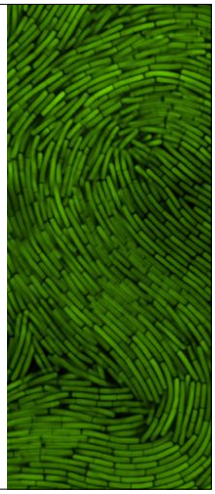
Genomics, Epigenetics & Synthetic Biology

Lecture 3:
Engineered logic and the control of gene expression.



Jim Haseloff
www.haseloff-lab.org (Education)

1

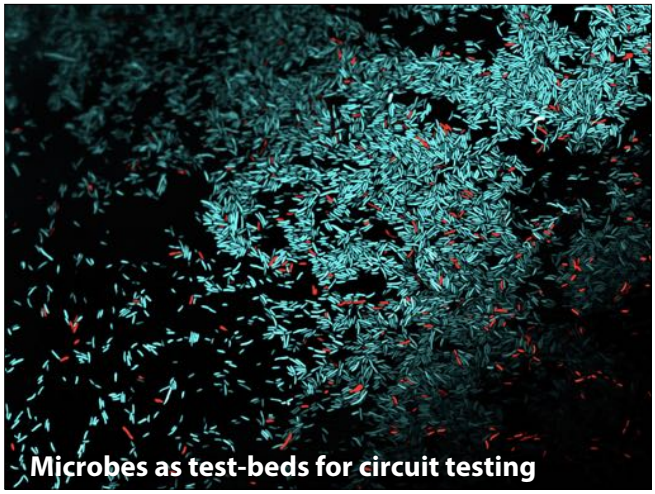
Applications of Synthetic Biology

Lecture 3
Cell autonomous genetic circuits with self-regulating properties
e.g. microbial engineering,
enviromental and biomedical sensors
engineering novel metabolic pathways

Lecture 4
Morphogenetic circuits with self organising properties
e.g. microbial biofilms or self-organising communities for
bioremediation and bio catalysis
engineering novel plant varieties for bioproduction

2

Genetic circuits can be divided into two classes. The first consists of circuits which act cell autonomously. These include regulatory circuits that might act as sensors, or metabolic circuits that affect local cell properties. The second includes circuits that allow communication between cells and might have self organising properties.



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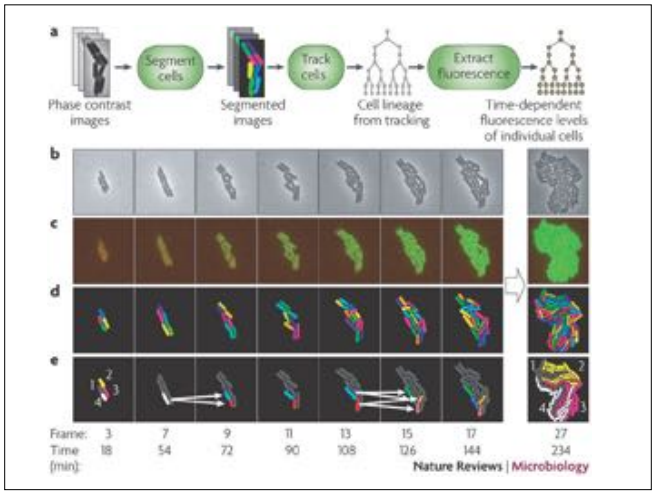
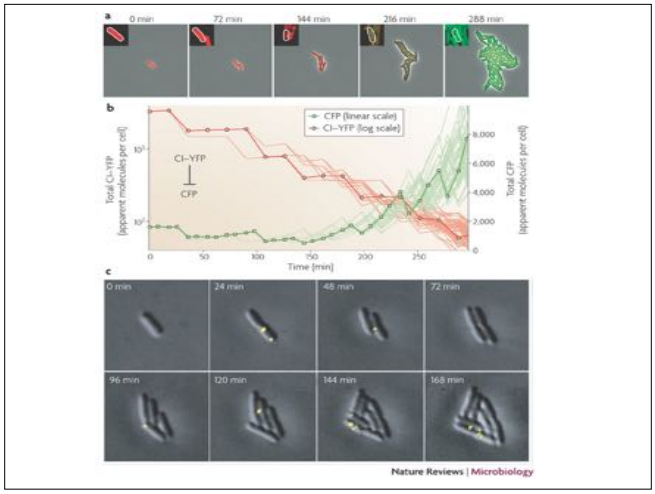
The first experiments in synthetic biology have focused on the use of microbes and in vitro expression systems - a simple and facile testbeds for testing new circuits.



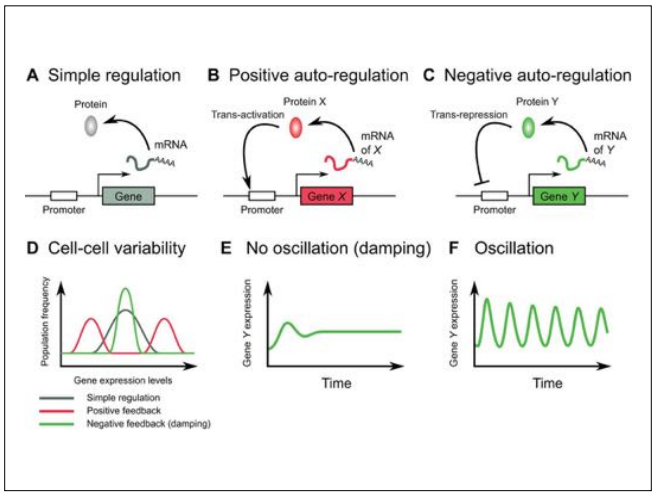
4

Quantitative imaging allows tracking and measurement of individual cells. **a.** Schematic of data flow for a cell tracking and segmentation system. During tracking, cell shapes must first be identified in images (segmentation) and then tracked over time. Finally, the fluorescence values must be extracted. **b**–**e.** Segmentation and tracking input and output. **b.** Phase contrast images over time. **c.** Fluorescence images of the micro-colony. In this example, filters for yellow and cyan fluorescent proteins were used (shown in red and green, respectively). **d.** Segmentation was performed on the phase contrast images to determine the locations of each cell. Arbitrary colours were used for labelling. **e.** Shows the descendents of cell 4. The panel on the far right shows the descendants of each of the four initial cells after approximately four generations.



