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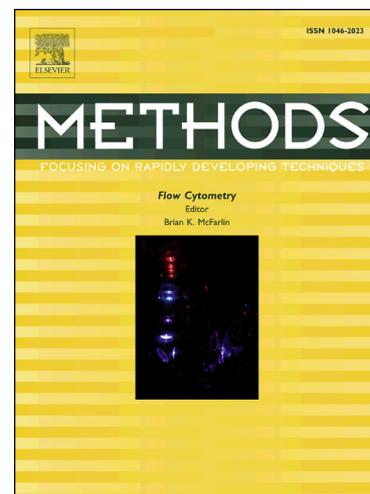
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Elucidating virus entry using a tetracysteine-tagged virus.

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Abstract:

Fluorescent tags constitute an invaluable tool in facilitating a deeper understanding of the mechanistic processes governing virus-host interactions. However, when selecting a fluorescent tag for *in vivo* imaging of cells, a number of parameters and aspects must be considered. These include whether the tag may affect and interfere with protein conformation or localization, cell toxicity, spectral overlap, photo-stability and background. Cumulatively, these constitute challenges to be overcome. Bluetongue virus (BTV), a member of the *Orbivirus* genus in the *Reoviridae* family, is a non-enveloped virus that is comprised of two architecturally complex capsids. The outer capsid, composed of two proteins, VP2 and VP5, together facilitate BTV attachment, entry and the delivery of the transcriptionally active core in the cell cytoplasm. Previously, the significance of the

endocytic pathway for BTV entry was reported, although a detailed analysis of the role of each protein during virus trafficking remained elusive due to the unavailability of a tagged virus. Described here is the successful modification, and validation, of a segmented genome belonging to a complex and large capsid virus to introduce tags for fluorescence visualization. The data generated from this approach highlighted the sequential dissociation of VP2 and VP5, driven by decreasing pH during the transition from early to late endosomes, and their retention therein as the virus particles progress along the endocytic pathway. Furthermore, the described tagging technology and methodology may prove transferable and allow for the labeling of other non-enveloped complex viruses.

Highlights:

Visualization of live-virus trafficking can be very informative regarding the interactions between a host-cell and infecting virus. While fluorescent protein markers have been utilized extensively to study the trafficking of enveloped viruses, non-enveloped, architecturally complex capsid viruses pose a challenge simply due to practical limitations imposed by their structure. Described here are the methodology and techniques utilized in the generation and validation of a fluorescently tagged non-enveloped, architecturally complex capsid virus, BTV, and how this approach facilitated the refinement in understanding of the BTV entry pathway.

Keywords:

Bluetongue virus, Fluorescent protein, biarsenical tetracysteine, double-stranded RNA, reverse genetics.

Abbreviations:

Fluorescent protein (FP), biarsenical tetracysteine (TC), Bluetongue virus (BTV), double-stranded RNA (dsRNA), reverse genetics (RG), Complementary DNA (cDNA), nonstructural protein 2 (NS2), early endosome marker (EEA1), late endosome marker (CD63), fluorescence resonance energy transfer (FRET), ethane dithiol (EDT₂), 2,3-dimercaptopropanol or British anti-Lewisite (BAL).

Introduction:

Since its inception, visualization of live-virus trafficking in host cells has been highly informative, furthering our understanding of the interactions between virus and host cells. To date, enveloped viruses have been at the forefront of such studies through the insertion of fluorescent markers into the viral genomes, enabling the study of their trafficking (1-3), or the labeling of lipid envelopes using lipophilic tracers, such as 1,1'-Dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI), 3,3'-Dioctadecyloxycarbocyanine perchlorate (DiO), 1,1'-Dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine perchlorate (DiD), 4-(4-(Dihexadecylamino)styryl)-N-Methylpyridinium Iodide (DiA), and 1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindotricarbocyanine Iodide (DiR) (4). In all cases, it is paramount that any modification does not inhibit cellular functions, nor affect viral infectivity (5). To date, two general strategies exist for the labeling of viruses. It is possible to label a target viral protein by fusing it with a fluorescent protein (FP) or by chemical labeling using a fluorescent dye (6).

