Supplementary Information

Self-Assembly of Precisely Defined DNA Nanotube Superstructures Using DNA Origami Seeds

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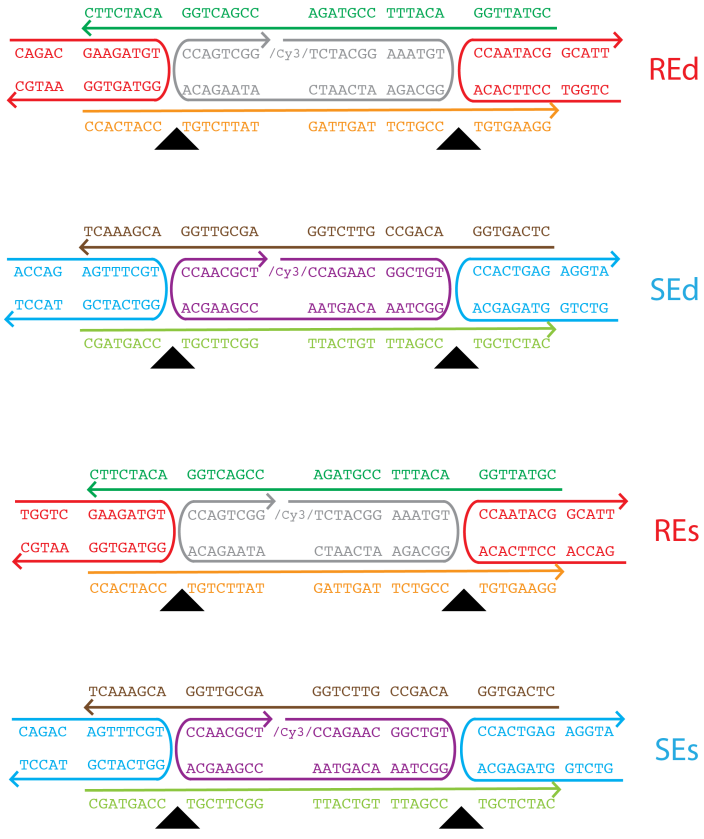
Supplementary Note S1: Nanotube tile sequences

The DAE-E tile motif design was used as the building block for all nanotubes in this study. The tile design and sequences are adopted from Rothemund *et al.*[1] The homogeneous nanotube nunchucks have diagonally striped nanotubes made up of two types of DNA tiles, REd and SEd, on both arms presented. The heterogeneous nanotube nunchucks have a REs single tile nanotube on one arm and a SEs single tile nanotube on the other.



Supplementary Figure S1: Schematic showing the architecture of the REd, SEd, REs and SEs tiles. Different colors with a single tile show different strands. Black triangles indicate crossover points. Arrowheads indicate 3' ends.

REdSEd nanotube tile sequences:

```
RE-1:       CGTATTGGACATTTCCGTAGACCGACTGGACATCTTC
RE-2EE01:   CTGGTCCTTCACACCAATACGGCATT
RE-3Cy3:    /Cy3/TCTACGGAAATGTGGCAGAATCAATCATAAGACACCAGTCGG
RE-4:       CAGACGAAGATGTGGTAGTGGAATGC
RE-5:       CCACTACCTGTCTTATGATTGATTCTGCCTGTGAAGG

SE-1:       CTCAGTGGACAGCCGTTCTGGAGCGTTGGACGAAACT
SE-2DIAG:   GTCTGGTAGAGCACCACTGAGAGGTA
SE-3Cy3:    /Cy3/CCAGAACGGCTGTGGCTAAACAGTAACCGAAGCACCAACGCT
SE-4DIAG:   ACCAGAGTTTCGTGGTCATCGTACCT
SE-5:       CGATGACCTGCTTCGGTTACTGTTTAGCCTGCTCTAC
```

/Cy3/ denotes a Cy3 fluorophore covalently attached to the 5' end of the DNA.

REs nanotube tile sequences:

```
RE-1:       CGTATTGGACATTTCCGTAGACCGACTGGACATCTTC
RE-2s:      GACCACCTTCACACCAATACGGCATT
RE-3_A647:  /5ATTO647NN/TCTACGGAAATGTGGCAGAATCAATCATAAGACACCAGTCGG
RE-3_A488:  /5ATTO488N/TCTACGGAAATGTGGCAGAATCAATCATAAGACACCAGTCGG
RE-4s:      TGGTCGAAGATGTGGTAGTGGAATGC
RE-5:       CCACTACCTGTCTTATGATTGATTCTGCCTGTGAAGG
```

/5ATTO647NN/ denotes an ATTO647N fluorophore covalently attached to the 5' end of the DNA. /5ATTO488N/ denotes an ATTO488 fluorophore covalently attached to the 5' end of the DNA.

SEs nanotube tile sequences:

```
SE-1:       CTCAGTGGACAGCCGTTCTGGAGCGTTGGACGAAACT
SE-2DIAG:   GTCTGGTAGAGCACCACTGAGAGGTA
SE-3Cy3:    /Cy3/CCAGAACGGCTGTGGCTAAACAGTAACCGAAGCACCAACGCT
SE-4s:      CAGACAGTTTCGTGGTCATCGTACCT
SE-5:       CGATGACCTGCTTCGGTTACTGTTTAGCCTGCTCTAC
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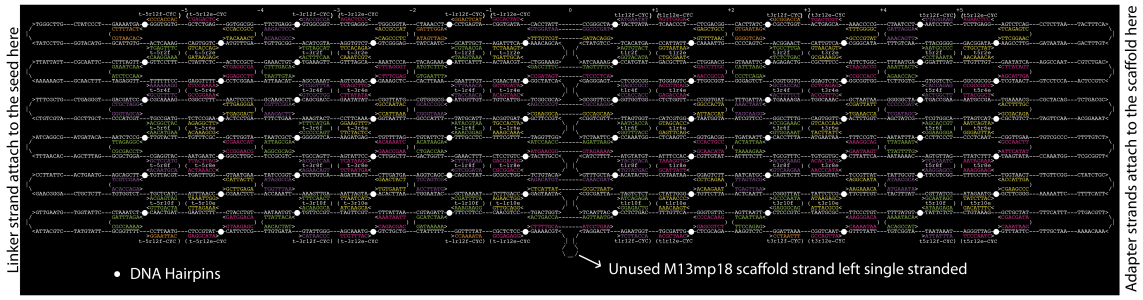
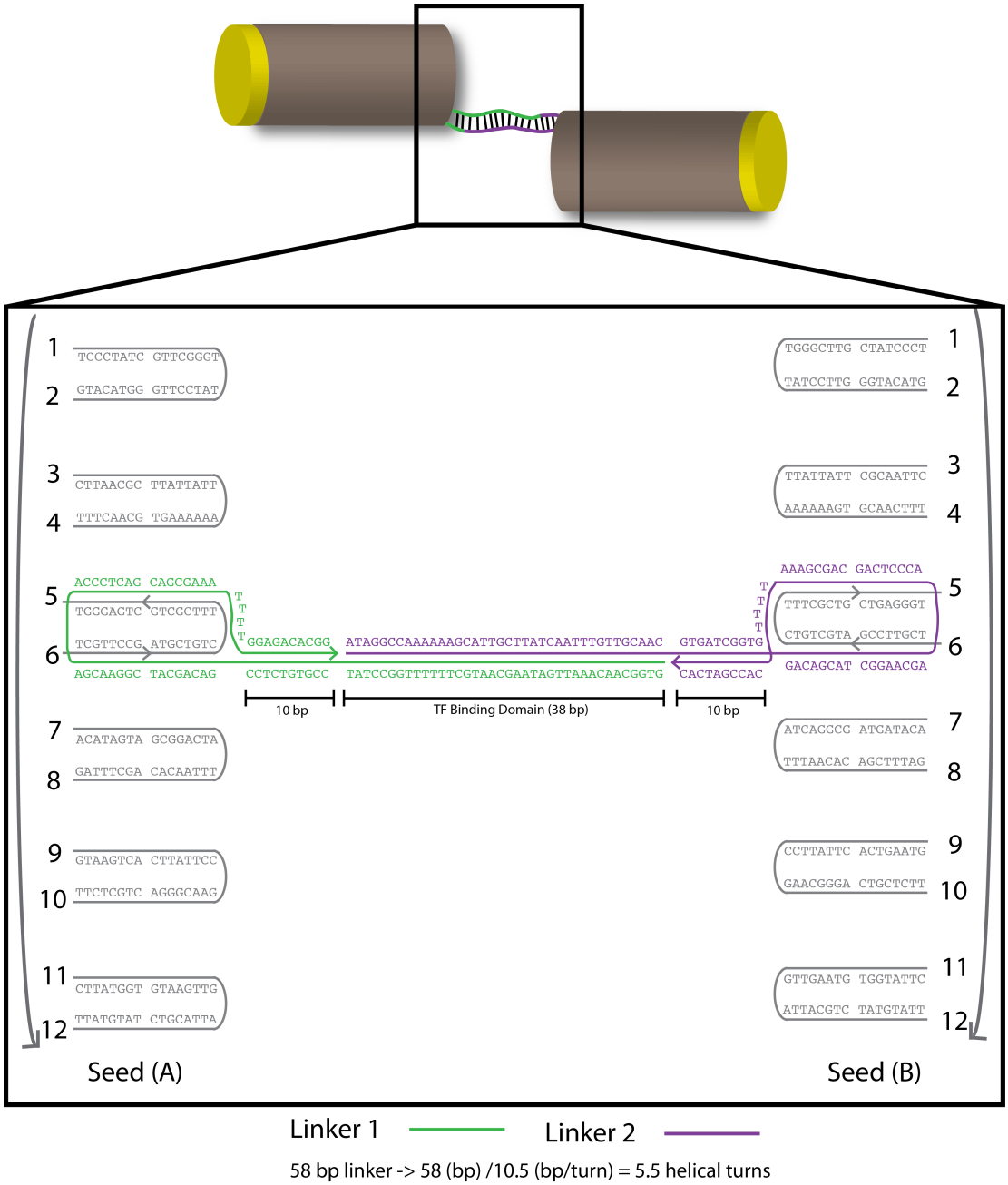
Supplementary Note S2: Design and sequences of nunchuck seeds

We used the seed design presented in the paper Mohammed *et al*[2] shown in Supplementary figure S2. Sequences are also typed in list form in the above work.

A nunchuck seed consists of two origami seeds each having a linker strand that is partially complementary to itself and that also binds to the nanotube seed on the end of the seed's cylindrical structure opposite to where the adapter strands attach. The adapter strands bind to the right edge of the structure as diagramed in Supplementary Figure S2 (Supplementary Figures S4-S6). The linker strands attach to the single stranded M13mp18 region on the left of Supplementary Figure S2 as shown in Supplementary Figure S3.



Supplementary Figure S2: Detailed staple map of origami seeds showing individual staples, location of hairpins, adapter strands and linker strand binding sites.



Supplementary Figure S3: Schematic showing linker binding to individual seeds and linker hybridization. Gray strands represent M13mp18 scaffold strands of the two individual seeds comprising a nanotube seed nunchuck.

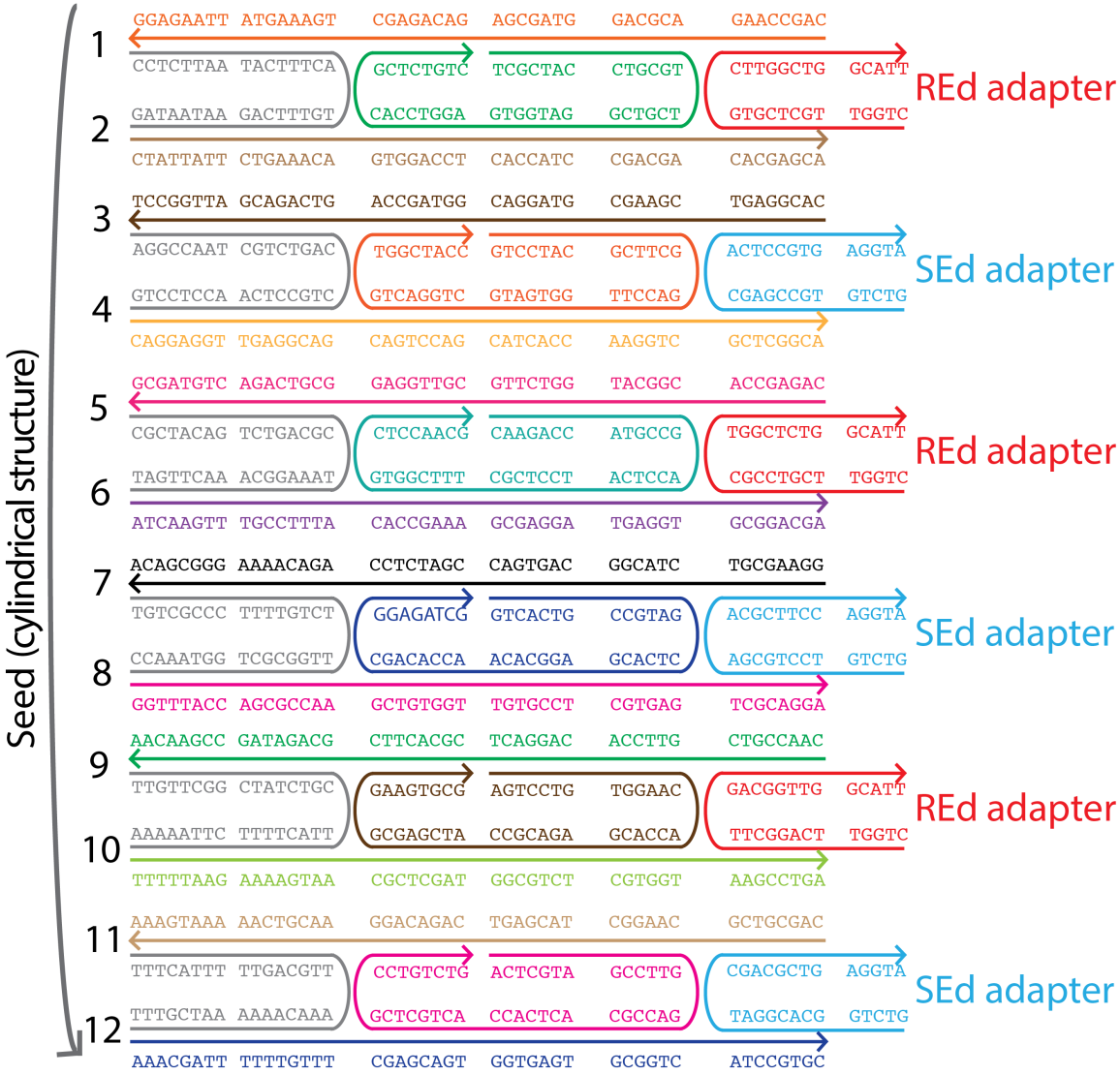
Linker strand sequences:

`Linker-1:`

GTGGCAACAAATTGATAAGCAATGCTTTTTTGGCCTATCCGTGTCTCCGACAGCATCGG
AACGAACCCTCAGCAGCGAAATTTTGGAGACACGG

Linker-2:
ATAGGCCAAAAAAGCATTGCTTATCAATTTGTTGCAACGTGATCGGTGGACAGCATCGG
AACGAACCCTCAGCAGCGAAATTTTCACCGATCAC

Adapter strand designs:



Supplementary Figure S4: Structure of assembled REdSEd adapter tiles. The gray strand represents the M13mp18 scaffold.

REdSEd adapter tile sequences

