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REVIEW ARTICLE

Optical biosensors to analyze novel biomarkers in oncology

Valentina Donzella[*,1] *and Francesco Crea*[*,2]

[1] Scuola Superiore Sant'Anna, Piazza Martiri della Liberta' 33, 56100 Pisa, Italy
[2] Division of Pharmacology, Department of Internal Medicine, University of Pisa, Italy

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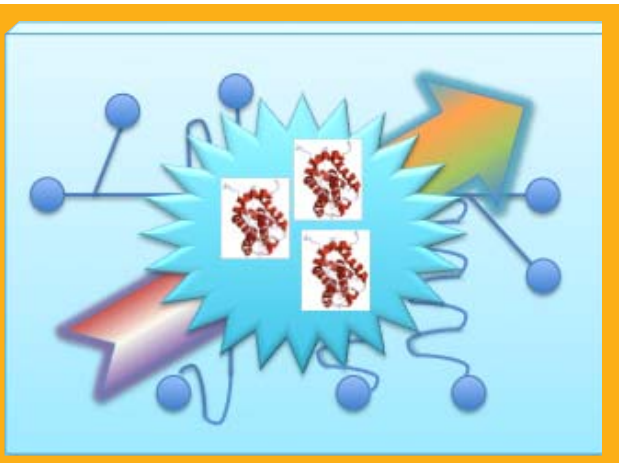
Many cancer types are characterized by poor survival and unpredictable therapy response. Easy-to-perform molecular analyses may help patient stratification and treatment tailoring. Several integrated devices have been proposed to overcome current analysis equipment limitations. They offer improved sensitivity and easy availability of parallel detection. Particularly, unlabelled optical biosensors combine the manifold advantages of integrated sensors (e.g. easy handling, portability and low-volume requirement) with detection of target molecules in their original form. Here, we review integrated optical biosensor current features, and discuss their possible application to the detection of protein variants from body fluids, with particular regard to histone modifications. Indeed, histone post-translational modifications are a set of epigenetic markers frequently deregulated in cancer. Available technology does not allow a comprehensive analysis of all histone modifications in a single patient. Thus, label-free optical biosensors may pave the way to the discovery and detection of a novel class of biomarkers in oncology.



Label-free optical biosensors: a new frontier in detecting proteins from body fluids.

1. Introduction

Using molecular tests in oncology has significantly improved clinical practice. Dosage of molecules from body fluids allows early detection, treatment tailoring and prognosis prediction [1, 2]. These tests are mainly based on the identification of specific proteins, or genetic alterations. Despite this, cancer prognosis remains dismal in many cases, and clinical outcome is often unpredictable [3]. In addition, treatment tailoring based on molecular profiling is still a challenge [4]. The main obstacles to overcome

* Corresponding authors: e-mail v.donzella@sssup.it, Phone: +39 050 5492194, Fax: +39 050 5492194;
fcrea@sssup.it, Phone: +39 050 992632, Fax: +39 0502218758

are the complex nature of molecular alterations occurring in cancer patients, as well as the accessibility of tumour derived biological samples [5].

Laboratories on a Chip (LOCs) can dramatically improve analysis tools available for oncologists, in particular in terms of high degree of analysis parallelization. They can integrate and automate all needed sample process functionalities into an inexpensive, portable and disposable, thumb-size device [6]. In addition, sample handling automation can include, in principle, direct LOC handling of available biological fluids, such as blood, urine, saliva. Thus, they can represent a breakthrough in personalized medicine.

The term LOC has been used to identify devices in which microelectronic, photonic and microfluidic structures are integrated together to perform biological analysis. They are the direct descendants of micro Total Analysis Systems, which were introduced for the first time by Manz et al. in 1990s [7]. The basic concept of these devices is the automation of analytical chemistry procedures, which has been quickly extended to the automation of more complex procedures, involving biological and chemical processes [8]. In these devices, streams of fluids, rather than human hands, take over the role of sample transport from one process to the other, driven by suitable voltages applied at channel ends [9]. Indeed, flow paths are defined by interconnected microchannels, integrated into planar substrates, with cross-section dimensions of the order of micrometers and length of few centimetres.

In particular, we will focus on a specific part of LOCs, which is the sensing one. This is the core block of a LOC, since it is the place where target bio-molecules are somehow 'trapped' and where interaction between biological fluids/solutions and electronic/optical signals happens (Figure 1). This interaction has to be properly controlled and detected in order to maximize and optimize device output and its sensing capabilities. In addition, sensors exploited for biological and chemical applications have to fit some specific requirements, such as biocompatibility, lack of sample degradations (e.g. due to sensor self-heating), etc. [10].

