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Nucleus Developer Notes

Testing Integration of a TetR Biosensor with a Toehold Exchange Riboregulator in Developer Cells with Nucleus Cytosol

CSHL Synthetic Biology 2026

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AbstractKeywords TetR biosensor, Toehold exchange riboregulator, Developer Cells, Nucleus Cytosol, Anhydrotetracycline

Note

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1. Overview

Through the 2026 CSHL Synthetic Biology Summer Course, the students, instructors, and teaching assistants helped optimize an anhyrotetracycline (aTc) sensor, which uses a toehold exchange (THE) riboregulator RNA circuit derepressed in the presence of aTc via the TetR transcription factor. This aTc sensor was first optimized in bulk cell-free expression reactions using PURExpress. The unregulated toehold exchange riboregulator circuit and the full aTc sensing circuit were then tested in Developer Cells (DevCells), an encapsulated cell-free expression system.

2. Bill of Materials

2.1. DNA templates and sequences

DNA templates for the full toehold exchange (THE) riboregulator transcription (TX) unit. **Bold** sequences indicate promoters. Underlined sequences indicate the regions amplified using the specified primers. *Italic* sequences indicate terminators. Annotated sequence maps are available on Benchling. Sequence files in GenBank format are included in `sequences/`.

The full-length `G_1 sCFP3A`, `G_1 sfGFP`, and `T7p_TetR` sequences are long and are provided verbatim as GenBank files in `sequences/` rather than inline. Plasmid maps for all six constructs are shown below.

Io

Table 1. *Reagents and materials*

Reagent	Product Name	Manufacturer	Catalog No.	Price	Storage Conditions	Link
CPRG	Chlorophenol red-β-D-galactopyranoside	Roche	10884308001	$160	–20 °C in water at 10 mg/mL	link
Cytosol (SMix, PMix, ribosomes, tRNA)	b.next	b.next	—	—	–80 °C	—
PURExpress	NEB PURExpress in vitro protein synthesis kit	New England Biolabs	E6800S	$307.00	–80 °C	link
aTc	Anhydrotetracycline hydrochloride	Sigma-Aldrich	37919-100MG-R	$275.00	Room temp	link
RNase Inhibitor	RNase Inhibitor, Murine	New England Biolabs	M0314L	$347.00	–20 °C	link
Lipid-in-Oil Kit	16:0-18:1 PC (POPC) + Cholesterol + Cy5.5	b.next	Composition in Table 5	—	4 °C	—
Optiprep	Optiprep	Serumwerk Bernburg	1893	—	Room temp	—
Glucose	D-(+)-Glucose, 99.5%	Sigma-Aldrich	G8270-1KG	—	Room temp	—
Nuclease-free water	Nuclease-free water	ThermoFisher Scientific	AM9916	—	Room temp	—
384-well glass bottom plates	384-well glass bottom plate, 1.5 cover glass	Cellvis	P384-1.5H-N	$423.00	Room temp	link
Optical Adhesive Film	MicroAmp Optical Adhesive Film	ThermoFisher Scientific	4311971	—	Room temp	—
Osmometer	Freezing-point osmometer	Advanced Micro-Osmometer	Model 3300	—	Room temp	—
PCR tubes	Thin-walled, RNase-free PCR tubes	ThermoFisher Scientific	AM12225	—	Room temp	—

seqviz

This block will be replaced with **./sequences/Io.gb** when deployed to Curvenote.

Options:

Figure 1. *Plasmid map of Io, the non-complementary input (OFF).*

Table 2. *Primers*

Name	Sequence (5′ to 3′)	Purpose
T7fwd	TTCTAATACGACTCACTATAGGGAG	Forward primer for T7 promoter (used in all PCRs except `IN_1_TetR`)
fwd_U1	CATTACTCGCATCCATTCTCAGG	Forward primer for `IN_1_TetR` PCR
T7rev	CAAAAAACCCCTCAAGACCCGTTTAG	Reverse primer for T7t terminator (For Gate / riboregulator template PCRs)
rev_Ux	GGTGGAAGGGCTCGGAGTTG	Reverse primer for `IN_1_TetR` PCR
Thyb10rev	AAAAAAAAACAGATAGCCGCGCGAAC	Reverse primer for Thyb10 terminator (For Input template PCRs)

IN_1

seqviz

This block will be replaced with **./sequences/IN_1.gb** when deployed to Curvenote.

