# Tap the Full Potential of Split Fluorescent Protein Technology by Using ChromoTek's Nano-Traps

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## Abstract

To understand the biology of proteins and with that cell function in health and disease, it is crucial to be able to visualize proteins *in situ* and to analyze their protein-protein interaction networks *in vivo*. To this end, fluorescent proteins (FPs) have proven an invaluable tool as protein tags and have literally thrown light upon many scientific questions. Fluorescent proteins were further developed into split FP variants, spurred by the need for dedicated tools for the analysis of protein-protein interactions. Here, (1) we give a review about split fluorescent protein technology and (2) present five case studies how ChromoTek's Nano-Traps can be applied to tap the full potential of this technology.

## Summary

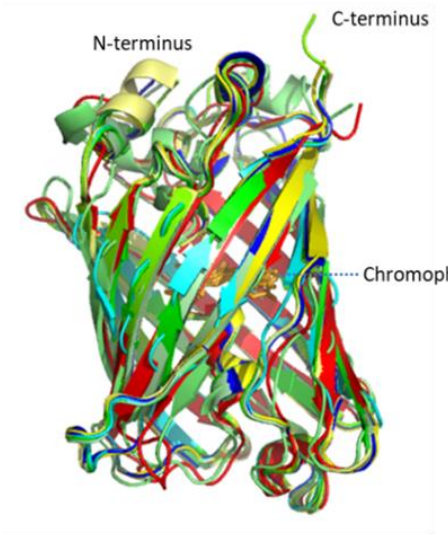
1. Jellyfish-derived GFP variants and most other fluorescent proteins from various species of coral or sea anemone share a common β-barrel fold composed of 11 single β-strands. Split fluorescent protein technology uses non-fluorescent fragments of this β-barrel, obtained by truncation between two or three β-strands to study single proteins or protein-protein interactions. Reconstitution of the full-length FP can be obtained either by conditioned (protein-protein interaction of fusion partners) or unconditioned (self-complementation) fragment association, recovering fluorescence. All aerobically grown cells and organisms that can be genetically modified have the potential to be used in split FP technology.
2. Split FP technology is frequently applied to cellular assays. ChromoTek's Nano-Traps constitute attractive research tools for the biochemical validation of such experiments. For instance, the ChromoTek GFP-Trap has been successfully applied to different assay types such as protein self-complementation, BiFC, TriFC, or BiCAP, involving several different split GFP variants.

## Introduction

A large number of fluorescent proteins (FPs) has been discovered and developed since the discovery of green fluorescent protein (GFP) in *Aequorea victoria* in 1962. Most FPs are derived from marine organisms such as jellyfish, coral, and sea anemone. These FPs bear an intrinsic chromophore.

Additionally, there are fluorescent proteins that bind an exogenous chromophore (e.g. Halo, or Snap) but these are less frequently used (Sanford and Palmer, 2017) and will not be discussed here. For more details about FPs in general, see also our informative ChromoBlog.

A




B

| FP | Mutation | PDB ID |
|---|---|---|
| wtGFP/avGFP | Wild-type | 1ema |
| eGFP | V2ins, F64L, S65T, H231L | 2y0g |
| sfGFP | αGFP (Q80R, F99S, M153T, V163A) + frGFP (F64L, S65T) + S30R, Y39N, N105T, Y145F, I171V, A206V | 2b3p |
| BFP | Y66H, Y145F | 1bfp |
| eYFP | V2ins, S65G, V68L, S72A, T203Y, H231L | 2jad |
| Venus | eYFP + seYFP (F64L, M153T, V163A, S175G) + F46L | 1myw |
| *CFP* | Y66W | n.a. |
| eCFP | CFP + V2ins, N146I, M153T, V163A, F64L, S65T, H231L | 2wsn |
| mCherry | Not GFP derived | 2h5q |
| mNeonGreen | Not GFP derived | 5ltr |

