

# 1 Extreme heterogeneity of influenza 2 virus infection in single cells

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8 **Abstract** Viral infection can dramatically alter a cell's transcriptome. However, these changes  
9 have mostly been studied by bulk measurements on many cells. Here we use single-cell mRNA  
10 sequencing to examine the transcriptional consequences of influenza virus infection. We find  
11 extremely wide cell-to-cell variation in the productivity of viral transcription – viral transcripts  
12 comprise less than a percent of total mRNA in many infected cells, but a few cells derive over half  
13 their mRNA from virus. Some infected cells fail to express at least one viral gene, but this gene  
14 absence only partially explains variation in viral transcriptional load. Despite variation in viral load,  
15 the relative abundances of viral mRNAs are fairly consistent across infected cells. Activation of  
16 innate immune pathways is rare, but some cellular genes co-vary in abundance with the amount of  
17 viral mRNA. Overall, our results highlight the complexity of viral infection at the level of single cells.

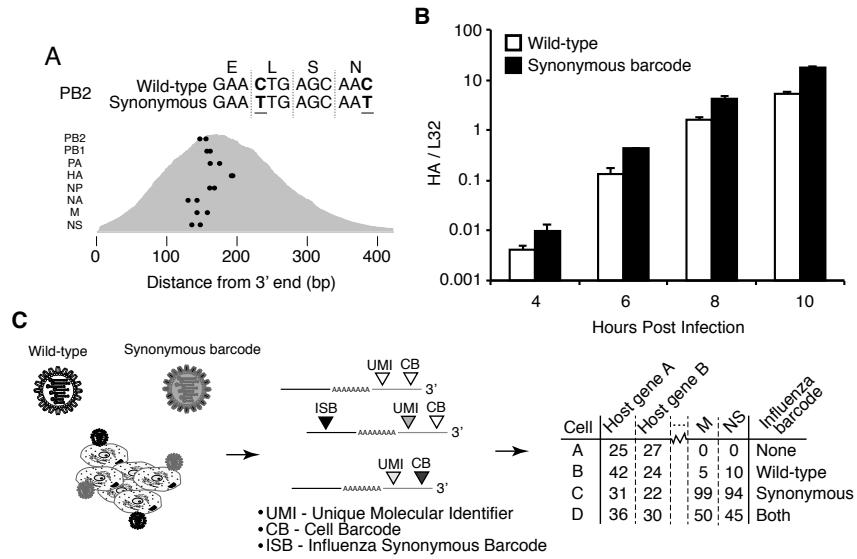
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## 19 Introduction

20 Viruses can cause massive and rapid changes in a cell's transcriptome as they churn out viral mRNAs  
21 and hijack cellular machinery. For instance, cells infected with influenza virus at high multiplicity  
22 of infection (MOI) express an average of 50,000 to 100,000 viral mRNAs per cell, corresponding  
23 to 5 to 25% of all cellular mRNA (Hatada et al., 1989). Infection can also trigger innate-immune  
24 sensors that induce the expression of cellular anti-viral genes (Killip et al., 2015; Iwasaki and Pillai,  
25 2014; Crotta et al., 2013). This anti-viral response is another prominent transcriptional signature of  
26 high-MOI influenza virus infection in bulk cells (Geiss et al., 2002).

27 However, initiation of an actual influenza infection typically involves just a few virions infecting  
28 a few cells (Varble et al., 2014; Poon et al., 2016; Leonard et al., 2017; McCrone et al., 2017). The  
29 dynamics of viral infection in these individual cells may not mirror bulk measurements made  
30 on many cells infected at high MOI. Over 70 years ago, Max Delbrück showed that there was a  
31 ~100-fold range in the number of progeny virions produced per cell by clonal bacteria infected  
32 with clonal bacteriophage (Delbrück, 1945). Subsequent work has shown similar heterogeneity  
33 during infection with other viruses (Zhu et al., 2009; Schulte and Andino, 2014; Combe et al., 2015;  
34 Akpinar et al., 2016), including influenza virus (Heldt et al., 2015).

35 In the case of influenza virus infection, targeted measurements of specific proteins or RNAs  
36 have shed light on some factors that contribute to cell-to-cell heterogeneity. The influenza virus  
37 genome consists of eight negative-sense RNA segments, and many infected cells fail to express  
38 one more of these RNAs (Heldt et al., 2015; Dou et al., 2017) or their encoded proteins (Brooke  
39 et al., 2013). In addition, activation of innate-immune responses is inherently stochastic (Shalek  
40 et al., 2013, 2014; Bhushal et al., 2017; Hagai et al., 2017), and only some influenza-infected cells  
41 express anti-viral interferon genes (Perez-Cidoncha et al., 2014; Killip et al., 2017). However, the  
42 extent of cell-to-cell variation in these and other host and viral factors remains unclear, as does the



**Figure 1.** Experimental design. **(A)** We engineered a virus that carried two synonymous mutations near the 3' end of each mRNA. At top are the mutations for PB2. At bottom are locations of the synonymous mutations relative to the typical distribution of read depth for our 3'-end sequencing. **(B)** The wild-type and synonymously barcoded viruses transcribe their genes with similar kinetics. The abundance of the viral hemagglutinin (HA) transcript relative to the cellular housekeeping gene L32 was assessed by qPCR in A549 cells infected at an MOI of 0.5 (as determined on MDCK-SIAT1 cells). Error bars  $\pm$  S.D., n=3. **(C)** For the single-cell mRNA sequencing, A549 cells were infected with an equal mixture of wild-type and synonymously barcoded virus. Immediately prior to collection, cells were physically separated into droplets and cDNA libraries were generated containing the indicated barcodes. The libraries were deep sequenced, and the data processed to create a matrix that gives the number of molecules of each transcript observed in each cell. Infected cells were further annotated by whether their viral mRNAs derived from wild-type virus, synonymously barcoded virus, or both.

**Figure 1-source data 1.** Sequences of wild-type and barcoded viruses are in [viralsequences.fasta](#).

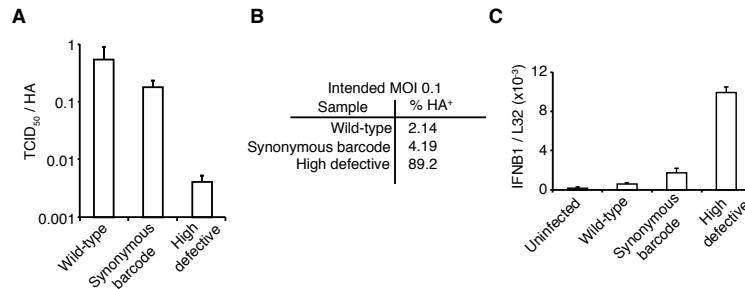
43 association among them in individual infected cells.

44 Here we use single-cell mRNA sequencing to quantify the levels of all cellular and viral mRNAs  
45 in cells infected with influenza virus at low MOI. We find extremely large variation in the amount  
46 of viral mRNA expressed in individual cells. Both co-infection and activation of innate-immune  
47 pathways are rare in our low-MOI infections, and do not appear to be the major drivers of cell-  
48 to-cell heterogeneity in viral transcriptional load. Individual infected cells often fail to express  
49 specific viral genes, and such gene absence explains some but certainly not all of the cell-to-cell  
50 heterogeneity. A variety of cellular genes, including ones involved in the oxidative-stress response,  
51 co-vary with viral transcriptional load. Overall, our work demonstrates remarkable heterogeneity in  
52 the transcriptional outcome of influenza virus infection among nominally identical cells infected  
53 with a relatively pure population of virions.

## 54 Results

### 55 Strategy to measure mRNA in single virus-infected cells.

56 We performed single-cell mRNA sequencing using a droplet-based system that physically isolates  
57 individual cells prior to reverse transcription ([Zheng et al., 2017; Macosko et al., 2015; Klein et al., 2015](#)).  
58 Each droplet contains primers with a unique *cell barcode* that tags all mRNAs from that  
59 droplet during reverse-transcription. Each primer also contains a *unique molecular identifier (UMI)*  
60 that is appended to each mRNA molecule during reverse transcription. The 3' ends of the mRNAs are  
61 sequenced and mapped to the human and influenza virus transcriptomes to determine transcript  
62 identities. This information is combined with that provided by the UMIs and cell barcodes to



**Figure 2.** The viral stocks in our experiments are relatively pure of defective particles. **(A)** Our viral stocks have a higher ratio of infectious particles to HA virion RNA compared to a high-defective stock propagated at high MOI. HA viral RNA was quantified by qPCR on virions. Error bars  $\pm$  S.D., n=6 (qPCR replicates). **(B)** Our viral stocks have a higher ratio of infectious particles to particles capable of expressing HA protein. A549 cells were infected at an MOI of 0.1, and the percentage of cells expressing HA protein at 9 hours post-infection was quantified by antibody staining and flow cytometry. **(C)** Our viral stocks are less immunostimulatory than virus propagated at high MOI. Measurements of *IFNB1* transcript by qPCR normalized to the housekeeping gene L32 in A549 cells at 10 hours post infection at an MOI of 0.5. Error bars  $\pm$  S.D., n=3. Note that MOIs were calculated by TCID50 on MDCK-SIAT1 cells, whereas the experiments in this figure involved infection of A549 cells.

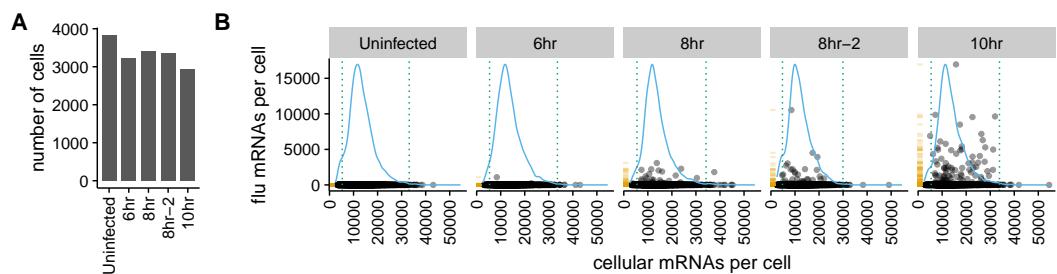
**Figure 2-Figure supplement 1.** Full flow cytometry data for panel B .

63 quantify the number of molecules of each mRNA species that have been captured for each cell.  
 64 Infected cells will express viral as well as cellular mRNAs – however the cell barcodes and UMs  
 65 cannot distinguish whether a cell was initially infected by one or multiple viral particles. We therefore  
 66 engineered an influenza virus (strain A/WSN/1933) that additionally carried *viral barcodes* consisting  
 67 of synonymous mutations near the 3' end of each transcript (Figure 1A). Critically, these synonymous  
 68 mutations did not greatly impact viral growth kinetics (Figure 1B). We infected A549 human lung  
 69 carcinoma cells with an equal mix of the wild-type and synonymously barcoded viruses. Cells  
 70 infected by a single virion will exclusively express mRNAs from either wild-type or synonymously  
 71 barcoded virus, whereas cells that are co-infected with multiple virions will often express mRNAs  
 72 from both the wild-type and synonymously barcoded viruses (Figure 1C).

