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Submitted date: 11/06/2019 • Posted date: 12/06/2019
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Citation information: Jones, Krysten A.; Kentala, Kaitlin; Beck, Michael; An, Weiwei; Lippert, Alexander; Lewis, Jared C.; et al. (2019): Development of a Split Esterase 1 for Protein-Protein 2 Interaction Dependent Small Molecule Activation. ChemRxiv. Preprint.

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Development of a split esterase for protein-protein interaction dependent small molecule activation

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Abstract

Split reporters based on fluorescent proteins and luciferases have emerged as valuable tools for measuring interactions in biological systems. Relatedly, biosensors that transduce measured input signals into outputs that influence the host system are key components of engineered gene circuits for synthetic biology applications. While small molecule-based imaging agents are widely used in biological studies, and small molecule-based drugs and chemical probes can target a range of biological processes, a general method for generating a target small molecule in a biological system based on a measured input signal is lacking. Here, we develop a proximity-dependent split esterase that selectively unmasks ester-protected small molecules in an interaction-dependent manner. Exploiting the versatility of an ester-protected small molecule output, we demonstrate fluorescent, chemiluminescent, and pharmacological probe generation, each created by masking key alcohol functional groups on a target small molecule. We show the split esterase system can be used in combination with ester-masked fluorescent or luminescent probes to measure a protein-protein interactions and protein-protein interaction inhibitor engagement. We demonstrate the esterase-based reporter system is compatible with other commonly-used split reporter imaging systems for the simultaneous detection of multiple protein-protein interactions. Finally, we develop a system for selective small molecule-dependent cell killing by unmasking a cytotoxic molecule using an inducible split esterase. Presaging utility in future synthetic biology-based therapeutic applications, we also show
the system can be used for intercellular cell killing via a bystander effect, where one activated cell unmask a cytotoxic molecule and kills cells physically adjacent to the activated cells. Collectively, this work illustrates that the split esterase system is a valuable new addition to the split protein toolbox, with particularly exciting potential in synthetic biology applications.
**Introduction**

Protein-protein interactions (PPIs) are critical regulators of diverse cellular processes\(^1\)–\(^4\) and are increasingly recognized as viable therapeutic targets for the treatment of multiple disease states\(^5\)–\(^7\). Synthetic biology-based biosensor systems that drive cell fate changes based on measured PPIs are increasingly critical components of engineered gene circuits. For example, split proteases can be used to engineer cell receptors\(^8\)–\(^9\) and drive gene expression, and split Cas9\(^10\) can be used to control gene editing, both based on fused sensor domains triggered by a PPI. Our group has developed proximity-dependent split T7 RNAPs as a versatile strategy for encoding PPIs in RNA signals for applications in biosensing, cell engineering, and directed evolution\(^11\)–\(^14\). In a similar approach, a bioluminescence resonance energy transfer-based system that enables transcriptional activation with improved PPI specificity was recently engineered\(^15\). While methods to generate genetic output responses based on PPI inputs are continuing to improve, a simple and general method to create a small molecule output based on measured PPIs is lacking, despite the versatility of small molecules as biological indicators and mediators.

Aside from exploiting PPIs for engineering purposes, methods for detecting the interactions between proteins and the disruption of those interactions due to therapeutic target engagement in live cells are critical for understanding PPIs\(^16\). One of the most widely used approaches to measure PPIs in live cells is protein fragment complementation assays (PCA), which involves fusing interacting proteins to complementary fragments of a split protein reporter\(^17\)–\(^19\). Interactions between the fused proteins drives assembly of the split reporter, which in turn generates an output signal. In the context of analysis, a variety of reporters have been developed, including luciferases,\(^20\) fluorescent proteins,\(^21\) and horseradish peroxidase,\(^22\) which produce photons, fluorescence, or reactive molecule outputs, respectively.
We envisioned a new split reporter system that would be capable of unmasking caged small molecules in a PPI-dependent manner. Small molecules provide substantial flexibility as an output signal for both analysis and generating bioactive molecules for synthetic biology purposes. Selective enzyme/substrate pairs that are orthogonal to endogenous cellular machinery have found utility for neuronal imaging\textsuperscript{23}, cell-specific pharmacology\textsuperscript{24-26}, and imaging of cellular interactions\textsuperscript{27,28}. For example, porcine liver esterase (PLE) was discovered to be able to process 1-methylcyclopropyl (CM)-masked phenol substrates when expressed in human cells, whereas endogenous esterases are not capable of processing this bulky protecting group\textsuperscript{23}. We reasoned that selective enzyme/substrate pairs may also be amenable to the creation of biosensor platforms by identifying proximity-dependent split sites of the enzymes that can ultimately unmask caged small molecules based on PPI-driven enzyme assembly. While it is conceptually possible to use the pre-existing split beta-lactamase\textsuperscript{29} with beta-lactam prodrugs\textsuperscript{30}, this has not yet been demonstrated. We envisioned using a selective esterase-ester pair for this strategy. Exogenous esterases have previously been expressed in mammalian cells for prodrug metabolism\textsuperscript{31}, analysis of calcium signaling\textsuperscript{32}, and neuronal imaging\textsuperscript{23}. In addition, ester masking strategies for a variety of functional groups, including CM-masked phenol substrates, are well established\textsuperscript{23,33-36}. Based on this work, we aimed to engineer a selective split esterase-ester substrate pair that would be capable of unmasking small molecules in a PPI-dependent manner (Figure 1a).