C

| | wtGFP | eGFP | BFP | CFP | eCFP | sfGFP | eYFP | Venus | mCherry | mNG |
|---|---|---|---|---|---|---|---|---|---|---|
| wtGFP | | 98 | 99 | 99.6 | 97 | 95 | 97 | 95 | 28 | 21 |
| eGFP | 98 | | 97 | 98 | 98 | 95 | 98 | 97 | 29 | 22 |
| BFP | 99 | 97 | | 99 | 96 | 95 | 97 | 95 | 28 | 21 |
| CFP | 99.6 | 98 | 99 | | 97 | 95 | 97 | 95 | 28 | 21 |
| eCFP | 97 | 98 | 96 | 97 | | 95 | 96 | 97 | 28 | 22 |
| sfGFP | 95 | 95 | 95 | 95 | 95 | | 93 | 93 | 28 | 21 |
| eYFP | 97 | 98 | 97 | 97 | 96 | 93 | | 98 | 29 | 23 |
| Venus | 96 | 97 | 95 | 95 | 97 | 93 | 98 | | 29 | 23 |
| mCherry | 28 | 29 | 28 | 23 | 28 | 28 | 29 | 29 | | 31 |
| mNG | 21 | 22 | 21 | 21 | 22 | 21 | 23 | 23 | 31 | |

*Figure 1. Comparison of wtGFP with seven of its variants (eGFP, sfGFP, eYFP, Venus, BFP, CFP and eCFP) and two GFP homologs from other organisms (mCherry and mNeonGreen). A: Structural alignment of all selected FPs (except CFP), shown as cartoon presentation with the chromophore drawn as orange sticks in the center of the β-barrel. Color code and PDB IDs are listed in B. The structures were aligned using PDBeFold (http://www.ebi.ac.uk/msd-srv/ssm/) and adapted using PyMOL B: Overview of the selected FPs showing their mutations compared to wtGFP. C: Sequence identities in [%] between the selected FPs. "mNG" is mNeonGreen. The protein sequences were retrieved from http://www.fpbase.org, and sequence identities were calculated using the software Geneious.*

A vast number of fluorescent proteins has been structurally characterized. Figure 1 gives an overview of ten such fluorescent proteins that have been selected because of their published use in split FP assays (see above and Table 1).

These jellyfish-derived GFP variants (first eight in Figure 1B), mCherry from a sea anemone, and mNeonGreen from a lancelet share a highly conserved β-barrel fold with a chromophore in its center. This chromophore is formed by three consecutive amino acids at positions 65, 66, and 67 in an autocatalytical process called maturation.

The high structural conservation between the ten selected FPs is illustrated by the structural alignment in Figure 1A (confirmed by an overall root mean square deviation (rmsd) of only ~1.2 Å over 207 compared backbone Cα atoms). In contrast, the shared sequence identity may be as low as 20-30 % (Figure 1C).

In practical terms, the sequence variety of fluorescent proteins derived from different organisms means that most anti-GFP antibodies exclusively bind to various sets of GFP variants derived from jellyfish. Other FP homologs such as mCherry or mNeonGreen require other, dedicated tools. ChromoTek offers such research tools for fluorescent proteins derived from many different species.

## Protein-Protein Interaction Assays

Since the discovery of GFP, fluorescent proteins have become wildly successful

tools in biological and biomedical research, as recognized by the Nobel Prize in chemistry in 2008. The tagging of a protein of interest (POI) by fusing it to a FP is widely used to visualize a POI's subcellular location *in vivo* or *in vitro*, to study its biochemistry (using methods such as immunoprecipitation), or to analyze fluorescent cells by flow cytometry (Leonetti et al., 2016).

Usually, it is not the single protein in isolation that is of interest, but rather its network of interactions with other proteins. These protein-protein interactions (PPI) are fundamental to all processes of life, e.g. in signal transduction or gene expression. Many methods have been developed to study protein-protein interactions in cells, some of which are based on or can be used with (split) FPs.

One of the most frequently applied methods is Förster resonance energy transer (FRET). It allows real-time detection of complex formation and dissociation. However, the method has low sensitivity and works only if the fluorescent reporter proteins are placed within 10 nm of each other (Kerppola, 2008, Miller et al., 2015).

Another PPI method is ChromoTek's Fluorescent Two-Hybrid (F2H®) assay. This assay enables real-time monitoring and quantitative analysis of interactions between GFP- and RFP-tagged proteins in live mammalian cells.

### Protein Complementation Assays

Protein-protein interactions can also be analyzed using protein complementation assays. These assays all rely on the use of fragments of a reporter protein, e.g. an enzyme or FP, that are fused to interacting proteins. Upon interaction of the fusion partners, the fragments of the reporter protein are brought into close proximity and are thus able to reconstitute an active protein. The reconstitution of a reporter protein from its fragments is also termed protein complementation. However, protein complementation cannot only be mediated in a conditioned way by interacting fusion proteins (as just described above), but also in an unconditioned way (self-complementation). The binding route highly depends on the kind of split FP fragments used.

