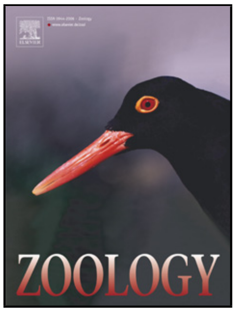

Title: Molecular techniques and their limitations shape our view of the holobiont

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Molecular techniques and their limitations shape our view of the holobiont

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Highlights

- We identify four categories of molecular technique according to the questions they answer in holobiont research

- All techniques have biases and limitations which may shape our view of holobionts
- Tackling the complexity of holobionts will depend on continued technological advances especially in DNA sequencing and imaging.

Abstract

It is now recognised that the biology of almost any organism cannot be fully understood without recognising the existence and potential functional importance of associated microbes. Arguably, the emergence of this holistic viewpoint may never have occurred without the development of a crucial molecular technique, 16S rDNA gene amplicon sequencing, which allowed microbial communities to be easily profiled across a broad range of contexts. A diverse array of molecular techniques are now used to profile microbial communities, infer their evolutionary histories, visualise them in host tissues, and measure their molecular activity. In this review, we examine each of these categories of measurement and inference with a focus on the questions they make tractable, and the degree to which their capabilities and limitations shape our view of the holobiont.

Keywords: Holobiont; Imaging; Metagenomic sequencing; Evolutionary inference; Model system; Multi-omics

1. Introduction

Most, if not all, animals and plants exist as part of complex multi-organismal assemblages (Bang et al., 2018; Bosch and McFall-Ngai, 2011; Bosch and Miller, 2016) comprised of the host and associated microorganisms. These associations, together called holobionts or metaorganisms (for a differentiation between both terms, see (Jaspers et al., 2019)) may include members that interact in a manner that contributes to the fitness of the whole as well as others without any identifiable functional impact. Adopting a holistic view of biology that acknowledges the existence of holobionts and metaorganisms raises fundamental questions specific to the multi-organismal condition. One such question is whether a core microbiome or a set of core microbiota exists (Hernandez-Agreda et al., 2017; Qin et al., 2010) and whether this should be defined by taxonomic or metabolic composition (Cho and Blaser, 2012). More mechanistic questions, such as whether microbial composition is influenced by the host (Augustin et al., 2017; Franzenburg et al., 2013; Fraune and Bosch, 2007; Rawls et al., 2006) and the degree to which partner organisms are interdependent (Fraune et al., 2015; Russell et al., 2013) are being addressed through experiments in model metaorganisms such as *Hydra*, zebrafish and pea aphids. Finally, the close association and potential interactions between organisms gives rise to a suite of evolutionary questions, such as whether key microbial taxa have co-evolved with their hosts (Baumann, P, Moran, N. A., and Baumann, L., 1997; O'Brien et al., n.d.; Pollock et al., 2018), how the microbiome has influenced speciation and emergence of novel traits in hosts (Brucker and Bordenstein, 2012), and how metabolic cooperation has shaped partner genomes (Russell et al., 2013).

These questions are challenging to answer, but advances in molecular techniques are rapidly making them tractable. Perhaps the most notable advance from the past two decades has been the development and widespread adoption of marker-gene targeted amplicon sequencing. This has revolutionised detection, classification, and quantification of microorganisms, making it possible to rapidly and cheaply profile the microbiota of a holobiont. The adaptability of this technique has allowed it to be employed in answering questions related to the variability of microbial communities across a wide range of species, tissues, and environmental conditions. In contrast, many molecular techniques form part of a toolbox that is closely tied to a particular organism or model system. Several such model systems are being developed for use in metaorganism research with toolboxes that include the removal or manipulation of important microbes (Fraune and Bosch, 2007; Moran and Yun, 2015; Voolstra, 2013), the ability to alter gene expression or edit genomes (Dunn et al., 2007; Franzenburg et al., 2013; Ikmi et al., 2014) and the ability to monitor metabolic interactions between components (Hillyer et al., 2017; Rädecker et al., 2018). Experiments in these model systems provide insights that are otherwise intractable in non-model systems and which form a framework for understanding inter-organismal interactions. Extending this framework to understand the biology of non-model metaorganisms is now a key focus for research on an increasingly broad range of taxa including keystone marine species such as corals, sponges, and reef fishes, agricultural and aquacultural species, and plants.

