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New baculovirus expression tools for recombinant protein complex production

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ABSTRACT

Most eukaryotic proteins exist as large multicomponent assemblies with many subunits, which act in concert to catalyze specific cellular activities. Many of these molecular machines are only present in low amounts in their native hosts, which impede purification from source material. Unraveling their structure and function at high resolution will often depend on heterologous overproduction. Recombinant expression of multiprotein complexes for structural studies can entail considerable, sometimes inhibitory, investment in both labor and materials, in particular if altering and diversifying of the individual subunits are necessary for successful structure determination. Our laboratory has addressed this challenge by developing technologies that streamline the complex production and diversification process. Here, we review several of these developments for recombinant multiprotein complex production using the MultiBac baculovirus/insect cell expression system which we created. We also addressed parallelization and automation of gene assembly for multiprotein complex expression by developing robotic routines for multigene vector generation. In this contribution, we focus on several improvements of baculovirus expression system performance which we introduced: the modifications of the transfer plasmids, the methods for generation of composite multigene baculoviral DNA, and the simplified and standardized expression procedures which we delineated using our MultiBac system.

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1. Introduction

There is growing evidence to support the concept of the eukaryotic cell as a collection of multisubunit protein machines. These assemblies participate in most cellular activities such as replication, transcription, gene regulation, RNA metabolism, translation and many other processes (Alberts, 1998; Nie et al., 2009; Parrish et al., 2006; Rual et al., 2005; Wahl et al., 2009). Although some complexes can be isolated from cells, many other biologically important assemblies are present in very low amounts and, if at all, can only be purified with enormous investments from native source material. Therefore, recombinant protein production techniques have become increasingly indispensable for studying these complexes at the molecular level (Bieniossek and Berger, 2009; Nie et al., 2009; Palomares et al., 2004).

Eukaryotic protein complexes often contain many subunits which depend on each other for proper folding and solubility. If produced separately, their activity may be compromised due to the absence of key interaction partners. Overexpression in *Escherichia coli* is the method most commonly used to produce recombinant proteins for structural studies, and significant advances have been made in the field of recombinant protein complex production in this cheap and versatile host (Bieniossek et al., 2009; Perrakis and Romier, 2008; Romier et al., 2006; Tan et al., 2005; Tolia and Joshua-Tor, 2006). However, many eukaryotic proteins and their complexes may fail to produce properly in *E. coli*, due to particular requirements for chaperone systems or post-translational modifications that *E. coli* cannot support. Overproduction of such specimens then necessitates a eukaryotic expression system.

The baculovirus/insect cell system (also called baculovirus expression vector system, BEVS) more recently has gained particular prominence for producing such eukaryotic targets. Methods and vectors for generating recombinant baculoviruses for infecting insect cell cultures have emerged more than 20 years ago when the first foreign gene expression with a baculovirus was demonstrated (Smith et al., 1983). BEVS is robust and well suited for producing eukaryotic proteins for many applications including the production of pharmaceuticals, pesticides, vaccines and more recently of gene therapy vectors (Kost et al., 2005). A number of features of BEVS

Abbreviations: *Ac*NPV, *Autographa californica* nuclear polyhedrosis virus; BAC, bacterial artificial chromosome; BEVS, baculovirus expression vector system; DNA, deoxyribonucleic acid; ds, double stranded; *E. coli*, *Escherichia coli*; YFP, yellow fluorescent protein; kb, kilo bases; MOI, multiplicity of infection; ORF, open reading frame; p10, p10 baculoviral late promoter; pa, proliferation arrest; PCR, polymerase chain reaction; pfu, plaque forming units; polh, polyhedrin baculoviral very late promoter; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SLIC, sequence and ligation independent cloning; Sf21, *Spodoptera frugiperda* cell line 21; ss, single stranded; TFIID, general transcription factor IID; VLP, virus-like particle.

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add to the advantages of this method. Importantly, baculoviruses do not replicate in eukaryotic cells besides their insect cell hosts, therefore, insect cell expression in the laboratory does not require particular safety measures (Murphy and Piwnica-Worms, 1994a,b; Murphy et al., 2004). Large proteins with several hundred kilodalton molecular weight can be produced by BEVS, and the proteins are often authentically processed. If required, insect cell cultures are easily grown in bioreactors (Weber et al., 2002). However, cultures grown in regular Erlenmeyer shaker flasks often yield 1–100 mg per 1 liter insect cell culture, which is sufficient for high-resolution structural biology projects including X-ray crystallography (Fitzgerald et al., 2006, 2007; Bieniossek et al., 2008). To date, hundreds of eukaryotic proteins, mainly single proteins or domains, have been successfully produced using baculoviral expression vector systems (Kost and Condreay, 1999; Kost et al., 2005; Possee, 1997).

Recent genome- and proteome-wide studies have led to biological research efforts increasingly focusing on large multiprotein complexes. As a consequence, baculovirus expression systems for producing eukaryotic multiprotein assemblies have become a method of choice in many laboratories. However, a technical drawback of the baculovirus/insect cell system was the lack of straightforward and easy-to-implement procedures to generate recombinant baculoviruses containing many foreign genes. Furthermore, once a composite baculovirus was constructed, it could not be modified easily, partly due to its large size (>130 kb). Exchange of genes and/or diversifying them by truncation or mutagenesis, however, is often a prerequisite for successful structural studies especially by X-ray crystallography. Proteins often need to be extensively truncated or mutated before they can be coaxed into forming highly ordered single crystals. We have developed strategies that address these shortcomings of BEVS. We implemented methods that improve protein production and facilitate protein diversification. Here, we review strategies that allow rapid and flexible multiprotein production, and furthermore are adaptable for high throughput approaches in a robotic setup.

2. Background

Baculoviruses, such as the *Autographa californica* nuclear polyhedrosis virus (*Ac*NPV) of the Baculoviridae family, have three distinct classes of genes, which are expressed in a chronologically regulated, sequential manner (Smith et al., 1983; Pennock et al., 1984). The first class of genes comprises the early genes, which have host-like promoters and can be transcribed by the host transcriptional machinery (Friesen, 1997). After the onset of viral DNA replication the late genes are expressed, such as the p10-coding gene, which require the virus-encoded transcriptional machinery (Lu and Miller, 1997; Passarelli and Guarino, 2007). Closer to the end of the infectious cycle the very late genes are expressed which code for several proteins including polyhedrin. Polyhedrin is the most abundantly produced protein and forms the characteristic polyhedra or occlusion bodies in the nuclei of insect cells infected with wild-type virus. Although late and very late promoter elements share many similarities, an additional downstream sequence, which leads to extremely high levels of transcription, is present in very late promoters (Ooi et al., 1989).