In this work, we develop a split BS2 esterase system using a fluorescence-based screen to identify interaction-dependent esterase fragments in E. coli. We then demonstrate that the split esterase is capable of detecting interactions between multiple PPIs in mammalian cells, including small molecule-induced dimerization domains, leucine zipper peptides, and medically-relevant proteins of the B cell lymphoma (Bcl-2) family of apoptotic regulatory proteins and Bcl-2 homology 3 (BH3)-only interacting domains. We validate that the split esterase system is capable of measuring time-dependent engagement of a small molecule PPI inhibitor in live cells. Moreover,
we go on to multiplex our split esterase reporter with existing PCA technologies to simultaneously monitor two PPIs in both the intracellular and extracellular environment. In addition to demonstrating outputs from fluorescent and chemiluminescent probes, we go on to show the split esterase can generate a bioactive molecule output to control cellular cytotoxicity, highlighting the versatility of small molecule signals. Taken together, these results establish the split esterase as a versatile new addition to the PCA toolbox.

Results

Development of a proximity-dependent split esterase.

In order to develop a split esterase reporter, we aimed to leverage the fluorescein α-cyclopropyl ester (fluorescein-CM₂) fluorogenic molecule to rapidly screen esterase cut sites for PPI-dependent esterase activity in \textit{E. coli}. We selected BS2 esterase from \textit{B. subtilis} as our exogenous esterase, which like PLE, acts on sterically-hindered esters. BS2, however, can be efficiently expressed in \textit{E. coli} and has been successfully used in plate-based screens with high enzymatic activity\textsuperscript{37}. First, to confirm BS2-mediated unmasking of fluorescein-CM₂, we incubated fluorescein-CM₂ with \textit{E. coli} expressing BS2, PLE, or a negative control protease. Both BS2 and PLE showed enhanced fluorescent signal due to fluorescein-CM₂ unmasking (Figure S2a), but BS2 showed significantly greater activity, likely due to improved expression in \textit{E. coli}. More importantly, when expressed in mammalian cells, both BS2 and PLE show robust fluorescent signal (Figure S2b-d). Based on these results, we moved forward with BS2 as our target for split esterase development.

To develop a split esterase, we screened eight potential split sites on BS2, each located on surface-exposed loop regions (Figure 1b). We fused the split esterase fragments via flexible linkers to the rapamycin dimerization domains, FRB and FKBP\textsuperscript{38}, coexpressed the fragments in \textit{E. coli}, and measured the activity on fluorescein-CM₂ in the absence and presence of rapamycin
(Figure 1c-d). Splitting of BS2 at two of the eight sites, positions 94 and 96, produced robust enzymatic activity, which was enhanced in the presence of rapamycin (Figure 1e). We selected cut site 94 as our lead and further validated esterase assembly with an abscisic acid-inducible dimerization system by fusing the fragments to the ABI and PYL proteins. Similarly, esterase assembly was enhanced with the addition of the small molecule dimerization trigger in E. coli (Figure S3). Given the performance of the system, we imaged lysate from E. coli cotransformed with N-terminal split BS2 (BS2\textsubscript{N})-fused FRB and FKBP-fused C-terminal split BS2 (BS2\textsubscript{C}) as a proof-of-principle experiment towards a cell-free synthetic enzymatic detection system. Indeed, the split esterase assembly was robust enough to detect fluorescein-CM\textsubscript{2} cleavage by eye and to discern the presence of rapamycin (Figure S4).

