Supplementary Information

Single-Molecule DNA Origami Aptasensors for Real-time Biomarker Detection

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Materials: All oligonucleotides and modified oligonucleotides were purchased from Integrated DNA Technologies (IDT) with the exception of the ATTO655 modified sequence which was purchased from Eurofins. M13mp18 viral DNA was purchased from Tilibit. Neutravidin and DPBS and TAE buffers were purchased from ThermoFisher. BSA-biotin and cortisol were purchased from Merck. $MgCl_2$ and NaCl were purchased from Fisher Scientific.

Time-dependent fluorescence experiments: Experiments were performed on a Cary Eclipse Fluorescence Spectrophotometer. For the ATTO 488 dye and BHQ-1 black quencher, the excitation and emission were set at 503 nm and at 522 nm, respectively. Slits for excitation and emission were set at 5 nm. Data was obtained every 10.2 sec. For cortisol detection in Figure 1B, 90 nM ATTO 488-labelled aptamer in 300 µl of 1x DPBS was introduced and allowed to equilibrate. Then, the quencher (BHQ-1) was added at a final concentration of 100 nM (black arrow), mixed and let equilibrate. Finally, 1 µl of 3 mM cortisol in ethanol was added to 300 µl 1x DPBS solution, which was added and mixed by pipetting. For the orange curve: 1 µl ethanol was added and mixed by pipetting. It is important to note that the duplexed aptamer system is very sensitive to mechanical perturbations and solution changes, i.e. that even simple solution mixing with pipette led to duplex dehybridisation as shown by small changes in intensity in Figure 1B at 6 min, 36 min and 63 min, although these would quickly re-equilibrate. Moreover, excessive additions of ethanol in the control experiment led to a significant intensity change similar to cortisol detection; therefore, we limited the amount of ethanol in solution to a maximum of 0.3% v/v in the total buffer solution.

Experiments in solution at other ionic concentrations: In order to understand the duplexed aptasensor's dynamic nature, we performed a series of experiments in solution at different ionic strengths at 25 °C. We evaluated the effect of ionic strength at intermediate concentrations between 1x DPBS and 1x DPBS/1.5 M NaCl and at 1× TAE/$MgCl_2$ (40 mM Tris, 20 mM acetic acid, 2 mM EDTA, 12.5 mM $MgCl_2$) (see Figure S1). As 1x DPBS buffers contains ~150 mM NaCl, additional NaCl was supplemented to reach final testing concentrations. For all curves: the ATTO 488-labeled aptamer in buffer was introduced and allowed to equilibrate. Then, the quencher was added after 10 min, mixed by pipetting and incubated for more than 1 hour. Finally, cortisol (10 µM) was added (black arrow) and mixed by pipetting, resulting in an aptamer to cortisol ratio of 1:111. Experiments at all tested salt concentrations show formation of the dye/quencher duplex, despite partial duplex dehybridisation before the addition of cortisol, as seen from the slow increase in fluorescence over long periods (see scheme in Figure 1A in main text). At 1× TAE/$MgCl_2$, hybridisation is complete within 5 min followed by a dehybridisation faster than in the case of 1x DPBS/1.5 M NaCl. In 1x DPBS, after adding the quencher, hybridisation takes at least 5 min more than the rest of tested cases but dehybridisation has a similar rate. We concluded that even though detection in 1x DBPS is possible, the dehybridisation rate alters the sensing capabilities of the system.

