Win MN, Smolke CD. 2008. Higher-order cellular information processing with synthetic RNA devices. Science. 322: 456-60. DOI: 10.1126/science.1160311

0. Introduction

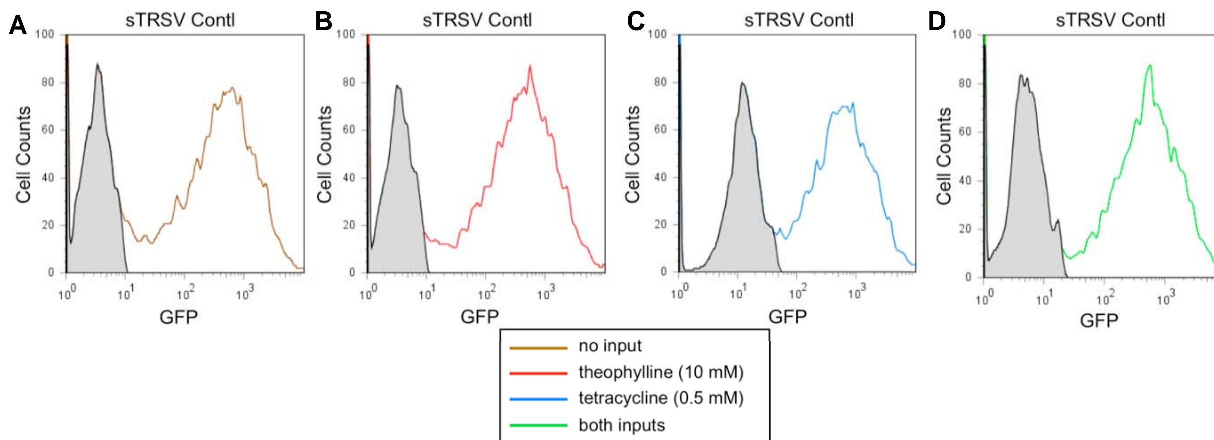
This supplement provides additional detail on the data analysis methods and the raw data for the RNA devices presented in the published manuscript and supporting online material. The information provided in the current supplement is organized as follows:

1. Gating methods for the raw flow cytometry data
2. Correction methods for nonspecific effects on fluorescence of chemical effectors controlling the RNA devices
3. Selection of standard against which to report device performance
4. Relevance to conclusions published in the Science paper
5. Example calculations
6. Raw data for RNA devices

1. Gate for induced cell populations from the flow cytometry histograms. Analyze the mean fluorescence of the induced cell populations.

Under the induction conditions used here (2% galactose), the galactose-inducible promoter system results in both induced and uninduced cell populations. We have observed that the distribution between these two populations can vary in a manner that is not correlated with media conditions or overall gene expression activity of the RNA device-containing construct. Since we are only concerned with the gene expression activity of the induced population, we run an uninduced sample (0% galactose) under identical media conditions for each construct and use this sample to set the gate for the induced population (see Figure 1 for an example). As described in the methods section of the published paper, the gene expression activity of an RNA device is reported as the mean fluorescence value of the induced population.

Figure 1. The activity of RNA devices is analyzed from the induced cell population. Gates are set to analyze the induced cell population, based on running an uninduced sample under identical media conditions (histograms shown in grey). An example of the gating method is demonstrated here for sTRSV Contl, where the panels represent histograms for cells under different effector conditions: 0 mM theo, 0 mM tc (A); 10 mM theo (B); 0.5 mM tc (C); and 10 mM theo, 0.5 mM tc (D).

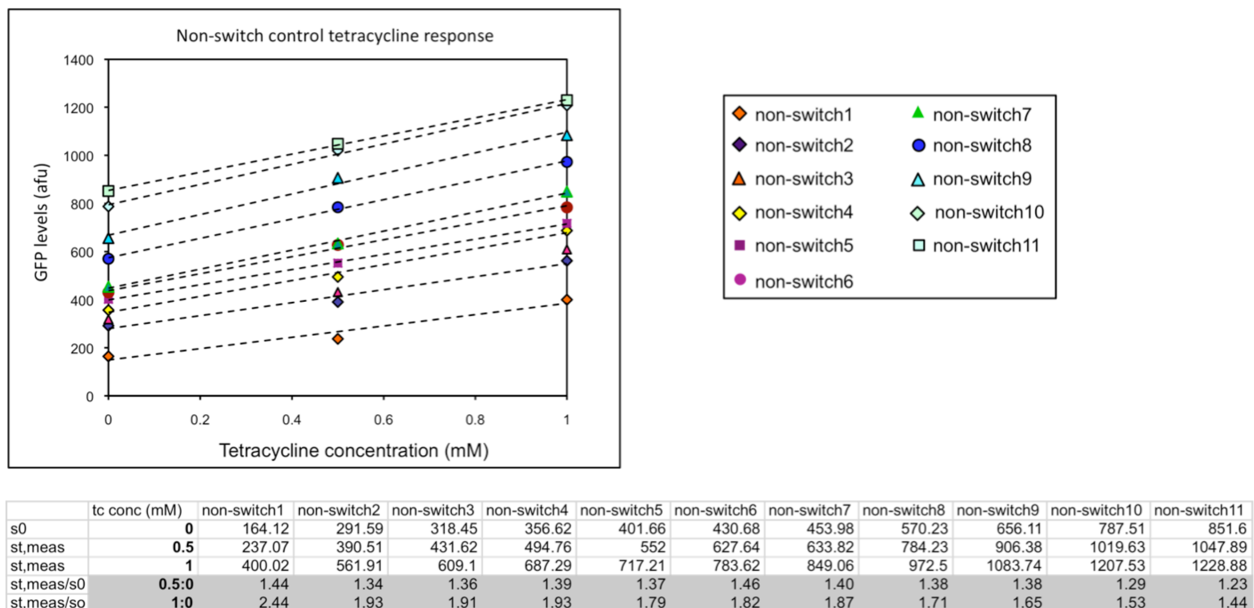


2. Correcting for nonspecific effects on fluorescence of chemical effectors for the RNA devices.

The chemical effectors controlling the RNA devices (theophylline, tetracycline) can have different nonspecific effects on the fluorescence of cells. These effects are dependent on the concentration of the effectors (ligands) used and also depend on the specific cell types the devices are characterized within. Under the concentrations used here, theophylline exhibits minor nonspecific effects on cellular fluorescence in yeast. However, tetracycline exhibits significant concentration-dependent nonspecific increases on the fluorescence of cells (especially at higher concentrations) that must be corrected for in order to obtain an accurate measurement of the gene regulatory effects specific to the activity of the RNA devices. All ligand concentrations used in this study do not result in significant effects on cell growth rates.

We corrected for the nonspecific effects of tetracycline by determining the increase in fluorescence as a function of tetracycline concentrations for non-switch controls that exhibit different basal levels of expression (Figure 2). The non-switch controls are ribozyme constructs harboring sensors that do not bind to tetracycline, in which their transmitter sequences have been modified to exhibit different basal expression levels.

Figure 2. Fluorescence increase of non-switch controls as a function of tetracycline concentration. Each non-switch control is an RNA device that is unresponsive to tetracycline and exhibits a different basal expression level. Gene expression activities for non-switch controls are determined as described in section 1. Fluorescence values at each point are reported in the table below the graph. Ratios between the fluorescence signal in the presence and absence of ligand are also shown to highlight that the relative increase in fluorescence due to nonspecific effects of tetracycline are dependent on expression level.

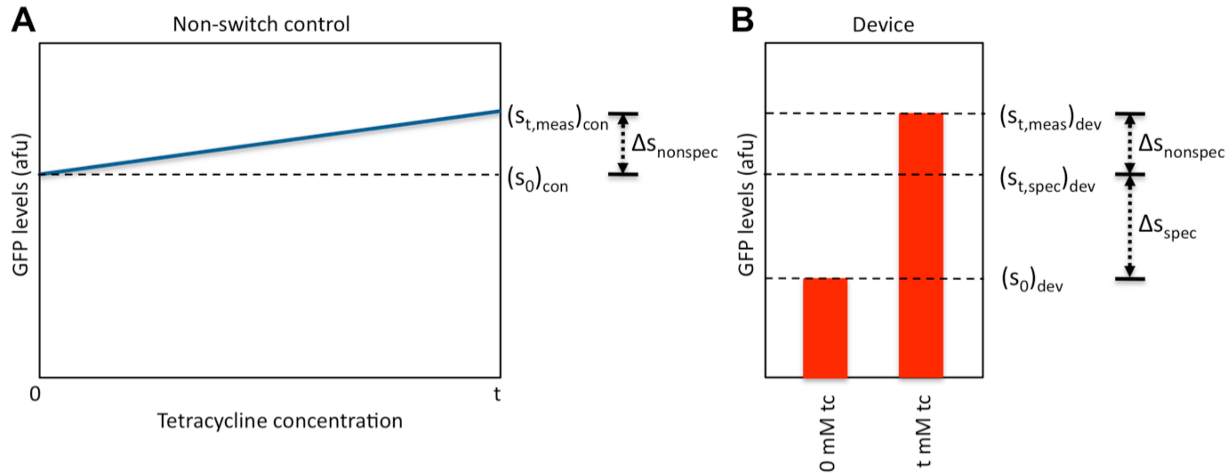


	tc conc (mM)	non-switch1	non-switch2	non-switch3	non-switch4	non-switch5	non-switch6	non-switch7	non-switch8	non-switch9	non-switch10	non-switch11
s0	**0**	164.12	291.59	318.45	356.62	401.66	430.68	453.98	570.23	656.11	787.51	851.6
st,meas	**0.5**	237.07	390.51	431.62	494.76	552	627.64	633.82	784.23	906.38	1019.63	1047.89
st,meas	**1**	400.02	561.91	609.1	687.29	717.21	783.62	849.06	972.5	1083.74	1207.53	1228.88
st,meas/s0	**0.5:0**	1.44	1.34	1.36	1.39	1.37	1.46	1.40	1.38	1.38	1.29	1.23
st,meas/so	**1:0**	2.44	1.93	1.91	1.93	1.79	1.82	1.87	1.71	1.65	1.53	1.44

The amount of increase in fluorescence observed for a non-switch construct provides a measure of the nonspecific increase in fluorescence due to tetracycline. It should be noted that the correction for the tetracycline effect is dependent on the basal expression of the construct (as

observed from the data in Figure 2). As such, the appropriate correction was determined for each gate construct by matching to the appropriate non-switch control at a similar expression level (Figure 3).

Figure 3. Schematic illustrating the correction for the nonspecific effects of tetracycline on fluorescence. (A) Schematic graph illustrating the relationship between cell fluorescence and tetracycline concentration for a non-switch control. The nonspecific contribution of tetracycline to fluorescence ($\Delta s_{nonspec}$) is determined as the difference between the measured fluorescence from the nonswitch control in the absence ($(s_0)_{con}$) and presence ($(s_{t,meas})_{con}$) of tetracycline. (B) Schematic bar graph illustrating the relationship between measured fluorescence from an RNA device in the presence ($(s_{t,meas})_{dev}$) and absence ($(s_0)_{dev}$) of tetracycline and the nonspecific ($\Delta s_{nonspec}$) and specific (Δs_{spec}) contributions of tetracycline to gene expression activity.



As described in the methods section of the published paper, the gene expression activity of an RNA device is reported as the mean fluorescence value of the GFP-expressing cell population. The gene expression activity of an RNA device (s) following a correction for nonspecific effects from tetracycline fluorescence is determined by a subtraction method as:

$$s_{t,spec} = \left(s_{t,meas}\right)_{dev} - \Delta s_{nonspec} = \left(s_{t,meas}\right)_{dev} - \left(s_{t,meas} - s_0\right)_{con}$$

where $s_{t,spec}$ represents the expression activity of the device at a tetracycline concentration of t when nonspecific effects of tetracycline are removed; $s_{t,meas}$ represents the expression activity of the device measured under that concentration of tetracycline; s_0 represents the expression activity of the device measured in the absence of tetracycline; $\Delta s_{nonspec}$ represents the change in expression activity of the device as a result of nonspecific effects of tetracycline on fluorescence and is measured from a non-switch control exhibiting similar expression levels as the device of interest at that tetracycline concentration; $s_{t,meas}$ and s_0 can also be reported for the non-switch controls and are indicated with a subscript 'con'.

As described in the methods section of the published paper, data on RNA device performance is reported as either device signal (S_d) or device response (R_d; defined lower on this page). The device signal is the gene expression activity of an RNA device (s) relative to that of sTRSV

Contl (c) (S_d = s/c). When using activities corrected for nonspecific effects of tetracycline using the subtraction method this is calculated as:

$$S_d = \frac{s_{t,spec}}{c_{t,spec}} = \frac{(s_{t,meas})_{dev} - \Delta s_{nonspec}}{c_{t,meas} - \Delta c_{nonspec}} = \frac{(s_{t,meas})_{dev} - (s_{t,meas} - s_0)_{con}}{c_{t,meas} - (c_{t,meas} - c_0)_{con}} \qquad \text{(eq. 1)}$$

where similar definitions apply to c for the inactive sTRSV Contl ribozyme (which is a non-switch control and standard used in our system, see section 3 below). As described in the methods of the published paper, device signal is reported as a percentage by multiplying with 100%; device signal is reported in units of expression by dividing S_d (as calculated above) by U_{ex} (which represents the gene expression activity of the parental ribozyme sTRSV relative to that of the inactive ribozyme sTRSV Contl in the absence of ligands). Device response ($R_{d,L}$) is the arithmetic difference between the device signals in the absence and presence of the appropriate ligands and represented as $R_{d,L} = S_{d,L} - S_{d,0}$.

