Endosomes supporting fusion mediated by vesicular stomatitis virus glycoprotein have distinctive motion and acidification

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Abstract
Most enveloped viruses infect cells by binding receptors at the cell surface and undergo trafficking through the endocytic pathway to a compartment with the requisite conditions to trigger fusion with a host endosomal membrane. Broad categories of compartments in the endocytic pathway include early and late endosomes, which can be further categorized into subpopulations with differing rates of maturation and motility characteristics. Endocytic compartments have varying protein and lipid components, luminal ionic conditions and pH that provide uniquely hospitable environments for specific viruses to fuse. In order to characterize compartments that permit fusion, we studied the trafficking and fusion of viral particles pseudotyped with the vesicular stomatitis virus glycoprotein (VSV-G) on their surface and equipped with a novel pH sensor and a fluorescent content marker to measure pH, motion and fusion at the single particle level in live cells. We found that the VSV-G particles fuse predominantly from more acidic and more motile endosomes, and that a significant fraction of particles is trafficked to more static and less acidic endosomes that do not support their fusion. Moreover, the fusion-supporting endosomes undergo directed motion.

KEYWORDS
endocytosis, endosomes, fluorescent pH sensor, organelle motility, vesicular stomatitis, viral trafficking, virus entry

1 | INTRODUCTION

Viruses infect host cells after binding to host receptors at the cell surface. For most enveloped viruses, infection requires internalization and trafficking through the endocytic pathway. Receptor binding occurs via glycoproteins on the viral surface and conformational changes of these proteins mediate fusion between the viral and host membranes, leading to infection. Some viruses fuse early in the endocytic pathway, while others must be trafficked to late endosomes to encounter conditions required to trigger fusion. These may include processing by proteases, binding to endosomal receptors, as well as favorable conditions of pH, lipid composition and ionic milieu. Late endosomes are more acidic than early endosomes, and endo-lysosomes and lysosomes are more acidic still. In addition, each progressively more acidic compartment in the pathway has its own lipid and protein components. In many cases endosomal pH is the primary fusion trigger, though in some cases there are other essential triggers. Viruses that fuse in late endosomes include influenza virus and lymphocytic choriomeningitis virus. In contrast, Semliki forest virus and avian sarcoma and leukemia virus (ASLV) fuse in early endosomes. Early endosomes can be further distinguished as subpopulations with differing characteristics and rates of maturation. These subpopulations...
can be important for cargo sorting and fate. While some cargos preferentially sort into faster-maturing and more motile “dynamic” early endosomes, other cargos are preferentially sorted to more slowly maturing and less motile “static” early endosomes (nomenclature as per²). Influenza virus is preferentially sorted to a subset of motile endosomes. In contrast, ASLV nonpreferentially sorts to static and dynamic endosomes in proportion to the abundance of those endosomes while reportedly preferentially fusing with less motile, slow-moving compartments in a cell line expressing TVA950, the transmembrane form of the ASLV receptor. Hence among viruses studied with respect to trafficking through subpopulations of endosomes, one virus (influenza) has been reported to preferentially sort to dynamic endosomes, while the other (ASLV) has been reported to preferentially fuse in static endosomes. This stark difference led us to ask whether particles bearing the glycoprotein of another virus, that is, vesicular stomatitis virus (VSV), fuse in dynamic or static endosomes. We addressed this question by single particle fluorescence microscopy correlating fusion with the pH state and motility of endosomes in live cells. We also characterized the fate of nonfusing particles. Although we chose to study trafficking and fusion directed by the well characterized VSV glycoprotein⁹-¹¹ as a model, the methods developed in this study should be applicable to study trafficking and fusion of other enveloped viruses at the single particle level in live cells.

VSV is an enveloped virus commonly used as a model for the study of the trafficking and fusion of enveloped viruses. It is a member of the family Rhabdoviridae, primarily causes acute illness in hoofed animals, and can cause mild symptoms in humans.¹² Cell entry and fusion of VSV is mediated by its envelope glycoprotein G. VSV-G is a class III fusion protein known for undergoing reversible conformational changes.¹³,¹⁴ The primary host receptor for VSV is the low-density lipoprotein receptor (LDL-R),¹⁵,¹⁶ which has previously been found to sort into dynamic endosomes. After binding its receptor, VSV undergoes a mode of clathrin-mediated endocytosis (CME) requiring actin,¹⁷,¹⁸ is trafficked through the endocytic pathway encountering progressively lower pH, and is triggered to fuse with an endosomal membrane at low pH.¹⁹,²⁰

To examine trafficking and fusion mediated by VSV-G and address the question of whether the virus fuses in dynamic or static endosomes, we use murine leukemia virus (MLV) pseudotyped with VSV-G. Our VSV-G pseudotype particles contain gag-mKate2 as a content marker to demonstrate full fusion and a membrane anchored FRET-based ratiometric pH-sensor to monitor changes in acidification as the virus particles are endocytosed and trafficked in live cells. This system allows us not only to correlate fusion with endosomal pH at the single particle level, but also to follow the velocity and directedness of motion of virus-containing endosomes. By using this approach, we describe differing characteristics in the populations of endosomes that support fusion events versus those that do not. Endosomes that are more motile and more readily acidified support VSV-G driven viral fusion events, whereas more static endosomes do not appear to support fusion of VSV-G pseudoviral particles.