As a future application of LOCs, we will discuss the possibility of sensing epigenetic markers exploiting optical signals and label-free detection. As we will show in the final sections, epigenetics is a complex mechanism of gene expression regulation, driving cancer initiation, metastasis and drug resistance. Thus, epigenetic alterations are promising novel biomarkers in oncology. LOCs allow for quick bio-molecule detection, without a previous treatment of target molecules. They offer high degree of analysis parallelism with the requirement of biological sample small volumes. If sensing blocks are properly designed, high sensitivity can be easily achieved. For example, it is possible to detect the resonant wavelength shift due to target bio-molecule binding, and in this case the output signal intensity will not depend on molecule concentration [11]. A quick comparison between commercial optical biosensors, the proposed technique, based on optical biosensors to be integrated in LOCs, and current laboratory instrumentation, such as Enzyme-linked immunosorbent assay (ELISA), and newly proposed multiplex assays, will explain how simple and cheap analysis can be performed. Thus, in a not so far future, LOCs based on label-free optical sensors could allow quick diagnosis and tailored therapy.

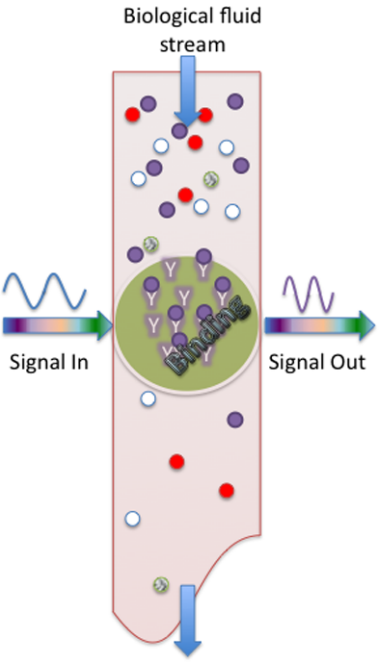


Figure 1 (online color at: www.biophotonics-journal.org) Schematic representation of a label-free optical biosensor. Biological fluid stream is driven by proper voltages at channel ends. Fluids are made up by different biomolecules (coloured circles), and only one kind is trapped on sensing surface. Input light signal propagation is altered by molecule binding and produces a detectable output signal.

2. Biomarkers in oncology: an overview

Cancer biomarkers are defined as detectable biological molecules, whose levels are correlated to individual cancer risk, prognosis, or treatment response [5]. From a chemical point of view, a biomarker may be a protein, a nucleic acid variant (DNA, RNA, micro-RNA) or a whole cell. Since cancer is a complex phenomenon, emerging biomarkers are often composed by multiple parameters. For example, the expression profile of 70 genes is required to predict breast cancer response to a chemotherapy regimen [12].

Biomarker identification is a complex process, requiring a clear clinical question, the selection of detectable molecules and possibly non-invasive procedures, the validation of analytical methods and the demonstration of clinical utility.

First, it is essential to define a clinical problem. In general, cancer biomarkers may be employed for early detection of cancer patients, or as prognostic/predictive. Early cancer diagnosis is associated to better prognosis and less invasive treatments [13]. After cancer diagnosis, biomarkers may be useful to predict response to a specific therapy or risk of cancer progression [4].

Cancer biomarkers can be detected from primary tumor or body fluids (blood, urine). In the first case, cancer-specific alterations are more likely found. However, primary tumors are not often accessible in the clinical setting, especially for metastatic disease. Thus, the identification of biomarkers from body fluids is generally preferred [3] and it can be of crucial importance. It is worth mentioning that body fluids are a complex mixture of cells and macro-molecules that may affect the analysis of a single marker.

With this background in mind, it is clear that an analytical method for cancer biomarkers should be:

1. designed to detect cancer-specific biological molecules
2. able to measure its target in complex biological fluids (specificity and detection limit are critical)
3. multi-parametric.

In the next sections, we will compare performances of label-free optical biosensors and other available biological analysis systems. In the conclusive chapters (Sections 4 and 5), we will explore the possible application of these sensors to the detection of epigenetic modifications. Along with genetic modifications and protein dosage, the discovery of epigenetic alterations in tumor cells is an emerging area of research [14]. Indeed, epigenetic modifications have been correlated to cancer risk, prognosis and treatment response [15]. However, for their nature, epigenetic modifications require highly sensitive and highly multiparametric diagnostic tools. Thus, we think that they could be better investigated by developing novel dedicated devices, and particularly devices based on label-free optical biosensors.

