nature portfolio

Peer Review File

Design and evaluation of a tripartite chemogenetic fluorescent reporter for visualizing ternary protein complexes

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This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Attachments originally included by the reviewers as part of their assessment can be found at the end of this file.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The author designed a method for protein visualization, which allows for the observation of ternary protein interactions in living cells using a triad pFAST. However, the author only demonstrated the technique with patterned protein, which is essentially only a proof-of-concept verification experiment without additional biological new findings involved.

Major issue:

1.Why is the complementation (%) in Figure 3b expressed as a percentage, and what is it based on? Is it the intensity of fluorescence or another metric? The author should clarify the rationale behind this.

2.In Figure 4b, for the same ternary complex using HMBR as the fluorescent group, why is the complementation significantly lower (less than 50%) compared to the other three groups? The underlying principles are lacking.

3.Why did the author use two different types of cells, HeLa and 293t cells, in the experimental setup? It is necessary to explain the impact and reasons for changing cell types on the experimental results.

4.In Figure 6, why use the fluorescence ratio of mcherry/ECFP as the y-axis, and how does this ratio prove correlation? From the confocal fluorescence images, it seems more appropriate to use the AU values of green fluorescence to demonstrate differences between experimental control groups.

5.Why do negative values appear in the ratio of AU fluorescence intensity in Figure 6c,f,j, but not in Figure 6i?

6.Are the three proteins selected by the authors, namely Tom20, cb5, and lyn11, indicative proteins of mitochondria, endoplasmic reticulum, and the inner membrane of the cell membrane? References supporting this choice need to be provided, and the biological significance of selecting these three proteins should be explained.

7.In the fluorescence images of PM in Figure 6e and 6k, there are no apparent nucleus-like void, while in the fluorescence image of Figure 6b, there are clear nucleus-like void at the same location. The inconsistency in the morphology of fluorescence images in the same area is problematic.

8.In the experiments in Figures 3 and 4, labeling was done at the C-terminus and N-terminus of two FRB proteins, respectively. What are the similarities and differences compared to labeling at both the C-terminus and N-terminus of the same FRB protein? Is it necessary to set up different experiments to compare the effects.

Minor issue:
1.The first line indentation is inconsistent throughout the entire text. For instance, the abstract has no indentation, while the introduction only has the first line indented. The first four paragraphs after "Results" are indented, but the following

paragraphs are not.

2.The figure legends for Figure 6 should include the full name of the abbreviations used. (such as PM, MT, cyto, ER, etc)

3.In Figure 6i, there is a missing dashed line.

Reviewer #2

(Remarks to the Author)
In this study, the authors present the development of a tripartite split fluorescent reporter based on the fluorogen-activating protein FAST. The same group had previously developed a bimolecular fluorescent reporter called split-FAST for visualizing the association of protein complexes in live cells (Tebo et al. Nat. Comm. 2019). The reconstitution of FAST through split FAST complementation drives the binding of FAST to a small molecule, HMBR, which is inherently non-fluorescent. Upon the formation of FAST-HMBR complex, the fluorogen module emits fluorescence when excited by light.
The goal of the current study is to achieve the detection of trimeric complexes in cells. The paper is well-written and the methodology is appropriate. However, crucial control points related to protein association kinetics and the biochemical characterization of tripartite split-FAST complementation are lacking.
The application chosen to validate the system uses the same approach that was developed by Inoue's group (Wu et al. Nat Meth, 2020) for CIT to induce heterotrimerization of split FRB:FKBP at organelle contact sites. In contrast of colocalization presented in Inoue's paper, the present system exhibits a fluorescence signal when the cytosolic FKBP is recruited by ramapycin induced by association with FRB at contact sites. In terms of novelty, split fluorescent probes also allow the detection of contact sites without background but are irreversible (Cieri et al., Cell death differ., 2018). The novelty of the present application of the system could be enhanced by illustrating the dynamics of the detection of MCS (removal of rapamycin).
Regarding the general application of the system as a protein-protein interaction (PPI) reporter for trimeric complexes, the demonstration is weak. It has only been conducted with highly affine PPI proteins (split-FRB:FKBP), serving as a good proof of principle but not representing a true trimolecular complex. There is a substantial number of trimeric complexes in the literature that could be utilized to further validate the system, supporting its claim to be a general reporter of trimeric complexes, as suggested in the title of the article.

1- To convert it into tripartite, the authors conducted circular permutation analysis to identify an additional split site in the previously developed bipartite split-FAST (Tebo et al. Nat. Comm. 2019). The authors demonstrated that one configuration, cpFAST (98-99), was capable of binding to the HMBR probe with a Kd that is 10-fold lower than full length FAST (0.1 µM). Further engineering of bipartite or tripartite split-FAST, the HMBR concentration used varies between 1 and 10µM, established through serial dilution of concentrated HMBR. It is unclear why the fluorogen concentration has to be screened for each PPI reporter system if only part of the probe is converted to fluorescence upon binding to FAST? It could be sufficient to work in excess of the probe relative to the concentration of FAST. Under these conditions, the reconstitution of the signal should more likely depend on the interacting proteins rather than the probe, which would act as a detector of reconstituted FAST.
The authors should clarify this point in the text, despite the development of previous technologies based on that assay. Ideally, the authors should state if independent measurements of the binding of their probe to the FAST variants have been done, apart from fluorescence, such as ITC or other biophysical method. If not, conducting the experiment will help to compare the two measurements revealing the fraction of the protein that is active to promote the fluorescent analogue.

2- The FRB:FKBP:RAP association is widely used to validate the system. However, the methodology to characterize the interaction and the results are intriguing. In Fig.3b and 3d, the data states as an example that EC50,–interaction = 31 ± 4 µM and an EC50,+interaction = 7.5± 0.7 µM. These values are set to be relative to HMBR binding driven by the interaction and are not representative of the affinity of the FRB:RAP:FKBP complex, which is known to be around the nanomolar range. To accurately assess the response to rapamycin binding of FRB, the authors should monitor FRB:FKBP association at a fixed concentration of HMBR (optimal 10µM) while varying rapamycin concentrations from the µM down to nM range. This would reveal the correlation between protein binding and reporter signal. It will also enable the assessment of whether the association follows appropriate binding kinetics driven by rapamycin. The same remarks apply to other figures, specifically 4b-4f and 5b, in which this interaction is monitored.