Metaorganism research is now heavily dependent on molecular techniques and its direction is arguably shaped by their availability, limitations, and advancement. In this essay we explore the role of four core categories of molecular technique in shaping the questions we can address in metaorganism and holobiont research. For each, we describe the related set of questions for which it is most suitably applied (Table 1) and outline the challenges that must be overcome in order to obtain clear answers. Where possible, we also reflect on opportunities afforded by new technologies and observe that these sometimes cut across categories. While our review reflects current research trends, it is likely that as new techniques develop they will blur the lines between those that exist today.

2. Identification and quantification of microbial communities

The ability to identify, taxonomically classify and quantify the abundance of microorganisms or their gene products, is a foundational requirement of metaorganism research. It is the basic measurement that allows us to ask “what are the members?” and “what are they likely to be doing at the molecular level?”. This, in turn, leads to consideration of where to draw the boundaries of an individual association and how holobiont membership varies with time, environment and host genotype.

Answering these questions requires tools that can comprehensively assay microbial communities from within different holobiont assemblages and their surrounding environments. Molecular techniques based on next-generation sequencing are potentially able to accomplish this because they require only that DNA or RNA be extracted from the sample. These techniques have undergone rapid development over the past few decades and have now largely replaced microbial culture for surveying microbial communities, although the latter remains important for phenotypic characterisation, reference genome sequencing and taxonomic classification (Yarza et al., 2014).

Current techniques for sequence-based microbial profiling can be broadly categorised into amplicon-based and whole metagenome methods. Amplicon-based approaches identify organisms based on the amplification of a marker gene sequence (Woese and Fox, 1977; Yarza et al., 2014) chosen to provide phylogenetic signal across a wide range of taxa, whereas whole metagenome approaches sequence in an untargeted manner across all of the DNA present. Alternatively, RNA sequencing (metatranscriptomics, (Abu-Ali et al., 2018)) or mass spectrometry based proteomics (metaproteomics, (Starr et al., 2018)) can be used, especially where metabolic activities rather than taxonomic composition are of primary interest (see section 4).

Currently, all methods for surveying microbial composition, including traditional culture-based methods as well as amplicon and whole metagenome sequencing, are subject to biases and limitations which contribute significantly toward shaping our view of the holobiont. DNA sequencing based techniques generally provide less biased estimates of abundance than culture-based methods but still show strong biases due to differences in DNA extraction efficiency (Xue et al., 2018). Amplicon based methods introduce additional bias related to the use of PCR including the primer set (Klindworth et al., 2013), target region, GC content, and input DNA concentration (Brooks et al., 2015; Kennedy et al., 2014; Laursen et al., 2017; Rintala et al., 2017).

In addition to bias, another issue with sequencing-based microbial profiling techniques is that they produce compositional data, that is, abundance measurements as a percentage of a (potentially unknown) total. It is essential that this is accounted for when choosing statistical methods for data analysis (Gloor et al., 2017; Lovén et al., 2012). More fundamentally, the total abundance of microorganisms provides crucial context required to infer ecological scenarios (e.g. competitive exclusion, outgrowth or differential survival) that give rise to changes in composition (relative abundance) (Props et al., 2017). Measurements that are sensitive to total microbe abundance are also important in the verification of xenobiotic models and in determining the difference in microbial load between host tissues versus the environment, an important indicator of host selectivity or filtering. Techniques for measuring abundance in absolute rather than relative terms such as qPCR based assays (Jian et al., 2018), flow cytometry (Props et al., 2017) and fluorescence in situ hybridisation (Daims et al., 2001) will help to fill this gap.

