Simultaneous Tracking of Capsid, Envelope Protein Localization in Living Cells Infected with Double Fluorescent Duck Enteritis Virus

Liu Chen
Institute of Animal Husbandry and Veterinary Sciences, Zhejiang Academy of Agricultural Sciences
Zheng Ni
Institute of Animal Husbandry and Veterinary Sciences, Zhejiang Academy of Agriculutural Sciences
Jionggang Hua
Institute of Animal Husbandry and Veterinary Sciences, Zhejaing Academy of Agricultural Sciences
Weicheng Ye
Institute of Animal Husbandry and Veteerinary Sciences, Zhejiang Academy of Agricultural Sciences
Keshu Liu
Institute of Animal Husbandry and Veterinary Sciences, Zhejiang Academy of Agricultural Sciences
Tao Yun
Institute of Animal Husbandry and Veterinary Sciences, Zhejiang Academy of Agricultural Sciences
Yinchu Zhu
Institute of Animal Husbandry and Veterinary Sciences, Zhejiang Academy of Agricultural Sciences
Cun Zhang (cz65@foxmail.com)
Institute of Animal Husbandry and Veterinary Sciences, Zhejiang Academy of Agricultural Sciences

Research

Keywords: duck enteritis virus, double-labelled virus, fusion expression, location

Posted Date: September 1st, 2020

DOI: https://doi.org/10.21203/rs.3.rs-58706/v1

Abstract

Background

Duck enteritis virus can cause an acute, contagious and lethal disease of many species of waterfowl. An infectious bacterial artificial chromosome clone of duck enteritis virus (DEV) vaccine strain pDEV-EF1 has been constructed in our previous study. To visually studying DEV, a double-labeled recombinant virus have been constructed.

Methods

based on pDEV-EF1, a recombinant mutated clone pDEV-UL35(c)CFP-gC(c)mRFP which carries a red fluorescent protein(mRFP) gene attached to the viral envelope protein gC, combined with a cyan fluorescent protein (CFP) gene fused to the viral capsid UL35 gene was constructed by two-step Red/ET recombination and the recombinant virus rDEV-UL35(c)CFP-gC(c)mRFP was rescued from chicken embryo fibroblasts (CEFs) by calcium phosphate precipitation. Then protein expression were detected by Western blot analysis, and subcellular location of gC and UL35 were observed by confocal microscopy. Viral morphogenesis were observed by transmission electron microscopy (TEM).

Results

The recombinant virus rDEV-UL35(c)CFP-gC(c)mRFP was rescued from chicken embryo fibroblasts (CEFs) by calcium phosphate precipitation. Western blot analysis showed UL35-CFP and gC-mRFP are both expressed in fusion forms in rDEV-UL35(c)CFP-gC(c)mRFP-infected CEFs, and subcellular location study showed gC-mRFP was mainly localized in whole cell at 36 h.p.i. and 48 h.p.i.; and then mostly migrated to cytoplasm after 60 h.p.i.; UL35-CFP was localized in nucleus in all stages of virus infection. Transmission electron microscopy indicated that viral particles at different stages of morphogenesis (A capsids, B capsids, C capsids) were observed in virus-infected cells. However,mature C capsids was less in rDEV-UL35(c)CFP-gC(c)mRFP-infected cells than rDEV-dEF1GFP and rDEV-gC(c)mRFP-infected samples.

Conclusions

This study has laid a foundation for visually studying localization, transportation of DEV capsid protein and envelope glycoprotein, as well as virus assembly, virion movement and virus-cell interaction.

Background

Duck enteritis virus (DEV), also known as duck plaque virus, is the causative agent of duck viral enteritis, which causes an acute, contagious and lethal disease of many species of waterfowl within the order

Anseriformes. DEV (also referred to as Anatid herpesvirus 1) is classified into the genus *Mardivirus* and the subfamily *Alphaherpesvirinae* of *Herpesviridae* according to virus taxonomy reported in 2012 by the International Committee on Taxonomy of Viruses (ICTV) [1].

Herpesvirus is a large, enveloped virus with four structural components, including a linear double-stranded DNA, an icosahedral capsid, an amorphous tegument, and a bilayer lipid envelope. In general, the DEV genome is approximately 158 kb composed of a unique long (UL) region and a unique short (US) region flanked by an internal repeat sequence (IRS) and a terminal repeat sequence (TRS). It contains 78 open reading frames (ORFs) predicted to encode potential functional proteins. Among them, there are 11 envelope glycoproteins (gB/UL 27, gC/UL 44, gD/US6, gE/US8, gG/US4, gH/UL22, gI/US7, gK/UL 53, gL/UL1, gM/UL10 and gN/UL49.5) and 7 capsid protein (UL38/VP19C, UL18/VP23, VP26/UL35, UL21, UL25, UL19/VP5, UL6) [2, 3]. Although the structure and function of these proteins in some human herpesviruses have been studied to some extent, little is known about DEV protein. The predicted ORF of the DEV UL44 gene was expected to encode a protein comprising 431 amino acids with a putative molecular mass of 47.35 kDa[4], but in fact, Hu Y *et al*. demonstrated that the molecular mass of gC is 55 kDa. We speculate that post-translation modification leads to molecular mass increase of gC. Research indicates that gC plays a role in the adsorption of DEV to chicken embryo fibroblasts cells and in infectivity, gC plays a role in DEV egress and absence of DEV gC results in increased plaque sizes *in vitro* [5, 6]. VP26 is the smallest capsid protein in herpes simplex virus type 1[7], the ORF of DEV UL35 encodes VP26 comprising 117 amino acids, with molecular mass of 12.9 kDa. DEV VP26 was testified to be a component of extracellular mature DEV virions [8].

In this study, we have constructed a double-fluorescence protein labelled recombinant duck enteritis virus which carries a red fluorescent protein (mRFP) gene attached to the viral envelope protein gC, combined with a cyan fluorescent protein (CFP) gene fused to the viral capsid UL35 gene. This study has laid a foundation for visually studying localization, transportation of DEV capsid protein and envelope glycoprotein, as well as virus assembly, replication and virus-cell interaction.

Material And Methods

All research was approved by the relevant committees at the Zhejiang Academy of Agricultural Sciences.

Virus strain and cells

The bacterial pDEV-EF1/GS1783 which contains the full-length infectious BAC clone of DEV vaccine strain and corresponding virus rDEV-EF1 were constructed and stored in our laboratory [9, 10]. Chicken embryo fibroblasts (CEFs) were prepared from 10-day-old specific-pathogen-free (SPF) embryonated eggs (Zhejiang JianLiang Bioengineering Co., Ltd., Hangzhou, China) according to standard procedures and cultured in DMEM (Gibco-BRL) supplemented with 8% FBS, 100 U of penicillin/mL and 100 μg streptomycin/mL.

Construction of transfer vector pT-linker-mRFP-P1 and pT-linker-CFP-P2

The transfer vector pT-linker-mRFP-P1 was constructed as flows: primer pair mRFP-F1(gly) and mRFP-R1 (Table 1) was designed to amplify a red fluorescent protein (mRFP) gene prefixing with flexibility linker GGGGSGGG from plasmid pLVX-mRFP-C1 (Clontech). The PCR fragment linker-mRFP was cloned into a pMD19T-simple vector (Takara), and the forward inserted clone pT-linker-mRFP was selected by PCR amplification with primer pair M13(+) and mRFP-R1. Another primer pair kan_mRFP-for(PstI) and kan_mRFP-rev(PstI) (Table 1) was designed to amplify P1 fragment (containing *Pst* I-homologous arm a -kan-*Pst* I) from plasmid pEP-kan-S (a gift form Dr. Nikolaus Osterrieder, Freie Universität Berlin, Berlin, Germany).

And P1 fragment was cloned into the *Pst* I site present in pT-linker-mRFP to construct the transfer vector pT-linker-mRFP-P1 which containing linker-mRFP1 (the first part of mRFP gene)- *Pst* I -homologous arm a-kan-*Pst* I-mRFP2(the second part of mRFP gene) in sequence, that is, mRFP gene is divided into two parts by fragment “homologous arm a-kan-Pst I”, and homologous arm a (Hom A) matches exactly the front end sequence of mRFP2.

The construct strategy of transfer vector pT-linker-CFP-P2 was similar to pT-linker-mRFP-P1. Primer pair CFP-F1(gly-SacI) and CFP-R1(Table 1) was designed to amplify cyan fluorescent protein (CFP) gene prefixing with flexibility linker GGGGSGGG (linker-CFP) from plasmid pCMV-C-CFP(Beyotime Biotechnology). Primer CFP-F1(gly-SacI) was introduced flexibility linker GGGGSGGG and restriction enzyme *Sac* I site. The PCR fragment linker-CFP was cloned into a pMD19T-simple vector (Takara), and the forward inserted clone pT-linker-CFP was selected by PCR amplification with primer pair M13(+) and CFP-R1. Another primer pair kan_CFP-for(*Sac* I) and kan_CFP-rev(*Sac* I) (Table 1) was designed to amplify P2 fragment (containing *Sac* I -homologous arm b-kan- *Sac* I) from plasmid pEP-kan-S. And P2 fragment was cloned into the *Sac* I site present in pT-linker-CFP to construct the transfer vector pT-linker-CFP-P2 which containing linker-CFP1 (the first part of CFP gene)-*Sac* I-homologous arm b-kan-*Sac* I -CFP2 (the second part of CFP gene) in sequence. Homologous arm b (Hom B) matches exactly front end sequence of CFP2.

