Dynamic covalent DNA networks to translate multiple inputs into programmable outputs

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Abstract

Inspired by naturally occurring protein dimerization networks, in which a set of proteins interact with each other to achieve highly complex input-output behaviours, we demonstrate here a fully synthetic DNA-based dimerization network that enables highly programmable input-output computations. Our DNA-based dimerization network consists of DNA oligonucleotide monomers modified with reactive moieties that can covalently bond with each other to form dimer outputs in an all-to-all or many-to-many fashion. By designing DNA-based input strands that can specifically sequester DNA monomers, we can control the size of the reaction network and thus fine-tune the yield of each DNA dimer output in a predictable manner. Thanks to the programmability and specificity of DNA-DNA interactions, we show that this approach can be used to control the yield of different dimer outputs using different inputs. The approach is also versatile and we demonstrate dimerization networks based on two different covalent reactions: thiol-disulfide and strain-promoted azide-alkyne click (SPAAC) reactions. Finally, we show here that the DNA-based dimerization network can be used to control the yield of a functional dimer output, ultimately controlling the assembly and disassembly of DNA nanostructures. The dynamic covalent DNA networks shown here provide a way to convert multiple inputs into programmable outputs that can control a broader range of functions, including ones that mimic those of living cells.

Introduction

The living cell is an impressive and inspiring example of how highly developed functions can emerge from a system of reacting and interacting molecules. While the inner workings of a cell are still being unraveled, there is growing interest in the development and construction of systems that perform some of the many functions of life from scratch using the same basic ingredients: the formation of covalent bonds and non-covalent interactions. These efforts have shaped the field of systems chemistry[1–3] and have led to synthetic systems that can move,[4–6] replicate,[7–9] evolve[10,11] and metabolize.[12,13] Further development of this field requires systems that are capable of processing information and enabling communication between different components, which is necessary for their proper integration into higher-level systems.

The topic of how molecular networks that combine chemical reactions with non-covalent interactions can process information at the molecular level has received comparatively little attention. Examples include work on how molecular recognition events propagate through dynamic covalent reaction networks or combinatorial libraries[14–16] in which simple monomer units oligomerize and reversibly exchange monomers.[17–20] While these cases demonstrate the potential of dynamic molecular networks to respond to specific molecular inputs, they are somewhat limited in terms of programmability and predictability.[21] The advancement of these aspects places ever-increasing demands on the specificity and tunability of molecular recognition events.

More recently, the exquisite predictability and sequence-specificity of DNA/DNA hybridization has enabled the construction of DNA-based reaction networks,[22] nanostructures[23,24] and circuits[25,26] that can process different inputs to provide predictable and programmable outputs in a modular fashion.[27] In these systems,

multiple DNA-based reactions (e.g. strand displacement) are used to create logic gates[28,29] and neural networks[30,31] that can process inputs and deliver outputs through complex signaling pathways. Compartmentalization of these systems can also lead to a higher level of computation capabilities.[32-34] DNA-based constitutional dynamic networks (CDNs) that enable adaptive behavior, increased dimensionality, and communication between different catalytic networks by mimicking natural dynamic signaling processes have also recently been demonstrated.[35-40] The DNA-based networks described above are often based on non-covalent Watson-Crick interactions between the individual nucleic acid components, a property that enables predictable sequence-specific recognition and catalytic functionality (e.g. thanks to the use of DNAzymes) and allows different DNA-recognizing enzymes to be used as tools to control either the input or the output of the network.[35–40] However, despite the above advantages, the use of Watson-Crick interactions also entails an inherent limitation on the overall complexity that these networks can achieve, as each individual DNA-based component can only interact with a limited number of related components via complementary domains.

In nature, however, many naturally occurring circuits or networks consist of groups of components that interact with each other in an all-to-all, many-to-many or promiscuous manner, leading to greater programmability and versatility of the network's input-output computations.[41] For example, in a competitive dimerization network, families of monomeric proteins (inputs) compete with each other in various combinations to produce a series of dimer outputs.[42] Upstream signals or molecular cues can modulate the concentrations of the monomers and thus control the formation of the active dimers downstream. Such dimerization networks are ubiquitous in cells and often regulate genes involved in a variety of processes, including cell proliferation,

differentiation and hormone signaling.[43–46] Motivated by the above considerations, we demonstrate here a DNA-based competitive dimerization network in which, unlike other DNA-based networks, each monomer interacts in an all-to-all or a many-to-many manner through covalent reactions to produce a library of different outputs. By using sequence-specific inputs, this network can then perform complex input-output computation in a highly predictable and programmable manner.

