaerobic medium with reducing agents such as sodium sulfide precipitate causing issues for live imaging		Develop optimized anaerobic medium for microscopic observation	
Technical challenges (experimental conditions)	**Waste accumulation** Accumulation of waste products (e.g. fermentation products)		Continuous cultivation (e.g. continuous medium feed in microfluidic chemostat systems)	
	Prokaryotic cell tracking • Prokaryotic cells are challenging to track in brightfield conditions • Fluorescent proteins genetically encoded and/or/ require oxygen to fluoresce		• Improve cell tracking software (ideally open-source software) • Develop more fluorescent proteins that do not require oxygen (e.g. phiLOV) • Further development of dyes and tags that do not require a genetic system	
	Slow growth of anaerobic microorganisms Most anaerobic microorganism require long cultivation times (e.g. days to weeks) which are challenging to monitor for a number of reasons: • Waste accumulation (discussed above) • Cell tracking (discussed above) • Large computational power for image analysis • Changing of environmental conditions over the experimental timescales e.g. gases may need to be changed in the glovebox or the culture medium source may need to be refreshed		• Use of high-performance computing storage for image files • Greater environmental control of the sample either inside or outside the glovebox • Minimize disturbance to the experiment with large reservoirs of culture medium	
	Multi-disciplinary skillset The use of microfluidics and fluid handling requires skills not traditionally taught in microbiology programs e.g. • Use of CAD software • Nanofabrication for devices to hold prokaryotes and suitable for experimental design		• Collaborate with biomedical (or other) engineers • Increase training in microfluidic fabrication • Increase options for off-the-shelf microfluidic devices for prokaryotes (with reduced cost and greater design flexibility)	

FIG 8 Overview of the challenges for anoxic live-imaging and suggested solutions.

between medical and environmental anaerobic microbiologists). We need initiatives to share resources and protocols, more collaboration avenues, and to design potentially cheaper alternatives to anaerobic gloveboxes (such as 3D printed incubators for anoxic visualization). In addition, we need to work with engineers, chemists, industrial partners, and other researchers to build new anoxic experimentation systems, synthesize new dyes and labels, off the shelf microfluidic systems for prokaryotic cells and other tools. We have created an anoxic cultivation special interest group hosted on Discord which welcomes discussions on how to implement methodological advancements for the benefit of the environment and public health (the invite link is https://discord.gg/Gy5egqkAdV).

ACKNOWLEDGMENTS

The project was funded by Netherlands Ministry of Education, Culture and Science (SIAM Gravitation grant, project 0.24.002.002) and the Dutch Research Council (NWO). L.V. and K.F. were also supported by the Moore–Simons Project on the Origin of the Eukaryotic Cell, Simons Foundation 735929LPI, https://doi.org/10.46714/735929LPI. M.D. was supported by a VENI research grant (VI.VENI.212.112) of the Dutch Research Council (NWO). C.K. acknowledges funding from the SIAM talent grant which supported a research visit to NIOZ.

The authors wish to acknowledge Patrick Lane at Sceyence Studios for providing the final illustrations.

AUTHOR AFFILIATIONS

[1]Department of Engineering, Durham University, Durham, United Kingdom
[2]Department of Marine Microbiology and Biogeochemistry, Royal Netherlands Institute for Sea Research (NIOZ), Den Burg, the Netherlands
[3]Laboratory of Microbiology, Wageningen University & Research, Wageningen, the Netherlands
[4]Centre for Living Technologies, Alliance TU/e, WUR, UU, UMC Utrecht, Utrecht, the Netherlands
[5]Department of Biology, Utrecht University, Utrecht, the Netherlands

AUTHOR ORCIDs

Ciara Keating http://orcid.org/0000-0001-9199-3068
Kerstin Fiege http://orcid.org/0000-0002-1117-5602
Martijn Diender http://orcid.org/0000-0002-0408-688X
Diana Z. Sousa http://orcid.org/0000-0003-3569-1545
Laura Villanueva http://orcid.org/0000-0001-5659-8599

FUNDING

Funder	Grant(s)	Author(s)
Soehngen Institute of Anaerobic Microbiology (SIAM)	0.24.002.002	Laura Villanueva
Nederlandse Organisatie voor Wetenschappelijk Onderzoek (NWO)	VI.VENI.212.112	Martijn Diender
Soehngen Institute of Anaerobic Microbiology (SIAM)	Talent Grant	Ciara Keating Laura Villanueva
Simons Foundation (SF)	735929LPI	Kerstin Fiege Laura Villanueva

AUTHOR CONTRIBUTIONS

Ciara Keating, Conceptualization, Visualization, Writing – original draft, Investigation | Kerstin Fiege, Conceptualization, Investigation, Visualization, Writing – original draft | Martijn Diender, Conceptualization, Investigation, Visualization, Writing – original draft | Diana Z. Sousa, Funding acquisition, Supervision, Writing – review and editing | Laura Villanueva, Conceptualization, Funding acquisition, Supervision, Writing – review and editing

ADDITIONAL FILES

The following material is available online.

Supplemental Material

Supplemental file 1 (AEM01321-24-S0001.docx). List of references for Figure 2.

