



BLOCH ELECTROMAGNETIC SURFACE WAVE
BIOSENSORS FOR EARLY CANCER DIAGNOSIS

Final Publishable Summary Report

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1. Executive Summary

The small or medium scale focused research project (STREP) ***Bloch electromagnetic surface wave bio-sensors for early cancer diagnosis (BILOBA)*** developed and pre-clinically validated a multifunctional point-of-care platform capable of performing real-time cancer biomarker detection in a tandem configuration. The configuration exploits a label-free detection based on the resonance shifts, and the spectral analysis of enhanced fluorescence emitted by biomolecules immobilized on the surface. Utilizing both labelled and label-free analysis on one sensor increases the sensitivity and the reliability of optically read-out surface bound assays.

The standard optical label-free detection is the surface plasmon resonance (SPR) method. Its sensitivity suffers from the strong absorption of waves bound to the metal surface. Here, a similar concept has been advantageously implemented by applying the unique properties of Bloch Surface Waves (BSW) sustained on 1D Photonic Crystals (1DPC). Therein, a surface wave without absorption is excited, giving rise to a pronounced narrowing of resonances and an associated increase in sensitivity. Furthermore, fluorescence enhancement due to near field effects is exploited. By engineering the BSW dispersion both detection schemes are combined in the same platform.

The project has explored, designed, and set-up BSW systems optimized for analytical sensing, and developed a corresponding instrument. Immobilization protocols and biochemical assays have been established for optimizing the binding site surface density and for detecting the target biomarkers. The development of a sophisticated, robust fluidic system to ensure a high signal-to-noise ratio even in the case of lowest analyte concentrations accompanied this work.

The biosensing platform developed by the project partners has been applied to cancer biomarker detection and validated in tests in a pre-clinical environment. The target application is the detection of Angiopoietin-1, Angiopoietin-2 and Vascular Endothelial Growth Factor with these proteins being indicative of angiogenesis associated to human cancer progression.

The project started in October 2012 and lasted 39 months until December 2015. BILOBA was implemented by a consortium of nine European beneficiaries with particular competence in the field, consisting of four universities, one large research institution, and four enterprises from four different countries.

The main project achievements are reported on a dedicated website available at www.biloba-project.eu and continuously updated with new details.

2. Summary Description of project context and objectives

2.1 Project Context

Cancer is one of the major devastating diseases worldwide, and the astonishing impact of this plague at the global level is testified by the fact that this year over 7 million people will die worldwide, and at least 10 million will be diagnosed with this pathology.^{1,2}

Prevention, early diagnosis and more effective treatments are the natural recipe to counteract this spreading pandemic; while prevention and identification of healthy lifestyles with mitigation of risk factors is something that mostly requires focused communication activities and mass sensitisation campaigns. A strong research effort is needed to push the diagnostic performances of current methodologies used to detect cancer beyond their current limitations, and to devise more effective and less harmful therapeutic approaches.

Although equally important, these two research lines should be analysed also from another point of view, starting from two surprising evidences:

- Several studies report that cancer misdiagnosis occur in around 10% of cases, with tremendous consequences in terms of patient morbidity and cost.³
- At the same time, timely and accurate detection of cancer is associated with increased patients' life expectancy and quality of life; the survival rate for many cancers in fact approaches 90% if one is able to detect the pathology in its first stage of development.^{4,5,6}

Although a care driven approach with an emphasis on new drugs has dominated the scene in the last 30 years, with scant results in terms of drop in mortality rate, the real problem is that we currently lack the capability to systematically detect cancer at its very early stage, due to the technological limitations of the diagnostic approaches currently available; these are clearly evident in well-established and validated systems such as imaging techniques (Magnetic Resonance Imaging, X-Rays, Computer Aided Tomography) as well as in biomarkers based analysis, where the performances in terms of detection limit and accuracy still have to be improved.

Other elements confirm the need to improve state of the art cancer screening techniques:

¹ World Health Organization, Cancer Fact Sheet, October 2011, available online:

<http://www.who.int/mediacentre/factsheets/fs297/en/>

² World Health Organization, "Global cancer rates could increase by 50% to 15 million by 2020", 2003

³ Hardeep Singh, Saurabh Sethi, Martin Raber, and Laura A. Petersen, Errors in Cancer Diagnosis: Current Understanding and Future Directions, *Journal of Clinical Oncology*, vol. 25 N.31, November 2007

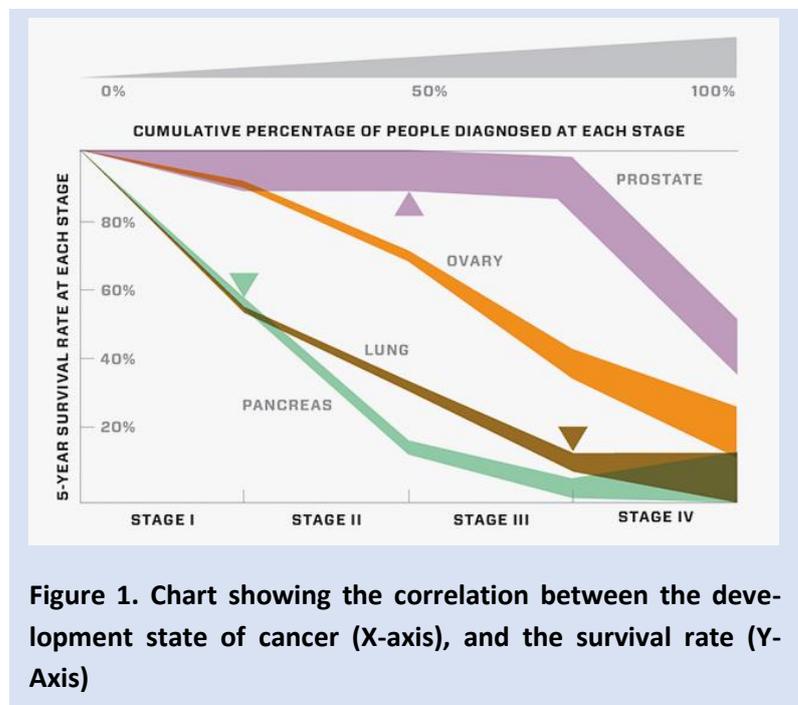
⁴ Horner MJ, Ries LAG, Krapcho M, Neyman N, Aminou R, Howlader N, Altekruse SF, Feuer EJ, Huang L, Mariotto A, Miller BA, Lewis DR, Eisner MP, Stinchcomb DG, Edwards BK (eds). SEER Cancer Statistics Review, 1975-2006, National Cancer Institute. Bethesda, MD, http://seer.cancer.gov/csr/1975_2006/, based on November 2008 SEER data submission, posted to the SEER web site, 2009

⁵ American Cancer Society. Cancer Facts & Figures 2009. Atlanta: American Cancer Society, 2009.

⁶ <http://seer.cancer.gov/statfacts/>

- Invasive techniques based on taking a biopsy and then examining the tissue using cell fixation and morphology approaches are not individually conclusive, as tissue removal can miss cancer cells in the earlier stage of the disease.
- More recent techniques use immunoassays (e.g. ELISA) to detect cancer biomarkers; however, in most cases only one biomarker is used as an indicator of the disease. Immunoassays are very sensitive and selective, but can be time consuming and expensive. In addition to this some ELISA tests are not sufficiently sensitive for the detection of low level marker concentrations which exist in the early stages of the disease. The same can be said for the most promising optical technique, Surface Plasmon Resonance (SPR) that has not yet succeeded in entering the diagnostics market in the cancer field.
- The use of molecular tools (genomic and proteomics) to create molecular signatures of the disease have been employed recently. These techniques are producing data which are also useful in understanding the disease, but are complex and require data management as well as linking to patient information.

This reality clearly suggests that there is an unmet societal need and a strong business case to boost the innovation in the early diagnosis of malignancies, through the development of easy to use robust instrumentation, which can be available to a large set of medical users. This result could also support the medical community with information on the underlying mechanism of the initiation of a disease, the process of ingravescence, and ultimately provide a method to diagnose and treat the disease with appropriate measures at the desired time.



