SRS-FISH: A high-throughput platform linking microbiome metabolism to identity at the single cell level

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One of the biggest challenges in microbiome research in environmental and medical samples is to better understand functional properties of microbial community members at a single cell level. Single cell isotope probing has become a key tool for this purpose, but the current detection methods for determination of isotope incorporation into single cells do not allow high-throughput analyses. Here, we report on the development of an imaging-based approach termed stimulated Raman scattering - two-photon fluorescence in situ hybridization (SRS-FISH) for high-throughput metabolism-identity analyses of microbial communities with single cell resolution. SRS-FISH offers an imaging speed of 10 to 100 milliseconds per cell, which is two to three orders of magnitude faster than achievable by state-of-the-art methods. Using this technique, we delineated metabolic responses of thirty thousand individual cells to various mucosal sugars in the human gut microbiome via incorporation of deuterium from heavy water as an activity marker. Application of SRS-FISH to investigate the utilization of host-derived nutrients by two major human gut microbiome taxa revealed that response to mucosal sugars tends to be dominated by Bacteroidales, with an unexpected finding that Clostridia can outperform Bacteroidales at foraging fucose. With high sensitivity and speed, SRS-FISH will enable researchers to probe the fine-scale temporal, spatial and individual activity patterns of microbial cells in complex communities with unprecedented detail.

With the rapid advances in both genotyping and phenotyping of single cells, bridging genotype and phenotype at the single cell level is becoming a frontier science (1). Methods have been developed to shed light on the genotype-metabolism relationship of individual cells in a complex environment (2, 3), which is especially relevant for an in-depth understanding of complex microbial communities in the environment and host-associated microorganisms. For functional analyses of microbial communities, single cell isotope probing is often performed in combination with nanoscale secondary ion mass spectrometry (NanoSIMS) (4–7), microautoradiography (MAR) (8, 9), or spontaneous Raman microspectroscopy (10–12) to visualize and quantify the incorporation of isotopes from labeled substrates. These methods can be combined with fluorescence in situ hybridization (FISH) using rRNA-targeted probes (13), enabling a direct link between metabolism and identity of the organisms. In addition, Raman-activated cell sorting has been recently developed, using either optical tweezers or cell ejection for downstream sequencing of the sorted cells (14–16). While these approaches have expanded the possibilities for functional analyses of microbiome members (17), all of the aforementioned methods suffer from extremely limited throughput. Consequently, only relatively few samples and cells per sample are typically analyzed in single cell stable isotope probing studies, hampering a comprehensive understanding of the function of microbes in their natural environment.

To overcome the limited throughput of Raman spectroscopy, coherent Raman scattering microscopy based on coherent anti-Stokes Raman scattering (CARS) or stimulated Raman scattering (SRS), has been developed (18, 19). Compared to CARS, the SRS signal is free of the electronic resonance response (20) and is linear to molecular concentration, thus permitting quantitative mapping of biomolecules (21, 22). Both CARS and

Significance Statement

Microbial communities play fundamental roles in the functioning of environmental and human-associated ecosystems. Yet, conventional experimental approaches to study the function of individual microbes in their native habitat are highly time-consuming, allowing only analysis of a few cells or samples. Here, we report the development of a high-throughput SRS-FISH platform to investigate the metabolism and identity of uncultivated microorganisms within microbiomes with single-cell level resolution. SRS-FISH enabled us to detect metabolic responses of over 30,000 cells from selected human gut microbiome taxa to mucosal sugars using heavy water as an activity marker. Metabolism-identity analysis of multiple samples revealed that the extent and pattern of microbiome response to mucosal sugars is individual-specific, and that Clostridia may have been overlooked as mucosal sugar degraders in the human gut.

X.G., F.C.P., M.W. and J.-X.C. conceived and designed the study. X.G., F.C.P. and M.M. performed the experiments and analyzed the data. X.G., F.C.P., A.S., M.W. and J.-X.C. wrote the manuscript. D.B. provided support and input to the microbiome experiments. M.Z. provided input on D labeling and cell fixation protocols. B.H. provided support with 16S rRNA gene amplicon sequencing and data analysis and deposition. J.Z. provided input on single cell data segmentation and analysis. A.S. provided support and input to the system precision and sensitivity characterization. All authors have given approval to the final version of the paper.

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SRS microscopy have successfully been applied for studying single cell metabolism in eukaryotes (23–26). In a label-free manner, SRS imaging has led to the discovery of an aberrant cholesteryl ester storage in aggressive cancers (27, 28), lipid-rich protrusions in cancer cells under starvation (29), fatty acid unsaturation in ovarian cancer stem cells (30) and more recently in melanoma (31). CARS and SRS have also been harnessed to explore lipid metabolism in live Caenorhabditis elegans (32–35). Combined with stable isotope probing, SRS microscopy has allowed the tracing of glucose metabolism in eukaryotic cells (36, 37) and the visualization of metabolic dynamics in living animals (25). Recently, SRS was successfully applied to infer antibiotic resistance patterns of bacterial pure cultures and heavy water (D$_2$O) metabolism (38). Yet, SRS microscopy has not been adapted for studying functional properties of members of microbiomes as SRS itself lacks the capability of identifying cells in a complex community.

Here, we present an integrative platform that exploits the advantages of SRS for single cell stable isotope probing together with two-photon FISH for the identification of cells in a high-throughput manner. To deal with the challenges in detecting low concentrations of metabolites inside small cells with diameters around one micron, we have developed a protocol that maximizes the isotope label content in cells and exploits the intense SRS signal from the Raman band used for isotopic detection.

Conventionally, FISH is performed separately by one-photon excited fluorescence microscopy (39). To enhance efficiency, we developed a system that implements highly sensitive SRS metabolic imaging with two-photon FISH using the same laser source. These efforts collectively led to a high-throughput platform that enables correlative imaging of cell identity and metabolism at a speed of 10–100 milliseconds per cell. In comparison, it takes about 20 seconds to record a Raman spectrum from a single cell in a conventional spontaneous Raman-FISH experiment (40).

