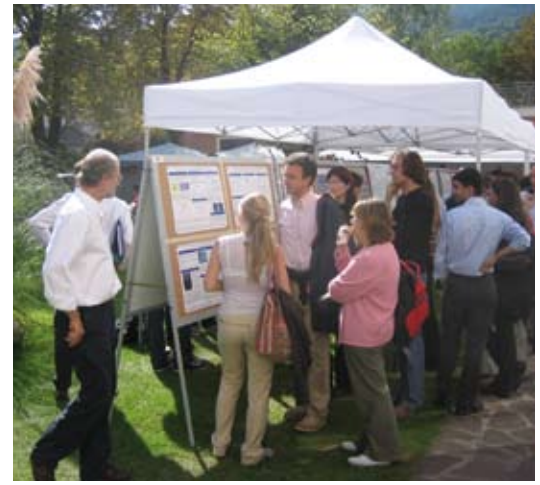
DOCTORAL PROGRAM IN BIOENGINEERING

Chair:
Prof. Maria Gabriella Signorini

The Doctoral Programme in Bioengineering trains graduate students through a strong interdisciplinary education on engineering, mathematics, medical and biological knowledge to develop high level engineering problem-solving abilities in life sciences inside a research group or in private or public industrial context.
Students are involved in research works in fields currently ongoing at the Bioengineering Department of Politecnico di Milano which organizes the PhD track.
PhD students in Bioengineering are about 15 per year, around 50 in the three year course.
Research themes include modelling and analysis of physiological data, signals and systems; biomedical imaging processing and technologies; technologies and instrumentation for movement analysis, rehabilitation, ergonomics and sports; therapeutic devices and life support systems in cardiology, cardio/surgery and pneumology; design and assessment of prostheses; computer aided surgery and surgery optimization through modelling; cardiovascular fluid dynamics; molecular, cellular and tissue engineering for biomaterials and prostheses; neuro-engineering and nanobiosystems; genomic and proteomic data analysis; bioinformatics. Stage periods in distinguished research institutes in Italy and abroad are an essential feature of the student training.

The educational offer includes ad hoc advanced courses specifically projected for the Ph.D. Among them, the school of the National Bioengineering Group is held every year since 1981 for one week in Bressanone(BZ). The content of the School is focused on themes of the bioengineering research and knowledge and it is organised with the support of national and international qualified teachers in the specific field coming both from academic and industrial research. The school is also a unique opportunity to put together students from different Doctoral Programs coming from the entire country. This allows exchanging ideas and experiences also representing a very useful educational event.

Some themes of the recent past editions:
2004 Advanced mthods of biomedical signal processing
2005 Biomaterials: from protesic implants to regenerative medicine
2006 Neuro-Robotics. Neuroscience e robotics for the development of intelligent machines
2007 Computational Genomics & Proteomics
2008 Wearable Intelligent Devices for Human Health and Protection



2009 Bioengineering for Cognitive Neurosciences
2010 Synthetic Biology

Scientific and research PhD activities receive a strong support by Laboratories located inside and outside the Department in cooperation with other research bodies and university hospitals:

- Laboratory of 2D-3D analysis and modelling of neural and sensory systems and bioelecromagnetism
- Biomaterials Laboratory
- Laboratory of biocompatibility and cell culture – BioCell
- Laboratory of Biological Structure Mechanics – LABS
- Laboratory of Computational Biomechanics
- The "Luigi Divieti" Posture and Movement Analysis Laboratory
- Laboratory of micro and bio fluid dynamics
- Biomedical Signal Processing Laboratory
- Medical Informatics Laboratory
- Biomedical Technologies Laboratories

The PhD in Bioengineering has an Advisory Board which has in charge all the student activities

The External Reference Committee is a fundamental link toward the industrial research, the clinical applications with an european and international perspective.

The interest toward the activities of the Ph.D in Bioengineering is demonstrated also by the external financing of 3 years PhD Fellowships. Some recent supporters, besides the Bioengineering Department, of our PhD are are listed in the advisory board.

In 2010 new PhD positions as **Executive PhD**'s have been created. They consist of a special PhD path organized in 4 years and dedicated to PhD candidates that already work in a company/society. The Bioengineering PhD opened 3 positions in 2010 (Fraunhofer Institute, Erlangen, Germany; Istituti Ortopedici Rizzoli, Bologna; SKE S.r.l. Milano) and in 2011

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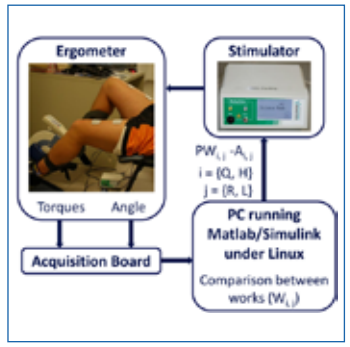
CYCLING INDUCED BY ELECTRICAL STIMULATION:
a sensorized cycle-ergometer to evaluate motor recovery and provide interventions for post-stroke lower limb rehabilitation

Emilia Ambrosini

Hemiparesis is a partial loss of motor function of one side of the body, mainly provoked by stroke, which ranks as the leading cause of long-term disability. The recovery of walking ability is the main goal of post-stroke lower limb rehabilitation. However, specific treatments for gait are limited, requiring extensive assistance for subjects with unstable balance and muscle weakness. Within this context, the main goal of the thesis is to enhance post-stroke lower limb rehabilitation by developing innovative treatments for the restoration of locomotion and by defining new assessment tools to quantify deficits and motor recovery. Pedaling has been chosen as the targeted motor task to define interventions aimed at the recovery of locomotion. Two rehabilitative methods, investigating different ingredients of the motor relearning process, have been proposed: a cycling training induced by Functional Electrical Stimulation (FES), and a biofeedback (BF) cycling training.
Concerning the first method, a double-blind randomized controlled trial was carried out to evaluate whether FES-cycling is an effective training in improving motor functions and walking ability on post-acute hemiparetic patients. 35 patients were recruited and randomized to receive FES-cycling or placebo FES-cycling. The 4-week treatment consisted of 20 sessions lasting 25 minutes each. Outcome measures were the Motricity Index (MI) and gait speed. Participants were assessed before, after training, and at 3- to 5-month follow-up visits. Repeated-measures ANOVA revealed significant increases in both outcome measures after training and at follow-up assessments for FES-treated patients (see Table 1). No outcome measures demonstrated significant improvements after training in the placebo group. A main effect favoring FES-treated patients was demonstrated by repeated-measures ANCOVA for MI. The study demonstrated that FES-cycling training significantly improves lower extremity motor functions and accelerates the recovery of locomotion in post-acute hemiparetic patients, providing a first clinical evidence about a carry-over effect from pedaling to locomotion.
To investigate the effect of patients' involvement in the therapy, a BF cycling training was proposed and tested on 3 chronic stroke patients. The training consisted of a 2-week treatment of 6 sessions. A visual BF helped the patients in maintaining a symmetrical contribution of the legs during pedaling. Participants were assessed before, after training and at follow-up (one week after treatment). Outcome measures were the pedaling unbalance (*U*) and a gait symmetry index (ie, the ratio between stance time in percentage of stride time computed for each leg). An intra-subject statistical analysis showed that all patients significantly decreased *U* after treatment and maintained a significant improvement with respect to baseline at follow-up (see Table 2). The intervention improved also gait symmetry in one subject. The study demonstrated that the treatment is feasible and may be effective in translating progresses from pedaling to locomotion. Naturally, a larger and controlled study is needed to confirm these results.
The final outcome of the thesis consists of a device able to provide different rehabilitation treatments, to assess motor recovery, and to help the physicians in choosing the optimal treatment for each patient. Starting from a pre-existing cycle-ergometer customized to measure the torque signals produced at the crank arms during pedaling, the proposed interventions (FES-cycling and BF cycling training) have been integrated on the same setup. This ergometer has been used also as a starting point for the definition of an automatic procedure to identify the stimulation parameters required for the application of FES-cycling every session on each patient. This procedure permits both to reduce the time needed for setting-up the training and to optimize the training performance, thereby having a potential impact on the clinical application of FES-cycling.
Besides, the system has been enriched by a symmetry controller for FES-cycling that makes the training more specific for hemiparetic patients, being the recovery of motor symmetry a crucial aspect of post-stroke lower limb rehabilitation.
This control system adjusts the stimulation parameters (pulse width and amplitude) delivered to both quadriceps and hamstrings in order to guarantee a symmetrical pedaling in terms of mechanical works produced at the pedals. Figure 1 shows the experimental setup. Preliminary trials on healthy volunteers and hemiparetic patients were performed to validate its operation.
Finally, the system can be used as a tool to assess patients' performance during pedaling, providing useful information both at the beginning (to choose the optimal treatment) and at the end (to evaluate progresses) of a rehabilitation program.
The resulting device, thanks to its flexibility, ease of use, and low cost, could have a strong impact on post-stroke lower limb rehabilitation. With the proposed interventions, the entire range of mildly to severely affected patients should be able to perform intensive and task-specific exercises characterized by active, repetitive movements. Moreover, the safety of the cycling movement, which does not require the constant supervision of a therapist, and the already listed features make the system an interesting option for the home rehabilitation of hemiparetic patients.



1. Experimental setup of the symmetry controller for FES-cycling (PW: pulse width; A: amplitude; W: FES-induced work; Q: quadriceps; H: hamstrings; R: right; L: left).

