Synthetic Control Systems for High-Performance Gene Expression in Mammalian Cells
Gabriele Lillacci, Yaakov Benenson, Mustafa Khammash

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S1 Analytical treatment of the controller steady-state behavior

To model the control topologies under consideration, we use the following set of ordinary differential equations (ODE).

\[
\begin{align*}
\dot{m}_R &= c k_1 - k_2 m_R \\
\dot{p}_R &= k_3 m_R - k_4 p_R \\
\dot{m}_A &= c k_5 - k_6 m_A - k_{15} m_A m_B \\
\dot{p}_A &= k_7 m_A - k_8 p_A \\
\dot{m}_B &= c \frac{k_9 p_A}{h_9 + p_A} - k_{10} m_B \\
\dot{m}_O &= c \frac{k_{11} p_A}{h_{11} + p_A} - k_{12} m_O - k_{16} m_O m_B \\
\dot{p}_O &= k_{13} m_O - k_{14} p_O
\end{align*}
\]

The variables \( m \) and \( p \) denote RNA and protein respectively, while the subscripts indicate the different genetic parts. \( R \) is the copy number reporter (mCitrine), \( A \) is the transactivator (tTA::Cerulean), \( B \) is the auxiliary repressor (miR-FF4) and \( O \) is the output protein of interest (DsRed), which we will also refer to as POI. Clearly, since \( B \) is a non-coding RNA, it has no protein counterpart. The model parameters are denoted by \( k_i, i = 1, \ldots, 16 \) and \( h_j, j = 9, 11 \), while \( c \) indicates the number of plasmid copies taken up by the cell. Throughout the main manuscript and the rest of these Supplementary Materials, we refer to this quantity as copy number or plasmid take-up. Note that the copy number scales the transcription rates only.

We are interested in looking at how the steady-state amounts of the different species in the fully induced synthetic controllers (i.e. in the absence of DOX) vary with the copy number. We refer to the fully induced steady-state amount of the POI as maximal output-level, or MOL. To do that, we set the derivatives equal to zero and solve the resulting algebraic system of equations. We then express the steady-state amounts of \( p_A, m_B \) and \( p_O \) as functions of \( c \). These functions are referred to as plasmid-takeup/TA function, plasmid-takeup/repressor function, and plasmid-takeup/MOL function respectively. We assume that all variables and parameters are strictly positive, except for \( k_{15} \) and \( k_{16} \), which may be positive or zero as required by the different topologies. Note that the first two equations above are decoupled from the following five. Therefore, given that at steady state we have:

\[
\begin{align*}
p_R &= \frac{k_1 k_3}{k_2 k_4},
\end{align*}
\]

it is equivalent to talk about \( p_A, m_B \) and \( p_O \) as functions of the plasmid copy number \( c \) or of the amount of reporter protein \( p_R \), as \( c \) and \( p_R \) are linearly related.

S1.1 OLP topology

For the OLP topology, we have \( k_{15} = k_{16} = 0 \). The steady-state value of \( p_O \), i.e. the plasmid-takeup/MOL function is:

\[
\begin{align*}
p_O^{OLP}(c) &= \frac{c^2 k_5 k_7 k_{11} k_{13}}{c k_5 k_7 k_{12} k_{14} + h_{11} k_6 k_8 k_{12} k_{14}},
\end{align*}
\]

We can rewrite this as:

\[
\begin{align*}
p_O^{OLP}(c) &= \alpha_1 \frac{c^2}{c + \alpha_2 h_{11}},
\end{align*}
\]

with:

\[
\begin{align*}
\alpha_1 &= \frac{k_{11} k_{13}}{k_{12} k_{14}}, \\
\alpha_2 &= \frac{k_6 k_8}{k_5 k_7}
\end{align*}
\]
As \( c \) becomes large, \( p_A^{OLP}(c) \) grows linearly with \( c \). The steady-state value of \( p_A \), also called the plasmid-takeup/TA function, is:

\[
p_A^{OLP}(c) = \frac{k_5 k_7}{k_6 k_8} c = \frac{1}{\alpha_2} c. \tag{S6}
\]

It is seen that the transactivator grows linearly with \( c \) for any value of \( c \). Finally, we look at the steady-state value of \( m_B \), also known as the plasmid-takeup/repressor function:

\[
m_B^{OLP}(c) = \frac{c^2 k_5 k_7 k_9}{c k_5 k_7 k_{10} + k_6 k_8 k_{10} h_9} = \frac{k_9}{k_{10}} \frac{c^2}{c + \alpha_2 h_9}. \tag{S7}
\]

The repressor grows also approximately linearly with the copy number for large \( c \).

### S1.2 IFF topology

In the IFF controller, we have \( k_{15} = 0 \) and \( k_{16} > 0 \). The plasmid-takeup/MOL function is:

\[
p_O^{IFF}(c) = \frac{N^{IFF}(c)}{D^{IFF}(c)},
\]

where:

\[
N^{IFF}(c) = c^3 k_5^2 k_7^2 k_{10} k_{11} k_{13} + c^2 h_9 k_5 k_6 k_7 k_8 k_{10} k_{12} k_{14},
\]

and:

\[
D^{IFF}(c) = c^3 k_5^2 k_7^2 k_9 k_{14} k_{16} + c^2 h_{11} k_5 k_6 k_7 k_8 k_9 k_{14} k_{16} + c^2 k_5^2 k_7 k_{10} k_{12} k_{14} + \ldots
\]

This can be rewritten as:

\[
p_O^{IFF}(c) = \frac{\beta_1 c^3 + \beta_1 \beta_2 h_9 c^2}{\beta_3 c^3 + (1 + \beta_1 \beta_2 h_9) c^2 + \beta_2 (h_9 + h_{11}) c + \beta_2^2 h_9 h_{11}}.
\tag{S11}
\]

with:

\[
\beta_1 = \alpha_1 = \frac{k_{11} k_{13}}{k_{12} k_{14}}, \tag{S12a}
\]

\[
\beta_2 = \alpha_2 = \frac{k_6 k_8}{k_5 k_7}, \tag{S12b}
\]

\[
\beta_3 = \frac{k_9 k_{16}}{k_{10} k_{12}}. \tag{S12c}
\]

Clearly, as \( c \) becomes large, the value of \( p_O^{IFF} \) approaches the ratio of \( \beta_1 \) and \( \beta_3 \), and becomes independent of \( c \) itself. This shows the adaptation to copy number adaptation feature that was described previously for the IFF topology [3]. However, the plasmid-takeup/TA and plasmid-takeup/repressor functions are no different from the one of the OLP topology, so we have:

\[
p_A^{IFF}(c) = p_A^{OLP}(c) = \frac{k_5 k_7}{k_6 k_8} c = \frac{1}{\beta_2} c. \tag{S13}
\]

and

\[
m_B^{IFF}(c) = m_B^{IFF}(c) = \frac{c^2 k_5 k_7 k_9}{c k_5 k_7 k_{10} + h_9 k_6 k_8 k_{10}} = \frac{k_9}{k_{10}} \frac{c^2}{c + \beta_2 h_9}. \tag{S14}
\]

The lack of negative feedback regulation implies that the transactivator and the repressor are free to grow with the copy number, so the attractive adaptation feature of the IFF topology comes at the price of very high transactivator and repressor levels. These impose a burden on the host cells that may be excessive in some applications, and is generally undesirable if one wants to build larger and larger synthetic circuits.
S1.3 FBK topology

For $k_{15} > 0$ and $k_{16} = 0$ (FBK topology), we have:

$$p^F_{FBK}(c) = \frac{2c^2 h_9 k_5 k_7 k_{11} k_{13}}{D^F_{FBK}(c)} \quad (S15)$$

with:

$$D^F_{FBK}(c) = h_9 h_{11} k_8 k_{12} k_{15} k_{14} \sqrt{\frac{4c^2 h_9 k_5 k_7 k_8 k_9 k_{15} + k_{10} (c k_5 k_7 + h_9 k_6 k_8)^2}{h_9^2 k_8^2 k_{10} k_{15}^2}} + 2c h_9 k_5 k_7 k_{12} k_{14} - c h_{11} k_5 k_7 k_{12} k_{14} + h_9 h_{11} k_6 k_8 k_{12} k_{14}. \quad (S16)$$

The function can be rewritten as:

$$p^F_{FBK}(c) = \frac{2\gamma_1 \gamma_4 c^2}{\sqrt{4\gamma_2\gamma_3 h_9 c^2 + (c + \gamma_2 h_9)^2 + (\gamma_4 - 1) c + \gamma_2 h_9}} \quad (S17)$$

where:

$$\gamma_1 = \beta_1 = \frac{k_{11} k_{13}}{k_{12} k_{14}} \quad (S18a)$$

$$\gamma_2 = \beta_2 = \frac{k_8 k_5}{k_5 k_7} \quad (S18b)$$

$$\gamma_3 = \frac{k_9 k_{15}}{h_9 k_{10}} \quad (S18c)$$

$$\gamma_4 = \frac{h_9}{h_{11}}. \quad (S18d)$$

We conclude that for large $c$, MOL will grow approximately linearly with the copy number. Hence, the feedback topology does not display adaptation to copy number.

