Supporting Online Material

S1. Design of the DX track

The design of the track centered around the stem-loop structures of *T1, T2, T3,* and *T4* (Fig. S1). The two sides of the stem-loops that react with the walker and fuel are spread out by two turns of duplex. This distance allowed for the spatial control of the fuel interaction, as described in main text. The spread-out structure of the stem-loops, and the fact that *T1, T2, T3,* and *T4* are single strands, generate this DX *(1, 2)* design, in which the crossover points in the center of the track lie four turns away from each other. The five crossover points are labeled *c1, c2, c3, c4,* and *c5* from left to right in Fig. S1.

To prevent dimers of the tracks from forming, the crossover points at the ends of the track *(c1* and *c5)* were altered in two ways: 1) Crossover point *c1* was placed one duplex turn (or 10 base pairs) from the leftmost edge of the track; and 2) A four thymine loop was added at crossover point *c5*. These two changes, made to the end strands, were equivalent in their ability to stop dimers from forming. We believe the two changes prevented these strands from engaging in domain swapping. Dimers and higher order structures formed before these two changes were made. [Note: Crossover point *c1* is nicked on the right hand side because it was moved from it's original placement at the leftmost edge of the track. Hence *c1* is not a two strand crossover point, unlike *c2*, *c3*, and *c4*]

Stem-loops *T1, T2, T3* and *T4* were designed to be single strands so we could assemble multiple stem-loops together onto the track. This required the stem region of *T1, T2, T3,* and *T4* to have a much higher melting temperature (79.3 ˚C predicted by mFold *(3)*)* than the rest of the track structure. This difference in melting temperature enabled us to implement an annealing procedure in which the stem-loops folded first, and then acted to scaffold the formation of the supporting DX strands.

The proposed kinetics of the annealing procedure are pictured in Fig. S2 for the 'front half' of the track without the walker on it: 1) The solutions containing the stem-loops and their supporting strands were heated to 90 ˚C to denature all the strands in the solution; 2) the solutions were brought directly to 65 ˚C, where the stem-loops were stable enough to fold, but the rest of the strands were denatured; 3) from 65 ˚C, the solutions were brought gradually to room temperature, to form the DX track around the folded hairpins. As can be seen in Fig. S2, the ends of the supporting strands that weave through the hairpins (*s2, s3, s4,* and *s5*) were designed to be free, even if they had already made pairings with their other complements. For example, if *s4* first makes pairings with *s1, s7* and *s6*, it's 5' end, designed to wrap through the loop of *T2* is still free to do so.

*(The melting temperature of the hairpin was predicted with mFold. To predict the melting temperature, we used the *T1* sequence (text S9), but replaced with poly-thymidine the 21 bases in the middle of the strand that pairs with the supporting strands. The poly-thymidine replacement was done so the predicted melting temperature was not taking into account the secondary structure in the 21 base region, but rather the stability

of the stem-loop structure.)

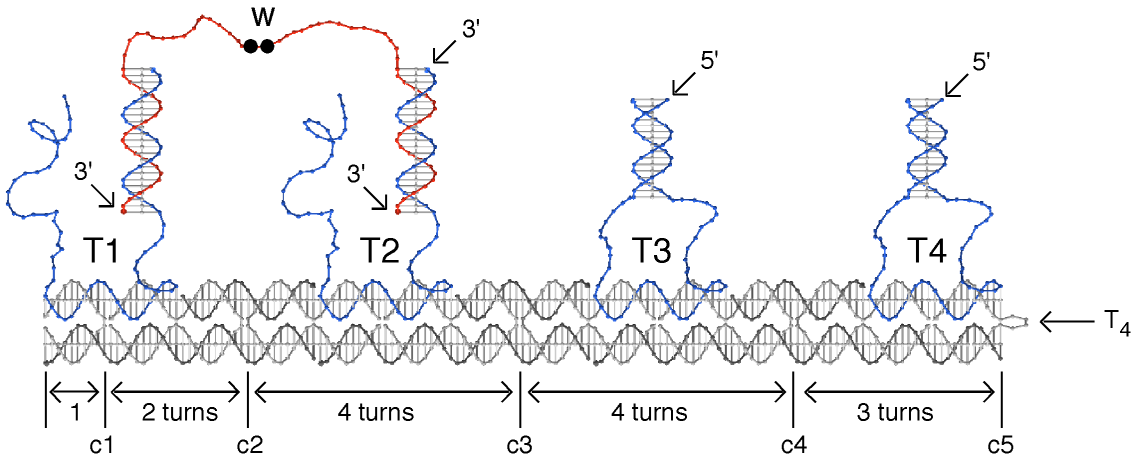

Fig. S1. 3D model of the walker and track drawn with GIDEON. The walker is drawn in red, the track stem-loops are drawn in blue, and the supporting strands are drawn in grey.

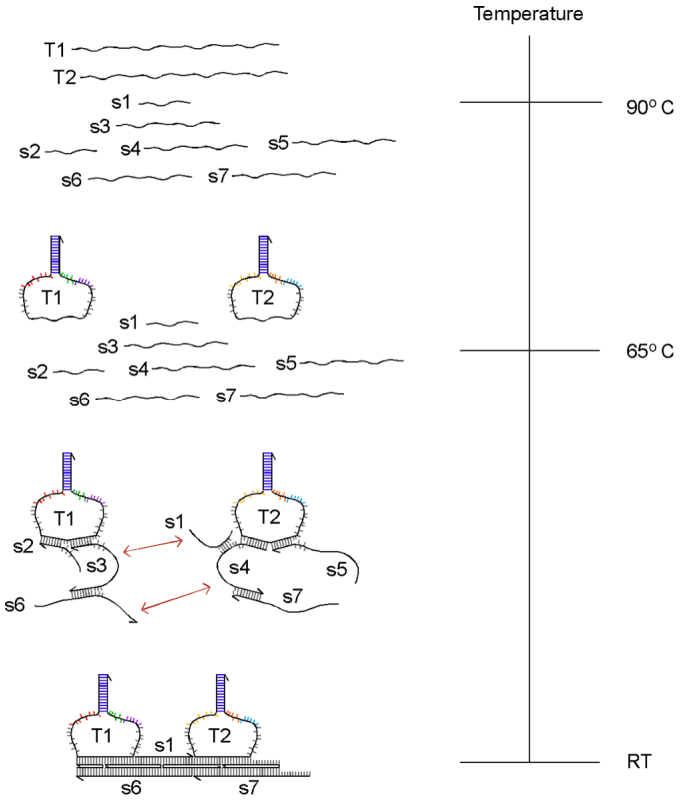

Fig. S2. Proposed kinetic path of the tile annealing. The track stem-loops and supporting strands are designed so that the stem-loops, having higher melting temperatures, form first and the supporting strands then form around them as the solution is brought directly from 90 deg to 65 and then gradually to room temperature. The track is also designed so

that the supporting strands (*s2, s3, s4, s5*) that pair with the track stem-loops can wrap their free ends through the stem-loops even if their other portions have already made pairings with the other support strands with which they are designed to pair.

S2. Sequence design

The sequences were first designed to minimize sequence symmetry with SEQUIN *(4)*. Some of the sequences were then altered by hand to minimize unwanted secondary structure in the hairpins predicted by mFold and NUPACK (http://www.nupack.org), and to maximize desired interactions between the strands predicted by NUPACK. ***Stem Region:*** The stem region has a much higher GC content (~ 71% - 10 of 14 bases) than the toehold sequences. and both ends terminate in two consecutive GC pairs. [The 3'-TA and 5'-AT ending in this experiment was added for psoralen crosslinking.] In contrast, the toehold single-stranded regions average to 35% GC content. This difference in GC content was intended to do several things: 1) strengthen the clamping function of the stem region by minimizing fraying and bubble formation; and 2) minimize the sequence similarity and hence base pairing between the stem region and the toehold regions. This is particularly important when the hairpins have been opened up and need to act downstream rather than fold on themselves. ***Toehold Sequences:*** The *leg-holding* sequences were chosen to be six bases long. The GC content is 33% in both *leg-holding a* and *leg-holding d* (2 of the six bases are either C or G). Neither a C nor a G lies at the base of the stem in the *leg-holding* sequences with the idea of making similar the kinetic and energetic features of the interactions of the walker legs with their respective track strands. It should be mentioned that both *leg-holding* sequences begin with a T, right at the base of the stem. While not desirable, as this is the first base (adjacent to the stem) where strand displacement can take place, we decided that the position of the base would serve only to allow strand invasion to continue through that position, rather than in the initiation of strand invasion. The *fuel-grabbing* sequences were chosen to be eight bases, two bases longer than the leg-holding sequences, so as to strengthen the reaction of the more difficult strand invasion that needs to take place for the track stem-loops to activate their fuels. The T_{16} flexibility of the fuel strands aids in opening the loops widely enough to allow for the invading track stem-loop strands to wrap more easily through the loops while they are invading. The *fuel-grabbing* sequences have GC content of 38% (3 of eight), with one G or C lying right at the base of the stem. The *leg-releasing* sequences are both six bases long with a GC content of 33% (2 of the 6), with no deliberate positioning of C or G's.

