Cells can be programmed to monitor and react to their environment using genetic circuits. Design automation software maps a desired circuit function to a DNA sequence, a process that requires units of gene regulation (gates) that are simple to connect and behave predictably. This poses a challenge for eukaryotes due to their complex mechanisms of transcription and translation. To this end, we have developed gates for yeast (Saccharomyces cerevisiae) that are connected using RNA polymerase flux as the signal carrier and are insulated from each other and host regulation. They are based on minimal constitutive promoters (~120 base pairs), for which rules are developed to insert operators for DNA-binding proteins. Using this approach, we constructed nine NOT/NOR gates with nearly identical response functions and 400-fold dynamic range. In circuits, they are transcriptionally insulated from each other by placing ribozymes downstream of terminators to block nuclear export of messenger RNAs resulting from RNA polymerase readthrough. Based on these gates, Cello 2.0 was used to build circuits with up to 11 regulatory proteins. A simple dynamic model predicts the circuit response over days. Genetic circuit design automation for eukaryotes simplifies the construction of regulatory networks as part of cellular engineering projects, whether it be to stage processes during bioproduction, serve as environmental sentinels or guide living therapeutics.
Results

Genetic parts to build insulated gates. NOT/NOR gates are based on a strong promoter that is turned off by a repressor. This promoter must not be sensitive to environmental or growth conditions. To this end, we designed synthetic constitutive promoters based on a TATA box and 20-bp transcription start site (TSS) gleaned from the ADH2 gene (Fig. 2a)\(^1\),\(^2\),\(^3\). We varied the location and sequences of the upstream activating sequence (UAS) and region between the TATA box and TSS (Extended Data Fig. 1). From these, we selected a promoter based on Gal4-binding UAS sites flanked by two 15-bp poly-T sequences and a 31-bp spacer between the UAS and TATA box (Fig. 2b) (repeated for lacI/lacO;\(^4\),\(^5\)).

To eliminate Gal4 as a regulatory input, we identified an alternative UAS that could achieve high expression levels. We found that the expression level was dominated by promoter choice (Fig. 2c). The promoters were fused to a 10-bp Kozak sequence and the yellow fluorescent protein gene (yfp) and cloned into a plasmid (Supplementary Fig. 15). Fluorescence measurements were made using strains carrying the promoter reporter in the presence or absence of the plasmid expressing TetR (Fig. 2b) (Methods). The strongest repression was consistently observed for promoters when cells are grown in diverse conditions. Like them, we demonstrated that it is the least affected natural constitutive promoter to 12 repressors (Extended Data Fig. 1 and Supplementary Figs. 2–12). For those repressors used to build gates, two variants of each promoter were built so that they could be used without invoking homologous recombination (Fig. 2c).

Readthrough of RNAP from a gate to its neighbours can cause circuit failures\(^6\). To block transcription between gates, 33 yeast terminators were characterized downstream from 35 promoters (Fig. 3a and Supplementary Tables 7 and 8) (Methods). All 1,155 combinations were evaluated and, while there were some outliers, we found that the expression level was dominated by promoter choice (Fig. 3a).

When terminators appear immediately adjacent to a promoter, they can change the nucleosome occupancy and promoter activity\(^7\). Spacers were identified to disrupt this effect that are based on ribozymes (RiboJ variants\(^8\),\(^9\)) placed between the terminator and promoter. While this does not stop an errant transcript from being produced, it is cleaved and not capped or exported from the nucleus, thereby blocking expression. When a ribozyme was placed after an inducible promoter and before a reporter gene, induction no longer led to expression (Fig. 3b and Supplementary Fig. 15). Insulators were then constructed by pairing the strongest terminators with a ribozyme and spacer, and evaluated in the genome for their ability to stop an upstream inducible system (Van) from influencing a neighbouring inducible system (anhydrotetracycline hydrochloride (aTc)) (Fig. 3c and Extended Data Fig. 3). All of the insulators were able to block interference, thus enabling the two neighbouring inducible systems to function independently.

Definition of a reference promoter. Several reference promoters have been defined for \(E.\) \(coli\) and used to report promoter activities in RPUs\(^{12,13}\). To define a similar reference promoter for \(S.\) \(cerevisiae\), we selected PFY1 based on an analysis by Ellis and co-workers\(^1\) demonstrating that it is the least affected natural constitutive promoter when cells are grown in diverse conditions. Like them, we inserted it into the \(ura3\) locus, but flanked it by strong terminators and an upstream ribozyme to buffer against transcriptional interference (Fig. 1).
The gate dynamics were quantified using a simple model that captured the characteristic times ($\tau$) of the circuit, and the algorithm avoided toxic combinations. CI434 readthrough from the genome (S. cerevisiae CY671int) (Fig. 4a). The fluorescence was measured by cytometry and defined as 1 RPU (Fig. 4b and Extended Data Fig. 4).

