Molecular Genetics and Genomics (2018) 293:1265–1277
https://doi.org/10.1007/s00438-018-1459-9

ORIGINAL ARTICLE

# Construction of a highly efficient display system for baculovirus and its application on multigene co-display

**Hao Zheng[1] · Xiong Wang[1] · Feifei Ren[1] · Shenglong Zou[2] · Min Feng[1] · Liangliang Xu[1] · Lunguang Yao[3] · Jingchen Sun[1]**

Received: 6 May 2018 / Accepted: 7 June 2018 / Published online: 19 June 2018


## Abstract

The classical baculovirus display system (BDS) has often recruited fields including gene delivery, gene therapy, and the genetic engineering of vaccines, as it is capable of presenting foreign polypeptides on the membranes of recombinant baculovirus through a transmembrane protein. However, classical BDS's high cost, complicated operation, low display efficiency and its inability to simultaneously display multiple gene products impede its practicality. In this study, we present a novel and highly efficient display system based on ires-dependent gp64 for rescuing gp64-null Bacmid of baculovirus construction without affecting the viral replication cycle, which we name the baculovirus multigene display system (BMDS). Laser scanning confocal microscopy demonstrated that eGFP, eYFP, and mCherry were translocated on the membrane of *Spodoptera frugiperda* 9 cell successfully as expected. Western blot analysis further confirmed the presence of the fluorescent proteins on the budded, mature viral particles. The results showed the display efficiency of target gene on cell surface is fourfold that of classical BDS. In addition, a recombinant baculovirus displaying three kinds of fluorescent proteins simultaneously was constructed, thereby demonstrating the effectiveness of BMDS as a co-display system.

**Keywords** Insect virus · Baculovirus multigene display system · Gene expression · High efficiency

## Introduction

*Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV) is a typical encapsulated double-stranded DNA virus. It is capable of transducing non-dividing cells (van

Communicated by S. Hohmann.

Hao Zheng and Xiong Wang contributed equally to this work.

✉ Lunguang Yao
lunguangyao@163.com

✉ Jingchen Sun
cyfz@scau.edu.cn

Hao Zheng
774926329@qq.com

Xiong Wang
284241559@qq.com

Feifei Ren
zzff43@outlook.com

Shenglong Zou
zoushl@163.com

Min Feng
hunanfengmin@163.com

Liangliang Xu
xv_liang_liang@163.com

[1] Guangdong Provincial Key Laboratory of Agro-Animal Genomics and Molecular Breeding and Subtropical Sericulture and Mulberry Resources Protection and Safety Engineering Research Center, College of Animal Science, South China Agricultural University, Guangzhou 510642, Guangdong, China

[2] Guangzhou Cynosure Bio-Technology Co., Ltd, Guangzhou 510080, Guangdong, China

[3] China-UK NYNU-RRes Joint Lab of Insect Biology and Henan Key Laboratory of Ecological Security for Water Source Region of Mid-Line of South-to-North Diversion Project, Collaborative Innovation Center of Water Security for Water Source Region of Mid-Line of South-to-North Diversion Project of Henan Province, Nanyang Normal University, Nanyang 473061, Henan, China

Loo et al. 2001) and has low cytotoxicity in mammalian cells even at a very high virus load (Yap et al. 1997; Sandig et al. 1996; Shoji et al. 1997). Due to its characteristic advantages of security, economy and convenience, the baculovirus display system (BDS) is regarded as the most versatile surface display system in eukaryotes (Hulst et al. 1994; Chen et al. 2007). The construction of BDS involves fusing the target protein with the type I or III envelope proteins of baculovirus, thus realizing the display of the target protein on the surface of the recombinant baculovirus envelope (Lin et al. 2008; Whitford et al. 1989; Yang et al. 2007). This method has been extended to develop the surface display of target proteins for eukaryotes. It has become a construction platform for gene therapy and genetic engineering vaccine with important application potential (Xu et al. 2008; Xu and Liu 2008; Lin et al. 2008; Tami et al. 2004; Yoshida et al. 2003).

A wide range of applications have been proposed since baculovirus was found to transduce into mammalian cells (Hu 2005). The attachment and endocytosis of baculovirus into insect cells are mediated by an envelope glycoprotein GP64. The mature structure of GP64 includes a transmembrane domain (TM) and cytoplasmic domain (CTD) (Monsma et al. 1996; Kitagawa et al. 2005). In addition, a signal peptide (sp) was located in the N-terminal of mature GP64 and directs its transport to the plasma membrane after expression in cells, where GP64 appears as homotrimers on the surface of infected cells. The CTD domain is responsible for contact with the nucleocapsid of baculovirus and guides its encapsulation by the envelope (Yang et al. 2007; Oomens and Blissard 1999). The significance of GP64 in virus budding has been used to enact the surface display of target peptides by inserting a heterologous peptide between the sp and mature domain of GP64 (O'Reilly 1997). After expression along with wild-type GP64, fusion GP64 is translocated to the cell membrane and assembled into the baculovirus envelope. In addition to GP64, fusions can be made to the N-terminus or C terminus of the major capsid protein VP39 without compromising the viral titer or functionality (Yoshida et al. 2003). Another strategy developed non-polar protein distribution on the baculovirus envelope, consisting of the expression of foreign peptide inserted between sp of GP64 and vesicular stomatitis virus (VSV) G protein (Peralta et al. 2013; Oker-Blom et al. 2003; Chapple and Jones 2002).

Since the GP64 fusion protein competes strongly with the wild-type GP64 envelope protein expressed by baculovirus, it leads to a low-efficiency of target protein display on the viral surface. The main objective of this study was to construct the envelope glycoprotein GP64 gene auxotrophic strain *E.coli* SW106 Bacmid, in the hope that internal ribosome entry site (ires)-dependent low-expression of wild-type gp64 (Mountford and Smith 1995; Pelletier and Sonenberg 1988) would improve the display efficiency of the target protein without affecting the baculovirus replication cycle. Here, we constructed a recombinant baculovirus AcMNPV-Δgp64-G64-M64-Y64-I64 with the ability of high display efficiency and multi-gene co-display.

## Materials and methods

### Bacterial strains, plasmids, viral Bacmid, reagents and insect cell

*E.coli* DH10B, BW23474, and TOP10 are used for the propagation of Bacmid, R6kγ origin-derived plasmids, and other general plasmids, respectively. *E.coli* SW106 Bacmid containing Bacmid, pHelper, and pGB2Ωinv were constructed previously (Yao et al. 2012, 2010). pUCDM and pFBDM were from Prof. Richmond (Berger et al. 2004). pFBDM and pUCDM, as well as the modified Bacmid with gentamycin or chloramphenicol resistance gene by mini-Tn7 or cre-loxp transposition, were from our previous study (Sun et al. 2009; Yao et al. 2007). pBac-IR-eGFP containing the 59-UTR internal ribosome entry site (ires) sequence was provided by Prof. Wu (Motohashi et al. 2005). pIZTV5 was purchased from Invitrogen.

Pfu *Taq*, restriction enzymes, and $T_4$ DNA ligase were purchased from NEB (New England Biolabs, England), while DL-α-ε Diaminopimelic acid (DAP) was bought from Sigma (cat.D1377, USA). Low salt (LS) medium (10 g of tryptone, 5 g of NaCl and 5 g of yeast extract in 1 L of broth, pH7.5) was used for cloning and growing the plasmids containing zeocin resistance gene. *Spodoptera frugiperda* 9 (Sf9) cells were maintained at 27 °C in Grace medium supplemented with 10% FBS (*Gibco*).

