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# Tools for the Microbiome: Nano and Beyond

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**ABSTRACT:** **The microbiome presents great opportunities for understanding and improving the world around us and elucidating the interactions that compose it. The microbiome also poses tremendous challenges for mapping and manipulating the entangled networks of interactions among myriad diverse organisms. Here, we describe the opportunities, technical needs, and potential approaches to address these challenges, based on recent and upcoming advances in measurement and control at the nanoscale and beyond. These technical needs will provide the basis for advancing the largely descriptive studies of the microbiome to the theoretical and mechanistic understandings that will underpin the discipline of microbiome engineering. We anticipate that the new tools and methods developed will also be more broadly useful in environmental monitoring, medicine, forensics, and other areas.**







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## MOTIVATION AND GOALS OF THE MICROBIOME INITIATIVE

The proposed Unified Microbiome Initiative seeks to develop and to apply new tools that enable understanding the microbiomes (and nanobiomes) of humans and other animals, plants, the earth, the ocean, and the atmosphere.[1−3] These coexisting populations are key components and sustainers of life as we know it and of our planet. Sometimes these populations are symbiotic and supportive; at other times, they are dangerous and destructive. Incredibly, we know little of their compositions, roles, interactions, and dynamics. Here, we motivate this project with the major issues and a number of tantalizing sketches of what we know, what we do not know, and what we would like to know.

Many different approaches will be required to read out the microbiome and its functions at different levels, as well as to synthesize these data into models that can improve our predictive understanding. In particular, we must determine the correct scales to read out microbial systems in order to understand processes from the intracellular to the planetary. This challenge is formidable because of the sheer diversity and number of microbes and their functions.

For example, microbial cells have been estimated to outnumber human cells in each of us by as much as ten to one and to constitute *ca.* 1 kg in typical adults.[4] The microbiome varies greatly among different body sites (for example, our stool and our skin harbor almost entirely distinct microbial communities),[5,6] and variation is also high within each body site from person to person.[6] Indeed, the differences between a person's gut and oral microbes are comparable to the differences between microbes in a soil and an ocean community.[7] In addition to the frequently studied bacteria and archaea, fungi, other microbial eukaryotes, and viruses play large and underappreciated roles. Most of the genes in the species that are known are unannotated, and the vast majority of species have not yet been cultured. Consequently, we lack even the parts list, let alone systems-level understanding of how the parts interact with one another to support physiological function. Similar considerations apply to the complex microbial communities that underpin function in the oceans, freshwater systems, the soil (including the rhizosphere of symbiotic root microbes that supports plant life), sediments, and perhaps even the air, where microbial transport is well-documented but microbial function less so.

**Earth Microbiomes.** Microbial communities play critical roles in many distinct processes across our planet. For example, microbes are essential in all nutrient cycles, including global carbon[8] and nitrogen[9] cycles, and provide key ecosystem services in both marine[10] and terrestrial[11] environments. Diversity in some ecosystems is tremendously high, with estimates of the soil microbial census exceeding 40,000 species per gram.[12] In the soil, in particular, microbes are essential for supporting plant life and play key roles in mediating nutrient uptake and entry into the food chain.[13,14] Therefore, substantial opportunities exist for biotechnological applications that alter microbes in order to improve crop productivity and environmental tolerance, especially to drought and salt. In addition to their role in plant growth, soil microbes, notably *Streptomyces*, provide a major source of antibiotics, and new antibiotics continue to be isolated from this source.[15] However, despite all this complexity, some relatively simple drivers, notably pH,[16,17] explain much of the variation. Spatial variation in the soil microbiome is immense, and even small areas such as the Hoosfield experimental strip at Rothamstead[18] or within a single biome such as the Arctic,[19] or even New York's Central Park,[20] can harbor a large fraction of the microbial community diversity. However, other factors such as plant cover tend to have small, although with sufficient sample size, detectable effects.[21] There is a substantial need to understand soil microbes on their own scale, for example, individual grains of soil, which are often dominated by single species,[22] and understanding how this heterogeneity scales to overall soil properties remains an important challenge.

The differences between a person's gut and oral microbes are comparable to the differences between microbes in a soil and an ocean community.

Earth is a microbial planet, with micro-organisms dominating virtually every ecosystem, ranging from soil to oceans, from the deep subsurface to the atmosphere, including extreme environments, such as hydrothermal vents. Although microbes are abundant and ubiquitous, we currently lack fundamental understanding of many of the key roles played by microbes in Nature, including cycling of carbon and other nutrients. It has been estimated that only a small fraction (≪1%) of Earth's microbes have ever been isolated and studied in pure culture. This limitation has resulted in expansive growth of non-culture-dependent molecular approaches to determine not only the identities of members of microbial communities in a variety of habitats but also their functional roles. Recent achievements include development of a range of molecular "omics" approaches. These include high-throughput sequencing of 16S rRNA genes as a conserved phylogenetic marker that is the current standard used to assign identities to largely unknown members of microbial communities.[23] In addition, sequencing of a microbiome's total DNA, or metagenomics, has been developed to determine both the phylogenetic and functional gene repertoire of microbial communities in environmental samples.[24] Metagenomics has already been successfully demonstrated for environments with relatively low species diversity and, as sequencing technologies continue to improve the depth of sequencing, is now becoming sufficient to apply metagenomics to highly complex microbial communities, such as soil.[25] Recently, it has been possible to achieve binning of complete to near-complete composite genomes from a range of environmental samples, including soil, from metagenome sequence data, many of which represent previously undescribed species.[26,27] One limitation with metagenomics is that the DNA sequence data do not provide information about what genes are expressed, and we understand that only a portion of genes are expressed at any given time, depending on the environmental conditions. Other omics technologies can compensate for this limitation, such as sequencing of RNA (metatranscriptomics) or identification of proteins (metaproteomics). Finally, characterization of metabolites (metabolomics) represents the ultimate signature of processes occurring in any particular environment.

Understanding the direction of some of these inputs is of critical importance. For example, the permafrost is one of Earth's largest carbon reservoirs, estimated at roughly 1600 Pg, about the same as the amount in the atmosphere and in vegetation.[28] Climate change is resulting in higher temperatures in the Arctic and increased fire frequency that is causing permafrost thaw, increasing the availability of carbon stored in permafrost to microbes that can degrade it, resulting in the release of greenhouse gases ($CO_2$ and methane).[29,30] Understanding the relative



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sizes of these inputs, and therefore whether lightning (which leads to fires) is a positive or negative factor in the dynamics of greenhouse gas emissions, requires a detailed knowledge of the activities of the microbes in permafrost.[31]

**Ocean Microbiomes and Nanobiomes.** Life in the oceans is supported by a community of extremely small organisms that can be called a "nanobiome." These nanoplankton particles, many of which measure less than 0.001× the volume of a white blood cell, harvest solar and chemical energy and channel essential elements into the food chain. A deep network of larger life forms (humans included) depends on these tiny microbes for its energy and chemical building blocks.

The importance of the oceanic nanobiome has only recently begun to be fully appreciated. Two dominant forms, *Synechococcus* and *Prochlorococcus*, were not discovered until the 1980s and 1990s.[32−34] *Prochloroccus* has now been demonstrated to be so abundant that it may account for as much as 10% of the world's living organic carbon. The organism divides on a diel cycle while maintaining constant numbers, suggesting that about 5% of the world's biomass flows through this species on a daily basis.[35−37] Metagenomic studies show that many other less abundant life forms must exist but elude direct observation because they can neither be isolated nor grown in culture.

The small sizes of these organisms (and their genomes) indicate that they are highly specialized and optimized. Metagenome data indicate a large metabolic heterogeneity within the nanobiome. Rather than combining all life functions into a single organism, the nanobiome works as a network of specialists that can only exist as a community, therein explaining their resistance to being cultured. The detailed composition of the network is the result of interactions between the organisms themselves and the local physical and chemical environment. There is thus far little insight into how these networks are formed and how they maintain steady-state conditions in the turbulent natural ocean environment.

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The serendipitous discovery of *Prochlorococcus* happened by applying flow cytometry (developed as a medical technique for counting blood cells) to seawater.[34] With these medical instruments, the faint signals from nanoplankton can only be seen with great difficulty against noisy backgrounds. Currently, a small team is adapting flow cytometric technology to improve the capabilities for analyzing individual nanoplankton particles. The latest generation of flow cytometers enables researchers to count and to make quantitative observations of most of the small life forms (including some viruses) that comprise the nanobiome. To our knowledge, there are only two well-equipped mobile flow cytometry laboratories that are regularly taken to sea for real-time observations of the nanobiome. The laboratories include equipment for (meta)genome analysis and equipment to correlate the observations with the local physical parameters and (nutrient) chemistry in the ocean. Ultimately, integration of these measurements will be essential for understanding the complexity of the oceanic microbiome.

The ocean is tremendously undersampled. Ship time is costly and limited. Ultimately, inexpensive, automated, mobile biome observatories will require methods that integrate microbiome and nanobiome measurements, with (meta-) genomics analyses, with local geophysical and geochemical parameters.[38−42] To appreciate how the individual components of the ocean biome are related and work together, a more complete picture must be established.

The marine environment consists of stratified zones, each with a unique, characteristic biome.[43] The sunlit waters near the surface are mixed by wind action. Deeper waters may be mixed only occasionally by passing storms. The dark deepest layers are stabilized by temperature/salinity density gradients. Organic material from the photosynthetically active surface descends into the deep zone, where it decomposes into nutrients that are mixed with compounds that are released by volcanic and seismic action. These nutrients diffuse upward to replenish the depleted surface waters. The biome is stratified accordingly, sometimes with sudden transitions on small scales. Photo-autotrophs dominate near the surface. Chemo-heterotrophs populate the deep. The makeup of the microbial assemblages is dictated by the local nutrient and oxygen concentrations. The spatiotemporal interplay of these systems is highly relevant to such issues as the carbon budget of the planet but remains little understood.

**Atmospheric Microbiome.** Microbes make up a sizable fraction of atmospheric aerosols, as they are suspended from soil, water, and plant surfaces.[44−52] The number of studies detailing microbial communities in terrestrial and ocean sources vastly exceeds those describing airborne microbes.[53] Understanding the sources contributing to the atmospheric nanobiome is an area of major recent growth, with recent studies demonstrating that atmospheric microbes can be ubiquitous, vary spatially, and remain suspended for days to weeks.[54] Average measured atmospheric concentrations of microbes have been reported to be $10^2-10^4$ cells $m^{-3}$ over land with lower levels over the ocean ($\sim 10^2$ cells $m^{-3}$).[55−57] However, large uncertainties exist in the atmospheric concentrations due to the limited number of measurements, low abundance, contamination issues, and the fact that most airborne bacteria cannot be cultured.[44]

The atmosphere serves as an effective conduit, transporting both free and particle-associated microbial life attached to dust and free microbes around the globe on time scales on the order of 2 weeks.[58] The global dissemination of microbes has important ramifications for agriculture, infectious disease, human health, clouds, precipitation, and our water supply. The extreme environmental conditions of the upper atmosphere in essence select which microbes will become dispersed as only certain microbes can remain viable under harsh irradiation and temperature conditions. However, viable microbes attached to soil dust can remain viable, even after transport across thousands of miles.[57,59,60]

At high altitudes, microbes can serve as cloud seeds and can profoundly impact climate and precipitation processes.[61−64] Viable microbes occur in cloud droplets and play significant roles in controlling aqueous-phase chemical processes.[65−69] Microbes can nucleate ice in clouds at warmer temperatures than any other particle type; thus, the atmospheric microbiome can enhance precipitation processes.[62,70] While most studies have focused on the ice-nucleating ability of mineral dust,[71,72] microbes such as *Pseudomonas syringae* are far more effective agents in this climate-driving activity.[73−75] Measurements of airborne ice crystals have shown that dust and biological particles from as far away as Africa can form ice in mixed-phase clouds and determine the amount of snowfall over California's Sierra Nevada mountains in California.[62]



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Many studies have focused primarily on terrestrial sources of atmospheric microbes, and thus, far less is known about oceanic sources.[76] Estimates of global microbial emissions assume negligible oceanic contributions and range from 40 to 1800 Gg dry weight year$^{-1}$.[77] However, oceans cover 71% of the Earth's surface and contain marine bacteria at concentrations of $10^6$–$10^8$ per m$^{-3}$. When waves break and bubbles burst at the surface of the ocean, microbes become highly enriched in the ejected sea spray droplets.[78] Thus, sea spray aerosol represents a significant but highly undetermined source comprising a complex mixture of microbial species. Given the large role in cloud formation, studies over many decades have investigated the ocean as a potential source of ice nuclei.[79,80] The paucity of knowledge regarding oceanic contributions to the atmosphere can be attributed to the difficulty associated with isolating solely oceanic sources given the complexity of the atmosphere and terrestrial impacts even in remote marine environments. Many studies have been conducted over the oceans, showing broad diversity, even at high altitudes (8–15 km).[81−83] It remains unclear whether sea spray production processes selectively introduce only certain types of microbes into the atmosphere. A recent study impinged a water jet on the surface of Arctic seawater and showed significant differences in the microbes that became aerosolized, suggesting selectivity can indeed occur.[84] The transfer of different species from seawater to the atmosphere strongly depends on the bubble size distribution and, hence, the physical mechanisms of bubble production.[85] Thus, in order to replicate sea spray aerosol composition more accurately, breaking waves were used to produce sea spray aerosol using realistic bubble sizes and hence physical production mechanisms.[86] Studies are now underway using more realistic ocean-in-the-lab breaking-wave approaches to isolate and to develop a library of ocean-derived microbes that will ultimately be used to determine the relative contributions of the ocean to the atmospheric nanobiome. Future studies will examine how phytoplankton blooms, as well as the chemistry and morphological properties of the microbes, impact their transfer into the aerosol phase, as well as the ability of the ejected microbes to remain viable under typical atmospheric conditions.[87]

**Microbes of Humans and Other Animals.** Microbiomes play important roles in the health of many animals, including humans, and disruption of these microbe–host interactions by exposure to certain diets or chemicals can lead to dysbiosis in the host (see Box 1). Host-associated microbial communities range from those involving a single bacterial species, such as the symbionts of certain insects, squids, and other invertebrates, up to the hundreds to thousands of species present in the mammalian gut. Different mammals contain microbiomes that range from relatively simple, for carnivores, to the complex, notably in herbivores that ferment cellulose in their hindguts (omnivores and foregut fermenters being intermediate in complexity). In addition to digestion, mammalian gut microbiomes perform many other functions, including producing essential amino acids and vitamins, regulating the immune system, providing resistance to disease (including diseases not localized in the gut, *e.g.*, liver disease and asthma), and even modifying appetite, circadian rhythm, and behavior.

Links between the human gut microbiome and disease have been of intense interest, with strong associations and high-quality predictive models being reported between the gut microbiome and conditions ranging from obesity to type-2 diabetes to cirrhosis to rheumatoid arthritis. Mouse models provide the opportunity to unravel possible mechanisms for some of these associations and, additionally, have linked the gut microbiome to models of anxiety, depression, and even autism.[103] The latter case is especially interesting because maternal immune activation *via* a double-stranded RNA virus induces autism-like behaviors including repetitive behavior, cognitive and social deficits, as well as gut barrier dysfunction often seen in humans with autism. These phenotypes can be reproduced in part using 4-ethylphenylsulfate (4EPS), which is produced by an aberrant microbial community in the autism model, and can be rescued with a *Bacteroides fragilis* probiotic strain.

