Electronic Supplementary Material (ESI) for ChemComm.

Supplementary information for:

Switchable and orthogonal gene expression control inside artificial cells by synthetic riboswitches

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Supplementary Figures

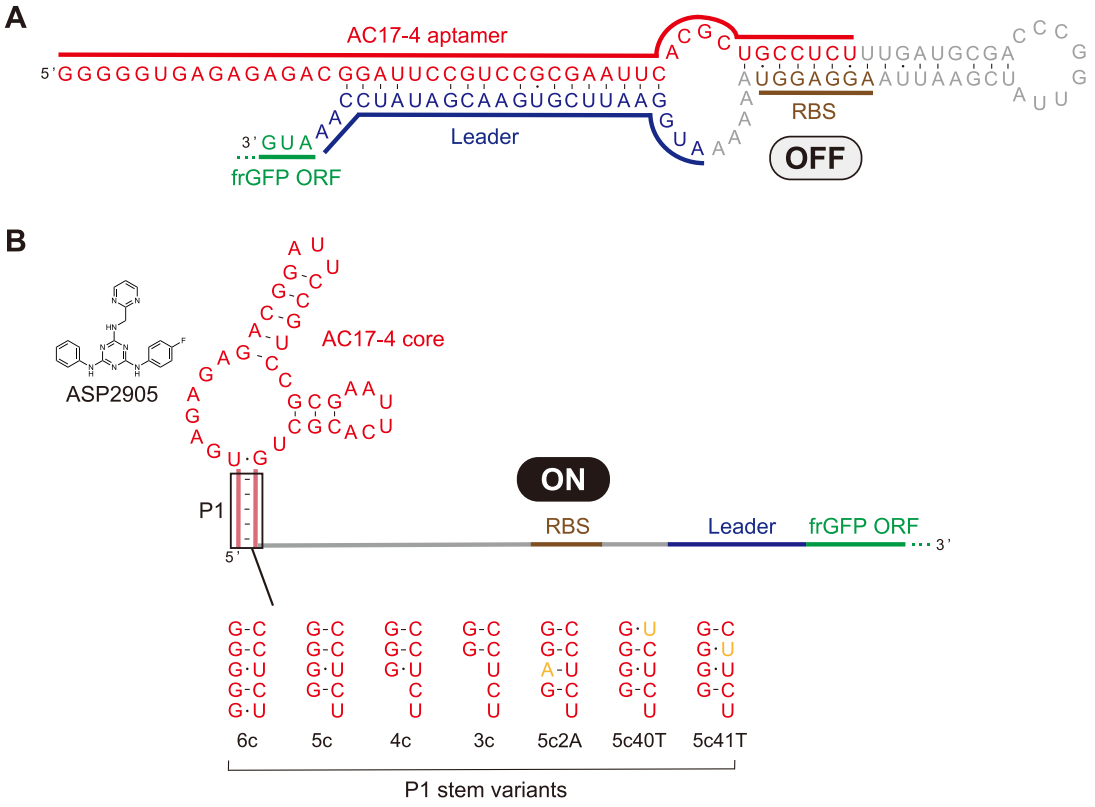

Figure S1 (related to Fig. 1A). Predicted secondary structures of ASP2905-responsive riboswitches (A) without or (B) with the cognate ligand. The predicted structures are based on mFold.[1]

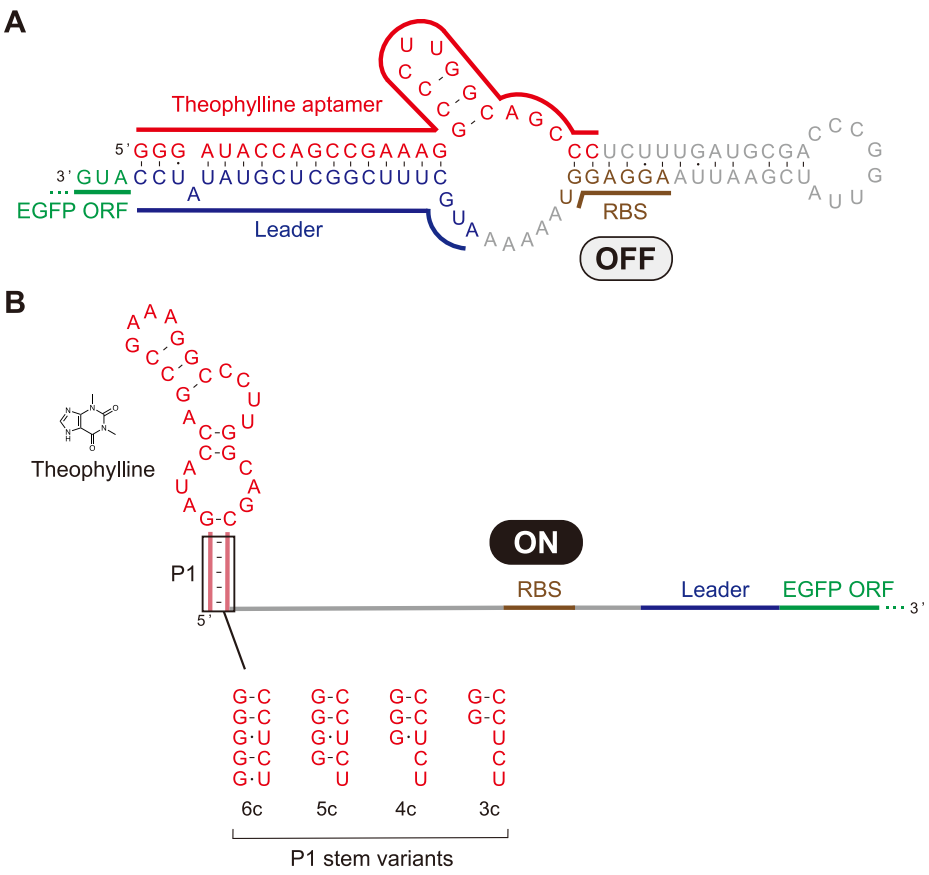

Figure S2 (related to Fig. 1C). Predicted secondary structures of theophylline-responsive riboswitches (A) without or (B) with the cognate ligand. The predicted structures are based on mFold.[1]

