

Supporting Information for:

Direct molecular detection of *SRY* gene from unamplified genomic DNA by metal-enhanced fluorescence and FRET

Danny Brouard,^{1,2} Olivier Ratelle,¹ A. Guillermo Bracamonte,¹ Maryse St-Louis,² and Denis Boudreau^{1,*}

¹*Département de chimie and Centre d'optique, photonique et laser (COPL), Université Laval, Québec (QC), Canada, G1V 0A6*

²*Héma-Québec, 1070, avenue des Sciences-et-de-la-Vie, Québec (QC), Canada, G1V 5C3*

*To whom correspondence should be addressed. E-mail: denis.boudreau@chm.ulaval.ca

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1. Materials

The cationic conjugated polymer (CCP), poly(1H-imidazolium, 1-methyl-3-[2-[(4-methyl-3-thienyl)oxy]ethyl]-, chloride), was provided by the group of Prof. Mario Leclerc and synthesized according to the procedure previously published.¹ Silver nitrate (AgNO₃), sodium citrate tribasic dihydrate (Na-Cit), tetraethoxysilane (TEOS), ammonia (NH₄OH), 3-(aminopropyl)triethoxysilane (APS), succinic anhydride (SA), N-hydroxysuccinimide (NHS), 2-(N-morpholino)ethanesulfonic acid buffer (MES), Triton X-100, ethylenediaminetetraacetic acid (EDTA), sodium acetate (CH₃COONa), ammonium acetate (CH₃COONH₄), sodium chloride (NaCl) and magnesium chloride hexahydrate (MgCl₂·6H₂O) were purchased from Aldrich and used as received. Eosin-5-isothiocyanate (EiTC) was obtained from Marker Gene Technologies. Trizma® base primary buffer and proteinase K were purchased from Invitrogen. All oligonucleotides were purchased from Integrated DNA Technologies in a standard desalting purification grade, except for the Cy5.5-labeled probes and targets, which were in a HPLC purification grade. All DNA sequences used in this work are shown in Table S1. For the detection of a specific sequence in the entire human genome to be quantitatively valid, it is very important to verify that this sequence is unique. The capture probe was tested against the human genome using BLAST calculations and the GenBank database and was found to be unique to the *SRY* mutation. Despite this uniqueness, it should be noted that several sites displaying one or two base differences were found. Deionised water (18 MΩ), anhydrous ethanol and isopropyl alcohol (Commercial Alcohols) and N,N-dimethylformamide anhydrous (DMF) (Aldrich) were used as dispersion media.

2. Synthesis of dye-doped core-shell nanoparticles

2.1. Synthesis of silver nanoparticles

A general description of the Ag nanoparticle synthesis method can be found elsewhere.² Briefly, in a 1-L Erlenmeyer flask, 500 mL of sodium citrate tribasic dihydrate aqueous solution (0.78 mM) was brought to a rolling boil and kept under vigorous stirring. 2 mL of a 0.27 M silver nitrate solution was then added rapidly and the reaction left to boil for one hour. After this time, the solution was allowed to cool to room temperature under vigorous stirring. The solution was completed to 500 mL with deionised water. A final nanoparticle (NP) concentration of $\sim 2 \times 10^{11}$ NPs/mL was determined by ICP-AES.

2.2. Synthesis of core-shell nanoparticles

Typically, several silica spacer shell thicknesses have to be prepared in order to optimize the enhancement of fluorescence. Therefore, 50-mL aliquots of the silver nanoparticle solution were diluted to 200 mL with anhydrous EtOH in 500-mL Erlenmeyer flasks and kept under continuous stirring. 5- to 9-mL aliquots of 9.9 mM tetraethoxysilane (TEOS) in anhydrous EtOH were added followed by 2 mL of NH₄OH, and the samples were left

to stir overnight at room temperature. The resulting Ag@SiO₂ nanoparticles were centrifuged at 11,000 RCF for 30 min and washed 3 times with EtOH before being dispersed in a final volume of 120 mL of anhydrous EtOH. These core-shell NPs are stable for at least 2 months if kept at 4°C.

