SUPPLEMENTARY INFORMATION

A Microfluidic Chip-Compatible Bioassay Based on Single-Molecule Detection with High Sensitivity and Multiplexing

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Supplementary information includes:

1. SUPPLEMENTARY METHODS

1.1. Materials

Reagents for Digital DNA

All PCR was performed with Platinum Taq DNA polymerase High Fidelity (Invitrogen, Carlsbad, CA) except for amplification of identification and receptor segments, for which Eppendorf TripleMaster PCR kit (Hamburg, Germany) was used. Ligations were performed with T4 DNA ligase, purchased either from NEB (Ipswich, MA) or Promega (Madison, WI). Restriction enzymes were purchased from NEB or Promega. Primers were synthesized by IDT (Coralville, IA). Two bisPNA tags were synthesized by Panagene (Korea): tag p368((N)-TMR-OO-Lys-Lys-TTCTTCTC-OOO-JTJTTJTT-Lys-Lys-(C)), and tag p2368 ((N)-TMR-OO-Lys-Lys-TCCTTCTC-OOO-JTJTTJJT-Lys-Lys-(C)). The tags include tetramethylrhodamine fluorophore (TMR) at their N-termini. The fluorophore, the Watson-Crick (T+C) strand, and the Hoogsteen (T+J) strand are connected with flexible hydrophilic O-linkers (8-amino-3,6-dioxaoctanoic acid). T, C, and J are thymine, cytosine, and pseudoisocytosine, respectively. N-Succinimidyl 3-(2-pyridyldithio)propionate (SPDP) and N-Succinimidyl S-acetylthioacetate (SATA) were purchased from Pierce (Rockford, IL). Alexa Fluor 647 succinimidyl ester (Alexa 647 SE) and nucleic acid dyes PicoGreen and POPO-1 were products of Invitrogen. All other reagents were purchased from Sigma-Aldrich (St. Louis, MO).

1.2. Digital DNA: design and preparation

Concept. With the conjugation chemistry described below, a suspension array can be built using natural DNA molecules with sufficiently different “barcodes” as scaffolds. We have successfully used this approach at the early development stages. Our goal, however, was not only

to demonstrate one more concept of a suspension array, of a single-molecule technology, nor of a DNA application, but to demonstrate the practicality of our approach. Therefore, we needed to develop a robust molecular system that meets all of the requirements for this analytical application. First, the system must provide a way to assemble identifiers with different lengths and multiple patterns that are easy to discriminate. We have achieved this requirement using a modular design. We developed two blocks (block ***1*** and block ***0***) and an approach to assemble many – up to 20 – of these blocks and to join them in any combination (Figure 1a, main text and Figure S1). Second, the system must ensure robust detection. Although we can simultaneously detect single fluorophores in different spectral ranges, this equipment employs top-end collection optics, detectors with single-photon sensitivity, and other features that make it delicate and expensive. Therefore, we used clusters of multiple fluorophores and receptors that provided considerably higher emission intensity. These extra photons can be traded for a simpler detector, higher sensitivity, or higher throughput. One of the approaches to simplify the detector is an integrated optics that is also more compact and vibration-resistant.[1] Integrated optics may employ diffraction-limited illumination[1] or near-field illumination[2] optics. The former and latter approaches have respectively worse or better resolution than the free-space optics we employ now. To accommodate for the resolution change, the blocks ***1*** and ***0*** can be made longer or shorter. To vary the number of fluorphores and receptors and to modify the block length, we have made the structure of the blocks themselves modular, so sections of different lengths and patterns of bisPNA-binding sites could be exchanged. Finally, reagent production must be fast and easy, although we can tolerate some complexity in the initial design and construction phase. Therefore, our molecular system was designed for propagation in a bacterial system and further isolation of the DNA with a simple, commercially available kit.

We have overcome several technical challenges to implement this approach. We have identified a combination of an appropriate plasmid vector and host bacterial strain to propagate DNA up to 200 kilobases long containing multiple tandem repeats without recombination or deleterious mutations.[3] We then ensured similar numbers of receptors in the DNA molecules with different identifying barcodes by building independent sites to bind the fluorophores and the receptors. These sites were carefully designed to avoid increasing the flexibility of the DNA molecule. BisPNA hybridization disrupts the tight double-helix DNA structure. Multiple loci with increased flexibility (which formally is described with decreased persistence length) would diminish the stretching efficiency of our microfluidic system.[4] We also had to establish that the antibody-antigen-antibody sandwich would survive the multistage procedure that included removal of the excess secondary antibody, dilution to the concentration compatible with single-molecule measurements, travel on the DNA into the microfluidic chip, and stretching in the accelerated flow. Finally, the molecular construct needed to be compatible with the methods to attach different types of receptors, such as antibodies, oligonucleotides, and aptamers.

The first generation of this molecular system included the specialized DNA – Digital DNA – and all required chemistries to assemble the whole capture unit (CU). This system was intended to study the potential problems discussed above. Therefore, it included more options and flexibility than required for the presented research. For example, we never used the specialized sites to attach the antibodies, but directly bound them to the fluorphore-carrying bisPNAs. The design of this adaptable research tool is described in detail below.

Design of blocks *1* and *0* for Digital DNA. We decided to use bisPNA to attach fluorophores and antibodies to DNA. We have extensive experience specifically hybridizing fluorescent bisPNA tags to long (hundreds of kilobases) DNA and to measure bisPNA-tagged

DNA.[5, 6] Also, the lysine amino groups present in our standard bisPNA structure offered convenient handles for attaching receptor molecules.

To moderate the increased flexibility of the Digital DNA after hybridization of multiple bisPNA tags, we grouped bisPNA-binding sites in short 0.33-0.34 kb binding segments separated by long 4.3-4.8 kb structural segments (Figure S1a). That way, only the binding segments become flexible after bisPNA hybridization, while the structural segments remain intact and as rigid as native DNA. With this design we expected to maintain sufficient DNA rigidity, even in completely assembled CUs. We prepared two versions of the blocks with 4 or 6 binding sites per segment to assess the effects of closely spaced bisPNA-binding sites. The 6-site version could be stretched as efficiently as the 4-site version; it also exhibited no extra cross-linking or any other adverse effects (data not shown). As the 6-site version produced considerably brighter barcodes, all presented data were obtained with it.

