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Single-molecule DNA biosensors for protein and ligand detection

Konstantinos Lymperopoulos[1,2], Robert Crawford[1], Joseph P. Torella[1], Mike Heilemann[1,2], Ling Chin Hwang[1], Seamus J. Holden[1] and Achillefs N. Kapanidis[1]

[1]Department of Physics and IRC in Bionanotechnology, Clarendon Laboratory, University of Oxford, Parks Road, Oxford, OX1 3PU, UK;

[2]Current address: BioQuant Institute, Cellnetworks Cluster; Ruprecht-Karls Universität Heidelberg, Heidelberg, D-69120, Germany

[3]Current address: Applied Laser Physics and Laser Spectroscopy and Bielefeld Institute for Biophysics and Nanoscience (BINAS), University of Bielefeld, Universitätsstr. 25, 66315 Bielefeld, Germany

FRET-based multiplexing. The concept of using 3-colour ALEX (Fig. S4c-d) for multiplexing is on an H2 half-site labelled with two fluorophores spaced in a way that results in distinguishable FRET efficiencies for the different TFs. In our design, "coding" is achieved by using a B→R FRET pair (where the "blue" fluorophore is ATTO488), with a dynamic range dictated by its Förster radius R_o of ≈56Å (see Ref. [1]). Specifically, the CAP-coding half-site is designed to yield low B→R FRET (19 bp separation, ≈7 nm B-R distance, $E \approx 0.2$) whereas the lacR-coding half-site should yield high B→R FRET for lacR (9 bp separation, ≈4 nm B-R distance, $E \approx 0.9$). The results show that in the presence of all 4 half-sites and CAP (Fig. S3d, top right), we obtain a BGR species with low B→R FRET efficiency ($E^*_{B\rightarrow R} \approx 0.3$), as expected from the large B-R separation within the $H2^{B\rightarrow R}_{CAP}$ half-site. In contrast, in the presence of all 4 half-sites and lacR (Fig. S3d, middle right), we obtain a BGR species with a high B→R FRET efficiency ($E^*_{B\rightarrow R} \approx 0.55$), as expected from the close B-R proximity within the $H2^{B\rightarrow R}_{lacR}$ half-site. (We note that the ALEX assay measures apparent FRET efficiencies, E^*; considering cross-talks and instrumental factors [2] should account for the difference observed between the uncorrected and accurate FRET efficiencies.) As with stoichiometry-based multiplexing, use of an equimolar CAP and lacR protein mixture (Fig. S3d, bottom right) results in a FRET distribution that reflects the sum of the individual distributions. Improved assay sensitivity should result in narrower distributions that discriminate better between different FRET-codes; this should be possible using a TIRF-based format.

Materials and Methods

DNA. Oligodeoxyribonucleotides were prepared by automated synthesis (IBA GmbH, Germany), labeled with fluorophores and hybridized to form half-sites.

Labeling at the 5'-end of DNA fragments was performed by labeling a 5'-amino-C6-modifying group; labeling at internal positions was performed by labeling amino-C6-dT residues or amino-C2-dT residues. DNAs were labeled using *N*-hydroxy-succinimidyl

esters of carboxytetramethylrhodamine (TMR, Invitrogen, Carlsbad, USA); ATTO647N or ATTO488 (ATTO-TEC GmbH, Siegen, Germany); or Cy3B (GE Healthcare, Uppsala, Sweden) using manufacturer's instructions, and HPLC-purified. Half-sites and dsDNA fragments were formed by hybridization of labeled DNA oligos with their complementary unlabelled strand at a final concentration of 1 μM in a 40 μl of annealing buffer (20 mM Tris-Cl pH 8.0, 1 mM EDTA and 500 mM NaCl) after heating for 4 min at 90°C and cooling to 25°C at a rate of 1°C/30 s.

DNA sequences. For all DNAs, **X**: amino-modifier C6 linker; **Y**: amino-modifier C2-dT

DNA naming convention:
5'-A—B-3'
3'-D—C-5'

DNAs for lacR-specific biosensor

```
LacR A          5′-XTGGTGTGTGGAATTGTGA-3′
LacR B          5′-GCGYATAACAATTTCACACAGG-3′
LacR C          5′-XCCTGTGTGAAATTGTT-3′
LacR D          5′-AYACGCTCACAATTCCACACACCA-3′
```

DNAs for CAP-specific biosensor

```
CAP A           5′-XAACGCAATAAATGTGA-3′
CAP B           5′-AGYAGATCACATTTTAGGCACCA-3′
CAP C           5′-XTGGTGCCTAAAATGTGA-3′
CAP D           5′-TCYACTTCACATTTATTGCGTT-3′
```

