

Review

Spatiotemporally resolved tools for analyzing gut microbiota

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SUMMARY

The gut microbiota is a hidden vital organ closely related to human health. Its spatial patterns and dynamics influence processes and functions including microbial colonization, community stability, host-microbe interactions, host metabolism, immune regulation, and neurodevelopment, but are not measurable via traditional techniques such as culturing, sequencing, and mass spectrometry, thus remaining largely unexplored. Here, we highlight emerging techniques such as spatially resolved sequencing and *in vivo* imaging, enabling the acquisition of spatial organizations and dynamic information of the gut microbiome, and discuss their contributions to our understanding of the gut microbiota including physiological activities, symbiotic functions, and spatial organization rearrangement under diverse physiological and pathological statuses, which provide new views for diet and clinical treatment via managing the gut microbiota.

INTRODUCTION

The gut microbiota plays key roles in host health, including nutrition,^{1,2} metabolism,³ pathogen resistance,⁴ immune regulation,^{5,6} and neurodevelopment.^{7,8} There are about 500–1,000 bacterial species in the gut intestine,⁹ and their composition and spatial distribution are intricate and dynamically changed. Diverse physiological and pathological functions of the gut microbiota are closely associated with the dynamics of the indigenous microbiome and their spatial organization within the host gut.¹⁰ Studies have shown that various diseases are related to the abnormal spatial distribution of gut microbiota, such as inflammatory bowel disease (IBD), colorectal tumors, and hepatic encephalopathy.¹¹ Besides, metabolism, growth, and spatial pattern change of the microbial communities can indicate bacterial colonization, pathogenic infection, and clinical treatment such as antibiotics and microbiota transplant therapy.¹² Therefore, tools to obtain spatial and dynamic information of the gut microbiota for elucidating its functions and disease associations are highly demanded.

Bacterial culturomics, sequencing, and mass spectrometry enable us to examine the genome, transcriptome, and metabolome of the microbial population and investigate the information with functions such as digestion, immune response, and disease occurrence, dramatically advancing our knowledge of the highly complex but versatile organ.¹³ We have cultured and identified about 30% of the bacterial species in the intestinal tract¹⁴ and analyzed the role of some microorganisms in disease occurrence by comparison, such as mucosal inflammation, impaired mucosal barrier function, and visceral hypersensitivity.¹⁴ In 2016, by culturomics, which increased the sample number by standardizing culture conditions, 531 new species have been isolated and discovered in the human gut.¹⁵ In addition, through large sequencing projects such as

THE BIGGER PICTURE

The gut microbiota is rich in diversity and function and plays key roles in human health. Their composition and spatial distribution are intricate and dynamically changed and would determine diverse physiological and pathological functions, including inflammatory bowel disease and colorectal tumors. The spatial and dynamic information of the gut microbiota is hard to measure because the gut is featured with acidity, thick walls, and plenty of intestinal villi. The coincident revolution of molecular probes, imaging technology, and sequencing techniques sheds light on the elucidation of gut microbiota in multiple dimensions. The review highlights the techniques for gut microbiota analysis in terms of spatial organizations and dynamic information and their contribution to the elucidation of structures, functions, and host disease occurrence linked to the gut microbiota and the potential to facilitate gut health maintenance, microbiota-based therapeutics, gut-stemmed diagnosis, and disease treatments.



European intestinal metagenomics (MetaHIT) and the Human Microbiome Project (HMP), enabled by next-generation sequencers, we have accumulated abundant metagenomics data on the human microbiome¹³ and elucidated the composition of the gut microbiota in different physiological and pathological statuses such as gastrointestinal disease and neurobehavioral development.^{16,17} Besides, methods based on mass spectra have been used to analyze the metabolic interactions between the host and gut microbiota, in which the two-way communication between the microbiota and the brain has attracted much attention.¹⁸ However, spatiotemporally resolved information is inaccessible through all these approaches. The gut presents a formidable sensing and inaccessible environment, due to its acidity, thick walls, and plenty of intestinal villi.¹⁹ Therefore, we often analyzed the fecal flora samples, rather than colonized microbes in the gut. The *ex situ* snapshot information obtained is suspicious to produce a deviation compared with that in the gut and cannot sufficiently inform the heterogeneity, cross talk, and functionality of the gut community.

The coincident revolution of molecular probes, imaging technologies, and sequencing techniques sheds light on the elucidation of gut microbiota in multiple dimensions. Here, we focus on the techniques for gut microbiota analysis in terms of spatial organizations and dynamic information, including their progress and remaining challenges. We highlight multiplexed imaging enabled by encoding fluorescence *in situ* hybridization (FISH), *in situ* sequencing, and expansion microscopy, which can parallelly investigate bacterial location, genetic mutations, and gene expression. We also discuss their limitations such as the multiplexing capacity and resolution to be improved for fulfilling the systematic investigation of gut microbiota. Then, we present *in vivo* imaging techniques including bioluminescence, near-infrared fluorescence, acoustic imaging, and positron emission tomography (PET), which have been designed to track bacteria and their metabolism in the gut, and propose potential solutions to circumvent their limitation for providing molecular information. In addition, we highlight the application of spatiotemporally resolved tools for the investigation of structures, functions, and host disease occurrence linked to the gut microbiota. We discuss how these techniques have improved our understanding of the gut microbiota in terms of spatial networks, interrelationships, physiological activities, and symbiotic functions, as well as their instructions on precise therapies.

SPATIALLY RESOLVED ANALYSIS OF GUT MICROBIOTA

The spatial organization of the gut microbiota that influences colonization, inter-microbial and host-microbe interactions, and community stability,¹⁰ however, remains largely unexplored. Current genetic information profiling is performed on homogenates or on populations of isolated single cells, whereas the histological context is lost in both approaches. To resolve the microbial interactions in the intestinal flora, it is necessary to obtain a high content of genetic information while preserving the bacterial location. We highlight the advances in analytical tools for studying the spatial organization of microbial communities (Table 1). From genetic labeling and FISH to the combination of diverse encoding methods and spatially resolved sequencing, the throughput and nucleotide resolution have been substantially improved. Besides, the defects of *in situ* techniques such as the low spatial resolution and the lack of three-dimensional (3D) information are optimized from the perspective of sample processing by the expansion microscope and sample clearing.

Genetic labeling

Genetic labeling based on synthetic biology techniques, using recombinant vectors and protein tags to localize bacteria, can be used for imaging closely related strains

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Table 1. Characteristics of spatially resolved approaches to analyze the gut microbiota

Technique	Principle	Multiplexity	Single-cell resolution	Species resolution	Acquired data	Host or microbe	Gut function, disease, and treatment	Reference
Tunable expression labeling	encoding via promoters	6 strains	yes	yes	taxon	microbe	colonization order	Whitaker et al. ²⁰
FISH	labeling via probe hybridization	1–3 strains	yes	no	taxon	microbe	inflammatory bowel diseases	Swidsinski et al. ²¹ and Nishida et al. ²²
CLASI-FISH	spectral encoding FISH	15 strains	yes	no	taxon	microbe	spatial arrangement of artificial gut microbiota	Mark Welch et al. ²³
HiPR-FISH	spectral encoding FISH	1,023 strains	yes	no	taxon	microbe	antibiotic treatment effect	Shi et al. ²⁴
par-seqFISH	sequential encoding FISH	105 genes	yes	no	taxon, gene expression	microbe	functional zonation in biofilm	Dar et al. ²⁵
Spatial transcriptomics integrated with scRNA-seq	using spatial transcriptomics to reconstruct spatial coordinates of scRNA-seq data	transcriptome	yes	–	gene expression	host	crypt-villus axis formation, mesenchyme differentiation and immune colonization	Fawkner-Corbett et al. ²⁶
LCM coupling with scRNA-seq	using landmark genes to reconstruct spatial coordinates of scRNA-seq data	transcriptome	yes	–	gene expression	host	zonation for antimicrobial, absorption, and immunity	Moor et al. ²⁷
MaPS-seq	sequencing 16 rRNAs of plot samples	metagenome	no	yes	taxon	microbe	dietary perturbation effect	Sheth et al. ²⁸

in the gut microbiota. Whitaker et al. used a new phage promoter and a translational regulatory strategy that yielded a high-level expression of fluorescent protein in *Bacteroides*, enabling the imaging of *Bacteroides* in the mouse gut with strain-level resolution.²⁰ Using these promoters, unique fluorescent protein tags were encoded and allowed six bacterial species to be distinguished. The high protein expression did not impose a detectable fitness burden in a complex community in the mouse gut, suggesting that an engineered microbe was compatible with long-term colonization *in vivo*. The strategy can be used to detect very similar or isogenic bacteria. In experiments comparing simultaneous and sequential colonization, it was found that the priority of arrival determined the capacity of bacteria for intestine colonization. The spatial location analysis indicated that crypt colonization played an important role in the entrenchment of *Bacteroides* in the gut. The study provided the first example of the investigation of the spatial organization of gut microbiota while providing strain-level resolution. A number of intestinal microorganisms are currently, however, difficult for culture and genetic modification. Besides, genetic labeling is limited to investigating exogenously modified bacteria, not applicable to endogenous intestinal flora.

Encoding FISH

FISH can determine the spatial location of specific DNA and RNA, label endogenous and exogenous cells with corresponding target sequences, and analyze the gene expression differences between cells.^{29,30} It has been widely applied to analyze the spatial organization of the gut microbiota³¹ and indicated the microbial regional diversity,³² the links between gut microbiota and inflammation and diet.^{33,34} However, FISH is limited by low-throughput due to the spectral overlap of fluorophores.³⁵ To realize the simultaneous identification of multiple bacteria, encoding strategies have been proposed to improve the multiplexing capacity for imaging gut microbiota.