Core photonic technologies possess a high potential for successfully addressing and solving the actual needs and problems outlined above. With the available know-how, ranging from rigorous calculation methods for the optical response of micro and nanostructures, to the fabrication/deposition techniques of thin metallic, dielectric organic and inorganic films, together with the ability to integrate organic functional films and microfluidics and Lab-on-a-chip on passive/active photonic structures, it should be possible to provide an integrated device for tumoral biomarker detection.

2.2 Project Objectives

BILOBA aimed to achieve the ambitious mission to reveal the cancer before it reveals itself by developing and pre-clinically validating a compact and multifunctional point-of-care platform that is capable of performing real-time cancer biomarker detection in a tandem configuration. The bio-sensing platform consists of disposable and low cost dielectric one dimensional photonic crystal (1DPC) biochips and an analytical instrument. The breakthrough is the capability of properly designed 1DPC to sustain Bloch electromagnetic Surface Waves (BSW) at their surface, whose peculiar properties can be exploited for overtaking the limitations to sensitivity, selectivity, and resolution of present point-of-care cancer diagnostic tools and leading to a new class of advanced industrial products.

The target application of the proposed sensing platform was the detection of biomarker proteins for early cancer diagnosis. In particular the project was focused on the detection at a very low concentration of angiopoietin -1 and -2 and vascular endothelial growth factor, which have been identified as tumor inducers⁷. Before the project start, such markers had already been studied by some of the project partners both from the medical and from the sensing application point of view. In particular at a medical level, the partners had set up preclinical and clinical strategies to correlate disease progression and disease-free survival with molecular and biological markers of angiogenesis.

BILOBA's main goal was to set a new standard in point-of-care cancer diagnosis, where Europe has a leading position, by developing an alternative to surface plasmon polaritons (SPP), currently used in surface polariton resonance (SPR) devices, by exploiting the potential of Bloch surface electromagnetic waves (BSW) at the surface of finite dielectric stacks. For cancer biomarkers' detection at a very low concentration, where superior sensing performance is needed, the absence of damping in dielectrics with resulting narrower resonances and the possibility to engineer the dielectric stack to tailor the surface waves' dispersion provides a relevant and significant improvement. New performances, otherwise impossible with SPP in metal based sensors, were made available, such as both polarizations accessibility (TM and TE), the pronounced dispersion of BSW for fluorescence detection and the exploitation of the variety of dielectrics' surface chemistry in order to improve the selectivity of the sensors.

2.3 Project Partners

The BILOBA project started in October 2012 and lasted 39 months until December 2015. It was implemented by a consortium of nine European beneficiaries (Table 1 and Figure 2) from four different countries with particular competence in the field, consisting on four universities, one large research institution, and four enterprises.

⁷ J.Folkman, Angiogenesis, *Ann. Rev. Med.*, **57**, 1-8 (2006); J.Folkman, Angiogenesis: an organizing principle for drug discovery?, *Nat. Rev. Drug Discov.*, **6**, 273-286 (2007).

Participant organisation name	Country
Università degli Studi di Roma "La Sapienza" - Department of Basic and Applied Sciences for Engineering	Italy
Politecnico di Torino - Department of Applied Sciences and Technology	Italy
Fraunhofer Gesellschaft zur Förderung der Angewandten Forschung <ul style="list-style-type: none"> • Unit a – IOF Institut für Ange-wandte Optik und Feinmechanik • Unit b - IWS – Institut für Werk-stoff- und Strahltechnik 	Germany
Imperial College London - Department of Materials	United Kingdom
Università degli Studi di Torino - Department of Oncology	Italy
Labor S.r.l.	Italy
Biotray	France
Horiba JobinYvon	France
KDS Radeberg	Germany

Table 1. Partners of the BILOBA project



Figure 2. Geographical map highlighting the countries involved in the project.

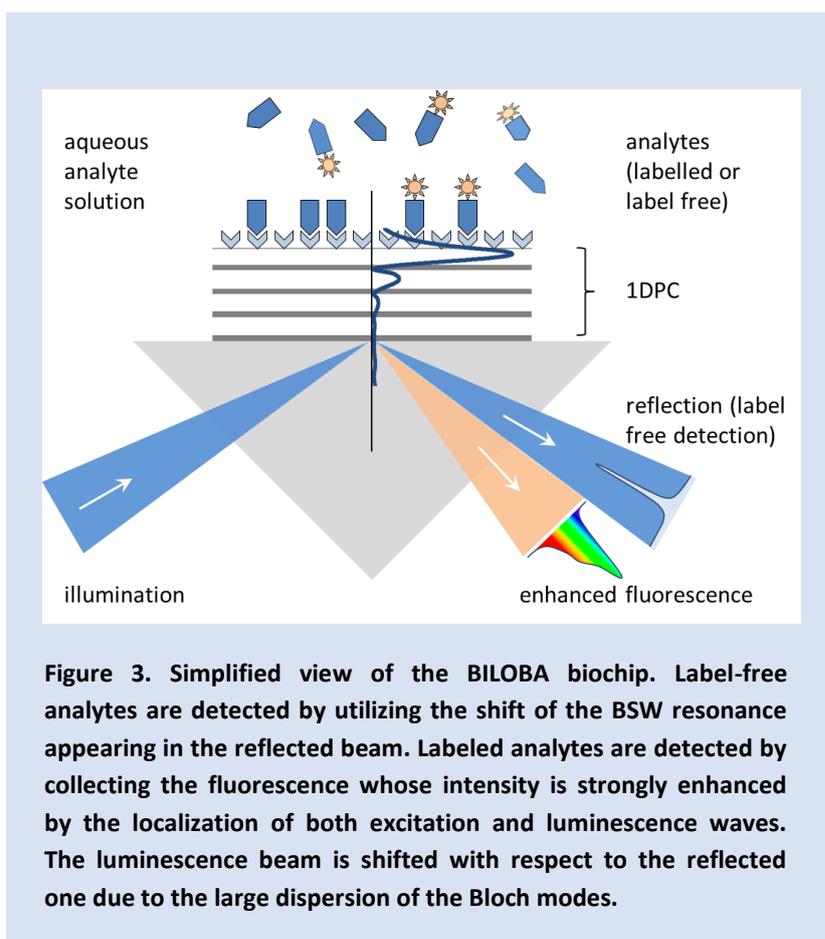
3. Description of the main S&T results/foregrounds

BILOBA developed and pre-clinically validated a compact and multifunctional point-of-care platform for real-time detection of cancer biomarkers in biological fluids with high sensitivity in a tandem configuration. The biosensing platform consists of disposable and low cost dielectric one-dimensional photonic crystal (1DPC) biochips and a reading device. A properly designed 1DPC sustains Bloch electromagnetic surface waves (BSW) at its surface. The ability of BSW to overcome the limitations of selectivity and resolution of the conventional point-of-care cancer diagnostic tools is a major breakthrough of the BILOBA platform, which would lead to a new class of advanced diagnostics. In addition, the BILOBA biochips are capable of analyzing 1D spot arrays, increasing the assay multiplicity and redundancy.

While the BILOBA platform enables efficient detection of diverse analytes, we targeted the detection of biomarker proteins for early cancer diagnosis. In particular, the project was focused on the ultrasensitive detection of vascular endothelial growth factor, angiopoietin 1 and 2 proteins, which are known to induce angiogenesis, thus providing important tumor markers.

The working principle of BILOBA is schematically presented in Figure 3. The two basic elements constituting the tandem approach are the label-free detection based on a BSW resonance shift, and the spectral analysis of enhanced fluorescence emitted by biomolecules immobilized on the biochip surface.

In the label-free approach, BSW are used in a SPR-like system exploiting the resonance shifts for detection (Figure 3, blue reflected beam). Owing to the tunability of the geometry of the 1DPC structures and the low absorption of the dielectric materials employed, BSW can be excited in a wide spectral range (from NIR to visible) and provide from ten to hundred times narrower resonances compared to those associated to surface plasmon polaritons (SPP)



and five to fifty times better resolution for surface modifications.

These advantageous features of BSW enable detection of low concentration of biomarker, leading to earlier cancer detection.

On the other hand, the fluorescence mode utilizes a strong field confinement at the very last interface of the photonic crystal to enhance the excitation of fluorescent labels placed in close proximity of the surface by 2 to 3 orders of magnitude. Again, this enhancement should enable detection of smaller concentrations of cancer biomarkers. As a complementary mechanism, the Stokes-shifted fluorescence emission from labels immobilized at the surface is preferentially coupled to BSW sustained by the 1DPC and leaks back into the prism, as shown in Figure 3 (pink beam). This results in a further improvement of fluorescence collection. The angular separation between the reflected and the fluorescence beam is guaranteed by the strong dispersion of BSW modes.