DNA origami synthesis: The DNA origami is a triangular-shaped origami previously reported,[1] based off a design by Rothemund.[2] It is a single-layer DNA sheet with 120 nm side length. It is synthesised from more than 200 short single strands (staples) and a 7249-nucleotide circular single-stranded DNA (scaffold). During origami preparation some staples were extended to allow hybridisation of functional components including the aptamer, biotin anchors, and fluorophore labels (see list of sequences at end of SI). The aptamer and biotin anchors were designed to extend from opposite faces of the DNA origami based on the number of turns in the DNA relative to each of their positions.[3] The staples (Integrated DNA Technologies, 100 µM in 1 × TAE buffer) and the 12 modified staples for biotin were mixed with a ratio of 5:1 with the scaffold (M13mp18 viral DNA, Tibilit) at a final concentration of 1 × TAE buffer/12.5 mM Mg_2^+ (annealing buffer). The modified A40-staple, surface aptamer strand and ATTO 655-strand, and biotin strand were added in a 10:1, 500:1, 50:1, 500:1 ratio with respect to the scaffold. The mixture was heated to 90 °C for 5 min and annealed from 90 °C to room temperature at the rate of 0.2 °C per min, which was performed in a PCR machine (Hybaid Sprint PCR Thermal Cycler, Thermo Scientific). The DNA origami was then purified by using 100 kDa MWCO spin filters (Amicon® Ultra, Ultracel-100 K, Millipore). The final concentration of origami was 1.2 nM in 100 µl annealing buffer.

Surface-based aptasensor preparation for single-molecule experiments: Glass coverslips were cleaned intensively to remove all florescence impurity. Coverslips were placed in a Teflon rack, rinsed with milliQ water (mQ) and sonicated in piranha

solution (3:1, sulfuric acid and hydrogen peroxide) for 2 hours. After this, coverslips were rinsed with mQ and sonicated in mQ for 10 min. Then, coverslips were sonicated in acetone for 10 min, rinsed with mQ, sonicated in ethanol for 10 min, and finally coverslips were blown dry with Argon. Cloning cylinders were glued to the cleaned microscope coverslips, where each cloning cylinder could be used as a reaction chamber or well. Coverslips were stored at -20°C in a Falcon tube filled with Argon. Before using, coverslips were equilibrated at room temperature. Then, plasma cleaned for 30 min. Coverslips were allowed to cool to room temperature as they were usually hot. Then, wells were passivated with 1 mg/ml BSA-biotin via physisorption for 10 min, rinsed with 1x DPBS, conjugated with 1mg/ml neutravidin for 10 min, rinsed with 1x DPBS and exchanged to 1× TAE buffer/12.5 mM Mg_2^+. Finally, the neutravidin-coated surface was sparsely conjugated with 240 pM biotinylated DNA origami in 1× TAE buffer/12.5 mM Mg_2^+ for 2 min and rinsed with 1× TAE buffer/12.5 mM Mg_2^+. Before the TIRFM experiments, the buffer in the wells was exchanged to 1x DPBS.

Total Internal Reflection Fluorescence (TIRF) Microscopy: Imaging for the single-molecule detection on a surface (Figure 3B) was performed using an LSM710 ELYRA PS.1 in TIRF mode with 642 nm laser excitation at 30% power, LP655 filter and 100 ms camera acquisition time. TIRF time series of the same area were performed at the following three states (See Figure S3 for snapshots of the acquired time series and Figure S4 for representative single-molecule traces): (1) recognition of DNA origamis via the single-molecule fluorescence of ATTO 655 where we confirmed single-molecules by photobleaching events (see representative event in Figure S4C); (2) addition of quencher (200 nM final concentration) in 1x DPBS with 10 min incubation where loss of fluorescence indicated dye/quencher duplex formation – after the 10 minutes this state was imaged for approximately 30 seconds to confirm that the quencher had bound; (3) addition of cortisol biomarker (1 µl; 500 µM - 10 µM final concentration) in 1x DPBS to the chamber without mixing - single step detection was observed 14 sec after cortisol addition. Only 2 individual aptasensors showed no detection after 1 min 47 sec of cortisol addition.