	GROUP	PRE*	POST*	FOLLOW-UP*	P†	P‖
MI	Placebo	45 (34)	55 (29)	63 (25)	§	<0.01
	FES	39 (26)	69 (29)	79 (24)	‡ §	
GS	Placebo	0.1 (0.2)	0.3 (0.3)	0.5 (0.5)	§	0.34
	FES	0.1 (0.2)	0.4 (0.3)	0.6 (0.3)	‡ §	

Table 1. Comparisons of pre-, post-training and follow-up outcome measures (FES-cycling study)

MI: Motricity Index; GS: gait speed (m/s)
*Mean(Standard Deviation)
†*P*: Significance level of repeated-measures ANOVA
‡ significant difference between Pre and Post
+ significant difference between Post and Follow-Up
§ significant difference between Pre and Follow-Up
‖*P*: Significance level of repeated-measures ANCOVA (baseline as covariate)

		PRE*	POST*	FOLLOW-UP*	P-VALUE†
S1	U (%)	32 (8)	25 (10)	18 (7)	<0.01 ‡ + §
	ST	0.9 (0.04)	0.9 (0.03)	0.9 (0.04)	0.32
S2	U (%)	45 (8)	29 (13)	40 (14)	<0.01 ‡ + §
	ST	0.6 (0.05)	0.7 (0.03)	0.8 (0.05)	<0.01 ‡ §
S3	U (%)	38 (9)	12 (10)	14 (10)	<0.01 ‡ §
	ST	0.8 (0.04)	0.9 (0.05)	0.8 (0.07)	0.15

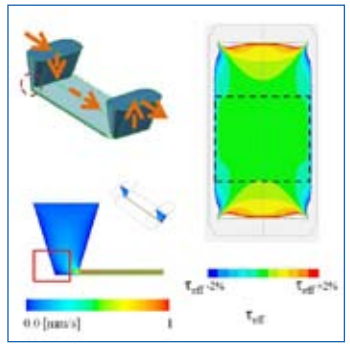
Table 2. Comparisons of pre-, post-training and follow-up outcome measures (BF cycling study)

U: pedaling unbalance; ST: gait symmetry index
* Mean (Standard Deviation)
† P: Significance level of repeated-measures ANOVA
‡ significant difference between Pre and Post
+ significant difference between Post and Follow-Up
§ significant difference between Pre and Follow-Up

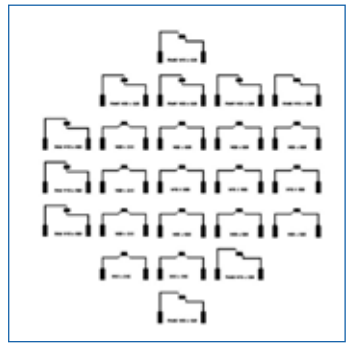
MICRODEVICES FOR HIGH-THROUGHPUT CELLULAR ADHESION ASSAYS

Elena Bianchi

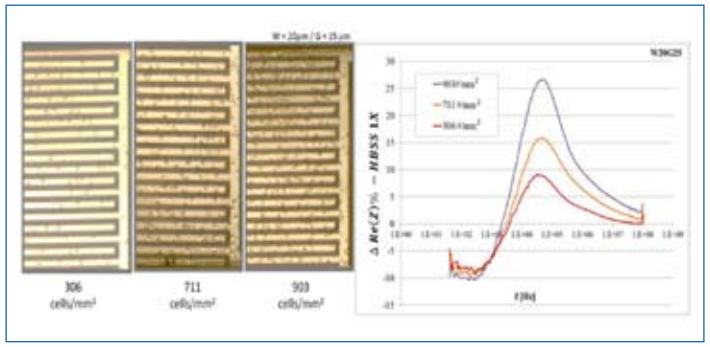
The essential role of leukocytes in inflammation and the knowledge of their adhesion cascade are crucial study areas in developing methods to control inflammation by modulating or blocking leukocyte adhesion to the endothelium. A defective leukocyte functionality often results in a decreased capacity for an inflammatory response or, on the opposite, in a chronic inflammation status. Development of anti-inflammatory drugs is based on the research on inflammation and in vitro model systems are the main tools used to mimic chemotactic gradients and the perform assays under flow condition.
A high-throughput approach can improve results in this field, enabling very rapid, intelligent, parallel experimentations, and increasing productivity by orders of magnitude over traditional approach. High-Throughput screening is an experimental scientific approach, to quickly conduct multiple biochemical, genetic or pharmacological tests. Active compounds, antibodies or genes, which modulate a particular biomolecular pathway, can be identified reducing time to result, the impact of the operator and of the variability of multiple samples on the final results of the assay.
Parallelization is a fundamental stone of high-throughput analyses although, in order to maximize the benefits that a high-throughput platform provides, the biological response must also be assessed in a high-throughput manner: advances in software and electronic systems are fundamental to acquire, deal with and interpret the huge amount of data generated for each assay.
In this context a device for parallel leukocytes adhesion assays under flow condition was proposed and designed.
Two microdevices dedicated to high-throughput biological analyses were considered, designed and studied: a multiple parallel flow-chamber for cellular adhesion and a microsensor to detect presence and adhesion of cells in a test chamber. A multiple flow-chamber microdevice was designed to contribute to the study of leukocytes adhesion and extravasation process through a membrane, one of the most interesting phenomena to observe for in vitro studies of the mechanisms ruling the inflammatory reaction. Such studies are usually performed in a parallel flow-chamber, where cells, forced to flow in suspension, are free to interact with a specifically coated surface. The aim of this study is the design of a high-throughput microdevice oriented to improve the efficiency and the efficacy of shear dependent adhesion and transmigration assays.
The considered microfluidic device is made of two main superimposed parts (40 x 15 x 0:7 mm, 45 x 15 x 0:7 mm). The two glass parts are aligned and constricted one on the other by means of a custom made PEEK (Polyether ether ketone)-steal holder. It consists of several parallel flow-chamber, three groups of three.
Each chamber shows a flow-section area of 1,5 mm width and 0,05 mm of thickness for 3 mm of length. The bottom glass part of the device includes wells interconnected by microchannels: all the wells face a corresponding chamber in the upper layer.
Two glass parts are combined in a micro-fabricated glass device and completed with a PDMS gasket and a polymeric membrane. The design is intended to save cell culture medium and reagents involved in a standard assay: using a flow rate of 450-600 ml/min, three high efficiency tests can be performed at the same time on the same device, each corresponding to a different level of wall shear stress and obtaining for each one a triplicated result. Resistive downstream channels, fixing the distribution of flow rate among the three test chamber groups, were dimensioned considering the pressure drop across the three total pathways from inlet to outlet.
Numerical fluid dynamic simulations have been carried out to improve the design (Figure 1) and experimental microPIV analyses have been performed to evaluate the local fluid dynamics in the test chamber and the distribution of flow rate among the chambers of each group: mechanical stimulus on the cells was found to be homogeneous on the adhesion membrane.
In order to obtain a high-throughput read out of cell adhesion inside the chambers, two different impedance spectroscopy systems of microelectrodes have been evaluated to be alternatively integrated into the device.
Both the systems, a Ti-Au interdigitated based sensor system or a Ti-Pt- facing electrodes system, have been evaluated by numerical analyses.
The Ti-Au interdigitated based sensor system was micro-fabricated (Figure 2) and experimentally tested (Figure 3). Test of cellular adhesion detection by means of impedance measurements were carried out. Impedance measurements in presence of cells were performed under the microscope: images of the electrodes were acquired and the number of cells on the sensor was estimated and assigned to each related impedance measurement.
Results suggest that the microdevice can improve the efficiency of the techniques and the reliability of the data commonly obtained with adhesion and transmigration assays.



1. Fluid dynamics inside a test chamber. Left: sketch of the chamber and velocity plot on the middle vertical plane. Right: wall shear stress on the adhesion membrane.



2. Technological mask for the microfabrication of the sensors



3. Several distribution of cells adhered to an interdigitated sensor: on the right the impedance variation due to the presence different concentration of cells on the sensor

INVESTIGATIONS ON PHYSIOLOGICAL AND PATHOLOGICAL SENSORY-MOTOR CONTROL

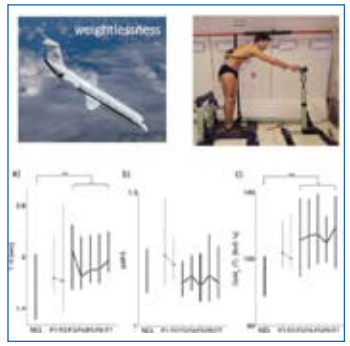
From biomechanics to brain functional imaging

Claudia Casellato

Motor behavior describes how the body moves during a motor task, reflecting the combined action of the neural circuits that control movement and the mechanical properties of the body. Since the huge amount of involved factors, the inference of neuromotor mechanisms underlying voluntary movements is very complex. A way to face this multiple-level investigation is to search for relationships between the produced movement and the neural substrates' mechanisms; they can be pointed out by comparing motor output in standard conditions and in presence of known alterations affecting motor control, such as a microgravity context or CNS dysfunctions. In this dissertation the following main experimental paradigms are contextualized into such scientific framework: the weightlessness environment, short-term and long-term, and the dystonic movement disorder. The approach of this work is primarily based on biomechanical analyses in healthy and neuropathological conditions. In a second step, brain functional imaging techniques are integrated in the attempt to define the neural correlates of the main processes involved in motor control and adaptation.

Moving in 0G environment means dealing with different mechanical constraints and altered sensorial information. Thus, the whole body reaching movement appears to be a perfect task to be studied to better understand the effect of weightlessness on CNS motor activity. We thus set out to analyze the movements of 12 standing subjects reaching for a target in front of them beyond arm's length in normal conditions and in transient weightlessness (parabolic flights). We used biomechanical modeling to compute focal-relevant and postural-relevant variables, synergism levels and torques estimation by inverse dynamic method (Fig.1). It was shown that our brain learns different internal models for a variety of contexts and they are organized in a parallel control architecture.

The persistence of a high joint coupling found in weightlessness reinforces the idea that joint covariation is a neural constraint in controlling multi-segment movements, mirroring the need of reducing the motor system redundancy. In terms of motor planning criteria, inverse dynamic analyses pointed out that the motor strategy selection process uses optimality criteria linked to the minimization of the interaction torque. To confirm or neglect these assumptions in a long-term motor learning,



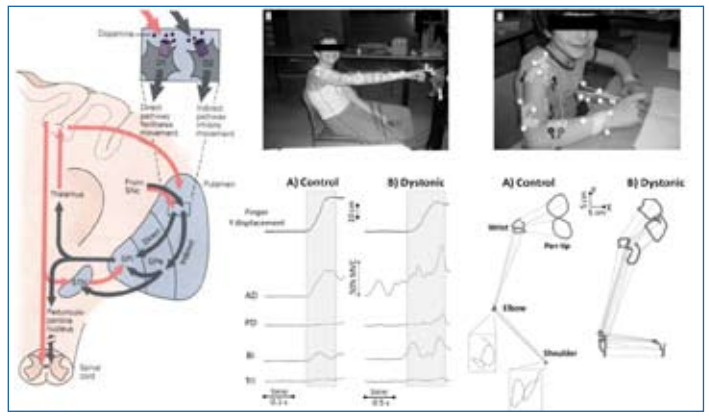
1. Top: parabolic flight technique for transient microgravity andthe whole body reaching protocol. Bottom: resultsfrom the biomechanical analyses (median among subjects). Movementduration (a), focal path (b),final position of Center of Mass along x-axis, % of Base of Support (c). NG= NormoGravity. P1:P7 = 0G Parabolas

we acquired similar protocols performed by two astronauts along space missions onboard the International Space Station, by the motion capture system ELITE-S2, ad-hoc developed. These preliminary results confirmed the hypothesis of a parallel modality of movement and posture coordination and the dynamic interaction torque as an element controlled by the movement planning mechanism to enhance the motion controllability.

