NMP4-CT-2005-017114

"RECEPTRONICS"

LABEL-FREE BIOMOLECULAR DETECTORS: AT THE CONVERGENCE OF BIOENGINEERED RECEPTORS AND MICROELECTRONICS

Instrument: STREP

Thematic Priority: NMP

FINAL PUBLISHABLE EXECUTIVE SUMMARY

1st Oct 2005 – 30th Sep 2008

Start date of project: Oct. 1st 2005   Duration: 3 years

Publishable executive summary

Introduction

RECEPTRONICS, is a project that has been financed by the VIth European Research WorkProgram involving 9 partners from European Institutions, namely: ARCES University of Bologna (Coordinator), Centre National de la Recherche Scientifique - CNRS Grenoble (FR), DSV and LETI laboratories at Commissariat à l'Energie Atomique - CEA Grenoble (FR), National and Kapodistrian University of Athens - NKUA (GR), Electrical Engineering and Chemistry Schools of University of Southampton (UK), Silicon Biosystems, S.p.A. (IT), and SPI-BIO (FR) .

The total cost of the RECEPTRONICS project has been about 3.5M€ with a grant of 2M€ from EC. The goal has been to develop low-cost, label-free biomolecular sensors by integrating concepts and methods from bio-nanotechnology and microelectronics. The RECEPTRONICS project is based on a strong multi-disciplinary platform where integration of knowledge from Biology, Physics, and Information Technologies is required.

Counting molecular events one-by-one

The project has been aimed to achieve the goal of estimating target concentrations by counting single molecular events. Due to technical limitations and sometimes convenience, biological analyses traditionally take averages over group of cells or molecules. However, averaging is sometimes problematic as it may mask important individual variations in structural and dynamics properties at both the cellular and molecular levels. Single molecule approaches have the advantage of avoiding averaging, enabling observation of transient intermediates and heterogeneity. Hence they might revolutionize the way many biological questions are addressed. This approach is true especially in sensing techniques. Unlike sensors aimed at detecting macroscopic quantities such as weight, pressure or acceleration, the input in biosensing is by definition a discrete quantity: the molecule. For this reason, by identifying single molecules in a sample might lead to the maximum resolution physically achievable. In other words, since concentration is made of a finite number of

elements, the best sensitivity can be achieved by counting molecular events of the same nature one-by-one. This is the reason why the single molecule detection challenge plays a fundamental role for implementing highly sensitive devices for both applications in diagnostics and drug discovery. Unlike conventional biosensors where concentration sensitivity is in the milli-molar range, the granularity of the input becomes apparent below the pico-molar order of magnitude. In molecular recognition, state-of-the-art artificial systems cannot compete with living organisms. As an example, the sensitivity of chemical senses in insects is much greater than conventional electronic systems such as the electronic nose.

State-of-the-art nose technology is currently based on electrical conducting polymers - materials which are similar to plastics but can conduct

electricity. These materials can be primed to absorb and respond to different odour molecules, and a typical artificial nose will feature an array of polymer sensors, each of which is responsive to a particular substance. However, odours affect many of the sensors in different ways, and the resulting pattern of responses needs to be analysed. Furthermore, their sensitivity is still relatively low. The RECEPTRONICS project has picked up the problem from a different angle. The project plan to replicate what goes on in biology. In the nose are cells with molecules embedded in the cell membrane. When these bind with an odour molecule, a hole opens in the molecule and an electrical current flows, creating a stimulus, which is transmitted to the brain.

The Project proponents are strongly convinced that the goal of molecular recognition could only be achieved using several strategies belonging to both Nanotechnology and Information

Technology. This is the reason why RECEPTRONICS is organized in a stack of technology objective layers (see figure below) where each task is integrated and developed in a strong synergy with others.
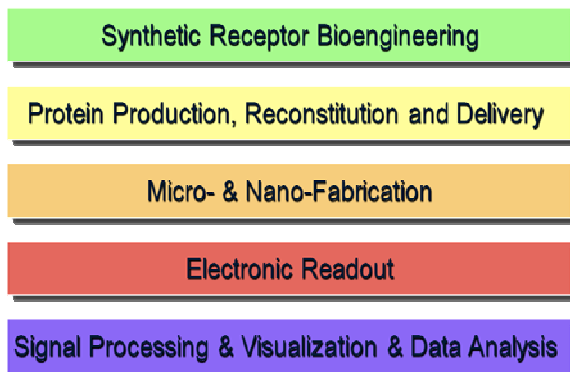
The way followed by the project to boost the sensor sensitivity is based on using affinity-binding paradigm employed by receptors. Receptors are proteins that are usually sitting on the cell membrane that are employing an extremely efficient molecular machinery for detecting specific molecules. The goal of the project is to combine efficiency of this bio-molecule with powerful flexibility of integrated electronics in a unique device.

The researchers will use molecular engineering techniques to create customized receptors that are sensitive to different substances. These receptors will be embedded in membranes in an array, with each receptor linked to an electronic interface, which can detect electronic signals transmitted when the receptor binds with its target molecule. The system will be mounted on a credit-card sized chip. The three-year project has been focused on designing a system, which could be used in medicine to detect hormones, and so help doctors to diagnose a wide range of diseases. In future years, molecular recognition will be one of the most important steps required for a deep understanding of mechanisms in living beings. Every active cell interacts each other and with the environment by means of a complex network of molecular messengers at very low concentration. There are perhaps thousands or millions of regulatory substances in the human body and any imbalance between them may have dramatic consequences for well-being and health.

Being able to detect specific biological molecules at very low concentrations is a new promising area of Medicine that aims to identify the onset or prediction of disease before the patient shows any symptoms. If the technology can be made cheap and simple enough for widespread use, it will enable the rapid identification and monitoring of proteins and pathogens. As a result, it will be possible not only to give appropriate treatment much more quickly but also to make treatment patient specific, leading to fewer side-effects and faster patient recovery. RECEPTRONICS introduced a technology that in future years could be used for sophisticated diagnostic tools in the field of early cancer diagnosis and hormone balance monitoring. Furthermore, the same technology could be employed for detecting contaminants at very low concentration for environmental safety in Agriculture and industrial processes.

The big picture

The approach is based on arrays of independent and electrically addressable micro-spots where functional lipid bilayers are self-assembled either in structured micron-scale apertures, or in artificial nanometric pores. Membrane functionalization is determined by embedding bioengineered ion channels or receptors in the lipid bilayers, to achieve highly specific interactions with target molecules. The array is organized so as to couple each spot with a smart and miniaturized electronic interface for the detection, amplification and conditioning of the signals produced by the transmembrane ion fluxes induced by the target molecules. An additional goal of this approach is to boost the overall system performance by means of advanced data processing and storage architectures.

The figure below shows the building blocks of the Receptronics technology at different levels of abstraction. More specifically, it shows the different scales on which the approach is developed: from nanometer to micrometer and above. The receptor bioengineering has been developed by CNRS. Protein production and delivery by the School of Chemistry of the University of Southampton. As log as the micro- and nano-fabrication is concerned, three modular platforms have been concurrently developed during the project: from School of Electrical Engineering of the University of Southampton, CEA, and NKUA. Electronic readout and signal processing have been developed by ARCES and Silicon Biosystems. Analysis on existing diagnostic kits have been done by SPI-BIO.
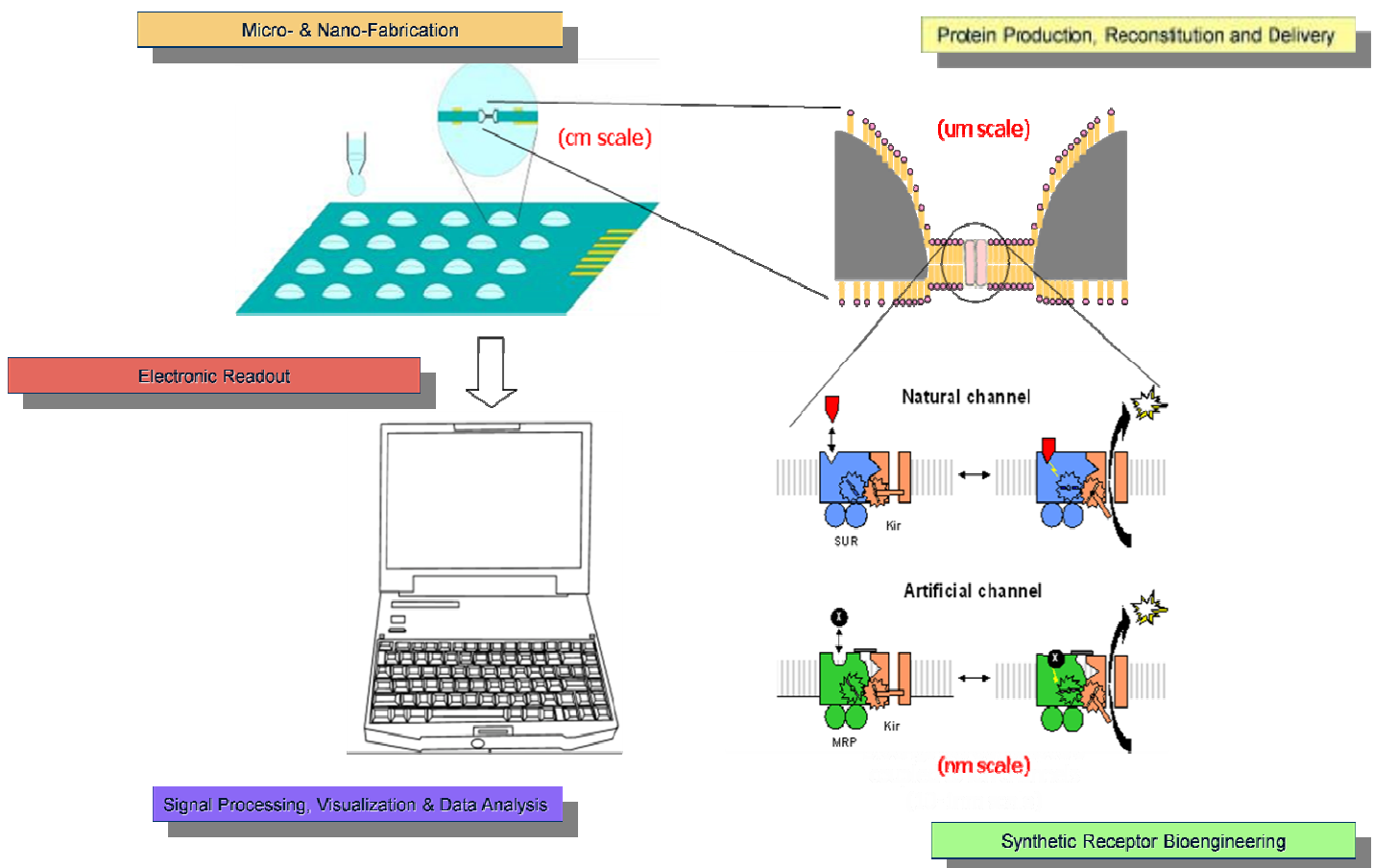

