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Transient transfection coupled to baculovirus infection for rapid protein expression screening in insect cells

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ABSTRACT

Baculovirus infected insect cells are widely used for heterologous protein expression. Despite the power of this system, the use of baculovirus techniques for protein expression screening is hampered by the time and resources needed to generate each recombinant baculovirus. Here, we show that a transfection/infection based expression system is suitable for screening of expression constructs in insect cells and represents a valid alternative to other traditional screening methodologies using recombinant baculovirus. The described method is based on gene delivery by transfection coupled to the induction of protein expression by non-recombinant baculovirus infection. Vectors that control expression by a combination of the baculovirus promoters *ie1* and *p10* and the enhancer element *hr5* are among the ones suitable for this method. Infection with non-recombinant baculovirus drastically increases the basal activity of these elements, leading to protein over-expression. Multiple vectors can be simultaneously co-transfected/infected, making transfection/infection amenable for screening of multiple co-expressed proteins and protein complexes. Taken together, our results prove that the transfection/infection protocol is a valid and innovative approach for increasing speed and reducing costs of protein expression screening for structural and functional studies.

1. Introduction

In the last decades, baculovirus infected insect cells have become one of the most widely used eukaryotic systems for heterologous protein expression (Berger et al., 2004; Kost et al., 2005; Jarvis, 2009; Trowitzsch et al., 2010). Ovarian cells from *Spodoptera frugiperda* (*Sf*21 and *Sf*9) (Vaughn et al., 1977) ensure protein production at very high yield, allow post-translational modifications (like *N*- and *O*-glycosylation, acylation, phosphorylation) similar to mammalian cells and can conveniently be grown in various scales in adherent or highly confluent suspension cultures. For gene transfer, infection with the double-stranded DNA virus *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV), belonging to the family of *Baculoviridae*, is most commonly used. In the baculovirus dependent insect cell expression system, the gene of interest replaces the viral *polh* gene. Upon infection, the target protein is then highly expressed under the control of the viral *polh* or *p10* promoter, while the loss of the endogenous gene does not affect survival, infectivity and propagation of the recombinant baculovirus (Murphy et al., 2004).

Several systems for the generation of recombinant virus have been developed and commercialized. Recombinant baculoviruses can be obtained by site-specific transposition in *E. coli* (Bac-to-Bac, Invitrogen). Here, a modified bacterial strain already containing the viral genome as a bacmid is transformed with a target gene cloned into an appropriate transfer vector (Luckow et al., 1993). Recombinant bacmid DNA can then be purified from the bacteria and introduced into insect cells by transfection for virus production. The read-out of the recombination event is easy in Bac-to-Bac, making the chance to amplify a non recombinant virus very low. The bacterial step, however, slows down the process and leads to high cost when a substantial number of constructs need to be tested.

Alternatively, generation of recombinant baculoviruses is achieved by homologous recombination of a co-transfected transfer vector and viral DNA directly in insect cells (Zhao et al., 2003). The system has been improved to increase the efficiency

Abbreviations: AcMNPV, *Autographa californica* multicapsid nucleopolyhedrovirus; *Sf*, *Spodoptera frugiperda*; polh, polyhedrin; p10, AcMNPV protein of 10 kDa; LIC, ligation independent cloning; EGFP, enhanced green fluorescent protein; $GABA_AR$, γ-amino butyric acid type A receptor; α7-nAChR, α7 nicotinic acetylcholine receptor.

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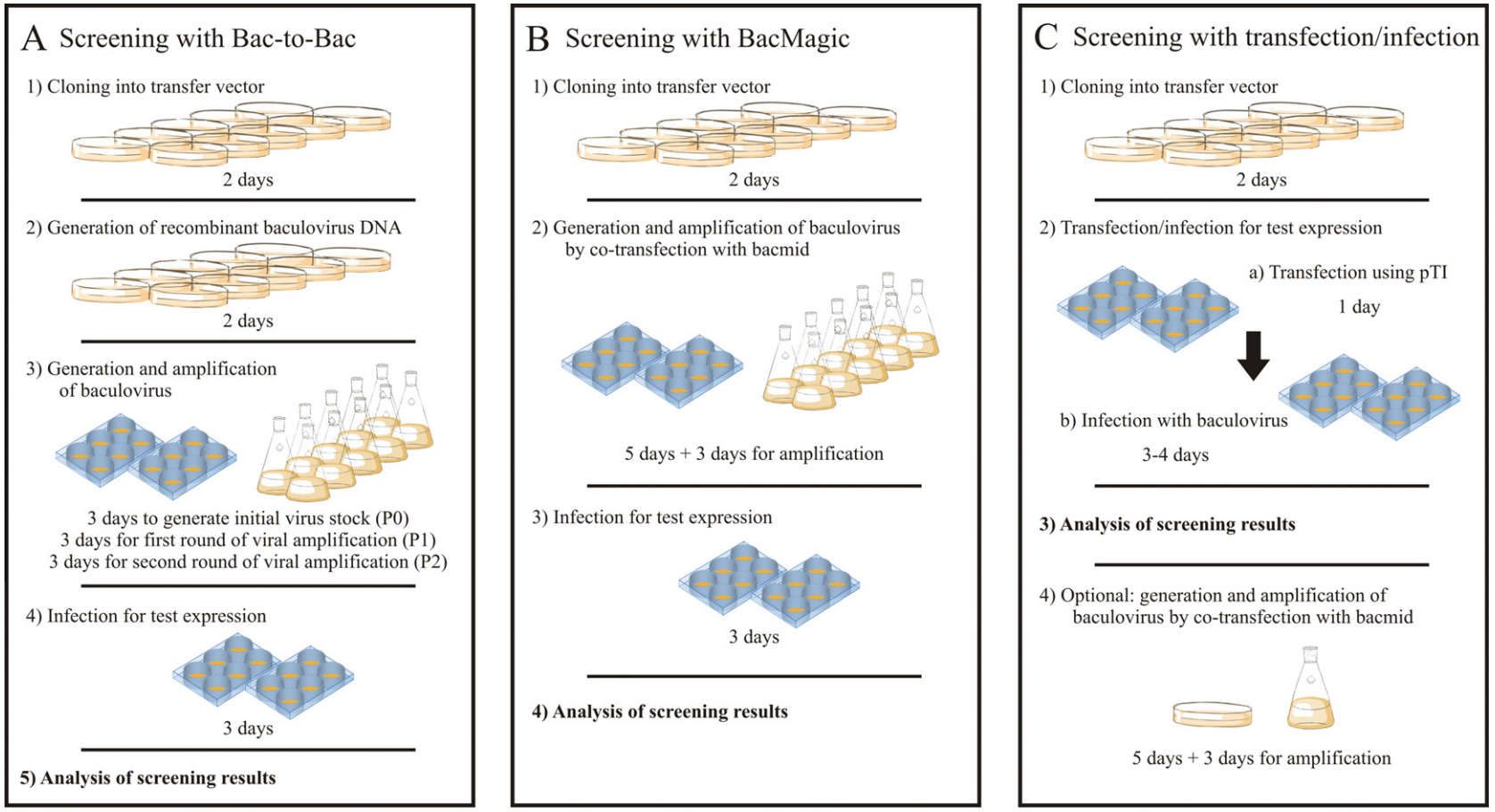
A Screening with Bac-to-Bac
1) Cloning into transfer vector
2 days
2) Generation of recombinant baculovirus DNA
2 days
3) Generation and amplification of baculovirus
3 days to generate initial virus stock (P0)
3 days for first round of viral amplification (P1)
3 days for second round of viral amplification (P2)
4) Infection for test expression
3 days
5) Analysis of screening results
Total: approx. 3 weeks
B Screening with BacMagic
1) Cloning into transfer vector
2 days
2) Generation and amplification of baculovirus by co-transfection with bacmid
5 days + 3 days for amplification
3) Infection for test expression
3 days
4) Analysis of screening results
Total: approx. 2 weeks
C Screening with transfection/infection
1) Cloning into transfer vector
2 days
2) Transfection/infection for test expression
a) Transfection using pTI
1 day
b) Infection with baculovirus
3-4 days
3) Analysis of screening results
4) Optional: generation and amplification of baculovirus by co-transfection with bacmid
5 days + 3 days for amplification
Total: approx. 1 week (up to step 3)
approx. 2 weeks (up to step 4)