2.3. Deposition of eosin-doped outer silica layer (Ag@SiO₂@SiO₂+eosin)

To incorporate eosin into the outer silica layer, the dye is first mixed with a silane coupling agent, 3-(aminopropyl)triethoxysilane (APS). In a 10-mL Sigmacote test tube (Aldrich), 7.1 μmol of EiTc was mixed with 21.4 μmol of APS and 1.67 mL of anhydrous EtOH and this solution was sonicated for 1 min then stirred for 3h at room temperature. The Ag@SiO₂/EiTc ratio giving maximum fluorescence and minimal self-quenching is determined by bracketing the proportion of these components, therefore varying amounts of EiTc-APS are reacted with aliquots of the Ag@SiO₂ nanoparticle solution. Typically, in 50-mL conical plastic tubes, 10-mL aliquots of Ag@SiO₂ solution were mixed to 0.24 mL of NH₄OH and stirred, followed by the addition of between 4 and 12 μL of the EiTc-APS solution. After 15 minutes, 300 μL of a 9.9 mM TEOS solution in anhydrous EtOH was added. The reaction was allowed to proceed for 15h at room temperature in the dark. Finally, 0.21 mmol of pure APS was added to the reaction mixture and allowed to react for 3h at room temperature in order to functionalize the surface of the nanoparticles with amine groups (-NH₂). The resulting nanoparticles were centrifuged (9000 RCF for 30 min), washed 3 times with anhydrous EtOH and finally redispersed in 10 mL of anhydrous DMF.

2.4. Surface modification and DNA functionalisation

In 50-mL conical plastic tubes, 10 mL of the Ag@SiO₂@SiO₂+eosin-NH₂ solution was mixed with 1 mL of 0.05 M succinic anhydride in DMF and allowed to react for 4h at room temperature under continuous stirring. The resulting carboxylated nanoparticles were centrifuged at 9000 RCF for 30 min, washed 3 times with sterilized water and finally redispersed in 5 mL of MES buffer (pH 5). 5 mL of this nanoparticle solution was then mixed in a 10-mL sterilized conical plastic tube with 1 nmol of capture probes in MES buffer and allowed to react for 30 min on an orbital stirrer. 50 μL of *N*-hydroxysuccinimide solution prepared in sterilized water (0.05 M) was then added and the reaction was allowed to proceed for 12h at 4°C in the dark on the orbital stirrer. The resulting nanoparticles were centrifuged in a sterilized tube (9000 RCF for 30 min), washed with sterilized water 3 times and finally redispersed in 5 mL of anhydrous EtOH. A final NP concentration of ~2×10¹⁰ NPs/mL was determined by ICP-AES. The surface coverage was estimated using 3'-Cy5.5 and 5'-amine-labeled probe sequences instead of the unlabeled probes and measuring the fluorescence from Cy5.5 in the supernatant before and after the functionalization step. A surface coverage of

$\sim 1.5 \times 10^3$ ssDNA/nanoparticle was determined, which is similar to surface coverage densities reported by other groups for similar nanoparticle surface chemistries.³⁻⁵

<i>Nanobiosensor</i>	
<i>SRY</i> capture probe	5'-AmMC12-CCT-AGT-ACC-CTG-ACA-ATG-TAT-T-3'
<i>SRY</i> complementary target	5'-AAT-ACA-TTG-TCA-GGG-TAC-TAG-G-3'
Non-complementary target	5'-GGC-AAC-GCT-AAT-TAC-AGG-TA-3'
<i>PCR</i>	
<i>SRY</i> sense primer	5'-TGGCGATTAAGTCAAATTCGC-3'
<i>SRY</i> anti-sense primer	5'-CCCCCTAGTACCCTGACAATGTATT-3'
<i>β-actin</i> sense primer	5'-CCTTCCTGGGCATGGAGTCCTG-3'
<i>β-actin</i> anti-sense primer	5'-GGAGCAATGATCTTGATCTTC-3'

Note: AmMC12: Amino modifier C12

Table S1. Base sequences of oligonucleotides used in this work.

