Single-cell virus sequencing of influenza infections that trigger innate immunity

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Abstract The outcome of viral infection is extremely heterogeneous, with infected cells only sometimes activating innate immunity. Here we assess how the genetic variation inherent in viral populations contributes to this heterogeneity. We do this by developing a new approach to determine both the transcriptome and full-length sequences of all viral genes in single influenza-infected cells. Infections that activate an innate-immune response in single cells are associated with viral defects that include amino-acid mutations, internal deletions, and failure to express key genes. However, immune activation remains stochastic in cells infected by virions with these defects, and sometimes occurs even in cells infected by virions that express unmutated copies of all genes. Our work shows that the genetic variation present in influenza virus populations substantially contributes to but does not fully explain the heterogeneity in infection outcome and immune activation in single infected cells.

Introduction

Infection with an acute virus such as influenza initiates a race between the virus and immune system. As the virus spreads, some infected cells trigger innate immunity and begin producing interferon (IFN). This IFN directs expression of anti-viral interferon-stimulated genes (ISGs) in the infected cell and its neighbors via autocrine and paracrine signaling, as well as helping launch a broader immune response (***Stetson and Medzhitov, 2006***; ***Honda et al., 2006***). If innate immunity is activated sufficiently rapidly, it can reduce viral replication and disease severity (***Solov'ev, 1969***; ***Treanor et al., 1987***; ***Beilharz et al., 2007***; ***Kugel et al., 2009***; ***Steel et al., 2010***)—although excessive immune responses later in infection can actually be associated with immunopathology and more severe disease (***La Gruta et al., 2007***; ***Iwasaki and Pillai, 2014***).

Unfortunately for the host, influenza infection only rarely triggers IFN production by infected cells early in infection (***Kallfass et al., 2013***; ***Killip et al., 2017***). This rareness of IFN induction is just one form of the extreme cell-to-cell heterogeneity that characterizes infection: cells also vary widely in their production of viral mRNA, proteins, and progeny virions (***Russell et al., 2018***; ***Steuerman et al., 2018***; ***Sjaastad et al., 2018***; ***Heldt et al., 2015***). Because viral growth and the IFN response are both feed-forward processes, early cell-to-cell heterogeneity could have significant downstream consequences for the race between virus and immune system—especially since natural human infections are typically initiated by just a few virions entering a few cells (***McCrone et al., 2018***; ***Xue and Bloom, 2018***; ***Varble et al., 2014***).

It is unclear why only some infected cells trigger innate-immune responses. Two possible contributors are pure stochasticity and pre-existing variation in cellular state. For instance, only some cells induce IFN even upon treatment with synthetic innate-immune ligands (***Shalek et al., 2013***, ***2014***; ***Wimmers et al., 2018***), and the frequency of IFN induction may depend on a cell's pre-existing chromatin state (***Bhushal et al., 2017***). But for influenza, a third possible contributor also looms large: viral genetic diversity. Because influenza has a high mutation rate, even if the *consensus* sequence of a viral population is "wild-type," individual virions will often have defects (***Parvin et al., 1986***; ***Suárez et al., 1992***; ***Suárez-López and Ortín, 1994***; ***Bloom, 2014***; ***Pauly et al., 2017***). Indeed, many studies have identified mutations that increase IFN induction when engineered into a viral population (***te Velthuis et al., 2018***; ***Du et al., 2018***; ***Killip et al., 2017***; ***Pérez-Cidoncha et al., 2014***), and viral stocks that are rich in internal deletions in the polymerase genes induce more IFN (***Baum et al., 2010***; ***Tapia et al., 2013***; ***Boergeling et al., 2015***; ***Dimmock and Easton, 2015***).

However, existing techniques are inadequate to determine how viral genetic diversity contributes to cell-to-cell heterogeneity during infection. Flow cytometry and fluorescent reporters only measure protein levels (***Brooke et al., 2013***; ***Guo et al., 2017***), and current single-cell transcriptomic techniques primarily measure abundance of transcripts and provide only fragmentary information on their sequences (***Russell et al., 2018***; ***Zanini et al., 2018a***,b; ***Steuerman et al., 2018***; ***Saikia et al., 2018***; ***O'Neal et al., 2018***). None of these techniques reliably reveal if the virion infecting a specific cell has some idiosyncratic mutation, despite the fact that such mutations are common even if cells are infected with a nominally "wild-type" stock of virus.

Here we develop a new approach to measure both the full transcriptome and sequences of all viral genes in single influenza-infected cells. To do this, we first enrich for rare IFN+ cells, and then perform both standard Illumina-based single-cell transcriptomics and full-length PacBio sequencing of viral genes. We obtain transcriptomes and sequences of all expressed viral genes in 150 infected cells, 40 of which express IFN. Even though we used a "wild-type" stock of virus, two-thirds of cells are infected by virions that have a mutation or defect in gene expression. This viral diversity is a major contributor to cell-to-cell heterogeneity, with cells infected by truly wild-type virions having a tighter distribution of viral transcriptional burden. Viral defects are especially common in IFN+ cells, and we identify at least four types of defects that cause increased IFN induction. However, viral genetic variation does not fully explain the heterogeneity, as even wild-type virions sometimes induce IFN. Therefore, viral diversity is an important but not exclusive cause of cell-to-cell heterogeneity during influenza infection.

Results

A system to identify and enrich for rare IFN+ positive cells

A challenge in studying IFN induction by influenza virus is the rareness with which infection triggers its expression. We previously performed single-cell transcriptomics on hundreds of influenza-infected A549 human lung epithelial cells at early times (6 to 10 hours) post-infection (***Russell et al., 2018***), and found that only 1 of 368 infected cells expressed detectable type I or type III IFN (***Figure 1***A). Prior studies using reporters have found that IFN expression is also rare in influenza-infected mice at 24 and even 48 hours post-infection (***Kallfass et al., 2013***), a conclusion supported by our own re-analysis of the single-cell transcriptomics of influenza-infected mice by ***Steuerman et al.*** (***2018***) (see ***Figure 1–Figure Supplement 1***).

To efficiently identify and enrich rare IFN+ cells, we created A549 cells that carried IFN reporters consisting of a type I (*IFNB1*) or type III (*IFNL1*) promoter driving expression of a cell-surface protein (LNGFRΔC; ***Bonini et al., 1997***; ***Ruggieri et al., 1997***) followed by a fluorescent protein (***Figure 1***B). Cells that activate the IFN reporter can be enriched by magnetic-activated cell sorting (MACS) or identified by flow cytometry. The reporters were efficiently activated by a strain of Sendai virus (***Strahle et al., 2006***) that potently induces IFN (***Figure 1–Figure Supplement 2***), and activation of the type I and type III IFN reporters was highly correlated in our cells (***Figure 1–Figure***

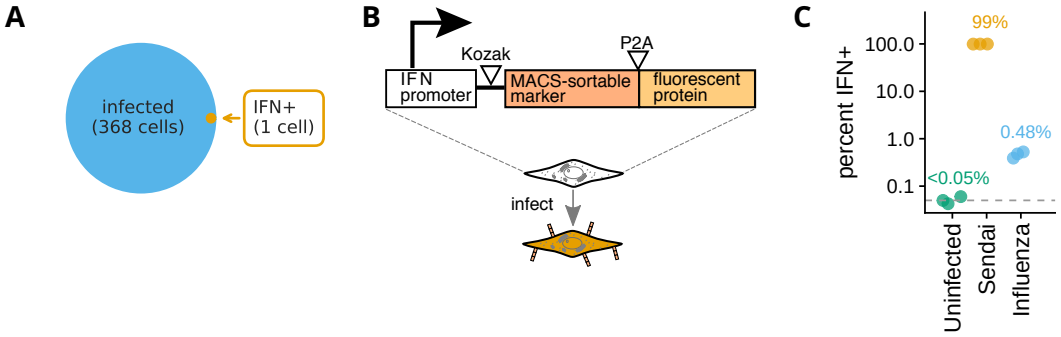

Figure 1. IFN expression is rare in influenza-infected cells. **(A)** Number of infected and IFN+ A549 cells in our prior single-cell transcriptomics (***Russell et al., 2018***). **(B)** An A549 reporter cell line to identify cells expressing IFN. The reporter consists of an IFN promoter that drives expression of a cell-surface protein amenable to MACS and a fluorescent protein. We created reporters with type I and type III IFN promoters (***Figure 1–source data 1***). The reporters were efficiently activated by an IFN-inducing strain of Sendai virus (***Figure 1–Figure Supplement 2***). **(C)** Frequency of IFN induction upon infection with the influenza virus stock used in the single-cell studies in this paper, as quantified using the reporter cell line (see ***Figure 1–Figure Supplement 4*** for details). The plot also shows uninfected cells, and cells infected with saturating amounts of Sendai virus. The limit of detection of 0.05% is indicated with a dashed line, and numbers show the median of three measurements.

Figure 1–Figure supplement 1. Few cells express detectable IFN in influenza-infected mice.

Figure 1–Figure supplement 2. Validation of IFN reporter cell lines.

Figure 1–Figure supplement 3. Expression of type I and type III IFN is highly correlated in A549 cells.

Figure 1–Figure supplement 4. Full flow cytometry data for ***Figure 1***C.

Figure 1–source data 1. Sequences of the reporters in ***Figure 1***B are at https://github.com/jbloomlab/IFNsorted_flu_single_cell/tree/master/paper/figures/IFN_stochastic/IFN_reporter/plasmids.

Supplement 3; further validated by the single-cell transcriptomics below). Therefore, for the rest of this paper, we use "IFN expression" to refer to combined expression of type I and III IFNs.

We generated a stock of "wild-type" A/WSN/1933 (H1N1) influenza (hereafter referred to as "WSN"), and found that it activated the IFN reporter in ~0.5% of infected cells—a frequency roughly comparable to that in our prior single-cell transcriptomics of influenza-infected cells (***Figure 1***A).

Combined transcriptomics and virus-sequencing of single infected cells

To determine if mutations or other features of the infecting virions contribute to the heterogeneous outcome of infection, we developed the approach in ***Figure 2*** to determine the entire transcriptome *and* the full sequences of all viral genes in single cells. First, we generated a stock of virus that consisted of a mix of wild-type WSN and a "synonymously barcoded" variant that contained two engineered synonymous mutations near each termini of each gene (***Figure 2–source data 1***). These viral barcodes allow us to identify co-infections from single-cell transcriptomic data (***Russell et al., 2018***), and provide an important control for PCR artifacts during full-length sequencing of viral transcripts (see below). We used this viral stock to infect A549 IFN reporter cells (***Figure 2***A) at a dose that led to detectable viral transcription in about a quarter of cells. From 12 to 13 hours post-infection, we used MACS to enrich cells that had activated the IFN reporter (***Figure 2–Figure Supplement 1***). To ensure the presence of IFN- cells, we added back non-enriched cells to ~10% of the total. We also added uninfected canine cells to ~5% of the total as a control for multiplets and to estimate the background amount of viral mRNA detected in truly uninfected cells.

We processed the cells on a commercially available platform (***Zheng et al., 2017***) that isolates cells in droplets and reverse transcribes polyadenylated mRNAs to append a unique cell barcode to all cDNAs in each droplet, and a unique molecular identifier (UMI) to each cDNA molecule (***Figure 2***B). Because influenza virus mRNAs are polyadenylated (***Robertson et al., 1981***), this process appends cell barcodes to both cellular and viral mRNAs. Furthermore, because virtually the entire

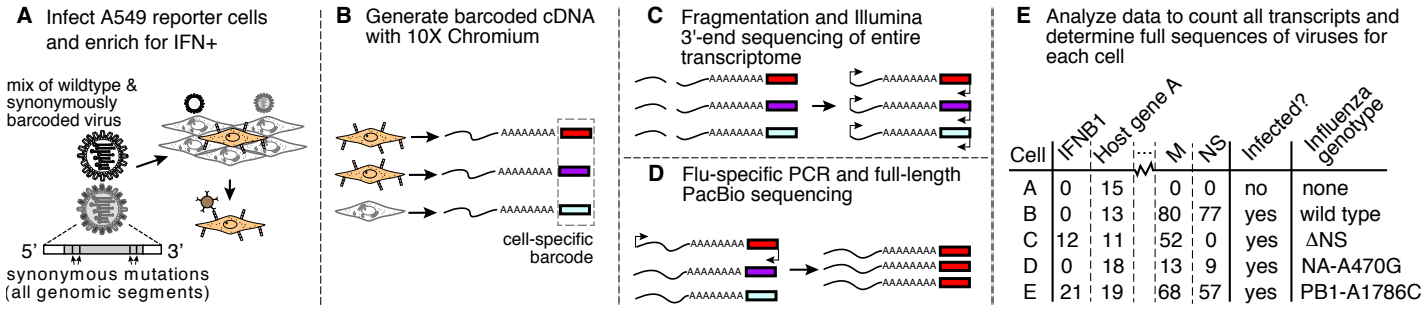

Figure 2. Approach for combined transcriptomic and viral sequencing of single influenza-infected cells that express IFN. **(A)** IFN reporter A549 cells are infected with a mix of wild-type and synonymously barcoded viruses. IFN+ cells are enriched by MACS (***Figure 2–Figure Supplement 1***), and pooled with non-enriched cells and uninfected canine cells that serve as an internal control for multiplets and mRNA leakage. **(B)** The mRNAs from individual cells are converted to cDNAs tagged with cell-specific barcodes. **(C)** Cellular transcriptomes are quantified using standard single-cell 3'-end Illumina sequencing, and **(D)** viral genes are enriched by influenza-specific PCR and fully sequenced by PacBio. **(E)** The result is a matrix giving the expression of each gene in each cell, as well as the full sequences of the viral genes in infected cells.

Figure 2–Figure supplement 1. MACS enrichment of IFN+ cells.

Figure 2–source data 1. Genbank files giving sequences of the wild-type and synonymously barcoded viruses are in https://github.com/jbloomlab/IFNsorted_flu_single_cell/blob/master/data/flu_sequences/flu-wsn.gb and https://github.com/jbloomlab/IFNsorted_flu_single_cell/blob/master/data/flu_sequences/flu-wsn-double-syn.gb.

influenza genome is transcribed, the cell-barcoded cDNA spans almost all 13,581 nucleotides in the segmented viral genome: the only portions not covered are one universally conserved nucleotide upstream of the transcription start site (***Koppstein et al., 2015***) and 17 to 22 highly conserved nucleotides downstream of the polyadenylation site (***Robertson et al., 1981***) in each of the eight viral gene segments.

We used a portion of the cell-barcoded cDNA for standard single-cell transcriptomics by Illumina 3'-end sequencing (***Figure 2***C). But we also took a portion and enriched for full-length viral molecules by PCR (***Figure 2***D). We performed PacBio sequencing on these full-length viral cDNAs to generate high-accuracy circular consensus sequences (CCSs; ***Travers et al., 2010***). These CCSs retain the cell barcodes, and with sufficient sequencing depth we obtain CCSs from multiple unique UMI-tagged cDNAs for each viral gene in each cell. Because most cells are infected by just one or two virions, we can build a consensus of CCSs for each viral gene in each cell to determine the sequence(s) of these virions. Combining this information with the 3'-end sequencing determines the entire transcriptome and the full sequences of the infecting virions in single cells (***Figure 2***E).

