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Genome-wide approaches to unravelling host–virus interactions in Dengue and Zika infections

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Genomics approaches are increasingly utilized to probe host–viral interactions and identify mechanisms of viral pathogenesis and host-subversion. Here we review recent studies that utilize Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-Cas9 screens, transcriptomics and epigenomics to gain insight into Dengue and Zika virus infections in humans. We discuss the benefits and limitations of recently utilized techniques that separate virally infected cells from neighboring uninfected cells to identify the mechanisms by which these viruses regulate host responses. We conclude by discussing how these approaches can best advance our understanding of Dengue and Zika virus pathogenesis in humans.

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Introduction

Dengue virus (DENV) and Zika virus (ZIKV) pathogenesis

DENV causes dengue fever (DF) and dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS), also known as Severe Dengue. It is the most prevalent viral illness transmitted by mosquitoes. Each year there are approximately 400 million DENV infections, of which 100 million are symptomatic [1]. There are over 2 million DHF/DSS cases and approximately 20 000 deaths each year [2]. DF is characterized by fever, arthralgia, myalgia, abdominal pain, and rash. In contrast, a hallmark of DHF/DSS is plasma leakage, which can lead to hemoconcentration, pleural effusion, ascites, and shock. Neurologic abnormalities, including encephalitis, are rare [3].

Similar to DENV, approximately 20% of ZIKV infections result in a DF-like, self-limited febrile disease. However, ZIKV infections *in utero* can cause Congenital Zika Syndrome (CZS), which includes microcephaly, cerebral malformations, ophthalmological and hearing defects, and arthrogryposis [4]. In a small number of adults, ZIKV may cause Guillain–Barre syndrome, an autoimmune peripheral neuropathy characterized by acute, symmetric limb weakness with decreased or absent deep-tendon reflexes [5–8].

DENV and ZIKV are transmitted to humans by the mosquitoes *Aedes aegypti* and *Aedes albopictus*, placing an estimated 3.6 billion people worldwide at risk for infection [1,9,10]. ZIKV can also be sexually transmitted [11,12]. Despite the significance of these pathogens, no specific therapies are available.

Genomic approaches to understanding DENV and ZIKV pathogenesis

All human pathogenic viruses must utilize host factors and disarm and/or avoid immune responses to survive. Therapeutics that inhibit viral utilization of essential host factors or viral suppression of critical immune responses could be effective anti-viral agents. Conventional approaches to studying antiviral responses have primarily focused on the function of a single or a limited subset of molecules. These studies have provided incredible insights into viral and host factors that influence virus replication and infection outcome. However, the response to viral infections is dynamic, complex and incompletely understood. Complementary, genome-wide methods, including CRISPR, transcriptomics and epigenomics, provide an unbiased and comprehensive way of expanding our understanding of host–viral interactions. However, bulk genomic approaches that examine mixed population of cells have important limitations that must be overcome, including the inability to differentiate responses originating from virally infected compared to bystander cells and distinguish cell-types to interogate cell–cell communication.

CRISPR–Cas9 loss of function screens

Viruses are obligate intracellular parasites that require host factors for survival. The CRISPR–Cas system is a prokaryotic immune defense engineered to serve as a genome editing tool [13–18]. Pooled CRISPR loss-of-function screens can identify host factors, practically genome-wide, required for viral infection that in turn could be targeted by therapeutics to prevent disease.

Compared to RNAi, CRISPR–Cas appears to result in a greater signal-to-noise ratio and the identification of fewer false-positives, and is continuously being optimized to decrease its off-target effects [15,19–21].

