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Controlling metabolic flux by toehold-mediated strand displacement

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To maximize desired products in engineered cellular factories it is often necessary to optimize metabolic flux. While a number of works have focused on metabolic pathway enhancement through genetic regulators and synthetic scaffolds, these approaches require time-intensive design and optimization with limited versatility and capacity for scale-up. Recently, nucleic-acid nanotechnology has emerged as an encouraging approach to overcome these limitations and create systems for modular programmable control of metabolic flux. Using toehold-mediated strand displacement (TMSD), nucleic acid constructs can be made into dynamic devices that recognize specific biomolecular triggers for conditional control of gene regulation as well as design of dynamic synthetic scaffolds. This review will consider the various approaches that have been used thus far to control metabolic flux using toehold-gated devices.

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Introduction

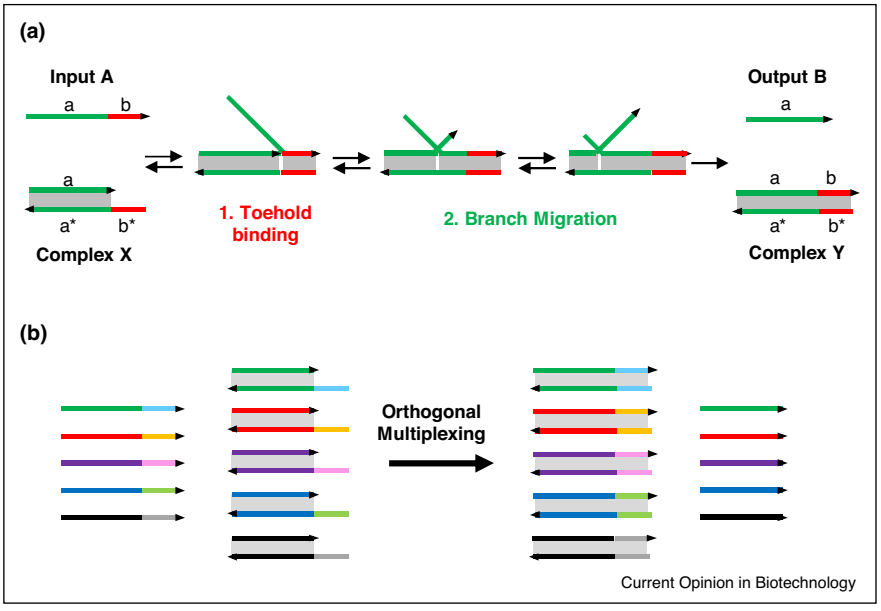
Controlling metabolic flux is key to maximizing the synthesis of desired products within engineered cellular factories. The overall flux along a metabolic pathway is determined by the individual enzyme activities catalyzing each reaction step [1]. Both the absolute cellular concentration and the local concentration of these enzymes can greatly affect metabolic flux. As a result, both genetic regulation of enzyme expression and enzyme co-localization through scaffolding have emerged as key approaches for engineering metabolic pathways. The optimized and dynamic gene expression of each enzyme allows metabolic activity to be maximized across changing conditions [2]. Meanwhile, enzyme co-localization through scaffolding decreases the diffusional path lengths between enzymes to allow for enhanced substrate channeling and enzyme clustering. Scaffolding is especially beneficial in limiting the accumulation of toxic metabolites, overcoming flux bottlenecks caused by pathway enzymes with low activity, and driving pathway equilibrium of reversible reactions in the desired direction [3].

Thus far, most strategies in developing genetic regulators and synthetic scaffolds for metabolic pathway enhancement have seen limited success due to the inability to generalize the strategy and/or extend its use for increasingly complex metabolic systems. Traditional control of gene expression relies on a limited component box of small molecule-regulated activators and repressors [4]. While operon design and protein engineering can be used to further fine-tune expression, ultimately, the difficulty in expanding the number of protein components, particularly those sensitive to cellular/metabolic signals, serves as the major limitation. A similar problem exists with protein-based scaffolds for enzyme pathways [3]. Predictable programming of proteins still lies in its infancy, and thus successful protein-based executions have required time-intensive design and optimization with limited generalizability and inability to scale up to more complex metabolic networks.

