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# Label-free Nanoplasmonic Sensing of Tumor-Associate Autoantibodies for Early Diagnosis of Colorectal Cancer

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## ABSTRACT

Colorectal cancer is treatable and curable when detected at early stages. However there is a lack of less invasive and more specific screening and diagnosis methods which would facilitate its prompt identification. Blood circulating autoantibodies which are immediately produced by the immune system at tumor appearance have become valuable biomarkers for preclinical diagnosis of cancer. In this work, we present the rapid and label-free detection of colorectal cancer autoantibodies directly in blood serum or plasma using a recently developed nanoplasmonic biosensor. Our nanoplasmonic device offers sensitive and real-time quantification of autoantibodies with excellent selectivity and reproducibility, achieving limits of detection around 1 nM (150-160 ng·mL<sup>-1</sup>). A preliminary evaluation of clinical samples of colorectal cancer patients has shown good correlation with ELISA. These results demonstrate the reliability of the nanobiosensor strategy and pave the way towards the achievement of a sensitive diagnostic tool for early detection of colorectal cancer.

## Keywords

nanoplasmonic biosensor; plasma; serum; colorectal cancer; autoantibodies; clinical diagnosis

## Abbreviations

CRC colorectal cancer

ELISA enzyme-linked immunosorbent assay

LSPR localized surface plasmon resonance

POC point of care

RI refractive index

SAM self-assembled monolayer

TAA tumor-associate antigen

# 1. Introduction

Colorectal cancer (CRC) is a worldwide health problem with an incidence over 1 million annual cases and being a major cause of morbidity and mortality in developed countries [1]. It is the third most common cancer and the fourth most common cause of death around the world. Despite the exact cause for CRC is not known, several risk factors have been established for the disease, including genetic and epigenetic parameters [2]. Familiar history of colon cancer or inflammatory diseases, age, lifestyle and environmental conditions are strongly associated to CRC development. CRC is easily curable when detected early [2], thereby prevention and regular screening play crucial roles in the fight against this cancer. However, CRC diagnosis is particularly challenging. The most reliable diagnosis technique is *via* sampling of colon biopsies suspected of possible tumor development, which is typically done during colonoscopy or sigmoidoscopy for the distal colon and rectum [3]. These procedures are highly invasive and present important limitations in terms of costs, available resources and low compliance. On the other hand, established noninvasive tests such as the guaiac-based fecal occult blood test (gFOBT) suffer from low specificity leading to inaccurate diagnosis results [4]. There is an evident need for novel screening tools, ideally, analytical techniques based on blood analysis, which permit the early and reliable identification and diagnosis of CRC. Development of blood biomarker assays that could indicate that a cancerous process is triggered would represent a great benefit. However, although a few serum proteins have been described as biomarkers in CRC (carcinoembryonic antigen (CEA), CA19.9 or CA125), none of them are recommended for early clinical diagnosis but for advanced stages and for monitoring recurrence of the disease [5].

Over the past decade, cancer research has made major advances in understanding the causes of developing CRC as well as the molecular mechanisms involved in the disease [6]. For instance many solid tumors such as breast, lung or colon cancer have revealed to be immunogenic. These tumors express aberrant levels of mutated or modified proteins known as tumor-associated antigens (TAA), which are related to the malignant growth. Such proteins can stimulate cellular and humoral immune response, triggering specific autoantibody production [7,8]. The role of autoantibodies in cancer is still unclear. It is not well-known whether they play a cancer-promoting role, an anti-tumor effect or if

1 they are an epiphenomenon associated to inflammation and tumor progression [9]. Nevertheless,  
2 autoantibody responses to TAAs hold promising characteristics to consider them as blood biomarkers  
3 for cancer detection and they are currently being investigated as potential diagnostic tools in multiple  
4 cancer types. Some reports have described the use of autoantibodies for early and preclinical detection  
5 of cancer, such as lung [10,11] or breast cancer [12]. The analysis of autoantibodies offers significant  
6 benefits when compared to direct determination of protein antigens associated to the tumor. Whereas  
7 detection of directly tumor-shed proteins in serum may be challenging due to their low abundance or  
8 to the difficulty of identifying simple mutations or structural modifications, serum autoantibodies are  
9 highly stable biomolecules and are produced in large quantities even after stimulation by a minimal  
10 amount of tumor antigen [12,13]. As a result, TAA-specific serum autoantibodies can constitute  
11 excellent circulating reporters for early and preclinical cancer diagnosis [14,15]. In the particular case  
12 of colorectal cancer, over 100 individual TAAs have been identified as target for autoantibody  
13 production, including full-length proteins, peptides, phage-peptides or glyco-peptides [14,16-18].  
14 Current efforts in CRC research are directed not only to define specific TAA panels but also to  
15 develop efficient and highly sensitive analytical methods capable of detecting TAA autoantibodies in  
16 serum with optimum accuracy and reliability [19,20]. Most commonly employed methodologies are  
17 based on ELISA or protein microarrays [21] which are usually aimed at finding relative cut-off  
18 values, so far providing qualitative or semi-quantitative results. Optical biosensors can offer a  
19 valuable alternative in terms of time and sample consumption and can provide accurate quantification,  
20 which may result useful from a diagnosis point of view. Photonic and plasmonic biosensors in  
21 particular have shown great promise for the development of high-throughput and miniaturized  
22 platforms capable of carrying out label-free and highly sensitive biochemical analysis [22,23].

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50 In this paper, we show the design and optimization of a nanoplasmonic-based biosensor for the direct  
51 detection and quantification of specific CRC-related TAA autoantibodies. We employ a  
52 refractometric nanoplasmonic biosensor whose configuration is based on the Localized Surface  
53 Plasmon Resonance (LSPR) of gold nanodisks [24]. The gold nanodisks are fabricated by hole-mask  
54 colloidal lithography [25] which is an easy, fast, low-cost and well-established methodology which  
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1 leads to reproducible results, with controlled density of disks on the surface. This device is highly  
2 sensitive to local refractive index (RI) changes occurring in close proximity to the surface of the  
3 transducer (in this case the gold nanodisks), such as the ones originated from biomolecular  
4 interactions. These RI changes can be detected as variations of the LSPR, which permits the real-time  
5 monitoring of the biorecognition events under label-free conditions. This nanoplasmonic biosensor  
6 has demonstrated excellent capabilities in terms of RI sensitivity improvement and signal-to-noise  
7 ratio enhancement, compared for instance with conventional Surface Plasmon Resonance [26] and  
8 more specifically its performance has also been validated for accurate detection of clinical biomarkers  
9 and antibodies in blood serum in few minutes[26,27]. The proposed biosensor strategy would allow  
10 rapid and simple analysis of TAA autoantibodies, providing a unique and innovative tool for CRC  
11 diagnosis.  
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## 25 **2. Experimental**

### 26 **2.1. Materials**

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31 Main chemical reagents and salts for buffer preparation and biofunctionalization procedure were  
32 acquired from Sigma-Aldrich (Germany): alkanethiols for self-assembled monolayer (SAM)  
33 formation (16-mercaptohexadecanoic acid (MHDA) and 11-mercaptoundecanol (MUOH)), reagents  
34 for carboxylate group activation (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride  
35 (EDC) and N-hydroxysulfosuccinimide (s-NHS)), ethanolamine and Tween 20. Poly-L-Lysine-graft-  
36 PEG (MW~70000 g·mol<sup>-1</sup> was purchased to SuSoS (Switzerland). Commercial serum was obtained  
37 from Sigma-Aldrich (Germany) and commercial plasma was purchased to Innovative Research  
38 (USA). cDNA encoding for full-length human genes EDIL3 and GTF2B in pDONR221 were  
39 obtained from the PlasmId repository (Harvard Institute of Proteomics) and, then, subcloned into  
40 pET28a (Novagen) for protein expression. TAAs were expressed in bacteria and purified according to  
41 previous studies [16,28]. The Institutional Ethical Review Boards of the Centro de Investigaciones  
42 Biológicas (CIB), the Spanish National Research Council (CSIC) and Hospital de Cabueñes (Gijón)  
43 approved this study on biomarker discovery in colorectal cancer. Serum samples were obtained from  
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1 the Hospital of Cabueñes previous informed consent of the patients. Antibodies anti-GTF2b and anti-  
2 EDIL3 were purchased to Santa Cruz Biotechnology (USA) and Abcam (UK), respectively.  
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## 5 **2.2. Description of the Nanoplasmonic Biosensor**

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8 The nanoplasmonic device is based on a recently implemented LSPR sensing scheme based on a  
9 waveguided electromagnetic mode that arises in thin monolayers of sparse and randomly distributed  
10 plasmonic nanoparticles. Nanoplasmonic chips consist of short-range ordered arrays of gold  
11 nanodisks (diameter  $D = 100$  nm, height  $H = 20$  nm (Ti/Au 1/19 nm), surface density  $F = 6-7\%$ )  
12 fabricated by hole-mask colloidal lithography (HCL) on glass substrates [25]. A detailed description  
13 of the fabrication process has been included in the Supplementary Material. Sensor chips are clamped  
14 between a trapezoidal glass prism ( $n=1.52$ ) contacting the samples through RI matching oil ( $n\approx 1.512$ )  
15 and a custom-made Delrin flow cell (volume= $4$   $\mu\text{L}$ ). The flow cell is connected to a microfluidic  
16 system consisting on a syringe pump (New Era, NE-1000, USA) with adjustable pumping speed that  
17 ensures a constant liquid flow and a manually operated injection valve (IDEZ Health and Science, V-  
18 451, USA). For LSPR excitation, gold nanodisks are illuminated with a collimated halogen light (HL-  
19 2000, Micro-pack, USA) set in transverse-electric (TE) polarization mode at an angle of incidence of  
20  $80^\circ$ . The reflected light is collected and fiber-coupled to a CCD spectrometer (Ocean Optics, Jazz  
21 Module, US). Reflectivity spectra are acquired every 3 ms, and 300 consecutive spectra are averaged  
22 to provide the spectrum to be analyzed. Excitation at  $80^\circ$  results in deep reflectivity dips at  $\lambda_{\text{LSPR}} \approx$   
23  $750$  nm that lead to optimal biosensor performance [24,26]. Biomolecular interactions taking place  
24 close to the gold nanodisks induce RI changes on the surface and, as a consequence, wavelength  
25 displacements ( $\Delta\lambda_{\text{LSPR}}$ ). Tracking of the real-time resonance peak position is achieved via polynomial  
26 fit using homemade readout software.  
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## 51 **2.3. Surface functionalization**

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54 Prior to surface functionalization, sensor chips were subjected to a cleaning procedure consisting of  
55 consecutive 1 min sonication cycles in acetone, ethanol and MilliQ water, respectively, dried with  $\text{N}_2$   
56 stream and placed in a UV/ $\text{O}_3$  generator (BioForce Nanoscience, USA) for 20 min, after which they  
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1 were rinsed with ethanol and water and dried with N<sub>2</sub>. Formation of the alkanethiol SAM was carried  
2 out by coating the chip with 250 μM MHDA in ethanol for 5h at room temperature. Then, surface was  
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4 rinsed with ethanol and water and dried with N<sub>2</sub> stream. For the activation of the carboxylic groups,  
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6 the chip was incubated with 0.2 M EDC/0.05 M s-NHS in MES buffer (0.1 M pH 5.5) for 20 min at  
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8 RT and then rinsed with water and dried. The surface was then immediately immersed in the TAA  
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10 solution in PBS (10 mM pH 7.4) and incubated overnight at 4°C. Finally, biofunctionalized sensors  
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12 were carefully rinsed with PBS and water, dried with N<sub>2</sub> stream and mounted in the platform. The  
13  
14 non-sensing glass areas were subsequently coated with PLL-g-PEG (0.5 mg·mL<sup>-1</sup>) to avoid non-  
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16 specific adsorptions. Figure S3 in Supplementary Material summarizes the biofunctionalization  
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18 protocol.  
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#### 21 22 23 **2.4. Antibody detection assays** 24

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26 For antibody analysis, PBST 0.5% (PBS + 0.5% Tween 20) was settled as running buffer. Different  
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28 concentrations of specific antibody were diluted in PBST 0.5% or in commercial serum/plasma and  
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30 flowed over the functionalized surface at 25 μL·min<sup>-1</sup> (See Figure S9 for an example of real time  
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32 senrograms for the detection of specific antibodies at different concentrations). Regeneration of the  
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34 surface was achieved by injecting 20 mM NaOH at 65 μL·min<sup>-1</sup>. Calibration curves were fitted to a  
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36 saturation total binding model. Limit of detection (LoD) was calculated as the concentration  
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38 corresponding to the blank signal plus three times its standard deviation (SD), while limit of  
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40 quantification (LoQ) was determined as the concentration corresponding to the minimum measurable  
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42 signal, set as the blank signal plus 10 times SD. Data analysis was performed using Origin Pro  
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44 software. ELISA was carried out as previously described [16,28]. Briefly, microtiter plates (Maxisorp,  
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46 Nunc) were coated overnight with 0.3 μg of the purified recombinant proteins, using GST and human  
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48 Annexin IV as negative controls in 50 μl of PBS. After washing three times with PBS, plates were  
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50 blocked with 3% skimmed milk in PBS (MPBS) for 2 h at room temperature. Then, serum samples  
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52 (dilution, 1:100 in 3% MPBS) were incubated for 2 h at room temperature. After washing,  
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54 peroxidase-labeled anti-human IgG (Jackson laboratories) (dilution, 1:500 in 3% MPBS) was added  
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56 for 2 h at room temperature. Then, the signal was developed with 3,3',5,5'-tetramethylbenzidine  
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1 substrate for 10 min (Sigma-Aldrich). The reaction was stopped with 1 M HCl, and absorption  
2 measured at 450 nm.  
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### 5 **3. Results**

