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PUBLISHABLE FINAL ACTIVITY REPORT

1. Introduction

The project objectives were directed towards the development of novel approaches for electrical stimulation of neurons aiming at the treatment of neurodegenerative pathologies for restoring visual sight functions. By building up artificial retinal cell interfaces using stimulation devices and by tuning the biocompatibility of the stimulation electrodes and their surface interactions with neurons, we proposed to fabricate microelectrodes and microelectrode arrays (MEAs) as well as Ion Sensitive Field Effect transistors (ISFETs) for medical applications in-vivo. For these applications long term stability of the implant device is required and a closer proximity neuro-electronic interface is required to lower the threshold of neuronal activation. DREAMS enabled to study and fabricate these novel types of nanotransducers, which are based on synthetic nanocrystalline diamond (NCD) and epitaxial single crystal CVD diamond thin films. The key objectives of the project were to take advantage of both the biocompatibility and the semiconducting properties of diamond in order to explore the feasibility of fabrication of novel artificial retina implants.

To achieve these goals, the project has succeeded in building up an expertise on the fabrication of retinal stimulation devices by the following achievements:

- diamond surfaces biocompatibility (INSERM)
- diamond MicroElectrode Array fabrication (UCL -nanotechnology)
- MEA biological performance validation (FZJ)
- patterning for direct diamond MEA fabrication (CEA)
- diamond ISFETs fabrication (WSI)
- cell recording using transistor arrays (WSI/FZJ)
- cell culture promoting and patterning with nanodiamond (UCL-pharmacology)
- in-vivo implant validation (INSERM)

2. Diamond surfaces biocompatibility for cell cultures (INSERM)

In the course of the DREAMS project, we have shown that mixed retinal cells can grow on nanocrystalline diamond. In these mixed retinal cells, we did find glial cells and retinal neurones, especially bipolar cells postsynaptic to photoreceptors. A specific staining of these different cells indicated that retinal neurones were directly adhering on the diamond surface with retinal glial cells present at a certain distance. Such features were present with or without polypeptide (polylysine and laminine) coating on the diamond material. Cell survival was confirmed by incubating the cultured cells in the live/dead assay showing calcein accumulation in the viable cells leading to a strong green fluorescence. To investigate if retinal neurones could survive on diamond independently of glial cells, adult retinal ganglion cells were purified and seeded on diamond. Again, these cells were found to survive and grow extended neurites on diamond. In the final quantification showed similar abilities to grow on diamond and glass. Finally, the preference between polypeptide coating and pure nanocrystalline diamond was compared by applying a polypeptide pattern on the diamond during this final semester period.

All these results indicated that nanocrystalline diamond is a remarkably biocompatible material for retinal cells and more precisely for retinal neurones. They therefore suggest that this diamond material could provide an excellent biocompatible material for interfacing retinal prostheses and retinal tissue or more precisely retinal neurones. These data are currently compiled in a large paper for publication in an international journal.

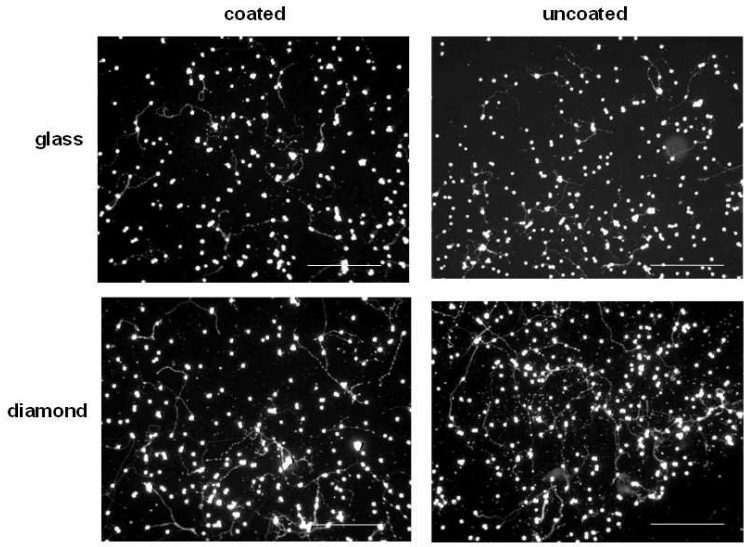

Figure 1 : retinal cell cultures on nanocrystalline diamond : as for glass, diamond exhibits a remarkable cell biocompatibility.

