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Personalized microbiome dynamics – Cytometric fingerprints for routine diagnostics

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ABSTRACT

Microbiomes convoy human life in countless ways. They are an essential part of the human body and interact with its host in countless ways. Currently, extensive microbiome analyses assessing the microbiomes' composition and functions based on sequencing information are still far away from being routine analyses due to the complexity of applied techniques and data analysis, their time demand as well as high costs. With the growing demand for on-time community assessment and monitoring of its dynamic behavior with high resolution, alternative high-throughput methods such as microbial community flow cytometry come into focus. Our flow cytometric approach provides single-cell based high-dimensional data by using only three parameters but for every cell in a system which is enough to characterize whole communities' attributes with high acuity over time. To interpret such complex cytometric time-series data, novel concepts are required.

We provide a workflow which is applicable for easy-to-use handling and measurement of microbiomes. Drawing inspiration from macro-ecology, in which a rich set of concepts has been developed for describing population dynamics, we interpret huge sets of community single cell data in an intuitive and actionable way using a series of bioinformatics tools which we either developed or adapted from sequence based evaluation approaches for the interpretation of single cell data. The developed evaluation pipeline tests for e.g. ecological measures such as community assembly, functioning, and evolution. We also addressed the meta-community-concept which is a well acknowledged idea in macro-ecology on how interconnected communities perform. The last concept discusses stability which is a metrics of paramount importance. A fast quantification of stability properties may not only detect disturbances and their impact on the organisms but also allow for on-time microbiome treatment.

The workflow's immanent ability to support high temporal sample densities below bacterial generation times provides new insight into the ecology of microbiomes and may also provide access to community control for microbiome based health management. The future developments will facilitate cytometric fingerprinting for human routine diagnostics to be as simple and meaningful as a blood count today.

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1. Introduction

The human meta-organism consists of eukaryotic human cells as well as a significant share of cells of microbial origin (Savage, 1977; Sender et al., 2016). These trillions of microbial cells represent our individual human microbiome and contain up to 300-fold more unique genes then the human genome (Yang et al., 2009; Blaser et al., 2013). The presence of our microbiome is vital for our wellbeing. The beneficial microbiota in the different habitats provided by our skin protect us against invasion of pathogenic microorganisms and modulate our innate as well as adaptive immune response (Grice and Segre, 2011). Up to 10^7 bacteria (number based on colony forming units, cfu) reside a cm^2 of our skin dependent on body site (Leyden et al., 1987) with up to 10^{12} cells in total on the skin of a single person (Wilson et al., 2002). And despite the exposure of the skin to the external environment, the skin microbiome is individual to location and person and relatively stable over time (Oh et al., 2016). In the same way our outer body is covered by microorganisms also our inner mucosal surfaces are populated by diverse microbiomes. The oral cavity contains up to

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10^7 cfu ml^{-1} saliva of *Lactobacillus* species (Dal Bello and Hertel, 2006) or up to 10^9 bacteria ml^{-1} saliva (Berg, 1996) and is the most variable body site regarding microbial community composition over time (Ding and Schloss, 2014). The highest cell density is reached in the colon with up to 10^{11}-10^{12} microorganisms per gram of colon content (Berg, 1996; O'Hara and Shanahan, 2006). The colonization of the human gastrointestinal tract starts directly after birth and following ecological succession a stable diverse gut microbiome develops within the first years of life (Lozupone et al., 2012). The gut microbiome not only determines the success of our daily digestion and hence the exploitation of nutrients from food and formation of micronutrients (Cassani et al., 2012; Crozier et al., 2010; Kau et al., 2011), it also severely impacts the health status of the human host and is associated to obesity-associated inflammation (Shen et al., 2013) and diabetes (Hartstra et al., 2015) and even a potential gut-brain axis has been identified (Benakis et al., 2016).

Altogether, our body constitutes numerous individual ecosystems with strong niche specialization (Human Microbiome Project, 2012). Thereby, the relationship between the human host and its microbiome is mainly symbiotic (Falk et al., 1998). However, temporal variations in the microbiome composition at different body sites can be found as a result of natural selection due to extrinsic (e.g. food, medication) and intrinsic factors (e.g. adaptive immune system) (Caporaso et al., 2011). The underlying mechanisms that shape our microbiome and the dependent effects on our health are not yet understood and require comprehensive studies that resolve the ongoing mechanisms.

The recent advances in DNA based culture independent methods have expanded our knowledge on the diversity of the human microbiome, its biogeography and its dynamics. Nevertheless, extensive microbiome analyses are until now only performed within research projects and still far away from being routine analyses due to the complexity of applied techniques. Although the development of sequencing based technologies regarding speed and costs are promising (Kuczynski et al., 2012; Goodwin et al., 2016), the main unresolved challenge is the automated data analysis and interpretation and especially the translation of the results into clinical actions and treatments (Cho and Blaser, 2012; Fournier et al., 2014). Further, one has to differentiate the clinical requirements. While the presence of a specific antibiotic marker or virulence is well targeted with sequencing based technologies (Fournier et al., 2014; Deurenberg et al., 2017), the demand for rapid and high-throughput microbiome monitoring can be perfectly met with microbial flow cytometry. Neither 16S rRNA gene affiliation (Bloom et al., 2011), gene association studies (Jostins et al., 2012), expression profiles of genes (Schulze et al., 2016) or metaproteomes (Schaubeck et al., 2016) are given by microbial flow cytometry. Instead, cellular characteristics such as morphology and physiology are recorded which describe the state of a microbial community being determined by its cytometric diversity and function. This review introduces the principle and analyzing routines of this single-cell based method for microbiomes (section 2) and illustrates its application and identification of underlying ecological patterns (section 3).

2. Cytometric characterization of microbiomes

Flow cytometry is a powerful technology that is widely applied in biomedical research and clinical routine diagnostics by now more than 30 years. It allows the characterization and discrimination of single human cells with high precision and in a high-throughput manner providing the classical foundation for disease monitoring from the standard blood count until advanced tumor biology and immune therapy (Muirhead et al., 1985; Perfetto et al., 2004).

A flow cytometer consists of a measurement chamber and the connected light source (one or several lasers of different wavelength) as well as the optical bank with the optical filters and photomultiplier that convert the optical into a digital signal. The cells are introduced in a fluid stream and pass the laser beam cell by cell. When the cells pass through the beam the light is scattered (forward and sideward scatter signal) and possible present fluorochromes are excited. In most cases, specific monoclonal fluorescently labeled antibodies for certain cell or cell-surface targets are applied. In this way, the specific optical characteristics of every individual cell are recorded and up to 10 000 cells s^{-1} can be easily measured. In addition to the high resolution and speed of analysis, cell sorting is a striking feature that allows to physically separate cells with a specific combination of optical characteristics into a test tube or on a microscopy slide facilitating further analysis. Recent advances in automated data analysis also enabled to work with more complex multi-parametric datasets (Bashashati and Brinkman, 2009; Lugli et al., 2010). In this way, flow cytometry represents an established powerful tool for monitoring states of health and disease in humans.