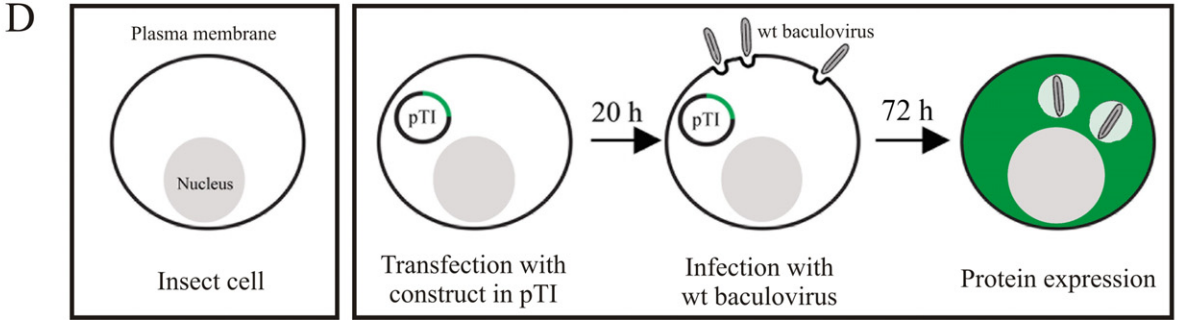
D
Plasma membrane
Nucleus
Insect cell
pTI
Transfection with construct in pTI
20 h
wt baculovirus
pTI
Infection with wt baculovirus
72 h
Protein expression

Fig. 1. Transient transfection coupled to baculovirus infection for rapid protein expression screening in insect cells. (A–C) Comparison between expression screenings of a pool of constructs in insect cells using standard approaches or the transfection/infection protocol. In (A), the pipeline follows a standard infection protocol, based on the Bac-to-Bac system (Invitrogen). A library of cDNAs is cloned into a transfer vector. Each recombinant baculovirus carrying a particular construct of the library has to be generated individually, making this step time consuming. After consecutive cycles of viral amplification, the recombinant viruses can be used for test expression. In (B), the screening follows the BacMagic system. Recombinant baculoviruses are generated for each single construct by co-transfection of insect cells with a transfer vector and bacmid DNA. One cycle of amplification is usually necessary to reach the required viral titer for screening. In (C), the screening follows the transfection/infection strategy. The library of constructs can conveniently be cloned into pTI by LIC. Each construct of the library can be used directly to transfect cells cultured in different multiwell formats. Protein production is then induced by infection with non-recombinant baculovirus, leading to transactivation and protein over-expression. Promising constructs can then directly be used for generating recombinant virus by homologous recombination. (D) Schematic representation of the transfection/infection protocol. Insect cells are transfected with pTI vector. 14–20 h after the transfection, baculovirus is added to the cultures to transactivate pTI. Protein expression can be investigated after 72–96 h.

of recombination by using bacmid DNA that is unable to produce virus without recombination (Possee et al., 2008; commercialized as BacMagic by Novagen and as flashBac by OET). Using BacMagic/flashBac, viral titers sufficient for test expression are often achievable already from the cultures in which the homologous recombination was performed. However, reported variability in the titers obtained by parallel co-transfected culture (Possee et al., 2008) makes the comparison between expression levels of individual construct difficult at this stage. Moreover, the relatively high cost of the required bacmid represents a disadvantage of this system.

In addition to viral approaches, systems for rapid expression screening following transient transfection of insect cells are commercially available (InsectDirect, Novagen). However, this system is expensive to up-scale and most plasmids (pIEX and pBiEX from Novagen) are not compatible with baculovirus generation. Positive hits from the screening therefore need to be re-cloned into an appropriate transfer vector if viral expression is desired, delaying the entire process and not ensuring the reproducibility of the expression in the new transfer vector or in the context of a viral infection.

Testing expression of multi-protein complexes is even more cumbersome. Complex formation can be achieved by simple co-infection with several baculoviruses, however, titer determination by plaque assays and systematic variation of the amount and ratio of individual viruses is required in most cases (Vijayachandran et al., 2011). Alternatively, several constructs can be integrated into the same virus, e.g. by the MultiBac system (Berger et al., 2004), which increases the success in protein complex formation, but limits the flexibility in construct combinations that can be tested without substantial investment of time and effort.

Recently published high-throughput insect cell expression systems have streamlined expression screening and protein analysis, but still adhere to generating and amplifying recombinant baculoviruses for each individual construct (Mancia and Love, 2011; Hanson et al., 2007; Buchs et al., 2009). In Fig. 1A, a pipeline for parallel protein expression screening using the Bac-to-Bac expression system is depicted. From 1 to 3 weeks (depending on the amplification step in which the virus has the requested titer) have to be invested before expression properties of each construct can be examined. For the BacMagic system in Fig. 1B, a reduced amount of time is needed (1–2 weeks), however, the cost of the bacmid DNA is often becoming considerable.

We therefore envisioned an expression screening method that combines the most beneficial features of transient and viral approaches. Such a method would be of great value and constitute an interesting supplement to established insect cells expression systems, if it: (a) is time- and cost-efficient in large screening approaches, (b) yields already in small scale sufficient quantities of proteins to perform analytical assays, (c) allows a proper comparison of expression between individual constructs, (d) facilitates co-expression of several proteins, (e) predicts the yield in large-scale infected cultures, and (f) allows the generation of a recombinant baculovirus without vector shuttling or re-cloning.

Protein expression in insect cells can be achieved by gene delivery via transient transfection followed by baculovirus trans-activation. In this so-called "transfection/infection" protocol (Fig. 1C), the gene to be expressed is located on a plasmid under the control of viral promoters that can be trans-activated without integration into the baculovirus genome (Fig. 1D). During the last decades, the method was used to investigate viral promoter activity and to express proteins to study their function and intracellular localization (Lo et al., 2002; van Anken et al., 2008; Jester et al., 2011). Despite this prior use, the method's applicability for the screening of heterologous protein expression has never been compared to existing structural biology protocols. Here, we prove that the transfection/infection protocol is suitable for fast screening of expression constructs in insect cells and represents a valid alternative to related methodologies using recombinant baculovirus. We show that transfection/infection allows rapid testing of a large number of single constructs or construct combinations before proceeding with the generation of recombinant virus. Parallel protein expression screening using transfection/infection is faster than Bac-to-Bac system while it is more economic than approaches like BacMagic/flashBac.

2. Materials and methods

2.1. Cell lines

Spodoptera frugiperda cells *Sf*21, (from ATCC) were grown either as adherent culture or in shaking flasks in serum free SF900II SFM medium (Gibco) supplemented with Penicillin/Streptomycin (Gibco). A stock of cells was maintained at confluency between 0.5×10^6 and 1×10^7 cells/ml at 28 °C in a shaking culture (80 rpm).

*Sf*9 (Invitrogen) were grown as above in serum free InsectXpress medium (BioWhittaker) supplemented with Penicillin/Streptomycin/Amphotericin (BioWhittaker). A stock was maintained as above at 27 °C in a shaking culture (140 rpm).

HEK293, human embryonic kidney cells, (from ATCC) were maintained in Dulbecco's Modified Eagle medium (GIBCO) supplemented with 10% FCS (Sigma) and Penicillin/Streptomycin at 37 °C in an incubator with 5% CO_2.

