

# Optochemical Control of Protein Localization and Activity within Cell-like Compartments

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## Supporting Information

**ABSTRACT:** We report inducible dimerization strategies for controlling protein positioning, enzymatic activity, and organelle assembly inside synthetic cell-like compartments upon photostimulation. Using a photocaged TMP-Haloligand compound, we demonstrate small molecule and light-induced dimerization of DHFR and Haloenzyme to localize proteins to a compartment boundary and reconstitute tripartite sfGFP assembly. Using photocaged rapamycin and fragments of split TEV protease fused to FRB and FKBP, we establish optical triggering of protease activity inside cell-size compartments. We apply light-inducible protease activation to initiate assembly of membraneless organelles, demonstrating the applicability of these tools for characterizing cell biological processes in vitro. This modular toolkit, which affords spatial and temporal control of protein function in a minimal cell-like system, represents a critical step toward the reconstitution of a tunable synthetic cell, built from the bottom up.

Attaining conditional control of biological organization and activity provides a unique strategy for interrogating the processes that govern cell behavior. Approaches based on optical or chemical control of dimerization exemplify this strategy, enabling regulation of protein localization and function in space and time.<sup>1</sup> Optogenetics is one such approach; it utilizes proteins whose oligomeric states are naturally sensitive to light, such as plant phytochromes (Pif/PhyB),<sup>2,3</sup> phototropins (Lov domains),<sup>4–6</sup> engineered fluorescent proteins such as Dronpa,<sup>7</sup> fungal photoreceptors,<sup>8</sup> and cryptochromes (Cry2).<sup>5,9</sup> However, these systems often require continuous illumination to maintain protein dimerization and have only moderate affinity in the illuminated state.

Optochemical approaches overcome limitations of optogenetic systems by using modular protein switches that dimerize in response to a high-affinity ligand and by photocaging the ligand through synthetic means to block binding in the absence of light.<sup>1</sup> For example, a bivalent ligand containing trimethoprim (TMP) linked to HaloTag ligand (Haloligand), termed TMP-Haloligand (TH), binds tightly to *Escherichia coli* dihydrofolate reductase (DHFR) and HaloTag protein (Haloenzyme). However, a coumarin-photocaged version,

called caged TMP-Haloligand (CTH), sterically hinders TMP, which blocks its binding to DHFR. In the dark, DHFR and Haloenzyme do not interact, whereas in the presence of violet light, CTH uncages and induces dimerization of these protein domains.<sup>12</sup> This strategy has been successfully implemented in vivo to optically control protein localization to organelles and kinetochores.<sup>10–12</sup> Similarly, rapamycin induces dimerization of the canonical FK506 binding protein (FKBP) and FKBP rapamycin binding domain (FRB),<sup>13</sup> and light-activated analogues have been generated. In particular, a light-cleavable rapamycin dimer called dRap allows FKBP to bind but sterically blocks association with FRB in the absence of light. Photoinduced association of FRB and FKBP is achieved by cleaving dRap with ultraviolet (UV) light to remove the steric constraints, allowing each FKBP:rapamycin to bind FRB.<sup>14</sup> By fusing these optochemically sensitive domains to a protein of interest, one can modulate enzymatic localization and function.<sup>15,16</sup>