The basic principle of protein complementation can be traced back to the 1950s, when Richards observed that subtilisin-cleaved fragments of ribonuclease are active again after self-complementation (Kerppola, 2008). Since then, various protein complementation assays using fragments of reporter proteins such as β-galactosidase, TEV protease, or luciferase have been developed.

However, these reporter proteins are not optimal for usage in protein-protein interaction assays, because the observed reporter enzyme products diffuse away from the investigated protein-protein interaction site. This diffusion leads to impaired colocalization of marker activity and PPI site (Kerppola, 2008, Cabantous et al., 2013). By contrast, the use of fluorescent protein fragments as tags in protein complementation assays, first described by Ghosh and coworkers (Ghosh et al., 2000), enables studies with the marker activity inherent to the PPI site. Ghosh and colleagues dissected a GFP variant (sg100GFP) between β-strands 7 and 8 and fused the resulting fragments to strongly interacting antiparallel leucine

zippers. They revealed that reconstitution of this split GFP variant is driven only by the interaction of both leucine zippers, works *in vivo* (e.g. *E.coli*) and *in vitro* (after GFP fragment purification from inclusion bodies), and that absorption and emission maxima are identical to parental full-length GFP (Ghosh et al., 2000, Kerppola, 2008).

## Progress in Split Fluorescent Protein Technology

In the following, we will describe key developments in the field of split FP assays. For an overview, commonly used split FPs are listed in Table 1; their split sites are mapped to a topology diagram of GFP in Figure 2.

In the early 2000s, Hu and coworkers identified the first eYFP fragments that form active complements only upon binding of their interacting fusion partners. They termed this mechanism of conditional complementarity "bimolecular fluorescence complementation" (BiFC) (Hu et al., 2002).

In 2003, Hu and Kerppola published designs for further variants of split FPs, which were intended for the use in (multicolor) BiFC (Hu and Kerppola, 2003). They generated fragments of eGFP, eYFP, eCFP, and eBFP truncated either between β-strands 7 and 8 or between β-strands 8 and 9 (Figure 2). The resulting fragments and combinations thereof were tested as fusions with interacting leucine zippers. Several combinations of fragments yielded indeed detectable fluorescence signals, but displayed varying tendencies for self-complementation. Such self-binding is not desired in BiFC assays and can be minimized by reducing expression of the fragment fusions to the lowest level that permits fluorescence detection.

While split eYFP had been suggested for usage in BiFC assays by Kerppola in 2008, the eYFP variant Venus is recommended as the split FP variant to use in BiFC assays by several authors today. (Kerppola, 2008, Miller et al., 2015, Zeng et al., 2017).

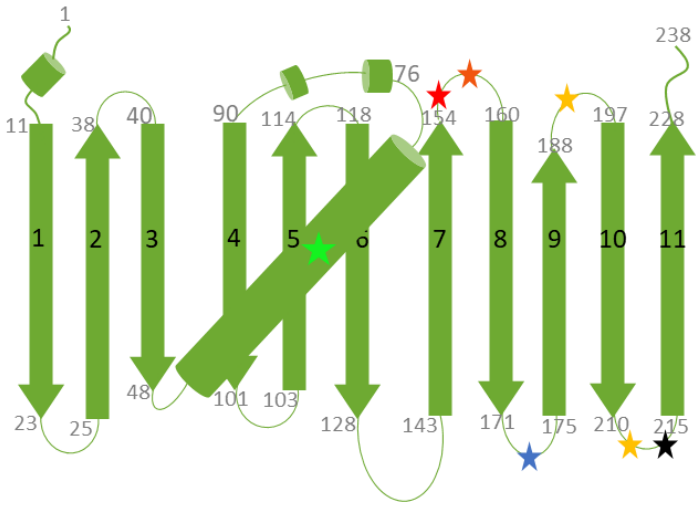



***Figure 2.*** *Secondary structure topology diagram of GFP and its variants. Shown are the 11 β-strands of GFP (or its variants) and the central α-helix. Black numbers on β-strands indicate the number of the respective β-strand, whereas the grey numbers on top or at the bottom of a β-strand mark individual amino acids (aa). The chromophore of any GFP variant is shown as a green star in the middle of the α-helix. Additional stars in red, blue, black and yellow mark split sites between β-strands for different split FP variants (see Table 1 for colour codes).*