3- FACS cytograms from Supp. Fig.6 show that a high concentration of HMBR (at least 1µM) are required to observe a shift of fluorescence between +RAP/-RAP conditions. It appears that this shift does not improve above 5µM. In line with this, the authors demonstrate in Fig. 3B a complementation curve on FRB/FKBP association with various HMBR concentrations "knowing that the efficiency of self-complementation and interaction-specific complementation depends on the concentration of fluorogen" (p5). If the fluorogen only fluoresces when bound on FAST and when complementation has occurred, the fluorescence should be independent of the fluorogen if it is not limiting to FAST protein abundance. Why do the results consistently present data in relation to increasing concentration of the reporter probe?
It is possible that self-association between split-FAST domains, independently of the interactions, needs to be reduced through mutagenesis. This point should be discussed. In term of proper use of the methodology, it it should be clarified by the authors whether, when investigating a new PPI pair, one should first monitor the binding curve of HMBR to determine the concentration that gives minimal background. Subsequently, the assay should be performed at only this given concentration combined with variable concentrations of rapamycin. The purpose of such an experiment is not clearly explained.

4- In term of presentation, the authors are progressively introducing different variants of FAST to enhance the split-reporters. This can be confusing for readers and the introduction of new acronyms requires frequent references to the literature. Adequate supplementary information should be added.
Data on RspA split-FAST are presented in the main body (Fig. 3). We are wondering why this variant has less self-association. Is it due to folding properties or association to the probe? This should be stated in the text. Additionally, it refers to an unpublished study, which requires the addition of characterization in supplementary information. How much homology does RspA FAST have with FAST to allow for a similar split site? A comparison of circular permuted RspA FAST (98-99) along with the CP FAST variant in supp. Fig. 2 will help to compare the two variants. This is an important point as this final product is used to further engineer the tripartite version, which is the focus of the study.
We find out later that another variant, pFAST, is used instead of RspA because the authors discovered that RspA had too much self-association when used in a tripartite mode. To make this clear, the figure title if Fig. 5 should state the variant used (ex: pFAST). A dedicated supplementary figure that summarizes the characteristics of the variants used for engineering split would be useful such as DNA sequence alignments and structural superimpositions of the three FAST variants used with split sites. A table listing their properties (affinities to the probes, fold change with rapamycin) would be appreciated.

5. According to the authors' statement (p. 9), the efficiency of tripartite reconstitution appears to be limited by the affinity of the probe to pFAST. Another possible reason could be that the fragments do not reconstitute efficiently. The assays in Fig. 5 were conducted in mammalian cells without controlling the expression levels of each fusions. The authors should investigate whether the pFAST fragments are easily purified in vitro and how the tripartite association behaves as a function of the concentration of each partner. Trimolecular association is complex as it depends on the relative affinities between the three partners.
The assay could be setup with equimolar amounts of each of the three fusions or with an excess of a third fragment at a fixed concentration of the fluorogen. This would help determine whether one of the three fragments should be in excess relative to the others. Furthermore, one should determine the order in which the split pFAST should be used (from N-terminal split internal and C-terminal fragments) and whether there are steric constraints to fusion tagging between the split domains.

Fig 5d. Reversibility of the system has been only proved for the bipartite system using FKBP homodimer dissociation experiments (ref 8 Tebo, A. G. & Gautier, A. Nat Commun 10, 2822–8 (2019). To add novelty to previously developed tripartite systems, the author should evaluate the reversibility of the tripartite system by removing Rapamycin to observe fluorescence decay.
Fig 5f. The example should favor first the split-FAST fused to FRB (pFAST99-114–NFRB + CFRB–pFAST1-98) for reassembly, then use FKBP to combine with rapamycin (creating a bimolecular complex). One needs to setup the background association if one of the three split-FAST fragments is expressed alone or with a non-relevant protein.

When the split fragments of FRB are directed to plasma membrane, a control should be one of the split-FRB fusion not directed to the membrane. Does the tripartite association is decreased? Or is the fluorescence signal fainter? (see Fig 5g). My concern here is that the detection is influenced by the strong localization signals on split-FRB proteins.

Figure 5g. control: Label FKBP with a specific antibody to show that FKBP is not recruited to FRB-PM in the absence of rapamycin.

Fig. 6 The experiments suggest that the junction between the different organelles is artificially induced by the addition of Rapamycin (e.g: FRB-CFP-CAAX is almost exclusively bound to the PM and massively relocalizes to the MT in the presence of Rap). If this is correct, it should be clarified in the text. One missing control is one of the fusion devoyed of split-FRB domain to evaluate the specificity of tripartite association (ex: CFP-CAAX and CFP-Cb5 without N-FRB).

Minor points

Fig. 3 For how long are the RAP and HMBR added in all of these experiments? Simultaneously or sequentially? (applies to fig. 4 ; fig. 5 and fig. 6). A method section regarding the use of the fluorogen with rapamycin induction is not provided.

Fig. 3f: Live assay. Line curves corresponding to complementation without Rapamycin is missing (applies to fig. 4h; fig. 5d and fig. 5h). Specify whether it is substracted to obtain final fluorescence values. Specify the terms used for fluorescence “Fmax”; “Fmin”. Define Fmax/Fmin in the legend (the same applies for Fig. 4 and Fig. 5)

Reviewer #3

(Remarks to the Author)
In this manuscript, the authors have developed a tripartite-split-FAST for the observation of three protein interactions. The development of the system is essential for the detailed study of protein-protein interactions. However, there are some fundamental issues that should be clarified. Here are my suggestions for the authors to improve their manuscript. Please find attached.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have adequately explained the significance of the work, revised the title, and supplemented Figure 7 to demonstrate its potential for biological applications. However, some issues still require clarification：
1. In Figure 4, the authors demonstrate that pFAST exhibits superior performance. However, it remains unclear why pFAST was not employed from the outset. A justification for this experimental design choice should be provided.

2. In Figure 6, the authors utilize a tripartite-split-FAST system by separating FRB into C-FRB and N-FRB. In contrast, Figure 4 employs pFAST fused to either the N- or C-terminus of FRB, representing a binary complex approach. These experimental designs should be made consistent, or the authors must provide a clear rationale for the differing methodologies.

3. The authors should clarify the theoretical basis for using PYP instead of FAST in the Circular Permutation site predictor (CPred) analysis. Additionally, it would be valuable to confirm whether crystal structure data for pFAST are available, as such structural insights could strengthen the mechanistic interpretation.