Monitoring small-molecule activated PPIs in Mammalian Cells with split BS2 esterase.

Given that the split esterase performed well in E. coli, we next sought to determine if the system could function in mammalian cells. We first fused the split esterase fragments to tightly-binding leucine zipper domains ZA and ZB\textsuperscript{11} (BS2\textsubscript{N}-ZA and ZB-BS2\textsubscript{C}) in order to optimize the deployment of the reporter in mammalian cells, including vector concentrations and experimental timing (Figure S5). Once we obtained optimized conditions, we then tested the rapamycin-dependent dimerization system in mammalian cells. Fluorescent signal, and therefore BS2 activity, was only observed in the presence of rapamycin in both imaging and plate reader-based cell assays (Figure 2a-b and Figure S6). While esterase activity was observed in the absence of rapamycin in E. coli, esterase assembly was fully PPI-dependent in mammalian cells. We hypothesized that this discrepancy was due to a substantial overexpression of esterase fragments in E. coli, as compared with the relatively lower concentrations achieved in mammalian cells, which enables purely PPI-dependent assembly. Similar to the rapamycin system, we also observed robust PPI-dependent BS2 activity in mammalian cells with the ABA-inducible dimerization system (Figure S7).
Monitoring Bcl-2 Family PPIs and Inhibitor Engagement.

Having established small molecule-dependent activation of split BS2 in mammalian cells, we next examined whether the system can detect therapeutically-relevant PPIs. We selected the Bcl-2 family of apoptotic regulatory proteins to detect both the interaction network with BH3 domains and their pharmacological engagement. We generated fusions of BS2\textsubscript{C} to the antiapoptotic proteins Bcl-2 and Mcl-1, and cloned fusions of BS2\textsubscript{N} to the BH3 binding domains of tBID, which binds Bcl-2 and Mcl-1, NOXA, a selective Mcl-1 ligand, or deadBID, a control peptide without the necessary BH3 domain (Figure 3a). Cotransfection of BS2\textsubscript{N}-fused deadBID with either of the antiapoptotic protein-fused BS2\textsubscript{C} showed no significant signal over non-transfected cells incubated with fluorescein-CM\textsubscript{2} as measured by either imaging or plate reader assays (Figure 3b-c and Figure S8). As expected, Mcl-1-fused BS2\textsubscript{C} showed significant esterase assembly when combined with either tBID- or NOXA-fused BS2\textsubscript{N}, while Bcl-2-fused BS2\textsubscript{C} only showed robust esterase activity in combination with tBID-fused BS2\textsubscript{N}. Collectively, these results indicate that split BS2 is capable of measuring biologically-relevant PPIs in mammalian cells in a manner that recapitulates the well-studied selectivity profiles of these PPI targets.

We next aimed to measure pharmacological engagement of PPI inhibitors, a common use of PCA systems, which would also allow us to test the time-dependency of split BS2 disassembly. We selected the FDA approved Bcl-2 inhibitor, ABT-199 (Venetoclax), and monitored tBID-fused BS2\textsubscript{N} and Bcl-2-fused BS2\textsubscript{C} disassembly over time (Figure 2d and Figure S9a-b). Treatment resulted in significantly decreased esterase activity within 1 h with no appreciable esterase activity detected after 6 h. We compared this to Nanobit, a structurally optimized split NanoLuc (Nluc) luciferase reporter, which has been used to monitor numerous PPIs and their modulation by small molecules (Figure S9c). While both systems reported on the ABT-199-mediated blockade of tBID-Bcl-2 assembly with approximately 10-fold reduction in signal on comparable time scales, a
high level of signal remained with Nanobit. Nonetheless, these results confirm that split BS2 performs similarly to an established PPI inhibitor screening system, such as the state-of-the-art Nluc system.