Figure 1: Building blocks of the Receptronics technology

In the picture below the data processing flow of the Receptronics technology is shown. The single molecular event at nanoscale is revealed in a spot site by extremely small current variations that should be first amplified by ultra-low noise embedded amplifiers. Spots are arranged in an array so as to readout concurrently single molecular events. Signals are converted by special Sigma-Delta amplifiers and stored on a portable PC platform for data processing and analysis.
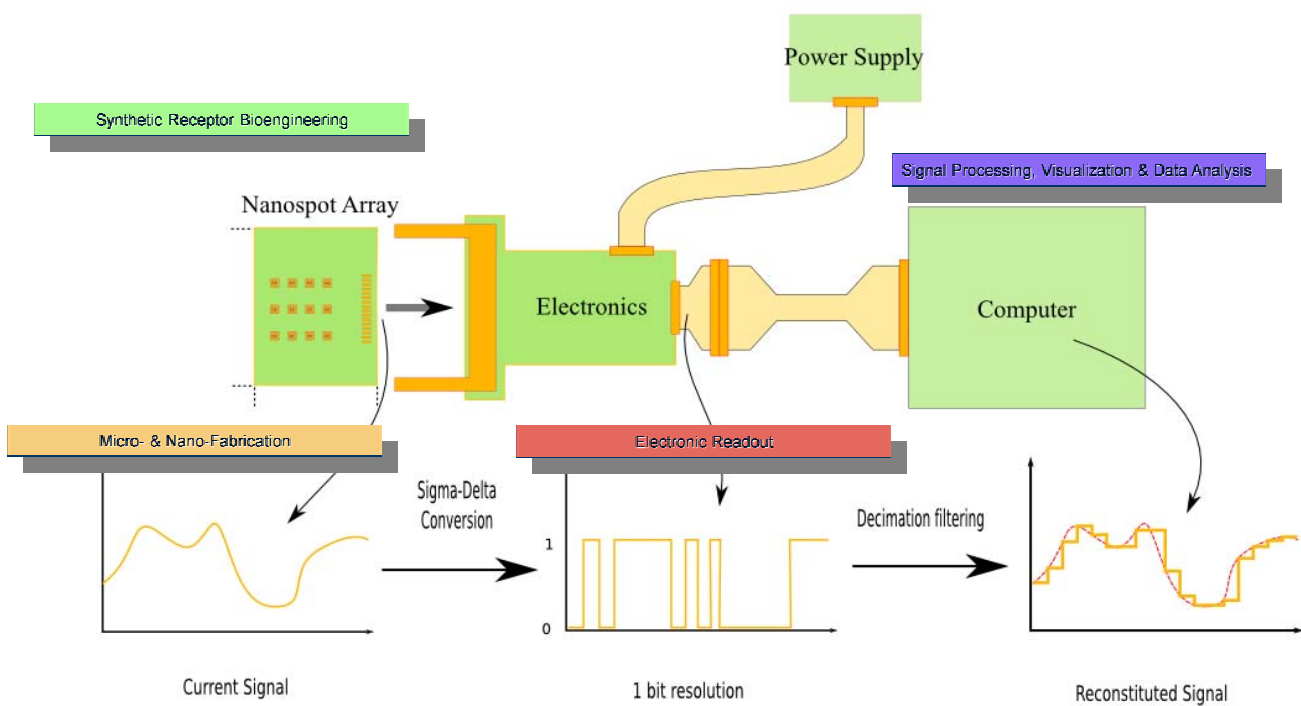

Figure 2: Data flow of the Receptronics technology

Syntetic Receptor Bioengineering

The CNRS partner from the Institut de Biologie Structurale in Grenoble, France, was in charge of designing the sensing elements, or biosensors, at the core of the Receptronics project. The goal was ambitious since these elements had to combine seemingly irreconcilable properties: a miniature size in the nanometric scale, the ability to detect and identify tiny amounts of molecules, the capacity to produce an electrical signal readily understood by electronic equipments.

To reach this goal, the original idea was to take advantage of the natural devices perfected by evolution that are used throughout the body to detect biochemical signals and produce electrical signals. These natural devices are receptors and channels, respectively (**Figure 3**). Cells being delimited by an impermeant lipid membrane, the flow of informations or substances is handled by specialised membrane proteins. Among those, receptors identify chemical signals arising from other cells or the environment and ion channels control the ion flux responsible for electrical signal generation.
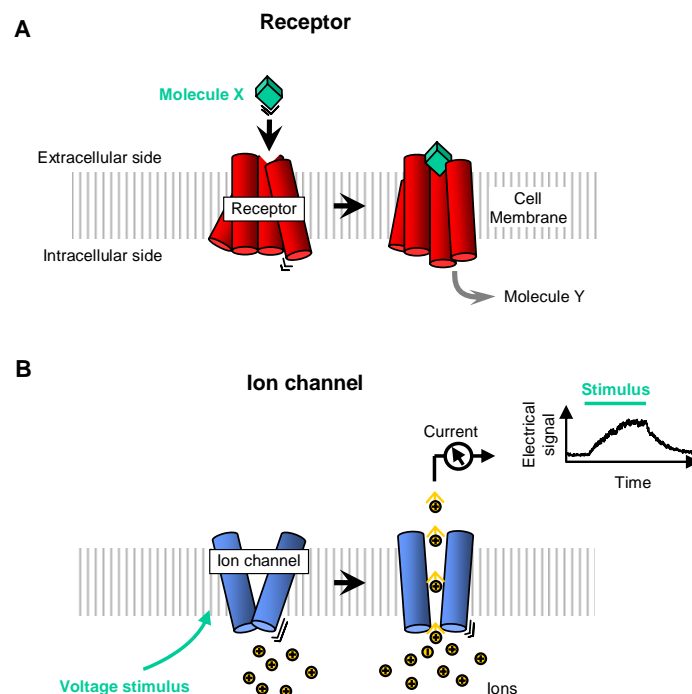

Figure 3: Receptors and channels
A. A cell detects the presence of external chemical signals, arising from other cells or from the environment, by means of receptors embedded in its membrane. The binding of a given molecule X to a receptor, such as a G-protein coupled receptor (GPCR) induces a change in the structure of the receptor which propagates across the membrane and triggers release of a molecule Y inside the cell. This allows conversion of external chemical signal X into internal chemical signal Y without any substance transfer through the membrane.
B. Ion channels are distinct types of membrane proteins that form holes through the membrane. These holes are very selective since they let pass only the smallest biomolecules, ions. Because ions carry a charge, the flux of ions through a channel produces an electrical current that, although small (of the order of a few picoamperes or 10^{-12} amperes), is readily measured with electronics. An ion channel, like a receptor, is a tiny mechanical device that changes its structure moves in response to various stimuli, such as a change in transmembrane voltage. This motion controls opening and closing of the ion pathway.

It was thought that, by combining these two elementary building blocks into a single protein, one could construct novel biological objects combining two properties: chemical signal recognition and its transduction into an electrical signal. This is the basis of Ion-Channel Coupled Receptors or ICCRs as illustrated in **Figure 4**A. The key point is to realise that both receptors and channels are mechanical devices: A receptor

moves when it binds a molecule and a channel moves when it opens and closes. By bringing them close enough together, these motions could be synchronized and the opening/closing of the channel could be coupled to the presence of biomolecules.
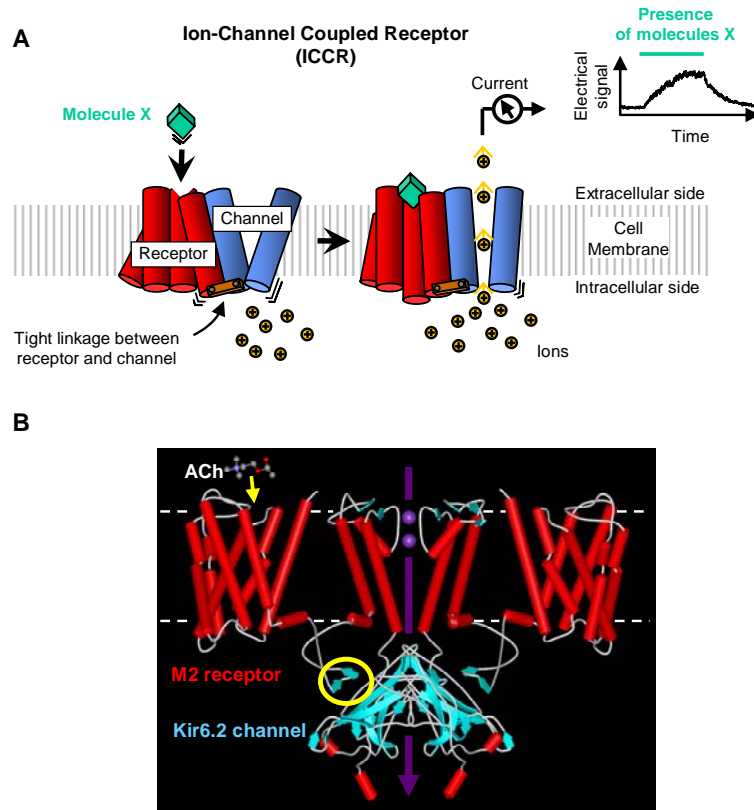

Figure 4: The Principe of ICCR
A. The principle of ICCRs (Ion-Channel Coupled Receptors). In an ICCR, a natural receptor is attached to an ion channel so that a rigid mechanical link couples the 2 proteins. When the receptor recognizes a molecule X, it undergoes a structural change that is transmitted to the channel. The degree of opening of the channel is consequently modified and the ion flux through the channel is altered. This ion flux is easily detectable as an electrical current. In essence, this artificial couple acts as a chemical-gated transistor with a very large gain (1 ligand-binding event = ~10^7 ions/s through channel).
B. Side view of a molecular model of an ICCR tetramer with front and back monomers removed. The channel and receptor are fused in the circled region. It is postulated that ligand binding to the receptor triggers a structural change that results in motion of the receptor extremity. Being tightly linked to the receptor extremity, the channel extremity is in turn displaced and pulls the channel open.

However, if this should work in theory, proving whether, and how, this could function in practice required significant effort. The structures and the dynamics of these proteins are not known and they can only be predicted with very little confidence (**Figure 4**B). Rational design is therefore not possible and one must rely on a time-consuming trial-and-error approach. The work consisted in using protein engineering techniques, based on molecular biology, to create artificial proteins associating receptors and channels in various arbitrary ways, and in testing these proteins to identify the best way to obtain a functional coupling between receptor and channel (**Figure 3**). After much effort, this approach was successful in defining the optimal ways to construct Ion-Channel Coupled Receptors. Basically, receptor and channel were fused together through a link and the size of the linking region was found critical to achieve proper coupling.
As a first step, biosensors for two major pharmacological targets were designed and tested to provide the proof-of-concept of ICCRs. These could serve to build new drug screening assays. They could also be used in other applications such as in vitro diagnostics or detection of toxic agents. These results were published in Nature Nanotechnology (Moreau et al. 2008) with a detailed editorial review (Abbas & Roth, 2008).

In summary, artificial proteins were created that associate receptors and ion channels. Designated ICCR (Ion-Channel Coupled Receptor), these ~10-nanometer-wide objects use their receptor component to detect biological molecules (hormones, neurotransmitters) and their channel component to produce an electrical current. These biosensors can detect and signal the presence of exceedingly small quantities of molecules. Their ability to directly generate electrical signals is a key feature for integration within microelectronic systems.
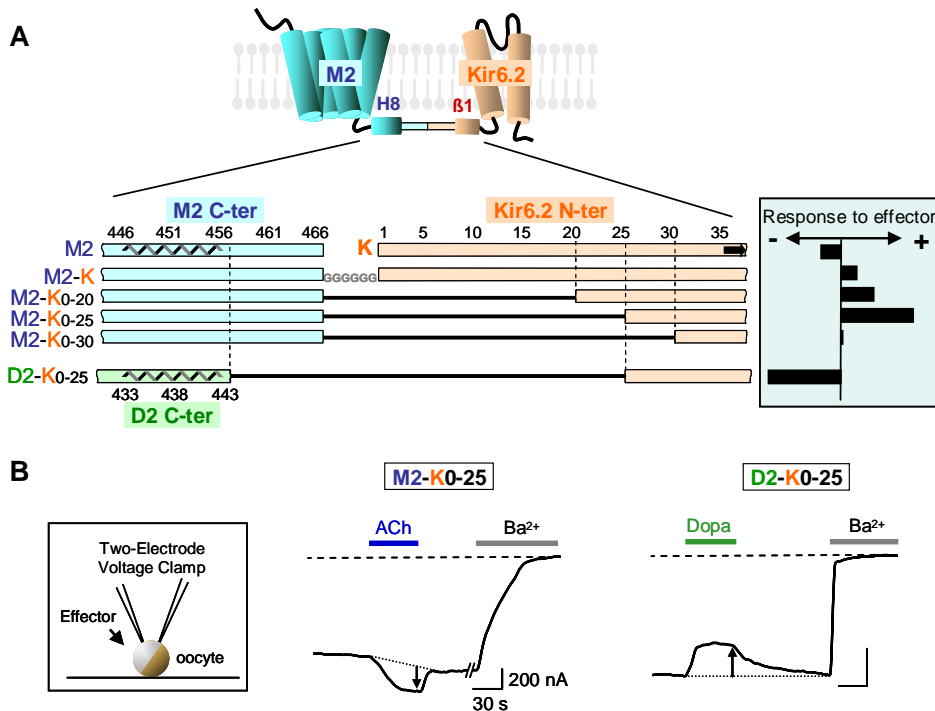
Figure 5: Design and tests of ICCRs based on the M2 and D2 receptors.