However, if we look at the plasmid-takeup/TA function, we find:

$$p^F_{FBK}(c) = \frac{2c h_9 k_5 k_7}{h_9 k_8 k_{15} \sqrt{\frac{4c^2 h_9 k_5 k_7 k_8 k_9 k_{15} + k_{10} (c k_5 k_7 + h_9 k_6 k_8)^2}{h_9^2 k_8^2 k_{10} k_{15}^2}} - c k_5 k_7 + h_9 k_6 k_8} \quad (S19)$$

$$= \frac{2c h_9}{\sqrt{4\gamma_2\gamma_3 h_9 c^2 + (c + \gamma_2 h_9)^2 - c + \gamma_2 h_9}}.$$ 

For $c \to \infty$, the above expression approaches:

$$\frac{2h_9}{\sqrt{4\gamma_2\gamma_3 h_9 + 1 - 1}}. \quad (S20)$$

Due to the feedback regulation, the transactivator levels are not free to grow unbounded as in the case of the IFF topology.

The plasmid-takeup/repressor function is:

$$m^F_{FBK}(c) = \frac{\sqrt{4c^2 h_9 k_5 k_7 k_8 k_9 k_{15}}}{k_{10}} \cdot \left(\frac{c k_5 k_7 + h_9 k_6 k_8}{c k_5 k_7 - h_9 k_6 k_8}\right)$$

$$\quad = \frac{k_5 k_7}{2h_9 k_8 k_{15}} \left[\sqrt{4\gamma_2\gamma_3 h_9 c^2 + (c + \gamma_2 h_9)^2} - c - \gamma_2 h_9\right]. \quad (S21)$$

It is seen that for large $c$ the repressor grows linearly with the copy number.
S1.4 HYB topology

So far we established that the IFF topology displays adaptation to copy number, but operates with high transactivator levels. On the contrary, in the FBK topology the transactivator levels are bounded, but MOL grows linearly with the copy number. It is therefore natural to ask whether one can combine the two desirable properties by creating a new topology with both feedback and feedforward regulation. The plasmid-takeup/MOL function of the new proposed HYB topology is the following:

\[
p_O^{HYB}(c) = \frac{2c^2h_9^2k_5k_7k_8k_{10}k_{11}k_{13}k_{15}}{D^{HYB(c)}},
\]

where:

\[
D^{HYB(c)} = -c^2h_9k_5^2k_7^2k_{10}k_{14}k_{16} + c^2h_{11}k_5^2k_7^2k_{10}k_{14}k_{16} + 2c^2h_9h_{11}k_5k_7k_8k_{10}k_{14}k_{15}k_{16} + \sqrt{4c^2h_9k_5k_7k_8k_{10}k_{15} + k_{10} (ck_5k_7 + h_9k_6k_8)^2} - \frac{h_9^2k_5^2k_{10}k_{15}^2}{k_5^2k_{10}k_{15}^2}
\]

\[
+ c_0h_9h_{11}k_5k_7k_8k_{10}k_{13}k_{15}k_{16} + \sqrt{4c^2h_9k_5k_7k_8k_{10}k_{15} + k_{10} (ck_5k_7 + h_9k_6k_8)^2} + 2c^2h_9k_5k_7k_8k_{10}k_{12}k_{14}k_{15} - c_0h_9h_{11}k_5k_7k_8k_{10}k_{12}k_{14}k_{15} - c_0h_9h_{11}k_5k_6k_7k_8k_{10}k_{14}k_{16} + h_9^2h_{11}k_6k_7^2k_{10}k_{12}k_{14}k_{15}.
\]

The function can be rewritten as follows:

\[
p_O^{HYB}(c) = \frac{2h_9^2\delta_1\delta_2 k_{15} c^2}{a_2c^2 + \left(1 + \frac{h_9}{h_{11}}\right)F(c)c + h_9h_{11}\delta_2 F(c) + a_1c + h_9^2h_{11}\delta_2 F(c)},
\]

where:

\[
\delta_1 = \frac{k_{11}k_{13}}{k_9k_{14}} \quad (S25a)
\]

\[
\delta_2 = \gamma_2 = \frac{k_9k_8}{k_5k_7} \quad (S25b)
\]

\[
\delta_3 = \gamma_3 = \frac{k_9k_{15}}{k_9k_{10}} \quad (S25c)
\]

\[
\delta_4 = \frac{k_9k_{16}}{k_{12}k_{15}} \quad (S25d)
\]

\[
a_2 = h_{11} - h_9 + 2h_9h_{11}\delta_2 \delta_3 \quad (S25e)
\]

\[
a_1 = h_9\delta_2 \left(2 - \frac{h_{11}}{h_9} - \delta_2 + \frac{h_{11}}{h_9}\delta_4\right) \quad (S25f)
\]

\[
F(c) = \sqrt{4\delta_2\delta_3h_9c^2 + (c + \delta_2h_9)^2} \quad (S25g)
\]

It follows that MOL remains bounded for \(c \to \infty\).

It is easy to see that the plasmid-takeup/TA and plasmid-takeup/repressor functions are no different than the ones of the FBK topology. Hence, we have:

\[
p_A^{HYB}(c) = \frac{2ch_9}{\sqrt{4\delta_2\delta_3h_9c^2 + (c + \delta_2h_9)^2} - c + \delta_2h_9},
\]

(S26)
and
\[ m^\text{HYB}_B(c) = \frac{k_5 k_7}{2h_9 k_8 k_{15}} \left[ \sqrt{4\delta_2 h_9 c^2 + (c + \delta_2 h_9)^2} - c - \delta_2 h_9 \right]. \]  \hspace{1cm} (S27)

We conclude that the HYB topology displays the desired adaptation to copy number feature (just like the IFF topology), but with bounded transactivator levels (like the FBK topology).
S2 Transcriptional versus post-transcriptional feedback

Previous work [2, 5] has shown that in the case of feedback regulation the MOL of the controller grows with the square root of the copy number. However, for our FBK topology we found that the growth is approximately linear. Why is that?

The key difference lies in the fact that in our implementation of the circuits the repression is post-transcriptional and not transcriptional. As a consequence, the transcription rate of the repressor scales with the copy number. To see this, we will consider a simple example: a gene that codes for a protein that can repress its own transcription. The corresponding ODE model is:

\[
\begin{align*}
\dot{m} &= ck_1 \frac{h_1}{h_1 + p} - k_2 m \\
\dot{p} &= k_3 m - k_4 p
\end{align*}
\] (S28a)

The steady-state value of \( p \), assuming all variables and parameters are strictly positive, is:

\[
p(c) = -\frac{h_1}{2} + \frac{1}{2} \sqrt{h_1 \left( \frac{4ck_1 k_3}{k_2 k_4} + h_1 \right)}.
\] (S29)

We find the square-root growth that was shown before.
S3 Numerical simulations

S3.1 Parameter values

The parameter values that were used to generate the plots in Figure S1b,c are listed in the Supplementary Table S1. The plots in Figure S1a were generated with the same values except for $k_{15}$ and $k_{16}$. The HYB plot (blue) was generated with $k_{15} = 1$ and $k_{16} = 0.5, 0.1, 0.05$. The IFF plot (red) was generated for $k_{15} = 0.5$ and $k_{16} = 0.5, 0.1, 0.05$. The FBK plot (yellow) was generated for $k_{15} = 50, 10, 0.05$ and $k_{16} = 0.08$. The OLP plot (green) clearly had $k_{15} = k_{16} = 0$. In all the simulations reported in Figure S1 the copy number $c$ was varied between 1 and 55.

S3.2 Plasmid-takeup/MOL and plasmid-takeup/TA behavior of the four controllers

Figure S1 shows simulated plasmid-takeup/MOL and plasmid-takeup/TA functions for the four proposed controller topologies. The simulations were carried out based on the models described in Section S1 with the parameter values reported in Section S3.1.

S3.3 Plasmid-takeup/repressor behavior of the controllers

In the Supplementary Note S1, we established that the plasmid-takeup/repressor functions of all the four controllers grow approximately linearly with the copy number. To study whether there are any significant differences in the growth rates, we plotted the plasmid-takeup/repressor relationships as a function of the transactivator dose-response curve. For simplicity, we set $k_9 = k_{11}$ and $h_9 = h_{11}$, thereby considering identical activation profiles for both $m_B$ and $m_O$. We vary the steepness of the curve by varying $h_9$, while keeping $k_9$ constant. We plot the plasmid-takeup/repressor functions for 5 different values of $h_9$ (Supplementary Figure S2). We observe that as $h_9$ increase, and the transactivator dose-response curve becomes less sharp, the difference in the growth rates of the plasmid-takeup/repressor functions of IFF and OLP and those of FBK and HYB becomes more pronounced. In such cases, the resource-saving effect of the post-transcriptional feedback is increased.