Heterologous genes driven by *Ac*NPV late and very late promoters are typically abundantly expressed (Roy et al., 1997). This circumstance was originally exploited for producing the first recombinant baculoviruses by standard homologous recombination procedures using transfer plasmids carrying the foreign genes. These baculoviruses were designed to express chimeric genes consisting of the polyhedrin promoter and the foreign coding sequence. Expression cassettes comprising the gene of choice flanked by baculoviral sequences of the polyhedrin region were provided on the transfer plasmids and integrated into the circular baculovirus genome by homologous recombination in *Spodoptera frugiperda* insect cells (usually Sf9 or Sf21 cell lines). Integration occurred into the polyhedrin locus, thereby eliminating the native polyhedrin gene, and thus giving rise to occlusion-incompetent recombinants. A recombination frequency of ~0.1% and a tedious isolation procedure of recombinant clones by their distinctive occlusion-negative plaque phenotype (visualized in plaque assay), however, made the integration process of foreign genes laborious and difficult.

Integration of DNA fragments into the baculoviral genome was significantly improved by using linearized rather than circular baculoviral DNA in the co-transfection experiment with the transfer plasmid harboring the gene(s) of choice (Kitts et al., 1990). Homology regions present on the baculoviral DNA and the transfer plasmid allowed integration of the expression cassettes via recombination within the insect cell. Heterologous gene products were only produced from re-circularized, replication competent viral DNA. This strategy increased the efficiency of recombinant baculovirus production from ~0.1% to ~20%. Later, this approach was further improved by using not only one but several restriction sites for linearization, thereby reducing background. One restriction site was placed within an essential viral gene, which was thus truncated. The missing piece (i.e. a complete gene) was then replenished from the transfer plasmid upon productive homologous recombination. Multiple-site linearization of parental virus DNA and concomitant functional inactivation of this essential viral gene lead to an increase in efficiency of recombinant virus production to over 90% (Kitts and Possee, 1993). A number of companies undertook to commercialize linearized baculoviruses and the corresponding transfer plasmids (Pharmingen Baculogold, Novagen BacVector series, OET FlashBac systems and others). Still, the baculovirus plaque assay to identify positive recombinants remained an essential part of the method, somewhat complicating its handling.

An elegant way to eliminate the tedious plaque assay for clonal separation and purification of recombinant viruses relies on *in vivo* bacterial transposition (Luckow et al., 1993). Here, baculoviral genomic DNA isolated from native virus was engineered into an artificial bacterial chromosome (BAC) containing a resistance marker and a single-copy bacterial origin of replication. Integration of DNA fragments into this BAC was accomplished *in vivo* via a Tn7 attachment site embedded in a *lacZα* gene on the BAC (Invitrogen, Bac-to-Bac). Recombinant BACs could be identified in their *E. coli* hosts by fast and convenient blue/white screening of bacterial clones harboring the BAC. Foreign genes flanked by the Tn7L and Tn7R sequence elements of the Tn7 transposon system, were provided on the transfer plasmid. Development of a bicistronic transfer vector, pFastBacDUAL, facilitated sequential sub-cloning of two foreign genes into two separate cassettes for co-expression. A helper plasmid provided the Tn7 transposon enzyme complex for catalyzing the transposition event. This Tn7 transposition-based gene integration principle and its more recent improvements probably remain most widely used in the community to date (Airenne et al., 2003; Berger et al., 2004; Laitinen et al., 2005).

Two further approaches to generate recombinant baculoviruses by transposition were described. In an *in vitro* transposition system (BaculoDirect), a gene of choice is transferred from a plasmid into viral DNA utilizing purified transposase. Upon transposition, a negative selection marker gene is eliminated from the parental viral DNA, thus allowing only insect cells transfected with recombined viral DNA to survive. In an alternative approach, viral DNA carrying a lethal mutation in a gene product (ORF1629) essential for virus replication is propagated in *E. coli* as a BAC and purified. A recombination event in insect cells co-transfected with the mutated baculovirus genome and a transfer plasmid carrying the gene of interest and the wild-type viral ORF, reconstitutes the essential

gene activity upon integration into the viral DNA (Zhao et al., 2003). In both cases tedious plaque assays are in theory no longer necessary. Apart from purifying clonal viral populations, the plaque assay is also commonly used to determine viral titers, i.e. the number of infectious viral particles (plaque forming units, pfu) present in a defined volume of viral supernatant. Also for this purpose, useful alternatives to the time intensive (5–7 days) plaque assay were developed based on an immunological assay or a PCR reaction, which can also be used on automated platforms (Bahia et al., 2005; Chambers et al., 2004; Kitts and Green, 1999; Kwon et al., 2002; Lo and Chao, 2004; Shen et al., 2002).

Initially, BEVS was used mainly to produce single proteins or protein domains. Useful concepts for simultaneously integrating many genes into a single baculovirus were largely lacking. A few rather make-shift transfer plasmids were commercially available (Pharmingen, Novagen), that offered single restriction sites in three, or four, expression cassettes to serially subclone genes of choice. These plasmids, themselves already around 10 kb in size, were inconvenient to use in particular if large genes needed to be integrated. In addition, they did not offer simple means to exchange or alter individual genes easily once the vector was assembled, thus severely constraining their utility. An alternative way to produce complexes is by co-infecting insect cells with several recombinant baculoviruses at the same time, with each virus providing one or two heterologous genes encoding for subunits of the complex of choice. This strategy certainly has its merit for complex production in small-scale, which may be sufficient for many biochemical analyses. Reproducible large-scale production, in contrast, is a serious challenge with this method, in particular if the complex contains many subunits and therefore requires many viruses for simultaneous co-infection. All viruses need to be produced and maintained at high titer simultaneously. Even then, it is difficult to ascertain in the experiment if all cells are infected with all viruses at the same ratio in the culture. In short, co-infection is not practical for reproducible complex productions on the scale required for more ambitious structural biology projects aimed at complex structure elucidation.

Complex production from a single baculovirus, which provides all genes required, is a viable alternative to co-infection experiments using many different viruses. Evidence suggested that multigene expression from a single baculovirus indeed is the superior method for complex production (Bertolotti-Ciarlet et al., 2003; Miller, 1988; Roy et al., 1997). Virus-like particles, for instance, were produced successfully in this way (Belyaev and Roy, 1993; Emery and Bishop, 1987; Noad and Roy, 2003). A prerequisite for the multigene baculovirus strategy for structural biology of complex eukaryotic systems is that the assembly of the multigene baculovirus be quick and efficient. Likewise, simple means needed to be put in place to allow for rapid exchange and alteration of genes encoding for individual subunits. Ideally, these changes implemented should be compatible with automated procedures, which are becoming increasingly indispensable in structural biology to handle the throughput required.

