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Genetically encoded split biosensors based on protein fragment complementation are a sensitive and robust tool for monitoring dynamic protein–protein interactions and activities of druggable targets in cell-based assays.

Split protein biosensor assays in molecular pharmacological studies

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Cellular signalling is commonly mediated through dynamic protein–protein interactions (PPIs). When pivotal PPIs are deregulated, cellular signalling can be altered; it is therefore attractive to monitor regulated PPIs to understand their role in health and disease. Genetically encoded biosensors that rely on protein fragment complementation have made it feasible to monitor PPIs in living cells precisely and robustly. In particular, split protein biosensors using fluorescent proteins or luciferases are frequently applied. Further, split TEV and split ubiquitin biosensor platforms flexibly allow using readouts of choice, including transcriptional barcode reporters that are amenable to multiplexed high-throughput formats and next-generation sequencing. Combining these technologies will enable assessing drug target activities and cellular response profiles in parallel, thereby opening up new avenues in drug discovery.

Introduction

Studying dynamic protein–protein interactions (PPIs) is crucial for understanding cellular signalling and ensuing responses. These regulated PPIs mediate distinct cellular signalling activities, such as proliferation, differentiation, apoptosis and inflammation. Often, a regulated interaction event can cause several proteins to aggregate transiently into larger complexes, which operate as functional signalling modules, or signalosomes, and initiate precisely regulated downstream signalling events. In terms of signalling, cell surface receptors are of special interest, because they respond to extracellular cues by relaying this information across the membrane into the cytosol. Initially, cell surface receptors transmit the signals through conformational changes, increased phosphorylation levels and altered binding affinities to adapter proteins that represent the first step of downstream signalling. Once the signal is received in the cytosol, defined intracellular signalling cascades are activated, which often share various components leading to crosstalk through multiple mechanisms [1]. Finally, cellular signalling culminates in a differential target gene response that defines overall cellular output behaviour, which might be hard to extract [2,3]. In addition, the altered activity of cell surface receptors is frequently

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GLOSSARY

BiFC bimolecular fluorescence complementation; a split biosensor assay (SBA) using fragments of fluorescent proteins such as GFP and derivatives.
BiLC bimolecular luciferase complementation; a split biosensor assay (SBA) using fragments of luminescent proteins such as firefly and Renilla luciferase.
BRET bioluminescence resonance energy transfer assay; a modified proximity assay based on FRET. For BRET, a bioluminescent luciferase (typically from Renilla reniformis) rather than CFP is used as the donor to produce an initial photon emission to excite YFP.
FRET Förster resonance energy transfer or fluorescent resonance energy transfer; proximity assay between a donor chromophore and an acceptor chromophore. Commonly, CFP and YFP are used as biological chromophores in a FRET assay. The donor and the acceptor are fused to interaction partners. When an interaction occurs, the donor, initially in its electronic excited state, transfers the energy onto the acceptor, which in turn emits a fluorescent signal. This signal is proportional to the distance between donor and acceptor, and therefore allows inferring interaction strength.
LUMIER assay luminescence-based mammalian interactome mapping assay; proximity assay between a luciferase-tagged (such as Photinus pyralis from the firefly–commonly termed firefly luciferase) protein and a FLAG-tagged protein. FLAG-tagged proteins are purified using immunoprecipitation techniques, and interacting proteins are quantified by luciferase readings.
NGS next-generation sequencing; NGS platforms use non-Sanger-based high-throughput DNA sequencing technology, during which millions of fragments of DNA from a single sample are sequenced simultaneously. This massively parallel sequencing technology allows an entire genome to be sequenced in less than one day.
PCA protein complementation assay; proximity assay in living cells to monitor protein–protein interactions, see SBA.
SBA split biosensor assay; a proximity assay in living cells to monitor protein–protein interactions. Protein biosensor fragments that are inactive by themselves are fused to interaction partners. The biosensor fragments only functionally complement when brought into close proximity owing to an occurred interaction event, resulting in regaining of its biological activity.
TEV protease tobacco etch virus protease (also termed TEV nuclear inclusion a endopeptidase); a highly specific viral cysteine protease cleaving at EXLYΦQ′φ sites, where X is any amino acid, Φ any large or medium hydrophobic residue (i.e. phenylalanine) and φ any small hydrophobic or polar residue (i.e. glycine or serine). Typically, the peptide sequence ENLYFQ′G or ENLYFQ′S is used. The wild-type TEV protease suffers from autocatalytic inhibition due to internal cleavage at residue S219 and folding of the C-terminal tail into the substrate pocket. Therefore, autocatalytic resistant (S219V, S219P) and truncated (1–221 with S219P) mutants were developed that display enhanced proteolytic activity.

associated with diseases, such as cancer and neurodevelopmental disorders. Therefore, receptors still represent prime candidates for pharmacological modulation using chemical compounds or biologics [4].

Much attention has been directed at monitoring the activities of major drug targets, such as the G-protein-coupled receptor (GPCR) and receptor tyrosine kinase (RTK) families [5,6]. Indeed, considerable progress has been made in the development of cell-based assays that use genetic biosensors to monitor the activities of cell surface receptors and regulated PPIs in general. Through various cell-based approaches, it has been established that the accurate monitoring of receptor activities, and other pivotal intracellular signalling events as well, would be of great advantage not only for the basic understanding of cellular biology but also for the rational design of therapeutic compounds [7,8]. Notably, cell surface receptors, such as GPCRs and RTKs, are accessible for cell-based assays that use genetic biosensors. These assays use biosensor moieties, which are either fused to the receptors or the cognate adapter binding partners, and provide high signal-to-noise ratios as well as a spatial and, if wished, a temporal separation between the interaction event and the readout itself. In addition, cytosolic drug targets are attracting further attention by drug discovery programmes, because crucial intracellular PPIs are often deregulated in disease [9,10].

In this review, we will discuss the variety and applicability of cell-based assays that use genetically encoded split protein complementation approaches. These split biosensor assays (SBAs; see Glossary), also termed protein fragment complementation assays (PCAs) as introduced by Michnik and colleagues [11], and reviewed in [7], allow monitoring of regulated protein interactions and receptor activities, and prove to be valuable for high-throughput and small compound screening approaches. Notably, there are two main categories of genetically encoded technologies used to study PPIs in living cells: SBAs and classical two-hybrid methods [12]. SBAs are based on the PPI-induced refolding of two previously nonfunctional and unstructured protein fragments to reconstitute a functional protein reporter. For example, non-fluorescent protein fragments only become functional when brought into close proximity, which prompts correct folding. By contrast, classical two-hybrid methods use the co-localisation of two protein fragments that are correctly folded and functional by themselves, such as the DNA-binding domain and the activation domain of GAL4. These domains, which are fused to interaction partners, do not need to be in physical contact to activate a reporter. Whereas two-hybrid assays are artificially localised to a particular compartment of the cell, usually the nucleus, SBAs do not require a specific subcellular location and occur in the native environment of the PPI.

Rationale of split biosensor assays

Dynamic interactions among proteins are central building blocks of cellular signalling. Perturbations induced by small molecules can cause a targeted disruption of a defined PPI within a cellular pathway, thereby linking a given cellular effect to a specific mechanism of action. Furthermore, it is reasoned that any perturbation, for example the regulated binding of an extracellular stimulus (e.g. a hormone) to a receptor or the genetic inactivation of an upstream pathway component, would propagate through the cellular signalling network, causing spatially and temporally distinct changes in downstream protein interactions. To measure subtle changes of the interaction behaviour between proteins in a reproducible, sensitive and robust manner, reporter proteins were engineered into non-active fragments. These moieties only functionally re-complement into a functional biosensor if they are

brought into close proximity as a result of an interaction event between bait and prey proteins (Fig. 1a). Molecularly, each fragment is fused to one of the candidate proteins, with a linker sequence in between to guarantee optimal polarity to fold and flexibility for the candidate proteins and split biosensor fragments.

Biosensor types

Various types of biosensors have been developed for split protein complementation assays. These biosensors offer a wide range of readouts, such as bioluminescence, fluorescence, cell survival, radiolabelled probes, artificial colour substrates, fluorogenic substrates and molecular barcode reporters (Fig. 1b). Notably, the molecular barcode reporters allow the integration into multiplexed cell-based assays using next-generation sequencing (NGS) as the readout technology. The assays developed differ with respect to readout, kinetic properties, limitations to localisation of detected PPIs, applicability to high-throughput approaches, steps of amplification and flexibility in use (summarised in Table 1).