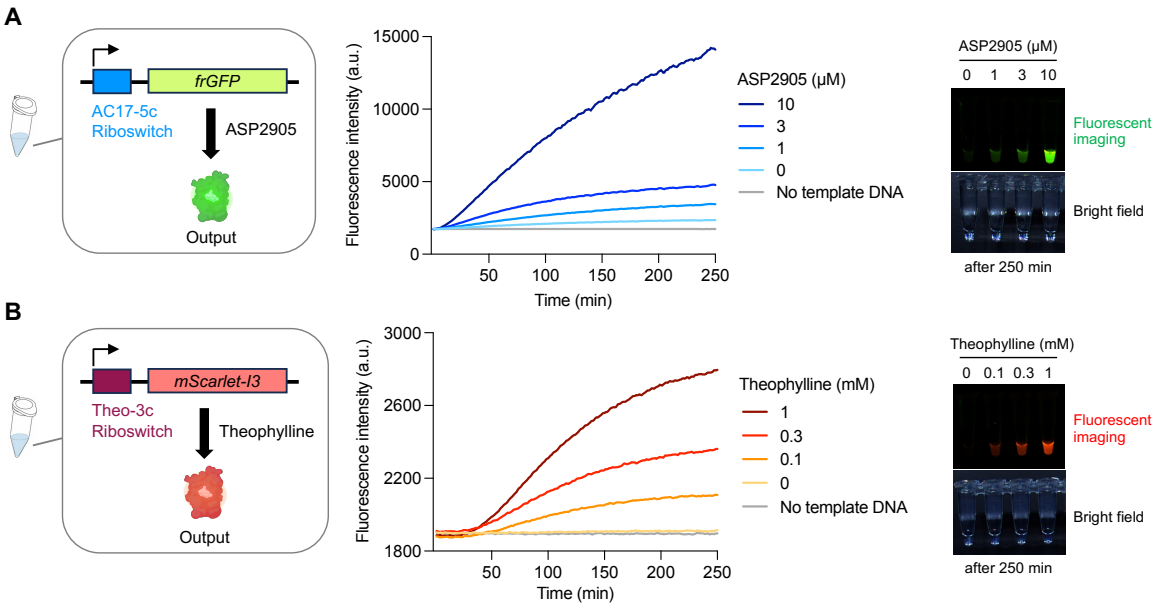

Figure S3. Real-time monitoring of (A) AC17-5c riboswitch-regulated frGFP and (B) Theo-3c riboswitch-regulated mScarlet-I3 expressions in PURE*frex* 1.0. Fluorescent images of tubes after a total 250 min reaction (right panels) were taken by a fluorescent gel imager (WSE-5400 Printgraph Classic, ATTO) equipped with an excitation light source (505 ± 25 nm) and 560 nm long path filter (YA-3). We have confirmed that the fluorescence signal rises as quickly as AC17-5c-frGFP when frGFP reporter was used for Theo-3c riboswitch (data not shown).

