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Analysis of IAV Replication and Co-infection Dynamics by a Versatile RNA Viral Genome Labeling Method

SUMMARY

Genome delivery to the proper cellular compartment for transcription and replication is a primary goal of viruses. However, methods for analyzing viral genome localization and differentiating genomes with high identity are lacking, making it difficult to investigate entry-related processes and co-examine heterogeneous RNA viral populations. Here, we present an RNA labeling approach for single-cell analysis of RNA viral replication and co-infection dynamics in situ, which uses the versatility of padlock probes. We applied this method to identify influenza A virus (IAV) infections in cells and lung tissue with single-nucleotide specificity and to classify entry and replication stages by gene segment localization. Extending the classification strategy to co-infections of IAVs with single-nucleotide variations, we found that the dependence on intracellular trafficking places a time restriction on secondary co-infections necessary for genome reassortment. Altogether, these data demonstrate how RNA viral genome labeling can help dissect entry and co-infections.

INTRODUCTION

Advancements in sequencing technology have increased the available information about viruses and made host responses to infection more accessible (Brister et al., 2015; Capobianchi et al., 2013; Law et al., 2013; Westermann et al., 2017). These improvements have been particularly useful for examining how heterogeneous RNA viral populations or quasispecies, generated by error-prone replication, contribute to viral fitness (Bordería et al., 2016; Xue et al., 2016). They have also made it easier to determine the origin of new RNA viruses and mutations responsible for changes in pathogenicity (Gire et al., 2014; Herfst et al., 2012; Imai et al., 2012). However, sequencing techniques generally provide limited spatial information about RNA viral genomes, which is necessary to examine processes related to genome trafficking, co-infections, and reassortment.

Due to technical limitations, cells infected by RNA viruses are typically identified with antibodies, which do not account for population heterogeneity. Approaches using single-molecule fluorescence in situ hybridization (smFISH) have shown promise for detecting the localization of RNA viral genomes in cells. Although these techniques are not easily modified to distinguish between RNA viruses that are highly similar, using the genome for detection provides several advantages. First, viral genomes are a defining feature of an infection and can be used to monitor viral entry. Second, genome replication is an accurate infection reporter, because it occurs early and in defined cellular regions. Third, sequence-based methods have the potential to achieve the specificity needed to co-analyze cell infections by viruses that differ by as few as one nucleotide.

Since single-molecule RNA localization techniques are relatively new (Crosetto et al., 2015; Gaspar and Ephrussi, 2015), much of the information about RNA viral genomes and trafficking has come from biochemical assays and electron and immunofluorescence microscopy. This especially applies to influenza A viruses (IAVs), for which these approaches helped define the genome as eight negative-sense, single-stranded, viral RNA (vRNA) gene segments (Arranz et al., 2012; McGeoch et al., 1976; Palese and Schultman, 1976). They also were used to establish the general entry pathway to the nucleus (Herz et al., 1981; Jackson et al., 1982; Martin and Helenius, 1991; Matlin et al., 1981) and to identify cellular proteins involved in gene
segment nuclear export (Elton et al., 2001; Ma et al., 2001). Recent studies have analyzed certain aspects of IAV gene segment trafficking using vRNA-associated proteins as reporters (reviewed in Breen et al., 2016; Hutchinson et al., 2010) and smFISH labeling (Chou et al., 2012, 2013; Huang et al., 2012; Lakdawala et al., 2014). However, these methods have not been extensively applied and many of the remaining questions about IAV genome trafficking require alternative approaches with high sequence specificity and multiplex capacity.

A rarely used technique for analyzing RNA viruses involves padlock probes (PLPs), which were initially designed to detect DNA sequences and later developed for labeling RNA sequences in cells and tissues (Andersson et al., 2012; Gyarmati et al., 2008; Ke et al., 2013; Larsson et al., 2010; Lizardi et al., 1998; Nilsson et al., 1994). PLPs function in a variety of biological samples, have high specificity and a short nucleotide recognition requirement, and can generate a localized amplified signal alone or in multiplex reactions (Banér et al., 1998; Hardenbol et al., 2003; McGinn et al., 2016; Nilsson et al., 1997; Zieba et al., 2012). In this study, we used the versatility of PLPs to develop an in situ RNA labeling method for analyzing IAV infections. The approach is capable of detecting all eight IAV vRNA gene segments, can monitor the stochastic aspects of IAV entry, and can be used to define the infection stages across cell populations. By using this methodology to analyze co-infections of IAVs with single-nucleotide variations, we obtained evidence that productive IAV cell co-infections occur in a limited time window, defined by the replication stage of the primary infection.

