Highly multiplexed spatial mapping of microbial communities

Mapping the complex biogeography of microbial communities in situ with high taxonomic and spatial resolution poses a major challenge because of the high density and rich diversity of species in environmental microbiomes and the limitations of optical imaging technology. Here we introduce high-phylogenetic-resolution microbiome mapping by fluorescence in situ hybridization (HiPR-FISH), a versatile technology that uses binary encoding, spectral imaging and decoding based on machine learning to create micrometre-scale maps of the locations and identities of hundreds of microbial species in complex communities. We show that 10-bit HiPR-FISH can distinguish between 1,023 isolates of Escherichia coli, each fluorescently labelled with a unique binary barcode. HiPR-FISH, in conjunction with custom algorithms for automated probe design and analysis of single-cell images, reveals the disruption of spatial networks in the mouse gut microbiome in response to treatment with antibiotics, and the longitudinal stability of spatial architectures in the human oral plaque microbiome. Combined with super-resolution imaging, HiPR-FISH shows the diverse strategies of ribosome organization that are exhibited by taxa in the human oral microbiome. HiPR-FISH provides a framework for analysing the spatial ecology of environmental microbial communities at single-cell resolution.

Microbial communities often exhibit rich taxonomic diversity and striking spatial organization. Although the taxonomic diversity of complex microbial communities can be readily analysed using metagenomic sequencing, assessing their spatial organization is very difficult. Fluorescence in situ hybridization (FISH) assays that target ribosomal RNA (rRNA) for taxonomic identification and visualization have been developed but are limited in their taxonomic resolution and multiplexity. These studies highlight the spatial organization of several abundant taxa, but are insufficient for unbiased analysis of microbial biogeography at the microbiome scale in the mammalian gastrointestinal tract, where hundreds to thousands of species coexist. Moreover, studies so far have largely been qualitative, and the few quantitative analyses that have taken place used coarse-grained approaches, which do not provide spatial information at the single-cell level.

Here we describe HiPR-FISH, a microbiome mapping technology that is capable of 1,000-fold multiplexity in the identification of taxa with a single round of imaging on a standard confocal microscope. HiPR-FISH achieves this high multiplexity through a binary barcoding scheme and machine-learning-based classification of the combined spectra of up to 10 fluorophores. For binary barcoding of species, HiPR-FISH implements a two-step hybridization scheme, with a first step that uses taxon-specific probes modified with DNA flanking sequences, and a second step with fluorescently labelled readout probes that target the flanking sequences. Unlike previous approaches that have used species-specific probes tagged with fluorophores, HiPR-FISH uses unmodified probes that can be synthesized with array technologies, leading to a higher throughput and lower cost. To apply HiPR-FISH to quantitative analyses of microbial communities at single-cell resolution, we developed a procedure for automated image segmentation. We used HiPR-FISH to study how antibiotics disrupt spatial networks in the gut microbiome, and to explore the temporal stability of microarchitectures in the oral microbiome. Finally, we combined HiPR-FISH with super-resolution imaging to reveal the diverse strategies for intracellular ribosome organization that are used by taxa in the oral microbiome.

Implementation of HiPR-FISH

For the practical implementation of HiPR-FISH, we used barcodes composed of up to 10 distinct fluorophores, leading to 1,023 unique combinations. For binary barcoding of species, HiPR-FISH implements a two-step hybridization scheme, with a first step that uses taxon-specific probes modified with DNA flanking sequences, and a second step with fluorescently labelled readout probes that target the flanking sequences. Unlike previous approaches that have used species-specific probes tagged with fluorophores, HiPR-FISH uses unmodified probes that can be synthesized with array technologies, leading to a higher throughput and lower cost. To apply HiPR-FISH to quantitative analyses of microbial communities at single-cell resolution, we developed a procedure for automated image segmentation. We used HiPR-FISH to study how antibiotics disrupt spatial networks in the gut microbiome, and to explore the temporal stability of microarchitectures in the oral microbiome. Finally, we combined HiPR-FISH with super-resolution imaging to reveal the diverse strategies for intracellular ribosome organization that are used by taxa in the oral microbiome.
classification for 99.7% of barcodes using reference spectra simulated with multivariate normal distributions, in which the mean and covariance were extracted from experimentally measured spectra. We used photon counting measurements to estimate the ribosomal density in *E. coli* cells and simulated the classification error with decreasing ribosomal density (Methods). We simulated over 7.16 million spectra and found that the classifier accurately predicted barcodes for cells with around $10^3$ ribosomes (96% of barcodes with less than 5% classification error rate for around 790 ribosomes per cell) (Extended Data Fig. 3).

**Proof of principle**

To test the robustness of HiPR-FISH, we characterized predefined mixtures of the 1,023 *E. coli* barcode isolates. We first created and imaged a mixture of all barcode strains at an equal concentration. We performed barcode decoding for a total of 65,534 cells (Fig. 1e) and determined that all barcodes were represented in the mixture, with a median fractional abundance of $8.9 \times 10^{-4}$ and full width at half maximum (FWHM) of $3.7 \times 10^{-4}$—close to what is expected for a multinomial distribution (median relative abundance of $9.8 \times 10^{-4}$ and FWHM of $3.1 \times 10^{-4}$). We next randomly divided the 1,023 aliquots into 8 groups, each comprising 127 or 128 barcodes, and mixed barcodes in the same group at varying abundance. We measured 35,000 to 40,000 single-cell spectra for each group and quantified the relative abundance of different barcodes in each mixture. We found close agreement between the expected and the measured abundance for all groups, with a median slope of 0.95 and an average $R^2$ value of 0.83 (Fig. 1f). Barcode misassignment was rare, with gross error rates (defined as the proportion of barcodes that do not belong in a group of barcodes) ranging from 2.5% to 6.6%.

To further demonstrate the principle of HiPR-FISH, we probed and imaged a set of 11 species of bacteria (Methods, Supplementary Tables 3, 4), including both Gram-positive (*n* = 4) and Gram-negative (*n* = 7) species. Probe sequences were designed using thermodynamic modelling and selected on the basis of stringent hybridization criteria (Methods, Extended Data Fig. 4). We generated three sets of probes (A, B and C), whereby each probe set comprised a common list of target sequences specific to the 11 species in the synthetic community but had different flanking encoding sequences. In particular, target sequences in sets A, B and C were encoded with the least complex, the most complex and a random selection of barcodes, respectively, in which barcodes composed of more true bits and therefore a greater number of fluorophores were considered more complex (Fig. 1g). To evaluate the specificity of each custom-designed species-specific probe, we recorded single-cell spectra for each probe set—species combination. To account for variable ribosomal density in cells from different taxa, we also developed a second classifier that incorporates a Förster resonance energy transfer (FRET) model to refine training spectra (Methods). This approach to barcode classification is easier to implement than a classifier that is based on the pure reference spectrum, because it requires only measurements of the emission spectra of the individual fluorophores as input for model training. We applied this classifier to determine which barcode out of the 1,023 candidates was most likely to correspond to each measured single-cell spectrum, and found strong agreement between the assigned and the expected barcodes for all barcode–species combinations (median error rate $1.4 \times 10^{-4}$) (Fig. 1g). In cases of spectral misclassification, the misclassified barcode was most often just one bit away from the correct barcode (Hamming distance 1) (Extended Data Fig. 5). Overall, we find that we can attain species-specific detection and flexible binary barcode encoding using HiPR-FISH.

**Image processing and single-cell segmentation**

To extract quantitative information with single-cell resolution from HiPR-FISH images, we developed local neighbourhood enhancement (LNE), an algorithm to define a seed for watershed segmentation (Fig. 2a). LNE classifies each pixel as part of a cell or the background using information contained in the local neighbourhood of the pixel.
Fig. 2 | Algorithm for single-cell segmentation. a, Key steps for image analysis. The contrast for denoised images is enhanced using LNE. Watershed seed masks are generated on the basis of the LNE image, and segmentation is performed using watershed. b, Example of a volumetric segmentation of a human oral biofilm. Different colours correspond to different cells. c, Example segmented and identified images of a section of a mouse colon after clindamycin treatment, with a few taxa highlighted in enlarged views (right). The segmentation and identification was repeated for 28 FOVs (ciprofloxacin-treated mice) and 30 FOVs (healthy control mice), with similar results. Scale bars, 25 μm. d, PCFs for Hespellia (orange) and Bacteroides (blue) show that Bacteroides cells are likely to form clusters at short ranges, whereas Hespellia cells exhibit a random spatial distribution. The distances are normalized with respect to the average semi-minor axis length \( \langle a_{\text{semi}} \rangle \) of each cell type. e, distance between cells. f, Relationship between bacterial density and distance to the mucosal barrier. The data show that Bacteroides cells are enriched near the mucosal boundary, whereas Hespellia cells are more evenly distributed away from the mucosal boundary. f, Tile scan of the edge of a faecal pellet from a ciprofloxacin-treated mouse, covering an area of approximately 266 μm × 546 μm. Scale bar, 50 μm. g, Volumetric rendering of a z-stack image from a ciprofloxacin-treated mouse, demonstrating the compatibility of HiPR-FISH with three-dimensional (3D) characterization of tissue samples.

