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Advances towards reliable identification and concentration determination of rare cells in peripheral blood

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ABSTRACT

Through further development, integration and validation of micro-nano-bio and biophotonics systems FP7 CanDo is developing an instrument that will permit highly reproducible and reliable identification and concentration determination of rare cells in peripheral blood for two key societal challenges, early and low cost anti-cancer drug efficacy determination and cancer diagnosis/monitoring.

A cellular link between the primary malignant tumour and the peripheral metastases, responsible for 90% of cancer-related deaths, has been established in the form of circulating tumour cells (CTCs) in peripheral blood. Furthermore, the relatively short survival time of CTCs in peripheral blood means that their detection is indicative of tumour progression thereby providing in addition to a prognostic value an evaluation of therapeutic efficacy and early recognition of tumour progression in theranostics. In cancer patients however blood concentrations are very low (=1 CTC/1E9 cells) and current detection strategies are too insensitive, limiting use to prognosis of only those with advanced metastatic cancer. Similarly, problems occur in therapeutics with anti-cancer drug development leading to lengthy and costly trials often preventing access to market.

The novel cell separation/Raman analysis technologies plus nucleic acid based molecular characterization of the CanDo platform will provide an accurate CTC count with high throughput and high yield meeting both key societal challenges. Being beyond the state of art it will lead to substantial share gains not just in the high end markets of drug discovery and cancer diagnostics but due to modular technologies also in others. Here we present preliminary DNA hybridization sensing results.

Keywords: Circulating Tumour Cells, silicon nitride waveguides, Mach Zhender Interferometry, Multimode Interference, Arrayed Waveguide gratings, AlOx sacrificial layer, azide organosilanes, DNA hybridization,

1. CTC DETECTION TECHNOLOGIES

1.1. A Point of Care Diagnostic Challenge

Some diagnostic applications are extremely demanding, such as those requiring both the identification and concentration determination of extremely low levels of cells in peripheral blood, that to date no such point of care (PoC) diagnostic technology generic or otherwise exists for these. One such application is for the recognition and the concentration measurement of rare circulating tumour cells (CTCs) for early diagnosis staging and monitoring of cancer. Pancreatic cancer is one such target, and is of strong socio-economic interest as currently it is usually diagnosed at an advanced stage when rapid progression has occurred for widespread disease dissemination and so a 96% chance of death for some 68000 Europeans diagnosed every year. A powerful tool for correct diagnosis and staging of pancreatic cancer would permit not just advances in cancer management, where clinicians could prescribe a suitable early treatment and monitor its progress within theranostics, but also in drug discovery for quick and accurate determination of the effectiveness of new anti-cancer drugs in development tests.

1.2. State of art

The detection and molecular characterization of CTCs are one of the most active areas of translational cancer research, with > 400 clinical studies having included CTCs as a biomarker [1]. There is a battery of analytical tools available for

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the analysis and characterization of cells once isolated, including immunochemistry and nucleic acid analysis. However, CTCs are rare even in patients with advanced cancer, as low as 1 CTC in 1 billion blood cells. Furthermore, the techniques currently used to isolate these CTCs require laborious manual sample preparation steps that result in highly variable results and low sensitivity. A reliable rare cell sorter technology for CTCs is therefore exceedingly challenging. Some of the current commercially available CTC isolation methods are described in Table 1.

Table 1: CTC detection technologies

Technology Name	**Company Name**	**Isolation Method/ Assay**	**Detection Method**	
			CTC Count	**Molecular Analysis**
AdnaTest	AdnaGen,	Immuno/Enrichment + Characterization	No	Yes
ApoStreamTM	ApoCell, Inc	Electrical + Immuno/Enrichment	Yes	No
IsoFlux	Fluxion Bioscience, Inc	Electrical + Immuno/Enrichment + Characterization	Yes	No
CellSearchTM	Veridex	Immuno/ Enrichment + Enumeration	Yes	No
CaptorTM	Abnova, Taipei,	Size/Enrichment + Enumeration + Characterization	Yes	No
DEPArray	Silicon Biosystems,	Electrical/ Enumeration	Yes	No
Epic HD-CTC Assay	Epic Science, Inc	Immuno/Enumeration + Characterization	Yes	No
CellCollector	GILUPI	Invivo (immuno)/Enrichment + enumeration Enumeration + Characterization	Yes	No
OncoCEE-BR TM	Biocept, Inc	Immuno/Enumeration + characterization	Yes	No
Cytotrack CT4	CytoTrack ApS	Immuno/Characterization	Yes	No
ScreenCell	Screen Cell,	Size/Enrichment + characterization	Yes	No
CanDo platform	***CanDo Consortium***	***size + Immuno/Enrichment + enumeration + characterization***	***Yes***	***Yes***

