Received: 18 July 2023 | Revised: 2 September 2023 | Accepted: 4 September 2023

DOI: 10.1111/mmi.15165

MINI REVIEW

WILEY

Illuminating bunyavirus entry into host cells with fluorescence

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Funding information
Agence Nationale de la Recherche, Grant/Award Number: ANR-11-IDEX-0007, ANR-11-LABX-0048, ANR-21-CE11-0012 and ANR-22-CE15-0034; Chinese Scholarship Council, Grant/Award Number: 202106790021

Abstract

Bunyavirales constitute the largest order of enveloped RNA viruses, many members of which cause severe diseases in humans and domestic animals. In recent decades, innovative fluorescence-based methods have paved the way to visualize and track single fluorescent bunyaviral particles in fixed and live cells. This technological breakthrough has enabled imaging of the early stages of infection and the quantification of every step in the bunyavirus cell entry process. Here, we describe the latest procedures for rendering bunyaviral particles fluorescent and discuss the advantages and disadvantages of each approach in light of the most recent advances in fluorescence detection and monitoring of bunyavirus entry. In this mini-review, we also illustrate how fluorescent viral particles are a powerful tool for deciphering the cellular entry process of bunyaviruses, the vast majority of which have not yet been analyzed.

KEYWORDS
bunyavirus, cell entry, endocytosis, fluorescent viral particles, intracellular trafficking, viral fusion

1 | INTRODUCTION

Bunyavirales is the largest and most diverse order of RNA viruses, encompassing 28 viral families, and over 500 named isolates worldwide. *Arenaviridae*, *Hantaviridae*, *Nairoviridae*, *Peribunyaviridae*, *Phenuiviridae*, and *Tospoviridae* are undoubtedly the most representative viral families in terms of the number of isolates and pathogenic bunyaviruses. The name *Bunyavirales* originates from the town of Bunyamwera in western Uganda, near where the first bunyavirus, Bunyamwera virus (BUNV), was isolated in 1943 (Elliott, 2014). The diversity of bunyaviruses is manifested not only in the wide variety of viruses, geographical distributions, and diseases but also in the large spectrum of vectors and hosts, which includes vertebrates, invertebrates, and plants. Hantaviruses and arenaviruses are harbored in small mammals such as rodents and bats and are mainly transmitted to humans through the inhalation of contaminated aerosols from the feces of infected animals. All the other known bunyaviruses are arthropod-borne viruses (arboviruses); they usually spread to vertebrates via various blood-feeding arthropods, such as mosquitoes, sandflies, and ticks (Boshra, 2022). The only exception is tospoviruses, which are plant-specific and transmitted by nonhematophagous vectors, namely, thrips.

Many bunyaviruses cause severe diseases, often fatal, in both humans and livestock. With an escalating number of outbreaks, bunyaviruses must be seriously considered as potential agents of emerging and reemerging diseases (Boshra, 2022). No effective vaccines or treatments for bunyaviruses have yet been approved for human use. However, our knowledge of the early stages of bunyavirus infection remains limited to only a few isolates, and their transmission, tropism, receptors, and cell entry remain poorly understood overall. In this mini-review, we examine the key experimental strategies for fluorescence labeling of authentic bunyaviruses that have proven highly effective in analyzing the cell entry of these viruses, beyond traditional immunofluorescence techniques. Moreover, we highlight how fluorescent viral particles, in combination with appropriate analytical methods, serve as indispensable tools for investigating pathogen entry, not only for the numerous emerging bunyaviruses that remain unexplored but also for other viruses that share similar molecular characteristics.

2 | GENOMIC AND STRUCTURAL ORGANIZATION OF BUNYAVIRUSES

The genome of most bunyaviruses consists of three single-stranded, mainly, negative-sense, RNA segments, the names of which refer to their size in nucleic acids, i.e., small (S), middle (M), and large (L) (Figure 1). A minority of bunyaviruses have a bipartite genome, such as most arenaviruses, which have only the S and L segments. The genomic RNA segments replicate exclusively in the cytosol of infected cells and encode just a few proteins, the number of which can vary not only from one isolate to another but also according to the species from which the virus is amplified (Boshra, 2022). A large majority of bunyaviruses have one or two nonstructural proteins, usually named NSm and NSs, which are expressed only in infected cells, i.e., the nonstructural proteins are not part of the viral particles. At least four viral structural proteins are expressed upon infection and necessary for the assembly of infectious viral progeny. They typically include RNA-dependent RNA polymerase L, nucleoprotein N, and two transmembrane glycoproteins (Figure 1).