Bridge DNAs for anisotropy measurements:

```
FL-CAP/LacR-T
5′-AGTAGATCACATTTTAGGCACCATTAGGCACCCCAGGCTTTACAT
TTATGCTTCCGGCTCGTATGTTGTGTGGAATTGTGA-3′

FL-CAP/LacR-B
5′-TTACGCTCACAATTCCACACAACATACGAGCCGGAAGCATAAATG
TAAAGCCGTGGGTGCCTAATGGTGCCTAAAATGTGA-3′
```

DNAs for 3-color ALEX (FRET-based coding)

```
Long LacR B     5′-GCGTATAACAATTTCACACAGGTGAYATGTTGTGTA-3′
Long LacR C     5′-XTACACAACATATCACCTGTGTGAAATTGTT-3′
Long CAP B:
5′-AGTAGATCACATTTTAGGCACCAYAACTGGATCCCTGCATCCTA-3′
Long CAP C
5′-XTAGGATGCAGGGATCCAGTTATGGTGCCTAAAATGTGA-3′
```

DNAs for TIRF studies

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Biotinylated LacR-D  5′-ATACGCTCACAATTCCACACACCA-Biotin-3′
Biotinylated CAP-D   5′-TCTACTTCACATTTATTGCGTT-Biotin-3′
```

Proteins. LacR was prepared essentially as described in [3]. The activity of purified lacR was ≈90%, measured using an electrophoretic mobility shift assay (EMSA). CAP-His_6 was prepared essentially as described in [4]. The activity of purified CAP-His_6 was ≈30%, measured using EMSA.

Preparation of complexes. Binding reactions (20 μl) between half-sites and TF (LacR as a tetramer or CAP dimer) were performed using 0.5-100 nM of each half-site and 0.5-400 nM TF at 14°C for 10-60 min in KG7 buffer (20 mM HEPES-NaOH pH 7.0, 100 mM potassium-L-glutamate, 10 mM $MgCl_2$, 1 mM DTT, 100 μg/ml BSA, 1 mM MEA, 5% glycerol). The reaction mixture was then diluted in KG7 (supplemented with 0.2 mM cAMP in cases of CAP detection) and single-molecule measurements were started within 5 min (see also [5]). For detection of 200 pM lacR, complexes were formed in low salt buffer, KG7-LS (20 mM HEPES-NaOH pH 7.0, 10 mM potassium-L-glutamate, 10 mM $MgCl_2$, 1 mM DTT, 100 μg/ml BSA, 1 mM MEA, 5% glycerol).

For IPTG detection, 200 nM lacR was incubated with 80 nM $H1^G$, 20 nM $H2^R$ and 0.2-100 μM IPTG at 14°C for 40 min in KG7-LS. The sample was diluted 200-fold in KG7-LS plus an IPTG concentration identical to the initial incubation, and was examined using ALEX spectroscopy.

For experiments on surfaces, 50 pM of biotinylated $H1^G$ half-site was immobilised on a glass coverslip coated with PEG-Biotin and PEG (1.25:100 ratio) after it had been coated with 0.5 mg/ml neutravidin [6]. Subsequently, 0-5.8 nM lacR (or CAP) and 1 nM of $H2^R$ half-site were incubated for 15-30 min in KG7 at room temperature; KG7 was then replaced by imaging buffer (1% Glucose, 165 U/ml glucose oxidase, 2170 U/ml catalase, and 2 mM Trolox diluted in Phosphate Buffered Saline [PBS]) and sealed with a glass coverslip before taking measurements. Each measurement consisted of 1-s movies with 20 Hz frame rate. For each sample, a total of 30 movies were collected.

For experiments in HeLa cell extracts, 10 nM of $H1^G$ and $H2^R$ were added to a final volume of 10 μl KG7 buffer containing 1 μg HelaScribe Nuclear Extract (Promega) and 10 nM CAP. The mixture was incubated for 10 min at 14°C, diluted to ≈20 pM DNA and was examined using ALEX spectroscopy.

