Supporting information

The Use of Minimal RNA Toeholds to Trigger the Activation of Multiple Functionalities

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Sequences used in this project

RNA and DNA sequences used to assembly RNA-DNA hybrids containing split asymmetric 25/27mer Dicer substrate RNA (DS RNA) duplex designed against eGFP[1].

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DS RNA sense:                    5` - pACCCUGAAGUUCAUCUGCACCACcg
sense labeled with Aexa-488:     5` - pACCCUGAAGUUCAUCUGCACCACcg-Al488
DNA for sense(8nts RNA toehold)  5` - CAGATGAACTTCAGGGTca
DNA for sense(6nts RNA toehold)  5` - TGCAGATGAACTTCAGGGTca
DNA for sense(4nts RNA toehold)  5` - GGTGCAGATGAACTTCAGGGTca
DNA for sense(2nts RNA toehold)  5` - GTGGTGCAGATGAACTTCAGGGTca

DS RNA antisense:                5`-  CGGUGGUGCAGAUGAACUUCAGGGUCA
antisense labeled with Alexa546: 5`-Al546-CGGUGGUGCAGAUGAACUUCAGGGUCA
DNA for ant(8nts RNA toehold)    5` - TGACCCTGAAGTTCATCTG
DNA for ant(6nts RNA toehold)    5` - TGACCCTGAAGTTCATCTGCA
DNA for ant(4nts RNA toehold)    5` - TGACCCTGAAGTTCATCTGCACC
DNA for ant(2nts RNA toehold)    5` - TGACCCTGAAGTTCATCTGCACCAC
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RNA and DNA sequences used to assembly RNA-DNA hybrids containing split asymmetric DSRNA duplex designed against HIV[2-5]. The names of corresponding DS RNAs are indicated for each DS RNA: Capsid (GAG) and Primer Binding Site – Matrix (LDR).

Capsid (Gag)

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Sense:                                5′-pGAAGAAAUGAUGACAGCAUUUCAGG
DNA for GAG sense (RNA toehold):      5`-GCTGTCATCATTTCTTCTT
Antisense:                            5′-CCUGAAAUGCUGUCAUCAUUUCUUCUU
DNA for GAG ant (RNA toehold):        5`-AAGAAGAAATGATGACAGC
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Primer Binding Site – Matrix (LDR)

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Sense:                                5′-pGGAGAGAGAUGGGUGCGAGUUCGUC
DNA for LDR sense (RNA toehold):      5′-CGCACCCATCTCTCTCCTT
Antisense:                            5′-GACGGACUCGCACCCAUCUCUCUCCUU
DNA for LDR ant (RNA toehold):        5′-AAGGAGAGAGATGGGTGCG
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RNA nanorings 3'-side functionalized with DS RNA antisenses against Green Fluorescent Protein[6].

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A:    5′-GGGAACCGUCCACUGGUUCCCGCUACGAGAGCCUGCCUCGUAGCUUCGGUGGUGCAGAUGAACUUCAGGGUCA
B:    5′-GGGAACCGCAGGCUGGUUCCCGCUACGAGAGAACGCCUCGUAGCUUCGGUGGUGCAGAUGAACUUCAGGGUCA
C:    5′-GGGAACCGCGUUCUGGUUCCCGCUACGAGACGUCUCCUCGUAGCUUCGGUGGUGCAGAUGAACUUCAGGGUCA
D:    5′-GGGAACCGAGACGUGGUUCCCGCUACGAGUCGUGGUCUCGUAGCUUCGGUGGUGCAGAUGAACUUCAGGGUCA
E:    5′-GGGAACCACCACGAGGUUCCCGCUACGAGAACCAUCCUCGUAGCUUCGGUGGUGCAGAUGAACUUCAGGGUCA
F:    5′-GGGAACCGAUGGUUGGUUCCCGCUACGAGAGUGGACCUCGUAGCUUCGGUGGUGCAGAUGAACUUCAGGGUCA
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Supporting Figures

