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Supplementary Information

Exploiting dCas9 fusion proteins for dynamic assembly of synthetic metabolons

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MATERIALS AND METHODS

Construction of expression vectors

The nuclease-null Cas9 proteins S.*pyogenes* (SpdCas9), S.*thermophilus* (ST1dCas9) and S.*aureus* (SadCas9) were purchased from Addgene. The dCas9 protein sequences were PCR amplified, double digested, and inserted into pET24a using SpeI and bamHI. Fluorescent protein markers were added into the vectors using the restriction sites bamHI and MfeI for the following final constructs: pET24a SpdCas9-mCherry-6xhis, pET24a pET24a SadCas9-mCherry-6xhis and St1dCas9-Lgbit-6xhis. The fluorescent proteins were swapped out for cellulose binding module (CBD) and the endoglucanase CelA5 using bamHI and MfeI to form pET24a SpdCas9-CBD-6xhis and pET24a St1dCas9-Cel5A-6xhis.

Protein expression and purification

All constructs were transformed into *E.coli* BLR [F- ompT hsdSB (r-B m-B) gal dcm(DE3) Δ(srl-recA)306::Tn10(TetR); Novagen, Madison,WI] cells for protein expression. Expression cultures were grown to OD 0.7 as 37°C, induced with 250 µM isopropyl-thiogalactopyranoside (IPTG), and

grown overnight at 20°C. The cells were collected via centrifugation, resuspended in NiNTA column buffer and then sonicated. The soluble fraction was separated by centrifugation at 20,000xg for 30 minutes and transferred to a Ni-NTA His column (Pierce Biotechnology, Rockford, IL) for purification utilizing the C-terminal 6xhis tag. Purified proteins were eluted with 250 µM imidazole. The remaining imidazole as removed by dialysis into PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4, pH 7.4). The cell lysates and the purified proteins were loaded onto a 7.5% SDS-PAGE gel and stained with coomassie blue for analysis. When relevant protein expression was also monitored via fluorescence protein markers.

RNA preparation

All RNAs (sgRNA and thgRNA) were transcribed in vitro via HiScribe T7 Quick High Yield RNA Synthesis Kit (New England BioLabs, Inc., Ipswich, MA, USA) and purified via phenol-chloroform extraction and ethanol precipitation. The purity, quality and quantity of RNA was analyzing by spectrophotometry using a NanoDrop 2000 UV-Vis spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Then subsequently using a denaturing urea PAGE electrophoresis with a 6% polyacrylamide gel containing 8 M urea.

Single/ Dual Beacon Assay

All DNA probes were prepared using unmodified and fluorophore or quencher labeled oligonucleotides synthesized and purified by the commercial vendor Integrated DNA Technologies (Coralville, IA, USA). Target Cas9 beacon DNA shown in Figure 1A, Figure 2A/B and Supplemental Table 1 were created by mixing equimolar amounts of complementary DNA strands at a final concentration of 1 µM in a beacon hybridization buffer (40 mM Tris, pH 7.9, 100 mM

NaC); heating for 5 minutes at 90°C then cooled to 12°C at a rate of 0.1 /s using a S1000 Thermal Cycler (Bio-Rad Laboratories, INC., Hercules, CA, USA). All beacon binding assays were carried out in beacon binding buffer (20 mM Tris–HCl, 120 mM NaCl, 5% v/v glycerol, 0.1 mM DTT, 1 mM MgCl2, 0.02% v/v Tween 20, pH 7.9) and fluorescence measured using a Synergy H4 Hybrid microplate reader (BioTek Instruments, Inc., Winooski, VT, USA). Final assay mixtures contained 1 nM hybridized beacon target DNA, 10 nM dCas9 protein(s), and 10 nM sgRNA(s). Excitation and emission wavelengths were dictated by the fluorophore in use for each target (A: FAM, ex: 498nm, em:520 nm; B: Cy5 ex: 648nm, em: 668nm).