A, Region linking GPCR C-ter and Kir6.2 N-ter for selected M2 and D2 constructs. The putative α-helix VIII of GPCRs and a Kir6.2 β-sheet, are predicted from the structures of the β2 adrenergic receptor and Kir3.1. Construct names 'G-K xx-yy' indicate the GPCR name (G), the residues deleted in the GPCR N-ter (xx) and in the Kir6.2 C-ter (yy). Boxed insert at right summarizes the change in current induced by GPCR agonists (ACh or dopamine) for each constructs. The best response is obtained when the full-length receptor is fused the channel lacking its first 25 aminoacids.

B, Currents due to movement of potassium ions through ICCRs were measured in *Xenopus* oocytes expressing ICCRs using the two-electrode voltage clamp technique. Examples of the time course of currents in response to binding of agonists are shown for M2 and D2 ICCRs (adapted from *Moreau et al, 2008*).

Protein Production, Reconstitution and Delivery

This work was carried out by the group of School of Chemistry, University of Southampton.

Expression of Bacterial Ligand Gated Ion Channels.

Two prokaryotic ligand gated ion channels have been expressed and purified from *E. coli*; MthK, a calcium gated potassium channel from *Methanobacterium thermoautotrophicum* and MloKI, a cyclic nucleotide gated potassium channel from *Mesorhizobium lot* (**Figure 6**).
Both proteins contain a His$_6$-tag to allow simple Ni affinity purification. The typical optimised yield of MthK was 1.5 mg/L cell culture and for MloKI was 4 mg/L cell culture.
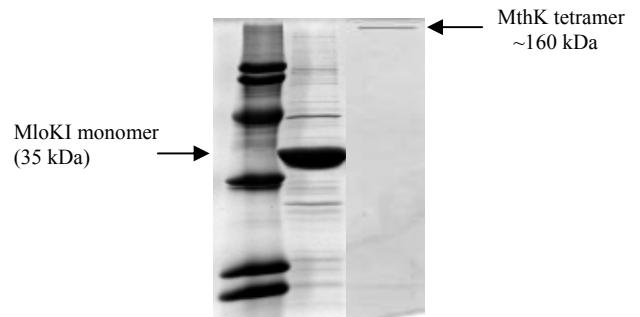

Figure 6: Example of expression of ligand gated ion channels in E. coli. *Mesorhizobium loti* MloKI

Potassium assay.

A method for assaying the activity of the protein prior to supported bilayer delivery has been developed. Firstly the lipid components of the vesicles used for reconstitution were optimised to produce stable vesicles. To test activity, a K+ indicator dye, PBFI was entrapped within the purified vesicles which contained no potassium and were buffer exchanged into an isocratic high [K+] buffer. The addition of K+ transporter valimomycin enabled the transport of K+ across the vesicle membrane, a process which could be observed by a related increase in PBFI fluorescence. This assay is suitable for the validation of active protein reconstitution.
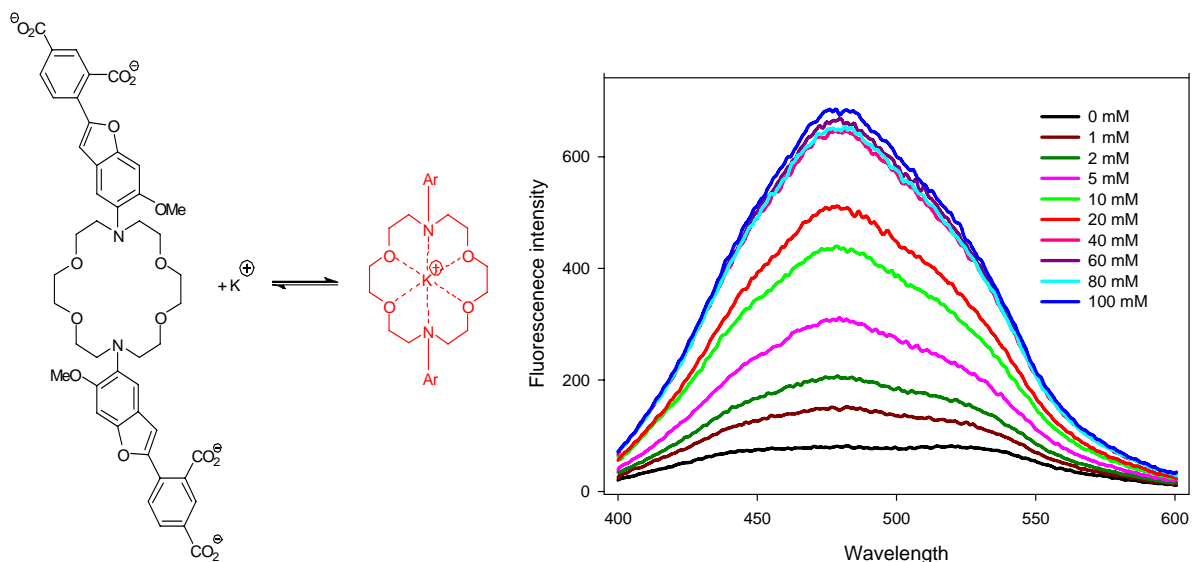

Figure 7: PBFI assay. Upon complex formation with potassium ions, the ligand becomes fluorescent

Methods for the reconstitution of ligand gated ion channels into vesicles.

The method of reconstitution which has led to some limited success in terms of the observation of active protein post delivery into a supported BLM is the biobeads method. In this technique, the ion channel is first reconstituted into mixed detergent / lipid micelles. The removal of the detergent using Bio-Beads results in the protein being reconstituted into ~100 nm vesicles. MthK reconstituted using this technique has twice shown activity afer supported BLM delivery (**Figure 8**), but the ability to express, purify, and reconstitute MthK maintaining activity is not yet consistent. It is currently unclear which step in the process is the point at which the protein loses activity, the use of the PBFI assay to test activity post reconstitution has not identified active protein. An alternative approach, detergent saturation has also been used for protein reconstitution. This procedure involves the preparation of 400 nm vesicles first by freeze-thawing and extrusion, followed by the addition of detergent to the maximum concentration at which the vesicle is stable. The protein is then added and detergent removed using biobeads. This method has been useful for the reconstitution of protein into PBFI filled vesicles, for fluorescence based activity assays, however no protein activity has been observed using this method.
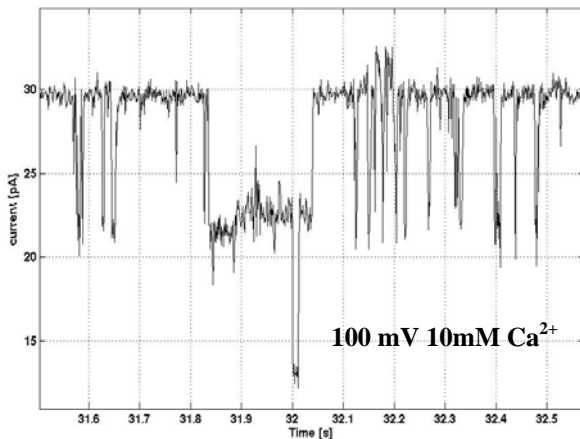
Figure 8: Electrophysiological analysis of MthK channels in a BLM. Recording was acquired at 1 kHz and a digital low-pass filter at 500 Hz was then applied.

Expression of SUR2A:Kir6.2 Fusion proteins.

The PCR amplification of the SUR2A:Kir6.2 construct (from the Vivadou group) was optimised, the optimal polymerase was found to be Bio-X-act long. The aim was to clone this construct into a yeast expression plasmid; either pYeDP60, for expression in Saccharomyces cerevisae, or pPICZA for expression in Pichia pastoris. An alternative cloning method, recombination cloning proved to be successful. This method involves the transformation of E. coli XL10 gold with linearised pPICZA and the N-His6SUR2A:Kir6.2CΔ36 PCR product which has been modified to contain 17 bp overlaps with the 5' and 3' ends of the linearised plasmid. This resulted in pPICZA_SK1, the SUR2A:Kir6.2 expression plasmid.
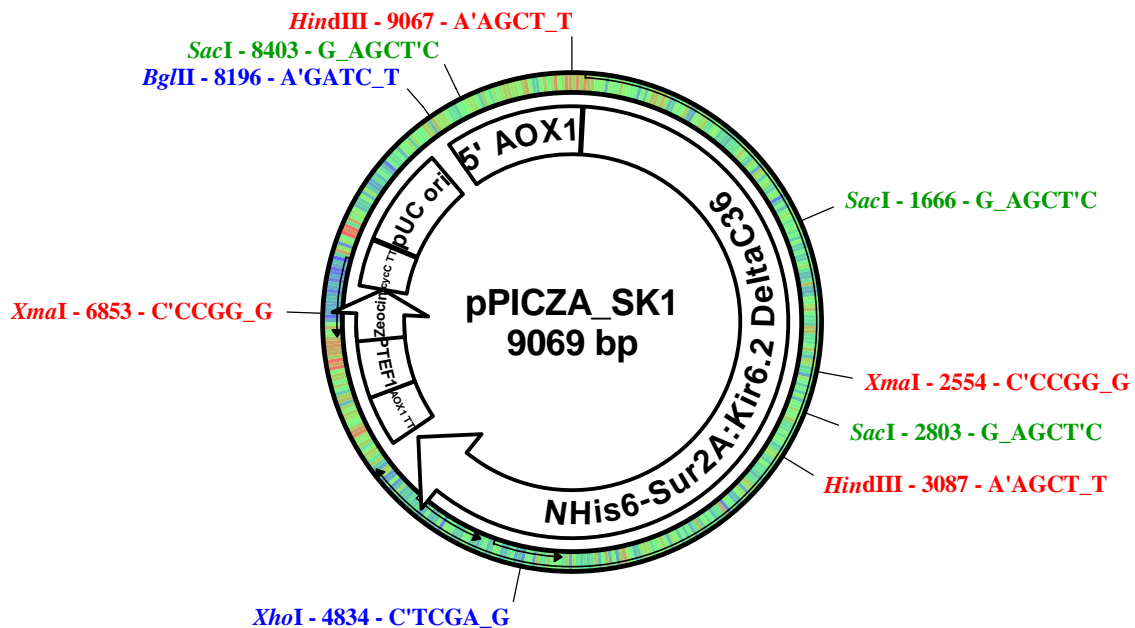
The plasmid was then transformed into *P. pastoris* strains GS115 and KM71H and once the recombination of the plasmid into the *P. pastoris* genomic DNA had been confirmed by colony PCR, the expression of the N-His$_6$SUR2A:Kir6.2CΔ36 construct from pPICZA was investigated. Optimisation of this expression system will be undertaken in future experiments.

Micro- and Nano-fabrication

Micro-fabrication (University of Southampton platform)

The bilayer lipid membrane (BLM) is the fundamental component of all living cells. It defines the cell boundaries and provides the matrix for the necessary sensing and channel proteins needed by the cell to survive and interact with its environment. Lipids are amphiphatic molecules - each molecule has a hydrophobic tail and and a hydrophilic head. In aqueous physiological conditions, these molecules self-assemble into an impermeable bilayer membrane with the hydrophobic tails in the interior and the hydrophilic heads exposed.

The focus of the Nano Group at the University of Southampton is to introduce natural and synthetic lipids onto very small apertures on microfluidic devices and incite them to self assemble into a defect- free bilayer. These microfabricated devices are designed for easy integration with electrical and optical measurement systems and thus the the suspended BLM formed across the aperture can be readily characterised. As BLM mimics the natural cell membrane, it can be exploited for the production of single molecule biosensors and the creation of model biological systems for fundamental biophysical studies. More importantly, membrane proteins, which constitute more than 60% of drug targets, can be reconstituted into these BLMs with enormous benefits for drug discovery and screening.