3. Detection of markers from body fluids

3.1 Clinically available analysis equipments

Enzyme-Linked Immuno-Sorbent Assay (ELISA)

The best validated and most widely used (in clinical laboratories and biomedical research) method to measure single protein, antigens or antibodies from biological fluids is ELISA, that was introduced in the seventies [16]. It is the direct descent of Radioimmunoassay (RIA), described for the first time by Yalow and Berson in 1959 [17], but it avoids radioactive target exploitation. Some advantages offered by ELISA include highly quantitative results and reproducibility. Nowadays ELISA kits for commonly measured proteins (e.g. Prostate-Specific Antigen, PSA) are commercially available, often from multiple vendors. On the other hand, ELISA allows for measuring only one biological molecule at a time, and performance is largely dependent on antibody quality, kit manufacturer, as well as operator skills and experience [18]. This feature hinders the possibility of revealing several soluble molecules from one biological sample. In addition, samples of ELISA-based assays have to be suitably diluted in order to fit the dynamic range (range over which there is a linear relationship between target molecule concentration and the absorbance reading [19]). This can be an issue when using serum samples, exaggerating differences between samples that have target molecule levels within the dynamic range (do not require dilution in the assay) and samples above the dynamic range (do require dilution).

Multiplex arrays

Even if ELISA's analyses still represent the gold standard in evaluating the concentration of a target bio-molecule from biological fluids, multiplex arrays are attracting more and more interest. This technology offers lower cost, requires less sample volume and has the possibility of studying multiple interactions. Nevertheless, it has not been accepted in the clinical practice and it is still less exploited.

Multiplex arrays can measure multiple target molecules in the same sample at the same time. The most common multiplex arrays are bead-based with flow cytometry. The beads are coated with specific capture antibodies, and fluorescent detection antibodies bind to the target captured molecules. Thus different bead – conjugated antibody sets allow for measuring different target molecules in a biological fluid by revealing the fluorogenic emission.

Even if this technique presents several advantages with respect to ELISA, experience with multiplex arrays remains limited. Very few studies appeared in peer-reviewed literature between their conceptual introduction in the late 1970's [20] to the development of commercial instruments in the late 1990's [21]. In addition, the most relevant studies about multiplex arrays and their comparison to ELISA are concentrated in the last decade [22, 23]. Concordance between ELISA and multiplex arrays is generally good [24], but it is much less robust

when using serum or plasma samples [25]. One drawback affecting multiplex arrays is cross-reaction phenomenon, which must be strongly reduced; non-reactivity to all other antibodies must first be established. Indeed, a reliable uniplex assay cannot just be simply added to a functioning multiplex array, since multiple potential interactions are involved. Another drawback is related to array dynamic range, since target molecules with largely different concentrations can have too different dynamic ranges. Furthermore abundant circulating molecules in serum or plasma samples may affect multiplex results since reactions take place among molecules and antigens, which are freely mobile in solution. As a result, these multiplex arrays are more sensitive than ELISAs to altered levels of circulating proteins and inhibitors [24].

Multiplex ELISA

As already said, ELISA is the most established method for evaluating protein concentration in fluids. Thus, a very powerful way to multiplex this method is to use different antibodies for each well. Indeed, as presented in [26], it is possible to spot at least nine antibodies on each well; four of the nine assays were not statistically different to the individual assays. Sensitivities and ranges for the nine chosen bio-molecules are comparable (sometimes even improved) with respect to traditional ELISA, showing the great potentiality of multiplex ELISA. On the other hand, there is a lack of published works on this topic and it not clear how many antibodies can be spotted on the same well without altering measurements. Although multiplex ELISA seems promising, it is only an upgrade of traditional ELISA, and in our opinion its disadvantages are very similar to multiple assay ones, such as different dynamic ranges and cross-reaction phenomenon.

3.2 A way towards Lab on Chip: optical biosensors

Recently, several integrated devices have been proposed in order to overcome current biological analysis equipment limitations. Indeed, integrated devices can offer improved performance in terms of sensitivity and of easy availability of multiple simultaneous detections. In particular, LOCs are miniaturized devices in which all the functionalities should be integrated, from sample handling to signal delivery and processing, from electronics to optics, from microfluidics to thermodynamics. This will allow the automation of all needed sample processing stages into portable and disposable, miniaturized devices.

