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Invited Review

New technologies to study helminth development and host-parasite interactions

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ABSTRACT

How parasites develop and survive, and how they stimulate or modulate host immune responses are important in understanding disease pathology and for the design of new control strategies. Microarray analysis and bulk RNA sequencing have provided a wealth of data on gene expression as parasites develop through different life-cycle stages and on host cell responses to infection. These techniques have enabled gene expression in the whole organism or host tissue to be detailed, but do not take account of the heterogeneity between cells of different types or developmental stages, nor the spatial organisation of these cells. Single-cell RNA-seq (scRNA-seq) adds a new dimension to studying parasite biology and host immunity by enabling gene profiling at the individual cell level. Here we review the application of scRNA-seq to establish gene expression cell atlases for multicellular helminths and to explore the expansion and molecular profile of individual host cell types involved in parasite immunity and tissue repair. Studying host-parasite interactions in vivo is challenging and we conclude this review by briefly discussing the applications of organoids (stem-cell derived mini-tissues) to examine host-parasite interactions at the local level, and as a potential system to study parasite development in vitro. Organoid technology and its applications have developed rapidly, and the elegant studies performed to date support the use of organoids as an alternative in vitro system for research on helminth parasites.

1. Introduction

Transcriptomic studies have revolutionized many aspects of biology and, in the parasitology field, have progressed our understanding of organism development, host-parasite interactions and host responses to infection. Microarray hybridization was initially used to profile gene expression by detecting specific mRNAs in test samples using probes of known gene sequence. These studies provided the first large-scale differential gene expression data on parasites at different developmental stages or sexes, as well as information on host gene expression pre- and post-infection, and host responses across species, strains or breeds. The first helminth microarray studies were reported for *Schistosoma* spp., making use of available expressed sequence tag (EST) information (Hoffman et al., 2002). Subsequently, RNA-seq improved the sensitivity of gene expression analysis and provided a profile of expression at a genome-wide level, as well as being a tool for de novo gene identification. In recent years, the development of single-cell RNA sequencing (scRNA-seq) technologies has stimulated a new area of research - identifying and profiling gene expression of individual cells. scRNA-seq was named Method of the Year in 2013 (Nature Methods, 2013) and is widely used, for example in immunology, developmental biology, oncology and neurology. In the parasitology field, it has identified transcriptome heterogeneity across single-cell parasites, established gene expression cell atlases of various stages of helminths, and detailed individual host cell responses to infection. While microarray and bulk RNA-seq detect transcripts from thousands of cells in a tissue or whole organism and average the gene expression, scRNA-seq provides the fine detail of expression in each cell at the time of sequencing. This single-cell resolution allows profiling of rare transcripts and cell types present in heterogeneous samples. Although not every mRNA is detected in all cells subject to scRNA-seq (drop out), by capturing a sufficient number of each cell type, a detailed profile of that cell can be established at unprecedented resolution.

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2. scRNA-seq methodology

2.1. Single-cell isolation and sequencing

The first scRNA-seq study was reported by Tang et al. (2009) on a mouse embryo and detected not only greater numbers of mRNAs compared with previous transcriptomic studies, but also novel splice variants. Variations of scRNA-seq have been developed, which differ in the methods used to isolate and label single cells from tissues, and the sequencing method employed. Cell isolation can be achieved by several approaches, including microscope-based micromanipulation, fluorescent based cell sorting (FACS) using fluorescent cell tags, laser capture microdissection, limiting dilution of cells plated in multiwells, and microfluidic or droplet-based capture of single cells (see Hwang et al., 2018). Each has advantages and disadvantages (detailed in See et al., 2018) and the approach used depends on the organism being studied and the research question. Micromanipulation and microdissection are time-consuming and low-throughput, while FACS can be particularly useful for specific cell types but requires antibody to a specific surface marker, or cells expressing an appropriate tag. One of the first scRNA-seq technologies was SMART-Seq, which performs a library preparation on single cells sorted in a plate. Droplet-based cell capturing and barcode labeling have advanced in recent years and are used in the commercially-available Chromium (10x Genomics) system, which provides high efficiency capture and sequencing from a low amount of input sample. Labeling cells by plate-based limiting dilution has been less widely used due to relatively poor efficiency. However this technology has recently become commercially available (Parse Biosciences, (USA), using Split Pool Ligation-based Transcriptome sequencing (SPLiT-seq) (Rosenberg et al., 2018), which benefits from being less expensive than Chromium droplet-based scRNA-seq, with no requirement for specialist equipment and can also be carried out on fixed cells. A summary of droplet- and plate-based scRNAseq methodology, data analysis and validation is shown in Fig. 1.

Droplet-based technologies partition cells into individual gel beads containing unique barcodes (overview of Chromium 10x Genomics scRNA-seq in Fig. 1). This enables the cell from which the mRNAs originated to be identified. Additionally, each mRNA within the cell is labeled with a barcode, referred to as a unique molecular identifier (UMI), to identify transcripts. As each transcript has a cell barcode identifying its cellular origin, cDNAs generated from each cell by reverse transcription can be merged in one tube for the downstream processes (cDNA amplification and library preparation), simplifying the procedure. SPLiT-seq can also label transcripts with UMIs, but requires multiple rounds of barcoding, pooling and re-plating of cells. For parasites, SPLiT-seq remains untested. Once generated, libraries are sequenced using an Illumina platform. As it does not select for transcripts with a polyA tail, a higher number of ribosomal transcripts and other RNAs may be captured. For 10x Genomics, it is recommended that each cell should be sequenced to a depth of at least 25,000 reads on average, however in practice this number should be higher, as not all reads map to expressed sequences.

