Chapter 4

Biosensors Based on Cantilevers

Mar Álvarez, Laura G. Carrascosa, Kiril Zinoviev, Jose A. Plaza, and Laura M. Lechuga

Summary

Microcantilevers based-biosensors are a new label-free technique that allows the direct detection of biomolecular interactions in a label-less way and with great accuracy by translating the biointeraction into a nanomechanical motion. Low cost and reliable standard silicon technologies are widely used for the fabrication of cantilevers with well-controlled mechanical properties. Over the last years, the number of applications of these sensors has shown a fast growth in diverse fields, such as genomic or proteomic, because of the biosensor flexibility, the low sample consumption, and the non-pretreated samples required. In this chapter, we report a dedicated design and a fabrication process of highly sensitive microcantilever silicon sensors. We will describe as well an application of the device in the environmental field showing the immunodetection of an organic toxic pesticide as an example. The cantilever biofunctionalization process and the subsequent pesticide determination are detected in real time by monitoring the nanometer-scale bending of the microcantilever due to a differential surface stress generated between both surfaces of the device.

Key words: Microcantilever, Nanomechanical biosensors, MEMs, Immunoassay, Pesticide detection, Surface stress, Biofunctionalization, Biorecognition.

1. Introduction

The working principle of the family of biosensors based on nanomechanical transducers, and specifically on microcantilevers, involves the translation of biochemical reactions into a mechanical movement in the nanometer range. In microcantilever sensors, the biochemical receptor layer is directly in contact with one of the cantilever surface. The biomolecular recognition process between the receptor layer and its corresponding analyte induce

Avraham Rasooly and Keith E. Herold (eds.), *Methods in Molecular Biology: Biosensors and Biodetection, Vol. 504*

DOI:10.1007/978-1-60327-569-9_4

quantitative changes in the mechanical properties of the cantilever beam, allowing the detection of such interactions by measuring the cantilever bending or the change in its resonance frequency *(1)*. The potential of this new kind of sensor relies on their tiny area and their ability to detect molecular interactions without labels (reducing the time and cost of sample preparation), and its low-cost fabrication and mass production by using standard semiconductor technology. In addition, their production can be easily scaled up (*see* **Note 1**).

The cantilevers can be fabricated of any shape and from substantially any material utilized in microelectronics industry, i.e. crystalline or poly-silicon, silicon nitride, silicon oxide, polymer materials (*see* **Note 2**). The rectangular shape beams are the most frequently used in biological research. In biological sensors based on the bending method, it is important to have the cantilevers flat and in plane with the base surface. Initial offset or curvature of the beams complicates adjustment of the experimental setup, especially, if working with arrays of cantilever. For this reason, the most common material for cantilevers fabrication nowadays is single crystalline silicon. A large variety of biomolecular interactions have been detected with silicon microcantilevers.

Our work is focused on nanomechanical biosensors based on the cantilever bending method (*see* **Note 3**), which measures the microcantilever deflection due to differences in the surface stress between the opposite sides of the microcantilever. For that method, just one side of the cantilever is sensitized with a bioreceptor layer. This specific functionalization is carried out by covering one side of the cantilever with a thin layer of gold and then using the well-known self-assembled monolayers (SAMS) chemistry for the covalent immobilization of the bioreceptor over the gold surface. The formation of consecutive biolayers over the cantilever surface can be sensed in real time due to the cantilever deflection shift produced by each layer. The cantilever motion is detected using the laser beam deflection readout technique (commonly used in atomic force microscopy), where the displacement of a laser beam reflected from the cantilever free end is registered in a position sensitive photodetector (PSD), *see* **Note 4**. In **Fig. 1**, a scheme of the cantilever bending method and the laser beam deflection readout is showed. The gold layer initially deposited over the cantilever surface helps to enhance the reflected beam intensity, increasing the signal to noise ratio. The sensitivity of the method depends both on the resolution of the readout electronics and on the cantilever mechanical properties, which are carefully optimized (by simulation) before fabrication to obtain maximum cantilever deflection in response to the expected bioreaction. The cantilevers are fabricated using standard silicon technology in single crystalline silicon as its

[Au1]

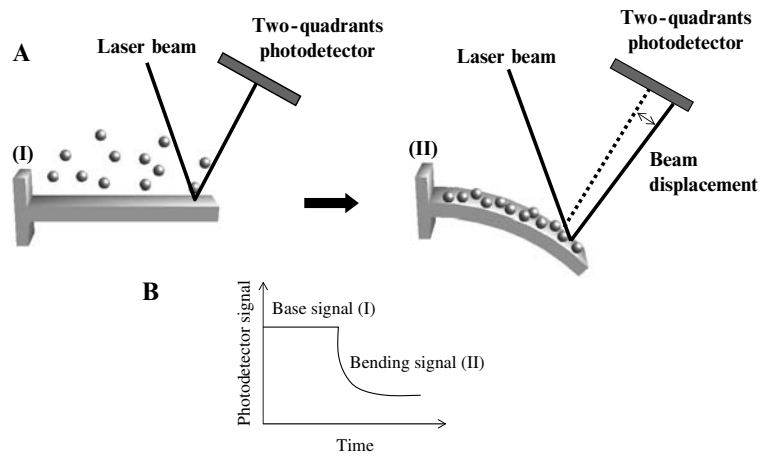

Fig. 1. (**A**) Simple scheme of the cantilever bending method and the laser beam displacement readout. The reflected laser beam suffer a displacement respect its original position (reflection over the flat cantilever), due to the adsorption of molecules over only one cantilever side. (**B**) Photodectector signal read in real time before and after the adsorption of molecules over one cantilever surface.

characteristics match the parameters of the simulated devices with the real ones.

During the last years, the application of nanomechanical biosensors has been expanded to many different fields, such as genomic, proteomic, environmental control, food quality control, clinical analysis, etc. Specifically, in the proteomic field, the immunological detection is one of the most employed, for example, for detecting pathogens, discovering protein expression patterns, or isolating proteins from cellular extracts. Using an immunological detection scheme, we present the detection of a toxic chemical compound (a pesticide) as an example of the application of cantilevers biosensors in the environmental control field.

There are several techniques suitable for the cantilever response read-out as the optical beam deflection, piezoresistivity, piezoelectricity, interferometry and capacitance, among the most important. However, the majority of the biochemical applications carried out with the cantilever-based sensor are based in the *optical beam deflection method*, because of its high sensitivity and its simplicity.

But for making practical devices, this readout implementation in array platforms is technologically challenging, as it requires an array of laser sources with the same number of elements as the cantilever array. The lasers displacement could be measured by using an array of photodetectors, adding alignment complications, or using just one photodetector and sequentially switching on and off each laser source to avoid the overlapping of the reflected beams. Additional engineering work is still needed in this direction before to deliver devices for real applications.