Construction of recombinant BAC clones

Mutated clone pDEV-UL35(c)CFP-gC(c)mRFP carrying a red fluorescent protein (mRFP) gene attached to the viral envelope protein gC combined with a cyan fluorescent protein (CFP) gene fused to the viral capsid UL35 gene was constructed by three times of two-step Red (*en passant*) recombination [11, 12]. Firstly, pDEV-dEF1GFP deleting open reading frame (ORF) of green fluorescent protein gene (GFP) (pEF1-GFP) was constructed, then mRFP gene was inserted to C terminus of DEV gC gene to construct recombinant clone pDEV-gC(c)mRFP, at last, pDEV-UL35(c)CFP-gC(c)mRFP was obtained by inserting CFP gene to C terminus of DEV UL35 gene. In details, to construct pDEV-dEF1GFP, ORF of pEF1-GFP was deleted from pDEV-EF1 by two-step Red (*en passant*) recombination. Brief, PCR was done with primer pair

DEV-dEF1-gfp-for and DEV- dEF1-gfp -rev (Table 1) using pEP-kan-S as template. Then, 100 ng of purified PCR product (1200 bp in length) was electroporated into competent GS1783 bacteria containing pDEV-EF1 and bacteria of transformation were plated on LB plates containing chloramphenicol (34 μg/mL) and kanamycin (50 μg/mL) and incubated at 32°C for 48 h. Double-resistant colonies were selected for the 2nd Red recombination to remove the kanamycin resistance gene. Colonies resistant to chloramphenicol but sensitive to kanamycin were selected. Following, positive clonies were determined by RFLP (restriction fragment length polymorphisms) with *Xba* I and sequencing the PCR products amplifying with primer pairs dgfp-JD-F and dgfp-JD-R flanking the region of pEF1-GFP deletion. Following, to construct pDEV-gC(c)mRFP, PCR was done with primer pair mRFP-gC(c)-F1 and mRFP-gC(c)-R1 (Table 1) using pT-linker-mRFP-P1 as template. And 100 ng of purified PCR product (1856 bp in length) was electroporated into competent GS1783 bacteria containing pDEV-dEF1GFP to carry out two-step Red (*en passant*) recombination as described earlier. Positive clones were determined by RFLP with *Bgl* II and sequencing the PCR products amplifying with primer pair gC(c).mRFP-JD-F and gC(c).mRFP-JD-R (Table 1) flanking the region of linker-mRFP insertion. At last, to construct pDEV-UL35(c)CFP-gC(c)mRFP, PCR was done with primer pair CFP-UL35 (c)-F1 and CFP-UL35 (c)-R1 (Table 1) using pT-linker-CFP-P2 as template. And 100 ng of purified PCR product (1898 bp in length) was electroporated into competent GS1783 bacteria containing pDEV-gC(c)mRFP to carry out two-step Red (*en passant*) recombination in the same way. Positive clones were determined by RFLP with *Bgl* II and sequencing the PCR products amplifying with primer pair CFP-UL35-JD-F and CFP-UL35-JD-R flanking the region of linker-CFP insertion.

Rescue of recombinant viruses

BAC DNAs of pDEV-dEF1GFP, pDEV-gC(c)mRFP and pDEV-UL35(c)CFP-gC(c)mRFP were extract from bacterial by alkaline lysis method. Next, 4 μg of BAC DNA was transfected into CEFs by calcium phosphate precipitation according to Chen L [9]. The cells were then cultured with DMEM supplemented with 8% FBS for 3 ~ 6 days until 70 ~ 80% cytopathogenic effect (CPE) occurred. The virus collected were separately named rDEV-dEF1GFP, rDEV-gC(c)mRFP and rDEV-UL35(c)CFP-gC(c)mRFP.

Measure sizes of virus plaque

The plaque sizes of rDEV-UL35(c)CFP-gC(c)mRFP, rDEV-gC(c)mRFP, rDEV-dEF1GFP and rDEV-EF1 were measured as follow. The viruses were serially diluted and plated onto CEFs seeded in a 12-well plate, and 2 h later, the culture medium was replaced with DMEM containing 1.5% methylcellulose (Sigma). After a 2-day-incubation at 37 °C, for every virus, 100 fluorescent plaques were randomly selected and taken photo under fluorescence microscope (Nikon, Japan), then their sizes were measured using Image J software (http://rsb.info.nih.gov/ij/). Statistical analyses to compare the differences in the plaque sizes between the four strains were conducted using one-way ANOVA with SPSS 11.5 software.

Determination of the growth curve

The growth curve of those reconstructed virus were determined and compared with the parent virus rDEV-EF1. Briefly, CEFs were infected with approximately 0.02 MOI((multiplicity of infection) of cell-free viruses of rDEV-EF1, rDEV-dEF1GFP, rDEV-gC(c)mRFP and rDEV-UL35(c)CFP-gC(c)mRFP. The cells and culture

supernatant were harvested at different time (6, 12, 24, 36, 48, 60, 72, 84, 96, 108 h) after virus infection. And cells were collected by trypsin digestion following washing twice with phosphate-buffered saline (PBS, pH7.0) and then suspended with 1 mL DMEM-2% FBS as the equal volume of supernatant. Then the collected cells and supernatants were stored at -70℃until all samples have been collected. Before titrated on CEFs, cells were treated by Tissuelyser-24 at 65 Hz for 90 s and centrifuged at 6000 rpm for 5 min, then 100 μL of lysis supernatant and culture supernatant were taken to titer by $TCID_{50}$ test according to standard virological method. The growth curves were computed from three independent experiments.

Western blot analysis of UL35-CFP and gC-mRFP protein expressed in recombinant virus-infected CEFs

Expression of UL35-CFP and gC-mRFP protein was verified by Western blot analysis using rabbit anti-GFP polyclonal antibody (Beyotime Biotechnology), rabbit anti-mRFP polyclonal antibody (Abcam) and mouse anti-gC polyclonal antibody (Preparation in our laboratory) and rabbit anti-UL35 polyclonal antibody (Preparation by Genscript Co.). In details, the cells infected with 1.0 MOI rDEV-EF1, rDEV-dEF1GFP and rDEV-gC(c)mRFP, rDEV-UL35(c)CFP-gC(c)mRFP which occurred 80% CPE were collected and treated with sample buffer, then subjected to 10% SDS-PAGE and electrophoretically transferred to a PVDF membrane (Millipore). To eliminate possible non-specific binding, the membranes were block with 10% skimmed milk in PBST (0.5% Tween20 in PBS) overnight at 4℃ and incubated with rabbit anti-GFP polyclonal antibody (1: 500) or rabbit anti-mRFP polyclonal antibody (1:500) or mouse anti-gC polyclonal antibody (1: 500) or rabbit anti-UL35 polyclonal antibody (1: 250) for 1 h at 37℃. The horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG or HRP- conjugated rabbit anti-mouse IgG (Santacruz) was used as the secondary antibody. And the protein bands were visualized by SuperSignal® West Pico Chemiluminescent Substrate (Pierce) and chemiluminescence was observed using the CCD camera-equipped Chemi Lux detection system by Intas.

Protein Localization observed by confocal microscopy

The viruses were plated onto the slides of CEFs seeded in a 6-well plate, and 24 h, 36h,48 h, 60h□72 h, 96 h after infection, the cells were staining with SYTO® Green-Fluorescent Nucleic Acid Stains (Life technologies)and visualized by laser scanning confocal microscopy (Leica TCS-SP5) with a 100 × oil immersion objective.

Transmission Electron Microscopy

CEFs were infected at a MOI of 0.01 and processed for sectioning at 84 hpi. Briefly, cell pellets were collected and washed three times with PBS,then were fixed in a solution containing 2.5% glutaraldehyde plus 1% paraformaldehyde in 0.1 M cacodylic buffer for 2–4 h at 4 ºC, further fixed with 1% buffered O_SO_4 (Sigma) for 2 h at RT, washed three times in 0.1 M PBS. Samples were then dehydrated in an ethanol-water series(25–100% ethanol) and embedded in resin using an Embed 812 kit (SPI, USA), and polymerized at 60℃ for 48 h. Then ultrathin sections were obtained by sectioning the polymerized samples at 60 ~ 80 nm with a diamond knife Ultra 45 ° (Diatome) using a Leica UC7 (Leica

Microsystems,Wetzlar, Germany). Sections were stained with uranium acetate and lead salts. Then cells were examined with a transmission electron microscope HT7700 (hitachi) at 120 kV.

Results

Construction of recombinant BAC clones

RFLPs were determined to confirm that BAC mutated clones were indeed generated. DNAs of BAC mutated clones and intermediate were digestion by *Xba* I or *Bgl* II (Fig. 3b) and taken to electrophoresis. And the results of RFLP patterns indicated that the obtained *Xba* I or *Bgl* II pattern almost matched those of *in silico* predictions with slight difference which might be attributed to the difference between the reference sequence and the actual sequence (Fig. 3a). The sizes and sequencing results of PCR product amplified by identification primers from the recombinant BAC DNAs were also accord with prediction (Fig. 3c). Those results demonstrate that exogenous genes are inserted in expected place of DEV genome.

Measure sizes of virus plaque

When the plaque areas of recombinant viruses rDEV-dEF1GFP, rDEV-gC(c)mRFP, rDEV-UL35(c)CFP-gC(c)mRFP were separately compared to parental virus rDEV-EF1, it was discovered that the plaque areas were approximately 100%, 113%, 41% increase respectively, than those formed by rDEV-EF1 when measured on day 2 p.i, but there were no significant differences between the groups ($P = 0.201$; $P = 0.05$; $P = 0.435$) (Fig. 4).