A crucial requirement of cancer diagnostics is to attain specific binding of a biomarker to the sensor surface. Therefore, functionalisation of the sensor biochips was a key point and was efficiently achieved by BILOBA, leading to the fabrication of a sensitive and selective biosensor device. Different surface functionalization routes were developed in the project to achieve broader applicability.

Apart from the BSW biochips, the BILOBA platform includes a purposely designed and fabricated analytical instrument that carries out the optical detection process in a microfluidic environment. The fluidic chamber allows the automated and uniform mixing of the assay solutions to facilitate interaction of the biomarker with the properly functionalized biochip surface.

3.1 Description of the work performed since the beginning of the project and the main results achieved

The BILOBA project has concluded its technical activities after 39 months, and throughout the project duration an outstanding collaborative effort has been dedicated under the supervision of the Executive Committee, the Coordinator, the Management Support Team and the work package Leaders, making it a smooth and successful STREP project.

During the initial phase of the project, all RTD activities were carried out in the frame of a "System Requirements" work package. All partners worked in close collaboration to define system specifications related to the three key elements of the BILOBA project: biomarkers recognition protocol, analytical instrument, and disposable BSW biochips. The System Requirements work package ended producing the three related deliverables and the BILOBA requirements/specifications matrix. Such deliverables aimed at building a user friendly decision support system for the design phase that started subsequently.

The three core BILOBA work packages attributed to the preparation of BSW Chips, the Analytical Instrument and the Interfaces and Biochemistry started following this common

specifications. Target of these three work packages was the development and set-up of technical hard- and software, patterned surface functionalization and workflows for performing biomarker detection assays.

In the work package related to BSW Biochips, the partners carried out the following work and achieved the related results:

- Development of physical/mathematical tools and related software to be used to design and optimize 1DPC sustaining BSW for optical bio-sensing
- Design, fabrication and integration of optimized plastic substrates for the final biochips
- Design and test of optimized low loss, BSW supporting thin film systems on polymers
- Fabrication of the biochips operating at several different wavelengths
- Test of the BSW biochips performance for label-free and fluorescence operation

The work package related to the development of the Analytical Instrument was focused on the following major tasks and obtained the related results:

- Design, fabrication and testing of BSW biochips working with a preliminary optical system for the label-free and fluorescence operation
- Final design and fabrication of an optical system capable of working in both label-free and fluorescence modes
- Development and fabrication of an integrated analytes handling system
- Fabrication of two types of microfluidic cells (two components injection molded and multilayer) to be integrated with the plastic biochip
- Development and fabrication of the system electronics including firmware and user software;

The partners involved in the Interfaces and Biochemistry work package carried out the following work with the related results:

- Development of two different routes to deposit functional coatings on the surface of the BSW biochips by means of plasma-polymerisation and of wet functionalisation protocols
- Optimization of the assays based on standard fluorescence readout
- Production of functionalized BSW biochips by plasma-polymerization
- Production of functionalized BSW biochips by optimized wet techniques

Following the initial tasks, a test phase completed the development of the Analytical System. It

was devoted to carry out the physical and chemical characterization of the complete BILOBA platform utilizing the technical solutions developed in the core technical work packages BSW Chips, Analytical Instrument, and Interfaces and Biochemistry. Moreover, comparative measurements were performed with the aim of testing the performance of the BILOBA platform versus state of the art commercial SPR assays. The significant results obtained were:

- Determination of the overall system performance on standard samples, in terms of label-free and fluorescence detection;
- Comparison and rating of the results from the BSW platform with those of a standard SPR platform;
- Implementation of first assays on the BILOBA platform.

The work package related to Pre-Clinical Tests started its activities already at an early stage of the project. It was focused in its initial period on the ethical management of the project activities and on the design of the assay protocol to be used for detecting the bio-markers targeted by BILOBA. During the second period, the work package operated in parallel to the core technical work packages and produced the following results:

- Setting up procedures to concentrate analytes and to eliminate albumin and immunoglobulin from plasma that could interfere in the measurements
- Preparation of ELISA assays for VEGF, Ang-1 and Ang-2
- Genetically engineer tumor cell lines overexpressing Ang-1, Ang-2 and VEGF

Once the BILOBA platform was finalized, it was used in performing the pre-clinical assays for cancer biomarker detection and validating the system. For this purpose the growth factors VEGF, Ang-1 and Ang-2 have been analyzed in

- buffered solutions
- cell cultures
- plasma from transgenic mouse models
- sera from patients with metastatic colorectal cancer

All the research work in the project was accompanied by a work package devoted to the Dissemination and Exploitation of the results. The scientific results were disseminated through publication of articles in peer-reviewed journals and participation at scientific conferences. A list of accepted/submitted papers and conference proceedings has been regularly updated on the BILOBA web-site (www.biloba-project.eu). BILOBA published Project Fact Sheets and a foldable flyer was distributed at conferences focusing on biosensing. A complete Plan for the Use, Dissemination and Exploitation of the Foreground of the project BILOBA was prepared.

3.1.1 1DPC stacks and biochips

During the project, the partners SAPIENZA, POLITO and FRAUNHOFER purposely developed numerical simulation tools that were used to design and optimize 1DPC, in order to simultaneously maximize the performance of the complete biochips for both the label-free and fluorescence operation. Several different families of 1DPC were designed, fabricated and tested during the first period of the project before getting to a final optimized performance.



Figure 4. Molded disposable polymer BILOBA biochips with two-components microfluidic element.

Two different 1DPC deposition technologies were developed in parallel in the frame of the project, one based on Ta₂O₅, SiO₂, TiO₂ dielectrics deposited by plasma ion assisted deposition under high vacuum conditions (PIAD, FRAUNHOFER) and one based on binary and ternary silicon alloys deposited by plasma enhanced chemical vapor deposition (PECVD, POLITO). These technologies produced families of high quality 1DPC biochips operating at several different wavelengths (532 nm, 546 nm, 632 nm, 670 nm, and

804 nm), demonstrating the flexibility of the 1DPC approach to electromagnetic surface wave propagation.

At the end of the first period, the project activities converged to one deposition technology (PIAD) and one specific 1DPC design, giving rise to a final generation of optimized biochips that were used, during the second period, in comparative experiments with a state-of-the-art SPR biosensing platform and in the final cancer biomarker detection assays carried out in a pre-clinical environment.

Injection molded polymer chips made of TOPAS® were purposely developed and manufactured by KDS (Figure 4). These comprise a prism shaped cross section in order to excite the surface wave in Kretschmann configuration. Additionally, a two-component flow cell has been molded. It consists of a hard polymer cover that can be clicked by hand onto the chip. Inside this cover an elastomer defines a straight micro channel of 800 μm x 100 μm cross section as well as perpendicularly aligned fluid connectors.

The injection molded chips were coated by PIAD with 1DPC sustaining BSW at their surface in aqueous environment, at the label-free wavelength of operation $\lambda_0=670$ nm. The final structure of the 1DPC stack is shown in Figure 5(a). In this stack, a 20 nm thin TiO₂ layer is combined

with a periodic $\text{SiO}_2/\text{Ta}_2\text{O}_5$ stack comprising 275 nm and 120 nm thicknesses, respectively ($n_{\text{SiO}_2} = 1.474 + i 5 \times 10^{-6}$, $n_{\text{Ta}_2\text{O}_5} = 2.160 + i 5 \times 10^{-5}$, $n_{\text{TiO}_2} = 2.28 + i 1.8 \times 10^{-3}$).

Depending on the chemical surface functionalization route, the last layer terminating the 1DPC can be either a 20 nm thin SiO_2 layer, providing the starting surface for silane based functionalization protocols, or a 40 nm thin plasma polymerized poly-acrylic-acid layer, directly providing the carboxylic groups needed to bind biomolecules at the surface. In Figure 5(a) the case of biochips fabricated for silane based functionalization is shown.