2. Materials

2.1. Set-Up Components

1. The cantilevers are fabricated from Silicon-On-Insulator (SOI) 100 mm standard UNIBOND™ wafers purchased from Soitec Company. They bear layers of single crystalline silicon, silicon dioxide (also called buried oxide layer), and a silicon substrate. The back side of the wafer may contain silicon dioxide. The fabrication of the cantilevers is done at Clean Room (class 100) facilities using standard microelectronics optical photolithography, reactive ion etching, wet etching, and various deposition methods. The procedure and materials used for each process are specific for different clean rooms. The dimensions of the fabricated cantilevers are: 200 μm long, 40 μm width, and 0.334 μm thick. Alternatively, the cantilevers could be commercially purchased. In this case, the ones chosen had dimensions of 200 μm length, 40 μm width, and 0.8 μm thick (ORC8-PS-W, from Olympus).
2. Fluid cell is made of glass with a cell volume of 50–100 μL (Digital Instruments, Veeco), where the cantilever is fixed.
3. Peristaltic pump (Gilson) for maintaining a constant flow during all the biochemical detection process.
4. An injection valve (model 5020, Sigma-Aldrich) allows the injection of different solutions without changing the flow speed.
5. Optical detection system composed of a diode laser of 3 mW (λ = 630–670 nm), for focusing in the free-end of the cantilever and a four quadrants photodetector (model S4349, Hamamatsu), for collecting the reflected light.
6. Two x–z positioning stages, and a rotatory one, for simplifying the laser-cantilever-photodetector alignment.
7. A digital acquisition card (DAC) connected to a computer (National instruments) for reading the photodetector signal in real time. The signal acquisition is controlled by a Labview-based application.

2.2. Surface Functionalization Based on Sams and Pesticide Detection

1. Trichloroethylene and ethanol absolute can be purchased from Panreac (no 161749); acetone from Sigma-Aldrich (179124-2L).
2. Piranha Solution contains a mix 70:30 of sulphuric acid 96% (Merk KGaA no 100732) and hydrogen peroxide 30% (Panreac, no 121076). The solution should be prepared 5–10 min before used, adding the sulfuric acid first, and followed by the peroxide. Never store piranha solutions. Piranha stored in a closed container will likely explode. *Caution: Piranha solution is very dangerous. It is extremely energetic and may result in explosion or skin burns if not handled with extreme caution.*

3. Mercaptoundecanoic acid (450561-5G), *N*-hydroxysuccinimide (NHS) (130672-25G), *N*-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide hydrochloride (EDC) (E1769-1G), and ethanolamine (236381-50G) can be purchased from Sigma-Aldrich. EDC quickly oxidizes under air atmosphere. A good way of handling EDC is to separate it into aliquots immediately after opening the container for the first time, then store aliquots at –20°C.
4. Phosphate buffer (PBS) can be purchased from Sigma-Aldrich (P7059-1L) or home-made using monosodic and disodic phosphated salts. We recommend buying or preparing a PBS 10× (pH 7–7.5) as stock solution. Then, prepare a working solution of PBS 1× by dilution from the stock solution. When diluted to 1×, working concentration contains 137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, and 2 mM KH_2PO_4, respectively.
5. Hydrochloric acid 0.1 M can be performed by dilution of hydrochloric acid fumant 37% (Merck, no. 1.00317.1000).

3. Methods

One of the first steps to develop cantilever-based biosensor of high sensitivity toward biomolecular interactions is to simulate the cantilever response depending on its dimensions. In general, making thinner and longer cantilevers increases the flexibility, but it also amplifies the thermomechanical noise. For that reason, a compromise between the bending amplitude and the resolution needs to be reached. Once the optimum dimensions have been defined, the cantilevers can be fabricated and characterized *(2)*. The fabrication process must produce cantilevers with well-controlled and reproducible mechanical properties, such as the spring constant, as well as initially stress-free. The spring constant can be characterized by exerting a controlled force on the cantilever with an atomic force microscopy cantilever, with a calibrated spring constant. For detecting the cantilever displacement, a home-made set-up, on the basis of the laser deflection technique, is used, which is able to detect the cantilever movement due to Brownian excitation (i.e., the motion induced in the cantilever by the random hits of thermally excited particles of the surrounding medium). The read-out configuration (e.g., laser spot size, photodetector resolution, cantilever-photodetector distance, etc.) affects the final sensitivity of the system.

The next step is the application of the microcantilever as biosensor in the environmental control field, by following an immunological detection scheme. For this purpose, we present the inhibition immunoassay performed to detect the highly toxic organochlorine insecticide compound dichlorodiphenyltrichloroethane (DDT).

Main applications of biosensors are based on antigen–antibody specific interactions. The detection of this specific interaction can be achieved (a) directly, by means of the specific immobilization of the antibody onto the transducer surface and monitoring the interaction with the antigen, or (b) indirectly, by means of an inhibition assay, where the immobilized molecule is the antigen and monitoring the inhibition of the antibody signal when mixtures of a fixed amount of antibody and variable amounts of antigen are introduced on the biosensor. For the DDT detection, an inhibition format was preferred instead of the direct detection due to the small molecular weight of the pesticide, which made the direct detection more difficult. For that reason, a hapten (DDT5) molecule, which consists of a molecule chemically equivalent to the DDT, is synthesized and coupled to a carrier protein (e.g., BSA) to be immobilized over the surface. The cantilever surface functionalization process that will be explained describes the immobilization of a hapten (or an antigen) that displays amine groups all over its surface. The receptor should be a protein, peptidic molecule, or any other molecule displaying free amine groups, or in the case of organic compounds as pesticides, if they are previously coupled to a protein carrier that supports the amine groups for the linkage. Inhibition format gives the advantage that a more stable monolayer is obtained, as proteins display several recognition sites for the antibody and an oriented immobilization is not required. In addition, they are more resistant to denaturalization than antibodies and typically, monolayers tolerate several regeneration cycles. But the main advantage is that inhibition formats have demonstrated better detection levels than direct detection formats for small analytes. Surface functionalization of cantilevers is one of the trickiest issues for the correct performance of the biosensor to reach high detection levels. The bio-receptor (BSA-DDT5) immobilization requires a selective and specific immobilization of molecules rending a densely and ordered monolayer, which must be stable enough under several regeneration cycles and free of nonspecific adsorption. This type of monolayers can be achieved using the well-known method of self assembled monolayers (SAMs). Self assembly takes place by the direct link of a thiol functional group of an alkanethiol molecule with the Au atoms of the sensor surface, rendering in a densely, ordered and very stable monolayer. Proteins are afterwards covalently attached to the surface previously functionalized with this alkanethiol monolayer. One of the main advantages of SAMs is the possibility of varying the alkyl chain length and ending group, generating different interface layers between the gold metal surface and the immobilized element. To perform the inhibition assay, samples containing a fixed concentration of monoclonal antibody (named LIB-DDT) are mixed with DDT known solutions at different concentrations. After a short incubation step, only the antibody that remained free in the mixture may couple to the bioreceptor. The signal observed in the sensor is an inversely measurement of the pesticide concentration in the sample.