Of particular interest is fecal transplantation, a procedure in which stool is transferred from a healthy donor to an unhealthy recipient (perhaps the same person, in the case of autologous stool transplant). This procedure has been 90–95% effective for treating *Clostridium difficile*-associated disease *versus* only 20–30% efficacy for antibiotics. Understanding which other diseases associated with dysbiosis of the microbiome could be corrected remains a major goal of microbiome research. The current regulatory framework in the United States, regulating stool as a drug and requiring an investigational new drug (IND) application for any application other than *C. difficile*, however, is a

**Box 1. Antibiotics: Dysbiosis, Resistance, and the Need for Precision.**

Perhaps the most frequent and profound examples of the consequences of microbiome perturbation involve the use of antibiotics. Worldwide, an estimated 1% to 3% of people living in developed countries are being treated with antibiotics at any given time.[88] In the United States, a large-scale study recently showed that outpatient antibiotics were prescribed at a rate of 842 courses per 1,000 individuals.[89] Antibiotic exposure early in life is a particular concern,[90] especially considering that the average US child receives three courses of antibiotics by age two, and 10 courses by age 10.[89] Understanding exactly what happens when human microbiomes are exposed to antibiotics is of critical importance.

It has long been known that antibiotics disrupt gastrointestinal microbiomes, and recent studies are beginning to provide population-level insights regarding the effects of perturbation. For example, treatment of adults for 5 days with ciprofloxacin, a widely used broad-spectrum fluoroquinolone, significantly decreased taxonomic diversity and altered the abundance of one third of the bacterial taxa in the gut.[91] Diversity returned to a level that resembled the pretreatment state within 4 weeks, but several taxa were still missing 6 months after treatment. Although this study used a small cohort of volunteers, and numerous variables are known or expected to determine the effects of antibiotic exposure, a growing literature shows that antibiotics induce the decline and expansion of specific bacterial taxa, leading to an overall decrease in diversity with the potential for short-term and lasting effects on both the microbial community and the host.[92,93]

A life-threatening consequence of the community disruption, or dysbiosis, that can follow antibiotic use is the expansion of opportunistic pathogens that are normally kept in check by a healthy, diverse microbiome.[94] Such infections are particularly problematic in the hospital setting, and the growing list of pathogens includes *Enterococcus faecium*, carbapenem-resistant Enterobacteriaceae, and *Clostridium difficile*. *C. difficile*, which causes antibiotic-induced diarrhea and a potentially fatal condition, toxic megacolon, is responsible for an estimated 250,000 hospitalizations and 14,000 deaths per year in the US.[95] A combination of drug resistance and the ability to form spores allows *C. difficile* to persist in the host and/or local environment during periods of antibiotic treatment. When treatment stops, the lack of a protective microbiome promotes *C. difficile* colonization and disease.[96] Since antibiotics were the cause of infection in the first place, the use of antibiotics to treat it are predictably associated with recurrent bouts of re-infection. In light of the ecological principles at play, it is not surprising that fecal microbiome transplantation, in which a patient is provided with a healthy gastrointestinal microbiome by ingestion or gavage, is significantly more effective than antibiotics in treating recurrent *C. difficile* infections.[97,98]

The gut microbiome is a reservoir of antibiotic resistance genes, and antibiotic therapy provides a powerful selective advantage for their expansion and dissemination.[92] In a study of patients with *Helicobacter pylori*-associated peptic ulcers, treatment with a standard combination of antibiotics that included clarithromycin, a macrolide, corresponded to a 1,000-fold increase in the fecal copy number of the *ermB* gene, which confers macrolide resistance.[99] Antibiotic resistance can be acquired by mutation or, as in the case of *ermB*, by horizontal transmission. Broad host range conjugative plasmids that can transfer between and replicate within a broad spectrum of bacteria are prototypical transmissible elements, and they are especially problematic for two reasons. First, they tend to encode arrays of genes that encode resistance to different antibiotics, so the use of one antibiotic co-selects for resistance to others.[100] Second, broad spectrum antibiotics select for transmission of resistance genes to a correspondingly broad spectrum of bacteria.

The problems of how to eliminate or to prevent *C. difficile* and similar infections without harming beneficial microbes, and how to avoid selecting for the dissemination of antibiotic resistance to diverse bacteria, have a common potential solution - precision antimicrobials. The ability to design and to deploy precision antimicrobials that target specific bacterial species, strains, or even isolates with high specificity and efficiency has been recently demonstrated in human oral biofilms[101] and murine models of *C. difficile* infection.[102] As highlighted below, similar types of precision tools will provide approaches for precisely manipulating microbiota to test hypotheses and to develop approaches for engineering microbiomes to generate or to restore beneficial effects.



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substantial barrier to research. Understanding more generally how various therapies including antibiotics, probiotics, prebiotics (essentially, fertilizer for the microbiome), phage therapy, *etc.* can reshape the microbiome remains a major technological and theoretical challenge.

As noted above, other mammals also have species-specific microbiomes, and many veterinary applications exist, from curing diseases such as inflammatory bowel disease and irritable bowel syndrome, and perhaps obesity, that plague domestic pets (obese owners tend to have obese pets, and sharing of microbes among family members including nonhuman family members has been demonstrated).[104] Additionally, manipulation of the microbiome to promote growth, to feed conversion efficiency, to manipulate body composition, and to improve disease resistance in livestock is a major area of interest. One could also imagine manipulating the microbiome for performance enhancement, for example, in horses and greyhounds, although little information on this topic is available in the literature to date.

Microbiomes outside the gut also play important roles, although most investigations have focused on either defining a healthy baseline community (without specific regard to function)[105] or on looking for differences associated with specific diseases.[106] The oral microbiome has received intense scrutiny in the context of tooth decay and gum disease,[107−109] the vaginal community is associated with bacterial vaginosis,[110,111] as well as susceptibility or resistance to yeast and viral infections.[112] The skin community has been linked to acne,[113] psoriasis,[114] and atopic dermatitis[115] and may play a role in resisting infections,[116] stimulating the immune system,[117] and potentially even risk of melanoma.[118]

The dynamics of the human microbiome remain relatively poorly understood, as few high-resolution time-series studies have been conducted.[119] The microbiome is initially seeded with different microbes depending on delivery mode, either vaginal microbes from passing through the birth canal or skin microbes acquired from the environment or from people touching the baby after C-section.[120] After that, the approach to the adult state takes about 3 years in the gut,[121,122] but the time scale is unknown in other body habitats. Development often appears chaotic early on, although whether this is an intrinsic property of the system or due to extrinsic forcing is not known at present. Similarly, whether early life events have relatively little impact because of the profound changes during this period or have a large impact in shaping the microbiome for life-long health is also unknown. However, exposure to allergens such as pets seems to have highly time-dependent effects (prenatal to first 3 years of life exposure reduces asthma and pet allergies later; exposure as a teenager increases risk[123]), and early life antibiotics increase the likelihood of obesity in a range of species.[124] Understanding how the microbiome can change and how we can develop predictive models that allow responses to everything from artificial sweeteners to diets to drugs affect the individual is a major goal of the field. Antibiotic resistance is a major threat; diagnostics and surveillance at the clinical and public health levels are required.[125,126] The tools developed here will be applicable to these efforts, and as noted above, the origins of resistance are also of intense interest in understanding microbiomes.

**Microbial Ecology.** Microbial communities are a pervasive and central part of every ecosystem on the planet, including our own bodies, as discussed above. Since their discovery, developing methods to detect and to characterize the bacteria, archaea, viruses, and protists that constitute the microbial world has driven technological research for microbiologists and microbial ecologists.[127] What started as a need to "see" the microbial world led to the development of microscopes and has now escalated into an explosion of technologies aimed at classifying the species that exist, their cellular metabolism, their impact on the chemical world around them, and the biosphere at large.[128,129] While many techniques exist to understand the microbial world, these can be divided into two themes: culture-dependent and culture-independent, the difference being whether the microbe is grown *in vitro* or characterized in its environmental state. The use of DNA as a marker of microbial diversity or function in an environment was realized in the 1970s.[130] This led to the development of technologies aimed at characterizing the microbial world using DNA or RNA. Culture-dependent analyses leveraged polymerase chain reaction (PCR) and genomic sequencing to determine the phylogeny and evolutionary history of the bacteria growing on plates, while PCR and metagenomics were being applied to characterize the microbes from DNA extracted from their native environment.

Currently, microbial community characterization is predominantly performed using specific growth media and sequencing for genomics, metagenomics, metatranscriptomics, as well as an array of molecular detection technologies.[131] Using 1 ng of DNA extracted from a cotton swab sample, we can now sequence the metagenome of the bacteria found there and assemble their genomes in a routine manner.[132,133] This detection threshold and data resolution are unparalleled in the history of our field. However, the methods used to acquire and to process samples and to sequence this genetic material have considerable limitations. Chief among these is the ability to sample and to characterize microbial communities remotely in a cost-effective manner, at spatial and temporal densities of observations that enable researchers to assess the natural gradients of diversity, the longitudinal fluctuations in community structure, and the routine detection of particular organisms in a quantifiable manner.

Microbial ecology research has generated many hypotheses that require dense spatial and temporal sampling to test and to refine. For example, determining the connectivity of microbial communities in river systems requires genotype-level resolution observation of bacteria at regular temporal and spatial frequencies. Only with a dense grid of simultaneous observations are we able to capture the dynamic dispersal and interaction of assemblages of bacterial taxa. In the absence of technology that enables this set of measurements, studies use two approaches: deep observation of few samples or shallow observation of many samples. For example, ultradeep sequencing of taxonomic or functional marker genes provides insights into the composition of specific microbial ecosystems, but without analyzing larger numbers of samples, limitations arise: the statistical significance of observed patterns cannot be determined, the patterns of co-occurrence between genes and taxa are difficult to assess, and the dominant biotic or abiotic factors structuring communities across time and space remain undetermined. Thus, for microbial ecology, many samples from related or contrasting communities must be studied in parallel.[127,128]

Currently, collecting these samples is nearly always a manual activity, requiring many hours of human effort. Additionally, processing of these samples occurs in a laboratory, often long after the sampling has occurred, so that all data generated are retrospective. While the data streams generated from these studies are rich and vast, to elucidate ecological processes at the scale of an ecosystem relevant to landscape-scale processes or specific applied utility requires continuous, remote, and spatio-temporally dense measurements. An example of this challenge is



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monitoring river systems. The state of the art requires manual sampling, DNA preparation, and analysis, which limits the depth and breadth of observations and hence the ecological significance of the findings. A recent study focusing on the water catchment scale biogeography of microbial distribution examined 23 samples across a river basin (Figure 1A) from a single time point

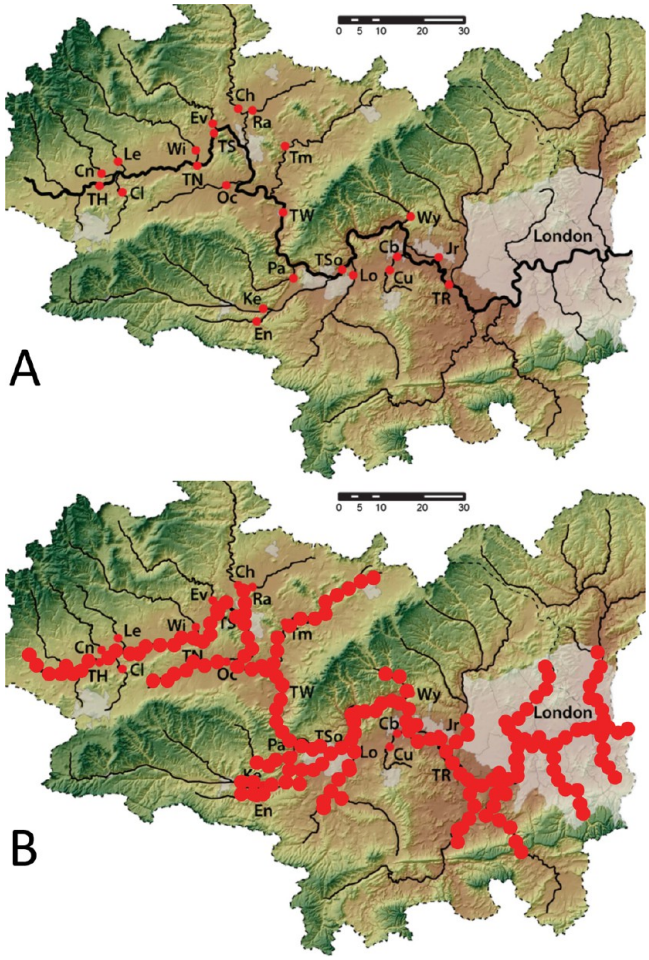


**Figure 1. (A) Map of regional study design for a state-of-the-art exploration of microbial diversity across a river catchment. Reproduced with permission from ref 134. Copyright 2015 Nature Publishing Group. (B) Example of coverage provided by dense grid of sensors for automated water-borne microbiome sampling.**

and used the data to describe the ecological dynamics across this system.[134] This study provided an observational density of one sample per 432 km$^2$ of the river basin. To get two orders of magnitude improvement in the observational density of this river basin would require analysis of ~2000 water sampling locations every 6 h for a month. This requirement would enable an observational density of 5 km$^2$ per sample (Figure 1B) but would also add a temporal element with 120 time points. This change multiplies the observational density to create 242,000 observations within a single month. With this data set, we would be able to determine the diurnal fluxes in microbial community density and composition, explore pulses in river community structure in response to river flow, allochthonous input (including waste discharge events), and weather patterns, and understand the true stability and biogeography of the river system. This study would provide us with the opportunity to use the microbial assemblages as an early warning system of shifts in ecological function and stability, to create a dynamic distribution model of river activity and its impact on biogeochemistry and response to pollution, and to devise novel ecological principles about the connectivity of microbial assemblages in these fluid systems.

A more applied example would be to develop the ability to monitor combined sewage overflow outlets or human stool or oral samples to determine the temporal flux in microbial community structure. Developing sensor platforms that could be used to monitor microbial communities routinely would be useful for detecting pathogen release into river systems or shifts in microbial community in stool or saliva that could be used as biomarkers for disease and health. One space in which monitoring could have enormous impact on human activities and health would be in urban built environments. The melting pot of humans, animals, plants, and water in cities creates a complex microbial meta-ecosystem that critically influences human health. In the urban environment, diverse microbes can migrate rapidly by water or air, colonize new niches, transfer between animal and human hosts, develop new functional capabilities, and transform urban metabolites. Environmental infectious disease transmission is a central problem for high-density urban environments. One hundred years ago, concerns about disease triggered the International Joint Commission study of trans-boundary microbial contamination in the Great Lakes, one of the largest urban efforts ever conceived. Today, key questions remain unanswered. How do urban sources and ecosystem dynamics affect microbial pathogen distributions? Is microbial water quality improving or deteriorating? What actions are needed to make soil, air, and water quality sustainable? Under increasing pressure from growing urban populations, renewed interest in addressing these questions is creating an impetus to develop novel technology to create data sets capable of tracking and monitoring the microbial health of our cities.

