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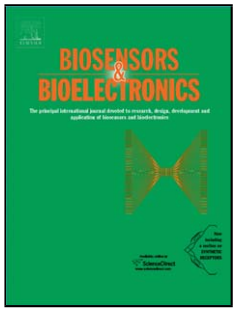

Title: SURFACE PLASMON RESONANCE IMAGING FOR AFFINITY-BASED BIOSENSORS

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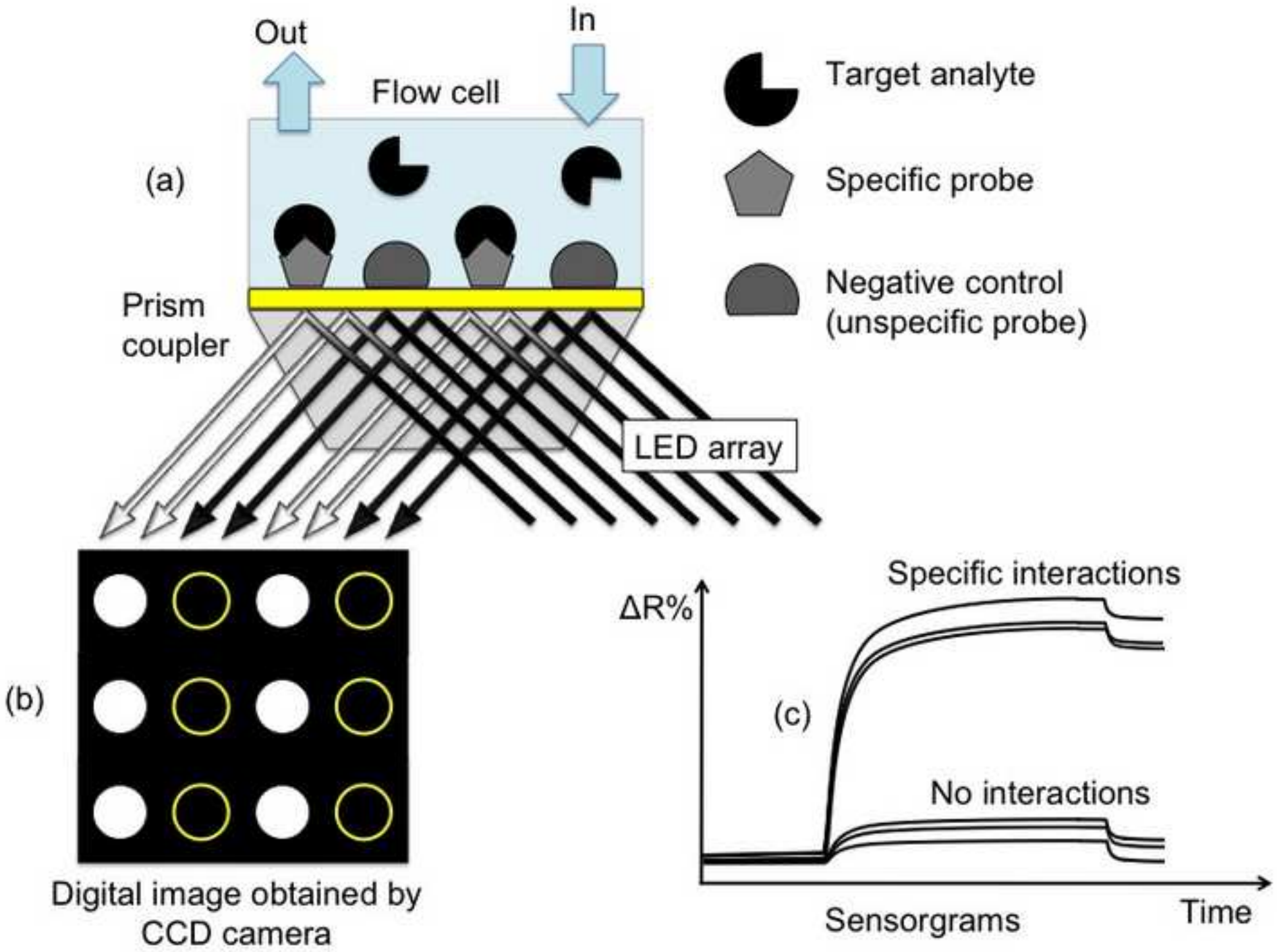
Figure(s)


Out
In
Flow cell
(a)
Prism coupler
LED array
Target analyte
Specific probe
Negative control (unspecific probe)
(b)
Digital image obtained by CCD camera
ΔR%
Specific interactions
(c)
No interactions
Sensorgrams
Time

Figure(s)

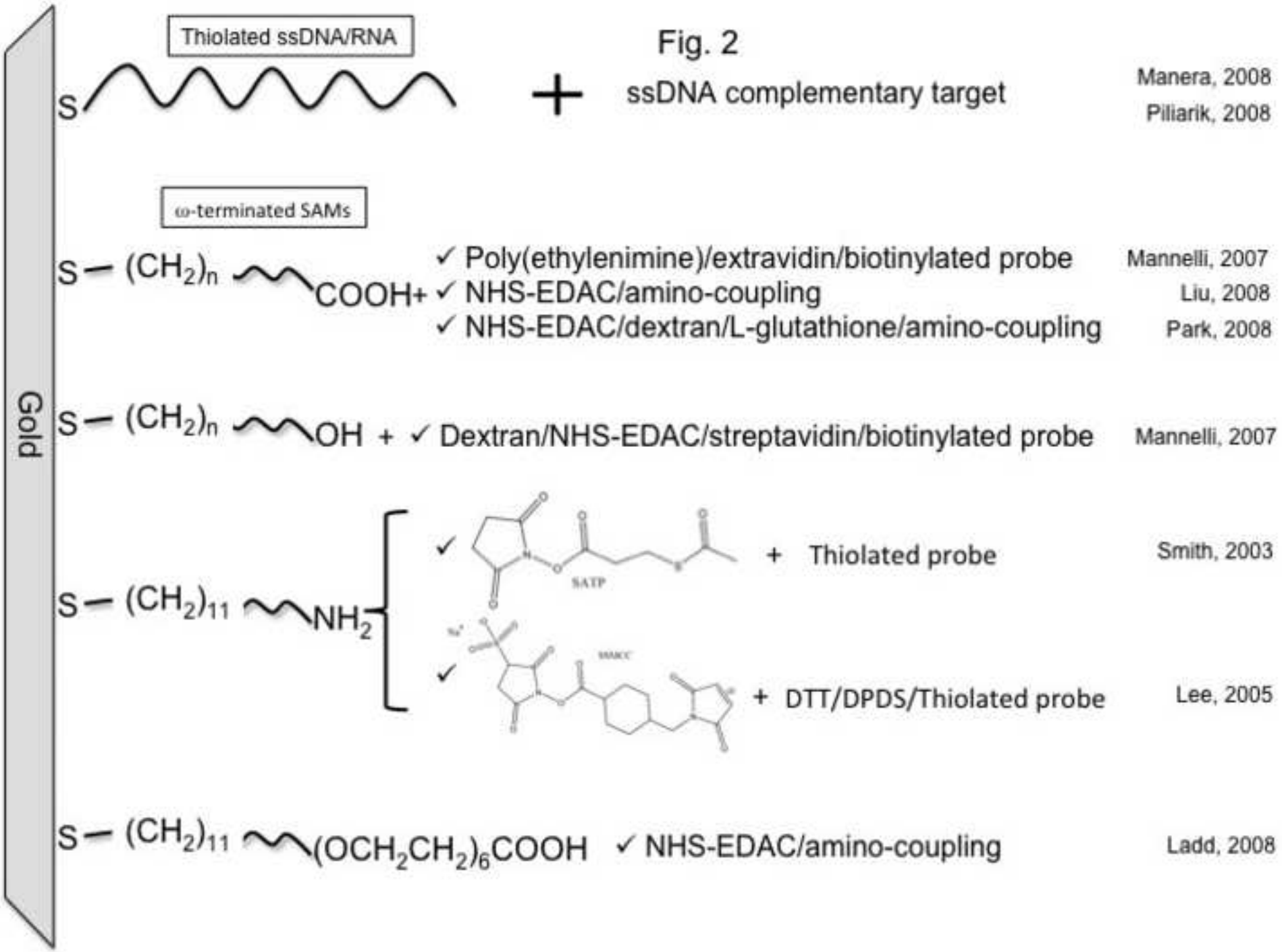


Fig. 2

Figure(s)

