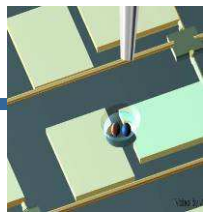
COCHISE



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1. Introduction

The **C**ell-**O**n-**CHI**p bio**SE**nsor for detection of cell-to-cell interactions (COCHISE Project) is a Specific Targeted Research Project which addresses integrated systems for point-of-care diagnosis, monitoring, and drug delivery.

COCHISE is the first step of an activity aimed at the development of enabling micro-technologies to monitor physiological cellular interactions at the single cell level with a high throughput. One of the primary applications of this technology is the immunological monitoring of anti-tumor vaccinations, singling out the rare effector cells (in the order of 1 cell among 1000 cells) that are actually active against tumor cells. The platform developed within the project consists of an orderly matrix of up to 1536 microwells where living cells are deposited. The platform is created in a biocompatible substrate that also serves as a high-density circuit board. The microwells are monitored by an external microscope and external electronics allow to control the biosensor electrodes and to perform impedance measurements.

The key point is that each microwell can force contact between individual cells, and detect consequences of these contacts. The project integrates on the same platform several technologies such as electronic sensing, microfluidic interfaces for cell dispensing, control of osmotic balance of nutrients, management of evaporation, surface nano-modifications for management of fluid flows (e.g. hydrophilic and/or hydrophobic surfaces tend to drive or repel droplets) and avoidance or induction of surface cell adhesion. An important side of the research is the definition of new therapeutic and diagnostic protocols for the immunotherapy of cancer. As a first step, we applied our technology to the analysis of anti-tumor lytic effector cells, for a precise detection of lytic events which happen in the array at certain locations and timings. A major advantage is that the cells are kept alive and can be retrieved individually for further analysis, such as gene expression profiling.

2. Design and test of the biosensor

Idea of the project is implement an array of microwell using printed circuit board (PCB) technologies for the construction of the multiwell plate. Each microwell integrates actuation and sensing features by electrodes realized as structured conductor lines. The envisioned structure that is used for determining the cell-cell interaction by means of impedance change maintains the cells in equilibrium via dielectrophoresis. Figure 1 shows the principle of the sensor. For the final device additional features are added to the structure. The nutrient solution for the cells will flow from the bottom side into the microwells. Furthermore, a hydrophobic funnel on top of the PCB will ease the cell positioning into the microwells.

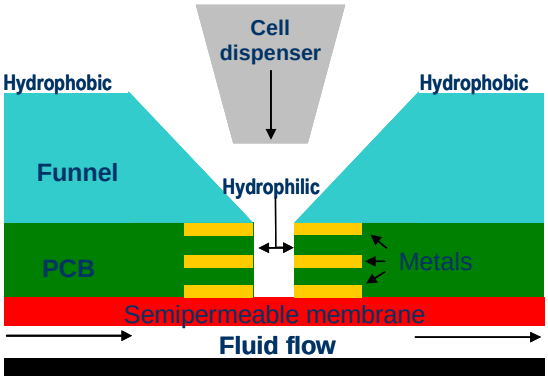

Figure 1 Sketch of sensor principle

2.1. Biosensor architecture

Each microwell contains three annular electrodes at top, middle and bottom levels (TOP, MID and BOT electrodes). In trap mode, the microwell levitates cells keeping them in the center of the structure. All possible configurations are obtained by applying the same sinusoidal signal on two electrodes and a counter-phase signal to the third one. The trap configuration is achieved by phase-shifting the MID electrode, thus creating an almost spherical cage at the level of the MID electrode and in the horizontal center of the microwell. To guarantee this shape, the distance between TOP/MID and MID/BOT electrodes must be equal to the microwell diameter. Figure 2B shows a contour plot of the mean square electric field for a 2D structure representing a vertical cross section along one of the diameters of the hole.

The minimum amplitude of applied signals to guarantee bead levitation was extracted from the simulator and found to be 1.9V for a microwell with a diameter of 125µm.

The reference particle for these simulations is a polystyrene bead, with a relative permittivity of 2.5 and a density of 1062Kg/m^3. With these values, the gravitational force acting on the particle is about 5pN.

As one can observe from Figure 2B, the trap mode also creates a semi-spherical cage on the top side of the microwell, thus preventing a particle dispensed from the top from entering the well. A different electrode polarization scheme is then used to create a load configuration by applying the counter-phase signal on BOT electrode (Figure 2A), thus creating an electric field minimum, in the region above MID electrode. Finally, the eject configuration (Figure 2C) opens the cage from the bottom, allowing particles to be removed from the microwell.

When implementing microwell arrays, independent addressing of single microwells is possible by changing the phases of the applied signals.

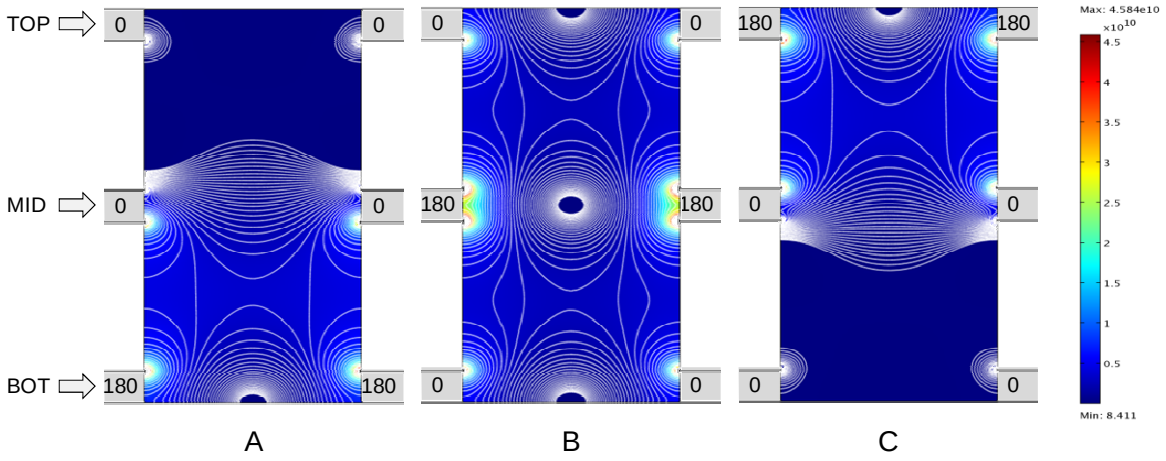

Figure 2 2D contour plot of mean square electric field on the vertical plane of a microwell. Load configuration (A), trap configuration (B) and eject configuration (C) are obtained by applying a 180° phase-shifted signal on BOT, MID and TOP electrodes respectively and a 0° phase-shifted signal on the two remaining electrodes.

An important consideration for cells controllability is related to the electrodes thickness, with particular reference to the MID electrode. Observing Figure 2B, the region close to MID electrode has two electric field maxima corresponding to the electrode edges, while in the middle region the electric field becomes null. As a consequence, the effect of MID electrode of repelling a particle towards the central region in negative dielectrophoresis (nDEP) conditions is reduced for particles with a small size compared to the electrode thickness. These particles, in fact, will find a region corresponding to the level at the center of MID electrode where the horizontal DEP force becomes null or small.

2.1.1 Fluid carrier

To provide a fluid flow to fill the microwells and change the supernatant, a fluid carrier was designed; the carrier is formed by two parts (the top frame and the bottom frame) between which is placed the biosensor. In addition, a PCB where spring loaded connectors are mounted is used for contacting the biosensor (see paragraph 2.1.2). A cross section of the entire package is shown in Figure 3.

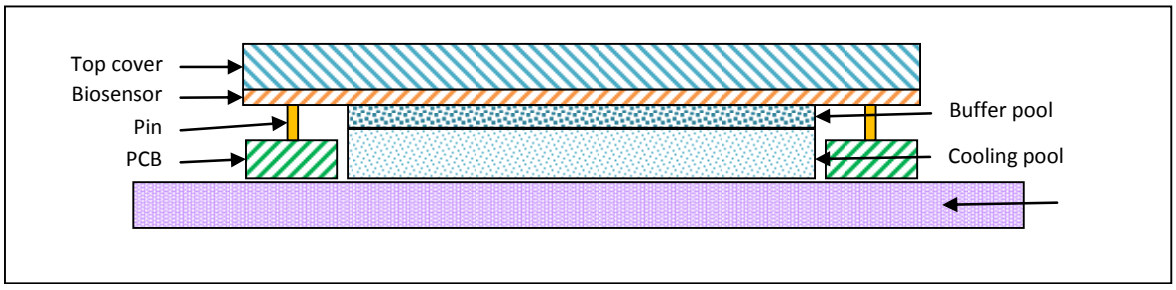

Figure 3 Cross section of the package including the fluid carrier, the electrical contacts and the biosensor

The bottom frame includes a chamber with a typical height of 2mm, a volume of 4ml and fluid inlet and outlet on the two sides, in order to provide fluids to the microwell. Moreover, a recess permits the positioning of a gasket. To allow the electrical connection to the biosensor pads, 4 holes arrays were squarely placed underneath the bottom frame.