Bunyaviruses are enveloped and roughly spherical with an average diameter of 80–160 nm (Boshra, 2022) (Figure 1). Most bunyaviral progeny particles assemble and bud in the Golgi (Barker et al., 2023), from which they acquire their lipid–bilayer envelope and, in general, the two viral transmembrane glycoproteins, Gn and Gc. Only arenaviruses differ in that they exit producer cells from the plasma membrane (Hulswit et al., 2021). Within viral particles, the N protein is associated with the RNA genome and, together with the viral polymerase, forms ribonucleoproteins (Figure 1). The N protein plays an important role in protecting the virus genome, as bunyaviruses have no classical matrix or rigid internal structure. On the surface of these viruses, viral glycoproteins constitute protrusions responsible for the attachment of bunyaviruses to target cells and subsequent penetration into the cytosol (Boshra, 2022).

3 | ENTRY OF BUNYAVIRUSES INTO MAMMALIAN CELLS

Research into the cell entry of viruses, including bunyaviruses, has long been hampered by the lack of means to explore the early virus–host cell interactions in real time. The methods employed until recently were essentially limited to the quantification of infectious progeny or the detection of newly synthesized viral proteins, hours or days after the entry and penetration of virions. However, a growing body of evidence indicates that bunyaviruses can use multiple cellular receptors for infectious entry, which certainly contributes to the broad tropism and species spectrum of these viruses in vivo. Some of these viruses have been identified in humans and other vertebrates, and a few have been identified in plants, but none have been identified in arthropods. Following binding to mammalian cells,