S3. Toplogical Gating: preventing the walker from walker falling off the track

Gating, or preventing the walker from being released prematurely from the track, emerges from the two metastable topologies that the walker and track create together when either one or two legs are attached. These two topologies protect the *leg-releasing* sequences directly below each leg from premature attack by their fuel strands. *Single-leg gating* is pictured in Fig. S3A: Above *leg-releasing* sequence *b,* the walker is shown in an intermediate position between resting states *RS-1* and *RS-2*. *L-E* is attached to *T2* and *L-O* is dangling. Below *b* the rest of the track is shown. Both of these complexes (colored in grey), shown above and below *b,* are too large to wrap through *F2,* and protect *b* from attack by *F2* while *L-O* invades *T3*. *Double-leg gating* is pictured in Fig. S3B: When both legs of the walker are attached to the track, the *leg-releasing* sequences *b* and *e* are

protected by the closed loop formed by the walker and the track. No matter what size or shape it is, a closed loop cannot wrap through another loop, so in this case it is the designed topology that performs the *gating*.

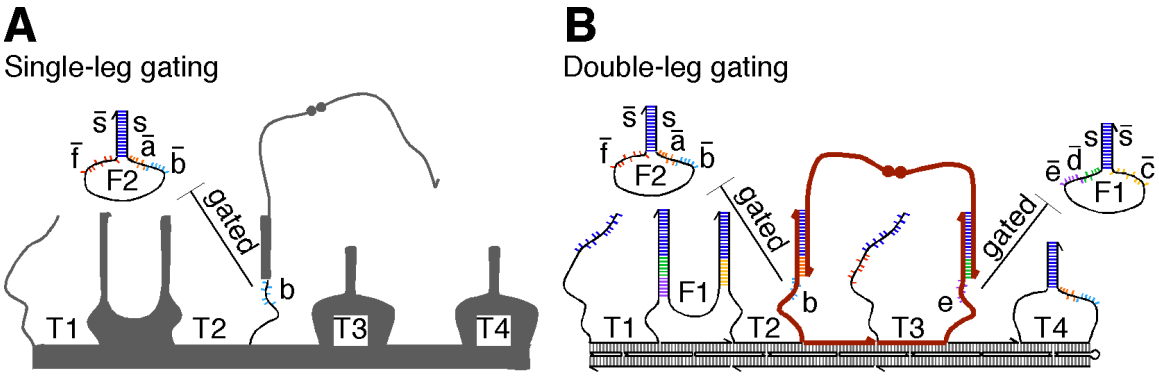

Fig. S3. Topological gating. (A) *Single-leg gating*. Above *b,* outlined in grey, the duplex formed by the walker and *T2* and the dangling leg are too large to wrap through the loop of *F2*. Below *b*, the track is too large to wrap through *F2*. (B) *Double-leg gating*. The bold red line outlines the closed loop topology that protects *b* and *e* from attack by fuel.

S4. Assembly and purification of the "front half " of the track

To assemble the "front half" of the track we initially annealed a molecule that did not include the walker. This molecule (named *H1*) contained *T1* and *T2* and their supporting DX strands. We then added the walker to *H1* for one hour at room temperature. This reaction resulted in a solution containing the target molecule (the "front half"), which consisted of one walker attached to *H1* (named *H1-W*), as well as many failure products (Fig. S4). We reasoned that the failure products were a combination of *H1*, and larger molecules containing multiple *H1's* linked together by the walker to form chains of *H1* (Fig. S5). Based on this reasoning we devised what turned out to be a successful method to purify the target H1-W.

Two unreacted sequences common to all the failure products were used to separate the failure products from *H1-W*. Those two unreacted sequences were the two *leg-holding* sequences on *T1* and *T2* that were left untouched in *H1*, and in the daisy chain's of *H1*. Two biotin functionalized strands (equivalent to the two legs of the walker not linked to one another), complementary to the two unreacted *leg-holding* sequences on *T1* and *T2*, were added to pair with the two *leg-holding* sequences. Once these biotin strands were paired with *T1* and *T2*, they provided a biotin handle to separate the failure products from *H1-W* with the use of streptavidin coated magnetic beads.

The procedure to purify *H1-W*, which is pictured in Fig. S5, went as follows: 1) The walker was reacted with *H1* for one hour; 2) The two biotin labeled strands that were complementary to the *leg-holding* sequences on *T1* and *T2* were added to the solutions and left to react to react for one hour; 3) The streptavidin coated magnetic beads were then added to the solutions and left to react for 30 minutes. During these 30 minutes, the biotin strands paired with the failure products attached to the streptavidin coated beads; 4) The solutions were then placed on a magnetic stand, which pulled the magnetic beads coated with the failure products to the walls of the tube. This left the target product *H1-W* suspended in solution. While still on the magnetic stand, this

remaining solution containing *H1-W* was pipetted into a new tube, isolating *H1-W* from the failure products.

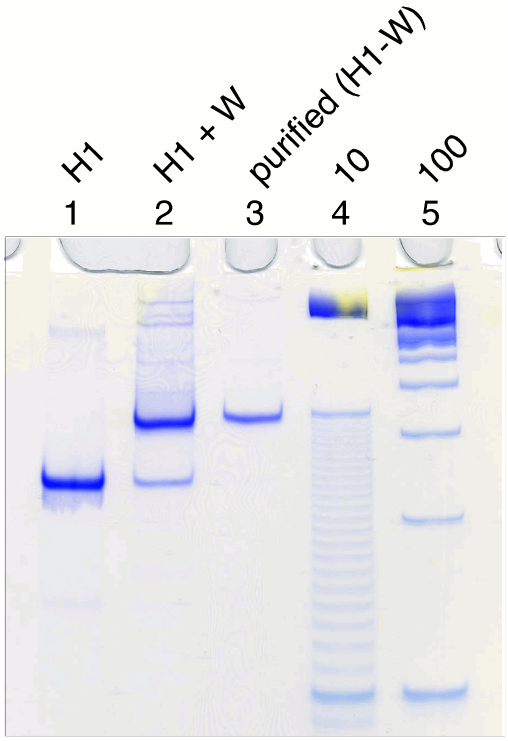

Fig. S4. Non-denaturing gel showing the purification of *H1-W* (stained with stains-all). The purification process is pictured in fig S3. (Note: This gel depicts the purification of a smaller and older version of *H1* not used in these experiments. This purification process became routine enough that we no longer ran diagnostic gels to compare the unpurified with the purified *H1-W* tile.)