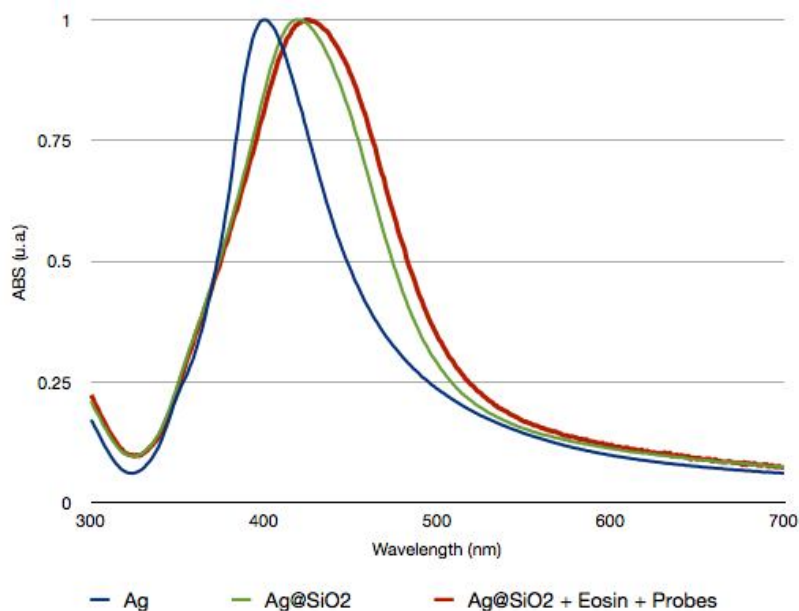


Figure S1. UV-VIS spectra showing the plasmonic red-shift of Ag cores due to the outer silica layers (Ag@SiO₂ and Ag@SiO₂@SiO₂+eosin+DNA nanoparticles).

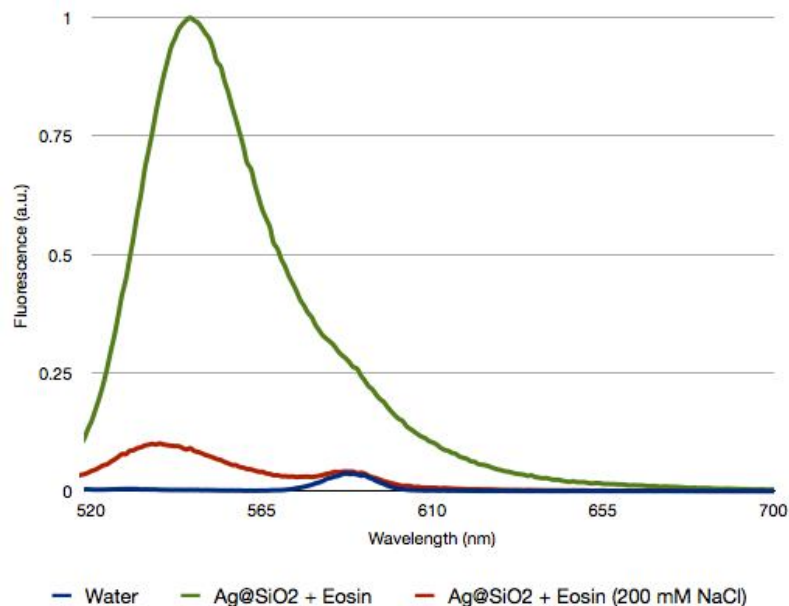


Figure S2. Comparison of NP fluorescence intensity of eosin before and after etching the silver with chloride (i.e., with and without the silver core). A fluorescence enhancement factor (EF) of ~8 is measured, indicative of an adequate silica spacer thickness (the spectral feature ~575 nm is the solvent Raman signal).