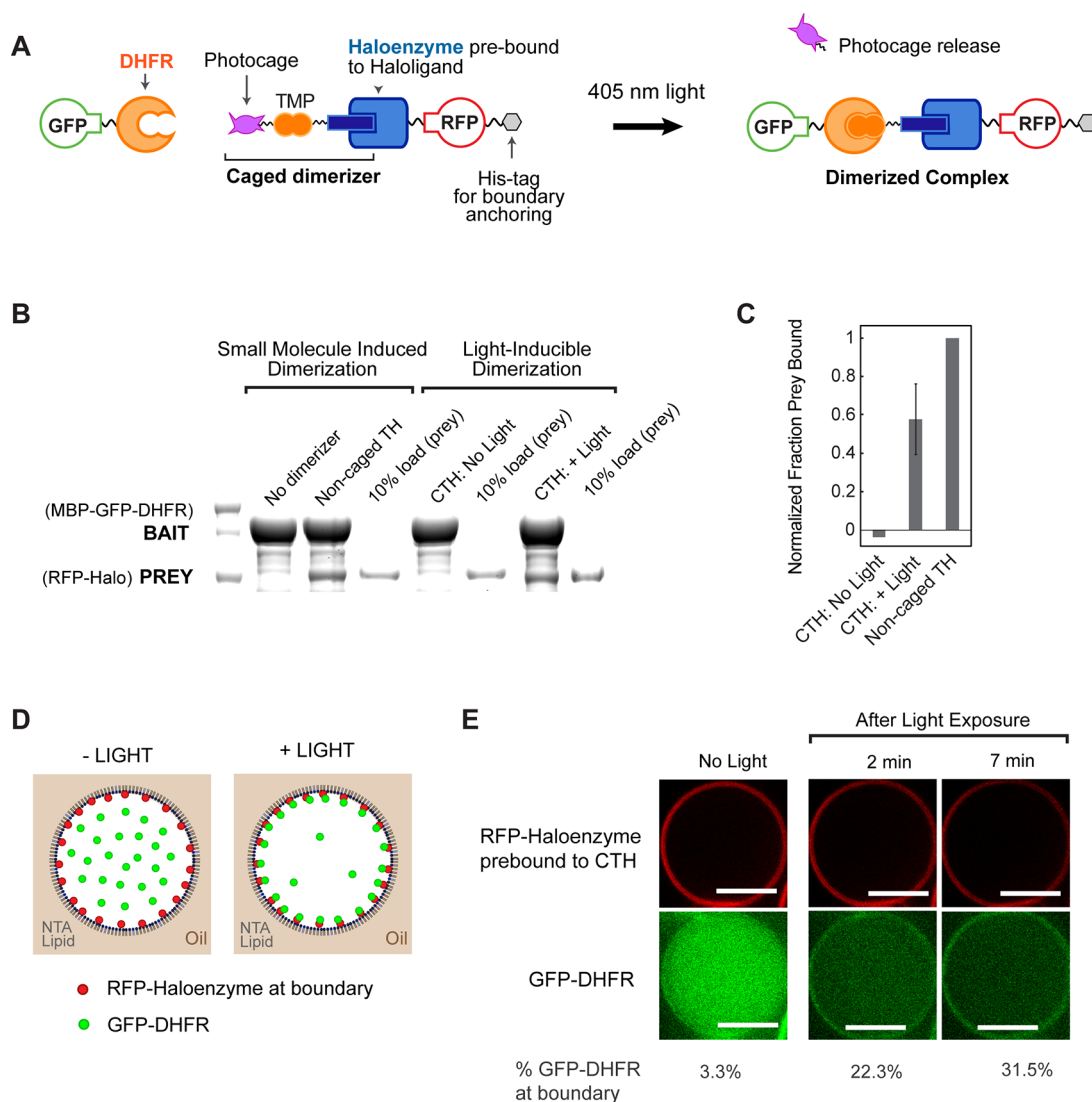
The complexity of the cellular milieu has motivated efforts to characterize biological processes in a minimal and carefully controlled context, such as through biochemical reconstitution of purified components.<sup>17</sup> Recently, it has become possible to perform more complex “cellular reconstitutions” to study chemical reactions in a cell-like context.<sup>18,19</sup> For example, cell-like compartments, such as liposomes and emulsions, have been used to encapsulate proteins and cytoplasm.<sup>20–22</sup> The geometries and picoliter volumes of these compartments mimic those of a natural cell. However, several limitations have hampered further development of these cell-like systems: only a handful of tools are available to pattern protein localization,<sup>23</sup> and the compartment boundary, whether a lipid monolayer or bilayer, is impermeable to hydrophilic molecules. The latter makes it challenging to chemically regulate enzymatic processes housed within these cell-like compartments. Light-inducible control of protein localization and activity promises to be a critical advance for cell biological studies in synthetic cell systems. Optical triggering is ideal because light can be readily controlled with temporal precision, localized irradiation can convey spatial control, and light easily penetrates into cell-like