S3.4 Dependency of plasmid-takeup/MOL functions on transactivator dose-response curve

To gain a better understanding of how the controller plasmid-takeup/MOL functions vary with important model parameters, we investigated their dependency on the transactivator dose-response curve. In the following, for simplicity we set $k_9 = k_{11}$ and $h_9 = h_{11}$. We vary the steepness of the curve by varying $h_9$, while keeping $k_9$ constant. We plot the plasmid-takeup/MOL functions for 5 different values of $h_9$ (Supplementary Figure S7). We note that the qualitative behavior of the controllers is largely unaffected. The only notable feature that is lost if the transactivator curve is not steep enough is the ability of the FBK topology to express the output as strongly as OLP. As $h_9$ increases, we notice an increasing drop in MOL between OLP and FBK and between IFF and HYB.
S4  miR-FF4 knockdown tests

To assess whether miR-FF4 was functional in knocking down tTA::Cerulean, we cotransfected the parts indicated in Supplementary Figure S3a (on 3 separate plasmids) into HEK293T cells and HeLa cells, and we compared the samples in which tTA::Cerulean had miR-FF4 targets in the 3'-UTR to the ones containing the tTA::Cerulean variant with mir FF5 targets. We included an additional source of tTA-Advanced to ensure that an excess amount of the transcription factor was present and that the activation of P_TRE did not depend on tTA::Cerulean only. This could have potentially affected the levels of DsRed and miR-FF4. After 48 hours, a time that was previously reported as sufficient to achieve quasi steady-state conditions in transient transfections [3], we analyzed the blue and red fluorescence using flow cytometry. Since in this experiment DsRed is constitutively expressed, we used it as a copy number reporter and we looked at the levels of Cerulean as a function of the levels of DsRed. The data for both HEK293T (Supplementary Figure S3b) and HeLa cells (Supplementary Figure S3c) clearly show that the samples with miR-FF4 targets display much lower levels of tTA::Cerulean for the same amount of DsRed. These results indicate that the DsRed-FF4 cassette is properly spliced and that the intronic miR-FF4 is correctly processed into a functional mature microRNA.

We then asked whether miR-FF4 could successfully target its own transcript. To address this question, we cotransfected the parts indicated in Supplementary Figure S4a (on 2 separate plasmids), comparing the samples with the DsRed-TFF4 gene to the ones with the DsRed-TFF5 gene. We now used tTA::Cerulean as copy number reporter and looked at the levels of DsRed as a function of the levels of Cerulean. Supplementary Figures S4b and S4c show the results for HEK293T and HeLa cells respectively. Again, the samples with miR-FF4 targets display much lower levels of DsRed for the same amount of tTA::Cerulean, suggesting that the self-knockdown is functional.
S5 Fitting of plasmid-takeup/MOL functions

To confirm that the experimental plasmid-takeup/MOL functions of our circuits are consistent with the ones that were predicted computationally, we performed curve fitting on the data shown in Figure 4(c) in the main text. Each experimental plasmid-takeup/MOL function was fit to approximate analytical plasmid-takeup/MOL functions of the following classes:

\[ f(c) = \frac{ac}{b+c}, \]  
\( (S30) \)

or:

\[ f(c) = \frac{ac^2}{b+c}. \]  
\( (S31) \)

The results are reported in the Supplementary Figure S6. We note that the OLP and FBK topologies are fit very well by both classes of functions. This is not surprising, as both are capable of generating approximate linear growth with copy number for suitable parameter values. On the contrary, the IFF and HYB topologies are better fit by functions of the class \((S30)\). This is because only class \((S30)\) can generate the saturation behavior that is required for copy number adaptation.
S6 Sensitivity to doxycycline

Our circuits make use of tTA::Cerulean as a transactivator. Therefore, Dox can be used as a parameter to tune the output of the motifs. To investigate whether the four topologies differ in their response to Dox, we considered again the single-plasmid motifs (Fig. 4b), and we transfected them in presence of different concentrations of Dox. This experiment was done in HEK cells plated in 24-well plates. The cells were seeded at the density of $10^5$ cells in 500 mL of complete medium containing one of the following concentrations of Dox: 0, 0.1, 0.2, 0.3, 0.5, 0.7 or 1.0 ng mL$^{-1}$. For transfection, we used 500 ng of DNA per well and 1.5 µL of Lipofectamine 2000 per well. The experiment was otherwise carried out as described in the general procedures of Supplementary Text S7.2. For each topology and condition, we then measured (in triplicates) the average DsRed fluorescence in the population of mCitrine-positive cells. This was normalized by the mean of mCitrine fluorescence (Supplementary Fig. S8a,b). As expected, the DsRed fluorescence decreases as a function of Dox in all circuits.

We fitted the data to Hill functions and found that the feedback motif displays a 3.2-fold increase in sensitivity to Dox compared to the open loop motif, as shown by the half maximal inhibitory concentration (IC$_{50}$) in the two cases (Supplementary Fig. S8b and Supplementary Table S2). This effect can explained by the fact that the feedback topology expresses much less tTA::Cerulean than the control, and therefore the same concentration of Dox leads to less free tTA::Cerulean. On the contrary, the incoherent feedforward circuit was found to have a 1.8-fold decrease in sensitivity to Dox (Supplementary Fig. S8b,c). In the hybrid controller these two effects appear to cancel out, and the IC$_{50}$ is similar to that of the open loop configuration (Supplementary Table S2).

The experimentally observations are also well captured by model simulations (Supplementary Fig. S8d-f). The plots were generated with the same parameter values reported in the Supplementary Table S1, except for $h_9$, $h_{11}$, $k_{15}$ and $k_{16}$, which were set to 0.2930, 0.8828, 0.0984, 0.3233 respectively. These values were found by fitting the model-generated IC$_{50}$ values to the experimental IC$_{50}$. The copy number $c$ was fixed to 20. The effect of Dox is modeled by a Dox-dependent increase of the degradation rate of tTA::Cerulean, i.e. of $k_8$, which is equivalent to saying that tTA::Cerulean bound to Dox is inactive. We used the following formula:

$$ k_{8,DOX} = 2.0601 + \frac{[DOX]^{1.5106}}{0.6031^{1.5106} + [DOX]^{1.5106}}, \quad (S32) $$

with $[DOX] = 0, 0.1, 0.2, 0.3, 0.5, 0.7, 1$. The parameters in (S32) were also found by fitting the IC$_{50}$ values.
S7 SI Materials and Methods

S7.1 Plasmid construction

The plasmids used in this study are derivatives of five constructs that were supplied by YB, and resulted from previous work done in his lab. These are as listed below.

- pGLM43 (formerly pKH025), containing \( P_{\text{EF1}}\alpha\)-mCitrine terminated by the rabbit \( \beta\)-globin polyadenylation signal (RbGpA).
- pGLM44 (formerly pKH024), containing \( P_{\text{EF1}}\alpha\)-Cerulean-RbGpA.
- pGLM45 (formerly pEL0089), containing a promoter-less mCitrine flanked by two insulator sequences (INS). The 5' insulator is followed by a bovine growth hormone polyadenylation signal (bGhpA).
- pGLM46 (formerly pTRE-tight-bi-DsRed-FF3wolac-CMV-Cyan-FF3), with the bidirectional promoter \( P_{\text{TRE-tight-bi}}\). One side of the promoter drives a cassette in which the synthetic mir-FF3 has been placed into an intron of DsRed, while the other side drives AmCyan. The 3'-UTR of DsRed contains a target sequence for mir-FF3. Both genes are terminated by SV40 polyadenylation signals.
- pGLM131 (formerly pJS014), containing \( P_{\text{UbC}}\)-TetR-SV40pA.

The inert plasmid that was used to adjust the DNA mass in some experiments is U6+27-cxcr4, and was a gift from Phil Sharp (Addgene plasmid no. 22498) [4]. It encodes a transcript with repeated binding sites complementary to an artificial microRNA based on a sequence from the CXCR4 gene, but not complementary to any known microRNA. The plasmid pGLM180, containing \( P_{\text{EF1}}\alpha\)-iRFP670-SV40pA was created by Timothy Frei in the group of MK. The sequences of the oligonucleotides that were used in the cloning are listed in the Supplementary Tables S6 and S7.

S7.1.1 Genetic part toolbox

We now describe the construction of the parts shown in Figures 1 and 4(a) of the main text. The plasmids mentioned in this subsection are summarized in the Supplementary Table S3.

The copy number reporter (pGLM49) was constructed by inserting \( P_{\text{EF1}}\alpha\) between the NheI and NcoI sites of pGLM45. The promoter was amplified from pGLM43 using primers oGL56 and oGL57.

