# Development of new sensors for detection of organic chemicals

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# Development of new sensors for detection of organic chemicals

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# Preface

This thesis has been written as a part of the requirements of obtaining the Ph.D. degree at the Technical University of Denmark (DTU). The Ph.D. project has been carried out at the DTU Nanotech department from October 2007 to December 2010 as a member of the Surface engineering group and in close collaboration with the Nanoprobes group.

The Ph.D. project is a part of the SENSOWAQ project. This work was financed by the Danish consortium SENSOWAQ in collaboration with GEUS, grant no. 2104-06-0006 from the Danish Council for Strategic Research. The project was realized under supervision of:

**M.Sc. Ph.D. Mogens Havsteen Jakobsen**
Main supervisor
**M.Sc. Ph.D. Rafael Taboryski**
Co-supervisor

I thank my supervisors for the help and guidance during the project work. Former and present members of the Nanoprobes group and Anja Boisen deserve special thanks for their continuous positive spirit and enthusiasm; it has provided an invaluable corner stone support during the challenges of the project. Furthermore I thank Jens Aamand from GEUS and Hans-Jørgen Albrechtsen from DTU Environment for their good collaboration in the SENSOWAQ group.
A special thanks is given to my family, to my girlfriend Jeanette Udesen and my close friends for their love and support during the work on the project.

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## Abstract

The need for direct detection of pesticide in drinking water is in increasing demand, as water sources are getting increasingly polluted with harmful chemicals. Such a system must remain un-attended in a harsh environment for 3 months and be able to detect extremely low concentrations of chemicals.

The use of a cantilever based surface stress read out method was investigated as a possible method for the detection of a 2,6-dichlorobenzamide (BAM) pesticide residue in water. The cantilever surface stress principle was investigated using a piezo induced read out method and an optical laser deflection read-out system. Surface Enhanced Raman Spectroscopy (SERS) was investigated as a secondary alternative for the detection of pesticides in water samples.

Using a BAM antibody assay on a cantilever surface, 10 repeated differential signals of 5 to 20 μV magnitude was obtained from a Canti4 Cantion cantilever system. As a signal was also obtained from unspecific antibodies, the signal could not be regarded as specific for the BAM antibody. BAM ELISA, microarray technique, and a flow cell system were used to chemically optimize the assay conditions on the cantilever BAM assay. Surface quality pictures and resonance frequency and bending values of the cantilever showed that the micro spotting functionalization step did increase the variance on the mechanical properties of the cantilevers.

An alternative optical read out system did reproduce the differential signal values obtained Canti4 Cantion cantilever system, and was furthermore able to measure the surface roughness of the cantilever surface as a useful indicator of the quality of the functionalized surface.

The bulk Raman shift and SERS spectrum of BAM and Dichlobenil pesticide was recorded. A simulated Raman spectrum was calculated, and used for comparison of the data obtained from the bulk Raman spectrum and SERS spectrum. The SERS system proved to be a good candidate for a label free direct measurement of pesticides in water samples.

# Dansk Resumé

Behovet for direkte påvisning af pesticider i drikkevand er i stigende efterspørgsel, efterhånden som drikkevands boringer bliver hyppigere forurenet med skadelige kemikalier. Et sådant system skal fungere autonomt i et barskt miljø i minimum 3 måneder og være i stand til at detektere meget lave koncentrationer af kemikalier.

En cantilever baseret overflade stress sensor er undersøgt som en mulig metode til påvisning af 2,6-dichlorbenzamid (BAM) pesticidrester i vand. Cantilever overflade stress princip blev undersøgt ved hjælp af piezo resistiv system fra Cantion/Nanonord A/S, og en optisk laser udlæsning system udviklet på DTU Nanotech. Surface Enhanced Raman spektroskopi (SERS) blev yderligere undersøgt som et sekundært alternativ til påvisning af pesticider i vandprøver.

Ved hjælp af et BAM antistof analyse udført på en cantilever overflade, 10 gentagne differentiale signaler af 5 til 20 μV størrelsesorden, blev målt ved hjælp af Canti4 Cantion cantilever systemet. Idet et signal også fremkom ved tilsætning af et uspecifik antistof, kan signalet ikke anses for at være specifikt for BAM antistoffet. BAM ELISA, microarray teknik, og et ”flow celle” system blev brugt til kemisk optimering af analyse forholdene på cantilever BAM-analysen. Billeder af cantilever overfladen, resonansfrekvens og udbøjnings værdier af cantilevere, viste at ujævnheder i overflade funktionaliseringen øgede variansen på de mekaniske egenskaber af cantileveren.

En alternativ optisk udlæsning system har gentaget den differentierede signal værdier, der blev opnået ved Canti4 Cantion cantilever systemet. Det optiske udlæsning system var i stand til at måle overflade ruheden af cantilever overfladen, som en nyttig indikator for kvaliteten af den funktionaliserede overflade.

Raman skift og SERS spektrum af BAM og Dichlobenil pesticid blev målt. Et simuleret Raman spektrum blev anvendes til sammenligning af data fra Raman spektrum og SERS spektrum. SERS systemet vist sig at potentielt være egnet for direkte måling af pesticider i vandprøver.

# Contents

# List of figures

# 1 Introduction

The first known pesticide to be used was sulfur dust; this was used by the Sumerians 6500 years ago in Persia. In the 15 century chemicals such as lead, arsenic, mercury and nicotine sulfate were used to kill pests. In the 1940´s and 1950´s the industrial production of synthetic chemicals took off, producing a large and diverse library of chemicals used to battle unwanted plants and insects on crops, on a global scale. Pesticides are today a useful and wide spread tool that allows us to have a very high production efficiency of agricultural products on a very small piece of land, this can in turn be regarded as one of the cornerstones of our society.[1]

The downside is that the yearly use of approx. 2,5 million tons of pesticides also have an unwanted effect on our surroundings. In 1960 these effects became clear when Rachel Carson published the book “Silent spring” where he discovered that the use of DDT to kill mosquitoes on his farm, was found to be the cause of death of the fish eating birds that lived in the area. [2]

This started the awareness of what impact mankind has on our environment and formed what is the environmental movement of today. The wide spread use of DDT was eventually banned by the Stockholm Convention on Persistent Organic Pollutants, a convention now consisting of 169 countries. Currently the use of more than 25 different pesticide compounds are banned or restricted.[3]

As the availability of harmful pesticides far succeeds the banned list, the monitoring of our environment is increasingly important, if we are to avoid unwanted harmful effects.

## 1.1 Pesticide assays

Because of the potentially high toxicity of pesticides, pesticide sensors technology is present in such diverse fields as water and soil control, agriculture, food industry and the military field. Until now the field of environmental analysis has mostly consisted of sample analysis done in traditional laboratories. This method is costly and very time consuming, and in the time span between sampling and result, a great deal of water with a potential health hazard can be sent out to the consumers. There is a big need for sensors that are able to deliver fast results and be an on or in-line monitoring sensor at the production flow, or portable enough to be used directly in the field. The last decade a big advance has been achieved in trying to bring the lab to the field with the introduction of "lab on a chip" approach, where processes such as extraction, separation and spectroscopy are miniaturized enough to be done directly on a portable device using a "single use" chip with these features incorporated. [4][5]

Pesticide sensors are usually divided according to the physical properties of the analyte, i.e. gas phase compound, volatile organic compounds, heavy metal ions or an organic phase analyte. Many of these sensors are based on traditional physical analysis methods such as gas chromatography, HPLC, capillary electrophoresis and mass spectroscopy. Although many of these methods are also being made more compact and more automated, the sample preparation and result analysis is not a simple task. There is a need for a simpler and more direct measurement of pesticides. For this purpose immunology based sensors can be a potential improvement. The immunological reaction of a specific antibody towards a specific analyte can be detected by a color change, the clogging of antibody or the change of surface tension when an antibody-antigen reaction takes place. The production of pregnancy test dip-strips is such an example of a cheap and simple to read-out portable analysis system.

The main problem of using an immunological reaction for the detection of a pesticide is that most pesticides are too small a molecule to elicit an immunological reaction. It is therefore necessary to construct an analyte that resembles the pesticide sought after but gives an immunological reaction in the host animal from where the antibody is isolated from. This can be done by attaching the pesticide to a small protein or enlarging the pesticide with a hapten

“tail“. The hapten tail needs to be carefully designed so it will not hinder the antibody recognition of the pesticides active sites and it must have an ability to bind to a carrier molecule. Once produced, the antibody should have an equal affinity towards the “natural” pesticide as well as towards the pesticide with the hapten tail. This way an assay can be constructed in such a way that if there is an eventual content of the pesticide in a water sample, it will react with the antibodies in the assay by blocking to the active site of the antibody, when the antibodies are then mixed with a known amount of the altered hapten-pesticide complex (immobilized on a surface) the assay will yield a lower signal than normal. This is called a competitive immunological assay.[6]

Although most immuno sensor systems are used for clinical diagnostics there is a development of immuno sensors for environmental use. In order to monitor the immunological reaction a transducer is needed. Recent advances have been made in the development of suitable transducers for immuno assays. With the advances in Atomic force microscopy using small cantilever spring devices, mechanical transducers are able to detect minute changes on a cantilever surface and detect this change via a bending in the cantilever. This alternative use of the atomic force microscopy technique was initially so sensitive that no suitable applications could be found, if was then used to detect differences in highland whiskies due to its high sensitivity to physical changes in the environment of the cantilever. In 2002 IBM research in Zürich developed an optical laser setup that measured the deflection of a cantilever, and were able to measure the change in surface stress caused by the hybridization of a DNA string to a complementary string immobilized on the surface of the cantilever. The cantilever platform has since been studied and used to measure the alteration of surface stress used for a wide variety of chemistry such as proteins and DNA and pesticides such as atrazine. [6][7] [8][20]

## *1.2 BAM pesticide detection*

Although specific BAM pollution is more common in Europe than the US or the rest of the world, the scenario of pesticide pollution of drinking water can potentially cause serious health hazards and great economic consequences for the consumer. The Danish water wells have seen a steady increase in pesticide related problems over the last 20 years. The latest figures from

GEUS show that approx. one in four wells have a content of 0,1-0,01 µg/l of a pesticide or its break down products. And one in five of the wells are specifically polluted with BAM.
BAM is the breakdown product of dichlobenil, a common herbicide used in the products Prefix and Casoron. The use of Prefix and Casoron has been banned in Denmark since 1997, but its breakdown product is still accumulated in the soil above the groundwater reservoirs. Pesticides containing dichlobenil was used as general herbicide in gardens and private house holding, not by farmers. This tendency is clearly seen on the geographical survey of polluted wells as most pollution arises from BAM and is found in rural areas. [9] [10]

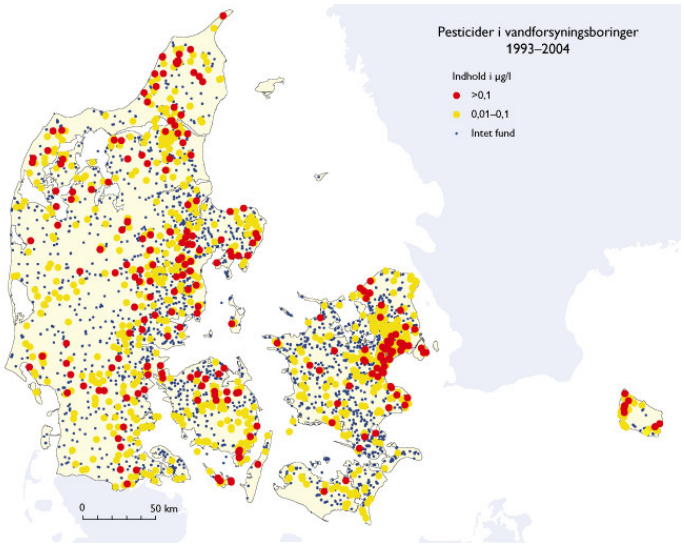



**Figure 1-1: Overview of pesticide polluted wells in Denmark (source: GEUS)**

The Danish law prohibits the use of drinking water with pesticide content higher than 0,1µg/l. So far the consequences of this have been that the polluted well has been closed, and in few cases the water is cleaned using a costly active carbon cleaning process. An alternative cleaning process using aminobacter bacteria as cleaning medium is currently being investigated at GEUS [7]. Currently water samples are being delivered to GEUS and analyzed for content of BAM via an ELISA method developed by Leif Bruun et.al. [21].

## *1.3 Dichlobenil and BAM*

The herbicide dichlobenil is used to kill unwanted weeds on the soil and in the aquatic environment. It has been a registered chemical in the US since 1964. Dichlobenil works through disrupting the formation of cellulose synthetase, a key component used in the formation of the cell plate structure used in plants during cell division. Thus this affects mainly

the fastest growing plants in the environment. The breakdown product of dichlobenil, BAM, inhibits processes in the chloroplast during synthesis and has an additional effect of uncoupling ATP fuelling reactions to the mitochondria in animal cells The $LD_{50}$ of laboratory animals is between 500 and 4250 mg/kg. Skin and reproduction organs are also affected by the pesticide. The most adverse effect of dichlobenil is the ability to do irreversible damage to the nasal cavity causing severe smelling loss in animals and humans [11] .

Although dichlobenil is fairly persistent in soil and also found in groundwater, it is BAM that is causing the biggest concern as it is more often found in ground water and is very poorly degradable in soil. The breakdown of dichlobenil to BAM is done by soil bacteria [10]

## *1.4 Developing a cantilever based pesticide assay*

The goal of the thesis is to contribute to the development of an autonomous system to measure the content of a pesticide in drinking water. The initial idea is to have an inline system that is to be placed in selected ground water pumping stations and is autonomous or very low maintenance. During the SENSOWAQ meetings a set of criteria was established for such a system:

- Sensitivity equal to established ELISA protocol (approx. 0,01 μg/l)
- Capable of 3 months in-line continuous run without maintenance in hostile environment
- Cost preferably below 20,000 d.kr. per unit
- Set in-line with water system and able to do at the least one measurement per day

If an immuno assay is chosen as the assay principle, the limiting factor of such a system would most likely be the cost of antibodies and available buffer solution. Lets suppose that a continuous running system uses 20 μl/min of buffer solution and 0,025 μg BAM antibody per analysis (as used in the Cantion BAM assay described in the thesis), if we include a washing step (1 hour), a surface regeneration step (1 hour) and the analysis of a water sample (2 hours), a full round of analysis could be done in a 4 hour period. The consumption over a 3 month period will thus be of at least 2,6 liter running buffer and 13.5 mg BAM antibody.

The consumption of antibody can probably be minimized, but it serves as an example to what limitations a possible system is facing. It is important to bear in mind the basics of these criteria's when designing the experiments for the development of a pesticide assay in general.
On many pumping stations today there are systems that monitor physical properties of the water such as conductivity, pH, turbidity, and coli form bacteria count. A working device for the detection of BAM and dichlobenil would use such a fluidic system.

## *1.5 The SENSOWAQ project*

Sensor for water control and monitoring of water quality (SENSOWAQ) is a project consisting of a diverse consortium of universities, companies and Danish water monitoring parties (4 universities, 3 research institutions, 5 private companies & 3 water companies). This thesis is a part of the SENSOWAQ project.

The overall goal of the project is to develop or investigate solutions that can lead to an online or in line water quality measurement device, through cross field collaboration and research.
SENSOWAQ is lead by Professor Hans-Jørgen Albrechtsen, DTU Environment and has a total budget of 18.3 million DKK; it is supported by the Danish Agency for Science, Technology and Innovation, Program Commission on Sustainable Energy, Environment and industry.

## 1.6 Overview of thesis

The Ph.D. project has been focusing on 3 main areas:

- The Cantion cantilever setup
- Optimizing surface chemistry involved in the BAM cantilever assay
- Alternative methods for detection of the BAM pesticide.

In order to perform and improve the BAM assay using the Cantion system, the biggest challenge in the Ph.D. has been to understand the factors affecting the Cantion BAM assay. Although the Cantion BAM assay has been improved, the assay never became specific enough to realistically be used as a real BAM assay. The cause of this is debated in the thesis.
Because the Cantion assay did not yield satisfactory results, it was decided to try and break down the assay in the sensing part and chemistry part. The chemistry involved in the assay was then characterized and optimized via microarray technique and via a flow cell containing a chip with microarray dots. This ensured that the chemical part was working in the assay.

Towards the end of Ph.D. project a new Cantion system with potentially better characteristics was given to the department for testing. The system is described briefly in the thesis. It was decided to continue to use the old system for experiments, partly because new chips were limited in numbers but mainly because a good knowledge of the old system was already established.
Two alternatives to the BAM assay was investigated, an alternative to the Cantion system but still using a cantilevers based surface stress sensing method (DVD setup), and an alternative label free sensor for direct measurement in water samples (Surface enhanced Raman spectroscopy). The suitability and potential of the sensor platforms are debated in the thesis.

**Chapter overview**

- **Chapter 2**. The relevant theory involved in sensors based on surface stress measurements is described. This includes a comparison of the internal piezo based sensing vs. an optical deflection sensing method.
- **Chapter 3**: The Cantion system is described, system characterization experiments and DNA assay experiments are presented.
- **Chapter 4**: The surface chemistry involved in the assay is presented. A series of different experiments involving BAM ELISA, microarrays, fluorescent imaging and a flow cell surface quality tool was used to investigate on the optimal working conditions for the assay.
- **Chapter 5:** Two rounds of the BAM assay done on the Canti4 setup are described .
- **Chapter 6:** A new version 8 cantilever device from Cantion/Nanonord is presented.
- **Chapter 7**: An advanced system based on hardware from a DVD player is investigated as an alternative to the Cantion system. The system uses the same surface stress principle as the Cantion system but is based on optical deflection rather than an internal piezo sensor.
- **Chapter 8**: Raman spectrum is investigated as direct label free detection system. This is a novel alternative to pesticide detection and to the Cantilever based surface stress system. The chapter describes computational modeling of Raman spectrum, experimental results of a traditional Raman spectrum and surface enhanced Raman spectrum of dichlobenil and BAM.
- **Chapter 9**: A conclusion and outlook of the work done in the thesis is presented
- **Appendix**: The protocols developed and used in the thesis are attached in order to clarify eventual specific questions about the methods of the experiments.

# 2 Theory and principles

In this chapter the fundamental principles behind the sensors used in the experiments are described briefly. The theory section describes the basic stress induced bending and the Wheatstone bridge. As the Ph.D. project was of experimental nature, an elaborate explanation of the equations is beyond the scope of the thesis.

## 2.1 Surface stress induced bending

The stress induced during bending of the cantilever can be calculated using Stoney´s formula:

$$\sigma = \frac{Eh^2}{6(1-\nu)R}$$

**Equation 2.1**

Where σ is the surface stress, v is Poisson´s ratio, R the radius of curvature, E is Young´s modulus and h is the thickness. The relation between the cantilever deflection (z) and radius of curvature (R) is given by

$$z = \frac{l^2}{2R}$$

**Equation 2.2**

Where z is the deflection at the cantilever tip, l is the length of the cantilever (120 µm) and R the radius of curvature.

The change of voltage as a function of voltage is given by:

$$\sigma = A\frac{V_{\text{output}}}{V_{\text{input}}}$$

**Equation 2.3**

Where A is the proportionality factor of the surface stress/voltage ratio. For the Cantion cantilevers A = 3125 N $m^{-1}$. V input and V output are the voltage values. If the above 2 equations are inserted in Stoney´s formula the relationship between deflection of the cantilever and output voltage is given by:

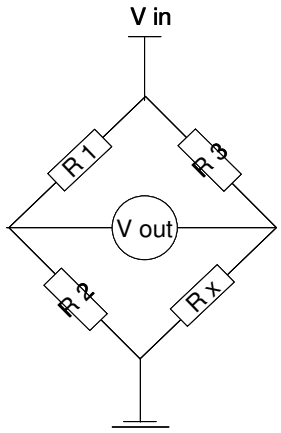
$$z = \frac{3(1-\nu)}{Eh^2} l^2 A \frac{V_{\text{output}}}{V_{\text{input}}}$$

**Equation 2.4**

The cantilever deflection can thus be calculated when the voltage input and output is determined. If young modulus is estimated to 220 GPa and Poissons ratio is 0.25, and the bridge voltage is set to 2.5 Volts, the deflection is z = 0.8 nm/µV. A 20 µV signal thus corresponds to a deflection of 16 nm on a 120 µm long cantilever [12] [13] [15] [16]

## 2.2 Wheatstone bridge setup

The Wheatstone bridge setup is a central element in understanding the Cantion setup.
The Wheatstone bridge was invented by Samuel Hunter Christie in 1833 and developed by Sir Charles Wheatstone in 1843. It is a circuit used to measure an unknown electrical resistance by balancing two legs of a bridge circuit, where one leg contains the unknown resistor value.
In the Cantion system the piezo element imbedded into each cantilever is the variable resistor that changes resistance when bended or elongated, and the corresponding fixed resistors are integrated into the chip body. Normally a voltage of 2.5 Volts is applied and the output signal of the system is a voltage difference from each cantilever. The Wheatstone bridge is given as:




**Equation 2.5**

When in balance the resistors are such as R2/R1 = Rx/R3. in the Cantion system only the Piezo resistor is variable, therefore the voltage needs to be adjusted in order to balance out the 2 sets of resistors. The output voltage is given as:

$$V_{out} = \frac{1}{4}\frac{\Delta R}{R} V_{in}$$

**Equation 2.6**

The sensitivity of the system is given by $\Delta R / R = K\varepsilon$, where $K$ is the gauge factor and $\varepsilon$ is the strain of the piezo resistor. In the Cantion system the minimal measurable stress is given by the voltage noise of the system. The voltage noise arises from thermal mechanical noise, thermal voltage noise and the spectral noise 1/*f*. For the Canti4 system the minimal measurable stress is around 1 μV under a 2.5 V input.[14][15].

# 3 Cantion cantilever system

## *3.1 Introduction to the canti4 cantilever system*

A cantilever system CantiChip4® from NanoNord/Cantion A/S was chosen for the assay [28] [29]. The bending of the cantilever causes a proportional change in voltage between the piezo layer in the cantilever and a fixed resistor embedded in the chip measured via a Wheatstone bridge setup. The system consists of 4 silicon based cantilevers with integrated piezo resistive readout. All 4 cantilevers are 120µm length x 50µm width x 0.45µm thickness, coated with a 40 nm gold layer, electrically grounded, and flip chip bonded to a polymer contact pad that is inserted in the CantiLab4© that converts the voltage signal to a proprietary recording software. As the CantiLab4© is a closed system manufactured by Cantion, no additional information is available about the content of the electronics. At the start of the project 77 pieces of 2$^{nd}$ quality chips and 20 pieces of 1$^{st}$ quality chips were available. The Canti4 chip and the canti4 setup are seen below.

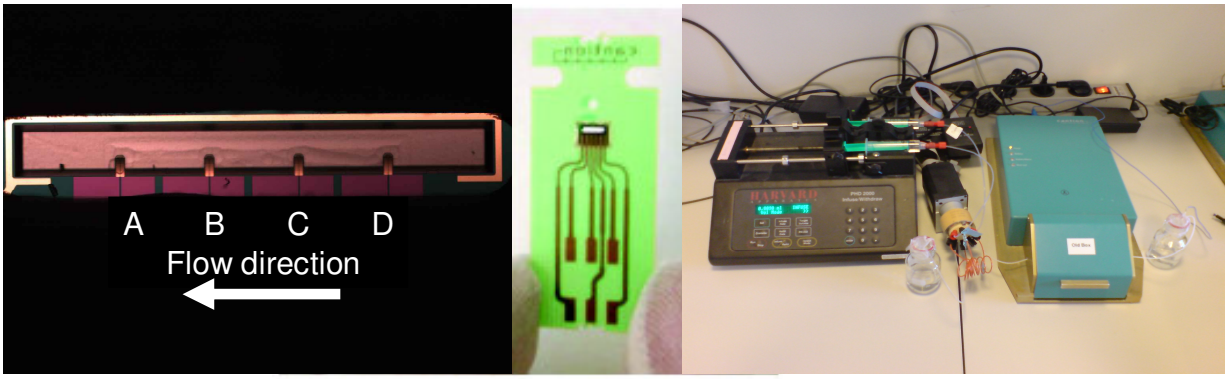



**Figure 3-1: The Canti4 system (left) The 4 cantilevers aligned inside the flow piece. (middle) A canti4 chip, the cantilevers are placed in the top of the chip. (right) The Canti4 system setup with the electronic readout CantiLab4© box, a switch box for the fluidics and a syringe pump.**

## 3.2 Functionalization of cantilevers

There are several reasons as to why a specific functionalization is needed to each cantilever. First, to achieve a differential signal from the system, it is necessary to use one or more of the cantilevers as reference, coating and activating only one side of the cantilever is expected to yield the highest bending effect from the system. To functionalize the surface of each cantilever a micro-spotter from Cantion A/S was chosen. Another common method is to insert each cantilever into micro capillaries with the coating solution; this is not possible as the cantilevers are situated down in a flow pit that is bonded with the electronic contacts.

The Canti$^{TM}$Spot is basically a platform consisting of an inkjet printer head, a PC interface camera, a lightning system and a manual X,Y stage holder designated to hold 5 pieces of the Cantion 4 chip. The system is mounted with a piezo electric controlled pin head (GESIM Sub-micro liter Piezoelectric Dispenser A010-006 SPIP) and capable of delivering ~0.1 nl drops to each cantilever surface while being monitored via a camera on a PC. A proprietary software records the spotting action and controls the voltage, pulse width and frequency settings of the Piezoelectric dispenser. The dispenser is actuated by an electronic box supplying the voltage pulse to the piezo element. The electric pulse contracts or expands the piezo layer, enabling drop suction or drop delivery from the dispenser head. To clean the dispenser, a closed syringe pump is also attached to the system. This allows the continuous flushing of the tip with Milli-Q water or ethanol to ensure that no salt crystals block the fragile dispenser tip. Although the Canti$^{TM}$Spot is a simple setup, the dispenser head is a very sensitive system that is easily clogged, highly concentrated salt dilutions are a challenging task to spot. In general a successful coating of only the top surface of a cantilever was achievable with training. The exact procedure for functionalization of cantilevers can bee seen in the appendix. The Canti$^{TM}$Spot platform is seen on the next page.[18][19]

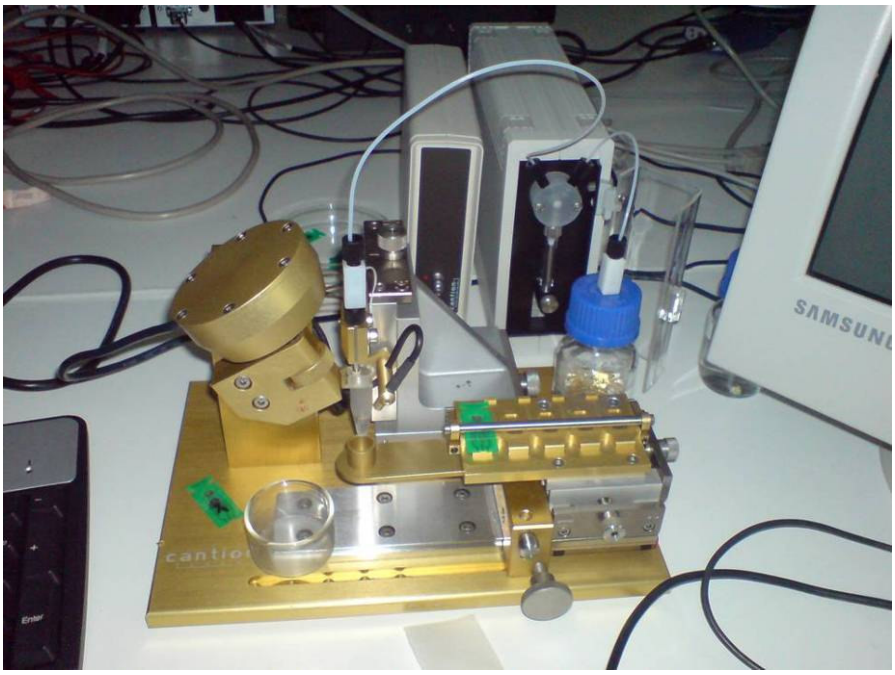


**Figure 3-2: The Canti$^{TM}$Spot with a syringe pumping station and piezo pulse box in the background**

## 3.3 Cantion cantilever experimental setup

The Cantion setup consist of the CantiLab4© box connected to a fluidic system that consists of a syringe pump and an 8 channel switchbox. The syringe pump (Harvard PHD2000) can be loaded with 2 syringes and set to push or pull. Although the pump is generating some noise and some fluctuation in flow speed, it was considered adequate for the system. The fluidic setup of the switch box (from Microlab Aarhus) consisted of 2 parallel channels where 2 inlets (one from the pump and one from a sample) can switch between 2 loops of 100µl each. The 2 outlets were connected to the CantiLab4© box and a waste beaker. This way, loop no.2 could be filled by suction with a sample, while buffer is continuously running into the CantiLab4© box. Using a 20µl/min flow the fluid from the loop would touch the cantilever 180 seconds after the switch was done. To avoid an electric battery effect between the chip and fluidic system, the waste bottle was grounded to the CantiLab4© electronic box with a gold wire.

After experiments, both channels were washed and filled with Milli-Q water at least 15 min. No specific cleaning of the fluidic system was done; this was to avoid unwanted effects from surfactants. The tubing and fittings were of polymer without traces of silicone grease as this

would cause a thin film of silicone in the cantilever chamber, rendering experiments impossible. Below is seen a schematic diagram of the fluidic system. The system remained unchanged in all the experiments (except the ground wiring was added in the BAM assay).

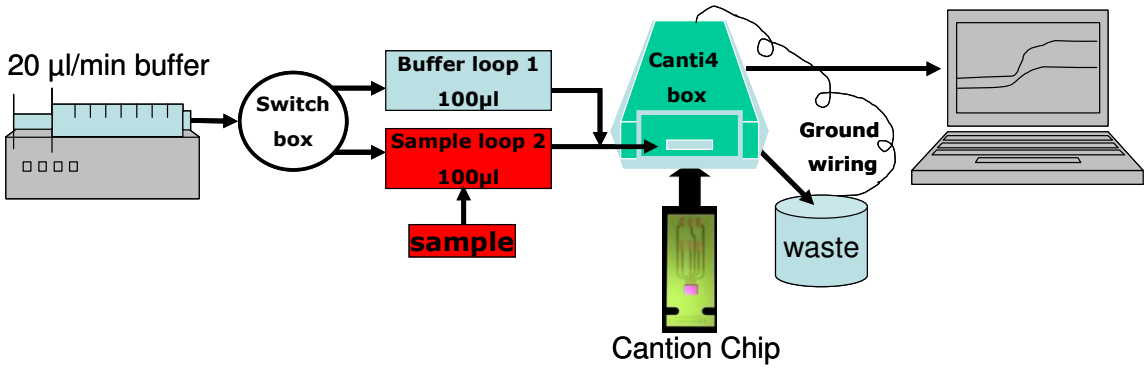



**Figure 3-3: A schematic overview of the fluidic setup.**

The data was recorded in text files in 0.2 seconds intervals containing the 4 resistances from the 4 cantilevers, and a temperature reading. The temperature readings were not used in the experiments, as they were not representative of the actual temperature in the chamber. The text files were converted in excel into graphs showing the differential values of the cantilevers. The actual values were often not suitable for presentation, due to large signal variations over time. In the DNA experiments, cantilever A and C were signal and B and D were reference. In the BAM assay, cantilever B and C were signal and A and D reference. In the flow system the fluid hits cantilever D first, it was speculated that this could cause more noise on D compared to the following cantilever C, B and A. This could eventually not be determined from the experiments.

## 3.4 DNA hybridization assay on Canti4 setup

The characterization of the Cantion system was done while working on the DNA assay. The DNA assay was used as a benchmark tool to test the Cantion system. The DNA hybridization assay has been repeated by many research groups and is therefore chosen for comparison. Although successful signal reports are available [17][28] few groups have done repeated measurements using a cantilever surface stress readout system, and no data on the success rate is available. The DNA assay consists of 2 sets of complimentary DNA strings (denominated B1 and B2). The cantilevers are spotted with a thiol modified DNA (B1SS and B2SS) and reacted with the complementary strand (B1C and B2C)

### 3.4.1 Materials and Methods

Prior to use, the oligonucleotides were reduced from 5´-disulphide modified oligonucleotides to oligonucleotides with a free thiol group, as the reactive thiol group bond strongly to the gold surface.

Using the Canti$^{TM}$ Spotter, the DNA string B1SS and B2SS was added to the cantilever A and C. Cantilever B and D were untouched and kept as reference. The functionalized chips were placed in a NaCl Humidity chamber to allow the thiol chemistry to react with the gold layer. To avoid unspecific binding the chips were then blocked in a 6 mercapto 1-hexanol mix for one hour.

The chip was inserted in the Canti4 box and a stabilization run using 20µl/min. flow was recorded. The non specific DNA (NS-DNA) was added to the chamber. 100µl of the specific DNA (0.11 mg/ml B1C and 0.1475 mg/ml B2C) was added to the Canti4 box. Each action was recorded min. 1500 seconds in a separate file and data processed using excel. A more detailed protocol can be seen in the appendix.

### 3.4.2 Results and Discussion

12 chips were used on DNA experiments from November 2007 to march 2008, of these only 6 experiments were able to complete the assay properly. A positive signal was only achieved in one, possibly two cases (40 μV on chip 51 and 10 μV on chip 52). All experiments had a large noise floor of ~ 5 μV. The complementary DNA is expected to enter the chamber at ~360 seconds and exit at ~660 seconds. In the results below the reference cantilever B and D can be seen as maintaining a relative constant value, whereas the cantilevers coated with B1 SS (cantilever A) and B2 SS (cantilever C) reacts towards the B1C and B2C complementary strand, keeping a signal difference past the 660 seconds. It is debated whether the signal should be expected to return to the initial level or remain altered, as the cantilever is expected to de-stress the surface by rearranging the molecules on the surface.

The next page shows the results from the addition of specific DNA and non-specific DNA from chip no. 52. The data is presented as signal – reference; reference –signal; and reference – reference in order to avoid overlap of the graphs and ease of read-out.

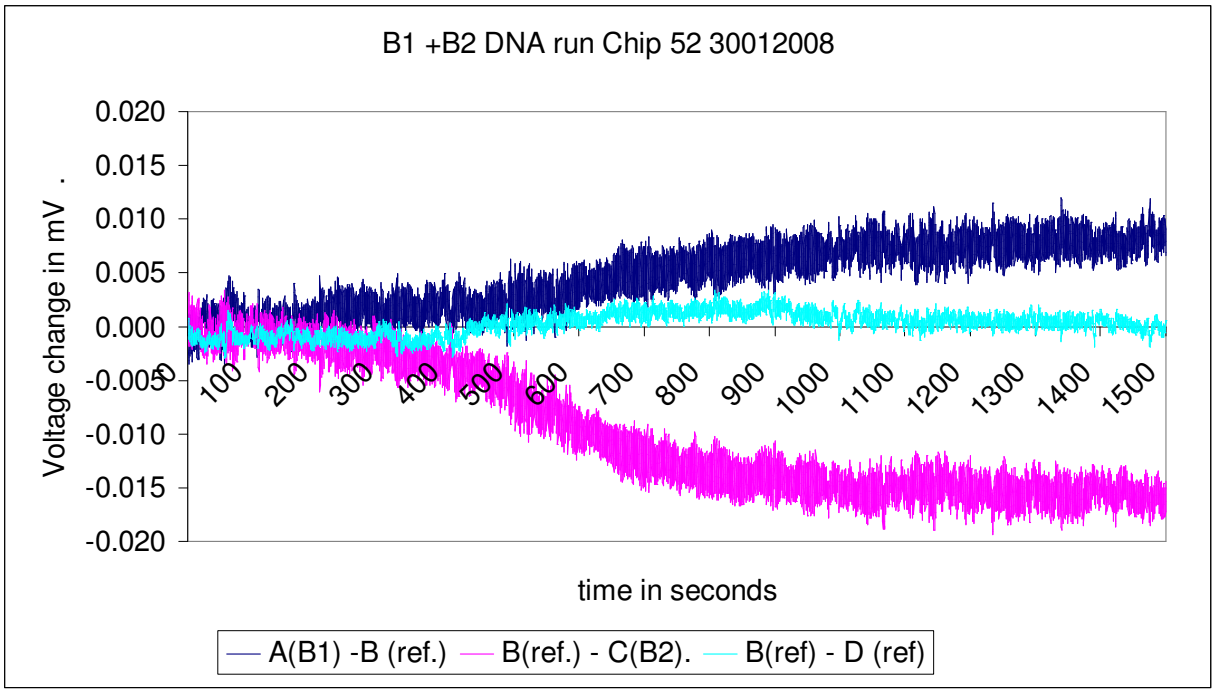



**Figure 3-4: Specific DNA results showing the differential values from chip no.52, cantilever A (B1 dna) – B (reference); cantilever B (reference) – C (B2 dna); cantilever B (reference) – D (reference)**

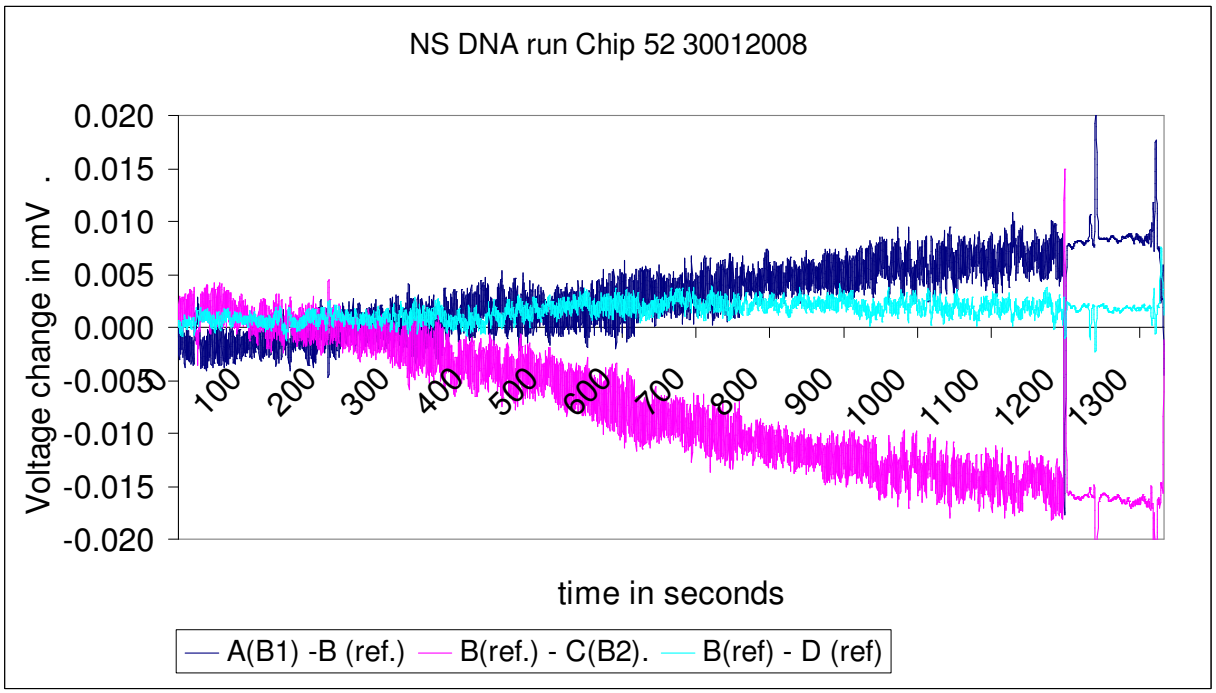



**Figure 3-5: Non-specific DNA results showing the differential values from chip no.52, cantilever A (B1 dna) – B (reference); cantilever B (reference) – C (B2 dna); cantilever B (reference) – D (reference)**

## 3.5 Canti4 system characterization

To learn the limitations and possible errors of the system, a series of experiments and observations were done on the Canti4 system. The experiment results were achieved through designated characterization experiments, but also through observation from experiments on the DNA assay and BAM assay.