Analysis of TIRF Microscopy: Using ImageJ, we analysed a TIRF microscopy time lapse (see Figure S3 for representative snapshots). Initially, a time lapse was loaded into ImageJ as a Stack. Then, bright spots were visually identified. A 2x2 pixels region was selected on the bright spot and shifted around the spot in such a way that the maximum intensity was obtained using the ImageJ's Plot Z-axis Profile tool. For each bright spot we also obtained a nearby background signal.
In order to determine if a fluorescent spot came from an individual aptasensor, we looked at photobleaching and blinking of the dye, where a single-step decrease in intensity equivalent to the measured background would indicate a single-molecule aptasensor (example seen in figure S4D). This behaviour was distinct from single molecule binding events, where the loss of intensity would not decrease to the background intensity and was instead dimmed.
For single-molecule binding events the observed intensity first decreased in a single step after the quencher was added and hybridised. In general, all the bright spots in the time lapses decrease their intensity after adding the quencher and waiting the 10 minutes incubation. Among those quenched single-molecules, we chose those with signal to noise ratio of at least 1.44 (calculated as $signal^2/noise^2$). When cortisol was detected the signal recovered, again in a single step, to the initial intensity recorded prior to adding the quencher. For a detection cut-off after adding cortisol, we considered a binding event to occur when the intensity recovered to the initial intensity recorded before the quencher was added. We confirmed 1 single-molecule bright spot per 36.4 μm^2 after evaluating 68 bright spots in an area of 1310.5 μm^2.

Atomic Force Microscopy (AFM): AFM characterisation in liquid was carried out on a Bruker Dimension FastScan in Peak Force Tapping mode with Fast Scan D tips from Bruker and was performed on a freshly cleaved mica substrate. Scanning buffer was the origami annealing buffer (1× TAE buffer/12.5 mM Mg_2^+).

Theoretical melting temperature calculations: The melting temperatures were calculated using OligoAnalyzer software from IDT where the DNA was set at 1 µM with varied concentrations of NaCl and $MgCl_2$. Calculated melting temperature for the trigger (highlighted in green in the sections related to DNA sequences for the in-solution aptasensor and single-molecule surface-based aptasensor: 1 µM 150 mM (1x DPBS) 35.7°C, 1 µM 300mM NaCl 38.2°C. Calculated melting temperature for the quencher duplex (highlighted in light blue in the DNA sequences section): 1 µM 150 mM NaCl (1x DPBS) 56.7°C, 1 µM 300 mM NaCl 59.6°C. Other DNA segments such as the "ATTO 655-strand" and the surface aptamer capturing strand (highlighted in purple in the DNA sequences section) were designed using simulation packages such as NUPACK and OligoAnalyzer from IDT for the tested 150mM NaCl in such a way that 100% hybridisation rate was predicted at 25°C.

DNA sequences for the in-solution aptasensor:
in-solution aptamer: /5ATTO488N/ ctctc**gggacgac** GCCCGCATGTTCCATGGATAGTCTTGACTA **gtcgtccc**
quencher-BHQ1: gtcgtcccgagag /3BHQ_1/
blocker: **gggacgac**TAGTCAAGACTATCCATGGAACATGCGGG

DNA sequences for the single-molecule surface-based aptasensor:
Surface aptamer: CACGCTGTTGAATGTCTGAC ctctc**gggacgac** GCCCGCATGTTCCATGGATAGTCTTGACTA **gtcgtccc**
modified A40-staple: CGTGATTAGGTAAGGCCTTA GTCAGACATTCAACAGCGTG TTTTT
TTAGTATCGCCAACGCTCAACAGTCGGCTGTC
ATTO 655-strand: /ATTO655/ TAAGGCCTTACCTAATCACG
quencher-iowa: gtcgtcccgagag /3IAbRQSp/

