Methods in microbiome research

Namrata Iyer

From the freezing lakes of Antarctica to the thermal vents deep within the oceans, microbes have managed to survive and thrive in the most inhospitable of conditions. Unsurprisingly, the relatively luxurious abode of the human body is chockablock with microbial life. We have coevolved over centuries with these microorganisms, collectively referred to as our microbiota, resulting in a relationship that is mutually beneficial. However, it has only been in the past few decades that the importance of the microbiota to human health has been uncovered. It is now well-known that imbalance in the microbiota—or dysbiosis—is strongly linked with a number of pathologies, such as Crohn's disease, inflammatory bowel disease, cancer and many others[1]. In order to understand the link between these microbes and various diseases, it becomes imperative to find the answers to several important questions: What are the specific microbes that inhabit our body? What functions do these microbes perform? Where are they located with respect to the host and each other? And finally, how do changes in microbiota lead to disease?

Recent times have seen vast leaps in modern technology that allow us to begin answering some of these key questions.

The diversity of microbes

Any site in the body can be considered as an ecosystem, inhabited by different microbial species, ranging from bacteria and archaea to fungi and viruses. To accurately define this ecosystem, assigning an identity to its members is of paramount importance. The methods employed for this purpose must be accurate, capable of detecting even rare species, fast and cost-effective. 'Next Generation' sequencing has largely satisfied most of these requirements. The most widely used method in microbiome analysis is 16s rRNA sequencing[2]. The 16s rRNA gene is highly conserved in all bacteria and sequencing of its regions of hyper-variability allows the identification of different bacterial species. But this technique suffers from inaccuracies at species level classification[2]. Furthermore, it limits the analysis to only bacterial species while ignoring other members in the community. Platforms are now being developed that can couple this technique with other genetic markers, allowing detection of eukaryotic members of the microbiota as well[3].

While 16s rRNA sequencing looks at a single gene in every bacterium, whole metagenome sequencing looks at the entire genomic content of the microbiota in an unbiased manner. This provides accurate species-level identity of the microbes as well as their complete genome. Analysis can reveal the metabolic potential of these communities, giving a better idea of their function as a whole.

Community function

Community composition has been the primary tool for microbiome research in the past decades. As more is revealed about the microbiota in different disease states, researchers are now trying to understand how these communities contribute to the physiological state of the host. Metagenome information does not tell us which subsets of genes are expressed at any given time. Keeping this in mind, whole metagenome sequencing is now being coupled with metatranscriptome sequencing to reveal the community gene expression profile[4]. This dynamic picture of community function can help identify the consequences of dysbiosis. Studies are now being taken to the next level, analyzing not only transcriptomes but also proteomes and metabolomes of the community as a whole[5]. Metaproteomics and metametabolomics are more technically challenging, given the sheer diversity of proteins and metabolites that each microbe can produce. While these techniques are still in their infancy, they promise to reveal unique insights into the communication and cooperation between the different members of the microbiota, and also host-microbiota interactions.

Microbiota structure

Community structure is a function of who is present in the microbiota community and how these members interact with each other. The behavior of each microbe is determined by the microbes and host cells in its immediate vicinity. Several pathologies, such as irritable bowel syndrome (IBD) and Crohn's disease, are now known to arise not only from an imbalance in the microbiota, but also in their spatial organization within the host. Visualization of this spatial organization is performed using FISH (fluorescent *in situ* hybridization). Using fluorescent probes against 16s rRNA sequences, FISH can specifically label various bacterial communities in host tissues and reveal how these different microbes are organized within their niche (**Fig. 1**)[6]. These imaging techniques can further be coupled to mass spectrometry imaging to reveal the spatial location of a bacterium as well as its metabolic status at the time.

Mechanistic link between microbiota and host health

Studies have uncovered startling correlations between changes in the microbiome and diseases ranging from diabetes to autism. While most of these studies reveal correlation, causation is yet to be comprehensively established.

Answers to these puzzles are now being found using *in vitro* models of microbiota research. These models simulate conditions in the specific niche to dissect out

Department of Molecular Microbiology and Immunology, Brown University, Providence, RI. Correspondence should be addressed to N.I. (namrata_iyer@brown.edu)

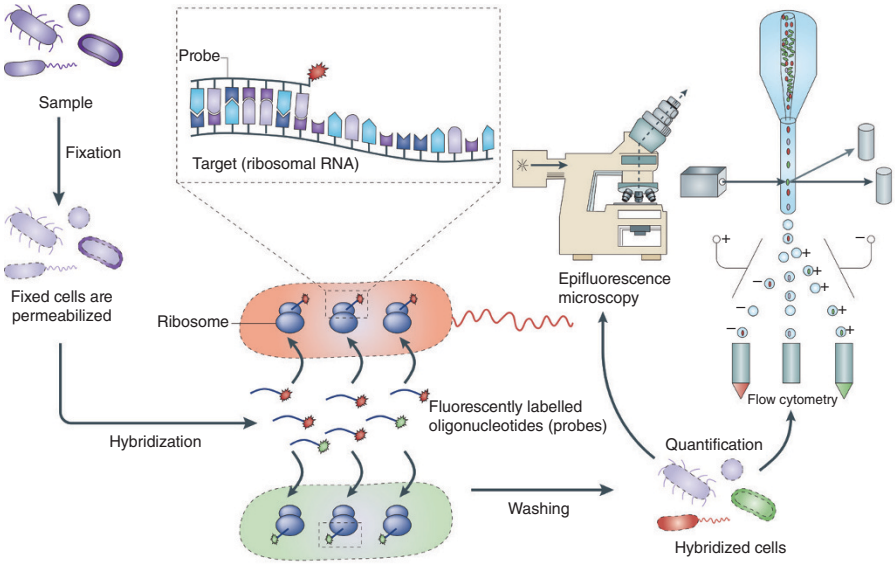

FIGURE 1 | Basic steps of fluorescence *in situ* hybridization (FISH). The sample is first fixed to stabilize the cells and to permeabilize the cell membranes. The labeled oligonucleotide probe is then added and allowed to hybridize to its intracellular targets before the excess probe is washed away. The sample is then ready for single-cell identification and quantification by either epifluorescence microscopy or flow cytometry. From *Nat. Rev. Microbiol.* **6**, 339–348 (2008).