Conventional systems for bilayer measurements only allow for a single bilayer site to be probed at any one time making them low throughput and unportable. Moreover, because these systems require large buffer reservoirs and employ discrete components involving long electrical connections, they are very susceptible

to noise which is detrimental to ultra-sensitive biomolecular measurements. A small microfabricated device requires only microlitre quantities of physiologically relevant liquid and picomolar biomolecule concentrations thus allowing for single molecule measurements. Also, a scaled down device can be conveniently interfaced with the ASIC-based BLM measurement system developed by ARCES-Bologna.

Polymeric materials can be used for single aperture BLM devices. These microfluidic devices are fabricated using simple, rapid, and hence low cost methods that can be scaled up to large-scale microfluidic systems involving multiple bilayer sites. The basic device design involves three polymer materials bonded together (**Figure 9**). Microfluidic trenches hot embossed on two polymer plates which are then solvent bonded to form sealed channels. An aperture is formed on one polymer by laser micromachining. A large hole is made on the third polymer and aligned to form a well around the aperture. When the suspended BLM is formed across the aperture the channels are split into two reservoirs and AgCl probes are inserted on each side of the bilayer for electrical measurements.
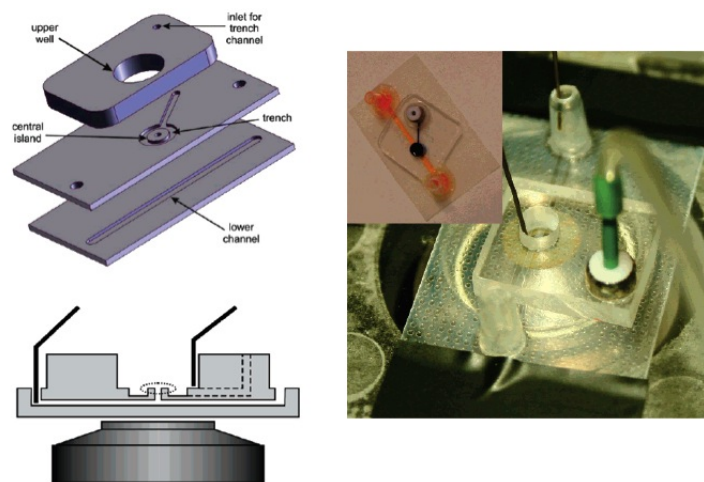

Figure 9: Schematic of the basic microfluidic design *(left)* and its actual implementation *(right)*. Lipid molecules dissolved in decane are injected as close to the aperture as possible. Silver-chloride electrodes for electrical excitation and measurement are inserted on two buffer reservoirs that are separated by the suspended BLM

The volume of lipid-solvent solution introduced to the aperture is very critical for the formation of a stable bilayer. The lipid solution can form a plug of lipids that does not thin fast enough to form a single bilayer. Our group has discovered that when the upper well is emptied of buffer following initial lipid injection the lipid film begins to thin spontaneously. A short exposure to air produces a lipid bilayer that is stabilised by the introduction of fresh buffer into the well. Several trials of the bilayer formation sequence by air exposure shows that it is a highly reliable (success rate 88%) and reproducible method for making BLMs that remain stable for 3-4 hours. This air exposure technique can be automated and thus lends itself well for devices involving multiple bilayer arrays (**Figure 10**).
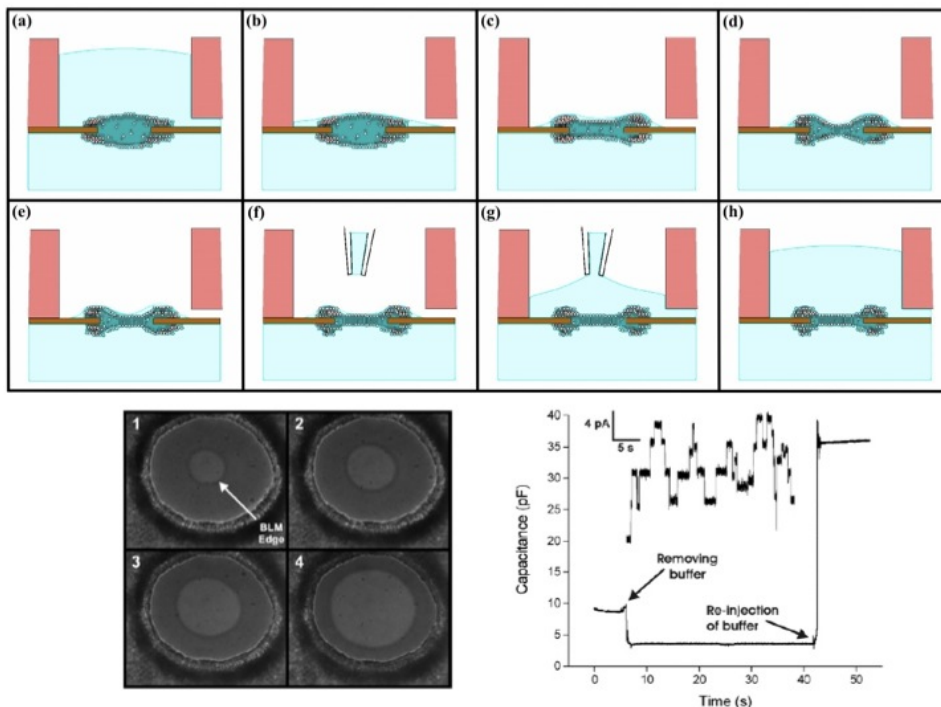
Figure 10: The air exposure technique sequence *(top)* for forming stable bilayers across the aperture. As viewed through an optical microscope *(bottom)* the lipid solution is shown to thin spontaneously into a bilayer as shown upon exposure to air and stabilising upon the re-injection of buffer as shown in the sharp increase in capacitance across the aperture.

The long-term stability of the air-exposed bilayers permits the delivery of proteins to the buffer and protein reconstitution across the bilayer (**Figure 10**). Ion channel measurements can thus be made (**Figure 11**).
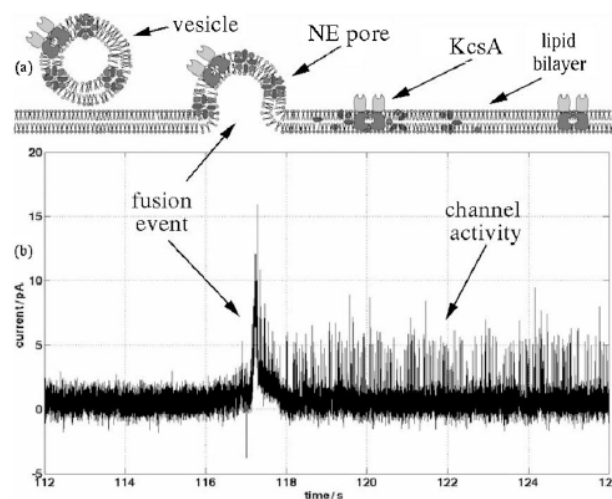

Figure 11: The potassium channel protein KcSA is delivered by vesicle fusion across the suspended BLM. The current trace across the BLM shows the fusion of the vesicle with the BLM and then the reconstitution of the protein across the bilayer. The transient current spikes indicate channel activity and thus successful incorporation of KcSA.

The main disadvantage of polymer-based microfluidic devices is that the polymer lacks chemical and thermal resistance. Applications involving biomolecules at very low concentrations require a contaminant-free microfluidic system prior to use and polymeric materials cannot be cleaned chemically and hence the devices are single-use. Silicon is chemically and thermally resistant but is very fragile and expensive to

manufacture. On the other hand, glass is a cheap and robust material that is optically transparent and chemically resistant making it reusable. Using standard but optimised lithographic processes the requisite microfluidic trenches can be etched on glass substrates. However, because of its crystalline structure, laser micromachining of the apertures is difficult and expensive. The apertures for bilayer formation are made using spark assisted chemical etching (SACE) (**Figure 12**). The aperture edges are then made hydrophobic by silanisation.
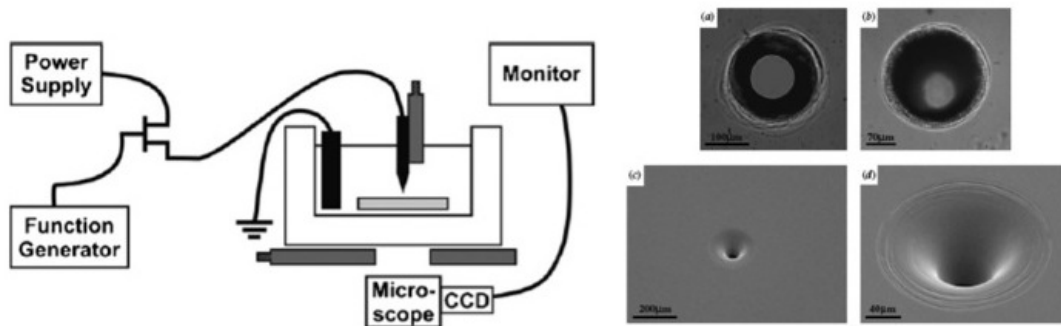

Figure 12: The set-up *(left)* for spark assisted aperture formation. The glass and tool electrodes are immersed in a strong alkali solution for electrolysis. Electrical discharge from the tool electrode initiates chemical and thermal reactions that result in etching of the glass surface. The resulting aperture has a conical profile *(right)*, which is beneficial for bilayer formation, with the through-hole diameter in the range of 100- 200 microns.

The glass-based microfluidic devices retain the basic design and the air exposure technique is used to form a stable BLM.
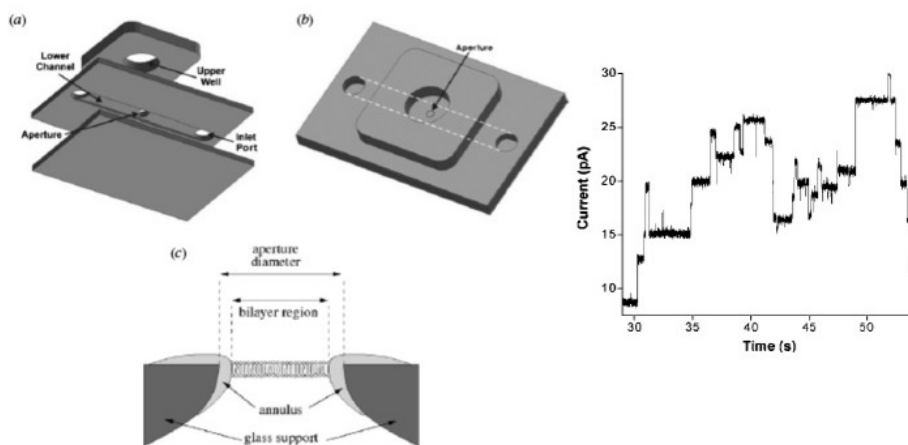

Figure 13: The glass-based microfluidic device *(top left)*. When a stable BLM is formed *(bottom left)* it is also possible to introduce an antibiotic (gramicidin) to the BLM forming pores that increase the permeability of the BLM as shown in the current trace *(right)*.