Growth properties of the reconstructed viruses

The growth curve of the reconstructed viruses rDEV-dEF1GFP, rDEV-gC(c)mRFP, rDEV-UL35(c)CFP-gC(c)mRFP were compared with the parental rDEV-EF1 by infecting CEFs with each virus at an MOI of 0.02, harvesting total virus from the infected cells (Fig. 4A) and cell culture supernatants (Fig. 4B) at the indicated times postinfection, and assaying each sample by standard $TCID_{50}$ test determination on CEFs. The growth kinetics results, as shown in Fig. 4, suggested that, overall, the reconstructed viruses rDEV-dEF1GFP and rDEV-gC(c)mRFP (both harvesting from the infected cells and cell culture supernatants) had similar growth kinetics to the parental virus rDEV-EF1; However, the viral titers of rDEV-UL35(c)CFP-gC(c)mRFP were at least 100-fold lower compared to the parental virus.

Western blot analysis of UL35-CFP and gC-mRFP protein expressed in recombinant virus-infected cells

The gC and mRFP polypeptides are 55 and 27 kDa in size, respectively; therefore, a gC-mRFP fusion protein would be expected to have a mobility of 82 kDa in SDS-PAGE gels. And UL35 and CFP polypeptides are 12.9 and 27 kDa in size, respectively; therefore, a UL35-CFP fusion protein would be expected to have a mobility of 39.9 kDa in SDS-PAGE gels.

First, we detected gC and mRFP expressed in rDEV-gC(c)mRFP -infected cell samples. The results show that rDEV-gC(c)mRFP -infected cell sample was occurred two specific protein bands with molecular mass between 75 ~ 90 kDa compared to rDEV-dEF1GFP -infected cell sample (Fig. 6A) detecting with anti-mRFP antibody; When detecting with anti-gC antibody, rDEV-dEF1GFP -infected cell sample was occurred two specific gC protein bands about 55 kda, while rDEV-gC(c)mRFP -infected cell sample was occurred specific two protein bands between 75 ~ 90 kDa (Fig. 6B). This result indicates that gC and mRFP were expressed in fusion protein form in rDEV-gC(c)mRFP-infected cell sample, while in natural form in control rDEV-dEF1GFP-infected cell sample. In this experiment, two gC or gC-mRFP bands were detected, we deduce that DEV UL44 (gC) also expresses variants in according with Marek's Disease Virus [13].

Then expression of UL35-CFP and gC-mRFP were detected in rDEV-UL35(c)CFP-gC(c)mRFP infected-CEFs with rabbit anti-GFP polyclonal antibody (also recognized CFP), rabbit anti-mRFP polyclonal antibody and anti-UL35 polyclonal antibody. Results showed that rDEV-UL35(c)CFP-gC(c)mRFP -infected cell sample was occurred specific band with molecular mass 39.9 kDa compared to rDEV-dEF1GFP and rDEV-gC(c)mRFP -infected cell samples detecting with anti-GFP polyclonal antibody (Fig. 7A), and the 39.9 kDa protein was also recognized by UL35-specific antisera (Fig. 7B); when detecting with anti-mRFP polyclonal antibody, rDEV-gC(c)mRFP and rDEV-UL35(c)CFP-gC(c)mRFP -infected cell samples were occurred specific protein band between 75 ~ 90 kDa compared with rDEV-EF1 and rDEV-dEF1GFP -infected cell samples (Fig. 7C). These results demonstrate that UL35-CFP and gC-mRFP were both expressed in fusion form in rDEV-UL35(c)CFP-gC(c)mRFP -infected cells.

Location analysis of mRFP-gC and CFP-UL35 proteins

The virus rDEV-UL35(c)CFP-gC(c)mRFP was plated onto the slides of CEFs seeded in a 6-well plate until fluorescence occurred, the cells were staining with SYTO® Green-Fluorescent Nucleic Acid Stains (Life technologies)and visualized by laser scanning confocal microscopy (Leica TCS-SP5). Result show that no obvious fluorescent could be observed before 24 h.p.i., gC-mRFP was mainly localized in whole cell at 36 h.p.i. and 48 h.p.i.; and then mostly migrated to cytoplasm after 60 h.p.i., but UL35-CFP was localized in nucleus in all stages of virus infection (Fig. 8).

Subcellular distribution of rDEV-UL35(c)CFP-gC(c)mRFP-infected cells were also analyzed by Western blot. Subcellular components of rDEV-UL35(c)CFP-gC(c)mRFP-infected cells were extracted by Membrane and Cytosol Protein Extraction Kit (Beyotime Biotechnology), and gC-RFP was detected by Western blot analysis as above with anti-mRFP antibody. Result shows that gC-RFP located in both cellular membrane and cytosol (Fig. 9).

Transmission electron microscopy

To further investigate the effect of inserting exogenous protein sequence on virion morphogenesis. Virion morphogenesis was observed by thin-section transmission electron microscopy (TEM) in rDEV-dEF1GFP, rDEV-gC(c)mRFP, rDEV-UL35(c)CFP-gC(c)mRFP-infected cells. Result showed that viral particles at

different stages of morphogenesis (A capsids, B capsids, C capsids) were observed in virus-infected cells. However,mature C capsids was less in rDEV-UL35(c)CFP-gC(c)mRFP-infected cells than rDEV-dEF1GFP and rDEV-gC(c)mRFP-infected samples.

Discussion

Little is understood about the viral gene products of duck enteritis virus. In this study, subcellular localization of DEV gC and UL35 protein has been illuminated. The amino acid sequence analysis revealed that DEV-gC contains a transmembrane domain [4] and therefore should be located predominantly in cellular membranes of infected cells.

In this study, two gC protein bands were detected, and gC were localization in both cellular membrane and cytoplasm. This could be explained by two points: variant gC proteins realeased from the cell by protease cleavage; Alternatively, it was possible that alternative mRNA splice variants that lack the transmembrane domain could be expressed. Several herpesviruses, such as HSV-1 and MDV, expresses UL44 variants through mRNA splicing [13, 14]. We speculate that the formation way of DEV gC isomers is similar to MDV gC.

In our study, mRFP have also been inserted to N terminus of gB, gD and gC, as well as C terminus of gB and gD, but it is either failed to rescue recombinant virus, or failed to obtain mRFP-envelop fusion protein. Thus, it is rather important for choosing of exogenous gene insertion site. In the process of cultivation, the recombinant virus with red fluorescence could almost infect all cells demonstrating that mRFP is highly expressed. *Alphaherpesvirus is* a kind of viral live vector with obvious advantage, which has large genome, many non-essential genes, large capacity of inserting exogenous genes, stable heredity, low interference from maternal antibodies and long survival time *in vivo*. Several gene-deleted or recombinant PRV or MDV vaccines have been successfully applied in prevention and control of virus infection, and DEV is also tried to be developed as viral live vector [15–23], thus, as reference, the C terminus of gC is a preference site for exogenous gene insertion when developing bivalent vaccines.

With florescence protein as a marker, the life cycle of virus can be monitored in living infected cells. Desai P(1998) *et al* have first reported that a GFP could be incorporation into the herpes simplex virus type 1 capsid UL35 (VP26)[24], but expressing GFP fused to N terminus of pUL35 capsid component of pseudorabies virus(PRV) impairs virus replication in cell culture and neuroinvasion in mice [25]. In this study, we tried to express CFP fused to C terminus of UL35 of DEV which connected CFP and UL35 protein by a flexible linker GGGGSGGG. Plaque size measure demonstrates that fusion of CFP with UL35 resulted in 36.7% decrease of rDEV-UL35(c)CFP-gC(c)mRFP virus-plaque area, when compared with that of rDEV-gC(c)mRFP-dEF1GFP. While simultaneous addition of a 27 kDa mRFP-tag to the C terminus of the 55 kDa gC protein and a 27 kDa CFP-tag to the C terminus of 12 kDa UL35 protein does not completely damage virion assembly. However, according to TEM, mature C capsids was less in rDEV-UL35(c)CFP-gC(c)mRFP-infected cells than rDEV-dEF1GFP and rDEV-gC(c)mRFP-infected one, maybe the capability of UL35 binding to virus DNA were reduced by adding CFP-tag to the C terminus of UL35.

In this study, there is less effect on virus titer when single fluorescent protein was inserted into C terminus of gC or UL35, but when mRFP and CFP were simultaneously inserted into C terminus of gC and UL35, virus titer was decreased sharply. We postulated that fusion expression with the two exogenous labeled proteins forms steric hindrance that obstructs assembly of viral capsid and envelope protein.

Conclusions

In conclusion, we have constructed a strain recombinant duck enteritis virus expressed mRFP and CFP which are seprately fused with viral envelop protein gC and capsid protein UL35. This study facilitated the ability to visuallize virus entry, cell to cell spread and capsid translocation on the cellular cytoskeleton.

Abbreviations

DEV: duck enteritis virus; mRFP: red fluorescent protein; CFP: cyan fluorescent protein; CEFs: chicken embryo fibroblasts; RFLP: restriction fragment length polymorphisms; CPE: cytopathogenic effect; HRP: horseradish peroxidase; MOI: multiplicity of infection; TEM: transmission electron microscopy

Declarations

Ethics approval and consent to participate

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

Availability of data and materials

The dataset(s) supporting the conclusions of this article is(are) included within the article (and its additional file(s))."

