Zika infection of neural progenitor cells perturbs transcription in neurodevelopmental pathways.

Lynn Yi[1, 2], Harold Pimentel[3], Lior Pachter[4]*

1 Division of Biology and Biological Engineering, Caltech, Pasadena, CA, USA

2 David Geffen School of Medicine, University of California, Los Angeles, CA, 90095, USA

3 Department of Genetics, Stanford University, Palo Alto, CA, USA

4 Departments of Mathematics, Molecular & Cell Biology and Computer Science, University of California at Berkeley, Berkeley, CA, USA

*Corresponding author: lpachter@math.berkeley.edu

Abstract

Background

A recent study of the gene expression patterns of Zika virus (ZIKV) infected human neural progenitor cells (hNPCs) revealed transcriptional dysregulation and identified cell-cycle-related pathways that are affected by infection. However deeper exploration of the information present in the RNA-Seq data can be used to further elucidate the manner in which Zika infection of hNPCs affects the transcriptome, refining pathway predictions and revealing isoform-specific dynamics.

Methodology/Principal Findings

We analyzed data published by Tang *et al.* using state-of-the-art tools for transcriptome analysis. By accounting for the experimental design and estimation of technical and inferential variance we were able to pinpoint Zika infection affected pathways that highlight Zika's neural tropism. The examination of differential genes reveals cases of isoform divergence.

Conclusions/Significance

Transcriptome analysis of Zika infected hNPCs has the potential to identify the molecular signatures of Zika infected neural cells. These signatures may be useful for diagnostics and for the resolution of infection pathways that can be used to harvest specific targets for further study.

Introduction

As infection with Zika virus (ZIKV) is associated with increasing cases of congenital microcephaly and adult Guillain-Barre Syndrome, a characterization of its pathophysiology becomes crucial. A molecular characterization of the effects of infection may help in the development of fetal diagnostics and can accelerate the identification of crucial genes and pathways critical in disease progression. RNA-Sequencing (RNA-Seq) is an effective technology for probing the transcriptome and has been applied to study the effects of ZIKV infection of human neuroprogenitor cells (hNPCs) [1].

While initial analyses of the data have been used to conduct a general survey of transcriptome changes upon infection [1-3], they have used a method, Cufflinks/Cuffdiff [4], that fails to take advantage of the experimental design used in Tang et. al [1].

We apply the recently developed kallisto [5] and sleuth [6] programs to improve the accuracy of quantification and to extract information from the data that was previously inaccessible. We find that sleuth's improved control of false discovery rate results in differential transcript and gene lists that are much more specific and that are significantly enriched in neurodevelopmental pathways. They reveal ZIKV's neural tropism and the host's response to viral infection. Furthermore, we demonstrate that the combination of accurate kallisto quantification, assessment of inferential variance and the sleuth response error model allows for the detection of post infection isoform-specific changes that were missed in previous analyses.

The sleuth Shiny app drives a freely available website that allows for reproducibility of our analyses, and provides tools for interacting with the data. This makes the dataset useful for analysis by infectious disease experts who may not have bioinformatics expertise.

Methods

We ran kallisto and sleuth on a total of eight samples of ZIKV infected and mock infected hNPCs (GEO: Series GSE78711). The runs were performed on a laptop and can be repeated using the provided scripts at http://www.github.com/pachterlab/zika/. We used kallisto to pseudo-align the RNA-seq reads, building an index using the ENSEMBL GRC38 release 85 *Homo sapiens* transcriptome and using default parameters (kmer size = 31, fragment length = 187 and sd = 70 for the single end reads), quantifying transcript abundances, and performing 100 bootstraps per sample. To identify differentially transcripts and genes we first modified sleuth to be able to take advantage of the technical replicates performed by Tang et. al [1]. This was done by replacing an estimate of inferential variance from an average of bootstrap estimated variances to a weighting based on the number of fragments in each replicate. The response error model of sleuth was then used to identify statistically significant differential genes and transcripts.

Table 1a: Experimental design and inferential variance estimation weights

Sample	Accession Number	Condition	Seq method	Seq machine	Reads	Fragments / weights
Mock1-1	SRR3191542	mock	paired-end	MiSeq	15855554	7927777
Mock2-1	SRR3191543	mock	paired-end	MiSeq	14782152	7391076
ZIKV1-1	SRR3191544	zika	paired-end	MiSeq	14723054	7361527
ZIKV2-1	SRR3191545	zika	paired-end	MiSeq	15242694	7621347
Mock1-2	SRR3194428	mock	single-end	NextSeq	72983243	72983243
Mock2-2	SRR3194429	mock	single-end	NextSeq	94729809	94729809
ZIKV1-2	SRR3194430	zika	single-end	NextSeq	71055823	71055823
ZIKV-2-2	SRR3194431	zika	single-end	NextSeq	66528035	66528035

Results

We detected 4610 transcripts across 3646 genes that are differentially expressed between ZIKV and mock infected samples (false discovery rate of 0.05) (Fig 1: principle component analysis. S1 Table: differentially expressed transcripts, sorted by significance level). 2895 of the 3646 differentially expressed genes were also reported in Tang et. al [1], but they report an additional 3969 genes that we failed to find containing a significant transcript (they found a total of 6864 significant genes), whose 18423 transcripts have an average qval = 0.55. Furthermore, we found 751 differentially expressed genes corresponding to 5426 transcripts not detected by Cufflinks.

