A Near-Infrared BiFC Reporter for In Vivo Imaging of Protein-Protein Interactions

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SUMMARY

Studies of protein-protein interactions deep in organs and in whole mammals have been hindered by a lack of genetically encoded fluorescent probes in near-infrared region for which mammalian tissues are the most transparent. We have used a near-infrared fluorescent protein iRFP engineered from a bacterial phytochrome as the template to develop an in vivo split fluorescence complementation probe. The domain architecture-based rational design resulted in an iSplit reporter with the spectra optimal for whole-body imaging, high photostability, and high complementation contrast, which compares favorably to that of other available split fluorescent protein-based probes. Successful visualization of interaction of two known protein partners in a living mouse model suggests iSplit as the probe of choice for noninvasive detection of protein-protein interactions in vivo, whereas its fast intracellular degradation enables time-resolved monitoring of repetitive binding events.

INTRODUCTION

Protein-protein interactions (PPIs) underlie many intracellular processes. Initial investigations of PPIs utilized in vitro techniques such as a communoprecipitation and later advanced to more elaborate approaches capable of PPI detection in live cells. A bimolecular fluorescence complementation (BiFC) is one of the later approaches and is based on the tagging of two proteins with half of a fluorescent protein (FP) each. Upon interaction of these proteins, the two halves of the FP (denoted as split FP) associate with each other to form a fluorescent complex, thus reporting on the PPI. The split protein approach was first proposed and tested for ubiquitin reconstitution (Johnsson and Varshavsky, 1994) and after proving the general concept was applied to a number of enzymes and FPs. The fluorescent split reporters have been engineered using ten different FPs and their mutants (Table 1). Many of them have been applied for studying various PPI events in live cells (Kerppola, 2009); however, certain properties of the current split FPs impose limitations to their use. Split FPs have a tendency for self-association, which decreases the BiFC contrast (Table 1). This has limited the highest reported contrast for cultured cell expression to ~17 for a Venus FP derivative (Kodama and Hu, 2010). Another drawback of many split FPs is a poor maturation at 37 °C, limiting their applicability to cells of nonmammalian origin. The latter property worsens in split constructs derived from red FPs, thus hindering multicolor BiFC for the detection of several PPIs simultaneously. Recently, some progress has been made in the engineering of a split mLumin protein (Chu et al., 2009); however, its further validation in mammalian cells is necessary.

Studying PPIs with BiFC in living mammals puts stringent requirements on the properties of the split FP reporter. In addition to good maturation at 37 °C, the reporter should possess both excitation and emission maxima inside of a so-called “near-infrared optical window” (NIRW: 650–900 nm) where mammalian tissues are relatively transparent because of low absorption of hemoglobin, melanin, and water in this region. Otherwise, good BiFC contrast observed in vitro will be unacceptably low in vivo due to high tissue autofluorescence caused by endogenous compounds, such as NADPH, flavins, collagen, and elastin. To date, no far-red FPs have been engineered into a BiFC reporter suitable for in vivo imaging. Split mLumin might provide some advance in this direction, although its spectral properties are suboptimal for applications in mammals. Notably, whole-body split reporter-based imaging was successfully facilitated using luciferase (Luker et al., 2004, 2012; Paulmurugan et al., 2002) and thymidine kinase (Massoud et al., 2010), resulting in chemiluminescence and positron emission signals, respectively. However, both types of reporters require injection of exogenous substrates and, in the latter case, provide low contrast and nonspecific signal in vivo.

The high demand for functional in vivo BiFC reporters prompted a search for novel types of protein templates. Recently, two near-infrared fluorescent proteins with both excitation and emission spectra in NIRW engineered from bacterial phytochromes have been reported and expressed in mammals (Filonov et al., 2011; Shu et al., 2009). One of them, iRFP protein (Filonov et al., 2011), appeared to be a good candidate for designing an in vivo PPI probe because it possesses high in vivo brightness and low cytotoxicity and utilizes endogenous concentrations of biliverdin (BV) chromophore to acquire fluorescence. Here, we describe the development, characterization in mammalian cells, and validation of in vivo applicability of a near-infrared split reporter based on iRFP protein.
RESULTS

iRFP Protein as a Template for the Split Reporter

iRFP has retained two distinct domains PAS and GAF from its parental bacterial phytochrome RpBphP2 (PHY and HisK domains were excluded from the protein on the first stage of the iRFP engineering). This suggested that the products of separating iRFP between the two domains might demonstrate split FP properties. Having aligned the sequence of iRFP with that of RpBphP3 (a close homolog of the iRFP’s parental RpBphP2 with a known crystal structure), we proposed that the optimal position for the introduction of polypeptide break would be in an unstructured loop spanning between amino acid residues 120–123 (numbering as in iRFP) between the PAS and GAF domains (Figure S1 available online). To test this in bacteria, independent production of these two domains was facilitated by a plasmid bearing two different promoters (rhamnose and arabinose dependent) (Figure S2A). To induce complex formation of the separated PAS and GAF domains, they were fused to coils (named E-coil and K-coil) previously reported to be capable of interacting with each other with a high affinity (Kd = 63 pM) (De Crescenzo et al., 2003) (Figure S2B). Cotransformation of the dual-promoter plasmid encoding the PAS-E and K-GAF fusion constructs with a plasmid encoding heme oxygenase allowed simultaneous expression of the fusions and production of BV. BV is an exogenous chromophore for iRFP that is necessary for the formation of the fluorescent adduct. Upon induction of transcription of the split fusions, the producing bacterial cells demonstrated a clear fluorescent signal. The fluorescent signal was 10-fold lower than that of bacteria expressing full-length iRFP yet 40-fold higher than the signal from the cells producing the PAS and GAF domains not fused to coils (Figure S3A), providing the BiFC contrast of 40-fold. BiFC contrast is a conventional notion in the field of split reporters that describes the difference between the real, induced signal, and background fluorescence, originated from a nonspecific split reporter complementation. Thus, the separated PAS and GAF domains of the iRFP protein retained their ability to reacquire fluorescent properties upon induced close proximity and had a low nonspecific interaction.