Results and discussion

In this work, we first consider a competitive dimerization network consisting of m interacting monomers (M_a, M_b, …, M_m) that can covalently connect to each other to form a library of dimer outputs (D_{ab}, $D_{ac,\ldots}$, D_{mm}) (Figure 1a). Each pair of monomers has the same equilibrium constant for the formation of a dimer and (unless otherwise stated) each monomer has the same concentration, so that random formation of all possible dimer outputs can be expected (i.e. each dimer has a similar statistically determined probability of forming). In this situation, the number of possible dimer outputs increases as the size of the network increases according to the following equation (Figure 1b):

Eq. 1
$$\sum D_{ij} = \binom{m+k-1}{k}$$

where *m* is the network size (i.e. the total number of different monomers) and *k* is the output size (i.e. the number of monomers composing each output). So, in case of a dimerization reaction the output size is 2 and Eq. 1 can be simplified as:

Eq. 2
$$\sum D_{ij} = \frac{m^2+m}{2}$$

As the network size increases, the yield (%) of a specific dimer (defined here as the target dimer output, D_{ij}) thus decreases according to the following function (Figure 1c):

Eq. 3 $$D_{ij}\ Yield\ (\%) = \frac{\Omega}{m^2} * 100$$

Where Ω is 1 in case of a homodimer output and 2 in case of a heterodimer output.

The addition of molecular cues (i.e. inputs) that specifically bind and sequester some of the monomers would reduce the overall size of such competitive dimerization network and the number of possible dimer outputs (Figure 1d). Thus, the addition of inputs (in this case we consider saturating concentration of each input) leads to an increase in the overall yield of the target heterodimer output as described by the following equation (Figure 1e-h):

Eq. 4 $$D_{ij}\ Yield\ (\%) = \frac{2}{(m - x * i)^2} * 100$$

Where x is the number of different monomers excluded by each input, i.

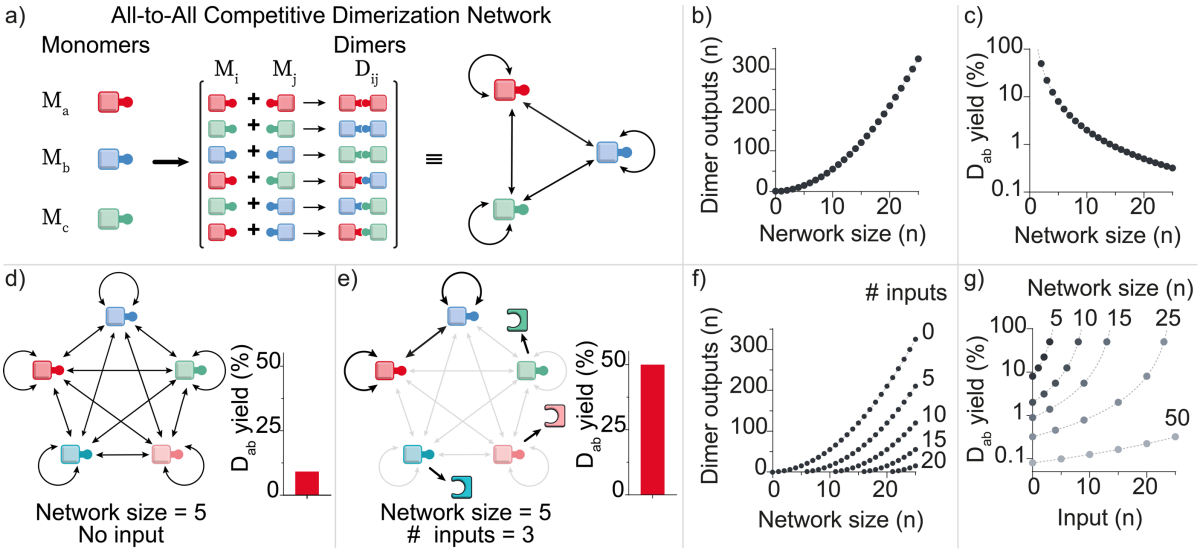

Figure 1. *a) A competitive all-to-all dimerization network consists of a series of monomers, each of which is capable of reacting with each other to form a library of dimers. b) The network size (number of different monomers) determines the number of possible dimer outputs and c) the theoretical yield (%) of a particular dimer output (here the heterodimer D_{ab}). d) An all-to-all dimerization network with 5 monomers leads to an expected yield of the heterodimer output D_{ab} of about 8% (bar chart). e) The same all-to-all dimerization network (5 monomers) with 3 inputs that sequester 3 monomers from the network leads to an expected yield of the same heterodimer D_{ab} output of about 50%. f) Diagrams of the number of possible dimer outputs compared to network sizes with different numbers of inputs. g) Diagrams of the yield of the heterodimer output D_{ab} compared to the number of inputs for a fixed network size (5, 10, 15, 25 and 50).*