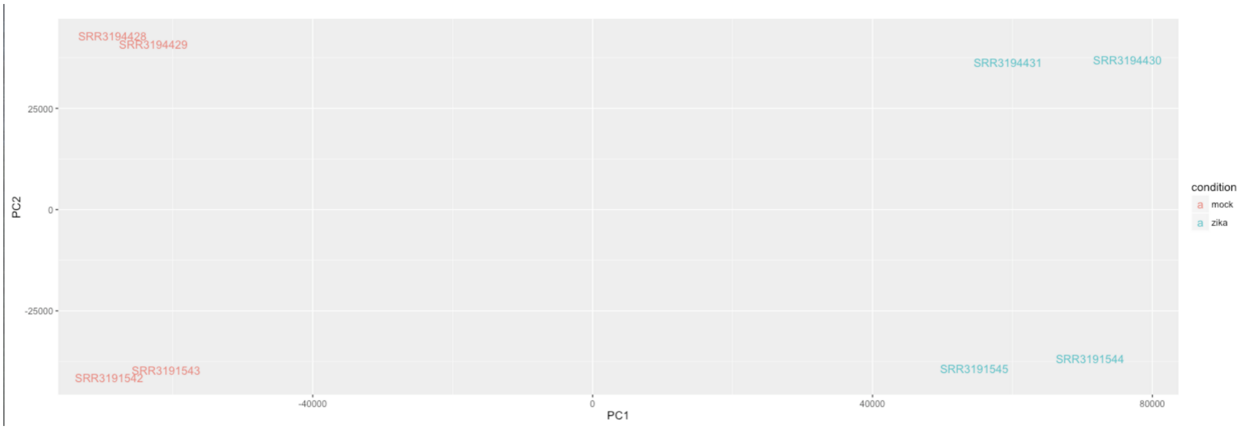


Figure 1: Principle component analysis shows that the primary contributor to variance is whether the sample is ZIKV-infected or mock-infected. The secondary component is method of sequencing, i.e. paired-ends or single-end.

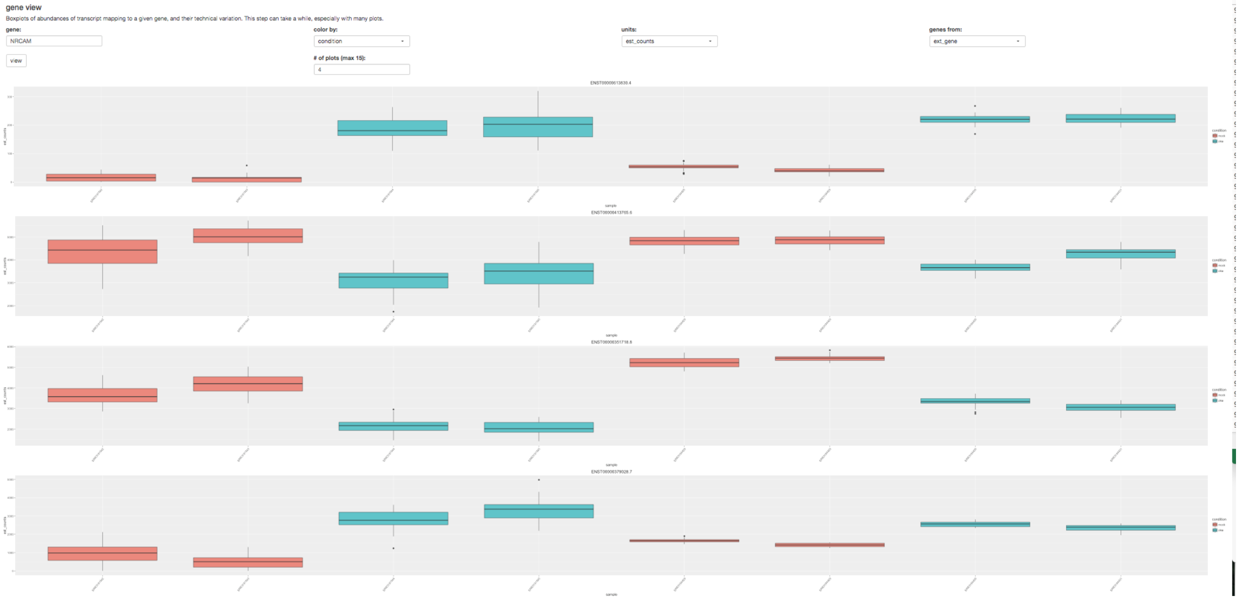
The statistics and figure above, along with interactive data visualization tools, can be found via Sleuth's Shiny app: http://lair.berkeley.edu/tang16/.