3. Diamond microelectrode array fabrication (UCL -nanotechnology)

Multi electrode arrays (MEA) were fabricated at UCL using silicon substrate with a SiO2 layer acting as a barrier. The MEAs were constructed using boron-doped nanocrystalline diamond (BNCD) as the conductive contact pads (~350nm) with titanium disilicide (TiSi2) as tracks (~140nm). Those MEAs were fabricated and used for neuron cell cultures and recordings. Arrays of 64 pixel electrodes were developed. For example, they were used for the monitoring of the action potentials in cultures of electrically coupled cardiomyocytes.

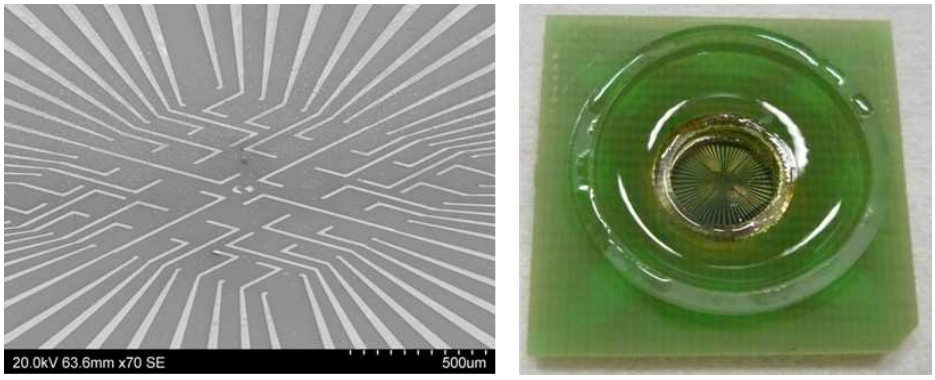

Figure 2 : 64 pixel nanocrystalline diamond MEAs used for neuron cell cultures.

4. MEA validation

BNCD MEAs were found to reliably detect electrical cell signalling from the cardiomyocyte-like cell line HL-1. The relative consistency of performance of this cell line compared to

neuronal cultures provides a system for comparison between the different chip designs. The signal to noise ratio was improved by 60% by adjusting the contact method between carrier and chip, and by selecting superior passivation methods. Though further refinement is necessary to achieve suitable bandwidth of signal transfer in NCD passivated BNCD MEAs, the surface was shown to be cell adhesive and conducive to culture growth. Thus, once electrical performance is tuned, a device with all diamond surfaces exposed to cells can be achieved.

5. Patterning for direct diamond MEA fabrication (CEA)

CEA has developed aside the project and in collaboration with ESIEE-Paris a novel approach enabling the direct selective growth of nanocrystalline diamond on patterned surfaces. This approach has enabled the fabrication of novel implants that could also be adapted on flexible substrates. Those implants were implanted in laboratory animals and demonstrated

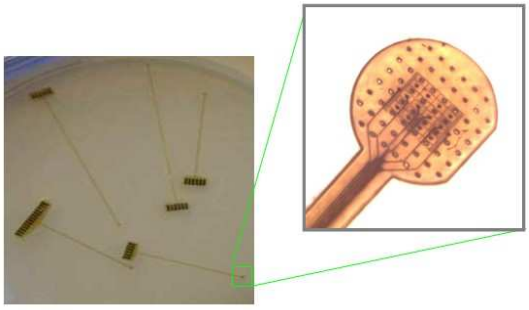

Figure 3 : Diamond MEAs fabricated on polyimide as soft implant prototypes.

6. Diamond ISFETs fabrication (WSI)

We have developed the technology for the fabrication of ISFETs arrays based on diamond surface conductive films. 4x4 arrays of transistors, with active gate dimensions 10x40 μm^2 and 20x40 μm^2, where fabricated using UV-photolithography on H-terminated single crystalline synthetic diamond substrates. The fabricated devices exhibit a high interfacial capacitance ($2\mu F/cm^2$) which leads to a high transconductance (i.e. sensitivity). Furthermore, the diamond ISFETs show an extreme stability in electrolyte, after more than 50 days of tests in physiological conditions. The noise performance of diamond ISFETs is shown to be superior to ISFETs based on other materials such as Si and AlGaN/GaN, which are also considered as the material of choice for ISFET devices. Diamond ISFETs exhibit 1/f-type noise, which is tentatively attributed to the effect of dislocations. Our diamond ISFETs exhibit an rms effective gate noise below 15µV, which is expected to be further reduced upon improvement of diamond crystal quality. The combination of facile fabrication, high stability, good transconductance, and low noise make of diamond ISFETs one of the most promising candidates for bioelectronic applications.