To establish a DNA-based all-to-all competitive dimerization network, we designed and synthesized a set of single-stranded DNA oligonucleotide monomers (length between 8 and 22 nts), each with a specific sequence and modified with a thiol group (i.e. R-C_6-SH) either at the 3'-end or at the 5'-end (Figure 2a). Under oxidizing conditions, such a dimerization network can induce the formation of a library of disulfide dimer outputs. We have identified one of the possible dimer outputs (here the heterodimer D_{ab}) as the "target" output. We can measure the formation (and thus the overall yield) of such a target output under different experimental conditions by a strand displacement reaction with an optically labeled DNA duplex (Figure S1-S2). For example, we performed experiments with DNA-based dimerization networks of different sizes (different number of monomers) using equimolar concentrations of each

monomer (i.e. 1.0 μM) and found that the yield of the target dimer D_{ab} decreases from 350 ± 60 nM to 70 ± 20 nM when we increase the network size from 2 to 10 monomers (Figure 2b). As expected, the observed yield of the target for the different network sizes agrees well with the theoretical yield (Figure 2b, dotted line).

We can rationally control the yield of the dimer output D_{ab} by introducing into our dimerization network different molecular inputs that act as specific sequesters of certain monomers. To this end, we designed and synthesized DNA strands with a sequence that is fully complementary to that of two monomers, such that a single input is able to exclude two monomers from the dimerization network (Figure 2c). With a dimerization network size of 10 (using an equimolar concentration of monomers and inputs of 1.0 μM), by adding 1 to 4 inputs we were able to control the yield of the target dimer D_{ab} from 70 ± 20 nM to 390 ± 70 nM, respectively (Figure 2d). Also in this case, the observed yield of the dimer output agrees very well with the expected yield under each experimental condition used (Figure 2d, dotted line).

The network size can also be controlled by tuning the concentration of each monomer in the reaction mixture. To demonstrate this, we prepared a dimerization network with 4 different thiol-DNA monomers in which two monomers (M_c, M_d) display a 4-fold higher concentration compared to the two output-forming monomers (M_a, M_b). Under these conditions the expected yield of D_{ab} will be the same as that expected in a network size of 10 monomers under equimolar conditions. We can control the yield of the dimer output D_{ab} by varying the concentration of the single input sequestering the two non-functional monomers. More specifically, by increasing the input concentration from 0.50 to 10 μM we were able to increase the yield of the target dimer D_{ab} from 60 ± 10 nM to 270 ± 30 nM (Figure S3).

Our DNA-based dimerization network allows to achieve highly programmable input-output computation by the rational design of different sets of inputs. To this end, we employed the same dimerization network (size = 10) described before (Fig. 2d) and we selected 3 different target heterodimer outputs (i.e. D_{ab}, D_{cd}, D_{ef}). We then synthesized 3 different sets of inputs (each set displaying 4 inputs) to induce the controlled upregulation of such dimer outputs in the same dimerization network (Figure 2e-f). The upregulation is specific and orthogonal, so a different upregulated dimer output can be achieved by simply changing the input set (Figure 2g). We can also upregulate in the same solution two different dimer outputs, although with a slightly lower efficiency, by reducing the number of inputs (i.e. 3) in each input set (Figure S4). Similar orthogonal and programmable upregulation of different dimer outputs using different set of inputs can also be achieved with larger network size (i.e. 30 monomers) providing a further demonstration of the computational ability of such DNA-based dimerization networks (Figure S5).