3.1. Cantilever Simulation and Fabrication

3.1.1. Cantilever Simulation

1. The cantilever deflection, Δz, generated by a surface stress change, $\Delta\sigma$, (due to a biorecognition process, for example) depends on the microcantilevers' dimensions (length L and thickness h) and its material's properties (Young's modulus E and Poisson coefficient ν) following the Stoney's equation:

$$\Delta z = \frac{3L^2(1 \square \nu)}{Eh^2}\Delta\sigma.$$

That means that for a produced $\Delta\sigma$, the cantilever bending will be higher for longer and thinner cantilevers, i.e., with low stiffness. The parameter that characterizes the cantilever stiffness is the spring constant, which depends on the cantilever dimensions and Young's modulus (*see* **Subheading 3.2**). The biomolecular interactions could be difficult to detect with commercial microcantilevers due to the low surface stress induced on such microcantilevers. To improve the cantilever deflection, this parameter, and its dimensions dependency, needs to be simulated before fabrication. The simulation was performed using a finite element analysis (FEA) technique (with ANSYS software).

2. One of the most important issues for simulation of microsystems is to know the properties of the materials. In the case of microcantilevers, young modulus, Poisson's ratio, and density are the main parameters to be considered. If monocrystalline silicon is used, as reported in this work, the mechanical properties are well-known (*see* **Table 1**). For an anisotropic material such as silicon, the Young's modulus, Poisson's ratio, and shear modulus depend on which crystal direction the material is being stretched. The appropriate values for each direction have to be introduced in the model.

3. Once the material is defined, the generated cantilever deflection will be larger when L/h increases (*see* Stoney's equation). For that reason, a thickness of only 0.334 μm was selected. As the limit of sensitivity is determined by the signal to noise

Table 1
Silicon mechanical properties

Density	ρ	2.33 × 10^3	kg/m^3
Young modulus (110)	Y_{110}	1.6895 × 10^{11}	Pa
Young modulus (100)	Y_{100}	1.3002 × 10^{11}	Pa
Poisson's ratio (110) (100)	$\nu_{110\,100}$	0.2785	–
Poisson's ratio (110) (110)	$\nu_{110\,110}$	0.0625	–
Shear modulus (110) (100)	$G_{110\,100}$	0.7951 × 10^{11}	Pa
Shear modulus (110) (110)	$G_{110\,110}$	0.5085 × 10^{11}	Pa

ratio, the length and width proposed was 200 µm and 40 µm, respectively, to avoid increasing the thermomechanical noise. For these chosen dimensions, the calculated deflection produced by a surface stress change of 1 mN/m (with $E = 169$ GPa and $\nu = 0.2$) would be about 5 nm. This value is five times larger than the one obtained for the alternatives commercial cantilevers proposed (ORC8-PS-W Olympus: 200 µ m length, 40 µm with and 0.8 µm thick, with $E = 175$ GPa and $\nu = 0.25$).

4. In spite of this modeling could be more time consuming, and a 3D geometrical model was chosen for the simulation of the microcantilevers, because of its more precise results.
5. The structural materials that conform the cantilevers can have intrinsic stresses and stress gradients because of the fabrication processes. These can change the mechanical performance of the microcantilevers. If stresses and stress gradients are high, they must be previously measured by using specific test structures and their values should be included in the simulations. As monocrystalline silicon will be used in the fabrication of our devices, these stresses can be neglected.
6. The spring constant of the cantilever could be simulated by applying a known force, F, at the free end of the cantilever. The force should be centered at the end of the cantilever to avoid any tilt. The spring constant, $k = 0.0077$ N/m, is determined by the applied force, F, and the simulated maximum displacement, $z = 0.13$ µm, by using the formula $k = F/z$.

3.1.2. Cantilever Fabrication

1. The processing starts with a SOI 4 in. wafer, which is bearing a 334 nm of single crystalline silicon on 1 µm thick silicon dioxide buried layer over the silicon substrate (*see* **Fig. 2A**).

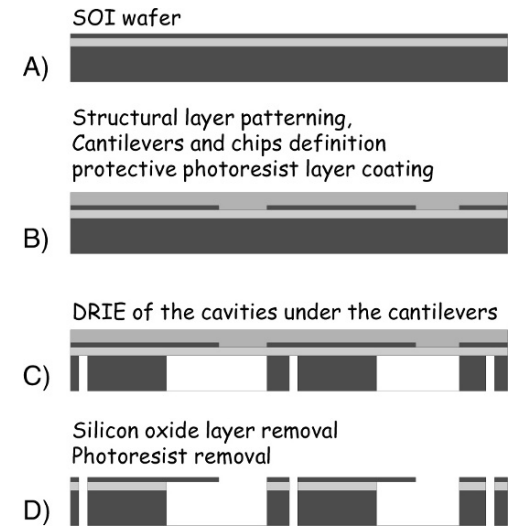

Fig. 2. Scheme of the microcantilever fabrication process.

The substrate is 425 µm thick (*see* **Note 5**). The cantilevers were fabricated in separate cavities with a pitch of 250 µm between them. The cavities are the through-wafer holes drilled using a deep reactive ion etching (DRIE) technique. Each cantilever was 40 µm wide and 200 µm long, the thickness of each cantilever was 334 nm (which was fixed by the thickness of the structural layer) (*see* **Note 6**).