Disruption of the mouse gut microbiome

The effect of antibiotic treatment on the spatial organization of the mucosa-associated gut microbiome has not been studied in detail. To fill this knowledge gap, we created HiPR-FISH maps of the mouse gut microbiome in the presence and absence of antibiotic treatment. We designed HiPR-FISH probe sets based on full-length 16S sequences that were generated using PacBio sequencing (Methods, Supplementary Tables 5, 6, Extended Data Fig. 4). We designed two sets of probes consisting of 115 and 264 probes, which target up to 47 genera, and used these probe sets to test the robustness of HiPR-FISH. We compared the fluorescence intensity measured for each barcode across multiple fields of view and tissue sections (Supplementary Table 7) and found a strong correlation in the total intensity measured across all barcodes, indicating that probe hybridization and imaging are reproducible (Methods, Extended Data Fig. 9). We next compared our HiPR-FISH imaging results with metagenomic sequencing of laser-capture-microdissected tissues obtained from formalin-fixed, paraffin-embedded (FFPE) sections from the same tissue block, and found agreement for the imaging and sequencing measurements (log10-transformed Pearson correlation 0.4, \( P = 0.007 \)) (Extended Data Fig. 9).

In the colon of a mouse that was treated with clindamycin, we detected cells from the genera of Bacteroides, Macellibacteroides and Longibaculum (Fig. 2c). We calculated the pair correlation function (PCF) for Bacteroides and Hespellia, two common genera in the gut microbiome (Fig. 2d). We observed a slow decay in the PCF for Bacteroides cells, indicating that Bacteroides tend to form clusters at short distances (around 3.3 μm). By contrast, the PCF of Hespellia cells was consistent with a random distribution. We next measured distances to the mucosal boundary and found that Bacteroides, but not Hespellia, are enriched near the boundary (Fig. 2e), in line with previous observations. We performed a large-area tile scan of the terminal area of a faecal pellet and found that faecal pellets are coated with a dense layer of microorganisms (Fig. 2f), even at locations that have no apparent contact with the host epithelial tissue. Finally, we generated a volumetric rendering of a z-stack image (Fig. 2g).

We next examined changes in species abundance and the number of physical contacts between any two taxa as a result of treatment with ciprofloxacin (Fig. 3a). We observed an increase in the ratio of Bacterioidetes/Firmicutes compared to control mice (Fig. 3b), consistent with previous studies. There was a small but significant
increase in Shannon diversity associated with ciprofloxacin treatment (independent t-test, two-tailed \( P = 0.002 \)) (Fig. 3c). The \( \beta \)-diversity of small patches of cells decreased as the patch size increased for both ciprofloxacin-treated and control mice (Fig. 3d). To examine variations in local composition, we calculated the Bray–Curtis dissimilarity between small patches of cells. The Bray–Curtis dissimilarity increased slightly with distance between the centre of patches but remained small even at a large distance (Fig. 3e). We observed similar trends for clindamycin-treated mice—except for the Shannon diversity, which was decreased in the antibiotic-treated group (independent t-test, two-tailed, \( P = 1.4 \times 10^{-6} \)) (Extended Data Fig. 9)—again suggesting that microstructures in the gut microbiome are conserved over large distances. Finally, we found that antibiotic treatment disrupted the spatial associations between several genera (Methods, Fig. 3f, Extended Data Fig. 9). The altered spatial association with the largest fold change occurred between Oscillibacter and Veillonella, both of which have been linked to altered inflammatory responses and metabolic activities in the host\(^{29,30}\).

### Structural stability of human plaque biofilms

The oral microbiome is one of the most diverse microbiomes that are present in humans, comprising more than 600 prevalent species\(^\text{24}\). We performed HiPR-FISH on plaque biofilms collected at 7 time points from a healthy donor over the course of 27 months (Fig. 4a, b). We first used a HiPR-FISH panel consisting of 233 probes that target 54 bacterial genera, and observed corn-cob-like structures composed of Streptococcus, consistent with previous observations (Fig. 4c). To further benchmark HiPR-FISH, we designed two additional sets of probes with different encoding sequences, barcode assignments and probe-selection criteria (Methods), consisting of 390 and 319 probes at the genus level that target 65 and 61 genera. In experiments with all three sets of probes, we observed clusters of cells from the Lautropia genus (which are known for their pleomorphic cocoid morphology) and we found that the cell sizes and morphologies of Lautropia were consistent across all three sets of probes (Extended Data Fig. 10).

Microbial communities in oral biofilms appeared to be more spatially structured than those in the mouse gut (Fig. 4b). We observed recurrent microarchitectures with prominent morphology—such as clusters of Lautropia cells (Fig. 4d)—across longitudinal samples, suggesting that the spatial structure of the oral microbiome remains stable over time\(^\text{24,25}\). To investigate this in more detail, we measured the Shannon diversity and the Bray–Curtis dissimilarity between patches of cells as a function of time, and found that both were longitudinally stable (Fig. 4e, f) \( (P = 0.28) \) (one-way analysis of variance). The Bray–Curtis dissimilarity of the oral microbiome was higher than that of the mouse gut microbiome, supporting our visual evaluation that the oral microbiome is more spatially organized than the gut microbiome. The \( \beta \)-diversity, as a function of the size of the local neighbourhood, followed similar trends (Fig. 4g). To examine the longitudinal stability of the spatial interactions between different microbial taxa, we calculated an aggregated spatial adjacency matrix for each time point and determined the Frobenius matrix distance as a measure of network similarity. The spatial association networks measured at all time points were more similar to each other than to a random network with the same taxa abundances, again indicating that oral microbiome architectures are stable over time (Fig. 4h).

We next considered microbial consortia and species of interest that have not previously been visualized in their native context. We observed
Fig. 4 | Biogeography of human oral biofilms. a, Experimental workflow of the oral microbiome experiment using 7-bit encoding probe sets. b, Example FOV of a human oral microbiome. Scale bars, 25 μm. c, Corn-cob-like structures formed by cells from the Streptococcus genus observed in human oral plaque biofilms (top, denoised; bottom, identified). Scale bars, 5 μm. Images in b, c are examples selected from 12 FOVs. d, Example FOV of Lautropia observed at three time points (0, 9 and 15 months), showing pleomorphic cocoid cluster morphology. Scale bars, 5 μm. Examples selected from 88 FOVs. e, Shannon diversity (measured using HiPR-FISH) remains stable in human oral biofilms over 27 months. f, Bray–Curtis dissimilarity between patches with the same number of cells increases with intra-patch distance and remains higher than those in the mouse gut at long length scales. g, β-diversity of patches in the same cells to measure the intracellular distributions of ribosomes. For Lautropia, Airyscan imaging revealed excluded regions within each cell with low ribosomal density, as well as apparent subcellular

multiple occurrences of a consortium of *Pseudopropionibacterium, Cardiobacterium* and *Schwartzia* (Fig. 4i, Extended Data Fig. 10). These three taxa are anaerobes, suggesting that the association is probably driven by metabolic interaction rather than by oxygen requirements. *Cardiobacterium* is commonly found in the mouth and upper respiratory tract, and accounts for 5–10% of cases of infective endocarditis. The observation of an association of *Cardiobacterium* with *Pseudopropionibacterium* and *Schwartzia* in a healthy oral microbiome may provide an area for investigation with regard to the pathogenesis of *Cardiobacterium*-associated endocarditis. In addition, we detected *Phocaeicola* cells embedded among clusters of *Rothia* cells (Fig. 4j), suggesting that these two genera interact metabolically. We also observed cells from *Lachnoanaerobaculum* and *Prevotellamassilia*—two genera that were not targeted in previous imaging experiments of the human oral microbiome, probably because of their low prevalence. The observed cell morphology for *Lachnoanaerobaculum* cells was consistent with that reported previously in culture-based studies (cell lengths 5–20 μm).