Electromobility shift assay (EMSA)

Single, dual and triple beacon assays were qualitatively verified using electromobility shift experiments carried out in 4.5% non-denaturing acrylamide gels. Final assay mixtures contained 10 nM hybridized beacon target, 100 nM dCas9 protein(s) and 100 nM sgRNA(s). The fully hybridized beacon DNA only, as well as the beacon DNA bound by the dCas9;gRNA complex were loaded and run for 90 minutes at a constant 90V. The gel was then imaged using a (Imager)

Triple Beacon Assay

All DNA probes were prepared using unmodified and fluorophore labeled oligonucleotides synthesized and purified by the commercial vendor Integrated DNA Technologies (Coralville, IA, USA). Target Cas9 beacon DNA shown in Figure 5 and Supplemental Figure 4 were created by mixing equimolar amounts of complementary DNA strands at a final concentration of 1 μM in a beacon hybridization buffer (40 mM Tris, pH 7.9, 100 mM NaC); heating for 5 minutes at 90°C then cooled to 12°C at a rate of 0.1 /s using a S1000 Thermal Cycler (Bio-Rad Laboratories, INC., Hercules, CA, USA). All beacon binding assays were carried out in beacon binding buffer (20 mM Tris–HCl, 120 mM NaCl,

5% v/v glycerol, 0.1 mM DTT, 1 mM MgCl2, 0.02% v/v Tween 20, pH 7.9). Final assay mixtures contained 10 nM hybridized beacon target, 100 nM dCas9 protein(s) and 100 nM sgRNA(s). Assay mixtures were loaded and run on a 4.5% non-denaturing acrylamide gels and fluorescence measured using a (imager). The triple target was labeled with a FAM fluorophore and with excitation and emission wavelengths at ex: 498nm and em: 520 nm.

Artificial cellulosome assembly and reducing sugar assay

Phosphoric acid-swollen cellulose (PASC) was prepared as described previously.[1] Final assay mixtures contained 20 nM dCas9 proteins, 20 nM sgRNA(s) with or without 2 nM target DNA in the presence of PASC. Synthetic cellulosome assembly was assayed at 30°C in 20 nM Tric-HCl buffer pH 6.0. with shaking. Samples were collected periodically over a 40 hour time period and immediately mixed with 0.25 mL DNS reagent (10 g/L dinitrosalicylic acid, 10 g/L sodium hydroxide, 2 g/L phenol, 0.5 g/L sodium sulfite) and incubated at 95°C for 10 minutes. After incubation, 0.5 mL of 40% Rochelle salts were added to fic the color before measuring the absorbance using a spectrophotometer at 575 nm.

Conditional beacon assay and cellulosome assembly

Conditional beacon assay and cellulosme assembly were run as described above with the exception of the sgRNA being replaced with a thgRNA. Toehold mediated strand displacement reaction was performed as described previously.[2] Final assay mixtures contained 1-2nM beacon DNA target, 10-20 nM dCas9 protein(s) and 10-20 nM sgRNA or thgRNA with or without 100-200 nM trigger strand.

Supplemental Results

Table S1. DNA sequence of single beacons, dual beacons and triple target.

