Chapter 14

Characterizing Dynamic Protein–Protein Interactions Using the Genetically Encoded Split Biosensor Assay Technique Split TEV

Jan P. Wintgens, Moritz J. Rossner, and Michael C. Wehr

Abstract

Dynamic protein–protein interactions (PPIs) are fundamental building blocks of cellular signaling and monitoring their regulation promotes the understanding of signaling in health and disease. Genetically encoded split protein biosensor assays, such as the split TEV method, have proved to be highly valuable when studying regulated PPIs in living cells. Split TEV is based on the functional complementation of two previously inactive TEV protease fragments fused to interacting proteins and provides a robust, sensitive and flexible readout to monitor PPIs both at the membrane and in the cytosol. Thus, split TEV can be used to analyze interactomes of receptors, membrane-associated proteins, and cytosolic proteins. In particular, split TEV is useful to assay activities of relevant drug targets, such as receptor tyrosine kinases and G protein-coupled receptors, in compound screens. As split TEV uses genetically encoded readouts, including standard reporters based on fluorescence and luminescence, the technique can also be combined with scalable molecular barcode reporter systems, allowing the integration into multiplexed high-throughput assay approaches. Split TEV can be used in standard heterologous cell lines and primary cell types, including neurons, either in a transient or stably integrated format. When using cell lines, the basic protocol takes 30–96 h to complete, depending on the complexity of the experimental question addressed.

Key words Protein–protein interaction, Split TEV, Biosensor, Split biosensor assay, RTK, GPCR, Phosphorylation-dependent interactions, Dose-response assay, Compound profiling

1 Introduction

1.1 Genetically Encoded Biosensor Assays for Studying Protein–Protein Interactions

Studying protein–protein interactions (PPIs) is paramount to understand cellular signaling and responses thereof. The majority of PPIs are precisely regulated both in time and space, and mediate distinct cellular activities, including differentiation, proliferation, apoptosis, and inflammation. In terms of signaling, cell surface receptors and highly interlinked intracellular proteins, or hubs, are of special interest, as these proteins integrate key signaling activities to regulate global cellular responses. For cytosolic proteins, pivotal associations with a strong implication on downstream signaling are

Viktor Stein (ed.), *Synthetic Protein Switches: Methods and Protocols*, Methods in Molecular Biology, vol. 1596,
DOI 10.1007/978-1-4939-6940-1_14,

frequently found at sub-membranous localizations. Here, signal transduction activities are commonly initiated through the dynamic formation of protein complexes that are composed of multiple PPIs [1]. For cell surface receptors, receptor tyrosine kinases (RTKs) and G protein-coupled receptors (GPCRs) are intensively studied protein classes, as deregulated signaling caused by these receptors is implicated in various human diseases, such as cancer and neurodevelopmental diseases [2, 3]. RTKs and GPCRs represent major drug targets, supporting their significant role in clinical research and drug discovery [4]. Both the associations between two proteins as well as the activities of RTKs and GPCRs can be reliably studied in split biosensor assays (SBA, also called protein complementation assays [5]). SBA are particularly suitable to monitor interactions of cell surface receptors and cytosolic proteins with a sub-membranous localization as SBA facilitate an analysis in the natural habitat of these proteins [6].

RTKs are single transmembrane receptors, with a total number of 58 encoded in the human genome [3]. RTKs respond, with few exceptions, to extracellular cues, and have an extra-cellular ligand-binding domain, a cytosolic tyrosine kinase domain, and a cytosolic tail, which contains interaction motifs that establish, once phosphorylated through ligand-driven activation, docking sites for cytosolic adapter proteins. Upon ligand binding, RTKs can homodimerize or heterodimerize, leading to a conformational change that transmits the signal into cytosol, where the kinase domains phosphorylate target tyrosine residues in trans [7]. In turn, Src homology 2 (SH2) domain- and phosphotyrosine binding (PTB) domain-containing adapters are recruited to phosphorylated docking sites, a process that can be robustly and precisely monitored by SBA [8–10]. An established interaction between an RTK and an adapter protein initiates downstream signaling activities, as these adaptors link RTK activation to downstream signal transduction pathways, such as the MAP kinase or PI3K/AKT signaling cascades.

GPCRs are the largest class of receptors encoded in the human genome, comprising more than 800 receptors in total [11]. Like RTKs, deregulated GPCR signaling is strongly implicated in various human diseases. Strikingly, 30–40% of all marketed drugs target GPCRs, making them the largest class of druggable receptors supporting their prominent role in drug discovery [4, 12]. GPCRs are seven-transmembrane receptors with three intracellular and three extracellular loops of varying length, an extracellular N-terminus, and a cytoplasmic C-terminal tail, which can associate with effector proteins in an activity-dependent manner [12]. Heterotrimeric G proteins are the major binding partners for GPCRs and trigger defined pathway responses, such as cAMP-mediated or Calcium-dependent pathway activities. Prolonged activation of GPCR activation results in its desensitization, which

is induced by the regulated recruitment of G protein-coupled receptor kinases (GRKs) that phosphorylate residues on the C terminus of the GPCR and thus provide a platform for β-Arrestin binding [13]. The binding of β-arrestin sterically obstructs G protein coupling and triggers the internalization of the GPCR. The regulated association between a given GPCR and β-arrestin2 can be exploited to reliably monitor GPCR activities and is regularly used by SBA and related proximity assays [14–17].

To assess dynamic interactions among proteins, various approaches for genetically encoded SBA are available, including, but not limited to, GFP and variants thereof [18–20], luciferases (firefly [21, 22], *Renilla* [23, 24], *Gaussia* [25], click beetle luciferases [17, 26, 27]), β-lactamase [28], β-galactosidase [29, 30], ubiquitin [8, 31, 32], and the tobacco etch virus (TEV) protease [33]. In addition to SBA, proximity assays were developed, such as bioluminescence resonance energy transfer (BRET) assays comprising firefly luciferase and GFP [34, 35] and the Tango method that uses a full-length TEV protease fused to an interacting partner like β-Arrestin to monitor GPCR activities [16].