Design of sensors and gates. A sensor array was constructed at the LChXV locus, from which regulatory proteins for three small molecule sensors were expressed (Fig. 4c). The xylose sensor is based on XylR from Bacillus licheniformis, which we identified from libraries of regulatory proteins and promoters (Supplementary Fig. 13). Three strains were constructed to measure the output promoter of each sensor integrated into IIA. ChV. The $\alpha$Tc, isopropyl-β-D-1-thiogalactopyranoside (IPTG) and β-d-1-thiogalactopyranoside (IPTG) and xylose sensors generated 1,200-fold, 120-fold and 600-fold inductions, respectively (Fig. 4d and Extended Data Fig. 5). The sensors showed no cross-reactivity with respect to each other's small molecules (Fig. 4e).

We constructed nine NOT gates based on the repressors and their cognate minimal promoters. The input to the gates was the IPTG-inducible $P_{\text{lac}}$ promoter ($P_{\text{lac}}$) from S. cerevisiae (Fig. 5a). The gate was encoded at the I.ChXV locus, from which regulatory proteins for three promoters were integrated into IIA.ChV (sensor promoters) or IIB.ChIII genomic loci (gate promoters). The step-by-step design of each promoter is described in Extended Data Fig. 6, which were then fit to:

$$y(x) = y_{\text{min}} + \frac{(y_{\text{max}} - y_{\text{min}})K^n}{K^n + x^n}$$

(1)

The resulting fits are shown in Fig. 5b and parameters are shown in Supplementary Table 2 (see also Extended Data Figs. 7 and 8). We selected Kozak sequences and output promoters to generate similar response functions. The ranges of $y_{\text{max}} \in [0.001, 0.2]$ and $y_{\text{min}} \in [1.5, 5.1]$ spanned the response threshold of the gates (~1 RPU), thus making them easier to connect. To test whether the repressors were orthogonal, we built $9 \times 9 = 81$ strains that varied the repressor expressed from IIA.ChV and the output promoter carried in IIB.ChIII, and no cross-reactions were observed (Fig. 5c). Finally, the growth impact of each gate was measured as a function of the strength of the input promoter (Extended Data Figs. 7 and 8). These data were used by Cello 2.0 to predict the growth impact of the circuit, and the algorithm avoided toxic combinations. CI434 and QaCg slowed growth before reaching maximum repression and were therefore avoided by the algorithm.

The gate dynamics were quantified using a simple model that captured the characteristic times ($t_{\text{ON}}$ and $t_{\text{OFF}}$) for a gate to switch OFF $\rightarrow$ ON or ON $\rightarrow$ OFF (Methods)²⁸. To perform these measurements, strains were grown under one set of conditions (with...
or without inducer) until reaching steady state. Then, they were transferred into fresh media with the opposite conditions (Methods and Extended Data Figs. 7 and 8). These data were fit to differential equation models to calculate $\tau_{\text{ON}}$ and $\tau_{\text{OFF}}$ (Supplementary Table 2). Note that the times required for inducer to be taken up by the cells and YFP to express and fold were separated from these values.

A NOR gate architecture was developed for the *S. cerevisiae* genome (Fig. 5d). Two copies of the repressor gene were designed with different codon usages. Each gene was paired with the same Kozak sequences and was driven by input promoter 1 or 2. To test this NOR gate design, we used the IcaR repressor and the aTc and IPTG sensors (Fig. 5e). The gate produced the expected two-dimensional NOR response function (Fig. 5f) independent of whether the copies of the repressor genes were co-located (Fig. 5g).

It is simpler for Cello to compute how to connect gates if the NOR gate response function can be described as a one-dimensional...
NOT gate response function, the input to which is the sum of the input promoters. We tested this for the nine gates where the repressor genes were encoded in the IIA.ChV locus, transcribed by Ptet and Plac. Different combinations of aTc and IPTG were added and the resulting fluorescences were measured (‘measured output’ in Fig. 5h). This was compared with the predicted output, which was obtained when the two input promoters were summed and used with the NOT gate response function (taken from Fig. 5b). While there was some systematic error, the correlation was sufficient to use the NOT response functions to connect gates.

Genetic circuit design automation. Cello 2.0 maps the desired circuit to a linear DNA sequence to be inserted in a specific genetic location. For S. cerevisiae, we selected two genomic loci to carry the circuit (Fig. 6a) (Supplementary Fig. 1). In practice, we found that maintaining the insulator order resulted in more reliable circuits. Therefore, we constrained the order of the insulators, as opposed to an order of repressor genes/gates. This is shown in Fig. 6a, where the transcription units follow the progression of 1–16, but the order of the repressor genes in the progression is random. To evaluate the difference in expression at these sites, 16 strains were built where a Ptet-yfp construct was inserted at each position and the variation in expression was less than twofold, which was small enough to not disrupt gate function (Fig. 6b).

To validate design automation, a test set of three-input logic circuits were constructed (specified in Verilog, Supplementary Note 1). The sensors were defined along with their OFF/ON response (red lines in Fig. 4d) and the UCF SC1C1G1T1 was selected.
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Input 1 (RPU)

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into the HO locus (Fig. 7c,d and Supplementary Table 4). Each output promoters of each intermediate gate in the circuit integrated this, strains were constructed that contained than the other can turn on after this change in the inducers. To test 7b). The last gate is an OR function where one input turns off faster to + (Fig. / −10A1 circuit, looking at the transition from −/−/− to ++/+ (Fig. +/ +). The last gate is an OR function where one input turns off faster than the other can turn on after this change in the inducers. To test this, strains were constructed that contained rfp reporters for the output promoters of each intermediate gate in the circuit integrated into the HO locus (Fig. 7c,d and Supplementary Table 4). Each gate was accurately modelled using the simple ordinary differential equation (ODE), including the observed glitch.