Spectral encoding FISH increases the flux through combinatorial labeling and spectral imaging. Multiple fluorescent probes are used to label bacteria in combinations

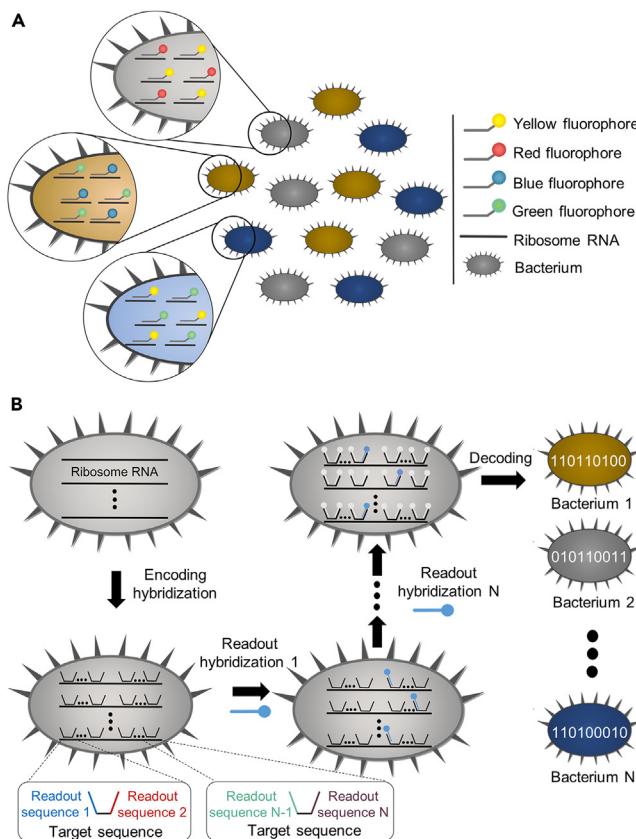


Figure 1. Encoding FISH principles

(A) Spectral encoding FISH. Each bacterial ribosome RNA is hybridized with multiple encoded DNA probes labeled with different fluorophores. Combinatorial labeling using the spectrally distinct probes increases the number of identifiable microbes.

(B) Sequential encoding FISH. Each bacterial ribosome RNA is first hybridized with DNA probes hanging with terminal readout sequences. The readout sequences are then sequentially labeled by probe hybridization, imaging, and probe removal. Bacterial taxon is identified based on the unique barcode composed of the sequential labeling.

(Figure 1A). Microbe-sized bacteria labeled with binary combinations of fluorophores with highly overlapping emission spectra could be further distinguished in spectrally acquired microscope images by linear algorithm, thus increasing the number of microbial species detected from a few to dozens.³⁶ Theoretically, 28 binary combinations can be generated via using 8 fluorophores. Moreover, Deng et al. proposed a sequence encoding strategy via adjusting probe hybridization, enabling to encode the fluorescence signal via both spectra and intensities, which increased the flux to 36 using 3 fluorophores.³⁷ Based on multiplexing imaging, Valm et al. utilized FISH to investigate the inter- and intra-taxon cell-to-cell relationships and their roles in the biofilm complexity.³⁶ Mark Welch et al. used spectral encoding FISH to examine a community of 15 bacterial populations at multiple spatial scales in germ-free mice introduced with human gut bacteria, including spatial distributions and metabolic interactions between its members.²³

Recently, by increasing the number of spectrally resolved fluorophores based on machine learning, the De Vlaminck group proposed a versatile technology termed high-phylogenetic-resolution microbiome mapping by FISH (HiPR-FISH),²⁴ for the first time, creating a micrometer-scale map of hundreds of microbial species in the

gut microbial communities. HiPR-FISH leveraged the throughput allowing us to visualize and identify up to 1,023 isolates of *Escherichia coli* by labeling the bacterial populations with up to 10 different fluorophores. Besides, De Vlaminck et al. showed the first use of super-resolution imaging to resolve the ribosome organization differing by taxa in the human oral microbiome. HiPR-FISH substantially improved the multiplexing capacity, detection speed, and measurable scale of intestinal flora.

Nevertheless, spectrally resolved fluorophores are usually limited to a number of 3–5 and cannot sustain spectral encoding to fulfill the throughput to cover gut microbiota that are expected to be over 500 species. In comparison, sequential encoding FISH could improve the labeling flux over 1,000.^{38,39} In the sequential encoding strategy, each transcript is identified through multiple cycles of *in situ* hybridization, imaging, and probe removal, so that a single sequence would form a different color hybrid in different cycles of detection (Figure 1B). The method exponentially increases the number of measurable bacterial species by adding labeling cycles, thus promising to profile microbiomes in the gut due to its designable and high throughputs.

Based on the sequential encoding principle, Dar et al. reported a transcriptome imaging approach, termed parallel sequential FISH (par-seqFISH).²⁵ Compared with HiPR-FISH, par-seqFISH can not only profile taxonomic information but also measure gene expression with single-cell resolution. Spatially resolving each mRNA inside cells is challenged by the small size of bacteria and diffraction limit. To occur the underestimation of gene expression by counting the number of spots inside cells due to the overlapping of mRNA signals, the fluorescence intensity of spots was used to estimate the mRNA counts in each spot. par-seqFISH was used to image 105 genes of *P. aeruginosa* related to physiology and virulence in biofilm and showed the heterogeneity and subpopulations with different physiological activities in individual multicellular biofilms. Besides, profiling gene expression, based on imaging, par-seqFISH can simultaneously acquire bacterial phenotypes such as cellular morphology, chromosome copy, and other markers through nucleic acid labeling or immunolabeling. The extended application of par-seqFISH for investigating the gut microbiota paves a way to elucidate gut biogeography, and the relationship between genotype and phenotype with single-cell resolution and spatial correlation.

Despite the progress, increasing labeling cycles, however, is technically at the cost of measurable region and bacteria, boosting the encoding error and the processing complexity. In a compromise, using spectral encoding to increase the labels at each time would dramatically reduce the labeling cycles, although yield required throughput for imaging the gut microbiome. In addition, the exploration of encoding FISH methods to simultaneously chart both host cells such as enterocytes, and gut microbiota with taxon and gene expression information in the intestinal epithelium region such as crypts and villi, would enable the elucidation of spatially resolved host-microbe antibiosis and symbiosis interactions.

Spatially resolved sequencing

In addition to the taxon information of intestinal flora measurable via FISH, we also need to acquire the genetic and gene expression information of bacteria without destroying the spatial organization of microbial communities. These requirements can be fulfilled by recently developed *in situ* sequencing.

In situ RNA sequencing, first proposed in 2010, allowed the direct sequencing of 4 bases of RNAs in the intact tissue samples.⁴⁰ In 2014, the Church group proposed a

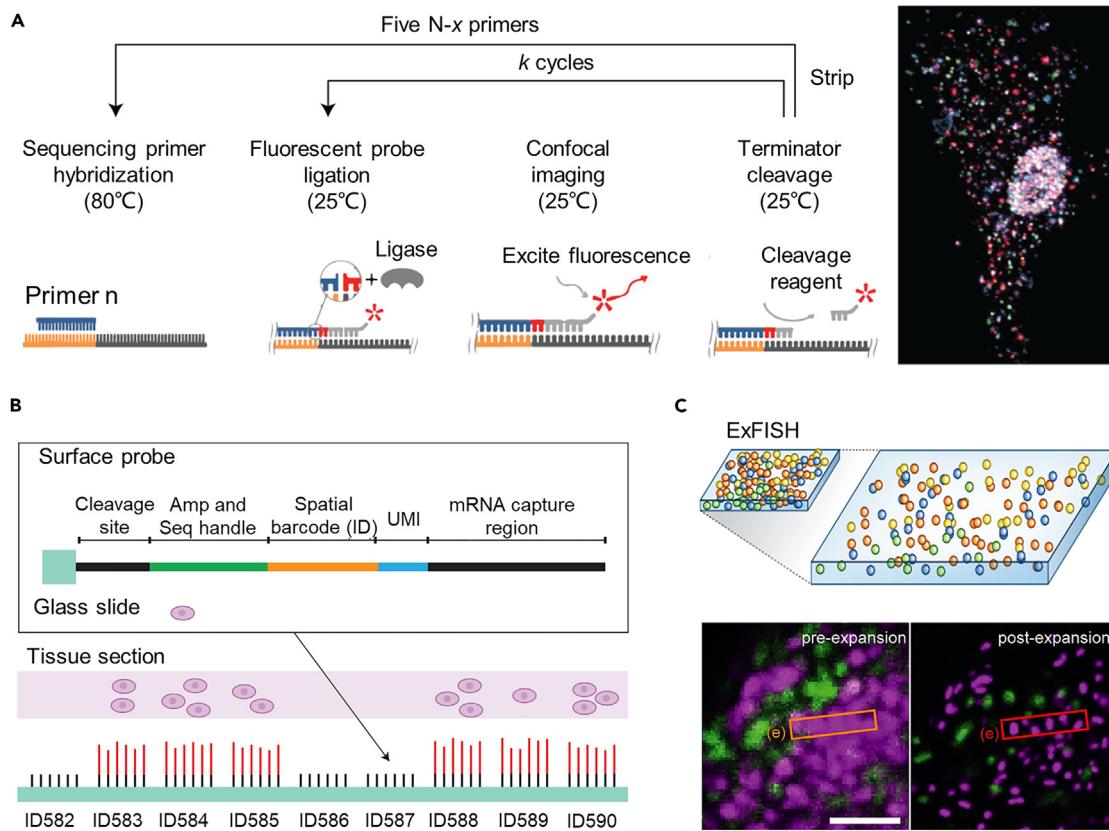


Figure 2. Spatially resolved sequencing principles and expansion microscopy

(A) Targeted *in situ* sequencing. Sequencing-by-ligation procedures (left), an example image for a primary fibroblast cell via 15 cycle reaction (right). Reprinted with permission from Lee et al.⁴¹ Copyright 2014 AAAS.