Above the biosensor, the top frame is pressed down and fastened by screws to the bottom frame. The gasket assures the sealing and avoids any kind of leaking without using glues or tapes. A prototype of this carrier was implemented using a prototyping machine a structuring PMMA sheets as required (Figure 4).

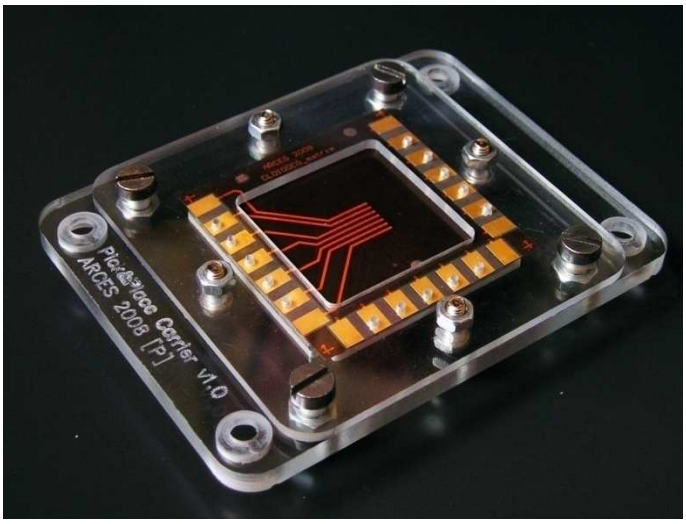

Figure 4: An 8x8-well microwell array mounted on the fluid carrier

2.1.2 Electrical interface

In order to send electrical signals to the biosensor pads, a PCB board (named “PCB Host”) was realized (Figure 5).

The board is equipped with 4 spring-loaded connectors arrays (H1, H2, H3, H4); the fluid carrier is positioned on the board pushing it through 4 snap-lock mounts that assure a mechanical and secure lock: the spring-loaded connectors apply pressure against the bottom sided biosensors pads allowing the electrical contact (Figure 6).

Two connectors (H5 and H6) permit to force the electrical signals from the outside.

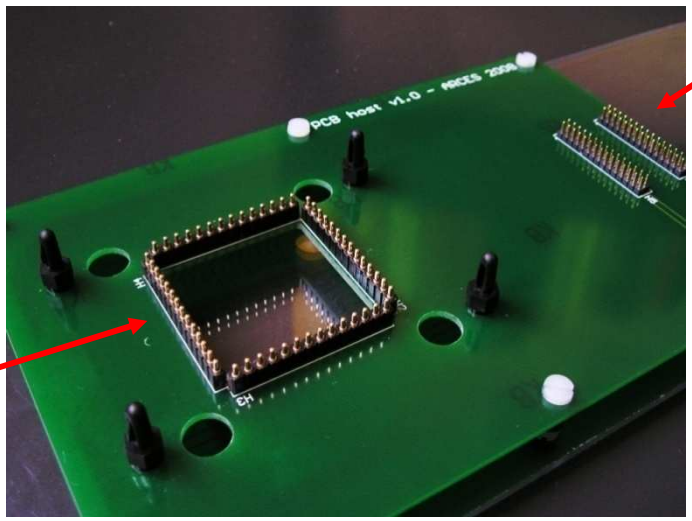

Figure 5: The “PCB Host” board.

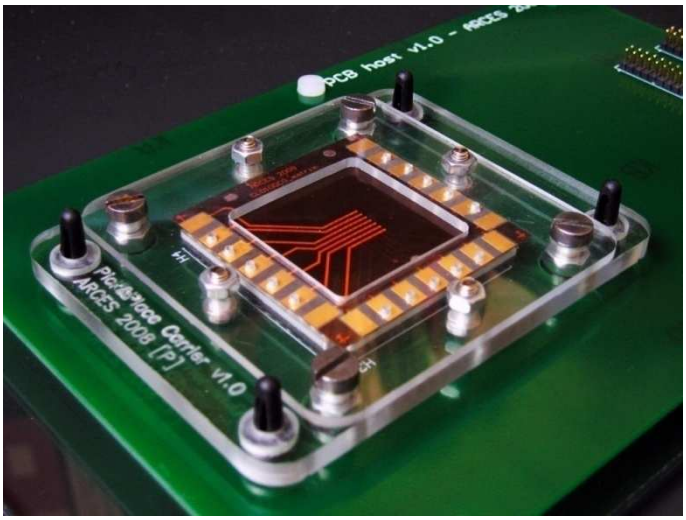

Figure 6: The fluid carrier mounted on the “PCB Host” board.

2.2. System architecture

The system is centrally controlled by a software application running on a PC where Labview software is installed. The system includes the biosensor with all its interfaces, a microscope with an XYZ motorized stage, horizontal and vertical cameras, a micro-dispenser and a micro-pipette. Most of this document will be focused on the biosensor architecture and on electrical and fluidic interfaces specifically designed to connect electrical signals and to provide fluids to the disposable biosensor, maintaining an easy way to disassemble the package, remove the biosensor when necessary, replacing it with a new one and perform the required cleaning procedures. A scheme of the system is reported in Figure 7, while Figure 8 shows a 3D view of the system.

