PROPOSAL

for a

Joint IIT-Sapienza LAB on Life-NanoScience

Roma, November 14th, 2010
Prof. L. Frati

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Introduction

Following preliminary discussions between the Scientific Director of IIT and the Rettore of the Sapienza University (hereafter called "Sapienza") and their delegates, a common desire to define an agreement aimed at establishing a "Unità operativa IIT" (hereafter called "the Unit") in the premises of the Sapienza University has emerged. It was established that the project was to focus on Life nanosciences and cover different technological aspects of common interest. Specifically i) The development of advanced instrumentation; ii) New techniques for structural determination and molecular imaging ; iii) Methodologies for drug delivery; iv) New genomic, proteomic and cellular diagnostics and therapies.

The project will develop in three phases: in the first phase (Phase 1) that will start in 2011, the Unit will occupy about 1700 square meters in premises close to the University Campus. Phase 2 will start around 2016, following a positive evaluation of the results obtained by the Unit in Phase 1. A this stage the Unit will be provided with increased space (about 5000 m2 within the Citta' della Scienza of the Sapienza Universty currently under planning). In Phase 3 the Unit activities will focus on projects exploiting the synchrotron radiation produced by the new accelerator for e^+-e^- collisione (SuperB) for advanced biomedical applications. The implemntation of Phase 3 is obviously subject to the actual construction of the accelerator.

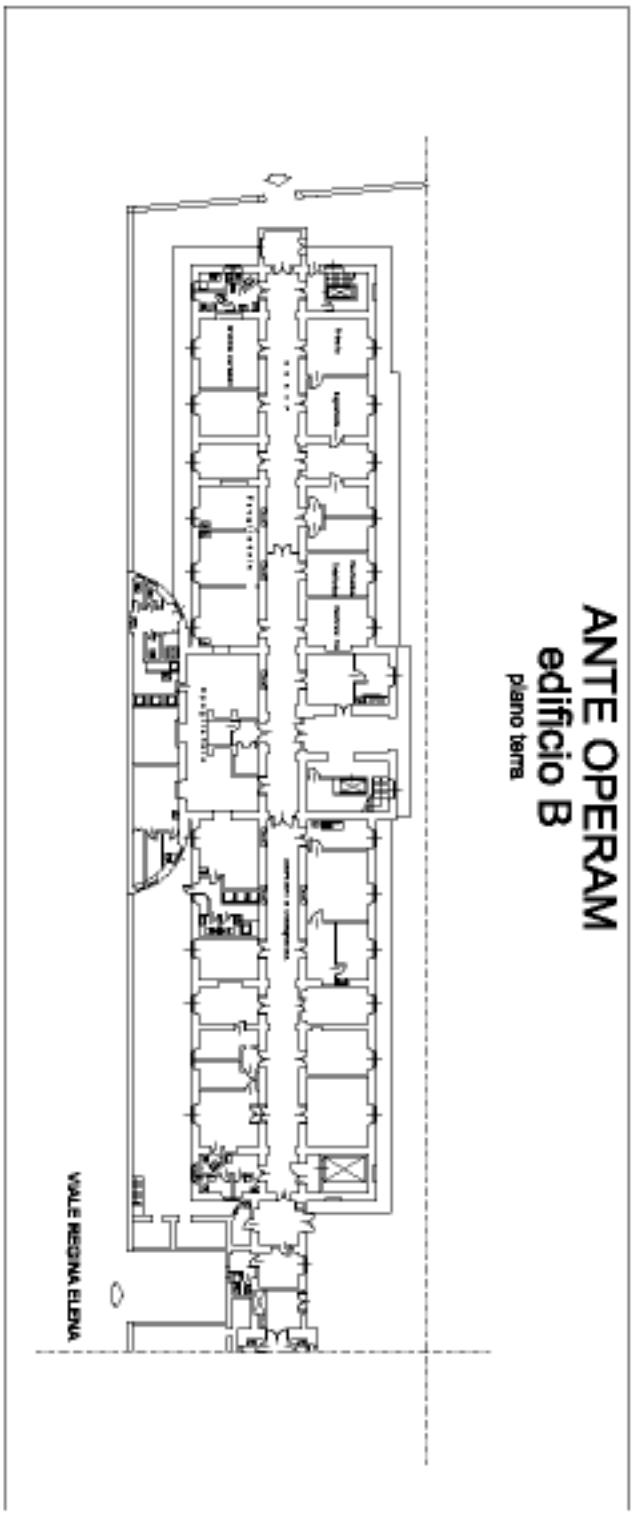
This document describes in detail the planned activities of Phase 1 in order to foster and accelerate the conclusion of an agreement between IIT and Sapienza.

Location

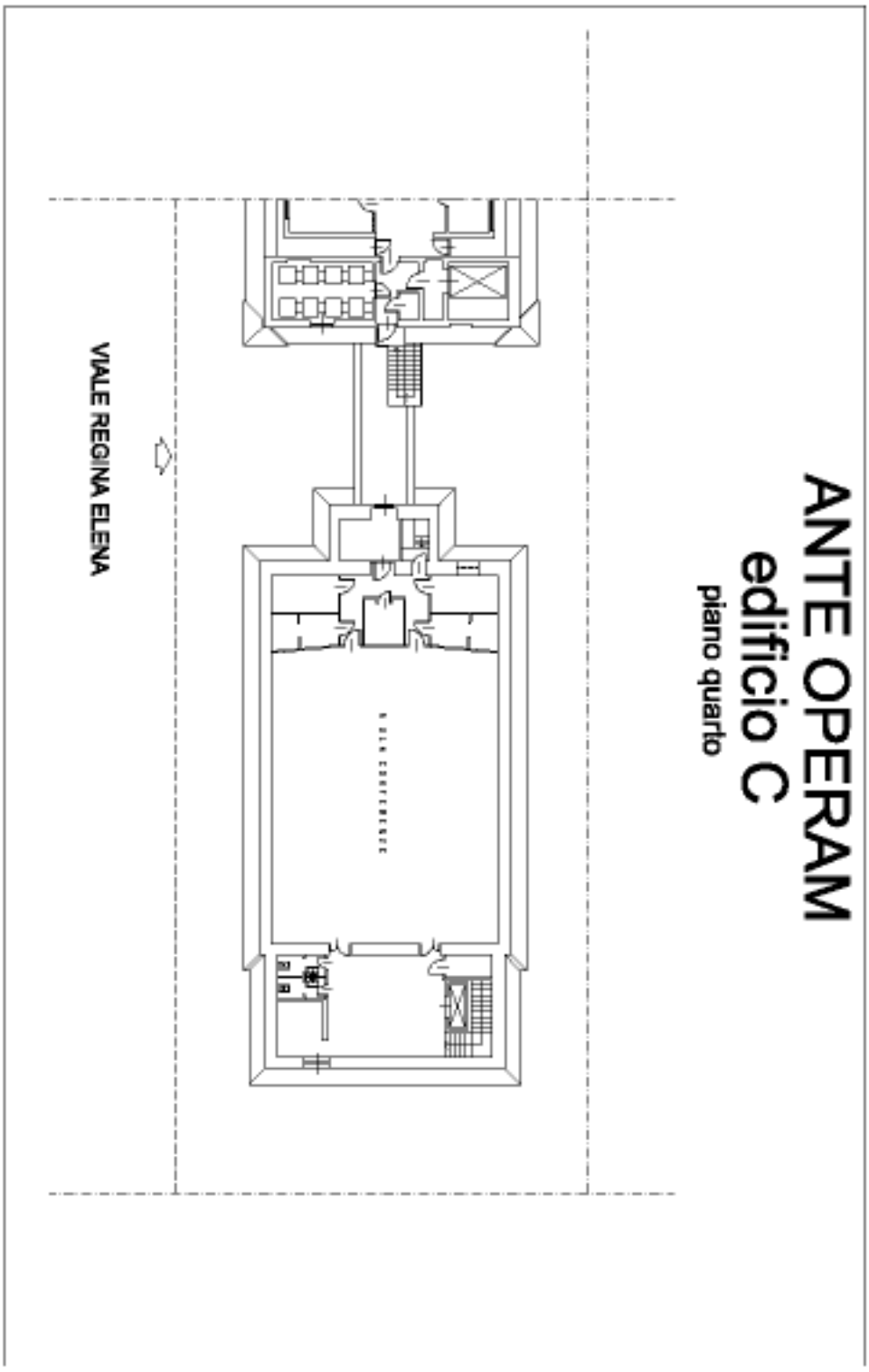
Sapienza has identitifed an appropriate space to lend to the Unit which can use it free of charge ("comodato d'uso gratuito"). The space is located on the ground floor of Edificio B of the former Regina Elena Institute (1100 m^2) and on the fourth floor of Edificio C of the same building (about 400 m^2) (see enclosed map). The space is being refurbished and will be ready and completed with essential services (electricity, network, air conditioning and ventilation) in 6 months from now.