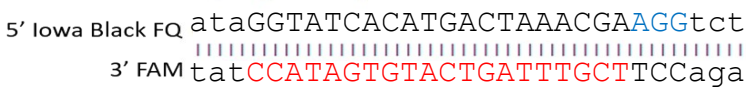
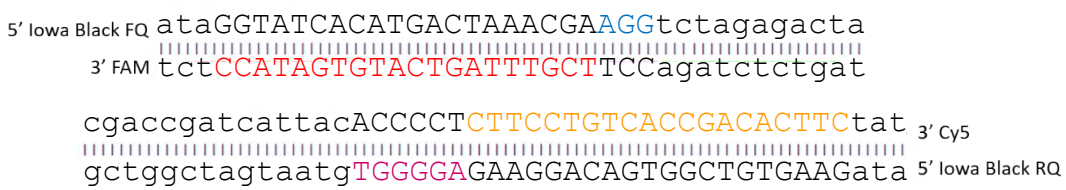
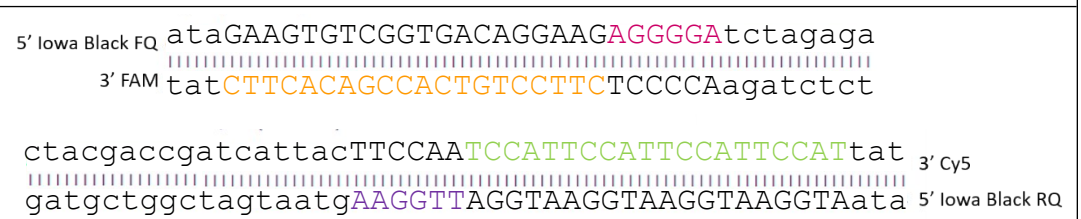
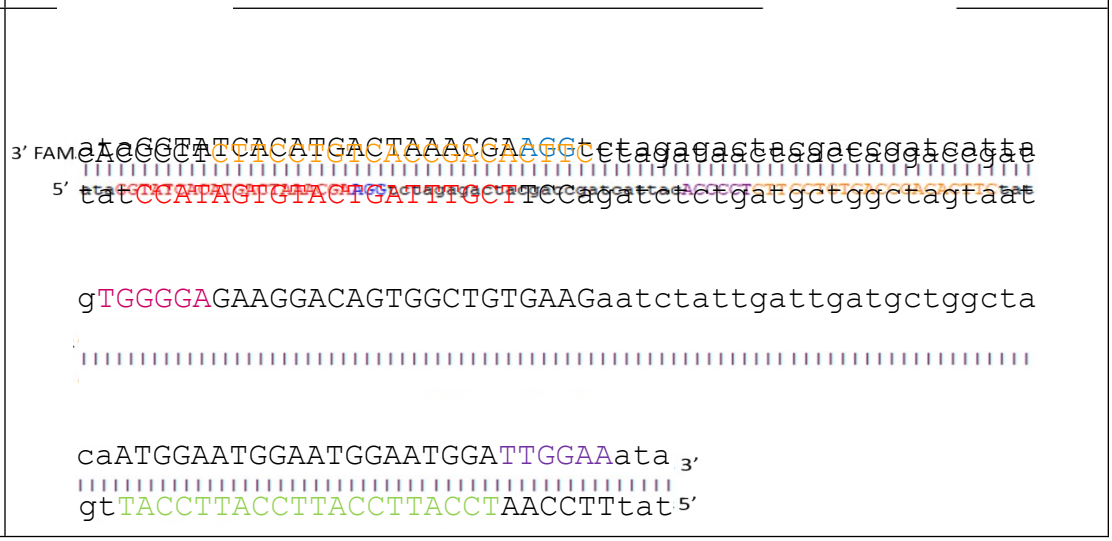
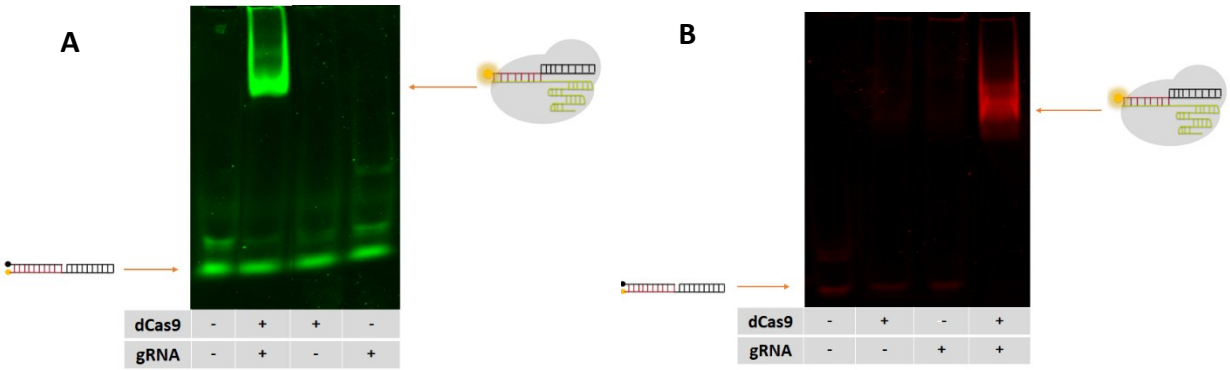
Sp Single Beacon	
St1 Single Beacon	
Sa Single Beacon	
Sp/St1 Dual Beacon	
Sp/Sa Dual Beacon	
Sa/St1 Dual Beacon	
Triple Beacon	