Options:

Figure 2. *Plasmid map of `IN_1`, the complementary input (ON).*

Table 3. *DNA templates*

Name	Sequence (5′ to 3′)	Purpose
`Io`	gaagtctaacgctgctctgggctaactgtcCATTACTCGCATCCATTCTCAGGCTGTCTCGTCTCGTCTTC TTC **TAATACGACTCACTATA** GGGAGATTCGTCTCCCAATCAATAACACACATA *ataatcAGATAACAGATACttcgGtatctgttatctgttTTTTTcAACAGATAGCCGCGttcgCGCGGCtatctgttTTTTTt* agctgtcaccggatgtgctttccggtctgatgagtccgtgaggacgaaacagcctcCAGGATACATAGATTACCACAACTCCGAGCCCTTC	Complementary input with maximum expression (OFF). Plasmid map: Figure 1.
`IN_1`	gaagtctaacgctgctctgggctaactgtcCATTACTCGCATCCATTCTCAGGCTGTCTCGTCTCGTCTTC TTC **TAATACGACTCACTATA** GGGAGATTCGTCTCCCAAATACTTAATACAAAATAGTCAATCTTCGAAGAGA *ataatcAGATAACAGATACttcgGtatctgttatctgttTTTTTcAACAGATAGCCGCGttcgCGCGGCtatctgttTTTTTt* agctgtcaccggatgtgctttccggtctgatgagtccgtgaggacgaaacagcctcCAGGATACATAGATTACCACAACTCCGAGCCCTTC	Complementary input with maximum translation rate; maximum expression (ON). Plasmid map: Figure 2.
`IN_1_TetR`	CATTACTCGCATCCATTCTCAGGCTGTCTCGTCTCGTCTTC **TAATACGACTCACTATA** GGTCCCTATCAGTGATAGAGACCAAATACTTAATACAAAATAGTCAATCTTCGAAGAGA *ataatcAGATAACAGATACttcgGtatctgttatctgttTTTTTcAACAGATAGCCGCGttcgCGCGGCtatctgttTTTTTt* CCAGGATACATAGATTACCACAACTCCGAGCCCTTCCACC actgtgtacttgttataacatctgacagttaaagtcgggagaataggagccgcaat	DNA template with the TetR operator sequence downstream of the T7 promoter. Plasmid map: Figure 3.
`G_1 sCFP3A`	see Figure 4	Toehold exchange riboregulator that controls cyan fluorescent protein (CFP) expression in response to `IN_1`
`G_1 sfGFP`	see Figure 5	Toehold exchange riboregulator that controls green fluorescent protein (GFP) expression in response to `IN_1`
`T7p_TetR`	see Figure 6	DNA template for T7-driven expression of TetR
`pOpen-deGFP`	see pOpen-deGFP.gbk	DevCell encapsulation control plasmid. Module documentation: docs.nucleus.engineering/docs/modules/reporter-degfp/spec

IN_1_TetR

seqviz

This block will be replaced with **./sequences/IN_1_TetR.gb** when deployed to Curvenote.

Options:

Figure 3. *Plasmid map of `IN_1_TetR`, the TetR-regulated input.*

G_1 sCFP3A

seqviz

This block will be replaced with **./sequences/G_1-sCFP3A.gb** when deployed to Curvenote.

Options:

Figure 4. *Plasmid map of `G_1 sCFP3A`, the CFP-reporting gate.*

G_1 sfGFP

seqviz

This block will be replaced with **./sequences/G_1-sfGFP.gb** when deployed to Curvenote.

Options:

Figure 5. *Plasmid map of `G_1 sfGFP`, the GFP-reporting gate.*

T7p_TetR

seqviz

This block will be replaced with **./sequences/pT7_TetR.gb** when deployed to Curvenote.

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Figure 6. *Plasmid map of `T7p_TetR`, the TetR expression template.*

3. Design

The mechanistic details of how toehold exchange riboregulators work are described in another DevNote: devnotes.nucleus.engineering/articles/ss-20260712.

The mechanistic details of TetR repression of transcription and derepression of transcription in response to aTc are described in the Nucleus documentation: docs.nucleus.engineering/docs/modules/detector-tetr-atc/spec.

Key design notes for toehold exchange riboregulators are described in another DevNote: devnotes.nucleus.engineering/articles/ss-20260712.

The sequences for the toehold exchange riboregulators are based on those discussed in the following publication: biorxiv.org/content/10.1101/2025.09.25.678518v2.