### Generation of the envelope glycoprotein *gp64* gene auxotrophic strain *E.coli* SW106 Bacmid

The genomic DNA of *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV) was extracted using a genomic DNA extraction kit (*TaKaRa*). Deletion of gp64 gene results in a failure of budding and invasion by baculovirus. The gp64 gene homologous recombinant arms contain gp64F (located upstream 400 bp of the promoter p64) and gp64R (located downstream 400 bp of p64), which were obtained by polymerase chain reaction (PCR) from AcMNPV DNA (Table 1). The zeocin resistance cassette was amplified with primer *Zeo-F* and *Zeo-R* (Table 1) from pIZTV5, which was inserted between gp64F and gp64R by over-lap PCR. The homologous recombination fragment (gp64F-Zeo-gp64R) was verified by sequencing and electroporated into the 42 °C-induced electro-competent *E.coli* SW106 Bacmid prepared according to the

**Table 1** Primers used in the study

| Label | Direction and primer sequences | Target genes |
|---|---|---|
| gp64F | F: 5′-tcatgagattgccaatcaaacgctcg | Homologous recombinant arm gp64F |
| | R: 5′-acacgtgctgcaaacaaaagtctacgttca | |
| Zeo | F: 5′-gtagacttttgtttgcagcacgtgttgacaa | Zeocin resistance cassette |
| | R: 5′-gcgtgttcgatgttcagtcctgctcctcg | |
| gp64R | F: 5′-aggagcaggactgaacatcgaacacgcgcaac | Homologous recombinant arm gp64R |
| | R: 5′-aaatagcctcgttgagactctcctga | |
| egfp | F: 5′-ggatccatggtgagcaagggcgaggagctgt; *Bam*H I | Enhance green fluorescent gene |
| | R: 5′-gagctcttacttgtacagctcgtccatgccg; *Sac* I | |
| ires | F: 5′-gagctcaataaaagaacctataatcccttcg; *Sac* I | Internal ribosome entry site, |
| | R: 5′-tctagagttactagatataaatagataaagct; *Xba* I | |
| spgp64 | F :5′-gagctcatggtaagcgctattgttttatatgtgcttttggcggcggcggcgcattctgc ctttgcggcgaataaaagaacctataatcccttcgc; *Xba* I | Signal peptide and wide-type gp64 |
| | R: 5′-actgcagttaatattgtctattacggtttctaa; *Pst* I | |
| p64sp | F: 5′-actagtctgagcgtccgtgttcatgatccc; *Spe* I | Promoter p64 with signal peptide |
| | R:5′-cccgggcgccgcaaaggcagaatgcgccgccgccgccaaaagcacatataaaa caatagcgcttaccatcgccgcaaaggcagaatgcgccgc; *Xma* I | |
| R-lucΔTAA | F: 5'-cccgggatgacttcgaaagtttatgatcca; *Xma* Ⅰ | *Renilla* luciferase without terminate codon |
| | R: 5′-ctcgagttgttcatttttgagaactcgctc; *Xho* I | |
| gp64 | F: 5′-gtcgacctcgaggagcactgcaacgcgcaaatgaagacg; *Sal* I/*Xho* Ⅰ | gp64 ORF for fusion |
| | R: 5′-gagctcgcatgcttaatattgtctattacggtttctaat; *Sac* I/*Sph* I | |
| F-luc | F: 5′-ggatccatggaagacgccaaaaacataaagaaag: *Bam*H I | *Firefly* luciferase |
| | R: 5′-gtcgacttacacggcgatctttccgcccttcttg; *Sal* I | |
| egfpΔTAA | F: 5′-ggatccatggtgagcaagggcgaggagctgt; *Bam*H I | Enhance green fluorescent gene without terminate codon |
| | R: 5′-gtcgaccttgtacagctcgtccatgccgag; *Sal* I | |
| mCherryΔTA | F: 5′-cccgggatggtgagcaagggcgaggaggat; *Xma* I | mCherry fluorescent |
| A | R: 5′-ctcgagcttgtacagctcgtccatgccgccgg; *Xho* I | gene without terminate codon |
| eyfpΔTAA | F: 5′-ggatccatgtccaagggcgaggagctgttca; *Bam*H I | Enhance yellow fluorescent gene without terminate codon |
| | R: 5′-gtcgaccttgtagagctcgtccatgccgtgg; *Sal* I | |

The 5′ ends of primers were designed to create restriction enzyme sites (underlined) and signal peptide sequences (boxed), respectively, after polymerase chain reaction amplification

previous work (Warming et al. 2005). The transformed cells were spread on LS plate supplemented with 50 μg/mL kanamycin, 10 μg/mL spectinomycin, 10 μg/mL tetracycline, 25 μg/mL zeocin, 0.5 mM DAP, and cultured at 32 °C overnight. The positive colonies grown on the kan/Spe/Tet/Zeo/DAP plate were picked and identified by PCR (primer *gp64F-F* and *gp64R-R*, Table 1). The zeocin cassette was then removed from the Bacmid by Flp-mediated excision (Cherepanov and Wackernagel 1995). The resulting Bacmid gp64 auxotrophic strain was named *E.coli* SW106 Bacmid-Δgp64.

## Construction of donor vectors

The coding sequence for egfp was amplified by PCR using primer *egfp-F* and *egfp-R* (Table 1). To follow transfection and infection processes, the PCR product was digested with *Bam*H I and *Sac* I, and ligated to the downstream of the promoter polyhedrin (polh) on the pFBDM, forming a donor vector pF-polh-G (Fig. 1a). Primers *ires-F* and *ires-R* (Table 1) were used to amplify the ires gene by pBac-IR-eGFP, which was ligated into the *Sac* I and *Xba* I of pF-polh-G. The N-terminal of gp64 gene fusing with sp was amplified from AcMNPV DNA by PCR using *spgp64-F* and *spgp64-R*. The PCR product was ligated to the downstream of the ires by *Xba* I and *Pst* I, to form another vector pF-polh-G-I64 (Fig. 1b), in which the spgp64 was dependent by ires element.

The primers *Fluc-F* and *Fluc-R* were used to amplify the *Firefly* luciferase (Fluc) gene (Table 1), and were cloned into the downstream of the polh on the pFBDM by the *Bam*H I and *Sal* I sites to generate the vector pF-polh-F. The C-terminal of promoter p64 fusing with sp (p64sp) was amplified from AcMNPV DNA by PCR using *p64sp-F* and *p64sp-R*. The PCR product was digested with *Spe* I and *Xma* I, and then ligated to the same sites of pF-polh-F to replace promoter p10. The flexible linker-peptide sequence (GGGGSGGGGSGGGGS) was fused to the C terminus of *Renilla* luciferase ΔTAA (deletion of termination codon, Rluc$(G_4S)_3$ΔTAA), and cloned