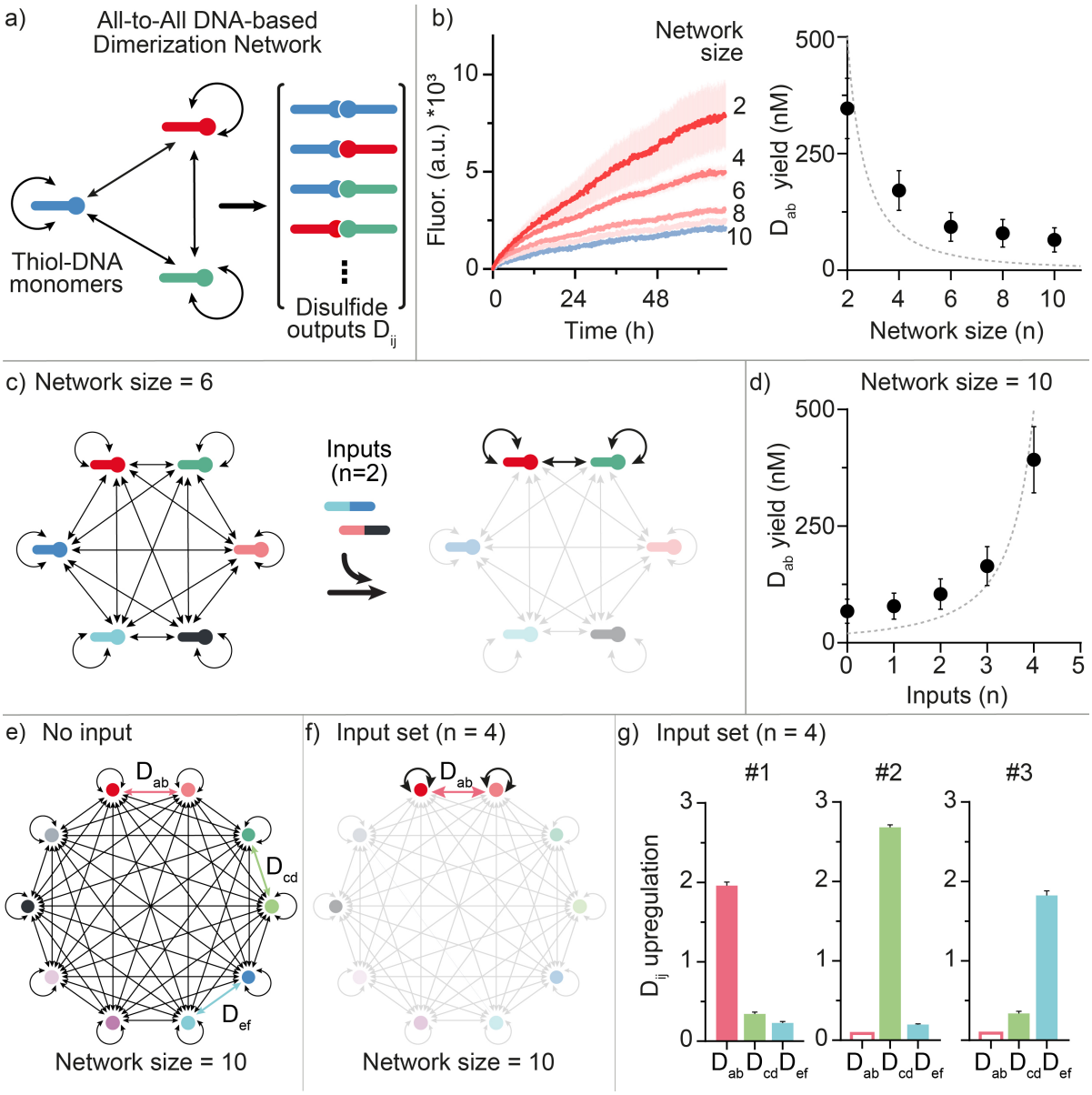

Figure 2. DNA-based dimerization network by disulfide formation. *a) Each monomer of the network is a thiol-modified single-stranded DNA oligonucleotide. The formation of the disulfide bond generates the dimer outputs. b) (left) Kinetics of the formation of the target dimer output (D_{ab}) for different network sizes (2, 4, 6, 8 and 10 monomers); (right) yield of the target dimer output (nM) as a function of network size. The dashed line represents the theoretical yield. c) The input strand hybridizes a monomer pair and excludes it from the network so that only the free monomers can react and form the dimer outputs. d) Yield (nM) of the target dimer (D_{ab}) as a function of the number of inputs (network size = 10). The dashed line represents the theoretical yield. e) Schematic of an all-to-all dimerization network formed by 10 different thiol-DNA monomers. f) For a fixed network size (n = 10), it is possible to use different sets of inputs (each set contains 4 different inputs) to induce upregulation of a different target dimer output. g) Yield of 3 different dimer outputs (D_{ab}, D_{cd}, D_{ef}) using 3 different sets of inputs. The experiments shown in this figure were performed in 1×TAE, 12.5 mM $MgCl_2$, pH 8.5. Each thiol-DNA monomer and each input was used at a concentration of 1.0 μM and the dimerization reaction was started adding 1.0 mM of $NaBO_3$. Reaction mixtures also contain the reporters for quantification of the dimer output yield. Error bars represent the standard deviation based on triplicate measurements.*

To demonstrate the versatility of DNA-based dimerization networks, we designed and synthesized new modified DNA sequences to create a many-to-many dimerization network that employs a different chemical reaction. More specifically, we synthesized ss-DNA monomers modified with either a dibenzocyclooctyne (DBCO) group or an azide group at one of the two ends of the strands, such that a spontaneous and irreversible Strain-promoted azide-alkyne cycloaddition (SPAAC) reaction between these two reactive groups would lead to a dimer output formed by an azide-DBCO conjugate (Figure 3a). In this case, the formation of homodimers is not possible, so the total number of possible interactions is reduced and the dependence of the number of possible dimer ouputs on the size of the dimerization network follows the equation below:

Eq. 5 $$\sum D_{ij} = m\,n$$

Where *m* is the number of monomers modified with DBCO and *n* is the number of monomers modified with azide.