## Results

### 2.1 Calibration and characterization of a FRET-based pH sensor in viral particles

Previous study used a FRET-based pH sensor consisting of mTFP1 and eYFP as a chimera with the ICAM1 transmembrane domain (ICAM1-TMD) to monitor pH during the trafficking of MLV bearing the envelope protein from ASLV. The paper’s authors identified two key limitations of the mTFP1-eYFP pH sensor: the particles were not observable in the perinuclear region due to noise contributed by cellular autofluorescence exceeding the signal of the sensor, and the sensor could not reliably detect pH values below 5.2. To improve on these issues, we designed a new ratiometric pH sensor using mTFP1 and mCitrine as a FRET pair attached to ICAM1-TMD (Figure 1AB). mCitrine is approximately 1.5-fold brighter than eYFP and has a lower pKa of 5.7 compared with 6.9 of eYFP. Both mTFP1 and mCitrine are translated from a single open reading frame with ICAM1-TMD. The fluorescent proteins are thus expressed in a one-to-one ratio permitting the fluorescence intensity ratio of mTFP1 over mCitrine to be used as a pH indicator (Figure 1B). The use of a ratiometric probe is key for live cell imaging because changes in focal plane affect both emissions wavelengths equally, permitting the measurement of pH even when the probe is on a moving object such as a viral particle or endosome. In unfused particles, ICAM1-TMD anchors mTFP1-mCitrine in the membrane of the viral particle topologically oriented toward the extracellular environment or, following endocytosis, into the endosomal lumen. Following fusion, the FRET pair is expected to sense endosomal pH via its luminal orientation with the TMD now embedded in the endosomal membrane.

MLV particles pseudotyped with VSV-G and containing the FRET pH-sensor were produced as described in Section 4. The particles were also labeled with a content marker, Gag-mKate2, which is cleaved to Gag and mKate2 during capsid maturation. To calibrate the sensor, particles were deposited on a poly-lysine coated glass coverslip and imaged in citrate–phosphate buffers of known pH. Individual, triply labeled particles were selected and the mean emission intensities from 8.9 nm wide spectral bands were extracted from each particle region. Particle regions were background subtracted and the emission intensity ratios of mTFP1 to mCitrine (I₄₃₉₄⁻⁴₅₇ / I₅₂₅₀⁻₅₃₈₉) were calculated for each pH. The sensor fluorescence ratio undergoes a transition from a high to a low FRET state as the pH is decreased from 7.4 to 4.2 (Figure 1C). Based on the measured fluorescence, the FRET sensor is well-equipped to distinguish pH in the range of 4.5–6.2 but is less discriminating between pH 6.2 and 7.4. At very low pH (4.2–4.5) there is high particle-particle variability in fluorescence ratio (Figure S1A,B). When buffers of decreasing pH were applied sequentially, most (10/13) individual virus particles’ fluorescence ratio responses increased with decreasing pH as expected. However, three of 13 particles were unresponsive or displayed a scattered fluorescence ratio response (Figure S2A,B). We do not know why the fluorescence of a few particles displayed these unexpected pH dependencies, but one explanation could be that one or the other fluorescent protein is not properly folded or their folding is differently sensitive to pH in these outlier particles.
To assess the performance of the pH sensor over repeated exposures and the variation of the fluorescence ratio for a single particle on a frame-to-frame basis, virus particles were imaged on a coverslip for 16 min in neutral live cell imaging buffer before imaging was paused, a buffer of pH 5.2 was introduced, and imaging was continued (Figure S2C). While some particles could be observed for many minutes after the pH change, several particles were visible only briefly after the introduction of low pH buffer or were no longer visible at all. As expected, the fluorescence ratio increased with the addition of low pH buffer for all particles still observed after buffer replacement (Figure S2C). The mean values (Figure S2D) and standard deviations (Figure S2E) of the fluorescence ratios over the first six frames (in pH 7.4 buffer) were calculated for each of these particles. The median value of the mean ratio for all particles over the first six frames was 0.58, which corresponds according to the calibration curve of Figure 1C to pH 6.8. The median value of the standard deviation was 0.045, which is equivalent to a range of pH 6.6–7.2 around the median initial ratio of all particles, slightly below the pH 7.4 buffer surrounding the particles. Once the buffer was shifted to pH 5.2, the median fluorescence ratio value of the mean fluorescence ratio for all particles in the six subsequent frames was 1.38, which corresponds to a pH of 5.1 according to the calibration curve. This is approximately the same as the pH 5.2 buffer surrounding the particles, further verifying the accurate readout of the sensor at low pH.

2.2 Visualization, pH dependence and time-course of fusion of VSV-G mediated fusion events

Having established the characteristics of the new FRET-based pH sensor in isolated viral particles, we used this sensor to track the measured pH (as calculated from the calibration curve in Figure 1C) of endosomes harboring VSV-G pseudotyped viruses and their eventual fusion by live cell imaging. Parameters to describe these endosomes included their pH, changes in pH, and their displacement and velocity of motion within the cell. Pseudoviral particles were spun onto A549 cells in the cold, a field of view was selected for imaging, and the cold buffer was replaced with warm (37°C) imaging buffer immediately prior to commencing imaging, which was conducted at 37°C. Fusion events were identified by a sudden drop of the mKate2 signal while the mTFP1 and mCitrine signals persisted, as indicated in the representative example shown in Figure 2A by the magenta arrow at 28.3 min. Fusion events, initially assessed by direct visualization, were confirmed by quantitation of the raw and background subtracted signals of all three channels (Figure 2B,C) as described in the Section 4.

The displacement and velocity of the particle shown in panel A were plotted as a function of time in Figure 2D and E, respectively. The displacement and velocity results from this example particle, as well as from many other particles will be described in a later section.