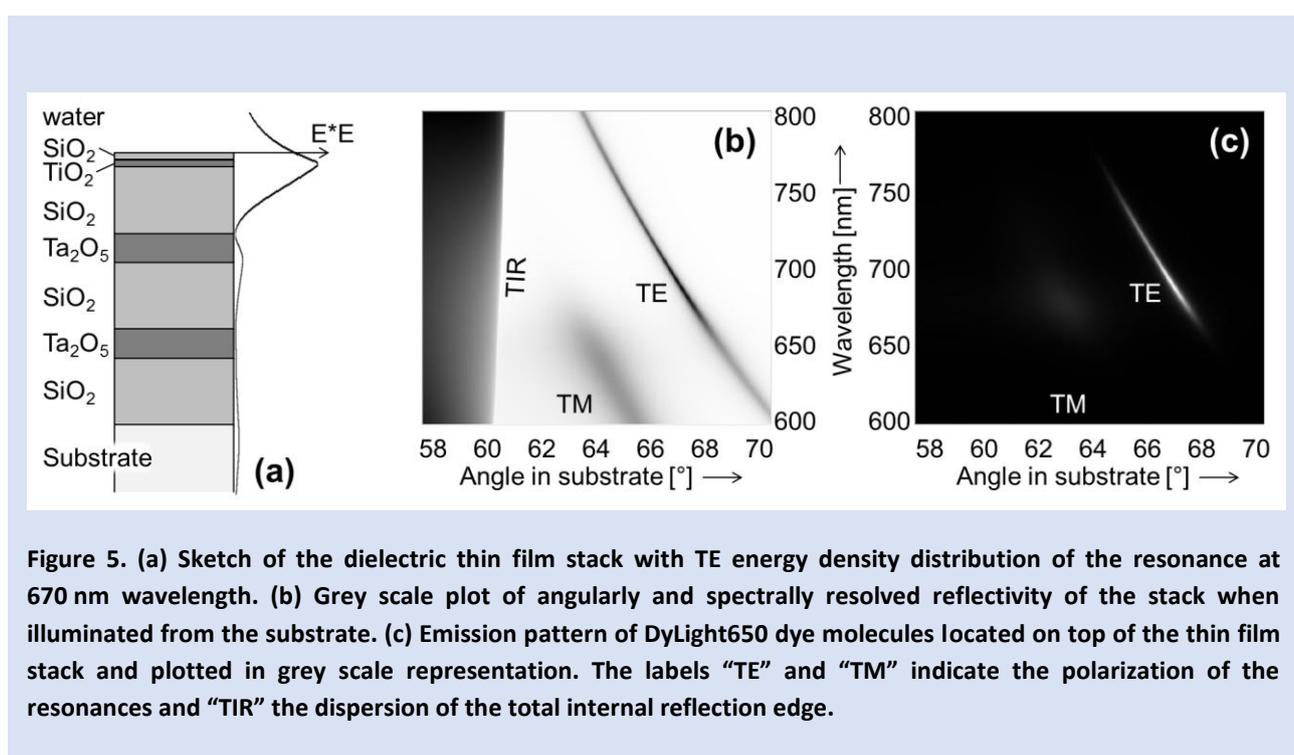


Figure 5. (a) Sketch of the dielectric thin film stack with TE energy density distribution of the resonance at 670 nm wavelength. (b) Grey scale plot of angularly and spectrally resolved reflectivity of the stack when illuminated from the substrate. (c) Emission pattern of DyLight650 dye molecules located on top of the thin film stack and plotted in grey scale representation. The labels “TE” and “TM” indicate the polarization of the resonances and “TIR” the dispersion of the total internal reflection edge.

As it can be seen from Figure 5(a) under resonant conditions the electromagnetic field is strongly confined at the biochip surface. The field shows an evanescent tail in the external medium, with a penetration depth of the order of one hundred nanometers, which is used for biosensing. Figure 5(b) illustrates the dispersion of the resonance in the wavelength-angle-plane. Two surface modes exhibiting different polarization are apparent. The TE resonance has been optimized for label-free sensing, but the stack design supports a rather wide and shallow TM mode at lower angles (wavelengths). Only the TE polarized mode is utilized for label-free operation, although both polarizations might be exploited, for example in ellipsometric approaches.

The fluorescence emitted by randomly oriented dye labels located on top of such stack was simulated rigorously by means of a transfer-matrix algorithm frequently applied for the modelling of Organic LED. In Figure 5(c) we show the results when the emission spectrum of the DyLight650 dye (Abcam, UK) is assumed. It is observed that the fluorescence energy is coupled preferentially into the surface modes, giving rise to a sharp resonance enhancement. The dispersion of such spontaneous emission is identical to the dispersion of the resonance. Thus, each wavelength is emitted at a slightly different angle as defined by the thin film stack’s dispersion. Combining this effect with the optical concept of angularly resolved emission yields

an expected fluorescence angular distribution as shown in Figure 6. Each polarized component of the emission (TE or TM) is detected as a separate peak, comprising an angularly dependent wavelength as given by the dispersion of the surface wave in Figure 5(c). It should be noted that the angular dispersion as well as the angular separation of polarized emission is due to stack design and might be altered by adapting the thin film system.

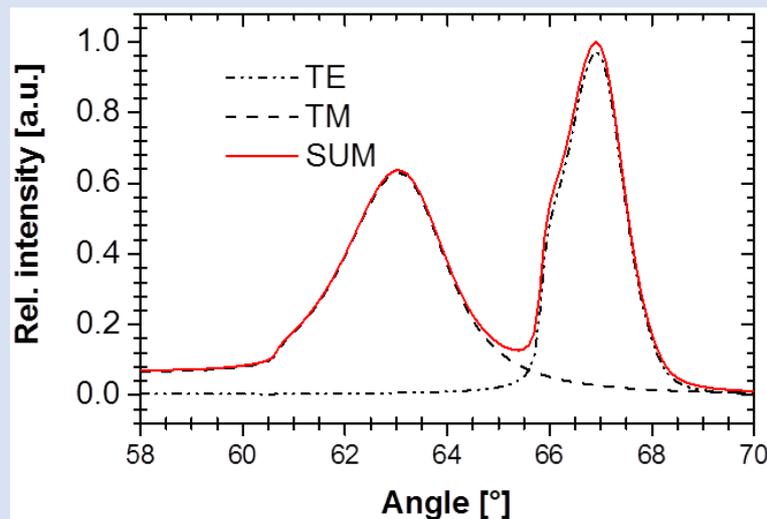


Figure 6. Angular spectrum of fluorescence simulated for the emission pattern in Figure(c) when taking into account the spectral transmittance of filters inserted in the fluorescence collection optics.

3.1.2 Analytical Instrument

The general sketch of the analytical instrument is depicted in Figure 7. It illustrates that the biochip is the key element to integrate the functionalities of fluidics (top, BIOTRAY and FRAUNHOFER) and optics (bottom, FRAUNHOFER) by combining the optical chip (POLITO, FRAUNHOFER, SAPIENZA, KDS) with an appropriate microfluidic flow cell (KDS). Supplementary electronics and human machine interface components are supporting the operation of the instrument and making it user friendly (LABOR). All the different hardware components have been developed in their respective tasks.

A simplified overview of the optical system for BSW based analysis is presented in Figure 8. A collimated light beam B is focused by means of a cylindrical lens C1 into the biochip (Figure 4 and Figure 5). The prismatic shape is required to illuminate the sensor surface above the critical angle for total internal reflection (TIR). As indicated in Figure 8 the surface is coated with the 1DPC. This approach creates an illuminated line along the direction x on the surface. Several measurement areas ("spots") can be arranged in a row along this illuminated line. Parallel readout of these spots is achieved by using a cylindrical detection optical system that consists of objective (C2) and tube lens (C3) systems in order to image the direction x onto one coordinate of a CCD detector. The design of the optical detection system ensures that the same angular range is observed for all spots along the illuminated region on the sensor surface.