Fusing a FP to the target viral protein is achieved by inserting the FP gene into the open reading frame of the viral protein (7,8). When the virus replicates within host cells it is thus labeled. However, while this allows for the study of virus replication (9), or the dynamics of viral entry (10), a caveat that can arise is a significant amount of background signal due to overexpression of the protein in infected cells.

An alternatively method to introducing a FP into the open reading frame is chemical labeling, such as the introduction of a biarsenical tetracysteine (TC) tag with a CCPGCC motif into the open reading frame of the target viral protein (11-13). Proteins with the TC tag are recognized and specifically bound by membrane-permeable biarsenical dyes, such as FIAsh (green) and ReAsH (red), which fluoresce when bound to the TC motif (11,12).

When labeling architecturally complex non-enveloped capsid viruses, there are physical constraints and limitations. Alterations, such as the introduction of a FP, could disrupt the structure-function relationship of the virus particles, interfering with their assembly or infectivity. This was evidenced by the lack of similar developments for the capsid virus members of the *Reoviridae* family. Recently, it was shown that such limitations could be overcome by the successful genetic engineering of the segmented RNA genome of

Bluetongue virus (BTV), a complex non-enveloped Orbivirus belonging to the *Reoviridae* family. This facilitated the generation of fluorescent virus particles that could be visualized in virus entry studies for both live and fixed cells (14).

BTV, with multiple serotypes (27 recognized to date) is endemic in most parts of the world, often resulting in high morbidity and mortality in ruminants. The BTV viral genome, comprising of 10 double-stranded RNA (dsRNA) segments (S1 to S10) encodes 7 structural (VP1 to VP7) and 4 non-structural proteins (NS1 to NS4) (15,16). In the virus particles, the structural proteins are organized in two capsids that form concentric protein shells. The outer capsid is comprised of VP2 and VP5, while an inner capsid, or “core”, comprised of VP7 and VP3. The core encloses the viral transcription complex (VP1, VP4 and VP6), in addition to the viral genome (15,17).

The constitutive components of the outer capsid (VP2 and VP5) have demonstrated a functional division to facilitate membrane attachment and membrane penetration. Three-dimensional structural studies of virions by cryo-electron microscopy (Fig. 1) revealed that VP2 arranges as trimers on the virion surface protruding as spike-like structures from the surface of the virus particles, facilitating viral attachment to the host cell (18,19). The second outer capsid protein, VP5, is less exposed than VP2, but is also arranged into globular trimers that are mostly covered by VP2 (19). Structurally, VP5 resembles the fusion proteins of enveloped viruses and consists of amphipathic α -helical regions on its external surface that facilitate penetration of endosomal membranes to release BTV cores into the cytoplasm (20).

A recent cryo-electron microscopy study at atomic resolution of BTV virus particles, under both physiological and low-pH conditions, has further elucidated how the VP2 and VP5 proteins sense endosomal pH. Furthermore this study revealed how VP2 and VP5 coordinate during cell entry and how VP5 acquires membrane-penetration activity by a dramatic conformational change induced by low pH (21), supporting and validating earlier observations.

The 10 segmented dsRNA molecules comprising the BTV genome, ranging in size from 0.8 to 3.9kb, have a limited capacity to accommodate foreign genes (22). Thus, to avoid disruption of the overall structure of the protein by introducing a FP, an alternate strategy of

introducing a small TC tag with a CCPGCC motif was selected (11-13). Previously, this technique had been successfully applied to enveloped viruses (23-26), but not for any complex capsid virus such as BTV. In this report we described a methodical approach that required combinations of sequence comparison data, biochemical methods and the BTV reverse genetics (RG) system (27) to generate a virus with the TC tags introduced into VP2 in such a way that they do not disrupt the structure-function relationship of the virus particles. The power of this technology was demonstrated through the enabling of the delineation of the dynamics of the two outer capsid proteins, which were shown to dissociate from one another during the early stages of virus entry into mammalian cells (14). Here we describe the prerequisite methodology that led to this observation.