#### 6 **3.1. Description of the Nanoplasmonic Biosensor Platform**

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11 The nanoplasmonic device is based on a recently implemented LSPR sensing scheme based on a  
12 waveguided electromagnetic mode that arises in thin monolayers of sparse and randomly distributed  
13 plasmonic nanoparticles [24]. We previously reported that the in-plane LSPR excitation strongly  
14 enhances the polarizability of the nanodisks, creating an effective RI that is sufficiently large to  
15 support a guided electromagnetic mode inside the plasmonic monolayer. Both the nanoparticle surface  
16 density (F) and the incidence angle of light are key aspects that affect this sensing performance.  
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18 Surface density was precisely chosen so that the optimal mode excitation (light coupling efficiency  
19 close to 100%) occurs at angles where the sensitivity was maximized (angle close to 90°). In  
20 particular we employ short-ordered arrays of gold nanodisks (diameter  $D = 100$  nm, height  $H = 20$   
21 nm, surface density  $F = 6-7\%$ ) fabricated by hole-mask colloidal lithography (HCL) (See  
22 experimental details in Supplementary Material). This nanofabrication technique allows simple, cost-  
23 efficient and wafer-scale production of the nanoplasmonic chips. This waveguided mode results not  
24 only in a large increase of the RI sensitivity, but also strongly improves the signal-to-noise ratio. Both  
25 effects assure an overall improved RI sensing performance that is up to one order of magnitude better  
26 than that of isolated non-interacting nanodisks. Thus, RI changes occurring close to the nanodisk  
27 surfaces are much easier to detect. A schematic representation of the biosensor can be seen in Figure  
28 1a. For LSPR excitation, the nanoplasmonic chip is illuminated with a broadband polarized light at a  
29 determined angle of incidence ( $\Theta = 80^\circ$ ) [24,26], and the reflected light is collected with a  
30 **spectrophotometer**. The obtained spectra show a deep reflectivity dip at  $\lambda_{\text{LSPR}} \approx 750$ . Biomolecular  
31 interactions occurring on the nanodisks surfaces generate RI changes that, in turn, cause  
32 displacements of the spectral LSPR peak ( $\Delta\lambda_{\text{LSPR}}$ ) (red shifts when binding occurs and blue shifts  
33 during a desorption process). The real-time interrogation of this  $\Delta\lambda_{\text{LSPR}}$  enables the extraction of  
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1 quantitative information related to the biomolecular interactions taking place in a label-free manner  
2 (Figure 1.B). In addition, the designed optical platform has very small dimensions (all optical  
3 components are mounted on a 20 x 20 cm<sup>2</sup> portable breadboard), exemplifying its facile  
4 miniaturization and potential portability.  
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### 8 9 **3.2. Design and Optimization of the Sensor Biofunctionalization**

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11 Among the numerous TAAs defined for colorectal cancer [18], we selected GTF2b (general  
12 transcription factor IIB) and EDIL3 (EGF-like repeats and discoidin I-like domain 3 protein) since  
13 both proteins have been previously evaluated as possible TAA targets for autoantibody production in  
14 colorectal cancer [14]. A study performed in cancer-induced animals provided evidences of  
15 immediate production of GTF2b and EDIL3 autoantibodies, among others [14]. The presence of  
16 autoantibodies was detectable at a very early stage in tumor development, even before adenoma  
17 formation. Especially, GTF2b could be detected before clinically observable symptomatology while  
18 EDIL3 is characterized by a more homogeneous but late response. This makes GTF2B more  
19 appropriate to enhance sensitivity while EDIL3 would enhance specificity.  
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34 In order to directly detect the autoantibodies for these two TAA, a biosensing strategy based on the  
35 direct immobilization of the TAA on the surface of the gold nanodisks has been addressed. A  
36 schematic representation of the proposed biosensor strategy is showed in Figure 1C.  
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38 Biofunctionalization of the nanoplasmonic sensor chip was based on the formation of a functional  
39 alkanethiol self-assembled monolayer (SAM) specifically onto the gold nanodisks *via* thiol  
40 chemisorption, which act as linker for the covalent attachment of the antigens. We employed 16-  
41 mercaptohexadecanoic acid (MHDA) to create a tight and uniform SAM where the carboxylic groups  
42 of the MHDA are activated to readily react with lysine (Lys) residues available in the proteins. The  
43 reaction generates an amide bond between the protein and the SAM. The grafting density of antigen  
44 molecules on the surface can also be controlled by introducing a lateral spacer during the formation of  
45 the SAM (i.e. 11-mercaptoundecanol, MUOH). In parallel, glass substrate was coated with the  
46 copolymer poly-L-lysine PEG (PLL-g-PEG 0.5 mg·mL<sup>-1</sup>) to prevent and minimize possible undesired  
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1 adsorptions. The PLL-g-PEG coating generates a highly hydrophilic layer that has demonstrated to  
2 effectively reduce nonspecific binding of proteins and other compounds present in biological  
3 matrices. This step facilitates the direct measurement in these biological fluids when using label-free  
4 biosensors [26,29].  
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9 To establish the best immobilization conditions for both TAAs, the nanoplasmonic chips were  
10 independently biofunctionalized with recombinant human GTF2b and EDIL3 proteins employing  
11 different molar ratios of mixed alkanethiol SAM (MHDA/MUOH 1:0, 1:1, 1:10) at a total thiol  
12 concentration of 250  $\mu\text{M}$ . The immobilization procedure was carried out *in situ* over the SAM-  
13 functionalized chip already mounted on the sensor platform, by flowing the protein solution and  
14 monitoring the covalent coupling process (Figure S4 in Supplementary Material). We compared the  
15 immobilization signals obtained with a fixed protein concentration ( $50 \mu\text{g}\cdot\text{mL}^{-1}$ ) over the different  
16 SAM ratios. We selected  $50 \mu\text{g}\cdot\text{mL}^{-1}$  based on preliminary experiments performed with a  
17 conventional SPR biosensor. We observed that although higher concentrations of protein (i.e. 100  
18  $\mu\text{g}\cdot\text{mL}^{-1}$ ) rendered higher immobilization signal, the subsequent detection of the autoantibody (at a  
19 fixed concentration of  $1 \mu\text{g}\cdot\text{mL}^{-1}$ ) remained similar to the one obtained with a protein concentration of  
20  $50 \mu\text{g}\cdot\text{mL}^{-1}$  (see Figure S5 in the Supplementary Material). This was indicative of a more optimum  
21 coverage of the surface for detection purposes with  $50 \mu\text{g}\cdot\text{mL}^{-1}$  than with  $100 \mu\text{g}\cdot\text{mL}^{-1}$ . Based on  
22 these results and on previous conditions optimized for protein attachment to SAMs [27] we selected  
23  $50 \mu\text{g}\cdot\text{mL}^{-1}$  of protein for further experiments. The highest amount of protein was attached to the  
24 sensor surface when maximum carboxylic density was employed (Figure S6a in Supplementary  
25 Material), inducing wavelength shifts around 3 nm. This was observed for both GTF2b and EDIL  
26 proteins. Introduction of spacer molecules (MUOH) to the SAM resulted in lower signals thereby  
27 indicating less amount of TAA immobilized. However, the optimum TAA layer, which will lead to  
28 better detection levels, does not necessarily require maximum coverage with proteins. The appropriate  
29 distribution of antigens on the surface reveals itself as an important factor to favor the accessibility of  
30 antibodies. Control of the spacing between TAA molecules can *a priori* modulate possible steric  
31 hindrance effects and improve the ability of the antibodies to interact. We evaluated the detection  
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1 efficiency by flowing a  $1 \mu\text{g}\cdot\text{mL}^{-1}$  specific antibody solution over the corresponding GTF2b and  
2 EDIL3 functionalized surfaces. This concentration was selected for screening purposes in order to  
3 choose the best immobilization conditions before performing a full calibration curve. In addition,  
4 TAA immobilization procedure was also carried out *ex situ*, by coating the functionalized sensor chip  
5 with the protein overnight, rinsing it with buffer and then installing the protein-modified chip in the  
6 biosensor. The antibody detection signal achieved for both *ex situ* and *in situ* procedures carried out  
7 with mixed alkanethiol SAMs at different molar ratios was compared and is summarized in the Figure  
8 S6b in the Supplementary Material). No steric hindrance effects appeared to be relevant for the  
9 concentration of TAA tested ( $50 \mu\text{g}\cdot\text{mL}^{-1}$ ) as maximum antibody detection was obtained in all cases  
10 when maximum carboxylic density was used (MHDA/MUOH 1:0). This suggests that the  
11 immobilization of TAAs at the selected concentration on alkanethiol SAMs formed exclusively with  
12 MHDA actually provides highly efficient bioreceptor layers, as also previously reported [30]. It can  
13 also be appreciated that *ex situ* immobilization resulted in significant higher antibody signals for the  
14 same concentration of immunoreagents. This could be attributed to a more efficient coupling yield  
15 and also to a more efficient protein rearrangement on the surface facilitated by longer reaction times  
16 (protein coupling overnight vs. 30 min when it is done *in situ*). From the above results, overnight  
17 immobilization of TAA over a 100% MHDA SAM was selected as optimum biofunctionalization  
18 strategy for further experiments. Regeneration for a potential reutilization of the TAAs functionalized  
19 surfaces was also evaluated. For both TAA-modified surfaces – GTF2b and EDIL3 – basic conditions  
20 (20 mM NaOH) disrupted the TAA-antibody interaction without altering or modifying the  
21 immobilized proteins (Figure S7 and S8 in Supplementary Material). Under these conditions it was  
22 possible to reuse the functionalized surface with good repeatability for up to 100 cycles before  
23 progressive decrease of the antibody detection signals.

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52 Calibration curves for both anti-GTF2b and anti-EDIL3 (Figure 2A and 2B) were then performed in  
53 standard buffer conditions (i.e. PBS with 0.5% of Tween 20 (PBST)), which according to previous  
54 works helped drastically reduce nonspecific adsorptions onto the sensor chip) [26,27]. Different  
55 antibody concentrations ranging from  $50 \text{ ng}\cdot\text{mL}^{-1}$  to  $1 \mu\text{g}\cdot\text{mL}^{-1}$  were flowed by triplicate through the  
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1 specific TAA functionalized surfaces, respectively, and the resonance shift was obtained (Figure 2A  
2 and 2B). Limits of detection (LoD) were determined as the minimum antibody concentrations that  
3 provide an observable signal (i.e. blank signal plus 3 times its standard deviation). The LoD for anti-  
4 GTF2b assay was  $10 \text{ ng}\cdot\text{mL}^{-1}$  (66 pM) and  $5 \text{ ng}\cdot\text{mL}^{-1}$  (33 pM) for anti-EDIL3 assay. Limits of  
5 quantification (LoQ) were determined as the minimum measurable signal, being  $34 \text{ ng}\cdot\text{mL}^{-1}$  (227 nM)  
6 and  $19 \text{ ng}\cdot\text{mL}^{-1}$  (127 pM) for GTF2b and EDIL3, respectively. Besides, the specificity of the assays  
7 was confirmed by using nonspecific antibodies as control. Measurements of anti-GTF2b over an  
8 EDIL3-functionalized surface and *vice versa* led to negligible signals (Figure 2A and 2B, red lines),  
9 which corroborates the signal contribution solely comes from the specific antibody recognition and  
10 confirms the feasibility of the methodology for antibody quantification. Excellent reproducibility and  
11 stability of the biosensor-based assays were finally demonstrated by performing intra- and inter-  
12 assays (curves performed in the same nanodisk-functionalized surface and in different surfaces,  
13 respectively). As it can be seen in Table 1, the coefficient of variation (CV) for both GTF2b and  
14 EDIL3 was below the maximum variability recommended for clinical analysis (~15%) [31], both for  
15 the LoD and for the maximum signal ( $S_{\text{max}}$ ), taken at the maximum antibody concentration tested  
16 ( $[\text{Ab}] = 1 \text{ }\mu\text{g}\cdot\text{mL}^{-1}$ ).  
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### 36 **3.3. Analysis of TAA Antibodies in Serum and Plasma**

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40 In order to evaluate the influence of the matrix, commercial serum (undiluted, and diluted 1:1 and  
41 1:10 in PBST) and commercial plasma (undiluted, and diluted 1:1 and 1:10 in PBST) were flowed  
42 over the biofunctionalized nanoplasmonic surfaces (i.e. TAA layer + PLL-g-PEG coating) (Figure 3).  
43 As it can be seen in the sensorgrams, in both cases a significant background signal was observed due  
44 to the binding of fluid components onto the bioactive layer, being slightly higher for undiluted plasma  
45 (whose difference compared to serum is the presence of fibrinogen). A 1:10 dilution in PBST was  
46 necessary to achieve a complete reduction of nonspecific adsorptions for both fluids, resulting in  
47 virtually no background signals.  
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1 Besides the influence of the nonspecific binding onto the surface, possible matrix effects on the  
2 interaction of the antibody with the TAA-coated layer can occur, altering the analysis features. In  
3 order to assess this undesired effect, both GTF2b and EDIL3 calibration curves were obtained by  
4 spiking serum or plasma with several known concentrations of antibodies and then diluting them in  
5 PBST (1:10) (Figure 4). The curves were analogous to those obtained with standard buffer conditions  
6 although a slight increase of the LoDs was observed. This minor worsening can be attributed to a  
7 possible hindrance of the antibody/TAA interaction. For the GTF2b antibody, the LoD was  $16 \text{ ng}\cdot\text{mL}^{-1}$   
8 in diluted serum and  $15 \text{ ng}\cdot\text{mL}^{-1}$  in diluted plasma, compared to a LoD of  $10 \text{ ng}\cdot\text{mL}^{-1}$  in buffer. In  
9 the case of EDIL3 antibody detection, LoDs were  $12 \text{ ng}\cdot\text{mL}^{-1}$  in diluted serum and  $11 \text{ ng}\cdot\text{mL}^{-1}$  in  
10 diluted plasma, compared to  $5 \text{ ng}\cdot\text{mL}^{-1}$  in buffer. Overall the sample dilution (1:10) has inevitably  
11 decreased the sensitivity over one order of magnitude, with detectabilities around  $150\text{-}160 \text{ ng}\cdot\text{mL}^{-1}$  for  
12 GTF2b and around  $110\text{-}120 \text{ ng}\cdot\text{mL}^{-1}$  for EDIL3, respectively depending on the fluid.  
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27 Clinical serum samples from CRC patients were analyzed with the nanoplasmonic biosensor for a  
28 preliminary assessment of the viability of our approach. Serum samples collected from patients from  
29 the Hospital of Cabueñes (Gijón, Spain) with diagnosed CRC and samples from healthy individuals  
30 were evaluated employing the optimal conditions selected before (1:10 dilution with PBST). All  
31 samples were previously analyzed for the presence of GTF2b autoantibodies using semi-quantitative  
32 ELISA, so the absolute concentration of the target biomarker was unknown. Table 2 compares the  
33 concentration values obtained with the nanoplasmonic biosensor (in  $\mu\text{g}\cdot\text{mL}^{-1}$ ) to absorbance values  
34 (in optical density units, OD) obtained with the ELISA (quantitative data is not commonly determined  
35 with the ELISA for autoantibodies). A consistent correlation was observed in terms of relative signals.  
36 Negative samples from healthy subjects resulted below the LoD established for our biosensor  
37 technique ( $160 \text{ ng}\cdot\text{mL}^{-1}$ ) while positive samples lead to relatively high signals (i.e. high concentration  
38 of GTF2b autoantibodies).  
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#### 54 **4. Discussion**

55 A major challenge in colorectal cancer research focuses on the development of novel diagnostic  
56 techniques for simple, rapid and accurate detection of the disease at earlier stages. In this regard,  
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1 cancer-associated autoantibodies generated by the immune system during tumor appearance have  
2 evidenced its high value as blood-circulating biomarkers for preclinical cancer diagnosis. We propose  
3 the use of an innovative nanoplasmonic biosensor for the direct and label-free quantification of CRC  
4 autoantibodies in serum or plasma. Our nanoplasmonic biosensor offer real-time detection of TAA  
5 antibodies without the need of any labels or sample pretreatments, which simplifies the analysis and  
6 provides interesting alternatives to develop small, fast and user-friendly point-of-care (POC) devices  
7 that could be used directly at doctor's office. The implementation of POC biosensors for rapid and  
8 reliable CRC screening could substantially afford a breakthrough towards non-invasive and highly  
9 specific diagnostic tools for this disease which in turn would help to improve patient survival rates.