Table 1. Comparison of Major Properties for Available BiFC Reporters Based on Fluorescent Proteins

<table>
<thead>
<tr>
<th>Parental FP Used for Engineering BiFC Reporter</th>
<th>Ex/Em Maxima of Parental FP Identity (nm)</th>
<th>Interacting Pair Used</th>
<th>BIFC Contrast in Cultured Mammalian Cells (Fold)</th>
<th>Temperature Sensitivity of Maturation of BiFC Reporter</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ECFP</td>
<td>452/478 (yes)</td>
<td>ZIP domains of Fos and Jun</td>
<td>~10 at 30°C</td>
<td>yes (poor maturation at 37°C)</td>
<td>Hu and Kerppola, 2003</td>
</tr>
<tr>
<td>EGFP-sg25</td>
<td>475/505 (yes)</td>
<td>Leucine zippers</td>
<td>ND</td>
<td>ND</td>
<td>Ghosh et al., 2000</td>
</tr>
<tr>
<td>EGFP</td>
<td>488/507 (no, 490/524)</td>
<td>biotinilated DNA oligonucleotides</td>
<td>ND</td>
<td>ND</td>
<td>Demidov et al., 2006</td>
</tr>
<tr>
<td>mKG</td>
<td>494/506 (ND)</td>
<td>leucine zippers, PX with PB1 of p40&lt;sup&gt;αcox&lt;/sup&gt; TCF7 with β-catenin, PAC1 with PAC2, PAC3 homodimerization</td>
<td>ND</td>
<td>no</td>
<td>Ueyama et al., 2008</td>
</tr>
<tr>
<td>Dronpa (activated form)</td>
<td>503/518 (ND)</td>
<td>hHus1 with hRad1</td>
<td>ND</td>
<td>no</td>
<td>Lee et al., 2010</td>
</tr>
<tr>
<td>EYFP</td>
<td>514/527 (yes)</td>
<td>ATF2 with Jun, p50 with Fos, p65 with Jun ZIP domains of Fos and Jun</td>
<td>~10 at 30°C</td>
<td>Yes (poor maturation at 37°C)</td>
<td>Hu et al., 2002</td>
</tr>
<tr>
<td>Venus (V150A)</td>
<td>515/528 (ND)</td>
<td>ZIP domains of Fos and Jun</td>
<td>10 at 37°C</td>
<td>no</td>
<td>Shyu et al., 2006</td>
</tr>
<tr>
<td>Venus (I152L)</td>
<td></td>
<td></td>
<td>10 at 37°C</td>
<td></td>
<td>Nakagawa et al., 2011</td>
</tr>
<tr>
<td>mRFP1-Q66T</td>
<td>549/570 (ND)</td>
<td>CPC with GL3, BP/KNAT1 with BLH7</td>
<td>15–20 (in plant cells only)</td>
<td>ND</td>
<td>Jach et al., 2006</td>
</tr>
<tr>
<td>mCherry</td>
<td>587/610 (ND)</td>
<td>SV40 large T antigen with p53</td>
<td>ND</td>
<td>yes (poor maturation at 37°C)</td>
<td>Fan et al., 2008</td>
</tr>
<tr>
<td>mLumin</td>
<td>587/621 (ND)</td>
<td>ZIP domains of Fos and Jun, EGFR with Grb2, STAT5a with STAT5B</td>
<td>ND</td>
<td>no</td>
<td>Chu et al., 2009</td>
</tr>
<tr>
<td>iRFP</td>
<td>690/713 (yes)</td>
<td>E-coil with K-coil, FRB with FKBP</td>
<td>50–80 at 37°C</td>
<td>no</td>
<td>This paper</td>
</tr>
</tbody>
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ND, not determined; RT, room temperature.
Directed Evolution of the Improved Split Reporter

To increase the brightness of the split iRFP complex in bacteria, we next subjected one of the domains to random mutagenesis. The GAF domain is larger and contains a BV chromophore binding pocket, so we chose to mutate it first. One round of random mutagenesis substantially increased the fluorescent brightness of bacteria producing the split iRFP complexes (Figure S3B).

Because the fluorescence brightness of bacteriophytochrome-based FPs in mammalian cells depends on their affinity to endogenous BV (Filonov et al., 2011), we further tested the obtained mutants of the GAF domain in HeLa cells. To accomplish this, the PAS-E and K-GAF (mutant) genes were cloned into two plasmids under the same promoter. Fluorescence-activated cell sorting (FACS) used to compare the mutants in mammalian cells showed that the reconstitution of the split iRFP complexes resulted in bright cell fluorescence at an endogenous BV concentration below $10^{-5}$ M (Filonov et al., 2011) (Figure S3C). Although further rounds of random mutagenesis of either the PAS or GAF domains slightly increased brightness of the split complexes, it decreased the iRFP complementation contrast. Thus, we decided to continue with the complex obtained after the first mutagenesis round. This complex, named iSplit, consists of the original PAS domain and GAF domain with three substitutions, which we denoted GAFm (Figure S1).

