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Inducible Biosynthetic Nanoscaffolds as Recruitment Platforms for Detecting Molecular Target Interactions inside Living Cells

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Supporting Information

ABSTRACT: We present a novel phenotypic readout using inducible, biosynthetic nanoscaffolds to directly visualize dynamic molecular interactions within living cells at the single-cell level with high sensitivity and selectivity. Labeled ferritin is used to form biological nanoparticles inside cells. Specific supramolecular assembly of ferritin-derived nanoparticles induces highly clustered nanoscaffolds. These inducible biosynthetic nanoscaffolds are used as the artificial recruitment/redistribution platform for monitoring interactions of a small molecule with its target protein(s) inside living cells.

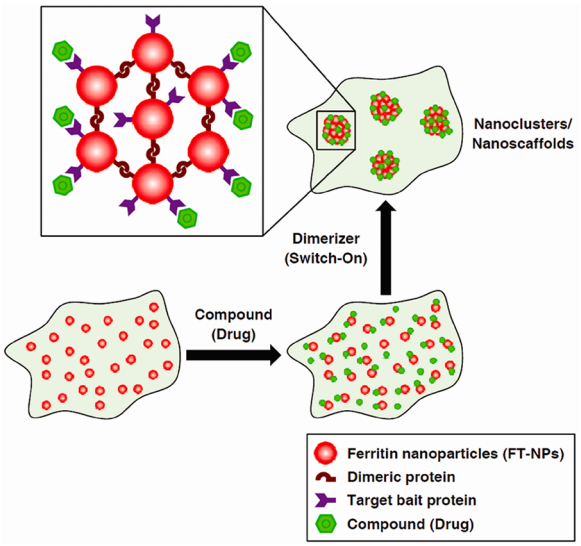
Many drugs and bioactive natural products are discovered fortuitously without prior knowledge of their physiological targets or underlying molecular mechanisms. Elucidation of their molecular targets is essential for understanding their therapeutic and adverse effects.[1] Moreover, discovering novel targets of small molecules from phenotypic screening in chemical biology allows development of new molecular probes. However, compared to relatively well established technologies for genome-wide protein−protein interaction screens, determining target proteins of bioactive small molecules, whose interactions are very weak and transient in nature, remains a formidable challenge.

In vitro binding technologies like affinity chromatography are the most widely used for molecular target identification.[1] However, use of an artificial milieu such as *in vitro* binding conditions or non-mammalian cells often gives erroneous experimental outputs.[1,2] The binding partner proteins of a bioactive small molecule are different in *in vitro* and cellular environments.[3] Thus, it is critical to analyze these molecular target interactions inside live cells under physiologically and pharmacologically relevant conditions.

Several technologies have been used to identify molecular targets of bioactive small molecules inside living mammalian cells, including gene expression/phenotypic profiling and three-hybrid systems.[1d,e] However, they suffer from diverse intrinsic problems, including complicated analyses, high false positives/negatives, and indirect or delayed readouts.

Other methods may be useful for molecular target identification, especially large-scale, like redistribution and co-localization assays, employing endogenous intracellular compartments such as plasma membrane, nucleus, and endosome as recruitment/redistribution platforms to monitor translocation and co-localization of interacting molecules inside cells.[1e,4] However, despite their simplicity, they suffer from intrinsic limitations, including high background and false positives/negatives from endogenous proteins already located at specific intracellular compartments.

Scheme 1. Inducible Biosynthetic Nanoscaffolds for Detecting Molecular Target Interactions inside Living Cells



To address these problems, we present a novel phenotypic readout using inducible, biosynthetic artificial nanoscaffolds inside living mammalian cells. Ferritin protein is known to be self-assembled into nanoparticles of 24 subunits in mammalian cells.[5] By genetic engineering, we designed ferritin nanoparticles (FT-NPs) displaying specific bait or (hetero)dimeric proteins inside living cells, where specific interactions of (hetero)dimeric proteins on FT-NPs cooperatively induce interconnected assembly of FT-NPs into nanoclusters (NCs) (Scheme 1).[6] Under these conditions, bait protein can be

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displayed with high densities on clustered FT-NPs containing multivalent moieties. The ability to form easily visualized artificial nanostructures inside cells led us to propose that these NCs may provide an inducible biosynthetic nanoscaffold as the artificial recruitment/redistribution platform to directly visualize translocation and co-localization of bait and prey within living mammalian cells. To visualize these phenotypic alterations and co-localizations with conventional fluorescence microscopy, we labeled bait or prey with a fluorescent probe, so specific binding of a prey small molecule to its target bait protein(s) can be effectively detected in individual living cells.

