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Bidirectional peripheral-nerves interfaces for hand prosthesis control. In human validation and analysis of the induced neuroplasticity and of the foreign body reaction

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Bidirectional peripheral-nerves interfaces for hand prosthesis control. In human validation and analysis of the induced neuroplasticity and of the foreign body reaction

A thesis presented by
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…to the strength of the constancy
like a reed in the storm,
waiting for the calm to return.

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Nowadays top quality research cannot be anymore the result of the activity of a single man, especially when the choice of investigating a field of frontier requests the joint collaboration of multidisciplinary knowledge. Indeed this tract would not be in this way without the fundamental support of many individuals that enlightened my path and bridged my lacks.

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As happened for the acknowledgments of my medical degree thesis, almost eight years ago, I cannot forget to mention the support outside the Lab that daily I receive from my mother, my family and from Gianluca. I hope to be able to thanks all of them again in my next thesis and later on.

Finally, and with sadness, my thought is for two living beings that gave me the strength to go ahead during those years and that recently, for different unexplainable reasons, are no more with me.

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List of Abbreviations

ADC — Analogue-to-Digital Converter
AFM — Atomic Force Mictoscope
AICs — Anti-inflammatory Cytokines
BBM — Blood-based Matrix
BCI — Brain Computer Interface
BMI — Brain Machine Interface
CNS — Central Nervous System
DoF — Degrees of Freedom
ECM — Extracellular Matrix
EEG — Encephalography
EMG — Electromyography
ENG — Electroneurography
ERD — Event Related Desynchronization
ERS — Event Related Synchronization
FBGC — Foreign Body Giant Cell
FBR — Foreign Body Reaction
FIB — Focused Ion Beam
fMRI — functional Magnetic Resonance Imaging
GABA — gamma Amino Butyric acid
IL-n — Interleukin n.
LIFE — Longitudinally-implanted IntraFascicular Electrode
M1 — Primary Motor Cortex
MEA — Multi-electrode array
MEG — Magnetoencephalograpy
MEPs — Motor Evoked Potentials
MMPs — Metalloproteinases
NMDA — N-methyl-D-aspartate
PDGF — Platelet-derived Growth Factor
PECs — Pro-extravasation Cytokines
PICs — Pro-inflammatory Cytokines
PLP — Phantom Limb Pain
PNS — Peripheral Nervous System
PPCx — Posterior Parietal Cortex
PPI — Pain Intensity Scale
S1 — Primary Somatosensory Cortex
sfMcGill — Short form McGill Pain Questionnaire
SMA — Shape Memory Alloy
SoA State of the art

TGFβ — Transforming Growth Factor β
TMS — Transcranial Magnetic Stimulation
TNFα — Tumor Necrosis Factor α
VAS — Visual Analogue Scale
VEGF — Vascular Endothelial Growth Factor
VP — Ventro-Posterior Nucleus
VPCx — Ventral Premotor Cortex

Chapter 0

PhD program

0.1. Candidate

Giovanni Di Pino received the Medical Degree (cum laude and honour mention) in 2003 at Campus Bio-Medico University of Rome. He worked in Italian and American Laboratories mainly involved in studying the mechanisms and molecules proper of neurodevelopment, apoptosis and neurophysiological intracellular recording. In 2001 and 2002 he was internal research fellow in the Neurobiology Department at the University of Pittsburgh.
His current fields of interest regard neuroscience and bio-engineering and all the possible matches.
In 2007 he started a PhD program in Bioengineering, primarily involved in developing bidirectional brain to computer interfaces to control sensorized cybernetic prostheses and to improve bio-electronic hybridity, while dedicating particular attention to the interconnection among the use of such interfaces and the consequent neuroplastic phenomena.

0.2. Introduction

In agreement with the common trend that goes against the division among sciences, the recent progresses of medical research should be framed together with a wider advancement in technological knowledge, proper of the current ages, and with the broad dissemination of this knowledge. Nowadays the physician cease to be the only one accountable for the improvement of the patients quality of life and he/she has to accept to be member of a professional team that is able to manage the care needs in multimodality.
Along this vein, the growing necessity to be supported by high-tech devices in both diagnosis and therapy as well as in research, open the doors of medical research centers and hospitals to biomedical engineering. Paradigmatic example of the collaboration among medicine and engineering is the newborn union of interests of neuroscientists and roboticists that give good reason for the creation of a novel branch called Neurorobotics. Aim of Neurorobotics is to develop robotic systems with motor, sensitive and cognitive abilities, starting from a solid base of knowledge about the brain and others neural systems. On the other hand neuroscientists can take advantage of robots to validate neuroscientific models and theories.
The increase in average life expectancy rises the prevalence in the population of disabling illness typical of elderly, such as stroke and other neurodegenerative disorders, resulting in a further demands for those technologies that replace functions or support during function recovery. Similar technologies are shown to be fundamental also in therapy and management of young patients with multiple sclerosis or with diseases of traumatic origin, such as spinal

cord injuries and amputations, that thanks to mechatronic devices can improve their performance in daily life activity and even reacquire the ability to work. Main challenge that the neurorobotics is facing is the development of biomimetic artifact that could satisfy the need of function replacement and especially the development of better way of interfacing those devices with the user.
The passion for this field and the decision of attempting a PhD program about neural interfaces for prosthesis control came from my belief that exploiting a new path where sciences fuse together is a winning strategy per se, from my medical and neuroscientific background of knowledge and from the chance to contribute to the validation in human of a new intraneural bidirectional interface for hand prosthesis control.

0.3. Objectives

1. In human validation of the control of a cybernetic sensorized multi degree of freedom hand prosthesis through longitudinally-implanted peripheral-nerves intraneural multielectrode.

   CyberHand project pretends to develop a cybernetic hand prosthesis able to be controlled with a low cognitive load, directly through the delivering of neural signals and to furnish to the user sensorial information. Final aim is to implement a human hand-like prosthesis that can be embodied in the user body scheme. This project reached the fundamental phase of first clinical validation, called LIFEHAND, during the PhD course. A volunteer subject received the implant of intraneural electrodes into his severed upper limb nerves and has been trained for controlling several types of grip made by the prosthesis. Afferent stimulation and efferent control were investigated through various modalities. Cortical plasticity in the user has been subject of further evaluations. In the following are collected my primary contributes to the experimentation:
   Specific Objectives
   a. Specific clinical protocol definition taking into account all the needs coming from physicians and bioengineers.
   b. Stimulation protocol definition.
   c. Stimulation/recording system (SRS) settings and integration.
   d. Electrodes-SRS wiring and shielding .
   e. Neuroplastic changes evaluation and functional analysis.

2. Requirements, specifications and designing of innovative bidirectional neural interfaces.

   Starting from the experience achieved with the LIFEHAND experimentation the follow research activities should lead to new insights toward the development of a new generation of intraneural interfaces respecting:
   a. The possible implications of neuroplastic changes in neural interface user.
   b. Mechanical tissue-electrode mismatch
   c. Foreign Body Reaction against implanted electrodes

0.4. Timecourse

The PhD course is structured in three main temporal phase:

1. Pre-LIFEHAND experimentation
   Analysis of the SoA of brain-machine interfaces, of hand prosthesis, brain and nervous system plasticity and body reactions to implanted material.
   Volunteer subjects selection and protocol development
2. LIFEHAND experimentation
   Patient training sessions, data collecting and neuroplasticity evaluation.
3. Post LIFEHAND experimentation
   Critical data analysis, results dissemination and conceptualization of new neural interface.

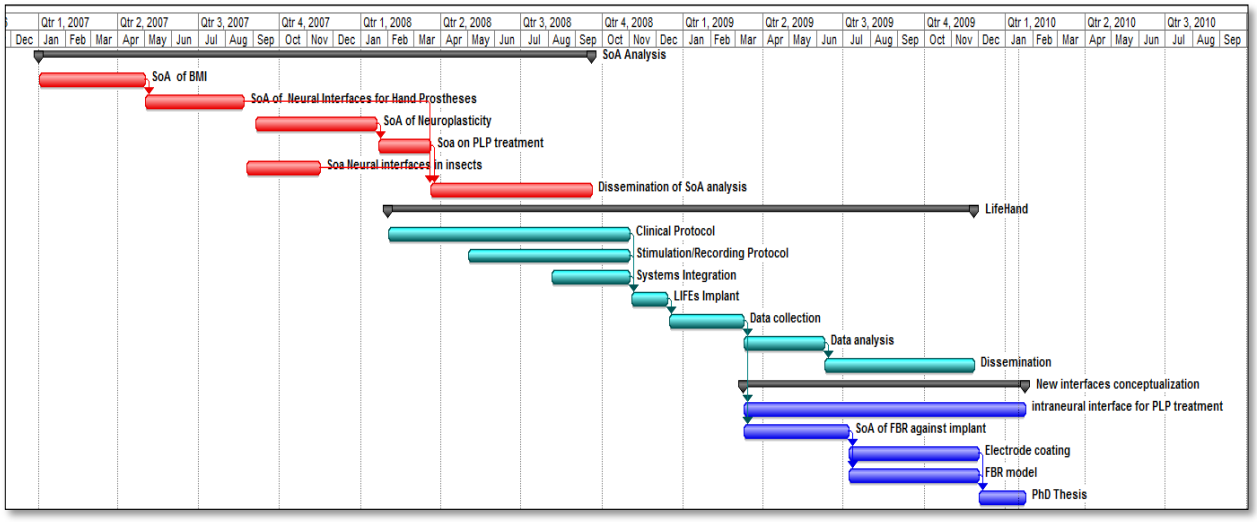

Figure 0.1 - Gantt chart of the PhD Program

Chapter 1

Brain Machine Interfaces

1.1 Introduction

The fascinating idea of artificially coupling the human nervous system with electronic devices, that for several years represented just a fortunate topic for science-fiction movies, in the last decades gained consideration as solid research theme that attracts significant amount of funds. In physiological condition a human being is able to interact with the environment through various modalities, but all of those involve neural signal passing through the nerves to/from the periphery, muscles activity and integrity of the organs of sense. Brain Machine Interfaces (BMI) propose themselves as revolutionary tools that, bypassing some or all the above obliged steps, allow to directly pick up motor intentions from the nervous system and relay them to an external electronic or robotic effector, that physically acts instead of the subject body. Moreover a class of BMI, called bidirectional, adds to the ability of extracting motor volitions the talent to manage neural signal flows in both afferent and efferent directions, thus translating and consigning, ready to be used, to the nervous system sensitive information that multimodal artificial sensors have extracted from the surroundings.
In few words the most commonly accepted definition of BMI says that are artificial devices that allow the direct communication among the nervous system and the environment through a computer and/or a mechatronic machine and that do not involve the use of muscles (Wolpaw et al., 2000). Their creating purpose is to mainly act in two areas of proficiency; replacement of lost functions in injured people, bypassing possible pathway interruptions due to nervous system or muscles accidents and augmenting, increasing the normal amount of brain inputs and outputs.
BMI systems are composed by all the components that electrically connect the neural tissue with a machine processor, such as recording and stimulating apparatus and signal processing unit, but have as main actor of their function the neural electrode that is the artifact that stays closer with the biological counterpart and establishes the contact.
Depending on the place where the electrode has to be settle, if it is inside or outside the body of the user, the neural interface gains the adjective of invasive or non-invasive. Is easy to imagine how invasive neural interfaces, to be allocated in their working site, in the central nervous system (CNS) or the peripheral nervous system (PNS), and to achieve a direct tight contacts with few clusters of neurons or with their neurites, need surgery, of various grade and with different risks. Microwire electrodes, consisting of a thin wire made by steel or tungsten and insulated material, that have been implanted in monkeys cortex for decades, have been mostly replaced by silicon microprobes mainly developed starting from the University of Michigan probe, planar with iridium recording sites on a silicon wafer, or from the University of

Utah probe, from the etching of a tridimensional silicon block obtaining an high density of platinum recording sites on needles tips in a 4X4mm square (Schwartz, 2004).
Concepts of modern assistive technological platforms such as functional electrical stimulation (FES) systems that replace central muscles control, prostheses and exoskeletons that substitute human limb or support in moving them and teleoperated robots rely on BMIs for a pleasant and intuitive interaction with the user, especially in situation when other ways are precluded.
Since today the evolution of most of those platforms is still in its experimental phases, non-invasive BMIs are often a very attractive solution for application in humans, because spare to the user any surgical procedures. Among several brain imaging techniques electroencephalography (EEG), magnetoencephalography (MEG), functional magnetic resonance (fMRI) and near-infrared spectroscopy have been implemented as BMIs allowing an easy access to experiments on human subjects. A wide literature about the use of non-invasive BMIs for the control of assistive technologies, such as electric wheelchairs or basic computer interfaces for enabling paralyzed patients to move a cursor on a screen, or of functional electrical stimulation systems is available - for a comprehensive review see (Birbaumer and Cohen, 2007).
On the other hand, non invasive BMIs, at least at the moment, suffers for the low portability of the equipments that work only in a high structured environment, except for EEG-based interfaces that are characterized by a very low spatial resolution. Moreover they are not able to retransmit sensory feedback respecting the physiological modalities.

1.2 History of BMI

Neural interfacing technologies had a common origin almost a century ago when neuroelectrophysiologist started to use external and implanted electrodes to monitor neural system electrical activity and later, to reconstruct functional maps of the brain.
With some approximation we can consider the devices developed within the first comprehensive study of electroencephalography in humans, made by the German physiologist Hans Berger (Berger, 1929) as the prime ancestors of non invasive BMI. The electroencephalographic techniques were born primarily as diagnostic tools for epileptic seizures, being able to record the rhythms of oscillation in the electrical potential of cortical areas close to the scalp. When after the work of DiCara and Miller (DiCara and Miller, 1968a, b), that introduces the concept of biofeedback, the scientific community was ready to accept the idea that most of the physiological activities that before were supposed to be only under the control of autonomic system could be consciously and voluntarily modified, the field was ready for the studies of Karima and coworkers. They, interested in understanding the weight of consciousness in the mind-brain dichotomy, demonstrated the ability of several human subjects to voluntarily modulate the amount of alfa rhythm during a trial period (Nowlis and Kamiya, 1970). Since was established that humans were able of voluntary modulating the outputs of devices developed only to investigate their brain activity, the further step in creating the brain-machine, or brain-computer, interface was to use the electronic output of those devices to control effectors that in some way act on the environment. Almost ten years earlier Licklider

and Engelbart were already hypothesizing a kind of symbiosis among man and computer and stressed the importance of the communicative interface (Licklider, 1960; Engelbart, 1962).
Invasive BMIs have their roots in the studies of Fetz and colleagues that during the 60s and 70s demonstrated that monkeys, conditioned with a reward, were able to control the firing rate of cortical neurons if aided with a biofeedback meter (Fetz, 1969; Fetz and Finocchio, 1975). Especially in United States research studies that target to establish a direct communication among the nervous system and electronic devices immediately attract the interest of the scientific community and of the government. The term brain computer interfaces, used in principle instead of BMIs, and today still in use, appeared for the first time in a scientific paper published on Annual review of biophysical engineer in 1973 (Vidal, 1973) at the end of project cofounded by the National Science Foundation and the DARPA, that took place at the University of California Los Angeles. In 1980 Schmidt first theorized that similar brain computer interfaces could be in the future utilized to control prosthetic arm in patient with motor disabilities (Schmidt, 1980). Nevertheless several illustrious scientist, such as Philip Kennedy, Miguel Nicolelis, Andrew Schwartz, John Donoghue, Jonathan Wolpaw, Niels Birbaumer and Gert Pfurtscheller actively worked in invasive and non-invasive neural interface field in the last twenty-five years, the idea to control a prosthesis with a neural interface had to wait twenty years to be experimented in animal (Chapin et al., 1999), five years more for the first attempt in human (Dhillon et al., 2004) and we are still a little far from a comprehensive system that could be delivered to the public for hand prosthetic application. Nowadays progresses in neural interfacing technology could be only the end-products of a real multidisciplinary research that take together neuroscientists, biomedical and electronic engineers and expert in computer science and in science of biomaterials. Efforts have been spent in various areas that regard the innovation of the electrodes itself, but also the signals analysis algorithms, the communication among electrode and recording unit and the inner control of the robotic actuator that reduces the needed amount of information coming from the brain of the user.

1.3 BMI for improving the biological-electronic hybridity

BMIs aim at establishing a direct connection with the nervous system of a living being and it involves the creation of new outputs for the brain that could be exploit also for scopes that go above the biomedical applications. It's already a matter of fact that gaming and entertainment companies look at the brain computer interfaces for the most modern ways of interfacing with the users and that, unfortunately, research on BMIs attracts the interests of armies and arms industries.
Final aspiration of a neural interface is to ameliorate the cybernetic performance of a mixed system where the word cybernetics regards, as proposed by who first coined the term, the ability to achieve a proficient communication among human and machine (Wiener, 1948). This engages the improvement of the biological-electronic constitutional hybridity and, if the biological component of the ensemble is a human, the formation of what is called an hybrid bionic system. It is defined as the tight complex composed by a robotic artifact directly interfaced with the brain of a human being. Machine-biological hybridity achievable with the

use of BMIs could lead to new insights in interfacing science and is a resource that should not be underestimated. The main advantages of an hybrid system are due to the fact that each of the two different elements brings its strengths to the system and compensates the deficiencies of the counterpart. In biomedical science this paradigm is usually exploited in terms of the machine that is employed by the user for function replacement and the rest of this dissertation is about this interpretation, but it is good to know that in some circumstances the hybridity is pursued to allow the biological fraction of the ensemble to support the function of the machine. This is the case of an insect-machine hybrid controller conceived for space exploratory vehicles (Di Pino et al., 2009) that involves the use of neural interfaces. Astronaut's capabilities has been theorized to be possibly enhanced by interfacing brains with machines (Rossini et al., 2009), but human presence drastically limits space travel and non-human missions still lack important capabilities, such as autonomous navigation, decision making, learning and adapting. One possible approach is the integration of animal brains into unmanned spacecraft for creating a animal-machine hybrid controller and insects seems to be suitable for the scope, because they developed navigation mechanisms which are optimized in terms of simplicity and robustness. In this scenario the hybridity permits that high level tasks like navigation, exploration would be handed over to the insect, while obstacle avoidance and locomotory issues would be dealt with the engineered controller. Along this vein other interesting examples of hybrid system that adopt neural interfaces could be found in the literature where are assembled cyborg beetles (Diorio and Mavoori, 2003; Takeuchi and Shimoyama, 2004; Sato et al., 2008) or small mobile robots are controlled by neurons cultured upon a microelectrode array (Novellino et al., 2007), by a moth (http://neuromorph.ece.arizona.edu/), or even by the brainstem of a lamprey (Reger et al., 2000).

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Chapter 2

Interfaces for human hand prosthesis control

2.1 Introduction to hand prosthesis for human amputees

Since thousands years ago people with post-traumatic limb amputation tried to compensate their cosmetic and functional deficiencies with the use of prostheses. Cosmetic prostheses were already used by the Egyptians, and prostethic-like devices specifically conceived with the aim of functional restoration are described during the early 1500's (Fumero and Costantino, 2001). It is quite obvious that the level of performances theoretically required to an upper-limb/hand/fingers prosthesis is remarkably more complex than for the leg. This problem has not been solved by upper limb transplantation which does not seem –so far- to represent an acceptable and suitable solution, at least for the large scale (Schuind et al., 2007). Despite the last five centuries are characterized by huge innovations in science and technology, several barriers continue to inhibit successful application of high-tech prosthetic devices. The most advanced hand prosthesis commercially available are little more than just one dimensional pinch, with no or a few embedded sensors –therefore mainly operating under sight control-, and the vast majority of amputees still use only cosmetic prostheses or do not use prostheses at all. The overall user acceptability of the prosthetic device is depending on multiple factors, such as dexterity, anthropomorphism, control features, autonomy of operation, dependability, but most remarkably by the type of limb-prosthesis interface and the quality and amount of sensory information fed-back by the prosthesis to the user (Micera et al., 2006; Zollo et al., 2007); within the frame of these last points information transfer rate and the latency -i.e. the time delay between command and action, enabled by the interface- play a major role (Tonet et al., 2007).

To overcome all these obstacles, scientists are developing new generations of prostheses. Modern robotic technologies, such as mechatronic design methods, miniature, high weight\power ratio actuators, micro position and tactile\force sensors, are enabling the development of compact, light-weight, multi-fingered hand prostheses (Zollo et al., 2007) embedding low-level controllers that allow active grasping control and could be prone to perform dexterous manipulative tasks, if properly interfaced to the human brain. Ideally, a Brain-Machine Interface (BMI) to a hand prosthesis apt to implement a closed-loop control of the mechatronic prostheses while exchanging bidirectional (efferent and afferent) information with the nervous system of the amputee. An example of application of such approach is the CyberHand system, a cybernetic anthropomorphic hand specifically designed to be connected via a bidirectional neural interface to the peripheral and central nervous system, which is supposed to be therefore able to exchange afferent and efferent signals with the user (Carrozza

et al., 2006). Another important research framework aiming at the development of several innovative hand prostheses is the Revolutionizing Prosthetics project funded by US DARPA. In fact, one of the most intriguing and disruptive innovations that can have a dramatic impact in the application of such a new generation of hand prostheses is the enhancement of the exteroceptive and proprioceptive inputs that the device is able to feed-back to the patient in a physiological fashion in order to partially replace natural sensations and re-obtain full consciousness of the missing limb by embedding it again in the body scheme. Even though the Cyberhand platform is a paradigm of a real step forward with respect to the state-of-the-art, the bottleneck of the lack of a natural and intuitive bidirectional interface fully enabling the performance of a multifingered, robotic hand is still far from being overcome.

2.2 State-of-the-Art of interfaces for prosthesis control

Commercially-available and prototypes of human hand prostheses may be equipped with many different types of human-prosthesis interfaces in order to derive information on the intention of the user (motor commands) and, in some cases, to feedback some artificial sensory data from the prosthesis to the user. Such interfaces can be grouped in five main classes: 1) Mechanical (body-powered) interfaces; 2) Myoelectric (EMG-based) interfaces using non-homologous muscles; 3) Myoelectric (EMG-based) interfaces using homologous muscles; 4) Non-invasive Neural Interfaces and 5) Invasive Neural Interfaces. Devices of the last two classes are often referred in the literature as Brain-Computer Interfaces (BCI), or more properly Brain-Machine Interfaces (BMI) (Schwartz, 2004; Lebedev and Nicolelis, 2006; Micera et al., 2006).

2.2.1 Mechanical and myoelectric interface for prosthesis control

Body-powered interfaces mechanically copy the user's intentions from the movements of a segment of the body, i.e. one shoulder or foot, that is physically linked to the prosthesis so to translate real volitive movements into hand movements. Myoelectric interfaces allow the user to communicate her/his will through voluntary muscles contractions translated into electric signals by EMG surface electrodes. Frequently, as a consequence of limb amputation the muscles used to move the hand are no longer available so the EMG electrodes are located on non-homologous muscles. This implies that for interfaces that belong to both class 1 and 2 as previously defined the user needs to perform an unnatural expression of his volition to move the prosthesis. If the amputation is trans-radial, the forearm muscles, or only part of them, are candidate to establish an EMG-interface with homologous muscles that results to be perceived as more natural and user-friendly. Recently an innovative approach called targeted reinnervation has been developed. Through the transfers of both sensor and motor fibers components of residual ulnar and median truncated nerves from the stump to the ipsilateral pectoral muscles, this surgical procedure allows to create an homologous myoelectric interface even in a subject with a very proximal amputation (Kuiken et al., 2007).

2.2.2 Non invasive BMI for prosthesis control

Non-invasive BMIs are gaining an increasing popularity since they do not need to be implanted inside the nervous system. Consequently, they provide a very attractive solution for the users, as they are not supposed to undergo any surgical procedures. As a matter of fact, several imaging technologies have been adopted for implementing such interfaces, i.e. EEG, MEG, fMRI and near-infrared spectroscopy. However, only a few experiences faced the control of hand prosthesis by using non-invasive EEG interfaces. Based on the experimental data reported so far, no more than one degree of freedom prostheses have been controlled while generating a very high amount of undesired movement (Nirenberg et al., 1971) or requesting an high level of user's attention (Guger et al., 1999) that made the use of such interfaces quite unpleasant for the patient. Even if recent developments of EEG-based approaches are taken into account, a more natural control, that detects native signals from the homologous motor areas of the brain, could be achieved in the future only through a dramatic increase of the spatial resolution of this technology, that does not seem to be at hand in the short-medium term. Moreover, the integrated use of non-invasive interfaces, not only for extracting efferent commands but also to convey sensory feedback to the brain, has not been explored so far, and it could pose severe limitations to the development of real bidirectional interfaces based on these technologies.

2.2.3 Central invasive BMI for prosthesis control

With the use of invasive neural interfaces an electric direct connection between the nervous system and a robotic arm could be established at several levels.

Twenty years after the theorization of the extraction of voluntary motor commands from cortical activity to control a prosthetic device (Schmidt, 1980) arrived the first demonstration of a robotic arm that dispenses water, controlled by a rat brain through a multielectrodes array implanted in M1 (Chapin et al., 1999). The same group succeeded in extracting the frontotemporal electrical activity and making a monkey control a robot arm equipped with a gripper while closing the loop with a visual feedback of a computer cursor. The cursor position on the screen expresses the position of the arm while the gripping force was provided by changing the size of the cursor (Carmena et al., 2003). Recently Schwartz group from Pittsburgh University was the first that achieved a direct real-time physical interaction with the environment using cortical signals from a monkey to control a multi-jointed multi degrees-of-freedom (DoF) prosthetic device in a self feeding task (Velliste et al., 2008).

In the last five years experimental trials with invasive single electrodes or microelectrodes arrays implanted both in central and peripheral nervous system to control prostheses reached the phase of clinical application to humans.

In 2004 Donoghue's group implanted a 96 microelectrodes array in M1 of a patient three years after his spinal cord injury at C3-C4 vertebral level. During a nine months follow-up the patient was able to control a simple one DoF hand prosthesis and to control a PC cursor (Hochberg et al., 2006; Donoghue et al., 2007).

