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Supporting Information

CRISPR/Cas-directed programmable assembly of multi-enzyme complexes

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Experimental section

Protein expression and purification

The genes encoding dCas9-SpyCatcher, mCerulean3–SpyTag, mVenus–SpyTag, and VioA-E enzymes with SpyTag were synthesized as gBlocks gene fragments (Integrated DNA Technologies) and were inserted into the multiple cloning site of the pET-19b plasmid (Novagen) using the Gibson Assembly (New England Biolabs). The assembled plasmids were transformed into T7 Express competent cells (New England Biolabs), which were grown in 37°C in Terrific Broth (IBI Scientific) containing 100 μg mL^{-1} ampicillin until OD 600 reached 0.6. Protein expression was subsequently induced at 25°C for an additional 15 h by adding 100 μM IPTG; 1 mM δ-aminolevulinic acid and 40 μM ammonium iron-(II)-sulfate were additionally added when expressing the heme-containing VioB-SpyTag. The cells were harvested by centrifugation at 6000 × g for 10 min, suspended in phosphate buffer with 20 mM Tris-HCl, 1 M NaCl, pH 8.0, lysed by French press, and additionally centrifuged at 22 000 × g for 50 min to collect the soluble lysate.

dCas9-SpyCatcher was first purified by binding to Ni-NTA resin (Life Technologies) via gentle inversion for 3 h at 4°C, washing five times with 20 mM Tris-HCl, 20 mM imidazole, 1 M NaCl, pH 8.0, and eluting with 250 mM imidazole. Subsequently, dCas9-SpyCatcher was further purified by first exchanging the buffer with 20 mM Tris-HCl, 125 mM KCl, 5% glycerol, 1 mM TCEP, pH 7.5, binding to a HiTrap SP cation exchange column (GE Healthcare) and eluting in a gradient from 125 mM to 1 M KCl. Eluted dCas9-SpyCatcher was buffer exchanged with 20 mM Tris-HCl, 200mM KCl, 5% glycerol, 1 mM TCEP, pH 7.5, concentrated using Amicon Ultra 15 mL centrifugal columns (50 kDa MWCO, Milipore), and stored at -80°C.

All other proteins were purified in a single step by binding to Ni-NTA resin via gentle inversion for 3 h at 4°C, washing five times with 20 mM Tris-HCl, 20 mM imidazole, 1 M NaCl, pH 8.0, and eluting with 250 mM imidazole; after the washing step, enzymes VioA-SpyTag, VioC-SpyTag and VioD-SpyTag were further incubated with 4 mM FAD for 1 h at 4°C, washed five times again and eluted in the same way. The eluted proteins were buffer exchanged with 20 mM Tris-HCl, 50 mM NaCl, pH 8, concentrated using Amicon Ultra 15 mL centrifugal columns (10 kDa MWCO, Milipore), and stored at -80°C. All purified protein fractions were inspected using SDS-PAGE and SimplyBlue staining (Invitrogen) before storage. For VioC-SpyTag, all buffers were supplemented with 10% glycerol.

sgRNA and DNA template synthesis

To synthesize sgRNA, the dsDNA template was first PCR amplified from a DNA plasmid (pET-19b) containing the target sequence using a forward primer containing a T7 promoter. Primer sequences for each target site are shown below; note that the same reverse primer was used for synthesizing dsDNA templates for all five types of target sites. Subsequently the products were purified using a PCR cleanup kit (Qiagen).

Forward primers (5’ -> 3’)	Reverse primer (5’ -> 3’)
T1: TAATACGACTCACTATAGCTACC T2: TAATACGACTCACTATAGGGCACCA T3: TAATACGACTCACTATAGATATCGT T4: TAATACGACTCACTATAGATTGGA T5: TAATACGACTCACTATAGATCCAC	(Common for all target sites) AAAAGCACCGACTCGGTG

sgRNA for each binding site was then transcribed from a corresponding dsDNA template using a T7 Ribomax Express Large Scale RNA Production System (Promega), and purified using a Monarch RNA cleanup kit (New England Biolabs). The DNA scaffold was similarly PCR amplified from a DNA plasmid (pET-19b) containing the target sequence using a forward primer, 5'-TTCCACTCGGTTGAGCCGGCTAGGC-3', and a reverse primer 5'-CTACTAGGATGGGCACAGCGGAGA-3'. For the production of fluorescently labeled scaffold, a forward primer with Alexa fluor 488 conjugated to the 5' end was used. The products were subsequently purified by ethanol precipitation. First, the reaction solution was mixed with 10% volume of 3M sodium acetate and 100% volume of isopropanol and centrifuged at 17,000 x g for 15 min. After decanting the supernatant, the pellet was washed with 70% ethanol and centrifuged again to decant the supernatant. The remaining pellet was dried and resuspended in nuclease-free water.