Initially, dihydrofolate reductase (DHFR) was selected for the complementation approach, which reports an occurred PPI either through cell survival (using nucleotide-free medium because this selects for DHFR activity) or fluorescence (in the presence of the fluorogenic substrate methotrexate) [11,13]. This system set the basis for any complementation approach; however, it is limited by its readout kinetics. If applied in a survival assay format, it is only possible to monitor a binary on–off signal rather than gradual intensities of occurred PPIs. This also holds true for the fluorescence assay mode, because the pharmacologically relevant quantities of methotrexate severely affect cellular function. To improve these assay characteristics, other biosensors were developed and include β-lactamase [14], green fluorescent protein (GFP) and variants thereof [15,16], β-galactosidase [17–20], thymidine kinase

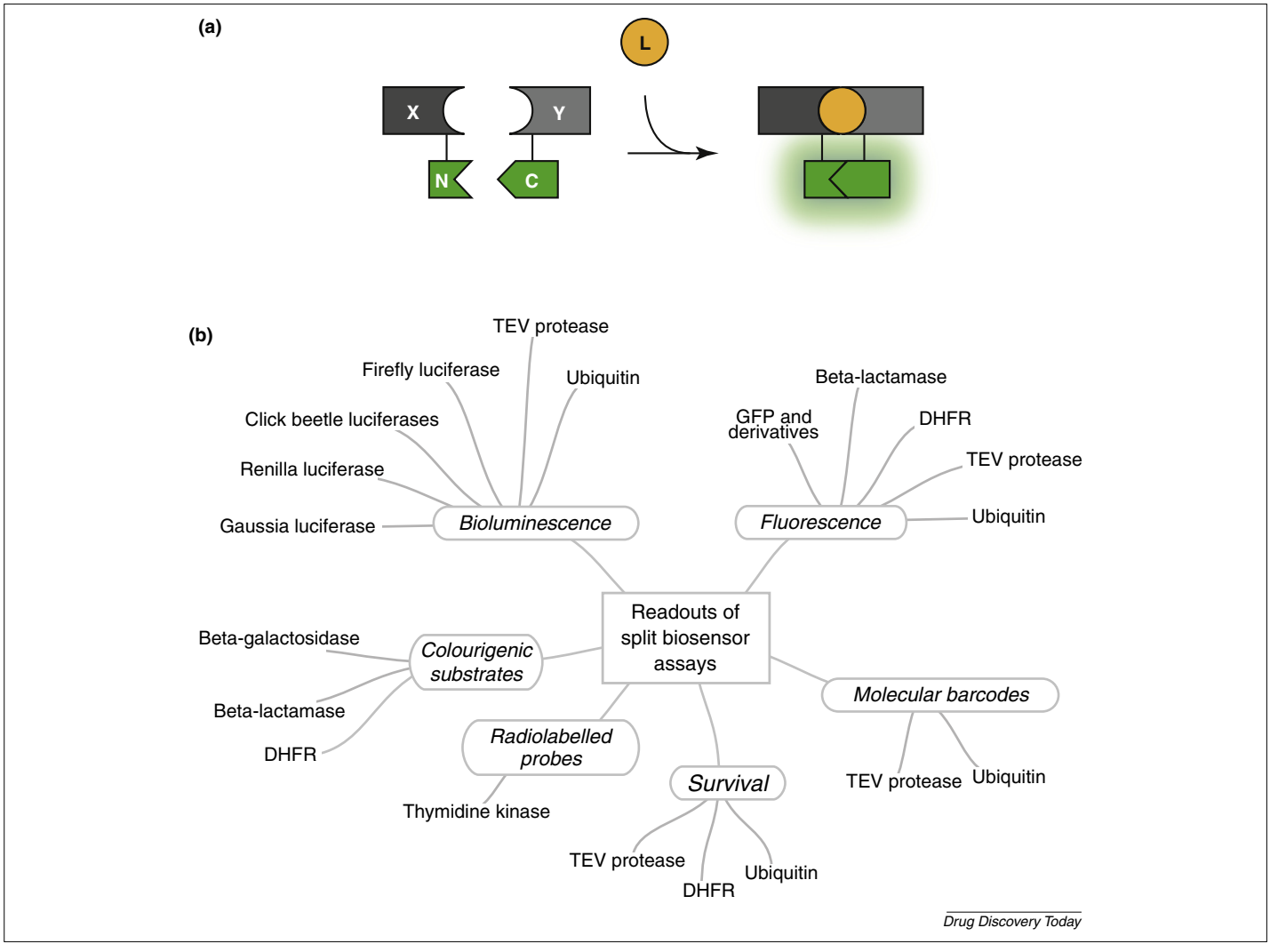

FIGURE 1

General principle and readouts of split protein biosensor assays. **(a)** Design of a split protein complementation assay to monitor a dynamic protein interaction event. Candidate proteins X and Y (depicted in brown) are fused to the N-terminal (N) and C-terminal (C) moieties of a protein biosensor (green). Upon addition of a ligand (L, red), X and Y interact causing the N and C fragments to reassemble and to reconstitute biosensor activity (indicated by outer glow). Well-known examples are split GFP and variants (Venus, Cerulean) as well as split luciferases (firefly, *Renilla*). **(b)** Variety of readouts available for split protein complementation assays. The readouts are grouped into six classes (in italics), with bioluminescence and fluorescence representing the major types. Note that the biosensors TEV protease and ubiquitin are applicable to four readouts.

TABLE 1

Key features of split biosensor assays and their application to various biological systems.

Split biosensor	Readout	Application					
		Detection of PPIs	Localisation of PPIs	Assay type	Kinetics	Amplification	Sensor size (kDa)
β-Galactosidase	Fluorescence, absorbance	Yes	–	Post-translational	–	1-Step	77
β-Lactamase	Fluorescence, absorbance	Yes	–	Post-translational	Limited	1-Step	29
Click beetle luciferases	Bioluminescence	Yes	Limited	Post-translational	Yes	1-Step	61
DHFR	Fluorescence, survival	Yes	Yes	Post-translational	–	1-Step	21
Firefly luciferase	Bioluminescence	Yes	Limited	Post-translational	Yes	1-Step	61
***Gaussia* luciferase**	Bioluminescence	Yes	Limited	Post-translational	Yes	1-Step	20
GFP and variants (BiFC)	Fluorescence	Yes	Yes	Post-translational	Limited	None	27
***Renilla* luciferase**	Bioluminescence	Yes	Limited	Post-translational	Yes	1-Step	36
TEV protease	Reporter gene activation [fluorescence, bioluminescence, molecular barcodes (i.e. RNA)]	Yes	Yes, but limited to membrane and cytosol	Transcriptional, post-translational	Limited for TF-based readouts; delay of 30–60 s in proteolysis-only	2-Step (fluorescence, barcodes); 3-step (luciferases)	25
Thymidine kinase	Positron emission tomography (PET), radiolabelled reporter probes	Yes	Yes	Post-translational	Yes	1-Step	41
Ubiquitin	Reporter gene activation [fluorescence, bioluminescence, molecular barcodes (i.e. RNA)]	Yes	Yes, but limited to membrane and cytosol	Transcriptional	Limited	1-Step (fluorescence, barcodes); 2-Step (luciferases); needs UBPs from host cell	9

Split biosensor	Organism						Refs
	Mice	Mammalian cells	Insect cells/*Drosophila*	*Saccharomyces cerevisiae*	Bacterial cells	Lysates	
β-Galactosidase	–	Yes	–	–	Yes	Yes	[17–20]
β-Lactamase	–	Yes	–	–	Yes	Yes	[14]
Click beetle luciferases	Yes	Yes	–	–	–	Yes	[27–29]
DHFR	–	Yes	–	Yes	Yes	–	[11,13,99]
Firefly luciferase	Yes	Yes	Yes	Yes	Yes	Yes	[22,23,71,100]
***Gaussia* luciferase**	Yes	Yes	–	–	–	Yes	[26,75]
GFP and variants (BiFC)	Yes	Yes	Yes	Yes	Yes	Yes	[15,16,56,57,59,61–64]
***Renilla* luciferase**	Yes	Yes	–	Yes	Yes	Yes	[24,25,101,102]
TEV protease	–	Yes	Yes	–	–	Yes	[32,37,41,95]
Thymidine kinase	Yes	Yes	–	–	–	–	[21]
Ubiquitin	Yes	Yes	–	Yes	–	Yes	[30,31,82]

Abbreviations: BiFC, bimolecular fluorescence complementation; DHFR, dihydrofolate reductase; GFP, green fluorescent protein; PPI, protein–protein interaction; TEV, tobacco etch virus; UBPs, ubiquitin-specific proteases.

[21], firefly luciferase [22,23], *Renilla* luciferase [24,25], *Gaussia* luciferase [26], click beetle luciferases [27–29], ubiquitin [30,31] and the TEV protease [32]. The biosensors are commonly dissected to yield separate functional subdomain structures (dissection points summarised in Table 2). Notably, for some biosensors, various dissection points are possible that have an impact on readout strength and robustness, as for example shown for bimolecular fluorescence complementation (BiFC) [33]. Below, we discuss the most widespread assays that are based on GFP and luciferases as well as the split ubiquitin and split TEV techniques,

TABLE 2

Split positions and protein fragments of split biosensors used in cell-based assays.

