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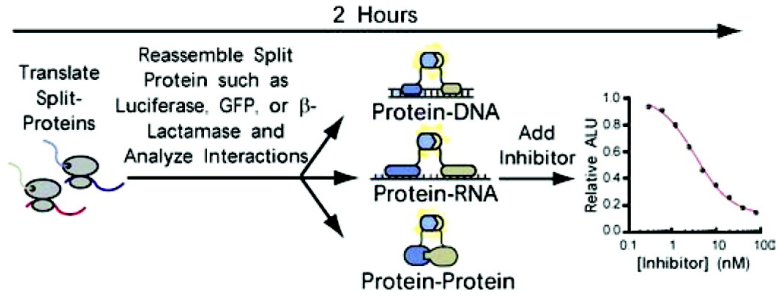

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A General and Rapid Cell-Free Approach for the Interrogation of Protein−Protein, Protein−DNA, and Protein−RNA Interactions and their Antagonists Utilizing Split-Protein Reporters

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Abstract: Split-protein reporters have emerged as a powerful methodology for imaging biomolecular interactions which are of much interest as targets for chemical intervention. Herein we describe a systematic evaluation of split-proteins, specifically the green fluorescent protein, β-lactamase, and several luciferases, for their ability to function as reporters in completely cell-free systems to allow for the extremely rapid and sensitive determination of a wide range of biomolecular interactions without the requirement for laborious transfection, cell culture, or protein purification (12−48 h). We demonstrate that the cell-free split-luciferase system in particular is amenable for directly interrogating protein−protein, protein−DNA, and protein−RNA interactions in homogeneous assays with very high sensitivity (22−1800 fold) starting from the corresponding mRNA or DNA. Importantly, we show that the cell-free system allows for the rapid (2 h) identification of target-site specificity for protein−nucleic acid interactions and in evaluating antagonists of protein−protein and protein−peptide complexes circumventing protein purification bottlenecks. Moreover, we show that the cell-free split-protein system is adaptable for analysis of both protein−protein and protein−nucleic acid interactions in artificial cell systems comprising water-in-oil emulsions. Thus, this study provides a general and enabling methodology for the rapid interrogation of a wide variety of biomolecular interactions and their antagonists without the limitations imposed by current *in vitro* and *in vivo* approaches.

Introduction

Protein−protein[1] and protein−nucleic acid[2] interactions are central to cellular function and are also emerging targets for pharmacological intervention when implicated in a particular disease pathway. Thus numerous *in vitro* and *in vivo* methods have been developed to target[3–7] and study these biomolecular interactions. Widely utilized *in vitro* methods for interrogating protein−protein and protein−DNA interactions and their antagonists include variations of enzyme linked immunosorbent assays (ELISAs), surface plasmon resonance (SPR), and fluorescence polarization (FP), which either require the use of antibodies or purified proteins and often require chemical derivatization. On the other hand powerful *in vivo* methods such as yeast two-hybrid[8] assays have the advantage of speed by eliminating the need for protein purification but can be subject to false positives and negatives due to the multifactorial nature of signal generation.[9] In between these two extremes lie protein fragment based methods, where a specific biomolecular interaction drives the reassembly of a previously split reporter protein[10] (Figure 1).

The reconstitution of a functional protein from split-peptide fragments was first demonstrated for ribonuclease in 1959.[11] Since then "split-protein reassembly" or "protein complementation" has been applied to the *in vivo* detection of a wide variety of protein−protein interactions utilizing numerous split-proteins including ubiquitin,[12] β-galactosidase,[13] dihydrofolate reductase,[14] β-lactamase,[15] GFP,[16] GFP-variants and analogues,[17–19] firefly luciferase,[20] and *Gaussia* luciferase.[21] Recently, we and others have also described methods for detecting nucleic acids and their chemical modification by the reassembly of ternary complexes of split-GFP and split-β-lactamase tethered to nucleic acid binding proteins.[22–27] Thus split-protein systems or "protein complementation assays" (PCAs) can directly image most

(1) Wells, J. A.; McClendon, C. L. *Nature* **2007**, *450*, 1001–1009.
(2) Hurley, L. H. *Nat. Rev. Cancer* **2002**, *2*, 188–200.
(3) Braisted, A. C.; Oslob, J. D.; Delano, W. L.; Hyde, J.; McDowell, R. S.; Waal, N.; Yu, C.; Arkin, M. R.; Raimundo, B. C. *J. Am. Chem. Soc.* **2003**, *125*, 3714–3715.
(4) Chin, J. W.; Schepartz, A. *Angew. Chem., Int. Ed.* **2001**, *40*, 3806–3809.
(5) Ding, K.; Lu, Y.; Nikolovska-Coleska, Z.; Qiu, S.; Ding, Y. S.; Gao, W.; Stuckey, J.; Krajewski, K.; Roller, P. P.; Tomita, Y.; Parrish, D. A.; Deschamps, J. R.; Wang, S. M. *J. Am. Chem. Soc.* **2005**, *127*, 10130–10131.
(6) Orner, B. P.; Ernst, J. T.; Hamilton, A. D. *J. Am. Chem. Soc.* **2001**, *123*, 5382–5383.
(7) Rajagopal, S.; Meyer, S. C.; Goldman, A.; Zhou, M.; Ghosh, I. *J. Am. Chem. Soc.* **2006**, *128*, 14356–14363.
(8) Fields, S.; Song, O. *Nature* **1989**, *340*, 245–246.
(9) Fields, S. *FEBS J.* **2005**, *272*, 5391–5399.
(10) Michnick, S. W.; Ear, P. H.; Manderson, E. N.; Remy, I.; Stefan, E. *Nat. Rev. Drug Discovery* **2007**, *6*, 569–582.
(11) Richards, F. M.; Vithayathil, P. J. *J. Biol. Chem.* **1959**, *234*, 1459–1465.
(12) Johnsson, N.; Varshavsky, A. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 10340–10344.
(13) Rossi, F.; Charlton, C. A.; Blau, H. M. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 8405–8410.
(14) Pelletier, J. N.; Campbell-Valois, F. X.; Michnick, S. W. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 12141–12146.

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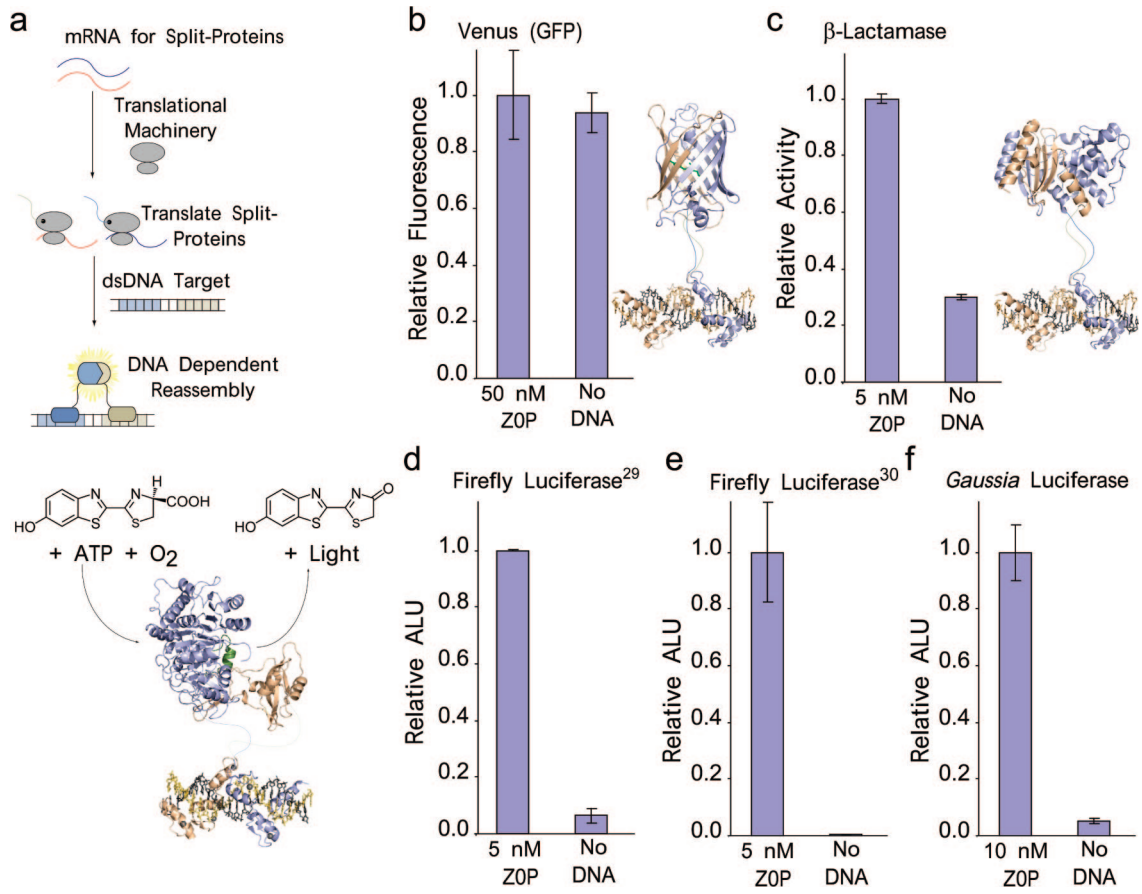