2.2.4 Peripheral invasive BMI for prosthesis control

New approaches in developing artificial architectures, that supply for the lost of motor and sensitive functions, involve the feasibility to directly interface with the nervous system at a peripheral level. It can happens thank to the skill belonged by specific implanted microelectrodes of exchanging information with the nerves. When comparing an invasive peripheral neural interface with the other possible interfaces suitable for neuroprosthesis control it seems to be a very good compromise among the extremely high invasiveness of cortical implants and the low selectivity of electroencephalograpyc (EEG) or electomiographic (EMG) interface, that mostly allow only unnatural control strategies and unfeasibility to release feedback. As an example see in Table 1 this comparison for an hand prosthesis control in amputees

Approach	Main Advantages	Main Disadvantages
Surface EMG	Non-invasive	Not natural control strategies must be used by the subject
Implantable EMG	Improved quality of EMG signals	Not natural control strategies must be used by the subject
Targeted reinnervation	More natural control strategies, effective sensory feedback	Requires a surgical implantation but works with non-invasive signals More suitable for very high level amputations
Implantable cortical interfaces	Direct connection with the Cortex	Too invasive for the disability related to amputation
Non-invasive cortical interfaces	Non-invasive approach	Limitations in the number of controllable degrees of freedom. Not natural control strategies
Implantable peripheral interfaces	Potentially selective and not very invasive (if compared with other implantable solutions)	Limitations in terms of controllable degrees of freedom and sensory feedback not clear

Table 2.1 - Comparison of the characteristics of the different human-machine interfaces for the control of hand prostheses in amputees. From (Micera et al., In Press)

Thanks to peripheral intrafascicular electrodes implanted in the stump nerves of amputees recently two different groups were able to record volitional motor nerve activity that has been used by the patient to control the grip of an hand prosthesis (Dhillon and Horch, 2005) and the flexion-extension of an artificial finger (Jia et al., 2007). We have recently succeeded in a similar approach by using intrafascicular electrodes chronically implanted in median and ulnar nerves for 28 days in a left arm amputee and driving a robotic hand for various, independent movements -see Chapter 4- and (Rossini et al., Accepted; Micera et al., In Press).

The choice of the interface adopted for the clinical trial came after a deep analysis of the different classes of peripheral neural interfaces, their advantages and disadvantages with a particular attention given also to their fabrication techniques.

Most peripheral nerve interfaces use an electrical coupling method both for detecting

the bioelectrical activity of the nerve fibers (recording) and/or to induce their excitation (stimulating).
Thus, the neural electrodes have to be implanted in the proximity of a peripheral nerve, even within, to reduce tissue impedance and the current intensity needed to stimulate. A simplified classification of PNS electrodes according with their spatial relation with the nerves, groups them into three classes: extraneural, intraneural, and regenerative.
Unfortunately the achieved selectivity of stimulation or recording individual nerve fibers increases with the invasiveness of the electrode implantation (Fig. 2.1).

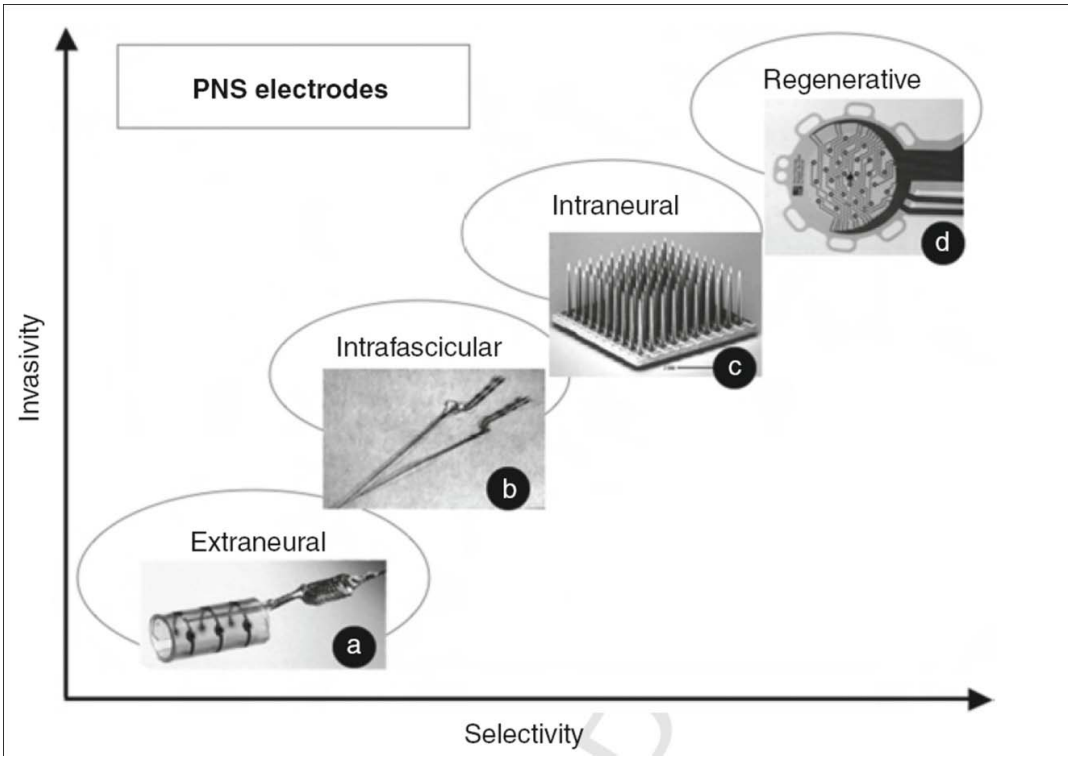

Figure 2.1 - Different types of electrodes applied to interface peripheral nerves classified regarding invasiveness and selectivity. Micrographs show examples of hybrid silicone–polyimide cuff electrode (Fraunhofer IBMT), polyimide tfLIFE (Fraunhofer IBMT), silicon-based Utah MEA (Cyberkinetics), and polyimide sieve electrode (Fraunhofer IBMT). From (Micera and Navarro, 2009)

Among all the extraneural electrodes Cuff represent the most advanced solution and probably the most investigated for research purposes. In Cuff electrodes an insulating tubular sheath encircles the nerve and contains the active sites that are exposed at the inner surface (Fig. 2.1a). Because of implanted around the nerves, they allow an accurate positioning (Loeb and Peck, 1996), and are less prone to damage the nerve than more invasive groups, but suffer for a reduced selectivity, recording only mixed sensorimotor activity from mixed nerves, especially from large myelinated and superficial fibers. Innovation in cuff structures with multisites and in processing algorithms have increased their selectivity.
To increase the contact area among the electrode and the nerve an extraneural electrode called flat interface nerve electrode (FINE) has been developed (Tyler and Durand, 2002). FINEs are

flattened around the nerve into a favorable geometry, thus reaching the deeper fibers. Chronically implanted FINEs in animals did not shown detectable changes in nerve physiology and histology if only small forces were applied, while high reshaping forces are prone to induce nerve damage (Tyler and Durand, 2003; Leventhal et al., 2006).

The aim of implanting an electrode inside the nerve instead than place it around can be resumed with the needs of increasing the selectivity of the contact in parallel with the signal to noise ratio.

Longitudinal intrafascicular electrodes (LIFEs), are inserted longitudinally into the nerve to lay in-between and parallel to the nerve fibers with micro-neurosurgery by using a tungsten needle that has to be removed after insertion(Lawrence et al., 2004). thin-film LIFEs (tfLIFE) (Fig 2.1b) represent the last generation of this electrodes and present several contacts in one device of polyimide substrate. They are able to perform invasive multi-unit peripheral nerve recording and stimulation, therefore allowing the combination of neural signals from multiple sites to better reconstruct the patterns of input or output information. Each single electrode is typically realized from thin insulated conducting Platinum or metal coated Kevlar wires with eight recording pads embedded in a polyimide substrate that can assure biocompatibility and flexibility (Citi et al., 2006; Hoffmann and Kock, 2005). tfLIFEs allow more selective interfacing than extraneural electrodes that wrap the whole nerve, because acquire signals from only few axons.

Moreover LIFEs are less invasive compared with intraneural multielectrode arrays (MEAs) that are composed of tens of needles inserted transversally into the nervous system and developed on materials such as silicon, glass, or polyimide (Fig 2.1c) (Kipke et al., 2003). They have been mainly implanted in the brain cortex (Hochberg et al., 2006; Velliste et al., 2008), but have been also tested in PNS in animal models and also in a human volunteer (Warwick et al., 2003). A modified version of MEA (named Utah Slanted Electrode Array, USEA) implanted in cat sciatic nerves achieved selective recording of single unit responses and low-current highly selective stimulation of motor fibers (Branner et al., 2001; Branner et al., 2004). While MEAs present the advantage of a high number of electrical contacts, they suffer for the rigidity of the structure that during movements may damage the nerves, and for the lack of electrical stability over the time (Warwick et al., 2003; Branner et al., 2004).

Sieve electrodes are part of the family called regenerative electrodes because are capable of interfacing with a elevated number of regenerating axons growing through an array of holes with electrodes around them (Fig. 2.1d). The tight relation with the axons allows to record action potentials and to stimulate individual or small groups of neurites. Unfortunately, regenerative electrodes can only be applied to transected peripheral nerves and need long time waiting for regeneration, thus precluding acute experiments. The characteristic of these electrodes make their main future purpose the implantation in severed nerves of an amputee limb for prosthesis control. Even though in experimental models axonal regeneration through polyimide sieve electrodes occurred in most of the animals implanted, we are still far away from their use in clinical practice (Navarro et al., 1998; Lago et al., 2005).

2.3 Microfabrication techniques of tfLIFEs

Among the described classes of neural electrodes for PNS implantation tfLIFEs represent at, the present knowledge, the better example of high selectivity/invasiveness ratio. They are the results of the innovation through several generations of intrafascicular electrodes and reached the phase of clinical evaluation in humans (Hoffmann et al., 2006; Rossini et al., Accepted; Micera et al., In Press). For those reasons tfLIFE-4s fabrication process, realized by the Department of Neuroprosthetic of the Fraunhofer Institute for Bio-Microtechniques, St.Ingbert, Germany, is present here as a paradigmatic example of the microfabrication techniques adopted for neural electrodes.

thin film - Longitudinal Intrafascicular Electrode (tf-LIFEs) combines a loop of a thin film electrode with a filament loop including a thin tungsten needle that can be used as guidance for the implant, while it is removed after the implantation procedure. The active part of the electrode is made of flexible polyimide thin-films.

The tf-LIFE4 micro structure consists of a polyimide substrate with overall thickness and length of 10 µm and 5 cm respectively. tf-LIFE4s present eight Pt 300 nm–thick recording or stimulating sites (four on each side of the loop, i.e., L1, L2, L3, L4, R1, R2, R3, R4) and proximal sites (reference and ground electrode on each side, i.e., L0, R0, GND, GND). On both ends of the structure, there are bond pads to contact the flexible part with an adaptor (Fig. 2.2).

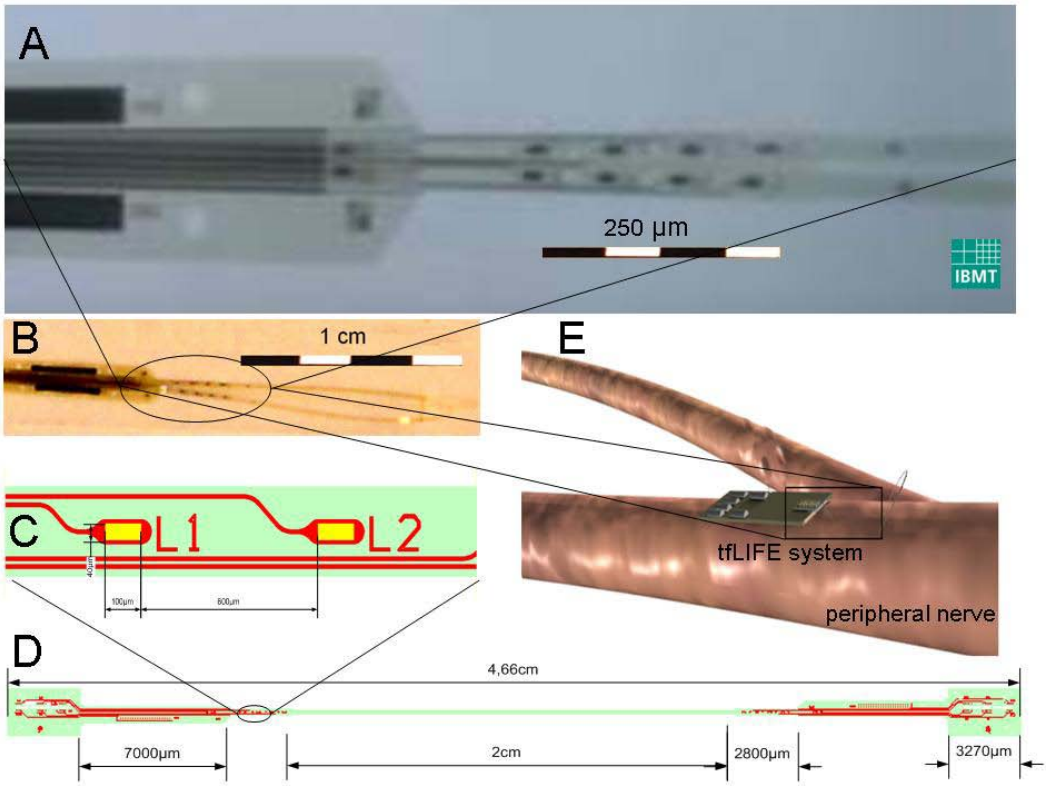

Figure 1.2 - A and B show optical microscope views of tf-LIFE4 at different magnifications, while C and D show illustrating schemes and measures. Note that only the encircled part remains within the nerve and contains the recording contacts (i.e., yellow rectangular L1 and L2 of Fig 3C), as schematised in E.

The polyimide acts as substrate and as insulator on which platinum tracks are sputtered and the active sites are realized. The mechanical properties of the polyimide give great flexibility to the active part of the electrode decreasing relative drifts between the tissue and the electrode together with fibrous encapsulations. The basic requirements for all components that have a direct interaction with the biological tissue are the biocompatibility of the used materials. Especially for electrodes the biocompatibility has to be investigated under stimulation as well as recording conditions. Electrode materials with stable electrochemical properties like electrochemical impedance and corrosion stability are needed (Hoffmann et al., 2006). Based on a micromachining process Polyimide (PI 2611, Du Pont) is used as carrier layer for connection lines and pads as well as the electrode material. After applying a 5 µm thick layer of polyimide on a silicon wafer a single metallization layer of 300 nm of Platinum will be sputtered on it and patterned by lift-off technique. Then a second polyimide layer of 5 µm thickness will be applied on the top of the structure and then coated with aluminium as etching mask for the afterwards dry etching process to open the electrode area and the connection pads. After the removal of the dry etch mask the wafer will be cleaned and the single structures (thin film electrodes) will be removed from it (Fig 2.3).

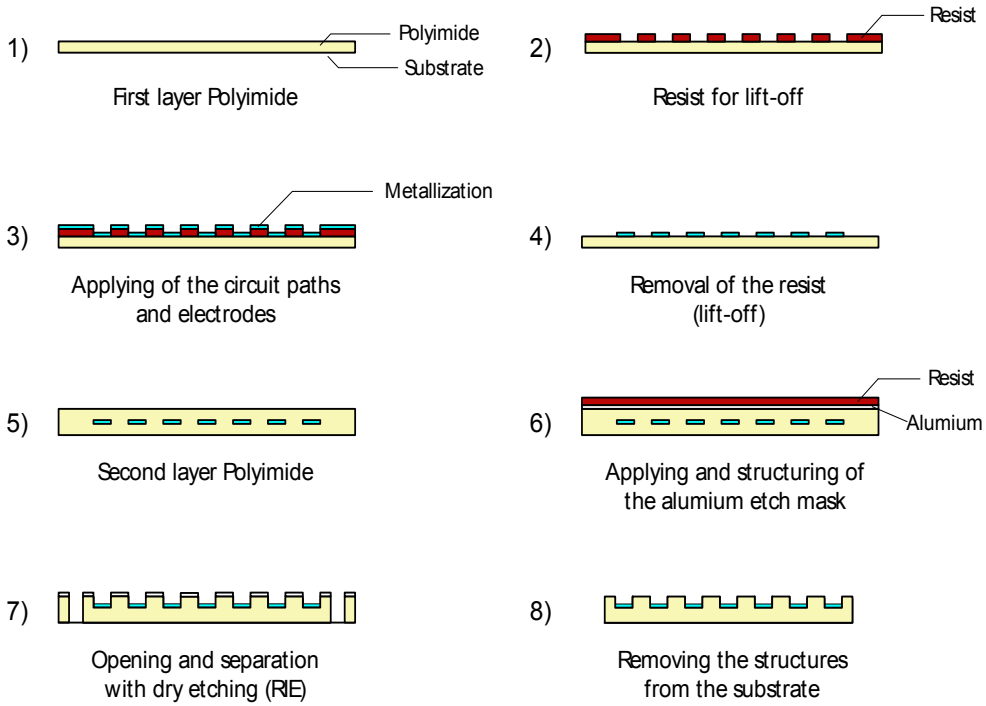

Figure 2.3 - General process sequence for the micromachining development of flexible thin film microelectrodes based on Polyimide substrate material. From (Hoffmann et al., 2006)

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Chapter 3

Amputation-induced plasticity of the nervous system

A fundamental contribution to derive important functional and technical specifications for novel human-machine interfaces for hand prosthesis and to properly model and predict all the complex phenomena that could be triggered by the implantation of the prosthesis and of different types of interfaces, can come from the systematic and in-depth analysis of what there is on the other side of the interface: the nervous system of the amputee and its reorganization following limb amputation.

3.1 General aspects of neuroplasticity after nerve injury

From a neuro-physiological point of view the issue of cortical and subcortical brain re-organization following limb amputation is part of a wider class of physiological phenomena including neural reorganization following peripheral input and actuator deprivation. It is well known that the neural representation of a body segment is continuously modulated by the 'external' and 'internal' world, as well as in response to activity, behavior and skill acquisition (Kaas, 1991); therefore, a perturbed sensory experience secondary to sudden loss of bidirectional nervous flow to-from a body segment induce a deprivation-dependent neural reorganization that affects its topographical and functional representation in the nervous system. Within this physiological frame such phenomena deeply involve cortical and subcortical areas as thalamus, brainstem and spinal cord and even the peripheral nervous system. The neural reorganization could follow different types of transient or permanent central and peripheral nervous system injuries, such as transient deafferentation (i.e. local anaesthesia or limb immobilization), peripheral nerve lesion, amputation, spinal cord and cerebral injury (Chen et al., 2002), and it regards not only the somatosensory and motor activities but also the visual and the auditory systems. Transient deafferentation is a useful model to demonstrate the rapid cortical changes due to short-term plasticity. In humans an ischemic block in the forearm provoked a manifold increase of the amplitude of motor-evoked potentials elicited by transcranial magnetic stimulation (TMS) in muscles proximal to deafferentation site, that returned to baseline within 20 minutes suggesting a temporary increase in motor cortex excitability for those muscles adjacent to the anesthetized ones (Brasil-Neto et al., 1992); partial hand cutaneous anesthesia was reflected in restriction of the motor cortical maps of muscles enveloped in the anesthetized skin, but not of those with normal skin sensation despite a common peripheral nerve innervation (Rossi et al 1998). Studies with magnetoencephalograpy (MEG) also show how somatosensory-evoked fields produced by finger stimulation can be modified by transient ischemic deafferentation with a shifting –strictly confined within the boundaries of hand cortical somatotopy- of the primary somatosensory

cortex (S1) representation of stimulated finger toward the deafferented one (Rossini et al., 1994a). The cortical representation changes not only follow sensorimotor deprivation, but can go in the opposite way as a result of intense use and experiences, as it was shown in a magnetic source imaging study in string players where the S1 representation of digits of the left hand was found to be larger than in controls and the amount of reorganization correlated with the age at which the person begun to play the instrument (Elbert et al., 1995).

3.2 Levels of Nervous System plasticity

BMIs, aimed to control prostheses or other devices, are interfaced with nervous system at different levels beyond the cortical ones (i.e. spinal interfaces) and even with PNS. In order to develop cybernetic prostheses with a high level of specificity and sensitivity, a deep knowledge of post-amputation plastic reorganization characteristics and mechanisms at different Nervous System levels is required.

Plasticity of somatosensory and motor systems following a peripheral nerve injury or an amputation has been extensively studied. For instance, initial reports on S1 reorganization in adult monkeys suggested an "upper limit" for cortical expansion of a body part representation of about 1-2mm along the cortical surface due to the maximum range of the projection area of a single thalamocortical axon (Merzenich et al., 1983). Thanks to a milestone study it is now known that cortical reorganization could involve a larger area within a distance up to 14mm (Pons et al., 1991) after long-term deafferentation and this suggests that changes in S1 are also secondary to other changes at lower -subcortical and spinal- levels. The contributions of brainstem and thalamic reorganization to explain mechanisms of cortical maps reorganization were for a long time underscored (Fig. 3.1). In example, the sensory cortex is at the end of amplification pathways arising from the most peripheral body segments with which we mainly explore the extracorporeal space (i.e. fingertips) where small modifications are magnified by the divergence of neuron projections (Kaas et al., 1999; Jones, 2000). On the other side similar considerations have been done for the alterations at subcortical level that receive the influence of strong efferent connections connecting the sensory cortex to the thalamic and brainstem/spinal relays (Ergenzinger et al., 1998; Kaas, 1999).

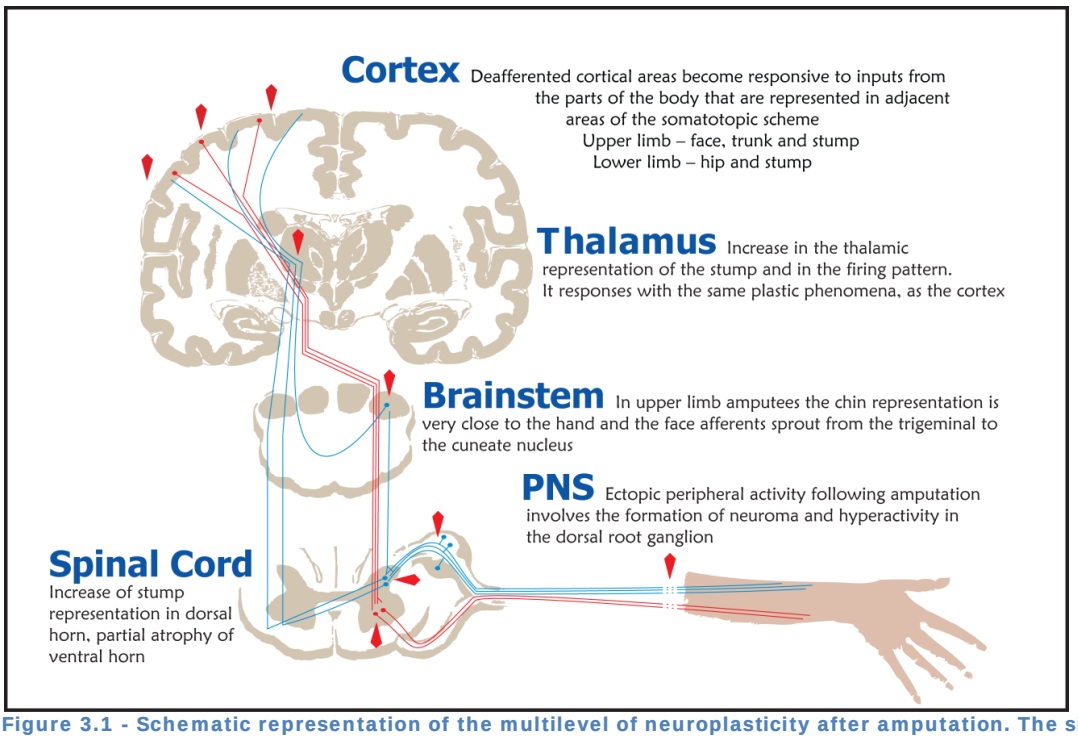

Figure 3.1 - Schematic representation of the multilevel of neuroplasticity after amputation. The sites that are involved are both in the central and peripheral nervous system and the changes regard the motor (red) and the somatosensory (blue) systems. The contribution of multiple sites from different levels of the neuraxis can explain the huge modifications that have been described in the cortical map of the lost limb. From (Di Pino et al .,2009)

3.2.1 Cortex

Cortex is the site where neuroplastic changes after nerves injuries have been most studied, both in somatosensory and motor areas. Cortical representation of the body is organized in well defined topographic motor and sensory maps where -from medial to lateral views- the lower limb, the trunk, the upper limb are represented, then followed by the hand with the fingers and the face (Penfield and Rasmussen, 1950). Strong evidences suggest that in S1 following amputation the deafferented cortical areas become responsive to inputs from the parts of the body that are adjacent each other in the Penfield homunculus. In fact, extensive cortical reorganization following somatosensory deafferentation has been demonstrated with microelectrode recordings in area 3b (deprived portion of the hand and arm representation in S1) of adult monkeys after digits amputation where the representations of palm and adjacent fingers invaded the area of lost fingers (Merzenich et al., 1984). In adult macaques with dorsal rhizotomy after 12 years the cortical reorganization was even wider than previously shown and the area of the cheek took the place of the deafferented upper limb map (Pons et al., 1991). The same kind of somatosensory reorganization has been also reported in others species such as cats, rodents, bats and raccoons (Kaas, 1991).

For what it regards the motor system, limb amputation produces an increase in the size of areas from which stimulation is able to obtain movements of the body parts adjacent to the stump,

trunk, shoulder, arm and face for upper limb, hip, stump and tail for the lower limb, and reduces the threshold to elicit them in rats (Donoghue and Sanes, 1988; Sanes et al., 1990) and in monkeys (Wu and Kaas, 1999).

Also in humans reorganization of cerebral cortex following amputation was extensively investigated. Ramachandran et al described that tactile stimulation of the face and skin around the line of amputation was referred by the patients as sensation in the missing hand or finger (Ramachandran et al., 1992). Shifting of cortical body map in both hemispheres of amputees have been shown with Magnetic Source Imaging following MEG recordings (Elbert et al., 1994; Flor et al., 1995; Knecht et al., 1996) and it is probably due to loss of input from the lost part combined and summed with increased use-dependent input from the contralateral extremity and from the stump (Elbert et al., 1997).

Cohen et al (1991), demonstrated that motor cortex stimulation via TMS in amputees evoke larger motor-evoked potentials and at a lower intensity of stimulation, and that muscles could be activated from a larger area than from the contralateral cortex controlling the intact limb, thus showing the enhancement of excitability in the motor cortex areas representing muscles contiguous to the amputation line. In fact, cortical motor area for the lower face and biceps muscles has been described to enlarge in a patient after traumatic arm amputation, compared with TMS study performed before the injury (Pascual-Leone et al., 1996).