The current "gold standard" for CTC isolation and CTC enumeration is the Cellsearch® System by Veridex, an affiliate of Johnson & Johnson. The CellSearch® System is the first and only 510(k) diagnostic test used to automate the capture and detection of CTCs, tumour cells that have detached from solid tumours and entered the patient's blood. The CellSearch System is focused on isolating CTCs from the peripheral blood using immunomagnetic beads conjugated with EpCAM [2]. This FDA approved technology sets the standard for patients with metastatic breast, prostate or colorectal cancer. Patients in the non-metastatic setting or with other tumour indications are not eligible to benefit from this technology. However, its success has been seriously hampered by very low yield and purity of isolated CTCs [3]. The low yield of immunomagnetic isolation of CTCs is likely to reflect the fact that these cells are very rare and do not readily survive the multi-step batch purification technique involved. A reliable clinical test that can confirm that a patient is free of circulating tumour cells is of great value for the patient in a staging situation or in a situation of follow up after successful treatment.

All-in-all current commercial technologies are semi-automated at best with multiple "bulk" enrichment steps such as magnetic particle sorting. As a result, the sensitivity and specificity of CTC detection and analysis is very low, highly variable, and not very useful for a clinical test.

1.3. Moving beyond state of art

In CanDo, through the integration of various components and sub-systems recently developed in other projects a diagnostic platform is targeted that will enable accurate and reliable early pancreatic cancer diagnosis, monitoring and prognosis determination based on efficient capture and recognition of CTCs in peripheral blood at unprecedented limits of detection.

The targeted approach (Figure 1) is based on a disposable cartridge system that combines a powerful cell separation technology; principally through a combination of the GILUPI system [4] or inertial microfluidics [5] and high yield sensitive immuno-capture, with Raman spectroscopy to identify/quantify the CTC and sensitive nanophotonic sensors for nucleic acid analysis from the lysed CTCs for molecular characterization. Not only should the sensors be sensitive but

they and their instrumentation should also be capable of multiplexing of up to 20 times and be of a suitably low cost to make the overall platform competitive. In this paper we present some preliminary results of some novel sensors that would meet such criteria.

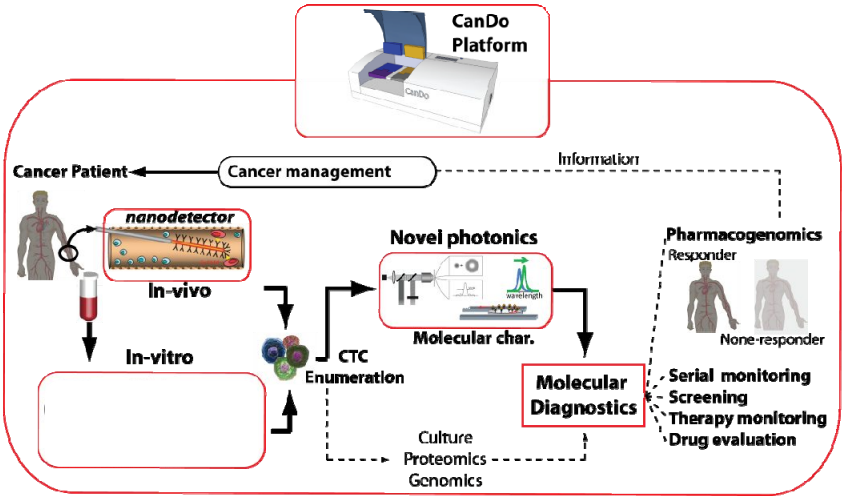

Figure 1. A schematic of the principally targeted CanDo platform with indicated components and sub-systems. The platform achieves cancer diagnosis through CTC capture and enumeration (cellular characterization) followed by nucleic acid analysis (molecular characterization). It takes whole blood from cancer patient and isolates the CTCs using a novel in-vivo isolation technology (whilst another in-vitro technology is considered as a backup) to deliver cell enumeration. The enumerated cells are then further processed for nucleic acid characterization using a label-free photonic sensor array.