Transcriptomic analyses of single IFN+ and IFN- influenza-infected cells

We obtained transcriptomes for 1,614 human (A549) cells, and 50 of the uninfected canine cells that were spiked into the experiment as a control (***Figure 3***A). We also obtained 12 transcriptomes with a mix of human and canine transcripts; from the number of such mixed cell-type transcriptomes, we estimate (***Bloom, 2018***) that ~11% of the transcriptomes are derived from multiple cells. To remove some of these multiplets, we filtered transcriptomes with unusually high or low numbers of cellular transcripts (***Figure 3***B), retaining the 1,490 human cells that passed this filtering.

To identify infected cells, we examined the fraction of each transcriptome derived from virus (***Figure 3***B). As expected, only a small fraction (~0.7%) of transcripts in the uninfected canine cells were from influenza; this low-level background is likely from lysed cells that release ambient viral mRNA that is captured in droplets during reverse-transcription. We tested whether each cell contained significantly more viral transcripts than expected under a Poisson model given this background fraction, and classified 290 human cells as definitively infected with influenza (***Figure 3***B). We classified the other cells as uninfected, although it is possible that some were infected with virions that produced too little mRNA to be detected in our experiment. The distribution of the amount of viral mRNA across infected cells is shown in the inset in ***Figure 3***B. As in our prior work (***Russell et al.,***

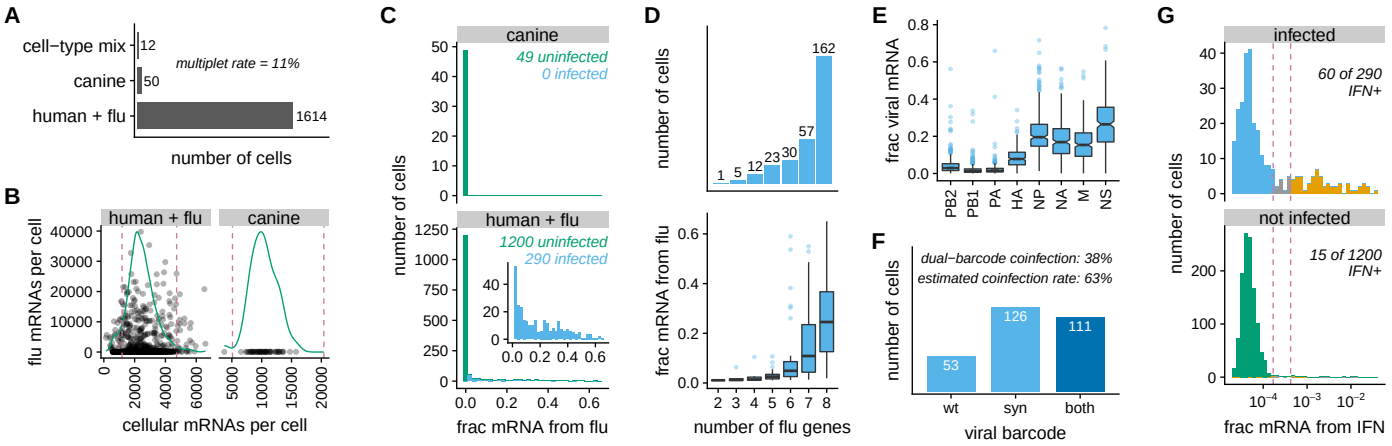

Figure 3. Single-cell transcriptomics of IFN-enriched influenza-infected cells. **(A)** Number of cells for which transcriptomes were obtained. From these numbers, we estimate (***Bloom, 2018***) that ≈11% of the transcriptomes are actually multiplets derived from multiple cells. **(B)** The number of cellular and viral mRNAs detected for each cell is plotted as a point. Green lines show the distribution of cellular mRNAs per cell. Cells outside the dashed magenta lines have unusually low or high amounts of cellular mRNA (likely low-quality emulsions or multiplets), and are excluded from subsequent analyses. **(C)** Distribution across cells of the fraction of all mRNA derived from influenza. Cells called as infected are in blue, while other cells are in green. The inset shows the amount of viral mRNA in the human cells that are called as infected. **(D)** Number of influenza genes detected per infected cell, and the amount of viral mRNA in cells expressing each number of viral genes. The majority of cells express all eight gene segments, but a substantial minority fail to express at least one gene. ***Figure 3–Figure Supplement 1*** shows the frequency that each viral gene is detected. **(E)** Relative expression of viral genes among infected cells. **(F)** Number of cells infected with wild-type virus, synonymously barcoded virus, or both. From the cells infected with both viral barcodes, we estimate (***Bloom, 2018***) that 63% of infected cells are co-infected. **(G)** Fraction of cellular mRNA from IFN across cells, faceted by whether the cells are infected. Cells to the left of the first dashed magenta line are classified as IFN-, and cells to the right of the second line are classified as IFN+. A pseudocount is added to the number of IFN transcripts detected in each cell, which is why none of the fractions are zero. Many cells that do not express IFN still express ISGs (***Figure 3–Figure Supplement 3***).

Figure 3–Figure supplement 1. Fraction of infected cells that detectably express each influenza gene.

Figure 3–Figure supplement 2. Expression of type I and type III IFN is highly correlated in single cells in our experiment.

Figure 3–Figure supplement 3. Expression of ISGs in single infected and uninfected cells.

Figure 3–Figure supplement 4. Unsupervised t-SNE clustering shows that cell-to-cell variation in expression of influenza, IFN, and ISG transcripts substantially contributes to the structure of the data.

2018), the distribution is extremely heterogeneous: many infected cells have only a few percent of mRNA derived from virus, but viral mRNA comprises over half the transcriptome of a few cells.

We then called the presence or absence of each viral gene in each infected cell, again using a Poisson model parameterized by background fractions estimated from uninfected canine cells. We called presence / absence of genes rather than transcripts, since the two influenza genes that encode multiple transcripts (M1 / M2 from the M gene, and NS1 / NS2 from the NS gene) do so via alternative splicing that leaves both isoforms with the same termini, making them indistinguishable by 3'-end sequencing. ***Figure 3***D (top panel) shows that most (162 of 290) infected cells express all eight genes, although a substantial minority fail to express one or more genes (see ***Figure 3–Figure Supplement 1*** for frequencies at which individual genes are present). This measured frequency of infected cells expressing all eight genes is slightly higher than in our own prior work (***Russell et al., 2018***) and studies by others (***Brooke et al., 2013***; ***Heldt et al., 2015***; ***Dou et al., 2017***), which have estimated that only 13% to 50% of infected cells express all genes.

The amount of viral mRNA tended to be lower in cells that failed to express viral genes (***Figure 3***D, bottom panel). However, viral burden remained highly variable even after conditioning on the number of viral genes: some cells that failed to express one or even two genes still derived >50% of their mRNA from virus, while other cells that expressed all genes had only a few percent of their mRNA from virus (***Figure 3***D, bottom panel). Consistent with our prior work (***Russell et al., 2018***), despite the wide variation in absolute expression of viral genes, their *relative* expression was fairly consistent (***Figure 3***E) and generally matched values from older bulk studies (***Hatada et al., 1989***).

By examining the synonymous viral barcodes near the 3' termini of transcripts, we determined that 38% of cells were co-infected with wild-type and synonymously barcoded virions (***Figure 3***F; cells called as co-infected if a binomial test rejected null hypothesis that ≥95% of viral mRNA is from one viral barcode variant). From ***Figure 3***F, we estimate (***Bloom, 2018***) that 63% of infected cells are co-infected, implying that 25% are co-infected with two virions with the same viral barcode (such co-infections cannot be identified from transcriptomic data). Interestingly, this co-infection rate is higher than expected from the relative numbers of infected and uninfected cells (***Figure 3***C) if infection is Poisson. This discrepancy could arise if the MACS for IFN+ cells also enriches co-infected cells, if infection is not truly Poisson, or if co-infection increases the likelihood that we identify a cell as infected given the thresholds in ***Figure 3***C. This moderately high rate of co-infection may also explain why more cells in our experiment express all eight viral genes compared to some prior studies, as a co-infecting virion can complement a missing viral gene (***Russell et al., 2018***).

We next examined expression of IFN and ISGs (***Figure 3***G and ***Figure 3–Figure Supplement 3***). Over 20% of infected cells were IFN+ given the heuristic thresholds in ***Figure 3***C, indicating that the MACS successfully enriched IFN+ cells far beyond their initial frequency of ~0.5% (***Figure 1***C) among infected cells. Few (~1.3%) uninfected cells were IFN+; the few that were present might be because the MACS enriched for rare cells that spontaneously activated IFN, or because some cells that we classified as uninfected were actually infected at low levels. Many more cells expressed ISGs than IFN itself: the ratio of ISG+ to IFN+ cells was 1.8 among infected cells, and 7 among uninfected cells (***Figure 3–Figure Supplement 3***A). The IFN+ cells were a subset of the ISG+ cells: IFN+ cells always expressed ISGs, but many ISG+ cells did not express IFN (***Figure 3–Figure Supplement 3***B). These results are consistent with the established knowledge that IFN is expressed only in cells that directly detect infection, but that ISGs are also expressed via paracrine signaling in cells that do not themselves detect infection (***Stetson and Medzhitov, 2006***; ***Honda et al., 2006***).

Finally, we qualitatively examined how expression of viral genes, IFN, and ISGs relate to the overall structure of the high-dimensional transcriptomic data. ***Figure 3–Figure Supplement 4*** shows unsupervised t-SNE clustering (***Maaten and Hinton, 2008***) of the cells. Cells expressing high levels of viral genes, IFN, and ISGs cluster together—and most of the structure in the t-SNE plot that is not associated with these genes involves uninfected and IFN- cells.

Full genotypes of viruses infecting single IFN+ and IFN- cells

We next used PacBio sequencing (***Figure 2***D) to determine the full sequences of the viral genes expressed in single infected cells. Using PCR enrichment (see Methods), we obtained over 200,000 high-quality PacBio CCSs that mapped to an influenza gene and contained a cell barcode and UMI (***Figure 4–Figure Supplement 1***). Crucially, the synonymous viral barcodes at both termini of each gene enabled us to confirm that PCR strand exchange (***Figure 4–Figure Supplement 2***) was rare (***Figure 4–Figure Supplement 1***), meaning that the vast majority of CCSs correctly link the sequence of the viral transcript to cell barcodes and UMIs that identify the cell and molecule of origin.

We called a mutation in a viral gene if it was found in least two CCSs originating from different mRNAs (unique UMIs) and at least 30% of all CCSs for that gene in that cell. For cells co-infected with both viral barcode variants, we called mutations separately for each viral variant. This strategy reliably identifies mutations in virions that initiate infection of cells infected with at most one virion of each viral barcode variant (~75% of infected cells), as well as high-abundance mutations in cells co-infected with multiple virions of the same viral barcode. It will not identify mutations that arise within a cell after the first few rounds of viral genome replication, since such mutations will not reach 30% frequency in that cell. Therefore, analogous to somatic variant calling in tumor sequencing (***Xu et al., 2014***; ***Cibulskis et al., 2013***), there is a limit to our detection threshold: we cannot identify mutations that occur on just a small fraction of transcripts in a cell.

We could call the sequences of all expressed viral genes in the majority of infected cells (***Figure 4–Figure Supplement 3***). We were most effective at calling full viral genotypes in cells that expressed high amounts of viral mRNA and were infected by only one viral barcode variant (***Figure 4–Figure***

Figure 4. Viral genotypes and infection outcomes in single cells. Arrows indicate presence of a viral gene from one (light blue) or both viral barcode variants (dark blue). Circles and boxes indicate mutations or indels as described in the legend, with circle areas and box heights proportional to the fraction of CCSs with that mutation. For dual-barcode infections, mutations / indels for the wild-type and synonymously barcoded viral variants are shown in the top and bottom half of the arrows, respectively. Green boxes at left show the percent of all mRNA in that cell derived from virus. Orange boxes show the percent of cellular mRNA derived from IFN, with orange box frames indicating cells classified as IFN+ in ***Figure 3***G.

Figure 4–Figure supplement 1. Number of PacBio CCSs and PCR strand exchange rate.

Figure 4–Figure supplement 2. Strategy for detecting strand exchange during sequencing of full-length viral genes.

Figure 4–Figure supplement 3. Number of cells for which genotype(s) of infecting viruses were completely determined.

Figure 4–Figure supplement 4. Genotypes and infection outcomes plotted separately for IFN+ and IFN- cells.

Figure 4–source data 1. A text file giving the primers used to amplify the influenza cDNAs for PacBio sequencing is at https://github.com/jbloomlab/IFNsorted_flu_single_cell/tree/master/paper/figures/WorkflowSchematic/PacBio_primer_list.txt

Figure 4–source data 2. A CSV file giving the genotypes is at https://github.com/jbloomlab/IFNsorted_flu_single_cell/blob/master/paper/figures/single_cell_figures/genotypes.csv.

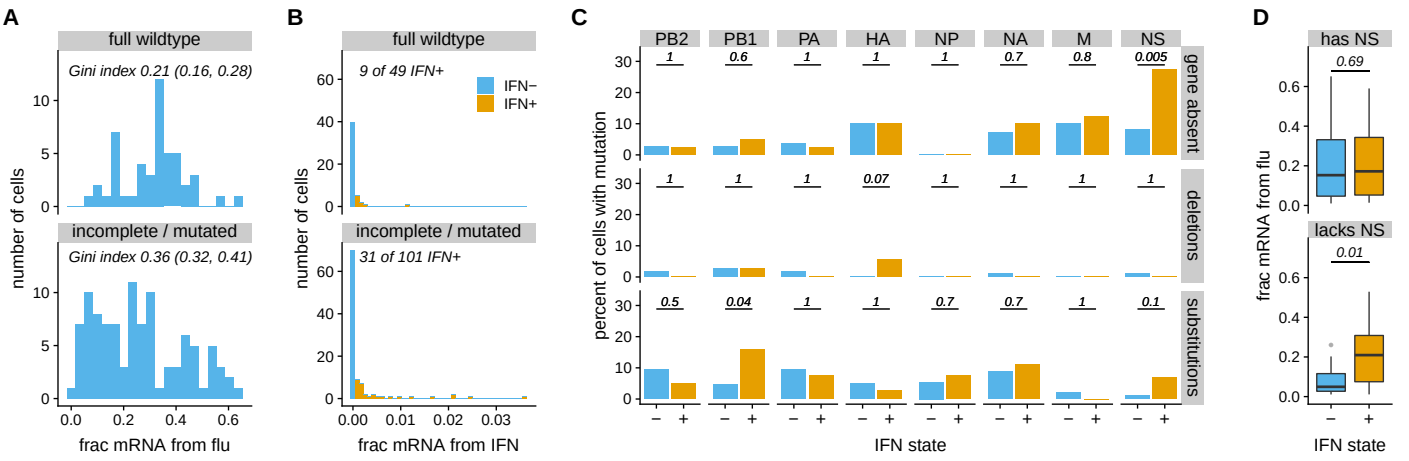

Figure 5. Viral features associated with heterogeneity in infection outcome among cells for which we sequenced all expressed viral genes. **(A)** Percent of all mRNA derived from virus, faceted by whether cells express unmutated copies of all eight genes. Cells infected by fully wild-type virus exhibit less heterogeneity in viral burden as quantified by the Gini index (95% confidence intervals are indicated). **(B)** IFN expression among cells expressing unmutated copies of all genes, and among cells with mutations or missing genes. **(C)** Specific viral defects associated with IFN induction. The top panel show the percent of IFN- and IFN+ cells that fail to express each viral gene. The middle and bottom panels show the percent of IFN- and IFN+ cells that have a deletion or amino-acid substitution in each gene, conditioned on the cell expressing that gene. Numbers give P-values (Fisher's exact test) for rejecting the null hypothesis that percents are equal among IFN- and IFN+ cells. ***Figure 5–Figure Supplement 1*** and ***Figure 5–Figure Supplement 2*** show that the trends are similar if we include infected cells with incomplete viral sequence information, or categorize cells by ISG expression rather than IFN expression. **(D)** There is no association between IFN induction and the amount of viral mRNA in cells that express NS, but viral burden is associated with IFN induction among cells that lack NS. Throughout this figure, we only consider substitutions that are non-synonymous. Insertions are not shown as a mutation type as they are extremely rare (***Figure 4***).