Toehold-mediated strand displacement provides promising solution

To this end, nucleic-acid nanotechnology has emerged as a promising solution to achieving programmable control of metabolic flux. Because nucleic acid base pairing and thermodynamic behavior are well characterized, they can be designed *de novo* to form complex structures [5]. Furthermore, through toehold-mediated strand displacement (TMSD) these structures can be programmed to behave dynamically in presence of specific biomolecular triggers [6,7]. TMSD is a thermodynamically driven process in which two nucleic acid strands hybridize to each other to displace a previously hybridized strand (Figure 1a). It is initiated at complementary single-stranded domains called toeholds. Upon toehold binding, a random walk process called branch migration results in

Figure 1



Toehold-mediated strand displacement.
(a) Basic mechanism of TMSD. Input A initiates binding to complex X through complementary toehold domains (red). Next, branch migration of the invading strand (input A) displaces the incumbent strand, resulting in a newly formed complex Y and output B. **(b)** Parallel and orthogonal multiplexing of TMSD is possible due to sequence specific nature of process.

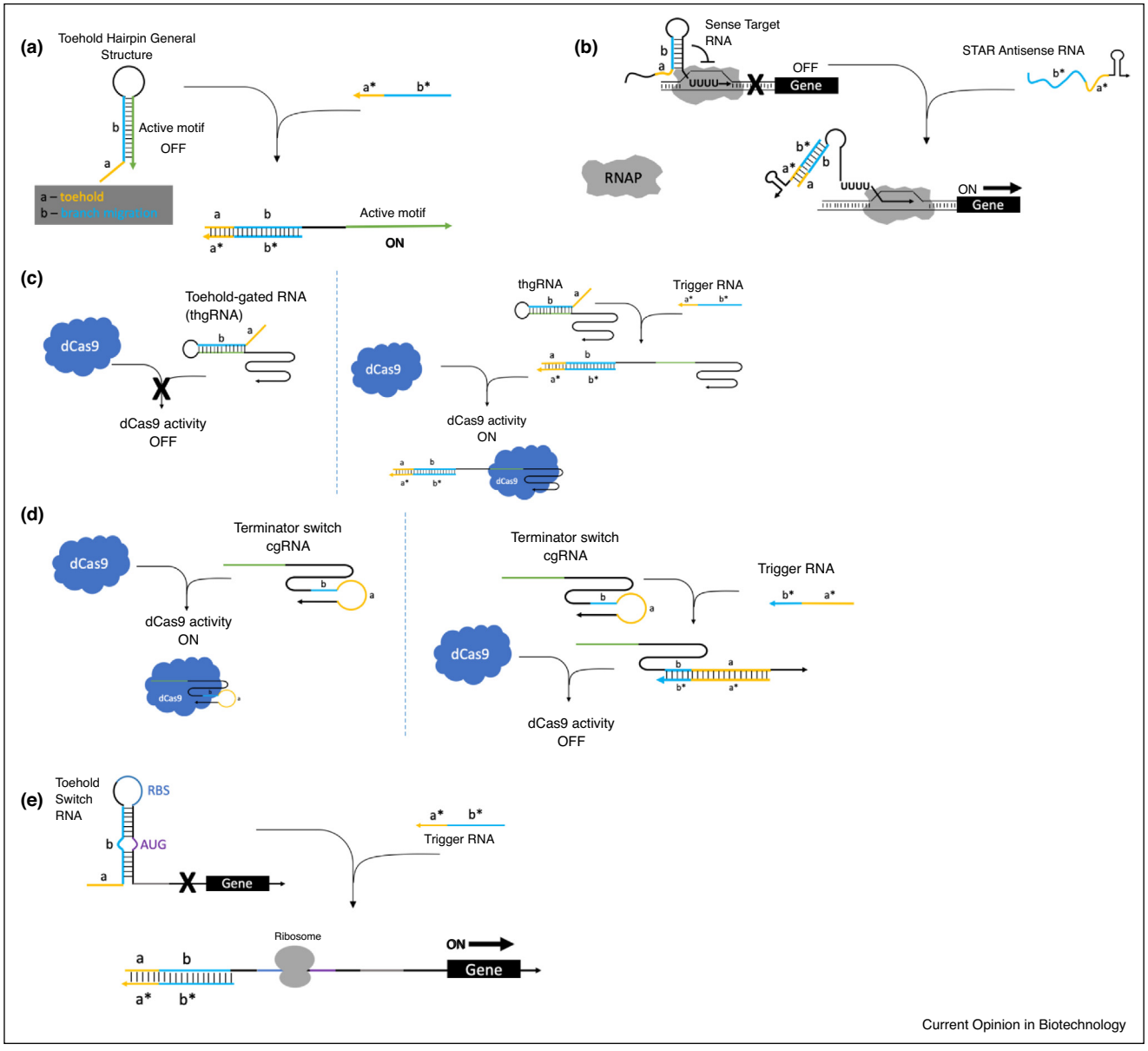
the displacement of the incumbent strand by the invader strand. By varying toehold length and composition, strand displacement rate constants can be modulated by over a factor of 10^6, and thus toehold-gated behavior can be finely tuned over a large dynamic range [8]. In addition, the sequence-specific nature of hybridization allows parallel and orthogonal multiplexing of toehold-gated devices (Figure 1b). By cascading strand displacement reactions, autonomous computation circuits can be rationally designed to exhibit a variety of behaviors that resemble digital circuitry [9–11]. Besides species-based reaction pathways, TMSD can be integrated into previously static nucleic acid nanostructures (tiled scaffolds [12], tweezers [13], origami [14], etc.) to give them dynamic behaviors. The power of TMSD has been unleashed with the advent of toehold-gated devices that dynamically control protein behavior and cellular machinery/processes.