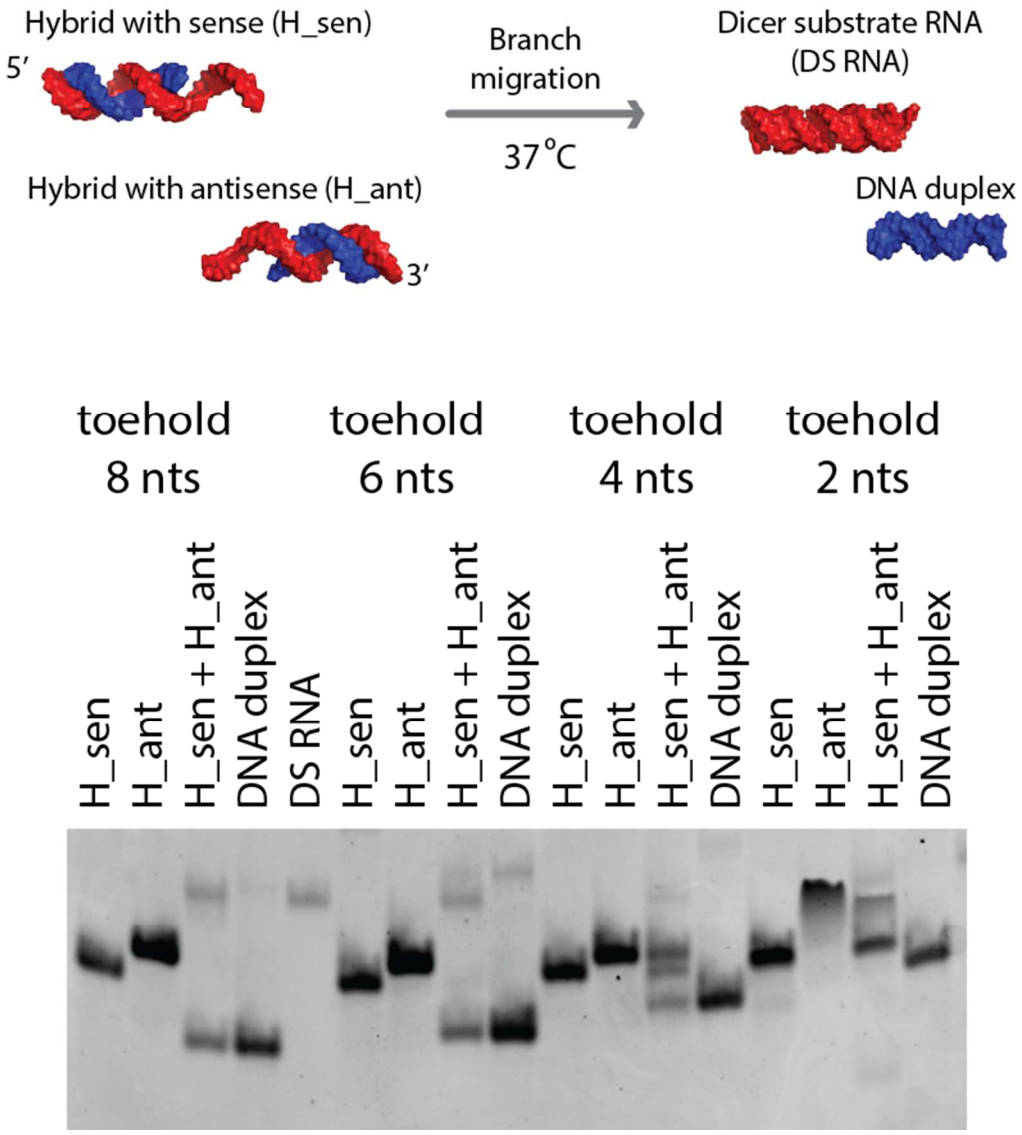

Figure S1. RNA toeholds initiate the re-association of RNA/DNA hybrids that leads to activation of RNAi. Schematic representation of re-association for RNA/DNA hybrids with 8-nts RNA toeholds. Depicted is total staining (with Ethidium Bromide) native-PAGE experiments that demonstrate the re-association of labeled hybrids with 2-, 4-, 6-, and 8-nts RNA toeholds (at 1 μM final). Please note that the hybrids with 4-nts ssRNA toeholds re-associate partially and hybrids with 2-nts do not re-associate at all.