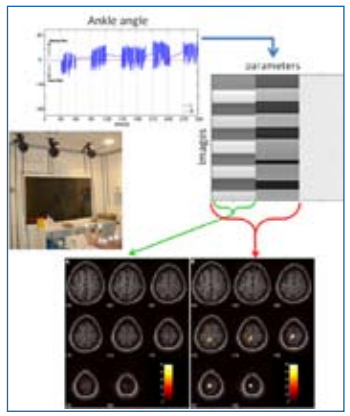
Dystonia is a movement disorder which represents a valuable model for understanding the role of basal ganglia circuits in normal motor control and their disruption in disease. We thus carried out a clinical study comparing 15 children with genetic dystonias and 9 age-matched controls.

The experimental acquisitions were focused on synchronized measures of kinematics and EMGs, during reaching and handwriting motor tasks. The findings support the hypothesis of a dysfunction at both direct and indirect pathways of basal ganglia, with an involvement also of coordination mechanisms (Fig.2). An ongoing study is now evaluating the motor adaptation in dystonia during upper limb protocols under force-field modifications.

Correlating the features of the actual executed movement with the associated cortical activations can represent a methodological approach useful to directly measure two levels of neuromotor control mechanisms. Thus, we have explored a new set-up combining fMRI with a motion capture system. Block-design movement protocols were recorded by the motion system cameras mounted within the MR room, along with fMRI acquisitions in healthy and hemiparetic subjects. The integrated measurement were reliable. The introduction of the kinematic regressor, which represents the actual executed movement, into the design matrix, besides the stimulus, leads to more consistent activation maps with respect to the standard analysis, allowing to better distinguish, especially during motor recovery, what is due to neural plasticity and what is due directly to the movement performance, quantified by the kinematic recordings (Fig.3). These studies represent valuable experimental paradigms and methodological approaches that could contribute to infer theories on neural control mechanisms and their adaptation, with possible clinical precious falls-out for neurorehabilitation.



2. Basal ganglia circuitry (GPi/e = globuspallidus pars interna/esterna; STN = subthalamic nucleus; SNc = substantianigracompacta). Reaching andhandwritingtasks. Resultsfor two exemplificative subjects (control and dystonic); reaching: finger vertical displacement and EMG envelopes of four muscles (Anterior Deltoid, Posterior Deltoid, brachial Biceps, brachial Triceps); writing: kinematics (shoulder, elbow, wrist, pen tip trajectories)



3. Simultaneous measurement of kinematics and fMRI. The recorded angle from ankle dorsal- plantar-flexion taskwas inserted as regressor into the design matrix, so as to compare the cortical maps from astandard model (only the stimulus, 1st column of design matrix) and adding the actually executed movement parameter (2nd column)

ADVANCED METHODS FOR IMAGE BASED TOMOTHERAPY OF HEAD-AND-NECK CANCER

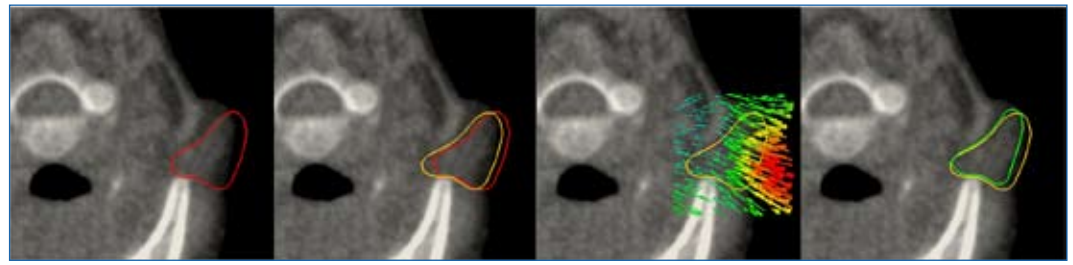
Elena Faggiano

Modern radiotherapy devices and in particular sophisticated systems as helical Tomotherapy, are able to provide high dose conformity and homogeneity for complex tumor target irradiation simultaneously avoiding proximal organs at risk. In this context the role of imaging has become increasingly important: multimodal image fusion improves the delineation of treatment plans while daily images of the patient acquired before the treatment session enable plan verification and anatomical patient changes individuation. Moreover the availability of patient daily images offers the possibility to extensively analyze anatomical changes of critical organs which may be indicative of compromise organ functionality. In particular, head- and-neck cancer patients may experience significant parotid glandes changes during the course of radiotherapy treatment in terms of parotid glands volume shrinkage and parotid migration towards the midline of the patient.
These deformations during radiotherapy, may predict acute and subacute/late parotid toxicity. For this reason the detection of anomalous deformations of parotid glands may be a powerful tool for head-and-neck cancer patients follow-up: an early detection could permit to selectively adapt the treatment in order to reduce the risk of toxicity or, if adaptation is not feasible, to better manage the toxicities and the supportive therapies.

To deeply exploit daily pre-treatment images advanced image based methods are more and more often used in this context, as image registration methods and contour propagation methods. Image registration, if rigid, gives an information about patient position error at the time of treatment while, if elastic, gives an information about anatomical modifications occurred to the patient from the moment of planning. Contour propagation from planning image to daily images produces organ contours on each daily image of the patient making easier organ changes analyses.
The aim of this work is to propose a complete methodology which consists in advanced image based methods for the processing and the analysis of head-and-neck Tomotherapy daily images for parotid glandes modifications evaluation.
In particular accurate elastic image registration and contour propagation methods are developed.
The proposed image registration method is based on the representation of the deformation as a trivariate cubic B-splines tensor product, to produce a smooth and locally controllable deformation. Moreover, mutual information is chosen as matching function to handle with different intensities in kVCT and MVCT images due to the different energy used in the two acquisitions and to the different type of noise. A new pre-processing is then introduced to constrain the algorithm in deforming only interesting areas.

Concerning contour propagation, our method consists of a surface 3D mesh generation and optimization step, and a subsequent mesh deformation step. Mesh deformation is performed through free-form deformation, transforming the mesh warping the space surrounding the mesh itself. The space warping is determined by the image registration result. The method thus conceived produces smooth contours with no need of post-processing adjustments in a short computational time. Furthermore the compact representation of the deformation results in an easy storage of data, which is important in radiotherapy context.
As the accuracy of image registration and contour propagation methods strictly depends on the specific application, an accurate validation of such methods was performed on 10 head-and-neck cancer patients treated with Tomotherapy. For this study we used the planning kVCT scans of each patient, the first MVCT image, the final daily MVCT image and three parotid glands contours for each image delineated by three expert operators. We investigated inter-observer variability in contouring parotid glands on MVCT images and compared the results of the automatic method with the protocol- related observer variability. Since validation strategies proposed in literature are all different and do not follow any guideline in this work we have proposed a validation that was as complete as possible both in terms of indices used (i.e. volume, center-of-mass, overlap index and surface distances) and in terms of statistical analysis performed. Concerning the inter-observer variability analysis we found a low variability in kVCT delineations and an increased significant variability in MVCT delineations indicating the importance, in this context, of having a good contour propagation method.
Concerning the contour propagation validation we found that our automatic method was never significantly different from the manual contours and we concluded that our method could successfully substitute manual contouring on MVCT images.
As new quantitative deformation measures, in this phd work a new technique for the analysis and visualization of parotid glandes deformation is also proposed. The idea consists in expressing the entity of deformation (in terms of compression or expansion of each single voxel) by the Jacobian transform (J) of the deformation field calculated by image registration. The mapping of J over the parotid sections provides a representation of the areas that are most involved in anatomical modifications. Moreover we proposed to group the distribution of the Jacobian values within specific structures to compose a Jacobian-volume histogram (JVH), in analogy with the familiar dose-volume histogram used in radiotherapy to optimize and calculate dose delivery; JVH assigns to each histogram bin the volume fraction value with a compression or expansion higher than that indicated by the bin itself. JVH was then studied in a study on 32 head-and-neck cancer patients in which we investigated the correlations between Jacobian parameters and dosimetry/geometric parameters. Our preliminary findings indicated that Jacobian and JVH could be used to improve our knowledge of parotid deformation and are consequently likely to become a robust surrogate of the early functional damage of parotid glands giving a potentially direct estimate of the reduced secretion capacity of each voxel.

The phd work was developed at the Institute of Bioimaging and Molecular Physiology - National Research Council (IBFM-CNR) of Milan (Italy) in collaboration with the Medical Physics Depatment of San Raffaele Hospital where an helical Tomotherapy device is used in clinical practice, and involved a multidisciplinary group of engineers, medical physicists and radiotherapists.
All the developed methods have been implemented in C++ language based on the Insight Segmentation and Registration Toolkit (ITK) and The Visualization Toolkit (vtk) libraries.



1. Contour propagation from kVCT to MVCT. From left to right, MVCT image with superimposed: kVCT parotid contour (red); roto-translated kVCT parotid contour (yellow), compared to the original kVCT parotid contour (red); roto-translated kVCT parotid contour (yellow) and the elastic deformation field; elastically deformed contour (green), compared to roto-translated

DEVELOPMENT OF NEW STRATEGIES FOR THE ANALYSIS OF HUMAN GENETIC VARIATION

Identification of natural selection signatures in the human genome

Matteo Fumagalli

During last decade, after completion of Human Genome Project, there has been a notably increased interest in studying human genetic variability, which refers to genomic nucleotide differences among human individuals. Complex patterns of genetic diversity in modern populations are the product of many layers of demographic and evolutionary events including natural selection. Natural selection is the process by which heritable traits that make it more likely for an organism to survive (increase its fitness) and successfully reproduce become more common in a population over successive generations.