The specificity and direct nature of this multifunctional PLP labeling approach extends how RNA viral infections can be analyzed and detected in vitro and in vivo.

RESULTS

Approach for IAV Gene Segment vRNA Labeling and Single-Cell Analysis

The IAV genome consists of eight gene segments, or ribonucleoprotein complexes, that contain a single vRNA with numerous copies of the nucleoprotein (NP) and one copy of the viral polymerase (Figure 1A). Due to the segmentation, productive infections only occur when all eight gene segments reach the nucleus for replication (Eisfeld et al., 2015; Herz et al., 1981; Te Velthuis and Fodor, 2016). Based on this requirement, we sought to analyze IAV infections by tracking the intracellular localization of the vRNAs in single cells. PLPs were chosen as a basis for the approach because of their ability to detect specific RNA sequences in situ and their multiplex labeling capacity (Ke et al., 2013; Larsson et al., 2010; Lizardi et al., 1998). Each PLP was designed for a specific vRNA from the H1N1 strain A/WSN/33 (WSN) by incorporating 5’ and 3’ arms that hybridize to adjacent ~20-nucleotide sequences, as well as two identification barcodes (Figure 1B) (Supplemental Information).

The labeling and single-cell analysis were established using infected Madin Darby Canine Kidney (MDCK) cells (Figure 1C). Following cell fixation, cDNAs were synthesized from each IAV gene segment using locked nucleic acid (LNA) modified primers (Supplemental Information). The template was then digested with RNase H, and PLPs were hybridized and ligated to enable synthesis of a rolling-circle product (RCP), which locally amplifies the signal by creating a concatemer with multiple complementary barcode copies. After RCP synthesis, cell nuclei were stained, fluorescently labeled barcodes were added (Supplemental Information), and the cells were imaged by fluorescence microscopy (Figure 1D). To accommodate all eight gene segments, the first barcode labels were removed and the cells were reimaged after labeling with the second set of barcodes. Images were then aligned and compiled, and nuclei staining combined with cell autofluorescence was used to define the cell segments used to assign the RCP-labeled gene segments.

Padlock Probe Labeling Efficiently Tracks IAV Gene Segment Localization in Situ

The general IAV cell entry pathway is well defined (Figure 2A) (de Graaf and Fouchier, 2014; Hutchinson et al., 2010; Skehel and Wiley, 2000). IAVs bind and traffic to the endosome where the low pH promotes hemagglutinin (HA)-mediated membrane fusion releasing the gene segments into the cytosol (Rust et al., 2004; White et al., 1982; Yoshimura and Ohnishi, 1984). From there, transporters transport them to the nucleus for replication by the viral polymerase through a positive-sense cRNA intermediate (Gabriel et al., 2011; Resa-Infante and Gabriel, 2013). The new gene segments then use the chromosomal region maintenance 1 (CRM-1) nuclear export pathway and Rab proteins to reach the plasma membrane for viral packaging in a gene segment-dependent manner (Amorim et al., 2011; Eisfeld et al., 2011; Elton et al., 2001; Gavaazzi et al., 2013; York and Fodor, 2013). To establish time parameters for this complex process in our system, vRNA levels in the cells and culture medium were determined when replication (~3 hr post-infection [p.i.]) and viral release (~7 hr p.i.) initiate (Figure 2B).

