Toward scalable parts families for predictable design of biological circuits

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Our current ability to engineer biological circuits is hindered by design cycles that are costly in terms of time and money, with constructs failing to operate as desired, or evolving away from the desired function once deployed. Synthetic biologists seek to understand biological design principles and use them to create technologies that increase the efficiency of the genetic engineering design cycle. Central to the approach is the creation of biological parts — encapsulated functions that can be composited together to create new pathways with predictable behaviors. We define five desirable characteristics of biological parts — independence, reliability, tunability, orthogonality and composability, and review studies of small natural and synthetic biological circuits that provide insights into each of these characteristics. We propose that the creation of appropriate sets of families of parts with these properties is a prerequisite for efficient, predictable engineering of new function in cells and will enable a large increase in the sophistication of genetic engineering applications.

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Introduction

Microbes are profoundly intertwined with our environment and our lives. They metabolize a wide variety of chemicals including elemental metals, and can synthesize an equally broad array of molecules including complex organics and drugs. Microbial communities play a critical role in global cycles of important elements such as carbon, nitrogen, and sulfur. For these reasons, understanding the molecular basis of microbial metabolic mechanisms and their regulatory control has been a central goal of molecular biology.

It was recognized early on that once genes and networks responsible for the broad array of microbial function were identified and understood, they could be exploited for technological benefit. Bacteria have been genetically engineered to produce commodity chemicals, pharmaceuticals [1,2], and recently fuel molecules [2–4]. Beyond the controlled culture environment of an industrial production bioreactor, bacteria have been cautiously engineered to aid in bioremediation [5], to support agriculture [6], and act as vehicles for macromolecular delivery [7], immunotherapy [8], and even cancer [9].

Despite its name, however, genetic engineering still remains an inefficient tool rather than an engineering science, and projects are plagued by multiple, costly cycles of design and testing as constructs fail to operate as desired [10], or evolve away from the desired behavior once generated [11]. To address these difficulties, synthetic biologists have recently designed and synthesized biological circuits aimed at uncovering, exploiting, and optimizing cellular components for use in predictable design and more safe and efficient construction of new complex function in organisms.

To construct biological circuits, synthetic biologists have focused on biological 'parts' — distinct encapsulations of biological function that may be wired together in different contexts to create new and sometimes predictable behaviors. A classic example that predates Synthetic Biology is the ability to attach promoters to arbitrary genes to express them in heterologous hosts which demonstrates a kind of discrete, rewirable containment of biological function that forms the basis of most metabolic engineering. In this case, a metabolic pathway may be viewed as a connection between enzyme gene parts that are used to produce specific metabolites, and gene expression control parts (promoters) that coordinate expression of the enzymes. Other examples of biological parts include regions of DNA (e.g. operator sites), RNA (e.g. ribosome binding sites), protein (e.g. domains), and even whole complex subsystems (e.g. secondary metabolic pathways) that can all be connected in synthetic circuits displaying myriad functions.

It might seem, then, that the major obstacle to engineering new function in microbes is assembling DNA encoding specific parts in the desired configuration into the cell.

This is indeed a challenge, but technological advances in DNA synthesis [12], and methods for transforming large DNA fragments into cells [13] are rapidly solving them. Instead, the main challenge seems to be uncertainty: uncertainty in our understanding of the precise mechanisms of internal part operation and part–part interaction, uncertainty in our understanding of environmental influence on part function, uncertainty in how to identify and measure characteristic part properties relevant to circuit design, and uncertainty in our understanding of the environment in which the cell must operate and survive.

Synthetic biologists seek to address these uncertainties directly as outlined in a number of reviews [2,14–17]. At the heart of these efforts are attempts to develop families of characterized biological parts that would interoperate in a predictable manner, even in complex biological circuits and across a variety of biological environments. In this paper, we define five fundamental properties that we seek in optimal biological parts: independence, reliability, tunability, orthogonality, and composability, which when combined, lead to scalable parts families that can be used in synthetic biological design (Box 1, Figure 1). Here, we emphasize recent work that is beginning to uncover the principles behind each of these fundamental properties. We then discuss how we can move these studies forward to produce a conceptual and physical infrastructure around a parts-based biological circuit design cycle that will create a dramatic increase in the efficiency of genetic engineering, and will improve our understanding of the principles behind biological design.