The tTA::Cerulean fusion genes were made as follows. First, we inserted \( P_{\text{EF1}}\alpha\) between the NheI and BsrGI sites of pGLM45. The promoter was amplified from pGLM43 using primers oGL69 and oGL70. The resulting construct was pGLM53. Next, we performed a one-step ligation of two inserts into pGLM53 cut with PacI and SpeI. The first insert, tTA-Advanced, was amplified (without stop codon) from pTet-Off-Advanced (Clontech) using primers oGL72 and oGL73. The second insert, Cerulean, was amplified from pGLM44 using primers oGL74 and oGL75. The resulting construct was pGLM60. Note that the primers introduced the following additional cut sites: Clal before \( P_{\text{EF1}}\alpha\), Pac after \( P_{\text{EF1}}\alpha\), Kpnl between tTA and Cerulean. It also introduced a TFF5 target sequence right after the coding sequence of Cerulean, flanked by BspEI and SpeI cut sites. Next, we replaced the backbone of pGLM60 with a new one containing the puromycin resistance gene. A first fragment containing the pUC origin of replication and the ampicillin resistance gene was amplified from pTet-Off-Advanced using primers oGL81 and oGL82. A second fragment containing the puromycin resistance gene was amplified from pMIR-REPORT (Ambion) using oGL83 and oGL84. These two were first assembled into a new fragment denoted OE-backbone using overlap extension PCR. Then, we performed a 2-part Gibson assembly using the OE-Backbone fragment and another one amplified from pGLM60 with oGL85 and oGL86. The result was pGLM65. Finally, we replaced the target sequenced in the 3'-UTR of pGLM65. Complementary synthetic oligonucleotides oGL163 and oGL164 (encoding 3 repeats of TFF4) were annealed, phosphorylated and ligated into pGLM65 digested with BspEI and SpeI to produce pGLM95. The same procedure but with oligos oGL167 and oGL168 (encoding 3 repeats of TFF5) gave pGLM96.

The DsRed constructs with intronic miR-FF4 were made as follows. First, pGLM64 was constructed by a one-step ligation of two inserts into pGLM45 digested with NheI and BsrGI. Insert 1, \( P_{\text{TRE}}\), was amplified from pGLM46 using primers oGL87 and oGL88. Insert 2, DsRed-Express with intronic mir-FF3 and FF3
target, referred to as DsRed(FF3)-TFF3, was also amplified from pGLM46 using primers oGL89 and oGL90. The primers introduced additional cut sites: EcoRV before P_TRE and NotI after P_TRE. Furthermore, they replaced the cut site at the 3’ side of TFF3 with AscI (original plasmid has SalI), so that the target sequence in now flanked by BstBI and AscI. Next, we performed site directed mutagenesis on pGLM64 to recover a SnaBI cut site that would allow us to change the micro-RNA encoded by the intron. The mutation was performed using the QuikChange Lightning kit (Agilent Genomics), and the mutagenic primers oGL153 and oGL154, and yielded pGLM88. At this point, we replaced the FF3 hairpin in pGLM88 with an FF4 hairpin. Complementary synthetic oligos oGL155 and oGL156 were annealed, phosphorylated and ligated into pGLM88 digested with SnaBI and EcoRI. This created pGLM89. Finally, we replaced the target sequences in pGLM89. Complementary synthetic oligomucleotides oGL161 and oGL162 (encoding 3 repeats of TFF4) were annealed, phosphorylated and ligated into pGLM89 digested with BstBI and AscI to produce pGLM91. The same procedure, but with oligos oGL165 and oGL166, (encoding 3 repeats of TFF5) gave pGLM92.

In order to have a constitutive red single-fluorescence control, we created a plasmid containing P_{EF1α}-DsRed(FF3)-TFF5. First, we replaced the FF3 target with an FF5 target in pGLM64 as follows. Complementary synthetic oligos oGL97 and oGL98 were annealed, phosphorylated and ligated into pGLM64 digested with BstBI and AscI. This created pGLM73. Next, the insert containing DsRed(FF3)-TFF5 was amplified from pGLM73 using oligos oGL134 and oGL136. The resulting PCR product was digested with PacI and SpeI and cloned into pGLM65 cut with the same enzymes. This yielded pGLM79. pGLM49 (mCitrine), pGLM79 (DsRed), pGLM65 (Cerulean) and pGLM180 (iRFP670) served as single-fluorescence controls for data compensation.

S7.1.2 Single plasmids

We now describe the construction of the single plasmids containing the full gene regulation controllers (main text, Figure 4(b)). The plasmids mentioned in this subsection are summarized in the Supplementary Tables S4 and S5.

The first single plasmid construct was obtained as a 4-part Gibson assembly as follows. Fragment 1 (containing INS-bGHpA-P_{EF1α}-mCitrine-SV40pA) was amplified from pGLM49 using primers oGL85 and oGL91. Fragment 2 (containing INS-bGHpA-P_{EF1α}-tTA::Cerulean-TFF5x1-SV40pA) was amplified from pGLM60 using oGL92 and oGL93. Fragment 3 (containing INS-bGHpA-P_TRE-DsRed(FF3)-TFF3-SV40pA-INS) was amplified from pGLM64 using oGL94 and oGL86. Fragment 4 was the same OE-backbone used to assemble pGLM65. The four fragments were mixed in equimolar amounts to a final volume of 5 µL. The DNA mix was added to 15 µL of 1.33x Gibson assembly master mix (Supplementary Tables S8 and S9) and the final 20 µL reaction was incubated at 50°C for 1 hour. Following this incubation, 10 µL of the reaction mixture was transformed into XL10-Gold ultracompetent cells (Agilent Genomics). The resulting construct was pGLM66.

Next, we replaced the mir-FF3 intron in pGLM66 with a miR-FF4 intron. The miR-FF4 intron was obtained by digesting pGLM89 with NotI and Bsu36I. The restriction fragment was gel-purified and ligated into pGLM66 digested with BstBI and AscI. The resulting construct was pGLM93. The same process, but with oligos oGL165 and oGL166 yielded pGLM94.

Next, we replaced the FF5 target with 3xFF4 targets in the 3'-UTR of DsRed in pGLM90. Complementary synthetic oligos oGL161 and oGL162 were annealed, phosphorylated and ligated into pGLM90 digested with BstBI and AscI. The resulting construct was pGLM93. The same process, but with oligos oGL165 and oGL166 yielded pGLM94.

Next, we replaced P_{EF1α}-tTA::Cerulean with P_{SV40}-tTA::Cerulean in pGLM97-100. To do that, we first replaced P_{EF1α} with P_{SV40} in pGLM96. The SV40 promoter was amplified from pGLM96 using primers oGL224 and oGL225. The product was digested with ClaI and PacI and ligated into pGLM96 digested with the same enzymes. This yielded pGLM125. Then, a fragment containing P_{SV40}-tTA was obtained from pGLM125 by digestion with ClaI and KpnI and subsequent gel extraction. Ligation of the purified fragment
into pGLM97-100 digested with the same enzymes yielded pGLM127-130 respectively.

Next, we replaced the P\text{EF\textsubscript{1}}\text{α}-tTA::Cerulean with P\text{UbC}-tTA::Cerulean in pGLM97-100. To do that, we first replaced P\text{EF\textsubscript{1}}\text{α} with P\text{UbC} in pGLM96. The UbC promoter was amplified from pGLM131 using primers oGL229 and oGL230. The product was digested with ClaI and PacI and ligated into pGLM96 digested with the same enzymes. This yielded pGLM139. Then, a fragment containing P\text{UbC}-tTA was obtained by digestion of pGLM139 with ClaI and KpnI, followed by gel extraction. The purified fragment was ligated into pGLM97-100 digested with the same enzymes to yield pGLM141-144 respectively.

To create the single plasmids pGLM204-209, which implement the feedback and open loop topologies with 3 different promoters driving tTA::Cerulean and were used to simulate the protein manufacturing scenario with the CHO-K1 transfections, we digested pGLM99, pGLM100, pGLM129, pGLM130, pGLM143 and pGLM144 (respectively) with HindIII and BstBI. The cut vectors were gel-purified and ligated with the complementary synthetic oligos oGL369 and oGL370, which were previously annealed and phosphorylated. This introduced a 6xHis tag and a V5 epitope at the 3’ end of DsRed, a modification that could be used to purify the output protein with affinity chromatography and to quantitate it using immunoblots.