Also in this case, the yield of a selected target dimer (D_{ab}) was measured across different network sizes with a specific strand displacement reaction, and the experimental results are in good agreement with the expected yield under each tested condition (Figure 3b). We can modulate and control the yield of the target dimer output by introducing DNA inputs that, by binding to specific monomers, exclude them from the network and thus upregulate the formation of the target dimer output. To demonstrate this, we designed inputs that can specifically bind to two monomers, and we chose a network size of 10 monomers (Figure 3c). By adding 1 or 4 input strands, we were able to up-regulate the D_{ab} dimer output from 60 nM $\pm$ 20 nM to 220 $\pm$ 20 nM

(Figure 3d). Also in this case, the input-output behaviour of this DNA-based dimerization network is highly programmable, so that the formation of different target dimer outputs can be upregulated by different input sets (Figure 3e). We were also able to upregulate in the same solution two different dimer outputs by reducing the number of inputs (i.e. 3) in each input set (Figure S6).

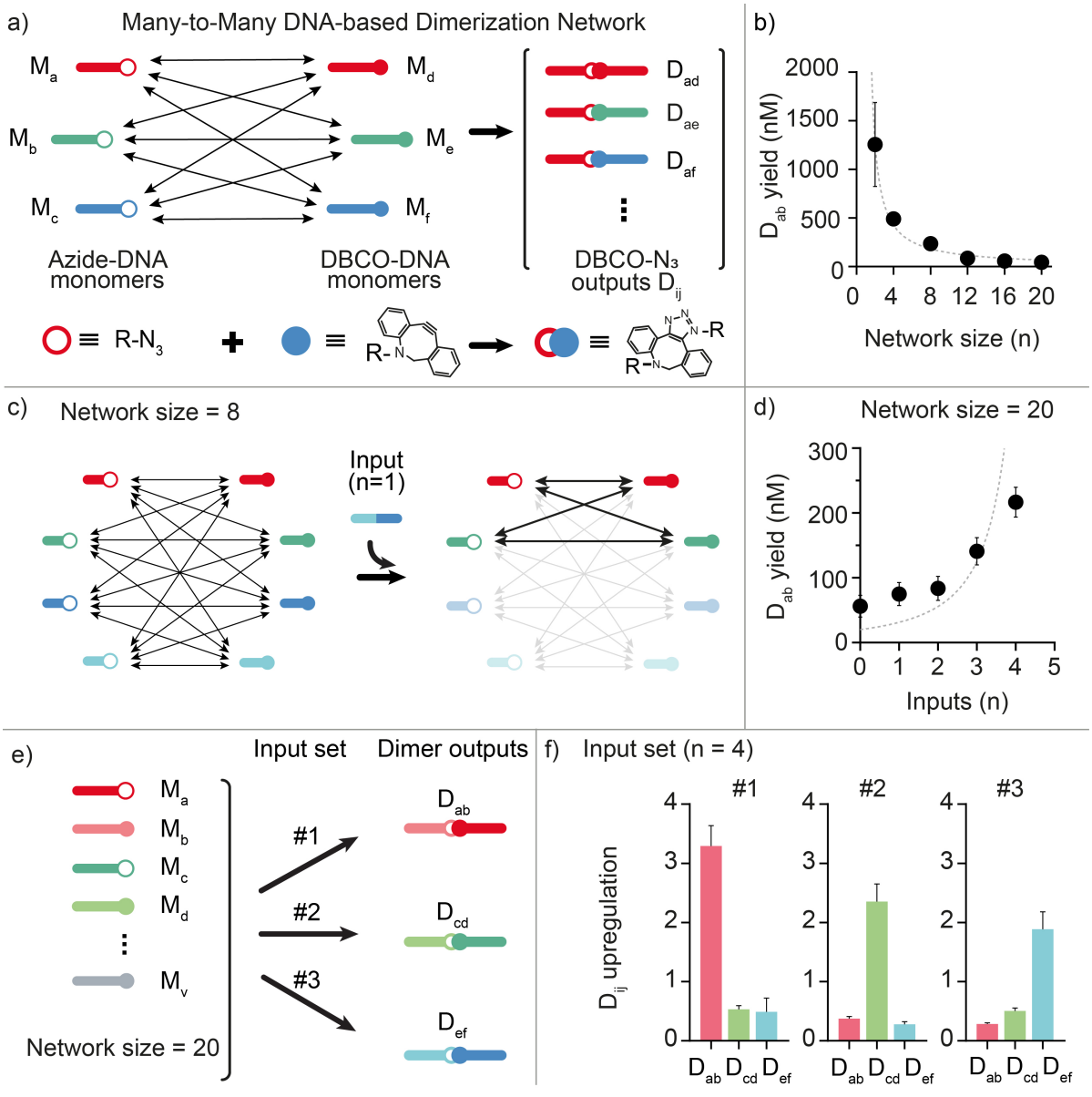

Figure 3. DNA-based dimerization network through SPAAC reaction. *a) Each monomer of the network is a single-stranded DNA oligonucleotide modified with either azide or DBCO. The formation of the DBCO-azide conjugate generates the dimer outputs. b) Yield of dimer outputs (nM) as a function of network size. The dashed line represents the theoretical yield. c) The input strand hybridizes two pairs of monomers and excludes them from the network so*

that only the free monomers can react and form the dimer outputs. d) Yield of target dimers (nM) as a function of the number of inputs. The dashed line represents the theoretical yield. Here the network size is 10. e) With a fixed network size (n = 10), it is possible to use different sets of inputs (4 different inputs in each set) to induce upregulation of a different target dimer output. f) Yield of 3 different dimer outputs (D_{ab}, D_{cd}, D_{ef}) after addition of 3 different sets of inputs. The experiments shown in this figure were performed in 1×TAE, 12.5 mM $MgCl_2$, pH 8.5. Each DNA monomer and each input was used at a concentration of 0.50 μM. Error bars represent the standard deviation based on triplicate measurements.