A laser diode at $\lambda_0 = 670$ nm is utilized for label-free analysis to achieve proper resolution of the narrow angular BSW resonances. Furthermore, the detection optical system features an

extended CCD detector to obtain 3388 (angular axes) and 2712 (position axes) pixels readout. The lateral field of view (coordinate x in Figure) is approximately 6 mm and 8 pixels binning is applied because of the limited spatial resolution of lateral imaging. The angular field of view is determined by cylindrical Fourier lens F to be approximately 2.9° . Full discretization of the CCD chip (no binning) is exploited in order to achieve proper sampling of the resonance. For label-free detection the region of interest (ROI) is reduced to approximately 1'200 pixels

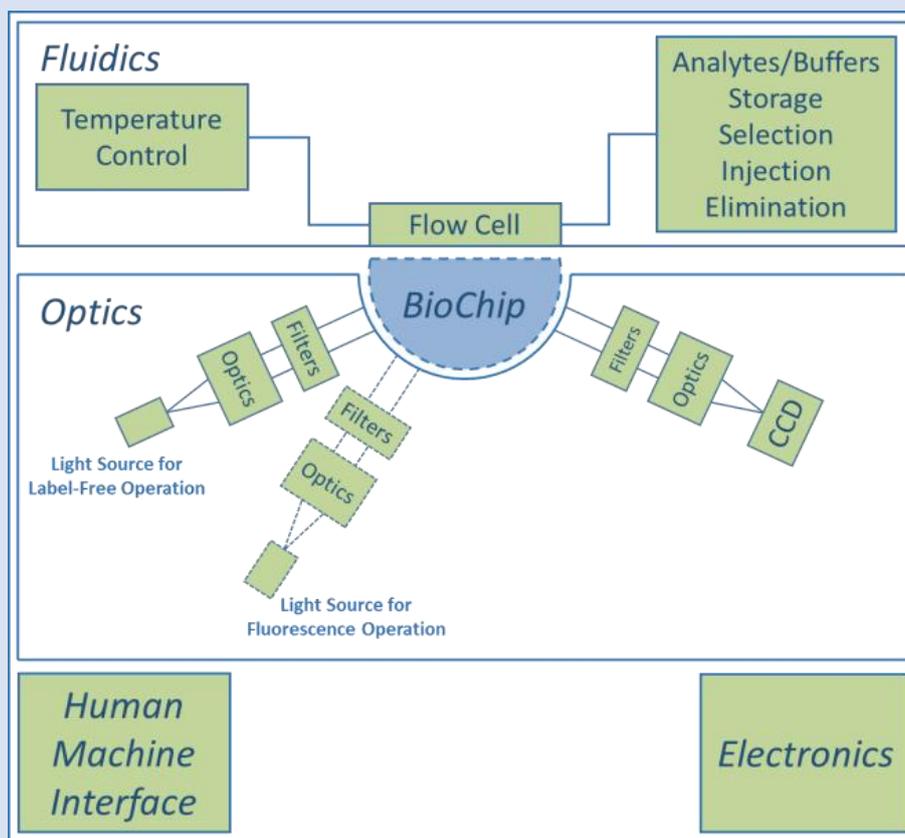


Figure 7. Overview of the analytical system.

around the resonance in order to decrease the time needed for CCD read out and data transmission.

Fluorescence detection is based on a 635 nm illumination. Spatial imaging and binning are identical to the label-free case. However, Figure 6 illustrates that the angular range of 2.9° is too small to detect both emission polarizations. Therefore, an additional cylindrical lens Z in Figure 8(b) is introduced into the light path in order to increase the angular field of view. The limited angular resolution associated with this approach is acceptable, and consequently angular binning of 8 pixels is utilized in fluorescence detection mode.

Filter elements for selecting a certain polarization and/or spectral range are arranged in-between the cylindrical objective (C2) and tube lens (C3) systems. The spectral fluorescence emission filter is fixed in the detection optics because the label-free operation wavelength of 670 nm is well within the fluorescence emission band and transmits such element.

The optical reading system is complemented by a fluid handling system. During the project two different approaches were carried on for fluid management.

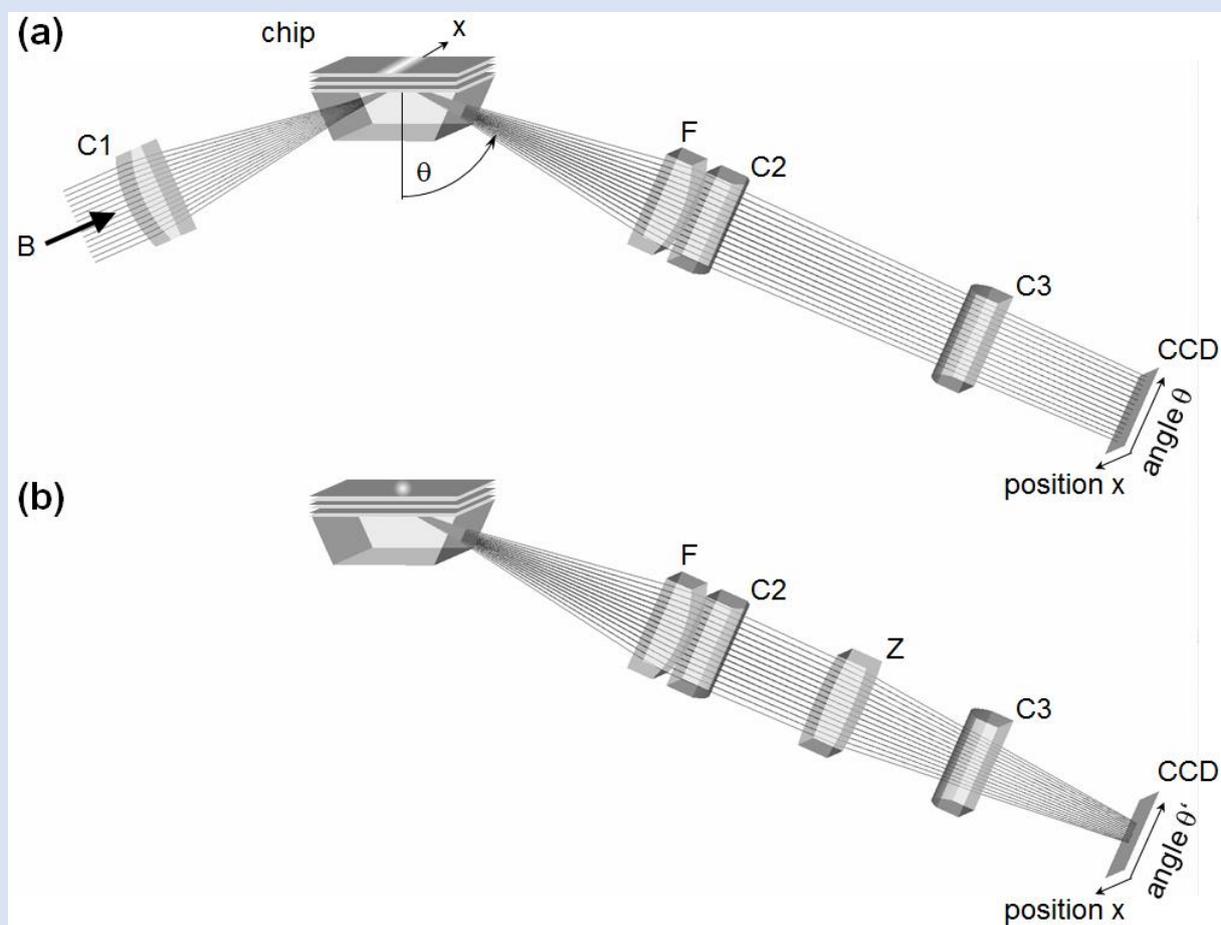


Figure 8. Simplified view of the optical system in label-free (a) and fluorescence (b) modes. In the label-free mode (a) a collimated beam (B) is focused by means of a cylindrical lens (C1) into the chip to create an illuminated line. Cylindrical objective (C2) and tube lens systems (C3) image the lateral coordinate onto one axes of the CCD detector. A Fourier lens F images the angular spectrum of light reflected at the sensor surface onto the CCD detector. An additional cylindrical lens (Z) is introduced for fluorescence detection (b) to increase the angular detection range.

In the first case BIOTRAY put forth a an integrated manifold approach in order to minimize the channel lengths and dead volumes of the system, embed microfluidic control inside the device, widen possible mixing operation, increase automation of protocol to get a user-friendly analytical instrument. It consists of a polymer element engineered to fit the fluidic contacts of the chip as well as to support all necessary components such as pumps, valves, injection block and thermal control system.

In the second case a simplified spring loaded and temperature controlled chip support was designed and manufactured at FRAUNHOFER. Such a fluidic handling system is coupled via Upchurch connectors to an external syringe pump that is used for pumping analyte solutions through the flow cell.