### S7.2 Cell cultures

#### S7.2.1 HEK293T and HeLa cells

HEK293T and HeLa cells were obtained from ATCC (cat. nos. CRL-3216 and CCL-2 respectively). The cells were grown at 37°C, 100% humidity, 5% CO\textsubscript{2}, and were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM, Sigma-Aldrich, cat. no. D5671), supplemented with 1% GlutaMAX (Invitrogen, cat. no. 35050-038), 10% fetal bovine serum (Sigma-Aldrich, cat. no. F7524), 1 mM sodium pyruvate (Gibco, cat. no. 11360070), and 1% penicillin-streptomycin solution (Sigma-Aldrich, cat. no. P4333). The adherent cultures were maintained in this medium by detaching them with 0.25% trypsin without phenol red (Invitrogen, cat. no. 15090-046, diluted 10-fold in PBS) and diluting in fresh medium upon reaching 50-90% confluence. Unless otherwise specified, HEK and HeLa cells were transfected in 6-well plates. The cells were plated at the approximate density of 4 · 10\textsuperscript{5} cells in 2 mL of complete medium without antibiotics. After 24 hours, the cells were transfected with Lipofectamine 2000 (Invitrogen, cat. no. 11668-019). We used 2.5 µg of total DNA and 7.5 µL of transfection reagent per well. The Opti-MEM reduced-serum medium (Invitrogen, cat. no. 31985-062) was used in the preparation of the transfection mixes, as detailed in the manufacturer-supplied protocol.

For sample collection after transfection, HEK293T cells were washed with PBS (Sigma-Aldrich), then resuspended in a solution of 1% bovine serum albumin (BSA, Sigma-Aldrich, cat. no. A9418) in PBS. HeLa cells were washed with PBS, detached with 0.25% trypsin, then resuspended in PBS + 1% BSA.

#### S7.2.2 CHO-K1 cells

CHO-K1 cells were supplied by Jiten Doshi in the lab of YB. The cells were grown in suspension cultures at 37°C, 100% humidity, 8% CO\textsubscript{2} in single-use baffled flasks (VWR, cat. no. 214-7211) with 100 rpm orbital shaking, and were maintained in Iscove’s Modified Dulbecco’s Medium (IMDM, Gibco, cat. no. 31980022). This medium formulation is supplied with GlutaMAX, HEPES buffer and sodium pyruvate, and was additionally supplemented with 10% FBS, 0.002% hypoxanthine-thymidine (ATCC, cat. no. 71-X), and 1% penicillin-streptomycin solution. The cells were maintained in this medium by diluting them to 1 · 10\textsuperscript{5} cells mL\textsuperscript{-1} upon reaching the density of 1 · 10\textsuperscript{6} cells mL\textsuperscript{-1}.

CHO-K1 cells were also transfected in 6-well plates. Polyethylenimine PEI was purchased from Polysciences, Inc. (cat. no. 24765). The powder was dissolved in water at the concentration of 1 µg µL\textsuperscript{-1}, the pH was adjusted to 7 with NaOH, and the resulting solution was filter-sterilized. On the day of transfection, the cells were seeded at the density of 8 · 10\textsuperscript{5} cells in 2 mL of complete medium without antibiotics. We used 2 µg of total DNA and 6 µL of PEI solution per well. For each well, DNA and PEI were first diluted separately, each in 150 µL of Opti-MEM. The DNA and PEI dilutions were then mixed and incubated at room temperature for 30 minutes. Finally, the mixture was added to the cells, and the transfected plates were incubated at 37°C, 100% humidity, and 8% CO\textsubscript{2} with 100 RPM orbital shaking.

For sample collection after transfection, CHO-K1 cells were pelleted to remove the transfection medium and then resuspended in PBS + 1% BSA.
S7.2.3 Human induced pluripotent stem cells (hIPSCs)

The hIPSC cell line BC1 was received from Dr. Laura Prochazka in the lab of YB. The cells were grown at 37°C, 100% humidity, and 5% CO\textsubscript{2} in a feeder- and serum-free adherent culture. The cells were maintained in Essential 8 medium (Gibco, cat. no. A1517001), which was prepared as per manufacturer’s instructions and supplemented with 0.5% penicillin-streptomycin solution. The cultures were grown in 6-well plates coated with Geltrex (Gibco, cat. no. A1413301) according to the manufacturer’s instructions. The adherent cultures were passaged upon reaching 70-80% confluence by incubating them with the Gentle Cell Dissociation Reagent (STEMCELL Technologies, cat. no. 07174). Following a 3-minute treatment at room temperature with the reagent, the cells were detached using a cell scraper. The resulting small clumps were then diluted 1:6 to 1:10 in fresh medium and seeded onto a fresh coated plate. The medium was replaced every 24 hours, except on the day after passaging.

hIPSCs were transfected in 24-well plates coated with Geltrex. For transfection, the cells were detached by incubating them with the Gentle Cell Dissociation Reagent at 37°C for 7 minutes. Following this treatment, the cells were resuspended in complete medium without antibiotics to obtain a single-cell suspension. This was then diluted with additional medium to the approximate density of 1.6 \times 10^5 cells mL\textsuperscript{-1}. Finally, the ROCK inhibitor Y-27632 (Selleck Chemicals, cat. no. S1049) was added at the final concentration of 10 µM, and 500 µL of suspension was added to each well. After 24 hours, the cells were transfected with DNA-In Stem (MTI-GlobalStem, cat. no. GST-2130) according to the manufacturer’s recommended protocol. We used 250 ng of DNA and 1.5 µL of reagent per well. The medium was replaced with fresh Essential 8 with antibiotics approximately 24 hours after transfection.

S7.3 Data analysis and binning

The raw flow cytometry data were gated and compensated with FlowJo (Treestar). Each experiment was compensated individually using single-fluorescence controls. The data were then further processed using custom R scripts. The cells were divided into bins according to the fluorescence of the constitutively expressed fluorescent protein. This was taken as an indirect measure of plasmid take-up. The splitting points of the bins were set equal to quantiles of the fluorescence distribution: the first bin goes from the 5% quantile to the 5.5% quantile, the second from the 5.5% quantile to the 6% quantile, and so on, with the last bin going from the 94.5% quantile to the 95% quantile. In this way, the binning becomes automatically finer where the distribution has more mass, and every bin contains the same number of cells. For each bin, the means of all the fluorescence channels were calculated. The plasmid-takeup/MOL and plasmid-takeup/TA functions were obtained by plotting the binned means of the output fluorescent protein (or of the transactivator fluorescent protein) as a function of the binned means of the constitutively expressed fluorescent protein.

Supplementary References

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Supplementary Figure S1: Controller plasmid-takeup/MOL and plasmid-takeup/TA behavior. (a) Numerical steady-state plasmid-takeup/MOL functions of the motifs. The functions show how the MOL of the circuits vary as a function of plasmid take-up. For HYB, FBK, and IFF, we plotted the functions for three different values of repressor binding affinity. (b,c) Comparison between the plasmid-takeup/MOL and plasmid-takeup/TA functions of the motif. The proposed hybrid topology has the same plasmid-takeup/MOL function of the incoherent feedforward circuit and the same plasmid-takeup/TA function of the feedback circuit. The different colors indicate different topologies: HYB (dashed blue), IFF (solid red), FBK (solid yellow), OLP (dashed green).

| Parameter | Units | Value | Interpretation |
|-----------|-------|-------|----------------|
| $k_1$     | µMh^{-1} | 1     | Transcription rate of reporter mRNA (per plasmid copy) |
| $k_2$     | h^{-1}  | 1     | Degradation rate of reporter mRNA |
| $k_3$     | h^{-1}  | 1     | Translation rate of reporter protein |
| $k_4$     | h^{-1}  | 0.029 | Degradation rate of reporter protein |
| $k_5$     | µMh^{-1} | 0.05  | Transcription rate of transactivator mRNA (per plasmid copy) |
| $k_6$     | h^{-1}  | 1     | Degradation rate of transactivator mRNA |
| $k_7$     | h^{-1}  | 1     | Translation rate of transactivator protein |
| $k_8$     | h^{-1}  | 0.029 | Degradation rate of transactivator protein |
| $k_9$     | h^{-1}  | 0.05  | Maximal transcription rate of repressor RNA (per plasmid copy) |
| $h_9$     | µM     | 0.1   | Transactivator concentration for half-maximal repressor RNA transcription |
| $k_{10}$  | h^{-1}  | 0.029 | Degradation rate of repressor RNA |
| $k_{11}$  | h^{-1}  | 0.1   | Maximal transcription rate of POI-coding mRNA (per plasmid copy) |
| $h_{11}$  | µM     | 0.1   | Transactivator concentration for half-maximal POI-coding mRNA transcription |
| $k_{12}$  | h^{-1}  | 1     | Degradation rate of POI-coding mRNA |
| $k_{13}$  | h^{-1}  | 1     | Translation rate of POI |
| $k_{14}$  | h^{-1}  | 0.029 | Degradation rate of POI |
| $k_{15}$  | µM^{-1}h^{-1} | 0.5   | Repressor binding affinity for transactivator mRNA |
| $k_{16}$  | µM^{-1}h^{-1} | 0.08  | Repressor binding affinity for POI-coding mRNA |

Supplementary Table S1: Model parameters for the numerical simulations.
Supplementary Figure S2: Controller plasmid-takeup/repressor behavior as a function of trans-activator dose-response curve. The top-left panel shows transactivator dose-response curves of different sharpness corresponding to the values of $h_9$ indicated. The remaining panels show the plasmid-takeup/repressor functions of the four controllers for each of the $h_9$ values. The different colors indicate different topologies: HYB (dashed blue), IFF (solid red), FBK (solid yellow), OLP (dashed green).