Modified staples for the biotin anchor and biotin strand:
A52-staple: CCCATCCTCGCCAACATGTAATTTAATAAGGC CTGATGATTGATACCG
A44-staple: TCAATAATAGGGCTTAATTGAGAATCATAATT CTGATGATTGATACCG
A07-staple: AAAGACAACATTTTCGGTCATAGCCAAAATCA CTGATGATTGATACCG
A15-staple: GGAGGGAATTTAGCGTCAGACTGTCCGCCTCC CTGATGATTGATACCG
B52-staple: GTACAACGAGCAACGGCTACAGAGGATACCGA CTGATGATTGATACCG
B44-staple: ATTGTGTCTCAGCAGCGAAAGACACCATCGCC CTGATGATTGATACCG
B07-staple: AACCAGACGTTTAGCTATATTTTCTTCTACTA CTGATGATTGATACCG
B15-staple: GATTAGAGATTAGATACATTTCGCAAATCATA CTGATGATTGATACCG
C52-staple: CGCGCGGGCCTGTGTGAAATTGTTGGCGATTA CTGATGATTGATACCG
C44-staple: CCAGGGTGGCTCGAATTCGTAATCCAGTCACG CTGATGATTGATACCG
C07-staple: GGACATTCACCTCAAATATCAAACACAGTTGA CTGATGATTGATACCG
C15-staple: TGACCTGACAAATGAAAAATCTAAAATATCTT CTGATGATTGATACCG
Biotin strand: /5Biosg/CGGTATCAATCATCAG

Staple strands for the triangular DNA origami:
A01: CGGGGTTTCCTCAAGAGAAGGATTTTGAATTA
A02: AGCGTCATGTCTCTGAATTTACCGACTACCTT
A03: TTCATAATCCCCTTATTAGCGTTTTTCTTACC
A04: ATGGTTTATGTCACAATCAATAGATATTAAAC
A05: TTTGATGATTAAGAGGCTGAGACTTGCTCAGTACCAGGCG
A06: CCGGAACCCAGAATGGAAAGCGCAACATGGCT
A07: AAAGACAACATTTTCGGTCATAGCCAAAATCA
A08: GACGGGAGAATTAACTCGGAATAAGTTTATTTCCAGCGCC
A09: GATAAGTGCCGTCGAGCTGAAACATGAAAGTATACAGGAG
A10: TGTACTGGAAATCCTCATTAAAGCAGAGCCAC
A11: CACCGGAAAGCGCGTTTTCATCGGAAGGGCGA
A12: CATTCAACAAACGCAAAGACACCAGAACACCCTGAACAAA
A13: TTTAACGGTTCGGAACCTATTATTAGGGTTGATATAAGTA
A14: CTCAGAGCATATTCACAAACAAATTAATAAGT
A15: GGAGGGAATTTAGCGTCAGACTGTCCGCCTCC
A16: GTCAGAGGGTAATTGATGGCAACATATAAAAGCGATTGAG
A17: TAGCCCGGAATAGGTGAATGCCCCCTGCCTATGGTCAGTG
A18: CCTTGAGTCAGACGATTGGCCTTGCGCCACCC
A19: TCAGAACCCAGAATCAAGTTTGCCGGTAAATA
A20: TTGACGGAAATACATACATAAAGGGCGCTAATATCAGAGA
A21: CAGAGCCAGGAGGTTGAGGCAGGTAACAGTGCCCG
A22: ATTAAAGGCCGTAATCAGTAGCGAGCCACCCT
A23: GATAACCCACAAGAATGTTAGCAAACGTAGAAAATTATTC
A24: GCCGCCAGCATTGACACCACCCTC
A25: AGAGCCGCACCATCGATAGCAGCATGAATTAT
A26: CACCGTCACCTTATTACGCAGTATTGAGTTAAGCCCAATA
A27: AGCCATTTAAACGTCACCAATGAACACCAGAACCA
A28: ATAAGAGCAAGAAACATGGCATGATTAAGACTCCGACTTG
A29: CCATTAGCAAGGCCGGGGGAATTA
A30: GAGCCAGCGAATACCCAAAAGAACATGAAATAGCAATAGC
A31: TATCTTACCGAAGCCCAAACGCAATAATAACGAAAATCACCAG
A32: CAGAAGGAAACCGAGGTTTTTAAGAAAAGTAAGCAGATAGCCG
A33: CCTTTTTTCATTTAACAATTTCATAGGATTAG
A34: TTTAACCTATCATAGGTCTGAGAGTTCCAGTA
A35: AGTATAAAATATGCGTTATACAAAGCCATCTT
A36: CAAGTACCTCATTCCAAGAACGGGAAATTCAT
A37: AGAGAATAACATAAAAACAGGGAAGCGCATTA
A38: AAAACAAAATTAATTAAATGGAAACAGTACATTAGTGAAT
A39: TTATCAAACCGGCTTAGGTTGGGTAAGCCTGT
A40: TTAGTATCGCCAACGCTCAACAGTCGGCTGTC
A41: TTTCCTTAGCACTCATCGAGAACAATAGCAGCCTTTACAG
A42: AGAGTCAAAAATCAATATATGTGATGAAACAAACATCAAG
A43: ACTAGAAATATATAACTATATGTACGCTGAGA
A44: TCAATAATAGGGCTTAATTGAGAATCATAATT
A45: AACGTCAAAAATGAAAAGCAAGCCGTTTTTATGAAACCAA
A46: GAGCAAAAGAAGATGAGTGAATAACCTTGCTTATAGCTTA
A47: GATTAAGAAATGCTGATGCAAATCAGAATAAA

