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 \(\times\) 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 \(\times\) 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, 200 mM 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).

<table>
<thead>
<tr>
<th>Forward primers (5’ -&gt; 3’)</th>
<th>Reverse primer (5’ -&gt; 3’)</th>
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<tr>
<td>T1: TAATACGACTCACTATAGCTACC</td>
<td>(Common for all target sites)</td>
</tr>
<tr>
<td>T2: TAATACGACTCACTATAGGGGCACCA</td>
<td>AAAAGCACCACCGACTCGGTG</td>
</tr>
<tr>
<td>T3: TAATACGACTCACTATAGATATCGT</td>
<td></td>
</tr>
<tr>
<td>T4: TAATACGACTCACTATAGATTGGA</td>
<td></td>
</tr>
<tr>
<td>T5: TAATACGACTCACTATAGATCCAC</td>
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</tr>
</tbody>
</table>
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′-CTACTAGGATGGCAGCGGAGA-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 MgCl₂, 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 \( \varepsilon_A \) and \( \varepsilon_D \) 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:

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

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

### 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.

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<tr>
<th>Title</th>
<th>Sequence (5’ -&gt; 3’)</th>
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<td></td>
<td>TCATAGGGCGGTTCTAGGATTGGAAGTTAGACCACGTGGATCACGTTACCACCAT</td>
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dCas9-SpyCatcher (173.9 kDa)

MGKKYSIGLA IGTNSVGAV ITDEYKVP SK KFKVLGNTDR HS1 I9NPLIGA LLFDGSE TAE
ATORLKLRTARR RTYRRKRRIC YLQEISFNEM AKVDDSFFHR LEESSFVVEED KKH Do RHPF I
NIVDVEMAYHE KYPITYHLR KLV DSTD KAD LRLY IYLAH MIKFRGHFLI EGD LNPDNSD
VDKLFQILVQ YTNQLFEE N I NASAGVDAK ILSRALS KSR RELNLIAPLQ GEKKNGLFGN
LIALSLGLTP NFKNFSDLAE DAKLQLS KDT YDDDLDNLLA QIGDQYADF LAAKNLSDAI
LLSDILRVN IET I KAPL S A M I KRY DEH HQ QLGLKALV R QQPEK YKE EI FFDQSINKGYA
GYIDGGAQZE EFY K FKP P IL EKMDGTE ELLKVLNRED LRR Q KRTF DNGS I PHQ IHLGELH
AILRRQEDFY PFL D NRE KEI K I I TFRIP Y YVGPLARGNS RFAM TRKSE ETITPWNFEE
VVDKGASAQS FIERM TNFDK NLPNKEKVL PK HSSL EYFVTV YNELTKVKV Y TEGMRKPAFL
SGEQKKAIVD LLFKTNK KVT YVK QLKE D YFK KIECFDSVEI SGVDE RFNAS LGTYHDD LKI
IKDKDFLDNE UNEDILEDIV LTDLLFEDRE MIEERLKYA HLFDKVMQK LKRRTYTWG
RLSRKLINGI RDQSGK TIL DFL KSDF GAN RNFMLIHD D SLTFKEDIQK A QVSGQGDSL
HEHIANL AGS PAI K KI G L OT K VVDKV LEGN RPKENV IEMAREN Q T Q KQKNSRER
MKRIEEGIKE LGSQILKE HEP VENTQL NKE LLYLYQNGR DMYDVQELDI N RLS D YVDVA
IVPQSFLLK Q S DNRGKSDNV PESE EVVKKM NYWQRLNNK LI TQRKFDNL
TCAERGG LSE LDKAGFI KRQ LVETQI TKH VAQILDSSRM NKD Y ENDKL REVKVITLKS
KLVSD FRKD F Q F YK VRE INN YHHAHAD YLAVV GTALIKK YPKLESEFVY GDYKVDV VRK
MIAS E QEGY S N M NFKTE L TAN GEIRK R SLIE TNE G EIVWDKGDRF
ATVRKVL SMP QQVIVK KTEV QT GGS FKE SI LPKRNSDKL ARKKWD PKK YGGFDS PTVA
YSV L V VAK VE KGS KKLKS V KEL L G T I M R SSF E KNP ID FLEAKGYKEV KKD LIIKLP
YS LFELENGS R KARL SAGEL Q KGN E ALP S KHY NL L YAS HYE KL KSG P E DNEQKQLFVE
QKH YLD NEI E IQ IS F SKRV ILAD ANLDK V ASYANK RD PK RIE Q AEN I HLFT LTN LG
PAFAQYFD TTT IDR KRYST KK E V LDL A L H Q S TT G Y T E RI D LS Q LG D DG GSGG SSYDI
PTTENLYFQG AMV D T LSG LS SE QG Q S GDMT IEB DSA THIK FS KRDE D GKE L AGATMLERD
SSGKTISTTWI SDGQVSDKYL Y P Y GKYTFVET AAPDG Y EVAT ATIFTVNE QG Q VTVNGKATK
GDHAGSGHH HHHH