Supplementary Figure S3: miR-FF4 knockdown test. (a) Experiment design. The diagram shows the genes that were cotransfected and their interactions. Each of the indicated genes is on a separate plasmid. TFF4 and TFF5 indicate the target sequences for miR-FF4 and miR-FF5 respectively. (b, c) Results in HEK cells (b) and in HeLa cells (c). Cerulean level as a function of DsRed level when tTA::Cerulean carries either miR-FF4 targets (green) or miR-FF5 targets (purple). Different shades of the same color indicate different replicates of the same experimental condition. The functions were obtained by binning the flow cytometry data according the quantiles of the DsRed distributions.
Supplementary Figure S4: Self knockdown test. (a) Experiment design. The diagram shows the genes that were cotransfected and their interactions. Each of the indicated genes is on a separate plasmid. TFF4 and TFF5 indicate the target sequences for miR-FF4 and miR-FF5 respectively. (b, c) Results in HEK cells (b) and in HeLa cells (c). DsRed level as a function of Cerulean level when DsRed carries either miR-FF4 targets (green) or miR-FF5 targets (purple). Different shades of the same color indicate different replicates of the same experimental condition. The functions were obtained by binning the flow cytometry data according the quantiles of the Cerulean distributions.

| Topology | a              | b              | n              | Adjusted $R^2$ |
|----------|----------------|----------------|----------------|----------------|
| HYB      | 0.01461 (0.01415, 0.01507) | 0.1484 (0.1387, 0.1582) | 1.63 (1.486, 1.769) | 0.9987         |
| IFF      | 0.01004 (0.00958, 0.01049) | 0.2773 (0.2502, 0.3043) | 1.56 (1.289, 1.825) | 0.9947         |
| FBK      | 0.1223 (0.1196, 0.125)    | 0.05157 (0.04912, 0.05403) | 1.96 (1.81, 2.111) | 0.9995         |
| OLP      | 0.1024 (0.1014, 0.1034)   | 0.159 (0.1555, 0.1624) | 1.52 (1.481, 1.566) | 0.9999         |

Supplementary Table S2: Fitting of doxycycline (Dox) response data to Hill functions. The data points presented in Fig. S8 were fit to repression Hill functions of the following form: $y = a \frac{b^n}{b^n + x^n}$, where $y$ denotes the normalized DsRed fluorescence, $x$ denotes Dox concentration, and $a$, $b$ and $n$ are the parameters to be fit. $a$ is the maximal unpressed expression, $b$ is the concentration of Dox corresponding to the half-maximal expression (IC50) and $n$ is the Hill coefficient. The table reports the best fits for each topology, along with the coefficient of determination $R^2$. The values in parentheses are 95% confidence intervals. Prior to the fitting, the Dox concentrations were transformed using a hyperlog transformation [1], which is defined (for nonnegative values) as the inverse of the function: $z = e^{x} + x - 1$. The concentrations of Dox used were the following: 0, 0.1, 0.2, 0.3, 0.5, 0.7 and 1.0 ng/mL, resulting in the following hyperlog-transformed values respectively: 0, 0.0494, 0.0975, 0.1445, 0.2350, 0.3212, 0.4429.
Supplementary Figure S5: tTA-Advanced titration. To ensure that the inverse correlation of transactivator dosage and $P_{TRE}$ activity was not due the fusion of tTA with Cerulean, we performed a titration experiment in HEK293T cells with the commercially available tTA-Advanced (Clontech). We cotransfected $P_{EF1\alpha}$-mCitrine (700 ng), $P_{TRE}$-DsRed(FF4)-TFF5x3 (900 ng) and varying amounts of $P_{CMV}$-tTA (pTet-Off-Advanced, Clontech). The amounts used were 900 ng, 700 ng, 500 ng, 300 ng, 100 ng and 0 ng. To make sure that the total amount of DNA in each transfection well was the same, we added the inert plasmid U6+27-cxcr4 to the mix [4]. The amounts of inert plasmid used were 0 ng, 200 ng, 400 ng, 600 ng, 800 ng and 900 ng respectively. The plot shows the DsRed level as a function of mCitrine level in samples with varying dosages of tTA-Advanced. The numbers in the legend indicate the nanogram amounts of tTA-Advanced plasmid used in the transfection mixes. The functions were obtained by binning the flow cytometry data according the quantiles of the mCitrine distributions.
Supplementary Figure S6: **Fitting of controller plasmid-takeup/MOL functions.** The dots are the experimental data shown in Figure 4(c) in the main text. The continuous lines show the best fits with the analytical function class reported in each panel.

Supplementary Figure S7: **Dependency of controller plasmid-takeup/MOL functions on the transactivator dose-response curve.** The top-left panel shows transactivator dose-response curves of different sharpness corresponding to the values of $h_9$ indicated. The remaining panels show the plasmid-takeup/MOL functions of the four controllers for each of the $h_9$ values. The different color indicate different topologies: HYB (dashed blue), IFF (solid red), FBK (solid yellow), OLP (dashed green).
Supplementary Figure S8: Response of the synthetic controllers to doxycycline. (a,b) Population output fluorescence as a function of Dox concentration. The output fluorescence is the mean of DsRed fluorescence in the population of mCitrine-positive cells normalized by the mean of mCitrine fluorescence. The Dox concentrations are reported in a hyperlog scale (Supplementary Table S2). The dots indicate the experimental data, while the lines are fits with Hill functions. Error bars denote standard deviation ($N=3$). The different colors indicate different topologies: hybrid feedback-feedforward (blue), feedback-only (yellow), feedforward-only (red), and open loop (green). (c) Half maximal inhibitory concentration ($IC_{50}$) of Dox applied to the synthetic motifs by topology. The $IC_{50}$ was calculated by fitting the experimental data to Hill functions (Supplementary Table S2). Error bars denote a 95% confidence interval; p-values are based on such intervals. (d, e) Simulation of the Dox response. Output protein amount as a function of Dox concentration. The action of Dox is modeled as a Dox-dependent increase in the tTA::Cerulean degradation rate (Supplementary Note S6). (f) $IC_{50}$ of the simulated response to Dox by topology.
Supplementary Figure S9: **Distribution of mCitrine fluorescence as a proxy for plasmid take-up variability.** Kernel density estimates of the distributions of mCitrine fluorescence for the same data as in Figure 4(E, F) in the main manuscript (single-plasmid implementation of the controllers). Each triplicate is plotted independently. The distributions are very wide, suggesting a very large plasmid variability induced by the process of transient transfection.
Supplementary Figure S10: **Fano factor as a measure of cell-to-cell variability in the synthetic controllers.** The figure shows the same data as in Figure 4 in the main manuscript, but plotting the Fano factor of the POI (a,c) and of the transactivator (b,d) as a function of plasmid take-up in the multi-plasmid (a,b) or single-plasmid (c,d) implementations of the controllers. Feedforward regulation results in a noticeable decrease in the Fano factor of the POI, while feedback regulation significantly reduces the Fano factor of the transactivator. This is consistent with both intuition and previous reports that micro-RNA regulation can suppress gene expression noise [6].
| Designation | Description | Figures |
|------------|-------------|---------|
| pGLM43     | P_{EF1α}-mCitrine-RbGpA |         |
| pGLM44     | P_{EF1α}-Cerulean-RbGpA |         |
| pGLM45     | INS-bGHpA-mCitrine-SV40pA-INS |         |
| pGLM46     | SV40pA-AmCyan-P_{TRE-tight-bi}-DsRed(FF3)-TFF3-SV40pA |         |
| U6+27-cxcr4| See [4]. | 3, 5(a-b), S5 |
| pTet-Off-Advanced | PCMV-tTA-Advanced | S3, S5 |
| pGLM49*    | INS-bGHpA-P_{EF1α}-mCitrine-SV40pA-INS | 3, 4(c-d), 5, S5 |
| pGLM53     | INS-bGHpA-P_{EF1α}-SV40pA-INS |         |
| pGLM60     | INS-bGHpA-P_{EF1α}-tTA::Cerulean-TFF5x1-SV40pA-INS |         |
| pGLM65*    | P_{SV40}-puromycin-SV40pA-INS-bGHpA-P_{EF1α}^* tTA::Cerulean-TFF5x1-SV40pA-INS | S4 |
| pGLM95     | P_{SV40}-puromycin-SV40pA-INS-bGHpA-P_{EF1α}^* tTA::Cerulean-TFF4x3-SV40pA-INS | 4(c-d), 5, 6(e-f), S3 |
| pGLM96     | P_{SV40}-puromycin-SV40pA-INS-bGHpA-P_{EF1α}^* tTA::Cerulean-TFF5x3-SV40pA-INS | 3, 4(c-d), 6(e-f), S3 |
| pGLM64     | INS-bGHpA-P_{TRE}-DsRed(FF3)-TFF3x1-SV40pA-INS |         |
| pGLM88     | INS-bGHpA-P_{TRE}-DsRed(SnaBI-FF3-EcoRI)-TFF3x1-SV40pA-INS |         |
| pGLM89     | INS-bGHpA-P_{TRE}-DsRed(FF4)-TFF3x1-SV40pA-INS | S3 |
| pGLM91     | INS-bGHpA-P_{TRE}-DsRed(FF4)-TFF4x3-SV40pA-INS | 4(c-d), 5(c-d), 6(e-f), S4 |
| pGLM92     | INS-bGHpA-P_{TRE}-DsRed(FF4)-TFF5x3-SV40pA-INS | 3, 4(c-d), 5(a-b), 6(e-f), S4, S5 |
| pGLM73     | INS-bGHpA-P_{TRE}-DsRed(FF3)-TFF5x1-SV40pA-INS |         |
| pGLM79*    | P_{SV40}-puromycin-SV40pA-INS-bGHpA-P_{EF1α}-DsRed(FF3)-TFF5x1-SV40pA-INS |         |
| pGLM180*   | INS-bGHpA-P_{EF1α}-iRFP670-SV40pA-INS | 6(e-f) |