The sequences were generated using the ctRSD and THE riboregulator sequence compiler:

- Documentation: ctrsd-simulator.readthedocs.io/en/latest/SeqCompiler.html
- Colab: colab.research.google.com/drive/1Bd-ZugIWnc7PMiFkpaia_UnWA24KF3Mv

4. Protocol

- Linear DNA templates for the circuit were prepared as described in a previous DevNote: devnotes.nucleus.engineering/articles/ss-20260712.
- The protocol follows the CSHL Synthetic Biology 2026 - Nucleus Base Cell protocol: docs.nucleus.engineering/docs/processes/build-base-cell/main.
    - With respect to DevCell preparation, the protocol did not deviate from the Nucleus protocol, other than the outer solution osmolarity, which was 1050 mmol/kg here.
    - The Cytosol reaction setup table shows the concentrations and volumes used for each of the samples. The Membrane composition and Outer solution tables show the mixtures used to prepare the DevCells.
    - For DevCell experiments that included TetR, TetR was expressed in a bulk reaction of Nucleus Cytosol using 4 nM of linear `T7_TetR` template (Figure 6). The reaction was incubated at 37 °C for at least 3 h.
    - This TetR-enriched cytosol was then mixed at a 1:4.56 volume ratio with fresh Nucleus Cytosol containing the circuit DNA templates, which was then encapsulated.
- After encapsulation, a 50 µL volume of DevCells for each sample was transferred to a 384-well glass bottom plate (Table 1; see also the Nucleus materials reference). Each sample was sufficiently diluted to prevent overcrowding of DevCells in the imaging wells. After initial imaging, the samples were incubated at 37 °C for 1.5 h or 2 h in a benchtop incubator before the first post-incubation imaging.
- After this first image, the samples were returned to the incubator and incubated at 37 °C for another 3 h before taking a final image.
- Imaging was conducted on a Cephla Squid fluorescence microscope with a 20x air objective (Olympus).
    - Green fluorescent protein (GFP) was imaged at 488 nm excitation. The DevCell membranes, which were labeled with Cy5.5, were imaged at 638 nm excitation. The microscope camera has no emission filter, so emission was captured as the integration of all wavelengths. For both fluorescence channels, the exposure times and excitation laser intensities were kept constant across all conditions within each experiment. Specific exposure time varied between experiments, depending on excitation laser power, to provide sufficient contrast across all samples.
    - For each sample, the entire area of the well was captured with 25 non-overlapping images at each time point, and all images were used for analysis and quantification.
    - This microscope did not have onboard temperature control, which is why the DevCells were incubated in a benchtop incubator and imaged at only a few time points.

Table 4. *Cytosol reaction setup*

Component	Stock Concentration	Final Concentration	Negative Control Volume (µL)	Positive Control Volume (µL)	EXP OFF Volume (µL)	EXP ON Volume (µL)
SMix	3.33x	1.09x	7.2	7.2	7.2	7.2
PMix	15 mg/mL	0.95 mg/mL	1.4	1.4	1.4	1.4
Ribosomes	10 µM	0.36 µM	0.8	0.8	0.8	0.8
tRNA	35 mg/mL	3.5 mg/mL	2.4	2.4	2.4	2.4
DNA template for Gate (`G_1 GFP`, Figure 5)	100 nM	2 nM	0.44	0.44	0.44	0.44
DNA template for complementary input (`IN_1`, Figure 2, or `IN_1_TetR`, Figure 3)	100 nM	18 nM	0	3.96	3.96	3.96
Non-complementary input DNA (`Io`, Figure 1)	100 nM	18 nM	3.96	0	0	0
TetR-enriched cytosol	1x	0.215x	0	0	4.73	4.73
RNase Inhibitor	40 000 U/mL	2000 U/mL	1.4	1.4	1.4	1.4
Opti-Prep	—	3.6%	0.8	0.8	0.8	0.8
Water	—	—	4.77	4.77	0	0

- Automated image analysis of DevCell intensities was conducted using the Nucleus Cell Development Kit (nucleus-cdk), software developed by b.next for Nucleus.

4.1. Cytosol reaction setup table

The last four columns indicate the volumes of each reagent to add for the four different samples tested, for encapsulating cell-free reactions in DevCells. These samples are detailed in Figure 8.