Figure S1. EMSA analysis showing qualitative single binding of (A) SpdCas9;SpgRNA (B) St1dCas9;St1gRNA and (C) SadCas9;SagRNA. Each single target beacon was used and denoted by target B while the positive control (the beacon hybridized without the quencher strand) is denoted by target C.



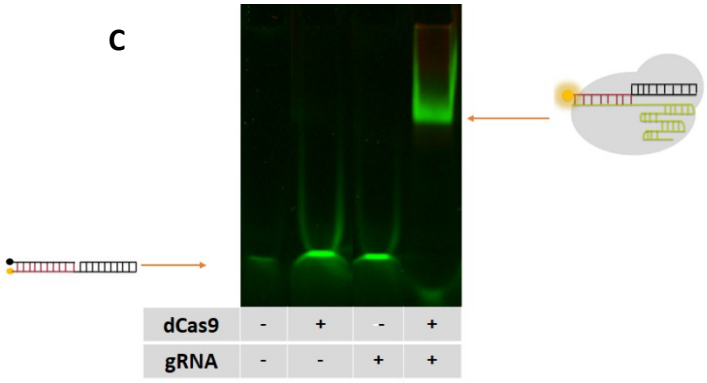
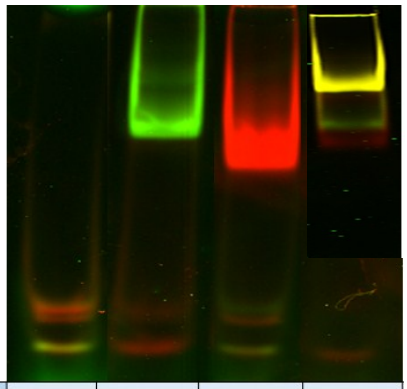


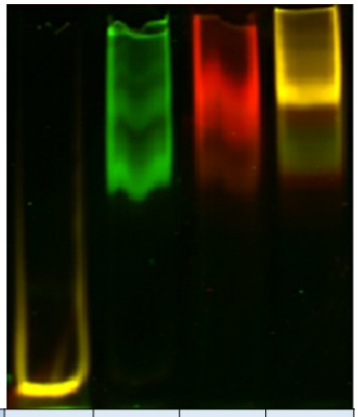
Figure S2. EMSA analysis showing orthogonal and dual binding of (A) SpdCas9 and ST1dCas9, (B) SpdCas9 and SadCas9, and (C) SadCas9 and ST1dCas9 to their respective dual beacon. Displacement of both quenchers by dual dCas9 binding resulted in the detection of a yellow fluorescent protein –DNA complex band.

A



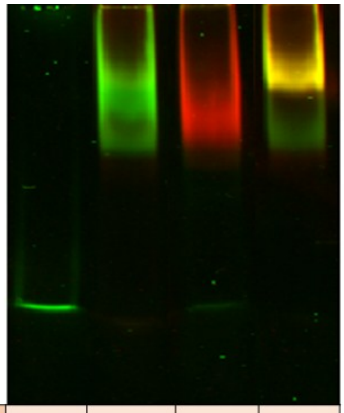
Spcas9	-	+	-	+
SpgRNA	-	+	-	+
ST1dcas9	-	-	+	+
St1gRNA	-	-	+	+