Split biosensor	Split position(s)	N-terminal fragment	C-terminal fragment	Refs
β-Lactamase	196/198	26–196	198–290	[14]
β-Galactosidase	788/789	1–788 (α)	1–1024 (Δ 11–41) (ω)	[17,18]
β-Galactosidase	51/55	5–51 (H31R) (α)	1–1024 (Δ 11–41) (ω)	[19,20]
BiFC, GFP	157/158	1–157	158–238	[15]
BiFC, GFP, YFP, CFP, Venus, Cerulean	154/155; 172/173	1–154; 1–172	155–238; 173–238	[16,55]
BiFC, mCherry	159/160	1–159	160–237	[53]
BiFC, optimised mVenus	210/211; 209/210	1–210	210–238	[61]
Click beetle luciferase, green	413/414; 393/394	1–413	394–542	[28]
Click beetle luciferase, green	415/416; 394/395	1–415	394–542	[29]
Click beetle luciferase, red	439/440	1–439	440–542	[27]
Click beetle luciferase, red	414/415; 394/395	1–414	395–542	[28]
DHFR	105/106	1–105	106–186	[13]
Firefly luciferase	437/438	1–437	438–554	[23]
Firefly luciferase	416/417; 397/398	2–416	398–550	[22]
***Gaussia* luciferase**	93/94	1–93	94–185	[26]
GFP, superfolder	214/215	1–214	215–229	[65]
GFP, superpositive	157/158	1–157	158–238	[62]
GFP, tripartite split	212/213, and GFP detector (1–193)	194–212	213–233	[64]
***Renilla* luciferase**	91/92	1–91	92–311	[24]
***Renilla* luciferase**	229/230	1–229	230–311	[40]
***Renilla* luciferase**	110/111	1–110	111–311	[36]
TEV protease	118/119	1–118	119–221 (S219P)	[32,37]
Thymidine kinase	265/266	1–265	266–376	[21]
Ubiquitin	37/38; 34/35	1–37 (13I)	35–76	[30,31,76]

Abbreviations: BiFC, bimolecular fluorescence complementation; DHFR, dihydrofolate reductase; GFP, green fluorescent protein; YFP, yellow fluorescent protein; CFP, cyan fluorescent protein; TEV, tobacco etch virus.

which can be flexibly adapted to various readout options and allow integration into multiplexed cell-based assays.

Controls

A crucial feature of split biosensors is that the dissected fragments do not spontaneously associate in the absence of a binding partner, because this would render the method useless owing to a high number of false positives. Studying protein interactions using protein complementation often involves overexpressing the biosensor fusions at rather high levels, because the biosensors are commonly introduced into cells by transfection, infection or stable integration. Therefore, it is advisable to include appropriate controls (i.e. proteins that do not bind to the candidate of interest) into an SBA, and to express the biosensor fusions at low levels, possibly close to those of endogenous counterparts.

Linkers between biosensor fragments and candidate proteins

The interaction partners of interest and the biosensor fragments are usually separated by a short linker sequence to guarantee optimal flexibility for the biosensor fragments to fold. Flexible linkers are rich in nonpolar glycine and polar serine residues [34]. The small size of these amino acids provides flexibility, whereas the incorporation of serine residues confers stability of the linker in aqueous conditions by establishing hydrogen bonds with water molecules. The GS linkers typically used for SBAs comprise short amino acid sequences covering roughly eight to ten amino acids or a few more, such as GGGSGGGS [35] or GGGGSGGGGS [36,37]. Semi-flexible linkers that exhibit a slightly more rigid structure and read ASPSNPGASNGS have also been applied to reduce background activity levels in SBAs [38,39]. For an intramolecular SBA, a rigid linker containing two or even four repeats of the sequence EAAAR was used to maintain a distance between interaction domains, a setup that significantly improved the signal-to-noise ratio of the assay [40]. Notably, flexible linkers tested for maintaining the distance did not prove successful. In detail, the fusion protein for the intramolecular SBA was constructed using the rapamycin-inducible interaction partners FK506-binding protein (FKBP) and FKBP-rapamycin binding domain of mammalian target of rapamycin (mTOR) kinase (FRB), which were separated by the rigid linker sequence repeats EAAAR. FKBP and FRB were each fused at its other end to split *Renilla* or split enhanced GFP (EGFP) fragments that were separated from the interacting partners by flexible GGGGSGGGGS linkers to guarantee optimal split biosensor complementation. These findings demonstrate that an adequate linker with defined characteristics (i.e. flexibility vs rigidity) is required for a specific function. Thus, flexible linkers allow

complementation of biosensor fragments fused to interaction partners whereas rigid linkers maintain distances between interaction partners.

Gateway recombination cloning is frequently used to swap cDNAs encoding the open reading frames of interaction partners into expression plasmids for PPI studies. When using Gateway® technology, a defined sequence of attB sites produces, at least in part, the linker sequence. Notably, split TEV-based approaches worked with attB sites in combination with either semi-flexible ASP linkers (ASPSNPGASNGS) or flexible GS linkers (GGGGSGGGGS) [32,37,41]. Likewise, split YFP sets were reported to work in combination with flexible GS linkers (GGGSGGGS) [42].

Methods complementary to split biosensors

Regulated protein interactions can also be monitored using alternative genetic methods that are complementary to SBAs. These methods often use full-length versions of the genetically encoded biosensors introduced above and include Förster or fluorescence resonance energy transfer (FRET) assays [43], bioluminescence resonance energy transfer (BRET) assays, for example between GFP and *Renilla* luciferase [44], a luminescence-based mammalian interactome mapping assay (LUMIER) based on co-immunoprecipitations of FLAG-tagged and luciferase-tagged proteins [45], mammalian PPI trap (MAPP-IT) assays, based on cytokine I signalling and defective cytokine receptor chimeras [46] and full TEV assays [47]. In addition, the co-immunoprecipitation (co-IP) approach, which is frequently applied as a complementary biochemical assay, uses either tagged or untagged proteins for the pull-down and the probing steps [48]. Although co-IPs are strongly dependent on the epitope-binding capabilities of the antibodies used and have a number of other limitations, such as sensitivity, robustness, reproducibility and automation capacities, they enable the study of endogenously expressed proteins.

Split biosensor assays for targeted DNA recombination and gene regulation

Split protein complementation approaches were also developed for DNA recombinases, such as Cre and Cas9 [38,49,50]. These split recombinases provide further options spatially and temporally to control the expression of genes, in particular when the N- and C-terminal fragments are fused to inducible PPI systems, such as the rapamycin-inducible interaction of FKBP and FRB. Recombination-deficient split Cas9 variants might not only be combined with the FKBP–FRB system but also with artificial transactivation or repressor domains to regulate genes of interest specifically [50].

Split protein approaches to study regulated PPIs in living cells

SBAs based on GFP (or its variants) and luciferases became the most popular approach used in laboratories, because fluorescent or luminescent reporter activities can be easily captured and quantified and also allow studying interaction events *in vivo*. In particular, luciferase-based SBAs are suitable to monitor reversible interactions and provide online kinetics of interaction events [22,36]. By contrast, SBA strategies that use transcriptional readouts, such as split TEV or split ubiquitin, are very robust and provide a flexible selection of readouts, which can, for example, be based on but not limited to fluorescence or bioluminescence [30–32]. These approaches also have the advantage of separating the actual interaction event from the readout itself. In addition, users could opt for molecular barcodes as transcriptional reporters, making the assay amenable to highly multiplexed formats by using NGS as the readout tool, thus providing the capabilities to monitor multiple signalling events in parallel. Based on these popular cutting-edge characteristics described above, we therefore focus on SBAs using fluorescent proteins, luciferases, ubiquitin and the TEV protease within the following sections.

Split GFP assays and variants

The split protein technique for GFP and its variants is known as BiFC, and it became the most widespread SBA technique because of its ease in technology transfer. GFP was first split into N-xFP 1–157 and C-xFP 158–238 [15]. In more advanced versions of BiFC, the GFP, YFP, CFP, Venus and Cerulean were split either between residue 154–155 or 172–173. The signal-to-noise ratios of blue- and green-shifted BiFCs were improved when biosensor fragments with a small overlap region were used (i.e. N-xFP 1–172 and C-xFP 155–238) [16,33,51,52]. For a red BiFC system, the red fluorescent protein mCherry is split between positions 159 and 160 [53]. The BiFC method proved very useful to monitor the interaction of binding partners and the subcellular localisation of the event in living cells simultaneously. BiFC was also used to detect transient and weak interactions owing to the quick and irreversible formation of fluorescence intensity, a finding that was particularly reported for the split Venus biosensor [54,55]. Therefore, the split Venus sensor was successfully applied to PPI screening approaches. The BiFC technique also allows the simultaneous visualisation of various PPIs using a multicolour approach, which has been shown for mammalian cells and living animals [16,56,57]. This multicolour BiFC exploits the ability of the various fluorescent protein fragments to form a biosensor with different spectral characteristics. Kinetic analyses, however, are limited because of the delayed off-kinetics observed in this technique. Furthermore, other disadvantages such as photobleaching, phototoxicity and autofluorescence have been reported for fluorescent proteins [58]. Some BiFC biosensors, such as split YFP, exhibit temperature sensitivity and fold best at 30 °C, which precludes optimal results in mammalian cells. To improve these limitations of split YFP, mutations were introduced and enhanced folding was observed in the Venus and superfolder YFP biosensor variants [51,59,60]. The split Venus biosensor was further optimised by systematically testing N- and C-terminal Venus fragments for a more efficient and improved complementation. Fragments were constructed according to the β-barrel structure, with the Venus protein dissected between β-strands. The best pair of split fragments yielded a large N-Venus fragment (1–210) covering β-strands 1–10 and a small C-Venus fragment (210–238) covering β-strand 11 [61]. Using this split Venus biosensor, the signal-to-noise-ratio of measured PPIs was substantially improved, as demonstrated for the interaction between cofilin and actin. Likewise, the initial split GFP biosensor was optimised by improving folding efficiency and solubility as well as reducing self-assembly-caused background fluorescence, resulting in biosensor variants that displayed an enhanced signal-to-noise ratio, such as the split superfolder GFP, split-superpositive GFP and tripartite split GFP biosensors [62–65].