We combined HiPR-FISH with super-resolution imaging to connect phylogenetic information with ribosome organization (Fig. 4k). We used HiPR-FISH to establish cellular identity, and Airyscan imaging on the same cells to measure the intracellular distributions of ribosomes. For *Lautropia*, Airyscan imaging revealed excluded regions within each cell with low ribosomal density, as well as apparent subcellular
compartments within each cell. For *Ottowia*, ribosomes were mostly found along the periphery and at the cell poles. Ribosomes were uniformly distributed in *Cardiobacterium*, and in *Neisseria* they tended to be organized at the centre of the cell. *Leptotrichia* have a unique, rod–chain morphology that is easily distinguishable using phase contrast imaging. We observed four *Leptotrichia* cells in a chain formation, with variable ribosomal density within each cell. *Olsenella* has been shown to occur individually, in pairs or in serpentine chains in culture. We observed two instances of serpentine chains of *Olsenella* and super-resolution imaging revealed that ribosomes were distributed in a non-uniform manner within each cell. Finally, we observed a long chain of three cells of *Lachnoanaerobaculum* (total length 97.5 μm), with a variable ribosome distribution along the length of each cell and the occurrence of puncta along the cells. Together, these observations highlight the diverse strategies of intra cellular ribosomal organization that are exhibited by different taxa in the oral microbiome.

**Discussion**

In this study we have described HiPR-FISH, a versatile tool for mapping microbiomes. Compared to existing approaches, HiPR-FISH provides an improvement of more than tenfold in multiplexity. HiPR-FISH uses a two-step hybridization scheme that was previously exploited to spatially map mRNA molecules in tissues. This strategy, in conjunction with the high abundance and relatively uniform distribution of 16S rRNA molecules in microbial cells, enables the labelling of bacterial cells with combinations of up to ten fluorophores. HiPR-FISH requires only a single round of imaging on a commercial confocal microscope. A single field of view can be imaged in just five minutes—much faster than FISH procedures that rely on successive rounds of hybridization and imaging. Consequently, HiPR-FISH is compatible with projects that require fast acquisition of data; it could be used, for example, in the diagnosis of infectious diseases. For other applications, implementations of HiPR-FISH with multiple rounds of hybridization and imaging could be considered to further increase multiplexity or improve accuracy through the incorporation of error-correction strategies.

We expect HiPR-FISH to have broad applicability in human health. HiPR-FISH could open up avenues for investigations of complex microbial populations in the gut, in the oral cavity or on implanted devices—all of which are known to contain biofilms. HiPR-FISH could also be applied to study gut-related disorders such as inflammatory bowel diseases, in which signalling between the microbiota and the epithelial tissue of the gut has a role in reducing barrier integrity. Furthermore, HiPR-FISH will enable analyses of the role of the microbiota in the initiation and progression of tumours that form at epithelial barrier surfaces (colorectal cancers, for example). Finally, the quantitative single-cell measurements that HiPR-FISH enables will be a useful resource for testing soft matter theories of the principles that govern the assembly of microbial communities.

**Online content**

Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at https://doi.org/10.1038/s41586-020-2983-4.

1. Donaldson, G. P., Lee, S. M. & Mazmanian, S. K. Gut biogeography of the bacterial microbiota. Nat. Rev. Microbiol. 14, 20–32 (2016).
2. The Human Microbiome Project Consortium. Structure, function and diversity of the healthy human microbiome. Nature 486, 207–214 (2012).
3. Tropini, C., Earle, K. A., Huang, K. C. & Sonnenburg, J. L. The gut microbiome: connecting spatial organization to function. Cell Host Microbe 21, 433–442 (2017).
4. Mark Welch, J. L., Rossetti, B. J., Rieken, C. W., Dewhurst, F. E. & Borisy, G. G. Biogeography of a human oral microbiome at the micron scale. Proc. Natl Acad. Sci. USA 113, E701–E800 (2016).
5. Valm, A. M. et al. Systems-level analysis of microbial community organization through combinatorial labeling and spectral imaging. Proc. Natl Acad. Sci. USA 108, 4152–4157 (2011).
6. Valm, A. M., Mark Welch, J. L. & Borisy, G. G. CLASI-FISH: principles of combinatorial labeling and spectral imaging. Syst. Appl. Microbiol. 35, 496–502 (2012).
7. De Weert, R. & Van De Wele, T. Micromanagement in the gut. Microenvironmental factors govern colon mucosal biofilm structure and functionality. npj Biofilms Microbiomes 1, 15026 (2015).
8. Thais, C. A. et al. Microbiota-durnal rhythmicity programs host transcriptional oscillations. Cell 167, 1485–1510 (2016).
9. Valm, A. M., Oldenbourg, R. & Borisy, G. G. Multiplexed spectral imaging of 120 different fluorescent labels. PLoS One 11, e0158495 (2016).
10. Mark Welch, J. L., Hasegawa, Y., McNulty, N. P., Gordon, J. I. & Borisy, G. G. Spatial organization of a model 15-member human gut microbiota established in gnotobiotic mice. Proc. Natl Acad. Sci. USA 114, E9105–E9114 (2017).
11. Earle, K. A. et al. Quantitative imaging of gut microbiota spatial organization. Cell Host Microbe 18, 478–488 (2015).
12. Dagher, M., Kleinman, M., Ng, A. & Juncker, D. Ensemble multiplexed FRET model enables barcoding at extreme FRET levels. Nat. Nanotechnol. 13, 925–932 (2018).
13. Otsu, N. A threshold selection method from gray-level histograms. IEEE Trans. Syst. Man Cybern. 9, 62–66 (1979).
14. Sauvola, J. & Pietikäinen, M. Adaptive document image binarization. Pattern Recogn. 33, 225–236 (2000).
15. Francino, M. P. Antibiotics and the human gut microbiome: dysbioses and accumulation of resistance. Front. Microbiol. 6, 543 (2015).
16. Samad, T., Co, J. Y., Witten, I. & Robberecht, K. Mucus and mucin environments reduce the efficacy of polymyxin and fluoroquinolone antibiotics against *Pseudomonas aeruginosa*. ACS Biomater. Sci. Eng. 5, 1189–1194 (2019).
17. Antinopoulos, D. A. et al. Reproducible community dynamics of the gastrointestinal microbiota following antibiotic perturbation. Infect. Immun. 77, 2367–2375 (2009).
18. Panda, S. et al. Short-term effect of antibiotics on human gut microbiota. PLoS ONE 9, e90478 (2014).
19. Li, J. et al. Probiotics modulated gut microbiota suppresses hepatocellular carcinoma growth in mice. Nat. Protoc. Sci. 11, E3160–E3115 (2015).
20. Scheiman, J. et al. Meta-omics analysis of elite athletes identifies a performance-enhancing microbe that functions via lactate metabolism. Nat. Med. 25, 1194–1198 (2019).
21. Dewhirst, F. E. et al. The human oral microbiome. J. Bacteriol. 192, 5002–5017 (2010).
22. Rasiah, I. A., Wang, L., Anderson, S. A. & Sissons, C. H. Variation in bacterial DDGE patterns from human saliva: over time, between individuals and in corresponding dental plaque microcosms. Arch. Oral Biol. 50, 779–787 (2005).
23. Costello, E. K. et al. Bacterial community variation in human body habitats across space and time. Science 326, 1694–1697 (2009).
24. Li, K., Bhina, M. & Methé, B. A. Analyses of the stability and core taxonomic memberships of the human microbiome. PLoS ONE 8, e69339 (2013).
25. Zhou, Y. et al. Biogeography of the ecosystems of the healthy human body. Genome Biol. 14, R1 (2013).
26. Baddour, L. M. et al. Infective endocarditis in adults: diagnosis, antimicrobial therapy, and management of complications: a scientific statement for healthcare professionals from the American Heart Association. Circulation 132, 1435–1486 (2015).
27. Hedberg, M. E. et al. Lachnoanaerobaculum gen. nov., a new genus in the Lachnoopsiracea: characterization of Lachnoanaerobaculum umaeense gen. nov. sp. nov. isolated from the human small intestine, and Lachnoanaerobaculum orale sp. nov. isolated from saliva, and reclassification of Eubacterium saburreum (Prevot 1966) Holdeman and Moore 1970 as Lachnoanaerobaculum saburreum comb. nov. Int. J. Syst. Evol. Microbiol. 62, 2865–2890 (2012).
28. Stracz, M. et al. Live-cell superresolution microscopy reveals the organization of RNA polymerase in the bacterial nucleoid. Proc. Natl Acad. Sci. USA 112, E4390–E4399 (2015).
29. Sanamrad, A. et al. Single-particle tracking reveals that free ribosomal subunits are not excluded from the Escherichia coli nucleoid. Proc. Natl Acad. Sci. USA 111, 11413–11418 (2014).
30. Smid, M. C., Dotterse-Katz, S. K., Plongla, R. & Bogges, K. A. Leptotrichia buccalis: a novel cause of chorioamnionitis. Infect. Dis. Rep. 7, 5801 (2015).
31. Hou, H., Chen, Z., Tian, L. & Sun, Z. Leptotrichia trevisanii bacteria in a woman with systemic lupus erythematosus receiving high-dose chemotherapy. BMC Infect. Dis. 18, 661 (2018).
32. Kraatz, M., Wallace, R. J. & Svensson, L. Olsenella umbonata sp. nov., a microaerotolerant anaerobic lactic acid bacterium from the sheep rumen and pig jejunum, and emended descriptions of Olsenella, Olsenella ulii and Olsenella profusa. Int. J. Syst. Evol. Microbiol. 61, 795–803 (2011).
33. Moffitt, J. R. & Zhuang, X. RNA imaging with multiplexed error-resistant fluorescence in situ hybridization (MEFISH). Method. Cell Mol. Biol. 572, 1–49 (2016).
34. Chen, K. H., Boettiger, A. N., Moffitt, J. R., Wang, S. & Zhuang, X. Spatially resolved, highly multiplexed RNA profiling in single cells. Science 348, aaa0909 (2015).