In Figure 9 we show the photograph of the final BILOBA analytical instrument with its housing. The performance of the complete platform, constituted by the analytical instrument and the BSW biochips, was assessed in comparison with a state-of-the-art SPR platform commercialized by the partner HJY. In test assays carried out with the same biological model (antiovalbumin/ovalbumin) the limit of detection (LoD) for ovalbumin concentration in phosphate buffered saline was determined both in the label-free and fluorescence operation modes. While the label-free LoD is slightly smaller (1.45 nM) but comparable to that of SPR (2.1 nM), the fluorescence LoD was more than twenty times smaller (80 pM) demonstrating the efficacy of the BILOBA approach⁸. The LoD values obtained for BILOBA, which depend also on the chemical surface functionalization and on the assay conditions, were further improved in assays with cancer biomarkers as it will be discussed below (Table 2).

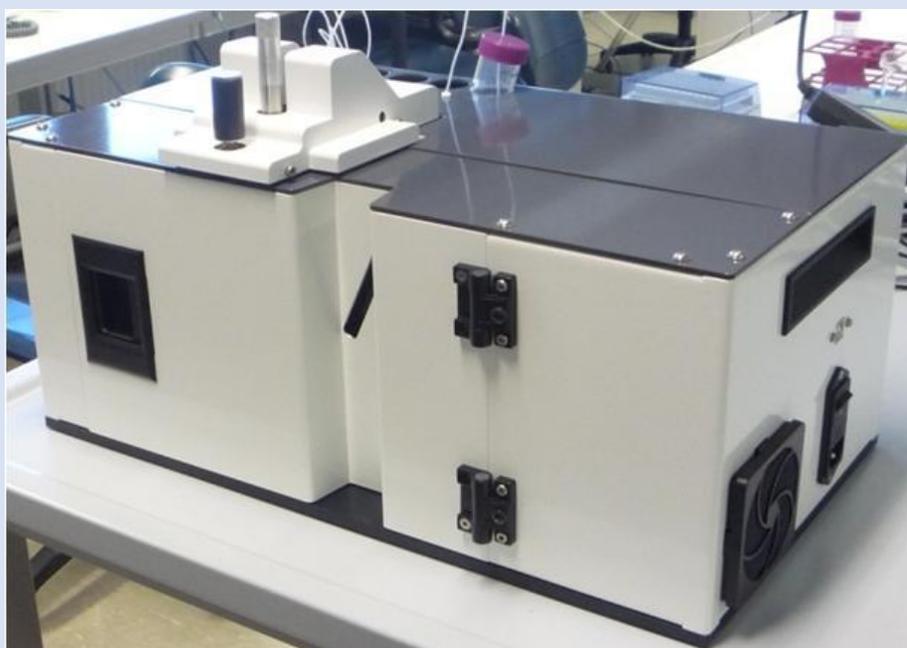


Figure 9. Complete BILOBA analytical instrument.

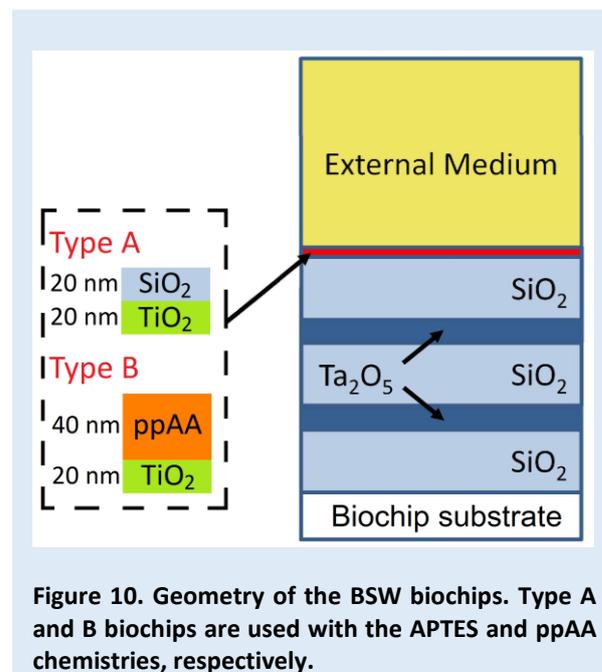
3.1.3 Chemical functionalization of the biochips' surface

Starting from the bare BSW biochips shown in Figure 4, it is necessary to chemically functionalize their surface in order to bind the biomolecules needed for the detection assay. A proper chemical functionalization guarantees a large and stable density of biomolecules that is the ideal starting condition for the subsequent assay steps.

⁸ The comparative assays were carried out using two different surface chemical functionalization technologies: silanization for the BSW biochips and thiol based chemistry for the SPR biochips.

During the project duration, the partners developed in parallel several different surface chemical functionalization routes. In particular they worked on wet techniques (IMPERIAL) and dry techniques (POLITO). In the first case, procedures for the wet deposition of nanometric chemical functionalization layers based on APTES ((3-Aminopropyl)-triethoxysilane) were developed by adapting to the case of the BSW biochips. In the second case, dry chemical functionalization with thin (40 nm) poly-acrylic-acid layers (ppAA) was achieved by plasma assisted deposition.

Depending on the chemical functionalization route used, the design of the 1DPC deposited on the BSW biochips was adapted, as shown in Figure 10. This was done to achieve in both cases a large sensitivity and field localization at the surface. While in the case of APTES the thickness of the chemical functionalization layer is nanometric and does not affect the BSW sustained by the 1DPC, in the ppAA case the functionalization polymer layer detunes the 1DPC. In the case of APTES the 1DPC is terminated by a thin (20 nm) silica layer that is used to provide the hydroxyl groups needed for the silanization. In the case of ppAA the last silica layer is missing, therefore compensating for the deposition of the ppAA layer itself and preserving the performance of the photonic crystal.



By the end of the project, the consortium converged to one of the two chemical functionalization technologies, APTES, which was used for the chemical functionalization of BSW biochip in all comparative tests and cancer biomarkers detection assays in pre-clinical environment carried out at UNITO until the end of the project.

The preparation of BSW biochips to be used in cancer detection assays follows the first three steps depicted in Figure 11 (UNITO). Starting from BSW biochips that were chemically functionalized by the wet APTES route, in the simplest case, two spots on the BSW biochip are defined and used for measurement and reference. During the project we worked with up to 5 spots on the same biochip, which however can operate with 25 spots in parallel.

Protein immobilization starts with the coupling of the linker with a glutaraldehyde solution in sodium bicarbonate buffer in the presence of sodium cyanoborohydride. A solution of Protein G in sodium bicarbonate buffer is brought in contact with the measurement spot. The reference spot is incubated with bovine serum albumin, which is used to block the surface. Finally, the whole biochip surface is immersed in BSA overnight at 4 °C in order to block remaining reactive sites. In result, capture antibodies can be coupled later on with Fc fragment to the protein G in the measurement spot surface in order to maintain their functional Fab sites for specific detection. On the other hand, in the reference spot, blocking guarantees that the

capture antibodies will not bind. The biochips with Protein G layer, when stored at 4 °C, were demonstrated to maintain their functionalities for more than 4 weeks after immobilization.