For experiments in bacterial lysates, *Escherichia coli* BL21(DE3) pLysS carrying plasmid pAKCRP was grown in 500 ml fresh LB at 37°C to an OD_{600} of ≈0.4. The culture was induced with 1 mM IPTG. Samples of 50 ml cells were taken at 0 min (before induction), 5, 10, 30, 60, 120, and 180 min after induction. Cells were collected by centrifugation, the supernatant was discarded and the pellets were kept at -20°C before resuspension. Pellets were resuspended in PBS and diluted to an OD of 0.5 in 2 ml KGE (20 mM HEPES-NaOH pH 7.0, 100 mM potassium-L-glutamate, 1 mM DTT, 100 μg/ml BSA, 1 mM MEA, 10 mM EDTA, 5% glycerol and supplemented with Roche Complete Protease Inhibitor). Cells were sonicated twice (30 s each) and cell debris was removed by centrifugation at 30,000 g

for 20 min, and 10% glycerol was added to the supernatant. Protein in the supernatant was quantified using 280-nm absorption using a Nanodrop ND-1000 spectrophotometer. A dilution factor was determined to find the protein concentration required using the 3-hr sample to achieve ≈90% saturation of the DNA bound fraction as measured by ALEX spectroscopy. The incubations were performed using 100 nM DNA half-sites. Each time point was then diluted to the same protein concentration before measurement.

Ensemble fluorescence measurements. Fluorescence intensity measurements were performed on a fluorescence spectrophotometer (PTI, Birmingham, NJ). For fluorescence-anisotropy measurements [4, 7], the spectrophotometer was fitted with L-format Glan-Thomson polarizers. To obtain equilibrium constant K_{D1}, we titrated 100 nM of $H1^G$ with 0-5 μM of bridge DNA, measured fluorescence anisotropy for each concentration, and fitted the concentration dependence of the relative anisotropy change to a rectangular hyperbola (titration was performed in triplicate; data not shown). The K_{D1} was 300±100 nM.

Single-molecule fluorescence spectroscopy: confocal detection of diffusing molecules. Single-molecule fluorescence spectroscopy was performed on a home-built microscopic ALEX setup [2, 8, 9]. Briefly, the microscopy system was equipped with two lasers, a 635-nm diode laser (Cube; Coherent, Santa Clara, CA) and a 532-nm Nd:YAG laser (Samba; Cobolt AB, Solna, Sweden). The individual laser beams were modulated at a frequency of 10 kHz, either directly through a TTL pulse, or by using an acousto-optical modulator (AOM; Isomet, Springfield, VA), and combined in a single-mode optical fibre (Thorlabs, Newton, NJ). The laser beams were then coupled into an inverted microscope (IX71; Olympus, Tokyo, Japan) and reflected into the back aperture of an oil-immersion objective (UPlanSApo 60x, 1.35NA, Olympus) by a