A timely project review (by the end of December 2010) could still allow changes aimed at an optimal usage of the laboratory space to be implemented.

It will be the responsibility of IIT to provide for the infrastructures necessary for the proper functioning of the lab and the furbishing of the latter with instrumentation and furniture.



Plan of the ground floor of the Edificio B of the former Regina Elena Institute. Dimensions are approximately 18 m x15 m.



Plan of the fourth floor of the Edificio C of the former Regina Elena Institute. The total area is about 400 m^2.

Lab Organization

The laboratory revolves around two very relevant activities of biomedical interest where technological innovation is key to reach the goals.

The first biomedical area of interest will cover neurodegenerative disorders, hereditary and sporadic conditions characterized by progressive nervous system dysfunction. The project will study the molecular, cellular and tissutal processes underlying nervous system homeostasis and differentiation and their misregulation.

The other area on which the efforts laboratory will focus is brain tumours, the most life-threatening diseases of adulthood and childhood. Primary aims here will be the understanding of the interplay between Cancer Stem Cells (CSC) and neoangiogenesis, the dynamics of the CSC population and the set-up of pharmacological screenings of available small drug and natural products libraries as well as of innovative immunotherapeutic strategies. At the same time the project will employ and further develop *in vivo* molecular imaging technologies to improve tumour detection

The laboratory will include two technological platforms (Genomics & Bioinformatics and Microscopy & Tracking) that will contain state-of-the-art commercial equipment and serve the technological needs of the projects. The platforms are also expected to provide support for the development of new techniques and instrumentation.

The lab is manned by IIT personnel, both at the scientific and technical level (for the management of the instrumentation).

A director who will be responsible for the overall activities of the laboratory will be hired/selected during the first year of operation.

Access to the Sapienza personnel[1] is granted on the basis of Associate membership to the Unit. A more generalized access and use of the technological platform is granted to other Sapienza or Sapienza-associated personnel to carry on specific short term IIT - Sapienza collaborative projects.

Affiliation

About 25/30 Sapienza scientists participating in the project will be associated to IIT. The association status will last for the length of the project.

Associate members of IIT will have the right to access the IIT laboratories and specifically the technological platforms according to a policy that will be established collegially.

[1] Sapienza personnel includes the scientific staff of the University and of the research Institutions having an active agreement with Sapienza.

The associate members of IIT must mention their affiliation to IIT, in conjunction with that of their own Department, in all the research products obtained as a consequence of the use of the IIT laboratories or achieved through the IIT-Sapienza collaboration. The rules for the exploitation of intellectual property will be established by a forthcoming agreement between IIT and Sapienza, which will also regulate launching and participating to spin-off companies.

Research activities

The scientific activity of the life nanoscience laboratory is devoted to interdisciplinary research targeting two major biomedical areas through the application of innovative technologies. The study of neuro/muscular degeneration and of brain tumours represents challenging health problems which can be addresses through a combination of technologies ranging from those aimed at developing new drug delivery strategies, novel techniques for structural determination, molecular imaging and diagnostic, genomic, proteomic and cellular therapies also through the development of novel advanced instrumentation.

The project is formed by two specific research activities in the field of nano-medicine, hereafter indicated as areas A1 to A2. The activities are:

A1 - Novel Nanotech-Based Approaches for the Study and Treatment of Neurodegenerative diseases

A2 - Novel strategies for the imaging and treatment of brain tumors through targeting cancer stem cell-specific signaling pathways.

The detailed project plans can be found at the end of this document.

The research activities in the bio-medical areas will be carried out in the last floor of Edificio C (about 400 m^2) in appropriately furnished and equipped spaces suitable for intensive human occupation.

A qualifying feature of the specific proposed projects is their innovative approach to health problems. These projects are also aimed at identifying, within the framework of relevant pathologies, innovative technological approaches required to answer to fundamental question to advance our knowledge and with an immediate fallout in diagnostic and therapy that will be developed in the R&D Laboratory.

Technological Platforms

A number of the activities described in the two specific projects A1-A2 can very much benefit from the establishment of common technological platforms that can effectively support the biomedical applications and their research teams. In order to exploit the synergy

among the various aspects of research and provide relevant added value to their development, the Laboratory will establish the following platforms:

- Genomics & Bioinformatics
- Microscopy

Each technological platform will be supervised by two scientific managers (one with technological and one with bio-medical expertise) and co-supervised by an IIT scientist.

The technological platforms occupy about 500 m^2 on the ground floor of Edificio B. The area is composed by lab spaces to be equipped with state of the art commercial instrumentation and will provide the technological services. The remaining part of the ground floor of Edificio B will be occupied by the technological R&D laboratories, as described in the A1-A2 projects.

Technicians are assigned to those instruments requiring special support. These units of personnel will take care of their maintenance and assist the users.

Whenever reasonable and possible, agreements will be sought with instrument providers in order to have "open versions" of the instruments that can be further developed by assembling and/or integrating them with novel instruments that can be *i)* used for the research activities carried out in the boiling pot and *ii*) exploited for the construction of new instrumentation of potential commercial interest. These activities will be carried out in agreement with the instrument providers.