Another suite of issues arises from the fact that most amplicon-based studies to date have relied on short (100-300 base pair [bp]) reads. One consequence of this is that it is often difficult to correctly separate reads arising from related taxa, which is important as it underpins estimates of microbial diversity, and because variation in the abundance of microbes that differ at the species or strain level may be related to functionally or ecologically significant differences in the host (Moeller et al., 2016; Neave et al., 2017b; Pollock et al., 2018). For whole metagenome approaches this process (called binning) is especially challenging because reads can originate from different genomic contexts as well as different taxa. The fixed genomic context used by amplicon sequencing means that differences between reads should arise only due to taxonomic differences and sequencing artifacts (e.g. single-base read errors or chimeras). Until recently many studies were based on the concept of OTUs (Operational Taxonomic Units), which are effectively clusters of marker gene sequences at a 97% similarity cutoff. Newer approaches explicitly model the difference between sequencing errors and biological variation to infer ASVs (amplicon sequence variants) representing biologically distinct sequences within the original sample (Callahan et al., 2016).

It is also important that biologically distinct sequences within the sample are correctly assigned to an agreed taxonomy. This provides a framework for communication, facilitates comparison between experiments and may also allow inferences to be made on the basis of homology. The most complete taxonomies are currently based on curated efforts to reconcile phylogenetic analyses of full length amplicon sequences with taxonomic assignments based on cultured strains (Yarza et al., 2014; Yilmaz et al., 2014). In principle, taxonomic assignments based on amplicon sequencing should be well placed to benefit from the relative completeness of these databases but since many studies only sequence a small region rather than the complete marker gene they are still prone to misclassification errors (Yarza et al., 2014). A recent development that promises to address this issue is the use of long read amplicon sequencing combined with software that models the error modes of long read sequencing technologies (Callahan et al., 2018). Notably, even with such sophisticated approaches, the challenge remains as how to denote distinct ‘species’ based on sequence diversity cutoffs (Konstantinidis Konstantinos T et al., 2006).

In principle, identification and quantification of microbes based on whole metagenome sequencing avoids many of the problems with the amplicon based approach. Not only is it less prone to biases due to PCR amplification, but it also sequences a far greater proportion of the genome that could be used for gene content analysis and more precise discrimination between taxa (Jain et al., 2018; Neave et al., 2017a; Shakya et al., 2013). In practice, this approach is currently limited by the availability of whole genome reference sequences (see http://gtdb.ecogenomic.org/ for an example of efforts to tackle this problem), high sequencing costs due to the fact that host DNA often dominates holobiont-derived samples, and high bioinformatic costs due to the far greater complexity and volume of data that must be analysed. Many of the challenges with whole metagenome sequencing data arise from the fact that raw sequencing reads are difficult to separate according to their taxon of origin (Lindgreen et al., 2016), a process that is much more complex for whole metagenome than for amplicon data. This reduces the accuracy of diversity profiles and effectively prevents assembly of complete genome sequences from mixed microbial data. Several new technologies and associated computational approaches are emerging as potential solutions to this issue.

A particularly promising approach is chromosome conformation capture (3C) and, specifically, the Hi-C method for preparing sequencing libraries (Liu and Darling, 2015). This technique captures information about the physical proximity of fragments of DNA, which can later be used to infer their co-location on a chromosome or within a cell (Liu and Darling, 2015), thereby improving the accuracy of metagenomic binning and assembly. Other new technologies, such as single-cell metagenomic sequencing (Xu and Zhao, 2018) and long read sequencing (Arumugam et al., 2018; Bertrand et al., 2018), offer alternative solutions to this issue (Nicholls et al., 2018). None of these new technologies are free from challenges (see (Liu and Darling, 2015) for a brief overview) but the rapid advances in this area suggest that it may soon be possible to accurately assemble microbial genomes directly from metagenomic sequencing data. This would not only allow for the accurate assessment of microbial communities in terms of metabolic capacity (via gene content), it would also provide the dense taxonomic sampling of bacterial genomes required to replace amplicon-based taxonomic classification with a whole genome-based system.

