Light it up: Highly efficient multigene delivery in mammalian cells

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Multigene delivery and expression systems are emerging as key technologies for many applications in contemporary biology. We have developed new methods for multigene delivery and expression in eukaryotic hosts for a variety of applications, including production of protein complexes for structural biology and drug development, provision of multicomponent protein biologics, and cell-based assays. We implemented tandem recombineering to facilitate rapid generation of multicomponent gene expression constructs for efficient transformation of mammalian cells, resulting in homogenous cell populations. Analysis of multiple parameters in living cells may require co-expression of fluorescently tagged sensors simultaneously in a single cell, at defined and ideally controlled ratios. Our method enables such applications by overcoming currently limiting challenges. Here, we review recent multigene delivery and expression strategies and their exploitation in mammalian cells. We discuss applications in drug discovery assays, interaction studies, and biologics production, which may benefit in the future from our novel approach.

Keywords:
cell-based assays; gene delivery and expression; high-content screening; high-throughput screening; tandem recombineering

Introduction

Multiprotein complexes and especially protein-protein interactions within these complexes are an emerging focus in current biology [1]. Fundamental questions about the structure, function, and regulation of these complexes are being addressed using highly purified material [2]. Cellular mechanisms, such as gene expression, cell cycle progression, signal transduction, metabolic pathways, and many others are often controlled by the generation or breakup of specific protein-protein interactions and networks [3, 4]. These molecular interactions are broadly studied in vitro using classical genetic, biochemical, and biophysical approaches.

Although prokaryotic expression systems are easy to use, fast and cost-efficient, eukaryotic systems are the systems of choice for production of therapeutically important human proteins in a functional form [5, 6]. The provision of eukaryotic chaperone systems and specific modifiers (i.e., kinases, phosphatases, glycosylases, and others) to allow for native post-translational modifications (PTMs) of the target protein has

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Abbreviations:
BiFC, bimolecular fluorescence complementation; **EGFR,** epidermal growth factor receptor; **FLIPR,** fluorescent imaging plate reader; **FRET,** fluorescence resonance energy transfer; **GABA,** γ-aminobutyric acid; **GAP,** GTPase activating protein; **GEF,** guanine nucleotide exchange factor; **GPCR,** G protein-coupled receptor; **GSM,** γ-secretase modulator; **HA,** hemagglutinin; **HCS,** high-content screening; **HE,** homing endonuclease; **HOS,** human osteosarcoma; **HP,** human papilloma; **HT,** high-throughput; **LIC,** ligation-independent cloning; **LTR,** long terminal repeat; **M1,** matrix protein 1; **NA,** neuraminidase; **PCR,** polymerase chain reaction; **PI,** phosphoinositide; **PtdIns,** phosphatidylinositols; **PTM,** post-translational modification; **shRNA,** short-hairpin RNA; **siRNA,** short interfering RNA; **SLIC,** sequence and ligation-independent cloning; **TR,** tandem recombineering; **VLP,** virus-like particle.

frequently proven to be critical for successful protein production [7]. Additionally, eukaryotic systems can be used to mimic specific environments in native tissues and are thus useful for acquiring data under near physiological conditions. In particular, native PTM patterns can be crucial for protein-protein networking and are essential in signaling cascades [8].

The interactions of a particular protein with different binding partners may also depend on the cellular/subcellular environment allowing a single protein to fulfill multiple tasks [9, 10]. In vivo studies of complex protein-protein interactions are increasingly complementing experiments with single proteins or protein domains as they can mimic additional aspects of the natural environment in which a protein acts. Simultaneous characterization of multiple interactions in a defined cellular environment will likely be instrumental for comprehensively deciphering the protein interactome of the cell. The information gained will then need to be translated into a corresponding and meaningful in vitro or in situ format. It is conceivable that most, if not all, components contributing to a particular biological process will need to be examined simultaneously as a prerequisite for developing innovative drugs. Promising approaches, for instance bimolecular fluorescence complementation (BiFC) assays to study protein-protein interactions in living cells, are now available and represent first steps toward addressing this complexity [11]. However, significant technological advances are still required to facilitate experiments involving several to many interacting protein partners.

Besides being potential future drug targets, multicomponent protein complexes are themselves powerful tools that can be engineered into potent therapeutics [12]. For example, virus-like particles (VLPs) are recombinantly produced components of the protein shell of a virus lacking its genetic material. VLPs are suitable candidates for vaccination, as they resemble live viruses from the outside but are not infectious [12]. A further exciting and promising area, which imperatively requires heterologous co-expression of multiple genes, is the reprogramming of somatic cells into stem cells. Reprogramming was recently demonstrated by generating induced pluripotent stem cells by co-expressing four specific transcription factors [13, 14]. Simultaneous monitoring of multiple parameters in living cells with several fluorescent protein-based sensors may be particularly useful for applications using photoactivatable, photoconvertable, or photoswitchable fluorescent proteins, fluorescence resonance energy transfer (FRET)-based biosensors, or fluorescent timer proteins [15]. The scope of these applications will be expanded considerably by simultaneous expression of multiple proteins in eukaryotic cells with controlled, homogeneous, and reproducible stoichiometry.