A48: CACCGGAATCGCCATATTTAACAAAATTTACG
A49: AGCATGTATTTCATCGTAGGAATCAAACGATTTTTTGTTT
A50: ACATAGCGCTGTAAATCGTCGCTATTCATTTCAATTACCT
A51: GTTAAATACAATCGCAAGACAAAGCCTTGAAA
A52: CCCATCCTCGCCAACATGTAATTTAATAAGGC
A53: TCCCAATCCAAATAAGATTACCGCGCCCAATAAATAATAT
A54: TCCCTTAGAATAACGCGAGAAAACTTTTACCGACC
A55: GTGTGATAAGGCAGAGGCATTTTCAGTCCTGA
A56: ACAAGAAAGCAAGCAAATCAGATAACAGCCATATTATTTA
A57: GTTTGAAATTCAAATATATTTTAG
A58: AATAGATAGAGCCAGTAATAAGAGATTTAATG
A59: GCCAGTTACAAAATAATAGAAGGCTTATCCGGTTATCAAC
A60: TTCTGACCTAAAATATAAAGTACCGACTGCAGAAC
A61: GCGCCTGTTATTCTAAGAACGCGATTCCAGAGCCTAATTT
A62: TCAGCTAAAAAAGGTAAAGTAATT
A63: ACGCTAACGAGCGTCTGGCGTTTTAGCGAACCCAACATGT
A64: ACGACAATAAATCCCGACTTGCGGGAGATCCTGAATCTTACCA
A65: TGCTATTTTGCACCCAGCTACAATTTTGTTTTGAAGCCTTAAA
B01: TCATATGTGTAATCGTAAAACTAGTCATTTTC
B02: GTGAGAAAATGTGTAGGTAAAGATACAACTTT
B03: GGCATCAAATTTGGGGCGCGAGCTAGTTAAAG
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B08: GAATACCACATTCAACTTAAGAGGAAGCCCGATCAAAGCG
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B15: GATTAGAGATTAGATACATTTCGCAAATCATA