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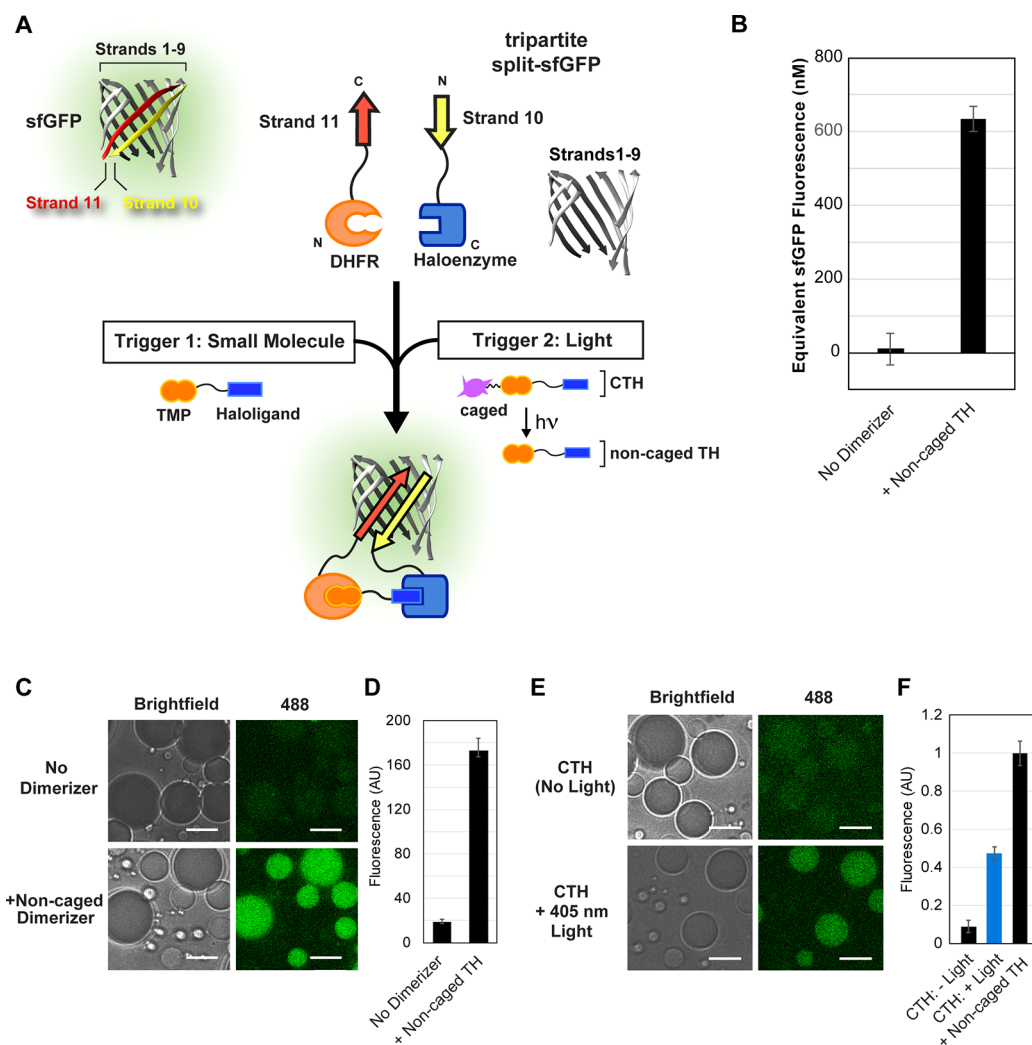
**Figure 1.** Optochemical control of protein spatial localization in cell-like compartments. (A) Schematic overview of the photocaged TMP-Halo-inducible dimerization system. (B) Haloenzyme and DHFR bind to one another in the presence of the noncaged TMP-Halo dimerizer, but not in absence of the dimerizer, in a pull-down assay. Photouncaging of caged TMP-Halo (CTH) leads to comparable binding to TH; there is undetectable binding in the dark. Caged and noncaged compounds were prebound to Haloenzyme protein. (C) Quantification of CTH and noncaged TH pull-down binding, normalized to the positive control, noncaged TH binding. The error bar shows the standard deviation from the mean ( $n = 3$ ). (D) Schematic of light-inducible protein recruitment to the boundary inside synthetic cell-like compartments: 5% DGS-NTA(Ni) lipid and 95% POPC in the decane phase and 1  $\mu$ M His10-RFP-Halo and 0.1  $\mu$ M GST-GFP-DHFR in the aqueous phase. (E) His10-RFP-Halo binds to the DGS-NTA(Ni) lipid in the droplet boundary. Recruitment of GST-GFP-DHFR to the boundary is triggered by 405 nm illumination, which uncages CTH. The scale bar is 10  $\mu$ m.