4. In Figure 6, the 20 μm scale bar appears inconsistent with the typical diameter of HeLa cells (~40 μm). The authors should verify this measurement and correct it if necessary. Did the authors investigate interactions at the subcellular level in Figure 6? Including higher-magnification confocal images would help elucidate finer details of these interactions. In Figure 6k, the green signal (indicating interactions) appears to colocalize with all mitochondrial regions, suggesting universal interaction with the plasma membrane and ER. The authors should discuss whether this observation is biologically plausible or if alternative interpretations exist.

5. In Figure 7, regions with intense green fluorescence correspond to areas where blue fluorescence is diminished. The authors should address the potential mechanistic basis for this inverse correlation.

Reviewer #2

(Remarks to the Author)
The authors addressed most of my comments appropriately. They included an additional example involving three different proteins, which confirmed the correct association of ternary H2B-Fos-Jun zipper pairs. Nonetheless, as the authors acknowledge in the discussion, the robustness of this protein–protein interaction complex does not preclude potential challenges that may arise in ternary PPI assays. The limited scope is appropriate, given the potential value of the new assay to the field. The revised Figures 3a and 5a now illustrate more clearly the actual sequence of protein association that may occur during tripartite reassembly. In light of the complexity of such engineered systems, the addition of a supplementary table detailing binding affinity constants of the various split pairs is a valuable and much-appreciated addition.

The authors clarified the specific requirements of FAST assays that utilize chemical probes—namely, the fluorogen and its dependence for visualizing protein complementation. Despite not showing the reversibility of the assay, they acknowledged previous publications.
Minor :
1-it is not mentioned whether the chemical probes HMBR and HBR are available through as open source or commercial suppliers.
2- The choice of FAST fragments and the position of the tags is not explained.

Reviewer #3

(Remarks to the Author)
The authors have addressed the previous reviews.

Version 2:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have addressed previous inquiries within this manuscript. pFAST represents a good tool for investigating protein-protein interactions and has been previously successfully employed in diverse intracellular systems. [Nature Methods volume 20, pages1553–1562 (2023)]. So, my only remaining concern is the novelty of pFAST strategy. I will leave this judgement to the editorial decision.

REVIEWER COMMENTS

Reviewer #1

The author designed a method for protein visualization, which allows for the observation of ternary protein interactions in living cells using a triad pFAST. However, the author only demonstrated the technique with patterned protein, which is essentially only a proof-of-concept verification experiment without additional biological new findings involved.

#1.0 We are grateful to reviewer 1 for their review and comments. We are very excited about the potential of our approach to uncover new biological insights. However, we would like to emphasize that the true innovation of our paper lies in the development and engineering of the tripartite complementation system. Designing tripartite split proteins presents significant challenges, with only a few examples in the literature. Our methodology for the design and characterization of this system is noteworthy and deserves thus recognition. We are confident that this unique tool will drive significant biological discoveries in the future. To emphasize our focus on engineering this tripartite system and its potential for visualizing ternary protein interactions and complexes, we have updated the title to “A tripartite chemogenetic fluorescent reporter: design and evaluation for visualizing ternary protein complexes”. To further demonstrate the potential of tripartite-split-FAST for the detection of ternary interactions with physiological relevance, we present in this revised version demonstration that tripartite-split-FAST enables the selective visualization of the Fos-Jun-DNA ternary interaction (see new Fig. 7).

Major issue:

1.Why is the complementation (%) in Figure 3b expressed as a percentage, and what is it based on? Is it the intensity of fluorescence or another metric? The author should clarify the rationale behind this.
#1.1 The analysis of the complementation was performed as follows: In the flow cytometry analysis, the evolution of the mean cell fluorescence in the function of the concentration of fluorogen in the presence and in the absence of rapamycin was fitted with a one-site binding model equation, sharing the maximal value for the experiments in the presence and in the absence of rapamycin. This allows us to determine the concentration of fluorogen for half-maximal complementation as well as the theoretical maximum fluorescence value. This latter value is then used to compute the percentage of complementation for each concentration of fluorogen. We added a text explaining our analysis in the Methods part.

2.In Figure 4b, for the same ternary complex using HMBR as the fluorescent group, why is the complementation significantly lower (less than 50%) compared to the other three groups? The underlying principles are lacking.
#1.2 We thank reviewer 1 for their remark. We call our system a tripartite system because it is composed of 3 protein fragments, but it is actually a four body system that also includes the fluorogen. The appearance of the fluorescence necessitates the assembly of 4 molecules, the three fragments of FAST and the fluorogen, making the complementation of the system a four-body problem, in which the concentration of fluorogen (and its affinity) is an experimental control parameter influencing the

complementation efficiency. In particular, our experiments on Figure 4b and 4c show that the use of a highly affine fluorogen like HBR-2,5DM rather than HMBR favors the complementation of the tripartite generated from RspA-FAST. We observed the same trend with the tri-partite system generated from pFAST (Fig 4d,e). We modified the text to better explain this point.

3.Why did the author use two different types of cells, HeLa and 293t cells, in the experimental setup? It is necessary to explain the impact and reasons for changing cell types on the experimental results.
#1.3 We used HEK293T cells for flow cytometry experiments and for microscopy-based imaging of cytosolic or membrane-localized interactions. We preferred HeLa cells for imaging interactions in specific localizations by confocal microscopy because HEK293T cells are a bit small and not very microscopy friendly.

4.In Figure 6, why use the fluorescence ratio of mcherry/ECFP as the y-axis, and how does this ratio prove correlation? From the confocal fluorescence images, it seems more appropriate to use the AU values of green fluorescence to demonstrate differences between experimental control groups.
#1.4 We thank reviewer 1 for this comment showing that we were not fully clear. On figure 6 we do not report the fluorescence ratio of mCherry and ECFP but a correlation coefficient enabling to evaluate the colocalization of the two proteins. The Y-axis was labeled 'mCherry/CFP correlation coefficient (arb. units)' and not 'mCherry/CFP ratio', and the legend of the figure says: 'Correlation of the ECFP and mCherry signals'. Correlation was assessed computing image correlation coefficient using Fisher's transform of Pearson's correlation coefficient as previously reported in Wu, H. D. et al. Rational design and implementation of a chemically inducible heterotrimerization system. Nat. Methods 17, 928–936 (2020). We modified the Y-axis legend to clearly state that we plot 'correlation coeff. of mCherry & ECFP signals'.

