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via orthogonal bioconjugation**

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A modular approach for dCas9-mediated enzyme cascading via orthogonal bioconjugation

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We report a new modular strategy to assemble dCas9-guided enzyme cascades by employing orthogonal post-translation chemistry. Two orthogonal SpyCatcher and SnoopCatcher pairs were used for the one-pot enzyme bioconjugation onto two different dCas9 proteins to enable their guided assembly onto a DNA scaffold. The resulting two-component cellulosomes exhibited 2.8-fold higher reducing sugar production over unassembled enzymes. This platform retains the high binding affinity afforded by dCas9 proteins for easy control over enzyme assembly while offering the flexibility for both *in vivo* and *in vitro* assembly of a wide array of enzyme cascades with minimal optimization.

Enzyme co-localization or metabolon formation is frequently employed in nature to enhance desirable enzymatic reactions^{1–4} by facilitating the direct passage of intermediates from one active site to the next one in a metabolic pathway.^{5–7} In addition, enzyme organization facilitates increased product titers due to its ability to limit the diffusion of transient toxic intermediates, prevent crosstalk between competitive pathways, and concentrate rate limiting reactants to drive unfavorable reactions. In most cases, a protein scaffold is used to organize multiple enzymes together rather than random clustering in order to provide precise control of enzyme spacing and ordering.⁸ Efforts have been made to create synthetic metabolons^{9–14} to mimic these natural designs to achieve the same level of enhancement in metabolic efficiencies.

Our lab has recently reported a new scaffolding platform that utilizes the high binding-affinity dCas9 proteins to guide the assembly of synthetic metabolons.^{15, 16} This dCas9-mediated assembly approach was used to demonstrate the formation of a synthetic cellulosome by fusing the endoglucanase CelA and a cellulose binding domain (CBD) to the C-terminus of two orthogonal dCas9 proteins. When scaffolded onto a DNA target, a 2.6-fold higher level of reducing sugars was produced as compared to free enzymes. While this synthetic platform offers great specificity, high binding affinity, and modularity, it is often difficult to generate larger dCas9 fusion proteins due to their folding issues.¹⁷ To this end, a new modular

framework is needed to decorate each orthogonal dCas9 protein with the enzyme of interest for site-specific assembly.

One simple way to bypass direct protein fusion is to exploit post-translational strategies for dCas9 decoration. Recent discovery of the SpyCatcher (12 kDa)/SpyTag (13 aa) pair provides a new exciting opportunity for covalent bioconjugation between the two protein domains.¹⁸ Because rapid ligation can be achieved under a wide range of physiological conditions, this technology is particularly useful for creating large protein conjugates *in vivo*. Lim and co-workers have recently exploited this strategy to generate dCas9-enzyme conjugates to improve violacein biosynthesis.¹⁹ However, they utilized the same SpyCatcher system to conjugate all five enzymes, making this strategy useful only for *in vitro* assembly. Fortunately, additional SnoopCatcher-SnoopTag²⁰ and SdyCatcher-SdyTag²¹ pairs have been identified to provide spontaneous and rapid isopeptide bond formation in a manner analogous to SpyCatcher-SpyTag. Because of the orthogonality of these additional catcher systems, a different catcher protein can be fused to each pathway enzyme to direct its specific conjugation to the target dCas9 protein carrying the corresponding peptide tag in a single conjugation reaction. Full-length chimeric proteins can be guided by their cognate sgRNAs for site-specific assembly onto a DNA scaffold (Fig. 1). This strategy not only minimizes improper folding, aggregation and truncation because of direct expression of larger dCas9 fusion proteins, it also offers a more modular synthetic scaffolding platform capable of being utilized for endless metabolic pathways with little optimization. The increased modularity of this new assembly framework was used to demonstrate the site-specific, co-localization of two cellulosomal components and the resulting impact on cellulose hydrolysis.

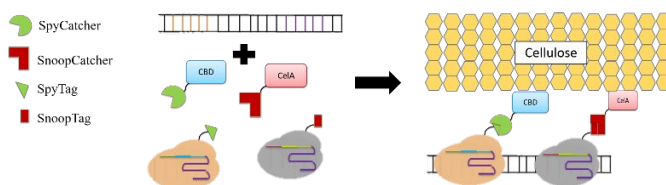


Figure 1. Formation of artificial cellulosome structures guided by full-length conjugated dCas9 chimeric enzymes onto a double-stranded DNA template. Each protein component is expressed individually and ligated post translation using a different Catcher pair to form the full-length chimeric protein. The orthogonality of the Catcher pairs enable the one-pot ligation and cellulosome assembly.