Cortical neuroplasticity after amputation of a limb was also evidenced by cerebral blood flow increment within contralateral sensorimotor cortex, assessed by positron emission tomography (Kew et al., 1994) and the increased activation in contralateral S1\M1 hand area during imagination of lost hand movement compared with controls assessed by functional magnetic resonance imaging (fMRI) (Lotze et al., 2001). Cortical neuroplastic changes are not restricted only to the cortex contralateral to the deafferentation, but result in bilateral reorganization that affect sensorimotor areas of both hemispheres. In flying fox, thumb anesthesia or amputation produces acute enlargement in receptive field of the sensory cortex dedicated to the opposite intact thumb and analogous evidences have been obtained in monkeys (Calford and Tweedale, 1990). In humans, ischemic nerve block in the forearm leads to increase motor cortical excitability of the representation of the unaffected opposite hand, further than of the areas of the arm adjacent to the site of the block, probably due to loss of GABA-ergic (gamma amino butyric acid) transcallosal inhibition of the homologous cortex. The specificity of this effect is testified by the absence of any change in excitability of other motor cortical regions such as those controlling thorax or leg muscles (Werhahn et al., 2002b). Moreover, plastic changes in the upper limb representation opposite to the deafferented one generate rapid increment in the tactile spatial acuity, reinforcing the idea that those changes reflect compensatory modifications for function maintenance and that, as suggested by their rapid time course, are not solely due to use-dependent plasticity from the unaffected arm (Werhahn et al., 2002a).

3.1.2 Thalamus

Strong evidences of expansion of receptive fields of thalamic neurons and nuclei following amputation have been described in rodents, primates and humans.

In human amputees using microelectrodes recording it was established that both thalamic representation of the stump and the firing pattern (Lenz et al., 1998) are increased. Neurons in

the ventro-posterior nucleus (VP) that would normally respond to stimuli from the amputated limb, instead appear to be responsive to touch on the stump (Davis et al., 1998) and -in a similar study- recordings from thalamus and cortical area 3b on two macaque monkeys (that had a long-standing forelimb amputation), VP neurons, once deprived of the sensory activation from the arm, showed clearcut responses to inputs from the stump and from the face. In the same study an equal reorganization pattern has been found in the 'orphan' cortex (Florence et al., 2000). It seems –therefore- that the thalamus exposed to input-deprivation reacts with the same early and long-term plastic phenomena, both topographically and at cellular level, as the cortex.

3.1.3 Spinal Cord and Brainstem

Neonatal forelimb removal in rats results in invasion of cuneate nucleus by sciatic nerve afferents (Lane et al., 1995). In monkeys following therapeutic amputation, subdermal injection of tracer in the stump produces a much more extensive labeled area, both in the dorsal horn of the spinal cord and in the cuneate nucleus of the brainstem, than in controls (Florence and Kaas, 1995; Wu and Kaas, 2002). The same group found evidence that, even though the ventral horn undergoes partial atrophy after long-term amputation, surviving motoneurons emit collaterals which innervate muscles proximal to the amputation site and that this mechanism might account for some of the M1 reorganizations (Wu and Kaas, 2000). At brainstem levels the chin representation is very close to the hand representation and the face afferents sprout from the trigeminal nucleus to the cuneate nucleus (Jain et al., 2000) contributing to the face expansion into area 3b in cortex.

3.1.4 Peripheral Nervous System

Ectopic peripheral activity following amputation, that does not originate from the nerve end, involves the formation of neuroma and hyperactivity in the dorsal root ganglion. Due to the injury at the end of the severed axons terminal, swelling and regenerative sprouting lead to the neuroma formation in the residual limb. The neuroma has an abnormal activity in response to mechanical and chemical stimulation and becomes a source of abnormal afferent sensory bombardment to the spinal cord and a possible trigger for stump pain and phantom limb syndrome (Wall and Gutnick, 1974; Fried et al., 1991). Absence of changes in the "H reflex/Motor potential" ratio elicited in the quadriceps femori of lower limb amputees suggests no modifications in the spinal alfa-motoneurons excitability, even though muscles ipsilateral to the stump show activation from a larger number of scalp positions and with a lower threshold (Fuhr et al., 1992).

3.3. Time-course of cortical changes

The evolution of the cortical reorganization after an amputation seems to go through three different epochs (Jones, 2000; Weiss et al., 2000). 1) a short-term stage of unmasking of already existing neural connections that were functionally silent, due to the lack of inhibitory influences from the ascending pathway previously connected to the missing limb. A recent study showed

short-term modifications in humans cortical receptive fields, with an alternate pattern, by applying a reversible index-to-little fingers artificial syndactily for 5 hrs. An initial decrease in the distance between the representations of D2 and D5 was evident in only 30 min, then followed by a subsequent increase that leads to a plateau in 2 hrs and by a final return to baseline at about 4 hrs of fingers webbing (Stavrinou et al., 2006). In M1 of rats reorganization has been described as early as 7 days following forelimb amputation (Sanes et al., 1990) and in human S1 cortex within a very short time of just 10 days after fingers amputation (Weiss et al., 2000). 2) An intermediate epoch, weeks to months after amputation, in which cortical areas deprived of peripheral input, modify their topographic organization and start to express new and different receptive fields as an effect of sprouting of the cortico-cortical projections. 3) A late, use-dependent, rearrangement of internal topography that produce more stable and sharpened cortical receptive fields, present only in long-survival amputees (Churchill et al., 1998).
The time-course of cortical changes was also demonstrated in monkeys with complete unilateral transection of the dorsal columns at C3/C4 level that, after 6 months of silenced registration from the hand area of the cortex, start to become responsive to stimulation of the face in the deprived cortical zone (Jain et al., 1997).

3.4. Mechanisms of plasticity

The idea of brain plasticity began to receive wide acceptance from the 1940's when the theory of neural plasticity got some popularity thanks to Donald Hebb, who was claiming that "cells that fire together, wire together". He described a theoretical mechanism for synaptic plasticity where an increase in synaptic efficacy arises from the presynaptic cell's repeated and persistent stimulation of the postsynaptic cell. Such a learning mechanism is now known as "Hebbian learning"(Hebb, 1949).
From a theoretical perspective in the following decades others supported the theory of the brain plasticity amongst whom Jacques Paillard. In his work (Paillard J 1976) recently republished in English (Will et al., 2008) he sustained that "The term plasticity is only appropriate in terms of the ability of a system to achieve novel functions, either by transforming its internal connectivity or by changing the elements of which it is made". This means that only those changes that are both structural and functional in nature can be defined 'plastic'. Structural modifications concern the constitutive elements of the system; for the central nervous system they are represented by neurons or by networks able to connect each other to form neural assemblies.
Later on, a wide range of neurobiological mechanisms have been discovered in line with the Hebb and Paillard's theoretical hypothesis, including cellular and anatomical phenomena that reflect synaptic efficacy and synaptic redundancy (i.e. synapses structurally existing, but functionally silent): synaptogenesis, dendritic arborization, and activity-dependent reinforcement of previous existing, but functionally silent synaptic connections (Calabresi et al., 2003).
Particurarly Hebb's theory received a new impetus by the fundamental description of the Long Term Potentiation/Depression mechanisms (Bliss and Lomo, 1973; Wigstrom and Gustafsson,

1986) and is now commonly evoked to explain some types of associative learning in which simultaneous activation of cells leads to pronounced increases in synaptic strength. Subsequent studies from Eric Kandel's lab - for a review see (Kandel, 2001; Bailey and Kandel, 2008)- provided evidence for the involvement of Hebbian learning mechanisms at synapses. However, much of the work on long-lasting synaptic changes between vertebrate neurons (such as long-term potentiation) involves the use of non-physiological experimental stimulation of brain cells, profoundly different from everyday experience in the human world.

Learning new skills as well as endogenous brain function repair following a lesion are based on neural plasticity (Elbert et al., 1995; Pascual-Leone et al., 1995; Rossi et al., 1998b; Rossini et al., 2003; Ween, 2008). External stimuli able to induce cerebral plasticity can be physiological or pathological in nature and the nervous system looks like a continuously changing structure of which plasticity is an inner property and the necessary result of each internal and external brain communication (Pascual-Leone et al., 2005).

However, besides the existence of a long-term, experience-dependent, compensatory neural plasticity in accordance with Hebbian rules, many studies witnessed that after the "perturbation of the system" also a short-term plasticity response arises.

In several original works short-term plastic effects following changes of the sensory input on cortical sensorimotor organization have been documented by our group (Rossini et al., 1994a; Kristeva-Feige et al., 1996; Rossini et al., 1996; Rossi et al., 1998a).

Insights into the functional and structural plasticity of the primary somatosensory cortex also come from studies on phantom limb; the amputation or deafferentation of a limb or another body part is usually followed by a global feeling that the missing limb is still present and site of specific sensory or painful sensations. Referring sensations in the phantom stimulating body areas adjacent to but also far from the amputated limb (Ramachandran and Hirstein, 1998) is a well known correlate of reorganizational processes in the S1 cortex also termed "topographical remapping" (Ramachandran, 1993) see also (Cronholm, 1951).

The reorganization of the CNS after amputation involves two main mechanisms operating at different times after the lesion; early changes involve mainly unmasking of anatomically present but functionally inactive connections while late changes are secondary to new connections formation. The loss of inhibition produces an increase in the size of receptive field in the cortical map of the territories close to the cortical representation of the lost part (Calford and Tweedale, 1988; Chen et al., 1998). Unmasking of connections could be the effect of changes in neurotransmitters, receptors or membrane conductance properties that lead to an increase of cortical excitation (Kaas, 1991), but most of all is due to a reduction of GABA fast inhibition of excitatory synapses. GABAergic neurons constitute about a quarter of the total amount of neural population and GABA major receptor class is regulated in an activity-dependent manner even in the adult (Jones, 1993). Reduction of GABA containing neurons and its synthesizing enzyme has been reported in somatosensory cortex after peripheral deafferentation (Welker et al., 1989). In the longer time period other mechanisms start to play their role, such as long-term potentiation, with its increase in N-methyl-D-aspartate (NMDA) receptor activation, and long-term depression (Buonomano and Merzenich, 1998). NMDA receptor blockade reduces cortical reorganization following peripheral deafferentation in cat (Kano et al., 1991) and monkey (Garraghty and Muja, 1996) sustaining the idea that glutamatergic activity is necessary for cortical reorganization. It is likely that all these

mechanisms -such as reduction in GABA inhibition and NMDA receptors activation- are not confined to the cortex, but also operate at subcortical levels (Jones, 2000). Long-term changes reflect also axonal regeneration and sprouting with modification in synapse architecture (Kaas, 1991), especially in the more distal regions of dendritic arborization in S1, as an attempt to maintain the normal level of signal-to-noise ratio and as an expression of homeostatic response to the input deprivation (Churchill et al., 2004).

3.5. Influence of age on neuroplasticity

The age of the patient at the time of amputation is one of the main factors in the determination and in the functional significance of neuroplastic changes. Monkeys that had a median nerve transection with a surgical repair in early ages maintain the ability to develop a correct hand representation in cortex showing a better recovery than the adult where an abnormal somatotopy in S1 is observed (Florence et al., 1996). In the motor system of forelimb amputated rats, cortical reorganization is more evident in neonatal than adult rodents (Donoghue and Sanes, 1988; Sanes et al., 1990). Concerning the cortical reorganization of congenital compared to traumatic amputation in humans there are conflicting data. Hall et al. in a study with TMS report increased excitability in congenital and early amputees (Hall et al., 1990), while Cohen et al. described sensation of movement in the missing hand following scalp magnetic stimulation in 7 patients with acquired, but not in congenital amputation (Cohen et al., 1991). A more recent work shows increased corticospinal excitability and cerebral blood flow both in deafferented M1 and S1 in traumatic, but not in congenital amputees (Kew et al., 1994). Magnetic source imaging revealed minimal reorganization and absence of phantom limb phenomena in congenital amputees (Flor et al., 1998). The age of amputation influences also the site of reorganization along the nervous system, thus forelimb removal in adult rats mainly leads to change in cortex, whereas at younger age leads mainly to reorganization in the neuraxis (Bowlus et al., 2003).

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Chapter 4

Robotic hand control via bidirectional multicontact, intraneural interface on a human amputee with double nerve implant and 4-weeks training

4.1. Persistence of neural pathways

Cortical reorganization that follows limb amputation is accompanied by an invasion of lost limb representation cortical areas from adjacent territories. In spite of such silencing, several recent evidences show how the deafferented hand-controlling cortex remains still responsive. Intraneural microstimulation of nerve fascicles, proximal to the nerve transection, in human amputees evoked sensations not different from those reported by subject with intact nerves (Moore and Schady, 2000) and somatosensory-evoked potentials studies, stimulating truncated nerves, demonstrated the presence of primary cortical component, showing persistence of thalamocortical input to S1 in long-term deafferented cortex of arm amputees (Mackert et al., 2003). The loss of limb cortical representation does not seem to correspond to a loss of perceptual representation, that is maintained in a form of phantom limb with its own sensory-motor properties. Similarly to the somatosensory system also in motor cortex, despite a strong post-amputation plasticity, the representation of amputated arm still survives, even if sometimes it cannot be directly demonstrated. TMS of the hand motor cortex elicits the sensation of movement of the phantom hand, with an amplitude of the movement positively correlated with the intensity of the stimuli and TMS is able to produce movements of the phantom even in subjects that cannot produce them voluntarily (Mercier et al., 2006). Voluntary movements of phantom hand trigger specific patterns of EMG activity in stump muscles, meaning that cortical neurons that previously targeted spinal motoneurons for the missing muscles became differentially activated for different movements of the phantom limb (Reilly et al., 2006). A discrete number of survived fibers and the redundancy of connections, with multiple parallel pathways, justify the retaining of at least some "memories" of sensory and motor functions of the lost limb.
In agreement with these findings Dhillon et al were recently able to record voluntary motor nerve activity and to elicit both exteroceptive and proprioceptive discrete unitary sensations stimulating with an electrode implanted in stump nerves –slightly proximal to the stump level- in long term amputees; the efficacy of those connections appeared to be improved by the training (Dhillon et al., 2004; Dhillon et al., 2005).

4.2. Introduction

The fact that amputation does not eradicate relays and connections in these areas allows them to be utilised by implanting a bidirectional intraneural electrode in the stump nerves to control artificial prostheses (Di Pino et al., 2009). Peripheral nerve interfaces aim to detect electrical activity of the nerve fibres and/or to excite them as selectively as possible(Navarro et al., 2005; Stieglitz et al., 2005; Tesfayesus and Durand, 2007; Micera et al., 2008). Intraneural electrodes have higher selectivity and better signal-to-noise ratio than extraneural ones due to a more intimate contact with afferent and efferent fascicles(Yoshida and Horch, 1993; Lawrence et al., 2004). Given the same fibre-contact distance, large myelinated fibres are picked up more effectively than small myelinated and unmyelinated fibres. Therefore, tactile or position sensations can be selectively and focally elicited without concomitant pain, while motor signals to extrafusal fibres are recorded much more easily than those to intrafusal and vegetative ones. If recording sites within the nerve are sufficiently dense, the probability of recording signals from the fascicles originally innervating the missing limb and conveying the relevant information on the desired movement is therefore high(Navarro et al., 2005).
Electroneurographic (ENG) signals from peripheral nerves have been recorded for periods of months in animals with implanted longitudinal intrafascicular electrodes (LIFEs) (Lago et al., 2007). Pattern recognition algorithms allowed differentiation of 4 to 5 units with up to 98% reliability and with a channel capacity ranging between 1.5 and 2 bit.
Novel thin film LIFE (tf-LIFE4) have been developed, assuring biocompatibility and flexibility for multiple-site recordings (Hoffmann and Kock, 2005; Citi et al., 2008).
The LifeHand experience is the first implant of tf-LIFE4s carried out on a human volunteer to test reliability and compatibility of these electrodes, for the 4 week period allowed by the European Health Authorities, to deliver sensory feed-back as a surrogate of action-driven perception, to record ENG signals from motor fibres during motor imagination of 3 distinct movements and rest, to implement classifiers with which to correctly interpret commands and to govern a robotic hand and finally to investigate the resulting brain plasticity.
I participated in all the aspects of the experimentation, while my primary direct contributes regarded mainly the definition of a specific clinical protocol taking into account all the needs coming from physicians and bioengineers, the definition of the stimulating parameters, the settings and integration of the Stimulating/Recording system its wiring and shielding, and the evaluation of Neuroplastic changes and their functional analysis.
This chapter offers a wide presentation of the whole experiment and emphasizes the above aspects. The activities to correlate performance and training-related changes with topographical reorganization of sensorimotor brain areas and with the eventual PLS modifications are described in the next paragraph.

4.3. Methods

4.3.1 Subject

A 26 year old right-handed male (P.P.) with left arm trans-radial amputation due to a car accident in 2007 (Fig 4.1), who had previously tried aesthetic and myoelectric prostheses, was selected. Previous medical history was silent. Full neurological and neurographic/electromyographic exams were normal. Neuropsychological and neuropsychiatric tests (MMPI-2, WAIS) demonstrated normal comprehension and intellectual capacity, and excluded personality disorders.
The study was approved by the local Ethics Committee and an informed consent was signed by P.P. in the presence of a witness from his family.

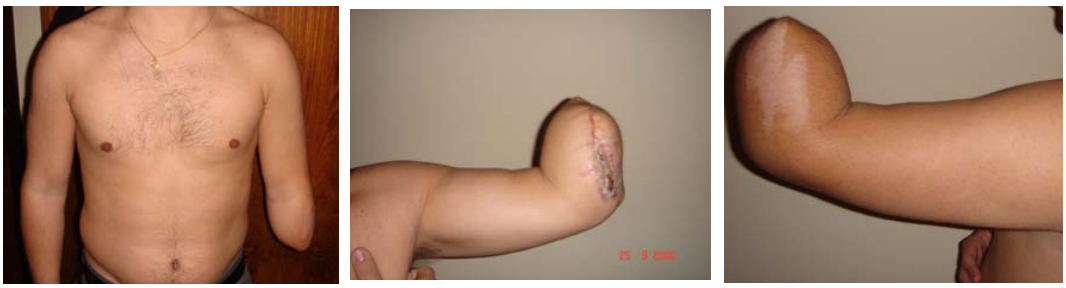

Figure 4.1 - Different view of the transradial amputation of left upper limb of subject that took part in the experiment after the car crash.

4.3.2 Surgery

After general anaesthesia, skin was incised along the medial edge of the biceps muscle for 10 cm to expose ulnar and median nerves in the distal upper arm; following epineural microdissection, two tf-LIFE4s (Hoffmann and Kock, 2005) (Fig. 4.2 and more in depth Fig. 2.2 of chapter 2) separated by 3 cm, were inserted in each nerve under surgical microscope (Opmi Vario/NC33, Zeiss) (Fig. 4.3A). A tungsten needle allowed electrode filament introduction into the nerve fascicle (Fig 4.2 and 4.3B).

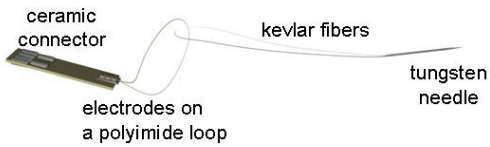

Figure 4.2 - tf-LIFE4 System with needle, polyimide fibre, electrode filament and ceramic connector.

tf-LIFE4s were introduced 45° obliquely to assure stability and to increase the probability of intercepting nerve fibres. The distal handle of the electrode was anchored to the epineurium by an 8.0 nylon suture (Fig. 4.3C). Four separate holes lateral to the incision allowed transit of the tf-LIFE4 cables (Fig. 4.3D). Four weeks later, tf-LIFE4s were removed.

P.P. worked on the project 4-6 hours/day for 6 days/weekly and did not report any complication during the 3 month follow-up period.

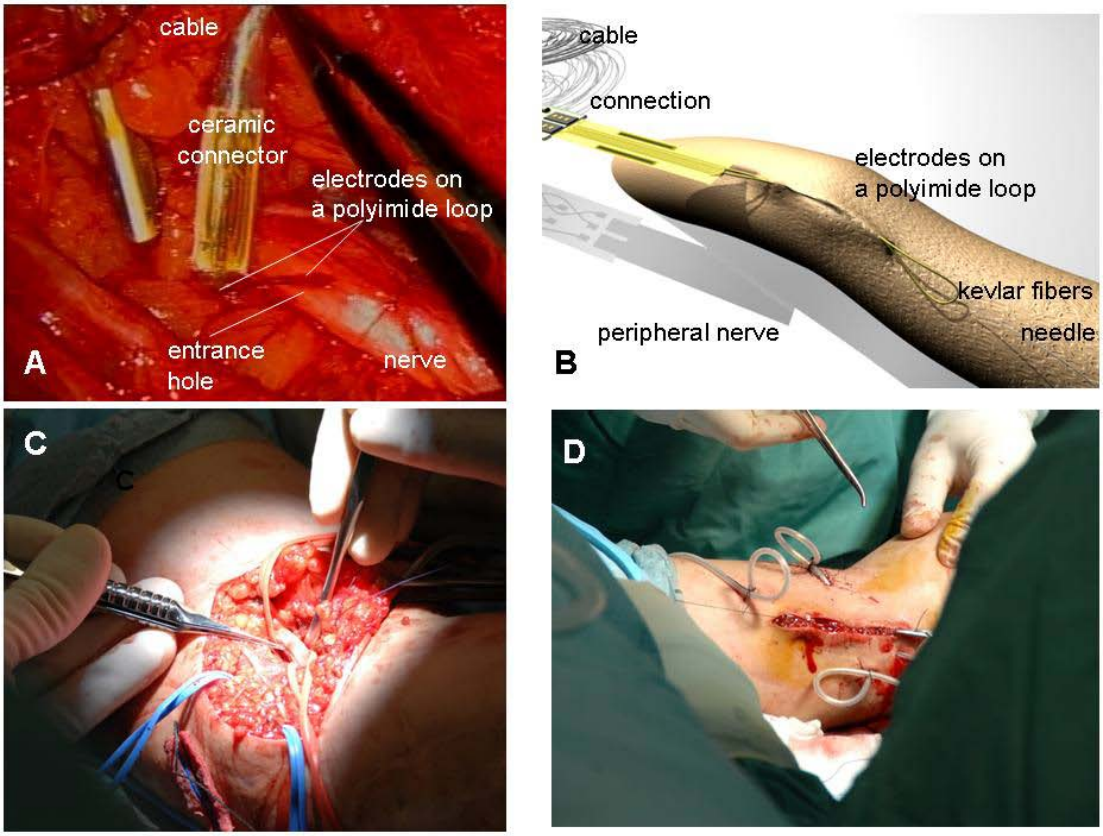

Figure 4.3 - A: intraoperative microphotographic view and B: descriptive illustration of the insertion of tf-LIFE4 in the median nerve. C: general view of the exposed median nerve and tf-LIFE4 cable; D: tf-LIFE4s skin transit cables. From (Rossini et al.,2010 In Press)

4.3.3 Prosthesis

A stand-alone version of the CyberHand prototype (Fig. 4.4), which approximates dimensions and grasping capabilities of the human hand with five underactuated fingers actuated by six motors (5 for the independent flexion/extension of the fingers, 1 for the opposition of the thumb), was employed (Carrozza et al., 2006). It was endowed with 6 position sensors and 5 tensiometers able to measure tension of the cables controlling finger flexion, similar to Golgi tendon organs. However, validation of real time artificial-sensor feed-back was not part of the present experiment; sensory stimulation and feed-back were delivered by the experimenters and not by the robotic hand sensors.

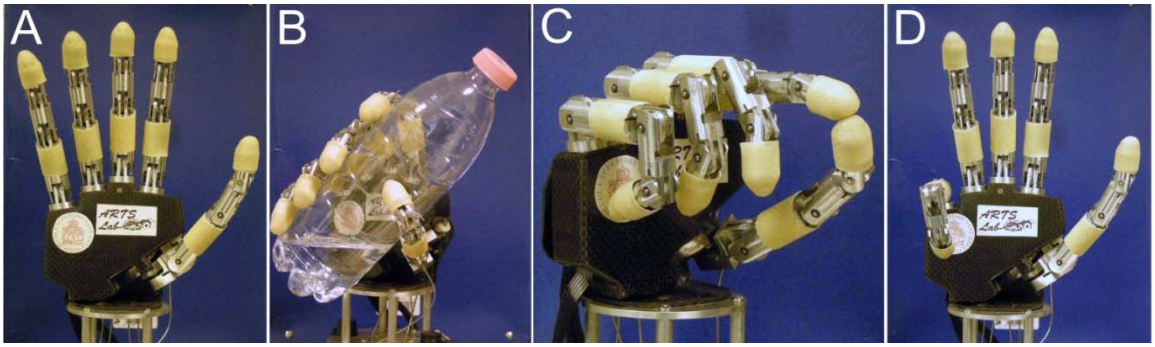

Figure 4.4 - Robotic hand experimental movements (A-Relax, B-Power grip, C-Pinch grip, D-Flexion of little finger)

4.3.4 Stimulation/recording system

The Recording and Stimulation System (Fig 4.5) is devoted to the amplification, filtering, acquisition, and the storage of the neural (ENG) and myoelectrical (EMG) signals, as well as to the electrical modulated stimulation of the neural fibres in contact with the tf-LIFEs.

Four integrated four channels amplifiers (Grass QP511 Quad AC Amplifier System for Evoked Potentials, EEG, EMG) simultaneously amplified ENG signals (amplified with a factor of 10.000, and band-pass filtered between 100-10k Hz), and EMG signals (amplified with a factor of 5.000 and band-pass filtered between 30-3k Hz).

A 16 channels, 16 bit, 1 Ms/s analogue-to-digital converter (ADC), rack mounted and connected to a rack mounted PC, was used.

A two channels stimulator (Grass S88X Dual Output Square Pulse Stimulator) was programmed for releasing trains of rectangular biphasic cathodic current pulses. Trains duration ranged between 300msec and 500msec, releasing between 3 to 250 pulses per train (with pulse frequency ranging between 10Hz to 500Hz). Pulse features as current intensity (10-100 microAmpere) and duration (20-300 microseconds) were set in accordance with tf-LIFEs intrinsic limitations on charge density.

The whole system is powered by a isolation transformer for medical application added to the system for safety reasons.

