Designs for Autonomous Unidirectional Walking DNA Devices

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Abstract

Imagine a host of nanoscale DNA robots move autonomously over a microscale DNA nanostructure, each following a programmable route and serving as a nanoparticle and/or an information carrier. The accomplishment of this goal has many applications in nanorobotics, nano-fabrication, nano-electronics, nano-diagnostics/therapeutics, and nano-computing. Recent success in constructing large scale DNA nanostructures in a programmable way provides the structural basis to meet the above challenge. The missing link is a DNA walker that can autonomously move along a route programmably embedded in the underlying nanostructure – existing synthetic DNA mechanical devices only exhibit localized non-extensible motions such as bi-directional rotation, open/close, and contraction/extension, mediated by external environmental changes.

We describe in this paper two designs of autonomous DNA walking devices in which a walker moves along a linear track unidirectionally. The track of each device consists of a periodic linear array of anchorage sites. A walker sequentially steps over the anchorages in an autonomous unidirectional way. Each walking device makes use of alternating actions of restriction enzymes and ligase to achieve unidirectional translational motion. We describe the construction of each walking device both using conceptual enzymes to illustrate the general design principle and using commercially available enzymes to demonstrate its practicality.

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1 Introduction

A major challenge in nanotechnology is to precisely transport a nanoscale object from one location on a nanostructure to another location following a programmable path. DNA has been explored as an excellent building material for the construction of both large scale nanostructures and individual nanomechanical devices [10]. The successful constructions of two dimensional DNA lattices and one dimensional DNA arrays made from DX molecules [15], TX molecules [5], rhombus molecules [7], and 4x4 molecules [16] provide the structural base for realization of the above goal. However, the existing DNA nanomechanical devices only exhibit localized non-extensible motions such as open/close [12, 13, 19], extension/contraction [1, 4, 6], and reversible rotation motion [8, 17]. Furthermore, these motions are not autonomously executed but rather mediated by external environmental changes such as the addition and removal of DNA fuel strands [1, 4, 6, 12, 13, 17, 19] or the change of ionic strength of the solution [8]. Autonomous unidirectional DNA devices executing linear translational motions are hence desirable.

There are already some exciting progress in this direction. Turberfield and colleagues have proposed to use DNA fuels to design autonomous free running DNA machines [14]. Reif has described theoretical designs of autonomous DNA walking and rolling devices that demonstrate random bidirectional translational motion along DNA tracks [9]. On the experimental side, Mao's group has recently constructed an autonomous DNA motor powered by a DNA enzyme [3]; Seeman's group has constructed a DNA walking device mediated by DNA fuel strands [11].

In the rest of the paper, we present two designs of autonomous DNA walking devices. Each device consists of a track and a walker. The track of each device contains a periodic linear array of anchorage sites. A walker sequentially steps over the anchor-

ages in an autonomous unidirectional fashion. Each walking device makes use of alternating actions of restriction enzymes and ligase to achieve unidirectional translational motion. The action of ligase consumes ATP as energy source. The walking devices described here make the following improvements over the walking device presented in [9]. Firstly, they demonstrate unidirectional motion rather that random bidirectional motion. Secondly, the moving part (walker) in each walking device is a physical entity with a flexible body size rather than a symbolic entity, and thus the walker can serve not only as an information carrier but also as a nanoparticle carrier. These walking device designs are also different from the walking device construction by Seeman's group [11] in that they are autonomous. A limitation of our first device is that it has a low probability of falling off the track. Our second device has zero probability of falling off the track, but it is a more complicated (hence less practical) construction and assumes a restriction enzyme property that has not yet been fully-substantiated. For each walking device, we first present its structure and operation, and then describe its implementation using conceptual enzymes followed by one or more concrete examples using commercially available enzymes. The design using conceptual enzymes illustrates the general principle of the design and reveals the essential information encoding of the device that dictates its operation, while the examples using real enzymes both validate the practicality of the design principles and illustrate some technical complications in mapping the conceptual design to real enzymes.

2 Definitions

A basic structural unit used in the construction of the walking devices is a *dangler*. A dangler is a duplex DNA fragment with single strand extensions at both ends: one end is the *fixed end* that is usually attached to another structural unit (*e.g.* the backbone of the track or the body of the walker); the other end is the *sticky end*. The flexible single strand DNA at the fixed end allows the otherwise stiff dangler to move rather freely around the fixed end. This property is crucial to the operation of the devices. The fixed end only serves to structurally join a dangler to another component of the device in a flexible fashion (*e.g.* the linkage of an anchorage to the backbone of the track/the linkage of a foot to the body a walker); the sticky end, in contrast, usually encodes information and participates actively in dictating the motion of the walker.

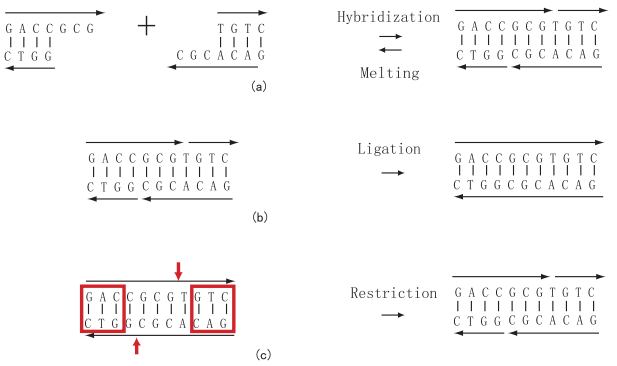


Figure 1: (a) Hybridization and melting. (b) Ligation. (c) Cleavage.

Two basic operational events driving the unidirectional motion of the devices are *ligations* and *cleavages*. Two neighboring danglers with complementary sticky ends can associate with each other via the hybridization of their sticky ends. Subsequent to this hybridization, the nicks at either end of the hybridized section can be sealed by a *ligase* and the two duplex fragments are joined into one in a process referred to as *ligation*. When the context is clear, the whole process of hybridization and subsequent ligation (joining of two DNA strands) is referred to as ligation, for simplicity. See Figure 1 (a) and (b) for schematic illustrations of hybridization and ligation, respectively. In *cleavage*, an approximately reverse process to ligation, a duplex DNA fragment is cut into two separate duplex parts (with each usually possessing a complementary sticky end) by enzymes known as *restriction endonucleases*. Following cleavage, the two duplex DNA fragments (each with a sticky end) can go apart in a process known as *melting*. When the context is clear, the whole process of cleavage and subsequent melting is referred to as cleavage. See Figure 1 (c) and (a) for schematic illustrations of cleavage and melting, respectively. Note that melting and hybridization are in dynamic balance as shown in Figure 1 (a). Cleavage by an endonuclease usually requires that the substrate DNA fragment contains *recognition site* (specific DNA sequences) corresponding to the endonuclease and that the cleavage happens at specific *cleavage site* along the DNA fragment. There are a rich set of restriction enzymes. Figure 2 illustrates three

types of restriction enzymes. Figure 2 (a), (c), and (e) describe the conceptual restriction enzymes that will be used in the construction of our devices. In this figure, r is the length of the recognition site in number of bases; d and e are parameters (in number of bases) that dictate the cleavage patterns. In Figure 2 (a), the value $d+e$ is also a parameter constituting the recognition site: $d+e$ has to be a specific value for a given restriction enzyme. Figure 2 (b), (d), and (e) show examples of corresponding real enzymes. In contrast to cleavage, ligation does not require specific recognition sites, but it requires complementary sticky ends from the two parts to be joined together. Cleavage uses no energy input from external environment while ligation consumes one molecule of ATP as energy source.