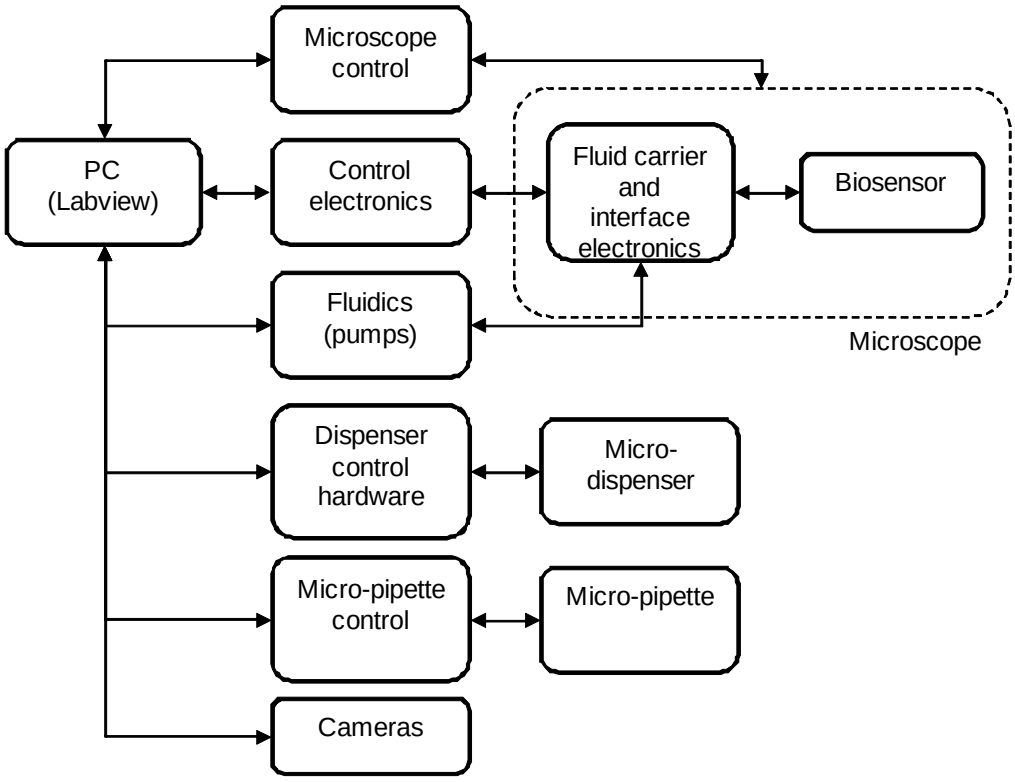

Figure 7: System diagram

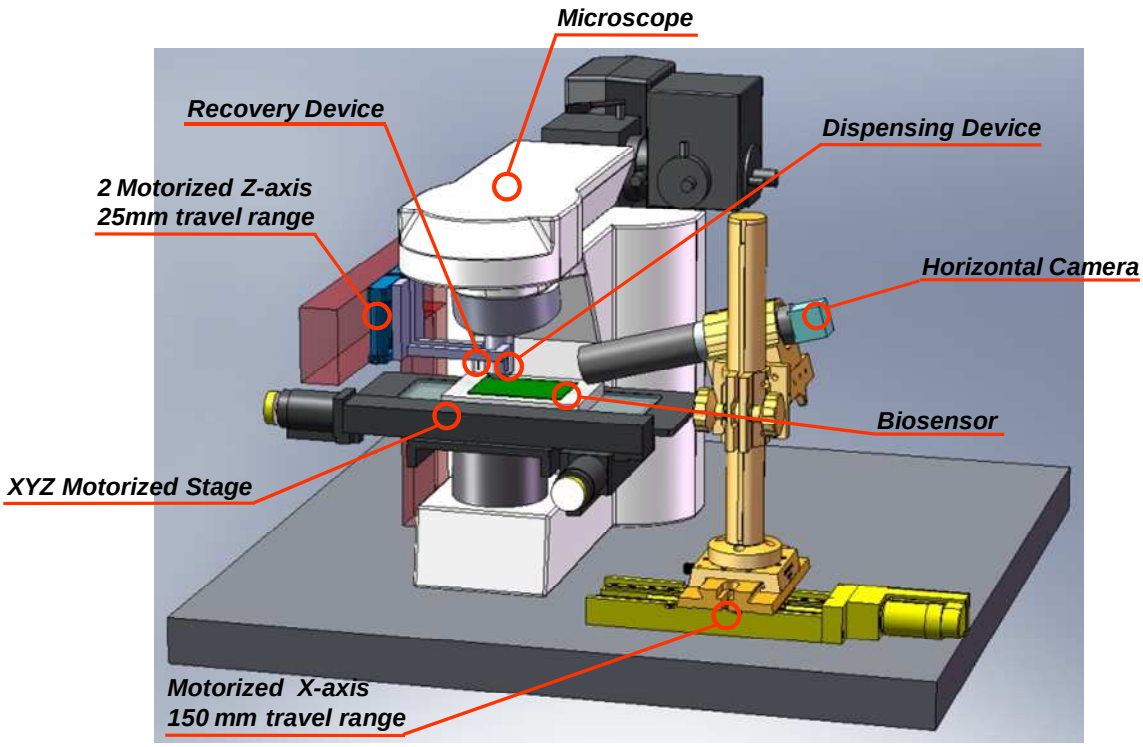

Figure 8: 3D view of the system

2.3. Cell and particle trapping

Polystyrene beads with different diameters were utilized as a first validation of the biosensor functionalities, and in particular of the ability to trap and levitate particles in the microwells by means of dielectrophoretic force.

After a first study, the biosensor was validated using K562 tumor cells. They were successfully levitated in physiological medium, as shown in Figure 9, and within microwells with a typical diameter of 70µm. When multiple cells are inserted in the well, the are all positioned towards the center of the structure and, consequently, come in contact. This allows the study of the effects of interactions between trapped cells. Optical inspection, including observation under fluorescent light, can be performed throughout the experiment to monitor the status of the microwells.

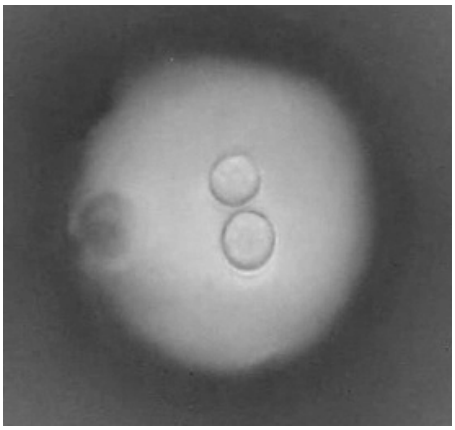

Figure 9 Two K562 cells trapped in a 70µm microwell

3. Cell delivery and recovery in microwells

Equipments and procedures for depositing cells into the microwells of the COCHISE biosensor, as well as for recovering cells of interest from selected microwells, were developed. Three dispensing techniques were investigated: a cell microdispenser previously developed at the Biochips Laboratory in CEA, a commercial piezoelectric jetting device, and cell handling with a machined ceramic micropipette. The latter device was fabricated and used both for depositing cells and pipetting them from the microwells. Instrumentation for cell handling includes some mechanical supporting structures, motorized stages, pressure control, optical devices, software, and development of operational loading methods (Figure 10). Viability of cells deposited and retrieved by these various means was demonstrated.

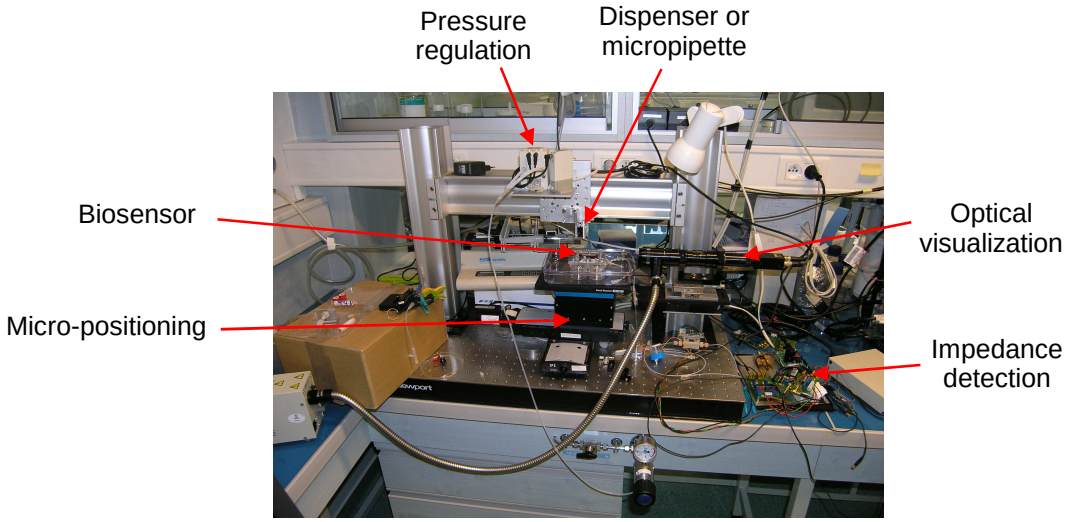

Figure 10 Integrated modules for dispense and recovery of cells towards microwells.

3.1. JetCell microdispenser

In the CellJet microdispenser, the cells are introduced into a microfluidic chip, transported and lined up along a microchannel, and detected by microelectrodes. For achieving cell dispense, some ~1 µL droplets are generated by a miniaturized solenoid valve so that the cells are brought out through the orifice of the microchannels. The cell-containing droplets are finally collected and absorbed by the microwell of the biosensor placed underneath (Figure 11). Cells deposited were proved to enter and pass through the microwells within few minutes.

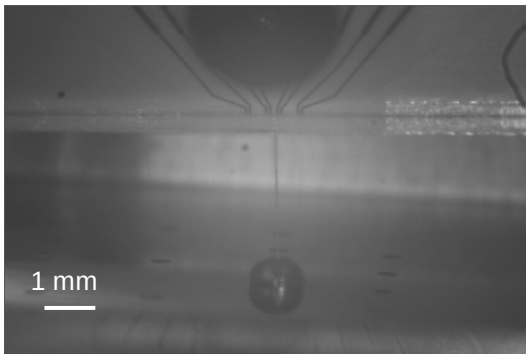

Figure 11 Spot dispensed onto a microwell by the CellJet microdispenser. Note that the jet is narrow and very straight, allowing the spot to be located in the near vicinity of the microwell. The spot is absorbed by the microwell within seconds or minutes according to the fluidic resistance of the underneath microwell.

A software program for the cell microdispenser was developed and a user's manual and a programmer's guide for the LabVIEW software were released. This software automatically drives the CellJet microdispenser. A report on the cell delivery of homogeneous cell suspension was created to describe the procedure to achieve a correct dispense of cells. Electrical profiles of cells flowing through the microfluidic chips were investigated to parameterize cell dispense.

3.2. Commercial jetting device

A commercial piezoelectric dispenser was also used for depositing cells. As the diameter of human cells is about 20 µm, a dispenser with an orifice of 60 µm was chosen. The droplet formed has generally a diameter slightly larger than the orifice diameter, *e.g.* 65 µm in diameter which results in an individual ejected volume of ~140 pL. A droplet generation requiring no pressure controller was implemented, and drivers using LabView software were programmed for controlling the electronic triggering of droplet jetting. Two modes of dispense were developed: a 'stop-on-spot' mode where the biosensor is moved and stopped under the microdispenser for dispense; and a 'in-fly ejection' mode where dispense is very rapid so that the ejected droplets can reach the underneath microwell while the biosensor is still moving at a constant velocity. Heterogeneity of the number of deposited cells is generally observed, which is likely due to sedimentation of cells upstream of the dispenser orifice. A classical limit dilution strategy, in which a low concentration of cells is introduced so that the total volume of cells statistically contains one single cell, was successfully investigated. Viability of spotted cells was demonstrated using Trypan blue and Live/Dead assays.