4.2. Membrane composition table

4.3. Outer solution table

5. Results and Observations

Building on a previous DevNote and bulk cell-free expression experiments (Piorino *et al.* 2025), we sought to test toehold exchange riboregulators (Piorino *et al.* 2025, Schaffter *et al.* 2025) and small molecule biosensors in DevCells. We first validated that the coupling of

Table 5. *Membrane composition*

Lipid	Target Percentage %	Molecular Weight	Concentration (mg/mL)	Volume to Add (µL)
1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC)	70	760.076	25	162.17
Cholesterol	29.95	386.66	50	17.65
Cy5.5	0.05	1366.32	1	4

Table 6. *Outer solution*

Component	Concentration
Glucose	1050 mM (for Nucleus Cytosol) or 870 mM (for NEB PURExpress)

a TetR-based biosensor and the toehold exchange riboregulators worked in bulk cell-free expression (Figure 7). Due to constraints on the available fluorescence channels on the microscopes available at the course, we had to switch from CFP expression in bulk reactions to GFP expression in the DevCell reactions. Although we do not expect this change to dramatically change system behavior, a detailed comparison would be warranted in a future study. Figure 8 shows the schematics of the different systems we encapsulated in DevCells for testing. In DevCells, we initially tested the same concentrations of Input template and Gate template that we used in the bulk cell-free experiments, but we did not observe much difference in expression levels between positive and negative controls (Figure 9, Figure 10), which contained the unregulated toehold exchange riboregulator circuit (Figure 8). We hypothesized that this might be because we were using multiple templates and perhaps many of the DevCells did not contain high enough concentrations of both templates. Thus, we retried the experiment and increased the Input template concentration to 10-fold of the Gate template. In microscope images, there were clearly many bright DevCells in the positive control samples (Figure 11), suggesting that this optimized ratio worked better. However, image analysis that quantified the average intensity of the DevCells revealed little difference between the positive and negative controls. There was just a much higher fraction of highly fluorescent outliers in the positive control samples (Figure 12, Figure 13, Figure 14), which may suggest that only a small percentage of the DevCells received enough of both the Input and Gate templates to activate protein expression. In the experimental samples, in which TetR and aTc were included, we were unable to assess whether the system worked because aTc exhibited a strong fluorescence signal in the GFP channel (Figure 9, Figure 11, Figure 13).

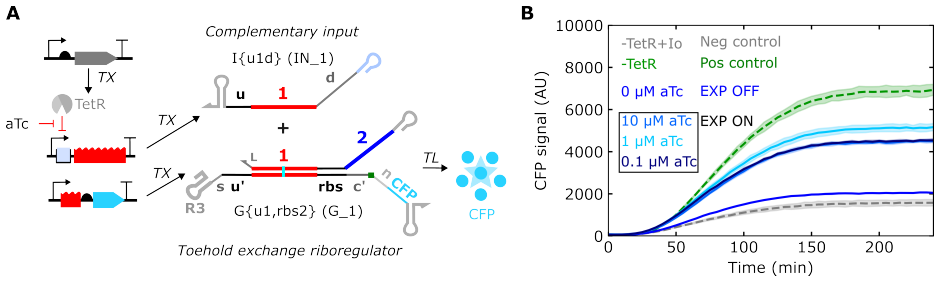


Figure 7. *Overview of system (**A**) and bulk plate reader results (**B**). Experiments were conducted in PURExpress at 37 °C as described in the following DevNote with the following conditions: 2 nM of `G_1` template (Figure 4), 4 nM of `IN_1` template (Figure 2) or `Io` template (Figure 1), and 4 nM of TetR template (Figure 6). The plot shows the mean CFP signal of three technical replicates, with the shaded regions indicating the standard deviation of the mean.*