Figure 5–Figure supplement 1. Analysis as in ***Figure 5***C but including infected cells with incomplete viral sequence information.

Figure 5–Figure supplement 2. Association of viral genetic variation and ISG expression.

Figure 5–source data 1. A CSV file giving the viral mutations and related information in ***Figure 5*** is at https://github.com/jbloomlab/IFNsorted_flu_single_cell/blob/master/paper/figures/single_cell_figures/mutations.csv.

Supplement 3). But we also called full genotypes for many cells that had low viral burden or were co-infected by both viral barcode variants.

The 150 cells for which we called the full viral genotypes are shown in ***Figure 4***. Simple visual inspection of this figure reveals a wealth of information. For instance, the cell with the highest viral burden (*cell 1* in ***Figure 4***, which has 65% of its mRNA from virus) was infected by a virion that expressed wild-type copies all eight genes and did not induce detectable IFN. But 12 of the other 13 cells with at least 50% of their mRNA from virus were infected by virions that had a mutation or failed to express at least one gene, and five of these cells expressed IFN. The two cells that expressed the most IFN (*cell 13* and *cell 123*) both lacked the viral NS gene, and many other IFN+ cells had different defects such as large internal deletions (e.g., *cell 5* and *cell 89*) or amino-acid mutations (e.g., *cell 9*, *cell 28*, and many others). However, expressing unmutated copies of all eight genes did not guarantee a favorable outcome for the virus: for instance, the wild-type virion that infected *cell 139* only managed to express viral mRNA to 6% of the total transcriptome, and the wild-type virion that infected *cell 105* still induced IFN.

Viral defects associated with infection outcome in single cells

To systematically assess viral features associated with infection outcome, we divided the 150 cells in ***Figure 4*** into those that expressed unmutated copies of all eight genes (disregarding synonymous mutations) and those that did not. ***Figure 5***A shows that the 49 cells infected by full wild-type viruses had a significantly tighter distribution of the amount of viral mRNA per cell than the other 101 cells as quantified by the Gini index (***Gini, 1921***). Therefore, viral defects are a major contributor to the heterogeneity in viral transcriptional burden among cells.

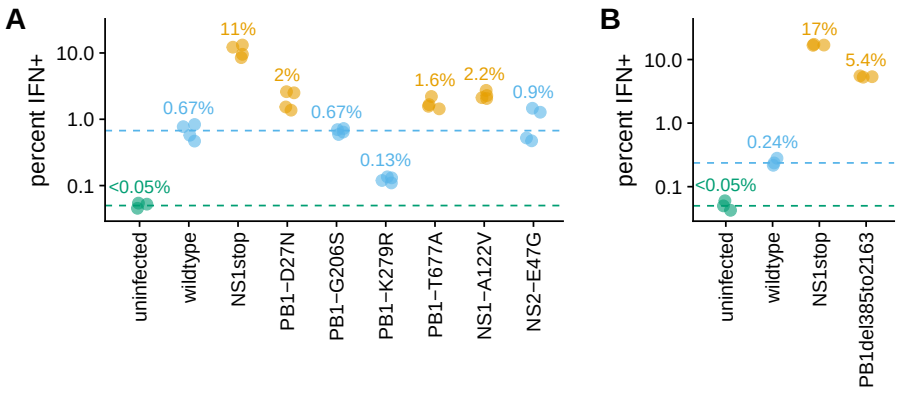

Figure 6. Validation that IFN induction is increased by some of the mutations identified in the single-cell virus sequencing of IFN+ cells. **(A)** Percent of cells that become IFN+ after infection with a bulk stock of the indicated viral mutant, as determined using a reporter cell line (see ***Figure 6–Figure Supplement 1*** for details). The numbers indicate the median of four measurements for each viral mutant. The limit of detection of 0.05% is indicated with a dashed green line, and the median value for wild type is indicated with a dashed blue line. Points are colored orange if the mutant virus stock induces IFN more frequently than the wild-type viral stock (one-sided t-test, $P < 0.01$), and blue otherwise. **(B)** Similar to the first panel, but validates the increased IFN induction for a large internal deletion in the PB1 gene. See ***Figure 6–Figure Supplement 2*** and ***Figure 6–Figure Supplement 3*** for more detailed experimental data. The experiments in the two panels were performed on different days, and so numerical values can be reliably compared within panels but not between panels.

Figure 6–Figure supplement 1. Flow cytometry data for ***Figure 6***A.

Figure 6–Figure supplement 2. Flow cytometry data for ***Figure 6***B.

Figure 6–Figure supplement 3. Validation of viral titer normalization for ***Figure 6***B.

Figure 6–source data 1. Genbank plasmid maps for the mutant genes cloned into the pHW* bi-directional reverse genetics plasmid (***Hoffmann et al., 2000***) are at https://github.com/jbloomlab/IFNsorted_flu_single_cell/tree/master/paper/figures/FluVariantPlasmidMaps

Cells infected by incomplete or mutated virions also expressed IFN more frequently than cells infected by full wild-type virions (***Figure 5***B), although this difference was not statistically significant ($P = 0.12$, Fisher's exact test). However, some specific viral defects were significantly associated with IFN induction: absence of NS and amino-acid mutations in PB1 were significantly enriched in IFN+ cells, and amino-acid mutations in NS and deletions in HA were weakly enriched (***Figure 5***C). Due to the low number of cells and large number of hypotheses in ***Figure 5***C, the only trend that remained significant at a false discovery rate of 10% was absence of NS.

One other interesting trend emerges from the single-cell data. There is no difference in the amount of viral mRNA between IFN+ and IFN- cells that express NS—but among cells that lack NS, the IFN+ ones have significantly more viral mRNA (***Figure 5***D).

Validation that viral defects in single IFN+ cells often cause increased IFN induction

To test if the viral defects identified in single IFN+ cells play a causal role in triggering IFN expression, we used reverse genetics to generate bulk stocks of viruses with some of these defects.

The viral defect most strongly associated with IFN induction was failure to express the NS gene (***Figure 4***, ***Figure 5***C). Although it is sometimes possible to use complementing cells to generate influenza viruses lacking a specific gene (***Fujii et al., 2003***; ***Marsh et al., 2007***), we were unable to generate viruses that lacked NS. The NS gene encodes two proteins (NS1 and NS2), the first of which is influenza's primary innate-immune antagonist (***García-Sastre et al., 1998***; ***Hale et al., 2008***). We therefore mimicked the effect of absence of NS by creating a mutant virus (which we will term "NS1stop") that had multiple stop codons early in the NS1 coding sequence.

The single-cell data also showed that amino-acid substitutions in the PB1 and NS genes were enriched in IFN+ cells (***Figure 4***, ***Figure 5***C), so we created mutant viruses with many of the amino-

acid substitutions found in these genes among IFN+ cells. Overall, we created six such point-mutant viruses: PB1-D27N, PB1-G206S, PB1-K279R, PB1-T677A, NS1-A122V, and NS2-E47G.

Finally, prior work has suggested that virions carrying internal deletions in the polymerase genes can induce higher levels of IFN (***Baum et al., 2010***; ***Tapia et al., 2013***; ***Boergeling et al., 2015***; ***Dimmock and Easton, 2015***). Although such deletions are not significantly enriched among IFN+ cells in our single-cell data (***Figure 5***C), there is an IFN+ cell that has a deletion in PB1 spanning nucleotides 385 to 2163 (*cell 5* in ***Figure 4***). We therefore created a virus carrying this deletion, and propagated it in cells constitutively expressing PB1 protein.

We tested the rate of IFN induction by each viral stock using the reporter cells. ***Figure 6*** shows that five of the eight mutant viral stocks induced IFN more frequently than wild type. The strongest IFN induction was by the NS1stop virus, but the PB1 internal deletion and three of the point-mutant viruses (PB1-D27N, PB1-T677A, and NS1-A122V) also induced IFN significantly more frequently than wild type. The other three point mutants (PB1-G206S, PB1-K279R, and NS2-E47G) did not increase IFN induction—an unsurprising finding, since we also expect some mutations without an IFN-enhancing effect to be found in IFN+ cells by chance. But overall, the results in ***Figure 6*** validate that the viral defects in single IFN+ cells often cause increased IFN production.

Discussion

We have determined the full sequences of all viral genes expressed in single influenza-infected cells, and examined how viral mutations and gene-expression defects associate with infection outcome in each cell. Methodologically, our major advance is to measure the *genotypes* of viruses in addition to the abundance of viral components (i.e., transcripts, proteins, or progeny virions) as has been done by prior single-cell studies (***Russell et al., 2018***; ***Zanini et al., 2018a***,b; ***Steuerman et al., 2018***; ***Saikia et al., 2018***; ***O'Neal et al., 2018***; ***Zhu et al., 2009***; ***Schulte and Andino, 2014***; ***Akpinar et al., 2016***; ***Heldt et al., 2015***; ***Brooke et al., 2013***). Our method builds on the observation by ***Saikia et al.*** (***2018***) and ***Zanini et al.*** (***2018b***) that fragmentary viral genetic information can be obtained by more standard single-cell transcriptomic techniques. To make this information complete, we have coupled single-cell transcriptomics with long-read PacBio sequencing of viral genes, a strategy analogous to that used by ***Gupta et al.*** (***2018***) to obtain full-length isoforms of some cellular genes in single cells.

This viral genetic information turns out to be crucial for understanding infection outcome and innate-immune induction. Despite the fact that we used a low-passage viral stock generated from plasmids encoding a "wild-type" influenza genome, most infected cells do not express wild-type copies of all viral genes. Although our study is certainly not the first to note that influenza has a high mutation rate (***Parvin et al., 1986***; ***Suárez et al., 1992***; ***Suárez-López and Ortín, 1994***; ***Bloom, 2014***; ***Pauly et al., 2017***) and sometimes fails to express genes (***Brooke et al., 2013***; ***Heldt et al., 2015***; ***Dou et al., 2017***; ***Russell et al., 2018***), it is the first to directly observe the full spectrum of these defects across single cells. Visual inspection of ***Figure 4*** shows how any experiment that does not sequence viral genes in single cells is averaging across a vast number of hidden viral defects.

It turns out that these viral defects substantially contribute to the heterogeneity observed in prior single-cell studies of influenza (***Russell et al., 2018***; ***Steuerman et al., 2018***; ***Heldt et al., 2015***; ***Sjaastad et al., 2018***), including helping determine if an infected triggers an IFN response. We identified four types of viral defects in single IFN+ cells that we validated to increase IFN induction. Two types of defects—absence of the NS gene and amino-acid mutation to the NS1 protein—presumably impair NS1's well-known ability to antagonize innate-immune pathways (***García-Sastre et al., 1998***; ***Hale et al., 2008***). The third type of defect, amino-acid mutations in the PB1 protein, is consistent with recent work by ***te Velthuis et al.*** (***2018***) showing that mutations to the viral polymerase affect generation of mini-viral RNAs that activate the innate-immune sensor RIG-I. Finally, we found an internal deletion in the PB1 gene that enhances IFN induction, consistent with a large body of prior work showing that such deletions are immunostimulatory (***Baum et al., 2010***; ***Tapia et al., 2013***; ***Boergeling et al., 2015***; ***Dimmock and Easton, 2015***). Given the extensive prior

work describing such deletions, it is perhaps surprising we did not identify more of them in our single IFN+ cells. There may be several reasons: we used relatively pure viral stocks (***Xue et al., 2016***) at modest MOI; our experiments preferentially captured cells with higher viral transcriptional load; and most prior studies have used techniques that can detect large deletions but not subtle point mutations. The relative importance of different types of viral defects for IFN induction likely varies across infection conditions, viral strains, and cell types—so it remains an open question as to which defects are most relevant for early immune detection of actual viral infections in humans.

But although viral variation is clearly important, it still only partially explains heterogeneity among influenza-infected cells. We find substantial breadth in viral transcriptional burden and occasional IFN induction even among cells infected with fully wild-type virions. Additionally, none of the viral defects in IFN+ cells induce IFN deterministically in our validation experiments—and the apparently most immunostimulatory defect (absence of NS) also occurs in multiple IFN- cells in our single-cell dataset. Of course, our single-cell virus sequencing only calls mutations present in a cell at relatively high frequency, and so could overlook low-frequency mutations that contribute to IFN induction. However, we suspect that stochasticity or pre-existing cellular states also affect the outcome of infection, since heterogeneity in IFN induction is present even among cells treated with synthetic innate-immune ligands (***Shalek et al., 2013***, ***2014***; ***Wimmers et al., 2018***; ***Bhushal et al., 2017***), as well as for other viruses (***O'Neal et al., 2018***).

Perhaps the most intriguing question is how the heterogeneity that we have described ultimately affects the macroscopic outcome of infection. Natural human influenza infections are established by just a handful of virions (***McCrone et al., 2018***; ***Xue and Bloom, 2018***; ***Varble et al., 2014***) that then undergo exponential growth, and early IFN responses are amplified by paracrine signaling (***Stetson and Medzhitov, 2006***; ***Honda et al., 2006***). It is therefore plausible that genetic variation in the founding viral population could have a large effect on downstream events. Extending our approaches to more complex systems could shed further light on how viral genetic variation interacts with cell-to-cell heterogeneity to shape the race between virus and immune system.

Methods and Materials

IFN reporter cell lines

We created IFN reporter variants of the A549 human lung epithelial cell line (***Figure 1***B). The parental A549 cell line used to create these reporters was obtained from ATCC (CCL-185), and was tested as negative for mycoplasma contamination by the Fred Hutch Genomics Core and authenticated using the ATCC STR profiling service. The cells were maintained in D10 media (DMEM supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 U of penicillin / ml, and 100 μg of streptomycin / ml) at 37°C and 5% carbon dioxide.

To create the type I interferon reporters, a 1kb promoter region upstream of the human IFNB1 gene were cloned into the pHAGE2 lentiviral vector (***O'Connell et al., 2010***), with a NotI site immediately downstream of the promoter serving as an artificial Kozak sequence. Downstream of this NotI site, each of the following reporter constructs was cloned: mCherry, mNeonGreen, and low-affinity nerve growth factor lacking the C-terminal signaling domain (LNGFRΔC) (***Bonini et al., 1997***; ***Ruggieri et al., 1997***) linked to mNeonGreen by a P2A linker (***Kim et al., 2011***). The sequence of the last of these constructs is provided in ***Figure 1–source data 1***.