Alternatively, one can correct for nonspecific effects of tetracycline through a ratio method as:

$$s_{t,spec} = (s_{t,meas})_{dev} \left(\frac{s_0}{s_{t,meas}} \right)_{con}$$

where terms are defined as above and the ratio of s_0 to $s_{t,meas}$ for the non-switch control represents a correction factor. We refer to this ratio method as the correction factor method. Either correction method ("subtraction" or "correction factor") yields similar results.

Using the correction factor method, device signal is calculated as:

$$S_d = \frac{s_{t,spec}}{c_{t,spec}} = \frac{(s_{t,meas})_{dev} \left(\frac{s_0}{s_{t,meas}} \right)_{con}}{c_{t,meas} \left(\frac{c_0}{c_{t,meas}} \right)_{con}} \qquad \text{(eq. 2)}$$

If the correction factors for the device and sTRSV Contl are similar in value or if the nonspecific effects on cellular fluorescence are small, the corrected device signal can be calculated as:

$$S_d = \frac{s_{t,spec}}{c_{t,spec}} \approx \frac{s_{t,meas}}{c_{t,meas}} \qquad \text{(eq. 3)}$$

The correction factors for the device and sTRSV Contl are not generally similar in value for tetracycline (see data in Figure 2 above), although they become closer in value at lower tetracycline concentrations. Equation 3 can be used for theophylline, as theophylline imparts relatively minor effects on cellular fluorescence.

We feel it is more accurate to carefully correct for the nonspecific effects of tetracycline using one of the methods described. Either correction may increase the reported function of devices that decrease expression activity in the presence of tetracycline and may decrease the reported function of devices that increase expression activity in the presence of tetracycline. We calculated the corrected device signal for each device construct in the presence of tetracycline

using the subtraction method described above (eq. 1), and checked the values with the correction factor method (eq. 2). We used equation 3 to calculate the corrected device signal in the presence of theophylline. In the presence of both ligands, we use equation 1 to correct for the effects of tetracycline and theophylline (see examples in section 5).

3. Characterization of device performance relative to a standard

We report device activity relative to a standard, which in this work is an inactive ribozyme. As described in the published supporting information for the paper, this standard (sTRSV Contl) acts as a HIGH reference for the system against which devices can be compared (where HIGH represents the maximum potential gene expression activity from the device) and provides a relative measure that serves several purposes for device characterization. In particular, it provides a measure of the full potential dynamic range of the system when compared to the active ribozyme control (sTRSV). This measure is important in identifying the activity of the device relative to the full potential device activity and making comparative analyses of device performance across a series of devices. In addition, as recently described by Endy and colleagues (PMID: 19298678) reporting *in vivo* gene expression activities relative to reference standards serves the purpose of reducing variability introduced by differences in characterization instruments (and their settings), media, and chemicals for assays performed in different laboratories and over different days. While it is acknowledged that absolute measures are useful, particularly when implementing engineered devices in specific applications, relative measures serve as a more consistent and reproducible characterization metric, allow comparison between different devices, and can be used to more efficiently optimize the integration of a device into a larger engineered system.

4. Relevance to the conclusions published in the Science paper

We report device behavior by correcting for nonspecific effects of the ligands as appropriate and normalizing the expression activity of the device to that of the selected standard (sTRSV Contl). The behavior of 48 of the 50 devices presented in the published manuscript is apparent in the raw cytometry data (unnormalized and uncorrected) for each device (see section 6). In addition, the determination of device behavior and relative performance is not affected by the processing or correction method. For two of the devices reported in the paper, NAND1 and NAND2, the behavior of the gate is not observed directly in the raw data. Our reported behavior depends on applying either the subtraction or correction factor method for nonspecific effects of tetracycline. This is a result of the small dynamic range of these devices, the nature of these gates resulting in a decrease in device signal in the presence of tetracycline (and theophylline), and the nature of tetracycline resulting in nonspecific increases in fluorescence. As observed in the raw data (see below), there is a measurable decrease in expression activity in the presence of both ligands as opposed to just tetracycline alone where this effect is significant and reproducible, supporting the NAND gate function. We acknowledge that this device exhibits a relatively low dynamic range (~20% change from its basal level (characteristic of typical reporting practices that internally normalize response of a given device to its high signal), and ~10% change across the full dynamic range of the promoter system), and the application of this gate to regulating a specific cellular function may require optimization of this dynamic range or connection to other devices such as signal amplifiers. We are currently conducting experiments to confirm the activity of this gate through a separate characterization method.

5. Example calculation for RNA device activity

First example:

We work through a calculation of device activity on a NAND gate (Figure 3B from published manuscript) as an example. The raw flow cytometry histogram data are shown on page 13 of this supplement. The gene expression activities are determined as the mean fluorescence of the induced population as described earlier and reported on page 13 as well.

The uncorrected gene expression activities of the RNA device under different input concentrations are reported in absolute fluorescence units (afu) as:

s_{meas} (no input) = 681.66
s_{meas} (10 mM theo) = 633.14
s_{meas} (1 mM tc)= 1122.69
s_{meas} (10 mM theo, 1 mM tc) = 947.02

Based on the expression levels of the device we use non-switch9 to correct for the nonspecific effects of tetracycline in the presence of tetracycline and non-switch8 to correct for the nonspecific effects of tetracycline in the presence of both ligands:

$\Delta s_{nonspec} = (s_{1,meas}-s_0)_{non\text{-}switch9} = 1083.74-656.11 = 427.63$
$\Delta s_{nonspec} = (s_{1,meas}-s_0)_{non\text{-}switch8} = 972.5-570.23 = 402.3$

It should be noted that while we are using a correction term based on the expression level from the device at the ligand concentrations of interest, the values are similar if the same correction term is used and if the correction term is determined based on the basal expression level of the device (expression level in the absence of input; which in this case would be based on non-switch9).

The tetracycline corrected gene expression activities of the device are:

s_{spec} (no input) = 681.66
s_{spec} (10 mM theo) = 633.14
s_{spec} (1 mM tc) = 1122.69 – 427.63 = 695.06
s_{spec} (10 mM theo, 1 mM tc) = 947.02 – 402.3 = 544.72

A similar calculation is performed on the gene expression activities for the standard sTRSV Contl as:

c_{meas} (no input) = 1263.55
c_{meas} (10 mM theo) = 1224.05
c_{meas} (1 mM tc) = 1747.47
c_{meas} (10 mM theo, 1 mM tc) = 1722.59

sTRSV Contl is a non-switch control and the data in the absence of tetracycline for this construct can be used to correct for the nonspecific effects of tetracycline through the subtraction method as:

c_{spec} (no input) = 1263.55
c_{spec} (10 mM theo) = 1224.05
c_{spec} (1 mM tc) = 1747.47 – 483.92 = 1263.55
c_{spec} (10 mM theo, 1 mM tc) = 1722.59 – 483.92 = 1238.67

It should be noted that we selected to correct the sTRSV Contl sample with both inputs by the subtraction method for consistency. The calculated device signals are similar in the case where the sample with both inputs is corrected by the sample with theophylline only (i.e., c_{spec} (theo) = c_{spec} (theo, tc)).

The device signals are then calculated as the ratio of the device expression activities to the sTRSV Contl expression activities as:
$S_{d,0}$ (no input) = 0.54
$S_{d,theo}$ (10 mM theo) = 0.52
$S_{d,tet}$ (1 mM tc) = 0.55
$S_{d,theo/tet}$ (10 mM theo, 1 mM tc) = 0.44

These numbers are multiplied by 100 to report device signal as a percentage (as reported in Table S1). These numbers are divided by U_{ex} ($U_{ex} \approx .02$) to report device signal in units of expression. The device response is reported as the difference between device signal (in units of expression) in the presence of both inputs and that at the indicated ligand conditions (as reported in Figure 3B).

Second example:
We work through a second example calculation of device activity on a NOR gate (Figure 2F from published manuscript). The raw flow cytometry histogram data are shown on page 12 of this supplement. The gene expression activities are determined as the mean fluorescence of the induced population as described earlier and reported on page 12 as well.

The uncorrected gene expression activities of the RNA device under different ligand concentrations are reported in absolute fluorescence units (afu) as:
s_{meas} (no input) = 321.52
s_{meas} (10 mM theo) = 204.58
s_{meas} (0.5 mM tc) = 226.69
s_{meas} (10 mM theo, 0.5 mM tc) = 200.15

Based on the expression levels of the device we use non-switch1 to correct for the nonspecific effects of tetracycline where:
$\Delta s_{nonspec} = (s_{0.5,meas}-s_0)_{non\text{-}switch1} = 237.02-164.12 = 72.9$

The tetracycline corrected gene expression activities of the device are:
s_{spec} (no input) = 321.52
s_{spec} (10 mM theo) = 204.58
s_{spec} (0.5 mM tc) = 226.69 – 72.9 = 153.79
s_{spec} (10 mM theo, 0.5 mM tc) = 200.15 – 72.9 = 127.25

A similar calculation is performed on the gene expression activities for the standard sTRSV Contl as:
c_{meas} (no input) = 1200.72
c_{meas} (10 mM theo) = 1403.06

c_{meas} (0.5 mM tc) = 1459.59
c_{meas} (10 mM theo, 0.5 mM tc) = 1460.49

sTRSV Contl is a non-switch control and the data in the absence of tetracycline for this construct can be used to correct for the nonspecific effects of tetracycline as:
c_{spec} (no input) = 1200.72
c_{spec} (10 mM theo) = 1403.06
c_{spec} (0.5 mM tc) = 1459.59 – 258.87 = 1200.72
c_{spec} (10 mM theo, 0.5 mM tc) = 1460.49 – 258.87 = 1201.62

The device signals are then calculated as the ratio of the device expression activities to the sTRSV Contl expression activities as:
$S_{d,0}$ (no input) = 0.27
$S_{d,theo}$ (10 mM theo) = 0.15
$S_{d,tc}$ (0.5 mM tc)= 0.13
$S_{d,theo/tc}$ (10 mM theo, 0.5 mM tc) = 0.11

These numbers are multiplied by 100 to report device signal as a percentage (as reported in Table S1). These numbers are divided by U_{ex} ($U_{ex} \approx .02$) to report device signal in units of expression. The device response is reported as the difference between device signal (in units of expression) in the presence of both inputs and that at the indicated ligand conditions (as reported in Figure 2F).

6. Raw data for RNA devices

We present raw data (representative flow cytometry histograms and averaged mean fluorescence values over triplicates) for all gates presented in the main manuscript text and supporting information. Note, that legends for the raw data reference the function of the devices and the figure names in the Science paper.