2.2. Baculovirus

Recombinant baculoviruses were generated with the Bac-to-Bac System (Invitrogen) or ××BacMagic system (Novagen). Co-transfection of transfer vector and BacMagic baculovirus bacmid DNA was performed following manufacturer instructions. In the experiment shown in Supplementary Fig. S2, the baculovirus is recombinant for the gene coding the mouse protease Furin (GI: 125347022 Radner et al., unpublished). Non-recombinant baculoviruses were generated with the Bac-to-Bac system using bacmid DNA from untreated DH10Bac cells (Invitrogen). Virus stocks obtained after several cycles of amplification were titrated using standard plaque

assay. The virus was stored in SF900II or InsectXpress medium at 4 °C in the dark. Virus stored at 4 °C was infective up to 4 months after the generation. For long-time storage, virus was supplemented with 10% fetal calf serum (Sigma).

2.3. Transfection/infection

1×10^6 *Sf*21 or $1–2 \times 10^6$ *Sf*9 cells were seeded into each well of a standard 6-well plate. Alternatively, between 3 and 6×10^6 cells were seeded into 10 cm dishes. Cells were allowed to get adherent and media was replaced by antibiotic free media. Cells were then transfected with plasmid using Insect GeneJuice (Novagen). The transfection reagent was diluted 10× in antibiotic free media followed by vigorous mixing. After 5 min, DNA was added drop-wise to the solution, which was then mixed vigorously. The ratio of DNA to Insect GeneJuice was chosen according to the manufacturer's suggestions (in this study: 10 μl reagent and 1.2 μg DNA for each well of a 6-well plate). Complex formation was allowed for 15–30 min and the solution was distributed evenly onto the cells. Transfection solution was replaced by antibiotic containing media (with or without baculovirus) after 14–20 h. Infections were performed at a multiplicity of infection of 0.1–2 infecting particles per cell, unless otherwise stated. DNA used for transfection was purified from bacteria by Maxi-prep (Qiagen).

$0.5–1 \times 10^6$ HEK293 cells were seeded in 35 mm plates. Cells were then transfected using a polyethylenimine (PEI) transfection procedure. For each well, 10 μg of PEI and 4 μg of DNA were mixed in 150 mM NaCl. After incubation of 15–30 min, the solution was diluted in serum free medium and added to the cells for 16–24 h to be then replaced with complete medium.

2.4. Expression screening

For EGFP expression screening in insect cells, adherent insect cells were harvested after washing 2 times with cold PBS by scraping them off the plate in cold harvesting buffer (25 mM Tris–HCl, 150 mM NaCl, 10 mM imidazole, pH 8.0). Cells were lysed by repetitive freeze and thaw cycles and cleared by centrifugation at min. 14,000×*g* for 15–30 min at 4 °C.

For the quantitative measurement of EGFP yield, cells were prepared as above and the fluorescence of the cleared lysate was measured at 535 nm (530 nm cut-off, λ exc. 488) in a SpectraMAX Gemini XS plate reader (Molecular Devices). Affinity-purified (His_6)–GFP–uv (Crameri et al., 1996) was used to generate a standard curve. EGFP concentration was calculated from the GFP–uv curve and then corrected for the different molar extinction coefficient of the two variants of the protein (εGFP–uv (488 nm) = 29,000 M^{-1} cm^{-1}, εEGFP (488 nm) = 61,000 M^{-1} cm^{-1}).

For the expression of $GABA_A$ receptors, adherent *Sf*21 cells were harvested after washing 2 times with cold PBS by scraping them off the plate in cold lysis buffer (25 mM Tris–HCl, 150 mM NaCl, 1 mM imidazole, 0.5% (v/v) Triton X 100, and protease inhibitors (Roche Applied Sciences), pH 8.0). After centrifugation at 14,000×*g* for 30 min at 4 °C, cleared supernatant was incubated with agitation with Talon beads (Clontech), which were equilibrated in wash buffer I (25 mM Tris–HCl, 150 mM NaCl, 10 mM imidazole, 0.1% (v/v) Triton X 100, pH 8) at 4 °C for 1 h. Beads were then harvested by centrifugation at low speed and washed extensively in wash buffer I and wash buffer II (25 mM Tris–HCl, 150 mM NaCl, 80 mM imidazole, 0.1% (v/v) Triton X 100, pH 8). Protein was eluted from the beads with elution buffer (25 mM Tris–HCl, 150 mM NaCl, 150 mM imidazole, 0.1% (v/v) Triton X 100, pH 8). Protein was precipitated with 10 volumes of cold acetone and dried pellets were resuspended in SDS PAGE loading buffer.

For the expression of the α7 nAChR, adherent *Sf*21 cells were harvested after washing 2× with cold PBS by scraping them off the plate in cold lysis buffer (25 mM Tris–HCl, 150 mM NaCl, 0.5% (v/v) Triton X 100, and protease inhibitors (Roche Applied Sciences), pH 8.0). After centrifugation at 14,000×*g* for 30 min at 4 °C, cleared supernatant was incubated with biotinylated alpha-bungarotoxin (100 nM, Invitrogen) over-night. Streptactin–agarose beads (Novagen) were then added and samples were left for 45 min at 4 °C. Beads were spun down and two washes were performed in wash buffer III (25 mM Tris–HCl, 500 mM NaCl, 0.1% (v/v) Triton X 100, pH 8) at 4 °C for 10 min. Beads were then resuspended in SDS PAGE loading buffer.

3. Results

We engineered a LIC (ligation independent cloning) variant of pIEX/Bac-1 (Novagen) which is referred to as pTI (plasmid for transfection/infection) in the following (Fig. 2). Since pTI contains sites for homologous recombination, it can be directly used as a transfer vector for the generation of a recombinant baculovirus in insect cells, e.g. with the BacMagic system (Novagen). Several regulatory elements known to be transactivated by baculovirus infection (Kovacs et al., 1991; Scheper et al., 1997; Lo et al., 2002) are located upstream the expression cassette of pTI: the *hr5* enhancer followed by an *ie1* promoter, and a very late viral *p10* promoter. The *p10* promoter has strong activity that depends entirely on viral factors (Todd et al., 1996), whereas the *ie1* promoter is weaker than *p10*, but can be recognized by host cell factors, together with the *ie1* terminator. The enhancer *hr5* promotes the activity of cis-linked *ie1* promoter in the absence of viral factors (Pullen and Friesen, 1995). The LIC overhangs make pTI compatible with a family of *E. coli* vectors designed for high throughput cloning (Luna-Vargas et al., 2011).

3.1. Infection with non-recombinant baculovirus enhances protein expression after transient transfection: the transfection/infection system

First, we tested the ability of baculovirus to transactivate protein expression from pTI. cDNA for EGFP was cloned into pTI as described in the supplemental methods and *Sf*21 cells were transiently transfected with pTI–EGFP. 14–20 h after transfection, the transfection solution was replaced by fresh medium and incubation was continued in the presence or absence of non-recombinant baculovirus. Approx. 72 h later, cells were either fixed or lysed and EGFP expression levels were analyzed by fluorescence microscopy or Western Blot, respectively. *Sf*21 cells that were transfected and subsequently infected showed an unambiguous increase in EGFP-fluorescence (Fig. 3A, GFP channel,+BV), if compared with uninfected cells (Fig. 3A, GFP channel, -BV). Transfected cells cultured in the presence of baculovirus (Fig. 3A, bright field + BV), but not the ones that were cultured in the absence of virus (Fig. 3A, bright field −BV), showed the typical morphology of infected insect cells, such as increase in size and loose attachment to the culture plate. In all the performed experiments, the number of living cells was lower in infected cultures due to the lytic properties of baculovirus. Similar results were obtained when using *Sf*9 cells instead of *Sf*21 cells, indicating that both cell types are suited for transfection/infection (Supplementary Fig. S1A). Transfection efficiency reached approx. 20–30% in all experiments. In cells that were co-transfected with pTI–EGFP and bacmid DNA (BacMagic) only a minor increase in fluorescence was observed (Fig. 3A, GFP channel,+bacmid).