To create the type III interferon reporters, a 1.2kb region upstream of the human IL29 (IFNL1) gene was cloned into the pHAGE2 vector, with the native Kozak sequence retained at the 3' end. Downstream of this promoter we cloned LNGFRΔC linked to ZsGreen via a P2A linker. The sequence of this construct is provided in ***Figure 1–source data 1***.

We used these constructs to generate lentiviral vectors and transduce of A549 cells in the presence of 5 μg polybrene. We then sorted single transduced cells and expanded them. A portion of the expanded cells were tested for reporter activity by transfecting poly(I:C) (a potent agonist of the RIG-I pathway), and we retained clones with strong activation. Importantly, the cells that we retained for further use were not the same portion that were tested by poly(I:C) treatment, but rather a separate split of the same population—this avoids any selection on the cells from transient activation of IFN. For the dual type I / type III reporter used in ***Figure 1–Figure Supplement 3***, a single-cell clone of the type III reporter cell line was transduced with the type I reporter bearing the mCherry fluorescent marker, and then isolated and propagated as a single cell clone for the other cell lines. All reporter lines tested negative for mycoplasma contamination by the Fred Hutch Genomics Core.

Figure 1–Figure Supplement 2 shows validation of the reporter cell lines using infection with saturating amounts of the Cantell strain of Sendai virus (obtained from Charles River Laboratories). For detection of the cell-surface bound LNGFRΔC, cells were stained with PE-conjugated anti-LNGFR (CD271) antibody from Miltenyi Biotec.

Viruses for single-cell experiments

We performed the single-cell experiments using the A/WSN/1933 (H1N1) strain of influenza virus. We used both the wild-type virus and a variant of the virus where synonymous mutations were added within a few 100 nucleotides of each termini of each gene segment. We have used a similar synonymous viral barcoding strategy in our prior single-cell work (***Russell et al., 2018***) as it allows us to detect about half of co-infected cells based on the expression of both viral barcode variants. In the current work, we extended this approach by placing synonymous barcodes near *both* termini of the gene segments in order to quantify strand exchange during PacBio sequencing (***Figure 4–Figure Supplement 2***). The sequences of all gene segments from the wild-type and synonymously barcoded viral strains are in ***Figure 2–source data 1***. These genes were cloned into the pHW2000 (***Hoffmann et al., 2000***) reverse-genetics plasmid.

Both viral strains were generated by reverse genetics using the pHW18* series of bi-directional plasmids (***Hoffmann et al., 2000***). We controlled the durations and MOI during viral passaging since these factors can greatly affect the accumulation of defective viral particles (***Xue et al., 2016***). The viruses were generated by reverse genetics in co-cultures of 293T and MDCK-SIAT1 cells in

influenza growth media (Opti-MEM supplemented with 0.01% heat-inactivated FBS, 0.3% BSA, 100 U of penicillin/ml, 100 μg of streptomycin/ml, and 100 μg of calcium chloride/ml) and then propagated in MDCK-SIAT1 cells in influenza growth media using the same basic procedures detailed in ***Russell et al.*** (***2018***). Specifically, after generation by reverse genetics, the wild-type variant was expanded at an MOI of 0.001 for 72 hours twice in MDCK-SIAT1 cells, and the synonymously barcoded variant was expanded once at an MOI of 0.01 for 60 hours. The MOIs for this passaging are based on titers determined using TCID50 assays via the formula of ***Reed and Muench*** (***1938***) as implemented at https://github.com/jbloomlab/reedmuenchcalculator.

Flow cytometry analyses for HA expression

For the single-cell experiments (which only examine the transcriptional results of a single cycle of infection), we were most interested in the titer of viral particles that are transcriptionally active for a single round of infection of A549 cells. We estimated titers of transcriptionally active virions by staining for HA expression in virus-infected A549 cells. Specifically, we infected A549 cells (or one of the A549 reporter cell line variants as indicated) in influenza growth medium, and at 13 to 14 hours post-infection, we trypsinized cells, re-suspended in phosphate-buffered saline (PBS) supplemented with 2% heat-inactivated fetal bovine serum (FBS), and stained with 10 μg/ml of H17-L19, a mouse monoclonal antibody previously shown to bind to the HA from the A/WSN/1933 strain of virus (***Doud et al., 2017***). After washing in PBS supplemented with 2% FBS, the cells were stained with a goat anti-mouse IgG antibody conjugated to APC, washed, fixed in 1% formaldehyde in PBS, washed again, and then analyzed by flow cytometry to determine the fraction expressing detectable HA protein.

Single-cell transcriptomics of IFN-enriched infected cells using 10X Chromium

The single-cell transcriptomics and virus sequencing was performed using the A549 cells with the *IFNB1* LNGFRΔC-P2A-mNeonGreen reporter. A schematic of the experiment is shown in ***Figure 2***.

The wild-type and synonymously barcoded viruses were mixed with the goal of adding equal numbers of transcriptionally active HA-expressing virions of each virus strain. The cells were then infected with this mixture at a dose designed to infect about half the cells (***Figure 3***C suggests that the actual rate of detectable infection was slightly lower). Infections were allowed to proceed for 12 hours. The cells were then trypsinized, the trypsin was quenched with D10 media, and cells were resuspended in de-gassed PBS supplemented with 0.5% bovine serum albumin and 5 mM EDTA. To enrich IFN+ cells, the cells were then incubated with anti-LNGFR MACSelect Microbeads (Miltenyi Biotec) and twice passed over an MS magnetic column (Miltenyi Biotec), retaining the bound (and presumably IFN-enriched) population each time. This MACS sorting is expected to give approximately the enrichment for IFN+ cells shown in ***Figure 2–Figure Supplement 1***. The original, unsorted, population was then added back in to ∼10% of the final cell fraction in order to ensure the presence of interferon negative cells. At this point, uninfected canine (MDCK-SIAT1) cells were also added to ∼5% of the final cell fraction to enable quantification of the cell multiplet rate (***Figure 3***A) and background viral mRNA in uninfected cells (***Figure 3***C). We began this entire process of cell collection and enrichment at 12 hours post-infection, but the process (which was performed at room temperature) took about an hour, and thus we consider the cells to have been analyzed at 13 hours post-infection. The final cell suspension was counted using a disposable hemocytometer and loaded on the 10x Genomics Chromium instrument (***Zheng et al., 2017***), targeting capture of ∼1,500 cells.

This sample was then processed to create libraries for Illumina 3'-end sequencing according to the 10X Genomics protocol using the Chromium Single Cell 3' Library and Gel Bead Kit v2 with one important modification: rather than process all full-length cDNA through enzymatic fragmentation, several nanograms were retained for targeted full-length viral cDNA sequencing as described below. The single-cell transcriptomics library was sequenced on an Illumina HiSeq 2500, and the data analyzed as described below.

Enrichment and preparation of viral cDNA for PacBio sequencing

We amplified virus-derived molecules from the small amount of cDNA retained from the 10X Genomics protocol for PacBio sequencing of the full-length cDNA. All molecules in this cDNA have at their 3′ end the cell barcode and UMI plus the constant adaptor sequence that is added during the 10X protocol (see ***Figure 2*** for simple schematic, or the detailed analysis notebook at https://github.com/jbloomlab/IFNsorted_flu_single_cell/blob/master/pacbio_analysis.ipynb or Supplementary file 1 for at detailed schematic). However, we only wanted to PacBio sequence cDNA molecules derived from virus, since it would be prohibitively expensive to sequence all molecules. We therefore needed to enrich for the viral molecules, a process made challenging by the need to also retain the 10X adaptor / UMI / cell barcode at the 3′ end (the primer at the 5′ end can be specific, but the one at the 3′ end must bind the common 10X adaptor shared among all molecules).

We first performed a multiplex PCR reaction on 1 ng of the full-length 10X cDNA using a 3′ primer complementary to the common 10X adaptor, and a multiplex mix of eight 5′ primers, one specific for the mRNAs from each of the eight viral gene segments (although some viral segments produce multiple splice forms, all mRNAs from a given segment share the same 5′ end). The sequences of these primers are in ***Figure 4–source data 1***. A major concern during these PCRs is strand exchange (see ***Figure 4–Figure Supplement 2***) which would scramble the cell barcodes and mutations on viral cDNAs. To reduce strand-exchange and hopefully obtain more even PCR amplification across segments, we performed emulsion PCRs using the Micellula DNA Emulsion Kit (Roboklon). Emulsion PCR involves encapsidating DNA template molecules in a reverse-phase emulsion, with each template positive droplet serving as a microreactor. This process physically separates disparate template molecules, preventing strand exchange and allowing each molecule to be amplified to exhaustion of its droplet's reagents without competing with the broader pool of PCR-amenable molecules (***Boers et al., 2015***). We performed the PCRs using Kapa HiFi Hotstart ReadyMix, supplementing the reactions with additional BSA to a final concentration of 0.1 mg/ml and using a volume 100μl . Both the common 3′ primer and the multiplex mix of eight 5′ primers were added to a final concentration of 0.5μM. We performed 30 cycles of PCR, using an extension time of 2 minutes 15 seconds at 67°C, and a melting temperature of 95°C. This melting temperature is lower than the standard 98°C melting step suggested by the manufacturer for Kapa HiFi because we wanted to avoid collapse of emulsion integrity at high temperature. We performed 30 cycles in order to saturate the reagents in each emulsion—unlike for open PCR reactions, the physical occlusion of amplified material in emulsion PCR reduces artifacts at high cycle numbers.

Because were were concerned that the multiplex PCR would still result in highly uneven amplification of different influenza cDNAs due to differences in their expression levels and lengths, the product of this multiplex PCR was subjected to eight additional individual emulsion PCR reactions, each using only a single segment-specific 5′ primer as well as the common 3′ primer, using 1 ng of material in each reaction. The material from these eight segment-specific PCRs was then pooled with the goal of obtaining a equimolar ratio of segments, and sequenced on one SMRT Cell in a PacBio RS II and one SMRT Cell of a PacBio Sequel. Detailed results from the analysis of these first two sequencing runs is shown in the PacBio analysis notebook at https://github.com/jbloomlab/IFNsorted_flu_single_cell/blob/master/pacbio_analysis.ipynb and Supplementary file 1. These results showed that although the PCRs substantially enriched for influenza molecules, the relative coverage of the different viral genes was still very uneven, with the longer genes (especially the polymerase genes) severely under-sampled.

To try to improve coverage of the polymerase genes, we produced two new sequencing pools: one consisting of the five shortest viral segments (HA, NP, NA, M, and NS) from the aforementioned segment-specific emulsion PCRs, and the other consisting of the three longer polymerase segments (PB2, PB1, and PA). The former was sequenced on one cell of a single SMRT Cell of a PacBio Sequel, and the latter on two additional SMRT Cells of a PacBio Sequel. As is shown in the PacBio analysis notebook at https://github.com/jbloomlab/IFNsorted_flu_single_cell/blob/master/pacbio_analysis.

ipynb (see also Supplementary file 1), even with these new sequencing runs the coverage remained relatively low for the polymerase genes—and most of the reads we did obtain were dominated by shorter internally deleted variants of the polymerase genes, which arise commonly during influenza replication (***Xue et al., 2016***) and are presumably preferentially amplified during PCR.

To obtain more reads for longer full-length polymerase variants, we therefore subjected 10 ng of our amplified material for each polymerase segment to a bead selection using SPRIselect beads at a volume ratio of 0.4 to select for larger molecules. This selection removes most low-molecular weight DNA species including internally-deleted defective segments. Material from this selection was then amplified using 16 (PB1) or 14 (PB2 and PA) cycles of a non-emulsion PCR using the standard conditions recommended by the Kapa HiFi Hotstart ReadyMix (extension at 67°C for 2 minutes 15 seconds, and melting at 98°C). The use of relatively few PCR cycles was designed to prevent the occurrence of the artifacts (including strand exchange) that occur in non-emulsion PCRs as the reactions approach saturation. We pooled the products of these reactions from this size-selection and sequenced on a SMRT Cell of a PacBio Sequel. As is shown in the PacBio analysis notebook at https://github.com/jbloomlab/IFNsorted_flu_single_cell/blob/master/pacbio_analysis.ipynb (see also Supplementary file 1), this sequencing yielded modestly more full-length polymerase variants, but they were still heavily undersampled compared to other viral genes.

To further to improve recovery of full-length PB1, PB2, and PA, we therefore took an alternative approach that allowed us to perform a specific PCR for full-length polymerase variants. Specifically, we circularized the template molecules (including the full length of genes plus the cell barcode, UMI, and 10X adaptor), and then used two segment-specific primers that annealed in apposition near the center of each polymerase gene to linearize these circular molecules. Only molecules that contain the middle of the polymerase genes (which are typically full-length) are linearized by this process. In the downstream computational analysis, we can then determine the full sequence of the gene as well as the cell barcode of the initial molecule from which the linearized molecule is derived. Specifically, we first used 2.5 ng of our already-amplified segment-specific material in a 10-cycle PCR to append circularization adapters (see ***Figure 4–source data 1*** for sequences), and cleaned the resultant mixture using SPRIselect beads at a volume ratio of 0.4. We then used 10 ng of this amplified material in a 20μl NEBuilder reaction using an extended reaction time of 50 minutes in order to circularize the molecules. We next incubated these reactions for 1 hour at 37°C with exonuclease V and additional ATP to a final increase in concentration of 1 mM to digest all non-circularized molecules. The circularized and digested material was then cleaned using SPRIselect beads at a volume ratio of 0.4. This material was then used as template for three non-emulsion PCRs specific to PB2, PB1, or PA, using two segment-specific primers that align to the central portion of each gene but in apposition to each another (see ***Figure 4–source data 1*** for sequences). These linearization reactions used 20 (PB2) or 26 (PB1 and PA) PCR cycles, and the resulting products were cleaned using SPRIselect beads at a volume ratio of 1.0. This material was pooled to produce an equimolar mixture of full-length PB1, PA, and PB2 and sequenced in an additional SMRT Cell of PacBio Sequel. As is shown in the PacBio analysis notebook at https://github.com/jbloomlab/IFNsorted_flu_single_cell/blob/master/pacbio_analysis.ipynb (see also Supplementary file 1), this process efficiently yielded many full-length polymerase variants.

The computational analyses of the full-length viral gene sequences described below used the combination of the data from all of these reactions. The number of sequences obtained for each gene after pooling the data from all reactions is shown in ***Figure 4–Figure Supplement 1***, which also indicates that the net rate of strand exchange is very low (see ***Figure 4–Figure Supplement 2*** for an illustration of how this is determined). A more detailed breakdown of the coverage of each gene and data showing a consistently low rate of strand exchange for all PacBio runs is at https://github.com/jbloomlab/IFNsorted_flu_single_cell/blob/master/pacbio_analysis.ipynb (see also Supplementary file 1). Importantly, the various PCR biases and enrichment schemes mean that the coverage of molecules by the PacBio sequencing is not proportional to their original abundance in the starting mRNA. However, as described in the computational analysis section below, the final

analyses use the cell barcodes and UMIs in conjunction with the standard 10X Illumina sequencing to ensure that none of the conclusions are affected by the disproportionate amplification of some molecules during the PacBio library preparation (for instance, duplicate UMIs are removed from the PacBio data, and all conclusions about gene abundance or absence are based on the Illumina data).

qPCR for viral genes and IFN

We performed qPCR on reverse-transcribed mRNA for influenza HA (to quantify viral transcription), IFNB1 (to quantify IFN induction), and L32 (a cellular housekeeping gene for normalization). For the qPCR, we used the SYBR Green PCR Master Mix (Thermo Fisher) according to the manufacturer's protocol using oligo-dT primers. The qPCR primers were: HA primer 1, 5'-GGCCCAACCACACATTCAAC-3'; HA primer 2, 5'-GCTCATCACTGCTAGACGGG-3'; IFNB1 primer 1, 5'-AAACTCATGAGCAGTCTGCA-3'; IFNB1 primer 2, 5'-AGGAGATCTTCAGTTTCGGAGG-3'; L32 primer 1, 5'-AGCTCCCAAAAATAGACGCAC-3'; L32 primer 2, 5'-TTCATAGCAGTAGGCACAAAGG-3'.