REFERENCES

1. Brusa T, Del Puppo E, Ferrari A, Rodondi G, Andreis C, Pellegrini S. 1997. Microbes in deep-sea anoxic basins. Microbiol Res 152:45–56. https://doi.org/10.1016/S0944-5013(97)80023-7
2. Canganella F, Wiegel J. 2011. Extremophiles: from abyssal to terrestrial ecosystems and possibly beyond. Naturwiss 98:253–279. https://doi.org/10.1007/s00114-011-0775-2
3. Elias DA, Krumholz LR, Tanner RS, Suflita JM. 1999. Estimation of methanogen biomass by quantitation of coenzyme M. Appl Environ Microbiol 65:5541–5545. https://doi.org/10.1128/AEM.65.12.5541-5545.1999
4. Santiago-Martínez MG, Ferry JG. 2023. Methods for culturing anaerobic microorganisms, p 231–238. In Oxygen sensing: methods and protocols. Springer.
5. Murphy EC, Frick IM. 2013. Gram-positive anaerobic cocci--commensals and opportunistic pathogens. FEMS Microbiol Rev 37:520–553. https://doi.org/10.1111/1574-6976.12005
6. Veloo ACM, van Winkelhoff AJ. 2015. Antibiotic susceptibility profiles of anaerobic pathogens in the Netherlands. Anaerobe 31:19–24. https://doi.org/10.1016/j.anaerobe.2014.08.011
7. Keating C, Chin JP, Hughes D, Manesiotis P, Cysneiros D, Mahony T, Smith CJ, McGrath JW, O'Flaherty V. 2016. Biological phosphorus removal during high-rate, low-temperature, anaerobic digestion of wastewater. Front Microbiol 7:226. https://doi.org/10.3389/fmicb.2016.00226
8. McHugh S, Carton M, Mahony T, O'Flaherty V. 2003. Methanogenic population structure in a variety of anaerobic bioreactors. FEMS Microbiol Lett 219:297–304. https://doi.org/10.1016/S0378-1097(03)00055-7
9. Nzeteu C, Joyce A, Thorn C, McDonnell K, Shirran S, O'Flaherty V, Abram F. 2021. Resource recovery from the anaerobic digestion of food waste is underpinned by cross-kingdom microbial activities. Bioresour Technol Rep 16:100847. https://doi.org/10.1016/j.biteb.2021.100847
10. Hungate R. 1969. Chapter Iv a roll tube method for cultivation of strict anaerobes. Meth Microbiol 3:117–132. https://doi.org/10.1016/S0580-9517(08)70503-8
11. Hungate RE. 1950. The anaerobic mesophilic cellulolytic bacteria. Bacteriol Rev 14:1–49. https://doi.org/10.1128/br.14.1.1-49.1950
12. Balch WE, Wolfe RS. 1976. New approach to the cultivation of methanogenic bacteria: 2-mercaptoethanesulfonic acid (HS-CoM)-dependent growth of methanobacterium ruminantium in a pressurized atmosphere. Appl Environ Microbiol 32:781–791. https://doi.org/10.1128/aem.32.6.781-791.1976
13. Plugge CM. 2005. Anoxic media design, preparation, and considerations. Methods Enzymol 397:3–16. https://doi.org/10.1016/S0076-6879(05)97001-8
14. Laso-Pérez R, Krukenberg V, Musat F, Wegener G. 2018. Establishing anaerobic hydrocarbon-degrading enrichment cultures of microorganisms under strictly anoxic conditions. Nat Protoc 13:1310–1330. https://doi.org/10.1038/nprot.2018.030
15. Kim HJ, Boedicker JQ, Choi JW, Ismagilov RF. 2008. Defined spatial structure stabilizes a synthetic multispecies bacterial community. Proc Natl Acad Sci U S A 105:18188–18193. https://doi.org/10.1073/pnas.0807935105
16. Momeni B, Brileya KA, Fields MW, Shou W. 2013. Strong inter-population cooperation leads to partner intermixing in microbial communities. Elife 2:e00230. https://doi.org/10.7554/eLife.00230
17. Hatzenpichler R, Krukenberg V, Spietz RL, Jay ZJ. 2020. Next-generation physiology approaches to study microbiome function at single cell level. Nat Rev Microbiol 18:241–256. https://doi.org/10.1038/s41579-020-0323-1
18. Carini P. 2019. A "cultural" renaissance: genomics breathes new life into an old craft. mSystems 4:e00092-19. https://doi.org/10.1128/mSystems.00092-19
19. Shah P, Fritz JV, Glaab E, Desai MS, Greenhalgh K, Frachet A, Niegowska M, Estes M, Jäger C, Seguin-Devaux C, Zenhausern F, Wilmes P. 2016. A microfluidics-based *in vitro* model of the gastrointestinal human-microbe interface. Nat Commun 7:11535. https://doi.org/10.1038/ncomms11535
20. Schouten S. 1911. Pure cultures from a single cell, isolated under the microscope, p 1910–1911. Konigl. Akad. Wetensch. APSS.
21. Barber MA. 1914. The pipette method in the isolation of single microorganisms and in the inoculation of substances into living cells: with a technique for dissection, staining and other processes carried out under the higher powers of the microscope. Bureau of Science of the Philippines Government.
22. Chambers R. 1922. New micromanipulator and methods for the isolation of a single bacterium and the manipulation of living cells. J Infect Dis 31:334–343. https://doi.org/10.1093/infdis/31.4.334
23. Barber MA. 1920. Use of the single cell method in obtaining pure cultures of anaerobes. J Exp Med 32:295–311. https://doi.org/10.1084/jem.32.3.295
24. Avery RC, Leland SJ. 1927. A simple method for the isolation of pure cultures from single bacterial cells. J Exp Med 45:1003–1007. https://doi.org/10.1084/jem.45.6.1003
25. Comandon J, Fonbrune P. 1938. Oil chamber for study of living microorganisms, tissue culture and micromanipulation. Ann Inst Pasteur 60
26. Richter KM. 1948. An improved moist chamber slide for use in micromanipulation. Science 108:192. https://doi.org/10.1126/science.108.2799.192
27. Thomas S. 1941. A technique for continuous microscopic observations. Science 93:577–578. https://doi.org/10.1126/science.93.2424.577
28. Afrizal A, Hitch TCA, Viehof A, Treichel N, Riedel T, Abt B, Buhl EM, Kohlheyer D, Overmann J, Clavel T. 2022. Anaerobic single-cell dispensing facilitates the cultivation of human gut bacteria. Environ Microbiol 24:3861–3881. https://doi.org/10.1111/1462-2920.15935
29. Yin J, Chen X, Li X, Kang G, Wang P, Song Y, Ijaz UZ, Yin H, Huang H. 2022. A droplet-based microfluidic approach to isolating functional