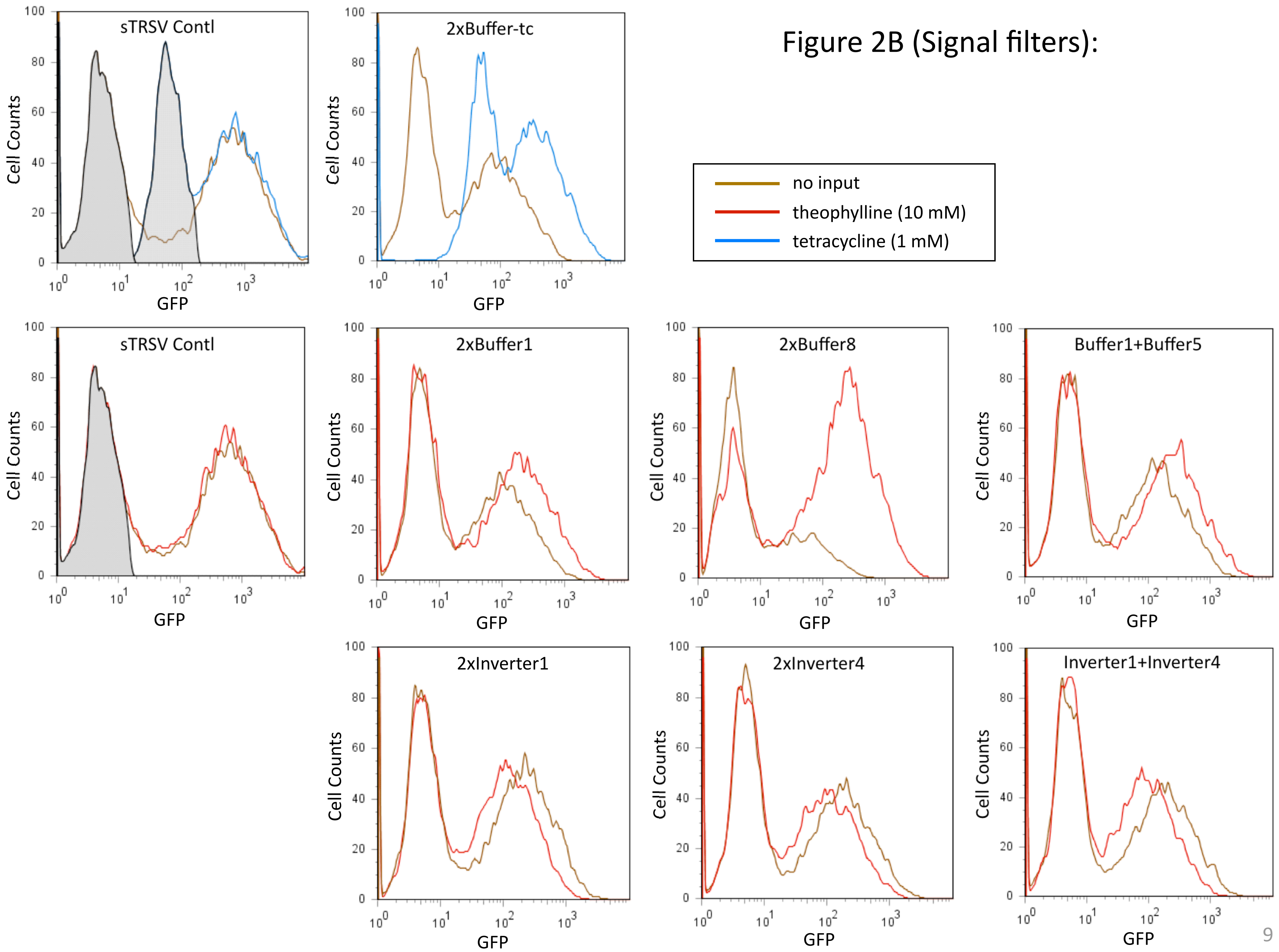
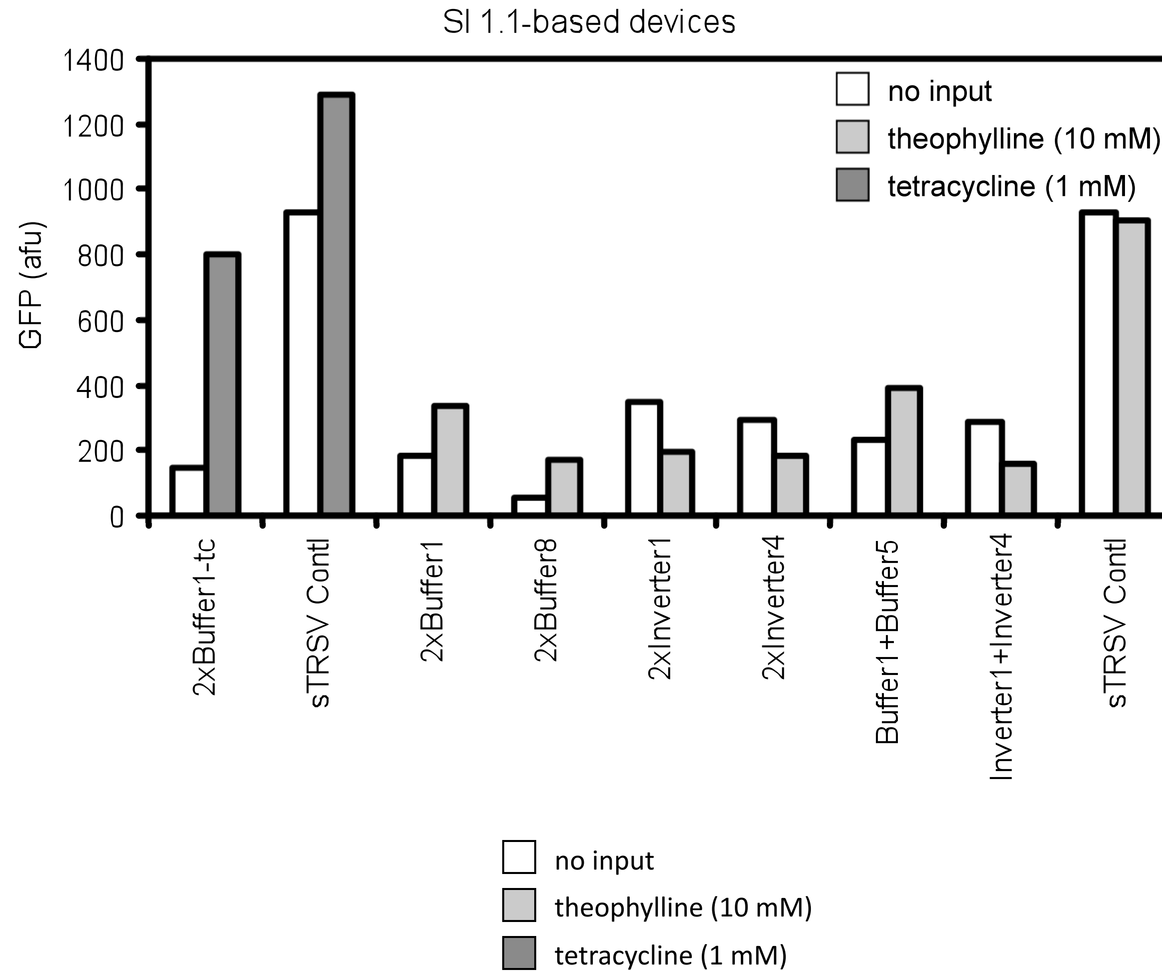


Figure 2B (Signal filters):
no input
theophylline (10 mM)
tetracycline (1 mM)
sTRSV Contl
2xBuffer-tc
sTRSV Contl
2xBuffer1
2xBuffer8
Buffer1+Buffer5
2xInverter1
2xInverter4
Inverter1+Inverter4
Cell Counts
GFP

Figure 2B (Signal filters):



Note, that raw data for single gates are available in supporting information of *Proc Natl Acad Sci USA. 2007. 104:14283-8.*

Figure 2D (AND gate):

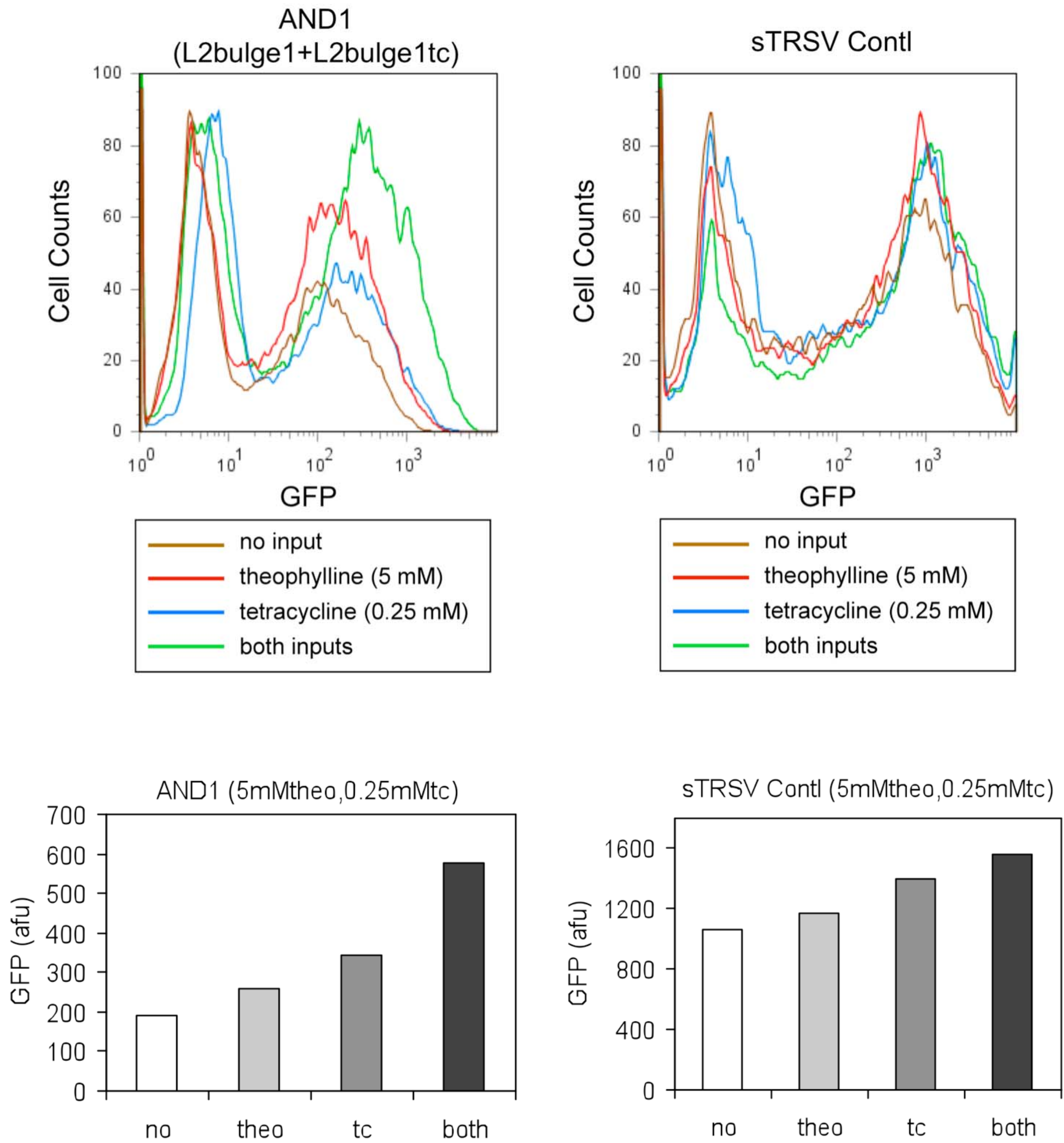
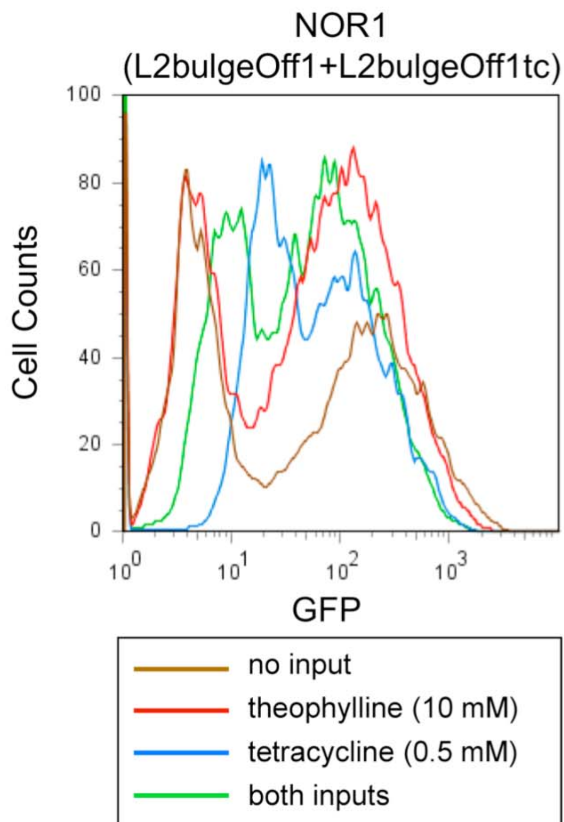
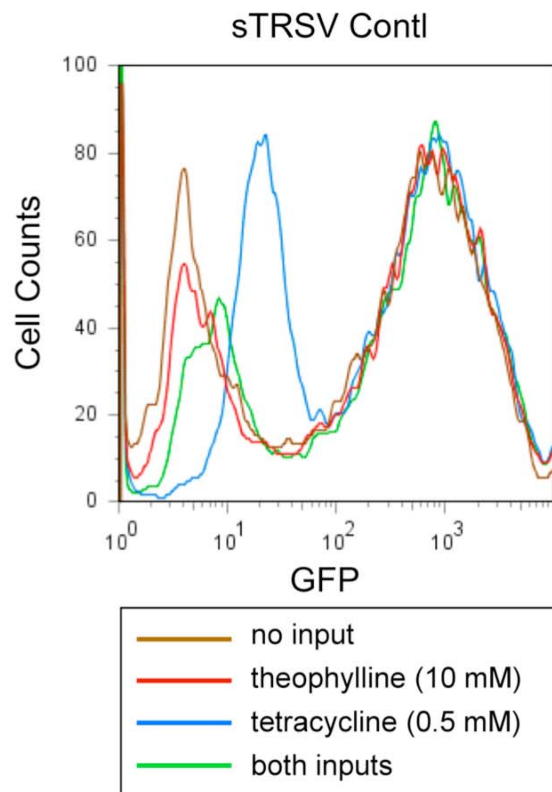
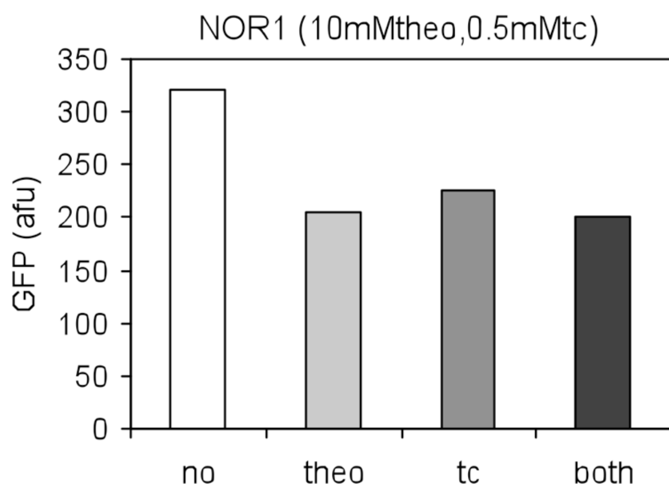


Figure 2F (NOR gate):