dual-band (FF545/650-Di01; Semrock, Rochester, NY) or triple-band (z488/532/633rpc, Chroma, Rockingham, VT) dichroic beamsplitter. The fluorescence signal from diffusing molecules was collected by the same objective and spatially filtered through a 100-μm pinhole aligned in the image plane. The signal was spectrally separated by a dichroic mirror (630DRLP, Omega, Brattleboro, VT) and directed on two avalanche photodiodes serving as single-photon detectors (SPCM-AQR14; Perkin Elmer, Waltham, MA) with additional filters in front (HQ585/F70 and HQ650LP, respectively; Chroma). Photon arrival times were recorded with a PC counting board (PCI-6602; National Instruments, Austin, TX), and data processing was done with custom-made software written in LabVIEW (National Instruments). Single-molecule experiments were performed using typical excitation powers of 20-80 μW (635 nm) and 50-200 μW (532 nm).

Three-color ALEX experiments were performed similarly to a published report [1]. Briefly, we added a 3rd laser (473-nm, Blues; Cobolt AB) for the excitation of the "blue" fluorophore and an additional acousto-optical modulator (AOM; Isomet, Springfield, VA) to produced modulated blue light; we then combined all beams in a single-mode optical fibre and reflected them into the objective using a triple-band (z488/532/633rpc, Chroma, Rockingham, VT) dichroic beamsplitter. The emitted signal was directed to a second

dichroic mirror (560DRLP, Chroma), which reflected the fluorescence of the blue fluorophore onto a third detector equipped with a corresponding bandpass filter (505BP30, Chroma).

The fraction of the observed bound complexes can be maximised by reducing the excitation laser power to minimize photobleaching; by increasing the ratio of G-labelled to R-labelled DNA half-sites from 1:1 to 4:1; and by minimising the dissociation rate of the TF-DNA complex by using KG7-LS for sample observation.

Data analysis for confocal detection. Data analysis was performed with custom-written LabVIEW software, as described [2, 10]. Photon arrival times recorded by the software carry information on the detector channel and the excitation cycle, such that photon streams F_{Xex}^{Yem} were generated, representing the photon count rate of emission of fluorophore *Y*, resulting from excitation of fluorophore *X*. For a detailed description, see Refs [2, 10]. Subsequently, the apparent stoichiometry *S* of a green-red fluorophore pair was calculated according to $S = \left(F_{Gex}^{Rem} + F_{Gex}^{Gem}\right) / \left(F_{Gex}^{Rem} + F_{Gex}^{Gem} + F_{Rex}^{Rem}\right)$, as well as the energy-transfer-efficiency parameter *E**, $E^* = F_{Gex}^{Rem} / \left(F_{Gex}^{Rem} + F_{Gex}^{Gem}\right)$. Two-dimensional *E**-*S* histograms were plotted and used to identify populations of different molecular species that were distinguished by *E** and *S*. Analysis of the 3-color ALEX data was performed as in Ref [1].