Figure 1. Cell-free detection utilizing split-proteins. (a) Cartoon representation of a split-protein system with zinc fingers tethered to split-proteins in the presence of a target dsDNA oligonucleotide. Different split-protein reporters tethered to sequence specific zinc fingers in the presence and absence of target dsDNA; (b) split-Venus (a GFP variant), (c) split-β-lactamase, (d) split-firefly luciferase as described by Luker *et. al*,[29] (e) split-firefly luciferase as described by Paulmurugan *et. al*,[30] and (f) split-*Gaussia* luciferase as described by Remy *et. al*.[21]

biomolecular interactions. Although the current methods are of great utility, all of the current split-protein methods have certain limitations for interrogating protein−protein and protein−nucleic acid interactions and their inhibitors in a very rapid and high-throughput fashion. For example, current *in vitro* methods require extensive protein purification[16,27] and also rely on proper folding of recombinant proteins, while *in vivo* methods require lengthy transfection and propagation of cellular cultures prior to analysis, both approaches being time intensive.[25,28] The current methods are also prone to problems arising from potential proteolysis of intracellularly expressed proteins and peptides as well as a lack of control over interfering coexpressed cellular factors as is also the case with yeast n-hybrid methods.

To provide a rapid and general method that circumvents many of the limitations discussed above, we hypothesized that fragmented reporter proteins fused to functional proteins could be rapidly generated directly from mRNA utilizing cell-free translation methodologies and immediately interrogated for biomolecular interaction-dependent signal generation. The use of split-proteins in cell-free translation takes advantage of fast protein synthesis rates, from 60 to 90 min, and easy adaptation to homogeneous assays avoiding immobilization and washing protocols. Herein we demonstrate how this cell-free approach provides a general platform for rapidly detecting protein−protein, protein−small molecule, protein−DNA, protein-methylated DNA, and protein−RNA interactions starting from mRNA or directly from DNA corresponding to the desired interaction pair in less than 2 h. Moreover, we demonstrate how this approach aids in determining the specificity of protein−nucleic acid

(15) Galarneau, A.; Primeau, M.; Trudeau, L. E.; Michnick, S. W. *Nat. Biotechnol.* **2002**, *20*, 619–622.
(16) Ghosh, I.; Hamilton, A. D.; Regan, L. *J. Am. Chem. Soc.* **2000**, *122*, 5658–5659.
(17) MacDonald, M. L.; Lamerdin, J.; Owens, S.; Keon, B. H.; Bilter, G. K.; Shang, Z.; Huang, Z.; Yu, H.; Dias, J.; Minami, T.; Michnick, S. W.; Westwick, J. K. *Nat. Chem. Biol.* **2006**, *2*, 329–337.
(18) Hu, C. D.; Kerppola, T. K. *Nat. Biotechnol.* **2003**, *21*, 539–45.
(19) Jach, G.; Pesch, M.; Richter, K.; Frings, S.; Uhrig, J. F. *Nat. Methods* **2006**, *3*, 597–600.
(20) Paulmurugan, R.; Umezawa, Y.; Gambhir, S. S. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 15608–15613.
(21) Remy, I.; Michnick, S. W. *Nat. Methods* **2006**, *3*, 977–979.
(22) Ghosh, I.; Stains, C. I.; Ooi, A. T.; Segal, D. J. *Mol. BioSyst.* **2006**, *2*, 551–560.
(23) Stains, C. I.; Porter, J. R.; Ooi, A. T.; Segal, D. J.; Ghosh, I. *J. Am. Chem. Soc.* **2005**, *127*, 10782–10783.
(24) Porter, J. R.; Stains, C. I.; Segal, D. J.; Ghosh, I. *Anal. Chem.* **2007**, *79*, 6702–6708.
(25) Ozawa, T.; Natori, Y.; Sato, M.; Umezawa, Y. *Nat. Methods* **2007**, *4*, 413–419.
(26) Rackham, O.; Brown, C. M. *EMBO J.* **2004**, *23*, 3346–3355.
(27) Ooi, A. T.; Stains, C. I.; Ghosh, I.; Segal, D. J. *Biochemistry* **2006**, *45*, 3620–3625.
(28) Remy, I.; Campbell-Valois, F. X.; Michnick, S. W. *Nat. Protoc.* **2007**, *2*, 2120–2125.
(29) Luker, K. E.; Smith, M. C. P.; Luker, G. D.; Gammon, S. T.; Piwnica-Worms, H.; Piwnica-Worms, D. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 12288–12293.
(30) Paulmurugan, R.; Gambhir, S. S. *Anal. Chem.* **2007**, *79*, 2346–2353.

interactions as well as in determining small-molecule antagonists of protein−protein interactions, which are currently of much interest.

Results and Discussion

Evaluation of Split-Protein Reporters for the Cell-Free Interrogation of Biomolecular Interactions. Initially, we evaluated the ability of our previously reported split-GFP[23] and split-β-lactamase[24,27] systems appended to specific zinc fingers to reassemble in the presence of target DNA utilizing *in vitro* transcribed mRNA in a purified wheat germ extract translation system (Figure 1b,c). Signal from the DNA-dependent reassembled GFP[23] was too low to observe over background using standard fluorescence measurements, while DNA-dependent β-lactamase activity[27] yielded measurable but low signal-to-background ratios. Thus, we turned to recently reported *in vivo* split-luciferase systems which have the significant advantage of negligible background from the translation system due to the generation of a bioluminescent signal (Figure 1a). We chose to first examine the fragmented firefly luciferase (Fluc) reported by Luker *et. al*[29] which, when appended to our zinc fingers, showed significant signal over background luminescence upon addition of target DNA (Figure 1d). This comprises the first demonstration of the bioluminescent read-out of a specific nucleic acid sequence, and accordingly this split-luciferase system was chosen for further studies in cell-free systems. The very recently described split-*Gaussia* luciferase[21] and alternatively split-firefly luciferase complementation systems[29,30] were also tethered to our zinc fingers and displayed extremely good signal over background bioluminescence (Figure 1e,f) in a DNA-dependent fashion and are currently being evaluated for their relative merits in a number of cell-free assays.