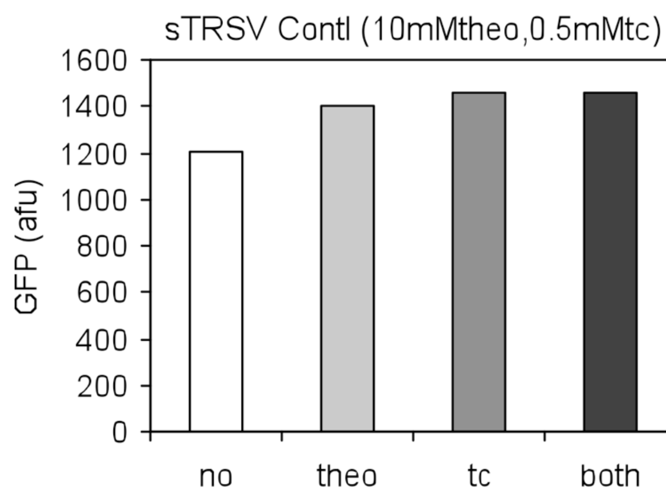
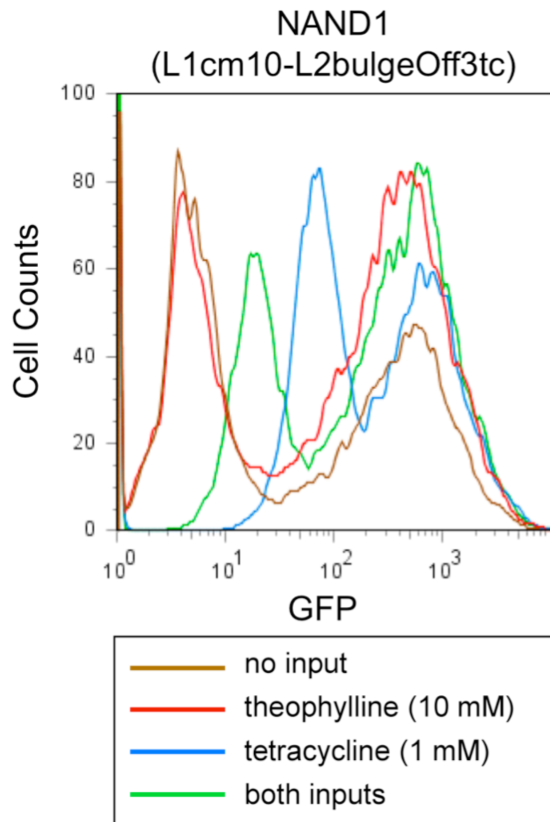
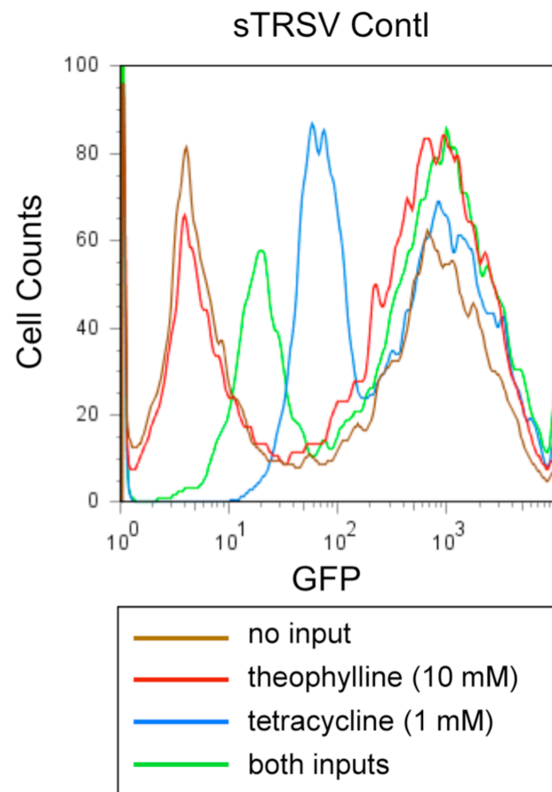
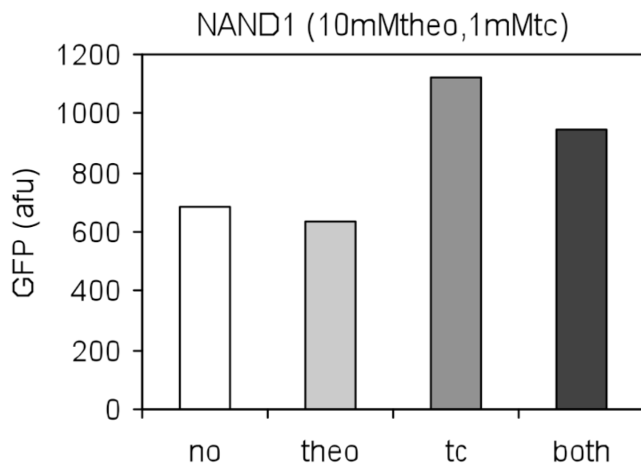


Figure 3B (NAND gate):







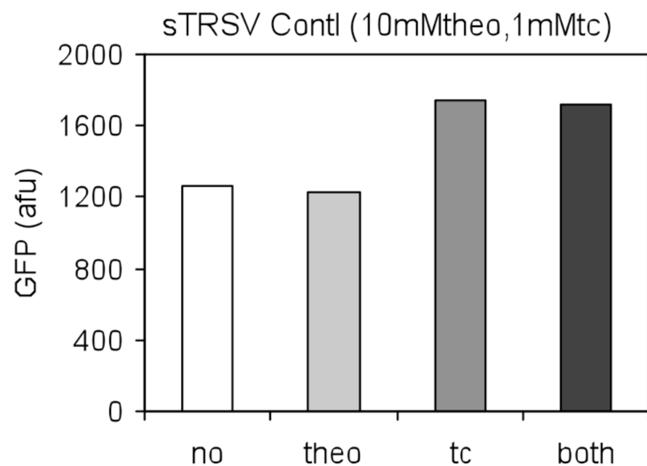
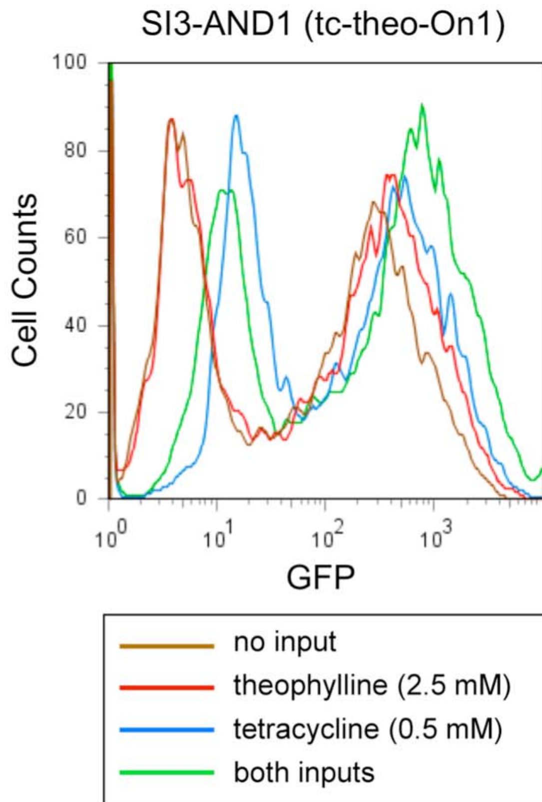
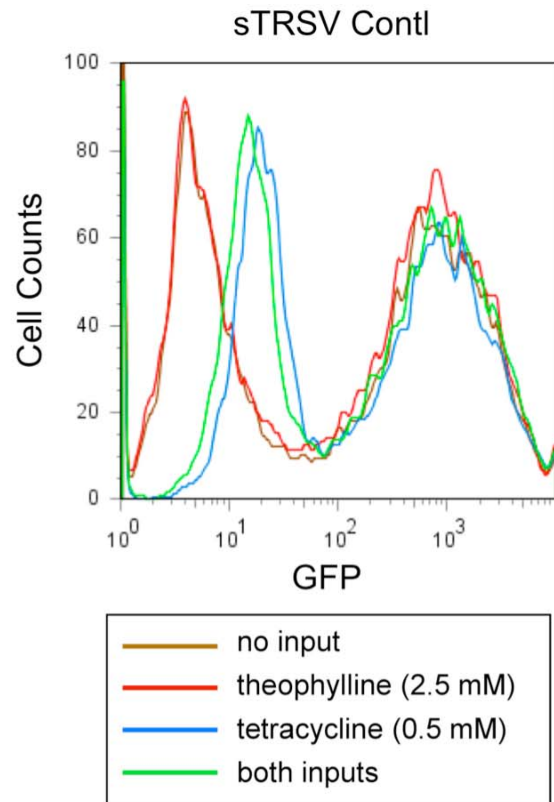
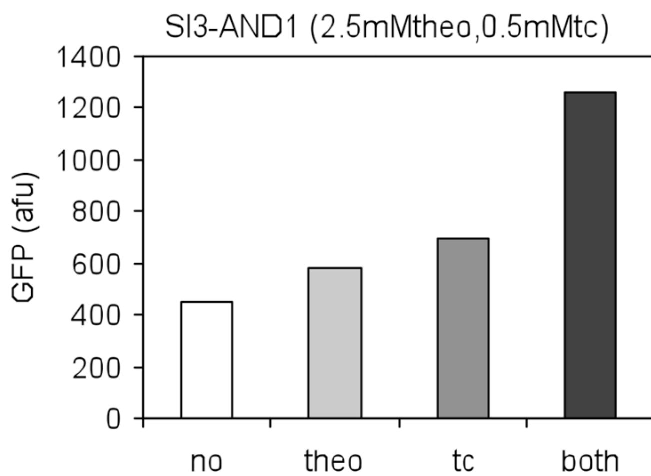


Figure 4B (AND gate, SI 3):







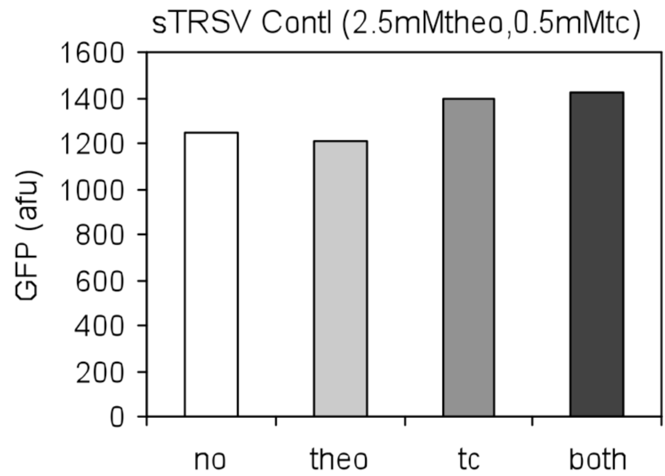


Figure 4D (Buffer gates, SI 3):

theo-theo-On1

theo-theo-On10

theo-theo-On11

theo-theo-On12

theo-theo-On13

sTRSV Contl

Cell Counts

GFP

no input

theophylline (10 mM)

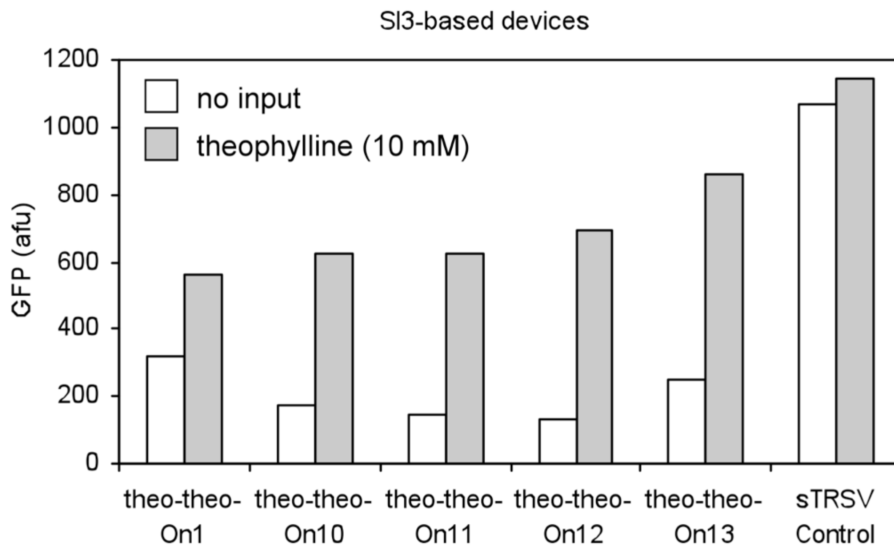
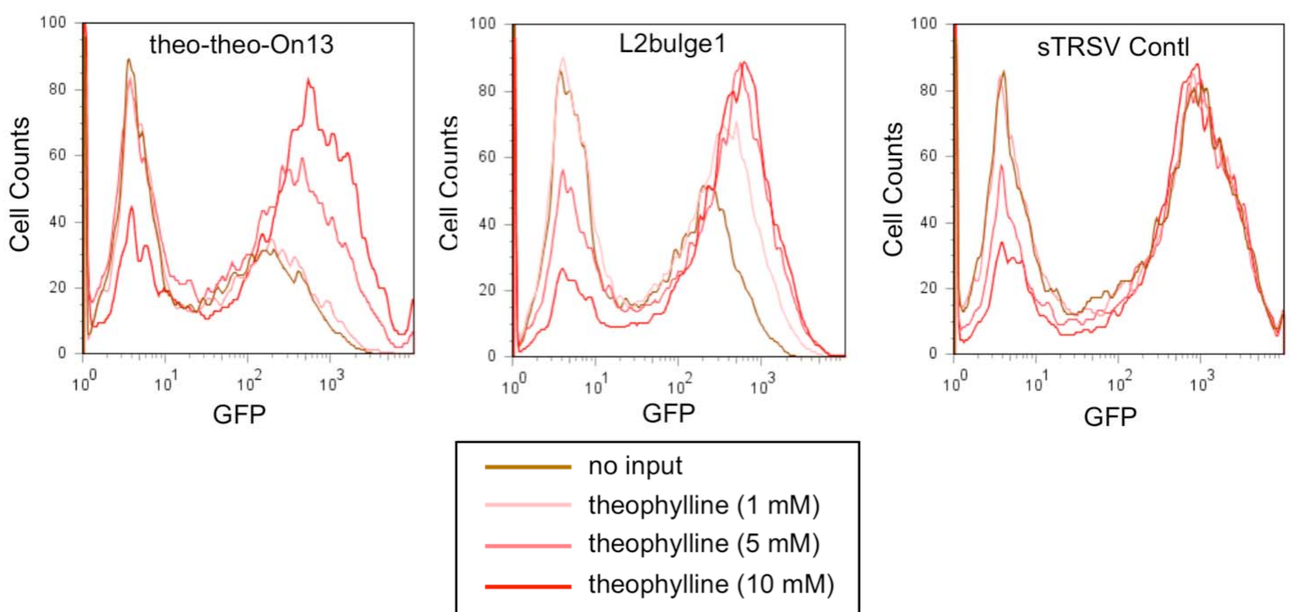
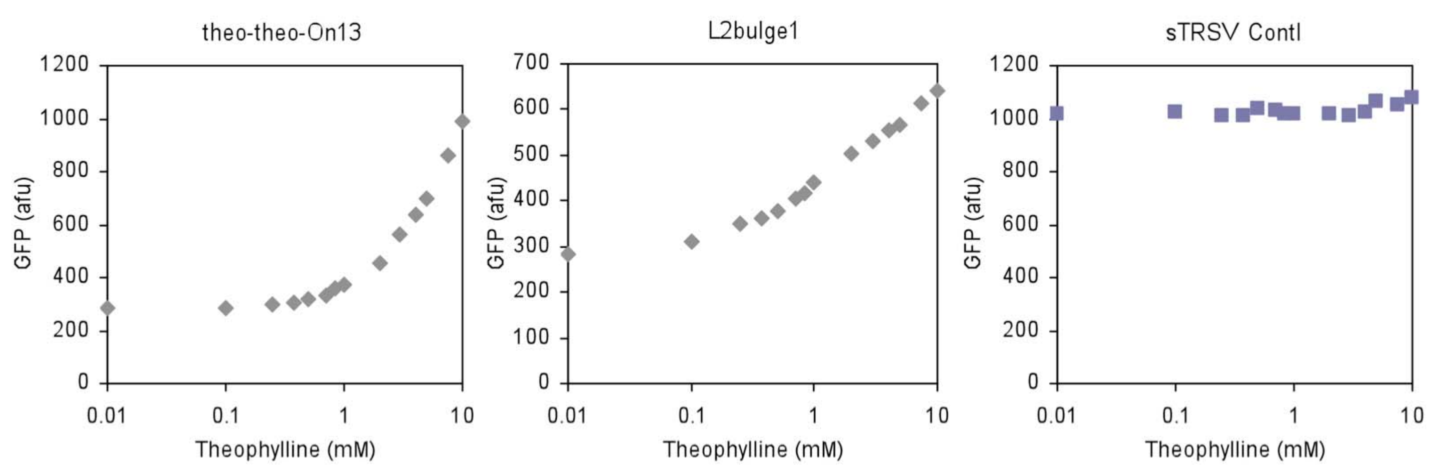
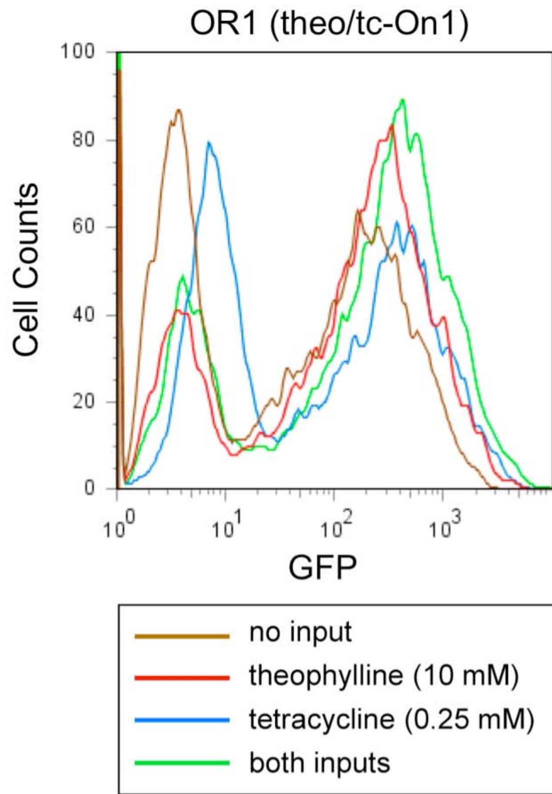