Coverage of the reads relative to the gene will depend on the scRNA-seq protocol used. For example, full-length transcript is generated using SMART-Seq2, while tag-based methods sequence the 3′ end (10x Genomics, massively parallel RNA single-cell sequencing (MARS-seq)) or 5′ end (single-cell tagged reverse transcription sequencing (STRT-seq)) of mRNAs. Droplet-based 3′ end sequencing methods provide data on a large number of cells (up to 10,000 cells per run), useful for profiling cell populations or sub-populations in complex samples. In contrast, full-length scRNA-seq can provide data on isoform or allele expression and RNA editing, due to the greater coverage of transcripts. Sequencing the 5′ end of transcripts is required for some genes, such as those encoding B or T cell antigen receptors, where the variable region is encoded from the 5′ end (Attaf et al., 2020). It is important to bear in mind that scRNA-seq provides a snapshot of mRNAs expressed above a threshold level in each cell at the specific time-point sampled and that only approximately 20–40% of transcripts will be reverse-transcribed and sequenced (approximately 20% for 10x Genomics, approximately 40% for SMART-seq2). Therefore, greater detail and depth of data will be obtained from a greater number of cells in the input sample. While 10X Chromium is currently the most commonly used method, some challenges remain: it is expensive relative to other methods, it requires high cell viability (90% recommended), and there is variability in how many of the loaded cells will be labeled with barcodes, potentially resulting in too many labeled cells for downstream analysis (doublets produced, see below). While a few publications suggest that sample preparation may result in cells subject to scRNA-seq having specific stress signatures (Denisenko et al., 2020), the bulk of publications include verification of scRNA-seq findings, giving confidence to the methodology (see below).

A further evolution of scRNA-seq technology is single-nucleus RNAseq (snRNA-seq). This approach sequences mRNAs associated with isolated cell nuclei rather than whole cells, and has several advantages. Firstly, nuclei are more resistant to mechanical disruption than whole cells and can be isolated from frozen tissues, meaning the technique is applicable to archived tissues and is not dependent on isolating intact live cells for sequencing. Secondly, as different cell types are more sensitive to tissue dissociation processes and could be lost in scRNA-seq analyses, snRNAseq may generate a more representative picture of the diversity of cell types within a tissue sample. Direct comparison of scRNA-seq and snRNA-seq indicates comparable gene detection between the two methods (Bakken et al., 2018; Wu et al., 2019; Selewa et al., 2020). snRNA-seq has been applied to mammalian tissues but has yet to be reported for parasites.

2.2. Data processing

Analysis of scRNA-seq data requires bioinformatics, coding expertise and, if larger datasets are analysed, also computational resources with sufficient memory. Quality control (QC) involves removing low quality bases and adaptor sequences, followed by mapping reads to the relevant genome, and identifying reads mapping to exonic sequences and untranslated regions (UTRs), for methods such as 10x Genomics where reads map to the end of 3′ UTRs. Therefore the quality of genome annotation is important, especially for UTRs, which are often omitted in annotations (Haese-Hill et al., 2022). Once the reads are mapped, read matrices can be loaded into analysis pipelines, for example Seurat using R (http://satijalab.org/seurat/). Additional QC often removes reads from cells showing high expression of mitochondrial (mt) genes, which most likely represent damaged or dead cells in which mtRNAs are retained, but cytoplasmic RNAs are lost (Bacher and Kendziorski, 2016). Cells with very low and very high numbers of mapped reads can also be filtered out as part of the QC process, to remove cells in which RNA may be degraded or that represent doublets (two or more cells captured in the same droplet), respectively. Normalisation can be carried out to correct for within-sample and between-sample biases (see Hwang et al., 2018; Luecken and Theis, 2019).

scRNA-seq can provide data on the expression of thousands of genes across many cells. Two types of reducing dimensionality are needed: the first to capture components that combine information across a correlated set of features and perform a Principal Component Analysis (PCA) to reduce the noise of scRNA-seq data.