Access to the technological platform is granted, through a policy to be defined, to the IIT personnel, to the IIT associated personnel working in lab. Access is also granted - on the basis of IIT-Sapienza collaborative short term projects - to Sapienza researchers. The latter can in total use the instrumentation for no more than 30% of the time. The proposals for short term projects will be evaluated in a timely fashion (within no more than 7 working days) by a committee whose members are designated, in equal number, by IIT and Sapienza.

IIT and Sapienza will have equal weight in choosing the instrumentation of the technological platform. This will be purchased using the Start up fund of the first year of the project (see budget)

A detailed explanation of the specific activities for each platform is given below.

Technological platform P1

Genomics & Bioinformatics

Coordinating PIs: Proff. Massimo Levrero and Anna Tramontano

Synopsis

The Integrative Genomic and Epigenomic Platform (IGEP) collaborates with scientists at the IIT-Sapienza Life-NanoScience Laboratory to identify and characterize patterns of genetic variation and gene expression. These patterns can yield a deeper understanding of how genetic factors influence disease risk and treatment outcomes in a wide range of human diseases, with a particular focus to neurodegenerative diseases and brain tumours.

IGEP aims to provide a wide range of genomic technologies and platforms, including genotyping, chromatin dynamics, gene expression and directed re-sequencing, and routinely apply these technologies to studies of both human and non-human samples. In addition, the IGEP platform will work to test out emerging genetic technologies, develop informatics tools and infrastructure to enhance scientific research and coordinate projects involving the IIT-Sapienza Life-NanoScience Laboratory.

The platform's major technological activities include:

High-throughput sequencing. IGEP NGS activity will produce the massive quantities of genomic data needed to investigate the whole genome structure, genome wide genetic associations (GWAS) of SNPs, genome wide protein/chromatin associations (ChIPSeq), the epigenome (histone modifications) and the DNA methylome, miRNA and sncRNAs genome wide profiling and the complete transcriptome (RNASeq). In some cases, the rough drafts of genome wide information are sufficient to achieve the research and clinical objectives but directed activities will be set up when appropriate.

Libraries preparations. IGEP will coordinate with users to design the “best fit” experiments and to provide the appropriate sample processing technologies in order to maximize the effort to gathered information ratio and thus minimize overall the costs (“sustainable genomics”). Genome wide technologies are rapidly evolving and a major focus of the IGEP will be to track/test/adapt/evolve new technologies as well as develop new applications.

Sequencing informatics (in collaboration with the bioinformatics section). These activities will provide the infrastructure and tools necessary to process, store, analyze and track the millions of samples handled by the platform.

Altogether these integrated activities will generate a large collection of genomic data sets amenable to further cross referencing with similar or complementary data sets produced elsewhere. Ultimately these efforts will advance discovery in biology and medicine and will hopefully shorten the lag time to the application of new knowledge to diagnosis and cure.

We are well aware that the development of tools for biological data analysis is a moving target as new technologies appear continuously. Each will require novel approaches and will deliver new data types that must be integrated with available ones.

The platform will be populated with commercial state-of-the-art instrumentation using the "Start Up P2" budget (1,500,000 Euro) during the first year of operation of the project.

- Illumina Hi-Seq 2000 NGS platform or higher
- Covaris S2 (DNA shearing instrument for NGS fragment library preparation)
- Caliper Lab ChIP XT (Automated nuclei acids fractionation apparatus for NGS)
- Agilent Microarray Station
- AB 7900 HT Fast (real-time PCR thermocycler)
- Production Computer cluster with disk space for storing laboratory data
- Development Computer cluster
- Phosphoimager, chemidoc and luminometer
- High-capacity radiographic processor

Genomics platform set-up

The advent of next-generation technologies has fuelled an explosion in the quantity of raw DNA sequence that can be generated. The chips (flow-cells) that are currently utilized for the GAIIx have eight channels or lanes, allowing eight sample libraries to be simultaneously analysed. Additional samples can be analysed using the so called multiplexing or indexing to mix different samples in a single lane of the chip; these samples can be subsequently separated in software using their unique sequence barcodes. Typically, all eight lanes of a 100-cycle run generate about 30 Gb of sequence in paired-end mode (sequencing sequentially 100 bases, e.g., from each end of the molecules).

The IGEP platform will both operate as a project-focused service for the IIT-Sapienza Life-NanoScience Laboratory A1 and A2 projects and will independently develop datasets and tools. The facility will assist users all along the research pipeline workflow from the experiments design to their actual performance to end with data analysis (in concert with the bioinformatics section of this platform) and, eventually, to results validation by providing an integrated access to high throughput PCR validation assays.

Running as a facility requires a great amount of support and integration in three critical areas: a) library preparation, b) maintenance and c) sequencing informatics. All these activities require the presence of qualified dedicated personnel.

The IGEP platform will hire one to two full time technicians devoted to the maintenance and running of the facility as well as one senior post-doc to coordinate projects design and management and one to two post-docs / PhD students to provide coordination/advice and eventually assist users in the pre-sequencing (project oriented) phase as well as in supervising/performing the NGS workflow. The actual data analysis will be performed in concert with the bioinformatics platform. Expert assistance for the NGS results validation phase will be provided according to needs / request.

The facility will be initially centered around one Illumina GAIIx apparatus. The acquisition of a second NGS apparatus is planned to year 2/3 of the project in order to best accommodate the growth curve of users demand and the consequent increase in the facility workload as well as to cope with the continuous development of both the technology and the hardware. The facility will also include support technology for sample preparation and fragments length selection (i.e. Covaris and Caliper LabChip) as well as an Agilent Array platform and a Bead Express for results validation phase. The Illumina high throughput sequencing techniques and microarrays-

based techniques will be used to generate mRNAs and miRNAs profiles, to study chromatin dynamics and epigenetic histone modifications (ChIP-Seq) and assess DNA methylation (me-DIP). Standard datasets analysis will be integrated with the refined clustering and classification algorithms for gene expression and chromatin immuno-precipitation (ChIP) analysis generated to identify robust interaction and regulatory patterns. The facility will also provide assistance for the extensive validation of new mid-to large size molecular signatures using the DASL (cDNA-mediated Annealing, Selection, Extension, and Ligation) multiplex expression profile protocol that allows robust quantitative analysis from small quantities of input RNA from both frozen tissues or paraffinated sections.

Research & Development

Besides performing the genomic analysis described in projects A1 and A2, IGEP will develop independent R&D mainly in the field of pre-NGS procedures (target selection, libraies preparation).