### 3.5.1 Influencing factors

A series of observations were made on the Canti4 system.

- **Flow speed:** The flow speed of the fluidics has a linear effect on the noise of the system. It was thus considered to use stop flow measurements, but as noise from the fluidic system was not the main influencing factor, a continuous flow was chosen. A flow speed of 20 µl/min was found to be adequate, giving a 100µl sample 5 min to react with the cantilevers. Although 10 min. is probably ideal regarding the reaction time, the data from the BAM assay and DNA experiments showed that, in all cases the biggest signal effect was achieved within the first 300 seconds.
- **Buffer type**: The buffer had an effect on the noise level of the system, the SSC buffer used in the DNA assay had a significantly larger noise level of up to 5 µV compared to the low noise level of 1 µV of the 1xPBS buffer used in the BAM assay. A higher conductance of a high salt buffer could mean a larger effect of an electric field from cantilever to cantilever.
- **System start up**: The system needed min. ½ hour to stabilize the resistance values of the cantilever. The resistance values had large signal slope amplitude in the start up period. The cause of this was regarded as being caused by heating of the electronics of the CantiLab4© box. It was also critical that the fluidic system was flushed in the start up period with 100µl/min speed, to eliminate build up of a buffer salt or pH gradient.
- **Fluidics battery effect**: When an air bubble was present in the fluidic system, or if the system was cut off outside the CantiLab4© box, the signal on all 4 cantilevers would have a large signal alteration and noise. This could be caused by the fluidic system acting as a small battery, where a minute current was acting through the system, from the waste bottle to the buffer in the syringe. This effect was eliminated by adding a gold wire to the waste bottle and connecting it to the CantiLab4© box. Before each

experiment, the waste bottle was filled half up with the same buffer as in the syringe, to minimize additional differences in fluids.

- **Signal slope**: In the duration of an experiment, that often lasted 3-4 hours, the receptivity values of the cantilevers would gradually slope in either negative or positive direction, this slope would often reverse after 1½ hours. The cause of this is speculated to arise from a difference in resistance over time in the fixed resistors of the Cantion chip used to measure the voltage difference in the Wheatstone bridge setup. No solution was found to eliminate this effect although a long startup period did seem to minimize the drift after a 1½ hour period. However, the drift values would often increase again after 4-5 hours run.
- **Loop switch**: Using buffer as a sample, revealed that if the fluid in the second sample loop was allowed to stand more than one hour before injection into the CantiLab4© box, a signal of 5-100 μV was detected on all 4 cantilevers or even as a differential signal. The signal lasted approx 5 min. until the sample had cleared off. To avoid this effect, the loops were switched and flushed 4-6 times before the experiments began, and also during daily maintenance of the system.
- **Temperature**: The laboratory temperature was not constant during the day (7-8 ºC difference) or during the year (from 23ºC to 30ºC). 24 hours experiments were done that confirmed the temperature difference, but as the experiments were done in day time and as only the differential signal was used, the room temperature did not have a noticeable effect on any experiments. However the Harvard pump used, did produce heat up to 45ºC on the surface, enough to change the temperature of the buffer in the syringe. The pump was therefore left on, so no additional heating of the pump could give a temperature gradient during the experiment.

These findings led to the conclusion that the performance of the Cantion Canti4 system is very sensitive towards minute changes and minor changes. A set of procedures and equipment was used to minimize the risk of these unwanted artifacts.

### 3.5.2 Chip traceability and documentation

The amount of available cantilever chips were limited (less than 70 chips) and no more chips could be made available from Cantion A/S. A goal of the BAM assay was to re-use the same chip for at least 10 rounds of experiments. It was therefore important to establish a traceability of the chips used, in the beginning of the experiments.

Each chip was tested for signal quality on all 4 cantilevers, and was examined for visible damage using a microscope. The chips used were numbered, and its use noted in an overview log book document. Additionally an experiment scheme was made for each experiment, where the settings used and general outcome of the experiment was noted. The spotting functionalization was recorded for each experiment with a video of the action. Many of these files were erased later as they only served as an immediate check of the spotting action.

The documentation method was developed gradually during the experiments. For the second round of BAM experiments an extensive report was made for each successful experiment. This was done to evaluate and cross check the experiment results by examining the fluorescence pictures with the 5 signal results (stabilization, switch test, buffer sample test, unspecific IgG, BAM antibody) and the settings used in the experiment. In addition each experiments had a film of the spotting action, microscope pictures of the cantilever surface before and after spotting, 5 files of data from the CantiLab4© box, fluorescent pictures of Cy3 and Cy5 signal, mass/stiffness values and bending values of each cantilever before spotting, after spotting, and after experiment. Although very labor intensive, this ensured a good foundation to evaluate the outcome of each experiment.

### 3.5.3 Initial voltage difference of internal resistor and cantilevers

As the output of the system is the difference in resistance between the cantilevers and a fixed resistor in a Wheatstone bridge setup, it is interesting to know the variation between the fixed resistor and cantilevers in between chips. The voltage value of the fixed resistor is given with 8 decimals, but the value changes slightly each time the chip is inserted in the CantiLab4© box, therefore only 3 decimals are feasible as readout. Unfortunately it is not possible to see the voltage variations of the fixed resistor during operation, as this stay fixed once the chip is set in

the CantiLab4© box. Likewise, the voltage values for the 4 cantilevers is changing significantly during the first 30 min of operation , the values are therefore given as a 2 decimal number, although recorded in the text file as a 5 decimal number.

When chips did malfunction during the experiments, it was observed that prior to the malfunction the resistor values would increase, until finally surpassing the 26.67 volt threshold. The absolute voltage values of 18 chips were therefore measured to see if the voltage values could reveal information on the quality of the performance of the chip. The values are read 2-3 min after inserting the chip in the CantiLab4© box. This revealed a very high standard deviation in between cantilevers on the same chip (average of 1.51 V) and also in between chips. Cantilevers that had a high initial voltage difference, were prone to malfunction during the assay run, resulting in a voltage overload ( +/- 26.67 V) or very high noise. As the internal resistor value is given when the chip is inserted in the Cantion box, the correlation of faulty Cantion chips with a high internal resistor voltage difference was examined.

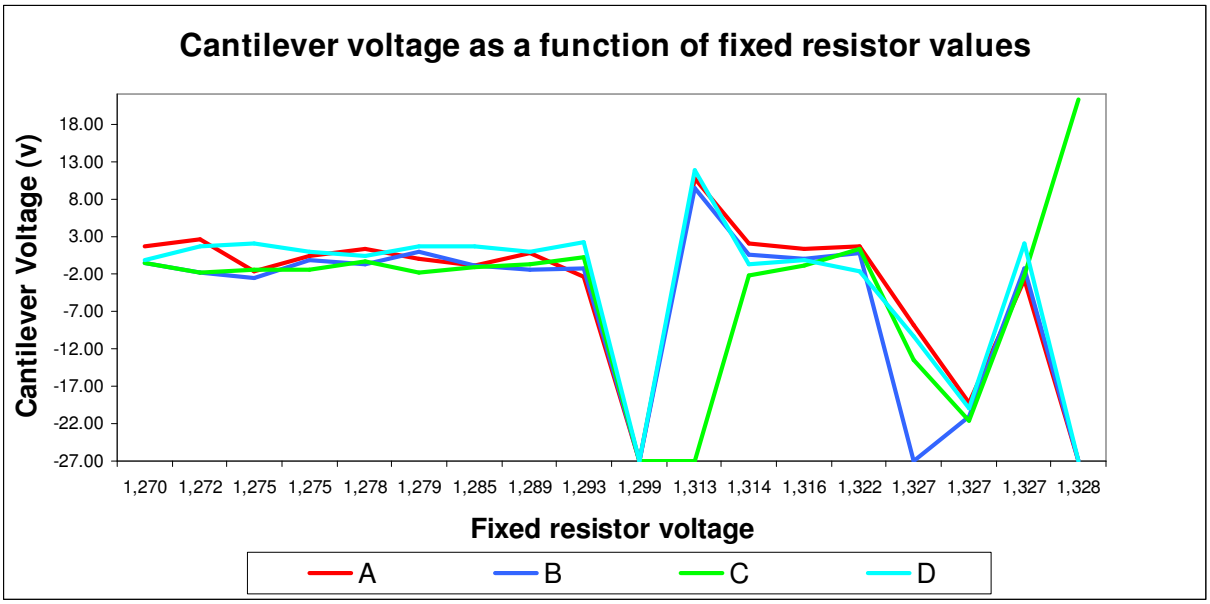



**Figure 3-6: Voltage values from 18 Cantion chips plotted as a function of the internal resistor value, note the large variation in cantilever voltage as the fixed resistor voltage increases.**

Above is seen a graph showing the large difference of the absolute voltage values of the cantilevers as a function of the internal resistance voltage difference. As the fixed resistor voltage increased to over 1,3 V the difference between the cantilevers grew considerably. No

explanation for this was found for this effect. On 8 chips the thin contact pad layer was worn off or had a slight rust layer, this was fixed by applying aluminum glue to the contact pad; these chips were not included in the above graph.

### 3.5.4 Recycling of Cantion 4 chips

As the overall goal of developing the BAM assay on a cantilever platform, is to have a usable autonomous device in the field, the idea of using a mono use chip for each experiment would bring the system to a very high cost. It is therefore interesting to analyze whether the chip can be cleaned, and re-used after an experiment.
A cleaning procedure for the Canti4 chip was developed by the Cantion company during the development of the chip and was followed. The procedure consisted of a 6 stage cleaning method:

- Milli-Q water rinse step
- Deconex cleaning, a common alkaline detergent
- Milli-Q water rinse step
- HNO3 acid cleaning
- Milli-Q water rinse step
- UV ozone oven cleaning incinerating organic leftovers on the surface

The chip was inspected after the final UV ozone step under a microscope to check for visual damage on the surface. To ensure proper quality of used chips, a set of pictures were taken after the cleaning. Furthermore, a dew point test was performed after cleaning, the chip was cooled down using an electric peltier cooling element, a dirty chip will have the dew drops dispersing on the surface vs. a clean surface will have small separate drops because of the higher surface tension.

A single chip was reused up to 5 times (chip 53) while still working on all 4 cantilevers. However the majority of reused chips did eventually malfunction after only 2 cleaning processes. The surface of the cantilevers on 3 different chips were inspected under an electron microscope in the Danchip cleanroom, and showed that the repeated cleaning process damaged

significantly the gold layer on the cantilever surface. Most cleaned chip suffered from damaged gold layer grounding around the chip pit. The UV ozone step is probably the harshest step of the cleaning process. Below is seen an electron microscope picture of the gold surface of some of the damaged cantilevers.

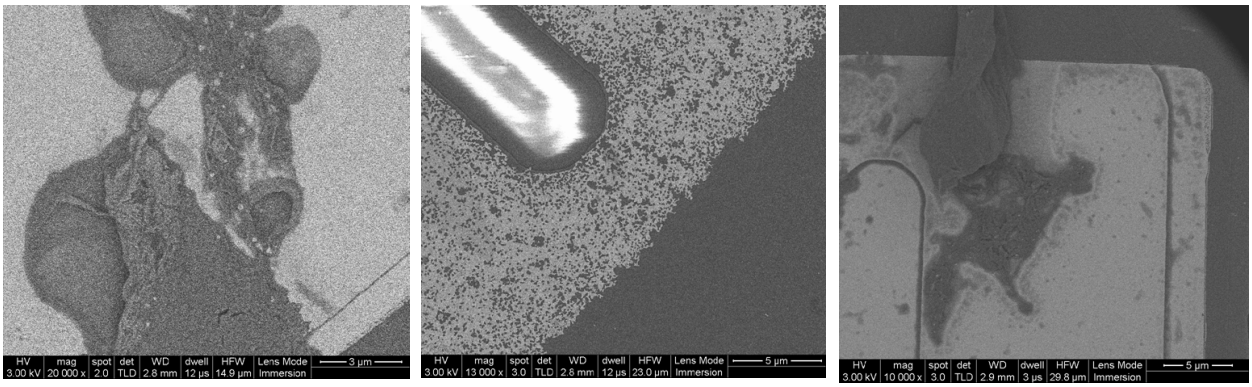



**Figure 3-7: Surface quality of 3 used cantilevers. The gold layer is seen peeling off, the middle picture show the tip with grooves from etching damage to the gold layer**

# 4 Surface chemistry optimization

The optimization of the Cantion assay was found to be too time consuming and yielding too little information about the optimal conditions for acquiring a sufficient signal. Also the number of available chips was limited, and the re-use of chips did not prove reliable as the gold surface deteriorated rapidly after few rounds of wash.

In order to understand the actions behind the Cantion cantilever BAM assay, a division had to be made between the influencing factors arising from the chemical part and the sensor part. The surface chemistry involved had to be optimized in order ensure that a maximum surface stress could be induced on the cantilever surface. A second goal was to minimize unspecific binding on the surface and possibly minimize noise on the sensor results. To achieve these goals a series of experiments in the following fields were done.

- Alternative chemistry: Alternative hapten chemistry was investigated to alter the antibody affinity towards the surface bound BAM. The use of Aptamers as a more robust alternative to antibodies was also discussed.
- BAM ELISA: The chemistry involved in the BAM assay was investigated; BAM conjugates were tested in the analysis.
- Microarray for chemical analysis: Here the blocking chemistry was tested.
- Flow cell device: The regeneration possibilities were tested using continuous flow conditions.

## 4.1 Alternative BAM hapten tails

There is currently no possibility to control the affinity ration of the BAM antibody between the surface bound BAM and free BAM in the sample, nor is there any knowledge regarding the individual binding constants between the antibody and BAM antigen. A lowering of the binding affinity towards the surface bound BAM will increase specificity towards the free BAM in the sample, as fewer antibodies are attached to the surface. This lowers the need for high concentrations of BAM in the sample, in order to detect the change in surface adhesion of the BAM antibody.

Another use can be the freedom of choice for binding possibilities of BAM to different surfaces ($SiO_2$, gold, polymer). Different choices of chemistry can increase the combination possibilities using different devices with diverse surface characteristics. This would expand the freedom of choice of the binding material, and also possibly the binding durability of the BAM hapten antigen, if the hapten is constructed specifically for the surface. In order to change the affinity of the antibody, an obvious choice would be to search for new BAM antibodies with different binding affinity towards BAM, but as this work has been done sufficiently by Leif Bruun et.al.[21].

An alternative option is to change the affinity of the BAM antibody through changing the hapten tail or the BAM molecule itself (i.e. removing or substituting a chlorine atom). The hapten tail complex properties can roughly be divided into polar/non-polar and steric hindrance haptens (shorter or large molecules chains). Changing the length can have a profound effect on how the BAM antigen is available for the BAM antibody, the BAM antigen can be attached so close to the surface that the BAM antibody can not close on it, alternatively the length can be so long that the BAM groups folds or turns on each other, thus also lowering the binding possibilities of the BAM antibody. The hapten complex can either bind to a carrier protein (using a COOH group), directly to a TsT activated SiO2 surface (using a primary NH2 group), or even a thiol group binding to a gold surface.[25][26][27]

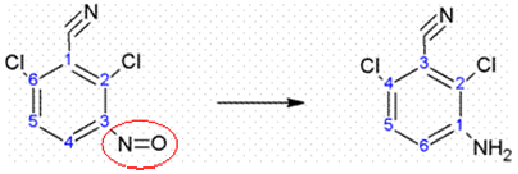
It was therefore decided to synthesize both a new batch of BAM hapten and 2 different versions of the BAM hapten. For this a series of alternative haptens and binding chemistry

were investigated. The synthesis planning was done in plenum in the Surface engineering group, and the synthesis carried out by Basil Uthuppu from Surface engineering group (also part of the SENSOWAQ project). As the author did not take part in the laborious synthesis lab work, the details of the synthesis is not described in the thesis. The main steps in the synthesis of the BAM hapten can be seen in the next chapter.

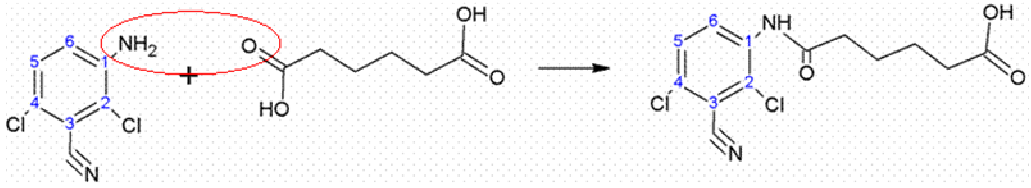
### 4.1.1 BAM hapten synthesis

The synthesis of the EQ0031 is a 3 step process. In the first step the nitro group is substituted by reduction reaction to an amino group, in the second step the carboxylic acid on the hapten linker is attached to the amino group. In the third step the Cyano group on the BAM molecule is oxidized to an amide group by addition of a water molecule. The synthesis is based on the article by Leif Bruun et.al. [22]. A more detailed synthesis scheme for EQ0031 can be seen in the appendix. The tree main reaction steps are illustrated below.

- 2,6 Dichloro -3-Nitro cyano benzene to 2,4 – Dichloro-3-Cyanoaniline




- 2,4 – Dichloro-3-Cyanoaniline to N-(5-Carboxymethyl-pentyl)-2,4-Dichloro-3-cyanoaniline




- N-(5-Carboxymethyl-pentyl)-2,4-Dichloro-3-cyanoaniline to N-(5-Carboxymethyl-pentyl)-2,4-Dichloro-3-amidoaniline

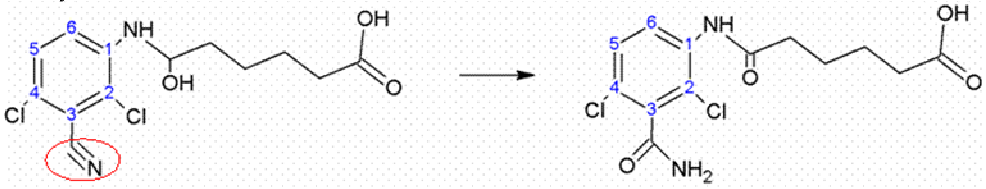



**Figure 4-1: Synthesis of BAM hapten**

## 4.2 Aptamers as an alternative to antibodies

In recent years an artificial alternative to immunological antibodies is gradually gaining popularity. The alternative is DNA or RNA based strings that through their secondary structure are able to bind selectively to a specific antigen. These alternatives are denominated Aptamers. There are several advantages in using Aptamers as an alternative to biological antibodies. The Aptamers have a known primary sequence, the secondary and tertiary sequence can be calculated using structural sequence database programs. Given the smaller size compared to antibodies, the structural conformational change is expected to be bigger, causing a larger surface stress if Aptamers are immobilized on the cantilever surface.

The Aptamer structure is generally more stable than antibodies towards harsh conditions. And lastly the production of Aptamers are cheaper and less complicated once the selection is completed. The selection is done using a systematic evolution of ligands by exponential enrichment (the SELEX method) [23]. However the SELEX selection process is very time consuming and requires high molecular biology skills. Thanks to a collaboration between Daniel Dupont and Jørgen Kjems from iNANO Aarhus and the Nanoprobes group at DTU Nanotech, 2 sets of Aptamers and corresponding antigens (against uPA and PAI-1 breast cancer marker proteins) were donated. The Aptamers are currently being tested using the DVD IBM cantilever setup described in section 7 in the thesis. As the Aptamers testing is not completed it is not included in the thesis.

## *4.3 BAM ELISA*

Quantitative detection of BAM pesticide is currently done at GEUS with a competitive ELISA. The use of antibodies for the detection of BAM pesticides is the key element in the BAM assay. It was therefore necessary to optimize the chemistry involved in the ELISA, to ensure that the signal obtainable on the cantilever was maximized. The assay principle is a competition between the BAM in the sample, and bound BAM on the surface. The BAM ELISA was used to learn the handling of the chemistry on the BAM assay, and investigate whether BAM ovalbumine conjugate could be used as a substitute to the Anthraquinone photo chemistry (EQ0028) for the attachment of BAM on a glass surface.

### 4.3.1 Materials and Methods

To determine the amount of BAM in drinking water, GEUS is using a competitive ELISA assay, with a horse radish peroxides (HRP) labeled BAM antibody produced by Statens Serums Institute. [21] The ELISA experiments were mainly done on GEUS as the plate scanner and plate washing station was of better quality compared to that available at DTU. The exact procedure for the ELISA method is described in the protocol section of the appendix.

**BAM ELISA principle**

The BAM bound to the surface of the well is bound using a photo linker developed by Exiqon A/S (Anthraquinone AQ-0028). The photolinker covalently bounds a BAM-Ovalbumine-AQ conjugate to a polysorb 96 well ELISA plate using a 30 min UV activation step. The BAM antibody is mixed with the water sample and is added to the well. The BAM antibody reacts with either free BAM from the water sample, or BAM bound to the ELISA plate. As the BAM antibody is Horse Radish Peroxidase (HRP) marked, the bound HRP converts a TMB substrate into a color substrate. The concentration of the color substrate is measured using a spectrophotometer. The concentration of the converted TMB substrate is proportional to the bound BAM antibody. If BAM is present in the water sample this will result in less bound BAM antibody, thus yielding a lower concentration of converted colored TMB substrate.

**BAM-OA compared to BAM-OA-AQ**

For the attachment of BAM to a glass or gold surface, a new batch of BAM ovalbumine (BAM-OA) was produced. The BAM-OA was compared to the existing BAM-OA-AQ by comparing the BAM antibody adhesion to the surface.

Plates coated with 0,075 mg/ml BAM-OA were passively coated overnight, whereas the plates with 0,05 µg/ml BAM-OA-AQ were coated using active photochemistry by ½ hour UV light exposure, activating the Anthraquinone linker to the polymer surface. Using the standard protocol, a dilution series of the BAM conjugate was added to the plates. The standard GEUS protocol for BAM water sample detection was followed. The standard used an 8 step dilution with 5x dilution between each step. The standards and control samples were added in repetitions of 4 on the plate.

**Linear range using BAM-OA and BAM standard**

The linear range of a plate using a BAM-OA passive coating was examined. A dilution series of BAM-OA were added to the plate and replicated on 2 plates. A 10 fold dilution step of 0,75 mg /ml BAM-OA was used in the first step, and a 2 fold dilution series with a total of 11 dilutions steps from 0.075 to 0.00007 mg/ml were made and compared with the BAM standard.

### 4.3.2 Results and Discussion

**Passive bound BAM-OA compared to active photo-linked bound BAM-OA-AQ**

2 plates of the passive coating of BAM-OA (plate no. 19b+ 20b) were compared with 2 plates of the UV activated coating of BAM-OA-AQ (plate no. 19a+ 20a) to 96 well polysorb plates.

The control samples from the passively absorbed BAM-OA showed a significantly higher standard deviation compared to the actively coated BAM-OA-AQ. The average increase in absorbance was 2.7 when using BAM-OA compared to the BAM-OA-AQ. This means more BAM antibody was bound to the surface, yielding a higher conversion of the TMB substrate.

Unfortunately, the concentration of BAM-OA may be too high, as the absorbance value goes beyond the linear range of 2 (optimal is an absorbance of 1 to 2)

The concentration of BAM-OA is: 35.3 µmol BAM (EQ0031) added to 1.5 ml DMSO, and 5 µl DMSO mix added to 1 ml of 1 mg/ml ovalbumine, making the BAM-OA ~ 0.12 mM. As the BAM-OA product was dialyzed to remove un-reacted small size chemicals, and not all BAM can be expected to react with ovalbumine, the actual concentration is not known and is probably lower. The concentration of BAM-OA-AQ is ~ 0.0006 mM as the third production step dilutes the concentration further by 200. (see the synthesis protocol in appendix).

Below is seen the standard curves from 4 plates where the intensity of the wells are plotted as a function of the BAM standard dilution series.

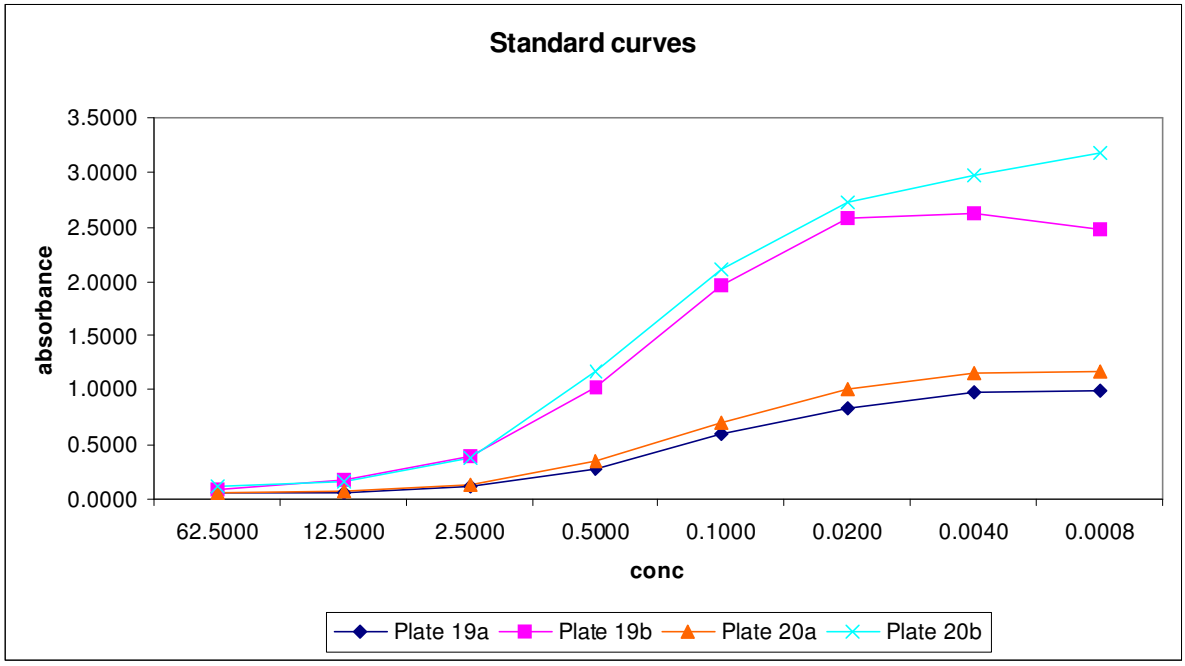



**Figure 4-2: Comparison of BAM-OA (plate 19b and 20b) potency to BAM-OA-AQ (plate 19a and 20a). Absorbance as a function of concentration of free BAM added to the plate.**

**Linear range using BAM-OA and BAM standard**

The results show good uniformity in the data on both plates, with a reasonable dose dependency of the concentration of added BAM, and the concentration of BAM-OA on the plate. Below is seen the 2D plot showing the absorbance intensity as a function of BAM standards (X) and the 11 curves from the dilutions of BAM-OA coating. The results are average values from 2 plates.

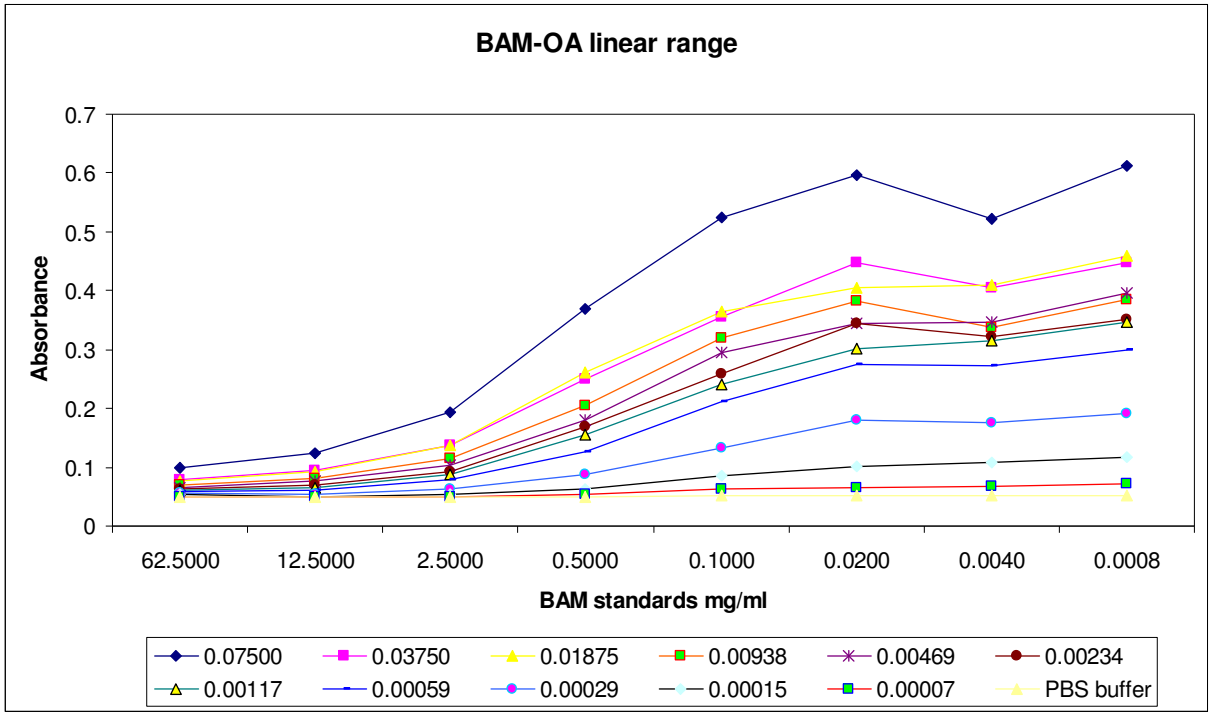



**Figure 4-3: BAM-OA linear range as a factor of BAM standard solutions**

The absorbance increases as the concentration of BAM standard decreases, meaning that more anti-BAM antibody is bound, thus converting more TMB substrate to a color reaction.

The optimal linear range of BAM-OA is probably slightly above the 0,075 mg/ml dilution (blue line), as an absorbance between 1 and 2 is optimal. The low BAM standard value of 0.004 mg/ml show a dip in absorbance, this could be due to variations in the low steps of the dilution series. Although ELISA uses a polymer surface attachment, and a cantilever uses a gold or $SiO_2$ surface, ELISA proved to be a reliable and straightforward tool to test the strength of new chemistry with the known standards.

## *4.4 Microarrays as a surface chemistry tool*

The goal of using microarrays was to investigate on the optimal surface pre-treatment and minimize unspecific binding by the use of blocking agents in the BAM assay. As one slide can contain several thousand spots, microarray technique is a statistically powerful tool to optimize chemical conditions. A large number of parameters can be evaluated using dilution series of BAM spotted on the glass surface. The glass slides were then allowed to react with fluorescently marked BAM antibody, the BAM antibody in excess is washed off, and the slide is analyzed in a fluorescent scanner. The analyte (in this case BAM) can also be spotted on several glass slides where the effect of different treatments on the BAM assay can be analyzed. However, the quality of the technology depends on how well the spots and hybridization steps are performed. An emphasis was therefore made on learning and understanding the different parameters in microarray spotting and analysis. Three different methods with increasing difficulty were used for this purpose.

### 4.4.1 Materials and Methods

The microarray experiments were divided in to a 3 stage process in order to learn the handling and spotting method. BAM was attached using only the BAM ovalbumine (purified egg white) conjugate. The adhesion was tested using the microarrays, and was an alternative for the photo linker Anthraquinone AQ-0028 conjugation, as this was only possible on polymer surfaces.

**Biotin-Cy3streptavidin hand spotting**

The main purpose was to learn the basic reaction chemistry involved in microarray experiments. A 5 step 2 fold dilution series of biotin in 2 equal columns (from 100µg/ml to 6,25 µg/ml) was hand spotted on a TsT and TsT-PEG-TsT coated glass slide. The slides were then added Cy3-streptavidin and coated to prevent unspecific binding and scanned in a microarray slide scanner.

**Biotin-Cy3 streptavidin microarray**

In the second round the microarray spotter machine was used; the main purpose was to learn the basics of microarray spotting and the general microarray experiment. A 4 step 2 fold dilution series of biotin was repeated in 5 rows, and again repeated in 40 blocks (2000 spots total) and spotted on a TsT and TsT-PEG-TsT coated glass slide. The slides were then added Cy3- streptavidin mix and coated to prevent unspecific binding and scanned in a microarray slide scanner.

**BAM antibody microarray**

Here the BAM assay was tested properly against the Cy3 marked anti-BAM. A set of 10 dilution steps (columns) of BAM-ovalbumine in a 2 fold dilution series (from 0.75mg/ml to 0.0007 mg/ml) was repeated in 5 rows. This group was repeated 20 times in 2 columns, giving a total of 2000 spots per slide. The slides were then added Cy3 marked anti- BAM and coated to prevent unspecific binding, and scanned in a microarray slide scanner. The spot intensity of the pictures was then measured.

**Glass slide treatment**

The spotting was done on glass slides with an activated surface, mainly TsT (cyanuric chloride) and Tst-PEG-TsT (cyanuric chloride-Poly ethylene glycol-cyanuric chloride). To avoid unspecific binding later in the BAM assay, the surface was tested with 1% skimmed milk and 1% ovalbumine as blocking agents for 1 hour and 24 hours, respectively. The handling of the spotting, BAM antibody hybridization, and washing step, was done using several protocols. Further information about the assay can be found in the protocol section of the appendix.

**Cy3/Cy5 labelling**

In order to detect the BAM antibody bound to the microarray surface it was fluorescently labeled using Amersham Cy3 mAb labelling kit from GE healthcare (code PA33000) for BAM antibody (Statens Serum Institut Hyp 273-3) in 1 mg/ml. For comparison, a non specific antibody was chosen, a mouse origin Immunoglobulin G mix (IgG). One kit Amersham Cy5 mAb labelling kit from GE healthcare (code PA35001) was used to label 1 mg dry Sigma Mouse IgG antibody, reagent grade (prod no. I5381). The non specific antibody was later used for the BAM assay.

The Cy3 marked anti-BAM had a final concentration after elution of 0.625 mg/ml and a dye/antibody ratio of 20 (by spectroscopy at 280/552 absorbance method). The unspecific mouse IgG had a concentration of 0.67 mg/ml and a dye/antibody ratio of 33. The detailed protocol and calculations can be seen in the protocol section of the appendix.

**Micro spotter**

To deliver the small quantities of BAM-ovalbumine on the glass slides, an automated micro spotter was used. The apparatus consist of the same basic components as the Canti$^{TM}$Spot but is made for a programmable robot delivery of the spots onto a glass slide surface. The micro spotter uses the same pump station and piezo actuated nano tip from GeSim A/S as the Canti$^{TM}$Spot. In addition, the temperature and humidity of the spotting chamber can be set. The spotting is programmed for each round using a designated pc program. The micro spotter allows for the spotting of up to 55 chips. The slides were read out using a microarray reader.

**Slide scanner**

The microarray scanner is a fluorescent confocal microscope with built in filters for Cy3 and Cy5, and scans the surface of the slide moving the glass slide using automated XY motor stages. The reader is controlled by a proprietary program on a pc. To calculate the intensity of the spots, a freeware program ScanAlyze was used. The program calculates the intensity values within a defined grid, set directly upon the picture from the scanner. The output is given as arbitrary values corrected from background intensity, and can only be used as comparison values within the slide.

**Microarray procedure**

The slides were are activated with TsT. Using the micro spotter, an array of 5x10 dilution series were spotted on the slides (20 groups in 2 columns). The slide was incubated 1 hour or overnight. A plastic cover was added to the microarray slide creating a small chamber on the surface; the antibody was then added in the chamber and incubated 1 hour. After a brief wash and drying step the slide was read in the slide scanner. More detailed procedure can be seen in theprotocol section of the appendix.