3. Evolutionary inference

Molecular sequence data from holobionts encodes rich information about the evolutionary forces that have shaped the association. Inferences based on these data can answer questions such as the extent to which co-evolution has occurred and for which taxa this is true. In addition, genes or other genomic features that have been shaped by co-evolution may be identified, which can provide insights into the mechanisms underpinning long standing interactions between taxa.

Molecular signatures indicative of co-evolution (O'Brien et al., n.d.) include phylosymbiosis (Brooks et al., 2016; Sanders et al., 2014), codivergence and the existence of genomic changes such as metabolic complementarity (Poulsen et al., 2014; Russell et al., 2013). In phylosymbiosis, the host phylogeny is correlated with a divergence pattern based on the microbiome profile distances (e.g. from amplicon or whole genome sequencing). Phylosymbiosis is frequently (Brooks et al., 2016; Pollock et al., 2018; Sanders et al., 2014) but not universally (Chandler et al., 2011; Kelley and Dobler, 2011) observed in holobiont systems and is not necessarily an indicator of co-evolution since it can also arise purely due to differential dispersal and establishment (filtering) of microbes in response to phylogenetically or geographically correlated host traits (Sanders et al., 2014; Sieber et al., 2018). Stronger evidence of co-evolution can be obtained by observing codivergence which represents the congruence of phylogenies between host and microbe or between divergent groups of microbes. It is typically observed only for a subset of dominant or key organisms within the microbial community. In a recent study of the microbial composition of skeleton, mucus and tissue in scleractinian corals, codivergence was observed in only four out of hundreds of microbial genera (Pollock et al., 2018).

Comparative genomic analyses may reveal changes in genome size, gene content or rates of gene evolution that are indicative of co-evolution. Reductions in genome size in ciliate endosymbionts (Boscaro et al., 2017), the aphid symbiont *Buchnera aphidicola* (van Ham et al., 2003) and dinoflagellates in the family Symbiodiniaceae (LaJeunesse et al., 2018) are all thought to be the result of co-evolution with their respective hosts (LaJeunesse et al., 2005). Analysis of microbial and host gene content can also reveal metabolic complementarity whereby both partners contribute to a shared function such as the synthesis of key amino acids (Russell et al., 2013) or complete digestion of a primary energy source (Poulsen et al., 2014).

Commented [CI1]: Dinoflagellates in the family Symbiodiniaceae (LaJeunesse et al., 2018) are intracellular symbionts of a range of marine invertebrates but most maintain the ability to survive outside their hosts. Their genomes exhibit features such as reduced size (LaJeunesse et al., 2005) and high levels of divergence that are consistent with a symbiotic lifestyle, however, difficulties in obtaining genomic data and rearrangement between genera the drivers behind this remain unclear because

The expansion of genes required for inter-species interactions, changes in the evolutionary rate of these genes, and horizontal gene transfer (HGT) may all also be observed where co-evolution is taking place (Friesen et al., 2006; Husnik et al., 2013). The close association and potential for metabolic interaction between species in a metaorganism suggests that HGT might be prevalent in such systems (Degnan, 2014; Keeling and Palmer, 2008) but the confident identification of instances of HGT is difficult. This is because the elimination of alternate hypotheses (e.g. widespread gene loss) requires a dense and high quality sampling of the genomes of related taxa, a condition that can rarely be fulfilled with current genomic databases.

The major bottleneck to the use of comparative genomics in the study of co-evolution comes from limitations in current genome and metagenome sequencing techniques. Sequencing of large eukaryotic genomes remains expensive and difficult, meaning that publicly available genomes are often sparsely distributed across high level taxa and, where genomes are available, they often contain assembly errors and are highly fragmented (Salzberg et al., 2012). Gene annotations in such draft genomes may also be fragmented, missing or falsely duplicated (Denton et al., 2014; Zhang et al., 2012). It is recognised that interpretation of de novo assembled genomes is challenging and multiple high quality genomes are required for fruitful comparative studies (Richards, 2018). Overall, these problems greatly complicate inferences based on gene content and arrangement which could otherwise be used to infer inter-organismal partnerships or horizontal gene transfer.