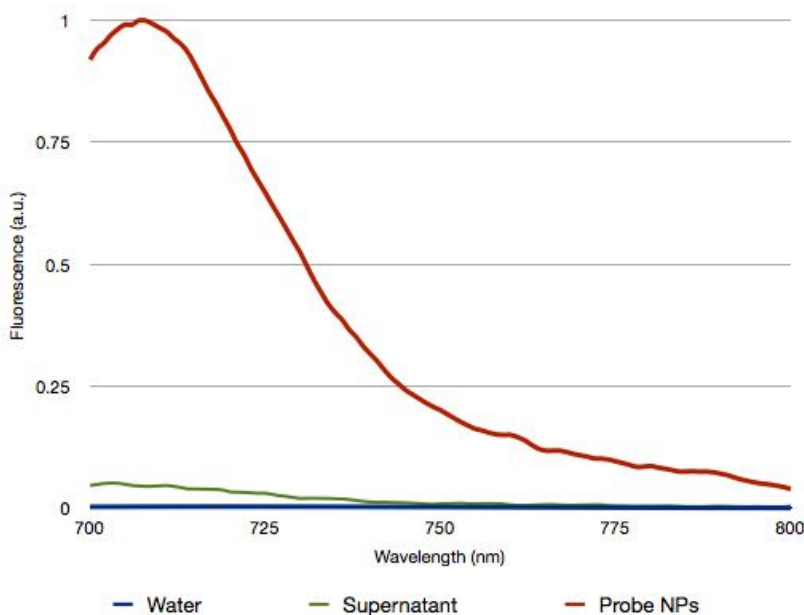


Figure S3. Evaluation of hybridization efficiency using an excess of 5'-Cy5.5-labeled complementary ssDNA target sequence. A fluorescence intensity ratio of 9 was recorded between the NPs and the supernatant after a 10-min hybridization step at 55°C in pure water. No signal was observed when the same experiment was realized without the polymer.

3. Preparation of target-ready NPs

The biosensor (“target-ready nanoparticles”) is prepared by mixing probe-grafted dye-doped core-shell nanoparticles with the CCP. The optimal ratio between probe-grafted NPs and CCP must be such that the total number of negative charges from the DNA probes (one per phosphate group and 1500 ssDNA/NPs) must be *ca.* twice the total number of positive charges from the CCP (one per repeating unit, with $MW_{units} = 262.83 \text{ g mol}^{-1}$, $\bar{x}_n = 40$). As a typical example, 400 μL of a $8.6 \times 10^{-6} \text{ M}$ CCP aqueous solution (*ca.* 2×10^{15} positive charges) is mixed with 1 mL of probe-grafted NP solution (1.8×10^{11} NPs/mL), resulting in a negative:positive charge ratio of 3. The mixture is allowed to react overnight, then washed and resuspended in pure water.

4. Extraction of DNA from blood

4.1. Composition of buffers

Tris-HCl (1 M, 50 mL): 6.05 g of Trizma® base dissolved in 35 mL of water, pH adjusted to desired value with HCl 10 N, completed to a final volume of 50 mL. Lysis buffer: 2.5 mL Tris-HCl (pH 9.5) + 1.23 mL MgCl_2 1 M + 2.5 mL Triton X-100, completed to 1 L. Dropping buffer: 1.5 mL NaCl 5 M + 4.8 mL EDTA 0.5 M, completed to 100 mL. TE 1X: 500 μL Tris-HCl (pH 8.0) + 100 μL EDTA 0.5 M (pH 8.0), completed to 50 mL. TE⁴: 500 μL Tris-HCl (pH 8.0) + 10 μL EDTA 0.5 M (pH 8.0), completed to 50 mL. TBE 1X (Tris-boric acid-EDTA): 10.8 g Trizma® base + 5.5 g boric acid + 0.75 g EDTA dissolved in 1 L of water. All buffers are prepared using sterilized deionised water.