5.Why do negative values appear in the ratio of AU fluorescence intensity in Figure 6c,f,j, but not in Figure 6i?
#1.5 As stated above, these graphs do not show ratios of fluorescence but correlation coefficient that can take negative values.

6.Are the three proteins selected by the authors, namely Tom20, cb5, and lyn11, indicative proteins of mitochondria, endoplasmic reticulum, and the inner membrane of the cell membrane? References supporting this choice need to be provided, and the biological significance of selecting these three proteins should be explained.
#1.6. We thank reviewer for this comment. TOM20 is the mitochondrial import receptor Tom20 subunit, Cb5 is the cytochrome b5, and lyn11 is the N-terminal 11 amino acid sequence of the Lyn kinase allowing targeting at the plasma membrane. These proteins are very classical to target the surface of mitochondria, the ER and the inner plasma membrane. We modified the text to introduce additional information on these proteins. About the biological relevance, we chose proteins expressed at the surface of various organelles enabling to artificially induce (with the splitFRB/FKBP trimerization system) and image (with tripartite split FAST) junctions between different organelles. MT-ER, ER-PM and MT-PM membrane contact sites/junctions were chosen as archetypes of membrane contact sites because of their biological relevance in the regulation of various biological processes. We added

a sentence in the main text to precise this point.

7.In the fluorescence images of PM in Figure 6e and 6k, there are no apparent nucleus-like void, while in the fluorescence image of Figure 6b, there are clear nucleus-like void at the same location. The inconsistency in the morphology of fluorescence images in the same area is problematic.
#1.7. The observed difference comes from the fact that we image different combination of organelles each time and therefore we do not always use exactly the same z-position to be able to image properly the different organelles.

8.In the experiments in Figures 3 and 4, labeling was done at the C-terminus and N-terminus of two FRB proteins, respectively. What are the similarities and differences compared to labeling at both the C-terminus and N-terminus of the same FRB protein? Is it necessary to set up different experiments to compare the effects.
#1.8. We are not sure to fully understand this comment. We choose in general tagging topologies for obtaining optimal complementation.

Minor issue:
1.The first line indentation is inconsistent throughout the entire text. For instance, the abstract has no indentation, while the introduction only has the first line indented. The first four paragraphs after "Results" are indented, but the following paragraphs are not.
#**1.9** We corrected first line indentation to be consistent throughout the manuscript.

2.The figure legends for Figure 6 should include the full name of the abbreviations used. (such as PM, MT, cyto, ER, etc)
#1.10 Since the full names of these abbreviations are conventional, already mentioned in the text, and to ensure an easy readability of the figures, we opted to keep the naming as such.

3.In Figure 6i, there is a missing dashed line.
#1.11 There is no missing dashed line in Figure 6i. The dashed line on the other graph indicates the 0 value, as there are negative values. On figure 6i, there is no negative value so the graph starts at 0, so there is no need for a dashed line.

Reviewer #2

In this study, the authors present the development of a tripartite split fluorescent reporter based on the fluorogen-activating protein FAST. The same group had previously developed a bimolecular fluorescent reporter called split-FAST for visualizing the association of protein complexes in live cells (Tebo et al. Nat. Comm. 2019). The reconstitution of FAST through split FAST complementation drives the binding of FAST to a small molecule, HMBR, which is inherently non-fluorescent. Upon the formation of FAST-HMBR complex, the fluorogen module emits fluorescence when excited by light.
The goal of the current study is to achieve the detection of trimeric complexes in cells. The paper is well-written and the methodology is appropriate. However, crucial control points related to protein association kinetics and the biochemical characterization of tripartite split-FAST complementation are lacking.

The application chosen to validate the system uses the same approach that was developed by Inoue's group (Wu et al. Nat Meth, 2020) for CIT to induce heterotrimerization of split FRB:FKBP at organelle contact sites. In contrast of colocalization presented in Inoue's paper, the present system exhibits a fluorescence signal when the cytosolic FKBP is recruited by ramapycin induced by association with FRB at contact sites. In terms of novelty, split fluorescent probes also allow the detection of contact sites without background but are irreversible (Cieri et al., Cell death differ., 2018). The novelty of the present application of the system could be enhanced by illustrating the dynamics of the detection of MCS (removal of rapamycin).
Regarding the general application of the system as a protein-protein interaction (PPI) reporter for trimeric complexes, the demonstration is weak. It has only been conducted with highly affine PPI proteins (split-FRB:FKBP), serving as a good proof of principle but not representing a true trimolecular complex. There is a substantial number of trimeric complexes in the literature that could be utilized to further validate the system, supporting its claim to be a general reporter of trimeric complexes, as suggested in the title of the article.

#2.0. We are grateful to reviewer 2 for their review and comments. We are very excited about the potential of our approach to uncover new biological insights. However, we would like to emphasize that the true innovation of our paper lies in the development and engineering of the tripartite complementation system. Designing tripartite split proteins presents significant challenges, with only a few examples in the literature. Our methodology for the design and characterization of this system is noteworthy and deserves thus recognition. We are confident that this unique tool will drive significant biological discoveries in the future, in particular for the study of membrane contact sites, for which reporters based on bipartite splitFAST have been recently developed (see García Casas et al Nat. Commun 2024). To emphasize our focus on engineering this tripartite system and its potential for visualizing ternary protein interactions and complexes, we have updated the title to "A tripartite chemogenetic fluorescent reporter: design and evaluation for visualizing ternary protein complexes". To further demonstrate the potential of tripartite-split-FAST for the detection of ternary interactions with physiological relevance, we present in this revised version demonstration that tripartite-split-FAST enables the selective visualization of the Fos-Jun-DNA ternary interaction (see new Fig. 7).

1- To convert it into tripartite, the authors conducted circular permutation analysis to identify an additional split site in the previously developed bipartite split-FAST (Tebo et al. Nat. Comm. 2019). The authors demonstrated that one configuration, cpFAST (98-99), was capable of binding to the HMBR probe with a Kd that is 10-fold lower than full length FAST (0.1 μM). Further engineering of bipartite or tripartite split-FAST, the HMBR concentration used varies between 1 and 10μM, established through serial dilution of concentrated HMBR. It is unclear why the fluorogen concentration has to be screened for each PPI reporter system if only part of the probe is converted to fluorescence upon binding to FAST? It could be sufficient to work in excess of the probe relative to the concentration of FAST. Under these conditions, the reconstitution of the signal should more likely depend on the interacting proteins rather than the probe, which would act as a detector of reconstituted FAST.