5

Visualising gene expression in individual cells. **a**,**b.** Measuring the gene regulation function of a repressor–promoter interaction in individual *Escherichia coli* cell lineages. Here, CI–YFP (lambda repressor fused to yellow fluorescent protein) represses expression of cyan fluorescent protein (CFP). In the regulator dilution experiment, cells are transiently induced to express CI–YFP and are then observed using time-lapse microscopy as this repressor dilutes out during cell growth. **c.** Monitoring transcriptional bursts in single cells. Frames from film footage of the expression of a membrane localised fluorescent protein under the control of a repressed *lac* promoter (yellow) and is overlaid with simultaneous DIC (differential interference contrast) images (grey). Note the burst-like expression pattern.



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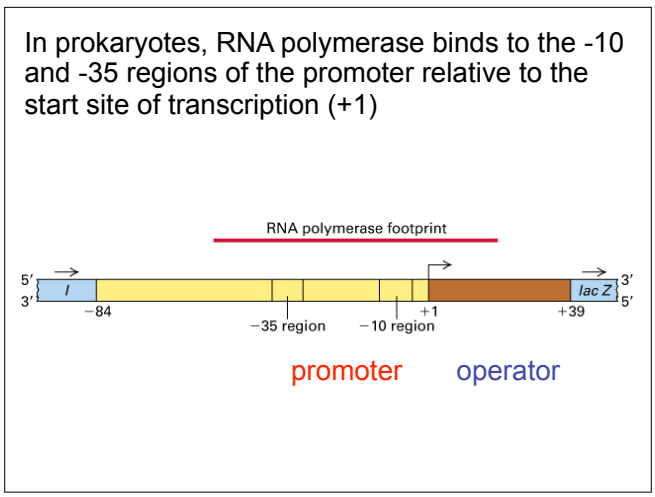
Regulatory elements can be programmed into DNA to produce circuits capable of a wide range of behaviours. These include simple gene expression or autoregulation to induce or repress gene activity. These basic regulatory activities can be combined to control cell to cell variability, induce gene expression and create oscillatory behaviour.



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The building blocks for the circuits come from components of normal microbial gene expression. Transcriptional control plays a major part in regulation of bacterial gene expression. Recruitment of RNA polymerase is regulated by sequences adjacent to the start site for transcription.

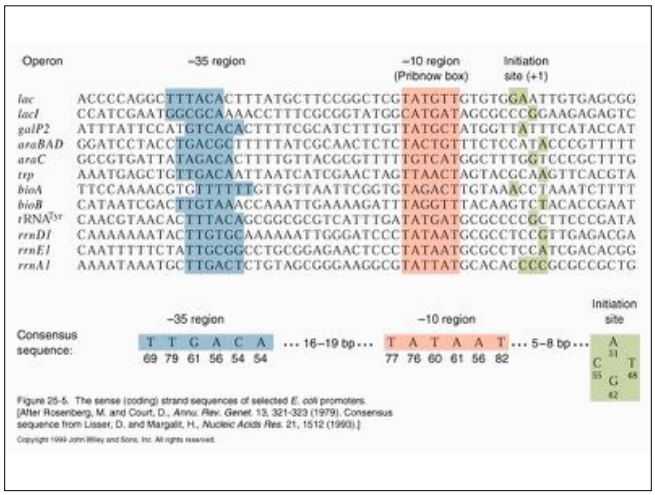
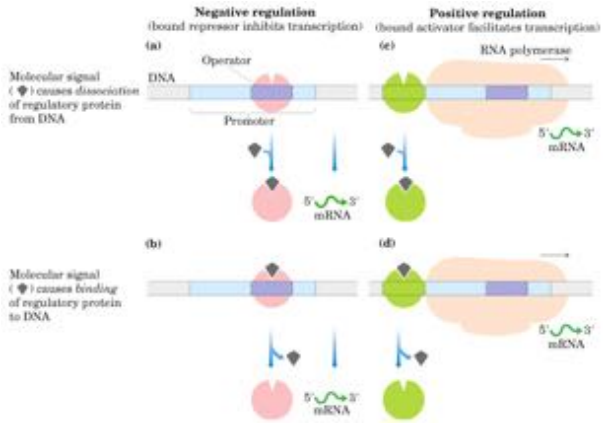


Figure 28-5. The sense (coding) strand sequences of selected *E. coli* promoters. [After Rosenberg, M. and Court, D., *Annu. Rev. Genet.* 13, 321-323 (1979). Consensus sequence from Lisser, D. and Margalit, H., *Nucleic Acids Res.* 21, 1512 (1993).]

8

These five prime proximal sequences share conserved domains around 35 and 10 nucleotides upstream of the initiation site for RNA synthesis.

Regulation of bacterial gene expression



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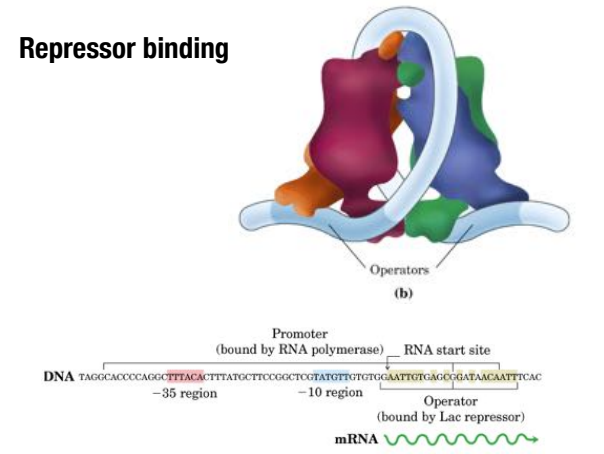
Regulatory proteins can either inhibit binding or recruit the RNA polymerase to the promoter sequence. These repressor or activator proteins bind to what are termed operator sequences. The addition of an operator to a promoter will confer ectopic regulation to that gene.