### 4.4.2 Results and Discussion

Getting a repeated stable spotting was challenging because of the clogging of the spotter tip, which was due to the BAM ovalbumine conjugate that had a high surface adhesion. The result was often misaligned spots. High concentration fluids were more susceptible towards misalignment because of a higher density, thus sticking more to the tip before being released. The problem was eventually solved by keeping the tip submerged in Milli-Q water when not in use. Below can be seen the effect of a misaligned dilution series, on the left picture the intensity drop of the dilution curve can be clearly seen. The misalignment of the spots rendered the intensity scanning difficult, as the intensity measurement of each spot had to be done manually [32].

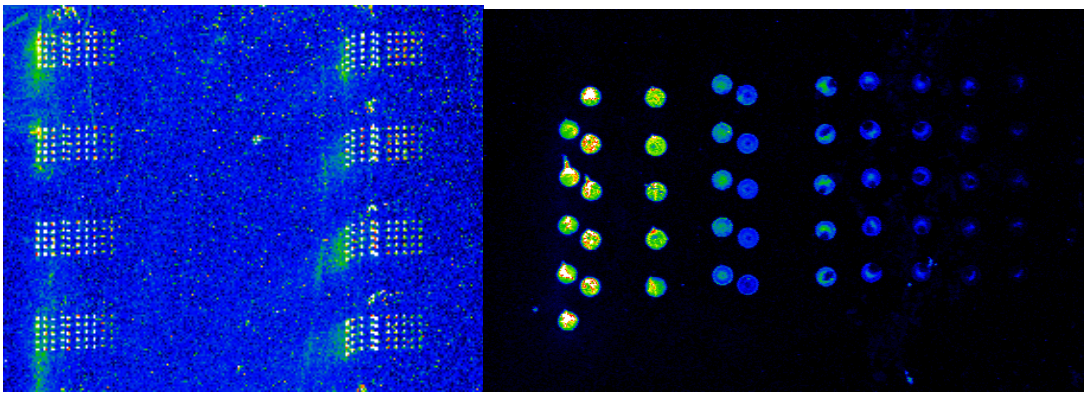


**Figure 4-4: Microarray series. Left: Over exposed slide picture, showing uniform groups and background signal. Right: Close-up of a group on slide 9 showing the decreasing signal intensity but poor alignment of spots**

The use of ovalbumine on glass surface proved very effective and sturdy for the attachment of BAM, the slides had little loss of signal after washing. The scanner intensity had to be kept on the same level for the comparison of slides, a laser intensity of 70% and PMT power of 70% was found adequate, as it produced clear spots with little background. The right picture above shows an in-homogenous drop structure cause by the drying process of the drop. This effect was seen on all slides.

**Blocking results**

The immediate blocking after spotting procedure in 1 hour, 1% ovalbumine was tested on 4 untreated glass slides, but revealed no fluorescent signal (data not shown). This is probably because the BAM ovalbumine layer was washed off before it had a chance to attach to the glass slide. 10 slides testing different blocking techniques were made. 2 slides used no blocking (slide 23+24), 2 slides used one hour 1% ovalbumine blocking (slide 19 +20), 2 slides used 24 hour 1% ovalbumine blocking (slide 15+16), 2 slides used 1 hour 1% skimmed milk powder (slide 22+21), and 2 slides used 24 hour 1% skimmed milk powder (slide 17+18). Below is seen the results of the blocking experiments.

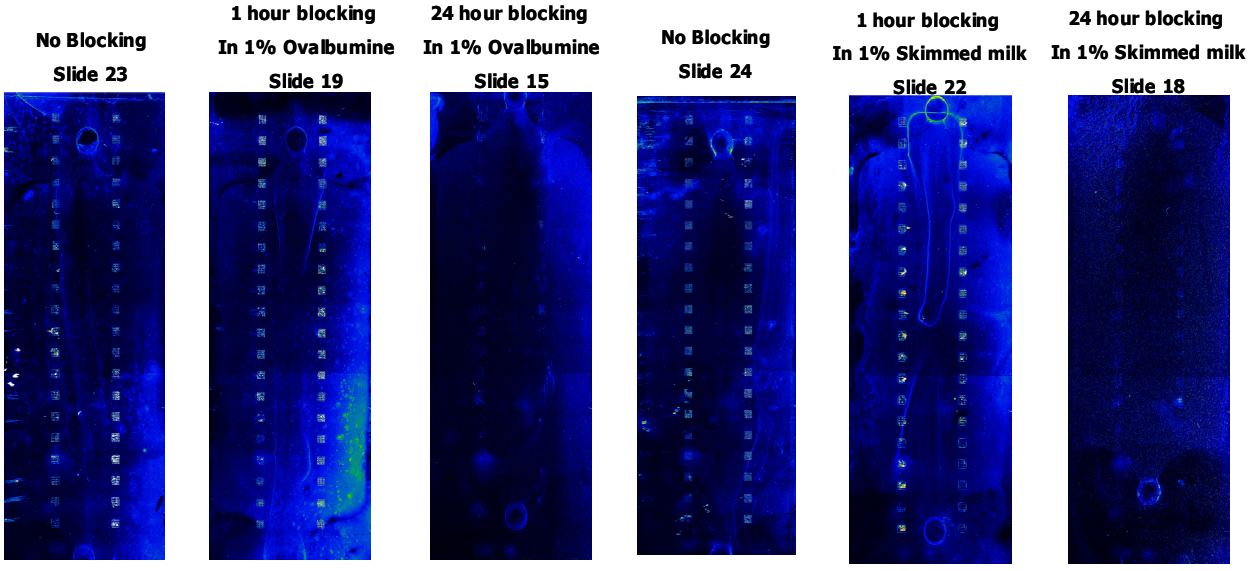



**Figure 4-5 : Testing of blocking agents on spotted slides. The blocking of skimmed milk or 1 % ovalbumine did not have a significant effect on the unspecific binding. The 24 hour blocking coated the whole surface and stopped the subsequent hybridization of cy3 marked anti-BAM.**

The 24 hour ovalbumine blocked slides had a lower signal compared to un-blocked slides as the BAM dots were covered with ovalbumine, thus also blocking the antibody adhesion. The 1% skimmed milk seemed to increase background signal slightly. No significant advantage of using ovalbumine or skimmed milk was found in the microarrays. The use of microarray provided vital knowledge about the BAM-OA hapten and the chemistry in the BAM assay. Furthermore, as the same spotting tip was used in microarrays and BAM assay, the microarray technique helped obtaining a better quality of functionalization on the BAM assay.

## 4.5 Break apart chip tool for surface regeneration experiments

This section describes the development of a flow cell for a single use "break-apart" chip device, and the experiments done using the flow cell.

In a working device, the surface of the cantilevers needs necessarily to be regenerated after each experiment to avoid single use of the chip, as this would increase the cost of the system considerably. The surface could be regenerated in between each experiment in the field using an automated pumping fluidic system. It was therefore important to investigate on the possibility of removing and re-attaching the antibodies on the BAM-ovalbumine conjugate. As the assay was done using continuous flow, and as the actual reaction time window was 5 min. (at 20µl/min.) for the BAM antibody to react with the BAM on the surface, a system that mimicked the chemistry involved in the Cantion BAM assay was needed. The system should be able to monitor the BAM assay reaction in real-time, as this may reveal the speed of reaction and changes in surface chemistry.

A device developed by Mogens Havsteen Jakobsen and Anders Greve in summer 2008, was found to be useful as a platform. The "Break-apart" device was initially constructed for the investigation of crystal structure of hemoglobin in malaria investigated cells. The device consists of a series of 2x40 small (125 x 25 mm) chips that can be broken off from a slide with a normal microscope slide format. The slide can be placed in the holder for microarrays, and be printed as standard microarray slides. The unit cell also fits into a PCR tube. One wafer contains 2 sets of microscope sized slides, containing 2x40 chips each. The chips are produced using cleanroom processes in the Dan Chip Laboratory at DTU. The process is a relatively simple 11 step process where a 2 step photolithography process defines the structure of the "break apart" chip; the chip is then etched using plasma etching and KOH etching techniques. The wafer can be gold coated after the last etching step, thus obtaining the same surface as a gold coated cantilever. The whole process can be done in a day and is relatively inexpensive. A new design has also added numbering to each "break-apart" chip, making it a versatile tool for analyzing chemistry or biological material, that can be micro spotted on the chip surface.

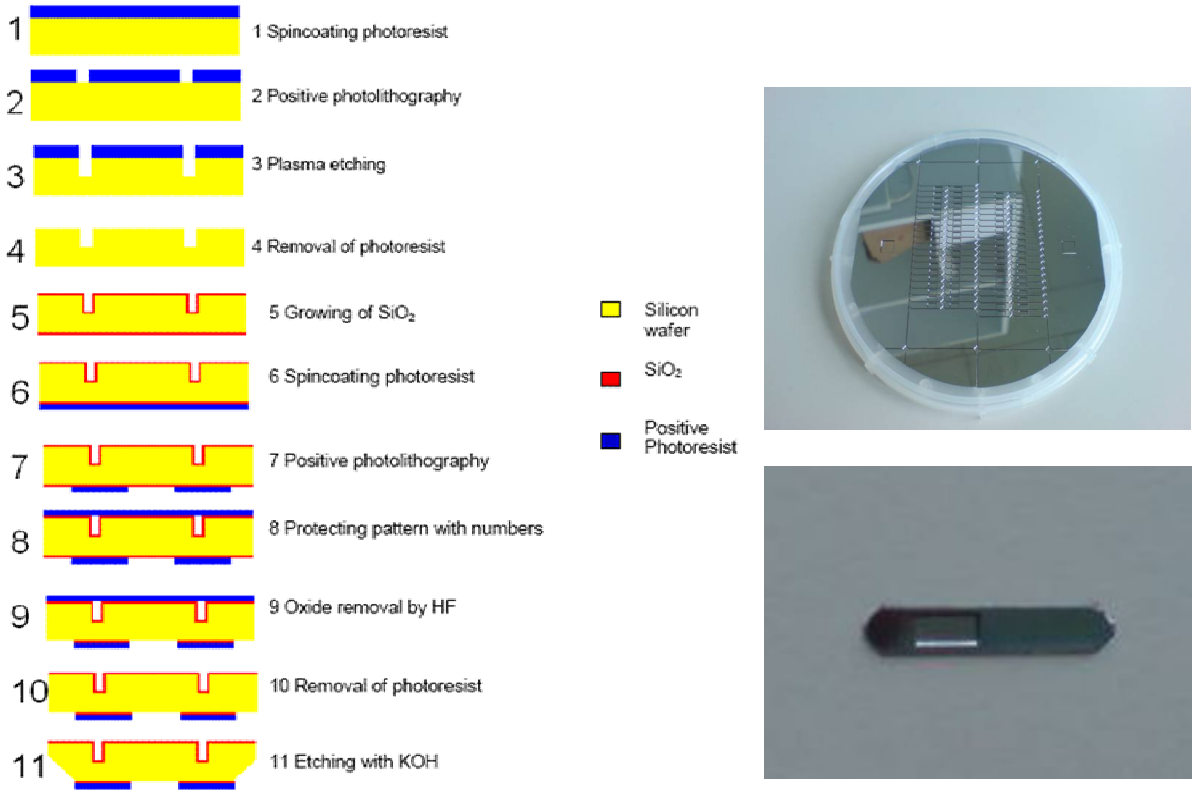



**Figure 4-6: Basic production process of the "break-apart" chip device. (Courtesy of Anders Greve). Bottom right picture shows a single break-apart unit.**

Above is seen the outline of the production process, the wafer and single unit of the "break-apart" chip. As the "break-apart system" can be broken into single units after printing, the system can be used to examine different treatment effects on the chemistry of the chip surface.

In order to mimic the flow conditions in the BAM assay, a designated flow cell with a transparent lid was constructed for this "break apart" system. The flow cell had the following set of criteria

- Must contain and clamp in place one unit of "Break-apart" chip.
- The chip in the flow cell should be able to be exchanged easily.
- Lid must be transparent to see fluorescent surface coatings under a microscope.
- Has to have a flow in and outlet, with tubes that can be exchanged and connected without the use of silicon grease or other glues.
- Reduced internal volume not exceeding 100 µl.
- A simple design that can be produced and altered relatively easy.

Three different flow cell designs were constructed from PMMA polymer plates and tested. The best design consisted of 3 parts, a bottom holder for the chip, a PDMS sealing and chamber middle part, and a top part with built in screw fittings for the tubing's.

### 4.5.1 Designing the flow cell device

The chip design idea was first drawn up using PowerPoint. The chip consists of layers of structure screwed together with 4 screws and bolts. Each layer was then drawn in 2D using AutoCAD 2000i. The dimension and structures of the device was defined in the AutoCAD 2000i program using mm coordinates. The CAD drawing was then imported and converted to a micro milling program (EZ-Mill express). Here each operation was defined using different mill sizes and actions for the milling machine. The middle reaction chamber was defined by just a line in the CAD and micro milling program, as the width of the chamber was then defined by the diameter of the mill used. Below can be seen drawings of the parts of the flow cell.

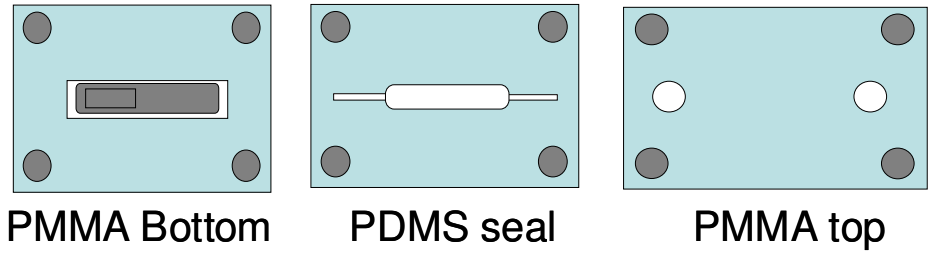



**Figure 4-7: Flow cell parts**

### 4.5.2 Micro milling the flow cell device

The different parts were fabricated on a micro milling machine using PMMA plastic plates in different widths depending on which structure was made. Each milling operation was defined (avoiding unnecessary mill head changes) and the mill speed settings set to a speed low enough to avoid overheating of the PMMA material, as it would otherwise gum up the drill and destroy the device. The final program code was then exported as a simple text file, to a third micro milling machine operation program. A printout of the different working steps was needed when operating the micro milling machine, in order to place the right mill head sizes in the right sequence, and to set the correct mill speed.

The micro mill consists of a table that can move in the x,y,z directions. The mill head was fixed in place and its speed was controlled by a separate control box. The milling machine was operated via a PC with a milling program (Mach2 CNC control program). Here the generated text file from the EZ-Mill express was imported. The PMMA plate was fixed on the table using double sticking tape and the mill head was then loaded in the machine socket. The mill head was lowered until touching the PMMA plate. This defined the zero point of the Z-axis in the mill program. The corner of the device was then chosen and was defined by the zero point of the X and Y axis. After this the milling was started, pouring water on the contact point between the mill head and substrate to avoid overheating of the PMMA plastic. Each time the mill head was changed, the Z zero point was defined again (keeping the X and Y point fixed). The PMMA plate was removed and the device cleaned, sanding off rugged edges and removing PMMA shreds, the device was then cleaned lightly in ethanol.

### 4.5.3 Making a PDMS seal

The seal was made from a 2 component fluid PDMS. The PDMS was added a hardening catalyst in the ratio 10:1. In order to make the seal, a negative stamp needed to be made from a PMMA plate. The negative was therefore designed as a normal device, only without cutting the device off the PMMA plate. A lid for the negative was also fabricated (a simple PMMA plate with 4 holes). The PDMS mixture was made and then placed in a vacuum chamber for 30 min to remove unwanted air bubbles from the mixing. The mixture was then poured in the mould and the lid screwed in place avoiding the formation of air bubbles, alternatively the mixture could be squeezed in via a syringe in the mould. The PDMS was allowed to cure overnight. The cured PDMS membrane/seal was then removed from the form, and edges were cut away using a surgical scalpel. PDMS is reported not to be polluting in liquid, but pollutes silicon surfaces in air, probably due to evaporation of fumes left over from the curing.

### 4.5.4 Making threads for tubing in/outlet

The in/outlet for the fluidics was milled by a 1 mm diameter groove. The hole/groove was enlarged to 2 mm for approx. 3-4mm length by turning a special cutter tool (combination boss tool). The thread was then cut in by turning a screw with the dimensions of the ferrules used. Finally the end of the groove was cut in a V- Shape in order to ensure the ferrules were closing

tightly when screwed in place. The tubes, screws and ferrules were provided by Upchurch Scientific.

## 4.6 Break apart Flow cell experiments

The main goal of the sensor is to autonomously perform successfully for at least 3 months. If one daily measurement is required and at least 3 measurements are required to perform one sample measurement (blank buffer + BAM standard + BAM sample), the total amount of unattended cycles is around 90. This means stripping the BAM antibody from the BAM-OA surface on the sensor and re-applying BAM antibody 90 times without decreasing signal intensity significantly.

A confidential DHI report produced at Cranfield University [24] showed that all chemicals used in the BAM ELISA are stable at least one month. The surface quality of BAM-OA-AQ deteriorates significantly during the regeneration of the surface after 4 stripping and re-attaching rounds of BAM antibody, using 50 mM Glycine HCl stripping buffer with pH 1.9. However the chemistry has not been optimized for regeneration. It is therefore interesting to investigate the quality of the regeneration and surface of a BAM-OA or BAM-OA-AQ coated surface during flow conditions. The Flow cell in use under the fluorescent microscope can be seen below.

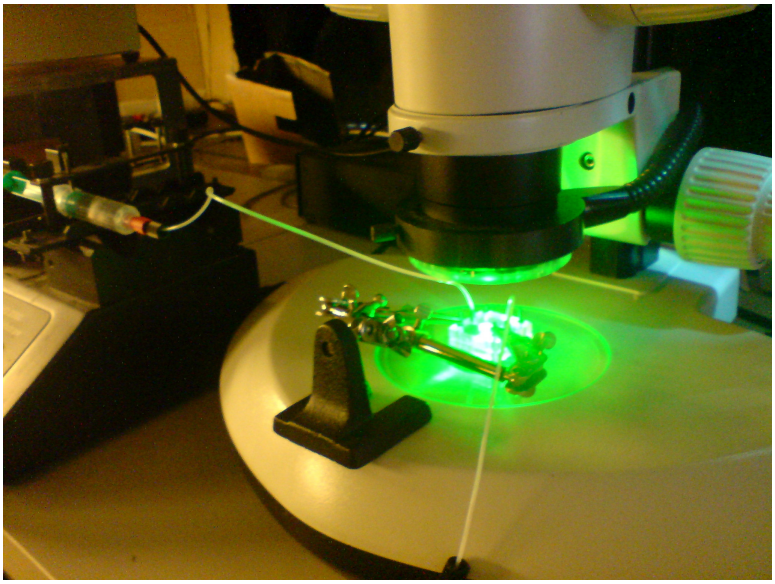


**Figure 4-8: Flow cell in use under Cy3 fluorescent light.**

### 4.6.1 Materials and Methods

Pictures were taken at 0, 15, 30 min. and 1 hour. The following procedure was used:

**Attachment of BAM antibody**

- A "break apart chip" slide was spotted with BAM-OA using the micro spotter apparatus, and left overnight in NaCl humidity chamber.
- The chip was dried and placed in the Flow cell chamber.
- 10 µl of a 100x dilution of Cy3 marked BAM antibody solution was placed directly on the surface of the chip, coating the whole chip surface. This was done to save on the amount of Cy3 anti BAM used.

**Stripping of BAM antibody**

- The flow cell was closed tightly and connected to a syringe pump and filled up with 0,1 M PBS pH 7,4 buffer solution.
- Chamber was flushed through with running buffer at 200 µl/min for up to 30 min.
- Stripping buffer was added using 20µl/min. flow.
- Pictures were taken at 0 min, 5 min, 15 min and 30 min under UV light and no other light sources. A 5x magnification was used, 1 sec. exposure and saturation level of 200. The light source was fluorescent light (with 650 light hours on a 100 light hour lamp) and a Cy3 filter.

### 4.6.2 Results and Discussion

The results from the flow cells consisted of fluorescent pictures of the surface. In order to compare different experiments the same equipment and intensity had to be used. This proved difficult, as the UV lamp quality was not consistent.

**Attachment of BAM antibody**

The flow cell showed a relatively fast Cy3 anti-BAM signal, showing the adhesion of BAM antibody to the BAM-OA on the chip surface. Spots could be seen after 5 min stop flow hybridization time. Below are seen pictures of 0, 15 and 30 min. reaction time with the flow cell.

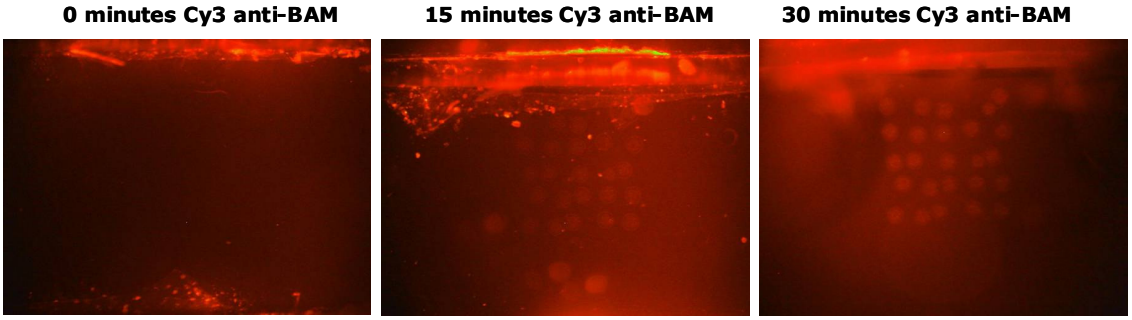



**Figure 4-9: The gradual attachment of the Cy3 marked Anti-BAM antibody to the spotted BAM-OA spots on a SiO2 break-apart chip.**

**Stripping of BAM antibody**

The report from DHI/Cranfield university showed that the addition of 50 mM Glycine HCl pH 1.9 to the BAM antibody would release the antibody, and leave the BAM layer relatively intact (within 4 rounds of stripping and re-attachment). This method was replicated on the flow cell by the continuous flow of 20µl/min 50 mM Glycine HCl stripping buffer. However, up to 24 hours continuous flow with this stripping buffer, revealed no loss of signal strength of the attached Cy3 anti BAM. The experiment was repeated 3 times, and repeated with colleague Basil Uthuppu one year later with the same negative outcome. The signal pictures of Cy3 from 3 different chips are shown on the next page. Pictures were taken using 1 sec exposure, saturation of 200, and 5X zoom.

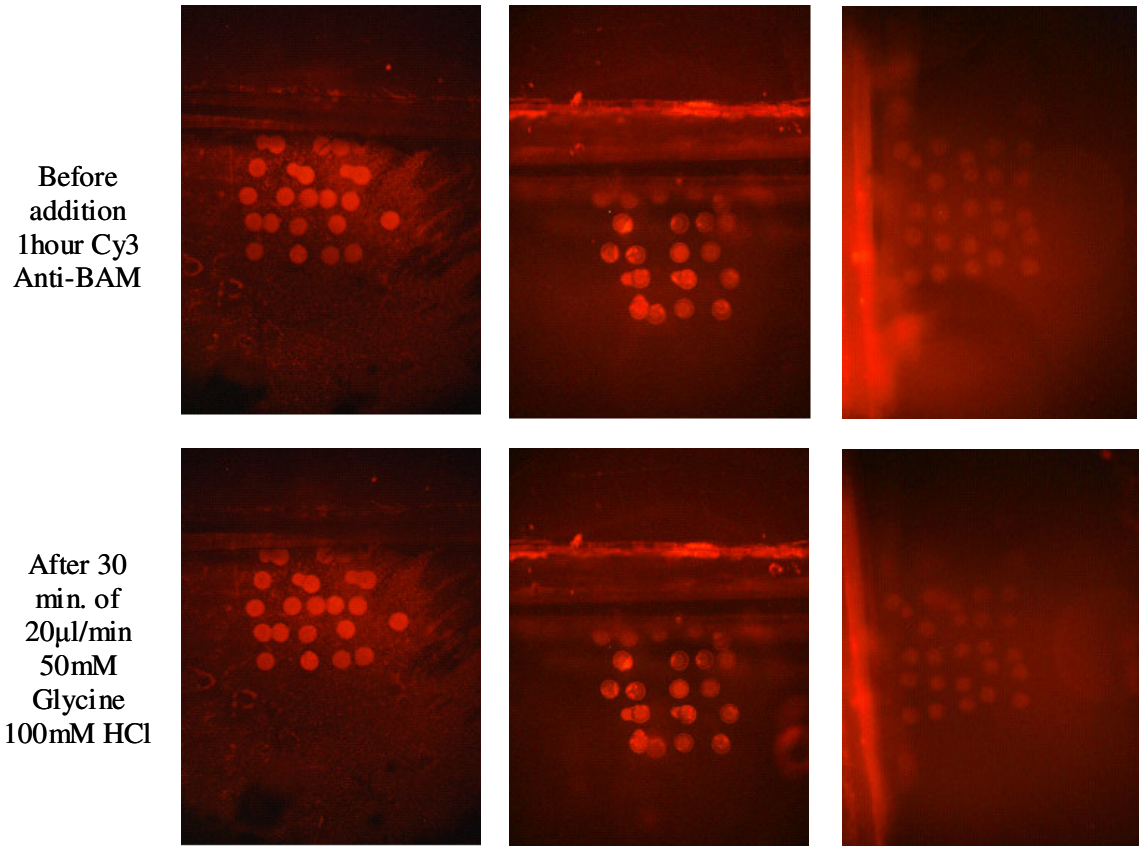



**Figure 4-10: Testing the removal of BAM antibody using 50 mM Glycine and 100mM HCl.**

Basil Uthuppu from surface engineering group at DTU Nanotech, showed that the stripping method did remove the antibodies when using the ELISA method, as described in the report from DHI/Cranfield University [24]. It is not understood why the BAM antibody was not removed using the flow cell method. The DHI/cranfield university report uses the ELISA method, where the ability of the HRP enzyme to convert the TMB substrate into a color dye, is used to record the presence of the antibody, whereas the flow cell uses a direct fluorochrome to see the presence of the antibody.

It is speculated that the 50 mM Glycine HCl buffer may denature the BAM antibody, still leaving Cy3 fluorochrome on the BAM ovalbumine spot surface. Alternatively the stripping buffer is not strong enough to unfold the antibody, due to the high binding affinity of the antibody to the BAM-OA. A third explanation could be that the stripping buffer in the ELISA method may render the HRP enzyme of the BAM antibody incapable of converting the TMB substrate, thus leading to “no signal” misinterpretation.

As the 50 mM Glycine HCl buffer did not remove the bound BAM antibody, alternative stripping buffers were tested. Western blotting techniques re-use radioactive probes by washing the membrane with a stripping buffer. Finding the right stripping buffer is often done by empirical knowledge, as a too harsh buffer may denature the proteins in the assay, and a too weak stripping buffer may not release the probes. The binding of antibodies to antigens is based on a multitude of non covalent bonds, as antigen-antibody bonds can vary greatly, so can the composition of a suited stripping buffer. In general there are acidic, alkaline and high salt concentration buffers. The removal of BAM antibody was done using 8M guanidine HCl, 100mM NaOH and 4M Urea. Although no signal was acquired after the repeated addition of Cy3 anti BAM, as the BAM ovalbumine conjugate surface was also removed in the process. The pictures below of NaOH stripping buffer addition, showed that the background also did fade gradually, meaning that some unspecific binding of Cy3 anti BAM was taking place.

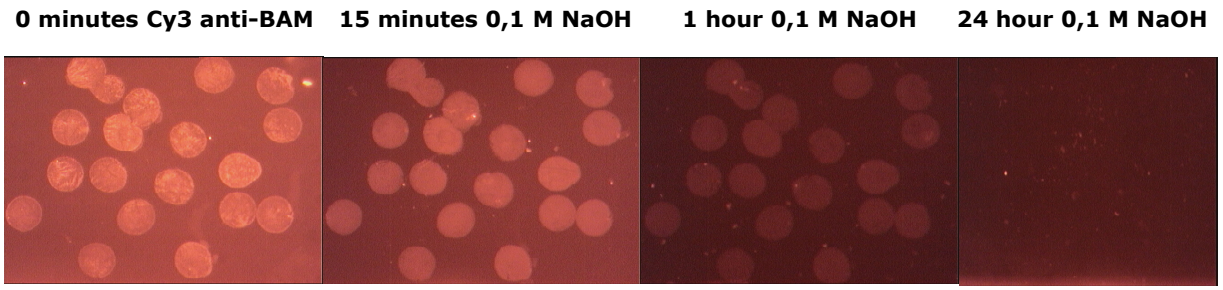



**Figure 4-11: Removal of Cy3 AntiBAM after 15 min , 1 hour and 24 hour washing with 0,1 M NaOH at 20 ml/min.**

Obtaining comparable pictures of good quality from the flow cell, proved challenging as the micro spotting did not align the arrays properly, and the fluorescent lamp used, did not provide light of constant intensity. Apart from occasional drop leaking from the tube ferrules, the flow cell operated well. The repeated opening and closing of the flow cell did over time break the PDMS sealant layer, and it had to be replaced.

# 5 Cantion Canti4 BAM assay

The Canti4 BAM assay was used to detect the BAM antibody binding, not the competitive BAM assay reaction. Based on the experiments done using the DNA assay, the characterization experiments and BAM ELISA, a tentative BAM assay was initiated on the Cantion system. The BAM assay experiments were conducted in 2 rounds of experiments. In the first round the goal was to achieve a signal result from the addition of BAM antibody and prove that BAM antibody had attached to the cantilever surface by using fluorescent pictures of the Cy3 signal. The goal of the second round was to get repeated positive signals on the BAM assay using a larger number of Canti4 chips (20 pieces). In order to investigate the outcome of the experiments the mass/stiffness ratio and bending of each cantilever were additionally investigated in some of the experiments.

## *5.1 Canti4 BAM assay first round*

This section describes the initial experiment on the BAM assay and focuses on the reasons behind the decision of the used settings in the experiments.

### 5.1.1 Materials and Methods first round

A tentative BAM assay procedure was formulated where the main objective was to eliminate unwanted artifacts from the fluidic system and minimize the electronic noise. To avoid build up of gas or crystals in the fluidic system, the syringe pump was set to pump 3-5 µl/min of 1xPBST buffer through the system with a dummy chip inserted. This also avoided long settling time due to a salt or temperature gradient in the fluidic system.

**Cantilever functionalization**

It was decided to coat the middle cantilevers B and C with 0.75 mg/ml BAM-OA and keep the "outer" cantilevers A and D coated with 1 mg/ml ovalbumine. This was done to avoid the eventual increased disturbance of drift from the outer cantilevers, as cantilever D is the first cantilever in the flow direction. For comparison, the DNA assay used cantilever A and C as signal and cantilever B and D was kept blank as reference.

**Cantion Cantilever BAM assay experiment**

All cantilever experiments began with a start up period of 30 min. to ensure warm up of the electronic system and minimize chip drift/error. The chip was placed in the flow cell and PBS /PBST was flushed at 20 µl/min in the system. The flow system was switched between sample and buffer to ensure all air bubbles have been flushed out. The experiment began when a steady baseline over 5 min had settled. Each run was recorded for 1000 seconds and filed under initial run, chip no. and date. To ensure a proper foundation for the comparison of the data an experiment was divided into a set of 5 different sub-experiments.

**Stabilization run Step 1**:1500 seconds run with 20 ml/min of 1x PBS 0,05 % Tween 20 buffer. This was to test the characteristics of each Cantion chip, and to ensure the setup and chip was fully functional. The stabilization period had to be stable in order to continue to step 2.

**Loop switch test Step 2**: A switch was done between loop 1 and loop 2 (sample loop) to ensure that the switching itself was not causing any voltage change artifacts. If a signal did appear the step would be repeated until no signal was seen.

**Buffer sample test Step 3**: 100µl of 1x PBS 0,05 % Tween 20 buffer was added as sample in the sample coil. This was to test that no signal arises from adding a sample with equal consistency as the running buffer. If a signal did appear the step would be repeated until no signal was seen.

**Unspecific IgG antibody Step 4**: 100 µl of 0,1 mg/ml of an unspecific Cy5 marked Immunoglobulin G antibody from mouse was added to test the specificity of the assay towards an unspecific monoclonal antibody. This was to investigate whether any antibody caused a signal change. There was some debate on how this would affect the setup, as a very high concentration may un-specifically cover the surface of the cantilevers diminishing the following adhesion of the complementary BAM antibody.

**Specific BAM antibody**: 100 µl of 0,1 mg/ml of the Cy3 marked BAM antibody (HYB-273) was added as sample. This was the test that the BAM assay could be performed using the Cantion Canti4 setup and should give maximum differential signal.

**Fluorescent Cy3 and Cy5 signal pictures**

The Cantion chip was removed from the fluidic chamber after the experiment and briefly rinsed in Milli-Q water to remove PBS salts. The chip was kept dry and later scanned for fluorescent signal of Cy5 and Cy3 fluorochrome in a scanner.

A flow chart guide of the main actions in the BAM assay is seen below.

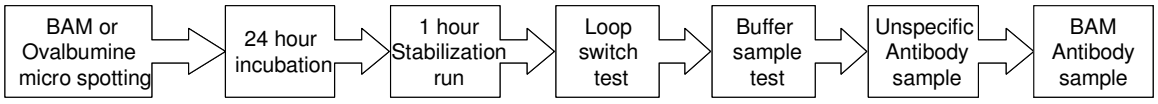



**Figure 5-1: A flowchart of the main actions of the BAM assay on the CantiChip4® system.**

Below is seen a microscope picture of the Cantion chip with the functionalization type of the 4 cantilevers indicated.

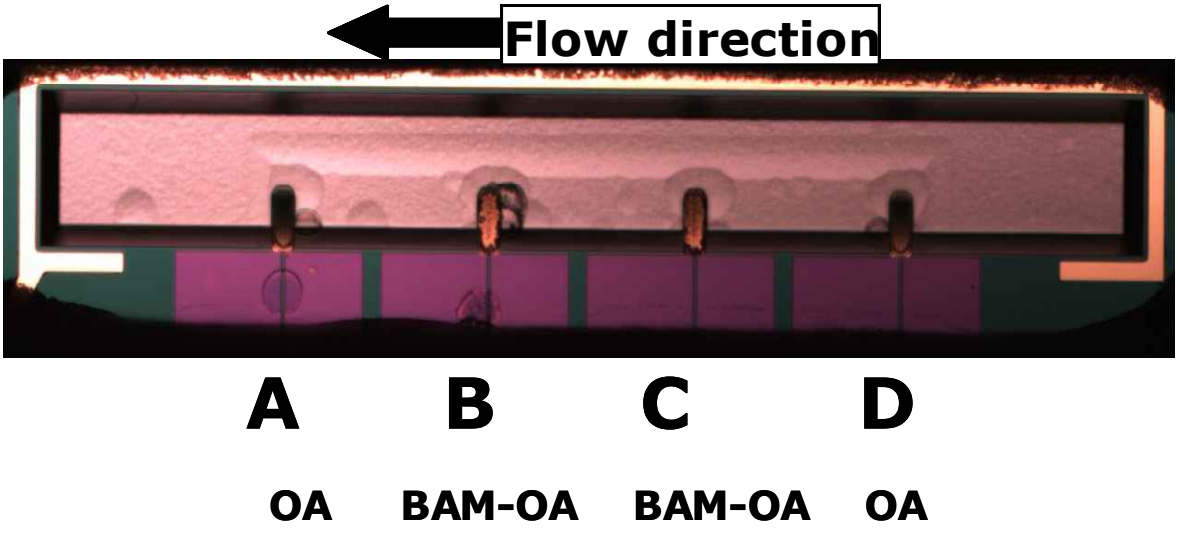



**Figure 5-2: Cantion cantilevers close-up with BAM-OA and ovalbumine spotted on the surface.**

### 5.1.2 Results and Discussion first round of BAM assay

14 chips were spotted with BAM-ovalbumine conjugate and ovalbumine as reference. Of these 14 chips, 5 experiments were completed, 5 had to be aborted due to too high voltage difference between the cantilevers and the built resistor, and 4 had to be aborted due to too high noise or artifacts from the fluidic system. Of the 5 performed experiments, none did achieve a clear signal above the noise level of the system. The fluorescent scan of these chips revealed that chip 22, 51 and 101 had fluorescent Cy3 anti BAM on cantilever B and C and none or low on the reference A and D (see next page). An overview of these experiments can be seen below.

| Chip nr. | Exp date | Exp. outcome | Fluorescent pictures |
|---|---|---|---|
| 10 | 13082009 | Too high voltage | |
| 22 | 12082009 | Performed | positive |
| 50 | 13082009 | Too high voltage | |
| 51 | 14082009 | Performed | positive |
| 52 | 13082009 | Too high voltage | |
| 53 | 14082009 | Performed | unconclusive |
| 54 | 13082009 | Too high voltage | |
| 100 | 21/22-082009 | Buffer and loop switch effect | |
| 101 | 17082009 | Performed | positive |
| 102 | 16082009 | Not stable, Too high noise | |
| 103 | 18082009 | Not stable, Too high noise | |
| 85 | 14082009 | Too high voltage | |
| 87 | 10082009 | Performed | unconclusive |
| 88 | 08082009 | Not stable, Too high noise | |

**Figure 5-3: Overview of results from the first round of Canti4 BAM experiments.**

The most common failure was a too high voltage on the differential program mode, this happened although the voltage difference of the cantilever chips was checked in dry air run, prior to selection for spotting. The addition of moisture to the chip or salt deposits on the contact pads was on some occasions seen to increase the voltage. Also the absolute voltage was seen to vary from day to day on the same chip. This is discussed more in detail under the characterization section.