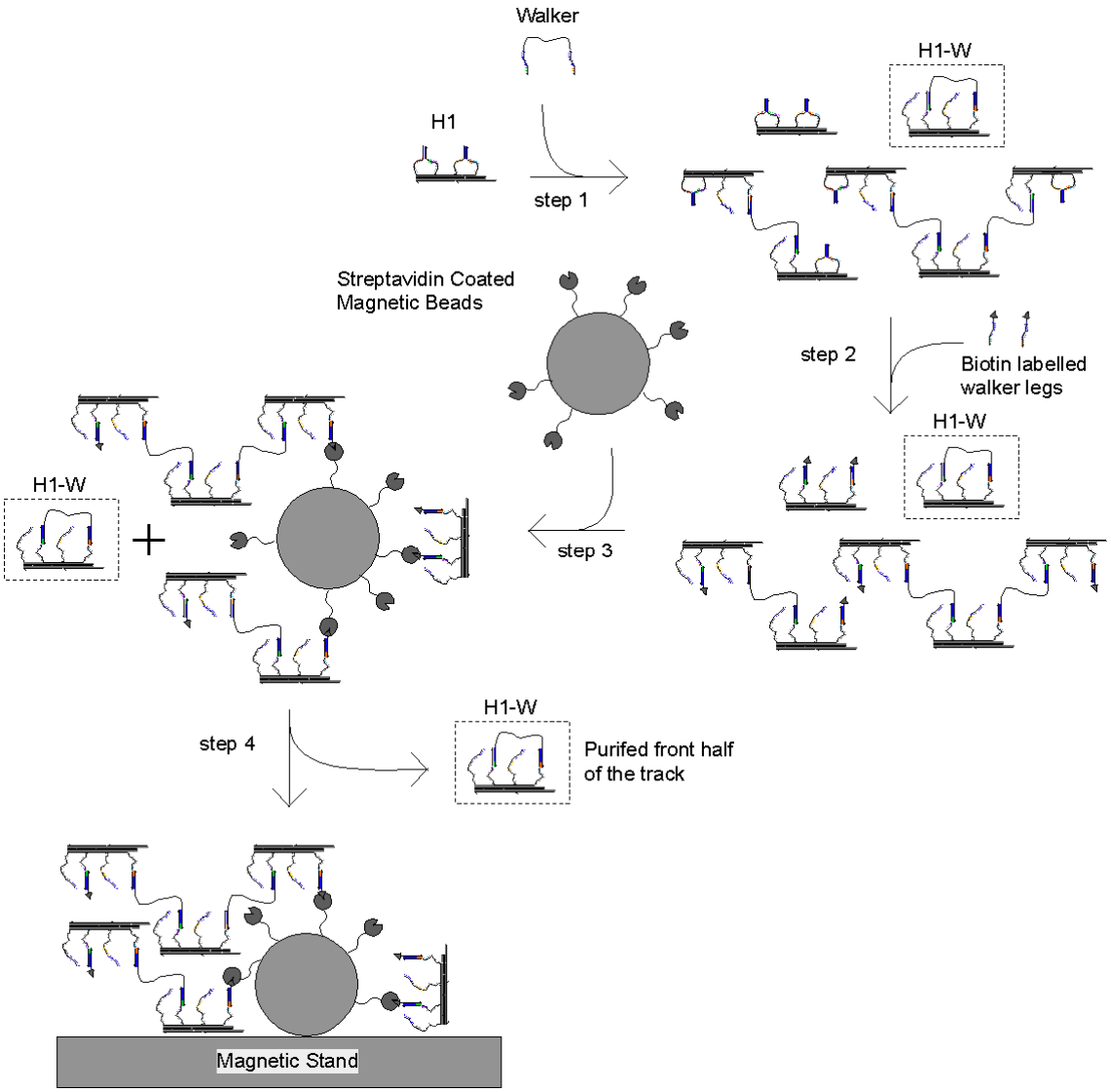

Fig. S5. Purification of the front half of track *H1-W*. (Step 1) The walker and *H1* are combined at room temperature. (Step 2) Biotin functionalized walker legs are added to the solution. (Step 3) Streptavidin coated magnetic beads are added to the solution. (Step 4) After being placed on a magnetic stand the failure products are separated from the target molecule *H1-W*.

S5. Pictorial summary of the track assembly

We assembled the five radioactive versions of the whole track (*W*, T1*, T2*, T3** and *T4**) in three steps that are summarized in Fig. S6. The bold red lines represent the strand that was radioactively labeled in each molecule. The assembly was performed as follows: 1) The front half of the track without the walker *H1*, and the back half of the track (named *H2*) were annealed separately; 2) The walker was combined with *H1* and the target product *H1-W*, consisting of one walker on *H1,* was isolated (text S4 and Fig. S4); 3) *H1-W* and *H2* were combined at room temperature to form the complete track.

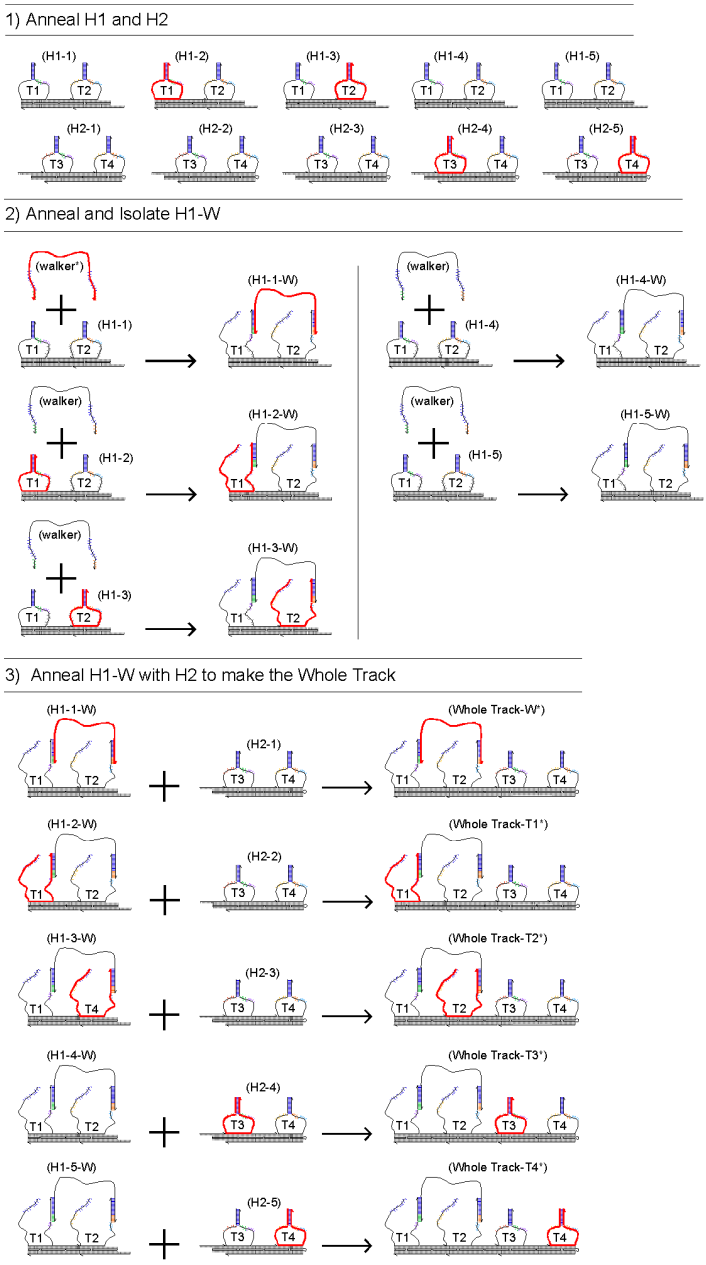

Fig. S6. Summary of the steps taken to assemble the five radioactive versions of the track (W*, T1*, T2*, T3*, T4*). The bold red strands represent the strand that is radioactively labeled in a particular construction. **Step 1:** Five H1 molecules (the front of the track without the walker) were annealed and five H2 molecules (the back half of the track) were annealed separately. Two of the H1 molecules were radioactive: H1-2 (radioactive stem-loop T1); and H1-3 (radioactive stem-loop T2). And two of the H2 molecules were radioactive: H2-4 (radioactive stem-loop T3) and H2-5 (radioactive stem-loop T4). **Step 2:** The walker was added to all five H1 molecules and the target molecules (H1-1-W, H1-2-W, H1-3-W, and H1-4-W) were purified as described in SOM S4. A radioactive walker was added to make H1-1-W. **Step 3:** The five versions of the two halves were annealed together to make the whole tracks in which only one strand was radioactively labeled.

S6. Description of the secondary crosslink products on the denaturing gels

The other bands on the denaturing gels consist of three other crosslinked products: 1) *s-f*: the track stem-loops crosslinked to the fuel hairpins; 2) *s-s*: the track stem-loops crosslinked to a neighboring track stem-loop; 3) *s*: the track stem-loops crosslinked to themselves, creating a circular product. Products *s-f* and *s* should reflect the state of the system as the walker progresses from *RS-1* to *RS-3* or *RS-4*. Product *s-s*, on the other hand, is a purely a failure product. Fig. S7 shows all the target products forming in all the states of the system.

All three of the secondary products (*s*, *s-f*, and *s-s*) result from the psoralen on the 3' end of the stem-loops crosslinking with the thymine penultimate to the end of the duplex on the 5' end (Fig. S7B). With UV irradiation, psoralen preferably crosslinks to the face of thymine in the 5' T-A direction (the *w-t* and *w-l* reactions), and much less in 3' T-A direction (the s, s-s and s-f reactions) (6). As a result, the *s*, *s-s* and *s-f* crosslink products formed in lower yields and showed up less intensely on the autoradiograms than the *w-t-l*, *w-t* and *w-l* products. Both *s-s* and *s-f* travel according to their size on the denaturing gels, as the two strands in the complexes are joined at their end points. This is in contrast to products *w-t-l*, *w-t* and *w-l*. These three products form from the stem-loops crosslinking to the walker at midpoints in the walker (Stem-loops crosslink to the walker on the two thymidines 23 bases in from the walker's 5' and 3' ends). Midpoint crosslink sites lead to more complex topologies that travel much slower on a gel than their number of nucleotides (7). Product *s* is a circular product, hence it also travels slower than its number of nucleotides would suggest under these conditions. Figs. S8 and S9 show complete pictures of all the super-denaturing gels shown in the main text (4B, 4C, 4E left and right, and 4G). The secondary products have a box drawn around them, and cartoons of their topologies are shown on the left hand side of the gels.

The existence of *s-s* (*T3-T4*) is evidence that *T3* and *T4* interacted with one another before the addition of fuel, presumably through their stem regions. While *T3* and *T4* did interact, their metastable topologies were still preserved. This is supported by the control in which *F2* was added alone to the track. If *T3's* stem-loop topology had been broken before the addition of *F2*, or by the addition of *F2*, *F2* would have been activated by *T3's fuel-grabbing* site *f*. The activated *F2* would have then released the leading leg of the walker (*L-E*) from *T2* on most of the tracks (Fig. S10). This result was not seen in our experiments. Rather, a majority of the walker remained on *T1* and *T2* after 1 hour being in solution simultaneously with *F2*. This indicates that *T3* mostly maintained it's fold (Fig. S9 - 4E left, lanes 6-10; and Fig. 4E in the main text, lanes 6-10). While the interaction of *T3* and *T4* did not greatly affect the operation of the system, it suggests that the spacing and flexibility of the stem-loops should adjusted in future designs, to preclude this interaction.