*Table 1. Overview of selected split fluorescent proteins. Selection was based on estimated relevance of split FPs. Numbers after each split FP variant refer to respective β-strands included in fragments. Colored stars refer to split sites in Figure 2.*

| **Split FP** | **Split (sg100) GFP 1-7/8-11** ★ | **Tripartite Split GFP 1-9/10/11** ★ ★ | **Split eYFP * 1-7/8-11** ★ | **Split Venus 1-7/8-11** ★ | **Split eCFP * 1-7/8-11** ★ | **Split mLumin 1-7/8-11** ★ | **Split (sf) GFP 1-10/11** ★ | **Split mNG2 1-10/11** ★ | **Split sfCherry2 1-10/11** ★ |
|---|---|---|---|---|---|---|---|---|---|
| **N-term fragment**<br>**C-term fragment(s)** | GFP1-7<br>GFP8-11 | GFP1-9<br>GFP10, GFP11 | eYFP1-7<br>eYFP8-11 | Venus1<br>Venus2 | eCFP1-7<br>eCFP8-11 | mLumin1-7<br>mLumin8-11 | GFP1-10 opt<br>GFP11 (M3) | mNG2 1-10<br>mNG2 11 | sfCherry2 1-10<br>sfCherry2 11 |
| **Based on:** | **wtGFP** | **sfGFP** | **eYFP** | **eYFP --> seYFP --> Venus** | **eCFP** | **tagRFP** | **sf GFP** | **mNeonGreen** | **mCherry --> sfCherry** |
| **Split FP Mutations:** | **GFP1-7:** F64L, S65C, Q80R, Y151L **GFP8-11:** I167T, K238N | **GFP1-9 opt:** S2R, V16E, S28F, N39I, T43S, S99Y, N149K, K158N, K166T **GFP10 (T10):** L194D, N198D, S205T, V206I, P211L **GFP11 (S11):** F223Y, insertion: DAS231-233 | (no mutations) | (no mutations) | (no mutations) | **mLumin1-7**: R67K, N143S **mLumin8-11:** S158A, F174L, H197R | **GFP1-10 opt:** N39I, T105K, E111V, I128T, K166T, I167V, S205T **GFP11 (M3):** L221H, F223Y, T225N | **mNG2 1-10:** K128M, S142T, R150M, G172V **mNG2 11:** V228M | **sfCherry2 1-10:** E118Q, T128I **sfCherry2 11**: G219A |
| **Split site(s) (aa)** | 157/158 | 193/194 and 212/213 | 154/155, (173/174) ** | 154/155 | 154/155 (173/174) ** | 151/152 | 214/215 | 214/215 | 207/208 |
| **Applications *** ** | BiFC | TriFC | BiFC | BiFC, BiCAP | BiFC | BiFC | Self-complemen-tation | Self-complemen-tation | Self-complemen-tation |
| **Research tools offered by ChromoTek** | a-GFP $V_HH$ as used in GFP-Trap (predicted) | a-GFP $V_HH$ as used in GFP-Trap (e.g. Foglieni et al 2017) | a-GFP $V_HH$ as used in GFP-Trap (predicted) | a-GFP $V_HH$ as used in GFP-Trap (e.g. Trevelyan et al 2019) | none **** | a-RFP $V_HH$ as used in RFP-Trap (predicted) | a-GFP $V_HH$ as used in GFP-Trap (e.g. Leonetti et al 2016) | a- mNeonGreen $V_HH$ as used in mNeonGreen-Trap (predicted) | a-RFP $V_HH$ as used in RFP-Trap (predicted) |
| **Ex/Em (nm)** | 485/510 | 485/510 | 500/535 | 500/530 | 436/470 | 587/621 | 485/510 | 500/520 | 590/610 |
| **Reference** | Ghosh et al 2000 | Cabantous et al 2013 | Hu et al 2002, Hu et al 2003 | Trevelyan et al. 2019 | Hu et al 2003 | Miller et al 2015 | Cabantous et al 2005 | Feng et al 2017 | Kamiyama et al 2016, Feng et al 2017 |
| **Origin** | *Aequorea victoria* | *Aequorea victoria* | *Aequorea victoria* | *Aequorea victoria* | *Aequorea victoria* | *Entacmea quadricolor* | *Aequorea victoria* | *Branchiostoma lanceolatum* | *Discosoma sp.* |

***Split eYFP and split eCFP show poor maturation rates at 37°C (Miller et al., 2015); ****This split site is functional, but site 154/155 is recommended, generally;
*****Only successfully tested applications are listed (not exhaustive); ******Only weak binding of GFP-Trap due to N146I mutation in eCFP (Rothbauer et al., 2008);

In 2005, the Waldo laboratory developed a split GFP variant optimized for self-association of its fragments and minimal fusion partner disturbance. This split FP system is not intended to be applied to PPI studies, but to high-throughput solubility screens of libraries of protein mutants. In contrast to the split GFP variants of Ghosh or Hu, Cabantous' split variant was based on superfolder (sf) GFP, which was truncated between β-strand 10 and 11. N- and C-terminal fragments both were mutated to optimize their suitability for fluorescence complementation and to reach high solubility (Cabantous et al., 2005).