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21 The overall performance of the biosensor assay has been demonstrated for the determination of  
22 autoantibodies against two important CRC antigens: GTF2b and EDIL3. Both autoantibodies were  
23 selected as representative for the disease as they are generated at very early stages of CRC  
24 development and can be detected in blood serum before the onset of tumor lesions [14].  
25 Biofunctionalization of the nanoplasmonic sensors has been designed to create a highly stable TAA  
26 layer that ensures an efficient capture of the specific antibodies. The immobilization strategy is based  
27 on the material-selective functionalization of the gold nanodisks through the formation of an  
28 alkanethiol SAM, which guarantees the biomolecular interaction to take place solely on the sensor  
29 spots. The PLL-g-PEG coating of the glass areas (which represents over the 94% of the surface)  
30 prevents the nonspecific binding to substrate. The covalent coupling of the TAA *via* the terminal  
31 amine groups of Lys residues is a simple and robust procedure that can be applied to immobilize  
32 virtually all proteins. Several biofunctionalization conditions have been optimized (e.g. alkanethiol  
33 SAM ratios, binding time, etc.) for the enhancement of the antibody capture efficacy reaching a LoD  
34 of  $10 \text{ ng}\cdot\text{mL}^{-1}$  ( $\sim 66 \text{ pM}$ ) for GTF2b antibody and  $5 \text{ ng}\cdot\text{mL}^{-1}$  ( $\sim 33 \text{ pM}$ ) for EDIL3 antibody in  
35 standard buffer, showing in both cases high selectivity and reproducibility. A complete assay cycle  
36 (including regeneration) is accomplished in 30 min. Besides, the bioactive surface with the  
37 immobilized TAA has proven to be reusable for more than 100 cycles with good repeatability.

1 An ultimate goal in POC development relies on the ability to readily detect the biomarkers in  
2 biological fluids. Here, we have exploited the unique hydrophilic properties of the PLL-g-PEG  
3 coating layer together with the use of a dilution buffer containing a high concentration of surfactant  
4 (Tween 20) in order to minimize possible interferences coming from serum or plasma matrices. In  
5 particular, a simple dilution of the biological sample 1:10 with PBST has led to a complete removal of  
6 background signals enabling the direct quantification without requiring purification or other extra  
7 pretreatments. This dilution factor inevitably increases the limit of detection. However, it is much  
8 lower than the minimum dilution required in the ELISA assay. Overall the features of the resultant  
9 calibration curves, both in diluted serum and plasma, offer a highly reliable analysis method to  
10 quantify the TAA autoantibodies with elevated selectivity and reproducibility. Moreover, clinical  
11 samples analysis further demonstrates the potential of using this device in comparison to conventional  
12 ELISA methods, according to the good correlation observed for the detection of GTF2b antibodies in  
13 CRC-diagnosed patients and healthy individuals. In this regard, a more complete quantitative clinical  
14 validation will be required in the future. However, to our knowledge, serological concentration levels  
15 of CRC autoantibodies have not been fully established so far, as most research articles in the field  
16 especially focus on their identification and the assessment of their diagnostic and/or prognostic value.  
17 Nevertheless we cannot obviate the evident usefulness of knowing this concentration value compared  
18 with other semi-quantitative or qualitative methods, not only from a perspective of early diagnosis but  
19 also for disease follow-up. Besides, the possibility of quantifying autoantibodies concentration in  
20 serum samples may allow further comprehension of the humoral response triggered by the tumor and  
21 harness the basis for the improvement of prognosis of the disease. On-going work currently focuses  
22 on the improvement of biofunctionalization strategies and the use of antifouling agents that permit  
23 direct measurements of undiluted serum and plasma, therefore enhancing the detectability at least 10-  
24 fold. Moreover, the biofunctionalization methodology developed can be easily adjusted for any TAA  
25 with potential interest. This facilitates the eventual expansion of the biosensor strategy to elaborate a  
26 multiplexed compact analytical platform for the simultaneous detection of a CRC-specific panel of  
27 autoantibodies, which is ultimately necessary to fully cover the patient variability and to maximize the  
28 sensitivity and specificity of the method.  
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## 5. Conclusions

We have developed a novel analytical label-free strategy for the detection of tumor-associated autoantibodies in blood serum and plasma based on an innovative nanoplasmonic biosensor technology. This strategy could provide a reliable and non-invasive screening and diagnosis of colorectal cancer at early stages. Our biosensor allows the label-free quantification of specific CRC-related autoantibodies in few minutes, without requiring any sample purification or pretreatment. Several biofunctionalization parameters have been optimized, reaching a limit of detection of 1 nM (150-160 ng·mL<sup>-1</sup>) for direct measurements in human serum or plasma. Selectivity and reproducibility of the assay have been also evaluated demonstrating the excellent accuracy and robustness of the biosensor. The analysis of clinical samples from colorectal cancer patients has shown good correlation with ELISA. Overall, the results obtained highlight the exceptional potential of our nanoplasmonic biosensor as a tool for the early detection of colorectal cancer and current efforts are focused on establishing a multiplexed approach to expand the strategy to a CRC-panel of autoantibodies.

## Acknowledgements

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**Figure Captions:**

**Figure 1. (a)** Schematic representation of the miniaturized nanoplasmonic biosensor. A picture of the nanodisks and a SEM image of the actual shape of the gold nanodisks fabricated on the glass substrate are also shown. **(b)** Graphs showing the resonance peak (photon counts vs.  $\lambda$ ) (*left*) and the shift of the resonance peak over time ( $\Delta\lambda_{\text{LSPR}}$  vs. time) (*right*); **(c)** TAA biofunctionalization methodology based on covalent coupling to an alkanethiol SAM and subsequent antibody detection.

**Figure 2. (a)** Calibration curve for anti-GTF2b detection performed over GTF2b-biofunctionalized nanodisks (black). Red dashed line indicates nonspecific adsorption of a control antibody (anti-EDIL3); **(b)** Calibration curve for anti-EDIL3 detection performed over EDIL3-biofunctionalized nanodisks (black). Red dashed line indicates nonspecific adsorption of a control antibody (anti-GTF2b).

**Figure 3. (a)** Background signal corresponding to nonspecific adsorption of undiluted serum (black), serum diluted 1:1 in PBST 0.5% (purple) and serum diluted 1:10 in PBST 0.5% (green); **(b)** Background signal corresponding to nonspecific adsorption of undiluted plasma (blue), plasma diluted 1:1 in PBST 0.5% (orange) and plasma diluted 1:10 in PBST 0.5% (pink).

**Figure 4. (a)** Calibration curves for anti-GTF2b antibody detection in PBST buffer (black), serum diluted 1:10 in PBST (green) and plasma diluted 1:10 in PBST (pink); **(b)** Calibration curves for anti-EDIL3 antibody detection in PBST buffer (black), serum diluted 1:10 in PBST (green) and plasma diluted 1:10 in PBST (pink).

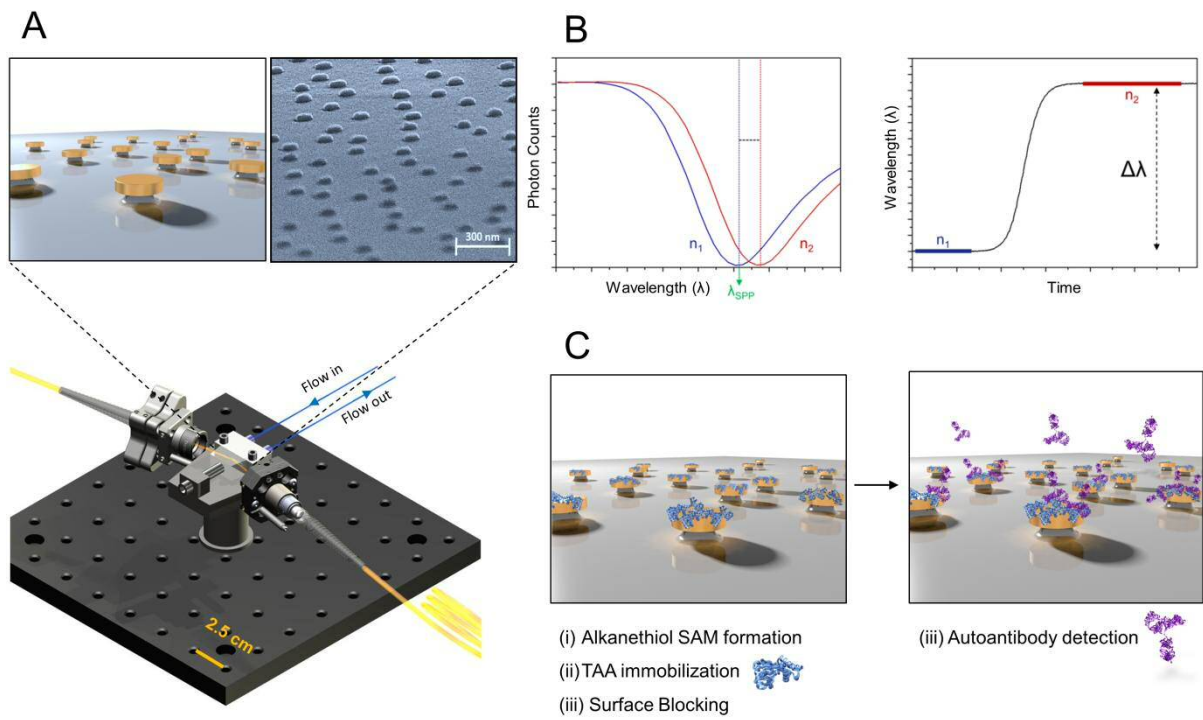


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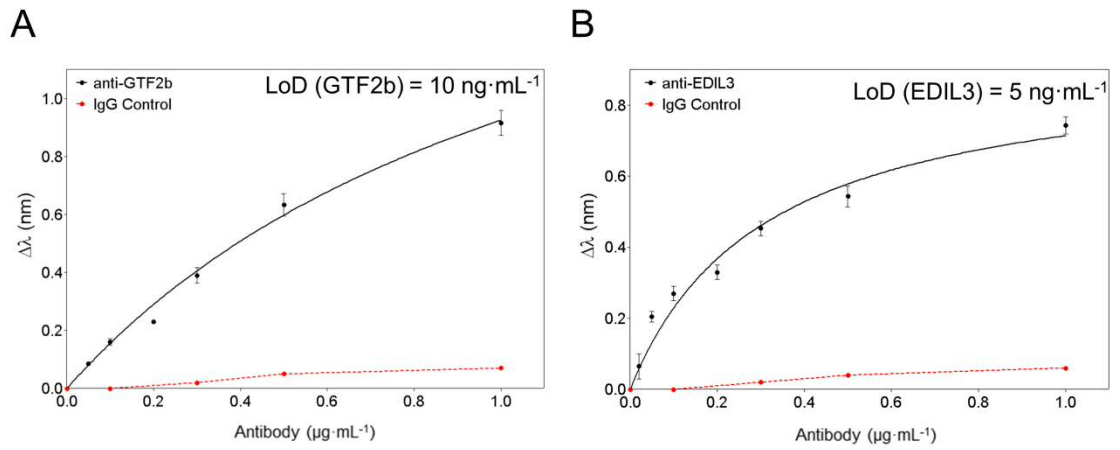
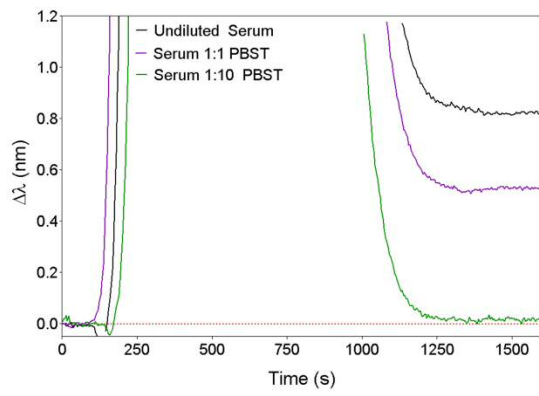