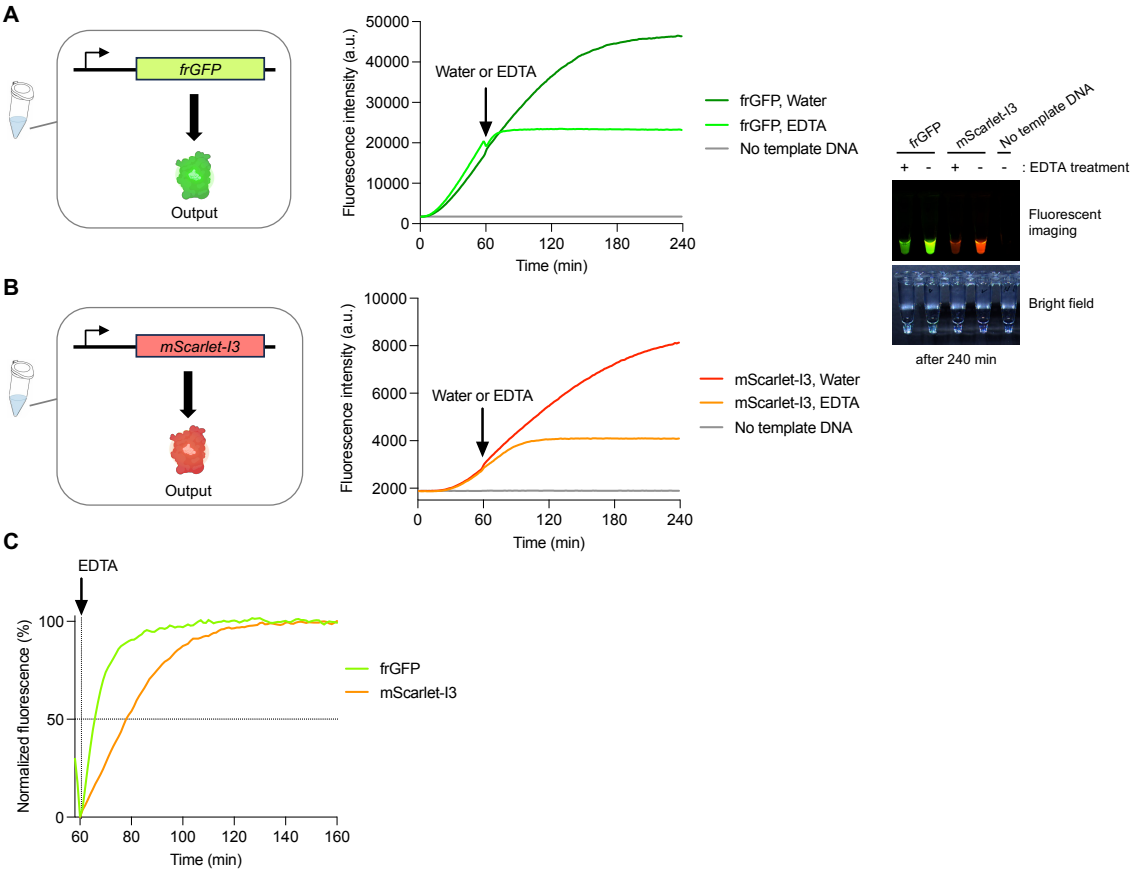

Figure S4. Real-time monitoring of chromophore maturations of (A) frGFP and (B) mScarlet-I3 fluorescent proteins. The fluorescent proteins were synthesized in PURE*frex* 1.0, and 45 mM ethylenediaminetetraacetic acid (EDTA) was added to the CFPS reaction mixture at a time point of 60 min to terminate the translation reaction. Fluorescent images of tubes after a total 240 min reaction time (right panel) were taken by a fluorescent gel imager (WSE-5400 Printgraph Classic, ATTO) equipped with an excitation light source (505 ± 25 nm) and 560 nm long path filter (YA-3). (C) Comparison of chromophore maturation rate between frGFP (light green) and mScarlet-I3 (orange). The maximum fluorescence intensity after 60 min was normalized as 100% and the fluorescence intensity at the 60 min time point was set as the minimum value.

