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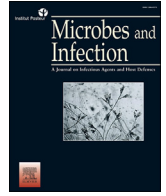


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## Unraveling the intricacies of host-pathogen interaction through single-cell genomics

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### ABSTRACT

Single-cell genomics provide researchers with tools to assess host-pathogen interactions at a resolution previously inaccessible. Transcriptome analysis, epigenome analysis, and immune profiling techniques allow for a better comprehension of the heterogeneity underlying both the host response and infectious agents. Here, we highlight technological advancements and data analysis workflows that increase our understanding of host-pathogen interactions at the single-cell level. We review various studies that have used these tools to better understand host-pathogen dynamics in a variety of infectious disease contexts, including viral, bacterial, and parasitic diseases. We conclude by discussing how single-cell genomics can advance our understanding of host-pathogen interactions.

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A pathogenic infection involves a dynamic and complex interaction between a pathogen and its host. Bilateral assessment of both host and pathogen biology is necessary to understand infection outcomes and to develop appropriate therapeutic strategies. This is especially critical considering that a single infectious agent can cause diverse disease phenotypes and outcomes in different individuals. For example, upon infection with *Yersinia pestis*, the bacterial etiologic agent of plague, individuals expressing a full-length isoform of endoplasmic reticulum aminopeptidase 2 (*ERAP2*) were better protected from severe disease outcomes, such as death, compared to those expressing a truncated isoform of the transcript [1]. Variability in response to a single infectious agent can also manifest at the gene level. For instance, a single germline gene mutation can redirect immune differentiation [2], resulting in cells from the same lineage to exhibit significantly different phenotypes. These examples reveal that infectious disease outcomes are partially governed by host derived mechanisms. Infectious disease outcomes are also partially governed by the virulence of the infecting pathogen. The variable response to infection by human cells, combined with the differences in cell biology observed

within- and across-pathogens poses a challenge to infectious disease therapeutic strategies, many of which are inadequate in part due to a failure to account for the biology of both host and pathogen in their design.

Single-cell genomics can reveal molecular signatures of various cell types in both the host and pathogen and can therefore further enhance our understanding of infection outcomes and help inform therapeutic strategies. In the host, they can be used to characterize the immune response to pathogens, determine the immune repertoire, and, in some cases, distinguish infected from uninfected cells. Researchers have successfully used single-cell genomics technologies and workflows for robust assessment of human and various model system. Single-cell genomics can also be used to characterize pathogens, including bacteria, viruses, and parasites. For this goal, researchers likely need to optimize aspects of the single-cell genomics pipeline (both technical and computational) to account for the cellular and molecular biology of the pathogen being assessed.

Our review highlights advancements in technologies and computational workflows that have significantly increased our understanding of host-pathogen interactions at the single-cell level. We feature studies of gene expression in individual cells (i.e., single-cell transcriptomics) and on the quantitative measurement of molecular signatures at the cellular level beyond

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transcription (i.e., chromatin accessibility, immune repertoire) in various infectious disease contexts (Fig. 1). Most research at the single-cell level has focused on the molecular signatures of either the host or the pathogen. Here, we include recent studies that have pushed the boundaries of single-cell profiling through bilateral assessment of host and pathogen at the gene expression level, and which can be brought to bear on other infectious disease contexts. As an example, these studies show how cells infected with Plasmodium parasites, the causative agents of malaria, can be discerned from non-infected cells at a previously inaccessible resolution. Finally, we discuss the current limitations of single-cell genomics, and explore the exciting prospects of multi-modal and spatial workflows that support the new frontier of research on infectious disease biology.

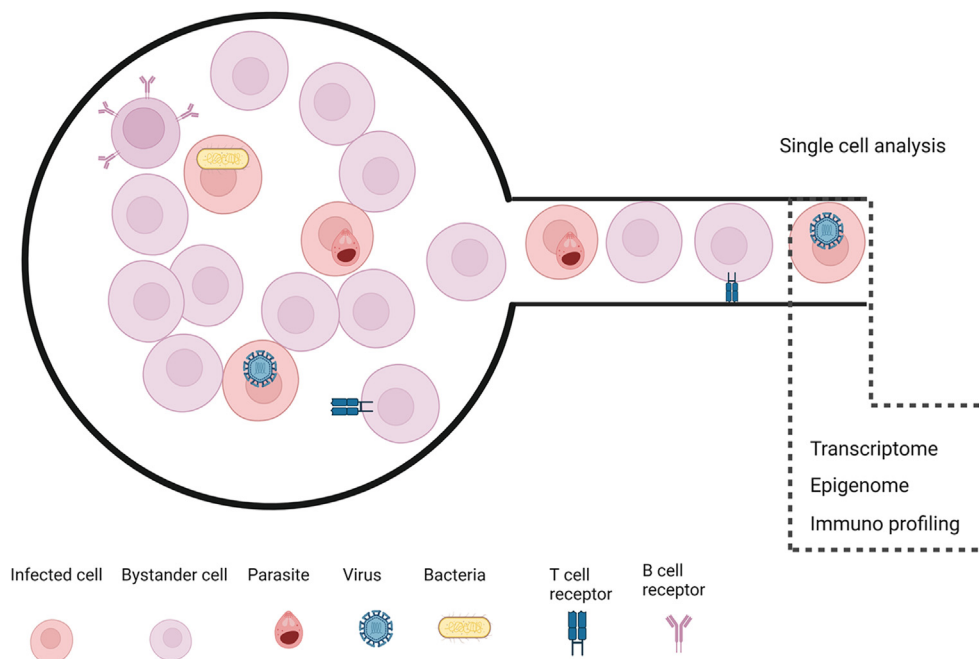
## 2. Single-cell genomics methods for the study of host-pathogen interactions