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To create a modular approach to decorate dCas9 proteins with any desirable enzyme of interest, we generated SpdCas9-SpyTag (ST) and St1dCas9-SnoopTag (SnT) for orthogonal conjugation. To ensure that the fused ST and SnT retain their full functionality, we investigated the ability of our dCas9 fusions to react with the appropriate catcher-enzyme fusions. Each dCas9 fusion was purified using the C-terminus his6 tag and mixed with the corresponding purified catcher fusion partner (either CBD-SpyCatcher (SC) or SnoopCatcher (SnC)-CelA) in a 1:1 ratio (Fig. 2). After overnight conjugation, the reaction products were analyzed by gel electrophoresis. For the ST/SC pair, the reaction reached nearly 100% completion with almost no SpdCas9-ST leftover. Similarly, ligation was observed for the SnT/SnC pair. However, the conjugation efficiency was lower, as reported in literature, with only 70% of the ST1dCas9-SnT reacted.²⁰

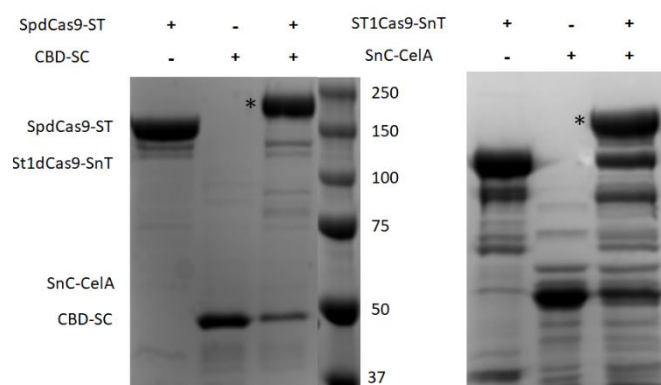


Figure 2. Purified dCas9-tag proteins spontaneously form isopeptide bonds with the corresponding Catcher proteins. (A) Purified SpdCas9-ST and CBD-SC formed a ligated product at 192 kDa with near 100% efficiency. (B) St1dCas9-SnT and SnC-CelA reacted to form a product band at 188 kDa. The ligation efficiency of SnT/SnC is less as reported before in literature.

We next tested the ligation reactions directly in cell lysate. As shown in Fig. 3, proper ligation was observed when the appropriate components were mixed in an equal 1:1 ratio. When components from different species were mixed together, no product was detected. This result indicates that these Catcher/Tag systems are highly specific and can be utilized for orthogonal conjugation of pathway enzymes to different dCas9 proteins. While several smaller product bands were observed for the SC/SnT pair due to ligation with truncated ST1dCas9-SnT, these truncation products are not capable of DNA binding as only a single band was observed using the electrophoretic mobility shift assay (Fig. S1).²²

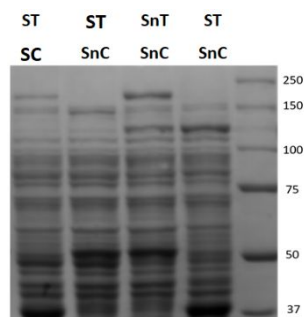


Figure 3. Specific ligation using two orthogonal Catcher pairs. Ligation was detected in cell lysates only when the corresponding Catcher/Tag pair was mixed together. When the components from different pairs were mixed, there was no product formation indicating the pairs are orthogonal.

To evaluate whether this modular scaffolding platform can be utilized for rapid assembly, the reaction kinetics of the Catcher/Tag pairs were investigated. Both reactions proceeded rapidly shortly after mixing, approaching full ligation after 2h (Fig. S2). Since both catcher reactions can be executed in complex cell lysates, we are confident that these orthogonal reactions can be employed for efficient enzyme conjugation for both *in vitro* and *in vivo* applications.

We next exploited the orthogonal catcher reactions for dCas9-guided assembly of a two-component cellulosome in either a stepwise or one pot fashion. For the stepwise assembly, we first conjugated CBD-SC to SpdCas9-ST and SnC-CelA to ST1dCas9-SnT in two separate reactions. The ligated products were mixed at a 1:1 ratio before incubating with their cognate sgRNAs and target DNA. DNA binding was first confirmed using our previously reported dual Cas beacon assay (Fig. S3). Next, the assembled enzyme-DNA complex was mixed with phosphoric acid-swollen cellulose (PASC) and the overall reducing sugars level was compared with free enzymes. As shown in Fig. 4, DNA alone as well as DNA bound by SpdCas9-ST and St1dCas9-SnT alone showed only background levels of reducing sugars production. While free enzymes (either direct fusions or ligated dCas9-enzyme conjugates) produced a higher level of reducing sugar, the overall PACS hydrolysis was improved by 2.8-fold when the dCas9-enzyme conjugates are co-localized onto a DNA scaffold. This enhanced is slightly improved than using the direct dCas9 fusion enzymes possibly due to the improved protein quality as well as the extended enzyme accessibility granted by the Catcher/Tag system.

