Electronic Supplementary Material (ESI) for Chemical Science.

This version of the ESI published 23/11/2022 replaces the previous version published 08/11/2022. Figure S22 is replaced with a corrected version.

Electronic Supporting Information

Live-cell RNA imaging using the CRISPR-dCas13 system with modified sgRNAs appended with fluorescent RNA aptamers.

This PDF file includes:

Experimental Section

Design of CRISPR-dPspCas13b sgRNA scaffolds: To design a stable RNA scaffold that is compatible for the insertion of multiple RNA aptamers into sgRNA, the aptamers were linked by two-way junctions, in loop or in the 3′ end of the sgRNA. To randomize the framework of the sgRNA-3′-F-2xBroccoli, we used the consensus sequence F30. The detailed design of sgRNA-In-2xBroccoli, sgRNA-3′-F-2 x Broccoli and its variants is shown in Figure S1. mFold[1] was used to fold the RNA sequence and to compute the minimum free energy and suboptimal free energy (SFE).

Plasmid construction: pmRuby3-HSF1, pmRuby3-24xGCN4, and pHAGE-dPspCas13b-3xEGFP-2xNLS-IRES-puro were from Ling-Ling Chen. pHAGE-dPspCas13b-2xNLS-IRES-puro and pHAGE-dPspCas13b-RFP-2xNLS-IRES-puro were constructed from pHAGE-dPspCas13b-3xEGFP-2xNLS-IRES-puro by removing 3xEGFP or replacing it with RFP. A series of modified sgRNA cassettes (synthesized by Genscript) was cloned into the pLKO.5 plasmid after the U6 promoter between the BmgBI and EcoRI restriction enzyme cutting sites, and a TagBFP sequence was inserted downstream to modify the sgRNA cassettes after the EF1α promoter. Then, we carried out gold gate assembly generation of these modified sgRNAs into the PC0043 vector, which resulted in two BbsI sites between the U6 promoter and the transcription start site of each cassette. The details of the cloning strategy are shown in Figure. S21. All the primers used in gene cloning procedure were synthesized by Sangon Biotech. The dPspCas13b and modified sgRNA expression vector reported here will be deposited at Addgene. All repetitive gRNA spacers of the target sites are listed in Table S1.

***In vitro* characterization of modified sgRNA cassettes:** The modified sgRNA was synthesized by *in vitro* transcription with T7 RNA polymerase according to the manufacturer's protocol. To generate the template for the modified sgRNA, we designed separate shorter overlapping sequences. In each forward primer, a T7 promoter sequence was flanked on the 5′-end of the target region of Pepper. Transcription reactions were performed at 37 °C for 4.0 hr in 1× transcript reaction buffer containing 100 mM HEPES-KOH (pH 7.9), 20 mM $MgCl_2$, 30 mM DTT, 2 mM each NTP, 2 mM spermidine, 0.1 mg/mL T7 RNA polymerase, and 300 ng PCR fragments. Following DNA degradation using DNase I at the end of transcription, the transcribed RNA products were then purified using the NaOAc/phenol/chloroform method. The purified Pepper RNA was resuspended in RNase-free H_2O. For the aptamer folding and *in vitro* fluorescence study, 5 μM RNA was incubated with 20 μM DFHBI-1T (0.5 μM RNAs with 1 μM HBC 530)

in 100 μL 1 × folding buffer (40 mM HEPES at pH 7.4, 100 mM KCl, 5 mM $MgCl_2$). After a 1-hr dark incubation at 37 ℃, the emission spectrum was measured in a 1-cm path-length cell by using an F-4600 FL Spectrophotometer (Hitachi). Slit width: excitation = 5 nm, emission = 5 nm, DFHBI-1T (λex = 488 nm), HBC 530 (λex = 485 nm).

Cell culture and transfection: Human embryonic kidney 293T cells were cultured on 20-mm glass-bottom dishes (Nest Corporation) in DMEM (Gibco) containing high glucose and supplemented with 10% (vol/vol) FBS at 37 °C and 5% CO_2. For transfection, 200 ng each of dPspCas13b-RFP-2xNLS, dPspCas13b-3xEGFP-2xNLS dPspCas13b and dPspCas13b-2xNLS and 1 μg of plasmid DNA for the desired guide RNAs were cotransfected using Lipofectamine 3000 (Thermo Fisher Scientific), and the cells were incubated for another 24-48 h before imaging. siRNAs (sequences in Table S2) were transfected using Lipofectamine 3000.

Cells and Virus: The 293T cells were infected with SFTSV at an MOI of 1 and cultured for 48 h. SFTSV (applied by Wuhan Institute of Virology, Chinese Academy of Sciences) was cultured on Vero cells with the DMEM medium containing 2% FBS, supernatants were collected through a 0.45um syringe filter and cells were passaged 7 day after infection. The virus titer was determined by indirect immunofluorescence assay and measured by Karber's method. Briefly, Vero cells were cultured in a 96-well plate at a density of 1 × 104 cells/well and infected with 100 μl 10-fold serially diluted virus solution for 48 h. Then the cells were washed and permeabilized with 0.2% Triton X-100 for 20 min, blocked with 5% BSA in PBS for 1 h, then incubated with the NP antibody (applied by Wuhan Institute of Virology, Chinese Academy of Sciences) at 4°C overnight. After incubation with the Fluorescent secondary antibody (Proteintech), cells were incubated with 4,6-diamidino-2-phenylindole (DAPI) solution for 5 min and washed three times with PBS. The cells were analyzed with a fluorescence microscope (Olympus IX73).

Flow cytometry: HEK 293T cells expressing the desired fluorescent dPspCas13b and/or guide RNA were subjected to MA900 Multi-Application Cell Sorter (Sony) equipped with 405-, 488-, and 561-nm excitation lasers. The emission signals were detected by using filters at 450/45 nm (wavelength/bandwidth) for BFP, 525/40 nm for Broccoli or Pepper, and 610/20 nm for RFP. All data were processed with FlowJo software. For Broccoli (Pepper) signal analysis, 5 μM DFHBI-1T (1 μM HBC 530) was added before FACS.

RT-qPCR: Cells were transfected as described in the previous sections. Briefly, 1.5 μg of dPspCas13b plasmid DNA and 1 μg of total guide RNA plasmid DNA were cotransfected using Lipofectamine 3000 (Thermo Fisher Scientific), and the cells were incubated for another 48 hr before collection. RNA was extracted with TRIzol reagent (Invitrogen) following the manufacturer's instructions, and reversed to cDNA by Hiscript III Reverse Transcriptase (Vazyme). Quantitative RT-PCR was performed using the Biorad CFX96 and Taq Pro Universal SYBR qPCR Master Mix (Yeasen), in a 10μl volume reaction mixture of 5μl SYBR Green PCR master mix, 1μl cDNA diluted template 1μl 1μM forward and reverse primers, and RNase-free water. RT-qPCR using the following primers and probe (Genecreate DNA Technologies) for sgRNA-In-2xBroccoli: forwards primer, 5′-GTTGTGGAAGGTCCAGTT-3′; 5′-GTCGGGTCCAGATATTCGTA-3′; and reverse primer, 5′-GCCCACACTCTACTCGACAG-3′. The forwards primer for sgRNA-In-8xPepper was 5′-GTTGTGGAAGGTCCAGTT-3′ and 5′-GTCGGGTCCAGATATTCGTA-3′, and the reverse primer was 5′-GCCCACACTCTACTCGACAG-3′. For BFP RNA from the same plasmid carrying guide RNA, the primers and probes were as follows: forwards primer, 5′-CGCCAAGACCACATATAGATCC-3′; probe, 5′-ACCCGCTAAGAACCTCAAGATGCC-3′; and reverse primer, 5′-TGGCCTCCTTGATTCTTTCC-3′. BFP RNA produced from the same plasmid as guide RNA was used as the calibration standard for transfected plasmid DNA. All data were normalized to the cell number using GAPDH mRNA as the internal reference.

The genome of SFTSV comprises three negatively-stranded RNA segments, the small (S), medium (M), and large (L) segments. Notably, the nucleoprotein (NP) encoded by the S segment play a crucial role in the replication of SFTSV, by protecting the genomic RNA from degradation by exogenous nucleases or the immune systems in the host cells and. Normally, the NP mRNA RT-PCR amplification is used to confirm the SFTSV infection[2,3]. The primer sequences for SFTSV-NP and GAPDH are as follows: SFTSV-NP-Forward: ACTTCTGTCTTGCTGGCTCC; SFTSV-NP-Reverse: CTAATTCCAACTGCGGGGGT; GAPDH-Forward: GTCTCCTCTGACTTCAACAGCG; GAPDH-Reverse: ACCACCCTGTTGCTGTAGCCAA. All data were normalized to the cell number using GAPDH mRNA as the internal reference.