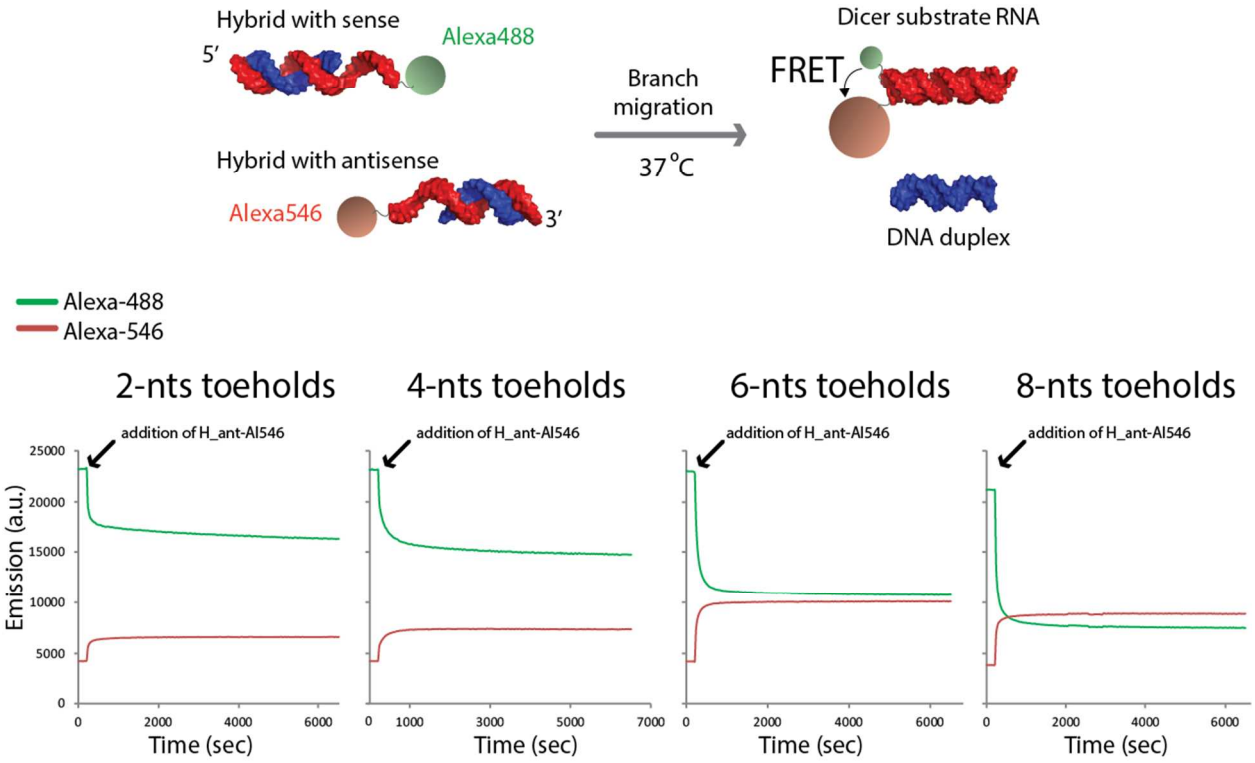

Figure S2. *In vitro* analysis of hybrids with 2-, 4-, 6-, and 8-nts ssRNA toeholds re-association using FRET.

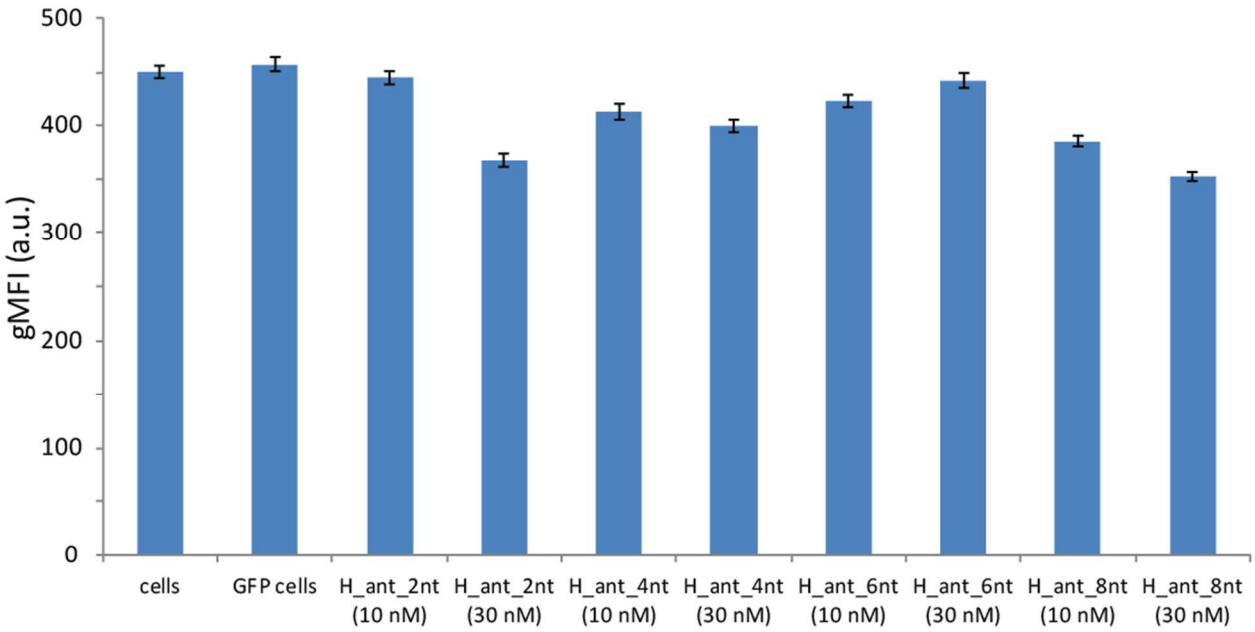

Figure S3. GFP knockdown assays for GFP expressing human breast cancer cells show no silencing with individual antisense carrying hybrids. Three days after the transfection of cells with individual hybrids, GFP expression was statistically analyzed with flow cytometry experiments. Hybrid concentrations (in nM) used in the silencing experiments are indicated. Error bars denote +/- S.E.M.