```
AD1REd_1:          CAGCCAAGACGCAGGTAGCGAGACAGAGCTGAAAGTATTAAGAGG
AD1_2REd_3:        TCGCTACCTGCGTTCGTCGGATGGTGAGGTCCACGCTCTGTC
AD1_2REd_5:        CTATTATTCTGAAACAGTGGACCTCACCATCCGACGACACGAGCA
AD2REd_2:          CTGGTTGCTCGTGCTTGGCTGGCATT

AD3SEd_1:          CACGGAGTCGAAGCGTAGGACGGTAGCCAGTCAGACGATTGGCCT
AD3_4SEd_3:        GTCCTACGCTTCGGACCTTGGTGATGCTGGACTGTGGCTACC
AD4SEd_5:          CAGGAGGTTGAGGCAGCAGTCCAGCATCACCAAGGTCGCTCGGCA
AD3_4SEd_2:        GTCTGTGCCGAGCACTCCGTGAGGTA

AD5REd_1:          CAGAGCCACGGCATGGTCTTGCGTTGGAGGCGTCAGACTGTAGCG
AD5_6REd_3:        CAAGACCATGCCGACCTCATCCTCGCTTTCGGTGCTCCAACG
AD6REd_5:          ATCAAGTTTGCCTTTACACCGAAAGCGAGGATGAGGTGCGGACGA
AD5_6REd_2:        CTGGTTCGTCCGCTGGCTCTGGCATT

AD7SEd_1:          GGAAGCGTCTACGGCAGTGACCGATCTCCAGACAAAAGGGCGACA
AD7_8SEd_3:        GTCACTGCCGTAGCTCACGAGGCACAACCACAGCGGAGATCG
AD8SEd_5:          GGTTTACCAGCGCCAAGCTGTGGTTGTGCCTCGTGAGTCGCAGGA
AD7_8SEd_2:        GTCTGTCCTGCGAACGCTTCCAGGTA

AD9REd_1:          CAACCGTCGTTCCACAGGACTCGCACTTCGCAGATAGCCGAACAA
AD9_10REd_3:       AGTCCTGTGGAACACCACGAGACGCCATCGAGCGGAAGTGCG
AD10REd_5:         TTTTTAAGAAAAGTAACGCTCGATGGCGTCTCGTGGTAAGCCTGA
AD9_10REd_2:       CTGGTTCAGGCTTGACGGTTGGCATT

AD11SEd_1:         CAGCGTCGCAAGGCTACGAGTCAGACAGGAACGTCAAAAATGAAA
AD11_12SEd_3:      ACTCGTAGCCTTGGACCGCACTCACCACTGCTCGCCTGTCTG
AD12SEd_5:         AAACGATTTTTTGTTTCGAGCAGTGGTGAGTGCGGTCATCCGTGC
AD11_12SEd_2:      GTCTGGCACGGATCGACGCTGAGGTA
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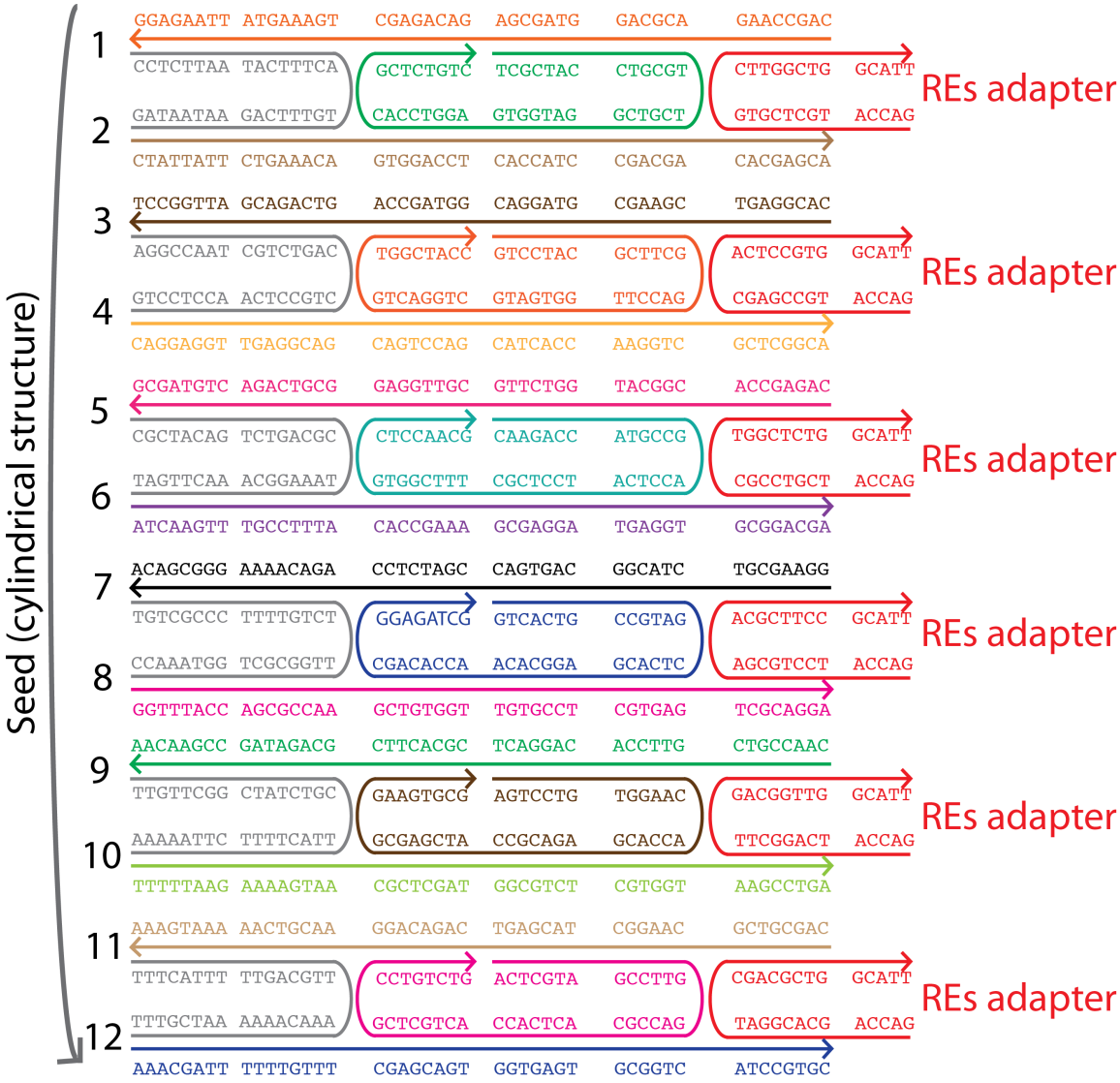
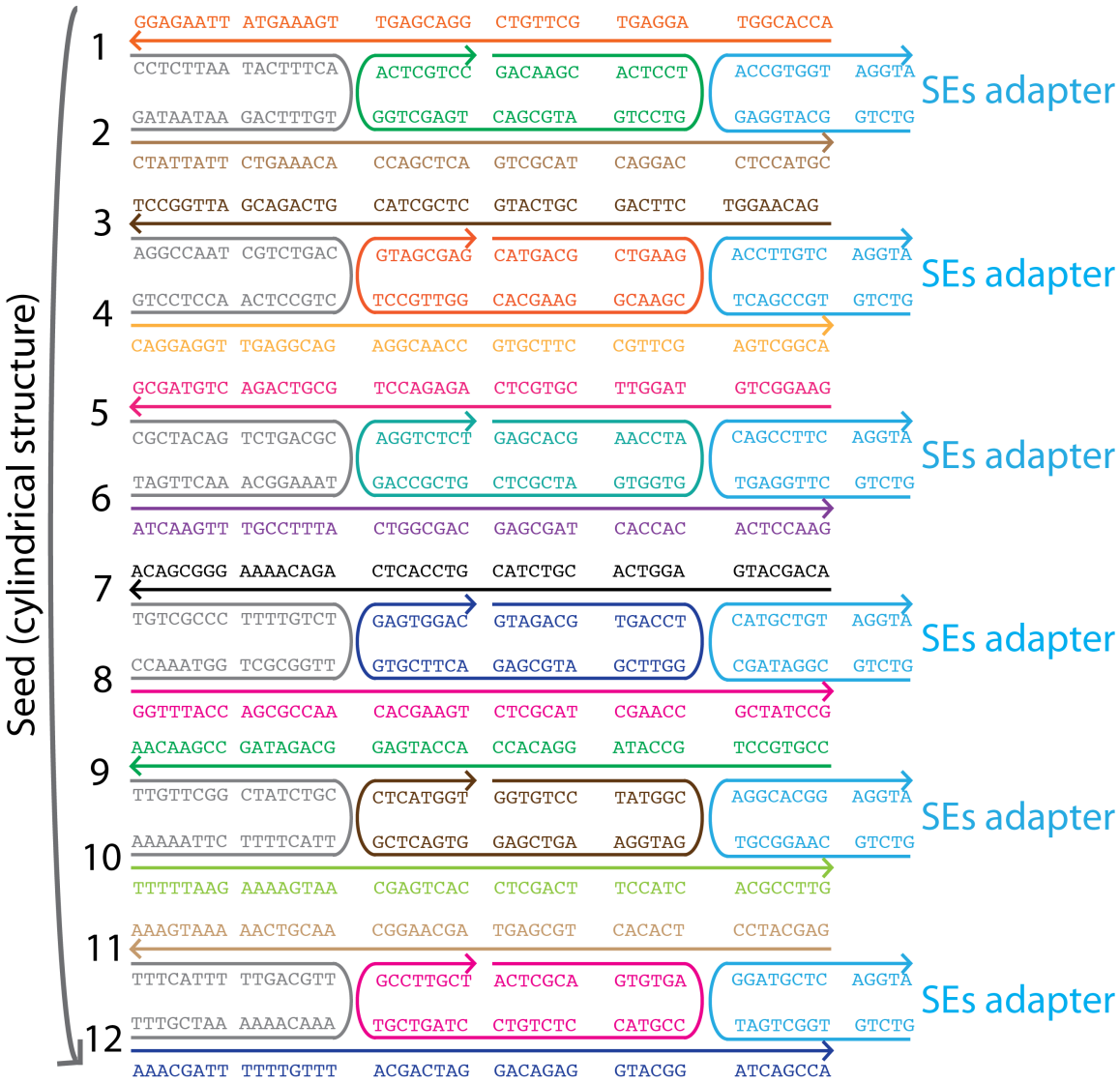


Supplementary Figure S5: Structure of the assembled REs adapter tiles. The gray strand represents the M13mp18 scaffold.

