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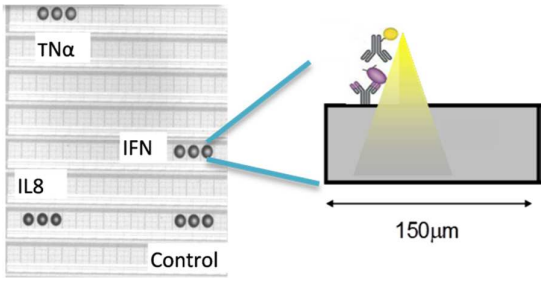

Individual CMOS Bio-pixels with triplicate assay for cytokines TNFα, IL8 and IFN. Expansion shows a schematic of light emission confinement.

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Large area CMOS bio-pixel array for compact high sensitive multiplex biosensing

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A novel CMOS bio-pixel array which integrates assay substrate and assay readout is demonstrated for multiplex and multireplicate detection of a triplicate of cytokines with single digit pg/ml sensitivities. Uniquely designed large area bio-pixels enable individual assays to be dedicated to and addressed by single pixels. A capability to simultaneously measure a large number of targets is provided by the 128 available pixels. Chemiluminescent assays are carried out directly on the pixel surface which also detects the emitted chemiluminescent photons, facilitating a highly compact sensor and reader format. The high sensitivity of the bio-pixel array is enabled by the high refractive index of silicon based pixels. This in turn generates a strong supercritical angle luminescense response significantly increasing the efficiency of the photon collection over conventional farfield modalties.

Introduction

Minaturised and simplified analysis systems are important tools for biological diagnostic applications. Recent developments in Lab-on-a-chip (LOC) diminutive devices have shown the potential to bring the quality of laboratory based assays to on-site and point-of-care settings. Extensive developments have enabled micron scale, low-volume replication of the primary techniques and features prevalent for biodetection applications.[1,2] This progress has resulted in the continued adoption of LOC devices for commercial clinical diagnostic products. Luminescence-based LOC remain the mainstay bio-assay technique, with fluorescent labels routinely employed to transduce biomolecular binding events. Multi-analyte and/or multi-replicate measurement capabilities can be provided by LOC array biosensors, comprising patterned arrays of biorecognition elements[3,4]. However, a number of LOC device limitations remain, including complexity of devices due to light collection challenges, constraints in engineering contact between the assay and the sensing reader, and the requirement of additional analysers.[3,-6] The poor signal collection provided by conventional far-field cameras, which at most collect a few percent of the generated light signals, decrease detection sensitivities. In many state-of-the-art LOC devices, elaborate read-out instrumentation are required such as expensive low noise photon multiplier tubes/back thinned cooled CCDs, long signal integration times and cumbersome parasitic light protection, result in the loss of portability and simplicity. Herein, a novel, large-area CMOS (complementary metal-oxide-semiconductor) bio-pixel array is presented which overcomes the separation between sample and signal detection, effectively integrating the assay and reader functions. This bio-pixel array incorporates a powerful method based on supercritical angle luminescence (SAL)[7], for efficient, high-precentage signal detection, serving to greatly increase the sensitivity and simplicity for LOC devices.

CMOS technology presents itself as a powerful tool for diagnostics/biosensing, benefiting from low-cost and mass-production, a capacity for scalable sensor design and provision for extensive parallel analyte detection.[8-11] The CMOS photodetector arrays employed here comprise specifically designed, large 150 µm x 150 µm area pixels (bio-pixels), developed by ST Microelectronics, to accomodate standardised liquid spotting and biofunctionalisation methodologies. The array consists of 128 photosensitive bio-pixels, each individually addressing a separate bio-spot without further image processing. Their large size resolves complications associated with liquid spots overlapping several pixels which occurs on conventional CMOS arrays with smaller pixel size (typically 2 - 10 µm)[11]. The large area biopixels importantly provide amenability to standardised and accessable assay substrate preparation and biofunctionalization techniques. Here, antibodies are coated onto individual bio-pixels using non-contact printing, which can be readily adopted for commercial assay development. Conventional light collection issues are solved by using the light detecting bio-pixels as the direct surface upon which the assays are is performed. Additionally, by taking advantage of the high refractive index of the silicon