73 We took care to generate stocks of virus that were relatively “pure” of defective particles.  
 74 Stocks of viruses typically contain an array of biologically active viral particles, some of which are  
 75 defective for replication owing to mutations or deletions in essential viral genes (*von Magnus, 1954;*  
*Huang et al., 1970; Brooke, 2014; Fonville et al., 2015; Lauring and Andino, 2010; Dimmock et al., 2014; Saira et al., 2013*). These defective particles become prevalent when a virus is grown at  
 76 high MOI, where complementation permits the growth of otherwise deleterious genotypes. To  
 77 minimize the levels of defective particles, we propagated our viral stocks at low MOI for a relatively  
 78 brief period of time (*Xue et al., 2016*). We validated that our stocks exhibited greater purity of  
 79 infectious particles than a stock propagated at high MOI by verifying that they had a higher ratio  
 80 of infectious particles to virion RNA (Figure 2A) and to particles capable of inducing expression of  
 81 a single viral protein (Figure 2B). In addition, viral stocks with many defective particles are more  
 82 immunostimulatory (*Tapia et al., 2013; Lopez, 2014*). We confirmed that our viral stocks induced  
 83 less interferon than a stock propagated at higher MOI (Figure 2C).

#### 86 Single cells show an extremely wide range of expression of viral mRNA.

87 We infected A549 cells at low MOI with a mixture of the wild-type and synonymously barcoded  
 88 viruses, and collected cells for sequencing at 6, 8, and 10 hours post-infection, including a replicate  
 89 a 8-hours. We replaced the infection inoculum with fresh media at one-hour post-infection, thereby  
 90 ensuring that most infection was initiated during a narrow time window. The exception was the  
 91 replicate 8-hour sample, for which we did *not* perform this media change and instead left the



**Figure 3.** There is a very wide distribution in the amount of viral mRNA per cell. **(A)** Number of cells sequenced for each sample. **(B)** The number of cellular and viral mRNAs detected for each cell is plotted as a point. The blue lines show the overall distribution of the number of cellular mRNAs per cell. The orange rug plot at the left of each panel shows the distribution of the number of viral mRNAs per cell. Cells outside the dotted green lines were considered outliers with suspiciously low or high amounts of cellular mRNA (possibly derived from two cells per droplet), and were excluded from all subsequent analyses. Figure 3-Figure supplement 1 shows the exact distributions of the fraction of viral mRNA per cell.

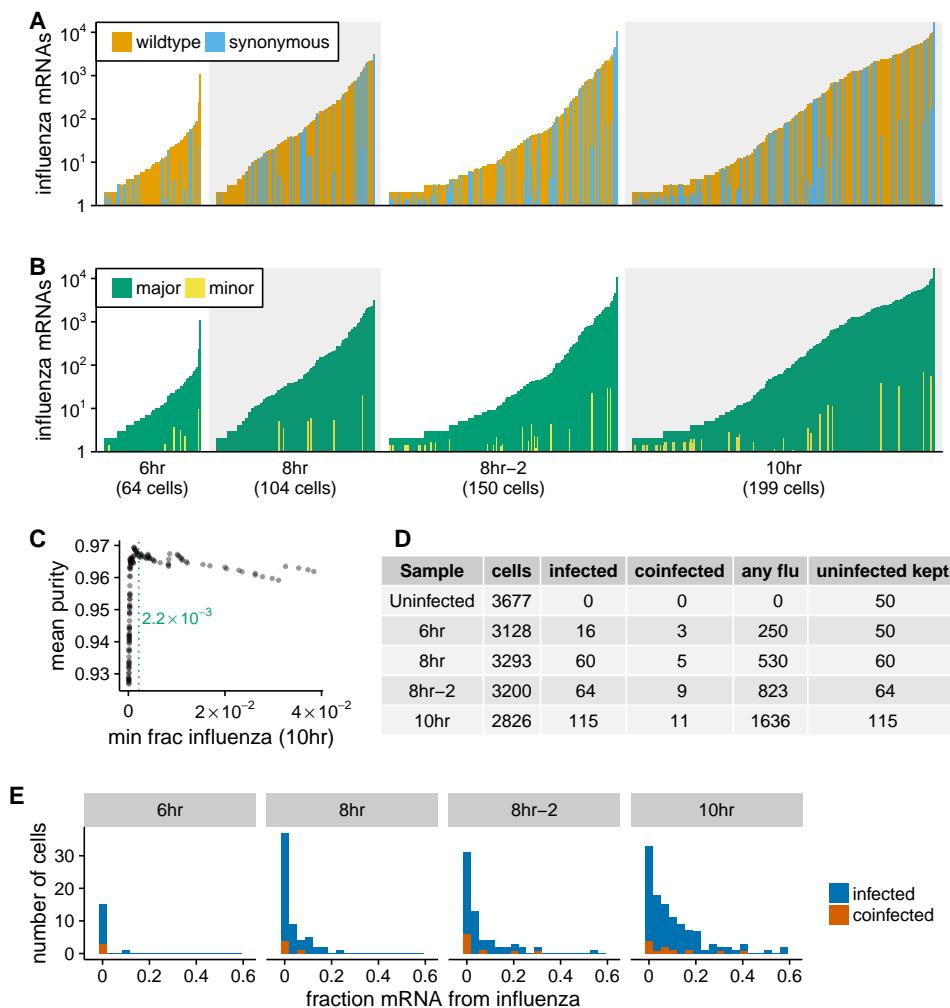
**Figure 3-Figure supplement 1.** Cumulative fraction plot of proportion of total mRNA from virus.

92 cells in the original infection inoculum. We recovered between 3,000 and 4,000 cells for each  
93 sample (Figure 3A). As expected for a low-MOI infection, most cells expressed little or no viral mRNA  
94 (Figure 3B and Figure 3-Figure supplement 1). Also as expected, the amount of viral mRNA per cell  
95 among infected cells increased over time (Figure 3B and Figure 3-Figure supplement 1). But what  
96 was most notable was how widely the number of viral mRNA molecules varied among infected cells.  
97 While the fraction of mRNA derived from virus was <0.1% for most cells, viral mRNA constituted  
98 half the transcriptome in a few cells at 8 and 10 hours (Figure 3B).

99 A complicating factor is that uninfected cells could have small amounts of viral mRNA due  
100 to leakage of transcripts from lysed cells. It is therefore important to establish a threshold for  
101 identifying truly infected cells. We can do this by taking advantage of the fact that roughly half the  
102 infecting virions bear synonymous barcodes. Reads derived from lysed cells will be drawn from  
103 both wild-type and synonymous barcoded viral transcripts. However, most cells are infected by at  
104 most one virion, and so the reads from truly infected cells will usually derive almost entirely from  
105 one of the two viral variants. Figure 4A shows the fraction of viral reads in individual cells from each  
106 viral variant, and Figure 4B indicates the fraction of viral reads from the most abundant variant in  
107 that cell. Most cells with large amounts of viral mRNA have viral transcripts exclusively derived from  
108 one viral variant – indicating non-random partitioning as expected from viral infection. However,  
109 cells with a small amount of viral mRNA often have viral transcripts from both variants, as expected  
110 from the random partitioning associated with simple mRNA leakage. Finally, a few cells with large  
111 amounts of viral mRNA have viral transcripts from both variants, likely reflecting co-infection.

112 We determined the threshold amount of viral mRNA per cell at which the barcode partitioning  
113 clearly resulted from infection rather than leakage (Figure 4C), and used this threshold to annotate  
114 cells that we were confident were truly infected. We also annotated as co-infected cells above this  
115 threshold that had mRNA from both viral variants. Figure 4D shows the number of cells annotated  
116 as infected and co-infected for each sample – these cells are just a small fraction of the number of  
117 cells with any viral read. These annotation thresholds are conservative, and will miss some true  
118 low-level infections, as well as any co-infections with the same viral variant. However, it is important  
119 that the analyses below are restricted to cells that are truly infected with virus, so we accepted  
120 the loss of some low-level infections in order to avoid false positives. Because most cells are not  
121 infected, we subsampled the uninfected cells to the numbers shown in Figure 4D to balance the  
122 proportions of infected and uninfected cells for all subsequent analyses.

123 Strikingly, the extreme variation in the number of viral transcripts per cell remains even after we  
124 apply these rigorous criteria for annotating infected cells (Figure 4E). The fraction of viral mRNA per  
125 infected cell follows a roughly exponential distribution, with many cells having few viral transcripts



**Figure 4.** Synonymous barcodes on the viral mRNAs distinguish true infections from cells that contain viral mRNAs derived from leakage of lysed cells. **(A)** Cells with at least two viral mRNAs for which the barcode could be called, arranged in order of increasing influenza transcript counts. Bar heights denote the number viral mRNAs on a  $\log_{10}$  scale, bar coloring is linearly proportional to the fractions of viral mRNAs derived from wild-type and synonymously barcoded virus. **(B)** Same as (A), but each bar is colored according to the relative fraction of the more common (major) and less common (minor) virus variant. At low levels of viral mRNA there is often a roughly equal mix, suggesting contamination with viral mRNAs leaked from lysed cells. At higher levels of viral mRNA, cells generally have only one viral variant, suggesting infection initiated by a single virion. A few cells are also obviously co-infected with both viral variants. **(C)** We determined a cutoff for calling “true” infections by finding the amount of viral mRNA per cell at which the viral barcode purity no longer increases with more viral mRNA. The purity is the fraction of all viral mRNA in a cell derived from the most abundant viral barcode in that cell. We fit a curve to the mean purity of all cells with more than the indicated amount of viral mRNA, and drew the cutoff at the point where this curve stopped increasing with the fraction of total mRNA derived from virus. See the Methods for details. **(D)** The number of cells identified as infected and co-infected for each sample, as well as the number of cells with any viral read. For all subsequent analyses, we subsampled the number of uninfected cells per sample to the greater of 50 or the number of infected cells. **(E)** Distribution of the fraction of mRNA per cell derived from virus for both infected and co-infected cells.

**Figure 4-Figure supplement 1.** Number of viral barcodes called.

**Figure 4-Figure supplement 2.** Fraction of total viral mRNA derived from a given fraction of infected cells.

**Figure 4-Figure supplement 3.** Flow cytometry analysis of viral protein production of cells contingent on infectious dose or coinfection state.

126 and a few cells having many. At 6 and 8 hours <10% of infected cells are responsible for over half  
 127 the viral transcripts, while at 10 hours ≈15% of infected cells produce over half the viral transcripts

128 (Figure 4-Figure supplement 2). Notably, co-infected cells do not express dramatically more viral  
 129 mRNA than other cells (Figure 4-Figure supplement 3), suggesting that the initial infectious dose is  
 130 not the major driver of cell-to-cell variation in our low-MOI infections.