Discussion

Many groups have shown that you can combine regulatory interactions to build a circuit function that performs correctly at a digital level. However, the quantitative response is not predicted. We have found that making more precise predictions requires the careful design of insulated gates and selection of where they should be carried in the cell. Philosophically, we define the UCF for a highly specified context (strain, loci and growth conditions) for which the predictions are valid. While gates may ‘work’ when inserted in a different locus, strain or media, these are not the conditions for

Fig. 5 | NOT/NOR gates. a. The NOT gate design is based on repressors that contain a carboxy-terminal nuclear localization signal (black). b. NOT gate response functions. The data for the response of each gate were fit to equation (1) (colour coded by repressor). The data used for these fits are shown in Extended Data Figs. 7 and 8. The concentrations of IPTG (from left to right) were: 0, 0.1, 0.25, 0.5, 1, 2, 2.5, 3, 5, 10 and 20 mM. c. Orthogonality of the gates. The promoters and reporters were integrated into IIB.ChIII and the repressors (P Tet controlled) were integrated into IIA. ChV under aTc control (0 or 100 ng ml−1 aTc added). The fold-repression was calculated as RPU (−aTc)/RPU (+aTc), d. The design of the NOR gate is shown, where each input promoter transcribes a different copy of the repressor gene with different codon assignments. e. The two copies of the repressor were integrated at the same genomic locus (top) or at two loci (bottom). f. The response of the IcaR NOR gate at IIA.ChV is shown as a function of both inducers. IPTG was added at 0, 0.5, 2, 20 and 200 mM (input 1) and aTc was added at 0, 20, 30 and 100 ng ml−1 (input 2). g. The IcaR NOR gate was compared when the repressor genes were on the same chromosome or split between two (as shown in e). h. Comparison of the output of a NOR gate with that predicted by using the corresponding NOT gate response function. For all nine gates, the two-dimensional response was measured (as in f) and compared with that predicted using the one-dimensional response function (b) and summing the activities of the input promoters. The data represent the average of three experiments (c and f) or individual experiments (g and h) performed on different days.
which they were designed, and this may produce a subtly different quantitative response that propagates to other gates, with the potential of ultimately leading to a circuit failure. Achieving precise predictions through design automation requires that the gates be re-characterized in the new strain or genetic location of interest and these data are then used to build a new UCF. While going through the process of building new gates and a new UCF is initially difficult, after it is complete, it simplifies the design of genetic circuits.
for that host. In fact, a circuit function can be re-mapped to DNA for different hosts in seconds just by selecting a new UCF in Cello 2.0. We now have examples where the circuit specified with Verilog code has been automatically mapped either to an E. coli plasmid (Eco1C2G2T2) or a yeast chromosome (SC1C1G1T1). The Verilog files that appear in this manuscript are relatively simple, but one can imagine a future where genetic designs are shared at this higher and more abstract level and then re-compiled to new organisms as needed.

Large genetic engineering projects will require regulatory networks to coordinate responses; for example, turning on metabolic valves at the right times during growth, managing energy and materials resources, dividing tasks among a population and staging processes. These networks must function precisely in different states and implement dynamic responses. As the need for more complex regulatory networks grows, they will become too large to construct in an ad hoc manner and they will have too many states to be optimized by directed evolution. Design automation will be relied on increasingly frequently to balance the constraints and put together the multitude of DNA parts required.

**Methods**

**Strain, media and reagents.** *S. cerevisiae* BY4741 MATA his3Δ1 leu2Δ0 met15Δ0 ura3Δ0, an S288C derivative, was the parent strain unless otherwise noted. *S. cerevisiae* CENPK113-7D was used to measure the native promoter–terminator combinations. Cells were grown in either YPD broth (Sigma–Aldrich; Y1375) or synthetic drop-out medium (SD; Sunrise; 1701), SD–ura (Sunrise; 1703), SD–Leu (Sunrise; 1707), SD–His (Sunrise; 1705) and SD–ura–Leu (Sunrise; 1721) media to select the correct clones for genome integration or maintain plasmids with corresponding markers. When indicated, 2% galactose (Sigma–Aldrich; G0750) was added to SD without glucose (Sunrise; 1797) medium. The antibiotic 100 μg ml⁻¹ nourseothricin (Gold Biotechnology; N-500) was used for NatMX marker selection. 1.8% agar (Bacto; 214010) was used for agar plates. The inducers used were: IPTG (Sigma–Aldrich; I6758), aTc (Sigma–Aldrich; 37919), β-xylose (Xyl; Sigma–Aldrich; X1500) and vanillic acid (Sigma–Aldrich; 94770). The reporter genes used for these experiments were yfp and rfp codon optimized for yeast⁺⁻⁻.