Next, we investigated the virally induced up-regulation of EGFP levels in cells transfected with pTI–EGFP by performing FACS (Fluorescence assisted cell sorting) analysis. FACS showed an overall right shift towards higher fluorescence in cells that were transfected/infected (Supplementary Fig. S1B, +BV), compared to the

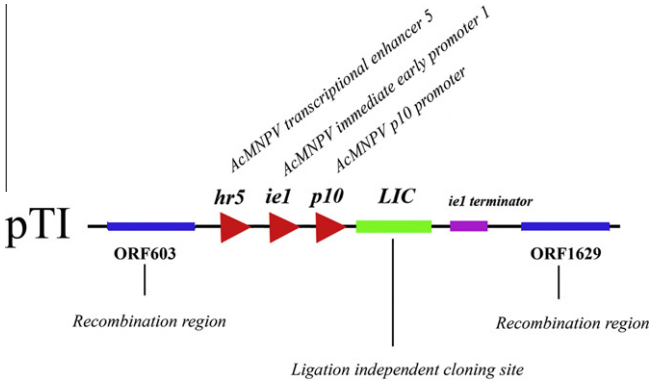

Fig. 2. pTI vector. Schematic drawing of the pTI vector used for transfection/infection (derived from pIEX/Bac-1 (Novagen)) with its main elements. The baculovirus early immediate promoter *ie1*, the enhancer element *hr5* and the late strong promoter *p10* are located upstream the ligation independent cloning site LIC. The *ie1* terminator is located at the 3′ of the expression cassette. The flanking recombination sites ORF603 and ORF1629 make pTI suitable for recombination with the baculovirus genome.

uninfected control (Supplementary Fig. S1B, −BV). Remarkably, there is a drastic increase of cells with very high EGFP fluorescence (more than 10^4 arbitrary units, AU) after transfection/infection.

Quantitation of EGFP yield obtained by transfection/infection in *Sf*9 cells was performed by recording EGFP fluorescence emission. Purified GFP–uv (Crameri et al., 1996) produced in *E. coli* was diluted in lysis buffer and used to create a standard curve, correlating the amount of protein to fluorescence emission. A transfected/infected *Sf*9 culture in a single well of a 6-well plated yielded 17 μg of EGFP per 10^6 cells, compared to 2 μg of EGFP that was produced by cells that were transfected, but not infected. pTI–EGFP was used to generate by homologous recombination (Bac–Magic) a baculovirus that expresses EGFP. This virus was infective for up to 3 months and no difference in yield of protein produced between young and old viral stock or between small and big scale was observed (data not shown). When we used this virus to infect *Sf*9 cells, we obtained a yield of 48 μg of EGFP per 10^6 cells ($n = 2$, Fig. 3B) confirming the ability of pTI to generate a functional baculovirus.

Western Blot confirmed the up-regulation of EGFP levels in transfected *Sf*21 cells upon infection (Fig. 3C, compare lanes 1 and 2) and, as expected, that transactivation efficiency is dependent on the amount of virus used to infect the cells. A multiplicity of infection of 2 (lane 2) to 0.2 (lane 3) infectious particles per cell yielded the highest expression (Fig. 3C).

To exclude that the burst in EGFP expression obtained by transfection/infection would result in misfolded and/or insoluble protein, cells were lysed and insoluble material was removed by ultracentrifugation. A comparison of total and soluble fractions of lysates obtained from transfected or transfected/infected cells indicated no difference in solubility, confirming the quality of the EGFP produced by transfection/infection (Fig. 3D, compare lanes 5 and 6). In order to optimize the transfection/infection protocol, time course experiments were performed. *Sf*21 cells transfected with pTI–EGFP were infected with non-recombinant baculovirus at different time points after the transfection. Increased expression could be achieved by infection 6–18 h after the transfection (Fig. 3E).

In all experiments depicted in Fig. 3, non-recombinant baculovirus was used for the transfection/infection protocol. We also addressed the possibility of using recombinant virus for the infection of transfected cells. For all proteins tested, baculovirus transactivation efficiency was similar for recombinant and non-recombinant baculovirus, demonstrating that expression from the pTI vector is not affected by the presence of heterologous gene(s) in the virus (Supplementary Fig. S2).

As a control, the commercial vectors pFastBac (Invitrogen) and pBac (Novagen) were tested for their ability to be transactivated by baculovirus. In both vectors, the open reading frame is located downstream of the strong polyhedrin promoter *polh*. Due to its strength, this very late viral promoter is widely used in baculovirus-mediated protein expression in insect cells. EGFP cDNA was cloned into pFastBac and *Sf*21 cells were transfected with pFastBac–EGFP and cultured in the presence or absence of baculovirus. Using pFastBac–EGFP, no fluorescence could be detected in transfected or transfected/infected cells, suggesting that this vector is not suitable for transfection/infection (Supplementary Fig. S3). The same results were obtained with pBac–EGFP (data not shown).

3.2. Expression of β3 $GABA_AR$ and α7 nAChR by transfection/infection

In order to test the applicability of the transfection/infection protocol on the expression of more challenging proteins than EGFP, two Ligand-Gated Ion Channels, the β3 subunit of the rat γ-amino butyric acid type A receptor ($GABA_AR$) (Kang et al., 2008) and the human α7 nicotinic acetylcholine receptor (α7-nAChR) (Anand et al., 1991) were cloned into vector pTI. *Sf*21 cells were transfected with pTI-(His_8-)β3 $GABA_AR$ (Fig. 4A) or with pTI-α7 nAChR (Fig. 4B) and cultured in the absence or presence of non-recombinant baculovirus. β3 $GABA_AR$ protein was pulled down using Talon Beads while α7 nAChR molecules were pulled down using the snake toxin alpha-bungarotoxin (α-bgtx) specific for α7 nAChR (Drisdel and Green, 2000). Western blots were performed using anti-β3 $GABA_AR$ specific antibody (Slany et al., 1995) and anti-α7 nAChR antibody (Benfante et al., 2011). Pronounced induction of β3 $GABA_AR$ expression after infection of cells transfected with pTI-(His_8-)β3 $GABA_AR$ could be observed (Fig. 4A). We could also see α7 nAChR expression after infection of cells transfected with pTI-α7 nAChR (Fig. 4B), and the observed affinity for alpha-bungarotoxin ensures the existence of a functional alpha-bungarotoxin binding domain in the expressed α7 nAChR protein (Wanamaker et al., 2003). Quantitation of Western Blots showed that a 7.4, 11.5, and 8.7-fold induction (relative to the expression achieved by simple transfection) could be obtained by using the transfection/infection protocol for EGFP, β3 $GABA_AR$ and α7 nAChR, respectively (Fig. 4C, $n = 3$). In summary, these results imply that the transfection/infection method using the pTI vector can be applied to several classes of proteins including those commonly regarded as "challenging", such as transmembrane proteins.

3.3. Co-transfected pTI vectors can be activated by a single infection step

A challenging step in heterologous protein expression is the production of soluble protein complexes. We therefore performed co-expression experiments in order to investigate whether co-transfected pTI vectors can be simultaneously activated by the same infection step. cDNA for EGFP, YFP and CFP was cloned into pTI. Upon transfection/infection, protein expression was analyzed by fluorescence microscopy using the appropriate filters. Like for EGFP, expression of YFP and CFP was strongly enhanced by baculovirus infection (data not shown). For cells transfected with pTI–CFP, signal was only observed with the CFP filter (Fig. 5A, CFP channel), whereas for cells transfected with pTI–YFP, fluorescence could exclusively be detected using the YFP filter (Fig. 5A, YFP channel). Upon co-transfection, however, signals obtained with either filter overlapped in most of the cells (Fig. 5A, merge), showing that both proteins can be over-expressed simultaneously using transfection/infection in *Sf*21 cells.