© The Author(s), under exclusive licence to Springer Nature Limited 2020
**Methods**

**Data reporting**
No statistical methods were used to predetermine sample size. Unless otherwise stated, the experiments were not randomized. The investigators were not blinded to allocation during experiments and outcome assessment.

**Sample collection**
Samples of human plaque biofilm were obtained from a volunteer. The study was approved by the Cornell Institutional Review Board (protocol 1910009101). The volunteer provided informed consent. Animal procedures were approved by the Cornell University Institutional Animal Care and Use Committee. Specific-pathogen-free C57BL/6j mice were acquired from the Jackson Laboratory and maintained under barrier conditions in ventilated and autoclaved cages.

**Bacterial cell culture**
All cultured cells were inoculated from frozen stock stored at −80 °C into 4 ml of appropriate growth medium. A loopful of the liquid culture was streaked on an appropriate agar plate. A single colony from the agar plate was inoculated into an appropriate liquid medium and grown to roughly mid-exponential phase. Cells were grown using growth media and growth times listed in Supplementary Table 3.

**Fluorescent readout probes**
Fluorophore conjugated readout probes listed in Supplementary Table 1 were purchased from Integrated DNA Technologies and Bioisotopes.

**PacBio sequencing**
Metagenomic DNA from plaque samples was extracted using the QIAamp DNA Mini Kit according to the manufacturer’s protocol. Ribosomal DNA was amplified from the extracted metagenomic DNA using the universal primers for the 16S rRNA listed in Supplementary Table 5, cleaned with the MinElute PCR Purification Kit according to the manufacturer’s protocol, and sequenced on a PacBio RSII or Sequel at the Duke Center for Genomic and Computational Biology. The PacBio sequence data were processed using rDNATools (https://github.com/PacificBiosciences/rDNATools), with a threshold of 99% accuracy. The output FASTA sequences of the rDNATools pipeline were used for probe design.

**Probe design and synthesis**
The 16S rRNA sequences for cultured bacteria were generated using Sanger sequencing, whereas those for environmental samples were generated with PacBio circular consensus sequencing (SMRT-CCS)\(^{35}\). Probes were designed using a custom pipeline in Python, using tools and packages listed in Supplementary Table 6. In brief, the 16S sequences were grouped by taxon and sequence similarity. A consensus sequence was generated for each taxon using usearch. FISH probes for each consensus sequence were design using primer3. Alternatively, probes were designed from each individual PacBio full-length sequence using primer3, and the unique set of probes was selected to proceed to the next stage of probe evaluation. The probes were then blasted against the database containing all 16S sequences from the community using blastn. A maximum continuous homology (MCH) score was calculated for each blast hit. The MCH score was defined as the maximum number of continuous bases that were shared between the query and the target sequence. Only blast hits above a threshold MCH score were considered significant and used for further analysis. The blast on-target rate, taxonomic coverage and maximum off-target GC count were calculated for each significant blast hit. Blast on-target rate was defined as the ratio between the number of correct blast hits and the total number of significant blast hits. Taxonomic coverage was defined as the ratio between the number of significant blast hits within the target species and the total number of sequences for the target species. The off-target GC count was defined as the number of G or Cs in any homology sequences between a probe and an off-target 16S sequence. Any probe with a blast on-target rate of less than 0.99 or maximum off-target GC count greater than 7 was excluded from the probe set to avoid mis-hybridization. For each taxon, the probe with the highest taxonomic coverage was then selected. Each probe was subsequently concatenated on both ends with 3-bp spacers, readout sequences and primer sequences. The 3-bp-long spacers for each probe were taken from the 3 bases upstream and downstream from the target region on the 16S sequence of the probe. As these blocking sequences were exactly the same as the target molecules, the blocking regions would not form hydrogen bonds with their corresponding nucleotides on the target molecule, minimizing any steric hindrance that the readout probes might otherwise encounter. For each probe, all blast hits were examined for potential mis-hybridization sites. For each potential mis-hybridization site, a blocking probe complementary to the off-target sequence was included\(^{36}\). Blocking probes were not conjugated to any readout sequences, and therefore did not contribute any fluorescent signals. The blocking probes were either purchased separately from Integrated DNA Technologies (www.idt.com) in plate format, or included in the complex oligo pool of encoding probes and purchased from CustomArrays (www.customarrays.com). Helper probes were included to improve ribosomal accessibility. All probes designed from primer3 upstream and downstream of the region targeted by the encoding probes were evaluated. The upstream and downstream regions were divided into five blocks, each spanning 20 bp of nucleotides. At most one probe from each region was selected. This limit was chosen to achieve broad coverage upstream and downstream of the encoding region on the target 16S molecule without exceeding the sequence number limit of commercially available complex oligo pools. The complex oligo pool was synthesized following the protocol as previously described\(^{35}\). In brief, the complex oligo pool was first PCR-amplified to incorporate T7 promoters. The PCR products were subsequently reverse-transcribed for 1h at 56 °C. The DNA–RNA hybrid products were subjected to alkaline hydrolysis for 15 min at 95 °C. The single-stranded probes were purified using Zymo spin columns. Finally, the purified probes were ethanol-precipitated, washed once in 70% ethanol and resuspended to 80 μl.

**HiPR-FISH on synthetic E. coli communities**
*Escherichia coli* cells were grown overnight on an LB agar plate. A single colony from the plate was inoculated into 800 ml LB broth supplemented with 40 μl of 1 M potassium phosphate buffer and 40 μl of 20% glucose solution. Cells were grown for 7 h to an optical density of 1.1. Cultured cells were fixed for 1.5 h by the addition of 800 μl of 2% freshly made formaldehyde. Fixed cells were aliquoted into 50-ml tubes, concentrated by centrifugation at 4,000 rpm for 15 min, resuspended in 1× PBS (1 ml per tube) and pooled together into two 50-ml tubes (around 16 ml of *E. coli* suspension each). Cell suspensions were washed 3 times in 1× PBS (50 ml per wash per tube), suspended in 50% ethanol and stored at −20 °C until use. Before the encoding hybridization experiment, cell suspensions were treated with lysozyme solution (10 mg/ml in 10 mM Tris-HCl, pH 8) for 30 min at 37 °C, washed once with 1× PBS and resuspended in 50% ethanol. Every 9.9 ml of encoding hybridization buffer includes 4 ml of cell suspension resuspended in 5.8 ml of ultrapure water, 1 ml of 20% SSC, 1 ml of Denhardt’s solution, 2 ml of ethylene carbonate and 100 μl of 1% SDS. Encoding hybridization buffers were aliquoted into 1.5-ml Eppendorf tubes at 99 μl per tube. Finally, 1 μl of the encoding probe for a barcode was added to each tube. The encoding hybridization suspension was briefly vortexed,
incubated at 46 °C for 4 h, washed once in washing buffer (215 mM NaCl, 20 mM Tris-HCl, 20 mM EDTA) for 15 min at 48 °C, washed twice in PBS and resuspended in 100 μl of 50% ethanol. The 1,023-plex synthetic community of barcoded E. coli was generated by mixing together 1 μl of each barcoded E. coli stock. The titrated community was generated first by dividing the 1,023 barcodes into 8 groups (7 communities with 128 barcodes each, and 1 community with 127 barcodes). Each 128-plex or 127-plex titration community was generated by mixing together a variable amount of barcoded E. coli stock. All synthetic community mixes were resuspended in 100 μl of 50% ethanol.