The optimised fabrication techniques for chemical glass etching and aperture formation by SACE have been used to scale up the single BLM test device into an array involving 8 separate BLM sites (Figure 14). An early prototype has been made and interfaced with the ASIC BLM Measurement System developed by ARCES (**Figure 15**)
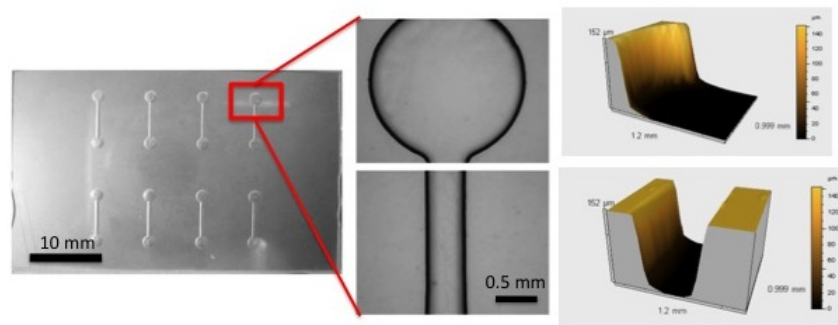
Figure 14: The glass microfluidic component of the BLM array. With chemical etching, the trenches can be made very deep thus reducing the number of components that need to be patterned.
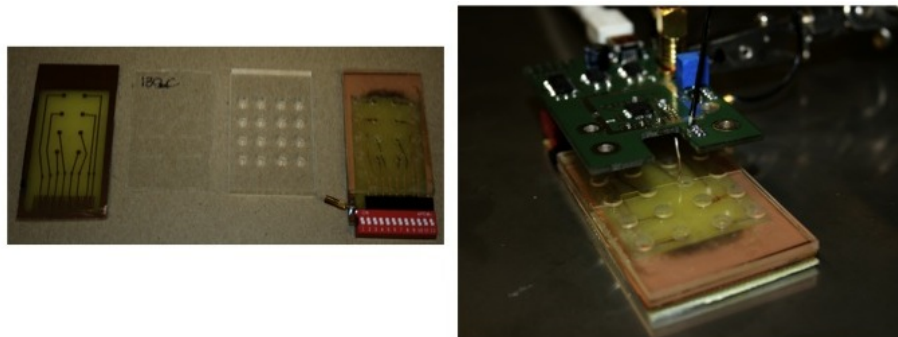

Figure 15: The components that make up a BLM array device with glass microfluidics *(left)* and the device interfaced with an ASIC Card for electrical measurements *(right).*

In summary the Nano Group has optimised an air exposure technique for promoting bilayer formation across hydrophobic apertures. This method is reliable, reproducible, and can be automated. Polymeric and glass based microfluidic devices were fabricated that are portable, use small quantities of buffer, and allow for the rapid exchange of electrolytes. Moreover, proteins have been successfully introduced across the BLM and single channel measurements have been demonstrated. These can be scaled up to a multiple bilayer array device that can be individually addressed by the ASIC BLM Measurement Electronics developed by ARCES-Bologna.

Nano-fabrication (CEA platform)

The proposed approach is composed of a free-standing silicon nitride (Si_3N_4) supporting membrane (**Figure 16**). This membrane features nanoapertures on top of which will be deposited a lipid bilayer which will host membrane receptors.
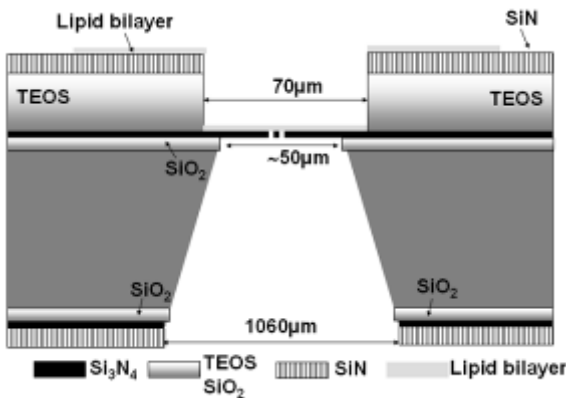
Figure 16: Cross-section view of the nanoporous membrane biochip covered by a lipid bilayer.

A process to fabricate 70*70μm² free-standing silicon nitride membranes featuring nanopores the diameter of which is ranging from 80nm to 1μm, was developed. The fabrication process of the nanopore membrane chips is performed within a 200 mm (8") wafer technology. Several chip configurations are investigated: number of nanopores per chip (1, 10, 100, or 1000), and nanopores diameter (80nm, 100nm, 250nm, 300nm, 500nm and 1μm). Finally, the wafer is diced to form 2*2mm² individual membrane chips that are inserted afterwards in a Printed Board Circuit (PCB). The biochips, organized in a 6*2 matrix, are embedded in wells designed in the PCB, the surface of which is functionalized preliminary by a "perfluorocarbon-like" PECVD shadow-mask coating to allow for droplet reservoirs at both sides of each chip (**Figure 17**). The wells are separated by a 9mm pitch in order to be compatible with the standard 96-well microplate format.
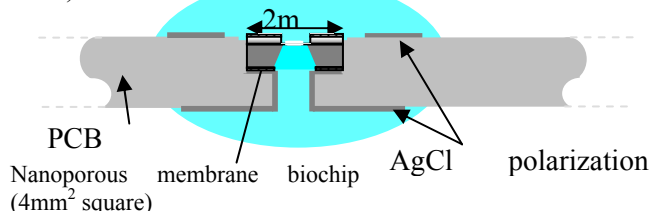
a) Droplet reservoirs on top (and bottom) of the

Figure 17: a) Host PCB into which are inserted the membrane chips, that are individually polarized. **b)** Cross-section view depicting a membrane biochip embedded into a well of the PCB.

Close-view SEM (scanning Electron Microscope) pictures of nanopores are represented in **Figure 17**. As a general tendency, the nanopores have a "bowl" shape, and their diameter is slightly widened compared to their design value. This slight difference may be attributed eventually to front-side RIE etching, the etch rate of which is locally higher at the membrane area (*e.g.* where the heat dissipation could be less efficient).
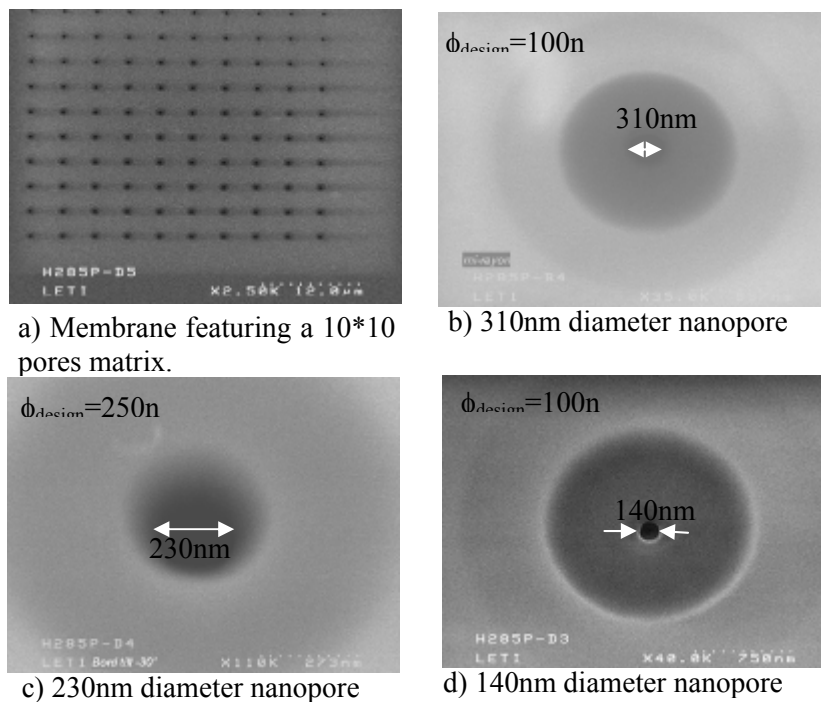
a) Membrane featuring a 10*10 pores matrix.

b) 310nm diameter nanopore

c) 230nm diameter nanopore

d) 140nm diameter nanopore

Figure 18: Close-view SEM pictures of nanopores.

Due to this problem of control of the nanopore shape, we launched a second silicon batch, in which the last step of TEOS layer is etched only using HF solution. Consequently we obtained the membrane with its nanopores. There is nevertheless a little overetching since the selectivity of the HF etching is limited between silicon dioxide and silicon nitride. The electrical properties of the chips, *i.e.* capacitance and resistance were tested, and are consistent with what was expected. In particular the thick TEOS layer ensures a capacitance in the order of 30 pF which allows single channels recording.

The strategy chosen to assemble bilayers was the fusion of giant unilamellar vesicles (GUVs) over the silicon aperture: the sucrose loaded vesicles sediment and spontaneously burst over the silicon membrane, to form a single lipid bilayer covering the aperture. The droplet conformation of the device (see **Figure 17**) has a pitch of 9 mm between each of the 12 measuring sites, making the dispensing of the GUVs compatible with an automated dispenser.

Furthermore, we have developed a new way of reconstitution of membrane proteins inside the GUVs to produce proteo-GUVs. This method is gentle for the proteins and easily automatisable, as it does not use any detergents, neither deshydration step. This process of proteo-GUVs formation was first validated by patch-clamp using a membrane protein model, the voltage dependent anion channel (VDAC). The ability of the proteo-GUVs to form spontaneously stable giga seals upon contact with a hydrophilic surface was further confirmed with planar silicon membranes.

Yields of stable seal formation over first time used and reused cards were compared. For first time used cards, the silicon membranes were washed three times with CARO, and then mounted into the PCB of the card. Prior to electrophysiological measurements, activation by oxygen plasma was performed. With first time used cards, stable seal formations were obtained for 25% of the sites with added GUVs whereas for reused cards, no stable seal was obtained, and only a transient increase of the resistance was observed for 10% of the tests (**Figure 18**).

Since no successful cleaning method was found to wash the silicon membranes once mounted into the card, it was decided that the card is a consumable. Comparing with free-standing silicon membranes (not inserted into the cards), a yield of 68% of stable seal formation was obtained (**Figure 19**). This difference could be explained by a very low risk of contamination of the silicon membrane that favours the seal quality.
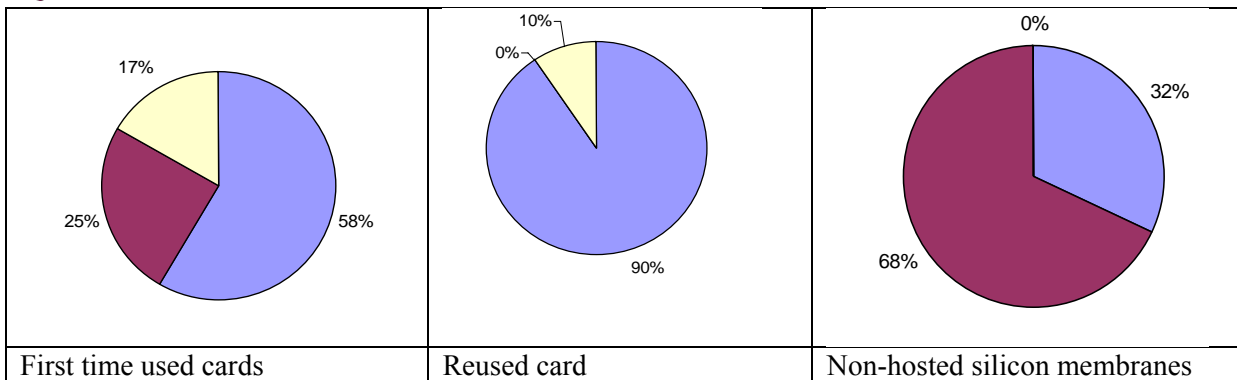
| First time used cards | Reused card | Non-hosted silicon membranes |

Figure 19: Yield of stable seal formation (red), unstable seal (white), and no seal (blue) formation.

In order to validate the functionality of the suspended lipid bilayers in the card, single channel currents were recorded with soluble proteins such as the peptide Gramicidin (**Figure 20**A) and the protein alpha-haemolysin (**Figure 20**B), that can insert a lipid bilayer to form ion channels.
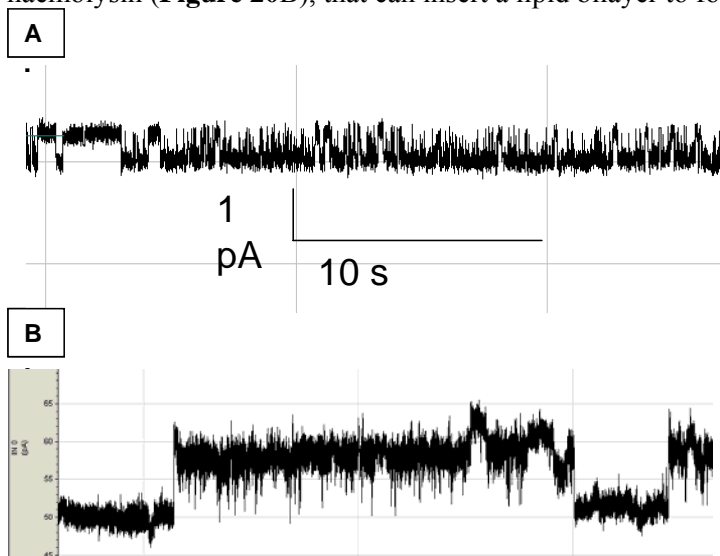

Figure 20: Electrophysiological recordings on cards. **A.** Filtered signal of 0.5 nM Gramicidin A at -80 mV and **B.** Filtered signal of 600 nM alpha-haemolysin at 100 mV.