Characterization of the Split Reporter in Cells

FACS analysis of the transiently transfected HeLa cells demonstrated that iSplit has a contrast of ~50-fold compared to the control PAS/K-GAFm pair (Figure 1A). We then chose another protein pair to drive a stimulus-dependent iSplit formation and fused PAS and GAFm to an N terminus of a FRB protein and a C terminus of a FKBP protein, respectively (Figure S2C). The FRB and FKBP partners interact upon addition of rapamycin (Luker et al., 2004). The induced interaction between the PAS-FRB and FKBP-GAFm fusion constructs yielded an ~35-fold increase in the HeLa cell brightness. The PAS and GAFm domains expressed separately or as the PAS/GAFm combination without induction had a very low fluorescent level close to background (Figure 1A). These results were supported by microscopy imaging of transfected HeLa cells (Figure 1B). An observed nuclear localization of the PAS-FRB/FKBP-GAFm complex was caused by a nuclear localization signal at the N terminus of the FKBP partner. Exclusion of the PAS-E/K-GAFm pair from nuclei was possibly caused by the large molecular weight of the iSplit.
complex of ~70 kDa that is beyond the diffusion limit of the nuclear pore (Marfori et al., 2011). Overall, the iSplit complexes did not form aggregates or localize to any specific compartment (Figure 1B). These results prompted us to study the iSplit spectroscopic properties in more detail. Split iRFP eluted from bacteria has the same absorbance, excitation, and emission peaks as parental iRFP (Figures 1C and 1D). The extinction coefficient, measured by comparison to the absorbance of free BV as previously described (Filonov et al., 2011), provided a value of 85,500 ± 830 M$^{-1}$cm$^{-1}$, which was slightly lower than an extinction coefficient of 105,000 M$^{-1}$cm$^{-1}$ for parental iRFP. The quantum yield of iSplit was 6.2% ± 0.5%, which is close to 5.9% of iRFP. The iSplit photostability in live mammalian cells was similar to that of iRFP with the half-time being 33.0 ± 5.2 min (Figure 1E), whereas for iRFP the half-time measured using the same conditions was 26 min (Filonov et al., 2011).

Next, we studied whether the PAS or GAF$\_m$ domains separately or combined could affect the binding efficiency of the FRB/FKBP partners. Transfected HeLa cells expressing the PAS-FRB/FKBP-GAF$\_m$ complex were incubated with different concentrations of rapamycin and then FACS analyzed to plot a titration curve (Figure 1F). The rapamycin-induced fluorescence intensity of these cells, fitted with a curve based on the Hill equation, showed saturable binding with the EC$_{50}$ being 11.6 ± 0.7 nM, close to the $K_d$ value determined in vitro (12 nM) (Banaszynski et al., 2005). This indicated that the PAS and GAF domains did not affect the binding properties of FRB and FKBP.

In order to evaluate the irreversibility of the iSplit complex formation, HeLa cells expressing the PAS-FRB and FKBP-GAF$\_m$ fusions and preincubated with rapamycin were washed and then treated with FK506, which is a competitive inhibitor of the FRB and FKBP interaction. Suitably, even high concentrations of FK506 did not cause a decrease in the fluorescence intensity, thus indicating the irreversible nature of the iSplit formation. BiFC irreversibility is common for other FP-based split partners as well (Shyu and Hu, 2008) (Figure 2A).

Because the main application of iSplit was conceived for in vivo imaging (see below), we made a stable preclonal mixture of the MTLn3 breast adenocarcinoma cells coexpressing E2-Crimson far-red FP (excitation 605 nm, emission 646 nm) (Strack et al., 2009) as a volumetric marker, and the PAS-FRB and FKBP-GAF$\_m$ fusions. The stably expressing PAS-FRB/FKBP-GAF$\_m$ cells provided a BiFC contrast of up to ~80-fold upon rapamycin addition and brightness of 25% of the MTLn3 cells stably expressing iRFP (Filonov et al., 2012) (Figure S4). We then measured the kinetics of cellular fluorescence upon addition of rapamycin using microscopy (Figure 2B). A BiFC contrast of 10-fold was reached as early as 4.5 hr after addition of rapamycin.

To study the intracellular stability of iSplit, the MTLn3 stable cells preincubated with rapamycin were washed and then either left in the medium only or in the medium with 30 mg/ml cycloheximide. The iSplit complex degraded rapidly with a half-time of 26 min (column D), 50 µM (column E), and 100 µM (column F). After incubation for additional 24 hr, all cells were harvested and analyzed with FACS.

Figure 2. Behavior of the iSplit Complex in Mammalian Cells
(A) Irreversibility of iSplit complex. HeLa cells were transfected with plasmids encoding either the PAS-FRB or FKBP-GAF$\_m$ fusion and incubated with 100 nM of rapamycin for 24 hr. Cells in column A were left untreated as a control. Then, these cells (except column B) were washed with PBS, medium was changed, and FK506 (specific competitor of rapamycin) was added to final concentrations of 10 µM (column C), 20 µM (column D), 50 µM (column E), and 100 µM (column F). After incubation for additional 24 hr, all cells were harvested and analyzed with FACS.