Single molecule RNA Fluorescence in situ Hybridization (smFISH): All smFISH probes were designed via Stellaris Probe Designer and labeled with Cy5 on the 3′ ends (Table S3). RNA FISH was carried out as described before[4]. Briefly, after the transfection of sgRNA and Cas protein plasmids for CasFAS imaging, cells were fixed with 4% PFA for 15 min and washed with DPBS for 3 times, followed by permeabilization with 70% EtOH overnight and washing with DPBS for

3 times. Cells were incubated in 10% formamide/2 x SSC for 15 min at 50 °C followed by hybridization at 30 °C for 16 h. The fluorophore was added after cells fixing and FISH probe hybridization.

Confocal laser scanning microscopy: Cells cultured in confocal vessels and transfected with desired plasmids were imaged after removal of medium and staining with fluorophore. Images were acquired with a microscope (DMIRB; Leica Biosystems) equipped with an EMC CD camera (iXon-897D; Andor Technology) and mounted with a 2 × magnification adapter and 63 × oil objective lens (NA 1.4). Basically, 405-, 488-, 552- and 638-nm excitation lasers were used for BFP/DAPI, Broccoli/Pepper, RFP/mRuby3, and smFISH fluorescence excitations, respectively. Correspondingly, 415-478 nm, 498-542 nm, 562-618 nm and 650-700 nm emission signals were acquired. Additionally, 3 washes with DMEM and alternative fluorescent stains were completed on the object stage of the microscope to capture the same cells after restaining. All fluorescence imaging data were analyzed by Fiji/ImageJ (https://imagej.net/Welcome).

Fluorophore Synthesis: DFHBI-1T was purchased from Sigma, and both HBC530/620 were synthesized following the protocols given in a previous report[5] (Figure S22).
HBC530. ^{1}H NMR (400 MHz, DMSO-*d6*) δ 8.01 (s, 1 H), 7.93 (s, 2 H), 7.90 (s, 2 H), 7.86 (d, *J* = 8.7 Hz, 2 H), 6.85 (d, *J* = 9.1 Hz, 2 H), 4.77 (t, *J* = 5.3 Hz, 1 H), 3.59 (t, *J* = 5.3 Hz, 2 H), 3.53 (t, *J* = 5.6 Hz, 2 H), 3.07 (s, 3 H). ^{13}C NMR (101 MHz, DMSO-*d6*) δ 152.09, 145.93, 140.15, 133.39, 132.43, 125.90, 120.51, 119.37, 119.22, 111.96, 110.08, 100.17, 58.67, 54.27, 39.23.
HBC620. ^{1}H NMR (400 MHz, DMSO-*d6*) δ 8.37 (s, 1 H), 7.88 (d, *J* = 8.6 Hz, 2 H), 7.84 (s, 1 H), 7.79 (d, *J* = 8.6 Hz, 2 H), 6.35 (s, 1 H), 4.88 (t, *J* = 5.4 Hz, 1 H), 3.65 (q, *J* = 5.6 Hz, 2 H), 3.45 (t, *J* = 5.7 Hz, 2 H), 3.09 (s, 3 H). ^{13}C NMR (101 MHz, DMSO-*d6*) δ 165.77, 148.99, 139.79, 139.03, 133.37, 131.30, 130.81, 125.51, 125.43, 119.31, 119.24, 109.42, 96.80, 93.82, 58.46, 57.52, 40.92.

Table S1. Primer sequences of generating a multiple targeting CasFAS system for SFTSV.

Sequences (5′-3′)	
F1	CACTATAGGGGAAGACAAGTTGGGCGTTATTGTAGTCTCGTCGTTGTGGAAGGTC CAGTTGGCCGAGACGGTCGGGTCCATCTGAG
F2	ACTATAGGGGAAGACAACTTTAATGCGGCCGTTGTGGAAGGTCCAGTTGGCCGAG ACGGTCGGGTCCATCTGAG
R1	GCTCAGCGGGAAGACTTAAAGCAACCGCGGTGTTTCGTCCTTTCCACAAGATA
R2	TCAGCGGGAAGACTTCAACTAACTCGAGGCAATAAGATCGGTGTTTCGTCCTTTCCACAAGATA

SFTSV guide

BbsI site

Table S2. sgRNA spacer sequences in sgRNA-aptamer to target intracellular RNA.

sgRNA spacer sequences (5′-3′)	
gNC spacer	Null
GCN4 spacer	GGTGGTAATTCTTTGAAAGCAG
MUC4 spacer	GTGACCTGTGGATGCTGAGG
SatIII spacer	GATTCCAATCCATGCCATTCCAC
NEAT1 spacer	AATTGTTTGCATCATCCCCAAGTCATTGGT
MALAT1 spacer	AAGTTGGCAAGTAACTCCCAAT
β-actin spacer	TGGAGCGAGCATCCCCCAAA

Table S3. siRNA sequences used in *MUC4* mRNA knock down in HEK 293T cells.

	Sence (5′-3′)	Antisence (5′-3′)
MUC4-Homo-1	CCCUCUACUUCACAGACAATT	UUGUCUGUGAAGUAGAGGGTT
MUC4-Homo-2	GCUGGAAUGACAAGCCCUATT	UAGGGCUUGUCAUUCCAGCTT
MUC4-Homo-3	GCACUCGCCCUCAGAAAUUTT	AAUUUCUGAGGGCGAGUGCTT

Table S4. NEAT1 smFISH probes and GCN4 smFISH probes (5′-3′).

NEAT1-sm-1	ACTGCTTACTTTATCTAAAGCT
NEAT1-sm-2	CACAACAAAATCCAAGGCATGC
NEAT1-sm-3	AAGTGAACCAGACACACAATGC
NEAT1-sm-4	CCATTCTTAAAAGTAGGCAAGC
NEAT1-sm-5	CCACACGAAACCTTACATCTTC
NEAT1-sm-6	AACTAATGATTTGTGCATTGCC
NEAT1-sm-7	ACTCCTTGGTAACTGTCACAAC
NEAT1-sm-8	TAAATACAGATGGAAGGCCTTC
NEAT1-sm-9	TAACCATTCTAACTAGGGTCAA
NEAT1-sm-10	ATCAATGAAGAAAGGGAGCCAC
NEAT1-sm-11	TGTGGAATGTGACAGAGAATCC
NEAT1-sm-12	CCAACAAAATGGGGAGGAACTG
NEAT1-sm-13	ATTACACAGGATATGGCAGATC
NEAT1-sm-14	AGTGACGGTGAGAATGCAACTC
NEAT1-sm-15	TGCATCAAAACACATGGAGCAG
NEAT1-sm-16	GTTAGTGTCGGGAATACCAATG
NEAT1-sm-17	GTCAACACCAGACACAGTGAAG
NEAT1-sm-18	CAGTGCAAACAAGAAGACAGCG
NEAT1-sm-19	GCAGTTGAAAATAACCCTCATA
NEAT1-sm-20	TAATTGTGTCCCCACATAGTAA
NEAT1-sm-21	AACCACAACAGGTGGATTATTT
NEAT1-sm-22	AAGCAAATGTGCCCATAACAAT
NEAT1-sm-23	CCCATGCATACAAAAGAAATCT
NEAT1-sm-24	CACCAGTGAGAACACTAATTGA
NEAT1-sm-25	AGGAGCAAAGGTTTTCAATGTG
NEAT1-sm-26	CAGACAAAGCAATGCTCACATC
NEAT1-sm-27	GACACAAGGTAGAGATGTTCAG
NEAT1-sm-28	TCCTTAAGATGTAAATGGCTTT
NEAT1-sm-29	ATCGTTTAACTTTTATCTACCT
NEAT1-sm-30	GATCTACACATAACAAGTTCAG
NEAT1-sm-31	AATATTTATGTACTCTTGGGGT
NEAT1-sm-32	ACCATACAAGTTCCATAAGAAA
NEAT1-sm-33	TCAAGGTCTTCTGAATTGAACC
NEAT1-sm-34	GTGAGAGCTCAACAAGGAGTAG

NEAT1-sm-35	GGTACAGGGAAATACTACTGGA
NEAT1-sm-36	AAAGCCTCTTCAAATGTGTTTG
NEAT1-sm-37	GAAAGTTATGCTTTCCACAGAA
NEAT1-sm-38	GATCTTTTTTGAGCACGAACAA
NEAT1-sm-39	GGCTACATATCCTTTAGTATAA
NEAT1-sm-40	AATTTACAGAGATGCTCACAGC
NEAT1-sm-41	AGTCATTTGAGTCTGTGAAGAG
NEAT1-sm-42	TACTCACAACAGGAAGTGTAGC
NEAT1-sm-43	TTAGTGGCTATGTAGGGAAATG
GCN4-sm-1	GCAGTTCTTCTCCACTGCCAGAA
GCN4-sm-2	GGTGGTAATTCTTTGAAAGCAG
GCN4-sm-3	GCTACCTCATTTTCCAGGTGG

Table S5. dPspCas13b, sgRNA vectors and scaffolds used in this study.