Independence

Independent parts do not interfere with their host circuitry and vice versa (Box 1). In an early example involving complex function, the multigene nitrogen fixation system from *Klebsiella pneumoniae* was shown to operate when transplanted into *E. coli*, albeit with somewhat diminished function [18]. That is, the transformed *E. coli* was able to fix nitrogen, implying that the system can function with some degree of independence from the context of the host in which it evolved. Independent parts also do not interfere with each other. Repressors that affect different promoters and do not interact with each other are independent, for example.

Part independence is far from guaranteed however. For example, different plasmid origins of replication can interfere with each other and form plasmid incompatibility groups that prohibit multiple members of the same incompatibility group from stably coexisting in the same cell [19].

Reliability

A reliable biological part functions as intended in a biological circuit in a suitable host (Box 1). Independence of part function is one aspect of reliability.

Another aspect of reliability is robustness in the face of noise in the cellular circuitry and fluctuations in the

Box 1 An informal glossary of synthetic biological part properties. Below we suggest definitions of commonly used terms in synthetic biology [2,14–17]. While a consensus for a formal definition of these terms is still being developed, we present one interpretation of the meaning of these emerging concepts.

Term	Definition	Illustrative examples
Independence	The degree to which a part does not interfere with other parts in the host or with the host machinery, and vice versa.	Nitrogen fixation in different hosts [18], Multiple repressors in the same cell [42,56]
Reliability	The degree to which a part functions as intended with respect to variability in its components and its environment. Implies independence.	Small RNAs increasing noise [23], feedback loops decreasing noise [25]
Tunability	The degree to which the function of a part may be controllably adjusted.	Ribosome-binding sites [36–39], mRNA stability elements [40], histidine kinases [45••], protein–protein-binding domains [46]
Orthogonality	The degree to which parts derived from a parent part can be tuned to the point of noninterference while maintaining the same basic conceptual function.	Designed ribosome–RBS pairs [47••]
Composability	The degree to which parts can be combined together to form units with composite function.	Zinc finger domains [51], chimeric proteins [52], Aptamer–ribozyme composites [53••,55] (Figure 2), protein-binding domains [43], repressor networks [22•]
Scalability	The degree to which the confluence of independence, reliability, tunability, orthogonality, and composability in a family of parts can be exploited to create many distinct, noninterfering instances of a function all otherwise having similar operating characteristics.	Aptamer–ribozyme composite parts [53••,55] (Figure 2)