(B) Illustration of spatial transcriptomics. Tissue section is placed in the assay featured with unique DNA-barcoded probes to indicate the cellular location. mRNAs in the tissue section are captured in the assay and turned to be cDNA via reverse transcription for sequencing. Reprinted with permission from Ståhl et al.⁴² Copyright 2016 AAAS.

(C) Visualization of densely located bacteria using expansion microscopy. Illustration of diluting cells via expansion (top), an example image of mCherry-labeled *Lactobacillus plantarum* and GFP-labeled *Acetobacter tropicalis* before and after expansion (bottom). Reprinted with permission from Wählby.⁴³ Copyright 2016 Springer.

next generation of the fluorescence *in situ* sequencing (FISSEQ) method, based on rolling-circle amplification and sequencing-by-ligation to generate cDNA amplicons in cells and achieved an improved detection speed (Figure 2A).⁴¹ By FISSEQ, they scaled up the sequencing to 30-base reads from 8,102 genes *in situ*. Particularly, a technique termed STARmap, integrated hydrogel-tissue chemistry, rolling-circle amplification, and *in situ* sequencing, can simultaneously boost the measurable gene number (1,020 genes) and spatial scale (millimeter-scale volumes containing ~30,000 cells).⁴⁴

Compared with the FISH method, *in situ* RNA sequencing can not only drive the taxon identification to be strain-level via resolving single-nucleotide polymorphisms but also provide the information of gene sequences and expression statuses in fixed tissues, providing an avenue to the elucidation of genotype to function of the gut microbial communities and the functionality of individual members in the ecosystem. For example, by analyzing the expression of genes associated with bacterial communications or genetic mutations related to drug resistance, it can be used to explore the specific microbial roles involved in the spatial and interaction

rearrangement of gut microbiota assembly related to processes such as colonization, quorum sensing, and antibiotics-response or resistance.

However, now the readable length of *in situ* sequencing is limited and can hardly cover microbial transcriptome. The emerging strategy called spatial transcriptomics enables the visualization and quantitative analysis of the transcriptome in tissue sections (Figure 2B). By mounting tissues on a chip for the localized capture of cellular RNAs, spatial transcriptomics can index and sequence RNAs with preserved two-dimensional location information.^{42,45} Its spatial resolution has been enhanced from about 50 μm to be below 10 μm via replacing microarrays with DNA-barcoded beads^{46,47} and to be sub-micrometer via using DNA nanoballs⁴⁸ or amplified barcoded oligonucleotides⁴⁹ as capturers. In principle, spatial transcriptomics holds the potential for profiling transcriptome at a single-cell or even a subcellular level. The lack of a cellular boundary marker, however, renders it hard to distinguish each cell in the tested spots. Deconvolution based on single-cell RNA sequencing (scRNA-seq) data sets would facilitate the determination of cellular composition in each spot and enable the reconstruction of spatial cell-type distribution with single-cell resolution.^{50,51}

Spatial transcriptomics has advanced cellular phenotype identification, functional zonation, and interaction assessment in the intestinal epithelium.^{49,52} By coupling with scRNA-seq, Fawkner-Corbett et al. charted intestinal morphogenesis.²⁶ The map of the geographical regions and transcriptional signatures shed light on the multiple events in the gut such as crypt-villus axis formation, mesenchyme differentiation, and immune colonization. The profiling of the transcriptomic landscape of the colon response to damage using spatial transcriptomics uncovered the dramatic variation of molecular regionalization of the colon during mucosal healing.⁵³ Using SpaceFold, a dimensionality reduction strategy, to map cell-specific transcriptomes along the crypt-villus axis based on the data from spatial transcriptomics, Niec et al. investigated the signaling between lymphatic cells and stem cell niches in small and large intestines and found that WNT-signaling factors and extracellular matrix protein serve as lymphatic signals to govern epithelial stemness and differentiation.⁵²

Despite the advance of spatial transcriptomics for investigating human and mouse intestines, its use for profiling the transcriptome of gut microbiota is still challenging. Bacterial transcripts are difficult to be freed and captured based on the current protocol due to the complexity of the bacterial walls and the lack of poly(A) tails. Besides, the abundant host eukaryotic RNAs would result in transcriptome data dominated by host sequences. Replacing poly(A)-based primers with random oligonucleotides enables the simultaneous capture of both host and bacterial RNAs at the cost of capture efficiency, and strategies for enriching bacterial RNAs may increase the sensitivity and coverage for profiling the bacterial transcriptome. Recently, Löstedt et al. proposed an analysis pipeline for spatially resolved profiling of host gene expression and microbial biogeography via sequencing polyadenylated RNAs and bacterial 16S rRNAs.⁵⁴ The simultaneous profiling of the transcriptome of both the host and microbe, enabled by spatial transcriptomics with further increased sensitivity via the optimization of the chip, amplification process, or sequencing protocols, can provide a comprehensive and precise analysis of the host-intestinal microbiota interaction.

Expansion microscopy and sample clearing

Besides improvements by the probe design and image processing, attempts are taken to deal with gut samples to acquire the high-resolution 3D image and intact spatial information.

In the gut, the microbiota is densely distributed, and the cells are overlapped, thus hardly distinguished as unicell by imaging. The gut sample can be physically expanded before imaging by using expansion microscopy, increasing the spatial resolution, and diluting the densely located microbiota.^{43,55–57} After sample fixing and labeling, the spatial density of the cells and molecular markers in the cells can be reduced by biochemically linking to a gel that is then expanded, maintaining the relative positions of molecular markers and bacteria while providing a resolution-improved image to be captured owing to the increase in spatial separation of the fluorophores (Figure 2C). Lim et al. demonstrated the first use of expansion microscopy for imaging bacteria and accurately identified and located the gut microbiota of model planarians by spectral labeling.⁵⁸ Particularly, the expansion process can also indicate the cell wall mechanics of the bacteria. Based on the principle, they innovatively measured the cell wall expandability to resolve nine species of commensal bacteria in the human gut. Contributed to its high sensitivity, the method enabled the detection of the damage to the cell walls of the gut bacteria by antibiotics such as vancomycin or the host's immune response that was difficult to be captured by the traditional imaging methods. The measurement of mechanical contrasts enabled by expansion microscopy would provide extra physiological information on the microbial communities in the gut besides taxonomy.

The clearing processing could facilitate the acquisition of high-resolution 3D images of samples by alleviating light scattering. Wang et al., for the first time, applied tissue-clearing techniques to elucidate the gut microbiota structures.⁵⁹ Combining the tissue-clearing clear, unobstructed brain imaging cocktails and computational analysis (CUBIC) strategy and D-amino acid (DAA) metabolically labeling, they got the first cellular level 3D imaging of the microbial communities transplanted in the gut and presented quantitative spatial details in relation to the host epithelium. The method, termed TiDaL, increased the thickness of the observable tissue sample from 5–10 μm to millimeters. By the quantitative 3D visualization in millimeter-thick tissue with single-bacterium resolution, they obtained clear images of bacteria in the crypts of the cecum and colon and first observed the bacterial overgrowth and translocation in the dextran sodium sulfate enteritis model.

Besides imaging-based methods, sample expansion strategies have the potential to be used for other spatial techniques for RNA profiling such as spatial transcriptomics. In principle, the expansion of the gut tissue section could increase the chip area and the number of barcoded primers for capturing bacterial RNAs in each cell, thus potentially improving the coverage of the bacterial transcriptome.

Spatially resolved sampling

Imaging approaches are still challenging to scale up to cover the analysis of the metagenome and transcriptome of the gut microbiota assembly.⁶⁰ Alternatively, by sampling the gut microbiota with location information and *in vitro* sequencing, we can obtain the biogeography and transcriptome information of the gut microbiome in the regions of interest (ROIs) and clarify interactions that underlie community functions.

Laser-capture microdissection (LCM), a technique with well-commercialized instruments, can selectively isolate cells in ROI with the precision of 1 μm using infrared or UV lasers.⁶¹ LCM is applicable to different tissue types such as frozen or fixed tissues. The isolated tissue or cellular samples are well preserved and can be used for sequencing. Based on LCM, Moor et al. investigated the zonation of enterocytes along the villus axis.²⁷ Enterocytes along the bottom to tops of villi were isolated

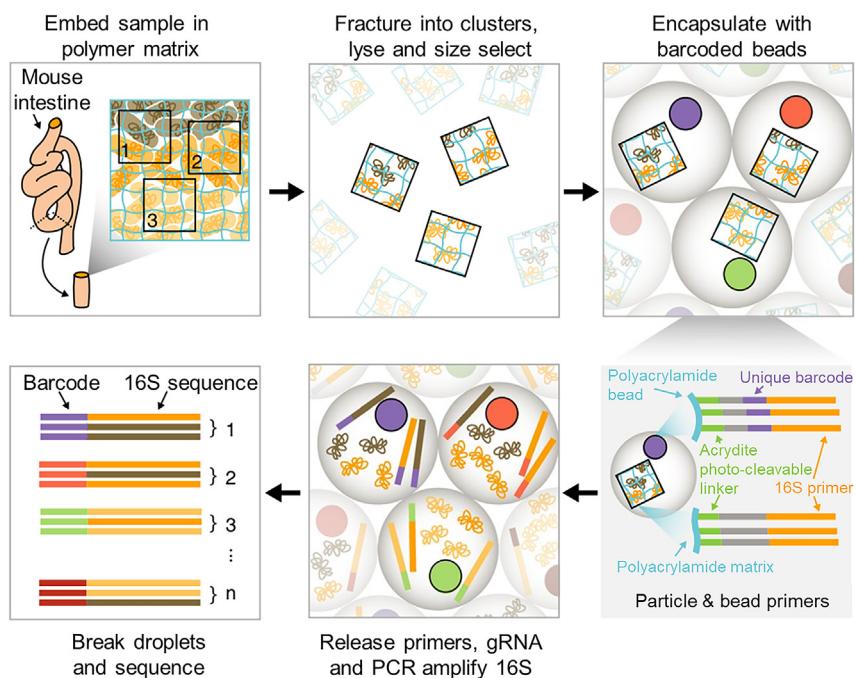


Figure 3. Illustration of the spatial metagenomic analysis by plot sampling and sequencing