Methods and Results:

Identification of putative exposed regions in VP2 for subsequent TC tag insertion. As the host attachment protein responsible for virus entry, the exposed outer capsid protein VP2 was the prime candidate for labeling the virus particle. The insertion of any tag had to minimize any potential disruptions of the protein conformation, as this was likely to have proven an impediment to virus infectivity. Optimally, the TC tag would have to be placed within flexible loop-linker regions. The incorporation of this tag was carried out prior to the elucidation of the atomic structure (21), thus requiring a more involved delineation process to identify a site for tag insertion.

To identify such exposed loop regions in VP2 in the absence of the atomic structure, the amino acid sequences (ExPASy) of two different BTV serotypes (BTV1 and BTV10) were analyzed. This analysis revealed the presence of putative trypsin cleavage sites that were conserved between the two VP2 sequences. It was hypothesized that these putative cleavage sites could potentially be exposed loop-linker regions that could be utilized for the insertion of TC tags, provided they were accessible in the VP2 protein conformation and that they were not internal.

To identify the presence of potential enzyme cleavage sites in VP2 trypsin cleavage profiles were generated by digesting purified virus particles (BTV1) and VP2 protein (of BTV10), with increasing concentrations of trypsin for 30mins at 37°C. Digestion of purified virus particles

and VP2 protein with 100ng of trypsin (~10,000 BAEE units/mg protein) yielded protein bands with smaller sizes on SDS-PAGE gel, signifying regions accessible to trypsin.

Subsequently, the larger fragment from digested VP2, the 100kDa product, was excised from the SDS-PAGE gel.

These 100kDa purified products were further digested by trypsin to generate smaller peptide fragments for subsequent analyzes by mass spectrometry, with the undigested 110kDa purified protein, treated in the same way, serving as control. SwissProt-based Mascot analysis of the peptide fragments confirmed that these peptides were BTV10 VP2 derived. Peptides derived from the 100kDa fragment mapped perfectly to the BTV10 VP2 sequence after amino acid residue 94; while the peptides derived from the full-length VP2 (110kDa) control mapped the entire length of the protein. This suggested that the 100kDa protein fragment lacked the first 94 amino acids. With an exposed loop identified, the TC tag was inserted, for subsequent determination of whether its introduction still preserved overall folding and biological activity (14).

Generation and characterization of recovered mutant viruses containing TC-tagged VP2.

The TC tag was subsequently inserted in the coding region of BTV1 VP2 between residues 94 and 95 (Fig. 2) within a T7 plasmid containing the VP2 open reading frame. The T7 plasmids containing all viral genes were used to generate RNA transcripts. Purified BTV single-stranded RNA (ssRNA) (S1 and S3 to S10) together with either wild-type BTV1-S2 or the tagged S2 transcripts were transfected into a replication permissive BSR cell (derived from baby hamster kidney [BHK] cells) monolayer before being covered with agarose overlay medium (22,27). This was followed by a 72 to 96 hour incubation to allow for amplification of the BTV virus and plaque formation. Both the wild-type and the mutant virus formed plaques and were therefore recovered successfully.

To validate the successful insertion and retention of the TC-tag in the recovered viruses, genomic dsRNA from infected cells was extracted from independent plaques. The analysis confirmed that 10 dsRNA segments synthesized by the tagged viruses had dsRNA profiles that were indistinguishable from that of WT BTV1. The presence and retention of the TC tag was subsequently confirmed using forward and reverse primers flanking the full-length S2. Complementary DNA (cDNA) of viral dsRNA from both WT and the tagged virus was

generated by RT-PCR and sequenced, confirming the presence of the TC tag sequence at the relevant position in the S2 segment of the tagged virus.