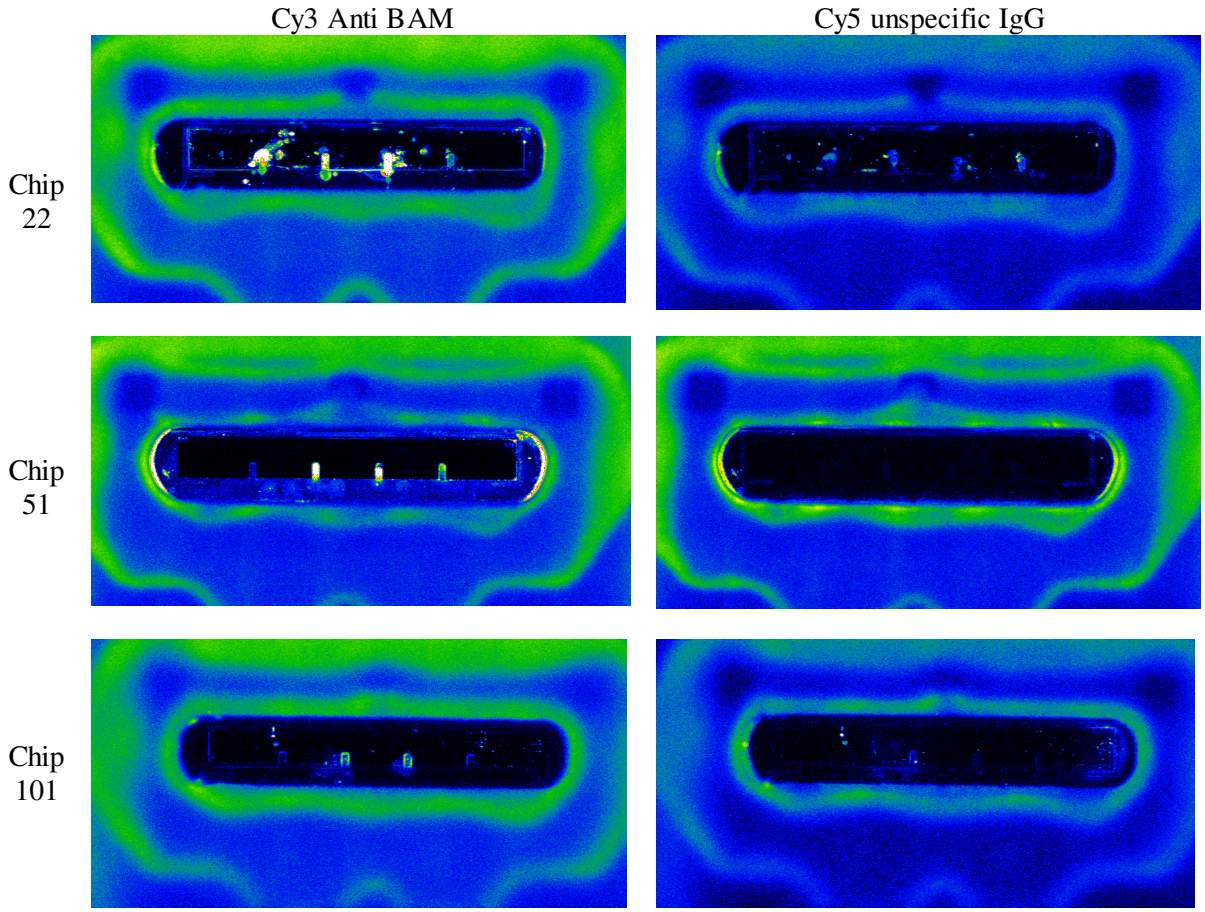



**Figure 5-4: Fluorescent Cy3 and Cy5 images of the cantilevers from chip 22, 51 and 101.**

Cy 3 Anti-BAM fluorescent signal appears from cantilever B and C on Chip 22, Chip 51 and Chip 101. On chip 22, some signal from the unspecific Cy5 was seen, and cantilever A had a clear Cy3 signal, although only ovalbumine was spotted on the surface. Likewise an erroneous weak signal was seen on cantilever D of chip 51. No signal was seen from the unspecific Cy5 IgG mouse antibody on Chip 51 and Chip 101. All pictures were taken with laser intensity at 80% and PMT at 80%. Focus length was taken at variable distance but usually ranging from 1800 to 1750 µm for both Cy3 and Cy5.

The first round of experiments made it possible to establish a protocol for a proper experiment procedure and evaluate when a chip was fit to be used for a BAM assay experiment.

## 5.2 Cantion BAM assay second round

This chapter describes the analysis of the BAM assay results, the fluorescent pictures and the mass/stiffness and bending values of the chip cantilevers. The first round of experiments showed a need for a strict experiment procedure and careful selection of Canti4 chips, if results from the BAM assay were to be compared from chip to chip. The fluorescent signal pictures used in the first round proved very useful in establishing whether the BAM antibody had attached to the surface and was also used in the second round. The acquisition of a new light interferometer and Vibrometer apparatus made it further possible to measure the absolute bending and resonance frequency of each cantilever on the chip. This data was interesting as it provided an idea of the absolute bending of each cantilever and the change in mass/stiffness before and after spotting and after the experiment. These measurements were done on the dry Cantion 4 chips.

### 5.2.1 Materials and Methods second round

The protocol described in the first round of BAM assay was used on all experiments. All chips used were inspected before and after spotting for visible damage using a microscope. A more detailed protocol for the second round of BAM assay can be seen in the protocol section in the appendix.

**Fluorescent Cy3 and Cy5 signal pictures**

In order to verify the binding of antibodies to the cantilever surface and control for unspecific binding a set of fluorescent pictures of Cy5 and Cy3 signal were taken after spotting and hybridization. This time the pictures were taken of laser intensity at 70, 80, 90% and PMT at 70, 80 and 90%.

**Mass/stiffness sensing and Light interferometer**

A light interferometer was used to analyze the absolute bending of the cantilevers on 5 of the experiments. To analyze the mass/stiffness values, a laser based Doppler Vibrometer (Polytech MSA 500) with a piezo actuator was used on 8 experiments. It was hoped that the data could provide an idea of the absolute bending of each cantilever and how the BAM assay functionalization and antibody addition changed the mass/stiffness values of the cantilever. The measurements could only be done on the dry Canti4 chips; this meant that the values of the bending and resonance frequency could not be expected to be compared directly to the data of the Wheatstone bridge voltage differences, as these were acquired in a wet environment during the BAM assay experiment. The bending was measured using the interference between 2 light beams, mapping the whole cantilever profile; the bending was then measured as the deflection from the base of the cantilever to the tip. The mass/stiffness values was measured by actuating the cantilever on a piezo vibrator, the vibration motion of the first order of the cantilever was measured by detecting the shift in the Doppler resonance of a laser beam with a known wavelength, using a light interferometer.

### 5.2.2 Results and Discussion second round of BAM assay

Twenty selected and new cantilever chips were used to carry out the BAM assay experiments. Ten experiments gave a clear signal, and five experiments gave a differential signal above 0,01 mV, with a single maximum signal of 0,02 mV. Seven chips used in the experiments had a chaotic signal or no signal, and three chips were discarded after functionalization, due to too high initial voltage difference between the cantilevers. A signal from the addition of specific BAM antibody, as well as from the addition of unspecific antibody appeared on all 10 experiments. The noise from the BAM experiments had modest amplitude of 1 to 2 µV, and did not interfere as the signals were higher than 5µV. On almost all experiments a signal slope effect was still observed during the experiment. The signal slope effect was not constant over time, and was varying from a few µV to several mV per hour. This is described more in detail in the characterization section. An overview of the outcome of the 10 experiments can be seen below.

| Chip nr. | Exp. Date | Exp outcome | Fluorescent Cy3 pictures |
|---|---|---|---|
| 104 | 31 08 2009 | 5 µV signal | Average signal on B and C, low on A |
| 105 | 01 09 2009 | 5 µV signal | Average signal on B and C, low on A |
| 106 (1) | 02 09 2009 | 12 µV signal | Average signal on B and C, low on A |
| 106 (2) | 07 01 2010 | 2 µV signal | Signal on B and C |
| 112 | 22 09 2009 | 20 µV signal | Strong signal on B and C |
| 113 | 23 09 2009 | 4 µV signal | Strong signal on B and C |
| 114 | 12 10 2009 | 12 µV signal | Average signal on B and C |
| 117 | 21 07 2010 | 20 µV signal | Strong signal on B and C, low on D |
| 118 | 22 07 2010 | 12 µV signal | Average signal on B and C |

**Figure 5-5: An overview of the outcome of the second round of BAM assays Canti4 experiments.**

The signal obtained in each experiment showed a very diverse signal profile, but had a similar signal profile between the specific and unspecific antibody within the same experiment. To test the reproducibility of a chip, Chip no. 106 was re-used using the same functionalization and experiment conditions, but this did not produce the same signal profile.

Below is seen a typical signal profile from chip no. 106, where cantilever B and C deviates from the reference cantilever A and D at t= 400, as the BAM antibody enters the chamber. The differential values from the 2 references A and D (light blue) and the 2 signal cantilevers B and C (orange) is expected to have zero differential value, however a small differential signal often appeared on all BAM experiments.

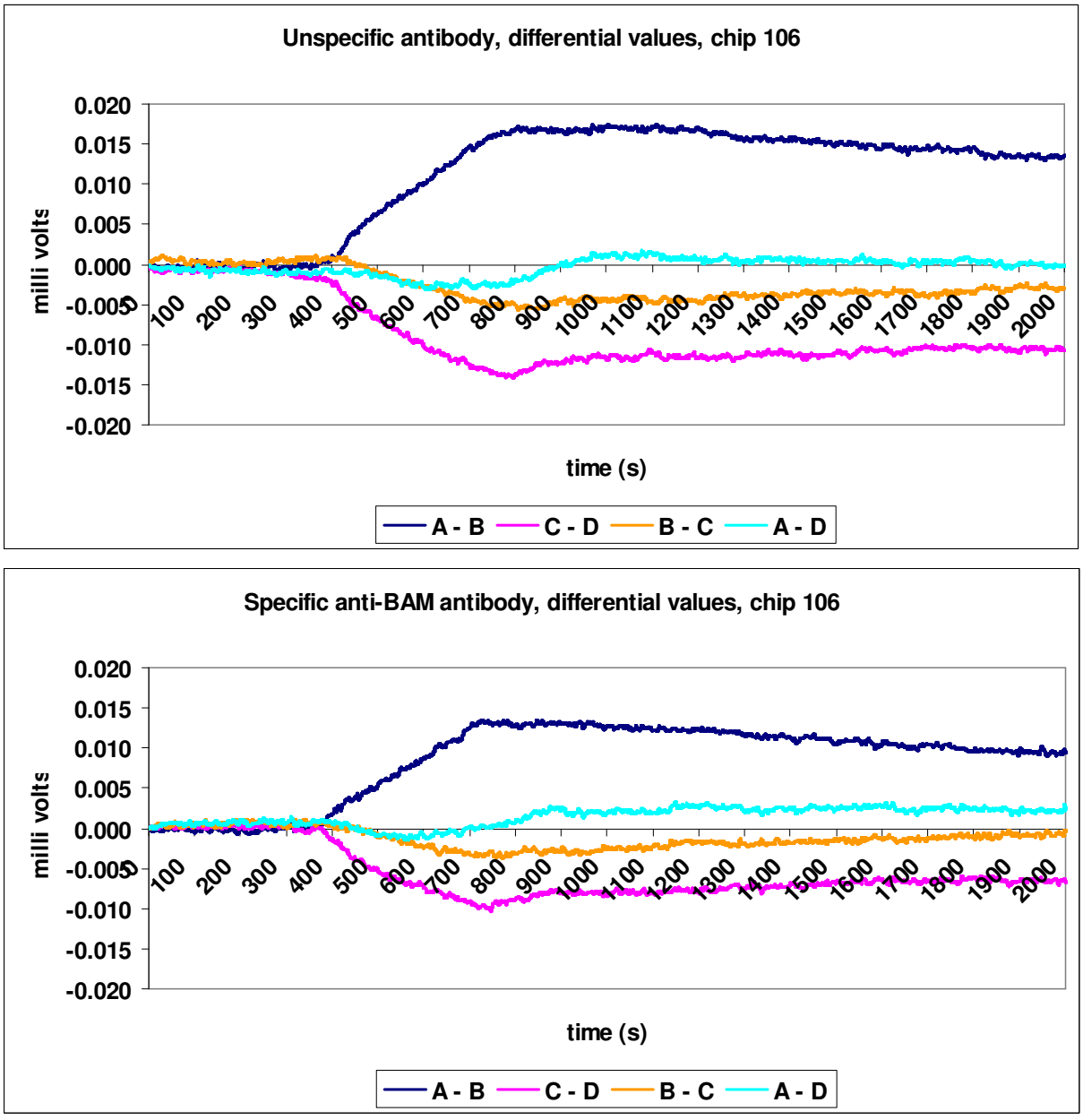



**Figure 5-6: A example of a bending signal from cantilever A,B,C,D from chip 106 under the addition of BAM antibody (Bottom) and the addition of an unspecific IgG mouse antibody (Top). The antibody is added at t =200 and enters the cantilever chamber at t=360 seconds.**

An overview of the differential signal from cantilever (B+C) – (A+D) is seen below; the plot shows the large variation in the response from the addition of unspecific and specific BAM antibody.

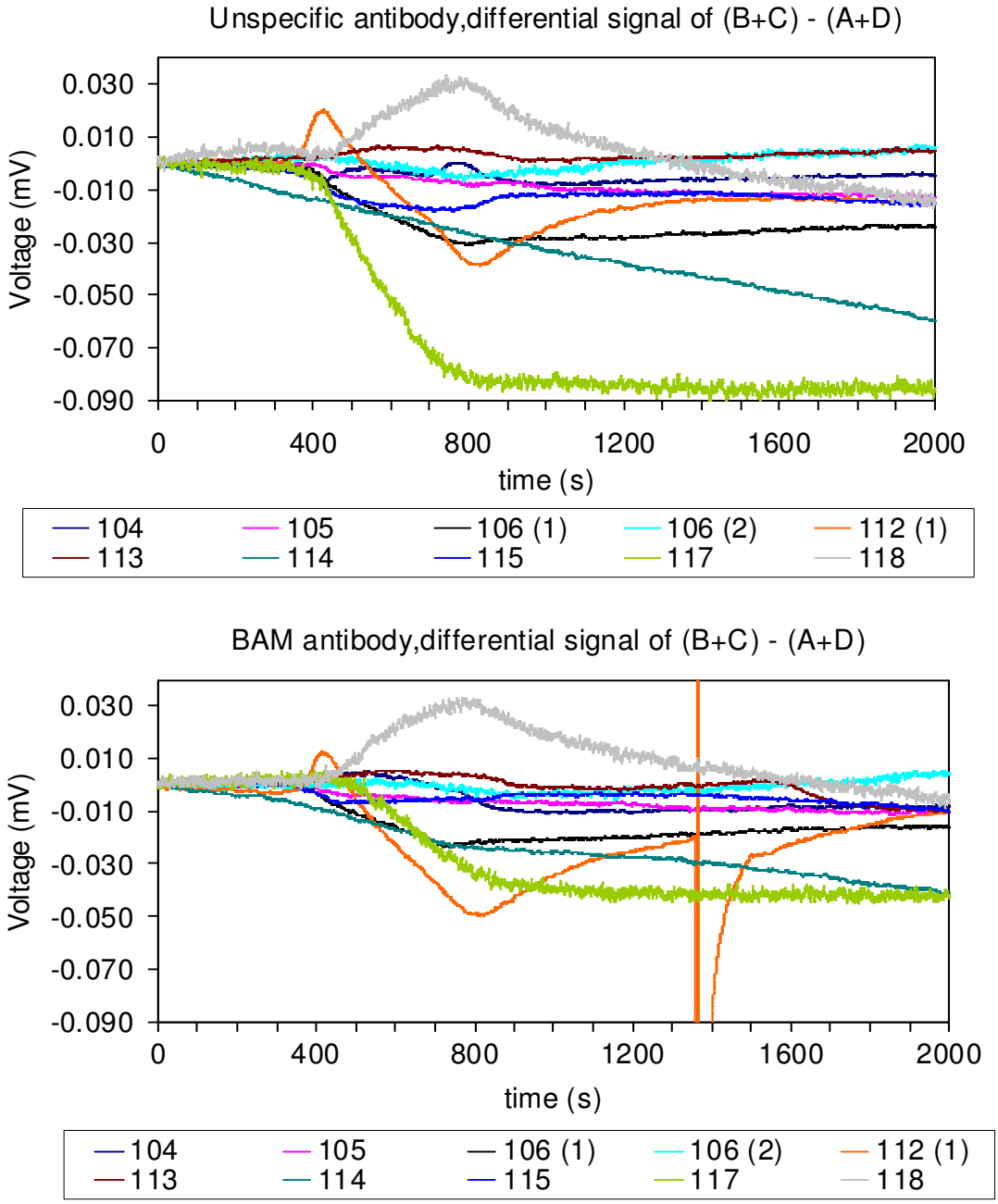



**Figure 5-7: Comparison of bending signals from all 10 experiments showing the differential signal between the 2 active coated (BAM) and 2 reference coated (Ovalbumine) cantilevers. Plotted as signal (mV) of (B+C) – (A+D) as a function of time (sec.) during the addition of BAM antibody (Bottom) and unspecific antibody (Top).**

**Fluorescent Cy3 and Cy5 signal pictures**

All 10 experiments had a similar Cy3 signal from BAM antibody on cantilever B and C and little or no signal on cantilever A and D. No significant amount of Cy5 marked unspecific antibody was found on any cantilever after the experiment. Below are seen fluorescent signal pictures from chip no. 106, 112 and 113, showing Cy3 signal from cantilever B and C on all 3 chips. Chip 112 had some unspecific signal on the whole chamber.

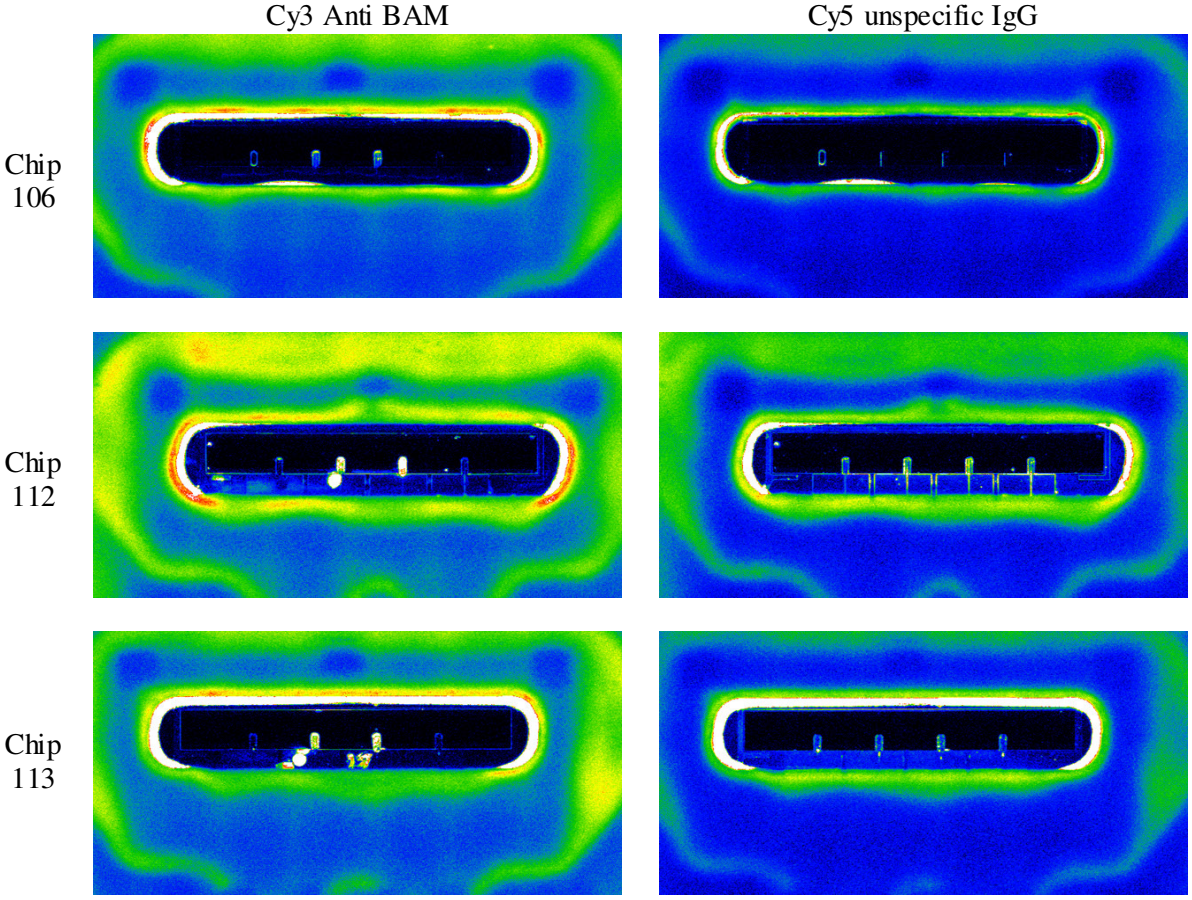



**Figure 5-8: Fluorescent pictures of Cy3 BAM antibody signal and Cy5 unspecific IgG signal from chip 106, 112, and 113.**

Chips no. 104, 105, 106, 112, 113 were used to test the stripping using 0,1 M Glycine and 0,1 M HCl buffer. All 5 dry cantilever chips were immersed in the strip buffer in 50 ml plastic tube and left 1 h on a shaking table followed by overnight incubation without shaking (approx 14 h) The chips were rinsed 5 min. in Milli-Q water and scanned at 70 PMT and 70 laser intensity at optimum distance (1750-1900 µm depending on loading of each chip). All 5 chips showed no loss of Cy3 signal on cantilever B and C after the addition of the stripping buffer.

### 5.2.3 Mass/stiffness sensing of cantilevers

The change of mass during the assay can be valuable information to the mechanical properties of the cantilevers.[30] The data acquired from the resonance frequency of the cantilevers can be regarded as a mass and/or stiffness ratio indicator. These 2 factors can not be isolated using only the resonance frequency. An increase in frequency means a decrease in mass or an increase in stiffness, and vice versa. The first order resonance frequency was used and recorded with a high Q factor for almost all 32 cantilevers except 2 (data not shown). A high Q factor indicates a narrow and high peak of the first order resonance frequency. The resonance frequency values had in general a high variance, this meant that no significant change could be concluded; although a tendency to an increase in frequency was observed after the micro spotting step, this can be translated to a stiffness increase probably caused by the PBS salt in the BAM/ovalbumine layer. As the data from the resonance frequencies are reliable, the data shows a large variation of the mass/stiffness properties of the cantilevers from chip to chip; this may explain the large variation in the signal profile from chip to chip. Furthermore the functionalization seemed to increase the variance of the resonance frequency of the cantilevers. Below is seen the average values of 8 experiments.

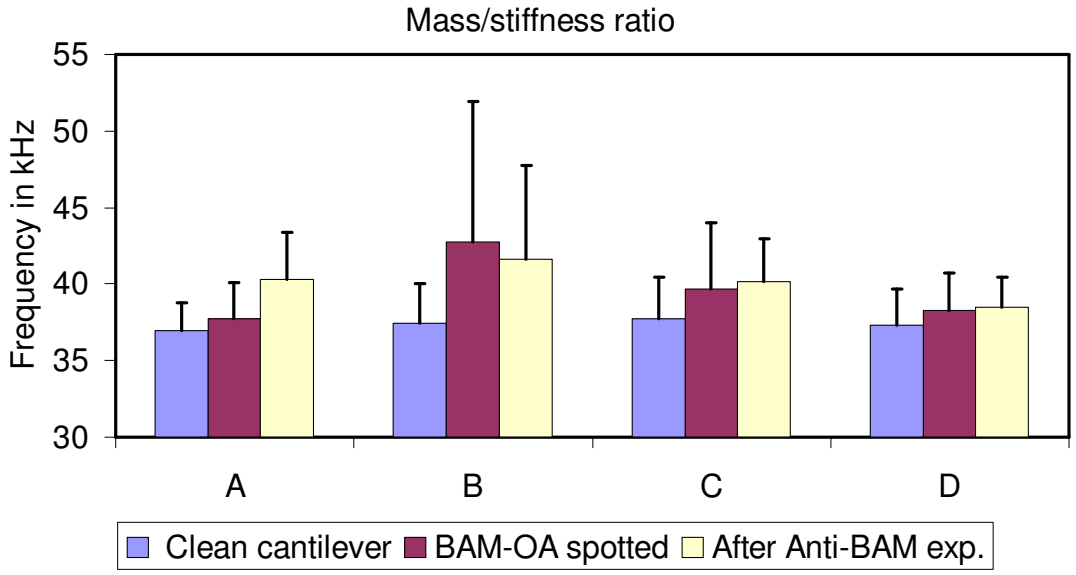



**Figure 5-9: Mass/stiffness ratios of each of the 4 cantilevers divided in 3 groups: clean chip, after micro spotting, and after the experiment. The values are an average of 8 experiments (chip 104, 108, 112, 116, 117, 118, 119, 120).**

### 5.2.4 Profilometer bending measurements of cantilevers

The deflection values showed an initial upward deflection of 8µm on average, and a downward bending (22-13µm) of all cantilevers after functionalization. This was probably caused by salt residue from the PBS buffer used in the micro spotting of BAM-ovalbumine conjugate and ovalbumine, the salt residue induced bending as it dried out on the cantilever surface. The cantilevers returned to their initial state after the experiment was performed, as a brief rinse in Milli-Q water removed the salt on the surface and de-stressed the cantilever. Although only 5 chips were used in the measurements, the data showed a relative low variation on the deflection of the clean cantilevers and after the BAM experiment, although the variation did increase during the functionalization step. This could indicate that the coating with BAM-OA and ovalbumine is not uniform on the cantilever. Below is seen the average bending values of cantilever A, B, C, and D, before and after functionalization and after the experiment, an example of the interferometer image obtained is seen on the right picture.

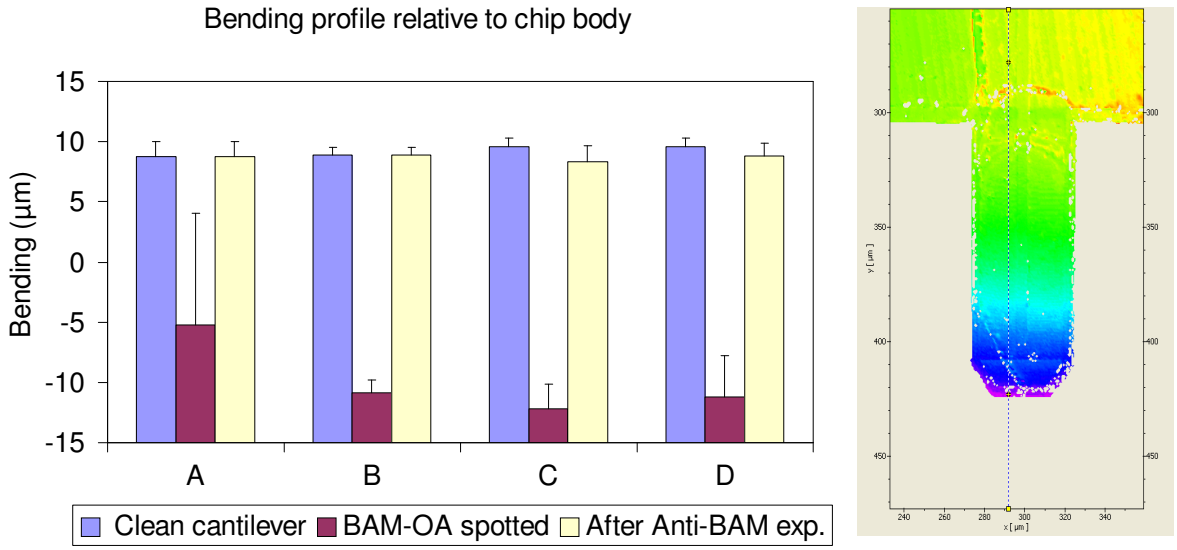



**Figure 5-10: Average bending values (µm) of cantilever tip relative to the chip body surface. Values are averages obtained from 5 experiments ( chip 116,117,118,119,120).**

### 5.2.5 Conclusion on the Cantion BAM assay

The second round of BAM assay experiments had a 50 % success rate and high signals in 5 experiments. This demonstrates that a repeated signal can be obtained. A signal from the specific, as well as the unspecific antibody was recorded in all experiments. The signal profile had very large variation from chip to chip. The noise level in the BAM assay was a low 1-2 μV compared to the 5 μV of the DNA assay. The Cy3 signal showed BAM antibody on cantilever B and C, however no significant unspecific Cy5 signal was seen on any cantilever. This indicated that the BAM antibody did attach to the signal cantilever and no significant unspecific Cy5 marked antibody remained attached to the cantilever surface.

The signal obtained can not be concluded to be specific for the BAM antibody, but is more likely that the addition of any antibody (or protein) in a concentration of 0.1 μg/ml, would cause a signal change when interacting with the cantilever surface. The addition of a lower concentration of antibodies (0.02 mg/ml) was tested on 2 chips but did not give a differential signal (data not shown). The bending and resonance frequency values of the cantilever were useful to monitor the general physical properties of the cantilever. It did not show the dynamic behavior of the cantilever during the BAM assay in the flow cell, as these measurements could only be done on dry Canti4 chips.

Visible light and fluorescent surface quality surface pictures of the cantilevers obtained in the DNA and BAM assay, showed an inhomogeneous surface caused by drops of micro spotted material. This error source was suspected to influence the cantilever properties. The increase in variation of the resonance frequency and bending of the cantilever, showed that the functionalization step using micro spotting of the cantilever surface, did have an influence on the mechanical properties of the cantilevers.

# 6 The Cantion 8 cantilever system

A new cantilever system from Cantion/Nanonord was developed during the PhD period. The system was characterized as a potential direct replacement of the Canti4 chip system. The system has the following improvements compared to the Cantion 4 system:

- Improved electronic box, where voltage noise is minimized, grounding of fluidic system is no longer necessary.
- Dynamic mode action, where each cantilever is actuated via a pulse in the piezo element, enabling measurement of resonance frequency in air and possibly also in water
- Heating control: The temperature of each cantilever can be individually controlled
- Magnetic field can be applied to the gold surface via a DC voltage on 2 cantilevers to attract or repulse magnetic particles.
- 8 cantilevers with dimensions of 50 or 25 μm width, 100μm length, 0,5 μm thickness, resistance 4,4 or 9 KΩ, electrically shielded and un-shielded circuit.
- Each chip has an EEPROM circuit (Electrical Ereasable Programmable Read Only Memory), to store information
- Sample frequency can be controlled (10 per sec to 0.01 per sec)

The electronic box functioned as its own server and was recognized by plugging it into the local area network. The software window controlled the many variable setting of the system. The data was accessed via a web browser and could be downloaded to the pc. The data format was accessed through a MATLAB program and not a text data file as in the Cantion 4 system. The data produced was very large compared to the Canti4 system (by a factor of 10), although this could be minimized by decreasing the sampling rate. The time span of an experiment was thus limited to 2 hours at 5 points per second. This made the data handling more complicated compared to the Cantion 4 system. As each chip had data storage capability, a system of tractability can be made. This was very useful for multiple experiments on the same chip. The many variable options of the Cantion 8 system made it a better system for research purposes, but also more time consuming for characterization and data analysis. Next is seen the Cantion 8 electronic box and chip.

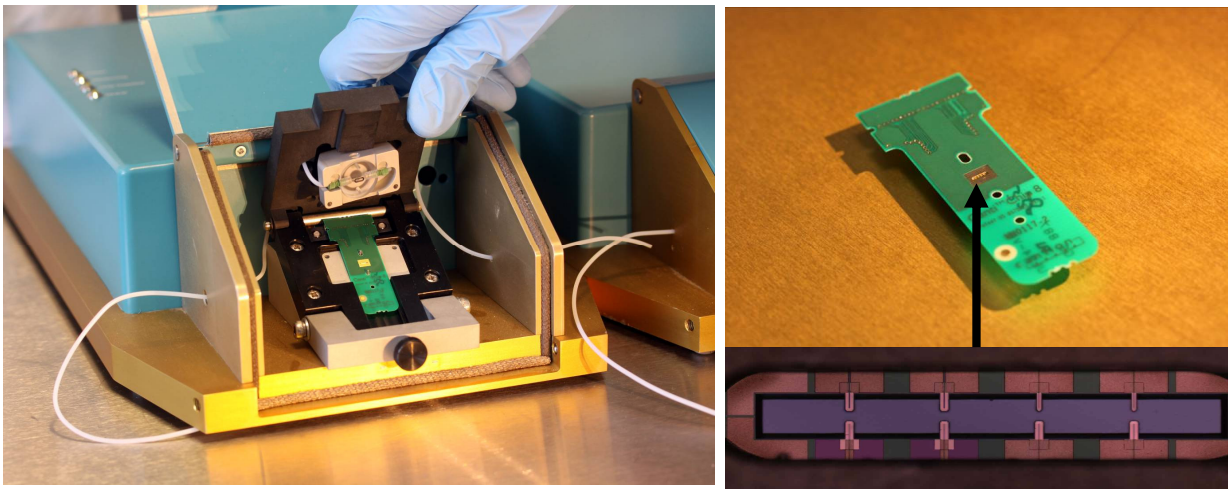


**Figure 6-1 : The new Cantion 8 setup (left) the electronic box with open fluidic cell. (Right) a Cantion 8 chip, the silicon chip is flip bonded to the circuit. Below is seen the pit with 8 cantilevers.**

To characterize the system, the Cantion 8 was tested using the BAM assay developed on the Cantion 4 system (see BAM assay on Cantion 4). As the cantilevers are divided in 2x4 groups with different characteristics, the 4 middle cantilevers were spotted with BAM-Ovalbumine and the outer 4 with ovalbumine alone. Pictures of the chip before and after the BAM assay are seen below.

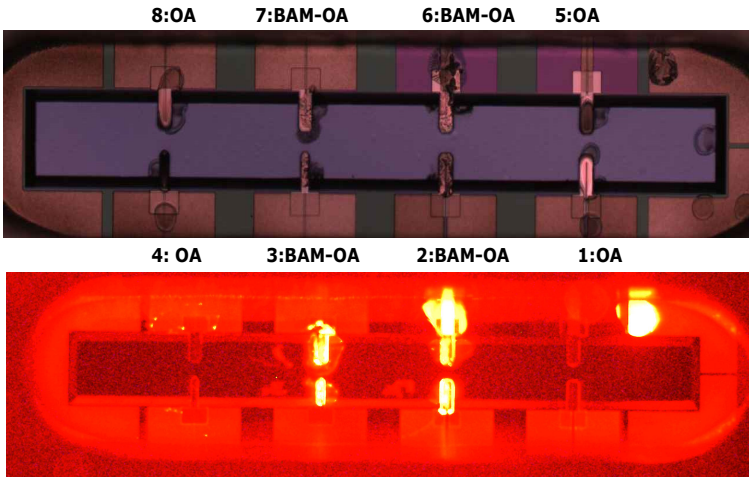



**Figure 6-2: A BAM assay performed on the Cantion 8 system. Above is seen a microscope picture of the spotted and ready Cantion 8 chip. Below is seen the Cy3 fluorescent image of the chip after the experiment. Note the Cy3 signal on chip 2,3,7,6 and none on the reference.**

The signal result from the BAM assay yielded a similar result as the Cantion 4 assay, in as much as both the unspecific and specific altered the voltage of the cantilevers. The main difference was a significantly larger noise with large signal jumps on all 8 cantilevers. Interpreting these results was noticeably more complicated compared to the Cantion 4 system. Below is seen the read out from the BAM assay, the data shown is not meant to be interpreted but is merely show to give the reader the impression of the complexity of the data output.

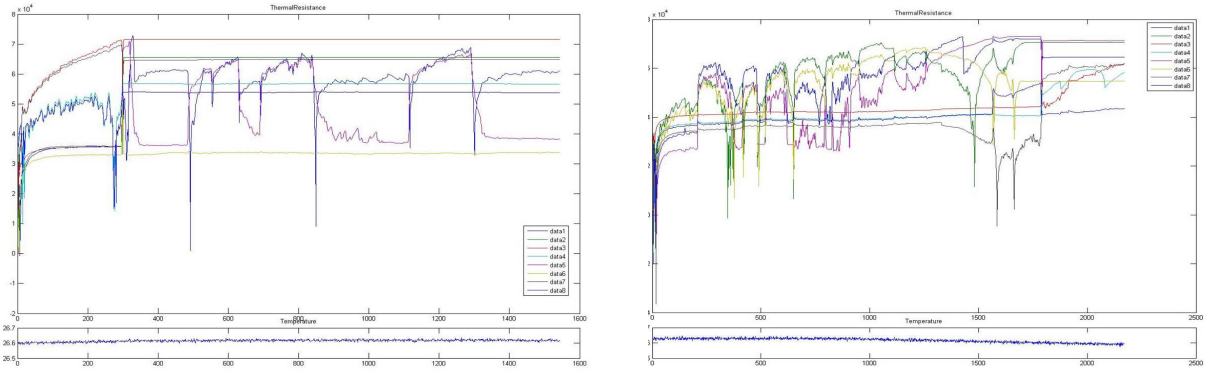



**Figure 6-3: The voltage readout from a BAM assay experiment. (left) Read out during unspecific Cy5 marked IgG. (Right) read out during the addition of the specific Cy3 marked BAM antibody. Both the specific and unspecific antibody gave a clear change of voltage in the system when the antibody entered the system.**

Although the Cantion 8 system had a higher sensitivity, and improved quality of the cantilever chips, the system was evaluated as having the same challenges as the Canti4 system. As the BAM assay had been established on the Canti4 system, and because the high cost of a single chip, meant that not enough chips were available for experiments; it was decided that the Cantion 8 system was not chosen as a platform for the BAM assay.