Each block ***1*** or ***0*** contained identification and receptor segments separated by structural segments (Figure S1a). The receptor segments in both blocks ***1*** and ***0*** included 6 binding sites with the sequences 5'-GAGAAGAA-3' where bisPNA tag p368 could hybridize. The signal segment in block ***1*** included 6 binding sites with the sequences 5'-GAGAAGGA-3'for bisPNA tag p2368, while signal segment in block ***0*** included no bisPNA-binding sites. Receptor and signal segments were separated by structural segments I and II. The length of the blocks was twice the current resolution of our detection system, which is about 4 kb, and allowed easy identification of the barcodes. Special care was taken to exclude the sequences differing from hybridization bisPNA sites by single mismatches at their termini to exclude non-specific binding of the tags.[6]

Preparation of single Digital DNA blocks in high-copy plasmids. Single Digital DNA blocks were constructed in the high-copy pBluescript II KS+ plasmid (Stratagene, La Jolla, CA) and propagated in STBL4 *E. coli* cells (Invitrogen) grown at 30 ºC. For block construction, we modified the multiple cloning site of pBluescript II KS+ with the Quick-change II site-specific mutagenesis kit (Stratagene) with primer set DD1 (Table S1) to construct the pUSG1 plasmid (Figure S2).

For easy selection of transformed cells, we introduced resistance to the antibiotic Zeocin into our block assembly plasmids (Figure S2). First, we used the DD2 primer set (Table S1) to eliminate the NcoI restriction endonuclease site from the Zeocin resistance gene in the pZErO-1 (Invitrogen) plasmid (Figure S2). The mutated Zeocin resistance gene was amplified by PCR with DD3 primers (Table S1) designed to incorporate BamHI and NotI recognition sequences at the 5' and 3' ends of the gene, respectively. The 3'-primer introducing the NotI site was designed to also incorporate the recognition sites of all restriction endonucleases required for Digital DNA block construction: NheI, PspOMI, SpeI, AsiSI, and XbaI (Figure S3a). The modified Zeocin resistance gene was ligated into the pUSG1 plasmid between the BamHI and NotI sites to produce pUSG2 plasmid (Figure S2).

Block ***1*** identification segment. The dsDNA fragment DD8 (Table 2) for the block ***1*** identification segment was constructed by PCR (Eppendorf TripleMaster PCR kit, Hamburg, Germany) from annealed overlapping oligonucleotides. It contained 6 binding sites with the sequences 5'-GAGAAGGA-3' for bisPNA tag p2368. This PCR product was digested with PspOMI and SpeI restriction enzymes producing a 339 bp-long identification segment, which was ligated into the corresponding sites of plasmid pUSG2.

Block ***0*** identification segment. The 343 bp-long dsDNA fragment for the block ***0*** identification segment was amplified by PCR (Eppendorf TripleMaster PCR kit) from pUC19 plasmid with DD4 primers (Table S1). The PCR product was digested with PspOMI and SpeI restriction enzymes and ligated into the corresponding sites of plasmid pUSG2.

Receptor segment. The dsDNA fragment DD9 (Table S2) for the receptor segment was constructed by PCR (Eppendorf TripleMaster PCR kit) from annealed overlapping oligonucleotides. It contained 6 binding sites with the sequences 5'-GAGAAGAA-3'for bisPNA tag p368. This PCR product was digested with AsiSI and XbaI restriction enzymes producing a 334 bp-long identification segment, which was ligated into the corresponding sites of plasmid pUSG2.

Structural segment I. A 4,302 bp-long dsDNA fragment for the structural segment I was amplified by PCR from *Autographa californica* viral genome (sequence between 85,363 and 89,665 bp) with DD5 primers (Table S1). The PCR product was digested with NheI and PspOMI restriction enzymes and ligated into the corresponding sites of plasmid pUSG2.

Structural segment II. A 4,842 bp-long dsDNA fragment for the structural segment II was amplified by PCR from *Autographa californica* viral genome (sequence between 16,536 and 21,378 bp) with DD6 primers (Table S1). The PCR product was digested with SpeI and AsiSI restriction enzymes and ligated into the corresponding sites of plasmid pUSG2.

Assembled blocks ***0*** and ***1***. As the result of the assembly, we have obtained plasmids pUSG3 and pUSG4 containing single full length blocks ***1*** and ***0***, respectively (Figure S3).

Assembly of Digital DNA. To simplify and accelerate the production of Digital DNA, we assembled all possible dimers of the blocks ***1*** and ***0*** in high-copy plasmids derived from pUSG2: plasmid pUSG5 with dimer ***00***, pUSG6 with dimer ***01***, pUSG7 with dimer ***10***, and

pUSG8 with dimer ***11*** (not shown). The dimer assembly was achieved with the procedure described below to assemble the whole Digital DNA. The assembly of the whole Digital DNA could include either attaching a single block or a dimer.

Construction of bacterial artificial chromosome (BAC) vector, pEW201. Due to the large size of the final expected constructs, Digital DNA blocks were assembled in bacterial artificial chromosome vectors (BACs). BAC vectors are capable of maintaining DNA in excess of 200 kilobases,[7] which is approximately the expected length of a 20-block Digital DNA molecule. The BAC plasmid pEW201 that was used as the backbone for Digital DNA construction was produced by modification of the BAC plasmid pCC1BAC (Epicentre Biotechnologies, Madison WI). To make it suitable for our task, we eliminated from pCC1BAC the sites of the restriction endonucleases that would interfere with Digital DNA assembly.

A plasmid that contained the 680 bp BstEII-KasI fragment, similar to the fragment of pCC1BAC, but in which the NotI site (at 3 bp position) was deleted, was purchased as a custom synthesis from DNA2.0 (Menlo Park, CA). We replaced the corresponding fragment in pCC1BAC with this fragment. Next, the PspOMI site was mutated by digestion with PspOMI and the overhanging ends were filled in with dNTPs by T4 DNA polymerase (NEB). The plasmid was recircularized by blunt-end ligation. Finally, the XbaI site located at 3,182 bp was eliminated by digesting pCC1BAC with XbaI, removing the 2,822 bp-long fragment, and replacing it with a 2,822 bp fragment generated from pCC1BAC by PCR amplification with DD7 primers (Table S1). The 5' forward primer retained the original XbaI cleavage site, while the 3' reverse primer replaced the second XbaI site with a SpeI site. The amplicon was then reintroduced by ligation into the plasmid to replace the fragment removed by double XbaI

cleavage. Colonies were screened with an NcoI digest to identify those in which the ligation occurred in the proper orientation.