For the qPCR in ***Figure 6–Figure Supplement 3***, A549 cells were seeded at a density of 10^4 cells/well in a 96-well plate in D10 media 24 hours prior to infection, with four independent wells seeded per experimental treatment. Immediately prior to infection D10 media was removed and replaced with influenza growth media and infected with the indicated influenza strains at a MOI of 0.4 based on TCID50 in MDCK-SIAT1 cells. For the cells with cycloheximide added to block protein expression (and hence secondary transcription), cycloheximide was added to a final concentration of 50 μg/ml (a concentration sufficient to block secondary transcription; ***Killip et al., 2014***) at the time of infection. After 8 hours, mRNA was harvested using the CellAmp Direct RNA Prep Kit for RT-PCR, reverse-transcribed using an oligo-dT primer, and qPCR was performed as described above.

Viruses and experiments for validation experiments

In ***Figure 6***, we tested the IFN inducing capacity of a variety of viral mutants identified in the single-cell experiments. For point-mutant viruses, we created variants for all amino-acid substitutions found in PB1 and NS among IFN+ cells that did not also lack NS. One of these mutants (amino-acid substitution S704P in PB1) did not reach sufficient titers in a single attempt to generate it by reverse genetics, and so was dropped from the experiment (note that we did not attempt replicates of the reverse genetics for this mutant, and so are *not* confident in drawing strong conclusions about its actual attenuation). This left six point-mutant viruses: four with point mutations in PB1, and two with point mutations in NS. We also created a mutant virus that contained the internal deletion in PB1 found in an IFN+ cell. In addition, we created a virus with an inactivated NS1 to mimic the infections that failed to express NS (we were unable to use complementing cells to generate a viral stock that completely lacked the NS segment). This NS1stop virus contained six nucleotide changes resulting in the addition of five in-frame stop codons in NS1 starting 10 nucleotides downstream of the 5' splice donor site, thereby disrupting NS1 while leaving NS2 (NEP) intact. All of these mutants were cloned into the pHW2000 bi-directional reverse-genetics plasmid (***Hoffmann et al., 2000***) in order to enable generation of viruses encoding the mutant genes. ***Figure 6–source data 1*** provides the full sequences for all of these plasmids.

We generated the wild-type and point-mutant viruses for the validation experiments in ***Figure 6***A by reverse genetics using the pHW18* series of WSN reverse genetics plasmids (***Hoffmann et al., 2000***), but substituting the appropriate mutant plasmid listed in ***Figure 6–source data 1*** for the wild-type plasmid for that gene. To generate the viruses from these plasmids, we transfected an equimolar mix of all eight plasmids into co-cultures of 293T and MDCK-SIAT1 cells seeded at a ratio of 8:1. At 24 hours post-transfection, we changed media from D10 to influenza growth media. At 50 hours post-transfection (for the replicate 1 viruses in ***Figure 6–Figure Supplement 1***) or 72 hours (for the replicate 2 viruses in ***Figure 6–Figure Supplement 1***), we harvested the virus-containing supernatant, clarified this supernatant by centrifugation at 300×g for 4 min, and stored aliquots of the clarified viral supernatant at -80°C. We then thawed aliquots and titered by TCID50 on MDCK-SIAT1 cells. For the infections in ***Figure 6–Figure Supplement 1***, we wanted to use equivalent

particle counts, so we normalized all viruses to an equivalent hemagglutination titer on turkey red blood cells (***Hirst, 1942***). Briefly, a solution of 10% v/v red blood cells (LAMPIRE Biological Laboratories, Fisher Scientific catalogue number 50412942) was washed in PBS and diluted to a final concentration of 0.5% v/v. Two-fold serial dilutions of virus were added to an equal volume of diluted red blood cells, and titer was measured as the highest dilution of viral stock at which complete hemagglutination of red blood cells was observed. We then performed infections of the A549 reporter cell line at equivalent hemagglutination titer and analyzed the data as described in ***Figure 6–Figure Supplement 1***.

To generate the NS1stop mutant virus and the wild-type and PB1del385to2163 mutant viruses in ***Figure 6–Figure Supplement 2***, we used slightly different procedures. The wild-type virus was generated by reverse genetics as described for the point-mutant viruses above, harvested at 48 hours post-transfection, and then passaged on MDCK-SIAT1 cells for 36 hours at an MOI of 0.05—conditions that we previously validated to lead to relatively little accumulation of defective particles (***Russell et al., 2018***). The NS1stop virus was similarly generated, but was passaged for 48 rather than 36 hours, since it had slower growth kinetics and so needed a longer period of time to reach high titers. The viruses with deletions in the PB1 segment could not be generated in normal 293T and MDCK-SIAT1 cells, since they required the exogenous expression of the PB1 protein. Therefore, these viruses were generated in previously described 293T and MDCK-SIAT1 cells that had been engineered to constitutively express PB1 (***Bloom et al., 2010***). These viruses were harvested from transfections at 72 hours, and passaged twice in the MDCK-SIAT1 cells constitutively expressing PB1 at a MOI of 0.001 for 72 hours and 0.01 for 48 hours. This passaging was necessary as viral titers from transfections were too low to generate sufficient virus from a single passage. The wild-type and NS1stop viruses were titered by TCID50 on MDCK-SIAT1 cells, and the PB1 deletion viruses were titered on the MDCK-SIAT1 cells constitutively expressing PB1. The infections in ***Figure 6–Figure Supplement 2*** were performed at equivalent TCID50s as described in the legend to that figure. That these equivalent TCID50s were also roughly equivalent in terms of particles capable of undergoing primary transcription is shown in ***Figure 6–Figure Supplement 3***.

Computational analysis of single-cell transcriptomic and viral sequence data

A computational pipeline that performs all steps in the data analysis is available at https://github.com/jbloomlab/IFNsorted_flu_single_cell (***Russell and Bloom, 2018***). This pipeline is orchestrated by `Snakemake` (***Köster and Rahmann, 2012***), and begins with the raw sequencing data and ends by generating the figures shown in this paper. The sequencing data and annotated cell-gene matrix are available on the GEO repository under accession GSE120839 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE120839).

Briefly, the raw deep sequencing data from the Illumina 3'-end sequencing were processed using the 10X Genomics software package `cellranger` (version 2.2.0). We built a multi-species alignment reference consisting of a concatenation of the human and influenza virus transcriptomes (the first "species") and the canine transcriptome (the second "species"). The human transcriptome was generated by filtering genome assembly GRCh38 for protein-coding genes defined in GTF file GRCh38.87. The influenza virus transcriptome consisted of the mRNAs for the wild-type A/WSN/1933 virus strain in ***Figure 2–source data 1*** (the `cellranger` alignment is sufficiently permissive that it aligns sequences from both the wild-type and synonymously barcoded viral variants to this transcriptome). The canine transcriptome was generated by filtering genome assembly CanFam3.1 for protein-coding genes defined in GTF file CanFam3.1.87. The `cellranger` software was used to align the Illumina 3'-end sequencing reads to this multi-species transcriptome, call human+influenza and canine cells (***Figure 3***A), and generate a matrix giving the expression of each gene in each single cell. We used a custom Python script to determine the number of influenza virus reads that could be assigned to the wild-type or synonymously barcoded virus, and added this information to the annotated the cell-gene matrix.

The PacBio sequences of the full-length viral genes were analyzed as follows. First, we used

version 3.1.0 of PacBio's `ccs` program (https://github.com/PacificBiosciences/unanimity) to build circular consensus sequences (CCSs) from the subreads files, requiring at least 3 passes and a minimum accuracy of 0.999. We further processed these CCSs using custom Python code and the `minimap2` (***Li, 2018***) long-read aligner (version 2.11-r797). The Python code has been implemented in the API of `dms_tools2` (***Bloom, 2015***, https://jbloomlab.github.io/dms_tools2/) package (version 2.3.0). A Jupyter notebook that performs these analyses is at https://github.com/jbloomlab/IFNsorted_flu_single_cell/blob/master/pacbio_analysis.ipynb, and is also provided in HTML form as Supplementary file 1. We refer the reader to this notebook for a detailed description and extensive plots showing the results at each step. Here is a brief summary: we filtered for CCSs that had the expected 5′ termini (from the influenza-specific primers) and 3′ termini (corresponding to the 10X adaptor), and for which we could identify the cell barcode, UMI, and polyA tail. We aligned the cDNAs flanked by these termini to the influenza transcriptome, and performed a variety of quality control steps. At this point, we examined whether cDNAs had the synonymous viral barcodes at both ends or neither end as expected in the absence of strand exchange (***Figure 4–Figure Supplement 2***), and reassuringly found that strand exchange was rare (***Figure 4–Figure Supplement 1***). The small number of CCSs with identifiable strand exchange were filtered from further analysis. We then further filtered for CCSs that contained valid cell barcodes as identified by the `cellranger` pipeline, and kept just one CCS per UMI (preferentially retaining high-quality CCSs that aligned to full-length cDNAs). We then removed from the CCSs the barcoding synonymous mutations that we had engineered into one of the two viral variants. Finally, we used the CCSs to call the sequence of the viral gene in each cell, calling mutations separately for each viral barcode variant. We called mutations (insertions, deletions, and substitutions) in the viral gene sequences as follows:

1. Mutations with accuracies less than 0.999 (which constitute <0.5% of all mutations) were ignored.
2. If all CCSs for a particular viral-barcode variant of a gene in a cell were wild-type, it was called as wild type.
3. If any CCSs for a particular viral-barcode variant of gene in a cell had a mutation, then require at least two CCSs to call the sequence.
4. If at least two and >30% of the CCSs had a specific mutation, then call that mutation as present and note its frequency among the CCSs. The exception was single-nucleotide indels in homopolymers, for which we required three CCSs to call a mutation (the reason is that the main mode of PacBio sequencing errors is short indels in homopolymers).

The plots in https://github.com/jbloomlab/IFNsorted_flu_single_cell/blob/master/pacbio_analysis.ipynb or Supplementary file 1 indicate that these are reasonable mutation-calling criteria. We could call the sequences of all expressed viral genes in about half of the infected cells (***Figure 4–Figure Supplement 3***). The mutations called using this pipeline are shown in ***Figure 4***, and ***Figure 4–source data 2*** gives the number of CCSs supporting each mutation call. The called sequences of the viral genes were added to the annotated cell-gene matrix.

Finally, we process the annotated cell-gene matrix in R to generate the plots shown in this paper. This analysis utilized a variety of R and Bioconductor (***Huber et al., 2015***) packages, including `Monocle` (***Qiu et al., 2017***; ***Trapnell et al., 2014***) and `ggplot2`. A Jupyter notebook that performs these analyses is at https://github.com/jbloomlab/IFNsorted_flu_single_cell/blob/master/monocle_analysis.ipynb, and is also provided in HTML form as Supplementary file 2. We refer the reader to this notebook for a detailed description and a variety of additional plots not included in the paper. Briefly, we first filtered cells that were extreme outliers in the amount of mRNA as shown in ***Figure 3***B. We used the uninfected canine cells to estimate the percentage of total mRNA in a cell that would come from influenza purely due to background (e.g., from cell lysis) in the absence of infection, and called as infected the human cells for which significantly more than this amount of mRNA was derived from influenza under a Poisson model (***Figure 3***C). We next used a Poisson model parameterized by the amount of expected background mRNA for each influenza gene to call

the presence or absence of each influenza gene in each infected cell (***Figure 3***D and ***Figure 3–Figure Supplement 1***). To identify cells that were co-infected with both viral barcodes (***Figure 3***F), we used a binomial test to identify cells for which we could reject the null hypothesis that at least 95% of viral mRNA was derived from the more common viral barcode. We called IFN+ and ISG+ cells using the heuristic thresholds shown in ***Figure 3***G and ***Figure 3–Figure Supplement 3***, respectively. We counted IFN mRNAs as any IFN-α, IFN-β, or IFN-λ transcripts. We counted ISG mRNAs as any of CCL5, IFIT1, ISG15, or Mx1. The plot in ***Figure 4*** summarizes all of the genotypic information, and was created in substantial part using `gggenes` (https://github.com/wilkox/gggenes).

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Competing Interests

The authors declare that no competing interests exist.

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Supplementary file 1. An HTML rendering of the Jupyter notebook that analyzes the PacBio data to call the viral sequences in infected cells is available at https://github.com/jbloomlab/IFNsorted_flu_single_cell/raw/master/paper/figures/pacbio_single_cell_figures/pacbio_analysis.html. This notebook contains detailed descriptions of each step and plots illustrating the results, and is the best way to understand this part of the analysis in detail. The actual Jupyter notebook rendered here is available at https://github.com/jbloomlab/IFNsorted_flu_single_cell/blob/master/pacbio_analysis.ipynb.

Supplementary file 2. An HTML rendering of the Jupyter notebook that analyzes the annotated cell-gene matrix to generate the figures in this paper is available at https://github.com/jbloomlab/IFNsorted_flu_single_cell/raw/master/paper/figures/single_cell_figures/monocle_analysis.html. This notebook contains detailed descriptions of each step and plots illustrating the results, and is the best way to understand this part of the analysis in detail. The actual Jupyter notebook rendered here is available at https://github.com/jbloomlab/IFNsorted_flu_single_cell/blob/master/monocle_analysis.ipynb.