Assembly of protein/enzyme-dCas9-DNA complex

For binding dCas9-SpyCatcher to the DNA template, first 10 µM dCas9-SpyCatcher was mixed with 10 µM corresponding sgRNA in a buffer containing 20 mM Tris-HCl, 100 mM KCl, 2mM $MgCl_2$, pH 8 and incubated for 10 min. Subsequently, each type of dCas9-SpyCatcher:sgRNA complex was mixed with the DNA template under the same buffer conditions for 1 h at room temperature; the reaction mixture contained 0.5 µM DNA template and 2 µM of each (dCas9:sgRNA) complex loaded with different sgRNA. For the electrophoretic mobility shift assay (EMSA) experiment to verify binding, a fluorescently labeled DNA template as described above was used; assembled

complexes were allowed to migrate for 2 h at 100 V in 0.7% agarose gel, and visualized under blue light.

For assembling the protein/enzyme-dCas9-DNA complex, 20 µM SpyTag-containing proteins/enzymes were mixed with 10 µM dCas9-SpyCatcher and allowed to conjugate for 1 h. Then, sgRNA was added to the reaction mixture at 10 µM and incubated for 10 min. Subsequently, each type of (protein/enzyme-SpyTag):(dCas9-SpyCatcher):sgRNA complex was mixed with the DNA template for 1 h at room temperature; the reaction mixture contained 0.5 µM DNA template and 2 µM of each complex loaded with different combinations of protein/enzyme and sgRNA. For downstream application, the complex was then purified by applying it to a Capto Core 700 size exclusion column (GE Healthcare), during which unbound excess proteins/enzymes below the MWCO of 700 kDa cutoff are trapped in the bead and removed, while the larger assembled complexes of interest are eluted. The concentration was determined from the absorbance of the final mixture at 260 nm, using the extinction coefficient calculated by summing those of all individual components assembled in the complex.

Fluorescence measurement

The assembled complexes containing 0.5 µM of each fluorescent proteins were transferred to a black 96-well plate to measure the fluorescence using a Spectramax M2 plate reader (Molecular Devices). The fluorescence was measured using a 412 nm excitation, 430 nm cutoff filter, and emission scan of 450–600 nm. Subsequently, ratiometric FRET was calculated by dividing the 475 nm mCerulean3 emission peak intensity by the 528 nm mVenus emission peak intensity.

Fluorescent spectra were integrated using a|e Spectral Software 1.2 (FluorTools), and the FRET efficiency was calculated using the equation:

$$E = \frac{I_{AD}\varepsilon_A - I_A\varepsilon_A}{I_A\varepsilon_D}$$

where I_{AD} and I_A are the intensities of the mVenus acceptor with and without the mCerulean3 donor, respectively, and ε_A and ε_D are the extinction coefficients of mCerulean3 and mVenus at the excitation wavelength. Subsequently, the average distance between the two fluorescent proteins undergoing FRET response was calculated using the equation:

$$R = R_0 \sqrt[6]{\frac{1-E}{E}}$$

where E is the calculated FRET efficiency and R_0 is the Forster distance of the mCerulean3 and mVenus pair, which was reported as 5.71 nm.[1]

Violacein enzyme assay

Violacein assay was carried out in a reaction mixture containing 0.5 µM of each enzyme in either free or scaffolded form, 500 µM L-tryptophan, 1 µM FAD, 2 mM NADPH and 5 units of catalase. The buffer solution was the same as for the assembly process described above (20 mM Tris-HCl, 100 mM KCl, 2mM $MgCl_2$, pH 8) in order to avoid the need for buffer exchange. The reaction mixture was incubated at room temperature for 2 h; at each time point, a 20-µL sample was taken and quenched with 4 µL DMSO and 40 µL methanol. Quenched samples were then centrifuged at 17,000 x g for 10 min to pellet down the aggregated enzymes, passed through a 0.45 µm filter, and 6 µL was injected into the HPLC column. For the analysis of the reaction product, an analytical 1100 Agilent HPLC with a diode array detector (DAD) and an autosampler

was used, with a Poroshell 120 column (SB-C18, 3.0 x 100 mm, 2.7 µm). Water and acetonitrile were both supplemented with 0.1% formic acid and used as the solvents. A gradient of 20-59% acetonitrile over 25 min was used for the analysis, and product detected at 590 nm. The concentration was calculated using commercially purchased violacein standard (Sigma-Aldrich).

References

1. D. J. Glover, S. Lim, D. Xu, N. B. Sloan, Y. Zhang and D. S. Clark, *ACS Synth. Biol.*, 2018, **7**, 2447.

Supporting tables and figures

Title	Sequence (5' -> 3')
Scaffold (30bp spacing)	TTCCACTCGGTTGAGCCGGCTAGGCCTCTCGCTACCATAGGCACCACGAGCGGCCTA TAACCCTTCTGAGAGTCCGGAGGCGGGGGCACCATACCGAGTGATGGGGTCATTAT TCCTATCACGCTTTCGAGTGTCTGATATCGTTTACCAAAACGGGGGTACATTACCCTC TCATAGGGGGCGTTCTAGGATTGGAGAGTTAGACCACGTGGATCACGTTACCACCAT ATCATTCGAGCATCGATCCACAAGTTACAATTGGTGGACACCATCTCCGCTGTGCCCA TCCTAGTAG
sgRNA for T1 site	GCUACCAUAGGCACCACGAGGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAG GCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU
sgRNA for T2 site	GGGCACCAUACCGAGUGAUGGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAG GCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU
sgRNA for T3 site	GAUAUCGUUUACCAAAACGGGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAG GCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU
sgRNA for T4 site	GAUUGGAGAGUUAGACCACGGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAG GCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU
sgRNA for T5 site	GAUCCACAAGUUACAAUUGGGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAG GCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU

Table S1. Nucleic acid sequences used in this study.
The sequences corresponding to each binding site is labeled in colors (Red, orange, green, blue and purple for T1-5, respectively), and the corresponding PAM sequences are underlined.

dCas9-SpyCatcher (173.9 kDa)

```
MDKKYSIGLA IGTNSVGWAV ITDEYKVPSK KFKVLGNTDR HSIKKNLIGA LLFDSGETAE
ATRLKRTARR RYTRRKNRIC YLQEIFSNEM AKVDDSFFHR LEESFLVEED KKHERHPIFG
NIVDEVAYHE KYPTIYHLRK KLVDSTDKAD LRLIYLALAH MIKFRGHFLI EGDLNPDNSD
VDKLFIQLVQ TYNQLFEENP INASGVDAKA ILSARLSKSR RLENLIAQLP GEKKNGLFGN
LIALSLGLTP NFKSNFDLAE DAKLQLSKDT YDDDLDNLLA QIGDQYADLF LAAKNLSDAI
LLSDILRVNT EITKAPLSAS MIKRYDEHHQ DLTLLKALVR QQLPEKYKEI FFDQSKNGYA
GYIDGGASQE EFYKFIKPIL EKMDGTEELL VKLNREDLLR KQRTFDNGSI PHQIHLGELH
AILRRQEDFY PFLKDNREKI EKILTFRIPY YVGPLARGNS RFAWMTRKSE ETITPWNFEE
VVDKGASAQS FIERMTNFDK NLPNEKVLPK HSLLYEYFTV YNELTKVKYV TEGMRKPAFL
SGEQKKAIVD LLFKTNRKVT VKQLKEDYFK KIECFDSVEI SGVEDRFNAS LGTYHDLLKI
IKDKDFLDNE ENEDILEDIV LTLTLFEDRE MIEERLKTYA HLFDDKVMKQ LKRRRYTGWG
RLSRKLINGI RDKQSGKTIL DFLKSDGFAN RNFMQLIHDD SLTFKEDIQK AQVSGQGDSL
HEHIANLAGS PAIKKGILQT VKVVDELVKV MGRHKPENIV IEMARENQTT QKGQKNSRER
MKRIEEGIKE LGSQILKEHP VENTQLQNEK LYLYYLQNGR DMYVDQELDI NRLSDYDVDA
IVPQSFLKDD SIDNKVLTRS DKNRGKSDNV PSEEVVKKMK NYWRQLLNAK LITQRKFDNL
TKAERGGLSE LDKAGFIKRQ LVETRQITKH VAQILDSRMN TKYDENDKLI REVKVITLKS
KLVSDFRKDF QFYKVREINN YHHAHDAYLN AVVGTALIKK YPKLESEFVY GDYKVYDVRK
MIAKSEQEIG KATAKYFFYS NIMNFFKTEI TLANGEIRKR PLIETNGETG EIVWDKGRDF
ATVRKVLSMP QVNIVKKTEV QTGGFSKESI LPKRNSDKLI ARKKDWDPKK YGGFDSPTVA
YSVLVVAKVE KGKSKKLKSV KELLGITIME RSSFEKNPID FLEAKGYKEV KKDLIIKLPK
YSLFELENGR KRMLASAGEL QKGNELALPS KYVNFLYLAS HYEKLKGSPE DNEQKQLFVE
QHKHYLDEII EQISEFSKRV ILADANLDKV LSAYNKHRDK PIREQAENII HLFTLTNLGA
PAAFKYFDTT IDRKRYTSTK EVLDATLIHQ SITGLYETRI DLSQLGGDGG GSGGGSDYDI
PTTENLYFQG AMVDTLSGLS SEQGQSGDMT IEEDSATHIK FSKRDEDGKE LAGATMELRD
SSGKTISTWI SDGQVKDFYL YPGKYTFVET AAPDGYEVAT AITFTVNEQG QVTVNGKATK
GDAHIGSGHH HHHH
```

mCerulean3-SpyTag (29.8 kDa)

```
MGHMHHHHHH GGVSKGEELF TGVVPILVEL DGDVNGHKFS VSGEGEGDAT YGKLTLKFIC
TTGKLPVPWP TLVTTLSWGV QCFARYPDHM KQHDFFKSAM PEGYVQERTI FFKDDGNYKT
RAEVKFEGDT LVNRIELKGI DFKEDGNILG HKLEYNAIHG NVYITADKQK NGIKANFGLN
CNIEDGSVQL ADHYQQNTPI GDGPVLLPDN HYLSTQSKLS KDPNEKRDHM VLLEFVTAAG
ITLGMDELYK GSGGGSAHIV MVDAYKPTK
```

mVenus-SpyTag (30.0 kDa)

```
MGHMHHHHHH GGVSKGEELF TGVVPILVEL DGDVNGHKFS VSGEGEGDAT YGKLTLKLIC
TTGKLPVPWP TLVTTLGYGL QCFARYPDHM KQHDFFKSAM PEGYVQERTI FFKDDGNYKT
RAEVKFEGDT LVNRIELKGI DFKEDGNILG HKLEYNYNSH NVYITADKQK NGIKANFKIR
HNIEDGGVQL ADHYQQNTPI GDGPVLLPDN HYLSYQSKLS KDPNEKRDHM VLLEFVTAAG
ITLGMDELYK GSGGGSAHIV MVDAYKPTK
```