For each particle undergoing fusion, the ratio of the mTFP1 to mCitrine fluorescence intensity was extracted and plotted at 20 s intervals, the frame rate of imaging, over all frames in which the particle was observed (Figure 3A). The time of fusion, determined as the time of disappearance of the mKate2 signal (Figure 2A,C), is shown as a vertical dashed line in Figure 3A. Several interesting phenomena of change in fluorescence ratio were observed immediately prior to or following fusion events. In several examples, the pH sensor signal disappeared or experienced significant frame to frame variation within 5 min following fusion (Figure 3A: examples ii, iv, v, ix, xii, and xiv). This is similar to the phenomenon observed when virus particles were rapidly acidified on a coverslip (Figure S2C) and may be due to loss of

**FIGURE 1** Design and calibration of a FRET-based pH sensor to measure cellular trafficking and the pH of fusion of viruses in cells. (A) Schematic of an MLV particle pseudotyped with VSV-G glycoprotein and bearing a FRET-based pH sensor with mKate2 as a content marker. (B) Schematic representation of the pH sensor construct. The preprotrypsin leader sequence (Leader) is followed by 3xFLAG sequences, followed by the fluorescent protein and ICAM1 transmembrane domain sequences. Linkers between components are (i), (ii), (iii) and (iv) where (iv) separates the first four amino acids of the ICAM1 cytoplasmic domain and the stop codon indicated by the red asterisk. The sequence of linker (ii) between mTFP1 and mCitrine is RSTLSQFGT. (C) The pH sensitive mTFP1-mCitrine FRET pair on the pseudotyped viral particle (as shown in “A”) is calibrated with buffers of known pH on coverslips. The ratio of the fluorescence emission intensities at 494 nm/530 nm is used as a proxy for FRET efficiency and plotted as a function of pH. The error bars represent the standard errors of the mean (SEM). Numerical values of the mean and SEM and numbers of individual particles evaluated at each pH are listed in Figure S1B.
signal from the FRET donor and acceptor, perhaps caused by quenching of the signal due to protonation of the chromophore or irreversible protein unfolding at very low pH (less than pH 4.2). Several fusion events occurred during periods of increasing fluorescence ratio indicating acidification (Figure 3A: examples iii, vi, vii and xviii.). Even transient shifts to lower pH may trigger viral content release. A decrease in ratio (alkalinization) immediately follows fusion (Figure 3A: examples vi, vii, x, xi, xiii.) for five of the fusion events. Temporary alkalinization of endosomes following fusion could be caused by a leaky fusion event in which there is a loss of contiguity in the endosomal membrane allowing the permeation of cytosolic contents into the endosome.

The mean time of fusion (content release), counted from the start of imaging and derived from the 18 events shown in Figure 3A, is distributed primarily in observation times <45 min with a mean value of 25.2 min (Figure 3B). The mean fluorescence ratio at fusion was 1.24 ± 0.08 (SEM), which is the equivalent of pH 5.3 (pH 5.2–5.4). The total range of pH values measured at viral fusion extended from a fluorescence ratio of 0.67 (pH 6.4) to 1.78 (pH 4.2) (Figure 3C).

For virus particles encountering progressively more acidic environments as they move along the endocytic pathway, the fluorescence ratio is expected to increase. For eight of the 18 fusing particles, the change in fluorescence ratio from the first time the particle is observed to the time of fusion was positive as expected, with a mean increase of 0.17 ± 0.77 over all particles observed (Figure S3A). Five of the 18 fusing particles had very small fluorescence ratio changes that fell within the expected frame-to-frame variability of the probe (2 SD of frame-to-frame ratio difference for single particles; Figure S2E) and thus cannot be interpreted as having a meaningful change in pH during fusion events. (Figure 3A: examples iii, vi, vii and xviii.).
the observed time before they fuse (Figure S3A). Five of the 18 fusing particles demonstrated increasing pH between the time the particle was first observed and the time of fusion. This latter result may both reflect physiologic fluctuations in the pH in the endosome and the possibility that observation of some particles may have only commenced soon before fusion (Figure 3A), when the particle may already have been in a moderately acidic environment.

Rapid fluctuations in pH during the trafficking of fusing virus particles were a particularly interesting phenomenon observed in several cases (Figure 3A: examples i, iii and xvii). Similar fluctuations have been observed during trafficking of influenza virus8,26 but were not observed to the same extent during the trafficking of ASLV.5 For trace i. in Figure 3A, the measured pH decreases from 5.7 to a local minimum of under 4.2 at a rate of at least \( \frac{0.4}{0.4} \) pH units per minute during the 4 min immediately following fusion. The pH is rapidly restored to a calibrated pH of 5.6 at the rate of at least 0.6 pH units per minute during the next 2 min (Figure S3B). This pattern of rapid acidification followed by alkalinization is also seen in trace xvii in Figure 3A and is quantified in Figure S3C. Fluctuations in pH over the course of minutes may reflect proton leak variability over time\textsuperscript{27,28} or other transient changes in endosomes. The very rapid rates of acidification following alkalinization are consistent with the previously observed general kinetics of V-ATPase when reacidifying endosomes.\textsuperscript{28}

2.3 Comparison of acidification and properties of motion for fusion permissive and nonpermissive endosomes

We next compared the features of fusion permissive endosomes and endosomes that did not permit fusion. The FRET-based pH sensor (Figure 1A, B) is topologically oriented toward the extracellular/luminal space both while embedded in the viral membrane during trafficking and in the endosomal membrane after fusion, permitting measurement of pH during viral trafficking as well as during and following fusion. Fusion events were identified as described in Figure 2 and in the Section 4 in 13 separate experiments conducted on seven separate days. All monodisperse, triple labeled virus particles (mTFP1, mCitrine, gag-mKate2) that were observable for at least 20 frames (\( \geq 7 \)) min were identified in experiments containing at least one fusion event. Of 88 total particles that were detected and tracked, 18 underwent fusion corresponding to a fusion efficiency of 20.4% for qualified triply labeled particles. We empirically observed a number of significant differences between fusion permissive and fusion nonpermissive endosomes.