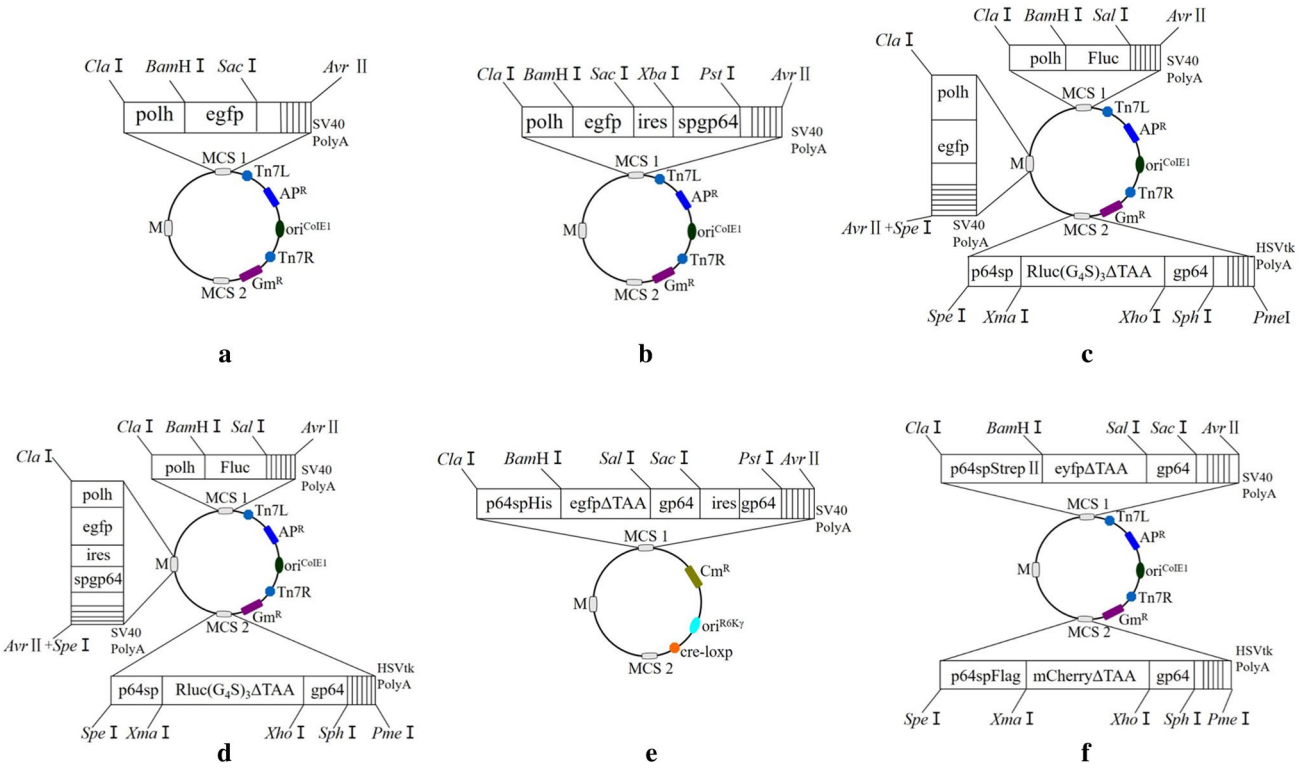


**Fig. 1** The gene arrangement on recombinant donor vectors. **a** The map of pF-polh-G shows an expression cassette including promoter polh and egfp. **b** The map of pF-polh-G-I64 shows an expression cassette including promoter polh, egfp, and ires-dependent gp64 expression cassette iresspgp64. **c** The maps of pF-p64sp-R64-polhF-polh-G show two expression cassettes including egfp and *Firefly* luciferase are driven by polh, and a fusion gene fragment of sp, *Renilla* luciferaseΔTAA and gp64. **d** The maps of pF-p64sp-R64-polhF-polh-G-I64 show two expression cassettes including egfp, iresspgp64, and *Firefly* luciferase are driven by polh, and a fusion gene fragment of sp, *Renilla* luciferaseΔTAA and gp64. **e** The maps of pU-p64spH-G64-I64 show a fusion gene fragment of sp, 6×His-tag, egfpΔTAA, gp64 and iresspgp64. **f** The map of pF-p64spF-M64-p64spS-Y64 show two fusion gene fragments of sp, strep II-tag, Flag-tag, eyfpΔTAA, mCherryΔTAA, gp64

under p64sp. The gp64 was amplified from AcMNPV DNA by PCR using primer *gp64-F* and *gp64-R*, and then was ligated under the Rluc$(G_4S)_3$ΔTAA to generate the transient vector pF-p64sp-R64-polh-F (Table 1). The pF-polh-G was digested with *Cla* I / *Avr* II to release DNA polh-egfp, and the fragment was cloned into pF-p64sp-R64-polh-F via *Cla* I and *Spe* I (*Avr* II and *Spe* I were isocaudamer) to construct donor vector pF-p64sp-R64-polh-F-polh-G (Fig. 1c). Similarly, the donor vector pF-p64sp-R64-polh-F-polh-G-I64 (Fig. 1d) was successfully constructed by pF-p64sp-R64-polh-F and pF-polh-G-I64.

The C-terminal of p64 containing sp and different label proteins (6 × His/ Sterp II/ Flag tag) were amplified from AcMNPV DNA by PCR to form three kinds of PCR products: p64spHis (6 × His-tag; sequence: 5′-CATCATCAC CACCATCAC), p64spSterp II (Sterp II-tag; sequence: 5′-TGGAGCCACCCGCAGTTTGAAAAG), and p64spFlag (Flag-tag; sequence: 5′-GATTACAAGGATGACGACGAT AAG), respectively. The p64spHis was ligated into pUCDM to replace polh via *Cla* I and *Bam*H I. The egfpΔTAA was amplified from pBac-IR-eGFP using primers *egfpΔTAA-F* and *egfpΔTAA-R* and ligated into the *Bam*H I and *Sal* I under p64spHis. gp64 was amplified from AcMNPV DNA using primers *gp64-F* and *gp64-R* and ligated into *Sal* I and *Sac* I under the p64spHis to generate the conditional replication transposition transient vector pU-p64spH-G64. pF-polh-G-I64 was digested with *Sac* I and *Pst* I to release DNA iresspgp64. The fragment was then cloned into the same sites of pU-p64spH-G64 to make donor vector pU-p64spH-G64-I64 (Fig. 1e).

To replace polh and p10, PCR products p64spSterp II and p64spFlag were respectively cloned into pFBDM through *Cla* I / *Bam*H I and *Spe* I / *Xma* I, forming another transient vector pF-p64spF-p64spS. The eyfpΔTAA and mCherryΔTAA were amplified using primers *eyfpΔTAA-F*/*eyfpΔTAA-R* and *mCherryΔTAA-F*/*mCherryΔTAA-R*, then cloned into pF-p64spF-p64spS via *Bam*H I/*Sal* I and *Xma* I / *Xho* I under the p64spSterp II and p64spFlag. The gp64 fragment was amplified from AcMNPV and successively introduced by *Xho* I/*Sph* I and *Sal* I/*Sac* I to construct donor vector pF-p64spF-M64-p64spS-Y64 (Fig. 1f).

## Introduction of multiple genes into Bacmid

Donor vectors pF-polh-G, pF-polh-G-I64, and pF-p64sp-R64-polh-F-I64 were transferred into *E.coli* SW106 Bacmid-Δgp64 through mini-Tn7 transposition (Sun et al. 2009; Yao et al. 2007) to generate three kinds of Bacmids, including Bacmid-Δgp64-G, Bacmid-Δgp64-G-I64, and Bacmid-Δgp64-p64sp-R64-polh-F-I64, respectively. Similarly, pF-polh-G and pF-p64sp-R64-polh-F were separately transferred into *E.coli* SW106 Bacmid to construct two other kinds of recombinant Bacmid-G and Bacmid-p64sp-R64-polh-F as control. Furthermore, egfp, eyfp, and mCherry were introduced from pU-p64spH-G64-I64 and pF-p64spF-M64-p64spS-Y64 into *E.coli* SW106 Bacmid-Δgp64 by cre-loxp and mini-Tn7 site-specific recombination to construct the recombinant Bacmid-Δgp64-p64sp-HG-FM-SY-I64. The positive recombinant Bacmid was identified by white–blue and PCR screening as per the previous report (Yao et al. 2012, 2010; Berger et al. 2004).

## Production of recombinant baculoviruses

*E. coli* SW106 cells with different recombined foreign genes were cultured until OD600 = 0.5–1 (attendance at 600 nm). These cells were collected by centrifugation (3000*g*) and resuspended in serum-free insect medium. The bacterial suspension was adjusted to different densities ($10^5$–$10^8$ cells/mL) with serum-free Grace's insect medium (Yao et al. 2012, 2010). Insect cells sf9 were cultured overnight in a 24-well plate until the cell density was approximately 70–80%. The supernatant was discarded and different concentrations of bacteria were added to the corresponding wells. After culturing at 28 °C for 4–5 h, bacteria in each well was washed out by serum-free Grace's insect medium. 500 μL of fresh insect medium (with 10% FBS and 0.075% of *penicillin*) was then added and incubated for 4–5 d.p.i. When the fluorescence in the corresponding well was observed by fluorescence microscope, indicating that sf9 cells were infected successfully, the supernatant was collected and infected again with sf9 cells. Fluorescence appeared again at 4–5 d.p.i, indicating that the recombinant baculovirus was successfully constructed and distributed in the cell supernatant. In this study, the purified Bacmids were respectively transfected into Sf9 cells to produce six kinds of recombinant baculoviruses, which we named AcMNPV-polhG, AcMNPV-Δgp64-polhG, AcMNPV-Δgp64-polhGI64, AcMNPV-Δgp64-p64spR64-polhF, AcMNPV-Δgp64-p64spR64-polhFI64, and AcMNPV-Δgp64-p64sp-HG-FM-SY-I64. The plaque assay technique was used to determine the recombinant virus titer (Roldao et al. 2009).

## Dual-Glo luciferase assay system

To prevent host cell apoptosis and break down, infected sf9 cells were collected by low-speed centrifugation (500*g*) and washed with PBS at 72 h.p.i. The expression of *Renilla* luciferase on the surface of the whole cell membrane was measured using the Dual-Glo luciferase assay

system (Cat NO.E1910, *Promega*). The termination solution and cell lysate was added, breaking the cell, after which the intracellular *Firefly* luciferase was released and determined.

## Baculovirus purification and titer determination

The supernatant of sf9 cells infected with recombinant baculovirus (MOI = 1) was collected at 4 d.p.i. Baculovirus in the supernatant was purified by two rounds of sucrose gradient ultracentrifugation according to standard methods (O'Reilly 1997). The baculovirus titers were measured by the $TCID_{50}$ method according to standard methods.