High-throughput (HT) methodologies for cloning, expression screening, and protein production in eukaryotic systems such as yeast, insect, and mammalian cells are available for the production of mainly single proteins or small, mostly binary systems in high quality and quantity [16]. Several transfer techniques to introduce genetic information into target cells have been described, which allow stable integration of transgenes into the host genome or transient introduction of episomally replicating plasmids [17]. Gene transfer can be accomplished via transfection or viral delivery systems to engineer mammalian cells genetically, but current technologies for generating mammalian cells, which express multiple heterologous genes simultaneously, suffer from inefficiency, inflexibility, and experimental tediousness. Further disadvantages of current methods reside in limited numbers of clonal selection markers, lack of precise regulation of individual expression levels, the presence of viral genes in infected or transduced cells, and the heterogeneity of cell populations.

In the following we will discuss a novel concept of multigene delivery for eukaryotic hosts [18, 19]. This approach facilitates rapid combinatorial construction of multigene delivery and expression vectors, and optionally allows single-step creation of transiently or stably transfected mammalian cells. We will furthermore outline how recent experimental approaches based on delivering genes in eukaryotic cells may benefit from this novel methodology for multigene transfer.

Rapid multigene delivery vector construction by tandem recombineering (TR)

Heterologous gene insertion into plasmid DNA is commonly achieved by cloning schemes relying on the activity of restriction enzymes and DNA ligases. This approach still dominates molecular biology to date. In the last several years, strategies have reemerged that do not rely on this classical cut-and-paste approach, but rather on ligation-independent cloning (LIC) or sequence and ligation-independent cloning (SLIC) methods, which enable seamless insertion of foreign genes independent of the presence or absence of restriction enzyme recognition sites [20]. In brief, DNA fragments are amplified via polymerase chain reaction (PCR) and overlapping regions are introduced. Single-stranded overhangs are generated by exonuclease activity of DNA polymerase in the absence of nucleotide triphosphates. DNA elements with overhangs are joined by annealing the complementary single-stranded DNA regions. LIC and SLIC methods enable the assembly of multiple DNA fragments in a single reaction in vitro into expression cassettes and plasmids. DNAs coding for tags, promoters and fluorescent protein fusions, e.g., can be concatamerized in this efficient and flexible way. Notably for HT applications, LIC or SLIC cloning is effectively replacing the cumbersome and less flexible conventional cloning methods.

For generating multigene delivery and expression constructs, we combined the SLIC method for heterologous DNA insertion with DNA concatenation catalyzed by Cre recombinase, which will conjoin DNA molecules containing the LoxP sequence [21, 22]. Small synthetic DNA plasmids, which we call acceptors and donors, contain only the sequences essential for plasmid propagation (replicon, resistance marker), the LoxP sequence and gene regulatory elements (promoter, terminator). The combination of SLIC DNA recombination and the Cre-LoxP fusion reaction we termed TR (Fig. 1) [21–23]. TR-based construction of multigene delivery vectors is independent of the eventual expression host and can be implemented for many applications in prokaryotic and eukaryotic expression hosts [10, 11, 14, 18, 23].

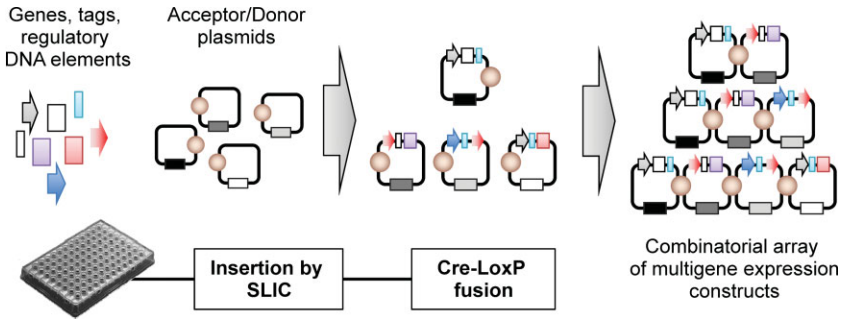

Figure 1. Tandem recombineering (TR): the assembly logic of multigene delivery constructs is shown. Multigene assembly is facilitated by combining a robust DNA insertion method (sequence and ligation independent cloning, SLIC) with an efficient DNA fusion reaction catalyzed by Cre recombinase. DNAs representing encoding genes, regulatory elements such as promoters and terminators, affinity tags, and fluorescent labels (left) are inserted by SLIC into an array of synthetic small plasmids called acceptors and donors which contain LoxP sequences (shown as balls). Cre recombinase concatenates acceptors and donors via their LoxP elements in a combinatorial fashion giving rise to an array of delivery vectors (right). All reactions can be carried out in an automated parallel format. TR is part of the proprietary ACEMBL suite installed at the EMBL Grenoble [22].

Multigene delivery in mammalian cells: The MultiLabel concept

The TR method was originally conceived for multigene transfer and expression in insect cells by using MultiBac, our baculovirus multiexpression system [7, 24–26]. The rationale was to provide a technology to combinatorially assemble multiprotein complexes for structural studies, an application where flexibility in construct design is critical. The approach further allows the co-production of modifiers such as phosphatases [7]. MultiBac has since developed into a widely used tool-kit for the production of heterologous protein complexes in insect cells [23]. The success of MultiBac prompted its expansion to complex expressions in mammalian cells. This was achieved by providing mammalian cell active promoters in the MultiBac expression cassettes instead of the corresponding baculoviral elements [18].