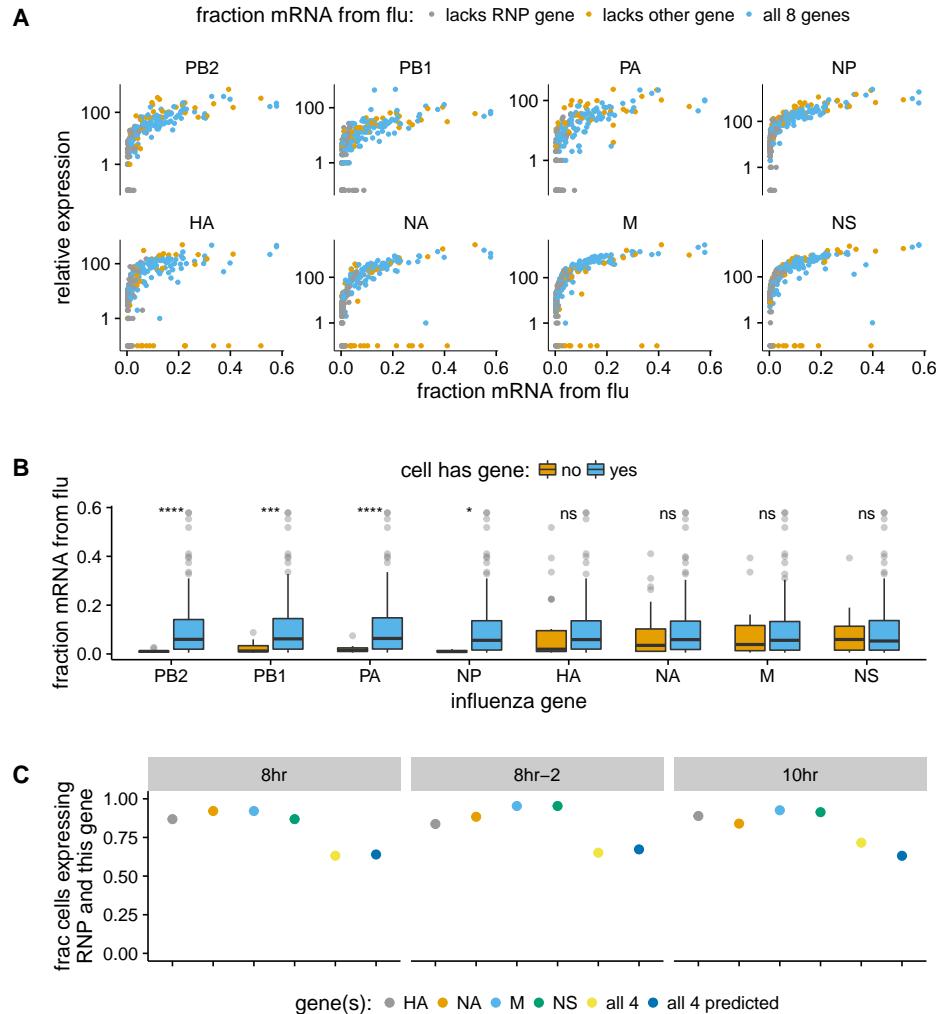
131 **Absence of viral genes partially explains cell-to-cell variability in viral load.**

132 The influenza genome is segmented, and cells can fail to express a viral mRNA if the encoding  
 133 gene segment is not packaged in the infecting virion or fails to initiate transcription after infection.  
 134 Indeed, several groups have reported that the majority of infected cells fail to express at least  
 135 one viral gene (*Brooke et al., 2013; Heldt et al., 2015; Dou et al., 2017*). We wondered if the  
 136 absence of specific viral genes might be associated with reduced amounts of viral mRNA within  
 137 single infected cells. In particular, transcription of influenza virus mRNAs is performed by the viral  
 138 ribonucleoprotein (RNP) complex, which consists of the three proteins that encode the tripartite  
 139 polymerase (PB2, PB1, and PA) as well as nucleoprotein (NP) (*Huang et al., 1990*). Each viral gene  
 140 segment is associated with one RNP in incoming infecting virions, but secondary transcription by  
 141 newly synthesized RNPs requires the presence of the viral genes encoding each of the four RNP  
 142 proteins (*Vreede et al., 2004; Eisfeld et al., 2015*). This secondary transcription is a major source  
 143 of viral mRNAs, as evidenced by the fact that blocking synthesis of the RNP proteins reduces the  
 144 amount of viral mRNA by several orders of magnitude in bulk cells (Figure 5-Figure supplement 1).

145 We examined the total amount of viral mRNA versus the expression of the genes from each viral  
 146 segment (Figure 5A, Figure 5-Figure supplement 2). Note that influenza virus expresses ten major  
 147 gene transcripts from its eight gene segments, as the M and NS segments are alternatively spliced  
 148 to produce the M1 / M2 and NS1 / NEP transcript, respectively (*Dubois et al., 2014*). However, an  
 149 inherent limitation of current established single-cell mRNA sequencing techniques is that they only  
 150 sequence the 3' end of the transcript (*Zheng et al., 2017; Macosko et al., 2015; Klein et al., 2015;*  
 151 *Cao et al., 2017*). Since the alternative spliceoforms M1 / M2 and NS1 / NEP share the same 3' ends,  
 152 we cannot distinguish them and therefore will refer simply to the combined counts of transcripts  
 153 from each of these alternatively spliced segments as the M and NS genes.

154 Cells that lack an RNP gene never derive more than a few percent of their mRNAs from virus,  
 155 confirming the expected result that all four RNP genes are essential for high levels of viral transcrip-  
 156 tion (Figure 5A, Figure 5-Figure supplement 2). However, we observe cells that lack each of the other  
 157 non-RNP genes but still derive  $\approx$ 40% of their mRNAs from virus, suggesting that none of the other  
 158 genes are important for high levels of viral transcription. These results are statistically supported by  
 159 Figure 5B, which shows that absence of any RNP gene but *not* any other viral gene is associated  
 160 with reduced amounts of viral mRNA. However, gene absence clearly does not explain all of the  
 161 variability in viral gene expression, since even cells expressing all viral genes exhibit a very wide  
 162 distribution in the amount of viral mRNA that they express. Specifically, at both 8 and 10 hours, the  
 163 amount of viral mRNA in individual cells expressing all eight viral genes still ranges from <1% to  
 164 >50% (Figure 5A, Figure 5-Figure supplement 2).

165 We also quantified the fraction of infected cells that completely failed to express a given gene. We  
 166 limited this analysis to examining the presence / absence of the non-RNP genes in cells expressing  
 167 all four RNP genes, since we might fail to detect viral transcripts that are actually present at low  
 168 levels in RNP-deficient cells due to the lower viral burden in these cells. At the 8- and 10-hour time  
 169 points, between 5% and 17% of cells fail to express any one of the four non-RNP genes (Figure 5C).  
 170 The absence of a given gene appears to be an independent event, as the probability of observing  
 171 all four non-RNP genes in a cell is well predicted by simply multiplying the probabilities of observing  
 172 each gene individually (Figure 5C). If we extrapolate the frequencies at which cells lack non-RNP  
 173 genes to the RNP genes, then we would predict that 40-50% of infected cells express mRNAs from all eight  
 174 gene segments. This estimate of the frequency at which infected cells express mRNAs from all eight  
 175 gene segments is slightly higher than previous estimates of 13% (*Brooke et al., 2013*) and 20% (*Dou*  
 176 *et al., 2017*). At least one difference is that *Brooke et al. (2013)* stained for proteins whereas we  
 177 examined the expression of mRNAs – it is likely that some cells contain mutated viral genes that fail



**Figure 5.** The absence of viral genes explains some of the variability in the amount of viral mRNA per cell. **(A)** The normalized expression of each viral gene as a function of the total fraction of mRNA in each infected cell derived from virus, taken over all time points. Cells with high viral burden always express all RNP genes, but some cells with high viral burden lack each of the other genes. **(B)** Box and whisker plots showing the per-cell viral burden among cells with >0.5% of their mRNA from virus, binned by whether or not the cells express each gene. A Wilcoxon signed-rank test was used to test the null hypothesis that absence of each gene does not affect viral burden: \*\*\*\* =  $P < 10^{-4}$ , \*\*\* =  $P < 10^{-3}$ , \* =  $P < 0.05$ , ns = not significant. **(C)** The fraction of cells that express each of the four other genes among cells that express all RNP genes, as well as the fraction that express *all* four of the other genes. The fraction that express all four genes is well predicted by simply multiplying the frequencies of cells that express each gene individually, indicating that gene absence is approximately independent across these genes.

**Figure 5-Figure supplement 1.** Secondary transcription is a major source of viral mRNA during bulk infections.

**Figure 5-Figure supplement 2.** Like panel (A), but shows samples individually.

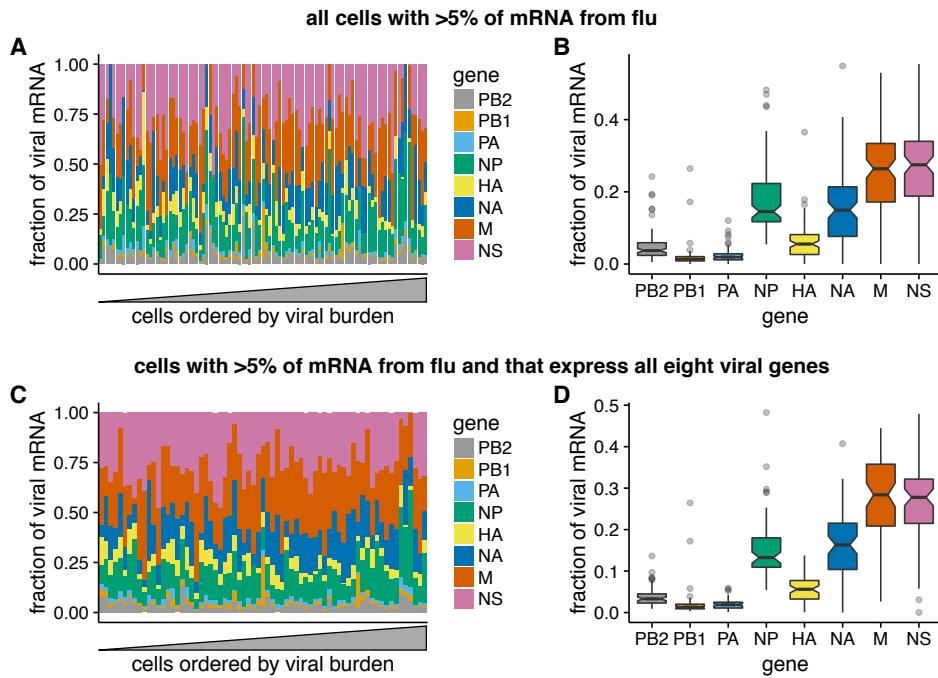
**Figure 5-Figure supplement 3.** Like panel (B) but for the 10-hr sample only.

**Figure 5-source data 1.** The numerical data for panel (C) are in `p_missing_genes.csv`.

178 to produce stable protein even when mRNA is expressed.

### 179 The relative amounts of different viral mRNAs are more consistent across cells.

180 The results above show that the amount of viral mRNA in infected cells varies over several orders  
181 of magnitude. Does the relative expression of viral genes exhibit similar cell-to-cell variability?



**Figure 6.** Relative expression of influenza virus genes in highly infected cells (>5% of total mRNA from virus). **(A)** The fraction of viral mRNA from each viral gene for each cell. **(B)** Box plots showing the distribution of the fraction of viral mRNA per cell from each viral gene. The black lines at the notches are the medians, and the tops and bottoms of boxes indicate the first and third quartiles. Whiskers extend to the highest or lowest data point observed within 1.5x the interquartile range, outliers shown as circles. Notches extend 1.58x the interquartile range divided by the square root of the number of observations. **(C), (D)** The same plots, but only including cells for which we observed at least one molecule of each viral gene.