Since both Nluc and split BS2 reporters measure pharmacological engagement in mammalian cells, and the two PCA systems should be orthogonal to one another, we reasoned that we could multiplex the reporters to simultaneously detect two PPIs and Bcl-2 inhibition (Figure 3e). We cotransfected one cell population with BS2N-fused tBID and Bcl-2-fused BS2C and another population with Nluc15S-fused tBID and Mcl-1-fused Nluc114. The two cell populations were then mixed and treated with ABT-199. As expected, only the Bcl-2 and tBID interaction was blocked, as esterase activity (as measured by fluorescence) was significantly decreased in the presence of the inhibitor while Nanobit activity (as measured by luminescence) remained constant (Fig. 3f). We also swapped the fusion partners on Nluc and split BS2, with Nanobit reporting on the Bcl-2/tBID interaction and split BS2 reporting on the Mcl-1/tBID interaction, and observed a decrease in luminescence, but not fluorescence, with ABT-199 treatment.

**Generation of a Chemiluminescent Signal Output for detecting extracellular PPIs.**

Based on the ability of the split esterase to sensitively and selectively monitor intracellular PPIs in mammalian cells, we next aimed to detect an extracellular PPI. Such an extracellular system could be useful for investigating both ligand- and receptor-mediated dimerization of transmembrane cell-surface receptors, such as receptor tyrosine kinases or G protein-coupled receptors. Additionally, measuring extracellular interactions also presented us with an opportunity to develop another signal output of split BS2. Specifically, we aimed to develop a caged chemiluminescent molecule that directly generates photons in an activity-dependent manner by split BS2. To this end, we first synthesized Chemilum-CM (Figure 4a, Figure S10, Supplementary Note 1-2), a methylcyclopropyl ester-masked pro-chemiluminescent substrate.
based on a previously reported scaffold\textsuperscript{45,46}. We reasoned that esterase activity on Chemilum-CM would release the chemiluminescent form of the probe, thereby generating a photon and resulting in luminescence. To confirm Chemilum-CM can act cooperatively with BS2 to generate luminescence, we incubated Chemilum-CM with HEK293T cells transfected with a glycosphatidyl inositol (GPI)-anchored\textsuperscript{47} full length-BS2 esterase and observed robust luminescence signal (Figure S11a). We next examined whether the split esterase could provide a sensitive and fast readout on extracellular PPIs. HEK293T cells cotransfected with GPI anchored BS2\textsubscript{N}-fused FRB and FKBP-fused BS2\textsubscript{C} were cultured in the presence and absence of rapamycin over time and subsequently incubated with Chemilum-CM (Figure 4b-c). Luminescence was observed 30 min post-rapamycin addition and increased over time, suggesting split esterase assembly is occurring at the cell membrane (Figure 4d, Figure S11b). Luminescence also corresponded to the amount of split esterase used for transfection (Figure S11c). The BS2/Chemilum-CM system therefore functioned as a “synthetic luciferase”, allowing us to perform side-by-side direct comparisons between split BS2 and Nluc.

We next tested whether we could monitor intracellular and extracellular PPIs simultaneously, using the selective luminescent signals generated from Nluc/luciferin and BS2/Chemilum-CM\textsubscript{2}. We used BS2\textsubscript{N}- ZA and ZB-BS2\textsubscript{C} with GPI-anchors to monitor extracellular interactions (Figure 4e), and used ZBneg\textsuperscript{41}, a triple mutant of ZB with weakened affinity for ZA, as a negative BS2\textsubscript{C} fusion control. To monitor intracellular PPIs, we used Nluc\textsubscript{11S}-fused FRB and FKBP-fused Nluc\textsubscript{114}. All four plasmids were simultaneously transfected into mammalian cells, followed by treatment with rapamycin or DMSO carrier as a control. After 24 h, we added Chemilum-CM\textsubscript{2} and measured luminescence, and observed robust esterase activity only in cells expressing the ZA/ZB interaction fusions (Figure 4f). Cells expressing the ZBneg fusion did not exhibit significant chemiluminescence over untransfected control cells, indicating esterase assembly was interaction-dependent, even on the cell surface. We then rinsed the cells and administered the
Nanobit substrate, furimazine, and again measured luminescence. As expected, cells that were treated with rapamycin showed significantly enhanced luminescence activity over DMSO-treated cells. While luminescence was also observed in DMSO-treated cells, the signal was comparable to cells expressing just Nluc11S-fused FRB or mismatched PPI controls and, moreover is a previously reported artifact attributed to the background of the N-terminal fragment of Nluc (Figure S12)⁴⁴. Collectively, these results not only confirm that split BS2 functions as a proximity-dependent split reporter at the cell surface, but also that the BS2/Chemilum-CM system is fully compatible with commonly-used split luciferase reporters.