2. The back side of the wafer is metallized with 1 µm of aluminum deposited by RF sputtering. This layer will be used as a mask material for the DRIE definition of the cavities.
3. The pattern for the cavities is defined on the wafer back side at the aluminum layer by using standard photolithography, dry and wet etching processes. Photoresist was cured for 30 min at 120°C to stand the steps of dry reactive ion etching and wet etching (**Fig. 2B**). A wet etching in SiOetch solution during 30 s was applied to remove the silicon dioxide from the windows.
4. The cantilevers were defined by photolithography and dry etching. The silicon dioxide buffer layer was kept to be employed as an etch stop layer for DRIE.
5. A protection photoresist layer is spun on the components side. The wafer is ready for DRIE (*see* **Note 7**).
6. The etching of the cavities (**Fig. 2C**) takes about 40 min in an Alcatel 601 DRIE machine (*see* **Note 8**).
7. After etching the cavities, clear silicon dioxide membranes are obtained, and a short, about 20 s, overetching without passivation is applied in an Alcatel 601 DRIE machine to remove the polymer formed in the cavities during the DRIE process.
8. The membranes of silicon dioxide are etched in vapors of Hydrofluoric (HF 49%) acid. It is a type of dry etching, because contact of the wafer with liquid HF must be avoided. The wafer is placed with the back side down on a pot with the acid. The pot has a diameter smaller than the diameter of the wafer, i.e., if the wafer has a diameter of 4 in., the pot should have a diameter of about 3.8 in. (*see* **Note 9**)
9. The wafer is rinsed with deionised water in a special bath with gentle filling and draining (*see* **Note 10**).
10. The wafer is dried at room temperature.
11. The photoresist is removed by oxygen plasma dry etching, *see* **Fig. 2D**.
12. The metallization of cantilevers is done in two steps: first, it is coated with an adhesion layer of Cr or Ti and after a gold layer is deposited by *e*-beam evaporation. E-beam allows a precise control of the coating thickness and the low temperature variation during evaporation makes the cantilevers less bended.

3.2. Cantilever Mechanical Properties Characterization

1. The cantilever spring constant, k, could be calculated from the expression $k = Ebh^3 / 4L^3$, where E is the Young's modulus, h the thickness, b the width, and L the length, respectively. Experimentally, the spring constant was determined by exerting a controlled force on the fabricated cantilever free end by using an atomic force microscope (AFM) with a calibrated cantilever (**Fig. 3A**). To measure the force that the AFM-tip applies over a surface, we used a force–distance curve, which is a plot of the AFM-cantilever deflection as a function of sample position, or piezoelectric displacement, along the z-axis. The fabricated cantilever spring constant is obtained from the force–distance curves slopes ($\Delta z / \Delta V$) measured when exerting force over the fabricated cantilever and over a hard surface, being ΔV the photodetector's voltage signal and Δz the piezoelectric's displacement, in the AFM. **Figure 3A** shows an example of the force–distance curves measured; the AFM-cantilever deflection is higher when pressing against the hard surface (high slope) than over the fabricated cantilever, because of both cantilevers coupled-deflection. The relation obtained between the cantilevers spring constants and the force–distance curves slopes is given by:

$$\frac{k_{\mathrm{AFM}}}{k} = \left[\frac{\Delta V_{\mathrm{hard}} / \Delta z_{\mathrm{hard}}}{\Delta V_{\mathrm{fab\_cant}} / \Delta z_{\mathrm{fab\text{-}cant}}} - 1 \right].$$

2. Because of the distance, d, from the cantilever free end to the point where the force is exerted, a correction must be included in the value of the spring constant previously obtained.

$$k^{\mathrm{final}} = k \left[\frac{L - d}{L} \right]^3$$

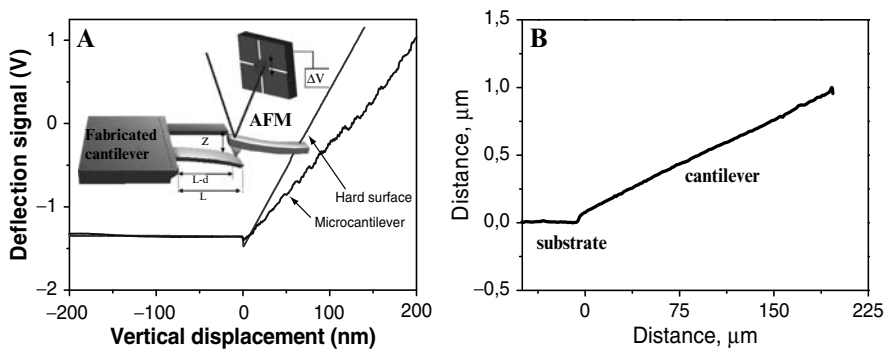

Fig. 3. (**A**) Static method for determining the cantilever spring constant. Force–distance curves obtained over a hard surface and over a fabricated microcantilever. The inset shows the method configuration scheme. (**B**) A profile of silicon cantilevers fabricated by the technology described above. The cantilever is flat, but the stresses at the anchoring area produce initial cantilever displacement. The profile has been measured by an optical confocal profilometer.

With this method, a spring constant value of 0.0067 N/m was obtained for microcantilevers of 200-μm length, 40-μm width, and 0.334-μm thick. The difference respect to the expected value (0.0079 N/m) is attributed to the compliance of the chip region where the cantilever is clamped (due to a thinning of this region, produced during the fabrication process) *(2)*.

3. The profile of the fabricated cantilevers was measured with a confocal microscope, to check the initial curvature generated during the fabrication process. A measured profile is shown in **Fig. 3C**. This profile shows that the cantilever is flat; however, a biasing due to stresses at the anchoring area can be observed (*see* **Note 11**).

3.3. Set-Up Configuration

1. All the biological interactions must be performed in a liquid medium. For that reason, the cantilever chip must be fixed into a fluid cell, by using a spring system. The fluid cell has an inlet and an outlet for liquid flow. The top part of the fluid cell is made out of glass, and the bottom is closed with a coverslip using a rubber "O"-ring.
2. The flow system is mounted by connecting the peristaltic pump to the injection valve and this one to the cell flow inlet by using Teflon tubes. The length of the tubes should be reduced as much as possible to avoid losses associated with the adsorption of protein on the tube walls and the dispersion effects of two different fluids inside the tube. The loop of the injection valve has the suitable length for injecting a solution volume of 200 μL. An extra tube is used from the cell outlet to the waste glassware (*see* **Fig. 4A**, **B**).
3. The flow cell is mounted over a rotatory stage that allows changing the reflection angle. This rotatory movement can compensate the reflected angle changes due to different mediums (air or liquid).
4. The diode laser and the photodetector are mounted over *x*–*z* positioning stages. In this way, it is possible to focus on a specific cantilever into an array and to adjust the position along the cantilever length where the laser will be reflected, increasing the bending sensitivity. At the same time, the photodetector position could be modified for centering the reflected laser beam. **Figure 4A** shows a scheme of the set-up configuration. A diagram showing the optical detection system is illustrated in **Fig. 4C**.
5. The distance between the diode laser, the cantilever, and the photodetector was chosen by weighing up the reflected beam spot size on the photodetector, because of the beam dispersion and the spot displacement magnification that could be obtained by increasing the cantilever–photodetector distance.
6. The photodetector signal is readout and processed with a digital acquisition card connected to a computer. A home-made