Microbial ecology is an important field for many aspects of human activity, from ecosystem restoration to food security, from urban sustainability to water resource management. To understand the systems that are vital for our health and well-being, it is essential that we develop mechanisms to observe the microbial world at sufficiently detailed *and* broad spatial and temporal resolutions. At a fundamental level, we need to parametrize the dynamics of microbial species, so that they can be modeled and predicted. Existing tools for characterizing the microbiome require either automated or manual collection of samples and use expensive, cumbersome apparati that require extensive personnel and time. In addition, devices need to be accessed regularly, cannot be remotely deployed, and cannot send back data without extensive robotization, which can be prohibitively expensive (*i.e.*, remote-operated submarines[133]). When they are collected, most samples need to be processed for DNA extraction, PCR, and sequencing in the laboratory, which, while generating a popular data product, is expensive and time-consuming. Although technology does exist for remote, inexpensive characterization of microbial communities, the samples still need to be manually collected and manipulated.[135] Addressing these challenges will require a cross-disciplinary program of technology development for automated sampling devices to enable the repeated characterization and remote data transfer of the microbiome. These technologies must enable integrated high spatiotemporal characterization of microbiomes with similar efforts to characterize the physicochemical and physiological properties of these environments. The ability to capture,


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to analyze, and to model these data also represent significant challenges.[136]

**Biofilm Formation.** In most biological, biomedical, and industrial circumstances, multiple microbial taxa typically form communities and can exist in biofilms. These microbial communities are highly structured and exhibit subtle patterns of spatial organization, as exemplified by microbial mats and soil communities in the environment and by communities that live within animal hosts, such as those in dental plaque, the gastrointestinal tract, and the lungs of cystic fibrosis patients. The distribution and behavior of species in these communities influence and are influenced by the role they play in a specific ecology or ecological trajectory. Important factors include patterns of cooperation and competition, environmental conditions, as well as the microbiology of individual species. It is in this last context, the microbiology of biofilms of individual species, where we have the most knowledge at present. This corpus of work provides us with a repertoire of concepts. There are five steps in the "standard model" of biofilm development: (1) free swimming planktonic bacteria attach reversibly to a solid surface and migrate on the surface; (2) bacterial cells adhere "irreversibly" to a surface and secrete extracellular polysaccharides; (3) microcolonies of 50−100 bacteria are formed, in a step that is sometimes described as the first social step in the development of a biofilm community; (4) a mature, spatially structured biofilm develops *via* a process of extracellular polymeric substances (EPS) production, signaling, motility, cell division; and (5) free swimming cells are released from the community and in turn repeat the process. Recent work on biofilm development invokes, refines, and sometimes modifies this paradigm. Illustrative examples without any attempt at completeness follow. Cells that land on a surface undergo complex processes of surface sensing,[137] which is just beginning to be understood. The transition from free swimming to biofilm physiology is often dependent on cyclic di-GMP, a bacterial secondary messenger, which has emerged to be kind of master regulator that impacts biofilm formation, EPS production, bacterial motility, virulence, and other processes.[138] Extracellular communication becomes more important; an important example with strong phenotypic impact is quorum sensing,[139−141] which remains an active area of study. Secreted extracellular polysaccharides are a defining characteristic of biofilms, but they are not simply secreted passive adhesins and can have important social functions in controlling surface motility in the early assembly of microcolonies.[142] Ultimately, biofilms produce a complex matrix of EPS, which include proteins, lipids, lipopolysaccharides, nucleic acids, as well as extracellular polysaccharides, that serves as scaffolds for the community. An outstanding challenge for our field is to generalize our existing ideas for single-species communities and/or to come up with the analogues of these guiding principles for multispecies communities.

The strategies we use for studying communities with two to three species will be different from those with 100 species, but both will be crucial for the development of our understanding. Model communities with simplified interactions will be helpful in this regard; Silver and co-workers have proposed and developed the tools to create a simplified gut microbiome that lives and interacts within a mouse model.[143−145] Special considerations are being built into such systems to enable manipulation of the microbiome and to contain it within the experimental model.

Solving this complex problem of understanding microbial communities is like learning a new language without a dictionary or knowledge of grammar: the fastest approach may be to learn a language like a child—through imitation and trend identification in complex examples of linguistic usage. However, to achieve the most powerful and nuanced linguistic expression, a framework of rules needs to be established. We will ultimately need to study realistic communities with the full diversity of species. Model systems with a small number of species can be used to identify predictive rules that help make sense of the trends. In this spirit, there are a number of interesting examples of studies that engage multispecies biofilm communities.

**Cooperation and Competition in Single-Species Biofilms.** Even in single-species biofilms, good examples of cooperative and competitive interactions have been reported. In *P. aeruginosa*, stratified patterns of protein synthesis and growth have been demonstrated.[146] Using green fluorescent protein (GFP) reporter gene constructs, it was found that active protein synthesis was confined to a narrow band in the part of the biofilm adjacent to the oxygen source. The zone of active GFP expression was approximately 60 $\mu$m wide in colony biofilms and 30 $\mu$m wide in flow cell biofilms. Mature *P. aeruginosa* biofilms contain active, growing cells. However, these biofilms also comprise large numbers of inactive cells. A recent example of the subtle interplay of cooperation and competition in these bifurcated populations can be seen in *Bacillus subtilis*. The complex relationship between peripheral cells and interior cells in a two-dimensional (2D) *B. subtilis* community leads to oscillatory growth.[147] A kind of mutualism emerges. Peripheral cells protect the interior from external cytotoxic agents but also starve the interior of nutrients. A novel phenotype with metabolic codependence emerges, one in which the growth halts periodically, which can, in principle, benefit interior cells and increase nutrient availability.

**Cooperation and Competition in Two-Species Biofilms.** The recurring theme of spatial structuring, already evident from the above, can be seen in two-species consortia. One example is cocultures of *Acinetobacter* sp. C6 and *Pseudomonas putida*, two soil-inhabiting bacteria that are members of a microbial consortium isolated from a creosote-polluted aquifer. In the presence of aromatic carbon sources, the two species enter into a symbiotic relationship, where *Acinetobacter* plays the role of host and *P. putida* plays the role of a commensal.[148] Acinetobacter metabolizes benzyl alcohol to benzoate. Since *P. putida* metabolizes benzoate produced by *Acinetobacter*, it mutates to have enhanced ability to attach to *Acinetobacter* and forms a "mantle" over *Acinetobacter* microcolonies. As a result, the two-species consortium has increased stability and productivity relative to their isolated counterparts. Implicit in this relationship is the adaptation of motility to facilitate this organization. It will be interesting to see how the rules for monospecies microcolony formation are altered in this two-species consortium. Recent work has suggested that small multicellular clusters rather than single cells can move on the surface and coalesce into microcolonies. Another demonstration of the role played by motility in generating spatially structured communities can be found in rRNA fluorescent *in situ* hybridization (FISH) confocal microscopy observations on cocultures of *Burkholderia* sp. LB400 and *Pseudomonas* sp. B13(FR1).[149] When the consortium was fed citrate as the carbon source, which can be metabolized by both *Pseudomonas* and *Burkholderia*, the two species formed separate microcolonies. However, when the carbon source was changed from citrate to chlorobiphenyl, *Pseudomonas* used their surface motility to form mixed microcolonies with *Burkholderia*, as the former can metabolize chlorobenzoate produced by the latter when grown on chlorobiphenyl.



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Of course, not all two-species consortia enter into symbiotic relations. Some relationships end in competition. The airways of cystic fibrosis patients are colonized with bacterial communities that evolve over time. *Staphylococcus aureus* is the most prevalent species in early childhood, but *Pseudomonas aeruginosa* dominates the ecology in early adulthood. In a recent coculture experiment on *P. aeruginosa* and *S. aureus* on monolayers of human bronchial epithelial cells with the ΔF508 cystic fibrosis transmembrane conductance regulator (CFTR) mutation,[150] it was shown that *P. aeruginosa* drives the *S. aureus* expression profile from that of aerobic respiration to fermentation. Although the reasons for this ecological shift are complex, two key factors have been identified: the production of Fe-chelating siderophores by *P. aeruginosa* and its preferential consumption of *S. aureus*-produced lactate over other carbon sources. It has been established that *S. aureus* induces the production of quinolone quorum sensors and subsequent biofilm formation in *P. aeruginosa*.[151] Although *S. aureus* and *P. aeruginosa* can initially coexist, *P. aeruginosa* eventually reduces *S. aureus* viability in extended cocultures.

**Cooperation and Competition in Three-Species Biofilms.** Generalizing from a two-species consortium to a three-species consortium greatly expands the range of possibilities in mutualistic relationships. A recent example is a ternary model biofilm community of *Pseudomonas aeruginosa*, *Pseudomonas protegens*, and *Klebsiella pneumonia*,[152] where the results are quite suggestive. The biomass of *K. pneumonia* KP-1 within the mixed consortium was significantly greater than the biomass of single-species KP-1 biofilms, despite having to contend, in principle, with the increased competition with *P. aeruginosa* and *P. protegens*. Moreover, the increased community-level resistance to aminoglycoside antibiotics is striking: *P. protegens* produces the infamous aminoglycoside-modifying enzyme *N*-acetyltransferase, which causes aminoglycoside antibiotics to bind inefficiently to bacterial ribosomes, so that it can no longer inhibit bacterial protein synthesis. Although *P. protegens* comprises only ∼15% of the total biomass, it is able to confer protection to both *P. aeruginosa* and *K. pneumoniae*, so that only 10% of the mixed biofilm is removed by the aminoglycoside antibiotic tobramycin. These model systems represent a large and underexplored frontier of microbiology and promise to attract and to reward attention.

## TECHNOLOGY STATUS AND NEEDS

Three areas of technology development will underline the breakthroughs needed to advance the biological questions described above: (1) sequencing and identifying microbial macromolecules and metabolites, (2) increased speed and standardization of bioinformatics tools, and (3) development and application of high-resolution imaging approaches to couple biochemical analyses with micron-scale microbe−microbe and microbe−tissue interactions. Although DNA sequencing technologies are currently high throughput and inexpensive, the same cannot be said for other omics technologies, such as metatranscriptomics, metaproteomics, and metabolomics, which are still primarily run one sample at a time and require special equipment and expertise. To elucidate the functional roles of members of complex microbial communities, new and better technologies are required for higher dynamic range, speed of analysis, and throughput. In addition, more complete and validated databases are needed for protein and metabolite identification. Advances in imaging technologies are needed to enable spatial localization of microbial cells and individual proteins in environmental matrices. Imaging tools for multiscale imaging, beyond conventional light/electron correlative microscopy tools, are needed, such that molecular, cellular, biofilm, and larger scales can be examined and inter-related.

**High-Speed, High-Throughput Genomic Sequencing and Annotation.** Genome sequencing, although insufficient for determining function, is still critical as a first step to understanding the function of novel organisms and ecosystems. Sequencing technologies for DNA have rapidly improved, achieving cost savings of over a million-fold in the past 15 years. However, there remain many barriers to overcome. First, the technologies that are currently highest throughput, such as the Illumina sequencing by synthesis platform, produce only short fragments of DNA.[153] Although these short fragments are extremely useful when mapped against a reference database, such as is available for the human genome and for model microbes, *de novo* assembly and annotation remain a major challenge, and many technical parameters such as insert size, read length, and depth of sequencing are not standardized across projects (nor are their effects on the final assembled genome known, in general). Second, long-read technologies, such as zero-mode waveguide sequencing (*e.g.*, Pacific Biosciences) and nanopore-based sequencing (*e.g.*, Oxford Nanopore), are both low-throughput and error-prone and cannot yet operate on very small amounts of input material.[154,155] Current methods for amplifying genomic DNA tend to introduce biases that greatly decrease the efficiency of sequencing because the parts of the genome that are amplified the most are read over and over again. However, these longer reads have proven useful for genome assembly, which is necessary for understanding higher-order structure, gene regulation, and revealing which components of the genome most likely work together in operons. A recent long-read method has extremely low error rates[156] (and is being commercialized[157]).

Single-cell sequencing (discussed below) remains a challenge and, despite many recent improvements, typically recovers only 70−90% of a genome. Understanding how to integrate partial signals from many cells, or to coax a cell through a few cycles of division before sequencing to reduce stochastic losses such as DNA attached to the cell wall and therefore lost to sequencing, remains largely unexplored. The dream system would be to separate single cells from complex matrix such as stool, soil, or biofilm, disrupt them *in situ*, and to separate each into a single library for highly multiplexed sequencing on a cheap long-read platform, ideally simultaneously reading out the DNA, the RNA, and any modified nucleotides at either level (for example, methylated nucleotides in the DNA are important for understanding gene regulation, and many types of modified bases in the RNA, including inosine, can alter the meaning of the transcript relative to what is encoded in the genome), all at low cost. However, the technology for performing these tasks has not been developed, although various microfluidic protocols show enormous promise in library preparation. Cellular isolation and disruption at high throughput remains a considerable challenge, as the impedance mismatch between a physical specimen and vast numbers of single-cell genomes is presently large.

Obtaining the DNA sequence is not sufficient: although most bacterial, archaeal, and viral DNA consist of protein-coding genes, the same is not true for microbial eukaryotes, and even in compact genomes, the regulatory elements (including siRNAs, miRNAs, CRISPR repeats, *etc.*) are important to understand both from functional and from evolutionary perspectives. Improved methods for understanding the functions of protein-coding genes, including structural genomics, computational structure prediction of sequences of unknown function that might be members of highly diverged protein families, and high-throughput expression followed


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by assays of biochemical function (that could be improved by mass spectrometry and other high-throughput chemical profiling techniques to identify novel biotransformations), are urgently needed. Expression data under different conditions, perhaps involving other organisms as well as pure cultures, may be necessary for understanding the functions of novel regulatory elements, although such studies are extremely time-consuming with existing techniques and impractical to apply to the vast majority of organisms that cannot yet be grown in culture. Additionally, compiling large databases of single-cell genomes and community metagenomes from environmental samples would allow us to understand the functions of unknown proteins and regulatory elements by identifying other genes, species, or environmental conditions with which they are associated (again, expression data at the RNA, protein, and/or metabolite level would be especially helpful here). An important side effect of these efforts would be improved understanding of how microbial communities function, which would assist in modeling efforts. Finally, many enzyme functions are known but not relatable to a single protein sequence, and many protein sequences have had their functions determined, but the annotations are in individual publications and not yet aggregated into machine-readable databases. Consequently, a focused effort to identify these missing enzymes and to apply sophisticated natural language processing (NLP) techniques such as IBM's Watson technology[158] to the biochemical literature could be especially useful in leveraging existing efforts in knowledge generation. One could also imagine nanosensors for enzyme function that might be able to read out a suite of possible enzyme activities better than currently available biochemical assays, each of which typically covers only a restricted range of chemistries.

**Nanoscience and Nanotechnology Opportunities.** The great advantage of nanoscience and nanotechnology in studying microbiomes is that the nanoscale is the scale of function in biology. It is this convergence of scales at which we can "see" and at which we can fabricate that heralds the contributions that can be made by developing new nanoscale analysis tools.[159−168] Microbiomes operate from the nanoscale up to much larger scales, even kilometers, so crossing these scales will pose significant challenges to the field, in terms of measurement, stimulation/response, informatics, and ultimately understanding.

Some progress has been made in creating model systems[143−145,169−173] that can be used to develop tools and methods. In these cases, the tools can be brought to bear on more complex and real systems. Just as nanoscience began with the ability to image atoms and progressed to the ability to manipulate structures both directly and through guided interactions,[162,163,174−176] it has now become possible to control structure, materials, and chemical functionality from the submolecular to the centimeter scales simultaneously. Whereas substrates and surface functionalization have often been tailored to be resistant to bioadhesion, deliberate placement of chemical patterns can also be used for the growth and patterning of systems, such as biofilms, to be put into contact with nanoscale probes.[177−180] Such methods in combination with the tools of other fields (*vide infra*) will provide the means to probe and to understand microbiomes.

Key tools for the microbiome will need to be miniaturized and made parallel. These developments will leverage decades of work in nanotechnology in the areas of nanofabrication,[181] imaging systems,[182,183] lab-on-a-chip systems,[184] control of biological interfaces,[185] and more. Commercialized and commoditized tools, such as smart phone cameras, can also be adapted for use (*vide infra*). By guiding the development and parallelization of these tools, increasingly complex microbiomes will be opened for study.[167]

Imaging and sensing, in general, have been enjoying a Renaissance over the past decades, and there are various powerful measurement techniques that are currently available, making the Microbiome Initiative timely and exciting from the broad perspective of advanced analysis techniques. Recent advances in various -omics technologies, electron microscopy, optical microscopy/nanoscopy and spectroscopy, cytometry, mass spectroscopy, atomic force microscopy, nuclear imaging, and other techniques, create unique opportunities for researchers to investigate a wide range of questions related to microbiome interactions, function, and diversity. We anticipate that some of these advanced imaging, spectroscopy, and sensing techniques, coupled with big data analytics, will be used to create multimodal and integrated smart systems that can shed light onto some of the most important needs in microbiome research, including (1) analyzing microbial interactions specifically and sensitively at the relevant spatial and temporal scales; (2) determining and analyzing the diversity covered by the microbial genome, transcriptome, proteome, and metabolome; (3) managing and manipulating microbiomes to probe their function, evaluating the impact of interventions and ultimately harnessing their activities; and (4) helping us identify and track microbial dark matter (referring to 99% of micro-organisms that cannot be cultured).