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B23: AACACTATCATAACCCATCAAAAATCAGGTCTCCTTTTGA
B24: ATGACCCTGTAATACTTCAGAGCA
B25: TAAAGCTATATAACAGTTGATTCCCATTTTTG
B26: CGGATGGCACGAGAATGACCATAATCGTTTACCAGACGAC
B27: TAATTGCTTGGAAGTTTCATTCCAAATCGGTTGTA
B28: GATAAAAACCAAAATATTAAACAGTTCAGAAATTAGAGCT
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B30: TGCTGTAGATCCCCCTCAAATGCTGCGAGAGGCTTTTGCA
B31: AAAGAAGTTTTGCCAGCATAAATATTCATTGACTCAACATGTT
B32: AATACTGCGGAATCGTAGGGGGTAATAGTAAAATGTTTAGACT
B33: AGGGATAGCTCAGAGCCACCACCCCATGTCAA
B34: CAACAGTTTATGGGATTTTGCTAATCAAAAGG
B35: GCCGCTTTGCTGAGGCTTGCAGGGGAAAAGGT
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Link-A2C: TTAATTTCATCTTAGACTTTACAA
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Link-A4C: TCAAGATTAGTGTAGCAATACT
Link-B1A: TGTAGCATTCCTTTTATAAACAGTT
Link-B2A: TTTAATTGTATTTCCACCAGAGCC
Link-B3A: ACTACGAAGGCTTAGCACCATTA
Link-B4A: ATAAGGCTTGCAACAAAGTTAC
Link-C1B: GTGGGAACAAATTTCTATTTTTGAG
Link-C2B: CGGTGCGGGCCTTCCAAAAACATT
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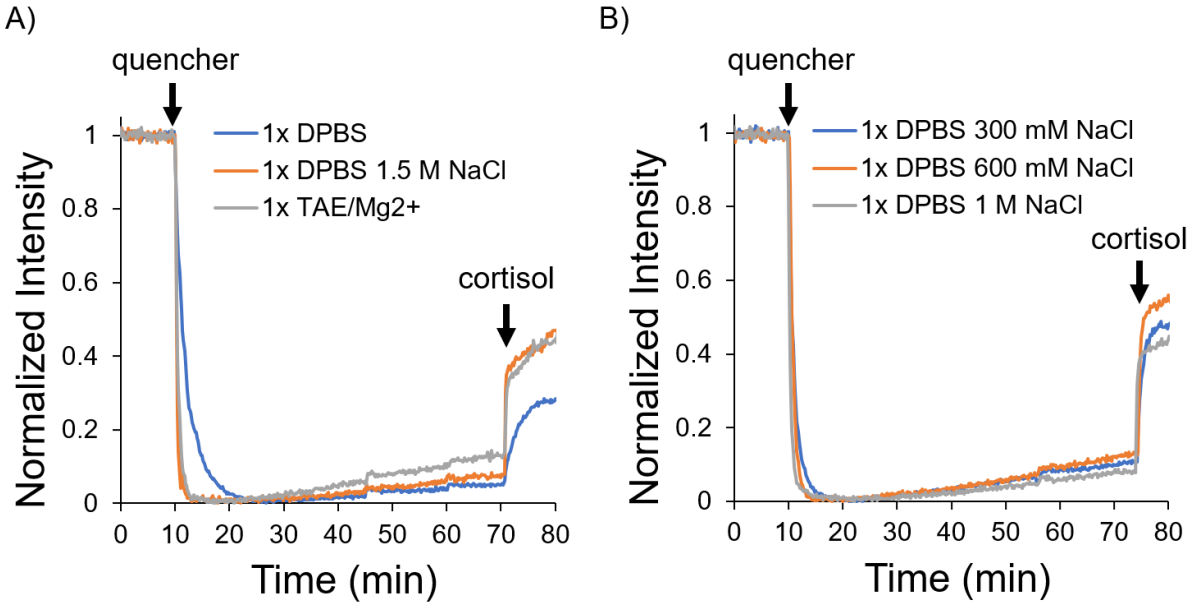