To verify this idea, we first examined whether specific heterodimeric protein interactions could induce NC formations inside living cells by exploiting rapamycin-induced FK506 binding protein (FKBP)-FKBP rapamycin binding domain (FRB) association.[7] To visualize formation of NCs within cells, monomeric cerulean fluorescent protein (mCerulean) was fused to FRB and FKBP (Figure 1a). HeLa cells were

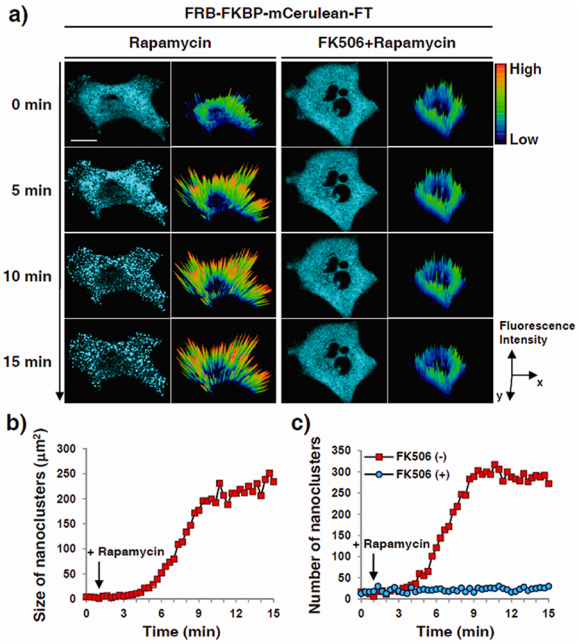

Figure 1. Dynamic nanocluster formation with FT-NPs induced by rapamycin-mediated heterodimerization of FKBP and FRB. (a) Time-lapse images for specific NC formation. HeLa cells co-transfected with FRB-FKBP-mCerulean-FT were treated with 500 nM rapamycin, with or without 25 μM FK506. (b,c) Quantitative analysis of NC formation (scale bar 20 μm).

transfected with expression plasmids for FRB-FKBP-mCerulean fused to the N-terminus of FT. Before induction, NC formation was barely detectable, and FRB-FKBP-mCerulean-FT fusion protein was mostly dispersed throughout the cytoplasm. In contrast, addition of rapamycin induced heterodimerization of FRB and FKBP displayed on FT-NPs, rapidly driving assembly of discrete punctated dots of fluorescence within minutes inside cells (Figure 1a; see also Movie S1). Under these conditions, NC formation was dynamically visualized at the subcellular level throughout the cytoplasm. These imaging data, together with previous data for nanoassemblies,[6a] indicate that specific interactions of rapamycin with both FKBP and FRB induced assembly of NCs.

We further examined the dynamics and specificity of NC formation inside living cells expressing FRB-FKBP-mCerulean-FT. For quantitative analysis, NC was defined by size over 0.2 μm^2 and circularity between 0.5 and 1.0. Treatment with rapamycin synergistically induced NC formations with increased sizes of NCs inside cells (Figure 1b). NC formation was initiated as a rapid response to rapamycin induction and became almost saturated within 5−10 min. Importantly, treatment with FK506, which binds to FKBP but not FRB, competed with rapamycin and consequently blocked this rapamycin-dependent NC formation, demonstrating the specificity of NC formation (Figure 1a,c).[7] This specific NC formation induced by FRB-FKBP heterodimerization was also observed after treatment with rapamycin in the presence of co-expressed FRB-EGFP (enhanced green fluorescent protein)-FT and FKBP-mRFP (monomeric red fluorescent protein)-FT fusion proteins in HeLa cells (Figure S1 and Movie S2). Furthermore, we did not detect toxic effects of NC formation on cell viability even in long-term incubation (Figure S2). These results support the specificity and dynamics of NC formation inside living cells. Furthermore, rapamycin may be useful as a small-molecule switch to induce NC formation for visualization. This inducible system with newly assembled NCs and an immediate readout may allow more reliable detection for molecular interactions by eliminating background and false positive/negative signals within living cells.