REs adapter tile sequences

AD1REd_1:	CAGCCAAGACGCAGGTAGCGAGACAGAGCTGAAAGTATTAAGAGG
AD1_2REd_3:	TCGCTACCTGCGTTCGTCGGATGGTGAGGTCCACGCTCTGTC
AD1_2REd_5:	CTATTATTCTGAAACAGTGGACCTCACCATCCGACGACACGAGCA
AD2REd_2:	GACCATGCTCGTGCTTGGCTGGCATT
AD3REd_1:	CACGGAGTCGAAGCGTAGGACGGTAGCCAGTCAGACGATTGGCCT
AD3_4REd_3:	GTCCTACGCTTCGGACCTTGGTGATGCTGGACTGTGGCTACC
AD4REd_5:	CAGGAGGTTGAGGCAGCAGTCCAGCATCACCAAGGTCGCTCGGCA
AD3_4REd_2:	GACCATGCCGAGCACTCCGTGGCATT
AD5REd_1:	CAGAGCCACGGCATGGTCTTGCGTTGGAGGCGTCAGACTGTAGCG
AD5_6REd_3:	CAAGACCATGCCGACCTCATCCTCGCTTTCGGTGCTCCAACG
AD6REd_5:	ATCAAGTTTGCCTTTACACCGAAAGCGAGGATGAGGTGCGGACGA
AD5_6REd_2:	GACCATCGTCCGCTGGCTCTGGCATT
AD7REd_1:	GGAAGCGTCTACGGCAGTGACCGATCTCCAGACAAAAGGGCGACA
AD7_8REd_3:	GTCACTGCCGTAGCTCACGAGGCACAACCACAGCGGAGATCG
AD8REd_5:	GGTTTACCAGCGCCAAGCTGTGGTTGTGCCTCGTGAGTCGCAGGA
AD7_8REd_2:	GACCATCCTGCGAACGCTTCCGCATT
AD9REd_1:	CAACCGTCGTTCCACAGGACTCGCACTTCGCAGATAGCCGAACAA
AD9_10REd_3:	AGTCCTGTGGAACACCACGAGACGCCATCGAGCGGAAGTGCG
AD10REd_5:	TTTTTAAGAAAAGTAACGCTCGATGGCGTCTCGTGGTAAGCCTGA
AD9_10REd_2:	GACCATCAGGCTTGACGGTTGGCATT
AD11REd_1:	CAGCGTCGCAAGGCTACGAGTCAGACAGGAACGTCAAAAATGAAA
AD11_12REd_3:	ACTCGTAGCCTTGGACCGCACTCACCACTGCTCGCCTGTCTG
AD12REd_5:	AAACGATTTTTTGTTTCGAGCAGTGGTGAGTGCGGTCATCCGTGC
AD11_12REd_2:	GACCAGCACGGATCGACGCTGGCATT



Supplementary Figure S6: Structure of assembled SEs adapter tiles. The gray strand represents the M13mp18 scaffold.

SEs adapter tile sequences

AD1SEs_1:	ACCACGGTAGGAGTGCTTGTCGGACGAGTTGAAAGTATTAAGAGG
AD1_2SEs_3:	GACAAGCACTCCTGTCCTGATGCGACTGAGCTGGACTCGTCC
AD1_2SEs_5:	CTATTATTCTGAAACACCAGCTCAGTCGCATCAGGACCTCCATGC
AD2SEs_2:	GTCTGGCATGGAGACCGTGGTAGGTA
AD3SEs_1:	GACAAGGTCTTCAGCGTCATGCTCGCTACGTCAGACGATTGGCCT
AD3_4SEs_3:	CATGACGCTGAAGCGAACGGAAGCACGGTTGCCTGTAGCGAG
AD4SEs_5:	CAGGAGGTTGAGGCAGAGGCAACCGTGCTTCCGTTCGAGTCGGCA
AD3_4SEs_2:	GTCTGTGCCGACTACCTTGTCAGGTA
AD5SEs_1:	GAAGGCTGTAGGTTCGTGCTCAGAGACCTGCGTCAGACTGTAGCG
AD5_6SEs_3:	GAGCACGAACCTAGTGGTGATCGCTCGTCGCCAGAGGTCTCT
AD6SEs_5:	ATCAAGTTTGCCTTTACTGGCGACGAGCGATCACCACACTCCAAG
AD5_6SEs_2:	GTCTGCTTGGAGTCAGCCTTCAGGTA
AD7SEs_1:	ACAGCATGAGGTCACGTCTACGTCCACTCAGACAAAAGGGCGACA
AD7_8SEs_3:	GTAGACGTGACCTGGTTCGATGCGAGACTTCGTGGAGTGGAC
AD8SEs_5:	GGTTTACCAGCGCCAACACGAAGTCTCGCATCGAACCGCTATCCG
AD7_8SEs_2:	GTCTGCGGATAGCCATGCTGTAGGTA
AD9SEs_1:	CCGTGCCTGCCATAGGACACCACCATGAGGCAGATAGCCGAACAA
AD9_10SEs_3:	GGTGTCCTATGGCGATGGAAGTCGAGGTGACTCGCTCATGGT
AD10SEs_5:	TTTTTAAGAAAAGTAACGAGTCACCTCGACTTCCATCACGCCTTG
AD9_10SEs_2:	GTCTGCAAGGCGTAGGCACGGAGGTA
AD11SEs_1:	GAGCATCCTCACACTGCGAGTAGCAAGGCAACGTCAAAAATGAAA
AD11_12SEs_3:	ACTCGCAGTGTGACCGTACCTCTGTCCTAGTCGTGCCTTGCT
AD12SEs_5:	AAACGATTTTTTGTTTACGACTAGGACAGAGGTACGGATCAGCCA
AD11_12SEs_2:	GTCTGTGGCTGATGGATGCTCAGGTA

Supplementary Note S3: Comprehensive protocol for the synthesis of homogeneous nanotube nunchucks

1. We prepared two mixtures containing seed staples, a linker strand, M13mp18 scaffold, REdSEd adapter strands in TAE Mg^{2+} buffer (40 mM Tris-Acetate, 1 mM EDTA and 12.5 mM magnesium acetate) in the amounts shown in the table below. The two mixtures differ only in the linker strand type included: the first mixture contained Linker strand 1 and second mixture contained Linker strand 2. When preparing the samples, we mixed each DNA stock solution (except the M13mp18 stock) by vortexing before drawing solution from the stock tube and adding it to the assembly mixture. The M13mp18 strand solution was mixed by pipette to avoid fragmentation due to shearing. We separately annealed these two mixtures, "Seed A" and "Seed B", from 90°C to 20°C at a cooling rate of 1°C/min.

Seed A (or B)	**Final desired concentration (nM or fold)**	**Stock concentration (nM or fold)**	**Amount of stock added (µl)**
H_2O	-	-	61.8
Seed staples mix	100	1389	7.2
Linker strand 1 (or 2)	10	1000	1.0
M13mp18	10	100	10.0
REdSEd adapter strand mix	100	1000	10.0
10x TAE Mg^{2+}	1x	10x	10.0
Total			100.0

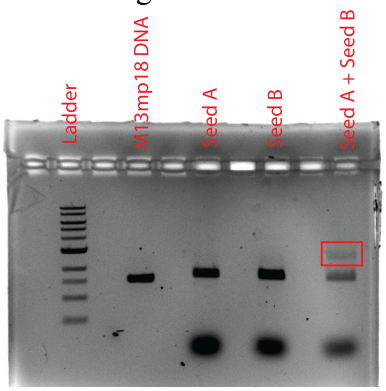
Seed staple mix: Mixture containing all 72 seed strands in equal concentrations. Mixing individual seed staple strands (each stock at 100 µM) in equal volume makes the final concentration of staples in the mixture to be 1.389 µM per strand (100 µM/72).

REdSEd adapter strand mix: Mixture containing all REdSEd adapter strands (see Supplementary Figure S4) at a concentration of 1 µM each, except the strands with sticky ends [AD2REd_2, AD3_4SEd_2, AD5_6REd_2, AD7_8SEd_2, AD9_10REd_2, AD11_12SEd_2] which are at 2 µM.

2. Following this anneal, we raised the temperature of each sample to 32°C and combined both solutions into the same tube, mixing well. This new sample remained at 32°C overnight.

3. We prepared a 100 ml 1% agarose gel containing ethidium bromide at a concentration of 1.25 µg/ml. We added 6X loading dye (Bromophenol Blue) to the sample, at a ratio of 1 mL to every 5 mL of sample, to dilute to a final concentration of 1X loading dye. We loaded this sample into the agarose gel immersed in pre-chilled TAE Mg^{2+} buffer and ran electrophoresis at a constant voltage of 100 V for 1 hour. After 30 minutes, we removed

the TAE Mg^{2+} buffer and added new chilled buffer to prevent the chamber from overheating.



Supplementary Figure S7: Gel image showing nunchuck seeds (within rectangle).

4. Once the dimer and monomer bands could be easily resolved (~ 1 hr at 100 V), we extracted the dimer band with a blade. We added this section of the gel to a Freeze N' Squeeze DNA Gel Extraction Spin Column (Biorad). We allowed this container to chill in a freezer for 5 minutes, and immediately centrifuged it at 13,000g for 3 minutes. The collection tube contains the gel-purified nunchucks.

5. We then prepared a nanotube growth mixture containing BSA-Biotin, REd-SEd adapters, REdSEd tiles, seed labeling attachment strands mix, ATTO647 strand in TAE Mg^{2+} buffer. We annealed this mixture from 90°C to 34°C (90 to 45°C at 1°C /min; held at 45°C for 1 hour; 45°C to 34°C at 0.1°C /min), adding the gel-purified nunchucks when the mixture reached 40°C.

REdSEd nunchuck nanotubes	**Final desired concentration (nM, fold or units as listed)**	**Stock (nM, fold or units as listed)**	**Amount of stock added (µl)**
H_2O	-	-	67.0
BSA-Biotin (mg/ml)	0.15	5	3.0
REdSEd adapter strand mix	0.4	100	0.4
REdSEd tiles (Cy3 labeled)	40	400	10.0
Seed labeling attachment strands mix	0.6	100	0.6
Labeling strand ATTO647	50	1000	5.0
10x TAE Mg^{2+}	1x	10x	10.0
Gel-purified nunchucks	0.04	1	4.0

Total			100.0

Seed labeling attachment strands mix: Mixture containing all 100 labeling attachment strands (see Supplementary Note S6) at a concentration of 100 nM for each strand.

REdSEd tiles (Cy3 labeled): Mixture containing all 10 strands that make up the REdSEd nanotube tiles (Supplementary Note S2). Similar to the tile mixture in Mohammed *et al*[2], the short strands (with sticky ends) [RE-2EE01, RE-4,SE-2DIAG, SE-4DIAG] were at twice the concentration of the long strands (without sticky ends) to ensure all tiles have sticky ends.