Excitingly, toehold-gated approaches have been successfully implemented in several compelling facets of metabolic engineering. Compared to traditional metabolite responsive transcription factors, toehold-gated devices respond to changes in shorter time scales, allowing for the development of more responsive control systems. As genetic transducers, toehold-gated devices can be multiplexed with high dynamic range to control expression levels of enzyme networks, and as physical actuators (i.e. dynamic scaffolds), they allow for fast and dynamic reorganization of enzyme assemblies post-translationally. This review will discuss how the programmable and modular nature of toehold-based design signals the emergence of a powerful class of control components for metabolic flux.

Toehold-gated devices as genetic transducers

As nucleic acid constructs, toehold-gated devices inherently carry genetic information in their base sequences that can readily interface with gene expression machinery by hybridization with DNA or RNA triggers [7]. These interactions are dictated by simple sequence complementarity and, hence, highly specific for the trigger sequences. By exploiting this sequence specificity, we can theoretically design toehold-gated devices that respond to any given metabolic and environmental states as reflected by the abundance of native RNAs for activation or repression of gene expression. The general design of these molecular devices includes a linear, single-stranded domain ('toehold'), followed by a hairpin structure sequestering an active motif, for example, terminator, RBS, or CRISPR-Cas spacer (Figure 2a). Hybridization of the trigger strand initiates displacement reaction with the linear region, followed by the unwinding of the hairpin structure and the release of the active motif that allows the activated molecular device to interface with endogenous or synthetic cellular machinery. This ability to interface enables toehold-gated devices to act as

Figure 2



Regulation of gene expression using toehold-gated devices.
(a) General design for toehold-gated molecular devices with a short, single-stranded 'toehold' region followed by a hairpin structure sequestering the active motif. Toehold region (a, yellow) binds to complementary region on trigger RNA, undergoes TMSD through the process of branch migration (b, blue) and results in newly output secondary structure that reveals the active motif for downstream functionality. **(b)** Small Transcription Activating RNAs (STARs) function through TMSD to disrupt the formation of terminator hairpins that prevent gene transcription. **(c)** CRISPR-Cas9 conditional gRNAs are created through addition of motifs on the 5′ end of the gRNA that function to sequester the spacer region and through TMSD become activated. **(d)** CRISPR-Cas9 conditional gRNAs are created through the addition of motifs on the scaffold region of the gRNA. TMSD occurs and disrupts the structure of the gRNA resulting in non-functional gRNAs. **(e)** Toehold switch riboregulators halt protein translation due to a hairpin structure which is unfolded through TMSD and protein translation can occur.

genetic transducers that can couple a great multitude of metabolic signals to any number of expression changes at both the transcription and the translation level to shift cellular metabolic states.

Control of transcription through toehold-gated terminator hairpins within mRNAs

Transcriptional regulation of gene expression can be achieved by incorporation of synthetic structural motifs,

specifically switchable terminators, within mRNAs. Exploiting TMSD, the Lucks group has developed transcriptional activators, termed Small Transcription Activating RNAs (STARs) that prevent formation of terminator hairpins through binding of a *trans*-acting synthetic RNA [15] (Figure 2b). Several iterations of STARs have gradually improved their dynamic range from ~10-fold to >1000-fold with essentially background-level leakage through computer-aided *de novo* designs [16,17••]. Crucially, these impressive riboregulators were applied to control the multigene violacein pathway and perform logic gate functions, demonstrating their potential in changing metabolic fluxes in a multiplexed setting.