The R&D activity will focus on the invention of novel and improved protocols to take better advantage of this new technology. This will be mainly applied to the pre-NGS procedures (target selection, libraries preparation). In spite of the high throughput of NGS it is not feasible to sequence large numbers of complex genomes in their entirety, because the cost and time taken are still too great. In addition to the burden in terms of actual time and funding the primary analysis, where the image files captured during the sequencing reaction are converted into nucleotide sequences, a huge burden would fall on the informatics infrastructure, for storage of the resulting sequence information. One way to cope with these limitations is to perform a 'target enrichment', where unwanted genomic regions are selectively depleted from a DNA sample prior to sequencing, as part of the sample preparation. IGEP will actively focus on the development of application oriented protocols aiming to ideally perform “personalized” pulldown target enrichments. Another area of development will be to invent/design/optimize/test new multiplexing procedures adapted to specific sequencing projects where the maximum high throughput of NGS runs would represent a massive excess. By combining barcoding and pooling of up to a hundred samples to be sequenced as a single sequencing library will make NGS more and more attractive in planning powerful NGS-based clinical investigation studies. To increace the barcoding design and reading capacity represent an additional area of possible R&D.

Bioinformatics platform set-up

Although there are several tools and systems available for the computational analysis of biological data, the ever growing amount of data and, especially, the continuous innovations in technology require that expertise is available to make the best use of the information.

The projects of the laboratory are challenging and will employ cutting edge technologies producing not only a vast amount of data, but also data of different types, from sequencing to imaging, to functional results. All of them need to be stored, retrieved, analysed and, especially, integrated. This implies that the computational aspects of the projects are challenging as well and will require extensive research and development activities that will be performed in collaboration with the computational biology scientists in the A1 and A2 projects.

The needs that we want to meet of this part of the technological platform are three:

- The data produced by the laboratory need to be integrated and easily accessible to scientists to perform "in silico" hypothesis testing (and to this end we will provide sufficient hardware resources to the lab).
- An integrated software system is required to guide the scientists, including clinical researchers, through the analysis of the data by mapping the results on genomes, proteomes and networks in an effective and easy to use fashion and by installing and customizing systems that combine several strategies for integrating the information. The data to be integrated include available functional information from public repositories and from orthologous genes from other organisms, SNPs mapping on sequences and (modelled) structures and information derived from biological networks, text mining and experimental data.
- All members of the laboratory must acquire the necessary knowledge to interpret their results correctly. This will be achieved both through the development of customized laboratory management systems and of a portal that will provide easy access to existing validated tools for sequence, structure, interaction and biological network analysis and by providing short hand-on courses, especially students and young post-docs, to make the most out of their data and to be able to objectively evaluate the effectiveness of newly appearing methods for their specific task.

It is essential that this is organized as soon as possible and for this reason we plan to hire two technicians at the very early stage of the project to ensure that the data analysis aspect of the projects are fully functional from the very beginning of the operations.

Research & Development

At the end of the start-up period, estimated in about 12 months from starting, the team will tightly collaborate with the computational biologists of the laboratory to develop innovative tools and computational instruments directed to the application areas of interest for the laboratory, in particular for the analysis of genomics data, target identification and characterization and biological network reconstruction.

In particular, we would like to explore novel solutions to substantially speed up the analysis of NGS data with the aim of processing them as fast as they are produced by exploring different hardware and software possibilities. This will be done in collaboration with CASPUR (Consorzio di Supercalcolo per Universita' e Ricerca) that is already exploring the possibility of using GPUs and that will provide us (through an official agreement) with additional power for data storage and handling.

We would also like to address the issue of the lack of integration of the tools to extract knowledge from the data. The main challenges are the extremely large data sets, the complex, multistep analysis procedures required to produce meaningful information, the multiple reference data needed to annotate and verify data and sample quality, the need of visualizing the datasets in multiple ways and in a comparative fashion to gain knowledge. The plan here is to develop an integrated system that will be designed in collaboration with the scientists of the laboratory in order to make it effective and suitable to their needs. We plan to explore the meta-server technology as well as the concept of web services to integrate results provided by different tools.

Technological platform P2

Microscopy

Coordinating PIs: Proff. Giancarlo Ruocco and Angela Santoni

Synopsis

This platform will provide basic support to the projects in the field of bio-oriented microscopy, as well as help in the development of novel instruments and methodology. The microscopy techniques requested by the A1-A2 project, as well as the new foreseen development, are mainly oriented to the study of intracellular distribution and of traffic of specific macromolecules (i.e. proteins and/or miRNA).

On one hand, the platform will host commercial state-of-the art instrumentation, which – according to specific agreement established with the provider - will be left "open" for technical implementations and new protocol definitions. On the other side, it will provide the natural environment for the development of new microscopy techniques, as detailed below.

Microscopy platform set-up

The platform will be populated with commercial state-of-the-art instrumentation using the "Start Up P2" budget (2,500,000 Euro) during the first year of operation of the project. Specifically, the following instruments are expected to be purchased:

- Multi-photon microscopy with STED capability
- Confocal microscopy, with FRET, FRAP,… capabilities
- Confocal microscopy combined to AFM / STM systems
- Cell sorter and flow cytometry systems

The operation of the platform will be guaranteed by four dedicated technicians and by a yearly budget of approx 100,000 Euro/year. The instrument time will be dedicated to three activities:

i) support to the A1-A2 project (approx 60% of the total instrument time)
ii) R&D for implementation and new protocols (approx 20%)
iii) Open to collaborative research project from the Sapienza community (approx 20%)

The time-slot allocation will be defined by the scientist(s) responsible for the Laboratory.

Development of new microscopy techniques

This part of the platform will support scientists involved in the A1-A2 project aiming to exploit innovative technologies and/or physical processes as yet unexplored. The budget for this activities is described in the A1-A2 project budget, and the platform will share the space for the

laboratories, allowing to create the proper synergy between existing commercial materials and new instrumentation.

Specific focus of the newly developed techniques will be towards single molecule imaging aimed at investigating macromolecular distribution and traffic at the intracellular level. In a nutshell (the details can be found within the A1-A2 project text) the foreseen activities are:

- *Development of digital holographic microscopy.*
- *Development of CARS microscopy.*
- *Development of total reflection microscopy.*
- *New scanning techniques"standard" confocal and/or multiphotons microscopy.*
- *Development of single molecule trapping techniques based on infrared radiation*
- *Development of a ready-to-use intracellular Raman single molecule detection system based on a plasmonic antenna*
- *Development of integrated AFM/spectomicroscopy based on photonic and plasmonic nanostructures for single molecules in both the intra and extra cellular context.*
- *Development of integrated spectro-photometric systems for multi spectra analysis (also on chip)*
- *Development of new nano-phosphorous labels which allow to go beyond the blinking and bleaching problems associated to fluorescent and nano-dots dyes.*
- *Development of techniques (software and hardware) for tracking and image reconstruction. Here the computational methodologies will be based on ab-initio methods, DFT, micro-nano fluidic modelization, FTDT for electromagnetic radiation and its interaction with matter.*

Budget Summary

The IIT funds will cover the StartUp phase expenses (5,500,000 Euro) in 2011, under the supervision of the Lab director. In this phase the technological platform and the laboratories will be equipped with state of the art commercially available instrumentation. The first three years of the research projects will be financed by IIT, the fourth and fifth year of these project will be financed about fourthy-sixty by IIT and by external, competitive, funds respectively. The **total IIT investment will be 21,000,000 Euro**, while the expected contribution from "competitive" funds will amount to 5,200,000 Euro, i.e. about 25% of the IIT contribution.