Zika induced isoform divergence.

Differentially regulated genes may be missed in gene-level differential analysis for several reasons. Noise in the measurement of a highly expressed transcript can mask expression changes in lowly expressed transcripts. In the case of isoform switching, the upregulation in one isoform can “cancel out” the effects of downregulation in another. We identified 108 genes that undergo isoform divergence as a result of infection, where isoform divergence is defined as a gene containing one or more transcripts that are are significantly upregulated and at least one other transcript that is significantly downregulated (see S2 Table of isoform diverging transcripts with statistics). Of these 108, 57 genes were not considered differentially expressed genes by Cuffdiff analysis, corresponding to 150 transcripts.

An analysis on these 108 isoform diverging genes using Reactome pathway analysis [7] show pathway enrichment in neuronal system (specifically transmission across chemical synapses and protein-protein interactions at the synapses), developmental biology (specifically axon guidance), immune system, DNA repair, chromatin modifying enzymes, gene expression (rRNA and transcriptional regulation), metabolism, signal transduction, transmembrane transport and vesicle-mediated transport.

One of these 57 isoform diverging genes not picked up by Cufflink is NRCAM, neuronal cell adhesion molecule, which according to Gene Cards is putatively involved in neuron-neuron adhesion and axonal cone growth. Another is CHRNA7, cholinergic receptor nicotinic alpha 7 subunit. [8] See Fig 2a and 2b for plots of the changes in their transcripts levels.



gene view

Boxplots of abundances of transcript mapping to a given gene, and their technical variation. This step can take a while, especially with many plots.

gene: NRCAM

color by: condition

units: est_counts

genes from: ext_gene

# of plots (max 15): 4

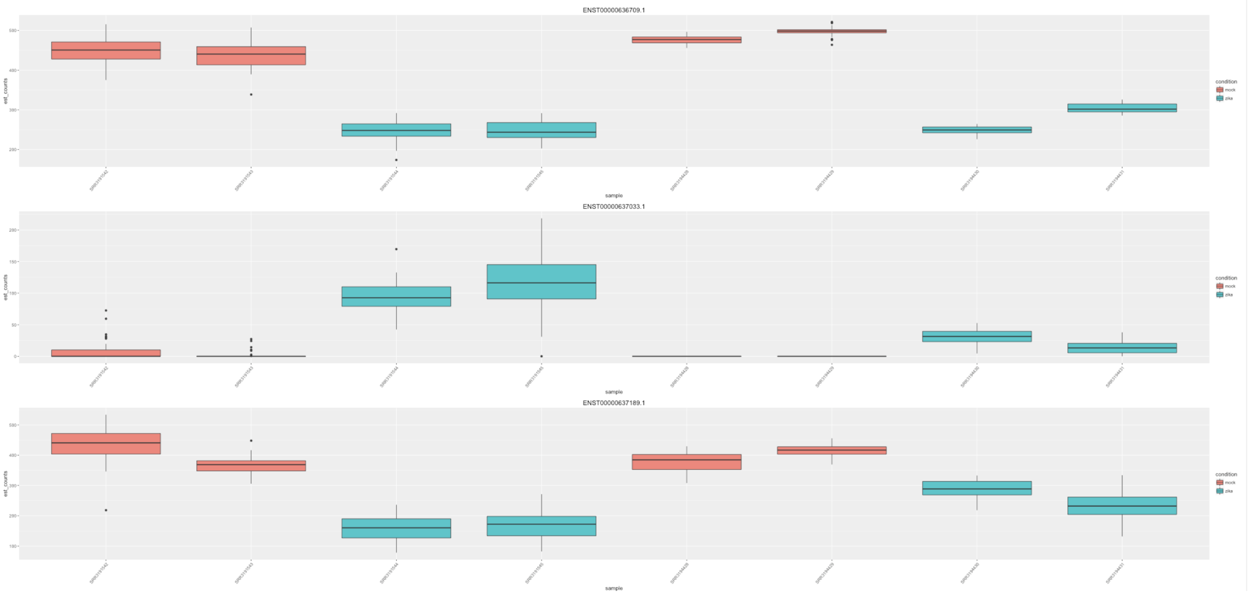
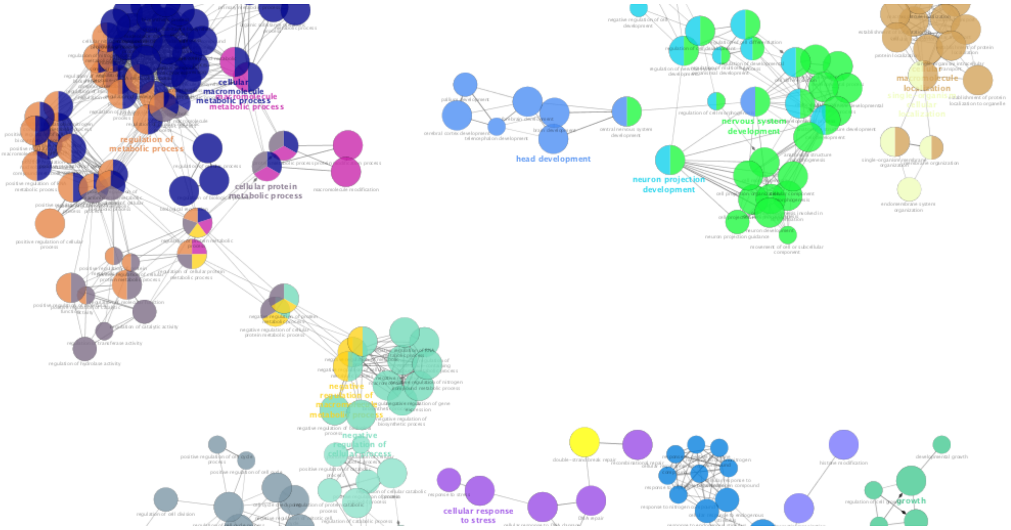