A strong limitation of split xFP techniques to consider is that, in conditions of elevated expression of N-xFP and C-xFP moieties, the xFP halves are prone to associate readily, resulting in false-positive fluorescent signals, as shown for split YFP in mammalian cells [66]. Therefore, it is essential to adjust the expression levels of both candidate fusions to avoid unspecific interactions. One potential strategy to circumvent excessive expression levels and the variability in gene dosage between candidates is the use of dual, or ratiometric, expression systems, which also include an internal fluorescence marker for optical expression control [42]. Conversely, the self-complementing feature of split GFP and its variants was exploited to analyse the topology of integral membrane proteins in plant cells [67].

Receptor activities can also be monitored using BiFC, as for example shown for the ERBB RTK family using the split Venus assay [68]. The first example reported for GPCRs was the measurement of regulated activities of the β2-adrenoceptor using a β-arrestin2 recruitment split YFP assay [59]. This particular assay used overlapping fragments of split Venus and proved to be very sensitive and robust in a HTS application. However, BiFC β-arrestin2-based assays for GPCRs are also largely irreversible and display delayed responses as a result of the association and maturation characteristics of the split biosensor fragments, thus precluding real-time kinetic monitoring of receptor activities [69]. Further, the split YFP assay was successfully applied to assess dimer formation of GPCRs, because this assay, in combination with a split Cerulean assay, was used to visualise the formation of adenosine A_{2A} and dopamine D_2 receptor homo- and hetero-dimers simultaneously in living neuronal cells and to monitor drug-induced changes in receptor dimers [70].

Split luciferase assays

Firefly and *Renilla* luciferases are the most widespread luminescent reporters. They can be applied to live-cell assays and, owing to their high sensitivity and simple technical implementation, are frequently used in high-throughput applications. For obtaining a bioluminescent signal, split luciferase assays require a substrate to be added to the assay, a prerequisite that contrasts with the BiFC assay. By contrast, major advantages of the luciferase assay are its superior sensitivity and reversible reassembly. Therefore, split luciferase assays are particularly suited to monitoring dynamic PPIs with close-to real-time kinetics [22,36].

The firefly luciferase (*Photinus pyralis*) converts the substrate D-luciferin in the presence of Mg, O_2 and ATP into light. The first split luciferases were designed by Paulmurugan *et al.* and Ozawa *et al.* [23,71]. However, for most sensitive split firefly luciferase assays, the N-terminal fragment covers residues 2–416 and 398–550 for the C-terminal fragment [22]. Optimisation experiments showed that these minimally overlapping open reading frames confer the best signal-to-noise ratio for split firefly SBAs, with a 1200-fold signal over background as determined by using the rapamycin-inducible interaction between FKBP and FRB. Importantly, unfused N-Fluc and C-Fluc moieties showed no enhanced activity compared with nontransfected cells, which supports the notion that background bioluminescence is very low because firefly luciferase fragments do not self-complement. The arrangement of the biosensor domain to the individual candidate protein could be crucial for monitoring a given interaction and should be tested to yield optimal readings, as demonstrated for the interaction between the Rho GTPase CDC42 and the GTPase-binding domain (GBD) of the effector WASP using a split firefly luciferase assay [72]. In addition, a self-complementing SBA was developed for the firefly luciferase. For such a setup, the optimally reconstituting firefly open reading frames were substantially overlapping for the N-Fluc (1–475) and C-Fluc (265–550) fragments [73]. Reconstituted firefly activity displayed 4% activity when compared with the full-length luciferase. This assay could be useful for the automated detection of two simultaneous promoter activities or the exploration of protein compartmentalisation, such as the monitoring of synapse formation, with the extracellular expression of membrane-bound N-Fluc in the dendrite and membrane-bound C-Fluc in the axon.

The *Renilla* luciferase (*Renilla reniformis*) requires coelenterazine as a substrate and O_2 to generate light photons. For a sensitive and robust complementation, the *Renilla* luciferase was dissected between residues 91 and 92, yielding N-Rluc (1–91) and C-Rluc (92–311) fragments [24]. The *Renilla* luciferase was also successfully split between residues 110–111 and 229–230, with the first one resembling the original dissection and the latter resulting in a larger N-Rluc (1–229) and smaller C-Rluc (230–311) fragment [36,40]. Notably, the bursting behaviour of *Renilla* causes a significant limitation on time-course-based image acquisition, in particular when applied *in vivo*. Conversely, the firefly luciferase is pH-sensitive and temperature-sensitive and undergoes a red-shift at lower pH values or increasing temperatures, precluding an optimal readout under adverse conditions. Therefore, alternative luciferases were engineered for SBAs and are based on the *Gaussia* luciferase (*Gaussia princeps*) and the click beetle luciferases (*Pyrearinus termitilluminans* emitting green light; *Pyrophorus plagiophthalamus* emitting red light) [26–28].

The *Gaussia* luciferase uses coelenterazine as a substrate, which is limiting to live-cell assays. Striking advantages are, however, its small open reading frame of 20 kDa and the 100-fold higher bioluminescent signal compared with *Renilla* luciferase [74]. Screening a library of potentially complementing fragments identified that the most effective SBA was achieved when the *Gaussia* was dissected between residues 93 and 94 [26]. Split *Gaussia* was later applied to monitor the activities of drug-inhibited chemokine receptor (CXCR)4 and CXCR7, in cell-based assays and in living mice [75].

The click beetle luciferases have the advantage that they are much brighter than the firefly luciferase and generate at least a tenfold higher bioluminescent signal using D-luciferin as the substrate [76]. Further, click beetle luciferases are pH-insensitive, and in particular the red click beetle luciferase is suited for live-cell imaging and *in vivo* studies, because this luciferase emits a stable red-shifted light, which is close to near-infrared and highly tissue transparent [77]. For SBAs, the sensitivity and signal-to-noise ratio of the enhanced green click beetle luciferase biosensor was optimised, resulting in the preferred combination of N-terminal enhanced green luciferase (N-ELuc) 1–415 and C-terminal C-ELuc 394–542 fragments producing the highest signals [29]. SBAs involving the combined use of green and red click beetle luciferases offer an important feature because two interaction events can be simultaneously assayed using a single substrate [78]. Activities of drug-relevant targets, for instance GPCRs using a β-arrestin2