Funding

The study was supported by grants from National Natural Science Foundation of China [grant number 31670150]; the National Key Research and Development Program of China (2016YFD0500107).

Acknowledgments

We want to thank Song xijiao for technical assistance. Plasmid pEP-kan-S was kindly provided by Dr. N. Osterrieder, Freie Universität Berlin, Berlin, Germany.

Author information

Affiliations

Institute of Animal Husbandry and Veterinary Sciences, Zhejiang Academy of Agricultural Sciences, Hangzhou, 310021, China

Liu Chen, Zheng Ni, Jionggang Hua, Weicheng Ye, Keshu Liu, Tao Yun, Yinchu Zhu, Cun Zhang

Contributions

LC planned and carried out virus recombinant, selection and identification of recombinant BAC clone, prepared figures, and drafted the manuscript. ZN constructed transfer vector for homologous recombination. JH and WY carried out rescue of reconstructed viruses and viral detection. KL performed protein expression analysis. TY and YZ performed characterization of viruses in cells. And CZ gave suggestion for this research and helped with overall planning and drafting of the manuscript. All authors read and approved the final manuscript.

Corresponding author

Correspondence to Cun Zhang.

References

1. King AMQ, Adams MJ, Carstens EB, Lefkowitz EJ. **Virus taxonomy**: **classification and nomenclature of viruses**: **Ninth Report of the International Committee on Taxonomy of Viruses**. http://ictvonline.org/ **virusTaxonomy**.**asp?version = 2012**&**bhcp = 1**. 2012.
2. Li Y, Huang B, Ma X, Wu J, Li F, Ai W, Song M, Yang H. Molecular characterization of the genome of duck enteritis virus. Virology. 2009;391:151–61.
3. Cardone G, Heymann JB, Cheng N, Trus BL, Steven AC. Procapsid assembly, maturation, nuclear exit: dynamic steps in the production of infectious herpesvirions. Adv Exp Med Biol. 2012;726:423–39.
4. Sun KF, Cheng AC, Wang MS. Bioinformatic analysis and characteristics of glycoprotein C encoded by the newly identified UL44 gene of duck plague virus. Genet Mol Res. 2014;13(2):4505–15.
5. Hu Y, Liu X, Zou Z, Jin M. Glycoprotein C plays a role in the adsorption of duck enteritis virus to chicken embryo fibroblasts cells and in infectivity. Virus Res. 2013;174(1–2):1–7.
6. Wang J, Osterrieder N. Generation of an infectious clone of duck enteritis virus (DEV) and of a vectored DEV expressing hemagglutinin of H5N1 avian influenza virus. Virus Res. 2011;159(1):23–31.

7. McNabb DS, Courtney RJ. Identification and characterization of the herpes simplex virus type 1 virion protein encoded by the UL35 open reading frame. J Virol. 1992;66(5):2653–63.
8. Cai MS, Cheng AC, Wang MS, Chen WP, Zhang X, Zheng SX, Pu Y, Lou KP, Zhang Y, Sun L, Wang LL, Zhu DK, Luo QH, Chen XY. Characterization of the duck plague virus UL35 gene. Intervirology. 2010;53(6):408–16.
9. Chen L, Yu B, Hua J, Ye W, Ni Z, Yun T, Deng X, Zhang C. Construction of a full-length infectious bacterial artificial chromosome clone of duck enteritis virus vaccine strain. Virol J. 2013;10:328.
10. Chen L, Yu B, Ni Z, Hua J, Ye W, Yun T, Zhang C. Construction and characterization of a recombinant duck enteritis virus expressing E protein of duck Tembusu virus. Acta Agriculturae Zhejiangensis. 2015;7(11):1889–95. **(in Chinese)**.
11. Tischer BK. **von** Einem J, Kaufer B, Osterrieder N: Two-step Red-mediated recombination for versatile high-efficiency markerless DNA manipulation in Escherichia coli. Biotechniques 2006, 40:191–7.
12. Tischer BK, Smith GA, Osterrieder N. En passant mutagenesis: a two step markerless red recombination system. Methods Mol Biol. 2010;634:421–30.
13. Jarosinski KW, Osterrieder N. Marek's Disease Virus Expresses Multiple UL44 (gC) Variants through mRNA Splicing That Are All Required for Efficient Horizontal Transmission. J Virol. 2012;86(15):7896–906.
14. Sedlackova L, Perkins KD, Lengyel J, Strain AK. **van** Santen VL, Rice SA: Herpes simplex virus type 1 ICP27 regulates expression of a variant, secreted form of glycoprotein C by an intron retention mechanism. J Virol 2008, 82(15): 7443–55.
15. Wang J, Osterrieder N. Generation of an infectious clone of duck enteritis virus (DEV) and of a vectored DEV expressing hemagglutinin of H5N1 avian influenza virus. Virus Res. 2011;159(1):23–31.
16. Liu J, Chen P, Jiang Y, Wu L, Zeng X, Tian G, Ge J, Kawaoka Y, Bu Z, Chen H. A duck enteritis virus-vectored bivalent live vaccine provides fast and complete protection against H5N1 avian influenza virus infection in ducks. J Virol. 2011;85(21):10989–98.
17. Liu X, Wei S, Liu Y, Fu P, Gao M, Mu X, Liu H, Xing M, Ma B, Wang J. **Recombinant duck enteritis virus expressing the HA gene from goose H5 subtype avian influenza virus.Vaccine** 2013, 31(**50**): **5953–5959**.
18. Zou Z, Liu Z, Jin M. Efficient strategy to generate a vectored duck enteritis virus delivering envelope of duck Tembusu virus. Viruses. 2014;6(6):2428–43.
19. Chen P, Liu J, Jiang Y, Zhao Y, Li Q, Wu L, He X, Chen H. The vaccine efficacy of recombinant duck enteritis virus expressing secreted E with or without PrM proteins of duck tembusu virus. Vaccine. 2014;32(41):5271–7.
20. Zou Z, Hu Y, Liu Z, Zhong W, Cao H, Chen H, Jin M. Efficient strategy for constructing duck enteritis virus-based live attenuated vaccine against homologous and heterologous H5N1 avian influenza virus and duck enteritis virus infection. Vet Res. 2015;46:42.

21. Wang J, Ge A, Xu M, Wang Z, Qiao Y, Gu Y, Liu C, Liu Y, Hou J. **Construction of a recombinant duck enteritis virus (DEV) expressing hemagglutinin of H5N1 avian influenza virus based on an infectious clone of DEV vaccine strain and evaluation of its efficacy in ducks and chickens.Virol J** 2015, 12: **126**.
22. Sun Y, Yang C, Li J, Li L, Cao M, Li Q, Li H. Construction of a recombinant duck enteritis virus vaccine expressing hemagglutinin of H9N2 avian influenza virus and evaluation of its efficacy in ducks. Arch Virol. 2017;162(1):171–9.
23. Chang P, Yao Y, Tang N, Sadeyen JR, Sealy J, Clements A, Bhat S, Munir M, Bryant JE, Iqbal M. **The Application of NHEJ-CRISPR/Cas9 and Cre-Lox System in the Generation of Bivalent Duck Enteritis Virus Vaccine against Avian Influenza Virus**. Viruses 2018, 10(**2**).
24. Desai P, Person S. Incorporation of the green fluorescent protein into the herpes simplex virus type 1 capsid. J Virol. 1998;72(9):7563–8.
25. Krautwald M, Maresch C, Klupp BG, Fuchs W. Mettenleiter TC:Deletion or green fluorescent protein tagging of the pUL35 capsid component of pseudorabies virus impairs virus replication in cell culture and neuroinvasion in mice. J Gen Virol. 2008;89(Pt 6):1346–51.

Table

Table 1 Primers used in this study

Primer	Sequence	Sequence introduced
DEV-dEF1-gfp-for	5- agtaaaacctctacaaatgtggtatggctgattatgatcagttaagcccggttaacgt gccggcac*AGGATGACGACGATAAGTAGGG* -3	
DEV-dEF1-gfp-rev	5-aaaatacctggttacccaggccgtgccggcacgttaaccgggcttaactgatcataatca gccata*CAACCAATTAACCAATTCTGATTAG*-3	
dgfp-JD-F	5-ccgcctactatcaaaccc-3	
dgfp-JD-R	5-atcagcgtgagactacga-3	
mRFP-F1(gly)	5-*ggcggaggcggatctggcggaggc*ATGGCCTCCTCCGAGGACGT-3	(linker GGGGSGGG introduced)
mRFP-R1	5- ggcgccggtggagtggcggcc-3	
kan_mRFP-for(PstI)	5- aaCTGCAGgacggcgagttcatctacaaggtgaagctgcgcggcaccaacttcccctc *aggatgacgacgataagtaggg*-3(*Pst*	I sites introduced
kan_mRFP-rev(PstI)	5-aaCTGCAGcaaccaattaaccaattctgattag-3 (*Pst*	I sites introduced
CFP-F1(gly-SacI)	5-*GgAggCggTggGtcCggAggTggT*Atggtgagcaagggcgaggagctcttc-3(linkerGGGGSGGG and *Sac*	I sites introduced
CFP-R1	5- cttgtacagctcgtccatgcc-3	
kan_CFP-for(Sac I)	5- aagagctcttcaccggggtggtgcccatcctggtcgagctggacggcgacgtaaacgg *AGGATGACGACGATAAGTAGGG*-3 (*Sac*	I sites introduced
kan_CFP-rev(Sac I)	5-aagagctccaaccaattaaccaattctgattag-3 (*Sac*	I sites introduced
mRFP-gC(c)-F1	5-gtcgtttatttatcaaaagctttattaaacattttatattaaaccagtattca *GGCGCCGGTGGAGTggcggcc*-3	
mRFP-gC(c)-R1	5-taattatggccgtttgtttctattattcgcgagataaagcagacaatattatt *ggcggaggcggatctggcggaggc*-3	
gC(c).mRFP-JD-F	5-tgctgataggacttgcaatcgc-3	
gC(c).mRFP-JD-R	5-cgaacactgactgcaacagtacg-3	
CFP-UL35 (c)-F1	5-atactcaataaaagcttttatgaatctttattgaatcatactcatttatttta *CTTGTACAGCTCGTCCATGCC* -3	
CFP-UL35 (c)-R1	5-tcaagcgcacctttttctccacgcatcatacgcgacgcgccagacgatcagcga *ggAggCggTggGtcCggAggTggT*-3	
CFP-UL35-JD-F	5- gttcgcacatcaataatagc-3	
CFP-UL35-JD-R	5-cttactaccatacgcagagc-3	

[a] restriction enzyme sites added to primers are in underlined, and sequences in italics indicate additional bases that are not present in the original DEV genome.