bacteria from gut microbiota. Front Cell Infect Microbiol 12:920986. https://doi.org/10.3389/fcimb.2022.920986
30. Ashkin A. 1978. Trapping of atoms by resonance radiation pressure. Phys Rev Lett 40:729–732. https://doi.org/10.1103/PhysRevLett.40.729
31. Ashkin A, Dziedzic JM, Yamane T. 1987. Optical trapping and manipulation of single cells using infrared laser beams. Nature 330:769–771. https://doi.org/10.1038/330769a0
32. Huber R, Burggraf S, Mayer T, Barns SM, Rossnagel P, Stetter KO. 1995. Isolation of a hyperthermophilic archaeum predicted by *in situ* RNA analysis. Nature 376:57–58. https://doi.org/10.1038/376057a0
33. Bashir R, Akin D, Gómez R, Li H, Chang WJ, Gupta A. 2003. From BioMEMS to bionanotechnology: integrated BioChips for the detection of cells and microorganisms. MRS Proc 773. https://doi.org/10.1557/PROC-773-N9.1
34. Beebe DJ, Mensing GA, Walker GM. 2002. Physics and applications of microfluidics in biology. Annu Rev Biomed Eng 4:261–286. https://doi.org/10.1146/annurev.bioeng.4.112601.125916
35. Desai TA, Hansford DJ, Kulinsky L, Nashat AH, Rasi G, Tu J, Wang Y, Zhang M, Ferrari M. 1999. Nanopore technology for biomedical applications. Biomed Microdevices 2:11–40. https://doi.org/10.1023/A:1009903215959
36. Inoue I, Wakamoto Y, Moriguchi H, Okano K, Yasuda K. 2001. On-chip culture system for observation of isolated individual cells. Lab Chip 1:50–55. https://doi.org/10.1039/b103931h
37. Wakamoto Y, Inoue I, Moriguchi H, Yasuda K. 2001. Analysis of single-cell differences by use of an on-chip microculture system and optical trapping. Fresenius J Anal Chem 371:276–281. https://doi.org/10.1007/s002160100999
38. Balagaddé FK, You L, Hansen CL, Arnold FH, Quake SR. 2005. Long-term monitoring of bacteria undergoing programmed population control in a microchemostat. Science 309:137–140. https://doi.org/10.1126/science.1109173
39. Diao J, Young L, Kim S, Fogarty EA, Heilman SM, Zhou P, Shuler ML, Wu M, DeLisa MP. 2006. A three-channel microfluidic device for generating static linear gradients and its application to the quantitative analysis of bacterial chemotaxis. Lab Chip 6:381–388. https://doi.org/10.1039/b511958h
40. Groisman A, Lobo C, Cho H, Campbell JK, Dufour YS, Stevens AM, Levchenko A. 2005. A microfluidic chemostat for experiments with bacterial and yeast cells. Nat Methods 2:685–689. https://doi.org/10.1038/nmeth784
41. Elfwing A, LeMarc Y, Baranyi J, Ballagi A. 2004. Observing growth and division of large numbers of individual bacteria by image analysis. Appl Environ Microbiol 70:675–678. https://doi.org/10.1128/AEM.70.2.675-678.2004
42. Wang P, Robert L, Pelletier J, Dang WL, Taddei F, Wright A, Jun S. 2010. Robust growth of *Escherichia coli*. Curr Biol 20:1099–1103. https://doi.org/10.1016/j.cub.2010.04.045
43. Huh D, Matthews BD, Mammoto A, Montoya-Zavala M, Hsin HY, Ingber DE. 2010. Reconstituting organ-level lung functions on a chip. Science 328:1662–1668. https://doi.org/10.1126/science.1188302
44. Steinhaus B, Garcia ML, Shen AQ, Angenent LT. 2007. A portable anaerobic microbioreactor reveals optimum growth conditions for the methanogen *Methanosaeta concilii*. Appl Environ Microbiol 73:1653–1658. https://doi.org/10.1128/AEM.01827-06
45. Fievet A, Ducret A, Mignot T, Valette O, Robert L, Pardoux R, Dolla AR, Aubert C. 2015. Single-cell analysis of growth and cell division of the anaerobe *Desulfovibrio vulgaris* hildenborough. Front Microbiol 6:1378. https://doi.org/10.3389/fmicb.2015.01378
46. Jalili-Firoozinezhad S, Gazzaniga FS, Calamari EL, Camacho DM, Fadel CW, Bein A, Swenor B, Nestor B, Cronce MJ, Tovaglieri A, Levy O, Gregory KE, Breault DT, Cabral JMS, Kasper DL, Novak R, Ingber DE. 2019. A complex human gut microbiome cultured in an anaerobic intestine-on-a-chip. Nat Biomed Eng 3:520–531. https://doi.org/10.1038/s41551-019-0397-0
47. Anderson MR, Padgett CM, Dargatz CJ, Nichols CR, Vittalam KR, DeVore NM. 2023. Engineering a yellow thermostable fluorescent protein by rational design. ACS Omega 8:436–443. https://doi.org/10.1021/acsomega.2c05005
48. Roberts TM, Rudolf F, Meyer A, Pellaux R, Whitehead E, Panke S, Held M. 2016. Identification and characterisation of a pH-stable Gfp. Sci Rep 6:28166. https://doi.org/10.1038/srep28166
49. Rodriguez EA, Campbell RE, Lin JY, Lin MZ, Miyawaki A, Palmer AE, Shu X, Zhang J, Tsien RY. 2017. The growing and glowing toolbox of fluorescent and photoactive proteins. Trends Biochem Sci 42:111–129. https://doi.org/10.1016/j.tibs.2016.09.010
50. Visone V, Han W, Perugino G, Del Monaco G, She Q, Rossi M, Valenti A, Ciaramella M. 2017. *In vivo* and *in vitro* protein imaging in thermophilic archaea by exploiting a novel protein tag. PLoS One 12:e0185791. https://doi.org/10.1371/journal.pone.0185791
51. McKinney SA, Murphy CS, Hazelwood KL, Davidson MW, Looger LL. 2009. A bright and photostable photoconvertible fluorescent protein. Nat Methods 6:131–133. https://doi.org/10.1038/nmeth.1296
52. Patterson GH, Lippincott-Schwartz J. 2002. A photoactivatable GFP for selective photolabeling of proteins and cells. Science 297:1873–1877. https://doi.org/10.1126/science.1074952
53. Subach FV, Patterson GH, Manley S, Gillette JM, Lippincott-Schwartz J, Verkhusha VV. 2009. Photoactivatable mCherry for high-resolution two-color fluorescence microscopy. Nat Methods 6:153–159. https://doi.org/10.1038/nmeth.1298
54. Fan JY, Cui ZQ, Wei HP, Zhang ZP, Zhou YF, Wang YP, Zhang XE. 2008. Split mCherry as a new red bimolecular fluorescence complementation system for visualizing protein-protein interactions in living cells. Biochem Biophys Res Commun 367:47–53. https://doi.org/10.1016/j.bbrc.2007.12.101
55. Chalfie M, Tu Y, Euskirchen G, Ward WW, Prasher DC. 1994. Green fluorescent protein as a marker for gene expression. Science 263:802–805. https://doi.org/10.1126/science.8303295
56. Shu X, Shaner NC, Yarbrough CA, Tsien RY, Remington SJ. 2006. Novel chromophores and buried charges control color in mFruits. Biochem 45:9639–9647. https://doi.org/10.1021/bi060773l
57. Heim R, Prasher DC, Tsien RY. 1994. Wavelength mutations and posttranslational autoxidation of green fluorescent protein. Proc Natl Acad Sci U S A 91:12501–12504. https://doi.org/10.1073/pnas.91.26.12501
58. Remington SJ. 2006. Fluorescent proteins: maturation, photochemistry and photophysics. Curr Opin Struct Biol 16:714–721. https://doi.org/10.1016/j.sbi.2006.10.001
59. Coralli C, Cemazar M, Kanthou C, Tozer GM, Dachs GU. 2001. Limitations of the reporter green fluorescent protein under simulated tumor conditions. Cancer Res 61:4784–4790.
60. Ransom EM, Ellermeier CD, Weiss DS. 2015. Use of mCherry Red fluorescent protein for studies of protein localization and gene expression in *Clostridium difficile*. Appl Environ Microbiol 81:1652–1660. https://doi.org/10.1128/AEM.03446-14
61. Chia HE, Marsh ENG, Biteen JS. 2019. Extending fluorescence microscopy into anaerobic environments. Curr Opin Chem Biol 51:98–104. https://doi.org/10.1016/j.cbpa.2019.05.008
62. Drepper T, Eggert T, Circolone F, Heck A, Krauss U, Guterl JK, Wendorff M, Losi A, Gärtner W, Jaeger KE. 2007. Reporter proteins for *in vivo* fluorescence without oxygen. Nat Biotechnol 25:443–445. https://doi.org/10.1038/nbt1293
63. Walter J, Hausmann S, Drepper T, Puls M, Eggert T, Dihné M. 2012. Flavin mononucleotide-based fluorescent proteins function in mammalian cells without oxygen requirement. PLoS One 7:e43921. https://doi.org/10.1371/journal.pone.0043921
64. Landete JM, Langa S, Revilla C, Margolles A, Medina M, Arqués JL. 2015. Use of anaerobic green fluorescent protein versus green fluorescent protein as reporter in lactic acid bacteria. Appl Microbiol Biotechnol 99:6865–6877. https://doi.org/10.1007/s00253-015-6770-3
65. Landete JM, Peirotén Á, Rodríguez E, Margolles A, Medina M, Arqués JL. 2014. Anaerobic green fluorescent protein as a marker of bifidobacterium strains. Int J Food Microbiol 175:6–13. https://doi.org/10.1016/j.ijfoodmicro.2014.01.008
66. Molitor B, Kirchner K, Henrich AW, Schmitz S, Rosenbaum MA. 2016. Expanding the molecular toolkit for the homoacetogen *Clostridium ljungdahlii*. Sci Rep 6:31518. https://doi.org/10.1038/srep31518
67. Shu X, Lev-Ram V, Deerinck TJ, Qi Y, Ramko EB, Davidson MW, Jin Y, Ellisman MH, Tsien RY. 2011. A genetically encoded tag for correlated

