One of the hallmarks of signal transduction circuit found in nature is asymmetric, asynchronous design. That is, there is little standardization of parts, e.g. all the promoters have different strengths and kinetics, transcription factors are designed to have different effects at different loci, and each enzymatic reaction has its own idiosyncratic mechanism and rates. In addition, all of the heterogeneous circuit elements are executing their functions concurrently and asynchronously; biological circuits are seemingly designed to deal with the fluctuating delays, different time-scales and energy requirements associated with each component process of the overall network. These factors also make design of novel biochemical circuitry from existent parts difficult to achieve. However, it is possible to achieve some level of homogeneity among the parts by exploiting the modular nature of yeast transcription factors and promoter organization. Using the yeast 'two-hybrid' system technology (9) it is possible to rationally design a relatively complex circuit from molecularly engineered components. To demonstrate this, we will build a digital-like flip-flop, a switchable memory element, into a yeast cell. The practical uses of such a switch are described following a discussion of the implementation. The techniques developed in the construction of this circuit demonstrate a general methodology for construction of such circuits.

Just as in the simplest electronic flip-flop, the yeast flip-flop is composed of two NOT-AND (NAND) gates made out of a small number of genetic components. The yeast two-hybrid screen is a method wherein the presence of an interaction between two proteins is queried by fusing one member of the pair to a DNA binding domain and the second member to a transcriptional activator. Functionally, the screen performs an AND function: only when *both* fusion proteins are present (and the two proteins bind to one another) is the activator brought proximal to the promoter to activate transcription. To achieve a NAND gate function, the activator is replaced by a repressor.

Figure 1A shows an electronic schematic for a flip-flop (see caption for explanation) and Figure 1B shows the yeast-genetic equivalent. In the yeast circuit, two inducible promoters, one induced by galactose and the other by a glucocorticoid (the strain of *Saccharomyces cerevisiae* that is responsive to the glucocorticoid also has a mammalian receptor engineered into it), serves as the 'high' inputs to the two genetic NAND gates. Each produces its own DNA-binding fusion protein that can interact with one of the gene products produced from each promoter. All the labeled components and their individual interactions have been reported in the literature. The network configuration is unique. This yeast circuit can be switched between green and red fluorescence by transiently lowering galactose or glucocorticoid concentrations respectively. This will be a very slow switch, however, simulations of the dynamics of this switch indicate that it is stable and switchable by the external environmental signals. The particular mechanisms of activation and repression of transcription in these circuits require only that the transcription modulation domain is brought into proximity to the polymerase complex bound at the promoter, thus, the same transcriptional modulation domains and promoters may be reused multiple times in the same circuit. Specificity is endowed by the particular protein-protein recognition pairs employed and the identity of the DNA binding domain. If DNA binding and protein-protein recognition are tight enough then most of the circuitry has been homogenized: all promoters and transcriptional modulators in the circuit are be the same. Degradation of the fusion proteins, however, is not yet controlled so asymmetry in these reactions can possibly lead to abnormal circuit behavior. Other sources of asymmetry include different efficiencies of transcription and translation and small differences in modulator/polymerase interaction due to different spacing with different protein interaction pairs.

The reasons for choosing to engineer a flip-flop into yeast are manifold: 1) First, it is a good test of molecular and network engineering skills. Multiple components must be designed by genetic manipulations such that their interactions are specific and there is no cross-talk among components. Small amounts of non-specific binding or transcriptional modulation can lead to circuit malfunction. 2) The circuit is a prime example of a genetic switch architecture, similar to those that underlie development and differentiation during the life-cycle of the cell. As such it provides a test bed for examining design criteria such as stability of the two states to genetic defects in any of the components (e.g. mutations in the binding sites, decreases in binding constant of the protein/protein interaction pairs) 3) It is a probe for stochastic gene expression in eukaryotes observed in the literature (1-8, 10-13). Noise in gene transcription can lead to a partition of the yeast cell population between the two stable states of the switch even after an initial population has been 'synchronized' in one state by lowering of one of the inputs. The statistics of appearance of the non-selected state and the final population partition are direct measures of the underlying stochastic kinetics of the genetic reactions. The effect of this noise on the maintenance of a given state will have relevance to the stability of other developmental switches (and thus to the probability of their failure). (4) The switch allows a cell-based biosensor to remember signals it has come across. For example, a population of yeast bearing a switch in which one input is repressed by exposure to galactose and the other repressed by a low-dose toxin could be seeded into a slowly flowing water supply and then recollected downstream. If the cells were all initially 'synchronized' by pulsing them with galactose, then any members of the population found downstream in the other state have most likely encountered a source of the toxin at a point upstream. (5) Finally, this circuit is the first step to

the building of complex sensing logic into a living cell. The design demonstrates the construction of both logic gates and memory devices, the two pieces necessary to build any computer. In the future this might lead to the ability to engineer cells to response only to complex mixtures of chemicals, i.e. chemical codes, in particular ways.