	dPspCas13b	sgRNA			Fluorophore	Applications
		Vector	Promoter	Scaffold		
1	dPspCas13b-RFP-2xNLS	pLKO.5-BFPnls	U6	3′-F-2xBroccoli	DFHBI-1T	sgRNA stability imaging, FACS & RT-qPCR, 3′-tagging vs In-loop structure. **Figure S2, S4, S15, S17**
2	dPspCas13b-RFP-2xNLS	pLKO.5-BFPnls	U6	In-2xBroccoli	DFHBI-1T	
3	dPspCas13b-RFP-2xNLS	pLKO.5-BFPnls	U6	3′-F-8xPepper	HBC530	
4	dPspCas13b-RFP-2xNLS	pLKO.5-BFPnls	U6	In-8xPepper	HBC530	
5	dPspCas13b-3xEGFP-2xNLS	pLKO.5-BFPnls	U6	In-8xPepper	HBC620	
6	dPspCas13b	PC0043	U6	GCN4-In-2xBroccoli	DFHBI-1T	*GCN4* Imaging. **Figure 1B,3C**
7	dPspCas13b	PC0043	U6	GCN4-In-8xPepper	HBC530	
8	dPspCas13b	PC0043	U6	MUC4-In-2xBroccoli	DFHBI-1T	*MUC4* Imaging. **Figure 2B, 3D**
9	dPspCas13b	PC0043	U6	MUC4-In-8xPepper	HBC530	
10	dPspCas13b-2xNLS	PC0043	U6	SatIII-In-2xBroccoli	DFHBI-1T	Visualizing stress granules. **Figure 2E & Video S1**
11	dPspCas13b-2xNLS	PC0043	U6	SatIII -8xPepper	HBC530	
12	dPspCas13b-2xNLS	PC0043	U6	NEAT1-In-2xBroccoli	DFHBI-1T HBC620	Labeling *NEAT1* and *MALAT1*, tracking RNAs interaction. **Figure 2D, 3D**
13	dPspCas13b-2xNLS	PC0043	U6	MALAT1-In-8xPepper		
14	dPspCas13b	PC0043	U6	MUC4-In-2xBroccoli-2xPepper	DFHBI-1T HBC620	Dual color labeling of single-RNA transcripts. **Figure 4A**
15	dPspCas13b-2xNLS	PC0043	U6	MUC4-In-2xBroccoli	DFHBI-1T HBC620	Orthogonal labeling of *MUC4* and *SatIII.* **Figure 4C**
16	dPspCas13b-2xNLS	PC0043	U6	SatIII-In-8xPepper		
17	dPspCas13b	PC0043	U6	In-8xPepper	HBC530 HBC620	Color switching of Pepper aptamer **Figure 3E & Figure S18**
18	dPspCas13b	PC0043	U6	β-actin-In-8xPepper		
19	dPspCas13b-2xNLS	PC0043	U6	β-actin-In-8xPepper	DFHBI-1T HBC620	Multicolor imaging. **Figure S20**
20	dPspCas13b-2xNLS	PC0043	U6	NEAT1-In-2xBroccoli-2xPepper		

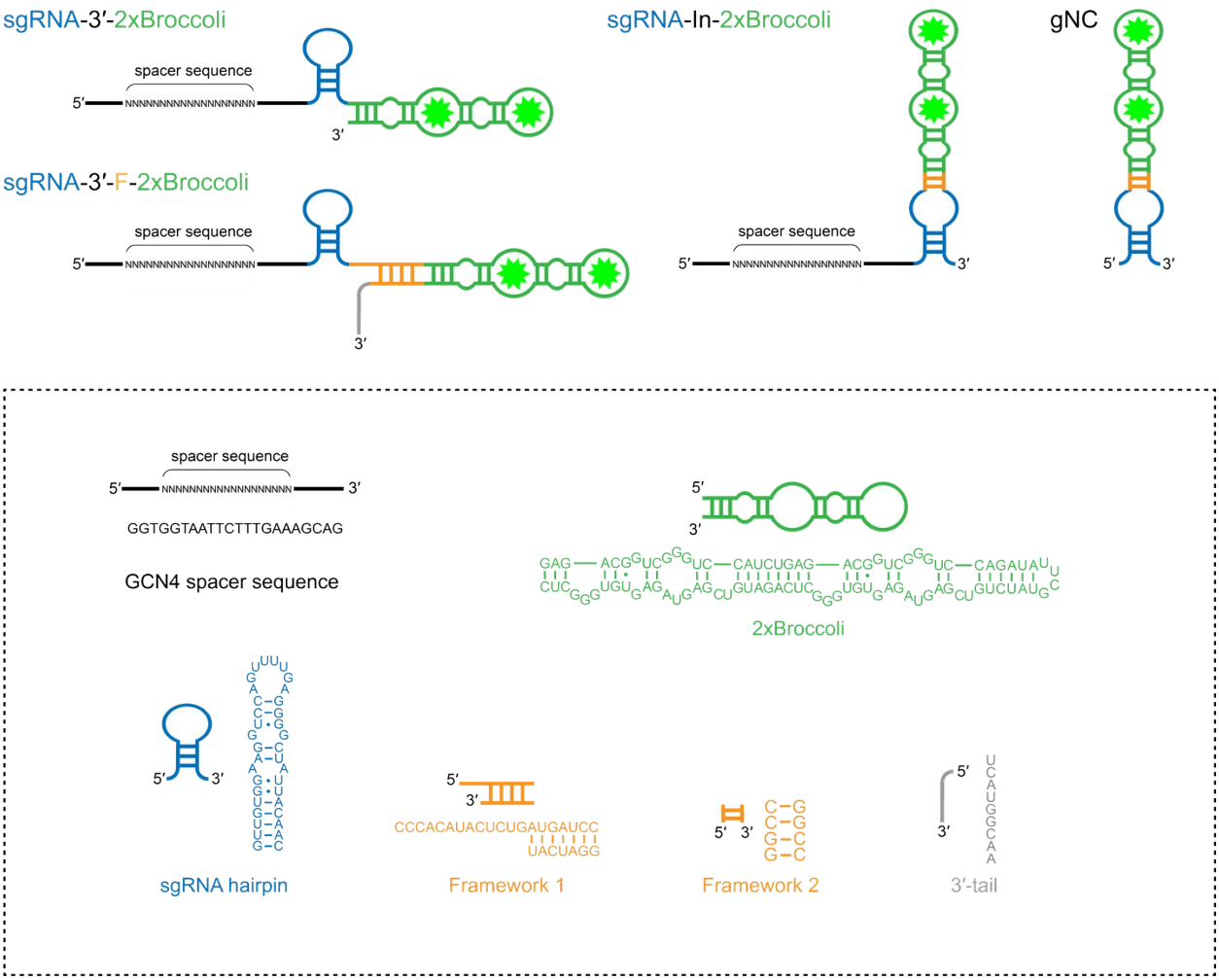


Figure S1. Overview of sgRNA-3′-2 x Broccoli, sgRNA-3′-F-2 x Broccoli, sgRNA-In-2 x Broccoli and gNC designs composed by modules including spacer sequence (black), dCas13b sgRNA hairpin (blue), 2 x Broccoli (green), framework (orange) and 3′-tail (gray) are showed. DFHBI-1T fluorophores are revealed as green stars. Modules and their sequence illustrations are showed respectively in the black dotted box below. GCN4 spacer sequence is taken as an example; Framework 1 refers to the framework in sgRNA-3′-F-2 x Broccoli; Framework 2 refers to the framework in sgRNA-In-2 x Broccoli and gNC.

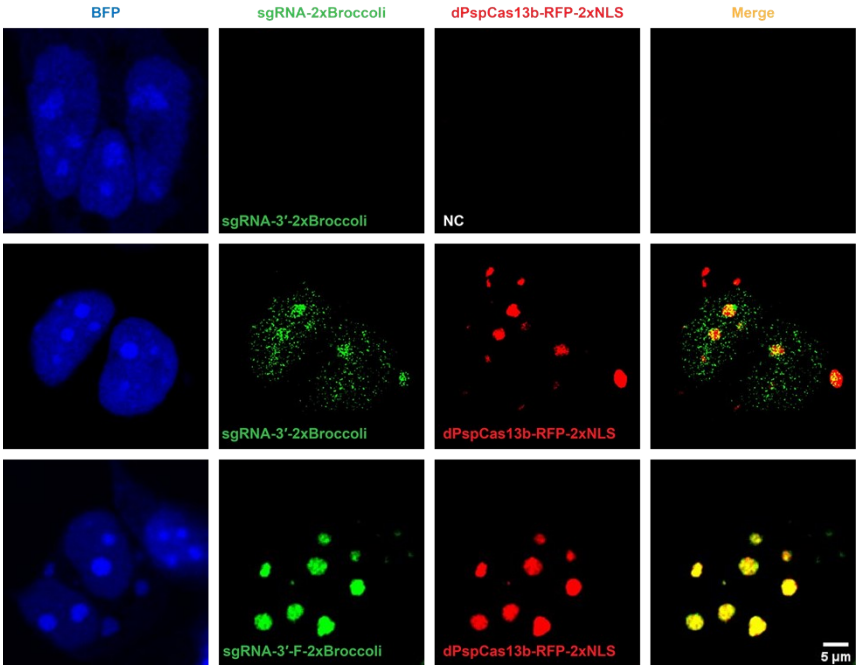


Figure S2. Representative images of sgRNA-3′-2 x Broccoli and sgRNA-3′-F-2 x Broccoli in presence of dPspCas13b-RFP-2 x NLS in HEK 293T cells. Data in all panels are representative of experiments performed at least three times.