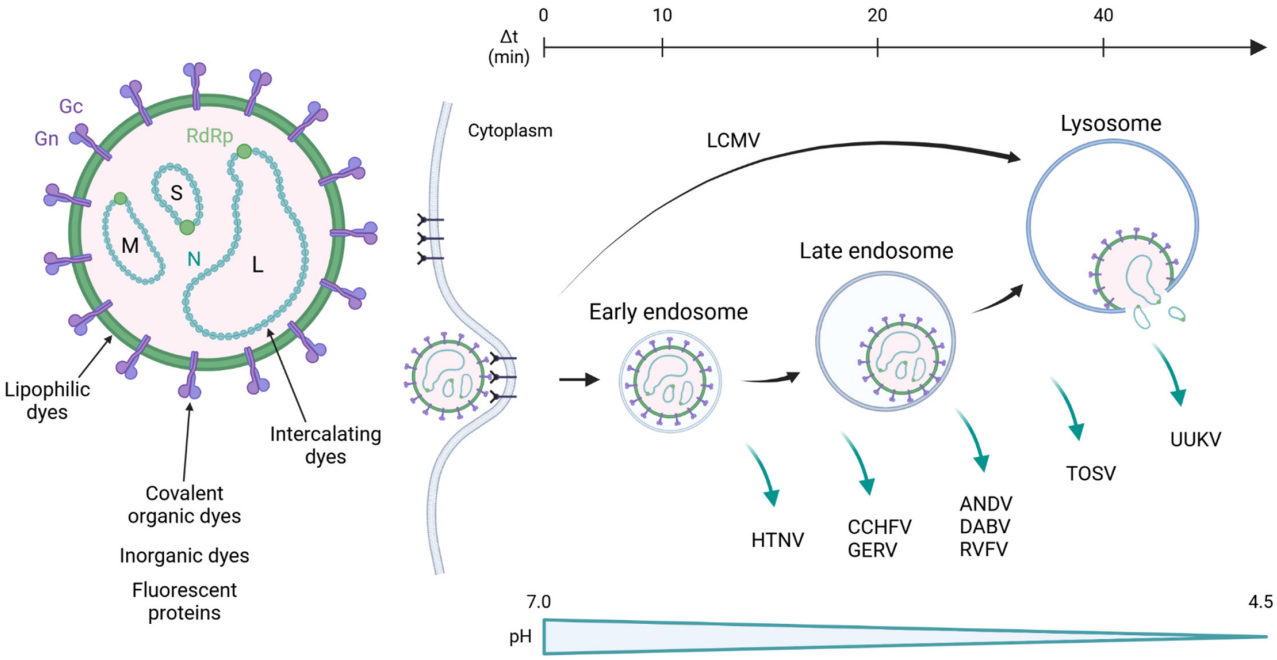

FIGURE 1 Bunyavirus entry into mammalian cells. This figure illustrates the structural organization of a prototype bunyavirus particle. The arrows indicate bunyavirus compounds that have been experimentally validated for labeling with fluorescent dyes or proteins (for details, see Table 1). This figure also summarizes the state of knowledge on bunyaviruses for which the endocytic pathways used to enter and infect host cells have been analyzed. At the top, the scales indicate the time required to transport the cargo from the surface to an organelle, and at the bottom, the corresponding pH inside endosomal vesicles. ANDV, Andes virus; CCHFV, Crimean Congo hemorrhagic fever virus; DABV, Dabie virus; Gc, glycoprotein Gc; GERV, Germiston virus; Gn, glycoprotein Gn; HTNV, Hantaan virus; LCMV, Lymphocytic choriomeningitis virus; N, nucleoprotein; RdRp, RNA-dependent RNA polymerase; RVFV, Rift Valley fever virus; S, M, and L, genomic RNA segments S, M, and L; TOSV, Toscana virus; UUKV, Uukuniemi virus. Created with BioRender.com.

bunyaviruses are sorted into the endocytic machinery, and many have been shown to penetrate the cytosol from late endosomal compartments via acid-activated membrane fusion (Figure 1). In this regard, most, if not all, bunyaviruses are late-penetrating viruses, which are viruses that share a dependence on late endosomal maturation for infectious entry (Lozach, Huotari, & Helenius, 2011).

Upon acidification in late endosomes, the viral glycoproteins undergo multiple conformational changes and ensure the fusion of the viral envelope with the endosomal membrane, a prerequisite for the subsequent release of the virus genome into the cytosol. A pH value below 6.0, typical of late endosomal organelles, is sufficient and necessary to trigger bunyavirus fusion in most cases. Nevertheless, the efficiency of the whole process relies on additional factors such as temperature and the lipid composition of the target membrane. For a more complete picture of bunyavirus entry, we recommend recent reviews (Cifuentes-Munoz et al., 2014; Hawman & Feldmann, 2023; Koch et al., 2021; Sarute & Ross, 2021; Windhaber et al., 2021).

Over the past decade, the study of bunyavirus entry has benefited greatly from advances in reverse genetics and chemistry, which paved the way to render authentic bunyavirus particles fluorescent. Membrane permeabilization and immunostaining were no longer required to detect virions, and the integrity of the cell and viral particles was preserved. From there, analyzing bunyavirus entry into fixed and live cells using fluorescence microscopy, flow cytometry, and fluorimetry was possible. Every stage of the bunyavirus entry process could thus be imaged and quantified. The overall picture of bunyavirus entry, however, remains largely incomplete. Only a handful of isolates have been examined, and even fewer, with the help of fluorescent viral particles (Table 1). In the following sections, these few examples are used to illustrate and discuss the different strategies employed to generate fluorescent bunyaviral particles. This information will undoubtedly prove useful in future work on emerging bunyaviruses that have not yet been analyzed.

4 | GENETIC ENGINEERING TO RENDER BUNYAVIRUSES FLUORESCENT

In the late 90s, reverse genetic techniques revolutionized research on bunyaviruses and other negative RNA viruses. This technology has made it possible to genetically engineer the bunyavirus genome and recover infectious viral progeny from DNA, paving the way for the study of bunyavirus gene function. BUNV was the first bunyavirus to be rescued from plasmids, and many others have followed (Ren et al., 2021). Extensive manipulation of bunyavirus genomes is, however, challenging, and the production of viral progeny is often compromised. The first recombinant bunyaviruses expressing reporter genes were only described in the 2000s (Billecocq et al., 2008; Ikegami et al., 2006).