Figure S1. Experiments in solution at different ionic concentration conditions

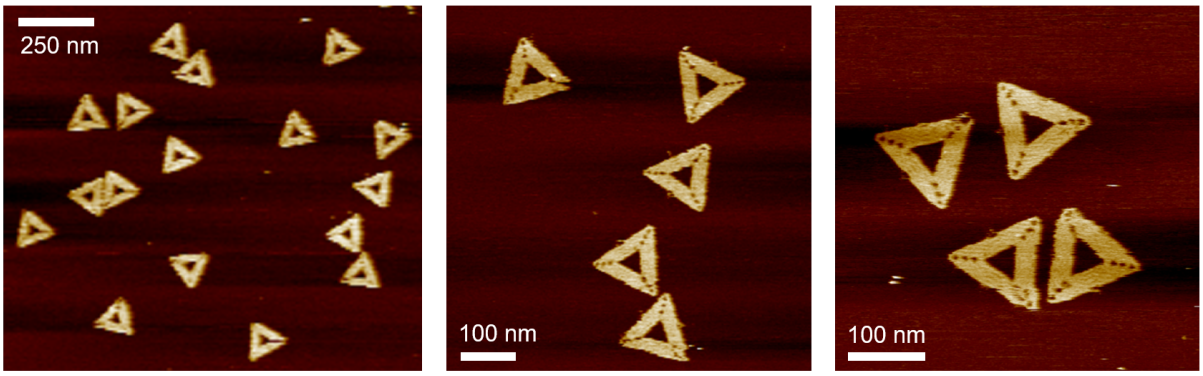

Figure S2. Representative AFM images in liquid of the triangular DNA origami

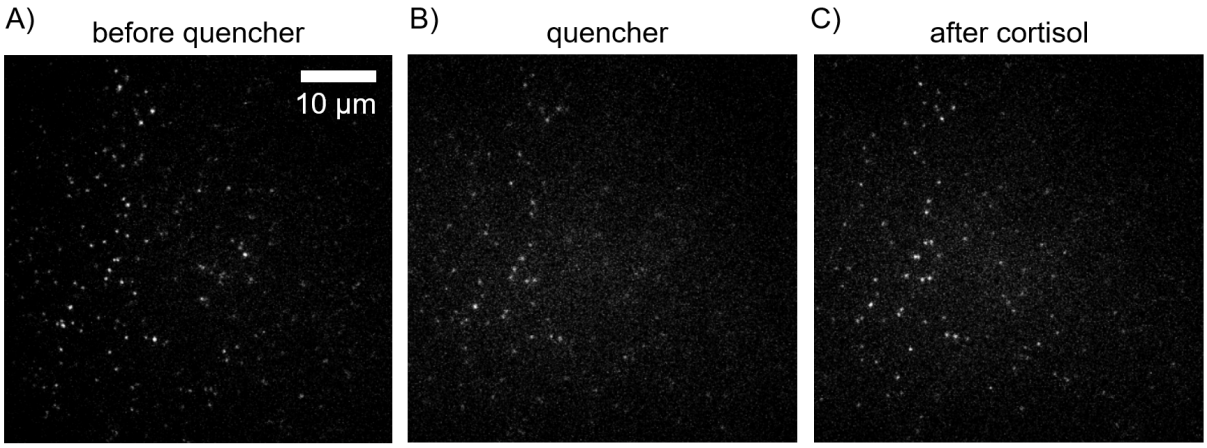

Figure S3. TIRF snapshots of the same area, where the bright spots indicate the presence of DNA origami with aptasensor. These are imaged in three different stages: A. “before detection”, where TIRF is used to locate the DNA origami with the aptasensor; B. “quencher”, where the origami is imaged 10 minutes after adding 200 nM quencher; and C. “after cortisol”, where 10 µM cortisol is added and the DNA origami is imaged approximately 14 seconds later.