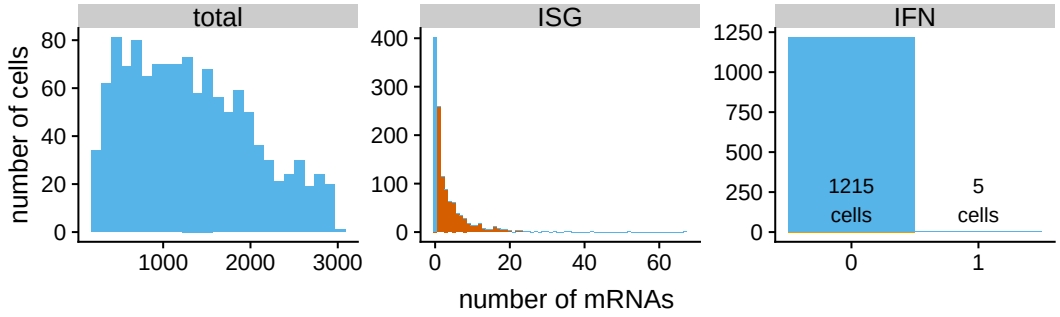

Figure 1–Figure supplement 1. A re-analysis of the data from ***Steuerman et al.*** (***2018***)'s single-cell transcriptomics of cells from influenza-infected mice shows that only 5 of 1220 virus-infected cells express *detectable* type I or type III IFN transcripts *in vivo*. Specifically, we downloaded the data from ***Steuerman et al.*** (***2018***) and identified influenza-infected cells using criteria similar to those described in ***Steuerman et al.*** (***2018***). Here we show statistics for the cells from the influenza-infected wild-type C57BL/6J mice, which were collected at 48 hours (two replicates) or 72 hours (one replicate)—we do not show cells from the control mice. As shown in the left-most panel, the sequencing depth of ***Steuerman et al.*** (***2018***) was quite low, with only ~1,500 mRNA counts per cell on average (this is 10 to 15-fold lower than the sequencing depth in ***Russell et al.*** (***2018***) and the current study, respectively). An important caveat is that this low sequencing depth could lead to a simple failure to detect IFN transcripts in some cells. Nonetheless, there is detectable expression of key ISGs—which are induced by autocrine and paracrine signaling (***Stetson and Medzhitov, 2006***; ***Honda et al., 2006***)—in the majority of infected cells (the middle panel shows the total counts of IFIT1, ISG15, CCL5, and Mx1). However, only 5 of 1220 cells express any detectable type I (IFN-α and IFN-β) or type III (IFN-λ) mRNAs, which are only induced by direct cellular detection of viral infection (***Stetson and Medzhitov, 2006***; ***Honda et al., 2006***). The full code that performs the re-analysis shown in this figure is at https://github.com/jbloomlab/IFNsorted_flu_single_cell/tree/master/paper/figures/IFN_stochastic/SteuermanReanalysis/.

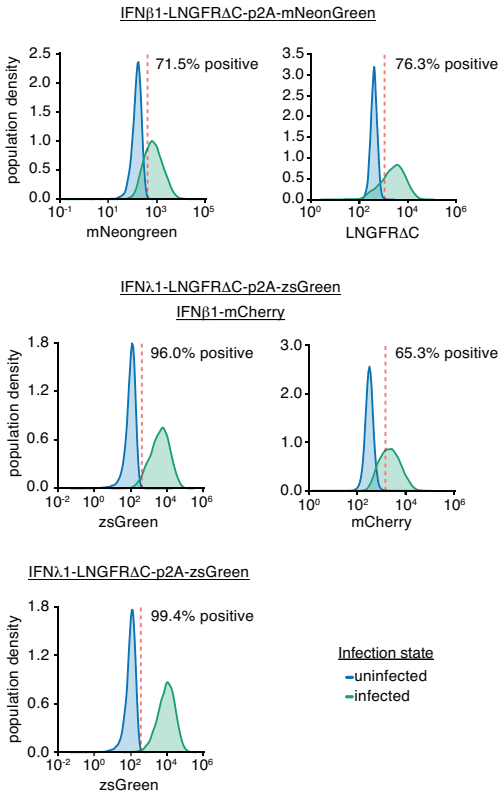

Figure 1–Figure supplement 2. To validate the IFN reporter cell lines, they were infected at high MOI with the Cantell strain of Sendai virus, which strongly activates IFN expression (***Strahle et al., 2006***). The name of each of reporter cell line is indicated at the top of each row of plots. At 13 hours post-infection, activation of the IFN reporter was then monitored by flow cytometry using the marker indicated at the bottom of each plot (either a fluorescent protein or antibody staining for the cell-surface LNGFRΔC using a PE-conjugated anti-LNGFR antibody from Miltenyi Biotec). Sendai infection efficiently activated the IFN reporter in all cases, with the strongest signal from the IFN-λ reporter driving ZsGreen.

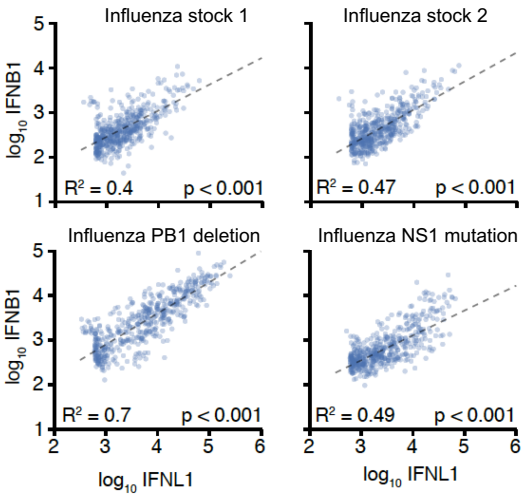

Figure 1–Figure supplement 3. An A549 cell line was generated by transduction with both the IFN-β and IFN-λ reporters driving expression of mCherry and ZsGreen, respectively. The cells were then infected with two different stocks of "wild-type" WSN influenza, or stocks with a deletion in PB1 or stop codons in NS1 (described later in the paper). After 13 hours, cells were analyzed by flow cytometry. Cells positive for either fluorescent reporter were further analyzed. As shown in the FACS plots, expression of the *IFNB1* and *IFNL1* reporters is highly correlated in all cases.

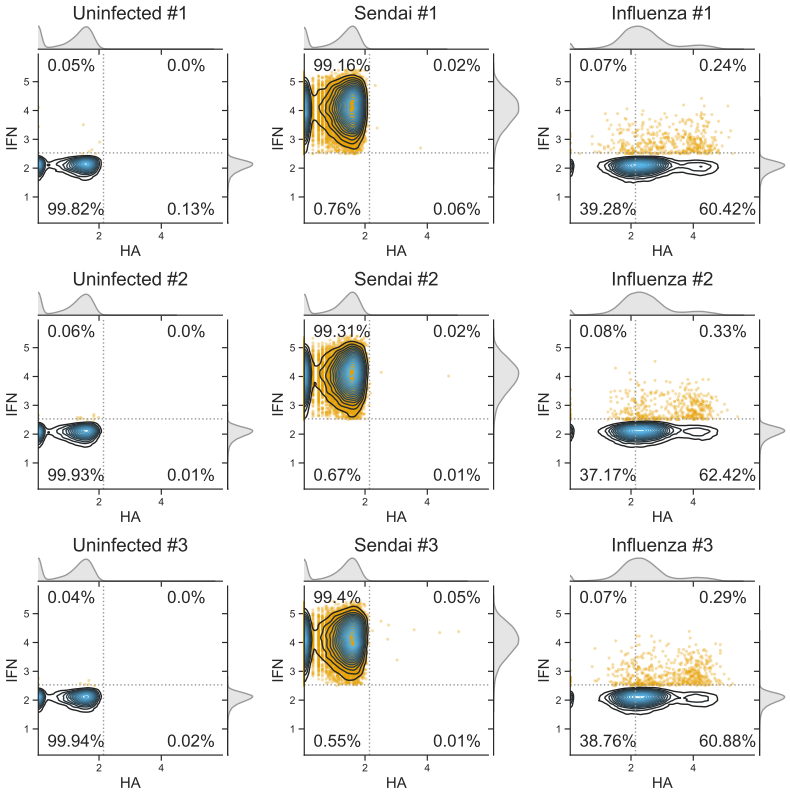

Figure 1–Figure supplement 4. Flow cytometry data for **Figure 1**C. The A549 cells with the *IFNL1* reporter driving LNGFRΔC-ZsGreen were not infected, infected with saturating amounts of the Cantell strain of Sendai virus (***Strahle et al., 2006***), or infected the same stock of influenza virus used in the single-cell experiment at a target MOI of 0.3. After 13 hours, the cells were stained for expression of HA protein and analyzed by FACS for HA and expression of the ZsGreen driven by the *IFNL1* reporter. Each condition was done in triplicate. The contour plots show the density of all cells, and all IFN+ cells are also indicated by orange dots. Cells were classified as HA+ or IFN+ based on gates set to put 0.05% of the uninfected cells in these populations. For the influenza-infected cells, the percentage IFN+ was calculated only among the HA+ cells (since these are the ones that are infected). For the uninfected and Sendai-virus infected, the percentage IFN+ was calculated among all cells, since these cells do not express HA.

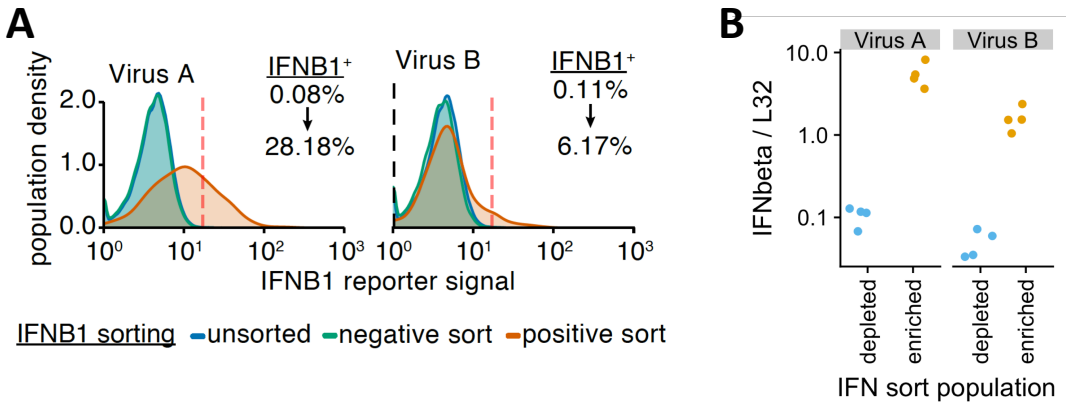

Figure 2–Figure supplement 1. Example MACS enrichments of IFN+ influenza-infected cells. A549 cells with the *IFNB1* LNGFRΔC-mNeonGreen reporter were infected with wild-type WSN influenza (two different viral stocks) at a target MOI of 0.1 TCID50 per cell. After infection had proceeded for 12 hours, the cells were twice magnetically sorted for LNGFRΔC expression over magnetic columns as detailed in the methods for the single-cell sequencing experiment. **(A)** After sorting, the populations were analyzed by flow cytometry for IFN expression using the mNeonGreen fluorescent protein. The plots show the distribution of fluorescence in the original population, the flow-through from the first column, and the MACS-sorted positive population after two columns. As indicated by the percentages shown for the original and MACS-sorted population, this process led to substantial enrichment in IFN+ cells. We expect that the IFN sorting for the actual single-cell sequencing led to similar enrichment, although we could not directly quantify this as the sorted cells in that case were immediately used for the sequencing and so could not be analyzed by flow cytometry. **(B)** Analysis of expression of IFNB1 (relative to the housekeeping gene L32) by qPCR in the positive (IFN enriched) and negative (IFN depleted) populations from panel (A). The qPCR validates a roughly 50- to 100-fold enrichment in total IFNB1 expression. The qPCR was performed in quadruplicate (hence the four points for each sample).

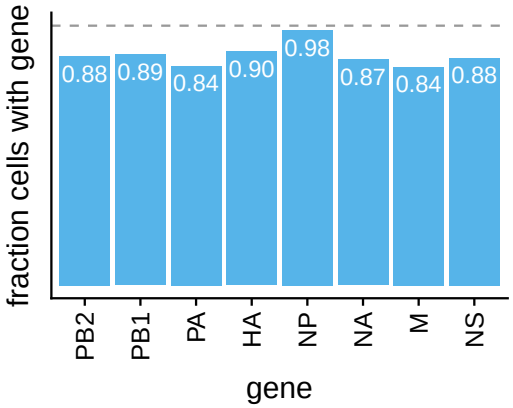

Figure 3–Figure supplement 1. The fraction of infected cells that are called as expressing each viral gene. The gray dashed line is at one (the fraction that would be observed if all viral genes are expressed in all infected cells). Each viral gene is detected in ~80-90% of the infected cells, roughly in line with prior estimates (***Brooke et al., 2013***; ***Heldt et al., 2015***; ***Dou et al., 2017***; ***Russell et al., 2018***). The exception is NP, which is detected in virtually all infected cells. The much higher frequency of detecting NP could reflect a biological phenomenon, but we suspect it is more likely that cells lacking NP tend to have much lower viral gene expression overall and so are not reliably called as being infected in our experiments because the number of viral mRNAs is below the detection limit.

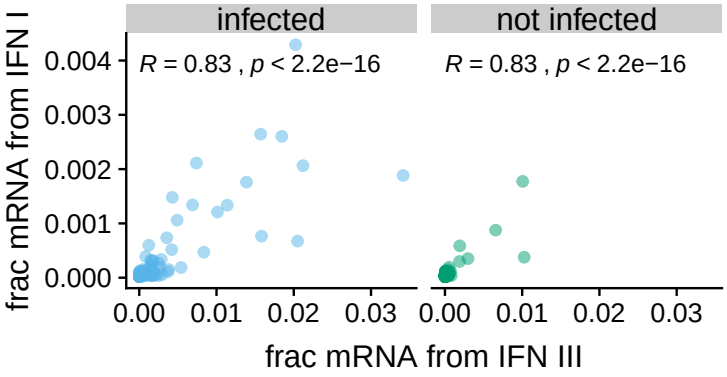

Figure 3–Figure supplement 2. The correlation between the fraction of cellular mRNA derived from type I and type III IFN in the A549 cells in our single-cell transcriptomics. Each point represents one cell. The plots are faceted by whether the cells are called as infected, and the Pearson correlation coefficient is shown. Because type I and type III IFN expression are highly correlated, for the remainder of the paper we group them together and refer to their combined expression as the level of IFN.

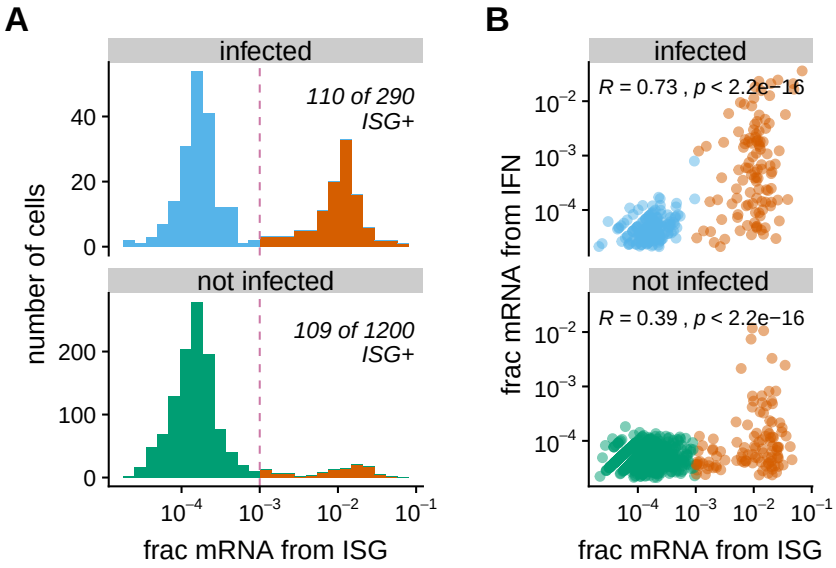

Figure 3–Figure supplement 3. For each cell, we quantified ISG expression as the total fraction of cellular mRNAs derived from four prototypical ISGs (IFIT1, ISG15, CCL5, and Mx1). **(A)** The histograms show the distribution of ISG expression taken across infected (top) and uninfected (bottom) cells. We heuristically classify as ISG+ cells with $> 10^{-3}$ of their cellular mRNA from ISGs, and color these cells red. Comparison to **Figure 3**G shows that substantially more cells are ISG+ than IFN+, both among infected and uninfected cells. This is probably because paracrine signaling can induce ISG expression in cells that are not themselves expressing IFN (***Stetson and Medzhitov, 2006***; ***Honda et al., 2006***). **(B)** Correlation between the fraction of cellular mRNA derived from IFN and ISGs. Each point represents one cell, and the Pearson correlation coefficient is shown. IFN and ISG expression are more correlated for infected than uninfected cells, probably because in the latter the ISG expression is more often due to paracrine signaling that does not induce expression of IFN itself. Among both the infected and uninfected populations, there are many cells with high expression of ISGs and little expression of IFN, but no cells that express high levels of IFN without also substantially expressing ISGs.