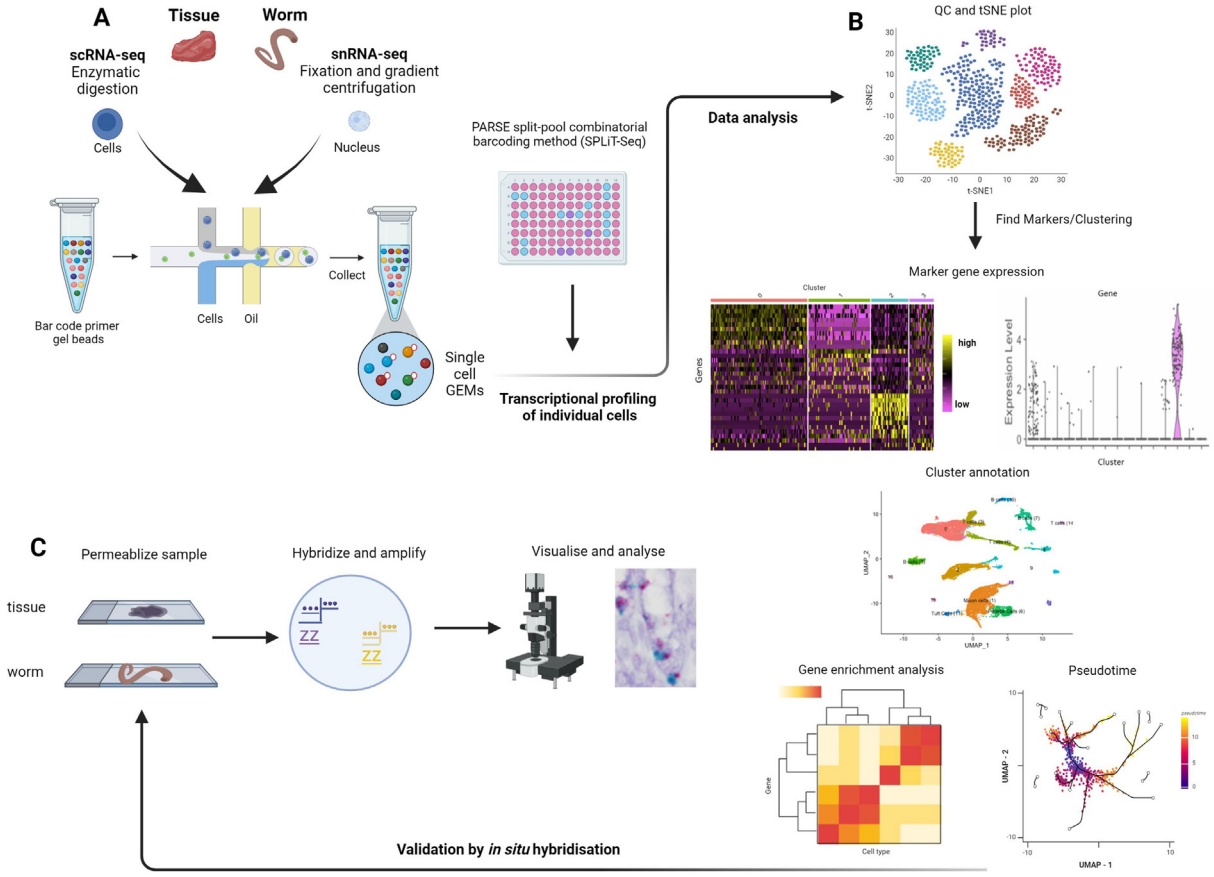

Fig. 1. Overview of single-cell (sc)RNA-seq or single-nucleus (sn)RNA-seq technologies, data analysis and validation. (A) 10x Genomics droplet-based technology partitions cells into barcoded gel beads (gel beads in emulsion; GEMs); plate-based methods separate cells into wells of multiwell plates. mRNAs are subject to reverse transcription, cDNA amplification, library preparation and sequencing. (B) scRNA-seq data is subject to quality control and analysed, for example, using the Seurat R package, to initially identify clusters of cell types based on expression of unique molecular identifiers (UMI) (t-distributed stochastic neighbor embedding (t-SNE) plot). Marker gene expression (heat map, violin plot) enables cluster annotation (uniform manifold approximation and projection (UMAP) plot). Differential gene expression and trajectory inference identify cluster sub-populations and developmental timing (heat map, pseudotime map). (C) Data is validated by in situ hybridization (e.g. RNAscope) using dye-labeled probes to co-localize specific genes. Image created with BioRender.com and own images (modified from Hildersley et al., 2021).

The second reduction involves two- or three-dimensional visualization of the data. The t-distributed stochastic neighbor embedding (tSNE) approach is non-linear and is appropriate for visualizing the high dimensionality and complexity of scRNA seq data. tSNE is available in the Seurat R package designed for scRNA-seq analysis (Butler et al., 2018). A faster reduction algorithm was more recently developed, uniform manifold approximation and projection (UMAP), which organizes and enables visualization of cell clusters based on expression of specific mRNAs. It is important to note that t-SNE and UMAP project the multidimensional data into two dimensions, while clustering and marker gene analysis is performed in the n-dimensional space. Therefore UMAP/t-SNE should not be over-interpreted.

Following initial visualisation, data can be filtered to focus on the most relevant features, using unsupervised feature selection tools. For example, the Find Markers algorithm enables identification of genes differentially expressed in one cluster relative to all other clusters and provides enrichment of expression values. Expression of specific genes of interest can be visualized on UMAP or violin plots and gene expression lists and heat maps generated, enabling identification of specific cell clusters based on marker gene expression. For example, immune cell types may be identified by enriched expression of Cluster of Differentiation (CD) and/or other immune cell markers. For generating cell-type atlases of helminth parasites, cells can be putatively identified based on their enriched expression of cell-type marker genes, such as *myoD* for muscle, cathepsin B (*ctsb*) for gut, or *nanos1* for stem cell types of *Schistosoma mansoni* (Wang and Collins, 2016; Wendt et al., 2020).

In addition to cell-enriched gene expression, scRNA-seq data can be mined further to identify more subtle differences between cells. This includes separation into subtypes followed by pseudotime analysis, using programs that can reveal progression of gene expression across subtypes, suggestive of transitions between developmental stages or phenotypes (Street et al., 2018). Alternative splicing and allelic expression can also be identified from full-length scRNA-seq (e.g. SMART-seq, SMART-seq2), as discussed in Arzalluz-Luque and Conesa (2018) and Chen et al. (2019). Finally, comparison of gene expression between different conditions or developmental stages requires differential expression analysis for each cluster, and downstream interpretation guided by enrichment analysis (for example, see Briggs et al., 2021).

3. Single-cell RNA-seq to study helminth biology

Within the parasitology field, scRNA-seq has been applied to unicellular, protozoan parasites and multicellular helminths. For *Trypanosoma brucei* for example, scRNA-seq has revealed diversity and development of life-cycle stages within the tsetse fly salivary gland (Vigneron et al., 2020), and identified different subtypes of slender and stumpy forms in the mammalian bloodstream (Briggs et al., 2021). For multicellular parasites, the steps involved, from cell isolation to bioinformatics analysis, are more complex

than for protozoa. However, details of helminth cell types and their gene expression using high throughput scRNA-seq have been increasingly reported in recent years. The first of these used the free-living model nematode *Caenorhabditis elegans*, and was quickly followed by scRNA-seq of the free-living planarian *Schmidtea mediterranea* and the parasitic fluke *S. mansoni*. For these species, synchronized developmental stages and clonal populations can be generated, and for free-living species, worms can be obtained in bulk, facilitating isolation of sufficient numbers of cells for sequencing. The difficulties of obtaining certain life-cycle stages, combined with parasite genetic diversity (Doyle et al., 2020), make scRNAseq of parasitic nematodes more challenging. In this section of the review, we focus on studies using scRNA-seq to establish helminth cell-type atlases (summarized in Table 1).

3.1. scRNA-seq of nematodes

Initial single-cell studies in nematodes focused on the early life stages of *C. elegans*, an organism for which the entire cell lineage is known (Sulston et al., 1983) and for which high quality genomic, transcriptomic and cell biology resources are available (WormBase, https://www.wormbase.org). The first reports used low input RNA-seq to study embryonic development in single cells manually separated from 1 to 16 cell stage embryos (Hashimshony et al., 2012, 2015; Tintori et al., 2016). A step-change in scale occurred in 2017, when Cao et al. developed a combinatorial indexing method termed 'sci-RNA-seq' to uniquely label and sequence thousands of single cells. This was initially optimized with human and mouse cells, and then used to sequence 42,035 cells from *C. elegans* L2s. The same approach was then applied to sequence 86,024 single cells from embryos at different stages of development (Packer et al., 2019). When integrated with the data from earlier studies (Hashimshony et al., 2015), and well-characterized marker genes from the literature, cell types or lineages could be identified for 93% of cells in the dataset. This allowed reconstruction of known (invariant) developmental trajectories at single-cell resolution for the entire nematode. Strikingly, differentiation of tissue sub-lineages appeared to be specified by only small numbers of genes, whereas terminal cell type expression was associated with large numbers of genes: hundreds to thousands of genes would typically be enriched in terminal cells from each major tissue and these would generally not be shared with cells from any other tissues. Consistent with this finding, there was also expansion in the number of transcription factors (TFs) that differentiated sister cells in the ectoderm (and mesoderm) lineages over five generations of cells, with 'reuse' of TFs in each generation of daughter cells in addition to expression of new sister-specific TFs. Collectively these findings highlighted that cell fate decision in *C. elegans* is an incremental process; while the timing of the specific event that leads to differentiation appears to vary for different tissues, many terminal cell types are only distinguished in the final division.