Here, we present a detailed protocol for the split TEV technique, a genetically encoded SBA that can be flexibly applied to sensitively monitor various types of regulated PPIs in living cells [33] (and reviewed in [6]). The technique is based on the interaction-induced fragment complementation of the TEV protease. Split TEV can be used to assess dynamic interactions at the membrane and in the cytosol, thus allowing various combinations among potentially interacting candidate proteins (Fig. 1). Notably, interactions in the nucleus cannot be monitored using the presented technique, as the method was specifically designed to analyze PPIs at the membrane and in the cytosol. The technique integrates various reporter systems, including fluorescent and luminescent reporters, making it implementable to many research laboratories. As split TEV flexibly allows using readouts of choice, scalable transcriptional barcode reporters that are amenable to multiplexed high-throughput formats and next-generation sequencing may also be used. Combining these technologies will enable assessing drug target activities and cellular response profiles in parallel, thereby opening up new avenues in drug discovery. Here, we centre on luciferase reporters, as these are widely used both in single interaction assays and in high-throughput applications.

For split TEV assays, an optimized form of the TEV protease is dissected into an N-terminal (NTEV, amino acids 1–118) and a C-terminal fragment (CTEV, amino acids 119–221, with a Ser- > Pro substitution at residue 219 for enhanced assay stability). The S219P mutation renders the TEV protease refractory to autocatalysis [36]. In addition, the optimized form is truncated after amino acid 221 to remove the inhibitory C-terminal tail, which

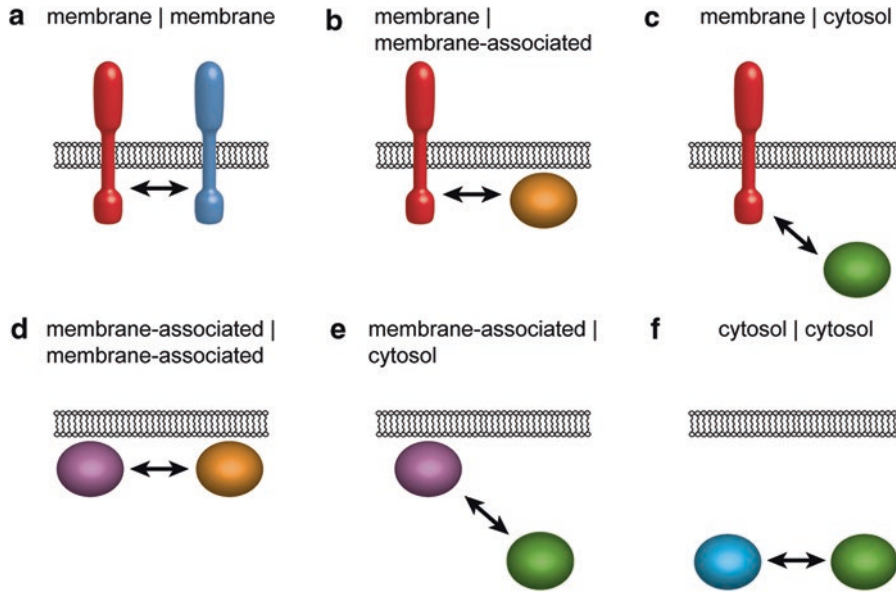

Fig. 1 Types of protein–protein interactions that can be monitored by split TEV. The candidates in a split TEV assay can be membrane proteins, membrane-associated proteins, and soluble proteins in the cytosol. Proteins localized to these subcellular areas can be tested for regulated interactions in all combinations, which are (**a**) membrane | membrane, (**b**) membrane | membrane-associated, (**c**) membrane | cytosolic, (**d**) membrane-associated | membrane-associated, (**e**) membrane-associated | cytosolic, and (**f**) cytosolic | cytosolic

blocks the active site of the TEV protease [37]. NTEV and CTEV fragments are fused to candidate proteins, preferably to the C-terminal end of the protein candidates. For NTEV, N-terminal fusions are also functional. Protein Interaction-induced reassembly of the NTEV and CTEV moieties leads to the reconstitution of TEV proteolytic activity, which activates TEV-specific reporters. These can be either of proteolytic or of transcriptional type, and they maybe fluorescent or luminescent-based. Notably, the luciferase-based transcriptional reporters proved to be most sensitive and robust as the readout is functionally uncoupled from the interaction event itself and background readings were commonly lower compared to other options. Transcriptional reporters based on luciferase exhibit another feature as they comprise three levels of signal amplification: In addition to the proteolytic cleavage and a transcriptional amplification step, enzyme-based (i.e., luciferase) reporters allow a third step of amplifying the initial signal resulting in a robust and sensitive readout.

1.2 Split TEV Assays for Membrane and Membrane-Associated Proteins

For monitoring dynamic PPIs at the membrane, a membrane protein or membrane-associated protein candidate is fused to the NTEV fragment along with a TEV protease cleavage site (tevS, encoded by the amino acids ENLYFQ'G; the TEV protease cleaves between Q and G) and the artificial transcriptional co-activator