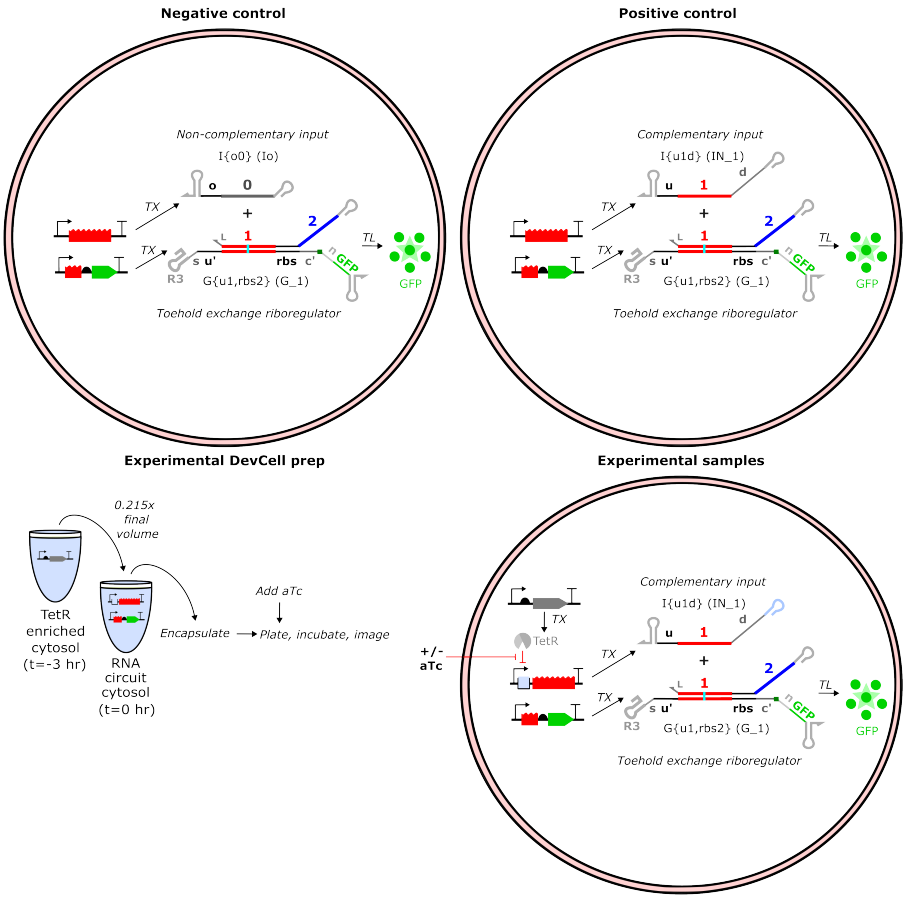


Figure 8. *Overview of different systems encapsulated and tested in DevCells. The negative and positive controls used input templates (`Io`, Figure 1, or `IN_1`, Figure 2) that did not contain the TetR operator downstream of the T7 promoter and thus are expected to have higher transcription rates for these inputs compared to the input regulated with the TetR operator (`IN_1_TetR`, Figure 3) in the experimental samples. The experimental samples were prepared by mixing TetR-enriched cytosol, in which TetR had been expressed for 3 h before the start of the experiment, with a freshly prepared cytosol mixture containing the RNA circuit DNA templates. This mixture was then encapsulated. These experimental samples thus had the same system encapsulated in the DevCells and aTc was either added (EXP ON) or not added (EXP OFF) to the solution surrounding the DevCells.*

5.1. Failure modes

A big failure mode was the green fluorescence of aTc. In future experiments, it is suggested to use a non-fluorescent read-out, such as luminescence, to mitigate this issue. At high concentrations, the aTc signal was so strong that it bled through channels other than the GFP channel, so switching to another fluorescent protein may not resolve the issue. We also

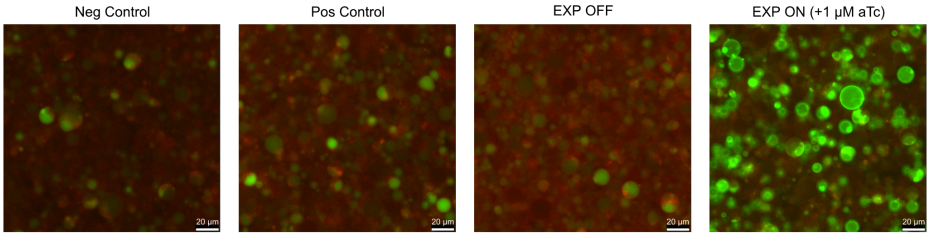


Figure 9. *Representative images of GFP fluorescence in DevCells after cell-free expression at 37 °C. Here, the input-gate template ratio was 4 nM:2 nM. Contrast and brightness adjustments were uniformly applied across all images for GFP (green) and Cy5 membrane dye (red) fluorescence channels. Scale bar: 20 µm.*