Figure 1
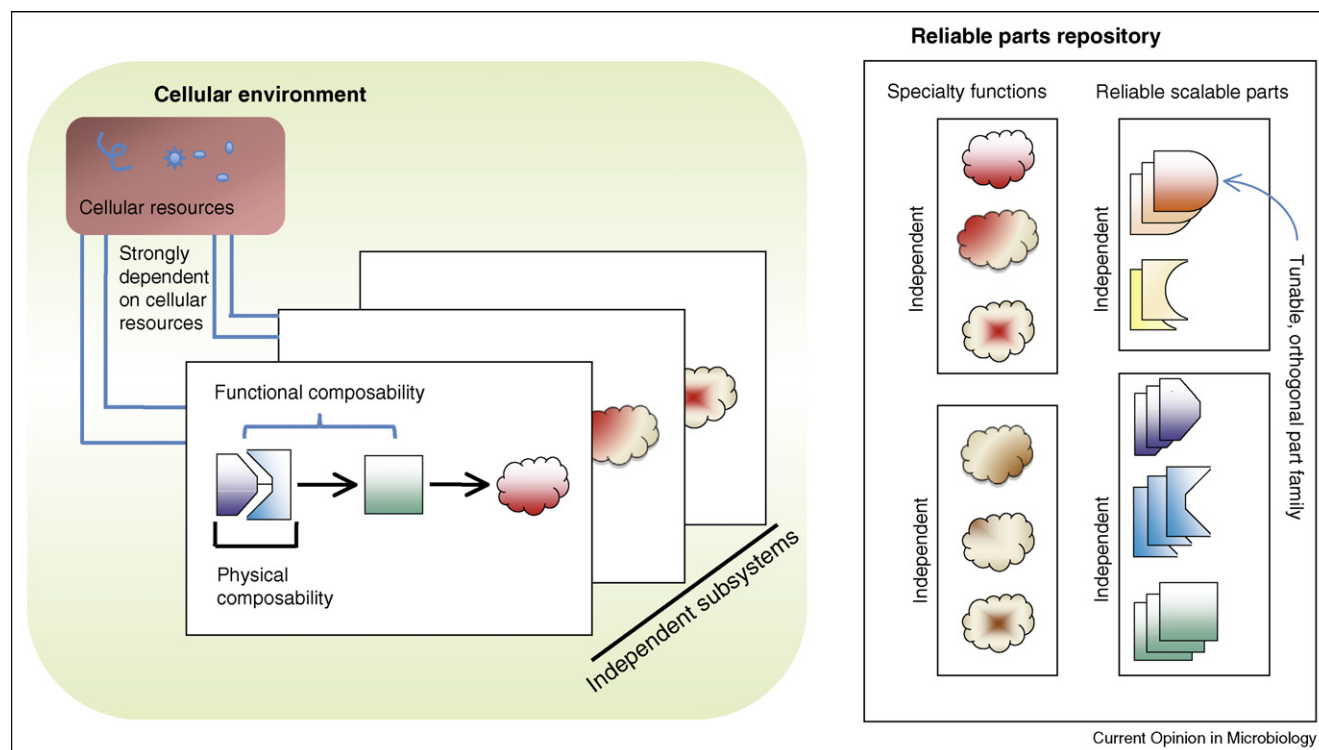
Biological circuits designed with parts from scalable families interconnecting specialty purpose parts. Multiple independent biological circuits are placed inside a single cellular environment (left panel), all sharing the same common pool of cellular resources and functioning more or less reliably with respect to variability in their components and the environment. The circuits are composed of interconnected parts (left panel, lines). Ideally, parts for circuits are drawn from a repository or knowledgebase (right panel) and consist of two types — parts that perform specialty activities, controlled by parts from scalable parts families (stacked groups). Within a single part family, there can be many orthogonal members indicated by shading. There can be many groups of scalable parts families, and those families whose parts function independently from each other are enclosed in rectangles.

cellular environment. Because of the fundamentally discrete and stochastic nature of chemistry, there is intrinsic noise in the dynamics of biochemical networks [20]. These effects can be quite large in certain cellular systems such as those involved in gene expression, and lead to very different dynamics that would be predicted from the classical deterministic picture [21]. In a study of noise propagation through gene expression cascades, the sharpness of the transition between off and on states, as well as the variability in the proportion of the cellular population that made the transition, was shown to increase with the length of the cascade [22•]. As a possible route to engineering this aspect of part reliability, a number of articles have demonstrated that engineering different molecular features and feedback mechanisms into gene expression circuits can alter the noise profile of parts and circuits [23–25]. This sort of intrinsic noise, however, does not always lead to unreliable function but can actually be a source of reliability [26–28]. Much like diversifying a stock portfolio, intrinsic noise in physiological function can be leveraged to bank against uncertainty in the environment. Blake *et al.* built synthetic circuits to test this experimentally in which noisy promoters connected to an antibiotic

resistance gene were shown to confer an advantage over more stable promoters for cells exposed to acute bursts of the antibiotic [29].