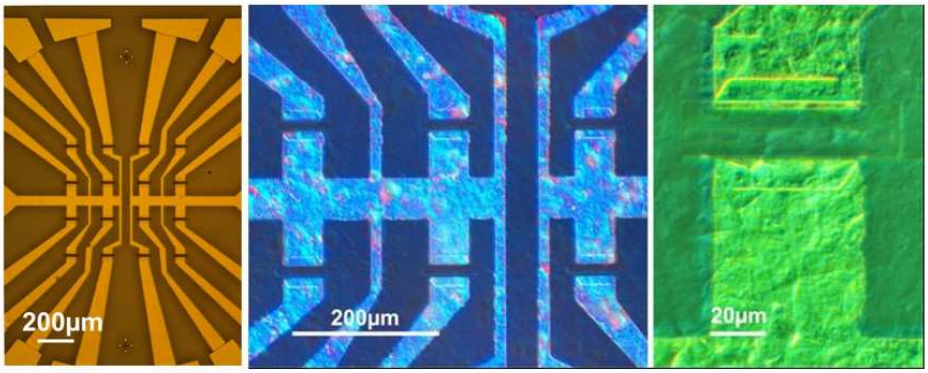

Figure 4 : (left)16 ISFET arrays as fabricated on diamond, (centre) and used for cell cultures with HL1 cardiac muscle cells, (right) zoom on one of the ISFET gates.

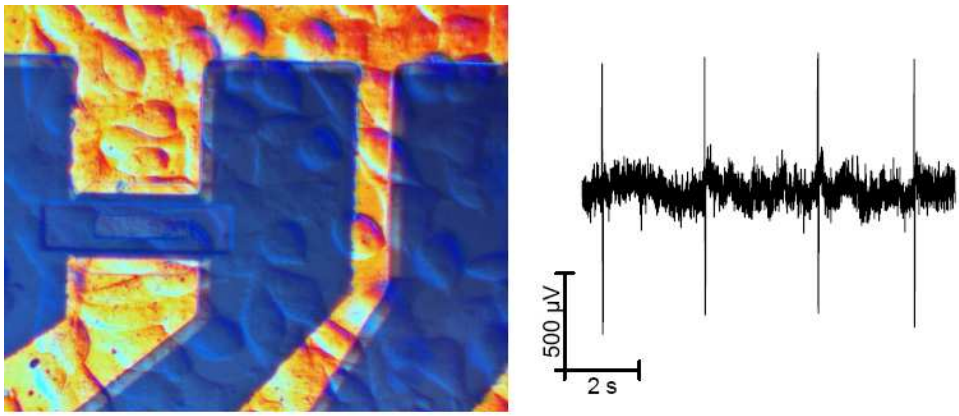

Figure 5. Left: Micrograph of cells plated on a diamond SGFET array. Right: extracellular signal recording of action potentials with a diamond SGFET array.

7. Cell recording using transistor arrays (WSI/FZJ)

Using the diamond ISFETs arrays developed in this project we have demonstrated for the first time the recording of the electrical activity of electrogenic cells which were cultured onto the devices. Electrical recordings from two different cell lines, both showing healthy growth and good adhesion to the substrate, have been successfully acquired. The diamond transistors are used to detect electric signals from both types of cells by recording the extracellular potential. The electrical activity from a three-days culture of HL-1 cells (cardiomyocytes, heart muscle cells) has been recorded, revealing the shape and the propagation of the spontaneously generated action potentials throughout the dense cell layer. In order to investigate the activity of individual cells, a better-controlled system has been investigated with patch-clamped Human Embryonic Kidney cells (HEK293), which stably expressed voltage-gated K^+ channels. In our cell-sensor coupling experiments, the cell's membrane potential is controlled by the patch pipette, which is used to induce the opening and closing of the potassium channels in the cell membrane, thus changing the transductive extracellular potential. The induced change of the extracellular potential is successfully recorded by the diamond transistor. Furthermore, The ion sensitivity of the diamond surface enables the detection of released potassium ions accumulated in the cleft between transistor and cell. The results from our diamond SGFETs attest to the promising capabilities of diamond for bioelectronics.