As flaviviruses, which include DENV, ZIKV and West Nile virus (WNV), are cytolytic, infecting a pooled group of cells in which Cas9 and whole genome gRNAs have been introduced and cultured together allows selection of rare mutant cells that do not support viral entry, translation, replication or virus-induced cell death under highly stringent conditions. Independent genome-wide CRISPR–Cas9 loss of function screens using DENV [22,23], WNV [24,25] and ZIKV [26] have identified functionally related ER complexes that are required for flaviviral replication. A CRISPR screen in DENV2-infected Huh7.5.1 cells identified multiple subunits of the translocon-associated protein (TRAP), ER-associated protein degradation (ERAD), and oligosaccharyltransferases (OST) complexes [22]. The translocon and TRAP complexes transport secreted proteins across, or integrate membrane proteins into, the mammalian ER membrane [27,28]. The OST can associate with the translocon and N-glycosylate translocating proteins [29,30]. In the ER lumen, the ERAD pathway provides a protein quality control mechanism that targets incorrectly folded proteins for retrotranslocation into the cytosol for proteasomal degradation [31,32]. Remarkably, in an independent CRISPR screen that used the same cell line, Huh7.5.1, and virus, DENV2 strain 16681, 9 out of top 10 genes identified as essential for DENV infection were also in the top 25 scoring genes in the previous screen [22], and included members of the OST complex [23].

A genome-wide CRISPR/Cas9-based cell death screen in WNV-infected 293T cells identified seven human genes that were later validated to reduce WNV, DENV and ZIKV infection [25]. These genes are members of the translocon as well as the OST, signal peptidase (SPase), and endoplasmic reticulum membrane protein (EMC) complexes. The SPases typically cleave the signal peptide from translocating proteins and were shown to be required for proper cleavage of the flavivirus structural proteins (prM and E) and secretion of viral particles [25,29].

Loss of a SPase subunit, *SPCS1*, alone suppressed many members of Flaviviridae family, including WNV, DENV, ZIKV, yellow fever virus (YFV), Japanese encephalitis virus (JEV), and hepatitis C viruse (HCV), but had little impact on unrelated positive-sense or negative-sense RNA viruses [25]. An independent genome-wide CRISPR screen in WNV-infected 293T cells, identified mutations in the ERAD machinery that conferred strong protection against WNV-induced cell death but did not appear to block viral replication [24].

The only ZIKV genome-wide CRISPR screen was performed in H1-HeLa cells infected with the African strain of ZIKV, MR766 [24]. In addition to identifying a putative ZIKV receptor (*AXL*), and genes involved in endocytosis, members of the TRAP, OST, and EMC complexes were identified as essential host genes for ZIKV replication.

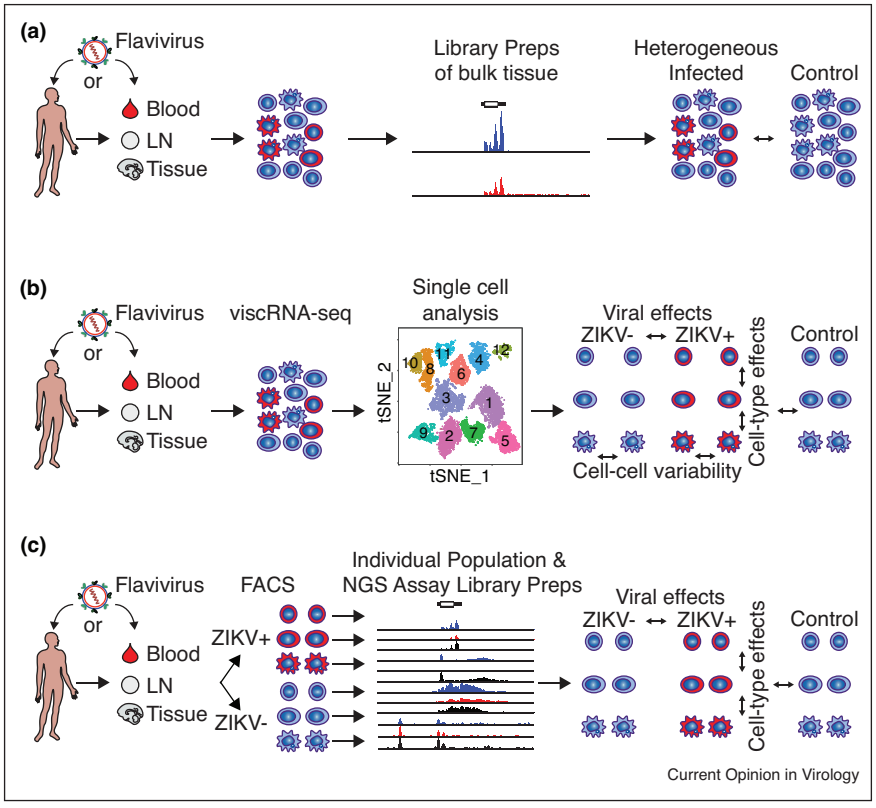
Together, these studies identified a critical role for several, often functionally related, ER complexes in flavivirus biology. These findings are consistent with the ER being the major site of flavivirus translation, polyprotein processing, replication, and virion assembly [33]. Importantly, the high reproducibility of genome-wide CRISPR screens contrasts with low overlap among results from siRNA-based screens for pro-viral factors. Thus at present, CRISPR–Cas9 represents an efficient, unbiased and reproducible way of identifying essential host factors in flavivirus infections. Alternatively, CRISPR screens performed in naturally resistant cells, or using CRISPR-mediated gene activation (CRISPRa) in susceptible cells, could identify key viral restriction factors important for flaviviral immunity [34–36].