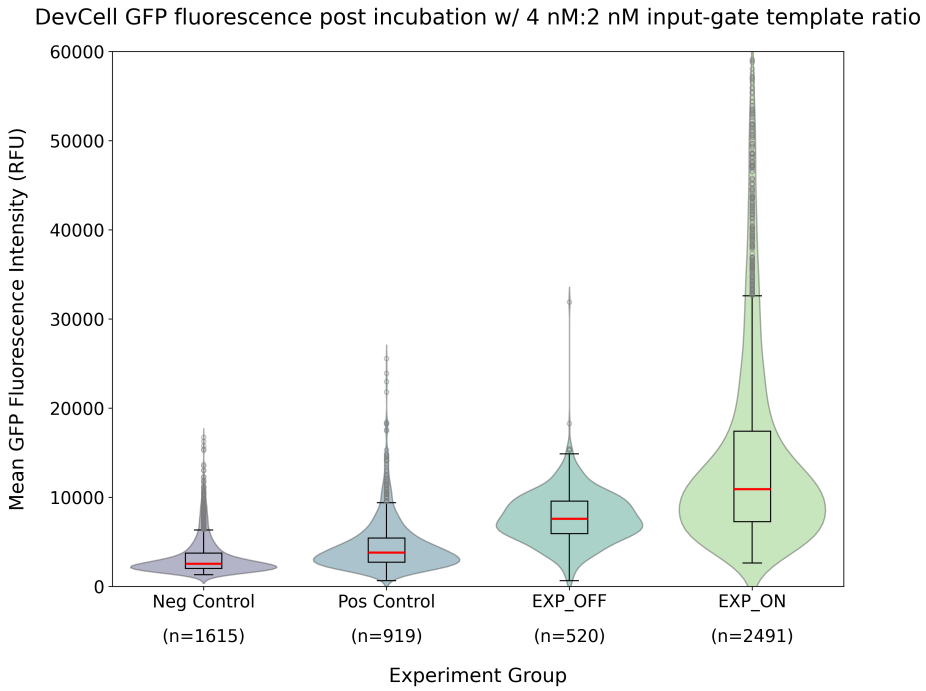


Figure 10. *Quantified GFP fluorescence of DevCells after 1.5 h incubation at 37 °C using bulk cell-free input-gate template ratio (4 nM:2 nM). This analysis was conducted on the images collected in the same experiments as shown in Figure 9, with n indicating the number of DevCells quantified for each sample. See Figure 8 for schematics of the different samples.*

found that aTc photobleached very quickly with our microscope setup, which can inactivate the molecule for binding (Baumschlager *et al.* 2020).

Not getting a high enough concentration of each of the templates in the DevCells is another potential source of failure. There is still more work that needs to be done around optimizing the concentrations of multi-template systems in DevCells.

6. Resources

- **THE riboregulator DevNote:** devnotes.nucleus.engineering/articles/ss-20260712
- **TetR/aTc detector module (Nucleus documentation):** docs.nucleus.engineering/docs/modules/detector-tetr-atc/spec
- **ROSALIND small-molecule biosensor DevNote (Khalid Alam):** devnotes.nucleus.engineering/articles/ka-30072024
- **THE riboregulators in cell-free:** biorxiv.org/content/10.1101/2025.09.25.678518v2
- **ctRSD and THE riboregulator sequence compiler**
  - Documentation: ctrsd-simulator.readthedocs.io/en/latest/SeqCompiler.html
  - Colab: colab.research.google.com/drive/1Bd-ZugIWnc7PMiFkpaia_UnWA24KF3Mv
- **Annotated sequence / plasmid maps from published THE riboregulator manuscripts**
  - Benchling: benchling.com/sschafft1/f_/mW2x3M1k3j-toehold-exchange-riboregulator-published-sequences