Visualizing mammalian cell compartments and their contents by fluorescently labeled proteins is widely used in cell biology for depicting protein localization and protein-protein interactions. For example, their perturbance by differential cell states, biological processes, or additives can be monitored with fluorophores coupled to proteins as sensors. For this purpose mammalian cells need to be transiently or stably transformed with genes encoding the fluorescent fusion proteins. Multigene delivery in this context, however, can be a considerable, even inhibitory challenge, if homogenous cell populations with a uniform ratio of the recombinant proteins have to be attained. This challenge was recently addressed by the creation of MultiLabel, a TR-based mammalian multigene delivery system particularly useful for labeling many proteins to study their actions in a cell simultaneously (Fig. 2). The MultiLabel technology and its potential applications are detailed in the following section.

MultiLabel vector design

MultiLabel uses a single plasmid containing all genes to be expressed for a one-step transfection of mammalian cells [19]. Gene transfer can be stable or transient. As for protein complex production in *Escherichia coli* and insect cells, construction of a multigene plasmid relies on a Cre/*LoxP* fusion-based donor-acceptor strategy [22, 24]. An Acceptor containing a common ColE1 derived origin of replication is fused to a variable number of donors, each with a conditional origin of replication and different resistance markers, by an in vitro Cre/*LoxP* reaction [27]. Productive multigene fusion products are selected by challenging transformants with combinations of antibiotics. Unfused donors cannot replicate in generic cloning strains, thus, only fused multigene acceptor-donor constructs are being propagated. During insertion, genes of interest can be fused to coding sequences for fluorescent proteins, as well as tags for purification, immunolabeling, or immunoprecipitation. In addition to SLIC procedures, the MultiLabel system also foresees conventional cloning strategies using restriction enzymes, notably homing endonuclease (HE) enzymes with long (>30 bp) and therefore unique recognition sequences.

Originally, a collection of regulatory DNA elements (promoter sequences, origins of replication, resistance markers, and tags) was created to facilitate the design of MultiLabel donors and acceptors [19]. The aggregate size of these DNA elements, when combined to acceptors or donors, was minimized to allow integration of large inserts while maintaining acceptable transfection efficiency. Standardization of junctions between DNA elements provides options for HT cloning and reprocessing of existing expression constructs, if for example origins or resistance markers are to be replaced. Notably, selectable markers facilitating site-specific integration (e.g. by the Flp-In system) can be embedded easily [19]. Customized acceptors or donors can be constructed in a single step reaction from these regulatory DNA fragments. Generation of multigene delivery constructs is achieved by applying our TR routines. The number of elements that can be assembled is theoretically unlimited, although the overall size of the DNA construct could become an issue because of reduced transformation efficacy of very large DNA molecules.

A multigene plasmid with five expression cassettes made up of four donors and one acceptor was assembled as proof-of-concept [19]. Stable cell lines were created by using a Flp-In cassette for site-specific integration into the genome [28] (Fig. 2). Integration efficacy in this experiment was maximized by linearization of the assembled DNA and introducing specific selection markers flanking the integration cassette [19].

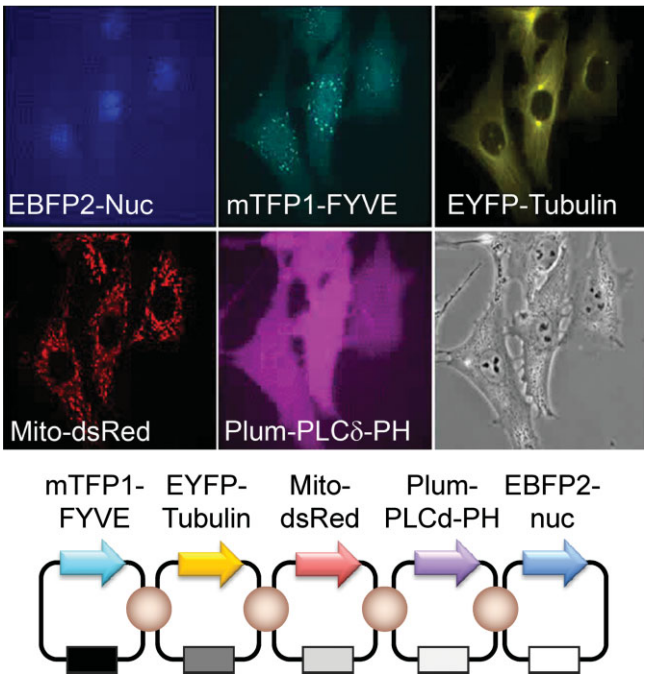

Figure 2. MultiLabel: highly efficient multigene delivery in mammalian cells. A MultiLabel proof-of-concept experiment involving five sensors containing fluorescent tags is shown (top). The five encoding genes were assembled into a single multigene delivery vector by TR (bottom). In addition this DNA construct contained the integration and selection elements for stable integration into the host cell genome. Sensors specifically visualize the nucleus (EBFP2-nuc), tubulin (EYFP-Tubulin), mitochondria (mito-dsRED); mTFP-FYVE interacts with a phosphoinositol-phosphate (PI-3-P), Plum-PLCd-PH interacts with a phosphoinositol-biphosphate (PI-4,5-P2). Porcine aortic endothelial cells were used in this experiment (shown in light field). All transfected cells contain all sensors and exhibit virtually identical signal levels across the entire cell culture population. Homogenous cell cultures of this kind were hitherto essentially unattainable, complicating many applications relying on multiple visual readouts simultaneously. Image is a frame from a time-lapse movie by P. Berger, PSI Villigen, Switzerland. Adapted from [19] with kind permission of the publisher.