Detecting natural selection signatures in the human genome has a twofold meaning. First, we can make inferences about humans evolutionary history and have a clearer picture of events that guided Homo Sapiens to conquer most of land masses after his appearance in Central Africa nearly 200k years ago. During their evolution, humans encountered a wide range of different environments and it is conceivable to think that they have been under strong selective pressures in order to adapt to them. Second, inferences about evolution of a genomic locus can provide important information on its biological functionality, especially when variants targeted by natural selection affect disease susceptibility for complex disorders. Thus identification of putative loci affecting disease susceptibility may be supported by genome scans of non neutrally evolving variants.

Over the last years, with the public availability of large-scale genotype data, studies concerning genome-wide scans for genes or genomic regions that have been subjected to selection have become achievable.

In this project we implemented a computational framework to analyze human genetic variation by applying population genetics methods combined with a novel strategy to specifically highlight signatures of genetic adaptation to local environments. We applied this approach to real data to detect and characterize signature of natural selection in the human genome and to correlate these findings with functional or phenotypic variation.

At a first stage, we investigated evolutionary history of candidate genes known to hold important biological and medical roles to search for signatures of selection by applying population genetics statistics. Then functional experiments or focused association studies on highlighted loci were performed to assess clinical implications of targets of natural selection.

In a second phase, we focused our attention on retrieving signatures of selection owing to adaptation to local environments, especially to pathogen load. By applying methods based on correlations between population allele frequencies and environmental variables we aimed at retrieving genes and pathways more likely to have been target of selection driven by adaptation, disentangling the contributions of different environmental factors.

We retrieved data for more than 500k genetic variants characterized in 55 human populations distributed worldwide. For each geographical location we assigned correspondent values measuring putative environmental features that acted as selective pressure through evolution: climate, diet and pathogen presence. We assessed relationship between population variants frequencies and environmental predictors using both a non-parametric statistics and a multiple regression strategy coupled with partial correlations. Projection to Latent Structures multiple regressions were used to model the relationship between population allele frequencies of each SNP and a matrix describing 14 distinct environmental factors describing evolutionary impact that climate, diet or pathogens had in human adaptation. We examined the relative abundance of genic versus intergenic SNPs in the upper tail of distribution of modeling prediction accuracy values. Notably we found a significant enrichment of genic SNPs compared to intergenic SNPs at great values of prediction accuracy, suggesting the action of natural selection owing to local adaptation driving the differential allele frequency shift among human populations.

We then aimed at identifying the relative fraction of loci whose population genetic variation is significantly correlated with specific environmental factors. These genes would be good candidate to be non neutrally evolving under a particular selective pressure. We assessed the relationship between the population genetic distance of each gene and the distance matrix for environmental variables via partial Mantel correlations. This is a non parametric statistical test for association between two distance matrices, controlling the effect of a third matrix to remove spurious correlations. Latter independent distance matrix is calculated as the overall population genetic distances among populations computed over all loci and therefore accounts for the non independence of populations and corrects for neutral demographic events. Assessing the statistical significance, we observed a striking predominance of genes whose variation is significantly associated with pathogens distance matrix.
Results pinpoint infectious agents as the main selective force which has driven population genetic differentiation through time. Furthermore we retrieved regulatory genic pathways specifically enriched with signals of adaptation to pathogens especially for genes known to modulate susceptibility of infection and autoimmune conditions.

Outcome of the current study shed a new light to past historical events that drove world colonization by humans. More importantly, identification of genes targeted by natural selection owing to adaptation to local environments allows a clear picture of relationship between common genetic variants and susceptibility to modern complex diseases with notable implications in a clinical-diagnostic perspective.
These results will guide further deep analyzes of genes subjected to environment driven natural selection and their effects on protein functionality and disease phenotypes that may help understanding the association between alleles and disorders.

MARKERLESS MOTION CAPTURE FOR HUMAN MOTION ANALYSIS

Emiliano Gambaretto

Introduction
This thesis represents one of the firsts attempts to bring Markerless Motion Capture (MMC) technologies to the study of human locomotion. Standard motion analysis requires positioning markers on the subject skin. The movement of the markers is then used to infer the underlying relative movement between two adjacent body segments with the goal of defining the joint movement. Besides being time-consuming markers positioning also requires specialized personnel and is subject to repeatability errors. On top of that the presence of markers can be perceived by the subject causing unwanted artifacts potentially compromising his natural pattern of motion.
In contrast to standard marker-based motion analysis, MMC aims at capturing the subject motion using video data only. The complete removal of markers brought by MMC lets the subject perform his motions freely, it also reduces the set-up time and helps reducing repeatability errors due to markers misplacement.
A complete MMC system was built from scratch as part of this thesis. Such system was tailored to fit clinical requirements. In particular advancements with respect to the state of the art were made on the articulated model definition and registration phases. In order for the reader to better appreciate the contributions of this Thesis a quick review of the MMC processing pipeline will now be provided. The assessment of the developed MMC system performances concludes this Thesis.

Methods
The MMC processing pipeline, that extracts the subject joint motion parameters from video data, can be divided in the following four stages:
Video Collection and Background Segmentation
The subject silhouettes are extracted from video data collected from multiple synchronized cameras. This is done in two steps: firstly video signals are brought into a suitable colour representation, then background segmentation is performed at both pixel and image level. At the pixel level each pixel is compared with a Gaussian background model. Pixels that don't fit such model are more likely to be considered as part of the foreground. At the image level spatial consistency between neighbouring pixels segmentation labels is enforced through a Markov Random Field model.

Shape from Silhouette
Video data coming from multiple cameras are merged together giving rise to a 3D representation of the volume occupied by the subject: the Visual Hull (VH). The VH reconstruction is performed in a multi-scale fashion for improved reconstruction speed. The VH surface is finally described as a point cloud with normals associated to each point.

Model Definition
An accurate, subject-specific, 3D animatable model consisting of a skeleton and mesh geometry is used to represent the subject surface as function of joint parameters. One contribution of the presented Thesis was to provide an automatic tool for the generation of such model. This tool accepts as input human meshes of both high quality (laser-scans) and poor quality (such as the one provided by video reconstructed VH). The presented solution is based on a statistical model of human shapes learned from examples (real humans' laser-scans with corresponding joints locations marked manually). Being based on a statistics of real human shapes, this approach is able to complete, fix and improve the quality of coarse input data.

Model registration
The Iterative Closest Point algorithm, used for registering rigid shapes, was extended to handle articulated bodies. Such algorithm was used to register, on every video frame, the subject model with the input VH point cloud in order to recover the subject pose. Our method includes joint-specific range of motion constraints enabling up to 6 degrees of freedom (dof) per joint. Such flexibility represents a further advancement on the state of the art and allows the clinician to customize the registration process to his specific needs. The registration problem formulation was made particularly compact by leveraging the exponential map representation of joint rotations.

Performance Assessment
A validation study that represents itself an interesting contribution to the evaluation of MMC for clinical applications was performed. In contrast to related MMC works, the performance of our method was validated not only in terms of errors in locating joint centers over time but also in terms of errors in angles and joint moments. We compared our method, applied to an 8 VGA cameras set-up, against a commercial marker-based system used as gold standard. Marker-based measures were obtained using a 6 marker protocol (iliac crest, great trochanter, lateral plateau, lateral malleolus, heel, 5th metatarsal). A force plate collected reaction forces at the feet enabling kinetic computations. The study was done on 3 subjects performing gait cycles at 3 different speeds with 3 repetitions per speed level (for a total of 27 sequences). The following table resumes the performance of the MMC system on the lower limb joints in terms of location, angular and moments RMS errors with respect to the marker-based gold standard (Moments were normalized with respect to the subject height and weight):

ERRORS IN JOINT LOCATION (CM)		
Hips	Knee	Ankle
2.13	2.00	2.04

ERRORS IN ANGULAR MEASUREMENTS (°)		
Knee flex/ext	Knee abd/add	Ankle flex/ext
5.34	2.43	4.48

ERRORS IN MEASURING MOMENTS (NM%(W*H))			
	Hips	Knee	Ankle
flex/ext	1.01	0.44	0.37
abd/add	0.34	0.32	0.26

Discussion and Conclusions
MMC joint location errors are compatible with inter-examiner errors in locating anatomical landmarks found in literature that affects standard marker-based approaches. Angular errors are slightly superior (especially for the knee flexion). Errors in moments are in percentage below 10% with the exception of the knee and in particular of the ankle abduction moments. These results are encouraging and show how MMC can be already considered as a valuable choice for many applications for which a slight increase in the measurement error is well worth the advantages related to markers removal.

CHARACTERIZATION OF STRUCTURES AND LESIONS USING SEGMENTATION AND REGISTRATION OF MAGNETIC RESONANCE IMAGES

Maria Ida Iacono

Magnetic resonance imaging (MRI) has many advantages over other diagnostic imaging modalities, such as high contrast between soft tissues, high spatial resolution and inherent 3D nature, and thus is utilized in a wide range of clinical applications. Visual inspection of the images is certainly the simplest approach to assess disease or to plan a pharmacological or a surgical treatment. This approach however is necessarily qualitative.
The growing demand of an evidence-based medicine requires that outcomes and the accuracy of information on which those outcomes depend to be measurable. Hence quantitative analyses are needed to improve the objectivity of the image interpretation procedure and to exhaustively characterize diseases across space and time.
In order to properly perform these tasks, two main issues should be solved: extraction and morphological characterization of regions of interest (ROIs) and quantitative evaluation of their changes over time. Characterization in space can be performed by segmentation of the ROIs, while the quantitative assessment of the pathology is determined by the registration of serial MRIs and by the analysis of the resulting deformation fields.