Proximity-dependent uncaging of bioactive molecules.

We then tested the utility of the split esterase to uncage a pharmacological agent in a proximity-dependent manner. The prodrug irinotecan (CPT-11) is converted to the cytotoxic active metabolite SN-38, a 1,000-fold more potent topoisomerase-1 inhibitor, by carboxylesterases³¹,⁴⁸,⁴⁹. Irinotecan is currently in clinical trials for neuroblastoma⁵⁰, colon cancer⁵¹, and other solid malignancies⁵²-⁵⁶. New strategies to more effectively activate irinotecan by modified rabbit and human carboxylesterases are also in development.³¹,⁴⁸ We reasoned our new split esterase system could potentially be used to activate irinotecan analogues in an orthogonal and programmable manner. We therefore synthesized a new methyl-cyclopropyl caged version irinotecan to generate SN-38-CM₂ (Figure 5a). Incubation of SN-38-CM₂ with BS2 in vitro showed >95% conversion to SN-38 within five minutes (Figure S13).

To test the PPI-dependent activity of the BS2/SN-38-CM₂ system, we incubated SN-38-CM₂ with MDA-MB-231 cells stably expressing luciferase and cotransfected with GPI anchored BS2N-fused FRB and FKBP-fused BS2C in the presence or absence of rapamycin. After incubation with the caged pharmacological agent over time, cells were rinsed and cultured for 40 h prior to measuring cell proliferation via luciferase assay. Increased cytotoxicity was observed in cells treated with
rapamycin over DMSO-treated cells and was comparable to activity observed with BS2 (Figure 5b, Figure S14). In addition, DMSO-treated cells and untransfected control cells showed no significant cytotoxicity with up to 1 µM SN-38-CM₂. The reduced cell survival observed at higher concentrations of SN-38-CM₂ for control conditions is likely due to slow background hydrolysis of the CM motif as shorter incubation times do not show any significant changes to cell proliferation (Figure S14). While careful tuning of the SN-38-CM₂ incubation is necessary to ensure maximum PPI-dependent esterase mediated cell death, our experiments suggest that further optimization of the BS2/SN-38-CM₂ system could effectively result in complete reduction of cell survival rate.

Since the spilt esterase/SN-38-CM₂ system induced robust cytotoxicity in cells expressing the esterase system, we aimed to visualize intercellularly-mediated cell death based on the relative location of a second cell population using a bystander effect (Figure 5c). Such an approach, if successful, could lead to “sentinel” cells that respond to activation signals and release cytotoxic molecules that kill adjacent cells only when activated. To test this idea, we used HEK293T cells cotransfected with GPI anchored BS2ₙ-fused FRB and FKBP-fused BS2ₖ suspended in extracellular matrix (Matrigel), which we then plated in the middle of a 3.5-cm² tissue culture dish in the presence or absence of rapamycin with SN-38-CM₂. After the matrix solidified, MDA-MB-453 luciferase expressing cells were plated around the Matrigel. The cells were then rinsed and assayed for cytotoxicity via light emission after 40 h. The reporter cells nearest the rapamycin-treated split BS2 cells showed significant cytotoxicity indicated by reduced light emission (Figure 5d-e, Figure S15). Light emission was comparable between control cells and split BS2 cells in the absence of rapamycin. These results confirm that cells engineered with the split esterase reporter system can generate a cytotoxic molecule and kill adjacent cells in a small molecule-dependent manner.
Conclusions

In this work, we developed a strategy for proximity-dependent uncaging of small molecules using an engineered esterase biosensor system. This approach comprises split versions of BS2 esterase that assemble to form a functional esterase when brought into contact by PPIs, which subsequently activates ester-caged small molecules. By utilizing small molecule products as the output of BS2 PPI-detection, we show we can generate fluorescent, chemiluminescent, and pharmacological output signals, thereby adding a versatile new addition to the PCA toolbox (Figure 5f). In principle, this method should be capable of uncaging any molecule that relies on an alcohol function group for bioactivity or function.