compartments, regardless of their permeability to small molecules.

By fusing split proteins to inducible dimerization domains, one can convert optochemical inputs into biological outputs. Split proteins, such as split GFP,<sup>24</sup> have been traditionally used to identify native interacting partners in protein complementation assays (PCA) and to characterize *in vivo* protein–protein interactions (PPI) and their inhibitors.<sup>25</sup> When fragments of split GFP are brought into the proximity of each other via interaction of binding partners, they reconstitute fluorescence.<sup>26</sup> Independent of their uses to identify constitutive binding interactions, split proteins have also been used to create triggerable switches. For example, a split version of tobacco etch virus (TEV) protease has been fused to FRB and FBKP domains to reconstitute TEV activity in the presence of

rapamycin.<sup>27,28</sup> Additionally, split enzymes have been optically reconstituted by illumination of caged compounds.<sup>14</sup>

Here we characterize the CTH photocaged dimerizer system as a tool for modulating protein localization and assembly in emulsion-based, synthetic cell-like compartments. We further test a second, complementary photochemical system, composed of a light-cleavable rapamycin dimer, dRap, paired with FRB and FKBP, as a strategy for transducing optical inputs into enzymatic function based on split protease reconstitution. We demonstrate the utility of this optochemically regulated protease through light-induced phase separation and formation of membraneless organelles within our synthetic cells.