We next determined whether induced NC formation can be used as the recruitment/redistribution platform to visualize small molecule−target protein interactions inside cells. We attempted to detect the intracellular target of a small drug molecule inside living cells by exploiting methotrexate, an anti-cancer drug, and its therapeutic target protein, dihydrofolate reductase (DHFR).[8] FT-NPs were designed to display FRB-FKBP and DHFR (Figure 2a). These engineered FT-NPs rapidly formed NCs after treatment with rapamycin. Fluorescein-labeled methotrexate can be recruited onto and co-localized with these NCs upon binding to DHFR. Rapamycin rapidly induced NC formations in living cells expressing FT-NPs displaying DHFR in addition to FRB-FKBP

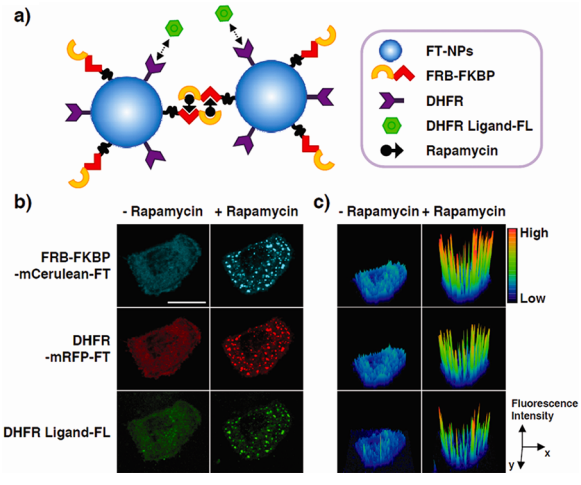

Figure 2. Visualizing specific interactions of DHFR with its small-molecule ligand on assembled biosynthetic nanoscaffolds. (a) HeLa cells co-expressing FRB-FKBP-mCerulean-FT and DHFR-mRFP-FT were treated with 500 nM rapamycin after addition of 10 μM fluorescein-labeled methotrexate (DHFR Ligand-FL). Images were captured before (0 min) and after (10 min) treatment with rapamycin. (b,c) Intensity surface plots for visualizing dynamic interactions of DHFR and DHFR Ligand-FL (scale bar 20 μm).

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(Figure 2b,c). Consistently, we observed rapamycin-induced co-translocation of FRB-FKBP-mCerulean-FT and DHFR-mRFP-FT from the dispersed cytoplasm to these newly detected punctated fluorescent dots of NCs (Figure 2a). Under these conditions, we observed specific recruitment of fluorescein-labeled methotrexate small molecules onto assembled NCs, while recruitment of methotrexate was not observed in NCs displaying no DHFR (Figure S3). Notably, high levels of induced recruitment signals onto easily visualized NCs with high signal-to-noise ratios and distinct phenotypic alterations may facilitate analysis of dynamic molecular interactions at subcellular levels within live cells (Figure 2a,b; see also Movie S3).

To further evaluate the specificity of our recruitment platform for DHFR small-molecule ligands, we tested interactions of trimethoprim with DHFRs from different species. Trimethoprim is known to bind to bacterial DHFR versus human DHFR with remarkably higher specificity (~3000-fold).[9] As expected, fluorescein-labeled trimethoprim was specifically recruited onto and co-localized with FT-derived NCs displaying *E. coli* DHFR, while recruitment of trimethoprim was not observed in NCs displaying human DHFR (Figure S4). These results suggest that recruitment of small molecules onto NCs can be used for monitoring specific interactions of a small molecule with its target protein(s) inside living cells.

We also examined whether our biosynthetic nanoplatforms could be used to probe other small molecule–target protein interactions by monitoring small-molecule ligands interacting with the target proteins haloalkane dehalogenase (HD) and O^6-alkylguanine-DNA alkyltransferase (AGT).[10] HeLa cells were co-transfected with expression plasmids for FRB-FKBP-mCerulean-FT and FT fused to HD (Figure 3a) or AGT

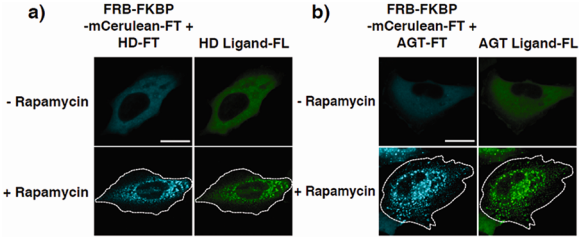

Figure 3. Visualizing specific interactions of small molecules with target proteins on biosynthetic nanoscaffolds: with haloalkane dehalogenase (a) or O^6-alkylguanine DNA alkyltransferase (b). HeLa cells co-expressing fusion proteins as indicated were treated with 500 nM rapamycin after addition of fluorescein-labeled ligand (Ligand-FL) according to the manufacturer's procedures. Dashed lines show cell boundaries (scale bar 20 μm).