substrate to evoke an effective SAL response, the light collection efficiency is greatly enhanced. The SAL phenomenon acts to strongly confine photons emitted in close proximity to the bio-pixel surface and direct them to the detection nodes within the pixel as indicated in Fig 1b).[7,12] In contrast to the isotropic radiation (Fig 1a)), the presence of the planar interface alters the radiation pattern of the fluorescence emitted from a molecular largely confining it in a cone whose angle $\theta = 61°$.[7,12-14] The very high refraction index of the silicon pixel sensor dramatically improves the SAL effect over conventional use of glass substrates. The case of the enzyme horseradish peroxidase (HRP), the extensively used chemiluminescent enzyme which has an emission spectrum centered at 430 nm, is modelled.

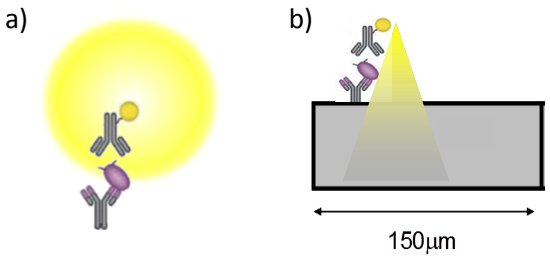

Fig 1. Illustration of efficient capture of chemiluminescent ELISA light emission due to the strong supercritical angle fluorescence effect of the silicon-based bio-pixels: Schematic of chemiluminescent ELISA light emission a) isotropic radiation pattern in a conventional bulk medium b) strong confinement above the surface of a high refractive index pixel sensor.

The biopixel–array has the potential to enable highly sensitive LOC detection of large numbers of targets in a simple diminutive all-digital format, without the need for optics or additional analaysers. Highly sensitive, multiplex and multi-replicate detection is demonstrated using the CMOS biopixel array to perform a classical chemiluminescent immunoassay (ELISA; enzyme linked immunosorben assay). The use of a chemiluminescent reporter avoids additional noise from excitation photons by using detection antibodies labelled with enzymes that catalyse a chemiluminescence-type reaction. As indicated in Fig. 1b, the resultant light generated from the chemiluminescent reaction is directly measured by the underlying bio-pixels. The statistically relevant detection of three different cytokines, small proteins which play important roles in the modulation of immune response pathways [15-17], are demonstrated. We report up to 100-fold improvement factors on detection sensitivity for the simultaneous chemiluminescent detection of three cytokines, TNFα, IFN and IL8 using the bio-pixel array compared to the same chemiluminescent assays carried out using a standard microplate reader for validation.

Experimental Methods

Materials

3-glycidyloxypropyl)trimethoxysilane (GOPTS), bovine serum albumin (BSA), glycerol and Tween20 were purchased from Sigma-Aldrich (France). Inteleukin-8 (IL8), Interferon-gamma (IFN) and Tumor Necrosis Factor-alpha (TNFα) and their capture and detection- antibodies and horseradish peroxidase (HRP) were obtained from R&D Systems (France). SuperSignal WestFemto Chemilumiscent substrate (ECL) was obtained from Thermo Fisher Scientific (France). Phosphate Buffered Saline (PBS, pH 7.4) and Tween 20 were purchased from Euromedex (France).

Bio-Pixel Array Packaging and Surface treatment The bio-pixel array is conditioned in a standard integrated circuit packaging which consists of a ceramic vessel with wire bonded gold-plated contact pads and a central cavity in which the sensor is glued. By embedding in an epoxy resin, mechanical integrity and water resistance is achieved (Fig. 2a)). Silanisation with (3-Glycidyloxypropyl)trimethoxysilane (GOPTS) was used to enable covalent bonding of capture antibodies on the surface of the bio-pixel arrays. O_2-plasma activation using an Oxford 80 Plasmalab RIE system (50 sccm O_2 flow, 2 min, 100 W) was first used to remove traces of organic contaminants and generate a hydrophilic surface. Chemical Vapour Deposition (CVD) of GOPTS was carried out by placing the bio-pixel arrays and GOPTS in separate chambers of an in-house developed CVD under vacuum up to $2\text{-}3x10^{-2}$ mbar. Upon heating to a temperature of 85-90°C the silane chamber was slowly opened to allow the silanisation of the bio-pixel array surface within a dynamic vacuum over a period of 1 h. The prepared bio-pixel arrays were stored in a dessicator in the dark for several weeks.