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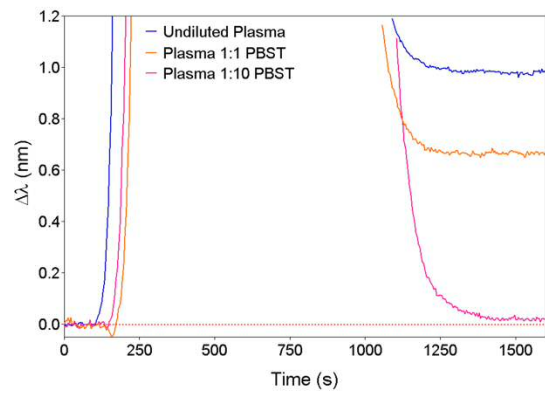
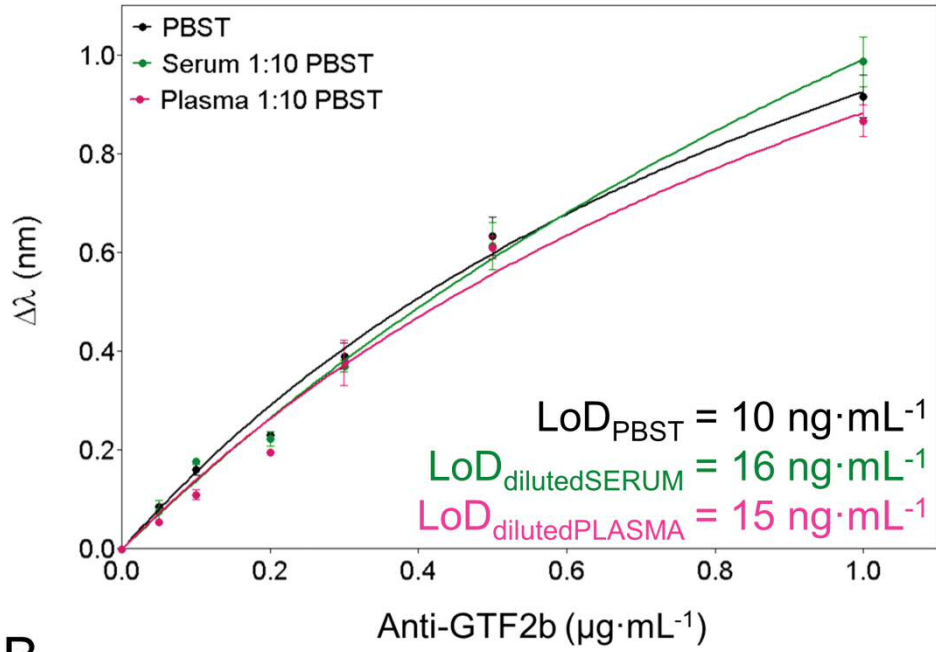


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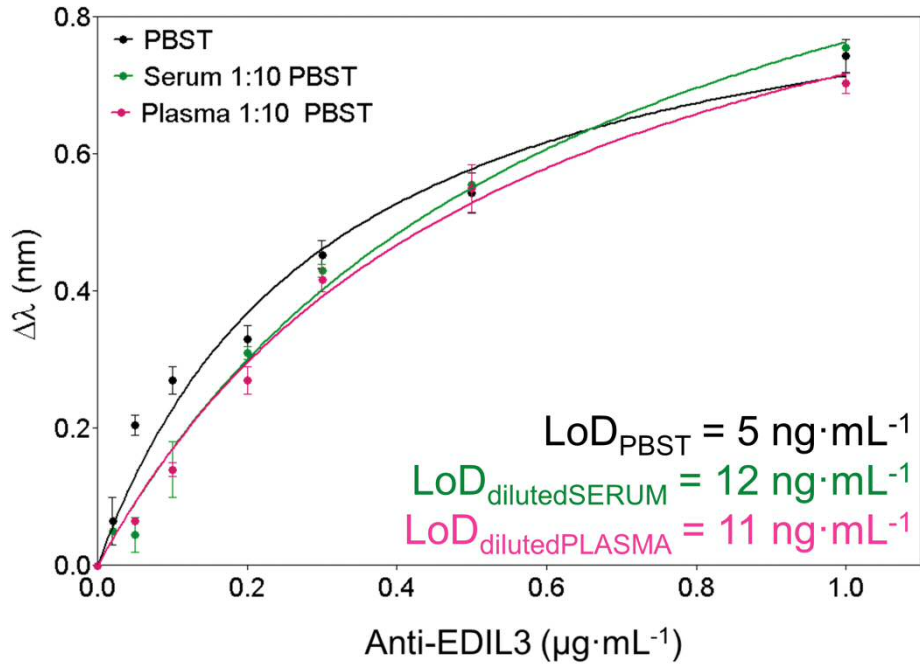


Figure 4



**Table 1.** Inter and intra-assay features for GTF2b and EDIL3 antibodies detection with the nanoplasmonic biosensor

		GTF2b antibody		EDIL3 antibody	
		Mean $\pm$ SD <sup>*</sup>	% CV	Mean $\pm$ SD	% CV
Intra-assay	LOD (ng·mL <sup>-1</sup> )	9.7 $\pm$ 0.5	5.15	5.2 $\pm$ 0.2	3.77
	S <sub>max</sub> (nm)	0.937 $\pm$ 0.0015	1.63	0.733 $\pm$ 0.016	2.08
Inter-assay	LOD (ng·mL <sup>-1</sup> )	10.1 $\pm$ 1.2	11.9	4.9 $\pm$ 0.4	8.16
	S <sub>max</sub> (nm)	0.917 $\pm$ 0.07	8.19	0.743 $\pm$ 0.04	5.60

\* Mean and standard deviation of 3 replicates

**Table 2.** Clinical serum samples analysis determined by ELISA and by the nanoplasmonic biosensor.

Sample	GTF2b Analysis Results		
		ELISA (OD)	Nanobiosensor (ng·mL <sup>-1</sup> )*
<b>G30</b>	Negative	0.18	ND <sup>†</sup>
<b>G42</b>	Positive	0.48	175 ± 8
<b>G56</b>	Positive	0.56	254 ± 10
<b>G101</b>	Negative	0.13	ND <sup>†</sup>

\* Mean ± SD for 3 replicates

<sup>†</sup> ND: No Detected (below limit of detection: 160 ng·mL<sup>-1</sup>)

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Figure 1  
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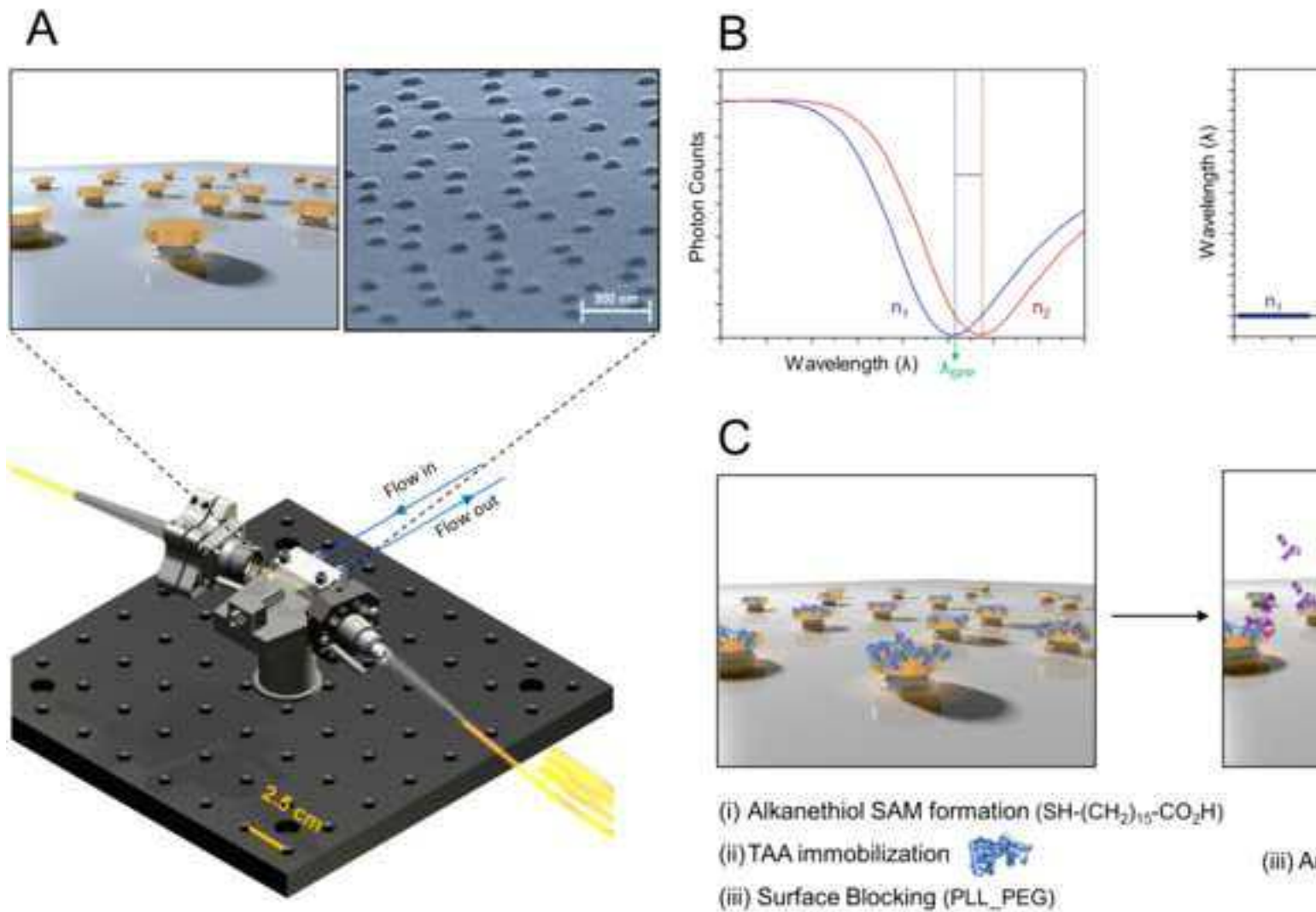
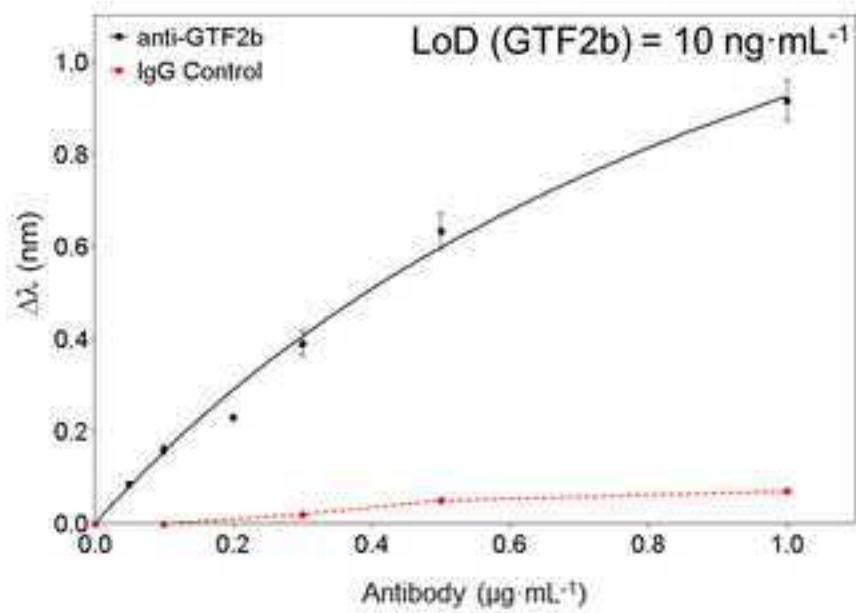


Figure 2

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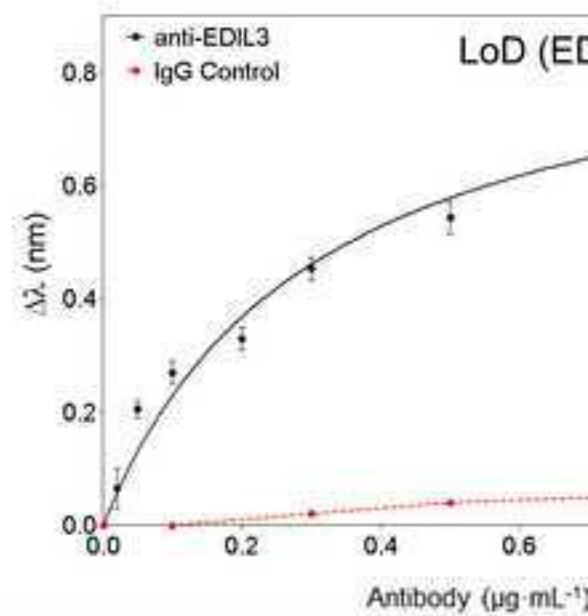
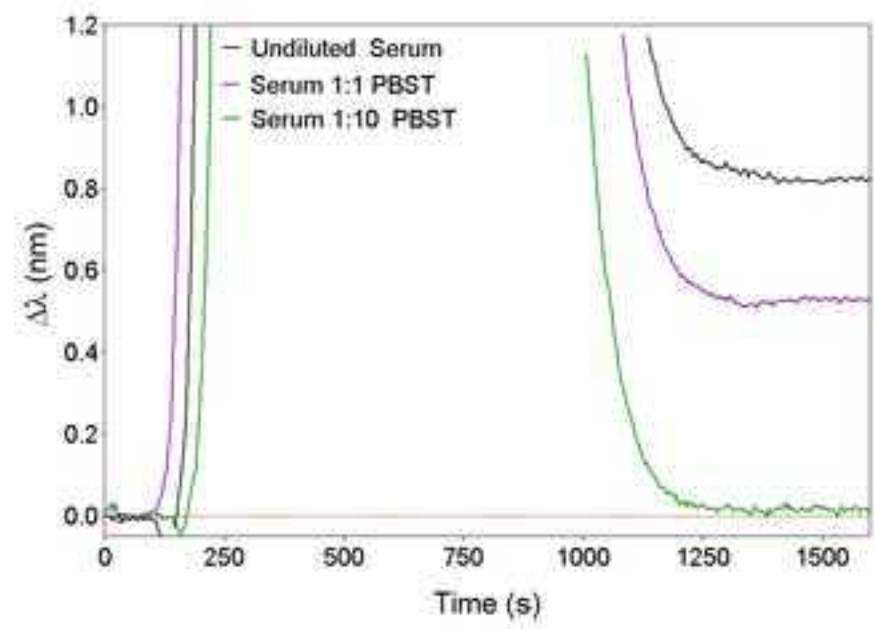