(B) Kinetics of the fluorescence increase in MTLn3 preclonal stable cells after addition of 100 nM rapamycin.

(C) Intracellular stability of iSplit in MTLn3 preclonal stable cells in comparison with iRFP. Cells were preincubated with 100 nM rapamycin for 24 hr and then washed (or left with rapamycin) and treated or not with 30 µg/ml cycloheximide (CHX).

(D) Fluorescence brightness of MTLn3 preclonal stable cells expressing iSplit after 100 nM rapamycin addition and washout cycles analyzed by FACS at the indicated time points. In all panels, error bars represent the 95% confidence intervals calculated based on either three samples in FACS analysis or several cells from three fields of view in microscopy. See also Figures S4 and S5.

Because the main application of iSplit was conceived for in vivo imaging (see below), we made a stable preclonal mixture of the MTLn3 breast adenocarcinoma cells coexpressing E2-Crimson far-red FP (excitation 605 nm, emission 646 nm) (Strack et al., 2009) as a volumetric marker, and the PAS-FRB and FKBP-GAF$\_m$ fusions. The stably expressing PAS-FRB/FKBP-GAF$\_m$ cells provided a BiFC contrast of up to ~80-fold upon rapamycin addition and brightness of 25% of the MTLn3 cells stably expressing iRFP (Filonov et al., 2012) (Figure S4). We then measured the kinetics of cellular fluorescence upon addition of rapamycin using microscopy (Figure 2B). A BiFC contrast of 10-fold was reached as early as 4.5 hr after addition of rapamycin.

To study the intracellular stability of iSplit, the MTLn3 stable cells preincubated with rapamycin were washed and then either left in the medium only or in the medium with 30 µg/ml cycloheximide. The iSplit complex degraded rapidly with a half-time of ~3.8 hr (Figure 2C). Addition of rapamycin to the cells with cycloheximide resulted in a similar fluorescence decay, indicating that intracellular complex degradation and not complex dissociation caused the fluorescence decrease. Without cycloheximide, the iSplit half-time increased to ~21.6 hr, possibly due to residual rapamycin left in the cells. The control iRFP-expressing cells demonstrated the expected high protein stability (Filonov et al., 2011).
These observations suggested that the fast intracellular turnover of the iSplit complex could provide the possibility of studying PPIs several times in the same cells, especially because the timescale of in vivo experiments is typically days. To validate this hypothesis, we used the same MTLn3 cells to turn the iSplit complex formation on and off several times (Figure 2D). The cells were rapamycin pretreated, FACS analyzed, washed out, FACS analyzed after additional time, and rapamycin pretreated again to complete the iSplit complex formation-degradation cycle. Indeed, the fluorescence intensity of the cells showed the anticipated cyclic behavior with some fluorescence increase as the cycles progressed, likely due to intracellular accumulation of rapamycin.

To test whether the iSplit formation is affected by exogenous BV, we added varying BV amounts to either MTLn3 cells coexpressing PAS-FRB and FKBP-GAFm pretreated with rapamycin or MTLn3 cells expressing iRFP, which possesses a high affinity to BV (Filonov et al., 2011). Both types of cells showed a low fluorescence dependence on the BV level, indicating that iSplit has an affinity to BV similar to that of iRFP and utilizes mostly endogenous BV to exhibit fluorescence (Figure S5).

**Figure 3. Expression of the iSplit Complex in Living Mice**

(A) Representative images of a mouse bearing a 3-week-old MTLn3 tumor coexpressing E2-Crimson and PAS-FRB and FKBP-GAFm pair before (left image) and 36 hr after (middle image) the rapamycin intraperitoneal injection (4.5 mg/kg) imaged in a 675/30–720/20 nm excitation-emission near-infrared channel for iSplit imaging (top) and in a 605/30–660/20 nm excitation-emission far-red channel for tumor volume assessment by means of E2-Crimson fluorescence (bottom). A mouse on the right is the autofluorescence control. Scale bar is 1 cm.

(B) Quantification of the near-infrared fluorescence intensities of the mice in (A). Fluorescence brightness before rapamycin injection (black bar), 36 hr after the injection (red bar), and that of the control mouse (green bar) is shown. Subtraction of the autofluorescence background from the fluorescence signals before and after the rapamycin injection results in ~23-fold difference. Error bars represent the 95% confidence interval calculated based on three subsequent images.

(C) Kinetics of the near-infrared fluorescence change in mice with MTLn3 tumors injected with either rapamycin (red diamonds) or vehicle (black squares). The near-infrared fluorescence intensity for each mouse was normalized to the E2-Crimson far-red signal, and the resulting value was normalized to a value at time 0 hr to plot the fluorescence changes. Error bars represent the SD values of the fluorescent intensity from three mice. Dashed line represents the b-spline fitting of the ratio of fluorescence from the mice injected with rapamycin and with vehicle. The latter normalization takes into the account spontaneously associated iSplit.

(D) Postmortem near-infrared images of the MTLn3 tumors cut in halves shortly after the isolation from mice. The tumors with noncomplemented iSplit (left image), complemented (middle image) iSplit, and expressing iRFP are shown. Scale bar is 1 cm.

(E) FACS analysis of the MTLn3 cells isolated from the tumors in (D).

See also Figures S6 and S7.