The research group of the late Richard Elliott succeeded in genetically engineering the peribunyavirus BUNV to produce infectious viral particles carrying a fluorescent protein (FP)-tagged Gc envelope glycoprotein (Shi et al., 2010) (Table 1). This approach was primarily employed for monitoring BUNV replication (Ter Horst et al., 2021), but it holds great potential for investigating peribunyavirus entry. The recombinant viruses are fluorescent and can be observed upon entering cells. Notably, tagging viral transmembrane glycoproteins with an FP appears to be limited to peribunyaviruses. Substituting nearly half of the N-terminal ectodomain of Gc does not hinder infectivity or propagation (Shi et al., 2010). In contrast, all regions within the glycoprotein ectodomains of other bunyaviruses are critical for virus attachment and membrane fusion. Moreover, the cytosolic tail of viral glycoproteins is likely unsuitable due to its importance for interactions with ribonucleoproteins during virion assembly. As a result, it may be worthwhile to explore the viral nucleoprotein and polymerase as alternative targets for the fluorescent labeling of viral particles.

Alternatively, the use of small peptide tags such as histidine (His) and tetracysteine (tc) to confer fluorescence is promising. Their compact size, consisting of only a few amino acids, minimizes any potential disruption to the viral genome. The fluorescence of both His and tc tags is activated upon the addition of nickel (Ni^{2+})-nitrilotriacetate-based fluorescent probes or specific dyes such as ReAsh (red) and FlAsh (green). The permeability of cell membranes to these dyes allows for real-time analysis, as illustrated by the recent visualization of NSs aggregate formation in live cells exposed to the phenuivirus Rift Valley fever virus (RVFV) (Leger et al., 2020). For optimal detection, the labeled viral protein should ideally be concentrated locally. In this regard, nucleoprotein N within ribonucleoproteins inside virions emerges as a perfect candidate.

Traditionally, the preferred method for incorporating a reporter gene into the bunyavirus genomes involves the deletion of sequences that encode nonstructural proteins. While this approach does not allow for the tracking of single particles, reporter gene expression can still serve as a readout of infectious entry. Notably, nonstructural proteins are essential for animal infection as they help viruses to evade immune responses and facilitate their propagation (Eifan et al., 2013). An example is recombinant RVFV expressing luciferase or green fluorescent protein (GFP) in place of NSs. The recombinant virus can be efficiently amplified in cell culture (Billecocq et al., 2008; Ikegami et al., 2006), but it can only propagate in mice deficient for interferon responses (Gommet et al., 2011). To overcome this limitation, genetic engineering of the S segment has been attempted, aiming to add the reporter gene while preserving the nonstructural protein sequence. Some success has been achieved, notably with the phenuivirus Akabane virus (Takenaka-Uema et al., 2015). Despite significant strides in monitoring bunyavirus infection and replication, reverse genetics techniques still often restrict direct analysis of virus entry, and their full potential in this area has yet to be fully realized.

5 | FLUORESCENT ORGANIC DYES

To make viral particles fluorescent, organic dyes offer significant advantages over FPs, as discussed in detail by Liu et al. (2020). A

TABLE 1 The different fluorescent dyes used to label bunyaviruses.