Such validation steps are essential for confirming that the tagged virus is functional, capable of successfully infecting cells, cell-to-cell spread and retaining the TC-tag. Had the virus been incapable of infecting, replicating or spreading to neighboring cells, no plaques would have been evident. This confirmed the viability of the tagged-virus for subsequent studies. Furthermore, by sequencing the viral genomes of recovered virus it could be confirmed that the TC-tag sequence was retained and not ejected from the genome. Had the TC-tag been lost from the genome, RT-PCR and sequencing would have resulted in absence of the tag.

Internalization of tagged viruses in cells. This tagged virus particle was then used to assess whether the fluorescent labeling with biarsenical dye FIAsh generated sufficient signal for the intended subsequent investigation of trafficking of tagged virus during entry into the host cells (Fig. 3). Live cell imaging of HeLa cells (BTV permissive cell line) mock infected (Fig. 3A) and infected (Fig. 3B) with the TC tagged virus stained with FIAsh, which binds specifically to tetracysteine tags, were imaged by confocal microscopy immediately after infection using the following reagents and protocol. Handling of all virus with regards to processing and containment followed local guidelines.

Reagents and buffers for tetracysteine staining of live and fixed cells.

- TC-FIAsh™ II In-Cell Tetracysteine Tag Detection Kit (T34561 - Thermo Fisher Scientific), containing: FIAsh-EDT2 labeling reagent (2 mM in DMSO) and BAL wash buffer (100X in ddH₂O (25 mM))
- Cell growth medium
- Opti-MEM®
- Phosphate buffered saline (PBS)
- Fixation buffer (4% formaldehyde in 1x PBS)
- Blocking buffer (1% BSA in 1x PBS)
- Permeabilization buffer (0.5% Triton X-100 in 1x PBS)
- Chamber slide (μ-Slide 4 Well - Ibidi)

Important – The main stain (FIASH) should not be frozen and thawed repeatedly.

Appropriate dilutions should be aliquoted and then stored at -20°C.

Note – all actions should be carried out on ice and all the reagents should be ice cold.

Protocol for tetracycline staining of live cells.

1. Cells were seeded in a μ -Slide 4 Well and grown overnight (number may depend on cell type).
2. The cells were washed with growth media that did not contain FCS.
3. The virus was adsorbed (Multiplicity of Infectious virus at 10) and incubated for 30 mins.
4. The cells were washed with fresh media once.
5. Cells were then washed once with Opti-MEM[®]. It is noteworthy that from the FIASH staining stage, everything is done in the dark, where possible, and samples should be protected from light for all subsequent steps.
6. For labelling with FIASH the master stain concentration is 2 mM and should be diluted to a final concentration of 2 μ M in cold Opti-MEM[®]. Sample and stain should be incubated together for 15 mins on ice. The incubation time can be adjusted to lower background staining. If 2 μ M gives a weak signal then this can be increased up to 5 μ M.
7. Cells were washed with BAL wash buffer once. The master buffer is x100 and should be diluted in Opti-MEM[®].
8. The BAL wash buffer was then replaced with normal growth medium (no FCS).
9. The chamber slide was then placed in a stage incubator at 37°C and live cell imaging commenced (optimum conditions may depend on cell type and may have to be adjusted accordingly). The FIAsH labeling Reagent has an excitation maximum of 508 nm and an emission maximum of 528 nm.
10. An appropriate objective was selected (100x) and the microscope software used to acquire single images of cells.

Note – The microscope stage must be preheated to 37°C for a minimum of one hour prior to imaging to allow for the system to stabilize to avoid temperature fluctuations affecting focusing. Further, between image gathering sessions, if required, the slide can be placed on a small heating block, in a carbon dioxide incubator or in a small heated humidity chamber (to minimize loss of growth medium).

Protocol for tetracysteine staining of fixed cells.

Note – all actions were carried out on ice and the reagents were ice cold.