The function of a synthetic part can also be affected by cell-to-cell variation in key cellular resources required for transcription, translation, and replication that, in turn, can be affected by changes in the cellular environment. A recent example that illustrates this problem is a synthetic genetic AND gate that utilizes both control of transcription and translation and requires two inputs to turn on gene expression [30]. The gate was shown to have a lower gain at low cell densities, an undesirable coupling to growth phase.

Finally, the load, either energetic or toxic, that a reliable part places on the host should be well understood and optimized so that it is not selected against over generations and does not add a diffuse 'metabolic' coupling among components in the cell. Addition of extra circuitry to a cell places an extra burden on the cell [31]. Use of selective markers can maintain a burdensome part in the right environment. In the absence of such markers,

mutants of the engineered cell that inactivate or rid the cell of the part will outgrow the original. For instance, in one recent study, You *et al.* discovered that cells started to escape their population-controlled cell death circuit three to six days after introducing the circuit into the cells [32], and Canton *et al.* found that the functioning of a gene expression controller at high induction decayed after 56 generations of being present inside the cells [33•]. While these examples may represent an actual resource load on the cell, other examples include high-production metabolic pathways that produce intermediates toxic to the cell [34]. In this case, reliable function may require additions and tuning of other parts such as enzymes immediately downstream of the toxic compound, or changing environmental conditions such as adding protective chemicals [35].

Tunability

'Tunability' refers to the ability to make controlled adjustment to a part's function (Box 1). For simple parts such as ribosome-binding sites (RBSs) on mRNA transcripts upstream of protein coding regions, this takes the form of varying the sequence of the RBSs to change both the structure of the transcript around the translation initiation site, and its interaction with the ribosome such that translational efficiency is affected. A number of groups have used this trick to adjust gene expression up to 1000 times the normal level [36–38] and some have tried to model the effect [39]. In addition to RBSs, the tuning of mRNA degradation was studied by Carrier and Keasling, who constructed a library of mRNA stability structures shown to confer mRNA half-lives in the range of 2–20 min [40]. Similar approaches have highlighted the role of intergenic regions in tuning the expression of multiple genes within a single operon [41].

The tuning of a molecular part function can, of course, alter the entire function of the circuit in which it is embedded. In early work, Gardner and Collins experimentally demonstrated that tuning RBSs in certain versions of a genetic toggle switch can affect whether the switch displays graded or bistable behavior [42]. Dueber *et al.* exploited the differential affinity of SH3, PDZ, and GBD peptide-binding domains to different peptide targets to construct proteins that function conditionally in the presence or absence of multiple environmental inputs [43]. Voigt *et al.* describe a theoretical 'evolvable' circuit motif in which it is possible to tune the strength of a