We addressed several of these issues by creating the MultiBac system for expression of eukaryotic multiprotein complexes in insect cells (Berger et al., 2004). We have since improved the system and protocols used with a particular view to structural biology (Fitzgerald et al., 2006, 2007; Bieniossek et al., 2008; Bieniossek and Berger 2009).

3. The MultiBac system

The MultiBac system utilizes an engineered *Ac*NPV baculovirus genome derived from the Tn7-based BAC variant described above (Luckow and Summers, 1988). The MultiBac baculoviral genome, like its progenitor, is also propagated as a bacterial artificial chromosome in *E. coli* cells, and contains the F factor as a (mostly) single copy origin of replication (occasionally, two copies of the same DNA with an F origin may exist in the same cell). MultiBac utilizes a Tn7 attachment site embedded in a *lacZ*α gene for integrating foreign genes, via specially designed multigene transfer plasmids into the baculoviral genome (Fig. 1A). Successful integration of expression cassettes leads to disruption of the *lacZ*α gene and positive clones are selected by blue/white screening. We further engineered a second entry site into the BAC for utilizing the Cre–LoxP recombination system. The system is based on LoxP imperfect inverted repeats which can be present on different DNA molecules (Ghosh and Van Duyne, 2002). These LoxP repeats are then recognized and combined in a site-specific recombination reaction by Cre recombinase, leading to fusion of the DNA molecules. To access this site in the MultiBac BAC, we created a second transfer plasmid (pUCDM) with a conditional origin of replication (derived from R6 Kγ phage). We carried out recombination of the MultiBac BAC and this transfer plasmid *in vivo* in a cell line we created (DH10MultiBac^{Cre}). These cells provide the MultiBac BAC, a plasmid for expressing Cre-recombinase, and, a second helper plasmid. This helper plasmid provides the Tn7 transposon complex for accessing the Tn7 site on the same MultiBac BAC (Berger et al., 2004).

The MultiBac baculovirus contains modifications to improve protein production. We eliminated the baculoviral genes *v-cath* and *chi*A by ET recombination (Berger et al., 2004; Muyrers et al., 2004) and in the process also integrated the said LoxP imperfect repeat sequence (Fig. 1B). *V-cath* codes for a viral protease which is activated upon cell death by a process depending on the juxtaposed gene, *chiA* (Hom and Volkman, 2000). Deletion of the protease from a *Bombyx mori* polyhedrosis virus was shown to improve protein production (Suzuki et al., 1997). Expression trials with our modified MultiBac virus showed a remarkable reduction of proteolytic breakdown of overproduced proteins (Berger et al., 2004). Interestingly, it also appeared as if the onset of cell lysis caused by the viral infection would be considerably delayed as compared to other baculoviruses available at the time, resulting in benefits to the heterologous product (Berger et al., 2004; Bieniossek and Berger, 2009). In fact, several commercial suppliers integrated these beneficial deletions (and others) into their BEVS (Novagen, OET) more recently.

3.1. MultiBac 2004: 1st generation transfer plasmids

For multiprotein expression, we engineered modular transfer plasmids specifically suited for multigene integration. The first generation of the MultiBac system consisted of two such modular transfer plasmids, pFBDM and pUCDM (Fig. 1B). pFBDM was derived from pFastBacDUAL (Invitrogen) and has Tn7 transposition sequences (Tn7R, Tn7L) and an origin of replication (ColE1) that allows propagation in standard *E. coli* cloning strains (such as TOP10, DH5α and HB101). pUCDM, on the other hand, has a LoxP recombination site and a conditional origin of replication derived from the phage R6 Kγ. Due to the conditional origin of replication, pUCDM requires for its propagation the presence of the *pir* gene product in special *E. coli* strains, such as BW23473 or BW23474 (Metcalf et al., 1994). Both pFBDM and pUCDM contained identical dual expression cassettes driven by polh and p10 viral promoters, as well as a so-called multiplication module. This multiplication module consists of a set of unique restriction enzyme sites in between and flanking the expression cassettes. These restriction sites were designed to facilitate iterative expansion of the expression cassettes to accommodate a theoretically unlimited number of genes in pFBDM and pUCDM (Berger et al., 2004).