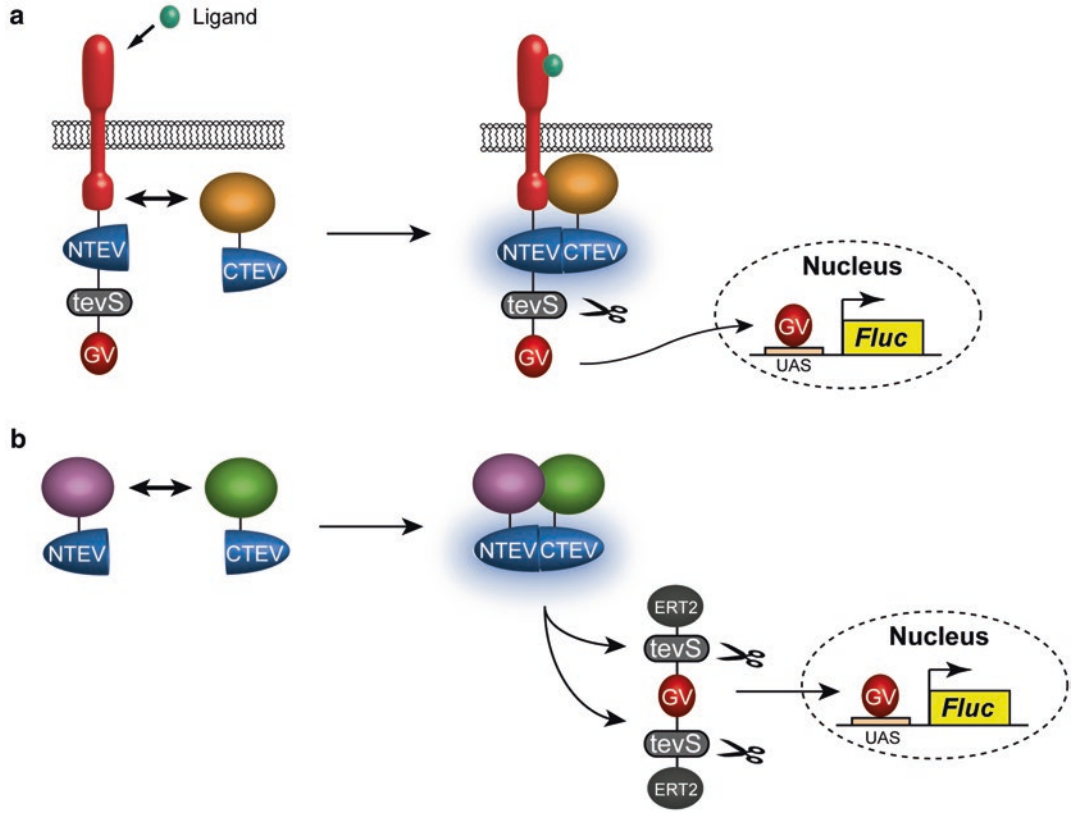

Fig. 2 Schematic representation of split TEV assays. (**a**) Split TEV assays for membrane and membrane-associated proteins. A candidate receptor is fused to the NTEV fragment, followed by the TEV protease cleavage site (tevS) and the co-transcriptional co-activator GAL4-VP16 (GV). A candidate soluble protein is fused to the CTEV fragment. Upon ligand stimulation, the receptor and the soluble protein associate with each other, bringing the TEV fragments into close proximity. This results in the reconstitution of TEV proteolytic activity, which is indicated by the outer glow shown in *blue* at the NTEV and CTEV fragments. Regained TEV protease activity cleaves at the tevS to release GV, as indicated by the scissors. Liberated GV translocates to the nucleus to transcriptionally activate a firefly luciferase reporter gene (Fluc) through binding to upstream activating enhancer sequences (UAS). (**b**) Split TEV assays for soluble proteins. A candidate soluble protein is fused to NTEV, a second soluble candidate is fused to CTEV. A cytosolic TEV reporter composed of a central GV unit that is trapped and flanked by ERT2 domains, each fused via a tevS, is additionally present in the cell. Association between the two candidates causes the TEV proteolytic activity to be reconstituted. Regained TEV protease activity cleaves at both tevS (indicated by the *scissors*) to liberate GV, which travels into the nucleus to activate the firefly luciferase reporter

GAL4-VP16 (GV), resulting in a NTEV-tevS-GV tag (Fig. 2a). As this hybrid tag contains a transcriptional co-activator, it is critical that the candidate protein is a membrane or membrane-associated protein, as a nuclear localization will readily lead to high background readings due to increased GV activity. The second candidate protein is fused to the CTEV fragment. This candidate may be a membrane, membrane-associated, a cytosolic protein, or

even a protein that shuttles between the cytosol and the nucleus. PPI-induced reconstituted TEV protease activity cleaves off GV, which translocates into the nucleus and activates a firefly luciferase reporter gene, which is placed under the control of upstream activating sequences that contain DNA sequences recognized by GV.

Split TEV assays can be used to monitor ligand-dependent interactions of cell surface receptors, such as RTKs and GPCRs, thus allowing to assess their activity. For instance, the Neuregulin1-induced association between ERBB4, an RTK of the ERBB family, and the regulatory subunit alpha of the PI3K, PIK3R1, was determined in a dose-response assay (Fig. 3a), as well as its inhibition by lapatinib (Fig. 3b). Sample constitutive interactions assayed using split TEV are shown for the membrane-associated protein KIBRA (Fig. 3c). For assessing GPCR activities, we have recently reported a detailed protocol on the split TEV-based GPCR/β-Arrestin2 recruitment assay, which uses a truncated version of β-Arrestin2 and a modified NTEV-tevS-GV tag for improved sensitivity [38]. We therefore refer the reader to this specified GPCR split TEV protocol. Notably, the split TEV method proved to be more sensitive than the full-TEV Tango approach when assaying selected GPCR activities [15].

1.3 Split TEV Assays for Soluble Proteins

Interactions between candidate proteins that stay strictly cytosolic and do not shuttle into the nucleus may be monitored using the hybrid NTEV-tevS-GV tag (*see* **Note 1**). For candidate proteins that show a certain degree of nuclear localization, candidates are fused to the NTEV fragment only (Fig. 2b). The other candidate protein is fused to CTEV. When pursuing the sole NTEV/CTEV tagging strategy, PPI-induced TEV protease activity leads to the activation of a cytosolically localized transcriptional TEV reporter (denoted GV-2ER), which contains a central GV unit that is trapped by two ERT2 domains, each fused to GV via a tevS. ERT2 domains are modified oestrogen receptor domains, that do not respond to endogenous oestrogen, but to 4-hydroxytamoxifen. Liberated GV translocates into the nucleus and activates the firefly luciferase reporter. MST1 dimer formation based on the C-terminal SARAH domain is shown as an example for soluble protein interactions (Fig. 3d). Dynamic interactions like AKT-induced and phosphorylation-regulated BAD/14–3-3 and rapamycin-regulated FKBP/FRB have also been described using split TEV [9, 33].