**Figure 6-source data 1.** The raw data for all cells are in `p_flu_expr_all.csv`.

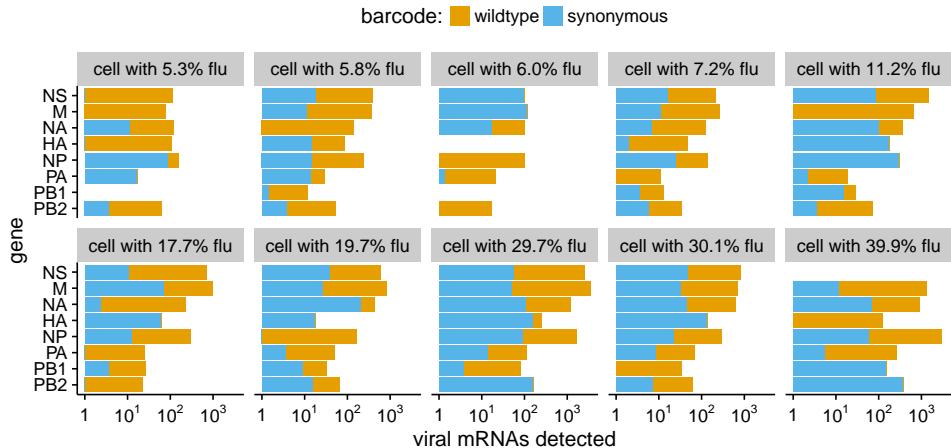
**Figure 6-source data 2.** The raw data for fully infected cells are in `p_flu_expr_fullyinfected.csv`.

182 To address this question, we focused on cells that derived >5% of their mRNA from virus, since  
183 estimates of relative viral gene expression will be less noisy in cells with more viral mRNAs.

184 In contrast to the extreme variability in the total viral mRNA per cell, the fraction of this mRNA  
185 derived from each gene is much more consistent across cells (Figure 6A). Total viral mRNA varies by  
186 orders of magnitude, but the fraction from any given viral gene is fairly tightly clustered around the  
187 median value for all cells (Figure 6B). The relative levels of each viral mRNA in our cells are similar  
188 to prior bulk measurements made by Northern blots (*Hatada et al., 1989*), which also found an  
189 expression hierarchy of M > NS > NP > NA > HA > PB2 ~ PA. The cell-to-cell consistency in  
190 the relative expression of different viral genes is even tighter if we limit the analysis only to cells  
191 that express all eight viral genes (Figure 6C,D). Therefore, with the exception of complete gene  
192 absence, the factors that drive the dramatic cell-to-cell variability in the amount of viral mRNA have  
193 roughly similar effects on all viral genes in a given cell. This finding is consistent with prior work  
194 showing positive correlations among the abundance of several viral genome segments in individual  
195 cells (*Heidt et al., 2015*).

#### 196 **Co-infection can provide infected cells with the full complement of viral genes.**

197 Our sequencing enables us to identify the rare cells that were co-infected with both wild-type and  
198 synonymously barcoded viral variants. Overall, we captured 10 such co-infected cells that had >5%  
199 of their mRNA derived from virus (Figure 7). Seven of these 10 cells expressed all eight viral genes.  
200 Remarkably, the majority (4 of 7) of these cells would *not* have expressed all the viral genes in the  
201 absence of co-infection, since they have at least one gene exclusively derived from each viral variant.



**Figure 7.** The abundance of each viral transcript in cells that are co-infected with the two viral variants and have >5% of their mRNA derived from virus. The bars show the logarithms of the numbers of each viral mRNA detected, and are colored in linear proportion to the fraction of that mRNAs derived from wild-type or synonymously barcoded virus.

**Figure 7-Figure supplement 1.** Co-infected cells express roughly equal amounts of a gene from each infecting viral variant.

**Figure 7-source data 1.** The raw data plotted in this figure are in `p_co-infection.csv`.

**Figure 7-source data 2.** The sequence of the HA viral RNA carrying the GFP gene is in `HAflank-eGFP.fasta`.

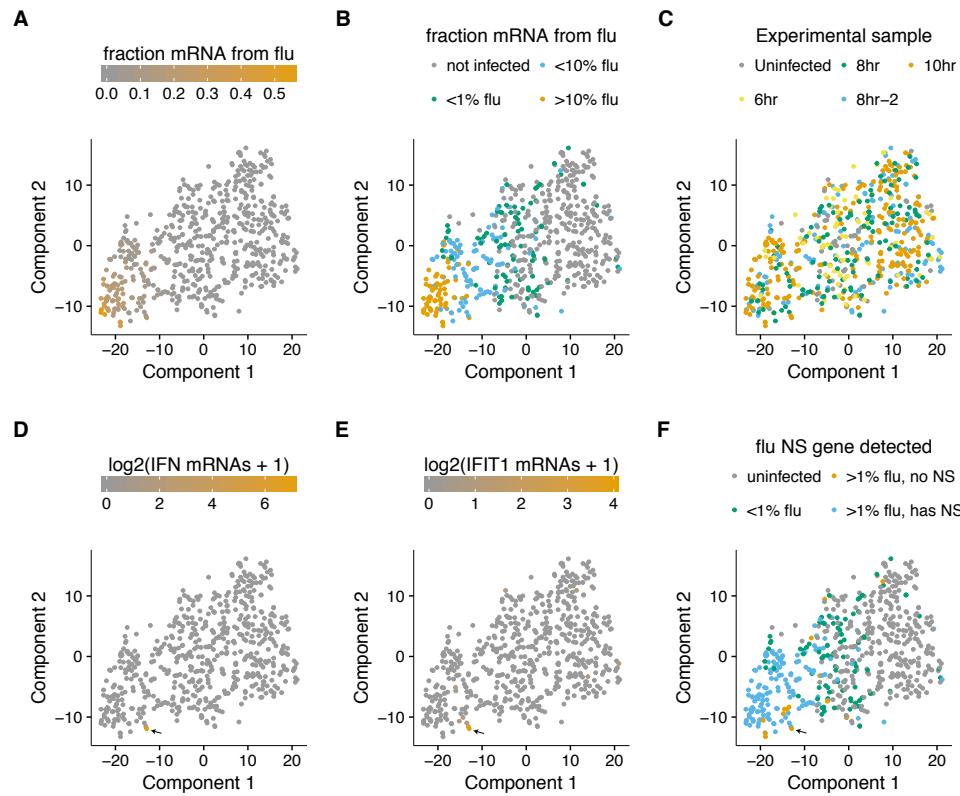
202 For instance, the cell with 11.2% of its mRNA from virus in the upper right of Figure 7 expresses  
 203 M only from the wildtype viral variant, and NP and HA only from the synonymously barcoded  
 204 variant. Our data therefore provide direct single-cell support for the idea that co-infection can  
 205 rescue missing viral genes (*Brooke et al., 2013, 2014; Fonville et al., 2015; Aguilera et al., 2017*).

206 Another observation from Figure 7 is that co-infected cells usually express roughly equal  
 207 amounts of transcripts from each of the two viral variants. This observation is consistent with the  
 208 finding by *Dou et al. (2017)* and *Huang et al. (2008)* that the temporal window for co-infection is  
 209 short – if both viral variants infect a cell at about the same time, then neither will have a headstart  
 210 and so each will have a roughly equal opportunity to transcribe its genes.

211 To support this idea with a larger dataset albeit at lower resolution, we generated a virus  
 212 in which the HA coding sequence was replaced by GFP. We then co-infected cells with a mix of  
 213 wildtype and ΔHA-GFP virus and used flow cytometry to score cells for the presence of HA only  
 214 (infection by wildtype virus), GFP only (infection by ΔHA-GFP virus), or both (co-infection) as shown  
 215 in Figure 7 Figure supplement 1. As in our single-cell sequencing data, we found that expression  
 216 of HA and GFP were highly correlated, indicating that co-infected cells typically expressed roughly  
 217 equal amounts of transcript from each viral variant.

### 218 Activation of the interferon response is rare in single infected cells.

219 Because our sequencing captured all polyadenylated transcripts, we can examine whether there  
 220 are prominent changes in the host-cell transcriptome in sub-populations of infected cells. Influenza  
 221 virus infection can trigger innate-immune sensors that lead to the transcriptional induction of  
 222 type I and III interferons, and subsequently of anti-viral interferon-stimulated genes (*Killip et al.,  
 223 2015; Iwasaki and Pillai, 2014; Crotta et al., 2013*). However, activation of the interferon response  
 224 is stochastic and bi-modal at the level of single cells (*Chen et al., 2010; Shalek et al., 2013, 2014;  
 225 Perez-Cidoncha et al., 2014; Bhushal et al., 2017; Hagai et al., 2017*). We therefore hypothesized  
 226 that we might see two sub-populations of infected cells: one in which the interferon response  
 227 inhibited viral transcription, and another in which the virus was able to express high levels of its  
 228 mRNA by evading or blocking this response.



**Figure 8.** A t-SNE plot created by semi-supervised clustering using genes that co-vary with viral infection status. Each point is a single cell, and each panel shows an identical layout but colors the cells according to a different property. **(A), (B)** Cells colored by the fraction of their mRNA derived from virus. **(C)** Cells colored by the experimental sample. **(D), (E)** Cells colored by the number of detected transcripts from type I and III interferons (IFN). Only one cell has detectable interferon expression (in orange, indicated with arrow). **(F)** Cells colored by the expression of the interferon-stimulated gene IFIT1. The one interferon-positive cell is lacking NS, but so are many interferon-negative cells.

229 To examine whether there were distinct sub-populations of virus-infected cells, we used a  
 230 semi-supervised t-SNE approach (*Van der Maaten and Hinton, 2008*) to cluster cells by genes that  
 231 co-varied with viral infection status. As shown in Figure 8A,B, this approach effectively grouped cells  
 232 by the amount of viral mRNA that they expressed. Sample-to-sample variation was regressed away  
 233 during the clustering, as cells did not obviously group by time-point, with expected exception that  
 234 the uninfected and 6-hour samples had few cells in the region of the plot corresponding to large  
 235 amounts of viral mRNA (Figure 8C).

236 But to our surprise, we did not see a prominent clustering of infected cells into sub-populations  
 237 as expected if the interferon response was strongly activated in some cells. To investigate fur-  
 238 ther, we annotated each cell by the total number of type I and III interferon transcripts detected.  
 239 Remarkably, only a single cell expressed detectable interferon (Figure 8D). We also examined  
 240 interferon-stimulated genes, which are induced by autocrine and paracrine interferon signaling.  
 241 Figure 8E shows expression of one such gene, IFIT1 (*Fensterl and Sen, 2011*). As with interferon  
 242 itself, expression of IFIT1 was rare and most prominent in the single interferon-positive cell, pre-  
 243 sumably due to the higher efficiency of autocrine versus paracrine signaling. Notably, interferon  
 244 and interferon-stimulated genes were also relatively ineffective at blocking viral transcription in the  
 245 single cell in which they were potently induced, since >10% of the mRNA in this cell was derived  
 246 from virus (Figure 8A,B,D,E).

247 We posited that the paucity of interferon induction might be due to the activity of influenza

248 virus's major interferon antagonist, the NS1 protein (*García-Sastre et al., 1998; Hale et al., 2008*).  
 249 We therefore identified cells that expressed substantial amounts of viral mRNA but lacked the  
 250 NS gene (Figure 8F). Consistent with the idea that NS1 is important for suppressing interferon,  
 251 the one interferon-positive cell lacked detectable expression of the NS gene. But other cells that  
 252 lacked NS expression still failed to induce a detectable interferon response, despite often having a  
 253 substantial amount of their mRNA derived from virus (Figure 8). This result is in line with other work  
 254 showing that NS1-deficient influenza virus does not deterministically induce interferon (*Killip et al.,  
 255 2017; Kallfass et al., 2013*). Therefore, many individual infected cells fail to activate innate-immune  
 256 responses even when the virus lacks its major interferon antagonist.