VioA-SpyTag (49.7kDa)

MHHHHHHGSG KHSSDICIVG AGISGLTCAS HLLDSPACRG LSLRIFDMQQ EAGGRIRSKM
LDGKASIELG AGRYSPQLHP HFQSAMQHYS QKSEVYPFTQ LKFKSHVQQK LKRAMNELSP
RLKEHGKESF LQFVSRYQGH DSAVGMIRSM GYDALFLPDI SAEMAYDIVG KHPEIQSVTD
NDANQWFAAE TGFAGLIQGI KAKVKAAGAR FSLGYRLLSV RTDGDGYLLQ LAGDDGWKLE
HRTRHLILAI PPSAMAGLNV DFPEAWSGAR YGSLPLFKGF LTYGEPWWLD YKLDDQVLIV
DNPLRKIYFK GDKYLFFYTD SEMANYWRGC VAEGEDGYLE QIRTHLASAL GIVRERIPQP
LAHVHKYWAH GVEFCRDSDI DHPSALSHRD SGIIACSDAY TEHCGWMEGG LLSAREASRL
LLQRIAAGGG SGGGSAHIVM VDAYKPTK

VioB-SpyTag (114.2kDa)

MSILDFPRIH FRGWARVNAP TANRDPHGHI DMASNTVAMA GEPFDLARHP TEFHRHLRSL
GPRFGLDGRA DPEGPFSLAE GYNAAGNNHF SWESATVSHV QWDGGEADRG DGLVGARLAL
WGHYNDYLRT TFNRARWVDS DPTRRDAAQI YAGQFTISPA GAGPGTPWLF TADIDDSHGA
RWTRGGHIAE RGGHFLDEEF GLARLFQFSV PKDHPHFLFH PGPFDSEAWR RLQLALEDDD
VLGLTVQYAL FNMSTPPQPN SPVFHDMVGV VGLWRRGELA SYPAGRLLRP RQPGLGDLTL
RVNGGRVALN LACAIPFSTR AAQPSAPDRL TPDLGAKLPL GDLLLRDEDG ALLARVPQAL
YQDYWTNHGI VDLPLLREPR GSLTLSSELA EWREQDWVTQ SDASNLYLEA PDRRHGRFFP
ESIALRSYFR GEARARPDIP HRIEGMGLVG VESRQDGDAA EWRLTGLRPG PARIVLDDGA
EAIPLRVLPD DWALDDATVE EVDYAFLYRH VMAYYELVYP FMSDKVFSLA DRCKCETYAR
LMWQMCDPQN RNKSYYMPST RELSAPKARL FLKYLAHVEG QARLQAPPPA GPARIESKAQ
LAAELRKAVD LELSVMLQYL YAAYSIPNYA QGQQRVRDGA WTAEQLQLAC GSGDRRRDGG
IRAALLEIAH EEMIHYLVVN NLLMALGEPF YAGVPLMGEA ARQAFGLDTE FALEPFSEST
LARFVRLEWP HFIPAPGKSI ADCYAAIRQA FLDLPDLFGG EAGKRGGEHH LFLNELTNRA
HPGYQLEVFD RDSALFGIAF VTDQGEGGAL DSPHYEHSHF QRLREMSARI MAQSAPFEPA
LPALRNPVLD ESPGCQRVAD GRARALMALY QGVYELMFAM MAQHFAVKPL GSLRRSRLMN
AAIDLMTGLL RPLSCALMNL PSGIAGRTAG PPLPGPVDTR SYDDYALGCR MLARRCERLL
EQASMLEPGW LPDAQMELLD FYRRQMLDLA CGKLSREAGS GHHHHHHGGG SGGGSAHIVM
VDAYKPTK

VioC-SpyTag (51.0kDa)

MHHHHHHGSG KRAIIVGGGL AGGLTAIYLA KRGYEVHVVE KRGDPLRDLS SYVDVVSSRA
IGVSMTVRGI KSVLAAGIPR AELDACGEPI VAMAFSVGGQ YRMRELKPLE DFRPLSLNRA
AFQKLLNKYA NLAGVRYYFE HKCLDVDLDG KSVLIQGKDG QPQRLQGDMI IGADGAHSAV
RQAMQSGLRR FEFQQTFFRH GYKTLVLPDA QALGYRKDTL YFFGMDSGGL FAGRAATIPD