Detection of Protein−Protein and Protein−Nucleic Acid Interactions. To test the generality of the cell-free split-luciferase approach we chose to investigate seven well-characterized and widely studied biomolecular interactions (Figure 2): (a) the catalytic subunit of cAMP-dependent protein kinase (PKA) with its inhibitor PKI (PKA/PKI)[31−33] and (b) the rapamycin-dependent interaction between the human FK506-binding protein 12 (FKBP) and the FKBP12-rapamycin binding (FRB) domain of human mTOR (FKBP/FRB).[34−36] Akin to the yeast three hybrid systems,[37] we also investigated the ternary association of (c) two sequence-specific zinc fingers[38] with a target DNA (Zif268/PBSII), (d) a zinc finger and methyl CpG-binding domain with a target CpG-methylated DNA (Zif268/MBD2),[39−42] and finally (e) two RNA-specific pumilio domains[43] with a target RNA (Pum1/Pum2). Additionally the widely utilized coiled-coil domains of the transcription factors Fos and Jun (Fos/Jun)[44−46] and the interaction between hypoxia inducible factor-1α (HIF-1α) and the CH1 domain of the transcriptional coactivator p300 (HIF-1α/p300)[47,48] were also tested (Supporting Information, Figure S1). The overall sensitivity (signal/background) of these systems (Figure 2a−e) was excellent and varied from 22 to 1800-fold, while the total assay time from translation to analysis was less than 2 h.

We also investigated whether it was possible to directly couple transcription and translation in a cell-free lysate system, which may eventually eliminate the need for the separate *in vitro* transcription step that we are currently employing. (Figure 3). These experiments were likewise successful for both DNA and small-molecule dependent interactions (Figure 3a,b). In addition to using purified lysate, we further interrogated whether we could detect the above interactions using a system composed entirely of purified translational components[49,50] (Figure 3c,e), the so-called "PURE System," thus demonstrating that reconstituted transcription and translation machinery is sufficient for detecting biomolecular interactions. This set of experiments clearly validates that a cell-free split-luciferase assay format allows for the rapid, sensitive, and direct detection of protein−protein, protein−small molecule, protein−DNA, protein-methylated DNA, and protein−RNA interactions starting from either mRNA or directly from DNA corresponding to the desired interaction pair. Having established that our methodology provides robust signal for a wide variety of biomolecular interactions we turned to investigating whether this system is amenable to reporting upon inhibitors of protein−nucleic acid and protein−protein interactions.

Detecting Antagonists of Protein-Nucleic Acid Interactions. In order to detect antagonists of protein−protein or protein−nucleic acid interactions, we first needed to demonstrate the thermodynamic reversibility of the ternary complex consisting of reassembled firefly luciferase fragments tethered to two zinc fingers (PBSII and Zif268) and target DNA (Figure 4a). Toward this goal, translations using mRNA encoding PBSII-NFluc and CFluc-Zif268 were initiated in the presence of the target oligonucleotide (Zif268-0-PBSII). Post DNA dependent firefly luciferase reassembly, a hairpin DNA (hpDNA-Zif268), which is a competitor for only Zif268 binding, was added at increasing concentrations followed by equilibration for 30 min. A concentration dependent decrease in luminescence was observed, clearly demonstrating that the ternary complex of firefly luciferase and dsDNA was reversible and could be inhibited by addition of the dominant-negative (hpDNA-Zif268) oligonucleotide (Figure 4b, TGG containing hpDNA). The generality

(31) Knighton, D. R.; Zheng, J. H.; Ten Eyck, L. F.; Xuong, N. H.; Taylor, S. S.; Sowadski, J. M. *Science* **1991**, *253*, 414–420.
(32) Narayana, N.; Cox, S.; Shaltiel, S.; Taylor, S. S.; Xuong, N. H. *Biochemistry* **1997**, *36*, 4438–4448.
(33) Taylor, S. S.; Buechler, J. A.; Yonemoto, W. *Annu. Rev. Biochem.* **1990**, *59*, 971–1005.
(34) Chen, J.; Zheng, X. F.; Brown, E. J.; Schreiber, S. L. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 4947–4951.
(35) Brown, E. J.; Albers, M. W.; Shin, T. B.; Ichikawa, K.; Keith, C. T.; Lane, W. S.; Schreiber, S. L. *Nature* **1994**, *369*, 756–758.
(36) Vanduyne, G. D.; Standaert, R. F.; Schreiber, S. L.; Clardy, J. *J. Am. Chem. Soc.* **1991**, *113*, 7433–7434.
(37) Vidal, M.; Legrain, P. *Nucleic Acids Res.* **1999**, *27*, 919–929.
(38) Wolfe, S. A.; Nekludova, L.; Pabo, C. O. *Annu. Rev. Biophys. Biomol. Struct.* **2000**, *29*, 183–212.
(39) Stains, C. I.; Furman, J. L.; Segal, D. J.; Ghosh, I. *J. Am. Chem. Soc.* **2006**, *128*, 9761–9765.
(40) Esteller, M.; Corn, P. G.; Baylin, S. B.; Herman, J. G. *Cancer Res.* **2001**, *61*, 3225–3229.
(41) Fraga, M. F.; Ballestar, E.; Montoya, G.; Taysavang, P.; Wade, P. A.; Esteller, M. *Nucleic Acids Res.* **2003**, *31*, 1765–1774.
(42) Ohki, I.; Shimotake, N.; Fujita, N.; Jee, J. G.; Ikegami, T.; Nakao, M.; Shirakawa, M. *Cell* **2001**, *105*, 487–497.
(43) Cheong, C. G.; Hall, T. M. *Proc. Natl. Acad. Sci. U S A* **2006**, *103*, 13635–13639.
(44) O'Shea, E. K.; Rutkowski, R.; Kim, P. S. *Cell* **1992**, *68*, 699–708.
(45) O'Shea, E. K.; Rutkowski, R.; Stafford, W. F.; Kim, P. S. *Science* **1989**, *245*, 646–648.
(46) Meyer, S. C.; Shomin, C. D.; Gaj, T.; Ghosh, I. *J. Am. Chem. Soc.* **2007**, *129*, 13812–13813.
(47) Freedman, S. J.; Sun, Z. Y.; Poy, F.; Kung, A. L.; Livingston, D. M.; Wagner, G.; Eck, M. J. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 5367–5372.
(48) Kung, A. L. et al. *Cancer Cell* **2004**, *6*, 33–43.
(49) Shimizu, Y.; Inoue, A.; Tomari, Y.; Suzuki, T.; Yokogawa, T.; Nishikawa, K.; Ueda, T. *Nat. Biotechnol.* **2001**, *19*, 751–755.
(50) Shimizu, Y.; Kanamori, T.; Ueda, T. *Methods* **2005**, *36*, 299–304.