**Collation of yeast part library.** Promoters and terminators were sourced from yeast part collections (+–/–/–/–, yeast transcriptomic data and other yeasts in the Saccharomyces genus using the Broad Institute Fungal Orthogroups Repository[102]. Parts were mutated to eliminate Bsal and BpiI restriction enzyme sites. For parts sourced from transcriptomic data, the sequence length was adjusted to capture the region of reduced nucleosome occupancy, as defined by Segal and co-workers[103], and to avoid including proximal genome features. Promoters and terminators were synthesized using GeneArt, or from the US Department of Energy Joint Genome Institute, or amplified from *S. cerevisiae* genomic DNA or yeast shuttle vectors. The sequences of all genetic parts are provided in Supplementary Tables 7 and 8.

**Fig. 7 | Circuit dynamics.** a. The five circuits were cycled between states for 400 h (17 d). Cells containing the circuits were cultured as described in the Methods, diluting into fresh media daily. Every 2 d, different combinations of inducers were added to the media (shown at the top). The experimental data points are shown and compared with the dynamics predicted with the ODE (Methods). The data points represent biological triplicates, collected concurrently. b. The circuit diagram of 0xA1 is shown, including the promoters corresponding to each wire. c. Cells containing the circuit were grown with the –/+/– transition of inducers until steady state and then transferred into fresh media with the +/+/+ combination of inducers. The responses of each of the sensors and valves to this transition are shown. The sensors were measured using strains that only contained their output promoter fused to yfp (no circuit) (*S. cerevisiae* CY637Int, CY639Int or CY928Int) (Supplementary Table 4). The other wires were analysed by fusing the promoter shown in b to rfp and integrating the reporter into the chromosome IV, 48031...46271, HO locus. The points show experimental duplicates collected over different days. The lines show the predictions from the ODE model. d. The output of the circuit (X) is shown for the –/+/– to +/+/+ transition, highlighting a fault. The circuit diagrams show the wires with active RNAP flux at the different time points. The middle diagram shows the delay leading to the fault when one of the inputs to the last NOR gate was turned off before the other could turn on. The data represent two experiments performed on different days.
Cell culture conditions for the characterization of parts, gates and circuits. The following protocol was used for all promoter, terminator and insulator experiments except those noted in the next section. Frozen stocks were streaked and grown overnight. A single colony was picked into 500μl SD medium (with auxotrophic selection) and inducers (if required) in a 2-ml 96-deep-well plate (Plate One; 1896-2000) sealed with AeraSeal film (Excel Scientific; BS25). The plates were incubated at 30°C and 900 r.p.m. in a Multitron shaker incubator for 24h. For circuits containing two or more layers, this culture was diluted 200-fold a second time and grown again under the same conditions for an additional 24h. Then, the overnight culture was diluted to OD600=0.005 (Synergy H1 plate reader; BioTek) by adding 2.5 μl culture to 497.5 μl fresh SD medium (without selection, but with inducers if required) in a 2-ml 96-deep-well plate and sealed with AeraSeal film. The cultures are grown at 30°C and 900 r.p.m. in a Multitron shaker incubator for 16h. After growth, a 20 μl culture was added to 180 μl phosphate-buffered saline (PBS; Omnipur; 6505-OP) with 10 μg/ml cycloheximide (Sigma–Aldrich; 227048) into a 96-well U-bottomed plate (Corning; 3367) and incubated for 1h at room temperature for cytometry analysis. In addition, 200 μl of this culture was transferred into 96-well black-walled optical-bottomed plates (Nunc; 165305) to measure the cell density.

Characterization of promoter–terminator pairs. The following protocol was used for promoter–terminator pair characterization in Fig. 3a. Note that the host strain of this experiment was S. cerevisiae CENPK.113-7D, which is different from the one used for the remainder of the work in this manuscript. The expression strengths of this experiment was determined using Venus YFP96 and the host strain S. cerevisiae CENPK.113-7D for promoter–terminator pair characterization in Fig. 3a. Note that the host strain was OFF; the gate output was lower than its steady-state value, and it would turn on at a rate dictated by the parameter τyss. In the opposite case, when the input was OFF and then turned ON, y would be higher than y0 and would have to turn off, which would do at a rate following the parameter τy0. This response was captured by the following differential equation describing a single gate:

\[
\frac{dy}{dt} = \frac{t^{ON}(y_m - y)}{\tau^{ON}} \text{if } y < y_m \\
= \frac{t^{OFF}(y_m - y)}{\tau^{OFF}} \text{otherwise}
\]

where the relationship between ym and the input x is provided by equation (1). Note that dy/dt describes the rate of change of the RNF flux from the promoter and not the reporter expression and maturation, which is mathematically separated. We developed a fitting procedure to extract the parameters τy0 and τyss using the empirical measurements described in the previous section. First, the dynamic response of the sensor was measured (Extended Data Fig. 5), from which we extracted the response time of the sensor’s output promoter upon addition or removal of the inducer and the rate associated with the expression and folding of the reporter protein. YFP degradation was assumed to be dominated by the cell doubling time. To fit the data for each sensor, the following equations were used:

\[
\frac{dx}{dt} = \tau_x(x_m - x)
\]

\[
\frac{dy}{dt} = \left\{ \begin{array}{ll} \frac{t^{ON}(y_m - y)}{\tau^{ON}} & \text{if } y < y_m \\ \frac{t^{OFF}(y_m - y)}{\tau^{OFF}} & \text{otherwise} \end{array} \right. 
\]

The results of the fitting procedure are shown in Extended Data Figs. 7 and 8 for the parameters for each gate are shown in Supplementary Table 2.