GSVSIAVCLP YSGSPSLTTT DEPTMRAFFD RYFGGLPRDA RDEMLRQFLA KPSNDLINVR SSTFHYKGNV LLLGDAAHAT APFLGQGMNM ALEDARTFVE LLDRHQGDQD KAFPEFTELR KVQADAMQDM ARANYDVLSC SNPIFFMRAR YTRYMHSKFP GLYPPDMAEK LYFTSEPYDR LQQIQRKQNV WYKIGRVNGG GSGGGSAHIV MVDAYKPTK
VioD-SpyTag (44.6kDa) MHHHHHHGSG KILVIGAGPA GLVFASQLKQ ARPLWAIDIV EKNDEQEVLG WGVVLPGRPG QHPANPLSYL DAPERLNPQF LEDFKLVHHN EPSLMSTGVL LCGVERRGLV HALRDKCRSQ GIAIRFESPL LEHGELPLAD YDLVVLANGV NHKTAHFTEA LVPQVDYGRN KYIWYGTSQL FDQMNLVFRT HGKDIFIAHA YKYSDTMSTF IVECSEETYA RARLGEMSEE ASAEYVAKVF QAELGGHGLV SQPGLGWRNF MTLSHDRCHD GKLVLLGDAL QSGHFSIGHG TTMAVVVAQL LVKALCTEDG VPAALKRFEE RALPLVQLFR GHADNSRVWF ETVEERMHLS SAEFVQSFDA RRKSLPPMPE ALAQNLRYAL QRGGGSGGGS AHIVMVDAYK PTK
VioE-SpyTag (24.8kDa) MHHHHHHGSG ENREPPLLPA RWSSAYVSYW SPMLPDDQLT SGYCWFDYER DICRIDGLFN PWSERDTGYR LWMSEVGNAA SGRTWKQKVA YGRERTALGE QLCERPLDDE TGPFAELFLP RDVLRRLGAR HIGRRVVLGR EADGWRYQRP GKGPSTLYLD AASGTPLRMV TGDEASRASL RDFPNVSEAE IPDAVFAAKR GGGSGGGSAH IVMVDAYKPT K

Table S2. Amino acid sequences of the proteins used in this study.

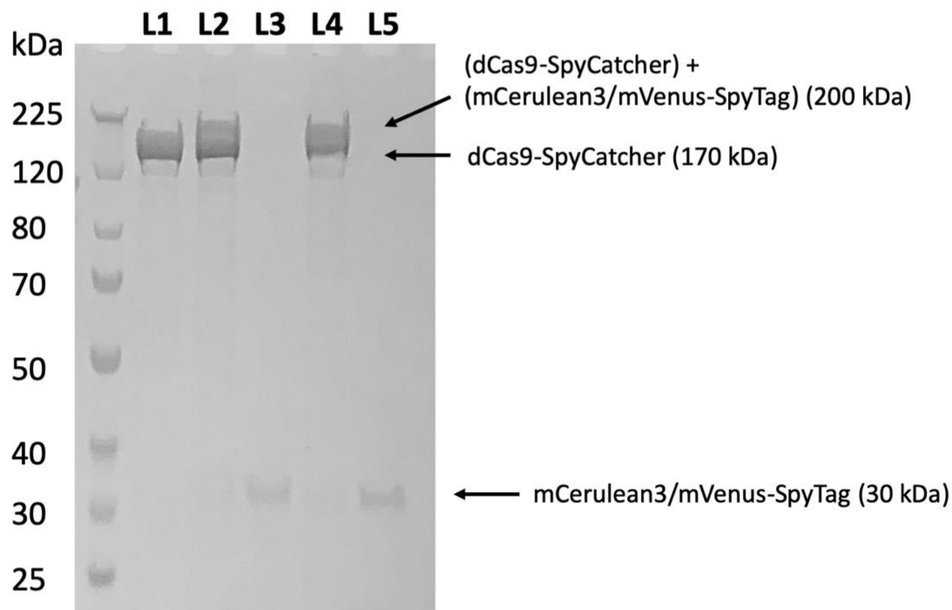

Figure S1. SDS-PAGE showing the conjugation of fluorescent proteins to dCas9-SpyCatcher. L1: dCas9-SpyCatcher (1μM); L2: dCas9-SpyCatcher (1μM) mixed with mCerulean3-SpyTag (0.5μM); L3: mCerulean3-SpyTag (0.5μM); L4: dCas9-SpyCatcher (1μM) mixed with mVenus-SpyTag (0.5μM); L5: mVenus-SpyTag (0.5μM). Upon mixing the two components, the upward shift in the band corresponding to dCas9-SpyCatcher as well as the disappearance of the band corresponding to mCerulean3/mVenus-SpyTag were observed, indicating successful conjugation. Note that the conjugation is unaffected by the SDS-PAGE conditions due to covalent isopeptide bond formation.