**Performance of the Split Reporter in Live Animals**

Having characterized the iSplit properties in vitro and in cultured cells, we utilized MTLn3 cells stably coexpressing E2-Crimson, PAS-FRB, and FKBP-GAFm to develop a tumor xenograft in living mice (see Experimental Procedures for details). Mice were first imaged 3 weeks after the cancer cell injection. Both the bright signal from E2-Crimson and the faint signal from non-induced iSplit were detected and spectrally resolved without interference (Figures 3A and S6), thus demonstrating the capability of iRFP and its derivatives to be used for two-color in vivo imaging. Upon rapamycin injection, a strong near-infrared fluorescence increase of up to 23-fold at 36 hr after the injection was observed in all animals (Figure 3B), whereas the vehicle injection did not cause fluorescence changes (Figures 3A and 3B).

**Figure 3C** shows kinetics of the rapamycin-induced fluorescence change normalized to the E2-Crimson fluorescence signal that takes into account differences in the proteins expression level and tumor size. The dashed line represents additional normalization to the spontaneous iSplit association. The iSplit signal reached an average of 18-fold contrast at 40 hr after...
rapamycin injection and then decayed during a week (Figure 3C). Another injection of rapamycin into the same mice caused the second wave of the fluorescence increase though with a lower contrast of ~7.5-fold due to the increased nonspecific signal from the accumulated iSplit complex. The decrease of BiFC contrast with time (Figure 3C) does not reflect the fluorescence intensity of the tumors, which, in fact, increased during the course of the experiment (Figure S7).

The postmortem fluorescence intensities of the several-week-old iSplit-expressing tumor, which was rapamycin-induced and excised at maximal brightness, and the iRFP-expressing tumor of similar size differed ~3.2-fold, with iRFP being the higher intensity tumor. Both tumors demonstrated even fluorescence distribution inside (Figure 3D). FACS analysis of cells isolated from these tumors showed decreased fluorescence compared to the original cultured cells (Figures 3E and S4). However, the relative brightness between the iSplit- and iRFP-expressing tumor cells (Figure 2E) remained similar to that obtained in cell culture, suggesting the same low cytotoxicity for iSplit as observed before for iRFP (Filonov et al., 2011).

DISCUSSION

The obtained results have demonstrated that two-domain bacteriophytochrome-derived iRFP is a good candidate for engineering split probes and does not require a laborious search for a position for the polypeptide break as in the case of GFP-like proteins. It is likely that other bacteriophytochrome-based FPs, all having multidomain structures, could be converted into BiFC reporters using similar positions.

iSplit has retained the majority of its parental near-infrared spectral and biochemical properties. With the BiFC contrast of 50- to 80-fold in cultured mammalian cells, iSplit compares favorably to available split reporters based on GFP-like FPs, which may also exhibit poor maturation at 37°C (Table 1), luciferases, and thymidine kinase with maximum intracellular contrast of 17-fold (Kodama and Hu, 2010), 20- to 75-fold (Villalobos et al., 2010), and ~3-fold (Massoud et al., 2010), respectively. iSplit is the BiFC reporter that has been imaged in a whole animal, moreover, with 18-fold contrast. Compared to luciferase and thymidine kinase, iSplit does not require injection of exogenous substrates. Moreover, fast degradation of iSplit could be beneficial for the visualization of the relatively slow dynamics of PPI in the body, because the fluorescent signal will fade soon after the specific stimulus is gone.

Similar to other PPI systems used in live cells, the iSplit reporter is not precisely quantitative because its fluorescent signal is determined by a number of factors, including PAS and GAF domains association, folding and BV-binding kinetics, rates of these proteins’ synthesis, and degradation. However, this is the only currently available in vivo PPI reporter system that does not depend on exogenous substrates. Ideally, the iSplit reporter should be used to assess or prove in vivo interaction of proteins, whereas precise measurements of the protein-partners binding properties should be done in vitro with more appropriate techniques.

The difference in the iRFP and iSplit intracellular stabilities can be a result of distinct folding pathways for these proteins. iRFP folds while translated as a single polypeptide chain and this way adopts its intrinsic structure. iSplit is synthesized as two different protein moieties, which likely prefold separately and reach their final conformation once brought into proximity. It is likely that the resulting folds of these proteins have differences, and iSplit possesses some structural elements, which confer its cellular instability. One of these apparent structural differences may be a trefoil knot (Wagner et al., 2005).

To overcome possible negative effects of the irreversibility of the current iSplit variant, a split protein reconstitution based on an intein-mediated protein splicing can be utilized (Paulmurugan et al., 2002). This approach involves incorporation of the intein split fragments into the protein-FP fusion. Once proteins in study interact, the intein halves are brought together and perform protein splicing, which results in ligation of two halves of FP and dissociation of the resulting full-length protein. Because iSplit is an unstable protein, its fluorescent signal should rapidly fade away, whereas the protein partners will now be set free for further biochemical events. This sketch reporter system could be more physiological than the iSplit reporter.

Alternatively, in case of the fast cellular events irreversibly building up, iSplit could allow imaging of transient and low abundant interactions. Also, other more sophisticated complementation strategies suggested for luciferase-based split reporter can also be applied to the iSplit system, thus further expanding its application range (Shekhawat et al., 2009).