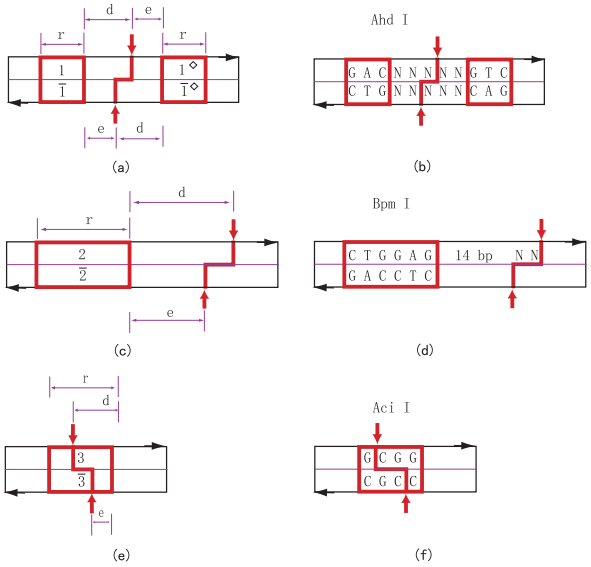
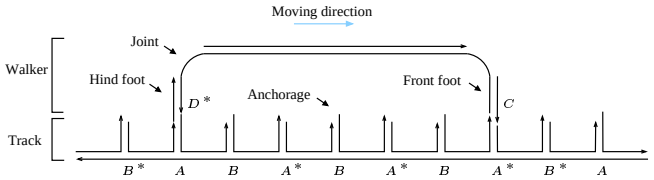


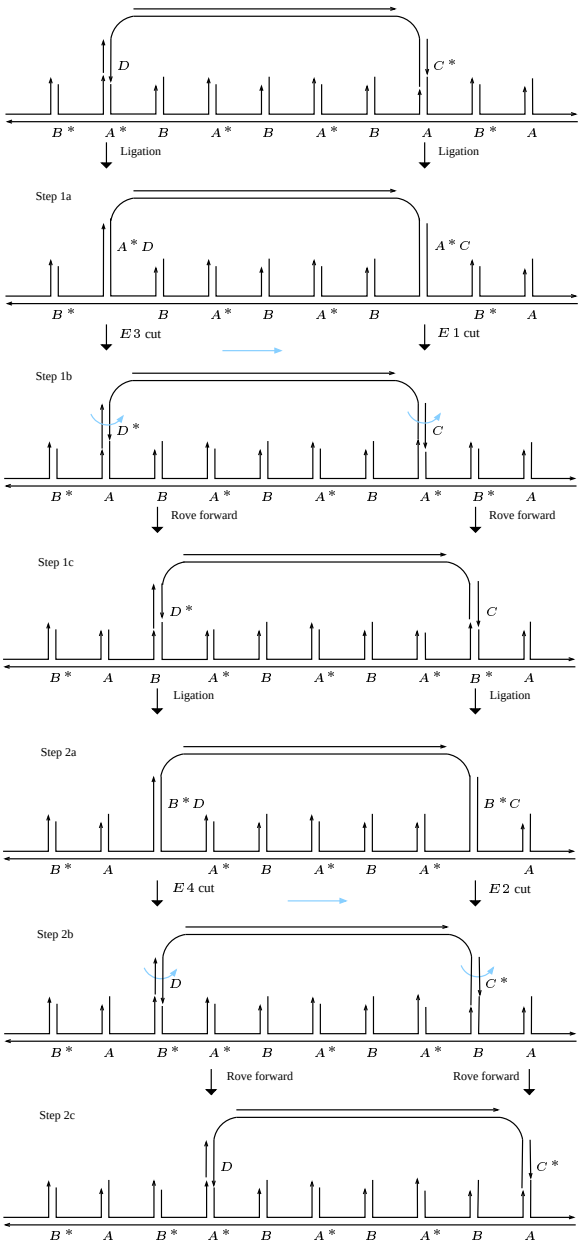
Figure 2: Panels (a), (c), and (e) illustrate conceptual endonucleases used in the construction of the walking devices. The sequences constituting the recognition site of the endonuclease in (a) are labeled with 1, $\bar{1}$, $1^{\diamond}$, and $\bar{1}^{\diamond}$; the sequences constituting the recognition site of the endonuclease in (c) are labeled with 2 and $\bar{2}$; the sequences constituting the recognition site of endonuclease in (e) are labeled with 3 and $\bar{3}$. Symbols r, d, and e are length parameters in number of bases. Panels (b), (d), and (f) show examples of real restriction enzymes corresponding to (a), (c), and (e). In panel (b), sequences GAC, CTG, GTC, and CAG correspond to sequences 1, $\bar{1}$, $1^{\diamond}$, and $\bar{1}^{\diamond}$ in panel (a), respectively. In this case, the values of r, d, and e are 3, 3, and 2, respectively. In panel (d), sequences $CTGGAG$ and $GACCTC$ correspond to sequences 2 and $\bar{2}$ in panel (c), respectively. In this case, the values of r, d, and e are 6, 16, and 14, respectively. In panel (e), sequences $GCGG$ and $CGCC$ correspond to sequences 3 and $\bar{3}$ in panel (f), respectively. In this case, the values of r, d, and e are 4, -3, and -1, respectively. Note that we use negative values for d and e to differentiate this cutting pattern from that in panel (c). In all the panels, recognition sites and cleavage sites are indicated with dark boxes and pairs of dark arrows, respectively. N indicates the position of a base whose value does not affect recognition by an endonuclease.

3 Device I

Design overview. Device I consists of two parts: the *track* and the *walker*. The walker is the moving part of the device while the track is the immobile part along which the walker moves. Figure 3 (a) gives a schematic drawing of the structure of device I. The track contains a linear array of anchorages, A and B. Each anchorage is a duplex DNA fragment with a sticky end on the top, and rigidly attached to the backbone of the track. The walker stands on top of the track. The walker consists of two parts, the body and the feet (a *front foot* C and a *hind foot* D). The body is a duplex DNA segment and each foot is a DNA dangler tethered to the body via a flexible single strand DNA joint. The flexible joint allows a foot of the walker to rove to and only to the two anchorages immediately neighboring the current anchorage on which it has been standing. The sticky end of a foot is complementary to the sticky end of the anchorage on which it is standing and hence the foot can hybridize with and be ligated with the anchorage. The ligation product between a foot and an anchorage will be cut by an endonuclease such that both the foot and the anchorage change their sticky ends. As a result, the foot will possess a sticky end that is complementary to the sticky end of the anchorage immediately ahead of the anchorage X on which the foot has been standing, but *not* complementary to the sticky end of the anchorage immediately behind X. Consequently, the foot can only hybridize with and be lig-



(a)



(b)

Figure 3: The structural design and step by step operation of device I. (a) Structural design of the device. (b) Step by step operation of the device.

ated with the anchorage immediately ahead of X, but not with the one immediately behind it. This guarantees the forward motion of the walker. The motion of the walker is described in more detail below (Figure 3 (b)).

A foot or an anchorage X can exist in two forms, X and X^*, where $X = A$, B, C, and D. X^* is derived from X by altering its sticky end. X and X^* are required to satisfy certain properties that will be described later. At any moment during the motion, the track in front of the front foot C and behind the hind foot D consists of alternating danglers A and B^* while the track between them consists of alternating A^* and B. Assume w.l.o.g. that at the start of the motion, both feet C and D are ligated with anchorages of type A, forming A^*C and A^*D respectively. Thus the initial configuration of the walker and track complex can be written as,

$$(AB^*)_i[A^*D]B(A^*B)_j[A^*C]B^*(AB^*)_k$$

where $[A^*C]$ (resp. $[A^*D]$) is the complex between anchorage A^* and the front foot C (resp. hind foot D). To make the walker move unidirectionally down the track, we implement the following reactions between a foot and an anchorage,

$$A + C^* \rightarrow A^*C \rightarrow A^* + C$$

$$B^* + C \rightarrow B^*C \rightarrow B + C^*$$

$$A^* + D \rightarrow A^*D \rightarrow A + D^*$$

$$B + D^* \rightarrow BD^* \rightarrow B^* + D$$

In phase a of each reaction, a foot is ligated with an anchorage; in phase b, the foot and the anchorage are cut separate by a restriction enzyme, each now possessing a new sticky end. Applying the reactions to the walker-track complex, we have the following motion of the walker along the track,

$$(AB^*)_i[A^*D]B(A^*B)_j[A^*C]B^*(AB^*)_k$$

$$\rightarrow (AB^*)_iA[B^*D](A^*B)_jA^*[B^*C](AB^*)_k$$

$$\rightarrow (AB^*)_{i+1}[A^*D]B(A^*B)_j[A^*C]B^*(AB^*)_{k-1}$$

The above is a full induction cycle of the motion of the walker, and hence the walker can (in principle) move forward along the track infinitely. We further require that phase a of each reaction is *not* reversible, thus the whole reaction is irreversible. Consequently, the walker can move along the track in only one direction.