1.4 Assay Controls

When studying PPIs using split TEV, tagged proteins of interest are commonly introduced into cells at rather high expression levels, either transiently by transfection or by stable integration. Therefore, assay components should be expressed at rather low levels, possibly close to those of endogenous counterparts. Further, each assay should be individually validated by specific

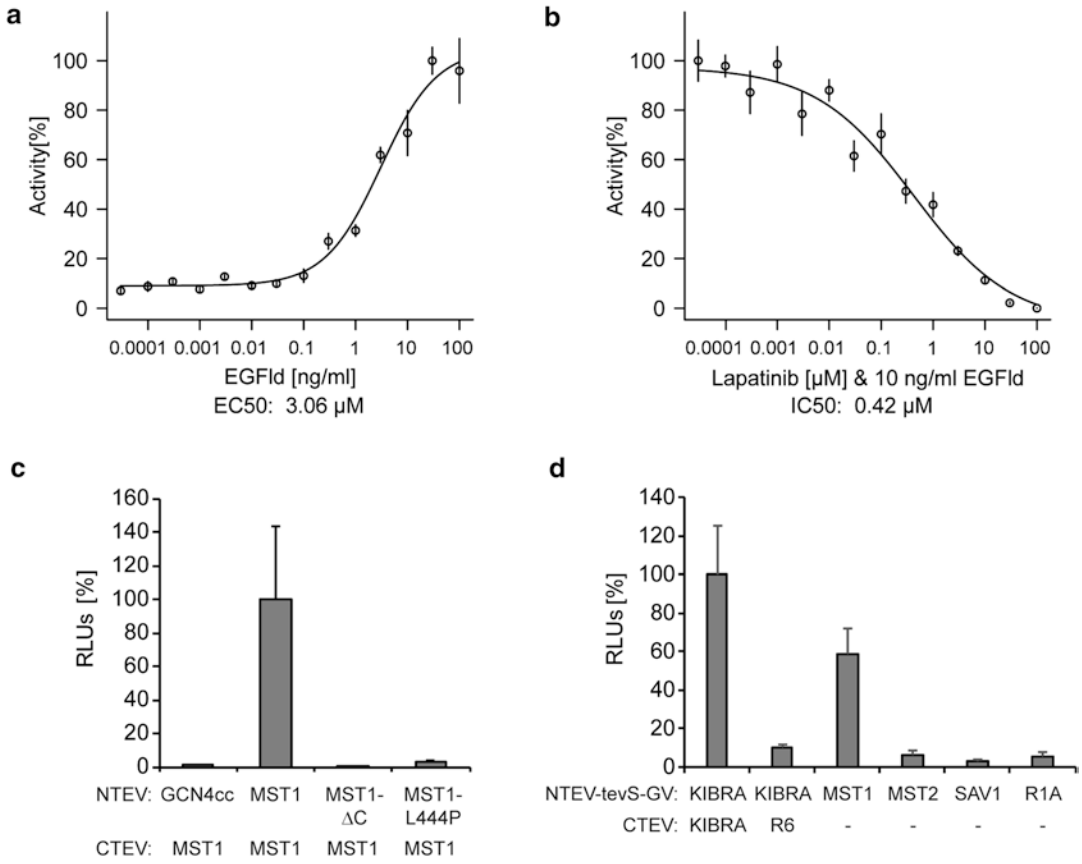

Fig. 3 Examples of constitutive and regulated split TEV assays. (**a**, **b**) Dose response split TEV assays for the ERBB4 receptor using (**a**) the agonist EGF-like domain (EGFld) and (**b**) the antagonist lapatinib. ERBB4-NTEV-tevS-GV and PIK3R1-CTEV fusions were transiently transfected into PC12 cells and treated for 20 h using the indicated compound concentrations. (**a**) ERBB4/PIK3R1-transfected assay cells were stimulated with increasing concentrations of EGFld. The EC_{50} value was calculated at 3.06 ng/ml EGFld. (**b**) ERBB4/PIK3R1-transfected assay cells were stimulated with increasing concentrations of lapatinib, followed 1 h later by the addition of a constant stimulus of 10 ng/ml EGFld. The IC_{50} value was calculated at 0.42 µM lapatinib (**b**). Six replicates per condition, error bars represent SEM. (**c**) Constitutive split TEV assays for the kinase MST1. MST1 dimerizes through its C-terminally located SARAH domain (*lane 2*). Note that MST1-ΔC, which contains residues 1–432 only and lacks the SARAH domain, does not interact with MST1 full-length, and thus only background signals are produced. Likewise, the missense mutation L444P disrupts the MST1 dimer formation [39]. (**d**) MST1 is not suitable for the hybrid tag NTEV-tevS-GV. MST1-NTEV-tevS-GV is cleaved and produces high background readings (*lane 4*). By contrast, MST1 interacting proteins MST2, SAV1 and RASSF1A (R1A) yield low background readings. Readings are compared to a strong PPI (KIBRA::KIBRA) (*lane 1*) and a background control (KIBRA::RASSF6 (R6)) (*lane 2*). *RLU* relative luciferase units; six replicates per condition, error bars represent SD

interaction controls. Therefore, it is strongly advisable to include positive controls (i.e., proteins that do interact with the candidate of interest) and negative controls (i.e., proteins that do not bind to the candidate of interest) for each assay designed.

1.5 Experimental Setups for Constitutive and Regulated Interaction Split TEV Assays

The split TEV technique can been applied to study constitutive and regulated interactions that are modulated by ligands, agonists, antagonists, or a combination thereof. Experimental setups for constitutive split TEV assays can be comfortably completed within 30 h (Fig. 4a). Dynamic interaction split TEV assays using agonists or agonist/antagonist combinations typically require up to 4 days, depending on the individual experimental design (cell seeding parameters, starving conditions, stimulation paradigms, etc.) (Fig. 4b, c). The effects of compound actions, either stimulatory or inhibitory, are optimally assessed in dose response assays, which allow the calculation of EC_{50} and IC_{50} values (concentrations of agonist/antagonist compounds at half-maximal stimulatory/inhibitory response).