Figures

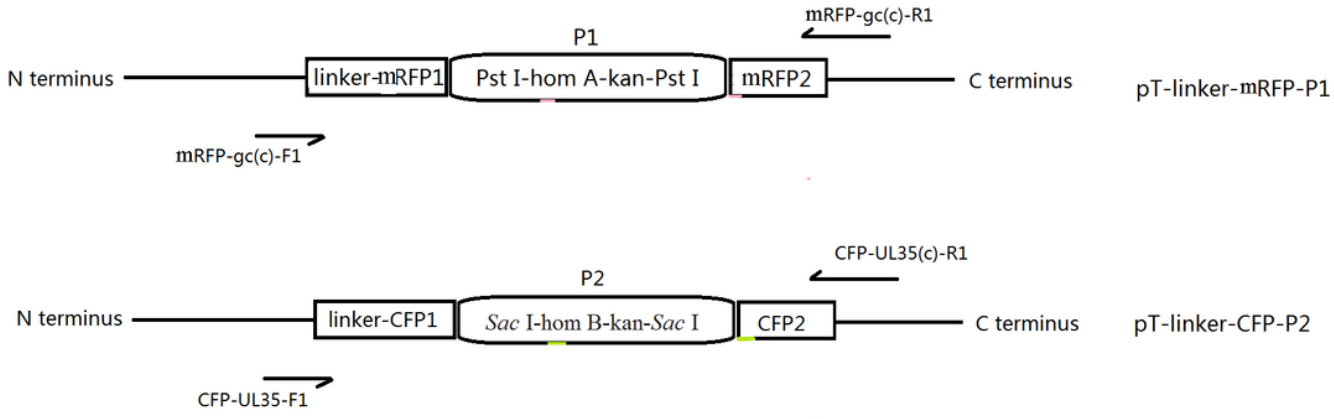

Figure 1

Construction of transfer vectors. In this procedure, mRFP gene is divided into two parts by P1 insertion in Pst I site. Homologous arm a (Hom A) matches exactly the front end sequence of mRFP2. In the same way, CFP gene is divided into two parts by P2 insertion in Sac I site. Homologous arm b (Hom B) matches exactly the front end sequence of CFP2.

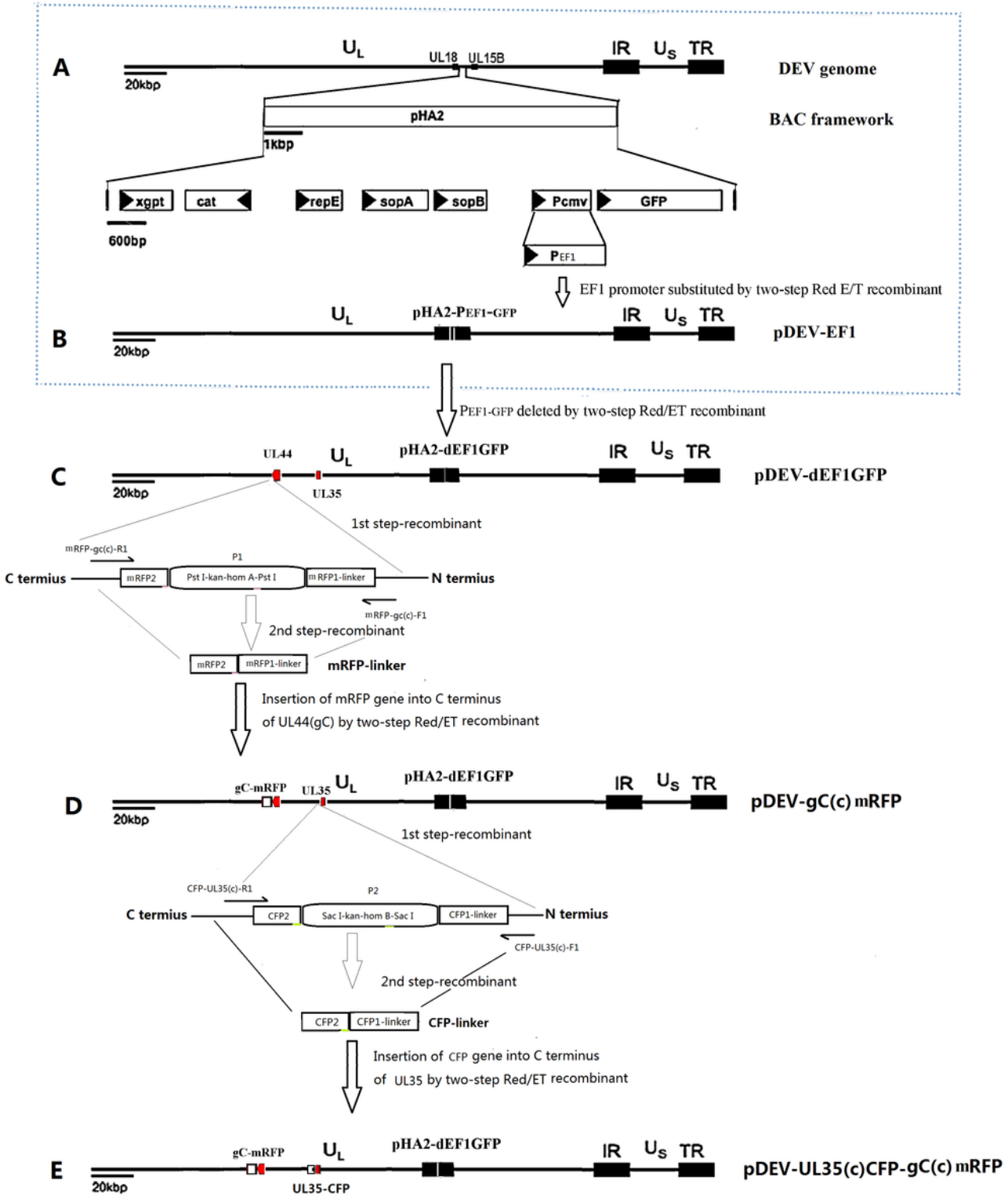

Figure 2

Construction of BAC clones. The flow shown in the dotted box (A and B) is complete in our previous study. A: A mini-F vector (pHA2) as a BAC that allows the maintenance of large circular DNA in E. coli was introduced into the intergenic region between UL15B and UL18 of a DEV vaccine strain by homologous recombination; B. pCMV promoter which control expression of GFP in pDEV-vac was substituted by pEF1 gene by two-step Red (en passant) recombination; C. Construction of BAC clone of pDEV-dEF1GFP: the

pEF1-GFP expression cassette was deleted from pDEV-EF1 genome by two-step Red/ET recombinant. D. Construction of BAC clone of pDEV-gC(c)mRFP by two-step Red/ET recombinant: the fragment containing linker-mRFP1-Pst I-Hom A-kan- Pst I-mRFP2 was inserted exactly to the C terminus of gC gene of DEV in 1st homologous recombinant, then the sequence of hom A-kan-Pst I will be removed by 2nd homologous recombinant, thus intact gC-mRFP gene will be obtained. E. Construction of BAC clone of pDEV-UL35(c)CFP-gC(c)mRFP by two-step Red/ET recombinant: the fragment containing linker-CFP1-Sac I-Hom B-kan- Sac I -CFP2 was inserted exactly to the C terminus of UL35 gene of DEV in 1st homologous recombinant, then the sequence of Hom B-kan- Sac I will be removed by 2nd homologous recombinant, thus intact UL35-CFP gene will be obtained. UL44 and UL35 gene is reverse complement in DEV genome.