4.2. Extraction protocol

In a normal blood donation procedure, the first 50-70 mL of blood is derived to a small pouch to avoid bacterial contamination from the skin before the blood stream is changed to the main bag. While the blood bag is being filled up (430-500 mL), the blood in this derivation pouch is separated into five 10-ml tubes dedicated to the qualification analyses (ABO/Rh blood grouping and transmissible diseases). Therefore 10 mL is the standard amount of blood available for blood group genotyping. In the present work, ten 10-mL blood samples were collected into 15-mL Falcon tubes from individuals after informed consent. Each tube was centrifuged (15 min, 390 RCF) and DNA was extracted, transferred to new 15-mL Falcon tubes and stored in the dark at 0°C until further use. Genomic DNA was extracted from lymphocyte nuclei according to a well-established method described elsewhere.⁶ Briefly, each sample is first dispersed into 10 mL of lysis buffer to break down the cells. After a washing step with lysis buffer, the nuclei are dispersed in dropping buffer and digested for 12h at 37°C with proteinase K (0.01 g in 1 mL). DNA is then isolated by several phase extractions followed by centrifugation

steps. The final aqueous volume (3 mL) containing DNA is transferred into a 15-mL Falcon tube and mixed with 300 μ L of aqueous sodium acetate (3 M), followed by the addition of 5 mL isopropanol to precipitate the DNA. The latter is transferred to a 15-mL sterilized Corex tube and mixed with 1 mL of TE 1X buffer and kept at 4°C for 12h or until complete dissolution. 0.5 mL of aqueous ammonium acetate (7.5 M) is added and the solution is mixed gently. 3 mL of cold ethanol is added to the solution to precipitate the DNA. Centrifugation at 9720 RCF and 4°C for 10 min yields a clear DNA pellet. This pellet is washed with 3 mL of cold ethanol and dried under vacuum for 10-15 min before being dissolved into 300 μ L of TE⁻⁴ buffer. The final DNA concentrations were measured on a NanoDrop 2000c UV-Vis instrument from Thermo Scientific (Table S2).

Sample ID	Sex	[DNA] (ng/ μ L)	260/280 nm
1	F	484	1.82
2	F	334	1.85
3	F	334	1.85
4	F	408	1.82
5	F	538	1.81
6	M	391	1.85
7	M	382	1.83
8	M	283	1.85
9	M	322	1.86
10	M	355	1.84

Table S2. Sample DNA concentration determined by UV-Vis.

5. Detection of SRY gene in genomic DNA sample

Each DNA sample was fragmented in an ultrasonic bath (Fisher Scientific Model FS20) for 10 minutes at ambient temperature and diluted 10 \times in sterilized water (dilution was necessary to lower the viscosity of the DNA samples which might otherwise affect the sample flow in the capillary of the IFC apparatus). It should be noted that no optimization study of the sonication step was performed, and the size distribution of the fragmented DNA was not measured. Shearing of genomic DNA by sonication is extremely efficient and yields a wide range of fragment sizes in a few minutes;⁷ since shorter fragments are expected to have a higher probability of capture by the nanoparticles, a conservative 10-min duration was used. In future implementations of this method, fragment analysis by gel electrophoresis might prove useful to determine an optimal fragmentation procedure for the nanobiosensor.

Following a heat treatment step to denature the DNA (10 min at 95°C), aliquots of 100 µL were mixed in triplicate with 50 µL of target-ready nanoparticle solution bearing SRY capture probes. Taking into account the concentration of DNA-grafted nanoparticles determined by ICP-AES ($\sim 2 \times 10^{10}$ NPs/mL) and neglecting possible losses during the addition of the polymer, the concentration of nanoparticles brought in contact with the fragmented genomic DNA is approximately $\sim 7 \times 10^9$ NPs/mL. Hybridization was allowed to proceed during 10 minutes at 55°C, then 10-µL aliquots were injected in triplicate in the IFC apparatus and the fluorescence signals were measured.

The independent-samples t-test was used to compare the means between the female (1-5) and male (6-9) samples. Since Levene's test showed that the two groups had unequal variances, the generalized independent t-test procedure was used. A probability value of 0.025 was determined. IBM SPSS Statistics (V22) software was used.