Primary objective of this project is the development of registration and segmentation methods in order to characterize the diseases across space and time.
In this Thesis, two main segmentation algorithms are used: a clustering-based segmentation and an atlas-based segmentation. Clustering was chosen since it results the most versatile and robust for MRI applications. In particular, fuzzy segmentation allows to classify voxels into more than one class with varying degree of membership. This behavior is beneficial for MRI segmentation, since noise, partial volume effect and the inhomogeneity of the structures make the exclusive assignment of voxels to a single class undesirable.
However, there are cases where the anatomical structures are very poorly contrasted on the images. Instead of intensity, structures can be distinguished by their location and their relationship to other structures. In such cases, location relative to their surrounding structures needs to be taken into account and included to guide the segmentation. A complete description of such relationships is an atlas. The standard atlas-guided approach treats segmentation as a registration problem. It first finds a transformation that maps a pre-segmented image (atlas) into a new image to be segmented. Then, it applies the resulting transformation on the atlas in order to project the labels onto the un-segmented image and to automatically segment on it the regions of interest.
For registration, two main strategies are considered as well to deal with MRIs.
The first registration procedure is a hierarchical non-rigid registration algorithm based on free-form deformations. The algorithm was chosen because it is known to be completely automatic and very robust for a number of MRI applications thanks to the measure used for the optimization: the normalized mutual information (NMI). NMI is independent of voxel intensity values. Therefore, it is well suited for images featuring different gray level ranges due to different image acquisition protocols or modality.
However, when MR images show very large differences due to the dissimilar intensity characteristics, scale, and most of all significantly different informative content (an image can include structures that are not visible on the other one), registration needs to be guided by a priori knowledge about the anatomy. We thus proposed a second registration procedure based on the iterative closest point (ICP). ICP requires that surfaces be segmented in the images prior to their registration and then it matches them by minimizing a distance measure.
The second part of the Thesis is focused on the application of the proposed methodologies on three different cases.

CASE 1: The localization of the globus pallidus internus for deep brain stimulation

Deep brain stimulation (DBS) of the internal part of the globus pallidus (GPi) have shown to significantly improve the motor symptoms of advanced PD patients. A process that significantly helps the neurosurgical DBS procedure is the accurate identification of the target using a pre-operative MRI. Currently, this is done by direct visualization, which is intrinsically limited because of the poor contrast and resolution of routine clinical images. To optimize this process we can use detailed information derived from high resolution human brain datasets in which the GPi is well defined and can be easily segmented. Herein we used these data in an atlas-based segmentation procedure to outline the GPi on the pre-operative low resolution MRIs of patients affected by PD. The mapping of the atlas on the low-resolution MRIs results in a highly accurate anatomical detail that is useful for target localization.

CASE 2: The morphological characterization of the dural ectasia in Marfan syndrome

Dural ectasia (DE) is considered one of the major criteria in the diagnosis of Marfan syndrome. Diagnosis of DE is based on the visual assessment of MRIs. However, the cases of mild ectasia represent often a diagnostic dilemma such that they cannot be easily evaluated solely by visual inspection. Then, the quantification of the degree of the DE could be useful as a discriminative index for an automatic identification of pathological cases.
The dural sacs of the patients were segmented by applying three different unsupervised clustering methods, fuzzy-C-means, Finite Gaussian Mixture Expectation Maximization, and Gaussian Hidden Markov Random Field- Expectation Maximization, and for each patient a tubular model of the dural sac was extracted by automatically detecting and removing the existent pathological extrusions.
The segmented images together with the resulting tube were then rendered using a marching cubes algorithm and compared to each other.

CASE 3 The monitoring of gliomas using serial follow-up MR images

High grade malignant gliomas require careful monitoring both during therapy and during follow-up for the evaluation of treatment effectiveness and for the early detection of a relapse. An appropriate characterization of the tumor is critical for a correct diagnosis and an accurate treatment planning.
Therefore we proposed the use of non-rigid registration methods for the detection of changes in serial MRIs of the same patient at different times.
The method consists of the registration of serial MRIs and the analysis of the deformation field. The registration was performed through a non-rigid registration using free-form deformations and normalized mutual information as a voxel-similarity measure. A subsequent analysis of the transformation is then performed by using the jacobian operator in order to extract information related to tumor evolution.
The novel approach leads to a quantitative description of tumor evolution. Therefore, it could be valuable for planning interventions and/or treatments.

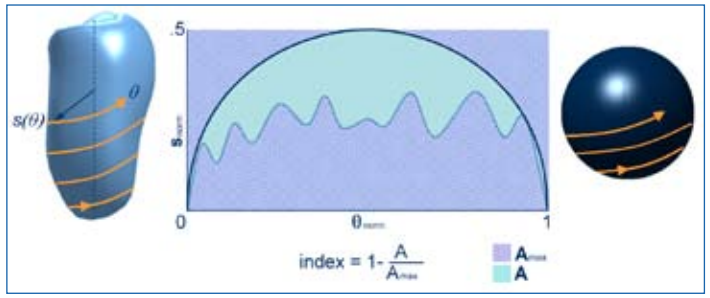
Conclusions

The goal of this PhD Thesis is to develop registration and segmentation methods for the discrimination, localization, and monitoring of structures to assist clinicians in clinical practice.
The proposed methodologies are applied to three different protocols with the goal of *i)* localizing the globus pallidus internus for DBS, *ii)* quantifying dural ectasia in Marfan syndrome, and *iii)* monitoring high grade gliomas.
Results show that the proposed methods copes with problems related to different structures of interest and different acquisition protocols of the images.
Particular appealing is the possibility of developing a unique tool integrating these complementary approaches in order to improve the diagnostic accuracy, to make the follow-up objective, rapid and robust and to enable better management and planning of the treatment.

NOVEL IMAGE BASED APPROACHES FOR THE QUANTITATIVE EVALUATION OF CARDIAC REMODELING

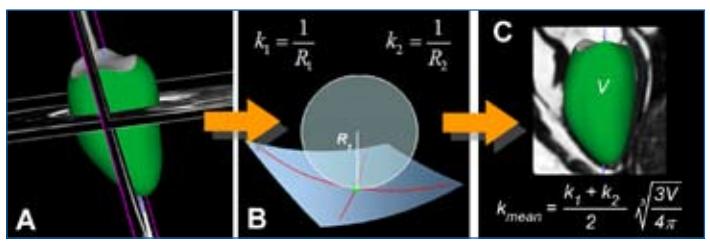
Francesco Maffessanti

Cardiac remodeling is a pathophysiological process, common to a large variety of disorders, in which cardiac chambers attempt to restore their normal performance by progressively changing their shape. Cardiac imaging is the ideal starting point to evaluate the remodeling, by means of a combined functional-morphological approach. However, while the novel 3D imaging modalities have already improved the functional evaluation, the morphological assessment is still performed qualitatively. The underlying hypothesis of this research project was that 3D echocardiography and cardiac magnetic resonance imaging techniques could serve as starting point to describe several nuances of cardiac remodeling. Accordingly, the aims were: 1) to define novel morphological indices of remodeling; 2) to test their ability in characterizing this process; 3) to evaluate the relationship between these indices and the severity of the pathology.
Novel algorithms for 3D left ventricular (LV) global and regional shape analysis were developed. A schematic of global shape analysis is shown in Figure 1: shape indices were defined by measuring the similarity between the signal obtained by helical sampling the left ventricle, and that obtained from a reference shape having the same LV principal moments of inertia.



1. Schematic of the computation of LV shape indices. The helical sampling of the LV endocardium is represented by the light blue line, while reference shape is shown by the dark blue line.

Global analysis may not be adequate when remodeling only affects the heart regionally. Therefore, custom software was developed for analysis of regional LV morphology (Figure 2): for each node of the mesh representing the LV surface, a quadric polynomial function was locally fitted and then curvature analytically obtained.



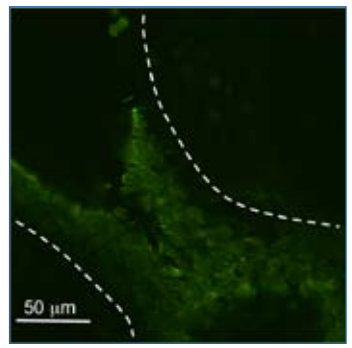
2. LV surface was reconstructed (A) and for each node the two principal curvature k1 and k2 were calculated (B). Local 3D curvature, kmean, was calculated as the mean of k1 and k2, normalized by mean LV curvature (C).

These algorithms were applied to three pathologic conditions: ischemic and dilated cardiomiopathies, mitral valve prolapse and pulmonary hypertension. Ad-hoc clinical protocols were designed and conducted at the Centro Cardiologico Monzino, Milan, and at the University of Chicago. The protocols involving ischemic and dilated cardiomiopathic patients were designed as in-vivo human validation studies of the algorithms. This population, largely described in literature, was considered optimal to test the capability of our methods to correctly describe the alterations in LV shape. Our results showed that LV dilation is associated with an increase in sphericity and decrease in conicity, while ischemic cardiomiopathy, reflecting a regional abnormality, is characterized by values of global shape indices intermediate between normal and dilated patients. When analyzing the percent change in regional curvature, ischemic segments showed abnormal patterns and similar behaviour to dilated segments. These results were consistent with the previously reported findings. The largest clinical protocol was designed to evaluate the remodeling associated with severe mitral regurgitation and following surgical repair. All the enrolled patients underwent early surgery: the surgical referral of asymptomatic patients aims at preventing the onset of LV dysfunction. From this perspective, the evaluation of LV remodeling could be useful to recognize subtle morphological changes before symptoms are evident. Our results showed that: 1) in presence of severe mitral regurgitation, the left ventricle already remodels, even in absence of symptoms; 2) prior to surgery, LV volumes are slightly dilated compared to controls; 3) as a consequence of surgical repair, the ventricle remodel towards a more physiological condition, with a significant reduction in volumes. Functional improvements were associated with favourable LV shape remodeling from an abnormally spherical to a more normal conical shape, anticipating the functional improvement. These results reinforce the clinical guidelines advocating for early repair of the mitral valve.
In the last protocol, curvature analysis was applied to characterize the behavior of the interventricular septum and its correlation with the severity of pulmonary hypertension, nowadays invasively assessed by right heart catherization. Findings demonstrated that septal shape is strongly related to the invasive data, with higher feasibility compared to echocardiography-derived indices. The integration of this method in the standard magnetic resonance protocol could allow a comprehensive evaluation of the pulmonary hypertension to be obtained.
In conclusion, the role of morphological quantification based on advanced imaging techniques has been underlined in this doctoral project, paying particular attention to the process of cardiac remodeling. Findings from extensive clinical protocols, clearly showed that this approach is feasible, has several clinical applications and its strength relies on the integration with functional analysis. This double point of view may give the clinicians complimentary information, further improving clinical practice and deepening the knowledge of pathophysiological conditions. Moreover, the flexibility and time-efficiency of the presented approaches are considered clinical requirements that have been proven satisfied, and may push their application outside the research field.