light and electron microscopy of intact cells, tissues, and organisms. PLoS Biol 9:e1001041. https://doi.org/10.1371/journal.pbio.1001041
68. Buckley AM, Jukes C, Candlish D, Irvine JJ, Spencer J, Fagan RP, Roe AJ, Christie JM, Fairweather NF, Douce GR. 2016. Lighting up *Clostridium difficile*: reporting gene expression using fluorescent lov domains. Sci Rep 6:23463. https://doi.org/10.1038/srep23463
69. Eichhof I, Ernst JF. 2016. Oxygen-independent FbFP: fluorescent sentinel and oxygen sensor component in *Saccharomyces cerevisiae* and *Candida albicans*. Fungal Genet Biol 92:14–25. https://doi.org/10.1016/j.fgb.2016.04.004
70. Mukherjee A, Schroeder CM. 2015. Flavin-based fluorescent proteins: emerging paradigms in biological imaging. Curr Opin Biotechnol 31:16–23. https://doi.org/10.1016/j.copbio.2014.07.010
71. Billinton N, Knight AW. 2001. Seeing the wood through the trees: a review of techniques for distinguishing green fluorescent protein from endogenous autofluorescence. Anal Biochem 291:175–197. https://doi.org/10.1006/abio.2000.5006
72. Cheeseman P, Toms-Wood A, Wolfe RS. 1972. Isolation and properties of a fluorescent compound, factor$_{420}$, from *Methanobacterium* strain M.o.H. J Bacteriol 112:527–531. https://doi.org/10.1128/jb.112.1.527-531.1972
73. Nikolaev A, Yudenko A, Smolentseva A, Bogorodskiy A, Tsybrov F, Borshchevskiy V, Bukhalovich S, Nazarenko VV, Kuznetsova E, Semenov O, Remeeva A, Gushchin I. 2023. Fine spectral tuning of a flavin-binding fluorescent protein for multicolor imaging. J Biol Chem 299:102977. https://doi.org/10.1016/j.jbc.2023.102977
74. Cserép GB, Herner A, Kele P. 2015. Bioorthogonal fluorescent labels: a review on combined forces. Methods Appl Fluoresc 3:042001. https://doi.org/10.1088/2050-6120/3/4/042001
75. Dean KM, Palmer AE. 2014. Advances in fluorescence labeling strategies for dynamic cellular imaging. Nat Chem Biol 10:512–523. https://doi.org/10.1038/nchembio.1556
76. Los GV, Encell LP, McDougall MG, Hartzell DD, Karassina N, Zimprich C, Wood MG, Learish R, Ohana RF, Urh M, Simpson D, Mendez J, Zimmerman K, Otto P, Vidugiris G, Zhu J, Darzins A, Klaubert DH, Bulleit RF, Wood KV. 2008. HaloTag: a novel protein labeling technology for cell imaging and protein analysis. ACS Chem Biol 3:373–382. https://doi.org/10.1021/cb800025k
77. Keppler A, Gendreizig S, Gronemeyer T, Pick H, Vogel H, Johnsson K. 2003. A general method for the covalent labeling of fusion proteins with small molecules *in vivo*. Nat Biotechnol 21:86–89. https://doi.org/10.1038/nbt765
78. Gautier A, Juillerat A, Heinis C, Corrêa Jr IR, Kindermann M, Beaufils F, Johnsson K. 2008. An engineered protein tag for multiprotein labeling in living cells. Chem Biol 15:128–136. https://doi.org/10.1016/j.chembiol.2008.01.007
79. Karunatilaka KS, Cameron EA, Martens EC, Koropatkin NM, Biteen JS. 2014. Superresolution imaging captures carbohydrate utilization dynamics in human gut symbionts. mBio 5:e02172. https://doi.org/10.1128/mBio.02172-14
80. Karunatilaka KS, Coupland BR, Cameron EA, Martens EC, Koropatkin NK, Biteen JS. 2013. Single-molecule imaging can be achieved in live obligate anaerobic bacteria. SPIE BiOS.
81. Charubin K, Streett H, Papoutsakis ET. 2020. Development of strong anaerobic fluorescent reporters for *Clostridium acetobutylicum* and *Clostridium ljungdahlii* using halotag and SNAP-tag proteins. Appl Environ Microbiol 86:e01271-20. https://doi.org/10.1128/AEM.01271-20
82. Nicolle O, Rouillon A, Guyodo H, Tamanai-Shacoori Z, Chandad F, Meuric V, Bonnaure-Mallet M. 2010. Development of SNAP-tag-mediated live cell labeling as an alternative to GFP in *Porphyromonas gingivalis*. FEMS Immunol Med Microbiol 59:357–363. https://doi.org/10.1111/j.1574-695X.2010.00681.x
83. Cassona CP, Pereira F, Serrano M, Henriques AO. 2016. A fluorescent reporter for single cell analysis of gene expression in *Clostridium difficile*. Methods Mol Biol 1476:69–90. https://doi.org/10.1007/978-1-4939-6361-4_6
84. Chia HE, Zuo T, Koropatkin NM, Marsh ENG, Biteen JS. 2020. Imaging living obligate anaerobic bacteria with bilin-binding fluorescent proteins. Curr Res Microb Sci 1:1–6. https://doi.org/10.1016/j.crmicr.2020.04.001
85. Chia HE, Koebke KJ, Rangarajan AA, Koropatkin NM, Marsh ENG, Biteen JS. 2021. New orange ligand-dependent fluorescent reporter for anaerobic imaging. ACS Chem Biol 16:2109–2115. https://doi.org/10.1021/acschembio.1c00391
86. Erapaneedi R, Belousov VV, Schäfers M, Kiefer F. 2016. A novel family of fluorescent hypoxia sensors reveal strong heterogeneity in tumor hypoxia at the cellular level. EMBO J 35:102–113. https://doi.org/10.15252/embj.201592775
87. Kwon J, Park JS, Kang M, Choi S, Park J, Kim GT, Lee C, Cha S, Rhee HW, Shim SH. 2020. Bright ligand-activatable fluorescent protein for high-quality multicolor live-cell super-resolution microscopy. Nat Commun 11:273. https://doi.org/10.1038/s41467-019-14067-4
88. Flaiz M, Ludwig G, Bengelsdorf FR, Dürre P. 2021. Production of the biocommodities butanol and acetone from methanol with fluorescent FAST-tagged proteins using metabolically engineered strains of *Eubacterium limosum*. Biotechnol Biofuels 14:117. https://doi.org/10.1186/s13068-021-01966-2
89. Hernandez E, Costa KC. 2022. The fluorescence-activating and absorption-shifting tag (FAST) enables live-cell fluorescence imaging of *Methanococcus maripaludis*. J Bacteriol 204:e0012022. https://doi.org/10.1128/jb.00120-22
90. Hocq R, Bottone S, Gautier A, Pflügl S. 2023. A fluorescent reporter system for anaerobic thermophiles. Front Bioeng Biotechnol 11:1226889. https://doi.org/10.3389/fbioe.2023.1226889
91. Plamont M-A, Billon-Denis E, Maurin S, Gauron C, Pimenta FM, Specht CG, Shi J, Quérard J, Pan B, Rossignol J, Moncoq K, Morellet N, Volovitch M, Lescop E, Chen Y, Triller A, Vriz S, Le Saux T, Jullien L, Gautier A. 2016. Small fluorescence-activating and absorption-shifting tag for tunable protein imaging *in vivo*. Proc Natl Acad Sci U S A 113:497–502. https://doi.org/10.1073/pnas.1513094113
92. Streett HE, Kalis KM, Papoutsakis ET. 2019. A strongly fluorescing anaerobic reporter and protein-tagging system for *Clostridium* organisms based on the fluorescence-activating and absorption-shifting tag protein (FAST). Appl Environ Microbiol 85:e00622-19. https://doi.org/10.1128/AEM.00622-19
93. Mie M, Naoki T, Kobatake E. 2016. Development of a split SNAP-CLIP double labeling system for tracking proteins following dissociation from protein-protein complexes in living cells. Anal Chem 88:8166–8171. https://doi.org/10.1021/acs.analchem.6b01906
94. Mie M, Naoki T, Uchida K, Kobatake E. 2012. Development of a split SNAP-tag protein complementation assay for visualization of protein-protein interactions in living cells. Analyst 137:4760–4765. https://doi.org/10.1039/c2an35762c
95. Tebo AG, Gautier A. 2019. A split fluorescent reporter with rapid and reversible complementation. Nat Commun 10:2822. https://doi.org/10.1038/s41467-019-10855-0
96. Wang XC, Wilson SC, Hammond MC. 2016. Next-generation RNA-based fluorescent biosensors enable anaerobic detection of cyclic di-GMP. Nucleic Acids Res 44:e139. https://doi.org/10.1093/nar/gkw580
97. Neumann-Staubitz P, Neumann H. 2016. The use of unnatural amino acids to study and engineer protein function. Curr Opin Struct Biol 38:119–128. https://doi.org/10.1016/j.sbi.2016.06.006
98. Nikić I, Plass T, Schraidt O, Szymański J, Briggs JAG, Schultz C, Lemke EA. 2014. Minimal tags for rapid dual-color live-cell labeling and super-resolution microscopy. Angew Chem Int Ed Engl 53:2245–2249. https://doi.org/10.1002/anie.201309847
99. Wang L, Xie J, Schultz PG. 2006. Expanding the genetic code. Annu Rev Biophys Biomol Struct 35:225–249. https://doi.org/10.1146/annurev.biophys.35.101105.121507
100. Grieb A, Bowers RM, Oggerin M, Goudeau D, Lee J, Malmstrom RR, Woyke T, Fuchs BM. 2020. A pipeline for targeted metagenomics of environmental bacteria. Microbiome 8:21. https://doi.org/10.1186/s40168-020-0790-7
101. Yilmaz S, Haroon MF, Rabkin BA, Tyson GW, Hugenholtz P. 2010. Fixation-free fluorescence *in situ* hybridization for targeted enrichment of microbial populations. ISME J 4:1352–1356. https://doi.org/10.1038/ismej.2010.73
102. Harris RL, Vetter M, van Heerden E, Cason E, Vermeulen J-G, Taneja A, Kieft TL, DeCoste CJ, Laevsky GS, Onstott TC. 2022. FISH-TAMB, a