Digital DNA assembly. Digital DNAs were assembled from multiple blocks by a modification of a tandem repeat cloning procedure[8] and introduced into DH10B or GeneHogs (Invitrogen) cells by electroporation[9] using Gene Pulser II (Biorad, Hercules, CA). Digital DNA was constructed in *recA1* mutant of *E. coli* in order to suppress possible RecA-dependent homologous recombination expected in cloning large DNA fragments with tandem repeats.[3, 10] Digital DNA was assembled in the pEW201 BAC vector and propagated as single copy plasmids.

The first step was the insertion of single blocks ***1*** or ***0*** from pUSG3 or pUSG4, respectively, into the pEW201 BAC vector at BamHI and NotI cut sites. Further steps were sequential additions of more blocks to the first; both single blocks and their dimers could be added with the same cloning strategy. Addition of whole dimers reduced the number of cloning steps by half. The individual blocks and the pEW201 vector were designed such that Digital DNA could be extended from either end. Further the procedure is described to add a single block to the Digital DNA within the pEW201 vector. Dimers can be added with the same procedure, using plasmids pUSG 5 through 8, instead of plasmids pUSG 3 or 4 with monomeric blocks.

To add a block to the growing construct, the block was excised from pUSG 3 or 4 plasmid with BamHI and XbaI restriction enzymes, which produced the sticky ends complementary to the ends of pEW201 plasmid cut with BamHI and NheI (Figure 2, main text). The block's termini were hybridized with the plasmid's ends and it was ligated to the plasmid. After ligation, the BamHI-specific site remained, while the sequences adjacent to the compatible cohesive ends of NheI and XbaI cuts were incompatible with either enzyme after ligation. In

"reverse" construction, the block was excised with NotI and NheI and ligated into the growing Digital DNA construct that had been digested with NotI and XbaI. Plasmids of the proper length were identified by pulsed-field gel electrophoresis with the Chef Mapper system (Biorad) (Figure S3b). EcoRI digestion was used to confirm the proper sequence of block order (Figure 2c, main text), which also was confirmed with the single molecule analysis. A total of 40 unique sequences have been generated using these techniques, as summarized in Table S3.

1.3. Capture Units: assembly and control

Concept. To produce capture units (CU), antibodies should be attached to Digital DNA molecules with a certain barcode (Figure S4). We excised linear Digital DNA from a corresponding circular plasmid and hybridized it with bisPNA tags. Each tag contained 4 lysines with positively charged side chains to accelerate the hybridization. Once the bisPNA was attached to DNA, the amino groups on these side-chains could be converted to activated thiol groups with the SPDP cross-linker, using standard protocols from the manufacturer (Pierce). The SPDP-modified DNA further reacted with thiolated antibody for subsequent sandwich assays. We also ran control reactions with antibodies containing both thiol groups and fluorescent dyes to confirm conjugation efficiency.

Assembly procedures. In the presented research, we used a simplified procedure and attached antibodies directly to the p2368 tags, which were hybridized to the signal segment of block ***1***. To assemble CUs exactly as designed, fluorophore-free p368 tags could be hybridized first to the receptor segments on the Digital DNA, and the antibodies attached to them as described below. Then this construct would be further hybridized with fluorescent TMR-containing p2368 tags that bind to the signal segments, at 37 °C[6] to protect the antibodies.

Assembly of CUs only with antibodies is described here. CUs with oligonucleotides or aptamers synthesized with thiol or amino groups has been performed using the same strategy.

Conjugation of antibodies with cross-linkers and fluorophores. Antibody stocks (4-10 mg/ml) were transferred into buffer #1 (0.1 M NaH_xPO_4, 0.15 M NaCl, 10 mM EDTA, pH 7.2) using a Sephadex G50 spin column (GE Healthcare, Piscataway, NJ). To produce the antibody derivative for cross-linking to bisPNA-hybridized DNA, we added a 4-fold molar excess of SATA cross-linker from a 5 mM stock in DMSO. To produce fluorescently-labeled secondary antibodies, a 10-fold molar excess of Alexa 647 SE was added from a 5 mg/ml stock in DMSO. In both cases, the reaction was allowed to proceed for 1 hour at room temperature. Then the excess cross-linker or fluorescent dye was removed with Sephadex G50 spin column equilibrated with buffer #1 containing 1 mM NaN_3.

Conjugation control experiments required antibody modified with both SATA and Alexa 647. In this case, antibody was transferred into buffer #1 as described above, allowed to react for 30 min with a 4-fold molar excess of SATA, then a 10-fold excess of Alexa 647 SE was added. The reaction was allowed to continue for another 30 minutes, and then the excess cross-linker and dye was removed from the antibody with a Sephadex G50 column as described above.

Assembly of capture units. Linear Digital DNA molecules were excised from the circular plasmids by restriction endonuclease digestion. In a typical reaction, 40 μg of plasmid DNA was incubated with 50 units of NheI and 200 units of XbaI in 1x NEB buffer #2 containing 0.1 mg/ml BSA (NEB). The reaction continued for 1 hour at 37 °C; the completion of the reaction was verified by agarose gel electrophoresis. The enzymes were heat-inactivated at 65 °C for 20 minutes. DNA was precipitated with 2.5 volumes of ethanol and 1/10 volume of 3 M sodium acetate (pH 5.5) and then resuspended in buffer H (10 mM NaH_xPO_4, 1 mM EDTA, pH 8.0).

For hybridization, DNA was combined with bisPNA in buffer H at final concentrations of 32 μg/ml and 2 mM, respectively. This mixture was incubated for 1 hour at 65 °C, placed on ice, mixed with an equal volume of 0.5 mM LC-SPDP in buffer #1, then incubated overnight at room temperature. Excess bisPNA and LC-SPDP were removed by 4 cycles of 10-fold concentration with a Microcon YM30 centrifuge filter unit (Millipore, Billerica MA) and addition of 10 mM NaH_xPO_4 (pH 7.2) buffer with 15 mM NaCl and 10 mM EDTA to the original volume.