The authors should clarify this point in the text, despite the development of previous technologies based on that assay. Ideally, the authors should state if independent measurements of the binding of their probe to the FAST variants have been done, apart from fluorescence, such as ITC or other biophysical method. If not, conducting the experiment will help to compare the two measurements revealing the fraction of the protein that is active to promote the fluorescent analogue.

#2.1. We thank reviewer 2 for their remark. The reason for testing the influence of fluorogen concentration is that the complementation efficiency depends on the fluorogen concentration (see Rakotoarison et al. ACS Chem. Biol. 2024). In the same way that the ligand concentration defines the fraction of protein:ligand complex formed at steady state in a simple two-body protein:ligand system, the concentration of fluorogen is an experimental parameter that controls the fraction of the three-body (resp. four-body) fluorescent complex formed by assembling the two (resp. three) fragments of FAST and the fluorogen. Performing the analysis at various HMBR concentrations enables us thus to determine the concentration of fluorogen for half-maximum complementation in the absence ($EC_{50,-interaction}$) or the presence ($EC_{50,+interaction}$) of interaction (see new Fig 3 and 5). Note that the interaction-induced preorganization of the two (resp. three) FAST fragments reduces the three-body (resp. four-body) system to a two-body system, and increases consequently complementation efficacy (see new Fig 3 and 5). The $EC_{50,-interaction}$ and $EC_{50,+interaction}$ values can thus be used to evaluate the efficacy of the self-complementation (obtained in absence of interaction) and of the interaction-dependent complementation: the higher the $EC_{50,-interaction}$, the lower the self-complementation, and the lower the $EC_{50,+interaction}$, the higher the interaction-dependent complementation efficacy. The change in EC_{50} allows us furthermore to evaluate the dynamic range of the system, the higher the change, the better the dynamic range, and thus the greater the system for the monitoring of interactions. The obtained complementation curves enable moreover to identify the optimal fluorogen concentration to maximize the interaction-induced complementation while minimizing self-complementation. These parameters are according to us the best way to characterize the behavior of the system in cells, in real-use conditions. In vitro characterization is not very relevant in this context according to us.
We modify the text of the main text to make this critical point clearer, and we added general schemes on Figures 3 and 5 presenting the different complementation processes, and the formula showing how the complementation efficiency depends on the fluorogen concentration.

2- The FRB:FKBP:RAP association is widely used to validate the system. However, the methodology to characterize the interaction and the results are intriguing. In Fig.3b and 3d, the data states as an example that EC50,–interaction = 31 ± 4 µM and an EC50,+interaction = 7.5± 0.7 µM. These values are set to be relative to HMBR binding driven by the interaction and are not representative of the affinity of the FRB:RAP:FKBP complex, which is known to be around the nanomolar range. To accurately assess the response to rapamycin binding of FRB, the authors should monitor FRB:FKBP association at a fixed concentration of HMBR (optimal 10µM) while varying rapamycin concentrations from the µM down to nM range. This would reveal the correlation between protein binding and reporter signal. It will also enable the assessment of whether the association follows appropriate binding kinetics

driven by rapamycin. The same remarks apply to other figures, specifically 4b-4f and 5b, in which this interaction is monitored.

#2.2. We thank reviewer 2 for their remark. There is however a misunderstanding on the meaning of the $EC_{50,+interaction}$ and $EC_{50,–interaction}$ values. These values do not refer to the binding of rapamycin. The $EC_{50,+interaction}$ and $EC_{50,–interaction}$ correspond to the concentrations of fluorogen for half maximal complementation in presence of interaction (+ rapamycin) or in absence of interaction (– rapamycin). It allows to characterize the complementation efficiency of the systems in the absence or the presence of an interaction. To determine the $EC_{50,+interaction}$ values, we use a saturating concentration of rapamycin (500 nM) to maximize the interaction of FRB and FKBP. $EC_{50,+interaction}$ and $EC_{50,–interaction}$ correspond thus to the two extreme scenarios, with and without interactions. Although, the proposed experiments is relevant for characterizing the binding affinity of rapamycin for FRB and FKBP, it does not allow to characterize the complemention efficiency of the split systems, which is the final goal of these series of experiments. About the kinetic analysis, our experiment aims at determining the kinetics of complementation of the split systems upon formation of an interaction, we therefore use saturating concentrations of rapamycin to go from no interaction to interaction rapidly, and assess the kinetics of complementation of the split systems. We believe that varying the concentration of rapamycin in this context would not provide any useful information.

3- FACS cytograms from Supp. Fig.6 show that a high concentration of HMBR (at least 1µM) are required to observe a shift of fluorescence between +RAP/-RAP conditions. It appears that this shift does not improve above 5µM. In line with this, the authors demonstrate in Fig. 3B a complementation curve on FRB/FKBP association with various HMBR concentrations “knowing that the efficiency of self-complementation and interaction-specific complementation depends on the concentration of fluorogen” (p5). If the fluorogen only fluoresces when bound on FAST and when complementation has occurred, the fluorescence should be independent of the fluorogen if it is not limiting to FAST protein abundance. Why do the results consistently present data in relation to increasing concentration of the reporter probe?

#2.3. We invite reviewer 2 to read our answer #2.1 that addresses this particular point. As said in our answer #2.1, the reason for testing the influence of fluorogen concentration is that the complementation efficiency depends on the fluorogen concentration (see Rakotoarison et al. ACS Chem. Biol. 2024). In the same way that the ligand concentration defines the fraction of protein:ligand complex formed at steady state in a simple two-body protein:ligand system, the concentration of fluorogen is an experimental parameter that controls the fraction of the three-body (resp. four-body) fluorescent complex formed by assembling the two (resp. three) fragments of FAST and the fluorogen. In the case of a two-body system, ones usually observe a binding curve that saturates to 100% binding at a certain concentration of ligand. One uses in general the dissociation thermodynamic constant K_D as a parameter for evaluating the efficacy of binding. This K_D value can be approximated when [protein]<<[ligand] to the concentration of ligand for half maximal binding. In the case of three-body or four-body systems such as our reporters, the fluorogen concentration defines also the fraction of the fluorescent

complex. Of course, for a given fluorogen concentration, the fraction of fluorescent assembly is different if the interaction of the two (resp. three) fragments of FAST is preformed (by induced or constitutive proximity) or not. The study at various concentrations enables to determine the concentration of fluorogen for half-maximum complementation in the absence ($EC_{50,-interaction}$) or the presence ($EC_{50,+interaction}$) of interaction, in order then to choose the right concentration to observe maximal interaction-dependent complementation and minimal self-complementation. The objective of our systematic study is to provide a comprehensive study of the influence of the fluorogen concentration enabling end-user to choose right concentration of fluorogen for their study. End-user can directly use our titration curve to determine the concentration of fluorogen to be used.