2. NANOPHOTONIC BIOSENSORS

2.1. State of art

Biosensors are by definition a combination of a biological receptor molecule and a physical or physicochemical transducer [6]. The main challenge for biosensor technologies in diagnostics and other biosensing applications is the ***rapid*** detection of ***low concentration*** of a specific analyte in ***small sample volumes*** typically in a complex mixture with large background signals (a problem known as ***non-specificity***).

Optical methods of transduction offer important advantages such as non-invasive/safe, label free and multi-dimensional (intensity, wavelength, phase, polarization) high sensitivity detection across optical frequencies that coincide with a wide range of physical properties of bio-related materials. Within the important category of label free biosensing, optical transducers have a significant presence: indeed, the dominant vendor of label free technology is Biacore, and their technology is a photonic one, based on surface plasmon resonance. However, the detection limit of this technology is inherently limited due to absorption effects in the metal. Nevertheless, with measurements of optical absorption and refractive index (RI) being well established analysis methods in chemistry and biology they have been incorporated in label-free biosensor based lab-on-chip solutions through technologies such as surface plasmon resonance (SPR) [7][8] as well as others based on thin-film interference spectroscopy [9], or optical waveguides [10].

In the quest for label free lab-on-a-chip systems with lower limits of detection an alternative optical biosensing technology to SPR and its metallic structures has arisen based on dielectric structures [11]. Specifically, recent maturity in silicon photonics technology that operates in the near infra-red (NIR) has led to advances in the employment for sensing of the evanescent field of guided modes in high-index core dielectric waveguides such as interferometers [12], photonic crystals [13], microsphere resonators [14] and silicon ring resonators – the latter having achieved detection limits down to the femtomolar range for proteins [15]. Their compatibility with mature silicon microprocessing technologies and materials have permitted a high integration of complex structures, on-chip integration of electronics and, via the addition of polymers, microfluidic channels for high-throughput arrays on a single-chip for simultaneous detection of multiple analytes. These devices with optical transmission lengths in the order of millimetres achieve

surface concentration detection limit (SCDL) values in the order of 0.1 - 1pg/mm^2 and so in recent years, the EU has funded relevant projects regarding optical biosensing systems based on these [16]. **Table 2** summarises the results from two of these, SABIO [17] and InTopSens [18].

Table 2: A summary of main achievements and problems encountered in two recent EC projects. UPV corresponds to the group of A. Maquieira at the Technical University of Valencia and IMEC to that of T. Stakenborg at IMEC.

Project	**Technology**	**Main Achievements**	**Problems encountered**
SABIO	SiN slot ring resonators with UPV chemistry	Methanol/Ethanol dilution series detection: 5x10^{-6} RIU. Glutaraldehyde-AntiBSA detection: successful detection of 0.9 pg/mm^2, a significant improvement on a previously published value of 28 pg/mm^2.	Poor resolution due to fabrication technology limitations (sidewall roughness).
InTopSens	Si slot ring resonators with UPV chemistry	Aqueous salt solution detection: 4.2x10^{-5} RIU Biotin-avidin detection: 5pg/mm^2	Lower resolution than SABIO device principally due to bending and mismatch losses.
InTopSens	Si ring resonators with UPV chemistry	Aqueous salt solution detection: 5x10^{-6} RIU Biotin-avidin detection: 2pg/mm^2. High degree of multiplexing achieved.	Instrumentation cost very expensive (tunable laser).
InTopSens	Vernier cascade Si ring resonators with UPV chemistry	Aqueous salt solution detection: 8.3x10^{-6} RIU. DNA detection: Cheaper readout but poorer LoD than regular ring resonators. Inherently temperature stable.	Surface chemistry issues prevented successful detection of on-chip hybridisation.
Post InTopSens	Regular Si ring resonators with IMEC chemistry	DNA detection: 100 nM 25 bp, to be compared to the best published result of 10pM of 58mers for a biomodal interferometer [19].	Limitation to multiplexing capability.