Our technology enabled high-throughput analysis of single cell metabolism in the human gut microbiome. In the human body, microbes have been shown to modulate the host’s health (41, 42). Analytical techniques looking into their activities and specific physiologies (i.e. phenotype) as a result of both genotype and the environment provide key information on how microbes function, interact with and shape their host. As a proof-of-principle, we used SRS-FISH to track the incorporation of deuterium (D) from D$_2$O into a mixture of two distinct gut microbiota taxa. Incorporation of D from D$_2$O into newly synthesized cellular components of active cells, such as lipids and proteins, occurs analogously to incorporation of hydrogen from water, during the reductive steps of biosynthesis of various cellular molecules (10, 43, 44). Importantly, D incorporation from D$_2$O has been shown to be reliable to track metabolic activity of individual cells within complex microbial communities in response to the addition of external substrates (10, 17, 45). When microbial communities are incubated in the presence of D$_2$O under nutrient-limiting conditions, individual cells display only minimal activity and only minor D incorporation (11, 17, 45). In contrary, when cells are stimulated by the addition of an external nutrient, cells that can metabolize this compound become active and incorporate D into macromolecules, which lead to the presence of C-D bonds into the cell’s biomass. Consequently, D incorporation from D$_2$O can be combined with techniques able to detect C-D signals, such as Raman based approaches, and to track metabolic activity at the single-cell level in response to a variety of compounds. Here we show that SRS-FISH enables fast and sensitive determination of the D-content of individual cells while simultaneously unveiling their phylogenetic identity. We applied this technique to complex microbial communities by tracking in situ the metabolic responses of two major phylogenetic groups of microbes in the human gut (Bacteroidales and Clostridia spp.) and of a particular species within each group, to supplemented host-derived nutrients. Our study revealed that (i) Clostridia spp. can actually outperform Bacteroidales spp. at foraging on the mucosal sugar fucose and shows (ii) a significant inter-individual variability of responses of these major microbiome taxa towards mucosal sugars. Together, our results demonstrate the capability of SRS-FISH to unveil the metabolism of particular microbes in complex communities, at a throughput that is two to three orders of magnitude higher than other metabolism-identity bridging tools, therefore providing a valuable multimodal platform to the field of single cell analysis.

Results and Discussion

An SRS-FISH platform to link cell metabolism with cell identity, SRS can visualize chemical information by coherently probing Raman active vibrations with two synchronized pulsed pump and Stokes beams. When the energy difference is tuned to the vibrational energy of the targeted chemical bond, a chemical concentration map can be generated. In our case, considering that C-H and C-D stretching vibrations are spectrally broad, we used femtosecond pulses to maximize the detection sensitivity (46). The use of femtosecond pulses facilitates efficient two-photon excited FISH on the same SRS microscope. The SRS and FISH signals are sequentially detected in the forward direction, using a photodiode and two silicon photomultipliers, respectively. The energy schematic and the setup are shown in Fig. 1A,B. Details can be found in Materials and Methods.

To retrieve information on the activity of single bacterial cells in pure culture or in complex samples, live cells present in simple (pure cultures) or complex (gut microbiome) samples were incubated in D$_2$O containing-media (10, 38), to enable incorporation of D into biomolecules of metabolically-active cells (Fig. 1C). Cells were subsequently fixed and subjected to FISH using fluorescently labelled oligonucleotide probes targeting rRNA, in order to reveal their phylogenetic identity (Fig. 1C,D). Samples prepared in this way were consecutively imaged to retrieve i) fluorescence signals from hybridized samples; and ii) chemical information that enables determination of cellular D enrichment levels for the different taxa targeted by FISH (Fig. 1D).

We have developed a two-photon FISH protocol to detect cyanine 3 (Cy3) and cyanine 5 (Cy5), two dyes that possess large two-photon cross sections (47) and are commonly used in FISH studies due to their brightness. Two silicon photomultipliers were used to selectively detect the fluorescence emitted by Cy3 and Cy5, respectively (Fig. 1D). We confirmed that two-photon-excited fluorescence (TPEF) retrieves accurate fluorescence information with comparable imaging quality and speed as achieved by a confocal microscope (further discussed below). Thus, two-photon FISH is a reliable tool for identity
Stimulated Raman scattering (SRS)-fluorescence in situ hybridization (FISH) platform to link phylogenetic identity (genotype) with metabolic activity (phenotype) of microbes. (A) SRS and two photon excited fluorescence (TPEF) mechanism. ES: excited state. VS: virtual state. GS: ground state. \( \omega_p \): pump beam laser frequency. \( \omega_S \): Stokes beam laser frequency. \( \omega_{SRL} \): stimulated Raman loss frequency. \( \omega_T \): TPEF excitation beam frequency. \( \omega_E \): fluorescence emission frequency. (B) SRS-FISH instrumental setup. M1-M3: mirrors. DM1-2: dichroic mirrors. SU: scanning unit. L1-4: lenses. OBJ: objective. COND: condenser. FM: flip mirror. PD: photo diode. SiPM1-2: silicon photomultiplier. (C) Typical sample procedure process for SRS-FISH experiments. Pure bacterial cultures or complex microbiome samples are incubated in D\(_2\)O-containing media to enable D incorporation into metabolically active cells. Samples are subsequently fixed and subjected to FISH. After FISH, samples are deposited in a glass cover slide and analyzed by SRS-FISH. (D) Schematic representation of SRS-FISH imaging results. Samples are hybridized with fluorescently-labelled oligonucleotide probes (double-labelled with either cyan or red fluorophores) targeting taxa of interest present in the sample. Fluorescence signal originating from hybridized samples (cyan and red) is then overlaid with the SRS C-D signal (yellow) and SRS the C-H signal (green) to reveal metabolic activity levels represented by %CD (magma) of each identified bacterial cell. Organisms not targeted by the probes will not display fluorescence (grey).

To determine D incorporation into bacteria, the pump and Stokes beams were tuned to target the C-D vibrational peak (2168 cm\(^{-1}\)). As bacterial cells have sizes that are comparable with the laser focus laterally and axially (Fig. S1), the different volumes exhibited by different bacterial species can influence the SRS intensity level. To compensate this effect, the pump beam was tuned to target the center of the C-H bond vibrational peak (2946 cm\(^{-1}\)) as a reference signal for intensity normalization. In terms of absolute concentrations, measurements of DMSO/H\(_2\)O mixtures revealed that as low as around three million C-D bonds or C-H bonds can reliably be detected by femtosecond SRS within the excitation volume. We have observed that some bacterial species generated signals in the silent spectral regions (from 1800 to 2800 cm\(^{-1}\)) that can be detected by SRS but not by spontaneous Raman spectroscopy (Fig. S3), which may originate from other nonlinear processes, such as transient absorption, photothermal lensing and cross phase modulation (48, 49). In order to correct this unspecific background, off-resonance images were recorded with the pump beam tuned to target 2479 cm\(^{-1}\) in the silent region, and subtracted from both C-D and C-H SRS images (Fig. S3, S4). Via combination of the individual signal intensities, a quantity can be defined that expresses the metabolic activity in D incorporation as %CD\(_{SRS}\), calculated from the SRS signal intensities at C-D, C-H, and off-resonance, according to the formula: %CD\(_{SRS}\) = (I\(_{CD}\) − I\(_{off}\))/(I\(_{CD}\) + I\(_{CH}\) − 2I\(_{off}\)), where I

mapping, although with slightly lower spatial resolution (\(~300\) nm) than confocal microscopy (usually \(~200\) nm) (Fig. S1).
SRS has sufficient sensitivity to detect single metabolically-active bacteria tagged by FISH. Though SRS has been used before (but without combining it with FISH) for imaging isotope incorporation in bacterial and mammalian cells (25, 38), a more comprehensive study about deuterium isotope labelling detected by SRS imaging on different bacterial species has not been conducted yet. In order to evaluate the optimum measurement settings, a first trial with two cultures of Escherichia coli (E. coli) cells grown in M9 medium, one without addition of D₂O (natural isotope abundance control) and the other one after addition of heavy water (50% D₂O), was performed. For each culture, eighty-one image scans, conducted with 10 µs per-pixel dwell time, were recorded within randomly selected fields of view. Analysis of these datasets revealed that 10 scans, corresponding to a measurement time of around 1 ms per single cell (Fig. S2), are sufficient for obtaining adequate precision in the determination of %CD values under maintenance of the high throughput of the technique (Supporting Information Text).