Transcriptomic analyses to understand ZIKV infection in iPSC-derived or primary human cells

The neurodevelopmental dysfunctions of CZS have been associated with ZIKV infection of neural progenitor cells (NPCs) [37–46]. Transcriptional profiling demonstrated that ZIKV infection in human NPCs (hNPCs) leads to downregulation of genes involved in cell-cycle progression [45,47] and neurogenesis [47]. These findings are consistent with many studies showing ZIKV impairs NPC proliferation [37,40,43], differentiation [47], and survival [37–46], all of which may contribute to CZS. During pregnancy, ZIKV productively infects placental macrophages and cytotrophoblasts which may allow dissemination of the virus into the fetal circulation [48–53]. Weisblum *et al.* analyzed the response of human maternal-decidual tissues grown *ex vivo* as three-dimensional (3D) organ cultures during infection with ZIKV compared to human cytomegalovirus (HCMV), another major cause of perinatal infections. Despite more robust ZIKV replication in decidual tissue, the genome-wide expression analysis revealed that ZIKV induced far fewer genes involved in immune cell activation and trafficking compared to HCMV [54]. Furthermore, the chorionic villus responses to ZIKV were selectively enriched for apoptosis, cell death, and necrosis when compared to HCMV.

Although these gene expression studies have provided important insights into ZIKV pathogenesis in clinically relevant cell types, they have been limited to measurements from whole tissues or bulk cell populations that include multiple cell types and both infected and uninfected bystander cells (Figure 1a). This approach masks

Figure 1



Genome-wide approaches to understanding host responses to flaviviral infection.
(a) Analysis comparing infected and uninfected tissue or bulk heterogeneous cell populations cannot differentiate signals from infected and uninfected bystander cells or cell type-specific expression patterns. **(b)** viscRNA-seq and **(c)** FACS-NGS approaches differentiate host responses in infected and bystander cells and between cell types providing insight into viral subversion of host responses and cell-specific reactions. Additionally, viscRNA-seq identifies transcriptional variability across single cells and measures viral abundance in individual cells while FACS-NGS is adaptable to many RNA and chromatin-based NGS approaches. Abbreviations: ZIKV, Zika virus; viscRNA-seq, virus-inclusive single cell RNA-seq; LN, lymph node; tSNE, T-distributed Stochastic Neighbor Embedding; FACS, Fluorescence-activated cell sorting.

gene expression differences between infected and bystander cells, and obscures cell type-specific expression patterns that play important functions in cell–cell communication [55–57]. Conventional approaches to overcome these obstacles include the use of reporter viruses that can be used to identify or enrich for infected cells or the infection of highly susceptible cell lines. Both of these approaches can produce confounding results, since insertion of reporter proteins into a relatively small flaviviral genome may alter their virulence and prohibit characterization of low passage clinical isolates. Additionally, transformed cell lines that support robust viral replication often lack important anti-viral signaling responses and likely do not reflect the responses in clinically relevant, primary cells. New genome-wide approaches, such as single cell RNA-seq (scRNA-seq) [57] and fixed cell fluorescence activated cell sorting for next-generation sequencing (FACS-NGS) [56], that can compare virally infected and uninfected cells and cell type-specific responses are emerging to analyze host–flavivirus interactions and improve our understanding of human responses to flavivirus infection and viral subversion mechanisms (Figure 1b and c).