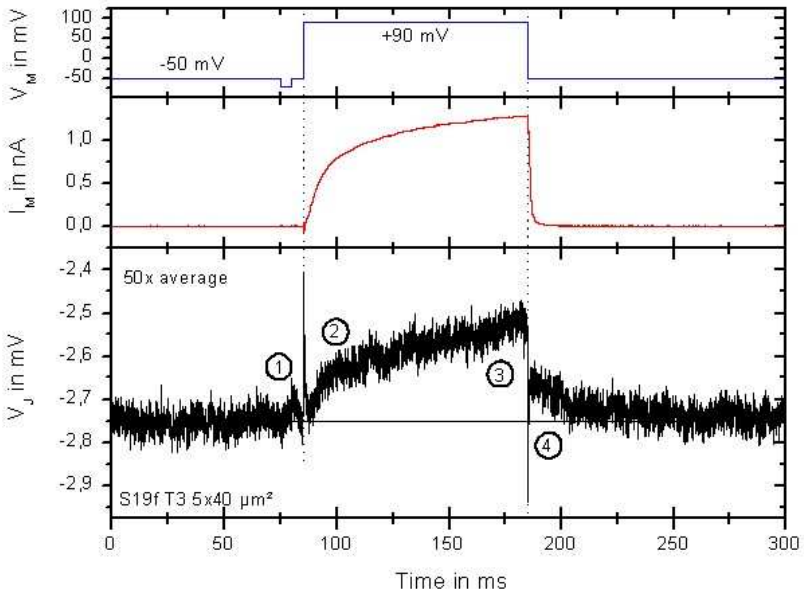

Figure 6 : Patch-clamp recording and corresponding transistor response of an HEK293 cell located on the gate area of the transistor. The cell potential as applied with the patch electrode is shown on top (blue), followed by the current across the membrane (red), and the transductive extracellular potential as recorded with the diamond transistor. The curves are averages of 50 recordings to improve the signal-to-noise ratio. The membrane current graph (red) does not show the sharp capacitive membrane current resulting from the change in membrane potential (dashed line at 85 and 185 ms, respectively) as this is compensated for by the amplifier electronics (20.8 pF membrane capacitance).[1]

8. Arrays of graphene solution-gated field effect transistors (WSI)

Graphene is a recent contender for the material of choice in the field of biosensors and bioelectronics. As an attempt to evaluate graphene's potential with respect to diamond, preliminary studies were conducted to evaluate the performance of graphene based solution gated field effect transistors. In fact, graphene possess unique electronic properties, such as a highly surface sensitive two-dimensional conductivity, high ambipolar carrier mobility, and low electronic noise. Furthermore, due to its growth and transfer technology, high quality graphene devices can be prepared on polymeric flexible substrates, paving the way for the integration of highly sensitive FET in flexible substrates for implants.

In DREAMS, we have started to explore the potential of graphene SGFETs. We have demonstrated a facile route for the scalable fabrication of transistor arrays that operate in aqueous environments using epitaxial graphene on SiC[2], as well as CVD grown graphene transferred to SiO_2 substrates. These two material substrates are scalable and can therefore take full advantage of our photolithographic processing method. The technology was developed at WSI during dreams towards the implementation of graphene devices on flexible substrates.

The fabrication of the graphene SGFETs is similar to the diamond SGFETs. The processing method allows the fabrication of micrometer range structures on a large scale (see Fig. 7c). The operation works similarly than in the case of diamond. Here, the monolayer graphene sheet is gated with an electrolyte solution and can be operated both in the hole and the electron accumulation region. The gate voltage U_G is applied between a reference electrode

[1] M Dankerl, S Eick, B Hofmann, M Hauf, S Ingebrandt, A Offenha¨usser, M Stutzmann, and JA. Garrido, Advanced Functional Materials, 2009, 19, 2915–2923

[2] M. Dankerl, M.V. Hauf, A. Lippert, L. Hess, S. Birner, I.D. Sharp, A. Mahmood, P. Mallet, J.Y. Veuillen, M. Stutzmann, J.A. Garrido, Adv. Funct. Mat., in press (2010)

in the electrolyte and a contact in the graphene, thereby fixing the potential drop between the graphene Fermi level and the reference electrode. Shifting the Fermi level with the gate potential below or above the Dirac point accumulates holes or electrons in the graphene sheet respectively (see Fig. 7b).