In summary, the split TEV technique is a powerful and sensitive tool providing a robust readout to monitor dynamic PPIs within their natural context. The split TEV method is characterized by various features as it enables (1) the detection of dynamic

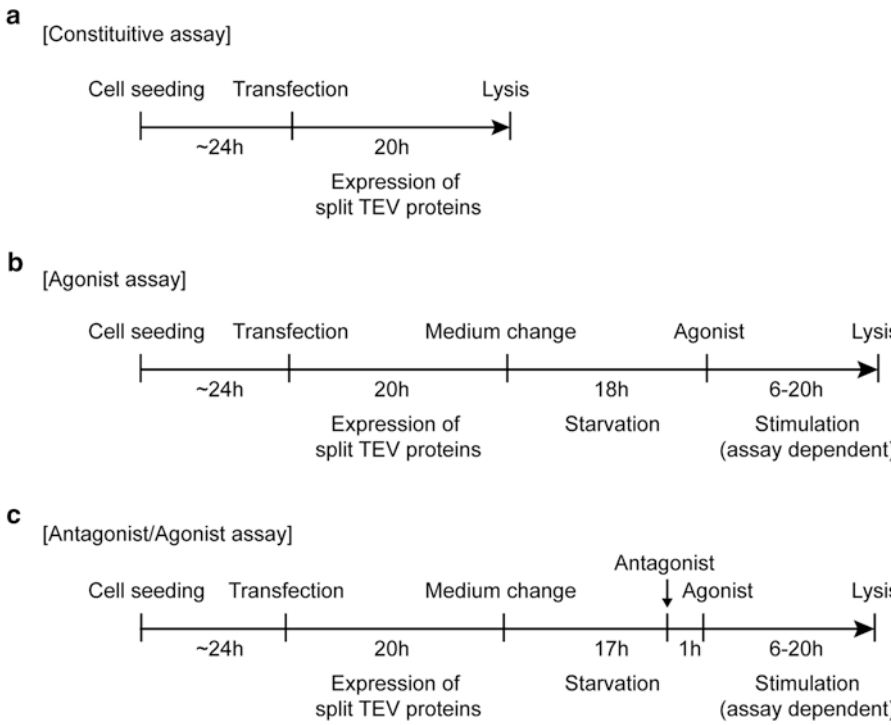

Fig. 4 Suggested timelines for split TEV assays. Cells are plated on the day before (or alternatively in the morning) and transfected with assay plasmids. (**a**) Constitutive assay. After transfection, cells are incubated for 20 h before lysis. (**b**) Agonist assay: After transfection, plasmids are allowed to express for 20 h, followed by a medium change to starve the cells in low-serum media. The following day, an agonist is added for 6–20 h, depending on the setup of the assay. (**c**) Antagonist assay: Transfection and medium change are performed as in (**b**). Before an agonist is added, cells are treated with an antagonist for 1 h. The stimulation time is dependent on the assay setup

interactions between full-length candidate proteins in living cells or in lysates thereof, (2) the detection of protein interactions localized at the membrane, sub-membrane compartment, and the cytosol, (3) the detection of protein interactions in heterologous mammalian cells, primary cell types including primary neurons and astrocytes, (4) the detection of regulated RTK and GPCR activities induced by extracellular stimuli, (5) the analysis of regulated, ligand or compound concentration-dependent interactions in dose-response assays assessing actions of agonists and antagonists, and (6) the setup as protein interaction readouts for high-throughput screening (HTS) purposes [9, 15, 33, 40–42]. Therefore, split TEV may also be adapted to industrial HTS setups to screen for RTK and GPCR activities, for example in drug discovery programs.

Below, we describe experimental setups for split TEV assays for membrane, membrane-associate and cytosolic proteins using firefly luciferase as a final reporter gene as this readout is widely used across laboratories, and allows adaptation to HTS approaches.

2 Materials

2.1 Plasmids

1. For membrane, membrane-associated and strictly cytosolically localized proteins:

   ORF(X)-NTEV-tevS-GV plasmid. The ORF of candidate X is fused to NTEV (amino acids 1–118), tevS (ENLYFQG), and GV (Fig. 2a). Use molecular cloning to generate candidate X fusion vectors (*see* **Note 2**). For a detailed description on GPCRs, see the protocol by Wehr et al. [40].

   For cytosolic protein candidates that shuffle between cytosol and nucleus:

   ORF(X)-NTEV plasmid. The ORF of candidate X is fused to NTEV (amino acids 1–118) only. In addition, the pGV-2ER plasmid is required as cytosolic TEV reporter (Fig. 2b).

2. ORF(Y)-CTEV. The ORF of candidate Y is fused to CTEV (amino acids 119–221, including a S219P mutation). Use molecular cloning to generate the fusion vector (*see* **Note 2**).
3. Firefly luciferase reporter plasmid. Use an upstream activating enhancer sequences (UAS)-driven firefly luciferase (e.g., pGL4.31[luc2P/GAL4UAS/Hygro], Promega, catalog no. C9351) (*see* **Note 3**).
4. *Renilla* luciferase control plasmid. Use a thymidine kinase (TK)-driven constitutive *Renilla* luciferase as internal control (pRL-TK, Promega, catalog no. E2241).
5. A GFP or GFP-derivative expressing plasmid for optical transfection control (e.g., mVenus-N1, Addgene, Plasmid #54640).

2.2 Cells

HEK293 (ATCC), PC12 cells (PC12 Tet-Off, Clontech), any other heterologous cell line, or primary cells that are amenable to efficient transfection and allow studying protein–protein interactions (*see* **Note 4**).