The application of flow cytometry is not restricted to human eukaryotic cells. Also microbial cells, which can be associated to the human body, can be measured. The general difference lies in the cellular characteristics of prokaryotic in contrast to human eukaryotic cells. Besides the cell size (0.3 to a few μm) and the cell volume (0.1–10 μm^3) being (an) order(s) of magnitude lower than the average eukaryotic cell, the high diversity of different microbial species results in a multitude of different cellular characteristics (e.g. differences in gram staining, presence of surface proteins, lectins, etc.) and impedes the establishment of general, antibody based markers for cellular surface characteristics. This challenge is further complicated regarding the development of protocols and the confirmation of their specificity of the chosen markers (e.g. membrane integrity, enzyme activity, presence of storage compounds; Müller and Nebe-von-Caron, 2010) as many microorganisms in environmental samples cannot be cultivated and thus protocols cannot not be verified. Despite their high diversity, the least common denominator in all microbial cells is the presence of DNA. Therefore, DNA labelling serves as a universal marker that can be applied to all microbial cells independent of their phylogeny, metabolic capacity or actual activity. Although being a universal marker, the DNA staining specifically mirrors the DNA content in the microbial cells being representative of their number of chromosome equivalents and their physiological state (Müller, 2007). In this way, also complex microbial communities can be characterized in a simple and rapid way (Koch et al., 2013c) and the state and dynamics of these communities interpreted as marker for healthy and disturbed ecosystems (Günther et al., 2016; Koch et al., 2013b; Zimmermann et al., 2016).

In the following sub-chapters the general procedure of microbial flow cytometry and cytometric fingerprinting is described, including routine laboratory as well as data analysis procedures which are also applicable to human associated microbial samples.

2.1. Microbial flow cytometry

Most microbial samples are suitable for cytometric characterization and an established step-by-step protocol with several variants for specific sample requirements (e.g. flocs and biofilms, anaerobic samples) is available (Koch et al., 2013c). First the samples are stabilized by fixation (e.g. 2% paraformaldehyde for 30 min at room temperature, followed by centrifugation at 3200*g*, 20 min, 15 °C and subsequent resuspension in 70% ethanol (1:8 volsample/volfixative) and storage at −20 °C (Günther et al., 2016)). Alternative fixation methods are described for different sample

types, e.g. environmental samples (Wallner et al., 1995; Günther et al., 2008), colon biopsy samples (Zoetendal et al., 2002), pathogenic bacteria (Depke et al., 2010), and faeces (Rigottier-Gois et al., 2003; Zimmermann et al., 2016). In this way, the cells can be stored until measurement. Dependent on the sample and the fixation procedure this stability can usually be given for some days until months and has to be tested for every new sample type. Sample disaggregation procedures are usually applied after fixation and prior staining (Koch et al., 2013c). Different DNA staining procedures are available and should be tested before application for new samples. To our experience, so far best results regarding resolution and single cell differentiation are achieved with DAPI. The staining is performed in a two-step procedure: First, the cells are shortly incubated in a solution containing citric acid and Tween20 to make them better accessible for the dye. Second, the cells are incubated with a low concentrated DAPI solution (0.24–0.68 μM) for 1–3 h to allow the dye to bind quantitatively to the DNA in the cells. Afterwards the stained cells are stable for up to 24 h and will result in highly reproducible cytometric pattern (schematic workflow in Fig. 1). Each cytometric measurement comprises then the analysis of 250 000 cells and requires about 3 min for routine sample analysis.

The standard parameters for cytometric fingerprinting are the forward scatter signal (related to cell size) and the DAPI-DNA fluorescence of the cells. Additional parameters such as the side scatter signal or further fluorescences can also be considered as cellular markers (Günther et al., 2009). Accordingly, every cell is characterized by a set of measured characteristics. Visualizing these results in a 2D-plot based on forward scatter signal and DAPI-DNA fluorescence every cell will have a specific position (Fig. 1 E and Supplementary Video). Cells with similar characteristics will be close to each other in the plot (i.e., within the same subcommunity), while cells with different characteristics will be more apart from each other (i.e., in Fig. 1 F subcommunity SC2 contains cells with a higher DNA content compared to subcommunity SC5, while both have the same cell size). In this way, the cytometric measurement represents a specific cytometric fingerprint of a particular microbial community structure at the time point of sampling, representing the variety of cells with different optical characteristics as well as their abundance and being representative of the state of the microbial community. Additionally, the overall cell number of the community in the sample can be determined by adding a defined number of fluorescent beads.

Supplementary video related to this article can be found at http://dx.doi.org/10.1016/j.mam.2017.06.005

2.2. Data analysis

While a single cytometric measurement represents the community structure based on the location of subcommunities in the 2D-plot, consecutive measurements of a microbial community over time or at different locations, e.g. different body sites, allow monitoring of microbial dynamics. These dynamics are characterized by changes in the number of subcommunities and the individual abundances of cells in each subcommunity. These differences are representative of functional changes in the microbial community due to individual physiological changes (the DNA content of the cells mirrors the state of the individual cell cycle and population growth conditions (Müller, 2007)) as well as changes in the phylogenetic composition of the microbial community (new species with different cell characteristics coming up or present cells disappearing). Therewith, microbial flow cytometry facilitates fast and high-resolution monitoring of changes in the community structure. The interpretation of the changes in the cytometric patterns requires quantitative data analysis procedures. These are available for different levels of analysis and will be described within the following sections.

The simplest tool for comparing a set of cytometric measurements is available with flowCHIC (Schumann et al., 2015a), a fast and nearly automated evaluation tool that is based on *c*ytometric *h*istogram *i*mage *c*omparison (CHIC, Koch et al., 2013a). The multiparametric single cell information of 250 000 cells in a measurement is reduced to a 2D-image with the individual pixel intensity in the image being representative of the cell distribution in the original measurement. Although individual cell information is lost in this way, the community structure is still well represented by the images which can be processed easily and operator independent. CHIC results in a dissimilarity matrix based on image comparison for all samples with each other. These differences can be further visualized with multidimensional scaling to decipher general trends in the community dynamics (Fig. 1 G). While the dissimilarity value between two samples does not represent the source of the difference, being either the result of many small differences over the complete microbial community or a drastic change in a single subcommunity, this question can be answered with segregated analysis provided by other analyzing tools.

Segregated microbial community analysis is realized with flowCyBar (Schumann et al., 2015b). Here, the individual subcommunities of a dataset, consisting of all cytometric measurements to be compared with each other, are identified and a combined gate template covering all of them is generated. Subsequently, the cell abundance in each gate is monitored over all samples. Using a heat map like visualization in the so called CyBar plot, the abundance changes in each subcommunity can be easily followed (Fig. 1 H). Although being manual and operator dependent at the step of the initial gating procedure (all follow up procedures are then automated in the respective R package), the flowCyBar procedure allows a detailed visualization of the ongoing dynamics and also enables to decide for subcommunities of interest for respective cell sorting. The characterization of the cytometric fingerprint regarding its subcommunities and their abundances is also crucial for the functional and ecological interpretation of microbial community dynamics. Looking at the schematic cytometric fingerprint in Fig. 1 F, 7 subcommunities are differentiated ($D_{cyt} = 7$) with between 1 and 5 cells detected per subcommunity. All subcommunities are consecutively numbered (SC1, SC2, etc.) and can then be administered in a sample SX as S_{Fig1F}(SC1; SC2; etc), with the relative cell abundance per respective gate being documented. As already said, this Figure represents only a schematic representation of a cytometric fingerprint. In reality, 250 000 cells are recorded (see also Supplementary Video) and usually between 1000 and 60 000 cells are found in each subcommunity dependent on the origin of the sample. This facilitates the identification of individual subcommunities and the generation of the gate template.