MultiLabel exploits

The potential of MultiLabel was demonstrated by homogenous expression of five different proteins from a single acceptor-donor fusion plasmid in transiently transfected HEK293 cells [19]. All components in all transfected cells are produced at uniform levels throughout the culture, in stark contrast to classical co-transfection of individual plasmids, which resulted in inhomogeneous mixtures of cells with strongly varying expression profiles [19]. Furthermore, a constant relationship between the expression levels of individual proteins at the single-cell level was achieved.

To demonstrate that cells expressing multiple proteins still exhibit normal physiology, a pathway of vesicle trafficking in live cells was visualized by tracking fluorescently labeled key components [19]. In this experiment, Rab GTPases, which are components of membrane trafficking processes in eukaryotic cells [29] were labeled. Ran GTPases cooperate with phosphoinositides (PIs), which are phosphorylated phosphatidylinositols (PtdIns), to define membrane identity by recruiting Rab effectors to specific areas of cellular membranes. Several PH domains are known to interact with specific lipid classes of PtdIns at different phosphorylation states and can be used as PI sensors [30]. GTPases Rab4, 5, 7, and 11 have been shown to associate with different vesicles in the endosomal compartment [29] and Rab5 is converted into its active form by the activation of epidermal growth factor receptor (EGFR).

Using MultiLabel, several PI sensor domains and fluorescently labeled Rab sensors were co-produced transiently in COS cells, which endogenously express EGFR. Intriguingly, time-lapse series showed that EGFR initially co-localized exclusively with Rab5 and only later with Rab5 and Rab7. We anticipate that similar approaches will prove to be instrumental in basic research and possibly also in next-generation drug discovery, in particular when used to expand the scope of existing technologies and approaches.

Baculovirus for efficient gene delivery: BacMam

About 20 years ago, the potential of baculovirus vectors containing expression cassettes controlled by mammalian cell active promoters for gene delivery and expression in mammalian cells was discovered [31, 32]. Efficient BacMam-based gene expression was shown for various cell types [33–36]. It appears that the baculovirus virion is sufficient for effective uptake into most mammalian cells and that the viral membrane glycoprotein gp64 plays a crucial role [37]. More recently, the efficacy of gene delivery by BacMam was considerably enhanced by providing a vesicular stomatitis virus envelope protein fused to gp64 on the virion surface [38]. BacMam eliminates the need for transfection reagents, which are typically lipid chemicals that would need to be combined with plasmid DNA used for gene delivery. The BacMam method has virtually no toxic effects on mammalian cells and automated processes are available [39–42].

BacMam can be used to combinatorially investigate systems with several components in cells. Combinations are currently achieved by administering multiple viruses, each delivering one gene to the cell culture investigated. Identical to gene delivery using multiple plasmids, this approach results in inhomogeneous cell populations, variations in expression levels from cell to cell and restrictions in reproducibility. All these disadvantages could be overcome by combining our TR approach with the efficient and chemical additive-free procedure of transfection of mammalian cells by the BacMam method. A "MultiBacMam" system will combine all advantages of MultiLabel in terms of homogenous cell populations and defined expression ratios with the superior handling DNA delivery characteristics of the BacMam approach (Fig. 3). The current limitation of MultiLabel with regards to low efficacy when delivering very large DNAs would likewise be abolished by "MultiBacMam" as, apparently, no decrease of transfer by BacMam occurs even if 30 kb or more of foreign DNA are inserted into the virus genome. "MultiBacMam" thus constitutes an interesting and attractive concept and work on such a system is being completed (I. Berger, unpublished).