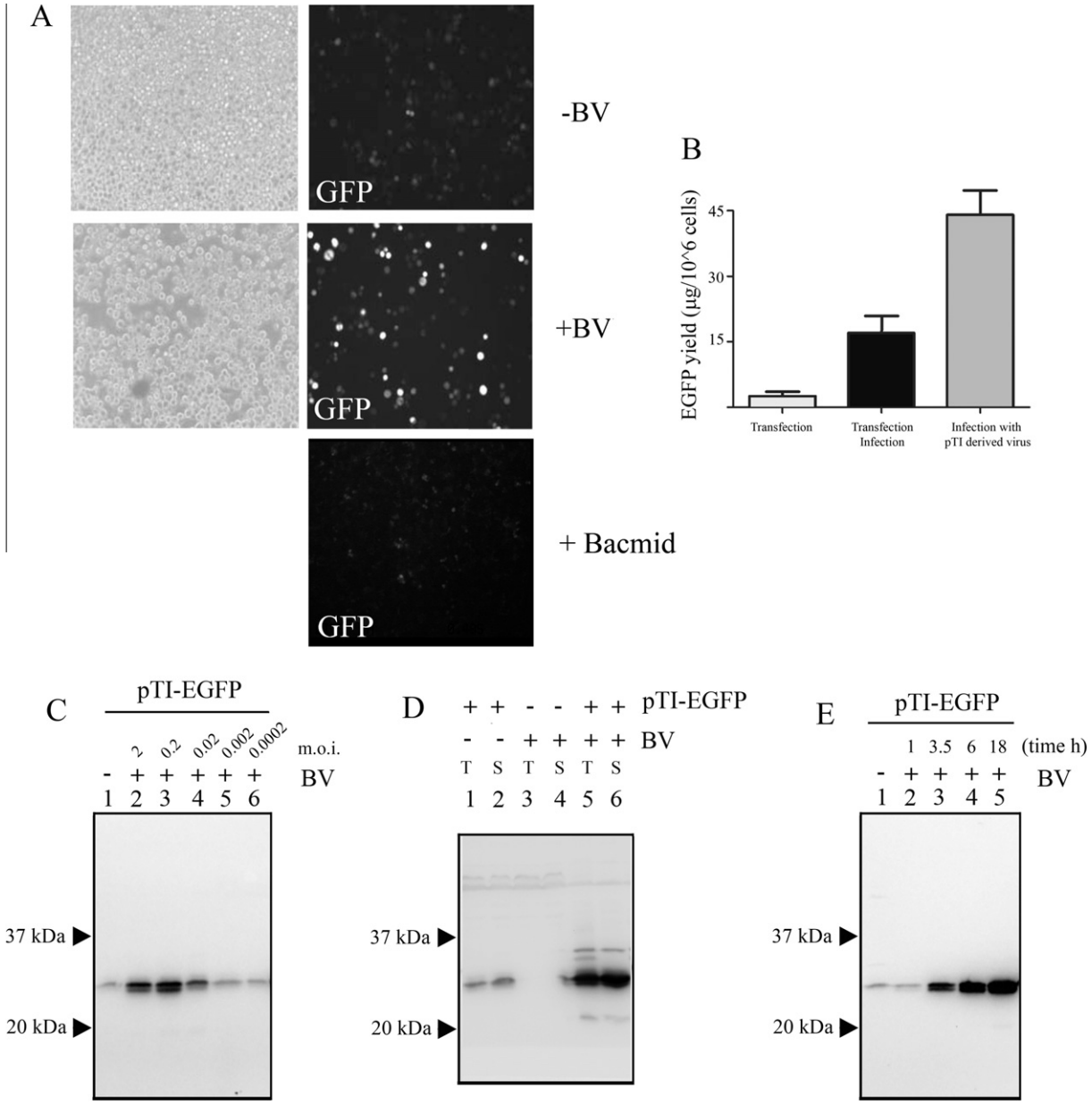

Fig. 3. Infection with non-recombinant baculovirus enhances protein expression after transient transfection. (A) EGFP expression is dependent on transfection/infection. EGFP cDNA was cloned into the vector pTI. *Sf*21 cells were transiently transfected with pTI–EGFP. After 14–20 h, non-recombinant baculovirus was added (+BV) to the cultures or omitted (−BV). Approx. 72 h after infection, EGFP expression was examined using an inverted fluorescence microscope (GFP channel, 20× magnification). As control, *Sf*9 cells were co-transfected with pTI–EGFP and BacMagic bacmid DNA and fixed after 5 days (+bacmid). Comparison of cell morphology between *Sf*21 cells transfected/infected (+BV) versus the only transfected ones (−BV) is shown in the bright field (20× magnification). (B) Yields of EGFP produced in *Sf*9 cells by transfection, by transfection/infection, or by infection with EGFP expressing virus that was generated from pTI by recombination. Quantitation was performed measuring the EGFP fluorescence emission of 2×10^6 cells as described in Section 2.4 ($n = 2$, error bars represent standard deviation). (C) *Sf*21 cells were transiently transfected with pTI–EGFP. Non-recombinant baculovirus was added (+BV) to the cultures at the indicated multiplicity of infection (m.o.i.) or omitted (−BV). Lysates were analyzed by Western Blot using anti-GFP antibody. (D) *Sf*21 cells were transiently transfected with pTI–EGFP and cultured in absence or presence of baculovirus. Total lysates (T) and soluble fractions (S) were analyzed as above. (E) *Sf*21 cells were transiently transfected with pTI–EGFP. Non-recombinant baculovirus was added (+BV) at the indicated time after the transfection, or omitted (−BV).

To further assess the versatility of the co-expression system, three vectors (pTI–YFP, pTI–EGFP, and pTI–CFP) were used for transfection/infection as described above. To avoid the problem of the EGFP cross-emission into the YFP and CFP channel, analysis was performed by FACS. Here, cells were gated for the expression of each of the chromophores (YFP, EGFP, and CFP) using the respective filters with the indicated cut-off (see supplemental methods). In Fig 5B, cells were always gated for one particular wavelength and the counts of the two other wavelengths were plotted on the *x*- and *y*-axis, respectively. As control, cells were transfected/infected with only one of the fluorescent proteins and gated for its wavelength. Here, fluorescence at the other two wavelengths was, if at all, only present at low intensity indicating cross-emission (Fig. 5B, upper panels, lower left sectors). Next, *Sf*21 cells were co-transfected with YFP, EGFP and CFP, followed by infection. When triple-transfected cells were gated for each wavelength, strong fluorescence at the other two wavelengths was predominantly observed (Fig. 5B, lower panels, upper right sectors).

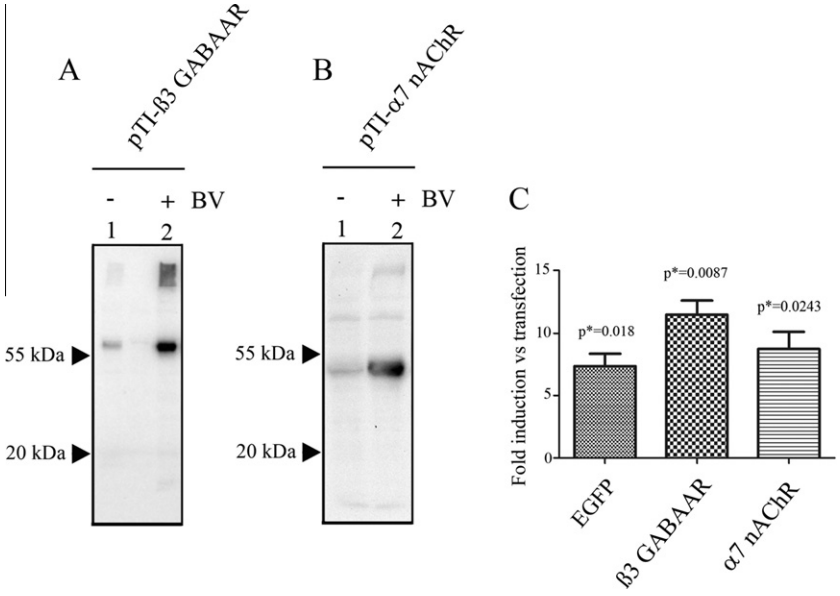

Fig. 4. Expression of β3 $GABA_AR$ and α7 nAChR by transfection/infection. (A) *Sf*21 cells were transfected with pTI-(His_8-)β3 $GABA_AR$. Non-recombinant baculovirus was added (+BV) at the indicated time after the transfection, or omitted (−BV). Protein expression was analyzed by Western blot using anti-β3 $GABA_AR$ specific antibody as described in the supplemental methods. (B) *Sf*21 cells were transfected with pTI-α7 nAChR and non-recombinant baculovirus was added (+BV), or omitted (−BV). Protein expression was analyzed by Western blot using an anti-α7 nAChR specific antibody. (C) Fold induction of protein expression after transfection/infection, relative to expression in absence baculovirus infection, was quantitated after Western Blotting ($n = 3$, error bars represent standard deviation).