Multiple techniques in molecular biology, microscopy, cytometry, and next generation sequencing (NGS) emerged to profile single-cells. For example, flow cytometry, fluorescence microscopy, and imaging flow cytometry (IFC) to date are some of the most used techniques for single-cell profiling [3] and have increased our understanding of host-pathogen interactions. These high-throughput techniques represent valuable tools for exploring host-pathogen interactions. However, they have limitations when it comes to the number of parameters that can be assayed per cell. With the advent of NGS workflows, single-cell analysis methods greatly expanded [4]. It has emerged as a powerful and flexible approach for generating, interpreting, and analyzing massive amounts of genomic data.

### 2.1. Single-cell methods for transcriptome analysis

Single-cell RNA sequencing (scRNA-seq) is an effective tool used to identify rare cell types, characterize gene regulatory networks, and trace developmental trajectories. In the context of infectious disease research, identifying entry receptors and their expression is key to developing targeted therapies aimed at preventing pathogen entry. The analysis of gene expression data generated by scRNA-seq workflows makes it possible to identify infection-susceptible cells and to determine the expression levels of pathogen entry receptors. For example, during the Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) outbreak that caused the coronavirus disease 2019 (COVID-19), single-cell gene expression data of 119 cell types were used to identify candidate peptidases co-receptors based on angiotensin I converting enzyme 2 (ACE2) expression [5]. Additionally, it is often required to identify rare cell populations like a subpopulation of infected cells or antigen specific lymphocytes. For instance, by integrating single-cell transcriptomic data with longitudinal antibody repertoire sequencing data and antibody binding measurements, Horns and colleagues compared the profile of a rare population of activated B cells after influenza vaccination with that of inactivated B cells [6]. They observed that multiple activated B cell clones were not vaccine-specific, suggesting the activation of non-specific bystander memory B cells.

To date, scRNA-seq is the most commonly used single-cell genomics tool. Various scRNA-seq methodologies exist, each with their own strengths. Some medium-throughput methods (i.e., plate-based) allow for transcriptome-wide profiling of full-length transcripts and therefore support the assessment of splice isoforms in individual cells. Other methods (i.e., microwell-based and droplet-based) support the assessment of hundreds to thousands of



**Fig. 1. Representation of the isolation of single-cells in a cell suspension comprised of a heterogeneous mix of infected and non-infected cells.** The three infectious agents of focus in this review (bacteria, parasites and viruses) and the immune cell receptors are highlighted. The figure also illustrates three main -omics approaches discussed in the review used to support infectious disease research and to better define host-pathogen interactions. Transcriptomics analysis will identify RNA transcripts within one single cell. Single-cell epigenomic methods provide a more profound understanding of the regulatory mechanisms that govern cell fate during infection. Immunoprofiling of B and T cell receptors characterizes receptor sequences of an expanded B-cell and T-cell clones provides insight into the developing immune response against pathogens and can aid in the development of vaccines, monoclonal antibodies, and engineered T cells for immunotherapies.

cells but are often limited to the capture of the 3' or 5' end of a transcript. Most single-cell methodologies support the incorporation of unique molecular identifiers (UMIs) to RNA molecules during library preparation. UMIs are random nucleotide sequences that tag individual mRNA molecules and improve quantification of mRNA molecules distinguishing different copies of the same transcript. The incorporation of UMIs has been implemented in several scRNA-seq protocols, such as Drop-seq [7] and MARS-seq [8], Smart-seq3 [9]. Several comprehensive reviews cover in detail the diverse scRNA-seq technologies [10,11]. scRNA-seq workflows require single-cell isolation, cell lysis, reverse transcription, cDNA amplification these steps are flexible and continuously adapted to meet emerging challenges. For example, "single-cell combinatorial fluidic indexing" (sci-fi)-RNA-seq [12], has facilitated the sequencing of millions of individual cells, thereby making this technology increasingly competitive with other single-cell profiling techniques. Additionally, combining CRISPR screening with scRNA-seq, CRISPR screening with single-cell transcriptome readout (CROP-seq) [13] or Perturb-seq [14] allow to assess gene expression phenotypes for each CRISPR perturbation in a reverse genetic approach.