3.3. Cell handling with a machined micropipette

A micropipette was machined from a capillary made in alumina (Al_2O_3) and toughened by zirconia (ZrO_2), as shown by Figure 12. This material is very resistant to mechanical shocks and thus is tolerant to abrupt contacts with the microwell walls. Moreover, the micropipette extremity was micromachined for providing inner and outer diameters of 56 µm and 150 µm respectively. As a result, the pipette can handle T, B and NK (lymphocytoid) cells of ~8 µm in diameter, and tumor cells of epithelial origin and melanomas of ~16 µm in diameter, as well as penetrate into the conical entrance of the microwell (300-400 µm in diameter) and collect the upper fraction of the microwell liquid. The microwell is supplied with liquid from the bottom fluidic chamber, and thus a prolonged aspiration of liquid by the micropipette is possible by continuous replacement of liquid during pipetting. Eventually, the meniscus height in the microwell can be increased beyond the microwell border by controlling the pressure inside the fluidic chamber. Meniscus upward movements can form a droplet above the microwell which facilitates the contact between the pipette and the liquid and thus favours the aspiration process.

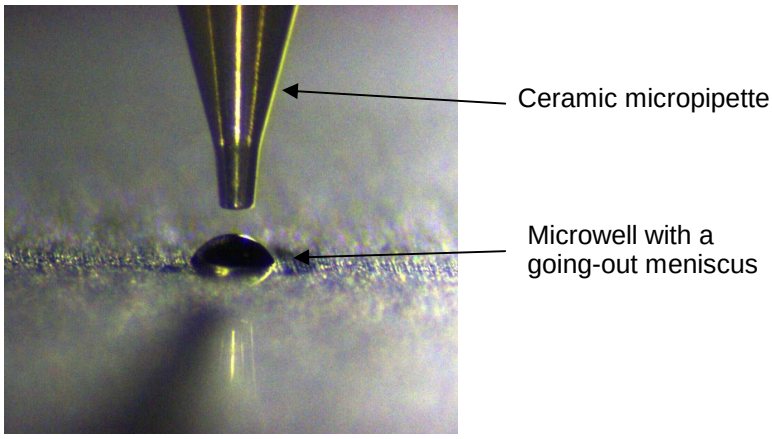

Figure 12 Handling of cell-containing liquid with a micromachined ceramic micropipette. The inner channel of the micropipette can be seen by transparency. A meniscus is formed above the microwell by enhancing pressure inside the fluidic chamber of the biosensor.

The ultrathin micropipette can be used both for depositing cells and for aspirating cells from microwells. By reversing direction of flow in the micropipette, the volume of liquid previously aspirated by the pipette can then be deposited into another microwell. Cells deposited in microwells can sediment inside the microwells within few minutes whatever the fluidic resistance of the microwell (Figure 13). Viability of pipetted lymphocytes was verified using a Trypan blue assay: no change in mortality ratio was observed compared to the initial cell suspension. A software program for the cell extraction system driving the pipeting of selected cells for the Cochise project was developed. A user's manual and a programmer's guide for the LabVIEW software were released. Some automatic inspection procedures of the presence of cells in the microwells were developed based on optical visualization with a vertical camera and an automatic image analysis software programmed with NI Vision.

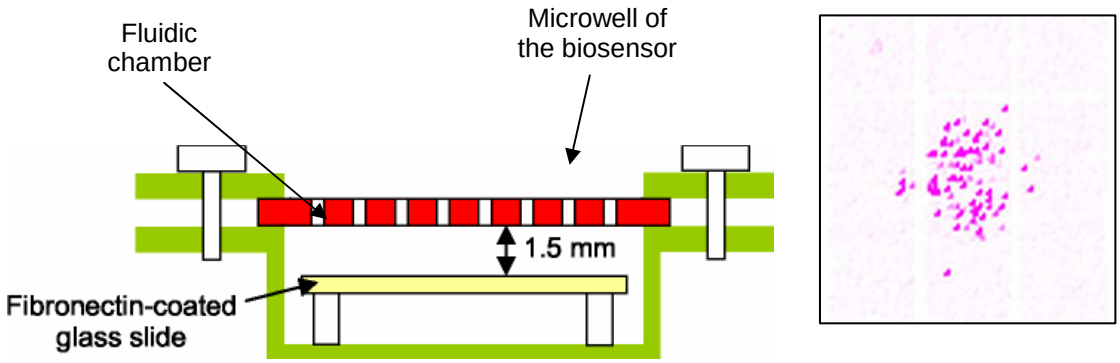

Figure 13 Collect of cells under the microwells after their dispense onto the well array of the biosensor. The cells are permitted to sediment along the microwell and then collected on a small glass slide coated with fibronectin. Cells are then attached to the glass slide, stained with a fluorescent label, and then detected using fluorescence microscopy.

4. Technology and manufacturing of the biosensor

4.1. Introduction

A key point in this innovative device is the fabrication technology of the microwell plate. Several technologies for microfluidic device fabrication and packaging have been proposed to integrate sensing capabilities and electrical interfaces into a single device. All of these approaches require complex steps or expensive facilities, and mostly are useful for prototyping only. Opposed to existing technologies, the biosensor developed within this project needs to be low cost and disposable to be applicable for the purpose intended. Due to these requirements standard printed circuit board (PCB) manufacturing processes have been selected as base technology. But as materials, with mainly copper as a conductive material, used for PCB manufacturing are not biocompatible new materials have to be identified and evaluated.

The PCB manufacturing process is a low cost mass production tool. It allows the realization of fine structures and vias with feature widths below 100 µm. Drawback of that technology is the default use of non-biocompatible materials as e.g. copper. Therefore, the first task was to identify biocompatible materials which can be processed with standard PCB technologies. But with new materials and new material combinations also processes as lamination, conductor line structuring, microwell drilling, via formation and via metallization need to be developed or at least adapted. Additionally surface modifications have to be developed for the sensor. Bottom side of the microwell sensor should have a hydrophilic surface for a bubble free wetting and flow of the nutrient solution for the cells. On the other hand the top side of the sensor should have a hydrophobic surface for easier cell positioning into the microwell and a defined nutrient solution meniscus over the microwell.

4.2. Materials

In standard PCB manufacturing process typically Resin Coated Copper (RCC) and glass fiber filled epoxy films are used. These materials are not applicable for the microwell realization due to non-biocompatibility of the copper and the poor processability of the fiber filled epoxies by laser structuring. Standard copper technology in combination with biocompatible metallization on top does not work due to the proposed manufacturing technology of the device (smearing of Cu particles during via drilling is contaminating also dielectric surfaces) and the metallization growth of an additional layer. Thus, no safe biocompatible device could be achieved using such a process combination.

Therefore, new dielectric materials had to be evaluated in combination with biocompatible metal layers. As possible metals for conductive layer formation palladium, aluminum and gold were taken into account. Pyralux (Py), a B-staged modified acrylic adhesive, in combination with Polyimide, printable epoxies and epoxy films with aramid fibers have been discussed as dielectric layers.

For realization of the final Lab-on-Substrate device also a hydrophobic / hydrophilic surface modification is needed. Therefore, different coatings and modifications have to be evaluated.

4.2.1 Biocompatibility Tests

Biological tests were performed on several different cell lines (LCL, K562 and IB3-1) and on several biological functions (cell growth, erythroid differentiation of K562 cells, CTL-mediated cell lysis of LCL cells, release of ICAM1 by IB3-1 cells). The results obtained firmly demonstrate that some materials exhibited strong inhibitory effects on most of the cell lines and biological functions assessed. When long-term culturing was carried out uncured Pyralux, printable epoxy fully-cured, Au over Ni, Au over Pd, Au over Pd + ODT, Cu and Cu + ODT inhibited biological functions. Aluminum and palladium were found to be not cytotoxic in most of the assays employed. Preparation of the materials appears to be a critical point. In this sense, both uncured preparation of Pyralux and Aramid displayed inhibitory activities in several assays, while cured Pyralux and epoxy films with aramid fibers did not. Most of the materials can be used for a short exposure of the cells, with the exception of Cu. A complete list of the materials tested and their results is described in detail in WP4.

4.2.2 Material Selection

Aluminum was selected as suitable metal layer due to low cost, biocompatibility, wide range of available foil thicknesses and good electrical conductivity. Different dielectric materials have been evaluated in a pre-study on their process potential for lamination and laser structuring and drilling. The combination of Pyralux / Polyimide has been finally chosen for further investigation due to the good availability, biocompatibility and good first process results. Table 1 summarizes materials discussed and their assessment.

Table 1: Possible materials for Lab-On-Substrate platform

Material	PCB-compatibility	Biocompatible	Processabilty	Availability	Costs
Conductor Material					
Cu	standard	no	good	good	low
Pt	non-standard	yes	to be evaluated	limited	high
Au	non-standard	yes	to be evaluated	limited	high
Al	non-standard	yes	to be evaluated	good	low
Dielectric Material					
RCC resin coated copper	standard	no	good	good	low
PCB core epoxy/glass fiber	standard	yes*	no laser cutting and drilling	good	low
Printable Epoxy	non-standard	yes*	with high effort	good	medium
Epoxy with Aramid Fibers	non-standard	yes*	to be evaluated	limited	medium
RCAl resin coated aluminum	non-standard	yes*	to be evaluated	not	-
Pyralux/ Polyimide	Flex: standard Al: non-standard	yes*	good	good	medium

*fully cured

4.3. Lab-On-Substrate Manufacturing Process Flow

The general process sequence for Lab-On-Substrate manufacturing with standard technologies is shown in Figure 14.