PECTIN-BASED MICROSPHERES FOR BONE TISSUE REGENERATION

Fabiola Munarin

Injectable microspheres are promising substrates for the regeneration of damaged tissues due to their ability to load, deliver and locally release cells, proteins, drugs or growth factors. Microspheres loaded with cells and, possibly, growth factors may be implanted in the organism soon after cell immobilization or later, allowing the *in vitro* proliferation and adhesion of cells, as the traditional scaffolds. From a clinical perspective, the use of such injectable scaffolds for regenerative purposes instead of traditional surgical procedures is very attractive as it minimizes patient discomfort, risk of infection, scar formation and the cost of treatments.
Other advantages of the use of microspheres are the possibility of injecting them directly in the pathological site and the possibility to conform to the irregular implant site, with more or less close packing.
Pectins are a family of polysaccharides found in the primary cell walls of plant cells. They are mainly composed of D-galacturonic acid residues partially methyl-esterified, and their chemical properties, i.e. molecular weight and degree of esterification, mainly depend on the source and extraction methods.
Traditionally, pectin is known for its gelling properties and it is usually employed in the industrial and household preparations of jams, jellies and marmalades. It is inexpensive, easily available and widely used for biomedical applications in drug delivery and wound healing.
In the last decade, pectin has been considered for tissue regeneration applications, as it forms gels via ionic interactions in the presence of divalent ions, with a fast cross-linking process, leading to a quick increase in the viscosity of the solutions and therefore to the possibility of obtaining *in situ* biocompatible gelling systems.
As a preliminary work, microspheres from alginate, chitosan, gelatin and pectin were obtained with the extrusion method using a coaxial air flow system and an electrostatic bead generator, using different formulations; cell immobilization was performed using line cells (C2C12, $1.6x10^5$ cells/mL and MC3T3-E1, $20x10^6$ cells/mL).
The experimental parameters affecting the microspheres diameter, i.e. the distance between the needle tip and the gelling bath, the velocity of extrusion and the pressure of the air flow or the electrostatic voltage applied were optimized to obtain microspheres with mean diameters in the range of 400÷450 mm, which was considered the optimal size for nutrient transport and easy *in vitro* handling of microspheres.
High encapsulation efficiency and cell viability were proven for each system, and pectin gels were considered attractive cell carriers. Pectin microspheres showed interesting characteristics for bone tissue regeneration, as they kept the cells viable, differentiating and releasing a mineral phase up to the end of the experiment (29 days). However, in this first experiment, a limited cells/material interaction and a long term stability of microspheres, which did not allow the progenitor cells to be released in the tested time period, were observed.
Two possible approaches were investigated to modulate pectin properties through chemical modifications to address cell adhesion and to improve the degradability of the polysaccharide. Degradation is fundamental in the case of stem cell carriers: despite giving the mechanical support during the first days after implantation, the biomaterial need to be gradually degraded by the biological environment, so to release cells for the regeneration of the damaged tissues. Taking into account the results obtained with alginate oxidation, in this work the same procedure of oxidation with sodium periodate were applied on pectin to obtain faster degradable pectin microspheres.
Osteoblast adhesion is essential for signal transduction and for the regulation of gene expression,



1. F-actin staining on RGD-pectin microspheres immobilizing MC3T3, 23 days after immobilization. From day 21 of incubation onwards, MC3T3 cells immobilized in RGD-pectin microspheres created 3D connections among different microspheres. The dashed lines indicate the borders of the microspheres

as the ECM-mediated cell spreading can stimulate the nuclear matrix, and therefore modify gene expression, inducing cell proliferation and differentiation. The biomimetic modification of biomaterials with RGD-containing peptides thus constitutes a promising approach for bone tissue regeneration.
The coupling of pectin with a RGD-containing oligopeptide was obtained following the procedure previously described for alginate, to form amide bonds between the carboxyl groups of the polymer backbone and the terminal amine of the oligopeptide $(Glycine)_4$-Arginine-Glycine-Aspartic acid-Serine-Proline.
Furthermore, treatments in SBF were performed to create a biomimetic environment, simulating the natural inorganic phase of the bones on pectin microspheres.
In vitro and in vivo studies performed on RGD- coupled pectin microspheres immobilizing preosteoblasts and mesenchymal stem cells demonstrated that cells spread inside the microspheres and organize in 3-dimensional structures, regenerating the natural extracellular matrix and showing osteogenic behaviour. Results indicated that cell morphology was influenced by the presence of the RGD groups on the pectin backbone: after 15 days of incubation, both MC3T3 and hMSCs were elongated and adherent to the microspheres. The elongation of MC3T3 cells in RGD-pect microspheres increased with time, and after 21 days of incubation MC3T3 cells began to grow on RGD-pect microspheres even on the surface, and to create 3D bridge-like structures connecting different microspheres (Fig. 1), whereas hMSCs remained inside the microspheres.
Mineralization and osteogenic differentiation were promoted by both cell types, as shown by cryo SEM images, Von Kossa staining and by the up-regulation of osteogenic markers (i.e. osteocalcin, alkalin phosphatase, RUNX2).
In vivo studies indicated the efficacy of mesenchymal stem cell immobilized in the hydrogel microspheres to promote the healing process in the nude mice model. For this aim, bilateral defects (0.9 mm diameter) were generated in femur metaphysis in nude mice. RGD-pectin microspheres, loaded and not loaded with hMSCs, were injected in the defects. As control, not filled defects were left to follow the physiological healing pathway. Bone formation was potentiated in the early post-surgery stages in the case of defects filled with stem cell loaded microspheres: at 14 days the formation of bone callus were observed; at 28 days post-surgery bone callus were resorbed and the gap filled. In absence of cell loaded microspheres, the gap was still open after 14 days, and bone formation was observed only at 28 days. Not loaded microspheres interfered with the healing process, and the closure of the defect was not accomplished at 28 days.
Finally, different sterilization techniques were evaluated to sterilize pectin powders and solutions. The obtained results indicate that gamma rays sterilization at low doses minimally affected pectin structure, and this sterilization method may provide interesting future advancements towards the industrial preparation of pectin-based medical devices.

AN OPTIMIZED OPTICAL MAGNETIC TWISTING CYTOMETRY SYSTEM FOR THE STUDY OF HUMAN AIRWAY SMOOTH MUSCLE CELL MECHANICS IN ASTHMA

Miriam Pastena

Living organisms are far more complex than engineered materials, they are dynamic and provide integrated functions that include metabolism, control, sensing, communication, growth, remodeling and apoptosi. Cells deform when external forces act on them, such behaviours are described by the mechanical properties of cells, and they are determined by cell compositions and structures as well as the surroundings with which the cells interact. Unlike inert substances, however, mechanical forces also induce biological responses in cell. There is a growing recognition that the mechanical properties of the cytoskeleton are the key determinant not only of the cell shape, but also of the other cellular function including spreading, crawling, polarity and cytokinesis.
Many normal and diseases conditions of cells are dependent on or regulated by their mechanical environment, and the deformation characteristics of cell can provide information about their biological and structural functions. The understanding of how cells sense and respond to mechanical perturbations and what changes take place in cells under pathological conditions requires a combination of biological, biophysical, and biochemical approaches.

Asthma is a chronic respiratory disorder characterized by a state of chronic inflammation, remodeling and by airway hyperresponsiveness (AHR) that is an exaggerated airway narrowing in response to many stimuli, hence the lung function could be impaired.
AHR is a key feature of asthma but remains largely unexplained. Airway smooth muscle (ASM) is now recognized as being the major end-effector of bronchospasm and acute airway narrowing in asthma. In recent years understanding of the relationship of responsiveness to muscle biophysics has changed. It has become well established, that muscle length is equilibrated dynamically rather than statically, and that non-classical features of muscle biophysics come to the forefront, including unanticipated interactions between the muscle and its time-varying load, as well as the ability of the muscle cell to adapt rapidly to changes in its dynamic microenvironment. These newly discovered phenomena have been described empirically, but a mechanistic basis to explain them is only beginning to emerge. Moreover even the AHR could be uncoupled with inflammatory processes; it is know that biochemical stimulations influence the mechanical properties of human airway smooth muscle (HASM) cells. Recent studies showed that a ubiquitous sphingolipid metabolite as sphingosine-1-phosphate (S1P) present in the cell environment in normal condition could have an important role in airway smooth muscle contraction but its mechanisms of action are still unclear.
The past decades has seen the development of instrumentations capable of mechanically probing and manipulating single cells and biomolecules at forces and displacements smaller than a piconewton and a nanometer. Viscoelastic measurement techniques must allow local tests on micrometer-nanometer scales and reproducible and reliable measurements. A common method to separate elastic from dissipative behavior for any material is to measure responses to oscillatory loads; this lead Maksym at al. 2000 to implement an new technique based on twisting ligand coated beads to the surface of living cell and to measure the measure the mechanical properties in terms of complex shear modulus, g*. Magnetic twisting cytometry with optical detection of the bead movement, known as OMTC, reduces the variability in the results since it allows to discharge unbound beads, moreover it has a better time resolution since is possible to reach high frequencies, up to 1 kHz. Since this technique was introduced it was applied to many studies involving the measurements of cell mechanics, but at the moment all the OMTC systems in use are self-made prototypes, since it is not a commercially available technique. Moreover, there are no detailed procedures to design OMTC systems and, most important, a standard protocol to calibrate and evaluate their accuracy in order to make OMTC measurements reproducible between different prototypes and centres is still missing.
The aims of the present work were the design and development of an OMTC system optimizing its components, the definition of a precise procedure for the system validation, in order to establish which are the real advantages of this technique and which are its intrinsic limits that have to be taken in account for applications and the application of OMTC system to the study of human airway smooth muscle cells mechanics. In particular we investigated the direct effect on cell mechanics of carbachol and S1P and then we evaluated whether a previous incubation with S1P modulates HASM cells response to a contractile stimuli. Hence we developed and optimized an OMTC system that is able to generate known low forces, allows the synchronization of image acquisition with the generated magnetic field, and is able to resynchronize every time either amplitude or frequency are change "on the fly". We established that the presence of ferromagnetic material does not influence the measurement and that the software is able to correctly perform the tracking of the particles; hence the system is able to detect bead displacements in a wide range of frequencies, with a good accuracy. We also characterized the system in term of total transfer function and this makes possible to know and interpret at all frequencies the system behavior and compensate possible deviation from ideal behavior. We identify a detailed protocol to characterize and validate the system that can be proposed as standard procedure to guarantee reproducible and comparable data between different laboratories. The in vivo test confirmed the sensitivity of the device in detecting changes of complex shear modulus subsequent to administration of contractile and relaxant stimuli. Moreover we analyzed the possible variables influencing the stiffness of HASM cell in culture to overcome the lack of standardization of measurements protocols and data analysis in OMTC tests on HASM cells. We shown that even if serum deprivation is essential for synchronizing the sample in the same cellular phase, a prolonged deprivation could lead to the impairment of the sample; we shown that the basal stiffness is very much influenced by the nature of the cell-bead binding and that the exclusion of the outlier on the basis of basal stiffness is a good methods to exclude not well bound beads. Moreover we identified the range of basal variability as reference to quantify the effect of different drug treatments. These results allowed to standardize and optimized the experimental procedure in order to obtain reliable and repeatable data and to reduce the measurements variability.
We applied the OMTC technique to the study of HASM cell mechanics; in particular obtain a dose response curve in terms of mechanical properties of both Carbachol and sphingosine-1-phospahte, and the comparison between Chc and S1P shown a S1P-induced contraction more than 8 times higher than the Chc-induced contraction. The results obtained incubating samples with sub-contractile S1P dose suggest that S1P could have a role in lowering the threshold for Cch-induced contraction and that this action in manly due intracellular Ca^{++}. These first results confirm the possible central role of S1P in the excessive airway narrowing characteristic of airway hyperresponsiveness in asthma and highlight the potentiality of OMTC technique for the study of drug induced changes in the mechanical properties of cell culture.