It is possible that self-association between split-FAST domains, independently of the interactions, needs to be reduced through mutagenesis. This point should be discussed. In term of proper use of the methodology, it it should be clarified by the authors whether, when investigating a new PPI pair, one should first monitor the binding curve of HMBR to determine the concentration that gives minimal background. Subsequently, the assay should be performed at only this given concentration combined with variable concentrations of rapamycin. The purpose of such an experiment is not clearly explained.

#2.4. We thank reviewer for this relevant remark. Self-complementation could be indeed further reduced although it is already pretty low, as shown by the value of $EC_{50,-interaction}$ = 91 µM. Self complementation could be further reduced by using a fluorogen of lower affinity or by using the tripartite system from RspA-FAST (see Fig. 4).
The systematic study shown in this paper should enable end-users to choose the right concentration of fluorogen to be used without the need to perform additional experiments.

4- In term of presentation, the authors are progressively introducing different variants of FAST to enhance the split-reporters. This can be confusing for readers and the introduction of new acronyms requires frequent references to the literature. Adequate supplementary information should be added.
Data on RspA split-FAST are presented in the main body (Fig. 3). We are wondering why this variant has less self-association. Is it due to folding properties or association to the probe? This should be stated in the text. Additionally, it refers to an unpublished study, which requires the addition of characterization in supplementary information. How much homology does RspA FAST have with FAST to allow for a similar split site? A comparison of circular permuted RspA FAST (98-99) along with the CP FAST variant in supp. Fig. 2 will help to compare the two variants. This is an important point as this final product is used to further engineer the tripartite version, which is the focus of the study.
We find out later that another variant, pFAST, is used instead of RspA because the authors discovered that RspA had too much self-association when used in a tripartite mode. To make this clear, the figure title if Fig. 5 should state the variant used (ex: pFAST). A dedicated supplementary figure that summarizes the characteristics of the variants used for engineering split would be useful such as DNA sequence alignments and structural superimpositions of the three FAST variants used with split

sites. A table listing their properties (affinities to the probes, fold change with rapamycin) would be appreciated.

#2.5. We thank reviewer 2 for this comment. We understand that the use of different FAST variants can be a bit confusing although we tried our best to justify the choice of every variant. We used RspA-FAST to optimize the splitFAST(98-99) because it allowed us to previously optimize splitFAST(114-115). The paper has been recently published in ACS Chem Biol (Rakotoarison 2024). References have been updated. Interestingly RspA-FAST has exactly the same length as FAST and shares 78 % sequence identity. In the case of splitFAST(114-115), this allowed us to generate two fragments with lower affinity and generate a system with lower self-complementation. We observe the same behavior when we used RspA-FAST to generate RspA-splitFAST(98-99). We assume that indeed it is due to a reduced affinity of the two fragments. We thus used first RspA-FAST to generate the tripartite system but realized that the complementation efficiency was too low in that case, very likely because of the entropic cost required to bring together four components. That's why we went back to pFAST, known to generate split-system with higher complementation efficacy. This allowed us to generate an efficient tripartite system. We added a supplementary figure 9 summarizing the sequences and data of the different systems developed.

5. According to the authors' statement (p. 9), the efficiency of tripartite reconstitution appears to be limited by the affinity of the probe to pFAST. Another possible reason could be that the fragments do not reconstitute efficiently. The assays in Fig. 5 were conducted in mammalian cells without controlling the expression levels of each fusions. The authors should investigate whether the pFAST fragments are easily purified in vitro and how the tripartite association behaves as a function of the concentration of each partner. Trimolecular association is complex as it depends on the relative affinities between the three partners.
The assay could be setup with equimolar amounts of each of the three fusions or with an excess of a third fragment at a fixed concentration of the fluorogen. This would help determine whether one of the three fragments should be in excess relative to the others. Furthermore, one should determine the order in which the split pFAST should be used (from N-terminal split internal and C-terminal fragments) and whether there are steric constraints to fusion tagging between the split domains.

#2.6. We thank reviewer 2 for this relevant comment. Indeed complementation efficacy depends also on the concentration of the different protein fragments. In the text we do not claim that the efficacy of tripartite reconstitution only depends on the affinity for the fluorogen. What we observed is that when using fluorogen such as HBR-2,5DM known to have higher affinity for FAST we observed accordingly higher efficacy of complementation for the tripartite system. This is logical when we consider a three-body or four-body system. What is interesting is that the fluorogen used and its concentration can be used by the experimenter to tune complementation efficacy. Although we agree that it could be interesting to make a full characterization of the four-body system in vitro, we believe that this is out of scope of this princept paper considering the challenge of fully characterizing all the binding affinity constants involved in the system. We believe that the demonstration and characterizing in cells is more useful for the application of the system. For reference, we performed in vitro characterization of bipartite split FAST in

Rakotoarison ACS Chem Biol 2024 and developed that corresponding theoretical framework.

Fig 5d. Reversibility of the system has been only proved for the bipartite system using FKBP homodimer dissociation experiments (ref 8 Tebo, A. G. & Gautier, A. Nat Commun 10, 2822–8 (2019). To add novelty to previously developed tripartite systems, the author should evaluate the reversibility of the tripartite system by removing Rapamycin to observe fluorescence decay.
Fig 5f. The example should favor first the split-FAST fused to FRB (pFAST99-114–NFRB + CFRB–pFAST1-98) for reassembly, then use FKBP to combine with rapamycin (creating a bimolecular complex). One needs to setup the background association if one of the three split-FAST fragments is expressed alone or with a non-relevant protein.