fixation-free mRNA fluorescent labeling technique to target transcriptionally active members in microbial communities. Microb Ecol 84:182–197. https://doi.org/10.1007/s00248-021-01809-5
103. Cezanne A, Hoogenberg B, Baum B. 2023. Probing archaeal cell biology: exploring the use of dyes in the imaging of *Sulfolobus* cells. Front Microbiol 14:1233032. https://doi.org/10.3389/fmicb.2023.1233032
104. Maslov I, Bogorodskiy A, Mishin A, Okhrimenko I, Gushchin I, Kalenov S, Dencher NA, Fahlke C, Büldt G, Gordeliy V, Gensch T, Borshchevskiy V. 2018. Efficient non-cytotoxic fluorescent staining of halophiles. Sci Rep 8:2549. https://doi.org/10.1038/s41598-018-20839-7
105. Westlund E, Bergenstråle A, Pokhrel A, Chan H, Skoglund U, Daley DO, Söderström B. 2023. Application of nanotags and nanobodies for live cell single-molecule imaging of the Z-ring in *Escherichia coli*. Curr Genet 69:153–163. https://doi.org/10.1007/s00294-023-01266-2
106. Bird RE, Lemmel SA, Yu X, Zhou QA. 2021. Bioorthogonal chemistry and its applications. Bioconjug Chem 32:2457–2479. https://doi.org/10.1021/acs.bioconjchem.1c00461
107. van Kasteren S, Rozen DE. 2023. Using click chemistry to study microbial ecology and evolution. ISME Commun 3:9. https://doi.org/10.1038/s43705-022-00205-5
108. Hatzenpichler R, Connon SA, Goudeau D, Malmstrom RR, Woyke T, Orphan VJ. 2016. Visualizing *in situ* translational activity for identifying and sorting slow-growing archaeal-bacterial consortia. Proc Natl Acad Sci U S A 113:E4069–E4078. https://doi.org/10.1073/pnas.1603757113
109. Hatzenpichler R, Scheller S, Tavormina PL, Babin BM, Tirrell DA, Orphan VJ. 2014. *In situ* visualization of newly synthesized proteins in environmental microbes using amino acid tagging and click chemistry. Environ Microbiol 16:2568–2590. https://doi.org/10.1111/1462-2920.12436
110. Babin BM, Bergkessel M, Sweredoski MJ, Moradian A, Hess S, Newman DK, Tirrell DA. 2016. SutA is a bacterial transcription factor expressed during slow growth in *Pseudomonas aeruginosa*. Proc Natl Acad Sci U S A 113:E597–E605. https://doi.org/10.1073/pnas.1514412113
111. Hajjo H, Bhardwaj N, Gefen T, Geva-Zatorsky N. 2023. Combinatorial fluorescent labeling of live anaerobic bacteria via the incorporation of azide-modified sugars into newly synthesized macromolecules. Nat Protoc 18:3767–3786. https://doi.org/10.1038/s41596-023-00896-7
112. Geva-Zatorsky N, Alvarez D, Hudak JE, Reading NC, Erturk-Hasdemir D, Dasgupta S, von Andrian UH, Kasper DL. 2015. *In vivo* imaging and tracking of host-microbiota interactions via metabolic labeling of gut anaerobic bacteria. Nat Med 21:1091–1100. https://doi.org/10.1038/nm.3929
113. Yuan X, Couto JM, Glidle A, Song Y, Sloan W, Yin H. 2017. Single-cell microfluidics to study the effects of genome deletion on bacterial growth behavior. ACS Synth Biol 6:2219–2227. https://doi.org/10.1021/acssynbio.7b00177
114. Anggraini D, Ota N, Shen Y, Tang T, Tanaka Y, Hosokawa Y, Li M, Yalikun Y. 2022. Recent advances in microfluidic devices for single-cell cultivation: methods and applications. Lab Chip 22:1438–1468. https://doi.org/10.1039/d1lc01030a
115. Ducret A, Maisonneuve E, Notareschi P, Grossi A, Mignot T, Dukan S. 2009. A microscope automated fluidic system to study bacterial processes in real time. PLoS One 4:e7282. https://doi.org/10.1371/journal.pone.0007282
116. Potvin-Trottier L, Luro S, Paulsson J. 2018. Microfluidics and single-cell microscopy to study stochastic processes in bacteria. Curr Opin Microbiol 43:186–192. https://doi.org/10.1016/j.mib.2017.12.004
117. Ashammakhi N, Nasiri R, Barros NR de, Tebon P, Thakor J, Goudie M, Shamloo A, Martin MG, Khademhosseini A. 2020. Gut-on-a-chip: current progress and future opportunities. Biomaterials 255:120196. https://doi.org/10.1016/j.biomaterials.2020.120196
118. Bein A, Shin W, Jalili-Firoozinezhad S, Park MH, Sontheimer-Phelps A, Tovaglieri A, Chalkiadaki A, Kim HJ, Ingber DE. 2018. Microfluidic organ-on-a-chip models of human intestine. Cell Mol Gastroenterol Hepatol 5:659–668. https://doi.org/10.1016/j.jcmgh.2017.12.010
119. Trujillo-de Santiago G, Lobo-Zegers MJ, Montes-Fonseca SL, Zhang YS, Alvarez MM. 2018. Gut-microbiota-on-a-chip: an enabling field for physiological research. Microphysiol Syst 2:7. https://doi.org/10.21037/mps.2018.09.01
120. Bossink E, Segerink LI, Odijk M. 2022. Organ-on-chip technology for aerobic intestinal host – anaerobic microbiota research. Organs Chip 4:100013. https://doi.org/10.1016/j.ooc.2021.100013