In this broad quest for creating next-generation imaging and sensing instrumentation to address the needs and challenges of microbiome-related research activities comprehensively, there are important issues that need to be considered, as discussed below.

**The Synthetic Biology Revolution.** The microbial world has been studied intensively since the invention of the microscope. With the advent of molecular genetics, recombinant DNA, and rapid DNA sequencing, many microbes are well understood and form the core of both basic and applied biological research. We are now ready to reap the benefits of this investment, and synthetic biology is poised to fuel this future. In broad strokes, synthetic biology provides the possibility of rapid, systematic, and predictable engineering of the microbial world. This includes the creation of microbes for varied purposes: (1) to act as biological computers to sense and to respond to events in their environment that cannot be directly observed; (2) to serve as chassis for chemical engineering to produce molecules of value more sustainably; (3) to produce new chemistries never seen before; and (4) to create synthetic microbes with a genetic firewall that prevents their genes escaping into other organisms in the environment. Increasing our ability to program microbes, especially in the context of complex microbiomes, will have vast implications for human and global health.

Synthetic biology draws from investments in molecular biology made over the past 50 years. Microbes provided the basis for much of this work, which ultimately led to sequencing the human genome, production of drugs such as human insulin and antibodies, and the growth of the biotechnology industry. Synthetic biology takes all of the prior knowledge to achieve faster, more predictable, and ultimately cheaper engineering of biology for the common good. The ultimate culmination of these efforts will be to synthesize entire microbial genomes that will perform as predicted without undesired impacts on the environment. Recent advances in the synthesis of DNA make this goal possible in the foreseeable future.



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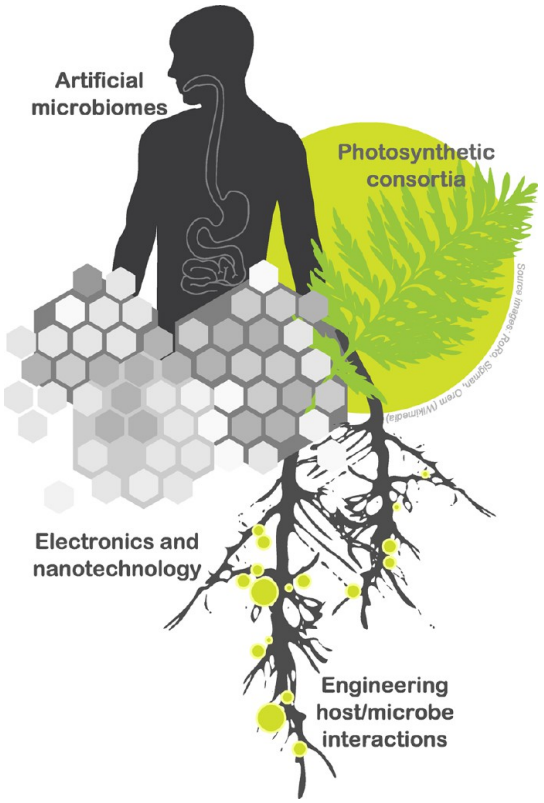


**Figure 2. High-risk projects driven by synthetic biology include a number of grand challenges, as follows. (1) Development of artificial microbiomes that can safely live with a human or animal host. These could, for example, make the use of antibiotics in farm animals unnecessary and provide new classes of therapeutics. Such synthetic microbes would become the gold standard in all of bioengineering. (2) Development of a microbial consortium driven by light collection that can produce commodity chemicals. This goal lies at the center of the dream of harnessing our greatest natural resource—sunlight—which Nature has accomplished but upon which we can improve. (3) Development of microbes that can function beyond biological systems in electronics, sensing, and nanotechnology. Biology is good at production at the mesoscale, which has been relatively refractory to bioengineers and chemists. Synthetic biology aims to learn from Nature to produce novel useful molecular structures to work at the interface between organisms and machines. (4) Engineering at the interface between microbes and their hosts. The same principles of synthetic biology can be applied to all cells, including those of animals and plants, to program their interactions with the microbial world for both discovery and applied research.**

**Work at the Interface of Basic and Applied Research.** To impact economic development and the average citizen, the microbiome initiative must support precommercialization research. This is particularly important in areas where the ultimate commercial product will require extensive and commercially risky testing or where the science that would underpin such a product is not yet established. Like other scientific revolutions that have transformed our understanding of our place in the universe—the Copernican revolution that made us realize that the Earth was not the center of the universe, the Darwinian revolution that made us realize that we were one twig of life among many, the Woeseian revolution that made us realize that most of life's diversity is microbial rather than contained in the plants and animals we see around us as multicellular organisms—the implications of the microbiome revolution, that we are outnumbered within our own bodies in terms of cell count and vastly so in terms of gene counts, will take time to understand at the level that permits far-ranging technological development. However, we are now poised to make rapid progress with appropriate investments in microbiome research.

Potentially ground-breaking areas include artificial microbiomes—engineered consortia of microbes that work together. One of the best methods of testing hypotheses about microbiome function will be to try to mimic or to replicate all or part of this function (*vide infra*). Exploring even a small part of the phase space of microbiome and function will help in the development of predictive understanding. As in other areas of such multidimensional complexity, theory and simulation can play critical roles in proposing key experimental tests of hypotheses and mechanisms, as well as in explaining observations. Consequently, improved models at all scales, as well as bridges across scales, such as appropriate quasi-static approximations, are required, just as improved modeling has been critical for understanding which materials in a vast search space are most likely to have promising properties if synthesized.[186]

Microbial electrochemical technologies (METs) are examples of such artificial microbiomes. Microbes have been used for electrolysis, chemical production, and generation of electrical power.[187] In the future, these technologies may further be coupled with photosynthetic microbes and produce or secrete advanced biofuels or high-value chemicals and drugs. Microbial fuel cells (MFCs) have been extensively researched but have not yet produced a commercial product.[188] Current MFCs suffer from low power densities and small scales.[189] Mechanistic insight into microbe interactions with one another and with fuel cell components remains elusive and hinders rational design of MFCs. Intercellular electron transfer, extracellular electron transfer (EET), and microbe electrode electron exchange are processes that continue to elude comprehensive mechanistic understanding.[190]

Extracellular electron transfer has been extensively studied in bacteria of the genera *Geobacter* and *Shewanella*. Several pathways have been proposed to explain how microbes move electrons beyond their outer membranes. It may be that electrons can be directly moved to or from an extracellular surface by contact with outer membrane proteins, shuttled to targets by secreted electron carriers, or transferred along the length of bacterial appendages such as pili or membrane extensions.[191,192] The design of microbial communities for maximal current output is difficult without understanding the EET pathway and the microbial components involved. To test how electrons are moved across length scales ranging from microns across biofilms to nanometers along individual cell appendages, nanoscience and nanotechnology methods are needed. Manufacturing nanoscale electrodes enables experiments to be performed that can probe electron transfer within larger communities.[193] Furthermore, scanning probe microscopies such as conductive atomic force microscopy and scanning electrochemical microscopy can be used to measure individual parts of cells, and recent enhancements enable measurements of key components of biological structures.[164,194,195]

Cocultures of multiple species have been shown to produce larger currents than any of the pure cultures in MFCs.[189] Understanding the full community of microbes that power MFCs in wastewater and benthic sediments will enable the efficient design of artificial microbiomes for energy production. Genome synthesis and targeted editing allow a far easier entry method for rapid engineering of microbes that have thus far only been


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sequenced but have not been cultivated, thereby drawing on the vast potential of the entire microbiome. Techniques that have primarily been used in homologous libraries can easily be applied with genes from the environment when those genes can be rewritten to optimize codon usage and other parameters for expression in a standardized chassis.

**Single-Cell Genomics.** Mechanism-based understanding of microbiome function (*vide infra*) and its interactions with host cells requires detailed characterization of the organisms that compose the microbiome, including their genetic contents. Complete genomic analysis of the microbiome implies determining the complete genome sequence of each constituent strain. Achieving this goal is technically challenging because of the complexity of microbiome communities, which may contain hundreds of operational taxonomic units (OTUs), the wide ranges of organism abundances, distorted representation of organisms in easily sampled fluids, fine-scale differences in genomes[196] that are functionally distinct but difficult to identify and to understand at the DNA sequence level, the diversity in conditions required for culture,[197,198] and lack of available culture conditions for most organisms.

Single-cell genomics can productively contribute to the genomic characterization of the microbiome, although technical improvements to single-cell methods are needed for these methods to have major impact on microbiome research.[199] Standard approaches to single-cell analyses involve the physical isolation of individual microbes, lysis of each cell, and the amplification of the cells' genomic contents by whole-genome amplification (WGA), commonly multiple displacement amplification (MDA).[200]

Single-cell genome sequences can be obtained directly from crude samples to generate reference sequences for organisms that are recalcitrant to laboratory culture.[201,202] Such reference sequences are valuable because they provide frameworks for interpreting metagenomic reads from these organisms and can be used to validate binning approaches that aim to create composite genome sequences directly from metagenomic data sets.[203−205] Single-cell sequencing can be used to obtain sequence information from rare community members in cases where analyses can be targeted to these organisms.[206]

The high sensitivity of single-cell sequencing opens the possibility of detailing the spatial structure of the microbiome, which is lost in conventional genomic analyses. For example, single cells obtained from microdissected tissues can be sequenced and assigned to the microsample. This approach has been effectively applied in tumor analyses.[207] An alternative approach to resolving spatial information is the use of single-cell workflows on small groups of physically associated cells to produce "mini-metagenome".[208] The co-occurrence of sequences from different organisms in mini-metagneomic data sets implies physical and functional association of those organisms, although care must be taken to exclude technical contamination artifacts. Future *in situ* single-cell analysis approaches may enable even higher resolution of spatial relationships.

The rise of extraordinarily high-throughput DNA sequencing makes comprehensive genomic analysis of the microbiome theoretically possible by a number of different approaches. In principle, high-throughput analyses of cultured isolates and/or single cells could be used provided that such isolates and/or amplified single-cell samples could be obtained. If such samples were available, the major challenges would be cost-effective sequence library construction from such a large number of samples and data analyses.

The major opportunities in single-cell analysis for microbiome studies are streamlining experimental workflows, improving data quality, and designing experiments that utilize single-cell data in synergy with specialized sample collection methods and other genomic analyses.

Currently available single-cell methods produce data quality that is distinctly inferior to standard genomics methods. Early on, contamination was a major impediment to single-cell analysis;[208] extreme care remains critical to the production of contaminant-free single-cell data sets.[209] Typically, single-cell data sets enable recovery of 10−60% of the genome due to the extremely uneven amplification of the genome by WGA.[209] Another issue with single-cell genomic data quality is the high frequency of chimeric reads generated in WGA, which can dominate coverage at specific loci.[210−212] Chimeric reads interfere with *de novo* analyses and analyses of horizontal gene transfer. In particular, the high incidence of artifactual chimeras, commonly occurring once per 5000 raw bases in single-cell MDA data, makes long-read analyses of typical single-cell data useless.[213] The coverage bias and location of chimera artifacts are nonreproducible from cell to cell, enabling substantial gains in genome coverage and assembly contiguity if data from several closely related cells can be pooled.[199,214] Finally, techniques for analysis of RNA (other than abundant rRNA) in single microbial cells are not yet established,[215] although many groups are actively working on this problem using strategies ranging from reducing the input biomass in standard bulk protocols to using *in situ* sequencing or probes for specific transcripts.

Significant work on improving WGA chemistries is underway.[199] To date, efforts have focused on reducing amplification bias in mammalian cells to enable improved genome recovery at lower sequencing effort and to enable accurate assessment of gene copy number. Today, WGA of microbial cells is performed almost exclusively using MDA, with few exceptions,[216] despite the advent of alternatives that promise improved performance.[217]

Other work focuses on novel reaction formats for single-cell WGA to improve throughput and data quality. There are two ways to improve throughput for single-cell-resolved microbiome analysis. The first is simply to enable WGA of a larger number of cells. The second is to preselect cells of interest for WGA, which is functionally equivalent to higher reaction throughput when subpopulations are of interest. Lab-on-chip-based microfluidic cell-sorting methods, including optical tweezers-based approaches, can be used for cell selection based on observed morphologies.[204,206,218] Nucleic acid probes can be used to identify cells based on lineage markers, although staining protocols may further compromise data quality.[219] Improved approaches may apply nucleic acid probes to WGA products after the amplification reaction is complete. Microdroplet approaches with the potential for very high throughput and/or improved data quality are also being developed.[216,220,221] In addition, there are alternative approaches like in-gel amplification that offer unique capabilities for single-cell studies.[222]

Despite extensive development of microfluidics for microbial single-cell analysis by individual research groups, established methods require specialized and/or expensive equipment, and there is no commercially available system for microbial single-cell genomics sample preparation.

Single-cell genomics fills important gaps needed to understand the contents and structure of the microbiome. Single-cell genomics may be the best way to characterize and to quantify microbiome composition at the strain level due to its high taxonomic resolution and lack of culture bias. However, further improvements



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in data quality, throughput, and ability to target organisms of interest are needed for single-cell genomics to make major advances in microbiome research. In the intermediate term, incremental advances in single-cell technology and improved integration with other tools and approaches like advanced analysis of metagenomic data and long-read sequencing will keep single-cell analysis at the forefront of microbiome research.

**Proteomics and Metabolomics.** To characterize the proteome and metabolome composition of myriad microbiomes comprehensively will require transformative developments to increase analytical sample throughput and biomolecule coverage and the generation of computational tools necessary to analyze, to integrate, and to visualize the obtained omics information. Ultimately, improved analytics and analyses should lead to tackling the ultimate goals of understanding microbial functions, microbe−microbe and host−microbe interactions, networks, and potentially the creation of predictive networks necessary to manipulate and to control microbial systems.