## 2.2. Single-cell methods for epigenome analysis

Beyond transcriptional information, single-cell epigenomic methods such as single-cell assay for transposase-accessible chromatin with sequencing (scATAC-seq) can provide a more profound understanding of the regulatory mechanisms that govern cell fate during infection. Epigenetic modifications may underlie cell-to-cell differences in gene expression in both humans and several pathogens. The adaptability of many pathogens is under control of epigenetic regulation and humans can maintain an altered epigenomic state for prolonged time during and after an immune response [15]. DNA transcription requires loosening of the chromatin structure to spatially allow regulatory factors like transcription factors to bind DNA. scATAC-seq can identify heterogeneity in chromatin accessibility, identifying DNA regions that are accessible to regulatory factors. For example, via scATAC-seq, Wimmers and colleagues [15] observed that influenza vaccination modified the chromatin accessibility to AP-1 and IRF targeted loci in a portion of myeloid cells that lasted 180 days. These modifications were associated with resistance to unrelated Zika and dengue viruses.

DNA methylation involves transferring a methyl group to cytosine, generally suppressing gene expression. DNA methylation influences gene expression and can play a significant role in host-pathogen interactions. Pathogens can have differences in DNA methylation, affecting their gene expression [16]. Additionally, they can alter the DNA methylation patterns of host cells via regulation of ten eleven translocation (TETs) and DNA methyltransferase (DNMTs) or via induction of inflammatory mediators [17]. The gold standard for defining DNA methylation status is considered the bisulfite conversion process. Current methods include single-cell bisulfite sequencing (scBS-seq), single-cell reduced representation bisulfite sequencing (scRRBS), single-cell whole-genome bisulfite sequencing (scWGBS), single-cell combinatorial indexing for methylation analysis (sci-MET), single-nucleus methylcytosine sequencing (snmC-seq), and single-cell post-bisulfite adaptor tagging (scPBAT) [18]. Whereas single-cell methylation analysis alone provides limited information about the overall cell status, combining these approaches with other modalities, such as scRNA-seq, offer a more comprehensive understanding of cellular state. For example, single-cell genome-wide methylome and transcriptome sequencing (scM&T-seq) can integrate RNA sequencing and methylation single-cell sequencing

data [19], while single-cell Triple Omics Sequencing 2 (scTriO-seq2) integrates single-cell genome, DNA methylome, and transcriptome sequencing [20,21]. While these methods are informative for investigating regulatory relationships, like epigenome-transcriptome interactions, their application in the context of infectious diseases has been somewhat limited. Due to the novelty of these techniques combined with the high cost and the complexity in data integration and interpretation, these methods have not yet been applied broadly. Despite these challenges, the field of single-cell epigenomics is rapidly evolving [22].

## 2.3. Single-cell methods for immune profiling

The adaptive immune response relies on the ability of B and T cells to recognize antigens and clonally expand to mount an effective immune response. Given the wide diversity of immunoglobulin and TCR genes, the use of gene-specific primers targeting their respective constant regions allows the enrichment across the variable, diversity, and joining (VDJ) gene segments [23]. The sum of B and T lymphocytes receptors in an individual is called the immune repertoire. Single-cell genomics approaches are particularly fitting to identify the characteristics of the immune repertoire and the immune receptor diversity occurring during VDJ recombination and somatic hypermutation. Since every B or T cell expresses one specific receptor clonotype, identifying the receptor sequence of an expanded clonotype can aid in the development of vaccine, monoclonal antibodies, and engineered T cells for immunotherapies, among other applications [24]. Furthermore, monitoring immune repertoire development longitudinally enables researchers to assess the evolution of immune diversity and expansion, providing insight into the adaptive immune response [25].

A commonly used approach in immunology research is to combine single-cell immune profiling techniques, such as single-cell B-cell receptor (BCR) sequencing and single-cell T-cell receptor (TCR) sequencing, with scRNA-seq. This multi-omic approach allows researchers to capture this information from the same cell, providing a more robust linkage between gene expression and immune repertoire. Recent methods and software packages supporting this integrated analysis are reviewed here [26,27].

For example, by sorting of hemagglutinin-specific B cells with a combination of scRNA-seq and scBCR-seq in multiple murine organs, Matthew and colleagues [28] were able to identify persistent germinal center B cell subpopulations. Moreover, by integrating measurements of monoclonal antibody (mAb) expression and affinity, they were able to identify a subset of memory B cells derived from both low and high affinity precursors.

### 2.3.1. Spatial transcriptomics

Spatial transcriptomics allows for the detection of gene expression in histological sections. This technique allows the observation of cells characteristics in their tissue context. The most commonly used method involves placing in contact a permeabilized tissue with a glass surface containing thousands of barcoded spots, each with an array of mRNA capturing probes. Each spot can contain information of a handful of cells, enabling a near single-cell resolution. A recent method called spatial total RNA-sequencing (STRS) [29] uses poly(A) polymerase to add poly(A) tails to RNA molecules *in situ*, thus enabling the detection of coding and non-coding RNA molecules in their spatial context. Using STRS, McKellar and colleagues also demonstrate that the method supports the detection of viral RNA. The ability to identify various RNA molecules from both the host and infectious agents allowed for the researchers to spatially resolve host transcriptional responses

associated with local viral RNA abundance in heart tissue of mice with viral-induced myocarditis, with near-cellular resolution [29].