To further investigate the suitability of SRS-FISH to detect the incorporation of deuterium in cells with different physiologies and cell wall structures, we performed SRS measurements on four bacterial cultures that have been hybridized with fluorescently-labelled probes (FISH): Bacteroides thetaiotaomicron (B. thetaiotaomicron, Gram-negative, Fig. 2A), Clostridium scindens (C. scindens, Gram-positive, Fig. 2B), E. coli (Gram-negative, Fig. 2C) and Blautia producta (B. producta, Gram-positive, Fig. 2D). These cultures were grown in either rich (BHI or LB), semi-minimal (BMMs) or minimal (BMM or M9) media containing various concentrations of D₂O and therefore covering a wide range of cellular D contents (Fig. 2). The lowest mean deuterium labelling content (%CD_{SRS}) that could be detected in single cells grown under all the above conditions was around 2.4% (B. thetaiotaomicron cultured in BHI medium with 30% D₂O), which still showed statistical significance against the negative control (two-sided Mann-Whitney U test, p < 10⁻¹⁰), cultured in the same medium but in the absence of D₂O. In addition, a linear relationship between the cellular %CD_{SRS} values and the D₂O concentration in the applied media was observed. A few exceptions occurred at 70% D₂O (Fig. 2B,D), which can be explained by the inhibitory effect exerted by elevated concentrations of heavy water on metabolic activity. As expected from previously published data (50), cells grown in complex rich media or semi-minimal media displayed lower levels of D incorporation compared to cells grown in minimal media containing equivalent concentrations of heavy water (Fig. 2). This is likely caused by the higher need for de novo biosynthesis of monomeric biomolecules (which will become labeled during synthesis), such as amino acids, nucleotides, or fatty acids, which are absent in minimal media but readily available in unlabeled form for direct uptake and incorporation from complex (or semi-minimal) media.

We further studied the impact of FISH on the D enrichment level measured by SRS. Both cell fixation and FISH protocols have been previously shown to elute material (e.g. lipids) from microbial cells, which can impact the D content as assessed by spontaneous Raman (4, 10, 40). Using SRS-FISH, we could observe a relative decrease by 13.65% ± 7.81% in the C-D level of E. coli hybridized cells (FISH) compared to fixed but non-hybridized E. coli cells (no FISH) (Fig. S5, p<0.05, Mann-Whitney U test) for both media tested. This reduction in %CD is approximately three times smaller than reported for a similar comparison performed with spontaneous Raman measurements (10) and might be explained by the fact that in the FISH protocol used here the exposure time of the cells to ethanol was reduced compared to the previously applied protocol Materials and Methods). Therefore, our optimized FISH protocol causes a comparatively small loss in cellular biomass, and thus minimizes the impact single of FISH on cell metabolic activity analyses.

Overall, SRS microscopy enabled efficient detection and discrimination of both Gram-positive and Gram-negative hybridized cells displaying a wide range of D content, with mean %CD_{SRS} values ranging from as low as 2.4% up to 30% (Fig. 2A, C; p<0.05, two-sided Mann-Whitney U test). To validate the accuracy of femtosecond SRS for detection of bacterial activity, we compared %CD_{SRS} with %CD values measured by spontaneous Raman microspectroscopy (%CD_{Raman}) (10). Under our conditions, SRS displayed similar sensitivity as spontaneous Raman (Fig. 2). However, SRS analysis is two to three orders of magnitude faster than achieved with spontaneous Raman (10-100 ms per cell vs 20 s per cell) (40). This high speed enables SRS measurements of a much larger number of cells, therefore increasing the power of statistical analysis and throughput. It is challenging to quantitatively compare the %CD value distribution patterns of SRS and spontaneous Raman due to the difference in the measurement principle (see Supporting Information Text). However, the data from our pure culture experiments clearly revealed that SRS-FISH is a fast and versatile platform to track metabolic activity in FISH-targeted bacteria.

SRS is compatible with two-photon FISH to link microbial metabolism with identity. As both SRS and TPEF are multiphoton processes, the compatibility of these two schemes requires further validation. Cellular contents labelled with fluorescent dyes can lead to background signals detected by SRS, impacting the %CD_{SRS} values. This can occur when a fluorophore absorbs one photon from the pump and one photon from the Stokes beam. The simultaneous absorption of photons from the two distinct beams could interfere with the SRS signal through a phenomenon known as non-degenerate two-photon absorption. Although two-photon absorption can be calculated based on the absorption cross-sections of the utilized dyes, there is scant data of non-degenerate two-photon absorption to estimate its influence on SRS. In our setup, we could not detect any interference on SRS attributable to the fluorophores in the sample, as we did not observe any significant differences in the %CDs values of hybridized compared with non-hybridized cells grown in the absence of D₂O for the two different media tested (Fig. S5; 0% D₂O). Therefore, the lower %CDs values obtained after FISH in D labelled cells are not caused by an interference with the dyes used for FISH with SRS imaging (Fig. S5).

We then evaluated the compatibility of using the pump beam at 852 nm and the Stokes beam at 1045 nm to excite the Cy3 and Cy5 dyes attached to the FISH probes (Fig. 3A). The two silicon photomultipliers sensitive to 3A). The two silicon photomultipliers sensitive to
Fig. 2. Sensitivity of the SRS-FISH platform to detect D₂O metabolic incorporation into bacterial cells hybridized with fluorescently-labelled rRNA-targeted oligonucleotide probes. SRS imaging and single cell statistics on %CD by SRS and spontaneous Raman of (A) B. thetaiotaomicron cells grown in BHI or BMMs media; (B) C. scindens cells grown in BHI or BMMs media; (C) E. coli cells grown in LB or M9 media; (D) B. producta cells grown in BHI or BMMs media, containing increasing concentrations of D₂O. Cell contours are indicated by gray lines. Scale bar: 5 µm. %CD_SRS scaling: (A) min 0, max 30%; (B) min 0, max 20%; (V) min 0, max 40%; (D) min 0, max 20% per-pixel dwell time: 100 µs. For details regarding data processing please refer to Fig. S4. The combined dot and box plots to the right of the images refer to the single cell %CD values measured with either SRS or spontaneous Raman spectroscopy. NS: non-significant, p > 0.05; *: 10⁻² < p < 0.05; **: 10⁻⁴ < p < 10⁻²; ***: p < 10⁻⁷ (two-sided Mann-Whitney U test). Each dot represents a cell. Boxes represent median, first and third quartile. Whiskers extend to the highest and lowest values that are within one and a half times the interquartile range. The white circles in the center of the boxes indicate the mean value of the data distribution. Please note that the negative %CD_SRS values originate from off-resonance background correction (Materials and Methods).