Next, we tested whether our DNA-based dimerization network can be used to control downstream reaction pathways. To do this, we used an all-to-all dimerization network with 10 thiol-modified DNA monomers similar to that shown in Figure 2. We designed two of the monomers so that their dimerization generates an output strand that can trigger the downstream disassembly of a DNA-based nanostructure (Figure 4a). More specifically, we used as DNA nanostructure a tubular object formed by the self-assembly of DNA "tiles" through hybridization of their complementary "sticky ends".[46] These structures self-assemble at room temperature and can be disassembled by introducing a DNA strand that binds to the tiles and "invade" the sticky end.[47,48] This type of assembly and disassembly mechanism can be easily monitored by labelling a tile-forming strand with a fluorophore so that the DNA structure can be visualized by fluorescence microscopy. Under competitive dimerization conditions, our dimerization network generates a dimer invader concentration (i.e. 40 $\pm$ 10 nM) that is not sufficient to observe significant disassembly of DNA structures (considering that the concentration of DNA tiles in solution is 100 nM) (Figure 4b). This is consistent with control experiments showing that under the experimental conditions used, a minimum concentration of 300 nM of invader DNA strand is required to observe disassembly of the DNA structures (Figure S8). Only with the addition of the set of input strands (n=4) required to upregulate the dimer invader strand, can the complete disassembly of the DNA tubes be observed over time (Figure

4b). The successful disassembly is demonstrated both by the reduced number of DNA structures (i.e. defined here as count per mm^2), which changes from 8 ± 1 to 2.0 ± 0.6 in the presence of the input set, and the reduced density of assembled tiles (from 1.4 ± 0.2 to $0.18 \pm 0.06 \times 10^7$ count / mm^2) (Figure 4b).

The versatility of the DNA dimerization network is again demonstrated by using the same network (size = 10) with three different input sets to allow orthogonal upregulation of three different output dimer invaders, each capable of specifically disassembling a different DNA structure (Fig. 4c). To this end, we first designed and characterized three DNA-based structures, each formed by the self-assembly of three orthogonal DNA tiles. To allow easy characterization of these structures, each tile was labelled with a different fluorophore with non-overlapping emission and excitation wavelengths. Each of these structures can be disassembled by a specific dimer output invader strand (i.e. D_{ab}, D_{cd}, D_{ef}). By introducing the different inputs into the dimerization network, we were able to upregulate only one of the three specific dimer invaders and thus disassemble one of the three coexisting DNA structures in solution (Figure 4d).