2.3. Functional interpretation

The next step for a functional interpretation of the recorded dynamics is based on multivariate statistics and similar to data evaluation procedures used by other culture independent methods applied in microbial ecology (e.g. high-throughput sequencing technologies or DNA fingerprinting methods (Lozupone et al., 2012; Paliy and Shankar, 2016; Ramette, 2007)).

First, the data can be analyzed in an exploratory way to identify potential underlying trends in the flow cytometric data. The combined data (data matrix resulting from flowCHIC or flowCyBar analysis) can be visualized in a way that more similar samples are displayed closer to each other while more dissimilar samples are more apart. This kind of grouping can reveal if a certain degree of

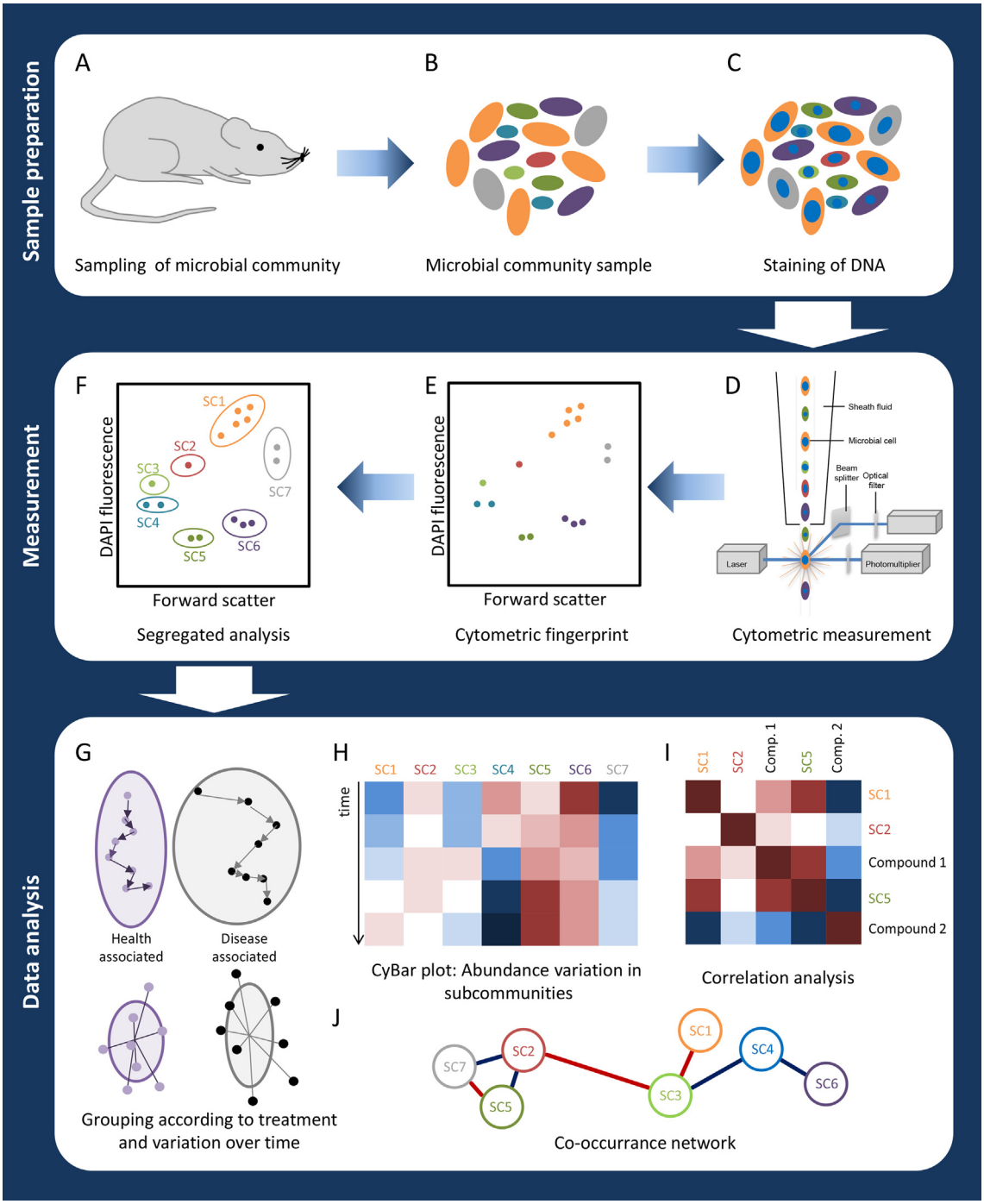

Fig. 1. The routine procedure for microbial flow cytometry is widely applicable and comprises the following steps: A) Sampling of the microbial community. This can e.g. be a swab of the skin or the saliva, a faeces sample or a direct gut microbiome sample but also any other kind of environmental microbiome sample. B) These microbiome samples are diverse microbial communities consisting of species with different phylogenetic background, cellular characteristics and functions. C) For routine microbial flow cytometry the DNA in the cells is quantitatively stained with DAPI. D) A cytometric measurement comprises the analysis of 250 000 cells regarding forward and sideward scatter as well as DAPI-DNA fluorescence and takes about 3 min. Accordingly, every cell is characterized by a set of measured characteristics. E) These characteristics can be visualized in a 2D-plot. Depending on forward scatter signal and DAPI-DNA fluorescence every cell will have a specific position in the plot and cells with similar characteristics will be closer to each other. In this way, the cytometric measurement represents the cytometric fingerprint of the microbiome. F) The cytometric fingerprint of the microbiome represents the variety of cells with different optical characteristics as well as their abundance in respective subcommunities (SCs) at the time point of sampling. The SCs can be identified based on segregated analysis and are marked by gates. After measuring a number of samples collected over time of the same sampling site or from different sampling sites data analyzing routines are applied to reveal community dynamics and underlying trends based on quantitative data analysis. G) Community dynamics can be combined with functional information that categorizes samples according to their specific treatment, clinical indication or sample origin. H) Segregated analysis based on flowCyBar reveals individual abundance variations in the SCs over time. Cell number increases (shades of red) and decreases (shades of blue) in a SC can be differentiated. I) Correlation analysis reveals potential SC functions. J) Co-occurrence analysis reveals interconnections between the individual SCs.

categorization, e.g. regarding a specific treatment, clinical indication or sample origin, is mirrored by the microbial community structure. Further, the evolution in the microbial community structure over time can be envisioned. Fig. 1 G displays potential variants in a schematic way being representative for variation in community structure over time as well as grouping into health and disease associated microbial communities. Also the speed of changes in the microbial community structure and its degree of variation is indicative for a functional interpretation as will be discussed in more detail in section 3. Ecological concepts of microbiome behavior.

A more detailed analysis of the correspondence of general changes in the community structure or the individual changes in the subcommunities is possible if the correspondent experimental or clinical data as well as information on sample related environmental gradients and specific environomic factors is available. Then correlation analyses can be performed using ready-to-use protocols included in the available R packages (providing e.g. Spearman and Pearson correlation analyses) as well as further measures for environmental interpretation based on multivariate statistics (see e.g. Ramette (2007) for methods overview). The outcome can then provide a general interpretation, e.g. the microbial community structure changes in response to a change in feed/food composition, but also more specific, e.g. the subcommunities SC1 and SC5 increase their abundance 5fold after a change of the feed/food composition, while SC4 disappears nearly completely (10fold decrease in abundance) (Fig. 1 H). Combined with Spearman correlation analysis (Fig. 1 I) a significant and highly positive correlation between SC1 and SC5 (red color) as well as both with compound 1 can be revealed. In contrast their correlation with compound 2 is negative (blue color).