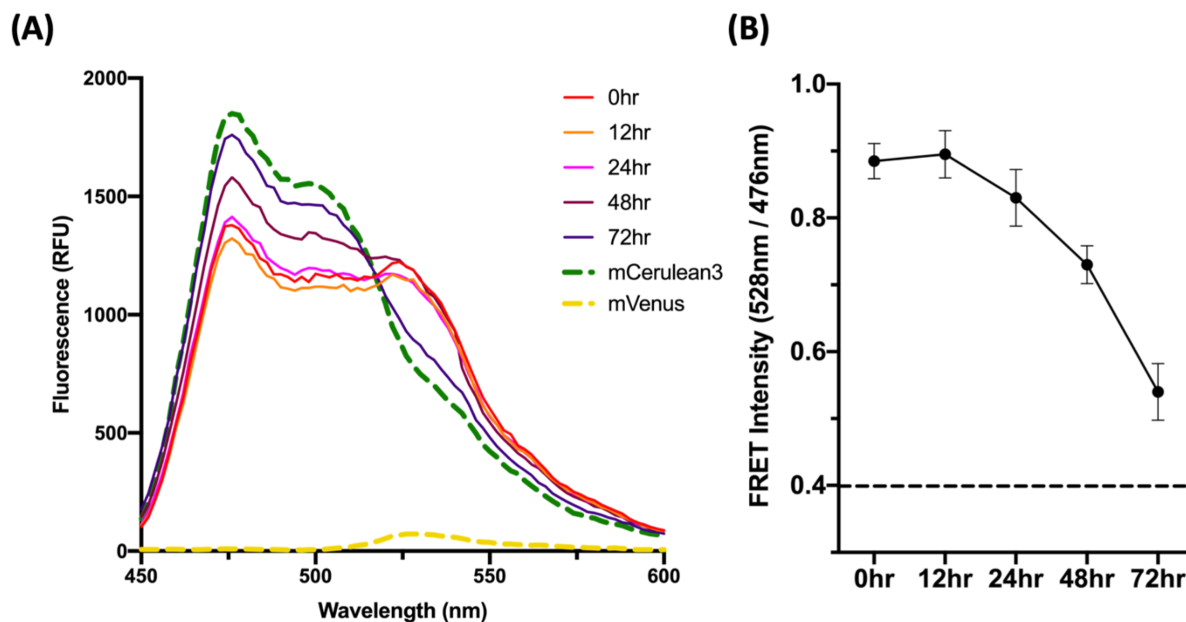

Figure S2. Stability of the assembled protein-dCas9-DNA complex assessed by monitoring the FRET response over time.

mCerulean3 and mVenus was placed at T1 and T2, respectively, and their (A) fluorescence emission spectra upon excitation at 412 nm and (B) ratiometric FRET intensity were monitored for 72 hours. Total concentration of the protein-dCas9-DNA complex was 0.5 μM for each measurement. Strong FRET was maintained until 24 hours after dCas9 binding to the template, whereas the weakening of FRET was observed at 48 hours; 72 hours later the emission spectrum and the (A_{528nm}/A_{476nm}) ratio were largely indistinguishable from that of mCerulean3 alone, indicating possible dissociation of the DNA-dCas9 complex. In (B), the dashed line at 0.4 represents the value measured from mCeruean3 alone, and the error bars represent the standard deviation (SD) from at least two independent experiments.