Fusion permissive endosomes display a higher maximum degree of acidification than fusion nonpermissive endosomes. The pH range falling within each histogram bin is shown in blue; fluorescence ratios were converted to pH range using the calibration curve (shown in Figure 1C). 18 fusion events were analyzed (out of 88 particles meeting the criteria described in the Section 4) and are represented here (\( n = 18 \))

**FIGURE 3** Kinetics and pH characteristics of VSV-G mediated viral particle trafficking and fusion. (A) Fluorescence emission intensity ratio plotted over time for all particles that fuse. The dashed vertical line represents the time of fusion as determined by the spreading or disappearance of the mKate2 signal. (B) Frequency histogram of the time from the beginning of imaging to the time at which fusion occurs. (C) Frequency histogram of the 494 nm/530 nm emission intensity ratios at the time of fusion using a six-frame moving average. The pH range falling within each histogram bin is shown in blue; fluorescence ratios were converted to pH range using the calibration curve (shown in Figure 1C). 18 fusion events were analyzed (out of 88 particles meeting the criteria described in the Section 4) and are represented here (\( n = 18 \))
a median minimum pH of 5.3 in fusion nonpermissive endosomes (Figure 4A); fluorescence ratios were converted to pH values using the calibration curve of Figure 1C. The pH sensor can best differentiate between pHs in the range of 4.5–6.2 and the upper plateau of the fit for the calibration curve is at pH 4.2, meaning that values at or below 4.2 represent highly acidic values and cannot be interpreted precisely. Despite the greater acidification of fusion permissive endosomes, both categories of endosomes had similar pH values when they were first observed (pH ~5.5, Figure 4B), and similar values for the most alkaline pH they ever experienced during the whole observation period (pH ~6, Figure 4C). Our observation that most virus particles, regardless of their ability to fuse, appear to start in endosomes with calibrated pH values below seven may be indicative of an initial rapid trafficking period into mildly acidic early endosomes during the 1 to 2 min required to refocus the microscope after the addition of warm buffer. Prior study found that up to 50% of ASLV viral particles reach a compartment of pH 6.2 or lower within 2 min of the initiation of viral trafficking.5

We also calculated the fluorescence ratio difference over the entire time the FRET pair could be observed in a given endosome. According to this analysis, fusion permissive endosomes became more acidified from their starting state than fusion nonpermissive endosomes. The median ratio changes were +0.24 and +0.01 units, respectively (Figure 4D). The difference between the initial and final pH was −0.4 units for fusion permissive and −0.1 units for fusion nonpermissive endosomes (Figure 4E).

Fusion permissive and nonpermissive endosomes displayed different displacements and velocities of displacement during the observed trafficking times in the cell. Velocities were measured from particle localizations in consecutive frames and a three-frame (1 min)

**FIGURE 4** Fusion permissive and fusion nonpermissive endosomes exhibit different extents of acidification. (A) Maximum emission intensity ratios (494 nm/530 nm) achieved in tracks based on a six-frame rolling average for fusion permissive and fusion nonpermissive endosomes. The right Y axis displays reference pH values (note that the relationship between pH and ratio is not linear; see Figure 1C). All selected particles were nonoverlapping with other particles, had apparent diameters less than ~1.5 μm, and were triple labeled. Nonfusing particles are from experiments, in which fusion events were observed in other locations. (B) Initial emission intensity ratios over the first six observable frames for fusion permissive and fusion nonpermissive endosomes. (C) Minimum emission intensity ratios in tracks based on a six-frame rolling average for fusion permissive and fusion nonpermissive endosomes. (D) Difference between emission intensity ratios at the time at which the particle is first observable (start) and the time at which the pH sensor from the particle is last observable (end) for fusion permissive and fusion nonpermissive endosomes. (E) The initial and final emission intensity ratios from “D” converted to pH using the fit of the calibration curve (Figure 1C) and plotted as pH differences over the observable time for fusion permissive and fusion nonpermissive endosomes. No change in pH is indicated with a horizontal dashed line. In all panels, points represent the values for individual particles and horizontal lines represent the median value of each group. Numbers of fusing particles and nonfusing particles are n = 18 and n = 70, respectively. A Mann–Whitney test (nonparametric assumption) was used to assess the significance of the differences in the distribution of fluorescence ratio values (A–D) or pH (E) (*p < 0.05, **p < 0.01, ****p < 0.0001, ns, not significant). In E, observations where the initial and final values fell above or below the lower and upper limits of the calibration curve fit were excluded from analysis and the numbers are n = 17 for fusing and n = 68 for nonfusing particles.
rolling average was taken for each time point. While the motions of both fusion permissive and nonpermissive endosomes were mostly slow and included stationary segments, leading to median velocities of all segments in a track of \( \mathbf{0.01} \) \( \mu \text{m/s} \) (Figure 5A), the fastest steps in fusion permissive endosomes had higher velocities (\( \mathbf{0.05} \) \( \mu \text{m/s} \)) compared with those observed in nonpermissive endosomes (\( \mathbf{0.01} \) \( \mu \text{m/s} \)) (Figure 5B). The total displacement, defined by the distance traveled between the coordinate points at which the particle was first and last observed, was determined for both subpopulations of endosomes. The median total displacement of fusion permissive endosomes was higher than that of nonpermissive endosomes (Figure 5C). This result is somewhat surprising in light of the similar median velocities of the two subpopulations of endosomes, but suggests that the high maximum velocities found in fusion permissive endosomes make an outsized contribution to their total displacement. Finally, FRET signals from tracks of fusion permissive and nonpermissive endosomes could be observed for a similar length of time (Figure 5D), demonstrating that the difference in total displacement and maximum velocity did not depend on the total time for which the particle was observed.
2.4 | Mean squared displacement and trajectory analysis of fusion permissive and nonpermissive endosomes

Given the different maximum velocities and total displacements between fusion permissive and nonpermissive endosomes, a mean-squared displacement (MSD) analysis was conducted to determine whether the two subpopulations of endosomes exhibit different modes of motion. We used plots of MSD versus time to distinguish between random (diffusive) and directed motion. To facilitate this analysis, we took a randomly chosen 18-member subset from the nonfusion permissive endosomes such that equal numbers of the two subpopulations of endosomes were analyzed. The MSD (μm²) at each time step up to 400 s (6.7 min) was calculated and plotted for the 18 fusion permissive and 18 nonpermissive endosomes (Figure S4A, B). For better visualization, we also averaged the MSD values for all 18 tracks in each set at each time lag and plotted the averaged MSD at each time lag (Figure 6A). This averaged MSD plot is dramatically different for fusion permissive and nonpermissive endosomes, with fusion permissive-endosomes showing an upwards bending curve indicative of directed motion, while the fusion nonpermissive endosomes exhibited a more linear MSD versus time relationship indicating free (Brownian) diffusion (Figure 6A). As expected, there is higher variability at longer time steps since these are derived from fewer measurements. The same trend observed in the averaged data can be seen in many curves of individual endosomes (Figure S4A,B).