Last, the dimeric nature of iRFP, likely inherited by its iSplit derivative, may hinder novel PPI screening but should not be an obstacle in transferring protein complex studies from cell culture to animal models. Other in vivo applications of iSplit could include studies of PPIs in processes at cellular and tissue borders including wound healing, host-pathogen interactions, and organ development. One of the promising applications of iSplit can be its use to extend the GRASP technology and image cell-cell contacts in live mammals (Feinberg et al., 2008).
inserted into the latter plasmids via Ncol and Kpnl sites to generate pWA-PAS-BAD-GAF and pWA-PAS-E-BAD-K-GAF plasmids, respectively. The E-coil and K-coil sequences were synthesized de novo based on the amino acid sequence (De Crescenzo et al., 2003), and a -ggs- linker was inserted between the coil fragments and the PAS and GAF domains.

A pWA21cBP-EFP-mTagBFP plasmid was constructed as follows. The gene coding the AraC DNA-binding domain was inserted into the pWA21 plasmid (Wegerer et al., 2008) at BglII/HindIII restriction sites after PCR amplification from the pBAD/HisB plasmid (Invitrogen). A pWA21c-AvrIINotI plasmid was next generated by site-specific mutagenesis of the pWA21c plasmid to insert AvrII and NotI sites. The pWA21c-AvrIINotI plasmid was converted into a pWA21cBAD plasmid by cloning the PCR-amplified DNA AvrII/NotI fragment encoding a pBAD promoter into the pWA21c-AvrIINotI plasmid. Also, in the pWA21cBP plasmid was engineered into the pWA21cBP-EFP-mTagBFP plasmid via inserting of the mTagBFP gene (Subach et al., 2008) at Ncol/Kpnl sites. The EFP gene was present in the original pWA21 plasmid.

A pWA23h plasmid was engineered to provide expression of a heme oxygenase for biliverdin production in bacteria. It contained the rhombohine promoter from the pWA21 plasmid (Wegerer et al., 2008), Kan resistance, and COLA origin parts from a pCOLADuet-1 plasmid (Novagen). The AvrII/PciI fragment containing Kan resistance and COLA origin was PCR amplified from pCOLADuet-1 plasmid and inserted into a pWA21h-AvrIINotI vector. Then, an hmuO gene encoding Bradyrhizobium ORS278 heme oxygenase was PCR amplified from plasmid pBAD(hisB)-RbpBPhP2-hmuO and swapped with the EGF gene in the pWA21-AvrIINotI plasmid.

Mammalian expression of the PAS-E and K-GAF domains for the constitutive complex formation was driven from the plasmids pPAS-E and pK-GAF generated as follows. A pEGFP-C1 plasmid (Clontech) was digested with Nhel and BglII and ligated with PCR fragments encoding either the PAS and K-coil sequences were synthesized de novo based on the amino acid sequence (De Crescenzo et al., 2003), and a -ggs- linker was inserted between the coil fragments and the PAS and GAF domains.

To generate eight plasmids for the rapamycin dependent interaction of the PAS and GAF containing various fusions, the pC4-RHE and pC4EN-F1 plasmids (ARRAD Pharmaceuticals) encoding FRB or FKBP partners, respectively, were digested with either XbaI or SpeI and ligated with the PCR amplified PAS or GAF domains digested with the same enzymes. A control pPAS containing no coil was generated after ligation of the rIPRF plasmid (Filonov et al., 2011) digested with Nhel and BamHI, and the PCR amplified PAS domain was digested with Nhel and BglII.

To study brightness in mammalian cells, the respective pairs of plasmids were cotransfected. In case of one relevant plasmid needed, the pEGFP-C1 plasmid (Clontech) was used as a mock. In all cases, the pmTagBFP2-C1 plasmid (Subach et al., 2011) was added in a ratio of 1:5 to total plasmid amount to normalize for transfection efficiency. Rapamycin (LC Laboratories) was typically added 24 hr before analysis to the final concentration of 100 nM. Fluorescence intensity of cells was analyzed using a LSRII cytometer (BD Biosciences) equipped with 404, 561, and 640 nm lasers and utilizing 405/50, 610/20, and 730/45 nm filters. To quantify cell fluorescence, a mean fluorescent intensity of the nonnegative population in the near infrared channel was divided by a mean fluorescent intensity of the same population in the blue channel, thus normalizing the near-infrared signal to the transfection efficiency. The analysis also included a compensation of the leakage of the E2-Crimson fluorescence into the near infrared channel. All FACS calculations were performed using FlowJo software (Tree Star).

Characterization of Mammalian Cells

To study brightness in mammalian cells, the respective pairs of plasmids were cotransfected. In case of one relevant plasmid needed, the pEGFP-C1 plasmid (Clontech) was used as a mock. In all cases, the pmTagBFP2-C1 plasmid (Subach et al., 2011) was added in a ratio of 1:5 to total plasmid amount to normalize for transfection efficiency. Rapamycin (LC Laboratories) was typically added 24 hr before analysis to the final concentration of 100 nM. Fluorescence intensity of cells was analyzed using a LSRII cytometer (BD Biosciences) equipped with 404, 561, and 640 nm lasers and utilizing 405/50, 610/20, and 730/45 nm filters. To quantify cell fluorescence, a mean fluorescent intensity of the nonnegative population in the near infrared channel was divided by a mean fluorescent intensity of the same population in the blue channel, thus normalizing the near-infrared signal to the transfection efficiency. The analysis also included a compensation of the leakage of the E2-Crimson fluorescence into the near infrared channel. All FACS calculations were performed using FlowJo software (Tree Star).