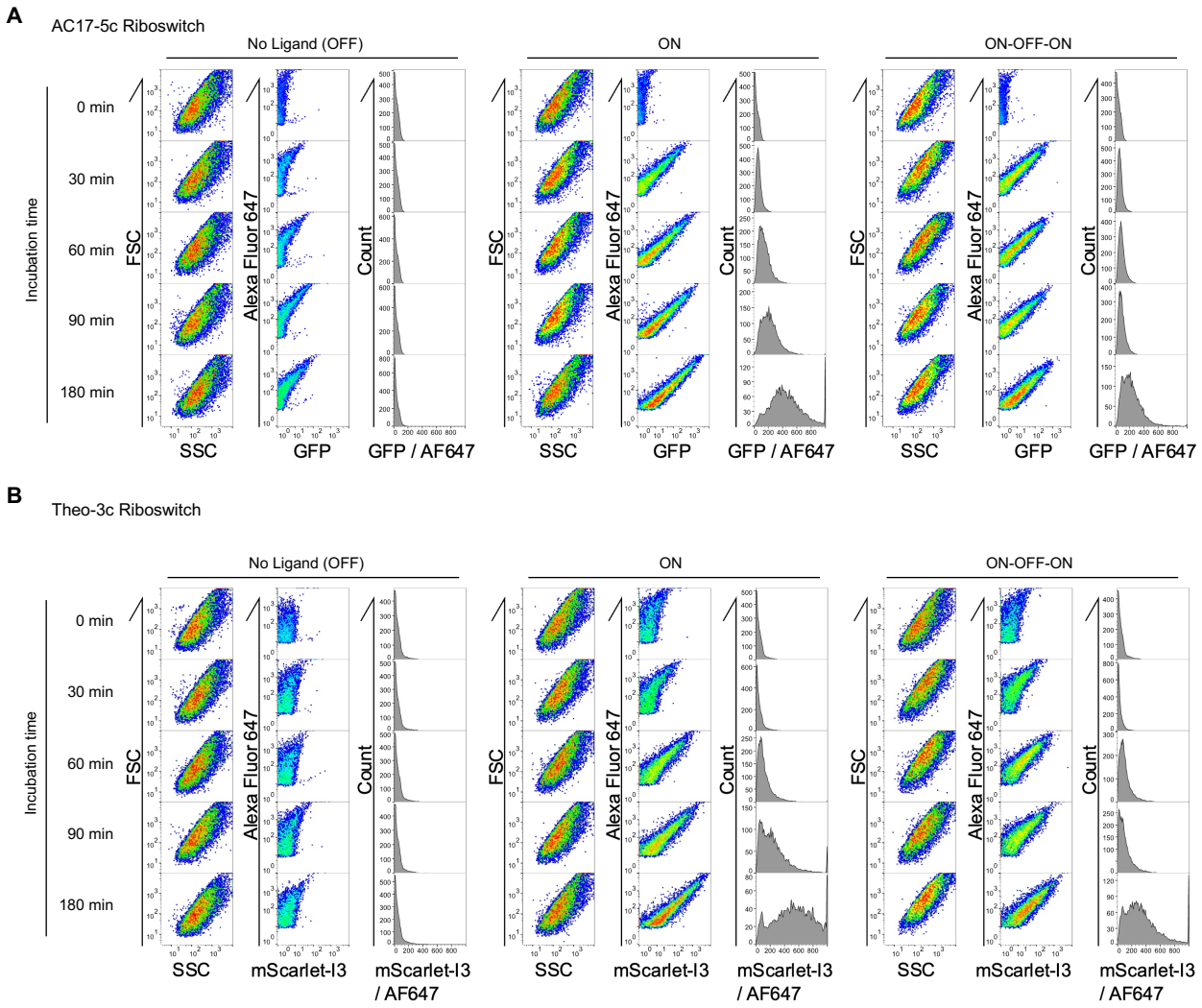

Figure S5 (related to Fig. 2). Flow cytometric analyses of reporter gene expressions in liposomes. (A) AC17-5c riboswitch-regulated frGFP expression. (B) Theo-3c riboswitch-regulated mScarlet-I3 expression. 10,000 particles were analyzed in each experiment. FSC: Forward Scatter. SSC: Side Scatter. Ovalbumin conjugated with Alexa Fluor 647 (AF647) was encapsulated in all liposomes and its fluorescence intensity should correlate linearly with the volume of each liposome. To obtain the representative value from this data, GFP or mScarlet-I3 fluorescence of the liposome were divided by AF647 fluorescence of the same liposome. Because AF647 fluorescence is linearly proportional to the aqueous volume of the GUV, this corresponds to calculating the relative GFP or mScarlet-I3 concentrations. Then the histograms of GFP/AF647 or mScarlet-I3/AF647 were obtained, and the geometric means were calculated and used as a value for the bar plots (Fig. 2).