Supplementary Table S3: **Plasmids used in this study, part 1.** For the constructs that were used in transfection experiments reported in this manuscript, the corresponding figures are referenced. The fluorescent biological parts are marked with the colors of the corresponding emission wavelengths. The plasmids marked with * were also used as single-fluorescence controls for data compensation whenever the corresponding fluorescence channel was involved.
| Designation  | Description                                                                 | Figures          |
|-------------|-----------------------------------------------------------------------------|------------------|
| pGLM66      | **pSV40**-puromycin-SV40pA-INS-bGHpA-P**EF1α**-mCitrine-SV40pA-INS-bGHpA-P**P**TRE-DSRed(FF3)-TFF3x1-SV40pA-INS |                 |
| pGLM90      | **pSV40**-puromycin-SV40pA-INS-bGHpA-P**EF1α**-mCitrine-SV40pA-INS-bGHpA-P**P**TRE-DSRed(FF4)-TFF4x1-SV40pA-INS |                 |
| pGLM93      | **pSV40**-puromycin-SV40pA-INS-bGHpA-P**EF1α**-mCitrine-SV40pA-INS-bGHpA-P**P**TRE-DSRed(FF4)-TFF4x3-SV40pA-INS |                 |
| pGLM94      | **pSV40**-puromycin-SV40pA-INS-bGHpA-P**EF1α**-mCitrine-SV40pA-INS-bGHpA-P**P**TRE-DSRed(FF4)-TFF5x3-SV40pA-INS |                 |
| pGLM97      | **pSV40**-puromycin-SV40pA-INS-bGHpA-P**EF1α**-mCitrine-SV40pA-INS-bGHpA-P**P**TRE-DSRed(FF4)-TFF5x3-SV40pA-INS |                 |
| pGLM98      | **pSV40**-puromycin-SV40pA-INS-bGHpA-P**EF1α**-mCitrine-SV40pA-INS-bGHpA-P**P**TRE-DSRed(FF4)-TFF5x3-SV40pA-INS |                 |
| pGLM99      | **pSV40**-puromycin-SV40pA-INS-bGHpA-P**EF1α**-mCitrine-SV40pA-INS-bGHpA-P**P**TRE-DSRed(FF4)-TFF5x3-SV40pA-INS |                 |
| pGLM100     | **pSV40**-puromycin-SV40pA-INS-bGHpA-P**EF1α**-mCitrine-SV40pA-INS-bGHpA-P**P**TRE-DSRed(FF4)-TFF5x3-SV40pA-INS |                 |
| pGLM125     | **pSV40**-puromycin-SV40pA-INS-bGHpA-P**EF1α**-tTA::Cerulean-TFF5x3-SV40pA-INS |                 |
| pGLM126     | **pSV40**-puromycin-SV40pA-INS-bGHpA-P**EF1α**-tTA::Cerulean-TFF4x3-SV40pA-INS |                 |
| pGLM127     | **pSV40**-puromycin-SV40pA-INS-bGHpA-P**EF1α**-tTA::Cerulean-TFF4x3-SV40pA-INS |                 |
| pGLM128     | **pSV40**-puromycin-SV40pA-INS-bGHpA-P**EF1α**-tTA::Cerulean-TFF5x3-SV40pA-INS |                 |
| pGLM129     | **pSV40**-puromycin-SV40pA-INS-bGHpA-P**EF1α**-tTA::Cerulean-TFF4x3-SV40pA-INS |                 |
| pGLM130     | **pSV40**-puromycin-SV40pA-INS-bGHpA-P**EF1α**-tTA::Cerulean-TFF5x3-SV40pA-INS |                 |

**Supplementary Table S4: Plasmids used in this study, part 2.** For the constructs that were used in transfection experiments reported in this manuscript, the corresponding figures are referenced. The fluorescent biological parts are marked with the colors of the corresponding emission wavelengths.
| Designation | Description                                                                 | Figures |
|-------------|-----------------------------------------------------------------------------|---------|
| pGLM139     | $P_{SV40}$-puromycin-SV40pA-INS-bGHpA-$P_{UbC}$-tTA::Cerulean-TFF5x3-SV40pA-INS |         |
| pGLM141     | $P_{SV40}$-puromycin-SV40pA-INS-bGHpA-$P_{EF1\alpha}$-mCitrine-SV40pA-INS-bGHpA-$P_{UbC}$-tTA::Cerulean-TFF4x3-SV40pA-INS-bGHpA-$P_{TRE}$-DsRed(FF4)-TFF4x3-SV40pA-INS | 6(b,d) |
| pGLM142     | $P_{SV40}$-puromycin-SV40pA-INS-bGHpA-$P_{EF1\alpha}$-mCitrine-SV40pA-INS-bGHpA-$P_{UbC}$-tTA::Cerulean-TFF5x3-SV40pA-INS-bGHpA-$P_{TRE}$-DsRed(FF4)-TFF5x3-SV40pA-INS | 6(b,d) |
| pGLM143     | $P_{SV40}$-puromycin-SV40pA-INS-bGHpA-$P_{EF1\alpha}$-mCitrine-SV40pA-INS-bGHpA-$P_{UbC}$-tTA::Cerulean-TFF4x3-SV40pA-INS-bGHpA-$P_{TRE}$-DsRed(FF4)-TFF5x3-SV40pA-INS | 6(a,d) |
| pGLM144     | $P_{SV40}$-puromycin-SV40pA-INS-bGHpA-$P_{EF1\alpha}$-mCitrine-SV40pA-INS-bGHpA-$P_{UbC}$-tTA::Cerulean-TFF5x3-SV40pA-INS-bGHpA-$P_{TRE}$-DsRed(FF4)-TFF5x3-SV40pA-INS | 6(a,d) |
| pGLM204     | $P_{SV40}$-puromycin-SV40pA-INS-bGHpA-$P_{EF1\alpha}$-mCitrine-SV40pA-INS-bGHpA-$P_{EF1\alpha}$-tTA::Cerulean-TFF4x3-SV40pA-INS-bGHpA-$P_{TRE}$-DsRed(FF4)::6xHis::V5-TFF5x3-SV40pA-INS | 6(b,d) |
| pGLM205     | $P_{SV40}$-puromycin-SV40pA-INS-bGHpA-$P_{EF1\alpha}$-mCitrine-SV40pA-INS-bGHpA-$P_{EF1\alpha}$-tTA::Cerulean-TFF5x3-SV40pA-INS-bGHpA-$P_{TRE}$-DsRed(FF4)::6xHis::V5-TFF5x3-SV40pA-INS | 6(b,d) |
| pGLM206     | $P_{SV40}$-puromycin-SV40pA-INS-bGHpA-$P_{EF1\alpha}$-mCitrine-SV40pA-INS-bGHpA-$P_{SV40}$-tTA::Cerulean-TFF4x3-SV40pA-INS-bGHpA-$P_{TRE}$-DsRed(FF4)::6xHis::V5-TFF5x3-SV40pA-INS | 6(a,d) |
| pGLM207     | $P_{SV40}$-puromycin-SV40pA-INS-bGHpA-$P_{EF1\alpha}$-mCitrine-SV40pA-INS-bGHpA-$P_{SV40}$-tTA::Cerulean-TFF5x3-SV40pA-INS-bGHpA-$P_{TRE}$-DsRed(FF4)::6xHis::V5-TFF5x3-SV40pA-INS | 6(a,d) |
| pGLM208     | $P_{SV40}$-puromycin-SV40pA-INS-bGHpA-$P_{EF1\alpha}$-mCitrine-SV40pA-INS-bGHpA-$P_{UbC}$-tTA::Cerulean-TFF4x3-SV40pA-INS-bGHpA-$P_{TRE}$-DsRed(FF4)::6xHis::V5-TFF5x3-SV40pA-INS | 6(c,d) |
| pGLM209     | $P_{SV40}$-puromycin-SV40pA-INS-bGHpA-$P_{EF1\alpha}$-mCitrine-SV40pA-INS-bGHpA-$P_{UbC}$-tTA::Cerulean-TFF5x3-SV40pA-INS-bGHpA-$P_{TRE}$-DsRed(FF4)::6xHis::V5-TFF5x3-SV40pA-INS | 6(c,d) |