6. The mixture is then held at 34°C for 15 hours to complete nanotube growth.

Supplementary Note S4: Comprehensive protocol for synthesis of heterogeneous nanotube nunchucks

1. We prepared two mixtures containing seed staples, a linker strand, M13mp18 scaffold, adapter strands in 1X TAE Mg^{2+} (40 mM Tris-Acetate, 1 mM EDTA and 12.5 mM magnesium acetate). The two mixtures differ in the linker strand and the type of adapter strands. For "Seed A" and "Seed B", we added REs and SEs adapter strands respectively. When preparing the samples, we mixed each DNA stock solution except M13mp18 well by vortexing before pipetting it from the stock solution into the seed solution. The M13mp18 solution was mixed by pipetting to avoid fragmentation due to shearing.

Seed A or B	**Final desired concentration (nM or fold)**	**Stock concentration (nM or fold)**	**Amount of stock added (µl)**
H_2O	-	-	13.2
Seed staples mix	100	1389	1.8
Linker strand (1 or 2)	10	100	2.5
M13mp18	10	100	2.5
REs or SEs adapter strand mix	100	1000	2.5
10x TAE Mg++	1x	10x	2.5
Total			25.0

REs adapter strands mix: Mixture containing all REs adapter strands for Seed A (see Supplementary Figure S5) at a concentration of 1 µM except the strands with sticky ends that are at 2 µM.

SEs adapter strands mix: Mixture containing all SEs adapter strands for Seed B (see Supplementary Figure S6) at a concentration of 1 µM except the strands with sticky ends that are at 2 µM.

2. We separately annealed these two mixtures, "Seed A" and "Seed B", from 90°C to 20°C at a rate of 1°C/min. Following this anneal, we raised the temperature of each sample to 32°C and combined both of them into the same tube, mixing well. This new sample remained at 32°C for 4 days.

3. We prepared a 100 ml 1% agarose gel containing ethidium bromide at a concentration of 1.25 µg/ml. We added a 6X loading dye (Bromophenol Blue) to the sample, at a ratio of 1 mL to every 5 mL of sample, to a final concentration of 1X loading dye. We loaded this sample into the agarose gel that is immersed in pre-chilled TAE Mg^{2+}buffer and placed the apparatus on ice. Electrophoresis was performed at a constant voltage of 100 V for ~1 hour.

4. Once the dimer and monomer bands could be easily resolved (~ 1 hr at 100 V), we extracted the dimer band with a blade and sliced the band into smaller pieces. We added this section of the gel to a Freeze N' Squeeze DNA Gel Extraction Spin Column (Biorad). We allowed this container to chill in a freezer for 5 minutes, and immediately centrifuged it at 13,000g for 3 minutes. The collection tube contains the gel-purified nunchucks.

5. We prepared a new mixture containing BSA, REs adapter strand mix, SEs adapter strand mix, REs tiles (ATTO488 labeled), SEs tiles (Cy3 labeled), seed labeling attachment strands mix, labeling strand ATTO 647 in TAE Mg^{2+} buffer. We annealed this mixture from 90°C to 34°C (90°C to 45°C at 1°C /min; held at 45°C for 1 hour; 45°C to 34°C at 0.1°C /min), adding the gel-purified nunchucks when the sample reached 40°C.

REs-SEs Hetero-nunchuck nanotubes	**Final desired concentration (nM, fold or units as listed)**	**Stock concentration (nM, fold or units as listed)**	**Amount of stock added (µl)**
H_2O			3.75
BSA (in mg/ml)	0.15	1	3.75
REs adapter strands mix	0.4	100	0.1
SEs adapter strands mix	0.4	100	0.1
REs tiles (ATTO488 labeled)	50	400	3.125
SEs tiles (Cy3 labeled)	50	400	3.125
Seed labeling attachment strands mix	1.8	25	1.8
Labeling strand ATTO 647	150	1000	3.75
10x TAE Mg++	1x	10x	2.5
Gel-purified nunchucks			3
Total			25

REs tiles (ATTO488 labeled): Mixture containing all 5 strands that make up REs nanotube tiles (Supplementary Note S1). The short strands (with sticky ends) [RE-2s,

RE-4s] were at twice the concentration of the long strands (without sticky ends) to ensure all tiles have sticky ends.

SEs tiles (Cy3 labeled): Mixture containing all 5 strands those make up SEs nanotube tiles (Supplementary Note S2). The short strands (with sticky ends) [SE-2DIAG, SE-4s] were at twice the concentration of the long strands (without sticky ends) to ensure all tiles have sticky ends.

6. The mixture is then held at 34°C for 15 hours to complete nanotube growth.

Supplementary Note S5: Guard strands to prevent further tile assembly

In our experiments, we grew nanotube nunchucks at 34°C. However, it was much easier to characterize the assembled nanotube nunchucks *via* fluorescence microscopy and AFM at room temperature. However, at room temperature, the critical concentration of free tiles is lower than at 34ºC, so if the solution were cooled, nanotubes would continue to grow during imaging. Further, unseeded nanotube nucleation can occur readily at room temperature at the tile concentrations used in this study. Spontaneous nucleation of new nanotubes at room temperature could interfere with the visualization of nanotube nunchucks and bias yield statistics. To prevent continued nanotube nucleation and growth during room temperature imaging, we added 'guard strands'[2] to the reaction mixture at 34°C before cooling the mixture to room temperature. Guard strands are designed to stop the assembly reaction. They have sequences complimentary to the sticky end strands (strands 2 and 4 of each tile type). When guard strands are mixed with tiles, the guard strands are expected to bind to exposed sticky end strands and remove them from the tiles *via a* toehold-mediated strand displacement process[3], thus preventing these tiles from assembling further (Supplementary Figure S8). Because free tiles and nanotube facets that have reacted with guard strands do not react, the addition of guard strands to the reaction mixture before imaging resulted in cleaner background images and presumably also prevented new nanotubes from nucleating during the imaging process. Tiles within nanotubes react with guard strands much more slowly because their sticky ends are not exposed and thus do not present a toehold that can enable quick displacement of the 2 and 4 strands.

For homogeneous nanotube nunchucks composed of REdSEd nanotubes, we used a mixture of 4 guard strands: RE-2EE01C, RE-4C, SE-2DIAGC and SE-4DIAGC in equal concentrations (sequences below). Each guard strand inactivated one corresponding sticky end strand. Together the 4 guard strands inactivated all of the sticky end strands on the two tiles we used. This guard strand mix was added to the reaction mixture at 34°C such that the final concentration of guard strands in the reaction mixture was 400 nM (10 times the concentration of tiles, 40 nM) and incubated at 34°C for 1 min before imaging.

For heterogeneous nanotube nunchucks composed of REs and SEs nanotubes, we used a mixture of 4 guard strands (2 guard strands for each tile type): RE-2sC, RE-4sC, SE-2DIAGC and SE-4sC in equal concentrations that also reacted with all of the sticky end strands in that system (sequences below).

Guard strand sequences for REdSEd nanotubes:

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RE-2EE01C:      AATGCCGTATTGGTGTGAAGGACCAG
RE-4C:          GCATTCCACTACCACATCTTCGTCTG
SE-2DIAGC:      TACCTCTCAGTGGTGCTCTACCAGAC
SE-4DIAGC:      AGGTACGATGACCACGAAACTCTGGT
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Guard strand sequences for REs nanotubes:

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RE-2sC:         AATGCCGTATTGGTGTGAAGGTGGTC
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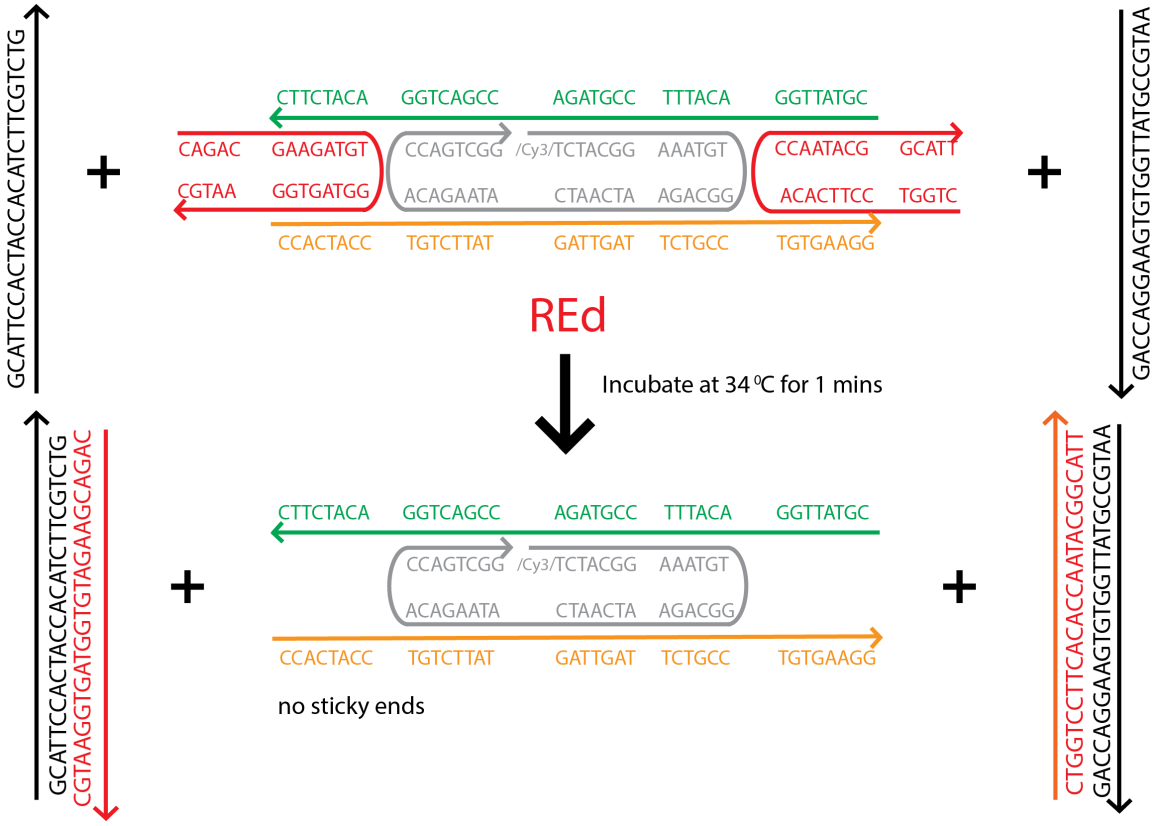
RE-4sC: GCATTCCACTACCACATCTTCGACCA

Guard strand sequences for SEs nanotubes:

SE-2DIAGC: TACCTCTCAGTGGTGCTCTACCAGAC
SE-4sC: AGGTACGATGACCACGAAACTGTCTG

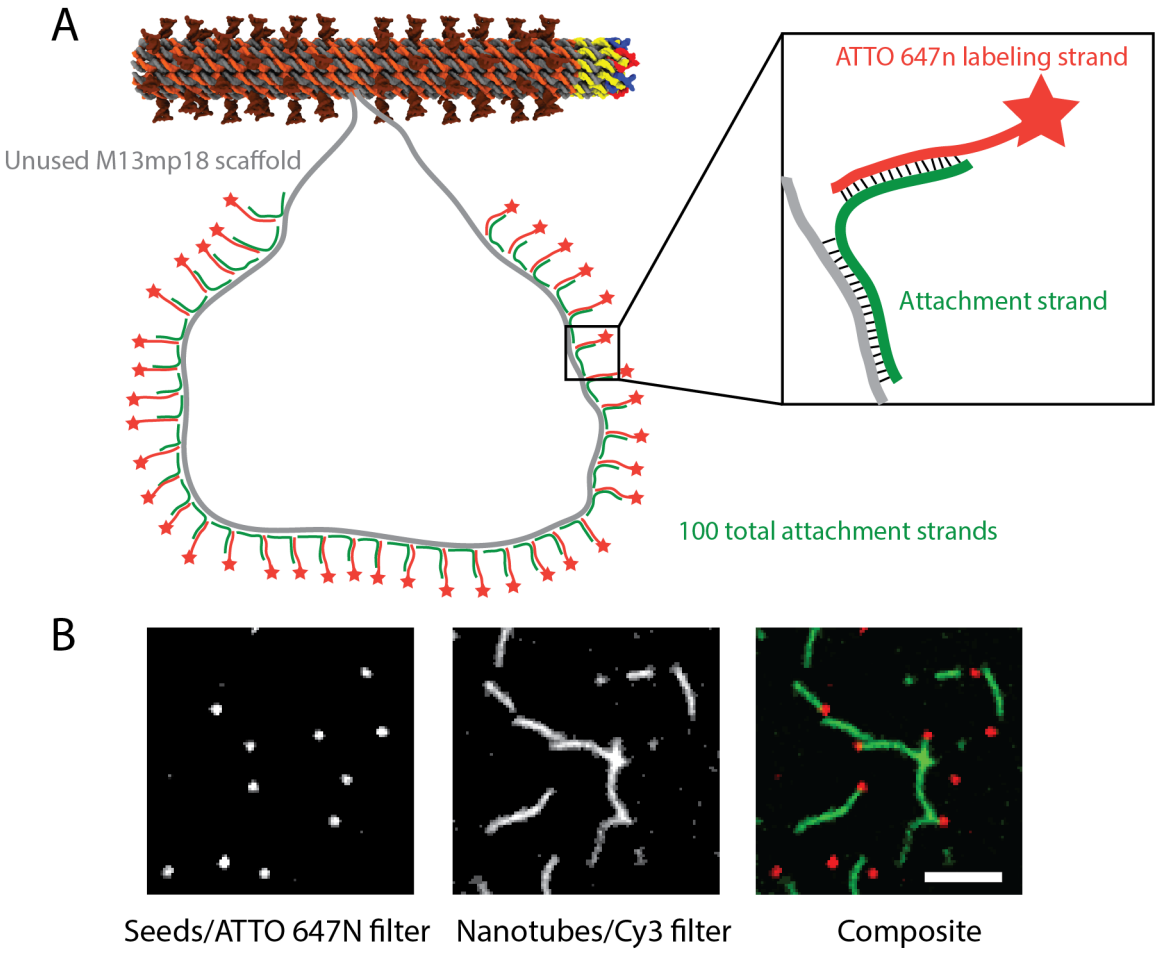


Supplementary Figure S8: REd tile inactivation by guard strands. Similar tile inactivation mechanism for SEd, REs and SEs tiles via addition of the corresponding guard strands prevents further tile assembly at room temperature.

Supplementary Note S6: Labeling seeds with fluorophores

To confirm that nanotubes grow from seeds, we used different fluorescent labels for nanotube tiles and nanotube seeds (Supplementary Figure S9). Nanotube seed structures were labeled with ATTO647N fluorophore dyes. The labeling system consists of 100 *attachment strands* that bind partly to the unused section of the M13mp18 scaffold. The reminder of the attachment strand binds to an *ATTO 647N labeling strand* that has an ATTO647N fluorophore dye on one of its ends.

Note: ATTO488 dye instead of ATTO647N was used to label the seeds in heterogeneous nunchuck nanotube experiments.



Supplementary Figure S9: (A) Schematic showing labeling of seeds with ATTO647N fluorophore dyes. (B) Fluorescence microscopy images showing seeds (in ATTO 647n filter), nanotubes (in a Cy3 filter) and composite from left to right. Scale bar: 5 μm