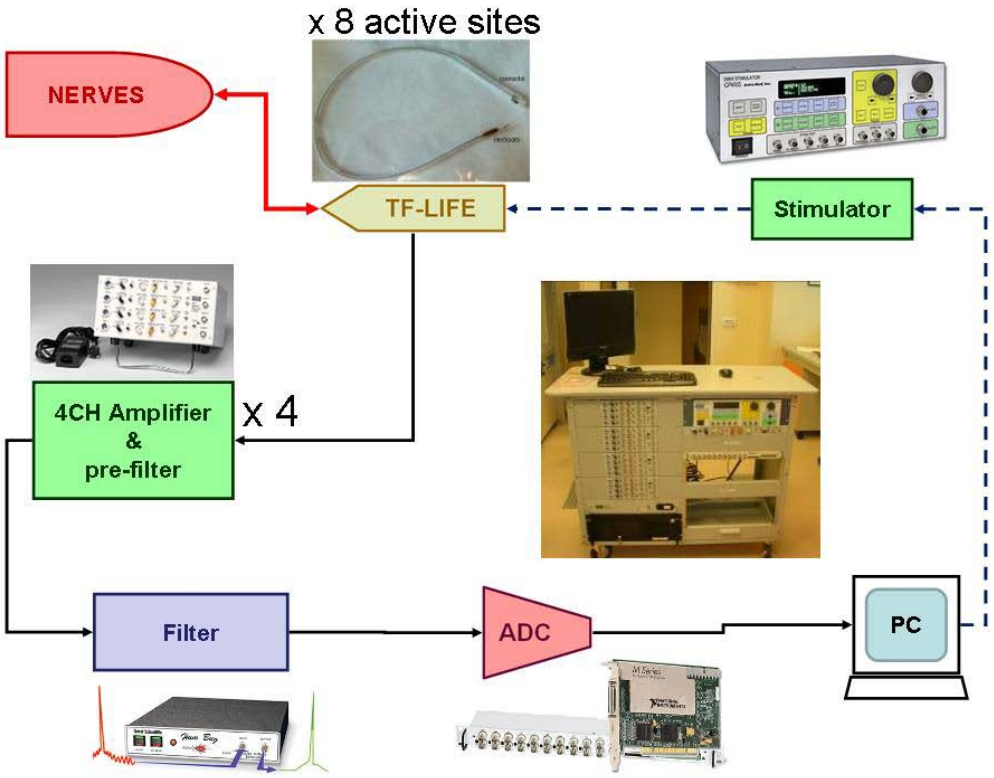

Figure 4.5 - Block scheme of the stimulation/recording system

tf-LIFEs implant on human is an absolutely innovative experiment, thus no commercially available devices specifically developed for such an application have been found. This implied a series of arrangements in the acquired instrumentation, as regard cables and adapter, grounding referencing and shielding the signals that required the joint work of medical doctors, bioengineers and the technical staff of the product seller.

The instrumentation uses proprietary cables and connectors and the producer provided technical support for adapting them according to the application needed. In particular, in order to limit the obstruction of the wires - more than 30 - to the patient and for safety reasons, a connector box has been identified as the optimal solution. It allows to group all the single-pole wires into a bigger multi-pole cable that could be simply and quickly plugged/unplugged without particular care. This box was connected to the electrodes through M/F Safelead connectors. So, depending on the particular interconnection cable, Safelead connectors have been directly mounted on it or adapted to standard 2mm banana connectors.

Moreover each tf-LIFE is axial-symmetric and each one of the two sides presents one reference pad and one ground pad. Since the connector box had only one input plug for the ground (GND) and reference (REF) and signal input plugs are coupled for each channel, an adaptation stage was needed to correctly connect the instrumentation to the electrodes. Given that 4 electrodes have been simultaneously 8 ground pads on the electrodes were connected to a single GND plug on the connector box and the same reference pad on the electrode should be connected

to the four REF plugs on the connector box related to it. These issues have been solved by assembling a set of purpose-made adapting cables (Fig 4.6) allowing that ground was unique for the whole instrumentation, while the references were kept unique for each amplifier. Reference grouping was chosen because of the characteristics of tf-LIFEs, fabricated for working with a single reference for each four channels.

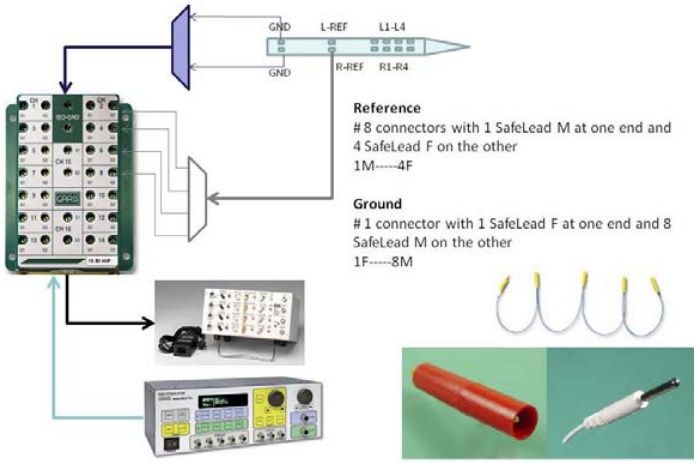

Figure 4.6 – Custom made wires adaptor for grounding and referencing

4.3.5 Motor output recordings

The protocol included the following phases: 1) Pre-implant training with a virtual hand for standardizing movement imagination; 2) Post-implant training to control output of tf-LIFE4s during motor imagination; 3) On-line prosthesis control designed to train the subject to control and standardise tf-LIFE4 output induced by movement imagination; 4) Off-line development of a classifier-algorithm for optimal prosthesis control.

During phase 1), P.P. practiced imagining three individual movements with the missing hand as shown in dedicated videos: (i) power grip; (ii) pinch grip; (iii) flexion of the little finger. These three actions were considered representative of the variety of movements controlled by the nerves under investigation: mostly median fibres for the pinch, ulnar for little finger flexion, and both for the power grip.

Following tf-LIFE4s implant, phase 2) began, in which the same videos were used to trigger P.P. motor intents while recording neural signals. Videos showed alternating open-relaxed hand movements and were synchronised with the recording system. Signals from tf-LIFE4s, biceps and triceps EMG electrodes were simultaneously recorded using a 48 kHz sampling rate, and were data-windowed in 1000 samples for mean rectified value calculation.

In phase 3), ENG channels with the best signal-to-noise ratio were selected while analyzing the recordings from the previous phase. The online activities of the best channels, together with EMG activity, were shown to the subject, who was asked to modulate them while keeping the EMG silent. Of the procedures tested, this one resulted in the most effective training (Table 4.1).

Feedback Protocols		**Outcome**
Acoustic	Audio from amplifiers	Rapid mental fatigue
	P.P. with eyes closed and verbal report of outcome	Excessive interference in focusing attention
Tactile	tf-LIFE4 electrical nerve stimulation (but on an electrode site different from the recording one)	After 10 days stimulation decayed
Visual	Virtual hand on video screen performing the 'prime' movement	No significant improvement
	Monitoring tf-LIFE4 and EMG signals associated with movement imagination	Best training procedure in obtaining a silent EMG and a reliably modulated LIFE output.
	Monitoring *cyberhand* movement	Optimal for maintaining performance after training

Table 4.1 - During the study, different feedback protocols were compared to determine the best protocol for reinforcing and maintaining subject's control, as summarised in the table.

Once a stable level of training was achieved, LIFE signals were translated into robotic hand actions and the subject had direct visual feed-back on the correct execution of the intended movement. Each movement type was triggered by the signal level of a proper single channel. In order to exclude activities caused by unwanted muscle contraction or environmental noise, only rectified values greater than 3÷8 μV in a time window ranging from 5 to 20 ms were used. Channels were chosen depending on their signal-to-noise ratio and anatomo-functional location (i.e., channels from the median nerve for power or pinch grip, channels from the ulnar for little finger flexion).
For phase 4), off-line examination of the original ENG signals and their processing was carried out to optimise the prosthesis control in order to avoid 'false' positive (unwanted movement) and negative classifications (no movement performed despite the intention). For efferent signals processing, an approach developed by the ARTS Lab of Scuola Superiore Sant'Anna has been used. It consists of extracting selected features as input to an artificial neural network, wavelet denoising and spike-sorting using a template creation and matching approach (Citi et al., 2008). Support vector machines were trained to use waveforms of the identified spikes to infer the type of imagined movement. Analysis was applied to a progressively higher number of active sites in order to test whether correct classification improves.
Whenever one type of action was classified, the robotic hand began and completed a movement after a time lag appropriate to a natural condition.

4.3.6 Sensory stimulation

To identify afferent fibres eliciting sensations, full mapping of all 32 contacts within the tested nerves was carried out. Rectangular cathodal pulses of 10-300 μs duration and 10-100 μA current intensity were employed. To avoid electrode damage, all stimulation trials were below 75% of the maximum charge (~4 nC), in the form of 300-500ms 10-500Hz pulse trains. The best active sites for sensation were characterised, beginning with short and low-current stimuli (10

µA, 10 µs) which were progressively increased in order to elicit different sensations; either the electrode's safety limits or subjective discomfort determined maximal stimulus intensities. A psychometric staircase method was used to quantify sensation, ranging from the minimal perceived threshold (score=1) to discomfort (score=5).
Stimulation was also tested the 7th and the 8th day after implant as feedback during a control motor task, in which P.P. was asked to produce a power grip every 5 seconds. In a set of trials, an operator triggered a stimulus train (0.3s train of 70Hz, 10 µ A, 10 µs pulses) after each burst of efferent activity recorded by tf-LIFE4s; success rates with or without sensory feed-back were then measured.

4.4. Results

tf-LIFE4s recorded a progressive improvement of signal-to-noise ratio which stabilised within about 10 days following surgery. All the contacts in all electrodes recorded properly during the entire 4 week experimental period. Contacts belonging to one electrode stopped working on the last day; the electrode was found dislodged from the nerve during LIFEs removal.
Three of the four electrodes appropriately stimulated for 10 days.
Off-line signal processing using the algorithms previously described allowed up to 85% of grip type correctly identified in the phase of mental activity immediately following the resting period.

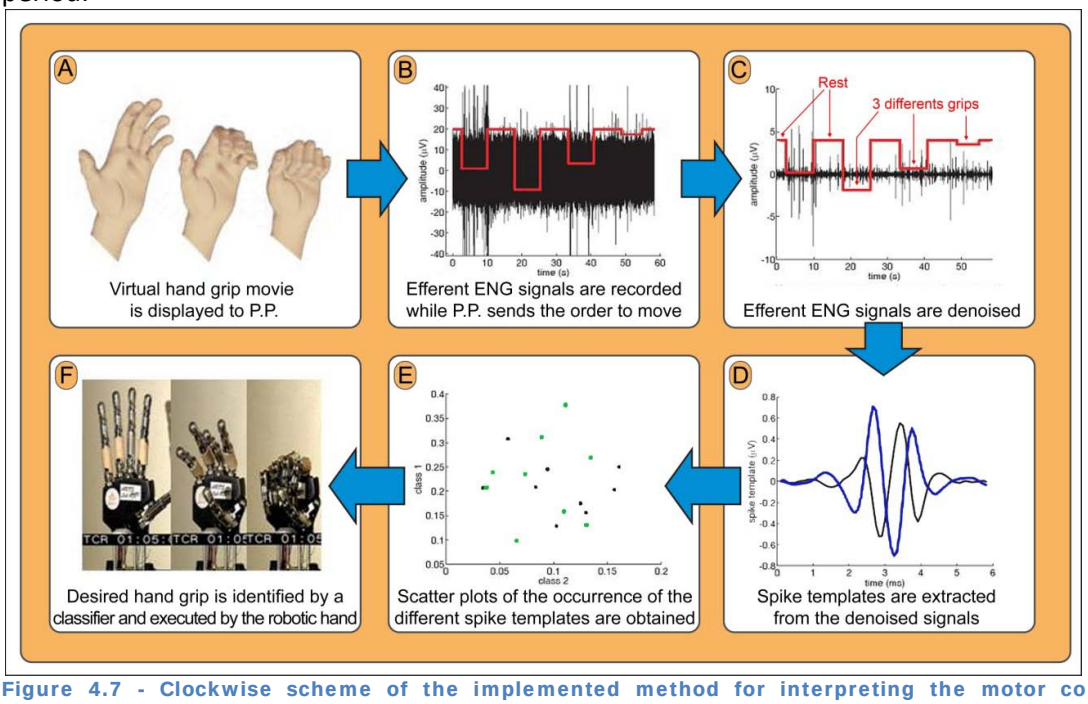

Figure 4.7 - Clockwise scheme of the implemented method for interpreting the motor command embedded in tf-LIFE4s efferent signals: A- video of virtual hand grasping; B- recording and pre-processing; C- denoising – In B and C black lines are the ENG recorded signals from which are extracted spike templates, while red lines represent the movements triggers presented in the movie as a function of time; D- features extraction; E- selection of the motor command using a classifier; F- control of the prosthesis. From (Rossini et al., 2010 In Press)

Evaluation of signals extracted from several contacts of different electrodes from either nerve improved discrimination performance when compared to information obtained from only one contact. Moreover correct classifications improved with time (75% on day 26 to >85% on day 28), indicating a learning effect. Optimal control can be achieved by combining information extracted from grip-related neural signals, together with fixed parameters embedded in the robotic controller and selected by a trial-and-error procedure. The level of "shared control" between the user's brain and the robotic controller can be modified according to the performance of the prosthesis (Cipriani et al., 2008).
Discrete tactile sensations were elicited from different stimulating sites of three electrodes (i.e., from 4 sites of EL1 and EL 2 in the median nerve and from 5 sites of EL3 in the ulnar nerve). In all cases, sensations were referred in the fascicular projection territories of the corresponding nerves. As an example, Figure 4.8A reports sensations related to median nerve stimulation through L1 and L2 sites of EL1 electrode: touch and tingling were referred by the subject both in the middle of the palm and near the base of the index and middle fingers (striped areas). Figure 4.8B reports sensations related to ulnar nerve stimulation through R1 site of EL3 electrode: touch sensation on the wrist was referred (black circle) that irradiated (black lines) towards 4th and 5th fingers. Gray shaded areas indicate the nerves' fascicular projection territories.
In agreement with the results of previous studies (Dhillon et al., 2004), stimulus frequency concurs to modulate the sensation intensity on a log scale (see Fig 4.8C).

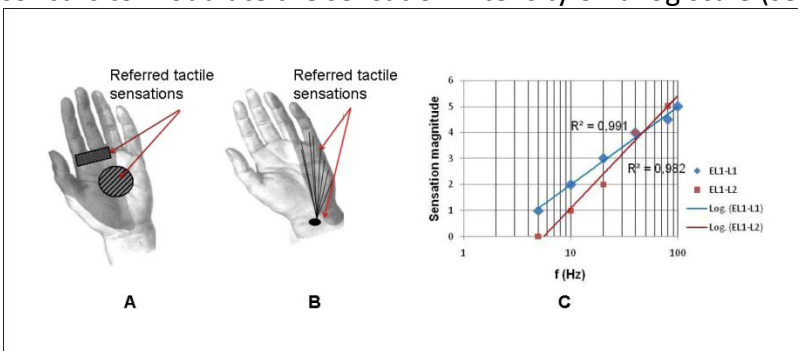

Figure 4.8 - A: Median nerve tf-LIFE4 stimulation; B: Ulnar nerve tf-LIFE4 stimulation; C: Estimate of sensation magnitude (1=threshold, 5= discomfort) vs. frequency for two channels of the electrode 1 in the median nerve (trendline and R2 are shown). From (Rossini et al., 2010 In Press).

When stimulation was added as sensory feedback for the motor control task, the success rate increased significantly and rapidly (Fig 4.9).

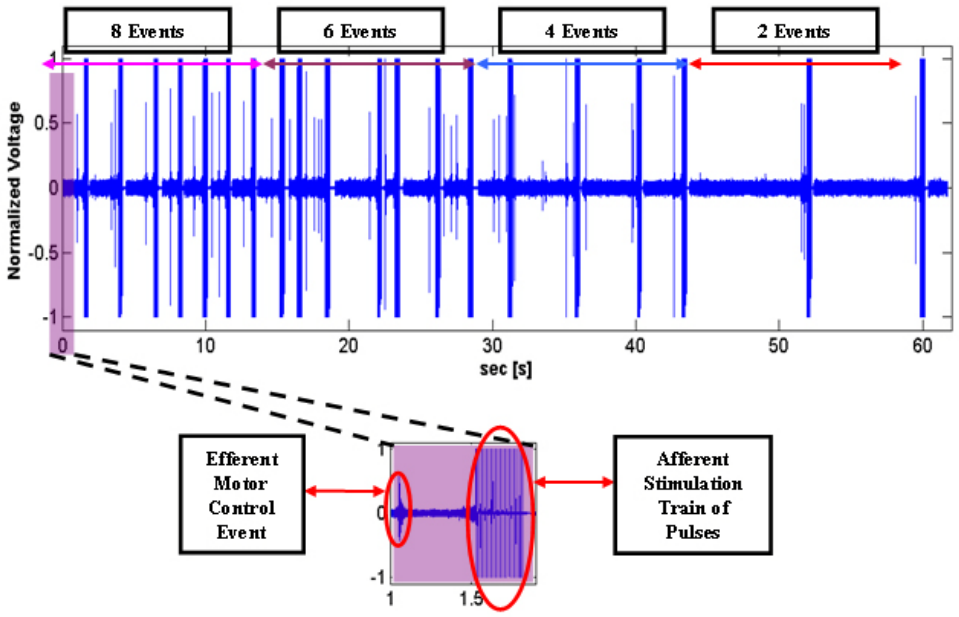

Figure 4.9 - Efferent / Afferent signals during a one minute trial, while stimulation through tf-LIFE4s was used as feedback. Each efferent movement control event was fed-back using a 0.3s train of 70Hz, 10µA, 10µs pulses. P.P. improved his control after less than one minute of movement-related feed-back. From (Rossini et al., 2010 In Press).

The electrical charge necessary to elicit sensations (minimal threshold) increased during the first ten days from 0.1 to 1 nC, until no sensation was elicited through any of the three electrodes despite delivery of the maximum allowed charge (~ 4 nC). In order to avoid Pt electrochemical irreversible processes with possible contamination of motor signal recordings, stimulation procedures were halted. Several, not mutually exclusive, explanations for this failure can be proposed: a) progressive 'habituation' of P.P., moving from an initial 'hypersensitivity' due to long-lasting sensory deprivation (subjective sensory threshold below maximum of stimulation) which then decreased and stabilised at a more physiological level (subjective sensory threshold above maximum stimulation); b) surface of the miniaturised contacts limiting the maximum applicable current charge, i.e., ~ 4 nC, well below parameters reported in (Dhillon et al., 2004); c) fibrotic tissue reaction which, however, was only suspected on visual inspection during LIFE removal but was not histologically demonstrated due to ethical restrictions.

4.5. LifeHand system overview

In the tab. 4.2 an overview of the LifeHand system divided in four different sections concerning the electrodes, the prosthesis, the stimulation/recording system and the clinical protocol is presented. For each component all the phases, from requirements to validation, are summarized.

	Requirements	Specifics	Design	Fabrication	Tests	Validation
Electrodes	Biocompatibility	Atoxic materials	Platinum/Polyimide electrode	Pt lift-off process on polyimide substrate	Test in small animals for 90 days(Lago et al., 2007) Test in pigs at Aalborg University, DK	The patient showed no adverse reaction or nerve damages reported 3-months after LifeHand implant
	Low tissue-device mechanical mismatch	Flexible Polymeric substrate	Thin Polyimide based structures	Two layer of polyimide (thickness: 5 µm) spinned on Silicon substrate Structure release by Reactive Ion Etching		
	Easiness of insertion	Needle as guidance to implant the thin film electrode longitudinally into the nerve.	Loop of a thin film electrode with a filament loop including a thin needle	Polyimide loop with tungsten needle	Surgical training on pigs at Aalborg University	One electrode broken during the insertion versus four correctly implanted in LifeHand implant
	Selectivity of stimulation	Multielectrode array	8 active ch. (4 on each side) of rectangular shape of 100 µm x 40 µm with a semi circle attached on each side of 40 µm diameter.	300 nm thick Pt sputtered on polyimide substrate and patterned with lift-off technique	Test in small animals	Discrete tactile reproducible sensation elicited for 10 days
	Appropriateness to Neural signal	Low impedance electrodes	Low impedance interconnection lines	300 nm thick Pt sandwiched between two Polyimide layers	Electrical characterization Impedance < 10 KOhm @1KHz	Signal recorded in the neural frequency band

	Requirements	Specifics	Design	Fabrication	Tests	Validation
Prosthesis	Dexterity	5 independent fingers	16 DoF, 6DoM (5 for fingers flex/ext and 1 for thumb abduction)	DC motors, Tendon-like transmission	Max grip force: 40N; Max tip to tip force 15N Faster closing time 3sec	Control of 3 different complex grips: fist, pinch, little finger flexion and rest
		Multi grip allowed			Cylindrical, spherical, lateral, bi and tridigital grips	
		Internal and user control	motor built-in position sensors, force sensors ad touch sensors in fingertips		Internal control of force, position and velocity of grip	Close loop with user not already validated
	Anthropomorphism	Size and shape of the human hand Aesthetic glove	Hand Weight 450gr; Hand + forearm weight 2,5 kgr	Aluminum and Carbon fiber		

	Requirements	Specifics	Design	Fabrication	Tests	Validation
Stimulation Recording System	Recording neural signals of few µV	Amplified factor 10.ooo-200.ooo	Commercial devices Grass Tech. No. 4 - ***QP511:*** 4-channel amplifier No. 1 - ***P511: Pre***amplifier No. 1 – ***AM10:*** Audio monitor		Recorded artificial test input similar to biological signal	Recorded enough signals to online control the prosthesis and to train the classifier
	High signal to noise ratio	Input noise 4 µV peak to peak				
	Multi-channels Recording	16 ch. parallel recording + 1 on audio			Cross-talking among channels test	Signal recorded in the neural frequency band
	Safely stimulation	In human use allowed	Commercial device Grass Tech. No. 1 - ***S88X:*** Dual channel square pulse stimulator		Test of maximal injected current in very low impedance condition	No nerve damages reported 3-months after LifeHand implant
		Function in constant current mode				
		Power supply by isolation transformer	Commercial device Grass Tech. No. 1 – ***IPS230:*** Isolated medical-grade power system			
	Fast switching among stimulation /acquisition ch.		Commercial device Grass Tech. No. 1 – ***SIU-BI:*** Stimulation isolation unit			achieved
	Isolation from environmental EM and conducted noise	Cables and connection shielding	Each stim/rec ch entirely and individually shielded			Still suffering for environmental EM noise
		Metal parts grounding	Custom made multi-grounding connection box			
		Hum bug - Noise eliminator	Commercial device Notch Filter (50-60Hz)			No 50Hz noise recorded
	Transportability	Multislide rack with trolley	Commercial device Grass Tech. No. 1 – ***iT3:*** Mobile rack cart			Achieved but the system need implantable stimulator
	Stim/rec controlled through custom made software	Integrated development environment (IDE) standard	Commercial device National Instrument. No. 1 –NI PCI-6251			achieved
	Compatible with surgery room	Power supply by isolation transformer	Commercial device Grass Tech. No. 1 – ***IPS230:*** Isolated medical-grade power system			Intrasurgical stimulation allowed
		Sterilization and encapsulation of wires				

	Requirements	Specifics	Design	Fabrication	Tests	Validation
Clinical Protocol	To elicit proprioceptive sensations	From SoA current in the range 10-100 µA Pulse width 10-250 µS Train 0.2-1 s	Catodic stimulation Square Pulse		Modulation of width, duration and frequency relation Progressive increase since pain sensation	Discrete tactile reproducible sensation elicited for 10 days
	Good settlement of electrodes	Longitudinal introduction			Intrasurgical stimulation	One of four electrodes was found displaced when explanted
	Evaluation of neuroplasticity	Changes before and after the	EEG, fMRI, TMS			Changes in severed limb proximal

		training				muscle TMS threshold
	Evaluation of changes in PLP	Changes before, after the training and 3 months follow-up	Questionnaire with sfMcGill, VAS, PPI and open section with PL awareness			Improvement in PLP and PLA lost after 3 months follow-up

Table 4.2 - Requirements / Specifics / Design / Fabrication / Validation Analysis

4.6. Discussion

It is worth noting that tf-LIFE4s 4-weeks implant duration was dictated by the European Authorities as an appropriate test period for an experimental medical device under scrutiny for biocompatibility. Moreover, the robotic hand was not directly wearable by the subject, being too heavy in its present form; when connected to tf-LIFE4s, the hand operated on a table in front of the subject. Despite such limitations, several new findings emerged from the study reported here. First, this generation of LIFEs can be implanted and used in humans for several weeks with a high rate of success in picking up signals with a good signal-to-noise ratio. Electrode positioning remained stable *in situ* even when carrying out everyday life activities (P.P. lived at home except for 3 days for surgical procedures). Multiple electrodes in different nerves with numerous contacts guaranteed a reliable flow of signals. Second, simultaneous recordings from several sites of 3 electrodes from two nerves improved the rate of correct classification for movement control with higher sensitivity and specificity (i.e., less false positive/negative) compared to a single-contact classification, particularly when discriminating independent movements. Quality and selectivity of efferent signals dispatched during movement imagination was augmented by concomitant sensory feed-back.
Tactile sensations were elicited and modulated by afferent stimulation during the first ten days following the implant.
When making an analytic comparison between the present and previous reports (Dhillon and Horch, 2005; Jia et al., 2007) some differences are found (Table 4.3)

	Dhillon et al	Jia et al	Present report
Numerosity	6-8	Case report	Case report
Implant duration	2 days (first study) 14 days (last study)	Acute study	4 weeks
Surgery	Implant	Intrasurgical study	Implant/explant
Pre-implant training	------	2 weeks	2 weeks
Interfacing protocol	recording/stimulation	Recording	recording/stimulation
Type of electrodes	Single filament LIFEs	Spring-like single filament	tfLIFE 4

Amount of electrodes	4-8 mono-electrode each subject	Best 4 among 6 monoelectrodes	4 multielectrodes 8 contacts each
Implanted nerves	Median and ulnar	Median, ulnar and radial	Median and ulnar
Stimulating electric charge	Min 4,7 nC Max 12,7 nC Progressive increase of the needed charge to evoke the same sensation	-	Min 0,1nC Max 3 nC Increase in the time of the needed charge to evoke the same sensation
Stimulation parameter	P. width 250 µs (300) P. ampl 200 µA(1-200) P. freq (randomly assigned by PC) Train duration 500 ms	-	P. width 20 µs (300) P. ampl 10 µA(1-200) P. freq 10-100 Hz Train duration 500 ms Train frequency 0.2 Hz
Stimulation duration	Whole study (15 days). Not all electrodes were tested for the full study due to limitations in subject availability.	-	Ten days
Motor control	Attempt to make one movement that results in maximum audible activity with loudspeaker	Subject was directed to make movements associated with the missing portion of the amputated limb as he performed on the healthy side.	Online control through amplitude/duration threshold Offline implementation with feature extraction, wavelet denoising and spike-sorting
Controlled movements	1 DoF (graded control)	Finger Flexion/extension with one electrode in radial nerve. No achieved control with ulnar and median nerve electrodes	3 movements and rest
Feedback	Loudspeaker noise	No feedback	see Tab 1
Clinical correlated features			Brain plasticity and PLS monitoring

Table 4.3 - Comparative analysis between the present study and two previous reports (Dhillon and Horch, 2005; Jia et al., 2007).