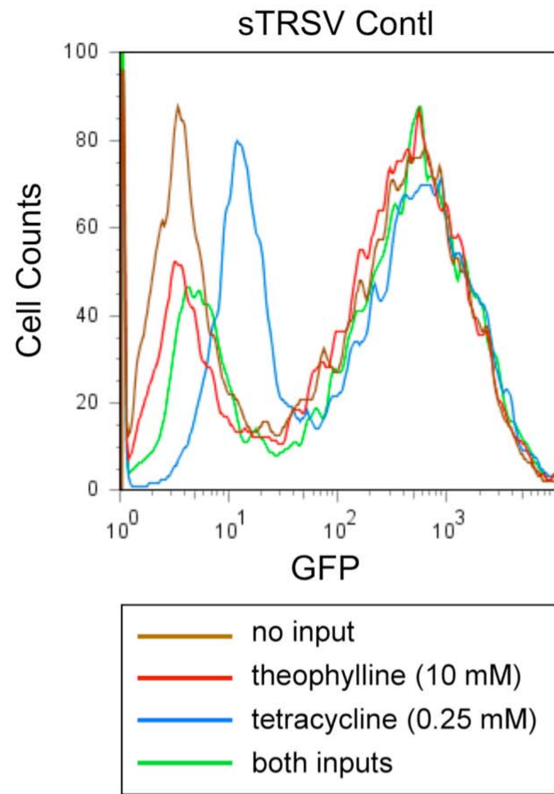
Figure 4E (Cooperative buffer gates, SI 3):

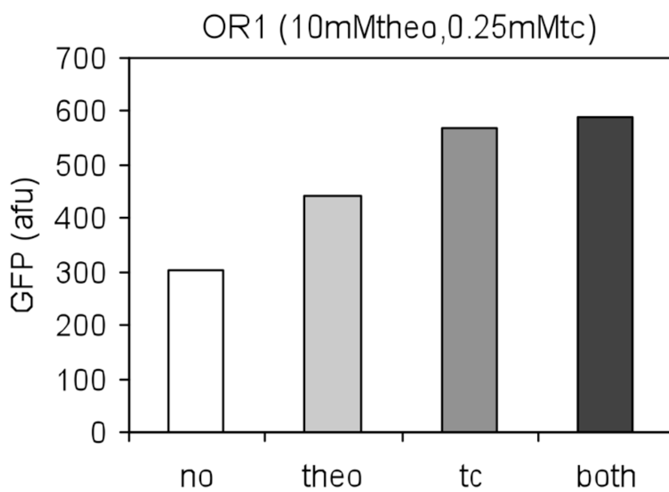


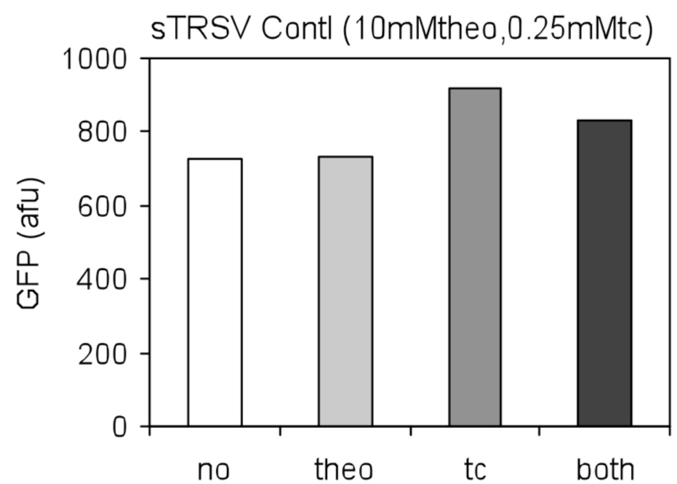


Supporting Material, Logic gates
Text S4 fig. 2 (OR gates):

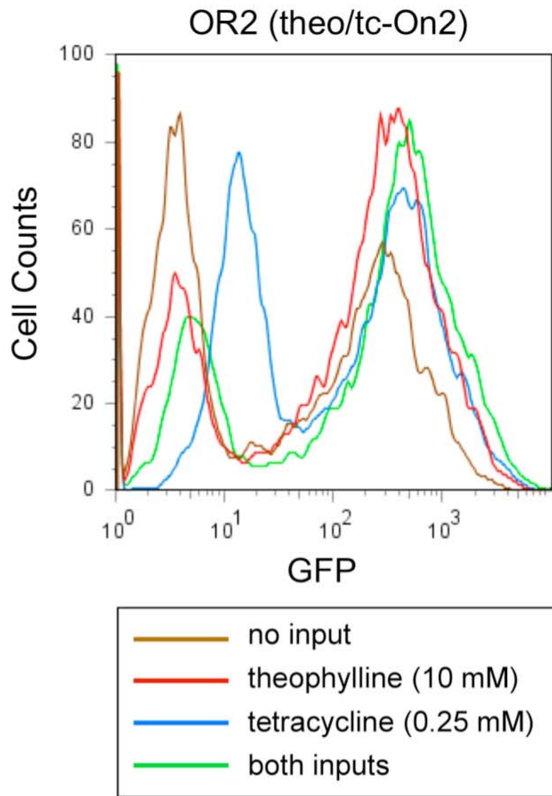


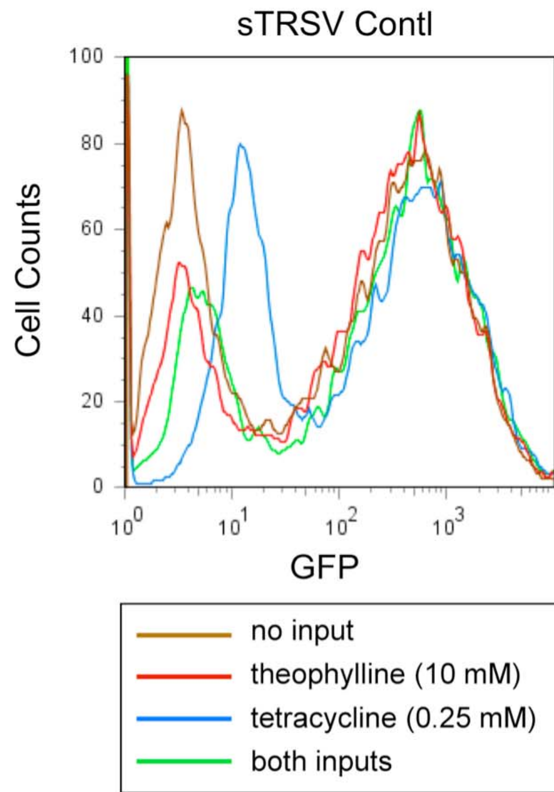


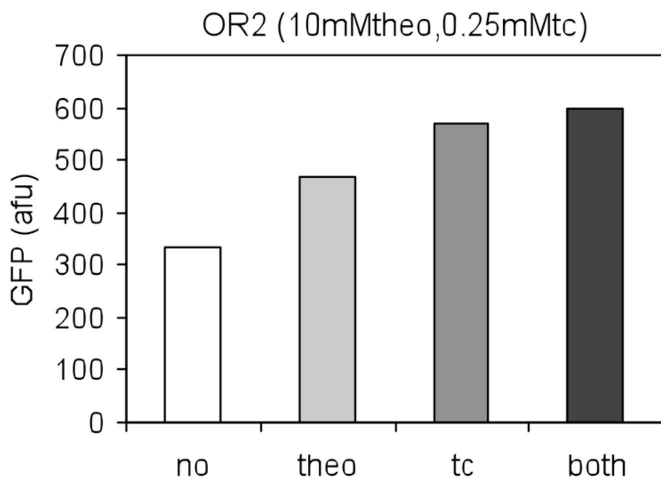


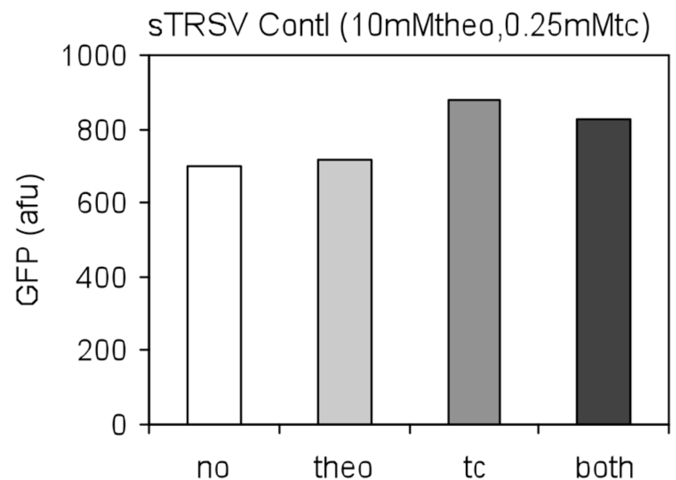


Supporting Material, Logic gates
Text S4 fig. 2 (OR gates, continued):