	Fluorescent proteins	Organic dyes			Inorganic dyes
		Covalent (Gn/Gc)	Intercalating	Lipophilic	
Fluorescent molecule	Gc-GFP[1] Gc-mCherry[2]	BODIPY-TR[3,6,8] AF488[3,5,8] AF568 AF594[3,8] AF647[3,7,8] Atto488[4] Atto647N[4,5]	SYTO 82[10,11]	DiD[10,11,15,16] DiI[16] DiOC18[14] Pyrene[13] R18[4,5,8,9,12,14]	QD605[15] QD705[15]
Color (emission)	Green, red	Green to far red	Orange	Blue to far red	Red, far red
Brightness	Low	Intermediate			High
Photostability	Low	Intermediate			High
Manipulation	Complex	Simple			Intermediate
Models	Fixed/live cells	Fixed/live cells			Fixed/live cells
Bunyavirus	BUNV[1,2]	GERV[4] JUNV[7] TOSV[5] UUKV[3,6,8,9]	BUNV[10,11]	BUNV[10,11] DABV[15] GERV[4] HRTV[14] PICV[12] PUUV[16] TOSV[5] UUKV[8,9,13]	DABV[15]
Abbreviations	AF, Alexa Fluor; BUNV, Bunyamwera virus; DABV, Dabie virus; GERV, Germiston virus; GFP, green fluorescent protein; HRTV, Heartland virus; JUNV, Junín virus; PICV, Pichinde virus; QD, quantum dot; TOSV, Toscana virus; UUKV, Uukuniemi virus				
References	1. Shi et al. (2010); 2. Ter Horst et al. (2021); 3. Lozach, Kuhbacher, et al. (2011); 4. Windhaber et al. (2022); 5. Koch et al. (2023); 6. Lozach et al. (2010); 7. Chou et al. (2016); 8. Meier et al. (2014); 9. Hoffmann et al. (2018); 10. Hover et al. (2018); 11. Charlton et al. (2019); 12. Spence et al. (2014); 13. Bitto et al. (2016); 14. Xia et al. (2023); 15. Liu et al. (2019); 16. Bauherr et al. (2020).				

large panel of these dyes is commercially available, and they generally provide higher brightness, better photostability, a wider range of colors (from ultraviolet to far red), and a smaller size (<1 kDa) than FPs. Small organic dyes can be finely tuned, as their fluorescence properties depend not only on the fluorophore structure but also on the chemical environment. In addition, they can be conjugated into various reactive groups, expanding the possible labeling strategies. Importantly, they eliminate the need for manipulating the viral genome when rendering virions fluorescent. Organic dyes are categorized into three groups according to their reactivity and can be used to label all components of bunyavirus particles: covalent dyes to target structural proteins, intercalating dyes to target genomic RNA segments, and lipophilic dyes to target the lipid bilayer envelope (Liu et al., 2020).

The limited quantity of virus that can be produced in cell culture often poses challenges for fluorescence-based labeling of viral particles. In this respect, most bunyaviruses have the advantage of efficient amplification in cell lines such as BHK-21 and Vero cells, even in the absence of serum, facilitating subsequent virus purification steps. Infectious titers typically reach 10^7 infectious particles per milliliter in cell supernatants, and higher numbers are achieved when considering both infectious and noninfectious particles (Hoffmann et al., 2018). The integrity of bunyaviral particles is largely preserved after labeling, although some isolates may require adjustments to reach a balance among the dye amount, virion fluorescence intensity, and infectivity (Koch et al., 2023; Lozach, Kuhbacher, et al., 2011; Windhaber et al., 2022). The fluorescent labeling of viral particles with small organic dyes has proven successful in analyzing each step of the entry program of several bunyaviruses, as described below.

6 | LABELING THE OUTER SURFACE OF BUNYAVIRUSES WITH COVALENT ORGANIC DYES

Cross-linking reactions are a simple method for conjugating viral proteins and other biomolecules with organic dyes via covalent bonds. Four functional groups can be targeted in proteins for covalent labeling: carbonyls, carboxyls, sulfhydryl, and amines (Liu et al., 2020). The latter two are present in cysteine residues and at the N-terminus of each peptide and lysine, respectively. In this context, the two viral transmembrane glycoproteins are ideal candidates for labeling bunyaviral particles with thiol- and amine-reactive dyes such as TS-Link BODIPY TR, Alexa Fluor (AF), or Atto NHS-esters (Hoffmann et al., 2018). Both viral glycoproteins cover the entire outer surface of bunyaviral particles and are abundant in cysteine and lysine

residues. This labeling technique enables high fluorescence of viral particles to be achieved using a minimal number of dye molecules while preserving the infectivity of virions and facilitating their detection. The first applications of this method for labeling bunyaviruses were reported for the phenuivirus Uukuniemi virus (UUKV) in the early 2010s (Lozach et al., 2010; Lozach, Kuhbacher, et al., 2011).