A few years later, the Waldo laboratory described an entirely new interaction assay based on tripartite split GFP (referred to as tripartite fluorescence complementation, TriFC) (Cabantous et al., 2013). In TriFc, three fragments are created by the double dissection of GFP between β-strands 9 and 10, and between β-strands 10 and 11, respectively. The advantage of TriFC over BiFC consists in the small size of the resultings tags, GFP10 (19 aa) and GFP11 (21 aa), which can be complemented by GFP1-9. GFP10 and GFP11 are also called T10 and S11 and are optimized for N-terminal tagging of a fusion partner. It was shown that fluorescence complementation only occurs upon interaction of the fusion partners of GFP10 and GFP11, rendering TriFC a promising tool to study PPIs *in vitro* and *in vivo*. In addition to PPI studies, TriFC allows the screening of libraries of interacting proteins or of libraries of small compounds inhibiting PPIs.

To diversify the range of colors of split FPs, additional variants based on yellow-green mNeonGreen2 or red sfCherry2 were introduced by Feng and coworkers (Feng et al., 2017). Using these variants, the authors created two-color or super-resolution images of endogenous proteins. Red, far-red and near-infrared split FPs have been the focus of further research efforts. However, the current red fluorescent protein (RFP) variants, split mCherry and split mRFP1 (Q66T), are functional only at relatively low temperatures (<30°C).

In the far-red range, two mutants of mKate, mLumin (Ex: 587 nm/Em: 621 nm, Table 1) and Neptune (Ex: 600 nm/Em: 650 nm) seem to be suitable for BiFC assays also at 37°C. Even longer excitation maxima (690 nm) can be targeted with the near-infrared FP iRFP, a bacterial phytochrome unrelated to the FPs discussed so far, which was successfully tested in BiFC assays (Miller et al., 2015).

It would exceed the scope of this whitepaper to describe all the recent advances in the field of split fluorescent proteins. Notwithstanding, we will describe some specific applications in more detail in the applications part below.

**Assay Types Based on Split FP Variants**

As already mentioned, the split FP variants discussed above can be applied to three basic assay types, FP self-complementation, bimolecular complementation and tripartite FP complementation. Here, we will summarise the basic characteristics of these assay classes:

FP self-complementation assays use FP fragments that self-associate independently of any fusion partner (Figure 3A).

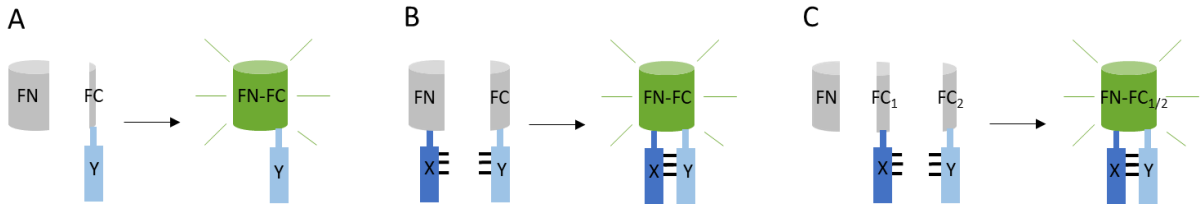



***Figure 3.*** *Schematic representation of fluorescent protein complementation assays. FN is the N-terminal and $FC_{1/2}$ the C-terminal fragment(s) of a fluorescent protein (FP). X and Y are interacting proteins fused to fragments of a FP.*
***A:*** *Fluorescent protein self-complementation,* ***B:*** *Bimolecular fluorescent complementation (BiFC),* ***C:*** *Tripartite fluorescent complementation (TriFC).*

C-terminal fragments of FPs used in self-complementation assays are generally smaller than those used in BiFC. The main aim of self-complementation assays is to study a single POI, e.g. its expression level, and not its interaction network.