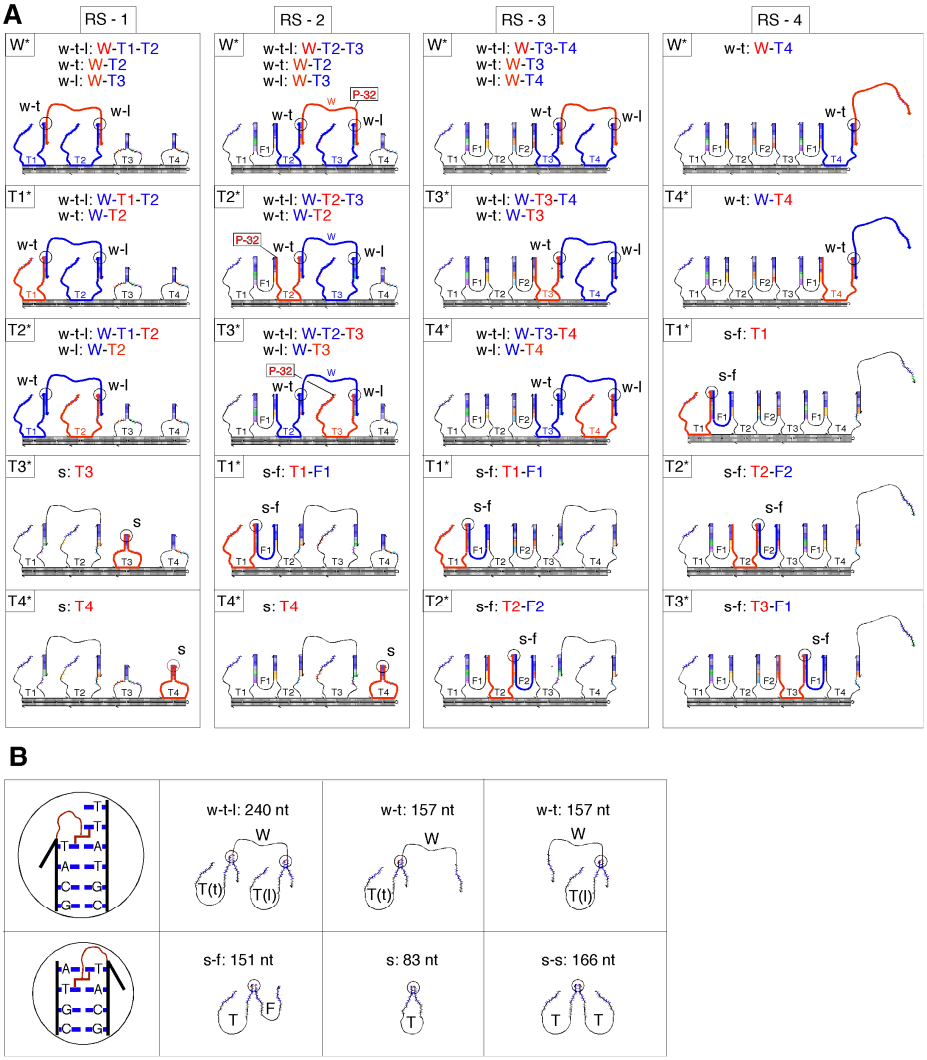

Fig. S7. Visualization of all the crosslink products with ^{32}P and topology of all the target products (A) This picture shows all the target crosslink products that form and are visible in all the states of the system according to which strand is radioactively labeled. Radioactively labeled strands are drawn in bold red and strands that crosslink to the radioactive strand are drawn in bold blue. The resulting crosslink products are listed in the boxes. (B) Detailed picture of the two crosslink reactions and topologies and sizes of the denatured crosslink products that form from them. The top row shows the crosslink reaction for products *w-t-l*, *w-t*, and *w-l*, and their denatured topologies and sizes to the right. The bottom row shows the crosslink reaction for products *s-f, s* and *s-s*, and their denatured topologies and sizes to the right.

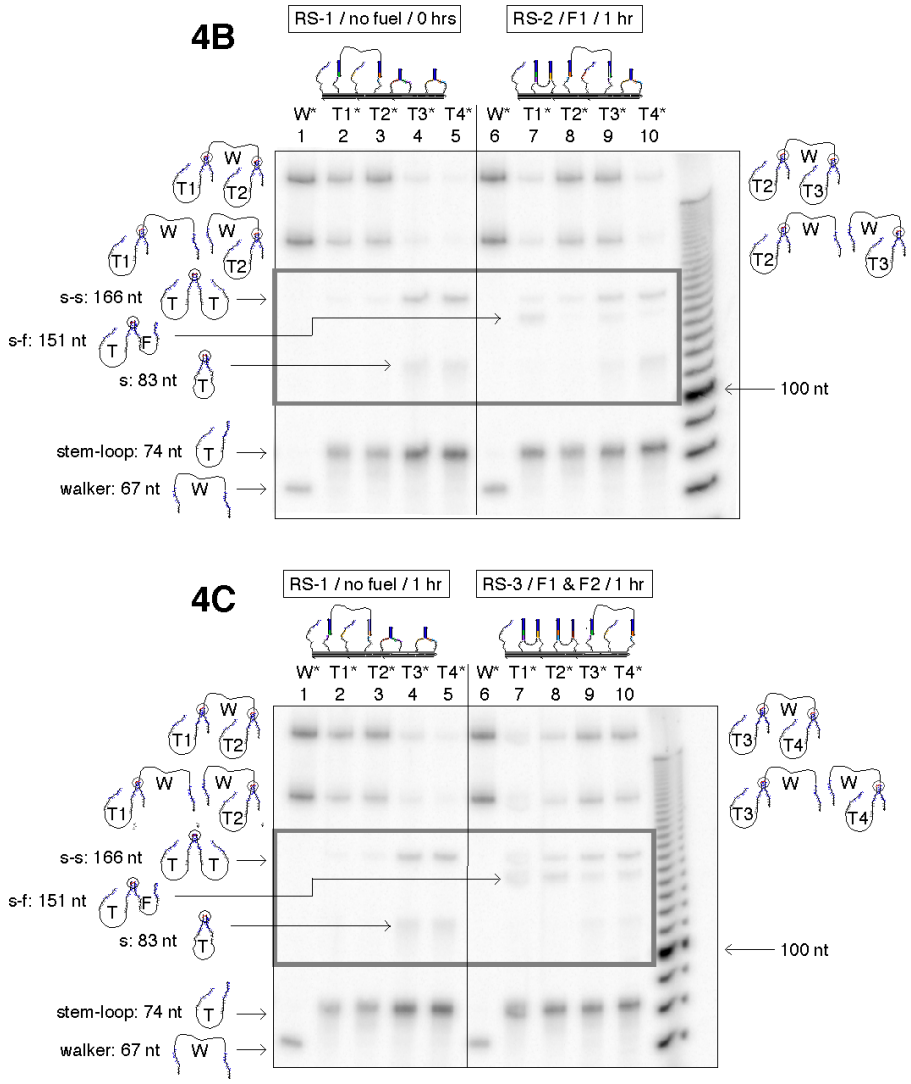

Fig. S8. Full pictures of main text denaturing autoradiograms fig 4B and 4C with the box outlining the secondary crosslinked products. The secondary products are pictured on the left hand side of the gels.