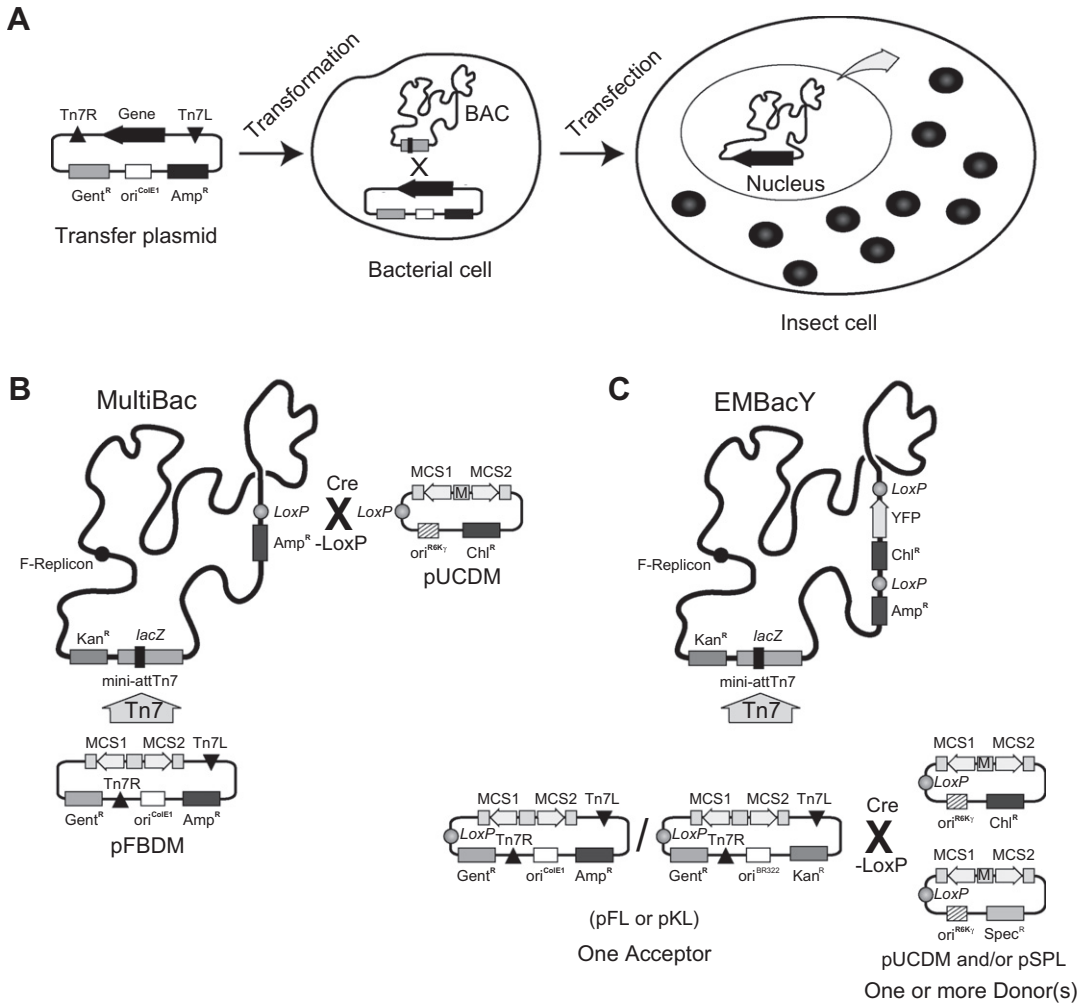

Fig. 1. The MultiBac System. (A) The principle of protein production by BEVS relying on Tn7 transposition is shown. The gene of interest, present on a transfer vector, is integrated via Tn7 transposition into a baculovirus genome maintained as a BAC in special *E. coli* cells. Composite BAC with the integrated gene of interest is isolated from the bacterial host and used to transfect insect cell cultures, often resulting in high-level heterologous protein production. (B) Central to protein expression by the MultiBac system is a BAC carrying a minimal Tn7 attachment site embedded in a *lacZα* gene and the LoxP imperfect inverted repeat sequence (gray sphere). Both sites are used for gene integration via transfer plasmid constructs (here pFBDM and pUCDM). Baculoviral genes *v-cath* and *chiA*, coding for a cathepsin protease and a chitinase, were eliminated, and the LoxP imperfect inverted repeat introduced together with an ampicillin resistance marker instead of *v-cath*. Derivatives of pUCDM are integrated *in vivo* via Cre recombination (marked by cross). Derivatives of pFBDM are integrated via Tn7L and Tn7R transposition sequences (black triangles) into the Tn7 attachment site (gray arrow). Expression cassettes can be generated using multiple-cloning sites (MCS1 and MCS2) and a so-called multiplication module (M) for expression cassette multiplication. Origins of replication (ColE1, R6 Kγ and F-replicon) are indicated. Genes mediating resistance to kanamycin (Kan^R), chloramphenicol (Chl^R), gentamycin ($Gent^R$), spectinomycin ($Spec^R$) and ampicillin (Amp^R) are shown as boxes. Gentamycin is used for selecting composite BACs upon productive Tn7 recombination. (C) EMBacY is a more recent BAC constructed by integration of an expression cassette for enhanced YFP production via Cre recombination. Expression of the YFP-coding gene (arrow marked YFP) is under control of the polh promoter and allows for efficient monitoring of virus performance and expression of other heterologous proteins driven by polh from the same BAC. All transfer plasmids utilized in conjunction with EMBacY contain a LoxP sequence. Assembly of multigene transfer plasmids is performed by using the MCS and the multiplication module to integrate genes, and subsequent Cre-mediated fusion of Acceptors (derivatives of pFL or pKL) and Donors (derivatives of pUCDM and/or pSPL) *in vitro* prior to Tn7 transposition into EMBacY. Acceptors pFL and pKL differ in the origin of replication (ColE1 or BR322, respectively) and the resistance marker (ampicillin or kanamycin, respectively). pSPL and pUCDM are identical except for the resistance marker (spectinomycin or chloramphenicol, respectively). Derivatives of pUCDM and pSPL can be used separately or simultaneously for Cre–LoxP fusion with derivatives of either pFL or pKL.

The concept of modular assembly was likewise extended to the integration of expression cassettes from pFBDM derivatives and/or pUCDM derivatives into the recipient MultiBac baculoviral genome. Integration could be carried out *in vivo* via Cre recombination and/or Tn7 transposition either simultaneously or sequentially in $DH10MultiBac^{Cre}$ cells, with the Tn7 transposon complex and Cre recombinase provided on two helper plasmids in *trans* (Berger et al., 2004). This explains also the need for the conditional origin present on pUCDM. During Tn7 transposition, only the DNA in between the Tn7L and Tn7R sites is integrated into the MultiBac BAC, and the ColE1 origin of replication, which is located elsewhere on pFBDM, is not. The Cre reaction, in contrast, results in plasmid fusion, which leads to the integration of the entire pUCDM derivative, including the replication origin, into the LoxP site on the MultiBac BAC. The R6 Kγ origin is not recognized as a replicon in $DH10MultiBac^{Cre}$ cells, therefore, the copy number of the composite MultiBac BAC remains under control of the F factor.

The Tn7 transposition site is embedded in a *lacZα* gene allowing the selection of positive MultiBac recombinants by blue/white screening. Since pUCDM carries a chloramphenicol resistance marker gene, productive MultiBac recombinants can be selected by challenging with this antibiotic on the selection plate (Berger et al., 2004). For virus production, we then used the isolated composite MultiBac multigene baculoviral DNA for transfecting Sf21 cells (Sf9 cells or others can likewise be utilized).

Due to its modular nature, the MultiBac system already in its original conception was adaptable to combinatorial applications for protein complex production (Berger et al., 2004). Further, low

expression levels of a particular protein subunit could be compensated for by introducing multiple copies of the same gene by using the multiplication module. The MultiBac system also allows for the combinatorial co-synthesis of modifying enzymes, such as kinases or phosphatases and their substrates, in order to enable post-translational modifications of expressed gene products (Fitzgerald et al., 2007).

3.2. MultiBac 2006: 2nd generation transfer plasmids

While useful beyond the state-of-the-art for multiprotein complex expression at that time, certain shortcomings of our system nevertheless soon became evident, particularly when we became interested in possibly automating multigene assembly. We found that the concept of the multiplication module still lacked sufficient flexibility as it relied on cumbersome restriction enzyme reactions and ligations. Also, the assembly of the multigene baculoviral genome was dependent on two *in vivo* events in the DH10MultiBac^{Cre} cells, namely the Cre–LoxP fusion and the Tn7 transposition. Furthermore, due to the size of the BAC being too large for sequencing or standard restriction mapping (>130 kb), it was not trivial to verify productive integration events into the LoxP site. However, we realized that we could instead actually use the Cre–LoxP reaction before the Tn7 integration step into the baculoviral genome, simply by providing a LoxP site somewhere in between the Tn7L and Tn7R sites on the pFBDM transfer vector. By integrating pUCDM derivatives into such a modified pFBDM variant rather than directly into the virus, the resulting fusion plasmid could be verified easily by standard procedures (PCR, sequencing, restriction mapping). The entire region between the Tn7L and Tn7R sites containing the complete pUCDM construct and the genes present on pFBDM, would then be integrated into the MultiBac BAC by a single *in vivo* Tn7 reaction (Fig. 1C). When we used this new approach, we also noticed that we sometimes integrated two rather than one copy of the pUCDM derivative into the pFBDM plasmid fitted with the LoxP site. This multiple insertion would usually occur when we used a comparatively large excess of pUCDM derivative in the fusion reaction.