The principle of bimolecular fluorescent protein complementation (BiFC) is shown in Figure 3B. A fluorescent protein (FP) is split into two parts, FN and FC, both fused to interacting proteins X and Y. Ideally, the FP complementation is driven only by the interaction of both fusion proteins. In such an optimal case, X and Y bind to each other and thus bring their FP fragments into proximity. Consequently, fragment reconstitution occurs quite fast and is virtually irreversible. Eventually, the fluorophore forms via maturation and gives rise to a fluorescence signal (Foglieni et al., 2017, Kerppola, 2008). The main aim of BiFC is the analysis of protein-protein interactions *in vivo*.

Tripartite fluorescent protein complementation (TriFC, Figure 3C) requires the splitting of an FP into three parts (FN, $FC_1$ and $FC_2$). Both interacting proteins (X, Y) are tagged with one of the small C-terminal fragment tags, $FC_1$ or $FC_2$ (~20 aa) (Cabantous et al., 2013). Small tags can be advantageous for some target proteins as they are expected to exercize minimal influence on the tagged protein. As in BiFC, the initial binding event occurs between the fusion proteins. Subsequently, the C-terminal fragments ($FC_1$ and $FC_2$) associate, but will only emit fluorescence upon binding of the third fragment, the N-terminal FN, which reconstitutes the full FP.

By using BiFC or TriFC, PPIs can be visualized in cells or *in vitro*. For both assays, minimal background fluorescence of the FP fragments in the absence of interacting fusion partners is essential, which translates to a minimum in self-association.

All three assay formats have in common that the reconstitution of FP fragments is virtually irreversible. For BiFC and TriFC assays, this means that even weak or transient protein interactions can be detected (Cabantous et al., 2013, Foglieni et al., 2017, Kerppola, 2008).

## Combining split FP with $V_HH$ Technology

Split FPs enable the visualization of PPIs, which can then be validated using biochemical methods, e.g. (co-) immunoprecipitation using FP-binding antibodies. In recent years, a remarkable class of single-domain antibodies called $V_HHs$ (or nanobodies) has emerged as an ideal tool for immunoprecipitation and other biochemical assays. $V_HHs$ constitute the heavy chain variable domain derived from camelid heavy chain-only antibodies and are among the smallest known

functional antibody fragments (13-15 kDa). Like conventional antibodies, $V_HHs$ bind to their cognate antigen with high affinity, i.e. with dissociation constants ($K_D$) in the nanomolar to low picomolar range. Unlike most conventional antibodies, however, $V_HHs$ tend to be extremely stable and remain functional at high temperatures and under harsh chemical conditions (van der Linden et al., 1999, Dumoulin et al., 2002, Muyldermans, 2013).

Of special interest is ChromoTek's GFP-Trap, which comprises an anti-GFP $V_HH$ immobilized to agarose-beads for one-step immunoprecipitation. The ChromoTek GFP-Trap binds to full-length GFP and GFP derivatives like eGFP, sfGFP, eYFP, Venus, CFP (less so eCFP) or BFP with very high affinity (dissociation constant $K_D$ of 1 pM[1]). In the context of split GFP or its variants, it captures only fully reconstituted GFP, but not its fragments, which enables the combination of BiFC with immunoprecipitation (see below).

## Case studies of the application of ChromoTek's GFP-Trap to split FP experiments

ChromoTek's GFP-Trap has been cited in more than 1800 publications for the use in immunoprecipitation, a number of which also refer to split FPs. In the following, we will present five case studies, in which ChromoTek's GFP-Trap was used together with split FPs. Common to these five studies is that each add an innovative approach to the field of split FPs, emphasizing how this field is still evolving.

### Foglieni et al. 2017 – an example for TriFC

Foglieni and coworkers employed split GFP to structurally characterize and quantify functional biomolecular interactions in the context of neurodegeneration (frontotemporal dementia (FTD)) (Foglieni et al., 2017). The investigation of how, when, and where certain proteins (self-) interact to form possibly toxic aggregates is crucial to better understand neurodegeneration. As model proteins, they used Tau and TAR-DNA-binding protein (TDP-43), which are both associated with FTD.

By using TriFC, they showed that the trimolecular complex of GFP10-TDP-43, GFP11-TDP43 and GFP1-9 reflects the subcellular localization of nuclear TDP-43 assemblies. They also successfully stained post-fixed cells, co-transfected with GFP10-TDP-43 and GFP11-TDP-43, by the simple addition of recombinantly produced GFP1-9. This experiment confirmed that TDP-43 self-assembly occurs independently of the presence of GFP1-9.