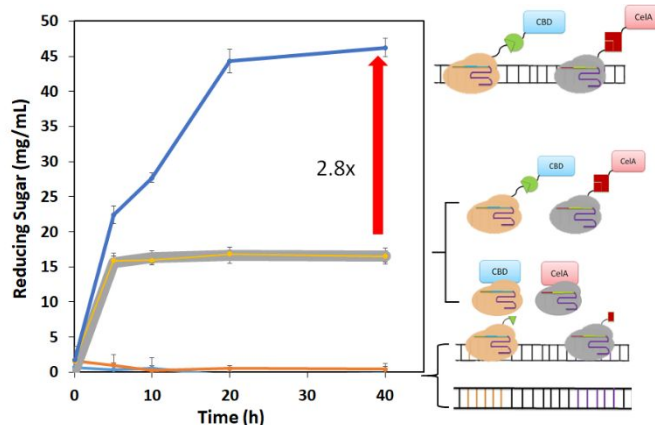


Figure 4. Stepwise assembly of a two-component cellulosome based on orthogonal Catcher reactions. The level of reducing sugars production from PACS using the assembled cellulosome was compared with DNA only, SpdCas9-ST and ST1dCas9-SnT co-localized on a DNA scaffold, proteins as direct fusions, and proteins ligated to dCas9 proteins. The assembled cellulosome exhibited a 2.8-fold increase in reducing sugars production over the unassembled components.

While the stepwise procedure is useful for *in vitro* enzyme assembly, the orthogonality of the Catcher/Tag pairs also allows for direct protein conjugation and DNA binding for *in vivo* applications. To demonstrate this feasibility, we first prepared the dCas9-enzyme conjugates in a one-pot reaction and confirmed DNA binding using the dual Cas beacon assay (Fig. S4). The assembled cellulosome structure again demonstrated the same 2.8-fold increase in reducing sugars titer (Fig. 5), indicating that the one-pot assembly approach is equally efficient in promoting enzyme assembly when compared to the stepwise approach. The key added advantage with the one-pot approach is the feasibility of *in vivo* enzyme assembly due to its

ability to execute both enzyme conjugation and dCas9-guided enzyme assembly onto a target DNA scaffold inside a living cell.

In conclusion, we demonstrated here a modular approach to create enzyme cascades using post-translational ligation and dCas9-mediated assembly. By expressing dCas9 proteins separately from the pathway enzymes before ligating them together using orthogonal catcher reactions, we have the flexibility to optimize individually both protein expression and enzyme assembly. This platform retains the high binding affinity afforded by dCas9 proteins for easy control over enzyme stoichiometry, order, and spacing while offering increased modularity for multiple enzyme assembly. The ability to execute this platform in either a stepwise or one-pot fashion allows flexibility of applications for both *in vivo* and *in vitro* assembly of a wide array of enzyme cascades with minimal engineering.

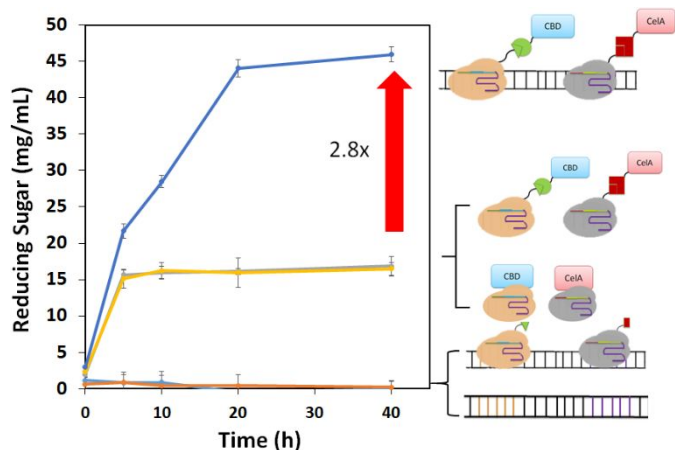


Figure 5. One-pot conjugation and assembly of a two-component cellulosome based on orthogonal Catcher reactions. The level of reducing sugar production from PACS using the assembled cellulosome was compared with DNA only, SpdCas9-ST and ST1dCas9-SnT co-localized on a DNA scaffold, proteins as direct fusions, and proteins ligated to dCas9 proteins. The assembled cellulosome exhibited the same 2.8-fold increase in reducing sugar production when compared to stepwise assembly.

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Conflicts of interest

There are no conflicts to declare.

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