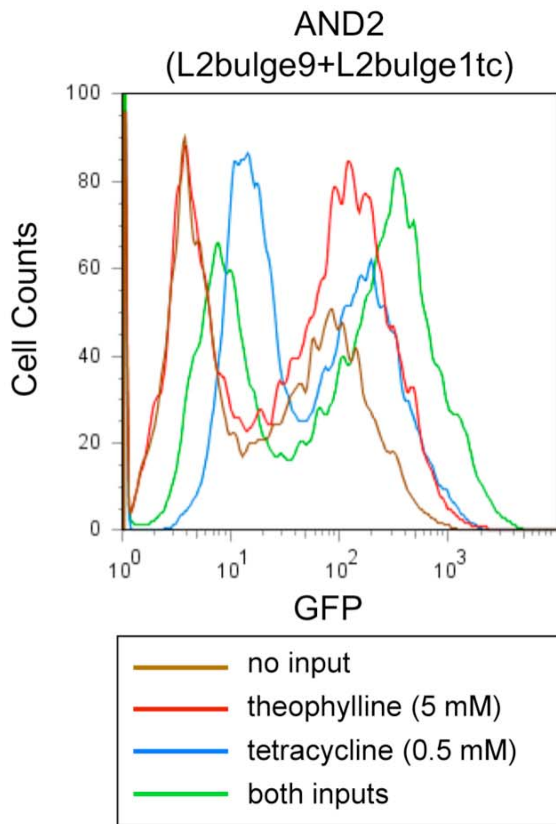


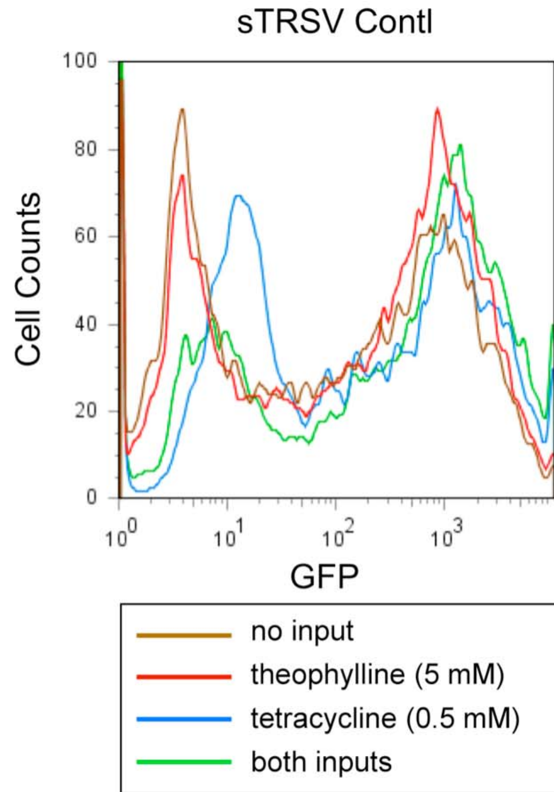


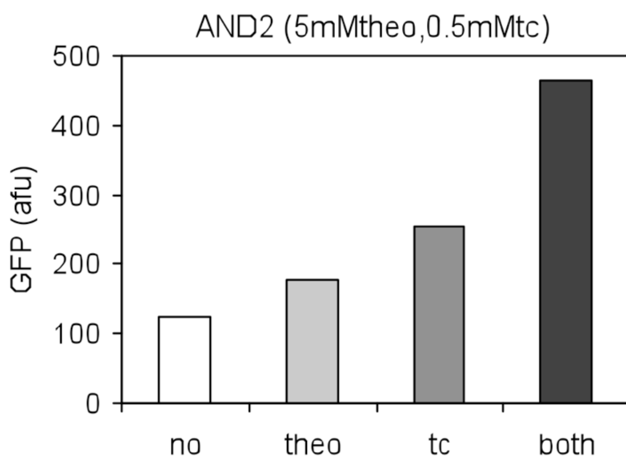


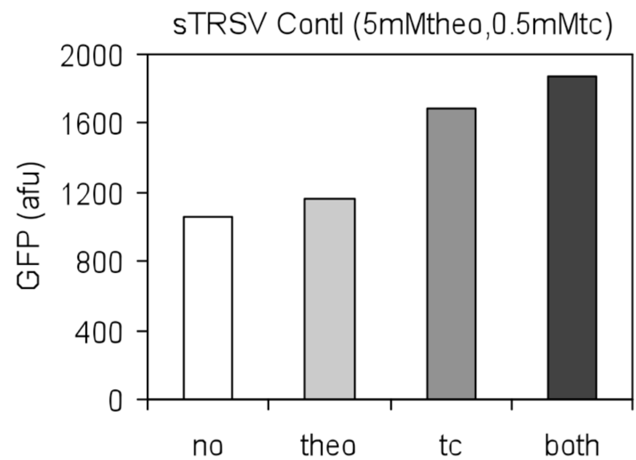


Supporting Material, Logic gates
Figure S1 (AND gate):

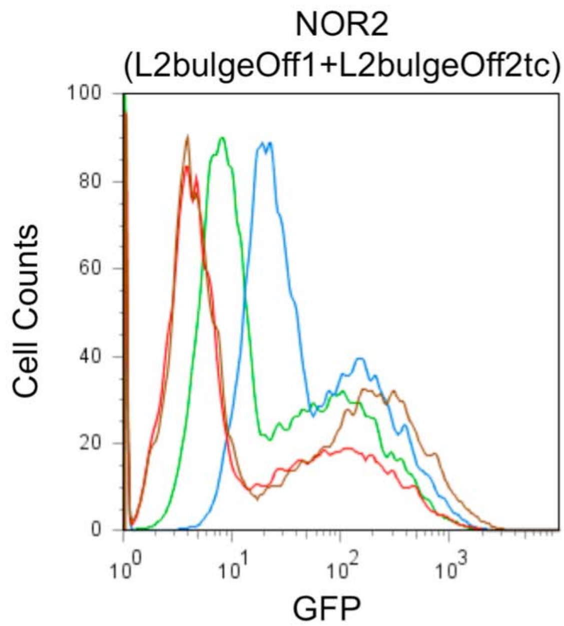


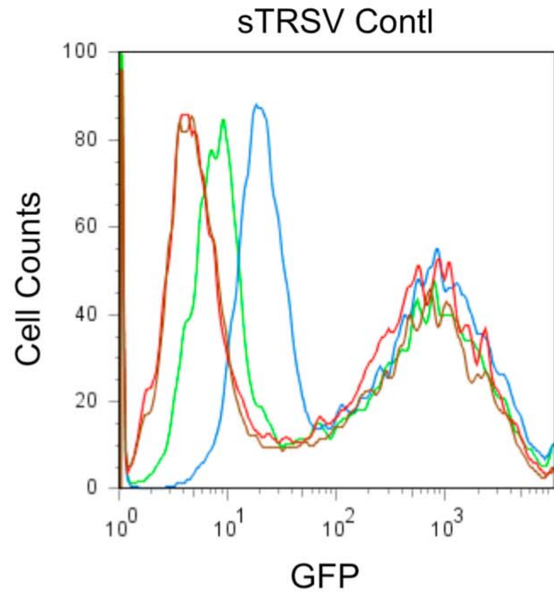


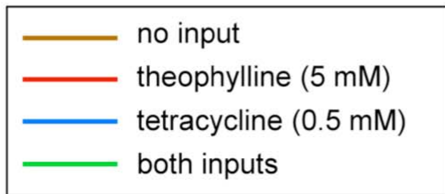


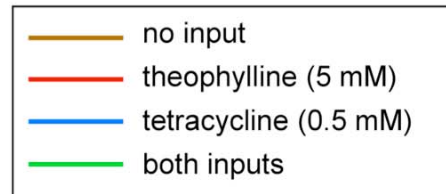


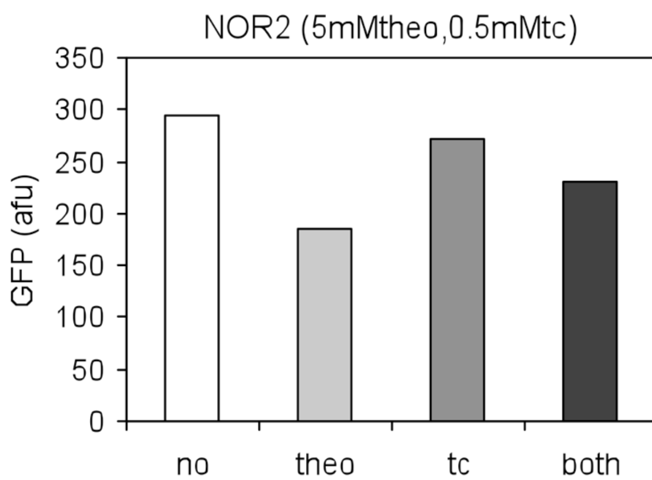
Supporting Material, Logic gates
Figure S3 (NOR gate):

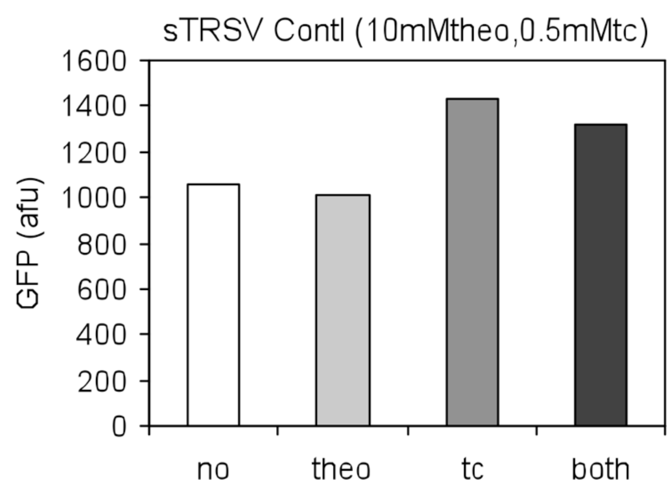




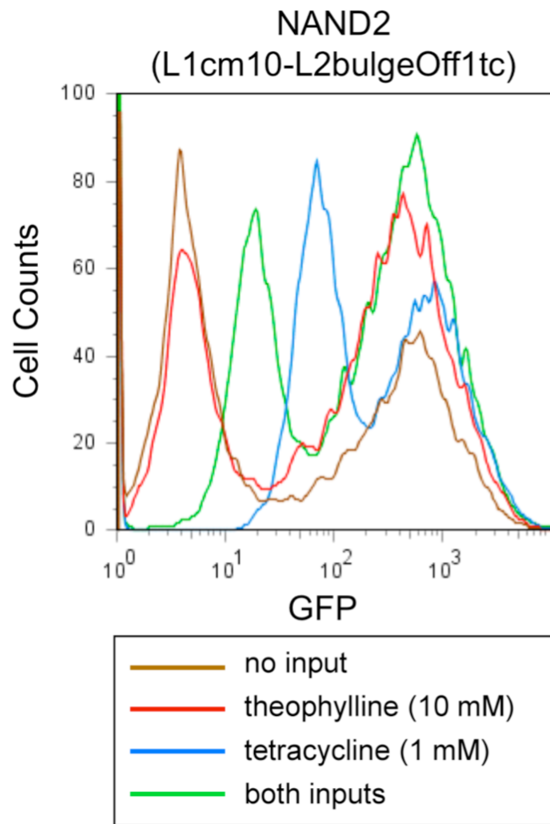


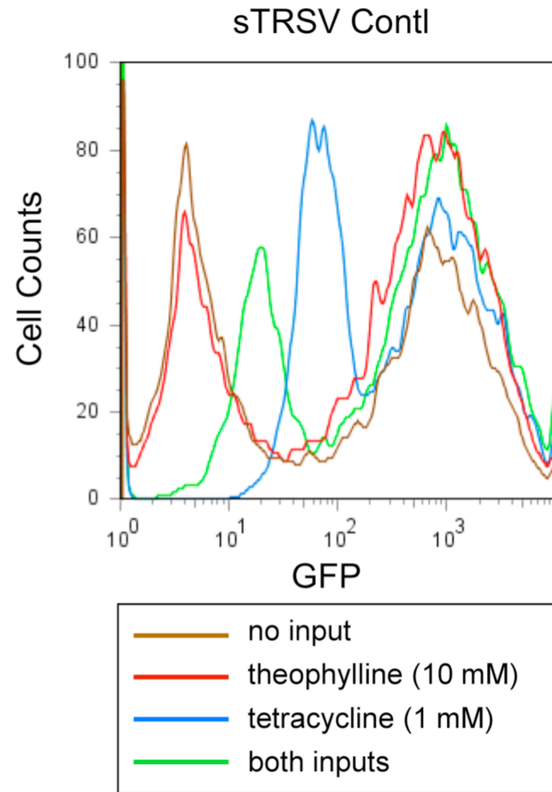


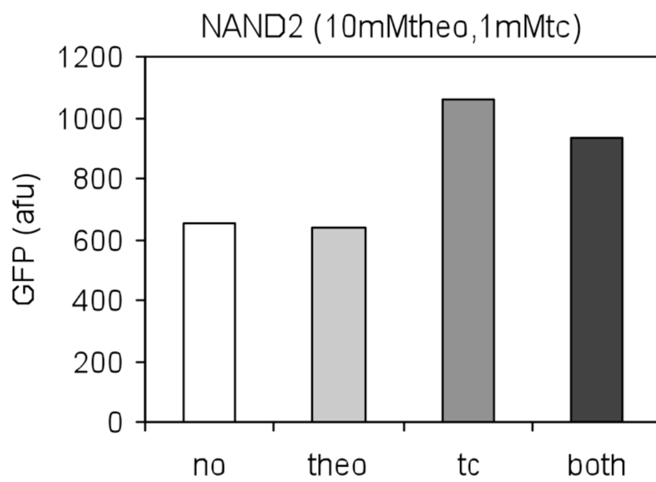


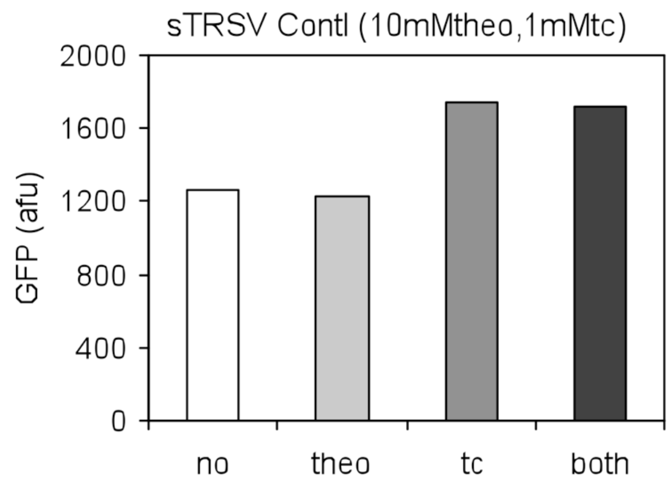


Supporting Material, Logic gates
Figure S5 (NAND gate):