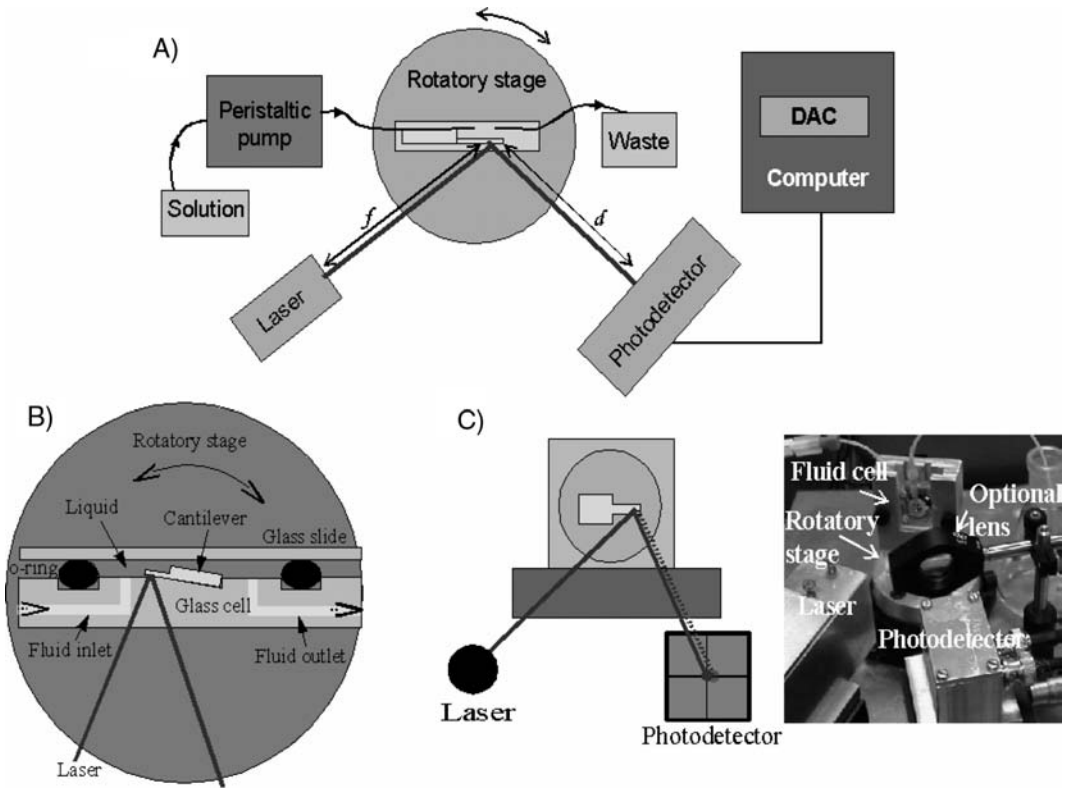

Fig. 4. (A) A schematic diagram of the experimental set-up (top view). The distance between the laser and the cantilever was fixed to the laser focal length, f, while the distance between the cantilever and the photodetector, d (d = 12 cm), was adjusted to fit the maximum spot displacement to the photodetector size (a lens can also be used for that purpose). (B) Schematic diagram (top view) of the cantilever-fluid cell, mounted over the rotatory stage. (C) Diagram and picture (front view) of the optical detection system (laser, photodetector, rotatory stage, and beam path).

software allows the continuous photodetector signal acquisition in real time.

3.4. Real-Time Detection: Surface Functionalization and Pesticide Detection

3.4.1. Gold Cantilever Cleaning Procedure

1. Gold surfaces must be thoroughly cleaned to avoid contamination of the surface and to enhance reproducibility from one measurement to another. This procedure must be performed immediately prior to performing the measurement. First, submerge consecutively for 2 min the cantilever on 2 mL of tricloroethylene, acetone, ethanol, and deionized water, respectively.
2. Dry carefully the cantilever under nitrogen flow and submerge it for 1 min on fresh piranha solution (70% H_2SO_4–30% H_2O_2).
3. Quickly rinse generously with water and again dry carefully the cantilever under nitrogen flow.
4. Cantilevers are ready to be positioned inside the flow cell to start the measurement. Once cleaned, cantilevers cannot be stored as they are susceptible to suffer contamination. New cleaning

steps cannot be performed as successive rinses with piranha dramatically reduce the thickness of the gold layer.

3.4.2. Immobilization of the Receptor

We have demonstrated good results of DTT detection on cantilevers, using a chemistry based on glutaraldehyde *(3)*. This molecule displays an aldehyde group in both the extremes of the molecule, and it is possible to couple the free amine groups of the bioreceptor to those from a previously immobilized SAM with amine groups (such as cystamine) by using this molecule. This renders an easy immobilization method, showed in **Fig. 5A**, where the first signal displacement corresponds to the formation of the SAM, the second to the glutaraldehyde bonding, and the third to the bio-receptor immobilization (the last deflection is due to the blocking of all the unreacted groups with ethanolamine). However, later experiments performed at our laboratory using SPR biosensor with the same gold-based chemistry demonstrated that this methodology displays an important drawback, as glutaraldehyde tends to form multilayers that can be partially removed during regeneration, giving less reproducibility from measurement cycle to cycle. For that reason, we shifted to a carbodimide (EDC)-mediated coupling chemistry, as enhanced results are achieved using this immobilization method *(4)*. We strongly recommend using the EDC chemistry, and it will be detailed here. Similar cantilever behavior to the one showed in **Fig. 5A** has been observed with this immobilization method.

Self-Assembly of a Carboxylic-Alkanethiol onto the Gold-Coated Cantilever Surface

1. Prepare a 50 µM solution of mercaptoundecanoic acid on ethanol. The final volume might vary from one flow system to another, however, typically ranges from 200 to 400 µL.