# 7 DVD IBM cantilever detection

This chapter describes a device that uses the readout hardware from a commercial DVD player and cantilever chips produced by IBM. The system can be regarded as a potential alternative to the Piezo electric read out of surface stress of the Cantion Canti4 system. The device has been tested using a Biotin-Streptavidin assay as a benchmark assay [31], and the BAM assay developed on the Canti4 system for comparison.

The Canti4 BAM assay experiments have shown that there is a large variance on the data from chip to chip. It is speculated that some of this variance is caused by small variances in the piezo resistive layer. Also the piezo layer could possibly induce a magnetic field, causing an electromagnetic induction from one cantilever to another; this could explain the large differences in noise level between experiments with SSC buffer and PBS buffer.

To eliminate eventual effects arising from the electrical piezo readout principle, an alternative method of detection is to measure the cantilever deflection using a laser on the cantilever surface. As the laser is reflected on the cantilever surface, the angle of bending of the cantilever affects the reflection of the laser. The angle difference can be detected very precisely using optics and a CCD camera for readout. Until now this has been done using a specialized custom setup, with capability of reading a few cantilevers in series. As a cantilever is very sensitive to alterations, obtaining a uniform layer on the cantilever surface is crucial if the variance on repeated experiments is to be lowered. As seen in the BAM assay on the Canti4 system this can be quite challenging. There is therefore a need for a device that can do multiple parallel measurements eliminating the inevitable variance in surface quality and variance on the results.

## *7.1 DVD IBM experimental setup*

The DVD IBM setup has been constructed by Filippo. G. Bosco and Stephan Keller from the Nanoprobes group in close collaboration with E.-T. Hwu and C. H. Chen from the Institute of Physics, Academia Sinica, Nankang Taipei in Taiwan. The project is a part of the X-sense project led by Anja Boisen, Nanoprobes group at Nanotech DTU.

Using the optics and mechanics from a DVD player, a device has been constructed that enables the parallel readout of up to 90 chips containing 8 cantilevers each. It allows for the readout of over 720 cantilevers per second, scanning the width of each cantilever with over 1000 points. This generates a large statistical data pool that is unprecedented. The setup is able to record the deflection, resonance frequency and roughness of each cantilever with a 1 nm resolution in the Z direction. The chips are placed on a DVD disc like holder and can be rotated. The spinning of the disc doubles as the fluidic pump as centrifugal forces push the liquid over the cantilever surface through channels embedded in the DVD holder. Below is seen the schematic picture of the DVD holder and a close up of the cantilever chip pit.

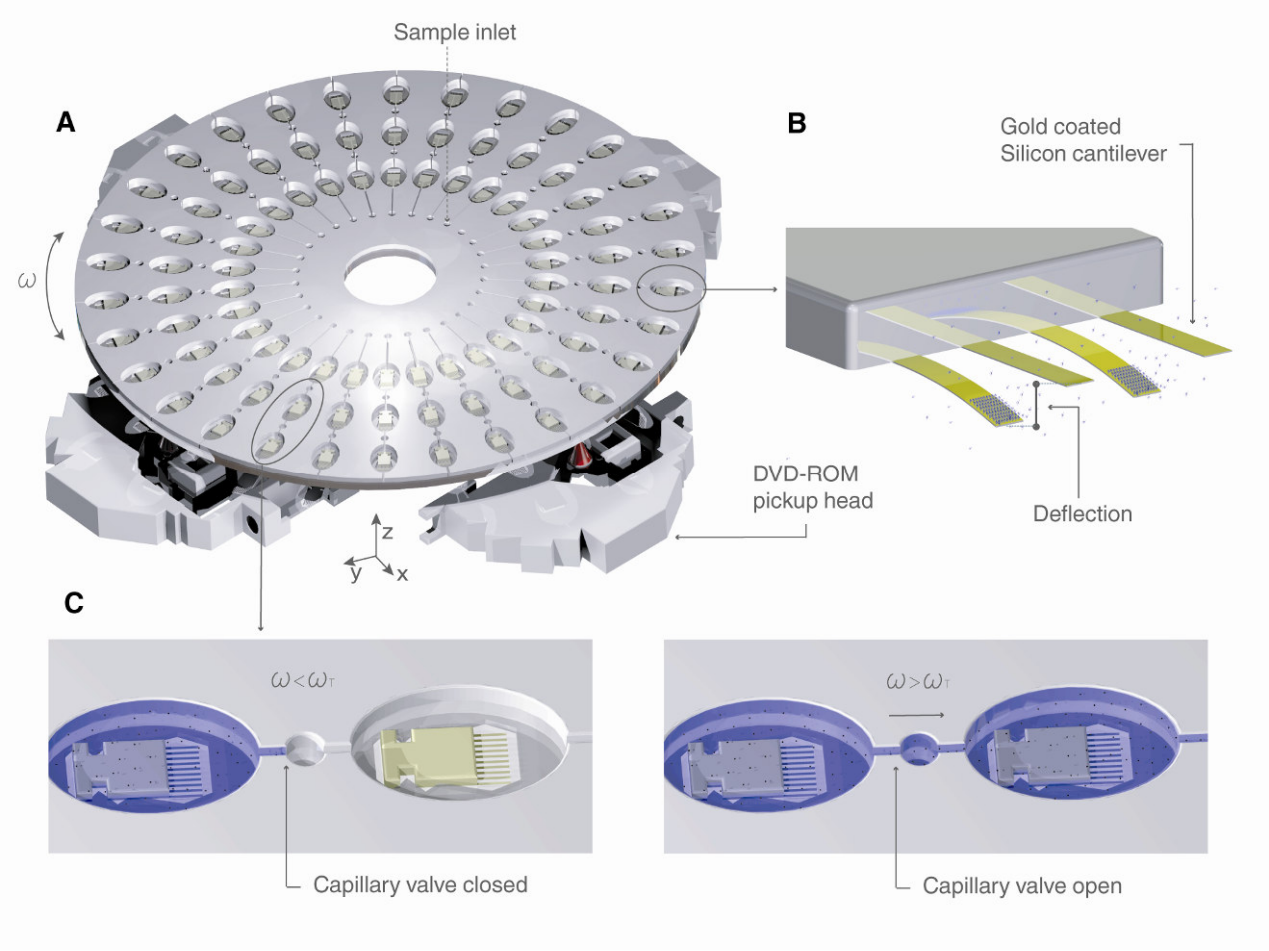



**Figure 7-1: Drawings of the DVD IBM setup. (A) The DVD disc with integrated flow channels and holder for 3 rows of cantilevers (B). An IBM style disc with gold coating on one side (C) Zoom of the flow channel with capillary valve.**

## 7.2 Material and methods

The IBM chip is a silicon device with 8 equal cantilevers with 500 µm length, 100 µm width and 1 µm thickness, each cantilever is coated with gold only on one side. The chips were cleaned using a piranha wash prior to use. The chips were placed in a holder and the cantilever bending values were recorded using four DVD-ROM optical pickup heads placed 1 mm below the disk, measuring the non gold coated underside of the cantilever. The disk was moved illuminating the cantilevers by a laser with a wavelength of 650 nm and a spot diameter of 0.56 µm. The deflection profiles were measured using the astigmatism-based detection mechanism normally used for auto focusing in a DVD player. Apart from the deflection of the cantilever, this also allows for the recording of the surface roughness of the cantilever, as the optical heads are able to scan along the length of the cantilever. The data was treated in MATLAB and a program written in LabView controlled the data acquisition. As the lid for the fluidic system was not fully developed, the experiments were done by adding the BAM antibody or Streptavidin solution directly onto the IBM chip using a 10 µl pipette. The measurements were done on dry cantilevers, before the addition of fluids, and after the added BAM antibody or Streptavidin. Measuring the bending of the wet cantilevers was not possible, as the signal had too much dispersion through the water layer. The data are not presented as a function of a continuous time scale as in the Canti4 system, but as rounds of revolution of measurements. One revolution lasted approx. 10 seconds. The bending values of all cantilevers were set to zero in the first revolution round, showing only the differential values. The 2 assays are described more in detail in the appendix protocol section. Pictures of the setup are seen below.

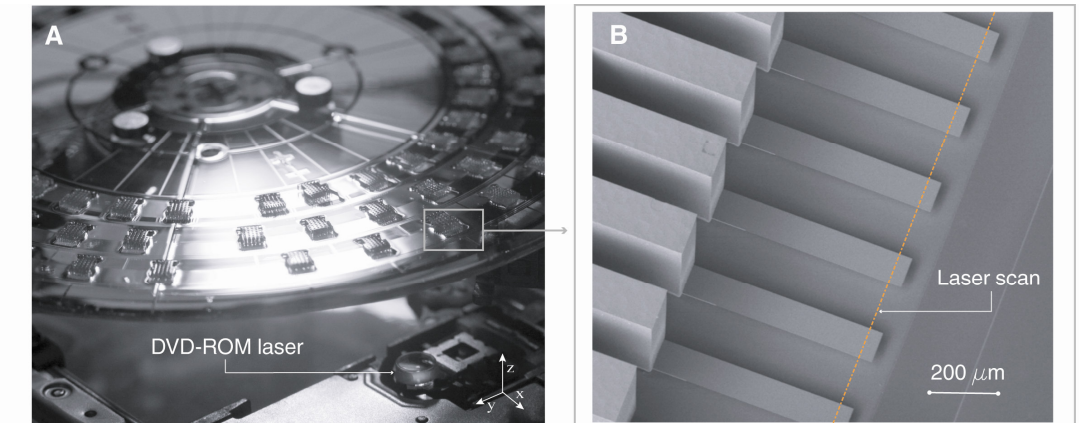



**Figure 7-2: Images of the DVD IBM setup. A: The holder for the IBM chips, the DVD-ROM optical pickup heads can be seen below. B: Close up of the IBM chip. (Courtesy Filippo Bosco)**

### 7.2.1 IBM DVD BAM assay experiments

The IBM chips were functionalized using the same Cantion micro spotter used for the Canti4 BAM assay. The outer cantilevers 1+2+7+8 were used as reference by coating the surface with 1 mg/ml ovalbumine in PBS buffer, and the inner cantilevers 3+4+5+6 as signal by coating with 0.75 mg/ml BAM-ovalbumine in PBS buffer. Additional IBM chips with 8 untreated cantilevers were used for blank reference measurements.

After an overnight incubation the chips were placed in the holder and a baseline recording of approx. 20 revolutions were done. 2µl of 0.1 mg/ml BAM antibody was added directly to the IBM cantilever pit covering the whole chip, after a 10 min. incubation time the antibody was removed and washed 3 times with PBS buffer and briefly with Milli-Q water. The bending values and roughness scan was recorded once the cantilevers were dry (approx. 10 min.). The IBM chips were scanned for Cy3 signal after the experiment revealing Cy3 anti-BAM signal on cantilever 3+4+5+6.

### 7.2.2 IBM DVD biotin/streptavidin experiments

All cantilevers of an IBM chip were functionalized by immerging the chip in a thiolated biotin solution overnight, another IBM chip with 8 untreated cantilevers were used for reference measurements. The chips were inserted into the DVD platform and exposed to a buffer solution containing streptavidin. After exposure, all cantilevers were washed in Milli-Q water to remove residual salt from the buffer solution. After washing, the water was left to evaporate and the cantilever responses were measured continuously.

## 7.3 IBM DVD experiment results

### 7.3.1 BAM assay results

Although the measurements are done on dry cantilevers, there is a significant difference between the signal and the reference cantilevers. The addition of BAM antibody caused a bending of 10 µm of the BAM functionalized cantilevers, ovalbumine coated cantilevers bended 5 µm, and a set of 8 non coated reference cantilevers bended 4 µm. The experiment was repeated twice with the same outcome. The bending values before and after addition of BAM antibody is seen below. The BAM antibody is added before revolution 21.

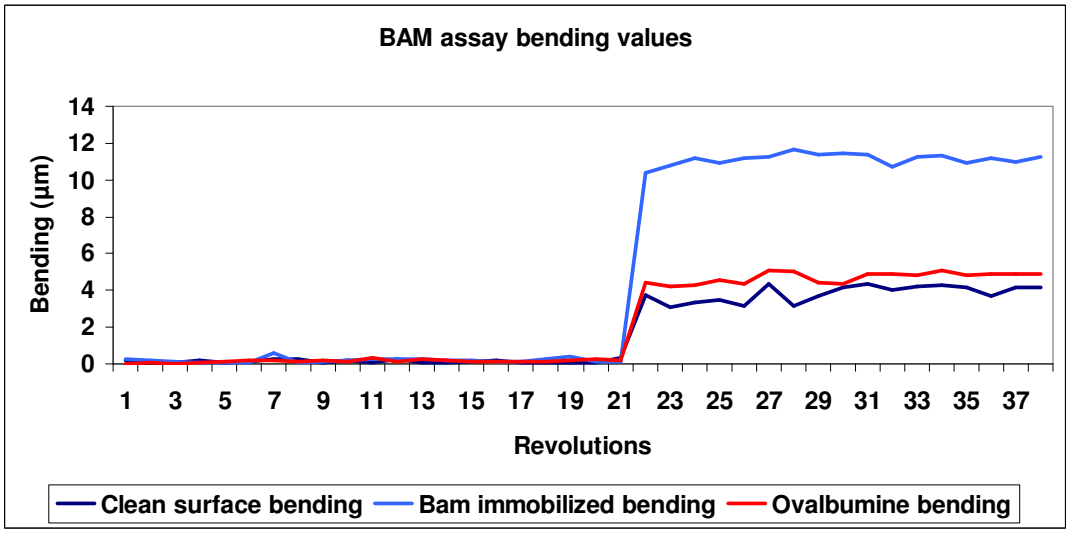



**Figure 7-3: Average bending values of 4 BAM-OA coated, 4 ovalbumine coated and 8 clean cantilevers.**

An additional feature of the DVD IBM setup is the ability to measure the surface roughness. The surface roughness is calculated as a sum of variance of the profile of the cantilever when scanned along its length, the system scans by measuring the deflection 1000 times along the width, and is repeated every 100 nm along the length of the cantilever. The surface roughness of the cantilevers showed a large increase of the variance on the clean gold surface. As the data is relative to the previous state of the surface, this could indicate that the clean gold layer was polluted with BAM antibody, and no significant increase in roughness could be recorded on the already functionalized surface of the BAM-OA and ovalbumine cantilevers. The surface roughness values are seen on the next page.

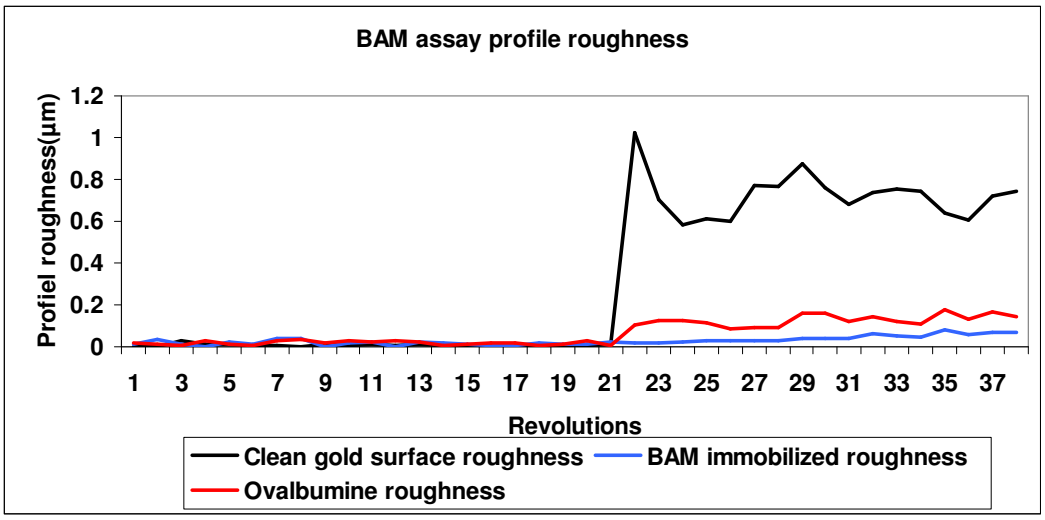



**Figure 7-4: Profile roughness of BAM antibody assay**

### 7.3.2 Biotin/streptavidin assay results

The addition of streptavidin show a surprising tendency of continuous bending after the cantilever had dried sufficiently for optical measurements. The Biotin functionalized cantilevers bend significantly less than the BAM assay signal cantilevers. The reference gold coated cantilevers have an initial higher bending of 3.8 µm that decreases to the initial state. The data can be explained by unspecific binding of streptavidin to the reference cantilever gold coated surface.

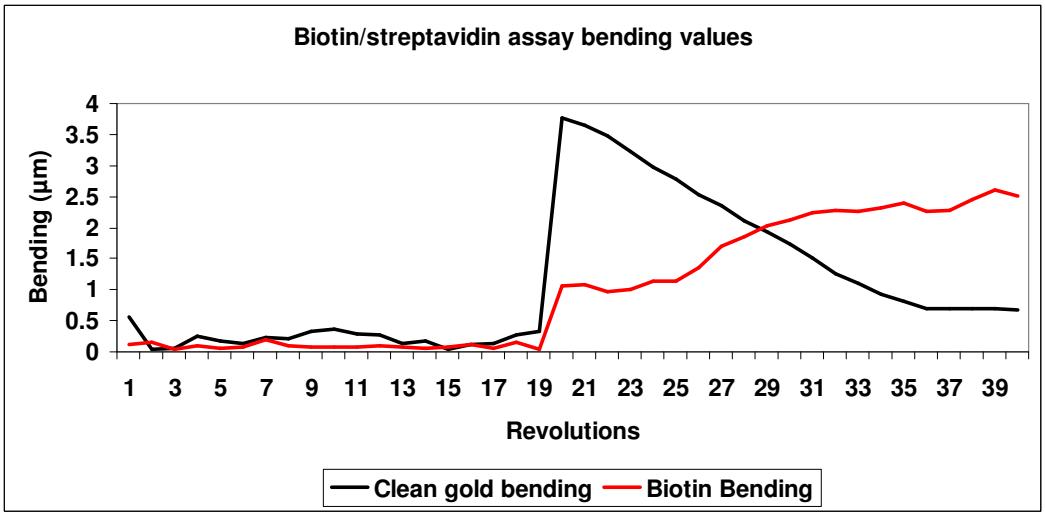



**Figure 7-5: Bending values of cantilevers in the biotin/streptavidin assay**

As seen in the DVD IBM BAM assay, the surface roughness of the Biotin functionalized cantilevers is relatively unaltered whereas the surface of the clean gold surface increases the

roughness with up to 0.4µm. This can be translated as the streptavidin attaching to the biotin layer in a controlled monolayer, while the clean gold layer is coated with streptavidin, but as an inhomogeneous multilayer.

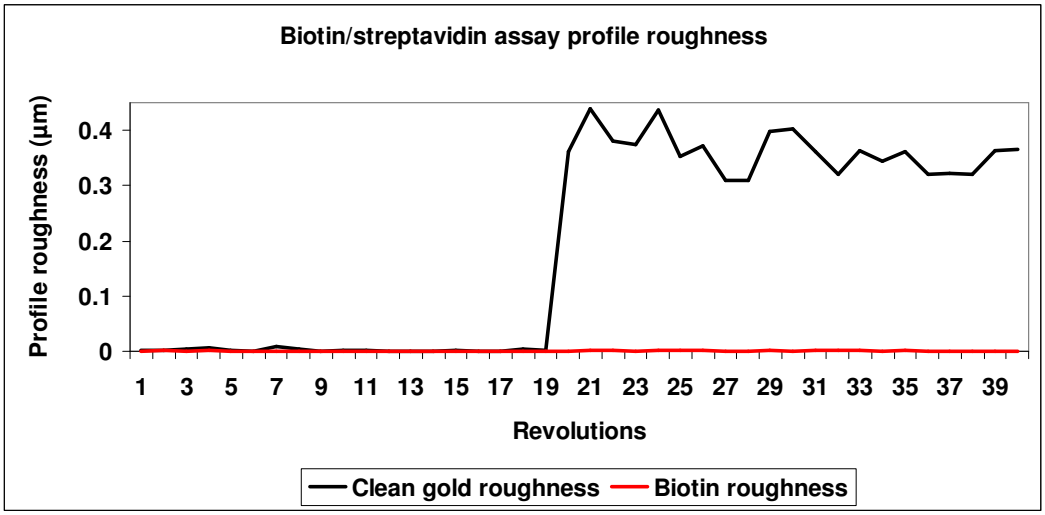



**Figure 7-6: Profile roughness of cantilevers in the biotin/streptavidin assay**

## *7.4 Concluding remarks on the IBM DVD setup*

The differential bending signal obtained in the Canti4 system is repeated using the BAM assay on the DVD IBM setup. Although the BAM and Biotin-Streptavidin assay was only repeated twice, and without testing the assay with an unspecific antibody or protein, the setup has 2 noticeable detection potentials. The ability to measure a lasting bending difference on dry cantilevers, and the use of surface roughness as an additional information parameter, for the indication of the quality of the cantilever surface. Measurements of cantilevers in air also eliminate errors and drift caused by air bubbles and other changes of the fluidic system.

# 8 Raman and SERS detection of pesticides

Raman detection is here presented as an alternative to the cantilever surface stress readout principle. The detection is label free and is suitable for detection of small organic and inorganic compounds in water samples. The classical Raman, surface enhanced Raman and Raman spectrum simulations is presented in this section.

When light hit dense matter most photons are scattered, but a few of these photon particles interact with the electron bonds and nucleus of the matter. This interaction can excite the bonds to a higher or lower energy virtual state. The exited energy state eventually returns to the native ground state by emitting photons with a higher or lower frequency than the initial state. If the photons are of a lower frequency than the initial state, this shift is defined as the stoke shift, if the frequency is higher the shift is defined as anti-stokes shift. This shift produces a unique fingerprint signature of frequency shifts that are dependent on the structure of the bonds in the matter. This effect was observed by Sir Chandrasekhara Venkata Raman in 1928 and awarded with the Nobel prize in 1930. The acquisition of the Raman shift signal allows for the creation of a library of unique Raman shift signals for each material. This can be used to directly identify unknown samples by comparing to known Raman shift profiles.

Unfortunately as the Raman signal is very weak, the detector and hardware needed to the record the spectra makes this a very expensive and cumbersome technique. Analyzing the data is also not a trivial matter and is often done by Raman experts as a range of factors can influence the spectra. Currently there are also some limitations to the complexity of the matter studied as large complex molecules such as proteins and nucleotides give a too blurred Raman spectrum as the signal peaks overlap each other. Inorganic and Simple organic compounds are ideal materials for a Raman spectrum as the peaks are distinct and often well separated, making the spectrum recognizable.[33]

## 8.1 Surface enhanced Raman spectroscopy

Raman spectroscopy is here presented as an alternative to the surface stress induced cantilever principle. The method uses a direct detection principle suitable for the detection of small organic and in organic substances in water samples.

In 1974 M. Fleischmann discovered that when pyridine was placed on a silver electrode the Raman effects were significantly magnified up to a factor from $10^6$ up to $10^{10}$. This method has since been known as Surface Enhanced Raman Spectroscopy (SERS). The cause of the signal enhancement is still not fully understood. The enhancement cause is generally regarded as a combination of factors from electromagnetic and chemical enhancement. The electromagnetic factors are contribution from localized surface plasmon resonance of the nanostructured metal surface, and are regarded to account for $10^4$ of the enhancement of the signal. Another electromagnetic factor is believed to be the induction of a dipole moment on the metal surface by the Raman scattering, and some of the radiated light from this dipole oscillation is stoke shifted causing a signal boost. The chemical factors contributing are charge transfer complexes, from material forming a bond with the metal surface, thus enhancing the Raman signal by a factor of 100. The quality of SERS depends strongly on the quality of the surface used and on the interaction of the material studied with the surface. The reproducibility of the results depends therefore on the uniformity of the SERS substrate. [33]

In recent years a great deal of development has been made to achieve better reproducible and more effective SERS substrates with higher Raman signal enhancement. These substrates have been used to detect signals from as little as single molecules on the surface. Since the needed surface area is quite small, the whole system can be incorporated in a compact package, with a fiber optic light source with filter attached and small CCD. This makes the system a potentially very portable and effective device for direct identification of a very diverse number of substances such as explosives, inorganic pollutants, drugs, pesticides.[35][37]

## 8.2 Raman experiments overview

In order to investigate the potential to use a Raman spectrograph for the detection of Dichlobenil and BAM in groundwater, 3 diverse series of experiments have been made:

- Bulk Raman: the spectrum of Dichlobenil and BAM has been made using a classic Raman setup in close collaboration with Rolf Berg
- The SERS spectrum has been measured using a range of dilutions of Dichlobenil and BAM on a silver coated nanostructured surface produced by Michael Stenbeck Schmidt. The experiments were done in collaboration with Michael Stenbeck Schmidt. A computer simulation of Dichlobenil and BAM has been made where the theoretical shift values that each bond in the molecule can induce is calculated. The simulations were done by Rolf Berg and analyzed by the author.

The purpose of analyzing these 3 different fields of Raman detection was to elaborate on the reproducibility of the SERS data and ensuring that the data is a good representative of the Dichlobenil and BAM spectrum, and not caused by a the specific interaction with the SERS surface. It was also hoped that the bulk Raman data and simulations can be used by future researchers in the field of pesticide detection using the Raman technique. Recent advances in SERS have opened up the possibilities of a SERS device for water quality monitoring. The main advantage is direct measurements of the analyte, no chemicals involved, large temperature range of operations, sensitivity far below µg/l, large dynamic range and long shelf life of a closed system. The disadvantages are that a previous knowledge needs to be acquired regarding the interpretation of the spectrum and the method is currently not adapted to quantitative measurements although some quantitative measurements have been made [36].

## 8.3 Bulk Raman measurements of BAM and dichlobenil

The bulk Raman spectrum was recorded for Dichlobenil and BAM in collaboration with Rolf W. Berg from Energy and Material Group at DTU Chemistry.

### 8.3.1 Bulk Raman experimental setup

The setup consist of a monochromatic laser light source shining on the matter of interest (in this case a 532.4 nm laser) the light reflected is filtered to block the wavelength of the monochromatic light source (see figure below), the light is then spilt up by a "grater" filter spitting the light according to the frequency and is detected by a Charged coupled device detector (CCD). As very few photons cause the Raman effect, a very sensitive and large detector is needed to record the emitted photons (2.5 cm x 2.5 cm), also the filters used have to be of good quality in order to not damp or allow photons of an unwanted frequency through the filter. The setup is placed in a basement and no external light sources are on during the experiment to avoid unwanted photon effect. Below is seen the schematic setup of a Raman detector.

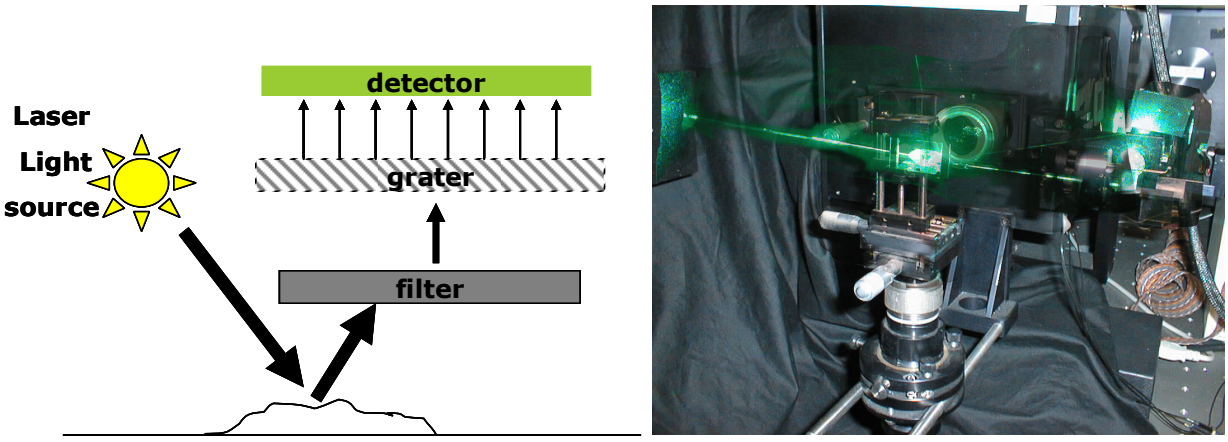



**Figure 8-1: (left) principle of Raman detection. (Right) the laser mirrors of the actual Raman apparatus guiding the laser source to the substrate (courtesy Rolf W. Berg).**

In the bulk Raman experiments the solid Dichlobenil or BAM matter was placed under the laser light source with a wavelength of 532.4 and an intensity of 0.100 mW and a probe spot diameter of approx. 100 µm. The Raman spectrum was recorded and repeated 3-4 times in order to check for noticeable differences on the spectrum. The spectrum of Dichlobenil was

recorded in 1 second aperture of the detector. BAM was recorded in 5 to 40 seconds intervals due to significantly higher background and less distinct peaks. Longer aperture may damage the substance by heating from the laser due to the small the focal point, this may in turn lead to Raman signals from breakdown products of the substance and not the pure substance. No significant difference was found in between repeated measurements when recording the spectrum for Dichlobenil and BAM.

### 8.3.2 Bulk Raman results

Below is seen the results of both pure BAM and dichlobenil (top) and BAM and dichlobenil on a SERS substrate (bottom). BAM had in general higher but also broader peaks compared to the Dichlobenil. Note that as the data are summary of multiple plots, the x scale is not given as whole integers and can not be plotted using ordered numbers.

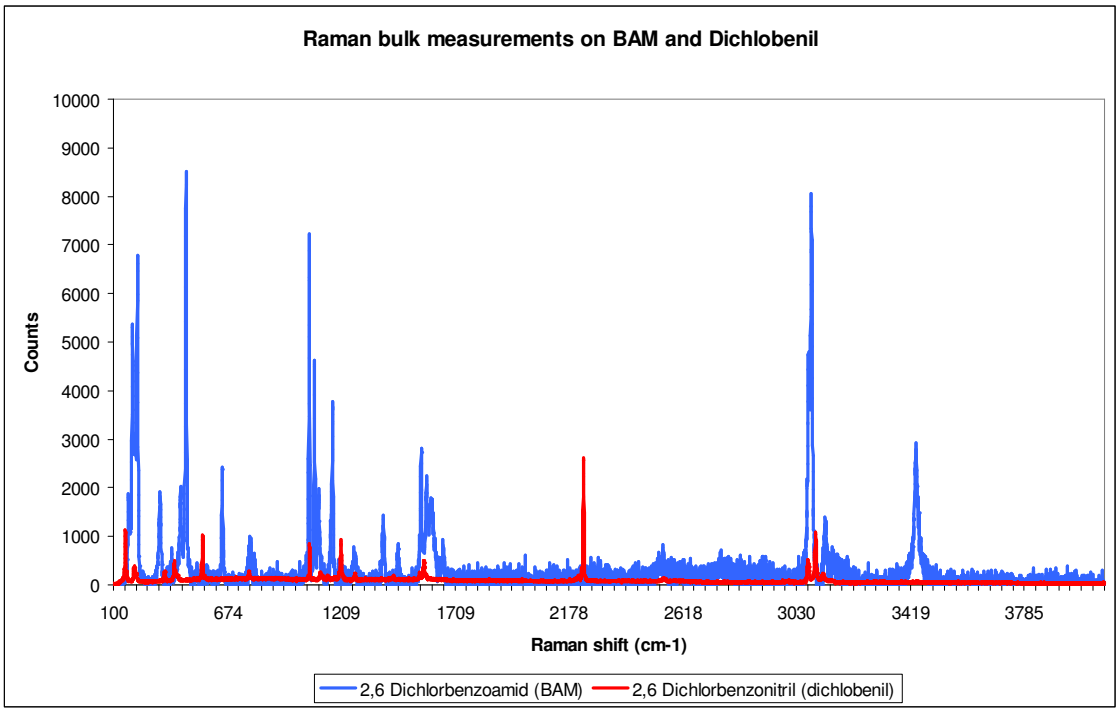



**Figure 8-2: Raman bulk measurements, BAM and Dichlobenil, 532.4 nm 100 mW laser, 40 sec exposure**

To investigate the effect of the SERS substrate, the Raman spectrum of BAM and dichlobenil on the SERS substrate was also measured. A 1 µl drop of 10000 µg/l of BAM and dichlobenil dissolved in acetonitrile/water solution was added to the substrate and allowed to dry. The BAM was first dissolved in acetonitrile as BAM has an extremely low solubility in water. On

the next page can be seen the spectrum results of BAM and Dichlobenil on a SERS surface using the classical Raman detector.

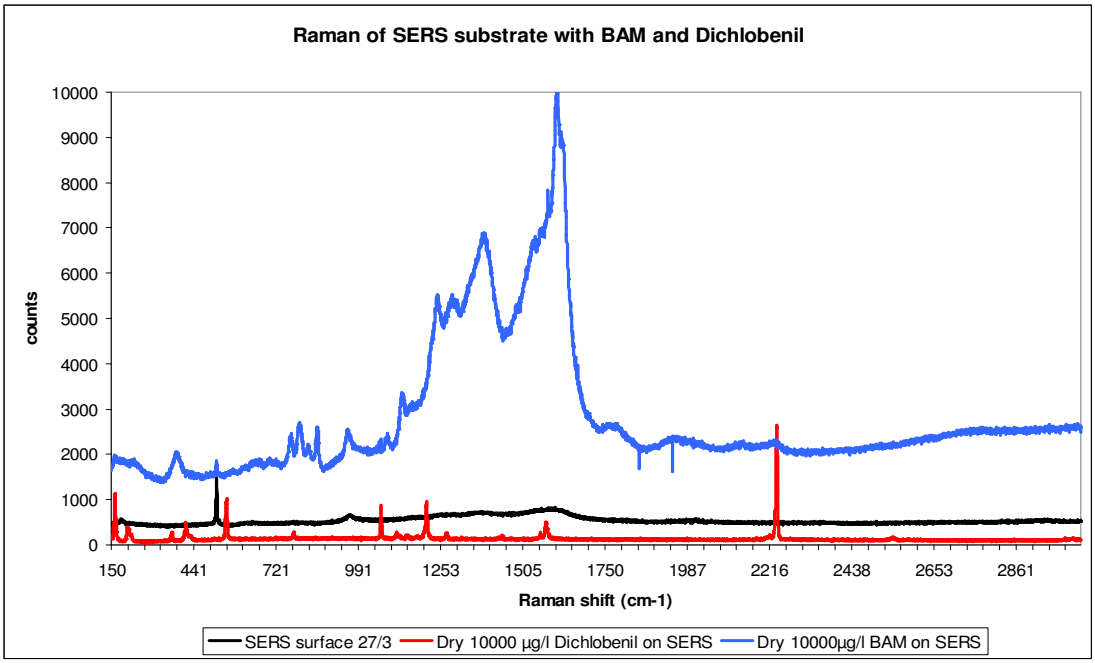



**Figure 8-3: Raman measurements of dry BAM and Dichlobenil on SERS substrate, 1 µl of 0,01g/l of BAM and Dichlobenil dissolved in 1ml/l Acetonitrile in water is added to the substrate and allowed to dry.**

Note that the amplitude of the signal counts is difficult to compare as it is dependent on aperture opening of the detector. The data acquired from bulk Dichlobenil shows clear and distinct peaks with low noise. The Raman spectrum of dichlobenil on SERS substrate have exactly the same peaks as the bulk spectrum, the highest signal peak is at 2236 $cm^{-1}$ for both bulk and SERS substrate dichlobenil.

BAM has a much larger noise level grouped less distinct peaks. This is probably due to the interaction of BAM molecules with the SERS surface. The large signal group seen from 1200-1600 $cm^{-1}$ on the BAM bulk Raman, could be polluted by residues of acetonitrile used to dissolve BAM in the solution. The Raman spectrum of acetonitrile has a broad peak from 1300 to 1500 (source: Chemfinder). Although most acetonitrile is considered to be evaporated at the time of measurement, some acetonitrile could remain trapped in the large surface area of the SERS surface. The full spectrum was recorded up to a frequency of 4000 $cm^{-1}$ but was truncated at 3000 $cm^{-1}$ as the SERS detector used in-house, only had a detector range of 300 to 2100 $cm^{-1}$. The clean SERS substrate only shows a peak at 520.