HiPR-FISH on synthetic multi-species microbial communities
Cultured cells were fixed by adding an equal volume of 2% freshly made formaldehyde to the liquid culture for 1.5 h. Fixed cells were washed 3 times in 1× PBS, permeated in absolute ethanol for 15 min, suspended in 50% ethanol and stored at −20 °C until use. For each species control experiment, 1 μl of 1 to 10 diluted pure culture was deposited onto an UltraStick slide and air dried. Cell walls were permeated by deposition of 20 μl of 10 mg/ml lysozyme suspended in 10 mM Tris-HCl onto the slide and incubation at 37 °C for 3 h. After lysozyme digestion, the slides were washed in 1× PBS for 15 min, dipped in pure ethanol, briefly rinsed with pure ethanol to remove any residual PBS and air dried. The encoding hybridizations were performed in a 9 × 9-mm Frame-Seal hybridization chamber with 1 μl encoding hybridization buffers per slide at 46 °C for 6 h. The slides were then washed in the washing buffer at 48 °C for 15 min, dipped in room temperature pure ethanol, rinsed with pure ethanol and air dried. Lysozyme digestion, encoding, and readout hybridizations were carried out as described in ‘HiPR-FISH on synthetic multi-species microbial communities’.

HiPR-FISH on human oral biofilm samples
Subgingival plaque was collected using a stainless-steel dental pick, gently deposited into 1 ml of 50% ethanol and stored at 4 °C until use. For each human plaque experiment, 20 μl of plaque material was deposited onto an UltraStick slide and air dried. The slides were then fixed in 2% freshly made formaldehyde for 1.5 h or overnight, washed in 1× PBS for 15 min, dipped in pure ethanol, rinsed with pure ethanol and air dried. Lysozyme digestion, encoding, and readout hybridizations were carried out as described in ‘HiPR-FISH on synthetic multi-species microbial communities’.

HiPR-FISH on mouse tissues
Mice received the antibiotics at six weeks of age. Mice in the treatment groups received one dose of ciprofloxacin (10 mg/kg) via oral gavage or clindamycin (10 mg/kg) via intraperitoneal injection, and the remaining sham-antibiotic groups received acidified water or PBS in a 200 μl volume. Mice were euthanized with carbon dioxide 7 days after antibiotic treatment for tissue collection. The entire digestive tracts posterior to the stomach were fixed in Carnoy’s solution (60% ethanol, 30% chloroform and 10% glacial acetic acid) for 48 h at room temperature. Fixed tissues were rinsed three times in ethanol and stored in 70% ethanol at −20 °C until paraffin embedding and sectioning. Tissues were embedded in paraffin and sectioned to 5-μm thickness. For deparaffinization, tissue sections on glass slides were incubated at 60 °C for 10 min, washed once in xylene substitute for 10 min, then in xylene substitute at room temperature for 10 min, once in ethanol at room temperature for 5 min, and air dried. To reduce autofluorescence, deparaffinized slides were washed with 1% sodium borohydride in 1× PBS on ice for 30 min, with a buffer change every 10 min, followed by three washes in 1× PBS on ice for 5 min each. Slides were briefly dipped in ethanol and allowed to air dry. Lysozyme digestion, encoding and readout hybridizations were carried out as described in ‘HiPR-FISH on synthetic multi-species microbial communities’.

Laser-capture microdissection and metagenomic sequencing on mouse gut tissue
FFPE tissue blocks were sectioned using the standard protocol, with blade changes between each tissue block to minimize cross contamination. Zeiss laser-capture microdissection (LCM) membrane slides were pretreated under ultraviolet light for 30 min. Sectioned tissues were then placed on sterilized LCM membrane slides and stored at room temperature until LCM. Biofilm tissue sections from the FFPE tissues were cut and catapulted into capture tubes. For each tissue section, multiple regions were cut and collected to ensure adequate starting material for library preparation. DNA was extracted using a Qigen DNA FFPE Tissue Kit following the manufacturer’s protocol. Extracted DNA was repaired using NEBNext DNA Repair Mix following the manufacturer’s protocol. Sequencing libraries were prepared using the NEBNext Ultra DNA Library Prep Kit following the manufacturer’s protocol. Prepared libraries were sequenced on an Illumina NextSeq run. Adaptor sequences were trimmed using Trim Galore with default parameters. Low complexity reads were removed using prinses-q-lite with parameters -lc_method entropy -lc_threshold 70.

Human and mouse host contaminant reads were removed sequentially using bbmap with parameters minid = 0.95 maxindel = 3 bw = 0.16 bw = 12 quickmatch fast minhits = 2. Contigs were assembled using metaspade with default parameters. Reads were mapped to assembled contigs using bwamem. Reads with mapping quality below 30 were removed from further analysis. Contigs were blasted against the NCBI nt database using blastn. The length of the corresponding genome for each contig blast result was retrieved using epost, summary and xtract. Genomic equivalent abundance was estimated as read counts mapped to each contig normalized by the genome length corresponding to that contig.

Spectral imaging
Spectral images were recorded on an inverted Zeiss 880 confocal microscope equipped with a 32-anode spectral detector, a Plan-APOCHROMAT 63×/1.40 oil objective and excitation lasers at 405 nm, 488 nm, 514 nm, 561 nm and 633 nm, using acquisition settings listed in Supplementary Table 7. The microscope is controlled using ZEN v.2.3.

Flat-field correction
One microlitre of each readout probe was added to 90 μl of Prolong Glass embedding medium. The solution was vortexed and briefly centrifuged. Finally, 15 μl of the mixture was deposited onto an UltraStick slide, and a #1.5 coverslip was gently placed on top of the embedding medium. The flat-field correction slide cured in the dark overnight and was imaged using the acquisition settings listed in Supplementary Table 5. Two FOVs of the flat-field correction slide were averaged to generate the flat-field correction image.

Photon counting and simulation of barcode prediction error rate
Escherichia coli cells were imaged on an inverted Zeiss 880 confocal microscope in photon counting mode. Laser power and pixel dwell time were reduced until no photon bunching occurred to enable accurate photon counting. Images were segmented, and photon counts per pixel were calculated for each cell. To simulate the effects of ribosomal density, the number of photons that would be collected in each spectral bin was multiplied by a dilution factor, and imaging noise was simulated using Poisson statistics. A total of 1,000 spectra per barcode per dilution factor were simulated, resulting in 7.16 million spectra in total.

Estimation of ribosomal density
Ribosomal density was conservatively estimated by assuming that at each voxel, each photon emission event corresponds to an individual ribosome. The total number of ribosomes for each cell was then estimated to be \( V_{cell} \times N_{photon} \), in which \( V_{cell} \) is the volume of a cell, \( N_{photon} \) is the number of photons, and \( V_{cell} \) is the volume of the cell, \( N_{photon} \) is the number of photons.
Simulation, measurement and classification of reference spectra

The reference spectrum for each barcode was measured using E. coli cells encoded with the corresponding barcode. For each barcode, around 300–500 single-cell spectra in a single FOV were recorded. For each barcode, the mean and covariance of the spectra were computed and used to simulate 1,000 new spectra. Linear ramps were applied to the emission spectra to simulate the effect of FRET. These spectra were added to the database of reference spectra. All simulated spectra were used to train a generalized classification scheme using a combination of uniform manifold approximation and projection (UMAP) and a support vector machine (SVM) (Extended Data Fig. 2). Spectra were first projected onto three dimensions using UMAP with a custom excitation-channel-wise cosine distance metric. An SVM trained on the three-dimensional representation of the reference spectra was then used for de novo barcode prediction. For each cell, the emission spectrum $f_{	ext{em}}$, $i \in [0, n_e]$, was measured, in which $n_e = 4$ for 7-bit experiments and $n_e = 5$ for 10-bit experiments. In the first stage, SVMs were trained to ascertain whether there was fluorescence signal in a given single-cell spectrum $f_{	ext{em}}$ acquired using each of the lasers, which was denoted as channel signatures $S_i, i \in [0, n_e]$. The channel signatures were appended to the spectra as additional features to be used in UMAP projection. In the UMAP projection, a custom metric was defined using excitation-laser-dependent cosine distance (equation (1)):

$$d(i, j) = \begin{cases} 1, & \sum_{k=0}^{n_e-1} S_i[k] - S_j[k] > 0 \frac{1}{n_e} \sum_{k=0}^{n_e-1} 1 - \frac{f_{	ext{em}}[k]}{f_{	ext{em}}[j] - f_{	ext{em}}[j-1]} \sum_{k=0}^{n_e-1} \frac{|S_i[k] - S_j[k]|}{n_e} > 0 \end{cases}$$