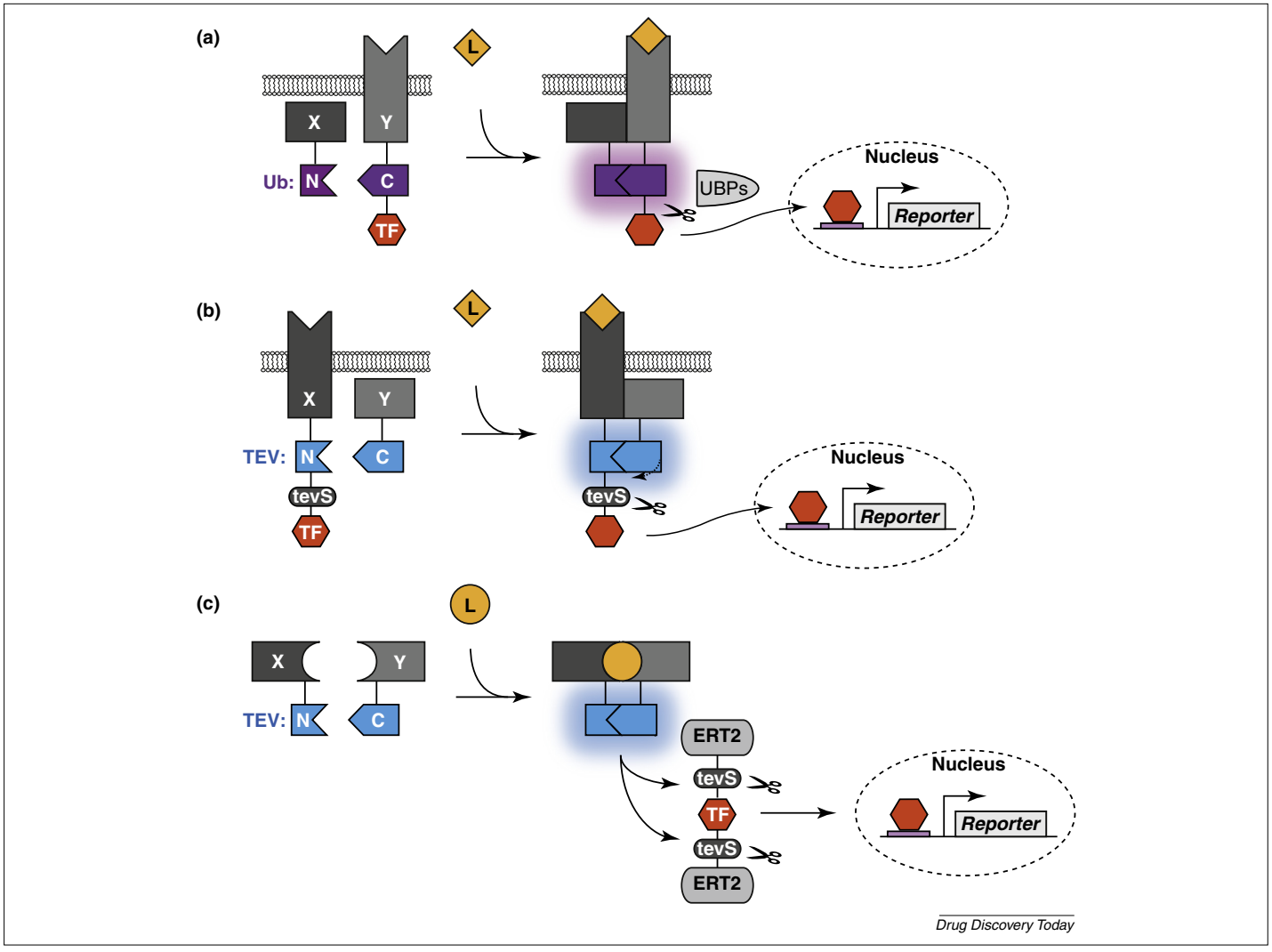

FIGURE 2

Split protein biosensors using transcriptional reporters for a sensitive, robust and flexible readout. **(a)** The split ubiquitin system. N- and C-terminal fragments (N, C) of ubiquitin (Ub) are fused to candidate proteins X and Y, respectively. Y is preferably a receptor or membrane-bound protein. A chimeric transcription factor (TF) is fused to the C-terminal fragment of ubiquitin. When ubiquitin is reassembled upon a ligand (L)-induced interaction event (indicated by outer glow), ubiquitin-specific proteases (UBPs) cleave off TF, which migrates into the nucleus to activate a reporter gene of choice. **(b)** The split TEV technique for protein–protein interactions (PPIs) at the membrane. N- and C-terminal fragments (N, C) of the TEV protease are fused to candidate proteins X and Y, respectively. An artificial TF is fused via a TEV protease cleavage site (tevS) to the N-terminal TEV protease fragment. Once the TEV protease activity is reconstituted (indicated by outer glow), TF is released and travels into the nucleus to activate a reporter gene. **(c)** The split TEV technique for soluble PPIs. To capture PPIs occurring in the cytosol, the TEV reporter comprises a separate molecule, wherein the central TF unit is flanked by two oestrogen receptor T2 (ERT2) domains and each linked via a tevS. The ERT2 domains cause TF to be sequestered in the cytosol. When both ERT2 domains are cleaved off by TEV proteolytic activity (indicated by outer glow), TF is released to activate a reporter gene. ERT2 is the mutated ligand-binding domain of the oestrogen receptor that does not respond to oestrogen any more.

recruitment assay, were monitored using split firefly luciferase [79] or split green click beetle luciferase [29], covering somatostatin 2 receptor (SSTR2), β2-adrenergic receptor (ADRB2), endothelin receptor type B (EDNRB) and cholecystokinin B receptor (CCKBR).

Split ubiquitin assays

Transcription-based SBAs offer flexible readouts, including fluorescence and bioluminescence, but preclude online kinetic measurements of PPIs. Currently, however, the most promising application for these assay types is their potential integration into multiplexed cell-based assays, which simultaneously allow the monitoring of many regulated protein interactions (including PPIs reporting receptor activities) and the assessment of downstream signalling events.

Split ubiquitin assays were introduced to analyse interactions of soluble proteins in living yeast cells [80]. Later, the technique was engineered to monitor regulated PPIs of membrane proteins in yeast, termed membrane yeast two-hybrid assay (MYTH) [30] (Fig. 2a). A similar approach using split ubiquitin was applied to detect PPIs of transcriptionally active proteins in the cytosol, now called cytosolic yeast two-hybrid system (cytoY2H) [81]. In this setup, transcriptionally active proteins are tethered as baits to the endoplasmic reticulum membrane, thereby preventing their transition to the nucleus and enabling the screening of interactions independently of their transcriptional activity. Limiting here, however, is that the interactions monitored are not occurring in the native environment of transcription factors (TFs). The most recently reported development, termed mammalian-membrane

two-hybrid assay (MaMTH), was designed to assess regulated PPIs and activities of cell-surface receptors, such as RTKs and GPCRs, in mammalian cells [82]. We would like to draw the reader's attention to the terminology used here. MYTH, cytoY2H and MaMTH assays are, strictly speaking, SBAs, or PCAs, but nomenclature implies that these are two-hybrid approaches. Therefore, the names of these assays might cause some confusion (see introduction for detailed explanation) [12].

For MaMTH assays, human ubiquitin, a small conserved protein of 76 amino acids in size, is split into two fragments termed NubI (residues 1–37, with I denoting an isoleucine at position 13) and Cub (residues 35–76). Candidate membrane proteins are fused with their C terminus to the N terminus of Cub, which is also linked by its C terminus to a chimeric TF. NubI is either fused to a membrane or a cytosolic protein, allowing the study of dimerisation or adapter recruitment events, respectively. Notably, in mammalian cells, NubI performed better in control interaction assays than NubG, which is the preferred N-terminal fragment in the yeast system. Candidate proteins linked to ubiquitin fragments via glycine–serine linker (GGGGSGGGGS) showed increased efficiency in complementation. The two transcription factors identified to perform best comprised: (i) the GAL4 DNA-binding domain fused to the activation domain of nuclear factor (NF)κB (GAL4–NFκB); and (ii) a mutated LexA DNA-binding domain fused to the activation domain of VP16 (LexA–VP16). GAL4–NFκB binds to five GAL4 upstream activating sequences, and LexA–VP16 binds to eight *lexA* operator repeats to drive the expression of a *GFP* or firefly luciferase reporter gene. When an interaction of the candidate proteins occurs, NubI and Cub complement to form a ubiquitin moiety, which is recognised by ubiquitin-specific proteases (UBPs), resulting in the liberation of TF and the activation of the reporter gene.

Most protein complexes are probably oligomeric rather than formed of binary interactions between two proteins. To study trimeric protein complexes, the split ubiquitin technique was engineered into a split ubiquitin bridge assay, wherein two candidate proteins X and Y that do not interact are brought into one binding complex by a third candidate Z, termed the bridge [83]. Because candidates X and Y are fused to the split ubiquitin moieties, only the addition of the bridge facilitates the complementation of ubiquitin, resulting in reporter activity. This assay technique testing for ternary interactions was initially shown for the yeast proteins SYP121, KC1 and AKT1, with KC1 representing the bridge [84]. Further, a split ubiquitin-based three-hybrid system was developed to identify pharmacologically relevant interactions between proteins and small molecules [85]. This approach is based on a hybrid compound that acts as a chemical inducer of binding between two candidate proteins fused to split ubiquitin moieties. This method comes, however, with the major limitation that hybrid compound ligands have to be chemically synthesised, which might be difficult or unaffordable to achieve depending on the nature or availability of the compound of interest.

Apart from the limitations discussed on kinetic analyses, the split ubiquitin approach comes with the constraint of employing the endogenously expressed UBPs for cleaving TF, which in turn activates the reporter gene of choice. The activity of the UBPs can be hard to control, particularly when screening chemical compound libraries.

Split TEV assays

Split TEV, a method that is based on the protease fragment complementation of the tobacco etch virus (TEV), can be used robustly and sensitively to detect regulated PPIs in living cells [32]. The technique can be applied to analyse dynamic PPIs occurring in the cytoplasm and at the membrane using a modular approach of various reporters, including fluorescent and luciferase reporters. Notably, the split TEV technique was specifically designed to analyse PPIs occurring at the membrane and in the cytosol, but

BOX 1

Guideline for selecting reporters using split TEV biosensor assays

Split TEV biosensor assays are designed to select flexibly between two reporter classes, which are either of transcriptional or proteolysis-only (i.e. post-translational) nature (Figure Ia). Both classes can be applied to either membrane or cytosolic interactions, and should be chosen according to the biological and technical question addressed, such as sensitivity or kinetic measurements. Commonly, transcriptional reporters yield higher assay sensitivity and robustness by spatially uncoupling the readout event from the interaction. Conversely, proteolysis-only reporters provide a fast response of an occurred interaction, also enabling close-to online measurements.

When, for example, assessing protein interactions of receptors, membrane-associated proteins or candidates strictly localised to the cytosol, the user can opt for the transcriptional reporter type to guarantee best robustness and sensitivity of the assay. For the candidates mentioned above, it is also useful to link the NTEV fragment via a TEV protease cleavage site (tevS) to an artificial co-transcriptional activator [i.e. Gal4–VP16 (GV)], to form an NTEV-tevS-GV tag (Figure 2b). Furthermore, selecting a transcriptional reporter format enables flexible switching among readouts, (i.e. fluorescence, luminescence or molecular barcodes).