These concepts and observations lead us to the creation of the 2nd generation MultiBac system (Fitzgerald et al., 2006, 2007). It had now two families of modular transfer plasmids, which we denominated Acceptors (pFL and pKL) and Donors (pUCDM and pSPL). Acceptors are based on pFBDM and comprise the Tn7 transposition elements and regular origins of replication, whereas Donors contain a conditional origin of replication derived from the phage R6 Kγ and a LoxP site (Fig. 1C) Since we had seen that more than one Donor could be integrated in a single Cre reaction, we decided to use this to our advantage by creating two Donors which were identical except for the resistance marker (pUCDM: chloramphenicol, pSPL: spectinomycin). The system also provides two Acceptors, with either a high copy-number (ColE1, pFL) or a low copy-number (BR322, pKL) origin of replication. We made pKL because, occasionally, we observed plasmid instability with the high copy-number origin when sensitive genes were integrated. Fusion products made by using one Acceptor and one or optionally two Donors simultaneously are selected via the appropriate antibiotic resistance marker combinations in *pir*-negative bacterial strains (Bieniossek et al., 2008). Multigene cassette containing fusions can thus be assembled, analyzed and modified *in vitro* prior to integration into the baculoviral genome by Tn7 transposition. Both types of plasmids have independent expression cassettes into which further expression cassettes can be inserted via the multiplication module, or, alternatively, by seamless cloning procedures (Berger et al., 2004; Bieniossek et al., 2008). *In vitro* fusion reactions of one Acceptor and several Donors can be carried out sequentially or simultaneously, only requiring a combination of purified Cre enzyme, buffer and DNA (Bieniossek et al., 2008; Fitzgerald et al., 2006).

Notwithstanding the relative ease of combining several to many genes by taking advantage of multiplication modules, seamless cloning, Cre fusions and Tn7 transposition in combination, we still advise to test expression from individual transfer constructs also before generating the ultimate fusion constructs and moving prematurely to large-scale protein complex production (Fig. 2). Such a stepwise validation provides a convenient means to identify "problematic" (in terms of expression) subunits early on, and allows for designing counterstrategies (such as provision of several copies of that gene). Acceptor derivatives can be directly used for expression tests by Tn7 transposition into the MultiBac BAC. Donor derivatives can and should be likewise tested. We recommend testing the expression of genes in Donor derivatives by fusing with an "empty" Acceptor (by Cre–LoxP reaction) and then integrating the fusion by Tn7 transposition into the MultiBac BAC (Fig. 2). Composite MultiBac BACs, each carrying expression cassettes encoding for parts of the multiprotein complex of choice, can be tested in turn as described. This strategy provides a convenient option to identify and produce sub-assemblies of a multiprotein complex of choice, which may be of interest for structural analysis. Virus performance and protein production should be monitored for all constructs (Fig. 2). Additionally, we typically prepare glycerol stocks of all positive bacterial clones carrying composite MultiBac BAC.

More recently, we observed that Acceptor–Donor fusions could be easily deconstructed by making use of the excision activity of Cre recombinase (Bieniossek et al., 2009). Selective deconstruction of fusion plasmids enables specific modification of DNA fragments coding for single subunits of a complex. Vectors carrying the modified DNA can be readily reintegrated by Cre–LoxP fusion into the multigene transfer construct and used for expression experiments. This possibility is especially attractive when multiple versions of a complex should be tested, for example when limited proteolysis experiments indicate that certain regions of the subunits should be altered or eliminated to enhance crystallization prospects. The simplicity of the combination of various Donors with an Acceptor by Cre fusion allowed us to script the procedure into a simple routine which can be easily implemented on a robot, which is useful for example if many Cre-mediated Acceptor–Donor assembly (or deconstruction) reactions need to be carried out in parallel (Bieniossek et al., 2009).

3.3. MultiBac 2008: EMBacY virus and standard expression procedures

One of the reasons why *E. coli* expression is so successful is the availability of simple standard protocols to carry out expression experiments even by non-specialist users. We endeavored to design similar accessible, standardized protocols for protein complex production using the MultiBac system. We felt that the "classical" protocols for baculovirus expression could be significantly streamlined to make them more suitable for structural biology applications at the throughput required. Towards this goal, we integrated an enhanced yellow fluorescence protein-coding gene (YFP) under the control of the polyhedrin promoter into the LoxP site present on the MultiBac BAC (Fig. 1C). The availability of the new Acceptors with LoxP sequences for *in vitro* Donor/Acceptor fusions essentially had made the LoxP site on the MultiBac BAC superfluous. The resulting BAC is called EMBacY. The presence of YFP serves the purpose of directly observing virus performance by a very sensitive means, namely by using a fluorescence spectrophotometer (Bieniossek et al., 2008). YFP is under control of a very late promoter (polh) as, typically, the heterologous genes of choice. We observed that when YFP expression reaches a plateau, expression of other heterologous proteins under the same promoter (and