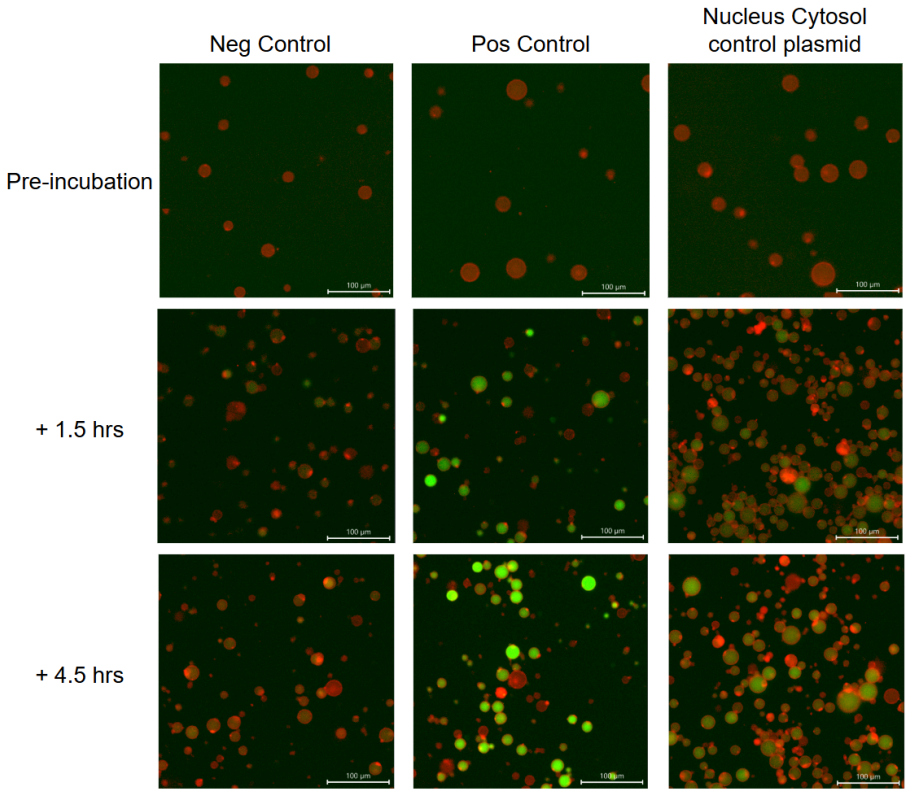


Figure 11. *Representative images of GFP fluorescence in DevCells after cell-free expression at 37 °C. Here, the input-gate template ratio was (20 nM:2 nM). Contrast and brightness adjustments were uniformly applied across all images for GFP (green) and Cy5 membrane dye (red) fluorescence channels. Scale bar: 100 µm.*

7. Participants

CSHL Week 2 participants: Gabriela Garza, Luis Montalvo-Gonzalez, Rocky Choi, Corynne Adanho, Sushmita Halder, Dwon Jordana, Grace Montgomery.

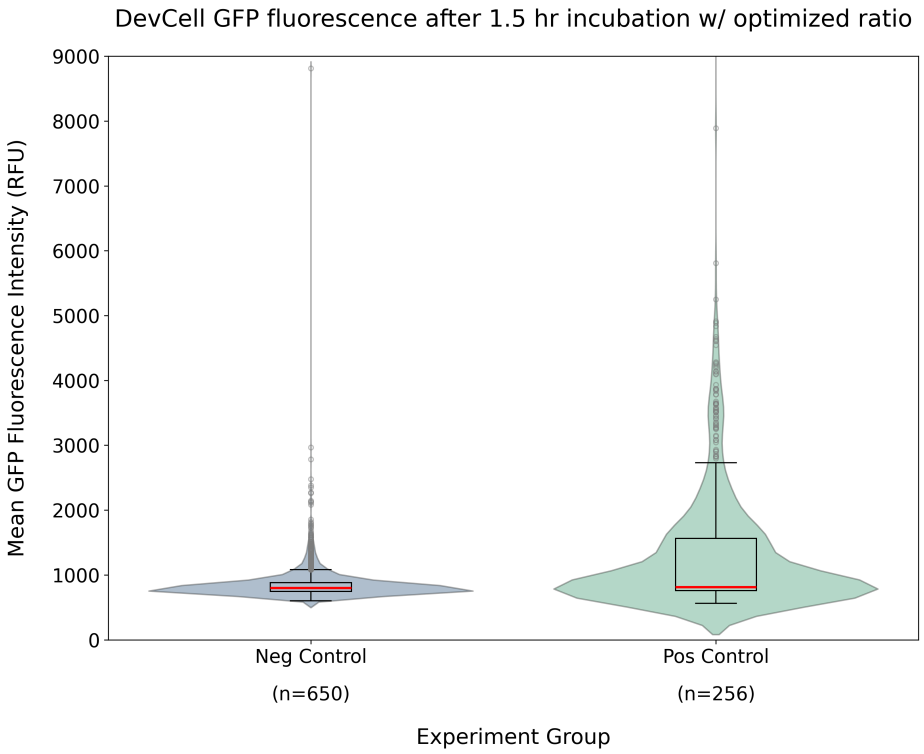


Figure 12. *Quantified GFP fluorescence of DevCells after 1.5 h incubation at 37 °C using optimized input-gate template ratio (20 nM:2 nM). This analysis was conducted on the images collected in the same experiments as shown in Figure 11, with n indicating the number of DevCells quantified for each sample. See Figure 8 for schematics of the different samples.*