microbiota function and host response. Many systems recreating the human intestine are now available. Examples such as the SHIME (Simulator of the Human Intestinal Microbial Ecosystem) make use of linked reactors that mimic the human gut, starting from the stomach to the colon. A recent model, known as 'Gut-on-a-chip', is a microfluidic system that co-cultures gut cells with microbes to study various host-microbe and microbe-microbe interactions[7]. These models could provide valuable insight into the physico-chemical processes that occur at the gut interface.

A comprehensive understanding of the link between microbiota and health, however, requires the use of live animals. The gold standard for such studies is gnotobiotic mice (mice with defined microbiological status). Germ-free mice, born and bred in completely sterile isolators (**Fig. 2**), reveal how the complete absence of microbiota affects host physiology. The lack of microbiota can have systemic effects on these animals, ranging from an inability to efficiently digest food to an under-developed immune system.

These mice can be further selectively colonized with defined microbiota to assess their effect. Such studies have made important revelations about the influence of our microbiota on our metabolic status. Obese and lean body types are now known to be transmissible between mice via a transfer of just their microbiota[8]. Gnotobiotic mice have also been instrumental in disclosing the complex relationship between our microbiota, diet and health. Diet is now thought to be a factor that dominates over our genome in determining which microbes colonize our gut[9].

The translational impact of these studies has further improved with the use of humanized mice (mice colonized with microbes from the human gut). Research integrating gnotobiotic mice, dietary variations and mathematical modeling have allowed scientists to predict the effects of diet on the composition of our microbiota and thereby our health[10]. This has wide implications for the growing fields of prebiotics and probiotics.

The dynamic nature of the microbiota, however, also means that minor changes in diet and other environmental conditions can significantly affect the microbiome, causing groups of mice that are genetically identical to display different phenotypes. The variability in husbandry practices at different animal care facilities can, therefore, cause problems with the reproducibility of these studies[11]. Greater uniformity in animal maintenance and comprehensive reporting of these conditions are required to generate a stronger foundation for microbiota research.

FIGURE 2 | Example of a typical gnotobiotic facility with sterile isolators. All components (such as food and bedding) have to be sterilized before being placed in each isolator, creating a strict set of requirements for maintaining gnotobiotic colonies. Image from NIAID.

Future perspectives

The Human Genome Project started with a goal to gain complete knowledge of our genome; the vision being that our genomic code, once unlocked, would demystify human physiology. Despite now having the genomic code firmly in our grasp, this vision is far from fulfilled. The microbiota is one of those missing links; an entity outside of our genome that exerts a profound effect on almost every aspect of our physiology. Deciphering the human metagenome, while seemingly a herculean task, has the potential to significantly improve our understanding of health and homeostasis. Combining the strengths of sophisticated technology, gnotobiotic models, and inter-disciplinary approaches might hold the key to breakthroughs in therapeutics for a gamut of diseases.

1. Turnbaugh, P.J. *et al.* The human microbiome project: exploring the microbial part of ourselves in a changing world. *Nature* **449**, 804–810 (2007).
2. Rajendhran, J. & Gunasekaran, P. Microbial phylogeny and diversity: Small subunit ribosomal RNA sequence analysis and beyond. *Microbiol. Res.* **166**, 99–110 (2011).
3. Morgan, X.C. & Huttenhower, C. Meta'omic analytic techniques for studying the intestinal microbiome. *Gastroenterology* **146**, 1437–1448 (2014).
4. Bikel, S. *et al.* Combining metagenomics, metatranscriptomics and viromics to explore novel microbial interactions: towards a systems-level understanding of human microbiome. *Comput. Struct. Biotechnol. J.* **13**, 390–401 (2015).
5. Pérez-Cobas A.E. *et al.* Gut microbiota disturbance during antibiotic therapy: a multi-omic approach. *Gut* **62**, 1591–1601 (2013).
6. Amann, R. & Fuchs, B.M. Single-cell identification in microbial communities by improved fluorescence in situ hybridization techniques. *Nat. Rev. Microbiol.* **6**, 339–348 (2008).
7. Kim, H.J., Huh, D., Hamilton, G. & Ingber, D.E. Human gut-on-a-chip inhabited by microbial flora that experiences intestinal peristalsis-like motions and flow. *Lab Chip* **12**, 2165–2174 (2012).
8. Zhao, L. The gut microbiota and obesity: from correlation to causality. *Nat. Rev. Microbiol.* **11**, 639–647 (2013).
9. Carmody, R.N. *et al.* Diet dominates host genotype in shaping the murine gut microbiota. *Cell Host Microbe* **17**, 72–84 (2015).
10. Faith J.J. et al. Identifying gut microbe–host phenotype relationships using combinatorial communities in gnotobiotic mice. *Sci. Transl. Med.* **6**, 220ra11 (2014).
11. Hufeldt, M.R. *et al.* Variation in the gut microbiota of laboratory mice is related to both genetic and environmental factors. *Comp. Med.* **60**, 336–347 (2010).