The MSD data from 17 out of 18 fusion tracks could be reasonably fit with a model for directed motion (Equation 1 in Section 4) with the velocity coefficient \( v \) as one of the fitting parameters. The particles were binned according to their velocity coefficients and moved with an average velocity coefficient of 0.0027 μm/s (Figure 6B). The velocity coefficients plotted for the fusion-permissive endosomes are consistent with previously published values for slow directed motion of endosomes containing influenza virus along microtubules or microfilaments. The difference in characteristics of motion and acidification between fusion permissive and nonpermissive endosomes are indicative of two distinctive populations of endosomes, one of which undergoes acidification and trafficking, and one of which is relatively static on the time-scale of trafficking and fusion of MLV particles bearing VSV-G (Figure 7).

3 | DISCUSSION

In this study, we have investigated the fusion of single virus particles mediated by the surface glycoprotein VSV-G in whole, live A549 cells and discovered that two subpopulations of endosomes with different characteristics of acidification and intracellular motion play a role in the fate of individual viral particles. Endosomes that did support fusion of internalized viruses were more acidic, more motile and underwent directed motion to a greater extent than endosomes that did not support fusion (Figure 7A). Based on our findings, we propose a model whereby the sorting of viral particles into more dynamic endosomes disposes particles to undergo fusion; conversely the sorting of virus particles to a more static subpopulation of endosomes traps these particles in locations where they are strongly disinclined to fuse (Figure 7B).

3.1 | Distinctive pools of endosomes and viral fusion

The presence of different pools of early endosomes has previously been established, with some less motile early endosomes maturing more
slowly, as defined by a longer time lag to the acquisition of Rab7, and some more motile early endosomes maturing more rapidly. Maintenance of these separate populations of dynamic and static early endosomes is dependent on intact microtubules and the early endosomal tethering complex CORVET, while sorting of cargo into subpopulations relies on differential clustering of the cargo at the cell surface. The significance of these separate pools to viral infectivity has only begun to be studied, with differing results as to whether viruses are more likely to be sorted to or fuse from dynamic or static early endosomes. We found that viral particles pseudotyped with VSV-G had a fusion efficiency (as indicated by content release) of approximately 20%. Fusion permissive endosomes moved a median 3.8 μm during their entire observation times (Figure 5C) with a mean velocity of 0.16 μm/min (Figure 6B). They also decreased their pH on average by ~0.4 units during the entire observation time (Figure 4E) and reached an average pH of 5.3 immediately before fusion (Figure 4C). Most of the remaining 80% of particles, consisting of the nonfusing subset, were contained in endosomes that had a lower motility and lower overall acidification than endosomes bearing particles that fused. For comparison, endosomes bearing virus particles that did not fuse moved on the median only 1.0 μm (Figure 5C), moved diffusively with no discernible component of directed motion (Figure 6A), and lowered their pH on average only by ~0.1 units during the entire observation time (Figure 4E). Only 10 of the 70 fusion nonpermissive endosomes achieved a change in pH above the median change in pH for fusion-permissive endosomes (Figure 4E).

The sorting of viruses and physiological cargoes to subpopulations of early endosomes may rely on a number of factors including cargo size and qualities of the receptor. The static subpopulation of endosomes is in the majority (65%) and the minority of endosomes are dynamic (35%) in BSC-1 cells. In the same cells, influenza virus is preferentially sorted to the dynamic endosome subpopulation as compared with static endosomes. When bound with its endogenous ligand, LDL, LDL-R, which also serves as the VSV-G receptor, is preferentially sorted to dynamic endosomes as well. In contrast to influenza virus, ASLV pseudotyped viral particles were found to sort predominately (70%) to less motile endosomes in CV-1 cells expressing the transmembrane receptor for ASLV, TVA. The 70% of ASLV sorted to less motile endosomes is consistent with the overall proportion (65%) of static endosomes. This suggests that ASLV is sorted to static and dynamic subpopulations in proportion to the abundance of each endosome subpopulation, without preference for either subpopulation. In our current study, VSV-G pseudotyped particles are also sorted without a clear preference for dynamic endosomes.

The indiscriminate sorting of VSV-G pseudotyped particles as compared with the preferential sorting of LDL-bound LDL-R to dynamic endosomes may be due to the smaller size of LDL compared with MLV particles. MLV particles are approximately five times larger in diameter than LDL, the physiological cargo for LDL-R. The importance of cargo and viral particle size with respect to trafficking has previously been demonstrated. VSV particles required an actin dependent mechanism of clathrin-mediated endocytosis as compared with small, defective viral particles that were otherwise similar. The MLV particles bearing VSV-G used in the present study are more spheroid in comparison to the bullet shape of VSV. They are ~25% smaller in length than the maximum dimension of VSV particles, but they are almost twice as wide, suggesting that they, like VSV, are large enough to potentially undergo clathrin-mediated endocytosis in a different manner than relatively small cargo such as LDL. Smaller particle size has also been shown to have important downstream effects in motility; smaller cargo are more likely to undergo fast directed motion.

Binding avidity for ligands to receptors has also previously been shown to affect cargo fate. Therefore, it is possible that the number of glycoproteins on a single viral particle that bind to multiple LDL-Rs in a single clathrin coated pit could also determine to which type of endosome the internalized virus is directed.