More recently, Taylor et al. profiled the entire nervous system of the *C. elegans* L4 at single cell resolution (Taylor et al., 2021). This study used fluorescent reporter strains to identify subgroups of neuronal cells, which were isolated by FACS, then sequenced to profile 70,296 cells, representing the 302 neurons in the mature nervous system. Consistent with the findings of the molecular atlas for embryogenesis, Taylor et al. identified a unique 'code' of neuropeptide and receptor expression in every neuron class.

The highly differentiated nature of gene expression in terminal cells in different tissues, as demonstrated by the above studies, suggests that data from *C. elegans* will be valuable for comparative studies in related parasitic nematodes. For example, the conserved set of ancestral nematode genes within the *C. elegans* datasets should provide markers for many terminally differentiated cell types in different species, which in turn may reveal novel/diverged gene expression profiles in tissues with roles in parasitism. It is also likely that protocols for cell isolation and sequencing developed in *C. elegans* will be adapted for parasites, helping avoid technical issues related to, for example, the highly cross-linked nature of the nematode cuticle and the polyploid intestinal cells, which would not be apparent in work from non-nematodes. The availability of reference quality genomes for a small number of parasitic nematodes will also facilitate the incorporation of genomic data with scRNA-seq for expression quantitative trait locus (eQTL) mapping at cellular resolution, as recently demonstrated in *C. elegans* (Ben-David et al., 2021).

Recent elegant studies on *Brugia malayi* have shown the feasibility of applying spatial and tissue transcriptomics (Airs et al.,

Table 1
Single-cell (sc)RNA-seq studies of helminths.

Helminth	Stage	Aim	Reference
Caenorhabditis elegans	Embryonic blastomeres	Method establishment and gene expression map of blastomeres	Hashimshony et al., 2012
	Embryo	Gene expression map of embryonic development	Hashimshony et al., 2015
	Early embryo	Gene expression map of 1–16 cell stage embryos	Tintori et al., 2016
	L2	Cell atlas of gene expression and regulation	Cao et al., 2017
	Embryo	Gene expression and lineage analysis	Packer et al., 2019
	L4	Gene expression map of nervous system	Taylor et al., 2021
	L2	Expression quantitative trait loci (eQTL) cellular map	Ben-David et al., 2021
		Cell-type transcriptional profile in response to injury	Wurtzel et al., 2015
Schmidtea mediterranea	Adult		
	Adult (head only)	Neural stem cell lineaging	Molinaro and Pearson, 2016
	Adult sections	Cell atlas of gene expression	Fincher et al., 2018
	Adult	Cell atlas and lineage trees of wild-type and regenerating tissue	Plass et al., 2018
Dugesia japonica	Adult	Cell atlas and comparison with *S. mediterranea*	Garcia-Castro et al., 2021
Schistosoma mansoni	Mother sporocyst	Stem cell gene expression and heterogeneity	Wang et al., 2018
	Adult male, mature female & virgin female	Cell atlas of gene expression in different reproductive states	Wendt et al., 2020
	Juvenile schistosomula	Germ-line stem cell characterization	Li et al., 2021
	2-day in vitro schistosomula	Cell atlas of gene expression	Diaz Soria et al., 2020
Brugia malayi	Microfilariae	Cell atlas; mapping of secretory products and drug targets	Henthorn et al., 2022

2022), as well as scRNA-seq (Henthorn et al., 2022), to a parasitic nematode. Airs et al. (2022) profiled gene expression for the head region of *B. malayi* using a combination of low input RNA sequencing with histological sectioning (termed 'RNA tomography') and microdissection. The head region of *Brugia* contains many structures with key roles in host-parasite interactions (including the buccal cavity, amphids, pharynx, nerve ring and excretory-secretory (ES) apparatus), which are greatly under-represented in whole-worm sequencing data. By sequencing RNA extracted from the head, serial sections of the head, and the pharynx after microdissection, high-resolution transcriptomes of these important structures could be generated. These data revealed distinct expression patterns for genes encoding filarial antigens with known immunomodulatory properties and for putative drug targets, as well as identifying possible marker genes for single-cell studies.

Subsequent work by Henthorn et al. (2022) adapted single-cell protocols for *C. elegans* to *B. malayi* microfilariae, accounting for differences such as contamination with host tissues and the chitin-rich sheath of the microfilaria, to generate a gene expression atlas from 46,621 cells. As expected, it was possible to use one-to-one orthologues conserved between *C. elegans* and *B. malayi* to annotate a significant proportion of cells (39%; 17 UMAP clusters of 18,317 cells) including those derived from muscle and neuronal tissues. A particular focus of this study was the origin of ES products, which have long been implicated in modulation of the host immune response by parasitic nematodes. In contrast to structures with more conserved functions, the transcriptome of the *B. malayi* secretory cell (identified morphologically and isolated by FACS) showed no overlap with the *C. elegans* excretory cell. Interestingly, the *B. malayi* secretory cell expressed an abundance of Cys_2His_2 TFs, which the authors speculate may regulate gene expression to allow for adaptation of microfilariae moving between the highly divergent environments of a mammalian host and an insect vector. This work confirms the utility of a *C. elegans* model for single-cell studies in parasitic nematodes but highlights the need for innovative approaches to study important parasite-specific aspects of nematode biology.

3.2. scRNA-seq of planaria

While *C. elegans* is a powerful model system for studying nematode development and gene function, for flatworms (flukes and tapeworms), the free-living planarian *S. mediterranea* provides an alternative model system (Collins and Newmark, 2013). This species is also of great interest to the study of stem cell biology and tissue regeneration. Approximately 20% of cells in adult *S. mediterranea* are stem cells (neoblasts), capable of generating all types of new differentiated cells (Collins et al., 2013). Wurtzel et al. (2015) carried out the first scRNA-seq of *S. mediterranea* using 619 cells to identify cell types responding to tissue injury and the dynamics of the transcriptional response. Their findings revealed a general stress response, including induction of heat shock protein genes, in most cell types, soon after damage. Cell-specific transcriptional responses also occurred, but these were limited and only observed in neoblast, muscle and epidermal cells. After the initial response, specific differentiation factors were switched on to direct tissue regeneration. These findings were supported by in situ hybridization (ISH), and a gene expression cell atlas for *S. mediterranea* was established.