2.3 Reagents

1. Medium, depending on the cell line chosen (e.g., for HEK293 cells: DMEM 4.5 g glucose/l without L-glutamine, each with and without phenol red). See complete media formulations for HEK293 and PC12 cells below.
2. Fetal bovine serum (FBS), heat-inactivated, 500 ml (Life Technologies)
3. Horse serum (HS), heat-inactivated, 500 ml (Life Technologies) (only needed for PC12 cells).
4. GlutaMAX, 100×, 100 ml (Life Technologies).
5. Penicillin and Streptomycin, 100×, 100 ml (Life Technologies).
6. Opti-MEM (Life Technologies).
7. Poly-L-lysine, mol wt 70,000–150,000, diluted in a final concentration of 0.02 mg/ml in H_2O (Sigma).
8. Lipofectamine 2000 transfection reagent (Life Technologies).
9. Passive lysis buffer, 5× (Promega, E1941).
10. Dual-Luciferase® Reporter 1000 Assay System (Promega, E1980) (for an alternative using self-made substrates, *see* **Note 14**).

2.4 Equipment

1. 96-well plates: white with flat bottom for luciferase-based assays (Falcon).
2. 96-well plates: clear with flat bottom for optical transfection control and fluorescence-based assays (Falcon).
3. 75 cm^2 flasks (Falcon) or 15 cm dish (Falcon) (for maintenance of cell lines).
4. Cell culture facility including flow hood and incubator set to 5% CO_2 and 37 °C.
5. Luciferase reader (e.g., Mithras, Berthold Technologies or Envision, PerkinElmer).
6. Fluorescence microscope (to check for transfection efficiency).

2.5 Media Formulation

Prepare for each cell line used the appropriate media for maintenance and assay conditions.

1. HEK293 cells, assay medium: DMEM (4.5 g glucose/l, without L-glutamine and without phenol red), 500 ml, supplemented with 0.5% FBS and 2 mM GlutaMAX.
2. HEK293 cells, medium for maintenance: DMEM (4.5 g glucose/l, without L-glutamine, with phenol red), 500 ml,

supplemented with 10% FBS, 2 mM GlutaMAX, and 100 U/ml of each penicillin and streptomycin.

3. PC12 Tet-Off cells, assay medium: DMEM (1 g glucose/l, without L-glutamine and without phenol red), 500 ml, supplemented with 1% FBS, and 2 mM GlutaMAX.
4. PC12 Tet-Off cells, medium for maintenance: DMEM (1 g glucose/l, without L-glutamine, with phenol red), 500 ml, supplemented with 5% FBS and 10% HS, 2 mM GlutaMAX, and 100 U/ml of each penicillin and streptomycin.

3 Methods

1. Design appropriate ORF(X)-NTEV-tevS-GV and ORF(Y)-CTEV fusion expression plasmids (*see* guidelines in Subheading 2.1, **items 1** and **2**, and **Note 2**). When testing interactions between candidates X and Y, it is strongly advisable to design appropriate interaction controls (*see* **Note 5**).
2. Clone and prepare sufficient amount of DNA for the assays, set the concentration for each plasmid to 100 ng/μl (*see* **Note 6**).
3. Prepare 96-well plates for split TEV luciferase assays and fluorescence controls.
   (a) Coat a white/clear 96-well plate(s) with a 0.02 mg/ml poly-L lysine (PLL) solution for 30 min at room temperature.
   (b) Wash twice with double deionized water, then let plates air-dry.
   PLL-coated plates can be stored at 4 °C for up to 6 weeks without losing coating efficiency. This step is optional for HEK293 cells.
4. Seed 20,000 HEK cells resuspended in maintenance medium without antibiotics into each well of the coated 96-well plate. Place the 96-well plate(s) into the incubator. For PC12 Tet-Off cells, seed 40,000 cells per well (*see* **Note 7**).
5. Seed cells onto a clear 96-well plate for an optical control of transfection efficiency.
6. On the next day, prepare the plasmid mixes for each condition to be tested. Run 6 replicates per condition to obtain sufficient readings for statistical analyses. For each 96-well, use 15 ng per single DNA. Make a master mix for 6 + 1 replicates, which contains an extra amount needed to allow for pipetting errors (for example, when using 15 ng DNA/well, use 15 ng x 7 = 105 ng in total). For the *Renilla* luciferase plasmid, use the same DNA amount chosen. Prepare a constitutively expressing GFP plasmid (or variant thereof) (2 ng per well) for control

positions on the clear plate to guarantee an optical control of transfection efficiency (*see* **Note 8**).

7. Prepare the transfection mix. Dilute the Lipofectamine 2000 150-fold in Opti-MEM supplemented with 1% GlutaMAX, vortex for 1–2 s, and incubate for 5 min. For each 96-well, we use 0.2 µl Lipofectamine 2000 that is diluted in 30 µl Opti-MEM. We suggest preparing a 7× master mix for all conditions to be tested.
8. Pipette the Lipofectamine/Opti-MEM mix onto the DNA, mix (vortex), and incubate for 20 min at room temperature.
9. Remove medium from each well entirely (be careful not to remove the cells) and transfer the DNA/transfection mix (30 µl per well) onto the cells.
10. After 1 h, add 60 µl medium for maintenance without antibiotics.
11. Depending on whether a constitutive or stimulus-dependent protein–protein interaction assay is run,
    - proceed to **step 12** for constitutive, or
    - proceed to **step 13** for agonist and agonist/antagonist assays.
    - Common experimental paradigms are schematically displayed in Fig. 4.
12. *Constitutive assay*: Incubate for 12–24 h. Typically, we incubate constitutive assays for 20 h. Before lysing the cells, verify the transfection efficiency using the clear optical control plate. By this time, the transfected plasmids should express considerably, and transfection efficiency, as determined by GFP signals, should be at least 30–50% to obtain stable assay conditions, otherwise start optimizing transfection parameters (*see* **Note 9**).
    - Proceed to **step 17**.
13. *Agonist and agonist/antagonist assays*: Incubate overnight.
14. On the next day, verify transfection efficiency (c.f. **step 12**, *see* **Note 9**) and replace the medium with 80 µl assay medium to induce the starvation of cells.
15. After 16–24 h, add appropriate stimuli in 80 µl assay medium, with the agonists in double concentration. If studying agonist/antagonist effects, add the antagonists 1 h before the agonists (*see* **Note 10**).
16. Incubate for 6–24 h, depending on the assay setup. This step strongly depends on the individual interaction measured, the cell type used, or biological question addressed. For a first approach, we incubate for 18–20 h (*see* **Note 11**).
17. Aspirate the medium and add 30 µl of 1x Passive Lysis Buffer to each well. Incubate the 96-well plate on a horizontal rocking platform (100–150 rpm) for 10 min at room temperature.