Due to the limited IAV gene segment entry data, we asked whether our approach can label vRNAs during entry by trapping the virus in endosomes using the pH-dependent viral fusion inhibitor baflomycin A1 (BFLA1) (Figure 2A) (Ochiai et al., 1995). In both untreated and BFLA1-treated cells, gene segment RCPs were visualized 2 hr p.i. (Figure 2C). However, the RCPs in BFLA1-treated cells mainly resolved as single clusters, indicative of endosomes with a nuclear localization frequency matching the average image occupancy of the nucleus (~40%), whereas the RCPs in untreated cells showed high nuclear localization. Next, we assessed the labeling of newly synthesized vRNAs by treating cells with leptomycin B (LMB), which inhibits their nuclear export (Figure 2A) (Elton et al., 2001). The gene segments were resolved in both samples, and similar to the distribution of the viral protein controls NP and PA (Figure S1), LMB treatment increased the RCP nuclear localization to ~80% (Figure 2D). These experiments confirmed that the developed in situ PLP labeling approach is applicable to the entire IAV replication cycle, because it can detect vRNA localization during entry and after replication.

IAV Gene Segment Labeling Accurately Identifies Productive Cell Infections

In tissue culture systems, defective interfering viral particles often exceed the number of infectious particles (Brooke, 2014; Dimmock and Easton, 2014; Fonville et al., 2015). Therefore,
we used an MOI corresponding to 0.3, or an infection rate of \( \frac{1}{8} \), to further assess the labeling specificity (Figure 2E). Although the recognition sequences caused some label variation (Figure S2), gating by the number of distinct gene segment RCPs revealed that \( \frac{1}{8} \) of the cells had productive infections (all eight vRNAs), whereas most of the population was uninfected or subject to potential nonproductive infections (Figure 2F). We then measured the number of NP gene segment RCPs per cell over time and found a reasonable linear correlation with NP vRNA levels obtained by qPCR (Figure 2G), indicating the labeling also can provide semiquantitative data for the vRNAs.

**Design for Labeling IAV Gene Segments with Single-Nucleotide Substitutions**

Similar to many RNA viruses, IAVs frequently acquire genome mutations, and these can significantly alter viral growth and pathogenicity (Boni, 2008; Drake, 1993; Schrauwen and Fouchier, 2014). However, most classical assays for monitoring viral properties are indirect and do not accommodate heterogeneity in viral populations (Dubecq and Vogt, 1953; Reed and Muench, 1938). Consequently, IAVs with high identity are often analyzed by sequencing or independent comparisons. PLPs offer a potential solution to this problem, because the recognition is based on sequence rather than epitopes or cell death. To test the ability to resolve IAV infections with single-nucleotide specificity, we rescued an isogenic virus (WSNIso), which contained a silent (synonymous) point mutation in each WSN gene segment (Figure 3A), by reverse genetics (da Silva et al., 2015; Hoffmann et al., 2000). During the rescue, both viruses showed similar in vitro growth (Figure 3B), and no changes occurred in the sequences or the distributions of HA, NA, M1, and NP in the viral particles after passaging (Figure 3C).

A pair of PLPs was then designed for each gene segment such that the terminal nucleotide of the 3’ arm hybridized to the nucleotide substitution site (Figure S3A). As an initial test, the labeling was performed on vRNA isolated from WSN and WSNIso particles in solution and the RCPs were quantified using automated amplified single-molecule detection (Figure S3B) (Göransson et al., 2012; Jarvius et al., 2006). In solution, the PLP pairs detected each gene segment from the two IAVs with similar efficiency (Figure 3E), and several pairs showed low cross-reactivity, including the HA gene segment pair (Figure S3A). We then analyzed RNA isolated from MDCK cells and mouse lung tissue infected with WSN, infected with WSNIso, or co-infected with WSN and WSNIso. Using the HA PLP pair, the viruses responsible for the in vitro and in vivo infections were appropriately identified, and similar RCP ratios were observed for samples that received equivalent numbers of infectious WSN and WSNIso particles (Figure 3F).
Although deep sequencing is more suited to identify unknown RNA sequence variants, our labeling approach is inexpensive and uses a straightforward analysis that offers the advantage of spatial information in situ. This unique property has the potential to enhance the cellular analysis of viral genome trafficking, heterogeneity and selection, gene segment reassortment, and...
other aspects in which sequencing is not ideal. Therefore, we examined the single-nucleotide resolution in situ by labeling WSN- and WSNIso-infected MDCK cells with the gene segment PLP pairs individually (Figures 4A and S4) and together (Figure 4B). In both cases, RCPs were primarily generated to the appropriate virus. The specificity was confirmed using MDCK cells that were independently infected with WSN or WSNIso and combined before the labeling (Figure 4C). Finally, we tested the ability to analyze in vivo samples by labeling lung tissue sections isolated from mice infected with WSN, infected with WSNIso, or co-infected with WSN and WSNIso. While the labeling process, the replication rate is determined by the number of gene segment templates and bound polymerases that reach the nucleus.