Single-molecule fluorescence spectroscopy: total internal reflection fluorescence microscopy for detection of immobilised molecules. The ALEX setup was similar to one described earlier [11]. Briefly, the microscopy system was equipped with two lasers, a 635-nm diode laser (Cube; Coherent) and a 532-nm Nd:YAG laser (Samba; Cobolt). The individual laser beams were modulated at a frequency of 10 kHz, either directly through a TTL pulse, or by using an acousto-optical modulator (AOM; Isomet), and combined in a single-mode optical fiber (Thorlabs, Newton, NJ). The laser beams were directed through an oil-immersion objective (Olympus, 100x, N.A 1.4) at an angle larger than the critical angle and the generated evanescent wave excited fluorescent molecules within ≈100 nm from the surface of a modified coverslip. The protocols for slide modification and immobilization of biotinylated DNA have been described [11, 12]. Fluorescence photons were collected through the objective, filtered (to remove excitation wavelengths) through a long-pass filter (HQ545LP; Chroma) and a notch filter (NF02-633S; Semrock), and the two emission wavelength regions were spectrally separated by a dichroic mirror (630DRLP, Omega, Brattleboro, VT), and directed to the two halves of the chip of an EMCCD camera (iXon+, BI-887, Andor, Belfast, UK). Movies were recorded using manufacturer's software and were further processed using home-built MATLAB software for image alignment and registration.

Kinetic Model. TF binding was modeled using a set of coupled reversible reactions representing the equilibrium between free and transiently-bound half sites (Eq. 1, $K_{D1} = k_{-1} / k_1$):

$$H1^G + H2^R \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} H1^G - H2^R$$

and between the annealed half-sites and CAP (Eq. 2, $K_{D1} = k_{-2} / k_2$):

$$H1^G - H2^R + CAP \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} H1^G - CAP - H2^R$$

As half-sites in the quantitation experiments are supplied at equimolar ratios, we assume Eq. 3. Finally, Eq. 4 and Eq. 5 account for conservation of the R-only half-site and the CAP concentration, respectively:

(1) $$\left[H1^G\right]_{eq}\left[H2^R\right]_{eq} k_1 = \left[H1^G - H2^R\right]_{eq} k_{-1}$$

(2) $$\left[H1^G\right]_{eq}\left[H2^R\right]_{eq} k_1 + \left[H1^G - CAP - H2^R\right]_{eq} k_{-2} = \left[H1^G - H2^R\right]_{eq}\left(k_{-1} + \left[CAP\right]_{eq} k_2\right)$$

(3) $$\left[H1^G\right]_{eq} = \left[H2^R\right]_{eq}$$

(4) $$\left[H1^G\right] = \left[H1^G\right]_{eq} + \left[H1^G - H2^R\right]_{eq} + \left[H1^G - CAP - H2^R\right]_{eq}$$

(5) $$\left[CAP\right] = \left[CAP\right]_{eq} + \left[H1^G - CAP - H2^R\right]_{eq}$$

To relate ALEX data to the model, we determined the bound fraction, F_B, for a given experiment:

(6) $$F_B = \frac{\left[H1^G - CAP - H2^R\right]_{eq}}{\left[H1^G\right]_{eq} + \left[H1^G - H2^R\right]_{eq} + \left[H1^G - CAP - H2^R\right]_{eq}} = \frac{\left[H1^G - CAP - H2^R\right]_{eq}}{\left[H1^G\right]}$$

Together, Eq.1-6 allows us to calculate the total TF concentration as a function of F_B (Eq. 7):

(7) $$[CAP] = \left[H1^G\right] F_B + \frac{F_B K_{D2}\left(2\left[H1^G\right](1-F_B) + K_{D1} + \sqrt{K_{D1}}\sqrt{4\left[H1^G\right](1-F_B) + K_{D1}}\right)}{2\left[H1^G\right](1-F_B)^2}$$

Measurement of F_B. Because reactions must be diluted to ≈50 pM prior to the ALEX assay, we assume that half-sites with K_{D1} > 1 nM dissociate completely (for our CAP experiments, K_{D1} ≈300 nM). The observed R-only bursts, N_A, are therefore proportional to $\left[H1^R\right]_{eq} + \left[H1^G - H2^R\right]_{eq}$, while the coincident bursts, N_C, are proportional to $\left[H1^G - CAP - H2^R\right]_{eq}$. This also requires that $H1^G$-CAP-$H2^R$ does not appreciably dissociate during the experiment, which is reasonable under our conditions: given the dissociation kinetics for the CAP-DNA complex ($k_{off} \approx 10^{-4}$ s^{-1}; see Ref. [8]), F_B will

decrease by <7% due to dissociation of the $H1^G$-CAP-$H2^R$ complex during a 10-minute ALEX experiment.

Although the bound fraction in an ideal experiment would be $F_B = N_C/(N_C + N_A)$, in practice, our equation for F_B is:

(8) $$F_B = N_C/(N_C + N_A - c)$$

Parameter c is a constant describing the average number of "spurious" acceptor-only bursts per experiment. These occur as a result of G-fluorophore photobleaching of $H1^G$-CAP-$H2^R$ complexes, from improperly annealed half-sites, and other species incapable of participating in TF binding. Parameter c can be obtained easily, as it equals N_A when the total TF is in excess of half-site (where all $H1^G$ should, therefore, be bound in $H1^G$-CAP-$H2^R$ complexes).

"Normalized DNA coincidence" as described in the text refers to a double Gaussian fit to the one-dimensional S data, normalized to the saturation point (i.e., accounting for the spurious R-only bursts).

FIGURE S1

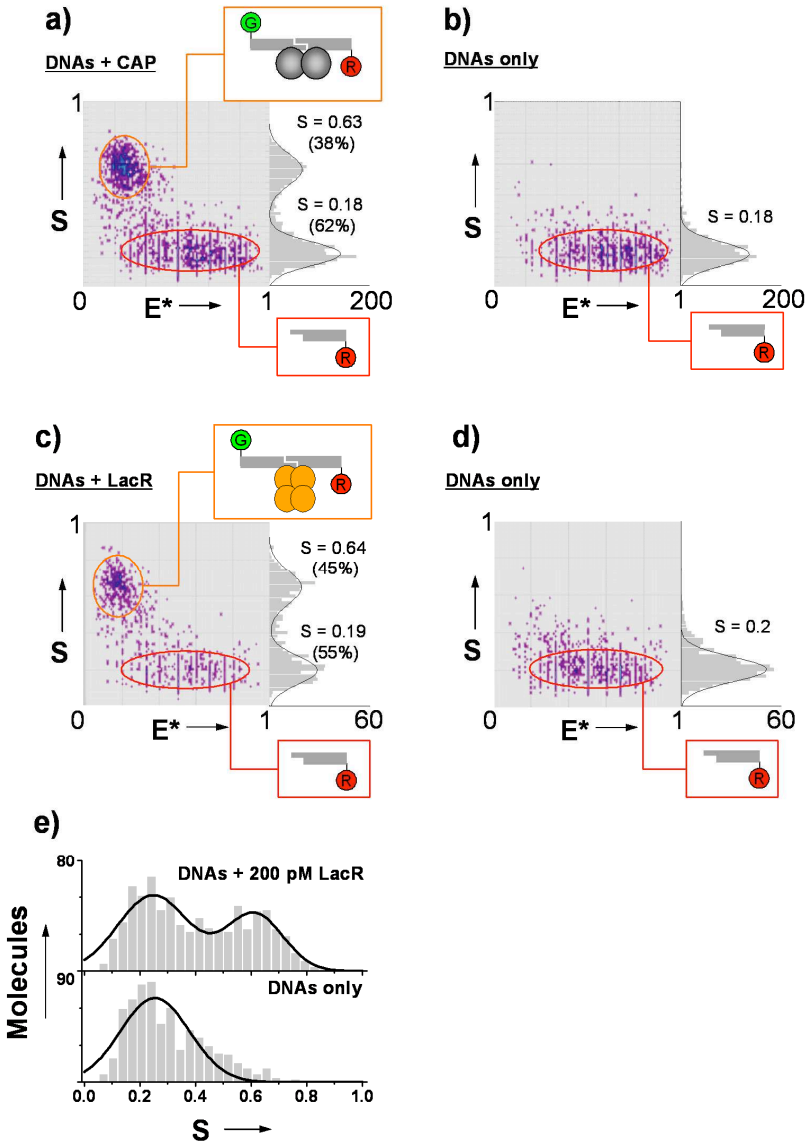

Figure S1. Application of the biosensing assay to the detection of transcriptional factors CAP and lacR. (a) Incubation of 200 nM CAP with 100 nM of each CAP half-site gives rise to a species with $S \approx 0.63$, corresponding to the complex of DNA half-sites with CAP (in orange rectangle); the second species is the free R-only half-site. (b) In the absence of CAP, only few counts due to random coincidence are present in the area previously occupied by the complex. (c-d) Similar results to a-b were obtained using 400 nM lacR with 100nM of each lacR half-site. (e) TF detection at sub-nanomolar concentrations without amplification. Incubation of 200 pM lacR with 1.5 nM H1 and 500 pM H2 in low salt buffer (see *Methods*) gives rise to a new population with $S \approx 0.6$ (*top panel*) when compared to the *S* histogram in the absence of lacR (*bottom panel*).