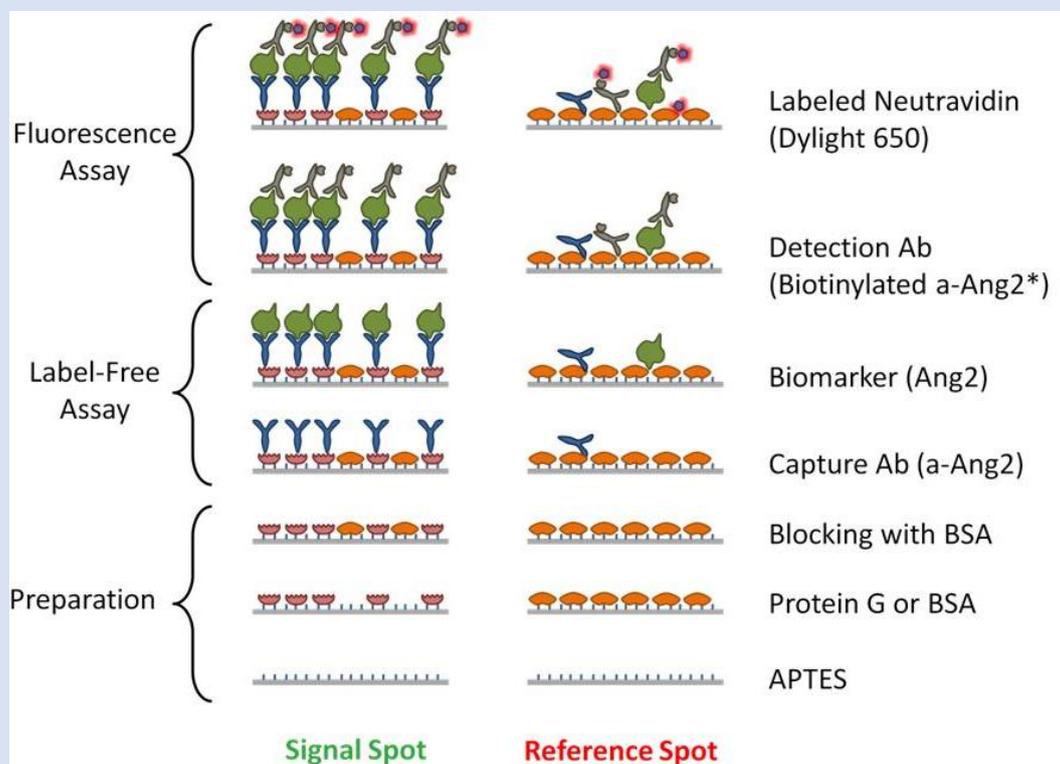


Figure 11. Sketch depicting the sandwich assay used for the detection of cancer biomarkers. In the biochip preparation phase, two regions on the same BSW biochip are functionalized with either Protein G or bovine serum albumin (BSA) to create one measurement and one reference spot. Blocking of the whole surface with BSA is performed to avoid nonspecific binding in the subsequent steps. During the label-free assay the biomarkers' binding is revealed through the shift of the BSW resonance. In the fluorescence assay binding of the detection antibody is revealed by the biotin/streptavidin reaction and measurement of the fluorescent emission.

3.1.4 Exemplary Cancer Biomarker Detection Assay

In the following we describe the procedures that were setup to perform the cancer biomarkers detection assay with the BILOBA platform⁹. Ang2, a factor playing an important role in embryonic and postnatal angiogenesis, has been selected for demonstration purposes.

⁹ The present report was written at the end of the BILOBA project, immediately after the end of the workpackage devoted to the use of the BILOBA platform for the detection of cancer biomarkers in a pre-clinical environment. By that time the results obtained had not yet been disseminated. For such a reason, in the present public report, we do not show details and graphs on such pre-clinical assays and the operation of the BILOBA platform is demonstrated by means of an assay carried out with a cancer biomarker in phosphate buffered saline solution.

In a typical assay, one biochip with Protein G is topped with the microfluidic element and then clicked in the analytical instrument. After injecting in the microfluidic channel the buffer, the label-free signal is tracked vs. time, as shown in Figure 12(a). In the present case the buffer is constituted by bovine serum albumin (0.1% wt) in phosphate buffered saline. In order to correct for drifts of the resonance position due to temperature fluctuations, bulk refractive index changes, pressure effects and laser wavelength instability, the reference measurement is subtracted from the signal obtained on the Protein G-coated spot. As Figure 8(b) illustrates, signal drifts can be removed and clear binding signals are obtained. The fast oscillations of the

signal are introduced by the recirculation of the solutions across the sensor surface during interaction.

Following a baseline measurement in the buffer, the capture antibody (monoclonal anti-human angiopoietin 2 antibody (a-Ang2⁽¹⁾) in the buffer) is injected in the fluidic channel (t~2 min). The temporal behavior can be well associated to binding of the a-Ang2⁽¹⁾ Fc fragment onto the Protein G coated measurement spot and, as expected, no specific interaction is observed on the reference spot that was blocked with bovine serum albumin (Figure 11, step 4).

After such immobilization step and rinsing with buffer (t~16-22 min) the biochip is ready to be used in Ang2 cancer biomarker

detection assays. Note that, for the optimization of the protocols, the preceding step is performed in the fluidic cell in order to monitor the immobilization of a-Ang2⁽¹⁾ in the Protein G measurement spot and to show the efficiency of the process. For the biochips that were used in the pre-clinical assays during the final phase of the project, the capture antibody immobilization was carried out before mounting the biochips on the platform. In that case the step 4 in Figure 11 is a part of the biochip preparation.

Figure 12 shows that injecting a solution of Ang2 in the buffer at a concentration (50 ng/ml) close to the LoD in the label-free detection mode (t~23 min) yields a positive slope of the

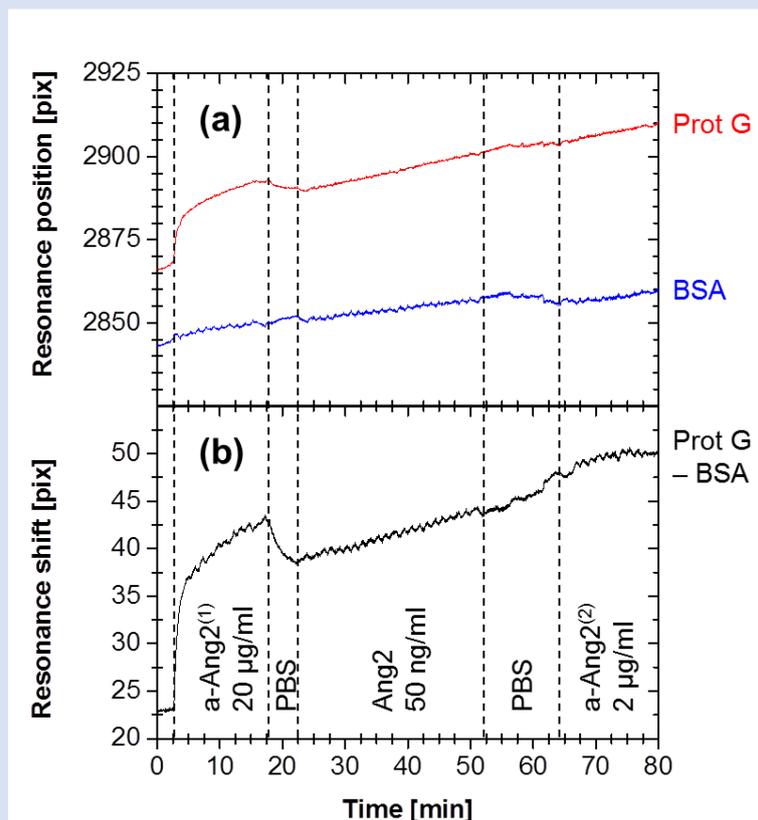


Figure 12. Label free assay detecting Ang2: (a) Raw data of BSW resonance position vs. time for one measurement (Prot G) and one reference (BSA) spot; the reference signal has been shifted for proper scaling of the diagram. (b) Difference signal of the two curves shown in (a).

differential signal, which might indicate a net increase of mass density at the measurement surface due to the specific detection of Ang2 via the capture antibody. However such signal can be hardly distinguished from the baseline drift, replicating a situation that is typical, for example in SPR, in optical label-free assays. This is the situation in which the fluorescence operation of the BILOBA platform, carried out according to the last three steps depicted in Figure 11, permits to increase the resolution and to give a clear response.

In order to carry out the fluorescence assay (Figure 11), a solution of a further specific monoclonal detection human angiotensin 2 biotinylated antibody (α -Ang2⁽²⁾) in the buffer is injected in order to recognize the surface bound antigen. Biotinylation enables one to introduce fluorescent labels in a last step by injecting a solution of a dye (DyLight 650) conjugated neutravidin in phosphate buffered saline. Thus, fluorescence emission will indicate the presence of antigen at the biochip surface. In between all steps, the sensor surface is typically washed with the buffer to remove any proteins or antibodies that are non-specifically bound.