Labeling strand sequences

```
Labeling_strand_ATTO488  /5ATTO488N/AAGCGTAGTCGGATCTC
Labeling_strand_ATTO647N /5ATTO647NN/AAGCGTAGTCGGATCTC
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Attachment strand sequences

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Unused_m13mp18_05       TTTAATGGTTTGAAATACCGACCGTTTTTGAGATCCGACTACGC
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Unused_m13mp18_07       ACGCGAGAAAACTTTTTCAAATATATTTTGAGATCCGACTACGC
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Unused_m13mp18_10       ACTACCTTTTTAACCTCCGGCTTAGTTTTGAGATCCGACTACGC
Unused_m13mp18_11       AATTTATCAAAATCATAGGTCTGAGTTTTGAGATCCGACTACGC
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Unused_m13mp18_59	TGGCAAGTGTAGCGGTCACGCTGCGTTTTGAGATCCGACTACGC
Unused_m13mp18_60	AAGCGAAAGGAGCGGGCGCTAGGGCTTTTGAGATCCGACTACGC
Unused_m13mp18_61	CGAACGTGGCGAGAAAGGAAGGGAATTTTGAGATCCGACTACGC
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Unused_m13mp18_74	AGCTGATTGCCCTTCACCGCCTGGCTTTTGAGATCCGACTACGC
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Unused_m13mp18_93	CGCTTCTGGTGCCGGAAACCAGGCATTTTGAGATCCGACTACGC
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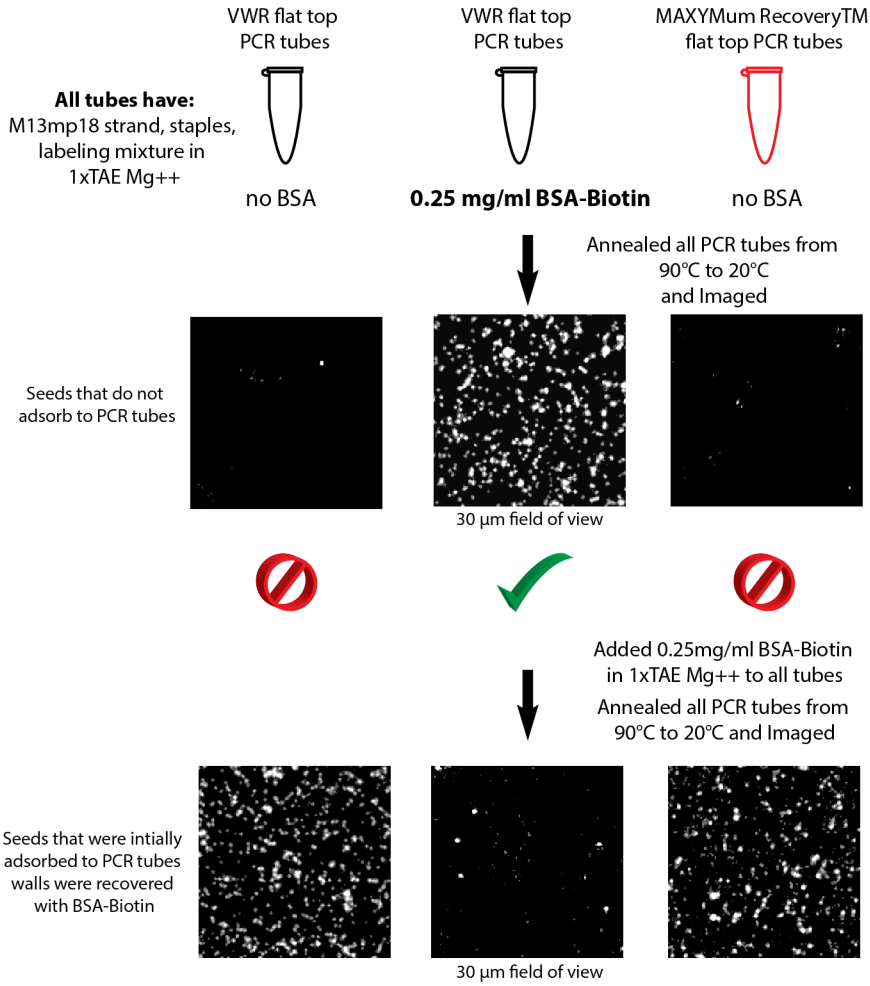
Unused_m13mp18_99	ACAACCCGTCGGATTCTCCGTGGGATTTTGAGATCCGACTACGC
Unused_m13mp18_100	TTCATCAACATTAAATGTGAGCGAGTTTTGAGATCCGACTACGC

Supplementary Note S7: Biotin-BSA prevents DNA nanotube seed adsorption to PCR tubes

In initial fluorescence microscopy images of labeled seeds on glass slides, a very low density of seeds was observed (<10 in 87 μm x 87 μm field of view for 6 μl of 40 pM seeds deposited on a 18mm x 18mm glass cover slip) as compared to what we should expect based on our calculations (~3000 seeds in 87 μm x 87 μm field of view). We hypothesized that few seeds were observed because the majority of the seed structures were adsorbed to the walls of PCR tubes. Other studies suggest the use of BSA to prevent sticking of cells or peptides to glass/lab plasticware.[4] To test whether BSA could reduce nanotube seed absorption to PCR tubes, we annealed labeled seeds in the presence and absence of BSA. Because it was what we had on hand at the laboratory, we used BSA with conjugated biotin. We mixed labeled seeds and BSA–Biotin (A8549, Sigma Aldrich) in two types of PCR tubes: standard *VWR* flat top PCR tubes and MAXYMum Recovery™ PCR tubes that *Axygen Scientific* claimed to have low binding of DNA to them. We found that the presence of BSA-Biotin prevents DNA absorption to the walls of the *VWR* flat top PCR tubes (Supplementary Figure S10). MAXYMum Recovery™ PCR tubes did not show significant improvement as compared to *VWR* flat top PCR tubes in the absence of BSA- Biotin. We further examined DNA adsorption in the presence of non-biotinylated BSA from various stocks (B4287 and A0281, Sigma Aldrich). These BSA stocks did not reduce DNA adsorption to PCR tubes as compared to solutions without any BSA or BSA-Biotin.

In the experiments described in this paper, we annealed our samples in the presence of BSA- Biotin protein unless mentioned otherwise.



Supplementary Figure S10: Addition of BSA-Biotin prevents the adsorption of seeds to the walls of *VWR* flat top PCR tubes.

Supplementary Note S8: Finding the optimal temperature at which pre-annealed seeds can be added to tiles

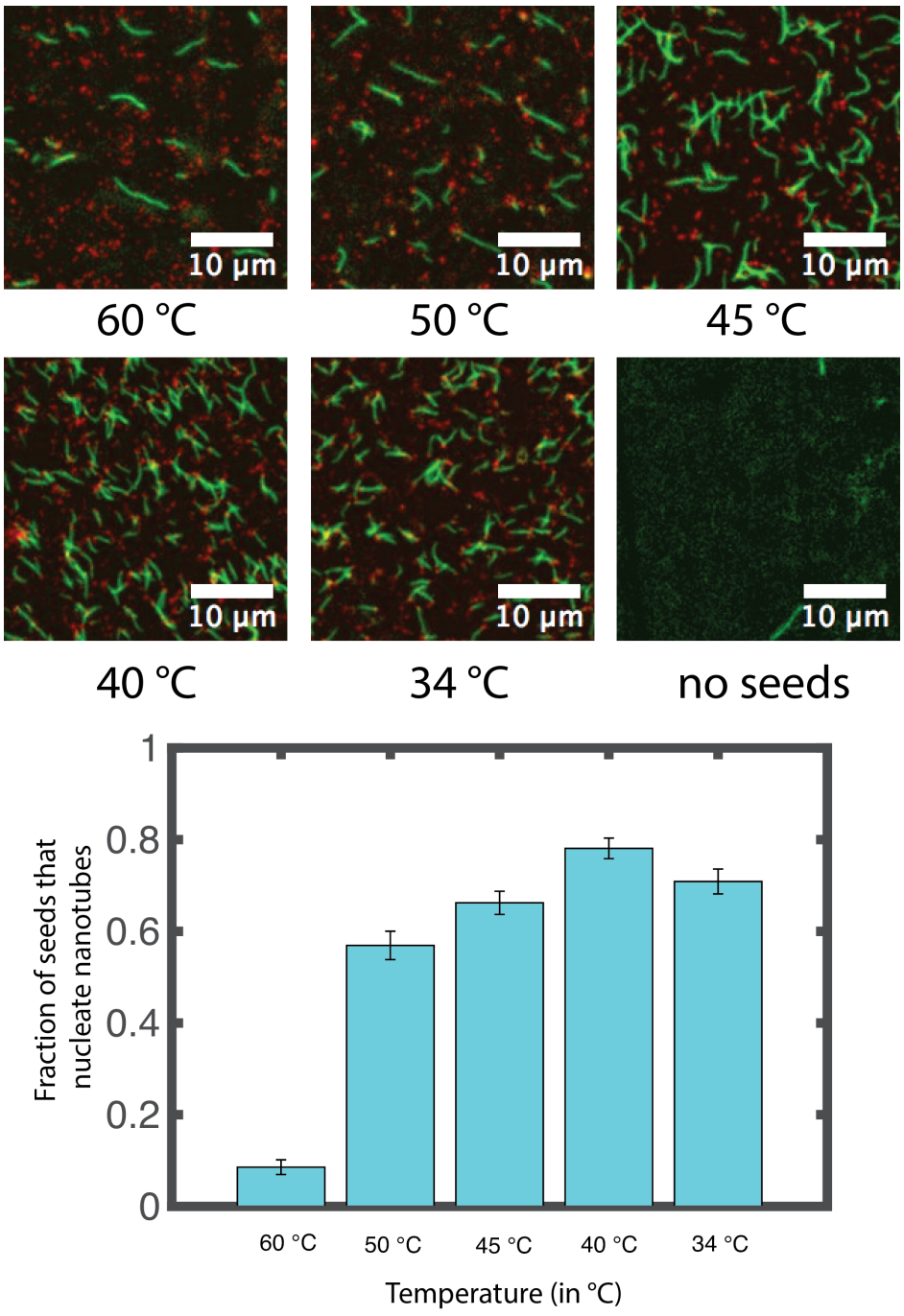
Nanotube nunchucks were grown by adding pre-assembled, purified nunchuck seed structures to a mixture of nanotube tiles at 34ºC, a temperature at which nanotubes are stable, but nanotubes nucleate and grow without seeds relatively rarely at the tile concentrations used here. The addition of seeds allows nanotubes to grow by providing a template on which nanotubes can grow with a small nucleation barrier.

To maximize the yield of nunchucks, we performed a series of experiments in which we varied when nunchuck seed structures were added to the tile mixture during the anneal. For each temperature where the seeds were added, we measured the seed efficiency, defined as the percentage of seeds from which nanotubes grow.

The tile annealing protocol was as follows:

1. 90°C – 5 mins
2. 90°C to 45°C - 1°C/min
3. 45°C – 60 mins
4. 45°C to 34°C – 0.1°C/min
5. Hold at 34°C for 15 hours

We added seeds at 60°C, 50°C, as soon as the sample reached 45°C *(i.e.* the beginning of step 3), 40°C and 34°C and measured the seed efficiency for each assembly process (Supplementary Figure S11).

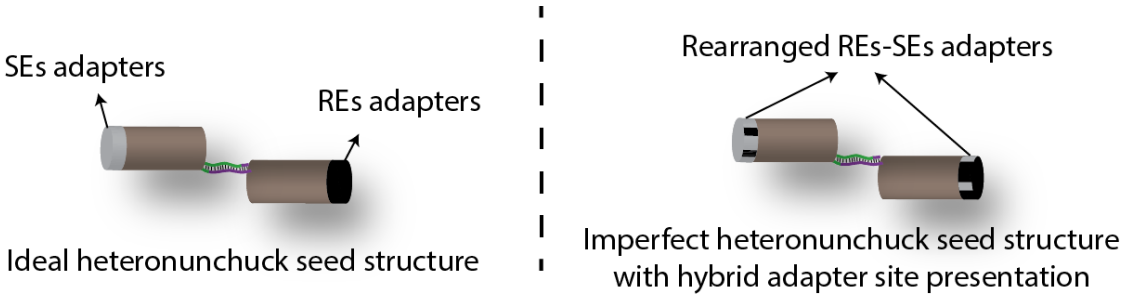


Supplementary Figure S11: Fluorescence microscopy images of seeded nanotubes when pre-formed purified seeds were added to annealing tile mixture at different temperatures. The highest percentage of pre-formed purified seeds having nanotubes is observed when seeds were added to tile anneal at 40°C.

Supplementary Note S9: Interference of SEs adapters with REs seeded nanotube nucleation and vice versa due to adapter swapping.

Nanotube nunchucks were synthesized by adding pre-assembled, purified nunchuck seeds to a mixture of nanotube tiles and excessive adapter strands. Additional adapter strands were included with the mixture of tiles as we found their presence increased the yield of homogeneous nanotube nunchucks. One reason that adapters could increase yields is that excess adapters could bind to sites were adapter tiles fell off seeds during the purification of nunchuck seed structures. During the synthesis of heterogeneous nanotube nunchucks, excess adapters for both of the growth fronts were provided.