Antibodies were conjugated to the DNA by combining 32 μg/ml SPDP-modified DNA with 2 μM SATA-modified antibody in buffer #1. This mixture was incubated at room temperature overnight, then excess antibody was removed from the DNA-antibody conjugate by 4-8 hours dialysis on 0.1 micron filter disks (Millipore, catalog #VCWP 025 00) against 10 mM NaH_xPO_4 (pH 7.2) buffer with 50 mM NaCl and 1 mM EDTA, changing disk and buffer once.

Quality Control. During their preparation, the quality of CUs was controlled at different steps. We used both pulsed-field gel electrophoresis (data not shown) and single-molecule measurements (Figure S5). With the former technique we monitored integrity of the CU DNA; with the latter technique we monitored completeness of the removal of excess fluorescent material to maintain low background. The starting material – linearized Digital DNA – had no significant signal in either the green or red channels (Figure S5a). Hybridization of fluorescent p2368 bisPNA tags to the block ***1*** positions on the DNA introduced the identification barcode that could be detected in green channel; the barcode remained after the conversion of amino groups with the SPDP cross-linker and removal of excess bisPNA and SPDP (Figure S5b). Then thiolated antibodies were attached to SPDP-modified DNA (Figure S5c). To evaluate the efficiency of the antibody conjugation step, we used dye-labeled antibodies (Figure S5d).

1.4. Data analysis

Classification of single-molecule traces. In addition to *GeneEngineer* used in this work, we have another software package, *Molecule Classifier*, for analysis of complex mixtures of different Digital DNA molecules. It analyzes every single molecule trace individually and compares it with theoretical templates. Pearson's linear correlation is calculated between each observed green barcode and the expected templates calculated from the DNA sequences of the Digital DNA used in that experiment. The molecule is classified as the template that is most correlated to the observed signal. *Molecule Classifier* also can filter out molecules that have uneven patterns of backbone fluorescence (indicative of stretching anomalies) or those which barcodes did not correlate well with any template. The single molecule traces sorted in the groups with the same templates could be aligned and averaged (Figure S6). This universal software package can be used for analysis of any data set, including mixtures of different DNA molecules. The algorithm of *Molecule Classifier* will be published in detail elsewhere.

Extraction of oriented maps. Even homogeneous samples generate two types of molecule images because half of the molecules travel head-first (say NheI site passes the laser spot first), while the other half travels tail-first (XbaI site first). These traces are mirror images of each other and, if all of them are averaged without sorting, they produce unoriented maps. For asymmetric DNA sequences, the orientation of individual DNA molecules can be determined by either selection of DNA molecules containing a peak unique to one orientation[6] (performed with *GeneEngineer*), or by correlating the barcode to calculated templates for head-first or tail-first sequences (performed with *Molecule Classifier* in Figure S6). Once the molecules have been separated, the averages clearly mirror the expected pattern (100111010100 in Figure S6).

2. Supplementary Tables S1-S3

Table S1. Sequences of primers used in Digital DNA design.

Code	Sequence	Purpose
DD1	5'-CTTATCGATACCGTCGGTACCCAGCTTTTG-3' 5'-CAAAAGCTGGGTACCGACGGTATCGATAAG-3'	Modification of pBluescript II KS+ plasmid to make pUSG1
DD2	5'-GTGAGGAGGGCCAGCATGGCCAAGTTG-3' 5'-CAACTTGGCCATGCTGGCCCTCCTCAC-3'	Removal of the NcoI restriction endonuclease site from the Zeocin resistance gene
DD3	5'-GTGTGTGGATCCTATTTTCTCCTTACGCATCTGTG-3' 5'-TGTGTAGCGGCCGCTACATGTCTAGAATCGTA GCGATCGCTCGATAACTAGTACTGTTGGGCCCTA ACGTGCTAGCAAAAATGAAGTTTTAGCACGTGTC-3'	Addition of BamHI and NotI restriction enzyme sites at the 5' and 3' ends of the Zeocin resistance gene, respectively
DD4	5'-GTGTGTGGGCCCAGTTACCTTCGGAAAAAGAGTTG-3' 5'-GTGAGTACTAGTGGATGAACGAAATAGACAGATCG-3'	To generate a 343 bp dsDNA fragment from pUC19 plasmid for block ***0*** identification sequence
DD5	5'-ATTGGCGCTAGCGAACGGTGGAACCTTCGC TGATAAT-3' 5'-GCCATTGGGCCCAAGCTTCTGAGCGTTTTG TCGTCAACAATCA-3'	To generate a 4,302 bp dsDNA fragment from *Autographa californica* for structural segment I
DD6	5'-GTGTGTACTAGTGTCGCAATAATGTTCATAAGATGC-3' 5'-GTGAGTGCGATCGCGATTTTGATAGATTGTT CGAAATGG-3'.	To generate a 4,842 bp dsDNA fragment from *Autographa californica* for structural segment II
DD7	5'-GTGTGTGTTCTAGAGTCGACCTGCAGGC-3' 5'-GAGTGAGTACTAGTACCAGCATGGATAAAGGCC TAC-3'	To generate a 2,822 bp fragment from pCC1BAC plasmid

Table S2. Sequences of receptor and identification bisPNA-binding segments used in the blocks *0* and *1*.

Code and segment	Sequence
DD8, Block ***1*** Identification segment	SpeI 5'- *GTGAGTA*CTAGTCATGGACGGTGAGC**GAGAAGGA**GTGGGTCTCGCGGATT GCAGCACTGGCCAGATGGTAGTCTACACGACGGAGTC**GAGAAGGA**AGGC AACTAATGGTGAACGATCGCTGAGAGATTAAGCATTGGTAACTGTCA G**GAGAAGGA**ACCAAGTACTCGATTGATTAACTTCATGGTTAATCCGGAAG GATCTAGGTGAA**GAGAAGGA**GATAGGCCTTGACTCATGACCAATCGACG TTCCACTAGCGTCAGACCGTAGAAA**GAGAAGGA**ATTCAAAGTCTTCTTGA GATCCATCTGCTGCTTGCAAACAAACGCTACCAGC**GAGAAGGA**GGTGGT TG*GGCCCACACAC*-3' PspOMI
DD9, Receptor segment	XbaI 5'- *GTGAGTT*CTAGACAGCTGTTCGCC**GAGAAGAA**CCGAAGCCAATGATGAG CACTTCTGCTATGTGCGGTATCCCGTATTGACGC**GAGAAGAA**CGGGCA AGAGCACTCGGTCTACACGACGGTTGAGTACTCAAGTCACAGAGC AT**GAGAAGAA**CATGGCATGACAGGAGCCATAGAGTGATAACACTGCGCT TCTGACAACGAG**AGAAGAA**TCGGAGGACCGAAGGAGCACCGCTTGGA TACTCGCCTTGATCGTTGGGAACC**GAGAAGAA**GCTGAATGAAGCCATA CCAAACGTAGCAGGCAACAACGTTGCGCAAACTAT**GAGAAGAA**TAACT GGGCGAT*CGCACACAC*-3' AsiSI

Underlined are the portions removed during the restriction digestion of the fragments prior to insertion into a block. The corresponding restriction enzymes are marked at the termini of the sequences.