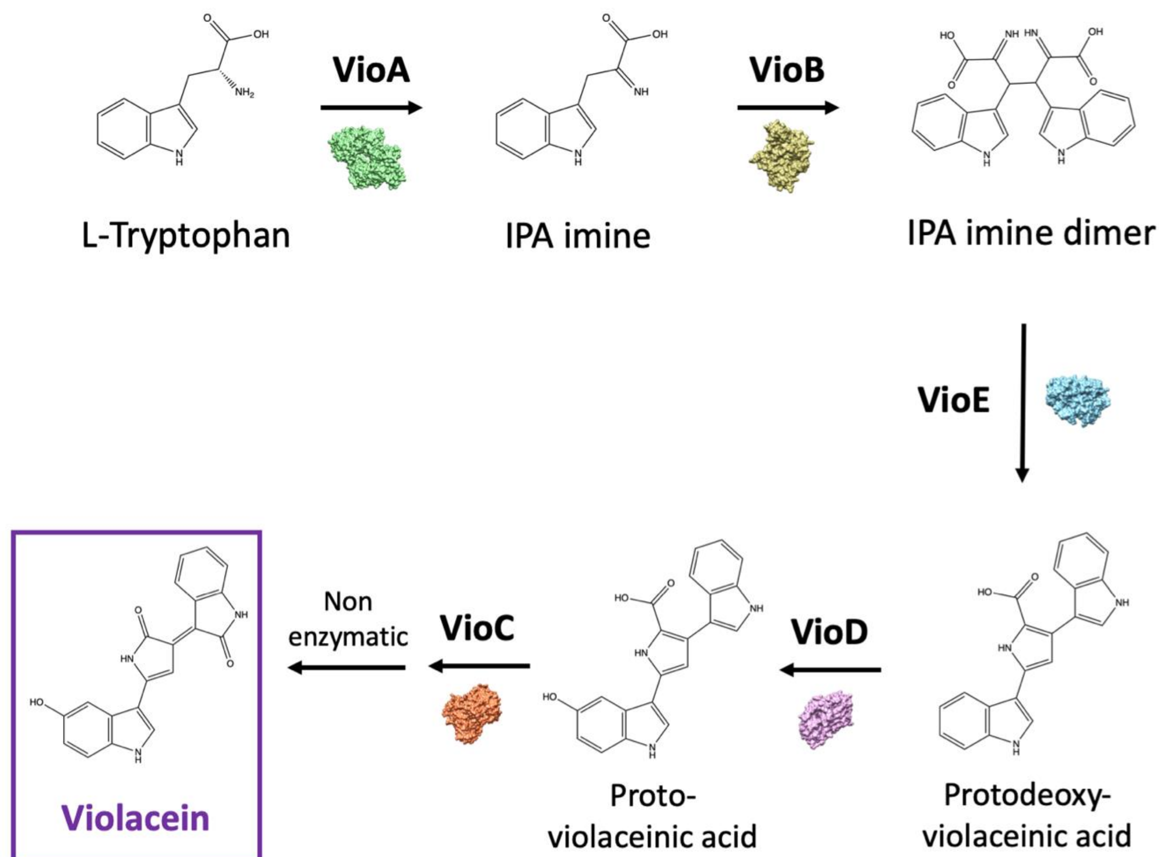

Figure S3. Scheme of the violacein biosynthesis pathway.

L-tryptophan is converted to the purple pigment violacein in a series of reactions involving the five enzymes VioA-E. Note that the last step that yields the final product proceeds spontaneously.

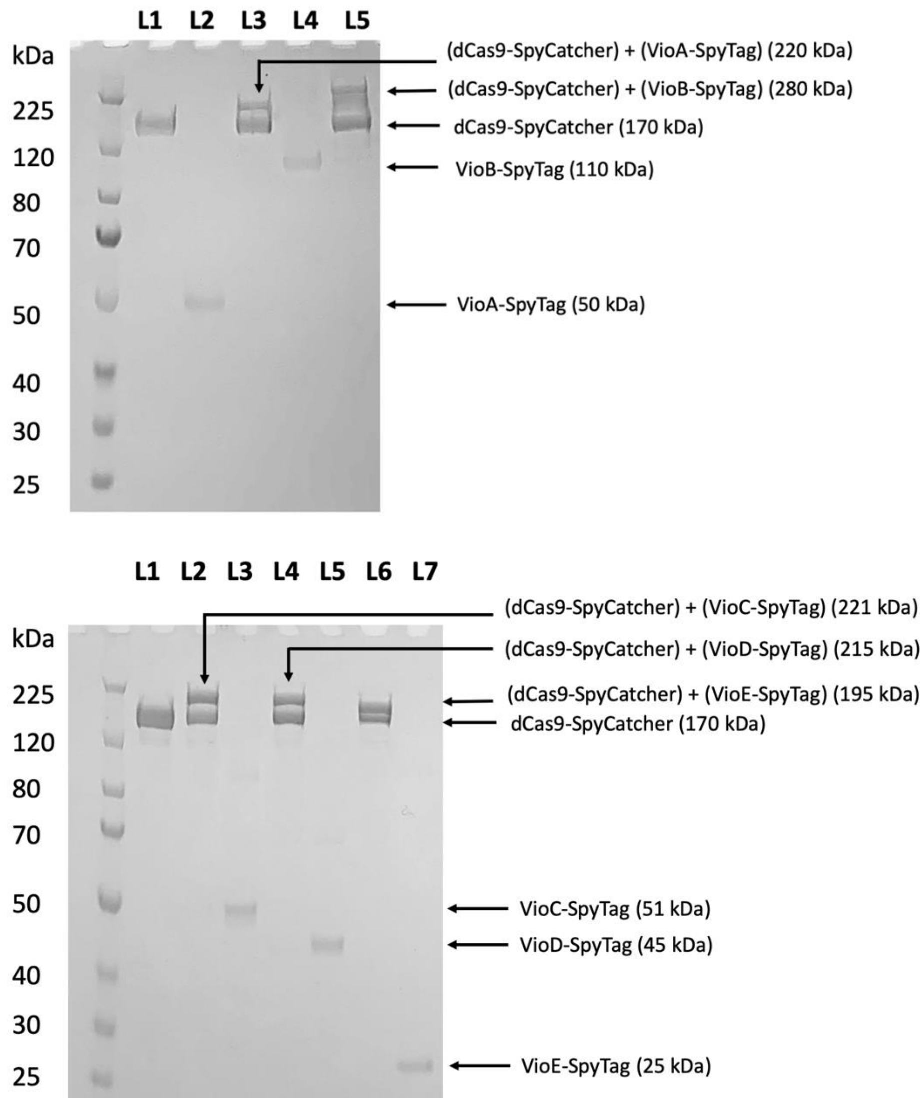

Figure S4. **SDS-PAGE showing the conjugation of the Vio enzymes to dCas9-SpyCatcher.**
(Up) L1: dCas9-SpyCatcher (1μM); L2: VioA-SpyTag (0.5μM); L3: dCas9-SpyCatcher (1μM) mixed with VioA-SpyTag (0.5μM); L4: VioB-SpyTag (0.5μM); L5: dCas9-SpyCatcher (1μM) mixed with VioB-SpyTag (0.5μM); (Down) L1: dCas9-SpyCatcher (1μM); L2: dCas9-SpyCatcher (1μM) mixed with VioC-SpyTag (0.5μM); L3: VioC-SpyTag (0.5μM); L4: dCas9-SpyCatcher (1μM) mixed with VioD-SpyTag (0.5μM); L5: VioD-SpyTag (0.5μM); L6: dCas9-SpyCatcher (1μM) mixed with VioE-SpyTag (0.5μM); VioE-SpyTag (0.5μM). Upon mixing the two components, the upward shift in the band corresponding to dCas9-SpyCatcher as well as the disappearance of the band corresponding to the Vio enzymes were observed, indicating successful conjugation.