In general accordance with our findings that fusion nonpermissive endosomes did not achieve the same extent of acidification as fusion permissive endosomes, endosomes containing ASLV particles that failed to fuse showed a pH distribution shifted toward more neutral pH values compared with fusing particles. However, while ASLV was observed to undergo an initial period of acidification to a pH of approximately 6.2–6.0 and then stay close to that pH, we observed continued further acidification as well as significant physiological fluctuations in pH in endosomes bearing VSV-G viral particles (Figure 3A, 4C-E). One limitation of the prior study, as identified by the authors, with respect to measuring pH during trafficking of ASLV particles was difficulty in observing pH values below 6.0 due to cellular autofluorescence and, potentially, the higher pKa of the pH sensor used in that study. We did not observe significant cellular autofluorescence and utilized a FRET acceptor fluorescent protein that was 1.5-fold brighter than the acceptor protein used in the previous study. Moreover, mCitrine, used in our study as the acceptor fluorescent protein, has a pKa of 5.7, permitting measurements down to pH 4.2 in live cell imaging.

Determining the compartment identity of both fusion permissive and nonpermissive populations of endosomes would be fertile ground for future study. While the current experimental system has the advantage of a ratiometric pH probe in combination with a content marker, allowing the measurement of both pH and content release, the combination of fluorophores occupies a broad spectral range (approximately 475–750 nm emission wavelength). Recently developed near infrared fluorescent proteins may offer an exciting opportunity to express fluorescent chimeras of compartment markers (such as APPL1, EEA1, Rab5, Rab7 or Rab11) that will not interfere with imaging of the FRET-based pH probe and viral content marker described in this study. One intriguing possibility is that fusion nonpermissive endosomes recycle viral particles to the cell surface, a phenomenon recently described for Zika and influenza viruses, thus providing an additional layer of defense to the host cell.

### 3.2 Characteristics of VSV-G mediated fusion

Viral internalization and trafficking through the endocytic pathway are key requirements for productive fusion and infection by most enveloped viruses. We identified individual full fusion events based on content release of mKate2 (Figure 2A–C, Figure 3A). By simultaneously tracking the pH sensors on individual particles prior to, during, and following
fusion, we were able to quantify pH changes as well as the pH at which full fusion occurred. The mean lag time from initiating trafficking via addition of warm medium to fusion was 25 min for the 18 fusing particles observed (Figure 3B). Although the time course of fusion for viral particles may depend on cell type and viral model, it is interesting that our results are substantially similar to at least three previously published studies, that is, similar values of approximately 20–35 min were obtained in bulk assays for half-maximal infection or fusion of viral particles bearing VSV-G.41,42 This indicates that the FRET-based pH sensor does not appear to significantly change the kinetics of trafficking and/or fusion. The heterogeneity of the time course of fusion seen in our single particle experiments likely not only reflects biological variabilities of individual particles, but likely also reflects the heterogeneity of maturation of the endosomes harboring the viral particles, leading to a quite broad range of times to achieve fusion.

To our knowledge, this is the first single-particle measurement of the pH of VSV-G mediated full fusion in intact cells. Our data suggest that most full fusion and content release events occur at a mean pH ranging from 5.2 to 5.4 (Figure 3C) in whole cells. Interaction of VSV-G with liposomes, reflecting an early stage of fusion, requires a pH trigger reported to be anywhere from approximately 4.0 to 6.011,44 although the biggest structural changes in the VSV-G protein occurs between pH 5.5 and 6.5.14 Cell–cell fusion mediated by G protein expressed in the plasma membrane showed half maximal fusion at approximately pH 6.0–6.2,19,20 though the extent of cell–cell fusion has been demonstrated to be greater at pH 5.5.45 The efficiency of VSV fusion (lipid mixing) with a supported lipid bilayer is also greatest at pH 5.5.14 Differences in the target membrane with which VSV-G fuses and ionic conditions in the endosome likely influence the fusion of internalized viruses and environmental conditions in the endosome may differ significantly from these earlier model systems used to study membrane binding and fusion.

Previous findings suggest that VSV-G mediated virus entry includes an intermediate step of fusion whereby viral particles first fuse with intraluminal vesicles (ILVs) within endosomes.43 The endosomes containing these virus-bearing ILVs then mature, acidifying further and acquiring lysobisphosphatidic acid (LBPA). In the presence of the appropriate lipid composition and pH, ILVs then undergo retrofusion with the limiting membrane enabling the release of viral contents in the cytoplasm43,42 (Figure 7B). The findings that VSV-G may fuse with intraluminal vesicles is further supported by the fact that anionic lipids, such as LBPA, promote VSV-G content release46,47 and these lipids are potentially enriched in ILVs.48 The lower average pH of content release determined in our experiments compared with the interaction of VSV-G with plasma membranes (e.g., for cell–cell fusion) reported at higher pH could be explained by the proposed mechanism of viral content release requiring ILV retrofusion in the late endosome. Many of the content release events we observe may therefore represent retrofusion events that may require a lower pH than the initial fusion with ILVs and are known to depend on the lipid composition of the late endosomes.43,49,50 The small number (3/18) of content release events we observe at greater than pH 6.0 (Figure 3C) may represent direct virus fusion with the limiting membrane of the endosome. It is possible content release via retrofusion is the predominant mechanism while fusion with the limiting membrane only predominates when the retrofusion mechanism has been blocked in some way or is not advantageous within a given cell or endosome. This is supported by data showing that content release is markedly less efficient when ILVs cannot be formed or trafficked, but that some content release still occurs under these conditions.3,43

3.3 | General considerations of FRET-based pH sensors to track viruses and study membrane fusion

The FRET-based pH sensor consisting of mTFP1 and mCitrine enhanced our ability to observe the trafficking of viral particles to low pH compartments and the pH of endosomes following viral fusion. Important was the lower pKa and higher emission intensity of mCitrine compared with FRET donors in previously used sensors. This system in conjunction with a third soluble content probe included in the viral particle to measure content release (full fusion) should be useful to follow the trafficking and fusion of other viruses. Improved far red fluorescence proteins such as mCardinal51 or the recently developed FR-MQV52 could further enhance signal over noise, improving particle tracking and perhaps allowing identification of more fusion events in every field of view. The mTFP1-mCitrine sensor could also be used in future experiments to describe pH and motion of individual physiological cargos during trafficking, thereby solidifying the role of distinctive populations of endosomes on the fate of specific cargoes.