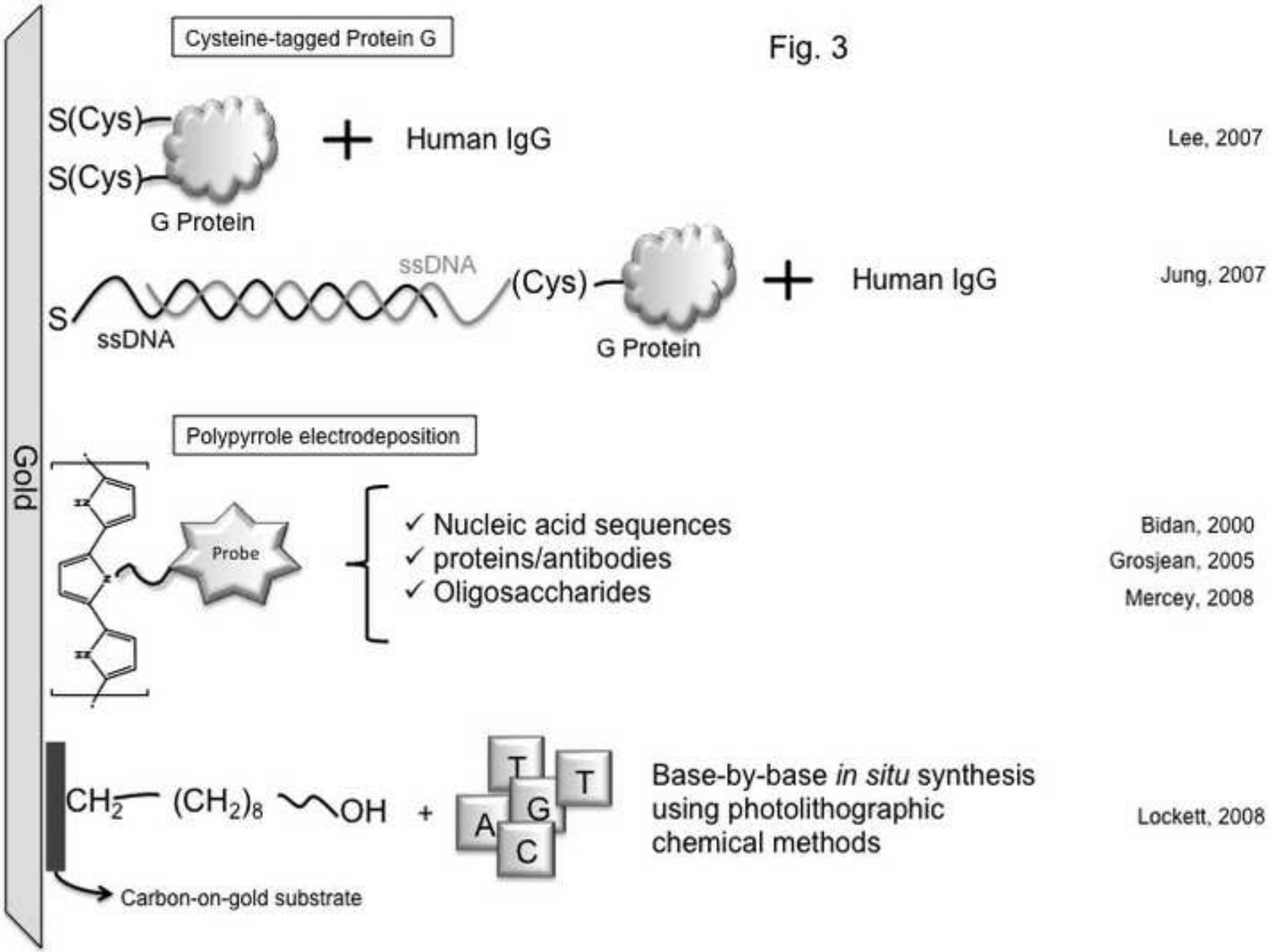
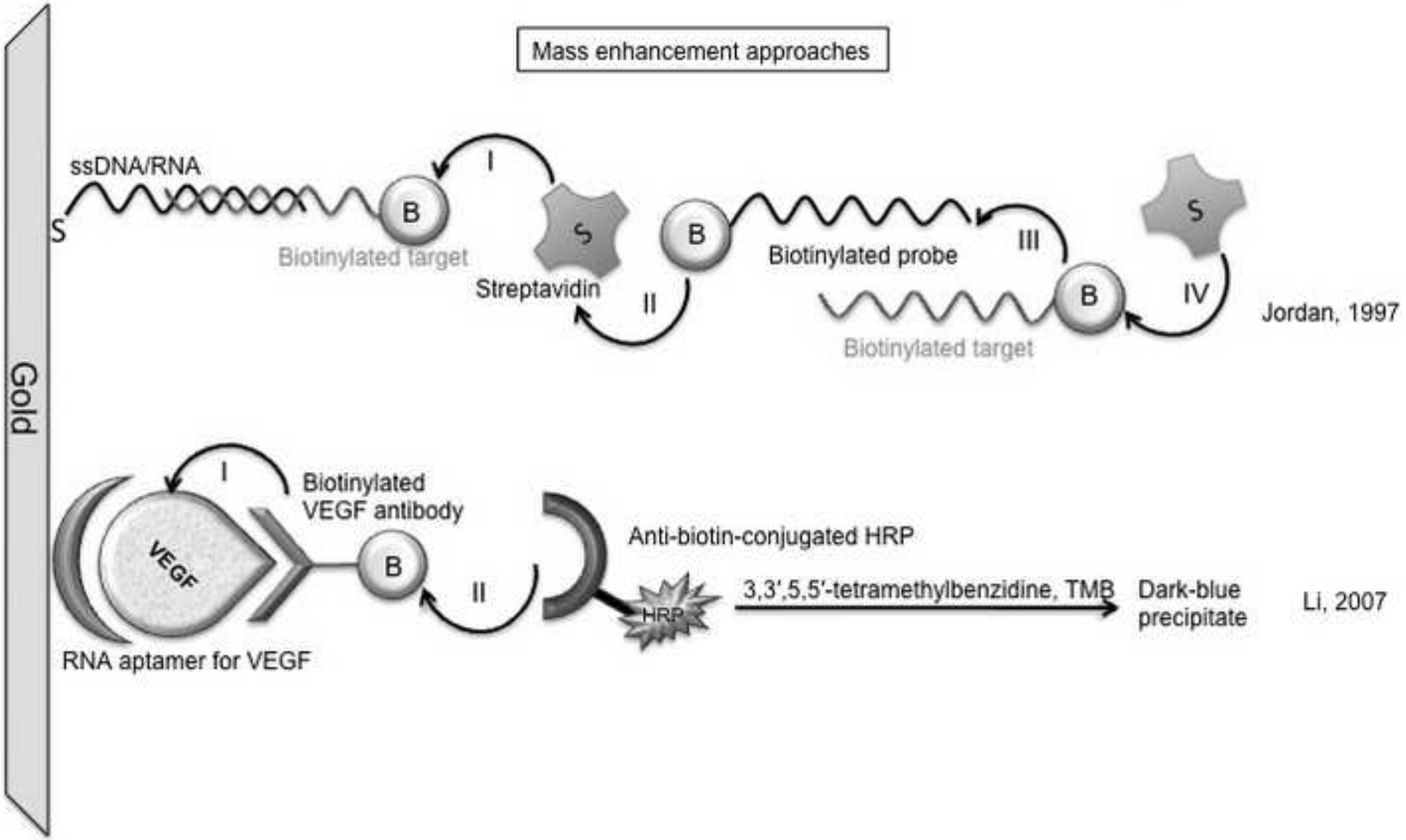


Fig. 3
Cysteine-tagged Protein G
S(Cys)
S(Cys)
G Protein
+
Human IgG
Lee, 2007
ssDNA
S
ssDNA
(Cys)
G Protein
+
Human IgG
Jung, 2007
Gold
Polypyrrole electrodeposition
Probe
✓ Nucleic acid sequences
✓ proteins/antibodies
✓ Oligosaccharides
Bidan, 2000
Grosjean, 2005
Mercey, 2008
CH_2 $(CH_2)_8$ OH
+
T
T
A
G
C
Base-by-base *in situ* synthesis using photolithographic chemical methods
Lockett, 2008
Carbon-on-gold substrate

Figure(s)

Fig. 4



Figure(s)