A major limitation of current proteomics and metabolomics capabilities is the need to compromise on either the scope of the experiment or the completeness of the measurements. Another significant limitation is the capacity of software to handle large volumes of data. Most mass spectrometry programs/algorithms/software break down when trying to analyze even *hundreds* of data files. Simple conversion of data in tab-delimited forms (*e.g.*, .mzML, .mzXML, or .MGF) so that data can be used easily with third-party tools performs poorly, often slowly and with errors. Batch processing requires skills at scripting. Similarly, we lack good tools and appropriate scoring functions that can address the complexity of the microbiome with proteomics. For metabolomics, the reference spectra, some 300,000 in the public domain covering some 20,000 spectra, do not cover microbial chemistries well.[223] Such limitations are areas of enormous growth potential if we want to understand the function *via* the molecular composition of the microbiome. To handle the vast numbers of samples that will need to be analyzed to understand microbiomes at the systems or population levels, traditional liquid chromatography−mass spectrometry (LC-MS) platforms, including the required analysis infrastructure, will need to be optimized or completely changed to obtain rapid sample throughput and high depth of coverage. A recent development that is a significant departure from traditional LC-MS is the construction of structures for lossless ion manipulation.[224] This technology has the potential to enable high resolving power in ion mobility separations, which exceed the resolving power of LC separations, and that in conjunction with MS-based platforms may increase sample throughput by as much as 100-fold. An additional LC-MS-based improvement with the potential to improve metabolomics and proteomics analyses greatly is multidimensional high-throughput separations for analyzing distinct ion characteristics simultaneously in a single analysis. Multidimensional separations can increase the overall measurement separation power, resulting in greater information content and more complete characterization of the complex samples.[225] It is also important to make the data interpretable for the end user. Data visualization is critical for understanding, and this need has only begun to be addressed for mass spectrometry. For example, the development of fragmentation trees for subclassification of chemistries,[226] cloudplots,[227] structural classification-based networking,[228] and molecular networking[229] will all make it easier for us to understand the data that are collected by mass spectrometry and other means. Visualization is critical for complex data sets. Although many improvements are needed to develop these capabilties in a common methodology, microbial imaging mass spectrometry has seen enormous advances.[230] Microbial imaging mass spectrometry can detect molecules, or fragments of molecules, at resolutions from 30 nm to millimeters. Imaging mass spectrometry of germ-free mice colonized with *B. theta.* or *B. longum* revealed how these bacteria are needed for the metabolism of complex carbohydrates, altering the immune system as evidenced by the increase in prostaglandin E2 and how they actively create new bile acid derivatives (Figure 3A). Three-dimensional cartography expands this range to centimeters, as shown for human skin (Figure 3B), and can be readily adapted to global scales.[231] The speed of technology development for omics analyses is rapid, and the next steps for microbiome research are to use new technologies

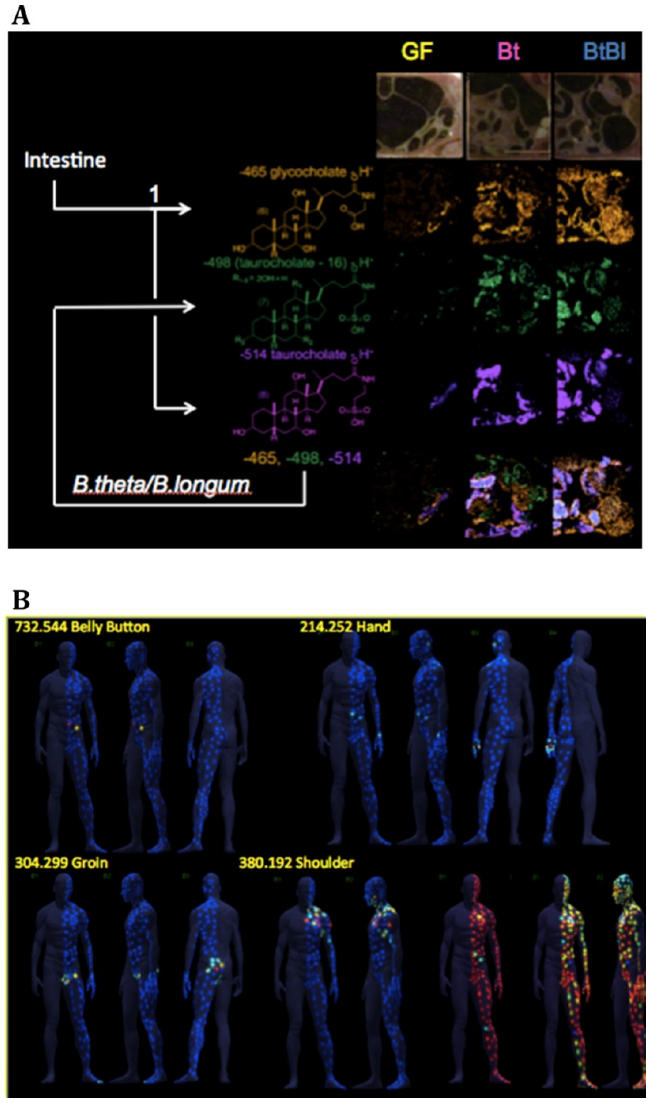


**Figure 3. Representative examples of imaging mass spectrometry and 3D cartography. (A) Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry image of bile acid metabolism by *B. theta* and *B. theta*/*B. longum*. Upon colonization, primary bile acids increase in concentration, specific bile acids have specific distributions, and the microbes actively metabolize bile acids. (B) Three-dimensional cartography of the surface of human skin. The signals shown are representative metabolomics masses detected in the experiments.**


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effectively to answer complex biological questions. Critically, the massive scale of data to be generated from these new technologies will challenge current bioinformatics capabilities. For example, we can currently only annotate less than 2% of the data that are collected in metabolomics experiments; computational solutions are key to overcome this annotation challenge.[232−234] New software and algorithms will be required to compile data sets, to overlay multi-omic analyses, and to visualize and to communicate important networks, interactions, functions, and other biological phenomena to emerge from the data. To our knowledge, at present, the only metabolomics analysis infrastructure where a large portion of the analysis is performed on microbiome data sets is the Global Natural Products Social Molecular Networking (GNPS),[235] a crowdsourced analysis infrastructure.

**Opportunities for Functional Microbiome Characterization by Activity-Based Protein Profiling.** Considerable challenges exist in determining the functional roles of human microbiomes or complex microbial communities in the environment. Due to the intrinsic complexity of these systems, the majority of our mechanistic insights into microbiomes and their enzymatic functions are either inferred from genomic sequence data or derived from experimental evidence using a relatively small number of culturable model organisms. More recently, metatranscriptomic data have been used to evaluate gene expression of the "active" fraction of microbiomes, providing greater insight into their potential aggregate functional capacities.[236,237] To advance our understanding of the functions of microbiomes, innovations in analytical approaches are needed that permit identification of the specific proteins associated with a given biochemical activity. Such innovations could, for instance, pave the path toward rational approaches to manipulate the human microbiome for therapeutic advantage or construct synthetic microbiomes to facilitate bioenergy production or bioremediation processes.

Activity-based protein profiling (ABPP) is a powerful chemical biology approach with the capacity to elucidate understanding of the specific protein activities that comprise a given microbiome phenotype. In ABPP, synthetic activity-based probes are used to report directly on protein function, regulation, and protein−small-molecule interactions within the native physiological context of systems under study. The probes target metabolite-binding sites of proteins (enzymes, transporters, or regulators) rather than DNA or RNA sequence, and thus their design is not constrained by sequence similarity or prior knowledge of DNA sequence, allowing identification of a broad variety of microbial organisms with the desired functional attributes. Specifically, probes can be developed to address: (1) targeting of enzymes with specific metabolic functions;[238] (2) chemical probes that mimic natural compounds, such as natural products or metabolites, that selectively label cells able to utilize the natural compound and bind to transporters and other proteins (enzymes, regulators) that are involved in metabolite detection, salvage, and disposition; and (3) probes that target protein regulator modifications, such as protein thiol redox events.[239] The probes consist of three elements: (*i*) a reactive group that forms an irreversible covalent bond with a target protein *via* direct catalytic reaction or photoreaction, (*ii*) a binding group (*e.g.*, protein substrate or metabolite) that biases a probe toward a protein or protein family and may also impart cell permeability, and (*iii*) a reporter group such as a fluorophore or biotin for enrichment and subsequent proteomic characterization. Alternatively, several studies employ probes containing an alkyne or azide moiety to enable the bio-orthogonal "click" chemistry reaction that permits the addition of a reporter tag following *in situ* or *in vitro* probe labeling; this chemistry also facilitates the facile exchange of reporter types applied based on the desired application and outcome of the study and properties of the sample being assayed.

To understand the function of the microbiome in different habitats, it is necessary to look across different spatial and temporal scales as well as to determine function at different levels of expression.

To date, ABPP has been applied to a diverse array of cultured microbes, from pathogenic organisms such as *Mycobacterium tuberculosis* and *Staphylococcus aureus* to cyanobacteria.[240] However, application to complex uncultivated microbiomes has yet to be reported; these challenges include optimizing sample labeling and processing, obtaining genomes or metagenomes representative of the microbiomes under study to be used for proteomic data analytics, and challenging analysis. However, these challenges are representative of microbiome proteomic analyses in general and need to be overcome to enable comprehensive omic studies. Despite challenges, ABPP has significant potential to play key roles in resolving spatiotemporal microbiome dynamics by imaging and proteomics, in characterizing functional responses due to microbiome perturbation by the environment or host, and in translating genomes or metagenomes directly to functional profiles. Coupled to improved omic and imaging strategies, ABPP will be important to the functional characterization of microbiomes.

**Multiscale Multi-Omics.** Functional testing at the ecosystem rather than the enzyme level is not yet possible. Subsets of data are accessible. Examples from different fields include bioreactors, where concentrations of different chemicals and gases can be tracked, the gut, where nutritional and physiological status of the host animal can be assessed, and for soils, where direct turnover measurements or the nutritional and physiological status of plants can be determined. At larger scales, one could apply this concept to aqueous environments to define ocean or lake health. Understanding the emergent function of microbial ecosystems remains an unsolved challenge but is a target of this initiative.

To understand the function of the microbiome in different habitats, it is necessary to look across different spatial and temporal scales as well as to determine function at different levels of expression. Genome and metagenome data are valuable for making predictions about potential functions. However, not all genes are expressed and translated to proteins under all conditions. To assess function more directly, one could use a multi-omics approach. The combination of 16S rRNA gene sequencing, metagenome sequencing, metatranscriptome sequencing, metaproteomics, and metabolomics has been referred to as an "omics information pipeline" (Figure 4).[241] Each step along the omics pipeline provides different details as to potential function or expression. For example, a multi-omics approach was recently used to determine the impact of permafrost thaw on microbial community processes.[242] In this study, the ratio of genes in the metatranscriptome to metagenome was used to assess relative levels of expression and activity of specific species



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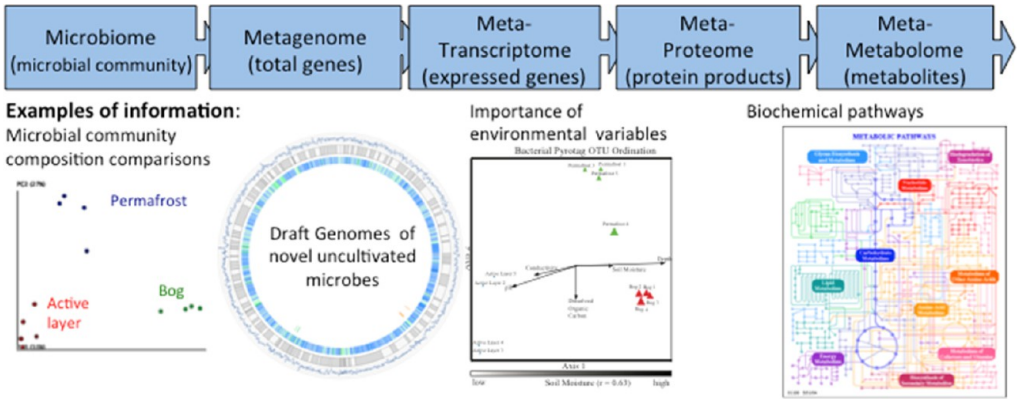


**Figure 4. Various stages of the multi-omics pipeline provide different levels of information.**

and functional genes in permafrost samples, compared to seasonally thawed-active layer samples and to a completely thawed thermokarst bog.

Another example of the use of a multi-omics approach was the use of 16S sequence analysis,[243−245] metagenomics and proteomics,[246] and metabolomics[16] to study twins that were discordant for Crohn's disease. In this series of studies, the exact same fecal samples were analyzed using different omics approaches. There were consistent differences between healthy and diseased individuals for all of the data sets, including lower diversity, depletion of *Faecalibacterium prauznitzii*, and lower levels of proteins involved in butyrate metabolism by *F. prauznitzii* in individuals with Crohn's disease inflammation in the ileum. Additionally, at the metabolite level, there were thousands of metabolites that differentiated healthy from Crohn's disease, including a higher amount of bile acids in the diseased individuals.[247] However, the majority of the metabolites could not be identified, highlighting the need for better understanding of and databases for metabolomics.

Each step along the omics pipeline provides different details as to potential function or expression.

After the Deepwater Horizon oil spill in the Gulf of Mexico, samples were taken from a deep plume of oil in the water column and analyzed by a combination of sequencing technologies, including 16S, metagenome, metatranscriptome, and single-cell sequencing.[248] The combined results revealed shifts in structure of the microbiome, shifts in transcription toward alkane degradation in the plume, as well as draft genomes of potential hydrocarbon-degrading members of the community.

A multi-omics approach has also been applied to the characterization of the complex molecular mechanisms employed in host−pathogen−commensal interplay during *Salmonella* intestinal infections.[249] Proteomics, metabolomics, metagenomics, and glycomics were used to reveal the dynamic disruptions to the intestinal microbial population and metabolite profile as a result of *Salmonella enterica* serovar Typhimurium infection. The emergent application of multi-omics strategies to microbiome research will continue to have significant impact, but bottlenecks remain in omics strategies. For instance, proteomics requires annotated genome files to associate experimental spectra to theoretical spectra for peptide/protein characterization. Metabolomics requires spectral libraries and metabolite standards for high-fidelity identifications. Several groups are addressing these and other challenges, thereby increasing the individual and combined utility of omics approaches.

**Multiscale Imaging and Functional Measurements.** Discovery-based approaches, such as microbial genomics, have been revealing the composition of the microbiome.[250] However, there remains a great need to define precisely the roles and nanoscale relationships of each member of a microbial community. To understand the interactions that make up the complex ecosystems in our bodies and our environment, these communities now need to be understood at the molecular scale. This advance will require the development of new tools for investigating the function of molecular processes within microbial communities.[251] Molecular-scale imaging can provide dynamical information from which to infer function. This cellular and subcellular appreciation will yield insight into human health and disease and enable advances from personalized medicine to control the human microbial community to engineering the ecology of natural and built environments.

Optical microscopy is a direct, noninvasive technique that can attain real-time information about microbial communities. Thus, imaging stands to have wide-ranging impact in the study of the microbiome. On the scale of thousands, millions, or more microbes, imaging will answer questions in real time about membership and spatial organization of cells in communities, about heterogeneity, and about diversity. A generalizable method, imaging promises to elucidate the cellular density and composition in water, on land, and within the body. On the single-cell and subcellular scale, imaging will fill in gaps in our knowledge about the biochemistry of microbiome members. Important questions will be answered, for instance: how do bacterial cells communicate with one another and with their host? How do microbes perform symbiotic functions like nutrient metabolism? How do cells communicate with and react to their physical environment?

**Challenges of Imaging the Microbiome.** The challenges of imaging the microbiome will push the limits of imaging technology. In particular, the community is of the utmost importance to the microbiome. Although models can be useful, communication between cells and interactions with the environment cannot be ignored. This community is not two-dimensional; complete, thick, three-dimensional (3D) assemblies must be considered, whether in animal hosts or in soils. Imaging the microbiome is therefore an inherently multiscale problem in both time and space. Visualizing the microbiome will require an arsenal of tools at different levels, from molecular-scale single-cell studies of prototypical microbes to large-scale, high-throughput/low-resolution techniques. Overall, new imaging tools need to be


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developed to resolve 3D materials in real time and to handle thick, opaque samples, including soil and tissue. Indeed, development of some of the needed imaging technology can benefit from solutions to other complex problems such as those being addressed in neuroscience.[168]

**Imaging Technologies.** Fluorescence and transmission microscopy are noninvasive methods ideally suited to resolve the dynamical processes in microbial communities. Confocal microscopy can achieve ideal diffraction-limited resolution, enabling optical sectioning and cell-level resolution. Still, optical microscopy is traditionally limited in its ability to visualize molecular-scale details and to probe inside bacteria cells. Super-resolution imaging has therefore been recognized as having the power to revolutionize biology.[252] In particular, single-molecule fluorescence tracking and imaging are sensitive to low copy number proteins, can disentangle heterogeneous behaviors, and bring the advantages of fluorescence microscopy to the high-resolution regime by enabling live-cell imaging with nanometer-scale resolution in conventional fluorescence microscopes.[253] Single-molecule microscopy has been extended to three dimensions, and modern developments based on adaptive optics, two-photon microscopy, and lattice light sheets now bring high-resolution imaging even to deep-tissue imaging.[254,255]

**Labeling Technologies.** Developments in protein-labeling technology will be important as we work toward the challenge of fluorescent imaging in the microbiome. Fusions to intrinsically fluorescent proteins are the traditional workhorse of optical microscopy, but these do not extend to the red, making it difficult to multiplex more than two fluorescent protein colors in high-sensitivity applications.[256] Furthermore, the chromophore in traditional GFP derivatives require oxygen to mature, making them unsuitable choices for imaging living anaerobic cells, such as most gut bacteria.[257] Enzymatic labeling systems like HaloTag[258] provide a useful alternative for specific covalent linkage of small molecules, and these have been used to label specifically a prototypical starch catabolism protein, the SusG $\alpha$-amylase, in the prominent gut symbiont *Bacteroides thetaiotaomicron*.[259] Looking forward, unnatural amino acid labeling is a promising way to tag proteins fluorescently in a less perturbative way, although this labeling scheme has not yet been demonstrated for single-molecule fluorescence applications.[260]

Although protein-labeling methodologies are now well-established for fluorescence and super-resolution microscopy, there remains an important need for fluorescent labeling techniques that are suitable for high-resolution, high-sensitivity imaging of other biomacromolecules. Beyond protein imaging, molecular-scale characterization of bacterial cell biology requires the identification of protein−gene interactions to elucidate regulatory responses. These can be inferred from gene knockouts,[261,262] and new technologies based on a repurposed CRISPR/Cas system now make it possible to label specific genetic loci directly.[263] Furthermore, fluorescent or fluorogenic probes for visualizing small molecules will be important to elucidate communication. Recently, Karuntilaka *et al.* demonstrated that the prominent human gut microbiota member *Bacteroides thetaiotaomicron* could grow on fluorescently labeled starch, allowing nanoscale detection of the mechanism of starch recognition.[259]

**Model Systems To Aid in Small- and Intermediate-Scale Studies of the Microbiome.** Because of the multiscale dynamics of the large-scale microbiome, community-level studies will need to be complemented by high-detail, high-resolution examinations of model systems. Fortunately, such model systems exist, for instance, the prototypical starch utilization system (Sus) in *B. thetaiotaomicron*,[264] which has tractable genetics and can be manipulated in culture or in mice.[265] Such studies have yielded tremendous insight into how diet shapes the composition of the human microbiome.[266] Larger scale, yet microbiologically manipulatable and transparent models like zebrafish provide interesting opportunities, in particular, for investigating mechanisms leading to intestinal injury and inflammation.[267,268] Finally, engineered environments such as mimic soils, epithelial monolayers,[269] and synthetic biofilms[270] provide well-controlled substrates that create simplified models of natural environments and host associations.