Several technologies are emerging that promise to improve our understanding of the mechanisms behind co-evolution. Long and linked-read sequencing (Ott et al., 2018; Wallberg et al., 2018) as well as Hi-C contact maps allow for substantial improvements in assembly contiguity and can account for haplotypic variation (Chin et al., 2016; Kronenberg et al., 2018; Ott et al., 2018), a major source of fragmentation and errors in short read assemblies (Goltsman et al., 2017; Kajitani et al., 2014). Furthermore, long read RNA sequencing has the potential to significantly improve genome annotation as it is capable of generating near perfect full length transcripts without an assembly step. These can be used as very high quality training data for gene prediction, greatly reducing the number of incomplete or incorrect gene models in draft genomes (Magrini et al., 2018).

Obtaining complete genome sequences for bacterial, archaeal and viral partners presents a separate set of challenges. Although these genomes are relatively compact and easily assembled when sequenced from pure cultures, such cultures are rarely available. Instead, sequencing is often done on complex community samples resulting in a mix of reads from many different organisms. As mentioned above (see "Identification and quantification of microbial communities"), there are several new technologies that facilitate the binning of metagenomic reads that should eventually allow for the assembly of complete genomes from uncultured microbial samples (Bishara et al., 2018).

4. Measuring molecular activity

The ability to measure molecular activity and track metabolic exchanges is key to understanding the interactions between members of a metaorganism. Such measurements have the potential to provide insights into what costs and benefits are incurred, how these are distributed between members, and what signals are used to allow organisms to avoid conflict with or exert control over others. There is growing recognition that answering these questions is an essential requirement for understanding animal and plant health (Berendsen et al., 2012; Cho and Blaser, 2012). For example, a complex picture of interactions between the human gut microbiome and the brain (gut brain axis) is emerging, which has been linked to psychiatric disorders as well as Multiple Sclerosis and the inflammatory bowel diseases, Crohn's disease and Ulcerative colitis (Collins et al., 2012).

Many of the best understood interactions between metaorganism members have been studied via experiments that manipulate the microbiota of a host (e.g. through removal and selective re-introduction). Notable examples include metabolic pathways shared between the bacterium, *Buchnera* and pea aphid hosts (Russell et al., 2013) to produce essential amino acids, fungal resistance in *Hydra* conferred by the presence of bacteria-bacteria interactions (Fraune et al., 2015) and the induction of intestinal Th17 cells in mice upon reintroduction of a single species of bacteria (Ivanov et al., 2009). While such experiments are often crucial to confirming that an interaction occurs, they typically rely on univariate assays to demonstrate the outcome (e.g. amino acid production, fungal immunity and cell growth) and additional techniques are required in order to explore the underlying molecular mechanisms at play.

One approach is to manipulate the molecular activity of the microbiota or host through experiments that knockdown or over-express specific genes. The molecular mechanisms by which *Hydra* are able to influence their microbiome have been revealed through experiments that knockdown the expression of specific antimicrobial peptides (AMPs) (Franzenburg et al., 2013) or that knockdown the expression of genes that regulate AMP expression such as FoxO (Boehm et al., 2012; Mortzfeld et al., 2018). This approach is extremely powerful but it is time consuming and is therefore only useful where prior information is available to generate well developed hypotheses related to specific genes and their effects.