Given two spectra, the channel signatures were compared first. Spectra with different channel signatures were assigned the maximum distance of 1. For spectra with the same channel signature, $n_e$ cosine distances were calculated, each corresponding to a cosine distance between spectra excited by the same laser. The final distance was calculated to be the average of all channel-specific cosine distances. For synthetic communities and environmental microbiomes, reference spectra were simulated using only the spectra of individual fluorophores, taking into account FRET. For each fluorophore pair, the Förster distance was calculated using the emission spectra of the donor fluorophore and excitation spectra of the receptor fluorophore (equation (2)). In equation (2), $R_0$ is the Förster distance, $\kappa^2$ is the dipole orientation factor, $Q_0$ is the donor fluorescence quantum yield, $n$ is the index of refraction, $f_{\text{em}}$ is the donor emission spectrum, $c$ is the wavelength-dependent receptor extinction coefficient and $\lambda$ is the wavelength:

$$R_0 = \frac{2.07}{128n^2} \kappa^2 Q_0 f_{\text{em}}(\lambda) c(\lambda) d\lambda.$$  

To simulate FRET effects between multiple fluorophores, an advanced FRET model was adapted to take into account FRET cascade, limited excitation effects and excitation-dependent fluorophore quenching. For each donor–acceptor pair, the dimensionless FRET number $\omega_{\text{FRET}}$ for a given physical distance $d$ between the donor and the receptor was calculated using equation (3). The FRET numbers were used to calculate the ensemble FRET efficiency $\xi$ for a given donor in the presence of multiple acceptors with equation (4), in which $l$ is the gamma function.

$$\omega_{\text{FRET}}(d) = \left( \frac{R_{\text{FRET}}}{d} \right)^2$$

$$\xi = 1 - \sum_{j=0}^{\infty} \left[ F \left( \frac{2}{3} \omega_{\text{FRET}} \right) \left( \frac{1}{3} \right)^j \right]^{\frac{l(y/3 + 1)}{jl}}$$

$$c_i^F(\lambda) = c_i^\text{max} f_i(\lambda) / f_i^\text{max}$$

$$c_i^F(\lambda_i^\text{max}) = c_i^\text{max} f_i(\lambda_i^\text{max}) / f_i^\text{max}$$

The molar extinction coefficient for each combination of fluorophore-laser pairs (equation (5)) as well as donor–acceptor pairs (equation (6)) was then calculated. These molar extinction coefficient matrices were used to calculate the photon absorption probabilities under direct excitation (equation (7)) and FRET transfer (equation (8)), in which $y_j$ is the labelling density for the $j$th fluorophore. The ensemble fluorescence of a fluorophore was calculated using equation (9), in which $F_j^c$ is the FRET-sensitized fluorescence calculated using equations (10) and (11) and $q_j$ is a random number in the interval $[Q_j^c, 1]$.

Cultured cell imaging

For each FOV, spectral images were collected at five excitation wavelengths (Supplementary Tables 2 and 4) and concatenated to form 95-channel images. The pixel size was 70 nm $\times$ 70 nm to ensure sampling at ≥Nyquist frequency. For image acquisitions.

Biofilm imaging

Biofilms were imaged using the same spectral setting as described above (‘Cultured cell imaging’) except for the laser power (Supplementary Table 7). All laser powers were changed by a common factor as necessary to compensate for the overall lower intensity of environmental microbiome samples. For biofilm experiments, only the 488 nm, 514 nm, 561 nm and 633 nm lasers were used. The pixel size was 70 nm $\times$ 70 nm to ensure sampling at ≥Nyquist frequency. For z-stack images, the confocal pinhole was set to 1 Airy unit for all image acquisitions.
Image processing for cultured cells
Images acquired with each excitation laser were concatenated, registered and denoised using non-local means or a convolution neural network. Denoised images were segmented using the watershed algorithm. Objects smaller than 60 pixels or objects with a maximum spectral intensity of less than 75% of the mode of the maximum spectral intensity across all cells were removed and excluded from further analysis. For each cell, an average spectrum was calculated and assigned to the corresponding barcode using the spectra classification scheme described above.

Image processing for biofilm samples
Biofilm images were acquired with each excitation laser. For volumetric images, multiple volumes of the same FOV were acquired with short pixel dwell time at low signal-to-noise ratio to reduce stage-drift induced motion blur. Raw volumes were computationally aligned to generate an average volume with minimal stage drift artefacts and high signal-to-noise ratio. For each voxel in the aligned volume, the line profile was extracted in multiple directions passing through the voxel under consideration. The structuring elements for the line profiles were parameterized by the azimuthal angle $\theta$ and polar angle $\phi$. For two-dimensional (2D) images, the azimuthal angle was set to zero. Each line profile was rescaled to the range $[0,1]$, and the quartile coefficient of variation was calculated for each voxel. To produce the preprocessed image, the neighbour profile image was voxel-wise multiplied with the $(1 - \text{quartile coefficient of variation})$. To distinguish signal pixels from background voxels in the pre-processed image, $k$-means clustering with $k = 2$ was used. A binary opening function was applied to the image to remove any residual connections between neighbouring objects that have a small number of connecting voxels. Objects that were less than 10 pixels in size were excluded for further analysis to remove sparsely segmented objects in the background, and binary filling functions were used to fill in any holes in the segmented objects. Finally, the objects in the resulting image were labelled and served as the seed image for the watershed algorithm. To generate a mask image for watershed, the natural log of the raw volume averaged along the spectral axis was computed and $k$-means clustering with $k = 2$ was implemented to distinguish cells from the background. The intensity image for the watershed algorithm was simply the enhanced image. Finally, watershed segmentation was implemented using the intensity image, seed image and the mask image generated above. Alternatively, 3D segmentations can be generated by concatenating 2D segmentations of each z-slice. For 3D volumes that were acquired with high signal-to-noise ratio (larger stage drift during acquisition), $k$-means clustering with $k = 3$ was used to distinguish signal pixels from background pixels in the pre-processed image.

Spatial-spectral detection of under-segmented objects
The spatial variation of spectral properties for objects consisting of two or more spectral barcodes was used to enable detection of under-segmented objects. The geometric centroid of the objects was first calculated using standard image-processing functions in skimage. Subsequently, for each spectral channel, the spectral centroid was calculated, in which the coordinate of each pixel was weighted by its associated spectral intensity. The spectral centroid for each spectral channel was calculated. The distance between the spectral centroid and the geometric centroid as a function of the spectral channel was then calculated. For objects with a pure barcode, the spectral centroid was aligned with the geometric centroid up to imaging noise, regardless of the spectral channel under consideration. For objects with multiple spectral barcodes (that is, under-segmented), the spectral centroid deviated relative to the geometric centroid as a function of the spectral channel. The distance between the spectral and the geometric centroid was therefore used as a metric to evaluate whether an object was under-segmented. Specifically, the median of the spectral–geometric centroid distances across all spectral channels was used as a filtering criterion. A threshold of 1 pixel was used in the biofilm image analysis.

Technical reproducibility analysis
To evaluate reproducibility across different FOVs, different FFPE sections and different mice, the maximum intensity along the spectral axis for every cell was measured. The average maximum intensity for all cells classified to a given barcode was then calculated. Finally, the Pearson correlation between the average maximum intensity for all the barcodes measured in different FOVs was calculated. This analysis was performed for images acquired from two different tissue sections from a ciprofloxacin-treated mouse (15 FOVs per tissue section), as well as for tissue sections from two ciprofloxacin-treated mice and two control mice (one tissue section per mouse, 14 to 15 FOVs per tissue section).

Spatial ecology analysis
The Shannon diversity was calculated using the standard formula of $S = -\sum p_i \log p_i$, in which $p_i$ was the relative abundance of each taxa. To calculate the Bray–Curtis dissimilarity between patches of cells, a pair of random points within an image area was first generated. For each point, the nearest $n$ cells were located ($n$ ranged from 10 to 200), and the centre of the patch was calculated as the mean of the centroids of the individual cells belonging to the patch. The Bray–Curtis dissimilarity between the two patches was then calculated using scipy.spatial.distance.braycurtis. To calculate the $\beta$-diversity of a small patch of cells relative to an image, a random point within the image was selected and the closest $n$ cells to that point were located. The $\alpha$-diversity of the entire image and the $\alpha$-diversity of the patch $a_w$ were then calculated. The $\beta$-diversity of the patch was then calculated as $P_B = a/a_w$.