Mouse intestine embedded in a polymer matrix is fractured into clusters and encapsulated with barcoded beads, allowing to be sequenced and spatial restructured. Reprinted with permission from Sheth et al.²⁸ Copyright 2019 Springer.

using LCM and sequenced to obtain a panel of landmark genes. Coupling with the scRNA-seq data, the spatial tissue coordinates along the villi were constructed and showed enterocytes located in the villi bottom and villi compartments yielded different gene expression patterns that were related to anti-bacteria and absorption. Cells with rare populations could be diluted by LCM and are more challenged to be profiled. To increase the capacity to analyze rare-type cells, such as secretory cells in the intestinal epithelium, Manco et al. reported a suboptimal dissociation strategy by replacing enzymes with EDTA to yield small clumps with 2–10 cells.⁶² Based on the secretory cell landmark genes, the technique, termed Clump sequencing, enabled the analysis of goblet, tuft, and enteroendocrine cells and yielded the zonation of 17%–35% of the highly expressed genes in enteroendocrine cells.

Besides surveying the microstructure in the intestinal epithelium, Sheth et al. proposed a plot sampling strategy, termed metagenomic plot sampling by sequencing (MaPS-seq), based on fracturing gel matrix, droplet, and deep sequencing techniques to map the geographical context of the gut microbiome at the macrolevel scale.²⁸ Metagenomic characterization of microbial cells in multiple plots was used to reconstruct the spatial organization of the gut microbiome. Specifically, intact colonic samples were first embedded in the acrylamide polymer matrix that was attached with primers for amplifying 16S rRNA. The colonic samples were then fractured and filtered to yield arranged clusters with a desired size, which can be indexed with location information. The clusters obtained by plot sampling were encapsulated using gel beads with bar-coded primers, followed by the amplification of 16S rRNA and sequencing (Figure 3). An analysis of multiple regions of the mouse intestine, compared them with fecal samples, first revealed a heterogeneous microbial distribution that was closely correlated between specific taxa. On the scale of tens of microns, individual groups in the gut

Table 2. Comparison of *in vivo* imaging methods for analyzing gut microbiota

Technique	Tissue depth ^a	Spatial resolution	Labeling strategies	Measurable targets	Investigation on gut microbiota	Reference
Bioluminescence imaging	several cm	200 nm	genetic labeling	exogenous bacteria	bacteria colonization	Foucault et al. ⁶⁴
Near-infrared fluorescence imaging	20 mm	200 nm	metabolic labeling	exogenous bacteria	effect of gut movement on microbiota biogeography	Wang et al. ⁶⁵
Ultrasound imaging	300 mm	30 μm	chemical labeling and genetic labeling	exogenous bacteria and protease activity	<i>E. coli</i> colonization	Bourdeau et al. ⁶⁶ and Lakshmanan et al. ⁶⁷
Photoacoustic imaging	80 mm	200 nm	chemical labeling and genetic labeling	–	–	Brunker et al. ⁶⁸
Positron emission tomography	no limit	1 mm	metabolic labeling	exogenous bacteria	infection diagnosis and evaluation of fecal bacterial transplantation	Weinstein et al. ⁶⁹ and Wang et al. ⁷⁰

^aTissue depth and spatial resolution are indicated based on the current best performance of their corresponding techniques. The spatial resolution is influenced by the imaging tissue depth.

microbiome were neither completely mixed nor highly structured but heterogeneous in the mixed patches.

Sample isolation techniques such as LCM and MaPS-seq could selectively collect both host and intestinal microbiota with high spatial precision. However, the risk of perturbations on the spatial structure and cellular gene expression induced by the sampling process should be evaluated. By coupling with dual RNA-seq, the spatially resolved collection of both host and microbe transcriptome holds the promise for elucidating host-intestinal microbiota interaction. In addition, due to the preservation of DNA, RNA, and protein in the dissected samples, joint profiling of transcriptome and protein would be beneficial for precisely inferring cellular and bacterial states in the gut which are featured with highly graded microenvironments.⁶³

IN VIVO DYNAMIC ANALYSIS OF THE GUT MICROBIOTA

To gain insights into the colonization, growth, and metabolic activities of gut microbiota, we need advanced technologies to obtain their dynamic information in living animals. Due to the development of imaging techniques, molecular probes, and synthetic biology, we can realize *in vivo* imaging of bacteria and their activity under the condition of thick intestinal walls, hypoxia, and high acidity. Based on the imaging principle, we highlighted *in vivo* analytical tools for gut microbiota, namely bioluminescence, near-infrared fluorescence, acoustic, and PET imaging (Table 2). Due to the availability of limited molecular and bacterial information, we also introduced the integration of *in vivo* and *in vitro* methods to simultaneously obtain dynamic, genetic, and metabolic information about the gut microbiota.

Bioluminescence *in vivo* imaging

Bioluminescence *in vivo* imaging is widely used because of its detectability and simple analytical formats.⁷¹ Luciferin is oxidized by luciferase to emit bioluminescence. Moreover, luciferin-luciferase systems could be adjustable for emitting luminescence with different spectral peaks, holding the potential for multiplexing labeling.⁷² Luciferase has been successfully expressed in both Gram-negative and Gram-positive bacteria, indicating its wide applicability. Bioluminescence imaging can be achieved by endogenous ATP and exogenous fluorescein substrate of bacteria. Bacteria such as *Escherichia coli* (*E. coli*), *Salmonella enterica*, and *Staphylococcus aureus* have been detected *in vivo* in the intestinal tract of mice by bioluminescence. Foucault et al. achieved long-term and stable fluorescence labeling in mice through the construction of the *E. coli* plasmid.⁶⁴ They examined differences

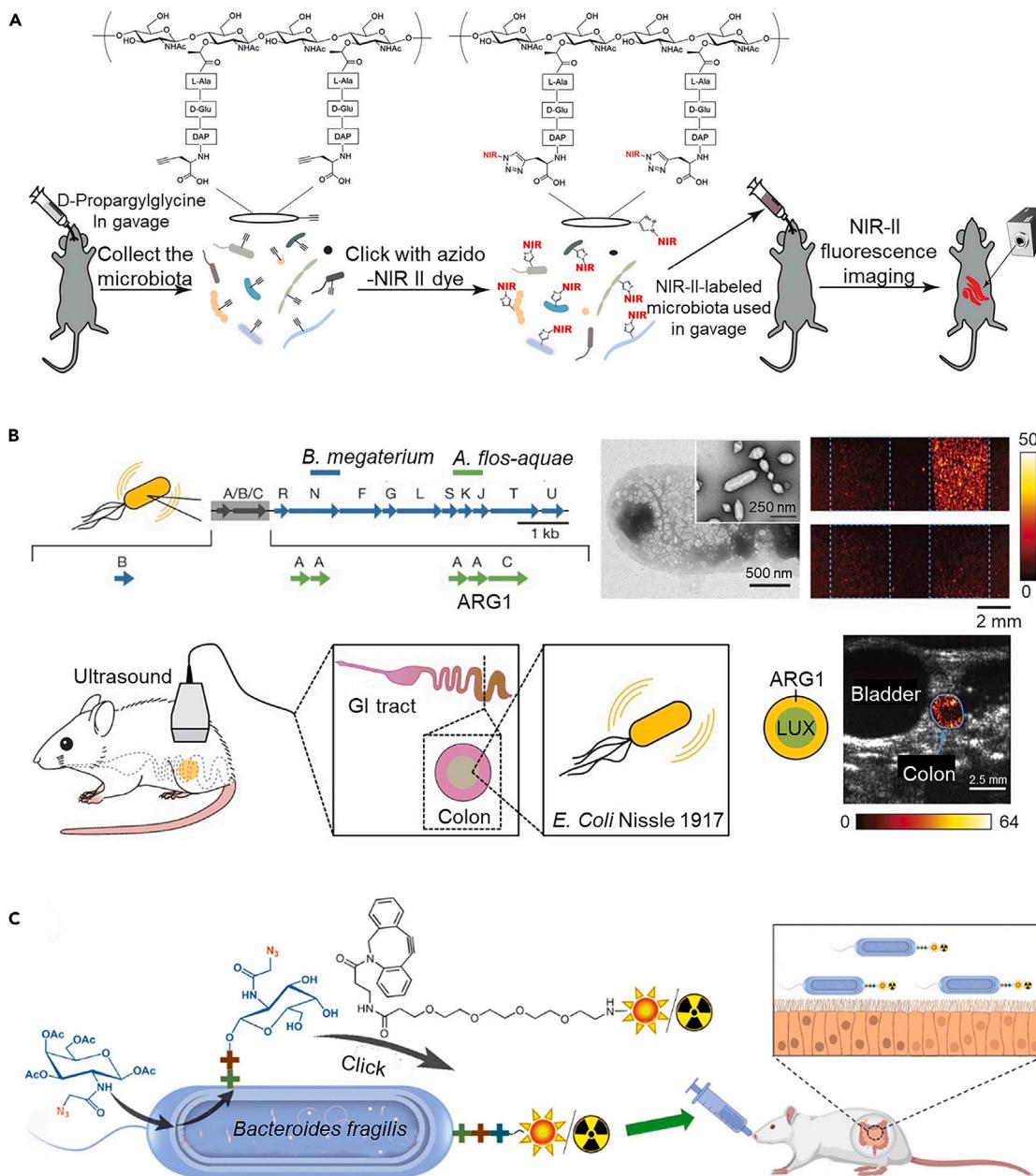
in the capacity of different strains of *E. coli* to colonize the large intestine and quickly screened for mutants and disease-causing strains. This method could be potentially used to examine antibiotic resistance of specific gut microbes and directly assess host effects involved in gut colonization, such as the role of the innate immune system. However, the hypoxic environment in the intestine is not conducive to the signal generation of aerobic bioluminescent proteins, and the reliance on bacterial engineering excludes the investigation of the endogenous gut microbiota. Conceptually, Ronda et al. reported a method based on an engineered exogenous bacterial donor and horizontal gene transfer,⁷³ enabling *in situ* labeling of endogenous bacteria in the gut. *In situ* engineering gut microbiota with genetic labels would enable the monitoring of indigenous microbiome and its interaction with exogenous bacteria, such as probiotics and pathogens.