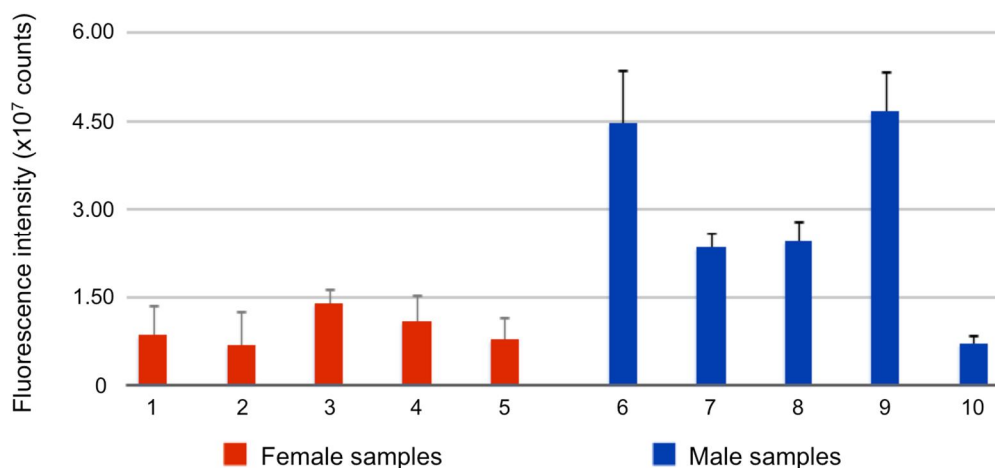


Figure S4. *SRY* genotyping results with the nanobiosensor for 10 unknown genomic DNA samples. DNA extracted from human blood samples was tested using a probe sequence specific to the *SRY* gene, and the fluorescence signals were correlated with PCR analysis. Samples 1-5 were female, and samples 6-10 were male. Overall, 9 out of 10 samples were correctly identified with the nanobiosensor.

6. PCR amplification and M/F differentiation

Each genomic DNA sample was diluted 10X in TE⁴ buffer. 1 µL of this solution was added to 2.5 µL AmpliTaq Gold 10X buffer (Invitrogen), 2.5 µL MgCl₂ 25 mM, 0.5 µL dTNP 10 mM (Invitrogen), 0.25 µL AmpliTaq Gold polymerase (Invitrogen) and 0.5 µL of *SRY* sense and anti-sense primers at 5 µM each (the PCR primer sequences are given in Table S1), and this mixture was diluted to 25 µL with sterilized water. An internal

control (β -actin) was also added, and a negative control was produced using 1 μ L of pure water in place of genomic DNA. Amplification was done in a PTC-200 thermocycler (Bio-Rad Laboratories, Hercules, CA) using the following conditions: 9 min at 95 °C, then 35 cycles of 30 s at 95 °C and 30 s at 60 °C followed by a 30 s extension step at 72 °C. Once amplification was completed, the amplified sample was examined by electrophoresis on an agarose gel (2% agarose + 2% Nusieve 3:1 diluted with TBE 1X buffer, $\Delta V=100V$) using GelRED™ as the fluorescent nucleic acid gel stain.

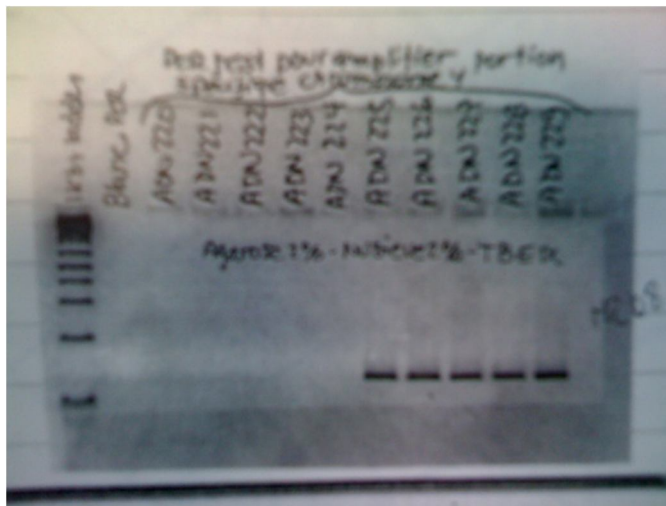


Figure S5: *SRY* genotyping by PCR and gel electrophoresis of the 10 genomic DNA samples. The 5 positive (male) samples are on the right.