### 3. Single-cell genomics for host-virus interaction

Detection of cellular heterogeneity in the context of viral infections is of great significance since viruses typically infect specific host cells to complete their life cycle. The encounter of a cell and a virus can have different outcomes based on the affinity in the interaction of the virus and host cell receptors. However, the infrequent occurrence of universal infection in a specific cell type suggests that other factors play a role in determining the outcome of the cell-virus encounter. This complexity is caused by viral mutations and cellular conditions like cell cycle, metabolism, activation, microenvironment, which collectively define the probability of a successful viral replication cycle [30].

Looking into these viral and host factors changes Hein and Weissman [31] modified single host and human cytomegalovirus cells via Perturb-seq. They observed that the transcriptional trajectory of infection changes velocity when perturbing host factors and can take alternative or abortive trajectory when perturbing viral factors.

Host factors like cell cycle, metabolic status, presence of co-infections, immune history, and underlying health conditions can lead to the production of different immune-regulatory molecules, directing viral replication. However, the relationship between host and virus is not always straightforward as observed by Russel and colleagues [32] in the context of influenza infection. Using scRNA-seq they observed that the activation of innate-immune interferon pathways was not a factor contributing to the heterogeneity of infected cells.

The adaptive immune response to viral infections is not limited to the generation of memory cells with antigen specificity, but also extends to the epigenetic level. For example, using scATAC-seq, You and colleagues observed that individuals recovered from COVID-19 have chromatin remodeling of both innate and adaptive immune cells [33]. Specifically, COVID-19 convalescing individuals had CD16+ and CD14+ monocytes with higher epigenetic activation and B/T cells with a characteristic differentiation program [33].

Viral factors are largely determined by their genetic sequence. Both short-read and long-read sequencing have their benefits in identifying viral particles. In short-read sequencing the genome is generally broken into fragments of 50–300 bases before being sequenced, while long-read sequencing allows for retrieval of longer (more than  $10^4$ ) base pairs. Short-read is the most utilized sequencing method due to the low error-rate and the high throughput. Long-read sequencing, however, can provide a more accurate sequencing of the viral genome. For example, the long-read sequencing allows the discrimination of polycistronic RNA, splicing isoforms, precise breakpoints in large scale genomic rearrangements like insertions or deletions and viral integration sites. Using the highly mutant influenza virus, Russel and colleagues enriched influenza virus reads via polymerase chain reaction (PCR) followed by long-read sequencing to determine the breadth of viral sequences infecting single-cells. They show that viral sequences modifications do not fully explain the cell-to-cell variability in infections and propose stochasticity and host cellular states as key players in the induction of cell specific immune response [34].

Most scRNA-seq tools (i.e., 10x Genomics, Drop-seq, and inDrop) use oligo-dT primers capture poly(A) of messenger RNA. Due to the frequent polyadenylation in viruses, these techniques can be used to capture both host and viral transcripts. By aligning the RNA library to a viral reference database like viruSITE [35], researchers can distinguish the presence of viral polyadenylated RNA in infected cells. This allows researchers to quantify host and viral

transcripts derived from the same cell and enables the comparison of gene expression profiles in infected and non-infected cells. For example, Wyler and colleagues [36] could show that fibroblasts in the S/G2/M cell cycle phases contain more viral transcripts of herpes simplex virus-1 (HSV-1) and support a more favorable condition to establish infection compared to fibroblasts in the G1 phase.

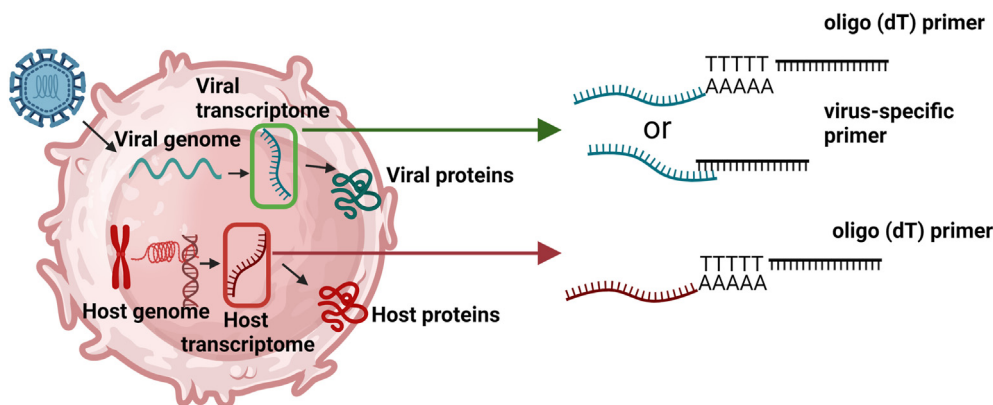
However, in some cases the capture of viral RNA is preferred over the corresponding viral transcript, which is polyadenylated mRNA. Recently, Chen and colleagues [37] utilized customized barcoded primers to detect influenza A virus (IAV) viral RNA instead of viral transcripts. This strategy aimed to enhance the detection of viral particles, to reduce detection of heterogeneous mRNAs, and to compensate for the incomplete viral gene expression during infection [38].