1. Cells were seeded onto coverslips and grown overnight (number may depend on cell type).
2. Cells were washed with growth media that does not contain FCS.
3. Virus was adsorbed (Multiplicity of Infectious virus at 10) and incubated for 30 mins.
4. Cells were washed with fresh media once.
5. Media was replaced with 2% FCS containing media and incubated at 37°C for the required time points.
6. Cells were washed with PBS thrice.
7. Cells were fixed in fixation buffer for 10 mins and washed with PBS thrice.
8. Cells were permeabilized using permeabilization buffer for a maximum of 10 mins (optional for co-localization studies) and washed with PBS thrice.
9. Cells were labelled with FIASH (as described in 5. For staining live cells) and washed with 1x BAL twice.
10. Cells were washed with PBS thrice.
11. Cells were blocked by adding blocking buffer for 1 hour (optional for co-localization studies).
12. Cells were washed with PBS thrice.
13. If co-localisation studies are being undertaken, then the primary antibody for the interacting partner can be now added and incubate with the cells for 1 hour (incubation times may vary depending on antibody used).
14. Cells were washed with PBS thrice to remove unbound primary antibody.

15. Cells were then incubated with the secondary antibody, for example, Alexa 594 (excitation at 590 nm, emission at 619 nm) and the nuclear stain (such as Hoechst 33342 (excitation at 352 nm, emission at 455 nm)) for 1 hour. The choice of secondary antibody fluorophore and nuclear stain should be made to minimize excitation/emission overlap between the individual staining components.
16. Cells were washed with PBS thrice.
17. The samples were then mounted, using mounting media onto glass slides and sealed with nail varnish, using standard protocols.
18. The slides were then placed on a confocal stage and imaged. Note that if storage is required, care should be taken to store the slides in the dark at 4°C.

This live cell staining protocol allowed for the detection of the movement of fluorescently labeled particles into the cellular cytosol and confirmed that insertion of the TC tag in VP2 could be used for the generation of fluorescently labeled BTV particles. Having confirmed functionality, the tagged virus could now be used to determine its applicability during modulated experimental conditions.

Previously, it was reported that BTV entered cells via the clathrin-mediated endocytic pathway (28). The cellular protein dynamin is known to mediate the pinching of the clathrin-coated pits to form the coated vesicles (29,30), which could be inhibited with the compound dynasore (31). As an example, tagged virus thus afford the opportunity to further delineate that process to determine whether virus entry was also affected by dynamin, as a role for dynamin in BTV entry had previously been suggested (32). As described above, tagged virus was adsorbed on both mock-treated and dynasore-treated HeLa cells. The cells were washed and either processed for zero time post-infection or incubated at 37°C for 30 mins. At both times the cells were fixed with 4% paraformaldehyde, stained with FIAsH and visualized by confocal imaging.

Analysis of the cells that had not been treated with dynasore at zero time post-infection showed the presence of the majority of the tagged BTV1 on the plasma membrane (Fig. 4, left panel.), while at 30 mins post-infection the majority of virus (~84%) could be observed within the cytoplasm (Fig. 4, middle panel). Conversely, however, dynasore-treated cells

analyzed at 0 min post-infection (~93%) and 30 mins (~84%) showed the presence of majority of tagged virus particles on the plasma membrane (Fig. 4, right panel).

The generation of this TC tagged virus also allowed for exploring a fundamental question of BTV virus entry. For example, do VP2 and VP5, which are juxtaposed on the surface of the virus particle and are shed in an early endosome, remain together during this process. Such an event is highly relevant for establishing BTV infection, since only after the shedding of VP2 and VP5, a transcriptionally active core is released into the cytoplasm. Once there, viral RNA transcripts are extruded, functioning as templates for the synthesis of viral genomic RNAs and proteins (19,33-36).