mCerulean3-SpyTag (29.8 kDa)

MGHMMMHHHH GGVSKGEELF TGV PILVEL DGDVNGHKFS VSGEGEGDAT YGKLT LK FIC
TTG KL LPVF WP TLV TTSWGV QCFARYPDHM KQH DFFKSAM PEGYQERTI FFKDDGNYKT
RAEVKFEGDT LVRNIELKGQ DPKEDGNLQG HKLEYNAIHG NYTADQKQ NGKANFGLN
CNIEDGSQG SQA ADHYQQNTPI GDGVPLLDPN HYLSTQSKLS KDP N ERD HM VLLEFVTAG
ITLGMDELYK GSGGGSAHV MV DAY KPTK

mVenus (30.0 kDa)

MGHMMMHHHH GGVSKGEELF TGV PILVEL DGDVNGHKFS VSGEGEGDAT YGKLT LKL CLIC
TTG KL LPVF WP TLV TTSWGYL QCFARYPDHM KQH DFFKSAM PEGYQERTI FFKDDGNYKT
RAEVKFEGDT LVRNIELKGI DPKEDGNILG HKLEYNAISH NYTADQKQ NGKANFKIR
HNIEDGGVQL ADHYQQNTPI GDGVPLLDPN HYLSTQSKLS KDPNEKRDHM VLLEFVTAG
ITLGMDELYK GSGGGSAHV MV DAYKPTK
**VioA-SpyTag (49.7kDa)**

MHHHHHHGSG KHSSDICIVG AGISGLTCAS HLDSAPCRG LSLRIFDMQQ EAGGRIRSKM
LDGKASIELG AGRYSPOQLHP HQFSSAMQYS QKSEVYFPFTQ LKFSHVQQQ LKRAMNELSP
RLKEHGHKESF LQFVSRYQGH DSAVMIRSM YGADLFLPDI SAEMAYDIVG KHPEIQSVTD
NDANQWFFAAE TGFAQLIQGQ KAKVKAAGAR FSLGYRLSV RTDGDGYLLQ LAGDDGKWLE
HRTRHILAI PPSAMAGLNV DFPEAWSGAR YGSLPLFKGF LTYGEPEWWDY YKLDQQVLLIV
DNPLRKYIFK GDKYLFYFTD SEMANYWRCG VAEGEDGYLE QIRTHLASAL GIVRERIPQP
LAHVVHKYWAH GVEFCRDSDI DHPSALSIRD SGIIACSADY TEHCGWMEGG LLSAREASRL
LLQRIAGGG SGGSAHIYM VDAYKPTK