## *8.4 SERS measurements of BAM and dichlobenil*

A nanostructured surface has been produced that enhances the Raman effect. The project is a part of the X-sense project lead by Anja Boisen from the Nanoprobes group at DTU Nanotech. The setup used and SERS surface is produced by Michael Stenbeck Schmidt from Nanoprobes group, DTU Nanotech. [38]

### 8.4.1 SERS experimental setup

A pattern of nanostructured pillars are created using reactive ion etching on a 4" inch siliceous wafer. The structures are etched using a gas mix SF6:O2 with a ratio of 1 to 1.12, and a power of F110-130 W with 8 to 56 mTorr. Adjusting the gas mix ratio, chamber pressure and effect greatly affects the morphology of the nanostructures. The wafer is then coated with a 50 to 400 nm layer of gold and/or silver metal, a layer of 250nm was found to give the most effective Raman signal using a Rhodamine 6G and Thiophenol assay. The wafer is diced out in 1x1 cm squares before use and placed in the setup with tweezers. The setup uses a 785 nm diode laser (Torsana Laser Technology) with a power of 130-245 mW, and a probe from In Photonics with a spot diameter of 160 μm. Different strategies were made for the measurement of BAM and Dichlobenil. The experiments were initially done using a pipette to deliver 1 μl drops of solution to the chip surface. The signal was measured directly without allowing the drop to dry out. It was later discovered that if the drop was allowed to dry out (approx 10 min.) the signal would be enhanced, probably because the water film is no longer dispersing the signal. To mimic an in-line flow device, a series of test were done using a flow cell for the SERS substrate, BAM and dichlobenil were measured under 20μl/min flow created by a syringe pump similar to the BAM assay Cantion assay. The method was abandoned as the Raman results were too dampened, probably due to the high damping of the water layer over the chip. [38]

### 8.4.2 SERS measurements of water samples

The following results present only the experiments made from measurements of dried drops on the SERS surface. Although measurements were obtainable from wet substrates, the dried surface had a higher more consistent Raman signal. The substrates used were named 22-3 and

27-3, the exact production settings can not be disclosed as the substrate is currently being part of a patent submission. Initially a solution of 0.1 g/l of BAM and Dichlobenil was used for testing (pure chemical grade from Sigma Aldrich). But as BAM and Dichlobenil dissolves poorly in water the absolute concentration was not known. To know the exact concentration of BAM and Dichlobenil, the substances had to be fully dissolved in a suitable organic solvent; BAM and Dichlobenil were therefore dissolved in 1 ml of acetonitrile and then in Milli-Q water using a step dilution series. A similar dilution of 1ml Acetonitrile in 1 liter of Mill-Q water was made as reference. The dilution series can be seen below.

| Dillution nr | Diluted | Conc. | | |
|---|---|---|---|---|
| 1, stock | 10 mg in 1 ml Acetonitrile | 10 g/l | 10000 mg/l | 100000 µg/l |
| 2 | 0.050 ml stock to 49.95 ml water | 0.01 | 10 mg /l | 10000 µg/l |
| 3 | 0.5 ml stock to 49.5 ml water | 0.0001 | 0,1 mg/l | 100 µg/l |
| 4 | 0.5 ml stock to 49.5 ml water | 0.000001 | 0,001 mg/l | 1µg/l |
| 5 | 0.5 ml stock to 49.5 ml water | 0.00000001 | 0,00001 mg/l | 0,01µg/l |

**Figure 8-4: Dilution series of BAM and Dichlobenil.**

A series of water samples were measured to investigate the sensitivity of the SERS system. In most cases 1 µl water sample was added and allowed to dry on the surface, a few SERS chip were dipped whole in the solution and allowed to dry to avoid an eventual "coffee stain" effect, but with no noticeable difference. Signals from 3 various water samples measured and used as reference, can be seen below. Note that water itself does not cause a Raman signal, but the addition of a fluid drop causes a change of signal that can be regarded as a change of the background signal, probably caused by altering the surface structure of the SERS substrate.

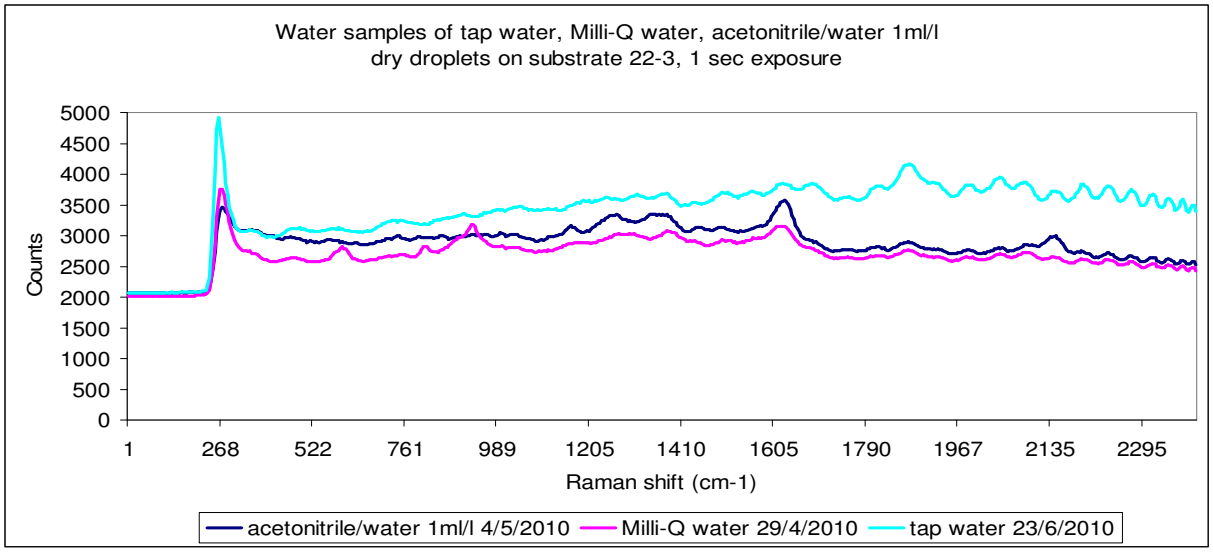


**Figure 8-5: SERS of Milli-Q water, acetonitrile/water in 1 ml/l, and tap water. Dry drop, substrate 22-3, 1 sec. exposure, 245 mW laser.**

A range of BAM solutions were added to the SERS substrate. The signal was clearly increased by a factor of 5 compared to the water reference values, the peaks of the spectrum were less distinct compared to the bulk Raman. Measurements from 4 different samples can be seen below. The background signals from the solvent (water or acetonitrile/water mix) have not been subtracted from the SERS data. Note that the less concentrated solution of 0.01 µg/l apparently has the highest value and the most distinct peaks, followed by the sample with an unknown concentration of BAM (up to 0.1 g/l).

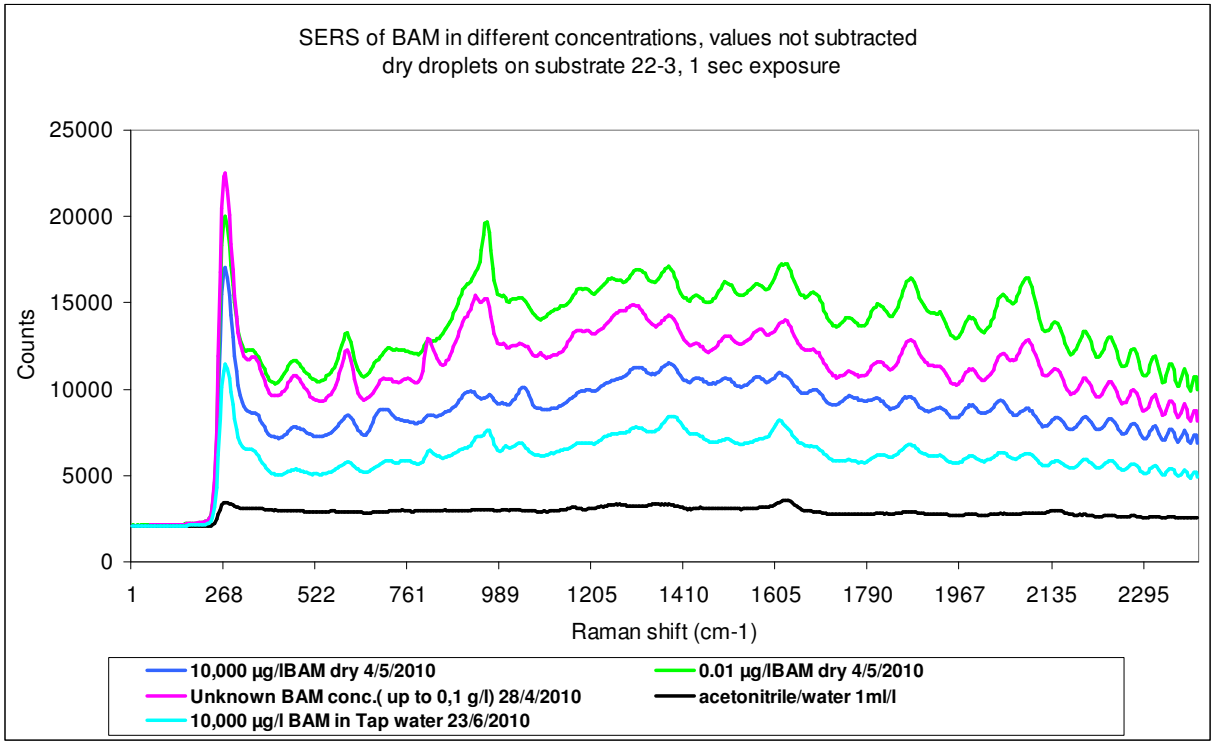



**Figure 8-6: Measurements of BAM in different solutions. There is a similar pattern for all 4 BAM samples but with a varying intensity.**

The experiments done with Dichlobenil showed a different profile. The signal was very high for the first round of experiments with a 0,1 g/l solution of Dichlobenil in Milli-Q water with very clear and distinct peaks. Note that the concentration can not be guaranteed to be accurate, as dichlobenil also has a low solubility of 2,5 x $10^{-4}$ g/l. The repeated measurements with the low concentration of 0,01 µg/l gave no signal above the water reference sample. The 10,000 µg/l concentration gave repeated measurements above the water sample level but still considerably lower than the first round. Signal peaks were broad but visible. Three different samples of dichlobenil are seen below.

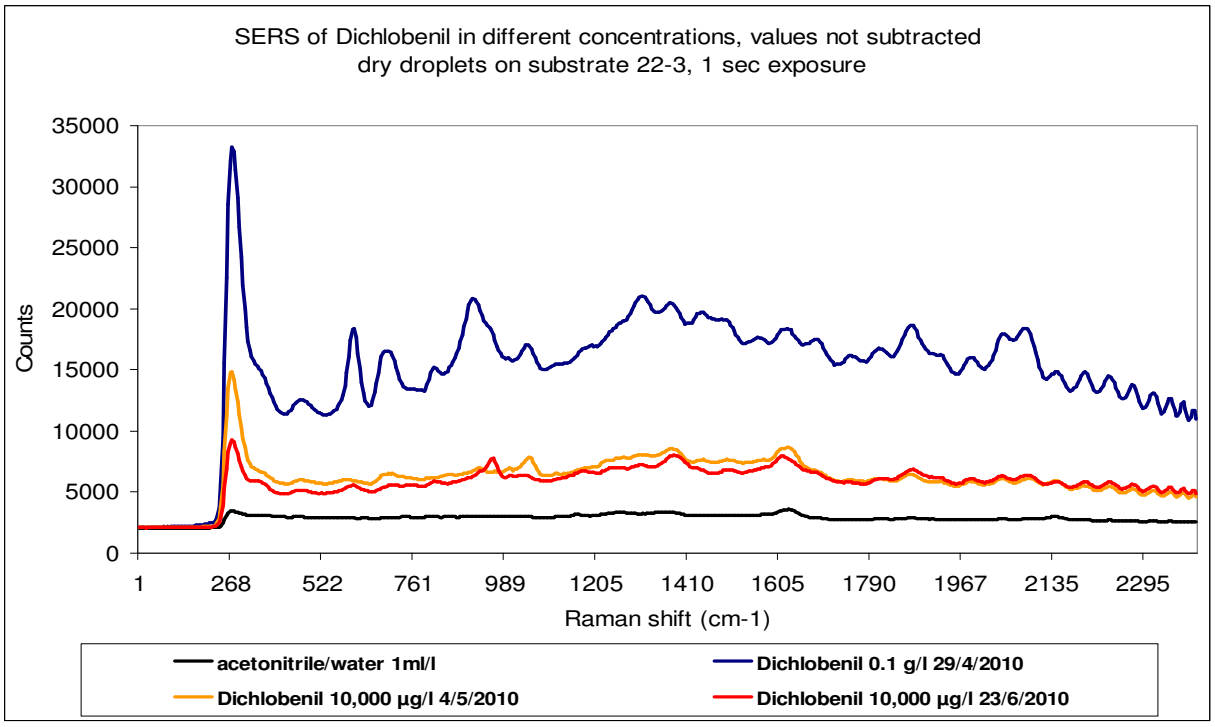



**Figure 8-7: Measurements of Dichlobenil in different solutions. There is a less similar pattern compared to the BAM samples. The 2 measurements of 10000 µg/l are done almost 2 months apart but has similar signal.**

### 8.4.3 Comments on SERS measurements

The measurements were generally done by measuring the reference first, followed by the samples and re- measuring the references again at the end. Each measurement was repeated 2-3 times on average to ensure that the value was representative of the sample.

Acquiring the correct focus length distance from the SERS surface to the detector was crucial, as a few millimeters off focus resulted in significant loss of signal. The measurements were of a higher value if the sample spot had been dried, although wet spot measurements were possible (with high noise) and the profile was roughly comparable to dry measurements. As stated previously, it is in general difficult to compare the amplitude of the results as quality of the surface and laser intensity can greatly influence the values. When comparing water samples with signal from BAM and dichlobenil, the signal has a general profile from 1900 $cm^{-1}$ and upwards to 2300. This is probably due to the quality of the detector and not caused by the sample. The effective detector range was evaluated to be from 300 to 2100 $cm^{-1}$.

There were a number of factors influencing the measurements. A few of the samples gave very low or no signal, this could be due to a damaged surface. However, the cause for this deviation could not be established. In general the first round of experiments gave the highest amplification of Raman signal. Possible pollution from the lab area could be a cause (dust or solvents) since the surface is extremely sensitive to changes. The experiments were repeated on a SERS substrate from a different batch (27-3) and gave a lower value compared to substrate 22-3. As stated in the literature the slightest change in production process can greatly affect the Raman enhancement effect. This clearly shows the need for well controlled process and storage conditions, if repeated measurements are to be made in a working device for pesticide identification.

## 8.5 Raman computer simulation of BAM and dichlobenil

The Raman shift and IR signal can be calculated and simulated if the structure of the material is known. The simulated values of the Raman spectrum can then be used to determine the origin of the Raman peak, and serve as guidance for the identification of the spectrum of unknown materials or to determine the signal quality of known substances. The program Gaussian 03, Revision E.01 by Gaussian, Inc., Wallingford CT, 2004 is used to do the calculations. The chemical structure is entered and the program calculates IR and Raman spectrum based on Gaussian orbital and quantum mechanics of atoms in a series of itineration. Depending on the complexity of the structure the calculations can take from few hours to months. Dichlobenil and BAM calculations were completed overnight.

The numbering of the denominations list follows the atom number on the structure picture, not the chemical nomenclature. Below is presented the results of the simulation of Dichlobenil showing a table of the frequency and Raman activity with the atom motion denominations in the right column.

| frequency | raman activity | denomination |
|---|---|---|
| 79.34 | 0.0433 | Skeleton wag |
| 139.6041 | 4.1319 | N1 rock |
| 197.8351 | 0.948 | Skeleton torsion |
| 201.436 | 2.137 | Cl 1 and Cl 2 scissor |
| 202.5232 | 0.0017 | C5 rock |
| 359.613 | 0.2687 | C7 to N1 wag |
| 361.9588 | 5.211 | Skeleton symetric strech |
| 421.1216 | 1.9764 | Skeleton rock |
| 424.2731 | 7.8498 | Skeleton torsion |
| 541.837 | 1.2672 | C4 and C6 torsion |
| 550.5046 | 20.6042 | Skeleton symetric strech |
| 583.2421 | 0.8208 | Skeleton twist |
| 604.7375 | 0.164 | C7 wag |
| 740.7358 | 0.1594 | Sleleton rock |
| 789.8201 | 4.3064 | Skeleton symetric strech |
| 790.6583 | 0.0144 | H1, H2, H3 rock |
| 798.7489 | 0.578 | Skelton torsion |
| 910.2879 | 0.1425 | H3 and H1 asymetric rock |
| 996.7289 | 0.0004 | H1, H2, H3 asymetric rock |
| 1087.3086 | 25.4142 | H1 and H3 scissor |
| 1113.314 | 13.0907 | Skeleton strech, Cl-C bonds |
| 1182.9503 | 2.1046 | H2-C2 wag |
| 1221.9526 | 3.0656 | H1 and H3 wag |
| 1225.7893 | 47.0289 | H1 and H3 scissor |
| 1300.2682 | 16.7948 | Skeleton twist |
| 1459.5308 | 9.0754 | C5-C7 strech |
| 1461.3519 | 0.6102 | H1, H2, H3 wag |
| 1589.9553 | 7.4 | Skelton torsion |
| 1616.4279 | 60.2419 | C3-C4 and C1-C6 strech |
| 2341.7933 | 337.8472 | N1-C7 strech |
| 3186.3416 | 86.097 | H1, H2, H3 asymetric strech |
| 3209.8325 | 64.5005 | H3-C3 and H1-C1 asymetric strech |
| 3213.8712 | 196.1049 | H1, H2, H3 symetric strech |

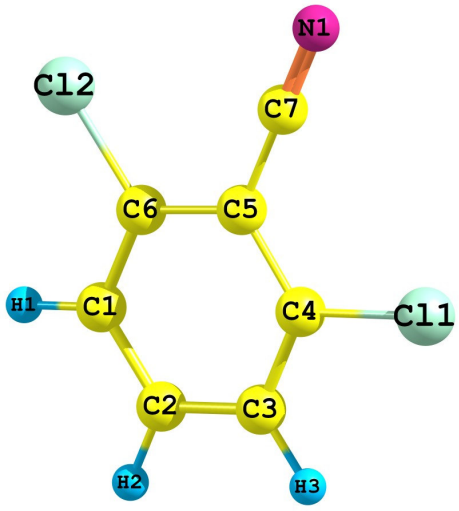



**Figure 8-8: (Left) Dichlobenil signal frequency and atom movement denominations. Structure of Dichlobenil (Right) visualized by Chemcraft based on data calculated by the Gaussian Inc. program.**

Dichlobenil has 33 Raman activity peaks arising from its 33 degrees of freedom. The most noticeable peaks is the Nitrogen to carbon stretch at 2342 $cm^{-1}$ that is not found on the BAM molecule, and various hydrogen atom signals (3214+3210+3186+1461+1226+1087 $cm^{-1}$). Hydrogen is in general causing high Raman signals because of the light and polarized structure. Likewise, the Raman simulation was done on the BAM structure, the structure and spectrum is seen below.

| frequency | raman activity | denomination |
|---|---|---|
| 32.0277 | 4.5629 | C7 twist |
| 74.0334 | 2.9943 | Skeleton Rock |
| 142.0769 | 1.8392 | C7 wag |
| 170.262 | 4.2211 | C5 angle bend |
| 184.9044 | 2.1239 | Cl 1 and Cl 2 scissor |
| 202.8868 | 1.6938 | C1 and C3 twist |
| 314.0911 | 6.0919 | Skeletom Strech |
| 372.7199 | 0.8306 | Skeleton wag |
| 391.0405 | 0.7574 | N1 rock |
| 434.3141 | 8.167 | Skeleton twist |
| 441.9519 | 18.4655 | Cl 1 and Cl 2 symetric strech |
| 521.6228 | 1.4192 | H5 wag |
| 544.8641 | 1.4637 | H1, H3, H5 wag |
| 586.038 | 2.4666 | Skelton rock |
| 622.3302 | 2.1346 | H4, H5 wag |
| 632.6487 | 5.3514 | Skeleton strech |
| 759.0708 | 0.0053 | Skeleton wag |
| 774.8552 | 1.6514 | Skeleton Rock |
| 779.9999 | 0.4104 | C7 wag |
| 790.1491 | 2.4218 | Skeleton strech |
| 823.9938 | 1.6882 | H1, H2, H3 symetric rock |
| 945.3725 | 0.7203 | H1, H3 asymetric rock |
| 1021.829 | 0.2305 | H1, H2, H3 asymetric rock |
| 1083.4537 | 14.6685 | H1, H3 symetric scissor |
| 1105.6447 | 13.3727 | Skeleton strech |
| 1120.732 | 5.7549 | NH2 angle bend |
| 1193.4824 | 6.7163 | C5 to C7 strech |
| 1201.2852 | 0.5055 | H2 wag |
| 1248.1665 | 2.7775 | H1 and H3 wag |
| 1346.9146 | 6.7184 | Skeleton strech |
| 1387.619 | 8.4238 | C7 to N1 strech |
| 1486.4908 | 0.2257 | Skeleton wag |
| 1487.0951 | 0.995 | C5 to C7 strech |
| 1610.4668 | 14.2593 | Skeleton wag |
| 1645.2669 | 20.4083 | Skeleton strech |
| 1671.1601 | 7.9501 | H4 H5 scissor |
| 1726.8687 | 10.0395 | C7 to O 1Strech |
| 3221.5663 | 78.451 | H2 to C2 strech |
| 3246.5974 | 70.6708 | H1 and H3 asymetric strech |
| 3252.6005 | 189.8729 | H1, H2, H3 symetric strech |
| 3602.74 | 156.9009 | H4 and H5 symetric strech |
| 3742.8742 | 69.8695 | H4 and H5 asymetric strech |

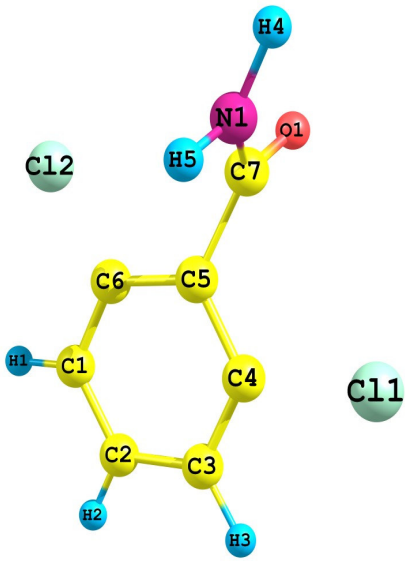



**Figure 8-9: (Left) BAM signal frequency and atom movement denominations. Structure of BAM (Right) visualized by Chemcraft based on data calculated by the Gaussian Inc. program.**

BAM has 42 signal sources. A specific signal for BAM is the oxygen to carbon stretch causing a modest signal at 1727 $cm^{-1}$, the hydrogen atoms are the cause of 15 distinct peaks, where the highest signal is from the triplet of Hydrogen nr 1, 2, 3 at 3743+3603+3253 $cm^{-1}$.

The simulation signal peaks of BAM and Dichlobenil can be compared on a graph, where the scaling is not absolute (as it depends on laser source and detector). The graph can be used to compare overlap on the BAM and Dichlobenil signal.

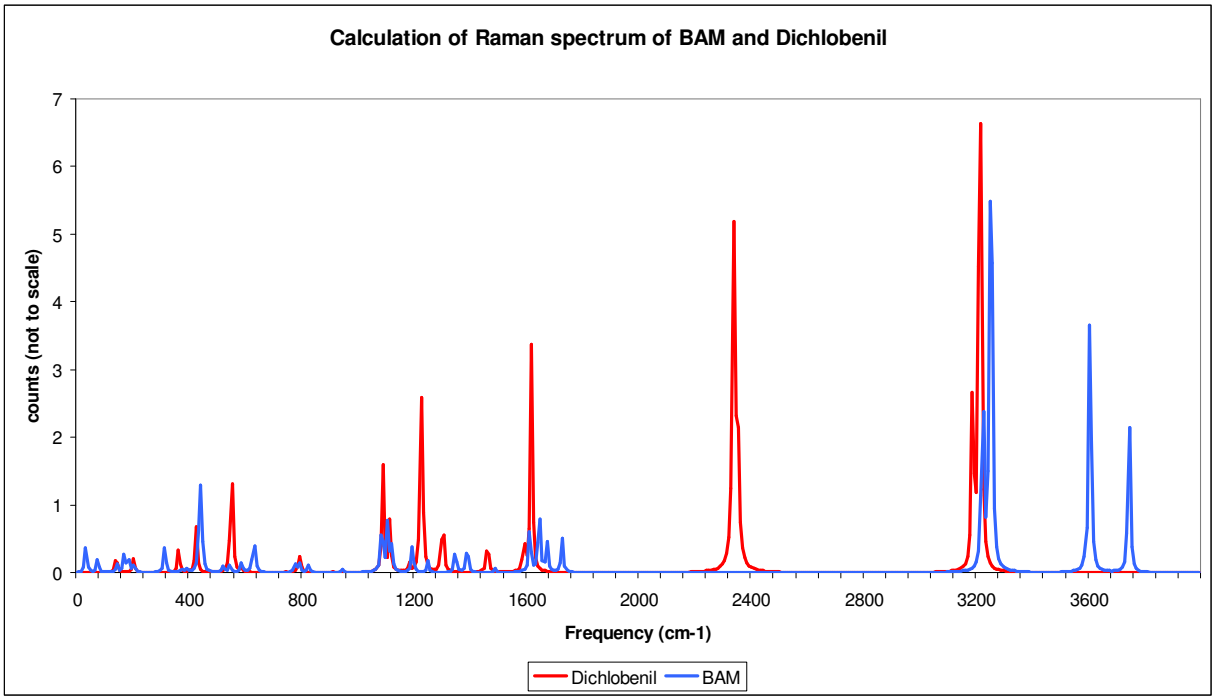



**Figure 8-10: Raman calculations of peak values of BAM and Dichlobenil, the counts are not absolute.**

Dichlobenil has higher and more distinct peaks compared to BAM from 1000 to 1600 $cm^{-1}$ and the nitrogen to carbon stretch movement cause a single large Raman signal at 2342 $cm^{-1}$, together with the peaks at 1616+1226+1087+550 $cm^{-1}$. It can be concluded that Dichlobenil has peaks that could be used for identification. Useful identification peaks for BAM could be the peak at 442 $cm^{-1}$ from the Chlorine atoms, triplet peaks from the aromatic ring (skeleton wag/stretch) and hydrogen at 1610 to 1671 $cm^{-1}$, and 2 large peaks at 3602+3743 from hydrogen no.1, 2, 3 on the aromatic ring. Although these are simulated values and the actual Raman signal values can be shifted, it is a useful tool for the preliminary investigation to determine whether the molecule is suitable for Raman identification. The program also produces simulation of the infrared spectrum, but it is not shown as it is beyond the scope of the thesis.

## 8.6 Conclusion on Raman detection

The classical bulk Raman measurements has shown a clear and recognizable spectrum of BAM and Dichlobenil. To the author best knowledge, no bulk Raman spectrum data of Dichlobenil and BAM has been publicized. The SERS spectrum of BAM and Dichlobenil acquired with the in house detector is not comparable to the data from the classical Raman setup, partly due to the different detectors used in the two systems. The spectrum of BAM show a triplet peak around 1600cm-1 on the calculated data, this is found in both the classical Raman data and SERS data, although the SERS data had a larger noise level and less distinct peaks, this could be used as a target peak for the detection of BAM in ground water samples.

The frequency shifts values of the calculated Raman spectrum, are poorly comparable to the spectrum obtained from the bulk Raman. The difference is partly caused by effects from the detector, and partly from the simulation quality of the spectrum. It can be debated whether the calculated, or the bulk Raman spectrum, can be regarded as most representative of the actual Raman shift spectrum of BAM and Dichlobenil.

# 9 Conclusion

The aim of the Ph.D. thesis was to investigate on the development of an autonomous system for the measurement of a pesticide in drinking water. A cantilever based surface stress read out method was chosen as the primary measurement principle for the detection of the 2,6-dichlorobenzamide (BAM) pesticide residue. As the chosen Cantion Canti4 system did not produce satisfactory results, an alternative optical cantilever system was investigated as surface stress read out method. Surface Enhanced Raman Spectroscopy (SERS) was investigated as a secondary alternative to the surface stress read out method for the detection of pesticides in water samples.

The Cantion Canti4 system was characterized using a DNA assay and a BAM assay. The characterization revealed a number of factors influencing the signal on the system. Re-use of the chip for multiple analysis, revealed that the gold surface of the cantilever rapidly deteriorated. Although functionality of the chip was intact, the damaged gold surface could not be expected to yield an optimal signal from a BAM assay. The characterization work eventually led to the conclusion, that the chemical part of the assay needed to be optimized separately and not in conjunction with the Canti4 system.

In order to optimize the induced surface stress on the cantilevers surface, and thus maximize the obtainable bending signal, a series of investigations were done on the chemistry used in the BAM assay. The optimization involved the use of an established BAM ELISA, microarray technique for surface quality analysis, and a flow cell system for the online monitoring of the BAM assay conditions.

The principle of the competitive BAM assay is the attachment of BAM antibody to BAM immobilized on the surface of a cantilever; the change of surface stress induced a bending that is detectable via an imbedded piezo layer [21]. The attachment of BAM antibody to a BAM functionalized cantilever was tested repeatedly on the Canti4 system in two rounds of experiments. The Cantion Canti4 system did yield 10 repeated signals from the addition of BAM antibody to the BAM functionalized cantilevers. The differential signals obtained were

varying from 5 to 20 µV. As a signal was also obtained from unspecific antibodies, the signal could not be regarded as specific for the BAM antibody. The signal profile was found to have a high variance from chip to chip, and had a poor reproduction of signal on the same chip. The use of fluorescent marked antibodies confirmed the attachment of BAM antibodies on the cantilever surface and low unspecific binding. Fluorescent and visible light pictures indicated that the functionalization step of the cantilevers was not fully homogenous on the cantilevers surface. The increase in variation of the resonance frequency and bending of the cantilever showed that this did indeed have an influence on the mechanical properties of the cantilevers. The summary of this work was submitted for publication in Nanoscale Research Letters journal.

As an alternative to the piezo induced read out system used by the Canti4 system, an optical read out system was chosen. The principle has been used in a large number of cases for the detection of antibodies [[33]and nucleic acid hybridization [7]. A platform developed in the X-sense project in the Nanoprobes group, was used as an alternative optical method for the readout of the surface stress on the cantilevers. The platform uses the optics from a DVD player and is capable of scanning along the length of the cantilever scanning up to 90 chips with 8 cantilevers each in parallel. The experiments were done on cantilevers in air (the Cantion system experiments were done in fluid). The results revealed a significant differential bending of the BAM functionalized cantilevers, when BAM antibody was added. The DVD system also revealed an increase in surface roughness of the reference cantilevers. This was found useful as an additional quality monitoring feature of the cantilevers surface. A description of The DVD system, including the results obtained with the BAM assay, was submitted for publication in “Nano Letters” journal.

As the SERS method has been successfully used for the quantitative detection of small analyte [36] and specifically for pesticide detection [39], it was investigated as a secondary principle for the direct label-free detection of pesticides in water samples. In the Ph.D. project, the bulk Raman shift and SERS spectrum of BAM and Dichlobenil was recorded. A simulated Raman spectrum was calculated, and used for comparison of the data obtained from the bulk Raman spectrum and SERS spectrum. This work has not yet been submitted for publication.

# 10 Outlook

As the Canti4 assay did not yield satisfactory results, and as no further Canti4 chips are available from Cantion/Nanonord A/S, no further use of the Canti4 system is currently planned.

The SERS method is to be further investigated for the use of BAM detection, in collaboration with Michael Stenbæk Schmidt from Nanoprobes group DTU Nanotech. Water samples donated from the Danish water work are to be analyzed using the SERS substrate.

The DVD IBM cantilever setup is planned to be used for dose dependent analysis of the BAM assay. Furthermore the Aptamer assays donated by Daniel Dupont and Jørgen Kjems from iNANO Aarhus, are to be used for analysis on the DVD IBM cantilever setup in collaboration with Filippo Bosco.

Both the DVD IBM system and SERS system is a part of the X-sense project at DTU Nanotech. The SERS substrate is planned to be integrated in the fluidic part of the DVD setup, accompanying the IBM cantilever laser readout system and providing an additional Raman spectrum analysis of the samples analyzed in the integrated DVD system. If successful, the integrated system can be a strong future candidate for a combined system for the detection of pesticides in water samples and other organic compounds in air.

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# Publications

Invited proceedings from Trends in Nanotechnology conference, Braga , Portugal 2010. Article submitted in October 2010 to be publicized in “Nanoscale Research Letters" special edition in may 2011. 2009 impact factor is 2.894.

- Bache Michael, Taboryski Rafael, Schmid Silvan, Aamand Jens, Jakobsen Mogens Havsteen. “Characterization of a cantilever system for a BAM pesticide assay”.

Co-author on submitted article to “Nano Letters” journal. Submitted December 2010. Manuscript ID: nl-2010-04375g. 2009 impact factor is 9.991.

- Bosco, Filippo; Hwu, En Te; Chen, Ching-Hsiu; Keller, Stephan; Bache, Michael; Jakobsen, Mogens; Hwang, Ing-Shouh; Boisen, Anja. "Towards a high throughput nanomechanical sensor platform for molecular recognition"

The articles are presented in appendix A and B.

# Appendix A

## Characterization of a cantilever system for a BAM pesticide assay

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**Abstract**

A 2,6 dichlorbenzamide (BAM) pesticide residue assay has been performed repeatedly using a cantilever based detection system. The stress induced by the binding of Anti-BAM antibody to BAM molecules immobilized on a cantilever surface, is measured using the CantiLab4© system from Cantion A/S with 4 gold coated cantilevers and piezo resistive readout. The detection mechanism is in principle label free, but fluorescent marked antibodies have been used to subsequently verify the binding on the cantilever surface. The bending and increase in mass of each cantilever has also been investigated using a light interferometer and a Doppler vibrometer. The system has been analyzed during repeated measurements to verify the BAM assay and characterize the CantiLab4© system.

**Key words:** Pesticide detection, cantilever, surface stress, characterization

### Introduction

During the last 10 years an increasing number of water wells have been polluted by pesticides or its break down products. BAM is amongst the most frequent found pesticide residues in European groundwater. As pesticide analysis of drinking water is currently being done by laboratory analysis, an in-line sensor will therefore be beneficial for water quality monitoring. Cantilever based assays for pesticide detection [[20],2] has been reported, but in general few description of repeated measurements of cantilever based detection systems are available. As a central principle of a possible cantilever based competitive assay, we have tested the binding of BAM antibody to a cantilever surface coated with BAM. In a working assay the BAM molecules in a water sample would compete with BAM attached to a cantilever surface for the binding to anti-BAM monoclonal antibodies, similar to a BAM ELISA described by Bruun et.al.[[21]]. The binding of anti-BAM antibodies to the surface of the cantilever will change the surface stress, causing bending of the cantilever. The bending is then detected by a change in resistance of the imbedded piezoelectric layer in the cantilever [[14],[15],6]. To investigate the effects induced by the antibody binding to the cantilever surface, the bending profile, resonance mass/stiffness and fluorescent signal was recorded from the clean cantilevers, after antigen spotting and after the antibody was added.

### Materials and Methods

A cantilever system CantiChip4® from NanoNord/Cantion A/S was chosen for the assay. The bending of the cantilever causes a proportional change in voltage between the piezo layer in the cantilever and a fixed resistor embedded in the chip measured via a Wheatstone bridge setup. The system consists of 4 silicon based cantilevers with integrated piezo resistive readout. All 4

cantilevers are 120µm length x 50µm width x 0.45µm thickness, coated with a 40 nm gold layer, electrically grounded, and flip chip bonded to a contact pad that is inserted in the CantiLab4© that converts the voltage signal to a proprietary recording software [[18]]. The functionalization of each cantilever was done using a micro-spotter from Cantion A/S with a piezo electric controlled pin head (GESIM Sub-Micro liter Piezoelectric Dispenser A010-006 SPIP) in a xyz stage setup monitored via a camera and a PC interface. A 2,6 dichlorbenzamide hapten (BAM hapten EQ0031) and ovalbumine conjugate was synthesized following Bruun et.al.[[21]]. The BAM ovalbumine conjugate was dialyzed 3x in 1x PBS buffer, and diluted to 0,75 mg/ml of ovalbumine in 1x PBS. The BAM-ovalbumine conjugate was determined to contain 5 units BAM/Ovalbumine via a UV-visual spectrophotometer method and was tested positive for BAM via an ELISA [[21]].