The small molecule output made possible by BS2 can be used to generate fluorescent or luminescent signals and is compatible with other common PCA detection tools such as luciferase for analysis purposes. By modulating the properties of the ester-masked imaging agents, it should be possible to rapidly alter the dynamic range and sensitivity of BS2 imaging. Indeed, cell permeability, pharmacokinetics, and emission maximum, have been investigated for D-luciferin enabling improved tissue distribution, sensitivity, and dynamic range in bioluminescence imaging\textsuperscript{57,58}. Fluorophores have also been extensively designed for enhanced brightness and photostability for cellular imaging\textsuperscript{59-61} and we are currently investigating ester-caging strategies for new imaging scaffolds. Finally, for in vivo imaging, caged red-shifted fluorophores should be easily accessible, as well as caged versions of other imaging modalities such as magnetic resonance-based imaging reagents.

In principle, any bioactive molecule that relies on an alcohol position for function can be caged and therefore unmasked by BS2 in an activity-dependent manner, which we demonstrated in this work through controlled cellular cytotoxicity of SN-38. Aside from activating killing in the cells expressing the biosensor, we also show that split BS2 in combination with an ester-masked
pharmacological agent induced intercellular cell killing based on the proximity of the second cell population to the esterase expressing cells. While robust cytotoxicity is also observed in cells expressing split BS2, this could be mitigated against by expressing known resistance proteins to SN-38\textsuperscript{62,63} and would enable a constant esterase source for adjacent cell killing. We are currently pursuing integrating split BS2 with cell engineering approaches, such as chimeric antigen receptor T cell therapy, to integrate activity-dependent small molecule activation at cancer sites. We also anticipate additional new synthetic biology opportunities for cell engineering and cell signaling-based prodrug release with PPI-dependent split esterase small molecule activation.
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Dickinson et al., Split esterase for protein-protein interaction dependent small molecule activation, 16
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ACKNOWLEDGEMENTS

This work was supported by the University of Chicago, the National Institute of General Medical Sciences (R35 GM119840) and the National Institute of Mental Health (RF1 MH114102) of the National Institutes of Health to B.C.D. Chemical synthesis and characterization was supported by the National Science Foundation (CHE 1653474). K.K. was supported by a Chemical Biology Training Grant from the National Institutes of Health (T32 GM008720).