### 257 **Some host genes co-vary with viral gene expression.**

258 We examined whether any host genes were differentially expressed in cells with more viral mRNA.  
 259 We restricted this analysis to infected cells with all eight viral genes in order to focus on cellular  
 260 genes that were associated with viral mRNA burden independent of effects due to the presence or  
 261 absence of particular viral transcripts. We identified 45 cellular genes that co-varied with viral gene  
 262 expression at a false discovery rate of 0.1 (Figure 9).

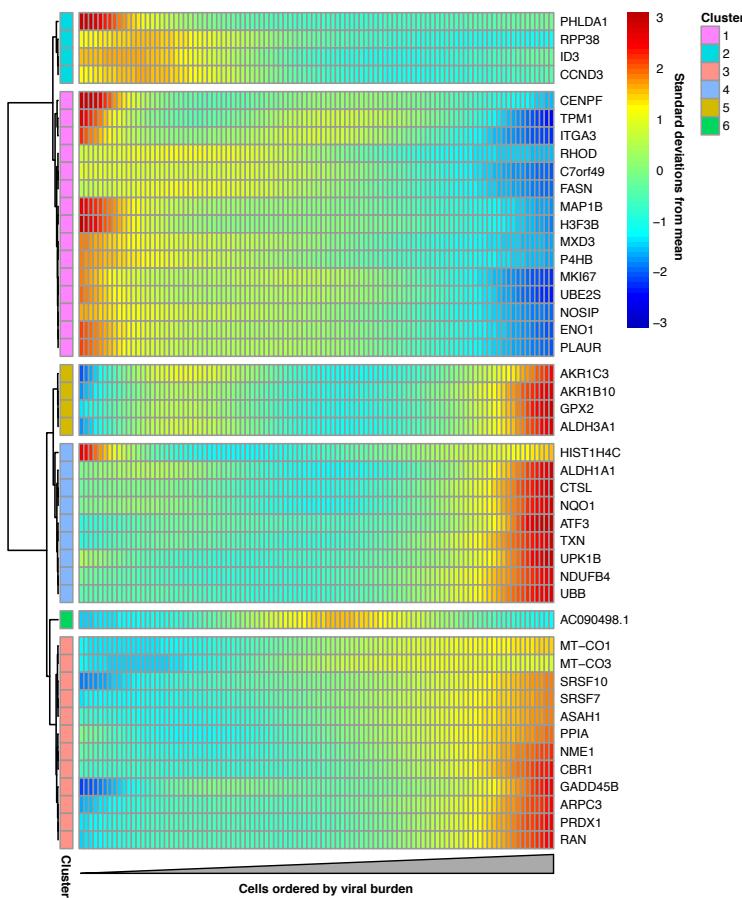
263 Many of the genes with increased expression in cells with more viral mRNA are known or  
 264 suspected to be regulated by the Nrf2 master regulator in response to oxidative stress. These genes  
 265 produce proteins that are involved in detoxification of reactive oxygen species or resultant products,  
 266 the management of misfolded proteins, the electron transport chain, or a general stress response  
 267 (Figure 9-Figure supplement 1). We additionally see reduced expression of the nitric oxide synthase  
 268 interacting protein (NOSIP). Transient oxidative stress is known to occur during viral infection, and  
 269 may act in a proviral fashion via MAPK activation driving vRNP export (*Amatore et al., 2014*). The  
 270 antioxidant response is thought to be largely antiviral, potentially through inhibition of MAPK activity  
 271 (*Lin et al., 2016; Sgarbanti et al., 2014*). Our data do not reveal whether the expression of genes  
 272 involved in the response to oxidative stress are a cause or a symptom of higher levels of viral mRNA,  
 273 and further investigation of this topic is an interesting area for future work.

## 274 **Discussion**

275 We have quantified the total transcriptome composition of single cells infected with influenza virus.  
 276 While we observe a general increase in the amount of viral mRNA over time as expected from  
 277 bulk measurements (*Hatada et al., 1989; Shapiro et al., 1987*), there is wide variation in viral gene  
 278 expression among individual infected cells.

279 The most obvious form of heterogeneity is the complete failure of some infected cells to express  
 280 one or more viral genes, which we estimate occurs in about half the infected cells in our experiments.  
 281 The absence of some viral genes in some infected cells has been noted previously (*Brooke et al.,  
 282 2013; Heldt et al., 2015; Dou et al., 2017*), and our work provides a holistic view by quantifying the  
 283 total viral transcriptional load as a function of the level of each mRNA. We find that cells lacking  
 284 expression of any of the four genes that encode the viral RNP express much less total viral mRNA,  
 285 consistent with prior bulk studies (*Vreeede et al., 2004; Eisfeld et al., 2015*). Interestingly, the reason  
 286 some cells fail to express some viral genes remains unclear. The prototypical influenza virion  
 287 packages one copy of each of the eight gene segments (*Noda et al., 2006; Hutchinson et al., 2010*),  
 288 but some virions surely package fewer (*Brooke et al., 2014*). However, it is also possible that much  
 289 of the viral gene absence is due to stochastic loss of viral RNPs after infection but prior to the  
 290 initiation of viral transcription in the nucleus.

291 The absence of viral genes only partially explains the cell-to-cell variation in amount of viral  
 292 mRNA, which still varies from <1% to >50% among cells expressing all the viral genes. It is likely  
 293 that other viral genetic factors explain some of this remaining heterogeneity. The 3'-end sequencing  
 294 strategy used in our experiments detects the presence of a viral gene, but does not identify  
 295 whether that gene contains a mutation that might hinder viral replication. However, viral mutations  
 296 are also unlikely to explain all the observed heterogeneity, since current consensus estimates of



**Figure 9.** Cellular genes that co-vary in expression with the amount of viral mRNA in cells expressing all eight viral genes. The columns are cells, ordered from left to right by the fraction of mRNA derived from virus. Each row is a gene that is differentially expressed as a function of the fraction of mRNA derived from virus at a false discovery rate of 0.1. Genes for which the color goes from blue at left to red at right are expressed at higher levels in cells with more viral mRNA. The scale bar indicates the number of standard deviations above or below the mean expression, truncated at 3-fold on both sides.

**Figure 9-source data 1.** The full results of the differential expression test is in `p_sig_cellular_genes.csv`.

**Figure 9-source data 2.** The results of a gene-set analysis are in `p_pathway_enrichment.tsv`.

**Figure 9-Figure supplement 1.** Many genes that co-vary with viral load are involved in the oxidative stress response.

influenza virus's mutation rate suggest that the typical virion in a stock such as the one used in our experiment should contain less than one mutation per genome (*Parvin et al., 1986; Suárez et al., 1992; Suárez-López and Ortín, 1994; Nobusawa and Sato, 2006; Bloom, 2014; Pauly et al., 2017*).

The rest of the heterogeneity must be due to some combination of cellular factors and inherent stochasticity. Some features of the cellular transcriptome co-vary with the amount of influenza mRNA. In particular, the viral load in individual cells is associated with the expression of genes involved in response to cellular stresses, including oxidative stress. It will be interesting to determine if these cellular transcriptional signatures are simply a consequence of the stress imposed by viral replication, or if their stronger activation in some cells is a causative factor that promotes viral transcription. However, it also would not be surprising if a substantial amount of the cell-to-cell heterogeneity cannot be ascribed to pre-existing features of either the viral genome or cellular state. Apparently stochastic heterogeneity is a common feature of many processes at a single-cell level (*Cai et al., 2006; Raj et al., 2006; Buganim et al., 2012; Shalek et al., 2013; Avraham et al., 2015*) – especially when those processes are initiated by very small numbers of initial molecules (*Elowitz*

311 **et al., 2002)**, as is the case for low-MOI viral infection.

312 Our data do suggest that the factors driving the heterogeneity in viral transcriptional load exert  
 313 relatively concordant effects on all viral genes in a given cell. Specifically, despite the extreme  
 314 heterogeneity in total viral mRNA per cell, the relative levels of the viral mRNAs are reasonably  
 315 consistent across cells, and generally reflective of classical bulk measurements (**Hatada et al., 1989**).  
 316 Therefore, despite the stochasticity inherent in initiating transcription and replication of each gene  
 317 from a single copy carried by the incoming virion, as long as a gene is not completely lost then the  
 318 virus possesses mechanisms to control its relative expression (**Shapiro et al., 1987; Hatada et al.,**  
 319 **1989; Perez et al., 2010; Heldt et al., 2012; Chua et al., 2013**).

320 One factor that surprisingly does *not* appreciably contribute to the heterogeneity in our ex-  
 321 periments is activation of innate-immune interferon pathways. Only one of the hundreds of  
 322 virus-infected cells expresses any detectable interferon, despite the fact that a number of cells fail  
 323 to express the influenza-virus interferon antagonist NS1. It is known that interferon activation is  
 324 stochastic at the level of single cells in response to both synthetic ligands (**Shalek et al., 2013, 2014;**  
 325 **Bhushal et al., 2017; Hagai et al., 2017**) and actual infection (**Rand et al., 2012; Perez-Cidoncha**  
 326 **et al., 2014; Avraham et al., 2015; Killip et al., 2017**). But interferon expression is a prominent  
 327 transcriptional signature of high-MOI influenza virus infection of bulk cells, including in the epithelial  
 328 cell line and at the time-points used in our experiments (**Geiss et al., 2002; Sutejo et al., 2012**). So  
 329 it is notable how rarely single cells express interferon. Interferon expression would surely be more  
 330 common at later times or with a viral stock passaged at higher MOI, since paracrine interferon  
 331 signaling (**Crotta et al., 2013**) and accumulation of defective viral particles enhance innate-immune  
 332 detection (**Tapia et al., 2013; Lopez, 2014**). However, the early events of physiological influenza  
 333 infection involve just a few virions (**Varble et al., 2014; McCrone et al., 2017**), and so it is interesting  
 334 to speculate whether rare events such as interferon activation during the first few cycles of viral  
 335 replication could contribute to heterogeneity in the eventual outcome of infection.

336 Overall, our work shows the power and importance of characterizing cellular infection at the  
 337 level of single cells (**Avraham et al., 2015**). The dynamics of viral infection in any given cell is shaped  
 338 by the genetic composition of the incoming virion, the host-cell state, the bi-modality of innate-  
 339 immune activation, and the inherent stochasticity of molecular processes initiated by a single copy  
 340 of each viral gene. We have shown how the confluence of these factors leads to extreme cell-to-cell  
 341 heterogeneity in the transcriptional outcome of influenza virus infection. Further deconstruction of  
 342 the contributions of each factor will enable a deeper understanding of how the bulk features of  
 343 infection emerge from the processes occurring within individual virus-infected cells.

## 344 Methods and Materials

### 345 Cell lines and viruses

346 The following cell lines were used in this study: the human lung epithelial carcinoma line A549  
 347 (ATCC CCL-185), the MDCK-SIAT1 variant of the Madin Darby canine kidney cell line overexpressing  
 348 human SIAT1 (Sigma-Aldrich 05071502), and the human embryonic kidney cell line 293T (ATCC  
 349 CRL-3216). All cells were maintained in D10 media (DMEM supplemented with 10% heat-inactivated  
 350 fetal bovine serum, 2 mM L-glutamine, 100 U of penicillin/ml, and 100 µg of streptomycin/ml) at 37  
 351 °C at 5 % CO<sub>2</sub>.