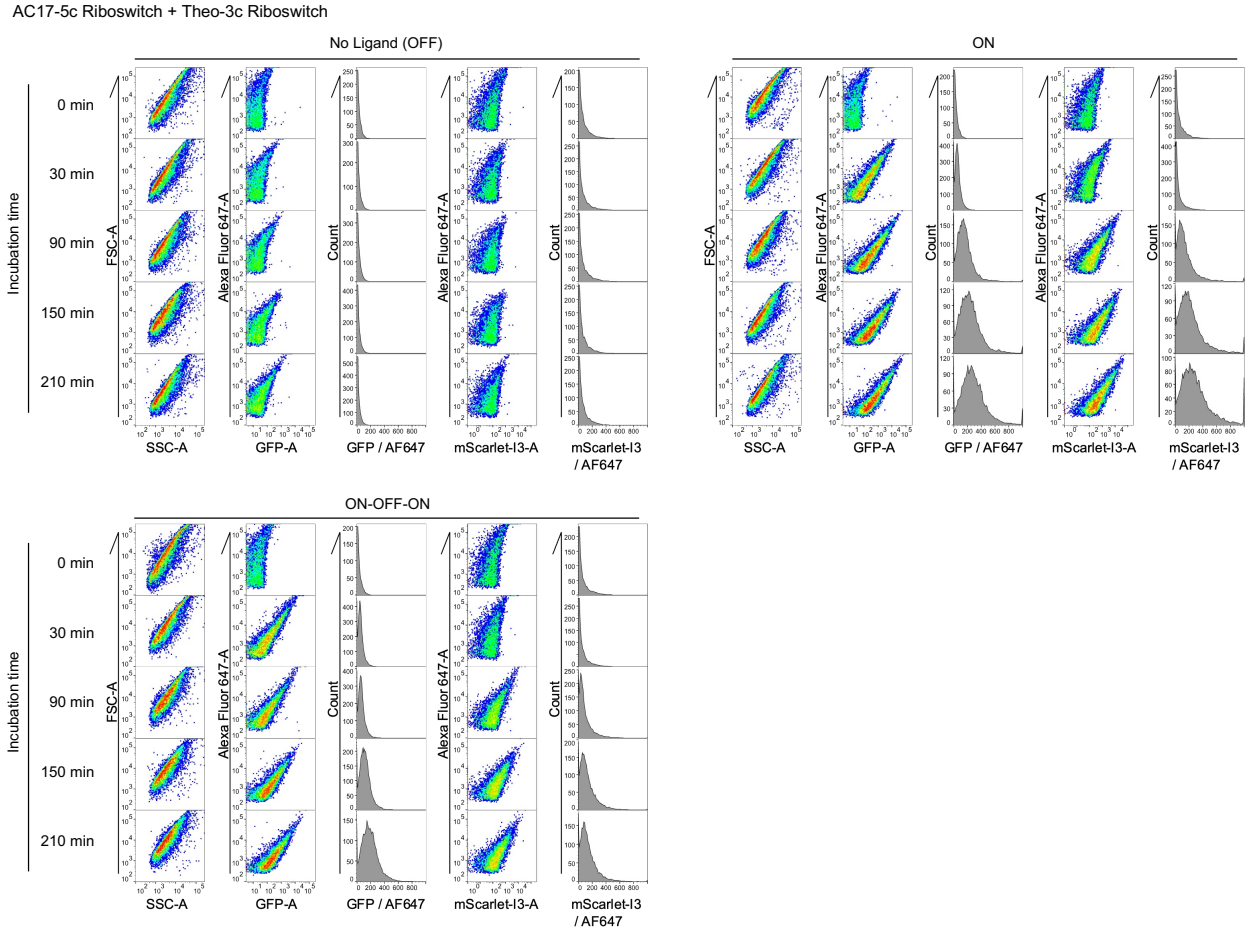

Figure S6 (related to Fig. 3A). Flow cytometric analyses of dual reporter (AC17-5c riboswitch-regulated frGFP and Theo-3c riboswitch-regulated mScarlet-I3) gene expressions in liposomes. 10,000 particles were analyzed in each experiment. FSC: Forward Scatter. SSC: Side Scatter. Ovalbumin conjugated with Alexa Fluor 647 (AF647) was encapsulated in all liposomes and its fluorescence intensity should correlate linearly with the volume of each liposome. The value used in Fig. 3 were obtained in the same way as described in the legend of Fig. S5.

Materials and Methods

1. Chemicals

ASP2905 (CAS No. 792184-90-8) and theophylline (CAS No. 58-55-9) were obtained from MedChemExpress (cat. No. HY-122015) and FUJIFILM Wako Pure Chemical (cat. No. 209-09932), respectively. The ligand stock solutions (10 mM ASP2905 and 100 mM theophylline) were prepared with dimethyl sulfoxide (DMSO, CAS No. 67-68-5). The ASP2905 stock solution was dispensed into 0.2 mL tubes, stored in the freezer at -80 °C, and used within 6 months, in accordance with manufacturer recommendations (https://medchemexpress.com/asp2905.html). The theophylline stock solution was stored in the freezer at -30 °C and used within 3 months. It should be noted that ASP2905 diluted with ultrapure water might absorb to the surface of certain types of microcentrifuge tube (e.g., DNA LoBind tubes). In our case, 1.5 mL microcentrifuge tubes (WATSON, cat. No. 131-7155C) were used for the preparation of aqueous ligand solutions. Ultrapure water was generated and supplied from a water purification system (Direct-Q UV5, Merck-Millipore). The reconstituted-type cell-free protein synthesis (CFPS) system (PURE*frex* 1.0) was purchased from Gene Frontier.

Egg phosphatidylcholine (CAS No. 97281-44-2) and cholesterol (CAS No. 57-88-5) were obtained from Yuka-Sangyo (cat. No. COATSOME NC-5) and Sigma-Aldrich, respectively. Alexa Fluor™ 647 hydrazide (cat. No. A20502) and Alexa Fluor™ 647-conjugated ovalbumin (cat. No. O34784) were obtained from Thermo Fisher Scientific.

2. DNA construction and riboswitch evaluation with the CFPS system (Fig. 1)

Riboswitch sequences (Table S1 and S2) were cloned upstream of the frGFP or EGFP reporter gene encoded in pIVEX2.3 vector and the DNA sequences were verified by Sanger sequencing. Plasmid DNAs encoding AC17-5c-frGFP or Theo-3c-EGFP are available in Addgene (https://www.addgene.org/, ID: 217525 and 217526). Plasmid DNA encoding Theo-3c-mScarlet-I3 was synthesized and purchased from Eurofin Genomics. Template DNAs (Appendix S1, S2, and S3) for the CFPS system were prepared by 25 cycles of PCR using KOD Plus Neo (TOYOBO, cat. No. KOD-401). The PCR products were purified with the NucleoSpin Gel and PCR Clean-up kit (MACHEREY-NAGEL) and eluted with ultrapure water. DNA concentrations were determined by absorption measurements using the NanoDrop 2000 or NanoDrop One photo-spectrometer (Thermo Fisher Scientific).

CFPS reactions (10 μL) were carried out using PURE*frex* 1.0 in a 0.2 mL PCR tube (Fig. 1) containing ultrapure water (1.5 μL), solution I (5 μL), solution II (0.5 μL), solution III (0.5 μL), 1 μM Alexa Fluor™ 647 hydrazide (0.5 μL), 10× ligand (1 μL), and 50 ng/μL template DNA (1 μL). Freshly prepared 10× ligand solutions (100 μM ASP2905 in 1% aqueous DMSO; 10 mM theophylline in 10% aqueous DMSO) were used every time. The tubes were incubated for 3 h at 37 °C in a thermal cycler (T100, Bio-Rad), and then equal volumes (10 μL) of Dulbecco's phosphate buffered saline (D-PBS) supplemented with 0.1% (v/v) polyoxyethylene(20) sorbitan monolaurate (Tween-20) were added. 18 μL of the mixture was transferred to a 384-well black plate and fluorescence (490 nm excitation, 520 nm emission for GFP; 650 nm excitation, 680 nm emission for Alexa Fluor™ 647) was measured by a microplate reader (Synergy H1, BioTek). Background fluorescence (control sample minus template DNA and Alexa Fluor™ 647 reference dye) was subtracted from the GFP and Alexa Fluor™ 647 fluorescence values. GFP fluorescence was normalized by Alexa Fluor™ 647 fluorescence to account for well-to-well variations. Relative fluorescence values were calculated from the GFP expression level without riboswitch as a standard. The ON/OFF ratio was calculated from the GFP/Alexa Fluor™ 647 value with ligand, divided by the value without ligand. Bar graph and data plots were generated using GraphPad