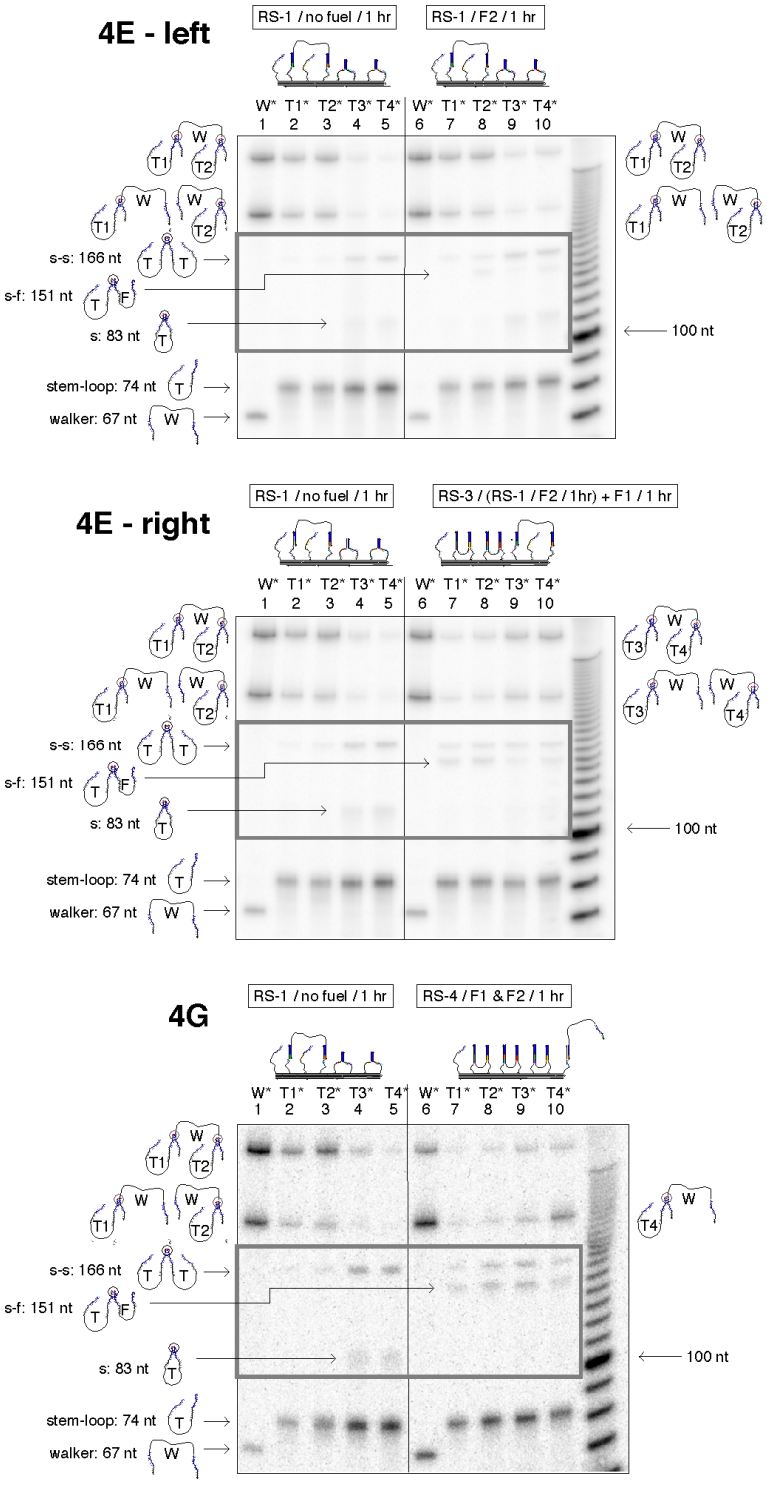

Fig. S9. Full pictures of main text denaturing autoradiograms Fig. 4E and 4G with the box outlining the secondary crosslinked products. Fig. 4E lanes 1-5 in the main text, are lanes 6-10 in 'fig 4E-left' above. Fig 4E lanes 6-10 in the main text, are lanes 6-10 in 'fig 4E-right' above. The secondary products are pictured on the left hand side of the gels.

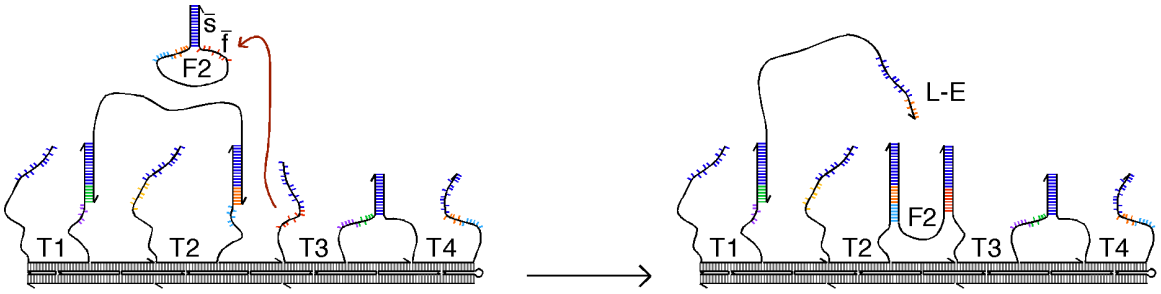

Fig. S10. A scenario in which *T3* was open by being paired with *T4* when *F2* was added, would lead to a state wherein *L-E* was kicked off of *T2*. This result is not seen in Fig. S6 (Fig. 4E left and Fig. 6E right). Instead a majority of the walker remains on *T1* and *T2* when *F2* sits in solution with *RS-1* for one hour (4E left, lanes 6-10). This result indicates *T3* maintains it's fold even while it interacts with *T4*.

S7. Complete native gels 4A, 4D, 4F, and 4H, and assembly gels for 4F and 4H

Complete pictures of native gels 4A, 4D, 4F, and 4H are shown below (Fig. S11). The track assembly gels associated with 4F and 4H are also shown. Fig. 4A, 4D, and 4F show results from tracks in which *T4's fuel grabbing* sequence *c* was deactivated. On the other hand, Fig. 4H shows results from tracks in which *T4's fuel-grabbing* sequence was restored. which allowed the walker to transition to *RS-4*.

A small amount of free walker can be seen in lane 7 of 4H (Compare to '4H assembly' lane 6). Lanes 7-11 of 4H show the native state of the system in state *RS-4*, in which only leg *L-E* of the walker is attached to the track on stem-loop *T4*. This small amount of free walker in lane 7 indicates that *single-leg gating gating* is less stable than the *double-leg gating*. Indeed, no free walker is seen in 4D and 4F, in which the *double-leg gating* topology was protecting the walker from release in state *RS-3* (lanes 7-11 of both gels). A difference in the stability of these two topologies is to be expected. Most importantly, the data supports the designed kintetic control of the system. In this kinetic control, the single leg gating topology is robust enough to protect the trailing leg's *leg-releasing* sequence from premature attack by fuel while the leading leg invades its target stem-loop and catalyzes the release of the trailing leg.

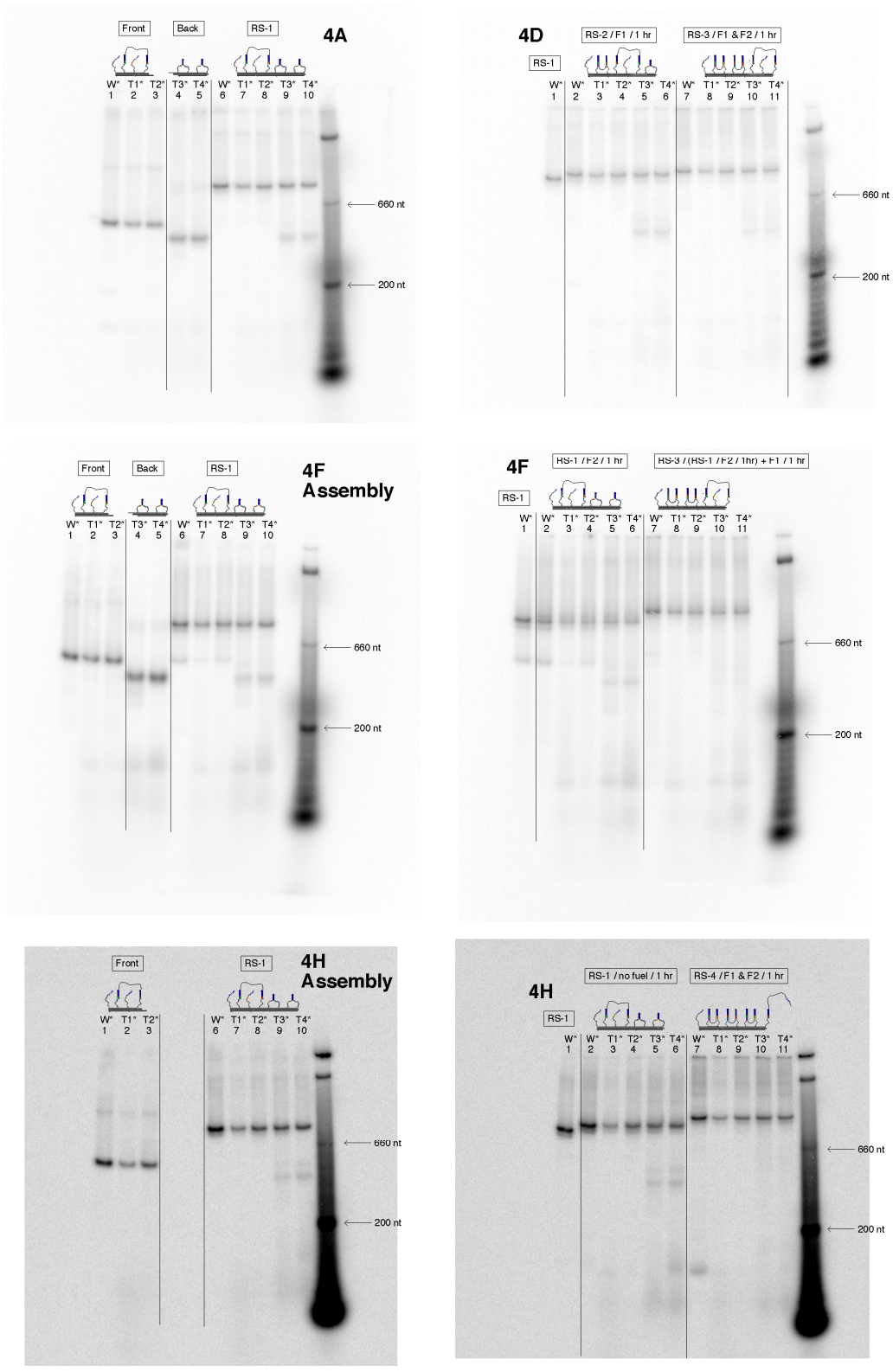

Fig. S11. Complete figures of all the non-denaturing autoradiograms. (Note: H2 was not loaded on gel 4H Assembly). All labeled bands in the marker lanes are labeled by the number of nucleotides they correspond to.