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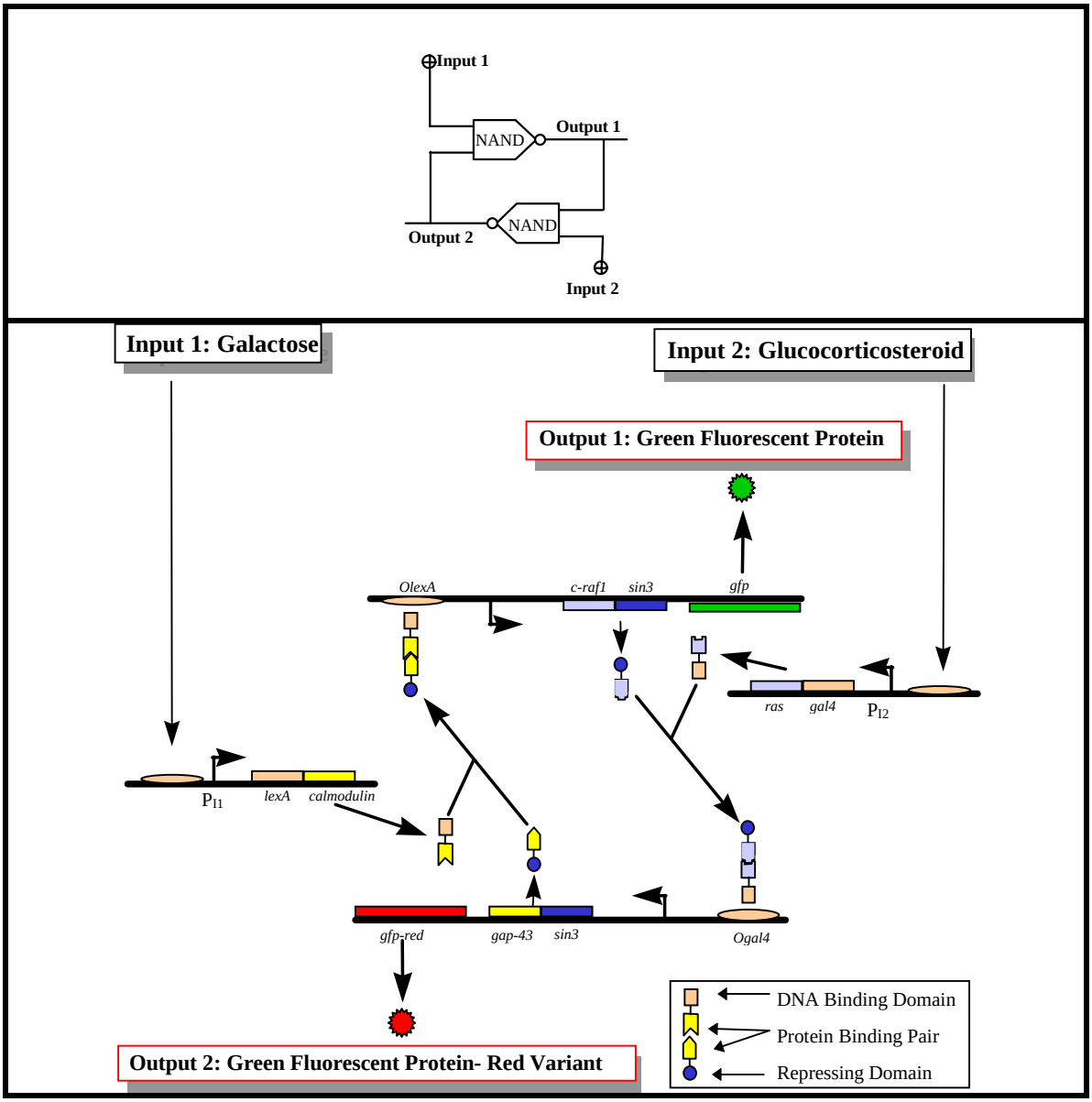

Figure 1. (A) A simple NAND gate based flip-flop. The symmetry of the circuit leads to a bistability in the circuit output. The states [Output1=1,Output2=0] and [Output1=0, Output2=1] are equally valid states of the circuit. A state may be selected by transiently lowering one of the inputs. (B) A genetic realization of the flip-flop in (A). Here, the two high inputs are represented by the fusion protein produced by induction of promoters P_{I1} and P_{I2} by galactose and glucocortocoid respectively. The c-raf1:sin3 fusion protein is analogous to Output 1 from (A) and the gap-43:sin3 protein is analogous to Output2. Note that the network structure of this genetic circuit is identical to the flip-flop in (A). It will have the same bistable behavior.