Prism software 10 (GraphPad Software) and non-linear curve fitting was carried out using the following equation.[2]

$$Y = Y_{min} + \frac{Y_{max} - Y_{min}}{1 + (\frac{EC_{50}}{X})^h}$$

Y_{min}: minimum fluorescence signal
Y_{min}: maximum fluorescence signal
EC_{50}: half maximal effective concentration of ligand
X: molar concentration of ligand
h: Hill coefficient

3. Real-time monitoring of CFPS (Fig. S3 and S4)

Real-time monitoring of fluorescent protein reporter was carried out by a real-time PCR machine (Stratagene, Mx3005P) equipped with appropriate filter sets (492 nm excitation and 516 nm emission for frGFP; 545 nm excitation and 568 nm emission for mScarlet-I3). CFPS reactions (20 μL) were carried out in a 0.2 mL 8-well PCR tube at 37 °C. For traces of fluorophore maturations of frGFP and mScarlet-I3 (Fig. S4), 2 μL of 0.5 M EDTA-Na (pH 8) was added to the 20 μL reaction mixture after 60 min incubation to terminate the transcription and translation, and then the incubation and trace were again started within 2 min.

4. Protein synthesis inside liposomes

Liposomes encapsulating PURE*frex* 1.0 were prepared using the water-in-oil (W/O) emulsion/transfer method as described previously[3-5] with some modifications. At first, 16 μL of egg phosphatidylcholine: cholesterol 8:2 mixture (100 mg/mL in chloroform) was added to a glass tube, and chloroform evaporated with nitrogen gas to give the lipid film. After addition of 200 μL of liquid paraffin (CAS No. 8042-47-5, FUJIFILM Wako Pure Chemical), the tube was vortexed and incubated at 70 °C for a few minutes. After additional vortexing for 30 s, the tube was further incubated at 70 °C for a few minutes. After cooling to room temperature, 20 μL of inner solution containing components for CFPS was injected into the liquid paraffin solution. The inner solution (in total, 20 μL) was prepared by mixing of the following reagents in a 1.5 mL microcentrifuge tube on ice: 1× PURE*frex* 1.0 solution I, PURE*frex* 1.0 solution II, PURE*frex* 1.0 solution III, 5 nM template DNA, 1.2 μM Alexa Fluor™ 647-conjugated ovalbumin, 20 mM HEPES-KOH (pH 7.6), 250 mM potassium glutamate, 120 mM sucrose, 280 mM glucose, and 0.8 U/μL RNase inhibitor (TOYOBO, cat. No. SIN-201). The indicated concentrations are final concentrations. The inner solution droplet in liquid paraffin was mechanically agitated along a 1.5 mL tube rack and vortexed for 30 s to obtain the emulsion. The emulsion solution was gently placed on top of 200 μL of outer solution (0.5 mM 20 amino acids, 1.5 mM ATP and GTP, 0.5 mM CTP and UTP, 1 mM spermidine, 1 mM creatine phosphate, 1 mM dithiothreitol, 20 mM HEPES-KOH (pH 7.6), 250 mM potassium glutamate, 18 mM $Mg(OAc)_2$, and 400 mM glucose) and centrifuged at 15,000 × *g* for 30 min at 20 °C with a microcentrifuge (TOMY, MX-307) equipped with a swing rotor. A hole was made at the bottom of the microcentrifuge tube with a hypodermic needle (21 G; 0.8 × 13 mm), and the aqueous layer containing the precipitated liposomes was collected from the hole and transferred into another 1.5 mL tube. The collected liposomes were resuspended by pipetting. To trigger protein expression of riboswitch-regulated reporter genes, the outer solution was exchanged to the solution supplemented with 20 μM ASP2905 or 2 mM theophylline (Fig. 2; ON). CFPS reactions were carried out at 37 °C and sampling (each 10 μL) was carried out at the indicated time points. For temporal gene expression control in liposomes (Fig. 2; ON-OFF-ON and Fig.3A), the riboswitch ligand inside liposomes was removed by buffer exchange: liposomes were precipitated by centrifugation at 15,000 × *g* for 5 min at 4 °C and outer solution with no ligand was

added to the liposomes. The procedure was repeated three times to ensure complete ligand removal.

5. Flowcytometric analysis

Flowcytometric (FCM) analysis was carried out using FACSAria III (BD). The liposome suspension was diluted approximately 10-fold with buffer solution (20 mM HEPES-KOH, pH 7.6, 250 mM potassium glutamate, 18 mM $Mg(OAc)_2$, and 400 mM glucose), and then subjected to FCM analysis. A total of 10,000 liposome particles were measured. The data were analyzed by FlowJo software (BD).

6. Confocal microscopy

Fluorescence imaging of riboswitch-regulated gene expression in liposomes was carried out using a confocal laser scanning microscope (LSM 900 with Airyscan 2, ZEISS) with a 40x oil lens and 90 µm pinhole. frGFP and mScarlet-I3 expressions were monitored using 480-nm and 561-nm laser excitation respectively, with lasers set to 2% using the Zeiss Zen software, and collected emission peaks at 511 nm and 594 nm. Samples were imaged on BSA-coated glass imaging slides. Slides were produced by 1 h incubation of 150 µL of 1% BSA in buffer solution (20 mM HEPES-KOH, pH 7.6, 250 mM potassium glutamate, 18 mM $Mg(OAc)_2$, and 400 mM glucose) on the viewing portion of the glass slide, followed by rinsing with ultrapure water and drying with $N_2(g)$ to leave behind a dry BSA coating. Vesicles were imaged in 1 mm-diameter silicon wells placed on the coated glass slides. The samples were incubated at 37 °C using the Stage Top Incubator (TOKAI HIT) and the buffer wash and exchange was performed at 30 min and 90 min timepoints. 10 µL of GUV sample was first imaged in buffer supplemented with 20 µM ASP2905. The ASP2905 was removed by dilution of the sample, by removing 9 µL of solution from the viewing chamber and replacing it with 9 µL of wash buffer containing neither ligand, 4 times. Finally, 10 µL of the 2 mM theophylline-containing buffer was added to initiate mScarlet-I3 expression. Images were taken at 30 s intervals. Images were analyzed and edited using ImageJ software (National Institute of Health, USA).