B



Spcas9	-	+	-	+
SpgRNA	-	+	-	+
Sadcas9	-	-	+	+
SagRNA	-	-	+	+

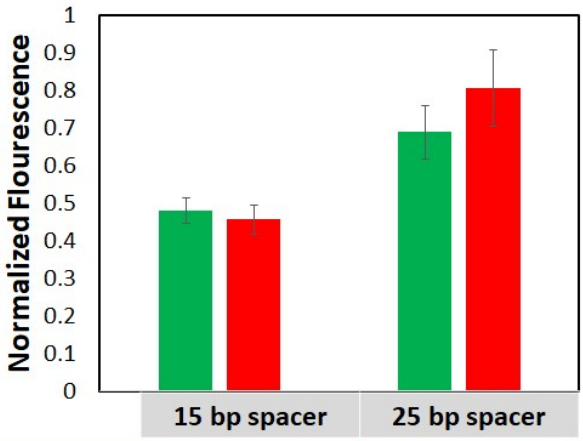
C



Sadcas9	-	+	-	+
SagRNA	-	+	-	+
ST1dcas9	-	-	+	+
St1gRNA	-	-	+	+

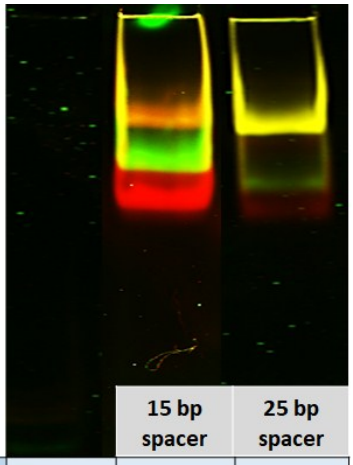
Figure S3. (A) Comparison of SpdCas9 and STIdCas9 binding onto dual beacons containing either a 15-bp or 25-bp spacer between the two binding sites. (B) EMSA analysis showing the less efficient binding of SpdCas9 and ST1dCas9 onto a dual beacon with a 15-bp spacer as compared to a dual beacon containing a 25-bp spacer.

A



	15 bp spacer	25 bp spacer
Spdcas9	+	+
SpgRNA	+	+
St1dcas9	+	+
St1gRNA	+	+

B



		15 bp spacer	25 bp spacer
Spcas9	-	+	+
SpgRNA	-	+	+
ST1dcas9	-	+	+
St1gRNA	-	+	+

Figure S4. The sequential binding of all three dCas9 fusion proteins to a FAM-labeled DNA scaffold. Binding of additional dCas9 fusion proteins results in the formation of DNA-protein complexes with decreased mobility.