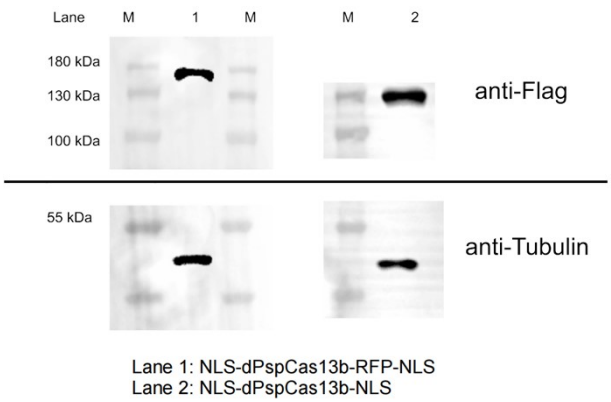


Figure S3. Western blot showing the expression of dPspCas13b-2 x NLS and dPspCas13b-RFP-2 x NLS, representing at least three independent experiments with similar results.

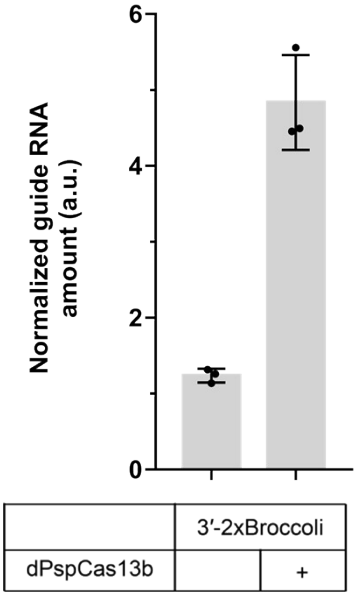


Figure S4. RT–qPCR analysis of sgRNA-3′-2 x Broccoli levels in the presence or absence of dPspCas13b in HEK 293T cells. All data are presented as the mean ± s.d.; n=3 independent experiments.

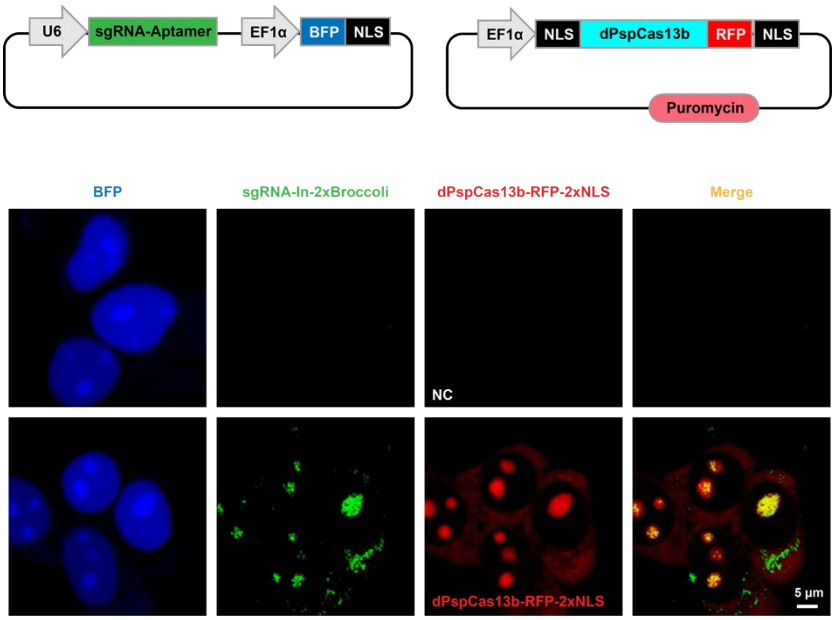


Figure S5. Representative images of sgRNA-In-2 x Broccoli in presence and absence of dPspCas13b-RFP-2 x NLS. Upper, schematic representation of expression plasmids of the sgRNA-aptamer with a reporting BFP-NLS protein and dPspCas13b-RFP-2 x NLS. Lower, representative images of sgRNA-2 x Broccoli (green) expressed in HEK 293T cells. Top row, negative control of dPspCas13b-RFP-2 x NLS; bottom row, sgRNA-In-2xBroccoli (green) with dPspCas13b-RFP-2 x NLS (red).

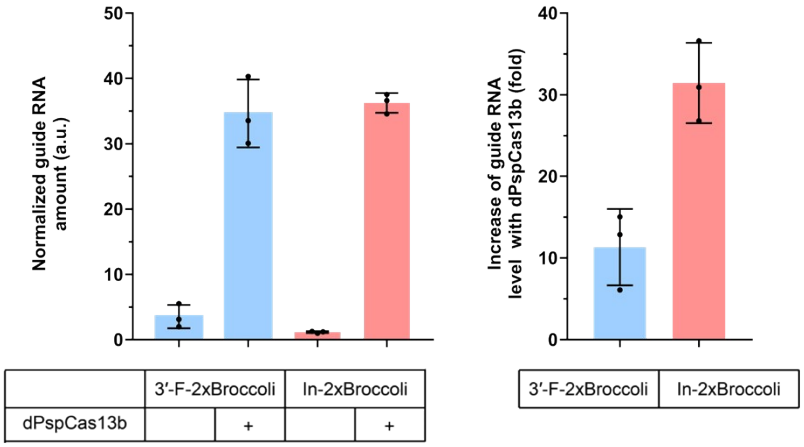


Figure S6. RT–qPCR analysis of sgRNA-Broccoli levels in HEK 293T cells. Left, the guide RNA levels of sgRNA-3′-2 x Broccoli, sgRNA-3′-F-2 x Broccoli and sgRNA-In-2 x Broccoli in the presence or absence of dPspCas13b were measured by RT–qPCR. Right, the increase of sgRNA levels in the presence of dPspCas13b. All data are presented as the mean ± s.d.; n=3 independent experiments.

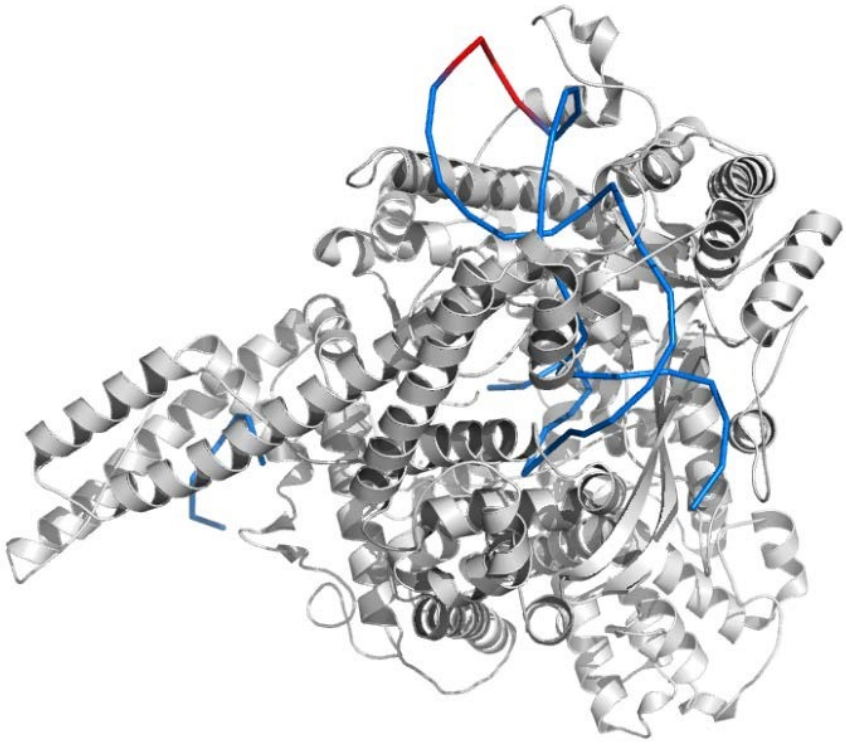


Figure S7. Three-dimensional simulation of the dPspCas13b and sgRNA complex.