Appendix S1. DNA sequence encoding AC17-5c riboswitch-regulated frGFP reporter gene.

ATGCGTCCGGCGTAGAGGATCGAGATCTCGATCCCGCGAAATTAATACGACTCACTATAGGGGTGAGAGAGAC
GGATTCCGTCCGCGAATTCACGCTGCCTCTTTGATGCGACCCGGTTATCGAATTAAGGAGGTAAAAAATGGAATT
CGTGAACGATATCCAAATGTCGAAAGGCGAAGAACTGTTTACTGGTGTAGTTCCGATTCTGGTGGAGTTAGACG
GTGACGTTAACGGTCACAAATTCAGTGTTTCCGGTGAAGGTGAAGGAGACGCTACTTACGGTAAGTTAACCCTG
AAATTCATCTGTACAACCGGTAAACTGCCGGTACCTTGGCCTACGCTGGTGACTACCCTGACTTACGGTGTACA
GTGCTTTAGCCGTTATCCCGATCACATGAAACGTCACGACTTCTTTAAATCTGCTATGCCAGAAGGTTACGTGCA
GGAACGTACCATTTCGTTTAAAGACGACGGTAACTACAAAACCCGTGCAGAAGTCAAATTCGAAGGTGATACTCT
GGTGAATCGGATTGAACTCAAAGGGATCGACTTTAAGGAGGACGGTAATATTCTGGGCCATAAACTCGAGTACA
ACTACAACAGCCACAACGTGTATATCACCGCGGATAAGCAGAAGAACGGCATCAAAGCGAACTTCAAAATTCGC
CATAACATCGAAGACGGTTCTGTCCAACTTGCCGATCATTACCAGCAGAATACACCGATTGGTGACGGACCTGTT
TTGCTTCCGGACAACCATTACCTGTCTACGCAATCAGCCTTGTCCAAAGACCCGAACGAAAAACGCGACCACAT
GGTCTTACTGGAGTTTGTTACGGCAGCTGGCATTACGCACGGTATGGACGAGCTGTATAAATAATAAAAGGGCG
AATTCCAGCACACTGGCGGCCGTTACTAGTGGATCCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCT
GCTGCCACCGCTGAGCAATAACTAGCATAACCCCT

T7 promoter
ASP2905-binding aptamer
Ribosome-binding sequence (RBS)
Leader sequence
Folding Reporter Green Fluorescent Protein (frGFP)
Sequences of riboswitch variants are summarized in **Table S1**
Underlined: primer-binding site

*Plasmid DNA encoding AC17-5c-frGFP is available in Addgene (https://www.addgene.org/, ID: 217525).

Table S1. ASP2905-responsive riboswitch variants

Variant	DNA sequence (5'-3')
AC17-6c	GGGGGTGAGAGAGACGGATTCCGTCCGCGAATTCACGCTGCCTCTTTGATGCGACCCG GTTATCGAATTAAGGAGGTAAAAAATGGAATTCGTGAACGATATCCAAATG…
AC17-5c	GGGGTGAGAGAGACGGATTCCGTCCGCGAATTCACGCTGCCTCTTTGATGCGACCCGG TTATCGAATTAAGGAGGTAAAAAATGGAATTCGTGAACGATATCCAAATG…
AC17-4c	GGGTGAGAGAGACGGATTCCGTCCGCGAATTCACGCTGCCTCTTTGATGCGACCCGGT TATCGAATTAAGGAGGTAAAAAATGGAATTCGTGAACGATATCCAAATG…
AC17-3c	GGTGAGAGAGACGGATTCCGTCCGCGAATTCACGCTGCCTCTTTGATGCGACCCGGTTA TCGAATTAAGGAGGTAAAAAATGGAATTCGTGAACGATATCCAAATG…
AC17-5c2A	GAGGTGAGAGAGACGGATTCCGTCCGCGAATTCACGCTGCCTCTTTGATGCGACCCGG TTATCGAATTAAGGAGGTAAAAAATGGAATTCGTGAACGATATCCAAATG…
AC17-5c40T	GGGGTGAGAGAGACGGATTCCGTCCGCGAATTCACGCTGTCTCTTTGATGCGACCCGG TTATCGAATTAAGGAGGTAAAAAATGGAATTCGTGAACGATATCCAAATG…
AC17-5c41T	GGGGTGAGAGAGACGGATTCCGTCCGCGAATTCACGCTGCTTCTTTGATGCGACCCGG TTATCGAATTAAGGAGGTAAAAAATGGAATTCGTGAACGATATCCAAATG…
Control w/o riboswitch	GGGAGACCACAACGGTTTCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATA CCATG…

Appendix S2. DNA sequence encoding Theo-3c riboswitch-regulated EGFP reporter gene.