121. Greenhalgh K, Ramiro-Garcia J, Heinken A, Ullmann P, Bintener T, Pacheco MP, Baginska J, Shah P, Frachet A, Halder R, Fritz JV, Sauter T, Thiele I, Haan S, Letellier E, Wilmes P. 2019. Integrated *in vitro* and *in silico* modeling delineates the molecular effects of a synbiotic regimen on colorectal-cancer-derived cells. Cell Rep 27:1621–1632. https://doi.org/10.1016/j.celrep.2019.04.001
122. Wang C, Dang T, Baste J, Anil Joshi A, Bhushan A. 2021. A novel standalone microfluidic device for local control of oxygen tension for intestinal-bacteria interactions. FASEB J 35:e21291. https://doi.org/10.1096/fj.202001600RR
123. Shin W, Wu A, Massidda MW, Foster C, Thomas N, Lee DW, Koh H, Ju Y, Kim J, Kim HJ. 2019. A robust longitudinal co-culture of obligate anaerobic gut microbiome with human intestinal epithelium in an anoxic-oxic interface-on-a-chip. Front Bioeng Biotechnol 7:13. https://doi.org/10.3389/fbioe.2019.00013
124. Espey MG. 2013. Role of oxygen gradients in shaping redox relationships between the human intestine and its microbiota. Free Radic Biol Med 55:130–140. https://doi.org/10.1016/j.freeradbiomed.2012.10.554
125. Grant J, Lee E, Almeida M, Kim S, LoGrande N, Goyal G, Sesay AM, Breault DT, Prantil-Baun R, Ingber DE. 2022. Establishment of physiologically relevant oxygen gradients in microfluidic organ chips. Lab Chip 22:1584–1593. https://doi.org/10.1039/d2lc00069e
126. Heller HH. 1921. Principles concerning the isolation of anaerobes: studies in pathogenic anaerobes Ii. J Bacteriol 6:445–470. https://doi.org/10.1128/jb.6.5.445-470.1921
127. Fröhlich J, König H. 2000. New techniques for isolation of single prokaryotic cells. FEMS Microbiol Rev 24:567–572. https://doi.org/10.1111/j.1574-6976.2000.tb00558.x
128. Lewis WH, Tahon G, Geesink P, Sousa DZ, Ettema TJG. 2021. Innovations to culturing the uncultured microbial majority. Nat Rev Microbiol 19:225–240. https://doi.org/10.1038/s41579-020-00458-8
129. Thompson AW, Crow MJ, Wadey B, Arens C, Turkarslan S, Stolyar S, Elliott N, Petersen TW, van den Engh G, Stahl DA, Baliga NS. 2015. A method to analyze, sort, and retain viability of obligate anaerobic microorganisms from complex microbial communities. J Microbiol Methods 117:74–77. https://doi.org/10.1016/j.mimet.2015.07.009
130. Bellais S, Nehlich M, Ania M, Duquenoy A, Mazier W, van den Engh G, Baijer J, Treichel NS, Clavel T, Belotserkovsky I, Thomas V. 2022. Species-targeted sorting and cultivation of commensal bacteria from the gut microbiome using flow cytometry under anaerobic conditions. Microbiome 10:24. https://doi.org/10.1186/s40168-021-01206-7
131. Jahn U, Gallenberger M, Paper W, Junglas B, Eisenreich W, Stetter KO, Rachel R, Huber H. 2008. *Nanoarchaeum equitans* and *Ignicoccus hospitalis*: new insights into a unique, intimate association of two archaea. J Bacteriol 190:1743–1750. https://doi.org/10.1128/JB.01731-07
132. Chen L, Zhao B, Li X, Cheng Z, Wu R, Xia Y. 2021. Isolating and characterizing translationally active fraction of anammox microbiota using bioorthogonal non-canonical amino acid tagging. Chem Eng J 418:129411. https://doi.org/10.1016/j.cej.2021.129411
133. Du Z, Behrens SF. 2021. Tracking *de novo* protein synthesis in the activated sludge microbiome using boncat-facs. Water Res 205:117696. https://doi.org/10.1016/j.watres.2021.117696
134. Kaster AK, Sobol MS. 2020. Microbial single-cell omics: the crux of the matter. Appl Microbiol Biotechnol 104:8209–8220. https://doi.org/10.1007/s00253-020-10844-0
135. Saito K, Ota Y, Tourlousse DM, Matsukura S, Fujitani H, Morita M, Tsuneda S, Noda N. 2021. Microdroplet-based system for culturing of environmental microorganisms using FNAP-sort. Sci Rep 11:9506. https://doi.org/10.1038/s41598-021-88974-2
136. Samlali K, Alves CL, Jezernik M, Shih SCC. 2022. Droplet digital microfluidic system for screening filamentous fungi based on enzymatic activity. Microsyst Nanoeng 8:123. https://doi.org/10.1038/s41378-022-00456-1
137. Baltekin Ö, Boucharin A, Tano E, Andersson DI, Elf J. 2017. Antibiotic susceptibility testing in less than 30 min using direct single-cell imaging. Proc Natl Acad Sci U S A 114:9170–9175. https://doi.org/10.1073/pnas.1708558114
138. Robert L, Ollion J, Elez M. 2019. Real-time visualization of mutations and their fitness effects in single bacteria. Nat Protoc 14:3126–3143. https://doi.org/10.1038/s41596-019-0215-x
139. Robert L, Ollion J, Robert J, Song X, Matic I, Elez M. 2018. Mutation dynamics and fitness effects followed in single cells. Science 359:1283–1286. https://doi.org/10.1126/science.aan0797
140. Lee KS, Landry Z, Pereira FC, Wagner M, Berry D, Huang WE, Taylor GT, Kneipp J, Popp J, Zhang M, Cheng J-X, Stocker R. 2021. Raman