352 Wildtype A/WSN/1933 (H1N1) influenza virus was generated by reverse genetics using the  
 353 plasmids pHW181-PB2, pHW182-PB1, pHW183-PA, pHW184-HA, pHW185-NP, pHW186-NA, pHW187-  
 354 M, and pHW188-NS (**Hoffmann et al., 2000**). The sequences of the viral RNAs encoded in these  
 355 plasmids are in Figure 1-source data 1. Reverse-genetics plasmids encoding the synonymously  
 356 barcoded WSN virus were created by using site-directed mutagenesis to introduce two synonymous  
 357 mutations near the 3' end of the mRNA for each viral gene. The sequences of the synonymously  
 358 barcoded viral RNAs are in Figure 1-source data 1.

359 To generate viruses from these plasmids, we transfected an equimolar mix of all eight plasmids

360 into cocultures of 293T and MDCK-SIAT1 cells seeded at a ratio of 8:1. At 24 hours post-transfection,  
 361 we changed media from D10 to influenza growth media (Opti-MEM supplemented with 0.01% heat-  
 362 inactivated FBS, 0.3% BSA, 100 U of penicillin/ml, 100 µg of streptomycin/ml, and 100 µg of calcium  
 363 chloride/ml). At 48 hours post-transfection we harvested the virus-containing supernatant, pelleted  
 364 cellular material by centrifugation at 300 x g's for 4 minutes, and stored aliquots of the clarified  
 365 viral supernatant at -80 °C. We then titered thawed aliquots of viral by TCID50 on MDCK-SIAT1 cells,  
 366 computing titers via the formula of *Reed and Muench* (1938). To generate our "high-purity" stocks of  
 367 viruses for the single-cell sequencing experiments, we then infected MDCK-SIAT1 cells at an MOI of  
 368 0.01, and let the virus replicate for 36 hours prior to harvesting aliquots that were again clarified by  
 369 low-speed centrifugation, aliquoted, stored at -80 °C, and titered by TCID50. The high-MOI passage  
 370 (high-defective particle) stock used in Figure 2 was generated by instead passaging in MDCK-SIAT1  
 371 cells twice at an MOI of 1 for 48 hours.

372 For the experiments in Figure 7-Figure supplement 1, we created a virus that carried an HA gene  
 373 segment in which GFP replaced most of the HA coding sequence, following a scheme first described  
 374 by *Marsh et al.* (2007). Briefly, we created a plasmid encoding a viral RNA with GFP in place of the  
 375 HA coding sequence in the context of the pH21 (*Neumann et al.*, 1999) reverse-genetics plasmid,  
 376 removing potential start codons upstream of the GFP (see Figure 7-source data 2 for the sequence  
 377 of the viral RNA). We then generated GFP-carrying virus by reverse-genetics in cells constitutively  
 378 expressing HA (*Doud and Bloom*, 2016). To obtain sufficient titers, this HA-eGFP virus was expanded  
 379 for 44 rather than 36 hours after initiating infection at an MOI of 0.01.

### 380 qPCR

381 For the qPCR in Figure 2 and Figure 5-Figure supplement 1, A549 cells were seeded at 3x10<sup>5</sup>  
 382 cells per well in a 6-well tissue culture plate in D10 the day prior to infection. On the day of  
 383 infection, a single well was trypsinized and the cells were counted in order to determine the  
 384 appropriate amount of virus to use to achieve the intended MOI. Immediately before infection,  
 385 D10 was replaced with influenza growth media. For cells incubated with cyclohexamide, the  
 386 compound was added to a final concentration of 50 µg/ml at the time of infection – previously  
 387 confirmed to be sufficient to halt viral protein production (*Killip et al.*, 2014). RNA was purified  
 388 using the QIAGEN RNeasy plus mini kit following manufacturer's instructions. cDNA was syn-  
 389 thesized using an oligoDT primer and the SuperScript™ III first-strand synthesis supermix from  
 390 ThermoFisher using the manufacturer's protocol. Transcript abundance was measured using  
 391 SYBR™ green PCR master mix, using a combined anneal/extension step of 60 °C for one minute  
 392 with the following primers: *HA*: 5'-GGCCCAACCACACATTCAAC-3', 5'-GCTCATCACTGCTAGACGGG-  
 393 3', *IFNB1*: 5'-AAACTCATGAGCAGTCTGCA-3', 5'-AGGAGATCTTCAGTTGGAGG-3', *L32*: 5'-  
 394 AGCTCCAAAAATAGACGCAC-3', 5'-TTCATAGCAGTAGGCACAAAGG-3'. Biological triplicates were per-  
 395 formed for all samples.

396 For the measurements of viral genomic HA content in Figure 2A, vRNA was harvested from 80  
 397 µl of viral supernatant by the addition of 600 µl of RLT plus before proceeding with the standard  
 398 QIAGEN RNeasy Plus Mini kit protocol. The cDNA was generated using SuperScript™ III first-strand  
 399 synthesis supermix using the manufacturer's protocol, and using the universal vRNA primers of  
 400 *Hoffmann et al.* (2001) with the modifications described in *Xue et al.* (2017). The qPCR was then  
 401 performed as for mRNA measurements. A standard curve was generated from three independent  
 402 dilutions of the HA-encoding reverse genetics plasmid. All vRNA values represent three independent  
 403 RNA extractions with two replicate qPCR measurements.

### 404 Flow cytometry titering and analyses

405 To determine viral titers in terms of HA-expressing units and for the flow cytometry in Figure 7,  
 406 Figure supplement 1, and Figure supplement 3 A549 cells were seeded in a 6-well plate and infected  
 407 as described above for the qPCR analyses. Cells were harvested by trypsinization, resuspended in  
 408 phosphate-buffered saline supplemented with 2% heat-inactivated FBS, and stained with 10 µg/ml

of H17-L19, a mouse monoclonal antibody confirmed to bind to WSN HA in a prior study (**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. Cells were then washed, fixed in 1% formaldehyde, and washed further before a final resuspension and analysis. We then determined the fraction of cells that were HA positive and calculated the HA-expressing units. For NS1 staining in Figure supplement 3, cells stained for HA as described above were permeabilized using BD Cytofix/Cytoperm following manufacturer's instructions, stained with anti-NS1 (GTX125990, Genetex) at 4.4 µg/ml, washed, stained with a goat anti-rabbit IgG antibody conjugated to Alexa Fluor 405, washed, and analyzed. For Figure 7, Figure supplement 1, and Figure supplement 3), after gating to exclude multiplets in FlowJo, data were extracted using the R package flowCore (**Le Meur et al., 2007**) and analyzed using a custom Python script. For Figure supplement 3) channels were additionally compensated in FlowJo. Gaussian kernel density estimates were obtained using the scipy stats package method, gaussian\_kde, using automatic bandwidth determination (**van der Walt et al., 2017**). For Figure supplement 3), the percentage of influenza-infected cells was determined by HA staining alone, and the top quantile of NS1-stained cells matching that percentage were taken as the NS1 positive population.

#### 425 Infections for single-cell mRNA sequencing

426 Single-cell sequencing libraries were generated using the 10x Chromium Single Cell 3' platform  
 427 form (**Zheng et al., 2017**) using the V1 reagents.

428 All time points except for the 8-hour replicate were prepared on the same day. For the infections,  
 429 A549 cells were seeded in a 6-well plate, with two wells per time point. A single well of cells was  
 430 trypsinized and counted prior to initiation of the experiment for the purposes of calculating MOI.  
 431 Wild-type and synonymously barcoded virus were mixed to an estimated ratio of 1:1 based on prior,  
 432 exploratory, single-cell analyses (data not shown). At the initiation of our experiment, the wells  
 433 for all time points were changed from D10 to influenza growth media. Cells were then infected  
 434 with 0.3 HA-expressing units of virus per cell (a determined by flow cytometry). The infections  
 435 were performed in order of time point: first the 10-hour time point, then the 8-hour, and then the  
 436 6-hour time point. At one hour after infection, the media for each time point was changed to fresh  
 437 influenza growth media. Note that the HA-expressing units were calculated without this additional  
 438 washing step, and so likely represent an overestimate of our final infectious dose (consistent  
 439 with the fact that fewer than 30% of cells appear infected in the single-cell sequencing data). All  
 440 cells were then harvested for single-cell analysis concurrently – ensuring all had spent equivalent  
 441 time in changed media. For our replicate 8-hour time point, cells were infected as above except  
 442 that the cells were infected at 0.1 HA-expressing units of virus per cell but no wash step was  
 443 performed, and the sample was prepared on a different day. After harvest, cells were counted using  
 444 disposable hemocytometers and diluted to equivalent concentrations with an intended capture  
 445 of 3000 cells/sample following the manufacturer's provided by 10x Genomics for the Chromium  
 446 Single Cell platform. All subsequent steps through library preparation followed the manufacturer's  
 447 protocol. Samples were sequenced on an Illumina HiSeq. The deep sequencing data are available  
 448 on the Sequence Read Archive under accession [[Upload in progress.](#)]

#### 449 Computational analysis of single-cell mRNA sequencing data

450 Jupyter notebooks that perform all of the computational analyses are available in Supplementary  
 451 file 1 and at [https://github.com/jbloomlab/flu\\_single\\_cell](https://github.com/jbloomlab/flu_single_cell) [*This GitHub repository will be made  
 452 public upon acceptance of the manuscript. If you are a reviewer who needs access, please contact the  
 453 editor.*].

454 Briefly, the raw deep sequencing data were processed using the 10X Genomics software package  
 455 CellRanger (version 2.0.0). The reads were aligned to a concatenation of the human and influenza  
 456 virus transcriptomes. The human transcriptome was generated by filtering genome assembly  
 457 GRCh38 for protein coding genes defined in the GTF file GRCh38.87. The influenza virus transcript-

tome was generated from the reverse-complement of the wildtype WSN viral RNA sequences as encoded in the reverse-genetics plasmids (Figure 1-source data 1). CellRanger calls cells based on the number of observed cell barcodes, and creates a cell-gene matrix. We used custom Python code to annotate the cells in this matrix by the number of viral reads that could be assigned to the wildtype and synonymously barcoded virus. Only about half of the viral reads overlapped the barcoded regions of the genes (Figure 1A) and could therefore be assigned to a viral barcode (Figure 4-Figures supplement 1). So for calculations of the number of reads in a cell derived from each viral barcode for each viral gene, the total number of detected molecules of that gene are multiplied by the fraction of those molecules with assignable barcodes that are assigned to that barcode. This annotated cell-gene matrix is in Supplementary file 2. A Jupyter notebook that performs these analyses is in Supplementary file 1.

The annotated cell-gene matrix was analyzed in R, primarily using the Monocle package (version 2.4.0) (*Qiu et al., 2017; Trapnell et al., 2014*). A Jupyter notebook that performs these analyses is in Supplementary file 1. For each sample, cell barcodes that had >2.5-fold fewer or more UMI counts mapping to cellular transcripts than the sample mean were excluded from downstream analyses (see red vertical lines in Figure 3B).