## SDS–PAGE and western blot

The purified baculoviruses or infected cell lysates were subjected to 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. Three primary antibodies including 6 × His / Sterp II/Flag tag (1:4000, *Beyotime*) were used to detect fusion protein in the western blot. The secondary antibodies were goat anti-porcine and goat anti-mouse IgG conjugated to HRP (1:3000 dilution, Invitrogen). The protein bands were visualized by the ECL chemiluminescence system Hyper-Max films, as recommended by the manufacturer.

## Laser scanning confocal microscopy and transmission electron microscope

The sf9 cells were cultured on sterile cover slips (placed in 6-well plates) and infected with baculoviruses at MOI = 10. At 72 h.p.i, infected sf9 cells were mounted on glass slides in 50% glycerol and examined under with confocal laser scanning microscopy (CLSM).

Purified baculoviruses were adsorbed onto glow discharge-activated carbon-coated grids for 2 min. And then,

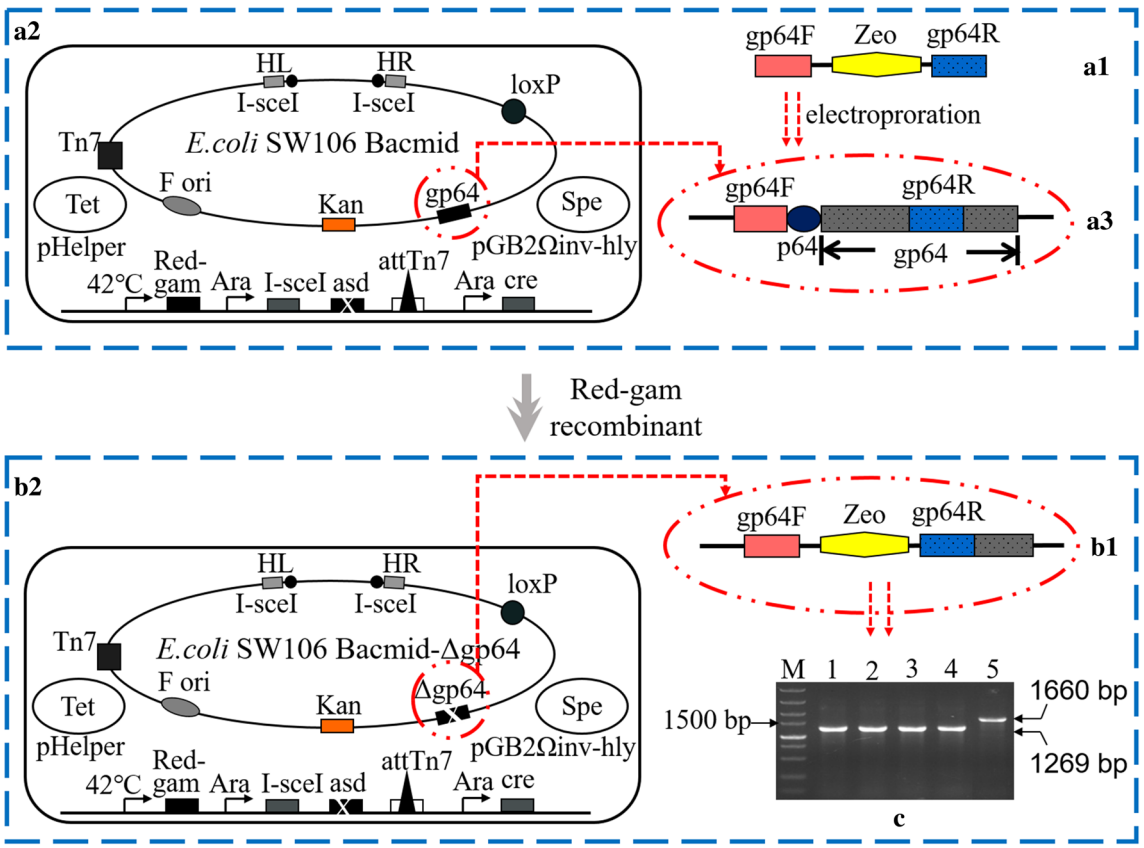


**Fig. 2** Construction of gp64-null Bacmid. **a1** The homologous recombination gene fragment gp64F-Zeo-gp64R; **a2** *E.coli* SW106 Bacmid; **a3** wild-type gp64 ORF with promoter p64. **b1** gp64 and p64 were replaced by a zeocin resistance cassette. **b2** *E.coli* SW106 Bacmid-Δgp64. **c** Identification the gp64 ORF and p64 were replaced successfully by PCR with primer *gp64F-F* / *gp64R-R* M: DL5000 Marker, 1–4: *E.coli* SW106-Bacmid-Δgp64; 5: *E.coli* SW106-Bacmid as control

the sample-coated grids were washed three times with distilled water, following a negative staining with 1% uranyl acetate for 45 s. Images were acquired using the JEM 100-CXII transmission electron microscope (TEM).

## Results

### Construction of gp64-null Bacmid

To improve the display efficiency of the baculovirus, the full gp64 gene was deleted. The homologous recombination fragment gp64F-Zeo-gp64R (Fig. 2a1) was electrotransformed into *E.coli* SW106 Bacmid (Fig. 2a2). Upon the completion of homologous recombination, wild-type gp64 ORF and p64 (Fig. 2a3) were replaced by a zeocin resistance cassette (Fig. 2b1).

The positive colony was further verified by PCR using primers *gp64F-F* and *gp64R-R* to amplify of 1,269 kb gp64F-Zeo-gp64R. However, a 1660 kb wild-type gene was amplified from the control with the same primers (Fig. 2c). The gp64-deficient strain was named *E.coli* SW106 Bacmid-Δgp64.

### gp64-null baculovirus was rescued using ires-dependent gp64 expression cassette

No infectious baculovirus was produced when using *E.coli* SW106 Bacmid-Δgp64 to infect sf9 cell, which proved GP64 was removed as expected (data not show). The extremely late promoter polyhedron (polh) was used to drive egfp, ires, and gp64. The ires-dependent GP64 was used to rescue the infectivity of gp64-null Bacmid. *E.coli* SW106 Bacmid-Δgp64-G-I64 was successfully constructed (Fig. 3a, b) and used to directly infect sf9 cells (Fig. 3c, d) for the production of recombinant baculovirus (Fig. 3e, f, g). Similarly, *E.coli* SW106 Ac-G containing the original wild-type gp64 was used as control (Fig. 3h).

48 h.p.i later, sf9 cells infected with *E.coli* SW106 Bacmid-Δgp64-G-I64 began to turn green (Fig. 3g1), which indicated that sf9 cells were infected successfully. Within 48 to 120 h.p.i (Figs. 3g1, 4), the number of green cells gradually increased, and the amount of fluorescence reached a maximum at 120 h.p.i (Figs. 3g, 4), indicating that the baculoviruses were propagating. The culture supernatant was collected and centrifuged at 80000*g*, after which the pellet was observed with TEM, and a number of mature baculovirus particles AcMNPV-Δgp64-G-I64 were successfully observed (Fig. 3, g5). When the viruses were collected and re-infected in sf9 cells (MOI = 1), green cells were observed at 24 h.p.i (Fig. 3, g5). Similar to the first generation strain's infection, fluorescence was observed to gradually increase within 24–96 h.p.i and reached a maximum at 96 h.p.i (Figs. 3g, 5g). This shows that recombinant baculoviruses undergo continuous production. Compared with the control (Fig. 3h), it was determined that the low expression of ires-dependent wild-type gp64 had no significant effect on the replication cycle of recombinant baculovirus. This method could be used to improve target protein display efficiency by decreasing the expression of wild-type gp64 by inserting the recombinant virus.

### Improvement of target protein display efficiency on the cell surface

Since the AcMNPV envelope membrane is extremely unstable, it is prone to breakage under unfavorable pH or metal ion concentration conditions. Thus, it was difficult to directly observe or account for integral proteins in the baculovirus envelope. To more conveniently and accurately determine the display efficiency of the target protein, we took advantage of the fact that baculovirus acquires the totality of its envelope from the host cell membrane during budding (Fig. 4a). Therefore, the display efficiency of the target protein on the surface of the host cell membrane is a direct reflection of that on the virus capsule.

The extreme early promoter p64 fused with sp and was used to drive the *Renilla* luciferase and gp64 fusion protein. *Firefly* luciferase, egfp and ires-dependent wild-type gp64 were driven by polh promoter. At 72 h.p.i., sf9 cells infected with AcMNPV-Δgp64-R64-F-G-I64 turned green, indicating the successful infection of sf9 cells and the expression of foreign genes (Fig. 4b). GP64 protein fused with *Renilla* luciferase was displayed on the surface of the cell membrane, while *Firefly* luciferase was expressed and distributed in the cell. Similarly, baculovirus AcMNPV-R64-F-G was used as a control.