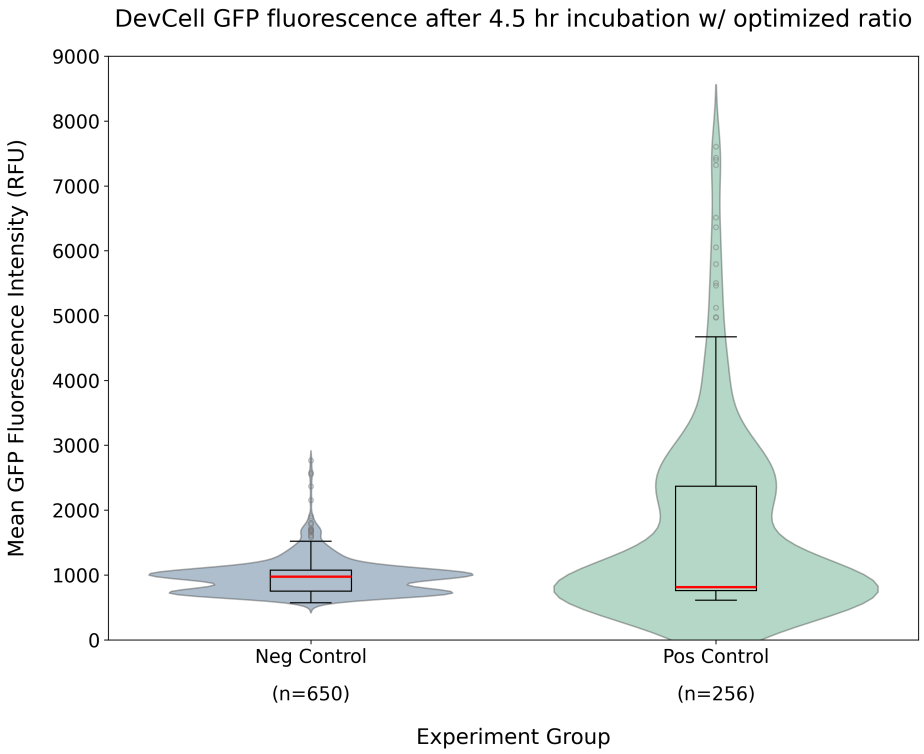


Figure 13. *Quantified GFP fluorescence of DevCells after 4.5 h of incubation at 37 °C using optimized input-gate template ratio (20 nM:2 nM). This analysis was conducted on the images collected in the same experiments as shown in Figure 11, with n indicating the number of DevCells quantified for each sample. See Figure 8 for schematics of the different samples.*

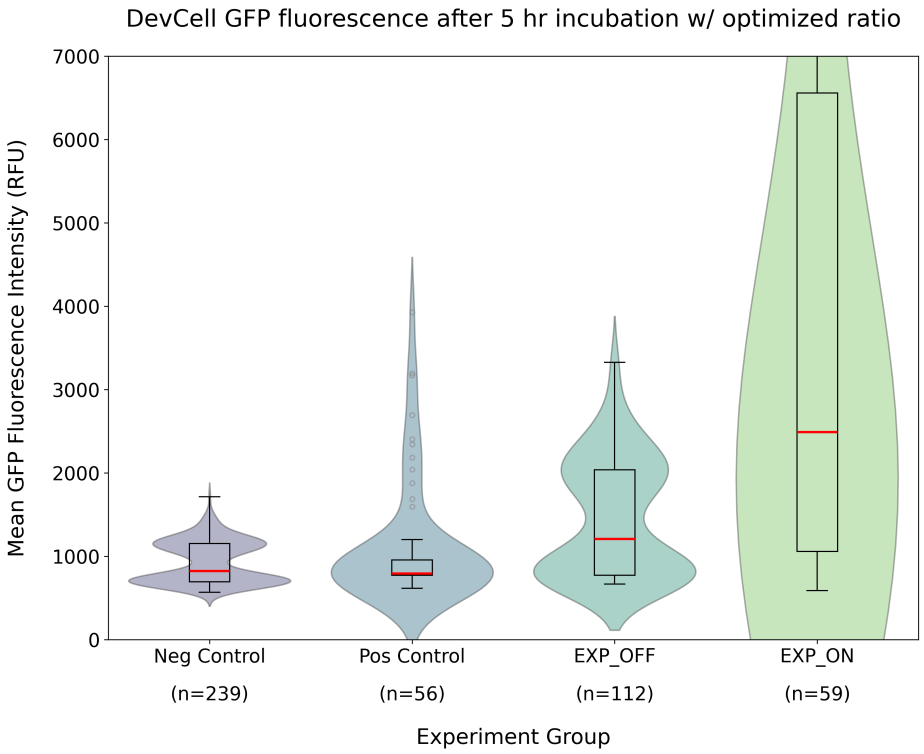


Figure 14. *Quantified GFP fluorescence after 5 h incubation using optimized input-gate template ratio (20 nM :2 nM). Here, all four conditions (Neg Control, Pos Control, EXP OFF, EXP ON) are explored. n indicates the number of DevCells quantified for each sample. See Figure 8 for schematics of the different samples. The high fluorescence in EXP ON is due to aTc's green fluorescence, making inference of any activation difficult.*