As Gramicidin and Alpha-haemolysin are partially soluble ion channels, and since the engineered proteins GPCR-Kir6.2 were not purified at this stage, an alternative non-soluble membrane protein, VDAC, was used to complete the validation of our protocol for the functionalisation of the card with membrane ion channel. Using our new method to form proteo-GUVs, the yield of incorporated active ion channels was determined around 50 %. With planar patch clamp on non-hosted silicon membranes, current variation was recorded after deposition of VDAC proteo-GUVs (**Figure 21**B). However the electrical profile was quite different from the one obtained with classical patch clamp, where many conductance levels were detected (**Figure 21**A). Further experiments of planar patch clamp on bilayers made of proteo-GUVs would be necessary to unravel if VDAC has different conductive characteristics compared to the ones observed in cell attached mode. This might be of importance since the different conductance levels of VDAC can be regulated by the physical characteristics of the membranes.
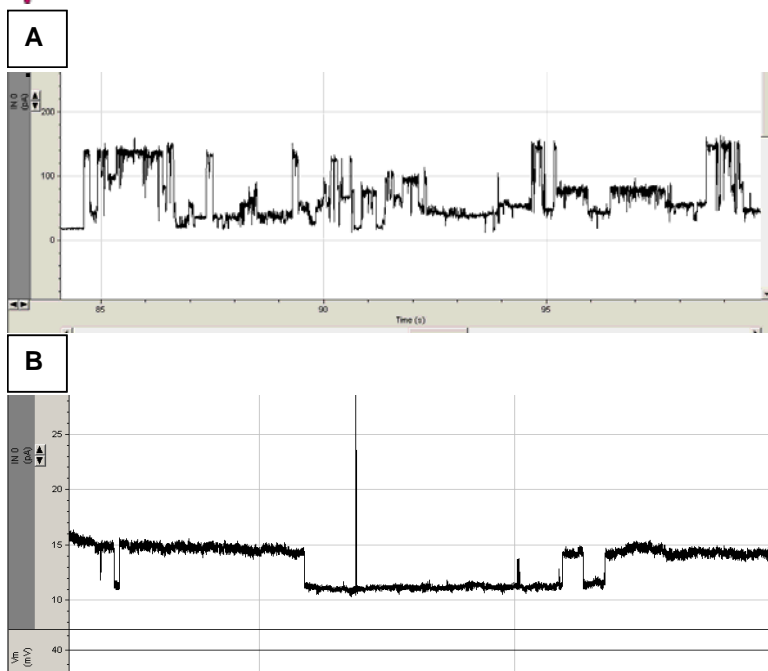
A

B

Figure 21: Evolution of current on VDAC proteo-GUVs at 40 mV **A.** with classical patch-clamp and **B.** with planar patch clamp.

Filter-based approach (NKUA platform)
This work was carried out by National Kapodistrian University of Athens.

The purpose of this approach is to:

- Assemble and characterize stabilized lipid bilayers on the Receptronics platform.
- Perform optical and calorimetric exploration of stabilized lipid films

This work was related to the preparation of a selective receptor for the rapid, selective and sensitive electrochemical flow injection analysis of carbofuran in foods using air stable lipid films supported on a methacrylate polymer on a glass fiber filter with incorporated artificial receptor. The selective receptor was synthesized by transformation of the –OH groups of resorcin[4]arene receptor into phosphoril groups. These lipid films were supported on a methylacrylate polymer (i.e., methacrylic acid was the functional monomer for the polymerization, ethylene glycol dimethacrylate was used as the crosslinker and 2,2'-azobis-2-methylpropionitrile as an initiator). A minisensor device was constructed for the electrochemical flow injection analysis of toxicants based on air stabilized lipid films supported on a polymer. The device can sense the analyte in a drop (50 µL) of sample. Carbofuran was injected into flowing streams of a carrier electrolyte solution. A host-guest complex formation between the calix[4]arene phosphoril receptor and carbofuran takes place through hydrogen bonding. This enhances the preconcentration of carbofuran at the lipid membrane surface which in turn causes dynamic alterations of the electrostatic fields and phase structure of membranes; as a result ion current transients were obtained and the magnitude of these signals was correlated to the substrate concentration. The response times were ca. 80 s and carbofuran was determined at concentration levels of nM. The effect of potent interferences included a wide range of compounds and other insecticides. The effect of interference of proteins and lipids was also examined. The reproducibility of the method was checked by recovery experiments in fruit and vegetable samples with satisfactory results.
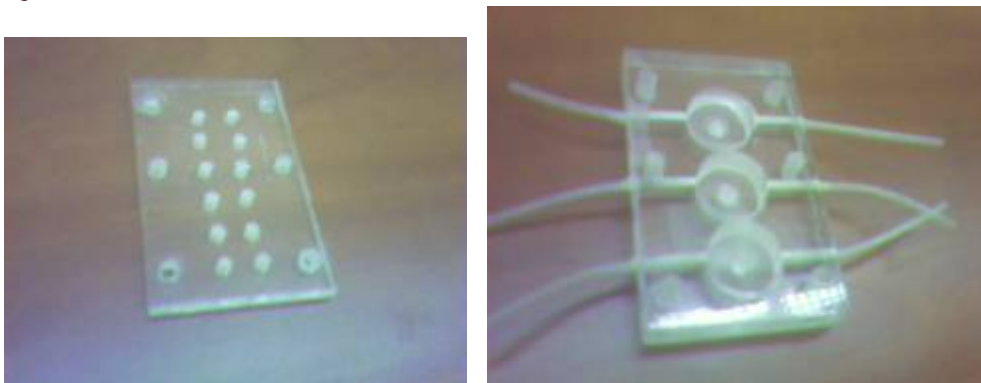
(A)   (B)

Figure 22: Upper (A) and lower (B) Plexiglass chamber cells used for the electrochemical measurements.

Further efforts were done on the development of a simple sensitive spot optical test and the construction of a portable biosensor for the rapid one-shot detection of naphthalene acetic acid (NAA) using stabilized lipid films supported on a methacrylate polymer on a glass fiber filter with incorporated auxin-binding protein 1 receptor. The lipid films without the receptor provided fluorescence under a UV lamp. The use of the receptor in these films quenched this fluorescence and the colour became similar to that of the filters without the lipid films. A drop of aqueous solution of naphthalene acetic acid provided a "switching on" of the fluorescence which allows the rapid detection of this stimulant at the levels of 10^{-9} M concentrations. It was also possible to have quantitative data based on a calibration graph. The effect of potent interferences included a wide range of compounds. The results showed no interferences from these compounds in concentration levels usually found in real samples. The method was applied for the determination of NAA in fruits and vegetables and the reproducibility of the method was checked in about 50 samples. These lipid films can be used as portable biosensors for the rapid one-shot detection of NAA in fruits and vegetables by non-skilled personnel in the field.
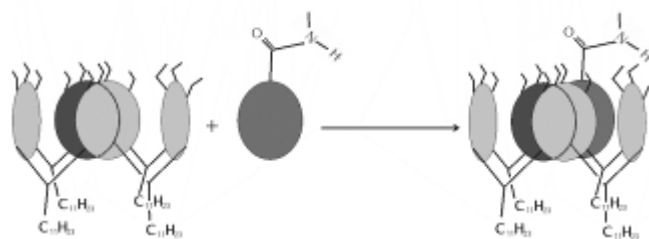

Figure 23: A schematic illustrating the interaction between receptor and carbofuran. . In the presence of zinc, the mechanism of signal generation is different than that when carbofuran interacts with the receptor. Presently, zinc interacts with the phosphate groups of the receptor and forms a complex.

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Electronic Readout, Signal Processing and Visualization

This work was carried out by Center of Excellence on Electronic Systems (ARCES), University of Bologna and Silicon Biosystems, S.p.A.

The objective of this section is:

1. To design a compact single channel recording readout structure to interface with the microfluidic interface. For this purpose, and integrated microelectronic structure has been designed and tested so as to reduce interface noise and to compete with existing bulky instruments in terms of signal to noise ratio.
2. To collect and elaborate signals from arrays of micro-spots where single molecule binding events occurs. In order to achieve this goal a compact readout system based on array of sigma-delta converters has been designed and tested.

Silicon design of electronic interface chip

The integrated readout scheme has been based on a current preamplifier followed by a switched capacitor $\Sigma\Delta$ converter having a noise floor as low as 150fA r.m.s. @1kHz at room temperature. The main advantages of this approach are: 1) it offers a digital output that can be easily multiplexed for nanoarrays; 2) the noise floor and bandwidth trade-off is easily set by using oversampling ratios; 3) implements a Correlated Double Sampling (CDS) scheme for reducing the low-frequency noise, very common in ion-channel recordings. The circuit blocks of the converter are shown in **Figure 24**. A single-ended charge integrator is followed by a CDS circuit, which helps to reduce the low-frequency noise and offset and also acts as a sample&hold (S/H). The prototype was fabricated using a 2P4M 0.35um CMOS process and a chip micrograph showing main blocks is shown in Figure 25. The amplifier occupies 0.5 mm^2 and the power consumption is below 23mW using a power supply of 3.3V. The plot shows great reduction of low-frequency noise due to the efficacy of the CDS scheme. The low frequency noise is as low as 5fA/$\sqrt{Hz}$ at room temperature and it is mostly due to the leakage of the reset transistor and of the testing board and package. This means that we can achieve a resolution of about 150fA@1kHz or 50fA@50Hz. The front-end has been specifically designed to work with nanosensor arrays such as in stochastic sensing approaches that are using single molecular events.
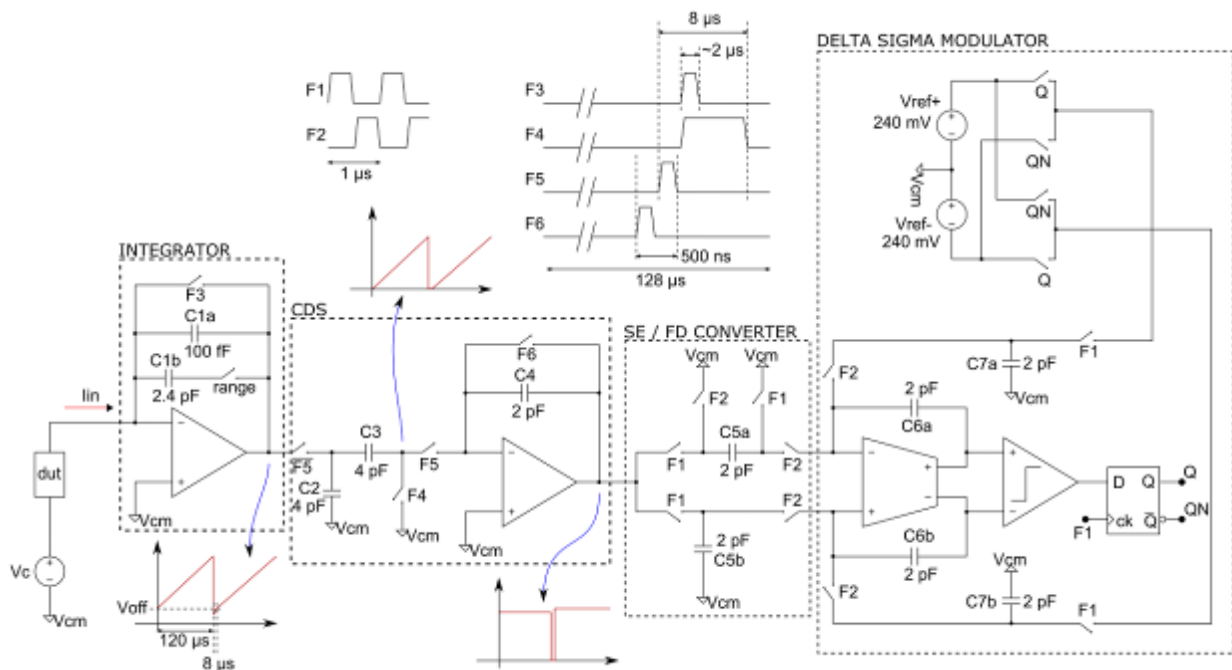

Figure 24: Silicon chip readout architecture.