Lysed cells can be stored at −20 °C for up to 4 weeks without losing significant levels of signal intensity (*see* **Note 12**).

18. Perform the Dual luciferase readout. Add 30 µl of Firefly substrate into each well (from commercial Dual Luciferase Kit prepared as indicated by the manufacturer), read the plate using a luciferase reader. Add 30 µl of *Renilla* substrate into each well, read the plate (*see* **Note 13**). Alternatively, self-made substrates may be used (*see* **Note 14**).
19. Calculate mean, standard deviations and standard errors of the mean using an Excel spreadsheet. Generate a bar graph to visualize the data. For dose-response analyses, we recommend using the dose response curve package 'drc' in R (*see* **Note 15**). Alternatively, the GraphPad Prism program can be used, which allows standard visualizations of dose response curves.
20. Advice for troubleshooting addressing critical steps can be found in Table 1.

Table 1
Troubleshooting advice

Step	Problem	Solution
4, 10	Low signals in general (both firefly and *Renilla*)	Do not use antibiotics as they might interfere with the transfection reagent
12, 16	Good transfection efficiency, good *Renilla* signals, but low firefly signals: • ORF-NTEV • ORF-NTEV-tevS-GV • ORF-CTEV fusion protein may not be expressed properly	Although the optical transfection control is fine as indicated by for example EGFP expression, the candidate fusion in question may be poorly expressed • Verify protein expression using Western blotting • Try to swap candidate proteins for NTEV and CTEV fragments. If using the cytoplasmic assay, you may also place the NTEV moiety to either end. Protein expression, however, also depends on the target protein chosen
16	Low firefly signals for inducible PPIs	Stimulation time may vary for a given receptor or any other inducible PPI. Therefore, we suggest to run an online luciferase split TEV assay to obtain the best activation window. If no appropriate reader is available, monitor assay activity from 6 to 20 h in an hourly or two-hourly mode after the agonist has been added
18	Low luminescence signals, in particular for firefly luciferase	Double-check the functionality of the luciferase buffers. This is of particular importance for the firefly luciferase buffer containing ATP, which can decay quickly. To do so, we prepare test lysates from cells that constitutively express both firefly and *Renilla* luciferases. These lysates can easily be stored at −80 °C for several months

4 Notes

1. When opting for the hybrid NTEV-tevS-GV tag, we recommend to first perform control assays that only contain the candidate-NTEV-tevS-GV to exclude high background readings. For example, activated Sterile20-like kinase MST1 (STK4) is cleaved [43] and produces high background readings, thus precluding the use of the hybrid tag. In a next step, the candidate may be tested with a protein that is known not to bind to the candidate to validate assay robustness, and to establish experimental parameters for a reference negative control.
2. Cloning of candidate split TEV fusions. Clone the candidate ORFs including all split TEV assay elements into a regular expression vector, such as pcDNA3.1. The fusions are constructed to contain a 10 amino acid flexible linker (GGGGS GGGGS) between the candidate protein and NTEV or CTEV. As expression plasmids, we regularly use pcDNA3-derived (Invitrogen) and pTag-derived (Stratagene) vectors. If required, N- and CTEV fusions can be stably integrated into the cells using the mammalian selection markers present on these plasmids (i.e., neomycin or zeocin resistance). We regularly use Gateway recombination cloning (Life Technologies) to quickly obtain candidate fusions. Gateway-compatible ORFs for more than 16,000 ORFs are available at Plasmid ID (http://plasmid.med.harvard.edu/PLASMID/Home.jsp). The TEV protease mutant S219P can be obtained from Addgene (plasmid no. 8830, pRK792).
3. When opting for fluorescence-based assays, use a UAS-EGFP plasmid (i.e., pJFRC7-20XUAS-IVS-mCD8::GFP, Addgene, plasmid no. 26220). In this case, normalization can be done using nuclear staining or by co-transfecting a constitutively expressing mCherry plasmid. In addition, a fluorescence plate reader is required for quantification.
4. For investigating membrane protein interactions, we start testing assays in PC12 Tet-Off cells (Clontech) and then proceed to HEK293 cells (ATCC). For membrane-associated and soluble proteins, we commonly start with HEK293 cells. Notably, U2OS cells proved to a valuable cell line to monitor GPCR activities. As we observed substantial differences in the activation ratios for some GPCRS, like AVPR1 and DRD2, and RTKs, like ERBB family receptors, we suggest to assess the optimal cell line for each individual assay.
5. Positive (known interacting protein) and negative (known non-interacting protein) controls should always be included in protein–protein interaction assays based on protein fragment complementation [33]. As a starting guideline, we suggest

using FKBP1A-NTEV or FKBP1A-CTEV as negative controls as FKBP1A represents a small protein with a defined number of interactions described [44]. FKBP1A was used in split TEV assays [40], and also by other split protein approaches. Further, we have successfully used the GCN4-coiled coil domain (GCN4cc) as interaction control [9, 41]. Another strategy may be applying a truncated form of an interacting partner that lacks the defined interaction domain(s). However, it is crucial to validate its correct use first.