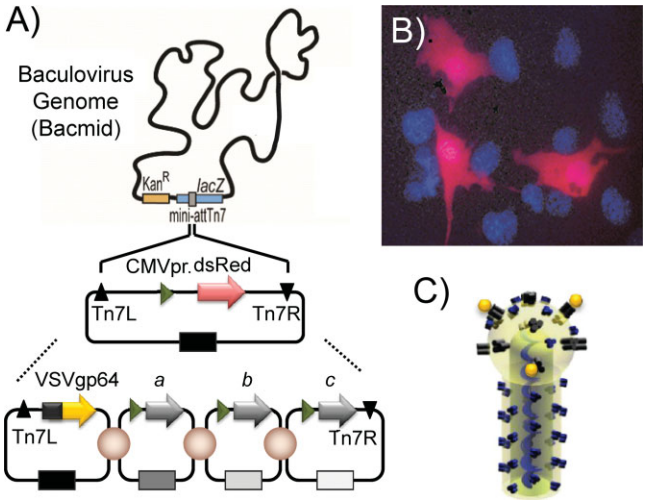

Figure 3. (Multi)BacMam: recombinant baculoviruses are a useful tool for gene transfer in mammalian cells. **A:** Conveniently, the baculovirus genome is provided as bacterial artificial chromosomes (Bacmid) in bacterial cells. Insertion of foreign DNA is catalyzed in these cells by Tn7 transposase via specific DNA elements present on a separate plasmid containing the gene of interest (Tn7L, Tn7R) and the bacmid (Tn7 attachment site, attTn7) disrupting a LacZ gene to identify positive recombinants. The composite bacmid is used to transfect mammalian cells. **B:** If the inserted gene (here dsRed) is controlled by a mammalian cell active promoter (here CMV), protein expression is observed in the target cells (nuclei are stained with DAPI). Image is adapted from [26]. Transfection efficiency is dramatically increased if a gene encoding for a fusion of a baculoviral surface glycoprotein (gp64) and a peptide derived from vesicular stomatitis virus (VSV) is present in the bacmid (A, bottom). This VSVgp64 fusion encoding gene, controlled by a baculoviral promoter, and numerous further heterologous genes (gray arrows labeled *a*, *b*, *c*) could be easily assembled by TR into a multigene delivery construct containing in addition also the Tn7L and Tn7R DNA sequences. **C:** This ''MultiBacMam'' approach would give rise to baculovirus particles with gp64 proteins displaying the VSV peptide (yellow balls) on the virion surface, for highly efficient delivery of the multigene DNA cargo in mammalian cells. Image of the virion is courtesy of K. J. Airenne, University of Kuopio, Finland.

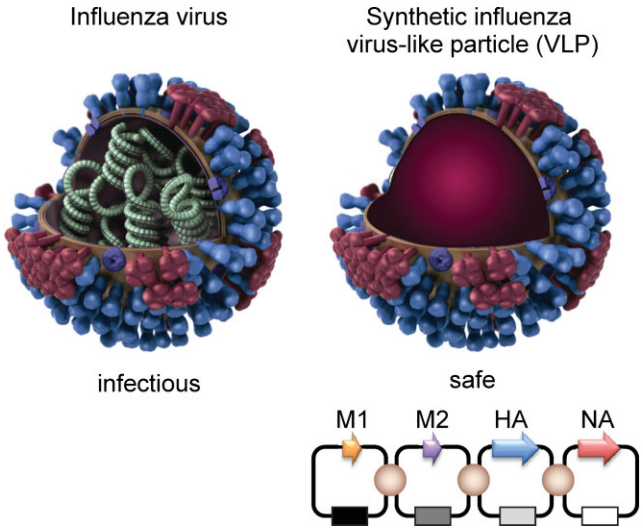

Figure 4. Multigene delivery for VLP vaccines. The influenza virus structure, modeled on data from electron microscopy, is shown (left). The genetic material (green), vital for infectivity, is protected by an envelope made of proteins HA (blue), NA (red), and two matrix proteins (M1, brown; M2, purple) and a lipid membrane. Synthetic influenza VLPs (right) can be produced efficiently by eukaryotic cells transfected with multigene delivery vectors containing the four encoding genes, for example assembled by TR (bottom right). The VLPs are structurally identical to the live virus however are not infectious. Influenza virus image is courtesy of D. Jordan.

Multigene delivery for virus-like particle production

VLPs resemble authentic viruses but do not contain infectious genetic material. For many viruses, co-expression of the individual capsid proteins or even a selection thereof leads to spontaneous and efficient assembly of VLPs [12]. VLPs were shown to be highly effective in stimulating immune responses and are thus prime candidates for vaccination [43–45]. VLPs could conceivably replace inactivated or attenuated viruses [46–49]. Several host expression systems including a number of mammalian cell lines have been described as potent expression systems for production of VLPs [12, 50]. Many of the VLPs synthesized for vaccines or vaccine development have already entered the preclinical stage, or phase I, or were licensed as vaccines [12, 50–56]. To achieve efficient VLP production of human papilloma (HP) viruses, a single shell protein had to be overproduced in lepidopteran cells from two identical genes [57]; this can be considered a special case of co-expression, where the MultiLabel approach could be useful. Influenza VLPs can be formed by co-expressing combinations of the matrix protein 1 (M1) with the three shell proteins [matrix protein M2, hemagglutinin (HA), and neuraminidase (NA)] (Fig. 4). Corresponding di-, tri-, and tetravalent subunit vaccines are now entering the pharmacological market as interesting and potentially cost-efficient alternatives to conventional viral production based on infected eggs [12]. Also here, controlled co-expression of the shell proteins of influenza VLPs from a single construct generated by TR is bound to be advantageous, particularly when considering the number of combinations of existing and emerging variants of influenza that need to be targeted.