Figure 3

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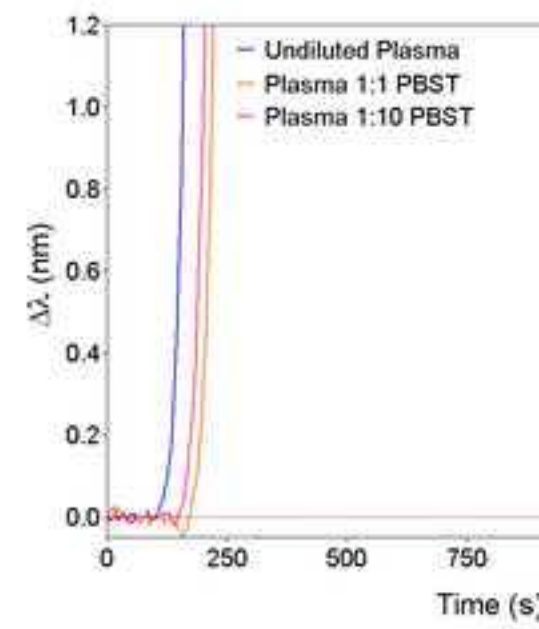
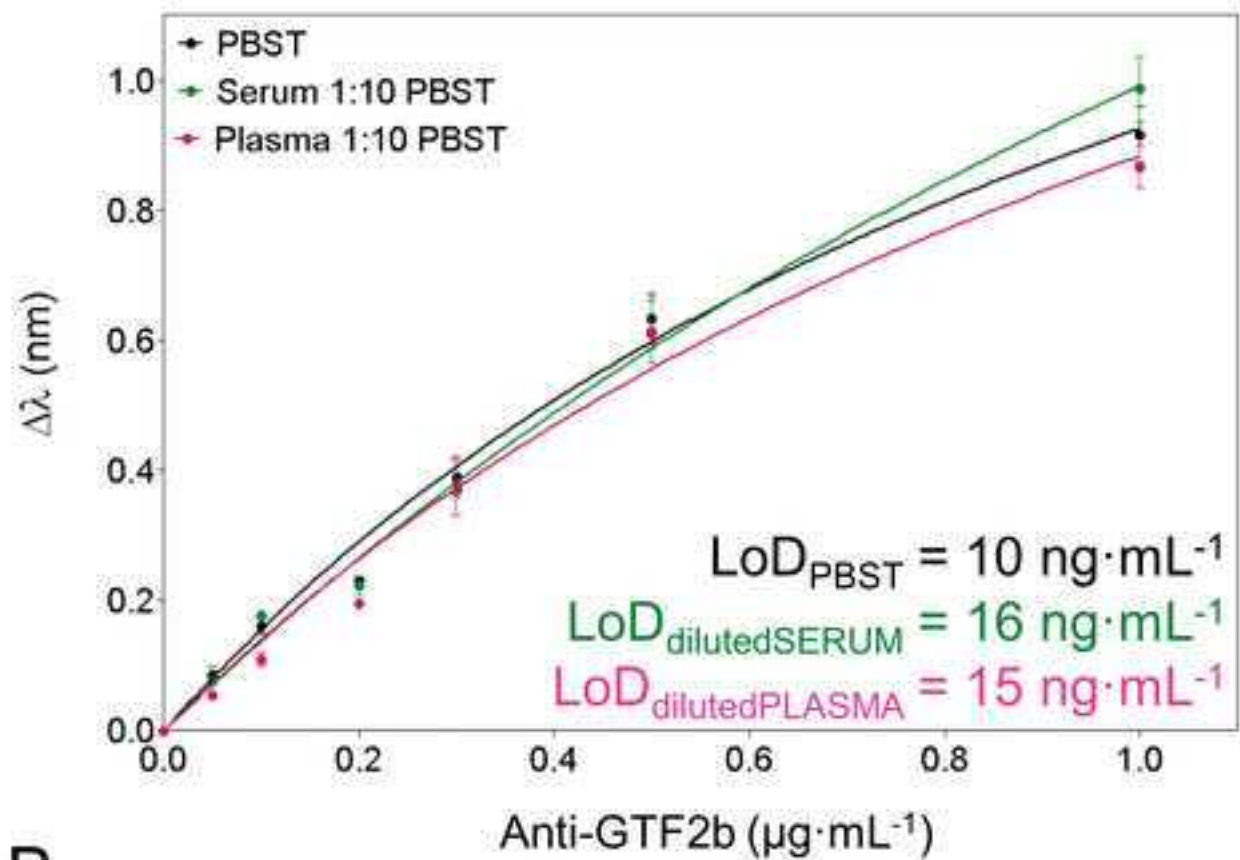


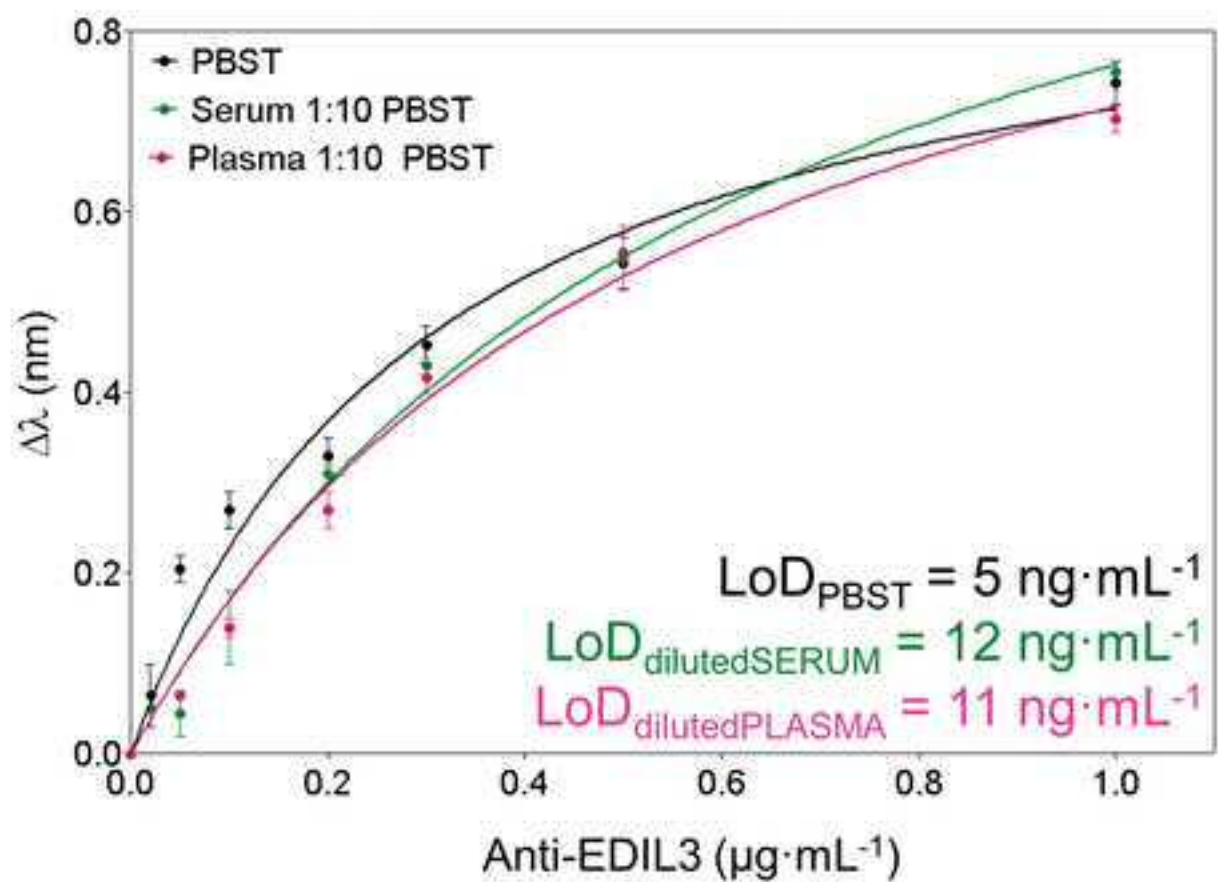
Figure 4

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**Electronic Supplementary Material (online publication only)**

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# Label-free Nanoplasmonic Sensing of Tumor-Associate Autoantibodies for Early Diagnosis of Colorectal Cancer

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## ABSTRACT

Colorectal cancer is treatable and curable when detected at early stages. However there is a lack of less invasive and more specific screening and diagnosis methods which would facilitate its prompt identification. Blood circulating autoantibodies which are immediately produced by the immune system at tumor appearance have become valuable biomarkers for preclinical diagnosis of cancer. In this work, we present the rapid and label-free detection of colorectal cancer autoantibodies directly in blood serum or plasma using a recently developed nanoplasmonic biosensor. Our nanoplasmonic device offers sensitive and real-time quantification of autoantibodies with excellent selectivity and reproducibility, achieving limits of detection around 1 nM (150-160 ng·mL<sup>-1</sup>). A preliminary evaluation of clinical samples of colorectal cancer patients has shown good correlation with ELISA. These results demonstrate the reliability of the nanobiosensor strategy and pave the way towards the achievement of a sensitive diagnostic tool for early detection of colorectal cancer.

## Keywords

nanoplasmonic biosensor; plasma; serum; colorectal cancer; autoantibodies; clinical diagnosis

## Abbreviations

CRC colorectal cancer

ELISA enzyme-linked immunosorbent assay

LSPR localized surface plasmon resonance

POC point of care

RI refractive index

SAM self-assembled monolayer

TAA tumor-associate antigen

# 1. Introduction

Colorectal cancer (CRC) is a worldwide health problem with an incidence over 1 million annual cases and being a major cause of morbidity and mortality in developed countries [1]. It is the third most common cancer and the fourth most common cause of death around the world. Despite the exact cause for CRC is not known, several risk factors have been established for the disease, including genetic and epigenetic parameters [2]. Familiar history of colon cancer or inflammatory diseases, age, lifestyle and environmental conditions are strongly associated to CRC development. CRC is easily curable when detected early [2], thereby prevention and regular screening play crucial roles in the fight against this cancer. However, CRC diagnosis is particularly challenging. The most reliable diagnosis technique is *via* sampling of colon biopsies suspected of possible tumor development, which is typically done during colonoscopy or sigmoidoscopy for the distal colon and rectum [3]. These procedures are highly invasive and present important limitations in terms of costs, available resources and low compliance. On the other hand, established noninvasive tests such as the guaiac-based fecal occult blood test (gFOBT) suffer from low specificity leading to inaccurate diagnosis results [4]. There is an evident need for novel screening tools, ideally, analytical techniques based on blood analysis, which permit the early and reliable identification and diagnosis of CRC. Development of blood biomarker assays that could indicate that a cancerous process is triggered would represent a great benefit. However, although a few serum proteins have been described as biomarkers in CRC (carcinoembryonic antigen (CEA), CA19.9 or CA125), none of them are recommended for early clinical diagnosis but for advanced stages and for monitoring recurrence of the disease [5].

Over the past decade, cancer research has made major advances in understanding the causes of developing CRC as well as the molecular mechanisms involved in the disease [6]. For instance many solid tumors such as breast, lung or colon cancer have revealed to be immunogenic. These tumors express aberrant levels of mutated or modified proteins known as tumor-associated antigens (TAA), which are related to the malignant growth. Such proteins can stimulate cellular and humoral immune response, triggering specific autoantibody production [7,8]. The role of autoantibodies in cancer is still unclear. It is not well-known whether they play a cancer-promoting role, an anti-tumor effect or if

1 they are an epiphenomenon associated to inflammation and tumor progression [9]. Nevertheless,  
2 autoantibody responses to TAAs hold promising characteristics to consider them as blood biomarkers  
3 for cancer detection and they are currently being investigated as potential diagnostic tools in multiple  
4 cancer types. Some reports have described the use of autoantibodies for early and preclinical detection  
5 of cancer, such as lung [10,11] or breast cancer [12]. The analysis of autoantibodies offers significant  
6 benefits when compared to direct determination of protein antigens associated to the tumor. Whereas  
7 detection of directly tumor-shed proteins in serum may be challenging due to their low abundance or  
8 to the difficulty of identifying simple mutations or structural modifications, serum autoantibodies are  
9 highly stable biomolecules and are produced in large quantities even after stimulation by a minimal  
10 amount of tumor antigen [12,13]. As a result, TAA-specific serum autoantibodies can constitute  
11 excellent circulating reporters for early and preclinical cancer diagnosis [14,15]. In the particular case  
12 of colorectal cancer, over 100 individual TAAs have been identified as target for autoantibody  
13 production, including full-length proteins, peptides, phage-peptides or glyco-peptides [14,16-18].  
14 Current efforts in CRC research are directed not only to define specific TAA panels but also to  
15 develop efficient and highly sensitive analytical methods capable of detecting TAA autoantibodies in  
16 serum with optimum accuracy and reliability [19,20]. Most commonly employed methodologies are  
17 based on ELISA or protein microarrays [21] which are usually aimed at finding relative cut-off  
18 values, so far providing qualitative or semi-quantitative results. Optical biosensors can offer a  
19 valuable alternative in terms of time and sample consumption and can provide accurate quantification,  
20 which may result useful from a diagnosis point of view. Photonic and plasmonic biosensors in  
21 particular have shown great promise for the development of high-throughput and miniaturized  
22 platforms capable of carrying out label-free and highly sensitive biochemical analysis [22,23].