There is nice dual property between front foot C and hind foot D. In the process of the motion, front foot C changes the configuration of the track from (AB^*) to A^*B; hind foot D moves on the modified track and restores it to its original configuration AB^*.

Implementation with conceptual endonucleases. To implement the designed reactions, we use four conceptual enzymes $E1$, $E2$, $E3$, and $E4$. The cutting patterns of these enzymes are similar to the one depicted in Figure 2 (a). Here we require that $d_1 - e_1 = d_4 - e_4 = e_2 - d_2 = e_3 - d_3$, where d_i and e_i are the length parameters for endonuclease Ei. Figure 4 describes the detailed step by step reactions that dictate the motion of the walker. Since only the region near the end of an anchorage or a foot is relevant for the reactions, we only depict the end regions in Figure 4.

Figure 4 (a) depicts reaction $A + C^* \rightarrow A^*C \rightarrow A^* + C$. In this reaction, the sticky end $\bar{u}$ of anchorage A is first ligated with the sticky end u (complementary to $\bar{u}$) of foot C^*, generating ligation product A^*C. This corresponds to the reaction of the front foot in Step 1a in Figure 3 (b): $A + C^* \rightarrow A^*C$. A^*C contains a recognition site for endonuclease $E1$ and is cut by $E1$ into A^* and C (Step 1b in Figure 3 (b): $A^*C \rightarrow A^* + C$). Note that now front foot C possesses a new sticky end $\bar{u}$. Recall that the anchorage immediately ahead of the anchorage A^*, on which front foot C is standing, is anchorage B^*. B^* possesses a sticky end u (complementary to $\bar{u}$). Thus C can rove forward and hybridize with B^* (Step 1c in Figure 3 (b)). This brings us to the reaction depicted in Figure 4 (b): $B^* + C \rightarrow B^*C \rightarrow B + C^*$. First, the hybridization product between B^* and C is ligated with form B^*C (Step 2a in Figure 3 (b): $B^* + C \rightarrow B^*C$). This ligation product is subsequently cut into B and C^* by endonuclease $E2$ (Step 2b in Figure 3 (b): $B^*C \rightarrow B + C^*$). Now front foot C^* possesses sticky u, and hence it will rove forward and hybridize with anchorage A down the track (Step 2c in Figure 3 (b)). This completes a full induction cycle for the front foot.

Note that the reactions $A + C^* \rightarrow A^*C$ is irreversible: there is no restriction enzyme that can cut A^*C back into A and C^*. This effectively establishes the irreversibility of the motion of foot C. However, we note that after A^*C is cut into A^* and C, the two can be religated into A^*C (which is subsequently cut

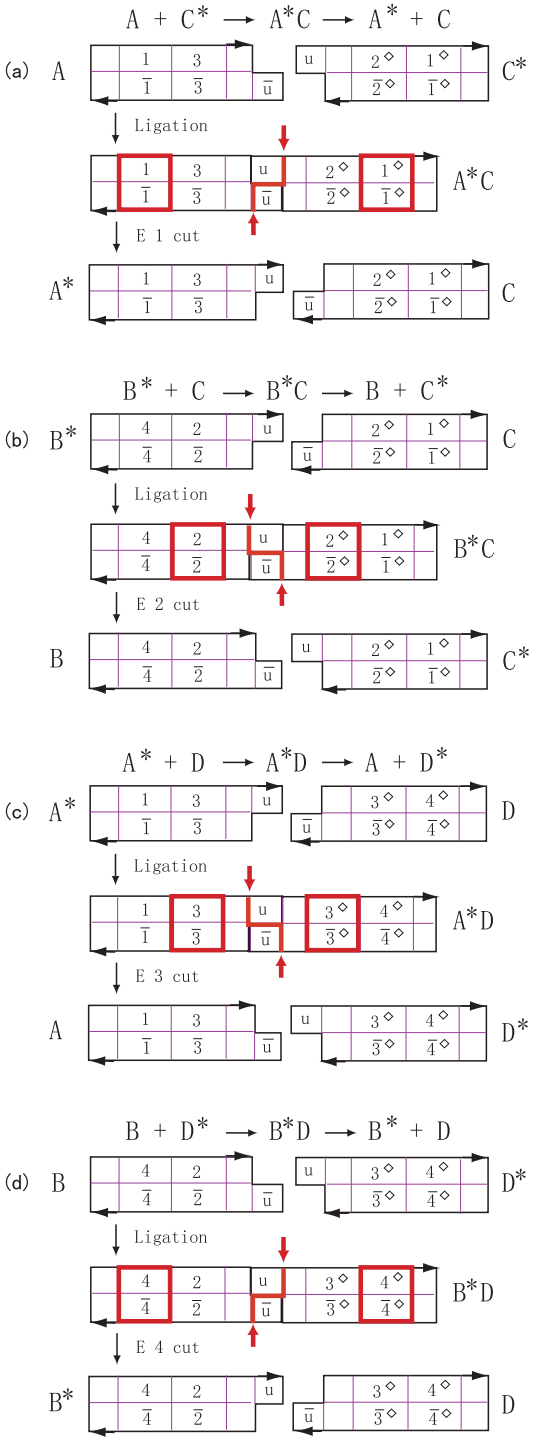


Figure 4: Implementation of device I using four conceptual restriction enzymes. Endonuclease recognition sites and cleavage sites are indicated with dark boxes and pairs of dark arrows, respectively.

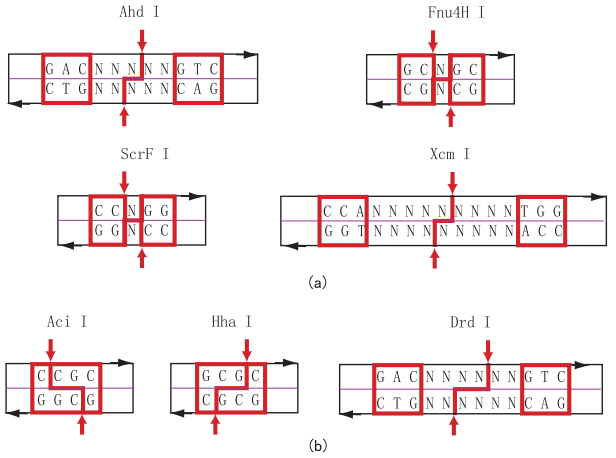


Figure 5: Real enzymes used in the construction of device I. Endonuclease recognition sites and cleavage sites are indicated with dark boxes and pairs of dark arrows, respectively. N indicates the position of a base whose value does not affect recognition by an endonuclease.

Reactions	Enzymes	DNA Sequences
$A + C^* \rightarrow A^*C$	Ligase	$5'$...gaccc-ngcgtc... $3'$ $3'$...ctgggn-cgcag... $5'$
$A^*C \rightarrow A^* + C$	Ahd I	$5'$...GACcc nˆgcGTC... $3'$ $3'$...CTGggˆn cgCAG... $5'$
$B^* + C \rightarrow B^*C$	Ligase	$5'$...ccanngcn-gcgtc... $3'$ $3'$...ggtnncg-ncgcag... $5'$
$B^*C \rightarrow B + C^*$	Fnu4H I	$5'$...ccannGCˆn GCgtc... $3'$ $3'$...ggtnnCG nˆCGcag... $5'$
$A^* + D \rightarrow A^*D$	Ligase	$5'$...gacccn-ggnntgg... $3'$ $3'$...ctggg-nccnnacc... $5'$
$A^*D \rightarrow A + D^*$	ScrF I	$5'$...gacCCˆn GGnntgg... $3'$ $3'$...ctgGG nˆCCnnacc... $5'$
$B + D^* \rightarrow B^*D$	Ligase	$5'$...ccanngc-nggnntgg... $3'$ $3'$...ggtnncgn-ccnnacc... $5'$
$B^*D \rightarrow B^* + D$	Xcm I	$5'$...CCAnngc nˆggnnTGG... $3'$ $3'$...GGTnncgˆn ccnnACC... $5'$

Table 1: Implementation of device I with endonucleases Ahd I, Fnu4H I, ScrF I, and Xcm I. Ligation sites and cleavage sites are denoted with $-$ and ˆ, respectively. The bases that determine recognition sites in action are in upper case.

back into A^* and C). This represents an idling step in the motion of the walker. Similar analysis applies to the reaction $B^* + C \rightarrow B^*C \rightarrow B + C^*$.