The dual-Glo luciferase assay results showed that the display efficiency of *Renilla* luciferase on cell surface infected with baculovirus AcMNPV-Δgp64-R64-F-G-I64 is fourfold greater than that of AcMNPV-Δgp64-R64-F-G (Fig. 4c). The assay also showed that ires-dependent gp64 used to rescue wild-type gp64-null Bacmid can significantly increase the display efficiency of the target protein.

### Displaying multiple target proteins simultaneously on baculovirus particles

Three different tags 6×His, Strep II, and Flag were fused with the N terminus of fluorescent proteins eGFP, eYFP, and mCherry, respectively. They were then fused with gp64 N-terminal to construct vectors pU-p64spH-G64-I64 (Fig. 5a) and pF-p64spF-M64-p64spS-Y64 (Fig. 5d). Recombinant baculovirus AcMNPV-Δgp64-G64-M64-Y64-I64,

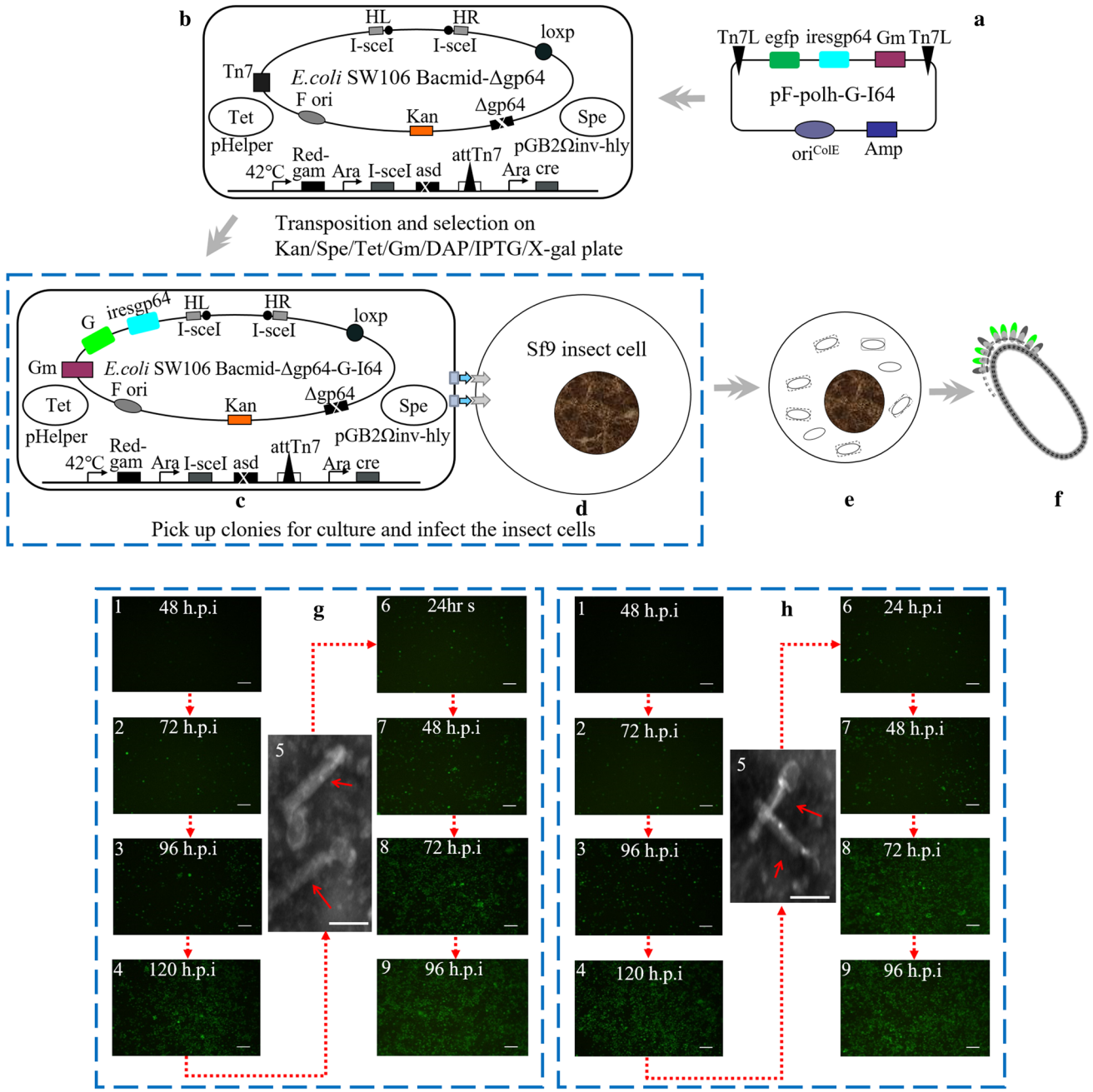


**Fig. 3** gp64-null baculovirus was rescued using ires-dependent gp64 expression cassette. **a** Donor vector pF-polh-G-I64. **b** *E.coli* SW106 Bacmid-Δgp64. **c** *E.coli* SW106 Bacmid-Δgp64-G-I64. **d** sf9 insect cell. **e** *E.coli* SW106 Bacmid-Δgp64-G-I64 was unable to synthesize cell walls in sf9 cells, thus rupturing and releasing Bacmid-Δgp64-G-I64. **f** Generation of recombinant baculovirus AcMNPV-Δgp64-G-I64. **g** Observation of insect sf9 cells infected by *E.coli* SW106 Bacmid-Δgp64-G-I64 under Nikon TS100 at 48 h.p.i (**g1**), 72 h.p.i (**g2**), 96 h.p.i (**g3**) and 120 h.p.i (**g4**), Bar: 200 μm. **g5** Purified recombinant baculoviruses (AcMNPV-Δgp64-polh-G-I64) were observed by TEM, Bar: 0.2 μm. Observation of insect sf9 cells infected by AcMNPV-Δgp64-G-I64 under Nikon TS100 at 24 h.p.i (**g6**), 48 h.p.i (**g7**), 72 h.p.i (**g8**) and 96 h.p.i (**g9**), Bar: 200 μm. **h** Using *E.coli* Sw106 Ac-G and AcMNPV-polh-G to perform the same treatment with Group g (as control)

which expressed three kinds of fluorescent proteins fused with GP64, was constructed successfully (Fig. 5f, g).

To determine if eGFP, mCherry, and eYFP proteins were properly translocated to the cell surface, sf9 cells were cultured on sterile cover slips, infected at an MOI of 10 by AcMNPV-Δgp64-G64-M64-Y64-I64, and subjected to LSCM at 72 h.p.i. Three fluorescing colors, green, yellow, and red, corresponding to fluorescent proteins eGFP,