Supplementary Table S5: **Plasmids used in this study, part 3.** For the constructs that were used in transfection experiments reported in this manuscript, the corresponding figures are referenced. The fluorescent biological parts are marked with the colors of the corresponding emission wavelengths.
| Designation | Sequence |
|-------------|----------|
| oGL56       | gatcgctageGCTCCGGTGCCCGTC |
| oGL57       | gatcccatggagTCTAGGGCACACCTGAAATGGAAGAAAAAACT |
| oGL69       | gatcgctagcatgatGCTCCGGTGCCCGTC |
| oGL70       | gatctgtacatacttagaaATCTAGGGCAGTTTATGTCACGACACCTGAAATGGAAGAAAAAACT |
| oGL72       | gatcttaataagcgACCAGTGTACTGACTAGACAGAGCA |
| oGL73       | gatcggtaceCCGGGGAGCATGTCAAGGG |
| oGL74       | gatcggtaceATGGTGAGCAAGGGCGAGG |
| oGL75       | gatcactagtttaatgtaagctatcggagacttaATACCTGGTACAGCTCGTCCATGCC |
| oGL78       | TGTGAGCAAAAAGCCAGCAAAAG |
| oGL82       | CAGGTGGCAGCTTTTCCGGGA |
| oGL83       | TCCCCGAAAAGTGCCACCTGAGGTTTTCACCGTCAATCCAC |
| oGL84       | tatcgccgctATGACATTAACCTTAAAAATAGGCGTATACG |
| oGL85       | TAATGTCATGgatgccgcatagtttaagccg |
| oGL86       | TTGCTGGCCTTTTTGTCACACTgctagttatcc |
| oGL87       | GATCGtcagtagattgaccttgactgacccag |
| oGL88       | GATCGccccgacgccagctgaaatctccagg |
| oGL89       | GATCGccccgacccagctgacctgctccagg |
| oGL90       | GATCGtacagttccgagttgcttaggttaatagagctgatttaac |
| oGL91       | TTCAATATCGAGAACAGCTGgctattttggtaaaaaatgctattttaac |
| oGL92       | GCTTGCCTGTATCTGTAAGGagttccccgagagactgatgtaac |
| oGL93       | GATCGTATCTTACGTCTACCGagttccgagagactgatgtaac |
| oGL94       | GTAAGTCATATGATAGCAGCTGgctattttggtaaaaaatgctattttaac |
| oGL97       | CGagagcacaagtgtgaaatctccagg |
| oGL98       | CGCGCCATCATGCTAATTTTATCGGG |

Supplementary Table S6: **Oligonucleotides used in this study, part 1.** All DNA sequences are listed in the 5' to 3' sense. Case has no particular meaning.
Supplementary Table S7: Oligonucleotides used in this study, part 2. All DNA sequences are listed in the 5’ to 3’ sense. Case has no particular meaning.

| Designation | Sequence |
|-------------|----------|
| oGL134      | GATCtaattaagccaccatggctcctcggag |
| oGL136      | GATCactagtaattgtcaatacagagctgtctgaagca |
| oGL153      | CAGGTTAACCAACAGAAGGTACGTATGCTGTTGACAGTGACGCA |
| oGL154      | GTGCGGCTCACCTGCCAACAGCATACGCTACCTTCTGTTGGTTAACCCTG |
| oGL155      | gtaTGCTGTGGACAGTGGAGCGCCGCCCTTTGAAAGTTTTTTAATTAAATAGTGAAGCCCAACAGTACAGTTAATTAAAGCTACCTCAGCTTACCTGTCCTACTGCTCCGGAG |
| oGL156      | AATTCTCCGAGGCAGTAGGCACCGCTTGAAAGCTCTTAAATTAATTAAACCCGCTTGAAGTCTCTTAAATTAACCAGCTTGAAGTCTCTTAAATTAAGCCTGCCTACTGTCACAGCACGATGTATTTAATTAAAGACTTCAAGCGGTGCCTACTG |
| oGL161      | cgaAGCGCTTGGAAGTCTTTTAAATTAAACCAGCTTGAAGTCTCTTAAATTAAGCCTGCCTACTGTCACAGCACGATGTATTTAATTAAAGACTTCAAGCGGTGCCTACTG |
| oGL162      | cgcgccCTTTAATTAAAGACTTCAAGCGGTGCCTACTGTCACAGCACGATGTATTTAATTAAAGACTTCAAGCGGTGCCTACTGTCACAGCACGATGTATTTAATTAAAGACTTCAAGCGGTGCCTACTG |
| oGL163      | cgggaCCGCTTGGAAGTCTTTTAAATTAAACCAGCTTGAAGTCTCTTAAATTAACCAGCTTGAAGTCTCTTAAATTAAGCCTGCCTACTGTCACAGCACGATGTATTTAATTAAAGACTTCAAGCGGTGCCTACTG |
| oGL164      | ctcgATTTTAATTAAAGACTTCAAGCGGTGCCTACTGTCACAGCACGATGTATTTAATTAAAGACTTCAAGCGGTGCCTACTGTCACAGCACGATGTATTTAATTAAAGACTTCAAGCGGTGCCTACTG |
| oGL165      | cgaAGACACTCTGATTTGGCAATTAAGCCTACTCTGATTTGGCAATTAAGCCTGCCTACTGTCACAGCACGATGTATTTAATTAAAGACTTCAAGCGGTGCCTACTG |
| oGL166      | cgcgccCTTTAATTAAAGACTTCAAGCGGTGCCTACTGTCACAGCACGATGTATTTAATTAAAGACTTCAAGCGGTGCCTACTGTCACAGCACGATGTATTTAATTAAAGACTTCAAGCGGTGCCTACTG |
| oGL167      | cgggaAAGCACTCTGATTTGGCAATTAAGCCTACTCTGATTTGGCAATTAAGCCTGCCTACTGTCACAGCACGATGTATTTAATTAAAGACTTCAAGCGGTGCCTACTG |
| oGL168      | ctcgATTTTAATTAAAGACTTCAAGCGGTGCCTACTGTCACAGCACGATGTATTTAATTAAAGACTTCAAGCGGTGCCTACTGTCACAGCACGATGTATTTAATTAAAGACTTCAAGCGGTGCCTACTG |
| oGL224      | gateATCGATGAAACGCCGAGACAGAAG |
| oGL225      | gateTCTTAAATTGGCAGAAGCGGTGCTCCTCC |
| oGL229      | gateATCGATgagcgctcgcgege |
| oGL230      | gateTCTTAAATTAGctgttgattgctctaaacaaaagcca |
| oGL369      | agtttacGCGCGTTGTAAGCCCTATCCCTCAACCTCCTCGGTCTCGATTCTACGGTGACGTGGTGTCATCACTCAACCTGACCAGCGCATG |
| oGL370      | cgaagcatgcTCAATGGTGATGGTGATGAGACCGGTACGCTGAGAACTCGAAGGCCAGAGGGGTAGGGTGAGACAGCAGCGGCgctga |

| Component                | Concentration | Amount to add |
|--------------------------|---------------|---------------|
| 5x isothermal buffer     | See Table S9  | 100 µL        |
| *Taq* ligase             | 40 U/µL       | 50 µL         |
| T5 exonuclease           | 1 U/µL        | 2 µL          |
| *Phusion* DNA polymerase | 2 U/µL        | 6.25 µL       |
| Nuclease-free water      | -             | to 375 µL     |

Supplementary Table S8: Composition of the 1.33x Gibson assembly master mix.
Supplementary Table S9: **Composition of the 5x isothermal buffer.**

| Component       | Concentration | Amount to add |
|-----------------|---------------|---------------|
| PEG-8000        | (powder)      | 0.75 g        |
| Tris-HCl pH 7.5 | 500 mM        | 1500 µL       |
| MgCl₂           | 50 mM         | 75 µL         |
| DTT             | 50 mM         | 150 µL        |
| dNTP            | 10 mM each    | 3µL           |
| NAD             | 5 mM          | 300µL         |
| Nuclease-free water | -       | to 3000 µL    |