While the presence of additional adapters during the growth of homogeneous nunchucks results in higher nanotube nunchuck yield, a similar addition of excess adapters during heterogeneous nanotube nunchuck growth could result in poor nucleation yields from the nunchuck seed structures because adapters for one type of nanotube nucleation site could swap or bind to the adapter attachment site of the other type of nanotube. We called this phenomenon adapter swapping.

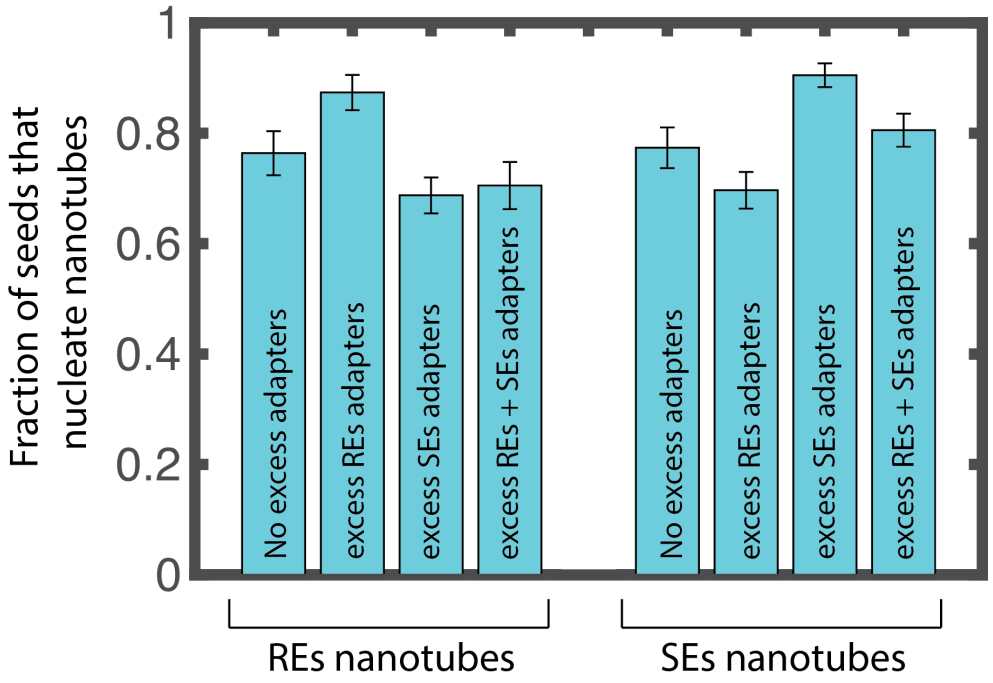


Supplementary Figure S12: Schematic illustrating the possibility of having imperfect nucleation sites on a heterogeneous nunchuck seed due to rearrangement of the individual REs or SEs adapters on the two seeds with the excess REs or SEs adapters in solution.

To test whether adapter swapping might decrease the nucleation rate of nanotubes from seeds when more than one set of adapter strands is present, we grew REs (and SEs) nanotubes in the absence and presence of a different set of adapters and compared yields when multiple sets of adapters were present with yields when only one set of adapters was present. In order to make these experiments directly relevant to understanding the low yields presented by heterogeneous nunchuck seeds, the seeds were purified using agarose gel in these experiments similar to our protocol for nunchuck seed structures before being mixed with tiles and adapters for growth. The seed concentration used in these adapter swapping experiments was 40 pM. Excess adapters were added to the reaction mixture to a final concentration of 0.4 nM, following the general methods in other nunchuck nanotube experiments. Micrographs were taken after 15 hours incubation at 34°C.

For both REs and SEs seeds and nanotubes, nanotube yields increased when excess adapters of the corresponding type of nanotube were mixed with tiles. Conversely, growth yields from seeds decreased slighted when excess adapters of the opposite type of nanotube were mixed with tiles (Supp. Fig. S13). However, changes in yield were small,

and the adapter switching observed in these experiments does not account for the low observed yields of heterogeneous nanotube nunchucks.



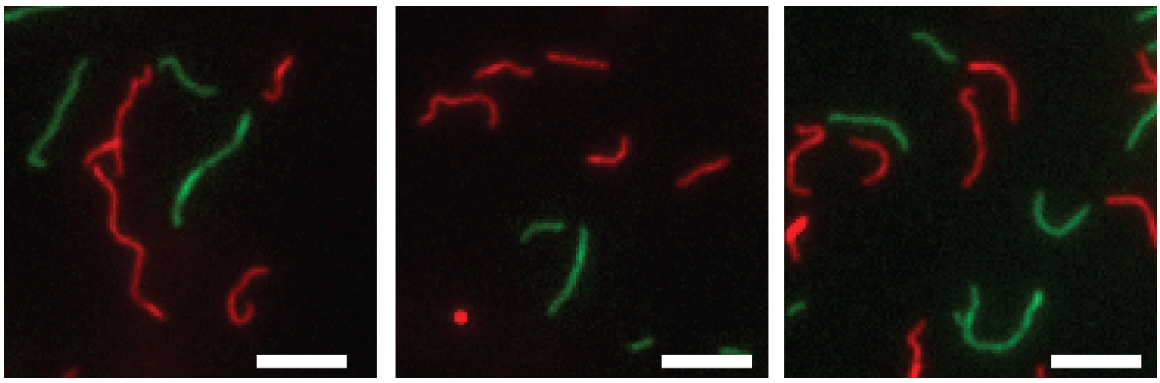
Supplementary Figure S13: The fraction of seeds that nucleate nanotubes in the absence of excess adapters, presence of excess REs adapters, presence of excess SEs adapters and presence of both REs and SEs adapters for REs nanotubes and SEs nanotubes.

Supplementary Note S10: Minimal crosstalk between REs and SEs tile nanotubes.

We synthesized heterogeneous nanotube nunchucks by growing both REs and SEs nanotubes simultaneously. This involved incubating both types of nanotubes tiles together with nunchuck seed structures. For reliable results, this assembly process requires minimal crosstalk between the REs and SEs nanotubes.

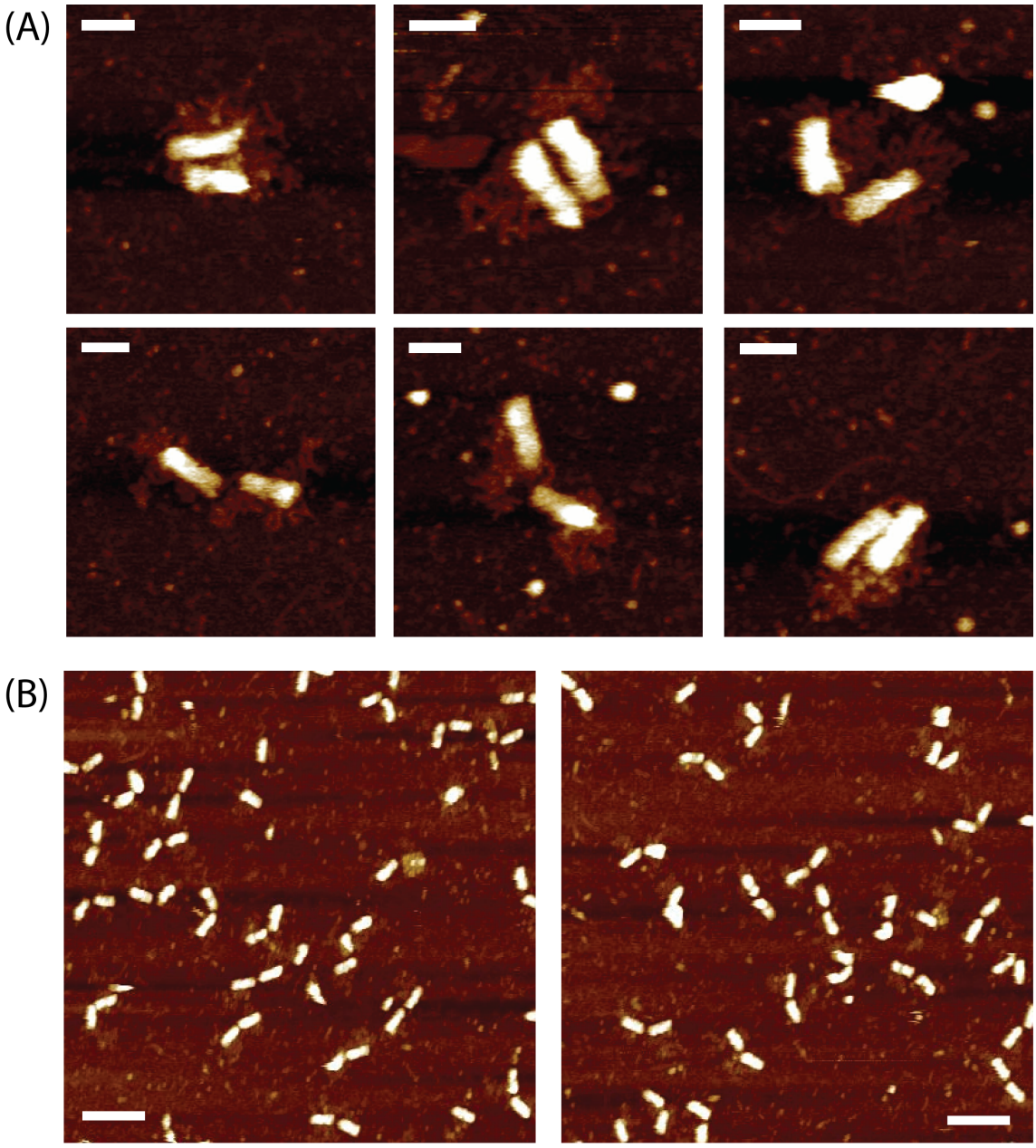
To test for crosstalk between REs and SEs nanotube tiles, specifically whether such tiles would interact by co-assembling, we characterized how these two types of tiles would assemble into nanotubes in the absence of seeds. We mixed REs tiles labeled with ATTO647N dye and SEs tiles labeled with Cy3 dye in one mixture along with TAE Mg^{2+} buffer. We then annealed the solution using the previously described annealing protocol (Supp. Note S8) but cooled the solution to 20°C instead of 34°C to so that homogeneous nucleation was rapid, allowing nanotubes to grow efficiently in the absence of seeds.

If REs and SEs tiles could co-assemble, we would expect to observe hybrid nanotubes consisting of both REs and SEs tiles. However, fluorescence microscope images of the co-annealed REs and SEs mixture showed no significant overlap of between ATTO647N and Cy3 dye channels (Supp. Fig. S14).

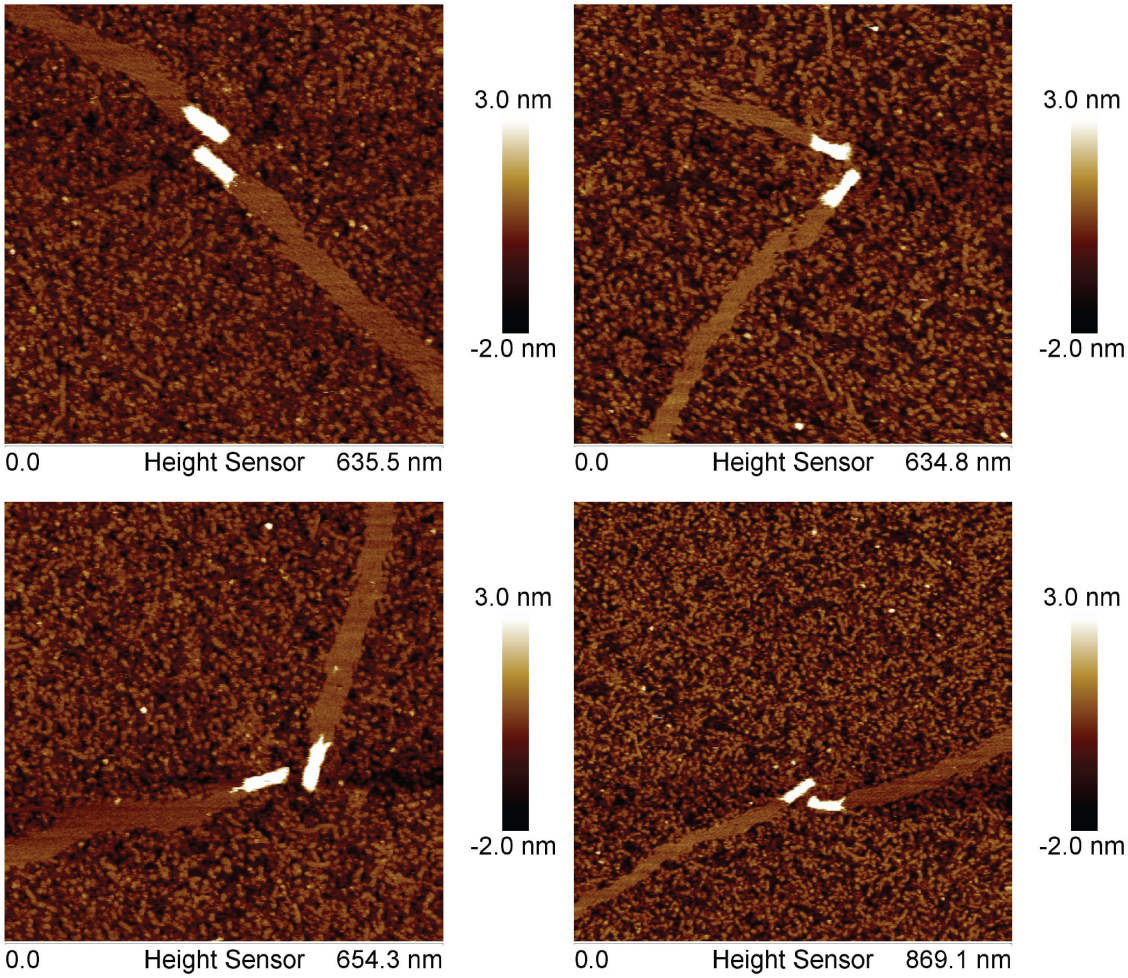


Supplementary Figure S14: Fluorescence microscopy images of REs and SEs nanotubes co-annealed in one pot. ATTO647N dye shown in red and Cy3 dye shown in green. Scale bars: 5 μm.

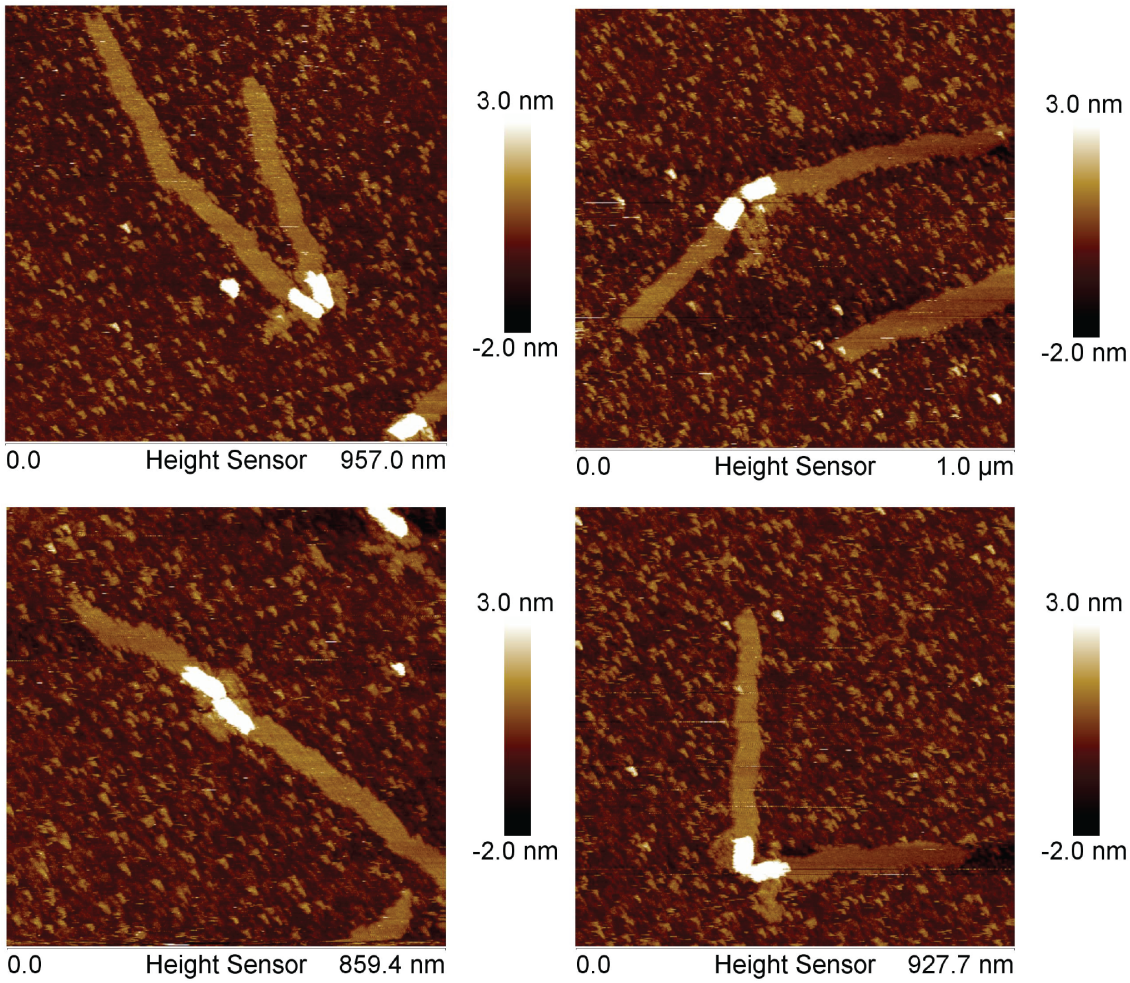
Supplementary Note S11: Additional images of nunchuck nanotube seeds, homogeneous nanotube nunchucks and heterogeneous nanotube nunchucks.



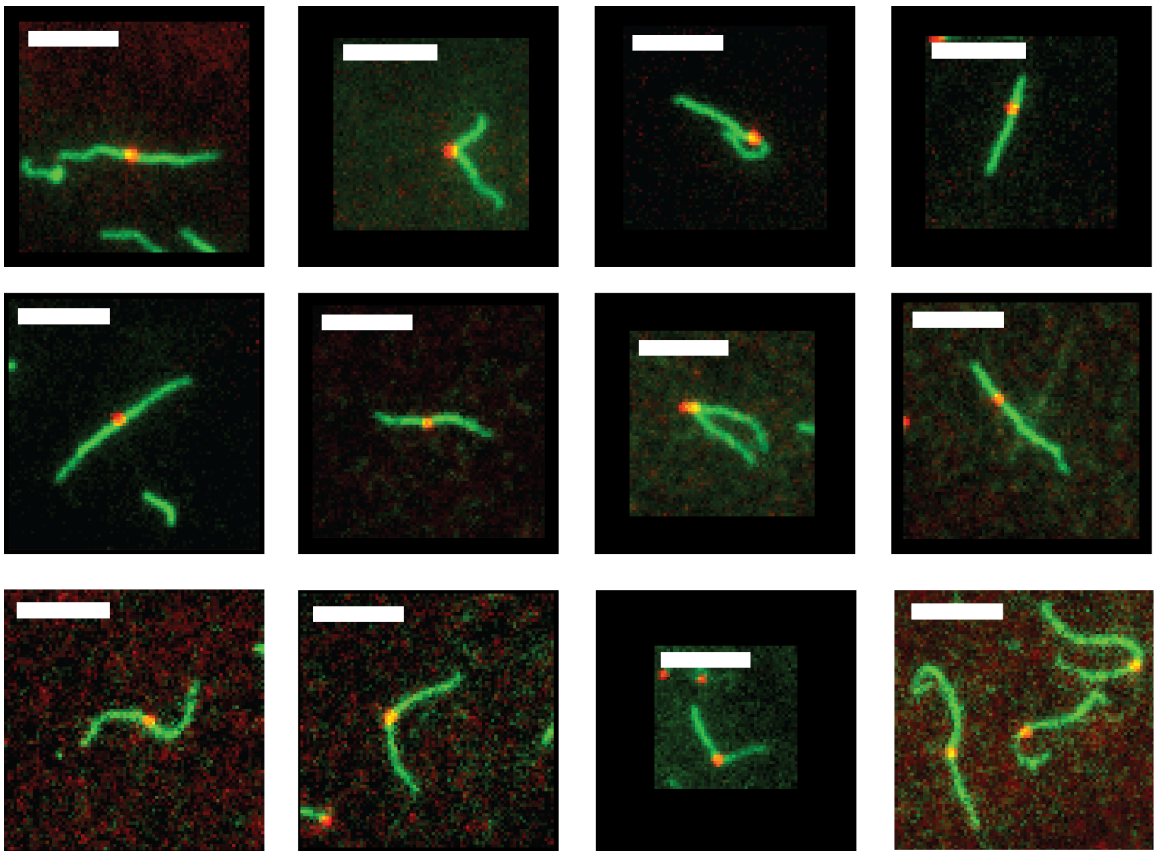
Supplementary Figure S15: (A) Sample AFM images of individual nunchuck nanotube seeds Scale bars: 50 nm. (B) Typical wide field AFM images of nunchuck nanotube seeds. Scale bars: 200 nm.



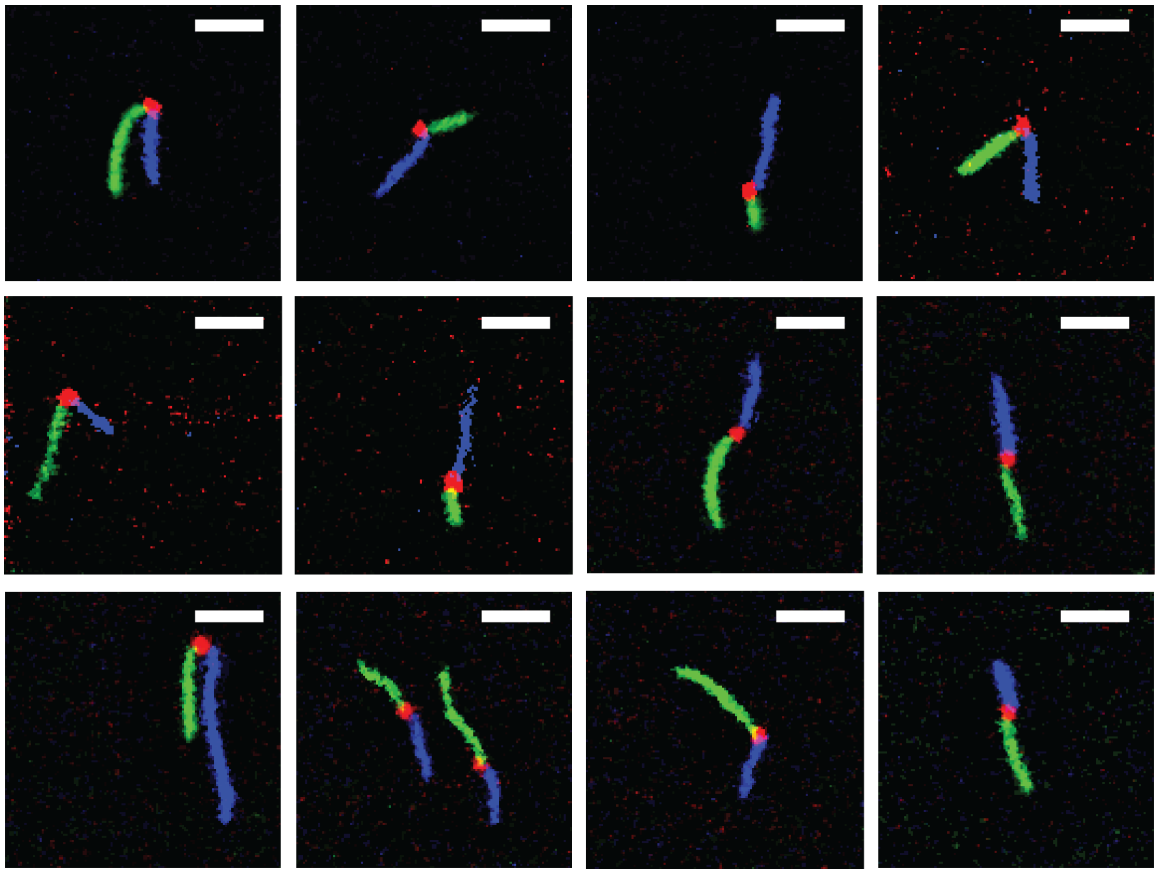
Supplementary Figure S16: Sample AFM images of homogeneous nanotube nunchucks. The samples were prepared according to the protocol described in Supplementary Note S3.



Supplementary Figure S17: Sample AFM images of heterogeneous nanotube nunchucks. The samples were prepared according to the protocol described in Supplementary Note S4.



Supplementary Figure S18: Sample fluorescence microscopy images of homogeneous nanotube nunchucks. The samples were prepared according to the protocol described in Supplementary Note S3. Scale bars: 5 μm



Supplementary Figure S19: Sample fluorescence microscopy images of heterogeneous nanotube nunchucks. The samples were prepared according to the protocol described in Supplementary Note S4. Scale bars: 2 μm