LOC core component is its sensing part, where target bio-molecules are somehow 'trapped' to produce a proper output signal (Figure 1). This part is named biosensor, and obviously it should be integrated with all needed components. A biosensor is defined by IUPAC as a self contained integrated device that is capable of providing specific quantitative or semi-quantitative analytical information using a biological recognition element (biochemical receptor), which is in direct spatial contact with a transduction element [27]. The recognition elements can be composed of antybodies, enzymes, hormones, nucleic acids, proteins, etc. Sensor structure and its working principle are of crucial importance for LOC realization. They determine device sensitivity, detection limit, specificity and all its characteristics.

It is worth noting that in the past biosensors based on different transduction mechanisms (e.g. optical [28], electrochemical [29], piezoelectric [30], thermal [31], etc.) have been proposed and demonstrated. Obviously, the exhaustive treatment of all these mechanisms is outside the aim of this review, and we focused on optical biosensors. Indeed, we thought that the long-term aim of optical biosensors is their integration in LOCs. In such case, it can be important to separate optical signals, used for molecule detection, from electrical ones, which should be used for fluid stream control, heating and also for the whole driving electronics. For example, electrochemical sensors are a mature technology and very well developed, but they have numerous drawbacks (sensitivity, electrode fouling, electrochemical stability of reagents, and side electrochemical reactions, [32]), that could be overcome by the use of optical detection methods.

Integrated optical biosensors can exploit several optical transduction mechanisms in order to produce the desired output. Here we report a brief list of the most common ones.

- Integrated sensors based on fluorescence, in which molecules of interest are labeled with bright fluorescent dye molecules and then can be located and tracked by optical microscopy [6, 33–36].
- Sensors exploiting plasmon resonance, in which the adsorption of target biomelecules by a surface bio-receptor is measured by tracking the change in incident light resonance coupling conditions to the propagating surface plasmon wave [37–39].
- Optical sensors based on Raman scattering [40, 41]. They use Raman effect that results from energy exchange between incident photons and scattering molecules.
- Biosensors based on the detection of solution absorbance change due to dissolved bio-analytes [42, 43].

Table 1 Comparison between different label free optical biosensors, commercial and custom-built (cb). Optical biosensing has been demonstrated for several molecules, oncological biomarkers, proteins, hormones, antibodies, DNA. The table is focused on results from complex human samples, when available. RIC, refractive index change; RAC, resonant angle change; RWS, resonant wavelength shift; SPR, surface plasmon resonance; WDM, wavelength division multiplexing. References in squared brackets.

Sensor	Working Principle	Analyte	fluid	Detection Limit	Ref.
Silicon microring resonator (cb)	RWS	Oncoprotein (carcinoembryonic antigen)	Undiluted serum	25 ng/ml	[50]
SPR (Biacore)	RAC	Cytokine (Interleukin-8)	Human saliva	1.5 ng/ml	[51]
SPR + WDM (cb)	RWS	Anti-Epstein-Barr virus antibodies	1% human serum	0.2 ng/ml	[52, 53]
SPR (cb)	RAC	Oncoprotein (alpha-fetoprotein)	Human plasma	<20 ng/ml	[54]
Microfl. module + SPR anal. (SPR670, ex NEL-LAB)	RAC	Hormone (insuline)	Insulin-impregnated serum samples	1 ng/ml	[55]
Opto-fluidic ring resonator (cb)	RIC	Cancer Growth Factor (HER2 extra-cellular domain)	Human serum	13 ng/ml	[56]
Opto-fluidic ring resonator (cb)	RIC	Methylated DNA	Buffer solution	1 nM	[57]

- Integrated biosensors, based on effective index change, due to bio-molecules binding in guiding structures [44–48]. We will discuss them in details in the following paragraph.

For a more detailed description of biosensors and their working principles, one can refer to [10]. Some biosensor configurations require a sample preventive treatment, for example labelling with fluorescent or radioactive substances, others require no preventive treatments, we will name them label-free biosensors.

3.3 Advantages of label-free optical detection

Label-free optical detection requires no preventive labelling procedure, thus target bio-molecules are detected in their natural form, without alterations. To the aim of this work, and also of the realization of stand-alone LOCs, our opinion is that label-free biosensors are more promising. Indeed, devices based on label-free detection allow for simply putting a sample drop in the chip, and then all needed operations can be performed within the chip itself. Thus, taking in mind the application we are interested in, i.e. sensing cancer biomarkers (and in the future, sensing epigenetic modifications) from biological fluids, this technique can be very straightforward. In addition to easy and quick usage, label-free based biosensors are more suitable to study molecular interactions and reaction kinetics.