Control of transcription through toehold-gated gRNAs for CRISPR/Cas activation or repression

One of the recent successes in engineered transcriptional regulators is based on CRISPR-Cas and its unique use of a short gRNA for binding with its target DNA [18]. As numerous studies have shown, the gRNA scaffold is amenable to many changes to its sequence, including substitution, insertion, deletion, and extensions [19–21]. This malleability allows our group and others to incorporate toehold-gated motifs into the basic scaffold to create conditional gRNAs that enable control over CRISPR-Cas transcriptional regulators (Figure 2c,d). Engineered gRNAs demonstrated activation by TMSD mechanisms and performed logic gate functions *in vitro* [22,23]. Guided by extensive computational design, Hanewich-Hollatz *et al.* showed activation and repression of Cas9-based regulators in *Escherichia coli* and HEK-293 cells [24•], while Oesinghaus and Simmel created an analogous scheme using the Cas12a system in *E. coli* [25]. Although these studies show that TMSD strategies to control CRISPR-Cas systems are viable inside complex cellular environments, it remains important to demonstrate that they can be used to couple endogenous signals to multiple expression changes. To that end, our group has recently designed toehold-gated gRNAs capable of responding to endogenous sRNA signals in *E. coli* as well as multiplexed regulation over multiple genes [26••]. We envision the possibility of applying these engineered gRNAs for control over CRISPR-Cas systems to enact large shifts in metabolic pathways through transcriptional regulation of multiple genes, which is a necessity for dynamic control of complex pathways.

Control of translation through toehold-gated riboregulators

Beyond transcription, toehold-mediated designs have been used prominently to regulate translation. The seminal work by Green *et al.* to create riboregulators *de novo* led to a new class of riboregulators termed 'toehold switches' for translational activation in *E. coli* [27] (Figure 2e). The general design of these riboregulators proved to be modular and highly versatile, enabling the creation of large libraries capable of performing complex logic computation [28]. Recently, the same group has extended the design to create translational repressors [29••]. Unlike previous riboregulators, these toehold switches achieved dynamic ranges similar to protein-based regulators (~600-fold for activation and ~100-fold for repression). These high-performance attributes make toehold switches ideal candidates to couple endogenous signals to changes in translation rates and thus gene expression.

There has been some success in designing toehold-mediated devices to interface with endogenous RNA to provide synthetic control over native systems. The primary challenge of using endogenous RNA as the trigger strand is the inherent secondary structures of many RNAs. For toehold-mediated strand displacement to occur efficiently, it is often necessary to minimize the secondary structure of the trigger strand. Some groups have been able to use ectopically delivered RNA to provide the toehold switch for their device [24•,26••,27]. Other groups have been able to take advantage of endogenous sRNA and create devices with the ability to interface with the endogenous system [25,26••,27]. Another challenge presented by the native cellular environment is the abundance of nonspecific RNAs. To address this concern, devices that require multiple inputs in different combinations for activation can be advantageous to create artificial circuitry capable of complex logic behaviors, such as AND, OR, or NOT gates, that mimic natural regulatory processes. Several of the toehold devices have been designed to recognize two-input logic, including AND, OR, NOR, NAND gates and demonstrated success through *in vitro* assays but have not been applied in a cellular context [22,23]. Other groups have been able to show complex two-input logic in *E. coli* successfully, generally separating the trigger strand into two distinct hybridization regions [15,25,28,29••]. Impressively, the Yin group created a 12-input logic circuit using a combination of AND, OR, and NOT gates with their translational regulators [28]. These successes suggest that toehold-gated devices can be designed to function in a multiplexed setting and are capable of shifting large metabolic changes inside the cell.