Transcriptional reporters for flexibly using fluorescent, luciferase-based and molecular barcode reporters

Transcriptional reporters offer the largest variety of readouts. For split TEV assays: fluorescence, bioluminescence and molecular barcodes were tested. However, it is conceivable, that other reporters, such as resistance genes, can be easily implemented as well. When choosing fluorescent reporters as the transcriptional readout, such as GFP or mCherry, the assay will include two amplification steps (proteolytic cleavage, transcriptional activation). Our experience indicates that for many interactions this procedure is sufficient to obtain a stable readout [32]. However, for weaker interactions, we suggest to include a third step of amplification using enzymes as final reporters (i.e. firefly luciferase). This approach has been shown to be invaluable for screening purposes [37].

Proteolysis reporters for close-to real-time assays

Proteolysis-only reporters are designed to quench the activity of fluorescence (DsRed) or bioluminescence (firefly luciferase) and to release the activity once activated through TEV-mediated cleavage (Figure Ib–e). As stated before, a transcriptional readout precludes online measurements. However, the proteolysis-only reporters allow close-to real-time measurements, because a signal of the rapamycin-induced FK506-binding protein (FKBP)/FKBP-rapamycin binding domain of mammalian target of rapamycin (mTOR) kinase (FRB) interaction was detectable within one minute using a cytosolic luciferase proteolysis-only reporter [32].

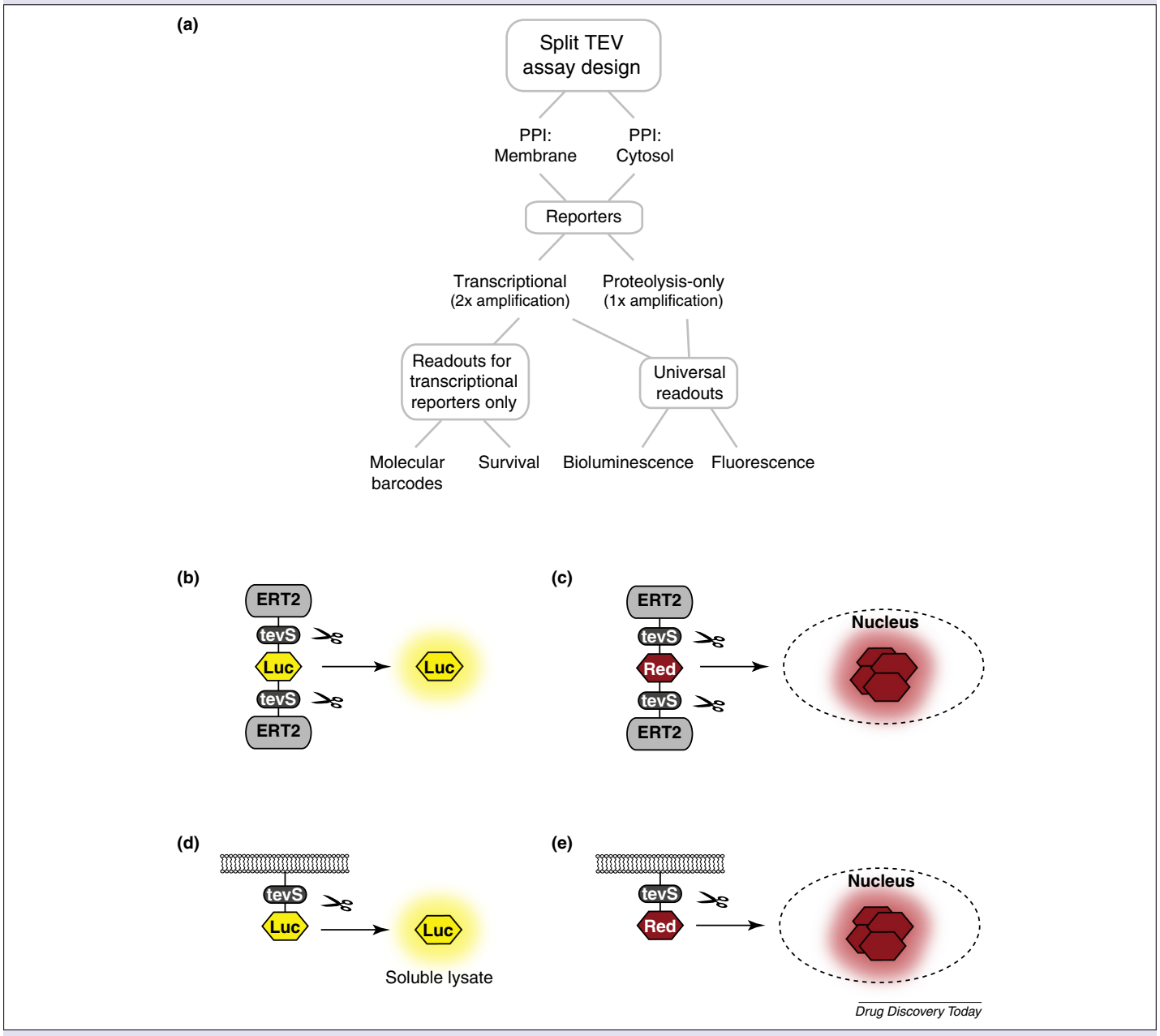

FIGURE I

A guideline for selecting reporters for split TEV biosensor assays. **(a)** Graphical representation of how to design a split TEV assay. Note that two different types of TEV reporters (i.e. transcriptional, proteolysis-only) are available, and that bioluminescence and fluorescence readouts exist for all reporters. **(b)** Luciferase proteolysis-only reporter for the cytosol. The activity of a firefly luciferase moiety (Luc) is quenched by two flanking oestrogen receptor T2 (ERT2) domains that are fused to Luc via TEV protease cleavage sites (tevS). Upon TEV proteolytic activity, the Luc is liberated and ready to convert D-luciferin and ATP into light signals (indicated by outer glow). **(c)** Fluorescence proteolysis-only reporter for the cytosol. Like in (b), the fluorescent activity of a DsRednuc moiety (Red) is quenched by the flanking ERT2 domains that are each linked via a tevS. Upon TEV proteolytic activity, Red is liberated, travels into the nucleus, forms a tetramer and emits a nuclear fluorescent signal (indicated by outer glow). **(d)** Luciferase proteolysis-only reporter for the membrane. A Luc moiety is fused via tevS to a membrane anchor or a membrane protein. Upon TEV proteolytic activity, Luc is liberated (indicated by outer glow). To obtain a good signal-to-noise ratio, only soluble firefly is measured from lysates. **(e)** Fluorescence proteolysis-only reporter for the membrane. A DsRednuc moiety (Red) is fused via tevS to a membrane anchor (or a membrane protein) and displays fluorescent signals at the membrane. Upon TEV proteolytic activity, Red is liberated, travels into the nucleus and emits a nuclear fluorescent signal (indicated by outer glow). *Abbreviation*: PPI, protein–protein interaction.

not in the nucleus. Split TEV allows the usage of one-step or two-step reporter systems, facilitating a flexible format that includes a strong amplification of signals.

For the split TEV assay, an optimised form of the TEV protease is dissected into an N-terminal fragment (NTEV, residues 1–118) and a C-terminal fragment (CTEV, residues 119–221, with residue 219 changed to proline for enhanced assay stability) [37]. The wild-type TEV protease has been further improved by introducing a stabilising mutation S219P to remove inhibitory autocatalysis [86]. In addition, the optimised form is truncated after amino acid 221 to remove the inhibitory C-terminal tail [87]. In agreement, a CTEV moiety truncated after residue 219 also displayed increased

assay sensitivity [88]. NTEV and CTEV moieties are fused to candidate proteins, preferably to the C-terminal end of the candidates. For NTEV, N-terminal fusions are also functional. PPI-induced reassembly of the NTEV and CTEV fragments leads to the reconstitution of TEV proteolytic activity, which activates TEV-specific reporters. These reporters are either protein reporters (termed proteolysis-only reporters) or transcriptional reporters, and can be either used for interactions at the membrane or in the cytosol. A detailed guideline of how to select the most sensitive reporter system for a given split TEV biosensor assay can be found in Box 1.

Compared to non-transcriptional SBAs, the split TEV method, like split ubiquitin, offers another degree of flexibility because any reporter gene of choice can be applied as the readout, also including molecular barcode reporters that allow the integration into multiplexed assays [89]. In addition to a transcriptional amplification step, enzyme-based reporters (e.g. luciferases) allow a second step of amplifying the initial signal. Until now, we have applied fluorescence, bioluminescence and molecular barcode reporters (see below).

Split TEV assays for membrane and membrane-associated proteins

For PPI assays at the membrane, an artificial transcription factor, such as GAL4–VP16 (GV), is trapped in the cytosol by fusing it via a TEV protease cleavage site (denoted tevS, amino acid sequence ENLYFQ'G, where the TEV protease cleaves between Q and G) and the NTEV moiety to the candidate protein of choice, resulting in an NTEV-tevS-GV tag (Fig. 2b). Importantly, this protein should be a membrane or membrane-associated protein to avoid immediate translocation of GV into the nucleus, because this will readily increase background signals. The second candidate protein, either a membrane, membrane-associated or cytosolic protein, is fused to the CTEV fragment. Through an occurred interaction event, TEV proteolytic activity causes GV to be cleaved off. In turn, GV translocates into the nucleus and binds to its cognate upstream activating sequences (UAS) to activate a final reporter gene of choice, such as GFP or firefly luciferase.