Fig. 3. Phylogenetic and phenotypic information can be acquired simultaneously by the SRS-FISH platform without interference. (A) TPEF visualization of D-labelled B. thetaiotaomicron, C. scindens, E. coli and B. producta cells hybridized (FISH) with Bac303-Cy3 (pseudo-coloured cyan), Erec482-Cy5 (pseudo-coloured red) or Gam42a-Cy5 (pseudo-coloured red) oligonucleotide probes or not (noFISH). No background in TPEF channels is detected for D-labelled, non-hybridized cells. Imaging was performed under dry conditions. Scale bar, 10 µm. (B) Simultaneous SRS and TPEF imaging of artificial mixtures of unlabelled (0% D₂O) B. thetaiotaomicron and E. coli cells (left panel) or of D-labelled (50% D₂O) E. coli and unlabelled (0% D₂O) B. thetaiotaomicron cells (right panel) cultured in minimal media. Bac: B. thetaiotaomicron. Ecoli: E. coli. %CD_SRS values are shown for both mixtures. In both cases, cells were hybridized with the taxa-specific probes Bac303-Cy3 (cyan) and Gam42a-Cy5 (red), enabling cells from the two populations to be distinguished by TPEF imaging. In the bottom panel, cell contours are shown with gray lines. The dashed circles highlight the different deuterium content of the two taxa. Scale bar, 15 µm. %CD_SRS scaling: min 0, max 60%. (C) Single cell %CD_SRS value distribution in the two different populations of the artificial mixtures presented in (B). Please note that the negative %CD_SRS values originate from off-resonance background correction (Materials and Methods).

High-throughput SRS-FISH for identifying mucosal sugar utilizers in the human gut microbiome. To demonstrate the applicability of the SRS-FISH setup to identify active taxa within a complex microbial community, we examined the responses of specific taxa from the human gut microbiota to additions of sugars contained in the mucus layer (54, 55) (Fig. 4A). Gut commensals able to forage on mucin play a pivotal role in resistance to pathogen colonization and in modulating the host immune response (54, 55). In previous work, D₂O combined with spontaneous Raman-activated cell sorting revealed that members of the families Muribaculaceae, Bacteroidaceae, and Lachnospiraceae are major mucosal sugar foragers in the mouse gut, and whole-genome sequencing revealed that the vast majority of these organisms indeed have genomic potential to catabolize these sugars (17). However, given the differences in microbiota composition of mice and humans, as well as differences in predominant types of mucus glycans that can be found in the two hosts (56, 57), it remains to be clarified if the same taxa are efficient mucosal sugar utilizers in the human gut, and what their substrate preferences are. For this purpose, freshly collected human fecal samples were incubated with the five different mucin O-glycan sugars (N-acetyleneuraminic acid:...
Next, we proceeded to identify which taxa respond to specific sugars in fecal samples from three different volunteers using the SRS-FISH platform. For this purpose, oligonucleotide probes targeting two of the most dominant and widespread phylogenetic groups of microbes in the human gut (58, 59) were applied: Bac303-Cy3 targeting *Bacteroides* and *Prevotella* (51), among other Bacteroidales, and Erec482-Cy5 targeting members of the family *Lachnospiraceae* (also denominated *Clostridium* clusters IVa and IVb) (60) (Table S1). Additionally, we selected these probes because a large percentage of organisms identified as efficient mucosal sugar foragers in the mouse and human gut are targeted or are closely related to organisms targeted by these probes (17, 61), and a large proportion of Bacteroidales spp. and Clostridia spp. have been shown to carry genes for mucosal sugar catabolism (61). High-throughput amplicon sequencing of the 16S rRNA gene of microbiome samples included in our study revealed that Bac303-targeted organisms belonged mostly to the *Bacteroides*, *Prevotella_9* and *Parabacteroides* genus within the Phylum Bacteroidota (Fig. 2C,D), which was the most abundant phylum in all samples. Organisms covered by the Erec482 probe largely belonged to several *Lachnospiraceae* genera, as well as to the genera *Roseburia*, *Ruminococcus* torques group and *Eubacterium* eligens group, within the phylum Firmicutes (Fig. 4C,D). Interestingly, microbiome samples clustered by volunteer (p<0.001, r²=0.85, PERMANOVA; Fig. 4E) rather than by supplementation of mucosal sugar, indicating that the short incubation time and conditions employed prevented major shifts in community composition due to the amended sugar. Nevertheless, amplicon sequencing revealed some fluctuations in relative abundances of taxa targeted by both the Bac303 and Erec482 probes in response to the different mucosal sugars amended (Fig. 4F,G). Fractions of Bac303- and Erec482-targeted taxa determined by amplicon sequencing differed from fractions determined by imaging of FISH labelled cells under dry conditions (Fig. 4F,G, see Materials and Methods and discussion below), which can be attributed to different biases and limitations of either method (62, 63). Despite this, shifts in fractions of both taxa in response to sugars detected by FISH correlated well with shifts detected by amplicon sequencing (Fig. 4F,G), therefore indicating that the TPEF-FISH approach is sensitive to capture the microbiome response to mucosal sugar amendment.