Repressor binding



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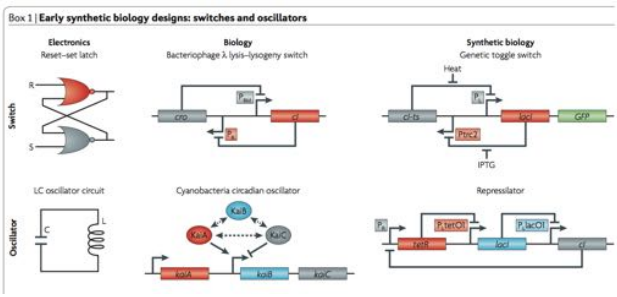
Lac repressor proteins binds as a dimer to adjacent inverted sequences in an operator. The resulting Lac dimer is capable of binding to another dimer. The presence of a second operator in a gene will allow the formation of a DNA loop structure, and tight repression of gene expression.



2 examples: genetic switches and oscillators

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These types of regulatory regions can be used to create synthetic circuits composed of DNA. We will look at two examples. The first shows switch-like behaviour, and second shows oscillatory behaviour. These synthetic circuits have counterparts both in electronic logic circuits and in natural biological circuits.

Construction of a genetic toggle switch in *Escherichia coli*

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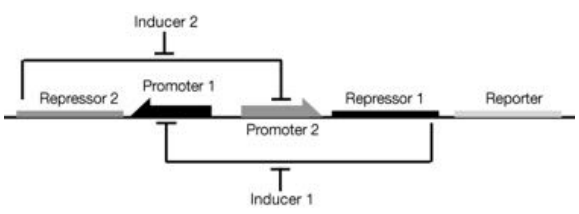
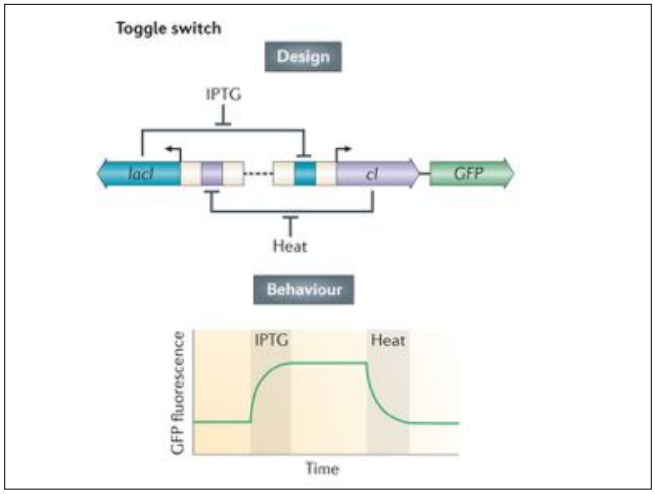
** Department of Biomedical Engineering, † Center for BioDynamics and ‡ Center for Advanced Biotechnology, Boston University, 44 Cummington Street, Boston, Massachusetts 02215, USA*



12

A simple switch can be built from two repressor genes, which are each configured to repress the expression of the other. In this circuit, small molecule inducers are used to regulate the behaviour of each repressor. For example, the presence of inducer 1 will allow expression from promoter 1 therefore accumulation of the repressor 2 protein - and therefore production of repressor 1 will be stably inhibited - even in the absence of the inducer. If inducer 2 is present, repressor 1 will be produced, and this will maintain a repressed state for repressor 2. In other words there are two metastable states, and gene expression can toggle between either.



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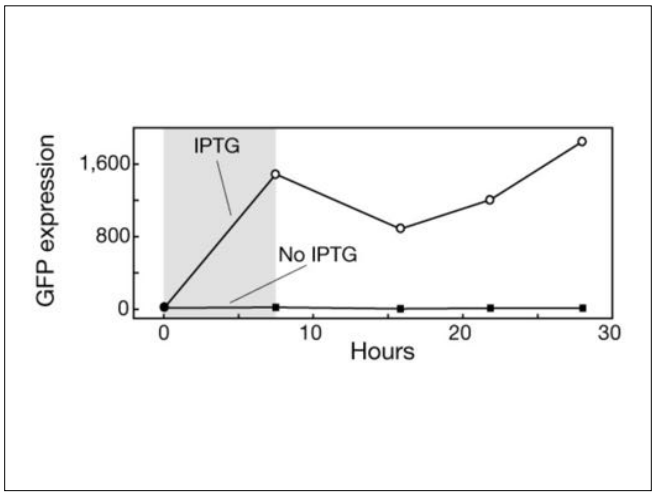
Schematic diagram of a genetic toggle switch built with genes for the lambda repressor (cI) and Lac repressor (lacI). Inducibility is provided by IPTG for the Lac repressor, and use of a temperature sensitive variant of cI. The state of the genetic switch is indicated by expression of a fluorescent protein linked to expression of the Lambda repressor. This is either on or off.



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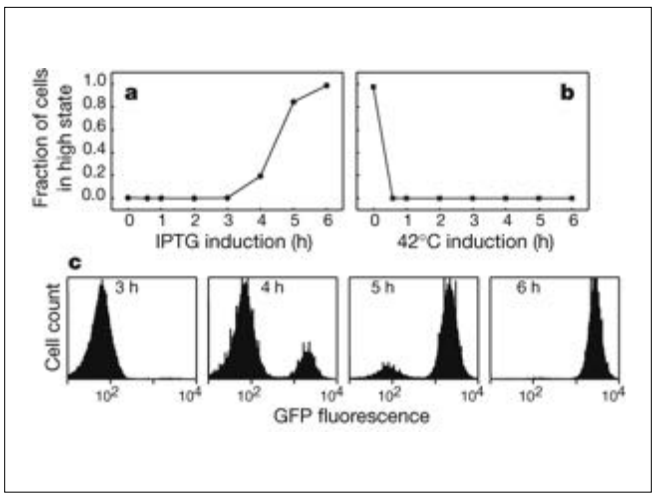
Exposure to IPTG inhibits the activity of the Lac repressor, and triggers expression of the Lambda repressor and GFP.