Since then, several other bunyaviruses have been successfully labeled with thiol- and amine-reactive fluorescent dyes to examine the entry process in fixed and live cells (Table 1). Confocal microscopy, flow cytometry, and fluorometry were used to monitor the binding of the phenuivirus Toscana virus (TOSV), the peribunyavirus Germiston virus (GERV), and the arenavirus Junín virus to the plasma membrane (Chou et al., 2016; Koch et al., 2023; Windhaber et al., 2022). Real-time imaging using total internal reflection (TIRF) microscopy revealed the motion of UUKV on the cell surface upon virus attachment and elucidated how phenuiviruses exploit the receptors DC-SIGN and L-SIGN, two human C-type lectins, to enter host cells (Leger et al., 2016; Lozach, Kuhbacher, et al., 2011). The attachment of phenuiviruses to DC-SIGN and L-SIGN on the cell surface occurs through a few initial contacts, followed by increased binding as more receptor molecules are recruited to the virus surface. DC-SIGN and L-SIGN are believed to play roles in the early stages of infection and in the liver tropism of many bunyaviruses.

Once attached to the cell surface, bunyaviruses undergo endocytosis. By using the BODIPY TR, AF, and Atto dyes, some bunyaviruses were directly observed in endosomes, and their journey through the endocytic machinery was examined using TIRF and confocal microscopy in both fixed and live cells (Chou et al., 2016; Koch et al., 2023; Lozach et al., 2010; Windhaber et al., 2022). In complementary approaches, differentiation between virions on the cell surface and those internalized within endosomes was achieved by exploiting the photophysical and biochemical properties of the BODIPY TR and AF/Atto dyes. Thiol-reactive dyes can be removed with reducing agents such as TCEP, while green-emitting covalent dyes can be quenched by trypan blue. Both TCEP and trypan blue are unable to penetrate the cell membrane. Consequently, after treatment, only internalized virions remain fluorescent and can be seen by microscopy or quantified by flow cytometry or fluorimetry (Lozach et al., 2010; Meier et al., 2014). With this method, bunyavirus binding to cells was found to be relatively inefficient but rapid, with the internalization process lasting 10–25 min depending on the viral isolate and family and the target cells (Koch et al., 2023; Lozach, Kuhbacher, et al., 2011; Meier et al., 2014; Windhaber et al., 2022).

7 | INTERCALATING DYES FOR VISUALIZING BUNYAVIRUS GENOMES AND PARTICLES

A less commonly used strategy to visualize viral particles involves labeling their genomic material. This tactic is often considered challenging due to the difficulty of accessing the viral genome. In intact virions, the virus genome is either fully coated with nucleoprotein or encapsulated, and in the case of bunyaviruses, it is also surrounded by a lipid bilayer envelope. While there are intercalating dyes capable of penetrating all barriers within viral particles, the most frequently employed technique is to label the viral genome during virus replication and assembly using dyes that can traverse cell membranes. To date, this approach has been reported for labeling the viral RNA genome from a single bunyavirus, i.e., BUNV with the orange fluorescent dye SYTO 82 (Charlton et al., 2019; Hover et al., 2018) (Table 1). One advantage of using intercalating dyes is to circumvent additional labeling and purification steps. There are various intercalating SYTO dyes, each possessing unique characteristics such as distinct optical properties and selectivity for staining DNA and RNA.

8 | TARGETING THE BUNYAVIRUS ENVELOPE WITH LIPOPHILIC DYES

Another aspect of bunyaviruses that can be exploited to induce fluorescence in viral particles is the lipid bilayer envelope. Several lipophilic dyes can be incorporated into the viral envelope (Liu et al., 2020). The labeling process is slightly simpler than procedures involving covalent dyes. Regardless of the number of virus particles, the concentration of the dye remains constant, and the dye can be inserted into virions during the amplification of viruses in infected cells or in the supernatant or after purification. The latter option reduces the labeling of cell membranes and vesicles, which could be mistaken for viral particles. However, lipophilic dyes are not covalently bound to particles, and viral stocks have a limited storage lifespan, usually only a few weeks.