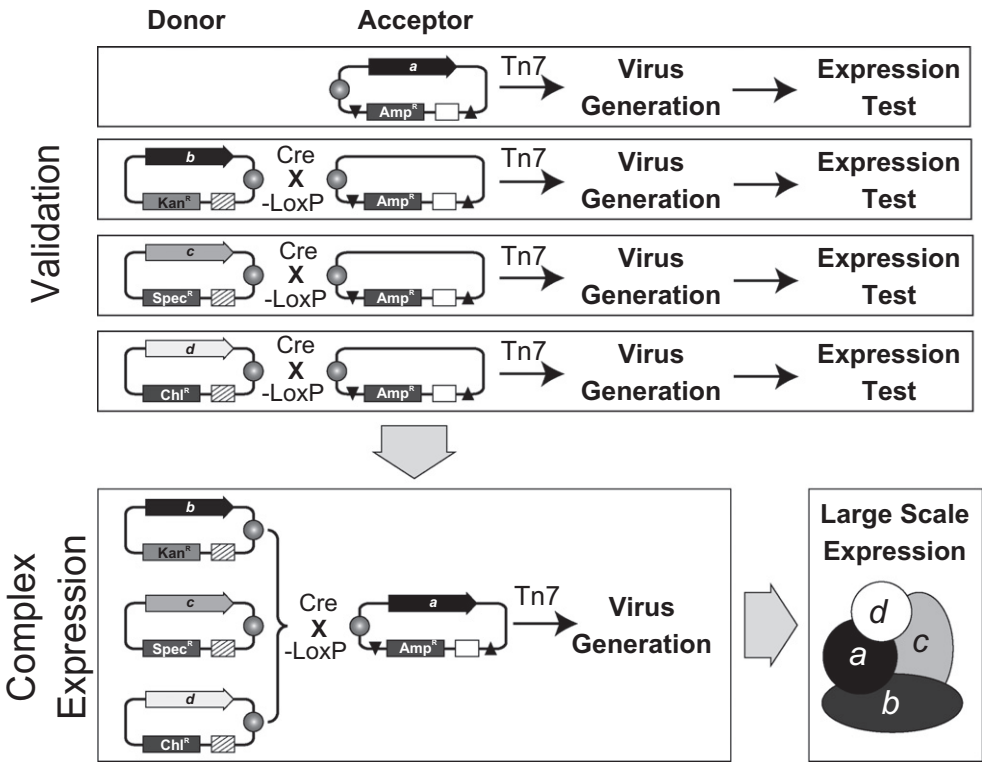

Fig. 2. Testing MultiBac Protein Expression. In a model complex expression experiment involving genes a, b, c, d (shaded arrows), each expression cassette can be tested by small-scale expression analyses on the way to building the full complex (top). Expression cassettes on Acceptors are integrated into MultiBac or EMBacY BACs via Tn7 transposition. Derivatives of Donors, in contrast, are lacking Tn7L and Tn7R sequences. Test expressions from Donors are therefore carried out by integrating Donor derivatives into an empty Acceptor by Cre–LoxP fusion *in vitro* followed by integration into the BAC of choice *in vivo* in *E. coli* cells via Tn7 transposition. Cre–LoxP fusion of all Donors (in this example three Donors, carrying genes b, c, d) with an Acceptor (carrying gene a) leads to a multigene fusion plasmid with the full complement of genes encoding for the complex of choice (bottom left). Successful expression of all components is followed by scale-up and purification of the complex (bottom right) for functional and structural analysis. Marker genes mediating resistance to kanamycin (Kan^R), chloramphenicol (Chl^R), spectinomycin ($Spec^R$) and ampicillin (Amp^R) are shown as boxes. The LoxP sites are shown as spheres. Regular origins of replication (ColE1 or BR322) present on Acceptors are marked as white boxes. Conditional origins of replication (derived from R6 Kγ) present on Donors are shown as shaded boxes.

by analogy also of p10, another frequently used promoter) also reach their peak production. Thus, we can follow heterologous protein production levels by following YFP expression. We had originally introduced YFP because we wanted to find out whether co-expression of many foreign genes would saturate our MultiBac expression experiments and thereby limit recombinant protein yields. Interestingly, YFP expression remained fairly constant irrespective of other heterologous protein products expressed from the same baculovirus (Berger et al., 2004). With the EMBacY virus we now were in a position to work out highly standardized protocols both for virus production and also for heterologous protein expression by taking advantage of YFP fluorescence (Bieniossek et al., 2008). In this new setup, we aimed to eliminate all steps we deemed unnecessary, including for example all virus titer measurements. In summary, we established simple standard protocols for routine use also by non-specialist users which lead to large-scale protein production in a reasonable short time frame of not more than 2 weeks (Fig. 3).

Briefly, selection and isolation of composite BACs requires roughly 4 days. To obtain initial virus (V_0), adhesive Sf21 cells are transfected with composite EMBacY BACs in a 6-well plate format. V_0 is harvested no later than 48–60 h post-transfection and immediately used to start virus amplification in an Erlenmeyer shaker flask. After V_0 is removed, the monolayers in the 6-well plate are overlaid with fresh medium and incubated for another 48 h. Then, these cells are harvested, the YFP signal is measured, and protein production is analyzed by SDS–PAGE analysis and/or Western blot.

Concomitantly, V_1 is amplified in suspension culture in a shaker flask. In our protocol, it is absolutely mandatory to maintain a low multiplicity of infection (MOI) during virus production and amplification. MOI is the number of infectious virus particles (plaque forming units, pfu) per cell in a cell culture. We experienced that a low MOI regimen is, in our hands at least, the best way to avoid detrimental gene deletions which can occur during baculovirus amplification, adversely affecting protein yields (Braunagel et al., 1998). Since we choose not to determine virus titers, we ascertain a low MOI by allowing at least one doubling of the cells in shaker flask after addition of V_0 (Bieniossek et al., 2008). Infected cell cultures in the shaker flasks are split every 24 h to a cell count of below 10^6 cells/ml until cell proliferation arrest (pa) occurs. After cell proliferation arrest, 10^6 cells are sampled from the culture every 12 h and the YFP fluorescence signal is recorded. Amplified virus (V_1) is harvested ~48–60 h after cell proliferation arrest and fresh medium is supplemented to the culture. Again, 10^6 cells are sampled from the culture every 12 h and the fluorescence signal of YFP is followed. Finally, cells are harvested when YFP signal has reached a plateau (typically after 3–4 days), and protein production is analyzed. Approximately 400 ml of V_2 virus are next produced in 2 L Erlenmeyer shaker flasks, strictly repeating the procedures outlined for generation of V_1. Rather than storing at 4 °C, we freeze V_2 by using the space-economic method of storing baculovirus-infected insect cell (BIIC) stocks in liquid nitrogen (Wasilko et al., 2009). Typically, 1–100 mg of pure protein/protein complex are obtained from 1 L culture by using the MultiBac system and our protocols. We experimentally determined that occurrence of defective virus, in which heterologous genes are preferentially eliminated, is significantly reduced when strictly adhering to our protocols (Bieniossek et al., 2008; Fitzgerald et al., 2006).