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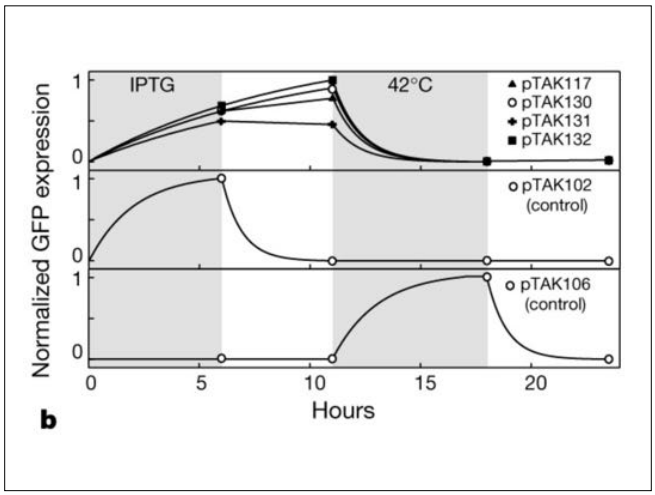
Similarly, exposure to heat-shock causes loss of lambda repressor function, switches on the Lac repressor, and switches off GFP expression.



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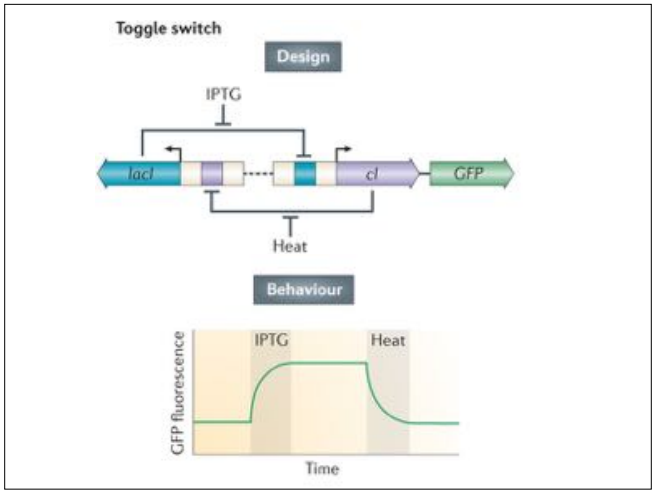
Once switched, the circuit retains its existing state until exposed to the opposite inducer. Hence it behaves like a conventional light switch.



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In the toggle switch circuit we have two repressor elements that are mutually antagonistic. What happens when we add repressor elements to this circuit?

The Repressilator

- Cellular clocks oscillate with defined periods
  - –Circadian clocks oscillate with 24-hour period
- Elowitz and Leibler set out to build oscillator with components not found in cellular clocks
- Used three transcription factors in mutual-repression network
  - –LacI
  - –TetR
  - –cI from lambda phage
- Readout: GFP controlled by Tet repressor

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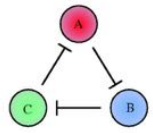
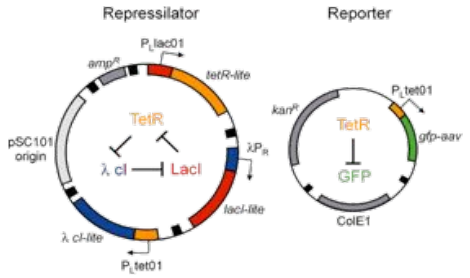
Elowitz and Leibler decided to try to build a synthetic oscillator without using any of the known components of existing clocks. They chose three well-characterized transcription factors: the lac repressor, which prevents transcription from the lac operon in *E. coli* bacteria, the Tet repressor that prevents transcription from the Tet operon, and the cI repressor acts in lambda phage to regulate the choice between the lytic and lysogenic phases. These three genes were organized in a mutual-repression network. As a readout for the oscillator, a TetR regulated GFP (green fluorescent protein) was used.

Repressilator design





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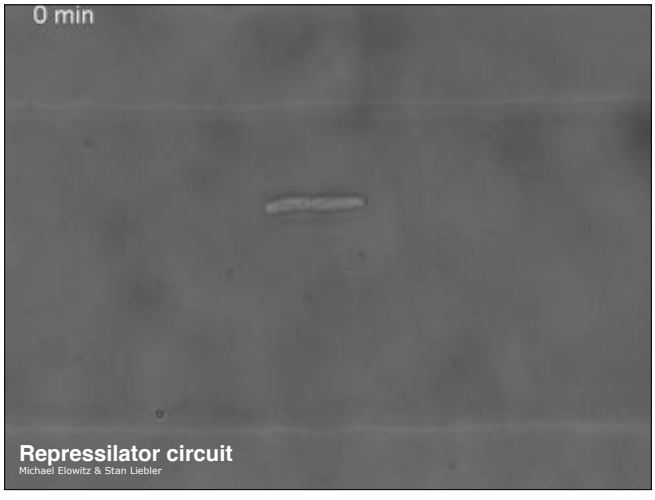
Each of the transcription factors acts to repress transcription from another transcription-factor gene: TetR represses lambda cI, lambda cI represses LacI, and LacI represses TetR. As the concentration of TetR increases, it shuts down expression of lambda cI, which reduces the concentration of the lambda cI repressor in the cell, which allows LacI expression to increase. However, the resulting increase in LacI repressor acts to shut down TetR expression, which in turn causes an increase in lambda cI, which represses LacI, etc. Thus, the system oscillates. To visualize the oscillatory behavior of the network, the investigators used GFP controlled by TetR. This construct was carried on a separate plasmid. To reduce the period of oscillation of the network, the repressor proteins were genetically destabilised.



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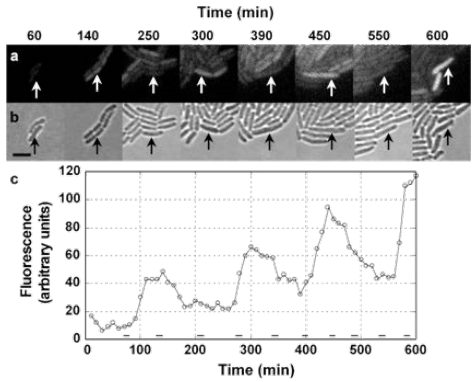
Visualisation of the repressilator circuit in action in individual cells. This first version of the repressilator showed poor synchronisation as the period and amplitude of the oscillations were susceptible to variation due to noise during cell proliferation.