microspectroscopy for microbiology. Nat Rev Methods Primers 1:80. https://doi.org/10.1038/s43586-021-00075-6
141. Cui L, Xin Y, Yang K, Li H, Tan F, Zhang Y, Li X, Zhu Z, Yang J, Kao S-J, Ren B, Zhu Y-G, Musat F, Musat N. 2023. Live tracking metabolic networks and physiological responses within microbial assemblages at single-cell level. PNAS Nexus 2:pgad006. https://doi.org/10.1093/pnasnexus/pgad006
142. Lee KS, Palatinszky M, Pereira FC, Nguyen J, Fernandez VI, Mueller AJ, Menolascina F, Daims H, Berry D, Wagner M, Stocker R. 2019. An automated Raman-based platform for the sorting of live cells by functional properties. Nat Microbiol 4:1035–1048. https://doi.org/10.1038/s41564-019-0394-9
143. Berry D, Mader E, Lee TK, Woebken D, Wang Y, Zhu D, Palatinszky M, Schintlmeister A, Schmid MC, Hanson BT, Shterzer N, Mizrahi I, Rauch I, Decker T, Bocklitz T, Popp J, Gibson CM, Fowler PW, Huang WE, Wagner M. 2015. Tracking heavy water (D_2O) incorporation for identifying and sorting active microbial cells. Proc Natl Acad Sci U S A 112:E194–E203. https://doi.org/10.1073/pnas.1420406112
144. Kalasinsky VF. 1996. Biomedical applications of infrared and Raman microscopy. Appl Spectrosc Rev 31:193–249. https://doi.org/10.1080/05704929608000570
145. Ge X, Pereira FC, Mitteregger M, Berry D, Zhang M, Hausmann B, Zhang J, Schintlmeister A, Wagner M, Cheng J-X. 2022. Srs-Fish: a high-throughput platform linking microbiome metabolism to identity at the single-cell level. Proc Natl Acad Sci U S A 119:e2203519119. https://doi.org/10.1073/pnas.2203519119
146. Lee K.S, Pereira FC, Palatinszky M, Behrendt L, Alcolombri U, Berry D, Wagner M, Stocker R. 2021. Optofluidic Raman-activated cell sorting for targeted genome retrieval or cultivation of microbial cells with specific functions. Nat Protoc 16:634–676. https://doi.org/10.1038/s41596-020-00427-8
147. Knoepp F, Wahl J, Andersson A, Borg J, Weissmann N, Ramser K. 2018. Development of a gas-tight microfluidic system for Raman sensing of single pulmonary arterial smooth muscle cells under normoxic/hypoxic conditions. Sensors (Basel) 18:3238. https://doi.org/10.3390/s18103238