6. We suggest to store DNA plasmid aliquots at −20 °C. Avoid unnecessary freeze-thaw cycles as this decreases DNA concentration and quality.
7. Seeding the cells can be done on the previous day or in the morning of the day the experiment is planned. When seeding on the same day, increase the amount of cells by roughly 50% and wait at least 3–4 h to let the cells attach to the surface of the plate.
8. It is also possible to prepare the DNA mix on the previous day. If done so, freeze the DNA as small volumes may evaporate at 4 °C.
9. To optimize transfection conditions, start with adapting plasmid amounts, i.e., increase/decrease DNA amounts, and/or change ratios of the candidate X vs. candidate Y vs. reporter plasmids. Further, we find changing cell densities and/or Lipofectamine 2000 concentrations helpful.
10. Agonists can be added in a 10× or 20× concentration diluted in assay medium. Note that both agonists and antagonists are frequently diluted in DMSO, which must not exceed 1% of the total volume.
11. For RTKs, we regularly incubate for 20 h to obtain stable inducible readings. For GPCRs, we noted, however, that shorter incubation periods of 6–8 h yield better readings. To determine the optimal time window for a given inducible PPI, we also suggest to run an online luciferase split TEV assay before investing into a dose response assay.
12. When opting for a fluorescent reporter, such as UAS_EGFP, do not lyse the cells, and proceed with acquiring the fluorescent signal using a fluorescence reader or an automated microscope.
13. When acquiring luciferase signal intensities using the Mithras device from Berthold Technologies, we apply a time integration protocol, which measures each well for 2 s. Substrates are added by automated injection across the entire plate, followed by an orbital shake for 10 s and an incubation time of 10 min, followed by reading the signals. This process is performed first

for the firefly luciferase substrates, and then for the *Renilla* luciferase substrates.

14. If using the self-made substrates for the Mithras device from Berthold Technologies, a different protocol for measuring the *Renilla* luciferase signals will be applied, as this substrate has a decreased stability. The substrate is injected, followed by an orbital shake of 2 s, and then immediately measured for 2 s. This Process is repeated well by well. The detailed protocol for making both firefly (a) and *Renilla* (b) substrates is listed below. We regularly order special reagents from p.j.k. GmbH, Kleinblittersdorf, Germany; these include coelenterazine, D-luciferin, co-enzyme A, ATP, DTT (1,4 dithiothreitol). c(0.001, 0.01, 0.1, 1, 10, 100),

    (a) Substrate for firefly luciferase

Chemical	Final concentration
Tricine	20 mM
$(MgCO_3)_4$*Mg$(OH)_2$*5H_2O (magnesium carbonate-hydroxide-pentahydrate)	1.07 mM
$MgSO_4$ * 7 H_2O	2.67 mM
EDTA	0.1 mM
DTT	33.3 mM
Coenzym A	270 μM
D-Luciferin, free acid	470 μM
ATP	530 μM

To solve magnesium carbonate, adjust the pH value using HCl (37%, use approximately 1 ml for 1 l) until solution becomes clear. Then, adjust the pH value to 7.8 using 5 M NaOH (use approximately 7.5 ml for 1 l).

(b) Substrate for *Renilla* luciferase

Chemical	Final concentration
NaCl	1.1 M
Na_2-EDTA	2.2 mM
K_xPO_4 (pH 5,1)	0.22 M
BSA	0.44 mg/ml
NaN_3	1.3 mM
Coelenterazine (solved in EtOH)	1.43 mM

K_xPO_4 (pH 5.1): 1 M KH_2PO_4, adjust pH value to 5.1 using 2 M KOH (use approximately 15 ml for 1 l). Adjust pH value to 5.0. Add coelenterazin afterwards.

For storage, make 50 ml aliquots and store at −20 °C in the dark. Aliquots can be stored up to 6 months.

15. The package 'drc' is used in R to calculate EC50 and IC50 values and to generate fitted dose response curves. The script uses the drm function and is amended to include error bars based on the standard error of the mean (sem). The assay data is saved as txt file using a tab delimited format. Luciferase activity (activity) is calculated in %, concentration (conc) in μM, and sem in %.
A sample assay data set is shown below:

activity	conc	sem
7.024253731	0.00003	0.969091058
8.789256841	0.0001	1.875653638
10.69690609	0.0003	1.271332669
7.650808458	0.001	0.991970081
12.68326337	0.003	1.117476168
9.120219216	0.01	1.537416736
9.865127488	0.03	1.367412567
13.02510883	0.1	2.670494192
26.97566853	0.3	3.160663768
31.31743626	1	2.324396584
61.8740283	3	3.084176905
70.77386505	10	9.107073314
100	30	5.429347986
95.9036847	100	13.04036002

The drc script including the addition of error bars reads:

```
# Loading package
library("drc")
# Loading data
drc <- read.delim ("D:/…/DRC_1.txt", header=T,
   dec=".")
# Returning data
drc
# Fitting the model
DR.1 <- drm(activity~conc, data = drc, fct =
   LL.4())
summary(DR.1)
```

```
# Calculating effective dose (ED) values
# first column: the estimates ED50, ED80, and
   ED90
# second column: the estimated standard errors
ED(DR.1, c(50, 80, 90))
EDout = ED(DR.1, c(50))
# Labelling of x axis, compound name & unit
DRxlab = "EGFld [ng/ml]" #or "Lapatinib [µM]
   & 10 ng/ml EGFld"
# Plotting graph with y axis from 0 to 100%
   activity
plot(DR.1, broken = FALSE, type = c("all")
   ,ylab = "activity[%]", xlab = DRxlab, log
   = "x", xt = c(0.001, 0.01, 0.1, 1, 10,
   100), ylim = c(0, 115), legend = FALSE,
   bty = "l", sub = paste("EC50: ",round(EDo
   ut[1,1],4),"µM",sep=" ")) #adjust EC/IC50
   and concentration
# Adding error bars as sem
for (i in 1:dim(drc)[1]){
segments(drc[i,2],drc[i,1]-drc[i,3],drc[i,2],
   drc[i,1]+drc[i,3],col="black")
}
```

Acknowledgment

This work was supported by the Ludwig Maximilian University of Munich and the DFG grant WE 5683/1-1 to M.C.W.

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