Split TEV assays for soluble proteins

Assays designed to occur in the cytosol either use the NTEV-tevS-GV fusion mentioned above (with a prerequisite that the soluble protein does not enter the nucleus) or engage a specialised TEV protease reporter, which is based on the modified oestrogen receptor ERT2 that stays strictly cytosolic and is not responsive to endogenous oestrogen but to 4-hydroxytamoxifen (4-OHT) (Fig. 2c). The specific responsiveness to 4-OHT also allows for performing a simple technical control, because the cytosolic TEV reporter can be artificially activated by the addition of 4-OHT to the culture medium. Structurally, the cytosolic transcriptional TEV reporter consists of a central transcription factor unit, such as GV, that is trapped in the cytosol because it is flanked on either side by a TEV protease cleavage site and an ERT2 domain. Both interaction candidates are cytosolic and are either fused to the NTEV or CTEV fragment. Once an active TEV proteolytic activity occurs as a result of an established PPI event, the ERT2 domains are cleaved off, allowing GV to translocate into the nucleus to activate the final reporter gene.

Applications of split ubiquitin and split TEV biosensors in pharmacological cell-based assays

GPCR and RTK signalling are frequently deregulated in human diseases [90,91]. As cell surface receptors, they thus represent major drug targets that are easily accessible for cell-based assays [5,6]. Ligand- and drug-induced activities of GPCRs can be assessed by the regulated recruitment of β-arrestin2 to an activated GPCR, which leads to desensitisation of the GPCR [92]. Likewise, the activity of RTKs can be sensed by SH2-domain-containing adapter proteins that are recruited to phosphorylated tyrosine residues of activated RTKs [91]. SH2 domains specifically recognise the phosphorylation state of tyrosine residues, and thus represent, either as full-length protein or clustered domains, a sensor module to be used in split biosensor cell-based assays [93,94]. Key SH2 domain-containing adapter proteins include SHC (Src homology 2 domain containing) transforming protein 1 (SHC1), growth factor receptor bound protein 2 (GRB2), phosphoinositide-3-kinase regulatory subunit 1 (PIK3R1), signal transducer and activator of transcription (STAT) proteins, SRC (sarcoma) proto-oncogene (SRC), phospholipase C gamma 1 (PLCG1), and insulin receptor substrate 1 (IRS1), with the last one specifically binding to the activated insulin receptor. For the implementation of cell-based assays using split ubiquitin and split TEV systems, a given GPCR or RTK is fused to one biosensor fragment, and the adapter protein (i.e. β-arrestin2 or SHC1) is linked to the other fragment (Fig. 2a,b).

The modified split ubiquitin system MaMTH was used to study activities of GPCRs and RTKs [82]. First, MaMTH was applied in a GPCR/β-arrestin2 recruitment assay that monitored the binding of β-arrestin2 to the activated β2-adrenergic receptor upon addition of the agonist isoproterenol. The specificity of the assay was confirmed using a β2-adrenergic receptor variant containing the mutated phosphorylation sites for the GPCR kinase (GRK)2 and GRK6 sites. Introducing both mutations simultaneously caused the agonist-induced activity of the β2-adrenergic receptor to be completely abolished. MaMTH was also used to assess regulated and phosphorylation-dependent interactions of RTKs, such as ERBB receptors. Further, MaMTH was applied as the screening tool to identify dynamic PPIs, and it allows mapping phosphorylation sites that mediate adapter binding [i.e. GRB2 binding to epidermal growth factor receptor (EGFR)].

Split TEV was also applied to monitor GPCR activities caused by drug-induced changes using a β-arrestin2 recruitment assay approach [39,95]. To do so, the assay was slightly modified as the C-terminal intracellular domain of the vasopressin receptor 2 (V2R, amino acids 343–371) was inserted between each GPCR of interest and the NTEV moiety. Several GPCRs, including the vasopressin receptor AVPR1, the dopamine receptor DRD2, the β2-adrenergic receptor ADRB2 and the serotonin receptor HTR5A, were successfully tested in heterologous and primary cells. In addition to split TEV, a full TEV assay termed Tango assay was specifically developed to assess dynamic PPIs of membrane proteins, using, for example, a GPCR/β-arrestin2 assay [47]. We compared the full TEV approach with our split TEV method using the vasopressin receptor AVPR1, the dopamine receptor DRD2 and β2-adrenergic receptor ADRB2 in transient transfection assays and found that split

TEV-based assays performed with an increased sensitivity and an improved signal-to-noise ratio, when applied in heterologous cells types (PC12 and U2OS cells) and primary neurons [95]. Furthermore, a combination of a full TEV assay and a permutated split luciferase, termed the LinkLight™ assay, was applied to study GPCR and RTK activities using cell-based recruitment assays [96].

Split TEV was also used to monitor ligand-regulated dimer formation of ERBB family members [32]. In addition, split TEV was applied in an adapter protein recruitment assay to assess the ligand-induced activity of ERBB receptors. In such an assay, the SH2 domain of the adapters mediates the binding to phosphorylated tyrosine residues of ERBB receptors with high specificity [41,89]. For dynamic PPIs in the cytosol, split TEV was used to monitor the rapamycin-inducible interaction between FKBP and FRB, and phosphorylation-regulated interactions between proapoptotic Bad and 14-3-3 as well as *Drosophila* Yorkie, the orthologue of the Hippo pathway effector YAP, and 14-3-3 [32,37,41,88]. The Yorkie–14-3-3 split TEV interaction pair was also used as the readout for a genome-wide RNAi screen in *Drosophila* cells to uncover new modulators of Hippo signalling [37]. Further, the method was applied to validate various PPIs present in the conserved Hippo signalling pathway [37].

For the integration of the split TEV system into multiplexed cell-based assays, molecular barcodes were used as transcriptional readout to capture a multitude of PPIs simultaneously [89]. Molecular barcodes are short RNA reporters with a defined sequence, which can be reverse-transcribed into DNA, amplified and analysed using NGS. In such a setup, a specific barcode reporter with a distinct sequence is invariably linked to one PPI event. Experimentally, a multitude of PPI events are performed in parallel and can be simultaneously analysed using NGS, allowing the acquisition of large datasets. A similar multiplexing approach was reported for the DHFR split biosensor in yeast, which combines SBA technology with molecular barcoding through a selection based on survival [97]. This integrated method was applied to understand the dynamic nature of 238 PPIs within the yeast PPI network and to identify perturbations caused by 80 chemical modulators. Each molecular barcode reports a given PPI indirectly, because PPI intensity is assessed by barcode abundance caused by yeast strain survival. Conversely, transcriptional readouts that are mediated by artificial TFs, for instance when using the GAL4/UAS or LexA system, directly monitor a PPI.

Taken together, the split ubiquitin and split TEV methods were applied to various experimental scenarios. They allow (i) detection of full-length PPIs in living cells or in lysates thereof, (ii) detection of interactions in heterologous mammalian cells, primary cell types including primary neurons, astrocytes, embryonic stem cells (split TEV only) and *Drosophila* cells (split TEV only), (iii) detection of interactions localised in the cytosol (split TEV only), (iv) detection of interactions at the membrane, (v) detection of regulated interactions induced by intra- or extra-cellular stimuli, (vi) monitoring of kinetic aspects of interactions, such as in dose–response assays, (vii) setup as protein interaction readouts for HTS purposes and (viii) implementation of molecular barcodes as transcriptional reporters for multiplexed cell-based assays. It is therefore conceivable that split ubiquitin and split TEV can be adapted to industrial HTS setups, for example in early drug discovery.

Split biosensor assays for the drug development process

Cell-based assays are frequently used within the early stages of the drug discovery process to understand how the activities of drug-relevant targets and cellular signalling is altered upon pharmacological perturbation [98]. In addition, the evaluation of intricately regulated individual cellular events requires assays that can capture different types of interactions using flexible formats and readouts, preferably in parallel. Split biosensor assays offer versatile formats and readouts and are therefore ideal to monitor the activities of cellular targets.

Major drug targets, such as cell surface receptors, are particularly amenable to split biosensor systems, because their activities can be sensitively and robustly addressed in pharmacological studies using adapter recruitment assays (Fig. 3). Therefore, receptor–adapter recruitment assays using split biosensor technologies could become increasingly important in compound screening campaigns [59]. Furthermore, assays that can be combined with a multiparametric approach using molecular barcode reporters and NGS as the final readout represent primary applications for early drug discovery [89,97], for instance when addressing the selectivity and specificity of a given compound for a selected target (Fig. 3, lower panel). Split biosensors based on fluorescence (BiLC) and luminescence (BiFC) could be selected for the *in vivo* validation of drug candidates. When opting for a particular split biosensor method, the following key criteria should be addressed to select the appropriate assay technique.