A tagged virus allows for an infection time course to be undertaken to delineate where the respective proteins are localized by fixing cells with 4% paraformaldehyde at 2, 5 and 15 mins post-infection. Dual labeling of the tagged virus VP2 (FIAsH labeled) and VP5 (immune-labeled with a polyclonal VP5 antibody) was carried out, as described above. Subsequent analysis of confocal microscopy images revealed that co-localization (yellow) of VP2 and VP5 was observed at 2 mins post-infection (~95.8%) and 5 mins post-infection (~90.5%) (Fig. 5, left and middle panel). However, from 15 mins post-infection onwards, VP2 and VP5 were seen as separate entities (Fig. 5, right panel) in the majority of the infected cells (~72.9%), suggesting that the two proteins disengage early in virus entry. This experiment validated that the FIAsH label could be successfully used in conjunction with immune-labeled proteins, without their juxtaposition interfering in a dual-labeling context.

Discussion:

When selecting a fluorescent tag for *in vivo* imaging of cells, a number of parameters and aspects must be considered. These include whether the tag may affect and interfere with protein conformation or localization, cell toxicity, spectral overlap, photo-stability and background. While fluorescent-labeled proteins (FPs) essentially have no background and are auto-fluorescent, they are large and their photophysical characteristics may not be optimal for their desired purpose. This is particularly relevant for visualizing a structurally complex capsid virus. Tight icosahedral structures have a limited capacity and tolerance for

accommodating large inserts due to the potential for interfering with protein-protein interactions and thus prove deleterious to viral viability.

Facing such limitations and constraints, only a small fluorescent tag can be accommodated, thus, a TC tag is required. The principal advantage of using a TC tag capable of binding biarsenical dyes is that the essential motif is small. This facet decreases the chance that the introduction of this sequence will disrupt the overall protein conformation and function. Furthermore, the capacity for visualizing an expressed protein *in vivo* by the addition of a fluorophore makes biarsenical dyes valuable tools for fluorescence resonance energy transfer (FRET), which has been utilized in mapping conformational changes of membrane receptors during ligand binding (37).

Clearly, while advantages exist, TC tags also entail major concerns that must remain in focus. This includes the effect of the tag on the protein's function or localization, overall cellular toxicity of the ligands, weak fluorescent signal, elevated levels of background labeling and most importantly, fluorescent bleaching. Firstly, it is possible to overcome or mitigate disruptions of protein localization or function concerns arising from the insertion of the TC tag into target proteins through untagged controls. As described in the Methods and Result section, it is essential to first validate the tagged virus, directly comparing its growth rates and biological attributes, where possible, with the original wild-type virus (14). Secondly, cellular toxicity concerns can be mitigated by controlling the duration of exposure to the biarsenical dyes. Thirdly, a weak fluorescent signal may be detected. Reasons for this may include low abundance of the TC-tagged protein, loss of activity of labelling reagent due to prolonged storage, the duration of the labelling time was too brief or that the labelling reagent was too dilute. Solutions to a weak fluorescent signal may include increasing cell culturing times to facilitate further tagged protein expression, increasing protein labelling times and the concentration of labeling solution (up to 10 μM). The loss of activity of the labelling reagent may be mitigated by freshly preparing labelling solutions when required and the aliquoting and storage at -20°C of the labelling solution while shielded from light. Fourthly, elevated levels of background labeling can occur where cells display a fluorescent haze. This may be due to non-thiol binding sites, such as membranes or hydrophobic pockets or the interaction of these dyes with off-target cellular proteins rich in cysteines (38). To address and reduce the fluorescence of background and off-target labelling,

samples can be treated and washed with free ethane dithiol (EDT_2), dye disperse blue 3 or the arsenic binding chemical such as British anti-Lewisite (BAL) (39-41) and with fresh OptiMEM® Medium. Concurrently, the labelling time and the concentration of the labelling reagent can be adjusted and optimized to conform to the unique experimental set-up. Similarly, the exposure times during imaging can be reduced. Furthermore, where feasible, tandem repeats of the TC tag can be inserted to increase the total number of dye molecules per target protein (42). Further, the increase in the number of tandem repeats may increase signal duration, temporarily stemming photo-bleaching effects, while background staining decreases during the exposure time.