Near-infrared fluorescence imaging

Fluorescence imaging yields high temporal and spatial resolution (~200 nm) for molecular monitoring; however, the low-tissue penetration of conventional fluorescence signals excludes its use for *in vivo* visualization. Nevertheless, the penetration of light is positively correlated with its wavelength.^{74,75} Fortunately, second near-infrared (NIR-II) (1,000–1,700 nm) fluorescence imaging could achieve deep tissue penetration (up to 20 mm),^{76,77} which enables real-time assessment of the gut microbial movement. Wang et al., for the first time, devised a feasible NIR-II imaging method to analyze gut microbes *in vivo* in mice (Figure 4A).⁶⁵ The light-up of bacteria in the gut was based on D-propargynoglycine and NIR-II dye containing azide that were subjected to click reaction to metabolically label the bacteria of interest. By dynamic monitoring of gut microbes, it is found that their distribution was closely related to the intestinal movement. This method provides the first protocol for *in vivo* visualization of gut microbiota within deep tissues using fluorescence imaging and can be used to explore the dynamic competition between different bacteria in the intestinal tract.

Encapsulated in the nanoparticle form can eliminate the encounter of dyes with harsh conditions and increase their fluorescence intensity. Jiang et al. designed an aggregation-induced emission luminogen (AIEgen) micelles to *in vivo* track probiotics in mice.⁷⁸ AIEgens would be lighted up by molecular aggregation in the micelles. By using a critical micelle concentration-switching strategy, the aggregation state and brightness of AIEgens were improved, yielding a bright contrast agent for deep-tissue imaging. By conjugating with DAA to the surface of AIEgen micelles, AIEgen micelles were accommodated to label and dynamically monitor probiotics including *Lactococcus lactis* and *Lactobacillus* in the mice's gastrointestinal tract.

By engineering with long conjugated chains and reduced intermolecular interactions, NIR-II fluorescent dyes yield substantially improved quantum yield (close to 10%) and photostability,⁷⁹ thus providing a high signal-to-noise ratio for molecular imaging in deep tissues or organs including tumors, brains, and gut.^{80–83} Metabolic labeling of the bacteria of interest by tagging amino acids and oligosaccharides with NIR-II fluorescent dyes can be feasible for *in vivo* tracking of the gut microbiota. Nevertheless, the labeling efficiency could be reduced by the modification of oligosaccharide and amino acid substrates with dyes due to the causing intolerance of biosynthetic enzymes. Thus, both the fluorescence features and compatibility for metabolic labeling should be considered during the optimization of the fluorescent dyes.

**Figure 4. In vivo dynamic imaging of gut microbiota**

(A) Scheme of the NIR-II-based fluorescence imaging of gut microbiota. Bacteria in the gut are metabolically labeled with D-propargylglycine probes via the surface bioorthogonal alkynyl group and imaged using a 730 nm laser and an InGaAs CCD. Reprinted with permission from Wang et al.⁶⁵ Copyright 2020 Wiley.

(B) Ultrasound imaging of bacteria in the gastrointestinal tract using acoustic reporter genes (ARGs). Organization of ARG clusters, an example TEM image of gas vesicles and an ultrasound image of *E. coli* expressing with ARG or green fluorescent protein (GFP) (upper). Illustration of ultrasound imaging of *E. coli* Nissle 1917 proximal to the colon wall (bottom). Reprinted with permission from Bourdeau et al.⁶⁶ Copyright 2018 Springer.

(C) Analysis of *Bacteroides fragilis* using click reaction and PET imaging. *Bacteroides fragilis* is metabolically labeled with ⁶⁴Cu via biorthogonal click chemistry and tracked in the mouse intestine. Reprinted with permission from Wang et al.⁷⁰ Copyright 2020 Springer.

Acoustic imaging

Ultrasound can penetrate deep tissues and enable *in vivo* and deep imaging with high spatiotemporal resolution (about 100 μm and 1 ms) based on measuring acoustic scattering in tissues. The sensitivity and contrast of acoustic imaging can be

enhanced by integrating with pulsed laser irradiation, principled as photoacoustic imaging (PAI).^{84,85} Different from ultrasound imaging, PAI yields an ultrasonic signal that carries the absorption information of the labels or intrinsic tissues by light-induced heat generation and thermoelastic expansion.^{86,87} By engineering chemical probes and genetically engineered reporters, both ultrasound and PAI are amenable for the non-invasive imaging of molecules and bacteria in deep tissues and recently being used to monitor bacteria and its activity in the gut.

Living bacteria can be monitored using PAI by labeling them with chemical dyes. Huang et al. proposed a PAI device to identify and quantify the bacteria in the mice intestine and colorectal tract.⁸⁸ By simple mixing, *Lactobacillus* and *E. coli* were labeled with indocyanine green and Prussian blue, respectively. In the PAI setting, the laser expanded through a concave lens in the mouse's gut, whereas the multiple data can be rapidly collected and reconstructed to be 3D PAI by a filtered back projection algorithm. They accurately identified and located *Lactobacillus* and *E. coli* and analyzed the ratio of probiotics to pathogenic bacteria in specific areas of the gut. However, bacteria were only imaged in the gut *ex vivo*. Besides, the cellular division would dilute the dye labeling; thus, the strategy cannot yield long-term microbial monitoring *in vivo*.

Bacteria can be genetically engineered with acoustic reporter genes to sustain *in vivo* and potential long-term track in the gut. To achieve deep *in vivo* imaging (> 1 mm), chromoproteins such as phycobiliprotein⁸⁹ and bacterial phytochromes⁹⁰ have been optimized with red-shifted absorption (600–950 nm) and enhanced nonradiative decay, which allow us to yield strong PA the *in vivo* status. Besides, an engineering photoswitchable reporter that changes its absorption on illumination by *cis-trans* isomerization can dramatically reduce the endogenous background⁹⁰ and yield an increased signal-to-noise ratio and measurable tissue depth for *in vivo* imaging. Genetically engineered PA reporters have been engineered into tumor cells for monitoring tumor metastases; however, currently, they have not been used for investigating gut microbiota. Recently, Bourdeau et al. constructed a genetically engineered acoustic reporter based on an air-filled protein nanostructure.⁶⁶ The hollow protein nanostructure, serving as a gas vesicle (GV), can scatter sound waves and yield nonlinear contrast by buckling mechanical deformations. Genetically engineered bacteria, including *E. coli* and *Salmonella typhimurium* with 8–14 genes that can collectively encode GVs, can be imaged in the gut with spatial and temporal resolutions of 100 μm and 1 ms, respectively (Figure 4B). Particularly, by shortening the outer scaffolding proteins, GVs can be adjusted with different collapse pressure, enabling the multiplexed labeling of genes in bacteria based on pressure spectrum unmixing. The Shapiro group further designed an acoustic molecular biosensor for detecting protease activity in the intestinal tract of the mice.⁶⁷ By incorporating the scaffold protein, GvPC with the substrate peptide sequences of protease, the GV-based acoustic biosensor yielded a specific response toward proteases including endopeptidase, calpain, and ClpXP by producing nonlinear ultrasound contrast. The sensing strategy allowed the monitoring of the protease activity in the engineered *E. coli* strains Nissle 1917 in the intestinal tract of the mice.

Acoustic imaging promises *in vivo* imaging of gut microbiota due to its deep penetration and high spatiotemporal resolution. Traditionally, ultrasound imaging is hindered by its low sensitivity for detecting low-abundance bacteria. Recently, by unmixing the temporal GV collapse signals from background scattering, the sensitivity of ultrasound imaging has been improved by 1,000-fold, thus allowing us to image gene expression in individual bacteria.⁹¹ Nevertheless, the *in vivo*

imaging methods, such as ultrasound imaging and PAI currently provide limited molecular information. The detectable molecular menu can be broadened by engineering cell-based biosensors. By coupling the acoustic,⁶⁶ photoacoustic,⁶⁸ and NIR-II fluorescence⁹² reporter genes with sensory receptors or actuators, such as transcription factors, promoters, and RNA switches,^{93–95} engineered cellular biosensors could allow to monitor the level, spatial pattern, and dynamic change of gene expression, microbial metabolites (such as neuroactive molecules and hormonal intermediates), metal ions, and antibiotics in the gut,⁹⁶ facilitating the investigation of microbial metabolism, cross talk, and treatment effects. Besides, by coupling with neuroimaging techniques, such as functional magnetic resonance imaging, the *in vivo* molecular imaging in the gut can be highly indicatable for the dynamic interaction between microbiome, gut, and brain, facilitating the elucidation of the relationship between gut microbiota and the brain-gut axis.