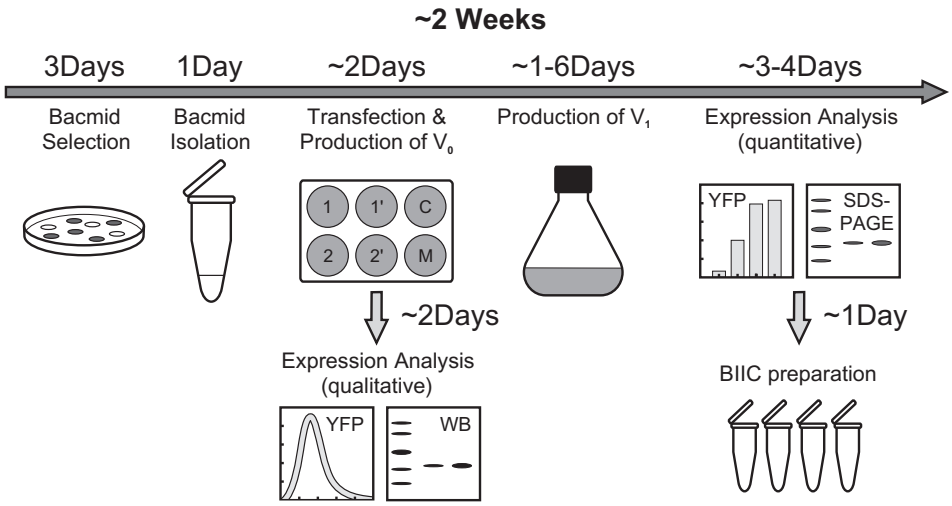

Fig. 3. Standard expression procedures with EMBacY. Multigene plasmids constructed by Donor–Acceptor fusions are integrated into EMBacY baculoviral DNA via Tn7 transposition. Positive clones are identified by blue/white screening. Positive transformants are optionally validated by re-streaking. After 3 days, composite EMBacY baculoviral DNA is isolated from cultures from single colonies and used to transfect Sf21 cells in 6-well plate format. Two clones obtained with the same multigene construct are processed in parallel (1, 1′ and 2, 2′). As controls, one well is charged with uninfected cells (C) and one with medium only (M). After 48–60 h, media containing initial virus (V_0) is removed from the wells and used for infecting an insect cell culture (25 ml volume) in an Erlenmeyer shaker flask. Fresh medium is added to each well of the 6-well plate and protein production is tested after 2 days by measuring the fluorescence signal of YFP, and by western blot (WB) with antibodies specific for the protein(s) produced. Infected cell cultures in shaker flasks are split every 24 h until cell proliferation arrest (pa) occurs. After proliferation arrest, 1 million cells are sampled every 12 h for measuring YFP fluorescence. Media containing amplified virus (V_1) is removed ~48 h after pa, and fresh medium is replenished instead. Cells are harvested when the YFP signal has reached a plateau (typically after 3–4 days). Protein production is analyzed by SDS–PAGE. Baculovirus-infected insect cell (BIIC) stocks are prepared for long-term storage of viruses (Wasilko et al., 2009). The whole procedure takes less than 2 weeks.

4. MultiBac exploits

In the years since its introduction, the MultiBac system has been put to good use in many laboratories (close to 300 by now) both in academia and industry, in addition to our own. The research interest of our laboratory is eukaryotic gene expression, and we have produced with MultiBac numerous multisubunit complexes that are involved in human transcription and its regulation, including chromatin remodeling enzymes and (sub)assemblies of human TFIID, a megadalton general transcription factor (Berger et al., 2004; Fitzgerald et al., 2006, 2007). Others have utilized MultiBac successfully to express a broad range of proteins and complexes with diverse functions, for biochemical and structural analyses, with a particularly prominent recent example being the crystal structure elucidation of the LKB1–STRAD–MO25 complex that revealed an allosteric mechanism of kinase activation (Zeqiraj et al., 2009) (Fig. 4). We had developed MultiBac for structural biology applications, and the system initially caught the interest mainly of other scientists in the structural biology community. Interestingly, however, the MultiBac system has in the meantime also been put to use by others whose main interest is not primarily structure. Thus, MultiBac has been used for efficiently producing virus-like particles (VLPs) from human papilloma virus serotypes. Here, it turned out to be crucial to integrate more than one copy of the encoding gene into the baculovirus used in the expression experiment to achieve efficient VLP formation (Senger et al. 2009). Among the most intriguing examples for MultiBac exploits beyond structural biology is its use for generating recombinant adenoviruses for gene therapy-based treatment of obesity in animals (Shapiro et al., 2008).

5. Outlook: towards automating MultiBac

Baculovirus expression vector systems have proven their worth over the years for many applications ranging from use as pesticides to gene therapy vectors (Boyce and Bucher, 1996; Cox and Hollister, 2009; Garcea and Gissmann, 2004; Hofmann et al., 1995; Jarvis, 2009; Kost and Condreay, 1999; Kost et al., 2005; Noad and Roy, 2003; Petry et al., 2003). BEVS is becoming increasingly utilized in many laboratories, particularly for producing eukaryotic proteins and their complexes. Illustrative examples for the power of the method include production of a wide range of virus-like particles which have been made by using BEVS, for structural and functional studies and also as promising vaccine candidates (Maranga et al., 2002; Noad and Roy, 2009; Roy and Noad, 2008; Roy et al., 2009).

Multiprotein complexes with many subunits are increasingly in the focus of biological research efforts and in order to study them recombinant overexpression is often required. The production of multiprotein complexes poses significant challenges in particular for structural biology applications, where a specimen of interest often needs to be appropriately tailored and diversified to reach the quality and homogeneity required for high-resolution analysis. This necessity is particularly the case in X-ray crystallography. Here, regions of low complexity may need to be eliminated to allow a sample to crystallize. Post-translational modifications may need to be removed or mimicked, or surface residues may need to be altered by mutagenesis. Such interventions have often been indispensable already for single proteins or small binary or ternary systems. It can be expected that they will be likewise crucial for analyzing large multisubunit complexes. Certainly, the workload is bound to increase exponentially when several to many subunits need to be diversified simultaneously in a multigene expression setup.