DEVELOPMENT OF SURGICAL PLANNING AND NAVIGATION TECHNOLOGIES IN BREAST PLASTIC AND RECONSTRUCTIVE SURGERY

Paolo Patete

The current clinical practice of breast reconstructive surgery envisages surgery following a tumor excision procedure (mastectomy or quadrantectomy) aiming at recovering morphology of the breast and ultimately patient's quality of life as soon as possible. Reconstructive surgery can be executed in a "one-stage" operation (when it is executed immediately after the excision) or in a "two-stage" protocol (when it is executed even months after the tumor excision).
The reconstructive surgical techniques vary from the use of expanders and prosthesis to the use of autologous tissue flaps or to the lipomodeling of the breast. Over the last years, the tendency has been to favor the use of autologous solutions in antithesis to the implant of prosthetic devices, which lead to chronic inflammatory problems, breast deformities due to capsular contraction and require replacement after few years.
Breast lipomodeling envisages a procedure in which fat tissue is removed from patient's adipose reserves (like lateral thighs), purified through centrifugation, and transplanted to the surgical target. Fat transplantation has been demonstrated to lead to striking results in radiation induced chronic ulcers, correction of breast deformities after breast conservative treatments (quadrantectomy) and in post-mastectomy for total breast reconstruction. In light of these results and of the easiness of fat tissue procurement, lipomodeling already represents a valid alternative to tissue flaps and a rapidly emerging technique in PRS.
From the general standpoint, the introduction of quantitative instruments in the PRS surgical workflow, needs to take into account three main critical issues:

1. breast deformability;
2. transfer of the surgical plan in the surgical outcome;
3. compliance of intra-operative navigation systems to the surgical procedures and environment.

For breast modeling, we decided to develop a mass-spring model of the thorax, in which all the important structures (skin, fat, mammary gland and chest wall) could be represented.
The main issue to be solved was the breast morphology dependency to gravity direction; this is a particularly critical issue, due to the fact that the MRI data for volumetric model definition are acquired with the patient in a prone position; conversely, PRS interventions are normally performed in supine position. In order to face this problem, a specific iterative algorithm was developed for the recover a of "rest" mesh configuration proper of the supine position, starting from the gravity deformed configuration characterizing the MRI prone position.
Surgical planning methods were developed for the lipomodeling surgical technique. The basic assumption was that the uniformity in fat tissue deposition within the target volume could improve the result, thus allowing for a lower number of surgical sessions for the breast reconstruction or the recovery of post-operative deformities.
Fat tissue uniform deposition is also believed to reduce post-operative complications such as liponecrosis and the formation of oil cysts, which normally originates from bulky tissue deposition.
The core of the surgical planning module for lipomodeling was based on a genetic algorithm, which is fed by the main breast anatomical structures (gland, skin, fat, rib cage layer) segmented on MR scans.
Geometrical parameters (number of the entry points, number of insertion pathways per entry point, etc.) are also required for the algorithm initialization. The genetic optimization optimizes the transplant geometry by minimizing the amount of breast volume which is not crossed by any insertion pathway. Specific graphic user interface was created for increasing application usability.
The technological effort related to surgical navigation was related to the development of innovative sensors for surgical tools real-time tracking. The work was based on recent attempts to develop alternative solutions to the spatial tracking of surgical tools, envisaging the use of inertial sensors and inertial measurement units (IMU).
The development of this kind of instruments is put forward to represent a considerable technological improvement for surgical navigation, allowing to reduce encumbrance and costs of navigation systems, thus favoring their introduction also in PRS.
The solution that was developed as a prototype is an accelerometer based IMU, in which 4 triaxial accelerometers are used to calculate both linear acceleration and angular velocity of the tool which the IMU is connected to.
The HW development was enriched by the identification and implementation of a dedicated algorithm based on an unscented Kalman filter for extracting the desired kinematic variables from the accelerations collected from the IMU.
The UKF was found able to correctly estimate the linear acceleration and the angular velocity of the IMU, so that orientation and position of the tool are calculated by a simple signal integration. As the integration requires a starting point, a calibrated "zero" position and orientation has to be defined, in order to correctly track the tool in the operating room.
Among the efforts produced for quantitative breast modeling was the development of an integrated system for breast surface acquisition and 3-D reconstruction, featuring a real-time correction of subject/patient's involuntary motion and breathing movements.
The proposed solution to overcome these problems is an integrated system made up by a static programmable laser pattern projector, a hand-held laser scanner and an optical tracking system.
The compensation of the rigid component of breathing movements was obtained by tracking a configuration of 6 markers fitted on the patient thorax. These data were used to correct surface points through a mapping algorithm and compensate for patient motion.
The acquisition procedure required patients to be fitted with the configuration of passive markers in correspondence of selected thoraco-abdominal landmarks and to sit on a chair with both arms stretched horizontally and a configuration of passive markers are placed on the patient thorax. The acquisition consisted of two steps:

1. a first scan lasting few seconds is made using the static laser spot projector;
2. the obtained model is refined by means of the hand-held scanner.

The laser projector generated a raster scanning of the surface and the scanning can be repeated as many times as desired in order to acquire a more dense point cloud.
The hand-held laser scanner consists of a spot laser source and a motion analyzer TV camera, rigidly mounted on an PMMA support; the device is tracked by localizing 5 passive markers placed onto the system frame. Tracking is performed by a couple of TV cameras of the optical device, which are also used for tracking patient motion.
Patient tracking allows one to count on a local reference system (Patient Co-ordinate System, PCS) with respect to which the surface points are expressed in 3D. This permits to compensate for patient motion thus granting extended acquisition time and reliable surface patches registration, when required.
Although much work is still needed for the definitive validation of the techniques developed during my Ph.D, the prototyped integrated system for surgical planning, navigation, and modeling in plastic and reconstructive surgery is put forward to provide an innovative contribution for breast reconstruction and deformities correction after conservative surgery. The work was organized to face the whole set of open issues related to the use of quantitative techniques for panning and navigation is PRS. In addition, the contribution in the field of surface laser scanning for pre-surgical breast modeling and post-surgical outcome assessment is proposed as a bleeding edge solution for the morphological evaluation of the patients in plastic surgery clinical practice.

IMPLEMENTATION OF FINITE ELEMENT MODELS FOR THE BIOMECHANICAL ANALYSIS OF THE ATRIOVENTRICULAR HEART VALVES

Marco Stevanella

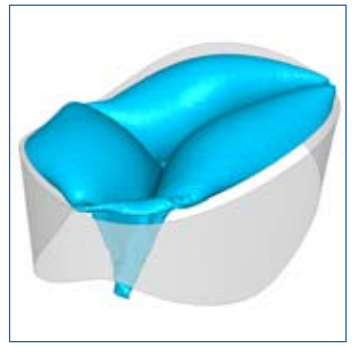
The atrioventricular valves, i.e. the tricuspid valve and the mitral valve (MV), separate the right and left atria from the respective underlying ventricles. Alterations in the synergic action of valvular substructures leads to high prevalence pathologies. In particular, mitral regurgitation is estimated to affect 2.5 million people in the USA, and this number is expected to almost double by 2030 because of population ageing and growth. The aim of the present work is the development of three-dimensional structural finite element (FE) models of the atrioventricular heart valves, in order to gain further insight into their physiological behavior, the alterations associated to pathological conditions, and the biomechanical effects of surgical repair procedures. Computational models based on the FE method allows the numerical solution of the equations of continuum mechanics applied to complex systems, and are suited to perform a flexible, repeatable and quantitative analysis of multifactorial scenarios, such as those characterizing heart valve function in pathological and post-operative conditions. However, the potential of this approach in supporting the clinical decision making process has not been fully explored. The possibility to simulate and predict the outcome of different valvular repair techniques can considerably improve the surgical planning and reduce the risk of persistent or recurrent valvular regurgitation.

To these purposes, in the present work two different modeling strategies have been adopted. On the one hand, paradigmatic models, representative of the average valve behavior, were used to identify the main biomechanical features characterizing valvular function. On the other hand, semi-automated strategies for the implementation of patient-specific models from *in vivo* imaging were developed and used to simulate different surgical procedures and predict the corresponding post-operative outcomes.