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Chapter 5

Neuro-rehabilitative effects on aberrant plasticity and PLS of LIFEHAND system

5.1. Introduction

Amputation of a limb is, for nearly all amputees, followed by the sensation that the lost body part is still present and kinesthetically perceived. Those phenomena are called respectively phantom awareness and phantom sensation (Hunter et al., 2003). In a variable percentage of 50-80% of amputees after amputation a painful dysesthesic perception in the lost limb called phantom limb pain (PLP) or syndrome is observed, that is a further cause of disability (Ephraim et al., 2005).
This chapter describes the work, realized in collaboration with the Department of Neurology of Campus Bio Medico, to find the correlation among the training in controlling the LifeHand system and in decoding the received sensorial feedback and the modification in brain plasticity, assessed through TMS and EEG, and in PLP and syndrome, assessed with clinical questionnaire.

5.2. Cortical reorganization and phantom limb syndrome

In order to better elucidate the neurophysiological substrate of PLP Authors reported a strong association for S1 changes, and PLP, but not with non-painful phantom sensation (Flor et al., 1995; Flor et al., 1998; Grusser et al., 2001). The association of cortical reorganization with PLP, but not with non-painful phantom sensation has been described also in motor cortex using steady-state movement-related cortical potential (Karl et al., 2004). fMRI exams showed shifts of lip representation in M1 and S1 and the activation of the face area following imagined phantom hand movement only in amputees with PLP (Lotze et al., 2001). Cortical reorganization in both motor and somatosensory systems, assessed with several methods, primarily manifests in patient with PLP, and probably represent its neuro-anatomical substrate. Several phantom phenomena could be evoked both ipsi- and contralateral to the amputation (Knecht et al., 1996) due to the contribution of bilateral pathways in amputees reorganization that goes further than the simple shifting in neighboring representation areas.

5.3. Effects of functional prostheses on Neuroplastic Reorganization.

Particular attention need to be devoted to studies that establish a relationship among neuroplasticity and active long-term (one to ten years) functional prosthesis use and even describe immediate plastic changes as soon as the prosthesis start to be used. In the following paragraphs chronic and acute effects in cortical organization are treated.

5.3.1. Influence of chronic prosthesis use on cortical reorganization

The frequency and type of prosthesis used by the amputee appear to be significantly correlated with presence and amount of cortical reorganization and also with PLP. In a fMRI study with long-term amputees both somatosensory and motor cortical reorganization and PLP negatively correlated with extensive use of myoelectrically-controlled prosthesis (Lotze et al., 1999). Furthermore a mean of nine years of a mechanical stump muscle-operated prosthesis use permits an increased affected limb utilization, thus leading to a PLP reduction, as assessed by a questionnaire, while it was unaltered in cosmetic prostheses using amputees (Weiss et al., 1999). Similar findings were observed with movement-related cortical potentials (Karl et al., 2004); it has been suggested that training of the stump muscles combined with visual feedback, both necessary for the prosthesis use, replace the lack of peripheral input to the somatosensory cortical area responsible for the missing hand, thus reducing the amount of reorganization in S1 and also in the motor areas seen as corollary changes to those in S1. Such considerations support the idea that reduction in cortical reorganization described after a sensory discrimination training in amputees, is due to amplification of inputs from the periphery (Flor et al., 2001). From all those findings we can infer that the daily and continuative use of a prosthesis that is able to feed-back a nearly physiological amount of both proprioceptive and exteroceptive sensorial inputs through its interfacing system, as a neural-interfaced prosthesis, will have in the long-term also positive effects in PLP reduction. The reduction could be improved if the neural interfaces that control the prosthesis are implanted in the severed nerves that had as original function to lead sensation and motion for the lost hand (Ulnar, Median and Radial nerves). This shrewdness will facilitate to furnish the increase in peripheral inputs directed to the hand-forearm brain's areas that are the sites mainly affected by aberrant plasticity.

5.3.2. Influence of acute prosthesis use on cortical reorganization

Convincing demonstrations of the influence of acute prosthesis use on cortical reorganization was provided by Maruishi et al by fMRI during use of EMG-guided prostheses creating a virtual electromyographic prosthesis. The images showed how the right ventral premotor cortex (VPCx) played an important role in manipulating the EMG prosthetic hand, while the right posterior parietal cortex (PPCx) might show a neural representation of the EMG prosthetic hand. Right VPCx and PPCx are clear dominant upon the left counterpart, even if the sequence is a right hand grasping. Focus of activation in the right PPCx shifted laterally when subjects manipulated EMG prosthetic hands (Maruishi et al., 2004). Such changes would be needed for perceptual assimilation of the EMG prosthetic hand and, correspond to changes in the brain

representation for the hand; however, they might underscore how even the acute use of an active prosthesis, alters the somatotopic body scheme. Recently an acute plastic reorganization of brain activity has been showed even in post-stoke patient after 13 to 21 sessions, one hour each, of magnetoencephalograpic control of an orthosis applied in their plegic hand with a visual feed-back (Birbaumer et al., 2008; Buch et al., 2008) sustaining the thesis that this form of BMI-guided activity-dependent plasticity is not limited to amputees.

Aim of the present work is to evaluate the changes in cortical reorganization due to neural plasticity and the following modification in subjective awareness of phantom limb and PLP after 4 weeks of training in active prosthesis control and sensory feedback perception through a bidirectional peripheral nerves interface. Phantom limb syndrome has been evaluated with a questionnaire while cortical reorganization through modification in electroencephalographic activation pattern during the intent of voluntary moving the phantom of the lost limb and through changes in the amount of cortical responding sites in transcranial magnetic stimulation.

5.4. Methods

Even if with the LifeHand systems implant has been developed a complex artificial intelligence classifier for signal analysis and transduction in motor command for the prosthesis the online control during the post-implant training was reached through a simple methods of amplitude threshold on the best channel for each single movement. Moreover, at that phase, the feedback for the user was delivered by the experimenter and not directly by the prosthesis (Fig. 5.1).

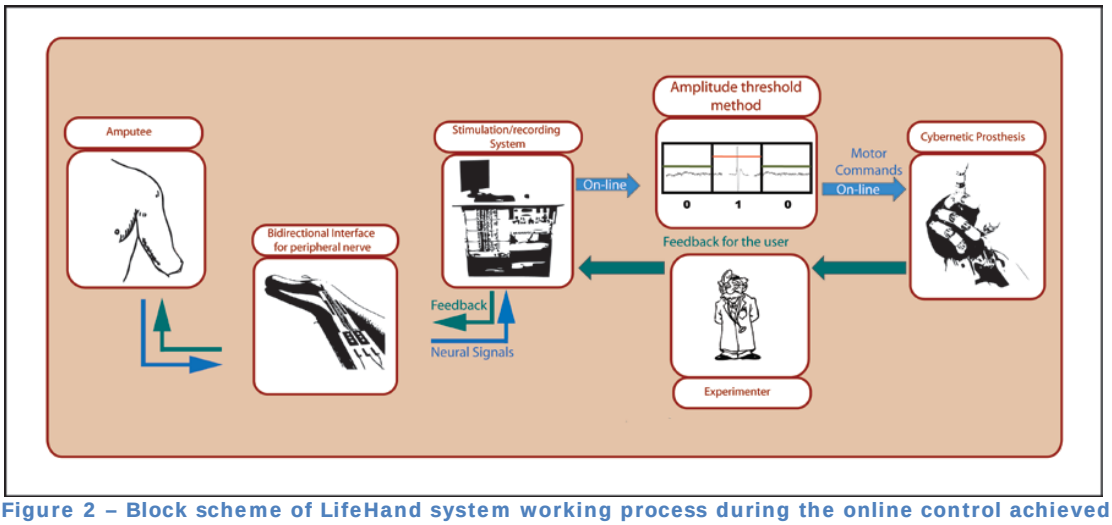

Figure 2 – Block scheme of LifeHand system working process during the online control achieved during the second phase of the experiment

5.4.1 Electroencephalography

Voluntary movements are accompanied by a definite pattern of changes in oscillatory firing of cortical neurons. While the presence of an ERD (event-related desynchronization or a power decrease in a frequency range) has been linked to the activation of cortical areas related to preparation of movement, the ERS (event-related synchronization) has been associated to inhibited or idling areas (28).

Topographical maps of ERD/ERS in alpha-1 (8-10 Hz), alpha-2 (11-14 Hz) and beta (15-25Hz) bands were obtained by averaging for each band the time-frequency representation in the period from 500 to 1500 ms. The topographical maps of the different sessions were compared.

EEG signals were recorded from the scalp in different sessions: i) before surgery (PRE) during voluntary command to perform left hand grasping, ii) after LIFEs implant (POST) and intensive training for motor commands control simultaneously to ENG acquisition, iii) during right hand movement. Thirty-two electrodes (scalp sites defined according to the international 10-20 system), mounted on an elastic cap and binaural reference, were used. Skin/electrode impedances were kept below 5 kOhms¶. Recordings were carried out utilizing a time constant of 0.1 s. EEG data were sampled at 1024 Hz (pre-sampling analogical filter 0.48–256 Hz, BrainAmp System).

5.4.2 Transcranial Magnetic Stimulation

Cortical motor output was mapped via TMS (Magstim200; eight-shaped coil with an inner wing diameter of 70mm; stimulus rate 0.1-0.2c/sec; intensity 10% above standardised excitability motor threshold(Rossini et al., 1994b)) for each hemisphere before surgery and at the end of the training period. To check for interhemispheric differences, Motor Evoked Potentials (MEPs) were recorded from proximal muscles of both limbs (biceps and deltoid) during separate mapping of right and left hemispheres. Intensities reported in Fig.7 refer to the excitability threshold for proximal muscles.

P.P. wore an elastic cap with a 99-square grid over the sensorimotor cortex. Square 1 corresponded to the point where the minimal intensity triggered MEPs of largest amplitude and shortest latency (*hot spot*); this was coincident for biceps and deltoid on both hemispheres.

5.4.3 Phantom Limb Syndrome

Phantom awareness and PLS were evaluated pre-surgically and were followed up at the end of the training period and 3 months after LIFEs removal using an abbreviated version of the McGill Pain Questionnaire (sfMcGill), the present pain intensity scale (PPI), the pain visual analogue scale (VAS), and an open section for description of PL awareness.

5.5. Results

5.5.1 Electroencephalography

In the first session (PRE) before surgery a slight power decrease in both alpha 1 (8-10Hz) and beta bands in the 'event' period after trigger onset was observed. Interestingly, in the second session three weeks after LIFEs implant (POST) and intensive training for motor control of the missing hand/fingers, a clear pattern consisting in an abrupt and intense power decrease (ERD) over the central sensorimotor areas contralateral to the missing hand in the time preceding voluntary movements was reliably observed in alpha 2 and beta bands (Fig 5.2), as widely documented by previous studies in healthy people (33-34). In alpha 1 band a diffuse desynchronization in bilateral central, frontal and parietal regions was also evidenced. In both sessions, in alpha 1 band a higher ipsi- than contralateral desynchronization was found in sensorimotor regions. The time preceding the real right hand grasping was associated with an ERD maximal to the contralateral central areas in all frequency bands. In alpha-1 band, a great involvement of the bilateral parietal areas was also observed.

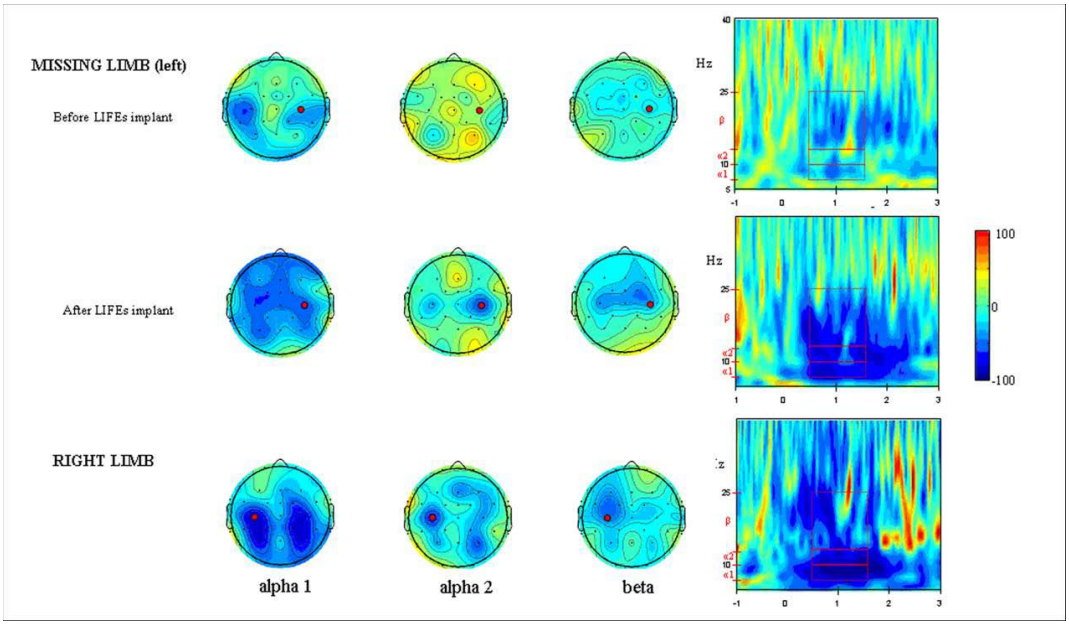

Figure 5.2 – In the first three columns is represented Scalp topography of ERD (blu)/ ERS (red) in alpha-1, alpha-2 and beta bands in the time period of 500-1500 ms after that the subject received motor trigger. C3 and C4 location are evidenced by red circles. In the last column there is the time-frequency representations of EEG power modulation in primary motor cortex (C4 on right and C3 on left). Boxes indicate the frequency bands and time period considered for scalp topography. Three lines correspond respectively to voluntary motor command of the missing limb before and after LIFEs implant (up) and for voluntary movement of right hand (bottom).

5.5.2 Transcranial Magnetic Stimulation

Stimulating left hemisphere while recording contralaterally in the distal (ADM e ECD) and proximal muscles of the healthy limb both the number of responding sites and the stimulating

threshold do not almost change before and 4 weeks after the implant (distal muscles PRE: responding sites 22, threshold 37%; POST: responding sites 20, threshold 36%; Proximal muscles PRE: responding sites 30, threshold 52%; POST: responding sites 35, threshold 53%). On the contrary as regard as right hemisphere stimulation, recording from the proximal muscles of the severed limb the number of responding sites significatively ($p<0.05$) decreased (PRE 36 vs POST 16). Stimulating threshold did not change (Fig 5.3).

Pre-surgical TMS motor maps showed an abnormal interhemispheric asymmetry of motor cortex excitability in the hemisphere governing the stump. Following training, post-surgical maps, in parallel with reduction of PLS, showed a clear reduction of this asymmetry and a trend toward balanced muscle representation in the two hemispheres, as in control subjects.

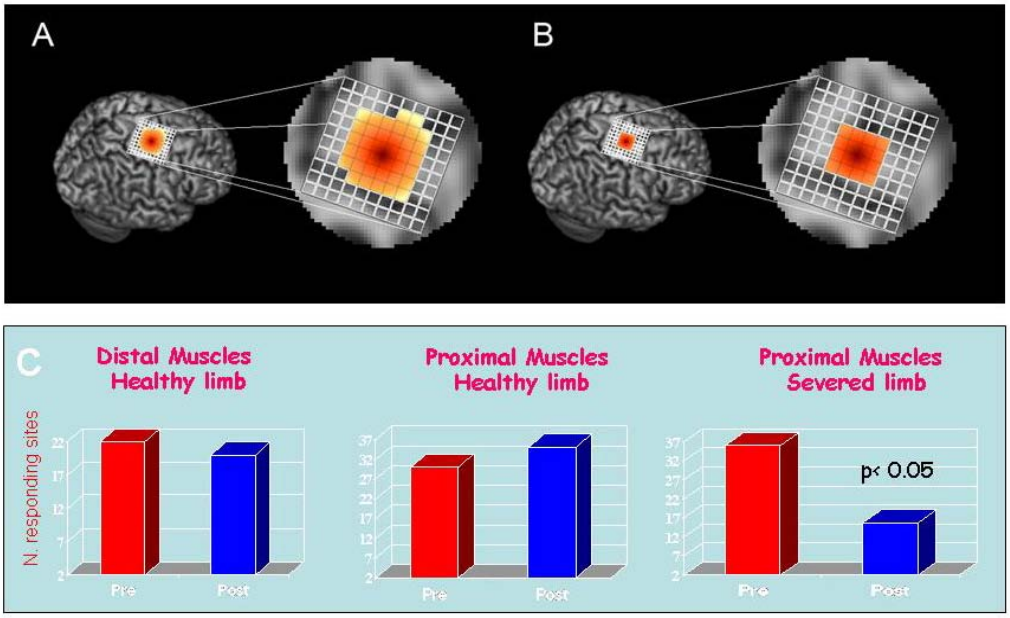

Figure 5.3 – Graphic representation of sites responding to transcranial magnetic stimulation upon right motor cortex controlling proximal muscles of amputated limb before (A) and four weeks after (B) tfLIFEs implant. C- Istogramms of responding sites before (red) and four weeks after (blu) the implantation recording respectively from distal and proximal muscles of the healthy limb and from the proximal muscles of the stump.

5.5.3 Phantom Limb Syndrome

Before implant, P.P. referred a moderate PLS and perceived the phantom of his left upper limb as if '*…the missing hand is directly attached to the stump, without any forearm, but blocked by an heavy load or tightly fastened by a belt that make impossible any movement*'. (fig 5.4).

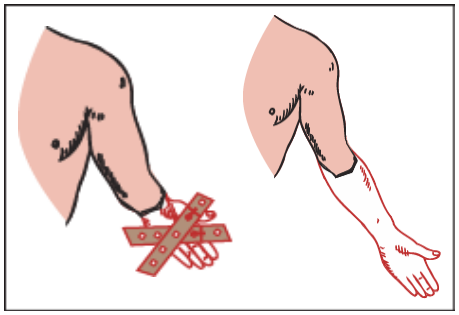

Figure 5.4 – Modification in subjective awareness of phantom limb before (left) and after fur weeks of training in active prosthesis control and feedback perception.

This weird perception underwent to a normalization when investigated the 4th week after the surgery, but was found again a 3 months of follow-up. Similar behaviour has been found for the improvement of PLP with all the three clinical scales adopted in the questionnaire (table 5.1).

	sfMcGill (0→45)	PPI (0→5)	VAS (0→100%)	Subjective description of PLS
Pre-implant	18	3	38	No forearm, blocked hand
Post-explant	11	2	23	Regain forearm, movable hand
3months Follow-Up	17	3	36	No forearm, blocked hand

Table 5.1 - Modification of phantom limb syndrome during the experimental period and after 3 months

5.6. Discussion

Training for robotic hand control and for sensory perception produced a normalization in the electroencephalographic activation pattern and a reorganization of the motor cortical maps distinguished by TMS with restriction of the cortical overrepresentation of muscles proximal to the stump. In parallel has been described a clinical improvement of PLS, with a progressive return to perception of full-length forearm and of the hand free of motion. Clinical improvement was no longer present at 3 months follow-up.

After this analysis of neuroplastic reorganization in amputees it arises the question about the presence or not of a functional significance of plasticity. Have we to assume that it is only an epiphenomena that follows the nervous injury or has it a specific 'functional' implication toward retainment of performance? Often neuroplastic changes lead to functional improvement; enlarging of somatosensory representation of the stump may increase sensory discrimination and the same can be said for the motor system, all in the attempt to partially compensate the loss of the limb. Similar considerations have been done in case of other kind of injuries where similar types of plasticity play a beneficial role, such as cross-modal plasticity in blind humans (Cohen et al., 1997) and in recovery of motor functions after stroke (Rossini and Pauri, 2000; Rossini et al., 2003). On the other hand, we have already reported that neuroplasticity in amputees leads also to maladaptive phenomena, such in the case of PLP (Flor

et al., 1995). Thus it seems that plasticity is not only an epiphenomenon of the damage but it mainly represents a compensatory response that could, in some circumstances, even have harmful consequences (Fig. 5.5) (Karl et al., 2001; Chen et al., 2002). In the equilibrium among maladaptive phenomena and compensatory responses the training for the control of an high interactive prosthesis bidirectionally interfaced at the neural level showed those neurorehabilitative qualities that could address the changes toward a curative plasticity (Fig. 5.5).

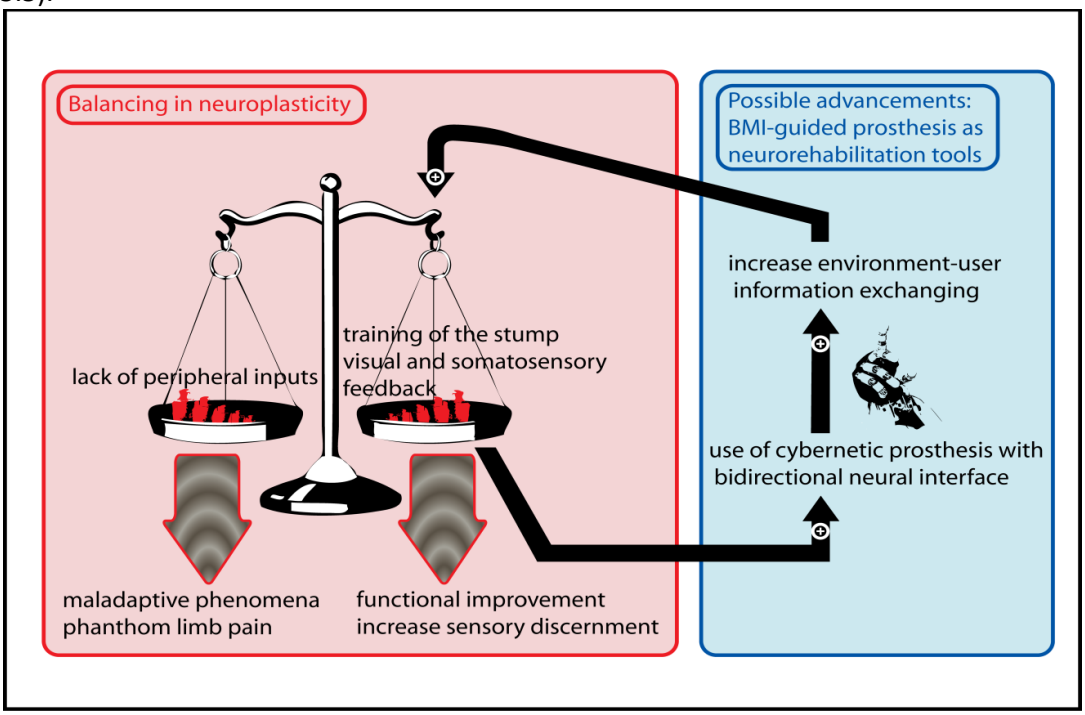

Figure 5.5 - In the pink box on the left main literature based evidences are summarized (Flor et al., 1995; Flor et al., 2001; Grusser et al., 2001; Karl et al., 2004) that clearly demonstrates how the maladaptive phenomena are due to the lack of input from the periphery. Training of the stump and provision to the brain of visual and artificial proprioceptive and exteroceptive sensorial inputs have been showed to increase the sensory discernment and to lead to a functional improvement on human amputees. As presented in the light-blue box on the right, as confirmed by the results of the present study, we envisage that the development of bidirectional interfaced cybernetic prostheses represent not only a more natural, acceptable and powerful solution for hand functional restoration, but also because their use generate a significant rehabilitative side-effect: the exchange of bidirectional information could allow to better guide neuroplasticity toward helpful adaptive changes, thus applying its additional load to the right plate of the balance in our figure. From (Di Pino et al.,2009).

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Chapter 6

Foreign body reaction to electrode implanted in neural tissue

6.1. Introduction

Electrodes and electrode arrays, that are mostly used today, showed an acceptable capability of recording high quality neural signals and of properly stimulating for sensorial feedback delivering, but what more deserves the attention of neural interfacing technology designers and producers is the long term endurance of performance that is still not acceptable for human prosthetic applications.

For instance, one of the weak points of the clinical experimentation of LifeHand system in human was that the stimulating properties of tfLIFEs, that soon after the implantation surgery seemed very satisfactory, decreased progressively and rapidly, until, after ten days, was not possible to elicit any perceptible sensation. Even if has been already establish that the surface of active contacts was not wide enough to support the necessary stimulating charge, as reported by the literature, should be some additional reasons that, together with the insufficient charge, can justify that decrement. A huge inflammatory and fibrotic reaction was observed both close to the implantation sites upon the nerves and around the cables and connectors in the perinevrial and connective tissue, but for ethical reasons has not been investigated histologically.

It's wide accepted that mechanical and electrical instability of the electrodes are often due to the inflammatory response to the foreign body operated by the nervous tissue, that encapsulate the electrode. This is a general response of the nervous system to implanted material that does not present main specificities depending on the type of implant. It affects both the short and even more the long-term performance of the device, in registration and in stimulation cues and it is strongly influenced by the electrode-tissue interface that has to reduce the electrical, mechanical and biochemical mismatch.

Moreover, the inflammatory reaction produces, further than electrode encapsulation, a progressive neuronal loss in the areas around the implanted structure, that result to be critical for chronic recording failure (Biran et al., 2005).