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50 In this paper, we show the design and optimization of a nanoplasmonic-based biosensor for the direct  
51 detection and quantification of specific CRC-related TAA autoantibodies. We employ a  
52 refractometric nanoplasmonic biosensor whose configuration is based on the Localized Surface  
53 Plasmon Resonance (LSPR) of gold nanodisks [24]. The gold nanodisks are fabricated by hole-mask  
54 colloidal lithography [25] which is an easy, fast, low-cost and well-established methodology which  
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1 leads to reproducible results, with controlled density of disks on the surface. This device is highly  
2 sensitive to local refractive index (RI) changes occurring in close proximity to the surface of the  
3 transducer (in this case the gold nanodisks), such as the ones originated from biomolecular  
4 interactions. These RI changes can be detected as variations of the LSPR, which permits the real-time  
5 monitoring of the biorecognition events under label-free conditions. This nanoplasmonic biosensor  
6 has demonstrated excellent capabilities in terms of RI sensitivity improvement and signal-to-noise  
7 ratio enhancement, compared for instance with conventional Surface Plasmon Resonance [26] and  
8 more specifically its performance has also been validated for accurate detection of clinical biomarkers  
9 and antibodies in blood serum in few minutes[26,27]. The proposed biosensor strategy would allow  
10 rapid and simple analysis of TAA autoantibodies, providing a unique and innovative tool for CRC  
11 diagnosis.  
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## 25 **2. Experimental**

### 26 **2.1. Materials**

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31 Main chemical reagents and salts for buffer preparation and biofunctionalization procedure were  
32 acquired from Sigma-Aldrich (Germany): alkanethiols for self-assembled monolayer (SAM)  
33 formation (16-mercaptohexadecanoic acid (MHDA) and 11-mercaptoundecanol (MUOH)), reagents  
34 for carboxylate group activation (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride  
35 (EDC) and N-hydroxysulfosuccinimide (s-NHS)), ethanolamine and Tween 20. Poly-L-Lysine-graft-  
36 PEG (MW~70000 g·mol<sup>-1</sup> was purchased to SuSoS (Switzerland). Commercial serum was obtained  
37 from Sigma-Aldrich (Germany) and commercial plasma was purchased to Innovative Research  
38 (USA). cDNA encoding for full-length human genes EDIL3 and GTF2B in pDONR221 were  
39 obtained from the PlasmId repository (Harvard Institute of Proteomics) and, then, subcloned into  
40 pET28a (Novagen) for protein expression. TAAs were expressed in bacteria and purified according to  
41 previous studies [16,28]. The Institutional Ethical Review Boards of the Centro de Investigaciones  
42 Biológicas (CIB), the Spanish National Research Council (CSIC) and Hospital de Cabueñes (Gijón)  
43 approved this study on biomarker discovery in colorectal cancer. Serum samples were obtained from  
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1 the Hospital of Cabueñes previous informed consent of the patients. Antibodies anti-GTF2b and anti-  
2 EDIL3 were purchased to Santa Cruz Biotechnology (USA) and Abcam (UK), respectively.  
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## 5 **2.2. Description of the Nanoplasmonic Biosensor**

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8 The nanoplasmonic device is based on a recently implemented LSPR sensing scheme based on a  
9 waveguided electromagnetic mode that arises in thin monolayers of sparse and randomly distributed  
10 plasmonic nanoparticles. Nanoplasmonic chips consist of short-range ordered arrays of gold  
11 nanodisks (diameter  $D = 100$  nm, height  $H = 20$  nm (Ti/Au 1/19 nm), surface density  $F = 6-7\%$ )  
12 fabricated by hole-mask colloidal lithography (HCL) on glass substrates [25]. A detailed description  
13 of the fabrication process has been included in the Supplementary Material. Sensor chips are clamped  
14 between a trapezoidal glass prism ( $n=1.52$ ) contacting the samples through RI matching oil ( $n\approx 1.512$ )  
15 and a custom-made Delrin flow cell (volume= $4$   $\mu\text{L}$ ). The flow cell is connected to a microfluidic  
16 system consisting on a syringe pump (New Era, NE-1000, USA) with adjustable pumping speed that  
17 ensures a constant liquid flow and a manually operated injection valve (IDEZ Health and Science, V-  
18 451, USA). For LSPR excitation, gold nanodisks are illuminated with a collimated halogen light (HL-  
19 2000, Micro-pack, USA) set in transverse-electric (TE) polarization mode at an angle of incidence of  
20  $80^\circ$ . The reflected light is collected and fiber-coupled to a CCD spectrometer (Ocean Optics, Jazz  
21 Module, US). Reflectivity spectra are acquired every 3 ms, and 300 consecutive spectra are averaged  
22 to provide the spectrum to be analyzed. Excitation at  $80^\circ$  results in deep reflectivity dips at  $\lambda_{\text{LSPR}} \approx$   
23  $750$  nm that lead to optimal biosensor performance [24,26]. Biomolecular interactions taking place  
24 close to the gold nanodisks induce RI changes on the surface and, as a consequence, wavelength  
25 displacements ( $\Delta\lambda_{\text{LSPR}}$ ). Tracking of the real-time resonance peak position is achieved via polynomial  
26 fit using homemade readout software.  
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## 51 **2.3. Surface functionalization**

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54 Prior to surface functionalization, sensor chips were subjected to a cleaning procedure consisting of  
55 consecutive 1 min sonication cycles in acetone, ethanol and MilliQ water, respectively, dried with  $\text{N}_2$   
56 stream and placed in a UV/ $\text{O}_3$  generator (BioForce Nanoscience, USA) for 20 min, after which they  
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1 were rinsed with ethanol and water and dried with N<sub>2</sub>. Formation of the alkanethiol SAM was carried  
2 out by coating the chip with 250 μM MHDA in ethanol for 5h at room temperature. Then, surface was  
3  
4 rinsed with ethanol and water and dried with N<sub>2</sub> stream. For the activation of the carboxylic groups,  
5  
6 the chip was incubated with 0.2 M EDC/0.05 M s-NHS in MES buffer (0.1 M pH 5.5) for 20 min at  
7  
8 RT and then rinsed with water and dried. The surface was then immediately immersed in the TAA  
9  
10 solution in PBS (10 mM pH 7.4) and incubated overnight at 4°C. Finally, biofunctionalized sensors  
11  
12 were carefully rinsed with PBS and water, dried with N<sub>2</sub> stream and mounted in the platform. The  
13  
14 non-sensing glass areas were subsequently coated with PLL-g-PEG (0.5 mg·mL<sup>-1</sup>) to avoid non-  
15  
16 specific adsorptions. Figure S3 in Supplementary Material summarizes the biofunctionalization  
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18 protocol.  
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## 23 **2.4. Antibody detection assays**

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26 For antibody analysis, PBST 0.5% (PBS + 0.5% Tween 20) was settled as running buffer. Different  
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28 concentrations of specific antibody were diluted in PBST 0.5% or in commercial serum/plasma and  
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30 flowed over the functionalized surface at 25 μL·min<sup>-1</sup> (See Figure S9 for an example of real time  
31  
32 senrograms for the detection of specific antibodies at different concentrations). Regeneration of the  
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34 surface was achieved by injecting 20 mM NaOH at 65 μL·min<sup>-1</sup>. Calibration curves were fitted to a  
35  
36 saturation total binding model. Limit of detection (LoD) was calculated as the concentration  
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38 corresponding to the blank signal plus three times its standard deviation (SD), while limit of  
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40 quantification (LoQ) was determined as the concentration corresponding to the minimum measurable  
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42 signal, set as the blank signal plus 10 times SD. Data analysis was performed using Origin Pro  
43  
44 software. ELISA was carried out as previously described [16,28]. Briefly, microtiter plates (Maxisorp,  
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46 Nunc) were coated overnight with 0.3 μg of the purified recombinant proteins, using GST and human  
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48 Annexin IV as negative controls in 50 μl of PBS. After washing three times with PBS, plates were  
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50 blocked with 3% skimmed milk in PBS (MPBS) for 2 h at room temperature. Then, serum samples  
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52 (dilution, 1:100 in 3% MPBS) were incubated for 2 h at room temperature. After washing,  
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54 peroxidase-labeled anti-human IgG (Jackson laboratories) (dilution, 1:500 in 3% MPBS) was added  
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57 for 2 h at room temperature. Then, the signal was developed with 3,3',5,5'-tetramethylbenzidine  
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1 substrate for 10 min (Sigma-Aldrich). The reaction was stopped with 1 M HCl, and absorption  
2 measured at 450 nm.  
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### 5 **3. Results**

#### 6 **3.1. Description of the Nanoplasmonic Biosensor Platform**

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10 The nanoplasmonic device is based on a recently implemented LSPR sensing scheme based on a  
11 waveguided electromagnetic mode that arises in thin monolayers of sparse and randomly distributed  
12 plasmonic nanoparticles [24]. We previously reported that the in-plane LSPR excitation strongly  
13 enhances the polarizability of the nanodisks, creating an effective RI that is sufficiently large to  
14 support a guided electromagnetic mode inside the plasmonic monolayer. Both the nanoparticle surface  
15 density (F) and the incidence angle of light are key aspects that affect this sensing performance.  
16 Surface density was precisely chosen so that the optimal mode excitation (light coupling efficiency  
17 close to 100%) occurs at angles where the sensitivity was maximized (angle close to 90°). In  
18 particular we employ short-ordered arrays of gold nanodisks (diameter D = 100 nm, height H = 20  
19 nm, surface density F = 6-7%) fabricated by hole-mask colloidal lithography (HCL) (See  
20 experimental details in Supplementary Material). This nanofabrication technique allows simple, cost-  
21 efficient and wafer-scale production of the nanoplasmonic chips. This waveguided mode results not  
22 only in a large increase of the RI sensitivity, but also strongly improves the signal-to-noise ratio. Both  
23 effects assure an overall improved RI sensing performance that is up to one order of magnitude better  
24 than that of isolated non-interacting nanodisks. Thus, RI changes occurring close to the nanodisk  
25 surfaces are much easier to detect. A schematic representation of the biosensor can be seen in Figure  
26 1a. For LSPR excitation, the nanoplasmonic chip is illuminated with a broadband polarized light at a  
27 determined angle of incidence ( $\Theta = 80^\circ$ ) [24,26], and the reflected light is collected with a  
28 spectrophotometer. The obtained spectra show a deep reflectivity dip at  $\lambda_{\text{LSPR}} \approx 750$ . Biomolecular  
29 interactions occurring on the nanodisks surfaces generate RI changes that, in turn, cause  
30 displacements of the spectral LSPR peak ( $\Delta\lambda_{\text{LSPR}}$ ) (red shifts when binding occurs and blue shifts  
31 during a desorption process). The real-time interrogation of this  $\Delta\lambda_{\text{LSPR}}$  enables the extraction of  
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1 quantitative information related to the biomolecular interactions taking place in a label-free manner  
2 (Figure 1.B). In addition, the designed optical platform has very small dimensions (all optical  
3 components are mounted on a 20 x 20 cm<sup>2</sup> portable breadboard), exemplifying its facile  
4 miniaturization and potential portability.  
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### 8 9 **3.2. Design and Optimization of the Sensor Biofunctionalization**

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11 Among the numerous TAAs defined for colorectal cancer [18], we selected GTF2b (general  
12 transcription factor IIB) and EDIL3 (EGF-like repeats and discoidin I-like domain 3 protein) since  
13 both proteins have been previously evaluated as possible TAA targets for autoantibody production in  
14 colorectal cancer [14]. A study performed in cancer-induced animals provided evidences of  
15 immediate production of GTF2b and EDIL3 autoantibodies, among others [14]. The presence of  
16 autoantibodies was detectable at a very early stage in tumor development, even before adenoma  
17 formation. Especially, GTF2b could be detected before clinically observable symptomatology while  
18 EDIL3 is characterized by a more homogeneous but late response. This makes GTF2B more  
19 appropriate to enhance sensitivity while EDIL3 would enhance specificity.  
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34 In order to directly detect the autoantibodies for these two TAA, a biosensing strategy based on the  
35 direct immobilization of the TAA on the surface of the gold nanodisks has been addressed. A  
36 schematic representation of the proposed biosensor strategy is showed in Figure 1C.  
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38 Biofunctionalization of the nanoplasmonic sensor chip was based on the formation of a functional  
39 alkanethiol self-assembled monolayer (SAM) specifically onto the gold nanodisks *via* thiol  
40 chemisorption, which act as linker for the covalent attachment of the antigens. We employed 16-  
41 mercaptohexadecanoic acid (MHDA) to create a tight and uniform SAM where the carboxylic groups  
42 of the MHDA are activated to readily react with lysine (Lys) residues available in the proteins. The  
43 reaction generates an amide bond between the protein and the SAM. The grafting density of antigen  
44 molecules on the surface can also be controlled by introducing a lateral spacer during the formation of  
45 the SAM (i.e. 11-mercaptoundecanol, MUOH). In parallel, glass substrate was coated with the  
46 copolymer poly-L-lysine PEG (PLL-g-PEG 0.5 mg·mL<sup>-1</sup>) to prevent and minimize possible undesired  
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1 adsorptions. The PLL-g-PEG coating generates a highly hydrophilic layer that has demonstrated to  
2 effectively reduce nonspecific binding of proteins and other compounds present in biological  
3 matrices. This step facilitates the direct measurement in these biological fluids when using label-free  
4 biosensors [26,29].  
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9 To establish the best immobilization conditions for both TAAs, the nanoplasmonic chips were  
10 independently biofunctionalized with recombinant human GTF2b and EDIL3 proteins employing  
11 different molar ratios of mixed alkanethiol SAM (MHDA/MUOH 1:0, 1:1, 1:10) at a total thiol  
12 concentration of 250  $\mu\text{M}$ . The immobilization procedure was carried out *in situ* over the SAM-  
13 functionalized chip already mounted on the sensor platform, by flowing the protein solution and  
14 monitoring the covalent coupling process (Figure S4 in Supplementary Material). We compared the  
15 immobilization signals obtained with a fixed protein concentration ( $50 \mu\text{g}\cdot\text{mL}^{-1}$ ) over the different  
16 SAM ratios. We selected  $50 \mu\text{g}\cdot\text{mL}^{-1}$  based on preliminary experiments performed with a  
17 conventional SPR biosensor. We observed that although higher concentrations of protein (i.e. 100  
18  $\mu\text{g}\cdot\text{mL}^{-1}$ ) rendered higher immobilization signal, the subsequent detection of the autoantibody (at a  
19 fixed concentration of  $1 \mu\text{g}\cdot\text{mL}^{-1}$ ) remained similar to the one obtained with a protein concentration of  
20  $50 \mu\text{g}\cdot\text{mL}^{-1}$  (see Figure S5 in the Supplementary Material). This was indicative of a more optimum  
21 coverage of the surface for detection purposes with  $50 \mu\text{g}\cdot\text{mL}^{-1}$  than with  $100 \mu\text{g}\cdot\text{mL}^{-1}$ . Based on  
22 these results and on previous conditions optimized for protein attachment to SAMs [27] we selected  
23  $50 \mu\text{g}\cdot\text{mL}^{-1}$  of protein for further experiments. The highest amount of protein was attached to the  
24 sensor surface when maximum carboxylic density was employed (Figure S6a in Supplementary  
25 Material), inducing wavelength shifts around 3 nm. This was observed for both GTF2b and EDIL  
26 proteins. Introduction of spacer molecules (MUOH) to the SAM resulted in lower signals thereby  
27 indicating less amount of TAA immobilized. However, the optimum TAA layer, which will lead to  
28 better detection levels, does not necessarily require maximum coverage with proteins. The appropriate  
29 distribution of antigens on the surface reveals itself as an important factor to favor the accessibility of  
30 antibodies. Control of the spacing between TAA molecules can *a priori* modulate possible steric  
31 hindrance effects and improve the ability of the antibodies to interact. We evaluated the detection  
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1 efficiency by flowing a  $1 \mu\text{g}\cdot\text{mL}^{-1}$  specific antibody solution over the corresponding GTF2b and  
2 EDIL3 functionalized surfaces. This concentration was selected for screening purposes in order to  
3 choose the best immobilization conditions before performing a full calibration curve. In addition,  
4 TAA immobilization procedure was also carried out *ex situ*, by coating the functionalized sensor chip  
5 with the protein overnight, rinsing it with buffer and then installing the protein-modified chip in the  
6 biosensor. The antibody detection signal achieved for both *ex situ* and *in situ* procedures carried out  
7 with mixed alkanethiol SAMs at different molar ratios was compared and is summarized in the Figure  
8 S6b in the Supplementary Material). No steric hindrance effects appeared to be relevant for the  
9 concentration of TAA tested ( $50 \mu\text{g}\cdot\text{mL}^{-1}$ ) as maximum antibody detection was obtained in all cases  
10 when maximum carboxylic density was used (MHDA/MUOH 1:0). This suggests that the  
11 immobilization of TAAs at the selected concentration on alkanethiol SAMs formed exclusively with  
12 MHDA actually provides highly efficient bioreceptor layers, as also previously reported [30]. It can  
13 also be appreciated that *ex situ* immobilization resulted in significant higher antibody signals for the  
14 same concentration of immunoreagents. This could be attributed to a more efficient coupling yield  
15 and also to a more efficient protein rearrangement on the surface facilitated by longer reaction times  
16 (protein coupling overnight vs. 30 min when it is done *in situ*). From the above results, overnight  
17 immobilization of TAA over a 100% MHDA SAM was selected as optimum biofunctionalization  
18 strategy for further experiments. Regeneration for a potential reutilization of the TAAs functionalized  
19 surfaces was also evaluated. For both TAA-modified surfaces – GTF2b and EDIL3 – basic conditions  
20 (20 mM NaOH) disrupted the TAA-antibody interaction without altering or modifying the  
21 immobilized proteins (Figure S7 and S8 in Supplementary Material). Under these conditions it was  
22 possible to reuse the functionalized surface with good repeatability for up to 100 cycles before  
23 progressive decrease of the antibody detection signals.