Genome-wide expression analysis at the single cell level using viscRNA-seq

DENV and ZIKV are positive-sense, single-stranded RNA viruses with genomes approximately 10.7 kb in length. The 5′ end of the positive strand genomic RNA has a N7 methylated (me7)-guanosine cap, and the 3′ end is non-polyadenylated and terminates in a stable stem-loop structure [33,58,59]. Typical scRNA-seq approaches rely on oligo (dT) priming, thus they would not be expected to distinguish flavivirus-infected from uninfected cells. Zanini *et al.* developed viral inclusive scRNA-seq (viscRNA-seq) approach that uses both oligo(dT) and virus-specific primers to amplify the whole transcriptome of single cells together with the viral RNA (vRNA) from the same cell (Figure 1b and Table 1) [57]. They performed a time-series of DENV and ZIKV infections in the human hepatocellular carcinoma

Table 1

Benefits and limitations of recently described viscRNA-seq (Zanini *et al.*) and FACS-NGS methods (Carlin *et al.*)

	viscRNA-seq	FACS-NGS
Special equipment	scRNA-seq platform with viral primers	FACS, antibodies
Sample and library preparation	Easy - hours	Medium - days
Compares single cells	Yes	No
Compatible NGS assays	RNA-seq	RNA-seq, ChIP-seq, ATAC-seq, Hi-C
RNA-seq library types	PolyA	PolyA, Ribo-depleted, miRNA, and so on
Viral abundance/cell	Yes	No

cell line huh-7. First they noted a large dynamic range for intracellular virus abundance. By correlating gene expression with DENV level in the same cell, they identified several cellular functions known to be involved in flavivirus replication, including ER translocation, N-linked glycosylation and intracellular membrane trafficking. Using loss-of-function and gain-of-function screens targeting highly correlated genes they identified novel proviral (*RPL31*, *TRAM1*, and *TMED2*) and antiviral (*ID2*, *CTNNB1*) factors involved in DENV infection [57]. In a subsequent study, the same group combined FACS with viscRNA-Seq using pan-DENV primers to profile the landscape of host transcripts and DENV RNA in thousands of single blood cells from six patients acutely infected with DENV [60]. This approach identified not only cell type-specific immune activation but also virus association with both naive B cells and monocytes. Although monocytes are well-accepted to be cellular hosts of DENV, the role of B cells in supporting productive DENV infection has been unclear [61]. Further studies using DENV patient samples and primary human cell culture models are now needed to validate B cell permissivity to DENV infection. Additionally, this study identified candidate predictive biomarkers of Severe Dengue that will also need to be validated in a larger cohort.

Understanding transcription at the level of chromatin

Epigenetics can help us understand why distinct cell types or environments lead to differential responses to the same viral infection, how the cellular responses during viral infections are coordinated by specific transcription factors (TFs) and chromatin states, and how human genetic variation can alter viral susceptibility. This knowledge is critical since viral responses are often cell-type and context-dependent. For example, ZIKV is highly cytotoxic to neural progenitors but glial cells can propagate ZIKV [62]. Additionally, ZIKV infection induces massive apoptosis of iPSC-derived NPCs but causes limited apoptosis and viral persistence in cells derived from primary neural progenitors or in NSCs within infected organotypic slice cultures [37,38,41,45,47,63]. These differential responses, with clear implications for ZIKV pathogenesis, could be driven by differences in gene expression determined by cell origin (iPSC-derived versus human *in vivo*) and/or the environment (monoculture versus organ culture), as both cell state and environmental stimuli can drive divergent gene expression programs by differentially activating a common enhancer repertoire [64–66].