On an inspected, tested and clean CantiChip4®, 3 drops of 0,75 mg/ml BAM-ovalbumine was micro spotted on cantilever B and C, using a tip voltage of 100 V and pulse width of 20 V. Cantilever A and D was used as reference and was equally micro spotted with a 1 mg/ml ovalbumine in 1x PBS buffer solution (Fig.1). The chip was incubated overnight in a humidity chamber. A functionalized chip was inserted in the CantiLab4© connected to a fluidic system that consisted of a syringe pump and an 8 channel switchbox. The pump flowed a 1xPBS 0,05% Tween 20 pH 7.4 buffer at 20 µl/min either through a primary or a secondary 100µl coil loop. This allowed for the continuous flow of buffer in the primary loop while the secondary loop was filled with a 100µl sample, thus avoiding air bubbles and possible temperature gradients. To avoid a battery effect between the chip and fluidic system, the waste bottle was connected to the CantiLab4© electronic box with a gold wire (figure 1). The system was allowed to heat up and stabilize with a continuous flow for approx. 1 hour, while a base line was recorded. The system was then tested against any signal induced by loop switching and against signal due to buffer injected as a sample. To test for any unspecific antibody hybridization signal, a sample of 100µl of 0.1 mg/ml unspecific Cy5 labeled (Amersham Cy5 Dye™ Antibody monofuctional Labeling Kit) mouse Immunoglobulin G (Sigma-Aldrich reagent grade I5381-1 mg, lot.no.025K7580 ) was injected. Following a 5 min. buffer, 100µl of the 0.1 mg/ml Cy3 (Amersham Cy3 Dye™ Antibody mono functional Labeling Kit) labeled BAM antibody (Statens Serum Institut, HYB 273-01, Batch no.03102P01/071008) diluted in 1x PBS 0,05%.Tween 20 was injected. The Cantion chip was removed from the fluidic chamber after the experiment and briefly rinsed in Milli-Q water to remove PBS salts. Each test and injection phase of the assay was recorded for 2000 seconds; the total experiment lasted typically 3½ hours, depending on quality of the output signal and the stabilization period

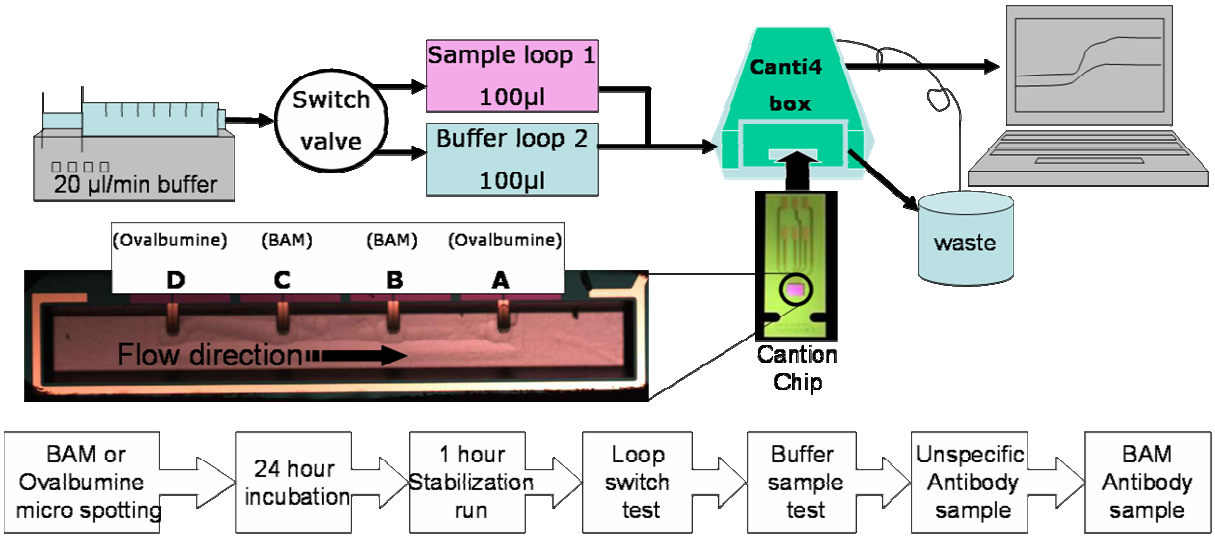


**Figure 1: (Above) A schematic overview of the fluidic setup. (Below) A flowchart of the BAM assay on the CantiChip4® system.**

In order to verify the binding of antibodies to the cantilever surface and control for unspecific binding, a set of fluorescent pictures of Cy5 and Cy3 signal were taken after spotting and hybridization. An optical surface profilometer (Polytech TMS-100), based on light interference, was used to analyze the absolute bending of the cantilevers on 5 experiments. To analyze the mass/stiffness values a laser based vibrometer with a piezo actuator (Doppler Vibrometer Polytech MSA 500) was used on 8 experiments [[30]]. All chemicals used in the assay were purchased via Sigma Aldrich; all glassware used was new and only rinsed in Milli-Q water to avoid any unwanted effect from surfactants.

**Results and Discussion**

Twenty chips were selected for the BAM assay based on signal stability while running in air mode and buffer. Of twenty experiments, ten gave a signal when adding BAM antibody (with five exp. giving a differential signal above 0,01 mV) (Fig.3). Seven experiments gave no signal, and three chips were discarded after functionalization, due to too high initial voltage difference between the cantilevers. A signal from the addition of specific BAM antibody, as well as from the addition of unspecific antibody appeared on all 10 experiments. Baseline noise was typically in the range of 0.004 to 0.002 mV. (Fig.2 left). As observed by V. Dauksaite et.al. [[15]] Almost all experiments had a signal drift effect during the experiment varying from a few µV to several mV per Hour, possibly caused by a variance in the resistance of the internal resistors of the chip. As the absolute bending signal was not suited to evaluate the experiment, four differential values were chosen (Fig.2 right).

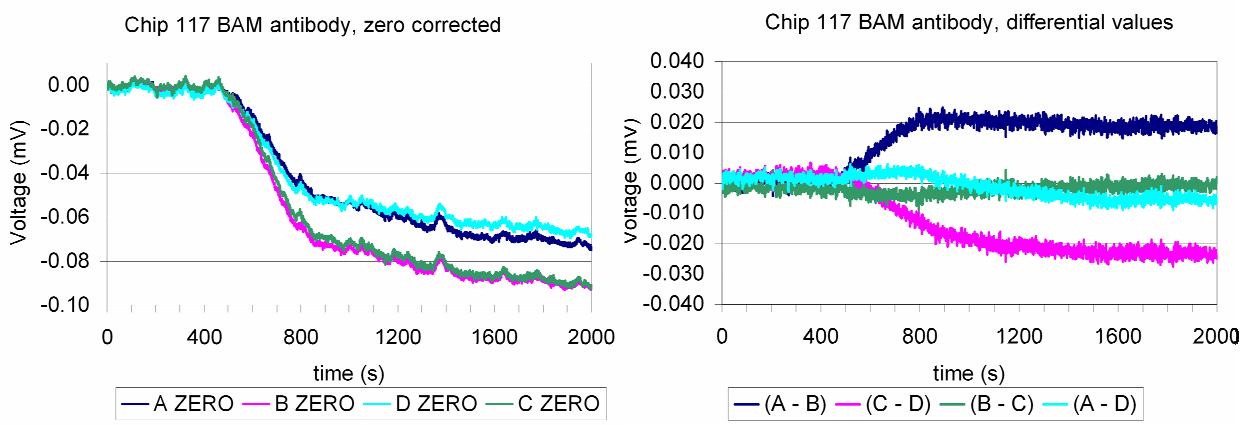


**Figure 2:(Left) A bending signal from cantilever A,B,C,D normalized to zero at t=0 under the addition of BAM antibody. (Right) The differential values of A (active) - B(reference), C(reference) - D(signal ), B(signal) – C(signal ), and A(reference) – D(reference). The antibody is added at t =200 and enters the cantilever chamber at t=500, gradually causing a lasting differential signal of ~0,02 mV for the specific antibody.**

Although only the differential values were used for evaluation, the signals obtained show a very diverse and distinct signal profile in between, but has a similar signal profile between the specific and unspecific antibody on each experiment (Fig.3).

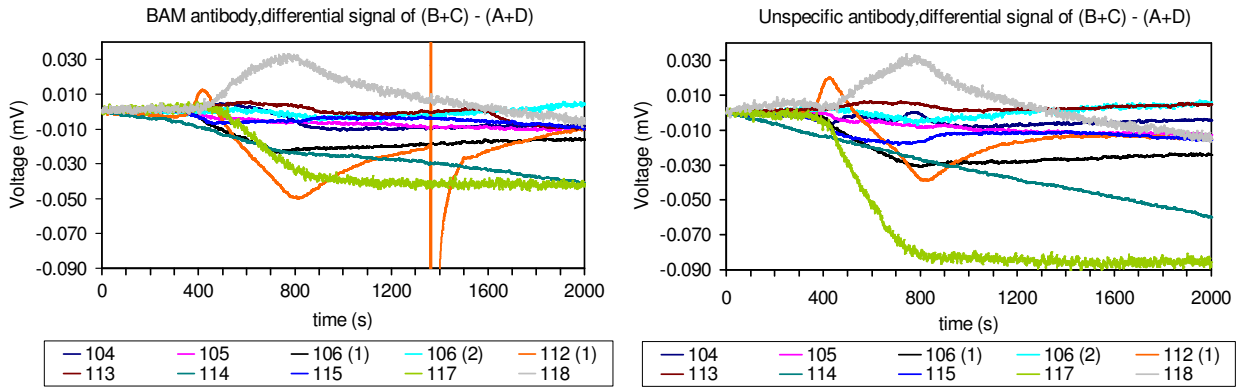


**Figure 3: Comparison of bending signals from 10 experiments showing the differential signal between the 2 active coated (BAM) and 2 reference coated (Ovalbumine) cantilevers. Plotted as signal (mV) of (B+C) – (A+D) as a function of time (s) during the addition of BAM antibody (left) and unspecific antibody (right).**

As the antibody binding is mainly controlled by electrostatic forces [[31]] and the bending signal was found to be very sensitive to minute changes in pH, salinity and temperature gradients [[32]], we wanted to investigate whether the unspecific antibody signal was caused by a high (0,1mg/ml) antibody concentration, 2 experiments with 1/10 (0,01 mg/ml) antibody were done, but unfortunately these showed no signal when antibody was added (data not shown). When an air bubble blocked the fluidic system, or if the waste bottle grounding to the CantiLab4© instrument was disconnected, a peak of 0.1-1 mV was observed. Occasionally a weak loop switch effect of 0,01-0,004 mV was also observed when switching the fluidic loop,

this was probably due to minute changes of pH or salinity in the sample buffer resting in the sample coil, the effect immediately disappeared after one loop switch. In order to assure that the BAM antibody was attached to cantilevers B and C and no unspecific antibody was attached, the antibodies were fluorescently marked respectively with Cy3 and Cy5. (Fig.4). All 10 experiments had a similar Cy3 signal from BAM antibody on cantilever B and C and little or no signal on cantilever A and D. No significant amount of Cy5 marked unspecific antibody was found on any cantilever after the experiment.

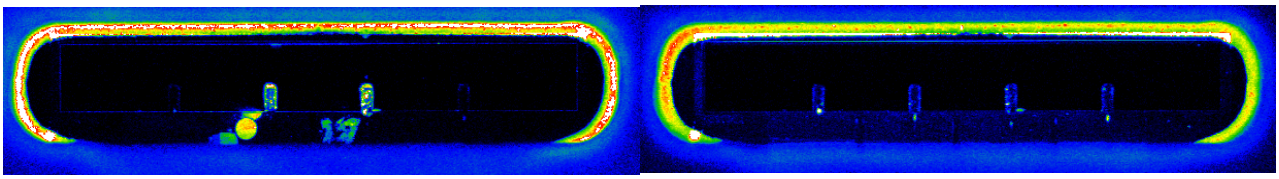

**Figure 4: (Left) A clear hybridization signal (ex. 550 nm, em. 570 nm) of Cy3 marked anti-BAM to cantilever B and C and none on A and D. (Right) Fluorescent picture of Cy5 signal (ex. 650 nm, em. 670 nm) from chip 113 showing a low background signal of Cy5 marked unspecific mouse antibody.**

A tendency to an increase in stiffness was observed after the micro spotting step, probably caused by the PBS salt in the ovalbumine layer (Fig.5, left), but was not significant. The deflection values showed a clear bending of all cantilevers after the antigen was added, probably caused by PBS buffer used in the micro spotting of BAM-ovalbumine conjugate and ovalbumine. The cantilevers bended back to the clean state after the experiment was performed (Fig.5 right), as the salts were washed off.

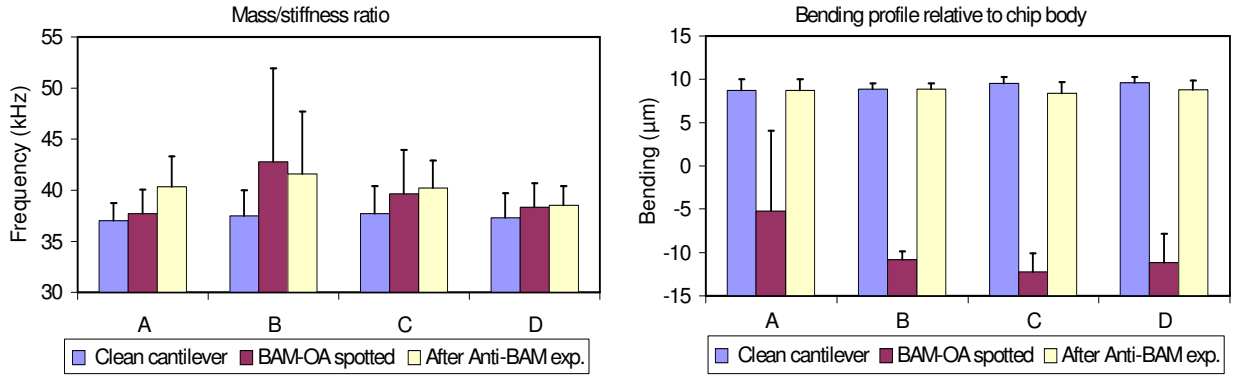


**Figure 5:(left) Mass/stiffness ratios of each of the 4 cantilevers divided in 3 groups: clean chip, after micro spotting, and after the experiment. The values are an average of 8 experiments (104, 108, 112, 116, 117, 118, 119, 120). (Right) Average bending values (µm) of cantilever tip relative to the chip body surface. Values are averages obtained from 5 experiments (116,117,118,119,120).**

Although 3 chips were discarded during the 20 experiments, the Cantion chips were able to perform continuous readout of several days with no increase of noise. Compared to known optical read out systems [[7],[33],[16]], the Cantion system was sturdy and more simple to handle. The Cantion chips could be re-used following a harsh rinsing protocol, this opens up the possibility of regeneration of the surface chemistry thus making repeated assays using only

one sensor in an automated system. However the sensitivity of the assay is not comparable to current ELISA standards; the piezo resistive readout is less sensible compared to the optical readout and a signal from the unspecific binding was obtained on all experiments.

**Acknowledgements**
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# Appendix B

# Towards a high throughput nanomechanical sensor platform for molecular recognition

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Sensors are crucial in many daily operations including security, environmental control, human diagnostics and patient monitoring. Screening and on-line monitoring require reliable and high-throughput sensing. We report on the demonstration of a high-throughput label-free sensor platform utilizing cantilever based sensors. These sensors have often been acclaimed to facilitate highly parallelized operation. Unfortunately, so far no concept has been presented which offers large data sets as well as easy liquid sample handling. We use optics and mechanics from a DVD player to handle liquid samples and to read-out cantilever deflection and resonant frequency. Also, surface roughness is measured and is discovered to hold valuable information on specific and unspecific binding events. In a few minutes, 90 liquid samples can each be analyzed in parallel by 8 cantilever-based sensors. The approach was used to detect the binding of streptavidin and antibodies. Micrometer and even nanometer sized cantilevers have since the mid-1990s been studied and used for label free molecular recognition (*1-9*). There, the cantilever is typically functionalized with probe molecules designed to specifically bind certain target molecules in solution. The specific binding of target molecules causes the cantilever to deflect due to a change in surface stress. Alternatively, the mass change of the cantilever can be monitored by measuring the resonant frequency change of the cantilever which is inversely proportional to the added mass. Cantilevers are unique probes for studying for example small molecule drug binding interactions (Rachel) and virus binding to cell membranes (hegner). Often the microscopic size of the cantilevers is highlighted as a virtue for parallelization. Parallelization and easy sample handling is needed for high throughput screening of thousands of liquid samples per hour. This has to date not been solved and a solution is crucial for a potential commercial breakthrough of the sensor technology.

**Methods for parallel cantilever sensning**

Today, the prevalent method of monitoring vibrational amplitudes and cantilever deflection is based on the optical leverage technique widely used in atomic force microscopy (*10*). Such systems are typically bulky because of the requirement for a long optical path. Also, the focusing of the laser spot on the cantilever and the alignment of the laser beam on the optical detector are tedious and time consuming. Alternatively, a CCD camera has been used for monitoring cantilever deflection and hereby large 2-dimensional cantilever arrays can be read simultaneously (*11*). However, all cantilevers have to be in the same focal plane which is extremely difficult to achieve in practice and both techniques only apply to micrometer sized cantilevers. In optical leverage the laser spot size is typically 20 μm or above and in the CCD system the amount of reflected light is too low for smaller devices. Integrated read-out has

been suggested by several groups. For example, cantilevers with piezoresistive (*12, 13*), piezoelectric (*14, 15*) and MOSFET-based (*16, 17*) read-out have been developed and applied for molecular recognition. Generally, these cantilevers have to be carefully insulated in order to be operated in liquid and the devices require significantly more packaging due to electrical interconnections.
Typically, the cantilevers are placed in small polymer or ceramic chambers and different liquids are introduced using i.e. syringe pumps. The pumps are a potential noise source and the liquid handling is performed in a serial manner and is tedious and slow. Because of primarily the instrumentation, few papers on cantilever-based sensing present statistically analyzed data sets. Sensing is normally performed on one or maybe two cantilevers at a time (one for reference) since measurements are rather elaborate and time consuming.

**A high throughput platform**
We report on a DVD based sensor platform that significantly reduces the aforementioned obstacles and challenges in cantilever based sensing and liquid handling. The concept is highly scalable and we can in this initial design analyze 90 different liquid samples in parallel. Also, the monitoring of three physical parameters (deflection, resonant frequency and optical roughness) increases the reliability of data. A DVD shaped disk is used to mount up to 90 cantilever chips, each with 8 cantilevers, in a radial symmetry, see Fig 1. In this work, silicon cantilevers with a length of 500 μm a width of 100 μm and a thickness of 1 μm have been used (*18*). All cantilevers are coated on one side with a 20 nm thick gold layer. The cantilever chips are clicked into individual reservoirs (*19*) after functionalization. Approximately 1 mm below the disk four DVD-ROM optical pickup heads (PUHs) provide the read-out system. The disk is spun and cantilevers are illuminated by the DVD lasers with a wavelength of 650 nm and a spot diameter of only 0.56 μm (Full Width Half Maximum). The deflection profiles are measured using the astigmatism-based detection mechanism normally used for auto focusing (*20*). Each PUH can measure the cantilever deflection with a resolution better than 1 nm in Z direction. We have measured cantilever deflections at rotating velocities up to 120 rpm, which equals approximately 500 cantilevers per second per PUH. The DVD disc format has in the past 10 years been widely used for liquid handling. The centrifugal forces generated by spinning the disc can be used to move liquid from the center of the disc and towards the edge(*21, 22*). In our design liquid can be handled using capillary valves which burst at well-defined threshold frequencies ($\omega_t$) (*23*). These allow precise sample dispensing to the reservoirs where the cantilever chips are clamped.
A photograph of the realized DVD platform with mounted cantilever chips is shown in Fig. 2A. A reflective aluminum pattern on the disk surface ensures that the DVD-ROM PUH maintains the focus distance. The laser scans from the bottom, passing through the glass substrate and focuses on the cantilever surface (Fig. 2B). Typical sampling rate corresponds to around 1000 measurement points across the width of each cantilever. We thus obtain a profile where data points are acquired every 100 nm along the width of the cantilever.

**Signal acquisition**
Raw signal acquired during one revolution of the disk is shown in Fig. 2C. The plot is composed of around 1.000.000 data points. Each distinguishable peak represents a chip with 8 cantilevers. Typical experiments consist of 30-50 revolutions, resulting in up to 50 million

measurement points. Zooming in on Fig. 2C we can extract the individual cantilever profiles, as seen in Fig 2D.
Before sensing experiments are performed, each cantilever is fully characterized by at least 10 revolutions of the disk. The variance of the measurements is used to evaluate the reliability of the data. After data processing, it is possible to obtain a detailed statistical analysis of the initial conditions of the cantilevers in air. The histogram in Fig. 2E shows the distribution of initial cantilever bending from 30 chips (240 cantilevers) measured over 10 revolutions. The average bending is 0.49 μm, with a standard deviation of 0.43 μm.
By combining cantilever scans at sequential radial positions it is possible to construct a 3D image of the cantilever surfaces, see Fig. 2F. In our work, the measured surface roughness is used to evaluate the distribution of biomolecules on the cantilever surface. When inhomogeneous binding of material occurs, the optical properties (refractivity, reflectivity) change, resulting in a "rough" surface. Monolayer-type binding gives an optically smooth surface.

**Molecular recognition**
For a first demonstration of biomolecular binding, 8 cantilevers were functionalized with thiolated biotin and 8 untreated cantilevers were used for reference measurements. The chips were inserted into the DVD platform and exposed to a buffer solution containing streptavidin. After exposure, all cantilevers were washed in water. The water was left to evaporate and the cantilever responses were measured continuously. Fig. 3A shows the averaged 3D reconstruction of 8 untreated cantilevers, measured before the injection of streptavidin into the cantilever reservoir. The surfaces have a roughness of a few nm, indicating that the gold layer is clean. The initial deflection (at the cantilever apex) is around 5 μm. After the injection of streptavidin and the washing step the same cantilevers show a high increase in surface roughness, suggesting that an inhomogeneous layer has formed. Additionally, the deflection of the cantilevers has changed approximately 1 μm. Both observations strongly indicate that streptavidin has bound unspecifically to the cantilever surface.
The cantilevers functionalized with biotin are initially bent 6-7 μm at the cantilever apex and the surface appears optically smooth, see Fig. 3C. This reflects that the biotin functionalization has created a monolayer on the gold surface of the cantilevers. After the biotin-streptavidin binding has occurred, the observed change in cantilever bending is approximately 3 μm and the roughness of the surface appears unchanged, indicating that streptavidin has been uniformly bound to the biotin layer.
In Fig. 3E a statistical analysis of the change in the bending of the cantilevers is shown. Each data point corresponds to the averaged value from 8 cantilevers. We notice, that after the injection of streptavidin the bending of the untreated cantilevers decreases, reaching an asymptotic value after around 15 disc revolutions (corresponding to approximately 5 minutes). At this stage the water has fully evaporated and stable measurement conditions can be obtained. Similar behavior (but opposite direction) is observed for the biotin functionalized cantilevers. These biotin functionalized cantilevers show an averaged deflection which is approximately 2 μm larger than for the untreated reference cantilevers when the measurements have stabilized. The averaged change in surface roughness (Fig. 3F) is significant for the untreated cantilevers compared with the functionalized ones suggesting that an irregular avidin layer is formed on the untreated cantilever whereas a uniform layer as expected is formed on the biotin-coated surface. Thus, we can easily detect the specific binding of avidin since it

results in significant differential changes (biotin functionalized minus untreated cantilever) in cantilever deflection and surface roughness.
Similar experiments have been performed for detection of the pesticide derivative 2,6-dichlorobenzamide (BAM)(*24*).The used protocol has been developed for a competitive assay which implies that the sensing cantilevers are initially coated with a layer of BAM (*25*). Two chips have been prepared for the measurements, each containing 2 cantilevers functionalized with BAM, 2 cantilevers with an ovalbumin blocking layer and 4 untreated cantilevers. The initial bending of the cantilevers is measured as above and specific antibodies against BAM are injected into the cantilever reservoirs followed by a rinse in water and subsequent water evaporation. Fig. 4A shows the induced averaged bending of the differently functionalized cantilevers. The BAM-functionalized cantilevers deflect approximately 10 μm compared with 3-5 μm for the blank and ovalbumin coated cantilevers. Probably, the antibodies bind strongly to the BAM functionalized surfaces causing a large change in surface stress whereas they bind unspecifically to the other cantilevers, illustrated in Fig. 4B. Cantilever profiles reveal that the untreated cantilevers become significantly rough, while the BAM and ovalbumin coated devices are unaffected by the introduction of antibodies. The ovalbumin coated cantilevers are initially rough reflecting the nature of the coating, see Fig 4C. We believe that this is once again an indication that specific binding results in ordered uniform layers whereas the unspecific binding results in a random and rough surface. The specific binding of BAM antibodies is easily detectable due to large differential signals in both deflection and surface roughness
In the BAM experiments we have also tested the capability of the system to measure changes in the resonant frequency using the thermal noise peaks of the cantilevers (*26*). Fig. 4D shows the change in resonant frequency of the 16 cantilevers after reaction with antibodies has taken place and the washing solution has evaporated. The BAM functionalized cantilevers have the highest change in resonant frequency (approximately 10 %) Compared to that, minor changes are observed for the ovalbumin blocked and the untreated cantilevers (1-2 %), which can be attributed to unspecific binding of antibodies as wells as solidification of salt present in the buffer solution. The observed frequency changes are positive which is probably a result of changes in both added mass and surface stress (*27*). The corresponding Q-factors of the cantilevers extracted from the resonant curves (Fig. 4E) generally follow the changes in resonant frequency.

**Conclusion**

The DVD platform offers a number of advantages over traditional cantilever sensing. It readily supplies large amount of data for statistical analysis facilitating the onset of statistical cantilever based sensing. Moreover, the platform allows for simultaneous measurements of deflection, vibrational amplitude and surface roughness increasing the amount of information to be achieved and consequently the reliability of data. We suggest that cantilever surface roughness is included as an additional parameter in cantilever based sensing as it strongly reflects the uniformity of the surface layer. Finally, the platform integrates an already well known and well characterized method of controlling liquids by centrifugal forces. We hope that our concept will be of use for fundamental as well as more applied studies and we believe that the platform opens up for a wide range of experiments in for example drug screening where reliability and statistical significance are crucial.

**Captions**

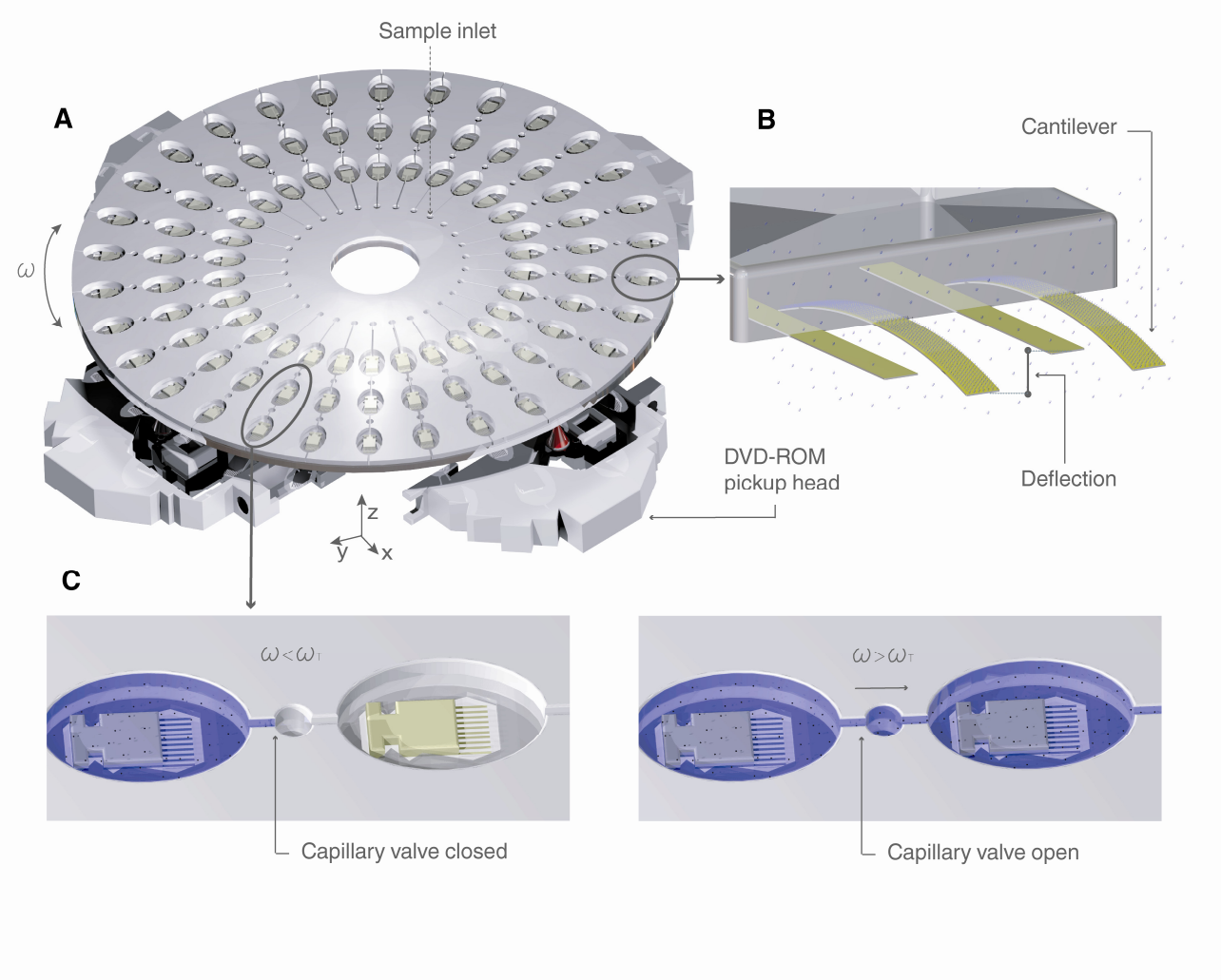
Fig. 1. (A) Schematic of the DVD platform for cantilever-based sensing. High throughput sensing as well as liquid handling are achieved by spinning the disk. (B) Chips, each containing eight gold-coated cantilevers, are mounted on the substrate. (C) The liquid flow is controlled by capillary valves.

Fig 2. (A) Photograph of DVD platform with integrated cantilever chips. The disc is fabricated in glass and the polymer SU-8. (B) Scanning Electron Microscope image of gold-coated silicon cantilevers with dimensions 100 μm x 500 μm x 1 μm. (C) Raw data from one revolution of the DVD. Each peak corresponds to one cantilever chip. (D) The obtained profiles from a single cantilever chip. (E) Distribution of the measured initial bending of the cantilevers. (F) Example of 3D reconstruction of eight cantilever surfaces.

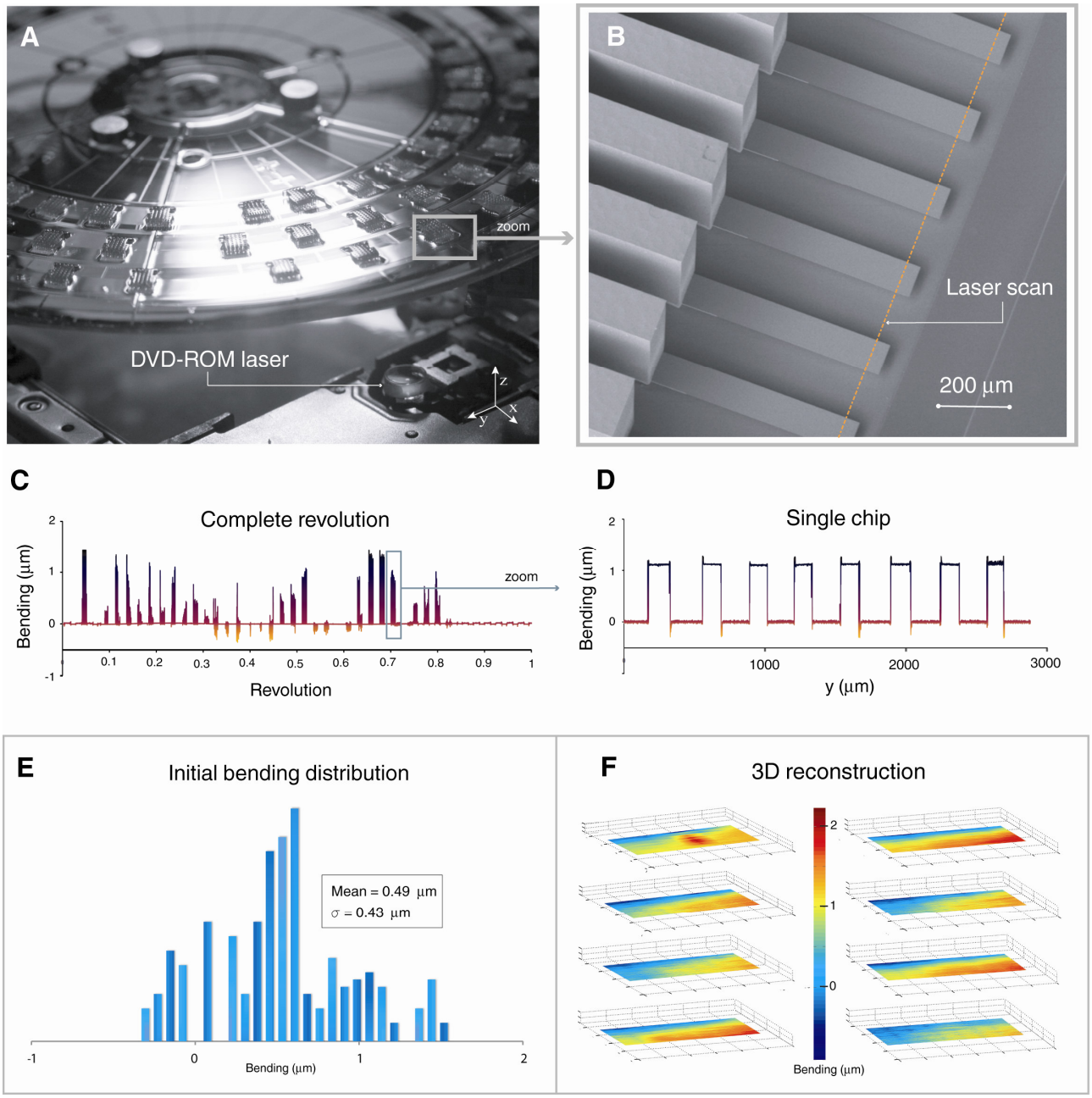
Fig 3. (A) Surface reconstruction of clean cantilever. (B) The same cantilever after exposure to streptavidin solution showing increased roughness. (C) Surface reconstruction of biotin functionalized cantilever and (D) of the same cantilever after reaction with streptavidin. (E,F) Averaged change in cantilever bending and surface roughness for an untreated surface (average value of 8 cantilevers) and a biotin functionalized surface (average value of 4 cantilevers). The blue region indicates the time the cantilever is in contact with the streptavidin solution.

Fig 4. (A) Averaged changes in cantilever deflections when exposed to BAM antibodies. All data points represent averaged values from either 4 (ovalbumin and BAM coated) or 8 (untreated gold-coated) cantilevers. (B) Graphical representation of the differently coated cantilevers. (C) Averaged changes in surface roughness after exposure to BAM antibodies. (D,E) Measured averaged changes in resonant frequency and Q-factor. The significant change for the BAM coated cantilevers indicates binding of the BAM antibodies. The blue region indicates the time the cantilever is in contact with the anti-BAM solution.