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52 Calibration curves for both anti-GTF2b and anti-EDIL3 (Figure 2A and 2B) were then performed in  
53 standard buffer conditions (i.e. PBS with 0.5% of Tween 20 (PBST)), which according to previous  
54 works helped drastically reduce nonspecific adsorptions onto the sensor chip) [26,27]. Different  
55 antibody concentrations ranging from  $50 \text{ ng}\cdot\text{mL}^{-1}$  to  $1 \mu\text{g}\cdot\text{mL}^{-1}$  were flowed by triplicate through the  
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1 specific TAA functionalized surfaces, respectively, and the resonance shift was obtained (Figure 2A  
2 and 2B). Limits of detection (LoD) were determined as the minimum antibody concentrations that  
3 provide an observable signal (i.e. blank signal plus 3 times its standard deviation). The LoD for anti-  
4 GTF2b assay was  $10 \text{ ng}\cdot\text{mL}^{-1}$  (66 pM) and  $5 \text{ ng}\cdot\text{mL}^{-1}$  (33 pM) for anti-EDIL3 assay. Limits of  
5 quantification (LoQ) were determined as the minimum measurable signal, being  $34 \text{ ng}\cdot\text{mL}^{-1}$  (227 nM)  
6 and  $19 \text{ ng}\cdot\text{mL}^{-1}$  (127 pM) for GTF2b and EDIL3, respectively. Besides, the specificity of the assays  
7 was confirmed by using nonspecific antibodies as control. Measurements of anti-GTF2b over an  
8 EDIL3-functionalized surface and *vice versa* led to negligible signals (Figure 2A and 2B, red lines),  
9 which corroborates the signal contribution solely comes from the specific antibody recognition and  
10 confirms the feasibility of the methodology for antibody quantification. Excellent reproducibility and  
11 stability of the biosensor-based assays were finally demonstrated by performing intra- and inter-  
12 assays (curves performed in the same nanodisk-functionalized surface and in different surfaces,  
13 respectively). As it can be seen in Table 1, the coefficient of variation (CV) for both GTF2b and  
14 EDIL3 was below the maximum variability recommended for clinical analysis ( $\sim 15\%$ ) [31], both for  
15 the LoD and for the maximum signal ( $S_{\text{max}}$ ), taken at the maximum antibody concentration tested  
16 ( $[\text{Ab}] = 1 \text{ }\mu\text{g}\cdot\text{mL}^{-1}$ ).  
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### 36 **3.3. Analysis of TAA Antibodies in Serum and Plasma**

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40 In order to evaluate the influence of the matrix, commercial serum (undiluted, and diluted 1:1 and  
41 1:10 in PBST) and commercial plasma (undiluted, and diluted 1:1 and 1:10 in PBST) were flowed  
42 over the biofunctionalized nanoplasmonic surfaces (i.e. TAA layer + PLL-g-PEG coating) (Figure 3).  
43 As it can be seen in the sensorgrams, in both cases a significant background signal was observed due  
44 to the binding of fluid components onto the bioactive layer, being slightly higher for undiluted plasma  
45 (whose difference compared to serum is the presence of fibrinogen). A 1:10 dilution in PBST was  
46 necessary to achieve a complete reduction of nonspecific adsorptions for both fluids, resulting in  
47 virtually no background signals.  
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1 Besides the influence of the nonspecific binding onto the surface, possible matrix effects on the  
2 interaction of the antibody with the TAA-coated layer can occur, altering the analysis features. In  
3 order to assess this undesired effect, both GTF2b and EDIL3 calibration curves were obtained by  
4 spiking serum or plasma with several known concentrations of antibodies and then diluting them in  
5 PBST (1:10) (Figure 4). The curves were analogous to those obtained with standard buffer conditions  
6 although a slight increase of the LoDs was observed. This minor worsening can be attributed to a  
7 possible hindrance of the antibody/TAA interaction. For the GTF2b antibody, the LoD was  $16 \text{ ng}\cdot\text{mL}^{-1}$   
8 in diluted serum and  $15 \text{ ng}\cdot\text{mL}^{-1}$  in diluted plasma, compared to a LoD of  $10 \text{ ng}\cdot\text{mL}^{-1}$  in buffer. In  
9 the case of EDIL3 antibody detection, LoDs were  $12 \text{ ng}\cdot\text{mL}^{-1}$  in diluted serum and  $11 \text{ ng}\cdot\text{mL}^{-1}$  in  
10 diluted plasma, compared to  $5 \text{ ng}\cdot\text{mL}^{-1}$  in buffer. Overall the sample dilution (1:10) has inevitably  
11 decreased the sensitivity over one order of magnitude, with detectabilities around  $150\text{-}160 \text{ ng}\cdot\text{mL}^{-1}$  for  
12 GTF2b and around  $110\text{-}120 \text{ ng}\cdot\text{mL}^{-1}$  for EDIL3, respectively depending on the fluid.  
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27 Clinical serum samples from CRC patients were analyzed with the nanoplasmonic biosensor for a  
28 preliminary assessment of the viability of our approach. Serum samples collected from patients from  
29 the Hospital of Cabueñes (Gijón, Spain) with diagnosed CRC and samples from healthy individuals  
30 were evaluated employing the optimal conditions selected before (1:10 dilution with PBST). All  
31 samples were previously analyzed for the presence of GTF2b autoantibodies using semi-quantitative  
32 ELISA, so the absolute concentration of the target biomarker was unknown. Table 2 compares the  
33 concentration values obtained with the nanoplasmonic biosensor (in  $\mu\text{g}\cdot\text{mL}^{-1}$ ) to absorbance values  
34 (in optical density units, OD) obtained with the ELISA (quantitative data is not commonly determined  
35 with the ELISA for autoantibodies). A consistent correlation was observed in terms of relative signals.  
36 Negative samples from healthy subjects resulted below the LoD established for our biosensor  
37 technique ( $160 \text{ ng}\cdot\text{mL}^{-1}$ ) while positive samples lead to relatively high signals (i.e. high concentration  
38 of GTF2b autoantibodies).  
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#### 54 **4. Discussion**

55 A major challenge in colorectal cancer research focuses on the development of novel diagnostic  
56 techniques for simple, rapid and accurate detection of the disease at earlier stages. In this regard,  
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1 cancer-associated autoantibodies generated by the immune system during tumor appearance have  
2 evidenced its high value as blood-circulating biomarkers for preclinical cancer diagnosis. We propose  
3 the use of an innovative nanoplasmonic biosensor for the direct and label-free quantification of CRC  
4 autoantibodies in serum or plasma. Our nanoplasmonic biosensor offer real-time detection of TAA  
5 antibodies without the need of any labels or sample pretreatments, which simplifies the analysis and  
6 provides interesting alternatives to develop small, fast and user-friendly point-of-care (POC) devices  
7 that could be used directly at doctor's office. The implementation of POC biosensors for rapid and  
8 reliable CRC screening could substantially afford a breakthrough towards non-invasive and highly  
9 specific diagnostic tools for this disease which in turn would help to improve patient survival rates.

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21 The overall performance of the biosensor assay has been demonstrated for the determination of  
22 autoantibodies against two important CRC antigens: GTF2b and EDIL3. Both autoantibodies were  
23 selected as representative for the disease as they are generated at very early stages of CRC  
24 development and can be detected in blood serum before the onset of tumor lesions [14].  
25 Biofunctionalization of the nanoplasmonic sensors has been designed to create a highly stable TAA  
26 layer that ensures an efficient capture of the specific antibodies. The immobilization strategy is based  
27 on the material-selective functionalization of the gold nanodisks through the formation of an  
28 alkanethiol SAM, which guarantees the biomolecular interaction to take place solely on the sensor  
29 spots. The PLL-g-PEG coating of the glass areas (which represents over the 94% of the surface)  
30 prevents the nonspecific binding to substrate. The covalent coupling of the TAA *via* the terminal  
31 amine groups of Lys residues is a simple and robust procedure that can be applied to immobilize  
32 virtually all proteins. Several biofunctionalization conditions have been optimized (e.g. alkanethiol  
33 SAM ratios, binding time, etc.) for the enhancement of the antibody capture efficacy reaching a LoD  
34 of  $10 \text{ ng}\cdot\text{mL}^{-1}$  ( $\sim 66 \text{ pM}$ ) for GTF2b antibody and  $5 \text{ ng}\cdot\text{mL}^{-1}$  ( $\sim 33 \text{ pM}$ ) for EDIL3 antibody in  
35 standard buffer, showing in both cases high selectivity and reproducibility. A complete assay cycle  
36 (including regeneration) is accomplished in 30 min. Besides, the bioactive surface with the  
37 immobilized TAA has proven to be reusable for more than 100 cycles with good repeatability.

1 An ultimate goal in POC development relies on the ability to readily detect the biomarkers in  
2 biological fluids. Here, we have exploited the unique hydrophilic properties of the PLL-g-PEG  
3 coating layer together with the use of a dilution buffer containing a high concentration of surfactant  
4 (Tween 20) in order to minimize possible interferences coming from serum or plasma matrices. In  
5 particular, a simple dilution of the biological sample 1:10 with PBST has led to a complete removal of  
6 background signals enabling the direct quantification without requiring purification or other extra  
7 pretreatments. This dilution factor inevitably increases the limit of detection. However, it is much  
8 lower than the minimum dilution required in the ELISA assay. Overall the features of the resultant  
9 calibration curves, both in diluted serum and plasma, offer a highly reliable analysis method to  
10 quantify the TAA autoantibodies with elevated selectivity and reproducibility. Moreover, clinical  
11 samples analysis further demonstrates the potential of using this device in comparison to conventional  
12 ELISA methods, according to the good correlation observed for the detection of GTF2b antibodies in  
13 CRC-diagnosed patients and healthy individuals. In this regard, a more complete quantitative clinical  
14 validation will be required in the future. However, to our knowledge, serological concentration levels  
15 of CRC autoantibodies have not been fully established so far, as most research articles in the field  
16 especially focus on their identification and the assessment of their diagnostic and/or prognostic value.  
17 Nevertheless we cannot obviate the evident usefulness of knowing this concentration value compared  
18 with other semi-quantitative or qualitative methods, not only from a perspective of early diagnosis but  
19 also for disease follow-up. Besides, the possibility of quantifying autoantibodies concentration in  
20 serum samples may allow further comprehension of the humoral response triggered by the tumor and  
21 harness the basis for the improvement of prognosis of the disease. On-going work currently focuses  
22 on the improvement of biofunctionalization strategies and the use of antifouling agents that permit  
23 direct measurements of undiluted serum and plasma, therefore enhancing the detectability at least 10-  
24 fold. Moreover, the biofunctionalization methodology developed can be easily adjusted for any TAA  
25 with potential interest. This facilitates the eventual expansion of the biosensor strategy to elaborate a  
26 multiplexed compact analytical platform for the simultaneous detection of a CRC-specific panel of  
27 autoantibodies, which is ultimately necessary to fully cover the patient variability and to maximize the  
28 sensitivity and specificity of the method.  
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## 5. Conclusions

We have developed a novel analytical label-free strategy for the detection of tumor-associated autoantibodies in blood serum and plasma based on an innovative nanoplasmonic biosensor technology. This strategy could provide a reliable and non-invasive screening and diagnosis of colorectal cancer at early stages. Our biosensor allows the label-free quantification of specific CRC-related autoantibodies in few minutes, without requiring any sample purification or pretreatment. Several biofunctionalization parameters have been optimized, reaching a limit of detection of 1 nM (150-160 ng·mL<sup>-1</sup>) for direct measurements in human serum or plasma. Selectivity and reproducibility of the assay have been also evaluated demonstrating the excellent accuracy and robustness of the biosensor. The analysis of clinical samples from colorectal cancer patients has shown good correlation with ELISA. Overall, the results obtained highlight the exceptional potential of our nanoplasmonic biosensor as a tool for the early detection of colorectal cancer and current efforts are focused on establishing a multiplexed approach to expand the strategy to a CRC-panel of autoantibodies.