The correlation of intensity of the plotted wavelengths is apparently linear. However, the fact that EGFP, CFP and YFP differ in their absorption and quantum yield (Patterson et al., 2001) prevents direct comparison of expression levels.

In summary, data from fluorescence microcopy and FACS clearly demonstrate that multiple pTI constructs can be expressed simultaneously in single cells, thereby making the transfection/infection protocol suited for screening of expression of multi-component protein complexes.

3.4. pTI is active in mammalian HEK293 cells

In addition to infection of insect cells, baculovirus is frequently used for gene delivery into a variety of mammalian cell types (Kost et al., 2005). Although mammalian cells are not permissive for baculovirus propagation, several viral promoters have been reported to be weakly recognized by host cell expression factors (Fujita et al., 2006). To assess whether our vectors are suited for protein expression in mammalian cells, we examined the activity of pTI upon transient transfection of HEK293 cells. Approx. 72 h after transfection, cells were fixed and analyzed using a fluorescence microscope. After transfection with pTI, no detectable fluorescence was observed in the cells while cells transiently transfected with pTI–EGFP exhibited clear fluorescence (Fig. 6). Western Blot using an anti-GFP antibody confirmed the fluorescence microscopy results (data not shown). This demonstrates that the pTI vector can be used for protein expression in both insect cells (*Sf*9 or *Sf*21) and mammalian (HEK293) cells without re-cloning.

4. Discussion

Insect cells are powerful hosts for the heterologous expression of recombinant proteins for multiple downstream applications. AcMNPV from the family of *Baculoviridae* facilitates both gene delivery into the cells and protein expression under the control of a group of exceptionally strong viral promoters. In many cases, protein expression in insect cells is straight-forward with high yields of correctly folded and processed proteins. However, some classes of proteins like transmembrane proteins, ion channels, kinases, multi-domain proteins, or protein complexes are challenging to overexpress in this heterologous system. Although several examples of transmembrane proteins that could be crystallized after expression in insect cells have been reported (Cherezov et al., 2007; Hibbs and Gouaux, 2011; Payandeh et al., 2011), production of the above-mentioned proteins remains notoriously difficult. For this specific class of proteins many different constructs (domain constructs, tags and mutants) need to be screened until a hit with the features suitable for structural studies (yield, oligomerisation, activity, sub-cellular localization, etc.) can eventually be found. For the expression of multi-protein complexes in insect cells, baculovirus carrying several constructs are commonly engineered, as testing construct combinations by co-infection with multiple viruses does not deliver reproducible results (Vijayachandran et al., 2011).

4.1. Transfection/infection is a rapid method for protein expression screening in insect cells

In this paper, we add the transfection/infection protocol to the repertoire of recombinant protein production technology. The method enables expression screening of multiple constructs or construct libraries in insect cells and combines advantages of transient transfection and baculovirus infection. The gene to be expressed is located on vector pTI, under the control of viral promoters that can be trans-activated by baculovirus infection (Fig. 2A).

We used the transfection/infection protocol to over-express the reporter proteins EGFP, CFP, and YFP, but also the transmembrane ion channels β3 $GABA_A$ receptor and α7 nAChR in small scale cultures of adherent insect cells (Figs. 3 and 4). The expression levels of EGFP per cell culture were found to be several times higher when transfection was followed by infection for 72 h (Fig. 3). Sim-