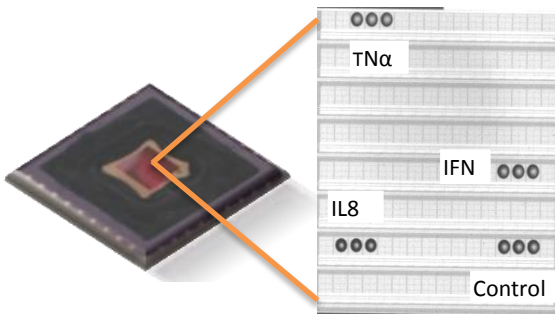

Fig 2. CMOS bio-pixel array, a) Epoxy resin-embedded CMOS bio-pixel array. b) Image of a section of the bio-pixel array individual pixels each available to address a specific bio assay. Selected pixels are spotted with capture antibodies for cytokines TNFα, IL8 and IFN spotted as well as 3 control spots.

Bio-Reagent deposition Bio reagents such as capture antibody probes were deposited with an automated piezo driven non-contact dispensing system of ultra-low volumes (SciFlexarrayer S3 from Scienion AG, Germany). Capture antibody solutions were prepared in PBS with 5% glycerol, with IFN at 1 mg/mL, and IL8 and TNFα at 0.4 mg/mL. For each of the three cytokines, three bio-pixels were spotted with one 220 pL drop under constant hygrometry (60%) and temperature (22 °C) conditions (Fig 2b)). Control bio-pixels were spotted with 1 % BSA in order to provide measurements of noise levels. Upon incubation for 1 hour the bio-pixel arrays were rinsed in PBS with 0.05% Tween20 (PBST-0.05) solution and followed by immersion in a PBS with 1% BSA solution for 1 h at room temperature in order to block the unreacted epoxy sites.

Multiplex Cytokine Chemiluminescent ELISA on CMOS biopixel array The chemiluminescent ELISA protocol was carried out by incubation at room temperature with the analyte solution (3 pg/mL in PBS-1% BSA mixture of the three cytokine analytes) on the bio-pixel array for 30 min, and rinsed with 5 x 200 µL of wash buffer (PBS-T0.05). This was

followed by incubation with the detection antibody solution (500 ng/mL in PBS with 1% BSA for the three detection antibodies) for 30 min and rinsing (5 x 200 µL of PBST-0.05). Incubation with Streptavidin-HRP conjugate (1/200 dilution from the commercial solution in PBS-1% BSA) for 20 min followed and a final rinsing step (5 x 200 µL of PBST-0.05). The bio-pixel array was then plugged into a commercial digital reader and ECL substrate was added. The chemiluminescent emission at 430 nm lies within the high sensitivity range of the bio-pixels.

Data acquisition An image of the experimental set up is shown in the supplementary information Fig 1, consisting of a USB port electronic circuit board connected to a labtop into which the bio pixel array chip to be read is inserted. The compact USB port bio-pixel array reader enables ready data transfer to a computer for analysis. For each bio-pixel, the accumulation of photons over time was measured; a representative is shown in Fig. 4b). Discarding the saturated values, a linear fit was applied to characterize the slope. The background noise, which is related to dark current, was measured prior to the addtion of ECL. The signal emitted by bio-pixels that do not bear probes provides a measurement of analytical noise and is used as control. The amount of analyte was measured by the signals difference between probes and background. The resulting signal, called the assay signal, was finally normalized with the control signal.

Characterization Techniques Absorbance and fluorescence measurements were collected using a microplate reader, Tecan Infinite 200 (Tecan, Switzerland).

Results

Principle of efficient light collection

The photosensitive bio-pixels are composed of silicon based materials which have a high refractive index (n=3.5) generating a strong supercritical angle luminescence (SAL) response. Under these conditions, photons emitted in a medium of lower refractive index such as air (n=1.0) or water (n=1.33), close to the high refractive index surface such as a bio-pixel, result in a substantial portion of the luminescence being radiated into the higher refractive index medium which here corresponds to the bio-pixel substrate. Hence, this mechanism acts as a 'photon pump' efficiently channeling photons generated, towards the photon-electron generation collection nodes located below the bio-pixel surface.