Figure 2a and b: Examples of genes with divergent isoforms, NRCAM and CHRNA7, viewed with our Shiny app. For a specific gene, it displays each transcript and the abundances corresponding to each sample.

A gene ontology (GO) analysis of sleuth-discovered genes showcase neural and head development networks.

We analyzed the set of 3656 genes with differentially regulated transcripts with a gene ontology tool, ClueGO a plugin for Cytoscape [9, 10], over the Biological Processes ontology network, using GO Term Fusion. We set the network specificity to global (GO tree interval: 1-4), using pathways with a minimum of 50 genes and kappa score of 0.5. The enriched nodes of particular interest include neuron projection guidance (pval = 2.7E-3 vs >0.05 with Cuffdiff), cerebral cortex development (1.6E-7 vs >0.05), neuron development (9.9E-6 vs 3.9E-4), neuron projection development (1.8E-6 vs 5.0E-5), nervous system development (3.0E-10 vs 1.0E-9), central nervous system development (6.9E-9 vs 1.0E-4), brain development (2.8E-9 vs 8.0E-4), forebrain development (1.9E-7 vs 4.1E-2), telecephalon development (2.7E-5 vs 5.2E-3), head development (pval = 1.3E-6 vs 3.2E-4), and cellular response to stress (9.4E-26 vs 7.3E-22).

Figure 3: A subgraph of the network resulting from ClueGO analysis of the differentially regulated genes discovered by kallisto and sleuth.


cellular macromolecule metabolic process
regulation of metabolic process
cellular protein metabolic process
head development
nervous system development
neuron projection development
macromolecule localization
negative regulation of macromolecule metabolic process
negative regulation of cellular process
cellular response to stress
growth

A GO analysis results tables and ctyoscape JSON file (see Supplementary materials) that can be used to render the network in cytoscape.

Discussion

RNA-Seq can provide rapid and highly resolved probing of infection response, and initial studies of Zika infection dynamics highlight neurally active isoforms, genes and pathways that may play an important role in disease etiology. However the simplicity of RNA-Seq library prep and cDNA sequencing belies the complexity of analysis. We have shown that a careful analysis of previously published data can reveal novel targets with higher confidence, and in the process rendering a valuable dataset usable by the community of Zika researchers.

The kallisto and sleuth tools we have used in our analysis are particularly powerful when coupled with the interactive sleuth Shiny application, and our publicly available server providing access to our analysis contains numerous interactive plots and analyses that cannot be reproduced in a static publication. This highlights the utility and importance of data sharing, and we hope that our analysis, aside from its usefulness for the Zika scientific community, can also serve as a blueprint for future data sharing efforts.

sleuth is a fast and accurate pipeline for analyzing RNA-Seq data that allows for testing at the isoform level. The alignment and quantification pipeline is feasible and compatible with a standard desktop computer. The interactive Sleuth application, made publically available, allows for informative data visualization, including those of library prep fragment size distributions, principle component analysis, and gene and transcript expression changes. We invite the scientific community studying Zika to utilize this toolkit.

Acknowledgements

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