An alternative approach that can complement these experiments is to use high throughput technologies such as RNA sequencing, and Mass Spectrometry to measure the expression of thousands of genes, transcripts, proteins or metabolites. In principle, it is even possible to measure changes in the molecular repertoires for multiple taxa within a holobiont simultaneously (cf DualRNA Seq; (Westermann et al., 2017)). Collectively termed ‘Omics approaches, these techniques can be used to generate plausible hypotheses for mechanisms of interaction between members of a metaorganism (Mohamed et al., 2016; Oakley et al., 2016). Although such ‘Omics approaches are now widely used in molecular biology, their use in a metaorganism context poses additional challenges. Increased sensitivity and dynamic range may be required to overcome a dominance of host signal or to detect metabolic activity from low abundance microbes. Data interpretation is also difficult because detailed information about molecular pathways and their associated genes and metabolites is largely derived from experiments on a small set of classical model taxa (eg E-coli, Yeast, Mouse, *Drosophila*, *C. elegans*). It is therefore important that ‘Omics approaches are adopted in conjunction with efforts to expand fundamental knowledge of the molecular biology of model metaorganisms and, more broadly, for non-model taxa.

5. Mapping microbiome components within host tissues

Holobionts are not homogenous associations but vary in composition and activity between host tissues. This has perhaps been most extensively studied in humans where body location dramatically affects microbiome composition (Byrd et al., 2018; Donaldson et al., 2016; Human Microbiome Project Consortium, 2012; Tropini et al., 2017), but such distinctions have also been observed for very simple animals such as *Hydra (Augustin et al., 2017)*.

The roles of individual microbiome components within the metaorganism often prove enigmatic. However, functions of microbe-microbe and microbe-host interactions may be revealed by locating the physical sites where these interactions occur (Figure 1). One way to approach this is through methods that reveal the location of specific microorganisms within host tissues such as Fluorescent In Situ Hybridization (FISH). Originally named “phylogenetic staining” (DeLong et al., 1989), FISH revolutionized the field by allowing detection and identification of microbes without the necessity of cultivation (reviewed by (Amann et al., 1995; Wagner et al., 2003)). This feature of FISH makes it particularly valuable for studies of non-model systems, including corals and sponges, where culture methods have not yet been developed. Despite difficulties associated with high levels of autofluorescence of coral tissues (Wada et al., 2016), FISH has successfully revealed an intimate physical association between *Endozoicomonas sp.*, considered a candidate mutualist within tissues of a coral host (Neave et al., 2017b; Pogoreutz et al., 2018). By detection of specific microorganisms within oocytes and larvae of marine sponges, FISH has confirmed vertical transmission of symbionts as had been suggested by sequencing-based methods (Schmitt et al., 2008; Webster et al., 2010).

While originally a relatively simple technique, FISH, often used in combination with other techniques, has become an increasingly sophisticated set of methodologies allowing characterization, quantification and co-localization of diverse microbes. For example, Combinatorial Labeling and Spectral Imaging FISH (CLASI-FISH), which can identify and differentiate up to 15 microbial taxa simultaneously (Valm et al., 2011), has been used to describe a highly spatially structured, multi-genus assembly of microbes in human plaque and provided insight into the function of individual components of this complex consortium (Mark Welch et al., 2016). On the other hand, combining FISH with electron microscopy imaging (fluorescence *in situ* hybridisation-correlative light and electron microscopy; FISH-CLEM) permitted identification and characterization of Poribacteria, common but uncultivated symbionts of marine sponges (Jahn et al., 2016). Combining FISH with laser microdissection, followed by amplicon sequencing of the isolated samples (Klitgaard et al., 2005), has the capacity to provide a high-resolution map of the microbiome within host tissues. One such study revealed that the dermis, the deepest layer of human skin and previously considered sterile, contains a diverse microbiome permitting direct communication with the host tissue (Nakatsuji et al., 2013).

While FISH, by its nature, can only be used on fixed specimens, live imaging of microbial-host interactions is possible in those experimental model systems where symbiotic microbes are amenable to culture and genome manipulation, and the host tissues are transparent. By introducing genes encoding green and red fluorescent proteins into *Aeromonas* and *Vibrio* strains isolated from the zebrafish intestinal tract, Wiles and colleagues generated bacteria which could be visualized in a minimally-invasive way in zebrafish larvae (Wiles et al., 2016). In this study, comparison of bacterial population dynamics in wild type and reduced gut motility mutant zebrafish demonstrated the importance of host (rather than simply direct bacterial competition) in structuring of the vertebrate gut bacterial community.