The motion of hind foot D is similar to motion of front foot C and we omit its detailed description for brevity.

Using an overlay technique, we can reduce the number of restriction enzymes to 2. The basic idea is to use $E1$ and $E2$ (in a "complementary reverse" fashion) in place of $E4$ and $E3$, respectively. However, in this construction, we need to put a further cleavage that $1 \neq \bar{1}^{\diamond R}$ and $2 \neq \bar{2}^{\diamond R}$, where $\bar{1}^{\diamond R}$ (resp. $\bar{2}^{\diamond R}$) is the reverse of $\bar{1}^{\diamond}$ (resp. $\bar{2}^{\diamond}$). In other words, neither of endonucleases $E1$ and $E2$ can have palindromic recognition site. Otherwise, there would be additional idling processes. However, the non-palindromic assumption generally does not hold for real endonucleases. The detailed description of this scheme can be found in Appendix I.

Molecular implementation using real enzymes. We give two implementations with real enzymes. The first one is a direct mapping of the implementation using the conceptual enzymes in Figure 4. The real enzymes used are shown in Figure 5 (a). Here, real endonucleases $AhdI$, $Fnu4HI$, $ScrFI$, and $XcmI$ correspond to conceptual endonucleases $E1$, $E2$, $E3$, and $E4$, respectively. The reactions are shown in Ta-

Reactions	Enzymes	DNA Sequences
$A + C^* \rightarrow A^*C$	Ligase	$5'$...gacnccg-c... $3'$ $3'$...ctgng-gcg... $5'$
$A^*C \rightarrow A^* + C$	Aci I	$5'$...gacnCˆCG C... $3'$ $3'$...ctgnG GCˆG... $5'$
$B^* + C \rightarrow B^*C$	Ligase	$5'$...c-cgc... $3'$ $3'$...cgc-g... $5'$
$B^*C \rightarrow B + C^*$	Hha I	$5'$...G CGˆC... $3'$ $3'$...CˆGC G... $5'$
$A^* + D \rightarrow A^*D$	Ligase	$5'$...gacnc-cggngtc... $3'$ $3'$...ctgnggc-cncag... $5'$
$A^*D \rightarrow A + D^*$	Drd I	$5'$...GACnc cgˆgnGTC... $3'$ $3'$...CTGngˆgc cnCAG... $5'$
$B + D^* \rightarrow B^*D$	Ligase	$5'$...gcg-gngtc... $3'$ $3'$...c-gccncag... $5'$
$B^*D \rightarrow B^* + D$	Aci I	$5'$...GˆCG Gngtc... $3'$ $3'$...C GCˆCncag... $5'$

Table 2: Implementation of device I with endonucleases Aci I, Hha I, and Drd I. Ligation sites and cleavage sites are denoted with $-$ and ˆ, respectively. The bases that determine recognition sites in action are in upper case.

ble 1 in a compact style.

The second implementation reduces the number of endonucleases to three by using a non-palindromic endonuclease (Aci I) and its slightly more involved construction is shown in Table 2. The real enzymes used are shown in Figure 5 (b). Note that Aci I shown in Figure 5 (b) is the same as the Aci I shown in Figure 2 (d): the latter figure is obtained by rotating the former one 180 degrees. The construction shown in Table 2 can be viewed as a partial realization of the conceptual design in Figure 9.

Processivity of device I. A key technical issue in the construction of device I is to assure that the walker is constrained to stay on or near the track. An isolated foot C or D would easily fall off the track and diffuse away. However, we can reduce the falling-off probability by constructing a multi-footed walker. Instead of possessing only two feet as in Figure 3, the walker has an array of alternate C and D feet. The feet are attached to a common backbone: if the backbone does not move then the feet have freedom to move up and down the track by one unit only. The walker is held to the track by multiple bonds - even if none are ligated (so all bonds are weak 1- or 2-base hydrogen bonds) then the probability of detachment is small. This is precisely what is needed - feet are held in the right place with the right amount of freedom to move - it introduces the constraint that no foot can move more than two anchorages forward until all feet have moved at least one anchorage.

Nanowheel. The design principle of device I allows flexible structural implementations and can result in nanorobotic devices of different morphologies yet based on essentially the same principles. One such structural variant is a nanowheel which rolls autonomously along the track unidirectionally. The construction of the nano-wheel is described in Appendix II.

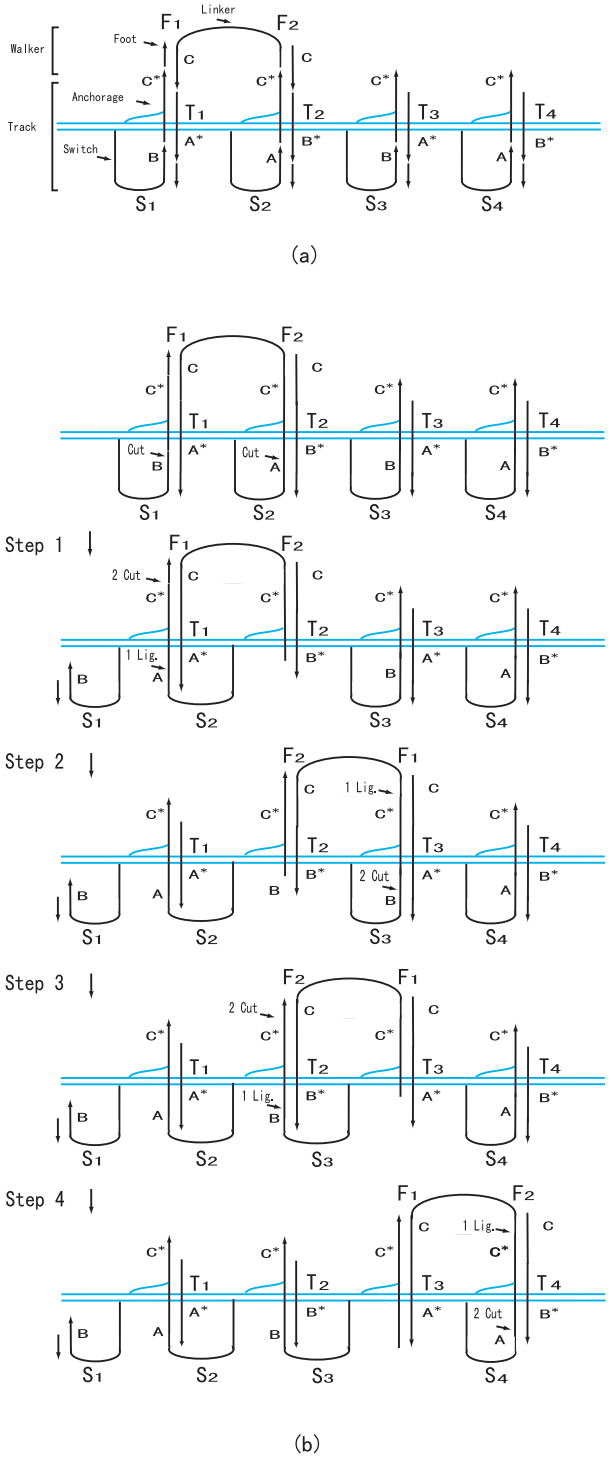


Figure 6: The structural design and step by step operation of device II. (a) Structural design of the device. (b) Step by step operation of the device.

4 Design II

Overview. A potential problem of device I is that it may fall off the track. Though a walker with more feet risks lower probability of falling off as argued above, we can not completely eliminate such risk. In contrast, the device we describe next is guaranteed

to stay on the track, though it has a more complicated (hence less practical) construction and assumes a restriction enzyme property that has not yet been fully-substantiated. In device II, a two-footed walker steps over the anchorages along a track unidirectionally. The design of device II is based on the following principle: the lifting of one foot off the track is conditional on the attachment (ligation) of the other foot to the track. This attachment principle can ensure that at any moment, at least one foot of the walker is attached to the track. We describe the structure and step by step operation of device II below.