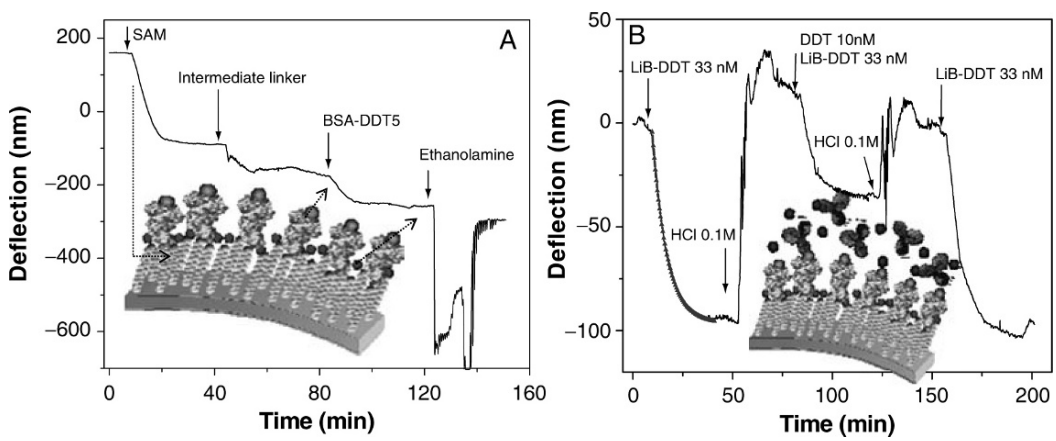

Fig. 5. (**A**) Example of a cantilever surface functionalization in real time. Each one of the layers formed over the surface produce a cantilever bending. (**B**) Real-time monitoring of an antibody direct detection and a competitive immunoassay. The number of antibodies free in solution able to binding the cantilever surface is reduced due to the binding with the DDT free in solution. The cantilever surface was regenerated with 100 mM HCl (100 ml) to break the hapten/antibody complex; (Reprinted from (3). Copyright (2003), with permission from Elsevier).

Hereinafter, this volume will be referenced as "one volume or 1× *V*" and half or double volumes as ½ *V* or 2× *V*, respectively.

2. Pump the mercaptoundecanoic acid solution through the injection loop and inject the sample into the flow cell. Flow speed must be adjusted and calibrated on each system. It is recommended a speed that keeps the sample volume in touch with the gold surface during at least 20–30 min; flow speeds in the range of 0.2–0.3 µL/s are strongly recommended. The SAM formation produce an initial deflection, mainly due to the covalent bond between the thiol group (–SH) and the gold atoms, and the interchains forces, which produce a compressive surface stress. The detected signal is equivalent to the one showed in **Fig. 5A**.
3. Pump 1× *V* of ethanol at 0.15 µL/s and proceed identically with a new injection of 1× *V* of a mixture ethanol/water 1:1 and finally 1× *V* of water.

EDC–NHS Mediated Antigen Coupling (*See* **Note 12**)

1. Stabilize the system with a continuous flow of water and wait until thermal drift is null. This water flow will be continuously pumped and only interrupted with samples injections in between by turning the injection valve from the "load position," to charge the samples into the injection loop, to the "injection position" to introduce the samples into the flow cell.
2. Prepare separately and then mix ½ V of an aqueous solution of EDC 0.4 M and ½ V of a solution of NHS 0.1 M. These solutions must be prepared immediately prior to their use. They are unstable and present short-lived in aqueous solution.
3. Pump the EDC–NHS solution into the flow cell at 0.3 µL/s. This step produce a second cantilever deflection, equivalent to the second one observed in **Fig. 5A**.
4. Pump immediately after the EDC–NHS solution, a previously prepared 1× *V* solution of the bioreceptor in the immobilization buffer (*see* **Note 13**) at 0.15 µL/s. Concentration must be optimized for each application; however, it typically ranges from 10 to 50 µg/mL. For DDT detection, 1× *V* of 6 µg/mL in PBS pH 7 should be used. The bioreceptor immobilization produce a new deflection, very similar to the one showed in **Fig. 5A**.
5. Pump 1× *V* of water or diluted acid (for example HCl 0.1 M) to remove the excess of the reagent and crosslinking products.
6. A second injection of the bioreceptor is recommended to ensure a maximum yield of immobilization.
7. Pump a 1× *V* of an aqueous solution of ethanolamine 1 M in such a way that all remaining unreacted NHS-esters could be deactivated (see last signal deflection in **Fig. 5A**).

3.4.3. Immunodetection by an Inhibition Format Assay

Calibration of the Antibody Signal

1. Restabilize the system in a continuous flow in the same buffer to the one that will be used to perform the immunodetection assay. For DDT inhibition assay, detection is performed under phosphate buffer saline solution (PBS) pH 7.
2. Prepare series of solutions (minimum 4–5) of different concentrations of the antibody in triplicate. For DDT detection, concentrations ranging from 0.5 to 10 µg/mL were used.
3. Depict the surface stress values to a calibration curve. Choose a value as high as possible, but below the saturation limit, able to give a high surface stress signal, so that at inhibition, signals would be still clear. This will be the antibody concentration that will be fixed to perform the inhibition assay.

Inhibition Assay: Detection of the Pesticide DDT

1. Ensure yourself that the system is equilibrated in the immunodetection assay buffer (*see* **Note 14**) and that the thermal drift is null.
2. Prepare and incubate for 10–15 min a series of mixture with the chosen concentration of the antibody, in our case, 5 µg/mL and different amounts of the antigen. Concentrations of the antigen to be measured depend on the limit of detection for each specific application and antigen type. Thus, for pesticides, they should be in the range of ppb or ppt and for other molecules in the nM–pM or ng/mL–pg/mL range.
3. Pump each of the series with a regeneration step in between. Monitoring of antigen–antibody interaction can be performed at a flow speed of 0.15 L/s, while regeneration can be performed faster, at 0.3 µL/s. Regeneration solution must be optimized for each particular application (*see* **Note 14**); however, excellent results have been achieved in our lab using diluted acid such as HCl 0.1 M or 1–5 mM HCl. In **Fig. 5B**, three sequential injections of antibody (5 µg/mL of antibody, 5 µg/mL of antibody with 3.5 ng/mL of antigen, and 5 µg/mL of antibody), with the corresponding surface regenerations, are represented. The antibody/receptor recognition over the cantilever is again translated into a compressive bending, while the regeneration step produces the opposite effect, recovering the initial level before the antibody bonding. A reduction in the deflection signal is observed when a combination of 5 µg/mL of antibody with 3.5 ng/mL of antigen is injected. The second injection of pure antibody produces the same change in the deflection than the first one, meaning that the reduction in the signal is effectively produced by competitive assay and not by degradation on the bioreceptor layer.
4. Depict the surface stress values for antibody inhibited signal for each of the series with different antigen amounts and the fixed antibody concentration to a calibration curve. Lower limit of detection should be in the desired range for the

application. If not, try to improve and optimize immobilization and immunoreaction conditions to reach higher signals.