The computational calculation of the redirection of light emitted from a dipole at various distances close to a silicon surface is given in Fig. 3 showing the high percentage of light concentration within the silicon substrate. The luminescent molecule, HRP, is assumed to have three emission dipoles of equal magnitude along the x,y and z axis. When the source is located on the surface (z=0 nm), most of the emitted power is found along two directions (145° and 215°) corresponding to substrate radiative modes that are trapped into the substrate. When the source is placed at z=25 nm and z-110 nm above the surface the radiation pattern still shows two maxima in the substrate radiative modes but a significant amount of energy appears in the radiative modes along directions 0° and 180°.
Integration over a solid angle of these radiation pattern reveals that ten times more power is emitted by a HRP molecule located at z=0 nm into a silicon substrate, than in water with 90% of the total emitted power being directed toward the substrate As the emitter is located further from the water/silicon interface the power emitted decreases to 75% for the case where emitter is located 490 nm above the silicon surface. These results confirm that a source located in the vicinity of a high refractive index sensor material emits most of its power into the sensor direction. This basic property is also used in SAL (the high index material being glass) but is dramatically improved here due to the very high refractive index of silicon.

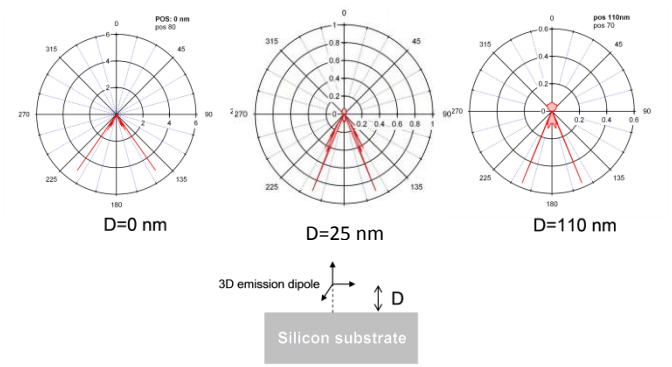

Fig. 3. Emission diagram of a 3D dipole located at a distance D from a bare silicon substrate. The high refractive index (n=3.5) of the silicon concentrates a high percentage of the light into the silicon substrate. The radiation pattern of the emission is illustrated at distances of 0 nm, 25 nm and 110 nm above the surface.

The assay constists of a stack approximately 28 nm in height with HRP (circa. 4 nm) at the summit, comprising, capture and detect antibodies typically 8 nm each, streptavidin (circa.6 nm) and cytokines which are estimated to be 2 nm. This estimated size most closely approached the simulation for 25 nm shown in Fig. 3. The computed radiation patterns confirms that a luminescent molecule located in the vicinity of the pixel sensor material emits most of its power into the sensor direction. This increased light capture feature of the bio-pixel array provides for the significant enhancement of bio-assays carried out directly on the surface and removes the need for external signal readout instrumentation.

Cytokine 3-plex Chemiluminescent ELISA

The chemiluminescent ELISA method, in widespread use in biological assay systems was used.

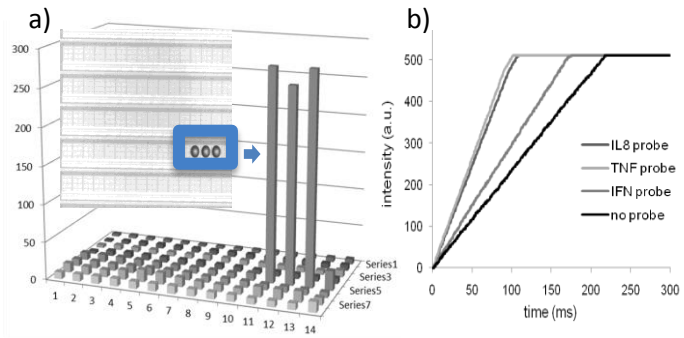

Fig. 4 a) Read-out from the bio-pixel array for the detection of 3 replicates of the TNFα cytokine. Inset shows image of a section of the bio-pixel array with 3 pixels upon which the TNFα assay was performed b) Representative curves showing data acquisition from 4 bio-pixels each containing either IL8, TNFα, IFN probe or no probe respectively during a 3-plex cytokine chemiluminescent ELISA assay.