The cytometric data analysis can be further expanded to network analysis based on co-occurrence (Günther et al., 2016) resulting in a complex visualization of the underlying interconnections between the individual subcommunities (Fig. 1 J) and also include experimental metadata. Altogether, there are no methodological limitations regarding data analysis and the result matrices can be introduced into established routines available for microbial ecology.

2.4. Cell sorting

Segregated microbial community data in combination with a functional interpretation can be used to identify the functional relevant subcommunities in a microbial community. Based on this pre-assessment these subcommunities can be physically separated from the rest of the community and further analyzed using standard molecular biology techniques (e.g. genomics (Zimmermann et al., 2016), proteomics (Jehmlich et al., 2010)). In this way, the functional subcommunities can be characterized for their phylogenetic affiliation but also the presence of specific functional genes or proteins can be investigated. This approach reduces the number of samples to be characterized with molecular techniques to functional relevant ones, while at the same time increasing the resolution of the performed analyses as not all cells contribute anymore. By this methodical combination a fast routine monitoring can be performed in combination with detailed functional and phylogenetic knowledge of individual subcommunities.

At this point we would like to highlight that the extent of the performed analyses with cytometric fingerprinting is highly flexible for different applications and research questions related to microbial community analyses including a high temporal resolution due to short sample preparation and analysis times. Nevertheless, we would also like to point to the fact, that all cytometric analyses, starting from the routine measurements until setting up a cell sorter, require a skilled operator and inclusion of respective controls to ensure a reliable and meaningful data interpretation. By this powerful combination even monitoring of the community stability of the intestinal microbiota considering its hour-scale fluctuations (Thaiss et al., 2015) can be well imagined and investigated with high resolution and in a resource efficient and rapid way based on cytometric fingerprinting.

3. Ecological concepts of microbiome behavior

3.1. Diversity and function

Diversity and function are parameters that describe vital characteristics of microbial communities. Both can be regarded as marker for community states and they thus play a crucial role as indicators for community changes. Microbial diversity is usually determined by sequence based technologies by addressing variable regions of the 16S rRNA genes or by using metagenomics approaches resulting in e.g. microbiome-wide association studies (Wang and Jia, 2016) which are also used for dynamic studies (Faust et al., 2015). Microbial functions are addressed on different levels as are functional genes, their transcripts, or the proteins using most often omics approaches. All levels provide bulk data. Also single bacterial cells can be addressed on the genome level by single-cell whole genome amplification and sequencing. But comparable applications on the single cell functional level are not yet routinely applied for bacteria although single cell transcriptomics and proteomics are well established in eukaryotic research (Junker and van Oudenaarden, 2014; Leclercq et al., 2017; Macaulay and Voet, 2014; Möllerström et al., 2015; Tang et al., 2011). Microscopic technologies such as NanoSIMS (Zimmermann et al., 2015) and Raman-microscopy (Heidari-Torkabadi et al., 2015) seem promising for measuring functional diversification but are slow with measuring only few cells at a time, thus limitedly suited for routine application with high-throughput. A much faster option is microbial community flow cytometry. Although no classical genetic or proteomic information is gained, cytometric fingerprinting can be used to decide on diversity and function of microbiomes (Koch et al., 2013c).

In macro-ecology, diversity is defined by both species richness and evenness where richness considers number of different species (but not abundance per species) while evenness considers the equal distribution of species and their respective abundance in a location. This concept translates in cell-based cytometric characteristics as well. Using only one of two intrinsic (forward or sideward scatter signal) and one extrinsic (DNA fluorescence) characteristics of cells the diversity D can be calculated by i) identifying distributions of cells in a 2D-plot which are called subcommunities (SCs), ii) counting the numbers of SCs in a 2D-plot and iii) counting the abundances of cells in each SC. Commonly, diversity is expressed as Dq using order-based Hill numbers (Hill, 1973) such as D_0 ($q = 0$), D_1 ($q = 1$), and D_2 ($q = 2$), which are explained in the following. A cytometric diversity D_0 ($q = 0$) considers only richness, being the number of identified SCs independent of the abundance of cells in each of them. Values $q > 0$ consider increasingly abundance data and the evenness of the cell abundance distribution between the subcommunities (Props et al., 2016).

For illustration, the cytometric diversity values are exemplarily applied to a small constructed example data set (Fig. 2) that could also be the result of a microbiome study. It visualizes the impact of an antibiotic treatment on the microbiome and the different alternatives for its recovery. The cytometric diversity values $D_{0\text{-}2}$ were calculated according to the formula of Props et al. (2016) which was adjusted for our example to gate-based instead of bin-