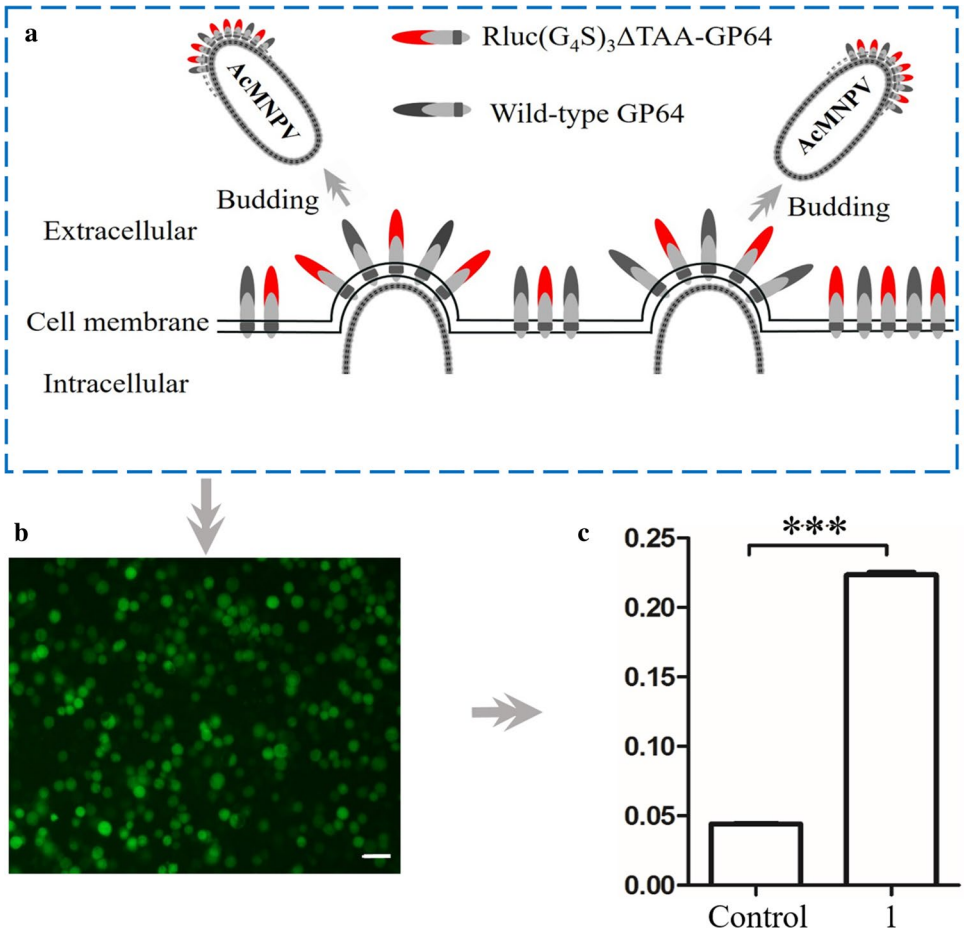


**Fig. 4** Improvement of display efficiency of target protein on cell surface. **a** The GP64 protein fused with *Renilla* luciferase was displayed on the surface of the sf9 cell membrane. The baculovirus AcMNPV-Δgp64-R64-F-G-I64 completely acquires the host cell membrane as the envelope when budding, resulting in the fusion protein being displayed on the envelope. **b** Infected 72 h.p.i by AcMNPV-Δgp64-R64-F-G-I64, the sf9 cells were observed by fluorescence microscopy (Bar: 50 μm). **c** The *Renilla* luciferase display efficiency (c1) was increased by fourfold compared to control (***$p < 0.01$)

eYFP, and mCherry were observed along the perimeter of the sf9 cells (Fig. 6a, b, c, d, e), indicating that eGFP, eYFP, mCherry fluorescent proteins were expressed and displayed on the cell membrane as expected. AcMNPV-Δgp64-G64-M64-Y64-I64 virus particles were then collected and purified, and western blot analysis results showed that the three fusion proteins were successfully and simultaneously inducted into the apical membrane of baculovirus (Fig. 6f). Furthermore, Z-section slices and measurements conducted by LSCM revealed that fluorescent proteins were correctly localized on the Sf9 plasma membrane, as opposed to remaining within intracellular structures. (Fig. 6g, h, i, j, k).

## Discussion

The baculovirus multigene display system (BMDS) is currently the most widely used eukaryotic display system (Boublik et al. 1995). Compared with other display systems, the system has a more complete protein folding and modification mechanism. It has a large capacity (greater than 50 kb) to accept foreign DNA fragments and allows fast and facile construction of high-titer recombinant viruses (Cheshenko et al. 2001; O'Reilly 1997; Davies 1994). Through the advancement of existing recombinant construction methods with novel strategies, BMDS is proving to be a powerful and efficient, yet low-cost method of constructing recombinant baculoviruses. BMDS successfully avoids complex operations such as the extraction of baculovirus genomes and liposome transfection (Yao et al. 2007, 2010, 2012), and exhibits a high degree of efficiency in co-presenting multiple target proteins, making the system an attractive new tool for receptor screening, gene therapy, and genetically engineering vaccines.

Since ires-dependent second gene expression was compared with cap-dependent first gene expression in several cultured cell lines (Mountford and Smith 1995; Pelletier and Sonenberg 1988). The expression of the ires-dependent second gene ranged from 6 to 100% (though in most cases between 20 and 50%) that of the first gene (Mizuguchi et al. 2000; Urabe et al. 1997; Adam et al. 1991). Thus, a single ires-dependent promoter can greatly reduce the competition from wild-type gp64 (Ghattas et al. 1991; Jang et al. 1989, 1988). The results also confirmed that the low-expression of GP64 did not significantly affect

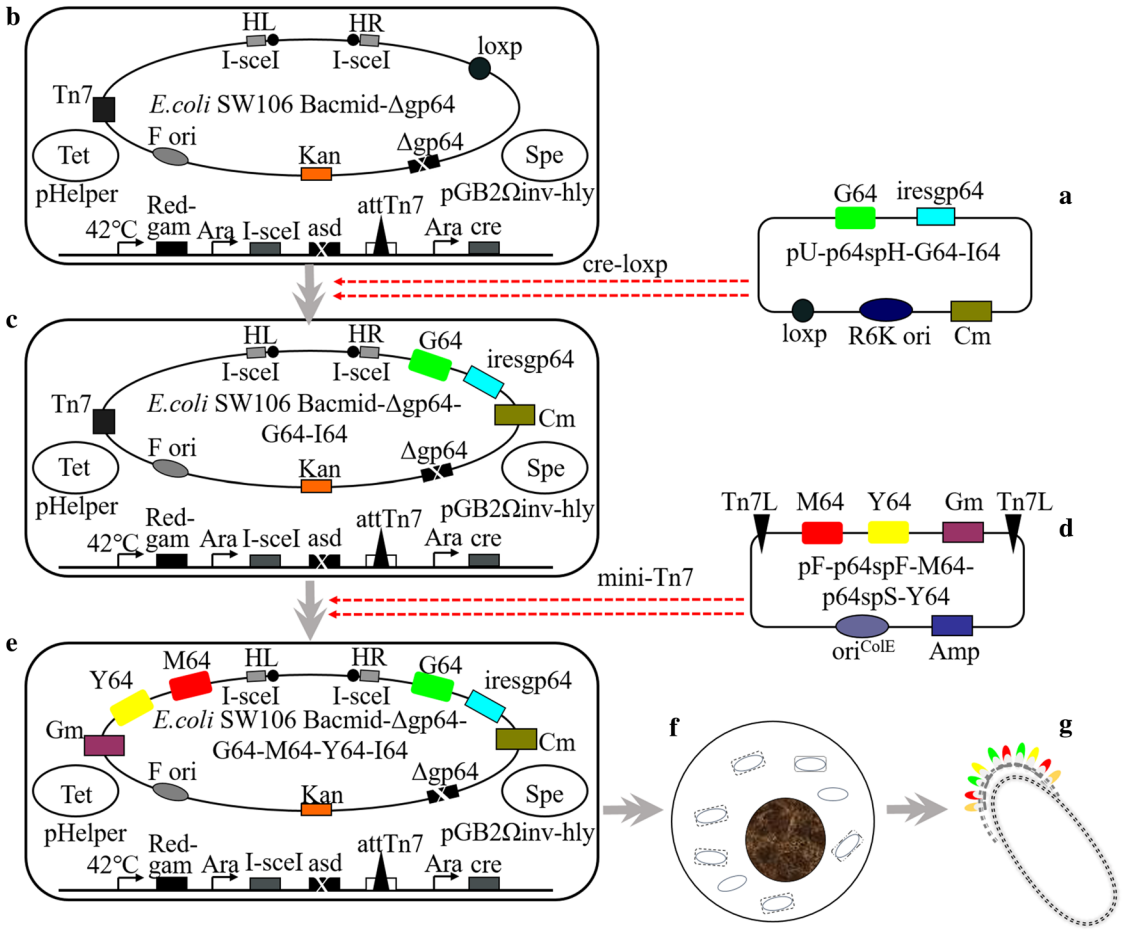


**Fig. 5** Diagrammatic sketch for recombinant Bacmid-Δgp64-G64-M64-Y64-I64. **a** Donor vector pU-p64spH-G64-I64; **b** *E.coli* SW106 Bacmid-Δgp64; **c** *E.coli* SW106 Bacmid-Δgp64-G64-I64. **d** Donor vector pF-p64spF-M64-p64spS-Y64. **e** *E.coli* SW106 Bacmid-Δgp64-G64-M64-Y64-I64. **f** The *E.coli* SW106 Bacmid-Δgp64-G64-M64-Y64-I64 was used to infect the sf9 cells; **g** recombinant baculovirus AcMNPV-Δgp64-p64sp-HG-FM-SY-I64

the replication cycle of baculovirus. Taken together, this method holds promising prospect in improving the display efficiency of target proteins. In addition, we found that p64 has high activity in early stages after initial virus infection through an analysis of the difference in activity of the baculovirus promoters. Namely its activity is about 20 times that of polh at 12 h.p.i. (data not shown). As such, the display efficiency of target protein can also be improved by p64 to some degree.