(Figures 3b and S5). As can be seen, rapamycin rapidly induced NC formations in living cells expressing FT-NPs displaying these molecular target proteins. Under these conditions, we observed specific recruitment of fluorescence-labeled small-molecule ligands onto assembled NCs displaying their specific target proteins. Prior to rapamycin induction, specific recruitment onto NCs was barely detectable, and FT-fused target proteins were mostly dispersed throughout the cytoplasm. These high levels of recruitment and co-localization signals on distinct NCs further support inducible biosynthetic NCs as nanoprobes for monitoring molecular interactions inside cells.

Furthermore, we evaluated the feasibility of NC formation as the recruitment/redistribution platform for probing protein–protein interactions in intracellular signaling processes using the NF-κB pathway.[11] Prior to activation of the pathway, members of the NF-κB family (e.g., RelA/p65) bind to an IκBα family member (e.g., IκBα) and are sequestered within the cytoplasm. To detect RelA-IκBα interactions in cells, HeLa cells were co-transfected with expression plasmids for IκBα-ECFP (enhanced cyan fluorescent protein)-FT and YFP-RelA (yellow fluorescent protein) along with FRB-/FKBP-mRFP-FT (Figure 4a). As

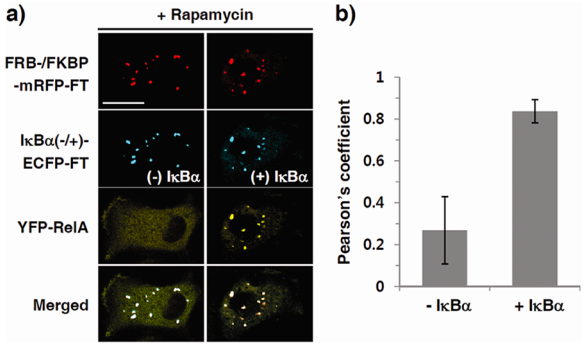

Figure 4. Visualizing protein–protein interactions on biosynthetic nanoscaffolds. (a) Interaction of IκBα with RelA on assembled NCs. HeLa cells co-transfected with expression plasmids as indicated were treated with 500 nM rapamycin. (b) Quantitative analysis for co-localization of IκBα and RelA on NCs (scale bar 20 μm).

indicated by co-localization of IκBα-ECFP with FRB-/FKBP-mRFP in punctated fluorescent dots of NCs, IκBα was efficiently displayed on NCs induced by rapamycin-dependent heterodimerization of FKBP and FRB in HeLa cells. Under these conditions, we observed specific recruitment of YFP-RelA onto these assembled NCs. In contrast, recruitment of YFP-RelA was not observed in NCs displaying no IκBα. These results suggest that NC formation will be useful for probing protein–protein interactions in addition to small molecule–protein interactions within cells.

To further evaluate the sensitivity of our biosynthetic nanoplatforms, we examined even weaker interactions by exploiting an anti-parallel leucine zipper interaction pair, with binding affinity known to be around 20 μM of K_d value.[12] Upon treatment with rapamycin, HeLa cells co-expressing FRB-/FKBP-mRFP-FT, Zip1-FT, and Zip2-EGFP showed NC formation and specific recruitment of Zip2-EGFP onto NCs with Zip1, but not without Zip1, demonstrating high sensitivity of our biosynthetic recruitment platform (Figure S6).

In summary, by monitoring specific recruitment of small molecules to target proteins on biosynthetic NCs/nanoscaffolds, we have developed an effective way to identify molecular targets inside living mammalian cells. This assay relies on inducible, biosynthetic artificial NCs/nanoscaffolds displaying bait proteins with high densities. High-density display is due to multivalent moieties of FT-NPs and their supramolecular clustering into discrete punctated dots induced by rapamycin-controlled heterodimeric interactions. Since high signal-to-noise ratios are observed and rapamycin-based inducible, rapid detection systems are feasible, biosynthetic recruitment/redistribution platform may be exploited in systematic screening of diverse molecular interactions inside living cells, efficiently eliminating intrinsic false positives/negatives or error-prone deviations. This method is also

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amenable to dynamic, systematic single-cell analysis of interactions with high sensitivites and selectivities.