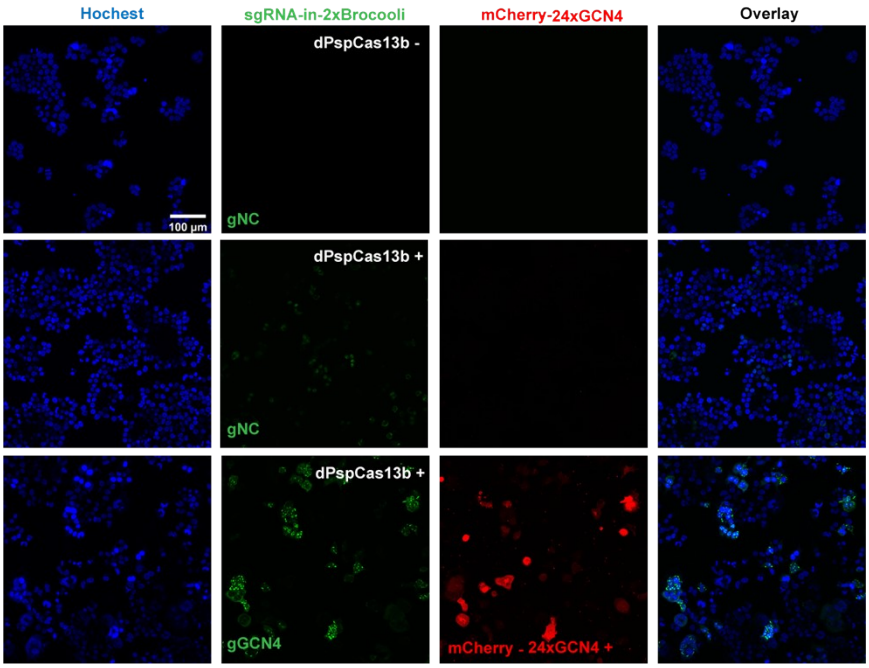


Figure S8. Representative images of sgRNA-In-2 x Broccoli in presence and absence of dPspCas13b-2 x NLS and mCherry-24xGCN4. Top row, representative images of the gNC-In-2 x Broccoli only; middle row, gNC-In-2 x Broccoli with dPspCas13b-2 x NLS; bottom row, representative images of gGCN4-In-2 x Broccoli in presence of dPspCas13b-2 x NLS ang mCherry-24xGCN4 in HEK 293T cells.

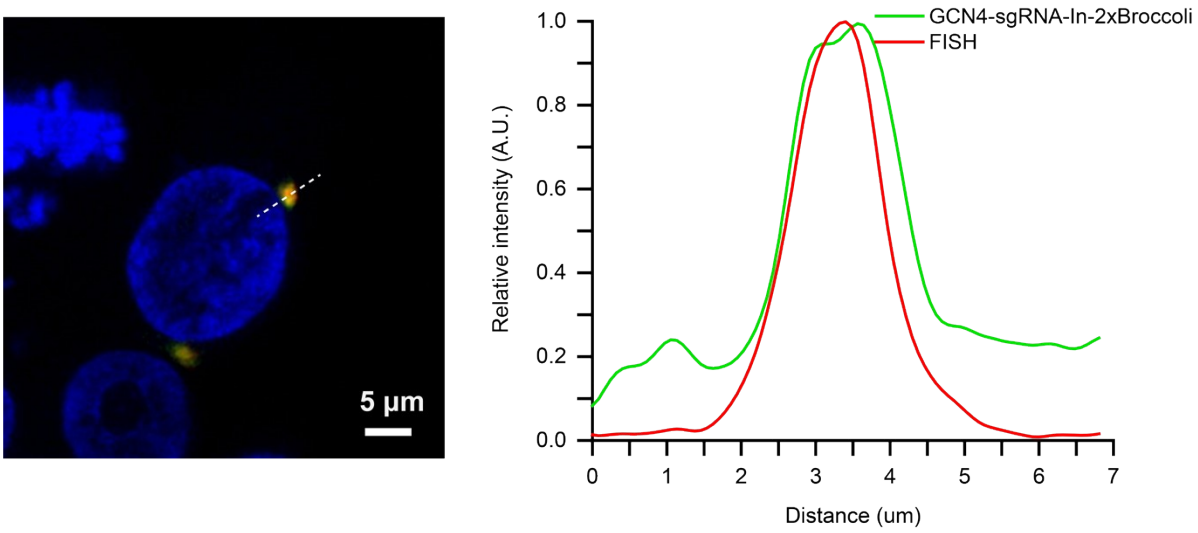


Figure S9. Colocalization analysis of GCN4-sgRNA-In-2 x Broccoli and smFISH signals in HEK 293T cells. Left, high-magnification image of the *GCN4* foci in Fig. 1b. Right, line scan of the relative fluorescence intensity of the signal indicated as the dotted line in the image on the left.

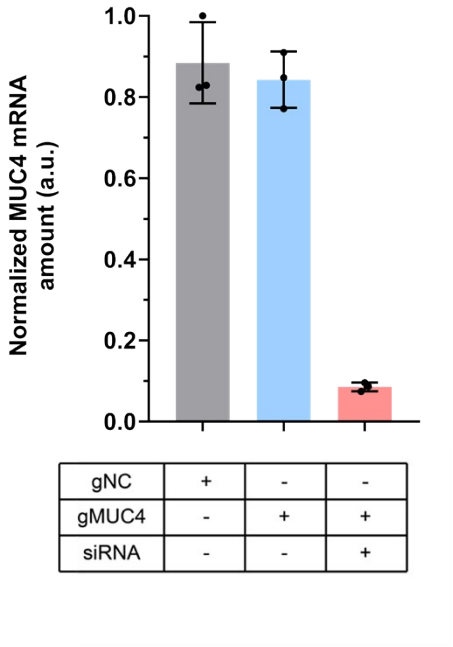


Figure S10. RT–qPCR analysis of *MUC4* mRNA levels in HEK 293T cells. *MUC4* mRNA levels in the presence or absence of siRNA (Fig. 1d, e) were measured by RT–qPCR. All data are presented as the mean ± s.d.; n=3 independent experiments.

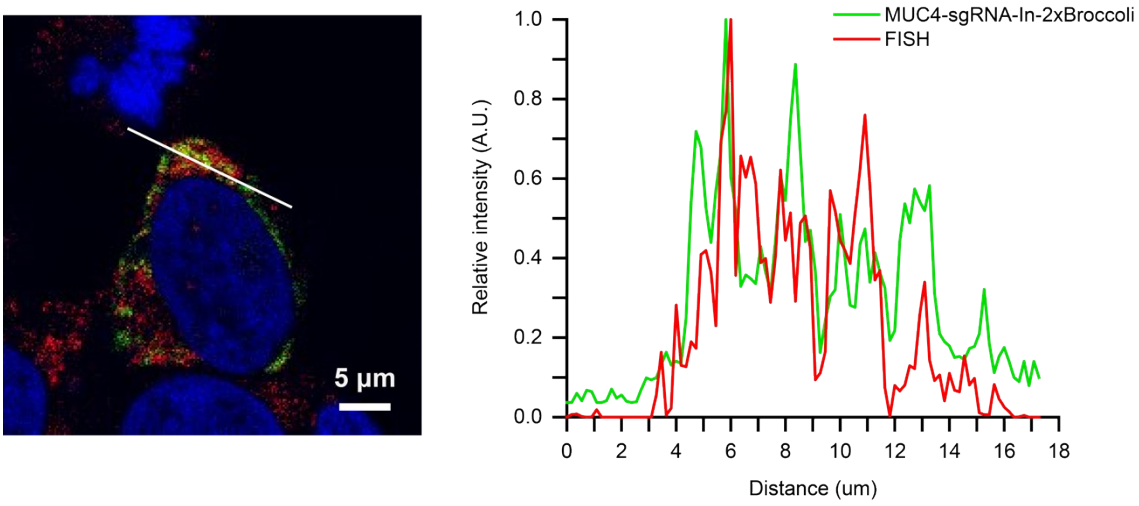
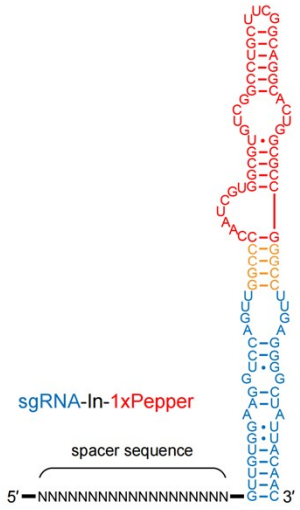
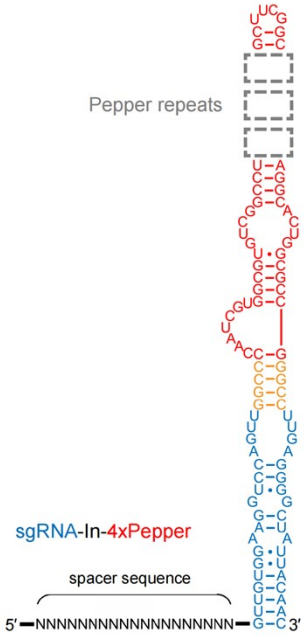
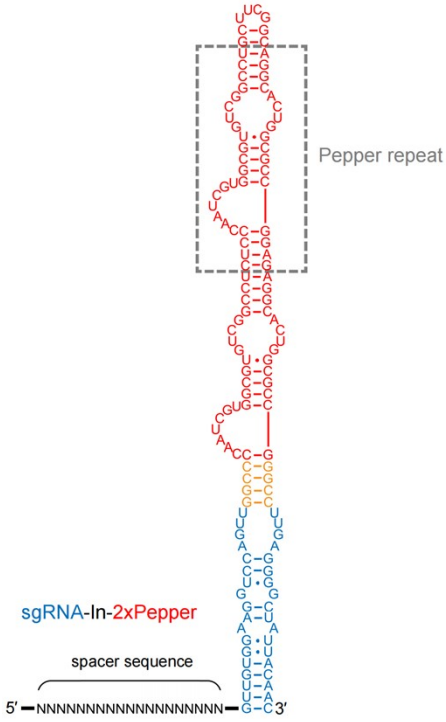