The silicon chip has been packaged using QFN32 with dimensions of 5x5 mm^2 and has 32 available pins with 0.5mm pitch. A test board has been designed and realized to measure the performances of the chip alone. Then silicon chips have been implemented in the boards in two options: chip-on-board and surface mount technology to further reduce parasitic capacitance.
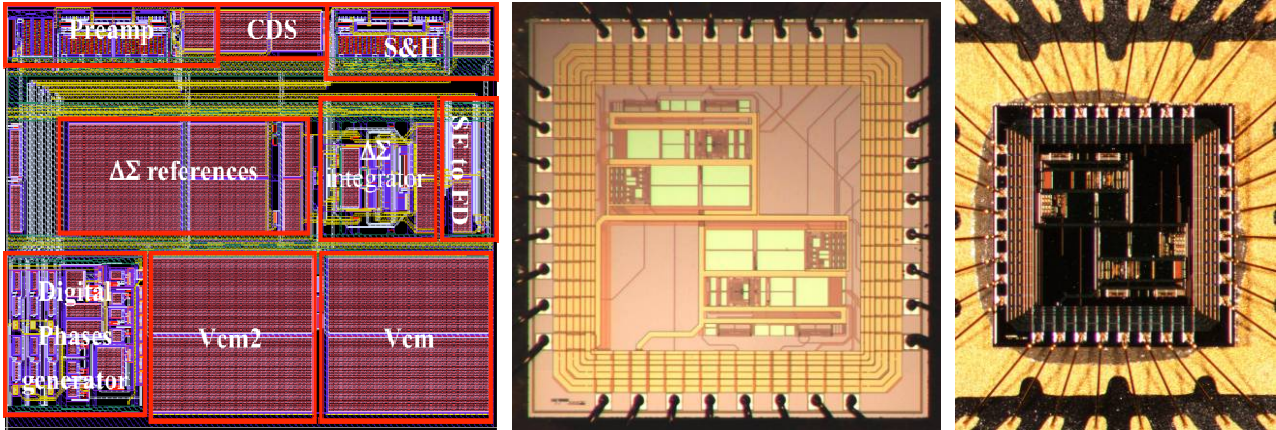

Figure 25: Silicon chip design (left), implementation (center) and packaging (right).

The noise performance is greatly enhanced with respect to previous prototypes of about a factor of three. This result is comparable with the state of the art low current measurement performances such as the Axopatch 200B by Molecular Devices. To minimize noise and external interference issues, an accurate design of the PCB especially around the chip footprint and the input path is necessary using four layers. All the digital signals are insulated between ground planes using internal layers and the input path are kept as short as possible. Moreover, the filter capacitors must be close to the chip pins. A photograph of the Board has already been shown in **Figure 25**.

Figure 26: Three silicon chips have been mounted using a SMT technology in a board to be interfaced with microfluidic structure that needs to be inserted in the left aperture.

Since current amplifiers are sigma-delta converters, their outputs consists of 1bit wide data streams that need to be processed. To achieve this, the stream of three outputs are concurrently decimated using a digital signal processing (DSP) chip as illustrated in **Figure 27**. Data are digitally processed by a $sinc^2$ filter first and by a Kaiser low-pass filter afterwards to achieve significant resolution in real time, requiring huge processing resources. However, this process allows to achieve extremely high selectivity (equivalent to that given by an analog filter with more than 40 poles). Moreover, this approach allows to easily trade-off noise floor for bandwidth by simply using digital setup. Using this approach, it is possible to readout concurrently 6 channels at 4kHz bandwidth with a floor noise that is four times smaller than previous versions.
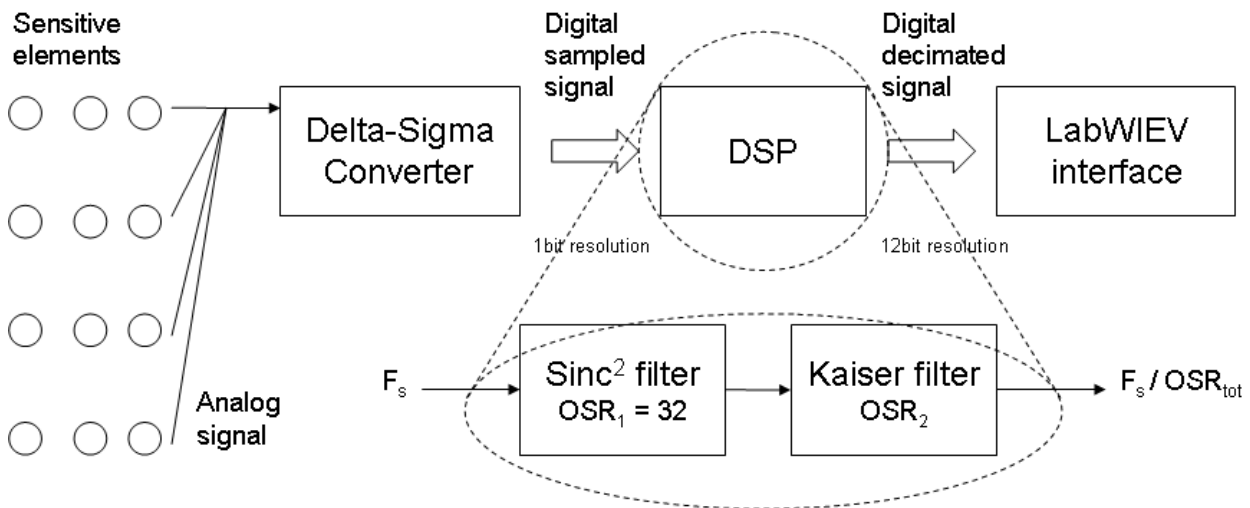

Figure 27: Data acquisition and visualization interface structure

Finally, data are transmitted in a PC platform by using a USB cable and displayed using a LabView® interface. A screen grab example of the visualization interface is shown in Figure 28.
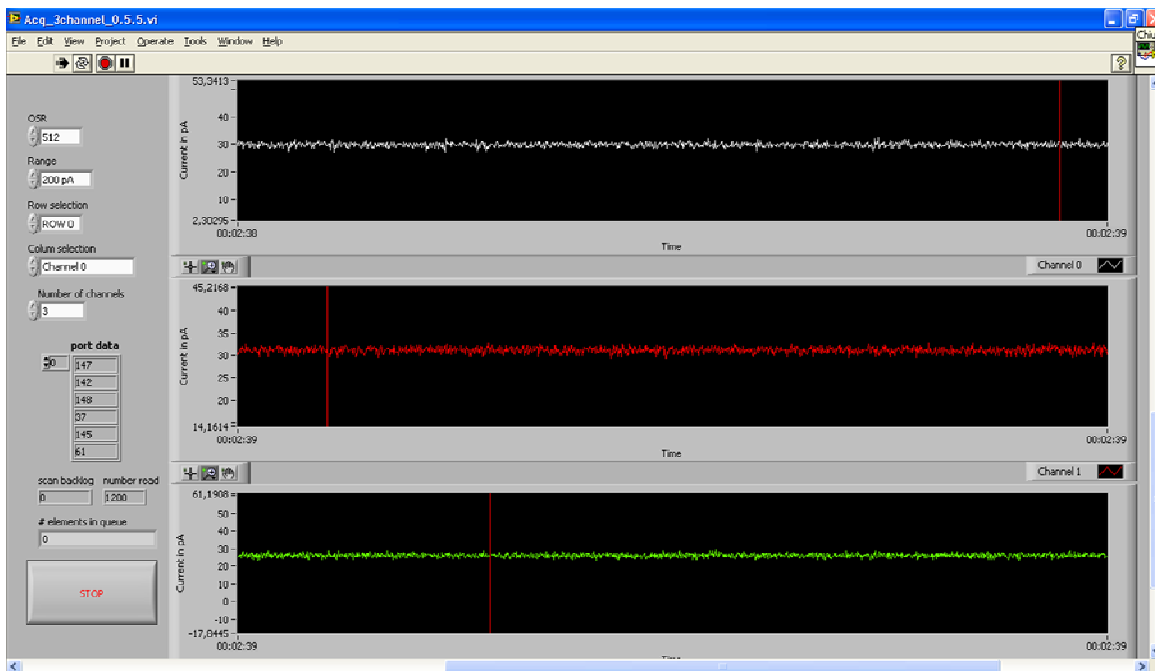

Figure 28: Visualization interface example, showing three concurrent readout channels. The graphical user interface can be customized in real time regarding range, timescale and bandwidth.

The interface visualizes concurrently three spots of the array. However it can set in runtime the following: spot selection, range (±200pA, ±5nA), bandwidth (4kHz, 2kHz, 1kHz, 500Hz, 100Hz). The complete readout systems structures developed during the project are displayed in Figure 29. The readout platform can also store data on files so as signals can be analyzed and treated during other times that that of acquisition.
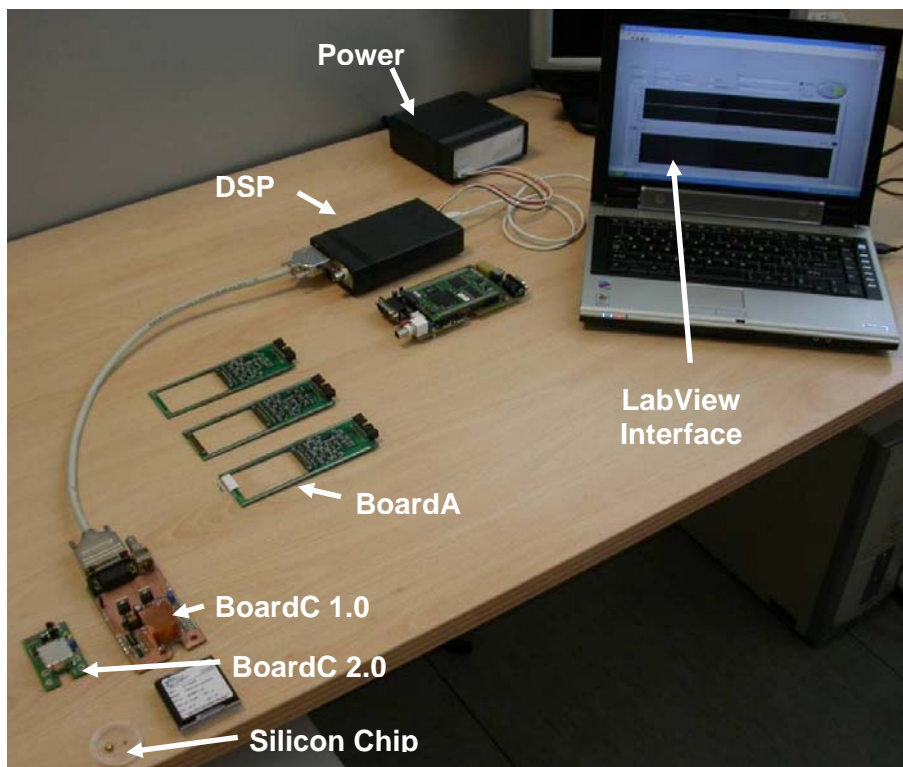

Figure 29: Complete readout system interfacing all the Boards of the project

Final prototype testing and validation

To validate the overall functioning of the system to detect single molecule events we have done acquisition of the current through an artificial lipid bilayer with an a-haemolysin protein which constituted a ionic channel, to demonstrate the work of our acquisition system in a real biological experiment.