This inducible biosynthetic nanoplatform with efficient protein displays may offer several advantages compared to traditional technologies: (1) In contrast to endogenous intracellular compartments (e.g. plasma membrane, nucleus, and endosome),[1e,4] this assay employs inducible, biosynthetic artificial NCs as recruitment/redistribution platforms, efficiently eliminating endogenous background signals and intrinsic false positives/negatives. Figure S7 shows one example of background signals from endosome-localized proteins in the case of a platform using intracellular endosome as a recruitment site. (2) This simple recruitment/redistribution assays seem better than three-hybrid systems due to the lack of geometric restrictions in molecular partner interactions.[1b] (3) Unlike indirect readout methods that rely on complex gene expression or biological phenotypic profiles,[1] co-localization on biosynthetic recruitment/redistribution platform directly translates physical interactions into clear readout signals. (4) In comparison with artificially synthesized NPs, which has to confront practical issues such as prerequisite of efficient and nondisruptive introduction throughout cells,[4b,13] this assay is simpler and easier to perform due to use of biologically self-assembled FT-NPs inside cells. (5) With distinct and clear phenotypic readouts, we expect this recruitment/redistribution assay may complement some fluorescence intensity-based readouts for probing molecular interactions with fluorescence resonance energy transfer (FRET) or bimolecular fluorescence complementation, which limit sensitivity/dynamic range or cause a long maturation time of fluorophore, respectively.[14] For comparison, using the same DNA constructs used in this study, FRET analyses of DHFR-methotrexate and IκBα-RelA interactions showed only 5−20% of dynamic ranges of fluorescence intensities (Figure S8). Furthermore, this biosynthetic NC formation probes molecular target interactions in a physiologically and pharmacologically relevant context, thereby greatly diminishing misleading outcomes produced by artificial experimental settings.[1−3] Table S1 summarizes the advantages and disadvantages of several technologies including our assay for molecular target interactions.

With these great advantages, our current efforts include genome-wide interaction screens for molecular target identifications of bioactive small molecules using diverse proteins biosynthetic nanoclusters and nanoscaffolds inside living mammalian cells.

■ ASSOCIATED CONTENT

Supporting Information

Experimental procedures and movies. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) (a) Stockwell, B. R. *Nature* **2004**, *432*, 846. (b) Burdine, L.; Kodadek, T. *Chem Biol* **2004**, *11*, 593. (c) Lin, H.; Cornish, V. W. *Angew. Chem., Int. Ed.* **2002**, *41*, 4402. (d) Terstappen, G. C.; Schlupen, C.; Raggiaschi, R.; Gaviraghi, G. *Nat. Rev. Drug Discov.* **2007**, *6*, 891. (e) Inglese, J.; Johnson, R. L.; Simeonov, A.; Xia, M.; Zheng, W.; Austin, C. P.; Auld, D. S. *Nat. Chem. Biol.* **2007**, *3*, 466.

(2) (a) Nguyen, T. N.; Goodrich, J. A. *Nat. Methods* **2006**, *3*, 135. (b) Chen, G. I.; Gingras, A. C. *Methods* **2007**, *42*, 298. (c) Luo, Y.; Batalao, A.; Zhou, H.; Zhu, L. *Biotechniques* **1997**, *22*, 350. (d) Deane, C. M.; Salwinski, L.; Xenarios, I.; Eisenberg, D. *Mol. Cell. Proteomics* **2002**, *1*, 349.

(3) Kim, Y. K.; Lee, J. S.; Bi, X.; Ha, H. H.; Ng, S. H.; Ahn, Y. H.; Lee, J. J.; Wagner, B. K.; Clemons, P. A.; Chang, Y. T. *Angew. Chem., Int. Ed.* **2011**, *50*, 2761.