S8. Stepping efficiency estimated from Fig. 4C (Fig. S12)

To estimate the stepping efficiency of the walker for step 1 (*L-O* moving from *T1* to *T3*), we calculated the percentage change in the amount of L-O crosslinked to T1 in states *RS-1* and *RS-3*. To estimate the efficiency for step 2 (*L-E* moving from *T2* to *T4*), we calculated the percentage change in the amount of *L-E* crosslinked to *T2* in states *RS-1* and *RS-3*. We used denaturing gel 4C from the main text (refer to Fig. S11) to perform this calculation. The calculations are given in Table S1. The estimates consist of calculating the percent of leg *L-O* dislodged from *T1* in step 1, and the percent of *L-E* dislodged from *T2* in step 2.

Three assumptions were made about the system to make this estimation: 1) the crosslinking efficiency was the same in all the cross-linking reactions between the walker and the track stem-loops; 2) all of *L-O* that was dislodged from *T1*, ended up on *T3*; and 3) all of *L-E* that was dislodged from *T2*, ended up on *T4*. Assumptions 2 and 3 can be made based on the fact that there is no free walker seen on non-denaturing gel Fig. 4D (main text and Fig. S11).

Image Quest (GE) gel analysis software was used to analyze gel 4C. The software integrated over designated gel lanes, calculating the percentage of counts in each band for that particular lane. The lanes and bands used to calculate the stepping efficiency are labeled in Fig. S11. For step 1, a 74% percent stepping efficiency was calculated by comparing line 1 to line 3. For step 2, a 74% percent stepping efficiency was calculated by comparing line 2 to line 4.

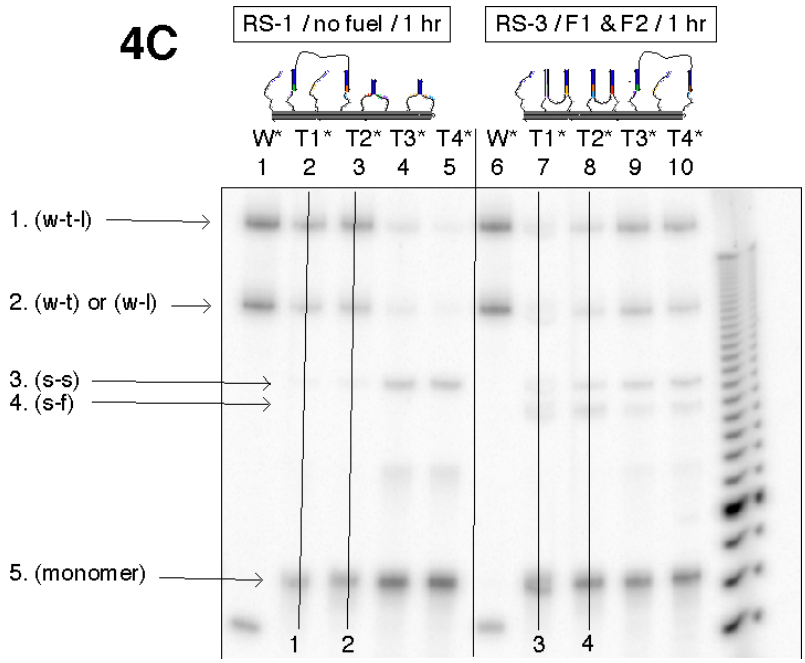

Fig. S12. Super-denaturing gel 4C from the main text. The percentage of counts in each band labeled on the left was determined for the four lanes labeled 1, 2, 3, and 4 on the bottom of the gel. See Table S1 for these numbers.

	RS-1		RS-3	
	$T1_{RS-1}$ (% of counts) (line 1)	$T2_{RS-2}$ (% of counts) (line 1)	$T1_{RS-3}$ (% of counts) (line 2)	$T2_{RS-3}$ (% of counts) (line 3)
1 (w-t-l)	35.07	35.08	7	8.06
2 (w-t) or (w-l)	19.43	19.94	7.04	5.26
3 (s-s)	1.04	1.92	2.71	3.36
4 (s-f)	0	0	5.93	6.29
5 (monomer)	44.47	46.06	77.33	77.03
	$T1_{RS-1}$ (1+2): % of W linked to T1 in RS-1	$T2_{RS-1}$ (1+2): % of W linked to T2 in RS-1	$T1_{RS-3}$ (1+2): % of W linked to T1 in RS-3	$T2_{RS-3}$ (1+2): % of W linked to T2 in RS-3
1+2	54.40	52.02	14.04	13.32
Stepping efficiencies for the transition from RS-1 to RS-3				
Step 1 efficiency $\frac{T1_{RS-1}(1+2)-T1_{RS-3}(1+2)}{T1_{RS-1}(1+2)} = \frac{54.40-14.04}{54.40} = 0.74$ or 74%				
Step 2 efficiency $\frac{T2_{RS-1}(1+2)-T2_{RS-3}(1+2)}{T2_{RS-1}(1+2)} = \frac{52.02-13.32}{52.02} = 0.74$ or 74%				

Table S1. Estimation of the stepping efficiency of the walker. Values used in this calculation were derived from gel 4C in the main text, which is shown in Fig. S5.

S9. Materials and methods.

DNA: All unmodified DNA molecules were ordered unpurified from Integrated DNA Technology (Coralville, IA). All modified DNA strands (*Psr, WL-O, RA,* and *RB*) were made on an Applied Biosystems 394 automatic DNA synthesizer, using phosphoramidites obtained from Glen Research (Sterling, VA). The names and sequences of all the strands are given at the end of this section.

Purification of DNA:: We purified the strands by denaturing PAGE. Extractions were done in elution buffer (500 mM ammonium acetate, 10 mM magnesium acetate, 2 mM EDTA) overnight at room temperature. This was followed by butanol extraction and ethanol precipitation. All stock solutions were prepared at 10 μM in double distilled H_2O.

Synthesis of walker: The walker has two 3' ending legs. To switch the polarity of the walker in the middle of the strand, we first synthesized from one end in the conventional 3' to 5' direction to the middle of the strand. We then switched polarity by synthesizing the rest of the strand in reverse, using 5'-CE phosphoramidites (Glen Research, Sterling VA).

Ligation of complete track stem-loops: The walker invades the 3' ends of the track stem-loops; hence, we needed our stem-loops to be functionalized with psoralen attached to the 3' end. Stem-loops with 3' psoralen molecules were made by first synthesizing a short 14 nt fragment (*Psr*) of the stem-loops in the 5' to 3' direction, with 5'-CE phosphoramidites (Glen Research) on a 5' support (Glen Research). Psoralen (Glen Research psoralen C2 phosphoramidite) was then added to the 3' end of *Psr* during

synthesis. *Psr* was finally ligated to the five 69 nt fragments (*T1p, T2p, T3p, T4p, T4p-b*) constituting the remaining portion of the track stem-loops.

Psr was phosphorylated in 100 μL 1X optikinase buffer containing 12.5 μL of 100 μM *Psr* stock, 10 μL of diluted optikinase (16 μL in 144 μl H_2O), and 10 μL of 10 mM ATP. (8 samples for each stem-loops was prepared). 25 μL of 50 μM linker strand *LT-odd* (for *T1* and *T3*) or *LT-even* (for *T2, T4* and *T4-b*) was added to the kinated *Psr* solutions and they were placed at 90 ˚C for five minutes then left to cool at room temperature for 20 minutes. After cooling we added 20 μL of 50 μM 69 nt fragments (*T1p* and *T3p* to *LT-odd* tubes; *T2p, T4p* and *T4p-b* to *LT-even* tubes), 6.11 μL of 10X optikinase buffer, and 10 μl of diluted ligase (USB corporation)(dilution – 16 μL stock in 144 μL dilution buffer) bringing the solutions to a total volume of 161.1 μL. They were all placed at 16 ˚C and left to react overnight.

After ligating overnight, the samples were dried down and 2 samples (~ 2000 pmoles) were purified on one 10 % denaturing gel. The target bands were cut out and eluted in buffer overnight at room temperature. This was followed by butanol extractions and ethanol precipitation's. Stock solutions were prepared at 10 μM in double distilled H_2O.

Super-denaturing gels:

The use of super-denaturing gels was required to get reliable results in these experiments. When standard denaturing PAGE gels were used, the walker/stem-loops crosslinked products did not consistently separate into defined bands. We think this was due to two properties of the crosslinked products: (1) The midpoint crosslink sites on the walker, which resulted in more convoluted topologies than those formed by end-point crosslink sites; and (2) the high melting temperature of the stem region, which prevented complete denaturation on the gels. When the crosslinked products were run on super-denaturing gels, they separated into clear bands.