4. Notes

1. This system can be easily scaled up allowing the detection of different concentrations and/or different analytes simultaneously. For biological experiments, arrays of cantilevers are normally employed *(5)* as one cantilever can be used as reference. Then the expected biointeraction can be diferenciated from any change of the experimental conditions. Actually, there are several commercial platforms available, based on cantilever-array sensors.
2. Other materials, such as polymers, are a cheaper alternative to increase the cantilever deflection, as the Young's module of polymers are lower than that of silicon *(6)*.
3. The bending detection method has a complex relation between the measured signal and the factors producing it, because of the different forces acting in the biorecognition processes. However, it reports a new type of information and represents a new alternative for biological discrimination, such as DNA single-base mismatch polymorphisms or single-molecule detection, otherwise not possible with other well-established biosensing techniques.
4. There are several techniques used for the cantilever bending detection, such as capacitive, piezoelectric, and piezorresistive; however, the laser beam deflection technique is maybe the most extended one, because of its simplicity and higher sensitivity. When working with arrays of microcantilevers, the detection of the bending by using the laser beam deflection read-out configuration could be a great challenge, because of the number of lasers and/or photodetectors required. Several strategies have been explored for solving these issues, and there are currently several systems commercially available on the basis of this read-out method. An alternative configuration has been proposed by using arrays of optical waveguide cantilevers *(7)*. The cantilevers act as a waveguide for conducting light. The exit light is collected by a second waveguide and finally with a photodetector. In this case, the cantilever bending is related to the reduction in intensity of the collected light with respect to the input light.
5. The technology for the fabrication of single cantilevers is basically the same as the technology for fabricating arrays *(8)*. Only some processes should be appropriately chosen to satisfy the requirements for the array geometry. In our example, the cavities under the cantilevers were formed using aniso-

tropic reactive ion etching instead of wet etching. This is due to the required distance of 250 µm between the cavities on 450 µm thick wafer, which cannot be maintained if anisotropic wet etching with bases were applied. In **Fig. 6A**, cantilevers extended from a border of a chip are shown. The fabrication procedure is similar to the technology for the fabrication of array of 20 cantilevers located in separate cavities and showed in **Fig. 6B**. The technology of array fabrication was developed to reach a 100% yield. Two thousand five hundred cantilevers per wafer were manufactured.

6. The silicon dioxide layer on the back side of the wafer may be not specified by the manufacturer. It is always recommended to measure the thickness of this layer before starting the process.
7. Protection layer of the photoresist is essential in this technological chain. If this layer is not used, the membranes of silicon dioxide would crack due to internal stress after being released from the substrate. Cracking the membranes results in a breaking on the cantilevers and brings technological yield close to zero.
8. The BOSCH deep reactive ion etching process is based on alternating etching and passivation cycles. (SF6 is used for etch cycle and C4F8 is used for the passivation cycle). With the appropriate recipe, it is possible to obtain structures with high aspect ratio and to form vertical walls in through-wafer etching. The imperfections of the technology as small lateral etching rate and notching *(9)* result in a small undercut from the back side and slight walls inclination by about 5°. The windows size in the photolithographic mask used for DRIE was corrected to obtain the desired cavity size at the components side. After the photolithography step and before the deep etching, it is also important to make sure that there are no impurities and rests of oxides in the openings on the back side. Otherwise, long spikes hanging from the silicon dioxide membranes, which are difficult to remove, can be formed by DRIE.

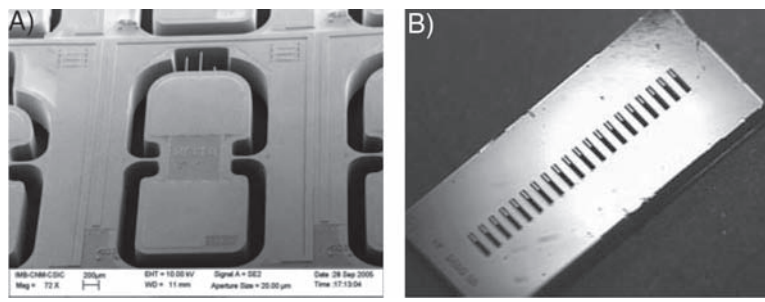

Fig. 6. (**A**) Photo of a chip with three cantilevers extended from the chip border. The photo has been taken before the wafer dicing. (**B**) An array of 20 cantilevers located in separate cavities. The cantilevers on both photographs were fabricated at the clean room facilities of CNM-IMB, Spain, from SOI wafers using the same technology.

9. The conventional wet processing does not work well here. If the wafer was immerged into the acid directly, then HF could penetrate under the photoresist protection layer and peel it off, which would lead to a cracking of the membranes. *Caution: HF should be carefully handled and could be necessary a previous training, because fluoride ion readily penetrates the skin, causing destruction of deep tissue layers, including bone.*
10. Generally speaking, starting from this step, where the cantilevers are released after etching of the membranes, wet processing of the cantilevers should be avoided. Manipulating the cantilevers in liquids may result in fracture of many of them. The wafer is placed into an empty bath, which is slowly filled with deionized water. Rinsing the cantilevers with water at this stage should be done carefully; the wafer should not be moved while it is inside water. After 30 min, the bath is drained slowly and the wafer is dried at room temperature in a clean room environment.
11. Initial bending/curvatures of the cantilevers must be low to avoid high divergence of the reflected beam when the optical beam deflection method is used. This requirement is stronger in arrays of cantilevers. Two phenomena that can induce initial deflection/curvatures of a microcantilever can be identified: (a) The most common one is the gradient stress within the structure. This phenomenon induces a cantilever deflection with a constant radius of curvature that can be predicted by Stoney's formula. This can be solved by using materials with low residual stress gradient or by compensation of the stresses with different layer materials. However, the best solution is to use single crystalline materials as monocrystalline silicon. (b) The second phenomenon is the stresses induced by the anchoring, which can induce an initial offset to the cantilever deflection. Several mechanisms cause stresses at the anchoring area that can induce the cantilever to rotate. The origin of this effect is difficult to determine and avoid. Several solutions have been presented: change the mechanical stresses at the anchoring area by the laser bending *(10)*, or the design of a special T-shape geometry for the cantilever *(11)*.
12. *EDC-mediated coupling:* This step is crucial for the success of the immobilization procedure. EDC reacts with the carboxyl groups to form an amine-reactive *O*-acylisourea intermediate. If this intermediate does not encounter an amine, it will hydrolyze and regenerate the carboxyl group. In the presence of *N*-hydroxysulfosuccinimide (Sulfo-NHS), efficiency of EDC coupling is increased by the formation of an amine-reactive Sulfo-NHS ester intermediate, which has

sufficient stability to permit two-step cross-linking procedures, as it shows in **Fig. 7**.