Focusing on the head region, Molinaro and Pearson (2016) isolated *S. mediterranea* cells using FACS and performed lineage analysis of stem cells and early progeny cells. Based on expression of stem cell and neural marker genes, they identified putative early and intermediate stem cells, and reported a novel neoblast subpopulation with neural characterisitics referred to as nu-Neoblasts (vNeoblasts). In 2018, Fincher et al. and Plass et al. characterised all cell types of *S. mediterranea* by performing high-throughput scRNA-seq on adult worms. Adults were cut into distinct regions, live cells were selected by flow cytometry, and Drop-seq was carried out to profile single cells. Distinct clusters were defined based on expression of known cell type markers and validated by ISH. Subclustering of distinct clusters enabled profiling of rare cell types not previously identified and revealed transition states between stem cells and different types of differentiated cells, based on expression of single or combinations of transcription factor genes (Fincher et al., 2018). Interestingly, regenerating samples showed a specific loss of parenchymal cells, suggesting that these cells may act as an energy reservoir to support tissue regeneration (Plass et al., 2018).

In a study to compare methods for metazoan cell dissociation for scRNA-seq, Garcia-Castro et al. (2021) compared the planarian species *Dugesia japonica* and *S. mediterranea,* and observed similar cell clusters in both species. Notably, a simple buffer that maintains RNA stability was reported for fixing cells rapidly in suspension, and cryopreserving cells for future library preparation and sequencing. This protocol may also prove appropriate for nematode cell isolation.

3.3. Schistosoma mansoni gene expression cell atlases

To date, single-cell transcriptome profiling has been achieved for various developmental stages of *S. mansoni* (see Table 1). scRNA-seq of mature adult male and female worms, as well as age-matched virgin female worms, identified 68 distinct clusters, including cells expressing markers characteristic of somatic stem cells, neuronal cells, tegument, flame cells, germ cells and muscle cells (Wendt et al., 2020). ISH confirmed the anatomical location of specific genes present within defined expression clusters. scRNA-seq data can help inform selection of genes for functional analysis by RNA interference (RNAi) gene silencing, leading to generation of a functional genomics map. For example, Wendt et al. (2020) identified a gut neoblast cell cluster expressing a specific marker gene, *hnf4*, RNAi silencing of which led to a decrease in gut gene expression, gut function and digestion of red blood cells. The availability of parasitic helminth cell atlases will clearly be a catalyst for new studies on vaccine and drug targets.

scRNA-seq of two-day old *S. mansoni* schistosomula, matured in vitro, identified 13 distinct cell clusters, that were characterized based on marker gene expression and validated by ISH to establish a cell-type atlas (Diaz Soria et al., 2020). Among other findings was the expression of previously identified micro-exon genes in a population of oesophageal gland cells. The function of these genes is currently unknown, however their potential to generate protein diversity and their expression in gland cells, which are involved in degrading host immune cells, identify them as potentially attractive vaccine targets (Diaz Soria et al., 2020). The same study also made use of gene expression data across cell clusters of *S. mediterranea* adults to identify *S. mansoni* schistosomula cell types, by employing a random forest model to average the outputs from multiple decision trees. Despite the differences in life-cycle stages, stem cells were found to map across the two species. There was also linkage of neuronal cells expressing *SmKK7* and a neuronal cell population in *S. mediterranea,* indicating conservation of some marker genes across the free-living and parasitic species.

scRNA-seq of *S. mansoni* germline stem cells has also been achieved (Wang et al., 2018; Li et al., 2021), identifying a set of genes that distinguish germline stem cells from other types of stem cell (Li et al., 2021). A specific homeobox transcription factor *onecut-1* (*oc-1*), which is required for balancing the proliferation and differentiation of germline stem cells, was identified by scRNA-seq and silenced by RNAi. The detail gained from scRNA-

seq studies on schistosomes highlights the potential of this methodology for progressing understanding of many aspects of development and the genes involved, and will be important in informing new potential strategies for limiting schistosome transmission and pathology (Li et al., 2021).

4. scRNA-seq to define host cell responses to helminth infection

Cell-type gene expression data has also detailed host responses to helminth infections. While much is known from bulk RNA-seq, scRNA-seq can identify the specific cell types involved, detail the dynamics of the cellular response, and determine differences between cell sub-populations that may be missed by bulk analyses (reviewed in Papalexi and Satija, 2018). Here we will focus on recent studies using scRNA-seq to detail host cellular responses to helminths infecting the gastrointestinal (GI) tract and characterise the key cell types and genes involved.

Work from our laboratories and others reported the importance of a rare epithelial cell type, the tuft cell, in initiating the characteristic type 2 response to gastrointestinal (GI) nematode infection (Gerbe et al., 2016; Howitt et al., 2016; von Moltke et al., 2016). Tuft cells are proposed to sense helminths in the GI tract and stimulate type-2 innate lymphoid cells (ILC2) to activate T helper 2 (Th2) cells. How tuft cells sense and respond to infection is currently unknown.

Haber et al. (2017) performed 10x Genomics scRNA-seq of mouse small intestinal (SI) epithelial and immune cells at homeostasis and following infection with the GI nematode *Heligmosomoides polygyrus* to determine their molecular profile. Distinct cell clusters, including Paneth, goblet, enteroendocrine, tuft and stem cells were identified, based on marker gene expression. Different sub-populations of cell types could be distinguished in different SI regions (duodenum, jejunum and ileum). For the first time, two subtypes of mature tuft cells (neuronal and immune) were identified from all three SI regions. Notably, the abundance of immune-type tuft cells, as well as goblet cells, increased significantly following *H. polygyrus* infection. This molecular and cellular analysis greatly progressed the previous immunological findings and identified genes encoding mediators, receptors and regulators of epithelial cell function.

We extended this work to infections caused by two economically important gastric nematode parasites of livestock, *Teladorsagia circumcincta* and *Haemonchus contortus* (Hildersley et al., 2021). Almost nothing was previously known of tuft cells in other host species and there were no ovine tuft cell surface markers to enable FACS purification for subsequent sequencing. scRNA-seq of ovine abomasal (stomach) mucosa identified 15 different cell types and their gene expression profile during *T. circumcincta* infection. In contrast to mouse SI, we did not identify neuronal and immune tuft cell types; however the more prolonged time course of ovine nematode infection and the application of trajectory analysis suggested populations of tuft cells at different states of maturation. Importantly, the data showed conservation of genes encoding mediators of tuft cell function (e.g. leukotrienes and prostaglandins involved in the inflammatory response). However, G-protein coupled receptors (GPCRs), proposed to sense parasites within the GI tract, differ between host species and/or GI regions (Hildersley et al., 2021). The data identified specific GPCRs enriched at the site of infection and these have been expressed in mammalian reporter cells. We are currently testing activation of ovine tuft cell GPCRs by parasite ES products and potential ligands therein, to better understand how type 2 responses may be initiated following nematode infection (Gillan et al., unpublished data).