Figure 2
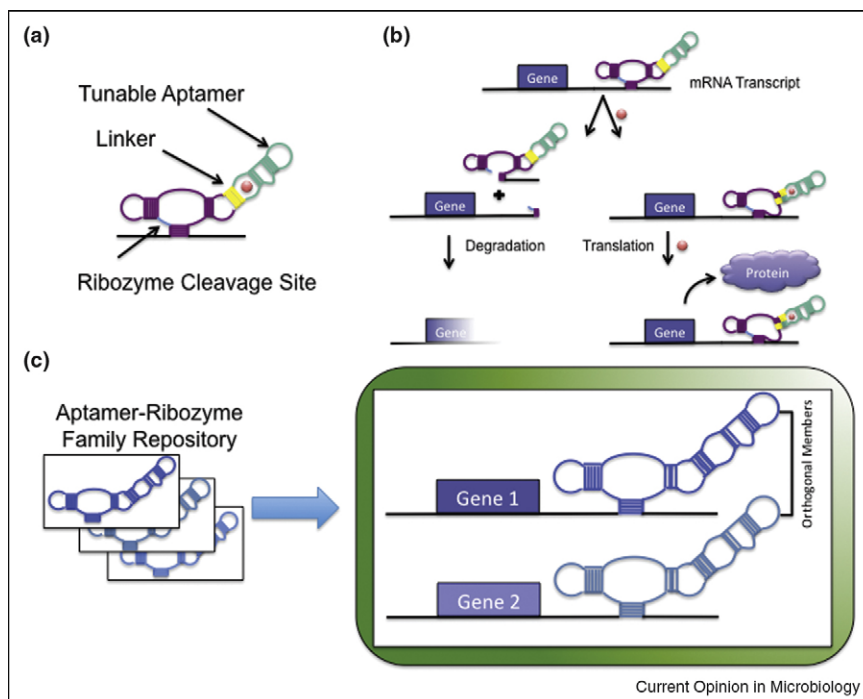
Specifics of a scalable parts family. **(a)** Self-cleaving ribozymes (purple) can be physically composed (yellow) to aptamer-sensing domains (green) which cause the inhibition of the self-cleaving reaction in the presence of a specific small molecule (red) (following [53••]). Aptamer sequences can be tuned to show specificity for different metabolites. **(b)** When the aptamer–ribozyme is placed downstream of a gene, a self-cleavage reaction in the absence of the small molecule destabilizes the transcript leading to degradation. Transcript destabilization is prohibited by the small molecule, which prevents self-cleavage through binding and RNA secondary structure rearrangement, enabling translation of the upstream gene. **(c)** Since they are part of the physical transcript, different aptamer-ribozymes composed behind different genes could act orthogonally. The combination of tunability, functional composability, and orthogonality make these parts an example of a scalable parts family for controlling gene expression [55].

promoter to switch the behavior of the circuit from a graded switch, to a bistable switch, to an oscillator, to a pulse generator [44].

In some cases, tuning a part's strength of function amounts to tuning its specificity toward its interaction target, and away from the multitude of other molecular species with which it could potentially interact (see Figure 2a). Skerker *et al.* demonstrated such specificity tuning by showing how the specific activity of a histidine kinase (HK) for a response regulator (RR) could be tuned by the mutation of rationally chosen amino acids [45**]. In some cases, it was possible to tune the interaction between two parts in the extreme and switch the specificity of one HK to a different RR and away from its natural partner. This type of tuning leads to parts whose specificities are so extremely tuned toward their target as to make them functionally orthogonal to each other.

Orthogonality
Orthogonal parts families are derived from parent parts that can be tuned to the point of noninterference with each other, while maintaining the same basic conceptual function (Box 1). The HK/RR example mentioned above demonstrates the possibility of designing multiple synthetic independent/orthogonal HK/RR pairs. Similarly, Reina *et al.* were able to tune three different PDZ domains to bind to new targets two orders of magnitude higher than to their cognate peptides [46].

Recently, there have been multiple examples illustrating the power of RNA designability for creating orthogonal parts families. By mutating the 16S ribosomal RNA sequence, Rackham and Chin created mutant ribosomes that each acted on a specific target RBS, independently of each other and the natural host ribosomes [47**]. Bayer and Smolke, and Isaacs *et al.* used RNA complementarity rules in their design of orthogonal translational lock systems that block translation initiation and can be unlocked by specific small molecules, or complementary RNA molecules expressed in *trans* [48,49]. Although achieved in mammalian cells, Rinaudo *et al.* were even able to exploit similar mechanisms to design RNAi logics that relied on orthogonally acting siRNA molecules to target multiple RNAi sites on mRNA transcripts to perform several combinations of Boolean operations controlling the expression of a reporter gene [50].

Each of these examples demonstrates the ability to create families of function in which multiple parts from each family can be used in the same cell without interference.