Table S3. Produced Digital DNA constructs with different lengths and barcodes.

Length	Barcode	Length	Barcode	Length	Barcode
4-mers	0111	11-mers	10011101011	12-mers	100111011101
	1111		10011101010		100111011010
5-mers	00111		10011101101		100111011000
	10111		10011101100		100111010010
	01110		10011101001		100111010000
6-mer	100111		10011101000		100111011110
7-mer	1001110		10011101111		100111011100
8-mer	10011101		10011101110		100111010001
9-mers	100111010	12-mers	100111010111		100111011111
	100111011		100111010110	14-mer	10011101101010
10-mers	1001110101		100111010101	16-mer	1001110110101011
	1001110110		100111010100	18-mer	100111011010101111
	1001110100		100111011011	20-mer	10011101101010111111
	1001110111		100111011001		
	1001110100		100111010011		

3. Supplementary Figures S1-S6 with Legends for Supplementary Methods

Figure S1. Digital DNA – structure of the building blocks and assembly schematic. a, Digital DNA is composed of blocks ***0*** and ***1***. The blocks contain receptor, signal, and structural segments. Receptor segments (red) are 328 bp-long and include 6 binding sites for bisPNA tag p368. The signal segment in block ***1*** (green) is 339 bp-long and includes 6 binding sites for bisPNA tag p2368, while the signal segment in block ***0*** (dark blue) is 343 bp-long and includes neither p368 nor p2368 binding sites. Receptor and signal segments are separated by structural segments I and II (light blue), which are 4,302 and 4,842 bp-long, respectively. **b**, Schematic of Digital DNA constructs, which are assembled from combinations of blocks ***0*** and ***1***. Digital DNAs carrying different antibodies carry different corresponding barcodes.

Figure S2. Production of the plasmid pUSG2 for the construction of Digital DNA blocks. Plasmid pUSG1 was obtained from plasmid pBluescript II KS+ (pBS II KS+) by elimination of PspOMI cut site using DD1 primers. Plasmid pUSG2 was obtained from plasmid pUSG1 by insertion of the modified Zeocin-resistance gene ZeoR at the BamHI and NotI cut sites. The modified ZeoR gene was obtained from the plasmid pZErO-1 by elimination of NcoI cut site using DD2 primers (plasmid pZeroNcoID) and addition of BamHI and NotI cut sites at the termini using DD3 primers (PCR amplicon). MCS, multiple cloning site.

Figure S3. Verification of correct assembly of the Digital DNA blocks *0* and *1*. a, Maps of restriction endonucleases' sites in the Digital DNA blocks. Positions of the sites (expressed in base pairs from 5'-terminus) are presented in parentheses at the names of the corresponding restriction enzymes. Note that Zeocin-reistance gene was added to the blocks to simplify the

selection of the cells infected with the construct plasmids. **b** and **c,** Restriction endonuclease digestion patterns of plasmids pUSG3 with block ***1*** and pUSG4 with block ***0***, respectively. Lanes 1 show the size standard (1-kb DNA ladder from NEB). Other lanes show digests with the following enzyme pairs: 2, NheI-PspOMI; 3, SpeI-PspOMI; 4, SpeI-AsiSI; 5, AsiSI-XbaI; 6, AsiSI-NotI; 7, NheI-SpeI; 8, PspOMI-AsiSI; 9, SpeI-XbaI; 10, PspOMI-XbaI; 11, BamHI-NotI; 12, BamHI-NheI. Digested DNA was fractionated by electrophoresis in a 1.0% agarose gel and stained with SYBR Green I.

Figure S4. Assembly of capture units (CU). BisPNA tags were hybridized to Digital DNA, and their amino groups were converted into activated thiol groups using SPDP reagent. The antibodies (Ig) were thiolated using SATA reagent to attach them to the converted tags. To verify conjugation efficiency, the antibodies were also fluorescently labeled with Alexa Fluor 647 succinimidyl ester (Alexa 647). See text for detail.

Figure S5. Different steps of preparation of capture units (CU). a, Linearized Digital DNA; **b**, Digital DNA with hybridized fluorescent p2368 bisPNA tags after their SPDP modification and removal of excess bisPNA and SPDP; **c** and **d**, assembled CUs with silent and fluorescent antibodies attached, respectively. Antibody against botulinum toxin was used. Digital DNA with sequence (100111011011) was used in these measurements. Signal intensity is plotted as a function of the distance from the center of DNA molecule. The numbers of molecules averaged for the traces in panels a, b, c, and d are 2916, 4040, 2109, and 1233, respectively.

Figure S6. Average maps of head-first (blue) and tail-first (brown) single molecule traces extracted from the measured data by *Molecule Classifier* software. The sequence of measured Digital DNA was (100111010100). Signal intensity is plotted as a function of the distance from the center of DNA molecule. Head-first and tail-first images include 1487 and 1592 single molecule traces, respectively. This is the data set used for Figure 3c, main text.