A challenge encountered in imaging of the complex gut microbiome samples by SRS-FISH was that the TPEF signal from fluorescently labelled cells bleached much faster than observed with pure cultures. To overcome this limitation, we acquired the TPEF signals from microbiome samples in a dried state to slow down bleaching while maintaining FISH imaging accuracy (Fig. S6, Fig. S7). Subsequently, the samples were immersed by the addition of water for maintaining the SRS intensity and sensitivity achieved in a liquid environment (Fig. S6, Supporting Information Text). Using the SRS-FISH protocol optimized for complex microbiome samples, we examined the response of cells targeted by the Bac303-Cy3 and Erec482-Cy5 probes to each mucosal sugar in fecal samples from three different volunteers (Fig. 5, Fig. S8). The response to mucosal sugars differed from volunteer to volunteer (One-Way Anova test, p<2.225×10−308) (Fig. S9), both in quantitative and qualitative terms. Quantitatively, the overall microbiome response to the amended sugars (with respect to the number of active cells and their %CD values) was the highest for volunteer 2, while lowest cellular activity was detected for volunteer 3 (Fig. S9, S10). This is not surprising, given that the human microbiome is highly individualized, and also that different fecal samples have been reported to contain different fractions of viable cells (64, 65). For volunteers 1 and 2, between 95% and 100% of the analyzed cells became active and isotopically labelled in response to galactose, which emphasizes that the incubation conditions applied in this study enable potentially all microbiome members to respond and get D labelled (Fig. S9, S10). Across the 3 samples, the highest average number of active cells (and higher %CD values) was recorded in response to the mucosal sugar GlcNac, followed by the response to galactose (Fig. S9, S10), which is in agreement with the results obtained by spontaneous Raman (Fig. 4B). Overall, we could detect a significant response of both FISH-targeted taxa to the mucosal sugars in all the
Fig. 5. SRS-FISH uncovers the ability of major gut microbiome taxa to forage on different mucosal sugars. (A) Microbiome samples of volunteer 1 were incubated with different mucosal sugars and hybridized with the oligonucleotide probes Bac303-Cy3 (cyan) and Erec482-Cy5 (red), respectively. Representative images obtained by TPEF (top row) and SRS (%CDERS with FISH contours in the middle row - %CDERS values of other microbes are not displayed to enhance clarity, C-H in log scale displaying all microbes in the bottom row) are shown. %CDERS scaling: min 0, max 25%. In the middle row, cells with FISH tag are shown with respective color contour lines. Negative control: H₂O±Glucose. Positive control: D₂O±Glucose. NA: no amendment. Scale bar, 10 μm. For details regarding data processing please refer to Fig. S4. (B) Single cell C-D level distribution in the two different targeted taxa presented in (A), measured by SRS for samples from 3 different volunteers. Box plots represent the median, first and third quartile with the extended lines represent the minimum and maximum value within 1.5 interquartile range from the first and third quartile. The white circles in the center of the boxes indicate the mean value of the data distribution. The deeper grey blue line (mean ±3SD of Bac303-Cy3 labelled cells in negative control samples) indicates the threshold value for consideration of Bac (Bac303-Cy3 cells) as significantly D enriched. The lighter grey blue line (mean ±3SD of Erec482-Cy5 labelled cells in negative control samples) indicates the threshold value for consideration of Erec (Erec482-Cy5) cells as significantly D enriched (refer to Fig. S10). The two-sided Mann-Whitney U-test was applied to compare the statistical significance between Bac and Erec bacteria for each amendment. NS: non-significant, *p > 0.05; **: 10⁻⁷ < p < 0.05; ***: 10⁻⁷ < p < 10⁻⁵; ** *: p < 10⁻⁷. Please note that the negative %CDERS values originate from off-resonance background correction (Materials and Methods).

samples analyzed, with the exception of Erec482-targeted taxa to GalNAc in the sample from volunteer 1 (Fig. 5B, Table S2). In none of the samples did the no control amendment lead to the stimulation of a significant number of cells (Fig. 5B, Table S2). Furthermore, the response of Bac303-targeted taxa was overall higher than the response from Erec482-targeted taxa across all volunteers for all supplemented sugars, with the exception of fucose, where the inverse was observed for two of the volunteers (Fig. 5, Fig. S10). These findings hold even by taking into consideration that the unspecific signals in the C-D region were higher in the control group (H₂O) for Bac303-targeted cells than for Erec482-targeted cells (Fig. 5). We further extended the SRS-FISH analysis by applying FISH probes targeting particular species within Bacteroidales (Bacteroides vulgatus, targeted by the Bvul1g1017-Cy3 probe (58)) and Lachnospiraceae (Aphthobacter rectalis, formerly Eubacterium rectale, targeted by Erec996+Erec1252-Cy5 probe (66)) that have been previously proposed to either contribute to mucin degradation (B. vulgatus) (67) or to preferentially associate with the mucin layer (A. rectalis) (68) (Table S1, Fig. S11). For volunteer two, we observed that the response from A. rectalis was low for all mucosal sugars tested, suggesting that it may be a poor mucus degrader despite its ability to colonize the mucus layer (Fig. S11). Importantly, this analysis also revealed that B. vulgatus is one of the most efficient sialic acid consumers within Bacteroidales, with Bvul1g1017 targeted cells overall displaying higher levels of metabolic activity in response to this sugar when compared to cells targeted by the broader Bac303 probe (Fig. 5C and Fig. S11B).

Discussion

Microbial communities are fundamental to the functioning of all ecosystems and the health of animals, plants, and humans. These microbiomes are typically investigated by meta-omic analyses that generate valuable annotation-based hypotheses regarding the metabolism of their members but are not suited for testing these hypotheses as gene annotations are often missing, wrong or incomplete (69). Furthermore, many microbes have cell cycles, show considerable phenotypic diversity within isogenic strains, and the activity of microbes is influenced by their spatial arrangement in their habitat. Thus, there is an urgent need for direct functional analyses of microbes within complex samples with single cell resolution.

SRS-FISH fills a gap among the available tools linking metabolism and identity in complex microbial communities due to its exceptionally high-throughput (10-100 millisecond per cell). Overall, SRS-FISH is at least two orders of magnitude faster than state-of-the-art methods: MAR-FISH (2-20 days per sample) (70), Raman-activated microbial cell sorting (>7.22 seconds per cell) (14), FISH-nanoSIMS (>10 seconds per cell) (71), which does not include the long preconditioning time) and spontaneous Raman-FISH (~20 seconds per cell) (40). Furthermore, implementation of FISH by TPEF can be advantageous when imaging thick biological specimens or live organisms, as near-infrared excitation enables deeper penetration into biological samples and causes less damage to cells (39).

The application of SRS-FISH to the gut microbiome demonstrated the suitability of our approach to link identity to metabolism within complex microbial communities, and at the same time revealed interesting new findings related to mucosal sugar foraging in the human gut. SRS-FISH measurements showed that Bacteroidales spp. tend to dominate the response to mucosal sugars over Clostridia spp. in all of the tested individuals (Fig. 5, Fig. S10). Indeed, the notion that Bacteroides spp. are major mucus degraders has been demonstrated by several studies (17, 61, 72). However, our results revealed that organisms from the Clostridium clusters XIVa/XIVb also substantially contribute to mucosal sugar degradation. Further, we show that larger fractions of Clostridia cells can forage on fucose compared to Bacteroidales cells (Fig. 5, Fig.S10). Fucose is an important sugar in the colon as it occupies a terminal position on host glycans, thus being at the interface of microbiota–mucus interactions (73). About 20% of human individuals naturally lack a functional copy of the FUT2 gene, and thus lack almost all gut fucosylation (74). Genome-wide association studies have shown that these individuals have an increased susceptibility to inflammatory diseases linked to the gut microbiota, such as Crohn’s disease (75, 76). Additionally, mice that lack the Fut2 enzyme have simpler gut microbiomes that are accompanied by a decrease in unclassified Clostridiales (76). These findings, together with our results, suggest that Clostridia may have been overlooked as fucose degraders in the gut (54). Elucidating which particular Erec482-targeted organisms use fucose may be key to design individualized probiotic interventions aiming to restore the homeostasis in humans lacking FUT2, and therefore in reducing their predisposition to gastrointestinal disease.