The epigenetic landscape of each cell type is established by pioneer TFs or lineage-determining TFs (LDTF) that initiate enhancer selection [67–76]. This epigenomic landscape allows transcription of constitutively expressed genes and provides the geography (chromatin state) that directs binding of signal-dependent TFs (SDTFs), like NFkB or interferon regulatory factors (IRFs) [68]. This means that the epigenomic landscape controls cell type-specific constitutive gene expression and the initial transcriptional response to stimuli, like DENV or ZIKV infections [67,68,72,76]. Viral infections can further change the epigenetic landscape and thus reprogram the host and viral transcriptional profile [77–80]. This epigenetic remodeling may involve activation or repression of enhancers, modifications of DNA or changes in chromatin architecture. Thus, simultaneous probing of epigenomic and transcriptomic responses can explain first, cell-type-dependent and context-dependent (for example, tissue environment) cellular responses to ZIKV/DENV infection while identifying second, how pro-viral and anti-viral cellular pathways are activated and suppressed in a genome-wide unbiased manner.

Genomics analyses in viral infected and bystander cells using FACS

We recently developed a FACS-based approach that distinguishes flavivirus-infected from uninfected cells for genome-wide studies (Figure 1c and Table 1) [56]. In this study, primary human macrophages from multiple donors were infected with two different strains of Asian ZIKV, including a low passage clinical isolate representing contemporaneous lineage. At different time points post-infection, the cells are fixed with formaldehyde and ZIKV-infected (ZIKV+) cells are separated from uninfected bystanders (ZIKV−) by intracellular antibody staining that labels the common flavivirus envelope antigen. High quality RNA or chromatin is recovered for genome-wide transcriptional and epigenetic studies. Using this method we observed strikingly divergent transcriptional and epigenetic responses between ZIKV-infected and uninfected bystander macrophages derived from human blood monocytes. In fact, we

revealed that studying mixed populations could produce misleading results about how ZIKV modifies cellular responses. In contrast, direct comparison of ZIKV+ infected to ZIKV− bystander cells that are exposed to the same environmental stimuli demonstrated how ZIKV manipulates human transcriptional responses. In particular, ZIKV+ macrophages exhibited a delayed and attenuated transcriptional response with suppression of multiple inflammation and immune response-related categories including IFN signaling, cytokine signaling, antigen presentation and pattern recognition receptor (PRR) response pathways, whereas ZIKV− bystander cells rapidly mounted and sustained a robust interferon-stimulated gene (ISG) response. ZIKV degrades STAT2 in ZIKV+ but not in ZIKV− bystander macrophages consistent with previous studies [81–83]. Thus, bystander macrophages can upregulate ISG expression by responding to secreted IFN-β using the canonical type I IFN pathway. A benefit of our approach is that sorted cell populations can be processed for epigenetic analyses such as ChIP-seq, ATAC-seq or genome 3D-structural analyses such as Hi-C. Using ChIP-seq, we identified genomic regions exhibiting gain or loss of H3K27ac in ZIKV+ or ZIKV− cells. Motif analysis of these regions demonstrated a reduction of enrichment of IRF/ISRE motifs as well as motifs for PU.1 and C/EBP, two macrophage LDTFs in ZIKV+ cells. In contrast, NF-κB and STAT1 motifs were equally represented. This unbiased approach showed that ZIKV specifically suppresses ISRE-dependent signaling but does not suppress signaling via NF-κB and STAT1 in macrophages. We then performed ChIP-seq for RNA polymerase II (RNApol2) in order to identify genes that are being actively transcribed during ZIKV infection. We observed a striking reduction of RNApol2 DNA occupancy in ZIKV+ cells, and in line with this result, decreased protein levels of RPB1, the largest RNApol2 subunit that plays critical roles in both DNA binding and transcriptional elongation. The mechanism by which ZIKV reduces RBP1 levels and RNApol2 DNA occupancy is as yet to be identified. Multiple viruses, including Influenza, Chikungunya and herpes simplex virus type 1 (HSV-1) produce viral proteins that interact with RNApol2 and suppress its function by various mechanisms [84–86]. A ZIKV protein may activate the degradation of RPB1 directly or degrade/inactivate one of the many host proteins involved in forming the RNApol2 pre-initiation complex. In our study, the loss of RNApol2 was particularly notable at genes important for core macrophage functions, many of which are associated with super enhancers (SEs), or stretch enhancers, in human macrophages. SEs are regions of disproportionately high densities of active chromatin regulatory marks and binding of TFs near genes that play essential roles in the identity and function of cell types [87–90]. Conceivably, loss of RNApol2 driven transcription of genes involved in core cellular identity could contribute to cell death and/or impaired differentiation of that particular cell type in various ZIKV-infected tissues, including NPCs in the brain. Importantly, changes in RNApol2 in ZIKV+ cells could not be identified using gene expression analyses alone, highlighting the need to integrate both transcriptomic and epigenetic information towards deciphering virus–host interactions. Future studies using simultaneous transcriptional and epigenetic analyses to study ZIKV-relevant primary human cells, including placental macrophages, trophoblasts and cells in the central nervous system, will help reveal how cell-type and context influence host responses to ZIKV.