Analysis of IAV Entry Kinetics by Gene Segment Labeling

Despite the central role of a viral genome in defining an infection, a comprehensive analysis of IAV gene segment trafficking during entry has not been performed due to technical limitations. Therefore, we used the labeling approach to directly monitor IAV gene segment trafficking to the nucleus using two MOIs. Because IAVs are endocytosed within minutes (Matlin et al., 1981), cell binding was synchronized at 4°C, and the gene segments were labeled before and after initiating entry by shifting the cells to 37°C (Figure 5A). With low and high MOIs, few RCPs were observed from bound IAVs (0 and 5 min) (Figures 5C–5E), likely because the cell-bound viral particles are inefficiently fixed. However, the fixation requirement enabled the measurement of IAV entry kinetics as the labeling increased upon internalization. With a low MOI, the percentage of cells associated with a gene segment RCP increased until 30 min post-binding (Figure 5B), reaching a higher level than predicted by the MOI, presumably due to defective particles. When a higher MOI was used, RCPs were assigned to most cells earlier, with a half-time of ~5 min (Figure 5B), consistent with entry measurements by single-particle live imaging (Lakadamyali et al., 2003; Rust et al., 2004).

The single-cell low MOI data also showed that the number of RCPs per cell plateaued from 30 min until replication began at 180 min (Figures 5C, 5D, and S5). When the RCPs from the high MOI were analyzed together or individually, the number per cell continuously increased even though replication was also initiated at 180 min (Figures 5C, 5E, and S5B). The combination of the results from the in situ labeling and qPCR indicated that two IAV entry steps are stochastic: (1) cell entry, when more viral particles are bound, the entry half-time for a single virus decreases but it takes longer for all particles to enter the cell, and (2) replication; although the replication initiation time is fixed because of the lengthy gene segment trafficking process, the replication rate is determined by the number of gene segment templates and bound polymerases that reach the nucleus.

Classification of IAV Infection Stages by Gene Segment Labeling

We next asked whether single-cell labeling can be used to classify IAV infection stages in cells. First, we established a RCP per cell cutoff (~20) that distinguishes early replication stages from later ones (Figure S6). We then combined this parameter with the nuclear co-localization values from the entry inhibitor BFLA1 (~40%) and nuclear export inhibitor LMB (~80%) to assign each cell to one of five stages, as shown in Figure 5F. As expected, most infected cells were initially assigned to stage 1 (cell entry), and over time, they shifted to stage 2 (nuclear
import) and stage 3 (replication), the latter of which combines cells in which the vRNAs reached the nucleus with cells actively replicating vRNAs (Figure 5G). At the low MOI, the stage 1 and 2 populations did not fully disappear, likely because they represent defective infections that are masked at higher MOIs. The higher MOI also caused a sharper stage 1, earlier stage 2 initiation, and more pronounced stage 3 population that dropped at 180 min when vRNA-replicating cells entered stage 4 (nuclear export). An extended analysis at the low MOI showed a later shift from stage 3 to stages 4 and 5 (exported) that correlated with viral release (Figures S7A–S7C). In agreement with previous work (Chou et al., 2013; Lakdawala et al., 2014), the gene segments did not show any bias in nuclear export (Figures S7D and S7E). These results demonstrate how IAV gene segment labeling can be used to analyze the entry process and to define cell infection stages within large populations.