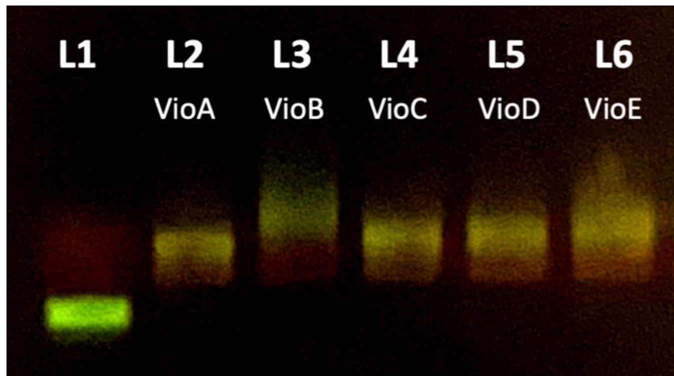

Figure S5. Effect of enzyme type on binding of enzyme:dCas9 complex to DNA scaffold.

We examined whether the binding of the enzyme:dCas9 complex to DNA is affected by the type and size of the enzyme used. dCas9-SpyCatcher bound to each Vio enzyme (VioA-E) was assembled on the T1 site of the fluorescently labeled DNA scaffold, and the migration was monitored using EMSA. L1: DNA scaffold control; L2: DNA bound to (VioA-SpyTag):(dCas9-SpyCatcher); L3: DNA bound to (VioB-SpyTag):(dCas9-SpyCatcher); L4: DNA bound to (VioC-SpyTag):(dCas9-SpyCatcher); L5: DNA bound to (VioD-SpyTag):(dCas9-SpyCatcher); L6: DNA bound to (VioE-SpyTag):(dCas9-SpyCatcher); total concentration of the DNA-bound complex was 0.5 μM in all lanes. Complete binding was observed for all Vio enzymes in L2-L6, indicating that the assembly efficiency is not affected by the type and size of the enzyme employed.

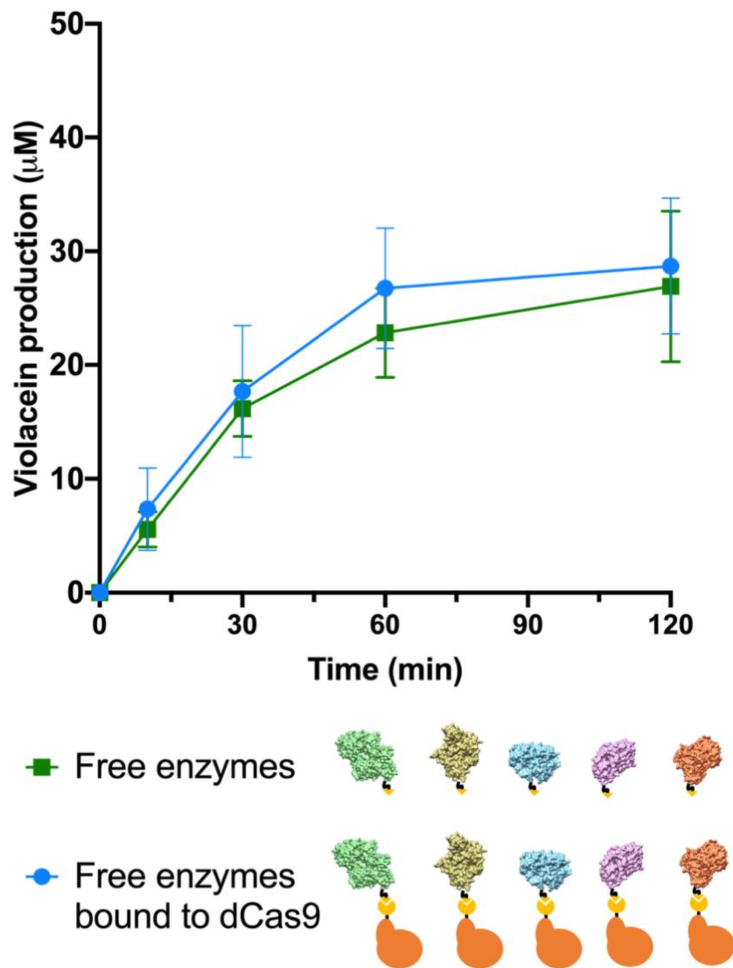

Figure S6. **Effect of dcas9-SpyCatcher conjugation on violacein production.**
Violacein production was monitored for 120 min for the free Vio enzymes in solution, with and without conjugation to dCas9-SpyCatcher. The reaction mixture contained 0.5 μM of each enzyme, and the experiment was performed as described in the Methods section. The difference in violacein production was insignificant for the two conditions, verifying the minimal effect of dCas9-SpyCatcher conjugation on overall productivity.