Multigene delivery applied to cellular assays

Cellular assays may likewise benefit from a synchronized delivery of multiple genes, especially when used as tools for compound screening and drug discovery (Fig. 5). High-throughput screening (HTS) technologies aim at identifying biologically active small molecules by testing the effects of thousands to millions of compounds in automated, large scale biochemical and/or cell-based assays, and are especially important in pharmaceutical applications [58, 59]. Although compound screening with primary cells undoubtedly is most predictive for substance behavior in vivo, practical limitations, such as appropriate supply of cells, often necessitates engineered cell systems or mechanistic cellular models instead. Engineered cells and

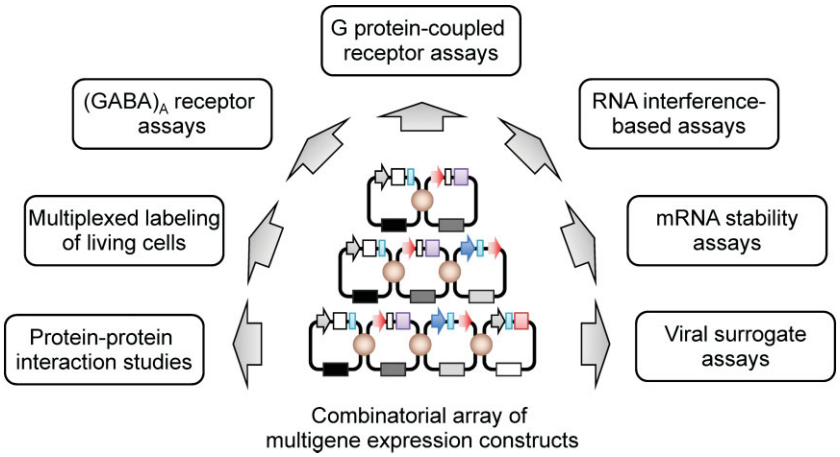

Figure 5. Multigene delivery for cellular assays. Combinatorial arrays of multigene expression constructs will be beneficial for modulating and optimizing a broad range of assays for compound screening and protein-protein interaction analyses (see text). Stable multigene delivery will also be crucial to render feasible assay formats with even higher complexity, since limitations and challenges resulting from inhomogeneous cell populations and/or from complexity of the system studied can be efficiently avoided or resolved. Combinatorial approaches, as multigene expression combined with RNA interference-based knockdowns, the generation of multiexpressor cell lines and the advancement in production of multiprotein drug targets, will be of high value for assay developments in the future.

mechanistic model systems are especially important in the early stages of drug discovery, when immense numbers of compounds have to be tested. High-content screening (HCS) is a multidimensional, multiparametric analytical tool to generate data on spatial and temporal changes on the basis of single cells, thus enabling a more sophisticated understanding of cellular responses to external signals [60, 61]. A vital contribution to the success of HCS approaches was the continuous development of proper biosensors, which are typically fluorescent molecules with a genuine plethora of properties [15, 62, 63]. Simultaneous fluorescence and morphological measurements on multiple cells labeled with various fluorescent probes or sensors are desirable and, as a consequence, efficient and easy-to-use tools to integrate these sensors are becoming increasingly important. We could imagine that simultaneous multigene delivery carries significant potential to advance many areas of cell-based assays in classical HT and HC screens by providing genes coding for sensors and probes in a synchronized and homogeneous manner.

Multigene delivery for protein-protein interaction studies

MultiLabel has the advantage that genes coding for different protein binding partners can be recombined in a simple and fast reaction and that transfected cell populations express all genes of interest. The technology can thus be used to address complex systems for protein-protein interaction studies. BiFC and FRET assays are efficient tools to monitor protein-protein interactions and/or changes in protein structure in living cells [11, 64]. BiFC occurs when two non-fluorescent fragments of a fluorescent protein bind to one another to form the mature fluorescent protein. Formation of the mature fluorescent protein in living cells is accomplished when the non-fluorescent fragments are fused to two proteins that interact with each other [11]. In order to achieve efficient protein fusion pairs of non-fluorescent fragments and binding partners, various combinations of fusions have to be tested. Because of its modular nature, MultiLabel could be an elegant way to meet these needs. Expression cassettes for fusion proteins can be modified and recombined easily and subsequently tested in an in vivo situation.

To date FRET assays are the methods of choice for analyzing short-term interactions of proteins in living cells [64]. As for BiFC, optimal FRET sensors have to be identified empirically. FRET pairs can be linked via bioactive segments, which change their conformation upon ligand binding or modification and thus alter the distance between the fluorophores [65]. Examples for the successful exploitation of this technique include FRET-based calcium or phosphorylation sensors, which were used to visualize intracellular calcium fluctuations or kinase activity in living cells. Furthermore, modifications of FRET pairs have expanded the application range drastically [63, 66–72].

Other examples where co-expression strategies could be optimized using a MultiLabel strategy, are studies on GTPase activities of members of the Ras-family [73–75]. Ras proteins are molecular switches, which are regulated by guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs) [76, 77]. Activities and specificities of GEFs and GAPs could be analyzed in cells, that were transfected with recombined constructs coding for fluorescently tagged GTPase and regulator.