However, none of these have been commercialized and it is debatable whether, the Maverick Technology, based on (non-slot waveguide) ring resonators fabricated by IMEC, of Genalyte will be a commercial success for the same reason – they require a high performance and therefore costly tuneable laser. In the case of the Genalyte system, its sale cost is anywhere between 50 and 100k€ which automatically limits it to niche applications, almost all being non-point of care, across healthcare, food safety, environment, security markets. Low cost ring resonator technologies such as the 'Verniers' [20] with their cheap broadband light sources have similar sensitivities but their on-chip spectrum analysers with resolutions of only 1nm are no match for the expensive external spectrum analysers, resulting overall in poorer limits of detection. Moreover, they are more limited for multiplexing.

2.2. Moving beyond state of art

2.2.1. Si_3N_4 integrated unbalanced Mach-Zehnder Interferometer

Photonic biosensors (Figure 2) based on a silicon nitride (Si_3N_4) integrated unbalanced Mach-Zehnder Interferometer (MZI) have been developed in the FP7 POCKET project by two of us (Mertens and Bienstman). Such biosensors have been shown to have both sufficient multiplex capability and limit of detection for the CanDo application and cost up to 15x less than the aforementioned technologies in section 2.1 that operate at wavelengths of 1300 or 1550nm. In order for their implementation to be low cost, an 850 nm free space SLED broadband source has been used as the incident light and, an on-chip arrayed waveguide grating (AWG) [21], filters the output MZI spectrum prior to the light exiting the chip. Specifically, the light is coupled into the on chip optical circuit through a 16º vertical grating coupler, before being guided along a monomode strip waveguide until its division between two arms using a MMI coupler. The whole circuit is covered with an oxide cladding layer except the sensing waveguide which is exposed to the analyte. The light from both arms are combined using a second MMI coupler, so there is an intensity modulation at its output depending on the phase shift introduced by the sample to be measured over the sensing branch. The MZI output is then filtered through the AWG into 30 channels with 1nm bandwidth each, and the light is coupled out of the circuit over 30 grating couplers. The output optical intensity from the couplers is measured by a CCD camera over a lens, and proprietary software fits peaks to the spectra and represents the MZI output modulation. The different designs of the MZI biosensors (MZI1, MZI2, MZI3 etc) on each chip in the reported experiments (Figure 3) permitted different sensitivities. Each chip contained 7 Input grating couplers, 6 different sensors, 7 AWG filters, 210 output grating couplers (6 sensors by 30 output AWG channels and 30 more for the reference) with the input and output couplers orthogonal for reducing scattering. Although each chip of biosensors used was identical those functionalised in one batch were labelled as HULKx and those in

another SPIDERMANy where x and y are numbers that indicate the sequence of use, first HULK1 and then HULK2 in the next experiment etc.

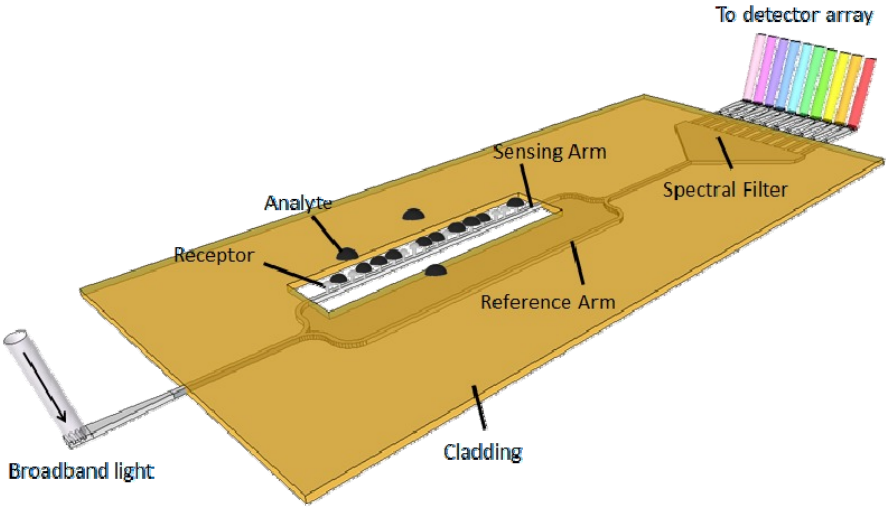

Figure 2. Schematic of the nanophotonic integrated biosensor circuit.