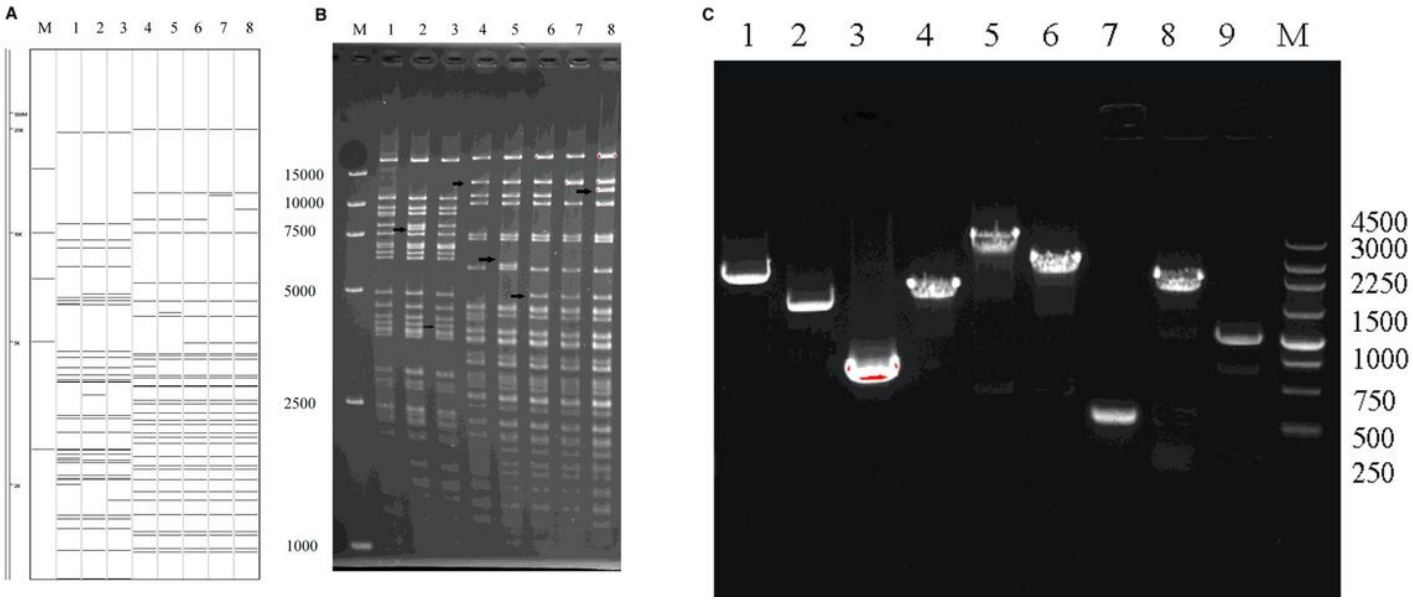

Figure 3

A and B. Analysis of recombinant BAC clones by Xba I or Bgl II digestion. 1. pDEV-EF1/XbaI□2. pDEV-kan.dEF1GFP/XbaI□3. pDEV-dEF1GFP/XbaI□4. pDEV-dEF1GFP/BglII; 5. pDEV-kan.gC(c)mRFP /BglII; 6. pDEV-gC(c)mRFP /BglII□7. pDEV-kan.UL35(c)CFP-gC(c)mRFP /BglII; 8. pDEV-UL35(c)CFP-gC(c)mRFP /BglII C. PCR identification of recombinant BAC clones 1. pDEV-EF1(2579 bp, dgfp-JD-F/R); 2. pDEV-kan.dEF1GFP(1682 bp, dgfp-JD-F/R); 3. pDEV-dEF1GFP(643 bp, dgfp-JD-F/R); 4. pDEV-dEF1GFP(1870 bp, gC(c).mRFP-JD-F/R); 5. pDEV-kan.gC(c)mRFP (3999 bp, gC(c).mRFP-JD-F/R); 6. pDEV-gC(c)mRFP(2569 bp□gC(c).mRFP-JD-F/R); 7. pDEV-gC(c)mRFP (293 bp, CFP-UL35-JD-F/R); 8. pDEV-kan.UL35(c)CFP-gC(c)mRFP (2085 bp, CFP-UL35-JD-F/R); 9. pDEV-UL35(c)CFP-gC(c)mRFP (1084 bp, CFP-UL35-JD-F/R)

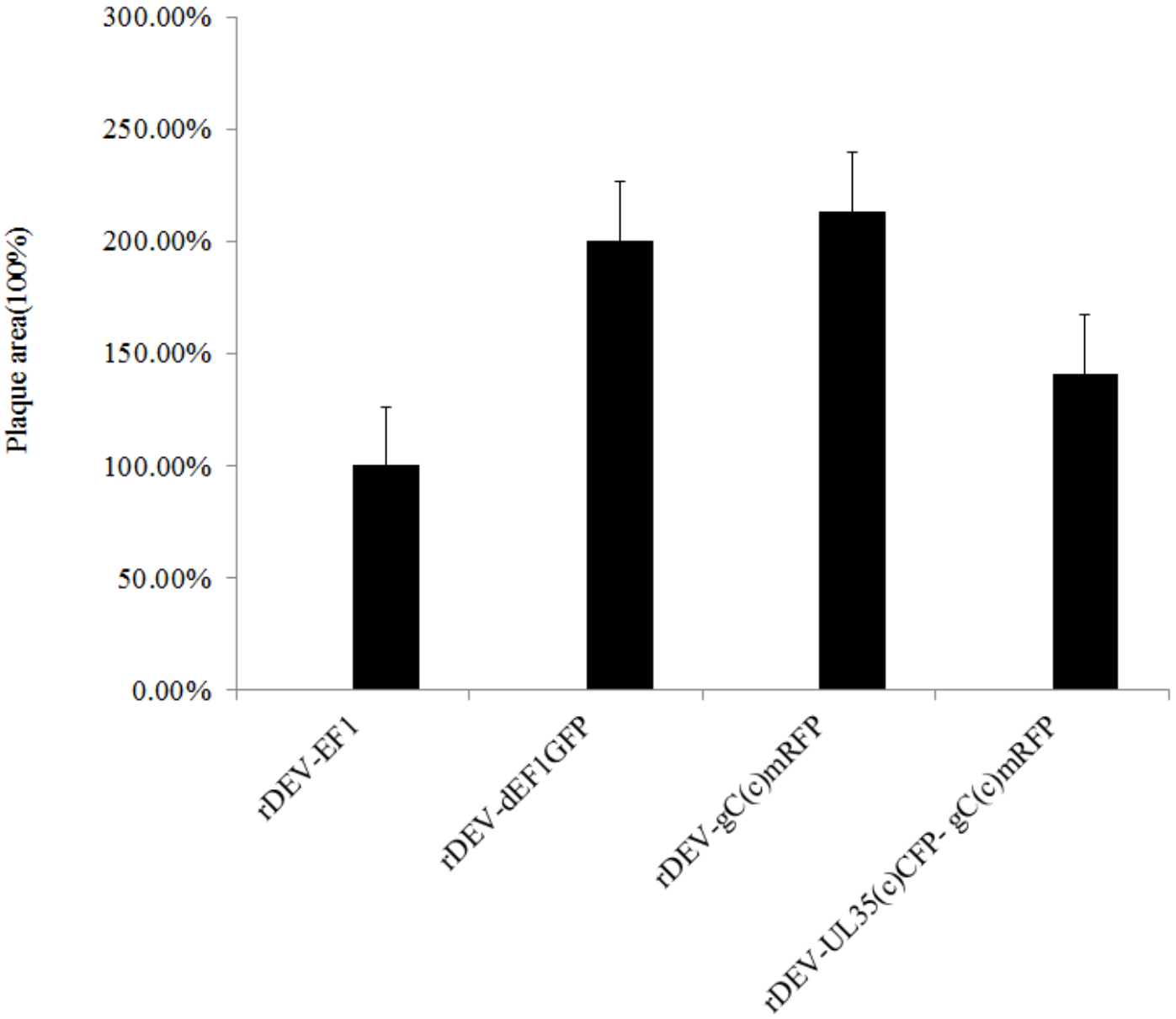

Figure 4

Plaque area measurement of rDEV-EF1, rDEV-dEF1GFP, rDEV-gC(c)mRFP, rDEV-UL35(c)CFP-gC(c)mRFP on CEFs The means and standard deviations of sizes of 100 plaques of each virus were measured with Image J software. The mean of the plaque area of rDEV-EF1 was set at 100%. Standard deviations are shown with the error bars.

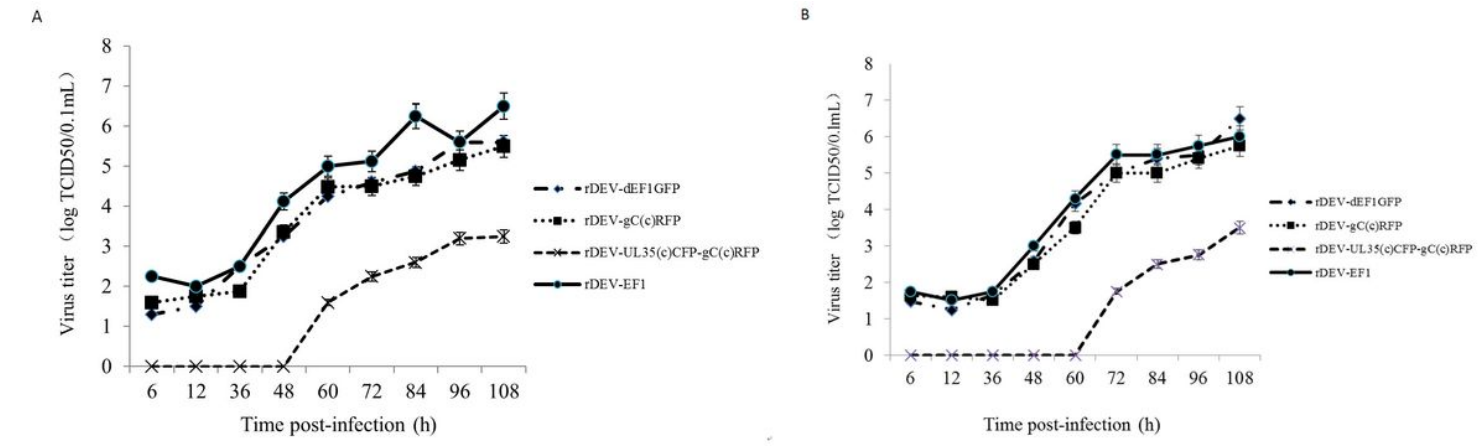

Figure 5

The growth curve of rDEV-EF1, rDEV-dEF1GFP, rDEV-gC(c)mRFP, rDEV-UL35(c)CFP-gC(c)mRFP on infected -CEFs(A) or supernatants of infected cells (B) Replication of parent and recombinant DEV in CEFs. CEFs monolayers in 12-well culture plates were infected at an MOI of 0.02 of the indicated viruses and incubated at 37°C with 5% CO2. Cells were harvested at times as indicated on the horizontal axis, and viruses collected from infected cells (intracellular infectivity) (A) or supernatants of infected cells (extracellular infectivity) (B) were titrated on CEFs. Shown are means of three individual and independent experiments, respectively. Standard deviations are given as error bars above symbols.