4. Supplementary Notes

References S1-S10 for Supplementary Methods and Supplementary Discussion

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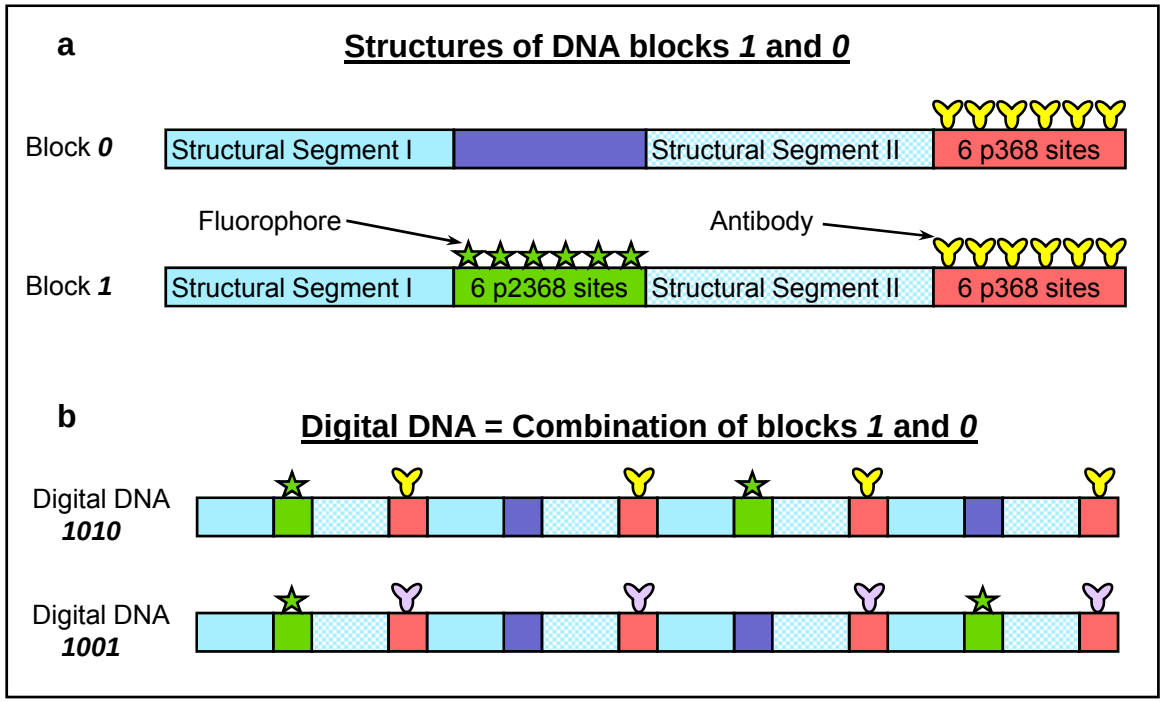

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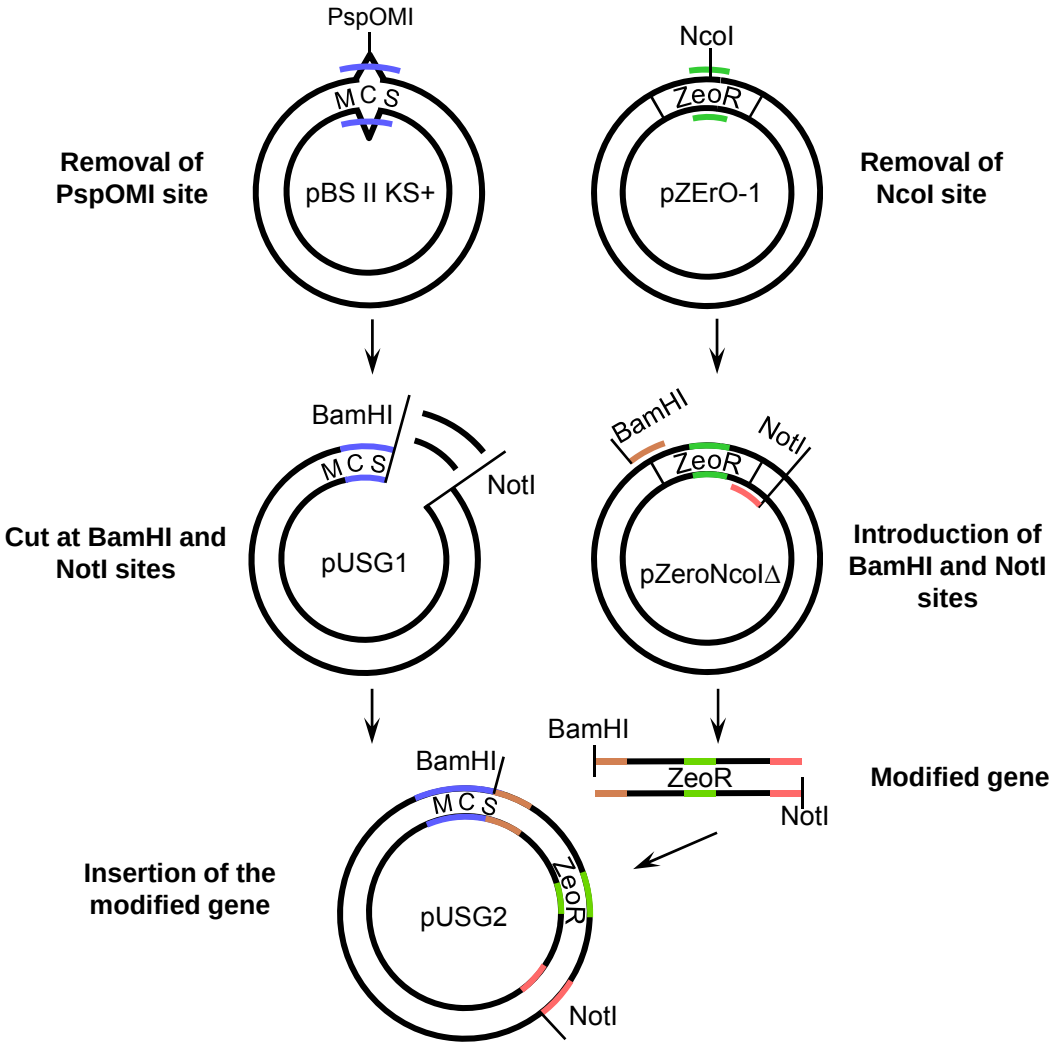