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**Figure Captions:**

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2 **Figure 1. (a)** Schematic representation of the miniaturized nanoplasmonic biosensor. A picture of the  
3 nanodisks and a SEM image of the actual shape of the gold nanodisks fabricated on the glass substrate  
4 are also shown. **(b)** Graphs showing the resonance peak (photon counts vs.  $\lambda$ ) (*left*) and the shift of the  
5 resonance peak over time ( $\Delta\lambda_{\text{LSPR}}$  vs. time) (*right*); **(c)** TAA biofunctionalization methodology based  
6 on covalent coupling to an alkanethiol SAM and subsequent antibody detection.  
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15 **Figure 2. (a)** Calibration curve for anti-GTF2b detection performed over GTF2b-biofunctionalized  
16 nanodisks (black). Red dashed line indicates nonspecific adsorption of a control antibody (anti-  
17 EDIL3); **(b)** Calibration curve for anti-EDIL3 detection performed over EDIL3-biofunctionalized  
18 nanodisks (black). Red dashed line indicates nonspecific adsorption of a control antibody (anti-  
19 GTF2b).  
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29 **Figure 3. (a)** Background signal corresponding to nonspecific adsorption of undiluted serum (black),  
30 serum diluted 1:1 in PBST 0.5% (purple) and serum diluted 1:10 in PBST 0.5% (green); **(b)**  
31 Background signal corresponding to nonspecific adsorption of undiluted plasma (blue), plasma  
32 diluted 1:1 in PBST 0.5% (orange) and plasma diluted 1:10 in PBST 0.5% (pink).  
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40 **Figure 4. (a)** Calibration curves for anti-GTF2b antibody detection in PBST buffer (black), serum  
41 diluted 1:10 in PBST (green) and plasma diluted 1:10 in PBST (pink); **(b)** Calibration curves for anti-  
42 EDIL3 antibody detection in PBST buffer (black), serum diluted 1:10 in PBST (green) and plasma  
43 diluted 1:10 in PBST (pink).  
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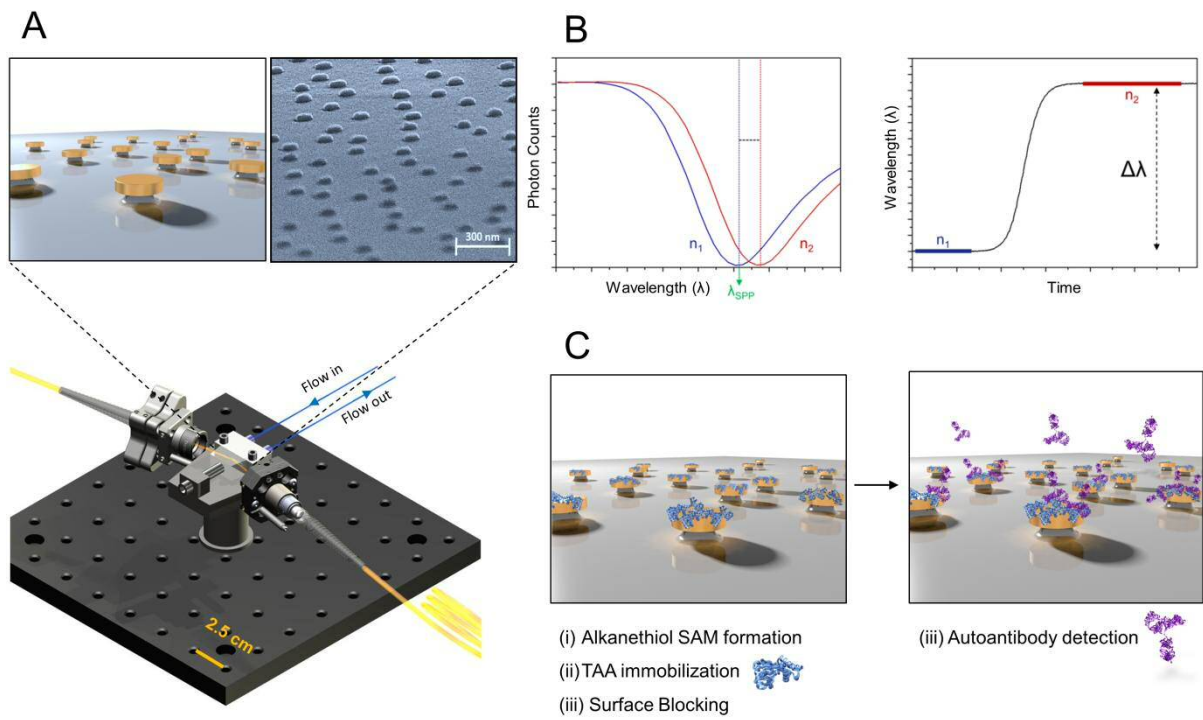


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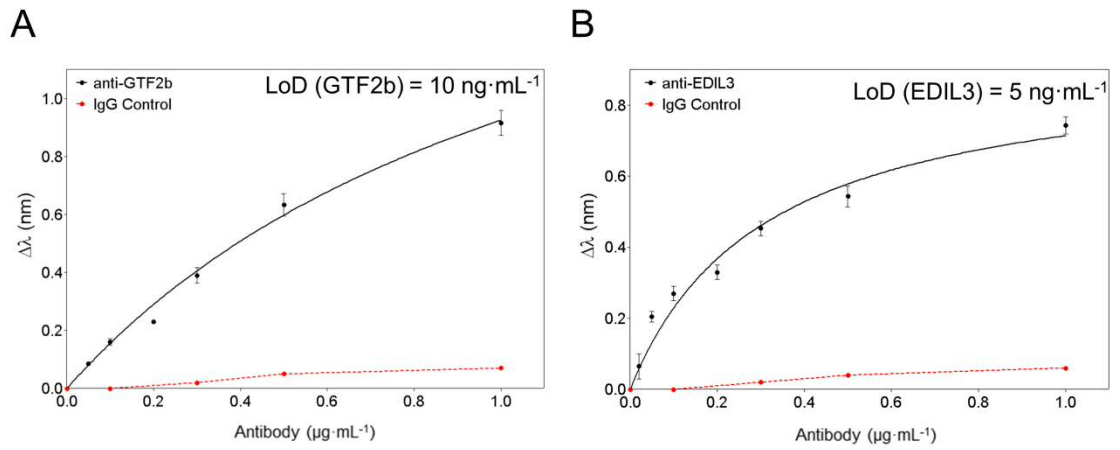
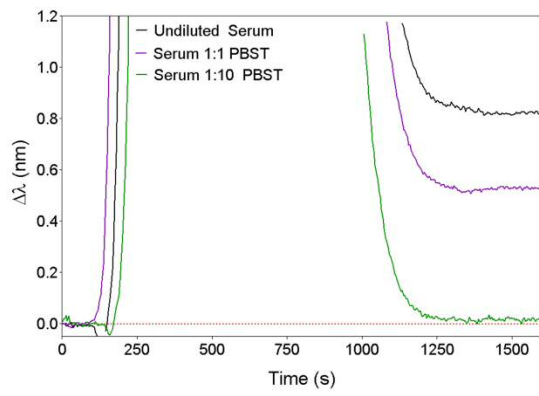


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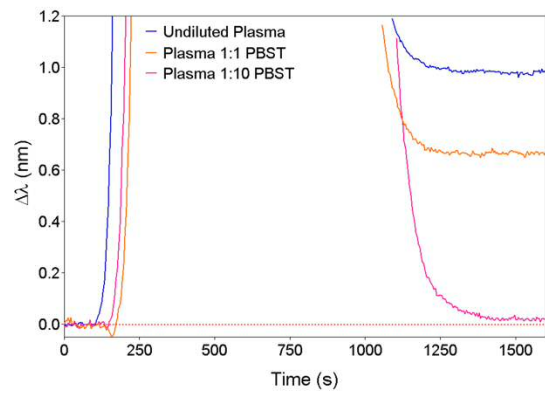
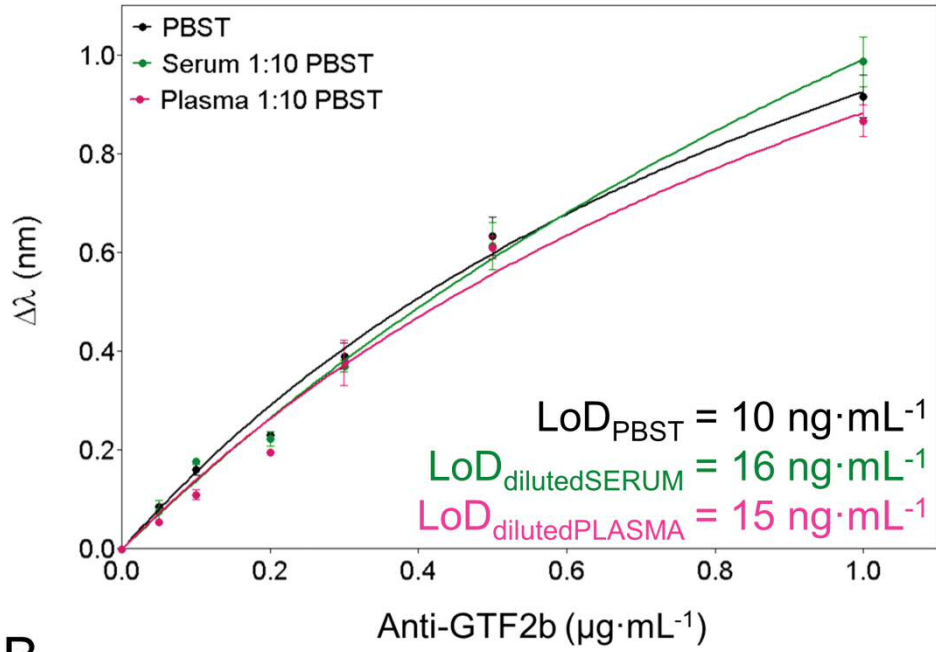


Figure 3

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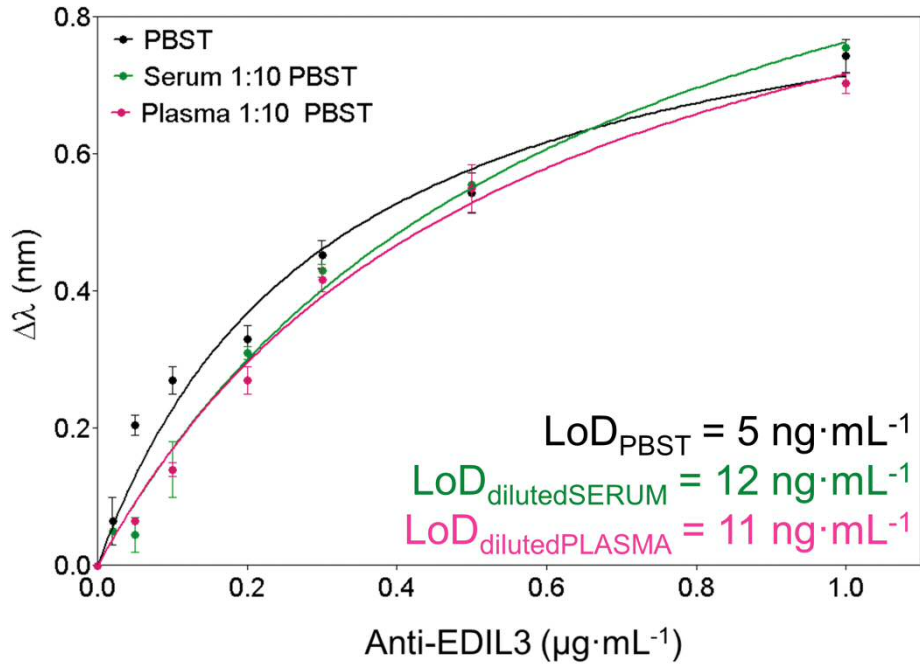


Figure 4



**Table 1.** Inter and intra-assay features for GTF2b and EDIL3 antibodies detection with the nanoplasmonic biosensor

		<b>GTF2b antibody</b>		<b>EDIL3 antibody</b>	
		<b>Mean <math>\pm</math> SD<sup>*</sup></b>	<b>% CV</b>	<b>Mean <math>\pm</math> SD</b>	<b>% CV</b>
<b>Intra-assay</b>	<b>LOD (ng·mL<sup>-1</sup>)</b>	9.7 $\pm$ 0.5	5.15	5.2 $\pm$ 0.2	3.77
	<b>S<sub>max</sub> (nm)</b>	0.937 $\pm$ 0.0015	1.63	0.733 $\pm$ 0.016	2.08
<b>Inter-assay</b>	<b>LOD (ng·mL<sup>-1</sup>)</b>	10.1 $\pm$ 1.2	11.9	4.9 $\pm$ 0.4	8.16
	<b>S<sub>max</sub> (nm)</b>	0.917 $\pm$ 0.07	8.19	0.743 $\pm$ 0.04	5.60

\* Mean and standard deviation of 3 replicates

**Table 2.** Clinical serum samples analysis determined by ELISA and by the nanoplasmonic biosensor.

Sample	GTF2b Analysis Results		
		ELISA (OD)	Nanobiosensor (ng·mL <sup>-1</sup> )*
<b>G30</b>	Negative	0.18	ND <sup>†</sup>
<b>G42</b>	Positive	0.48	175 ± 8
<b>G56</b>	Positive	0.56	254 ± 10
<b>G101</b>	Negative	0.13	ND <sup>†</sup>

\* Mean ± SD for 3 replicates

<sup>†</sup> ND: No Detected (below limit of detection: 160 ng·mL<sup>-1</sup>)

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