Figure S11. Colocalization analysis of MUC4-sgRNA-In-2 x Broccoli and smFISH signals in HEK 293T cells. Left, high-magnification image of the *MUC4* foci in Fig. 1c. Right, line scan of the relative fluorescence intensity of the signal indicated as the dotted line in the image on the left.


sgRNA-In-1xPepper
spacer sequence
5′ NNNNNNNNNNNNNNNNNNNN G C 3′


Pepper repeats
sgRNA-In-4xPepper
spacer sequence
5′ NNNNNNNNNNNNNNNNNNNN G C 3′


Pepper repeat
sgRNA-In-2xPepper
spacer sequence
5′ NNNNNNNNNNNNNNNNNNNN G C 3′

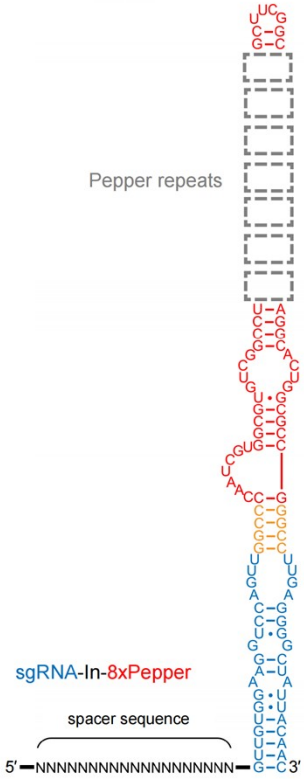

Pepper repeats
sgRNA-In-8xPepper
spacer sequence
5′ NNNNNNNNNNNNNNNNNNNN G C 3′

Figure S12. Illustration of sgRNA-In-1 x Pepper, 2 x Pepper, 4 x Pepper and 8 x Pepper sequences and secondary structures, including dCas13b sgRNA hairpin in blue, framework in orange and Pepper in red. Repetitive modules are illustrated by gray dotted boxes.

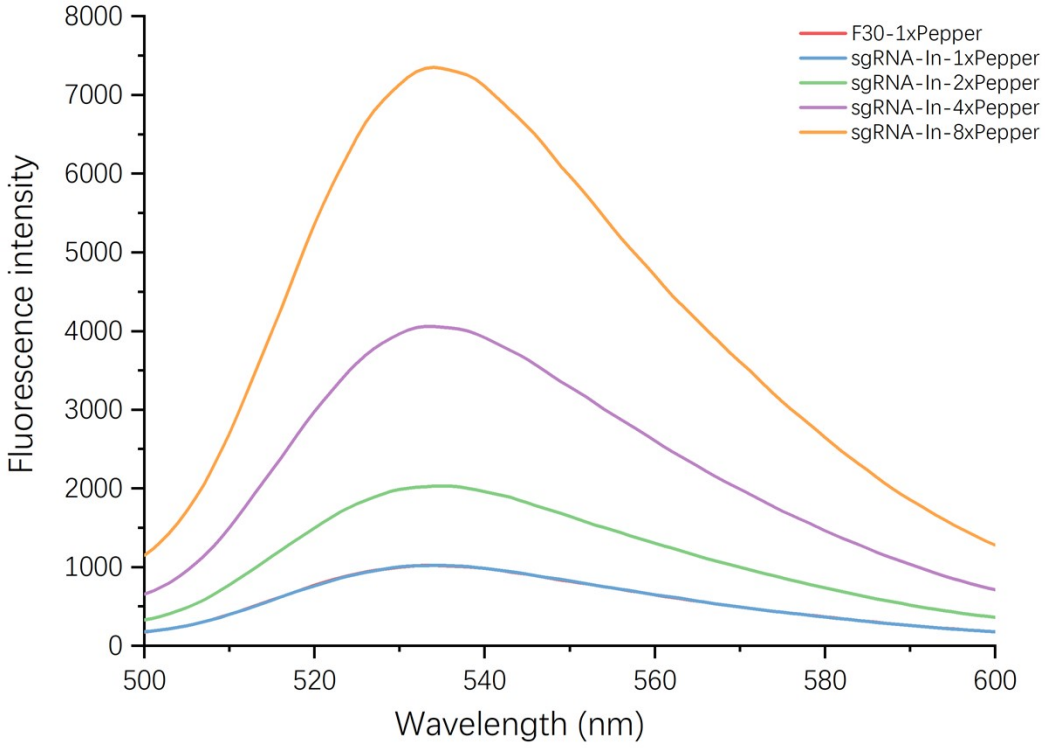


Figure S13. Aptamer folding and *in vitro* fluorescence study of sgRNA-In-Pepper series. 0.5 μM RNAs were incubated in 1 x folding buffer with 1 μM HBC530 for 1 h dark incubation at 37 °C. The emission spectrum was measured in 1 cm path-length cell using a F-4600 FL Spectrophotometer (Hitachi). Slit width: excitation = 5 nm, emission = 5 nm, HBC530 (λex = 485 nm).

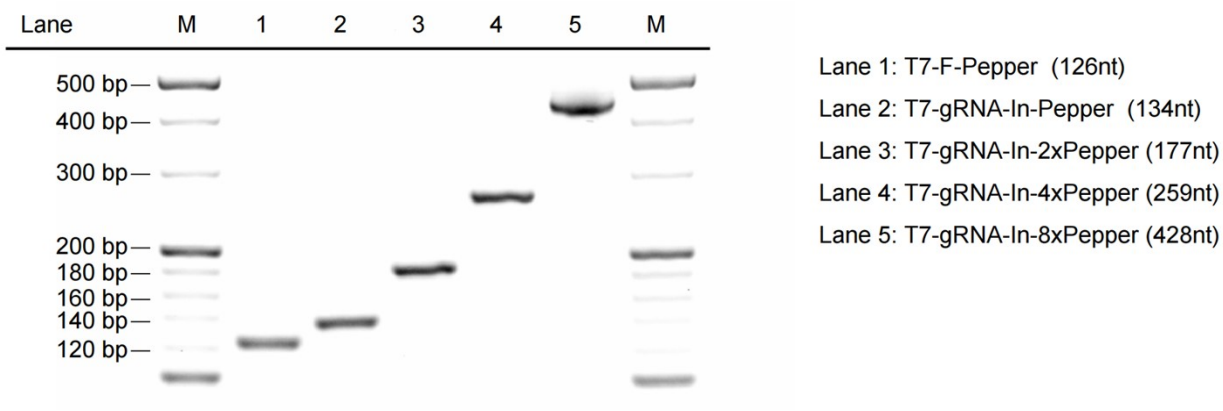


Figure S14. Agarose gel electrophoresis of sgRNA-Pepper DNA series used in T7 transcription for *in vitro* fluorescence assay. PCR solution of sgRNA-Pepper series after preliminary purification were subjected in 3% agarose gel electrophoresis. All data are representative of experiments performed at least three times. Marker: 20 bp DNA Ladder (Dye Plus, Takara).

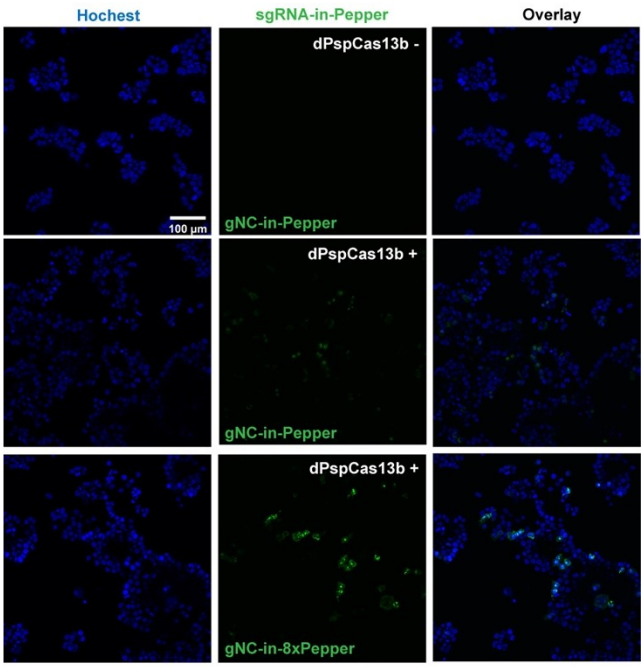


Figure S15. Representative images of sgRNA-In-Pepper and sgRNA-In-8 x Pepper in presence and absence of dPspCas13b-2 x NLS. Top row, representative images of the gNC-In-Pepper only; middle row, gNC-In-Pepper with dPspCas13b-2 x NLS; bottom row, representative images of gNC-In-8 x Pepper in presence of dPspCas13b-2 x NLS in HEK 293T cells, all experiment treated with 1 µM HBC530.