Another interesting finding from our study is that the pattern of mucosal sugar foraging differs between the murine and human microbiome: human gut bacteria preferentially metabolize GlcNAc (Fig. 5, Fig.S9), while the preferred sugar of the murine microbiome is galactose (17). This could reflect the different overall compositions of human and murine colonic mucins, i.e., while the human colonic mucin carries predominantly GlcNAc-containing core 3- and core 4-based O-glycans, the murine colonic mucin is mostly characterized by galactose-containing core 1- and 2-type structures (56, 57).
This finding may have important implications when translating results from mouse studies into humans.

There are several opportunities to further improve our SRS-FISH platform. These improvements include SRS-selective scanning of FISH targeted cells, which can even further improve the throughput of SRS-FISH when the taxa of interest appear in very low abundance. Besides, laser equipment with upgraded wavelength tuning speed will also provide the potential to gain higher throughput (77). Other than the throughput, the sensitivity and resolution of SRS-FISH can also be improved by implementing visible SRS (78, 79). Of note, the excitation beam in visible SRS can efficiently excite fluorophores from cells targeted by FISH and help avoid the TPEF bleaching issue when imaging cells in a liquid environment. On the other hand, the number of taxa simultaneously tracked by SRS-FISH can be substantially increased using spectral unmixing and custom-designed FISH probes (80–82). Regarding metabolism probing, besides using D2O as an activity marker to induce C-H peak shifts, deuterium-labelled substrates and other stable isotopes, such as 13C and 15N, could be used to track the metabolism of particular compounds and provide information on major catabolic activities and pathways. By targeting spectral features between 400-1800 cm⁻¹ (16), SRS could fingerprint major intracellular macromolecules that display shifts upon incorporation of stable isotopes. This could potentially be achieved by the implementation of hyperspectral SRS with ultrafast delay-line tuning and machine learning into the SRS-FISH platform (83). SRS-FISH would also be a useful tool to probe the distribution of several storage compounds and intrinsic biomolecules in diverse eukaryotic and prokaryotic cells (84–87).

In summary, we have developed an exceptionally high-throughput SRS-FISH platform and successfully applied this new tool to identify efficient mucosal sugar utilizers in the human gut microbiome. SRS-FISH can be applied to a broad range of environmental samples (e.g., marine sediments, soil) including those where some autofluorescence background is an issue because SRS is more resilient to sample autofluorescence than spontaneous Raman (20, 88). Meanwhile, SRS-FISH is not limited to microbiome samples. With the state of the art SRS metabolism imaging and versatile FISH techniques, such as probing abnormal proliferation of chromosomes or targeting mRNA, SRS-FISH will be broadly applicable to eukaryotes. By allowing the scanning of multiple samples in a fast and sensitive manner, SRS-FISH is well-suited to reveal fine-scale temporal, individual, and spatial patterns in a variety of specimens, which can otherwise be missed by existing methods due to their low-throughput.

Materials and Methods

SRS-FISH platform. A dual output, 80-MHz femtosecond (fs) pulsed laser (InSight X3, Spectra-Physics, USA) provides the pump beam (tunable from 680 nm to 1300 nm) and the Stokes beam (fixed at 1045 nm) for the fs SRS system (Fig. 1B). Stimulated Raman loss (SRL) provides the SRS intensity by detecting the modulation transfer from the Stokes to the pump beam. The Stokes beam was modulated by an acousto-optic modulator (1205c, Isomet Corporation, USA) at ∼2.26 MHz. After that, the AC signal was amplified by the build-resonant amplifier circuit centered at ∼2.26 MHz. Then the AC signal was further extracted by a lock-in amplifier (HF2LI, Zurich Instrument, Switzerland). The quantitative chemical maps were created when the energy difference between the pump and the Stokes beam matched the vibrational energy of the targeted chemical bond (C-D bond was centered at 2168 cm⁻¹ and C-H bond at 2946 cm⁻¹) (Fig. 1D). The off-resonance images were recorded when the pump beam was tuned to 830 nm (targeting 2479 cm⁻¹) for subsequent background subtraction in SRS (Fig. S3). As the femtosecond pulsed lasers have rather broad bandwidths, femtosecond SRS has a total covering range of 200 cm⁻¹ around the peak (29). Thus, in this study, the C-D and C-H signature peaks at 2040-2300 cm⁻¹ and 2800-3100 cm⁻¹ can be mostly covered by femtosecond SRS.

To incorporate FISH visualization into the platform, we implemented TPEF in the fs SRS system (Fig. 1A, right panel). Forward detection with a higher collection efficiency of the condenser better enabled the fluorescence signal. With a flip mirror, the light was directed into the fluorescence collection devices. Two silicon photomultipliers (C14455-3050GA, Hamamatsu, Japan) modules were implemented to provide better quality fluorescence images compared to photomultiplier tubes (HT422-40, Hamamatsu, Japan) with an external pre-amplifier (29). A 75 mm focal length lens focused the emission light onto the SPMs with a 605 nm cut-on dichroic mirror (DMLP605, Thorlabs, USA) that separated the emission into two paths. Two filters centered at 570 nm (ET570/20x, Chroma, USA) and 670 nm (ET670/50m, Chroma, USA) were used to detect the fluorescence from different FISH labeled cells with Cy3 or Cy5, which can be efficiently excited by the Stokes beam and the pump beam (for C-D, C-H or off-resonance due to the wide two-photon absorption bandwidth) in SRS respectively. A data acquisition card (PCL-e6363, National Instruments, USA) collected the final output to construct the images.

To evaluate the limit of detection for C-D and C-H bonds by our femtosecond SRS setup, measurements of DMSO/H2O mixtures in the concentration range from 0.004 to 4 M were conducted, each for isotopically unlabeled DMSO (purity ≥99.9%, D8418-500ML, Sigma-Aldrich) and fully deuterated DMSO (DMSO-d6, 99.96 atom % D, 156914-1G, Sigma-Aldrich) and C-D and C-H bond detectable by femtosecond SRS imaging amounts to 2.6×10⁶ per pixel. Thus, in this study, the C-D and C-H bond detectable by femtosecond SRS imaging amounts to 2.6×10⁶ per pixel.