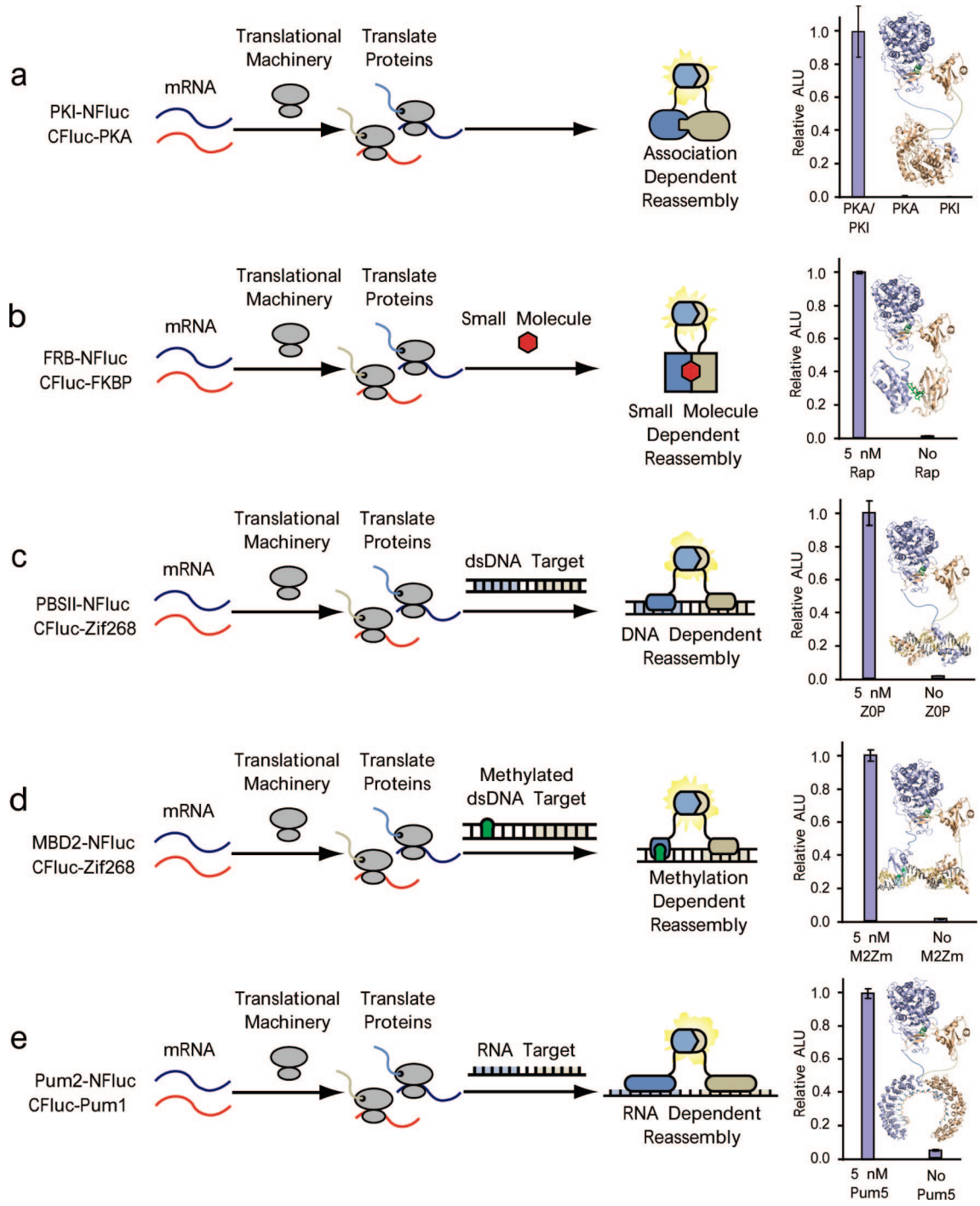


Figure 2. Cell-free detection of a wide variety of biomolecular interactions utilizing split-firefly luciferase starting from mRNA. Detection of (a) the protein−protein interaction between PKI-NFluc and CFluc-PKA; (b) the rapamycin-induced interaction between FRB-NFluc and CFluc-FKBP; (c) the protein−DNA interaction between PBSII-NFluc, CFluc-Zif268, and a target dsDNA oligonucleotide; (d) the methylation-dependent protein−DNA interaction between MBD2-NFluc, CFluc-Zif268, and a target methylated CpG dsDNA oligonucleotide; and (e) the protein−RNA interaction between Pum2-NFluc, CFluc-Pum1, and a target RNA oligonucleotide.

of utilizing the cell-free system for probing protein−nucleic acid inhibition was further demonstrated with translations containing mRNA encoding Pum2-NFluc and CFluc-Pum1 in the presence of target RNA. As earlier, a concentration-dependent decrease in luminescence was observed only upon the addition of increasing amounts of a competitor half-site RNA target that is known to selectively bind one of the pumilio domains[43] (Figure 4e). Building on these results, we envisioned that our split-luciferase-based cell-free system could be readily utilized to analyze the relative target site specificity of nucleic acid binding proteins through competitive binding experiments.

Identification of Protein−DNA Target-Site Specificity. A number of methods have been developed to interrogate the relative affinity of DNA-binding proteins for their target site, including traditional EMSAs and more recently DNA microarrays.[51,52] Although powerful, these techniques require the use of purified components, specialized equipment, or radioactive materials. Having established that ternary zinc finger−DNA