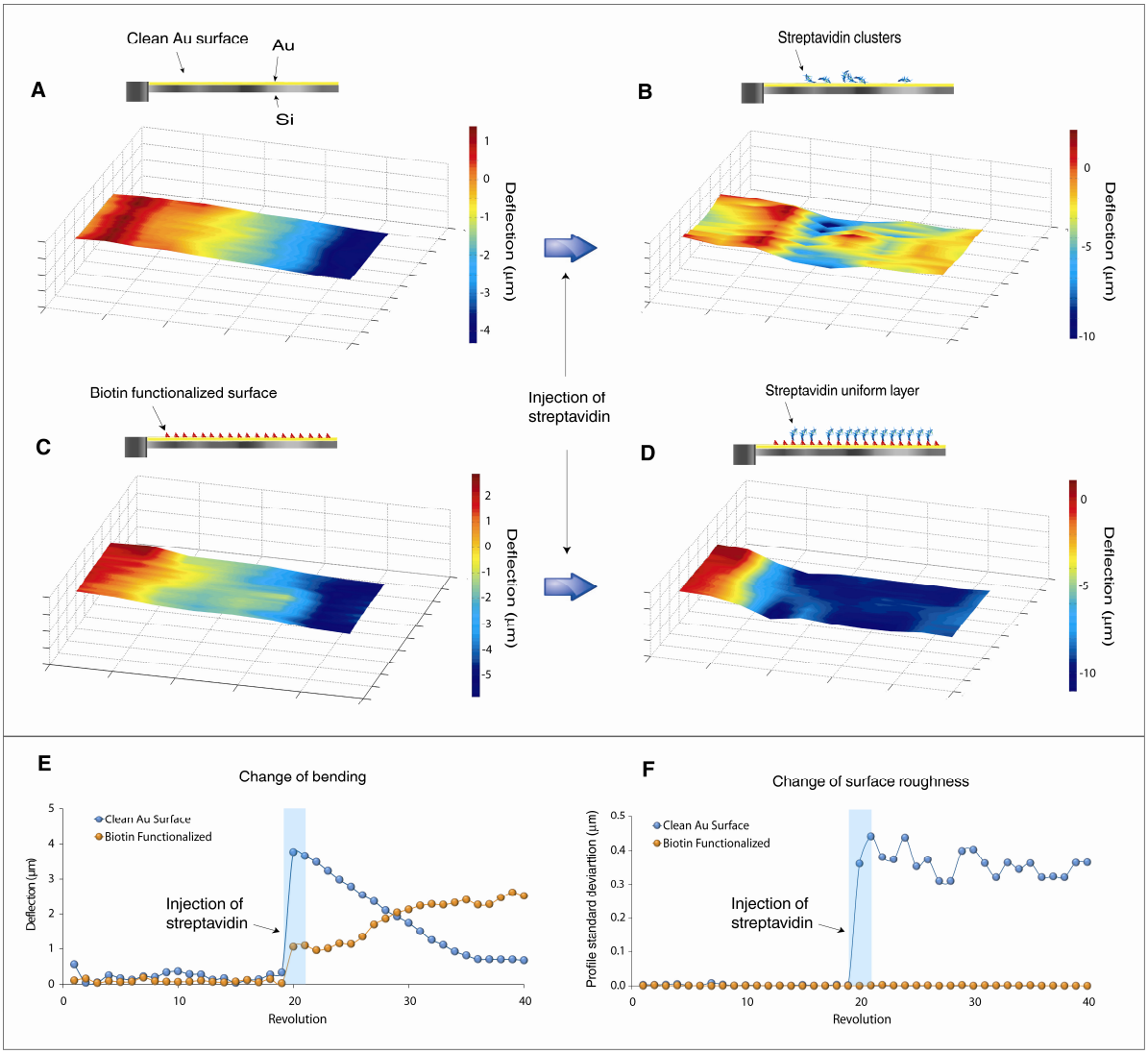
# Figure 1


Sample inlet
A
B
Cantilever
ω
DVD-ROM
pickup head
Deflection
z
y
x
C
ω<ω_T
ω>ω_T
Capillary valve closed
Capillary valve open

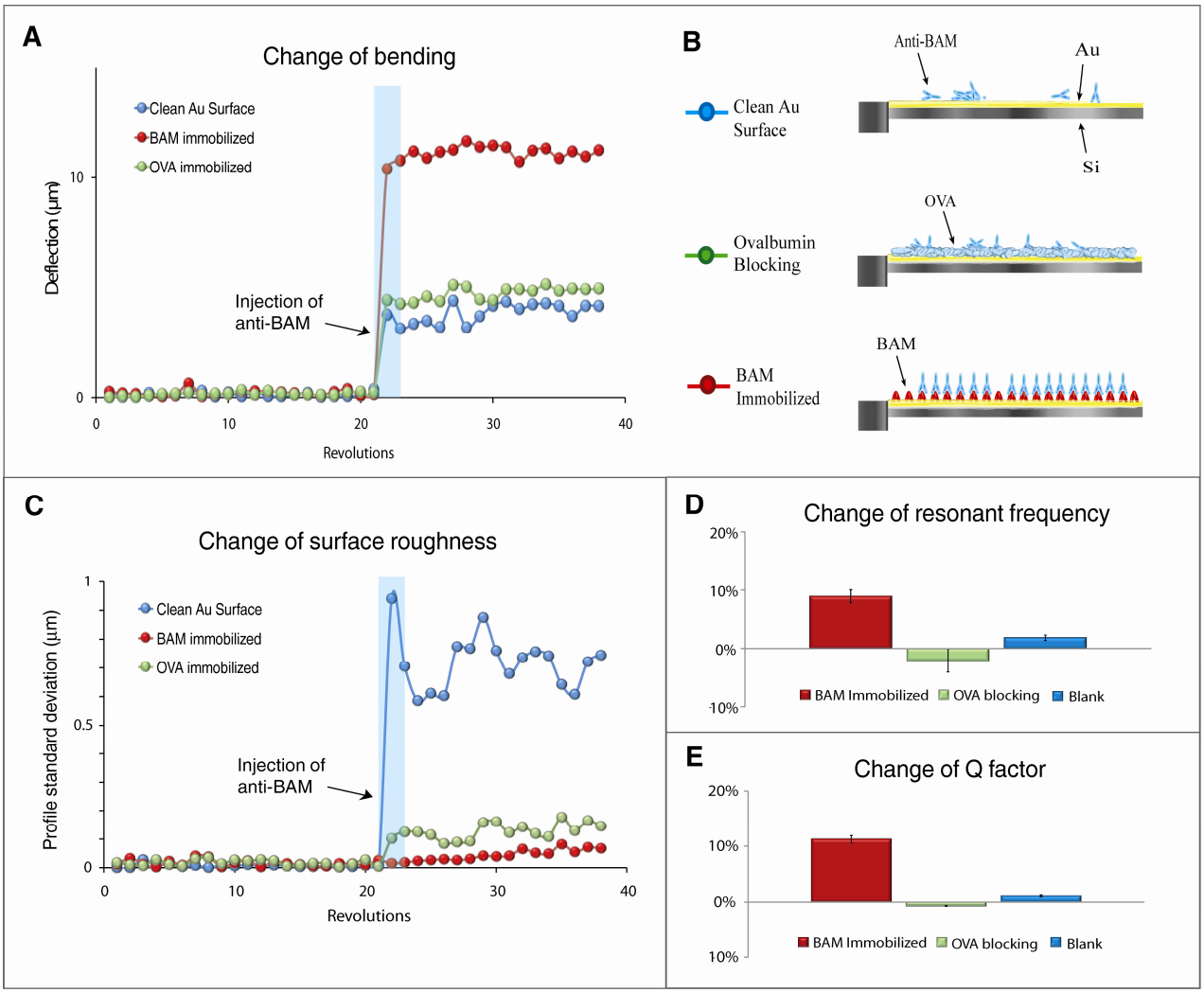
# Figure 2


A
zoom
DVD-ROM laser
z
x
y
B
Laser scan
200 µm
C
Complete revolution
Bending (µm)
zoom
Revolution
D
Single chip
Bending (µm)
y (µm)
E
Initial bending distribution
Mean = 0.49 µm
σ = 0.43 µm
Bending (µm)
F
3D reconstruction
Bending (µm)

# Figure 3


A
Clean Au surface
Au
Si
B
Streptavidin clusters
Deflection (µm)
Injection of streptavidin
C
Biotin functionalized surface
D
Streptavidin uniform layer
E
Change of bending
Clean Au Surface
Biotin Functionalized
Injection of streptavidin
Deflection (µm)
Revolution
F
Change of surface roughness
Profile standard deviation (µm)

# Figure 4


A
Change of bending
Clean Au Surface
BAM immobilized
OVA immobilized
Deflection (µm)
Injection of anti-BAM
Revolutions
B
Anti-BAM
Au
Si
Clean Au Surface
OVA
Ovalbumin Blocking
BAM
BAM Immobilized
C
Change of surface roughness
Profile standard deviation (µm)
Clean Au Surface
BAM immobilized
OVA immobilized
Injection of anti-BAM
Revolutions
D
Change of resonant frequency
BAM Immobilized
OVA blocking
Blank
E
Change of Q factor
BAM Immobilized
OVA blocking
Blank

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# Appendix C, Protocols

The following section provides the more detailed protocols of the assays used in the thesis. The protocols are presented in the same order as used in the thesis.

## *BAM hapten EQ0031 conjugation to Ovalbumine*

Conjugation buffer: dissolve 1,68 g natrium hydrogen carbonate in 200ml Milli-Q water.
EQ0031 is first activated by taking 35,3µmol EQ0031 (11.76 mg) and 35,3 µmol BOP (Benzotriazol-1-yloxy-tris (dimethylamino)phosphonium hexafluorophosphate) (15.61 mg) and dissolving it in 1,5 ml dry DMSO (Dimethyl sulfoxide). Subsequently adding 18,5 µl DIEA (N-Ethyldiisopropylamine) (13.69 mg).
5 µl of activated EQ0031 is added to 1ml (1 mg/ml) solution of Ovalbumine dissolved in conjugation buffer. Shake 2 hours in darkness. Keep at 4ºC. The product is dialyzed 48 hours in 1xPBS buffer with 3 changes of buffer, removing unreacted small size chemicals.

## *BAM hapten EQ0028 conjugation to EQ0031-Ovalbumine*

Conjugation buffer: dissolve 1,68 g natrium hydrogen carbonate in 200ml Milli-Q water
EQ0028 is first activated by taking 35,3µmol EQ0028 and 35,3 µmol BOP (Benzotriazol-1-yloxy-tris (dimethylamino)phosphonium hexafluorophosphate) and dissolving it in 1,5 ml dry DMSO (Dimethyl sulfoxide). Subsequently adding 18,5 µl DIEA (N-Ethyldiisopropylamine)
5 µl activated EQ0028 is added to 1ml (1 mg/ml) solution of Ovalbumin-EQ0031 dissolved in conjugation buffer. Shake 2 hours in darkness. Keep at 4ºC.

## *BAM hapten synthesis*

**Synthesis of 2,3 Dichloro-3-cyanoaniline**

Mix 10.0 g of 2,6 Dichloro-3-nitrocyanobenzene with 16.6 g Acetic acid and 200 ml absolute ethanol. Add 7.7 g iron powder in small portions to avoid too violent reaction. Reflux overnight under nitrogen gas at 74ºC in a round bottom refluxer. Add 10 g activated charcoal granules, and reflux for 30 min. Filter off charcoal granules and insoluble materials while the ethanol solution is still hot. Transfer the solution to an open container. Add 750 ml Milli-Q-water to the product, the product precipitates as a yellow product. Collect the product and let it

dry. Yield is approx 6,9 g. Test purity on TLC using a mix of ethyl acetate/acetic acid as carrier solvent.

**Synthesis of N-(5-Carboxymethyl-pentyl)-2,4-dichloro-3-cyanoaniline**

In a 2 liter reaction flask: Dissolve 6.0 g of the above produced 2,4-Dichloro-3-cyanoaniline in 75 ml DMF. Add 9.5 ml hexanoic acid, 28.4 g BOP and 27.0 ml Triethyl amine to the solution. Let it stand for 48 hours with magnetic stirring at room temperature. Check purity with TLC, if reaction is insufficient add a new portion of BOP, hexanoic acid and triethyl amine, and let it stand for a day at room temperature. Add 750 ml Milli-Q-water and 400 ml dichlormethane to the solution. The product dissolves in the dichlormethane. Transfer the dichlormethane to a new small flask that can fit in a vacuum container. Extract the dichlormethane by adding 100 ml 10% sodium carbonate 3 times (3x 100 ml) and 100 ml 6M hydrochloric acid. Remove water by adding 10 g magnesium sulphate to the mixture. Concentrate the mixture in vacuum, the product is the oily substance. Keep the product in the container for the next reaction steps.

**Synthesis of N-(5-Carboxypentyl)-2,4 –dichloro-3 amidoaniline HCl salt**

Dissolve the above product in 4.0 ml ethanol and 1.5 ml 25% sodium hydroxide. Add slowly 2.5 ml 30% Hydrogen peroxide to avoid a violent reaction, possibly keep on an ice bath. After the exothermic reaction is ended, heat the mixture to 50ºC for 3 days using an electric heating. Add 20 ml Milli-Q-water and extract with dichlormethane 3 rounds. Precipitate the product from the water phase by adding concentrated hydrochloric acid. Dry the mixture in an oven at 50ºC. The product is a white solid. Test the purity by TLC using ethyl acetate/acetic acid as carrier solvent. Melting point is 179-181ºC.

## *Cantion Cantilever DNA assay*

A DNA hybridization experiment on the Cantion Cantichip 4. B1SS or B2SS DNA is spotted on a canti4 cantilever. B1C or B2C is added in the Canti4 box and the change of surface stress recorded.

**Running buffer**: Dilute 100ml 20x SSC buffer with 200 ml Milli-Q- water.

**TCEP Spot buffer**: (1 M Sodium Phosphate + 100mM NaCl pH 7,0). Make 100 ml of 0,2 M (27,8 g/l) Sodium phosphate monobasic, monohydrate, cas.No.10049-21-5, mix 2,78 g Sodium phosphate monobasic with 100 ml Milli-Q water, mix well with clean stirrer, consider heating the solution slightly if salt does not dissolve.

Make 100 ml of 0,2 M (53,6 g/l) Sodium phosphate dibasic, heptahydrate,

cas. No.7782-85-6, mix 5,36 g Sodium phosphate dibasic with 100 ml Milli-Q water, mix well with clean stirrer, consider heating the solution slightly if salt does not dissolve.

Mix 39 ml 0,2 M Sodium phosphate monobasic, monohydrate, with 61 ml 0,2 M Sodium phosphate dibasic, heptahydrate. 100 mM NaCl is added by adding 1,168 g NaCl to the solution. Adjust to 200ml with Milli-Q-water. Take out a 10 ml sample and check the pH with a pH meter.

TE buffer: 0.2 M tris buffer + 2mM EDTA (TE-buffer), adjusted to pH 7,5 with HCl: For 50 ml TE buffer: 1,576 g Tris .HCl (Sigma T 5941, cas.no.: 1185-53-1) dissolved in 50 ml Milli-Q-water. Add 0,2 ml of 0,5M EDTA solution (Sigma E7889, cas.:6381-92-6). Check pH and adjust to pH 7,5 with 1 M NaOH (approx 20 drops). TCEP.HCl in TE buffer. For 1 ml 100mM TCEP.HCl in TE buffer: Mix 1 ml TE buffer with 28,6 mg TCEP (sigma C-4706, cas.no.: 51805-45-9)

**DNA bases used:** HPLC purified quality:

No. 518654 (12base) 0,2 micro mol B1SS, 8 AC ATT GTC GCA A

No. 518655 (12base) 0,2 micro mol B2SS, 8 TG CTG TTT GAA G

No. 518656 (12base) 0,2 micro mol B1C, TTG CGA CAA TGT (3628g/mol)

No. 518657 (12base) 0,2 micro mol B2C, CTT CAA ACA GCA (3688g/mol)

No. 518658 (12base) 0,2 micro mol NS-DNA, ACA CAC ACA CAC (3628g/mol)

**DNA preparation**

B1C DNA: 3.9 µl of 25 µM +1497µl running buffer; B2C DNA: 3.6 µl of 34 µM+1497µl running buffer; NS DNA 3,38 + 1497 µl running buffer.

**Reduction of DNA oligonucleotides**

The oligonucleotides needs to be reduced from 5´-disulphide modified oligonucleotides to oligonucleotides with a free thiol group, in order to bond better to the gold surface: Prepare 200µM solutions of each of the 5´- disulphide modified oligonucleotides in Milli-Q-water. Prepare a 100 mM solution of TCEP.HCl in TE buffer.Mix 50 µl TCEP solution with 50 µl 5´disulphide modified oligonucleotide solution in a micro centrifuge tube. Leave at room temperature for 1 hour. Purify the 5´- thiol modified oligonucleotides on spin columns according to the manufacturer instruction. Dispense the Purified 5´- thiol modified oligonucleotides in 2 µl portions in PCR tubes at -20°C.Determine the concentration of the DNA by UV measurement absorbance at 260nm.

**Spotting DNA**

Rinse the spotter 3 times with x µl Milli-Q-water. Prime the spotter. Set the spotter parameters to 50V tip voltage and 50V pulse width. Make sure the spotter head can dispense water droplets without any satellites. Add 8µl spot buffer to vial of 2 µl thiol modified B1SS DNA (B1SS, 8 ACATTGTC GCAA) and suck up into tip. Coat tip with B1 mix. Keep in NaCl humidity chamber overnight. Block chip in glass ware container with 1 µl 1 mM mercapto 6-hexanol (density 0,985 g/ml) in 7,5 ml Milli-Q water for one hour.

**Canti4 DNA experiment**

Insert chip and wait for stabilization of signal. Record baseline for 1500 seconds (Buffer run). Add 100µl of NS DNA (non specific NS-DNA ACA CAC ACA CAC) through loop and record for 1500 seconds. Flush loop and switch back. Add 100µl of specific B1C DNA (B1C, TTG CGA CAA TGT) and record for 1500 seconds.

## *BAM ELISA*

**Equipment**

ELISA absorbance reader. Polysorb 96 well plate. Multidrop dispenser or hand held dispenser. Pipette tips. Washing towels. Beakers and eppendorf tubes for dilutions

**Chemicals**

Ovalbumin, cas 9006-50-2. BAM Hapten EQ0031-EQ0028. 0,1X PBS buffer.10 X PBST buffer. BAM standards. Antibody HYB 273-1.1 M H2SO4.BOP :Benzotriazol-1-yloxy-tris (dimethylamino) phosphonium hexafluorophosphate. DMSO: Dimethyl sulfoxide. DIEA: N-Ethyldiisopropylamine. Conjugation buffer (Natrium hydrogen carbonate)

**Standard BAM-OA-AQ ELISA**

96 well NUNC polysorb plates are filled with 200µl EQ0031-Ovalbumin-EQ0028 conjugate diluted 1:5000 in 0,1x PBS buffer. The plates are placed in a Stratalinker UV oven for 30 min, washed in 1xPBST coat and dried at 37°C for 30 min. The water samples, standards and controls are temperature equilibrated 1 hour at room temperature before adding to the wells.

Add 150µl of samples/controls/standards, incubate for 30 min at room temperature in darkness

Dilute the BAM antibody (HYB-273-1HRP) 1:3500 in 10x PBST and mix well. Add 50µl BAM antibody to the whole plate with 12 channel BIOHIT pipette. Incubate 1 hour at room temperature at 300rpm shaking and in darkness (use alu foil).Wash plate 4 times with 1xPBST using the Wellwash Ascent 2x12 program. Add 200µl TMB color reaction substrate to each well. Incubate 25min at room temperature at 300rpm shaking and in darkness (use aluminum foil). Stop color reaction with 50µl 0,5M Sulfuric acid per well. Read color intensity on Thermomax Elisa reader using the protocol Pesticide assay 4 best. Save results.

Source: GEUS BAM ELISA protocol.

**Passive absorbed BAM-OA ELISA**

200µl of BAM-OA is added to each well in a 96 well polysorb plate (NUNC) and let stand overnight or longer. BAM-OA solution is removed and the plate is washed 3x in 1x PBST buffer. Add 150 µl of tempered standards and samples per Well. Incubate 30 min at room temperature. Add 50 µl peroxidase labeled rabbit anti mouse IgG, diluted 3500x in 10x PBST (4µl to 14 ml). Incubate 1 hour. Wash 4 X with PBST. Add 200µl TMB plus per Well. Time 25 min and stop reaction with 50 µl 1 M H2SO4 per Well. Measure intensity at 450nm.

## *Fluorescent labeling of BAM antibody*

One kit Amersham Cy3 mAb labelling kit from GE healthcare (code PA33000) is used to label BAM antibody (statens serumsinstitut Hyp 273-3) in 1 mg/ml.
One kit Amersham Cy5 mAb labelling kit from GE healthcare (code PA35001, lot.no.:) is used to label 1 mg dry Sigma Mouse IgG antibody, reagent grade (prod no. I5381).
Antibody conc. is 1 mg/ml so ½ is used adding 0,5 ml 50mM Phosphate buffer, giving a 1 ml solution with at least ½mg protein. Protein solution is added to coupling buffer vial and mixed gently. Coupling buffer solution is added to reactive dye buffer and let stand for 30 min, mixed every 10 min. Separation column is mounted and 13 ml fresh elution buffer is added to the column and flushed through. The labeled Antibody mixture is transferred to the column. 2 ml elution mixture is added to the column. When the leading pink band is near the edge add an additional 2.5 ml elution mixture. Collect the 2.5 ml elution mixture containing the labeled antibody in one tube.

**Determining Cy3 dye/antibody ratio using absorbance at 552 and 280**

As the absorbance of 280nm and 650/552 is different, an undiluted and 1:100 dilution was made of the marked cy3 anti-BAM and Cy5 IgG. The ratio of Cy3 dye/antibody is approx. determined using the absorbance of Cy3 at 552 nm and the protein absorbance at 280 nm. The concentrations are calculated using the following formula:

Cy3 = A552/150.000; Antibody = (A280 – (0.08 x(A552))/ 170.000; Ratio = Cy3 / antibody

Cy3 antiBAM ratio: (1,13 x 100 x 0,088) / (0,201 –(0,08 x(0,088 x 100)) = 20
Cy5 IgG ratio :(0,68 x 100 x 0,133) / (0,387 –(0,05 x(0,133 x 100)) = 33

**Protein concentration**

1mg of antiBAM antibody and IgG mouse unspecific antibody was used for the experiment. The Cy3 marked anti-BAM was dyed and eluted in 1,6 ml fluid, thus 0,625 mg /ml. The Cy5 marked IgG was eluted in 1,5 ml fluid, thus 0,67 mg/ml.

## *Cantion Cantilever BAM assay*

**Running buffer**: 1xPBST 0,05% Tween 20 (one capsule Phosphate buffered saline Fluka no.: 08057, dissolved in 1 liter Milli-Q-water in a new 1 liter Bluecap bottle, solution is degassed before use); A data sheet is available for the experiment. Added antibody samples are:

**250 µl of 0.1 mg/ml Cy5 IgG**:

Sigma-Aldrich unspecific IgG from mouse serum, reagent grade I5381-1 mg, lot.no.025K7580, freeze dried in vial. Marking: Done with Amersham Cy5 Dye™ Antibody monofuctional Labeling Kit for 1 mg antibody. Dilution: 37.5 µl of 0.67 mg/ml stock Cy5 IgG + 215 µl 1x PBS Tween 20 0,05% .

**250 µl of 0.1 mg/ml Cy3 AntiBAM**:

Made from mouse serum at Statens Serum Institut, HYB 273-01, Batch no.03102P01/071008 Marking: Done with Amersham Cy3 Dye™ Antibody monofuctional Labeling Kit for 1 mg antibody. Dilution:40 µl 0.625 mg/ml stock Cy3 AntiBAM + 210 µl 1x PBS Tween 20 0,05%.

**Experiment procedure**

The flow system runs overnight at low flow (3-5µl/min). A new 20 ml green syringe without silicone is loaded with 20 ml buffer and placed on the Harvard instruments PHD2000 syringe pump. The flow system is flushed with buffer at 200µl/min while switching in between loop1 and loop2 until no air bubbles is left. The sample loop is washed with 2-3 ml buffer. The waste container is filled to half with fresh buffer and gold ground wiring is inserted in the buffer and connected to the cantibox4. The cantibox4 was allowed to run 30 min at Flow rate 20 µl/min with the chip in place, prior to recording. The program is setup to show absolute values of cantilever A,B,C,D and differential values of A-B, C-D, B-C and A-D, from 0 to 2000 sec and +/- 80 µV.

The samples are diluted directly into a new brown glass vial with screw cap, and 100 µl is added to sample loop 2 via suction using a 5 ml syringe. Samples are added by switching from loop 1 to loop2 at 200sec after a stable run. If a stable run can not be obtained the experiment file is overwritten, and the experiment repeated until the base line of the differential signals are stable. After a sample is added and run from sample loop2, loop2 is washed with approx 2-3 ml

buffer. The flow system is left running constantly at low flow (3-5µl/min) with 1x PBS 0,05% Tween 20.

**Data acquisition**

Settings: Operator: MIB: Box: B; Input R.:± 26.67 mV; Bridge volt.: 2.50V; Fixed Voltage is noted on a separate sheme. Flow rate is 20 µl/min. Each experiment is recorded until 2000 sec. approx. The treated cantilever data can be seen in a separate Excel file as well as 5 raw data text files. The chip is tested using the following order of experiments.

| Exp. | Raw data file name (text file) |
|---|---|
| Stabilization | Mic0xxx_date_RUN1 |
| Switch from loop 1 to loop 2 | Mic0xxx_date _RUN101 |
| Buffer effect test | Mic0xxx_date _RUN102 |
| Unspecific Cy5 IgG | Mic0xxx_date _RUN104 |
| Specific Cy3 Anti-BAM | Mic0xxx_date _RUN105 |

**Assay quality control using fluorescent pictures**

To ensure that the antibodies have attached to the cantilever surface a set of pictures are taken at cy3 and cy5 using a micro array fluorescent scanner and a special holder for the chip. The pictures are first focused for max intensity at PMT 90% and laser 90% on the cantilever surface, then taken at 70, 80 and 90% PMT and laser intensity. Cy3 ex: 550 nm, em: 570 nm; cy5 ex: 650 nm, em: 670nm.

## *Piranha wash and TsT Glass surface activation*

To make the glass surface hydrophilic the surface properties are changed towards a more nucleophilic chemistry. Use the designated washing containers for the reactions

**For 10 glass slides:**

Each slide is marked with a diamond scratching pen (do not use ink as it will go off in the wash). The glass container for the slides is placed in an ice bath. Piranha mix: 40 ml H2O2 is slowly added to 120 ml H2SO4, be careful not to overheat the solution! Glass slides are placed in the piranha mix for 20 min with stirring. Glass slides are rinsed 3x 5 min in Milli-Q-water and once in acetone. 3,7 g cyanuric chloride is dissolved in 194 ml acetone. Glass slides are placed in the acetone solution and added 7 ml DIEA, and incubated for 1 hour with stirring.Glass slides are washed 3x 5 min in acetone and dried. Slides are kept in an airtight container until use, preferably within a week

## *Microspotter procedure for Nano-Plotter 2.1*

The Spotting program has 4 windows: I - E - R - S; **Interactive; Edit; Run; Simulation.**
In order to spot slides, two sub programs are needed: a program that defines the spot placement on the slides, and a program that defines the transfer from the 384 well to the slides.

**Start up**

Turn on heating, set to 25 °C + turn on humidity, set to 55% + turn on pc, + turn on Microspotter. Set Pico/Nano-tip in holder 1, screw lightly in place with umbraco key, Set wire in slot 1(the two live wires downwards), press fluid tube down on tip carefully, close port again with screw. Load your 384 well plate in the holder, align all four corners and press all the way down (its very tight!). Set all glass slides in place on table, check borders of slides to see that there are no differences in height. Run NpC applications, run initialize microspotter. Fill tubings, preferably use complete fill and empty tubing (removes unwanted bubbles), check for visual air bubbles in pump piston and tubings, check that waste container is not overfilled or water container empty. (Under **Interactive** Check tip type (Nano or Pico) in program) Under **Interactive** Use "Wash &Dry" 3-4 times, check stroboscope to see if satellite drops are present, fix with voltage and pulse width until only one drop appear. (voltage = shooting force, pulse width = drop size) To check the height of the slides place the Z-sensor in the holder next to the spotting tip. Under **Interactive** check off Z-sensor for height setting of slides, run "measure Z-levels". Remove Z-sensor.

**Loading programs**

Under **Interactive** run "open workplate settings" and load spot placement program from NpC V2.14.09/Bache folder, Under **Edit** adjust wanted spots with "edit plate", check spots placing with "zoom plate", Under **Interactive** remove or add slides by checking "available slide". Open the plate to slide transfer program outside the NpC program (a simple text file under NpC V2.14.09/DATA/bache folder), check your plate to slide placement, program layout is : t A1  1,1 , meaning transfer from well in row A and column 1 to spot in row 1 and column1

**Running a spotting program**

Under **Simulation** Load the NpC application, under programs use “Transfer Seq” program and choose the slide program you just made. Via simulation the spotter will test whether the 2 programs you made are compatible. Under **Run** and under programs use “Transfer Seq” program and choose the slide program you just tested. Don’t use well to slide text transfer, and uncheck stroboscope function, unless you want to sit and do all stroboscope alignment for each well applied. A slide with 25 spots in 40 clusters takes approx 30 min. After run, if failed spottings are present then redo malfunctioned spottings using “repair Seq” under the NpC application. End spotting using “Goodnight” under the NpC application list. Turn off heating, turn off humidity, turn off Microspotter, close NpC program, but leave the pc turned on. Remove 384 well plate from holder, remove slides and place them in NaCl humidity chamber overnight. Remove Pico/Nano-tip from holder, replace protection cap on both end and place in container. Place slides them in NaCl humidity chamber overnight.

**Microarray on glass slides**

Activate glass surface following the specific TsT activation protocol. Make a dilution series of chemicals that need to be spotted (note: high protein or salt content may cause problems in spotting due to high viscosity). Fill 40µl of each solution on a 384 well plate. Follow above microarray spotter protocol. Allow slides to incubate overnight in a humidity chamber with a saturated sodium chloride water solution at room temperature. Place slides in a staining jar with blocking solution, and incubate for 1 hour with stirring. Wash slides 3x 5 min. with 200 ml PBS Tween 20 (0.05% Tween 20).Rinse slides for 10 sec. in Milli-Q-Water. Dry slides at room temperature for 2 hours or at 37ºC. Read slides in micro slides scanner (Scan-array program) using 70 % pmt gain and 70% laser power, preferably at resolution 10. Use a suitable sub program to read the slide.

## *BAM flow cell assay*

A wafer of break apart chips is Piranha treated and TsT activated following the specific TsT activation protocol. Gently snap the wafer into 2 microscope slide format pieces, if one slide snaps in two it can still be spotted if reassembled and placed gently in the spotter slide slot. Follow the microarray spotting procedure.

Keep the chip in a dry dustproof container overnight; alternatively allow the spots to dry for 2 hours before blocking the chips. Block the chips as a microscope slide format in a staining jar (following microarray on glass slides protocol) or individually placed in small PCR tubes. Blocking should not exceed 1 hour as the whole surface will be covered by the blocking protein. Place the single chip in the groove of flow cell with the flat side upwards by unscrewing the 4 screws and removing the lid and PDMS seal.

The chip should fit in the small groove easily but often the corners of the chip still have a small strip of silicon that breaks off when placed in the groove. Replace the PDMS seal and lid, check the lid for impurities and grease before replacing. Tighten the bolts lightly on the 4 screws (using a pincer is enough), too tight fitting will squeeze the PDMS seal too much and block the in/out channel. Add the tubes to the flow cell and fix the cell under the microscope. Attach the tubes to syringes or to switch box, use a small beaker for the outlet tube waste. Check chamber for air bubbles, air bubbles can be removed by pressing the syringe piston a couple of times.

The microscope is set using a Cy3 filter and the fluorescent light source is lighted (do not switch on/off repeatedly as this will destroy the lamp). Let it run 2 min with buffer at min.20 µl/min. Before starting any experiment to be sure no air bubbles are left in the system. Take pictures before you start the experiment and note the camera gain and shutter time if possible
After the experiment wash the chamber in buffer solution and Milli-Q-water.

## *Antibody Stripping buffers*

**Alkaline high pH (NaOH):**0,1 M NaOH in Milli-Q-Water.

**High salt (8M guanidine HCl)** : (CN3H9.HCl) cas.no.: 50-01-1, 1 mol 95,54 g/mol; 8 M = 764,32 g /l; 7,65 g dissolved in 10 ml Milli-Q-Water.

**High salt (Urea):** 4M Urea: 1 mol: 60,07 g/mol; 4 M 240,28 g/l; 2,4 g dissolved in 10 ml Milli-Q-Water.

**Glycine HCl buffer:** 100 ml 50 mM Glycine : 75,07 g/mol; 0,3753 in 100 ml; 100 mM HCl from 37% HCl: 0,826 ml 37% HCl in 100 ml Milli-Q-Water. Adjust pH to1.9 with HCl.

## *DVD IBM cantilever assay*

**Purpose**

To perform a DNA hybridization experiment using single stranded DNA attached on a gold coated cantilever and adding a complementary strand

**General chemicals**

All following chemicals are purchased via Sigma Aldrich.

Sodium hydrogen carbonate, 99.5%, cas.no. 4-55-8, Sigma no. S6297.BOP (Benzotriazol-1-yloxy-tris (dimethylamino)phosphonium hexafluorophosphate), 97%, cas.no. 56602-33-6, Sigma no.226084.DMSO (Dimethyl sulfoxide). BioReagent, for molecular biology, ≥99.9%, cas.no. 67-68-5, Sigma no.D8418.DIEA (N-Ethyldiisopropylamine) 99.5%, biotech. Grade, .cas.no. 7087-68-5, Sigma no. 496219.Ovalbumine, lyophilized powder, ≥98%, cas.no.9006-59-1, Sigma no.A5503.1xPBS 0,05% Tween 20 pH 7.4 buffer salt pouches, Sigma no.P3563.

1x PBS pH 7.4, Sigma no. P3288.Absolute ethanol; cas.no.: 64-17-5; Sigma no. 459844

98 % $H_2SO_4$.; cas.no.: 7664-93-9, Sigma no. 320501.30% $H_2O_2$; cas.no.: 7722-84-1; Sigma no. H1009.

**Equipment**

All glassware used has been new and only washed in Milli-Q-water prior to use to avoid detergents. Cantion Cantispot micro spotter + Cantion spotting pc program. GESIM Sub-Microliter Piezoelectric Dispenser, pin head A010-006 SPIP, cylindrical housing. (~0.1 nl drops). Microscope for surface quality control. 6x 25 ml small clean glass beakers (5 for cleaning + 1 for coating). 5 ml, 10 ml glass pipette +1 ml,100 µl,10 µl pipette. Large glass container for piranha wash. Shaker table.

**Cantion Canti spotter procedure**

The spotter is tested with water until a steady series of drops can be obtained with the tip. The spotting quality is monitored via a web camera and recorded. The spotter head is placed in a vial containing the sample, and 4-5 µl of spotting solution is sucked up using 1µl increments. A tip voltage of 100 V and pulse width of 20 V is used. Each cantilever is spotted 5-7 times with ~0.1 nl drops fully covering only the top surface of the cantilever, the solutions are 1,0 mg/ml

Ovalbumine in 1x PBS on reference cantilever 1,2,7,8 and 0,75 mg/ml BAM-Ovalbumine conjugate in 1x PBS on the active cantilever no.3,4,5,6. After spotting the chip is kept in a NaCl/water humidity chamber overnight at 4ºC.

## Procedure for Biotin /streptavidin assay on DVD IBM setup

To coat gold surfaces with biotin via a thiol based sulphur-gold bond and subsequently monitor the surface effects of the binding of biotin to streptavidin.

As reference an alternative thiol coating of gold with 11-mercapto-1-unedecanol is made. The streptavidin binding can be confirmed with a fluorescence microscope by the Cy3 fluorochrome attached to streptavidin.

### Chemicals Streptavidin biotin assay

EZ-link Biotin-HPDP, 50 mg , mol. Weight: 539,78 g/mol, Pierce prod. No. 21341. Streptavidin–Cy3™ from Streptomyces avidinii, Sigma no.S6402. 11-Mercapto-1-undecanol 99%; CAS Number: 73768-94-2;, Sigma no. 674249 (stored at -20 °C).

### Piranha cleaning

Piranha mix: 7,5 ml 98 % $H_2SO_4$ is added to a 25 ml container. 2,5 ml of 30% H2O2. is added slowly and drop wise, heating up the mixture). The mix is preferably used within one hour. Beware: extreme acid and oxidizing agent, careful of spills, always use gloves and glass protection. For washing, 4 containers with 10 ml of Milli-Q-water and one with absolute ethanol is filled. The cantilever chips are placed in the piranha mix and let stand for 10 min. The chips are then transferred to Milli-Q-water wash and let stand 1-2 min in each container, followed by a 4 min wash in absolute ethanol. The chips are store in a closed container in a dry place (preferably in a desiccator). Gold get dirty fast so the reaction should be made within days from cleaning, the sooner the better.

### Biotin-HPDP Coating, 1mM EZ-link Biotin-HPDP:

10 mM Stock solution: 5.5 mg EZ-link Biotin-HPDP is transferred to an empty 1,5 ml eppendorf tube. 1 ml of Dimethylsulfoxide (DMSO) is added and dissolved by shaking. The vials are divided into 10x 100 µl aliquots (or less) and stored in the freezer.

**Coating of EZ-link Biotin-HPDP in ethanol**

50µl of 10 mM EZ-link Biotin-HPDP Stock solution is dissolved into 950 µl absolute ethanol into a 1,5 ml eppendorf tube. The cantilevers are placed into a 1,5 ml eppendorf tube , adding 1ml of 1mM EZ-link Biotin-HPDP solution, and placed on a shaking table for 24 hours at room temperature (low shaking!). The chips are transferred to another vial with ethanol and let stand for 10 min. The chips are then allowed to dry in air.

**Streptavidin Cy3 binding to biotin gold surface**

Stock streptavidin - Cy3 is 1 mg/ml, 0,1mg/ml is used (~1,6 nM). 10µl of 1 mg/ml Stock streptavidin-Cy3 is diluted with 90 µl 10 mM PBS buffer.

A baseline recording of the cantilevers on the chip is done. With a 10µl pipette 3-5 µl of 0,1mg/ml streptavidin-Cy3 is added to the chip submerging all cantilevers on the chip. The chip with streptavidin solution is incubated at room temperature for 10 min. The streptavidin solution is removed with a 10 µl pipette and washed 3 times with 1x PBS and once with Milli-Q-water to remove salts. The chip is allowed to dry (approx 5 min) and the bending values of each cantilever are recorded again.

**11 mercapto-1-unedecanol coating**

As reference to the biotin coating the chips are coated in 1 mM 11 mercapto-1-unedecanol in ethanol. 50µl of 11-mercapto-1-unedecanol is thawed and dissolved in 950 µl absolute ethanol into a 1,5 ml eppendorf tube. The cantilevers are placed in a 1,5 ml eppendorf vial containing 1 ml of 11 mercapto-1-unedecanol solution, and placed on a shaking table for 24 hours at room temperature (low shaking!). The chips are transfer to another vial with ethanol and let stand for 10 min. The chips are then allowed to dry in air.

## Procedure for BAM assay on DVD IBM setup

To coat gold surfaces with BAM pesticide via ovalbumine gold bond and subsequently monitor the surface effects of the binding of a BAM antibody to the BAM pesticide on the gold surface of the cantilever. The BAM antibody binding can be confirmed with a fluorescence microscope by the Cy3 fluorochrome attached to the BAM antibody.

**Cy3 BAM Antibody**

Made from mouse serum at Statens Serum Institut, HYB 273-01, Batch no.03102P01/071008. Marking: Done wih Amersham Cy3 Dye™ Antibody monofuctional Labeling Kit for 1 mg antibody. Diluted prior to use to 0,1 mg/ml as such :40 µl stock Cy3 Anti-BAM + 210 µl 1x PBS 0,05% Tween 20.

**BAM hapten EQ0031 conjugated to Ovalbumine**

Conjugation buffer: 1,68 g natrium hydrogen carbonate is dissolved in 200ml Milli-Q water. BAM hapten EQ0031 is first activated by taking 35,3µmol EQ0031 and 35,3 µmol BOP (Benzotriazol-1-yloxy-tris (dimethylamino) phosphonium hexafluorophosphate) and dissolving it in 1,5 ml dry DMSO (Dimethyl sulfoxide). Subsequently adding 18,5 µl DIEA (N-Ethyldiisopropylamine). 5 µl activated EQ0031 is added to 1ml (1 mg/ml) solution of freeze dried ovalbumine dissolved in conjugation buffer and shaken 2 hours in darkness. The solution is divided into aliquots and kept at 4ºC. The BAM-ovalbumine conjugate is tested positive by a BAM ELISA. See ref. [1]

**Ovalbumine solution**

The ovalbumine solution is made from freeze dried ovalbumine dissolved in Milli-Q-Water. A stock solution of 1 mg/ml is made; the solution is distributed in 100µl pcr tubes and kept in the freezer until use.

**BAM antibody-Cy3 binding to BAM-OA gold surface**

A baseline recording of the cantilevers on the chip is done. With a 10µl pipette 3-5 µl of 0,1 mg/ml BAM-OA is added to the chip submerging all cantilevers on the chip. The chip with antibody solution is incubated at room temperature for 10 min. The antibody solution is removed with a 10 µl pipette and washed 3 times with 1x PBS and once with Milli-Q-water to remove salts. The chip is allowed to dry (approx 5 min) and the bending values of each cantilever are recorded again.