Electrical characterization of these devices in electrolyte shows the expected behavior according to the operation principle described above (see Fig 8a). Both n- and p-type conductivity dependent on the gate voltage is observed with a V-shaped minimum around the Dirac point. The devices show very high transconductance (Fig.8b), one order of magnitude higher than diamond SGFETs, due to the high mobility of carriers in the graphene layer; the large transconductance reflects the high sensitivity of the graphene SGFETs to variations of the gate voltage, which offers a clear advantage for bioelectronic applications.

Furthermore, the low-frequency noise of graphene SGFETs operating in electrolyte has been investigated, revealing an effective gate noise of tens of µV, which compares very well with low-noise Si devices currently used in bioelectronic applications. This preliminary work demonstrates that graphene SGFETs, with their facile technology, the possibility of integration with flexible substrates, high transconductance, and low noise promise high benefit for future technologies in implant applications.

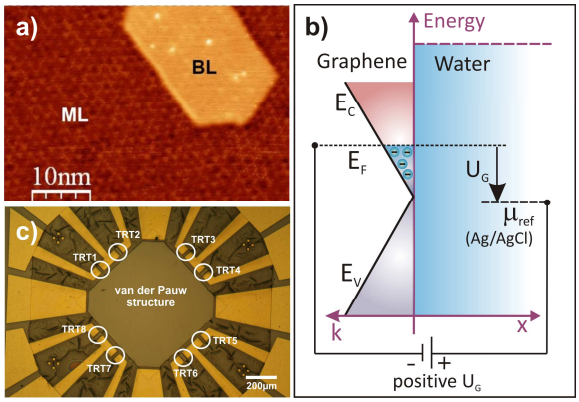

Figure 7 a) In-situ STM image revealing the morphology of the as-grown epitaxial graphene, with large terraces of single layer graphene (ML) and patches of bilayer islands (BL). b) Schematic drawing representing the modulation of the carrier density in the graphene film. c) Optical micrograph of graphene SGFETs. The Ti/Au structures (yellow) represent drain and source contacts for the transistors and contacts for the van der Pauw structure in the centre.

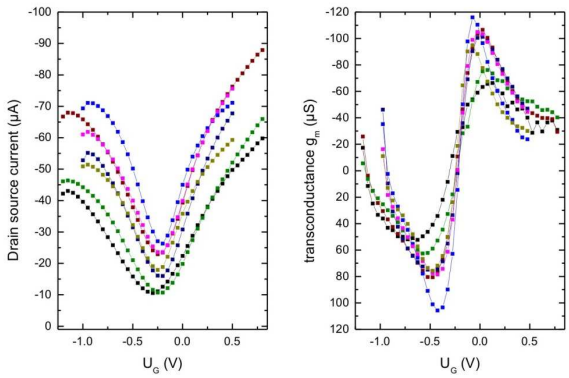

Figure 8 (a) Drain-source current curves (@ UDS =100 mV) measured for at different gate voltages, for graphene transistors with different active areas. The V-shape curve results from the ambipolar graphene. (b) Transconductance, $\partial I_{DS}/\partial U_G$, for the graphene SGFETs; the transconductance represents the modulation capability of the gate. The recorded transconductances are about one order of magnitude higher than for diamond SGFETs, due to the particularly high mobility of charge carriers in graphene.

