molecular systems biology

A fluorescent reporter for mapping cellular protein-protein interactions in time and space

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Review timeline:

Submission date:	22 November 2012
Editorial Decision:	13 December 2012
Revision received:	05 January 2013
Accepted:	28 January 2013

Editor: Thomas Lemberger

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 13 December 2012

Thank you again for submitting your work to Molecular Systems Biology. We have now heard back from the two referees who agreed to evaluate your manuscript. As you will see from the reports below, the referees find the topic of your study of potential interest. They raise, however, several concerns on your work, which should be addressed in a revision.

Both reviewers feel that more attention should be devoted to the discussion and explanation of the interpretation of the data generated with your reporter system. An additional very useful suggestion from reviewer #2 would be to test the reporter using a rapidly inducible expression system to obviate the need of the mating system. This could potentially significantly expand the general applicability of your reporter.

If you feel you can satisfactorily deal with these points and those listed by the referees, you may wish to submit a revised version of your manuscript. Please attach a covering letter giving details of the way in which you have handled each of the points raised by the referees. A revised manuscript will be once again subject to review and you probably understand that we can give you no guarantee at this stage that the eventual outcome will be favorable.

Referee reports:

Reviewer #1 (Remarks to the Author):

The elucidation of protein-protein interaction (PPI) is an important issue in systems biology and related fields. Several techniques, including high throughput methods have been developed to identify PPI. However, with the exception of FRET, not many of these methods have been established to elaborate the details of each PPI on spatial and temporal dimensions.

In this manuscript, the authors describe a novel tool, which attempts to tackle this issue. The approach described is based on the previously developed split-ubiquitn approach with a module of two different fluorescent proteins, namely Cheery-Cub-GFP, fused to the bait. The interaction with Nub-containing prey leads to cleavage of GFP. Thus, the location and occurrence of the target PPI can be followed by the loss of GFP signal through time-lapse microscopy in a clever system of yeast mating, similar to stopped-flow strategy utilized in enzyme kinetics.

Another advantage of this approach is that it can be easily transitioned from large-scale screening to specific detailed ("drill down") elaboration.

In this manuscript, the authors not only verified some PPIs obtained from their previous screening but also characterized their spatio-temporal features, which provide new information of these interactions.

In general, the manuscript is well organized. The reported method and results should also raise general interest to the audience of MSB. However, there are some issues, which need to be addressed by the authors.

My major concern is how to interpret the change of fluorescent signal to PPI kinetics of the late phase of studied PPI. Since the conversion of bait-CCG to bait-CC is an irreversible process, the signal change reflects only the association but not the dissociation process. Similar to the initial reaction rate in enzyme kinetics, the early phase of fluorescent signal change should accurately reflect the interaction. However, during the following phase, since GFP has already been cleaved from most bait molecules in many cases, no fluorescent change can be observed if there is dissociation/re-association of molecules occurring. Therefore, it may not appropriately represent actual PPI during this stage. The authors should discuss this issue in their revised manuscript.

Beside the above, there are two minor points as follow.
1. In fig. 7, the authors should describe how they define cytoplasm vs. nuclear signals.
2. Statistics should be added for Fig. 7.

Reviewer #2 (Remarks to the Author):

The manuscript by Moreno et al describes an expansion of the split ubiquitin method to detect protein-protein interactions using fluorescence microscopy.

It is well understood that protein-protein interactions can be regulated as a function of localization and/or cellular state. Nevertheless, not so many such examples have been studied in details, because of the lack of generic and sensitive methods to visualize the occurrence of protein-protein interactions inside cells with subcellular resolution. FRET based methods are relatively insensitive and usually good for the detection of interactions in areas with a high concentration of a protein, and they rely on a signal that requires that the two protein moieties tagged with fluorescent proteins are in close proximity. FCCS methods require co-mobility of proteins, and thus are suitable for the detection of soluble proteins, irrespective of the distance o the two fluorophores. But it cannot be used to report on protein-protein interactions at static localization sites. However, in contrast to split protein methods, and this includes the method in this manuscript, FRET and FCCS results are not biased by protein-protein interactions of the reporter itself, since they detect a signal that is independent of a second protein-protein interaction - the one of the reporters. Split-protein methods always score the interaction of two halves of some reporter as a function of their proximity, which

indeed is likely to be best promoted by a direct protein-protein interaction of bait and prey, but can also be caused by co-localization to a common structure, such as a membrane, since this removes one degree of freedom and hence promotes weaker interactions.