Fig. 5

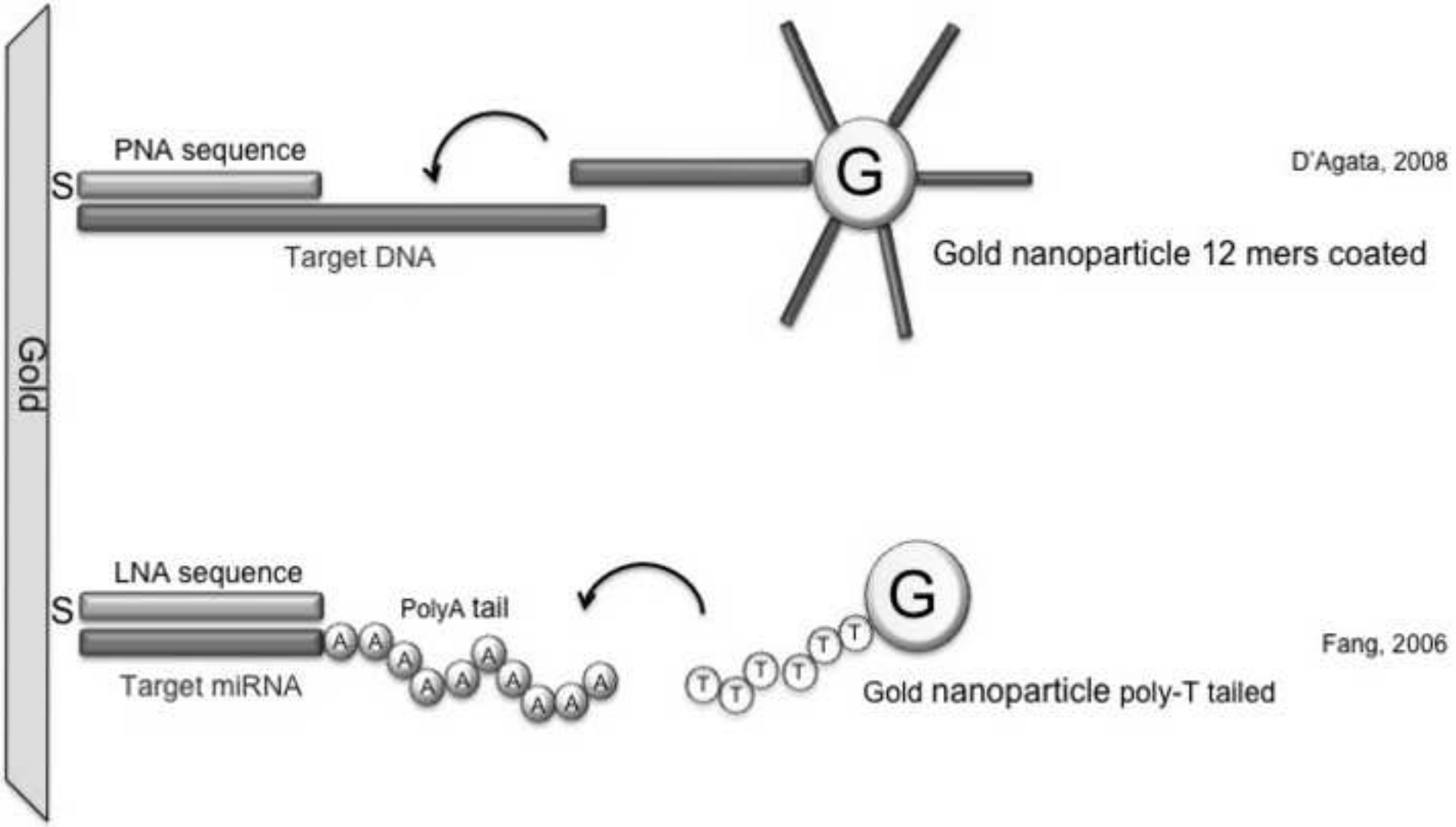
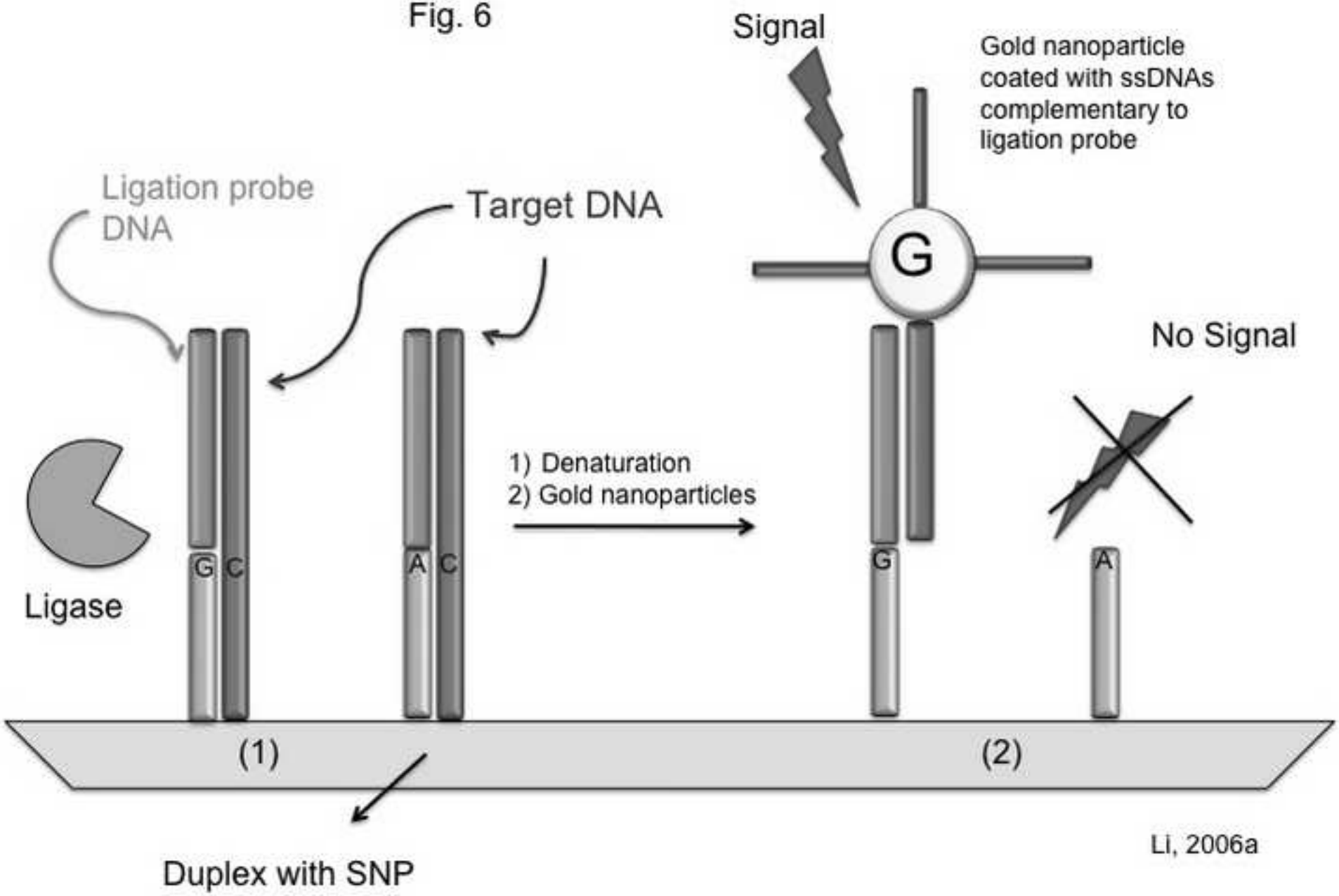


Fig. 6


Signal
Gold nanoparticle coated with ssDNAs complementary to ligation probe
Ligation probe DNA
Target DNA
G
No Signal
1) Denaturation
2) Gold nanoparticles
Ligase
G C
A C
G
A
(1)
(2)
Duplex with SNP
Li, 2006a