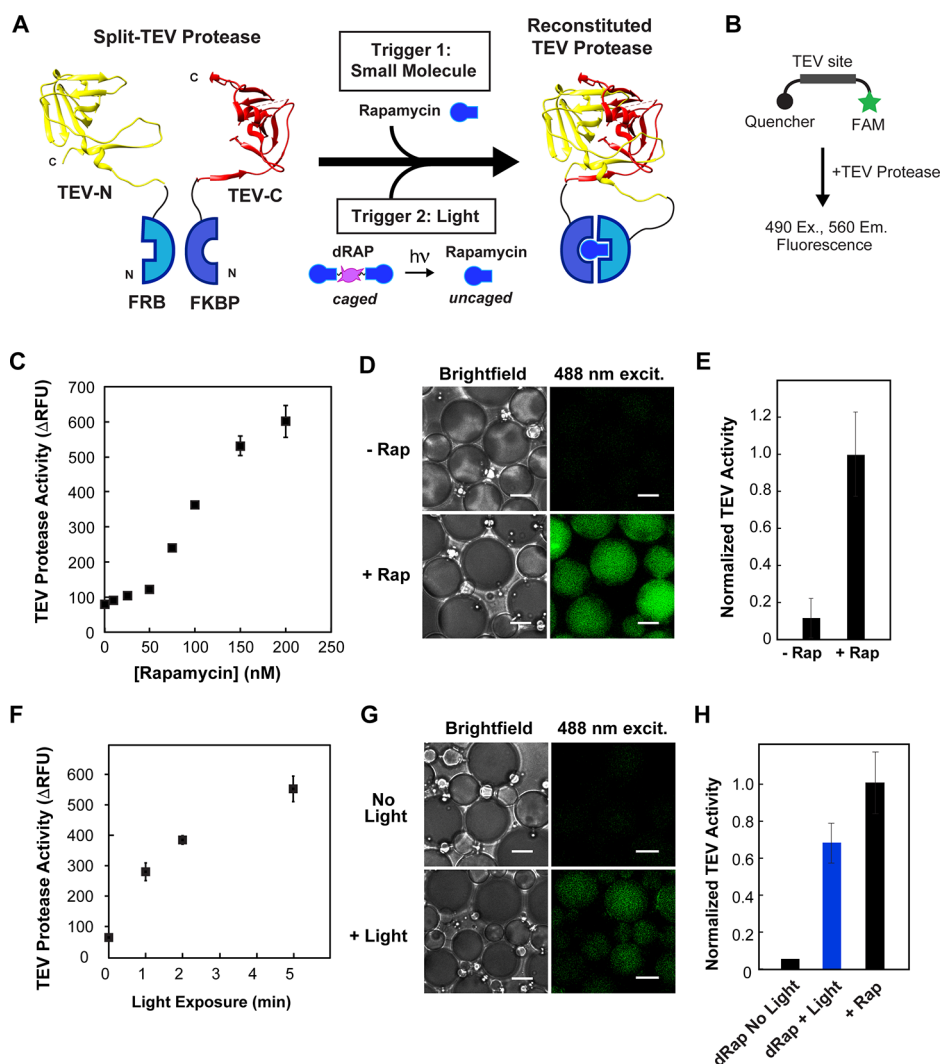
**Figure 2.** Inducible reconstitution of tripartite sfGFP assembly and activity. (A) Schematic of the tripartite sfGFP system and fusion to DHFR and Halo domains to enable chemical or optical control of sfGFP reconstitution. Noncaged and caged versions of the TMP-Halo compound are prebound to the sfGFP-strand10-Haloenzyme protein. (B) The noncaged dimerizer promotes reconstitution of sfGFP fluorescence in a plate reader assay. The increase in fluorescence over 12 h was interpolated to a dilution series of full sfGFP (Figure S3C), yielding concentrations of sfGFP reconstitution. (C and D) Small molecule-induced assembly of tripartite sfGFP inside cell-like compartments depends on the presence of a noncaged TH dimerizer. Fluorescence was quantified 18 h after addition of the dimerizer and encapsulation, to allow for sufficient chromophore maturation. (E and F) Light-induced reconstitution of sfGFP inside water-in-oil emulsions, normalized to the noncaged TH positive control. Approximately 50% uncaging was achieved using a 1 s exposure to 405 nm laser light. All experiments used 3  $\mu\text{M}$  DHFR-sfGFP-strand11, 3  $\mu\text{M}$  sfGFP-strand10-Haloenzyme, and 24  $\mu\text{M}$  sfGFP-strands1–9. The scale bar is 10  $\mu\text{m}$ .

## RESULTS

To engineer spatial control of protein positioning within synthetic cells, we tested whether proteins could be relocated from the lumen to the compartment boundary within a water-in-oil emulsion. The proteins included His10-RFP-Haloenzyme, whose His tag enables anchoring to DGS-NTA(Ni) lipid, and GFP-DHFR (Figure 1A and Figure S1A,B). The dimerizer, noncaged TH, and the caged dimerizer, CTH, were prebound to Haloenzyme through a covalent interaction with Haloligand. To determine whether the components were biochemically active, we tested whether Haloenzyme and DHFR could be chemically and optically dimerized *in vitro*. First, we conducted pull-down binding assays using MBP-GFP-DHFR immobilized on amylose beads as bait. We found that in the presence of noncaged TH, His10-RFP-Haloenzyme prey bound to MBP-GFP-DHFR (Figure 1B). In the absence of a dimerizer, an interaction could not be detected via the pull-

down assay (Figure 1B). Using the photocaged compound, we found undetectable interaction in the absence of light and significant binding after exposure to 405 nm light (Figure 1B,C). The level of prey binding was consistently much higher in the light than in the dark and within a factor of 2 of that observed in the positive control, noncaged TH dimerizer (Figure 1C and Figure S1C). These results demonstrated that the core components were functional and bind specifically to one another, only in the presence of a small molecule dimerizer or in response to photouncaging of the caged dimerizer.