The split ubiquitin method for screening applications is based on the degradation of a reporter (URA3) in order to confer resistance to a drug (5'-FOA). This implies that only interactions are detected where all URA3 is degraded, hence saturation with the binding partner, irrespective of the localization. To achieve this, the rather strong Cup1-promoter is used to express the Nub-module; hence no endogenous level expression is sampled. The SPLIFF method presented here again is based on the same expression conditions. This experimental setup thus does not likely allow to sample protein-protein interactions with spatial resolution.

In order to circumvent these problems the authors revert to a mating assay, where a large pool of Nub is released into a target cell tagged with the interaction sensor (mCherry-Cub-GFP) by means of cell-cell fusion. This permits kinetic measurements, and in several cases the authors could gain qualitative insights into primary interaction sites, and they could derive some qualitative estimates of the interaction strengths. This dependency on a kinetic approach however also precludes applications with other organisms where such tools are not available, and it makes the method rather complicated, which precludes it from being used as a simple high content imaging method. The up-regulation of the expression of the Nub may be an alternative - although slower - but no such experiment is shown.

The interpretation of these dynamic results may also not be straight forwards, and in most of the given cases it actually relies on a priori knowledge. For example the Spc72-Stu2 interactions can be understood as rapid interaction only at the SPB, while the signal at the spindle is likely due to released Stu2 that has entered the nucleus where it becomes visible at the microtubules. Since the authors know (from literature) that Spc72 is only at the outer side of the SPB, this is the only explanation.

The authors report on the plus-tip associated Stu2CCG signal, which becomes red upon NUb-Kar9, encounter, which is interesting. The ideal control for this experiment would be the analogous StuCCG experiment with Spc72, where this plus-tip Stu2-localization would be expected to show no interaction (hence: stay green). Can this be seen?

Another line of complication for the evaluation of results is exemplified in the Kel1-Spa2 interaction, and the claim for an interaction gradient. First of all, the authors should define exactly what they mean by a 'gradient'. There are two options: the interaction as such is regulated in a spatial manner, giving rise to alterations in the interaction-strength as a function of the position. Alternatively, the interaction my be constitutive, and the gradient simply reflects the underlying individual protein distributions, which in this case must be determined by factors (other binding sites) that do not depend on the interaction that is investigated. This latter option may also led to a read out that results from self-interaction of the split Ubi, which cannot simply be neglected. For example, it is well known from GFP, that the weak tendency of the early GFPs caused significant self-interaction if one degree of freedom with respect to mobility is removed (e.g. upon membrane binding, see work about monomeric GFP from the Tsien lab), and that this can cause artifacts that disappear when a monomerized version is used. Therefore, to conclude about a true interaction gradient (the first type mentioned before), a much more careful investigation is required; here a three fluorescent protein system may be an option (e.g. a system with CFP, YFP and mCherry). If Spa2 would not show a specific enrichment in the area where it Kel1 is activated, one may conclude about a localized interaction gradient. Moreover, direct testing would then be required - e.g. by mapping of the interaction domain and selective mutations to prevent interactions. In addition, obviously a model should be developed to explain the localized interaction.

Altogether, I think this system is a highly useful addition to the available methods to visualize protein-protein interactions and well worth to be published. But given the relatively difficult interpretation of the results, as well as the dependency on time course measurement and methods only available with yeast (mating), I think the method is best suited for single case studies conducted in yeast where a broad range of controls can be generated in order to obtain an experimental base to follow up new hypotheses. Furthermore, it is wishful to use the method in conjunction with other methods, such as photoconversion or photobleaching, in order to validate some of the conclusions

drawn from the SPLIFF method.

1st Revision - authors' response 05 January 2013

Thank you for your e-mail and for sending the comments of the reviewers.
Both reviewers are positive about our work and would like to see it published.
Beside a few very specific questions concerning technical issues that can be promptly addressed the reviewers point to certain shortcomings in the interpretation of our results and recommend to more directly discuss the limitations of our technique.
By incorporating some of the reviewers suggestions and responding to their comments in the discussion section we now better explain the advantages, the potential, and limitations of SPLIFF.
We hope that the revised version of our manuscript can now be accepted for publication.