Spatial association analysis
To enable spatial association analysis, an adjacency segmentation was generated using the watershed algorithm, but with the raw image averaged over the spectral axis as the intensity image. This approach produced a segmentation where objects were in contact with each other, which enabled the calculation of region adjacency graphs. Objects that were within areas labelled as host tissue, objects that were too large to be cells (area greater than 1,000 pixels for the mouse gut and 10,000 pixels for the oral microbiome) or objects that have a raw cosine spectra distance of greater than 0.02 were filtered out from further analysis. The spatial association matrix was calculated using the region adjacency graph. Each spatial association matrix element corresponded to the number of association instances between any two taxa. To calculate differential spatial association matrices between antibiotic treatment and control, the spatial association of images under each treatment was first compared to a random spatial association network. The random spatial association network for each image was generated by shuffling the assigned barcode to each cell while keeping the abundance of each taxon the same as the measured image. For each image, 100 random spatial association networks were simulated. The spatial association enrichment was calculated as the fold change of the measured spatial association network against the average of the simulated random matrices. Finally, the differential spatial association matrix was calculated as the ratio between the spatial association enrichment for ciprofloxacin-treated mice and that of the control mice. To calculate the significance of the observed differential spatial association, the differential spatial associations measured from each FOV were used as independent measurements. The log$_2$-transformed differential spatial association from each image of ciprofloxacin-treated mice was compared to those from the control mice using an independent $t$-test.
The significance threshold was corrected for multiple hypotheses testing using the Bonferroni criteria. The number of hypotheses tested was calculated as the number of unique genus-genus interactions detected across all images. For longitudinal comparison of the oral plaque microbiome spatial association networks, the spatial association enrichments against random networks with the same taxa abundance were calculated as above. The average of all simulated random spatial association matrices was used as a benchmark of comparison. The difference between two spatial association matrices was calculated using the Frobenius distance (element-wise squared difference).

Super-resolution image straightening
To straighten the super-resolution images, a segmentation mask was first generated from the raw image using k-means clustering with two clusters. A Gaussian filter with a kernel size of 20 pixels was then used to broaden the segmented area, and a k-means clustering with 3 components to separate foreground pixels from the background. This step ensured that the segmentation masks were extended well beyond the cell, so that the edges of the cells in the super-resolution images remained intact. The medial lines of the segmentation masks were then calculated. The medial lines were extended in the forward and reverse directions to capture the poles of the cell. To accomplish this, the gradients of the medial lines were calculated using the first and last five pixels (average of the first order difference between pixel coordinates) and used to extend the medial lines in both directions by 50 pixels (or 10, for smaller circular cells). Interpolated splines were then fit to the medial line coordinates to generate up-sampled medial line coordinates, such that the distance between each successive pixel in the up-sampled coordinates list was one pixel. Finally, the straightened image was generated by interpolating the raw image pixel intensity at the up-sampled medial line coordinates.

Three-dimensional volume rendering
The 3D datasets were segmented, identified and assigned false colours as described in ‘Image processing for biofilm samples’. The four-dimensional (4D) data cube (xyzc) was then rendered in ipyvolume using linear ramps of RGBA transfer functions.

Reporting summary
Further information on research design is available in the Nature Research Reporting Summary linked to this paper.

Data availability
PacBio sequencing data are available at the NCBI Sequence Read Archive (SRA) with accession number PRJNA665727. Metagenomic sequencing data of laser-capture-microdissected tissue samples are available at the NCBI SRA with accession number PRJNA665536. All microscopy data have been deposited to Zenodo. A full list of DOIs is provided in the Supplementary Information. Source data are provided with this paper.

Code availability
All code is available on GitHub at https://github.com/proudquartz/hiprfish.

Acknowledgements
We thank R. M. Williams and J. M. Dela Cruz for assistance with microscopy; A. Douglas, J. McMullen, T. Doerr, J. Peters and M. Petassi for providing materials; and P. S. Burnham, A. P. Cheng, T. Lan and E. Michel for discussions and feedback. This work was supported by an instrumentationgrant from the Kavli Institute at Cornell and by US National Institutes of Health (NIH) grants 1DP2AI138242 to I.D.V. and 1R33CA253520 to I.D.V., W.Z. and I.L.B. Imaging data were acquired in the Cornell Biotechnology Resource Center Imaging Facility using the shared, NYSTEM (CO29155)- and NIH (S10OD018516)-funded Zeiss LSM880 confocal and multiphoton microscope.

Author contributions
H.S. and I.D.V. conceived the study. H.S., W.Z., I.L.B. and I.D.V. contributed to the study design. H.S., performed the E. coli experiments. H.S. and J.L.S. performed the multispecies community experiments. Q.S. and I.L.B. performed mouse experiments. H.S. performed PacBio sequencing experiments and analysed data. H.S. and B.G. performed HiPR-FISH experiments in mice. H.S. performed HiPR-FISH experiments on the human oral microbiome. H.S. and I.D.V. analysed the data. H.S., W.Z., I.L.B. and I.D.V. wrote the manuscript.

Competing interests
H.S. and I.D.V. are inventors on the patent WO 2019/173555, which was filed in September 2019 by Cornell University Center for Technology Licensing and which covers the technical aspects of the manuscript.

Additional information
Supplementary information is available for this paper at https://doi.org/10.1038/s41586-020-2983-4.

Correspondence and requests for materials should be addressed to H.S. or I.D.V.

Peer review information
Nature thanks Anjum Raj, Carolina Tropini and the other, anonymous, reviewer(s) for their contribution to the peer review of this work.

Reprints and permissions information is available at http://www.nature.com/reprints.
Extended Data Fig. 1 | Workflow of HiPR-FISH experiments. Environmental microbial consortia are first split into two samples. One sample is used to generate full-length 16S amplicon sequences using PCR and PacBio sequencing. The resulting sequence file is used to generate a list of probes, which are purchased from a commercial vendor. The other sample is used for imaging experiments. Fixed samples are hybridized using an encoding hybridization buffer containing the amplified complex probes and read out using a readout hybridization buffer containing fluorescently labelled readout probes. Samples are then embedded and imaged on a standard confocal microscope in the spectral imaging mode. Resulting raw images are registered and segmented. The spectra of individual cells are measured using the raw image and the segmentation image and classified using a machine learning algorithm. Finally, classified images are used for downstream quantitative measurements of microbial spatial associations.
Extended Data Fig. 2 | Spectra construction and classification.

a. Fluorescence spectra measured using each laser are concatenated for classification. b. Example spectra for two different barcodes. The combined spectra can contain distinct peaks or broad peaks, depending on the fluorophores used for each barcode. c. Spectra for the 10 fluorophores used in this study. d. Classification algorithm for barcode assignment. Concatenated spectra are projected using UMAP before classified using support vector machines.
Extended Data Fig. 3 | Classification accuracy for *E. coli* 1,023-plex barcoding experiment. **a**, Classification frequency as a function of Hamming distance for all 1,023 barcodes. Insets show barcodes with detectable error (in orange) and an example of a barcode with no detectable error (in blue). **b, c**, Photon counting measurements for Alexa 488 for each pixel (**b**) and across each cell (**c**). **d**, Signal-to-noise ratio calculated using Poisson statistics for the *E. coli* cells under nominal experimental imaging conditions. **e**, Simulated classification error as a function of ribosomal density within individual cells. In box plots (**b, c**), the centre lines show the median value, the bounds of the boxes correspond to the 25th and 75th percentiles and the whiskers extend to 1.5 × IQR.
Extended Data Fig. 4 | Probe design pipeline and amplification strategy.

**a**, Full-length 16S sequences are first grouped by taxa. The consensus sequence for each taxon is used to design probes. Each probe within each taxon is then blasted against the database of full-length 16S sequences. Several probe quality metrics are calculated on the basis of the blast results and are used to select probes. All selected probes are conjugated to the appropriate readout sequences and blasted against the database of full-length 16S sequences to remove probes with any potential mis-hybridization sites owing to the conjugation of the readout sequences.