9. Cell culture promoting and patterning with nanodiamond (UCL-pharmacology)

We have demonstrated within the project that nanostructured diamond materials, so- called NanoDiamonds (NDs) provide a new dimension of interaction with biological systems that takes place on a sub-cellular level with a high degree of specificity. In the field of neuroscience the nanoscale corresponds to the size of synapses; the specific connections between brain cells. The suitability of nanodiamond (ND) monolayers, derived from coatings with highly dispersed NDs, to act as a platform for neuronal growth has thus been investigated. Neurons cultured on various ND-coated substrates perform remarkably well, and similar to those grown on standard protein-coated materials with respect to their initial cell attachment, sustained neurite outgrowth, cell-autonomous neuronal excitability and functionality of the resulting electrical networks (Fig 9), for the full testing period (DIV 12). Importantly, we have demonstrated that neurons can grow directly on the new surfaces without the need for interpositioned glia cells (Fig 10). Such direct contact is desirable for efficient electrical coupling between devices and neurons.

In conclusion, we have shown that ND layering converts unfavourable growth surfaces for the neurons we tested into most suitable platforms that provide an excellent growth substrate on various materials for functional neuronal networks and bypasses the necessity of protein coating, which promises great potential for chronic medical implants.

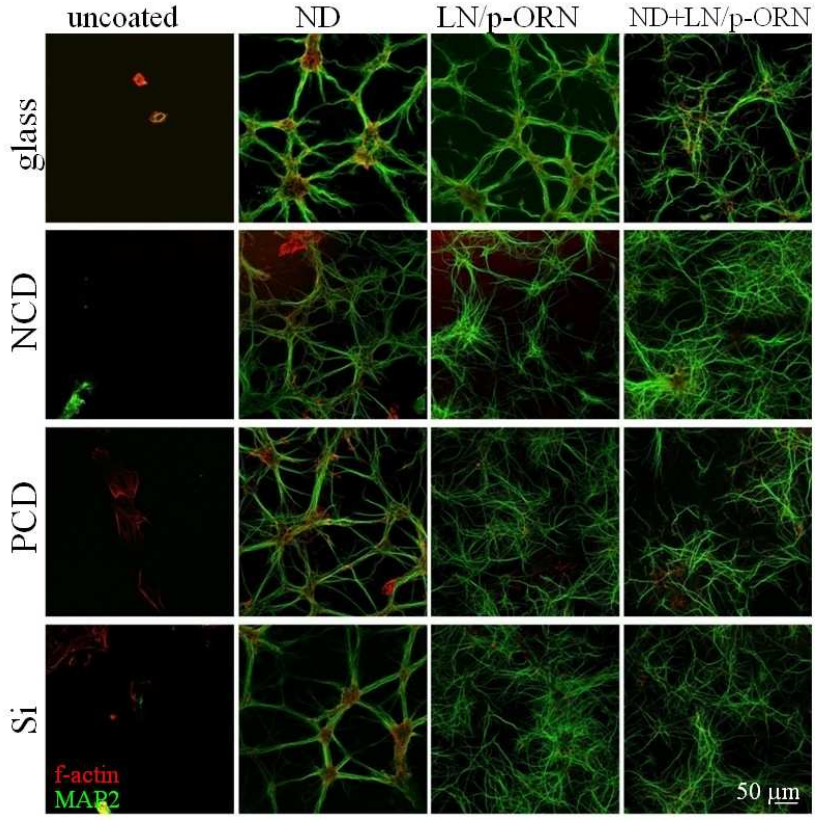

Figure 9 : Neuronal growth on ND-coated substrates. Confocal analysis of growth of hippocampal neurons after 2 days in culture (DIV2). Substrates glass, nanocrystalline diamond (NCD), polycrystalline diamond (PCD), and silicon (Si) were coated with nanodiamonds (ND) and/or laminin (LN)/poly-DL-ornithine (p-ORN); surfaces of uncoated substrates are oxygen terminated. Immunostaining reveals neurons via the dendrite-specific marker MAP2 (shown in green), while the cytoskeletal filaments of actin (f-actin) are stained for with rhodamine-phalloidin (red), highlighting structures rich in f-actin, such as growth cones at tips of neurites. ND coating (2nd column) promoted neuronal attachment and outgrowth similar to conventional protein coating (3rd column), whereas the uncoated substrates displayed no significant attachment and growth of neurons, where only few cells of apparent non-neuronal origin can be detected (1st column). Scale bar, 50 µm. 3 independent batches of cultures and coatings were tested.[3]

[3] A Thalhammer, RJ. Edgington, LA. Cingolani, R Schoepfer, RB Jackman, Biomaterials 31 (2010) 2097–2104