We have recently addressed this imposing bottleneck by designing experimental procedures for multigene assembly that were simple and robust enough to be carried out in a parallel fashion for example by using a liquid-handling workstation (Bieniossek et al., 2009). We have translated corresponding routines into robotics scripts and validated them by expressing many assemblies including membrane protein complexes in *E. coli* (Bieniossek et al., 2009). We chose *E. coli* expression as a model system for testing our automation development, since in this host the multigene con-

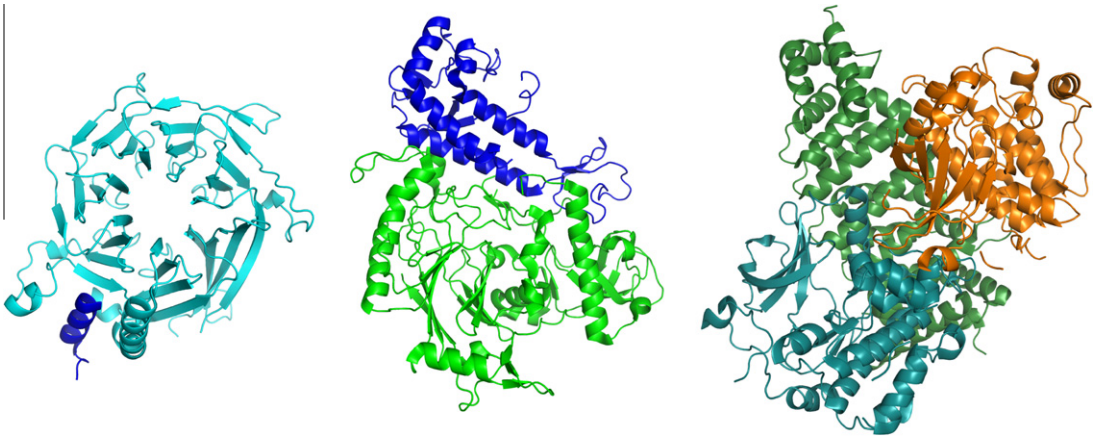

Fig. 4. Selected MultiBac structure exploits. MultiBac expression was successfully used to produce samples for X-ray structure elucidation of a number of complexes, including the RbAp46/H4 complex (left, Murzina et al., 2008; PDB code 3CFS); the yeast polymerase α/B subunit complex (center, Klinge et al., 2008; PDB code 3FLO) and human LKB1–STRAD–MO25 complex (right, Zeqiraj et al., 2009; PDB code 2WTK) and others. Subunits are shown in colors.

struction could be immediately used for expression trials bypassing the more intricate procedures for composite baculovirus generation and amplification. The routines we developed included gene insertion into Donors and Acceptors (fitted with bacterial promoters and terminators) by using sequence and ligation independent cloning procedures (SLIC, Li and Elledge, 2007), combinatorial Donor–Acceptor fusions using the Cre–LoxP reaction, small-scale expression of multigene constructs in *E. coli* and small-scale purification in multi-well plate format (Bieniossek et al., 2009).

Originally, our robotic approach was limited to *E. coli* as an expression host. Nonetheless, the same procedures with appropriate vectors containing baculoviral promoters and terminators can, by the same token, be applied to the generation of multigene transfer plasmids by using SLIC and Cre–LoxP reactions for MultiBac expression experiments. The resulting multigene transfer plasmids then simply will have to be integrated into the MultiBac or EMBacY baculoviral genomes by a robust transposition event that can be automated (Fig. 5). Several studies have emerged recently that investigated automation of baculovirus generation and small-scale expression for library screening (Airenne et al., 2003; Laitinen et al., 2005). We are currently evaluating these and other approaches for fully automating multigene assembly and small-scale expression by using our MultiBac system, including means for producing biological subunits other than proteins that are parts of complexes. We anticipate that the successful assembly of such a eukaryotic complex expression pipeline will prove to be invaluable for structurally addressing the complex proteome of eukaryotic organisms.

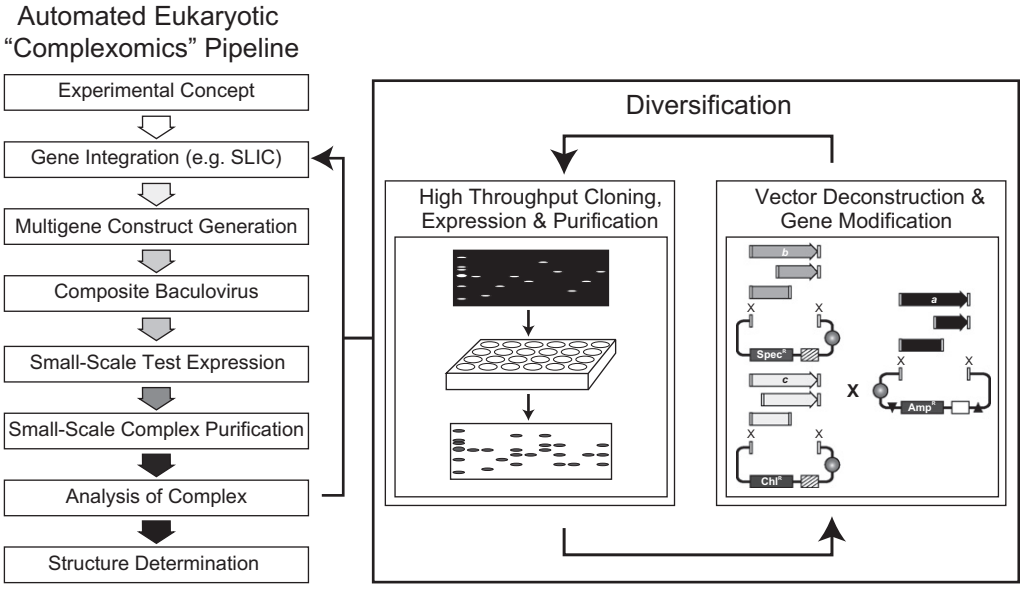

Fig. 5. Towards automating MultiBac. A future automated workflow from target selection to structural characterization of a protein complex, which is adaptable to automation, is shown schematically (left). After carefully choosing the co-expression strategy to be employed, genes are integrated into Donors and Acceptors by sequence and ligation independent cloning (SLIC). Multigene Acceptor–Donor fusions are generated by Cre–LoxP reaction and integrated into EMBacY via Tn7 transposition. Insect cell cultures are infected in small-scale (24-well plate format) for virus and protein production, and proteins are purified (48- or 96-well plate format). Complexes are analyzed biochemically and biophysically for integrity and functionality. Diversification of complex subunits may be required by mutating or truncating encoding genes to enhance success prospects for example for obtaining crystals for X-ray diffraction experiments. These can be easily integrated into the workflow in an iterative fashion (right). Fusions can be deconstructed by using the reverse Cre reaction (excision), and genes of individual expression cassettes are replaced with modified DNA fragments. All constructs are pre-purified via immobilized metal affinity chromatography in a 96-well plate format and complexes visualized by SDS–PAGE. Ideally, the steps involve only routines that can be translated into robotics scripts to create an automated "complexomics" pipeline on a liquid-handling workstation.

Conflict of interest

The authors declare competing financial interest. I.B. is author on patents (EP 1 723 246, EP 1 945 773) and patent applications describing parts of the technologies discussed in this contribution.

Submission declaration

The work here described has not been published previously and is not under consideration for publication elsewhere. Its publication is approved by all authors and tacitly or explicitly by the responsible authorities where the work was carried out. If accepted, it will not be published elsewhere including electronically in the same form, in English or in any other language, without the written consent of the copyright-holder.

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