**Title: Time to scale-up.**

**Standfirst:** The construction of modular and scalable synthetic gene networks is now a goal within reach. This advance in synthetic biology promises applications for biotechnology, medicine, nanoscience and nanotechnology.

**Guilhem Chalancon and M. Madan Babu**

The primary objective of developing synthetic gene regulatory networks is to implement predictable and controllable functions into cellular systems\(^1\)\(^-\)\(^3\). Such artificial networks typically consist of transcription factors (input) that either activate or repress the expression of a reporter gene (output) in a cell (**Figure 1a**). While several studies have designed simple circuits, construction of complex networks remains a challenge due to issues associated with scalability. Writing in Nature Nanotechnology, Leisner et al.\(^4\) describe a scalable approach to synthesize networks with an arbitrarily defined number of components and input-output behaviour. They present a proof of concept for an RNA-based approach to construct programmable and scalable logical integration of transcription factor inputs in mammalian cells. Such an approach has the potential to fuel applications in biotechnology and medicine.

**Main body (895 words long).** Molecular computing using synthetic circuits requires components that can sense combinations of inputs, resulting in a predictable output according to a particular logic. An ideal approach for constructing gene networks should be modular, predictable and scalable. Leisner et al.\(^4\) use the presence or absence of transcription factors (TFs) as inputs, each of which in turn regulates the expression of a microRNA. Each microRNA has target sequences in the 3' UTR of the reporter gene. Thus, the TF indirectly regulates the expression of the reporter through the microRNA (**Figure 1a**). The authors used immortalized human embryonic kidney cells (HEK293) and expressed three different TFs (two activators and one repressor) and combinations thereof. A yellow fluorescent protein was used as an output and was designed to be constitutively expressed from two separate transcripts, one of which has the target site for two microRNAs, and the other containing a site for the third microRNA (**Figure 1b**).

In the first set of experiments, synthetic networks with all possible combinations of pairs of TFs were designed in a plasmid and expected outputs were demonstrated. Next, they scaled up the system by constructing a circuit with three inputs to execute a pre-defined program (**Figure 1b**). Seven of the eight possible input combinations behaved as predicted. One expected “off” output still expressed the reporter at a higher level and this was attributed to the low performance of one of the modules. These results demonstrate the modularity and scalability of the framework to synthesize gene networks. It also highlights the need for designing highly robust modules, as an unstable module will affect any larger network that it is a part of.

While the authors demonstrate scalability of their framework, certain limitations must be overcome before it can be employed for developing specific applications (**Figure 1c**). Some technical considerations include plasmid copy-number variation across the cell population, the choice of promoter for gene expression, microRNA design, number of target microRNA sites, minimizing off-target binding of microRNAs and the TF (i.e., cross-talk with the host), effect of epigenetic modification and chromatin, and the number of transcripts encoding the reporter gene. All these parameters can be independently optimized to ensure a more predictable output. Finally, the intrinsic stochasticity\(^5\) of transcription and translation (i.e., noise) could affect predictability of individual modules, thereby hampering synthesis of more complex networks of much larger size. Thus, the effect of noise in complex gene circuits needs to be considered seriously as this may give rise to variation in the output in individuals within a cell population, which may be undesirable in most instances\(^6\).

One key objective of synthetic biology is to design synthetic networks that interfere minimally with the host regulatory system. This can be achieved by constructing orthogonal (i.e., independent)
systems\(^2\). In a recent study, An and Chin\(^7\) reported the construction of an orthogonal transcription-translation network which minimised interference with endogenous processes and at the same time made the circuit amenable to engineering the kinetics of the input-output behaviour. In the framework of Leisner et al\(^4\), orthogonality may be implemented by using zinc-finger transcription factors that bind uniquely to the promoter of a gene in the synthetic network. This might minimize off-target binding in the host genome and may also permit construction of hierarchical regulatory cascades where the reporter gene happens to be another TF.

In another direction of research, it is reasonable to consider transcription factors not only as inputs, but as intermediate elements controlled by small-molecules. Thus one could envision advancements wherein the TFs could be engineered to sense particular concentrations of certain ligands and thereby transduce this information. Such developments can have applications in sensing combinations of small-molecules. Similarly, this framework may serve as the starting point to combinatorially synthesize gene networks using a library of different elements (i.e., promoter sequences, microRNA, etc), as was recently done by Ellis et al\(^8\), to identify the right components in order to design modules with robust input-output characteristics.

The advancements described above can have applications in biotechnology, biomaterials, drug delivery, and medicine (Figure 1d). For instance, Ellis et al\(^8\) recently described the generation of gene circuits that control the timing of yeast sedimentation. Kemmer et al\(^9\) reported a synthetic circuit that senses uric acid levels and triggers a dose-dependent expression of a urate oxidase gene in mouse to counteract disease conditions such as gout and tumor lysis syndrome. The availability of scalable systems can serve as an additional layer to sense complex physiological conditions and then trigger an output response accordingly.

Scalable networks can also serve as a molecular tool to investigate the effects of perturbing the expression level of combination of genes that affect a cellular process of interest. For instance, in stem cell research and regenerative medicine, a combination of human TFs could be expressed through synthetic circuits in differentiated cells upon sensing a particular cellular state to study reprogramming or to induce pluripotency. A scalable approach can also be used to study the input-output characteristics of more complex networks that can compute a wide-range of inputs which in turn can be built from combinations of regulatory cascades, logic evaluators, hysteretic circuits and epigenetic toggle switches. Finally, with the recent publication of the first synthetic prokaryotic genome by Gibson et al\(^10\), we foresee that transplantation of large modular synthetic networks will become a reality, in both bacterial and eukaryotic organisms. These are definitely exciting times for synthetic biologists.

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**REFERENCES**


**Figure 1**: Towards scalable synthetic gene networks. *a,* A module from Leisner et al\(^4\) comprises of a transcription factor (TF) as an input, either activating or repressing the expression of a microRNA. The microRNA represses the expression of a downstream reporter gene (output)
which is constitutively expressed through a plasmid. By integrating the different modules, synthetic gene networks that compute a particular logic can be achieved. The asterisk denotes constitutive expression. 0/1 denotes absence or presence of an input. The truth table (i.e., input-output behaviour) for the gene network is shown. Factors influencing the construction of scalable synthetic gene networks. Scaling-up synthetic gene networks by interweaving such modules into more complex networks will enable the development of applications in biotechnology and medicine.

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