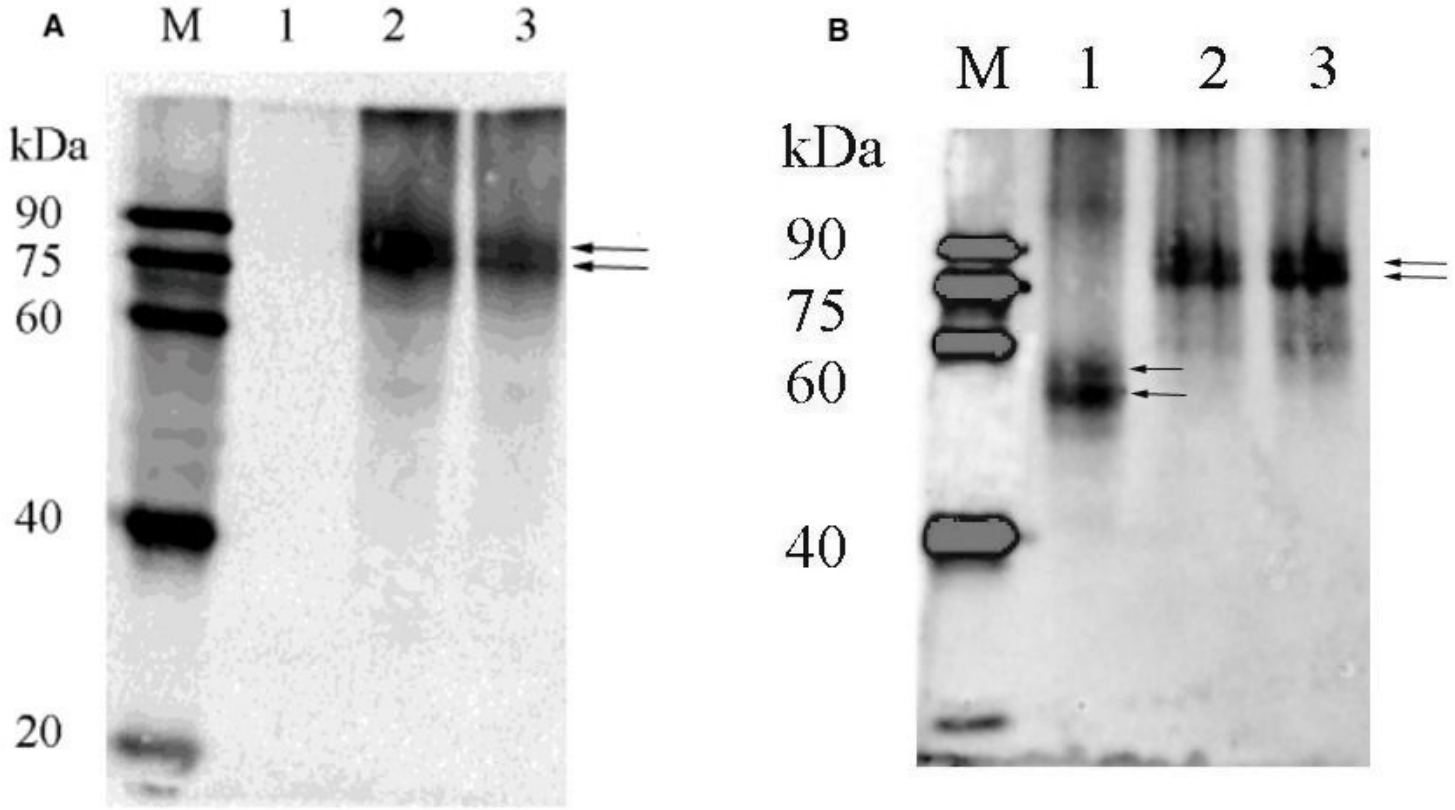

Figure 6

Detection of gC and mRFP protein expressed in rDEV-gC(c)mRFP -infected CEFs by Western blot analysis (A. anti-mRFP antibody; B. anti-gC antibody). M. ECL marker; 1. rDEV-dEF1GFP; 2. 3. rDEV-gC(c)mRFP

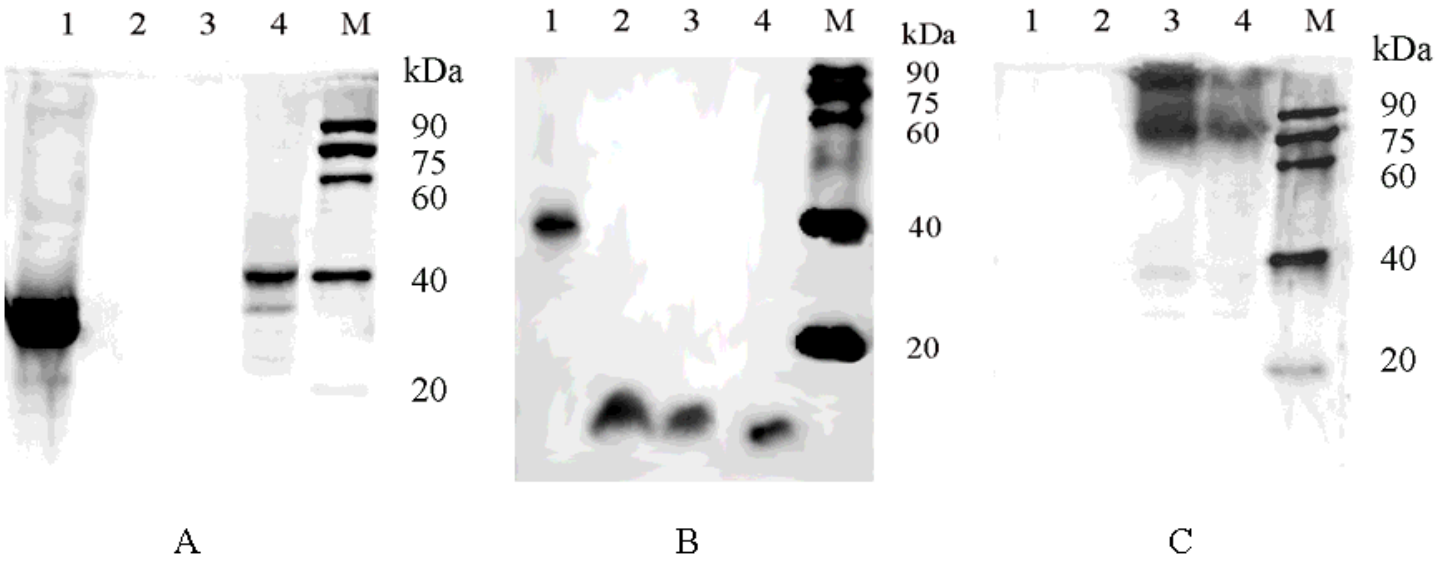

Figure 7

Detection of gC-mRFP and UL35-CFP protein expressed in rDEV-UL35(c)CFP-gC(c)mRFP -infected CEFs by Western blot analysis. (A. anti-GFP polyclonal antibody; B. anti-UL35 polyclonal antibody; C. anti-mRFP polyclonal antibody) 1. rDEV-EF1; 2. rDEV-dEF1GFP; 3. rDEV-gC(c)mRFP; 4. rDEV-UL35(c)CFP-gC(c)mRFP; M. ECL marker