Another class of model microbiomes is that provided by plants and invertebrate animals that have beneficial symbioses with one or a few species of bacteria. The longest and best studied of these associations is that between nitrogen-fixing bacteria and leguminous plants,[271] which have provided great insight into areas as diverse as the chemistry of cell−cell signaling and the mechanisms of coevolution. More recently, a number of natural invertebrate models have begun to produce similar insights into the principles governing bacterial−animal microbiomes. The phylogenetic diversity of these associations is exceeded only by the breadth of biological questions they have opened to investigation (Figure 5). Like nitrogen-fixing symbioses, the

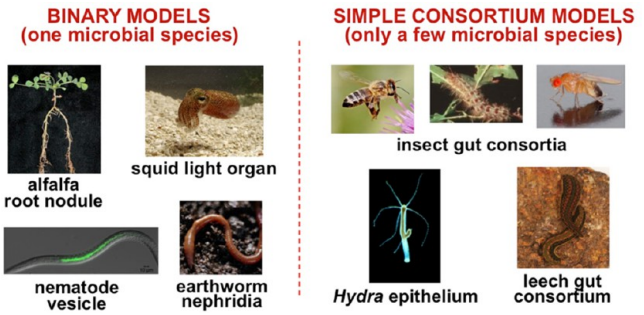


**Figure 5. Examples of simple model symbioses for microbiome research.**

monospecificity of the microbiome present in the bioluminescent organ of sepiolid squids has made possible fundamental discoveries like quorum signaling in a beneficial symbiosis[272] and provides a window into the experimental manipulation of population-level diversity in a microbiome.[273,274] In the past few years, studies of a number of simple consortia such as those present on the surfaces of hydra[275] and within the guts of leeches or honey bees[276,277] have begun to reveal the mechanisms of immunological and physiological communication between a host and its microbiota. Taken together, these simple, but natural, microbiomes have proven remarkably useful in providing windows into the workings of more complex and difficult to study consortia like that in the mammalian gut. For example, the roles of bacterial envelope molecules in inducing tissue development or of symbiont modulation of circadian rhythms were discovered in invertebrate model symbioses[278] and led to the recognition that bacteria regulate similar activities in the gut.[279,280] We have only begun to learn how natural, but simple, animal symbiotic systems will yield insight into how microbe−host and microbe−microbe interactions underlie microbiome function. The small sizes of invertebrate microbiomes will make them important platforms upon which to develop and to apply nanotechnologies that enable microbial


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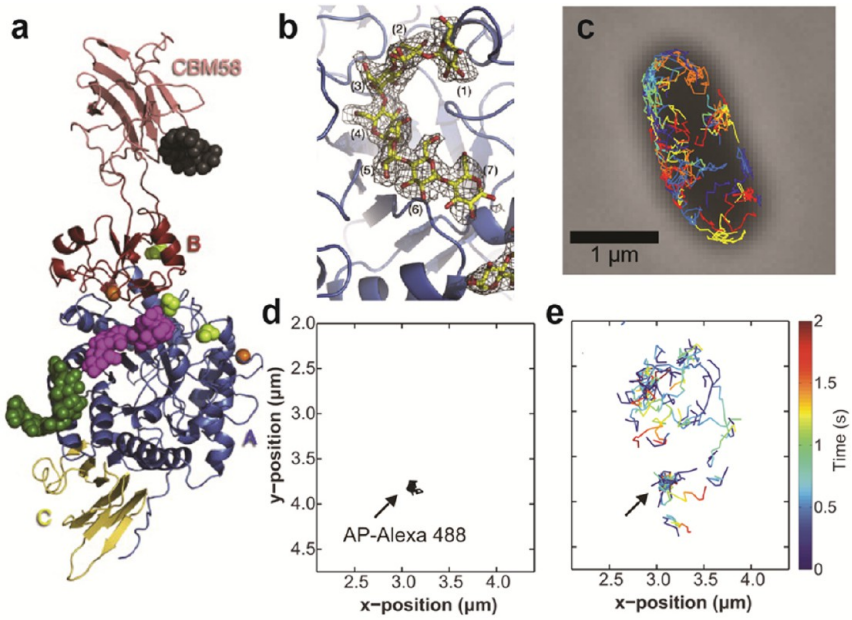


**Figure 6. Molecular-scale depiction of starch catabolism in gut symbionts is derived from single-molecule imaging and structural biology. (a) X-ray crystal structure of the prominent gut symbiont *Bacteroides thetaiotaomicron* starch utilization system α-amylase protein SusG. Ribbon diagram of SusG, colored by domain. Metal ions are displayed as orange spheres, and likely ethylene glycol molecules are in light green. The locations of maltoheptaose molecules bound to the active site, to the secondary starch-binding site, and to the carbohydrate-binding module CBM58 are represented by mauve, green, and gray spheres, respectively. (b) Malto-oligosaccharide bound to the active site of SusG. Electron density from an omit map at the SusG active site of the SusG-D498N mutant cocrystallized with maltoheptaose. The electron density is contoured at 3 s, and the stick model of the bound oligosaccharide is colored according to atom type. (c) Single-molecule trajectories of SusG-HaloTag-tetramethyl rhodamine (SusG-HTL) in glucose (random colors) reveal that the starch utilization protein SusG diffuses heterogeneously on the *B. thetaiotaomicron* outer membrane. (d) Single-molecule tracks show confined movement of Alexa 488-labeled amylopectin (AP-Alexa 488) bound to a cell. (e) Time-dependent single-molecule tracks of SusG-HTL in starch show high confinement of SusG at the position of AP-Alexa 488 (arrow). Panels a and b reproduced with permission from ref 284. Copyright 2010 Elsevier. Panels c–e reproduced with permission from ref 259. Copyright 2014 Karunatilaka *et al.***

community manipulation, chemical analysis, imaging, and other modes of investigation.

**Synergies with Biology, Biochemistry, Spectroscopy, and Bioengineering.** While imaging can provide high-resolution, real-time, 3D glimpses into the microbiome, these images and tracks can be assigned function only through integration with data from biology and biochemistry. Importantly, structural biology, which achieves atomic resolution, can probe the *in vitro* structure and binding sites of microbiome proteins.[281,282] This *in vitro* snapshot can then provide a context for the less tidy information that comes from live-cell and *in vivo* imaging.[259] Optical microscopy can also be easily integrated with other imaging methods, including electron tomography of cells and organisms, atomic force microscopy of surfaces, and X-ray tomography. Overall, synergy between biophysical and optical tools and cell biological approaches is needed to explore the molecular-scale aspects of the microbiome.[283]

Assigning function to microscopic data will further require spectroscopy and other functional assays. Comprehensive understanding of the molecular-scale behavior of microbiomes will therefore be achieved only by multiplexing functional and imaging data, for instance, by correlating images with readouts for nutrient uptake, communication, and quorum sensing. Such a large-scale effort will need to take advantage of computation and data repositories, and indeed, these applications will pose significant challenges that may motivate new computational approaches.

**Applying State-of-the-Art Microscopy to Questions about the Microbiome.** The human gut microbiome plays key roles in health and nutrition by metabolizing many host-indigestible carbohydrates. Indeed, the ability to recognize and to process carbohydrates strongly influences the structure of the gut microbial community. Karunatilaka *et al.* used nanometer-scale super-resolution imaging to explore the transient interactions, assembly, and collaboration of the proteins involved in starch processing by the starch-utilization system (Sus) in the prominent human gut symbiont *Bacteroides thetaiotaomicron* in real time and in live cells.[259] This project brought molecular-scale understanding to this aspect of the human microbiome, as well as demonstrated the power of single-molecule imaging of living anaerobes. Protein fluorescent labeling was performed based on information from X-ray crystallography about the structure of the α-amylase SusG (Figure 6a) and the SusG starch-binding site (Figure 6b).[284] Live-cell super-resolution imaging, single-molecule tracking (Figure 6c), simultaneous monitoring of starch and protein moieties (Figure 6d,e), and Sus protein knock-out strains provided unique mechanistic insights into a glycan catabolism strategy that is prevalent within the human gut microbial community. Overall, the results from this study provided a working model of Sus complex assembly and function during glycan catabolism and are likely to describe aspects of how other Sus-like systems function in human gut Bacteroidetes within the human gut.

On the organism scale, another state-of-the-art imaging technique, light-sheet microscopy,[285] has been used to visualize the colonization of a live, vertebrate gut by specific bacteria with sufficient resolution to measure activity over a population range from a few individuals to tens of thousands of bacterial cells.[268] By acquiring 3D images of living, initially germ-free zebrafish larvae inoculated with fluorescently labeled strains of *Aeromonas* bacteria over the course of several hours (Figure 7), the authors quantified bacterial growth kinetics, finding that the average


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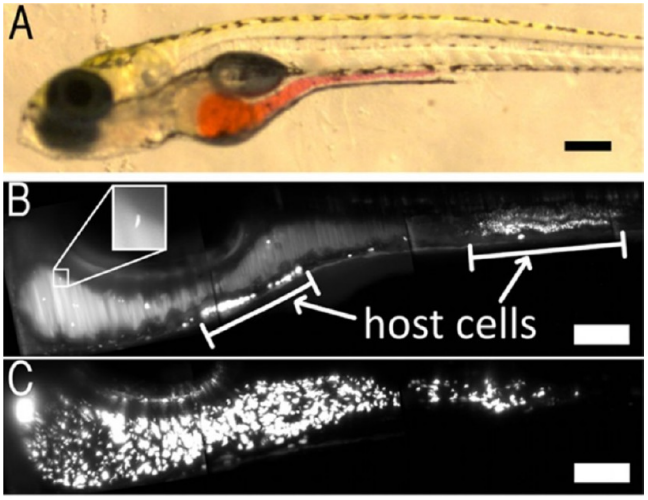


**Figure 7. Growth kinetics of a microbial species in the zebrafish gut monitored *in vivo* by light-sheet microscopy. (A) Image of a larval zebrafish 5 days postfertilization, with the intestine shown by phenol red dye (red). Scale bar: 250 $\mu$m. (B) Maximum intensity projection (MIP) at 1.6 h postinoculation of the initially germ-free zebrafish with *Aeromonas veronii*. Several individual bacteria are visible, and the inset shows a magnified view of a single bacterium (inset width: 22 $\mu$m). White bars indicate autofluorescent sources from the zebrafish host. Scale bar: 100 $\mu$m. (C) MIP of the same fish as shown in (B) at 9.1 h postinoculation, showing a large bacterial population at this time point. Scale bar: 100 $\mu$m. Orientation of all images is anterior to the left and dorsal to the top of the panel. Reprinted with permission from ref 268. Copyright 2014 Jemielita *et al.***

population growth followed a logistical model. Furthermore, the cell-level resolution of this method uncovered heterogeneities that would be masked by ensemble measurements. In particular, by resolving the spatial and temporal dynamics of the bacteria, these cells were found to be nonuniformly distributed throughout the gut, and bacterial aggregates were found to grow considerably faster than discrete individuals. These results highlight the importance of acquiring cell-level maps to predict host–microbe interactions and suggest that single-organism-level spatial characterization will help to describe host-associated microbial community assembly.

Bacteria in biofilm communities are phenotypically distinct from those in isolated, free swimming form; thus, new strategies for experimental characterization of these phenotypes will provide a powerful complement to genome-based and transcriptome-based approaches. Multidisciplinary approaches will be important since the new tools will be both experimental and conceptual.[166] While much has been learned, we are reaching the limits of traditional bacteriological methods since biofilm development depends strongly on epigenetic and communal factors such as individual responses to chemical gradients, proximity of neighbors and neighbor behavior, and heterogeneity of signals. For example, super-resolution microscopy can be used to delineate the distinguishable individuality of each cell in a community, as well as the heterogeneous environments that are created and felt by each cell, as exemplified in recent work on *Vibrio cholerae*.[169]

Present technology enables either analyses of only a small number of cells or analyses of entire cell populations that have been removed from the conditions of interest. Because bacteria in growing communities often display multiple phenotypes that are not discerned when observed collectively, what is needed are methods that track the behavior of individual cells at the community level. Recently, massively parallel techniques have been developed to track single-species assemblages at single-cell resolution.[286,287] Movies of bacteria imaged by microscopy are translated into full histories of individual cells and searchable databases of behavior, so that the information content extracted is ~100,000× greater than that from traditional methods. These methods must be generalized to address multispecies communities.

Examples of symbiosis within microbial consortia demonstrate the importance of motility in the self-assembly of these mixed-species assemblages. However, we will also need to generalize our experimental techniques to create surveillance systems for tracking lineage, signaling, and secretion, in addition to motility, so that we can determine how dynasties of cells of a given species interact with themselves and with others. A step in the right direction can be seen in recent work on combinatorial fluorescent labeling that combines confocal imaging with spectrometry.[288] While traditional fluorescence *in situ* hybridization traditionally only labels a small number of phylotypes in a community, the array of fluorescent signatures that can be deconvoluted from a single microscopy image has been expanded by using binary combinations of fluorophores. Using combinations of genus- and family-specific oligonucleotide probes targeted to microbial rRNA, 15 different taxa in human dental plaque were simultaneously imaged and analyzed.[288]

**Sample Preparation and Multimodal Imaging for Advanced Analysis.** Sample preparation is an important element that will determine the overall complexity and success of a given imaging or sensing instrument and its application to microbiome-related analysis and characterization. In cases where destructive or invasive sampling and analysis are acceptable, various mainstream labeling and sample preparation strategies, including fluorescence or isotope-based labels and multiplexed micro- and nanofluidic systems, can be utilized to bring sensitivity and specificity to detection and tracking of various chemical signatures to probe, for example, genes, enzymes, metabolites, *etc*. Recently developed fluorescence microscopy/nanoscopy super-resolution techniques such as photoactivated localization microscopy (PALM),[289] stochastic optical reconstruction microscopy (STORM),[290] stimulated emission depletion microscopy (STED),[291] structured illumination microscopy,[292] and light-sheet microscopy[293] will find critical uses in revealing spatial and temporal dynamics of nanoscopic processes within single microorganisms.[253,294–296] On the other hand, there is also an important need to create nondestructive or minimally invasive sampling interfaces without altering the natural habitat of the microbiota, and these types of imagers and/or sensors will need to be mostly label-free and require minimal front-end processing before a measurement is performed. For label-free systems, specificity can still be achieved by various means, through, for example, endogenous contrast mechanisms (which can be read, *e.g.*, by Raman spectroscopy,[297,298] autofluorescence imaging,[299,300] multispectral imaging,[301] optical scattering[302,303]) for 2D and 3D morphology as well as motion. The latter can be especially interesting for the analysis of airborne and waterborne microbiomes and would be an exciting research direction to create fundamentally new imaging designs that utilize *motion* as a key signature for microbiome analysis.