**The IAV Replication Cycle Defines the Time Window for Cell Co-infections**

Reassortment can occur when two IAVs infect and replicate within the same cell (Steel and Lowen, 2014). However, the complex trafficking of the gene segments to the nucleus is likely to limit the time window for productive secondary IAV infections. To investigate this concept, we designed a co-infection experiment in which cells receive a primary infection (WSN) followed by a secondary infection (WSNIso) at increasing time intervals and all gene segments were labeled 480 min (8 hr) after the primary infection (Figure 6A). This endpoint was chosen because the primary infection caused increasing cell loss at later times. To control for the decreasing duration of the secondary infection, a set of WSNIso-infected cells was generated without a primary infection (Figure 6B). As expected in the control samples, the decreasing infection times corresponded with
a lower number of RCPs per cell (4 hr) (Figures 6B and S8A) and a shift to a more pronounced stage 3 (replication) population (Figure 6D).

In the co-infection samples, the number of WSN RCPs per cell decreased when the virus was added more than 45 min after WSN (Figures 6C and S8B), whereas the number of WSN RCPs decreased when the virus was added more than 45 min after WSN (Figures 6C and S8B), whereas the number of WSN RCPs...
Figure 6. Co-labeling of IAV Genomes Reveals the Time Window for Productive Cell Co-infections

(A) Diagram of the co-infection experiment. Cells were bound with the primary IAV (WSN) at 4°C using an MOI of ~3, unbound virus was removed, and the secondary IAV (WSNIso) was added at an equal MOI at the indicated times (red arrows) after the 37°C temperature shift. Cells were labeled 8 hr p.i. with WSN, resulting in increasingly shorter durations of the WSNIso infections.

(B) Representative control images of the WSNIso gene segment labeling to account for the shorter durations of the infection. Cells were only infected with WSNIso.

(C) Representative images of the WSN (green) and WSNIso (red) gene segment labeling in co-infected cells. The secondary WSNIso infections were initiated at the indicated times after WSN binding (listed on the left). The durations of the WSNIso infections are listed on the right.

(D) Distribution of the WSNIso infection stages in the control samples (no WSN primary infection) as the duration of the infection becomes shorter.

(E) Left: distribution of the WSNIso infections with WSN primary infection. Right: combined distribution of the WSN infection stages in the cell populations from all samples.
remained largely unaffected (Figures 6C and S8C). The decrease in the WSNiso RCPs was accompanied by a small decrease in the percentage of WSNiso-infected cells, which was distinct from the one associated with shorter infection times (Figure 6E). As the time interval between the two infections increased, the WSNiso-infected cell population also showed a pronounced shift to stage 1 (cell entry), instead of the expected shift to stage 3 (replication) that is attributed to the shorter infection duration. Because the WSNiso gene segments were still visible in the cells, it implies the loss in productive WSNiso infections (stages 3–5) occurred at these times because the primary infection impairs the nuclear import of the WSNiso gene segments, but not their entry into the cell. By considering the temporal aspect of the replication cycle, these results show that productive IAV cell co-infections are restricted once an IAV genome has reached the nucleus and initiated the replication process (Figure 7).

**DISCUSSION**

All viruses use particular cell compartments for genome replication. Despite the advantages of monitoring cell infections through the viral genome, protein-based reporters are more commonly used due to technical limitations. This is especially true for RNA viruses. Therefore, we sought to develop an approach to detect the location of RNA viral genomes in situ and based the methodology on the versatile PLP labeling properties (Ke et al., 2013; Larsson et al., 2010). We used IAVs as a test case for the technique due to the challenges associated with labeling eight relatively short RNA gene segments and the limited data about the IAV life cycle before replication initiation. By combining the PLP labeling with a single-cell analysis, the approach provided semiquantitative data about the IAV gene segments in individual cells and could distinguish cell infections by IAVs that vary by single nucleotides. The addition of a temporal parameter made it possible to follow gene segment dynamics from entry through replication (Figure 7), monitor the stochastic aspects of IAV entry, and define the time window for productive cell co-infections.