New capabilities and features of Cell 2.0. Cello 2.0 encompasses a suite of features to expand circuit design to new species. This requires flexibility in how gates are defined, how organizational rules are written to map the gates to a DNA sequence, and where the circuit DNA is carried. Collectively, this enables Cello to map a Verilog design to multiple species with minimal user intervention. In addition, Cello 2.0 has been designed to be compatible with the greater Synthetic Biology software development community through compatibility with SynBioHub and SBOL data standards, with the former linking the Cello designs to a process workflow for construction and testing. The graphic user interface has also been modified so that a user can set up a project and store sensor collections and there is improved error reporting. Cello has been modified to use the logic synthesis tool Yosys, which implements the Verilog 2005 specification nearly in its entirety (Institute of Electrical and Electronics Engineers standard 1364-2005) so that the circuit design can be exchanged between different software tools and domains. This extended language support is required for the specification of large circuits, distributing circuits across multiple cells, and for the design of sequential logic.
this work). The user first creates a new project then selects from these UCFs or uploads a new one for their own species.

Gate architecture specification. Cello 1.0 was hardwired for NOR gates with two input promoters in series. The new UCF structure has a JSON gate_structure object type, which allows a user to define a gate architecture based on a collection of part types. This is critical for the S. cerevisiae gate architecture since: (1) two input promoters each drive a copy of the repressor gene with different DNA sequences; (2) the terminator is not part of the gate architecture; and (3) there are yeast-specific translation parts and insulators. Expanding beyond the yeast gates, this approach can be applied to other modes of regulation (for example, CRISPRi and invertsases), any Boolean gate as well as more than two input promoters.

Gate model flexibility. Previously, the response functions were defined to have a single mathematical form and associated fit parameters. However, different gate architectures and different classes of regulators require alternative models. To accommodate this, we created a new JSON object type gate_model that has two fields: function and parameters. The function identifier refers to a function (for example, for S. cerevisiae, it is Hill_function, defined as \((x, y) \to \left(\frac{x^n}{x^n + y^n}\right)\)), as well as each parameter (\(\text{name}\): \(\text{map}\): \('#'/model/parameters/y \alpha \beta'), where 'map' is a pointer to a numerical value. Variables are also defined (\(\text{name}\): \(\text{map}\): \('#'/model/functions/input_composition'), where 'map' points to a function that, in the simplest case, sums the RPU activity of both gate inputs, as for S. cerevisiae.

Expansion of rule sets for DNA mapping. Cello uses rules, defined in the UCF, to map the circuit design to a linear DNA sequence (for example, the order and orientation of repressor genes on a plasmid). These rules were expanded to encompass the need for gate designs in new species and for their encoding in the genome. A new object type, genetic_locations, defines the host genome, either by a link to a sequence in National Center for Biotechnology Information or by completely embedding the host genome sequence in the UCF, as well as the insert locations that can be referenced symbolically in the Eugene rule sets. Each genetic locus available for circuit inserts is assigned a symbol (for example, L1, L2 and so on). For S. cerevisiae, the gates can occur in 16 positions in two chromosomal locations. Rules in the UCF constrain not only the chromosomal location of each gate but also the relative positions of gates within a locus. The S. cerevisiae gates are separated by defined scars that are used to control gate placement; for example, in the rule 'P1_C1434_b AFTER Kesa AND P1_C1434_b BEFORE Oscar.' The S. cerevisiae NOR gates comprise two expression cassettes based on two copies of the repressor. The expression cassettes are intended to be separated into two different locations in the genome. For every gate, there is a rule such as OR[(AND ['GateX_a BEFORE L', 'GateX_b AFTER L2'], OR['GateX_a AFTER L', 'GateX_b BEFORE L'])], indicating that an expression cassette can be in either location, as long as it is not the location of the gate's other cassette. The parsing of these rules was also modified to make it easier for a user to compose a rule set that is interpretable by Cello.

UCF. The S. cerevisiae UCF (SC1C1G1T1) is provided in the Supplementary Data 1. The UCF contains the gate technology and associated data (response functions, including cytometry distributions, and OD\(\delta_{\text{in}}\) measurements). It also defines the strain, the genetic location of the circuit and the growth conditions for which the circuit design is valid. SC1C1G1T1 is based on S. cerevisiae strain BY4741 and the location of the circuits is inura3:: (IIA,Ch1V) and lexA:: (IIB,ChIIII) (Fig. 1). For each gate, the repressor has two versions of sequences in different codons to maximize potential homolog sequence, and the targeting promoter also has a mutated version for the same reason. Eugene\(\delta_{\text{in}}\) rules are also included to specify the organization of gates into a linear DNA sequence. This UCF uses the following layout rules for gates and type II cloning scars. Multiple gates that use the same repressor and different targeting promoters are prohibited from appearing in the same circuit. Cloning scars appear in the order A, B, C, D, E, F, G, H, I (IIA,Ch1V) and J, K, L, M, N, P, Q, R, S (IIB,ChIII) (Supplementary Figs. 14 and 17).