The track and the walker are depicted in Figure 6 (a). As in device I, the track contains a linear array of anchorages. But the anchorages in device II are different. As depicted, each anchorage is a duplex DNA fragment with single strand DNA overhangs at both ends and its midpoint is tethered to the backbone of the track via single strand DNA. Thus the anchorage is like a two-ended dangler. In addition, between every two neighboring anchorages is tethered another dangler, referred to as a *switch*. As we shall see below, the alternating arrangement of anchorages and switches are used to construct a signaling mechanism which ensures the unidirectional and non-falling-off-track motion of the walker. The anchorages and switches are denoted as T_i and S_i respectively, where $i = 1, 2, 3, \ldots, n$. A switch S_i can only be ligated with its immediate anchorage neighbors T_{i-1} and T_i. The upper ends of T are of type C^*, and the lower end of T_i is of type A^* and B^* for odd and even i-s, respectively. Note that since an anchorage is tethered to the backbone of the track via single strand DNA, the upper and lower ends of an anchorage can not be held constantly in upper and lower positions – we just denote the C^* type end as upper end the A^*/B^* type end as lower end for ease of exposition. In fact, we shall see that we do not need to fix the relative upper and lower positions of the ends for the valid operation of device II.

The walker consists of two danglers connected with a single strand DNA . The two danglers serve as the feet of the walker and are denoted as F_1 and F_2. The ends of both F_1 and F_2 are of type C. The walker stands on top of the upper ends of the anchorages and walks down the track unidirectionally, with the switch/anchorage complex of the road serving both as attaching points and as a signal transducing device to dictate the lifting and attaching of its feet in an alternating fashion such that it never falls off the track. In particular, at any point, if one foot is attached to anchorage T_i, the other foot can only be attached to T_i's immediate neighbors, T_{i-1} and T_{i+1}.

The ends of the feet of the walker, of the anchorages and of the switches have the following properties:

1. The complementary end pairs are: (A, A^*), (A, B^*), (B^*, A^*), (B, B^*), and (C, C^*). Two danglers with these complementary ends can be ligated.

2. The formation of CC^* ligation product at the upper end of the anchorage introduces a recognition site on the anchorage for endonuclease $E3$. Endonuclease $E3$ has similar cleavage pattern as the one depicted in Figure 2 (b). And this results in a cleavage at the other end of the anchorage such that the anchorage is cut from the switch currently ligated with it (if there is one). Similarly, the formation of A^*A (resp. B^*B) at the lower end of the anchorage will produce a recognition site on the anchorage for endonuclease $E1$ (resp. $E2$) and this will result in the cleavage of CC^* at the upper end of the anchorage if there is a foot end C ligated with C^*.

We will next see how these properties guarantee the desired motion of the walker as we go through a step by step description of the walker's motion.

Step by step motion. Now we describe the four steps of the walker's motion that completes a full inductional cycle. Initially, the walker and track complex is assembled in such a way that the feet F_1 and F_2 of the walker are ligated with anchorages T_1 and T_2, respectively; each switch S_i is ligated to the lower end of T_i, forming BA^* for odd i and AB^* for even i. Note that BA^* and AB^* are different.

Step 0. Upon introduction of enzymes into the system, switches S_1 and S_2 are cut from anchorages T_1 and T_2 respectively, since the CC^* sequences at the upper ends of T_1 and T_2 constitute endonuclease $E3$ recognition sites and result in cleavages at the lower ends of T_1 and T_2. Now S_2 (with end A) can explore its neighboring space and be ligated with either T_1 (with end A^*) or T_2 (with end B^*), since (A, A^*) and (A, B^*) both are compatible end pairs. Ligation between S_2 and T_2 is a just an idling step, since the ligation product will be subsequently cut again. In contrast, ligation of S_2 and T_1 brings the system to Step 1.

Step 1. The ligation of S_2 (with end A) and T_1 (with end A^*) introduces a recognition site for $E1$,

and results in the cleavage of F_1 from the upper end of T_1. Note that the ligation product between the lower end of T_1 and S_2 contains recognition site (AA^*) for endonuclease $E1$ while the ligation product between foot F_1 and the upper end of T_1 contains recognition site (CC^*) for endonuclease $E3$. As such, both $E1$ and $E3$ will compete to perform cleavage on the common ligation product. (See Figure 8 (a) for detail.) It is possible that endonuclease $E3$ cuts switch S_2 away from anchorage T_1, resulting in an idling step. However, there must also be non-zero probability that endonuclease $E1$ cuts foot F_1 away from anchorage T_1, advancing the system to Step 2.

Step 2. Now foot F_1 has free end C and can swing around the ligation product between foot F_2 and anchorage T_2 and get ligated with the upper end C^* of anchorage T_3. Note that now foot F_1 is in front of foot F_2. The ligation of CC^* subsequently results in the cleavage of S_3 from T_3.

Step 3. Switch S_3 has free end B and is ligated with the B^* end of anchorage T_2, and the newly formed recognition site BB^* leads to the action of endonuclease $E2$ and results in the cleavage between foot F_2 and anchorage T_2.

Step 4. Foot F_2 swings to in front of foot F_1 and is ligated with anchorage T_4, resulting in the cleavage of switch S_4 from the lower end of anchorage T_4.

Upon completion of Step 4, the walker has moved from anchorages T_1 and T_2 to anchorages T_3 and T_4. This finishes a full inductional cycle, and hence the walker can continue moving down the track.

Correctness. To show the correctness of the design, we prove the following three properties of the walker: 1) the motion of the walker is unidirectional; 2) the walker never falls off the track; 3) the motion of the walker is never blocked. We give high level intuition here, and present a rigorous proof in Appendix III.

To see the unidirectionality of the motion, first note that once a foot of the walker, say, F_1, is attached to an anchorage T_i, it can not be cut from anchorage T_i unless the other foot F_2 is attached to anchorage T_{i+1} further down the track. But once that has happened, the first foot is constrained to only explore the space where anchorages T_i and T_{i+2} lie. In particular, it can not reach anchorage T_{i-1}, which could have resulted in one step backwards.

The reason why the walker always stays on the track is because the detachment of one foot from an anchorage is conditional on the attachment of the other foot to another anchorage. Thus at any time point, at least one foot is attached to an anchorage.

Reactions	Enzymes	Sequences
$A + A^* :: C^*C \rightarrow$ $AA^* :: C^*C$	Ligase	$5'$...ctg-gag(n)$_{11}$ctcaag...$3'$ $3'$...g-acctc(n)$_{11}$gagttc...$3'$
$AA^* :: C^*C \rightarrow$ $AA^* :: C^* + C$	Bpm I	$5'$...CTGGAG(n)$_{11}$ctc aaˆg...$3'$ $3'$...GACCTC(n)$_{11}$gagˆtt c...$3'$
$AA^* :: C^* + C \rightarrow$ $AA^* :: C^*C$	Ligase	$5'$...ctggag(n)$_{11}$ctcaa-g...$3'$ $3'$...gacctc(n)$_{11}$gag-ttc...$3'$
$AA^* :: C^*C \rightarrow$ $A + A^* :: C^*C$	BpuE I	$5'$...c tgˆgag(n)$_{11}$CTCAAG...$3'$ $3'$...gˆac ctc(n)$_{11}$GAGTTC...$3'$
$B + B^* :: C^*C \rightarrow$ $BB^* :: C^*C$	Ligase	$5'$...gtg-cag(n)$_{11}$ctcaag...$3'$ $3'$...c-acgtc(n)$_{11}$gagttc...$3'$
$BB^* :: C^*C \rightarrow$ $BB^* :: C^* + C$	Bsg I	$5'$...GTGCAG(n)$_{11}$ctc aaˆg...$3'$ $3'$...CACGTC(n)$_{11}$gagˆtt c...$3'$
$BB^* :: C^* + C \rightarrow$ $BB^* :: C^*C$	Ligase	$5'$...gtgcag(n)$_{11}$ctcaa-g...$3'$ $3'$...cacgtc(n)$_{11}$gag-ttc...$3'$
$BB^* :: C^*C \rightarrow$ $B + B^* :: C^*C$	BpuE I	$5'$...g tgˆcag(n)$_{11}$CTCAAG...$3'$ $3'$...cˆac gtc(n)$_{11}$GAGTTC...$3'$
$AB^* :: C^* + C \rightarrow$ $AB^* :: C^*C$	Ligase	$5'$...ctgcag(n)$_{11}$ctcaa-g...$3'$ $3'$...gacgtc(n)$_{11}$gag-ttc...$3'$
$AB^* :: C^*C \rightarrow$ $A + B^* :: C^*C$	BpuE I	$5'$...c tgˆcag(n)$_{11}$CTCAAG...$3'$ $3'$...gˆac gtc(n)$_{11}$GAGTTC...$3'$
$BA^* :: C^* + C \rightarrow$ $BA^* :: C^*C$	Ligase	$5'$...gtggag(n)$_{11}$ctcaa-g...$3'$ $3'$...cacctc(n)$_{11}$gag-ttc...$3'$
$BA^* :: C^*C \rightarrow$ $B + A^* :: C^*C$	BpuE I	$5'$...g tgˆgag(n)$_{11}$CTCAAG...$3'$ $3'$...cˆac ctc(n)$_{11}$GAGTTC...$3'$