Activity of repressilator



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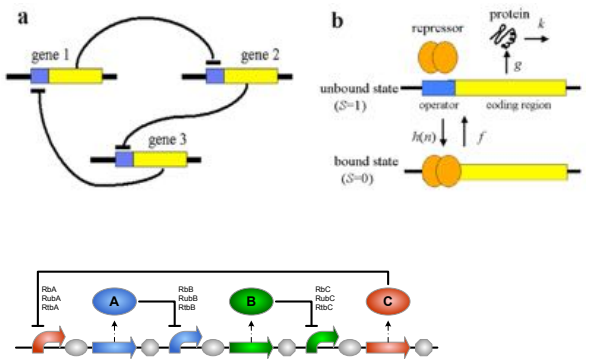
Oscillatory behavior of the repressilator. In the upper panel, a single *E. coli* bacterial cell is followed as it divides to form a microcolony. In the lower portion of this panel are bright-field images showing the increase in the number of bacteria. The upper part of the panel shows the GFP fluorescence readout of the repressilator, which has a period of approximately 150 minutes. Thus, at 60 minutes there is a low point of activity, at 140 minutes a peak of fluorescence, at 250 minutes another trough, at 300 minutes another peak, etc. In the graph in the lower panel, the level of fluorescence is plotted against time. The small bars at the bottom of the graph show the times when the bacteria were dividing. The period of the oscillations of the repressilator do not correspond to the bacteria's division cycle. Thus, the increases and decreases of the proteins involved in repressilator function continue independently of the division cycle of the bacteria.



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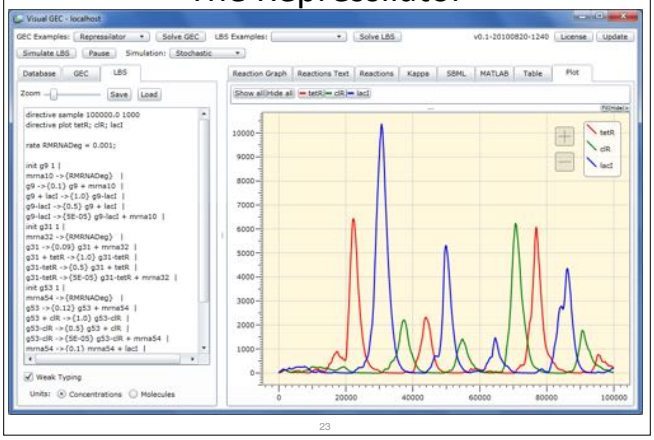
prom<neg(A,RbB,RubB,RtbB)>; rbs; pcr<codes(B)>; ter;
prom<neg(B,RbC,RubC,RtbC)>; rbs; pcr<codes(C)>; ter

| 0.4 < RubB | RubB < 0.6
| 0.4 < RubC | RubC < 0.6
| 0.4 < RubA | RubA < 0.6

22

The parameters associated with gene production rates, protein properties and DNA affinities can be used to create simple continuous models for behaviour of the repressilator circuit.

The Repressilator



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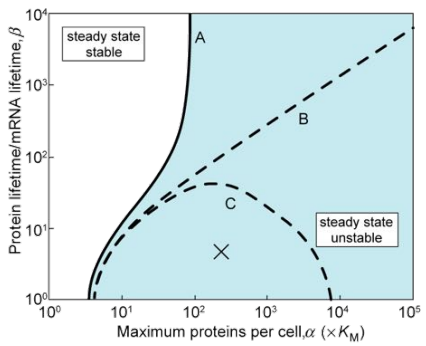
Computer models used to predict the behaviour of the circuit, and have also been used to improve the circuit.

Network behavior



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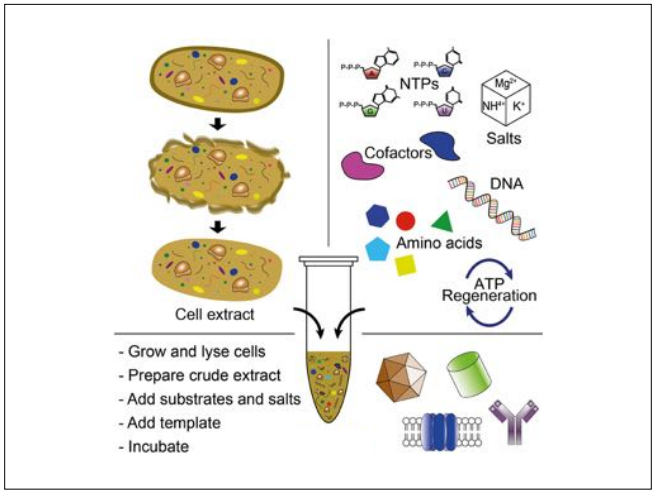
Network behavior is dependent on the parameters associated with each of the components of the network. In the case of the repressilator, the critical parameters include the stability of the proteins and mRNAs of the three repressors, as well as the maximum number of proteins per cell. Stability diagram for a continuous symmetric repressilator model is shown. The parameter space is divided into two regions in which the steady state is stable (top left) or unstable (bottom right). Curves A, B and C mark the boundaries between the two regions for different parameter values, corresponding to the strength of promoter leakiness and repressor binding. The unstable region (A), which includes unstable regions (B) and (C), is shaded.



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Cell-free extracts of *E. coli* have been used to characterise circuits in vitro. The cell-free extracts contain transcription and translation components from the bacterium, to which are added nucleotides amino acids salts and an energy production system. The extracts can then be programmed by added DNA.