Methods that detect and identify metabolites with subcellular spatial resolution are emerging as powerful tools for studying organismal interactions. Metabolic imaging at subcellular resolution with labelled or label-free methods permits the identification of a broad range of molecules as well as their localization. Live imaging (via introduced green fluorescent protein fluorescence) combined with mass spectrometry showed that an interaction between *Ralstonia solanacearum* and soil fungi may be responsible for the virulence, persistence and proliferation of the bacterial pathogen. Subsequent gene disruption experiments combined with confocal microscopy confirmed that the arrangement of the bacterium is primarily determined by a metabolite produced by the bacteria (ralsolamycin). Using mass spectrometry, the ralsolamycin was visualized at the interface between *R. solanacearum* colonies and approaching fungal hyphae (Spraker et al., 2016).

This direction of study promises to improve our understanding of the function and structural principles of inter-organismal relationships and to provide insight into an array of fundamental biology issues (e.g., cell-cell recognition, immunity, signaling, cell-cycle control). To this end, secondary ion mass spectrometry (SIMS), while originally mainly employed by material scientists, has developed into a technique capable of imaging tissues, single cells, and microbes revealing chemical species with sub-micrometer spatial resolution (Gamble and Anderton, 2016; Henss et al., 2013). One of these methods, NanoSIMS, provides nanometer-scale resolution able to resolve the location of specific molecules/metabolites within bacterial cells. On the other hand, ToF-SIMS instruments have a 'coarser' resolution (typically in the range of micrometers), but can be regarded as "molecular microscopes" that generate chemical maps either across an area or via depth profiling to allow for the three-dimensional reconstructions of cell and tissue structure and molecular composition (Gamble and Anderton, 2016). The high spatial resolution and ability to detect and quantify a wide range of compounds means that these techniques can trace metabolic activity to microbial aggregations or even to specifically labelled microorganisms (Alonso et al., 2012). This is particularly promising for the study of marine cnidarian invertebrates and their associated microalgae and bacteria (Neave et al., 2016), where nanoSIMS has traced key metabolic interactions between algae and bacteria (Raina et al., 2017) as well as between microalgae and cnidarian hosts (Rädecker et al., 2018).

In addition to these emerging high-tech methods, it is important to note that simply dissecting host tissues can also help to understand the distribution of microbial aggregations. Because of the small size of most coral polyps, this is not usually a feasible feat, but the mushroom coral Heliofungia offers an opportunity to look at the biology and physiology of the polyp on a wholly different scale than in other corals. Just as the giant axons of the squid Loligo - at that time, a relatively unknown organism - enabled major advances in neurophysiology (e.g.(Young, 1938)), being creative in selecting target species may permit insights into the spatial organisation of microbes in organisms such as corals. *Heliofungia* are solitary polyps which disassociate from the substrate after development and grow to a maximum size of 50 cm, a diameter several orders of magnitude larger than the average polyp from other corals. This allows for the dissection of tissue layers (a method originally developed for anemone (Richier et al., 2006)), sampling of different localities on the polyp (ie distal vs. central, base of tentacle vs. tip of tentacle, etc.), treatment or exposure trials, and sampling of the oral/gastric system. This coral is a promising model on which applying the molecular methods described in this essay in the ultimate goal to map the functional profile of microbes across a coral polyp. Understanding where microbes aggregate, how they function and how this function varies with microhabitat will illuminate the relationship between host and microbe at the molecular level, thus allowing for a deeper understanding of the relationship at the scale of organism or population.