To assess the FRB/FKBP binding constant, different amounts of rapamycin were added to HeLa cells 48 hr after the transfection and FACS analyzed 22 hr later. Imaging of HeLa cells was performed 48 hr after the transfection. The HeLa and MTLn3 cells were stained with DAPI and counterstained using a Olympus IX81 inverted epifluorescence microscope equipped with LumenPro200 200 W metal-halide arc lamp (Prior), a 60× 1.35 numerical aperture (NA) oil immersion objective lens (UPlanSApo, Olympus), and a standard Cy5.5 filter set (665/45 nm exciter and 725/50 nm emitter) (Chroma). To study fluorescence growth in MTLn3 preclinical mixture coexpressing PAS-FRBP/FKBP-GAF<sub>m</sub> complex upon 100 nM rapamycin treatment, an environmental chamber with the temperature control (Precision Control) was used to keep the temperature at 37°C. Photobleaching measurements of the nuclear localized PAS-FRBP/FKBP-GAF<sub>m</sub> complex (iSplit) in the stable preclinical mixture of MTLn3 cells were
Mixture of MTLn3 cells expressing the PAS-FRB/FKBP-GAFm complex were incubated with 100 nM rapamycin for 24 hr or stable preclonal mixture of MTLn3 cells expressing iRFP (Filonov et al., 2012) were washed with PBS and then treated with 100 nM of rapamycin for an additional 21 hr to start the next cycle. To check fluorescence dependence on BV concentration, the respective amounts of exogenous BV were added to the stable preclonal mixture of MTLn3 cells expressing PAS-FRB/FKBP-GAFm complex, preincubated with 100 nM rapamycin for 24 hr, or to the stable preclonal mixture of MTLn3 cells expressing iRFP (Filonov et al., 2012).

To study complementation-degradation cycling, the stable preclonal mixture of MTLn3 cells expressing the PAS-FRB/FKBP-GAFm complex were incubated with 100 nM rapamycin for 21 hr, washed with PBS, split in two parts, and either passed to another dish or FACS analyzed. Nonanalyzed cells were split in two 53 hr after that and either FACS analyzed or treated with 100 nM of rapamycin for an additional 21 hr to start the next cycle. Cell fluorescence was assessed with FACS analysis, as described above.

To study degradation-complementation cycling, the stable preclonal mixture of MTLn3 cells expressing PAS-FRB/FKBP-GAFm complex were incubated with 100 nM rapamycin for 21 hr, washed with PBS, split in two parts, and either passed to another dish or FACS analyzed. Nonanalyzed cells were split in two 53 hr after that and either FACS analyzed or treated with 100 nM of rapamycin for an additional 21 hr to start the next cycle. Cell fluorescence was assessed with FACS analysis, as described above.

In Vivo Fluorescence Imaging

One million MTLn3 cells stably expressing E2-Crimson with the PAS-FRB/FKBP-GAFm complex, E2-Crimson alone, or iRFP alone were injected into the mammary gland of SCID/Ncr mice (female, 5–7 weeks old) (Taconic) and imaged starting 3 weeks later using an IVIS Spectrum instrument (PerkinElmer/Caliper) in epifluorescence mode. Rapamycin was dissolved in 100% ethanol to a concentration of 9 mg/ml and stored at −20°C. Before its intraperitoneal injection, the concentrated stock of rapamycin was diluted in an aqueous solution of 5.2% Tween 80 and 5.2% PEG400. The typical injected volume was 200 µl and resulted in 4.5 mg/kg concentration. The IVIS Spectrum instrument was equipped with 675/30 and 720/20 nm excitation and emission filters for iSplit and iRFP imaging, and 605/30 and 660/20 nm excitation and emission filters for E2-Crimson imaging. Belly fur was removed using a depilatory cream. Mice were fed with AIN-93M Maintenance Purified Diet (TestDiet) to reduce intrinsic autofluorescence level. The iSplit- and iRFP-expressing MTLn3 tumors were excised postmortem, cut in half, and imaged using the above setup of the IVIS Spectrum instrument.

Quantitative measurements of fluorescence signal were made with a Living Image Software 4.0 (PerkinElmer/Caliper). Then, tumors were chopped into pieces, washed with PBS supplemented with 2% of bovine serum albumin, and subsequently filtered through sieves and a 35 µm filter. FACS analysis was performed with the LSR II cytometer using 610/20 and 730/45 nm emission filters.

All animal experiments were performed in an AAALAC accredited facility using protocols approved by the Albert Einstein College of Medicine Animal Usage Committee.

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and can be found with this article online at http://dx.doi.org/10.1016/j.chembio.2013.06.009.

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REFERENCES


Supplemental Information

A Near-Infrared BiFC Reporter for In Vivo Imaging of Protein-Protein Interactions
Grigory S. Filonov and Vladislav V. Verkhusha

Inventory of Supplemental Information
SUPPLEMENTAL DATA

Figure S1, related to Figure 1. Alignment of the amino acid sequences of iSplit with parental iRFP.

Figure S2, related to Figure 1. Schemes of the plasmid for iSplit mutagenesis and its protein fusions.

Figure S3, related to Figure 1. Initial assessment of split iRFP properties in bacteria and comparison the GAF domain mutants in bacteria and mammalian cells.