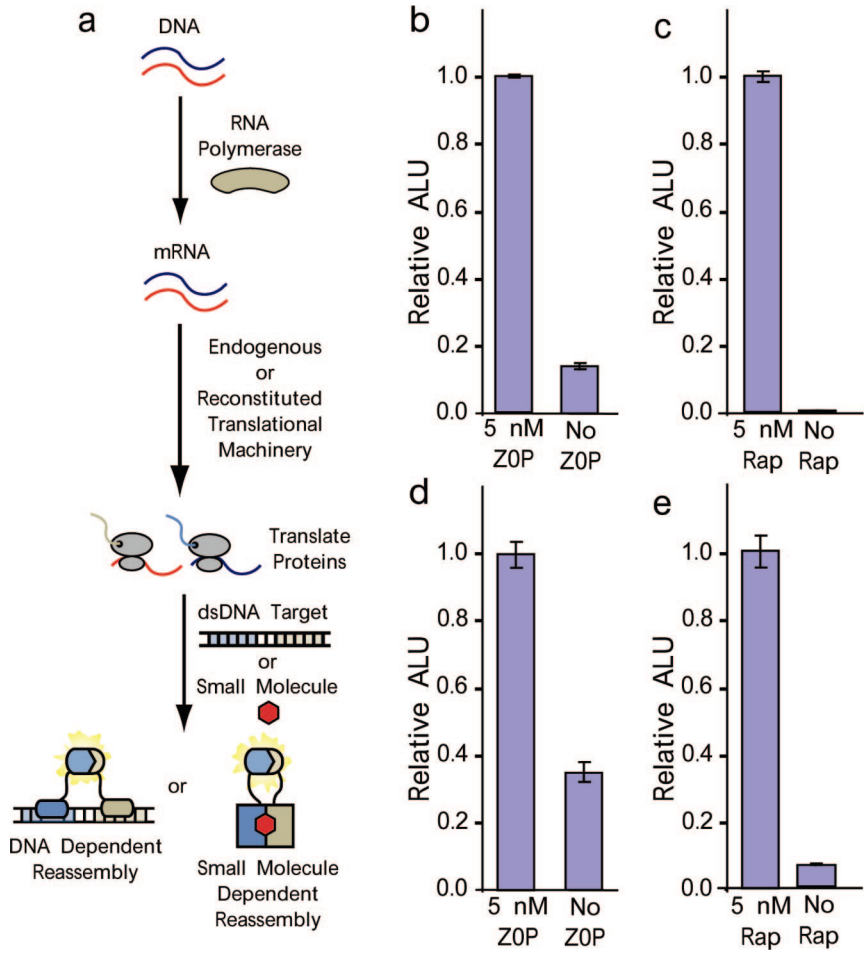


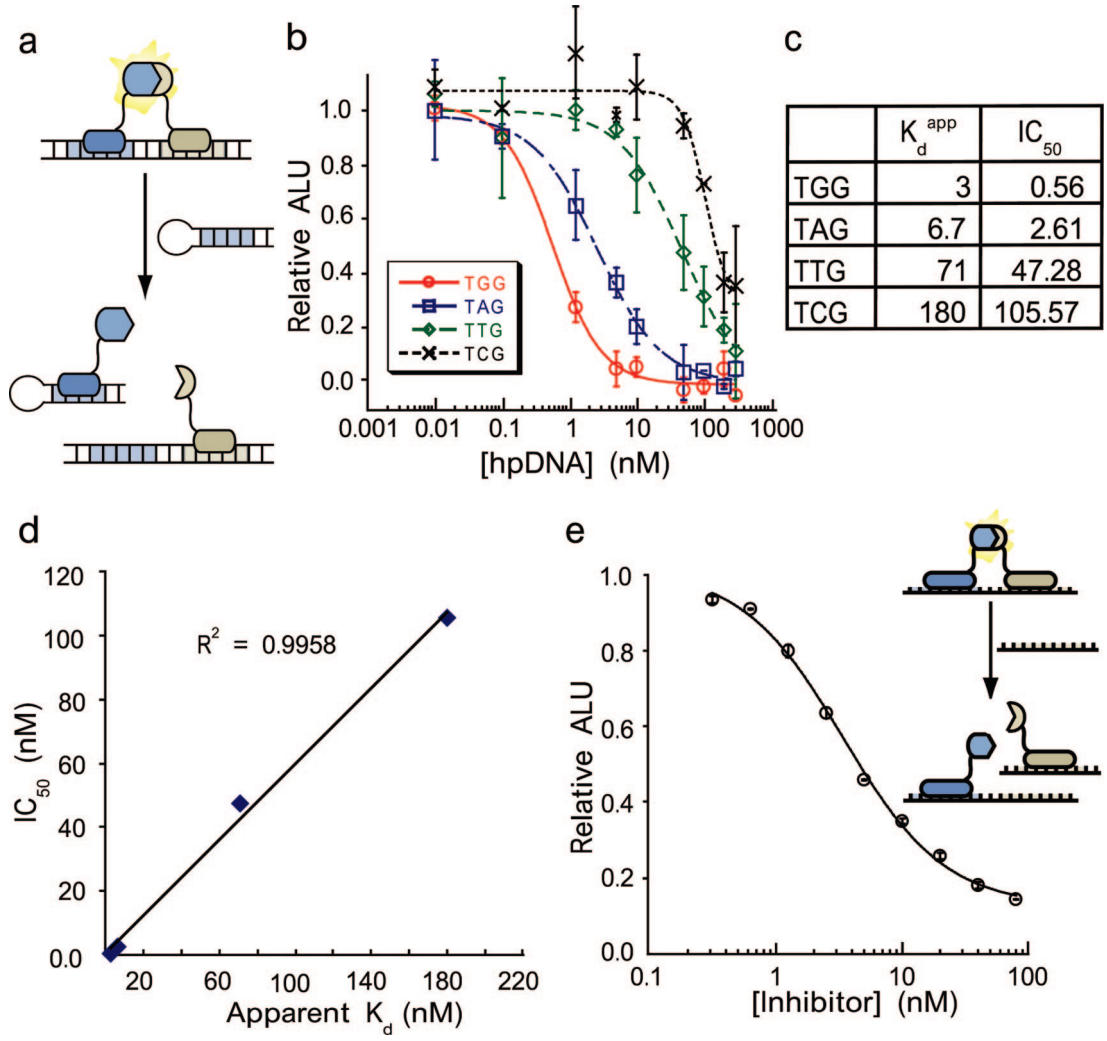
Figure 3. Cell-free detection of biomolecular interactions with split-luciferase starting from DNA utilizing a coupled transcription and translation cell-free lysate system: (a) the protein−DNA interaction between PBSII-NFluc, CFluc-Zif268, and 5 nM target dsDNA oligonucleotides and (b) the rapamycin-induced (5 nM) interaction between FRB-NFluc and CFluc-FKBP. Detection of biomolecular interactions utilizing purified DNA in the PURESYSTEM classic II system consisting of completely purified transcriptional and translational components: (c) the protein−DNA interaction between PBSII-NFluc, CFluc-Zif268, and 5nM target dsDNA oligonucleotide and (d) the rapamycin-induced (5 nM) interaction between FRB-NFluc and CFluc-FKBP.

complexes can be disrupted by a competitor oligonucleotide added in trans, we next attempted to correlate the known binding affinities of Zif268 for single-nucleotide changes in its binding site to IC_{50} values obtained from our cell-free firefly luciferase approach in a 96-well format. Separate translation reactions containing both PBSII-NFluc and CFluc-Zif268 mRNA in the presence of the dsDNA target oligonucleotide, Zif268-0-PBSII, were initiated. Duplicate experiments were allowed to translate and assemble for 90 min, forming ternary complexes, followed by the addition of increasing concentrations of a competitor hpDNA, containing one of four different Zif268 binding sites having either A, T, C, or G at the central position. In each case a competitor hpDNA concentration-dependent decrease in luminescence was observed (Figure 4b) within 30 min. IC_{50} values for each competitor hpDNA (Figure 4c) were shown to correlate extremely well (R^2 = 0.996) (Figure 4d) with previously reported relative affinities of these target sites.[52] These results serve to validate the application of the split-luciferase cell-free system for the determination of relative binding affinities of nucleic acid-binding proteins for their target sites and more generally in studying inhibitors of protein−nucleic acid interactions. Thus this cell-free system provides an attractive alternative to current methods for interrogating protein−nucleic acid binding specifities as it can be performed in a simple, rapid, high-throughput, and homogeneous format without having to purify or refold the protein(s) of interest.

Detection of Small-Molecule and Peptide Modulators of Protein−Protein Interactions. Having demonstrated the ability to measure antagonists of protein−nucleic acid interactions, we next sought to interrogate the ability of cell-free firefly luciferase reassembly to report on antagonists and agonists of protein−protein interactions. As a first test of small-molecule modulation of split-luciferase activity we chose the well-characterized rapamycin-dependent interaction between the human FK506-binding protein 12 (FKBP) and the FKBP12-rapamycin binding (FRB) domain of human mTOR (residues 2024−2113)[34] which has been a standard test for several split-protein reporter systems.[21,29,30] A rapamycin concentration-dependent increase in luminescence was observed as expected from the cell-free translations of the split-reporters (Figure 5a).[29]

As our first test for antagonism of protein−protein interactions, we chose the well-characterized interaction between the catalytic subunit of cAMP-dependent protein kinase (PKA) with its inhibitor PKI (residues 5−24).[31] Initial experiments had demonstrated that the fusion proteins PKI-NFluc and CFluc-PKA could be translated *in vitro* from mRNA and their

(51) Berger, M. F.; Philippakis, A. A.; Qureshi, A. M.; He, F. S.; Estep, P. W.; Bulyk, M. L. *Nat. Biotechnol.* **2006**, *24*, 1429–1435.
(52) Bulyk, M. L.; Huang, X. H.; Choo, Y.; Church, G. M. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 7158–7163.



	K_d^{app}	IC_{50}
TGG	3	0.56
TAG	6.7	2.61
TTG	71	47.28
TCG	180	105.57

Figure 4. Interrogation of protein−nucleic acid interactions utilizing a split-firefly luciferase cell-free assay. (a) Cartoon showing the dissociation of the reassembled dsDNA−firefly luciferase ternary complex by the addition of a competitor hairpin DNA containing one of the two zinc finger binding sites. (b) Dissociation of the reassembled PBSII-NFluc, CFluc-Zif268, and dsDNA ternary complex by the addition of Zif268 hairpin DNA targets containing TGG (wild type, middle finger), TAG, TTG, and TCG triplet base pairs. (c) Previously reported relative affinities[49] of target oligonucleotides with Zif268 with IC_{50} values derived from the cell-free firefly luciferase reassembly assay and their correlation (d). (e) Dissociation of the Pum2-NFluc, CFluc-Pum1, and RNA ternary complex by the addition of an RNA target containing a Pum1 binding site.