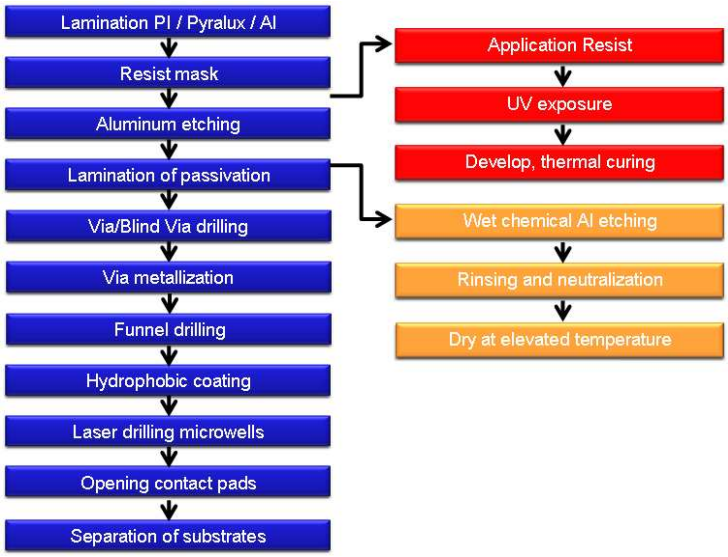

Figure 14 PCB Manufacturing Process Flow

Starting process is the lamination of the inner Al–Py–PI layer. In sequential steps Al structuring is done by wet chemical etching followed by lamination of the next dielectric and metal layer. Final layer on top and bottom side is a PI passivation. Microwells, through vias and blind vias are realized by laser drilling. Last process step would be the electroless metal deposition for via metallization.

4.3.1 Lamination

A Lauffer vacuum laminating press was used for all lamination processes described. This press allows the lamination under vacuum with defined parameters as pressure, temperature and time. Lamination process starts with a drying step at 50 ℃ under vacuum without pressure. The following lamination steps under force, heating and vacuum are adapted to the Pyralux cure. Figure 16 shows a cross section of a laminated Al-Py-PI stack. Homogeneous layer thicknesses and good adhesion between the different layers is achieved.

4.3.2 Aluminum Structuring

For the structuring of the conductor lines of the first samples a UV laser machine was used. The wavelength of the laser is 355 nm and the laser has a focus spot diameter of ~12 µm. Due to the characteristics of the machine, it is able to ablate different materials like polymers and metals. First samples were realized, producing just a single laser cut to isolate the conductor line from the remaining aluminum of the substrate. During the testing of these substrates problems with shortcuts of the conductor lines to the remaining Al and also in the area of the electrodes were observed, this is the reason for evaluating a combination of a laser photolithography and wet chemical aluminum etching.

A liquid photo resist is applied to the substrate by spin coating, realizing a nearly 6 µm thick resist layer. The structuring is done by UV exposure with a film mask and developing. A suitable thermal treatment is necessary to realize the needed stability in the etchant. The wet chemical etching was done using standard aluminum etching solution based on H_3PO_4, HNO_3 and CH_3COOH. By optimizing the etching process and the thermal treatment of the resist mask, it was possible to realize the structuring of the aluminum layer with a uniform under etching and a good reproducibility of the structures. Figure 15 depicts the resist mask after developing of a test structure (left) and the Al layer etched (right). Structures in the range of 50 µm could be reproducibly achieved.

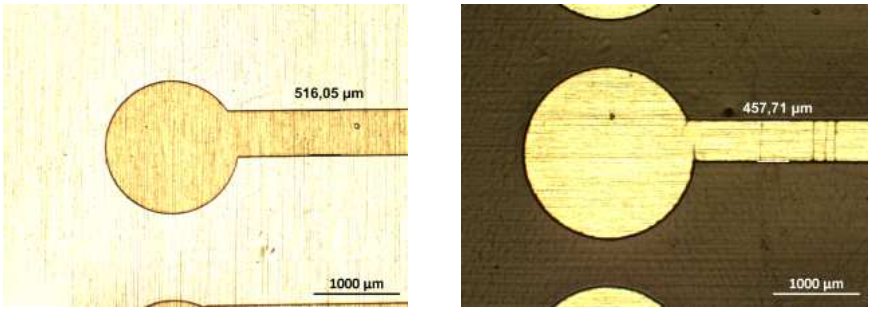

Figure 15 Developed resist mask (left) and etched Al layer (right).

4.3.3 Microwell and Via Formation

The formation of the laser via is done using the same UV laser system as for the first structuring trials. The drilling process is milling of the material by circling the laser beam. A typical aspect ratio of almost 4:1 could be realized. A 60 µm via in a stack of 210 µm overall thickness is shown in Figure 16 as an example.

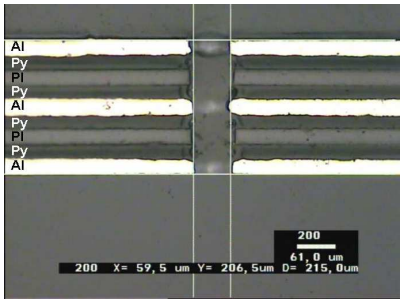

Figure 16 60 µm via in Al/Pyralux/PI stack.

Due to the different material properties of the Pyralux adhesive and the PI layer, the resulting via diameter of these materials is different. Vias in Pyralux are larger, possibly due to a combination of higher ablation rate and larger thermal shrinkage with this material. The protection of the substrate surface is crucial, since material removed during the laser drilling process can be re-deposited and thus contaminate the substrate surface. For further processing a suitable protective tape will be mounted to the substrate, which can be removed after the processing.

4.3.4 Via Metallization

An electroless Ni/Au process has been used to realize electrical connections between inner aluminum layers and solderable pads on top of the device. The basic process chain is a sequential treatment of the Al with different chemicals, which are needed to apply the desired pretreatment to the pads, followed by the electroless Ni and immersion Au deposition.

4.3.5 Funnel / Pool Drilling

For easier cell spotting into the microwells a funnel or a pool had to be realized above each microwell. Therefore on top of the final metal layer two thick polyimide layers (thickness ~300 µm) are laminated. The polyimide acts as passivation on the metal structures and as a base for the funnels/pools. Funnels are mechanically drilled before the microwells are drilled by laser. For drilling a standard programmable 3D milling machine from Datron is used. The optical unit of the machine allows an alignment of the drilling to the metal structures. Realized structures are shown in Figure 17.

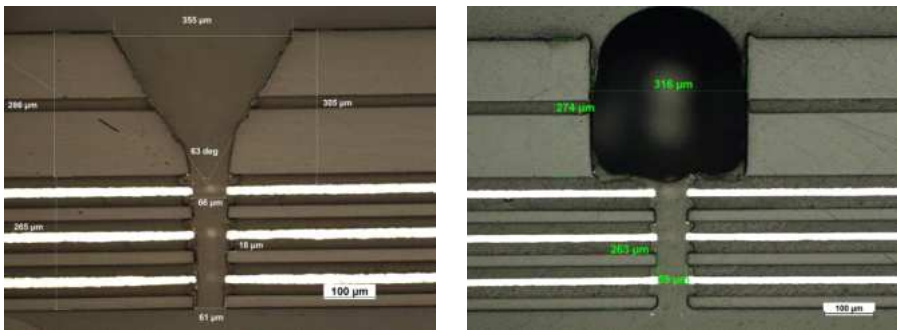

Figure 17 Funnel (left) and pool (right) realized by mechanical drilling in the final Polyimide top layer

4.4. Hydrophobic / Hydrophilic Surface Modifications

The materials used for the realization of the device (see Table 2) were characterized and classified according to their surface energy using a Dataphysics OCA 20 contact angle measurement unit with test liquids bromonaphthalene (nonpolar), glycerol (slightly polar) and water (highly polar). It was found that the surfaces of both polyimide and aluminum must be treated to allow a designing of a hydrophilic/hydrophobic microfluidic network required for a successful operation of sensor devices.

Surface modifications were tested with:

- fluoralkyl polymer, hydrocarbon polymer (technical release agent), octadecane thiol and SF6-plasma to increase hydrophobicity and
- organic acids, O2-plasma and several polar silanes to increase hydrophilicity.

Most of these modifications gave insufficient or inhomogeneous results, so experiments were focused on fluoralkyl and hydrocarbon to get hydrophobic surfaces and O_2-plasma for a hydrophilic treatment.