Using the ChromoTek GFP-Trap Magnetic Agarose (gtma), they validated these protein-protein interactions biochemically. Lysates of HEK293 cells, co-transfected with GFP10-HA-TDP-43, GFP11-β1-TDP-43 and GFP1-9, were analyzed by immunoprecipitation and Western Blot (IP/WB). Again, they observed interaction between the two TDP-43 constructs in the presence of GFP1-9. If any of the three components was missing, no protein was precipitated, which underlines the

[1] Kinetic parameters of the GFP-Trap have been determined using Dynamic Biosensors' switchSENSE® technology

specificity of the ChromoTek GFP-Trap for the fully reconstituted split GFP.

### Leonetti et al. 2016 – an example for self-complementation

Leonetti and colleagues combined CRISPR/CAS9 and split GFP technology (GFP1-10 & GFP11) to tag endogenous human genes (Leonetti et al., 2016). They used HEK293T cells stably expressing GFP1-10 as a parental cell line. Using CRISPR/CAS9 technology, they generated 48 cell lines by fusing GFP11 to different endogenous model proteins, representing various subcellular locations such as the cytoskeleton, endoplasmic reticulum, nucleus, or endosomes.

Crucially, the small size of the 16-aa GFP11 tag enabled the authors to transduce the parental cell line with single-guide RNA/CAS9 ribonucleoprotein complexes. In 30 of 48 cases, the resulting fluorescence signal was sufficiently high to be detected by flow cytometry analysis. In additional cases, successful albeit low expression of GFP11-tagged protein was observed using confocal microscopy. The overall knock-in efficiency (i.e. the fraction of green fluorescent cells) was determined to be approximately 36% using flow cytometry. The fluorescence signal could be increased using repeats of GFP-11 tags owing to a higher number of self-complemented GFP molecules. In addition, GFP self-complementation allowed the correlation of the GFP signal to protein expression levels by ribosome profiling.

In a next step, Leonetti et al. illustrated the benefit of their GFP1-10/GFP-11 complementation approach for the analysis of native, endogenous networks. Using the ChromoTek GFP-Trap Agarose (gta), they isolated four native, well-established multiprotein complexes, namely cohesin, SEC61 translocon, clathrin, and SPOTS sphingolipid synthesis complex. For each complex, a single subunit had been tagged using GFP11 and was used as bait-protein for the immunoprecipitation using ChromoTek GFP-Trap. Western blot analysis confirmed the presence of the bait as well as of its expected interactions partners.

Moreover, they modified this strategy to enable the specific and non-denaturing release of captured proteins from the ChromoTek GFP-Trap. Non-denaturing elution is desired for subsequent activity assays or structural studies, for example. To this end, they included a TEV protease cleavage site between the GFP11 tag and the POI. Thus, captured target protein was eluted using on-resin TEV protease cleavage, which yielded protein of high purity – despite the low abundance of the endogenous proteins in question. This example underlines the unusually high affinity of the ChromoTek GFP-Trap, which results in very efficient pulldown experiments even for proteins of low expression.

### Croucher et al. 2016 – combining BiFC with immunoprecipitation (BiCAP)

Croucher and coworkers introduced a new method, which they dubbed bimolecular complementation affinity purification (BiCAP). They combined conformation-specific nanobodies with a protein-fragment complementation assay and affinity purification (Croucher et al., 2016). Traditionally, affinity purification coupled with tandem mass spectrometry (AP-MS/MS) is used to isolate a single bait protein and its interaction partners. In contrast, BiCAP facilitates the specific

isolation and characterization of the interactome of a binary protein complex. In their report, the interactome of ERBB2 (also known as Her2), a member of the family of epidermal growth factors (EGFR), was characterized either in the form of a homo- or a heterodimer (with EGFR or ERBB3). The ERBB2 gene is amplified in many breast cancer cells, and ERBB2 dimers are targeted by several therapeutic agents.

The authors used split Venus (a variant of GFP) and specifically selected the ChromoTek GFP-Trap for immunoprecipitation analysis, as the GFP-Trap captures only reconstituted Venus, i.e. the ERBB2 homo-/heterodimers. MS analysis of BiCAP-isolated receptor dimers revealed a core interactome of ten protein for the three dimer pairs (ERBB2/ERBB2, ERBB2/EGFR, ERBB2/ERBB3), but identified also a set of proteins distinct to each dimer. Thus, the BiCAP approach in combination with the ChromoTek GFP-Trap provides a powerful method for the analysis of interactomes.