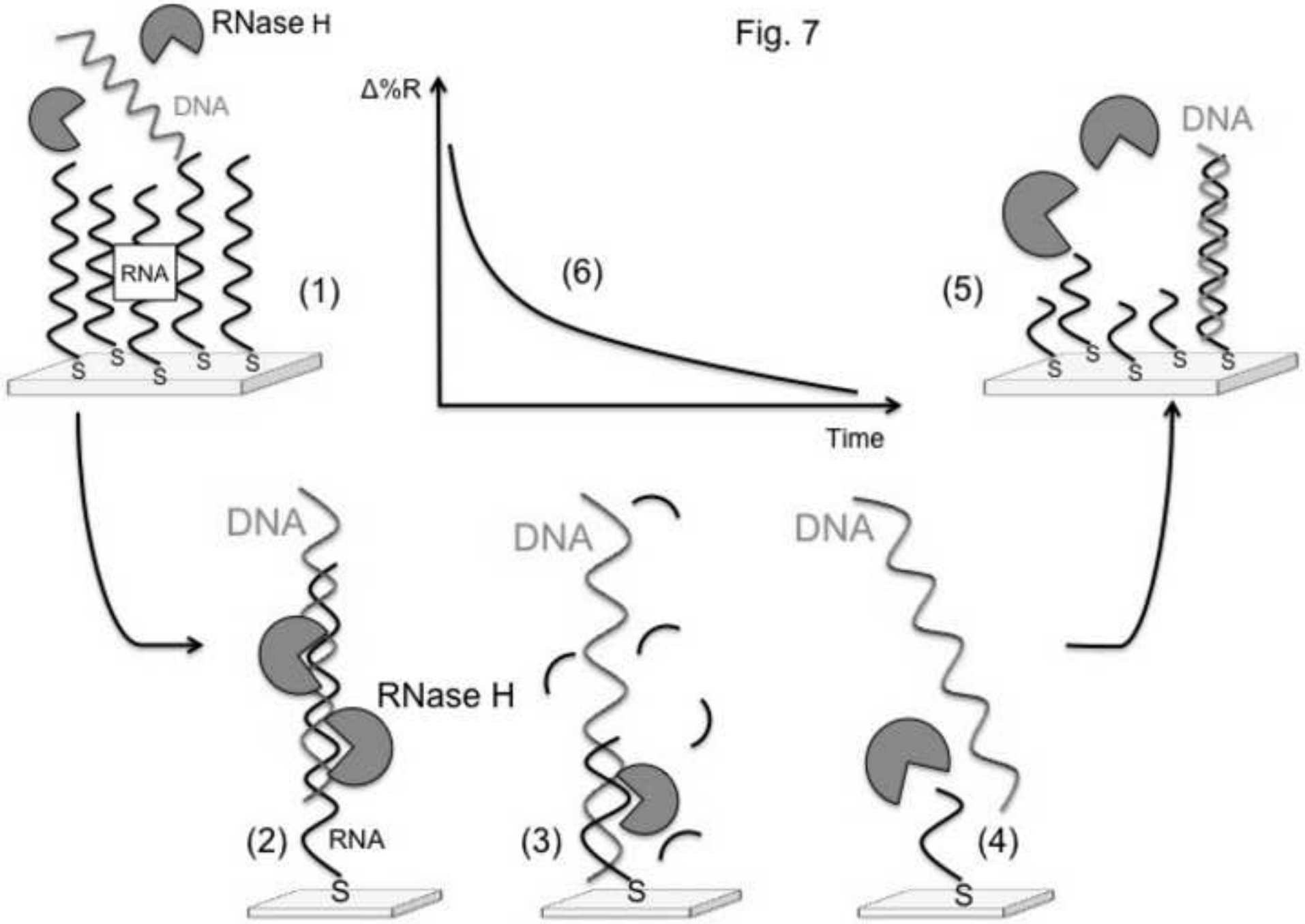


Fig. 7

SURFACE PLASMON RESONANCE IMAGING FOR AFFINITY-BASED BIOSENSORS

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SPR imaging (SPRi) is at the forefront of optical label-free and real-time detection. It offers the possibility of monitoring hundreds of biological interactions simultaneously and from the binding profiles, allows the estimation of the kinetic parameters of the interactions between the immobilised probes and the ligands in solution. We review the current state of development of SPRi technology and its application. Commercially available SPRi instruments are covered. Attention is also given to surface chemistries for biochip functionalisation and suitable approaches to improve sensitivity.

Keywords: surface plasmon resonance imaging, SPRi, affinity sensing, immobilisation chemistry, signal amplification immunosensor, aptasensor, optical sensor

Introduction

Following its commercial launch in 1990, Surface Plasmon Resonance (SPR) sensing has emerged as a key research tool for pharmaceutical development, food quality control, environmental monitoring and clinical analyses (Homola, 2008). An increasing number of reviews (Fan, 2008; Homola, 2008; Visser, 2008; Jason-Moller, 2006; Pattnaik, 2005), and books (Schasfoort and Tudos, 2008; Homola, 2006) dealing with the fundamentals and applications of SPR-based sensing have been published over the last decade. One of the most important advances in the field is the SPR imaging (SPRi), also called "SPR microscopy", which couples the sensitivity of scanning angle SPR measurements with the spatial capabilities of imaging. SPRi represents a promising and highly versatile affinity sensing platform suitable for an array format. SPRi has been reported for a variety of affinity systems, including DNA/DNA (Hayashi *et al.,* 2008; Piliarik, 2008; Lecaruyer *et al.,* 2006; Nelson, 2001), RNA/DNA (Li *et al.,* 2006; Nelson, 2002), DNA-binding protein (Jeong *et al.,* 2008; Bouffartigues *et al.,* 2007; Wegner *et al.,* 2003), RNA aptamers/protein (Garcia *et al.,* 2008; Li *et al.,* 2007; Li *et al.* 2006), antibody-antigen (Ladd *et al.,* 2008; Liu *et al.,* 2008; Rebe Raz *et al.,* 2008; Wong *et al,* 2008; Yuk *et al.,* 2006) and carbohydrate/protein (Grant *et al*., 2008; Mercey *et al.*, 2008; Wakao *et al*., 2008; Smith *et al*., 2003). SPRi technology enables affinity interactions to be monitored in real-time and without the use of any label, as in classical SPR sensing, but by using a CCD camera for signal detection both sensorgrams (*i.e.* resonance signal *vs*. time) and images of the chip can be recorded allowing simultaneous analysis of many interactions (up to hundreds).

This review describes SPRi technology and its applications. Commercially available SPRi instrumentation is included with a focus on some key technical features such as the fluidic

system, temperature control, cell and biochip design. Attention is also given to surface chemistry for biochip functionalisation and suitable approaches to improve sensitivity.

Traditional SPR *cf.* imaging SPR: features and breakthroughs

SPR transduction belongs to the class of refractometric sensing devices, which use evanescent waves to investigate surface phenomena. Changes in refractive index at the sensing surfaces due to analyte binding influence the resonant angle and this shift is used to generate a real-time signal. Thus labelling is not required. Commercial SPR instruments are available from numerous suppliers (Homola, 2008), the most frequently reported being the Biacore™ system (GE Healthcare). SPR platforms can be classified according to three modulation approaches: angular, wavelength and intensity (Homola, 2006).

In contrast to scanning angle SPR and scanning wavelength SPR (traditionally termed "SPR spectroscopy"), SPRi systems are generally based on intensity modulation, measuring the reflectivity of monochromatic incident p-polarised light at a fixed angle. The polariser permits measurements both with p-polarised and s-polarised light, but the latter is used only as reference signal to improve the image contrast and to eliminate artefacts. In the majority of cases, SPRi instruments use a high refractive index prism in the Kretschmann configuration (Kretschmann and Reather, 1968). An alternative is the use of grating couplers (Wassaf *et al.*, 2006). The sensing surface of the prism is coated with a thin metal layer (usually gold or silver, approximately 50 nm in thickness), on which surface plasmons (SPs) are excited by matching SPs and evanescent wave (EW) propagation constants. The resonance conditions depend on the characteristics of the prism, metal and dielectric medium. The best resonance conditions are usually achieved by varying the incident angle of the light on prism. A charge coupled device (CCD) camera collects the reflected light, allowing the visualisation of the whole biochip in real

time. If the sensor surface is divided into multiple sensing spots, , *i.e.* microstructured, the device can be used as a multichannel sensor.

SPRi offers two main advances over conventional SPR: the ability to visualise the entire biochip surface in real time and the chance to monitor up to hundreds of molecular interactions continuously and simultaneously in a multi-array format of molecular probes formed as circular or square spots. It is also possible to control the quality of the spotted array by viewing the surface image to accurately select the measurement area. These Regions of Interest (ROIs) are selected, for example, according to shape, size, and quality of the spot to enhance experimental results. Direct image control of the surface also helps identify and reduce the ubiquitous problem of non-specific binding by defining spots without receptors and spots on gold, to be used as negative control surfaces (negative control ROIs). The ability to immobilise many receptors (up to hundreds) on the surface and to monitor the kinetic parameters of biospecific interactions simultaneously in a real-time and label-free microarray format is a tantalising opportunity. Spots (currently from 50 μm^2 up to 1 cm^2) can be created both manually or by automatic spotters.