Table 3: Implementation of device II with endonucleases Bpm I, Bsg I, and BpuE I. Ligation sites and cleavage sites are denoted with $-$ and ˆ, respectively. The bases that determine recognition sites in action are in upper case.

To prove that the motion is never blocked, first note that there are always moments when both of the feet of the walker are attached to neighboring anchorages. This is because we have shown that the walker never falls off the track and hence the attachment of one foot will result in the attachment of the other foot to a neighboring anchorage since all the upper ends of the anchorages are of the same end type (C^*) which is compatible to the end type (C) of either feet of the walker. However, the attachment of both feet to the track will necessarily result in the ligation between the lower end of the anchorage, which the current hind foot is attached to, and the end of the immediate downstream switch. This event in turn results in the cleavage of the current hind foot from the anchorage and it has non-zero probability to explore the downstream neighbor of the anchorage that the current front foot stands on, and hence the motion moves on.

Implementation with conceptual enzymes. The

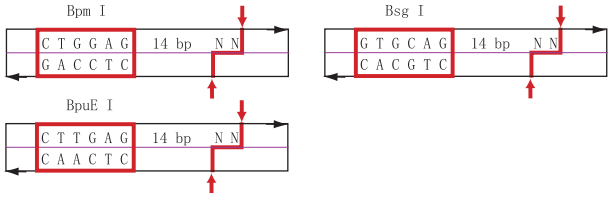


Figure 7: Real enzymes used in the construction of device II. Endonuclease recognition sites and cleavage sites are indicated with dark boxes and pairs of dark arrows, respectively. N indicates the position of a base whose value does not affect recognition by an endonuclease.

above reactions can be implemented with three conceptual enzymes $E1$, $E2$, and $E3$ that have similar cutting patterns as the one shown in Figure 2 (a). We require that $d_1 = d_2 = d_3$ and $e_1 = e_2 = e_3$, where d_i and e_i are the length parameters for E_i for $i = 1, 2$, and 3. Figure 8 describes the implementation of device II with these conceptual restriction enzymes. In Figure 8 (a), two anti-parallel flows of reactions are depicted. Starting from the top, end A (of a switch) has sticky end sequence complementary to end A^* (lower end of an anchorage) and hence the two are ligated together. This creates a recognition site for endonuclease $E1$, and results in the cleavage of end C (of a foot) from end C^* (upper end of an anchorage). This downward flow of reactions can be fully reversed into the anti-parallel upward flow starting from the bottom with C^* and C and ends at the top with A and A^*. We note that due to the fully reversible nature of reactions, the reaction system has non zero probability to explore all three states: the top one (A, $A^* :: C^*C$), the middle one ($AA^* :: C^*C$), and the bottom one ($AA^* :: C^*$, C), where :: represents the duplex portion of DNA connecting the two ends. Similar fully reversible anti-parallel flows of reactions involving $E2$ and $E3$ are depicted in Figure 8 (b). In contrast, reactions in Figure 8 (c) and 8 (d) are not fully reversible since neither ligation of AB^* nor that of BA^* can result in a recognition site for an endonuclease, and hence CC^* can not be cleaved. This irreversibility ultimately accounts for the unidirectionality of the motion of the walker. The downward reaction flow in Figure 8 (a), the upward reaction flow in (d), the downward reaction flow in (b) and the upward reaction flow in (c) correspond to Steps 1, 2, 3, and 4 in Figure 6, respectively.

Molecular implementation with real enzymes. The above conceptual enzymes can be mapped directly to real enzymes in Figure 7, where conceptual enzymes $E1$, $E2$, and $E3$ correspond to real enzymes Bpm I, Bsg I, and BpuE I, respectively. Table 3 describes the implementation with these real enzymes. Note that we have the following mapping from sequences in Figure 8 to the sequences in Table 3: $1 = C$, $u = TG$, $1^\diamond = GAG$, $2 = G$, $2^\diamond = CAG$, $\bar{3}^{\diamond R} = CTC$, $\bar{v}^R = AA$, and $\bar{3}^R = G$.

Practicality. One assumption we make about the enzyme is that the presence of a single strand between the recognition site and cleavage site of each endonuclease used above will neither alter the specificity nor totally inhibit the activity of that endonuclease. A theoretical modeling of the molecular structure of the enzyme and its interaction with the DNA strands would shed light on the practicality of this assumption. However, the final validation of this assumption relies on a rigorous experimental study. Though our preliminary experimental result is in agreement with this assumption, more work is still required to further substantiate this assumption.

5 Discussion

We have depicted the backbones of the walking devices as duplex DNA fragments for simplicity. However, this is not technically precise. One property we require of the backbone of a track is its rigidity, to ensure that the walker cannot skip anchorage(s) and "jump" ahead. Existing DNA lattices provide such a platform [5, 7, 15, 16]. We can easily embed the anchorages to a rigid DNA lattice and thus integrate a walking device to a lattice, with the latter provide the desired rigid backbone for the anchorages. In addition to the rigidity of the track, the structure and the size of the walker are also crucial factors in ensuring that the foot of the walker can only explore the immediately neighboring anchorages. In device I, though it is hard to ensure this property for a two-footed walker (since in such a walker one foot might swing around the other foot in a similar fashion as in device II), this property can be rather straightforwardly guaranteed in a multi-footed walker with a rigid body. In device two, the two feet of the walker alternate their order along the track by swinging around each other and

we hence only need to properly design the size of the body such that a foot can only reach a neighboring anchorage.

The designs of the devices assume that enzyme cleavage occurs only after the DNA strands are ligated. This is assumption is in agreement with the experimental results observed in our recent construction of a unidirectional autonomous DNA walker [18]. In this device, we use two class II enzymes PflM I and BstAP I and the system operates at 37 $^{\circ}C$. However, we note that this property does not hold true for all class II enzymes under all conditions. Indeed, Shapiro's group has observed that a class II enzyme Fok I can cleave GC rich DNA duplex strands with nicks present between Fok I recognition site and cleavage site under at low temperature (8 $^{\circ}C$) [2].

How practical are the designs? Though we have proved that each walker will behave in its designated way in a theoretical setting, closing the gap between a theoretical construction on the paper and a working device in the real world remains enticing. As an exciting first step, we have successfully constructed in the lab a prototype system based on similar design principles of the devices presented here [18].