Super-denaturing stock was prepared as follows: We combined 150 mL of formamide, 250 mL of 20% denaturing acrylamide solution containing 100 mM Tris.HCl, pH 8.3, 89 mM Boric acid, 2 mM EDTA (1X TBE), 25 mL of 10X TBE buffer, and 125 g of urea. The mixture was heated to dissolve the urea and divided into 20 mL volumes. Dividing the solution before storing was necessary because the urea slowly precipitated out of the solution. By dividing it before precipitation, each solution was guaranteed to have the correct amount of urea. Each 20 mL solution was heated before use to re-dissolve any crystalized urea. We also found it helpful to heat the gel plates before casting the gel, as cold plates sometimes caused the urea to crystallize while the gel was polymerizing.

Radioactive labeling and purification of the walker and track stem-loops:

K = Counts per minute/μCi, (CPM/μCi)

Walker*:* The walker was radioactively labeled in the middle of the strand by first radioactively labeling a portion of the walker named *WHA* that had a 5' end available for phosphorylation with ^{32}P. *WHA* was then ligated to the remaining portion of the walker (named *W6A*) that contained the 5'-5' linker and hence had two 3' ends with a bridging strand (named *WJN*). Labeling of *WHA* was done in 10 μL 1X optikinase buffer (USB corporation) that included 2 μL of 10 μM *WHA*, 2 μL of ^{32}P ATP (MP Biomedicals), and 1μL of optikinase (10X diluted) (USB corporation). The solution was left at 37 ˚C for 30 minutes and the reaction was killed by shifting it to 90 ˚C for 5 minutes. 2 μL of 10 μM

WJN was added and the whole solution was placed at 90 ˚C for five minutes then left to cool at room temperature for 20 min. 1 μL of 10 μM *W6A*, 0.4 μL of optikinase buffer, and 1 μL of ligase was added bringing the solution to 14 μL and the solution was left at 16 deg for 1 hour.

Track stem-loops: *T1, T2, T3,* and *T4* were radioactively labeled on their 5' ends. Because their stem regions have high melting temperatures, we found that the efficiency of labeling could be greatly increased by the addition of partial complements to their 3' ends. The partial complements opened up the stem-loops and made their 5' ends more accessible to optikinase. The stem-loops (*T1, T2, T3,* and *T4*) were first annealed with their opening strands [either *LT-odd* (for *T1* and *T3*) or *LT-even* (for *T2* and *T4*)] in 9 μL solutions. All four 9 μL solutions contained 1μL of optikinase buffer, 2 μL of ^{32}P ATP, 1 μL of 10 μM stem-loops (*T1, T2, T3* or *T4*), 2 μL of either *LT-odd* (for *T1* and *T3*) or *LT-even* (for *T2* and *T4*) and 4 μL of H_2O. They were placed at 90 ˚C for five minutes and then left to cool at room temperature for 20 minutes. 1μL of optikinase was then added to all four solutions and they were placed at 37 ˚C for 30 minutes for kination, and then 90 deg for five minutes to stop the reactions.

Purification of radioactive W, T1, T2, T3, and T4: After labeling, all the solutions were dried down, and run on a 10% denaturing gel. The gel was then exposed to film which was developed and used to locate the target products on the gel. The target bands were then cut out and eluted in buffer. After elution, salts were removed by ethanol precipitation. The solutions were then dried down and their activity was measured on a scintillation counter. Stock solutions were prepared to try and make 50K/μL solutions. Due to the small amount of DNA in the solutions, the initial measurements of radioactivity included non-recoverable material that was stuck to the walls of the tubes. Once water or buffer was added to make stock, the activity of aliquots of the stocks were measured, to establish the extent of the recoverable counts/μL were.

Detailed experimental procedures:

Stock solutions of all the non-radioactive strands were prepared at 10 μM. All solution were 10.5 mM TAE Mg^{2+}. Stock solution of the radioactive strands were prepared at approximately 50 K/μl. **[**K = Counts per minute/μCi, (CPM/μCi)]

Step 1 - Annealing (H1), the front half of the track without the walker

H1-1 (non-radioactive T1 and T2): 20 pm of the seven support strands and 20 pm of *T1* and *T2* were combined to make a 40 μL solution. *H1-2 (radioactive T1, non-radioactive T2):* 20 pm of the seven support strands and 20 pm *T2* were combined with 18pm of non-radioactive *T1* and 100 K of radioactive *T1* to make a 40 μL solution. *H1-3 (radioactive T2, non-radioactive T1):* 20 pm of the seven support strands and 20 pm *T1* were combined with 18 pm of non-radioactive *T1* and 100 K of radioactive *T1* to make a 40 μL solution. *H1-4 (non-radioactive T1 and T2):* Same as *H1-1*. *H1-5 (non-radioactive T1 and T2):* Same as *H1-1*. [Annealing procedure for all of the above: All solutions were annealed 90 ˚C (5 min), 65 ˚C (20 min), 45 ˚C (20 min), 37 ˚C (20 min), room temperature (20 min).]

Step 1B - Annealing (H2), the back half of the track: *H2-1 (non-radioactive T3 and T4):* 10 pm of all nine strands were combined to make a 20 μL solution. *H2-2 (non-radioactive T3 and T4):* Same as *H2-1*. *H2-3 (non-radioactive T3 and T4):* Same as *H2-1*. *H2-4 (radioactive T3, non-radioactive T4):* 10 pm of the seven support strands and 10

pm *T4* were combined with 9 pm of non-radioactive *T3* and 50 K of radioactive *T1* to make a 20 μL solution. *H2-5 (radioactive T4, non-radioactive T3):* 10 pm of the seven support strands and 10 pm of *T3* were combined with 9pm of non-radioactive *T4* and 50 K of radioactive *T4* to make 20 μL solutions. [Annealing procedure: All solutions were annealed 90 ˚C (5 min), 65 ˚C (20 min), 45 ˚C (20 min), 37 ˚C (20 min), room temperature (20 min).]

Step 2: Adding the walker and purifying H1-1-W, H1-2-W, H1-3-W, H1-4-W, H1-5-W: *H1-1-W (radioactive W):* 15 pm (3 μL of 5 μM stock in 10.5mM TAE Mg^{2+}) of non-radioactive walker was added and 100 K (2 μL of ~50 K/μL stock) of radioactive walker in 10.5 mM TAE Mg^{2+}. This brought the volume of *H1-1-W* to 45 μL. *H1-2-W, H1-3-W, H1-4-W, and H1-5-W:* 15 pm (3 μL) of non-radioactive walker was added to all four solutions bringing the volumes of the samples to 43 μL. [Annealing procedure: After adding the walker, all five solutions were left to anneal for 1 hour at room temperature.] *Purifying all H1-W's:* 60 pm (1.5 μL of 40 μM buffer solutions) of the two cleaning strands *RA* and *RB* (same sequence as the two walker legs modified with 5' biotins) were added to all five *H1-W* solutions and left to react for 1 hour. 240 μL of streptavidin coated magnetic bead solutions (cleaned three times with 10.5 mM TAE Mg^{2+} buffer) were added to all five *H1-W* solutions for 30 minutes stirring the solution every 10 min. The samples were then placed on magnetic stands and the solutions were removed yielding clean samples. (see text S4 and Fig. S5). The cleaning process reduced the volumes of the samples by about 3 μL bringing H1-1-W back to 45 μL and H1-2-W through H1-5-W back to 43 μL.

Step 3: Annealing H1-W's with H2's to make whole tracks (W*, T1*, T2*, T3*, T4*) All five samples of cleaned *H1-W* (*H1-1-W, H1-2-W, H1-3-W, H1-4-W,* and *H1-5-W*) were equalized to 45 μL (the volume of *H1-1-W*) by adding 2 μL of buffer to *H1-2-W* through *H1-5-W*. Eight portions [8 x (45 μL/10) = 36 μL] of all three radioactive samples of *H1-W* (*H1-W: H1-1-W, H1-2-W, and H1-3-W*) were combined with eight portions (8 μL) of non-radioactive *H2* (*H2-1, H2-2,* and *H2-3*) respectively and likewise 8 portions of the two non-radioactive samples of *H1-W* (*H1-4-W* and *H1-5-W*) were combined with 8 portions of the two radioactive samples of *H2* (*H2-4* and *H2-5*) for one hour to yield the five radioactive whole track samples *W*, T1*, T2*, T3** and *T4** with volumes of 44 μL. *H1* and *H2* were both initially prepared at 0.5 μM. The addition of the walker to *H1* and its isolation removed a significant amount of material. By titrating cleaned samples of *H1-W* (after magnetic bead cleaning) against *H2* to make the whole track, we determined that *H1-W* had to be combined with 4.5 times the amount of *H2* to get the correct stoichiometry. [e. g - Having started with 10 pm of *H1* without the walker we would end up with approximately 2.22 pm (or ~ 22 %) of purified *H1-W*]. Combining 36 μL of *H1-W* with 8 μL of 0.5 μM H2 gave us 44 μL solutions with a track concentration of approximately 91 nM (0.091 μM). In all experiments, 1 portion of whole track [(vol. of whole track solutions / 8) = (44 μL / 8 = 5.5 μL)] was eventually used for each analysis (each lane on a gel). To compensate for slight evaporation and pipetting errors, 10 portions (2.5 K each) of *H1-W's* and *H2's* were initially prepared.