**Measurement Throughput and Sampling Volume.** The spatial and temporal throughput of imaging and sensing technologies is important, especially if the diversity of microbial communities is large and their spatiotemporal patterns are not known. Multimodal and advanced microscopy/nanoscopy and


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spectroscopy tools, although extremely powerful with their multidimensional information, tend to be low throughput and can only probe rather small fields of view or sample volumes. This limitation can potentially be addressed by some of the emerging computational imaging and sensing techniques, which can analyze orders of magnitude larger volumes by using lens-free on-chip designs,[304] where the sample volume and field-of-view are dictated not by optics or lenses, as in the case of traditional imaging designs, but by the active area of an optoelectronic sensor chip, which can easily reach 10−20 $cm^2$ using modern CCD and CMOS technologies. Another interesting advantage of such computational imaging and sensing tools, over their traditional counterparts, is that they can also be made significantly more cost-effective and field-portable, which opens up opportunities currently beyond the reach of traditional advanced laboratory-grade imaging and analysis tools.[305]

**Field-Deployable and Ubiquitous Imaging and Sensing Tools.** For decades, microscopy has been the workhorse of a number of fields including medicine and biology. Over the past few years, however, cost-effective and compact microscope designs were developed such that even mobile phones could be converted into advanced microanalysis tools, capable of detecting single viruses or bacteria, conducting blood count, measuring molecular signatures of diseases in bodily fluids, high-resolution imaging of histopathology slides, malaria smears, among many others.[306−312] All of these mobile imaging, sensing, and measurement interfaces benefit from economies of scale, mostly due to mobile phones and other consumer electronics devices, and through these emerging platforms various imaging and sensing tasks that are normally performed in advanced laboratories can now be performed in field settings in extremely cost-effective ways.[313] Another dimension of this exciting development is that it opens up new opportunities for citizen scientists to make meaningful contributions to microbiome research. In other words, through these simple, cost-effective, ubiquitous, but powerful interfaces that now give interested and engaged citizens sensitive and specific measurement capabilities at the micro- and nanoscales, we are likely to see massive increases in the numbers of useful measurements that sample various microbiome-related signals.

Once successfully scaled up, this smart network of microscopes, measurement tools, and their users (*i.e.*, professional scientists as well as citizen scientists) could deliver an extraordinary bounty of microbiome data through innovative uses of this network and its expanding database. For instance, by creating massive libraries of various microbial communities, parasites, viruses, *etc.*, we can dynamically track the spatiotemporal evolution of different micro-organisms and investigate and identify the cause−effect relationships of these patterns at large scales. Such a network of microscopes and related databases could be a priceless global asset for research and for microbiome-related applications for both the developed and the developing world. On the other hand, one important potential challenge toward this vision is handling such large-scale data and creating standardized interfaces and repositories, as discussed below.

**Precision Tools for Manipulating Microbiomes.** In the context of naturally occurring microbiomes of high complexity, determining the roles of specific microbial species, biovars, genes, and gene products in community function is critical and will require tools for precise manipulation. Ideally, these tools would be useful for studying microbial communities in diverse environments, from agricultural soils to gingival pockets, but niche-specific approaches are also of value. A toolkit for deleting, adding, or genetically modifying specific microbes *in situ*, alone and in combination and without the need for prior cultivation, would be of extraordinary value. In addition to their utility for interrogating and managing microbiota, a subset of these tools could provide new approaches for treating infectious diseases in a way that reduces selection for transmissible resistance and leaves beneficial microbes unharmed (see Box 2).

**Box 2. Modeling approaches to capture microbial meta-omic data to develop neural-network learning models for forecasting ecosystem responses.**

**Bottom-Up Mechanistic Models:** *Genome-Enabled Metabolic models* consist of a stoichiometric matrix with all metabolic reactions, a mapping between metabolic genes and reactions, and an objective function such as cellular abundance.[331] Genomes acquired from traditional sampling and metagenome assembly provide a roadmap of the potential metabolic interactions that can be applied to predict microbe response and behavior using constraints-based flux balance analysis to capture the metabolic limits of an organism. *Cheminformatics models* enable the prediction of biochemical pathways from a mechanistic analysis of existing biochemical databases.[331-333] When applied to a set of substrates and products, these models can be used to predict novel compounds and pathways. *Agent-based models* can be developed that synthesize physicochemical, genomic, and metabolomic data into compartmentalized species distribution networks, capable of predicting interspecies metabolic interactions and biochemical activity within spatial and fluid dynamic models.

**Top-down statistical models.** *Species distribution models* test for associations between environmental parameters and microbial genes, species, transcripts, and metabolites, using discrete or continuous constructs. Site-specific effects are usually larger unexplained in the absence of longitudinal data, that is why to model the dynamics of the human microbiome or a marine ecosystem requires extensive time-series observations to capture the systematic and periodic trends that can be related across sites.[127,334-336]

*Dynamic Bayesian Networks* can capture interactions between entities across multiple system levels (metabolites, genes, species) to enable specific co-dependencies to be described that can help to connect top-down to the bottom-up approaches.[148,330-337]

*Convergent Cross-Mapping* elucidates codependences between non-linear interacting variables that are normally missed through traditional networking analysis.[338] Using longitudinal data, it is possible to create convergence manifolds to cross-map non-linear temporal co-associations.

**Integrating Bottom-Up with Top-Down.** By combining bottom-up with top-down systems-scale models, it is possible to control major cross-site differences observed between ecosystems, and therefore to develop forecasting neural networks to capture and predict the linear and non-linear emergent properties of each ecosystem construct. So the overall deliverables from these models would be a complete interaction-network representation of microbiome interactions with environmental nutrients overtime with single-gene resolution (where possible); novel biochemical pathways predicted by cheminformatics to fill knowledge gaps in food-webs; and a dynamic model of microbiome structural evolution capable of predicting responses to changes in environmental conditions, such as climate change, diet, or disease burden.

Design features for a precision antimicrobial capable of *ablating* specific members of a diverse microbial community *in situ* include (1) high specificity, definable at the species, strain, biovar, or other relevant level and (2) high efficiency, which includes gaining access to target cells in natural environments and killing activity upon arrival. Although still at an early stage, two approaches for engineering precision antimicrobials have shown efficacy in model systems of human disease. The first is a specifically targeted antimicrobial peptide (STAMP) that consists of a targeting sequence fused to an antimicrobial peptide (AMP). A STAMP called C16G2 selectively kills *Streptococcus mutans*, which is a predominant cause of tooth decay.[314] For C16G2, targeting is conferred by a 16 amino acid sequence derived from a strain-specific bacterial pheromone, competence-stimulating peptide, fused to a 16 residue broad-spectrum AMP designated G2. In an *in vitro*, saliva-derived biofilm model containing over 100 species representative of the diversity of the human oral microbiome,[315] C16G2 showed impressive antimicrobial activity against *S. mutans*, decreasing average abundance from 24 to 0.1%. A corresponding community-level shift in species composition and abundance was also observed. Although likely due to the ecological consequences of eliminating a predominant member of the multispecies biofilm, further studies are needed to determine if the broad spectrum of the G2 AMP also results in some level of off-target killing. Nonetheless, this is a promising approach for an anticaries drug, and STAMPs may provide broadly applicable tools for engineering microbiomes in other environments.



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Another strategy for designing precision antimicrobials is based on contractile nanotubes, ubiquitous tools used to penetrate bacterial surfaces in nature, often with exquisite specificity and efficiency. Their utility is greatly expanded by the ability to engineer specificity for different cell-surface receptors, combined with a generic mechanism of cell penetration. Myovirus bacteriophages, exemplified by phage T4, use contractile injection systems to translocate DNA into bacterial cells.[316] An adaptation of the same contractile mechanism is used by numerous bacteria to kill competitors, with the best studied example being the R-type bacteroicins produced by *Pseudomonas aeruginosa*.[317] In contrast to phage, these function as bactericidal particles by inserting ion-conducting channels across the envelopes of target bacteria. A recent cryo-transmission electron microscope (cryo-TEM) analysis of pre- and postcontracted particles provides a model for contraction that likely applies to ejection systems used by phage, bacterial type-VI secretion systems, and other related machines.[317] The contractile nanotube shown in Figure 8 consists of an outer sheath surrounding a hollow inner tube. The precontracted particle is assembled into a high-energy, metastable state in which sheath and tube proteins interact through charge complementarity. Contraction initiates when tail fibers, which are disordered and not resolved by cryo-TEM, bind to cognate receptors on bacterial cell surfaces. This recognition initiates a cascade of events that results in translational movement of sheath subunits that are intertwined by $\beta$-sheet augmentation. During contraction, the sheath increases in width, decreases in length, and electrostatic interactions between sheath and tube proteins are broken. The released energy powers the injection process, which occurs in the absence of ATP. In many cases, a single R-type bacteriocin is sufficient to kill a bacterial cell.

The ability to retarget bacteriocin specificity by substituting ligand-recognition domains of receptor-binding proteins (RBPs) from phage or other contractile bacteriocins has been demonstrated in multiple studies.[318−320] Since bacterial genomes are often replete with prophage sequences, the ability to culture a particular organism may not be required to design a precision ablating tool if sufficient genome sequence information is available.[321] Although the bactericidal spectrum of the structure shown in Figure 8 is likely confined to Gram-negative species, Gram-positive bacteria produce analogous bactericidal structures that can also be retargeted by substituting RBPs. The utility of this approach was recently demonstrated in a mouse model of antibiotic-induced infection by *Clostridium difficile*,[320] which has become a worldwide public health threat (Box 1). A contractile nanotube engineered for specificity against a hypervirulent strain of *C. difficile* efficiently prevented infection of antibiotic-treated mice, with no detectable effects on the normal gut microbiota.[320] In the context of infectious diseases, a unique benefit of precision antimicrobials is their potential for use not only as therapeutic agents but also for prophylaxis, which is rarely indicated for conventional antibiotics. A genetic approach with the potential for genome modification as well as precise ablation has recently been developed using CRISPR-Cas technology in which RNA-guided nucleases, delivered by transmissible plasmids or phage, are engineered to alter specific DNA sequences to modify or to kill a bacterial host.[322]

The above examples are based on engineering targeted bactericidal molecules or structures using naturally occurring components. The opportunity exists, however, to develop bio-inspired nanomaterials with desirable properties and utility as precision tools. Numerous types of nanoparticles have potent antibacterial killing efficiency, including nitric-oxide-releasing

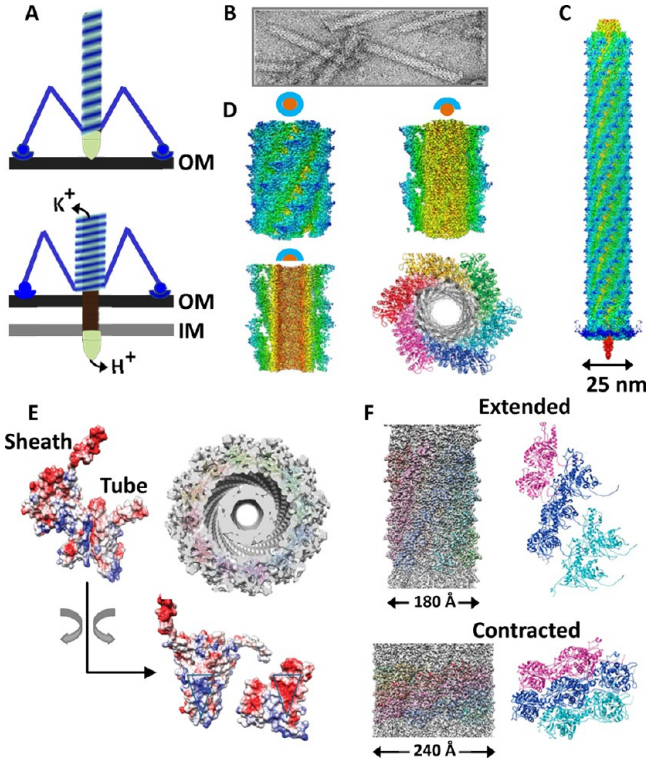


**Figure 8. Cell penetration by a bactericidal contractile nanotube (CTN). (A) CTN binds specifically to receptors on the outer membrane (OM) of a bacterial cell using its six tail fibers (two are shown, top). Binding induces conformational changes in the outer sheath (blue diagonals), injecting a hollow tube (black) across the cell surface, inducing ion flux, and depolarizing the inner membrane (IM, bottom). (B) Electron micrographs of CTNs stained with uranyl acetate. (C) Cryo-transmission electron microscopy-based reconstruction of the trunk of a CTN in the extended state at 3.5 Å resolution. (D) Segmented surface views of the extended CNT trunk are shown with a cross section (lower right) showing the hollow center of the tube. (E) Charged surface view of sheath−tube protein interactions in the extended state (top left), with open-book view (bottom right) highlighting charge complementarity. Sheath−tube protein interactions are broken during contraction (top right). (F) Precontraction (top) and postcontraction (bottom) sheath. Cryo-EM density map (left) and ribbon diagrams of sheath subunits (right) show rigid-body movement of subunits intertwined by $\beta$-strand augmentation. Adapted with permission from ref 317. Copyright 2015 Nature Publishing Group.**

nanoparticles, nanoparticles containing chitosan, and metal-containing Ag, Zn, Cu, Ti, Mg, or Au nanoparticles.[323] The ability to target these materials with phage-derived RBPs, pheromones, or other specific binding ligands and to engineer them to deploy only upon binding to bacterial cell surfaces would provide a platform for an array of new approaches for developing precision tools for microbiota management, as well as the treatment and prevention of infectious diseases.

**Observatories.** Microbial observatories are instrumented stations set in natural environments for long-term analyses of microbial communities while at the same time observing hydrological, geochemical, and other processes and interactions.[324] The first such observatories have been set in both marine and continental environments across a range of geographical and geochemical extremes. Integration of instruments to probe and to culture microbes *in situ* has been used to increase the microbiological experiments possible in these observatories.[325]



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Measurements of microbe catabolism and growth rates have been performed in marine sediments as an example.[326] Expansion of both the enabling tools to these observatories and the study of a greater range of environments such as topsoils will provide critical data to elucidate trends and features arising from microbiome habitats. Opportunities for nanoscience and nanotechnology include networks of sensor arrays to monitor chemical, physical, and biological environments, in addition to the microbiome itself. Microbial observatories enable experimentation on longer time scales and larger scales than typically observed in laboratories and can operate with minimal disruption from the microbiomes' natural settings.

## BIG AND/OR SMART DATA

**Data Standardization, Quality Control, and Repositories.** Another important task that needs to be addressed is the creation of unified data standards and integrated data repositories on microbiome-related imaging and sensing data that will be utilized and accessed by various research communities and organizations collecting, sharing, organizing, and analyzing vast ranges of biomarkers, molecular signatures, gene profiles, metabolites, *etc.* corresponding to different microbiomes and also as a function of both space and time. As discussed above, these data might be collected using cost-effective and massively scalable interfaces and technological solutions based on, for example, mobile phones or other consumer devices in addition to mainstream standard laboratory-grade instruments, multimodal advanced imaging, and sensing interfaces. For this broad aim, some important milestones that need be achieved include (1) standardization of data/metadata formats as well as new measurement hardware and peripherals (especially important for ensuring quality measurements from citizen scientists); (2) development of measures of and methods to assess data quality, which need to include automated data cleaning and/or correction techniques for elimination of false and/or contaminated data from data repositories; and (3) addressing data ownership and ethics-related issues, including but not limited to human−microbiome-related data.