Cumulatively, with both constraints and advantages taken into account, the methods described here detail how it proved possible to successfully label a structurally complex virus without compromising its genetic and functional integrity. This facilitated the delineation of the hitherto obscured entry pathway utilized by BTV, demonstrating that within mammalian cells, following infection of cells, VP2 dissociated from virus particle, while the BTV particles containing an outer layer of VP5 trafficked onward, in the absence of VP2.

Conclusions:

The methodology detailed here highlights the feasibility of introducing small molecular tags into structurally complex viruses without diminishing or influencing any structure/function relationships. However, safeguards must be maintained in ensuring that the undertaken modifications do not affect virus replication or other structure/function relationships, principally by validating all such attributes in parallel to an unmodified wild-type control virus.

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Figure legends:

Figure 1. BTV capsid structure. Electron-Cryomicroscopy high-resolution (7-Å) image of BTV. Outer-capsid coat comprised of VP2 (cyan and magenta) and VP5 (green). Inner capsid comprised of VP7 (black and red), whereby VP3 (not visible) is occluded by the VP7 layer (Adapted from Zhou, Roy & associates, PNAS, 2010).

Figure 2. TC tag insertion site into VP2. BTV1-VP2 amino acid sequence with trypsin cleavage site (*) and TC tag (green box) inserted downstream of cleavage site.

Figure 3. FIAsh labeling of BTV1 during live-cell imaging. HeLa cells mock infected (A) and infected (B) with TC-tagged BTV1 labeled with FIAsh immediately post-infection (Adapted from Du et al, JVI, 2014).

Figure 4. Inhibition of dynamin inhibits BTV entry into cells. HeLa cells either mock treated (-) or treated with Dynasore (+) were infected with TC-tagged BTV1 (FIAsh labeled – green)

and analyzed 0 min and 30 min post-infection (arrows indicate fluorescently labeled VP2), bars, 10 μ m. White lines depict margin of cells. (Adapted from Du et al, JVI, 2014).

Figure 5. VP2 and VP5 dissociate early during virus entry. HeLa cells were infected with TC-tagged BTV1. Localization of VP2 (FlAsH labeled – green) and VP5 (red) was assessed at 2, 5 and 15 min post-infection. Co-localization was visualized as yellow. Bars 10 μ m. (Adapted from Du et al, JVI, 2014).

Figure 1.

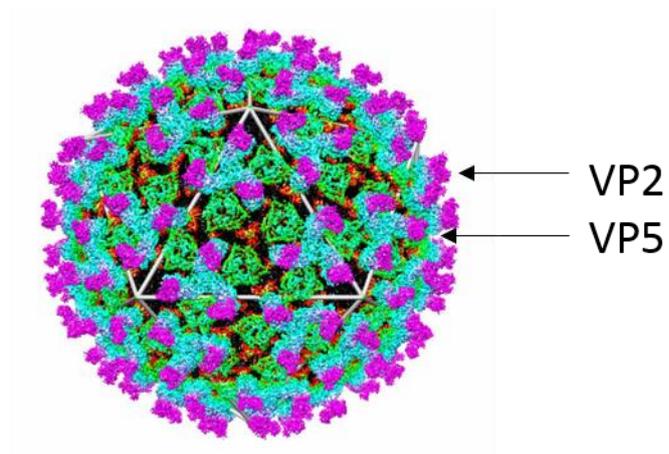


Figure 2.

BTV1-VP2 LRGYDERRAVVETR CCPGCC HKSFHTNDQ
* TC tag

Figure 3.