Composability
Composability is the property of certain parts that allows them to be combined to form a predictably functioning biological circuit (Box 1). Parts that are physically composable can be placed on the same molecule (DNA,

mRNA transcript, protein, or membrane) thereby displaying a composite behavior predictable from the individual functions. Promoters, RBSs, and genes are one of the most familiar examples of physically composable parts that can be placed in series on a DNA molecule to control expression of the gene. However, these have to be carefully chosen to prevent the formation of interfering secondary structures on the transcript, among other things, that could lead to poor expression. In proteins, Mandell and Barbas have shown programmable specificity of synthetic zinc finger binding domains, which can be physically composed together to create proteins that bind to desired target DNA sequences [51].

Physically composable parts can also be combined to form a composite part with chimeric function. Taz is a protein which combines the sensing domain from the aspartate receptor Tar from *E. coli*, with the kinase domain of EnvZ. EnvZ activity is normally regulated in response to osmolarity. *E. coli* cells containing Taz respond to the presence of aspartate with the activation of promoters that are normally regulated in response to osmolarity [52]. Win and Smolke composed self-cleaving RNA ribozymes with small-molecule sensing RNA aptamers [53**,54,55] (see Figure 2). When the aptamer domain is placed in the middle of the ribozyme sequence, changes in conformation of the aptamer domain upon binding a specific small molecule, either allow, or prohibit ribozyme cleavage. When this composite part is composed downstream of a gene, cleavage by the ribozyme results in transcript destabilization, thus controlling gene expression. Proper functioning of this composite part required the creation of a linking element that could preserve the functioning of the individual parts while coupling their function together [53**,55].

When two parts are not on the same molecule they can still be functionally composed. For example, a promoter–repressor encoded on one DNA molecule can couple to a promoter on another DNA molecule to repress its activity [56]. This requires matching part–part interaction parameters, a currently heterogeneous process requiring a great deal of tuning. Anderson *et al.* resorted to screening RBS libraries to find one that allowed leakless, inducible expression of a target gene above a target amount from a commonly used promoter over a desired range of inducer concentration [38].

Conclusions: the road to scalability and predictive design
In most engineering disciplines, there are specialty parts and there are generic parts. The specialty parts carry out application-specific function such as LCD screens and CCD cameras, and they are interconnected by generic parts — key parts that are used in nearly every design, such as transistors in electronics. There are many specialty parts in biology, for example,

enzymes and molecular machines such as photosynthesis, motility, protein secretion, and nitrogen fixation, evolved over billions of years. For now, we should essentially use these 'as-is', using our understanding of how to tune these parts [58] to match particular biological circuit designs.

In contrast, for the generic parts, we need an engineering science that can provide predictable and scalable design. The confluence of parts that are all independent, reliable, tunable, orthogonal, and generally composable, as outlined above, leads to scalable families of parts that can be readily combined together to form predictable and possibly complex new functions in cells (Figures 1 and 2). A central challenge of synthetic biology is how best to choose these scalable parts families to form a powerful basis set of biological function.

An initial effort ought to be the generation of scalable parts families that control transcription, translation, and the generic features of protein–protein interaction. These processes are central to nearly every application and generally provide the logic by which the application's key activities are deployed. There is evidence that early success will come from nucleic-acid-based gene expression regulators where Watson–Crick base pairing rules are a good starting point for design [36,40,41,47$^{\bullet\bullet}$,48–50,53$^{\bullet\bullet}$,55,57] (Figure 2). However, an organized program to characterize such parts in the seemingly immense and diverse number of contexts in which they may appear is needed to understand the fundamental principles behind part independence, reliability, tunability, orthogonality, and composability.

With sufficiently deep parts families covering a broad, but carefully selected array of function, we should have the tools to finally enable a predictable biological circuit design cycle, thereby dramatically increasing the efficiency, safety and sophistication of genetic engineering. This will make the small forays into design principles for pattern forming circuits [59], growth controllers [32], and other higher level designs [30] of greater general interest and use to other designers and scientists. Eventually, understanding these basic concepts will help us transit from the engineering of small biological circuits and pathways, to genome scale designs that operate beyond the bioreactor across the population, and ultimately ecological levels, all the while enabling a deeper identification and understanding of the design principles of biology.

Acknowledgements

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