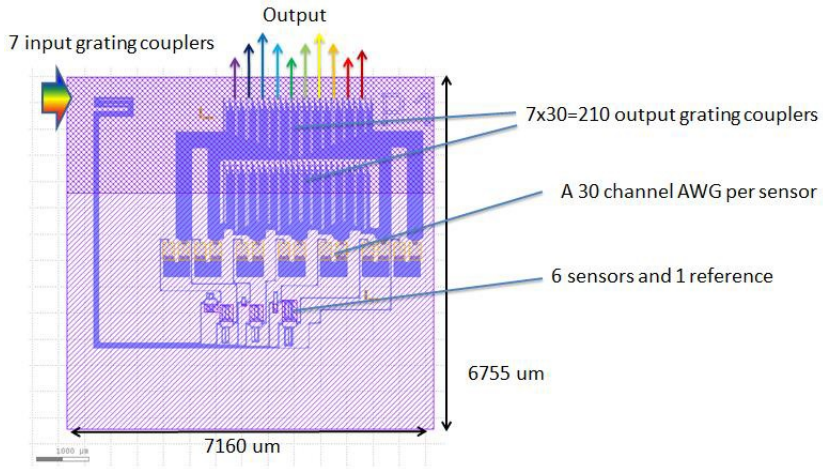

Figure 3. Components distribution of the fabricated and analysed chip

2.2.2. DNA sensor functionalisation

Synthetic hexynyl-modified single strand DNA (ssDNA) probes were covalently bonded to the functionalised surface of the MZI sensing waveguides through the use of standard click chemistry. The surface was functionalised with azide organosilanes previously immobilized using a vapour phase deposition method (145°C, 25mbar).

2.2.3. Experimental setup

The setup (Figure 4) includes a syringe pump to flow a hybridization buffer (1M NaCl, 10 mM Tris.HCl, and 2mM EDTA; pH 7) at 10 µL/min towards the chip via a valve which is switched to allow the ssDNA analyte to flow through an inlet tube and into a PDMS (PolidimetilSiloxane) microfluidic channel on the surface of the chip that conducts the sample over the surface of the sensing arm waveguide. The output of the microfluidic channel thereafter passes into an outlet tube and onto the waste chamber. The chip is mounted on an aluminium plate with a Peltier element which is connected to a temperature controller to fix the chip temperature to 303.45K. The optical source with a collimator is

mounted on a three axis micro-positioner to align the light spot onto the input grating couplers of the chip. The intensity of the 30 output light spots is measured by a CCD camera and the control computer software detects the wavelength shifts.