Influence of human genetic variation on flavivirus responses

Host genetics can strongly influence human immunity and disease severity during viral infections [91–96]. Accordingly, ZIKV infection differentially affects the neuronal differentiation of human fetal brain NSCs derived from different donors, thereby suggesting a role for human individual differences in susceptibility to ZIKV-neuropathology [47]. Consistent with this idea, Caires-Junior *et al.* infected NPCs from three pairs of dizygotic twins in which one fetus was CZS-affected and the other was CZS-unaffected [97]. NPCs from CZS-affected individuals had significantly higher ZIKV replication and reduced cell growth compared to CZS-unaffected. RNAseq analysis before infection revealed baseline differences in gene expression in CZS-affected versus CZS-unaffected NPCs. Whole-exome analysis of 18 affected CZS-affected babies compared to 5 unaffected twins and 609 controls did not suggest a monogenic model to explain CZS susceptibility. The authors suggested that the differences in gene expression and ZIKV susceptibility may therefore be related to oligogenic and/or epigenetic mechanisms. Thus, a potential benefit of epigenetic analyses of host–viral interactions is determination of the mechanisms by which human genetic variation alters viral susceptibility. The majority (∼88%) of risk loci for common diseases in genome-wide association studies (GWASs) are outside of the protein-coding genome [98], and functional variants for GWAS loci can reside in signal-dependent enhancers [93,99–102]. Thus, a significant fraction of disease-causing mechanisms originate in context-dependent regulatory states. Mapping epigenetics, especially in the appropriate context (i.e. during flaviviral infections), will be beneficial because it can be used to overlap with expression quantitative trait loci (eQTLs) or GWAS-risk associated variants to help elucidate their potential epigenetic mechanism of action.

Influence of flaviviral genetic variation on human cell responses

Flaviviral genotypes can also affect human cell responses during infection, thereby modulating disease severity. The association of Asian DENV serotype 2 (DENV2)