Figure S4, related to Figure 2. FACS analysis of the rapamycin induced iSplit formation in the stable preclonal mixtures of MTLn3 cells.

Figure S5, related to Figure 2. Assessment of BV binding to the iSplit complex.

Figure S6, related to Figure 3. Initial assessment of the iSplit performance in vivo.

Figure S7, related to Figure 3. Increase of raw fluorescent intensity in the rapamycin injected mouse.

SUPPLEMENTAL REFERENCES
**SUPPLEMENTAL DATA**

**Figure S1, related to Figure 1.**

Alignment of the amino acid sequences of iSplit with parental iRFP.

<table>
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<th></th>
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An original iRFP protein was cut in a loop between the PAS (blue font) and GAF (red font) domains determined according to a crystal structure of the close homologue <i>RpBphP3</i> (Yang, et al., 2007). The amino acid changes introduced into the GAF domain (resulting in GAF<sub>m</sub>) after random mutagenesis are highlighted in yellow.
Figure S2, related to Figure 1.
Schemes of the plasmid for iSplit mutagenesis and its protein fusions.

To express the PAS and GAF domains in bacterial cells both genes were cloned onto a single plasmid (a) under either arabinose- or rhamnose-induced promoters and introduced into *E. coli* along with another plasmid (see Experimental Procedures) encoding heme oxygenase to produce a BV chromophore. In the course of this study PAS and GAF domains were fused either to the E- or K-coils (De Crescenzo, et al., 2003) to promote constitutive binding following translation (b) or to the FRB or FKBP proteins (Banaszynski, et al., 2005) allowing inducible interaction upon addition of rapamycin (c).
Figure S3, related to Figure 1.

Initial assessment of split iRFP properties in bacteria and comparison the GAF domain mutants in bacteria and mammalian cells.

(a) Initial expression of the PAS and GAF domains (both with the sequences identical to the ones of iRFP) in bacteria showed that when brought into proximity these domains produce fluorescent complex with a brightness being one tenth of that of parental iRFP. (b) Random mutagenesis of the GAF domain generated a series of the mutants with the fluorescence brightness in bacteria being substantially higher than for the initial PAS / GAF pair (indicated as WT). (c) The PAS domain and mutants of the GAF domain, which provided the brightest signal in bacteria, were re-cloned into mammalian plasmids and their performance was assessed in HeLa cells to choose the optimal pair: PAS (identical to one in iRFP) + GAFm (mutant C4). Fluorescence in bacteria was measured in bacterial suspension and normalized to optical density. Fluorescence in mammalian cells was studied using FACS as described in the Experimental Procedures.
Figure S4, related to Figure 2.

FACS analysis of the rapamycin induced iSplit formation in the stable preclonal mixtures of MTLn3 cells.

The stable preclonal mixture of MTLn3 cells co-expressing E2-Crimson, PAS-FRB, and FKBP-GAF<sub>m</sub> fusions were generated as described in the Experimental Procedures. Cells were pre-incubated with 100 nM of rapamycin for 24 h and then compared to the untreated cells using FACS. The non-expressing cells were used as a negative control, and the stable preclonal mixture of MTLn3 cells expressing iRFP (Filonov, et al., 2012) was used as a positive control.
Figure S5, related to Figure 2.

Assessment of BV binding to the iSplit complex.

Effect of addition of exogenous BV on cellular fluorescence of MTLn3 preclonal mix expressing either iSplit (pre-treated with 100 nM rapamycin for 24 h) or iRFP was studied. Cells were incubated with the respective amounts of BV for 2 h before FACS analysis. Both iRFP and iSplit complex exhibited a low fluorescence dependence on the increasing amount of exogenous BV and efficiently utilized endogenous BV to achieve their steady fluorescence level. Error bars represent 95% confidence interval calculated based on either 3 samples.
Figure S6, related to Figure 3.
Initial assessment of the iSplit performance in vivo.

To ensure that there is no spectral crosstalk between E2-Crimson and iSplit signals we imaged a control mouse bearing two tumors. MTLn3 cells stably expressing E2-Crimson only were injected into a mammary gland on the right part of the body and iRFP only stably expressing MTLn3 cells (Filonov, et al., 2012) were injected into a mammary gland on the left part of the body. Imaging started 4 weeks later using the IVIS Spectrum equipped with the 605/30 nm and 660/20 nm (for E2-Crimson) and 675/30 nm and 720/20 nm (for iRFP) filter sets (a and b, respectively). One can see that both signals are spectrally resolved with no detectable crosstalk even when the display range of fluorescence intensity is broaden to visualize autofluorescence (right image in each panel). This experiment allowed to attribute the signal observed in E2-Crimson / PAS-FRB / FKBP-GAFm co-expressing tumor in mice before rapamycin injection to the real background signal from the spontaneously associated PAS-FRB / FKBP-GAFm pair and, thus, to quantify the signals appropriately (panel c, mouse on the right is a control one without a tumor).
Figure S7, related to Figure 3.
Increase of raw fluorescent intensity in the rapamycin injected mouse.

A representative mouse from Figure 3a was imaged using the IVIS Spectrum instrument as described in Methods, and raw fluorescent data from the near-infrared channel was plotted as a function of time after the first injection of rapamycin. The second rapamycin injection was made at the time point of 192 hours after the first one. Overall signal shows a steady increase due to the growth of the tumor volume and possible accumulation of non-specifically complemented iSplit complex.
SUPPLEMENTAL REFERENCES