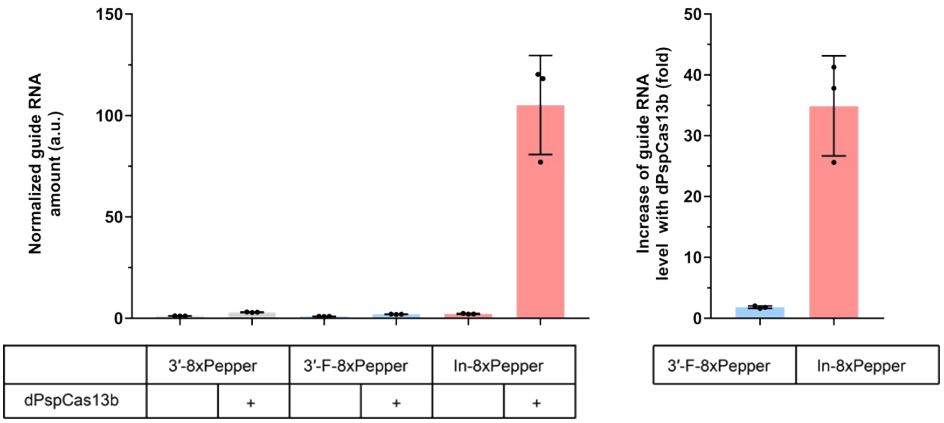


Figure S16. RT–qPCR analysis of sgRNA-Pepper levels in HEK 293T cells. Left, the guide RNA levels of sgRNA-3′-8 x Pepper, sgRNA-3′-F-8 x Pepper and sgRNA-In-8 x Pepper in the presence or absence of dPspCas13b were measured by RT–qPCR. Right, the increase of sgRNA levels in the presence of dPspCas13b. All data are presented as the mean ± s.d.; n=3 independent experiments.

HBC530	√	√	√	√
In-8xPepper	×	√	×	√
dPspCas13b-RFP	×	×	√	√

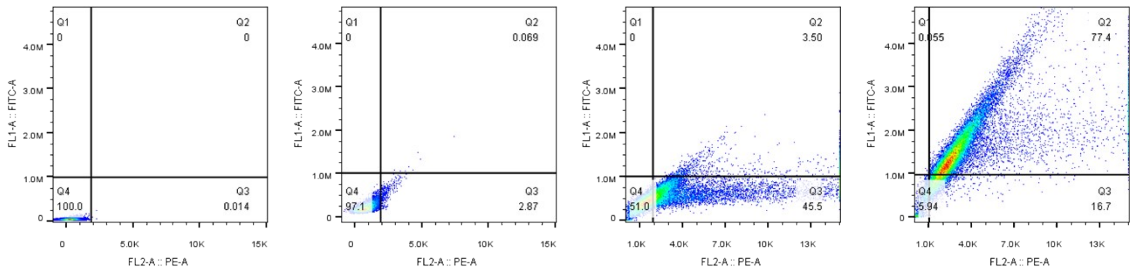


Figure S17. FACS analysis of sgRNA-In-8 x Pepper fluorescence with HBC530 in presence or absence of dPspCas13b-RFP in HEK 293T cells. The cellular level of sgRNA -In-8 x Pepper and RFP reporter were measured in the presence of 1 μM of HBC530 for FACS analysis. All data were representative of at least three parallel experiments.

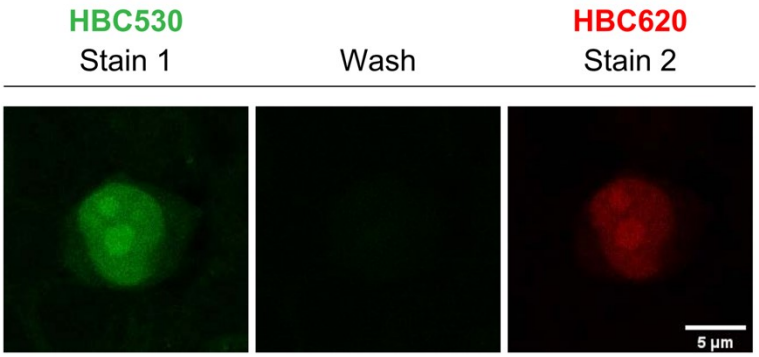


Figure S18. Color-switching of sgRNA-In-8 x Pepper using different fluorophores in live cells. HEK 293T cells expressing sgRNA-In-8 x 3epper and dPspCas13b-2 x NLS were firstly labeled with 1 µM HBC530 and imaged. The cells were then washed twice with fresh medium to remove HBC530, then the same cell was imaged with 1 µM HBC620. Data in all panels are representative of experiments performed at least three times.

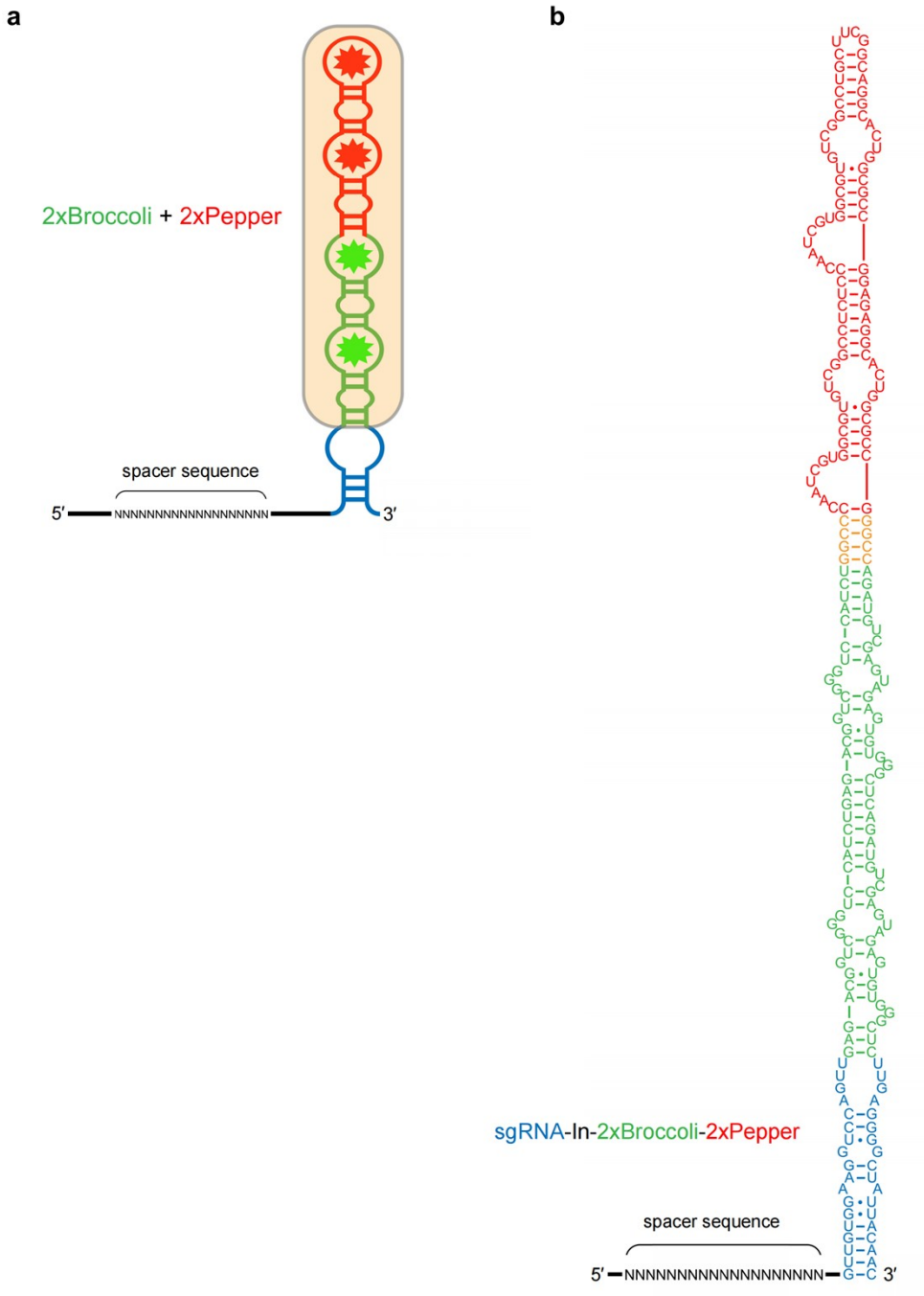

a
2xBroccoli + 2xPepper
spacer sequence
5′
NNNNNNNNNNNNNNNNNNN
3′
b
sgRNA-In-2xBroccoli-2xPepper
spacer sequence
5′
NNNNNNNNNNNNNNNNNNN
G-C
3′

Figure S19. Design of sgRNA-2 x Broccoli-2 x Pepper scaffold. a) Overview of sgRNA-2 x Broccoli-2 x Pepper designs. The structure of dCas13b sgRNA hairpin (blue), Broccoli with DFHBI-1T (green) and Pepper with HBC 620 (red) respectively are showed. b) Illustration of sgRNA-2 x Broccoli-2 x Pepper sequences and secondary structures, including dCas13b sgRNA hairpin in blue, framework in orange, Broccoli in green and Pepper in red.