Paralleling the substantial progress in creating toehold devices that can interact with endogenous or multiple RNAs, considerable successes in using these toehold devices to direct functional protein expression have also been made to create artificial devices that can control and direct native pathways. Many groups have shown the ability to design these devices regulating the expression of fluorescent reporters [15,16,17••,24•,26••,27,28,29••]. Furthermore, several groups used their toehold devices to successfully direct expression of an endogenous protein. The Lucks group used STARs to regulate the endogenous chemotactic regulator CheZ and control the multigene violacein pathway in *E. coli*, showing the

versatility of these regulators to function in diverse genetic contexts [17••]. Toehold switch riboregulators were able to control the expression of several different endogenous genes [27]. Both of the above toehold devices are required to be integrated into the mRNA strand of the desired target protein to function, as opposed to the toehold-gated motifs that are incorporated into the gRNA of the CRISPR/Cas-interfacing systems [22,23,24•,25,26••]. While they have yet to be used to regulate multigene metabolic pathways based on endogenous inputs, we expect these CRISPR/Cas-based devices to perform robustly in dynamic control schemes for metabolic engineering and other synthetic circuits.

Toehold-gated devices as physical actuators

The displacement or rearrangement of nucleic acids through TMSD can be harnessed to create physical actuators that can translate a DNA/RNA input into a change in metabolic flux by changing the physical proximity of proteins and cofactors to overcome the limits imposed by the diffusion rates of substrates. Control is achieved by designing complex DNA constructs whose structure changes through the initiation of TMSD to achieve the desired outcomes. *De novo* designs such as these allow for creation of fast response nanoreactors which are actuated through toehold-gated devices.

Spatial control of two proteins or cofactors through TMSD

The advent of DNA origami designs greatly contributed to the development of toehold-gated devices as physical actuators. Through DNA origami, complex 2D and 3D DNA structures can be created [5]. Site-specific control of enzymes, and cofactor localization on these nanostructures, allows precise enzyme activity and metabolic flux control. The most well studied DNA nanomachine utilizes a tweezer mechanism to bring two pieces of a metabolic pathway in close proximity to increase the flux. The DNA machine is designed with two DNA double crossover motifs which form two rigid arms (Figure 3a). The two rigid arms can open and close through a 4-way junction actuated by TMSD. By fusing glucose oxidase (GOx) and horseradish peroxidase to the two arms of the tweezer, the activity of the pathway could dynamically change by 30% from the open to the closed state multiple times [30]. Furthermore, the same nanomachine has been employed to increase NAD+ availability for GOx resulting in a fivefold increase in GOx activity between the open and closed states. With kinetics in the hour range, the nanoreactor offers precise control of biological reactions [31•]. Finally, through computational design, the tweezer on/off kinetics and tightness of states has been further improved up to 32% [32]. Simple DNA nanomachines using TMSD are straightforward to design and provide quick kinetics of physical actuation but are limited in complexity, often only having two locations on which proteins and cofactors can be attached.

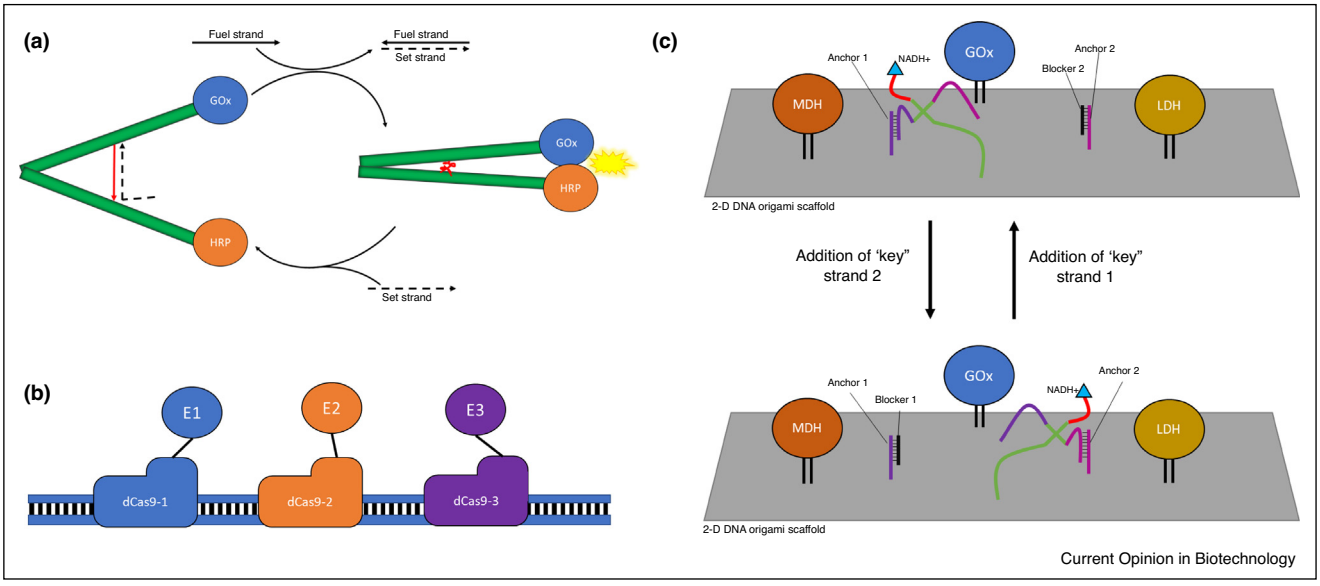
Spatial control through the use of linear DNA scaffolds expands potential uses