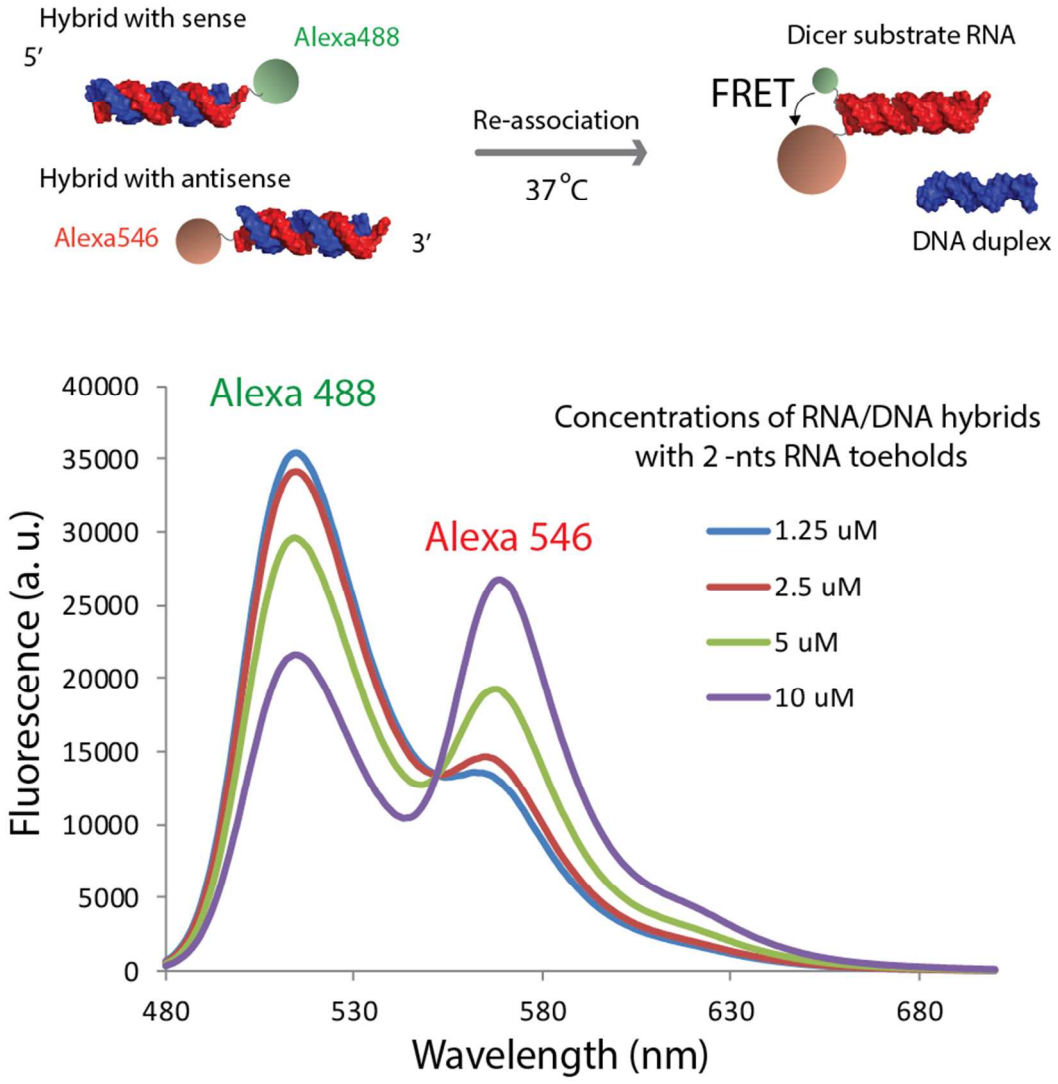

Figure S4. FRET analysis of concentration dependent re-association of hybrids with 2 nts ssRNA toeholds. The hybrids were incubated at the indicated concentrations for 5 hours in a water bath at 37°C. Upon incubation, they were diluted to the same final concentration of 90 nM final and then subjected to fluorescence analysis.