**b**, Schematic for probe synthesis. Complex oligo pools are amplified using limited-cycle PCR. The T7 promoter introduced during PCR allows the templates to be in-vitro-transcribed. Reverse transcription then converts RNA to cDNA. Finally, alkaline hydrolysis removes the RNA strand to generate the final single-stranded DNA probe pool.
**Extended Data Fig. 5** Classification accuracy in synthetic communities. Classification accuracy as a function of Hamming distance for each species of bacteria measured using different barcodes.
Extended Data Fig. 6 | Image segmentation workflow and comparison to other methods. 

a, A typical raw image of a human plaque biofilm sample averaged along the spectral axis was enhanced using the LNE algorithm before segmentation using the watershed algorithm. Spectra of segmented cells were then used to generate the identification image. Scale bars, 25 μm. 

b, Examples of segmentation comparisons between LNE and existing methods. Scale bars, 25 μm. 

c, Enlarged views highlight advantages of LNE over existing methods at segmenting closely packed cells. Scale bars, 5 μm.
Extended Data Fig. 7 | LNE can segment objects with diverse shapes in images collected using different modalities. a, Raw (left) and segmented (right) images of a longitudinal section of a km fibre in a partially contracted *Stentor coeruleus* cell imaged using transmission electron microscopy. b, Raw (left) and segmented (right) images of fluorescently labelled actin bundles from chicken muscle imaged using total internal reflection microscopy. Source images are from the Cell Image Library.
**Extended Data Fig. 8** | Spatio-spectral deconvolution accuracy of simulated merged objects. 

**a,** Heat map of merger detection rates across all 10-bit barcode combinations. Barcode–neighbour combinations not detected in the 1,023-plex *E. coli* mixing experiment are shown in orange. The diagonal corresponds to the correct identification of merged objects with the same barcode as single objects. 

**b,** Merger detection rate as a function of Hamming distance. The spatio-spectral deconvolution approach can detect 99.6% of all objects with spatially varying barcodes that are more than 1 bit away.
Extended Data Fig. 9 | Additional analysis of the gut microbiome images. 

a. Heat map of the Pearson correlation between maximum average intensity for all detected barcodes from different FFPE sections of the same mouse gut, with 15 FOVs per FFPE section. b. Heat map of Pearson correlation between maximum average intensity for all detected barcodes from different mice, with 14 to 15 FOVs per mouse. c. Comparison of imaging and sequencing measurements on mouse gut FFPE tissue sections. d. Phylum abundance measurements from images of a clindamycin-treated mouse compared to a control mouse. The clindamycin-treated mouse shows a lower Bacteroidetes to Firmicutes ratio than the control mouse. e. Measured Shannon diversity is lower in the clindamycin-treated mouse than the control mouse. The centre lines show the median value, the bounds of the boxes correspond to the 25th and 75th percentiles and the whiskers extend to 1.5 × IQR. f. β-diversity as a function of patch size shows similar trends in the clindamycin-treated mouse and the control mouse. The boxes correspond to 25th and 75th quartile, and the whiskers extend to the most extreme data points. g. Bray–Curtis dissimilarity increases as a function of intra-patch distance in both the clindamycin-treated and the control mice. h. Volcano plot of significance versus spatial association fold change between ciprofloxacin-treated mice and control mice. Altered spatial associations that are statistically significant after Bonferroni correction are listed.
Extended Data Fig. 10 | Reproducible and recurrent microarchitectures in the oral microbiome. a. Clusters of Lautropia cells observed using different panels of probes. b. Two-dimensional UMAP projections of the physical properties of Lautropia cells observed using different probe panels overlap in the reduced dimensions. c. Additional observed instances of the Pseudopropionibacterium—Cardiobacterium—Schwartzia consortium.
## Reporting Summary

Nature Research wishes to improve the reproducibility of the work that we publish. This form provides structure for consistency and transparency in reporting. For further information on Nature Research policies, see Authors & Referees and the Editorial Policy Checklist.

### Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

| Item                                                                 | Confirmed |
|----------------------------------------------------------------------|-----------|
| n/a                                                                 | ✓         |
| The exact sample size \( (n) \) for each experimental group/condition, given as a discrete number and unit of measurement | ✓         |
| A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly | ✓         |
| The statistical test(s) used AND whether they are one- or two-sided   | ✓         |
| Only common tests should be described solely by name; describe more complex techniques in the Methods section. | ✓         |
| A description of all covariates tested                               | ✓         |
| A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons | ✓         |
| A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals) | ✓         |
| For null hypothesis testing, the test statistic (e.g. \( F, t, r \)) with confidence intervals, effect sizes, degrees of freedom and \( P \) value noted | ✓         |
| Give \( P \) values as exact values whenever suitable.                | ✓         |
| For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings | ✓         |
| For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes | ✓         |
| Estimates of effect sizes (e.g. Cohen’s \( d \), Pearson’s \( r \)), indicating how they were calculated | ✓         |

*Our web collection on statistics for biologists contains articles on many of the points above.*

### Software and code

**Policy information about availability of computer code**

| Data collection | Imaging data were acquired using the Zen software from Carl Zeiss Inc. The version is Zen 3.2. |
|-----------------|------------------------------------------------------------------------------------------------|
| Data analysis   | All data analysis code are written in python, and are available at github.com/proudquartz/hiprfish. The probe design pipeline uses several third-party open source softwares, and are described in detail in the Extended Data tables. All python modules used can be found at the top of each python script. The python version is 3.5. Usearch version is v8.2.64_i86linux32. Primer3 version is 2.3.5. Blastn version is 2.9.0. Trim galore version is 0.6.5dev. Prinseq-lite version is 0.20.4. Bbmap version is 37.62. Metaspades version is 3.14.0. BWA version is 0.17.7. Samtools version is 1.0.2. |

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research [guidelines for submitting code & software](https://natureresearch.com/guidelines) for further information.

### Data

**Policy information about availability of data**

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

All imaging and sequencing data are available at Zenodo. The DOI for each data item is listed in the manuscript. All PacBio sequencing data was deposited to Zenodo under DOI 10.5281/zenodo.3459587. All microscopy data was deposited to Zenodo under the following DOIs: 10.5281/zenodo.3455774, 10.5281/zenodo.3455817, 10.5281/zenodo.3455819, 10.5281/zenodo.3455821, 10.5281/zenodo.3455823, 10.5281/zenodo.3455825, 10.5281/zenodo.3457243, 10.5281/zenodo.3457245, 10.5281/zenodo.3457247, 10.5281/zenodo.3457249, 10.5281/zenodo.3457251, 10.5281/zenodo.3457253, 10.5281/zenodo.3457255, 10.5281/zenodo.3457257, 10.5281/zenodo.3457261, 10.5281/zenodo.3457263, 10.5281/zenodo.3457265, 10.5281/zenodo.3457267, 10.5281/zenodo.3459331, 10.5281/zenodo.3457271, 10.5281/zenodo.3457273, 10.5281/zenodo.3458633, 10.5281/zenodo.3458635, 10.5281/zenodo.3458637.
Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

- Life sciences
- Behavioural & social sciences
- Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

| Sample size | For cultured cell experiments, the number of cells measured is routinely in the hundreds, so sample size calculation was not necessary. For mouse experiments, we chose sample sizes that are similar to previous similar studies on mouse gut microbiome. |
| Data exclusions | No data were excluded from the analysis. |
| Replication | All attempts at replication were successful. Generally multiple fields of view are collected for each sample. HiPR-FISH on mouse experiments were replicated using tissue sections from each mice for inter-individual comparisons and using two different tissue sections from one mouse for within-individual comparison. |
| Randomization | Allocation was random. |
| Blinding | Blinding was not needed for the descriptive and exploratory studies in figure 3. No specific hypothesis was tested. |

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

- Antibodies
- Eukaryotic cell lines
- Palaeontology
- Animals and other organisms
- Human research participants
- Clinical data

Methods

- ChIP-seq
- Flow cytometry
- MRI-based neuroimaging

Animals and other organisms

Policy information about studies involving animals: ARRIVE guidelines recommended for reporting animal research

Laboratory animals

The study used C57Bl/6j mice. All animals were 5-week old specific pathogen free female mice received from the Jackson Lab. All mice were housed under standard barrier conditions in individually ventilated cages with autoclaved food and autoclaved water ad libitum and monitored in accordance with the rules of the Weill Hall Barrier Animal Facility. Lights on at 6am and lights off at 8 pm. Temperature: 70F-74F and % RH: 30% - 70%.

Wild animals

The study did not involve wild animals

Field-collected samples

The study did not involve field-collected samples.

Ethics oversight

The Cornell University Institutional Animal Care and Use Committee approved the study protocol.

Note that full information on the approval of the study protocol must also be provided in the manuscript.
Human research participants

Policy information about [studies involving human research participants](#)

| Population characteristics | The study involved oral plaque samples donated from a healthy male volunteer. |
|-----------------------------|-------------------------------------------------------------------------|
| Recruitment                 | No recruitment was used for the study. One healthy volunteer donated samples. |
| Ethics oversight            | The Cornell Institutional Review Board approved the study design with protocol number 1910009101. |

Note that full information on the approval of the study protocol must also be provided in the manuscript.