The acute phase of inflammation is primarily triggered by the traumatic insertion of the electrode that provokes bloody vessels damage, severs extracellular matrix and neuronal processes and recruits macrophages and microglia. The acute phase still allow a complete restoration if the foreign body is timely removed (Csicsvari et al., 2003). Chronic response is mainly characterized in the CNS by a huge astrocytes activation, while in PNS by fibroblast activation, with a neo-fibrotic evolution, that coats the electrode surface, insulate the device and abnormally increasing its impedance.

Implantation procedure is determinant upon the amount of initial mechanical damage but the literature presents contrast opinion on this topic about manual or automatic insertion mode and the velocity of insertion. Slow insertion of 100 µm/s should permits to the tissue to compliance the electrode (Nicolelis et al., 2003) while fast insertion of 8.3 m/s (Campbell et al., 1991) should concentrate the cutting forces along the electrode track, thus preserving the areas in close proximity.

In the CNS mounting of FBR are primarily involved four cell types: the resident macrophages called microglia that coat the electrode and together with other leukocytes produce the pro-inflammatory cytokines (PIC), the astrocytes that respond to the PIC increasing the production of fibrillary proteins, such as vimentin and glial fibrillary acid protein (GFAP) and together with the fibroblast generate the chronic fibrotic reaction (Fig.6.1) (Turner et al., 1999; Szarowski et al., 2003).

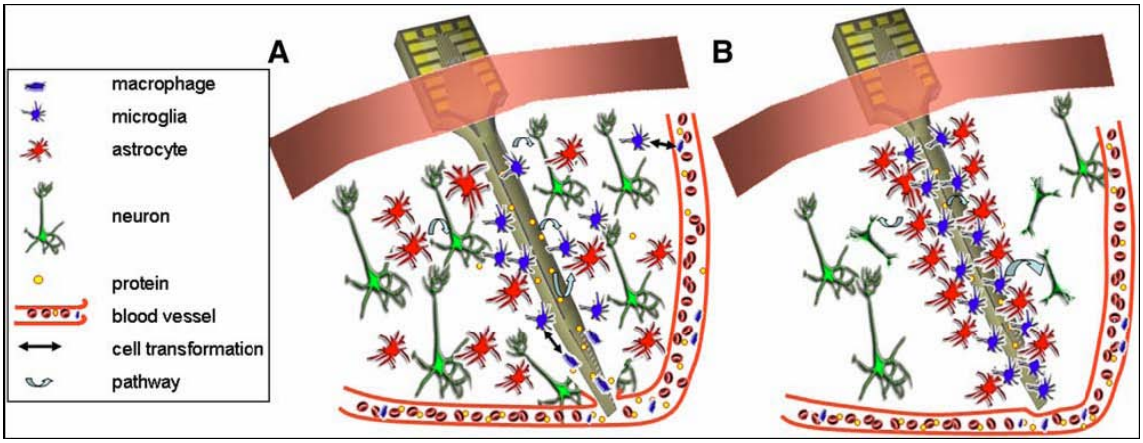

Figure 6.1 - Graphical overview of the acute (A) and chronic stages (B) and the involved cells, of the foreign body reaction of neural tissue around an implanted electrode in the Central Nervous System. From (Schwartz et al., 2006) .

Active macrophages presence is demonstrated with immunostaining again the cell marker CD68, recognized by the ED1 antibody, while GFAP is used for astrocytes. The encapsulation time-frame has been described by Turner and colleagues as present, extending about 500 µm, but not very organized at two weeks, continuous and compacted, 50-100 µm of thickness after 6 week (Turner et al., 1999), but commonly four weeks can be considered as a reasonable minimum time for stabilizing the reaction.

In the PNS the foreign body reaction against electrodes is mainly operated by activated macrophages in contact with the implant, circumscribed by a dense and compact layer composed by fibroblasts and collagen (Fig 6.2) (Lago et al., 2007)

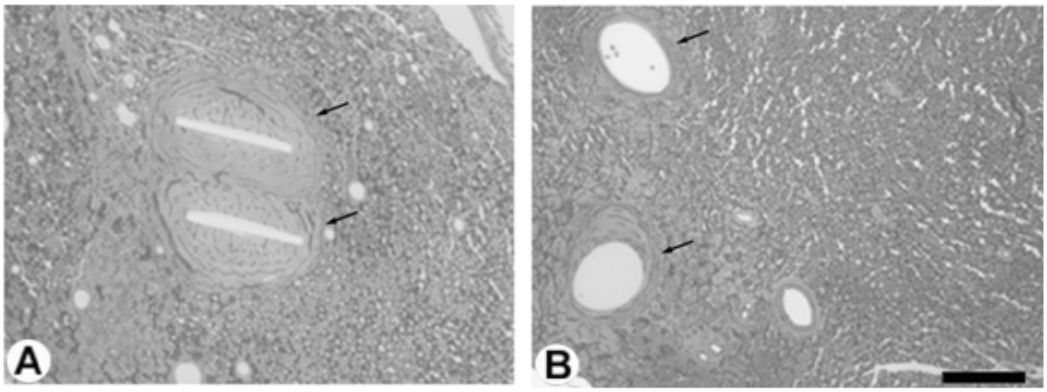

Figure 6.2 – Histological transverse sections of FBR capsule grew around laminar (A) and round section (c) electrodes after 3 months from implantation in a rat sciatic nerve. In the inner layer are visible FBGC circumscribed by concentric collagen fibers. Black bar in B is 100 μm long. Modified from (Lago et al., 2007).

Going towards a new generation of neural interfaces for prosthesis control, that could justify their wide dissemination in human in the clinical practice, the deeper study of the mechanisms of foreign body reaction (FBR) around a neural implant and the develop of strategies to reduce the electrode-tissue unphysiological mismatch deserve more efforts.

6.2. Cellular and molecular mechanisms of foreign body reaction

Foreign body reaction to implanted material is a primary, sterile reaction of nonspecific immune system that is still not deep known, but that shares mostly mechanisms, especially as regard as its onset, with the wound healing process. Being the wound healing more investigated in literature, it was a valid font from which is gathered part of this knowledge.
FBR presents several phases that changes during the time: an immediate onset followed by the phase of acute inflammation, the chronic inflammation that stabilizes the process with the arising of the granulation tissue and finally the resolution.
Cytokines, chemokines and metalloproteinases (MMPs) are soluble mediators that, produced in the neighborhood of the implanted tissue, interact in spatial and temporal order, thus regulating the cascade processes that take part of the FBR. The implantation implies vascular and tissue damages, with an increase in vascular permeability and new angiogenesis, that evolve in extravasation and migration of inflammatory cells. Those cells start a phagocytotic activity against the foreign body, while the final evolution of the procedure is the insulation of the implanted material with a fibrotic reaction.
In its stable form the capsule produced by this particular chronic inflammation, is composed by two concentric layers: an inner cellular layer made by macrophages fuse together to form foreign body giant cells (FBGC), and an outer fibrotic layer made by tight collagen fibers (Lago et al., 2007).
In reviewing this particular kind of inflammation we have to keep in mind that FBR is a complex, non-deterministic biological process that involves a huge amount of cells an mediators acting in

overlapping time windows, thus any attempt to schematize this process is a simplification that always discharges part of the information.
When a foreign material is inserted inside the body an injury to peripheral tissue and to the vascular capillaries occurs with a blood/material interaction. This interaction triggers two parallel process: the adsorption of proteins on the material surface and the formation of a thrombus (blood-based matrix) in the peri-material tissue.

6.2.1 Protein adsorption

The material used for human implant have to be nonimmunogenic and nontoxic, thus the immune response to the material is mediated by proteins that adsorb upon its surface and work as adaptors among phagocytes and foreign material.
Main adsorbent protein are fibrinogen, complement and antibody (IgG)(Jenney and Anderson, 2000b, a). Fibrinogen adsorbs directly to the implant and changes its conformation exposing its P1 fragment and enabling the binding with the phagocyte integrin complement receptor 3. Complement protein C3b spontaneously adsorb to the implant and leads to the alternative complement pathway activation. Antibodies are abundant in serum and aspecific binds the implant surface while its constant fragment its recognized by complement C1q factor, thus activating the classical component pathway. Both the complement pathways increases vascular permeability and leukocytes chemoattraction, increasing the inflammatory process (Fig. 6.3).
The meaning of the protein adsorption is to create an adaptor among the nonimmunogenic surface of the implanted material and the monocyte/macrophage cells that try to pagocytize the foreign body (Wilson et al., 2005).
The adsorption of the protein layer and its composition is strictly dependent upon the chemistry and the hydrophobicity of the surface and it's a key determinant of the the whole inflammatory reaction.

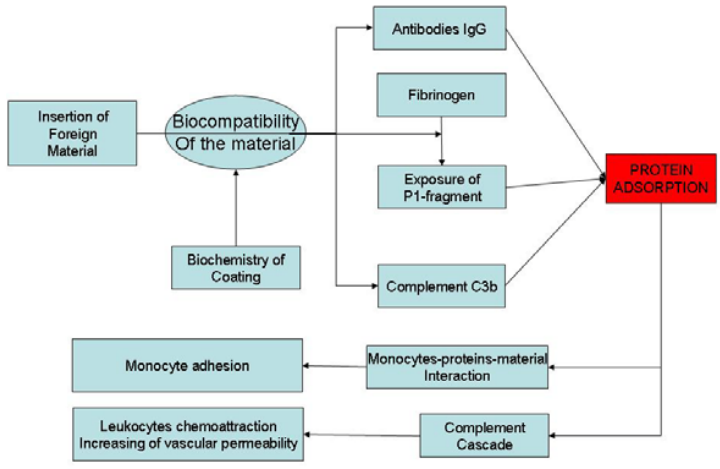

Figure 6.3 – Blocks scheme that summarize the protein adsorption process.

6.2.2 Blood-based matrix

The blood-material interaction, caused by the blood vessel rupture, leads to the activation of the extrinsic and intrinsic coagulation system, the kinins system, the complement and fibrinolytic systems and to the aggregation of platelets, with the formation of a thrombus. The interstitial environment become structured by a transient blood-based matrix mainly made by fibrin, but also by other extracellular protein such as collagens, laminin, fibronectins, elastin, proteoglycan and glycosaminoglycan, and caused by coagulation. Its network behaves as a structural scaffold for the cellular migration and adhesion. To allow the migration the network needs an optimum density grade and needs to be rearranged by the metalloproteinases (MMPs), zinc dependent endopeptidases such as the collagenases, that are produced by active leukocytes and fibroblasts, that are able to cut the mesh (Fig. 6.4) (Luttikhuizen et al., 2006).

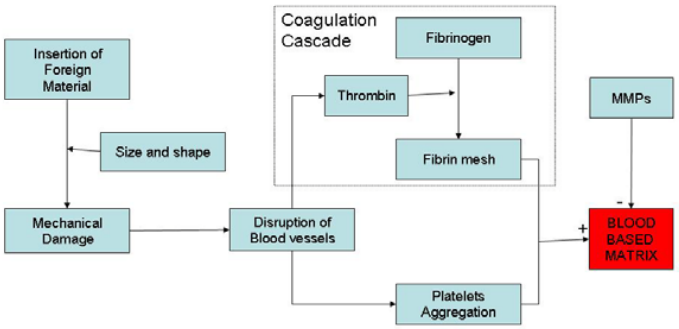

Figure 6.4 - Blocks scheme that summarize the blood-based matrix formation and rearrangement.

6.2.3 Vascular Permeability

The mechanical damage produced by the insertion of the foreign body leads to the platelets aggregation and to an activation of the surrounding tissue and the vasculature, with a consequent cytokines release. Activated platelets produce vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF), while mastcells degranulates histamine, defined together as pro-extravasation cytokines (PEC). PECs are able to promote new angiogenesis, through the activation of endothelial cells, and through the upregulation of cell adhesion molecules that allow the extravasation. Furthermore the fibrin, the product of the hydrolysis of fibrinogen in the coagulation cascade, is a potent vasodilatatory and pro-angiogenetic factor.

All together those effects result in an increase of the vascular permeability to the inflammatory cells (Fig. 6.5).

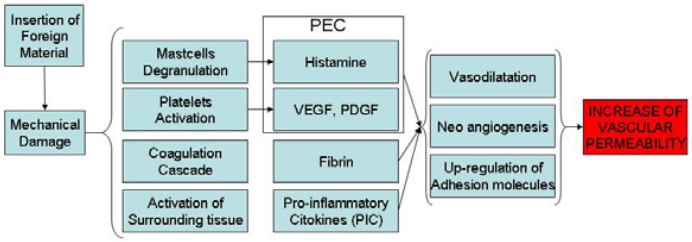

Figure 6.5 - Blocks scheme that summarize the processes that lead to the increase of vascular permeability

6.2.5 Chemokines Gradient

Main characteristic of an inflammatory response is the migration of inflammatory cells, monocytes, other leukocites and fibroblasts from the blood reservoir and the contiguous tissues toward the site where the phlogosis began. Chemokines are soluble mediators released by activated cells that presents strong chemoattractive properties and establish a chemical gradient of concentration that guides the cells, that express chemokine receptors, to their meta. The chemokines system and the chemokines-receptors match is flexible and redundant, thus often the same mediator acts on several different cells. Nevertheless, among the others, the chemokine CXCL-8, that coincide with IL-8, is the stronger for leukocites and fibroblasts attraction, while CCL-2 and CCL-3 (also called macrophage inflammatory protein MIP-1) are the most powerful for monocytes/macrophages.

6.2.5 Cytokines Network

The cytokines network is another pleiotropic and redundant system made by hormones, growth factors and interleukins, that regulates the proliferation, the activation level and the cell to cell interaction. Most of the functions overlap among different cytokines and cytokine receptors. Moreover our knowledge upon cytokines function in FBR is still poor and most of the insights comes from the wound healing process. Therefore this attempt to schematize the system and its function cannot aim at the whole description of each single mediator and action, but more at a functional simplification that summarizes how the system in toto works.

During the FBR cytokines are secreted primarily at two functional moments. The insertion of the foreign body produces the "first cytokines release" that increases the vascular permeability – see the PEC actions - and allows the initial migration of inflammatory cells. When a considerable amount of inflammatory cells reached the foreign body the "second cytokines release" starts to increase the activation state of the migrated cells. Phlogosis leads leukocytes, macrophages and fibroblasts to produce pro-inflammatory cytokines (PIC), mainly TNFα and IL-1, that enhances phagocytosis, chemotaxis, and induces edema and adhesion molecule production. PIC are counteract by the effects of anti-inflammatory cytokines (AIC), mainly IL-10 and IL-6, that inhibits both PIC and chemokines production and release (Luttikhuizen et al., 2006).

TGFβ is initially produced by activated platelets and after by macrophages and fibroblasts and it is a strong activator of monocytes and PMN, but its main action is to promote the collagen production by fibroblasts (Crowe et al., 2000). Both IL-4 and IL-13 are crucial for the macrophages fusion to form foreign body giant cells (Anderson et al., 2008), probably because upregulate the mannose receptors used by the fusing cells (DeFife et al., 1997).

6.2.6 From recruited monocytes to FBGC

A fundamental step in FBR is the recruitment of monocyte toward the site of implantation. Monocytes migration is due to the chemoatractant activity of several mediators, but mainly to chemokines belonging to CCL family. Once recruited the monocytes interact with the adsorbent protein layer on the foreign material surface trough their integrin surface receptor (Anderson et al., 2008). Adhered monocytes feel the effects of the balance among PIC and AIC and become activated to macrophages and increment their cytokines production. Moreover the adhesion

leads to a cytoskeleton remodeling with the formation of podosome and later the fusion of cytoplasmatic material. Foreign body giant cells (FBGC) are polinucleates cells born from the fusion of several macrophages in the attempt of insulating the foreign material from the body. It happens because of the big size of the particles that does not allow macrophages to phagocytate the material (frustrated phagocytosis).
An FBGC present from 25 to 300 nuclei an ellipsoidal shape bended around the foreign body and extensive cytoplasmic spreading of about 0.8-1.5 mm^2 (Anderson, 2000).
For the fusion is essential the binding with the mannose receptor, improved by the action of IL-4 and IL-13, secreted by T lymphocytes. The species of protein adsorbed on the material are of main importance, not only on the amount of adhered monocytes, but also on the ability of macrophage to fuse into FBGC (Shen et al., 2004; Jones et al., 2007) , probably because they can influences the T cells on interleukins production (Fig. 6.6).

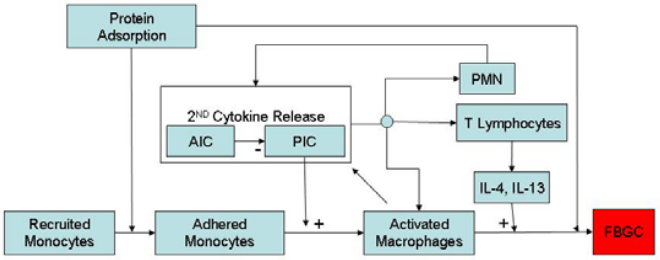

Figure 6.6 - Blocks scheme that summarize the processes that from monocytes recruitment lead to the formation of FBGC

6.2.7 Fibrotic ECM

Migrated and resident inactive fibroblasts are stimulated, mainly by TGF-β, to change their histo-type in activated mio-fibroblasts and start collagen production. The collagen fibers are concentrically organized in bundle around the foreign body and interlinked eachother. Moreover TGF-β decreases also the level of MMPs, thus limiting matrix degradation. Both TGF-β and MMPs are secreted by fibroblasts and by adhered and fused macrophages, that present indeed the ability to modulate extracellular matrix (ECM) fibrosis and remodeling in a material surface-dependent manner (Fig. 6.7).

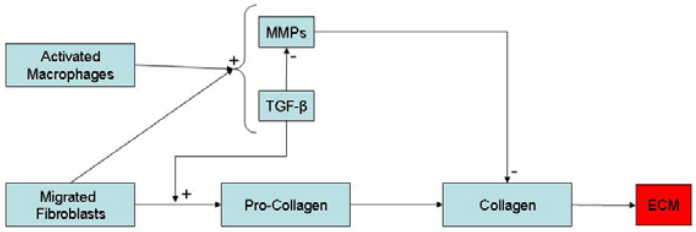

Figure 6.7 - Blocks scheme that summarize the processes that lead to the fibrotic ECM formation

6.2.8 Resolution

FBR is a peculiar chronic inflammatory reaction that is exposed to the equilibrium among PIC and fibrotic factors that push towards phlogosis resolution. The progression phases are characterized by a sustained cytokines and chemokines productions from macrophages and other leukocytes with high levels of IL-1 and continuative secretion of TNFα, that contribute to maintain the inflammation. Typical of the resolution phase is, on the contrary, the increase of TGF-β with its anti-inflammatory fibrotic activity and of the AIC while has been also described a decrease in CCL family chemokines and in IL-8 (Jones et al., 2007).

6.3. References

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Chapter 7

Bioactive coating of polyimide based electrode arrays

7.1 Introduction

Huge efforts have been spent in the research of optimal parameters for electrode size and shape that provoke the smallest tissue reaction and especially on the shape of the tip (Edell et al., 1992; Nicolelis et al., 2003), but Szarowski and colleagues did not describe any major differences among several electrode size and shape when, after six weeks, the FBR become chronic (Szarowski et al., 2003).
Aiming at minimizing the immune response to implanted electrodes, further than the electrodes design and the implantation techniques, a key factor that could allow achieving better long-term performance seems to be represented by the improvment of electrode-tissue interface characteristics.
Both microwires and thin-film microfabricated array are shielded by an insulation layer that is the one that closer interact with the neural tissue. Non-toxic material such as Teflon (Nicolelis et al., 2003), Epoxilite resin (Liu et al., 1999) or Polyimide (Campbell et al., 1991; Hoffmann et al., 2006) are the most commonly used. The high flexibility of polyimide reduces the mechanical electrode-tissue mismatch, but on the other hand, does not fulfill the minimum stiffness requested to pierce the neural tissue and involves the use of a rigid needle to create the implant hole (Hoffmann and Kock, 2005).
Unfortunately these materials do not stimulate attachment and growth of neurons toward the electrode, thus producing the reduction in perielectrode neuronal density. Moreover the chronic component of FBR has been linked with the repetition of tissue-electrode relative micromotions and employing a three dimensional finite element method model has been showed how a tight physical coupling, that could be achieved by neuro-integrative protein surface coating, reduces those micromotions (Lee et al., 2005).
To overcome the issue of the interface surface mismatch several strategies have been experimented: modifying the surface texture, depositing nanotubules that promote the neuronal growth (Moxon et al., 2004). Others approaches had implemented microfluidic structures in the electrode arrays (Chen et al., 1997; Wise, 2005) that release neurotrophines or anti-inflammatory drugs, such as dexamethasone and cyclosporin A (Shain et al., 2003; Retterer et al., 2004). The use of corticosteroid had shown good results in the acute reaction phase

(Spataro et al., 2005; Wadhwa et al., 2006), while in long term the short duration of drug release limits the effect (Zhong and Bellamkonda, 2007).
Although the most promising approach seems to involve electrode coating with active biomaterial that easily interact with the neural tissue cellular elements. Intact extracellular matrix proteins such as collagen, laminin or fibronectin (Ignatius et al., 1998) or only part of them with the property of adhesion polypeptides have been widely employed (Polikov et al., 2005). Among polypeptides have been tried RGD, YIGSR and IKVAV both in vitro (Kam et al., 2002) and in vivo (Cui et al., 2003) with the promising results of improving neuronal cell attachment and re-grown of neurofilaments (Cui et al., 2001). Electrode coating methods were also tested to attract glial cells, thus reducing micromotions (Cui et al., 2001; Kam et al., 2002) or, on the contrary, to repeal astrocytes and microglia adhesion through low protein binding surface coatings (Singh et al., 2003; Leung et al., 2008) and consequently reduce the encapsulation.

7.2 Laminin dip-coating deposition on dummy structures

Aim of this work, performed in collaboration with Scuola Superiore Sant'Anna, is to find a suitable material and procedure for creating a neuro-attracting coating layer for Polyimide-Platinum electrode arrays, such as tfLIFEs and the last generation prototype called TIMEs (Transverse Intrafascicular MultiElectrode) that are still in developmental phase. The coating layer should reduce fibrosis reaction around the electrode and tissue electrode relative micromotions.

7.2.1 Methods

Hexagonal shape disc made by a base of Polyimide and round Platinum spot of about two cm^2 of area, provided by Institute for Microtechnology of the University of Freiburg were used as dummy structure for the protein matrix deposition (Fig 7.1) being the evaluation of functional properties or the arrays outside the scope of this work.

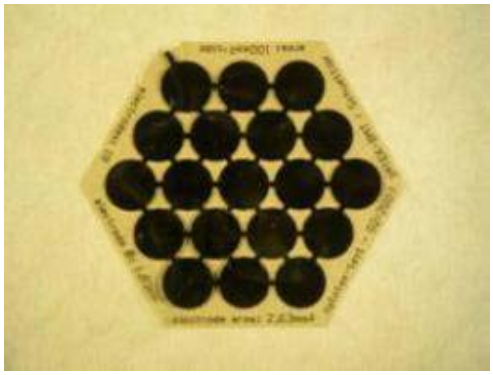

Figure 7.3 – Hexagonal Polyimide/Platinum dummy structure

Laminin stock solution of 1mg/ml (Invitrogen) has been diluted in polyphosphate buffer (PBS) at different concentration from 1:100 to 1:5. Dummy structures has been dip-coated with the laminin-PBS solutions incubated in CO_2 5% at body temperature for several time periods lasting from 30 min to 30 hours (Table 7.1).

Sample N°.	Dilution	Dip-coating Time
1	1:15	3 h
2	1:30	3 h
3	1:15	20 h
4	1:30	20 h
5	1:25	30min
6	1:100	30min
7	1:10	24h
8	1:5	24h
9	1:10	30h
10	1:5	30h

Table 7.1 - Dilution and dip-coating time incubation of the different samples.

After coating samples were rinsed in PBS and stored in refrigerator at 4°C. Coated structures have been analyzed at optical microscope and with FIB (focused ion beam) and AFM (atomic force microscope).

7.2.2Results

Deposited Laminin presents a fern-like shape and its coating has been found discontinuously in all the samples. 1:15 dilution presents the more homogenous laminin layer while samples incubated overnight present a ticker coating layer. In Figure the images for sample N^{o} 3 (1:15, 20h) (Fig microscope) are shown.

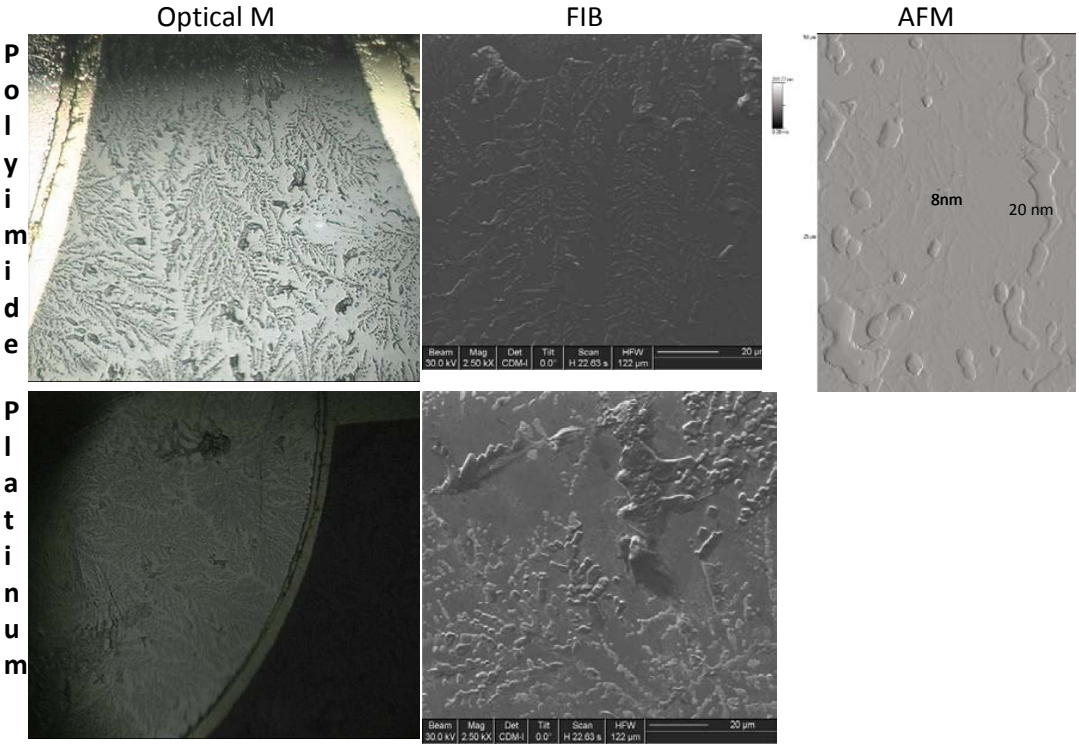

Figure 7.2– Laminin coating in sample No 3 (1:15, 20h) upon Polyimide (upper row) and Platinum (lower row) seen at optical microscope, FIB and AFM. Note in AFM the different thickness of laminin coating and background.