**Image Labeling, Computer Vision, and Crowd-Sourcing/Gaming Interfaces.** In general, there is much detail and subtlety associated with microscopic or nanoscopic multidimensional image data, and therefore, accurate analysis and interpretation of such images often become tedious and time-consuming, even for highly trained professionals and experts. This is probably one of the main reasons why machine-learning and automated image-labeling strategies are still not widely adopted for identification and characterization of micro-organisms through their microscopic/nanoscopic images. Even for biomedical diagnostic applications, such as identification of a parasite within a sample, machine learning is lagging mostly due to the lack of large-scale gold-standard image libraries, which makes it difficult to leverage the power of some of the emerging Big Data analytics tools that the industry (*e.g.*, Google, Facebook, Amazon) has been routinely utilizing for various image analysis and pattern recognition tasks.

Crowd-sourcing of microbiome-related microscopic analysis and image labeling/annotation is timely in several ways.[327] With rapid advances in mobile telecommunication and Internet technologies such as mobile phones, tablet PCs, *etc.*, we have hundreds of millions of active users in the cloud that are all connected to a global network. This current infrastructure and the state of connectivity make it feasible to create a self-learning data repository platform that leverages crowd-sourcing, gaming, and communications theory concepts to conduct accurate and sensitive analyses of microbiome image data in a distributed fashion, even using nonexpert users and gamers.[328] More importantly, by coupling microbiome image data repositories with machine-learning and crowd-sourcing strategies, we can also create a self-learning *hybrid* network, machine + human (*i.e.*, both professionals and citizen scientists) that gets much better in automated identification and classification of microscopic images of specimens. Through such large-scale data analysis, we can also better identify statistically significant parameters for individual members of each microbial community, which is extremely important to harness big data into "useful data" and "small data", also helping us to identify and to act on outliers.

All of these efforts will need substantial cross-disciplinary expertise to make decisions regarding the standards, regulations, types of data that will be collected and analyzed, and how data will be organized, processed, and accessed, so that the entire resulting framework will ultimately be as useful as possible for microbiome-related research at a global scale.

## MODELING AND SIMULATIONS

**Simulating Microbial Ecology.** Sensors that can enable the identification and analysis of microbial community structure and genotype distribution are essential to enabling us to view the microbial world at the appropriate resolution to understand the spatiotemporal dynamics we observe in soils, waters, and even human bodies. However, these sensors will significantly increase the volume and immediacy of data acquisition, and while this will improve statistical rigor and enable real-time validation of predictions, the data stream needs to be handled appropriately to provide input into predictive models at multiple scales.[329] Well-developed and controlled feedback between observation and modeling has provided humanity with sophisticated weather and climate system predictions and enabled ecological predictions that provide information to support focused restoration initiatives. Therefore, microbiome and metabolite forecasts, analogous to current weather forecasts, are required for multiple ecosystems, whereby new data are used to train and to refine existing models through a neural network that learns as data are acquired.[330] These microbial and metabolite forecasts must be able to inform end users to facilitate the design and maintenance of more resilient and productive ecosystems to support food production, health promotion, pollution remediation, and global environmental stability. Complementary bottom-up mechanistic and top-down statistical models can capture the discrete or continuous associations between environmental components and microbial ecology with sufficient predictive power to model changes and trends within an ecosystem. There are many different types of potential systems-scale models that enable us to predict either the cellular processes that support emergent ecosystem dynamics or the ecosystem processes that support global emergent properties. Bottom-up mechanistic modeling approaches include cellular systems-based prediction of metabolic processes, including flux-balance modeling of individual cells or communities, cheminformatic approaches to predict novel metabolic pathways, and agent-based models that leverage multifactorial quantitative parametrization of ecosystem properties (at both the cellular and community scale) to predict interspecies interactions and outcomes within a given system. Top-down statistical models include species distribution network models that use relationships between external and biological parameters to predict outcomes or extrapolate observations, while dynamic Bayesian and convergent cross-mapping



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network models capture linear and nonlinear interactions that support dynamic ecosystem properties. Examples of these systems are given in Box 2.

Mechanistic models can be calculated based purely on metagenomic and metabolomic data; these consist of genome-scale metabolic models with course regulatory components for each major species identified in a system, which can be assembled based on a combination of reference genome data and primary reconstruction of annotated sequencing reads.[339] Reference genomes can provide data on course species behavior and biology (*e.g.*, redox preferences of species, general growth rates, growth behavior) that can be used to facilitate simulation of communities using a combination of steady-state community flux modeling to identify possible species interactions, and dynamic flux modeling to predict dynamic growth of all species over time within a community.[136] As one might imagine, dynamic models require parameters, and generally, flux models are significantly underdetermined. Put another way, there are many alternative schemes for interspecies interactions that are initially equally feasible in these models. A means of identifying the most plausible interactions and dynamic parameters to use in constructing, testing, and validating dynamic community models is required. This nexus is where the conceptual and quantitative links with statistical models are applicable. Statistical models operate at low systems resolution, statistically fitting parameters that represent course nonmechanistic interactions between species, such that the statistical model can match observed changes in species abundance in a microbiome over time. These course parameters derived from the statistical models can be translated into high-level constraints imposed on the mechanistic models. For example, a statistical model may determine that species A and species B have a high probability for mutual dependency based on analysis of their changing abundance across a time series (using dynamic Bayesian or convergent cross-mapping predictions; see Box 2). Longitudinal data are highly important for the application of this technique, whereby the temporal dynamics of the community are translated into a constraint within a community metabolic model that filters possible schemes for species interaction to only those schemes that involve some form of mutual interaction between species A and species B. Of course, these are not hard constraints because the high-resolution mechanistic flux modeling is based on optimization approaches. Instead, the optimization algorithms will favor predicted interactions between species based on the probability for the interaction calculated by the statistical models. So how is the mechanistic model adding information if the statistical model has already predicted the species interaction? Well, the statistical model predicts which species are likely to be interacting, and the mechanistic model uses this information to predict how these species are interacting such that the entire community forms a single biochemically consistent system that conforms to observed dynamics.

The outcome of these combined modeling efforts based on data acquired from continuous high spatiotemporal density sensors monitoring microbiome dynamics will be the identification of the keystone taxonomic and metabolic components of these systems. These keystone components, as in the keystone in a bridge, support the whole system, helping to make it resilient, robust, and stable. These models will identify the feedstocks that form the base of any given microbial interaction network, as well as the intermediate metabolites exchanged between organisms in an interaction network. These interaction networks can form the basis for subsequent dynamic models of microbiome evolution. These models can predict the evolution of the microbiome community structure over time, including interactions that may involve exchange of a signal molecule or the removal of an inhibitory compound (*e.g.*, oxygen or fatty acid).

**Stimulation and Response of Microbiomes.** One long-term vision for applied microbial research is that genetically engineered microbes could be released into the wild, perform a useful task, and then disappear without environmental disruption or genetic contamination of the ecosystem. Applications could include probiotic microbes used as therapeutics and prophylactics for the health of the gut, skin, and lungs in humans and livestock; photosynthetic microbes living in open ponds that produce commodities; carbon-fixing microbes that capture $CO_2$ from coal-burning power plants; bacteria that compete with fungal parasites that endanger food crops (which is becoming more urgent due to global warming and crop monoculture); microbes engineered to metabolize insecticides and other toxins in cleanup sites; microbes that sense chemicals that may represent security threats, such as explosives or neurotoxins; and so on.

Synthetic biologists have conceptualized and developed a large number of such useful microbes, but deployment is always stymied by the same problem. If we release such organisms into the environment, what will happen after they carry out their function? Will they evolve into pests and disrupt an ecosystem? Will they exchange genetic information with other microbes to create hybrids in which genetically engineered modules become established in the wild?

Unless these worries are addressed, synthetic microbiologists may continue to create useful microbes, but these will remain in the realm of "toy systems". An important area of research will be in containment systems for engineered microbes. These "systems" would not be physical—rather, we need biologically based containment. As an illustration, one promising approach is the development of "recoded" organisms in which the genetic code—the correlation between bases in DNA and amino acids—is completely rewritten for an engineered microbe (Figure 9). In this way, if DNA is transferred in or out of the organism, the transferred DNA cannot be read and will provide no selective advantage. This type of technology is broadly enabling for commercial and defense applications, but it is not currently funded and needs support in order to reap the benefits of synthetic biology outside the laboratory.

One example of the potential advantages of manipulating microbiomes is in treating obesity, which has become a global epidemic. Studies in both mice and humans have implicated gut microbiomes in the ability to harvest energy from food.[340] Selectively replacing gut microbiomes in mice indicated the divergent paths that individuals could take in their synergy with and dependence on their microbiomes.

## ETHICS

Understanding how integrated microbial systems work within and with the environment, whether that environment is a living organismal "host", a biofuel reactor, or an agricultural soil, is a grand scientific goal. However, a number of ethical issues must be considered in basic microbiomes research and in association with manipulation of environmental and human microbiomes. For example, open data sharing has been proposed for the microbiome initiative, including for human microbiome data, yet human gut microbiomes can be traced to particular patients[341] and may indicate health status and age, among other private attributes.[342] The public will reap the benefit of insights gleaned from microbiomes research when information access is broad,



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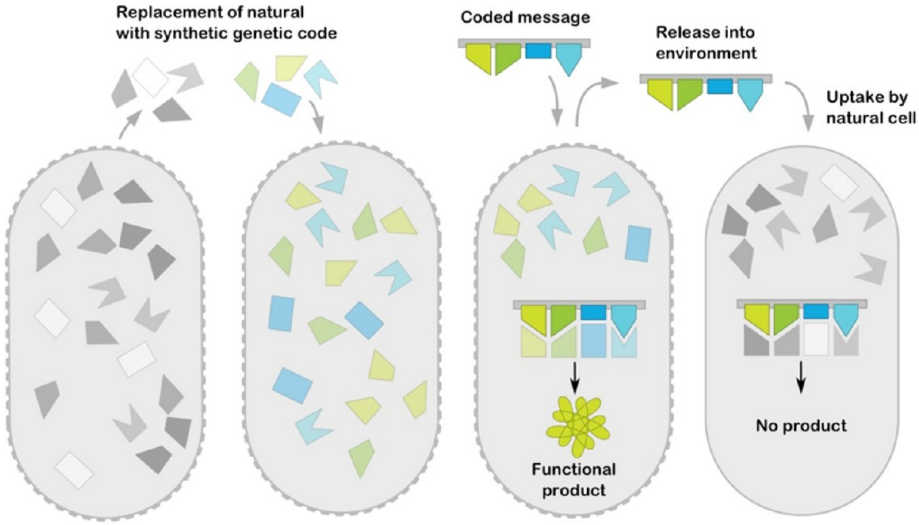


**Figure 9. Synthetic biology can be used to modify organisms that can interact with and thereby drive changes in microbiomes.**

but individuals will shoulder the risk to their privacy from misuse of information. Though human microbiomes are more fluid than whole human genomes, the fact that under some circumstances microbiomes can be tied to particular individuals suggests that similar ethical considerations[343] should be tackled.

In documents focused on whole-genome sequencing in humans and on synthetic biology, the Presidential Commission on Bioethical Issues emphasizes five ethical principles to guide research practices:[344,345] public beneficence, responsible stewardship, intellectual freedom and responsibility, democratic deliberation, and justice and fairness. These same basic principles can inform human and environmental microbiomes research. "Public beneficence" aims to maximize benefit and minimize harm to the public and entails continuous re-evaluation of promise and risk from safety, security, environmental, economic, and social perspectives.[346] "Responsible stewardship" requires that the global community consider long-term and widespread implications of actions for our shared environment, for the currently disenfranchised, and for future generations. "Justice and fairness" emerge from responsible stewardship—benefits of new knowledge should accrue to all of society. "Intellectual freedom" to pursue emerging technologies, though they are by nature continuously changing, must be delicately balanced with the development of a regulatory structure to ensure responsible action.[347] "Democratic deliberation" should be at the heart of community decision-making, with the goal of societal benefit outweighing individual interests, and with input from philosophy, social sciences, and the general public to maintain public legitimacy.[346]

Beyond the ethical issues associated with study and manipulation of human microbiomes, manipulation of organismal and environmental microbiomes to improve food and fuel supplies, or potentially even to counter global climate change, also requires scientific, ethical, political, and legal input. Microbes have tremendous promise as partners in Earth stewardship; they have shaped and maintained Earth's life support systems for billions of years. The first priority of scientists must be to obtain understanding deep enough to consider both the promise and the potential perils (unintended consequences) of attempts to harness microbial activities operating in ecosystems or at planetary scales. Even within organisms, manipulation of a single gene can lead to surprising results.[346] The complexity of microbiomes comprising multiple organisms interacting with their environment is far higher; the potential for unintended consequences of microbiome manipulation is thus a very real risk, particularly in the imperfectly understood natural Earth systems on which all humanity depends.[348] A highly visible example of proposed manipulation of an environmental microbiome is the decades-old, controversial (bio)geoengineering proposal to fertilize the ocean, in order to stimulate phytoplankton growth, bury resulting organic carbon deep in the ocean after it sinks from upper ocean layers, and thus mitigate increasing concentrations of atmospheric carbon dioxide.[349] The London Convention/London Protocol formed an international governance and assessment framework for research testing this geoengineering technique.[348] Similar frameworks will be essential before considering any large-scale manipulation of environmental microbiomes, as concerns will emerge over responsibility, liability for unintended ecological impacts, open and cooperative research, evaluation, assessment, and public consent.[348,350]

## PROSPECTS

There are tremendous opportunities for nanoscience and nanotechnology to contribute to understanding the microbiome. The ability to image, to sense, and to stimulate at the scale of function will be critical in measuring and ultimately understanding the microbiome. Hybrid synthetic/biological nanostructures will yield precise tools to manipulate the microbiome and also to address key challenges in medicine. Such advances will enable us to reshape microbiomes in systems ranging from the gut to the global rhizosphere. New ways of dealing with the information obtained from what will necessarily involve multiple measurement modalities will be required. Such data acquisition and data science opportunities can be used to broaden the community of those working on the microbiome. We anticipate that the combinations of nanotechnology with synthetic biology and other fields will generate fertile new science and applications, in health, agriculture, climate science, energy, and other areas.

We hope and anticipate that, like the BRAIN Initiative,[168] the microbiome initiative will leverage the worldwide investments in science, technology, and people in nanoscience and nanotechnology[1,3,351,352] to bear on exploring and understanding the microbiome and will generate a new world of scientific questions and opportunities.



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### Notes

The authors declare the following competing financial interest(s): P.C.B. is an equity holder in GALT, Inc. and 10X Genomics, and is a co-inventor of technologies for micro-analytical systems that may be commercialized. G.M.C. is consultant and shareholder of Seres Therapeutics and has other broader involvements (listed at http://arep.med.harvard.edu/gmc/tech.html). S.E.F. holds patents for imaging tools, some of which are deployed as imaging diagnostics (at Varocto, Inc.) or are licensed to Zeiss. J.F.M. is a cofounder, equity holder, and chair of the scientific advisory board of AvidBiotics Inc., a biotherapeutics company in San Francisco, California. R.K. is co-founder and CEO of Biota Technology, Inc., which uses data science and microbial DNA for oilfield applications. A.O. is the co-founder of a company that commercializes imaging and sensing technologies for medical diagnostics applications. S.R.Q. is a consultant and shareholder of Fluidigm and Karius. G.v.d.E. is co-inventor of flow cytometers and related instruments that have been licensed and for which he receives royalties related to cell-sorting technologies as tools for genome/biome analyses.

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