The lipophilic octadecyl rhodamine B dye R18 and the carbocyanine dyes DiD and DiI have been successfully used to label the arenavirus Pichinde virus and the hantavirus Puumula virus, respectively (Bauherr et al., 2020; Spence et al., 2014) (Table 1). Both bunyaviruses were imaged using fluorescence microscopy and observed to be bound to host cells and inside intracellular vesicles. Notably, lipophilic dyes generally do not exhibit high-fluorescence intensity and tend to bleach rapidly, which is detrimental to imaging. These dyes are better suited to the study of viral membrane fusion. Analysis commonly involves fluorimetry. Unlike microscopy, this technology does not provide information on cellular localization but is more sensitive and less harmful to fluorophores. Importantly, fluorimetry can be used to monitor the membrane fusion activity of enveloped viruses in live cells during their penetration process.

Typical assays for studying viral fusion rely on the autoquenching properties of certain lipophilic dyes, such as R18 or DiD, when present in high concentrations in the viral envelope. Following fusion, the release of R18 in the endosomal membrane leads to an increase in fluorescence that can be quantified using a spectrofluorometer. Capturing fusion events via microscopy is more challenging, as it requires intensive and prolonged imaging due to the late penetration of bunyaviruses in general. Although the subsequent release of the dye into the target membranes corresponds to the hemifused mixture of outer leaflets rather than the strict formation of a fusion

pore, it serves as a good correlate for fusion. This approach has been successfully employed to measure the fusion of UUKV, GERV, and TOSV directly in live cells (Hoffmann et al., 2018; Koch et al., 2023; Meier et al., 2014; Windhaber et al., 2022) (Table 1). These viruses typically require 15–40 min from the moment they bind to the cell surface to enter cells and fuse in late endosomal compartments.

Lipophilic self-quenching dyes on labeled viruses can also be utilized to monitor viral fusion with liposomes. Liposomes are commonly used to investigate the significance of lipid composition in target cell membranes or to determine the optimal pH for viral fusion. Typically, UUKV has been labeled with pyrene, which also exhibits autoquenching capabilities at high concentrations. Dye dequenching was measured using fluorimetry to assess fusion with target membranes with different lipid compositions (Bitto et al., 2016). This study revealed that UUKV fusion is facilitated by phospholipids with negatively charged head groups, such as the phospholipid bis(monoacylglycerol)phosphate found in late endosomes. Following a similar strategy, it was recently demonstrated that a low pH is both necessary and sufficient for TOSV fusion (Koch et al., 2023). The data showed that TOSV relies on progressive acidification in endosomes for activation and fusion within a pH range of 5.5–5.8 rather than at a specific pH threshold.

9 | INORGANIC DYES: THE FUTURE OF TRACKING SINGLE VIRAL PARTICLES?

An attractive alternative to organic dyes is nanoparticles, specifically quantum dots (QDs) and metal nanoparticles (Kang et al., 2021). QDs are inorganic nanocrystals made of semiconductor materials with remarkable optical properties (Wang et al., 2021). They are 100–1000 times more photostable than FPs and organic dyes, making them optimal for long-duration fluorescence recording in live cells. The use of QDs in the study of bunyaviruses is still limited, and only the phenuivirus Dabie virus (DABV) has been visualized using this technique (Liu et al., 2019) (Table 1). Although the infectivity of the virus seems to have been preserved, it is not possible to completely rule out the possibility that the large size of QDs (up to 20 nm) has implications for viral motion and the entry process. In this respect, metal nanoparticles can be much smaller (below 2 nm), but they are not visible under fluorescence microscopy as they scatter light. Their advantage is that they do not suffer from photobleaching; their disadvantage is that their use is limited to darkfield microscopy or electron microscopy.