AUTHOR CONTRIBUTIONS

K.A.J., K.K, M.W.B, J.L, and B.C.D. conceived of the experimental design. K.A.J. performed all biological experiments. M.W.B. synthesized and characterized SN-38-CM₂. W.A. and A.R.L. synthesized and characterized chemilum-CM. K.A.J. and B.C.D. wrote the paper with input from all coauthors.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.
Figure 1. Development of a split esterase sensor. (a) Schematic of PPI-driven split esterase assembly to unmask 1-methylcyclopropyl (CM)-masked molecules. Interacting proteins are fused to inactive fragments of BS2 esterase. Interaction between the fusion proteins results in assembly of the esterase which cleaves an inactive small molecule to generate an active molecule and output signal. (b) Mapping the cut sites onto a homologous *B. subtilis* esterase structure (PDB 1QE3). The BS2_N fragment (green) and BS2_C (magenta) from the lead split site, S94, are shown. Split sites occur after the designated amino acids. (c) Vector system to identify PPI-dependent esterase fragments in *E. coli*. (d) Chemical structure of masked fluorophore fluorescein-CM₂. (e)
Fluorescence output of split esterase fragments. *E. coli* expressing BS2\textsubscript{N}-fused FRB (tan), FKBP-fused BS2\textsubscript{C} (gray), or both in the absence (light green) or presence (green) of rapamycin were incubated with fluorescein-CM\textsubscript{2} for 4 h and then analyzed for fluorescence. Error bars are the standard deviation for $n = 3$ replicates.
Figure 2. Split BS2 can detect small molecule activated PPIs. (a) HEK293T cells cotransfected with BS2N-fused FRB and FKBP-fused BS2C or HEK293T control cells (white) were incubated with rapamycin (green) or a DMSO control (grey). After 24 h, fluorescein-CM₂ was applied and the cells were analyzed by plate reader for fluorescence. (b) HEK293T cells were treated identical to conditions in (a) and analyzed by fluorescence microscopy. Error bars are the standard deviation for \( n = 4 \) replicates. Unpaired t-test; ***\( P < 0.0001 \). 20 µm scale bars shown.
Figure 3. Split BS2 can detect a Bcl-2 family PPIs and inhibitor engagement. (a) Vector system to test Bcl-2 split esterase PPI detection. (b) HEK293T cells cotransfected with the plasmids shown in (a) were incubated with fluorescein-CM₂ and analyzed for fluorescence by plate reader. The normalized emission for interactions between deadBID/Bcl-2 protein (gray), tBID/Bcl-2 protein (green), and NOXA/Bcl-2 protein is shown. HEK293T control cells (white) were similarly analyzed (c) HEK293T cells cotransfected and incubated with fluorescein-CM₂ as in (b) were analyzed by fluorescence microscopy. (d) HEK293T cells cotransfected with BS₂ₙ-fused tBID and Bcl-2-fused BS₂ₙ were treated with DMSO (time = 0) or ABT199 for 0-3 h (green) followed by incubation with fluorescein-CM₂ and analyzed for fluorescence by plate reader. HEK293T control cells (white) were similarly analyzed. (e) Vector system to simultaneously detect two PPIs and Bcl-2 inhibition. (f) HEK293T cells were cotransfected with the split esterase plasmids or split Nluc plasmids shown in (e). The two cell populations were mixed and treated with ABT-199 or a DMSO control. After 24 h, the cells were incubated with fluorescein-CM₂ and analyzed (green). Immediately after analysis, furimazine was added and the cells were re-imaged.
(orange). The Bcl-2/tBID interaction was selectively blocked and detected with the esterase reporter (left) or Nluc reporter (right). HEK293T control cells were similarly analyzed. Error bars are the standard deviation for $n = 4$ (b), $n = 6$ replicates (d), and $n = 8$ replicates (f). Unpaired t-test; *$P < 0.01$, **$P < 0.0001$. 20 µm scale bars shown.
Figure 4. Multiplexed PPI analysis with split BS2. (a) Chemilum-CM is unmasked by esterase activity and generates a photon. (b) Vector system to monitor extracellular PPIs. (c) HEK293T cells were cotransfected with plasmids shown in (b). Rapamycin (blue) or a DMSO control (grey) were added to cells for 0-6 h. Media was replaced with Chemilum-CM and analyzed for luminescence. HEK293T control cells (white) were similarly analyzed. (d) HEK293T cells cotransfected as in (c) or HEK293T control cells (triangle) were incubated with rapamycin (blue) or a DMSO control (grey). After 6 h, the cells were analyzed with Chemilum-CM as in (c). (e) Vector system to simultaneously monitor extracellular and intracellular PPIs. (f) HEK293T cells were transfected with all four plasmids shown in (d). Rapamycin or a DMSO control were added to the cells for 24 h. Media was replaced with Chemilum-CM (10 µM) and analyzed for luminescence (blue). The cells were then rinsed, incubated with furimazine, and analyzed for bioluminescence (orange). Error bars are the standard deviation for n = 4 replicates. Unpaired t-test; *P < 0.01, **P < 0.001 ***P < 0.0001.
**Figure 5. Small molecule-induced intra- and inter-cellular cell death.** (a) Chemical structure of masked chemotherapeutic SN-38-CM$_{2}$. (b) MDA-MB-231 luciferase cells were cotransfected with GPI anchored BS$_{2n}$-fused FRB and FKBP-fused BS$_{2c}$. Rapamycin (blue square) or a DMSO control (grey square) were added to cells for 12 h prior to addition of SN-38-CM$_{2}$. After 6 h, the cells were rinsed, cultured for 40 h, and imaged with D-luciferin. MDA-MB-231 luciferase control cells (grey dashed circle) were similarly analyzed. (c) Schematic of coculture cytotoxicity assay. Split esterase or control HEK293T cells were plated in the center of a 3.5-cm$^2$ dish (dashed circle). MDA-MB-453 luciferase cells were plated around the split BS2 or control cells. PPI-mediated cleavage of SN-38-CM$_{2}$ by split BS2 results in an active molecule to induce killing of neighboring cells. (d) Representative bioluminescence images of cocultures after treatment as in (b). (e) Quantification of light emission from (d). Photon counts along a 1.8 cm horizontal line from

Dickinson et. al, *Split esterase for protein-protein interaction dependent small molecule activation*, 25
the Matrigel embedded cells (0 cm) to the right of each dish are plotted. (f) Commonly used PCA systems and their corresponding outputs. Error bars are the standard deviation for $n = 4$ replicates. Unpaired t-test; $*P < 0.01$, $**P < 0.001$, $***P < 0.0001$. 