For profiling of $CD4^+$ T cells responding to *Nippostrongylus brasiliensis* infection, Brown et al. (2021) carried out bulk RNA-seq and scRNA-seq of sorted cells. These were from lung or draining lymph node tissue, where *N. brasiliensis* L3s transit, rather than epithelial cells. Distinct clusters and gene expression profiles helped distinguish Th2 and T follicular helper (Tfh) cell types, while clusters showing overlap in gene expression between locations may represent circulating or transitory cells not yet committed to a specific fate. These experiments are important for understanding the finer details of T cell activation and differentiation in addition to their eventual phenotype.

While tuft cells are key in initiating a type 2 response to GI nematodes in the upper GI tract, the initial response in the caecum to *Trichuris* whipworm infection has also been characterized. Duque-Correa et al. (2022) carried out bulk and scRNA-seq on mouse caeca before and following infection with the mouse whipworm *Trichuris muris* and identified a cluster of enterocyte cells expressing IFN-stimulated genes, including the alarmin *Isg-15*. This cell cluster increased in size soon after *T. muris* infection, suggesting it may be involved in triggering an immune response and tissue repair. scRNA-seq is proving to be highly informative in extending our understanding of the development and dynamics of host immunity, particularly if deployed over multiple time-points during infection.

5. Organoids as an in vitro system to study parasites and host responses

Understanding of nematode infection and host-parasite interactions has greatly progressed in recent years through application not only of scRNA-seq, but through the development of tissue organoids or "mini-tissues". Organoids are in vitro, multicellular three-dimensional tissue constructs derived from tissue stem cells or induced pluripotent stem cells, that can recapitulate the features of their corresponding in vivo organ. In the presence of defined growth factors and nutrients, stem cells will grow and differentiate into three-dimensional structures comprising different cell types, including Paneth, goblet, enteroendocrine and tuft cells for SI organoids, spatially organised in a manner similar to the tissue of origin. Their cellular diversity and spatial organisation make organoids more physiologically relevant than immortalized cell lines, while sharing some advantages: they can be continually propagated and passaged as long as a stem cell population is present, they can be manipulated (e.g. CRISPR gene editing, addition of inhibitors or antibodies) for functional studies, and they can be cryopreserved allowing organoid tissue biobanks to be generated (Perrone and Zilbauer, 2021). The host specificity of many helminths means there are few in vivo or in vitro models for studying parasite development and host-parasite interactions. Numerous studies have shown the feasibility of organoid development using stem cells from different mammalian species and host organs (examples in Sato et al., 2011; Hamilton et al., 2018; Chandra et al., 2019; Smith et al., 2021; Faber et al., 2022), with each organ type requiring some variations in the growth factors and media conditions required (see Kaushik et al., 2018; Smith et al, 2022). We will focus here on the use of gastro-intestinal organoids to study tissue responses to GI nematodes and their products, and for nematode development (work to date summarized in Table 2).

Most information is currently available on using organoids to study the murine whipworm *T. muris*. Initial studies used mouse colonic stem cells to generate organoids and test uptake of extracellular vesicles (EVs) secreted from adult worms. Following micro-injection into the organoid lumen, labeled EVs could be seen in the cytoplasm of organoid cells. This occurred at 37 °C but not at 4 °C, indicating an active process (Eichenberger et al., 2018a). Stud-

Table 2
Published studies using organoids to examine host-parasite interactions and parasite development.

Helminth	Organoid tissue	Species	Aim	Reference
Ascaris suum	Small intestine	Dog	Uptake of EV	Chandra et al., 2019
Heligmosomoides	Small intestine	Mouse	Effect of infection & IFN-γ on epithelial cells	Nusse et al., 2018
	Small intestine	Mouse	Effect of ES & L3 on epithelial cells	Drurey et al., 2022
	Small intestine	Mouse	Effects of ES on epithelial stem cells	Karo-Atar et al., 2022
Nippostrongylus brasiliensis	Small intestine	Mouse	Uptake of EV	Eichenberger et al., 2018b
Ostertagia ostertagi	Abomasum	Cattle	Uptake of EV & uptake of L3	Faber et al., 2022
Teladorsagia circumcincta	Abomasum and small intestine	Sheep	Uptake of L3	Smith et al., 2021
Trichinella spiralis	Small intestine	Mouse	Effect of ES & extract	Luo et al., 2019
Trichuris muris	Colon	Mouse	Uptake of EV	Eichenberger et al., 2018a
	Caecum 2D	Mouse	Uptake of EV & immune effect	Duque-Correa et al., 2020
	Caecum 2D	Mouse	Cell invasion by L1	Duque-Correa et al., 2022

EV, extracellular vesicles.

ies from the same group also showed that EVs secreted from adult stage *N. brasiliensis* were taken up into SI organoid epithelial cells (Eichenberger et al., 2018b). Subsequent studies used caecaloids, caecal organoids derived from stem-cells, to examine effects of injected *T. muris* adult EVs on host cellular responses and observed down-regulation of nucleic acid recognition and Type I interferon signaling (Duque-Correa et al., 2020). The parasite mediators of these effects are currently unknown, and may involve protein and/or small RNA molecules within EVs.

Our previous work on *H. contortus* EVs identified a small number of microRNAs (miRNAs) within secreted vesicles (Gu et al., 2017). Using ovine abomasal organoids (Fig. 2), we recently demonstrated uptake of labeled EVs from *H. contortus* adult ES into organoid cells (Fig. 2). This was observed following incubation of labeled EVs with organoids rather than micro-injection, which is more technically challenging. We are currently examining the cellular localization of labeled EVs and of labeled miRNAs following incubation with organoids, and any influence these may have on host cell gene expression and organoid development (Perez, Gillan et al., unpublished data).