The materials used for the build up of the demonstrators were investigated intensively. Results of contact angle measurements of water droplets on aluminum and polyimide surfaces are shown in Figure 18.

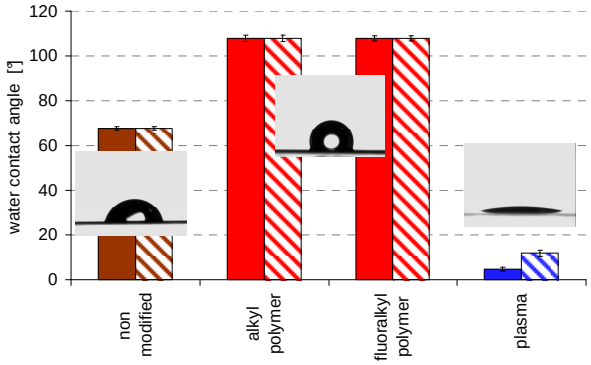

Figure 18 Contact angles of water droplets on hydrophilic and hydrophobic modified PI-surfaces. Fully colored columns are measured directly after surface modification; patterned columns show values after one week storage in air (normal atmosphere).

The measurements show hydrophobic as well as hydrophilic modifications work well and are stable at least for one week under normal conditions (room temperature, 60 % relative humidity). Long-term stabilities of these surface treatments under different storage conditions were also investigated; results are shown in Figure 19.

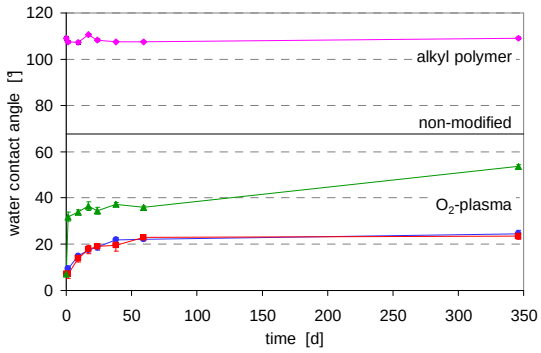

Figure 19 Contact angles of water on modified PI-surfaces versus storage time under different storage conditions: ● ◆ air, ■ dry N_2, ▲ distilled water

It was found that the hydrophobic modification by an alkyl polymer is stable under normal conditions for nearly one year. Hydrophilic modification also showed long term stability, only a slight increase of the water contact angle was detected during the first few weeks of storage of O2-plasma treated samples under air and dry nitrogen, indicating a marginal loss of hydrophilicity. Storage under water was found to reduce the hydrophilic treatment drastically during the first day and then tending slowly to the value of the non-modified polyimide.

Aluminum was also modified with several substances to get a higher hydrophobic character of the surface; results are shown in Figure 20. It can be seen that surface treatments with fluorcarbons as well as hydrocarbons gave highly hydrophobic surfaces, whereas the effect of the long chain thiol ODT is only minor.

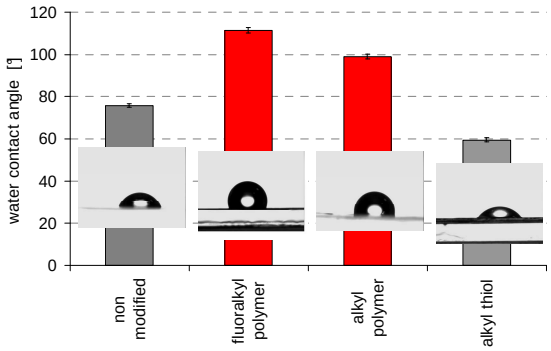

Figure 20 Contact angles of water droplets on hydrophobic modified PI-surfaces.

5. Biotechnological sensor validation

The first objective of WP4 was to perform careful analysis for the determination of the biocompatibility of materials employed for the fabrication of the COCHISE Biosensor platforms. This objective is related to the concept that the manipulation of single cells requires that the materials employed are non-toxic and defined as biocompatible. The second objective was the development of experimental cellular systems useful to validate the COCHISE platform. The third objective was the manipulation of single cells using the available COCHISE platform, performed with the following specific aims: (a) determine whether this procedure is not toxic, (b) determine whether the manipulated cells maintain their biological activity. The fourth objective was to develope highly sensitive methodologies for analysis of low numbers of cells, suitable for characterization of the target/effector cells recovered from the COCHISE platform. The fifth objective was to develop suitable in vivo experimental systems.

WP4 was the responsible of the culture, characterization, storage and expansion of all the cell lines used by the other WPs for the preliminary characterization of the COCHISE Biosensor platforms. The cell lines employed are the following (a) human IB3-1 tracheal cells producing a variety of interleukins and cytokines when treated with TNF-alpha (Task: secretomic analysis from low number of cells); (b) K562 cells induced to erythroid differentiation and production of hemoglobin by mithramycin (this cell line is the model system employed by UniBO and Mindseeds Laboratories to characterize the devices); (c) LCL cells (target of CTLs and used for the biosensor validation); (d) the target 221 and 221.G1 cells (target of the NK cell lines and used for the biosensor validation); (e) the NKL and YTS natural killer cell lines, used for the biosensor validation; (f) the RMA and RMA-S tumor cells, used by Angelini for developing the in vivo cell system; (g) several GFP-positive clones for in vivo experiments; (h) rat hippocampal primary cells.

5.1. Major achievements

5.1.1 Biocompatibility of materials used for Biosensor fabrication.

The precise analysis of the biological effects of materials used for the construction of LOAC platforms with PCB technologies for cell manipulation is a required pre-requisite for the design of the possible applications of the devices in biomedicine and biotechnology. The analysis of the effects of biomaterial on biological functions have been analysed on: (a) rat ippocampal cells differentiating in neurons and astrocytes; (b) human IB3-1 tracheal cells producing a variety of interleukins and cytokines when treated with TNF-alpha; (b) K562 cells induced to erythroid differentiation and production of hemoglobin by mithramycin and (c) CTL mediated LCL-lysis. We have conclusively and reproducibly analysed all the materials using long-term exposures, supporting the conclusion that among the 24 materials tested (7 employed in surface treatments, 14 dielectrics/adhesives, and 5 metals) some display strong inhibitory effects on biological functions. When the results obtained on cell growth parameters and morphology of rat hippocampal cells are considered together with those obtained with K562 and LCL, it can be concluded that all materials can be used for a short exposure to the cells, with the exception of aramid fiber filled epoxy uncured and, to a lower extent, Au over Pd, Cu, DAF, Chemlease 41-90 temp. Therefore, the use of these materials should be avoided, even if the device under construction is designed for short-term cell exposure, for instance in the case the Lab-on-a-chip platforms is simply dedicated to cell manipulation, isolation and sub-culturing. Of great importance for the COCHISE Project are the results of biocompatibility obtained using CTL as experimental model system. The results obtained support the concept that all materials are compatible with this biological activity for a short exposure, except aramid fiber filled epoxy uncured, Cu and, to a lower extent, Cu+ODT, Au over Pd and Tesa 4985. Therefore, all the other materials are compatible with preparation of platforms for analytical purposes.

5.1.2 Biosensor validation

In order to validate the Biosensor, generation of EBV-EBNA1 specific cytotoxic T-lymphocytes was performed, HPV specific CTLs were obtained from monocyte-depleted PBLs from EBV-seropositive

donors stimulated with synthetic peptide HPV (HPVGEADYFEY from EBNA1–EBV protein, aa 407-417). In addition, NK cells were employed exhibiting (a) differential lytic properties and (b) possibility to be identified by antibody-antigen interactions. For CTL-mediate lysis the LCL cell lines was employed. For NK-mediated lysis, two tumor cell lines were employed, the 221 and 221.G1 lymphoblastoid cells. NKL cells were shown to be able to lyse the 221 cells, but unable to efficiently lyse 221-G1 cells; NK-YTS cells were able to lyse with high efficiency both 221 and 221-G1 cells.

The first control experiment on LCL cells is depicted in Figure 21 and demonstrated the possibility to isolate these cells in microwells using the reversed microwell approach and to assess cell viability. Calcein fluorescence of target LCL cells was tested inside the device for more than 30 minutes. These results firmly demontrated that calcein fluorescence of target cells (LCL cells for CTL activity assays, and 221/221.G1 for NK cell activity assay) is stable within the COCHISE device, rendering feasible the analysis of CTL (or NK-) mediated cell lysis leading to calcein loss from target cells.