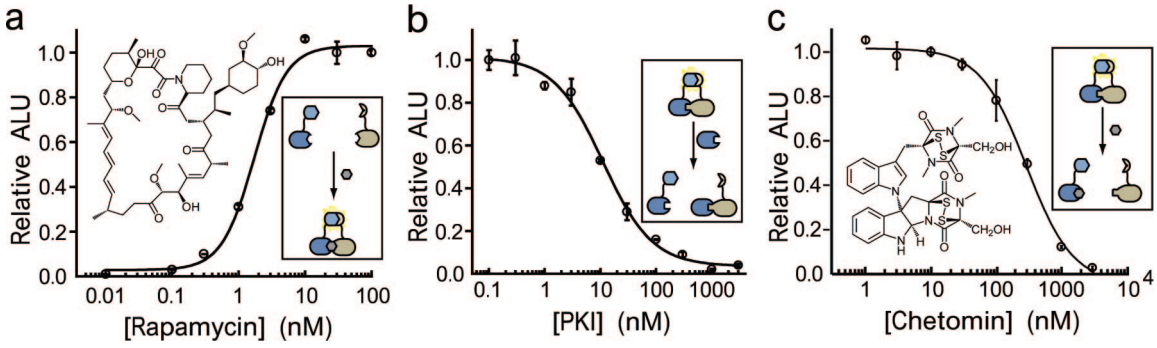


Figure 5. Interrogation of small-molecule and peptide modulators of protein−protein interactions utilizing the split-firefly luciferase cell-free assay. (a) Concentration-dependent association of FRB-NFluc and CFluc-FKBP mediated by rapamycin (inset). (b) Concentration-dependent dissociation of the PKI-NFluc/CFluc-PKA complex by PKI peptide. (c) Concentration-dependent dissociation of the reassembled p300-NFluc/CFluc-Hif1α complex by chetomin (inset).

association monitored via luminescence (Figure 2a). Knowing that the reassembly of fragmented firefly luciferase is dependent upon PKA/PKI complex formation, the inhibition of this interaction was interrogated by the addition of increasing concentrations of a PKI peptide[46] yielding an observed IC_{50} value of 11 nM (Figure 5b). As a further example of the generality of this cell-free format, we interrogated the inhibition of an emerging anticancer target, specifically the interaction between hypoxia inducible factor-1α (HIF-1α) and the CH1 domain of the transcriptional coactivator p300.[53] Initial experi-

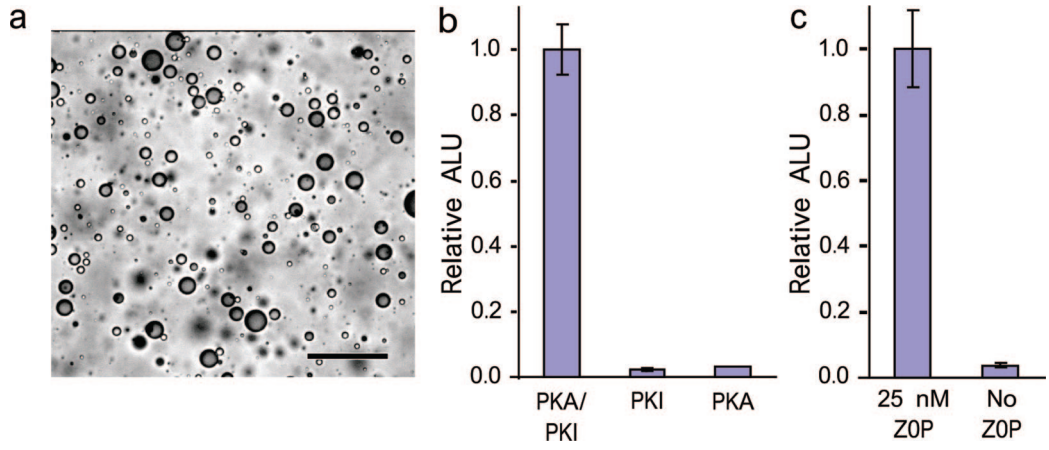


Figure 6. Protein−protein and protein−DNA interactions in artificial cells interrogated by split-firefly luciferase-dependent bioluminescence. (a) White light microscope image of a water-in-oil emulsion containing a PKI-NFluc/CFluc-PKA translation in wheat germ extract. Scale bar is equal to 75 μm. (b) Protein−protein association (PKI-NFluc/CFluc-PKA)-dependent split-luciferase reassembly and bioluminescence within water-in-oil emulsion. (c) Protein−DNA interaction-dependent firefly luciferase reassembly within water-in-oil emulsion.

ments (Supporting Information, Figure S1) demonstrated that the fusion proteins p300-NFluc and CFluc-HIF-1α could be translated *in vitro* from mRNA and their association monitored via luminescence. Recently, the small molecule chetomin has been identified as a first-in-class inhibitor of the interaction between HIF-1α and p300.[48] To evaluate if our method could aid in the identification of small-molecule inhibitors, mRNA encoding p300-NFluc and CFluc-HIF-1α fusion proteins were translated followed by the addition of increasing concentrations of the small molecule chetomin postreassembly. Luminescence measurements following chetomin incubation revealed a concentration dependent decrease in signal and yielded an IC_{50} value of 290 nM (Figure 5c). Importantly, control experiments with excess chetomin or PKI-peptide showed no effect on signal generation in the zinc finger/DNA cell-free assay (Supporting Information, Figure S2), which verified that the loss in signal was dependent upon the disruption of specific protein−protein interactions rather than off-target effects such as inhibition of luciferase activity. Thus, these three systems demonstrate that both peptide and small-molecule modulators of protein−protein interactions can be rapidly evaluated in the cell-free split-luciferase system without the need for transfection and cell-culture (current yeast n-hybrids and PCA methods), protein purification and selective fluorophore labeling (FP), or immobilization on solid surfaces (SPR and ELISA).

Application to Artificial Cell Systems. In addition to monitoring protein−protein and protein−nucleic acid interactions, the cell-free methodology described here could potentially be used for the selection of protein−protein and protein−nucleic acid interactions. Elegant experiments by Tawfik and Griffiths have recently established that water-in-oil emulsions of *in vitro* translation reactions can function as so-called artificial or "man-made" cells, which have been utilized for linking genotype with phenotype in protein evolution experiments.[54,55] Toward demonstrating that our cell-free split luciferase assay is compatible with *in vitro* compartmentalization, translations containing mRNA encoding PKI-NFluc and CFluc-PKA, or PBSII-NFluc, CFluc-Zif268 and target DNA were performed in wheat germ extract encapsulated in water-in-oil emulsions (Figure 6a), followed by luminescence analysis (Figure 6b,c). This set of experiments clearly demonstrates that the cell-free split-firefly luciferase system has the potential for being adapted in screens for protein−protein and protein−nucleic acid interactions in artificial cell-based methodologies and may also find applications in studying proteins incorporating unnatural amino acids where significantly greater control over translational machinery components is often desirable.[56–60]

Conclusion

Numerous *in vitro* and *in vivo* methods are being developed to interrogate the interactions between proteins and nucleic acids, including strategies utilizing the reassembly of split-protein reporters. Although powerful, current cell-based and *in vitro* strategies depend on a number of cumbersome and time-consuming steps including transfection, cell culture, purification, washing steps, and/or covalent modification, yielding overall experimental times in excess of 12−48 h when starting from appropriate clones. Herein we have described a general platform for interrogating biomolecular interactions in homogeneous assays based on cell-free split-protein systems within 2 h. This cell-free assay is capable of utilizing a variety of split-protein reporters providing both fluorescent (β-lactamase) and bioluminescent (luciferase) signal outputs. One drawback of the current method as also found in ELISA and *in vivo* approaches is that only relative affinities and IC_{50} values can be determined, unlike with methods such as FP and SPR. However, the speed and ease of implementation of this cell-free approach, which does not require cell culture, protein purification, or chemical derivatization, can be used to rapidly address biological and chemical questions with appropriate controls, as we have

(53) Kung, A. L.; Wang, S.; Klco, J. M.; Kaelin, W. G.; Livingston, D. M. *Nat. Med.* **2000**, *6*, 1335–1340.
(54) Tawfik, D. S.; Griffiths, A. D. *Nat. Biotechnol.* **1998**, *16*, 652–656.
(55) Miller, O. J.; Bernath, K.; Agresti, J. J.; Amitai, G.; Kelly, B. T.; Mastrobattista, E.; Taly, V.; Magdassi, S.; Tawfik, D. S.; Griffiths, A. D. *Nat. Methods* **2006**, *3*, 561–570.
(56) Forster, A. C.; Tan, Z. P.; Nalam, M. N. L.; Lin, H. N.; Qu, H.; Cornish, V. W.; Blacklow, S. C. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 6353–6357.
(57) Mendel, D.; Cornish, V. W.; Schultz, P. G. *Annu. Rev. Biophys. Biomol. Struct.* **1995**, *24*, 435–462.
(58) Rackham, O.; Chin, J. W. *Nat. Chem. Biol.* **2005**, *1*, 159–166.
(59) Seebeck, F. P.; Szostak, J. W. *J. Am. Chem. Soc.* **2006**, *128*, 7150–7151.
(60) Goto, Y.; Ohta, A.; Sako, Y.; Yamagishi, Y.; Murakami, H.; Suga, H. *ACS Chem. Biol.* **2008**, *3*, 120–129.

demonstrated with either dominant negative inhibition or a known small-molecule ligand.