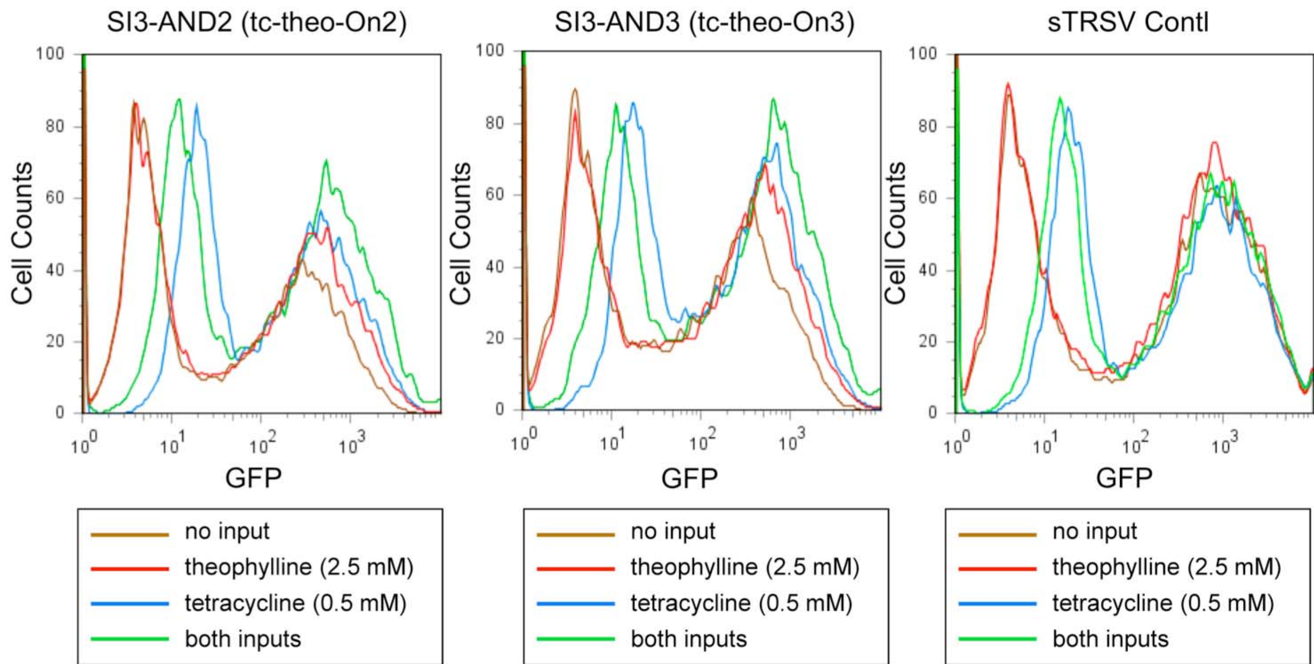


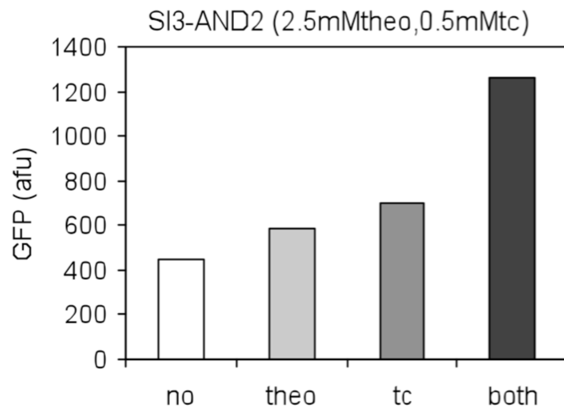


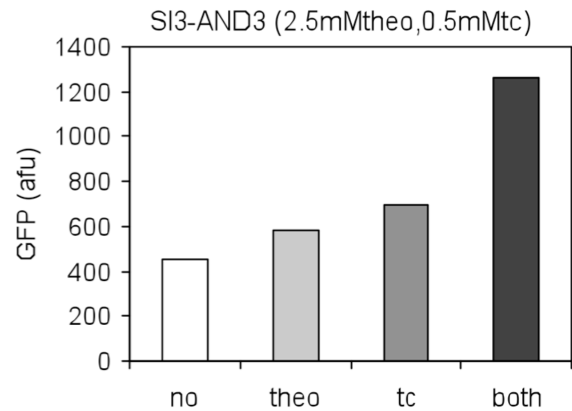


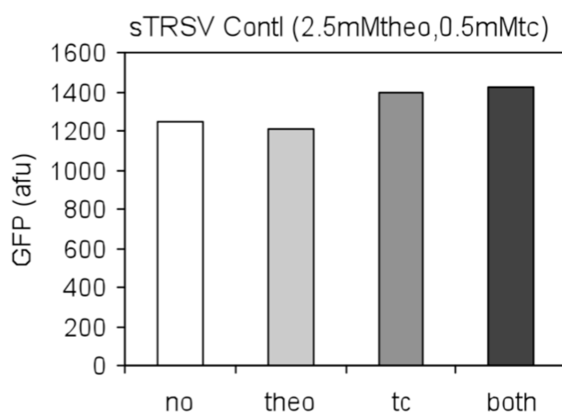


Supporting Material, Logic gates
Figure S6 (AND gate, SI 3):

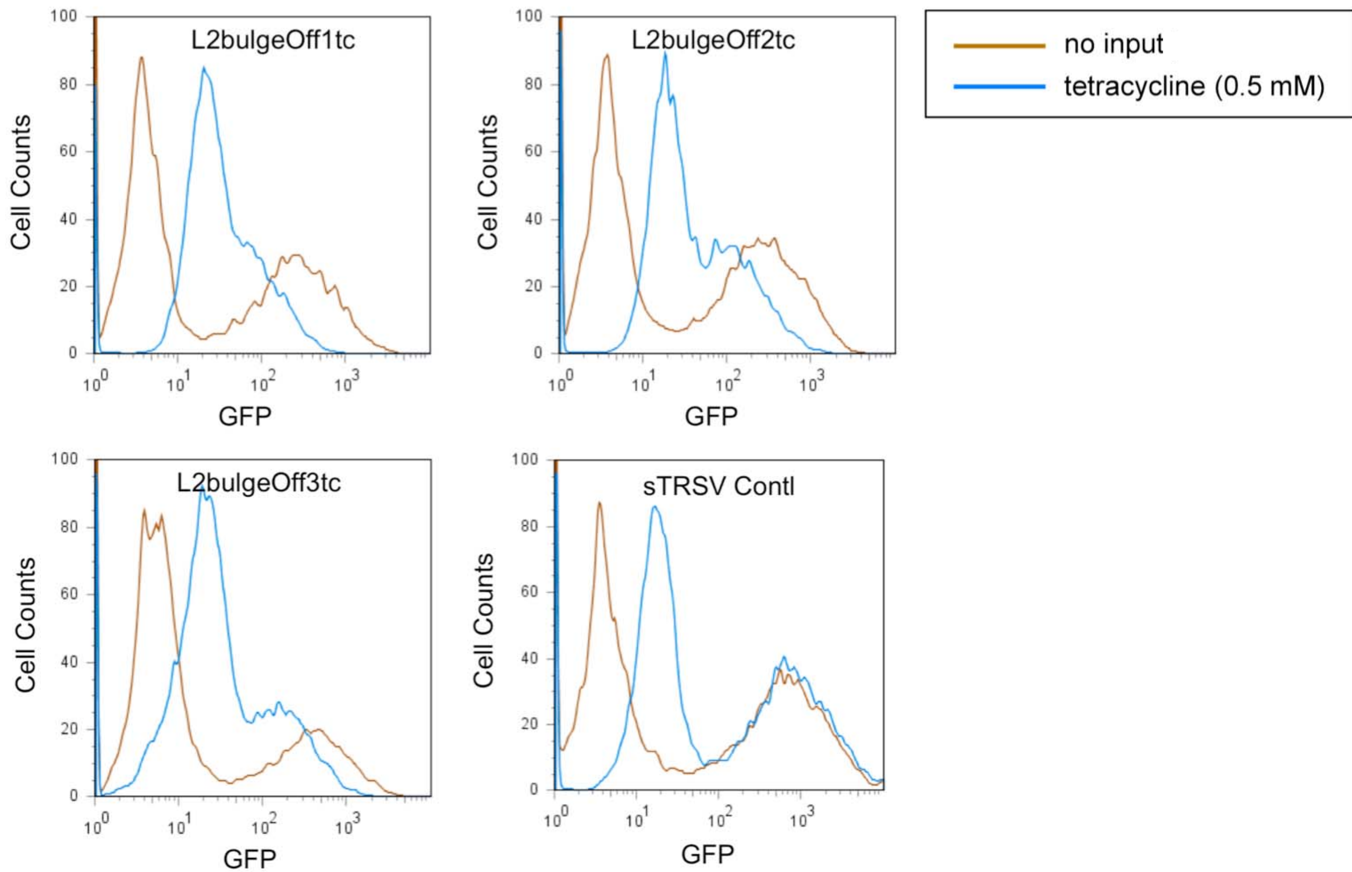


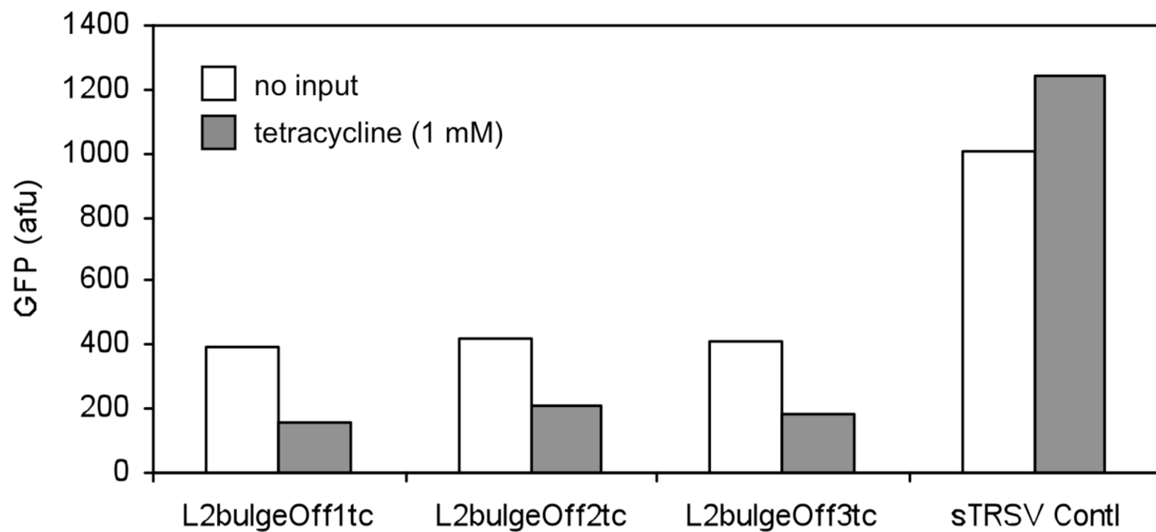




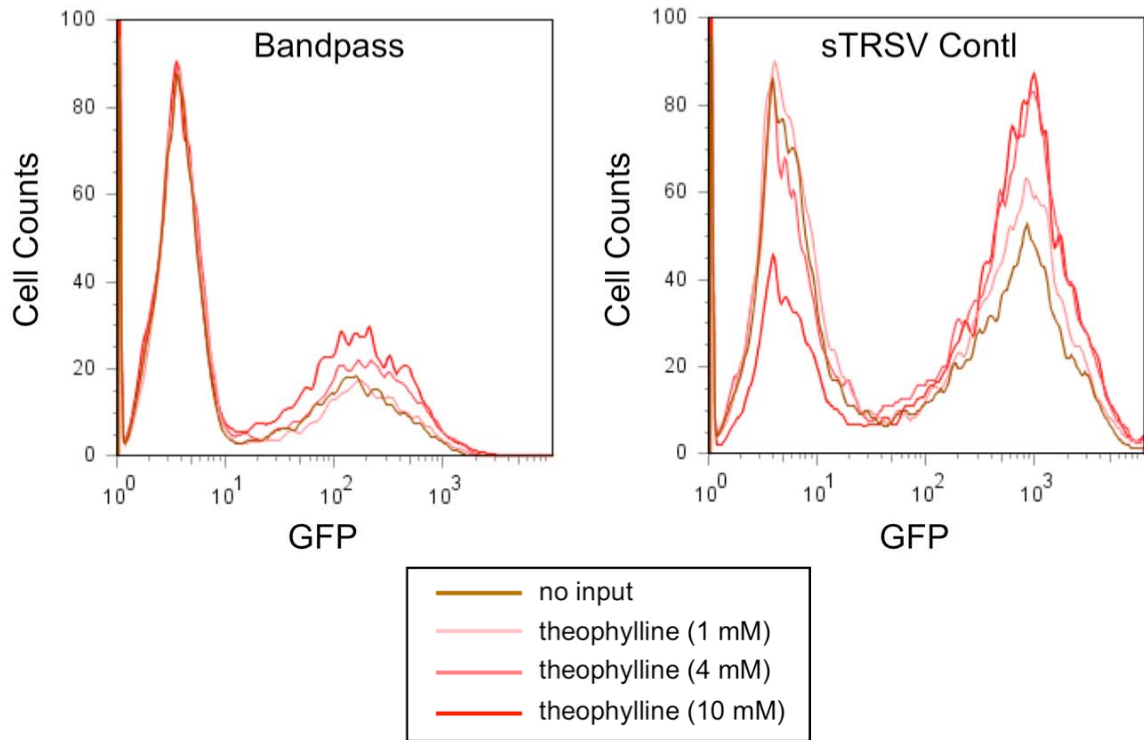


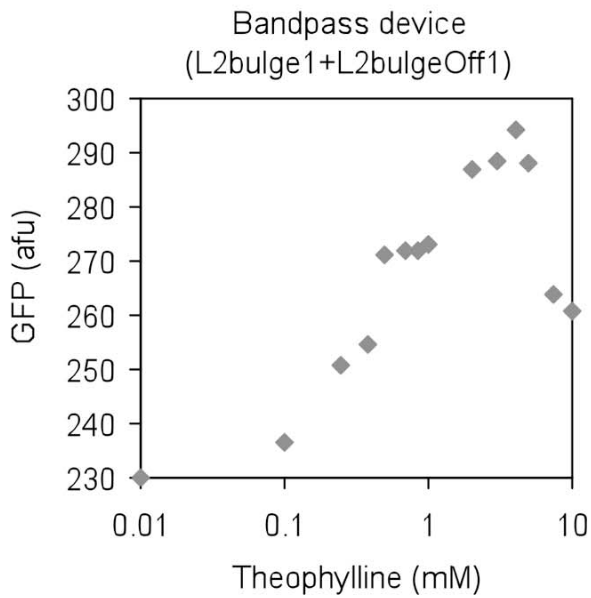
Supporting Material, Single-input gates
Figure S2 (tet inverter):