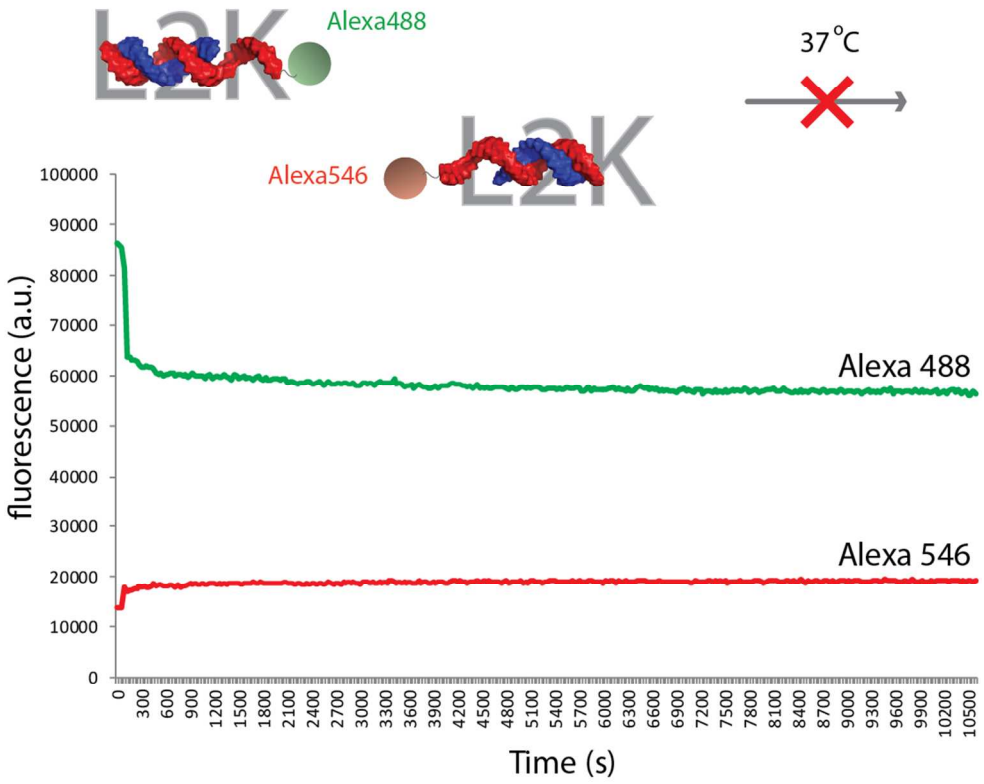

Figure S5. *In vitro* analysis (using FRET) demonstrates no re-association of hybrids (with 8 nts ssRNA toeholds) individually pre-incubated with Lipofectamine 2000 (L2K).

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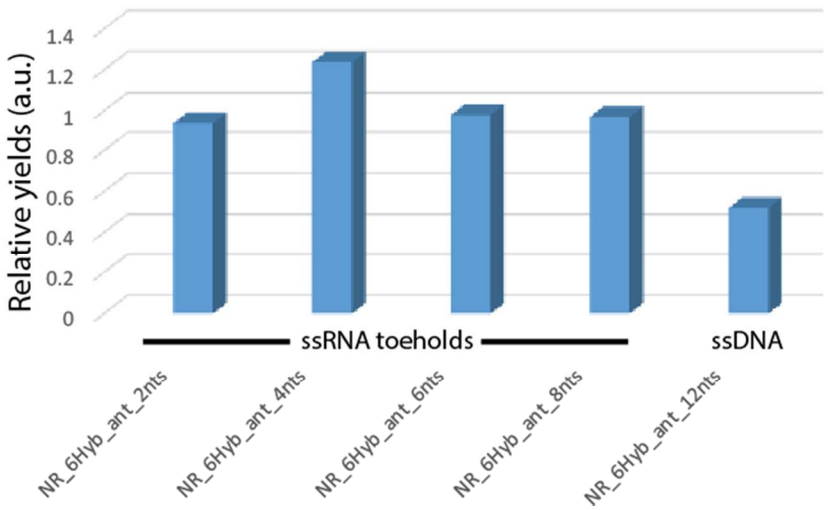

Figure S6. Relative yields of co-transcriptionally assembled RNA nanorings functionalized with RNA-DNA hybrids measured after native-PAGE purification and recovery. Each nanoring was functionalized with six RNA-DNA hybrids of varying ssRNA toehold lengths (8-nts, 6-nts, 4-nts, and 2-nts in length).