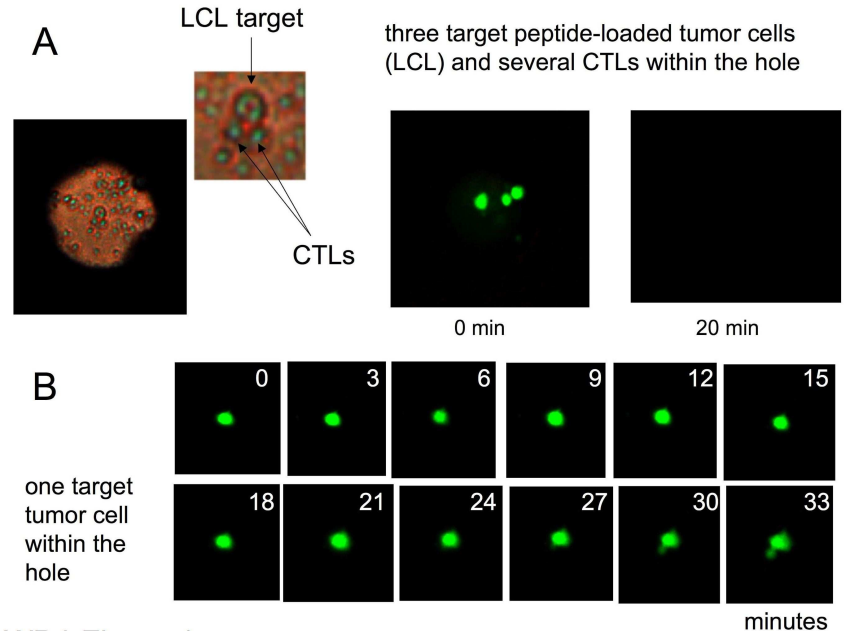

Figure 21

In a typical experiments aimed at determining the activity of CTLs, CTLs were delivered in microwells when single LCL cells were previously deposited. Pictures, taken every 5 minutes show the calcein disappearance in LCL cells pre-loaded with peptide (Figure 22) and consequently demonstrate the lysis of these cells by the surrounding CTLs. Repeated experiments indicate the lysis happen between 10 and 15 minutes.

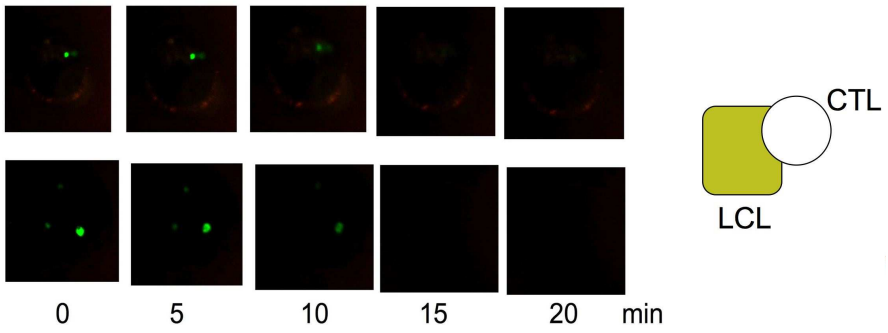

Figure 22

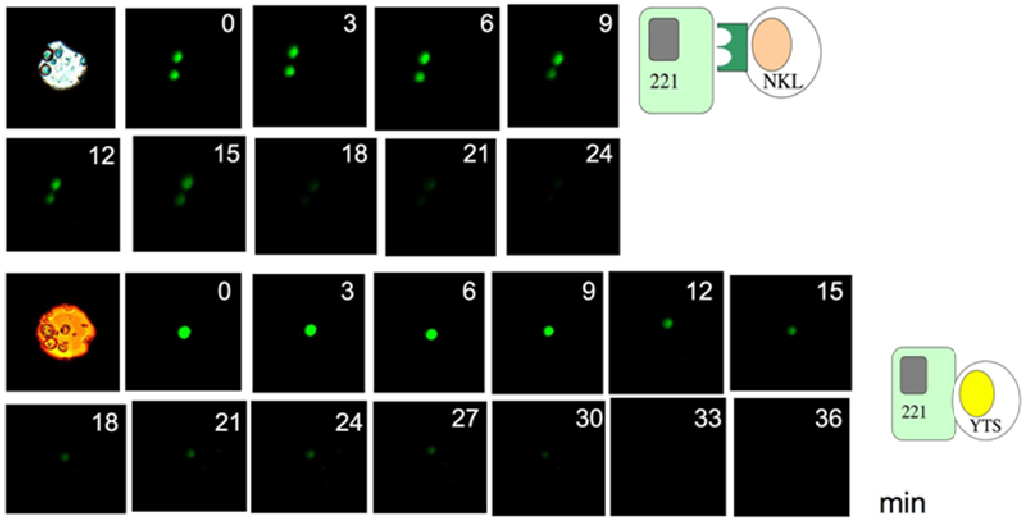

Figure 23

Figure 23 demonstrates that NK-mediated lysis is detectable on the COCHISE Biosensor using the 221 cell line as target and the NKL and YTS cells as effectors. In this particular representative experiment pictures were taken each 3 minutes.

These experiments firmly demonstrate that the COCHISE Biosensor is suitable for real-time detection of lysis of single target cells by CTL (Figure 22) and NK (Figure 23) cells.

5.1.3 Technical achievements

1. Methods to characterize biocompatibility of materials used for Lab-on-a-chip fabrication (UniFE, UniBO).

2. Technologies to identify NK cells exhibiting different lytic properties.

3. Set up of a NK cell-sensitive tumour model in C57BL/6 mice (Angelini, UniFE).

4. Protocol allowing the isolation and biomolecular characterization of single cells (ICP)

5. Protocol for isolation of single lytic tetramer-positive T cells (ICP).

6. Dissemination and exploitation

6.1. Dissemination activities

Dissemination activities within COCHISE project aimed at raising the level of public awareness about the perspectives of biosensor technology in the field of oncology.

6.1.1 Press Releases

The Consortium issued two press releases.

The first one (Annex 1) was issued in September 2008 in 3 countries (France, Germany and Italy) and it led to the appearance of 19 articles, 13 of which were online and 6 on papers. In Italy 11 articles appeared, 5 in France and 3 in Germany. The total readers audience was about 3 millions people. Prof. Guerrieri was interviewed by a couple of Italian magazines.

The second press (Annex 2) release was issued at the beginning of July 2009, just after the end of the project. It constitutes the main document to communicate the results achieved during the research as well as the plan for future activities towards the industrial community and the European Community. This press release was issued in four countries (France, Germany, Italy and the UK) and it was mailed to 33 journals in Italy, 35 in France, 29 in Germany and 55 in Great Britain.

6.1.2 Flyer

A flyer illustrating the COCHISE project to the wider scientific community interested in biosensor technology was prepared during the second year of the project (Annex 3). Its purpose was to raise interest and awareness in this field providing a set of basic information and contact references about the COCHISE project. The flyer was used during dissemination events, its purpose being to make the general scientific community aware of the project and of its implications in different areas.

6.1.3 Web Site

The COCHISE web site (http://cochise.arces.unibo.it) has been the main communication channel to the external world, providing a flow of information about the project. The web site describes the consortium participants, contains documentation and public project documents, provides training material, publishes news about the project main events and favours contacts among the project partners and between the partners and interested visitors.

During the project, the site has provided an updated view of the progresses made by the project and now it may evolve to become a reference web site about biosensor technology for diagnostic and therapeutic applications.

During the project the web site has been contacted by about 1500 visitors, half of whom were from Italy and the rest from France, Germany and the United States.

6.1.4 List of publications and dissemination events

Type	Nr	Place and Date/Journal	Authors, ”Title”	Contact Author	Status

a	1	MicroTAS, October 2007, Paris, France	B. Iafelice, F. Destro, D. Manessis, D.Gazzola, E. Jung, L. Bottcher, M. Borgatti, T. Braun, J. Bauer, R. Gavioli, R. Gambari, A. Ostmann, R. Guerrieri "Aluminium printed circuit board technology for biomedical microdevices"	B. Iafelice	Published
a	2	9th EPT Conference, November 2007, Singapore	T. Braun, L. Bottcher, J. Bauer, E. Jung, A. Ostmann, K.-F. Becker, R. Aschenbrenner, H. Reichl, R. Guerrieri, R. Gambari "Microtechnology for realization of dielectrophoresis enhanced microwells for biomedical applications"	T. Braun	Published
a	3	DTIP Conference, April 2007, Stresa, Italy	E. Jung, D. Manessis, A. Neumann, T. Braun, J. Bauer, H. Reichl, B. Iafelice "Lamination and laser structuring for a DEP microwell array"	E. Jung	Published
a	4	Biosensors 2008, May 2008, Shangai, China	M. Bocchi, M. Lombardini, A. Faenza, L. Rambelli, L. Giulianelli, N. Pecorari, R. Guerrieri "Dielectrophoretic trapping in microwell for manipulation of singlecells and small aggregates of particles"	M. Bocchi	Published
p	5	CMOS Biotechnology, H. Lee, D. Ham & R.M. Westervelt Eds., Springer-Verlag 2007	C. Nastruzzi, A. Tosi, M. Borgatti, G. Medoro, R. Guerrieri, R. Gambari "Applications of dielectrophoresis-based-on-a-chip devices in pharmaceutical sciences and biomedicine"	R. Guerrieri	Published
p	6	Microsystems Technologies, 14 (2008) S931-S936	E. Jung, D. Manessis, A. Neumann, L.Bottcher, T. Braun, J. Bauer, H. Reichl, B. Iafelice, F. Destro, R. Gambari "Lamination and laser structuring for a microwell array"	E. Jung	Published
p	7	Sensors and Actuators A: Physics, 145-146 (2008) 194-200	D. Gazzolla, B. Ianfelice, E. Jung, E. Franchi Scarselli, R. Guerrieri "An integrated meniscus sensor formeasurement of evaporative flow"	D. Gazzolla	Published
p	8	Microfluidic Nanofluidic, Springer Verlag, 2009, doi 10.1007/s10404-0090426-5	D. Gazzolla. E. Franchi Scarselli, R. Guerrieri "3D visualization of convection patterns in lab-on-a-chip with open microfluidic outlet"	D. Gazzolla	Published on line
p	9	Biosensors and Bioelectronics, 24 (2009), 1177-1183	M. Bocchi, M. Lombardini, A. Faenza, L. Rambelli, L. Giulianelli, N. Pecorari, R. Guerrieri "Dielectrophoretic trapping in microwells for manipulation of single cells and small aggregates	M. Bocchi	Published