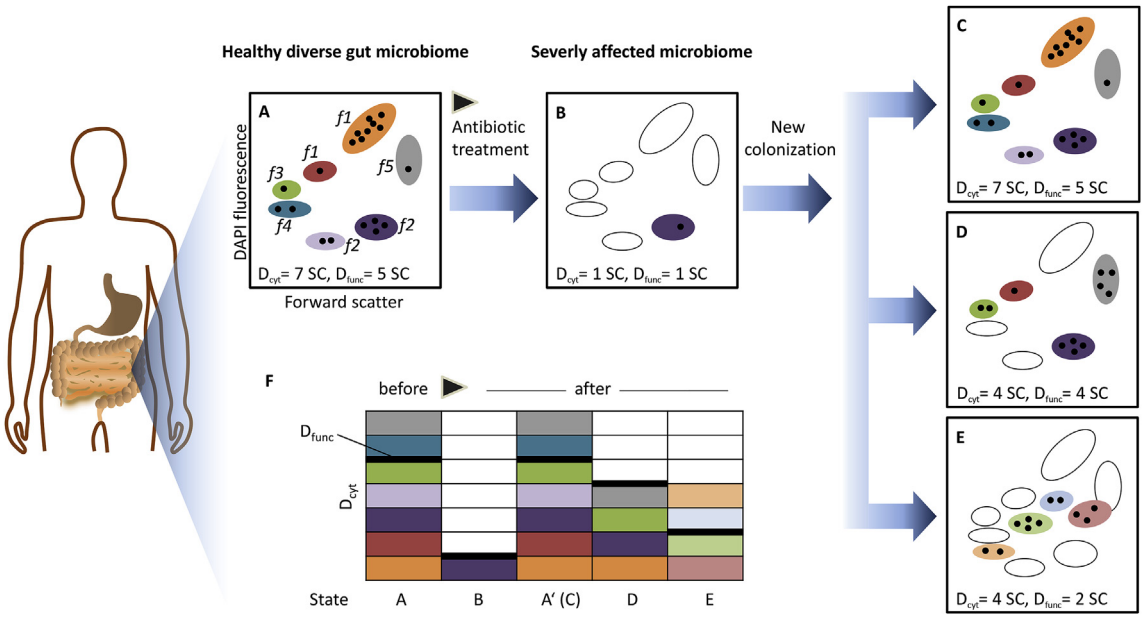

Fig. 2. Schematic illustration of the effect of antibiotic treatment on the human gut microbiome and its analysis based on microbial flow cytometry for a constructed example data set. A) A normal and healthy human gut microbiome is characterized by a characteristic cytometric diversity (D_{cyt}) which comprises several subcommunities (SCs) that differ in their abundance and function (D_{func}). B) After antibiotic treatment the microbial community is reduced leading to loss of phylotypes and functional diversity. A new colonization of the human gut occurs during patient recovery and can result in different microbial communities (C–E). C) The community is recovered with all SCs and functions exactly as before the treatment. D) The community is recovered in a reduced state compared to before the treatment. Three SCs are completely lost and two SCs changed cell abundance compared to before the treatment. The diversity is higher than directly after the antibiotic treatment but still lower than before. E) The recovered microbial community is very different from the community before the treatment. This can infer long-term loss of microbiome functions and the risk of colonization of pathogenic species. F) Visualization of the concept of community states based on the numbers of SCs(D_{cyt}) and their respective functional properties (D_{func}). The antibiotic treatment operates as disturbance after which new community states establish. For state A′ the community returns to its original state comprising all SCs and functions. Alternatively, the SCs and functions are only partly restored and result in state D, or a completely different community establishes with state E. For simplification, the cell abundance data were not included for visualization but would be part of the mathematical considerations.

based information. A normal and healthy human gut microbiome is characterized by a diverse microbial community consisting of several subcommunities (here $D_{cyt} = 7$, Fig. 2 A) that differ in their position in the 2D-plot and their cell abundance. Describing the community diversity mathematically, it reads $D_0 = 7$, $D_1 = 5.1$ and $D_2 = 4.0$. Beside the optical cell information provided by the cytometric measurement, also the functional information of the individual subcommunities (as described in section 2.3) can be included. The healthy microbiome contains five functionally different subcommunities (each labeled with fx in Fig. 2 A) leading to a functional diversity of $D_{func} = 5$.

Applying an antibiotic treatment, it is well known that the microbial community is reduced. This can also be depicted from the resulting cytometric pattern (Fig. 2 B). Most subcommunities are completely lost and only cells in a single subcommunity were able to survive the antibiotic treatment. Accordingly, the diversity is obviously reduced and now reads $D_0 = 1$. D_1 and D_2 are disregarded due to the low number of SCs.

In the process of patient recovery also the gut microbiome is restored. This can be partly induced through microorganisms originating from the oral cavity but also from food intake (Dal Bello and Hertel, 2006). Dependent on the species that survive the antibiotic treatment as well as the species newly colonizing the gut, a different community structure and function compared to the previous state of the patient is possible. While in Fig. 2 C the community recovered with all subcommunities and functions exactly as before the treatment, Fig. 2 D represents a reduced microbial community. Some sucommunities are completely lost and one subcommunity changed its abundance compared to before the treatment. The diversity is higher than directly after the antibiotic treatment but still lower than before. It now reads as $D_0 = 4$, $D_1 = 3.5$ and $D_2 = 3.3$. Due to the subcommunity loss also the functional diversity is affected and only four functions remain. Fig. 2 E represents a third case that is also often found in practice (Bäumler and Sperandio, 2016; Theriot et al., 2014): The recovered microbial community is very different from the original community. This can infer long-term loss of microbiome functions and especially for small children is often associated with permanent health problems (Cho et al., 2012; Marra et al., 2006). The described case was only exemplary, but comparable investigations and the respective calculations can be performed automatically as part of the data evaluation pipeline and give a quick overview on variation or trends in the diversity of microbial communities and are therewith representative of the individual health state of a patient.

Generally, loss in microbial community diversity is acknowledged as a sign for a disturbance as was e.g. described for induction of colitis in mice where cytometric microbial diversity was found to be reduced as compared with healthy mice (Zimmermann et al., 2016). Sequence based data supported these findings based on rarefaction curves and Shannon index of disturbed and undisturbed microbial communities associated with colitis and health of the mice. Since few years diversity calculations are frequently used for the diagnosis of dysbiosis such as colitis or rheumatoid arthritis (Wang and Jia, 2016). Therefore, microbial community cytometry may become increasingly comparable and competitive for routine diagnostics as the method can easily provide summation records on dynamic trends in community evolution while being inexpensive and rapid in application.

While a wide range of measures for the diversity of microbial communities is at hand (e.g. Props et al. (2016)), such clear ecological definitions for microbial community functions are not existent. We are aware that the wealth of metabolic traits of the

human associated microorganisms contributing to carbon, energy, and nutrient availability in our body are crucial for the human metabolism. Besides many other benefits of the human microbiome this functional diversity in terms of metabolic variety is probably the most important contribution to the health of human beings (Grice and Segre, 2011; Human Microbiome Project, 2012). As already discussed above the assignment of functions to single cells is still limited, relying till date on genome information of metabolic traits. Even microscopic observation of microbial cell activity demands bench top experiments which are not applicable in vivo in the original ecosystem. Labeled substrates (e.g. heavy isotopes in combination with NanoSIMS, Schreiber et al. (2016)) are fed to cells and labeled intermediates as well as labeled cells record the used traits for their metabolization. Very often, cascade traits of different organisms are active when a complex substrate is consumed. Apart from the human microbiome the ability of microorganisms to work together for their mutual benefit is exploited in biogas digesters where hydrolysis of complex fiber substrates is followed by acidogenesis with the production of short-chain fatty acids which are further degraded to acetate by the acetogenic step before methane and carbon dioxide are produced by methanogenesis (Weiland, 2010). While the presence of traits in the genome based on functional genes offers hints on possible connections between organisms in a community, verification on transcripts or proteome on the single bacterial cell level is still indicative at best (Schulze et al., 2016). Thus, for the affiliation of functions in microbial communities based on the current technologies bioinformatics tools are required and frequently involve both correlational and similarity/dissimilarity analyses. But these tools are generally applied on bulk data. Alternatively, cell functions can be made visible by cytometry due to autofluorescence (such as the F420 factor for methanogens, unpublished results) or by labeling cells with specific functional probes. The latter technique can be demanding because only few regions on the genome can be targeted and fluorescence intensity might thus not be enough for a good detection. Universal fluorescence probes for vitality assessment can also not be recommended here because they cannot be pretested for the unknown members of a community and would also give only general and unspecific information (Shi et al., 2007). Thus correlational and similarity/dissimilarity analyses are similar attractive for cytometric data as they are for sequence based bulk data. The Cytoscape plugin CoNet is an example software application to excavate functional relationships between members of a microbial community from sequences (Faust and Raes, 2016) and was also successfully applied to cytometric data which were preprocessed using the flowCyBar application (Günther et al., 2016). Thus, data handling on the functional level requires analogous workflows for sequence and cell based data sets.

In macro-ecology functionality is often seen *pari passu* to diversity because different aspects of complexity such as trophic structure, multifunctionality, spatial or temporal heterogeneity, and spatial population dynamics must be implemented in the functional biodiversity research (Carmona et al., 2016; Hillebrand and Matthiessen, 2009). The same concepts are valid for microbial communities but not yet considered with the same complexity. In microbial communities, functions are usually regarded as redundant which is different for the microorganisms' diversity. The scheme in Fig. 2 A schemes a situation with higher taxonomic than functional diversity. In case of loss in diversity, functions can still be maintained by other species and thus the function of the whole system does not change.