(4) (a) Zhang, J.; Campbell, R. E.; Ting, A. Y.; Tsien, R. Y. *Nat. Rev. Mol. Cell. Biol.* **2002**, *3*, 906. (b) Giepmans, B. N.; Adams, S. R.; Ellisman, M. H.; Tsien, R. Y. *Science* **2006**, *312*, 217. (c) Heydorn, A.; Lundholt, B. K.; Praestegaard, M.; Pagliaro, L. *Methods Enzymol.* **2006**, *414*, 513. (d) Lee, K. H.; Lee, S.; Lee, W. Y.; Yang, H. W.; Heo, W. D. *Proc. Natl. Acad. Sci. U.S.A.* **2010**, *107*, 3412.

(5) (a) Chasteen, N. D.; Harrison, P. M. *J. Struct. Biol.* **1999**, *126*, 182. (b) Cohen, B.; Ziv, K.; Plaks, V.; Harmelin, A.; Neeman, M. *Wiley Interdiscip. Rev. Nanomed. Nanobiotechnol.* **2009**, *1*, 181.

(6) (a) Lee, S.; Lee, K. H.; Ha, J. S.; Lee, S. G.; Kim, T. K. *Angew. Chem., Int. Ed.* **2011**, *50*, 8709. (b) Perez, J. M.; Josephson, L.; O'Loughlin, T.; Hogemann, D.; Weissleder, R. *Nat. Biotechnol.* **2002**, *20*, 816. (c) Li, M.; Wong, K. K. W.; Mann, S. *Chem. Mater.* **1999**, *11*, 23.

(7) (a) Heitman, J.; Movva, N. R.; Hall, M. N. *Science* **1991**, *253*, 905. (b) Brown, E. J.; Albers, M. W.; Shin, T. B.; Ichikawa, K.; Keith, C. T.; Lane, W. S.; Schreiber, S. L. *Nature* **1994**, *369*, 756. (c) Sabatini, D. M.; Erdjument-Bromage, H.; Lui, M.; Tempst, P.; Snyder, S. H. *Cell* **1994**, *78*, 35.

(8) (a) Schweitzer, B. I.; Dicker, A. P.; Bertino, J. R. *FASEB J.* **1990**, *4*, 2441. (b) Miller, L. W.; Sable, J.; Goelet, P.; Sheetz, M. P.; Cornish, V. W. *Angew. Chem., Int. Ed.* **2004**, *43*, 1672.

(9) Dauber-Osguthorpe, P.; Roberts, V. A.; Osguthorpe, D. J.; Wolff, J.; Genest, M.; Hagler, A. T. *Proteins* **1988**, *4*, 31.

(10) (a) Fernandez-Suarez, M.; Ting, A. Y. *Nat. Rev. Mol. Cell. Biol.* **2008**, *9*, 929. (b) Los, G. V.; Encell, L. P.; McDougall, M. G.; Hartzell, D. D.; Karassina, N.; Zimprich, C.; Wood, M. G.; Learish, R.; Ohana, R. F.; Urh, M.; Simpson, D.; Mendez, J.; Zimmerman, K.; Otto, P.; Vidugiris, G.; Zhu, J.; Darzins, A.; Klaubert, D. H.; Bulleit, R. F.; Wood, K. V. *ACS Chem. Biol.* **2008**, *3*, 373. (c) Keppler, A.; Gendreizig, S.; Gronemeyer, T.; Pick, H.; Vogel, H.; Johnsson, K. *Nat. Biotechnol.* **2003**, *21*, 86.

(11) Karin, M.; Yamamoto, Y.; Wang, Q. M. *Nat. Rev. Drug Discov.* **2004**, *3*, 17.

(12) Magliery, T. J.; Wilson, C. G.; Pan, W.; Mishler, D.; Ghosh, I.; Hamilton, A. D.; Regan, L. *J. Am. Chem. Soc.* **2005**, *127*, 146.

(13) (a) Duan, H.; Nie, S. *J. Am. Chem. Soc.* **2007**, *129*, 3333. (b) Ruan, G.; Agrawal, A.; Marcus, A. I.; Nie, S. *J. Am. Chem. Soc.* **2007**, *129*, 14759.

(14) (a) Fernandez-Suarez, M.; Chen, T. S.; Ting, A. Y. *J. Am. Chem. Soc.* **2008**, *130*, 9251. (b) Michnick, S. W. *Curr. Opin. Biotechnol.* **2003**, *14*, 610. (c) Nguyen, A. W.; Daugherty, P. S. *Nat. Biotechnol.* **2005**, *23*, 355. (d) Kerppola, T. K. *Nat. Protoc.* **2006**, *1*, 1278.