AUTHOR BIOS

Ciara Keating is an Assistant Professor in Environmental Engineering at Durham University, UK. Her research centers on the interactions between microorganisms and their environments. Specifically, she is interested in how we can harness this knowledge to solve global societal and environmental challenges. Her work has included for example, research on waste treatment solutions, developing methods in anoxic live-imaging, and soil microbial communities. She enjoys the challenges of method development and hopes to create innovative tools and techniques that advance our understanding of microbial processes, combining her expertise in engineering and biology.

Kerstin Fiege finished her PhD from TU Kaiserslautern, Germany, in 2019 in microbiology. During her PhD she analyzed the biochemical function and physiological role of sensor proteins from a methanogenic archaeon. After a subsequent year as a postdoctoral researcher in the same group, she joined the department of Marine Microbiology and Biogeochemistry at the Royal Netherlands Institute for Sea research as a postdoctoral researcher in 2020. Her main interest are membrane-based changes during the adaptation process of archaeal and bacterial partners to syntrophic growth.

Martijn Diender is assistant professor at the Laboratory of Microbiology at Wageningen University & Research. His research focuses on the anaerobic physiology of CO-utilizing microorganisms, and recently expanded his research area to microbial metal reduction. He aims to fundamentally understand the physiology of the microbes he works with, as well as develop related biotechnological processes. In addition, he works on improving and developing anaerobic cultivation and analysis methods, with the aim to improve our understanding of anaerobic microbes and their environmental interactions.

Diana Z. Sousa is Professor of Microbial Physiology at the Laboratory of Microbiology at Wageningen University & Research. Her research primarily focuses on anaerobic microbiology, specifically in the areas of one-carbon metabolism, syntrophic interactions and microbial co-cultivation. Over the years she has gained broad experience with different types of anaerobic physiology, often working with challenging and slow growing anaerobes. Combining her background in biological engineering and expertise in anaerobic microbiology, she seeks a better understanding of microbial communities and microbial interactions, while searching for biotechnological applications of these communities in circular economy approaches.

Laura Villanueva is a senior researcher at the Royal Netherlands Institute for Sea Research and professor of marine microbiology at Utrecht University, The Netherlands. Her research focus is on microbial lipids and marine anaerobic microorganisms. She is passionate about the use of different methodological approaches to study microorganisms in their natural habitat, from microscopy to molecular methods. Her group is known for advancing the field of microbial ecology in marine systems and to establish bridges between the fields of biology and organic geochemistry.