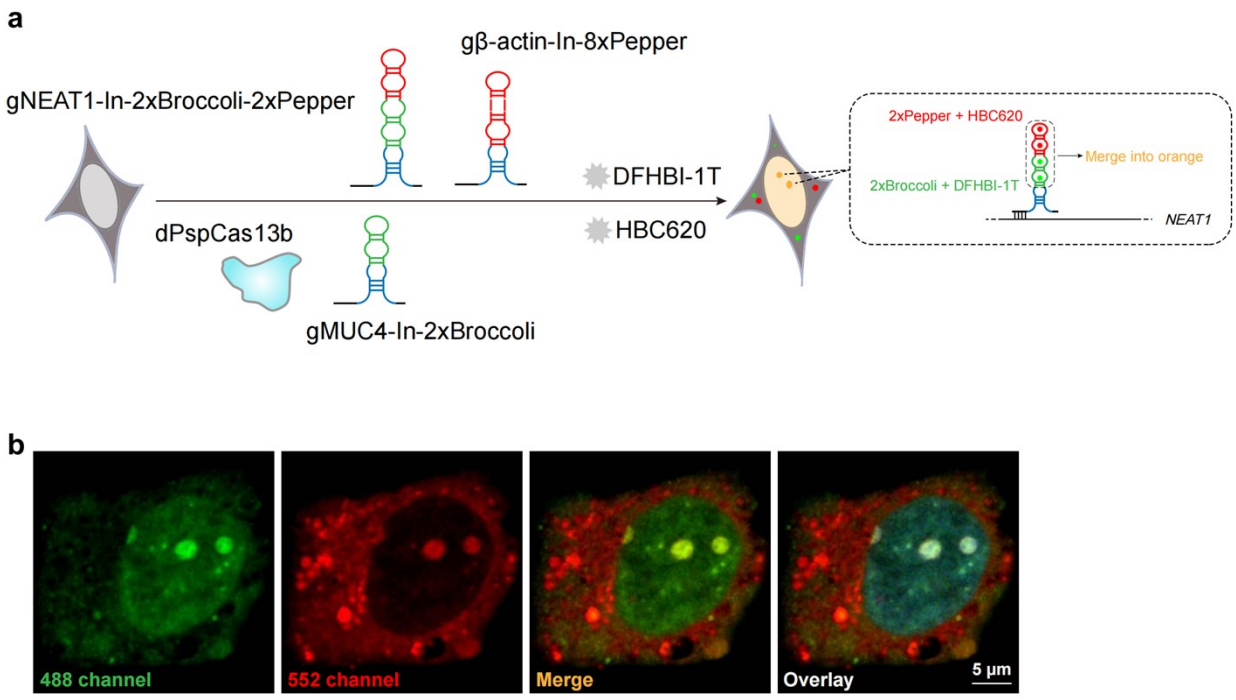


Figure S20. Multicolor labeling of *NEAT1*, *β-actin* and *MUC4.* a) Overview of multicolor labeling of *NEAT1*, *β-actin* and *MUC4*. b) Representative images for multicolor labeling. pNEAT1-sgRNA-In-2 x Broccoli-2 x Pepper, pβ-actin-sgRNA-In-8 x Pepper and pMUC4-sgRNA-In-2 x Broccoli were co-transfected into HEK 293T cells. Each color was dedicated to one RNA locus: orange (merged by red and green) for *NEAT1*, green for *MUC4*, red for *β-actin*. Data in all panels are representative of experiments performed at least three times.

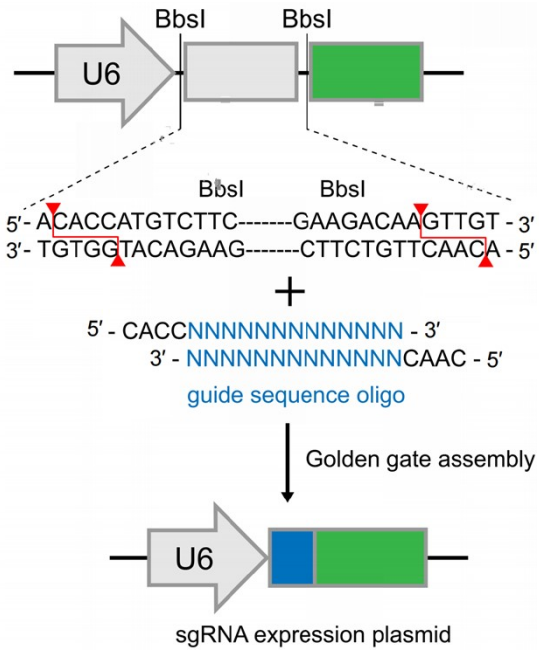


Figure S21. Single-step cloning for modified sgRNA cassettes with guide sequence to label RNA target. Guide sequence oligo (blue) was inserted after cutting of BbsI restriction enzyme to generate a seamless cloning of guide sequence in the upstream of sgRNA-aptamer (green).

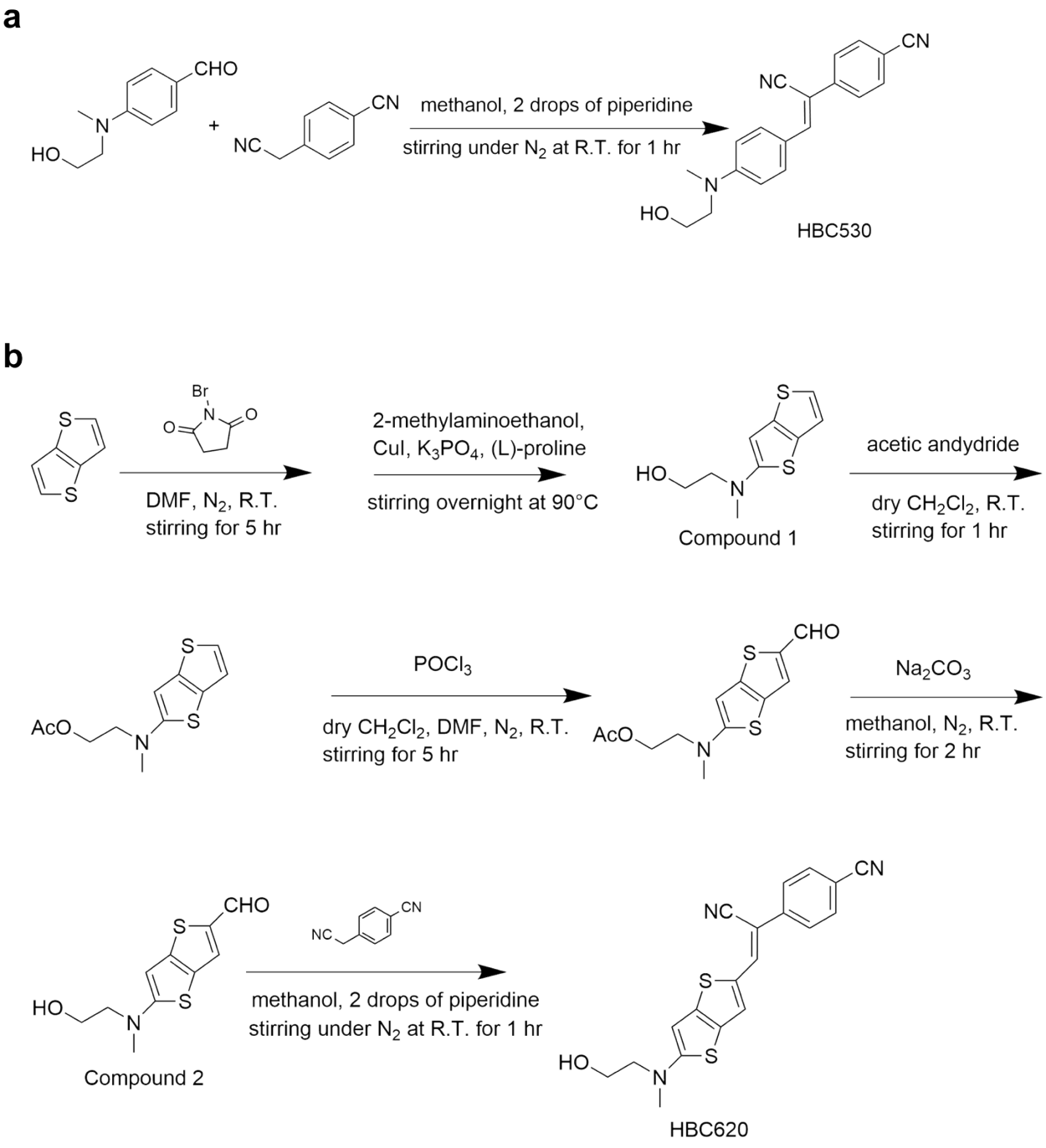


Figure S22. a) Synthesis route of HBC530. b) Synthesis route of HBC620.

Video S1. *SatIII* Tracking by SatIII - sgRNA-In-2 x Broccoli with 1-Min Intervals (40mins).

References

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