7. Spectrophysical characterization of nanoparticles

Transmission electron microphotographs (TEM, Model JEM-1230, JEOL Instruments) were used to characterize the morphology of the core-shell nanoparticles. Steady-state fluorescence measurements were performed using a Fluorolog spectrofluorometer (Model FL322) from Horiba Jobin-Yvon with double monochromators on the excitation and emission channels. The UV-Vis extinction spectra were taken with a Varian Cary 50 spectrophotometer.

8. Imaging flow cytometry

The imaging flow cytometry (IFC) experimental set-up has already been described in a previous report.³ This detection technique uses a square capillary to bring the sample flow perpendicularly across a planar light sheet shaped from a laser beam (Figure S6). Sample aliquots were flowed at 5 μ L/min into the capillary using a syringe pump. The

nanoparticles entering the excitation plane delimited by the light sheet were imaged onto a high sensitivity CCD camera using a high numerical aperture objective (60×, NA=1.2). The scattered light background was eliminated using a long pass band filter and the fluorescence signal from the nanoparticles was measured at 528 ± 20 nm using a band pass filter. A sequence of still images was recorded as the fluorescent particles eluted through the excitation plane.

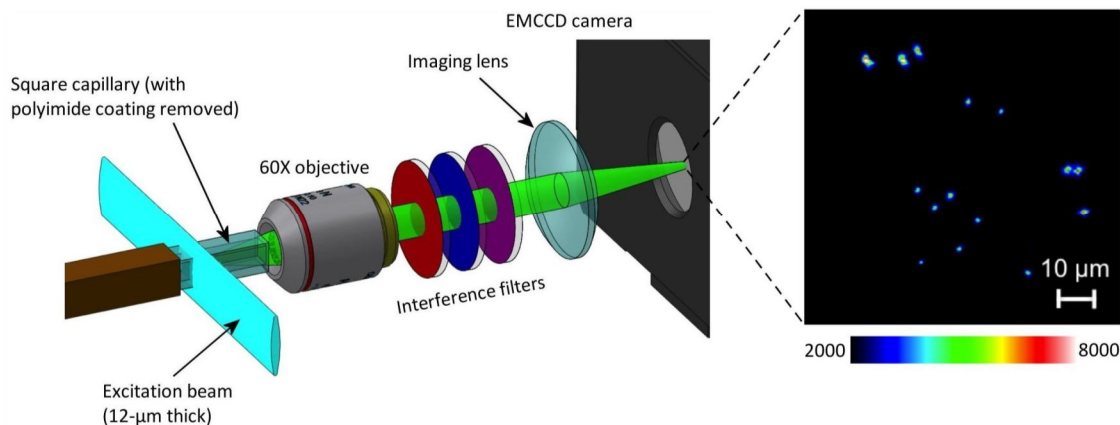


Figure S6. Imaging flow cytometry (IFC) experimental set-up. The nanobiosensor particles flowing in the capillary are intercepted by a laser light sheet and the fluorescent particles are imaged through an optical filter stack onto a CCD camera.³

References

1. H. A. Ho, M. Boissinot, M. G. Bergeron, G. Corbeil, K. Doré, D. Boudreau and M. Leclerc, *Angew. Chem.*, 2002, **114**, 1618.
2. M. Lessard-Viger, M. Rioux, L. Rainville and D. Boudreau, *Nano Lett.*, 2009, **9**, 3066.
3. D. Brouard, M. L. Viger, A. G. Bracamonte and D. Boudreau, *ACS Nano*, 2011, **5**, 1888.
4. S. Liu and M. Han, *Adv. Funct. Mat.*, 2005, **15**, 961.
5. S. Liu, Z. Zhang and M. Han, *Anal. Chem.*, 2005, **77**, 2595.
6. G. I. Bell, J. H. Karam and W. J. Rutter, *Proc. Nat. Acad. Sci.*, 1981, **78**, 5759.
7. M. A. Quail, "DNA: Mechanical Breakage", in: *Encyclopedia of Life Sciences (ELS)*, John Wiley & Sons, Ltd: Chichester.