Important to the success of the current study was also the software developed to extract the traces of trafficking particles and their fusion events. Summing the fluorescence from three spectrally well-defined channels and defining appropriate surrounding backgrounds around moving particles in live cells was critical for identifying fusion events (Figures 2 and 3). In addition, this software enabled trajectory analysis to distinguish different modes of motion of organelles or viruses in cells (Figure 6).

The current study should also serve as a helpful illustration of the virtue and limitations of current pH sensing and tracking methods. As illustrated in many of our figures, the spread of biological variations of individual particles and the uncertainties of the measured parameters can be quite large. Careful calibration of the probes and analytical methods including defining the appropriate statistical descriptions of error is important. Despite these challenges, it is possible to extract meaningful new biological data from single particle tracking and pH measurements in cells as demonstrated in the present study. We expect that the continued development of new technologies in microscopy will further improve signal-to-noise and enable the tracking of many particles in the same field of view at high time resolution, thereby increasing assay throughput.

4 | MATERIALS AND METHODS

4.1 | Cell lines

A549 cells came from the lab of Judy White and were verified by ATCC Human Cell Line STR Profiling Service. A549 cells were grown
in Ham's F12 Nutrient Mixture with 10% FBS v/v and 100 units/ml penicillin, 100 units/ml streptomycin. 0.25 micrograms/ml amphotericin B. HEK293T17 cells were grown in high glucose DMEM supplemented with 10% FBS v/v, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 units/ml penicillin, 100 units/ml streptomycin, 0.25 micrograms/ml amphotericin B.

4.2 | Plasmids and cloning

To construct the FRET-based pH sensor, the mTFP1-mCitrine fragment was synthesized by GENEWIZ. The linker between the two constituent fluorescent proteins was RSTSLQEGT. The gene fragment was then inserted in the place of ecto-pHluorin (EcpH) in EcpH-TM, a gift from Gregory Melikian (Addgene plasmid # 85389; http://n2t.net/addgene:85389; RRID: Addgene_85389). The mTFP1-mCitrine gene fragments and EcpH-TM were separately digested using HindIII HF and EcoRI HF (New England Biolabs) according to manufacturer instructions for 1 h. Digestions were gel purified; then EcpH-TM and the insert were ligated with T4 ligase (NEB). The ligation reaction was heat inactivated and transformed into DH5α competent cells (NEB). Colonies were selected on LB-carbenecillin plates. Plasmids were initially screened by digestion and then the sequence of the open reading frame was verified with primers corresponding to sequences in CMV (forward), TFP (forward), hGH poly(A) (reverse).

To construct the FRET-based pH sensor, mCitrine-mTFP1-ICAM1 and the content marker were prepared by transfecting HEK293 T17 cells where tracking was ambiguous in the analysis, particles were not considered. Only particles with fluorescence from gag-mKate2 (content marker), mTFP1 (FRET donor) and mCitrine (FRET acceptor) were included in analysis of calibration and live cell imaging.

4.4 | Particle and experimental analysis criteria for calibration and live cell imaging

Stringent quantitative selection criteria were applied to ensure that the pH readout was uniform between calibration and live cell imaging, as well as that tracking would be accurate. Only particles with fluorescence from gag-mKate2 (content marker), mTFP1 (FRET donor) and mCitrine (FRET acceptor) were included in analysis of calibration and live cell imaging. In addition, only particles less than approximately 1.5 μm in apparent diameter were included in the analysis as larger spots were considered to be part of aggregates.

Live cell imaging was performed on 27 plates of cells on 11 separate days. In each plate of cells, a single field of view was imaged, comprising one individual experiment of these 27 individual experiments, only 13 individual experiments conducted on seven separate days had ideal conditions in which the field of view had 5–20 viral particles visible in the collected image series and a viral content release event was observed in the field of view. For four of the individual experiments conducted, more than one fusion event was observed in the field of view. Individual experiments with too many particles in the field of view were excluded because individual particles could not be differentiated and tracked during trafficking.

For the 13 plates retained for analysis, the criteria described above for particle selection was applied (triply labeled, <1.5 μm in apparent diameter). As an additional measure to avoid including particles where tracking was ambiguous in the analysis, particles were not considered. Only particles that could be observed for at least 20 frames (6.67 min in the analysis). From a total observation of 5041 particle tracks from the 13 individual experiments, 930 tracks were retained based on the applied criteria of the particle/endosome being tracked for at least 20 frames. After the additional particle inclusion criteria (listed above) were applied, 88 tracks remained. Within these 88 tracks, 18 fusion events occurred. Intensity and trajectory analysis were conducted for all 88 tracks.

4.5 | Calibration and evaluation of FRET-based pH sensor

Six to eight microliters of suspended pseudoviral particles labeled with the FRET-based pH sensor, mCitrine-mTFP1-ICAM1 and the content
label, gag-mKate2 were spun for 20 min at 4°C and 200×g onto poly-L-lysine 0.1% V/V (Sigma-Aldrich) coated 35 mm imaging dishes with a #1.5 glass insert (Mattek), such that there were ~5–20 particles per field of view. The particles were observed on a laser scanning confocal microscope (LSM880, Carl Zeiss) at 37°C with a 63× (1.4 NA) oil immersion objective. The pH sensor was excited with an Argon laser at 458 nm and the content label was excited at 561 nm. Emissions intensities were collected in 15 × 8.9 nm-width spectral bands between 472 and 606 nm using the GaAsp spectral detector of the microscope. In addition, emissions above 606 nm were collected in a photomultiplier tube detector and that intensity was assigned to mKate2.