The fluorescence is analyzed prior and after labelling α -Ang2⁽²⁾ with the dye-conjugated neutravidin. The difference of the angular fluorescence intensities before and after labelling is shown in Figure 13, for the measurement and the reference spots. Figure 13 clearly shows that the detection antibody bound

on the Protein G-coated spot, while practically no fluorescence increase is obtained at the BSA-coated spots. This result unveils the uncertainty of the label-free result, with the same platform and within the same assay. The marked difference of the signals in the measurement and reference spots also indicates that the LoD in the fluorescence mode is well below that found in the label-free mode. In this particular case the LoD was found to be 88 pM, corresponding to 4.4 ng/ml. Development carried out during the last period of the project permitted to further decrease such a limit as it will be shown below.

The shape of the angular spectrum detected in Figure 13 agrees well with the prediction shown in Figure 6. Dye labels couple their excitation energy to both surface wave polarizations. Two peaks are observed in the emission angular spectrum. Due to the fact that the dispersion of the surface wave is known, such angular spectrum is a representation of the spectral emission.

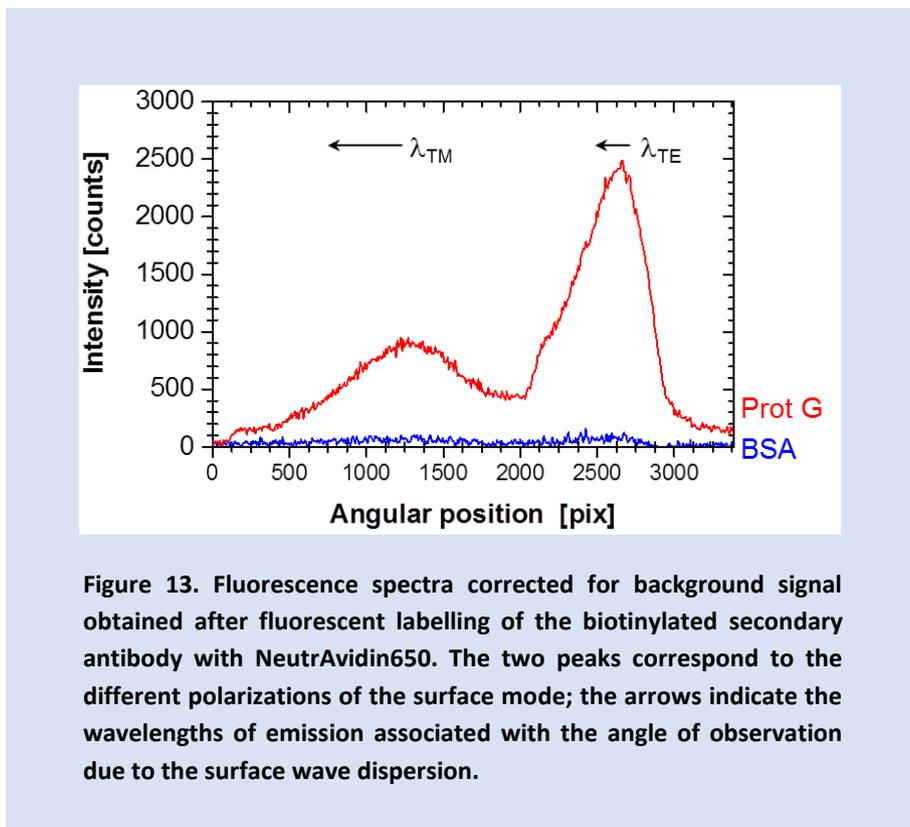


Figure 13. Fluorescence spectra corrected for background signal obtained after fluorescent labelling of the biotinylated secondary antibody with NeutrAvidin650. The two peaks correspond to the different polarizations of the surface mode; the arrows indicate the wavelengths of emission associated with the angle of observation due to the surface wave dispersion.

Note, that the wavelength – angle relation is not similar for both polarizations. Furthermore, it is in general non-linear (compare Figure 5). That's why the two arrows indicate the wavelength scale direction only, without showing a second axis.

We point out that the last two steps of the fluorescence assay (Figure 11) can be coupled by directly using dye-conjugated detection antibodies. This route was not followed during the project to maintain a certain degree of flexibility of the protocols. However in an industrial application this is the route to be implemented, since it reduces the duration of the assay and the complexity of the procedures.

During the BILOBA project, assays for the detection of the three target cancer biomarkers (angiopoietin 1 and 2 and vascular endothelial growth factor) were carried out in several different laboratories of the consortium with preliminary versions of the platform and at UNITO with the final platform and protocols. This permitted to determine the specifications of the final platform that we report in the next section. It was particularly important the last phase of the project that permitted to test the performance of the platform with pre-clinical models, namely buffered solutions, cell cultures, plasma from transgenic mouse models, sera from patients with metastatic colorectal cancer¹⁰.

3.2 Platform Specifications

At the end of the BILOBA project the consortium successfully achieved the following results:

- On a single biochip platform, integration of label-free detection based on the BSW resonance shift, and enhanced spectral intensity of fluorescence emitted by biomolecules immobilized on the surface
- Design and fabrication of biochips with increased sensitivity and resolution;
- Development of surface functionalization techniques to obtain biochips that are selective for specific cancer biomarkers;
- Design and fabrication of a user-friendly analytical instrument for biosensing assays;
- Validation of the performance of the point-of-care platform for the detection of specific cancer biomarkers at a pre-clinical stage.

The objectives that were pursued and achieved by the consortium are summarized in Table 2. The results obtained at the end of the project with the BILOBA system have been compared to the target values for the relevant parameters, and to the performance of the SPR platforms.

¹⁰ Collection, processing, storage and use of human blood samples strictly adhered to the BILOBA protocol approved by the Ethical Committees and reported in the Ethical Manual. The protocol complies with national and European Union legislation, and respects international conventions and declarations. The protocol respects the fundamental ethical principles applicable to the study, considering in particular the importance of patient autonomy and privacy.

Objective	Target value	Value of state of the art SPR platforms	Values obtained by the BILOBA project
Sensitivity of amplitude-based sensors to refractive index changes	30 RIU ⁻¹	150 RIU ⁻¹	211 RIU ⁻¹
Resolution for refractive index changes (baseline noise) ¹¹	$\Delta n < 5 \cdot 10^{-8}$ RIU	$\Delta n \sim 2 \cdot 10^{-7}$ RIU ¹²	<u>Top values achieved by BILOBA</u> label-free $\Delta n = 1.9 \cdot 10^{-7}$ RIU fluo $\Delta n_{\text{eff}} = 3.8 \cdot 10^{-8}$ RIU <u>Final Platform</u> label-free $\Delta n = 5 \cdot 10^{-7}$ RIU fluo $\Delta n_{\text{eff}} = 3.8 \cdot 10^{-8}$ RIU
Field enhancement factor for improving fluorescence intensity	5000	100	4800
Integration of label-free and enhanced fluorescence detection	YES	NOT EXISTING	YES
Resolution for detectable cancer biomarkers concentration (limit of detection) ¹³	10 ng/mL (label-free) <0.1 ng/ml (fluo)	10 ng/mL (PSA biomarkers ¹⁴) 64 ng/mL (ALCAM biomarkers ¹⁵)	<u>Final Platform</u> VEGF-A label-free 11 ng/ml fluo 0.65 ng/ml Angiopoietin-2 fluo 1.06 ng/ml

Table 2. Objectives of the BILOBA project in terms of the target values of the key parameters.

¹¹ In order to compare the BILOBA platform to SPR we make use here of the definition of the resolution given in the reference 12 (below), where the baseline noise is taken as the resolution (1 sigma confidence level).

¹² M. Piliarik, J. Homola, "Surface plasmon resonance (SPR) sensors: approaching their limits?", *Opt. Expr.*, **17**, 16505 (2009).

¹³ The resolution for detectable cancer biomarkers concentration is defined here as twice the baseline noise in a biological assay (2 sigma confidence level) in order to compare to the standard procedures used at HORIBA Jobin Yvon, partner of the BILOBA project.

¹⁴ P. D'Orazio, "Biosensors in clinical chemistry — 2011 update (invited critical review)", *Clinica Chimica Acta*, **412**, 1749–1761 (2011).

¹⁵ N. D. Braulta, C. Gaoa, H. Xuea, M. Piliarik, J. Homola, S. Jianga, Q. Yua, "Ultra-low fouling and functionalizable zwitterionic coatings grafted onto SiO₂ via a biomimetic adhesive group for sensing and detection in complex media", *Biosensors and Bioelectronics*, **25**, 2276–2282 (2010)