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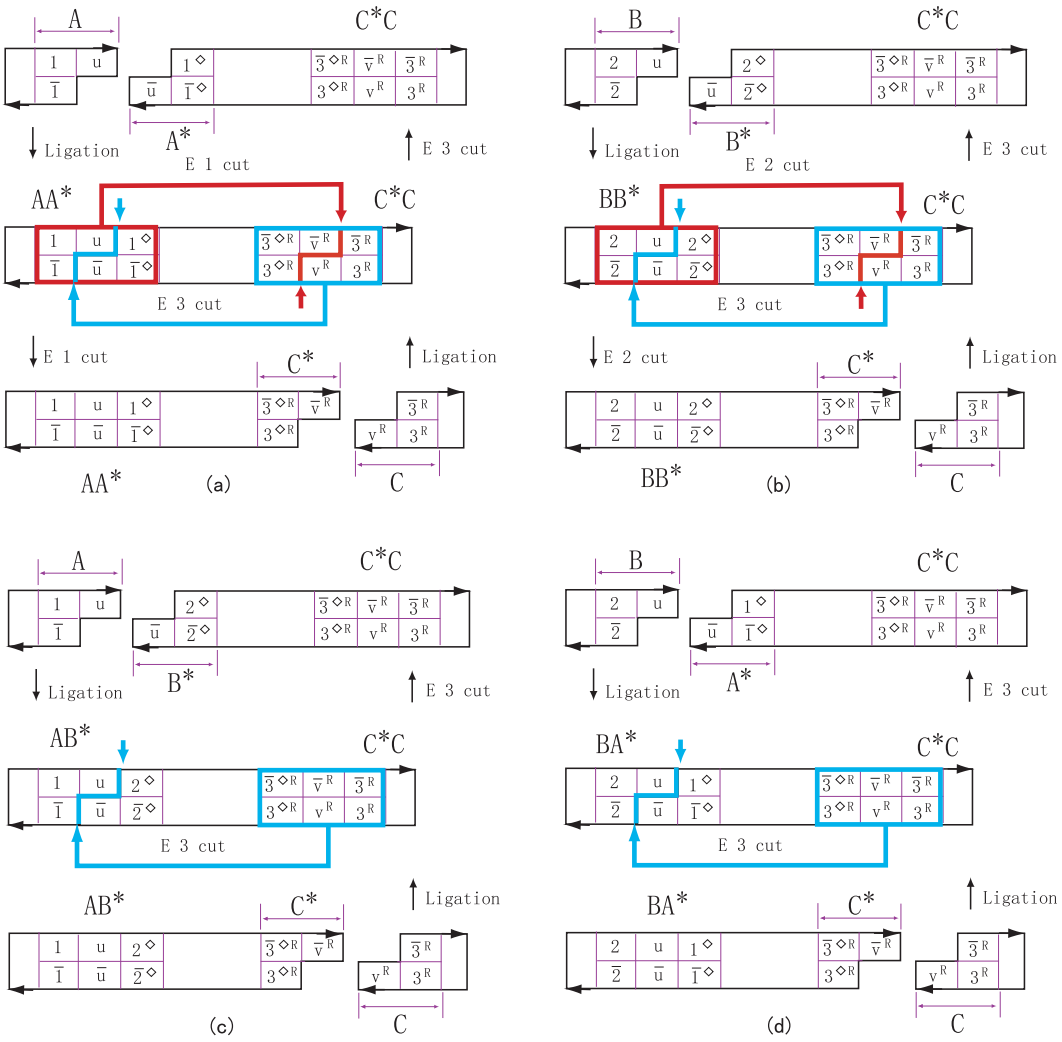

Figure 8: Actions of conceptual enzymes used in the construction of device II. (a) Sequences 1, u, $1^{\diamond}$, $\bar{1}$, $\bar{u}$, and $\bar{1}^{\diamond}$ (sequences of AA^*) together constitute the recognition site (red box) for conceptual endonuclease $E1$, whose cleavage site is indicated with a pair of dark arrows. Sequences $\bar{3}^{\diamond R}$, $\bar{v}^R$, $\bar{3}^R$ $3^{\diamond R}$, v^R, and 3^R (sequences of C^*C) together constitute the recognition site (light gray box) for conceptual endonuclease $E3$, whose cleavage site is indicated with a pair of light gray arrows. (b) Two anti-parallel flows of reactions by $E2$ and $E3$. (c) and (d) Neither ligation of AB^* or BA^* results in cleavage of CC^*.

Appendix I: Construction of device I using two conceptual restriction enzymes

Figure 9 illustrates how to exploit the overlay technique to reduce the number of restriction enzymes to 2 in the construction of device I. We use $E1$ and $E2$ in place of $E4$ and $E3$, respectively, by letting $4 = \bar{1}^{\diamond R}$ and $3 = \bar{2}^{\diamond R}$. However, in this construction, we need to put a further cleavage that $1 \neq \bar{1}^{\diamond R}$ and $2 \neq \bar{2}^{\diamond R}$. In other words, neither of endonucleases $E1$ and $E2$ can have palindromic recognition site. Otherwise, there would be additional idling processes: B^*C can also be cut by $E1$ into $B^* + C$; similarly, B^*D can be cut by $E2$ into $B + D^*$. However, these reactions would only count as idling reactions: the unidirectional motion of the walker can neither be reversed nor blocked.

Appendix II: Construction of nanowheel

Nanowheel. The design principle of device I allows flexible structural implementations and can result in nanorobotic devices of different morphologies yet based on essentially the same principles. One such structural variant of interest is a nanowheel which rolls autonomously along the track unidirectionally. The construction of the nano-wheel is shown in the Figure 10. The nano-wheel consists of $2k + 1$ ($k = 1$ in Figure 10) evenly spaced wheel feet D/D^* attached to a nano-disk and it rolls unidirectionally on a track of linearly arranged anchorages A/A^* and B/B^*. The feet are arranged in the counter clockwise order $D(DD^*)_k$; the anchorages are arranged in the order of $B^*(A^*B)_n$. The reactions are exactly the same as in device I,

$$A^* + D \rightarrow A^*D \rightarrow A + D^*$$

$$B + D^* \rightarrow B^*D \rightarrow B^* + D$$

We next describe the movement of the nano-wheel along the track. For the ease of exposition, denote the wheel's feet in counter clockwise order a_i where $a_0 = D$, $a_{2k-1} = D$, and $a_{2k} = D^*$. Denote anchorages from left to right as δ_i, where $\delta_0 = B^*$, $\delta_{2k-1} = A^*$, $\delta_{2k} = B$. We describe the motion for the case when $k = 1$. Initially, foot $a_0 = D$ is ligated

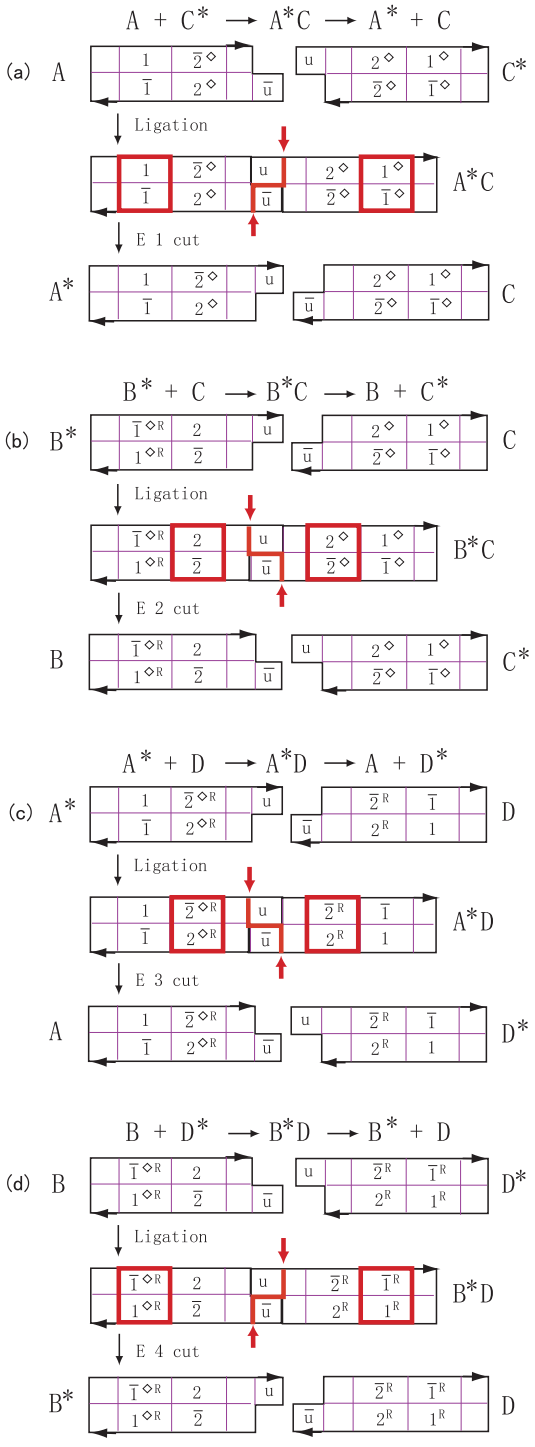

Figure 9: Construction of device I using two conceptual restriction enzymes.