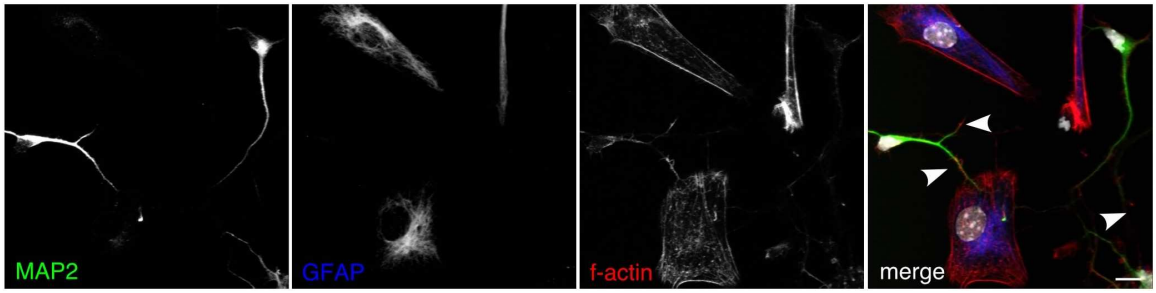

Figure 10 : Confocal imaging of cell types and cellular compartments revealed that neuronal outgrowth can take place in direct contact with ND-layer. Dendrites of neurons (MAP-2, green in false colour merge), glia cells (GFAP, blue), f-actin (rhodamine-phalloidin, red), cell nuclei (Hoechst33258, grey values in merge). Arrowheads indicate neuronal growth cones. Scale bar, 10 µm. .

10. In-vivo implant validation (INSERM)

Retinal implants with a diamond layer were produced by CEA with similar dimensions as those used in a previous study (Salzmann et al., 2006)[4]. They were implanted following a similar procedure in rats. During the in vivo period, an endoscope was used to visualize the retinal implant and control its position. Finally, animals were sacrificed one month after the operation, perfused with fixatives to maintain the retinal implant in the same position as in vivo. Retinal sections made with a cryostat enabled us to label the tissue with antibodies specific for reacting glial cells. These staining indicated that no major reactive gliosis was induced by diamond. However, in some instance, the diamond layer dissociated from polyimide, although this is likely to have occurred after this animal was sacrificed during the sample preparation.

These studies are consistent with the in vitro studies showing a good biocompatibility for diamond in contact to the retinal tissue. It further supports the potential use of diamond in neuroprostheses and more specifically in retinal prostheses.

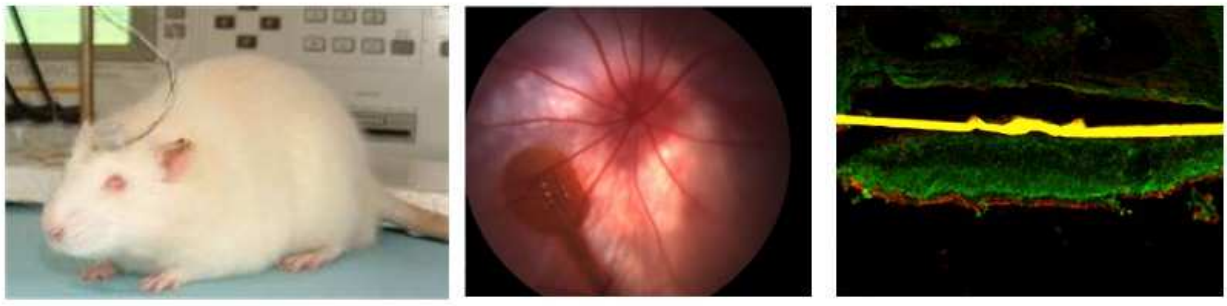

Figure 11 : Implant biocompatibility validation on laboratory animals: (centre) view of the implant in subretinal location, (right) after view of the retina bipolar cells (green) and glial cells (red). Hardly no form of gliosis is visible for the tissues in contact with the implant.

[4] Salzmann J, Linderholm OP, Guyomard JL, Paques M, Simonutti M, Lecchi M, Sommerhalder J, Dubus E, Pelizzone M, Bertrand D, Sahel J, Renaud P, Safran AB, Picaud S (2006) Subretinal electrode implantation in the P23H rat for chronic stimulations. Br J Ophthalmol 90:1183-1187.