In Table 1, modified from [49], we have summarized the most important results achieved with optical label-free biosensors. In particular, their applicability has been demonstrated for detection of cancer biomarkers, hormones, antybodies, drugs, and DNA at physiological levels. Reported results show not only that this technology can offer performance comparable with traditional methods, but also its direct applicability to biological fluids, such as serum, saliva, plasma. Some results were achieved thanks to commercial devices available on the market, but most of them concern custom-built devices.

Label-free detection can exploit several of the working principles listed in previous paragraph; to the aim of this work, we are particularly interested in integrated devices. Indeed, integrated devices offer the advantage of high degree of analysis parallelization in a miniaturized, automated and inexpensive chip. In particular, we will focus on detection of refractive index change (due to bio-molecule binding) in guiding structures.

Label-free biosensors based on several optical sensing mechanisms are already on the market, as reported in Table 2. The most affirmed ones are Biacore sensors, based on surface plasmon resonance [58]. Such sensors measure resonance angle change to detect protein binding. The instrument has comparable performance with traditional analysis tools (see Table 2), and has different models available on the market. Also DOT sensor, from AXELA [59], offers challenging performance, and it is based on diffracted light from the gratings where target molecules are trapped. On the other hand, the other reported devices present lower performance compared to current equipment. In addition, commercial de-

Table 2 Selected protein detection tool specifics. Detection limit range refers to different target proteins, except for multiplex assay (different available commercial kits). In the last column, number of possible simultaneous detections is in brackets. Sensitivity is the magnitude of sensor transduction signal change in response to analyte concentration change. Detection limit is the minimum detectable molecule concentration. Reported data are relative to specific target molecules, and different values may be found changing target. LFD, label free detection; not spec, not specified; molec, molecules. References in squared brackets.

Detection tool	Detection limit [ng/ml]	Sensitivity	Multiple detection?
Traditional ELISA	0.003–0.6 [26]	2.4 fmol/well [60]	No
Multiplex arrays	3.2–22.2 [61]	Not spec.	Yes (100 molec)
Multiplex ELISA	0.02–0.53 [26]	Not spec.	Yes (25 molec)
Biacore (Suface Plasmon resonance technology + LFD) [58]	0.01–10 [62]	0.1 RU	Yes
Farfield Analight (Dual polarization interferom. + LFD) [63]	1.7e3 [64]	Not spec.	Yes (up to 348 wells)
ForteBio Octet (biolayer interferometry + LFD) [65]	Tipically 1e3 [66]	Not spec.	Yes (8 wells)
Axela DOT (grating-based light diffraction + LFD) [59]	~0.04 [67]	~0.0025 diffractive intensity	Yes (40 molec)
CSEM WIOS (grating waveguide optically interrogated + LFD) [68]	~0.2 [69]	~0.02 of normalized immusensor signal	Yes (4–8 channels)
Slot based microring resonator	2–50 [50–70]	70 nm/RIU [70]	Yes (thousands)

vices, inserted in Table 2, are bulky instruments, some of them based on prism coupling, and they require quite expensive analysis chips. So even if they represented a remarkable innovation with respect to previous laboratory analysis equipment, they are still far away from the idea of integrated analysis chips. The reported commercial sensors are attractive in terms of performance, but they are not suitable to be integrated in a unique platform, together with optics, fluidics and electronics, since they generally cannot be fabricated using standard silicon fabrication technique and their working principles require bulky components, such as prisms.

An alternative sensing strategy is the use of waveguides as guiding structures, in order to be easily integrated in stand-alone LOCs. Such waveguides can be traditionally shaped [71] or based on slot structures [44, 48]. They can be fabricated by mature technologies, which have been investigated for decades, and their behaviour and their performance can be simulated and optimized using proper finite element based codes and commercial simulation tools.

As exhaustively reported in [72], there is the possibility to realize several biosensor structures based on refractive index change. The crucial point is to maximize as much as possible the interaction between light beam and the surface where target biomolecules should bind to bio-receptors (Figure 2). This is important to improve sensor sensitivity, since biomarker detection from body fluids can involve very small concentrations [73].

Promising solutions can arise from combining different sensor configurations, in order to merge their advantages and achieve optimized sensors. For example, it has been proposed and validated [44] that ring resonator sensors, combined with slot waveguides, can be used to realize optical biosensors. Even if this recently proposed sensors have to be improved (as can be seen from Table 2), they offer numerous features that make them promising as possible candidates for sensing elements in LOCs with an elevated degree of analysis parallelism. These advantages are briefly discussed in the following.