Web-based protein functional and structural prediction servers PROSITE (https://prosite.expasy.org/) and PredictProtein (https://open.predictprotein.org/) indicate a linker sequence $(GGGGS)_3$ was added to N terminus of *Renilla* luciferase to provide distance and flexibility for the N-terminal fusion proteins to fold correctly (Kukkonen et al. 2003). The results also showed that the *Renilla* luciferase still had a very strong enzyme activity, indicating that the system did not affect the activity of the target protein significantly while still displaying the target protein efficiently.

Our development of BMDS significantly improves the limitations of classical BDS, which lacks a donor vector system and mature technology, and is focused on the display of a single target protein or polypeptide. By mini-Tn7, cre-loxp transposition and red-gam homologous recombination, BMDS can display up to ten foreign genes together (Yao et al. 2010). In addition, baculovirus exhibits high titer and does not compete with target antigens, which provides an ideal method for the development of polyvaccine. Whether BMDS is capable of supporting the simultaneous expression of even more genes and/or the display of proteins with more complicated biological activities remains to be seen. In light of the results described above, we speculate that BMDS, with its wide range of use, high display efficiency, and multi-gene co-display capabilities, is of potentially important relevance to a variety of biological

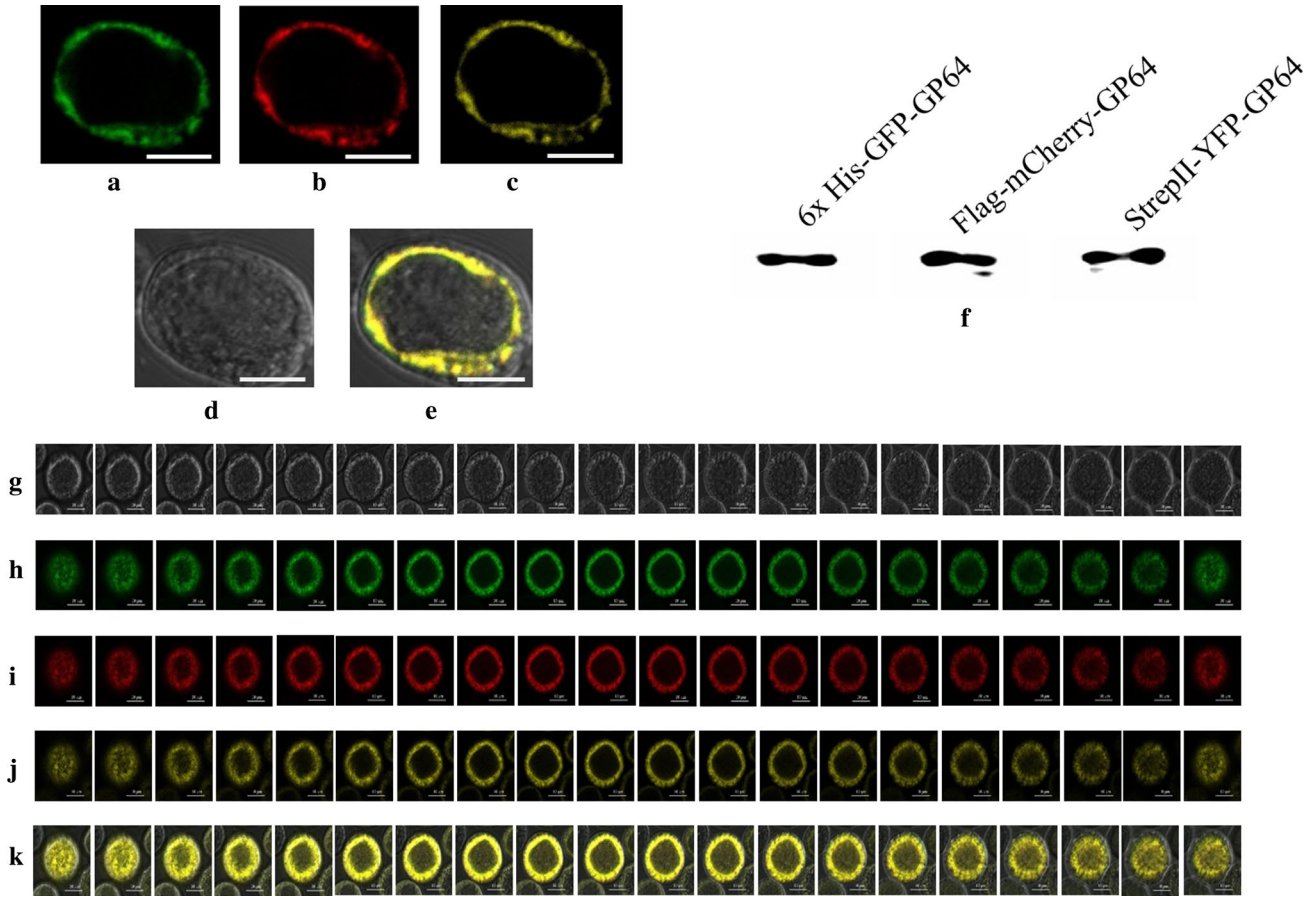


**Fig. 6** Sufficient and stable system of multigene co-display. 72 h.p.i., sf9 cells infected with AcMNPV-Δgp64-G64-M64-Y64-I64 (MOI = 10) were observed using CLSM. **a** green fluorescence. **b** red fluorescence; c: yellow fluorescence; **d** bright; **e** merge (**a** + b + **c** + **d**). **f** Purified AcMNPV-Δgp64-G64-M64-Y64-I64 by Western blot, reaction of fusion proteins with 3′ tag-antibodies 6 × His, Sterp II and Flag. Z-section slices and measurements conducted by CLSM showed that fluorescent proteins were correctly localized onto the Sf9 plasma membrane. **g** bright; **h** green; **i** red; **j** yellow; **k** merge (**g** + **h** + **i** + **j**), bar: 10 μm. (Color figure online)

and genetic applications, whether it is in industry or in the development of novel recombinant vaccine for prevention of epidemics.

**Acknowledgements** We would like to thank Jonathan Jih (University of California, Los Angeles) for revising the grammar of the manuscript. This study was funded by the National Natural Science Foundation of China (Grant Number 31372373, 31372381), the Natural Science Foundation of Guangdong Province, China (Grant Number 2016A030311018) and Science and Technology Planning Project of Guangzhou, China (Grant Number 201510010276).

**Author contribution** HZ, LY, and JS coordinated the project. HZ and XW performed the research. HZ and JS wrote the manuscript. LY and JS contributed new methods and improved the manuscript. FR, SZ, LX, and MF performed the data analysis. HZ, LY, and JS interpreted the context of results. All authors have read and approved the manuscript.

## Compliance with ethical standards

**Conflict of interest** All authors declare that they have no conflict of interest.

**Ethical approval** This article does not contain any studies with human participants or animals performed by any of the authors.

## References

Adam MA, Ramesh N, Miller AD, Osborne WR (1991) Internal initiation of translation in retroviral vectors carrying picornavirus 5′ nontranslated regions. J Virol 65(9):4985–4990

Berger I, Fitzgerald DJ, Richmond TJ (2004) Baculovirus expression system for heterologous multiprotein complexes. Nat Biotechnol 22(12):1583–1587