with severe disease in the Americas during 1980s and the increased virulence of the Asian strains relative to American DENV2 in human blood monocyte-derived dendritic cells and macrophages mapped to multiple changes in the viral genome, including substitutions at amino acid 390 of the envelope (E) protein and in the 5′ and 3′ untranslated regions (UTRs) [103–105]. Additionally, the epidemiological fitness of DENV2 clade PR-2B in 1994–1995 in Puerto Rico increased [106,107] due to substitutions in the 3′ UTR that enhanced production of subgenomic flavivirus RNA (sfRNA), which can bind tripartite motif 25 (TRIM25) and inhibit *IFNB1* expression [108]. Similarly, sequence comparison of the 2015–2016 epidemic ZIKV isolates to pre-epidemic ZIKV strains have identified multiple mutations of possible significance. A single amino acid serine-to-asparagine mutation, S139N, in the prM protein was found to increase ZIKV infectivity of human NPCs and neurovirulence in neonatal mice [109]. Additionally, an alanine-to-valine amino acid substitution, A188V, in the NS1 protein increases NS1 secretion thereby potentiating ZIKV infection of *Aedes aegypti* mosquitos [110]. This same mutation also enables NS1 to bind TBK1 and reduce *IFNB1* induction [111]. Thus, phylogenetic and molecular analyses of clinically important flaviviral strains can provide important insights into how viral mutations contribute to immune evasion, epidemic potential, and clinical outcomes.

Conclusions

Genomic technologies are increasingly used to study host–viral interactions because they can rapidly identify key pro-viral and anti-viral pathways in an unbiased genome-wide manner. However, many challenges remain to using these technologies to best understand human responses to flavivirus infections.

The reported flavivirus CRISPR–Cas9 screens appear to be very efficient, unbiased and reproducible methods of identifying essential host factors. Viral host factor requirements may be viral strain type-specific and cell type-specific. For example, African and Asian ZIKV isolates show phenotypic differences *in vitro* and *in vivo* with only recent Asian epidemic strains being associated with congenital abnormalities [112,113]. Additionally, specific flaviviral mutations can influence the antiviral response of the host cell. Ideally, CRISPR studies should use clinically and epidemiologically important viral isolates and confirm with multiple strains. Moreover, host-factors may be cell type-specific, especially in highly specialized cells such as neural stem cells during ZIKV infection. Cell type-specific factors that are important for understanding human pathology may only be revealed if CRISPR screens are performed in the appropriate cell contexts.

We lack mechanistic understanding of how human responses, especially responses in heterogenous tissues, to DENV and ZIKV contribute to morbidity and mortality. Here we describe two approaches, viscRNA-seq (Figure 1b) and FACS-NGS (Figure 1c) that overcome these limitations to provide deeper insight into host–DENV and host–ZIKV interactions. Some of the benefits and limitations of each method as currently described are presented in Table 1. Both viscRNA-seq and FACS-NGS techniques could be applied to better understand how cell–cell communication between infected and bystander cells as well as between different cell types in heterogenous tissues contributes to the observed phenotypes. By correlating intracellular virus abundance with gene expression in individual cells, viscRNA-seq can identify gene expression that is strongly correlated (positive or negative) with viral replication. In addition to assessing transcriptional responses, FACS-NGS is adaptable to many epigenetic analyses. This allows simultaneous comparison of the epigenetic landscape and gene expression profiles to determine how cell origin, differentiation method and tissue microenvironments establish context-dependent patterns of gene expression that drive differential outcomes to the same viral infection.

Direct application of the recently utilized techniques, viscRNA-seq and FACS-NGS, to study tissue responses during *in vivo* human infections is an important next step to understand human disease. Development of new genomics approaches, including those capturing 3D genome structures and requiring small cell numbers, and rigorous testing of both existing and new techniques will be needed to identify the best methods for sample isolation and preservation that most reliably capture *in vivo* human responses and can also be performed in DENV-endemic and ZIKV-endemic countries with limited resources. Additionally, improved computational methods for integrating large, diverse data-sets that extract maximal understanding are required (Box 1).

Box 1 Outstanding questions

How does cell type-specific and environment-specific enhancers control flavivirus responses and infection outcome?

Does human genetic and epigenetic variation control DENV and ZIKV susceptibility and if so where are these mutations located?

What are the best methods to isolate and preserve *in vivo* responses to viral infections for downstream genomics approaches?

What are the optimal computational methods for integrating multiple types of genomics data, including trascriptomic, epigenomic, and 3D organization of chromatin, towards identifying the dominant pro-viral and anti-viral pathways?

Author contributions

AFC and SS conceived of the review; AFC wrote the manuscript; SS edited the manuscript.

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