13. *Immobilization buffer:* Immobilization buffer conditions can dramatically vary from one antigen type to another depending mainly on their intrinsic isoelectric point. Good immobilization signals are typically achieved on immobilization buffers displaying an equal pH to that of the isoelectric point of the protein; however, there is not a universal rule and immobilization should be tested also for an upper and lower pH buffer.
14. *Immuno-detection assay buffer and regeneration conditions* **(12):** Procedures used for regeneration are mostly empirical. The most frequent procedure, i.e., the use of low pH with low ionic strength (e.g., 10 mM glycine, pH 1.5–2.5), probably works because most proteins become partly unfolded and positively charged at low pH. As a result binding sites no longer match and the molecules repel each other. Other procedures include the use of pH 10.5, high salt concentration, or chemicals such as urea or guanidine hydrochloride. When none of these conditions work properly, a trial-and-error search is conducted to find the suitable regeneration conditions.

The type of regeneration condition that is most effective for a particular antibody–antigen interaction depends on the specific mixture of ionic, hydrophobic, and hydrogen bonds involved. Other factors to be taken into account for regeneration are: the antibody type (polyclonal antibodies respond to different epitopes by slightly different mechanisms; therefore, effective removal of all polyclonal antibody from the immobilized antigen layer may require several different regeneration conditions) and affinity constant of the antibody for the antigen (the higher is the affinity constant, the hardest should be the regeneration condition, and the lower the number of regeneration cycles that can be accomplished). The protocol must be chosen according to its ability to target several binding forces simultaneously by mixing different chemicals into a regeneration cocktail. This is quite likely to increase the chances of reversibly breaking the interaction under

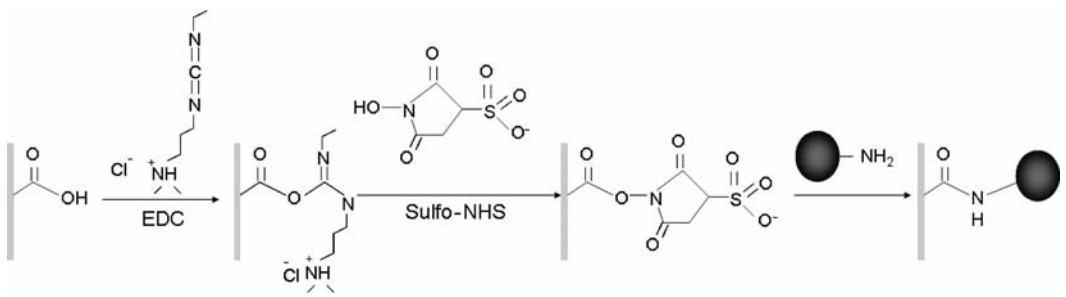

Fig. 7. Scheme of the chemical linkage of the bioreceptor to carboxylic groups through EDC–NHS mediated coupling.

more gentle conditions. The ideal regeneration buffer should effectively release the antibody without irreversibly denaturing or inactivating them. In practice, all regeneration buffers cause some loss of antibody or antigen function, limiting the number of cycles that the same functionalized cantilever can be reused.
pH and ionic strength conditions are gentle on antibody and protein function, while denaturants and organics are harsh. Usually, a low-pH condition is the best option to try first. If a low pH regenerates efficiently but causes irreversible functional damage, then consider testing an ionic strength buffer. If the low-pH buffer does not elute effectively, then consider testing one of the high-pH conditions. To reach a condition that allow repeated regeneration of antigen or antibody (especially when using monoclonal antibody), consider testing a variety of buffer conditions to find the one that is as gentle as possible while still being effective.

Acknowledgments

The authors acknowledge to the European Union (Project Optonanogen, IST-2001-37239). Authors thank Dr. Angel Montoya (Ci2B, University of Valencia, Spain) for the inmunoreagents.

References

1. Ziegler, C. (2004) Cantilever-based biosensors. *Anal. Bioanal. Chem.* 379, 946–959
2. Alvarez, M., Tamayo, J., Plaza, J.A., Zinoviev, K., Dominguez, C. and Lechuga, L.M. (2006) Dimension dependence of the thermomechanical noise of microcantilevers. *J. App. Phys.* 99, 024910
3. Alvarez, M., Calle, A., Tamayo, J., Lechuga, L.M., Abad, A. and Montoya, A. (2003) Development of nanomechanical biosensors for detection of the pesticide ddt. *Biosen. Bioelectron.* 18, 649–653
4. Mauriz, E., Calle, A., Lechuga, L.M., Quintana, J., Montoya, A. and Manclus, J.J. (2006) Real-time detection of chlorpyrifos at part per trillion levels in ground, surface and drinking water samples by a portable surface plasmon resonance immunosensor. *Analytica Chimica Acta* 561, 40–47
5. Zhang, J., Lang, H.P., Huber, F., Bietsch, A., Grange, W., Certa, U., Mckendry, R., Guntherodt, H., Hegner, M. and Gerber, C. (2006) Rapid and label-free nanomechanical detection of biomarker transcripts in human RNA. *Nat. Nanotech.* 1, 214–220
6. Ransley, J.H.T., Watari, M., Sukumaran, D., McKendry, R.A. and Seshia, A.A. (2006) Su8 bio-chemical sensor microarrays. *Microelectron. Eng.* 83, 1621–1625
7. Zinoviev, K., Dominguez, C., Plaza, J.A., Cadarso, V. and Lechuga, L.M. (2006) A novel optical waveguide microcantilever sensor for the detection of nanomechanical forces. *J. Lightwave Technol. V.24(5) (2006)* 24, 2132–2140
8. Lechuga, L.M., Tamayo, J., Álvarez, M., Carrascosa, L.G., Yufera, A., Doldán, R., Peralías, E., Rueda, A., Plaza, J.A., Zinoviev, K. et al. (2006) A highly sensitive microsystem based on nanomechanical biosensors for genomics applications. *Sens. Actuators B* 118, 2–10

9. Kumar, S. and Pike, W.T. (2005) Technique for eliminating notching in through-wafer etching. *16th MME Micromechanics Eur. Workshop* **P**15, 88–91
10. Zhang, X.R. and Xu, X.F. (2005) Laser bending for high-precision curvature adjustment of microcantilevers. *App. Phys. Lett.* **86**,021114
11. Plaza, J.A., Zinoviev, K., Villanueva, G., Álvarez, M., Tamayo, J., Domínguez, C. and Lechuga, L.M. (2006) T-shaped microcantilever sensor with reduced deflection offset. *Appl. Phys. Lett.* 89, 094109
12. Andersson, K., Areskoug, D. and Hardenborg, E. (1999) Exploring buffer space for molecular interactions. *J. Molec. Recogn.* 12, 310–315