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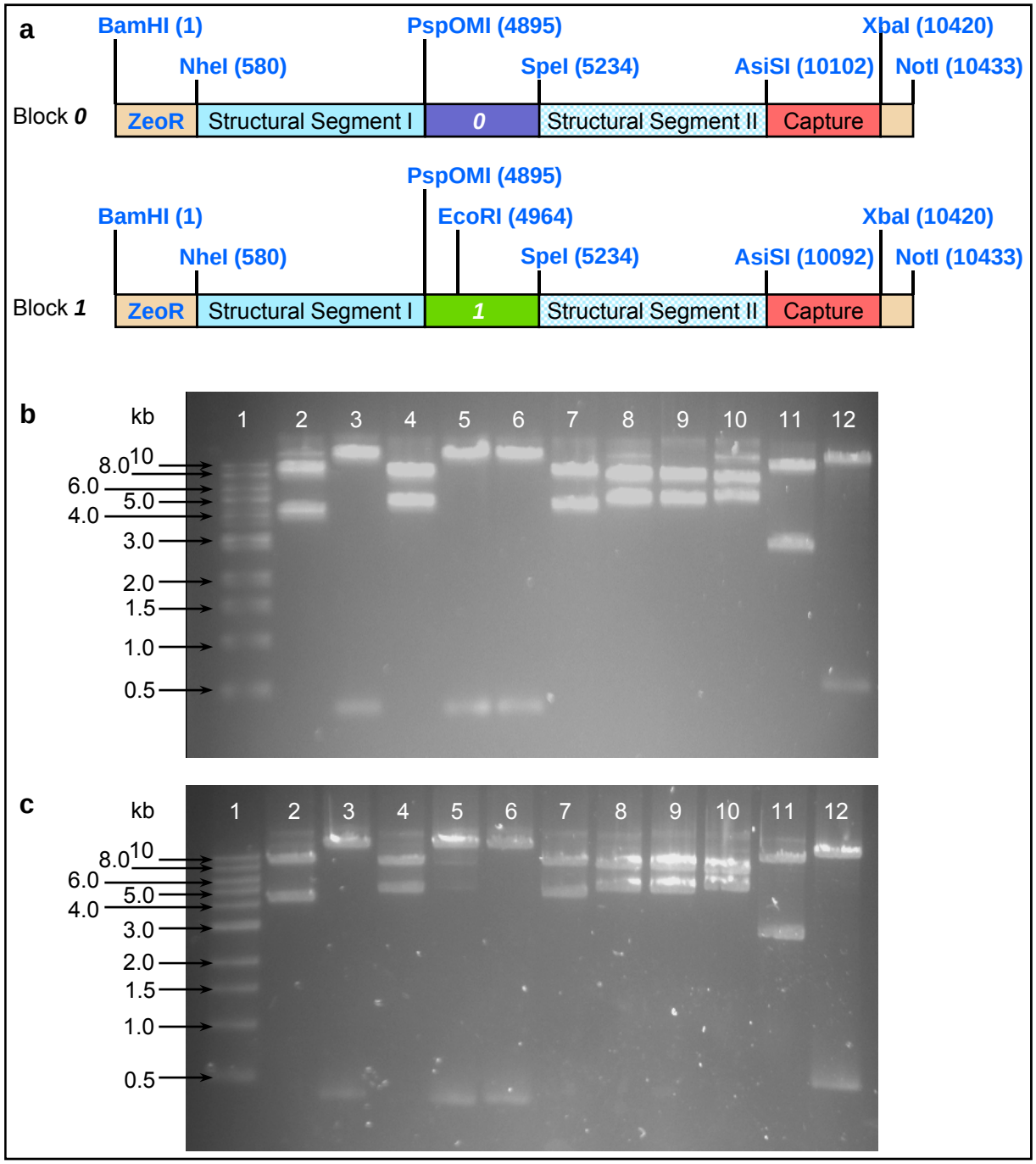
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ZeoR
Structural Segment I
0
Structural Segment II
Capture
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BamHI (1)
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Structural Segment II
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0.5
1 2 3 4 5 6 7 8 9 10 11 12
c
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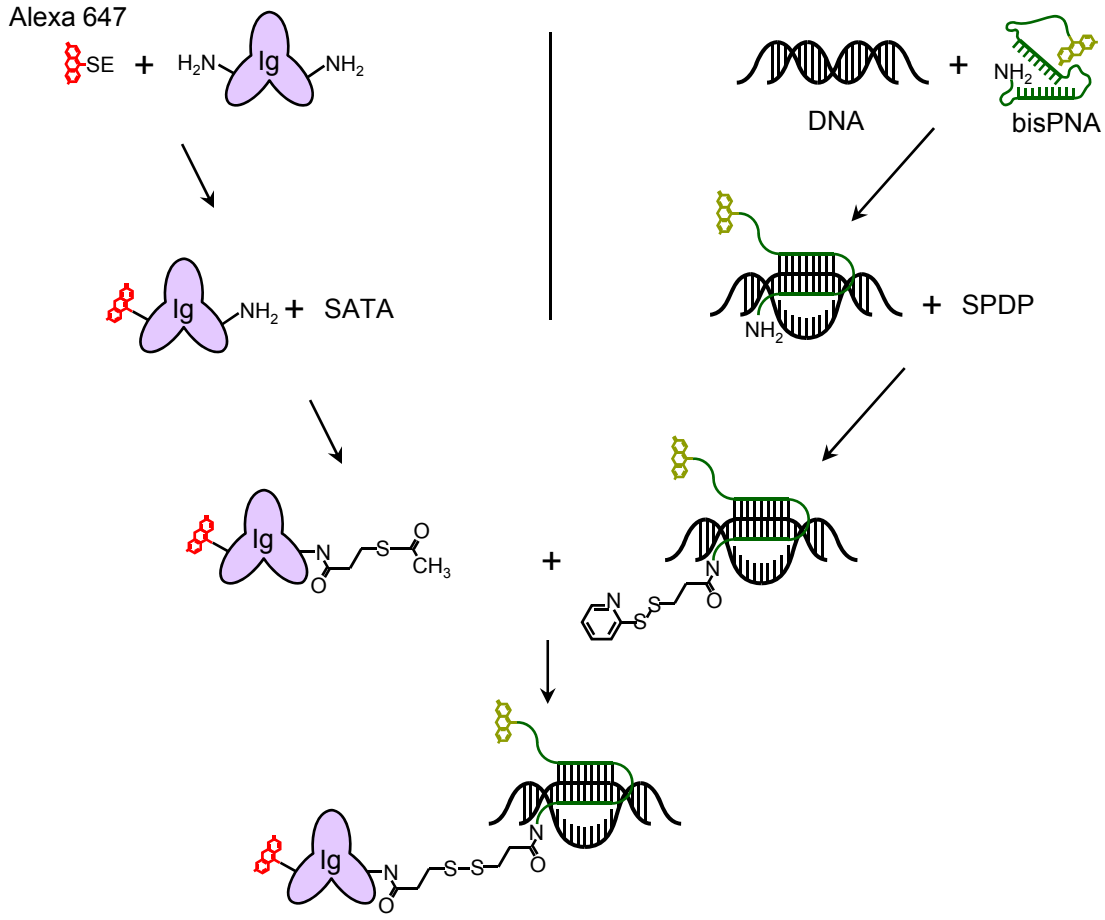
Figure S3