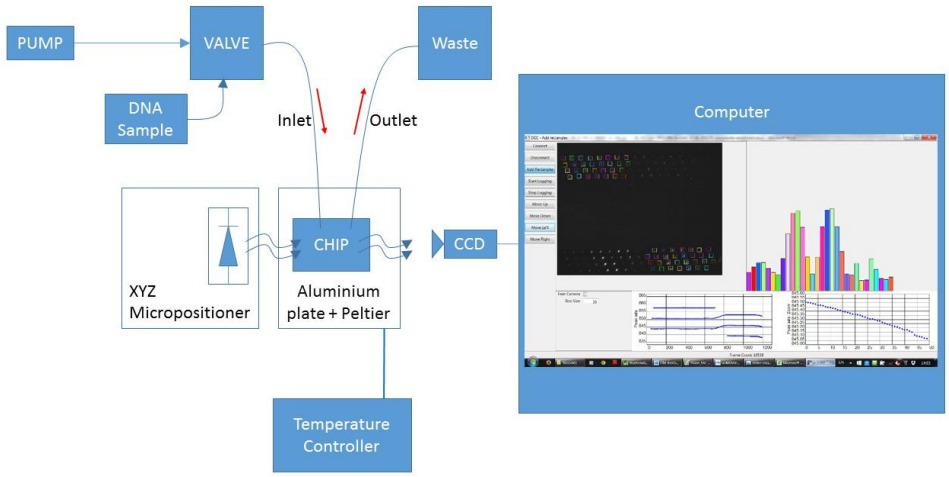

Figure 4. Schematic of the DNA detection setup

2.2.4. Experimental measurements

Before the start of DNA detection, the sensing surfaces within the microfluidic system were rinsed with injections of isopropanol and distilled water followed by HCl to regenerate the surface. Afterwards, the DNA hybridization buffer was injected at a rate of 10 µL/min to allow the surface of the waveguide to capture the complementary ssDNA analyte. Once the peak wavelength was stable, and with the buffer still flowing, injections of the analyte (a 25mers complementary ssDNA) at concentrations of 5pM, 50pM, 500pM, 5nM, 50nM, 400nM were introduced, each separated by HCl injections to regenerate the surfaces.

Figure 5 shows sensorgrams for four different DNA concentrations on four different sensors within chips Spiderman1, 2 and 3. Figure 5a shows the detection of just 5pM of ssDNA from a wavelength shift of 50pm although the noise level is around 18pm.

Figure 6a shows the measured peak wavelength shift over different DNA concentrations, from 50pM upwards, on MZI4 and MZI6 sensors (both on chip HULK4). From linear fits to this preliminary data a 23pm/pM sensitivity was derived for the sensor MZI4, and 37pm/pM for MZI6. Figure 6b shows measurements from an experiment with a sensor of the same design (MZI4) on a chip functionalised in a separate batch, Spiderman4. In this experiment HCl was not injected between samples, and so the DNA concentration range extends up to 455nM. From a linear fit over the first three data, the derived sensitivity is similar to that of that on Hulk4 at 17nm, with the difference possibly due to differences in surface activity being functionalised in two different batches.

The wavelength dependency on temperature has also been studied. Figure 7 shows that MZI4 on the Spiderman4 chip has a temperature sensitivity of -54.9pm/K.