Readout. The most widespread readouts are fluorescence and bioluminescence. However, the recent trend towards the simultaneous monitoring of multiple events requires the integration of molecular barcode reporters into SBA strategies. In addition, the user might want to have the opportunity to change between readouts types, a feature that is well represented in split ubiquitin and split TEV techniques. Notably, split TEV also allows the usage of transcriptional and post-translational reporters, which facilitate either an additional signal amplification step (transcriptional reporters) or close-to-online readouts (proteolysis-only reporters). By contrast, biosensors that are readily applicable *in vivo* (i.e. in mouse models) use bioluminescence, fluorescence or radiolabelled probes for readout.

Robustness. A strong signal-to-noise ratio is characterised by low background readings and a robust induction by a well-defined stimulus, as commonly determined using the rapamycin-inducible interaction between FKBP and FRB. Structure-assisted and systematic biosensor fragment screening resulted in optimised moieties used in various SBAs.

Kinetics. The assessment of real-time kinetics of PPIs is a prerequisite for understanding the overall dynamics of cellular signalling. Split luciferase assays are the preferred option here, although, with some limitations, BiFC assays (split Venus or optimised split GFP) and split TEV assays also allow close-to real-time monitoring of PPIs.

Localisation. BiFC approaches clearly provide the best option, because the fluorescence signal forms at the site of the interaction event and can be readily imaged using standard microscopy. Other methods, such as split luciferase and split TEV assays, only allow a limited discrimination between membrane and cytosol-localised PPIs.

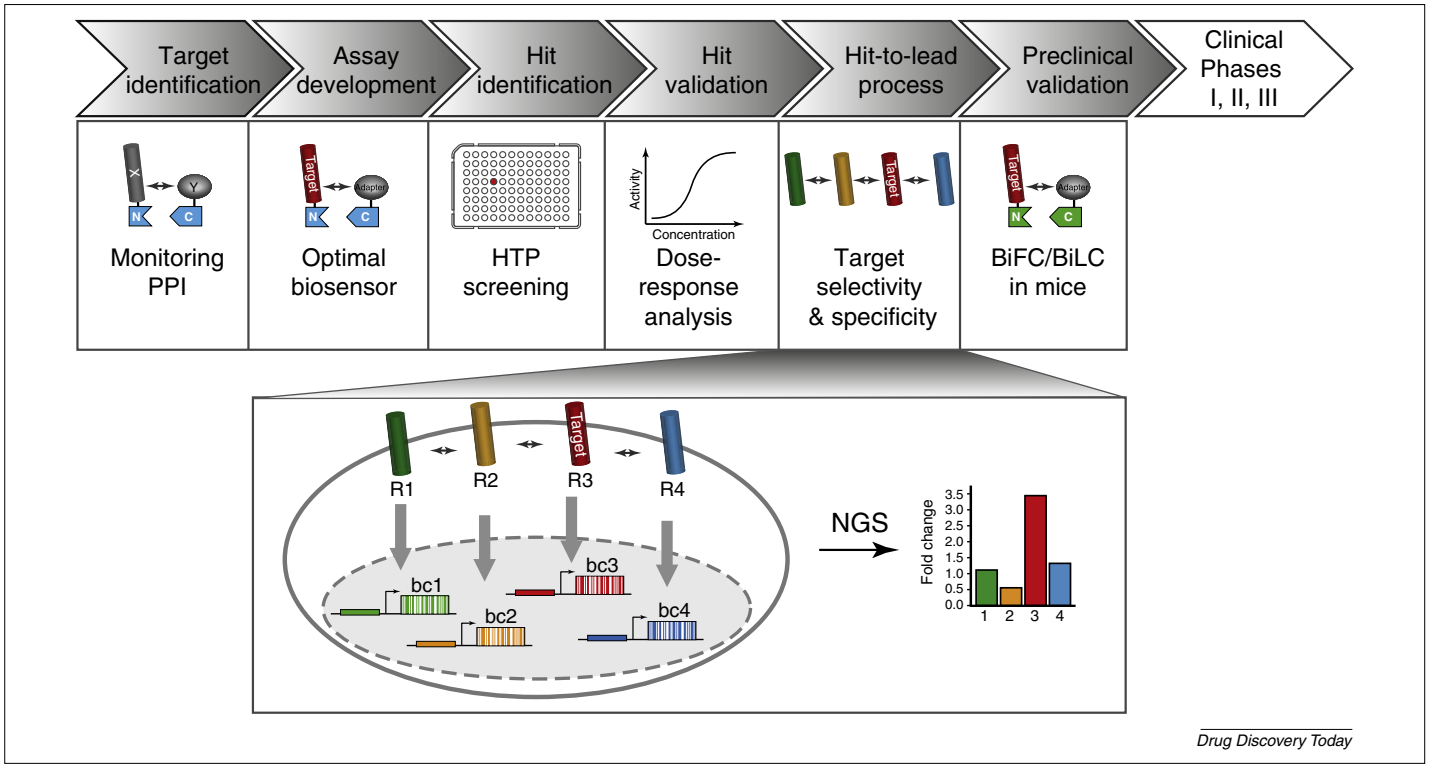

FIGURE 3

Applications for split protein biosensors in the drug development process. Split biosensors can be designed for various steps of drug discovery, including target identification, assay development, hit identification, hit validation, lead optimisation and preclinical validation. For target identification, split biosensor assays can help to understand how a potential target (X) is regulated within the cellular network by assaying protein–protein interactions (PPIs) with candidates (Y). When developing a cell-based assay, selecting a split biosensor assay with a broad window of activation or inhibition is paramount to run HTS campaigns and to validate candidates. Within the hit-to-lead process, the selectivity and specificity of a given target is characterised, employing split biosensor assays for the selected target and related ones. In the preclinical validation stage, bimolecular fluorescence complementation (BiFC) and bimolecular luciferase complementation (BiLC) can assist to corroborate target validity. Lower insert: more recently, split biosensors employing transcription-based readouts that combine monitoring PPI activity, molecular barcode (bc) reporters and next-generation sequencing (NGS) technology became attractive for an integrated target specificity analysis. Cell surface receptors (R1–R4) are prime candidates as targets. A selected target receptor (R3, red) and three related targets (R1, green; R2, yellow; R4, blue) are tested for their activity caused by chemical perturbation. Activities of each target is reported by specified molecular barcode reporters (bc1–4, matching colour code to targets) and analysed using NGS.

High-throughput applications. Luciferase-based strategies are currently the preferred choice, because luminescent substrates based on d-luciferin can be self-made and therefore provide a cost-effective solution. To capture weak and transient interactions as well, it is generally suggested to apply a technique that provides a strong amplification of signals and a robust signal-to-noise ratio in a 96-well or 384-well format, as established, for example, for the split TEV and split ubiquitin approaches that use transcriptional reporters [37]. In addition, split β-galactosidase-based assays and BiFC-based assays are applicable to high-throughput approaches. However, these assays have a limited degree of signal enhancement owing to a lacking amplification step based on transcription. For the pharmacological industry and academic high-throughput centres, it might also become invaluable to analyse the effects of small chemical compound libraries or numerous biologics in parallel. Therefore, molecular barcode reporter techniques have been developed that are amenable to NGS, and thus allow the simultaneous analysis of multiple cellular events, such as PPIs, in parallel. Such an application has been described for the split TEV technique [89], and is also conceivable for other SBAs using transcriptional reporters, such as split ubiquitin.

Flexibility of use. Split ubiquitin and split TEV techniques cover the largest collection of readouts available (Fig. 1). Whereas split ubiquitin is engineered to monitor membrane-based PPIs specifically, split TEV facilitates the monitoring of membrane and cytosolic interactions (Fig. 2). In addition, split TEV allows studying limited aspects of kinetics and localisation. The split TEV technique has been robustly applied using transient transfection, also to primary cultured cells including neurons, thereby providing the option to study the impact of a given PPI readily in the context of health and disease (i.e. in primary neurons generated from induced pluripotent stem cells, which are either derived from patients or healthy individuals). Therefore, the high flexibility of split TEV assays could represent a helpful feature to preserve the contextual specificity, which is largely determined by the cell type. For instance, the versatility of the technique appears to be highly important when studying GPCR pharmacology, because the vasopressin receptor AVPR1/β-arrestin2 split TEV assay performed robustly in PC12 cells and primary neurons, but not in U2OS cells [95]. By contrast, ligand-induced activation of dopamine receptor DRD2 was well observed in U2OS cells and primary neurons, but not in PC12 cells.

Concluding remarks

The precise measurement of protein interactions is essential for understanding the complexity of cellular signalling. Taken together, assay techniques based on split biosensor complementation represent an invaluable tool to monitor dynamic PPIs and receptor activities in cell-based assays. Future applications could involve the generation of more-effective split biosensors that would allow sensitive and kinetically robust measurements of protein interactions *in vitro* and *in vivo*. A first step towards increased output is the implementation of multiplexed assay formats, providing the simultaneous measurement of multiple interaction events. The current technologies and their projected improvements will foster the understanding of the underlying mechanisms of cellular signalling, protein interaction networks and drug interactions, potentially supporting new avenues towards better therapeutic interventions.

Conflicts of interest

M.C. Wehr and M.J. Rossner are co-founders and shareholders of Systasy Bioscience GmbH, Munich, Germany.

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