The PLPs, which facilitate the labeling, also provide in situ resolution of IAV sequences that is unattainable with conventional methods. We used the single-nucleotide specificity to alleviate bias in the co-infection experiments that showed the replication of a second IAV is restricted once the replication of the primary IAV initiates (Figure 7). While the time frame may vary among strains and cell types, it is reasonable to assume that reassortment events happen less frequently when replication of a secondary IAV genome is restricted. In our system, the restriction was observed when the time interval between infections reached ~2 hr, which supports previous results examining the temporal contributions to reassortment in IAVs (Marshall et al., 2013). However, the labeling approach revealed that secondary IAV gene segments still enter the cell but seem unable to reach the nucleus for replication. From a mechanistic aspect, impairing nuclear import once replication begins would benefit IAVs, because it would prevent new gene segments from recycling into the nucleus when they are needed for viral packaging at the plasma membrane.

In addition to being ideal for investigating the cell machinery that facilitates viral genome import and trafficking, this approach can potentially make several aspects of viral infections in vivo more accessible and be developed further for diagnostic applications. For instance, a sequence analysis revealed that only seven PLPs are needed to identify and differentiate among almost all known IAVs and influenza type B viruses (Figure S9). By including additional PLP permutations, HA and NA subtypes can be identified (Gyarmati et al., 2008), as well as the species of origin for each gene segment. However, this strategy would require further development for clinical specimens.

Viral protein labeling is a sufficient infection reporter in many cases, but genome labeling is more applicable for differentiating highly similar viruses, especially those with changes in inaccesible epitopes, such as transmembrane domains (da Silva et al., 2015; Nordholm et al., 2013), or epitopes that are removed (e.g., signal sequences) or shielded by glycans (Krammer and Palese, 2015; Stewart-Jones et al., 2016). Genome labeling is also more appropriate for analyzing different aspects of entry, which are...
for 20 min at room temperature, and 1.5 mL trypsinized 293T cells in OMEM at a density of $\sim 10^5$ cells/mL were added to each mixture. The mixture was incubated 15 min and transferred to the MDCK cell flask at 37°C. At 24 hr post-transfection, media was replaced with infection media (DMEM with 0.1% FBS, 0.3% BSA, 100 U/mL Penicillin-Streptomycin, and 4 μg/mL TPCK-trypsin). Viral titers were determined by calculating the median tissue culture infectious dose (TCID50/mL) on MDCK cells as described (da Silva et al., 2015; Reed and Muench, 1938). WSN and WSNiso sequencing (Eurofins MWG Operon) was performed using PCR-amplified cDNA copies of the vRNAs extracted from isolated particles.

**Viral Particle Isolation and Immunoblotting**

WSN or WSNiso viral particles were passaged in MDCK cells, the culture media was clarified by centrifugation (1,000 x g; 5 min), and the viral titer were measured. Equal infectious particle numbers were then sedimented (115,000 x g; 60 min) through a 0.2 M sucrose cushion as previously described (da Silva et al., 2015). The isolated particles were resuspended in equivalent volumes of Laemmli sample buffer, resolved by SDS-PAGE, transferred to a polyvinylidene fluoride (PVDF) membrane, and processed (da Silva et al., 2013) using the HA antiserum and antisera raised against purified recombinant NA, M1, and NP expressed in E. coli.

**MDCK Cell Infections, Co-infections, BFLA1 and LMB Treatment, and Fixation**

MDCK cells grown on 3.5 cm dishes (qPCR analysis), or chamber slides (NP, PA, and gene segment labeling), were incubated at 4°C with infection media containing WSN at a low (0.3) or high (3) MOI for 30 min to facilitate binding. Unbound virus was removed, cells were washed twice with 4°C PBS, new 37°C infection media was added, and the cells were placed at 37°C. For co-infection experiments, WSN was bound to cells at 4°C using a MOI of ~3, unbound virus was removed, and WSNiso was added at an MOI of ~3 to the cells before the 37°C temperature shift to initiate entry or at the indicated time after the shift. To prevent IAV fusion, cells were treated with 100 nM of the vacuolar ATPase inhibitor BFLA1 from 1 hr before binding until labeling 2 hr p.i. To inhibit CRM-1-mediated nuclear export, cells were treated with 10 μM LMB 5 hr p.i. At the indicated times, cells were fixed with cold 4% (w/v) PFA in PBS (pH 7.4) treated with DEPC (PBS-D) for 20 min, followed by two PBS-D washes and three 5 min fixations with increasing ethanol concentrations (70%, 85%, and 99%). Slides were stored at ~80°C.