#2.7. We thank reviewer 3 for their relevant comment. Considering that bipartite splitFAST is reversible, we can expect the tripartite splitFAST to be reversible as well. The reason why we have not performed the proposed experiment is that the rapamycin-based trimerization system has been shown to be fully irreversible even after extensive washout of rapamycin by Inoue et al. in their princept paper. Without a model system enabling to dissociate the ternary assembly, it is difficult to demonstrate the reversibility.
About the second part of the question, we believe that the experiment shown on figure 4 in which pFAST99-114 and pFAST1-98 are fused at the N and C ter of FRB protein already enables to demonstrate what happens if pFAST99-114 and pFAST1-98 are already pre-assembled and if pFAST115-125 is expressed alone. This experiment (in absence of rapamycin) enables to evaluate the background association in that case.

When the split fragments of FRB are directed to plasma membrane, a control should be one of the split-FRB fusion not directed to the membrane. Does the tripartite association is decreased? Or is the fluorescence signal fainter? (see Fig 5g). My concern here is that the detection is influenced by the strong localization signals on split-FRB proteins.

#2.8. In this experiment, the control experiment in absence of rapamycin is the control that no assembly occurs in absence of interaction. The overall interest of using rapamycin-induced dimerization of FRB and FKBP, and rapamycin induced dimerization of splitFRB and FKBP is that the conditions without rapamycin provide us with a negative control, and avoid us to do additional controls. The interesting result of this experiment is that even though pFAST1-98 and 99-114 are indeed localized at the plasma membrane as pointed out by reviewer 2, there is no self-complementation of the system in absence of rapamycin, demonstrating that complementation requires the proximity of the three proteins.

Figure 5g. control: Label FKBP with a specific antibody to show that FKBP is not recruited to FRB-PM in the absence of rapamycin.

#2.9. We believe that this control is not necessary. As there is no fluorescence in the tripartite splitFAST channel in the absence of rapamycin, we can reasonably assert

that FKBP-pFAST 115-125 is localized in the cytosol and not recruited to the plasma membrane

Fig. 6 The experiments suggest that the junction between the different organelles is artificially induced by the addition of Rapamycin (e.g: FRB-CFP-CAAX is almost exclusively bound to the PM and massively relocalizes to the MT in the presence of Rap). If this is correct, it should be clarified in the text. One missing control is one of the fusion devoyed of split-FRB domain to evaluate the specificity of tripartite association (ex: CFP-CAAX and CFP-Cb5 without N-FRB).
#2.10. We believe that this control is not necessary. The rapamycin-inducible trimerization system has been designed by Inoue for this particular application, induction of junction/contact between organelles. Our results are in agreement with the formation of interactions between the indicated proteins.

Minor points

Fig. 3 For how long are the RAP and HMBR added in all of these experiments? Simultaneously or sequentially? (applies to fig. 4 ; fig. 5 and fig. 6). A method section regarding the use of the fluorogen with rapamycin induction is not provided.

#2.9. We added additional details in the Methods part to specify more precisely this information.

Fig. 3f: Live assay. Line curves corresponding to complementation without Rapamycin is missing (applies to fig. 4h; fig. 5d and fig. 5h). Specify whether it is substracted to obtain final fluorescence values. Specify the terms used for fluorescence “Fmax”; “Fmin”. Define Fmax/Fmin in the legend (the same applies for Fig. 4 and Fig. 5)

#2.9. The information of the fluorescence in the absence of rapamycin is given by the initial point of our kinetics. No change is expected during a time-lapse in the absence of rapamycin as fluorogen-treated cells are at steady state.
We added the meaning of Fmax and Fmin in the legend.

Reviewer 3

In this manuscript, the authors have developed a tripartite-split-FAST for the observation of three protein interactions. The development of the system is essential for the detailed study of protein-protein interactions. However, there are some fundamental issues that should be clarified. Here are my suggestions for the authors to improve their manuscript.
#3.0. We thank reviewer 3 for their review.

1. The author declared that “To demonstrate that the tripartite split system could selectively complement when the three fragments are fused to three different proteins that interact together. We fused pFAST99-114 at the N-terminus of the N-terminal fragment of split-FRB (pFAST99-114–NFRB) and pFAST1-98 at the C-terminus of its C-terminal fragment (CFRB–pFAST1-98), and kept pFAST115-125

fused to the C-terminus of FKBP" However, the system still only represents the interaction between two proteins (FRB and FKBP). It would be better to develop the system among three proteins.

#3.1. We are a bit surprised by this remark. Split-FRB is composed of two proteins fragments, NFRB and CFRB, which interact together with a third protein FKBP in the presence of rapamycin. This corresponds thus to three proteins interacting together (2 fragments of FRB + FKBP). This system represents a very nice model system for us as it is composed of three protein domains that can interact in the presence of rapamycin, enabling to evaluate the complementation of our system with and without interaction. To further demonstrate the potential of our approach to detect the proximity of three species, we added new experiments showing the possibility to selectively visualize the Fos·Jun·DNA complex (see new Figure 7 and new text page 12 and 13).

2. Will the fluorescence sign disappear when the protein is dissociated?
#3.2. We thank reviewer 3 for this remark. As mentioned in our comment **#2.8**, considering that bipartite splitFAST is reversible, we can expect the tripartite splitFAST to be reversible as well. Demonstration of the reversibility would require a ternary interaction that can be conditionally dissociated.

3. "The fluorescent proteins mCherry and ECFP allowed to verify the proper localization of the proteins at the PM. We expressed in HEK293 cells the two proteins together with FKBP-pFAST115-125 as a cytosolic partner and treated cells with 10 μM HBR-2,5DM." However, it seems that the fluorescence sign of FKBP-pFAST115-125 appears in the cell membranes rather than in the cytoplasm (Fig.5g)
#3.3. The green fluorescence signal at the cell membrane corresponds to the complementation of the tripartite system after rapamycin-induced interaction of the two PM-localized proteins and the cytosol-localized protein. It is thus normal that the fluorescence appears at the plasma membrane, as expected.

4. Whether the targeting of mitochondria and endoplasmic reticulum has been confirmed (Fig.6)?
#3.4. We used classical targeting sequences for localizing proteins at the mitochondria, the ER and the plasma membrane. Each fusion contains an additional fluorescent reporter which allowed us to confirm their proper localisation by visual inspection.

5. The FQY value in Fig. 2 should be a numerical range
#3.5. The fluorescence quantum yields for the cpFAST(114-115) variants are values previously reported in Tebo et al. ACS Chem Biol 2018 as approximate numbers. Thus we just reported them here as published.