Different from the above described situations where species depend on each other to get access to their specific carbon source members of microbial communities can also compete for the same substrate. The winner of this situation is the one with the shortest generation time leading to the extinction of the competitor. Such scenarios are well described for first colonizers (Aujoulat et al., 2014) or in case of disturbances when pathogenic fast growers dominate the community shortly after their invasion (Bäumler and Sperandio, 2016).

Correlational based testing for functions reveal, in the best case, only the active networks between members of a community and provide a mean for modeling and prediction of systems functioning. Using sequence data such models are built on gene numbers and the probability of gene expression while cytometric data provide cell abundance data based on growth or extinction and thus may reveal competitional situations of community members much easier.

3.2. The metacommunity concept

Microbial communities can exist isolated as closed systems or connected in open systems. Depending on how closed systems are defined, isolated communities do not have access to other microorganisms. Examples can be laboratory microbial communities or biotechnological production microbes that are cultivated under aseptic conditions but also artificially mixed constructs from pure strains designed mostly to address scientific questions. However, in nature such situations seldom subsist. Microbial communities in natural ecosystems are extensively interconnected and exchange members frequently. A good example is the open see. Different from these two extremes microbial communities live in cavities that are interconnected and allow transport and exchange of organisms and nutrients between them which is, however, restricted by the environmental conditions of the interlinked ecosystems and their distance from each other. This situation of microbial life in interconnected cavities resulting in a metacommunity is probably the most common one and two well acknowledged examples are wastewater treatment plants and the human microbiome. Microbial exchange leading to species invasion or species loss can occur in any (e.g. the human skin microbiome) or in just one direction (e.g. the human gut microbiome). Setting microorganisms assemblies into a metacommunity context provides, in addition to the basic information on diversity and function described above, knowledge on stability and evolution of communities and can thus be of predictive value.

The metacommunity concept originates from macro-ecology and describes a region containing and supporting multiple local communities which are interlinked in one way or the other. As part of this region the habitat holding a local community is described by the term locality (Leibold et al., 2004; Wilson, 1992). Accordingly, a region consists of different localities which host individual communities that are interlinked with each other.

Four major paradigms were agreed upon which can constitute a metacommunity. The first paradigm describes adjacent but isolated patch localities establishing identical communities. The second paradigm refers to species-sorting conditions meaning that the milieu of localities and the species therein form the environment for the resident community. The third paradigm entails mass-transfer and assumes adjacent communities to be shaped by exchange between members of different communities. The forth paradigm has a unique view on communities as it renders all organisms equal (also with regard to the specific functions and taxonomy of the respective members) and community assembly random. This forth paradigm is called the neutral perspective on a metacommunity (Leibold et al., 2004). A phylogeny independent characterization of the diversity of a metacommunity can be performed based on diversity indices. For example, α-diversity defines the composition of a local community and β-diversity regards the differences in composition between localities. γ-diversity describes

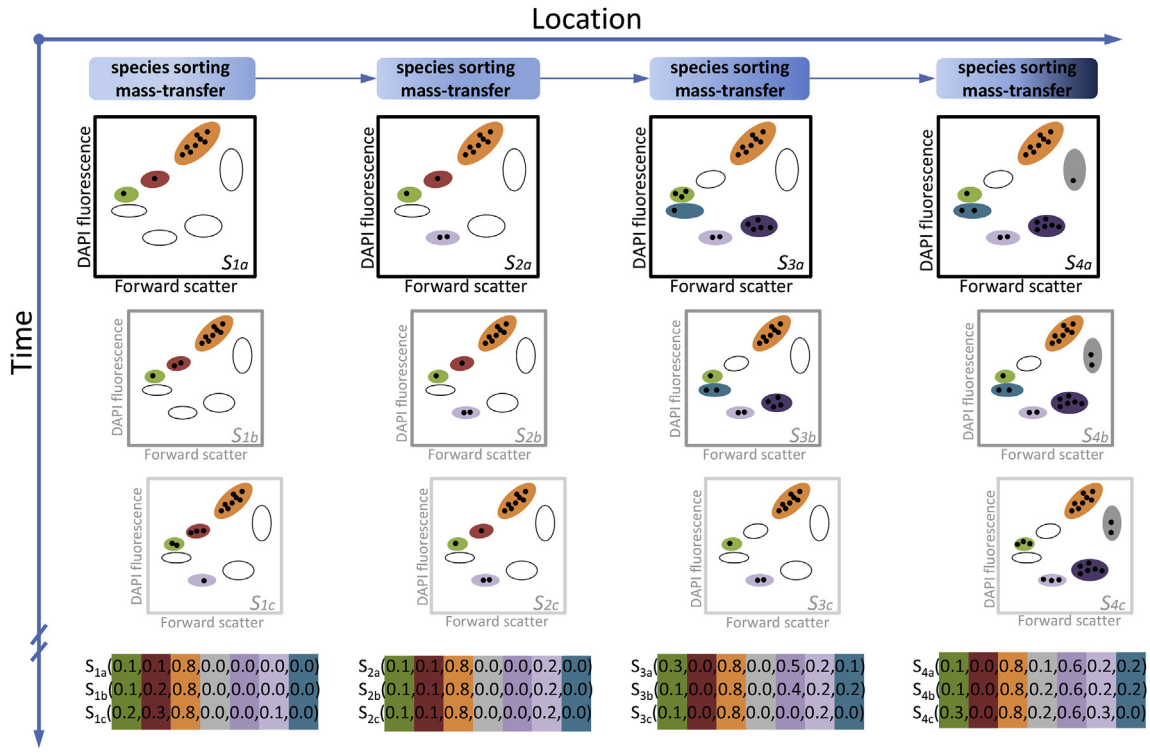

Fig. 3. Schematic illustration of an open metacommunity analyzed by flow cytometry. The metacommunity comprises four different localities that create their own environment due to the species sorting paradigm. At the same time these four localities are interconnected by the paradigm mass-transfer allowing immigration and emigration of phylotypes. The microbial communities of each locality have their own characteristic states (S1–S4). Flow cytometry was also used to follow community dynamics (a–c). While most communities changed their cytometric diversity over time the community in the second row (S2a to S2c) remained constant. The number of subcommunities (SCs) and cell abundancies per SC for each community state are listed on the bottom. The colors represent specific SCs while the black points mark cells.

the total diversity of the metacommunity (Günther et al., 2016). Fig. 3 schemes a cytometrically analyzed microbial metacommunity obtained from four interconnected locations such as the intestine where respective community states were analyzed at three different times (a,b,c). Using D_0 as representation of the number of subcommunities the variation in α-diversity reads for states S1: $D_0 = 3$, S2: $D_0 = 4$, S3: $D_0 = 5$, and S4: $D_0 = 6$ by counting SCs per location (S1–S4) at time (a). The β-diversity D_0 counts unique SCs that are not present in a compared locality which are for time (a) S1/S2: 1, S2/S3: 1, S3/S4: 1, S2/S4: 2, and S1/S4: 3 with the highest β-diversity value. The γ-diversity value is counted as number of unique SCs (5) within the metacommunity of each sampling day for the same sample set.

Various mechanisms can be responsible why local communities differ within a metacommunity and why there are dynamic changes within a locality. No change in community structure was schematically designed for the local communities S2a to S2c in Fig. 3. In situations like this a saturated community can be assumed that does not allow immigrants to establish themselves and also avoids extinction of community members. Such communities are also called end-point communities (Leibold et al., 2004) and are obviously less vulnerable to disturbances. Abiotic factors shape end-point communities such as reactor or other confined local environments as well as access to substrate, energy and nutrients but also the community members themselves contribute to those stable states by cascade or complementary metabolic traits.