To determine the SRS lateral resolution, 200 nm methylmethacrylate (MMA200, Degradex phospho col., USA) was used. By deconvoluting the SRS image with simulated nanoscale beads, we obtained the point spread function (PSF) of SRS (89), which was the product of pump and Stokes beam and had a FWHM of 2.6 µm (Fig. S1). With 210 nm yellow green fluorescence beads (17151-10, Polysciences, USA), the spatial and axial resolution of non-degenerate (ND)-TPEF, pump beam TPEF, and Stokes beam TPEF were measured in the liquid or dry conditions. The ND-TPEF was also the product of pump and Stokes beam, thus it is similar to SRS PSF measurement. Then ND-TPEF images were obtained by subtracting fluorescence images with pump and Stokes both on by pump only TPEF images and Stokes only TPEF images. Due to the broad two-photon absorption, we could detect fluorescence in imaging different samples, under both liquid and dry imaging conditions, although signal from the 1045 nm TPEF beam in dry conditions was too weak to see the fluorescence beads. The resolution was calculated following the steps of SRS resolution calculation. In the liquid environment, the lateral resolutions of pump only TPEF, Stokes only TPEF, and ND-TPEF by pump beams
Image acquisition. Fixed cells were spotted onto a poly-L-lysine coated coverslip, and covered and sealed by another coverslip with a spacer in between (38). Samples were prepared in this way to reduce the modulation signal while keeping the sample in the liquid environment that matches the refractive index of the water objective used for imaging. For imaging in the dry conditions, fixed cells were spotted onto a poly-L-lysine coated coverslip, dried, and then covered by another coverslip with spacers at two opposite sides. For each sample, three fields of view (FOV) were scanned by a motorized stage, or manually for SRS-FISH analysis. Three channels of SRS images (C-D, C-H, and off-resonance) and two fluorescence images (Cy3 and Cy5) were collected as a full image set for analyzing two populations targeted with FISH. Although fluorescence images could be acquired simultaneously with SRS-CD by splitting the output beam in the forward direction or collecting instead of dual-modal, limited acquisition time may not provide higher sampling speed for multichannel sampling. So all the images were acquired sequentially. Each FOV was 42.82 or 85.62 μm² with 214 nm per step and covered around 300~400 or 1200~1600 cells. Depending on the signal intensity level, 10 μs pixel dwell time with 1~10 frames average was applied for either SRS or fluorescence images. The laser wavelength tuning and stabilizing time for changing between different SRS frames was around 10 s. The throughput of SRS-FISH analysis is around 100~100 ms per cell (~10-100 cells per second) by taking into account the FOV moving time and laser wavelength switching time. For the microbiome test of 3 individuals’ samples in 8 different conditions, 3 randomly selected field of views were measured, which totally covered around thirty thousand cells.

Image processing. For bacterial cultures, three SRS images at C-D, C-H, and off-resonance were collected without fluorescence reference as their identities were known beforehand. For applying FISH masks in two targeted populations in the artificial mix or gut microbiome samples, two fluorescence images of Cy3 and Cy5 were recorded along with three SRS images. After all images were acquired (Fig. S4, Step 1), the illumination correction was applied to alleviate the uneven illumination causing intensity variation (Fig. S4, Step 2). Subsequently, SRS images were subtracted by the mean background intensity to eliminate the signal from the non-fluorescent areas (Fig. S3, Step 3). Three channels were rescaled according to the DC readout from the photodiode connected to the resonant amplifier circuit (Fig. S4, Step 4). The AC and DC signals were linear to the pump power within the power range used in this experiment. After that, the signal intensities from the off-resonance channels were subtracted from the rescaled C-D and C-H intensities which eliminated the other pump-probe background (Fig. S4, Step 5). Then the %CD_{SRS} images were calculated via \%CD_{SRS} = (I_{CD} - I_{LR})(I_{HC} + I_{LC} - 2I_{LR}) (Fig. S4, Step 6). Owing to the not perfect pixel-by-pixel off-resonance subtraction, resulting in over-correction, and the dominance of Moff at low C-D signal intensities, some bacteria with low D content exhibited %CD_{SRS} values of masked cells (Fig. S4, Step 10). By calculating the ratio between the number of bacteria in each FISH channel and in C-H channel, we quantified the percentage of FISH-stained cells labelling condition for each probe under different sugar amendment conditions (Fig. 4F,G). All imaging and statistical analyses were performed with CellProfiler and MATLAB (The MathWorks, USA).

Gut microbiome incubations. Human fecal samples were collected from three healthy adult individuals (one male and two females between the ages of 25 to 38) who had not received antibiotics in the prior 3 months. Study participants provided informed consent and self-sampled using an adhesive paper-based feces catcher (FecesCatcher, Tag Hemi, Zeijen, NL) and a sterile polypropylene tube with the attached sampling spoon (Sarstedt, Nümbrecht, DE). The study protocol was approved by the University of Wisconsin Ethics Committee (reference No.00161). All data was anonymized and compliant with the University’s regulations.

Samples were transferred into an anaerobic tent (Coy Laboratory Products, USA) within 30 min of collection. Samples were suspended in M9 medium (prepared with H2O and without glucose) to achieve a concentration of 0.1 g/ml-1, and further diluted 20 times in this medium. The homogenate was left to settle for 10 minutes, and the supernatant was then distributed into glass vials. An equal volume of M9 (without glucose) prepared with either D2O (99.9 at% D; Sigma Aldrich) or H2O (99.9 at% H; Sigma Aldrich) was added to each vial, and each vial was finally supplemented with different concentrations of mucosal sugar monosaccharides (N-acetylneuraminic acid: 2 mg/ml⁻¹; N-acetylglucosamine: 5 mg/ml⁻¹; fucose: 2.5 mg/ml⁻¹; galactose: 5 mg/ml⁻¹; N-acetylgalactosamine: 2.5 mg/ml⁻¹), D-glucose (5 mg/ml⁻¹) or nothing (no-amendment control) (all amend-