Point-by-point response:

Reviewer 1:
1. "My major concern is how to interpret the change in fluorescent signal to PPI kinetics of the late phase of studied PPI."
Response to 1: The identification of interaction is obtained through measuring a positive rate of conversion at a certain position in the cell. As long as this rate is positive a protein interaction is indicated at this position. This is a qualitative statement about the time and the place of interaction. The reviewer is right in pointing out that the change in the relative fluorescence intensity (RFI) and the calculated rate of conversion might get smaller as longer the interaction progresses. In cases where CCG-conversion is near completion it is thus possible that an interaction although it still occurs is no longer monitored (Page 15, line 4).
The reviewer is also right in pointing out that quantitative differences in the rate of CCG-conversion at different times of the interaction do not necessarily reflect quantitative differences in the underlying protein interactions. We stress these points by inserting an additional paragraph in the discussion section (page 15, line 22).

2. "The authors should describe how thy define cytoplasm vs. nuclear signals."
Response to 2: We added the sentence **"**The cytoplasmic fluorescence is defined as the difference between total cellular and nuclear fluorescence." to the legend of Figure 7 (page 33, line 4).

3. "Statistics should be added for Fig. 7."
Response to 3: Fig 7 B shows the analysis of the single experiment of Fig. 7A. The statistics for the complete set of experiments is shown in the Supplementary Figure 4.

Reviewer 2:

> **1.** **"**This dependency on a kinetic approach however also precludes applications with other organisms where such tools are not available, and it makes the method rather complicated, which precludes it from being used as a simple high content imaging method. The up-regulation of the expression of the Nub may be an alternative - although slower - but no such experiment is shown."
>
> **Response to 1:** The reviewer is right in pointing out that the approach is developed for investigating protein interaction networks in yeast and that the trick of kick starting the analysis of protein interactions by cell fusion is restricted to yeast (including other yeasts and fungi) or might require additional manipulations like virus mediated cell fusion etc..
> In its current version we do not envision this technique as a "stand-alone" high content imaging method. Instead we suggest that besides its applications for thorough *in vivo* characterizations of selected protein interactions, SPLIFF should be used as a tool to reliably follow up the hits of high-throughput screens (mass spectroscopy, split protein sensors, two-hybrid) to reveal the locations and time points of protein interactions. Current yeast databases contain for this organism alone thousands of protein interaction hits without further characterizations or validations. The resulting highly complex and global protein interaction networks need to be disentangled to be of interest for cell biologists. Two important criteria to structure these

networks are the when and where of the interactions. Our method thus helps to fill an important gap that still precludes the effective conversion of protein interaction data into a systems biology understanding of protein interaction networks.
We contradict the reviewer's statement that the SPLIFF method is complicated. When compared to other optical methods for measuring protein interaction the technical set up that is required and the procedure of acquiring and processing the images are standard for cell biology labs.
The reviewer suggested the use of tunable promoters as an alternative to the cell fusion assay. This is a good suggestion that we plan to implement in future studies in mammalian cells. In this study we focused our efforts to first establish this method in yeast. Yeast is one of the preferred model organisms for high throughput studies and has a rich history in developing and applying protein interaction technologies and in analyzing and computing protein interaction networks. We agree that one has to get used to the yeast mating assay as our choice to bring together the N_{ub}- and C_{ub}-fusion protein. The advantages are the precise definition of the starting point of the analysis, the reproducibility among different measurements, the automatic synchronization of the different measurements, and the instantaneous availability of the two reactants at the desired concentrations. Consequently, in yeast the cell fusion assay was in our hands always superior to the use of inducible promoters in determining location and timing of a protein interaction. For example, we alternatively monitored several interactions between CCG- and N_{ub}-fusion proteins using the TET on/off system for regulating the expression of the N_{ub}–fusion proteins. Although the system was very tight and interactions could be robustly measured, the induction of expression took longer than one cell cycle of yeast and thus prevented us to precisely define location and time point of interaction. In cell types whose cell cycles are significantly longer than the time to induce the expression of the N_{ub}-fusion, the TET on/off system or other expression systems should be sufficiently fast to reveal this information. We are confident that this or comparable expression systems will be of great value to apply SPLIFF in mammalian cells.