FIGURE S2

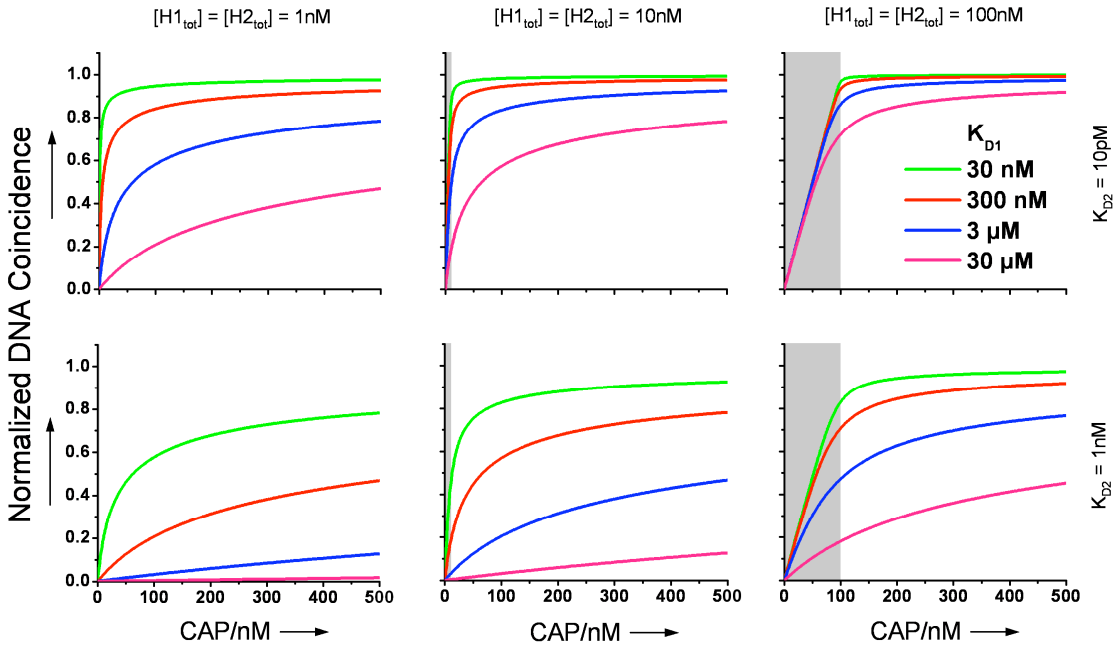

Figure S2. Model predictions for the dependence of the CAP-bound fraction (F_B) of DNA half-sites on CAP concentration, as a function of K_{D1}, K_{D2} and $H1^G$. Generally, lower K_{D1} values, lower K_{D2} values and higher $H1^G$ concentrations increase the bound fraction achieved for a given concentration of CAP. In the special case of low K_{D2} and high $H1^G$ (top right) the bound fraction increases in a nearly linear manner between 0 nM and $H1^G$ = 100 nM CAP. Above 100 nM, however, detection saturates. Increasing K_{D2} (bottom right) or decreasing $H1^G$ (top middle, left) generates more hyperbolic curves, which saturate for significantly higher CAP concentrations.

FIGURE S3

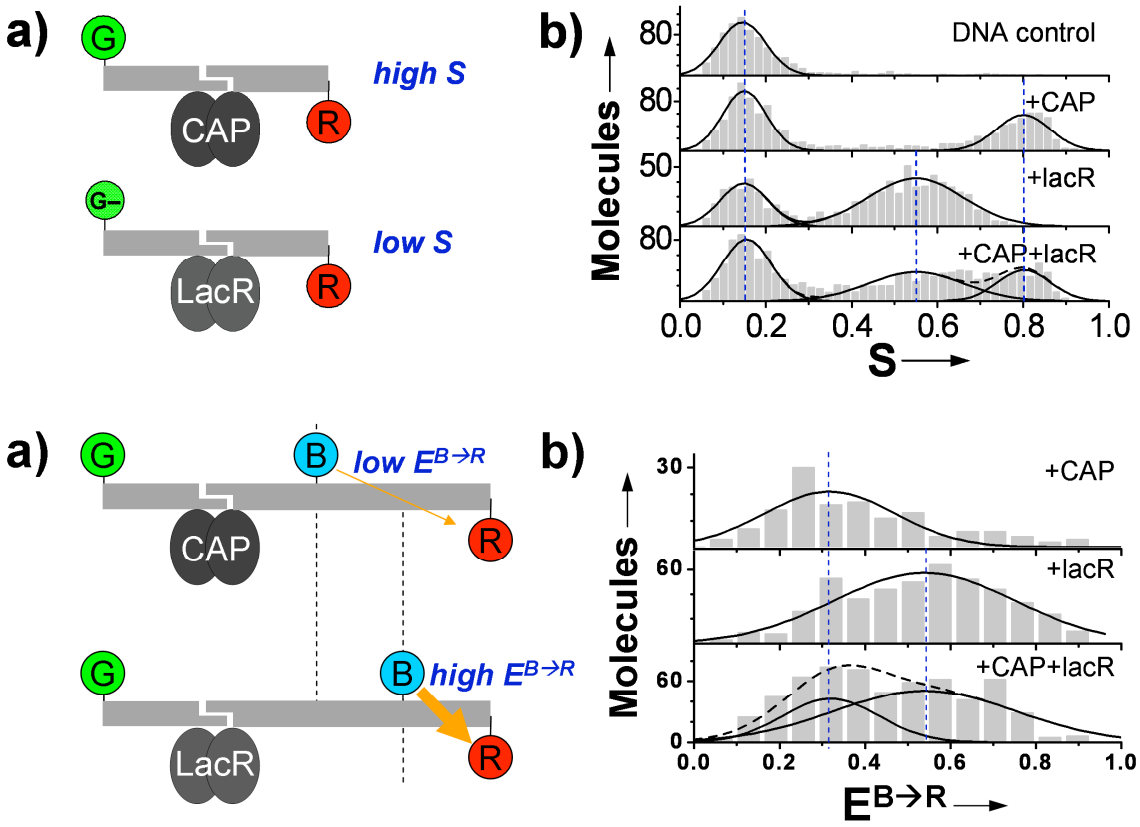

Figure S3. Stoichiometry- and FRET-based multiplexing for simultaneous detection of two TFs without solid supports.

(a) Schematic describing multiplexing using half-sites labelled with fluorophores of different brightness. Due to the definition of *S (see Data analysis for confocal detection, Supporting information)*, a G-R fluorophore pair with a "bright" G fluorophore will yield a higher *S* value than a G-R pair with a "dim" G fluorophore. Accordingly, we prepared CAP-specific half-sites using a bright G fluorophore (Cy3B) and lacR-specific half-sites using a dim G fluorophore (G–; tetramethylrhodamine, TMR).

(b) All panels refer to assays in solutions containing both CAP-specific and lacR-specific DNA half-sites. Top: In the absence of CAP and lacR, only free half-sites are observed (the $S \approx 0.15$ species is due to free R-only half-sites). In the presence of 20 nM CAP (2nd panel), CAP-specific half-sites (10nM) associate to yield a population with $S \approx 0.8$ (due to the use of Cy3B as the bright G fluorophore). In the presence of 20 nM lacR (3rd panel), lacR-DNA half-sites (10nM) associate to yield a population with $S \approx 0.55$ (due to the use of TMR as the dim G fluorophore). In the presence of both TFs (bottom panel), both CAP- and lacR-specific half-sites associate, resulting in a histogram that corresponds to the sum of the individual distributions and enabling simultaneous detection and quantification of both CAP and *lacR*. Dotted vertical lines: mean *S* values for the 3 main species observed.

(c) FRET-based multiplexing: concept. Here, the fully assembled DNA site contains three fluorophores (BGR representing Blue, Green and Red respectively). Coincidence of the G and B-R