Some viruses, for example flaviviruses like Zika virus and dengue virus, not generate polyadenylated transcripts and require a different capture strategy than the standard oligo-dT primers. Zanini and colleagues added virus-specific oligos for detection of viral transcripts in virus-inclusive single-cell RNA-Seq (viscRNA-Seq) [39] to profile peripheral blood mononuclear cells from dengue patients and identify cells expressing dengue virus transcripts (Fig. 2). They identified IgM B cells, followed by monocytes as cell types harboring DENV transcripts and could compare the profile of these cells to that of bystander cells. Via Droplet-assisted RNA targeting by single-cell sequencing (DART-seq), Saikia and colleagues [40] were able to sequence reovirus non-polyadenylated viral transcripts in single-cells using an alteration of the DROP-seq strategy [7]. Recently, methods like Smart-Seq-Total [41] and VASA-seq [42] have gone beyond the capture of polyadenylated transcripts and have included enzymatic polyadenylation of all transcripts after cell lysis. This enables single-cell total RNA-sequencing, including non-coding RNA.

Single-cell genomics approaches, particularly the integration of transcriptomic workflows with other modalities (i.e., methylation, chromatin accessibility, immune repertoire), have aided in advancing our understanding of the complex nature of antiviral responses. Despite the various of single-cell genomic tools and strategies available to assess host-virus biology, their usage in least developed countries lags. Using these tools to study viral diseases such as Zika, dengue, Chikungunya and others in these countries is needed in order to better understand disease outcomes in these settings which are often heavily burdened by viral diseases. It will be crucial to prioritize research efforts towards these diseases and to find ways to reduce the high costs associated with library preparation and sequencing.

### 4. Single-cell genomics for host-bacteria interaction

The human body is constantly exposed to diverse pathogenic or commensal bacteria. This brings about a crosstalk of complex cellular interactions in space and time at the single-cell level between human cells and these microorganisms. For over a decade, RNA-seq has been the primary tool to study such host–bacteria interactions at the transcriptome-wide level [43,44]. Initially, researchers employed techniques to separate host and bacterial cells from one another followed by separate transcriptomic analysis [43,44]. The technology advanced beyond one-sided RNA-seq in 2013, when studies started to appear in which transcriptomes of interacting organisms were simultaneously detected together in so-called “bulk” dual RNA-seq [45]. Finally, in 2015 a new era of transcriptomics was ushered in the field of bacterial infection biology with the introduction of scRNA-seq [46,47], which has led to profound new discoveries. Pioneering work using a *Salmonella enterica* serovar Typhimurium macrophage infection model revealed both a high level of transcriptional heterogeneity in



**Fig. 2. Schematic representation of sequencing requirements for the assessment of host and viral transcripts.** Standard detection of messenger RNA (mRNA) uses poly-deoxythymidines (poly-dT) primers to capture polyadenylated transcripts. The annealing of poly-dT to the polyA tails of mRNA guides the synthesis of complementary DNA (cDNA) that gets copied and is often the starting material for RNA sequencing. Not all viral transcripts are polyadenylated. For non-polyadenylated viral transcripts, specific virus-specific primers can be used for their isolation and subsequent analysis.

*Salmonella*-infected macrophages and the existence of complex signaling pathways with consequences for the intracellular behavior of the pathogen that would have been impossible to detect by “bulk” RNA-seq approaches [46,47].

While transcriptional analysis of individual cells by scRNA-seq has become routine for eukaryotic host cells, the development of approaches to infer the transcriptome of single bacterial cells has proven considerably more challenging [48]. When establishing single-cell transcriptomics workflows in bacteria, various significant technical challenges should be considered in the selection of suitable approaches:

- First, extracting bacterial mRNA requires highly efficient cell lysis, which is difficult to achieve in bacteria due to their complex multilayered cell envelopes (absent in mammalian cells) with diverse organization and composition. Presently, “bulk” lysis of bacteria for RNA extraction typically requires either mechanical or chemical lysis using enzymes, detergents, and chaotropic and reducing agents. Neither approach is compatible with existing single-cell protocols at microliter scales as (i) mechanical disruption with bead beating or sonication causes droplet instability and (ii) reagents typically used in chemical lysis necessitate subsequent RNA purification, as reaction components are incompatible with downstream amplification and library construction workflows.
- Second, bacterial transcriptional profiles are at a higher risk of being altered during time-consuming processing steps like cell sorting, as transcriptional turnover is much higher in bacteria compared to eukaryotic cells. Bacterial mRNA has a comparatively brief existence on the scale of minutes, compared with an mRNA half-life of hours in eukaryotes [49], requiring rapid bacterial cell lysis and subsequent RNA stabilization and fixation.
- Third, the total RNA abundance of a single bacterial cell infecting a single eukaryotic cell is two orders of magnitude lower than that of the infected cell, requiring extremely sensitive cDNA synthesis and amplification protocols [50].
- Fourth, unlike eukaryotic cells, bacteria do not typically polyadenylate their mRNA transcripts (when a poly(A) tail is added it serves as a tag for degradation). As a result, bacterial minority mRNA transcripts cannot be easily selectively enriched against ubiquitous bacterial rRNA (ribodepletion), which is generally not informative of cellular state and presents up to 98% of the bacterial transcriptome [51].