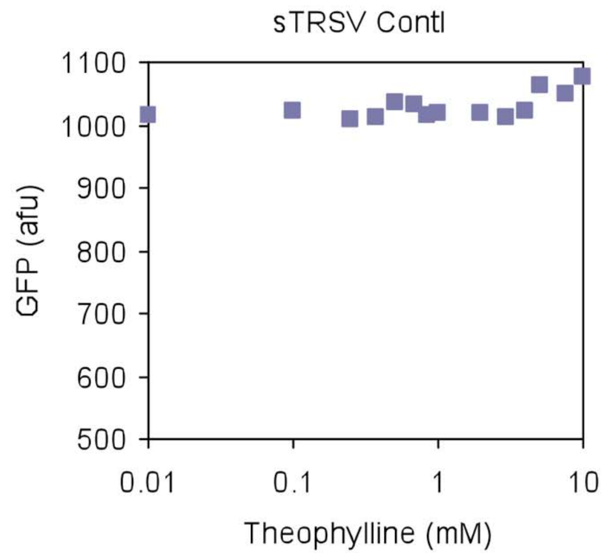




Supporting Material, Filters
Figure S4 (bandpass filter):

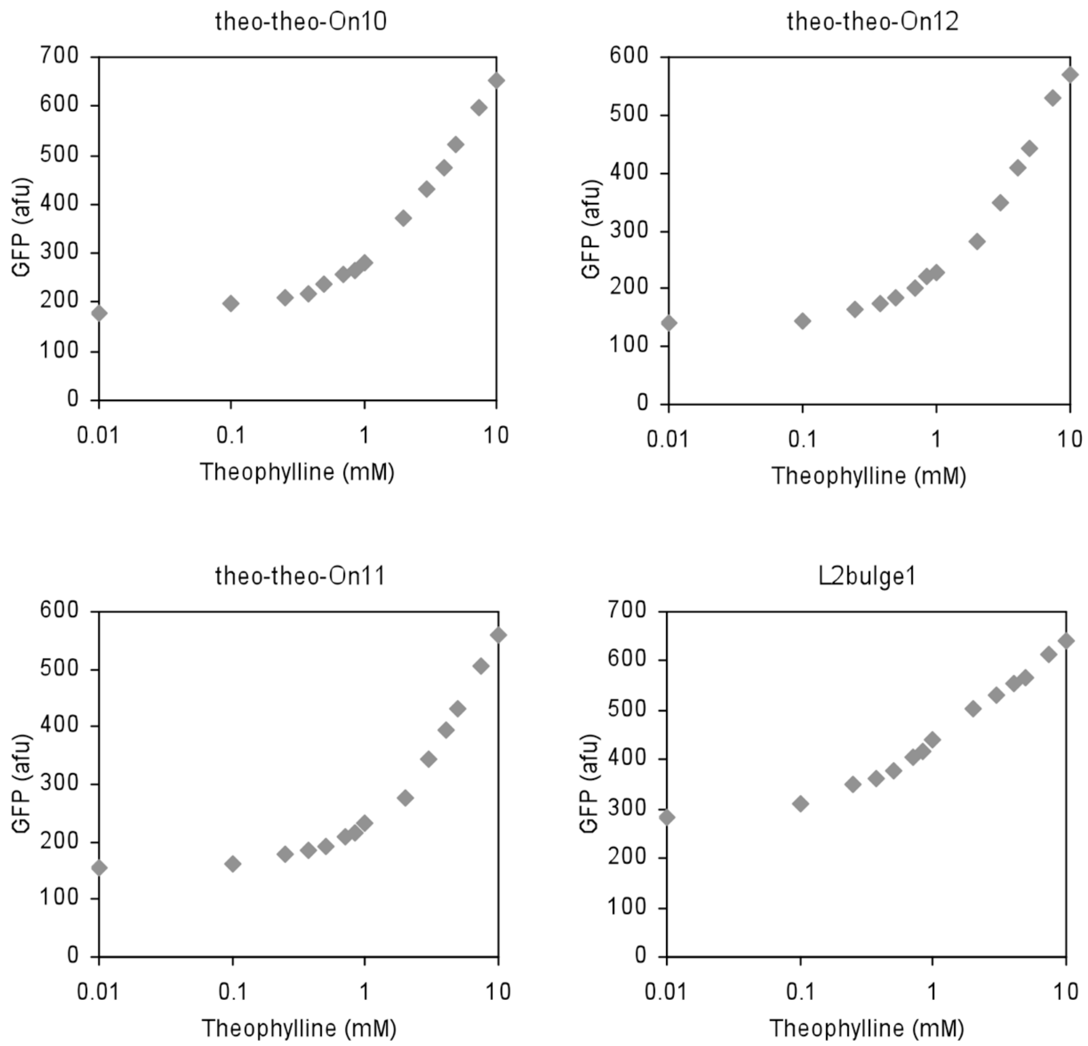




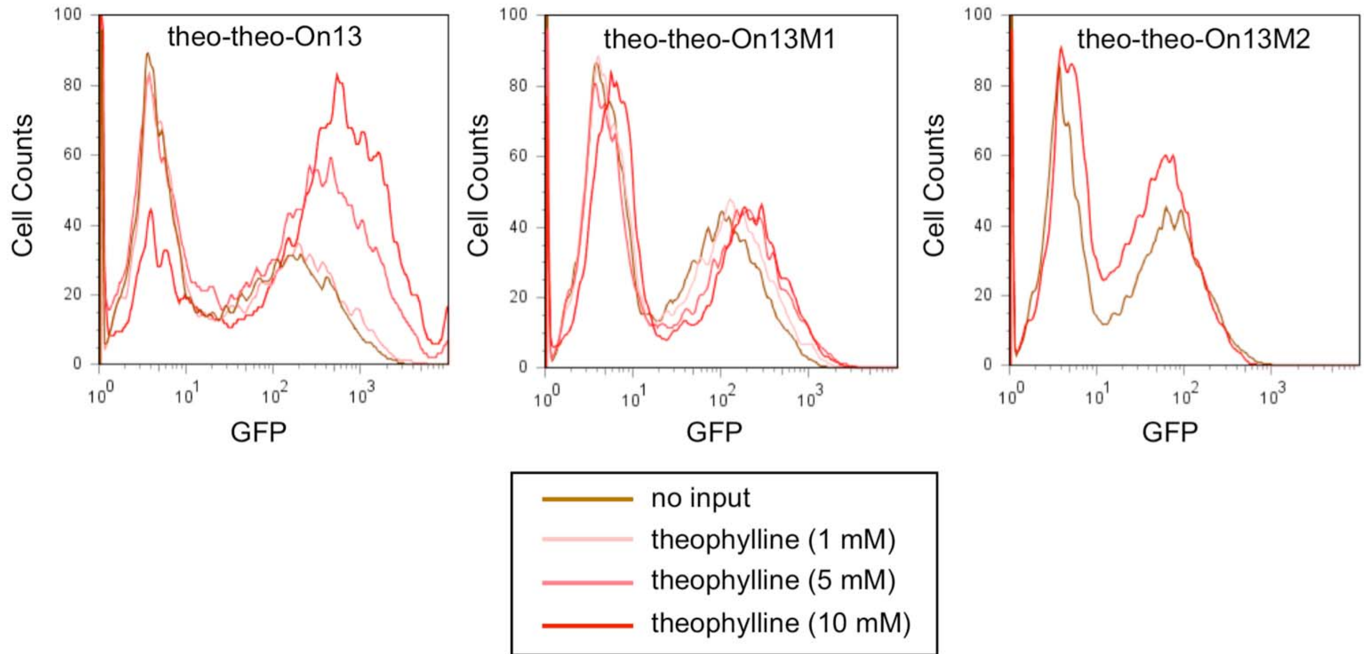


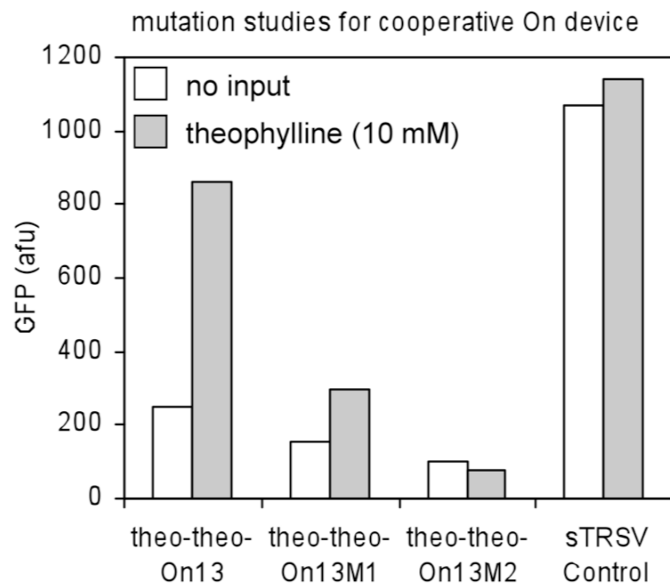
Supporting Material

Figure S7 (Cooperative buffer gates):

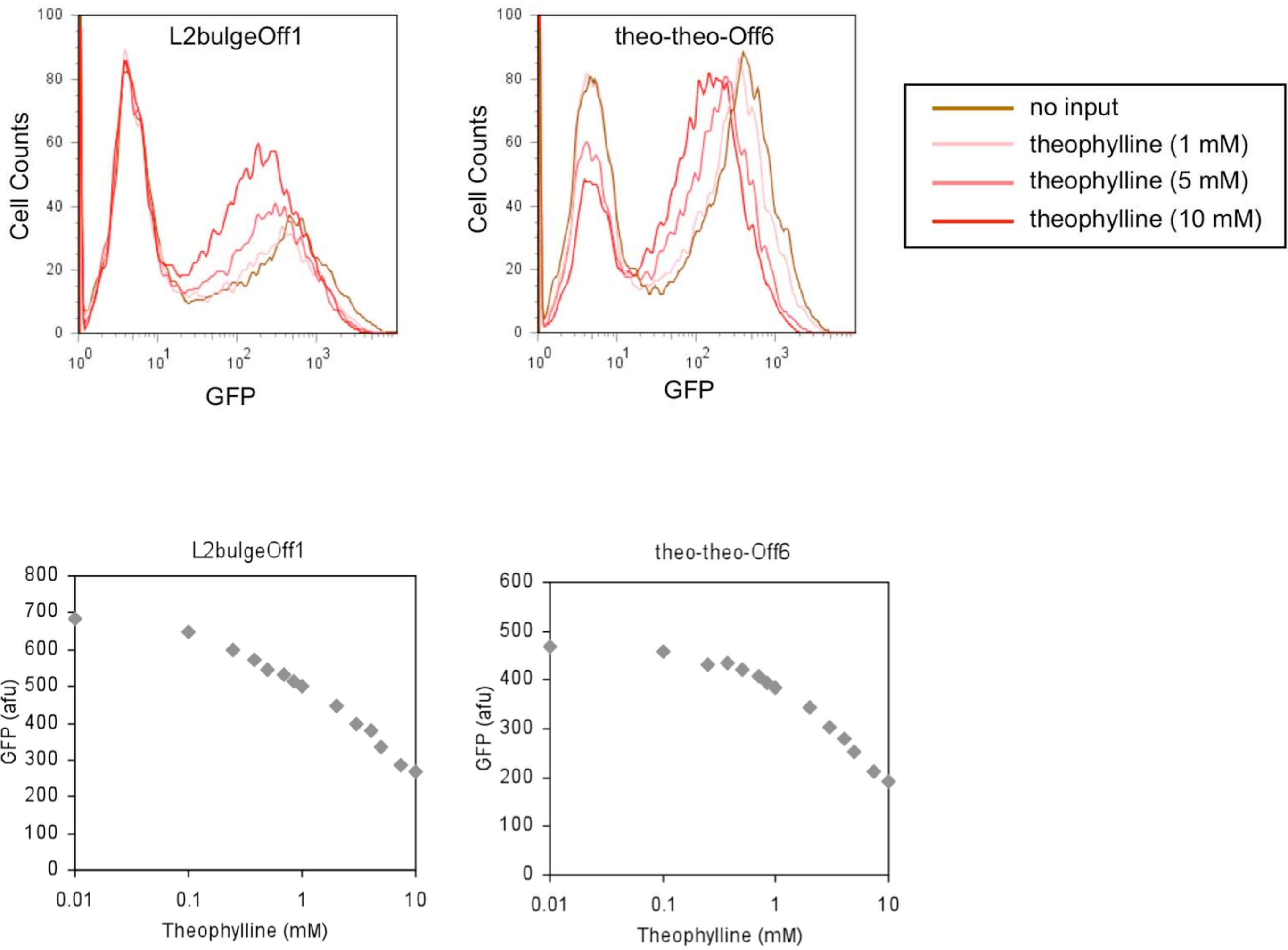


Supporting Material
Figure S14 (Mutational studies, Cooperative buffer gates):





Supporting Material
Figure S9 (Cooperative inverter gates):



Supporting Material
Figure S15 (Mutational Studies, Cooperative inverter gates):