Characteristics of SPR imaging instruments on the market

Many in-house SPRi instruments have been described (Ladd *et al*., 2008; Ruemmele *et al*., 2008; Chinowsky *et al.*, 2007; Lecaruyer *et al.*, 2006), but a number of commercial SPRi instruments have recently been launched on market by, for example, GWC Technologies (Madison, USA), IBIS Technologies B.V. (Hengelo, The Netherlands), Genoptics Bio Interactions (Orsay, France) and GE Healthcare (Uppsala, Sweden).

Except for the Biacore™ Flexchip (GE Healthcare), configured as a grating coupler sensor, the Kretschmann configuration is adopted (Fig. 1a). The measurement is carried out by fixing a

single working angle that corresponds to the greatest plasmon curves slope of interest and recording the analytical datum as the intensity variation of the reflected light at that angle for each ROI is detected (Fig. 1b). At the same time interactions are displayed as sensorgrams (Fig. 1c). Only the IBIS iSPR instrument operates in scanning angle mode; it directly records the so-called "SPR dip" angle shifts linked to analyte binding. The main difference between these two optical assets relies on the dynamic range of the recorded variable (angle shift or reflectivity change) *vs.* refractive index change (and, consequently, *vs.* mass density change). In particular, measurements conducted in fixed angle mode limit the linear relationship range between reflectivity and change in mass to the linear portion of the plasmon curve. This range corresponds to approximately 5% variation of reflectivity and about 50 mdeg of the SPR dip (Nelson *et al.,* 2001).

Performance comparisons have been reported (Beusink *et al.,* 2008; Rebe Raz *et al*., 2008; Lokate *et al.,* 2007). Beusink, *et al.* (2008), using the IBIS iSPR, developed a 24-spot biosensor by immobilising both a short peptide (2.4 kDa) and IgG (150 kDa), modified with biotin, to test the dynamic range, limit of detection and standard deviation of the measurement of anti-biotin mouse IgG. The experiment was conducted in parallel with the SPRimager®II system (GWC Technologies) and fluorescence microscopy. The results showed that IBIS iSPR angle scanning mode yielded a 10-fold larger dynamic range compared to the SPRimager®II system, allowing the detection of molecules of very different molecular weight simultaneously immobilised on the same sensor chip.

Liquid handling systems, flow cells and temperature control

The performance of a biosensor is strictly correlated with the control of the hydrodynamic conditions on the sensor surface and, consequently, the liquid handling system plays a crucial role. Flow-through and cuvette systems are available. The first system presents the benefit of well-defined hydrodynamic conditions during measurements without rebinding effects. Microfluidic networks have attracted recent attention and studies to minimise diffusion problems and on analyte mass transport effects have appeared (Kanda *et al*., 2004; D'Agata *et al*., 2008). Cuvette systems, allows multiple sample additions. Moreover the cuvette approach reduces sample volume require thus it is very suitable when low volumes are available and/or in case of analyte with low binding constants. Most of SPRi instruments offer both flow through and cuvette systems.

Another key feature in all SPRi platforms is the flow cell. Early work by Berger *et al.* (1998) reported one- and two-dimensional multichannel immunosensing using a homemade SPRi platform equipped with a four-channel flow cell governed by a four-channel peristaltic pump. Initially, the cell was placed in contact with the biochip and antibody was coated onto its surface by flowing through the channels. The four-channel cell was then turned 90°, thus creating 16 independent sensing areas of ~1 mm^2, and an equal number of antibody/antigen signals on a single biochip surface. This multichannel approach has been recently applied to commercial available instrumentation (ProteOn™ XPR36, Bio-Rad Laboratories, CA, USA) based on traditional SPR transduction. At present, the flow cell is designed to let the same analyte solution interact with an array of different probes, minimising the high variability exhibited by multichannel systems. The internal volumes of most flow cell range from few up to hundreds of nanoliters and only slight differences in shape can be found between the different instruments. The SPRimager®II system (GWC Technologies) can be equipped with flow cells of 24 and 12 µL internal volume and measurements can be achieved both in stop-flow and recirculation mode.

IBIS Technologies supplies the iSPR instrument with standard flow cells (30 µL) and cuvettes (100 µL) and the minimum sample volume is 50 µL. SPRi-Plex and SPRi-Lab^{+} (Genoptics Bio Interactions) not allow sample recirculation and the internal volume of the equipped cells is about 6 µL. The minimum volume/injection ranges from about 20 µL for SPRi-Lab^{+} up to 200 µL for SPRi-Plex model. The Biacore family of instrumentation has also recently launched the Flexchip (GE Healthcare) for up to 400 hundred simultaneous measurements. The minimum required volume is P/ DOWKRXJK WKH FHOO YROXPH LV RI / for application of the system to molecular biology research, but competitive for proteomic studies.

Some commercially available instruments have temperature control. The IBIS-iSPR has high precision temperature control and both the sample deck (with microtitre plate) and sensor can be cooled or heated with 0.01-degree precision (°C) facilitating thermodynamic kinetic analyses. SPRi-Plex, from Genoptics' has a Peltier temperature control system between 15°C and 40°C with a 0.1°C precision and a 0.01°C stability. Thermostatic control can be also obtained with the fully automated Flexchip in the range 25°-37° C.

To address the lack of temperature control some groups have reported in house developed temperature control from 25 to 70°C (Corne *et al.,* 2008) or temperature scanning to perform melting curves on DNA duplexes for point mutation detection (Fiche *et al.*, 2008).

SPRi Biochips

Immobilisation of the molecular probe should be reproducible, retain biological activity and orientate the receptor for optimal binding. Moreover the number and type of probes can vary, depending of the application under study. It is not easy to fulfil all these requirements thus ready to use chips are available from different companies commercialising SPRi instrumentation for

not experienced users. Alternatively many groups familiar with biosensors, report about interesting chemical approaches for the SPRi biochips development.

The common strategy for realising spots is to isolate small areas of the surface and to surround them by an antifouling environment, thus reducing non-specific SPRi signals from background. An example is the commercial SpotReady™ gold chip (GWC Technologies).

Pre-coated chips are also available with different types of chemicals (e.g. Z-terminated amine carboxylic functionalities from IBIS Technologies) suitable for further chemical modifications. Electropolymerisation of pyrrolated-conjugates prepared using pyrrole-NHS, pyrrole-phosphoramidite and pyrrole-hydrazide is also applied for biochip development by Genoptics

Behind ready to use biochips, one can develop suitable chemistry for bioreceptor immobilisation.

Immobilisation methods

In SPRi the sensor surface is almost always a thin layer of gold. Most immobilisation techniques involve a first layer of a chemical linker directly bound to the gold, allowing subsequent anchoring of molecules of interest. The main objective is to create a support structure for the receptor that ensures stability under working conditions and access to the analyte, while minimising receptor detachment. The use of a blocking agent prevents non-specific adsorption. A variety of chemical strategies have been reported for SPRi-based sensing.

A favoured immobilisation method is the use of a self-assembled monolayer (SAM) on the gold surface, which increases the degrees of freedom of the probe and, consequently, those of binding target molecules. SAMs can be formed by thiol-modified biomolecules, *e.g.* DNA (Manera *et al.*, 2008) and RNA (Piliarik *et al.,* 2008) sequences, directly attached to the gold surface by exploiting its high affinity with thiol groups (Fig. 2). Alternatively, the receptor can be covalently attached via alkanethiols and alkoxylanes containing Z-terminated amine (Lee *et al*., 2005; Smith *et al.*, 2003), hydroxylic (Mannelli *et al*., 2007) or carboxylic (Liu *et al.*, 2008; Park

et al., 2008; Mannelli *et al.*, 2007) functional groups (Fig. 2). Probe immobilisation to polyelectrolyte films via electrostatic interactions has also been reported. In particular, 11-mercaptoundecanoic acid (MUA) modified gold surfaces were exposed to a polyelectrolyte film of branched poly(ethylenimine) (PEI) (Mannelli *et al.* 2007), in which the amino groups bind the carboxylic end of the MUA electrostatically. The high-density extravidin layer then strongly binds the biotinylated probes (Bassil *et al.,* 2003) via the extravidin–biotin complex (Fig. 2).