- Ring (or racetrack [74]) resonators provide a compact sensing element (footprint of the order of few tens of μm^2), that can be easily integrated in a 2D platform with optics, electronics and fluidic. An important feature, offered by a ring resonator based sensors, is that the actual light propagation length in contact with biomolecules does not scale down with ring size. It rather depends on the number of revolution performed within the ring, thus on its quality factor. One can have an elevated interaction length (order of hundreds, thousands of microns) only increasing the Q factor, in a very miniaturized device, and sensor performance can be improved by simply improving the Q factor.
- The working principle of these sensors is a frequency shift of resonance wavelength due to waveguide effective index change. Such change is caused by target molecule binding on the outer waveguide surface (properly functional activated with bio-recognition molecules), which influences signal propagation within sensor

structure. The interesting aspect is that output signal intensity does not change due to the binding, and the event can be measured as a frequency shift of ring resonance wavelength. Thus, very few target molecules can be revealed since output signal intensity does not scale down proportionally to their concentration.

- Slot waveguide (SWG) is a relatively novel guide-wave configuration that was first introduced by Almeida et al. [75]. SWGs are able to confine and guide light in a low refractive index region (with dimensions of few hundreds of nm) using total internal reflection at levels that cannot be achieved by conventional waveguides. Using the slot as sensing region in a sensor enhances light/bio-molecule interaction, since light field actually propagates in the slot region, where the binding of biomolecule happens (Figure 2). On the other hand, other guided configurations do not offer such possibility and there is a weak interaction of field evanescent tails with biolayer. Sensor sensitivity can be improved by a proper slot region design, which allows for a complete filling of the slot region by the biological fluid [44].
- Recent results [76] have shown that a proper slotted ring resonator design allows for eliminating sensor response temperature dependence. This is an important advantage with respect of previously proposed sensor configurations.
- Different material systems can be chosen to fabricate slot waveguide based ring resonators, accordingly to chosen application and keeping in mind that one fundamental aspect is the compatibility with silicon platform. A CMOS compatible fabrication process can actually simplify sensor development, allowing for a unique, universal and cheap platform for optics, fluidics and electronics.

Integration of microfluidics and optics [77], of optics and electronics [78], and of microfluidics and electronics [79] has been already demonstrated. The next, and more important step is the integration of all the components together, and of more capabilities together (e.g. [80]), having a clear idea of final system requirements. Pointing out a challenging integrated optical sensor configuration can be very important for LOC development and affirmation.

4. A promising class of oncology biomarkers: histone modifications

In this section, we will briefly describe an emerging and promising class of cancer biomarkers: epigenetic alterations, with particular regard to histone modifications. As shown in the conclusion, we think that clinical application of these biomarkers may derive particular benefit from the development of label-free optical biosensors.

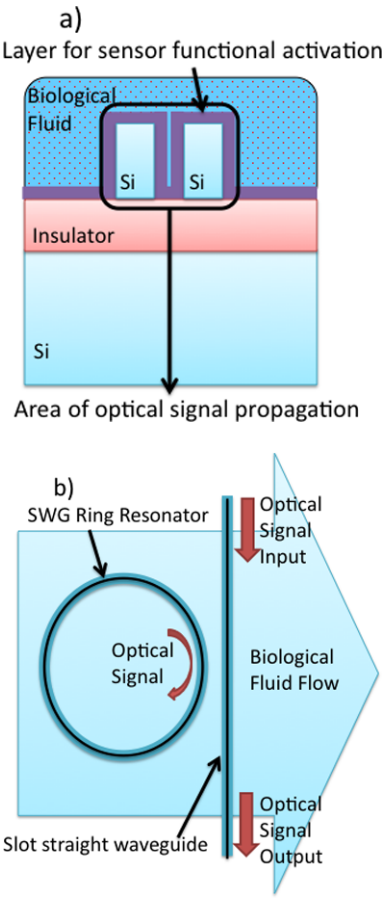


Figure 2 (online color at: www.biophotonics-journal.org) (**a**) Schematic representation of the slot waveguide (SWG) ring resonator transverse cross-section. The structure can be fabricated exploiting Silicon On Insulator technique. The black-edged box points out the area in which is concentrated the optical field. (**b**) Upper view of the ring resonator coupled with a straight waveguide for optical signal input/output.