Supplementary Note S12: Nanotube nucleation rates from yield measurements

Because nanotube nucleation is a stochastic process, it is possible for nunchuck seed structures to nucleate both, only one, or neither of the two-nanotube types. To determine the rate of nanotube nucleation on these constructs, fluorescence micrographs of both homogeneous and heterogeneous nunchuck samples were taken at 10 randomly selected locations.

Homogeneous nanotube nunchucks:

The observed proportions of homogeneous nunchucks were:

Species	Fraction observed
2-arm	0.50 ± 0.05
1-arm	0.45 ± 0.05
0-arm	0.05 ± 0.02

Taking T to be the probability for nanotube nucleation from one seed, the probability of forming each of the structures is

2-arm-nunchuck: $T \cdot T$
1-arm-nunchuck: $2 \cdot T \cdot (1-T)$
empty-nunchuck: $(1-T) \cdot (1-T)$

Setting these probabilities equal to the observed proportions and solving the resulting three simultaneous equations gives a best fit of T=0.70± 0.03. Although this fit is close to our direct measurement of T = 0.78 ± 0.02, there is a small but significant difference between the two values.

Two possible explanations for this discrepancy are 1) nucleation from seeds in nunchuck form occurred at a lower rate than nucleation from seed monomers, and 2) some seeds in the nunchuck mixture were in monomeric rather than dimeric form, which could arise due to either incomplete purification or spontaneous dissociation. To explore this second possibility, we assume the rate of nucleation from the nunchucks is the same as from monomeric seeds and define P as the proportion of seeds in the monomeric state. Then, the probability of forming each of the structures is

2-arm-nunchuck: $T \cdot T \cdot (1-P)$
1-arm-nunchuck: $2 \cdot T \cdot (1-T) \cdot (1-P)$
empty-nunchuck: $(1-T) \cdot (1-T) \cdot (1-P)$
1-arm-monomer: $T \cdot P$
empty-monomer: $(1-T) \cdot P$

Because nunchuck seeds and monomer seeds are not distinguishable in the fluorescence images, the expected probability of the three observable structures is

2-armed nunchuck: $T \cdot T \cdot (1-P)$
seeded nanotube: $[2 \cdot T \cdot (1-T) \cdot (1-P) + T \cdot P]$
empty seed: $[(1-T) \cdot (1-T) \cdot (1-P) + (1-T) \cdot P]$

Setting these probabilities equal to the observed proportions and solving the resulting three simultaneous equations for the two unknowns yields T=0.89 and P=0.36. These are not likely to be the correct values because (i) they are not consistent with our direct measurement of T = 0.78 ± 0.02, and (ii) the analysis performed fails to account for uncertainties in the observed proportions.

To include these constraints in the analysis, we instead solve for P as a function of T and the observed proportions:

$$P = (1 - N_{2arm}/T^2)$$
$$P = (N_{1arm} + 2T(T-1))/ (T + 2T(T-1))$$
$$P = (N_{empty} - 1 + 2T - T^2)/ (T - T^2)$$

Supplementary Figure S20 plots the three functions above with their 68% confidence intervals. From the overlap of the confidence intervals for the three different functions P(T) with each other and with the that of T, we find P = 0.16 ± 0.02.

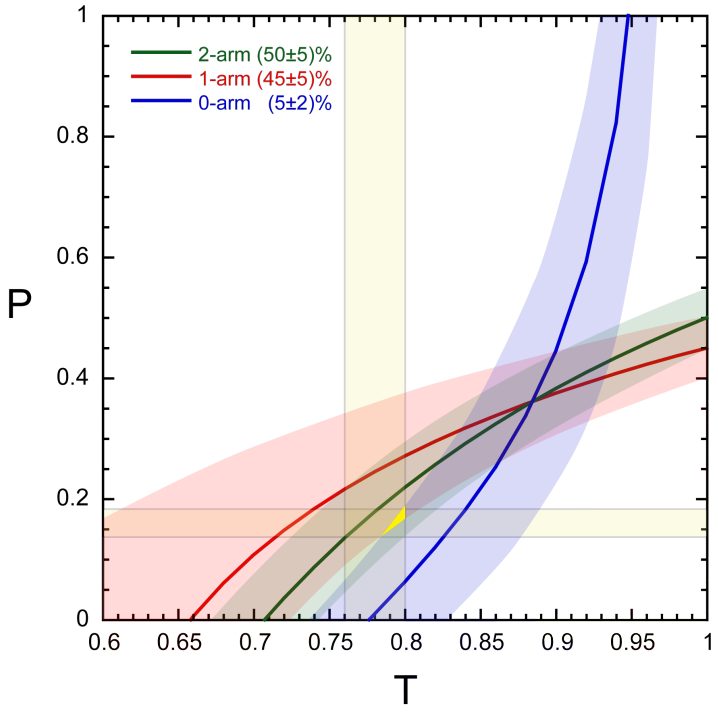


Figure S20: Possible values of P and T allowed by the observed proportions of seeds with two arms (green), one arm (red) and no arms (blue). Shaded regions indicate 68% confidence intervals. Given the probability of nanotubes nucleating from single seeds, T = 0.78 ± 0.02, which was observed independently under similar conditions (yellow, vertical), we find the 68% confidence interval for P = 0.16 ± 0.02 (yellow, horizontal).

Heterogeneous nanotube nunchucks:
Heterogeneous nanotube nunchucks consist of two different types of nanotube arms (REs and SEs), one on each seed of the nunchuck seed structure.

The observed fractions of seeded species in the synthesis of heterogeneous nunchucks were:

Species	Fraction observed
RS-nunchuck	0.30 ± 0.03
RR-nunchuck	0.025 ± 0.007
SS-nunchuck	0.015 ± 0.005
Seeded R nanotubes	0.28 ± 0.03
Seeded S nanotubes	0.29 ± 0.03
empty seeds	0.09 ± 0.01

Taking R to be the probability for REs nanotube nucleation on an R-type seed, S to be the probability for SEs nanotube nucleation on an S-type seed, the probability of forming each of the structures is

2-arm-nunchuck: $R \cdot S$
one REs-arm-nunchuck: $R \cdot (1-S)$
one SEs-arm-nunchuck: $(1-R) \cdot S$
empty-nunchuck: $(1-R) \cdot (1-S)$

Setting these probabilities equal to the observed proportions and solving the resulting four simultaneous equations gives $R = 0.61 \pm 0.03$ and $S = 0.62 \pm 0.03$. These values are in good agreement with direct measurements of nucleation yields when nunchuck seeds are exposed to only a single tile-type: $R = 0.67 \pm 0.06$ and $S = 0.60 \pm 0.05$. However, these yields are much lower than those observed on monomeric seeds: $R = 0.87 \pm 0.03$ and $S = 0.90 \pm 0.02$. The presence of a small number of homogeneous nunchucks in the heterogeneous preparation indicates that adapter strands on the two facets of the nunchucks are in dynamic equilibrium with adapter strands in solution. We therefore attribute the reduced yield of nucleation in the presence of heterogeneous nunchucks at least in part to the exchange of adapter strands, as discussed in the main text.

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