Growth and labeling of microbial pure cultures. Escherichia coli K12 (DSM 498) was grown aerobically at 37°C in Luria-Bertani liquid medium (LB; DSMZ medium 381) or M9 minimal medium containing per L of medium: 7.5 g Na₂HPO₄ 2H₂O, 3 g KH₂PO₄, 0.5 g NaCl, 0.5 g NH₄Cl, 1 mM MgSO₄, 0.3 mM CaCl₂, 1 mg thiamine hydrochloride (Sigma-Aldrich), 1 mg biotin (Sigma-Aldrich), and 0.4% (w/v) D-Glucose (Carl Roth GmbH). For D labeling of E. coli cultures, 50 µl of a stationary-phase culture were used to inoculate 5ml of LB or M9 medium containing different percentages (vol/vol) of D2O (99.9 at% D; Sigma Aldrich). Cells were grown until the late exponential phase (3 h in LB medium or 5 h in M9) by carbon dioxide (CO₂) gas flush and were fixed in 4% formaldehyde in phosphate-buffered saline (PBS) for 2 h at 4°C. Cells were subsequently washed once with PBS and stored at 4°C until further use. Bacteroides thetaiotaomicron (DSM 2079) cells were grown anaerobically (85% N₂, 10% CO₂, 5% H₂) in BHI broth (DSMZ medium 215c) or in Bacteroides minimal medium (92) containing different percentages (vol/vol) of D2O (99.9 at% D; Sigma Aldrich). After 6 hours of growth at 37°C, cells were harvested by centrifugation, resuspended in PBS, and fixed by adding formaldehyde in PBS as described above. All cells were stored in a PBS solution at 4°C until further use. Clostridium scindens (ATCC35704) and Blautia producta (isolate Y6.3- GenBank accession number OM489277) were grown anaerobically (85% N₂, 10% CO₂, 5% H₂) in BHI broth (DSMZ medium 215c) or in Bacteroides minimal medium (92) supplemented with 5 g per litre of casamino acids (MP Biomedicals™) (BMMs) containing different concentrations (vol/vol) of D2O (99.9% atom % (at%) D; Sigma Aldrich). Supplementation with casamino acids was necessary as for C. scindens and B. producta to grow on BMM, as these organisms were either predicted or shown to be auxotrophs for some amino acids (93, 94). After approximately 8 or 12 hours of growth at 37°C in either BHI or BMMs, C. scindens cells were harvested by centrifugation, resuspended in PBS, and fixed by adding formaldehyde in PBS as described above. B. producta cells were harvested following the same procedure, but after 5 or 8 hours of growth at 37°C in either BHI or BMMs.
ment chemicals were from Sigma–Aldrich, except D(+)-galactose which was purchased from Carl Roth GmbH. These concentrations were selected based on reported concentrations of the different monosaccharides in purified hog gastric mucin and mucin gels, as described in (17). Once all incubations were set, an aliquot sample from the negative control was immediately collected, pelleted and stored at -20°C until further use (T0 sample). After incubation for 6 h at 37°C under anaerobic conditions (5% H2, 10% CO2, 85% N2), two sample aliquots from each incubation were collected by centrifugation. One aliquot washed with 1× PBS to remove D2O and then fixed in 4% formaldehyde for 2 h at 4°C. Samples were finally washed twice with 1 ml of PBS and stored in PBS at 4°C until further use. The second aliquot was stored at -20°C until further processing.

FISH. Fixed cells (100 µl) were pelleted at 14 000 g for 10 min, re-suspended in 100 µl 96% analytical grade ethanol and incubated for 1 min at room temperature for dehydration. Subsequently, 100 µl of 900 mM NaCl, 20 mM TRIS HCl, 1 mM EDTA, 0.01% SDS and 100 ng of the respective fluorescein labeled oligonu- cleotide as well as the required formamide concentration to obtain stringent conditions (Table S1). After hybridization, samples were diluted 100× with cold PBS and transferred into iced centrifuge tubes with 2× PBS. The suspension was centrifuged at 14 000 g for 15 min at maximum allowed temperature (40°C), to minimize unspecific probe binding. Samples were washed in a buffer of appropriate stringency (95) for 15 min at 48°C, cells were centrifuged for 15 min at 14 000 g and finally resuspended in 20 µl of PBS. Cells (5 µl) were spotted on Poly-L-lysine coated cover glasses No. 1.5H (thickness of 170±5 µm, Paul Marienfeld EN) and allowed to dry overnight at 4°C, protected from light. Excess of salt was removed by dipping the coverslips 2× in ice-cold Milli-Q water and allowed to dry at room temperature protected from light.

Confocal fluorescence microscopy. Samples were spotted onto microslide slides (Paul Marienfeld EN) with Poly-L-lysine coating and visualized using an Olympus scanning confocal microscope (FV3000) with a 60X oil objective (PLAPON60XO, 1.42 NA, 0.15 mm WD, Olympus) and two high-sensitivity photomultiplier tubes (PMTs). Cy3 double-labeled probe Bac303 was excited by a 514 nm solid state diode laser and its emission was collected between 530 nm and 630 nm. Cy5 double-labeled probe Erec482 was excited by a 640 nm solid state diode laser and its emission was collected between 650 nm and 750 nm. Transmission images were also collected for validating the focus and the distribution of the whole gut microbiome sample. The ideal spatial resolution limit is around 221 nm×275 nm. The scanning step size of the confocal image was set to 1.5 µm for avoiding missing intensity in overlapping areas due to the Nyquist frequency for both excitation wavelengths. Each acquired field of view is 212×942 µm².

Spontaneous Raman microspectroscopy. Fixed cells were spotted on aluminum-coated slides (Al136; EMF Corporation). Excess of salt was removed by dipping the slide twice into ice-cold Milli- Q water. Individual cells were observed under a 100×/0.75 NA microscope air objective Single microbial cell spectra were acquired using a LabRAM HR800 confocal Raman microscope (Horiba Jobin-Yvon) equipped with a 532 nm neodymium-yttrium aluminum garnet (Nd:YAG) laser and 300 grooves/mm diffraction grating. Spectra were acquired in the range of 400–3200 cm⁻¹ for 30 s with 2.18 mW laser power. Spectra were then aligned according to the phenylalanine peak region and a background correction using the sensitive nonlinear iterative peak-clipping algorithm was applied as described before (10). Spectra were then normalized by dividing the spectral intensity at each wavelength by the total spectral intensity of the analyzed Cy3 double-labeled probe Bac303 was excited by a 514 nm solid state diode laser and its emission was collected between 530 nm and 630 nm. Cy5 double-labeled probe Erec482 was excited by a 640 nm solid state diode laser and its emission was collected between 650 nm and 750 nm. Transmission images were also collected for validating the focus and the distribution of the whole gut microbiome sample. The ideal spatial resolution limit is around 221 nm×275 nm. The scanning step size of the confocal image was set to 1.5 µm for avoiding missing intensity in overlapping areas due to the Nyquist frequency for both excitation wavelengths. Each acquired field of view is 212×942 µm².

Amplification and sequencing of the 16S rRNA genes. Pellets of microbiome incubation samples were resuspended in 1 ml of InhibitEX Buffer (Qiagen) and subjected to bead beating for 40 seconds at 6 m/s in Lysis matrix E (MPBiomedicals) tubes. DNA extraction was performed with the Qiaamp Fast DNA stool kit (Qiagen) according to the manufacturers protocol. Amplification of bacterial 16S rRNA genes from DNA extracts was performed with a two-step barcoding approach (UDB-H12 (96)). V1-V3 amplicons were made using primers which was smaller than half of the diffusion limit of both beams and ensured sampling spatial frequency higher than the Nyquist frequency for both excitation wavelengths. Each acquired field of view is 212×942 µm².

Analysis of 16S rRNA gene amplicon sequences. Amplicon pools were extracted from the raw sequencing data using the FASTQ workflow in BaseSpace (illumina) with default parameters (96). Input data was filtered for PhiX contamination with BBduk (BBTools, Bushnell B, sourceforge.net/projects/bbmap). DADA2 classifier (100) was used for demultiplexing amplicon sequencing variants (ASVs) from the negative control was immediately collected, pelleted and stored at -20°C until further use. The second aliquot was stored at -20°C until further processing.

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