Positron emission tomography

PET uses radionuclides for *in vivo* imaging. Using PET, we can acquire dynamic information and images quickly at the speed of seconds and quantitatively detect physiological parameters *in vivo*. It has been used in monitoring antimicrobial efficacy in animals that have been infected with drug-susceptible or drug-resistant *E. coli*.⁶⁹ Disaccharides and polysaccharides such as maltose and maltodextrin, amino acids, or their analogs such as methionine and para-aminobenzoic acid can be metabolically utilized only by bacteria, but not mammalian cells. They can serve as specific radiotracers for gut microbiota by labeling it with fluorine-18 and carbon-11. Besides, radiolabeling could be achieved by biorthogonal click chemistry. The labeling of *Bacteroides fragilis* with ⁶⁴Cu was achieved by metabolic oligosaccharide labeling of N₃ and click chemistry (Figure 4C).⁷⁰ Based on bacteria radiolabeling, PET tracking enabled by metabolic labeling allows for real-time monitoring and quantitatively tracking gut microbiota and have been used to investigate bacterial infection⁶⁹ and probiotics transplants.⁷⁰ PET is advantageous in the capacity for *in vivo* imaging at the scale of full bodies and temporal resolution. This method, on the other hand, uses radioactivity and requires constant injections of radionuclide tracers to yield images, hindering its application in regular labs.

Combination of *in vitro* and *in vivo* strategies

The substantially developed *in vivo* imaging technologies have realized the dynamic tracking and quantitative analysis of intestinal bacteria. However, currently, *in vivo* detection yields little molecular and cellular information. The development of genetically engineering or metabolically labeling strategies potentially broadens the measurable molecular and bacterial markers. Alternatively, combining *in vivo* imaging that provides dynamic spatial information with *in vitro* analytical strategies such as FISH⁹⁷ and mass spectra⁹⁸ that offer in-depth molecular information including taxon, genetic markers, and metabolites would facilitate the comprehensive analysis of gut microbiota.

To quantitatively analyze the intrinsic metabolic status of gut microbiota, Lin et al. used a combination of metabolic DAA labeling and FISH.⁹⁷ Bacterial genera were classified and identified in the intestinal flora of fluorescent DAA-labeled mice by intragastric administration. Then, the metabolic activities of the intestinal bacterial genera were quantified by flow cytometry and the corresponding FISH probe, using the FISH signals to distinguish the corresponding genera and fluorescent DAA signals to indicate the basal metabolic level of the bacteria. This method has been applied in the study of the diurnal metabolism of intestinal flora in mice and the evaluation of the viability of intestinal flora transplantation. The use of multiplexing FISH

to yield biogeography would further indicate the spatial rearrangement of gut microbiota induced by intestinal flora transplantation.

Besides imaging, exogenous substrates such as isotope-labeled small molecular probes and peptides have been engineered to serve as synthetic biomarkers to non-invasively detect endogenous, enzymes and bacteria *in vivo*.⁹⁹ Unlike imaging, the level of endogenous metabolites, enzymes, and bacteria is estimated by *in vitro* measurement strategies, that is, detecting the cleaved substrates in urine or exhalation. The strategy is exemplified by the ¹³C-urea breath test for *Helicobacter pylori* in the stomach and has been rejuvenated due to the boost of exogenous substrates and delivery strategies.^{99,100} These synthetic biomarkers can be highly specific due to the reactive selectivity of enzymes, no background due to its non-existence in the body, and stably deliverable to the target tissues or organs by using the targetable and protectable carrier. Continuous monitoring of the cleaved substrate *in vitro* enables the dynamic measurement of metabolites, enzymes, and bacteria *in vivo*. Synthetic biomarkers that have been demonstrated to be feasible for detecting tumors, respiratory diseases, and inflammation^{99,101} hold great potential to broaden the *in vivo* measurable molecular, bacterial, and cellular information in the gut.

INVESTIGATION OF GUT MICROBIOTA ECOLOGY, FUNCTION, AND DISEASE ASSOCIATION

Spatiotemporal resolved analytical tools facilitate our understanding of the structure, function, and disease association of gut microbiota by obtaining the spatial pattern, growth, and metabolic activities of microbes in the gut. Besides, attributed to molecular engineering and nanotechnology, the spatiotemporal regulation of bacteria in the gut is achievable and valuable in clinical treatment.

Dietary effect on the bacterial spatial structure

The lipid and polysaccharide content of the diet affects gut microbiota and the structure of the mucus layer.^{28,102} However, evidence directly explaining changes in spatial distribution and function influenced by dietary factors is not available. Nagar et al. investigated microscale habitats in the intestine using FISH and demonstrated that the starch granules served as a major habitat and potential nutritional niche for intestinal *Bifidobacterium pseudolongum*.¹⁰³ By integrating FISH with an image analysis pipeline, Earle et al. proposed a quantitative analysis strategy with single-cell resolution, allowing us to investigate the spatial relationships among the host and bacterial taxa across multiple planes of the gut.¹⁰² The quantification of the microbial organization indicated that monophyletic clustering was diet-dependent and the lack of dietary fiber drove the reduction of the thickness of protective mucus layer.

Although changes in the diet can rapidly alter the composition of the microbiota within days, the ecological mechanisms underlying the community-scale variation are unknown. Sheth et al. analyzed multiple regions of the mouse intestine, using MaPS-seq to detect microbiota changes caused by two different diets with distinct macronutrient profiles (Figure 5).²⁸ The experiment identified a strong association between *Bacteroidales* taxa in all gut regions and showed phylogenetically clustered local dietary perturbation. High-fat and high-sugar diets resulted in more phylogenetically diverse taxa within individual clusters and more frequently positive co-associations between diverse taxa. The investigation based on the microbiome

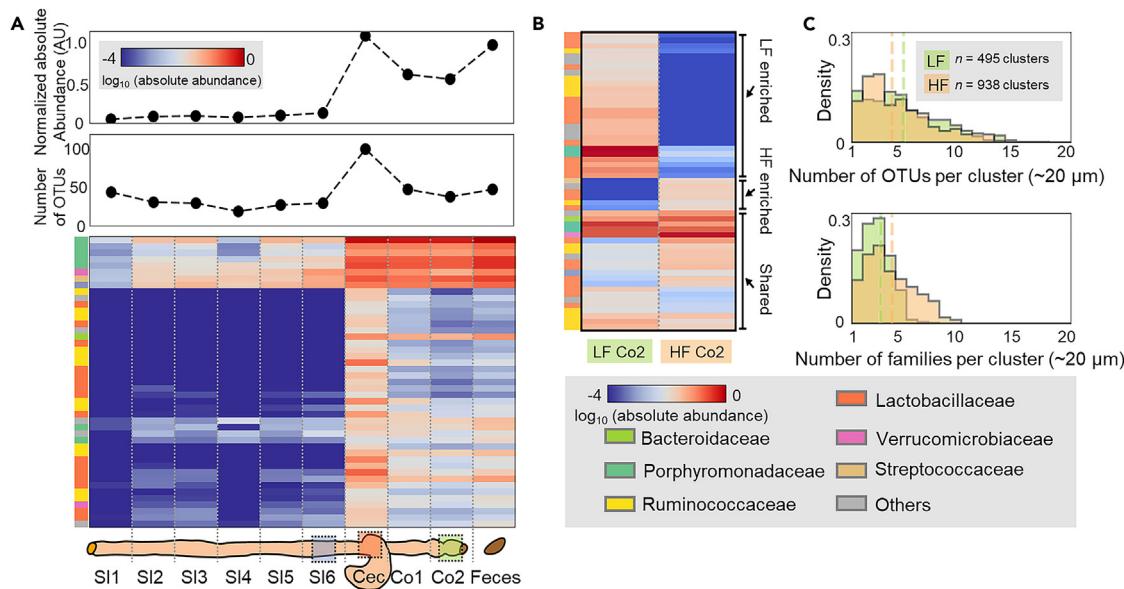


Figure 5. Effects of diets on the bacterial spatial structure

(A) Absolute abundance within gut intestinal regions.
 (B) Absolute abundance of dominant OTUs in the distal colon of cohoused mice fed an LF or HF diet for 10 days.
 (C) Histogram of the number of OTUs per cluster (top) and distinct families per cluster (bottom). OTUs, operational taxonomic units; LF, low-fat, plant polysaccharide-based diet; HF, high-fat, high-sugar diet. Reprinted with permission from Sheth et al.²⁸ Copyright 2019 Springer.

biogeography in the gut indicated that a diet rich in cellulose and other polysaccharides can be used to improve the systemic diversity of gut microbiota.

Collateral damage of antibiotics on gut microbiota

Antibiotics use can reduce gut bacterial diversity.^{104–106} However, exploring the destructive effect of antibiotics on the gut microbiota with high taxonomic and spatial resolution remains challenging. Using a high-throughput encoding FISH method (HiPR-FISH), Shi et al. created a map of the mouse gut microbiome response to antibiotic treatment. Clindamycin-treated mice were used to test the spatial organization of bacteria, *Bacteroides*, *Macellibacteroides*, and *Longibaculum* in the guts (Figure 6).²⁴ The spatial analysis showed that the pair correlation function of *Bacteroidetes* cells slowly decayed, suggesting that *Bacteroidetes* tended to form clusters at short distances. By contrast, *Hespalia* cells were randomly distributed. *Bacteroides* were enriched near the boundary. Moreover, they assessed the changes in species abundance and physical contact between two taxa after ciprofloxacin treatment. A similar trend of part index number was observed in clindamycin-treated mice. The result suggested that the microstructures of the gut microbiota were conserved over large distances. They also found that antibiotic treatment weakened the spatial associations between several genera. *Oscillators* and *Veillonella* yielded the highest fold change of spatial association, both of which were associated with altered inflammatory responses in the host. The results showed that not only the component but also the spatial pattern of the gut microbiota can be dramatically changed due to the use of antibiotics, posing a further risk to the balanced gut.