Figure 30 shows results from a typical experiment acquired using a prototype of the chip, where a current of some pAs has been successfully acquired in a bandwidth of 1 kHz. In this experiment, the device under test is a BLM formed across a 150-μm orifice separating two chambers in a Delrin® bilayer apparatus. In one chamber an α-haemolysin (αHL) protein is placed in contact with BLM. αHL is an exotoxin secreted by the bacterium Staphylococcus aureus that forms pores allowing ions and molecules to pass through the BLM with a conductance of about 1nS. Then a transmembrane potential of -80 mV was applied with Ag/AgCl electrode. αHL was added to the trans chamber to reach a final concentration of 5 ng/ml and so a current of about -80 pA is observed. Lately, we have introduced β-cyclodextrin (βCD), which binds the α-haemolysin protein and reduces significantly the ionic channel conductance, thus the measured current. This cyclic molecule, in fact, is able to get into the lumen of the pore from the stem side and to bind to it, partially blocking the channel and causing current spikes.
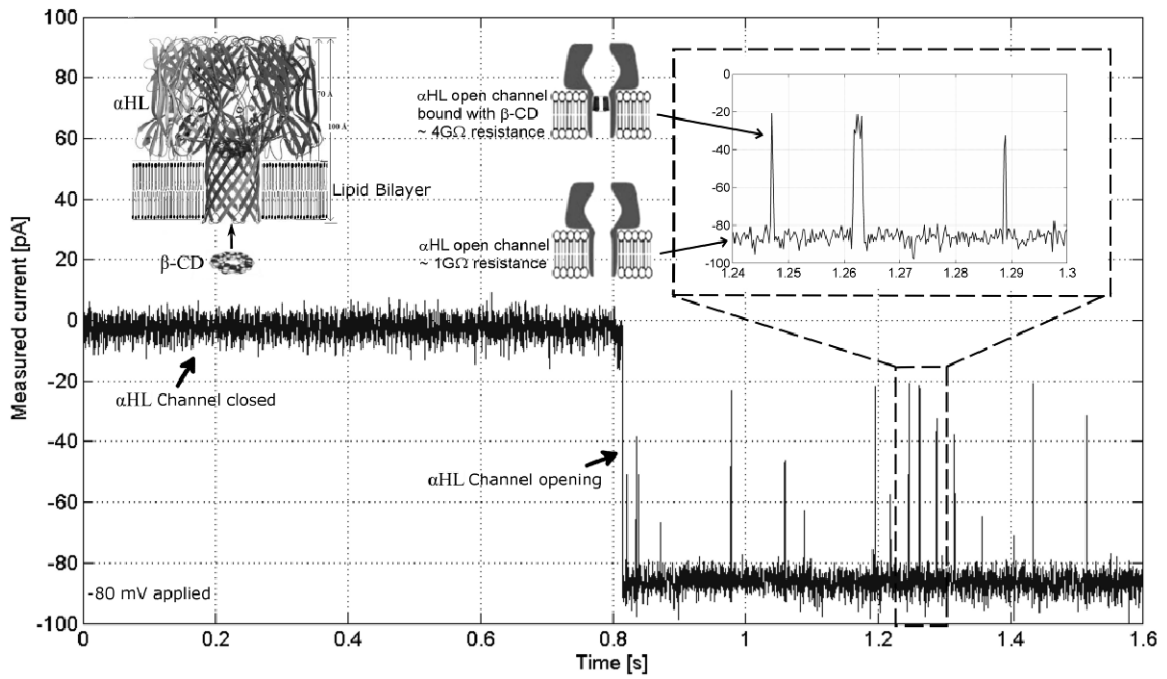
Figure 30: Single molecule detection using the readout technology developed during the Receptronics project.

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Conclusions and results

The project has been focused on the merge of several technologies aimed at realizing a new approach for detecting single target molecules, a very challenging task for large variety of applications. Most of the deliverables were (and still are) very challenging in the research arena of the last years and no significant experiments were done before the beginning of the project by partners. For the above reasons, in some cases it was very difficult to estimate the amount of time required to achieve the scheduled deliverables. For some it was as scheduled (i.e. data processing and receptor bioengineering) for others the time was longer than expected but within the project duration (i.e. silicon chip design and some microfluidics) and for few of them the required time resulted longer than the project duration (i.e. receptor structure overexpression and delivery). To summarize, the following table is shown, with respect to each layer[1].

The good	**The bad**	**The truth**
	Synthetic Receptor Bioengineering	
Very new approach	Adaptation to new targets is time-consuming. Will improve with a better understanding of the molecular mechanisms.	This can be adapted to a large number of cases
	Protein Production, Reconstitution and Delivery	
Ambitious goal. Once demonstrated it is easily applicable to new targets	Very difficult to achieve with ligand-gated ion channels (as required by the project).	Experimentation should take more time (years)
	Micro- & Nano-Fabrication	
It has been demonstrated on prototypes	Requires careful microfluidic design and fabrication	Could not be assembled using off-the-shelf components. Should be designed by skilled technicians.
	Electronic Readout	
The "Axon-amplifier in a chip" paradigm has been fully demonstrated.	Noise limits are even fixed by packaging setups. Careful assembling design is required.	Integrated electronics approach for nanosensors could be applied to new fields of single molecule detection.
	Signal Processing, Visualization & Data Analysis	
Single molecule detection has been demonstrated to be very effective for quantitative sensing.	The visualization platform should be better engineered for external users.	It is a new trend in biosensing

Table 1: Compact summary of results related to the technology layers at the formal end of the project.

The next table describes the goal achievements on each technology layers. It is important to point out that for "results" it is intended the scientific achievements with respect to the deliverables of the project, as estimated in the proposal. It is not related to the efforts in terms of men-power and budgetary considerations. It is also not related to the scientific excellence of the group(s) responsible for the deliverables. The tables also illustrates the achievements in terms of technology fusion between the different layers.

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[1] In the following tables is not present the task of comparing the proposed technology with existing kit since it is not a component of the technology but only a final proof of the whole system.

Layers	Technology fusion results	Notes	Level of uncertainty	Results	Estimated time to achievement
Synthetic Receptor Bioengineering			****	*****	On time
	A technology to overexpress bioengineered receptors is still under development	It is a very time consuming task (>1y)			
Protein Production, Reconstitution and Delivery			*****	**	>1y
	Delivery achieved with model channels but not with bioengineered receptors because of difficulties with production.	It is a very time consuming task (>1y)			
Micro- & Nano-Fabrication			***	****	On time
	Fusion of electronic readout and microfluidic structures has been fully demonstrated	Successful, but requires skilled developers			
Electronic Readout			**	*****	On time
	Electronic readout and visualization is integrated in a	Seems straightforward, but needs care to optimize the best performance			
Signal Processing, Visualization & Data Analysis			*	***	On time

Table 2: Inter-layer technology fusion related to the Receptronics project. The symbols: [symbol], [symbol], and [symbol] are related to the relationships results between layer technologies, referred to as very successful, successful and still missing, respectively.

However, as evidenced by the scientific production listed in the next section, the insights gained during the development of the Receptronics technology were extremely significant. As detailed in the Activity Report of the last period, most of the partners will continue to work on the technology using the support of the related Institutions for at least one more year.

Main results of the first year of the project
- Validation of synthetic fusion protein design principle. Natural ion channels are artificially coupled with specific receptors. The resulting protein is not existing in living beings, but it can be used to naturally detect target substances at molecular level;

- Design and test of several approaches to electrically address arrays of artificial lipid bilayers and methods for delivering fusion proteins into them. The design has been driven by the requirements to achieve high reliability and reproducibility for industrial applications; Structures with an yield of about 90% have been preliminary demonstrated in the Project.
- Design and test of an extremely compact electronic system for single molecule event detection. The system, as large as a credit card, is the first step for designing efficient and integrated electronics for interfacing bionanosystems;
- Data acquisition and statistical elaboration of molecular signals. This is needed since molecular signalling is intrinsically stochastic and it should be treated with proper tools. Precision below 1% of accuracy for ion channel open probability could be achieved with developed algorithms.

Main results of the second year of the project
- Single molecule events recorded on arrays of electrically addressable micro- and nano-spots using state-of-the-art laboratory instrumentation;
- Consolidation of the above technologies for achieving more robust testing data and yield;
- New generation of synthetic receptors based on the tandem principle to new target molecules and constructs;
- Consolidation of the reading out architecture based on Sigma-Delta amplifiers interfaced to DSP processing;

Main results of the third year of the project

- Receptor bioengineering procedures have been greatly optimized, thus reducing overall development time (few weeks instead of few months) with respect to target molecule. A paper on this methodology has been published in *Nature Nanotechnology* journal;
- The array of the artificial lipid bilayers has been completely redesign using a new in-house glass technology to better interface with electronics
- The silicon chip has been designed and successfully tested. Results (see plot on left) have shown performances that are very close to those given by state-of-the-art bulky instruments.
- The readout electronic system has achieved a definitive configuration. Using the developed platform it is possible to acquire and store concurrently up to twelve independent sites on a PC. The system is cross-platform and can be used with any version of the boards (BoardA, BoardB v1.0 and v2.0) developed in the project.

Ongoing status of the project can be found in the website: www.receptronics.org.

Scientific results[2]

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[2] This list has been collected at the formal end of the project, Oct 2008. Lots of other papers are under preparation or under referee process.

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[14] Varnier, T. Plénat, L. Ghenim, F. Marcel-Kermarrec, N. Picollet-d'Hahan, V. Agache, F. Sauter, F. Chatelain, and A. Fuchs, "Highly passivated silicon chips with micron aperture for the pA

measurements of single ion channels", Proceedings of Nanotech 2008: Life Sciences, Medicine & Bio Materials, Volume 2, Boston, June 1 -5, 2008, pp. 562 – 565.

[15] Varnier, T. Plénat, L. Ghenim,, D. Fajolle, V.Agache, F. Sauter, F. Chatelain and A. Fuchs, "ELECTRICAL DETECTION OF ION CHANNEL ACTIVITY IN AN ARRAY OF SUSPENDED LIPID BILAYERS", Proceedings of µTas 2007, 7 – 11 October 2007, Paris, FRANCE, pp. 491-493.

[16] V. Agache, F. Sauter, C. Pudda, R. Blanc, C. Chabrol, P. Caillat, T. Plenat, A. Varnier, L. Ghenim, and A. Fuchs, "Fabrication and packaging of nanoporous membrane chips for label- free ion-channel transducer based biosensor", Proceedings of the 14th IEEE International Conference On Solid-state Sensors, Actuators and Microsystems Transducers '07 & Eurosensors XXI., Lyon, France, June 10-14, 2007, pp. 819-822.

Invited Talks

- *"Towards Stochastic Molecular Sensing: the Receptronics Project"*. World Congress on Lab-on-a-chips, M. Tartagni, 2007.

- Talk session concerning "*Bright field and epifluorescence microscopy of lipid bilayer membranes*", at the Imaging Services Workshop, Biological Sciences, University of Southampton, Southampton, UK, 2007.

- Talk session concerning *"The formation of artificial lipid bilayers in microfabricated systems and the development of protein delivery methods"*, at the Bionanotechnology Seminars, University of Oxford, Oxford, UK, 2007.

- Talk session concerning *"The delivery of protein to artificial lipid bilayers within microfabricated systems"*, at the DTC/IRC bionanotech module, University of Oxford, Oxford, UK, 2007.

- Bioelectric sensors engineered by coupling of ion channels and receptors. International conference on *"Channels, Chips and Nanopores: Biosensing with Channels"*, Berder Island, France, 2007. M Vivaudou

- *Protein-based biosensors for diagnostics and screening of GPCRs*. Invited talk. Sanofi-Aventis, Vitry-sur-Seine, France, 2008. M Vivaudou

- Design of biosensors based on the assembly of membrane proteins. Forum of Innovation Gravit *"Modelisation, tool for competitivity"*, Grenoble, France, 2008. M Vivaudou