**VioB-SpyTag (114.2kDa)**

MSILDFPRHIH FRGWARNVAP TANRDPHGHI DMASNTVAMA GEPFDLARHP TEFRHLRSL
GPRFGLDERA DEPEGFSLAE GYNAAGNNHF WSEATVSHV QWDGEADRG DGLVLRALAL
WGHYNYLRT TFNRRAWVDS DPORTRDAQI YAGQFTISPA GAGPGLTWLF TADIIDSHGA
RNNTRGHHIAE RGGHFLDEEF GLARLFQFSV PKDHPFHLFHP PGPFSEAWR RLQLALEDDE
VLGLTQYAL FNMSPPQPN SPVFHMGVG VQLWRONGE SYPAGLRLLRP RPQPLGDLTL
RVNGGRRVALN LACAIPFSTR AAQPSAPDRL TPDLGAKLPL GDLRLREDLG ALLARVPQAL
YQDYWTNHGI VDPLLLEPR SGLTSLSELA EWREQDWVTQ SADSNLYLEA PDRHRGFFF
ESIALRSYFR GEARARPDPD HRIEGMGLVG VESRQDGDAA EWRITGLRPG PARIKLDGGA
EAIPRLVLPD DWALDAATVE EVDYAFYLRL VMAYELVYPF FMSKDFVLSA DRCKCETYAR
LMQJCMDDQQN RDKSYYMPPG RLSAPKRL FLKLYLAEQG QARLQAPPRA GPARIKQAL
LAAELRKAVID LELSVMLQYL YAAYSINPNA GQQQRVDRGA TWEAQLQLAC GSGDRRDRGG
IRAALLETAH EEMIHYLVN NLMALMEPEF YAGVPLMGEA ARQAGFSTDTE FALPFESEST
LARFVLREWLP HFIAPGKSKI ADCYAIRQKA FLDLDLFLGG EAGKRGEGHH LFLNELTNRA
HPQYQFLVFD RDSALFGIAF VTDQEGGGL DSPHYEHSHF QRLREMSARI MAQSAPEPEA
LPALRNPVLQD ESRGCQRVAD GRARALMDQ GVVYELMFM AMQHFAVPLK GSLRRSRLMN
AAIDLMTGLL RPLSCALMN PSHIAGRTAG PPLPGPVDTIR SYDDYALGCR MLARRCERLL
EQASMLEPGW LPDAQMELLD FYRRQMLDLA CGKLSREAGS GHHHHHHHGG SGGSAHIYM
VDAYKPTK

**VioC-SpyTag (51.0kDa)**

MHHHHHHGSG KRAIIIIVGGGL AGGLTAIYLAA KRGYEVHVE KRGDPLRDLS SYVDMSSSRA
IGVSMYRVTG KVSVAAGIPR AELDACGEP IYAMAFSVMQ GNYREKLPLF DFPRPLSLNRA
AFQKLLNMYA NLAVGVRVYFE HKCLDVLDDL KSVLQIQGKDQ PQRQLQGDMG IGAADGHAUSD
RQAMQSGLRR FEFQUTFRH GYKTLVLPDA QALGYRKDTL YFFGMDSSGGL FAGRAATIPD
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<td>MHHHHHHGSG KILVIGAGPA GLVFASQLKQ ARPLWAIDIV EKNDEQEVLG WGVLPGPGQ HPANPLSYL DAPERLPQF LEDFKLVHHN EPSLMSTGVL LCGVERRGLV HALRDKCRSQ GIAIRFESPL LEHGEPLAD YDLVVLANGV NHKTAHFTEA LVPQVDYGRN KYIYGTSQ FDQMNLFRT HGKDFIAHA YKYSVTMSTF IVECSEETYA RARLGMSEESAAYVAKVF QAELGGHGLV SQPGGWRFN MTLGHDRCGD GKLVLGDAL QSGHFSIGHG TTMAVVVAQL LVKALCTEDG VPAALKRDVEE RAPLVLQFLFR GHADNSRVWF ETVEERMHLS SAEFVQSFDA RRKSLPPMPAL ALAQNLRYAL QRGGSASGGG AHIVMVDAYKPTK</td>
</tr>
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**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 \((A_{528\text{nm}}/A_{476\text{nm}})\) ratio were largely indistinguishable from that of mCereulan3 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.