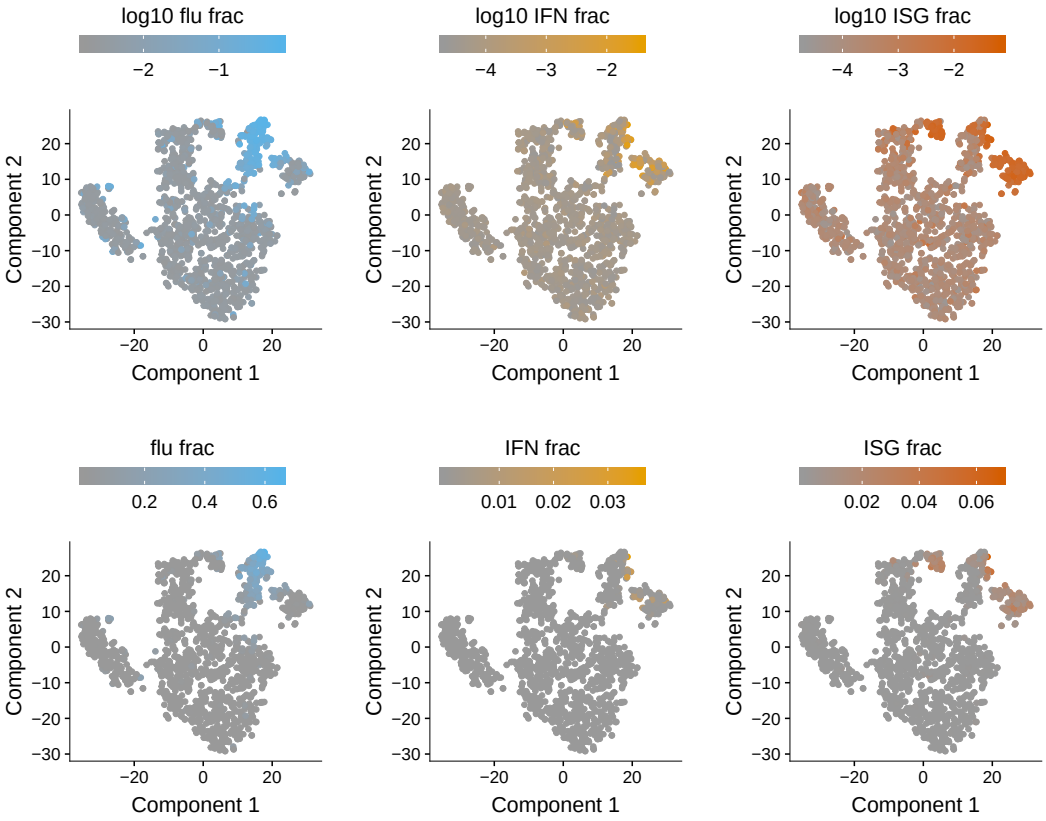

Figure 3–Figure supplement 4. To generate an unbiased representation of the factors that distinguished the transcriptomes of the cells in our experiments, we used unsupervised t-SNE clustering (***Maaten and Hinton, 2008***) as implemented in `Monocle` (***Qiu et al., 2017***; ***Trapnell et al., 2014***) to generate a two-dimensional representation of the data. In the t-SNE plot, each point is a different cell, and cells with similar transcriptomes are closer together. Each panel shows the same t-SNE plot, but the cells are colored differently in each panel based on the amount of viral, IFN, or ISG mRNA, shown on a log (top) or linear (bottom) scale. As is clear from this plot, expression of influenza, IFN, and ISG genes contributes substantially to the structure of the data, since cells with high expression of these genes clearly group together.

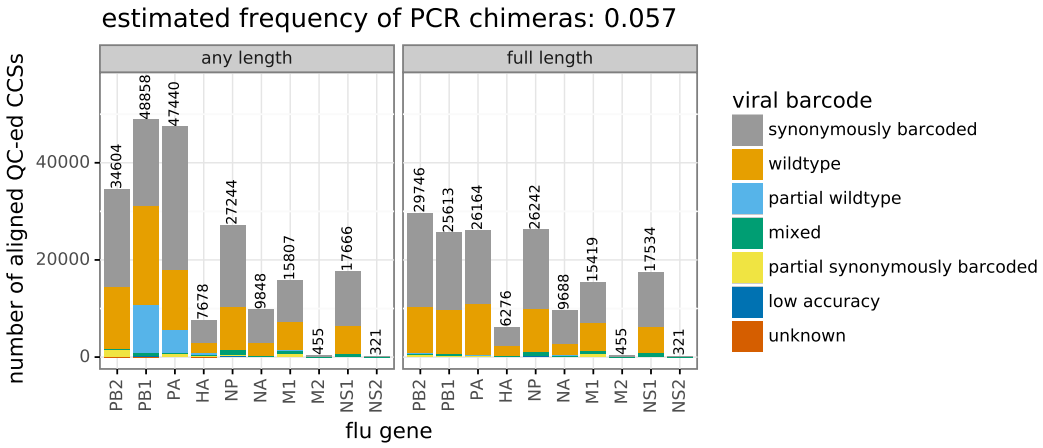

Figure 4–Figure supplement 1. The number of PacBio CCSs that passed quality-control steps and aligned to an influenza virus gene. Note that these sequences were obtained using several PacBio runs, most of which were intentionally loaded with different amounts of the various viral genes in order to increase coverage on genes that were needed in order to obtain the full sequences of virions infecting cells. (For exhaustive details, see the Jupyter notebook at https://github.com/jbloomlab/IFNsorted_flu_single_cell/blob/master/pacbio_analysis.ipynb and in Supplementary file 1.) Because of this unequal loading and the inherently different PCR amplification efficiencies of different viral genes, unlike the transcriptomic data in **Figure 3**, the numbers of CCSs for different genes should *not* be taken as an indicator of their abundance in the infected cells. Especially for the polymerase genes (PB2, PB1, and PA), many CCSs corresponded to genes with internal deletions, since these shorter forms of the genes were preferentially amplified during PCR. Therefore, the plot is faceted by the number of CCSs for any length of the gene, and for full-length genes. Note that the disproportionate sequencing of the shorter internally deleted genes should not greatly affect the genotype calling in **Figure 4** since UMIs were used to collapse duplicate sequences derived from the same cDNA, and cell barcodes were used to collapse duplicate sequences from the same cell. The bars in the plot are colored by whether the sequence is derived from the wild-type viral variant, the synonymously barcoded viral variant, or represents a mixed-barcode molecule (see ***Figure 4–Figure Supplement 2*** for more explanation). From the frequencies of these different forms, we estimate (***Bloom, 2018***) that 5.7% of molecules are chimeric due to PCR strand exchange. Note that about half of these PCR chimeras could be identified by the presence of mixed viral barcodes and removed from subsequent analyses, leaving ~3% un-identified chimeras. Note also that for some molecules (mostly polymerase genes with internal deletions) one of the barcode sites was deleted from the molecule and so the barcode identity could only be partially called. A negligible number of molecules have low-accuracy sequence or unexpected nucleotide identities at the sites of the viral barcodes.

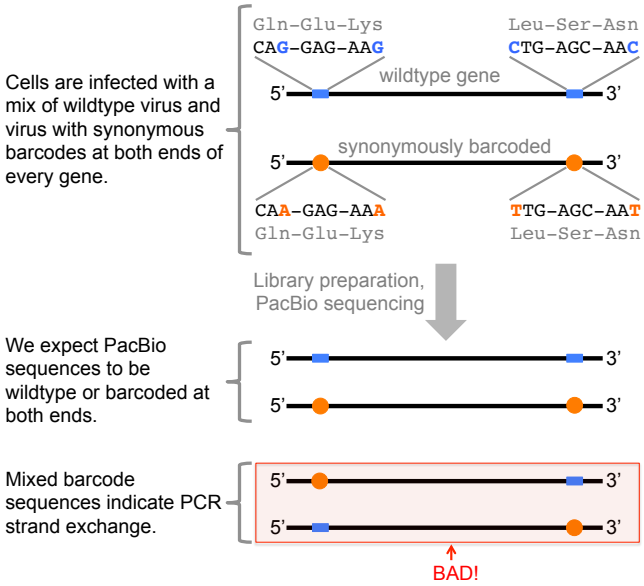

Figure 4–Figure supplement 2. The library preparation for PacBio sequencing of the cDNA for the full-length viral genes required many cycles of PCR. A major concern is that strand exchange during this PCR could scramble mutations and 10X cell barcodes / UMIs from different molecules. We can detect PCR strand exchange by leveraging the fact that our cells were infected with a mix of wild-type virus and virus carrying synonymous barcodes near both termini of each gene. If there is no strand exchange, all molecules should either be wild-type or have the synonymous barcoding mutations at *both* termini. But strand exchange will create some molecules that have wild-type nucleotides at one termini and synonymous barcoding mutations at the other termini. ***Figure 4–Figure Supplement 1*** shows the frequencies with which these different types of molecules were observed during the PacBio sequencing. Note that since the rate of homologous recombination in influenza virus in negligible (***Boni et al., 2008***), such mixed-barcode molecules are *not* expected to be generated naturally during co-infection.

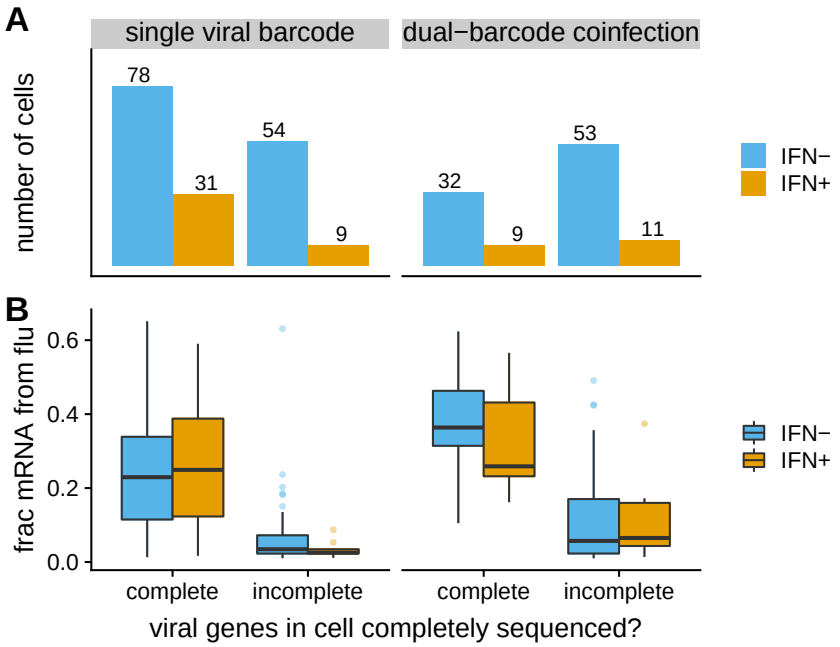

Figure 4–Figure supplement 3. Cells for which we could determine the full sequences of all genes expressed by the infecting virion(s). **(A)** We could call the complete genotypes of the infecting virion(s) for the majority of cells infected with just a single viral barcode variant, but only a minority of cells co-infected with both viral barcodes. **(B)** The cells for which we could call complete viral genotypes tended to have higher expression of viral mRNA than cells for which we could not call complete genotypes. Both facts makes sense. Cells with more viral mRNA are more likely to have their viral cDNA captured in the PacBio sequencing, which is only captures a small fraction of the total transcripts identified by the 3'-end sequencing transcriptomic sequencing. The lower calling rate for dual-barcode co-infections is probably because these co-infections have more viral genes that must be sequenced (potentially a copy of each viral gene from each viral variant), increasing the chances that one of these genes is missed by the PacBio sequencing. An important implication of this plot is that the cells for which we call complete viral genotypes are *not* a random subsampling of all infected cells in the experiment, but are rather enriched for cells that have high levels of viral mRNA and do not have dual-barcode viral infections. Note also that this plot is limited to the cells that were called as infected (**Figure 3**C) and could clearly be classified as IFN- or IFN+ (**Figure 3**G).

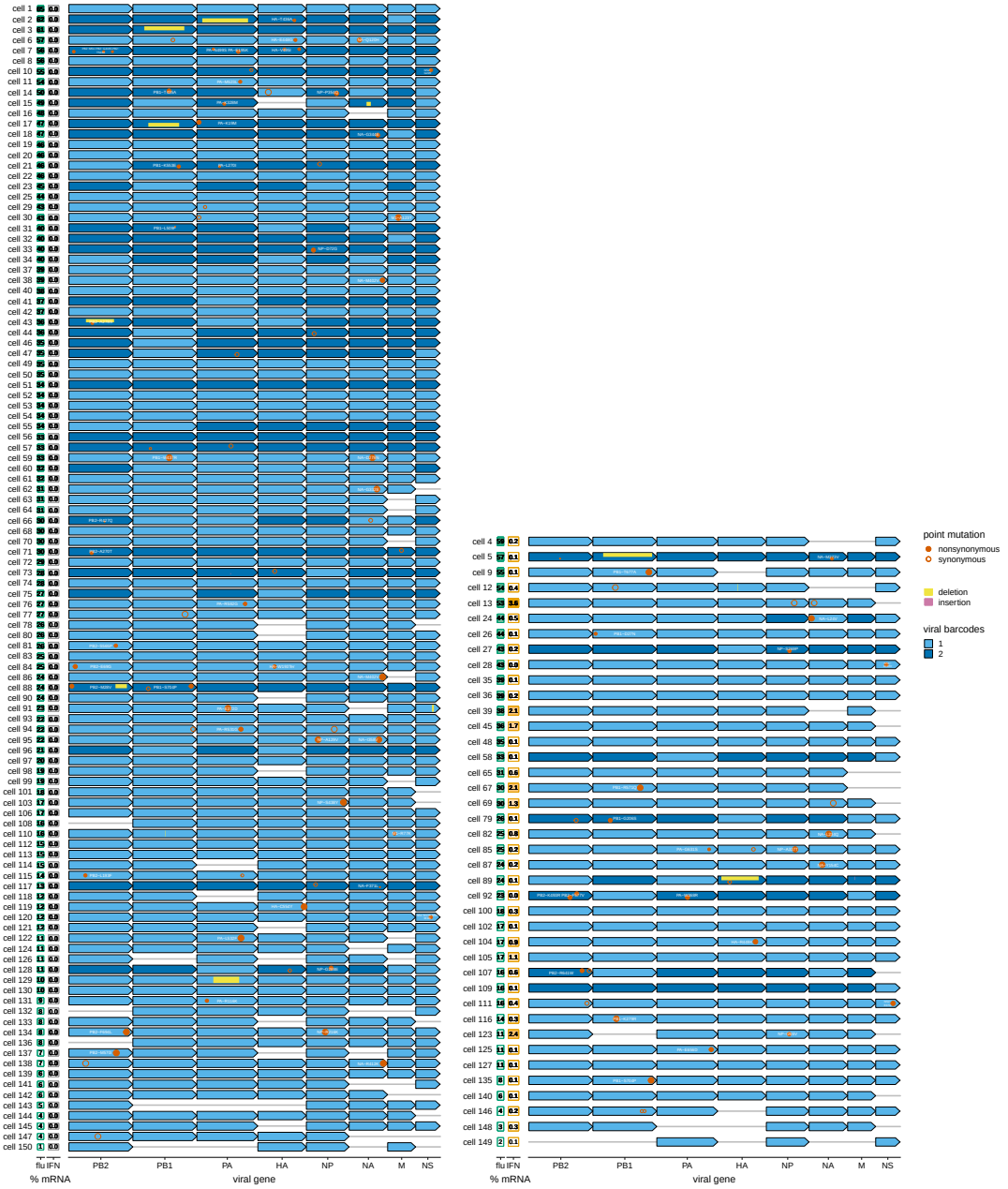

Figure 4–Figure supplement 4. This plot shows the same data as **Figure 4**, but with cells separated into columns based on whether they are IFN- (left column) or IFN+ (right column).