Rules followed for the addition of the fuel: Fuel molecules *F1* and *F2* were annealed separately at 9 μM in 10.5mM TAE Mg^{2+}. In all the cases when either *F1* or *F2* were added alone, the concentration of fuel in the solution with track was 1 μM. In all cases when *F1* and *F2* were added together, they were first combined from the individual 9 μM

solutions, then added to the track solutions, resulting in a fuel concentration of 0.56 μM for each fuel strand. *Adding F1 or F2 alone:* For an initial fuel concentration of 9 μM, 0.69 μL of either F1 or F2 was added for every 5.5 μL of whole track (where 5.5 μL was 1 portion of the whole solution). This gave a final volume of track plus fuel of 6.19 μL, in which the concentration of the track was approximately 0.065 μM, and the initial concentration of the fuel was approximately 1 μM. *Adding F1 and F2 together:* When *F1* and *F2* were added together they were first pre-combined at 9 μM and then 0.79 μL of the combination was added for each 5.5 μL portion or track. This gave a final volume of track plus fuel of 6.29 μL, in which the concentration of track was approximately 0.063 μM, and the initial concentration of fuel was 0.56 μM for each fuel strand.

Experiment 1: (main text figs. 4A , 4B, 4C, and 4D). After the final annealing step of the track solutions was completed, a non-denaturing gel was run containing one portion of *H1-1-W, H1-2-W, H1-3-W* and *H2-4* and *H2-5* and one portion of the fully formed track *W*, T1*, T2*, T3*, T4**, and *T5** (4C). After loading 4C, pre-annealed *F1* was added to two portions (11 μL) of all five whole tracks and pre-annealed and then pre-combined *F1* and *F2* were added to another two portions for one hour. The two portions of each of the samples were divided after the hour and one half was crosslinked at 4° C (UV 366 nm) for 10 minutes, denatured at 90 ˚C for 5 minutes, dried down, combined with denaturing dye and run on 10% super-denaturing gels (4B and 4C). The other half was run on a 4% non-denaturing gel (4D). Three portions had no fuel added as controls: One portion was UV exposed before fuel was added to any of the samples (Fig. 4B lanes 1-5); The second was exposed to UV an hour later with the other samples to which fuel was added (Fig. 4C, lanes 1-5); The third acted as a marker on the non-denaturing gel (Fig. 4D, lane 1). All gels were exposed to phosphoimager film for 30 min to an hour and scanned on a Storm Phosphoimager 840 (GE Healthcare).

Experiment 2: (main text Fig. 4E and 4F). Same procedure as in Experiment 1, except that *F2* was added to four portions of annealed track for one hour. Half (2 portions) was then removed, to which F1 was added for another hour.

Experiment 3: (main text Fig. 4G and 4H). Same procedure as in Experiment 1.

Sequences used in the construction and operation of device:

Refer to figure S13 for a picture of the track with all the supporting strands labeled by name. Except as noted, all the sequences below are given in 5' to 3' direction.

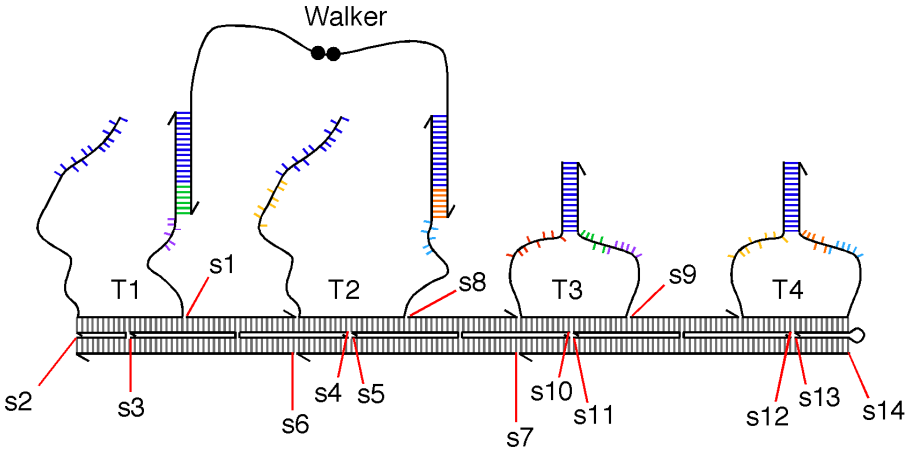

Fig. S13. Picture of the track with all the supporting strands labeled by name on their 5' ends. The color coding of the walker and stem-loops in the figure is consistent with the colors of the bases in the written out sequences.

Supporting track strands:

s1: 21
CCTCACACCAGGTCTTAATAC

s2: 20
TGATGAGGTGCCGACATTGG

s3: 42
ACTAATGGTTACATCGGTAGGTGGTGTGAGGATTCCTCTGCC

s4: 42
TGTCTCGATGGTATTAAGACCACAGCTCTCTGGAGTTACTGG

s5: 42
ACAAGAGGCTCGTTGTGTAGGTGGATAGCAGGTGCTCTAGCC

s6: 42
CAGAGAGCTGTCCTACCGATGTAACCATTAGTCCAATGTCGG

s7: 42
TCTTAGAAAGTCCTACACAACGAGCCTCTTGTCCAGTAACTC

s8: 21
CTGCTATCCAGGTCGTATGCT

s9: 21
CTAATTTGCAGGCTACATGAG

s10: 42
TGGTTTGAGAAGCATACGACCACTTTCTAAGAAGCTCAGCGG

s11: 42
ACACGTAGTTGTCACCGCAGGTGCAAATTAGGCGCGACTCCT

s12: 42
TGGCCTTTGACTCATGTAGCCACACTTACCTTGTTGCGGCGG

s13: 22
ACTGAGATTTCGACTGTGTTCT

s14: 63
GAAATCTCAGTCCGCCGCAACAAGGTAAGTGTCCTGCGGTGACAACTACGTGTCCGCTG
AGCT

Walker, track stem-loops, and fuel:

Walker: 74
(3′)GATAGACGACGCTCACCTGCTATTTTTTTTTTTTTTTT(5′-5')
TTTTTTTTTTTTTTTATCGTCCACTCGCAGCAAGTTC(3′)

T1: 83
ATCGTCCACTCGCAGCTTTTTTTTTTTTTCACCTCATCAGGCAGAGGAATTTTTTTCTG
TACTATCTGCTGCGAGTGGACGAT(psoralen)

T2: 83
ATCGTCCACTCGCAGCGTCATCTATTTTTCATCGAGACAGGCTAGAGCACTTTTTCATA
ACGAACTTGCTGCGAGTGGACGAT(psoralen)

T3: 83
ATCGTCCACTCGCAGCCACATAACTTTTTTCTCAAACCAAGGAGTCGCGCTTTTTTCTG
TACTATCTGCTGCGAGTGGACGAT(psoralen)

T4: 83
ATCGTCCACTCGCAGCGTCATCTATTTTTTCAAAGGCCAAGAACACAGTCTTTTTCATA
ACGAACTTGCTGCGAGTGGACGAT(psoralen)

F1: 68
ATCGTCCACTCGCAGCAGATAGTACAGATTTTTTTTTTTTTTTTTAGAT
GACGCTGCGAGTGGACGAT

F2: 68
ATCGTCCACTCGCAGCAAGTTCGTTATGTTTTTTTTTTTTTTTTGTTATGTGGCTGCGA
GTGGACGAT

Strands used to make the complete track stem-loops:

Psr: 14
TGCGAGTGGACGAT(psoralen)

T1p: 69
ATCGTCCACTCGCAGCTTTTTTTTTTTTTCACCTCATCAGGCAGAGGAATTTTTTTCTG
TACTATCTGC

T2p: 69
ATCGTCCACTCGCAGCGTCATCTATTTTTCATCGAGACAGGCTAGAGCACTTTTTCATA
ACGAACTTGC

T3p: 69
ATCGTCCACTCGCAGCCACATAACTTTTTTCTCAAACCAAGGAGTCGCGCTTTTTTCTG
TACTATCTGC

T4p: 69
ATCGTCCACTCGCAGCGTCATCTATTTTTTCAAAGGCCAAGAACACAGTCTTTTTCATA
ACGAACTTGC

T4p-b: 69
ATCGTCCACTCGCAGCTTTTTTTTTTTTTTCAAAGGCCAAGAACACAGTCTTTTTCATA
ACGAACTTGC

LT-odd: 28
ATCGTCCACTCGCAGCAGATAGTACAGA

LT-even: 28
ATCGTCCACTCGCAGCAAGTTCGTTATG

Strands used to make the radioactively labeled walker:

WL-E: 12
(5′)CGCAGCAAGTTC(3′)

WL-O: 62
(3′)GATAGACGACGCTCACCTGCTATTTTTTTTTTTTTTT(5′-5')
TTTTTTTTTTTTTTTATCGTCCACT(3′)

WJN: 22
GAACTTGCTGCGAGTGGACGAT

Biotin labeled strands used to purify H1-W:

RA: 20
(biotin)ATCGTCCACTCGCAGCAGATAG

RB: 20
(biotin)ATCGTCCACTCGCAGCAAGTTC

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