To implement control of protein localization in synthetic cells, we tethered His10-RFP-Haloenzyme to DGS-NTA(Ni) lipid present in the lipid monolayer boundary and tested light-induced recruitment of GST-GFP-DHFR to the boundary. The components were recombinantly expressed and then encapsulated in water-in-oil emulsions with a lipid monolayer composed of 95% POPC and 5% DGS-NTA(Ni) lipid (Figure 1D). The DGS-NTA(Ni) lipid recruited His10-RFP-Halo-



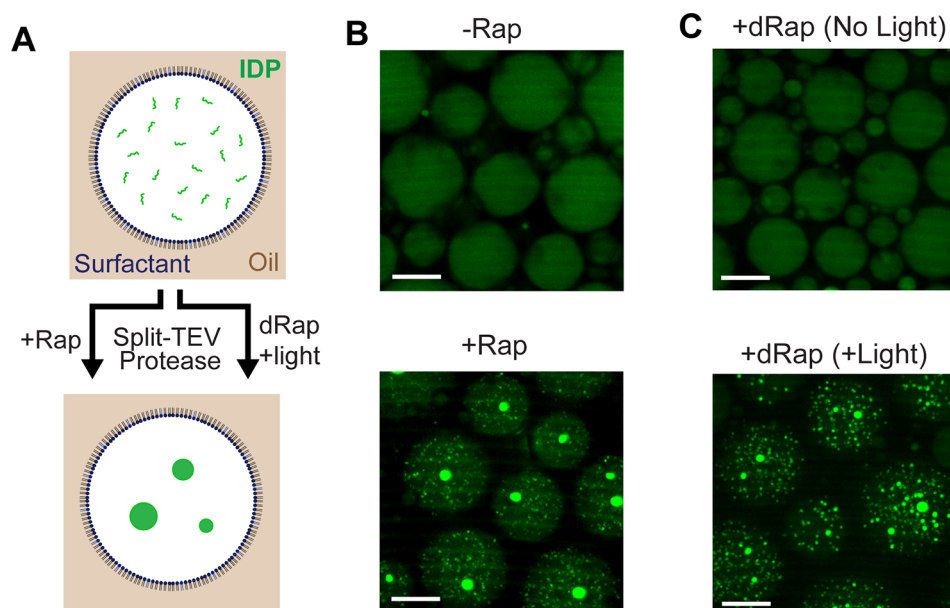
**Figure 3.** Activation of split TEV protease in cell-like compartments using small molecule and light-inducible dimerization. (A) Schematic of photocaged rapamycin, dRap, and split TEV fragments fused to FRB and FKBP. (B) Schematic of the TEV activity assay. Upon substrate cleavage, a FAM fluorophore is released from the quencher. (C) Dose dependence of rapamycin-mediated TEV reconstitution. The assay uses 125 nM split TEV proteins and varying concentrations of rapamycin. (D and E) Chemically induced reconstitution of TEV activity inside cell-like compartments. An equimolar concentration of rapamycin promotes TEV protease activity; there is low background activity in the absence of rapamycin. (F) Optical uncaging of dRap at various exposure times promotes TEV reconstitution in a plate reader assay (125 nM split TEV and 73 nM dRap). (G and H) Temporal triggering of TEV activation within cell-like compartments using light. A 10 min exposure to 365 nm ultraviolet light to uncage dRap within emulsions. Minimal background activity in non-illuminated samples. For panels D, E, G, and H, 500 nM split TEV proteins with equimolar rapamycin or equivalent dRap. For panels G and H, activity from a control without the dimerizer was subtracted from conditions with the dimerizer present. The scale bar is 5  $\mu$ m.

zyme bait to the boundary (Figure 1E). In the dark, only 3% of GST-GFP-DHFR prey is localized to the compartment boundary. After illumination using 405 nm light nearly one-third of the protein relocates to the compartment boundary, via light-inducible dimerization to the tethered bait (Figure 1E and Figure S2). These results demonstrate successful light-inducible protein localization in water-in-oil emulsions for patterning cell-like organization, such as cortical protein recruitment, with temporal control over this spatial organization.