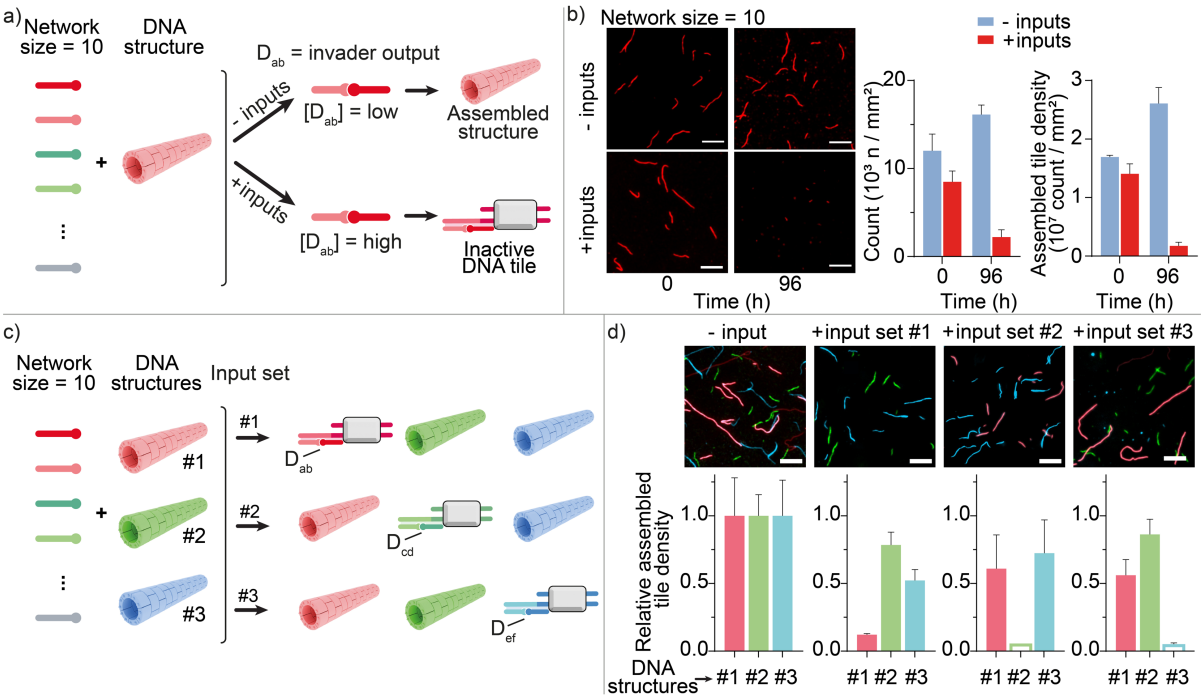

Figure 4. DNA dimerization networks for the control of DNA structures. *a) Schematic representation of a dimerization network of thiol-DNA monomers that produces, among other outputs, a dimer (Dab) that induces the disassembly of a DNA structure. Only in the presence of inputs, such an invader output is upregulated and DNA structures disassembled. b) Fluorescence images and histograms showing the count and the density of assembled tiles obtained with the dimerization network (size = 10) in the absence and presence of inputs (at 0 and 96 h). c) Upregulation of three different dimer invader outputs in the same dimerization network (n = 10) using three different sets of inputs in a mixture containing three different DNA structures (#1, #2, #3). Each dimer invader output is designed to specifically induce disassembly of only one of the three DNA structures. d) Fluorescence images and relative density of assembled tiles for each of the three structures obtained with the dimerization network (size = 10) in the absence and presence of the three sets of inputs (at 72 h). The experiments shown in this figure were performed in 1×TAE, 12.5 mM $MgCl_2$, pH 8.5. Each thiol-DNA monomer and each input was used at a concentration of 1.0 μM. The DNA structures were used at a concentration of DNA tiles of 100 nM and the dimerization reaction was started adding 1.0 mM of $NaBO_3$. Error bars represent the standard deviation based on triplicate measurements.*

Conclusions

It is now known that most cellular metabolic pathways consist of simple elements that interact with each other and can process input information (encoded as molecular or environmental signals) in a highly flexible and complex manner.[44,50] This type of input-output computation is very common in cells and can provide higher level functions in signal transduction,[50,51] adhesion[52,53] and transcriptional regulation. [54,55]

One of the most intriguing of these computational mechanisms, namely protein competitive dimerization, involves a set of proteins that are able to interact with each other in a many-to-many or an all-to-all manner. Such protein-based promiscuous networks can provide powerful computational capabilities that are particularly crucial in multicellular organisms,[43,56] and their importance in natural biological contexts is currently being uncovered [42].

Inspired by this mechanism, we demonstrate here a synthetic DNA-based dimerization network consisting of a series of DNA oligonucleotides modified with reactive moieties that can covalently bind to each other either in an all-to-all or in a many-to-many fashion. We have shown that such networks can be constructed with either reversible or irreversible covalent bonds. In this way, we demonstrate that highly programmable input-output computation can be achieved, allowing the yield of a given DNA dimer output to be controlled in a predictable manner. We also show that DNA-based dimerization networks can be used to modulate the yield of a functional dimer output to ultimately control the assembly and disassembly of synthetic DNA nanostructures.