4.1 Histone code: biological function and chemical complexity

For many decades, information flow in eukaryotic cells has been considered unidirectional. The so-called "central dogma" predicted that backup information stored in DNA is transcribed into RNA and eventually translated into proteins, which are the ultimate responsible for cellular functions [81]. Each gene produces a single protein. Emerging evidence is revolutionizing this simple view, showing that the

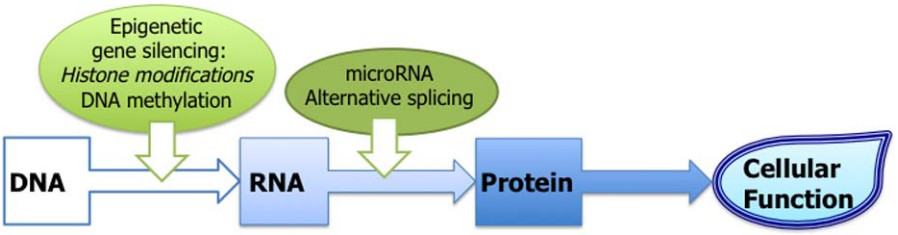


Figure 3 (online color at: www.biophotonics-journal.org) Information flow in eukaryotic cells. Squared boxes represent classical information flow, described by the central dogma of molecular biology. Circles represent non-classical mechanisms of gene expression modulation.

genome is a textbook that may be read in several ways, thereby generating many cell types in the same organism (Figure 3). First, DNA transcription is far from being an automatic process. DNA can be modified by specific enzymes that are able to add methyl groups to cytosine residues [82]. DNA methylation is able to turn off genes, thereby contributing to cell differentiation and embryo development [14]. In addition, DNA is wrapped around core proteins called histones. Histones-DNA complexes are organized in cylindrical structures called nucleosomes, that may be more or less tightly bound [83]. When histones are tightly bound to DNA, they do not allow gene transcription. When they are loosely bound, DNA transcription is possible. DNA-histone linkage is controlled by post-translational modifications occurring on histone tails, protruding from the nucleosome. Histone tails may be modified by adding methyl-, ubiquityl-, phosphoryl-, acetyl-groups. At present, tens of possible modifications have been described, and their interaction is regulated by a complex histone code, that is not completely understood [84]. For example, histone acetylation is always able to activate genes, while histone methylation may turn them on or off. In addition, histone modifications interact with DNA methylation to regulate gene function [14]. DNA methylation and histone modifications are called epigenetic modifications, a word coined by the embryologist Waddington [85]. In Greek, "Epi" means "above". Epi-genetic modifications represent a higher order-genetic code organization, allowing for quick adaptation to the environment. Epigenetic modifications interact with other gene regulation mechanisms, like RNA splicing and microRNA, which are beyond the scopes of the present review (Figure 3).

Epigenetic interactions regulate each gene function during embryonic development, tissue regeneration and many other physiological processes. Since human genes are approximately 23 000 [86], and since different histone modifications interact in distinct gene regions [87], the combinatorial complexity of human epigenome is at least 10^5. This explains why we need powerful multi-parametric tools to dissect them.

4.2 Histone post-translational modifications as biomarkers in oncology

Global levels of histone modifications have been correlated with clinical outcome in many cancer types. These analyses are not gene-specific, since they simply measure the expression level of a single histone modification. For example, histone H3 and H4 methylation and acetylation predict recurrence risk after prostatectomy in prostate cancer patients [88]. Similar results have been found in lung, kidney and esophageal cancers [88, 89]. In these studies, histone modifications have been measured through immunostaining performed on primary tumor specimens. Nevertheless, tumor specimens are not often available, and in the clinical setting, blood samples are more readily accessible. Thus, the identification of tools that may detect histone modifications from biological fluids is needed. The biological rationale for the development of these methods is the presence of tumor-derived DNA in the serum of cancer patients. Free circulating DNA is present in minimal amounts in the plasma of healthy individuals. In cancer patients, several tumor-dependent alterations allow the concentration of higher amounts of circulating DNA. Circulating DNA can be found at concentrations of 0.5–0.9 ng/ml in healthy individuals, and 1.5–50 ng/ml in lung cancer patients [90]. Interestingly, tumor-derived DNA has been employed to detect specific genetic [73] and epigenetic [91] alterations in cancer patients. Since histones are tightly bound to DNA, tumor-derived histone modifications can be found in the plasma of cancer patients. Indeed, plasma histone H3 Lys 27 trimethylation levels discriminated between localized and metastatic prostate cancer [92]. In this case, a single histone modification has been measured through ELISA assay.