In natural ecosystems, stable end-point communities were described for the different reactors of a wastewater treatment plant where the species-sorting mechanism qualified as the dominant paradigm in this context (Günther et al., 2016). Similarly, a stratification in diversity and cell number but also in the abiotic milieu point to comparable mechanisms within the consecutive localities of the human intestine. According to the reviewed overview of Donaldson et al. (2016) the small intestine shows low cell density of 10^2 cfu g^{-1}, higher oxygen levels, low pH and is colonized by facultative anaerobs such as Enterobacteriaceae and Lactobacillaceae. The cell number increases dramatically from Ceacum to Rectum (10^5 to 10^{11} cfu g^{-1}) and oxygen is found depleted and the pH increased. The community composition here is different from the first locality of the small intestine favoring now, among others, Bacteroidaceae, Prevotellaceae, Rikenellaceae, Lachnospiraceae, and Ruminococcaceae.

Besides species-sorting, other mechanisms can be hold responsible for stable end-point communities such as movement of community members from one locality to the next one due to population sizes or resource gradients (Leibold et al., 2004). While communities in closed systems can be prone to either competition or extinction such developments can be counteracted in a way that extinction or change is avoided by constant immigration from a source community. This is described as rescue effect (Weinbauer and Rassoulzadegan, 2007). Examples are the inflow of a wastewater treatment plant or the diet and immediate environment of human beings. In the view of the metacommunity concept these mechanisms can be allocated to the mass-transfer paradigm. In Fig. 3 the paradigms mass-transfer and species-sorting are presented by the horizontal increases in SC and cell numbers for times (a) and (b).

In case of disturbances, depending on how serious they are, even end-point communities can be imbalanced. Colonization, which describes the local settlement of previously absent species or the

upcoming of conditionally rare taxa can both dramatically change the composition and even the function of a community. While colonization refers to immigration from outer sources rare taxa are always present in an established community and only multiply when resource gradients vary. Both types can thus give indications for disturbances. In Fig. 3 colonization events are shown at time (c) from states S3c to S4c whereas the upcoming of conditionally rare taxa is exemplified in the schematic 2D-plots of Fig. 4. In this example, only two of the resident subcommunities survived the disturbance while four new ones established themselves being rare taxa before. Similarly, rare taxa that are kept low in abundance in a healthy gut community of mice fed with sterilized diet can suddenly dominate a community when colitis is induced (Zimmermann et al., 2016). Instead, an example for colonization events in the human gut microbiome is the invasion of pathogenic bacteria after antibiotic treatment which can be fast growers that use open local spaces for establishment (Bäumler and Sperandio, 2016; Theriot et al., 2014), similar to the example shown in Fig. 2 E.

3.3. Community stability

Stability of communities in diversity and function is of primary consequence. Stable healthy or productive microbial communities are beneficial for their host or a biotechnological process while stable pathogenic or non-productive communities can be a nuisance or even ruinous when staying unchanged over long time periods. Thus, directing interest on stability characteristics of communities is of utmost interest. Stability is a global term which is used by macro-ecology as well as socio-economy and thus causes much confusion when applied to microbiological background. In accordance to a response to this confusion by Grimm and Wissel (1997) we also prefer stability to be defined by specific properties such as constancy, resistance, and resilience and translated the accordant properties into interpretation of cytometric data (unpublished results). Constancy describes an original community state that does not change or remains in a defined constancy space while resistance describes unchanged states despite disturbance. If, however, a disturbance causes a change in a community state but the community is able to come back to its original state, this community is defined as resilient. If a different than an original state is established after a disturbance it is defined as an alternative stable state. There are also time dependent stability properties, such as displacement speed which describes the time a community needs to overcome its resistance in case of disturbance, or elasticity which is the time a community needs from a dislocated state to come back to its original state. All these different stability properties can be determined and calculated by microbial cytometry (unpublished results). Fig. 2 F schematically represents this concept for the already above described example of the impact of an antibiotic treatment on the human gut microbiome and the different alternatives for its recovery (Fig. 2A–E). The possible community states are described by the numbers of subcommunities and their respective functions (Fig. 2 F). State A reflects a normal and healthy human gut microbiome which is characterized by a diverse microbial community consisting of several subcommunities ($D_{cyt} = 7$) that comprises several functions ($D_{func} = 5$). Here, the term constancy describes an unchanged community structure and function over time (Fig. 2 A). The disturbance by the antibiotic treatment causes a loss of six subcommunities which results in both diminished cytometric and functional diversity (Fig. 2 B). This is the result of low resistance of the community towards this disturbance. A highly resistant community would not be altered. Nevertheless, the community recovers with patient recovery. The different options of recovery represent three possible new states of the microbial community. First, the community returns to its original state A' (C). All subcommunities and functions are exactly restored as before the disturbance. Alternatively, the subcommunities and functions are partly restored, state D, or a completely different community establishes, state E. In the last state new subcommunities came up that were not detected before. They can represent conditionally rare taxa that have not been detected before or new colonizers that could both also add new functions to the functional community diversity (state E). The scheme in Fig. 2 F does not mirror cell abundance changes in the SCs but this information is essential part when cytometric stability states are calculated (unpublished results).

In this review we do not want to discuss types of disturbances such as pulse or press disturbances, depending on their duration (Bender et al., 1984). However, one should be aware that in microbiology the term durations comprises another meaning compared to macro-ecology. Generation times of bacteria can be as low as 10 min, therefore, a short-time pulse in macro-ecology can place huge pressing strength on microorganisms. Microbial cytometry is highly applicable to fast dynamics and thus predestined to detect fast structural variations and changes in stability states overcoming detection gaps frequently transpiring when sequence based technologies are applied.

3.4. Examples

Ley et al. (2006) discussed two different ecological principles on how microbial communities may evolve in the gut. In interkingdom relationships by constraints the host selects for beneficiary functional stable communities which results in a low number

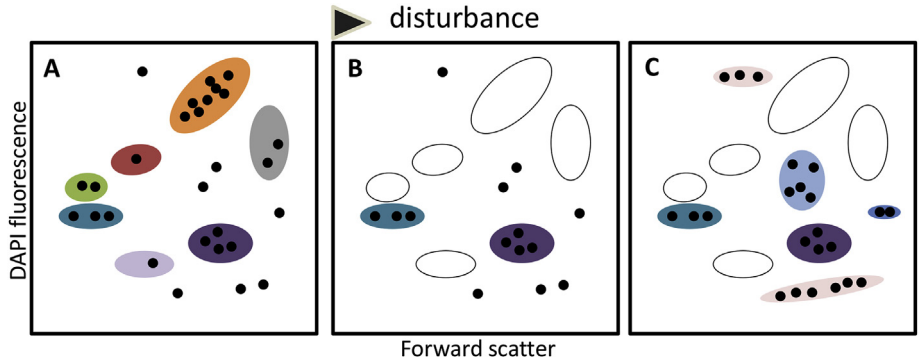

Fig. 4. Schematic illustration of upcoming of conditionally rare taxa. A) A cytometrically highly diverse community comprises cells that usually cluster in subcommunities (SCs) but some continue un-clustered at low abundancies. B) After a disturbance the majority of SCs is lost but un-clustered cells remain. C) Several generation times after the disturbance event the abundance of the formerly rare taxa increases and own SCs are formed.