In order to determine an appropriate cutoff for how many reads a cell needed to contain in order to be classified as infected, we calculated the mean viral barcode purity across all cells that contained at least a given fraction of viral mRNA and had multiple viral reads that could be assigned a barcode (Figure 4B,C). We then determined the threshold fraction of viral mRNA at which the mean purity no longer increased as a function of the amount of viral mRNA. This threshold represents the point at which we have effectively eliminated cells that have low barcode purity simply due to lysis-acquired reads sampled randomly from both viral barcodes. The 10-hour sample has the most viral mRNA, and so is expected to have the most lysis-associated influenza reads. We therefore determined the threshold using just this sample, and then applied this threshold to all samples. The threshold is shown in Figure 4C. This procedure is expected to be conservative, and may miss some truly infected cells with very low amounts of viral mRNA. For subsequent analyses, we retained all infected cells and a subsample of uninfected cells (the greater of 50 or the number of infected cells for that sample). The rationale for subsampling the uninfected cell is that the vast majority of cells are uninfected, and we did not want these cells to completely dominate the downstream analyses. Cells were classified as co-infected if both viral variants had an RNA level that exceeded the threshold, and if the minor variant contributed at least 5% of the viral mRNA.

For the semi-supervised t-SNE clustering, we used Monocle's cell hierarchy function to bin cells into those with no viral mRNA, <2% viral mRNA, between 2% and 20% viral mRNA, and >20%. Candidate marker genes for t-SNE dimensionality reduction were then determined using the Monocle function markerDiffTable, excluding the effects of sample variation and the number of genes identified in a given cell, using a q-value cutoff of 0.01. The specificity of these markers was determined using the function calculateMarkerSpecificity – the top 40 markers were retained, and used to place populations in a two-dimensional plane based on tSNE dimensionality reduction.

For the analyses of cellular genes that differed in expression as a function of the amount of viral mRNA, we only considered cells that expressed all 8 viral mRNAs to avoid effects driven simply by viral gene absence. We also only considered cellular genes in the differential gene analysis, since viral gene expression will tautologically co-vary with the amount of viral mRNA. Additionally, because influenza virus has the capacity to degrade or prevent the synthesis of host mRNAs (*Bercovich-Kinori et al., 2016*) and contributes significantly to the total number UMIs in some cells, we calculate size factors (a scalar value representing efficiency of UMI capture) based on cellular transcripts alone. Finally, we assigned all cells a ceiling fraction of mRNA from virus of 25% so that a few extremely high-expressing cells did not dominate. Cellular genes with expression that co-varied with the fraction of viral mRNAs in a cell were then determined using the Monocle differentialGeneTest, after removing variance explained by sample to sample variation. Figure 9 shows all genes that were significantly associated with the fraction of mRNA from virus at a false discovery rate of 0.1.

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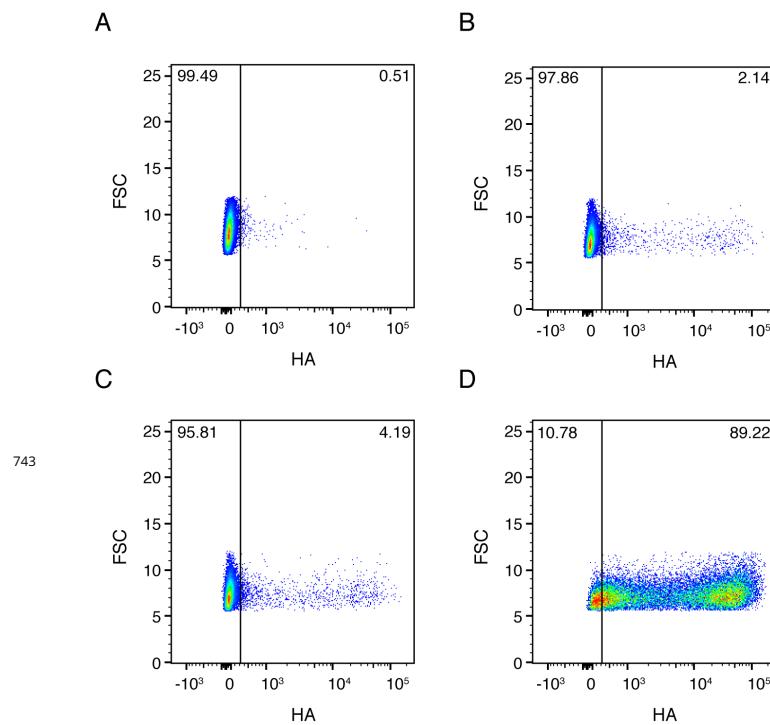
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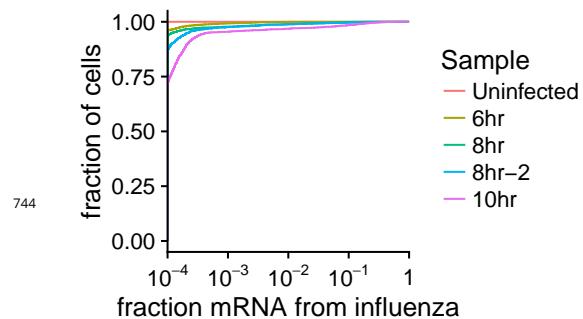
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**Supplementary file 1.** Computer code for the analyses. This ZIP file contains a Jupyter notebook that runs CellRanger to align and annotate the reads, and a Jupyter notebook that uses Monocle to analyze the cell-gene matrix. The ZIP file also includes associated custom scripts.

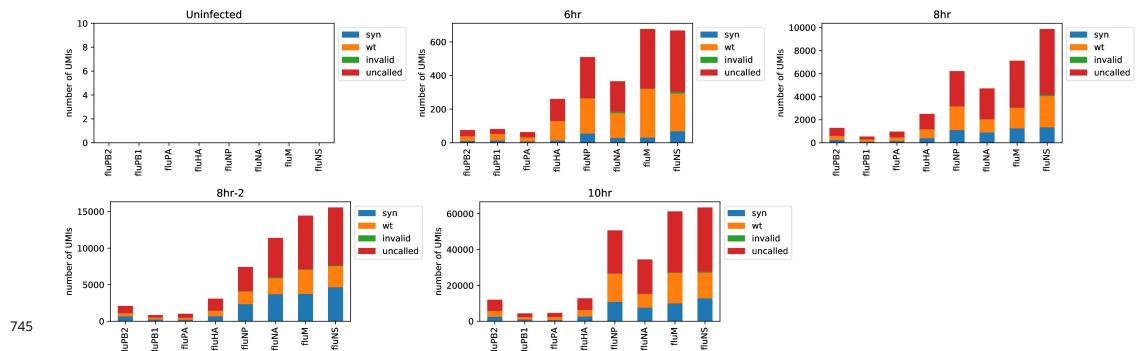
**Supplementary file 2.** The annotated cell-gene matrix in Matrix Market Format. *[This file is too large for the eLife submission system. We will communicate with the editors to get it uploaded for a final accepted version, or we will post it on DataDryad. If you are a reviewer and need access, please contact the editor.]*



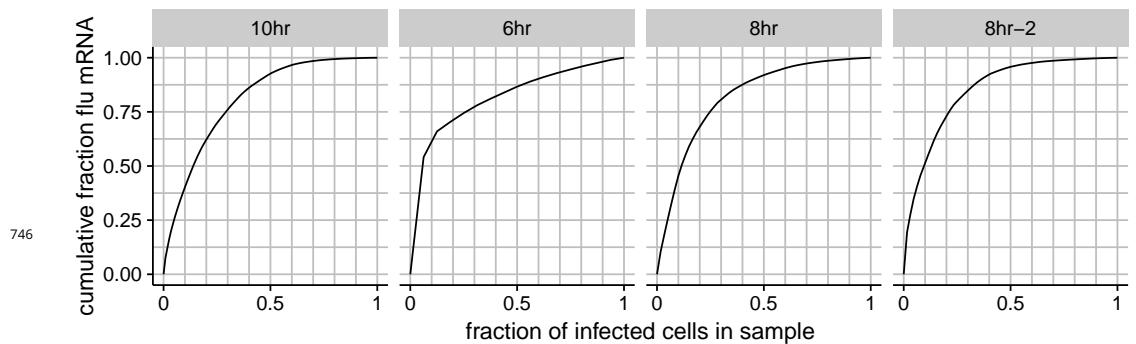
**Figure 2–Figure supplement 1.** Full flow cytometry data for Figure 2B. A549 cells were infected at an MOI of 0.1 as calculated by TCID<sub>50</sub> on MDCK-SIAT1 cells. **(A)** Uninfected gating control. **(B)** Cells infected with the wild-type virus stock used in our experiments. **(C)** Cells infected with synonymously barcoded virus stock used in our experiments. **(D)** Cells infected with a stock of wild-type virus propagated at a high MOI, and therefore enriched in defective particles.



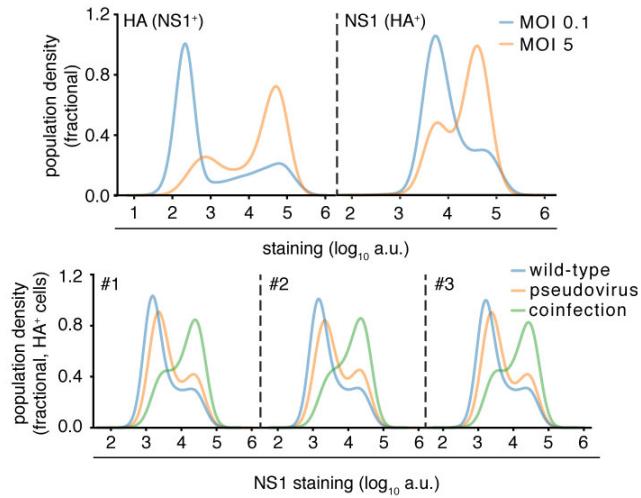
**Figure 3–Figure supplement 1.** For each sample, this plot shows the fraction of all cells that derive at least the indication fraction of their mRNA from influenza virus.



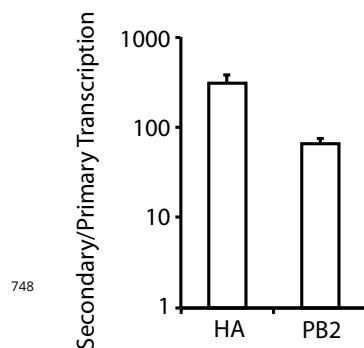
**Figure 4-Figure supplement 1.** The number of viral barcodes called for each sample and gene segment. Viral transcripts are classified as *syn* if they mapped to a synonymously barcoded influenza transcript, *wt* if they mapped to a wild-type influenza transcript, *invalid* if multiple reads for the same UMI differed on the status of the viral barcode, and as *uncalled* if none of the reads for that UMI overlapped the region of the viral transcript containing the viral barcode. For calculations of the number of reads in a cell derived from each viral barcode for each viral gene, the total number of detected molecules of that gene are multiplied by the fraction of those molecules with assignable barcodes that are assigned to that barcode.