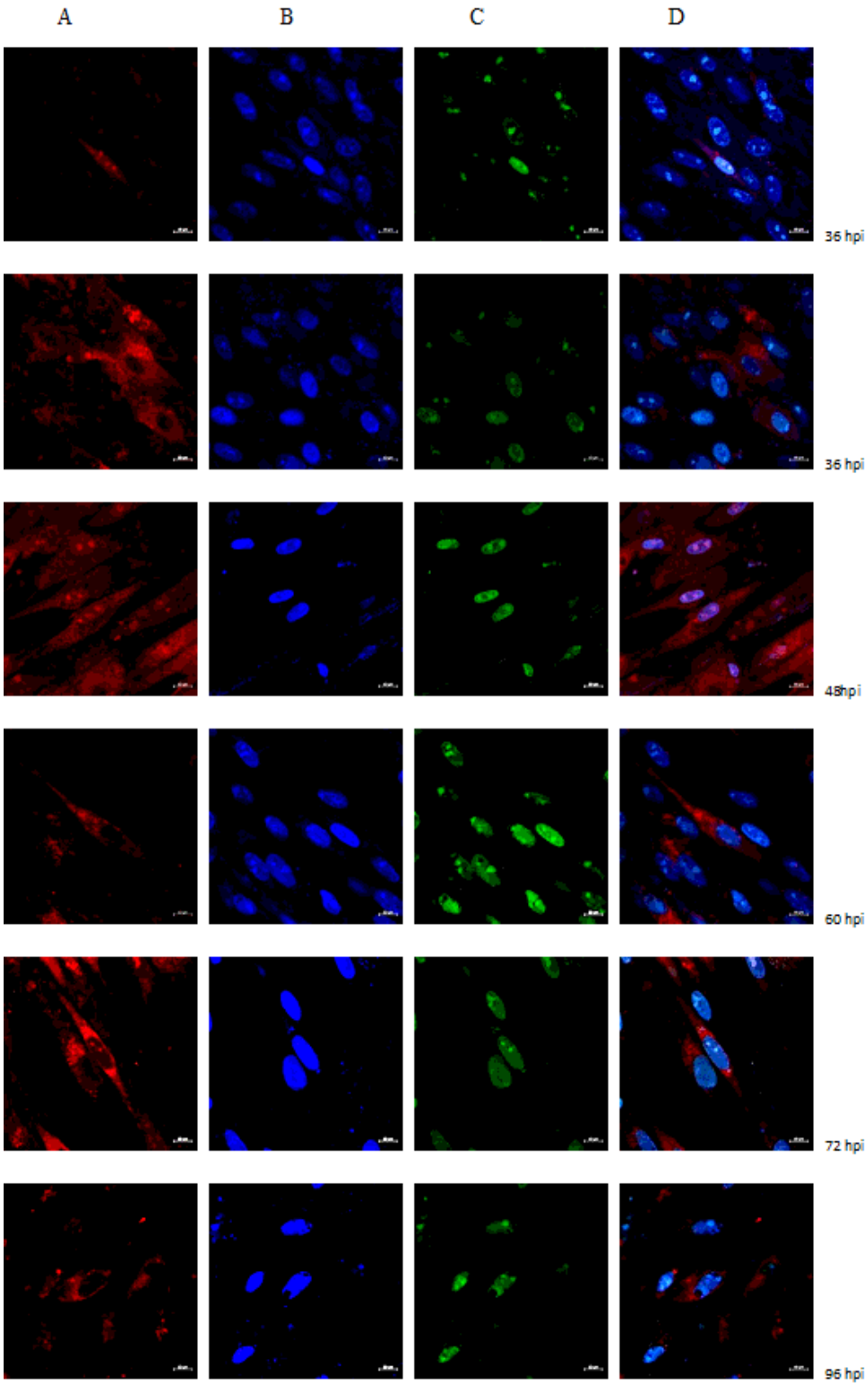

Figure 8

rDEV-UL35(c)CFP-gC(c)mRFP virus-infected cells was stained with SYTO and obsevation by confocal microscopy Location of gC-mRFP and UL35-CFP in rDEV-UL35(c)CFP-gC(c)mRFP virus-infected cells by confocal microscopy. A. gC-mRFP; B. UL35-CFP; C. SYTO; D. Merge

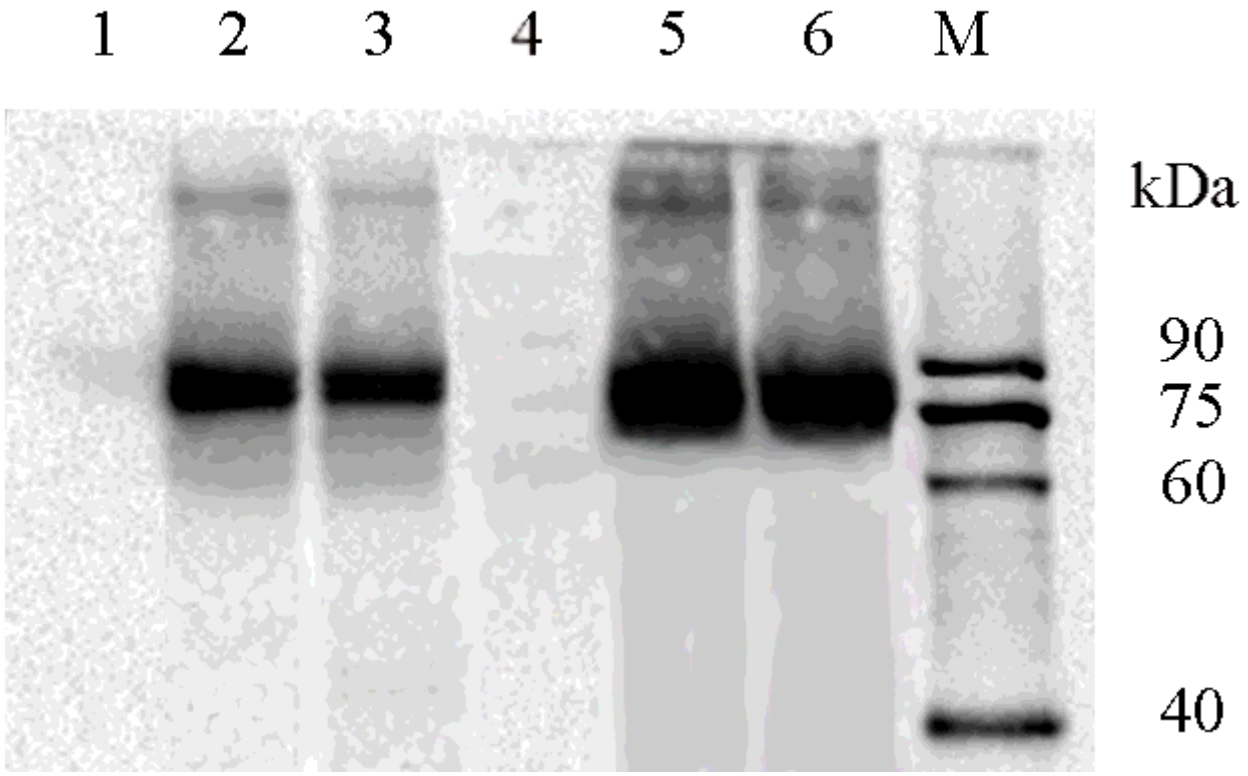

Figure 9

Analysis subcellular location of gC-RFP by Western blot 1-3 cytoplasmic fraction: 1. rDEV-dEF1GFP; 2. rDEV-gC(c)mRFP; 3. rDEV-UL35(c)CFP-gC(c)mRFP; 4-6 membrane fraction: 4. rDEV-dEF1GFP; 5. rDEV-gC(c)mRFP; 6. rDEV-UL35(c)CFP-gC(c)mRFP; M. ECL marker

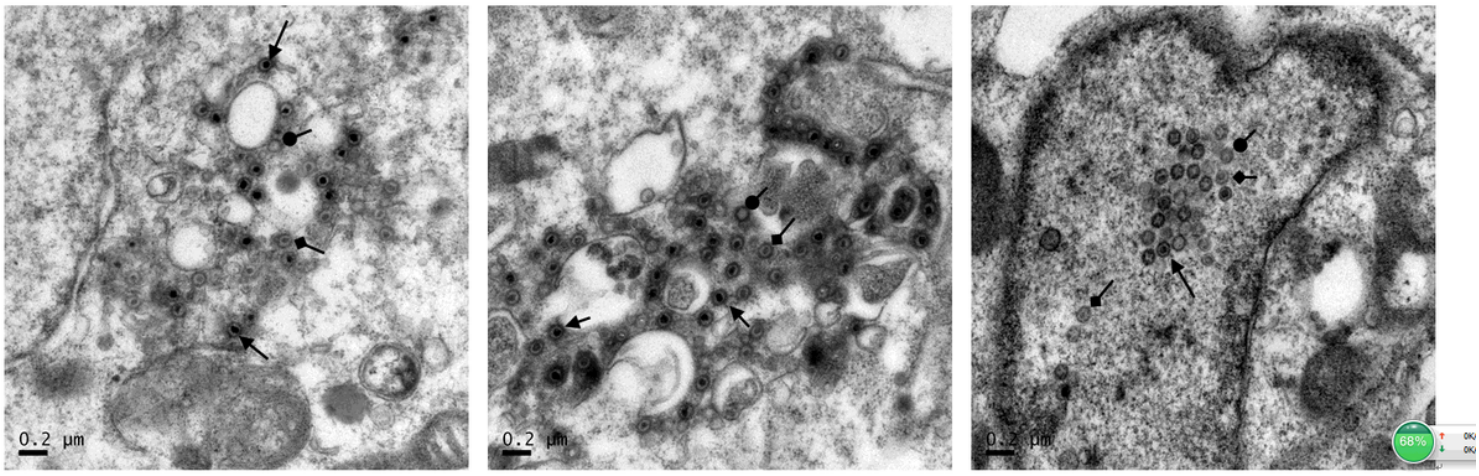

Figure 10

Electron micrograph of virions in CEFs infected with rDEV-dEF1GFP (a), rDEV-gC(c)mRFP(b) or rDEV-UL35(c)CFP-gC(c)mRFP(c). Scale bar represents 200 nm. A capsids (indicated with circular arrow); B capsids (indicated with the diamond arrow); C capsids (indicated with the black arrow)