Alternatively, SAMs were formed by mixtures of 7 parts to 3 1-mercapto-11-undecyltetra(ethylene glycol) (OEG) and carboxylic acid-capped hexa(ethylene glycol)undecanethiol (COOH-OEG). Carboxylic groups of COOH-OEG were then activated with N-hydroxysuccinimide (NHS) and N-(3-dimethylaminopropyl)-N-ethylcarbodiimide hydrochlorid (EDAC) for antibody immobilisation by amino-coupling (Ladd *et al.,* 2008) (Fig. 2). Finally, a SAM layer comprising DNA sequences and OEG was created on a gold surface for the simultaneous detection of DNA and protein analytes (Ladd 2008a). Thiol-modified single-stranded DNA sequences were spotted onto gold-coated glass substrates, which were then immersed in an OEG-terminated thiol solution. This last step created a protein-resistant surface background and improved the orientation of the DNA molecules. Antibodies conjugated to complementary single-stranded DNA sequences were immobilised on the surface through DNA hybridisation. By converting only part of the DNA array into a protein array, both nucleic acid and proteins could be immobilised.

Corn and co-workers have developed immobilisation protocols over the last decade for immobilising nucleic acids (DNA and RNA probes, RNA aptamers), peptides, proteins and carbohydrates. Their fabrication procedures uses a reversible protecting groups to manipulate surface properties during array construction (Wegner *et al.,* 2004; Brockman *et al.,* 2000) to

spatially confine aqueous solutions. A monolayer of 11-mercaptoundecylamine (MUAM) is adsorbed via self-assembly onto an evaporated gold thin film, which can then be reversibly protected using organic protecting molecules. In one of the most used methods, a reversible amine, 9-fluorenylmethoxycarbonyl (Fmoc), acts as protecting group to form a hydrophobic surface. By ultraviolet (UV) photo-patterning squares of bare gold are then obtained and further exposed to MUAM. Thiol-modified DNA is covalently attached to the MUAM squares using a bifunctional linker, *i.e.* the sulfosuccinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (SSMCC). The linker contains a N-hydroxysulfosuccinimide ester (NHSS) functionality (reactive toward amines) and a maleimide functionality (reactive toward thiols) (Smith *et al.,* 2003). Alternatively single-stranded, thiol-modified DNA is immobilised using SATP (*N*-Succinimidyl S-acetyl-thiopropionate). Deprotection of SH group on the surface is achieved using Dithiothreitol (DTT), a small redox molecule, leaving the sulfhydryl to react with dipyridyl disulphide (DPDS) to create a pyridyl disulfide surface. Finally a thiolated molecular probe can be bound to the surface by an exchange reaction between the pyridyl sulphide and the thiol group of the probe (Fig. 2).

The formation of a hydrogel film composed of carboxymethyl dextran covalently linked to the gold surface with the SAM layer is a widely used method to immobilise the probe for SPR and piezoelectric sensing (Tombelli *et al.,* 2005; Löfås *et al.,* 1995; Löfås and Johnsonn, 1990). By activating the caboxymethyl groups, amino-coupling of protein receptors can be achieved. This approach has been adopted in SPRi for peptide and antibody immobilisation (Beusink *et al.,* 2008). Alternatively, biotinylated molecular probes can be attached via avidin/streptavidin binding previously immobilised using the aforementioned dextran chemistry (Mannelli *et al.,* 2007) (Fig. 2).

One limitation of amino-coupling is the lack of control of receptor orientation, since coupling can occur randomly with any aminic group on the side-chains. In the case of antibody arrays this implies a reduction in the biosensor sensitivity and reproducibility. A number of papers deal with new immobilisation procedures to overcome these drawbacks. For antibody arrays, genetically engineered *Streptococcus* bacteria were employed to produce protein G with cysteine residues at the N-terminus allowing orientation (Lee *et al.,* 2007) (Fig. 3). Cysteine residues can be genetically introduced into the specific site of the target protein, and this modified protein forms a properly oriented protein layer through the thiol group adsorption on the gold surface in either thiolate or disulfide form (Kallwass *et al.,* 1993) for biosensor development (Di Felice and Selloni, 2004; Persson *et al.,* 1990). Protein G was used as an affinity receptor for antibody immobilisation affording better orientation of immobilised antibodies as well as a higher SPRi sensitivity against antigen targets. Recently, a modified antibody immobilisation introducing a novel linker, protein G-DNA conjugate, was reported (Jung *et al.,* 2007) that possesses the strengths of both protein G and DNA-directed immobilisation. DNA-directed immobilisation allows spatial assembly of target proteins on DNA-functionalised assay surfaces (Schroeder *et al.,* 2009), resulting in robust and stable chemical linkers (Fig. 3) for SPR transduction and offering possibilities for SPRi.

Another immobilisation method involves polymeric species, mainly based on polypyrrole (Cosnier *et al.,* 2003). Livache and colleagues developed an electropolymerisation technique for SPRi using the electrochemically directed copolymerization of pyrrole and oligonucleotides bearing on their 5' end a pyrrole moiety (Bidan *et al.,* 2000) (Fig. 3). The resulting polymer film on the electrode consists of pyrrole chains bearing covalently linked oligonucleotides (a mixed solution of DNA-pyrrole and pyrrole monomers). Initially developed for the construction of DNA chips, the polypyrrole approach has been then extended to other biochemical compounds,

mainly proteins (Grosjean *et al.*, 2005) and oligosaccharides (Mercey *et al.*, 2008). The electropolymerisation reaction is based on an electrochemical process allowing a very fast coupling of the probe directly to a gold working electrode, without the need for further chemical linkers.

Very recently, in order to synthesise oligonucleotide probes *in situ*, a quite different approach has been proposed by Lockett *et al.* (2008), to create a more resistant surface to oxidation and photodegradation. A lamellar structure in which a thin layer of amorphous carbon was deposited onto the gold thin film was developed. Carbon-based surfaces can be readily modified with biomolecules *i.e.* by attachment of alkene-containing molecules by UV light-mediated formation of carbon-carbon bonds. The surface was further functionalised with 9-decene-1-ol leading to hydroxylic terminated surface, which was modified via photolithographic chemical methods, using oligonucleotide bases modified with a photolabile 3'-nitrophenylpropyloxycarbonyl- (NPPOC-) protecting group (Fig. 3).

The best choice of surface attachment chemistry is not yet clearly established mainly because it depends on many factors such as the biomolecule type to be immobilised, the sample medium and the detection method.

4. Strategies for signal amplification

Typical DNA samples contain attomolar or femtomolar concentrations, which are well below the nanomolar detection limit SPRi. Hence, sample enrichment (PCR amplification), has been generally applied (Goodrich *et al.*, 2004a) although in few cases DNA sequences have been detected directly *e.g.* extracted from plant using piezoelectric sensing (Minunni *et al.*, 2005),

from human lymphocytes by traditional SPR (Minunni *et al.*, 2007) and from bacteria by SPRi (Nelson *et al.,* 2002).

In order to increase the sensitivity of SPRi, signal amplification has been developed by: a) increasing the SPR output signal through mass or enzymatic processes at the receptor/analyte adduct; b) increasing of the intensity of the evanescent field by structuring the chip surface. Mass enhancement is achieved by addition of molecules which selectively interact with the receptor-ligand complex formed at the surface. It was first applied to SPRi for DNA detection by Jordan *et al.* (1997), who combined the hybridisation step with subsequent biotin/streptavidin binding (Fig. 4). Both mass and enzyme-based enhancements have been applied to the detection of the signalling protein Vascular Endothelial Growth Factor (VEGF) at 1 pM physiological concentration (Li *et al*. 2007). A VEGF-specific aptamer bound the protein, followed by the addition of biotinylated anti-VEGF antibody (sandwich-type assay). The SPRisignal was further amplified using an anti-biotin conjugated horseradish peroxidase (HRP) which, in presence of an HRP substrate such as 3,3',5,5'-tetramethylbenzidine (TMB), created a localised dark-blue precipitation reaction with SPRi signal enhancement (Fig. 4).

DNA-linked gold nanoparticles have also been used for SPRi signal amplification with immobilised peptide nucleic acid (PNA) sequences to detect point mutations. The target sequences hybridise the PNA probe on the chip surface and amplification is achieved by DNA-modified nanoparticles binding to a different region of the target sequence (Fig. 5). Selective and ultrasensitive (1 fM) mismatch recognition was successfully achieved (D'Agata *et al.,* 2008). Fang *et al*. (2006) reported a similar approach using a polyadenine (polyA) tail, synthesised *in situ*, added to the target sequence to bind its complementary LNA (Locked Nucleic Acid) probe.

PolyT-modified nanoparticles, binding the polyA tails were then added to form a ternary surface complex. The reported detection limit for 19-23 mers miRNA was of 10 fM (Fig. 5).