Computational circuit design. The Cello 2.0 software was used to design the circuit DNA sequences (note that the UCF will not work with Cello 1.0). The toxicity cut-off is 0.7 for gates and 0.5 for the complete circuit. Each circuit was specified as a Verilog text file (Supplementary Note 1). All circuits were specified as truth tables and Cello 2.0 identified the wiring diagram using logic minimization. The SC1C1G1T1 UCF was used for the calculations and is provided as Supplementary Data 1. Data for the sensors are also provided (Supplementary Table 1). The output of Cello 2.0 includes DNA sequences for the circuit, which were constructed as specified, and predictions for cytometry fluorescence data and the impact on growth.

Circuit long-term growth and induction experiments. Colonies were picked into 500 µl SD medium (with auxotrophic selection) and inducers (initial states, if required) in 2-ml 96-deep-well plates (Plate One; 1896-2000) sealed with AeraSeal film (Excel Scientific; BS25). The plates were incubated at 30°C and 900 r.p.m. in a Multitron shaker incubator for 16 h. Aliquots of 500 µl of the culture at OD\(\delta_{\text{in}}\)=1.0 were taken and spun down by centrifugation at 4°C and 5000 for 5 min. After removing the supernatants, the cell pellet was resuspended in 500 µl fresh SD-glucose media (with final state inducers) and then 1:20 diluted to 500 µl fresh SD-glucose media (with final state inducers) at OD\(\delta_{\text{in}}\)=0.005 (Synergy H1 plate reader; BioTek) in 2-ml 96-deep-well plates sealed with AeraSeal film. The plates were incubated at 30°C and 900 r.p.m. in a Multitron shaker incubator. The same dilution process (without the spin-down step) was repeated every 12 h for continuous exponential growth. The cells were collected every 3 h for flow cytometer measurement. For the dynamic experiments performed over 400 h, cells were resuspended and diluted to OD\(\delta_{\text{in}}\)=0.005 (Synergy H1 plate reader; BioTek) in fresh SD-glucose media with inducers every 24 h. The fluorescence was measured by flow cytometry at 16 h after dilution.

Simulation of circuit dynamics. The RPU outputs for all gates under initial conditions were first obtained from Cello 2.0. Then, we performed a numerical analysis from time 0 using the ODEs for the circuit. For example, the equations for the 0x06 circuit are shown below:

For sensors

\[
\frac{d R}{dt} = \begin{cases} \frac{\gamma_{on} \left( x_{\text{Rup}} - x_{\text{Roff}} \right)}{\gamma_{on} + x_{\text{Rup}} - x_{\text{Roff}}} & \text{if } x_{\text{Rup}} < x_{\text{Roff}} \\ \frac{\gamma_{off} \left( x_{\text{Rup}} - x_{\text{Roff}} \right)}{\gamma_{off} + x_{\text{Rup}} - x_{\text{Roff}}} & \text{otherwise} \end{cases}
\]

For gates

\[
\frac{d y}{dt} = \begin{cases} \frac{\gamma_{on} \left( x_{\text{Rup}} - x_{\text{Roff}} \right)}{\gamma_{on} + x_{\text{Rup}} - x_{\text{Roff}}} & \text{if } y_{\text{Rup}} < y_{\text{Roff}} \\ \frac{\gamma_{off} \left( x_{\text{Rup}} - x_{\text{Roff}} \right)}{\gamma_{off} + x_{\text{Rup}} - x_{\text{Roff}}} & \text{otherwise} \end{cases}
\]

\[
\frac{d x}{dt} = \begin{cases} \frac{\gamma_{on} \left( x_{\text{Rup}} - x_{\text{Roff}} \right)}{\gamma_{on} + x_{\text{Rup}} - x_{\text{Roff}}} & \text{if } x_{\text{Rup}} < x_{\text{Roff}} \\ \frac{\gamma_{off} \left( x_{\text{Rup}} - x_{\text{Roff}} \right)}{\gamma_{off} + x_{\text{Rup}} - x_{\text{Roff}}} & \text{otherwise} \end{cases}
\]

\[
\frac{d y_{\text{diff}}}{dt} = \begin{cases} \frac{\gamma_{on} \left( x_{\text{Rup}} - x_{\text{Roff}} \right)}{\gamma_{on} + x_{\text{Rup}} - x_{\text{Roff}}} & \text{if } x_{\text{Rup}} < x_{\text{Roff}} \\ \frac{\gamma_{off} \left( x_{\text{Rup}} - x_{\text{Roff}} \right)}{\gamma_{off} + x_{\text{Rup}} - x_{\text{Roff}}} & \text{otherwise} \end{cases}
\]