10 | DUAL-LABELING STRATEGIES FOR MONITORING VIRAL FUSION

In recent years, dual-labeling strategies have also been employed to assess bunyavirus fusion. The principle behind this approach is that viral particles labeled with two colors, usually red and green, appear yellow when they are intact. Once the viral particles have fused and released their dyes into the target cell membranes, both red and green signals become visible. This technique was utilized to label the envelope of the phenuivirus Heartland virus (HRTV) using two lipophilic dyes, DiOC18 (green) and R18 (red), and to monitor HRTV-induced membrane fusion (Xia et al., 2023). Alternatively, the envelopes of BUNV and DABV were labeled with DiD, and instead of using a second dye to target the viral envelope, the STYO 82 dye was intercalated into the BUNV genome (Charlton et al., 2019; Hover et al., 2018) or the surface of DABV particles conjugated to QDs (Liu et al., 2019). This approach helped to define the roles of potassium and cholesterol in BUNV entry and shed new light on the early DABV-host cell interactions. In these four studies, microscopy was employed to visualize viral fusion. Nevertheless, it is more common to use flow cytometry or fluorimetry, which allows more sensitive and accurate quantification.

11 | CONCLUDING REMARKS AND PERSPECTIVES

Bunyaviruses pose a growing threat to humans and domestic animals worldwide. Preventing the spread of bunyaviruses requires strategies targeting the early stages of infection, i.e., virus entry. Tracking single viral particles enables qualitative and quantitative analysis of the bunyavirus entry program, from seconds upon virus binding until the time of fusion and penetration into the cytosol. This mini-review shows that a large number of protocols have been used to make bunyaviruses fluorescent. However, the expression of FPs is mainly limited to peribunyaviruses, and FPs are too invasive to be applied more widely. Inorganic dyes still need improvements to effectively track the smallest viral particles in the future. Organic dyes appear to be the most commonly used, offering the best compromise among photophysical properties, ease of labeling, and impacts on infectivity.

Research on bunyaviruses has been insufficient. More methods for visualizing single bunyavirus particles remain to be investigated. Clickable amino acids, a novel technology with minimal invasiveness, and probes derived from fluorescence in situ hybridization should be considered for labeling structural proteins and genomic RNA segments in virions. The use of pH-activated dyes could prove useful in analyzing bunyaviruses in endocytic vesicles, where acidification increases with the maturation of endosomes. Although not applicable to cells, dynamic light scattering is an interesting label-free strategy to characterize virions. The recent development of pH-sensitive or phototransformable FPs could prove helpful in designing new assays aimed at examining the entry process of bunyaviruses, particularly that of peribunyaviruses. Finally, all these advances need to be evaluated in physiological contexts, employing light-sheet and multiphoton microscopy to image infection in complex 3D cellular systems such as organoids and, ultimately, live animals.

The development of image analysis and deep-learning algorithms has enabled the tracking of single viral particles in microscopy images and, in turn, the quantification of each step of virus entry. Flow cytometry and fluorimetry represent interesting alternatives to

measure the binding, internalization, and fusion of fluorescent viruses. Both methods offer a good compromise between the number of samples and the rapidity of analysis. Only the analysis of more isolates, in combination with the expression of FP-tagged endocytic markers and inhibitors of endocytic pathways, will provide a comprehensive view of the bunyavirus entry process, and in turn, lay the foundation for the development of new antiviral strategies.

AUTHOR CONTRIBUTIONS

Pierre-Yves Lozach: Funding acquisition; conceptualization; writing – original draft; writing – review and editing; supervision. **Yu Gu:** Writing – original draft; writing – review and editing; conceptualization.

ACKNOWLEDGMENTS

This work was supported by funding from the Agence Nationale de la Recherche (ANR) (grant numbers ANR-21-CE11-0012 and ANR-22-CE15-0034) awarded to P.Y.L. This work was also supported by the LABEX ECOFECT (ANR-11-LABX-0048) of the Université de Lyon (UDL), within the program "Investissements d'Avenir" (ANR-11-IDEX-0007) operated by the ANR and by the RESPOND program of UDL. The Chinese Scholarship Council granted a fellowship (no. 202106790021) to Y.G.

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no competing interests.

DATA AVAILABILITY STATEMENT

This is a mini-review and data sharing does not apply.

ETHICS STATEMENT

No formal consent or approval was required for this mini-review.

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How to cite this article: Gu, Y. & Lozach, P.-Y. (2024) Illuminating bunyavirus entry into host cells with fluorescence. *Molecular Microbiology*, 121, 671–678. Available from: https://doi.org/10.1111/mmi.15165