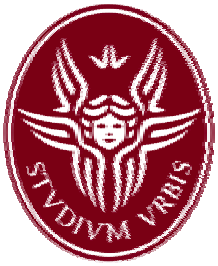
The Start Up budget for P1 and P2 is detailed above. The Activity budget for A1 and A2 is detailed in the two following projects. The P1+P2 activity budget is foreseen for the basic platform activities (consumables, 1,000,000 on five years) and for the technical staff associated to the platforms (12 persons overall, 1,800,000 Euro on five years) . The basic supplies budget, 1,500,000 is meant for the infrastructures (including a Cell Culture Facility, 500,000 Euro). Finally the lab management budget (700,000 Euro) is supposed to cover the live expenses (electric power, cleaning, heating ….).

Summary of budget by Activity

Start Up P1	**(2011)**	**1,500**
Start Up P2	**(2011)**	**2,500**
Activity P1+P2	**(2012-2016)**	**2,800**
Activity A1	**(2012-2016)**	**7,500**
Activity A2	**(2012-2016)**	**9,700**
Basic supplies	**(2011)**	**1,500**
Lab management	**(2011-2016)**	**0,700**

TOTAL **(2010-2015)** **26,200** **kEuro/6 years**

Project A1



Novel Nanotech-Based Approaches for the Study and Treatment of Neurodegenerative diseases

Coordinator:
Irene Bozzoni
Full Professor of Molecular Biology
Department of Genetics and Molecular Biology

Synopsis of the scientific proposal

Background and rationale

Neurodegenerative disorders are defined as hereditary and sporadic conditions which are characterized by progressive nervous system dysfunction. These diseases, that account altogether for more than 600 different species, are often associated with atrophy of the affected central or peripheral structures of the nervous system.
In the framework of this project we want to exploit and further develop the study of the molecular, cellular and tissutal processes underlying nervous system homeostasis and differentiation and at understanding their misregulation occurring in pathological conditions, building upon new discoveries in the field of molecular neurobiology and new opportunities arising from improvement in nano- and bio-technology applications.

Specific interest will be devoted to the study of neuromuscular diseases as model systems where to analyze the processes underlying neuronal degeneration as well as muscle function.
One of the most severe neurodegenerative disorders is Amyotrophic Latheral Sclerosis (ALS), a progressive and fatal disease in which motor neurons undergo degeneration, causing muscle atrophy, weakness and, ultimately, muscle paralysis. The disease has adult-onset with a typical course of 1 to 5 years. Most forms of ALS are sporadic, but ~10% of patients have inherited familial forms of the disease and a clear family history. Understanding of ALS pathogenesis began with the landmark discovery of dominant causative mutations in the gene encoding copper/zinc superoxide dismutase 1 (SOD1) in ~20% of familial ALS cases and ~1% of sporadic cases. More recently, our view of ALS has been improved by the recent discovery of two new mutations in the DNA/RNA-binding proteins TDP-43 and FUS, which have triggered new interest in ALS pathogenesis (Lagier-Tourenne and Cleveland, 2009). Even in these cases, where a well defined mutation has been linked to the disease, a clear correlation between the genetic defect and the physiopathology of the disease has not yet been disclosed.

The objective of this project will be to identify, in the genetic context of the above mutations, the deregulated molecular processes relevant for ALS initiation and progression and to understand how they affect neuromuscular junctions (NMJ) and motor neurons (MN) survival and activity. The integration of the molecular data with the morpho-functional alterations will eventually enable the identification of the molecular circuitries and cellular functions primarily involved in the disease.

As a next step, we plan to test whether the recovery of the correct expression or function of the identified molecular players in affected animals could confer any beneficial effect to the physiopathology of the disease. Initial tests will be performed *in vitro*, taking advantage of the cellular model systems described below, where we can test whether any of the identified genes is able to rescue a wild type-like MN phenotype/function. Subsequently, we want to set up conditions for MN-specific gene delivery in mice model systems by utilizing the *in vivo* transducing features of the Adeno-Associated Virus (AAV). This system has provided very good performances in transducing spinal MNs after a single unilateral intramuscular injection in muscles of adult mice and monkeys (Pirozzi et al. 2006; Towne et al., 2010). Recovery of correct expression of the ALS-associated genes in the affected MN of the mouse models

should allow to reach proofs of concept of whether any of them can have therapeutic value. In parallel, we intend to exploit the utilization of *ad hoc* functionalized nanoparticles for the delivery of selected nucleic acid molecules as an easy tool for *in vitro* manipulation of cells as well as vehicles for *in vivo* cell-type specific delivery.

A further aspect of ALS pathogenesis is that, as a consequence of nerve degeneration, muscles become severely compromised. Nevertheless, this is not a one way process, since adult muscle fibers are a source of signals that may influence neuron survival, axonal growth and maintenance of synaptic connections. Therefore, this project will also aim at identifying the molecular/metabolic/physiological alterations taking place in ALS muscles and to define how the progression of a pathological condition in skeletal muscle will eventually affect NMJ and motor neurons survival and activity.

Along this line, we will further deepen our knowledge on the molecular mechanisms controlling proper muscle differentiation as well as tissue homeostasis and integrity.
To achieve all these goals, the project will benefit of integrated and multidisciplinary actions including basic research and technologic development, genetic and bioinformatics approaches, as well as cell biology and functional assessments.

Therefore the aims of this proposal are:

1) Produce suitable *in vitro* and *in vivo* models for the ALS pathology.
2) Characterize the molecular circuitries controlled by ALS-associated proteins and identify crucial targets misregulated in ALS pathology.
3) Set-up appropriate bioinformatics tools to study the complex networks of gene expression regulation in normal and pathological neurogenesis.
4) Set-up and optimize sensitive and innovative methodologies for the in situ detection of specific macromolecules (RNA/proteins) along the MN axon or at the NMJ in *in vitro* ALS-reconstituted systems as well as for the study of body wide distribution of viruses or nanoparticles in mice model systems.
5) set-up appropriate electrophysiological tests and bio-mechanical measurements to study motoneurons activity and their impact on muscle activity.
6) Optimize strategies of gene delivery into muscles and/or in MN cells

Workpackage 1. Cellular and animal model systems

A crucial aspect of the project deals with setting up appropriate cellular and animal model systems where to study the activity of wild type and mutant ALS-associated proteins. The following biological material will be utilized:

Task 1. Cells engineered for ALS mutations

Mouse motor-neuron-like NSC-34 and human neuroblastoma cell lines will be engineered to alter
the function of the FUS or TDP-43 proteins. For loss of function experiments anti-FUS and anti
TDP-43 siRNAs will be delivered to the cells either as synthetic double stranded RNAs (Qiagen)
or as lentiviral constructs able to constitutively express shRNA transcripts. The same cell lines
will be modified for the overexpression of the FUS^{R521C} and $TDP\text{-}43^{A315T}$ mutations with ad hoc constructs. These cell lines, even if not representing appropriate systems for studying the functional effects of ALS-mutation on MN activity, are suitable *in vitro* systems for biochemical
and molecular analyses.