			of particles"		
a	10	Proceedings of Nanotech 2009, Houston (TX), vol. 2, pp 181-184	M. Lombardini, M. Bocchi, L. Giulianelli, R. Guerrieri "Precise positioning of particles in microwells within horizontal DEP cages"	M. Bocchi	Published
a	11	IEETransactions on Nanotechnology	M. Bocchi, E. Franchi, R. Guerrieri "Handling of rare cells for biomedical and environmental applications"	M. Bocchi	Submitted under invitation
p	12		M. Bocchi, E. Duqi, L. Giulianelli, N. Pecorari, R. Guerrieri "Macro-to-micro interface for a flexible lab-on-a-chip platform"	M. Bocchi	In preparation
p	13		M. Lombardini, M. Bocchi, L. Rambelli, R. Guerrieri "DEP cage within open microwells array for precise positioning of K562 cells"	M. Bocchi	In preparation
c	14	Micro-Nano-Bio convergence Systems (MNBS), March 12, 2009, Brussels, Belgium	M. Bocchi "The COCHISE project"	M. Bocchi	Oral presentation
c	15	Nanotech 2009, May 5, 2009, Houston (TX)	M. Lombardini, M. Bocchi, L. Giulianelli, R. Guerrieri "Precise positioning of particles in microwells within horizontal DEP cages"	M. Bocchi	Oral presentation
c	16	Techconnect Summit 2009, May 3-7, 2009, Houston, TX	M. Bocchi "Microtiters with integrated electronics for high-throughput single-cell manipulation and analysis"	M. Bocchi	Oral presentation
m	17	Inst. of Molecular Medicine, Houston, TX	M. Bocchi Presentation of MindSeeds Laboratories and its core technologies	M. Bocchi	Oral presentation
p	18	Phys. Status Solidi A, 1-6 (2009) doi 10.1002/pssa.200880480	T. Braun, L. Bottcher, J. Bauer, E. Jung, K.-F.-Becker, A. Ostman, R. Aschenbrenner, H. Reichl Lab-on-substrate technology platform"	T. Braun	Published on line
a	19	Proceedings ECTC 2009, San Diego (CA)	T. Braun, L. Bottcher, J. Bauer, M. Bocchi, A. Faenza, R. Guerrieri, R. Gambari, K.F.-Becker, E. Jung, A. Ostman, M. Koch, R. Kahle, R. Aschenbrenner, H. Reichl "Biocompatible Lab-on-substrate technology platform"	T. Braun	Published
a	20	IMAPS Microtech 2009 Workshop on Biosensors and MEMS packaging,	E. Jung et al. "Microtechnology platform for cell	E. Jung	Published

		March 2-3, 2009, Edinburgh (UK)	interaction detection"		
p	21	Int. J. Mol. Med.	R. Rizzo et al. Production of sHLA-G molecules by "in vitro" matured cumulus-oocyte complex	R. Gambari	In press
p	22	Biomaterials	M. Mazzuferi et al. Effects of materials for Lab-on-a-chip production with Printed Circuit Board technologies on different cell systems	R. Gambari	Submitted
p	23	J. Biomaterials Science, Polymer Edition	F. Destro et al. Effects of biomaterials for Lab-on-a-chip production on cell growth and expression of differentiated functions of leukemic cell lines	R. Gambari	In press
c	24	10th International Thalassemia Conference, May 6-7, 2009, Cairo (Egypt)	R. Gambari Novel trends for the discovery anf characterization of modifiers of globin gene expression	R. Gambari	Oral presentation
c	25	National Meeting of PhD course in Biochemical Sciences, June 9-12, 2009, Brallo – Pavia (Italy)	F. Destro et al. Effects of biomaterials for Lab-on-a-chip production on differentiated functions of leukemic cell lines	R. Gambari	Oral presentation
a	26	12th International Symposium on Molecular Medicine, October 2009, Athens (Greece)	R. Gambari et al. Set up of a NK cell-sensitive tumour model in C57BL/6 mice.	R. Gambari, C. Milanese	To be published in Int. J. Mol. Med.

a=abstract, p= paper; c= conference; m= meeting (general)

6.1.5 University Courses/Thesis

The COCHISE Project is included in 2 lessons within the course "Nanobiotechnology and Biosensors" (Pharmeceutical and Chemical Technology, Ferrara University, years 2007-2008 and 2008-2009, Faculty of Pharmacy, responsible Roberto Gambari). More than 100 students attended to the course.

Roberto Gambari presented the Conference "Nanotechnologies and Lab-on-a-chip devices: Biomedical applications" at Ferrara University U.T.E.F., University for the Permanent Education (29 April, 2008).

PhD Thesis: Lab-on-a-chip devices based on dielectrophoresis: applications in Pharmaceutical and Biomedical Sciences (PhD student: Dr. Enrica Fabbri, Tutor: Prof. Roberto Gambari).

6.2. Exploitation activities

The goal of exploitation activities within COCHISE has been the identification of the practical utilization of the results obtained during the research directly by the partners or by external companies and institutions interested in the biosensor technology.

6.2.1 Intellectual Property protection

The research carried on within the COCHISE project allowed to file two different patents.

The first one was filed by MindSeeds in 2007 with the title “Method and apparatus for manipulating single cells and small aggregates thereof” and it covers the main technical aspects and the applications of a platform developed for immune cell characterization (patent number WO/2007/138464)

The second patent was filed by University of Ferrara in 2007 with the title “Diagnostic and therapeutic application of CTL and NK cells functionally selected” which covers the application of Lab-on-a-chip platforms to CTL characterization (patent number WO/2007/116309) and covers the major biomedical issue of the COCHISE, i.e. the application of Lab-on-a-chip platforms to CTL characterization, was further reinforced and confirmed in its potentiality thanks to the results obtained within the COCHISE Project.

6.2.2 Training activities

University of Bologna organized a training activity within the “Solid state sensors” course for University students where applications of dielectric measurements of cell suspensions were explained and the architecture of a biosensor for the manipulation of single sells or small aggregates were explained.

Several Ph.D. students of the Bologna University worked within COCHISE project and part of this work will be included in their final Ph.D. theses.

Angelini hosted a Ph.D. student from Bologna University to train her in the use of new instrumentation for cell analysis and manipulation.

6.2.3 Exploitation plan

Exploitable knowledge	**Exploitable product measure**	**Target market**	**Development timeline**	**Owner and involved partners**
Platform for high-throughput single cell recovery	Isolation of rare cells, modification of cell strains	Health, Energy	Long term	MindSeeds
Identification of biocompatible materials	Standard PCB fabrication process	Bio-platform technology	Medium term	Fraunhofer IZM
Platform for high-throughput single cell recovery	Biomarker identification	Cancer immunotherapy	Long term	ARCES - UniBO
Platform for high-	Pharmacological high	Drug discovery	Medium term	Angelini

throughput single cell recovery	throughput screening			
Downsizing hole diameter and pitch size	Cell arrays and nozzle arrays	Research device manufacturers	Medium term	Micronit Microfluidics
Ultrathin alumina micropipette system	Smart pipette development to be used as mixing element and for cell imaging	Research device manufacturers	Medium term	CEA

6.2.4 Socio-economic impact of biosensor technology

Biosensors are analytical devices extensively employed in many fields such as clinical diagnosis and biomedicine, military applications, agricultural analysis, process control, fermentation control, pharmaceutical and food analysis, pollution monitoring, industrial gas analysis and control. The biosensor market has significantly increased and will be mushrooming in the next decade with a total biosensor market over $15 billion at the end of 2010.

We think that, in this favorable situation for biosensors, the long-term innovation provided by the COCHISE device will surely find social and economical applications in the field of medicine and diagnostics.