\[
\frac{d n}{dt} = \begin{cases} \frac{\gamma_{on} \left( x_{\text{Rup}} - x_{\text{Roff}} \right)}{\gamma_{on} + x_{\text{Rup}} - x_{\text{Roff}}} & \text{if } x_{\text{Rup}} < x_{\text{Roff}} \\ \frac{\gamma_{off} \left( x_{\text{Rup}} - x_{\text{Roff}} \right)}{\gamma_{off} + x_{\text{Rup}} - x_{\text{Roff}}} & \text{otherwise} \end{cases}
\]
where \( y/x \) is the output for gate/sensor i and \( y_i/x_i \) is the steady-state output (both in RPU). The term \( f(y, y') = y + y \) captures the input into each gate, in this case a simple sum of the input promoter activities. The equations were solved by MATLAB scripts, using the ODE solver ODE15S. All scripts are available on GitHub (https://github.com/VoigtLab).

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

Genetic part sequences and the UCF file SCIC1G1T1.ucf are available in Supplementary Data 1. The data that support the findings of this study are available from the corresponding author upon reasonable request. Plasmids and cloning strains generated in this study will be available from Addgene (https://www.addgene.org/Christopher_Voigt/) and the corresponding author upon request. Source data are provided with this paper.

Code availability

The Cello 2.0 software and code are freely available at http://www.cellocad.org/ and https://github.com/CIDARLAB/Cello-v2.