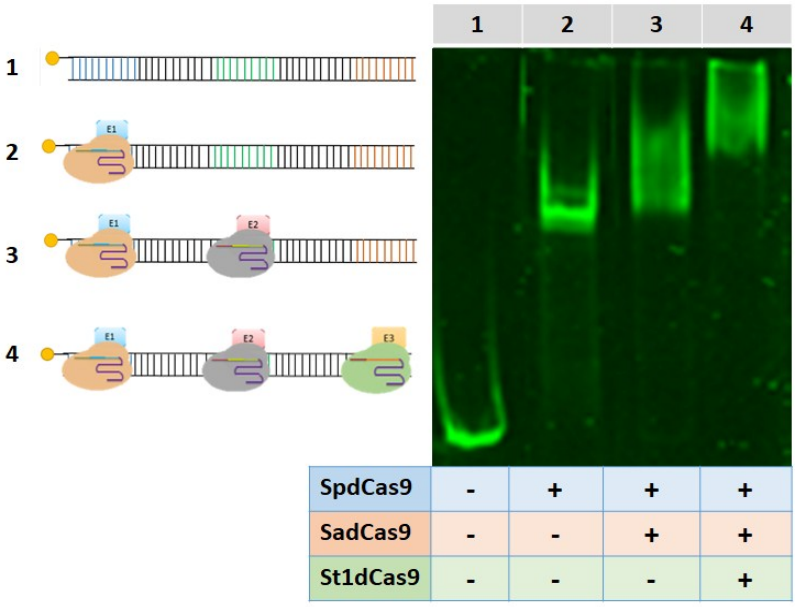
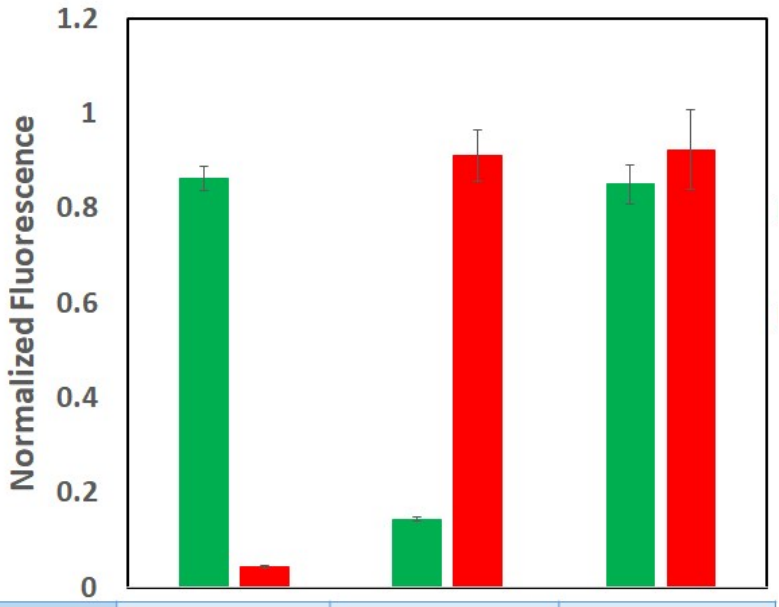
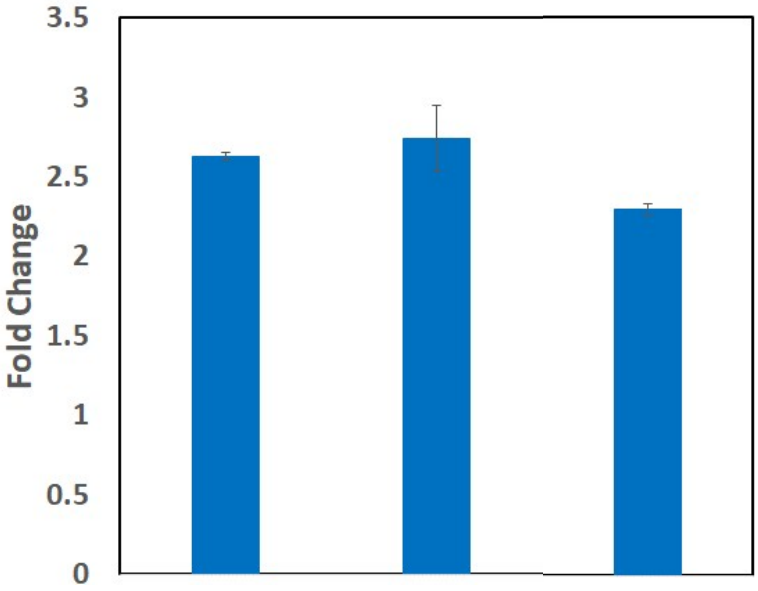


Figure S5. Binding efficiencies of SpdCas9-CBD/StidCas9-CelA onto a dual beacon.



Spdcas9	+	-	+
SpgRNA	+	-	+
St1dcas9	-	+	+
St1gRNA	-	+	+

Figure S6. Comparison of cellulose hydrolysis by different synthetic cellulosomes. Different DNA templates were used to assemble SpdCas9-CBD and St1dCas9-CelA.



Target	CBD-25bp-CelA	CelA-25bp-CBD	(CBD-25bp-CelA)$_3$
Fold Chang	2.6	2.7	2.3

1. F. Liu, S.-L. Tsai, B. Madan and W. Chen, Biotechnology and Bioengineering, 2012, **109**, 2829-2835
2. K.-H. Siu and W. Chen, *Nat. Chem. Biol.*, 2019, **15**, 217–220.