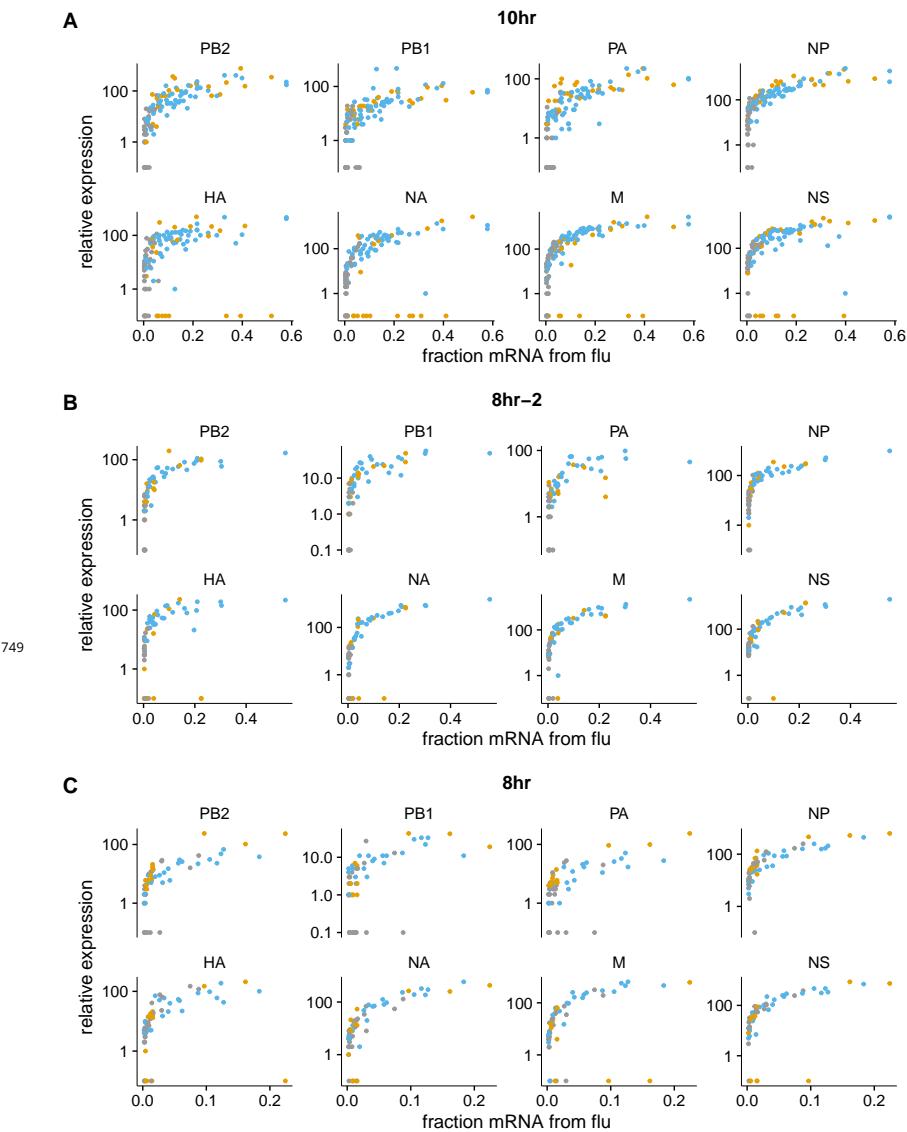
**Figure 4-Figure supplement 2.** The total fraction of all viral mRNA among infected cells that is attributable to a given fraction of these cells. For instance, the plot for the 8-hour sample shows that roughly 50% of all viral mRNA is derived from 10% of the infected cells.



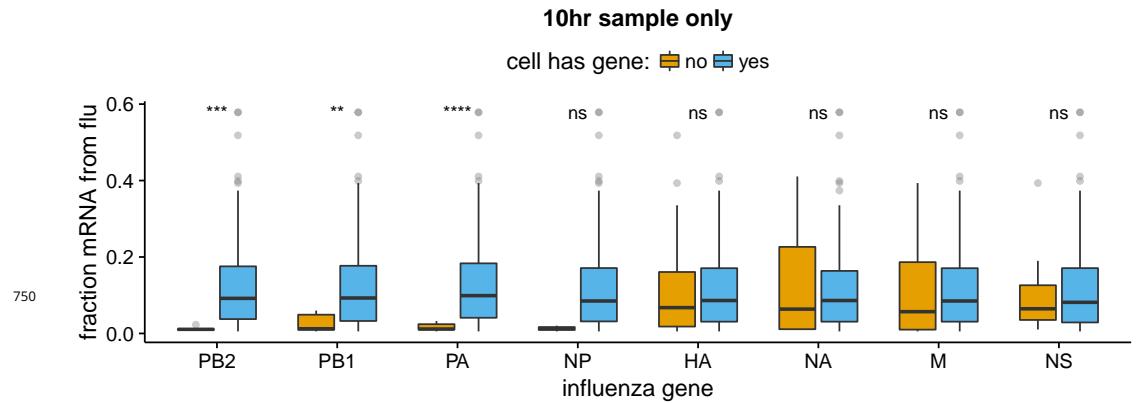
**Figure 4-Figure supplement 3.** (Top) Expression of a viral proteins in cells infected at high (MOI 5 as calculated by TCID50 on MDCK-SIAT1 cells) or low (MOI 0.1) initial infectious dose. Cells were concurrently stained for HA and NS1 10 hours post infection. HA staining was analyzed in cells positive for NS1 (left), and NS1 staining was analyzed in cells positive for HA (right). While a higher dose leads to more cells expressing high amounts of viral protein, it does not substantially increase the amount of viral protein in either the low-expressing or high-expressing cells. Therefore, higher viral dose does not lead to a large continuous increase in viral protein production among all cells – rather, it mostly changes the distribution of where cells fall in the highly heterogeneous distribution of viral protein production. (Bottom) Cells were co-infected with a mix of wild-type virus and virus in which the HA gene was replaced by GFP flanked by the terminal regions of the HA gene segment. At 10 hours post-infection, cells were stained for NS1 and HA expression and analyzed by flow cytometry. Cells could be annotated as infected by one or more infectious particles (wild-type or pseudovirus alone) or at least two infectious particles (coinfection). Coinfected cells, like cells infected at a higher infectious dose, occupy different positions in the distribution of viral protein production but do not exhibit a continuous increase in viral protein production. Replicates shown.



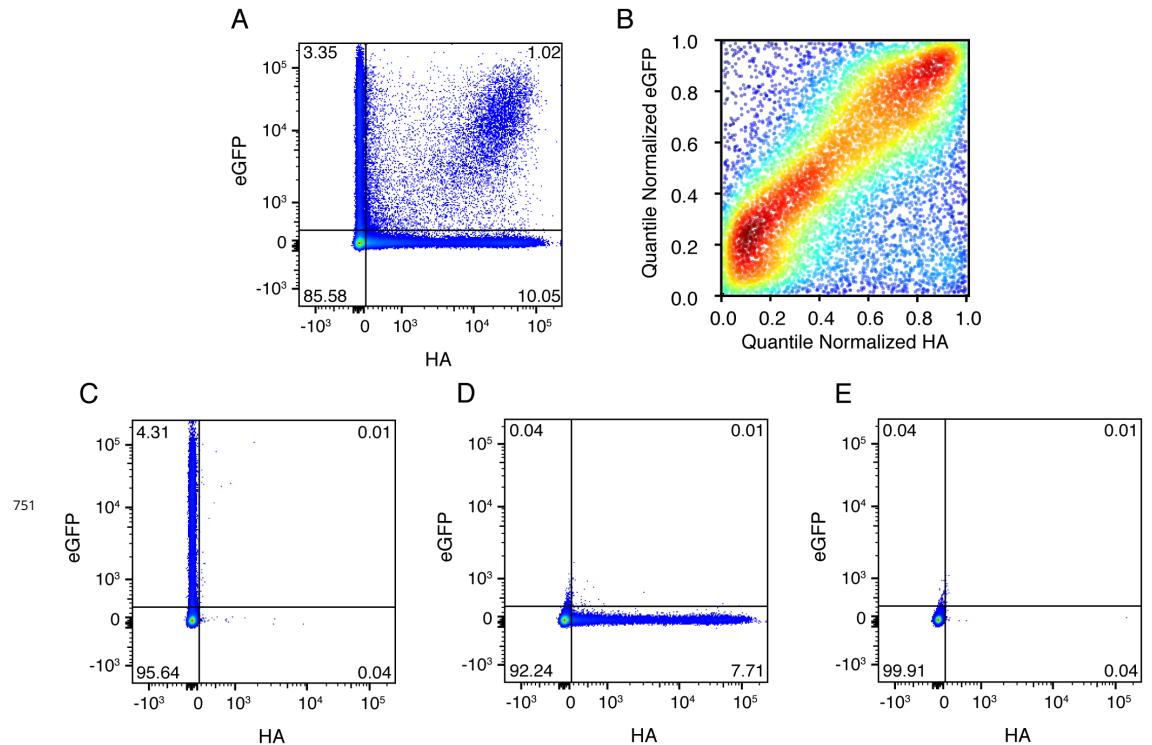
**Figure 5-Figure supplement 1.** A549 cells were infected at an MOI of 0.2 as calculated on MDCK-SIAT1 cells in either the presence or absence of the protein-translation inhibitor cyclohexamide, and viral mRNA was quantified at 8 hours post-infection by qPCR. The cyclohexamide prevents translation of new PB2, PB1, PA, and NP protein, and so prevents the formation of the new RNPs needed for secondary transcription. The bars show the relative amount of HA and PB2 mRNA in the absence versus the presence of cyclohexamide. Error  $\pm$  S.D. n=3.



**Figure 5—Figure supplement 2.** The fraction of mRNA in each infected cell derived as a function of the normalized expression of each viral gene, shown for the 10-hour and 8-hour samples individually (the other samples had too few infected cells for this analysis to be useful). Points are colored as in Figure 5A.



**Figure 5–Figure supplement 3.** The absence of viral RNP genes but *not* non-RNP genes remains significantly associated with reduced viral burden when we examine only the 10-hr sample, which is the single time point with the most data points. The difference for NP is no longer statistically significant due to low counts of infected cells lacking NP, but the trend remains. We do not show statistical analyses for other samples, as the number of infected cells is too low.



**Figure 7–Figure supplement 1.** **(A)** Cells were co-infected with a mix of wild-type virus and virus in which the HA gene was replaced by GFP flanked by the terminal regions of the HA gene segment. At 10 hours post-infection, cells were analyzed by flow cytometry for HA and eGFP expression. **(B)** The expression of HA and GFP are correlated in co-infected cells. Shown are the quantile-normalized HA and eGFP signals for double-positive cells. Cells are colored by density, using a Gaussian kernel density estimate. **(C),(D),(E)** Gating controls, single infection with eGFP virus, single infection with wild-type virus, and uninfected cells, respectively.

Category	Genes
Detoxification	AKR1C3, AKR1B10, GPX2, ALDH3A1, ALDH1A1, NQO1, CBR1, PRDX1
Protein folding	TXN, PPIA
Electron transport chain	NDUFB4, MT-CO1, MT-CO3
Regulators	ATF3, GADD45B
ROS-responsive relevance complex/unknown	UBB, NME1

**Figure 9–Figure supplement 1.** Table delineating genes in Figure 9 that are associated with the response to oxidative stress (*Duong et al., 2017; Jung et al., 2017; Lee and Ryu, 2017; Peuchant et al., 2017; MacLeod et al., 2016; Jiang et al., 2016; Gorrini et al., 2013; Miura et al., 2013; Kim et al., 2009; Banning et al., 2005; Murray et al., 2003; Doyle et al., 1999*).