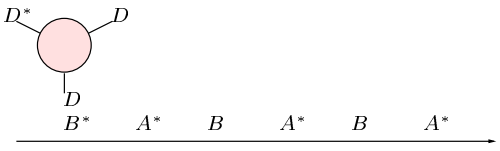

Figure 10: The construction of nano-wheel.

with anchorage $\delta_0 = B^*$. Then foot $a_1 = D$ is ligated to anchorage $\delta_1 = A^*$, forming A^*D. B^*D formed between danglers a_0 and δ_0 is subsequently cut into B^* and D and the wheel rolls $360/(2k+1)$ degrees to its next position down the track and foot $a_2 = D^*$ is ligated with anchorage $\delta_2 = B$. A^*D formed between a_1 and δ_1 is cut into A and D^*. Then foot $a_0 = D$ will be ligated with anchorage $\delta_3 = A^*$, and the motion goes on in an induction way (here we have only described half of the induction cycle, the full cycle has $2 \times (2k+1)$ steps). We note that to ensure smooth motion of the nano-wheel, an odd number of feet are required. It is not hard to see by the same token of argument as for device I that the wheel can oscillate backwards only to a limited number of steps in an idling process, which essentially guarantees the unidirectionality of the wheel's movement. Straightforward details are omitted for brevity.

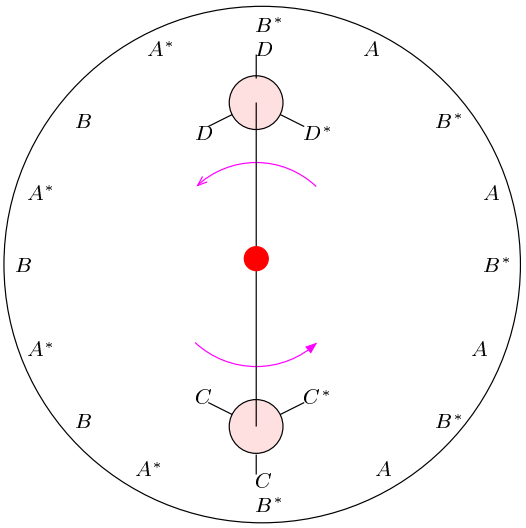

Figure 11: Rotor composed of two dual nano-wheels.

Observe that the track is changed from $B^*(A^*B)_n$ to $B^*(AB^*)_n$. This is an undesirable property that precludes the wheel from moving in cycles on the same track. As in device I, we address the problem by introducing a dual nano-wheel with danglers $C(CC^*)_k$. The two wheels move together on the same track. D wheel changes the track from $B^*(A^*B)_n$ to $B^*(AB^*)_n$ and C wheel changes $B^*(A^*B)_n$ to $B^*(AB^*)_n$. As such, the track changed by one wheel is repaired by its dual wheel. The dual D and C wheels can be combined to construct a rotor device as in Figure 11.

Appendix III: Proof of correctness for the motion of device II

To prove the correctness of the motion of the walker in device II, we need to prove the following,

1. The motion of the walker is unidirectional.
2. The walker never falls off the track.
3. The motion is never blocked.

Let W denote the walker. Recall that F_1 and F_2 denote the two feet of W; S_i and T_i denote the switches and anchorages respectively, where $i = 1, 2, 3, \ldots, n$. For the ease of exposition, we introduce some more definitions and notations. If an end of a foot, anchorage or switch is not ligated with some other end, then it is referred to as a *free end*. Denote a ligation between X and Y as $\sim$, and a cleavage that cuts a ligation product XY into X and Y as $X \nsim Y$, where X/Y can be one of F_i, S_j, and T_j, $i = 1, 2$ and $j = 1, 2, 3, \ldots, n$. By $F \sim T_j$, we mean either $F_1 \sim T_j$ or $F_2 \sim T_j$.

Unidirectionality of motion.

Lemma 5.1 *After the occurrence of $F \sim T_i$, ligation $F \sim T_j$ cannot happen, where $3 \leq i \leq n$ and $j \leq i-2$.*

Proof: Prove by induction. We first show that the claim holds for $i = n$.

Suppose we have $F_1 \sim T_n$. Note that cleavage $F_1 \nsim T_n$ cannot happen since T_n is the last anchorage and only a ligation between S_{i+1} and T_i can result in a cleavage on the T_i end. Due to the space constraint (only danglers in proximity of each other can interact), ligation $F \sim T_j$ cannot happen for $j \leq i-2$.

Next we prove that the claim holds for $i < n$. Suppose for $i > k$, the claim in Lemma 5.1 holds, we show that it also holds for $i = k$. Suppose w.l.o.g. that $F_1 \sim T_k$. Prove by contradiction. Suppose that ligation $F \sim T_{k-2}$ happens subsequent to ligation

$F_1 \sim T_k$. Then $F_1 \nsim T_k$ must have occurred. Otherwise, F_1 cannot be ligated with T_{k-2} since F_1 is not a free end; due to the space constraint, ligation $F_2 \sim T_{k-2}$ cannot happen either. Thus, cleavage $F_1 \nsim T_k$ must have occurred. But this means that ligation $T_k \sim S_{k+1}$ must have occurred. This further implies that cleavage $S_{k+1} \nsim T_{k+1}$ must have occurred. This is only possible if ligation $F_2 \sim T_{k+1}$ have occurred. But we know from the induction hypothesis that ligation $F \sim T_{k-2}$ cannot occur after ligation $F \sim T_{k+1}$. We have thus reached a contradiction. Schematically, we have shown the following causal relations,

$$F \sim T_{k-2} \Rightarrow F_1 \nsim T_k \Rightarrow T_k \sim S_{k+1} \Rightarrow$$

$$S_{k+1} \nsim T_{k+1} \Rightarrow F_2 \sim T_{k+1} \Rightarrow \nexists\, F \sim T_{k-2}$$

□

Attachment.

Lemma 5.2 *At any time point during walker W's motion, there is always a ligation $F \sim T_i$ for some T_i.*

Proof: Prove by contradiction. At the start of the reaction, the claim is obviously true. Now assume at time t, the first violation of the claim occurs. Suppose w.l.o.g. that the violation happens as the cleavage $F_1 \nsim T_i$ occurs. At time t, F_2 must be a free end; there must be a ligation $S_{i+1} \sim T_i$. By the same token of arguement as in Lemma 5.1, $S_{i+1} \nsim T_{i+1}$ must have occurred; $F \sim T_{i+1}$ must have occurred. But since at time t, F_2 is a free end, $F \nsim T_{i+1}$ must have occurred. Hence $S_{i+2} \sim T_{i+1}$, $S_{i+2} \nsim T_{i+2}$, $F \sim T_{i+2}$ must have occurred. Thus we must have that $F \sim T_i$ occurs after $F \sim T_{i+2}$, contradicting Lemma 5.1. □

Occlusion free movement.

Lemma 5.3 *In the case $F \sim T_i$ and $F \sim T_{i+1}$, where $i \leq n-2$, a cleavage on the ligation $F \sim T_i$ is guaranteed to occur.*

Proof: Suppose w.l.o.g. that $F_1 \sim T_i$ and $F_2 \sim T_{i+1}$. Since we have $F_2 \sim T_{i+1}$, there must be a cleavage on the ligation $S_{i+1} \sim T_{i+1}$. Now we only need to show that S_{i+1} can form a ligation with T_i, which will result in a cleavage on the ligation $F \sim T_i$. In turn, we only need to show that T_i can be a free end at this point. But this is obviously true because ligation $T_i \sim F_1$ introduces a cleavage site at T_i. □

Lemma 5.4 *Walker W can move down the track without occlusion.*

Proof: Study the time point when $F_1 \sim T_i$ and $F_2 \sim T_{i+1}$. According to Lemma 5.3, walker W can always lift its current hind foot F_1 at this point. We only need to show that it can attach F_1 to T_{i+2}, but this is trivially true since T_{i+2} is a free end compatible with F_1. □

Lemmas 5.1, 5.2, 5.3 and 5.4 lead immediately to the following theorem,

Theorem 5.5 *Walker W is guaranteed to move unidirectionally towards and reach T_n.*