2. "The interpretation of these dynamic results may also not be straight forward, and in most of the given cases it actually relies on a priori knowledge. For example the Spc72-Stu2 interactions can be understood as rapid interaction only at the SPB, while the signal at the spindle is likely due to released Stu2 that has entered the nucleus where it becomes visible at the microtubules. Since the authors know (from literature) that Spc72 is only at the outer side of the SPB, this is the only explanation."
Response to 2: We respectfully disagree with the notion that in most of the given cases a priori knowledge is needed for the interpretation of our results.
For the interactions in the nucleus (Figure 2, 3), the interactions at the nuclear membrane (Figure 4) the interaction at the sites of polar growth (Figure 5, 6), the interactions in the cytosol and the incipient bud site (Figure 7) we strictly obeyed our rule to assign sites of interactions to those places in the cell where the rate of CCG conversion is positive and above the average. These interpretations were derived without a priori knowledge. However we agree that many assignments fulfilled the expectations that could have been derived by the literature. We think that providing a mix of known and novel interactions is an allowed and rather useful approach to establish a new method.
In the example mentioned by reviewer 2 we assigned the site of the Spc72p/Stu2p interaction to the tip of the spindle (Figure 8). This interpretation was strictly derived from our measurements. It is however true and the reviewer is correct in pointing this out, that in the discussion section we went one step further and suggested that that this tip signal very probably reflects the interaction at the cytosolic site of the SPB. We rephrased the corresponding sentence in the discussion to make clear that this statement is partly based on previous knowledge about the localization of Spc72p (page 14, line 18).
The comment of the reviewer also prompted us to more precisely define the limits of the method to exclude a certain place as a site of protein-protein interaction (page 14, starting from line 23).
3. "The authors report on the plus-tip associated Stu2CCG signal, which becomes red upon NUb-Kar9, encounter, which is interesting. The ideal control for this experiment would be the analogous StuCCG experiment with Spc72, where this plus-tip Stu2-localization would be expected to show no interaction (hence: stay green). Can this be seen?"
Response to 3: We did not detect GFP-signal at the plus tip of the microtubules. We explain this lack by the earlier occurring conversion of the cytoplasmic Stu2CCG by N_{ub}-Spc72p and the

rapid exchange of cytoplasmic Stu2p between its different locales. The issue of strictly excluding the existence of interaction at a later time point when most of the CCG is already converted by the first encounter with its N_{ub}-labelled interaction partner should now be covered in the revised version of the manuscript (page 14, starting from line 23).

4. **"**Another line of complication for the evaluation of results is exemplified in the Kel1-Spa2 interaction, and the claim for an interaction gradient. First of all, the authors should define exactly what they mean by a 'gradient'. There are two options: the interaction as such is regulated in a spatial manner, giving rise to alterations in the interaction-strength as a function of the position. Alternatively, the interaction may be constitutive, and the gradient simply reflects the underlying individual protein distributions, which in this case must be determined by factors (other binding sites) that do not depend on the interaction that is investigated. This latter option may also led to a read out that results from self-interaction of the split Ubi, which cannot simply be neglected."

Response to 4: The comments of the reviewer are correct. However, the depth of the reviewer's considerations exceeds the intended and more moderate scope of our manuscript. In the context of introducing the method we considered it more important to show that SPLIFF can detect gradients of CCG conversion. We acknowledge that the original definition of "gradient of protein interaction" was too vague. We replaced the original description with the following: "The reconstituted differential interaction maps describe a gradient in the ratio of red to green fluorescence that peaks at the bud tip and trails off towards the mother cell (Fig. 6D, E). This distribution very probably corresponds with a gradient of interactions caused by the unequal distribution of Spa2p and Kel1p across the bud (Figure 5A, 6A)." (Page 9; line 18)

Using proper controls we always try to exclude self-interaction of the Ub-fragments as the cause of the observed effects. Before we embarked on mapping the distribution of GFP and Cherry fluorescence across the bud we first tested Kel1CRU and Kel1CCG against different N_{ub}-fusions including N_{ub}-Bni1p as a further component of the polar cortical domain. No interactions could be observed. We would like to keep Figure 6 as it is. Here the N_{ub}-fusion to a short peptide (N_{ub}-) serves as the representative of these "negative" controls.