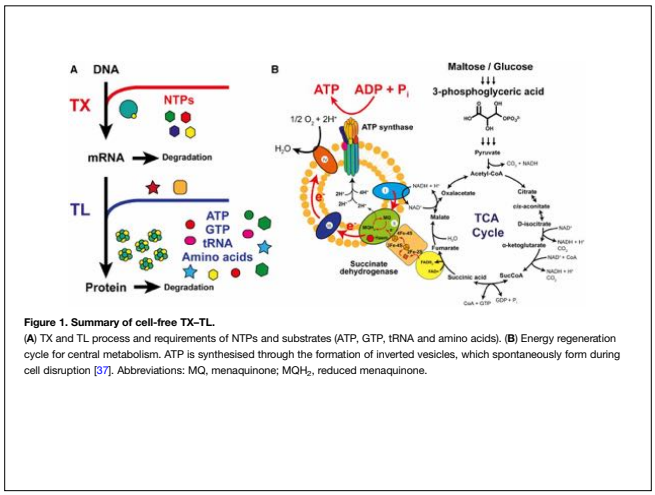

Figure 1. Summary of cell-free TX–TL.
(**A**) TX and TL process and requirements of NTPs and substrates (ATP, GTP, tRNA and amino acids). (**B**) Energy regeneration cycle for central metabolism. ATP is synthesised through the formation of inverted vesicles, which spontaneously form during cell disruption [37]. Abbreviations: MQ, menaquinone; MQH_2, reduced menaquinone.

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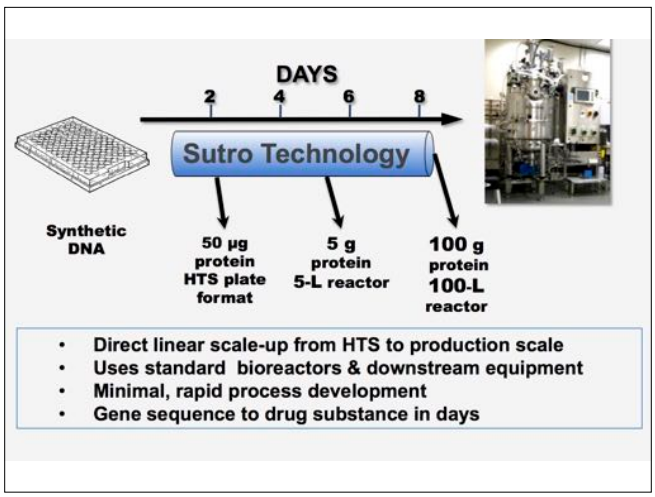
The extracts contain inverted vesicles derived from the plasma membrane. These act to sustain energy production in the extract by oxidative phosphorylation.



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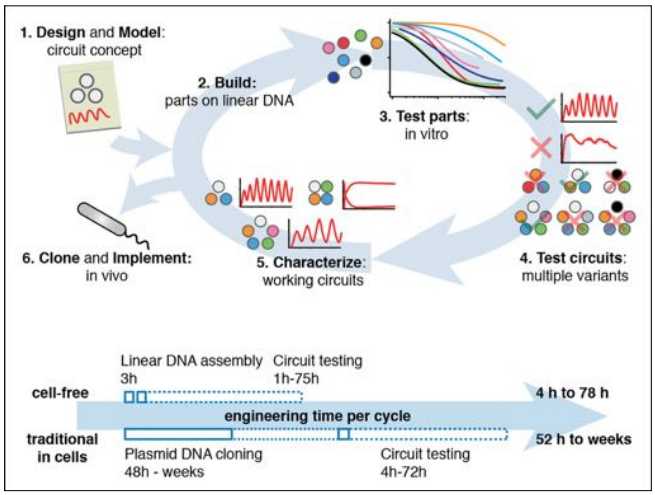
The extracts can be expanded to even the 100 L scale for *in vitro* production of DNA encoded synthetic proteins…



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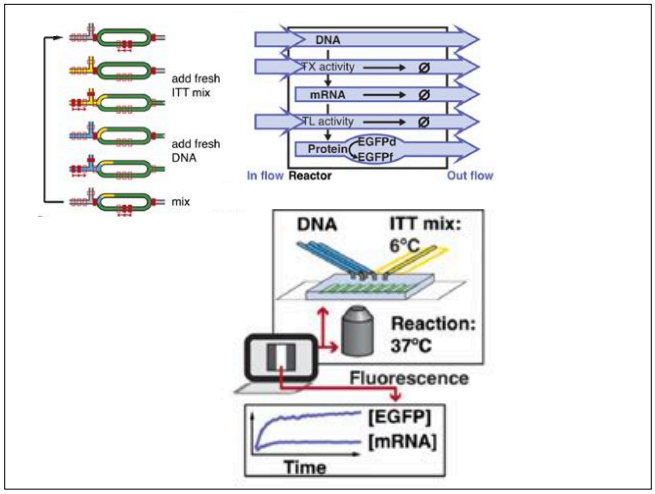
…and can be used to accelerate the testing of circuit variants at small-scale. *In vitro* cell extracts have been used to test and improve variants of the repressilator circuit.



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In work from the Maerkl Lab, has exploited use of microfluidic containers to feed reactants into a central chamber where repressilator circuit was active. Valves and pumps used to mimic the process of DNA replication and cytoplasmic dilution through cell division.

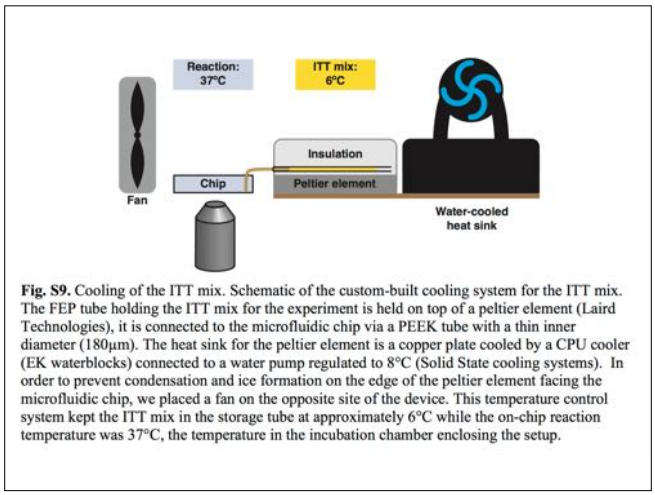

Fig. S9. Cooling of the ITT mix. Schematic of the custom-built cooling system for the ITT mix. The FEP tube holding the ITT mix for the experiment is held on top of a peltier element (Laird Technologies), it is connected to the microfluidic chip via a PEEK tube with a thin inner diameter (180µm). The heat sink for the peltier element is a copper plate cooled by a CPU cooler (EK waterblocks) connected to a water pump regulated to 8°C (Solid State cooling systems). In order to prevent condensation and ice formation on the edge of the peltier element facing the microfluidic chip, we placed a fan on the opposite site of the device. This temperature control system kept the ITT mix in the storage tube at approximately 6°C while the on-chip reaction temperature was 37°C, the temperature in the incubation chamber enclosing the setup.