Next, we wanted to test whether a small molecule or light stimulus could be used for temporal control of protein assembly and reconstitution within synthetic cells. With this goal in mind, we used a tripartite split superfolder GFP (sfGFP) (Figure 2A), which fluoresces only when strands 10 and 11 are dimerized in the presence of strands 1–9. This

variant of split GFP was developed to have a reduced level of autoreconstitution<sup>29</sup> compared to an older bipartite split sfGFP.<sup>30</sup> We fused sfGFP-strand10 to Haloenzyme and DHFR to sfGFP-strand11 and generated GST-sfGFP-strands1–9 (Figure S3A,B). Using these components, we tested whether assembly of the trimeric complex and ultimate generation sfGFP fluorescence could be triggered by the addition of noncaged TH or by uncaging CTH with 405 nm light. First, we tested whether we could stimulate tripartite sfGFP assembly and fluorescence in a bulk solution using the noncaged TH dimerizer. We found that noncaged TH led to significant reconstitution of sfGFP fluorescence, comparable to 21% of the intact sfGFP control (Figure S3C). Only minimal autoreconstitution in the absence of dimerizers was observed, yielding a >65-fold increase in the fluorescence of the tripartite split sfGFP in the presence of the dimerizer (Figure 2B).





**Figure 4.** Triggering formation of membraneless organelles in cell-like compartments using small molecule and light-inducible protease activity. (A) Schematic of fluorescently tagged IDP formation of membraneless organelles. The MBP solubilization domain is cleaved from the IDP, resulting in phase separation and formation of membraneless organelles. (B) Rapamycin-dependent formation of protein droplets within emulsions. In absence of rapamycin, IDP remains soluble and well mixed. Split TEV ( $1 \mu\text{M}$ ) with or without the equimolar dimerizer and  $30 \mu\text{M}$  IDP, in the presence of 25% *Xenopus* egg extract. (C) Light-induced TEV activation and formation of membraneless organelles in cell-like compartments. Compartments encapsulated with  $1 \mu\text{M}$  split TEV,  $500 \text{ nM}$  dRap,  $30 \mu\text{M}$  IDP, and 25% egg extract, either kept in dark or exposed to 365 nm UV light for 10 min. For panels B and C, emulsions were imaged 12 h post-induction. The scale bar is  $20 \mu\text{m}$ .

We further wanted to test whether such a strategy for inducing protein reconstitution would work inside cell-size compartments, so we encapsulated the reaction mixtures in water-in-oil emulsions. Because we were not interested in recruitment of protein to the compartment boundary, we selected a passivating surfactant, Cithrol DPHS, to prevent nonspecific interactions. We found that water-in-oil emulsions containing the three proteins and noncaged TH displayed an sfGFP fluorescence 9-fold higher than the fluorescence of those that lacked the dimerizer (Figure 2C,D). This result demonstrated that small molecule-induced split protein reconstitution was feasible within cell-like compartments. However, these experiments are limited because the dimerizer must be added prior to encapsulation; the boundary and continuous oil layer prevent noncaged TH from diffusing from the exterior environment into the lumen of the compartment. Therefore, we decided to test light as a trigger, because photons are able to transduce the compartment.

To determine whether an optical trigger was capable of reconstituting tripartite sfGFP in synthetic cells, we loaded sfGFP-strand10-Haloenzyme with CTH and encapsulated it along with DHFR-sfGFP-strand11 and sfGFP-strands1–9 in water-in-oil emulsions. In the absence of light, minimal fluorescence was observed. However, upon stimulation with light, we observed sfGFP reconstitution consistent with approximately half the fluorescence signal in the noncaged TH control sample (Figure 2E,F). This outcome demonstrates that we can achieve robust light-triggered protein assembly within our cell-like compartments using the CTH system.