6. Conclusions

Advances in molecular techniques over the past few decades have allowed the diversity of microbes in a wide variety of environments and biological systems to be estimated. It is now clear that in order to study host organisms (most animals, plants and fungi) one must also study their associated microbes. In this essay we reviewed four categories of molecular techniques related to this goal and identified several key technological developments, some of which may blur the lines between these categories in the future. One such advance is the ability to sequence whole communities of micro-organisms and reliably track sequences back to their cell of origin. This will vastly improve our ability to assemble whole microbial genomes and may accelerate the shift from amplicon based to whole genome based methods for microbial community profiling. Widespread adoption of whole genome based methods could have far-reaching effects on our ability to understand metabolic interactions by facilitating an accurate assessment of gene content within microbial communities. It would also improve our ability to understand the evolution of holobionts by providing dense taxonomic sampling for comparative genomic analyses. Another promising advance is the development of molecular imaging techniques that allow complex information to be gathered in a spatially resolved fashion. These include techniques that measure metabolites at sub-cellular resolution (nanoSIMS) which, when combined with techniques that image the the spatial distribution of microbial communities within a host, could greatly improve our understanding of metabolic interactions between partner organisms.

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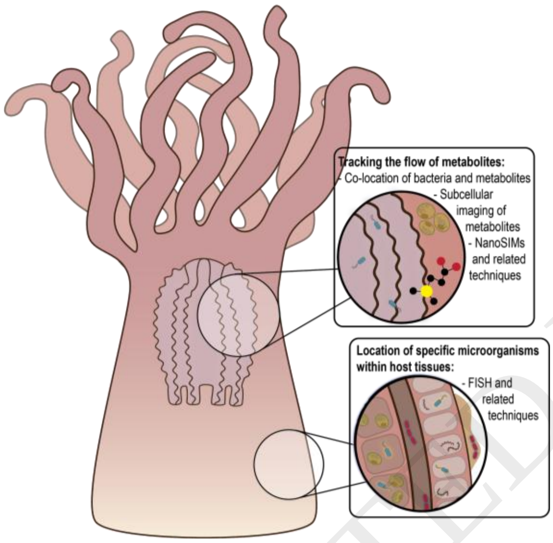

Figure 1: Methods for mapping microbiome components within host tissues include techniques such as nanoscale secondary ion mass spectrometry (nanoSIMS) that allow subcellular imaging of metabolites, and techniques such as fluorescence *in situ* hybridisation (FISH) that map the location of specific microorganisms. When applied in combination, these techniques can be used to make inferences about metabolic activity in microbe-microbe or microbe-host interactions.

Table 1: Important and emerging molecular techniques in holobiont research categorised according to the type of measurement or inference they provide. (FACS: Fluorescence Activated Cell Sorting; qPCR: Quantitative Polymerase Chain Reaction; SIMS: Secondary Ion Mass Spectrometry; GC-MS Gas Chromatography Mass Spectrometry)

Measurement or Inference	Techniques	Related Question(s)
Identification and quantification of microbial communities	Relative microbial community profiling using amplicon or metagenome sequencing Quantification using qPCR, Fluorescent staining, Gold labeling, FACS counts	Is there a core microbiota? How does the taxonomic composition and metabolic potential of the microbiome vary with factors of interest? What is the bacterial load/cargo of host organisms per unit of tissue/organ/compartment?
Evolutionary inference	Phylogenetics Comparative genomics	Has co-speciation occurred? Do genomic changes reflect metabolic interdependence? Have certain genes been subject to strong selection? Is there evidence of genetic drift in partner genomes?
Measure molecular activity	Profile gene expression with RNASeq or Mass Spectrometry Profile metabolite production with GC-MS Experimental manipulation of microbiota and/or gene expression	What are the benefits and costs of the association to different partners? Does nutrient exchange occur between partners? Do partners exert molecular control over each other?
Map (visualise, identify, quantify) microbiome components in host tissues	Dissection FISH FISH-CLEM SIMS	Is there a defined spatial organization/structure of the microbiome? Is the distribution of microbes affected by the host and/or environmental conditions?