Compared to other examples of DNA-based networks and circuits where hybridization of base pairs drives both input recognition and output formation,[26-35] here we present an alternative strategy that could improve the computational capability of these systems. Our approach exploits the predictability of DNA-DNA interactions to achieve input specificity. However, unlike previously demonstrated DNA networks, it utilizes covalent reactions between the DNA-based reactive units of the network to create a larger chemical space for output generation. Combining the programmability of DNA hybridization with the ability to explore different reactive functionalizations of DNA strands could provide a simple route to developing more complex promiscuous

architectures and networks that lead to a broader range of functions and can be used to develop synthetic systems that mimic functions of living cells. For example, it would be interesting to create DNA-based dimerization networks similar to those shown here, where the DNA output is capable of controlling a relevant biochemical pathway. Given the central role of DNA in genetic circuits, the most obvious application of a similar network could also be the development of synthetic genetic networks capable of converting specific inputs into the expression of an output protein.

Experimental Section

Chemicals

All reagent-grade chemicals, including $MgCl_2$, Trizma Base, ethylenediaminetetraacetic acid (EDTA), NaCl, sodium bicarbonate $NaHCO_3$, tris(2-carboxyethyl) phosphine hydrochloride (TCEP) and sodium perborate ($NaBO_3 \cdot 4H_2O$) were purchased from Sigma-Aldrich (Italy) and used without further purification.

Oligonucleotides

Oligonucleotides employed in this work were synthesized, labelled, and HPLC-purified by Metabion International AG (Planegg, Germany) and used without further purification. The DNA oligonucleotides were dissolved in phosphate buffer (50 mM, pH 7.0) and stored at -20 °C until use. All the sequences of the different systems are reported in the Supporting Information.

DNA-based dimerization network by disulfide formation

To ensure the absence of unnecessary thiol groups in the samples, disulfide-DNA dimers were used to generate thiolated-DNA monomers. Each disulfide-DNA dimer

(20 μM) was reduced overnight with a solution of 1.0 mM TCEP, prepared in TAE +12.5 mM $MgCl_2$, pH 8.5, at room temperature, to allow quantitative reduction of disulfide bonds. After reduction, these solutions were mixed and diluted to a final concentration of 1.0 μM in the same buffer, prior dimerization. The dimerization process is induced by adding an oxidizing agent to the sample (1.0 mM $NaBO_3$). Both TCEP and $NaBO_3$ were freshly prepared before use.

DNA-based dimerization network through SPAAC reaction.

The dimerization reaction is carried out at room temperature using a bicarbonate buffer (50 mM $NaHCO_3$ + 1.0 M NaCl, pH 8.6). Two separate solutions, each containing 1.0 μM of DBCO-DNA monomers and azide-DNA monomers, are prepared in the same bicarbonate buffer. Equal volumes of these solutions are then mixed in a 1:1 ratio to initiate the SPAAC reaction.

Fluorescence Experiments

Fluorescence kinetic measurements were carried out on a Tecan F200pro plate reader using the top reading mode with black, flat bottom non-binding 384-well plates and a 30 μL final volume. Detailed procedures employed are reported in the Supporting Information.

Self-Assembly of DNA nanostructures

The tile design and sequences employed in this study are described elsewhere.[46-48] Briefly, DNA tiles for all the systems were prepared as follows: tile-forming strands were mixed at a final concentration of 5.0 μM in H_2O/Mg^{2+} (12.5 mM $MgCl_2$), and annealed using a thermocycler (Bio-Rad T100™ thermal cycler) by heating the

solution to 90 °C and cooling it to 20 °C at a constant rate for a 6-h period. The concentrations employed and buffer conditions for DNA nanostructure disassembly are reported in the caption of the corresponding figure and in the supplementary information.

Fluorescence Imaging of DNA-based nanostructures

An Axio Observer 7 ZEISS microscope was used for fluorescence microscopy imaging. The images were acquired with a 100 × oil objective and a monochrome CCD camera (Axiocam 305 mono-ZEISS). Images were analyzed and processed to correct for uneven illumination and superimposed to produce multicolor images using ZEN-3.3 lite (ZEISS) software. Average length and count of assembled scaffolds were quantified by image metrology using SPIP software.

Acknowledgment

This work was supported by the European Research Council, ERC (project n.819160 to FR and n. 724863 to RS), by Associazione Italiana per la Ricerca sul Cancro, AIRC (project n. 21965) (FR) and by “PNRR M4C2-Investimento 1.4- CN00000041” financed by NextGenerationEU.

Associated Content

The Supporting Information describes the oligonucleotide sequences used, image analysis protocols and supplementary figures.

Notes

The authors declare no competing financial interest.

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