ATGCGTCCGGCGTAGAGGATCGAGATCTCGATCCCGCGAAATTAATACGACTCACTATAGGGATACCAGCCGAA
AGGCCCTTGGCAGCCCTCTTTGATGCGACCCGGTTATCGAATTAAGGAGGTAAAAAATGCTTTCGGCTCGTATAT
CCATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTGCCCATCCTGGTCGAGCTGGACGGCGACGTAA
ACGGCCACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGACCCTGAAGTTCAT
CTGCACCACCGGCAAGCTGCCCGTGCCCTGGCCCACCCTCGTGACCACCCTGACCTACGGCGTGCAGTGCTTC
AGCCGCTACCCCGACCACATGAAGCAGCACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGGAGC
GCACCATCTTCTTCAAGGACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTCGAGGGCGACACCCTGGT
GAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAGCTGGAGTACAAC
TACAACAGCCACAACGTCTATATCATGGCCGACAAGCAGAAGAACGGCATCAAGGTGAACTTCAAGATCCGCCA
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GCTGCTGCCCGACAACCACTACCTGAGCACCCAGTCCGCCCTGAGCAAAGACCCCAACGAGAAGCGCGATCAC
ATGGTCCTGCTGGAGTTCGTGACCGCCGCCGGGATCACTCTCGGCATGGACGAGCTGTACAAGTAATAAAAGG
GCGAATTCCAGCACACTGGCGGCCGTTACTAGTGGATCCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTG
GCTGCTGCCACCGCTGAGCAATAACTAGCATAACCCCT

T7 promoter
Theophylline-binding aptamer
Ribosome-binding sequence (RBS)
Leader sequence
Enhanced Green Fluorescent Protein (EGFP)
Sequences of riboswitch variants are summarized in **Table S2**
Underlined: primer-binding site

*Plasmid DNA encoding Theo-3c-EGFP is available in Addgene (https://www.addgene.org/, ID: 217526).

Table S2. Theophylline-responsive riboswitch variants

Variant	DNA sequence (5'-3')
Theo-6c	GGGGGGATACCAGCCGAAAGGCCCTTGGCAGCCCTCTTTGATGCGACCCGGTTATCGAATTAAGGAGGTAAAAAATGCTTTCGGCTCGTATATCCATG…
Theo-5c	GGGGGATACCAGCCGAAAGGCCCTTGGCAGCCCTCTTTGATGCGACCCGGTTATCGAATTAAGGAGGTAAAAAATGCTTTCGGCTCGTATATCCATG…
Theo-4c	GGGGATACCAGCCGAAAGGCCCTTGGCAGCCCTCTTTGATGCGACCCGGTTATCGAATTAAGGAGGTAAAAAATGCTTTCGGCTCGTATATCCATG…
Theo-3c	GGGATACCAGCCGAAAGGCCCTTGGCAGCCCTCTTTGATGCGACCCGGTTATCGAATTAAGGAGGTAAAAAATGCTTTCGGCTCGTATATCCATG…
Control w/o riboswitch	GGGAGACCACAACGGTTTCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACCATG…

Appendix S3. DNA sequence encoding Theo-3c riboswitch-regulated mScarlet-I3 reporter gene.

TAATACGACTCACTATAGGGATACCAGCCGAAAGGCCCTTGGCAGCCCTCTTTGATGCGACCCGGTTATCGAAT
TAAGGAGGTAAAAAATGCTTTCGGCTCGTATATCCATGGATAGCACCGAGGCAGTGATCAAGGAGTTCATGCGG
TTCAAGGTGCACATGGAGGGCTCCATGAACGGCCACGAGTTCGAGATCGAGGGCGAGGGCGAGGGCCGCCCC
TACGAGGGCACCCAGACCGCCAAGCTGAAGGTGACCAAGGGTGGCCCCCTGCCCTTCTCCTGGGACATCCTGT
CCCCTCAGTTCATGTACGGCTCCCGTGCCTTCATCAAGCACCCCGCCGACATCCCCGACTACTGGAAGCAGTCC
TTCCCCGAGGGCTTCAAGTGGGAGCGCGTGATGATCTTCGAGGACGGCGGCACCGTGTCCGTGACCCAGGAC
ACCTCCCTGGAGGACGGCACCCTGATCTACAAGGTGAAGCTCCGCGGCGGCAACTTCCCTCCTGACGGCCCCG
TAATGCAGAAGCGTACAATGGGCTGGGAAGCATCCACCGAGCGGTTGTACCCCGAGGACGTCGTGCTGAAGGG
CGACATTAAGATGGCCCTGCGCCTGAAGGACGGCGGCCGCTACCTGGCGGACTTCAAGACCACCTACAAGGCC
AAGAAGCCCGTGCAGATGCCCGGCGCCTTCAACATCGACCGCAAGTTGGACATCACCTCCCACAACGAGGACT
ACACCGTGGTGGAACAGTACGAACGCTCCGTGGCCCGCCACTCCACCGGCGGCTCCGGTGGCTCCTAATAAAA
GGGCGAATTCCAGCACACTGGCGGCCGTTACTAGTGGATCCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGT
TGGCTGCTGCCACCGCTGAGCAATAACTAGC

T7 promoter
Theophylline-binding aptamer
Ribosome-binding sequence (RBS)
Leader sequence
mScarlet-I3
Underlined: primer-binding site

Supplementary References

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