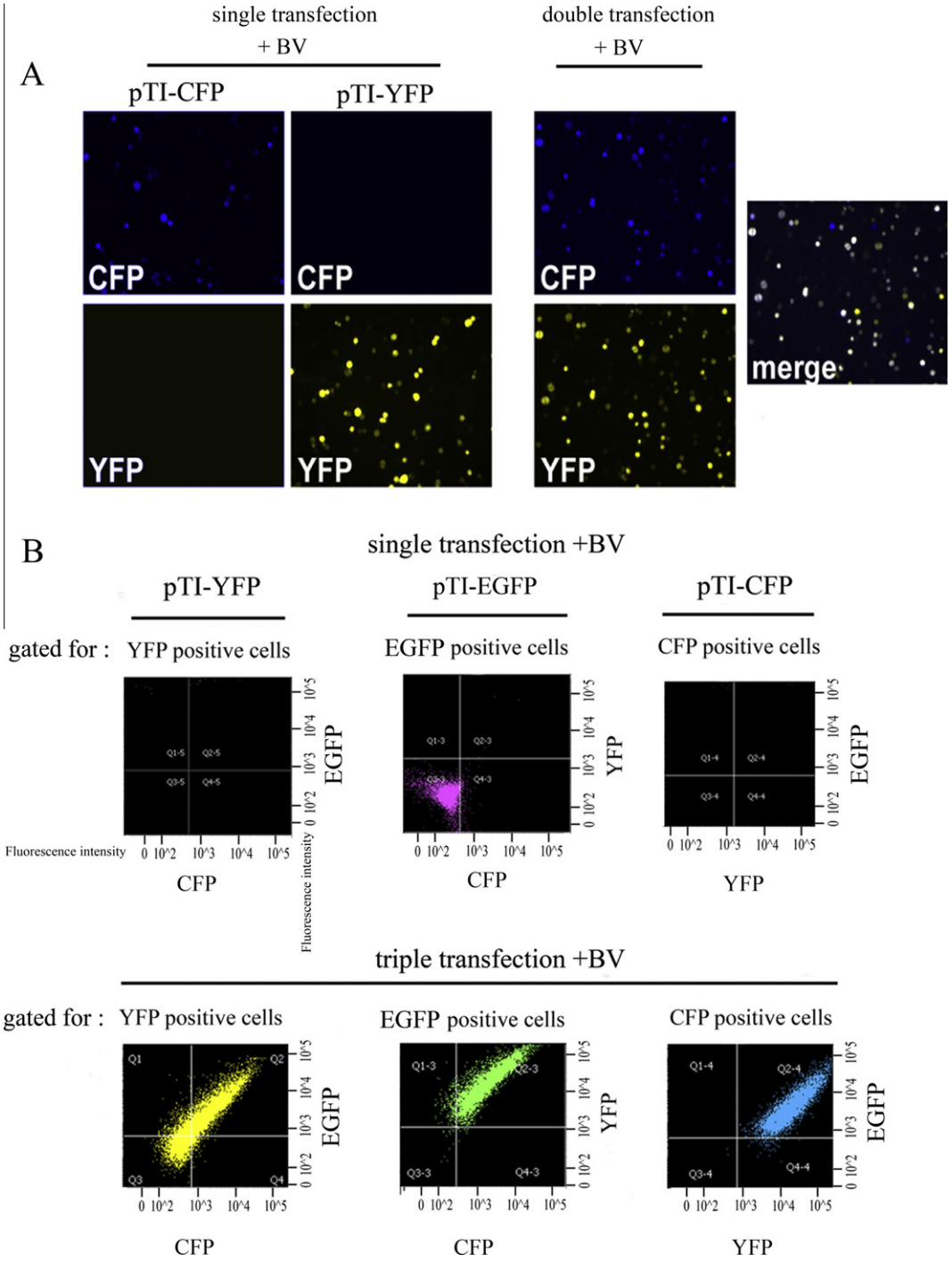

Fig. 5. Co-transfected pTI vectors can be transactivated by a single infection step. (A) cDNA for CFP and YFP was cloned into pTI to generate pTI–CFP and pTI–YFP. *Sf*21 cells were transiently transfected with one of the two constructs or with equimolar amounts of them. 14–20 h after transfection, non-recombinant baculovirus was added (+BV) to the cultures. Approx. 72 h later, CFP and YFP expression was examined using an inverted fluorescence microscope and analyzed using the indicated filter (20× magnification). The merge panel refers to the sample co-transfected/infected with both vectors and overlapping signals are shown in white. (B) Upper panels: pTI–YFP, pTI–EGFP or pTI–CFP were individually used for transfection/infection in *Sf*21. Approx. 72 h after infection, cells were trypsinized and sorted by FACS for the indicated fluorochrome. To determine cross-emission, fluorescence intensity (log scale, AU) of the two other chromophores is plotted on the *x*- and *y*-axis, respectively. Lower panels: *Sf*21 cells were co-transfected/infected with equimolar amounts of pTI–YFP, pTI–EGFP, and pTI–CFP. Triple-transfected cells were analyzed by FACS as above to measure simultaneous YFP, EGFP and CFP expression. Cells with high fluorescence of each wavelength appear in the top right sector.

ple co-transfection of pTI–EGFP and bacmid DNA, in contrast, induced increased expression only in a few cells (Fig. 3), suggesting that the infection step in the transfection/infection protocol can not be replaced by bacmid co-transfection. The yield of EGFP produced with transfection/infection in small scale in 4 days was in the microgram range.

The recombination regions present on pTI make the vector suitable for the generation of the recombinant baculovirus without further cloning (Fig. 2). When pTI–EGFP was recombined with baculovirus DNA, a culture infected with the resulting EGFP baculovirus produced 3 times more protein compared to the sample transfected/infected, consistent with the 30% transfection rate of

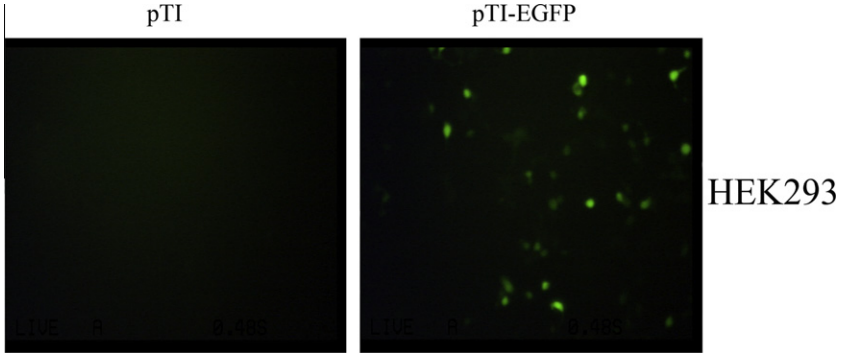

Fig. 6. pTI vector is active in mammalian HEK293 cells. HEK293 cells were transiently transfected with pTI or pTI–EGFP. 48–72 h later, cells were fixed and EGFP expression was examined using an inverted fluorescence microscope as described in the method section (20× magnification).

the insect cells and with a similar upregulation of protein expression by virus infection whether the virus was empty or contained the gene of interest. In addition, these data confirmed the expected ability of the pTI promoter cassette to drive expression when integrated into the viral genome (Fig. 3). The yield of EGFP produced with the pTI EGFP derived virus was is in the milligram/liter range, an order of magnitude suitable for structural studies and comparable with the yield of EGFP-expressing baculovirus that was generated using Bac-to-Bac.

4.2. Transfection/infection allows screening of co-expressed constructs and complexes

There is currently no ideal solution for rapid screening of co-expressing constructs using standard baculovirus techniques. We could demonstrate that three pTI vectors can simultaneously be used for transfection of insect cells and get activated simultaneously by the same baculovirus infection (Fig. 5). This marks a clear improvement to co-expression by mixing of recombinant virus, which is cumbersome and requires viral titer determination followed by testing of a variety of titer ratios (Vijayachandran et al., 2011). Co-transfection/infection is therefore especially applicable for the screening of (i) constructs for the expression of protein complexes, (ii) a library of mutants; or (iii) siRNAs libraries. Thus, we consider the transfection/infection method to have high potential for screening of co-expression of multiple proteins.

The protein expression repertoire can be further increased when recombinant baculovirus is used for the infection of transfected cells (Supplementary Fig. S2), so that additional proteins can be co-expressed by the baculovirus in *cis*. This feature can be employed for introducing, e.g. a further protein of a multi-complex, a chaperone essential for the folding of the protein of interest, an enzyme modifying the protein to express, etc.

4.3. Vector pTI enables shuttling between expression hosts

We showed that the promoter cassette of pTI is also active in HEK293 cells, rendering pTI a possible shuttle vector for insect and mammalian cells (Fig. 6). It has previously been reported that certain baculovirus promoters are weakly recognized by host cell factors after transduction of mammalian cells (Fujita et al., 2006). Since pTI carries the homologous sites for generating a recombinant baculovirus, not only the plasmid itself but also the derived recombinant virus could serve as vector for gene delivery into HEK cells, a method commonly known as "BacMam system" (Hofmann et al., 1995; Boyce and Bucher, 1996). In mammalian cells, gene delivery can be markedly increased using the BacMam system, especially in large suspension cultures of HEK293 (Condreay et al., 1999).

4.4. Transfection/infection is a useful strategy for expression screening

In Fig. 1, a comparison between three pipelines for expression screening of a construct library is depicted. In Fig. 1C, the screening follows the transfection/infection protocol. A construct library can conveniently be cloned into pTI via LIC. Following MiniPrep, each construct in pTI can immediately be used to transfect cells cultured in various multiwell formats. Protein production will then be induced by baculovirus transactivation. Transfection/infection requires fewer steps and a reduced number of baculoviruses to be tested than expression screening based on Bac-to-Bac (Fig. 1A) and Bac-Magic (Fig. 1B), respectively. It effectively allows filtering constructs prior to proceeding with generation of recombinant virus for up-scaled expression, if needed.

5. Conclusion

The transfection/infection protocol, together with vector pTI, provides excellent tools for the rapid investigation and comparison of protein (co-)expression of multiple constructs in a small scale in insect cells. Transfection/infection lacks the need of recombinant baculovirus generation and viral titer determination for protein expression screening, rendering the method time- and cost-effective. In single wells of a 6-well plate, the yield of protein produced is in the microgram range, which is sufficient for biochemical assays (e.g. analysis of folding, complex formation, activity, ligand binding, etc.), or preliminary structural analysis (quaternary structure determination, set up of purification protocols). When upscaling is desired, constructs in vector pTI can directly be used for virus generation without vector shuttling or re-cloning. Thus, the transfection/infection protocol constitutes a valuable supplement to established insect cells expression systems, in particular with respect to transmembrane proteins and multi-protein complexes.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jsb.2012.04.013.

References

Anand, R., Conroy, W.G., Schoepfer, R., Whiting, P., Lindstrom, J., 1991. Neuronal nicotinic acetylcholine receptors expressed in Xenopus oocytes have a pentameric quaternary structure. J. Biol. Chem. 266, 11192–11198.

van Anken, E., Sanders, R.W., Liscaljet, I.M., Land, A., Bontjer, I., et al., 2008. Only five of 10 strictly conserved disulfide bonds are essential for folding and eight for function of the HIV-1 envelope glycoprotein. Mol. Cell Biol. 19, 4298–4309.