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Extended Data Fig. 1 | Optimization steps for building the TetR-responsive minimal promoter. The annotated promoter sequences are provided in Supplementary Table 5, including intermediates, and all part sequences are provided in Supplementary Table 10. The promoters are evaluated through the transcriptional fusion with yfp and this cassette is carried on a plasmid (Supplementary Figure 15). The data are measured under identical experimental conditions (Methods). In the top graph, the carbon source is either 2% glucose or 2% galactose. The star indicated that there is no fluorescence detected over background. When TetR<sup>R</sup> is expressed from a second plasmid (Supplementary Figure 15), this is indicated by “+TetR<sup>R</sup> cassette”). The red arrows show the data corresponding to the promoter selected for the next round of optimization. The horizontal orange lines mark where mutations are made in order to diversify the part sequence to avoid homologous recombination in the context of a circuit. The data represent the average of three experiments performed on different days. The optimizations steps for other promoters are shown in Supplementary Figs. 2-12.
Extended Data Fig. 2 | Promoter designs with varying lacO5 operator spacing. The sequences of genetic parts are provided in Supplementary Table 5. The UAS binds to Gal4 and the TSS is a 20bp sequence from ADH2 (Extended Data Figure 1). The promoters are transcriptionally fused to yfp and cloned into a plasmid backbone (Supplementary Figure 15). A second plasmid is constructed where LacI is expressed from a constitutive promoter (Supplementary Figure 15). The cytometry distributions show the fluorescence from the reporter plasmid in the absence (black) and presence (grey) of the LacI plasmid and the reported “Repression” is the ratio of the medians of these distributions. The data represent the average of three experiments performed on different days.
Extended Data Fig. 3 | Upstream insulator impact on an inducible promoter. The fluorescence values are shown when $P_{tet}$ is maximally induced (100 ng/ml). Sb is a 450bp nonfunctional DNA sequence. When there is no insulator (none)-Sb, then the maximum expression is lower. The horizontal dashed line is the average of the fluorescence measurements from the strains containing an insulator. The strains are described in Supplementary Table 4 and insulator sequences are provided in Supplementary Table 5. The data represent the average of three experiments performed on different days.
Extended Data Fig. 4 | Cytometry distribution for the RPU reference standard. The cytometry distributions show the fluorescence from the strain containing the RPU standard (S. cerevisiae CY671int) (grey), S. cerevisiae BY4741 cells (white) and a reporter strain containing the strong native promoter P_{Tdh3} (S. cerevisiae CY676int) (black). Detailed strain information is provided in Supplementary Tables 3 and 8. Three experiments were repeated on different days with similar results.
Extended Data Fig. 5 | Sensor cytometry distributions and sensor dynamics. These data correspond to Fig. 4d and Supplementary Table 1. The detailed schematic for the reporter of each sensor shown to the left and the corresponding strains are provided in Supplementary Table 4. The sequences for the genetic parts are provided in Supplementary Tables 4 and 8. The experimental conditions are as described in the Methods. Two representative cytometry distributions are shown when cells containing the sensors are grown in (0 or 100 ng/ml) aTc, (0 or 20 mM) IPTG, or (0 or 10 mM) xylose. The dynamic data was measured as described in the Methods and fit to Equations 4 and 5. The data points combine two experiments performed on different days.
Extended Data Fig. 6 | Converting the x-axis of a NOT gate response function to RPU. **a,** An example is shown to demonstrate how data gathered using two strains are combined to create a NOT gate response function. The PhlF gate is strain *S. cerevisiae* CY960-CY663int and the IPTG sensor is strain *S. cerevisiae* CY639int (Supplementary Table 4). Both strains are evaluated by adding different concentrations of IPTG (left to right): 0.0, 0.1, 0.2, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 5.0, 10 and 20 mM. The growth conditions, cytometry measurements, and normalization to RPU are described in the Methods. The graph to the right is built using the RPU measurements for the input promoter (Plac) and output promoter (PphlF.1) at each inducer concentration. The data are then fit to Equation 2, the parameters for which are presented in Supplementary Table 2. **b,** Growth impacts are processed similarly so that the x-axis can be reported as promoter activity and not inducer concentration. As an example, the growth impact of the PhlF gate (*S. cerevisiae* CY1085-CY663int) is shown along with the strain used to determine the activity of the (Ptet + Plac) promoters. The strains were induced with 0, 1, 3, 5, 7.5, 10, 20 mM IPTG (0 ng/ml aTc) or 1, 2.5, 5, and 20 mM IPTG (100 ng/ml aTc). The data represent three experiments performed on different days.
Extended Data Fig. 7 | Detailed characterization of the NOT gates. These data correspond to Fig. 5b and Supplementary Table 2. The detailed schematic for the gate is shown to the left and the corresponding strains are provided in Supplementary Table 4. The sequences for the genetic parts are provided in Supplementary Tables 4 and 8. The data used to fit the response functions (Equation 2) were calculated as described in Extended Data Figure 6 and the resulting parameters are provided in Supplementary Table 2. The data represent three experiments performed on different days. The experimental conditions are as described in the Methods. Two representative cytometry distributions are shown when cells containing the gates are grown in 0 or 20 mM IPTG. The dynamic data was measured as described in the Methods and fit to Equations 6, 7 and 8. The data represent two experiments performed on different days.
Extended Data Fig. 8 | Detailed characterization of the NOT gates. These data correspond to Fig. 5b and Supplementary Table 2. The detailed schematic for the gate is shown to the left and the corresponding strains are provided in Supplementary Table 4. The sequences for the genetic parts are provided in Supplementary Tables 4 and 8. The data used to fit the response functions (Equation 2) were calculated as described in Extended Data Figure 6 and the resulting parameters are provided in Supplementary Table 2. The data represent three experiments performed on different days. The experimental conditions are as described in the Methods. Two representative cytometry distributions are shown when cells containing the gates are grown in 0 or 20 mM IPTG. The dynamic data was measured as described in the Methods and fit to Equations 6, 7 and 8. The data represent two experiments performed on different days.
Extended Data Fig. 9 | The failure of the 0x61 circuit. The logic diagram is shown with gate colors corresponding to the assigned repressor. The response of the circuit is shown for different combinations of inducer: 100ng/ml aTc, 10mM xylose, and 20mM IPTG. The experimental data are shown as cytometry distributions (black) and blue/red distributions show the ON/OFF output predicted by Cello. The states behave as predicted, except for the -/+/+ state, which should be OFF but is measured as being ON. To determine where this breakage originates the output promoters of intermediate gates are fused to rfp and inserted at the HO locus. The measured responses are then compared to those predicted. The population variability in the response of the CI gate, which causes errors that propagate to the final BM3R1 gate. The DNA sequence of the circuit is provided in Supplementary Table 11, the strains are provided in Supplementary Table 4, and the reporter constructs in Supplementary Table 4. Three experiments were repeated on different days with similar results.
Extended Data Fig. 10 | The impact on growth from carrying the circuits in different states. Each point represents a different combination of inducers for a given circuit. The prediction is made using Cello and is calculated by multiplying the empirically-measured growth impact of all the gates with input promoter activities corresponding to that state [7]. Details regarding the growth assay are presented in the Methods and normalized to S. cerevisiae BY4741 in the same growth conditions. The data represent three experiments performed on different days.
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- BD FACS DIVA software was used to collect all of the cytometry data in this study. A multi-mode microplate reader (Biotek, Synergy 526 H1) was used for the data collection.
- FlowJo version 10.4.1 (TreeStar Inc.) was used to analyze the cytometry data. MATLAB R2018b were used for simulating dynamics. MATLAB scripts were available at https://github.com/VoigtLab. Cello2.0 was available at cellocad.org. Cello2.0 codes were available at https://github.com/CIDARLAB/Cello-v2.

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Genetic parts and the UCF file SC1C1G1T1.UCF are available as Supplementary Information. Source data for Figs. 2–7 are in this article. The data that support the findings of this study are available from the corresponding author upon reasonable request. Plasmids and cloning strains generated in this study will be available on Addgene (https://www.addgene.org/Christopher_Voigt/) and the corresponding author on request.
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#### Flow Cytometry

- Plots:
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  - All plots are contour plots with outliers or pseudocolor plots.
  - A numerical value for number of cells or percentage (with statistics) is provided.
Methodology

Sample preparation: Yeast cells were diluted in either PBS with cycloheximide.

Instrument: BD LSR II Fortessa running based on FACSDIVA software.

Software: FACSDiva software for collection; FlowJo for data analysis.

Cell population abundance: Typical samples contained 20,000 or more cells.

Gating strategy: Cells in mid-log phase were gated as >500 SSA and >500 FSA to remove cellular debris. We also gated for un-budded cells by SSW and FSW.

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