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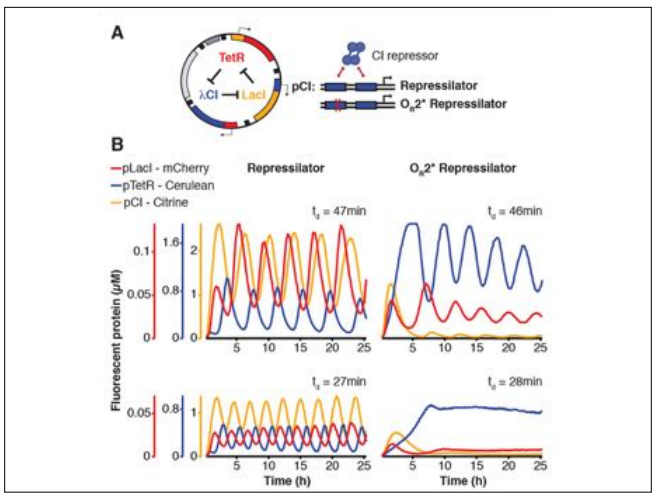
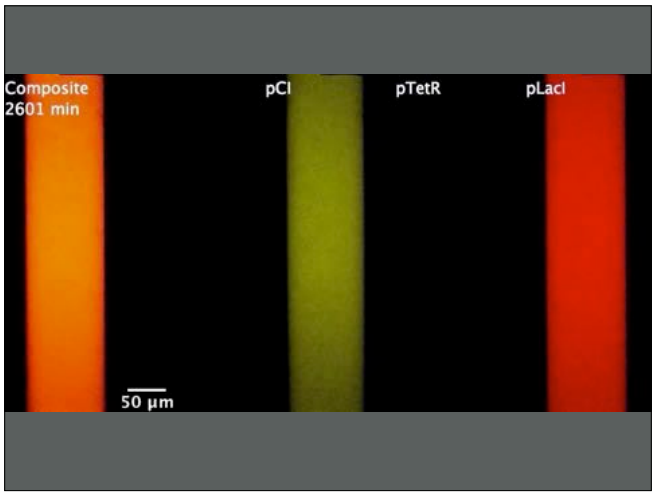
Reactants were warmed and fed into the reaction chamber for direct observation using microscope optics.



31

It was then relatively simple to adjust the reaction conditions and DNA templates to systematically explore the behaviour of many circuit variants and conditions. Here showing the behaviour of the repressilator when different strength operators were used. The biochemical observations and parameter measurements were paired with the use of mathematical models for the circuit.



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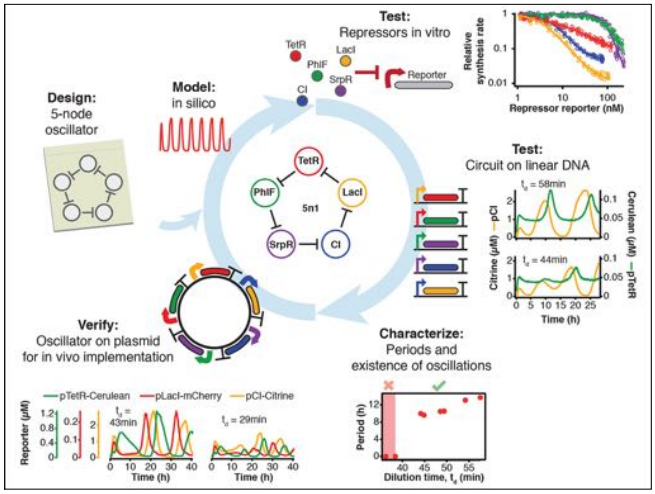
An example of improved repressilator activities, here shown with multispectral fluorescent protein outputs.



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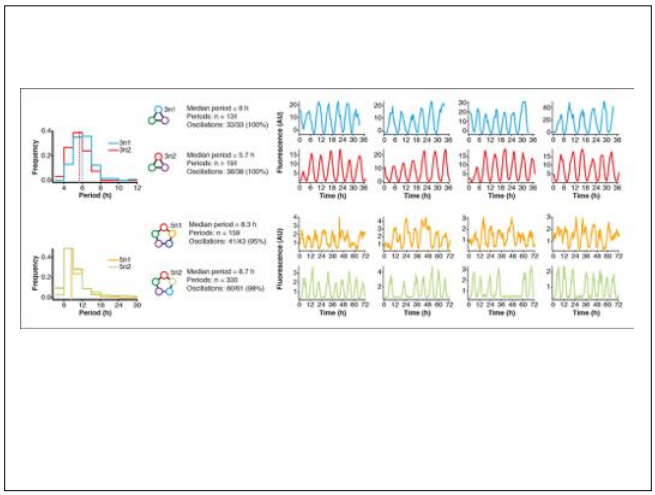
Extension of the *in vitro* Design-Build-Test approach used for 5 repressor circuits. An odd number of elements in the gene circuit gives rise to oscillations.



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Measurements of the behaviour of 3- and 5-repressor circuits, showing amplitude and period of regular oscillations.

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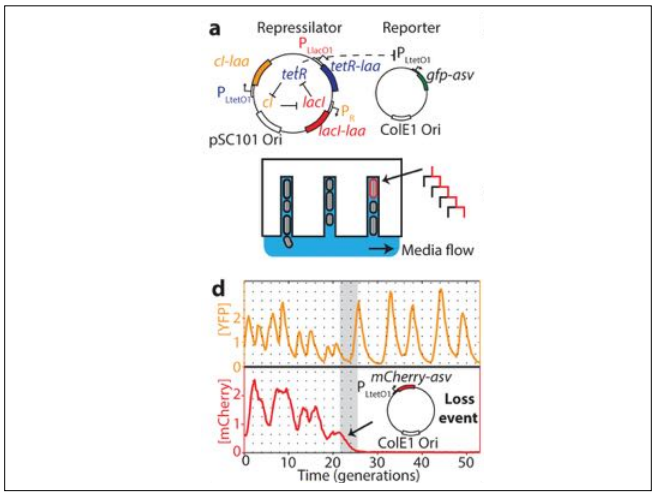
The circuits which showed improved robustness *in vitro* could be implemented in cells also. Here, the Maerkl lab used a "mother machine" - where cells are trapped in narrow chambers, and cell divisions and lineages can be tracked precisely. Dividing cells largely remain in synchrony.