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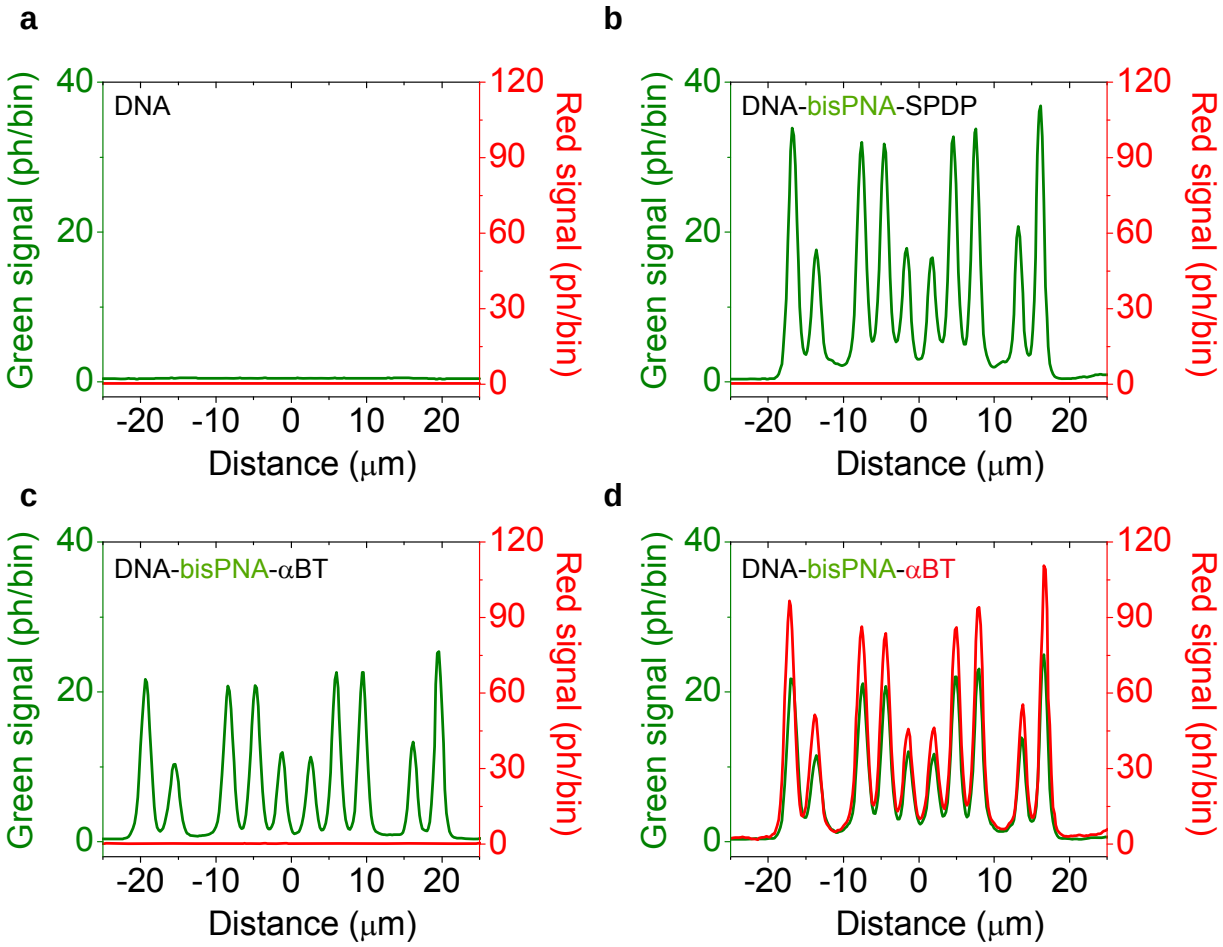

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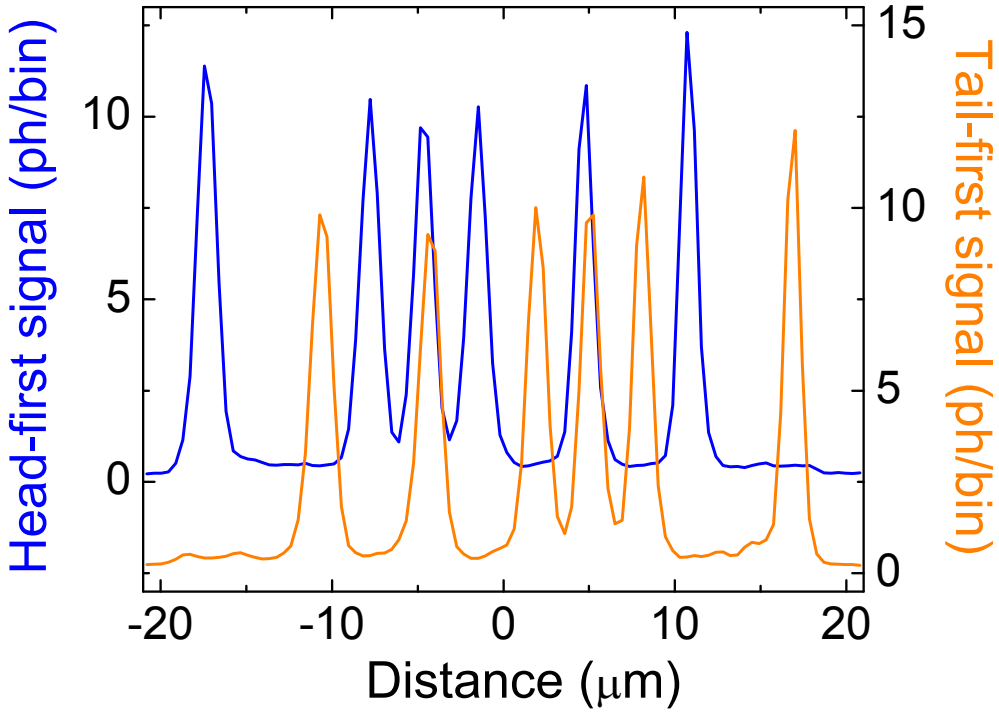

Figure S6