Task 2. Neuronal Stem (NS) cell lines

Functional analyses will be instead performed on primary cultures of motor-neurons and Neural Stem (NS) cells derived from both wild type and transgenic ALS mouse models (see below). When necessary, these cells can be genetically manipulated and differentiated *in vitro* to generate both motoneurons and astrocytes.

NS from mouse embryonic spinal cord can easily differentiate into neurons and, under specific culture conditions, retain the expression of motor neuron markers, such as the homeodomain transcription factor Islet1. These observations, in accordance with data from literature (Bohl et al., 2008), indicate that NS could be a convenient source for the generation of MN. We will test several protocols, including the overexpression of specific transciption factors (e.g. neurogenein-2 and/or Hb9 and/or Nkx6.1) and the exposure to specific morphogens (e.g. sonic hedgehog and/or retinoic acid) to efficiently derive MN from NS. The ability of NS lines, derived either from human ALS-derived fibroblasts or from the mouse model, to generate cells with MN identity will be assessed by evaluating the expression of appropriate markers, including choline acetyltransferase, acetylcholine transporter ISL-1, Hb9 and by the capacity to evoke electrophysiological properties.

Task 3. Reprogramming of patients' fibroblasts

A formidable approach named "genetic reprogramming" has been recently devised to generate induced pluripotent stem cells (iPS) from primary mouse and human fibroblasts. Fibroblasts from patients carrying ALS-associated mutations (or suitable controls) will be reprogrammed into iPS cells (Takahashi et al., 2007). Proliferating iPS cultures will be then induced toward a MN fate following protocols previously established (Karumbayaram et al., 2009).

iPS cells have been derived via excisable lentiviral and transposon vectors or through repeated application of transient plasmid, episomal, and adenovirus vectors. However, all these procedures entail some risk of genomic recombination or insertional mutagenesis.

Therefore, we propose to utilize, in conjunction with classical viral mediated gene delivery, a new methodology which makes use of synthetic mRNA for efficiently generating iPSCs or for directing their differentiation into MN without compromising genomic integrity (Warren et al., 2010).
MNs will be initially characterized by immunofluorescence using specific markers such as Isl1, Hb9 and ChAT and by their spine morphology. MN differentiation will be also followed by transducing iPS cells with a Hb9-GFP construct whose expression tightly correlates with MN markers such as Hoxa5 and ChAT (Singh et al., 2005). From the same iPS cells, multipotent Neural Stem (NS) cells can be also derived following the procedure already set up for the mouse (Onorati et al., 2009). This will provide very powerful material with the appropriate ALS genetic background in which to perform most of the molecular/functional analyses. The advances in such technology offer the unique possibility to generate NS lines also from reprogrammed fibroblasts derived from patients affected by a specific genetic disease. When necessary, post-mortem biopses of FUS mutant patients could be utilized (both types of material will be provided by Prof. A. Chiò of the Turin University, following standard patient's information procedure).

Task 4. Neuro-muscular co-cultures
An important aspect of the project will be to develop co-culture systems between myogenic and neuronal cells to first mimic in an ex vivo system the physiopathologic interplay between muscle and nerve, to study and better manipulate the critical signals emanating from the two tissues, and to define potential diagnostic tools for classifying the progression of the disease.
We plan to co-culture motor neuron with a recently developped 3 dimensional culture system (X-MET), able to reproduce the structure and function of adult skeletal muscle without using any scaffold or any specific chemical layer. This construct, could be used as a simplified model of skeletal muscle in vitro system to study muscle biology, to test the influence of pharmaceutical or toxic agents either on development and contractile functions and to study the functional interaction between muscle and nerve in a co-culture system (X-MET with neuronal cells). Immunocytochemical evidence for synapse formation between motor neuron and myotube will be represented by the close proximity of presynaptic markers, such as synaptophysin (synaptic vesicle protein) and the postsynaptic acetylcholine receptor clusters (e.g. identified by alpha-bungarotoxin labeling). On the other hand, patch clamp electrophysiological recordings will ascertain if motor neurons as well as myotubes maintain their electrical properties. Electrical stimulation of MN and recordings on myotubes will allow to evaluate synaptic activity.

Task 5. Transgenic mice
Several mouse models already exist which express the mutant SOD1 gene ubiquitously or selectively in skeletal muscle (Dobrowolny et al., 2008a, 2008b). A FUS-/- model mice exists (Fujii et al., 2005) but it is not clear whether it represents a good model of the human mutation. Therefore, in the context of this project we intend to produce transgenic mice with those site-specific mutations of FUS and TDP-43 proteins that in biochemical studies will show to have the most prominent altered phenotypes. Both ubiquitously and/or locally expressing mice will be raised. Morphological, functional and molecular analysis will characterize possible alterations at the level of muscle, MN and NMJ of novel transgenic mice. Moreover, these model systems can be utilized to test the recovery of the ALS phenotype upon delivery of putative "therapeutic" molecules.

Objectives

- Produce appropriate cellular model systems, and in particular reprogrammed patient-derived fibroblasts, where to study the activity of FUS and TDP-43 mutations and where to test the ability of identified molecular targets to rescue a functional MN phenotype.

- In parallel, transgenic mice for FUS and TDP-43 mutations will allow to carry out morphological, molecular and functional analyses both in muscles and spinal cord and to test whether any of the identified molecular targets of ALS-associated proteins can be exploited for possible ALS therapeutic interventions.

Workpackage 2. Identification of alterations induced by ALS-associated mutant proteins

The availability of the above model systems will allow to carry out the analysis of the cellular and molecular alterations occurring during the different stages of the disease at the level of both muscle and spinal cord.

Task 1. Monitoring intracellular components/organelles during the disease onset and progression.

We propose to monitor the time dependent functional and structural alterations of muscle, NMJ, and nerves and of major intracellular organelles (mitochondria, contractile elements, sarcoplasmic reticulum, transverse-tubules), using a combination of molecular and ultrastructural approaches. In addition, we will seek possible alteration in mitochondria - an organelle which has often been involved in pathogenic events of several diseases including ALS - in skeletal fibers, nerve endplates and spinal cord. We will then evaluate molecular markers of mitochondrial damage, such as cytosolic release of cytochrome C from the mitochondrial intermembrane space and will also monitor the mitochondrial fission/fusion dynamics in atrophying muscle fibers and in motor neurons as a function of time.

Task 2. Genome and epigenome expression profiles in both muscle and spinal cord.