### Trevelyan et al. 2019 – another example of BiCAP

Trevelyan et al. (Trevelyan et al., 2019) applied the BiCAP approach and split Venus to apoptosis signal-regulating kinases (ASK1-3), which are activators of the P38 and JNK MAP kinase pathways. ASK1, for example, is associated with melanoma, gastric cancers, or non-alcoholic steatohepatitis (NASH). Several inhibitors of ASK1 are the object of clinical trials. The kinases ASK1-3 form oligomeric complexes called ASK signalosomes, a process, which is not fully understood yet. Thus, Trevelyan and colleagues set out to further investigate the formation of these signalosomes.

In one of their experiments, they determined the stoichiometry of the complex comprising ASK1 and ASK2. Using the ChromoTek GFP-Trap Agarose (gta), they immunoprecipitated two split Venus fusion proteins, ASK1-Venus1 and ASK1-Venus2, from HEK293T cells followed by mass spectrometry analysis. Although ASK1 was overexpressed, the abundance of ASK2 was 75% of ASK1, which indicates a selective incorporation of near-equal ratios of ASK1 and ASK2. In addition, other proteins assumed to interact with ASK1 were identified, too (e.g., ASK3 or several members of the ubiquitin ligase family). As for Croucher et al., the key to this experiment was the use of the ChromoTek GFP-Trap, which only binds to reconstituted Venus protein and not to Venus1 or Venus2 fragments, facilitating interactome studies of ASK1 dimers only.

### Dáder et al. 2019

Using the split GFP system GFP1-10/11, Dáder and coworkers (Dáder et al., 2019) have conducted immunoprecipitation experiments with *Aradopsis thaliana* and a plant virus protein. In this specific case, P6 protein, also termed transactivator-viroplasmin (TAV), a key player in the viral replication cycle from *Cauliflower mosaic virus* was used (CaMV). As CaMV is a plant virus with a small circular DNA genome of 8 kb that does not tolerate genome insertions longer than a few hundred nucleotides, tagging of P6 with the 16 amino acid GFP fragment GFP11 (termed 11P6) was key to facilitate these studies.

GFP1-10 transgenic *Aradopsis thaliana GFP1-10* was infected with $CaMV_{11P6}$. Plants developed typical mosaic, yellowing and stunting symptoms like control plants inoculated with $CaMV_{wt}$, however, a little time-delayed. GFP reconstitution of $CaMV_{11P6}$-infected *A. thaliana GFP1-10* plants was successfully confirmed by whole plant imaging with a fluorescence scanner.

Cell lysates prepared from healthy and $CaMV_{11P6}$-infected *A. thaliana GFP1-10* leaves were subjected to immunoprecipitation using the ChromoTek GFP-Trap Magnetic Agarose (gtma). Subsequent SDS-PAGE and Western blot analysis showed that 11P6 was indeed captured by the ChromoTek GFP-Trap, indicating affinity-purification of 11P6 as part of the reconstituted split GFP complex.

## Conclusion

Split fluorescent protein complementation assays are broadly applicable to the visualization of target proteins or protein-protein interactions within their cellular setting, to the screening of libraries, or to cell sorting. In conjunction with other biochemical methods (e.g. immunoprecipitation and mass spectrometry) they are very powerful orthogonal validation methods.

As underlined by our small selection of case studies (Foglieni et al., 2017, Leonetti et al., 2016, Trevelyan et al., 2019, Dáder et al., 2019, Croucher et al., 2016), $V_HH$-based reagents such as the ChromoTek GFP-Trap are highly valuable tools in split FP assays. For example, in a bimolecular complementation and affinity purification assay (BiCAP), it was shown by Croucher et al. and Trevelyan et al. that the ChromoTek GFP-Trap exclusively binds to the reconstituted form of the split GFP variant Venus. This binding mechanism is predicted to hold true for other split GFP variants with similar split sites.

While there are no data available yet for non-GFP-derived split fluorescent proteins such as mNeonGreen2, sfCherry2 or mLumin, other ChromoTek NanoTraps, e.g. mNeonGreen-Trap or RFP-Trap, may also bind their reconstituted target FP and thus further facilitate biochemical experiments. In addition to the use of ChromoTek's Nano-Traps, other $V_HH$ formats might be applicable to split FP assays. For instance, one could envisage the use of unconjugated ChromoTek a-FP $V_HH$ in sandwich immunoassays or of the ChromoTek Nano-Boosters in immunofluorescence experiments with split FP variants.

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