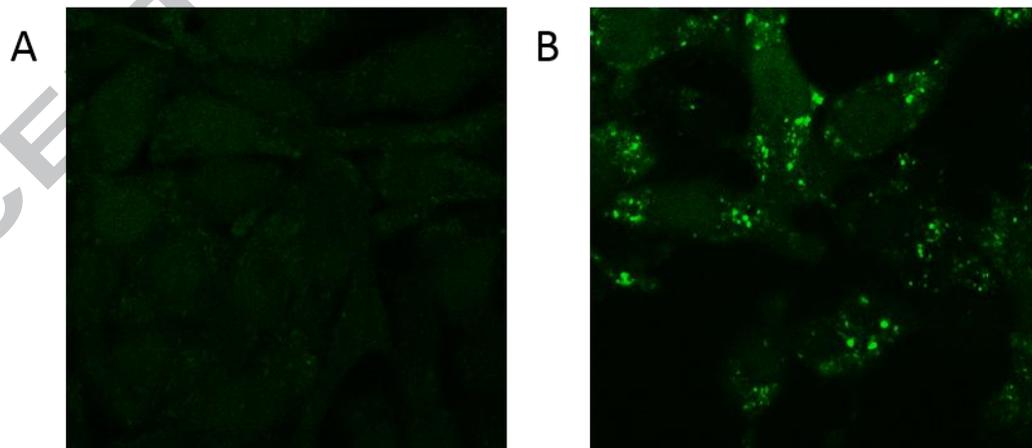


Figure 4.

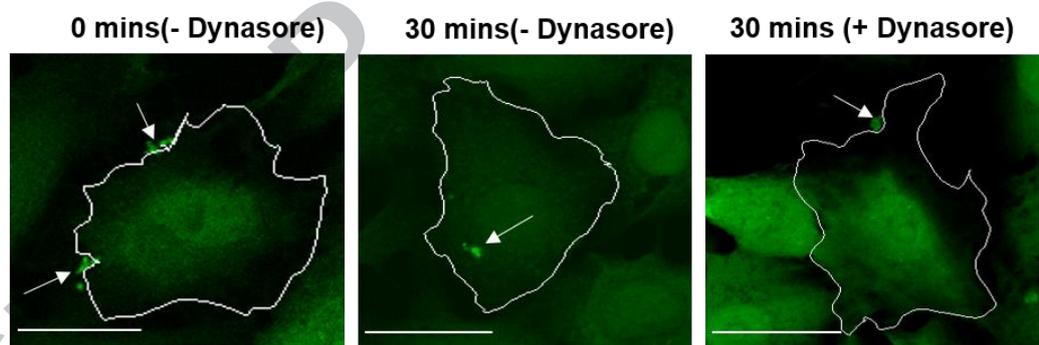
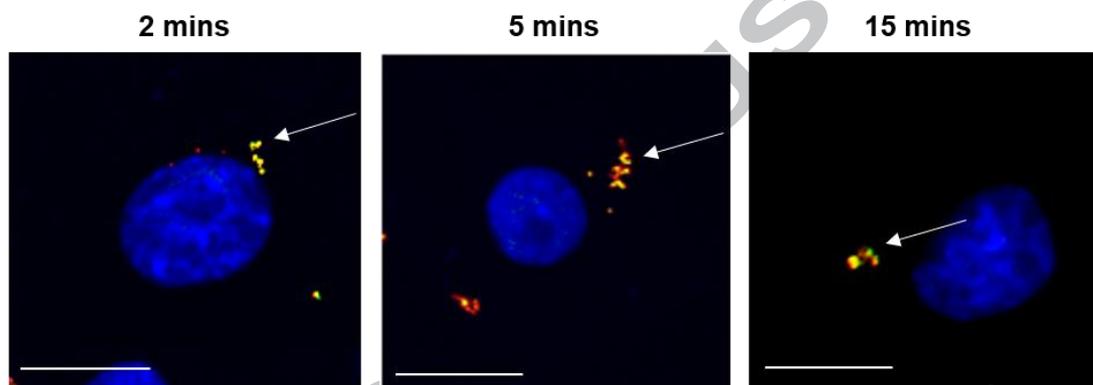


Figure 5.



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