Global levels of histone modifications may be less informative than modifications at specific genetic *loci*. For example, histone H3 Lys 27 methylation of 14 genes was shown to accurately predict clinical outcome in prostate cancer patients [93]. This evidence has been then extended to other cancer types. At present, this analysis is feasible only on primary

tumor specimens. A tool that may identify specific histone modification on single genes from biological fluids may significantly improve treatment tailoring and risk prediction in oncology. A locus-specific, genome wide histone modification profiling would provide a further improvement. To our knowledge, there is no report of similar analysis from clinical samples. However, this technique has been applied to cancer cell lines [94], an experimental condition providing much higher amounts of target biomolecules.

5. Conclusion: future application of LOC technology to cancer epigenetics

Whole-genome epigenetic profiling will be a goal of 21st century biomedical research [95]. In this context, the identification of cancer-specific histone modifications will be possible. Since cancer is a multi-genic disease, each cancer type could be likely identified by several histone modifications. In addition, a specific set of histone modifications may predict cancer risk, metastatic spreading or response to therapy. Thus, analysis instruments need to be highly multiparametric. Furthermore, primary tumor is not often available for molecular analysis, especially for advanced tumor stages. For these reasons, a specific tool, able to handle and directly analyze biological fluids, can improve available diagnostic techniques in oncology. Moreover, a technological breakdown is expected by the integration on a single chip of multiple functionalities, from fluid stream control into micro-channels, to sample heating or cooling, electronic chip driving as well as optical detection. This versatility can be very useful for our purpose. For example, a gene specific analysis of histone modifications may require several thermal cycles of polymerase chain reaction amplifications [96]. LOCs should be fabricated using current (or slightly upgraded) electronic micro-fabrication techniques. This can allow for commercializing low cost devices without requiring any expensive, bulky machine.

The technology described in this review has not yet been applied to detection of histone modifications in cancer patients. Despite this, LOCs developed for biosensing could provide the following advantages:

1) Identification of markers of cancer initiation, for early diagnosis. This could be achieved by simple blood tests, similarly to what is PSA dosage for prostate cancer.
2) Identification of prognostic or predictive markers, to tailor treatment decisions. Histone modification profiling could be particularly useful to predict response to an emerging class of cancer drugs, targeting histone acetylation and DNA methylation [97, 98].
3) Analysis of easily accessible biological fluids: blood samples [90] but also tumor-specific fluids (urine for genito-urinary neoplasms, saliva for head and neck cancer). This would allow for rapid and reproducible epigenetic profiling. For example, it could be possible to follow-up histone alterations at different time points during cancer therapy, thereby adjusting therapeutic interventions.

Despite these promising applications, some drawbacks need to be overcome, before applying this technology to the clinical setting.

1. Some technology issues should be solved, namely finding the suitable materials, which can be used not only for integrating optics, fluidics and electronics but also to guarantee bio-compatibility.
2. Devices should be optimized, by properly engineering, to improve performance (reproducibility, sensitivity, detection limit, analysis times) with respect to currently available techniques. In particular, detection limit should range between 1 and 50 ng/ml, according to circulating tumor DNA concentration [90].
3. Appropriate trials should be designed to validate label-free based LOCs effectiveness in specific clinical settings.

If these challenges will be met, cancer epigenome studies based on label-free optical biosensors may become a reality in the next decade.

Valentina Donzella, received her B.-Sc. (2003) and M.Sc. (2006) in electronic engineering, from University of Pisa and Scuola Superiore Sant'Anna (SSSUP). She obtained her Ph.D. from SSSUP (2010), with a dissertation about "Silicon Nanoclusters and Rare Earth sensitized Er doped waveguide amplifiers for telecommunication applications". During her Ph.D. she spent 8 months as visiting student at the department of Engineering Physics at McMaster University, ON, CA. She is currently a research fellow at SSSUP, working on integrated optical amplifiers and bio-sensors as well as on photovoltaic cells.

Francesco Crea obtained his MD degree in 2006 from University of Pisa (Italy). His dissertation was on "Epigenetic mechanisms of irinotecan sensitivity in colorectal cancer cells". He obtained his Ph.D. in Cancer Epigenetics in 2010, from Scuola Superiore Sant'Anna, Pisa (Italy). From August 2008 to December 2009, he was admitted at National Cancer Institute-Frederick (MD, USA) as visiting scientist. There, he followed a project on cancer stem cell epigenetics. He is actually resident in Clinical Pharmacology (University of Pisa), with a research project on epigenetic biomarkers in colorectal, pancreas and brain tumors.

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