Roles of gut microbiota on enteritis

One in a thousand people in developed countries are affected by enteritis.¹⁰⁷ Mislocalization of the gut microbiota potentiates enteritis. However, the relationship between the microscale ecological structure in the gut and the pathogenesis of enteritis remains

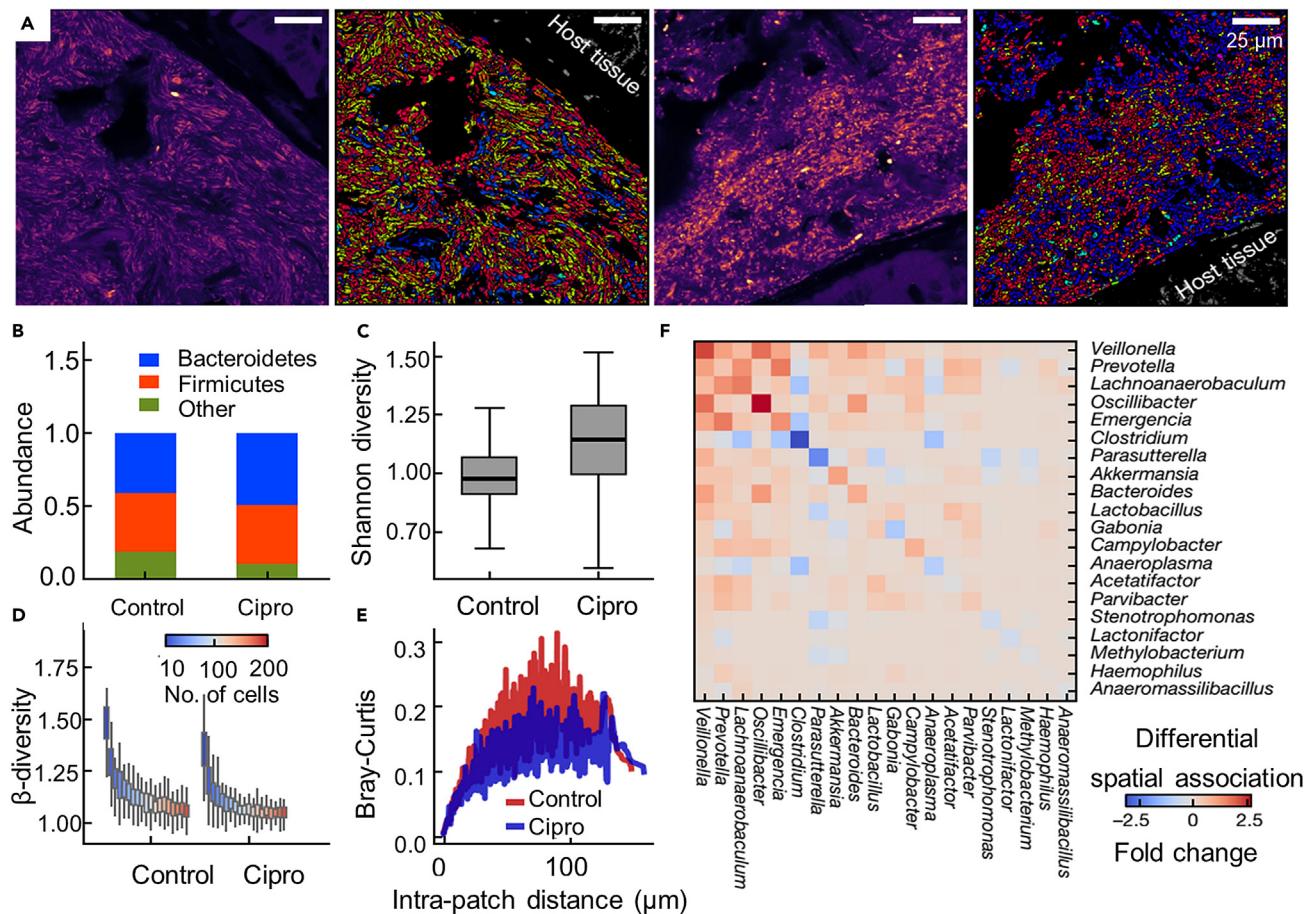


Figure 6. Disruption of the spatial organization of the gut microbiome due to antibiotic treatments

(A–D) (A) Fluorescence images of the gut microbiome with ciprofloxacin treatment. Phylum-level abundance (B), Shannon diversity (C), and β -diversity as a function of patch size (D) in control and ciprofloxacin-treated (cipro) mice.

(E) Analysis of Bray-Curtis dissimilarity between the same-size patches.

(F) Differential spatial association network analysis between ciprofloxacin-treated and control mice. Reprinted with permission from Shi et al.²⁴ Copyright 2020 Springer.

to be explored.^{21,22} FISH can be used to analyze the bacterial species and distribution characteristics in the intestines of patients with enteritis. By using nonaqueous Carnoy fixative to preserve the mucus layer, Swidsinski et al. performed *in situ* analysis of colonic mucosal bacteria in patients with IBD and healthy subjects, using rRNA-targeted FISH probes.²¹ The spatial analysis of bacteria in the mucus layer indicated that in contrast to the high diversity of the fecal bacteria, only six bacterial species were found to be adherent, and *Bacteroides fragilis* dominated the biofilm biomass in patients with IBD. *In situ* imaging allowed us to obtain the spatial pattern of the gut microbiota. Two types of adherence, coat- or string-like adhesion and a patchy adhesion were observed. Broad, highly concentrated bacterial bands were typical for Crohn's disease, whereas ulcerative colitis was featured with bead-like or string adhesion. *Eubacterium rectale*, *Enterococcus faecalis*, and *Fusobacterium prausnitzii* were patchily adhered. The spatial pattern of colonic mucosal bacteria varied in different enteritis, which was expected to influence the aberrant immune response and inflammatory damage to intestinal tissues; however, the mechanism still needs to be explored. Attributed to single-cell resolution, intracellular bacteria comprised

Bacteroides fragilis and *Eubacterium rectale* were found in epithelial cells next to the basal membrane. These investigations revealed the spatial characteristics of bacteria both in healthy and patients, which is helpful for us to further explore the pathology of enteritis and provide treatment strategies such as intestinal mucosal repair.

Fecal microbiota transplantation therapy

Fecal microbiota transplantation is an efficient way to treat diseases such as IBD, irritable bowel syndrome, and cancers by reconstructing the gut microbiota, and more than 200 fecal microbiota transplantation clinical trials are ongoing or finished.¹⁰⁸ FISH, along with a tissue clearing strategy, enabled the illustration of the distribution of transplanted bacteria with single-cell spatial resolution and millimeter imaging depth.⁵⁹ However, methods for the monitoring of *in vivo* bacterial behaviors after transplantation are still limited; thus, the survival, colonization, and function of transplanted bacteria remain difficult to be investigated.

The survival of transplanted bacteria *in vivo* could be achieved by metabolic labeling. Wang et al. developed a sequential tagging strategy using DAA probes (termed STAMP) to assess the viabilities of transplanted bacteria.¹⁰⁹ The surviving transplanted bacteria would emit fluorescence from the sequentially administered DAA probes, thus could be identified by fluorescence imaging. Based on the method, they found that the preconditioning using vancomycin, cefotaxime, and metronidazole could reduce the microbiota transplantation efficacy; by contrast, polymyxin B treatment improved the ratios of transplanted bacteria survivors. The result indicated that the choice of antibiotics for preconditioning is highly important for fecal microbiota transplantation.

The functioning of transplant bacteria was also explored by dynamic *in vivo* imaging. Wang et al. used PET imaging to dynamically analyze transplant bacteria associated with antitumor in the intestines of mice.⁷⁰ The antitumor effect of the anti-PD-1 blocker on fecal transplantation and broad-spectrum antibiotic imipenem was observed in low-response 4T1 cogene mice. After intragastric administration of mice, metabolizing *Bacteroides fragilis* in the intestinal tract was labeled, and then, PET imaging was used to track and quantitatively analyze the dynamic changes of the bacteria. *Bacteroides fragilis* was labeled with ⁶⁴Cu and yielded retention in the intestines for up to 24 h. Combined with *in vitro* 16S rRNA analysis, they found that gut microbiota disturbance reduced the efficacy of anti-PD-1 treatment, but the relative abundance of *Bacteroides fragilis* increased significantly after the administration of PD-1. These results suggested that the coupling of *Bacteroides fragilis* gavage and PD-1 blockade contributed to the rescue of the antitumor effect of anti-PD-1 therapy. Nevertheless, the used metabolically labeling strategy cannot detect proliferated bacteria, hindering the long-term monitoring of transplant bacteria and their function association.

Spatiotemporal manipulation of gut microbiota

The spatiotemporal manipulation of the transplanted bacteria with defined metabolism and physiological activities promises precise microbial theranostics. Although engineered bacteria can be used for the treatment in the gut, they are difficult to colonize in the gut or do not colonize in a precise manner and play their expected roles.¹¹⁰ Therefore, we need to intervene and control engineered bacteria's colonization and activities to achieve the purpose of using intestinal flora to address physiological health and clinical issues. Physical stimulation can be engineered to spatiotemporally control gut bacteria. Magnetic fields are ideal for providing the *in vivo* control because they can easily penetrate biological tissues. Buss et al.