Linear DNA scaffolds in conjunction with toehold-gated devices can be used to dynamically assemble proteins. Using linear DNA scaffolds allows the implementation of complex logic circuits to translate multiple inputs in successful physical actuation. Furthermore, the longer scaffolds can be used to control flux through more complex metabolic pathways due to the availability of more locations onto which proteins can be attached (Figure 3b). However, with more complexity, slower kinetics and more design constraints can become a problem. Dynamic protein assembly upon addition of the ON strand and disassembly though the addition of the OFF strand on a linear ssDNA scaffold was demonstrated through FRET [33••]. The same system was used to successfully assemble an artificial cellulosome for cellulose hydrolysis *in vitro* and a split yeast cytosine deaminase protein (yCD) in HeLa cell lysate for targeted cell death [33••]. The system uses the input DNA or RNA in order to alter the spatial arrangement of target proteins and shows promise for *in vivo* applications since it can successfully operate in cell lysate with RNA inputs. A linear DNA scaffold can also be used to colocalize enzyme cascades by fusing the enzymes to orthogonal dCas9 proteins that use gRNAs designed to bind in adjacent locations on the DNA scaffold [34•]. The assembly can be made conditional through the use of toehold gated gRNAs, thus allowing precise control over when the scaffold assembles [34•]. Linear DNA scaffolds and TMSD actuation increase the capabilities of physical actuation by allowing the use of more complex circuitry and the control of more complex pathways.

Spatial control of a branched reaction pathway using complex DNA origami

More complex DNA origami structures increase the capabilities of TMSD as a physical actuator through the creation of bigger and more complex nanoreactors. While designing such nanoreactors can be more difficult than even linear DNA scaffolds, the ability to colocalize different pathway branches and more copies of the pathway enzymes in the same DNA structure can yield more dramatic flux control. The flux through the branched reaction pathway of GOx with either malate dehydrogenase (MDH) or lactate dehydrogenase (LDH) was achieved by placing all three enzymes on a 2-D DNA scaffold with 2 small blocked anchors (between MDH and GOx and GOx and LDH) (Figure 3c). The NAD+ cofactor, which is reduced by GOx and then reoxidized by either MDH or LDH was attached to a four-way Holliday junction that can bind to either one of the anchors in the presence of the appropriate 'key' strand [35]. Substrate diffusion has also been controlled through TMSD by creating a DNA nanochannel containing the GOx and HRP enzyme cascade. The channel can be opened with a 'key' strand and closed with a 'lock' strand, thus

Figure 3



Toehold gated devices as physical actuators.
(a) Architecture of the DNA tweezer nanomachines. The green rectangles represent the two rigid arms of the nanomachine. They are composed of DNA strands that form two double helixes on each arm that are attached to each other in two locations. The two arms are connected by a regulatory oligomer (shown in red). In the absence of the set strand the regulatory oligomer adopts a stem-loop hairpin structure that closes the tweezer and brings the two arms in proximity. The set strand contains a toehold so upon the addition of the fuel strand the set strand is sequestered from the nanomachine through TMSD. Thus, the nanomachine can cycle between open and closed configurations. **(b)** Using a long DNA helix as a scaffold allows the colocalization of more than two proteins for increased flux through the pathway. Each protein of interest is fused to an orthogonal Cas9 protein. The gRNA binding sequences are designed to be close to each other. Assembly can be made dynamic by creating toehold gated RNAs that only allow the Cas9 proteins to bind them upon addition of the appropriate trigger strand. **(c)** Controlling flux through branched pathways using a 2-D scaffold. Proteins GOx MDH and LDH are attached to a 2-D DNA origami scaffold. The cofactor NADH+ necessary for both the GOx-MDH and the GOx-LDH pathway is attached via a 4-way Holliday junction to the 2-D scaffold as well. The cofactor can swing from one pathway branch to the other by addition of the corresponding 'key' strand that will open up the DNA anchor between the two enzymes of the pathway branch. Dynamic cycling between two branches of the pathway is thus achieved through TMSD.