Our experiments demonstrate the ability to detect a wide variety of protein−protein interactions, including the well-studied heterodimerization of the leucine zippers Fos and Jun, the interaction between the protein kinase PKA and its inhibitor PKI, and the small-molecule-dependent interaction between FKBP and FRB. Additionally, we provide the first example of a rapid method for interrogating the interaction between HIF-1α and p300, an emerging protein−protein target implicated in cancer progression. Furthermore, we detail the first examples of sensitive split-luciferase-mediated detection of a wide range of protein−nucleic acid interactions, including zinc finger domains with specific dsDNA, a methyl CpG-binding domain with specific methylated DNA, and RNA binding pumilio domains with target RNA. We have also demonstrated that this methodology can be utilized to interrogate the relative binding affinities of nucleic acid binding proteins for their target sites and the evaluation of small-molecule and peptide modulators of protein−protein interactions. In addition to using purified lysate and wheat germ extract, we have demonstrated the detection of protein−protein and protein−nucleic acid interactions using a system composed entirely of purified components that minimizes nonspecific interactions from cellular components and allows control over the translational machinery. This may find utility in numerous applications involving unnatural amino acid incorporation.[56–60] Finally, we have demonstrated that the split-protein reporters are functional in water-in-oil emulsions, providing artificial cell systems for studying protein−protein and protein−nucleic acid interactions that can potentially be utilized in screening methodologies.[54]

We envision that this cell-free format could potentially be used in a wide-variety of applications that include screening of DNA or RNA target sites for nucleic acid binding proteins and the determination of target site preference. More importantly perhaps, this approach can be used for screening small molecules, nucleic acids, peptides, or proteins for inhibition of specific protein−protein or protein−nucleic acid interactions.[1,2] Moreover, since split-protein approaches have been widely utilized in a cellular context, the initial hits from the rapid cell-free system can be rapidly tested in a cellular context.[10,20] Thus, we anticipate that this rapid, sensitive, and homogeneous assay system will be widely utilized for interrogating user-defined natural and unnatural biomolecular interactions and for evaluating agonists and antagonists of these interactions.

Materials and Methods

General Materials. All materials were obtained from Sigma-Aldrich unless otherwise noted. $ZnCl_2$ was obtained from EM Sciences. Restriction enzymes were obtained from NEB and *in vitro* translational products from Promega. Oligonucleotide primers and targets were from IDT.

Plasmid Construction and mRNA Production. The fusion protein constructs used in this study are shown in Supporting Information, Table S1. DNA coding for N-terminal and C-terminal reporter protein fragments (GFP, β-lactamase, and three luciferases) were generated by PCR with appropriate primers and subsequently cloned into either the pETDuet-1 vector (Novagen) or the pMAL-c2x vector (NEB) using standard techniques with verification by dideoxyoligonucleotide sequencing. Fragments encoding the nucleic acid-binding or associating proteins used in this study were generated by PCR starting from specific plasmids. The fusion protein constructs were generated using standard cloning techniques and verified by dideoxyoligonucleotide sequencing. The mRNA necessary for cell-free assays was generated as follows: PCR fragments corresponding to the desired fusion constructs were generated using a forward primer containing a T7 RNA polymerase promoter and a Kozak sequence and a reverse primer containing a 3′ hairpin loop. The purified PCR products were subsequently used as templates for *in vitro* transcription using the RiboMAX Large Scale RNA Production System-T7 (Promega) following the manufacturer's protocols.

Target DNA Preparation. All nucleic acid targets were obtained from IDT. A dsDNA target containing a zero base pair separation between the Zif268 and PBSII zinc finger sites (Z0P) was annealed as previously described.[23] Hairpin DNA targets were annealed in 1x *Bam*HI buffer by heating at 95 °C for 7 min followed immediately by cooling on ice.

Reassembly of the GFP Variant Venus. Duplicate 150 μL translations were carried out in Wheat Germ Plus extract (Promega) according to the manufacturer's protocol using 4 pmol of each mRNA encoding for NVenus(residues 1−157)-Zif268 and PBSII-CVenus(residues 158−238), 10 μM $ZnCl_2$, 0.5 μL of RNasin Plus (Promega), and either 50 nM Z0P target DNA or no DNA. Translations were incubated at 25 °C for 2 h (after which no fluorescence was observed) followed by interrogation for fluorescence after a 20 h incubation at 4 °C. Fluorescence spectra were acquired by exciting at 515 nm and monitoring emission at 528 nm.

Reassembly of Split β-Lactamase-Zinc Finger Fusions. Four duplicate 25 μL translations were carried out in Wheat Germ Plus extract (Promega) according to the manufacturer's protocol using 0.5 pmol of each mRNA encoding for NβLac(residues 26−196)-Zif268 and PBSII-CβLac(residues 198−290), 10 μM $ZnCl_2$, 0.5 μL of RNasin Plus (Promega), and either 20 nM Z0P target DNA or no DNA. Translations were incubated at 25 °C for 2 h and assayed by combining 25 μL of translation solution to 75 μL of PBS buffer containing a final concentration of 10 μM Fluorocilin Green soluble β-lactamase substrate (Invitrogen). The final concentration of DNA in the assay was 5 nM. The rate of Fluorocillin Green hydrolysis was determined by exciting at 495 nm and monitoring emission at 525 nm with a 515 nm emission cutoff using a Spectra Max Gemini plate reader. Emission was read every 30 s for 10 min.

Reassembly of Split-Firefly Luciferase. Duplicate 25 μL translations were carried out in Flexi-Rabbit Reticulocyte Lysate (Promega) according to the manufacturer's protocol using 2 pmol of each mRNA encoding for either PBSII-NFluc(residues 2−416) and CFluc(residues 398−550)-Zif268 or NFluc(residues 2−398)-Zif268 and PBSII-CFluc(residues 394−550), 10 μM $ZnCl_2$, 0.5 μL of RNasin Plus (Promega), and either 25 nM Z0P target DNA or no DNA. Translations were incubated at 30 °C for 90 min and assayed by combining 20 μL of translation solution with 80 μL of Steady-Glo Luciferase Assay System (Promega). The final concentration of DNA in the assay was 5 nM. Light emission was monitored 1 min after Steady-Glo addition using a Turner TD-20e luminometer with a 3 s delay and a 10 s integration time.

Reassembly of *Gaussia* Luciferase. Duplicate 25 μL translations were carried out in Flexi-Rabbit Reticulocyte Lysate (Promega) according to the manufacturer's protocol using 2 pmol of each mRNA encoding for NGluc(residues 17−109)-Zif268 and PBSII-CGluc(residues 110−185), 1 μM $ZnCl_2$, 0.5 μL of RNasin Plus (Promega), and either 50 nM Z0P target DNA or no DNA. Translations were incubated at 30 °C for 90

min and assayed by combining 20 μL of translation solution with 80 μL of PBS buffer containing a final concentration of 20 μM coelenterazine. The final concentration of DNA in the assay was 5 nM. Light emission was monitored 10 min after coelenterazine addition using a Turner TD-20e luminometer with a 3 s delay and a 10 s integration time.