6. "Correlation of the EGFP and mCherry signals before and after rapamycin addition"
EGFP should be modified to ECFP.
#3.6. We thank reviewer 3 for pointing out the typo and corrected it.

7. The authors are recommended to use cell membrane or nuclear stain for a better presentation of cellular outline expressing the binding protein (Fig. 3e and Fig. 4g).

#3.7. Although we agree that additional markers could be useful, it would be relatively challenging to add two additional fluorophores in terms of spectral channels availability, as we already have three channels used. Live-cell imaging with 5 colors would start to be a bit challenging to keep cells alive and avoid phototoxicity.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have adequately explained the significance of the work, revised the title, and supplemented Figure 7 to demonstrate its potential for biological applications. However, some issues still require clarification：

#1.0 We thank reviewer 1 for their review.

1. In Figure 4, the authors demonstrate that pFAST exhibits superior performance. However, it remains unclear why pFAST was not employed from the outset. A justification for this experimental design choice should be provided.

#1.1 We thank reviewer 1 for their relevant comment. As we demonstrated that RspA-FAST was the best variant to generate bipartite splitFAST by splitting either between 114 and 115 or between 98 and 99, it was natural to start with this variant for the design of the tri-partite version. As the performances of tripartite RspA-splitFAST were modest because of a lack of complementation efficiency, we then tested other variants known from our previous work to display higher self-complementation in their bipartite version. That is why we tested FAST and pFAST. We believe that the rational of our design is comprehensivenly explained in the paper from page 7 to 10.

2. In Figure 6, the authors utilize a tripartite-split-FAST system by separating FRB into C-FRB and N-FRB. In contrast, Figure 4 employs pFAST fused to either the N- or C-terminus of FRB, representing a binary complex approach. These experimental designs should be made consistent, or the authors must provide a clear rationale for the differing methodologies.

#1.2 We thank reviewer 1 for their comment. The rational of these experiments was to demonstrate the complementation efficiency of the tripartite system by testing it first on the simple bimolecular interaction FRB/FKBP fusing two fragments at the N-ter and C-ter of FRB, and the third fragments to have a preliminary evaluation of the complementation efficiency of the tripartiet system, and then in a second step on a true ternary interaction using splitFRB and FKBP. We believe that the rational of our experimental strategy is already comprehensively explained in the article.

3. The authors should clarify the theoretical basis for using PYP instead of FAST in the Circular Permutation site predictor (CPred) analysis. Additionally, it would be valuable to confirm whether crystal structure data for pFAST are available, as such structural insights could strengthen the mechanistic interpretation.

#1.3 We thank reviewer 1 for their comment. As mentionned in the article page 4, in absence of the crystal structure of FAST and pFAST, we used the crystal structure of PYP, their parental protein, for identifying the best split sites using CPred. FAST and pFAST being closely related to PYP (they differ by only 9 and 16 residues respectively), we believe that our CPred analysis is fully relevant. We added information in the main text on page 4.

4. In Figure 6, the 20 μm scale bar appears inconsistent with the typical diameter of HeLa cells (~40 μm). The authors should verify this measurement and correct it if necessary. Did the authors investigate interactions at the subcellular level in Figure 6? Including higher-magnification confocal images would help elucidate finer details of these interactions. In Figure 6k, the green signal (indicating interactions) appears to colocalize with all mitochondrial regions, suggesting universal interaction with the plasma membrane and ER. The authors should discuss whether this observation is biologically plausible or if alternative interpretations exist.

#1.5. We thank reviewer 1 for their comments. The size of the scale bars on Figure 6 is correct, and is, according to us, consistent with the size of HeLa cells.
About the second part of the comment, we have not investigated the interactions at higher magnification.
About the comment on the Figure 6k, as we force the junction between three organelles using chemically induced trimerization it is expected to observe the green signal colocalizing with the network of mitochondria.

5. In Figure 7, regions with intense green fluorescence correspond to areas where blue fluorescence is diminished. The authors should address the potential mechanistic basis for this inverse correlation.
#1.5. We thank reviewer 1 for their comment. mTurquoise2 being used as transfection reporter and being not physically linked to members of the ternary assembly has no reason to be colocalized. The ternary assembly seems to accumulate in the nucleoli, from which mTurquoise2 is excluded, explaining the difference in localization.

Reviewer #2 (Remarks to the Author):

The authors addressed most of my comments appropriately. They included an additional example involving three different proteins, which confirmed the correct association of ternary H2B-Fos-Jun zipper pairs. Nonetheless, as the authors acknowledge in the discussion, the robustness of this protein–protein interaction complex does not preclude potential challenges that may arise in ternary PPI assays. The limited scope is appropriate, given the potential value of the new assay to the field. The revised Figures 3a and 5a now illustrate more clearly the actual sequence of protein association that may occur during tripartite reassembly. In light of the complexity of such engineered systems, the addition of a supplementary table detailing binding affinity constants of the various split pairs is a valuable and much-appreciated addition.

The authors clarified the specific requirements of FAST assays that utilize chemical probes—namely, the fluorogen and its dependence for visualizing protein complementation. Despite not showing the reversibility of the assay, they acknowledged previous publications.

#2.0. We thank reviewer 2 for their review, and for acknowledging the improvement of our manuscript.

Minor :
1-it is not mentioned whether the chemical probes HMBR and HBR are available through as open source or commercial suppliers.

#2.1. The fluorogens used in this study are commercially available from the Twinkle Factory. This information was added in the Materials and Methods.

2- The choice of FAST fragments and the position of the tags is not explained.
#2.2. We thank reviewer 2 for their comments. The choice of the fragments and their positions is indeed non trivial. It should be carefully perfomed for each new interaction as discussed in the discussion. As mentionned on page 7, we used the assistance of Alphafold2 to predict the best topologies for complementation.

Reviewer #3 (Remarks to the Author):

The authors have addressed the previous reviews.

#3.0. We thank reviewer 3 for their review.

In this manuscript, the authors have developed a tripartite-split-FAST for the observation of three protein interactions. The development of the system is essential for the detailed study of protein-protein interactions. However, there are some fundamental issues that should be clarified. Here are my suggestions for the authors to improve their manuscript.

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7. The authors are recommended to use cell membrane or nuclear stain for a better presentation of cellular outline expressing the binding protein (Fig. 3e and Fig. 4g).