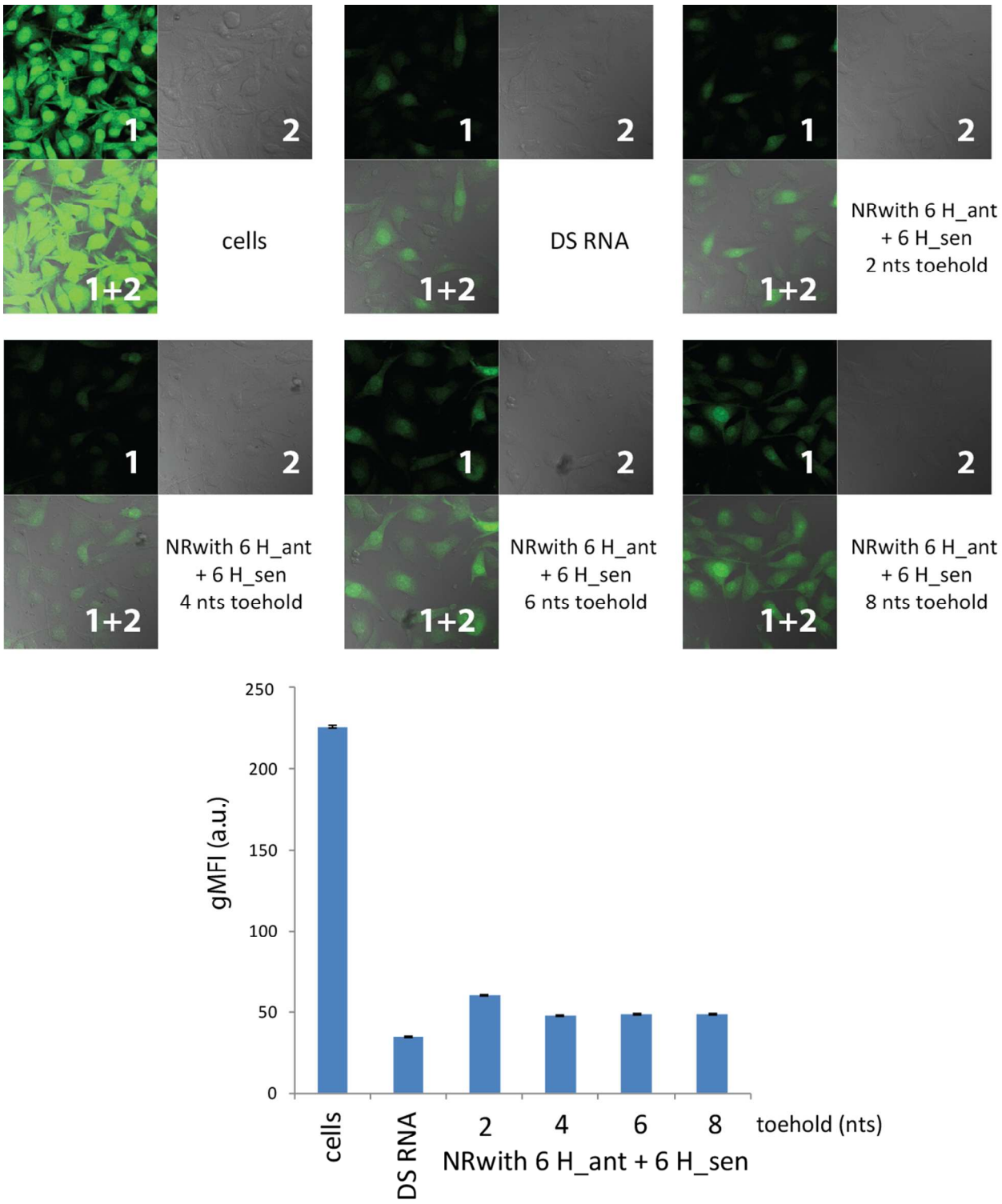

Figure S7. Intracellular re-association of hybrid nanorings (1 nM) and cognate hybrids triggers the GFP silencing. Three days after the co-transfection of cells with nanorings decorated with hybrids and cognate hybrids, GFP silencing was confirmed by fluorescent microscopy and statistically analyzed with flow cytometry experiments. Error bars denote +/- S.E.M. Image numbers correspond to: (1) - GFP emission, (2) - differential interference contrast images.