7.2.3 Discussion and future step

Even if this study presents only preliminary results it shows the feasibility of Polyimide/Platinum based structures coating with Laminin and the best dilution and incubation time to adopt. Going along this vein a similar procedure will used for coating with the adhesive polypeptide RGD from the integrin-ligand superfamily (Kam et al., 2002). To evaluate the foreign body reaction against the implant dummy structures coated with different bioactive proteins will be co-cultured with a monocytes enriched media as described by Shen and colleagues (Shen et al., 2004) and real coated electrode will be implanted in small rodents for achieving an histological picture of the FBR reduction in parallel with a test of their functional electrical activity. Moreover the efficacy of Mesenchimal Stem Cells (MSCs) in reducing FBR will be also investigated. The strategy of grow MSCs upon a Polyimide support coated with Laminin, represent a broad new possible solution never tested in the literature. MSCs form the bone marrow stroma supporting the hemopoiesis and contribute to the regeneration of injured

tissue by differentiation and by secreting a wide range of cytokines with an immunomodulatory and an antifibrotic effect. Based on these unique properties, MSC are currently under investigation for their possible use to treat immuno-mediated diseases such as graft-versus-host disease, rejection of organ allograft, Crohn's disease, and inhibition tissue fibrosis on tissue repair. In addition MSCs do not express cell surface markers that are highly stimulatory to the immune system, such as MHC class II and suppress many T cell, B cell and NK cell functions (Le Blanc and Ringden, 2007)

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Chapter 8

ODEs model of FBR against peripheral nerve implanted electrode

8.1. Introduction

Foreign body reaction (FBR) against implanted electrode is widely considered as a critical issue in long-term maintaining of electrode performances and consequently improving our comprehension about this response of the body could give us new insights in the development of more tissue-integrated devices. Further than from all the histological studies already described in chapter 6, the knowledge of FBR could be derived from a mechanistic understanding of the underlying processes, and their relations achieved through a mathematical model.

Biological complex systems have been one of the most common topic affronted by model experts in the last decades. Although the mathematical and computational tools can offer only a reduced interpretation of such intricate phenomena, they can allow an accurate formal representation of the problem that should not suffer for the subjectivity of the experimenter and for the impossibility of controlling all the variables at the same time during an experimentation. Aim of such models should be the generation of a simulation that matches the reality as much as possible and refer to real experimentation for calibration and validation (Vodovotz et al., 2009). Consequently the *in silico* and the *in vivo* methods are more strictly interdependent than it seems at first approach.

The behavior of a dynamic system could be represented as the evolution over time of variables involved in the system and described with mathematical functions. A model that is constituted by interlinked equations and use time as the unique independent variable, while ignoring the spatial contribute, is defined as ordinary differential equations (ODEs) model. ODEs model succeed in modulating complex systems in various fields, and also in particular situation close to the application of our interest (Kumar et al., 2004). With a good basal knowledge of the problem the definition of all the involved variables is almost easy, while the choice of the functions describing the interdependences among variables and the value of the parameters remain the hardest obstacle. Moreover the ODEs models are strictly deterministic and sometimes suffer when describing the stochasticity of a complex phenomenon. To overcome the neglect of spatial aspects partial differential equation models present spatial dependence of variables as well and to overcome the rigid deterministicity of ODEs model Gillespie used the concept of the probability to model discrete reaction (Gillespie, 2000). Other solutions to consider the spatial structure in a model involves FEM (finite elements models), where the space is compartmented in subdomains that present uniform characteristic describable through ODEs, and ABM (agent based model) that view a system as an ensemble of agents that follow rules basing on local condition (Bonabeau, 2002).

Inflammatory reactions of the body, and all the sub-processes that are involved in it, are optimal examples of high complex systems, with multiple interactions among several cells and soluble mediators, that evolve in the time and their study can benefit of the appliance of formal analytical and synthetic methods typical of the mathematical models.
A group from the University of Pittsburgh has been very active in the field of inflammation phenomena modeling, Kumar and colleagues drew an ODEs model of infectious inflammation and its possible positive or negative outcome (Kumar et al., 2004) and others Authors modeled the acute inflammation (Day et al., 2006; Reynolds et al., 2006) both with ODEs and ABM models (Vodovotz et al., 2004), the shock state (Chow et al., 2005) and the wound healing process (Mi et al., 2007). More related to our purpose of FBR modeling, can be found two works in literature modeling the collagen formation during the wound healing process (Dale et al., 1996; Dallon et al., 2001), two other studies modeled the fibrin gel formation during coagulation (Kuharsky and Fogelson, 2001; Guy et al., 2007) while Haugh modeled the PDGF chemoattractant in wound healing (Haugh, 2006). From such models can be gathered ideas and indications about the way of modeling part of the FBR, but given the peculiarity of this phenomenon, it result not possible to adopt whole sections of those models and integrate them in the FBR one. Although the recognized importance of FBR in determining the performance of electrodes and other implanted devices (i.e. orthopedic prosthesis and insulin dispenser), and its inclination to be modeled literature is unfortunately poor of FBR mathematical model except for an old work by Nichols and colleagues about FBR against aspecific material (Nichols et al., 1979), that is not very useful being missed all the used parameters, and a technical report that faces in particular the FBR to neural implants that introduce the argument, but remands to a future, not yet published, scientific article (Su et al., 2007).
An electrical selectivity model starts from the hypothetical size, shape and electrical properties of an implanted electrode arrays and from the histological features of the neural tissue that is planning to receive the implant and predicts the spatial selectivity of the stimulation. Such tool has been showed to be of fundamental importance when the developers design dimensions and location of active contacts in an electrode array.
An additional reason that justify the development of a FBR model is that all the electrical selectivity models do not take into account that the tissue fibrotic reaction alters the electrical characteristic of the perielectrode area, thus deserves to be modeled as well and inserted among the histological description of implanted tissue.

8.2. Blocks schemes and specifications of the model

In approaching to a *in silico* representation of FBR, focused, as regard as the desired output, upon the thickness of the capsule in a time frame when the reaction stabilizes (about 4 weeks), main aim was to obtain a simplified model, with a low computational demand, but capable at the same time to account for the parameters crucial for our interest, such as main geometrical (size, shape, insertion angle) and chemical (coating surface) properties of the implanting electrode.

To this aim we chose to adopt a lumped component model, described by ordinary differential equations (ODEs model), that assumes some theoretical main simplifications listed in the following:

- Peripheral nervous tissue that receives the implant is considered homogeneous
- In the set of points, laying on a section plane perpendicular to the longitudinal axis of the electrode, that are equally distant from the electrode the evolution of the FBR is equal
- Blood and tissues adjacent to the insertion site (following defined as region D and region C) are an unlimited reservoir of cells compared to the amount recruited in the FBR

Furthermore to be able to define each single equation we went through an intermediate step of blocks conceptual schematization describe in the following.

The model presents an initial on/off stimulus, corresponding to the electrode insertion, that start the inflammatory process through the mechanical damage produced at the insertion site. Variation in size, shape and insertion angle of the electrode modulates the amount of the damage, while, given a particular damage, the biochemistry of the surface coating modulates the further biological process producing the inflammatory reaction. Once this reaction is started, a balance among the reinforcement and the resolution of the inflammation is mainly due to the counter effects of pro and anti-inflammatory cytokines (PIC and AIC). The fibrotic evolution is responsible for the stabilization of the electrode encapsulation. Secondary micromovements of the electrode that act after the insertion push toward an increase and a chronicity of the inflammation. The whole process is schematized in Fig. 8.1

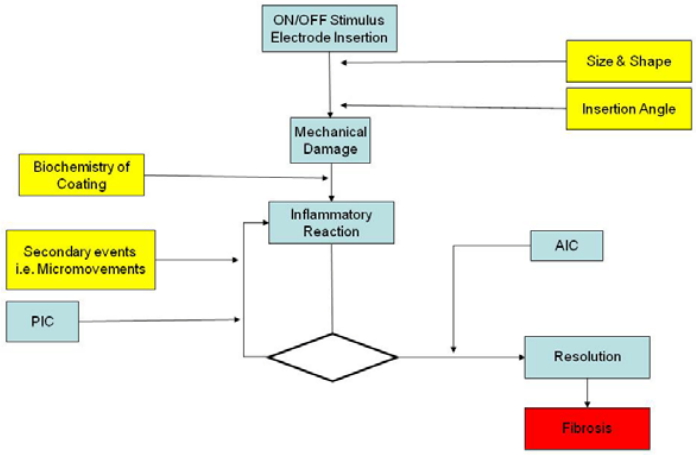

Figure 8.1 – Block scheme overview of the model. In yellow the significant parameters that we change to see how the model reacts, in red is the output of the model.

The strategy followed in the model needs to define four spatial regions where the variables are homogeneus. Going from the electrode surface to the periphery, region A is the tissue-electrode interface occupied by FBGC, region B the peri-electrode tissue made by fibrotic ECM, region C is the ensemble of tissues adjacent to the implant and region D represent the blood volume inside the peri-implant vessels. The electrode is in contact only with region A. Region A

and B are concentric and together form the granulation tissue of the capsule of which the model proposes to give the thickness as its output. Region B is in contact with both region D through the blood vessel wall barrier and with Region C (Fig. 8.2).

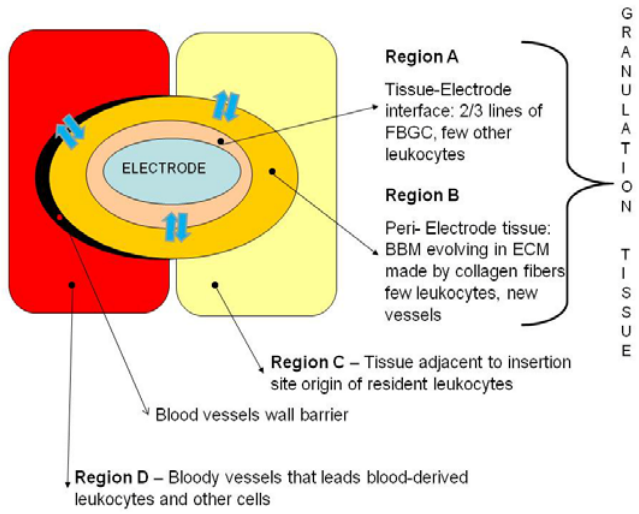

Figure 8.2 - Scheme of the four spatial regions involved in the model seen in a section plane perpendicular to the major axis of the implanted electrode.

In region A temporal evolution of inflammation sees the immediate adsorption of proteins on the electrode surface that work as adaptor for the proximal monocytes adhesion followed by the activation in macrophages cell-type and the fusion to form FBGC. In region B the insertion damage produce the blood vessels rupture and the consequent formation of a blood-based matrix (BBM) that during a four week time period will be remodeled in extracellular matrix (ECM), mainly through the deposition of collagen fibers by activated fibroblast (Fig. 8.3).

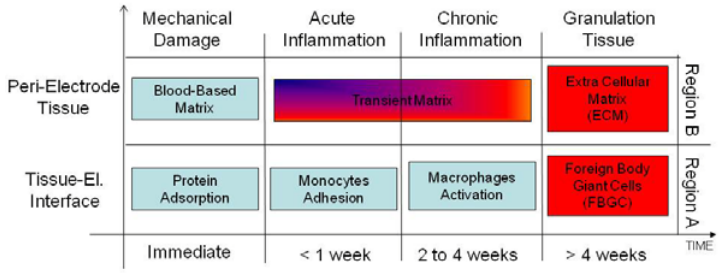

Figure 8.3 – Time evolution of the inflammatory states in the two region composing the fibrotic capsule around the electrode.

The model is based upon a schematization that sees the main cells flows among the different regions moved by the chemokines gradient through the permeability of blood vessels walls for the extravasation and of the blood-based/extracellular matrix for passing across the tissues

(Fig. 8.4). Once arrived in the destination areas, the fibroblast collagen production in region B and the monocytes/macrophages evolution in FBGC in region A are modeled.

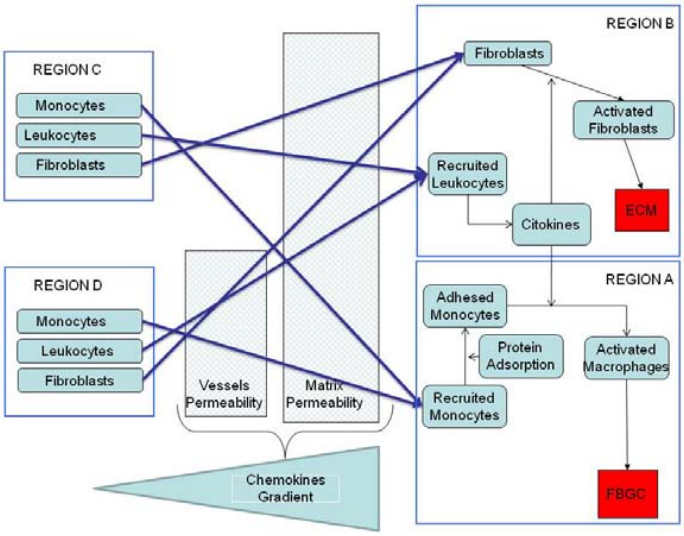

Figure 8.4 – Main blocks scheme of the model. Blue thick arrows represents cell flows.

8.3. Equations of the model

According to the block scheme in Fig. 8.4 the FBR mathematical model can be defined by the dynamic equations of the two main cells in the different model areas: monocytes/macrophages and fibroblasts in region A, B, C and D.

8.3.1 Monocytes dynamics and formation of region A

The dynamics of monocytes/macrophages in region B can be defined by the following equation:

$$\dot{M}^{B} = \dot{M}_{R}^{C} + \dot{M}_{R}^{D} - \gamma\dot{M}_{ADH} + \dot{M}_{BORN}^{B} - \dot{M}_{DIED}^{B} \qquad (1)$$

where:

- $\dot{M}_{R}^{C}$ is the incoming flow of monocytes recruited from region C;
- $\dot{M}_{R}^{D}$ is the incoming flow of monocytes recruited from region D;
- $\dot{M}_{BORN}^{B}$ is a positive flow of monocytes due to cellular proliferation;
- $\dot{M}_{DIED}^{B}$ is a negative flow of monocytes due to cell death;
- $\gamma\dot{M}_{ADH}$ is a negative flow of monocytes due to the adhesion of the cells to the electrode surface.

The elements of eq. (1) can be grouped in two different categories: the first three terms are due to cellular flows among different contiguous regions, while the last two are related to the cellular turn-over.

The $\dot{M}_R^C$ models the flow of monocytes going from region C to region B, generated by the chemokines gradient through the blood based matrix. Mathematically, this can be described with a mass transfer equation, having the chemokines gradient (G_{CCL}) as the motive force which moves the monocytes flow against the resistance of the blood based matrix (R_M):

$$\dot{M}_R^C = \frac{\dot{m}_R^C \cdot G_{CCL}(t)}{R_M} \tag{2}$$

In eq. (2), the term $\dot{m}_R^C$ is the specific monocytes flow which is proportional to the monocytes concentration in region C (M^C).

Analogously, the flow of monocytes going from region D to region B can be defined as follows:

$$\dot{M}_R^D = \frac{\dot{m}_R^D \cdot G_{CCL}(t)}{R_V + R_M} \tag{3}$$

where R_V is the resistance for extravasation through the vessel wall, and $\dot{m}_R^C$ is proportional to the monocytes concentration in region D (M^D).

The term $\dot{M}_{ADH}$, which represents the monocytes migration from region B to region A due to the adhesion with the adsorbed protein upon the electrode coating surface, is modelled as:

$$\dot{M}_{ADH} = (k_{IgG}^{adh} + k_{FIBR}^{adh}) \cdot M^B \tag{4}$$

where k_{IgG}^{adh} and k_{FIBR}^{adh} are the coefficients that represent the capability of induct monocytes adhesion of respectively IgG and fibrinogen, that are the two main proteins that adsorb upon a foreign material surface in the body; these coefficients depend on the material coating the electrode.

The last two terms in eq. (1) refer to natural cell proliferation and death; the proliferation is modeled by a logistic growth, described by the equation:

$$\dot{M}_{BORN}^B = r_M M^B \left(1 - \frac{M^B}{k_{M1}}\right) \tag{5}$$

where r_M is the monocytes growth rate and k_{M1} is the carrying capacity.

On the contrary, the cell death can be approximated with a first order dynamics as:

$$\dot{M}_{DIED}^B = k_D M^B \tag{6}$$

To have a complete description of the system modelled so far, we need to define the time course of some variables used in the previous equations; in particular G_{CCL}, R_V, R_M have to be mathematically defined.
As regards the chemokines gradient (G_{CCL}), its time variation has been defined using a Gaussian function fitting the experimental values for the chemokine MIP-1β taken from (Jones et al., 2007).
The resistance for extravasation R_V is the inverse of the vascular permeability (P_V), which mainly depends on the mechanical insertion damage (D), and can be expressed by the equation:

$$P_V = \frac{1}{R_V} = k_{EV} \cdot f_{PEC}(t) \cdot D \tag{7}$$

where $f_{PEC}(t)$ is a function of the simulation time, and was chosen according with the time-course trend of the pro-extravasation cytokines (PEC) concentration during the inflammatory response to the foreign body. This is justified by the fact that PEC are the major factors accountable for the increment of the vascular permeability. In particular, $f_{PEC}(t)$ reaches its peak quite immediately after the stimulus (electrode insertion) and then follows an exponential decay during the progression of the inflammatory response (see Fig. 8.5). As regards the mechanical insertion damage (D), it depends from the electrode size and shape, and from the insertion angle, defined as the angle between the longitudinal axis of the electrode and that one of the nerve:

$$D = \frac{\pi}{4} \cdot {d_H}^2 \cdot \frac{1}{\sin(\theta)} \tag{8}$$

where d_H is the *hydraulic diameter* of the electrode, defined as 4 times the cross sectional area divided by the perimeter, and θ is the insertion angle.

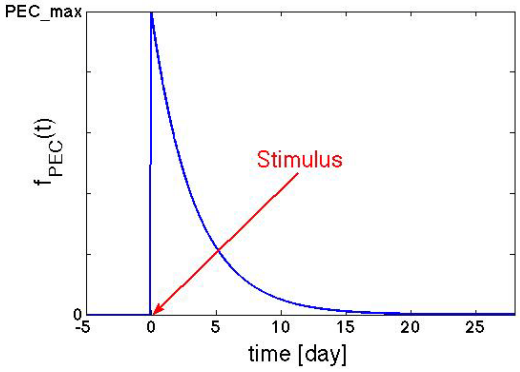

Figure 8.5 – Time evolution of the amount of pro-extravasation cytokines secreted after the insertion (stimulus).

Similar to the resistance for extravasation R_V, the resistance of the blood-based matrix R_M is the inverse of its permeability (P_M). To properly introduce the definition of this function, it could be useful to recall the main physiological mechanisms underlying the formation of the blood-based matrix.

The mechanical damage made by the insertion produces the rupture of blood vessels and an organization clots-like of the extracellular space occupied by a blood-based matrix. During the evolution of FBR the matrix progressively changes toward a fibrosis. This network works as structural scaffold for cells migration through the tissues [see also chapter 6], thus an optimal dimension of the loops of the net, reflected by an optimal matrix density (ρ_M^*), is required for achieving an high matrix specific permeability (p_M). A higher or a lower value of matrix density results in an overall reduction of the matrix permeability (p_M).

Thus, matrix specific permeability (p_M) can be described by a non-monotonic function of matrix density (ρ_M), with a peak value ($p_M^{\max}$) corresponding to the optimal matrix density (ρ_M^*). To this purpose we defined the function $p_M = r_M^{-1} = f(\rho_M)$ as the polynomial function of third degree represented in Fig. 8.6.

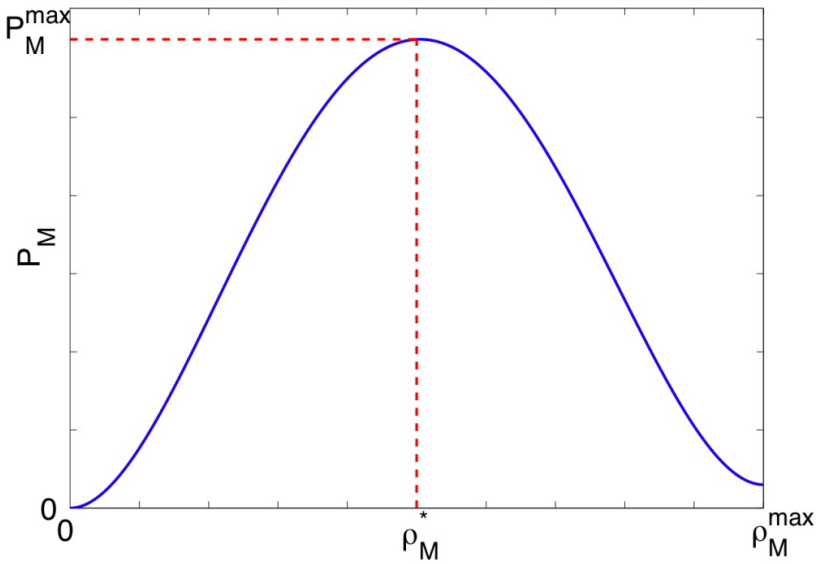

Figure 8.6 – Relation among the matrix specific permeability (p_M) to cellular migration and its density (ρ_M)

As regards the time course of the matrix density (ρ_M) during the inflammation process, it reaches its peak among day 2 and day 8 after the insertion, when it is constituted by platelets and organized fibrin. After the pick ρ_M start to decreases because of the cleaving action of the MMPs that attack the fibrin network. It is reasonable to say that wider and heavier is the damage (D) and more important is the activation of surrounding tissue and faster is the organization of the clot that reaches its density peak in few days and viceversa. Given that the p_M is maximal for an optimal ρ_M (ρ_M^*), that is reached both in the ascending and in the

descending part of the $\rho_M(t)$ curve, the trend of $p_M(t)$, according with this modeling strategy, presents a double-hump shape as showed in the second row of Fig. 8.7.
The value of the pick of the overall matrix permeability (P_M) is further influence by the damage(D), as showed in the last row of Fig 8.7, because the amount of damage influences the extension of matrix involved in the cellular migration: ($P_M = p_M \cdot hD$).

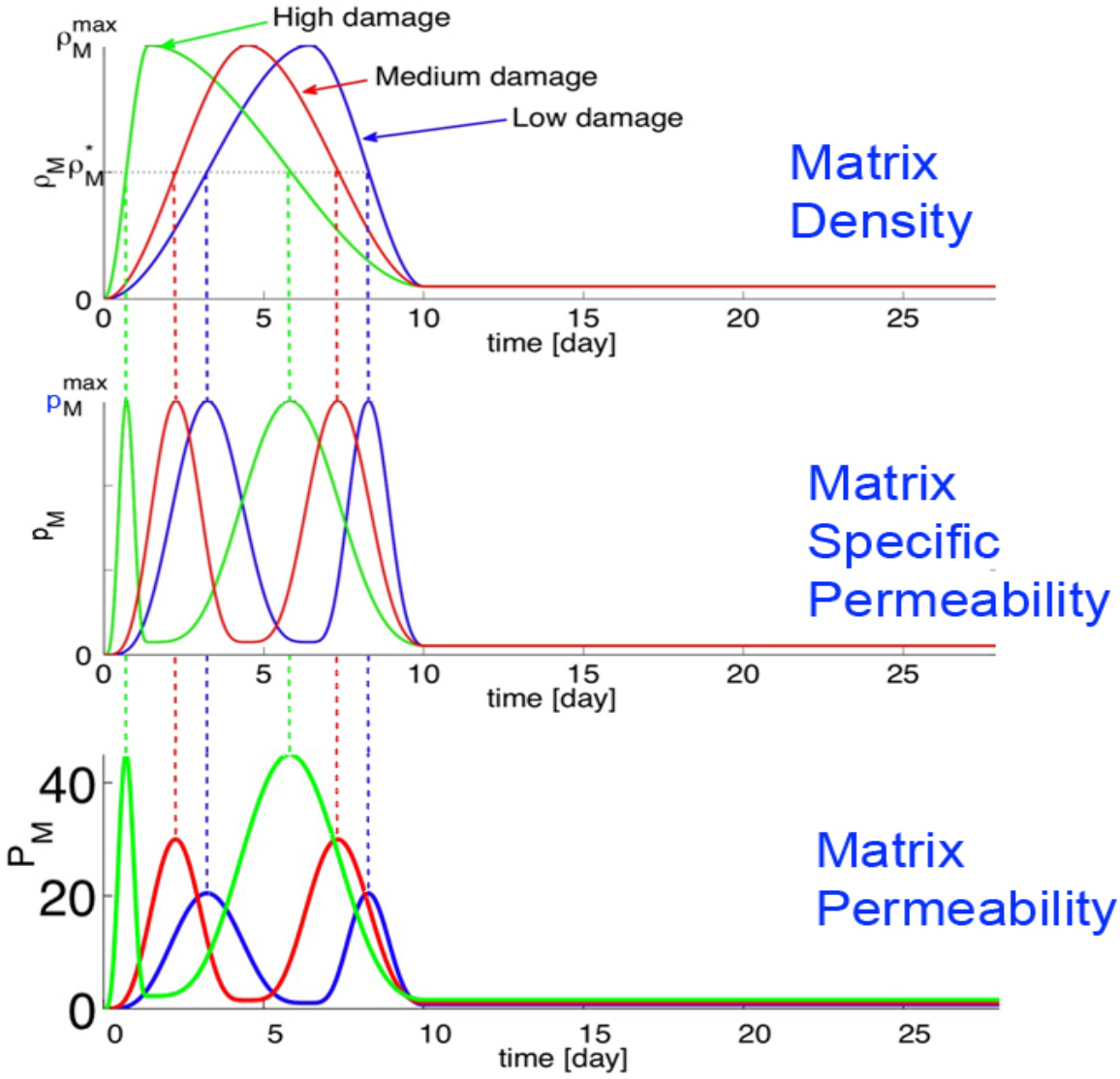

Figure 8.7 – In the upper row the time evolution of the density of the matrix (ρ_M), in the second row the matrix specific permeability (p_M) and in the lower row the matrix permeability (P_M). Different colors correspond to different grade of the damage (D).