Specific interest will be devoted to understanding the role played by FUS and TDP-43 proteins in RNA metabolism. Indeed, observations of a widespread mRNA splicing defect in diseases characterized by mutations of TDP-43 or FUS would reinforce the crucial role of splicing regulation in neuronal integrity and potentially could identify candidate genes whose altered splicing is central to ALS pathogenesis. However, due to growing interest in the non-coding components of the transcriptome, we propose to study also the profile of gene expression of coding mRNAs. High-throughput sequencing will be used to demonstrate whether these RNA-binding proteins can affect alternatively spliced transcripts or non coding RNAs. Deep sequencing analysis will be carried out with Illumina Solexa platform on specific interesting specimen in order to identify possible alterations in the biosynthesis of either type of transcripts.

TDP-43 and FUS proteins have been also shown to specifically interact with the Drosha protein (Gregory et al., 2004) thus suggesting that they may be involved in the regulation of miRNA expression by modulating the activity of this processing enzyme. Preliminary data of

one of the proponents indicate that the expression of several miRNAs, playing a key role in neuronal differentiation, is deregulated when the FUS protein is downregulated. Misregulation of some miRNA expression due to FUS mutations may likely result in major disruption of mRNA targets' regulation and neuronal functions in FUS-linked ALS forms.

miRNAs are plentiful in the nervous system where they fine-tune neuronal gene expression in a spatially and temporally restricted manner; moreover, their deregulation has been linked with the initiation and progression of several neurological disorders (Barbato et al., 2009). Deep sequencing and miRNA profiles performed in the different cellular and animal ALS model systems should allow to identify candidate miRNAs affected in ALS motoneurons and to define whether the FUS and TDP-43 mutations produce common patterns of deregulated miRNAs.

As a following step, we intend to set up *in vitro* assays where to study and compare the following activities of wt and mutated FUS and TDP-43 proteins: i) binding to pri/pre-miRNA species; ii) processing of miRNA precursor species; iii) chromatin association with different miRNA genes; iv) binding to basal constituents of the transcriptional and processing apparatus.

Instrumental to the project will be pSILAC experiments that will allow us to assess, at a genome-wide level, whether targets for specific miRNAs with possible important function in ALS pathogenesis exist. The data from deep sequencing and pSILAC experiments, in conditions of overexpression of specific miRNAs, will be analyzed to unravel the architecture of the underlying regulatory network, by identifying significant correlations between miRs levels, expression level of the target genes and avoidances (the tendency of every two miRs to avoid residing within shared 3′ UTRs). The definition of even a partially complete network will allow us to identify specific targets relevant for the neuromuscular physiology and pathology, and to design experiments to validate and complete the network. The identified protein targets will be analyzed using state-of-the-art techniques to predict, when unknown, their function, structure and interactions. Their tendency to aggregate will also be analyzed by computational methods.

The final objective of these studies should be the identification of the alterations produced by mutant FUS and TDP-43 proteins in the biogenesis and maturation of different types of transcripts. This will provide potential candidates whose de-regulation can be important for the ALS pathology. Lentiviral constructs, expressing candidate effectors, will be utilized to infect iPS cells, derived from fibroblasts' patients, and to study the recovery of MN activity upon *in vitro* differentiation. The recovery of morphological and functional parameters will be assessed as described below (WP 5). If interesting candidates will be indentified *in vitro*, they will be subsequently utilized in AAV-mediated MN transduction in the mutant mouse model and morphofunctional parameters will be tested for the recovery of wild type performances.

A similar approach will be adopted at the level of skeletal muscle. In particular, to reveal the molecular changes occurring in transgenic mice and verify whether adult myofibers are source of signals that can negatively impact the nerve, we will perform proteomic, epigenomic and genomic analysis on both skeletal muscle and spinal cord of ALS animal models, at different ages and stages of disease: before the clinical onset of the disease (1 months) and at paralysis stage (4 months). The comparison of the resultant profilings between the experimental models

will be the baseline to characterize specific factors involved in muscle atrophy and to clarify how altered skeletal muscle can potentially affect the nervous system. On the other hand it is known that skeletal muscle is a source of signals that influnce nerve activity, axonal growth and maintenenace of synaptic connection. Among these, insulin-like growth factor 1 has anabolic effects on both muscle and nerve. Based on these evidences and on our preliminary data (Dobrowolny et al, 2005; 2008a), the local form of IGF-1 (mIGF-1) represent a good candidate to counteract muscle wasting and activate survival mechansism at level of spinal cord.

Task 3. *In vitro* analysis of FUS and TDP-43 activity

For the most relevant miRNA candidates *in vitro* processing assay will be performed in order to identify the molecular mechanism underlying the involvement of FUS and TDP-43 in different steps of miRNA biogenesis. The processing reaction will be performed as described in Lee and Kim (2007); in particular, in vitro labelled pri-miRNAs will be incubated with total protein extracts prepared from ALS-derived fibroblasts and as control in cells either interfered for FUS and TDP-43 or overexpressing the mutant proteins. Processing products will be analysed by gel electrophoresis and the accumulation of precursor molecules will be considered as diagnostic mark for impaired Drosha or Dicer activities. In parallel, Chromatin Immuno-Precipitation (ChIP) analysis with FUS and TDP-43 antibodies will be carried out to test whether both the proteins are associated to specific miRNA loci thus acting during pri-miRNA transcription. After the crosslinking step the chromatin will be sonicated and then immunoprecipitated with the specific antibodies. The recovered DNA will be then analysed by quantitative real time PCR (Applied Biosystem) using specific oligo pairs spanning the miRNA promoter and transcribed regions.

Task 3. Novel lab-on-chip methodologies for easy and cheap detection of miRNAs as biomarkers of neurodegenerative disorders

One relevant aspect in the study of neurodegenerative disorders relates to the possibility of obtaining biomarkers diagnostic of cellular degeneration (both neuronal and muscular) and to correlate them with the severity and progression of the disease. Nowadays it has become quite clear that many diseases correlate with a specific signature of miRNA expression; moreover, it has been proposed that the observed differences in miRNA expression levels can be utilized as biomarkers to evaluate the extent of the disease in human patients as well as for measuring the outcomes of therapeutic interventions. In the case of muscle degeneration, it has been observed that the altered miRNA expression patterns found in degenerating fibers are reflected also in the blood stream. The possibility of analyzing such molecules directly in the blood provides a very rapid and sensitive way for measuring and comparing samples from individuals at different stages of the disease or under different therapeutic treatments. In line with these observations, we intend to proceed along two different directions: i) analyze whether in neurodegenerative disorders neuronal-specific microRNAs can be found in the blood stream; ii) set up appropriate lab-on-chip devises able to quantify such species from limited amount of blood/serum and in a cheap and rapid way.

Lab-on chip devices have been already set up for PCR amplification of DNA samples. It is part of the present proposal the goal of extending such methodology to the detection and quantification of microRNA species.