Notwithstanding these major technical challenges, over the course of the last three years, three new proof-of-concept approaches for bacterial scRNA-seq have been published: MATQ-seq [52], microSPLIT [53], and PETRI-seq [54]. These landmark studies have firmly demonstrated that transcriptomes can be read from both individual Gram-negative (*S. enterica*, *Pseudomonas aeruginosa*, *Escherichia coli*) and Gram-positive (*Bacillus subtilis*, *Staphylococcus aureus*) bacteria at the single-cell level. Furthermore, the three approaches capture two orders of magnitude more different mRNAs per single bacterium compared to previous approaches. These novel proof-of-concept studies therefore represent significant progress towards opening the microbial world to investigation of the scale of the bacterial cell. Even though bacterial scRNA-seq is still at its infancy, the ramifications of the technology in infection biology are clear. An example of such a consequence includes the potential to shed new light on bacterial ‘persister’ cells which are able to withstand antibiotic exposure by changing to a reversible state of dormancy and, as a result, can cause infection relapse [55]. Persister cells represent one of the clearest examples of reversible bimodal gene expression (bistability) in bacteria and establishing whether these dormancy switches represent stochastic spontaneous events or regulated responses is currently not understood. It has nevertheless been established that during experimental macrophage infection intracellular *S. enterica* serovar Typhimurium persisters are transcriptionally active and survive through modulating the macrophage immune response [55]. We expect that bacterial scRNA-seq of persisters isolated from infected patients might soon be able to provide insights into how and why reactivation occurs. Overall, we anticipate that through technical refining and automating the MATQ-seq, PETRI-seq, and microSPLIT workflows, bacterial scRNA-seq will gain popularity as a commonly utilized approach for investigating bacterial pathogens.

The ultimate goal for RNA-seq in bacterial infection biology remains ‘dual’ scRNA-seq, which would allow researchers to simultaneously profile and correlate gene expression changes in a host cell infected with a single bacterium [56]. ‘Dual’ scRNA-seq has the potential to revolutionize our comprehension of the molecular pathways that contribute to the success of either the host or the bacterial pathogen. By doing so, it could have a significant impact in comprehending the host’s protective responses when encountering bacterial pathogens. This understanding can then serve as a valuable guide for developing new interventions and identifying crucial infection biomarkers. Similarly, ‘dual’ scRNA-seq, has the potential to pave the way for targeted interventions against bacterial

pathogens, specifically by inhibiting crucial pathogen programs necessary for virulence or survival. Finally, through comprehending the specific cell types involved, their responses, and the instances of inappropriate responses, we can potentially uncover the reasons behind varying levels of susceptibility to bacterial infection among individuals. The Pathogen Hybrid-Capture method [57] represents a promising approach towards 'dual' scRNA-seq. Here, transcriptome-specific oligonucleotide probes are employed to simultaneously enrich bacterial mRNA and deplete bacterial rRNA from dual RNA-seq libraries enabling the capture of the transcriptome of a few bacterial cells within a single host cell. Another particularly promising method [58] isolates single infected eukaryotic host cells by FACS sorting and employs a random hexamers strategy to prime host and bacteria-derived transcripts, followed by a poly-A tailing step and *in vitro* transcription. As a result, the development of 'dual' scRNA-seq is poised to make significant advancements, but still needs to be brought to a genome-wide scale. This nevertheless seems achievable considering the existing MATQ-seq, PETRI-seq, and microSPLIT bacterial scRNA-seq protocols, which are not reliant on poly(A), enabling the capture of both eukaryotic and bacterial RNA, like bulk dual RNA-seq. With enhancements such as existing targeted enrichment [57], 'dual' scRNA-seq holds the potential to dissect host-microbe interactions with an unparalleled level of detail.

## 5. Single-cell genomics for host-parasite interaction

Parasites have evolved to on one hand, maximize the acquisition of their hosts' resources, and on the other, minimize being detected by their host, with the overarching goal of continued transmission. Single-cell methods have helped broaden our understanding of how parasites accomplish this goal. To date, researchers have used single-cell methods to profile various disease-causing parasites in humans, including but not limited to those responsible for African trypanosomiasis, leishmaniasis, and malaria [59–61]. Genome-wide profiling of these various parasites reveals that they exist on a spectrum of transcriptional states, both within- and across-the various stages of their respective life cycles. More broadly, these analyses lay the groundwork for parasite cell atlases which could help to inform new therapeutic strategies.