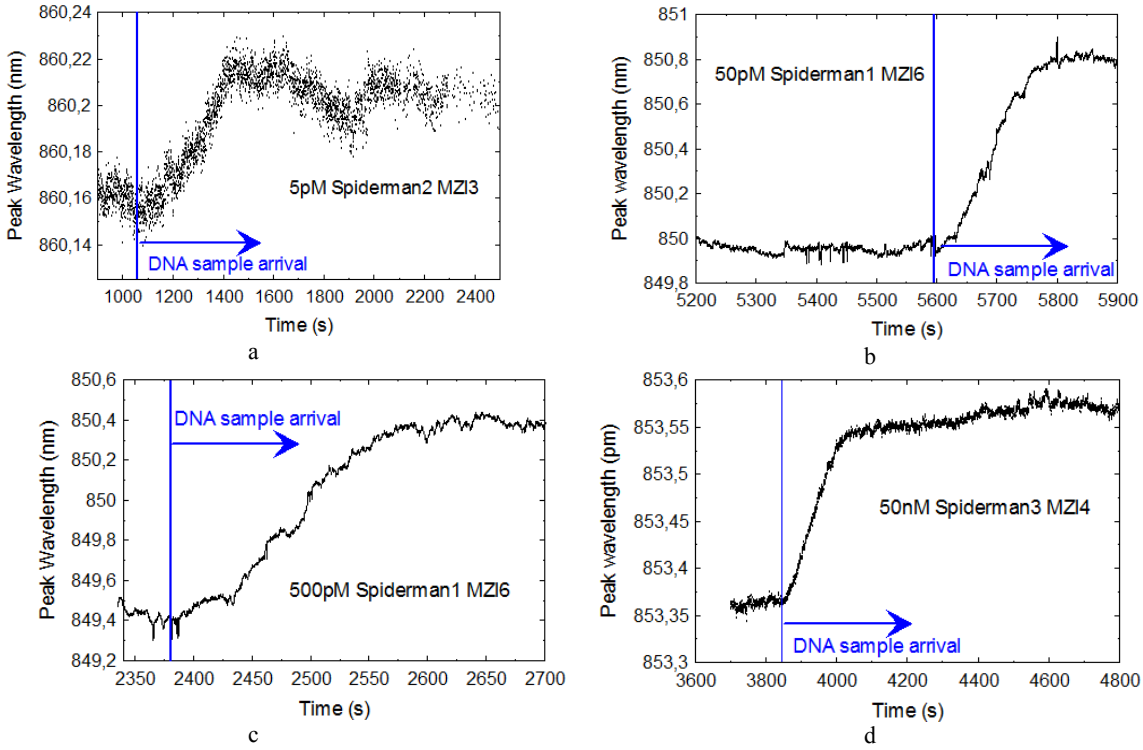

Figure 5. Measured sensorgrams at different concentrations of 25-mers DNA fragments on different sensors.

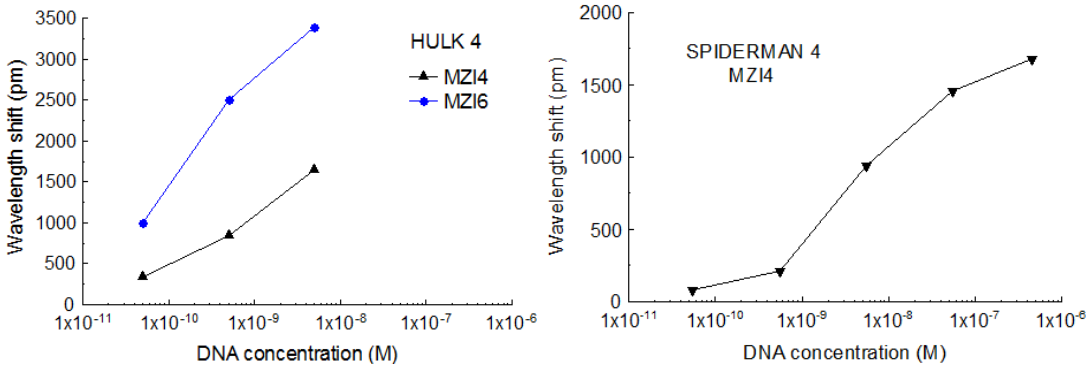

Figure 6. Measured wavelength shifts for given ssDNA analyte concentrations for sensors MZI4 and MZI6 on chip Hulk4 with HCl injections between samples (a), and MZI4 on chip Spiderman4 without HCl injections between samples (b).

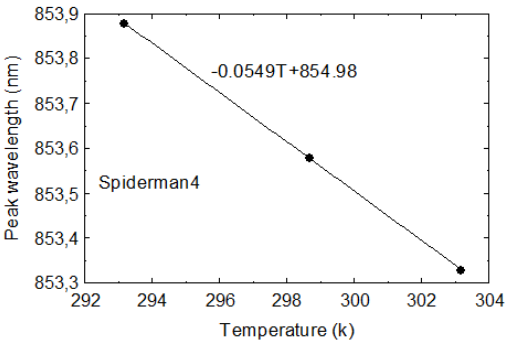

Figure 7. Peak wavelength over temperature for MZI4 on chip Spiderman4.

3. CONCLUSIONS

A low cost, potentially highly multipliexed biosensor design based on an integrated unbalanced silicon nitride nanophotonic Mach-Zehnder Interferometer integrated together with a spectrum analyser is presented, and its functionalization for DNA detection described. As far as we are aware this is the first time experimental results for DNA hybridization sensing have been shown for such a device. If the preliminary result of 5pM detection of 25mers ssDNA is confirmed in further experiments underway for full analytical data, it would be superior than what we understand to be the best published limit of detection to date of 10pM of 58mers. That with said the preliminary data is sufficient to demonstrate the suitability of the biosensors for their integration into the CanDo device.

4. ACKNOWLEDGMENTS

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