It does not require an outside excitation source and therefore eliminates the need to use optical filters on the surface of the bio-pixel array. The performance of the assay directly on the bio-pixel surface also ensures that the luminescence emitted on analyte binding is sufficiently close to the bio-pixel surface to fulfill the conditions for SAL, thus enabling highly efficient light capture. The electronic readout of the bio-pixel array is shown in Fig. 4a) for the triplicate detection of the TNF-α cytokine. A clear differentiation in pixel signal intensity between the assay pixels and the surrounding non-assay pixels is observed. Negligible cross talk, as observed in Figure 4a, is facilated by the relatively low number of pixels present compared to typical CMOS chips, enabling sufficient electronic isolation of the individual bio-pixels.

Simultaneous chemiluminescent ELISA assays for three cytokines, TNF-α, IL8, and IFNγ were carried out in triplicate on the bio-pixel array. As shown in Fig. 5 a), low pg/mL detection limits were achieved using the bio-pixel array. These results were 50 to 100 times more sensitive than carrying out the same assays by conventional methods using a TECAN 96 well plate reader. Excellent signal to noise ratios were achieved using the bio-pixel array, an example for which is given in Figure 5 b) for the detection of concentrations of 3 pg/mL.

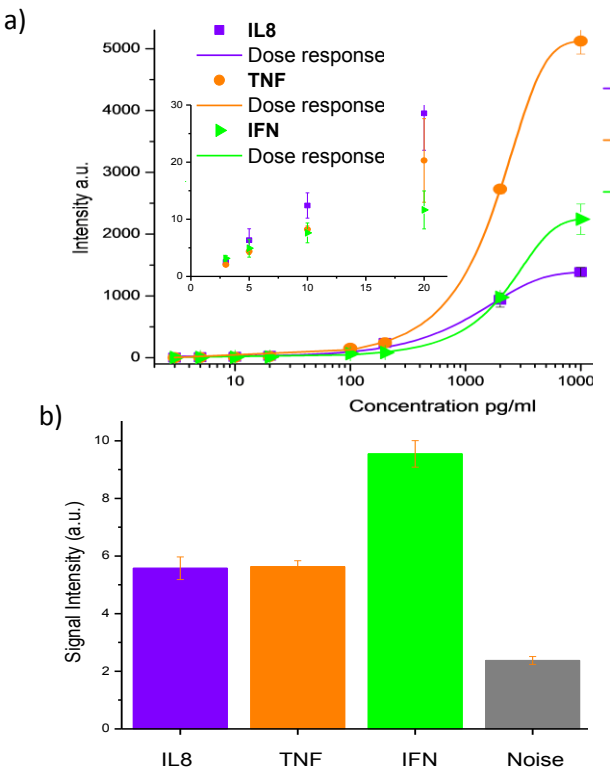

Fig 5. a) Dose response curves with inset showing single digit pg/mL sensitivities for the 3-plex detection of cytokines IL8, TNF and IFN. b) Signal to noise ratio for the 3 cytokines measured at a concentration of 3 pg/mL.

The unique combination of the bio-pixel array features including, large arrray bio pixels which enable a single assay to be completed on a single pixel, the capacity of the bio-pixels to serve as both substrate and reader removing the need for far field light collection and the use of the SAL phenomon on bio-pixels with such a high refractive index enables these high sensitivites with high signal to noise ratio to be achieved using CMOS technology. These high sensitive, multiplex, mulitreplicate results enabled using the diminutive low-cost large area bio-pixel platform, demonstrate its excellent potential for commercial applications, in particular for mainstay chemiluminescent immunoassays.

Conclusions

Compact, highly sensitive, multiplex detection using the CMOS bio-pixel array has been demonstrated for a trio of cytokines. The large area of the bio-array pixels enables individual assays to be dedicated to and addressed by a single pixel. The 128 available pixels provide the capability to simultaneously measure a large number of targets. The compact format of this sensor is facilitated by the capibility to carry out the assays directly on the pixel surface so that the pixel provides the integrated dual role of sensor substrate and sensor reader. Further, the high refractive index of the pixels enables effective SAL, greatly increasing the light collection efficiencies compared to conventional signal collection modalitities. These advantages combined with the inate low-cost mass production features of CMOS-based systems poise the CMOS bio-pixel array as a technology which has the potential to greatly the increase sensitivity and simplicity for LOC devices.

Notes and references

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