Once we obtain the concentration of adhered monocytes from Eq. 4 the next equation define the concentration of FBGC in region A (expressed as the number of FBGC per surface unit).

$$FBGC = (k_{IgG}^{fus} + k_{FIBR}^{fus}) \cdot M_{ADH} \tag{9}$$

where

k_{IgG}^{fus} and k_{FIBR}^{fus} are the constant of fusion for M_{ADH} toward generate FBGC relative to IgG and Fibrinogen respectively taken from (Shen and Horbett, 2001; Shen et al., 2004) and change during the time of simulation with a sigmoidal shape with plateau. (Jones et al.,2007; Rodriguez et al., 2009).

FBGC are the main component that constitute Region A of the FBR capsule and in this region are tight packed forming a dense component.
Their spatial organization can be geometrically schematize assuming that the shape of a giant cell is similar to a thin ellipsoid with its major area (axes: α_{FBGC}^{maj} and $\alpha_{FBGC}^{\min}$) smashed toward the electrode surface, with the aim of incorporating the foreign body, and the thickness twenty fold smaller than the minor axis ($\alpha_{FBGC}^{Thick} = \frac{1}{20}\alpha_{FBGC}^{\min}$). Indeed FBGCs in region A are packed with one of this α_{FBGC}^{Thick} axis directed toward the electrode. According to this schematization, the thickness of region A can be mathematically obtained from the next equation:

$$Thick^{A} = \alpha_{FBGC}^{Thick} \cdot L_{FBGC} = 1/20\, \alpha_{FBGC}^{\min} \cdot L_{FBGC} \tag{10}$$

where:

$\alpha_{FBGC}^{\min}$ is the minor axis of the ellipsoid representing the FBGC shape;

L_{FBGC} is the number of layers made by FBGC around electrode surface.

L_{FBGC} depends on FBGC concentration as defined in the following equation:

$$L_{FBGC} = FBGC \cdot \overline{S_{FBGC}} \tag{11}$$

Where $\overline{S_{FBGC}}$ is the mean value of the area of a FBGC maximal surface along a section plane perpendicular to the longitudinal axis of the electrode and passing through the major axis of the ellipsoid (α_{FBGC}^{maj}) and is reported in literature (Anderson 2000).
According to the geometrical schematization of FBGC given above, this area corresponds to:

$$\overline{S_{FBGC}} = \pi \cdot (\frac{\alpha_{FBGC}^{maj}}{2} \cdot \frac{\alpha_{FBGC}^{\min}}{2}) \tag{12}$$

And the minor axis is

$$\alpha_{FBGC}^{maj} = 2 \cdot \alpha_{FBGC}^{\min} \tag{13}$$

Thus we have that:

$$\alpha_{FBGC}^{Thick} = 1/20\, \alpha_{FBGC}^{\min} = 1/20 \sqrt{\frac{2 \cdot \overline{S_{FBGC}}}{\pi}} \tag{14}$$

Substituting the eq. (11) and (14) in (10) the thickness of region A is:

$$Thick^{A} = 1/20 \sqrt{\frac{2}{\pi}} \cdot \left(\overline{S_{FBGC}}\right)^{3/2} \cdot FBGC \tag{15}$$

8.3.2 Fibroblasts dynamics and formation of region B

The process involving fibroblast have been already described in chapter 6 paragraph 6.2.7.
Similarly as seen for monocytes, fibroblast are recruited both from contiguous tissues (region C) and from the blood (region D) thanks to a CXCL family chemokines, mainly IL-8, gradient (G_{CXCL})through the resistance of blood vessels wall (R_V) and the extracellular matrix (R_M).
The flow of recruited fibroblasts from region C are modelled as:

$$\dot{F}_R^C = \frac{\dot{f}_R^C \cdot G_{CXCL}(t)}{R_M} \tag{16}$$

Where:
$\dot{f}_R^C$ is the specific fibroblasts flow which is proportional to the fibroblast concentration in region C (F^C).
Analogously, the flow of fibroblast going from region D to region B can be defined as follows:

$$\dot{F}_R^D = \frac{\dot{f}_R^D \cdot G_{CXCL}(t)}{R_V + R_M} \tag{17}$$

where R_V is the resistance for extravasation through the vessel wall, and $\dot{f}_R^C$ is proportional to the fibroblast concentration in region D (F^D).
Henceforth fibroblasts dynamics and collagen secretion has been modeled by deriving most of the equations from the work proposed by Dale et colleagues about the collagen formation in dermal wound healing (Dale et al., 1996) . Main actors of this process are fibroblast, collagen and the family of its cleaving enzymes called metalloproteinases (MMPs) including the collagenase, and the pro-fibrotic cytokine TGFâ. Both MMPs and TGFâ are produced by fibroblasts and adhered monocytes activated in macrophages histotype (Fig. 6.7).

Fibroblast concentration in region B (F^B) is modeled with a logistic growth term, which represents mitotic generation (first term of equation 18). Cell growth is enhanced by TGFâ, where a_1 and a_2 are the parameters regulating the growth rate and k_c is the carrying capacity of the environment. Cells die at a constant rate a_3. The equation for fibroblast density is the following:

$$\dot{F}^B = (a_1 + a_2 TGF)F^B\left(1 - \frac{F^B}{k_c}\right) - a_3 F^B + \dot{F}_R^C + \dot{F}_R^D \tag{18}$$

Fibroblast proliferation and collagen synthesis are up-regulated by the cytokine called TGFâ. Concentration of TGFâ is governed by an autocrine mechanism in the fibroblasts described by the first term of Eq. 19. Natural decay of TGFâ is modeled as a first order process with time constant A_6 (Sporn and Roberts, 1990). The last term corresponds to TGFâ production by adhered monocytes.

$$\dot{TGF} = \frac{a_4 fTGF}{1+a_5 TGF} - a_6 TGF + a_7 M_{ADH} \tag{19}$$

MMPs bind to collagen breaking down the fibers. They are secreted by fibroblasts, but the secretion is inhibited by the presence of TGFâ (Jeffrey, 1992). Again, the natural decay is taken to be of first order. The last term corresponds to production by adhered monocytes.

$$\dot{MMP} = \frac{a_8 F^B}{1 + a_9 TGF} - a_{10} MMP + a_{11} M_{ADH} \tag{20}$$

Collagen concentration depends on the concentration of fibroblasts and TGFâ, as described by the first term of the following equation. Collagen is degraded by MMPs as described by the last term of the equation.

$$\dot{C} = (a_{12} + a_{13} TGF)F^B - a_{14} MMP \cdot C \tag{21}$$

where:
$a_{12}F^B$ is the basal production of collagen;
a_{13}TGF F^B is the amount of collagen production induced by TGFâ.

Assuming that the typical average concentration of collagen in region B ($\overline{C_B}$) is known, it is possible to estimate the superficial concentration of collagen as the product of this average concentration ($\overline{C_B}$) by the thickness of region B ($Thick^B$). At the same time, this superficial concentration has to be equal to the time integral of the superficial production of collagen, expressed by the product of collagen production ($\dot{C}$) by the same thickness $Thick^B$.
Thus, the following equation is able to describe the relation between collagen production ($\dot{C}$) and thickness of region B ($Thick^B$):

$$\int_{t_0}^{t} \dot{C} \cdot Thick^B \, dt = \overline{C_B} \cdot Thick^B \tag{22}$$

By differentiation both the terms of eq. (22), the following equation expresses the growth rate of the thickness of region B in function of collagen production ($\dot{C}$):

$$\dot{Thick}^B = \frac{\dot{C} \cdot Thick^B}{\overline{C_B}} \tag{23}$$

The comprehensive thickness of the capsule around the electrode ($Thick^{FBR}$) is given by the sum of the thicknesses of the region A and region B:

$$Thick^{FBR} = Thick^A + Thick^B \tag{24}$$

8.4. Simulation

For this preliminary simulation of the model has been considered an electrode with its geometrical properties resembling the tf-LIFE4s, but implanted perpendicularly to the nerve. Adhesion and fusion percentage of monocytes and chemokines gradients have been gathered from data reported by (Jones et al.,2007; Rodriguez et al., 2009) for polyethylene terephthalate (PET), that presents the same water contact angle than polyimide (about 70^{o}).
The model has been implemented in MATLAB/Simulink (The Mathworks, Natick, MA) using ODE45 function as solver for the differential equations (Fig 8.8)

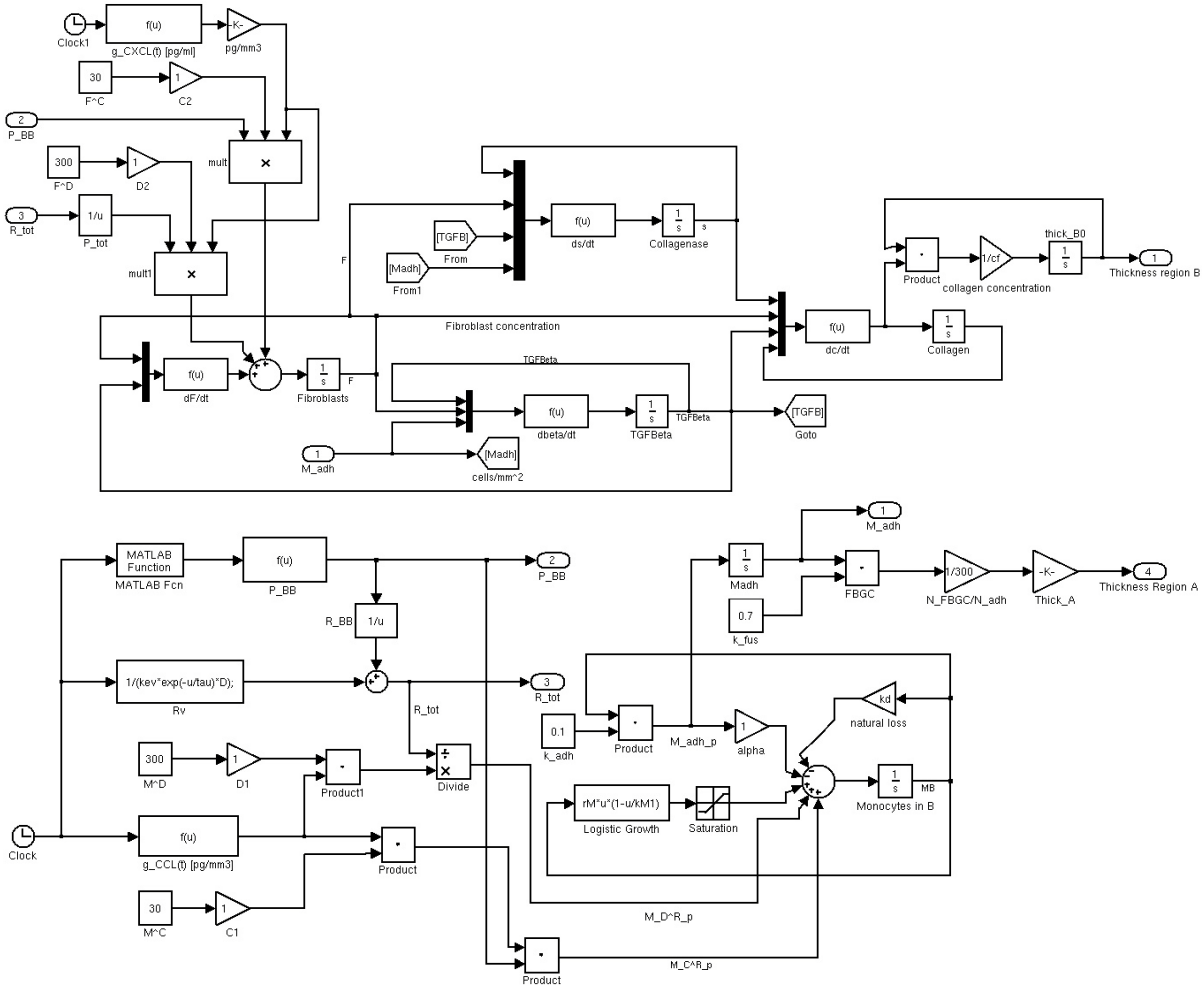

Figure 8.8 - Functional block scheme of the model as implemented in Simulink

8.5. Results

The follow graphs present the time course of the number of FBGCs for square millimeter, number of fibroblasts in region B for cubic millimeter and the thickness of region A, B and the total thickness of the capsule. In agreement with the fact that the reaction became stable after about 4 weeks since the insertion values tend to stabilize when overcome day 25 (Fig 8.9).

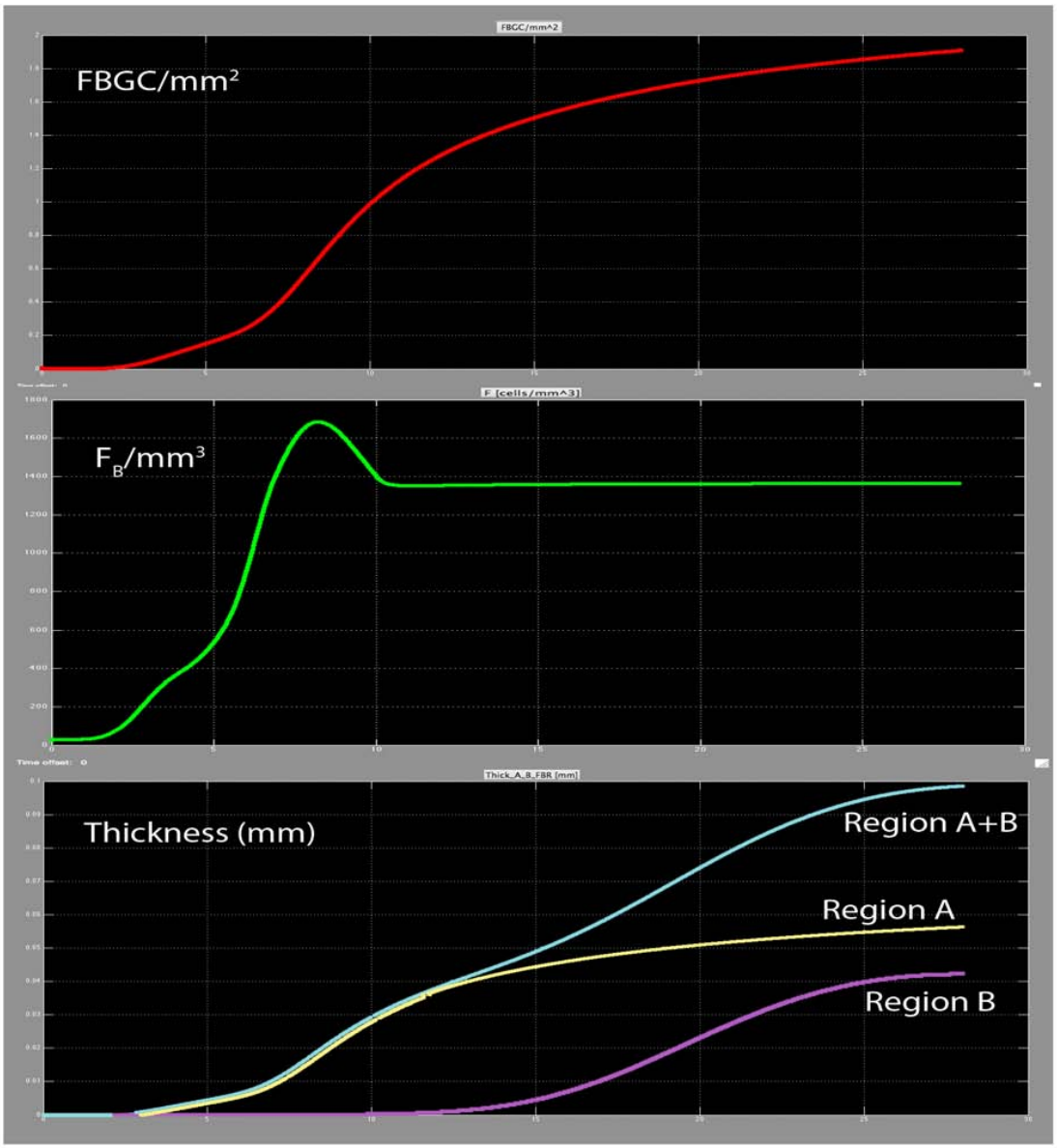

Figure 8.9 - Time course of the number of FBGCs for square millimeter, number of fibroblasts in region B for cubic millimeter and the thickness of region A, B and the total thickness of the capsule

The comprehensive thickness of the capsule becomes stable for values close to 100 µm as described by literature (Lago et al., 2007).

8.6. Discussion and future steps

Aim of this model is to gather an idea of the influence of geometrical and chemical parameters that characterize an electrode upon the foreign body reaction produced by the implantation of the electrode in a peripheral nerve. This tool is useful even before that the electrode is fabricated and tested, thus allowing electrode developers to spare time and economic resources and to evaluate several possible hypothetical solutions for accounting the tissue-electrode mismatch.

The dynamics of the processes have been modeled starting from a deep analysis of the literature about FBR, in particular in the nervous system. The parameters have been taken from published articles, favoring especially the data regarding the PNS, or have been settled according with the most plausible results of simulations. The output of the simulation with an electrode that resemble the characteristic of tfLIFE is consistent with the histological description reported by Lago et colleagues (Lago et al., 2007). Nevertheless the model will profit from a experimental validation in vitro and in vivo, adopting different materials as foreign body or different coating surfaces.

Because biocompatibility is a functional characteristic, which strictly speaking requires an analysis of tissue response using an electrically functional implant, the future version of the model should take into account also the electrical properties of a stimulating/recording working electrode.

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Chapter 9

Overview of the results of the PhD program and scientific literature production.

9.1. Summary of the results of the PhD course

The three years spent at the Laboratory of Biomedical Robotics & Biomicrosystem gave me the opportunity to work in a cloudless familiar environment, closely to young and qualified biomedical engineers, that braced me the value of acting with a systematic, rigorous method even when investigating such undeterministic system as the human living being and its pathologies. In my opinion this represents the more significant outcome of my PhD course.
Going more in particular, the achieved results involves the realization of a deep and critical analysis of the state of the art on neural interfaces, hand prostheses and nervous system plasticity that led to publish two review articles as first author in high impact factor journals. During the investigation of ways for brain-computer communication and of their possible applications, I went through the suitable appliances of such devices in space science and in particular an insect-machine hybrid controller, for improving autonomy of exploratory vehicle, that exploits neural interfaces for biological-artificial connection has been conceptualized.
All the aspects of the LifeHand experience, the implant in human of intraneural multielectrodes for the control of a cybernetic hand prosthesis and for sensory feedback delivery, have been personally and closely followed. My efforts have been spent in particular on the setting of the recording/stimulating system, the organization of the clinical protocol, the formulation of the stimulation protocol and the integration of the whole system, the evaluation of phantom limb pain and the management of the patient during the training sessions. A strong interconnection among the continuative use of neurally-interfaced hand prostheses, curative neuroplasticity and reduction in PLP has been first theorized and then demonstrated with the result of LifeHand. This achievements open new possibility for the phantom limb syndrome treatment, that up today still miss a resolutive therapy strategy, and sustain the feasibility of a research line of profound interest.
As regard the conceptualization of innovative neural interfaces, new bioactive proteic coating for polyimide-based electrodes has been realized for reducing the tissue-electrode mismatch and the foreign body reaction against the implant. Being the reduction of FBR a main issue for electrode developers a further analysis of the state of the art was dedicated to a deeper comprehension of this particular inflammatory reaction. The acquired knowledge gave the basis for the designing of an ODE model of foreign body reaction against an electrode implanted in a peripheral nerve, in order to be able to predict before the fabrication process, how changes the

thickness of the encapsulation depending on geometrical characteristics of the electrode and biochemistry of its surface. With its preliminary simulations the model gave outputs that have a satisfactory matching with the data collected from the literature.
Fig 9.1 resumes with a blocks scheme the path I followed within the PhD program.

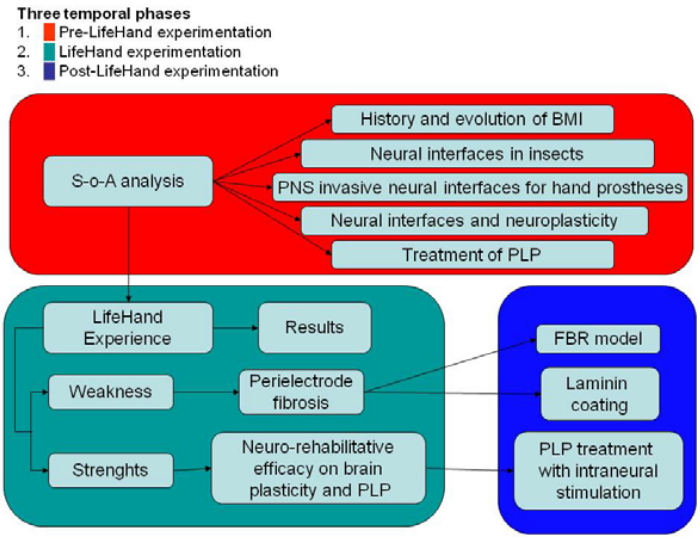

Figure 9.1 – Blocks scheme summarizing activities and results of the PhD program.

9.2. Scientific Literature Production

In the following the scientific literature production during the three years of PhD course is presented:

9.2.1 Journals

I.F.

Di Pino G, Guglielmelli E, Rossini PM (2009) Neuroplasticity in amputees: main implications on bidirectional interfacing of cybernetic hand prostheses. Prog Neurobiol 88:114-126. 9.130

Di Pino G, Seidl T, Benvenuto A, Sergi F, Campolo D, Accoto D, Maria Rossini P, Guglielmelli E (2009) Interfacing insect brain for space applications. Int Rev Neurobiol 86:39-47. 6.588

Micera S, Citi L, Rigosa J, Carpaneto J, Raspopovic S, **Di Pino G**, Rossini L, Yoshida K, Dario P, Rossini PM (2010 In Press) Decoding sensory and motor information from neural signals recorded using intraneural electrodes: towards the development of a neurocontrolled hand prosthesis. Proc IEEE. 3.820

Rossini PM, Micera S, Benvenuto A, Carpaneto J, Cavallo G, Citi L, Cipriani C, Denaro L, Denaro V, **Di Pino G**, Ferreri F, Guglielmelli E, Hoffmann KP, Raspopovic S, Rigosa J, Rossini L, Tombini M, Dario P (2010 In Press) Double nerve intraneural interface implant on a human amputee for robotic hand control.Clin Neurophysiol — 2.972

Benvenuto, F. Sergi, **G. Di Pino**, T. Seidl, D. Campolo, D. Accoto and E. Guglielmelli (2009), "Beyond biomimetics: towards insect/machine hybrid controllers for space applications", Advanced Robotics, 23 (7-8):939-953 — 0.737

Article in preparation:

- *Readdressing of neuroplasticity and improvement in PLP and syndrome due to the neuro-rehabilitative effects of Lifehand system*
- *Does intraneural multielectrodes design fulfill implantation requisites: Lessons stemming from the surgical act in human amputee*
- *Review of PLP treatments*
- *ODE model of FBR around an implanted peripheral nerve intrafascicular electrode*
- *Bioactive-coatings for FBR reduction*

I.F.

9.2.2 Conference Proceedings

S. Micera, J. Rigosa, J. Carpaneto, L. Citi, S. Raspopovic, E. Guglielmelli, A. Benvenuto, L. Rossini, **G.Di Pino**, G. Cavallo, M.Carrozza, P. Dario, P. M. Rossini,"On the control of a robot hand by extracting neural signals from the PNS: preliminary results from a human implantation", 31st Annual International Conference of the IEEE Engineering in Medicine and Biology Society (EMBC'09), Minneapolis, Minnesota, USA, 2nd - 6th September, 2009, 4586-4589 — 0.784

Benvenuto, F. Sergi, **G. Di Pino**, D. Campolo, D. Accoto, E. Guglielmelli and T. Seidl, "Conceptualization of an Insect/Machine Hybrid Controller for Space Applications", in Proceedings of the 2008 IEEE International Conference on Biomedical Robotics and Biomechatronics (BIOROB 2008), Scottsdale, USA, October 19-22, 2008, 306-310

9.2.3 Poster Presentation

Di Pino G, Ferreri F, Tombini M, Micera S, Carrozza MC, Benvenuto A, Cavallo G, Rossini L, Guglielmelli E, Rossini PM. Efficacia in neuroriabilitazione di una protesi cibernetica di mano con interfacciamento neurale bidirezionale: effetto sulla plasticita' corticale aberrante connessa alla sindrome da arto fantasma. In Neuroriabilitazione Robotica dell'arto superiore. Genova 14-15 Dic 2009.

G. Di Pino, L. Rossini, L. Zollo, E. Guglielmelli. Dependability analysis of Brain Machine Interface for Cybernetic Prosthesis control. Poster Presentation at the Sixth IARP-IEEE/RAS-EURON Joint Workshop on Technical Challenges for Dependable Robots in Human Environments

G. Cavallo, M. Tombini, **G. Di Pino**, G. Curcio, P. M. Rossini, E. Guglielmelli.Brain training softwares: is their efficacy real and influenced by age? A preliminary report. Poster Presentation at the 6 International Conference of the International Society for Gerontechnology 2008

A. Benvenuto, **G. Di Pino,** F. Sergi, D. Campolo, D. Accoto, G..Assenza, PM Rossini, E. Guglielmelli. "Machine/Animal Hybrid Controller for Space Applications", Poster Presentation at GNB'08

9.2.4 Report

Benvenuto A, **Di Pino G**, Sergi F, Campolo D, Accoto D, Assenza G, Rossini PM and Guglielmelli E. Machine/Animal Hybrid Controllers for Space Applications. Esa/Ariana 07/6301 Final Report http://www.esa.int/gsp/ACT/doc/ARI/ARI%20Study%20Report/ACT-RPT-BIO-ARI-07-6301-MachineAnimalHybridController-Rome.pdf

9.2.5 Book Chapter

G. Di Pino, T.Seidl, A. Benvenuto, F. Sergi, D. Campolo, D. Accoto, P.M. Rossini, E.Guglielmelli "Interfacing insect brain for space applications", in "Brain Machine Interfaces for Space Applications: Enhancing Astronauts' Capabilities", L.Summerer, D. Izzo, L. Rossini (eds.), Elsevier.

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