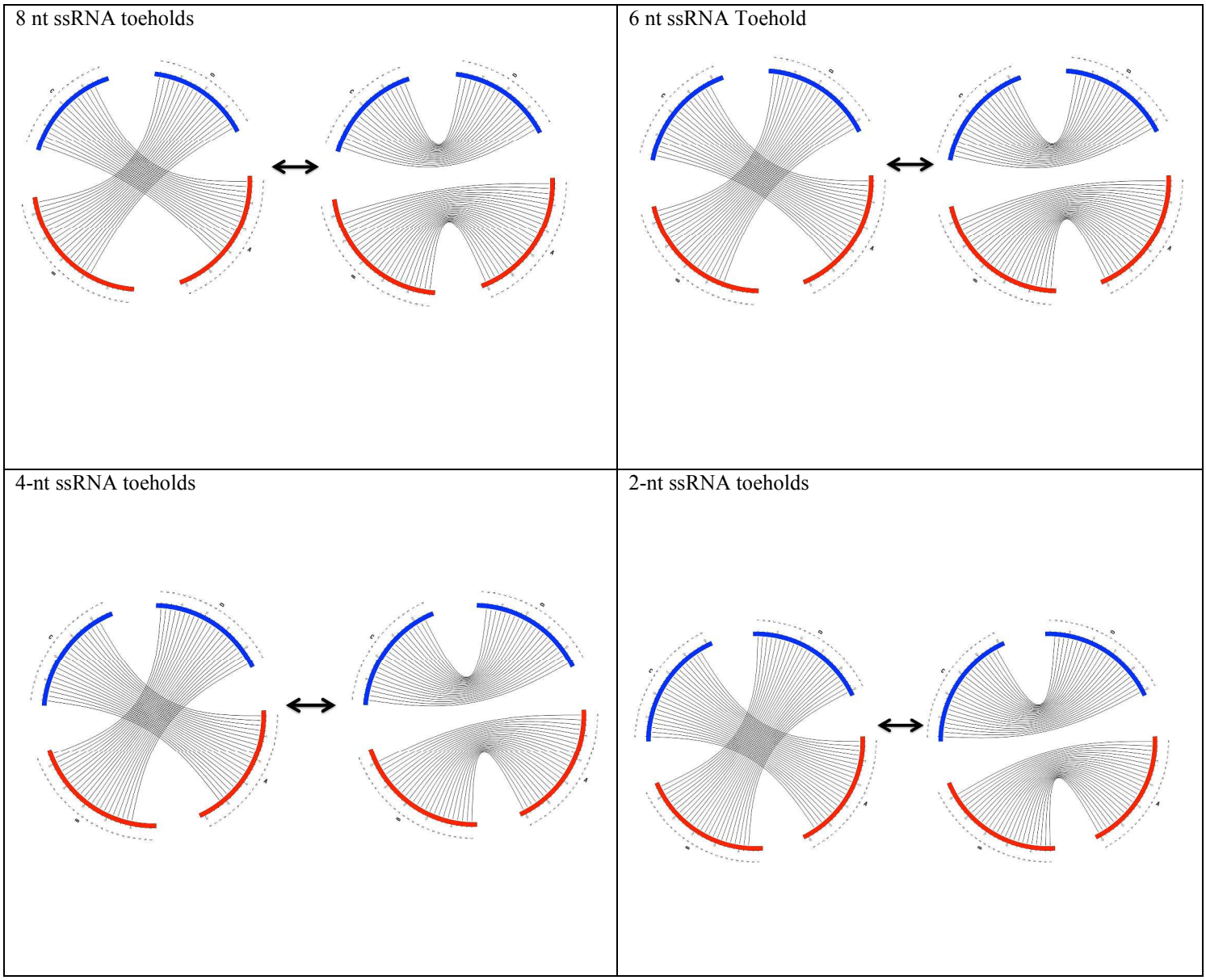

Figure S8. *In silico* predictions of re-association of RNA/DNA hybrids initiated by 2-, 4-, 6-, and 8-nts ssRNA toeholds. RNA strands are depicted as red segments; DNA strands are shown in blue. Each base pair corresponds to an arc shown in black.