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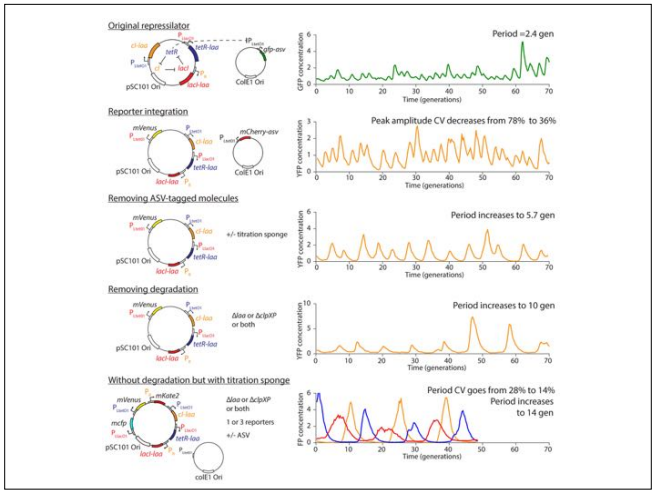
The Paulsson lab further used "mother machine" based measurements to improve the basic circuit. They discovered that various types of molecular competition and cross-talk in the circuit were sources of noise. For example, the reporter GFP gene was carried on a high copy number plasmid - separate from the repressilator circuit. Loss of the reporter plasmid was observed to cause more regular, higher amplitude cycles and longer period. This was shown to be due to competition for protein degradation machinery - as all repressors and GFP reporter shared 3-amino acid degradation tags.



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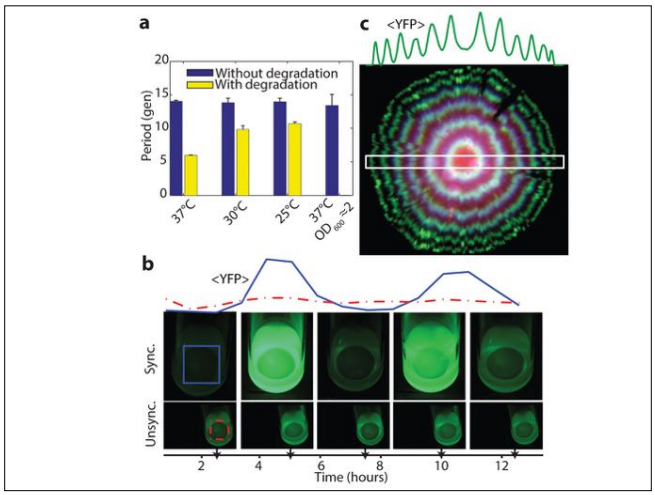
More robust cycles were seen when this and similar other sources of noise were removed. (i) Integration of the reporter gene on the low-copy repressilator plasmid. (ii) Removal of all destabilised (ASV C-terminus tagged) proteins). (iii) Use of a mutant E. coli strain with defective protein turnover. (iv) Addition of a plasmid borne "sponge" for binding excess TetR protein.



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The combination of these improvements gave rise to cell lines that maintained long period oscillations that remained in phase over many cell generations. Synchronised liquid cultures showed bulk oscillatory behaviour. Colonies grown on solid media showed ring-like features as the cells grew outward from teh point of innoculation.

Rational circuit design

- Problem: to understand design principles of biological networks
- Approach: Design and construct synthetic network

- Knowledge gained from the design of synthetic networks should help understand real networks
- Could be used to engineer new cellular behaviours in plants

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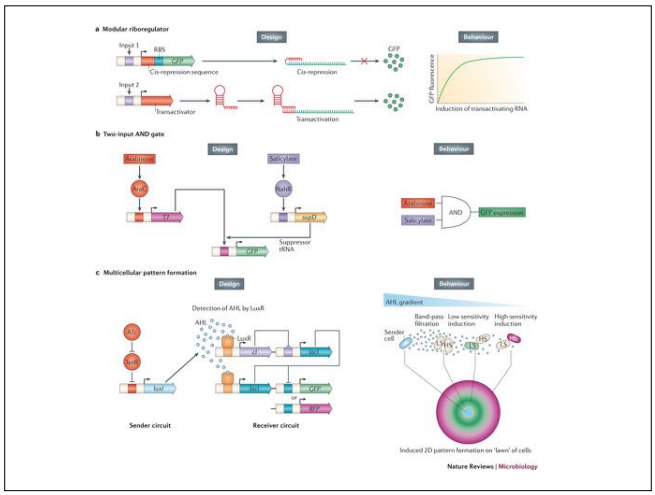
A key problem facing the field of systems biology is in understanding how biological networks are put together, or, in systems engineering terms, understanding their "design principles." One approach is to design and construct synthetic networks and then see how the synthetic networks function when introduced into cells. Once introduced into an organism, a synthetic network can be subjected to various perturbations to see how the network responds. In addition to helping us understand real cellular networks, synthetic networks have the potential to be used to engineer new cellular behaviors.



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The toggle switch and repressilator are simple examples of a growing collection of circuit motifs that are being assembled as building blocks to engineer larger scale synthetic biological systems. Not dissimilar to the way early digital electronic circuits were first characterised, and then used as modular components for larger scale systems.

Lecture 1: Genetic modification in agriculture and the advent of Synthetic Biology.
Lecture 2: Genetic circuits and genome scale DNA engineering.
Lecture 3: Engineered logic and the control of gene expression.

1. **Cell autonomous genetic logic**
2. **Microbial test systems**
3. **Feedback regulation: toggle switches**
4. **Transcription networks: Genetic oscillators**
5. ***In vitro* systems for rapid testing**
6. **Complex circuit design**

Lecture 4: Self-organisation and reprogramming of multicellular systems.

Additional resources: **http://www.haseloff-lab.org** (Education)