**In Situ PLP Ligation, Rolling-Circle Amplification, and Barcode Labeling**

The following procedure was carried out in 50 μL hybridization chambers created by attaching secure seals to the slide. Cells were washed with PBS-D containing 0.05% Tween 20 (PBST-D), treated with 0.1 M HCl-DEPC for 10 min, and washed. cDNA synthesis was then performed for 3 hr at 37°C using 20 μL/tRT tRT in 1× tRT buffer with 0.5 mM dNTPs, 0.2 μg/μL BSA, 1 μM LNA-modified cDNA gene segment primers, and 0.8 U/μL Ribonuclease inhibitor. After washing (PBST-D), cDNAs were fixed with 4% (w/v) PFA for 10 min and washed (PBST-D), PLPs (0.1 μM of each) were annealed and ligated using 0.5 U/μL Ampligase in 1× Ampligase reaction buffer containing 20% formamide, 0.2 μg/μL BSA, 50 mM KCl, and 0.4 U/μL RNase H for 30 min at 37°C followed by 45 min at 45°C. Cells were washed (PBST-D) and RCPs were synthesized using 1 U/μL phi29 polymerase in 1× phi29 buffer with 5% glycerol, 0.25 mM dNTPs, and 0.2 μg/μL BSA for 16 hr at 25°C. Cells were washed and RCPs were labeled after 20 min at 37°C using 0.1 μM of each barcode probe in 30 mM saline-sodium citrate buffer (pH 7.0) containing 300 mM NaCl and 20% formamide. After a final wash, cell nuclei were stained with 0.5 μg/mL Hoechst for 10 min, secure seals were removed, and the cells were dehydrated for 1 min in an ethanol series (70%, 85%, and 99%) and mounted. To exchange barcodes, coverslips were removed, new secure seals were placed, and three 5 min incubations were performed with 65% formamide at 65°C before washing (PBST-D).

**Mouse Infections, Lung Sectioning, and IAV vRNA Labeling**

Experiments were performed in accordance with the local ethical committee (Stockholms Norra djurförsöksetiska nämnd). Male 6- to 8-week-old C57BL/6...
mice (Janvier Labs) were sedated by intraperitoneal injection of 80 mg/kg ketamine and 5 mg/kg xylazine (Rompun, Bayer) and intranasally inoculated with \( \sim 5 \times 10^4 \) plaque-forming units (p.f.u.) of WSN or WSNiso or co-infected with WSN and WSNiso (\( \sim 2.5 \times 10^4 \) p.f.u. of each). Mice were sacrificed 2 days post-inoculation and lungs were inflated with a 3:1 mixture of 4% PFA to FSC22 cryosectioning media (Leica), tied off at the trachea, extracted, and fixed in 4% PFA for 4 hr. Lungs were placed in base molds (Leica) covered with FSC22 and frozen at \(-80^\circ\)C, and 20 \( \mu \)m airway sections were cut with a Biosystems CM3050 cryostat (Leica). Sections were attached to super-frost slides, washed twice (PBST-D), fixed with 4% PFA for 45 min, washed, and treated with 2 \( \mu \)g/ml pepsin in 0.1 M HCl-DEPC for 1.5 min. The sections were dehydrated with a 2 min ethanol series (70%, 85%, and 99%) and secure seals were mounted. PLP labeling was performed in 100 \( \mu \)l reactions as described for cells with the following changes: cDNAs were synthesized for 16 hr at 45°C and fixed with 4% (w/v) PFA for 30 min, and RCPs were synthesized at 37°C and labeled for 30 min.