Following our initial demonstration of light-inducible protein assembly, we wanted to expand our optochemical toolkit and add triggerable enzymatic activity. We decided to use a split version of TEV protease, a member of the cysteine family of proteases that recognizes and efficiently cleaves the ENLYFQG

recognition site.<sup>31</sup> N- and C-terminal domains of TEV were fused to FRB and FKBP (Figure S4A,B), respectively, to enable small molecule-based dimerization and activation of split TEV (Figure 3A), as shown previously.<sup>27</sup> Because split TEV had not been previously characterized outside cells, we were unsure whether the proteins would be functional and if the protease activity could be successfully triggered by rapamycin. Using an *in vitro* fluorimetric TEV protease assay (Figure 3B and Figure S4C), we tested the activity of a mixture of FRB-TEV-N and FKBP-TEV-C with varying concentrations of the dimerizer (Figure 3C). We observed TEV protease activity that was dependent on rapamycin dosage, with little background activity in its absence. Next, we tested whether we could introduce a similar strategy to chemically induce TEV protease activity in cell-size compartments. We found that water-in-oil emulsions containing split TEV constructs in the presence of rapamycin displayed activity that was 9-fold higher than that of emulsions without rapamycin (Figure 3D,E). To the best of our knowledge, these studies represent the first demonstration of a small molecule-activated protease in a cell-free system.

We also set out to implement a light-inducible version of TEV, because chemical triggers cannot permeate the emulsion compartment. As our optochemical tool, we selected a light-cleavable rapamycin dimer (dRap), which, upon illumination with 365 nm light, uncages and dimerizes FRB and FKBP proteins. The light-cleavable rapamycin dimer binds two FKBP proteins in the dark state and allows FRB to bind each FKBP:rapamycin complex only after irradiation to cleave the dRap linker.<sup>14</sup> Using the FRB-TEV-N and FKBP-TEV-C proteins described above, we added dRap and illuminated the samples for varying periods of time. TEV protease activity displayed a dose dependence to light exposure, with minimal signal in the absence of light (Figure 3F).

Next, we tested this strategy for light-induced TEV protease activation in cell-like compartments. FRB-TEV-N, FKBP-TEV-C, and dRap were encapsulated inside cell-size water-in-oil emulsions. Following illumination with UV light, TEV protease activity achieved a level corresponding to approximately 70% of the activity in the positive control containing rapamycin (Figure 3G,H). Minimal activity was observed in samples that were not exposed to light, illustrating that enzymatic activity can be optically triggered within a synthetic cell system.

To demonstrate that our optochemical protease platform could be used to interrogate cell biological phenomena, and in the presence of cell cytoplasm, we chose to test the formation of membraneless organelles from an intrinsically disordered protein (IDP) inside cell-like compartments (Figure 4A). We used a domain containing RGG repeats, which self-assembles and phase separates *in vitro*.<sup>32</sup> IDP domains, such as this one, demix from solution under physiological conditions but can be made soluble by tagging with a large globular domain.<sup>33</sup> We fluorescently tagged the IDP and tested whether phase separation and formation of organelles could be triggered by TEV-mediated removal of an MBP solubilization tag (Figure 5SA,B). In cell-like compartments, the IDP readily phase separates into protein droplets when encapsulated along with split TEV and rapamycin but remains well-mixed in the absence of rapamycin (Figure 4B). Next we tested whether formation of membraneless organelles could be triggered using light by encapsulating the components along with dRap. In the absence of exposure to light, cell-like compartments displayed no protein droplets. However, via exposure to 365 nm light, membraneless organelles were formed inside these compartments (Figure 4C). This finding demonstrates the successful use of our optochemical system for inducible control of cell biological processes such as membraneless organelle formation.

In summary, our study demonstrates the utility of optochemical approaches for inducing protein dimerization and identifies a robust set of tools for conditional control of protein localization and activity in a minimal cell system. Triggering protein patterning and function using light is particularly critical to the study of biochemical pathways in water-in-oil emulsions because these compartments, once formed, are impermeable to small molecules. Although these proteins were validated in water-in-oil emulsions, they will retain functionality within liposomes or polymersomes and thus represent a robust toolkit for studies within a broad variety of synthetic cell systems. By demonstrating optical triggering of protein-based membraneless organelle assembly, our work paves the way toward spatial and temporal perturbations of additional cell biological processes inside cell-like compartments.

## ■ ASSOCIATED CONTENT

### 📄 Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.8b00131.

Supplemental methods, Figures S1–S5, and Table S1 (PDF)

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### Notes

The authors declare no competing financial interest.

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