Mean fluorescence emission intensities at each wavelength were extracted from the region of each triple labeled, detected particle in each field of view and background subtraction was performed.

The donor intensity relative to the acceptor intensity was quantified by measuring the ratio of the intensities from the two respective bands according to $I_{494.0-502.9}/I_{530.0-538.9}$. The mean and standard deviations of all evaluated particles for a given prep at a given pH were plotted and fit with a sigmoidal function in GraphPad Prism 9.

4.6 Live cell imaging

A549 cells were seeded 24–32 h prior to imaging at a concentration of approximately 1 × 10⁵ cells/dish onto a 35 mm imaging dish with #1.5 glass slide insert (Mattek) coated with fibronectin. Prior to addition of virus, cells were chilled on ice for 10 min, then washed with cold HEPES imaging buffer (Life Technologies) supplemented with 4.5 mg/ml glucose. 8–12 µl of pseudoviral particles were spun onto cells for 20 min at 4°C and 200×g. A single pseudovirus preparation was used to generate the data derived from live cell imaging and displayed in Figures 2–6. Based on the similar infectivity and pH sensor calibration of viral particles from different preparations (Figure S5B,C), we expect that live cell imaging data from additional sensor calibration of viral particles from different preparations would produce very similar results to those shown in Figures 2–6. Cells were washed in 2 ml of cold imaging buffer, placed on the microscope with the stage warmed to 37°C and the field-of-view for imaging was selected. After field-of-view and focus were set, viral trafficking was initiated by removal of 4°C imaging buffer and replacement with 37°C imaging buffer. A 1–2 min period of re-focusing followed the addition of 37°C imaging buffer and time zero in all experiments is the initiation of imaging after this initial time lag.

Excitation and detector configurations were the same as described for calibration and evaluation of the FRET-based pH sensor. An additional photomultiplier detector was used to generate a pseudo-DIC image of the cells using the transmitted light from the 458 nm excitation. Frames were taken every 20s for at least 1 h in a z-stack of 13 planes with 0.535 nm between each plane. The image size was (800 × 800 pixel²) with a pixel resolution of 0.141 nm/pixel and an optical zoom of 1.2×. The Definite Focus feature of the microscope was used every five frames to avoid vertical drift.

4.7 Analysis of live cell imaging

Unless otherwise noted, particles were tracked in 2D using a maximum intensity Z-projection of the integrated intensities from emission bands 494–502 nm, 530–538 nm and 593–741 nm. The cell area was masked for analysis using the pseudo-DIC image to exclude particles directly adhered to the glass insert of the imaging dish. Particles were tracked using the TrackMate.55 Particles were detected using a Laplacian of Gaussian segmentation and an expected minimum particle radius of 700–800 nm and an intensity threshold appropriate to the individual experiment. The simple Linear Assignment Problem (LAP) tracker was used with a maximum search distance of 2 nm for consecutive frames and a search radius of 3 nm for missed frames with a maximum of two missed frames permitted. Tracks with <4 spots were eliminated. In cases where a particle was visible but below the selected threshold for two or more frames, tracks were manually connected. Manual connection of particle localizations was only performed in cases where based on the sparseness of particles we had a high degree of confidence the particle identified by the tracking program at two separated time points was the same.

Tracking results were compiled into tracks and intensities from 494.0–502.9 and 530.0–538.9 were extracted using the MATLAB (R2018a, MathWorks). The mean intensity of the background at all extracted emissions intensities was subtracted from the mean intensity of the particle area, with the background region being a 2 pixel width annular region around each 14 pixel (1.96 µm) diameter circular particle region. This data was used to calculate fluorescence ratios. An in-house designed MATLAB (R2020b, MathWorks) application extracted and stored image regions of 21 × 21 pixel dimensions around each tracked particle and from three spectral channels. This data was then visualized and analyzed to identify fusion events, displacements and motion by custom built software written in LabView (National Instruments). Emissions collected from wavelengths 494–511 were assigned to channel 1, 521–538 to channel 2 and 593–741 nm to channel 3. The extracted image regions corresponding to the trajectory of each file were read into LabView. Fusion was identified as a well-defined decrease of the mKate2 signal over 1 min or less while signal corresponding to the FRET pair persisted. Only well-centered particles in the extracted region that had an approximate apparent diameter of <1.5 µm were considered for fusion analysis and only particles satisfying the same criteria were selected for the comparison set of nonfusing particles.

The velocity coefficient ($v$), representing the velocity of directed motion apart from drift or diffusion, was obtained using a directed-motion model of mean-squared displacement (MSD, ρ)

$$ρ(τ) = 4Dτ + v^2τ^2$$ (1)

where $D$ is the observed diffusion coefficient and $τ$ is the time lag. Only time lags <60% of the total length of the entire tracks were used to fit to Equation (1) because of the degree of imprecision in time lags that are long relative to the length of the observation time of the particle.29
pH values were determined from the measured fluorescence emission intensity ratios $I_{480.0-502.5}/I_{530.0-538.9}$ using a standard table calculated from the fit in the calibration experiment of Figure 1C (see Section 4.8).

4.8 Statistical analysis and data fitting

Particle localization and emissions intensity data for all experiments was initially aggregated in R Studio (v. 1.1.453, R Studio Team 2020). Velocity, total displacement, fluorescence intensity ratio change and related values were calculated in R Studio unless otherwise indicated. Statistical analysis was conducted in GraphPad Prism (Version 9.1.2 for Mac) and compared with the distributions of groups without an underlying assumption of parametric distributions (Mann-Whitney test). The two-tailed $p$ values were calculated and the summary values are reported in the relevant figures.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

All authors designed the experiments. Maya Cabot carried out all experiments and wrote the initial draft of the manuscript. Volker Kiessling programmed several routines for data analysis. All authors contributed to the final writing and editing of the manuscript.

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DATA AVAILABILITY STATEMENT

The routines to analyze the virus particle tracking data are available from the authors upon request.

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