Multigene delivery for multiplexed labeling of living cells

Gilbert et al. reported a new HT approach using multiplexed labeling of viable cells to monitor glycine receptor function by flow cytometry [78]. Co-expression of a chloride-sensitive mutant of yellow fluorescent protein with wild type and mutant forms of the receptor were used to detect functional and non-functional receptor mutants. To allow for faster analysis of mutants, each cell population carrying a different receptor mutant was differently color-coded by cell permeable dyes. A similar labeling approach was used to study cell clonality [79, 80]. Living cells were color-coded via transduction with combinations of lentiviral vectors each carrying one of three fluorescent proteins, which facilitated tracking of clonal cell fates in vitro and in vivo [79, 80]. Such combinatorial approaches might be accelerated by using TR in a robotic setup for generating combinatorial vector libraries coding for defined sets of fluorescent proteins, into which different mutants of the target protein could be introduced. Each transfected cell population could then be identified via its homogeneous color code. Furthermore, genes of target proteins and mutants thereof could be easily shuffled into the vector library.

Multigene delivery for ion channels: γ-aminobutyric acid $(GABA)_A$ receptors

$GABA_A$ receptors are primarily involved in inhibitory neurotransmission in the nervous system, where they operate as ligand-gated Cl^- channels [81]. These molecules are pluripotent targets for a wide range of therapeutic agents [82–84]. $GABA_A$ receptors are built up from a repertoire of various subunits encoded by about 20 genes and are thought to be pentameric, with two α, two β, and one γ subunit [85]. The two most abundant subtypes of $GABA_A$ receptors in the brain are $GABA_{A1}$ and $GABA_{A2}$, which are composed of $(\alpha1)_2(\beta2)_2\gamma2$ and $(\alpha1)_2(\beta3)_2\gamma2$ subunits, respectively [86, 87]. An HT functional assay for the characterization of these $GABA_A$ receptor subtypes was reported recently [88]. Instead of electrophysiological approaches, the authors employed a new fluorescent imaging plate reader (FLIPR)-based functional assay for measuring channel activity, which is amenable to HT [88]. In this assay, genes coding for the receptors $GABA_{A1}$ and $GABA_{A2}$ were provided on separate expression constructs for transient co-transfections of HEK293 cells. Production of the channels with correct subunit stoichiometries was established by titrating appropriate ratios of construct DNA for co-transfections [88]. Since correct subunit composition of a $GABA_A$ receptor is crucial for its pharmacological properties, the authors stated that large batches of cells had to be co-transfected and cryopreserved in order to reduce assay variability over a long timeframe. The MultiLabel approach would simplify this complex expression system significantly by providing expression cassettes for the individual subunits assembled on a single plasmid. Different subunit stoichiometries could be realized within certain limits by introducing double or triple expression cassettes for the respective subunit in combination with promoters of varying strength. Ideally, parallel screening of various receptor subtypes, with or without mutated individual subunits, could give information on the effect of subunit exchange or mutation on drug binding specificity and receptor activity. In a similar fashion, other multisubunit targets of high pharmaceutical interest, like γ-secretases [89], would be conceivable for γ-secretase modulator (GSM) screenings and activity assays.

Multigene delivery for G protein-coupled receptors (GPCRs)

Similar to $GABA_A$ receptors, the MultiLabel approach could likewise catalyze the study of GPCRs and downstream effectors. GPCRs represent one of the most diverse groups of signaling proteins and mammalian cell lines expressing GPCRs were widely utilized to discover novel agonists and antagonists. New GPCRs and orphan receptors are continuously added to the existing pool, rendering transient expression approaches increasingly important for their study, in particular as more and more cell types are becoming accessible [90, 91]. To date, BacMam-mediated production of GPCRs is the method of choice for such GPCR assays [90, 91]. Several parameters that determine functional consequences of receptor engagement can be measured [92, 93]. Upon stimulation, a GPCR receptor interacts with effector proteins (G-proteins) [94]. Inactive G proteins are trimeric protein complexes composed of three subunits: α, β, and γ. Upon coupling with GPCRs the trimeric complex dissociates into the α subunit and the βγ dimer. The dissociated subunits interact with a variety of downstream effector systems to modulate cellular functions [95]. Many GPCRs seem to exist as homo- and/or hetero-oligomeric complexes or might even function as subunits of large multiprotein signaling complexes [96–98]. Applying MultiLabel to study GPCR activation and signal transduction would allow co-expression of functionally and pharmaceutically distinct receptor subtypes with combinations of G protein complexes. Signaling properties and susceptibilities of monomeric and heteromeric GPCRs to various drugs could thereby be studied. Adaptation of existing assays for readout of GPCR activation by exploiting the phosphorylation of p42/44 MAP kinase ERK1/2, e.g., could be instrumental in establishing HT assays with even higher complexity [92]. Furthermore, parallel, mutational analyses of individual units of GPCR signaling complexes using a MultiLabel approach could shed new light on the roles of single proteins in ligand binding and response activation.

Although functionally separated GPCR-mediated signaling pathways converge at the kinase ERK1/2, spatially distinct pools of active ERK 1/2 are found in a cell [94]. Exact visualization of the localization of activated ERK1/2 pools after GPCR stimulation using fluorescent marker proteins could thus be used to determine the functional outcomes of receptor stimulation assays. MultiLabel could be instrumental in developing assays in which receptor, effector molecules, and sensor molecules are homogeneously expressed in a cell population. GPCR activity studies with such a level of complexity will critically depend on efficient, controlled, and defined delivery of each encoding gene. MultiBacMam approaches, combining the advantages of MultiLabel with the proven utility of BacMam for studying GPCR function in mammalian cell hosts, may offer significant advantages in this context.