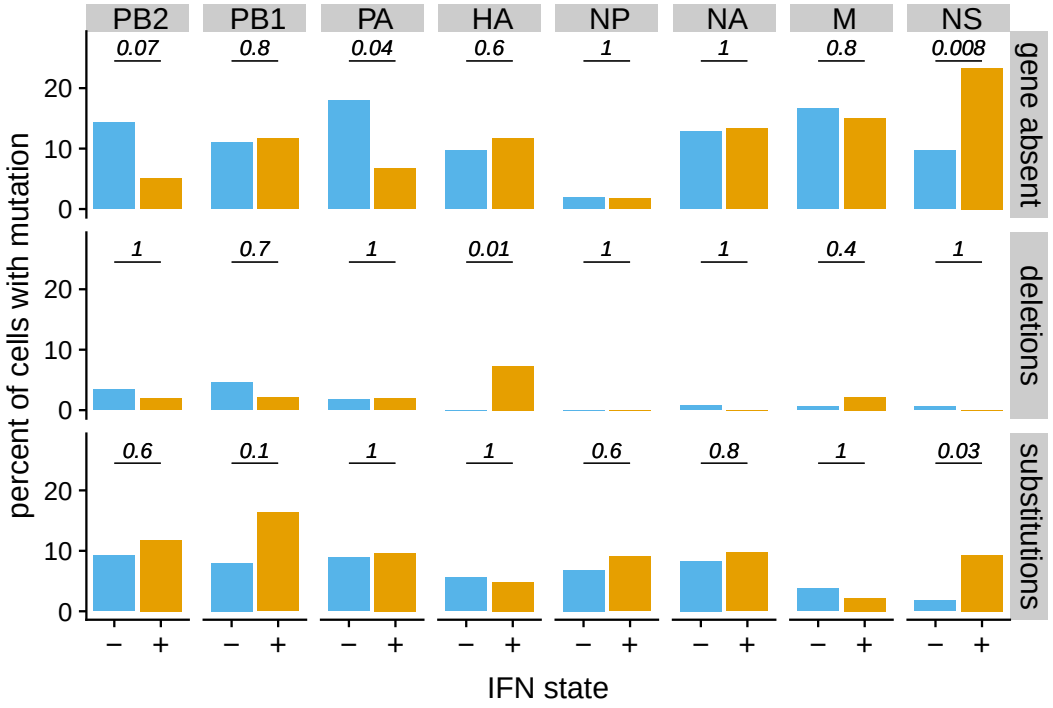

Figure 5–Figure supplement 1. This plot differs from Figure 5C in that it also includes data from cells for which some viral genes were not fully sequenced. For incompletely sequenced cells, deletions and substitutions are included in the counts when that particular viral gene is sequenced. The major trends in Figure 5C are also true for the larger dataset in this figure. In addition, there is now a modest trend for infected cells that fail to express PB2 and PA *not* to express IFN.

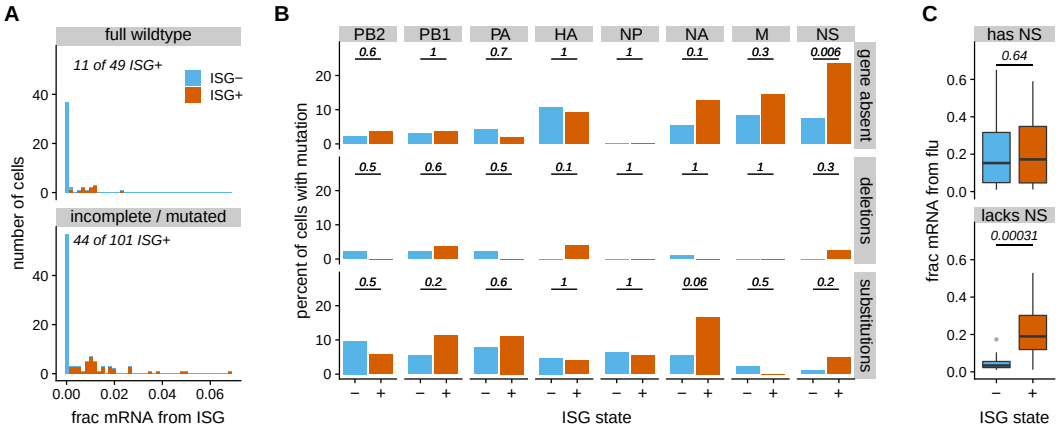

Figure 5–Figure supplement 2. This plot shows the same information as panels Figure 5B-D except that it shows the association of viral gene absence / mutations with ISG expression rather than IFN expression. Cells are classified as ISG+ or ISG- as in ***Figure 3–Figure Supplement 3***. The major viral features that associate with IFN induction also associate with ISG expression. Specifically, cells that express wild-type copies of all eight viral genes tend to express ISGs less frequently and at lower levels The absence of NS is strongly associated with higher ISG expression. Viral burden is not associated with ISG expression levels in NS-sufficient infections, but is in NS-deficient infections.

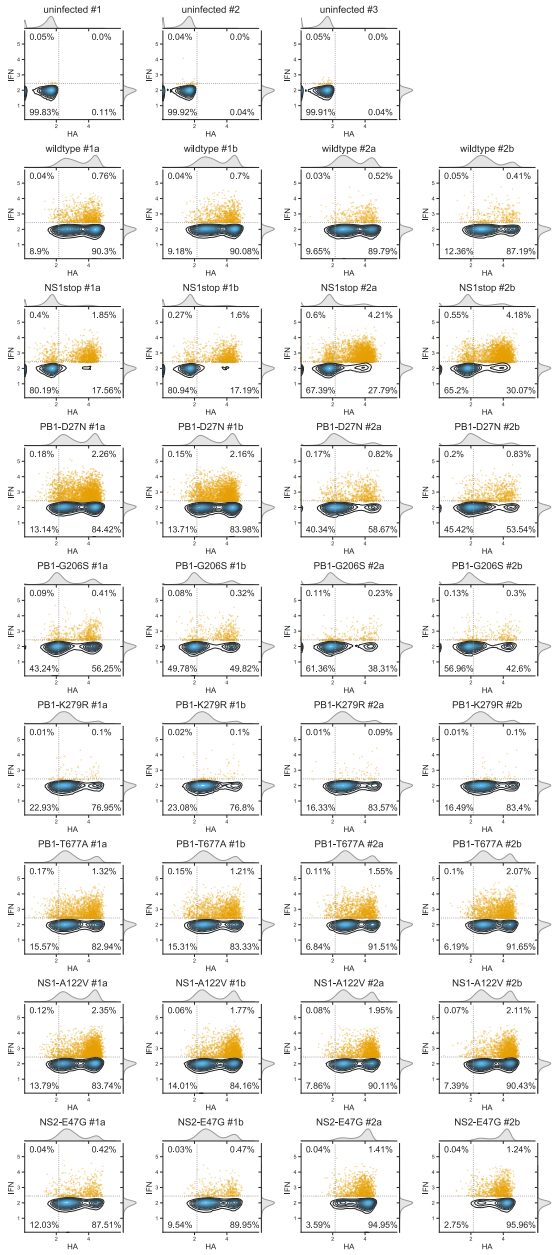

Figure 6–Figure supplement 1. A549 cells with the *IFNL1* reporter driving LNGFRΔC-ZsGreen were not infected (three replicates) or infected with stocks of the indicated viral mutant in quadruplicate. After 13 hours, cells were stained for expression of HA protein and analyzed by FACS for HA and the ZsGreen driven by the *IFNL1* reporter. The contour plots show the density of all cells, and IFN+ cells are also indicated by orange dots. Cells were classified as HA+ or IFN+ based on gates set to put 0.05% of the uninfected cells in these populations. For all influenza-infected cells, the percentage IFN+ was calculated only among the HA+ cells (since these are the ones that are infected). For the uninfected cells, the percentage IFN+ was calculated among all cells, since uninfected cells do not express HA. For each viral mutant, two independently generated stocks were each assayed in duplicate (i.e., #1a and #1b are replicates of one viral stock, and #2a and #2b are replicates of the other viral stock). The infections with replicate #1 of the wild-type virus were performed at an MOI of 0.1 as determined by TCID50, and all other viruses were infected at an equivalent particle number as determined by HI assay. The HA staining indicates that the number of transcriptionally active viral units per cell was similar across most mutants, and calculating percentage IFN+ only among HA+ cells largely corrects for modest variation in titer across mutants.

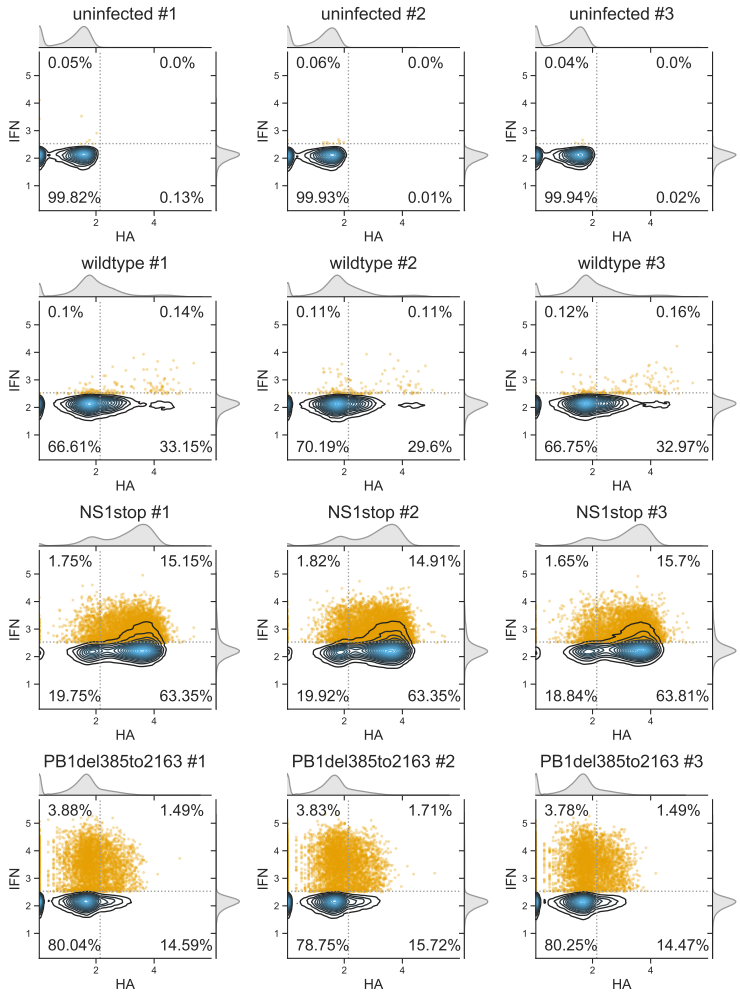

Figure 6–Figure supplement 2. This figure is similar to ***Figure 6–Figure Supplement 1*** except that it shows the flow cytometry data for **Figure 6**B. Each infection was performed in triplicate. A complicating factor for these data is that the virus with the deletion in PB1 (PB1del385to2163) cannot perform secondary transcription because it does not express PB1 protein, and so cannot be effectively normalized by HA expression since it expresses lower levels of HA due to the lack of secondary transcription. Therefore, for the data in this figure all cells were infected at an equivalent MOI of 0.3 as determined by TCID50 on MDCK-SIAT1 cells for the wild-type and NS1stop viruses, and on MDCK-SIAT1 cells expressing PB1 (***Bloom et al., 2010***) for the PB1del385to2163 virus. Finally, the approach in ***Figure 6–Figure Supplement 3*** was used to show that equivalent TCID50s, all viral variants had similar amounts of transcriptionally active virus in the absence of secondary transcription. All this normalization suggests that all infections were performed at a similar dose of particles active for primary transcription. Therefore, the percent IFN+ was calculated from the flow data in this figure for *all* cells (HA+ and HA-) since that is a more fair comparison for the PB1del385to2163 virus.

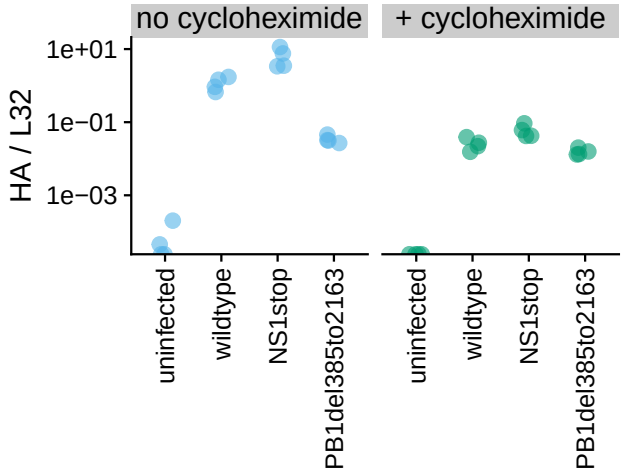

Figure 6–Figure supplement 3. Validation that the infections in **Figure 6**B and ***Figure 6–Figure Supplement 2*** were performed at similar doses of virions capable of initiating primary transcription. In this experiment, A549 cells were infected at MOI of 0.4 (based on TCID50 as described in ***Figure 6–Figure Supplement 2***), and then after 8 hours mRNA was harvested for qPCR on oligo-dT primed reverse transcription products. The y-axis shows the ratio of viral HA mRNA to the housekeeping gene L32. These infections were performed in the presence of absence of 50 μg/ml cycloheximide, which blocks protein synthesis and hence secondary transcription by newly synthesized viral proteins (***Killip et al., 2014***). In the absence of cycloheximide, the viruses with deletions in PB1 produced less viral mRNA presumably because they could not produce PB1 protein for secondary transcription. But in the presence of cycloheximide, all viruses produced similar amounts of viral mRNA, indicating that the dose of particles active for primary transcription is roughly equivalent across variants. Each measurement was performed in quadruplicate.