controlling the diffusion of glucose into the channel [36••]. The GOx and HRP cascade has also been assembled on a DNA triangle prism by hybridizing DNA-protein constructs on the triangle prism. Through TMSD the proteins can dissociate and associate with the triangle-prism in a dynamic cycling that exhibits a threefold change in activity [37]. These DNA origami structures allow the potential to control branched pathways and substrate transport at the tradeoff of even more complex design principles and limitations.

Most of the work in developing toehold-gated physical actuators has been limited to *in vitro* applications so far, however the future for *in vivo* applications is promising. There has been success in using TMSD as an activator of RNAi *in vivo* by delivering DNA–RNA complexes that can dissociate from one another and form active dicer substrate RNA once in the cells to silence GFP, and the HIV-1 coat protein group specific antigen (Gag) [38•]. In the past, RNA origami technology has mainly exploited RNA tertiary structures that are formed cotranscriptionally. However, recently it has taken inspiration from its DNA counterpart, and more complex RNA origami structures have been created [39].

Conclusion/future outlook

Taken together, the feasibility of using toehold-gated devices as genetic transducers or physical actuators have created an emerging class of synthetic regulators that is only beginning to expand its capabilities. Although toehold-gated devices have successfully directed global protein expression through activation or repression of either transcriptional or translational processes with an impressive fold change, they are limited by the kinetics of these respective processes in endogenous cellular environments. Furthermore, while toehold-gated genetic transducers provide a powerful opportunity to create artificial cascades that can interface with endogenous RNAs, sequence constraints when utilizing toehold gated devices, particularly in conditional gRNAs, serve as a major obstacle. Many of these conditional gRNAs require sequestering regions through complementary hybridization that by nature introduce sequence constraints [24•,25,26••]. Encouragingly, there have been

Table 1

Comparisons between general approaches of toehold-gated devices

Characteristic	Genetic transducers	Physical actuators
Dynamic range	~2 to ~1000-fold	<2-fold to ~5-fold
Response time	~Hour	~Seconds to minutes
Multiplex-ability	Yes	Yes
Logic functions	Yes	Yes
Endogenous triggers	Demonstrated	Not yet demonstrated

successful strategies to overcome typical sequence constraints, but these have yet to be shown in a cellular setting [22,23]. A particularly promising approach to address these limitations are computational programs that predict the folding of RNA structures, which have aided the design of orthogonal pairs of toehold devices that have been implemented for multiplexed protein expression [17••,26••,27,28,29••].

TMSD has also been applied towards physical actuators to alter the proximity of various enzymes. To date, much of this work has been done *in vitro* and has used DNA nanotechnology. However, with an increasing understanding of RNA tertiary structure and computational power, *in vivo* RNA toehold-gated physical actuators designed to control intracellular metabolic fluxes is a near reality. These post-translational designs offer the potential to operate on short timescales with fast responses, generally on the order of minutes to a few hours. Such devices will allow for direct control of intracellular metabolic fluxes to maximize non-native pathway product titer without having to sacrifice cell viability.

Using toehold-gated devices as either genetic transducers or physical actuators offers great promise for controlling metabolic flux. However, as with more traditional metabolic flux control tools, introducing more complexity in the cellular environment can result in overburdened cells. It is important to understand that the control offered by toehold gated devices comes with an energy burden to the cells, which should be taken into consideration when applying these tools in new systems. While control is necessary in systems with bottleneck steps, toxic intermediates/products, and substrates required by native metabolism, in some cases simpler metabolic engineering approaches could prove more effective. The decision to use one approach over the other depends on the desired application and related requirements which could include dynamic range, response time, multiplex-ability, interfacing with the endogenous cellular environment, or creating an entirely artificial cascade (Table 1).

Conflict of interest statement

Nothing declared.

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- • of special interest
- •• of outstanding interest

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