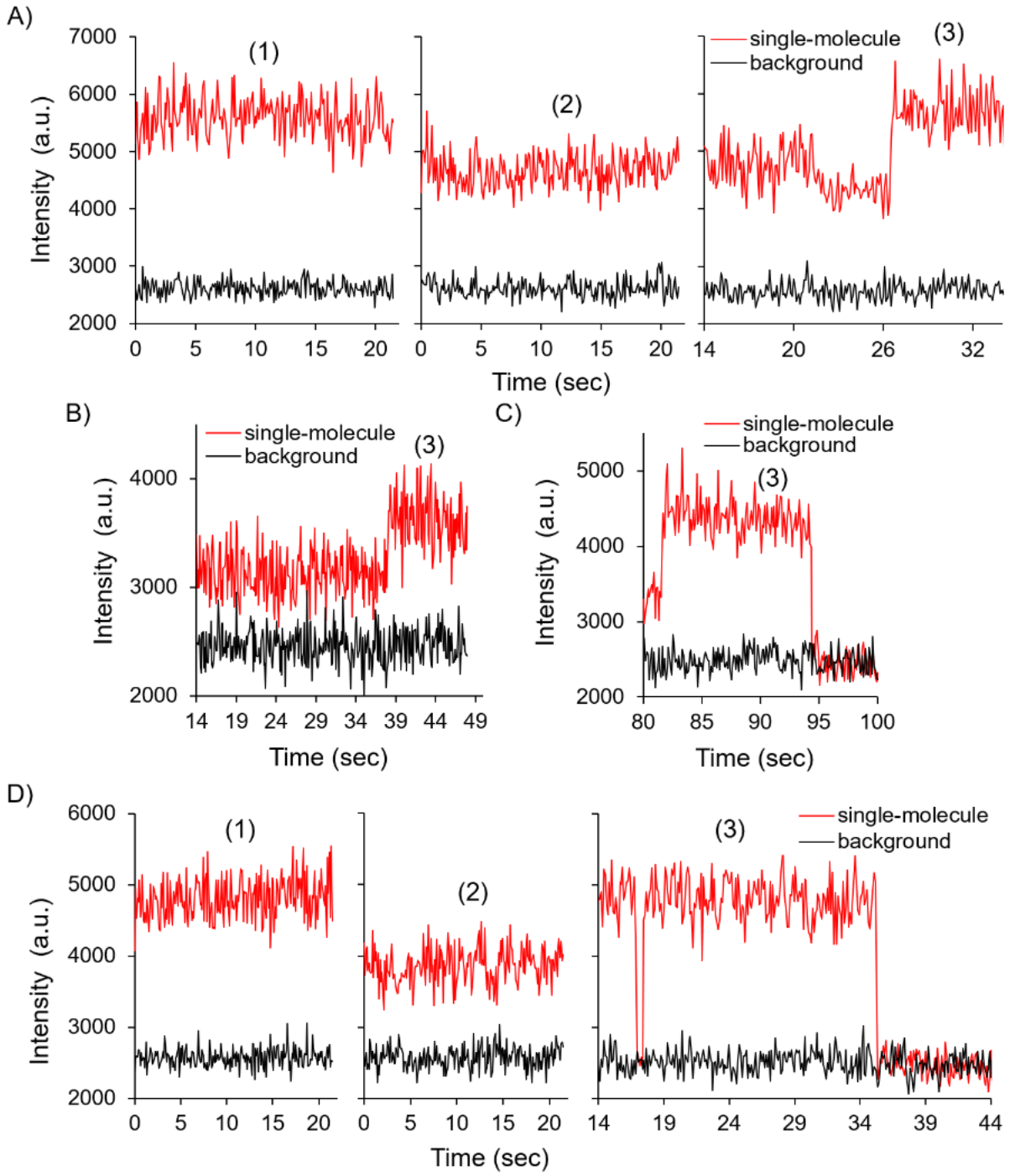

Figure S4. Representative single-molecule traces. A) Representative single-molecule detection event for 10 µM cortisol by TIRFM at three different states: (1) before adding the quencher, (2) after adding excess amount of quencher in solution, and (3) after addition of 10 µM cortisol. The states 1, 2 and 3 correspond to the states in the scheme in Figure 3C in the main text. Detection was achieved 26.5 sec after cortisol addition. B) Detection of cortisol after 37.9 sec after cortisol addition. C) Detection of cortisol after 81.8 sec after cortisol addition; it also shows a single step photobleaching event after 94.4 sec. D) Detection of cortisol within 14 sec after cortisol addition; it also shows a single step photobleaching event after 35 sec. Red traces indicate the single-molecule aptasensor, while black traces indicate the reference background. Camera acquisition time was set at 100 ms.

References

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