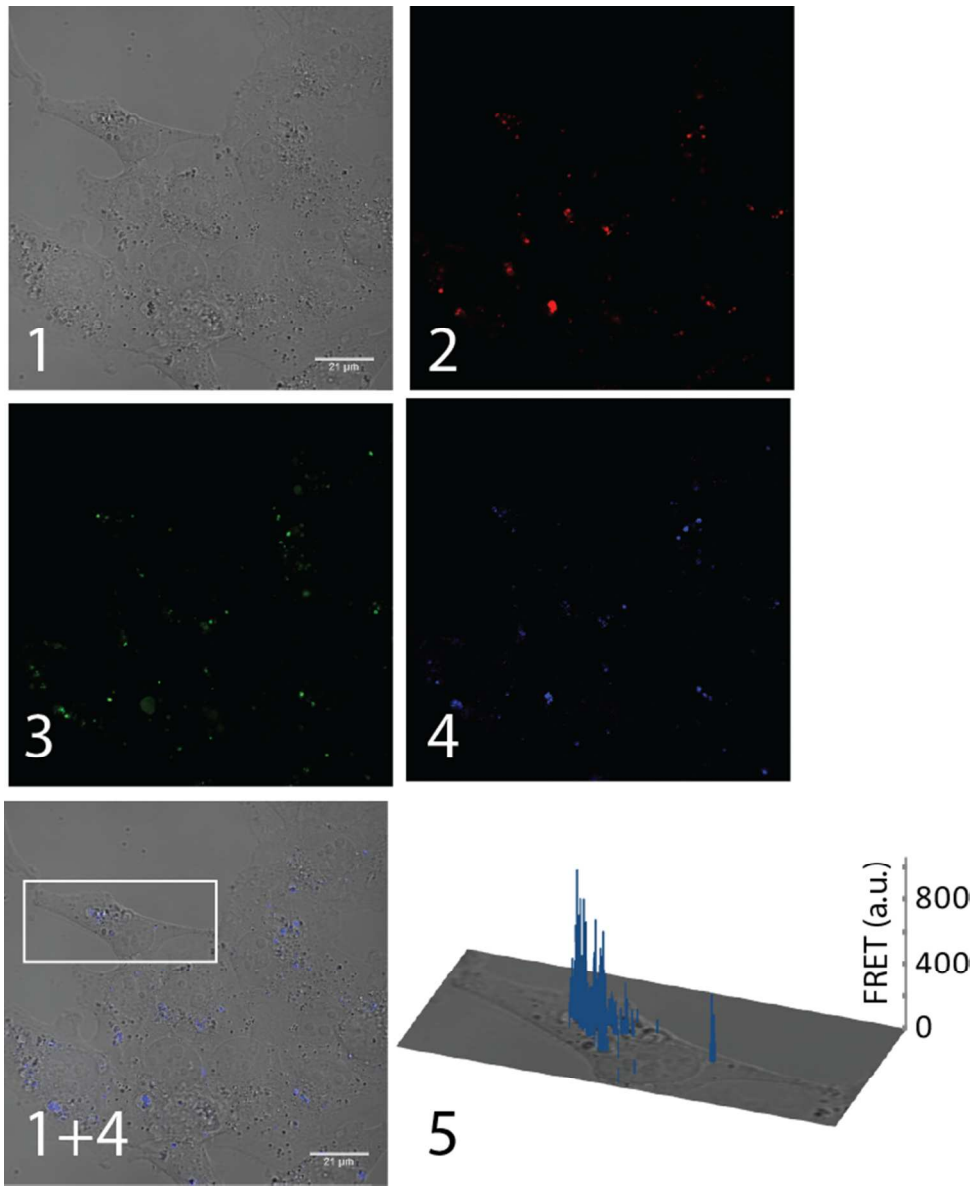

Figure S9. FRET experiments: cells were co-transfected with cognate hybrids, with 8 nts RNA toeholds (100 nM final), labeled with Alexa546 and Alexa488 and images were taken on the next day. Image numbers correspond to: (1) - differential interference contrast images, (2) - Alexa488 emission, (3) - Alexa546 emission, (4) - bleed-through corrected FRET image, (5) 3D chart representation of zoomed fragment indicated by a white box of bleed-through corrected FRET image

Command line parameters

For the predictions of the hexameric ring involving 6 or 12 nucleotide strands (6 RNA strands of the hexameric ring and 6 cognate DNAs), the following command line options were used for the hyperfold program:

-c 10 -q 10 -T 4 --pseudo 1 --hsort 2

Explanation:

-c <N> Concentration of each strand in units of micromol/l .

-q <N> Size of the involved search queue. Default is a queue size of 1000. A large queue size approaches exhaustive search and corresponds to thermodynamic equilibrium, a small queue size corresponds to a fast search and "kinetic folding".

-T <N> Temperature in Celsius. Default: 37 degree Celsius.

--pseudo 0|1|2 0: no pseudoknots; 1: only inter-strand pseudoknots are considered; 2: no strand-connectivity restriction on pseudoknots (default).

--hsort 1|2 Sorting of helices 1 (Default): helices are sorted by free energy. 2: Helices are sorted by free energy, but intra-strand helices are folded first. This case simulates "snap-cool" assembly protocol, where intra-strand folding precedes inter-strand folding.

Supporting references

(1) Rose, S. D.; Kim, D. H.; Amarzguioui, M.; Heidel, J. D.; Collingwood, M. A.; Davis, M. E.; Rossi, J. J.; Behlke, M. A. *Nucleic Acids Res* **2005**, *33*, 4140.

(2) Berkhout, B.; Sanders, R. W. *Antiviral research* **2011**, *92*, 7.

(3) Liu, Y. P.; von Eije, K. J.; Schopman, N. C.; Westerink, J. T.; ter Brake, O.; Haasnoot, J.; Berkhout, B. *Molecular therapy : the journal of the American Society of Gene Therapy* **2009**, *17*, 1712.

(4) Low, J. T.; Knoepfel, S. A.; Watts, J. M.; ter Brake, O.; Berkhout, B.; Weeks, K. M. *Molecular therapy : the journal of the American Society of Gene Therapy* **2012**, *20*, 820.

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(6) Afonin, K. A.; Viard, M.; Koyfman, A. Y.; Martins, A. N.; Kasprzak, W. K.; Panigaj, M.; Desai, R.; Santhanam, A.; Grabow, W. W.; Jaeger, L.; Heldman, E.; Reiser, J.; Chiu, W.; Freed, E. O.; Shapiro, B. A. *Nano letters* **2014**, *14*, 5662.