Protein−Protein Interaction-Dependent Reassembly of Firefly Luciferase. Duplicate 25 μL translations were carried out in Flexi-Rabbit Reticulocyte Lysate (Promega) according to the manufacturer's protocol using 2 pmol of each mRNA encoding the fusion proteins being analyzed, and 0.5 μL of RNasin Plus (Promega). Translations were incubated at 30 °C for 90 min and assayed by combining 20 μL of translation solution to 80 μL of Steady-Glo Luciferase Assay System (Promega). For the rapamycin-induced interaction between FRB and FKBP either 5 nM rapamycin or control (DMSO) was added after translation followed by a 30 min incubation at room temperature. Light emission was monitored 1 min after Steady-Glo addition using a Turner TD-20e luminometer with a 3 s delay and a 10 s integration time.

Reassembly of Split-Firefly Luciferase in a Coupled Transcription/Translation System. Coupled transcription/translation reactions were carried out in TNT T7 Coupled Rabbit Reticulocyte Lysate (Promega) according to the manufacturer's protocol. Coupled reactions using split-firefly luciferase−zinc finger fusions contained 0.5 pmols of each DNA encoding PBSII-NFluc and CFluc-Zif268, 10 μM $ZnCl_2$, 1 μL of RNasin Plus (Promega), and either 100 nM Z0P target DNA or no DNA in a total of 25 μL. Coupled reactions using split-firefly luciferase−FKBP and FRBP fusions contained 0.5 pmol of DNA encoding FRB-NFluc and CFluc-FKBP, and 1 μL of RNasin Plus (Promega) in a total of 25 μL. Solutions were incubated at 30 °C for 90 min. Reactions were diluted at a 1:4 ratio into PBS buffer containing 1% BSA (1% BSA and either 25 nM rapamycin or DMSO in the case of FRB/FKBP) and equilibrated at room temperature for 30 min. Samples were assayed for luciferase activity by combining 20 μL of translation solution with 80 μL of Steady-Glo Luciferase Assay System (Promega). Luminescence readings were taken on a Turner TD20e luminometer using a 3 s delay and a 10 s integration; the average of replicate experiments is shown. The final concentration of Z0P or rapamycin in the assay was 5 nM.

Reassembly of Split-Firefly Luciferase in a Purified Transcription/Translation System. Coupled transcription/translation reactions were carried out using the PURESYSTEM classic II system (Post Genome Inst. Co. Ltd.) according to the manufacturer's protocol. Coupled reactions using split-firefly luciferase-zinc finger fusions contained 0.5 pmols of each DNA encoding PBSII-NFluc and CFluc-Zif268, 10 μM $ZnCl_2$, 1 μL of RNasin Plus (Promega), and either 100 nM Z0P target DNA or no DNA in a total of 25 μL. Coupled reactions using split-firefly luciferase-FKBP and FRBP fusions contained 0.5 pmol of DNA encoding FRB-NFluc and CFluc-FKBP, and 1 μL of RNasin Plus (Promega) in a total of 25 μL. Solutions were incubated at 37 °C for 60 min, followed by the addition of either 25 nM rapamycin or vehicle in the case of FKBP/FRB. Samples were assayed for luciferase activity by combining 20 μL of translation solution with 80 μL of Steady-Glo Luciferase Assay System (Promega). Luminescence readings were taken on a Turner TD20e luminometer using a 3 s delay and 10 s integrations, the average of replicate experiments is shown. The final concentration of Z0P or rapamycin in the assay was 5 nM.

Competition Assay to Identify Protein−DNA Target Site Specificity. Duplicate 25 μL translations were carried out in Flexi-Rabbit Reticulocyte Lysate (Promega) according to the manufacturer's protocol using 0.05 pmol of mRNA encoding PBSII-NFluc and CFluc-Zif268, 10 μM $ZnCl_2$, and 0.5 μL of RNasin Plus (Promega) and allowed to incubate for 90 min at 30 °C in the presence of 750 pM Z0P dsDNA target. Following translation and firefly luciferase reassembly, increasing concentrations of each Zif268 hairpin DNA being tested were added followed by a 30 min incubation at room temperature. Samples were assayed for luciferase activity by combining 20 μL of translation solution with 80 μL of Steady-Glo Luciferase Assay System (Promega). Light emission was monitored 1 min after Steady-Glo addition using a Wallac 1420 VICTOR 3 V luminometer with a 1 s integration time.

Dissociation of the Reassembled Pum2-NFluc, CFluc-Pum1, RNA Ternary Complex. Duplicate 25 μL translation reactions were carried out in Rabbit Reticulocyte Lysate (Promega) according to the manufacturer's protocol using 0.1 pmol of mRNA encoding Pum2-NFluc and CFluc-Pum1 and 0.5 μL of RNasin Plus (Promega) and allowed to incubate for 90 min at 30 °C in the presence of 2.5 nM RNA oligonucleotide target. Following translation and firefly luciferase reassembly, increasing concentrations of a competitor RNA oligonucleotide were added followed by a 30 min incubation at room temperature. Samples were assayed for luciferase activity by combining 20 μL of translation solution with 80 μL of Steady-Glo Luciferase Assay System (Promega). Light emission was monitored 1 min after Steady-Glo addition using a Turner TD-20e luminometer with a 3 s delay and a 10 s integration time.

Detection of Small-Molecule and Peptide Modulators of Protein−Protein Interactions. Duplicate 25 μL translation reactions were carried out in Rabbit Reticulocyte Lysate (Promega) according to the manufacturer's protocol using 2 pmols of mRNA encoding either FRB-NFluc and CFluc-FKBP, PKI-NFluc and CFluc-PKA, or p300-NFluc and CFluc-HIF-1α and 0.5 μL of RNasin Plus (Promega) and allowed to incubate for 90 min at 30 °C. For analysis of the p300/HIF-1α interaction 10 μM $ZnCl_2$ was added to the translation reaction. Following translation, lysates were diluted 1:4 with PBS containing 1% BSA followed by the addition of increasing concentrations of either rapamycin in DMSO, PKI, or chetomin in DMSO followed by a 30 min incubation at room temperature. Samples were assayed for luciferase activity by combining 20 μL of translation solution with 80 μL of Steady-Glo Luciferase Assay System (Promega). Light emission was monitored 1 min after Steady-Glo addition using a Wallac 1420 VICTOR 3 V luminometer with a 1 s integration time.

Reassembly of Split-Firefly Luciferase in Water-in-Oil Emulsions. Water-in-oil emulsions were prepared in 2 mL round-bottom cryogenic vials by adding 50 μL of aqueous phase, over 2 min, into 950 μL of mineral oil containing 4.5% Span 80 and 0.5% Tween 80 while stirring at 1150 rpm using a 2 mm × 9 mm stir bar. Stirring was continued for one minute after the complete addition of the aqueous phase. Translations were prepared on ice using Wheat Germ Plus extract (Promega) according to the manufacturer's protocol using 4 pmol of each mRNA encoding either PKI-NFluc, CFluc-PKA, or both and 0.5 μL of RNasin Plus (Promega). For DNA-dependent reassembly, 4 pmol of each mRNA encoding for CFluc-Zif268 and PBSII-NFluc, 10 μM $ZnCl_2$, 0.5 μL of RNasin Plus (Promega), and either 25 nM Z0P target DNA or no DNA were mixed in a total of 50 μL. Emulsions were prepared using the ice-cold

translation as the aqueous phase. Emulsions were incubated at 25 °C for 2 h and assayed by combining 20 μL of emulsion with 80 μL of Steady-Glo (Promega). Luminescence readings were taken on a Turner TD20e luminometer using a 3 s delay and a 10 s integration time.

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Supporting Information Available: Details of cloning, assay conditions and protocols, and the complete ref 48 are available free of charge via the Internet at http://pubs.acs.org.

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