Studying parasite biology at the single-cell level is no easy task: to perform -omics analyses, researchers must obtain sufficiently pure samples to prevent excessive capture and amplification of host material during the preparation of sequencing libraries. Depending on the parasite and the tools available, researchers must decide on the experimental workflow that best supports their model and research goal (Fig. 3). In the case of extracellular parasites, enrichment can be accomplished by purification methods such as cell-sorting methods (i.e., fluorescence-activated cell sorting, magnetic cell separation, density gradient purification). For intracellular parasites, lysis protocols that rupture the host cell but keep the parasite intact may be suitable to minimize host material captured. When the host cell cannot be ruptured without compromising the parasite, or when both host- and parasite-information is desired, methods that maximize the number of infected cells should be considered. In either case, researchers must take into consideration the genome size differences when sequencing RNA derived from both parasite- and host-material [62]. For example, relative to the human genome, a parasite's genome is often orders of magnitude smaller, therefore researchers must plan to sequence at greater depths to ensure parasite read coverage and depth is sufficient.

Most of the genomic studies done at the single-cell level have focused on parasite molecular signatures, but a few studies that have taken single-cell profiling a step further by simultaneously

profiling gene expression levels in both parasites and the host. These studies on *Plasmodium* parasites (the causative agents of malaria) and infected human cells have demonstrated how infected cells can be discerned from non-infected cells. They provide us with biological insights at a previously inaccessible resolution that can be applied to other infectious disease contexts.

### 5.1. Single-cell transcriptomic analyses of hepatocytes during *Plasmodium* liver stage infection

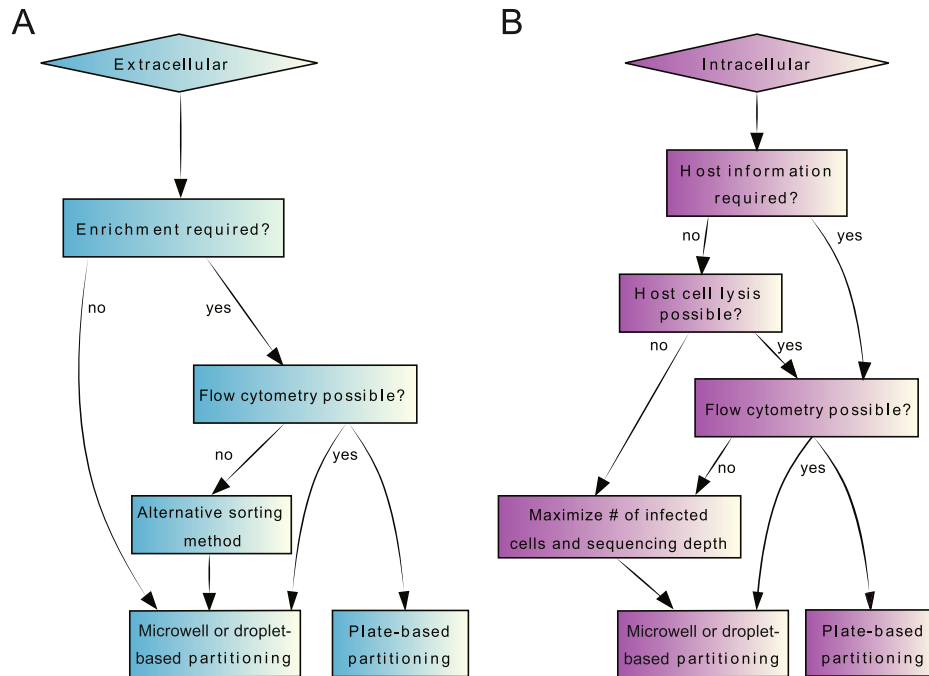
Upon mosquito inoculation, *Plasmodium* parasites must migrate to the liver and infect hepatocytes. After intra-hepatocyte multiplication, thousands of parasites are released in the bloodstream to continue their life cycle where they cause disease. In some *Plasmodium* species, such as *Plasmodium vivax* and *P. ovale*, not all liver forms undergo immediate intra-hepatocyte multiplication. These parasites (better known as hypnozoites) instead enter a dormancy-like state where they can persist for weeks to years before activating to cause a relapsing infection. The high uninfected to infected hepatocyte ratio makes it difficult to isolate and study *Plasmodium*-infected hepatocytes in sufficient numbers. Primary hepatocyte systems capable of harboring *Plasmodium falciparum* or *P. vivax* can partially overcome this problem. Platforms have recently been optimized to support the complete liver stage development of human malaria parasites, including *P. vivax* hypnozoite formation and activation [63,64]. Coupling these platforms with single-cell -omics methods has been critical for advancing our understanding of how *Plasmodium* liver stages alter the hepatocytes they infect.

Using an *in vitro* liver stage platform coupled with scRNA-seq, Marques Da-Silva and colleagues [65] assessed the transcriptional signatures of *P. falciparum*-infected and non-infected primary human hepatocytes. They observed greater expression of genes associated with hepatocyte injury, inflammation, and cell-death pathways in infected versus non-infected hepatocytes.