RNA interference-based assays

RNA interference is a useful tool to analyze cellular processes by reducing or eliminating specific mRNAs upon provision of small complementary RNA molecules. A system for expression of short interfering RNAs (siRNAs) in mammalian cells to efficiently and specifically down-regulate gene expression for the analysis of loss-of-function phenotypes has been introduced [99]. This approach uses short siRNA-like stem-loop structures (shRNA, short-hairpin RNA), which are expressed from a plasmid. The expression of p53, a transcription factor with a well-known role in tumor suppression [100], was suppressed successfully by transient and even stable transfections of shRNA over a long period of time [99]. MultiLabel technology could conceivably be extended to allow simultaneous expression of transcripts, which would lead to suppression of specific gene products. Expression could be achieved by providing cassettes transcribing corresponding siRNAs and could be coupled to complementation with mutants or variants of the suppressed protein for analyzing gain-of-function. This approach would enable direct readouts of resulting phenotypes. Combinatorial approaches would potentially even allow the knockdown and complementation of several target genes simultaneously in transfected cells.

Viral surrogate assays

HIV-1 targets cells by interaction of the viral envelope protein gp120 with the CD4 molecule of the target cell and via interactions with β-chemokine co-receptor CCR5. Blocking these interactions prevents entry into host cells in vitro and reduces viral fusion. To build up a cell/cell fusion assay that mimics the process of viral fusion and entry into the host cell, researchers have employed two cell types, namely stably transfected human osteosarcoma (HOS) cells and a transiently BacMam transduced HEK293 cell line, as recipient host cell and viral mimics, respectively [101]. Recipient host cell mimics were programmed to express CCR5, CD4, and a chimeric long terminal repeat (LTR)-luciferase reporter construct. Transduced viral mimics expressed four viral proteins, namely gp120, gp41, tat, and rev. Interaction of gp120 with CCR5/CD4 resulted in fusion of the host cell and viral mimics and transfer of the tat protein to the cytosol of the host. Tat protein, in turn, binds to the LTR region on the luciferase reporter and activates transcription, resulting in an increase in cellular luciferase activity. The authors state that their surrogate assay design allows reproducible HT quantification of fusion events enabling evaluation of effects of compounds on gp120/CCR5/CD4-mediated viral fusion into host cells. MultiLabel could considerably facilitate the construction of multiexpressor cell lines in similar experiments.

Multigene delivery for mRNA stability assays

Deregulation of mRNA stability emerges as an important factor for diseases in various therapeutic areas [102]. Specific *cis*-acting elements on the transcript and *trans*-acting factors are key regulators of mRNA stability, and are crucial for the expression of many proteins [103]. Specific targeting of mRNA stability might therefore open up novel methods of drug discovery.

Cell-based assays utilizing reporter genes to monitor mRNA stability after treatment of cells with low molecular weight compounds were proven to be suitable for HTS [104, 105]. It was also shown that mRNA stability can be modulated by targeting the RNA interference pathway with small molecules [106, 107]. Benjamin et al. used a reporter transcript containing the green fluorescent protein gene upstream of the destabilizing 3′-untranslated region of the murine IL-3 gene and could identify okadaic acid as a stabilizing compound [105]. To allow for proper controls, cells are usually co-transfected with reporter transcripts lacking destabilizing regions enabling selection of only motif-specific compounds. Chemiluminescence and fluorescence-based reporter systems mimicking nonsense-mediated mRNA decay have also been described [108, 109]. Those assays would benefit from MultiLabel technology, since all transfected cells would carry all reporter and control transcripts.

Conclusion and prospects: Enabling multigene delivery in mammalian cells

Delivery and expression of multiple heterologous genes in mammalian cells is becoming an indispensable key technology in contemporary biological research. We have developed customized reagents and reliable protocols that facilitate assembly of many DNAs including genes, fluorescent markers, and purification and detection tags into functional expression cassettes and their combinatorial assembly into multigene delivery constructs. The methodology relies on TR procedures, was successfully implemented in a robotic setup and is suitable for multigene expression plasmid constructions in HT. The utility of this approach to generate transiently and stably transfected mammalian cells that produce many proteins simultaneously at defined and constant ratios was demonstrated, giving rise to cell populations of hitherto unattainable homogeneity. Integrating this approach for multigene delivery vector construction with the systematic provision of fluorescence markers as fusion proteins resulted in MultiLabel, a system for efficiently marking up mammalian cells for various cell-based assays. Proof-of-concept experiments demonstrated the potential of our approach.

We have outlined in this contribution a broad range of applications that could potentially benefit from the MultiLabel technology concept in the future. The technology could be instrumental for the production of important multiprotein assemblies including essential multisubunit drug targets and multivalent assemblies of pharamacological value such as VLP vaccine candidates. Moreover, we anticipate that numerous methodologies in contemporary research and drug discovery applications, which rely on multiple sensors and/or the provision and tracking of cell metabolites or RNA molecules, will be considerably facilitated by applying our technologies, thereby significantly extending the existing toolbox for multigene delivery and expression in mammalian cells.

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