Boublik Y, Di Bonito P, Jones IM (1995) Eukaryotic virus display: engineering the major surface glycoprotein of the *Autographa californica* nuclear polyhedrosis virus (AcNPV) for the

presentation of foreign proteins on the virus surface. Biotechnology 13(10):1079–1084
Chapple SD, Jones IM (2002) Non-polar distribution of green fluorescent protein on the surface of *Autographa californica* nucleopolyhedrovirus using a heterologous membrane anchor. J Biotechnol 95(3):269–275
Chen L, Xia YH, Pan ZS, Zhang CY (2007) Expression and functional characterization of classical swine fever virus E(rns) protein. Protein Expr Purif 55(2):379–387
Cherepanov PP, Wackernagel W (1995) Gene disruption in *Escherichia coli*: $Tc^R$ and $Km^R$ cassettes with the option of Flp-catalyzed excision of the antibiotic-resistance determinant. Gene 158(1):9–14
Cheshenko N, Krougliak N, Eisensmith RC, Krougliak VA (2001) A novel system for the production of fully deleted adenovirus vectors that does not require helper adenovirus. Gene Ther 8(11):846–854
Davies AH (1994) Current methods for manipulating baculoviruses. Biotechnology 12(1):47–50
Ghattas IR, Sanes JR, Majors JE (1991) The encephalomyocarditis virus internal ribosome entry site allows efficient coexpression of two genes from a recombinant provirus in cultured cells and in embryos. Mol Cell Biol 11(12):5848–5859
Hu YC (2005) Baculovirus as a highly efficient expression vector in insect and mammalian cells. Acta Pharmacol Sin 26(4):405–416
Hulst MM, Himes G, Newbigin E, Moormann RJ (1994) Glycoprotein E2 of classical swine fever virus: expression in insect cells and identification as a ribonuclease. Virology 200(2):558–565
Jang SK, Krausslich HG, Nicklin MJ, Duke GM, Palmenberg AC, Wimmer E (1988) A segment of the 5′ nontranslated region of encephalomyocarditis virus RNA directs internal entry of ribosomes during in vitro translation. J Virol 62(8):2636–2643
Jang SK, Davies MV, Kaufman RJ, Wimmer E (1989) Initiation of protein synthesis by internal entry of ribosomes into the 5′ nontranslated region of encephalomyocarditis virus RNA in vivo. J Virol 63(4):1651–1660
Kitagawa Y, Tani H, Limn CK, Matsunaga TM, Moriishi K, Matsuura Y (2005) Ligand-directed gene targeting to mammalian cells by pseudotype baculoviruses. J Virol 79(6):3639–3652
Kukkonen SP, Airenne KJ, Marjomaki V, Laitinen OH, Lehtolainen P, Kankaanpaa P, Mahonen AJ, Raty JK, Nordlund HR, Oker-Blom C, Kulomaa MS, Yla-Herttuala S (2003) Baculovirus capsid display: a novel tool for transduction imaging. Mol Ther 8(5):853–862
Lin YH, Lee LH, Shih WL, Hu YC, Liu HJ (2008) Baculovirus surface display of σC and σB proteins of avian reovirus and immunogenicity of the displayed proteins in a mouse model. Vaccine 26(50):6361–6367
Mizuguchi H, Xu Z, Ishii-Watabe A, Uchida E, Hayakawa T (2000) IRES-dependent second gene expression is significantly lower than cap-dependent first gene expression in a bicistronic vector. Mol Ther 1(4):376–382
Monsma SA, Oomens AG, Blissard GW (1996) The GP64 envelope fusion protein is an essential baculovirus protein required for cell-to-cell transmission of infection. J Virol 70(7):4607–4616
Motohashi T, Shimojima T, Fukagawa T, Maenaka K, Park EY (2005) Efficient large-scale protein production of larvae and pupae of silkworm by *Bombyx mori* nuclear polyhedrosis virus bacmid system. Biochem Biophys Res Commun 326(3):564–569
Mountford PS, Smith AG (1995) Internal ribosome entry sites and dicistronic RNAs in mammalian transgenesis. Trends Genet 11(5):179–184
O'Reilly DR (1997) Use of baculovirus expression vectors. Methods Mol Biol 62:235–246
Oker-Blom C, Airenne KJ, Grabherr R (2003) Baculovirus display strategies: Emerging tools for eukaryotic libraries and gene delivery. Brief Funct Genom 2(3):244–253
Oomens AG, Blissard GW (1999) Requirement for GP64 to drive efficient budding of *Autographa californica* multicapsid nucleopolyhedrovirus. Virology 254(2):297–314
Pelletier J, Sonenberg N (1988) Internal initiation of translation of eukaryotic mRNA directed by a sequence derived from poliovirus RNA. Nature 334(6180):320–325
Peralta A, Maroniche GA, Alfonso V, Molinari P, Taboga O (2013) VP1 protein of Foot-and-mouth disease virus (FMDV) impairs baculovirus surface display. Virus Res 175(1):87–90
Roldao A, Oliveira R, Carrondo MJ, Alves PM (2009) Error assessment in recombinant baculovirus titration: evaluation of different methods. J Virol Methods 159(1):69–80
Sandig V, Hofmann C, Steinert S, Jennings G, Schlag P, Strauss M (1996) Gene transfer into hepatocytes and human liver tissue by baculovirus vectors. Hum Gene Ther 7(16):1937–1945
Shoji I, Aizaki H, Tani H, Ishii K, Chiba T, Saito I, Miyamura T, Matsuura Y (1997) Efficient gene transfer into various mammalian cells, including non-hepatic cells, by baculovirus vectors. J Gen Virol 78(Pt 10):2657–2664
Sun JC, Zhang EH, Yao LG, Zhang HL, Jin PF (2009) A high efficient method of constructing recombinant *Bombyx mori* (silkworm) multiple nucleopolyhedrovirus based on zero-background Tn7-mediated transposition in *Escherichia coli*. Biotechnol Prog 25(2):524–529
Tami C, Peralta A, Barbieri R, Berinstein A, Carrillo E, Taboga O (2004) Immunological properties of FMDV-gP64 fusion proteins expressed on SF9 cell and baculovirus surfaces. Vaccine 23(6):840–845
Urabe M, Hasumi Y, Ogasawara Y, Matsushita T, Kamoshita N, Nomoto A, Colosi P, Kurtzman GJ, Tobita K, Ozawa K (1997) A novel dicistronic AAV vector using a short IRES segment derived from hepatitis C virus genome. Gene 200(1–2):157–162
van Loo ND, Fortunati E, Ehlert E, Rabelink M, Grosveld F, Scholte BJ (2001) Baculovirus infection of nondividing mammalian cells: mechanisms of entry and nuclear transport of capsids. J Virol 75(2):961–970
Warming S, Costantino N, Court DL, Jenkins NA, Copeland NG (2005) Simple and highly efficient BAC recombineering using galK selection. Nucleic Acids Res 33(4):e36. https://doi.org/10.1093/nar/gni035
Whitford M, Stewart S, Kuzio J, Faulkner P (1989) Identification and sequence analysis of a gene encoding gp67, an abundant envelope glycoprotein of the baculovirus *Autographa californica* nuclear polyhedrosis virus. J Virol 63(3):1393–1399
Xu XG, Liu HJ (2008) Baculovirus surface display of E2 envelope glycoprotein of classical swine fever virus and immunogenicity of the displayed proteins in a mouse model. Vaccine 26(43):5455–5460
Xu XG, Chiou MT, Zhang YM, Tong DW, Hu JH, Zhang MT, Liu HJ (2008) Baculovirus surface display of E(rns) envelope glycoprotein of classical swine fever virus. J Virol Methods 153(2):149–155
Yang DG, Chung YC, Lai YK, Lai CW, Liu HJ, Hu YC (2007) Avian influenza virus hemagglutinin display on baculovirus envelope: cytoplasmic domain affects virus properties and vaccine potential. Mol Ther 15(5):989–996
Yao LG, Liu ZC, Zhang XM, Kan YC, Zhou JJ (2007) A highly efficient method for the generation of a recombinant *Bombyx mori* nuclear-polyhedrosis-virus Bacmid and large-scale expression of foreign proteins in silkworm (*B. mori*) larvae. Biotechnol Appl Biochem 48(Pt 1):45–53
Yao LG, Sun JC, Xu H, Kan YC, Zhang XM, Yan HC (2010) A novel economic method for high throughput production of recombinant baculovirus by infecting insect cells with Bacmid-containing diminopimelate-auxotrophic *Escherichia coli*. J Biotechnol 145(1):23–29

Yao LG, Wang SS, Su S, Yao N, He J, Peng L, Sun JC (2012) Construction of a baculovirus-silkworm multigene expression system and its application on producing virus-like particles. PLoS One 7(3):e32510. https://doi.org/10.1371/journal.pone.0032510

Yap CC, Ishii K, Aoki Y, Aizaki H, Tani H, Shimizu H, Ueno Y, Miyamura T, Matsuura Y (1997) A hybrid baculovirus-T7 RNA polymerase system for recovery of an infectious virus from cDNA. Virology 231(2):192–200

Yoshida S, Kondoh D, Arai E, Matsuoka H, Seki C, Tanaka T, Okada M, Ishii A (2003) Baculovirus virions displaying *Plasmodium berghei* circumsporozoite protein protect mice against malaria sporozoite infection. Virology 316(1):161–170