A combination of surface hybridisation, surface ligation and nanoparticle amplification has been applied to point mutation detection (Li *et al.,* 2006a) by exploiting signal magnification obtained only when DNA sequences were fully matched, *i.e.* not containing Single Nucleotide Polymorphisms (SNPs). Arrays of two sets of probes, differing only in the last nucleotide at their 3' ends specific to the SNP (Fig. 6, grey bars showed in step 1) were exposed to a target solution (blue bars) containing a second DNA sequence ("ligation probe DNA", orange bar) and Taq DNA ligase enzyme. Duplexes were formed (Fig. 6; Step 1) where Taq DNA ligase could only extend the probe sequences with the ligation probe if the receptor did not contain the SNP. After ligation, treatment with 8 M urea selectively denaturated the adducts containing the SNP. Finally, nanoparticles modified with oligonucleotides complementary to the ligation probe DNA were added. The SPRi signal, recorded only where the ligation occurred, revealed if the target DNA contained the SNP (Fig. 6, step 2). The detection limit was 1 pM using 36 mer oligonucleotides and the system was applied to point mutation detection in the BRCA1 gene, which is a breast cancer diagnostic.

A novel approach for DNA detection used RNase H in conjunction with RNA microarrays (Goodrich *et al.,* 2004 & 2004a) (Fig. 7). The exonuclease selectively destroys RNA sequences only in RNA-DNA heteroduplexes, releasing the DNA back to into solution. A single-stranded RNA (ssRNA) microarray was exposed to a solution containing both complementary DNA and RNase H (Fig. 7; Step 1). The DNA binds its RNA complement on the surface (Fig. 7, step 2). RNase H then binds to this heteroduplex, selectively hydrolysing the RNA probe (Fig. 7, step 3), and then releasing the DNA complement back into solution (Fig. 7, step 4). The released DNA

molecule is then free to bind to another RNA probe on the surface, so that a single DNA molecule can initiate the destruction of many surface-bound RNA probes (Fig. 7, step 5). With sufficient time, all of the RNA probe molecules are removed from the surface resulting in a decrease of the SPRi signal (Fig. 7, step 6). Hence, one sequence could in theory prime the enzymatic catalysis and lead to the destruction of the heteroduplexes on the surface to generate an observable SPRi signal. In practice, the detection limit was 1000 times improved allowing DNA detection down to 1fM.

Other forms of SPRi amplification focus on surface engineering. Long-Range Surface Plasmons (LRSPs) are surface electromagnetic waves that can be created on thin metallic films imbedded between two identical dielectric media. LRSPs have been known for almost thirty years (Quail *et al.,* 1983), but have only recently been used for the characterisation of thin-films or for bioaffinity measurements. Wark *et al.* (2005) used SPRi to demonstrate the suitability of LRSPs for molecular detection and for thin-film sensing in general. A micrometric layer of a commercially available inert, optically transparent material (Cytop, CTL-809M.) with a refractive index very close to water was deposited on a prism coupler surface. A gold layer was then vapour-deposited onto the prism surface and chemically modified for probe immobilisation. Results obtained showed that it is possible to enhance the SPRi output signal by about 20% due to the narrow LRSP resonance curve.

Improvement in sensitivity can also be obtained by ‘‘nanostructuring’’ the SPR interface. Lisboa *et al.* (2008) patterned the gold surface of SPRi chips using two organothiols, *i.e.* thiolated polyethylene oxide (PEO), which is unsusceptible to protein and cell adhesion, and mercaptohexadecanoic acid (MHD) with a carboxylic group suitable for coupling to the amino group of a bioreceptor. The surface was modified with active ‘‘nanospots’’ for subsequent probe

immobilisation, surrounded by an inert PEO surface. The nanoarrayed surface produced five times the sensitivity for the Human IgG/anti Human IgG immunoreaction compared to the standard uniform immobilisation.

5. SPR imaging transduction in affinity sensing

Shortly after its introduction by Yeatman and Ash (1987) SPRi was used in a biomolecular application for the imaging of phospholipid monolayer films (Hickel *et al.,* 1989). Since then, SPRi has also been used for surface morphological investigations of many systems, including self-assembled monolayer films (Evans and Flynn, 1995), mono- and multilayer films prepared by Langmuir-Blodgett techniques (Duschl *et al.,* 1996) and multilayer films by alternating polyelectrolyte deposition (Nelson *et al.,* 1999). More recently, SPRi was applied in array-type configurations for studying molecular recognitions between nucleic acids, nucleic acids and protein, immunoreactions and interaction with carbohydrates.

5.1 Nucleic acid arrays for SPRi

Over the past two decades, Corn's research group has optimised immobilisation chemistries for microarray development for SPRi, mainly focusing on DNA and RNA sequences. SPRi has been applied to hybridisation-based sensing for many applications, including bacterial genotyping, SNP identification and studing DNA repair. Nucleic acid arrays are also providing innovative tools for the survey of biomolecular interactions between DNA and RNA sequences and binding proteins (Jeong *et al.,* 2008; Bouffartigues *et al*., 2007; Wegner *et al*., 2003).

Opening up the field of genotyping for environmental and clinical applications, detection of 16S ribosomal RNA from *E. coli* with short, unlabelled DNA and RNA sequences by SPRi was successfully demonstrated (Nelson *et al.,* 2002; Nelson *et al.,* 2001). Other research groups

(Manera *et al.,* 2008a; Piliarik *et al.,* 2008, Hottin *et al.,* 2007) have obtained promising results for bacterial pathogens, but data are still scarce for real matrices.

Specific RNA arrays for SPRi have been designed to demonstrate the specificity and affinity between synthetic aminoglycoside antibiotics (kanamycins) and RNA sequences (Nishimura *et al*., 2005) to elucidate aminoglycoside-RNA recognition in order to overcome resistance and side effects of antibiotics. It was shown that kanamycins have non-specific, multiple interactions with RNA hairpins and that the binding strenght is not always proportional to the antimicrobial activity.

Due to its capability to discriminate between highly similar nucleotide sequences, SPRi has recently gained the attention of researchers investigating SNPs (Fiche *et al.,* 2008; Hayashi *et al.*, 2008) and gene mutation (Lecaruyer *et al.,* 2006). SPRi applicability for point mutations rapid screening, was demonstrated by Livache's, using in-house apparatus, performing temperature scans. DNA probe sequences carrying different point mutations were immobilised and distinguished perfectly matched duplexes from mismatched ones by virtue of their different melting temperature over ten temperature scans (Fiche *et al.,* 2008). The results showed that imaging coupled with temperature scans can be an efficient and low-cost tool for point mutation detection on DNA chips.

Some crucial enzyme activities have also been investigated using immobilised DNA sequences such as the binding and catalytic properties of DNA N-glycosylase towards damaged DNA sites (Corne *et al.,* 2008; 2008a). DNA N-glycosylases are enzymes involved in Base Excision Repair (BER), which is the major mechanism for correction of damaged nucleobases. A decrease of BER activity is correlated to carcinogenesis and aging and the study of biological interactions

between damaged DNA and repair enzymes have a crucial role in the search for new DNA repair inhibitors and the understanding of DNA repair mechanisms (Baute *et al.,* 2008).

The importance of microRNAs (miRNAs) in gene expression and regulation, as well as in cell function is well established (Williams *et al.,* 2008) and the synthesis of miRNA has been investigated by SPRi (Wark *et al.,* 2008; Fang *et al*., 2006) for possible clinical diagnostic applications. Promising high-quality miRNA profiling for simple, rapid and multiplexed analyses is emerging. Another class of nucleic acid sequences, aptamers, represents a competitive tool for proteomic research such as protein biomarker discovery (Tombelli *et al.,* 2007). Aptamers are a promising alternative to antibody microarrays for clinical analysis and SPRi aptasensing has already been reported for the detection of human Immunoglobulin E (Wang *et al.,* 2007), human factor IXa (Li *et al.,* 2006) and vascular endothelial growth factor (Li *et al.,* 2007).