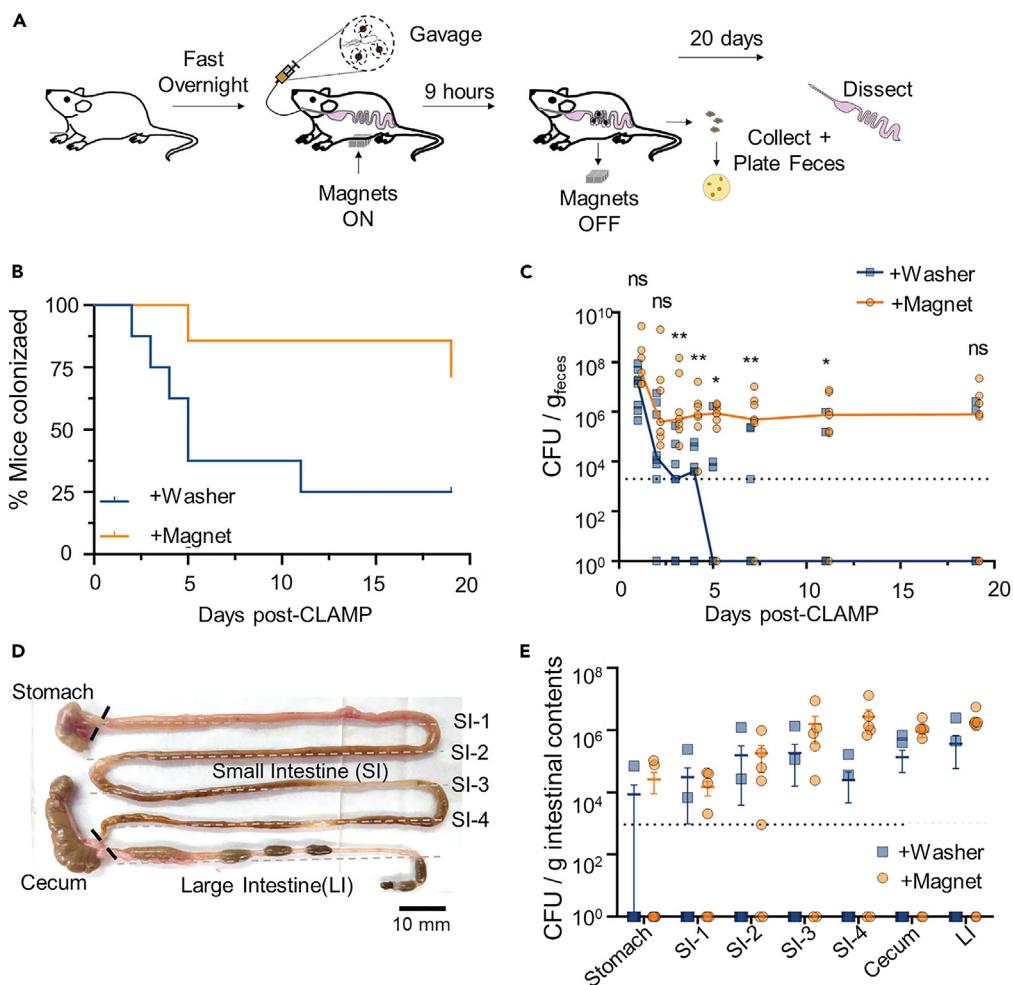


Figure 7. Precise colonization enabled by magnetic control

(A) Protocols for oral gavage and magnetic control.

(B) Percentage of mice with any detectable *E. coli* Nissle 1917 in the feces after gavage.

(C) Tendency of CFUs per gram of feces after CLAMP.

(D) Image of intestines from a mouse with magnetic control for 20th day after gavage.

(E) Distribution of *E. coli* Nissle 1917 in intestinal segments on 20th day after gavage. CFUs, colony-forming units; CLAMPs, cellular localization assisted by magnetic particles. Reprinted with permission from Buss et al.¹¹¹ Copyright 2021 Wiley.

demonstrated the manipulation of probiotic bacteria in the gut using magnetic fields by co-administered magnetic microparticles (termed the strategy as cellular localization assisted by magnetic particle [CLAMP]) (Figure 7).¹¹¹ The temporally applied magnetic force could trap and retain probiotics in the gut. Particularly, it enabled the accumulation of probiotics at specific locations in the gastrointestinal tract and the stable colonization without the need for antibiotic treatment. Focused ultrasound can noninvasively deliver energy to specific tissues and thus can be used to control the behavior of the engineered bacteria *in vivo* by the spatiotemporal control of thermal stimulation. By engineering *E. coli* Nissle 1917 with gene circuits that could be activated by thermal stimulation,¹¹² the probiotics enabled the temporally controlled release of immune checkpoint inhibitors in tumors. The ultrasound-controllable probiotics promise to precisely treat enteritis and intestinal cancers with spatiotemporal targeting capacity by replacing the genetic therapeutic payload.

CONCLUSIONS AND PERSPECTIVES

An emerging tool palette allowing for spatiotemporally resolved analysis of gut microbiota provides new dimensions to investigate gut microbiota ecology, function, and disease associations. Traditional genetic labeling and FISH methods have facilitated functional investigations with the location of a specific bacteria. Advances in encoding strategies with FISH substantially increase the multiplexing capacity, driving us to unravel the microbial communities and the interactions that underlie microbial spatial arrangement in the gut. The newly emerging *in situ* sequencing and spatial transcriptomics further increase the coverage of taxon identification and, particularly, offer us genetic, mutational, and gene expression information of the gut microbiome, thus potentially accommodating us for the elucidation of genotype to function of the gut microbial communities. The integration of genetically and metabolically labeling strategies with advanced imaging techniques, such as NIR-II, PAI, and PET enables us to *in vivo* monitor bacteria in the gut and determine the dynamics of bacterial localization. These spatiotemporally resolved tools, providing the spatial pattern, growth, and metabolic activities of microbe in the gut, have illustrated the spatial reprogramming of the microbiota under different diets, antibiotics treatment, enteritis conditions, and guided microbiota transplantation therapy.

Despite the substantial progress, there are technical issues to be addressed to fully explore these tools to investigate the complex gut microbial communities. First, the spatial pattern dissection of gut microbial is still challenged by compactly located microbiota, which is estimated to be 10^{11} bacteria per gram of content.⁹ Overlap of bacterial cells blurs the cellular spatial network. Based on bacterial dilution, expansion microscopy, precisely preserving the bacterial location, can facilitate the addressing of the densely located microbiota. By coupling with expansion microscopy and imaging computational tools based on deep learning, *in situ* sequencing and spatial transcriptomics promise to sustain a delineation of microbial biogeography with microbiome scale and single-cell resolution. Second, tools for spatially resolving protein markers and metabolites with a multiplexing capacity to fulfill the investigation of the gut microbiota are insufficiently developed. With the substantial development of ion sources and mass analyzers, mass spectrometry imaging has enabled mapping proteins and metabolites with single-cell resolution in 3D samples¹¹³; however, its low sensitivity still hinders its application for profiling low-level molecules in the gut yet. Besides, the fixation protocols compatible with different molecular imaging methods remain to be developed. Third, the high dimensionality of the dataset from spatial genetic and molecular atlases magnifies the difficulty of data interpretation and feature mining. The generated data are scalable and multi-modal but inherently noisy and sparse, thus require specialized computational tools for data filtering, integration, and statistical modeling to precisely indicate the composition, colocalization, and communication of the gut microbiome. Computational tools developed for spatial transcriptomics and single-cell sequencing, such as machine learning or deep learning algorithms, are applicable to integrate different omics and imaging data. However, either by supervised or unsupervised learning, knowledge of microbial metabolic pathways and microbiome-host cross talk is still a prerequisite for deconvolving complex signatures and developing artificial intelligence strategies, which allows us to identify the components involved in biological processes and microbial functions in interactional multicellular systems in the gut.

A comprehensive dynamic analysis of the gut microbiota is hindered by the limited bacterial and molecular coverage. To broaden the measurable molecular

biomarkers, cell-based sensors, equipped with diverse sensory receptors or actuators for metabolites, metal ions, and antibiotics, can be integrated with ultrasound, photoacoustic, and magnetic resonance genetic reporters to enable the *in vivo* sensing in the gut by imaging.¹¹⁴ Another strategy to cover more bacterial and molecular markers in the gut is the expansion of metabolic probes from amino acids to oligosaccharides, nucleic acids, and other metabolic substrates. The high diversity of carbohydrates can improve the coverage of the markable bacteria and enhance the labeling specificity to identify bacterial subgroups. Metabolic RNA labeling allows the estimation of bacterial activity in the gut by estimating the transcriptional level. Exploration of other specific bacterial metabolic substrates serving as probes would facilitate the selective labeling of endogenous specific taxonomic microbiota. Besides, the development of metabolically labeling probes based on turn-on, long-wavelength, and bright fluorophores, such as silicon rhodamines,¹¹⁵ eliminating the wash process can be a feasible strategy for *in vivo* monitoring indigenous bacteria in the gut. Apart from broadening the sensing scope, attenuation of labeling signals along with cellular division and metabolism is also an issue to be addressed. Signal attenuation of probes or sensors can hardly sustain long-term monitoring of bacteria in the gut. The gap between the duration of stable labeling and bacterial rearrangement response to diet change or microbiota transplantation may result in missing key events in a spatial reprogramming process of gut microbiota.

Overall, the emergence of spatiotemporally resolved tools offers us an unprecedented capacity to examine subtle spatial factors and temporal changes in the gut microbiome and provides structural, functional, and mechanistic insights into microbial ecosystems. Further work to mine the relationship between the spatiotemporal features of the gut microbiota and clinical data will dramatically facilitate gut health maintenance, microbiota-based therapeutics, gut-stemmed diagnosis, and disease treatments.

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DECLARATION OF INTERESTS

The authors declare no competing interests.

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