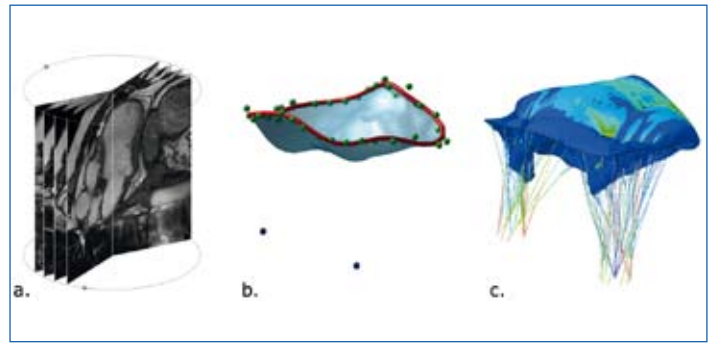
First, a paradigmatic model of the MV was developed, including several novel features: an accurate morphological description of leaflet shape and chordae tendineae arrangement, a non-linear anisotropic formulation for the tissue mechanical properties, and dynamic boundary conditions mimicking annulus and papillary muscle contraction. This model allowed to identify biomechanical features of general validity; in particular, it highlighted and quantified the importance of annular and papillary muscle contraction in regulating MV function. Moreover, from a methodological standpoint, the development of such model provided the basis of the subsequent activity, allowing to identify the critical aspects of the modeling strategy and to formulate possible solutions and improvements.

Further insight into MV function, and in particular into the contractile properties of the anterior mitral leaflet (AML), was obtained in collaboration with the Stanford University School of Medicine. These models focused on the assessment of the impact of AML recently discovered *in vivo* time-varying material properties and the importance of the time-dependent synergy of annulus, leaflets and papillary muscles. To this purpose, biplanar fluoroscopic acquisitions of an array of radiopaque markers silhouetting the mitral apparatus in the beating ovine heart were performed. This study quantified the importance of *in vivo* AML transient stiffening in preventing leaflet bulging and limiting deformations during systole in ovine models, thus providing a stable boundary for blood flow during ventricular ejection.

The paradigmatic modeling approach was then extended to the study of the tricuspid valve (Figure 1), given the similarities with the mitral valve in terms of function and structure.



1. FE model of the tricuspid valve in open (grey) and closed (blue) configuration



2. a. acquired long-axis multiple CMR cut-planes; b. annulus and leaflet 3-D profile reconstructed through our custom software; c. stress contour plot on the patient-specific FE model.

Even though data about the tricuspid valve in the literature are very few, this preliminary model was implemented combining them with the results of an experimental campaign performed in collaboration with the Luigi Sacco Hospital (Milan, Italy), and allowed to provide useful quantitative indications on the tricuspid biomechanical behavior and to set the basis for future more sophisticated approaches.

Finally, a framework for MV patient-specific modeling from *in vivo* imaging was implemented, setting the basis for a computational tool that may help the clinical decision making process through the patient-specific prediction of the outcome of common surgical procedures. The present framework already includes a semi-automated procedure for the reconstruction of MV FE models directly from medical images (Figure 2) acquired using different imaging techniques, i.e. real-time three-dimensional echocardiography (RT3DE) and cardiac magnetic resonance (CMR). This modeling approach was only slightly affected by operator's inaccuracies and image noise, proving flexible and solid with respect to input data from both data sources. CMR allowed for higher spatial and temporal resolution and the inclusion of papillary muscle motion throughout the cardiac cycle. However, since it has higher costs, is not routinely used in the clinical practice for the assessment of valvular diseases and thus requires the use of an *ad hoc* acquisition sequence, this technique is probably more suited for research studies than for the extensive use in surgical planning. Low cost, flexibility and ease of handling make RT3DE the primary clinical tool for evaluation of valvular heart disease. Thus, RT3DE is likely the most promising choice as input source for the present modeling framework, especially if we aim to use it as a predictive tool for supporting the surgical planning of early-stage valvular repair. This tool may also help to significantly reduce the costs due to re-interventions after recurrent disease by improving long-term valvular function through the prediction of the repair strategy with better post-operative performances.

COMBINING ELECTROENCEPHALOGRAM AND FUNCTIONAL MAGNETIC RESONANCE IMAGING FOR THE STUDY OF COGNITIVE PROCESSES AND PATHOLOGICAL DISORDERS

Maria Gabriella Tana

Neuroscientists have at their disposal a variety of tools for investigating human brain functions. Among the technologies of non-invasive functional imaging that have flowered in the last five decades, two techniques are particularly popular: the electroencephalogram (EEG) which records electrical voltages from the electrodes placed on the scalp and functional magnetic resonance imaging (fMRI) which records magnetization changes due to variations in blood oxygenation. The fusion of EEG and fMRI allows to study cognitive and pathological processes in terms of *spatial* localization of the regions involved in the investigated process and in terms of knowledge of the *temporal* dynamics of such a process. Four type of analysis were here proposed, the first two were aimed to the *spatial* definition of the brain network underlying neural activity and the last two were aimed to study the *temporal* dynamics of cerebral processes.
The first one has been called ***time EEG-informed fMRI analysis*** and consists in designing fMRI regressors by using information derived from EEG analysis in the time domain. This is particularly useful for spatially localizing of brain areas involved in spontaneously arising neural mechanisms such as epilepsy in which the stimuli causing the alteration of neural activity are task-free, random, spontaneous and endogenous (i.e. internally generated). EEG measurements are analyzed in the time domain in order to identify the time of onset of epileptic activity and this information is used to build regressors for General Linear Model (GLM) analysis of fMRI images. One of the most critical factors limiting the potential of the above mentioned technique and preventing it from becoming a clinical tool in the context of epilepsy is the few knowledge about the haemodynamic response function (HRF) to the epileptic spikes. The use of a fixed "a priori" HRF model, hence, can impair the accuracy and the sensitivity of the technique. We used a non-parametric approach where no prior hypothesis about the shape of HRF was done and no bias in the haemodynamic response estimation was introduced. HRF was estimated to the maximum-likelihood estimate using an expectation conditional maximization algorithm.
We showed that the shape of the HRF to the epileptic spikes may differ significantly from the standard model, it is variable across regions and it could be widespread and found in cortical region that are distant from the scalp EEG findings. This suggest an underlying biological process that extends beyond the area clinically assumed as focus and the possibility of effects of focal EEG spikes on remote but synaptically connected regions.
Information derived from EEG analysis in the frequency domain can be also used to construct a GLM design matrix in order to perform a ***frequency EEG-informed fMRI analysis***.
The construction of the design matrix modeling the link between oscillatory EEG activity and fMRI signal was based on the recent findings recording showing that the haemodynamic signal is related to frequency content of EEG signal in all frequency bands rather to a single frequency. We applied this model to fMRI data recorded during a motor task.
Our findings revealed that a model of the relationships between BOLD signal and EEG oscillatory activity comprising all frequency bands is effectively able to well describe the frequency-dependent neurovascular coupling.
In particular, the use of brain wave model allows to recover information about the fMRI response for brain processes not directly linked to the task itself but complementary to it, and, therefore, it can offer a continuous "additional signal", which goes beyond the mere on-off dichotomy of the movement task. Integrating frequency-dependent EEG oscillatory activity and haemodynamic signal allows to recover interesting information about the physiological mechanisms underlying rhythmic activity of neuroelectrical signal. More particularly, the most interesting result is that the alpha rhythm is linked predominantly to the somatosensory system, while the beta rhythm is more related to primary motor system.
The second general aim of the present work is the investigation of the *temporal* dynamics of neurophysiological or neuropathological process under investigation. In this context, an important issue is to obtain information about the ***temporal evolution of fMRI activation maps during prolonged task***. To solve this issue, we proposed a novel GLM design matrix in which the task is subdivided into a set of regressors, each one representing a given period of the esperiment. We applied this method to fMRI data recorded during sustained attention task. Our results showed that the proposed method for creating the design matrix, allows to apply fMRI also to a paradigm of prolonged stimulation and is able to provide a sort of "dynamical" statistical maps showing the temporal evolution of fMRI response. From a clinical point of view, using the proposed method we have been able to identify a complex network of brain regions consistent with the existing models of visual object processing and attentional control and the results of clinical literature obtained with other neuroimaging techniques.
Furthermore, analysis of the temporal evolution of the fMRI response during the task, has shown a progressive decreasing of BOLD response during the first half of the test due to the habituation effect and an increasing of the activation in second half of the test probably correlated with the deterioration of the performance that occurs at the middle of the test.
The investigation of dynamics of neurological processes, during the last decade, has put increasing emphasis on the analysis of ***temporal interactions within brain networks***. The last part of this thesis is devoted to the problem of investigating inter-region connectivity. In order to estimate directed casual influences between cerebral structures Granger Causality Analysis (GCA) was introduced on the basis of the Granger causality concept according to which the activity of a first region of interest "causes" the activity of a second region if the knowledge of past values of the first time series improves the prediction of the current value of the second time-series. Although GCA approaches opens new opportunities for the researches on brain networks there some fundamentals issue that need to be investigated more deeply than it have been by now.
One of the main concern of GCA is the definition of the nodes of the networks. The current common approach to define nodes consists in considering as nodes simply the cluster resulting from GLM analysis. The belonging of two voxels to a same cluster depends only on their spatial nearness and on the fact that an no-active voxel is interposed or not between them. To overcome these limitations we developed two methods of parcellation of fMRI clusters: the first one is based on information derived by anatomical atlas; the second one is based on functional information and works with algorithms of hierarchical clustering applied directly to fMRI data.
In this work we applied both methods to fMRI data of epileptic patients and we compared the connectivity results of the two networks obtained by the two parcellation techniques. We find that our novel parcellation methods are able to define nodes of the brain network in such a way to obtain interpretable results and useful information about the phenomenon under investigation. Both proposed approaches allowed to identify the source of the network and therefore the epileptogenic focus that is otherwise impossible to identify with only surface EEG.
With reference to the debate existing in literature about the reliability of GCA in the study of brain connectivity, our findings stated and confirmed the reliability of GCA to investigate direct influence inside brain networks, since they showed that it is possible to obtain similar information by applying different nodes definition strategies.