### Image Acquisition and Processing

To capture RCP signals, multiple focal plane images were acquired using an Axiosplast epifluorescence microscope (Zeiss) and merged using maximum-intensity projections (Zen software). Single-cell analysis was performed using CellProfiler software (Kamentsky et al., 2011). To increase the nucleus segmentation accuracy, a Gaussian filter correction was applied to the Hoechst staining and refined using shape descriptors. Individual cells were defined by automated thresholding using the segmented nuclei as a seed, followed by watershed segmentation using the cytoplasmic autofluorescence signal. For RCP counting, top-hat filtering was used to enhance the RCP resolution and each RCP signal, assigned by localization to a particular cell or nucleus, was tabulated.

### qRT-PCR

cDNAs of segment 5 (NP) and segment 6 (NA) were generated from equivalent amounts of cellular RNA using segment-specific primers (0.25 \( \mu \)M) with a vRNA tag (5'-GGCCGTACGTTGGCCGAAAT-3') at the 5’ end as previously described (Kawakami et al., 2011). qPCR was performed with a CFX96 Real-Time System (Bio-Rad) and iQ Universal SYBR Green Supermix according to the manufacturer’s instructions (Bio-Rad). Relative vRNA quantities were calculated using CFX manager software v.3.1 (Bio-Rad) and a standard curve for each vRNA. Cycling parameters were 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C for 30 s.

### In-Solution Labeling of vRNAs from IAVs, MDCK Cells, or Mouse Lung Tissue

RNA templates were extracted using an RNasey mini kit from sedimented WSN and WSNiso particles, or \( \sim 1 \times 10^6 \) MDCK cells or \( \sim 30 \) mg of mouse lung tissue. MDCK cells were infected with WSN or WSNiso (MOI of \(-0.5\)), or co-infected with equal infectious units of WSN and WSNiso for 16 hr. Lung tissue was isolated from 8-week-old female BALB/c mice, intranasally infected under isoﬂurane sedation with 5.8 \( \times 10^3 \) p.f.u. of WSN or WSNiso, or co-infected with 2.9 \( \times 10^6 \) p.f.u. of both WSN and WSNiso diluted in 50 \( \mu \)l PBS. Following cervical dislocation 12 hr p.i., lungs were harvested and transferred to RNAlater (QIAGEN) for 12 hr at 4°C and stored at \(-80^\circ\)C. The animal experiments were performed in strict accordance with the guidelines of the German animal protection law. All animal protocols were approved by the relevant German authority (Behörde für Stadtentwicklung und Umwelt Hamburg).

cDNAs were synthesized using equal RNA amounts, 0.1 \( \mu \)l of the gene segment primers, and RT according to the manufacturer’s instructions. Each cDNA (10 \( \mu \)l) was combined with a 10 \( \mu \)l ligation mix containing 200 \( \mu \)M PLPs, 0.4 \( \mu \)g/\( \mu \)l BSA, 2x Ampligase buffer, and 500 mU Ampligase enzyme for 40 min at 55°C. The ligated PLPs were mixed with 200 \( \mu \)M phosphororse in 1x phophorose buffer, 125 \( \mu \)M dNTPs, and 0.2 \( \mu \)g/ml BSA for 2 hr at 37°C. The reaction was terminated at 65°C for 5 min and the RCPs were labeled with 5 \( \mu \)M barcode probes in hybridization buffer (50 mM Tris-HCl [pH 7.5], 5 mM EDTA, 1 M NaCl, and 0.01% Tween 20) for 2 min at 75°C and 15 min at 65°C. RCPs from fixed volumes (20 \( \mu \)l) were quantified by automated amplified single-molecule detection (ASMD) with an Aquila 400 (Q-Linea).

### Statistical Analysis

Means from technical replicates of qPCR experiments were used for normalization, and the SD was calculated from the means of independent replicates. Box-and-whisker plots show the 5%–95% confidence interval (CI) for the indicated single-cell samples, and the percentages of nuclear localizations are presented as population means with the SD. Infection stage analysis was performed using all raw single-cell data collected for each condition. All statistical analyses were performed with GraphPad Prism 6.0.

### SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and nine figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2017.06.021.

### AUTHOR CONTRIBUTIONS


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