Intestinal organoids also recapitulate the type 2 response of epithelial cells to cytokines such as IL-4 and IL-13, which stimulate the differentiation of secretory cell types (goblet, Paneth, tuft cells). It was reported that *H. polygyrus,* which is able to establish a long-term intestinal infection, can inhibit the development of these specialised effector cells. In vitro, organoids co-cultured with adult ES

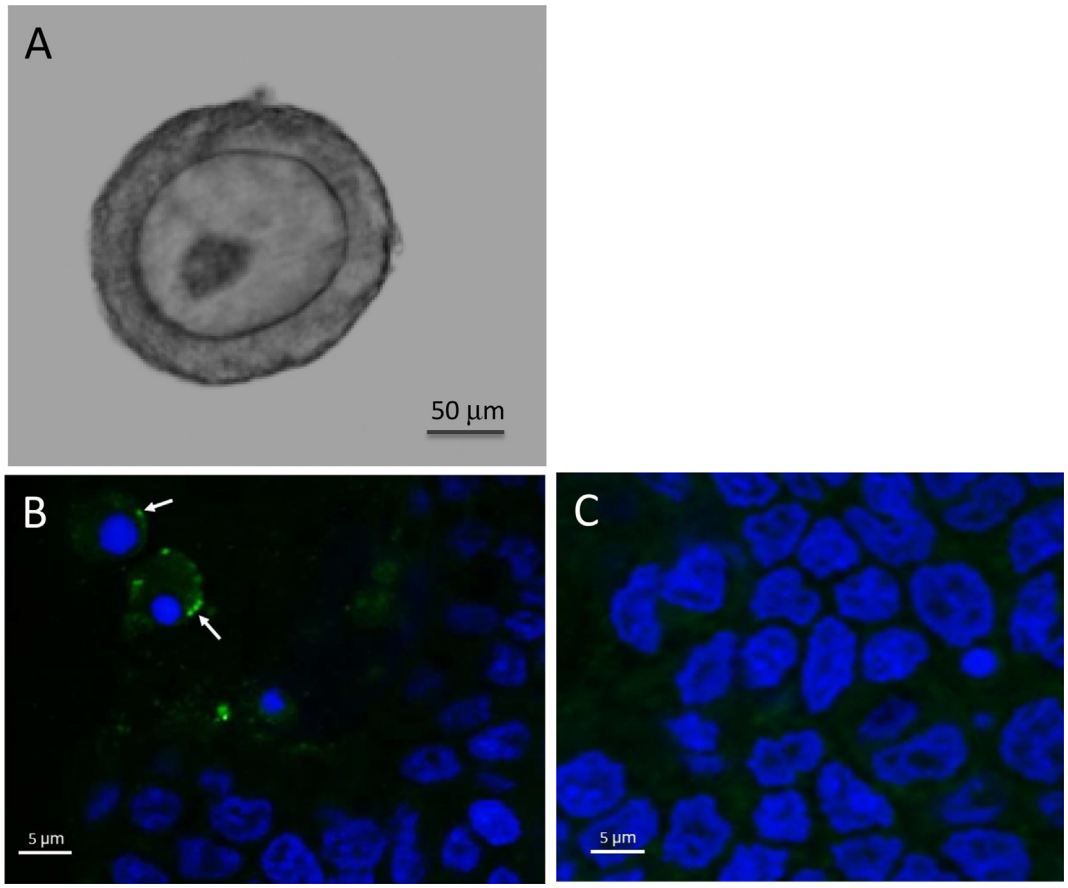

Fig. 2. Ovine abomasal organoids. (A) Three-dimensional ovine abomasal organoids after 3 days in culture at 37 °C, developed from crypt stem cells. (B) Uptake of adult *Haemonchus contortus* PKH-67 labeled (green) extracellular vesicles (EVs) into ovine abomasal organoid cells after 24 h of incubation at 37 °C. Arrows indicate labeled EVs inside cells. (C) No green fluorescence was observed following incubation of organoids with PKH-67 labeled EV-depleted supernatant. Blue indicates DAPI staining.

products or L3s developed fewer tuft cells in response to IL-4/IL-13 (Drurey et al., 2022). Moreover, in vivo both live infection or the ES products were able to suppress tuft-cell hyperplasia observed during *N. brasiliensis* infection, or in mice treated with the metabolite succinate, which other authors had shown previously can stimulate tuft cell outgrowth (Nadjsombati et al., 2018; Schneider et al., 2018). Interestingly, SI organoids exposed to *H. polygyrus* ES developed into large spheroid structures, rather than forming budding organoids with crypt-like niches required for differentiation. Spheroid, non-differentiating organoids were previously observed following growth of SI stem cells from *H. polygyrus*-infected mice or after IFN-γ treatment of SI organoids. Thus, this parasite infection can reprogram intestinal stem cells into a more de-differentiated fetal-like state that may represent either or both a fetal-like, or repair and regenerative phenotype. This observation was similar to the fetal-like granuloma-associated cells occurring in vivo during *H. polygyrus* infection (Nusse et al., 2018). More recently, it has been shown that reprogramming of the intestinal epithelium by *H. polygyrus* involves the emergence of Clusterin-expressing revival stem cells (revSCs) in vivo, and using organoids it was demonstrated that these cells can be induced directly by *H. polygyrus* ES (Karo-Atar et al., 2022).

A similar spheroid-like phenotype has been observed in bovine gastric organoids exposed to live *Ostertagia ostertagi* L3s or their ES products (Faber et al., 2022). Exposed organoids rapidly expanded in size within 1 h of culture, the rapidity of which suggested fluid influx into the lumen rather than epithelial de-differentiation. Interestingly this expansion preceded invasion of the organoid lumen by L3s, suggesting it may be involved in initial L3 invasion of the gastric glands in vivo. These findings support the suitability of organoids for studying parasite effects on host epithelial cells. Additionally, as tissue organoid systems lack immune cell types, they provide a relevant system for identifying direct effects of parasites or their products on the epithelium that may act independently of classical immune cells.

Maintaining most helminth parasites in vitro is challenging due to the lack of culture systems that support development through the life-cycle stages. Using organoids as a system to promote nematode development would be a huge advantage and, to date, some success has been achieved. Duque-Correa et al. (2022) showed that *T. muris* L1 stage can degrade secreted mucous and invade intestinal epithelial cells within caecal organoids, providing an in vitro system to examine establishment of infection and parasite interaction with host cells. Notably, this was achieved using caecaloids grown in an open two-dimensional conformation in transwells, in which the L1s could access the apical intestinal surface. Understanding how parasites infect host cells, using organoid systems, may help determine the specificity of many host-parasite interactions and could identify targets and possible inhibitors to block infection.