5.2 Protein and short peptide arrays for SPRi

Increasing interest in the analysis of peptides and proteins using array formats with surface-sensitive optical techniques like SPRi is prompted by the chance to detect the biomolecular interactions without labelling, which may adversely affect molecular structures. Jung *et al.* (2005) recently developed protein microarrays for high-throughput SPRi measurements and tested a SPRi platform for protein–protein interactions (Ro *et al.,* 2005; 2006). The approach facilitated the study of protein-protein interactions where multiple proteins are involved and demonstrated high-throughput monitoring of affinity-tagged proteins in expression and purification processes (Ro *et al.,* 2005). Moreover, they chose the Human Papilloma Virus E6 protein with its binding proteins, the ligase E6AP and the tumor suppressor p53, as a model study to investigate the triple protein interaction (E6/E6AP/p53) that takes place during the viral

infection (Ro *et al.,* 2006). The complex formation induces a degradation of p53 and, consequently causes the transformation of normal cells into malignant cells and work in this area supports the development of new anticancer drugs against HPV-related cervical cancer. More recently, they also reported the study of caspase-3 activation by SPRi (Park *et al.,* 2008). Caspase-3 is an intracellular cysteine protease and plays a crucial role in the apoptotic cell process. Its activation is commonly used as a biomarker for assessment and understanding of apoptosis, and is conventionally detected using an arti¿FLDO ÀXRUHVFHQW VXEVWU blotting. The new method measured a fluorescence signal adapted to SPRi. A chimeric caspase-3 substrate (GST:DEVD:EGFP) composed of glutathione S transferase (GST) and enhanced green ÀXRUHVFHQW SURWHLQ (*)3 ZLWK D VSHFLDO3 cleavage OLQN sequence (DEVD) was developed. Utilizing this caspase-3-dependent proteolytic reporter, they successfully monitored the proteolytic activity of caspase-3. These results confirm that SPRi offers new ways to study protein mechanisms without labelling.

Short peptides play an important role in identifying important residues in protein-protein recognition processes and in the understanding of peptide-DNA interactions and enzymatic modification of peptides. Katayama's group has recently developed SPRi to explore peptide probes as kinase substrates (Mori *et al*., 2008; Inamori *et al.,* 2005). The synthesis of drugs modulating the activities of particular protein kinases has become a prime focus of the pharmaceutical and biotechnology industry, thus development of high-throughput screening formats for these enzymes is of crucial interest. Mori and colleagues proposed a novel detection system for on-chip phosphorylation of peptide probes and a zinc(II) chelate compound, working independently of the amino acid residues on the array, using a single-probe complex, differing from in solution conventional methods (Sola-Penna *et al.,* 2002; Ross *et al.,* 2002).

SPRi immunosensor based on antibodies arrays, have been also reported. Work dealt with antibodies orientation for enhancing sensor performance (Jung *et al.*, 2007; 2008; Lee et al., 2007) and assays for candidate cancer biomarkers. Anti-activated cell adhesion molecule/CD 166 (anti ALCAM) and anti transgelin-2 (anti TAGLN2) antibodies were employed for the identification and quantitation of the relative ALCAM and TAGLN2 antigens (Ladd *et al.,* 2008) immobilised in array format. The detection limits in buffer were in the ppb range for both targets, which is compatible with physiological levels in human serum (typically 10 to 100 ppb), although high non-specific signals were found in spiked 10-fold diluted commercial serum samples. Better results can be expected by improving the surface chemistry to reduce matrix effects.

5.3 Carbohydrate microarrays for SPRi

As mentioned above, carbohydrates are interesting candidates for array format analysis, but publications are sparse due to the lack of reliable methods of fabrication. Smith *et al*. (2003) reported the fabrication of mannose and galactose carbohydrate arrays on gold films using poly-(dimethylsiloxane) (PDMS) microchannels and their use in SPRi to monitor the binding of the lectins jacalin and concanavalin A (ConA). They quantified the strength of lectin-carbohydrate interactions by determining the adsorption coefficients and the solution dissociation constants for the lectins ConA and jacalin, highlighting the potential of SPRi for weak protein-carbohydrate interaction studies. Karamanska *et al.* (2008) recently adopted a similar approach and their data confirms the feasibility of a label-free and selective lectin-glycan recognition using SPRi.

An interesting biotechnological application for screening phage display has been described by Weiss' and Corn's groups (Lamboy *et al.,* 2008) using a polyl-lysine-modified surface created by the photopatterning of adsorbed 11-mercaptoundecanoic acid. The peptides Lys_n (n=16 to 24)

emerged as optimal for wrapping the phage. The patterned surface was exposed to a solution of phage in water that was allowed to adsorb electrostatically onto the surfaces. Lys_n also provided effective wrappers for RNA binding in assays against the RNA-binding protein HIV-1 viral infectivity factor Vif. The oligolysine peptides blocked non-specific binding to allow successful selection and screening of the targets.

6. Concluding remarks

SPRi represents the forefront of label-free and real-time optical detection of many (up to hundreds) biological interactions simultaneously, furnishing binding profiles for the estimation of the kinetic parameters of the different interactions between immobilised probes and ligands in solution. Most of the immobilisation protocols originate from research on traditional SPR-based biosensing. Applications addressed by SPRi vary from protein-protein interactions, hybridisation reactions with DNA, RNA or *in vitro* selected nucleic acids such aptamers with the specific ligands or carbohydrate–lectin interactions. In order to improve sensitivity, signal amplification can be achieved by nanopatterning the sensing surface or by addition of enzyme or nanoparticle-labelled reagents, facilitating detection limits down to 1 fM of DNA for point mutation analysis. Given the wide applicability of this emerging technology, some commercial instrumentation has appeared on the market with dedicated ready-to-use chips. Interest in SPRi technology is rising quickly and we can expect that it will become important in areas ranging from environmental and food analysis to clinical diagnostics.

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Legend to Figures

Fig. 1:
a) Kretschmann configuration for SPRi; a high refractive index prism is in contact with the detection cell and couples the incident light to the surface plasmons by evanescent waves. p-polarised light is directed to the prism, on which the biomolecular probe is tethered, and a CCD camera collects the output signal as variations in reflectivity.

b) Data are recorded as intensity variation of the reflected light at a fixed angle for each ROI selected. A differential image (left) is produced in real time together with the relative sensorgrams. As example here are reported two sets of signals corresponding to the interaction with different chemically modified areas. During the specific interaction with the target analyte, only the relative specific probe will react, leading to a local change in intensity of reflected light. This translates into black/white contrast for the image. The unspecific receptor series (used as negative control) will give negligible or none signal.

c) Sensorgrams corresponding to the interactions of the analyte with the spots on the surface.

Fig. 2

Immobilisation methods using chemical linkers. A self-assembled monolayer is created as the foundation of the array comprising alkaneth L R O V F R Q W D L Q L Q J 8 terminated amine, hydroxylic or carboxylic functional groups. After the formation of the thiolated layer many immobilisation chemistries can be performed.

Fig. 3

Immobilisation methods exploiting direct attachment of the probe to the gold surface of the chip using thiol- and pyrrole-functionalised biomolecules.

Fig. 4

Signal improvement in SPRi based on mass/colorimetric approaches. The upper diagram shows the DNA hybridisation step followed by addition of streptavidin and biotinylated DNA for signal amplification. In the lower drawing, an aptamer array is exposed to the ligand protein (VEGF)

and then biotinylated antibody forms a sandwich-type assay. The SPRi signal was amplified using an anti-biotin conjugated horseradish peroxidase (HRP) that in presence of a suitable substrate creates a localised dark-blue precipitation reaction.

Fig. 5

Gold nanoparticles for SPRi signal enhancement. The upper drawing shows gold nanoparticles coated with oligonucleotide sequences able to bind the free moiety of the target DNA after the probe/target duplex formation. A similar approach is shown in bottom drawing, in which gold nanoparticles are coated with a poly-T tail able to bind the target sequence on a suitable poly-A end.

Fig. 6

Signal amplification by a "switch on/off" detection strategy: the SPRi signal is produced only if the target sequence fully matches to the specific probe sequence. The array consists of two probe types, differing in the presence or absence of a sequence specific to the SNP. The array is exposed to target solution (blue bars) that contains the ligation probe DNA (orange bar) and Taq DNA ligase (grey round shape). Duplexes that are formed comprise the combination of probe (grey bars), target sequence (blue bar) and ligation probe DNA (orange bar) regardless of the presence of the mutation (Step 1). Since ligation occurs only on fully matched duplexes (no SNP), the addition of nanoparticles carrying nucleotide sequence complementary to the ligation probe (Step 2) produces a SPRi signal only if target DNA does not contain the SNP.

Fig. 7

Diagram of the approach proposed by Goodrich and colleagues for the enhancement of SPRi sensitivity. Step 1: a RNA microarray is exposed to target solution containing the enzyme RNase H and the target DNA sequence; Steps 2 and 3: once the RNA/DNA heteroduplexes are formed, RNase H starts to selectively destroy RNA strands in heteroduplexes; Step 4: target DNA strands are released back to into solution and hybridise other free RNA probes on the surface, promoting the their subsequent destruction. The decrease in percent reflectivity becomes larger with time until all of the available RNA probes on the surface are destroyed (Step 6).