DNA fragments is used for TF detection, while individual TFs are identified via their specific B→R FRET signatures (which depend on the B-R fluorophore spacing on DNA).

(d) *Right panel (top)*: In the presence of CAP, association of CAP-specific half-sites gives rise to a B→R population with $E^*\approx 0.3$. The width of the distribution is large due to the limited number of photons available for the calculation of the E^* ratio.

Right panel (middle): In the presence of lacR, association of lacR-specific half-sites gives rise to a B→R population with $E^* \approx 0.55$.

Right panel (bottom): In the presence of both TFs, two overlapping peaks are obtained corresponding to the sum of the individual distributions obtained using CAP and lacR separately.

FIGURE S4

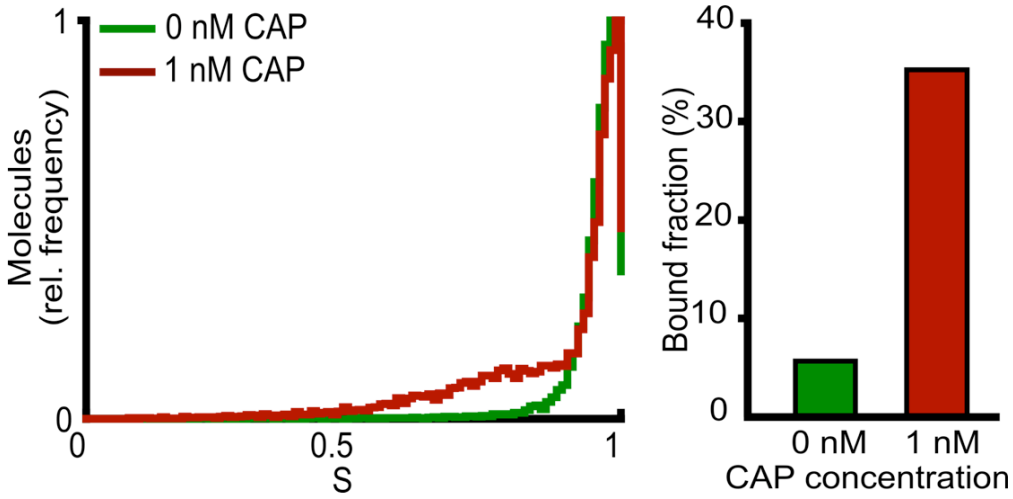

Figure S4. TF detection on the surface with high sensitivity: CAP detection.

Incubation, imaging and analysis were as in Fig. 3c-d, main text. *Left*: Relative molecular frequency vs. stoichiometry in the presence and absence of 1 nM CAP. *Right*: Bound fraction (the fraction of G fluorophores that co-localize with R fluorophores), calculated by summing the molecular frequency in the S histogram, for $S < 0.87$ (co-localized G and R) and $S > 0.87$ (G-only).

REFERENCES

[1] N. K. Lee, A. N. Kapanidis, H. R. Koh, Y. Korlann, S. O. Ho, Y. Kim, N. Gassman, S. K. Kim, S. Weiss, *Biophys J* **2007**, *92*, 303.

[2] N. K. Lee, A. N. Kapanidis, Y. Wang, X. Michalet, J. Mukhopadhyay, R. H. Ebright, S. Weiss, *Biophys J* **2005**, *88*, 2939.

[3] B. Sclavi, S. Woodson, M. Sullivan, M. R. Chance, M. Brenowitz, *J Mol Biol* **1997**, *266*, 144.

[4] A. N. Kapanidis, Y. W. Ebright, R. H. Ebright, *J Am Chem Soc* **2001**, *123*, 12123.

[5] A. N. Kapanidis, E. Margeat, T. A. Laurence, S. Doose, S. O. Ho, J. Mukhopadhyay, E. Kortkhonjia, V. Mekler, R. H. Ebright, S. Weiss, *Mol Cell* **2005**, *20*, 347.

[6] C. Joo, T. Ha, in *Single-molecule Techniques: a laboratory manual* (Eds.: P. R. Selvin, T. Ha), Cold Spring Harbor Laboratory Press, New York, **2008**.

[7] T. Heyduk, Y. Ma, H. Tang, R. H. Ebright, *Methods Enzymol* **1996**, *274*, 492.

[8] A. N. Kapanidis, N. K. Lee, T. A. Laurence, S. Doose, E. Margeat, S. Weiss, *Proc Natl Acad Sci U S A* **2004**, *101*, 8936.

[9] R. P. Goodman, M. Heilemannt, S. Dooset, C. M. Erben, A. N. Kapanidis, A. J. Turberfield, *Nature Nanotechnology* **2008**, *3*, 93.

[10] A. N. Kapanidis, M. Heilemann, E. Margeat, X. Kong, E. Nir, S. Weiss, in *Single-Molecule Techniques: A Laboratory Manual* (Eds.: P. R. Selvin, T. Ha), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, **2008**, pp. 85.

[11] E. Margeat, A. N. Kapanidis, P. Tinnefeld, Y. Wang, J. Mukhopadhyay, R. H. Ebright, S. Weiss, *Biophys J* **2006**, *90*, 1419.

[12] T. Ha, I. Rasnik, W. Cheng, H. P. Babcock, G. H. Gauss, T. M. Lohman, S. Chu, *Nature* **2002**, *419*, 638.