Single-cell workflows have also advanced our understanding of how *P. vivax* liver stages alter the hepatocytes they infect, which until recently, remained unknown. Similar to the strategy used by Marques-Da Silva and colleagues [65] for *P. falciparum*, researchers have relied on *in vitro* platforms to maximize the number of *P. vivax*-infected hepatocytes. Data from two studies using different single-cell profiling strategies reveal differential expression of genes encoding for host immune signaling pathways in infected versus non-infected hepatocytes [66,67]. Hepatocytes infected with *P. vivax* display greater expression of genes associated with innate immune response during early infection, whereas later in the infection, genes associated with interferon-related pathways display significantly lower expression [66]. Interestingly, the decrease in expression of genes encoding for interferon-related pathways was also observed in hepatocytes infected with non-replicating hypnozoites [67]. Thus, while a *Plasmodium*-infected hepatocyte is equipped with mechanisms to eliminate the parasite, these studies suggest that the parasite may re-program the hepatocyte it resides in to prevent recruitment of innate immune cells on the one hand, and to reduce the likelihood of their detection by the adaptive immune system on the other, all to enable progress through schizogony, and in the case of hypnozoites, to persist undetected.

## 6. Future directions and challenges

Advancements in single-cell genomics have revolutionized our understanding of cellular heterogeneity in the context of infectious disease research and have provided us with new avenues to study host-pathogen interactions. To date, most single-cell studies in this context use solely transcriptomic approaches. The integration of



**Fig. 3. Flow diagrams highlighting the various workflow considerations for single-cell assessment of host or parasite cells, or both.** (A) In the case of extracellular parasites, experimental workflows prior to sequencing include determining whether samples are sufficiently pure in order to minimize the chance of capturing non-parasite, 'contaminating', material. Capturing 'contaminating' material could minimize the number of sequencing reads aligning to the parasite and thus compromise downstream analyses. If samples are sufficiently pure, they can then be prepared for microwell- or droplet-based single-cell partitioning workflows. If cell enrichment is required and cell sorting via flow cytometry is available, this opens the door for preparing samples for either microwell- droplet- or plate-based single-cell workflows. If flow cytometry is not possible but cell enrichment is required, alternative sorting methods such as immunomagnetic or density separation can be used prior to microwell- or droplet-based single-cell partitioning. (B) In the case of intracellular parasites, experimental workflows prior to sequencing require additional considerations given the nature of the sample. Sample processing must be tailored according to whether researchers are interested in obtaining information strictly from the parasite or both the parasite and the host (i.e., dual single-cell RNA-seq). If host information is not required, a cell lysis protocol that eliminates the host cell but does not disrupt the parasite's cellular integrity could be used. From here, parasite-only samples can be processed further for either plate-, microwell-, or droplet-based single-cell sequencing workflows. In instances where both host- and parasite- material is desired, or when the host cell cannot be removed without compromising the parasite, experimental designs that maximize parasite material for sequencing should be considered (i.e., by sorting infected from non-infected cells via flow cytometry or an alternative method, if available). When cell sorting methods are unavailable and if working with an in vitro system, researchers may plan to maximize the number of infected cells for microwell- or droplet-based partitioning. By capturing thousands of cells and by increasing sequencing depth, researchers can overcome the difficulties of dual RNA sequencing efforts.

gene expression data with other modalities, such as DNA, chromatin, and protein, will enable a more comprehensive understanding of infection and disease outcomes. Pathogens have evolved survival strategies, often within a defined tissue environment of their host, to support continued transmission. Understanding the interaction between a pathogen its microenvironment and how this interaction changes over the course of an infection may support the development of host- or pathogen-directed therapeutics. Recent advancements in single-cell genomic approaches allow for the assessment of the spatial distribution cell populations within a tissue, and thus provides a more complete understanding of the infection microenvironment. Studies in rodent malaria models have already begun to unveil spatial signatures of infection both at the transcript and protein levels [68,69]. Evaluating the spatially resolved transcriptomic and proteomic landscapes during infection holds enormous potential in advancing our understanding of disease dynamics.

As the technologies supporting single-cell genomics advance, analyzing increasingly complex datasets remains challenging. More often than not, processing single-cell 'omics data requires dedicated high-performance computing infrastructure, limiting accessibility to a broader range of researchers, especially in least developed countries where the burden of infectious diseases is typically high. Additionally, the ability to make comparisons across single-cell datasets generated by different research groups can be difficult if differences in technical and computational pipelines

exist. While a 'one-size-fit-all' analysis pipeline would be difficult to implement (given the diverse nature of questions researchers pursue), the development of user-friendly software would remove roadblocks associated the integration and analysis of single-cell 'omics data generated using different analytical pipelines.

The studies reviewed here reveal the diversity of infection outcomes resulting from exposure to a pathogen. Both host and pathogen factors can contribute to infection outcomes. Furthermore, the heterogeneity in host and pathogen cells can contribute to the range of outcomes possible. Single-cell genomic approaches provide researchers with a means of assessing infectious disease dynamics at the cellular level. Our review highlights various single-cell genomics approaches that can be used to study host-pathogen interactions, and ultimately to support strategies for fighting infectious diseases.

#### Declaration of competing interest

After submission of the manuscript, Emanuele Gioacchino became an employee of Becton Dickinson. The remaining authors have no conflicts of interest to declare.

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