Smith et al. (2021) also showed that infective L3s of the important veterinary nematode *T. circumcincta* can survive for up to 2 weeks in the central lumen of ovine abomasal and ileum organoids. While L3s were active during this time, limited further development occurred. It will be interesting to test if use of open two-dimensional organoids, as employed for *T. muris*, facilitates development. The suitability of organoids to support in vitro culture and their use in successful development of *T. muris*, as well several protozoan parasites, is encouraging for further studies on much-needed helminth in vitro systems (Smith et al., 2022).

6. Conclusions and future directions

As with most new technologies, applications and variations of scRNA-seq and organoid systems are continuing to develop. scRNA-seq cell atlases are highly informative and provide a platform to compare gene expression in individual cells across helminth life-cycle stages, between isolates/strains and to begin to determine how expression is regulated. The use of these extensive datasets is facilitated by the provision of user-friendly web applications and R packages to visualize and access the data (for example, Worm Cell Atlas (https://atlas.gs.washington.edu/worm-rna/), VisCello and CengenApp for *C. elegans*). It will also be important to integrate scRNA-seq data from current and future work, and make these widely available, for example via WormBase and WormBase ParaSite (https://parasite.wormbase.org).

By combining different single-cell sequencing technologies, we can progress our knowledge further. Durham et al. (2021) used the scRNA-seq data available for *C. elegans* L2 stage (Cao et al., 2017) to map upstream regulatory regions to specific tissues and cells. This was achieved using the Assay for Transposase Accessible Chromatin followed by sequencing (ATACseq) (Buenrostro et al., 2015), which exploits the ability of Tn5 transposase to cut accessible (open) genomic regions. These regions were then tagged with adaptor sequences, for library construction and DNA sequencing. The results were comparable to previous bulk *C. elegans* studies from whole worm ATACseq or transcription factor chromatin immunoprecipitation (ChIP-seq). Additionally, by mapping to scRNA-seq data, active regions could be assigned to cell types.

Adapting ATACseq to parasitic species will shed light on mechanisms of parasite activation and development within the host. Mapping accessible regulatory regions and motifs pre- and post-infection, and assigning these to worm cell types via scRNA-seq, would build upon the current genome projects (Wormbase ParaSite) and help elucidate how cell-type-specific genes are regulated. It may be possible to identify specific transcription factors and perhaps host factors regulating parasite development within the host, and the responsive worm cell types involved.

ATACseq could also identify variations in promoter sequences or splicing events that may determine phenotypic differences within or between helminth populations. Such differences may also be informed by expression quantitative trait loci (eQTLs); scRNA-seq has recently been used to map eQTLs to cell types in genetically distinct *C. elegans* individuals (Ben-David et al., 2021). Extending such analyses to genetically heterogenous helminth parasites has the potential to advance understanding of a range of biological phenotypes, such as anthelmintic resistance and survival post-vaccination (Sallé et al., 2018).

The studies using *S. mansoni* scRNA-seq suggest that it should be feasible to adapt methodologies for cell extraction, single-cell sequencing and bioinformatic characterization to parasitic nematode species, and make use of available parasite and *C. elegans* cell marker information. Technologies are available for combining scRNA-seq with protein expression that facilitate cell identification, as well as profile protein levels within cells. For example, cite-seq (Stoeckius et al., 2017) adds a DNA tag to antibodies, followed by barcoding of the DNA tag and mRNA within the same cell. This enables co-detection of known marker proteins and mRNAs. This approach is currently feasible with surface proteins, but will require more invasive cell permeabilisation and optimisation for detection of intracellular proteins. Additionally, spatial transcriptomic approaches, to localize transcripts and proteins of interest using labeled probes in a multiplexed platform, are continuing to evolve. These include the NanoString GeoMx™ system (Zollinger et al., 2020), which employs barcoded probes to capture and sequence multiple transcripts within a cell or tissue, as well as a more recent fluorescence imaging-based system that can directly spatially profile transcripts and proteins (detailed in Vu et al., 2022).

'Omics' technologies have greatly progressed knowledge of helminths and host responses, however development of in vitro systems to study parasite development and host-parasite interactions

are more challenging. As detailed above, recent work using two-dimensional caecaloids has demonstrated *T. muris* larval invasion of epithelial cells in a manner consistent with host infection (Duque-Correa et al., 2022), while L3 stages of important veterinary GI nematodes can survive within SI and abomasal organoids (Smith et al., 2021; Faber et al., 2022). Specific questions and potential interventions can now be addressed in vitro, such as the ability of inhibitors to block the activity of parasite-secreted proteases thought to be required for tissue penetration (Duque-Correa et al., 2022). Host cells, molecules and pathways that may support invasion can also be examined using organoids derived from specific gene-knockout mice, or through CRISPR/Cas9 gene editing directly in organoids (Ringel et al., 2020; Teriyapirom et al., 2021). CRISPR-mediated gene knock-in is also feasible, enabling generation of reporter organoid lines (Artegiani et al., 2020).

Genetic variation in host susceptibility to helminth infection is well documented and many studies have identified differences in immune mechanisms (Piedrafita et al., 2010; Lins et al., 2022). A common finding is the earlier onset of host responses in resistant animals. Development of organoids from hosts of differing susceptibilities could help address questions such as: are there inherent differences in host epithelial cells at homeostasis, are there differences in epithelial cell responses or damage following infection in different strains/breeds, are immune cells involved in influencing outcome, how may local responses or repair be enhanced? While an advantage of organoids is their simplicity, comprising only those cells developing from tissue-specific stem cells, it is also possible to co-culture organoids with immune cell types. For example, intestinal organoids have been co-cultured as two-dimensional monolayers on transwell 'shelves', with primary macrophages or neutrophils residing under the basal surface (Noel et al., 2017; Staab et al., 2020). Such systems would allow parasites or their ES products to be applied to the apical surface, and responses in the immune cell compartment analysed.

The technologies discussed here are revolutionising molecular and cellular biology, and are very relevant to helminth research. Further adaptation and optimization will provide the tools to address some of the outstanding questions in parasite activation/development, gene regulation and host-parasite interactions. The fine detail provided by these systems will help identify novel ways to limit parasite survival and enhance host resistance, including identifying targets for future drug and vaccine development.

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