of different yet redundant functions trenched from high taxonomic diversity. On the level of intra-kingdom relationship, in contrast, competition between members of the microbiota is supposed to cause diversification of functions as niche colonization and speciation are preferred strategies to avoid competition. Both principles seem to be active in the human microbiome. Le Chatelier et al. (2013) demonstrated only a few species of a gut microbiome to cover at least 90% of the whole microbial community but varied highly in abundance among human individuals (10-10,000-fold). Ley et al. (2006) stated that up to 98% of all human gut microbiome derived 16S rRNA sequences are assigned to the phyla Firmicutes and Bacteriodetes in each mammalian host. Cytometric analysis of the mice microbiome confirmed an overall low diversity in species number because we found gates in mice feces that were solely subsumed with just one species which could therefore serve as indicator for health states (Zimmermann et al., 2016). In colitis the monotony in cytometric diversity further increased which enabled us for clear discrimination between healthy and colitis mice (Zimmermann et al., 2016). Independent from these general trends, we found successive feces acquired from the same mouse and day displaying very different cytometric patterns with respect to position and number of subcommunities and contained cell abundancies although the same mouse was nourished and kept under defined conditions (not shown). From such data it can be assumed that the influence of the host might get lost in the rear section of the gut system and competitive extinction in response to competition for limiting resources might become more dominating. In addition, niche-building to avoid extinction might increase diversification and thus be the cause of the different cytometric composition of successive feces. Based on sequencing data, Donaldson et al. (2016) argued that spatial sampling is preferable to sample homogenization because microorganisms grow in patches or clusters, have different access to local nutrients and their bioavailability, and local microenvironments are also suspected to imprint the resident microbiota as do the microorganisms themselves as part of the species-sorting paradigm.

Instead, a stark influence of the host can be assumed for the human salivary microbiota (Huang et al., 2011). Using cytometry we recorded a general low cytometric diversity each of which was obviously bound to the respective healthy host; and dynamic time-bound cytometric screening did not show huge intra-personal variations. Even disturbances, caused by short-term changes in the food-regime, briefly altered the cytometric pattern but did not transform the structure in the long run as always a rearrangement of original states was observed. Thus, the high resilience observed for the salivary microbiota (unpublished results) may be buttressed by the host although a constant inflow of seeding microorganisms from person-related food preferences must be considered. Backed by 16S rDNA sequencing studies, even healthy states could not be differentiated from gingivitis in the human salivary microbiome (Huang et al., 2011) which was, however, different for the plaque microbiome which allowed discrimination between two human host types susceptible to gingivitis (Huang et al., 2014).

4. Outlook

Microbiomes are highly difficult to categorize because of 1) varying taxonomic diversity vs. redundant functional states, 2) interconnections with other microbiota and their localities that allow a meta-community to be formed, 3) the degree to which taxonomic and functional diversity as well as metacommunity paradigms interpose stability properties or render a community vulnerable to disturbances, 4) the complexity of a microbiota subsistence where even locally as well as taxonomically and functionally close communities can obey separate ecological rules.

Resolving the ecology of a specific microbiota requires next to dense sampling in time and localities both sound data analyses and bioinformatic evaluation. However, it is the dynamic variation itself, of almost all parameters introduced before that is the key to the ecological classification of microbiota and their fate. All four points will not reveal considerable insight if only static data are available. Fast growth and extinction rates, upcoming of unconditionally rare taxa, or in case of open systems, mass-transfer are only few but flexible elements of many unknown variables whose classification demand strategic diligence. In this review we propose flow cytometry as a fast, cost-effective, near-online, and easy to apply tool for microbiome research and screening. The technique allows measurements every few minutes providing data based on only two or three physiological parameters of a microbial cell: forward or sideward scatter and DNA content. In addition to high sampling density high numbers of cells per sample are analyzed providing resolutions that can compete with sequence based technologies. Recently published data analysis pipelines (Günther et al., 2016; Koch et al., 2013a, 2013c) allow automatic evaluation of the data sets that fall under the category of big data (250 000 cells per sample where each cell is quantified by three characteristics, with e.g. several samples per day per host individual). The load of data can be diminished when already well known microbiota systems are to be screened or if only subcommunities with the highest impact on community behavior are of interest. We used the function MultiCoLA (Multivariate Cutoff Level Analysis, Gobet et al. (2010); Günther et al. (2016)) to differentiate between dominant and rare cytometric subcommunities by testing different cut-off levels as is shown exemplarily in Fig. 5 where only subcommunities with cell numbers of >1 (B) or >2 (C) as representatives of most abundant members of a community are regarded for

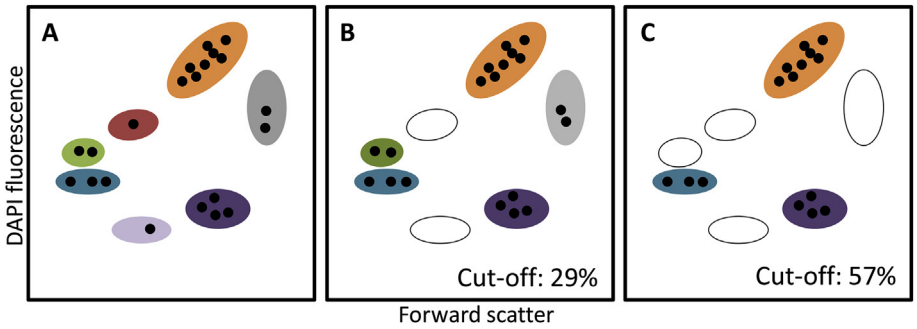

Fig. 5. Schematic illustration of testing for dominant subcommunities (SCs) using MultiCoLA analysis (Multivariate Cutoff Level Analysis, Gobet et al. (2010)). Here, the testing was done for SCs with the highest cell abundancies. SCs with low abundance were removed step by step (A to C) and the remaining SCs can now be tested for similarity and correlation with the initial SC assembly.

further data computation.

It is conceivable that cytometric microbiome data sets can become part of routine monitoring as it is tested at the time for biotechnological production processes where cell based data are recorded, mined, and modeled using process analysis technology (PAT)-systems on the basis of a guideline released by the Food and Drug Administration (FDA, 2004). Now PAT allows access to dynamic profiles of biomass and product formation rates as well as process parameters which can be implemented in dynamic approaches such as hybrid semi-parametric models that combine existing process knowledge and data related to the biological system (von Stosch et al., 2016). But even PAT is still not widely tested with regard to implementing single cell data. There are also efforts to establish individual based models (IBM) (reviewed by Hanemaaijer et al. (2015); Widder et al. (2016)) based on programs such as MetaFlux (component of the SRI's Pathway Tool software, Karp et al. (2016)) and iDynoMiCs 2 (Clegg et al., 2014; Lardon et al., 2011). But those models still simulate the function of only a few organisms at a time and still cannot handle the wealth of diversity and possible functions of members in natural microbial communities to date. All these examples show that there is ongoing research in the field of bioinformatics to unearth the vast information produced with microbial flow cytometry. The future developments will facilitate cytometric fingerprinting for human routines diagnostic to be as simple and meaningful as a blood count today.

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