Benfante, R., Antonini, R.A., DePizzol, M., Gotti, C., Clementi, F., et al., 2011. Expression of the α7 nAChR subunit duplicate form (CHRFAM7A) is down-regulated in the monocytic cell line THP-1 on treatment with LPS. J. Neuroimmunol. 230, 74–84.

Berger, I., Fitzgerald, D.J., Richmond, T.J., 2004. Baculovirus expression system for heterologous multiprotein complexes. Nat. Biotechnol. 22, 1583–1587.

Boyce, F.M., Bucher, N.L., 1996. Baculovirus-mediated gene transfer into mammalian cells. Proc. Natl. Acad. Sci. USA 93, 2348–2352.

Buchs, M., Kim, E., Pouliquen, Y., Sachs, M., Geisse, S., Mahnke, M., et al., 2009. High throughput protein expression and purification. Methods Mol. Biol. 498, 199–227.

Cherezov, V., Rosenbaum, D.M., Hanson, M.A., Rasmussen, S.G.F., Thian, F.S., et al., 2007. High-resolution crystal structure of an engineered human beta2-adrenergic G protein-coupled receptor. Science 318, 1258–1265.

Condreay, J.P., Witherspoon, S.M., Clay, W.C., Kost, T.A., 1999. Transient and stable gene expression in mammalian cells transduced with a recombinant baculovirus vector. Proc. Natl. Acad. Sci. USA 96, 127–132.

Crameri, A., Whitehorn, E.A., Tate, E., Stemmer, W.P., 1996. Improved green fluorescent protein by molecular evolution using DNA shuffling. Nat. Biotechnol. 14, 315–319.

Drisdel, R.C., Green, W.N., 2000. Neuronal alpha-bungarotoxin receptors are alpha7 subunit homomers. J. Neurosci. 20, 133–139.

Fujita, R., Matsuyama, T., Yamagishi, J., Sahara, K., Asano, S., et al., 2006. Expression of *Autographa californica* multiple nucleopolyhedrovirus genes in mammalian cells and upregulation of the host beta-actin gene. J. Virol. 80, 2390–2395.

Hanson, M.A., Brooun, A., Baker, K.A., Jaakola, V.-P., Roth, C., et al., 2007. Profiling of membrane protein variants in a baculovirus system by coupling cell-surface detection with small-scale parallel expression. Protein Expres. Purif. 56, 85–92.

Hibbs, R.E., Gouaux, E., 2011. Principles of activation and permeation in an anion-selective Cys-loop receptor. Nature 474, 54–60.

Hofmann, C., Sandig, V., Jennings, G., Rudolph, M., Schlag, P., et al., 1995. Efficient gene transfer into human hepatocytes by baculovirus vectors. Proc. Natl. Acad. Sci. USA 92, 10099.

Jarvis, D.L., 2009. Baculovirus-insect cell expression systems. Method. Enzymol. 463, 191–222.

Jester, B.C., Drillien, R., Ruff, M., Florentz, C., 2011. Using Vaccinia's innate ability to introduce DNA into mammalian cells for production of recombinant proteins. J. Biotechnol. 156, 211–213.

Kang, S.U., Fuchs, K., Sieghart, W., Lubec, G., 2008. Gel-based mass spectrometric analysis of recombinant GABA(A) receptor subunits representing strongly hydrophobic transmembrane proteins. J. Proteome Res. 7, 3498–3506.

Kost, T.A., Condreay, J.P., Jarvis, D.L., 2005. Baculovirus as versatile vectors for protein expression in insect and mammalian cells. Nat. Biotechnol. 23, 567–575.

Kovacs, G.R., Guarino, L.A., Graham, B.L., Summers, M.D., 1991. Identification of spliced baculovirus RNAs expressed late in infection. Virology 185, 633–643.

Lo, H.R., Chou, C.C., Wu, T.Y., Yuen, J.P.Y., Chao, Y.C., 2002. Novel baculovirus DNA elements strongly stimulate activities of exogenous and endogenous promoters. J. Biol. Chem. 277, 5256–5264.

Luckow, V.A., Lee, S.C., Barry, G.F., Olins, P.O., 1993. Efficient generation of infectious recombinant baculoviruses by site-specific transposon-mediated insertion of foreign genes into a baculovirus genome propagated in *Escherichia coli*. J. Virol. 67, 4566–4579.

Luna-Vargas, M.P.A., Christodoulou, E., Alfieri, A., van Dijk, W.J., Stadnik, M., et al., 2011. Enabling high-throughput ligation-independent cloning and protein expression for the family of ubiquitin specific proteases. J. Struct. Biol. 175, 113–119.

Mancia, F., Love, J., 2011. High throughput platforms for structural genomics of integral membrane proteins. Curr. Opin. Struc. Biol. 21, 517–522.

Murphy, C.I., Piwnica-Worms, H., Grünwald, S., Romanow, W.G., Francis, N., et al. 2004. Overview of the baculovirus expression system, in: Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., Struhl, K. (Eds.), Current protocols in molecular biology. John Wiley & Sons, Somerset, NJ, Chapter 16, Unit 16.9.

Patterson, G., Day, R.N., Piston, D., 2001. Fluorescent protein spectra. J. Cell Sci. 114, 837.

Payandeh, J., Scheuer, T., Zheng, N., Catterall, W.A., 2011. The crystal structure of a voltage-gated sodium channel. Nature 475, 353–358.

Possee, R.D., Hitchman, R.B., Richards, K.S., Mann, S.G., Siaterli, E., et al., 2008. Generation of baculovirus vectors for the high-throughput production of proteins in insect cells. Biotechnol. Bioeng. 101, 1115–1122.

Pullen, S.S., Friesen, P.D., 1995. Early transcription of the ie-1 transregulator gene of Autographa californica nuclear polyhedrosis virus is regulated by DNA sequences within its 5' noncoding leader region. J. Virol. 69, 156–165.

Scheper, G.C., Vries, R.G., Broere, M., Usmany, M., Voorma, H.O., et al., 1997. Translational properties of the untranslated regions of the p10 messenger RNA of *Autographa californica* multicapsid nucleopolyhedrovirus. J. Gen. Virol. 78 (Pt 3), 687–696.

Slany, A., Zezula, J., Tretter, V., Sieghart, W., 1995. Rat beta 3 subunits expressed in human embryonic kidney 293 cells form high affinity [35S]t-butylbicyclo-phosphorothionate binding sites modulated by several allosteric ligands of gamma-aminobutyric acid type A receptors. Mol. Pharmacol. 48, 385–391.

Todd, J.W., Passarelli, A.L., Lu, A., Miller, L.K., 1996. Factors regulating baculovirus late and very late gene expression in transient-expression assays. J. Virol. 70, 2307–2317.

Trowitzsch, S., Bieniossek, C., Nie, Y., Garzoni, F., Berger, I., 2010. New baculovirus expression tools for recombinant protein complex production. J. Struct. Biol. 172, 9.

Vaughn, J.L., Goodwin, R.H., Tompkins, G.J., McCawley, P., 1977. The establishment of two cell lines from the insect Spodoptera frugiperda (Lepidoptera; Noctuidae). In vitro 13, 213–217.

Vijayachandran, L.S., Viola, C., Garzoni, F., Trowitzsch, S., Bieniossek, C., et al., 2011. Robots, pipelines, polyproteins: enabling multiprotein expression in prokaryotic and eukaryotic cells. J. Struct. Biol. 175, 198–208.

Wanamaker, C.P., Christianson, J.C., Green, W.N., 2003. Regulation of nicotinic acetylcholine receptor assembly. Ann. N.Y. Acad. Sci. 998, 66–80.

Zhao, Y., Chapman, D.A.G., Jones, I.M., 2003. Improving baculovirus recombination. Nucleic Acids Res. 31, E6-6.