Following an initial design of miRNA-specific primers, the proposed LoC will implement all the analytical steps for the simultaneous analysis of the different microRNAs specific of neurodegenerative disorders:
1. mixing patient sample with appropriate solutions;
2. flow of solutions along microfluidic channels;
3. the initial RT reaction;
4. a conventional PCR amplification;
5. quantitative analysis of the microRNA presence through the on-chip detection.
All these steps will be achieved through functional modules integrating micro- and optoelectronics with microfluidics and biological surface treatments on the LoC. In particular, the on-chip detection will be performed through an amorphous silicon (a-Si:H) photosensors deposited on the glass substrate and aligned with the site where the PCR amplification occurs.
The timing, control and readout electronics will manage the entire LoC system acting as control unit for the heating modules and as signal conditioning and readout unit for the detection module. Moreover, the system will be able to transfer the results on an external display unit, eg. a personal computer.
The basic structure for a specific miRNA can be considered as a single element of an array of amplification and detection site for a parallel analysis for different sequences of different miRNA. In the proposed application the objective will be the implementation of a number of elements up to 16 that allow the identification of all the miRNA specific for the considered neurodegenerative desease.
Evaluation of the LoC analytical and diagnostic performances will be carried out and compared with standard equipment.

Objectives
- *The combined analysis of the morphological, biochemical and molecular pathways affected by the mutated ALS-associated proteins should allow to identify specific targets that in turn can be tested in reconstituted experiments in order to validate their role in the pathology and to test their possible use as therapeutic molecules.*
- *Reconstruction of the networks implied by the pSILAC data in combination with functional and genomic data in neuronal and muscles cells will allow to elucidate important molecular circuitries responsible for correct tissue homeostasis and their correlation with disease onset and progression.*
- *Further objective will be to obtain a rapid, cheap and reliable system for measuring different types of RNA molecules as biomarkers of the ALS pathology and for testing its severity.*

Workpackage 3. Computational and system biology

For the success of the project, it is essential that data are properly analysed and that all the available information is integrated and used to develop models useful for diagnostic and therapy, starting from the identification of the molecular players and the inference of their properties.
The data produced by the project will be used to reconstruct the implied regulatory network(s) integrating the information with that contained in publicly available resources.

Once the molecular players have been identified, and we expect that information will be available at different levels of details for each of them, we will analyse their structure and function via computational tools, some available and some developed on purpose, ranging from similarity searches for identifying homologous proteins in different organisms, methods for modelling their three-dimensional structure, docking tools, etc.
The combination of the knowledge of the regulatory network and of the molecular and structural details of its protein components, if sufficiently complete, will enable us to simulate the system in silico and to evaluate its properties (e.g. topological properties, presence of recurring motifs, robustness, etc.) and, ultimately, to model the effect of mis-regulation of its components relevant for neuromuscular pathologies.
The results obtained from the in situ detection of specific macromolecules (RNA/proteins) along the MN axon or at the NMJ in different systems that will be set-up in WP4 using Raman spectroscopy would be even more valuable if the specific macromolecules could be readily identified and this WP will attempt to do so by combining the spectroscopy results with computational methods for protein structure analysis and prediction.

Task 1. Network modelling

We will reconstruct the regulatory networks implied by the data produced within the project integrated with those contained in publicly available resources (genomic, proteomic, interactomic databases, ontologies, etc).
In detail we will combine information derived from:

- Level of the gene product obtained in SILAC experiments upon perturbation of miRs expression
- Presence/absence of the predicted binding site for miRs in the gene 3′ UTR
- Evolutionary conservation of the predicted binding site
- Gene annotation using Gene Ontology (GO)
- Presence of the cognate binding site on the candidate genes, if the gene codes for a known or predicted transcription factor

We will analyse the resulting network to decompose it into elementary building blocks, or network motifs, that recur in the network more than expected by chance, as these motifs are likely to perform local "computations," such as the detection of signal persistency or the coordinated gradual activation of a set of genes (Kalir S, 2005; Mangan S, 2003; Milo 2002; Shen-Orr,2 002). This will allow us to highlight specific targets for further analysis.
One important aspect will be the identification of feed forward loops composed of miRs and trascription factors (TF), in which these two regulators target the same genes, with the TF also exerting a regulatory effect on the miR with which it co-regulates the target genes (Hornstein 2006). This feed-forward loop is a well-known feature of many biological networks and we will devote special attention to the identification of such architectures in our network, since they could act as a switch for developmental and other programs in cells and highlight important molecular players in the system.

We also plan to develop and validate, on existing datasets, on the datasets generated by the project and on purposely designed dataset, refined clustering and classification algorithms for gene expression/protein abundance analysis in order to identify robust interaction and regulatory patterns. Regulatory elements may then be integrated into metabolic models to simulate the coupling of relevant signalling modules with the cellular system.

Task 2. **Protein structural analysis and prediction.**
This task will be devoted to gain information on the identified molecular targets.

For soluble proteins, we will take advantage of our extensive experience in protein sequence analysis and in building three dimensional models of soluble proteins for analysing the identified protein targets. Depending upon the evolutionary relationship of the identified target protein with the set of protein of experimentally determined structure, we will use comparative modelling techniques or fragment based methods.
Membrane proteins need to be treated differently since the quality of their models obtained though heuristic techniques is unsatisfactory. On the other hand, the prediction of the location of trans-membrane segments can be done quite effectively and this implies that the free energy of systems can be studied with a collective variable space of lower dimensionality. Even so, though, standard molecular dynamics methods cannot be used because the timescales involved are too long. Dome of us have contributed to the development of advanced activated event simulation that can be used to reconstruct the free energy, to find the different minima and assess their relative stability, and to investigate the transition mechanism among them. These methods have been applied both to biological problems and in other contexts (e.g. hydrogen storage). They will be tested and optimized for helical membrane proteins and applied to targets identified in the project.

Task 3. **Protein identification from Raman spectroscopy data.**

Our modelling expertise will also take advantage of a tight collaboration with WP4. The latter will employ and develop a new technique able to detect the presence of a specific, label free, macromolecule identified via its Raman signature in a small ($\approx$100 nm^3) region of space, below the cell membrane or in the membrane itself. This will allow to monitor morphological changes in live cells We will develop tools to identify the specific detected macromolecule . To this end, we will build a database of Raman spectra for known proteins and test several techniques to develop a tool (based on regression and/or machine learning) able to identify the observed molecular species out of a set of candidate molecules.

Objectives
Reconstruction of the network implied by the data delivered by the project
Identification of the key elements of the network and their functional assignment
Novel classification algorithms for gene expression/ protein abundance analysis and design of validation experiments
Simulation of the metabolic network
Prediction of the structure of the identified proteins
Construction of the database of Raman spectra of known protein
Implementation of methods for identifying proteins from their Raman spectrum

Workpackage 4. New microscopy technologies

Crucial steps in the project path are related to the possibility of identify specific macromolecules (RNA and proteins) in different cellular environment, steps that rely on the development of new imaging techniques. Specifically, within the project one needs to i) perform in situ analysis of specific macromolecules (RNA/proteins) along the motor-neuron axon or at the neuron muscle junction in different in vitro reconstituted systems and in co-cultures with miotubes; ii) follow nuclear migration and positioning during cell maturation and muscle regeneration and iii) perform in situ detection of low abundance compounds at synapses. To cope with these needs, we plan to push the frontiers of optical microscopies. This goal, beside being crucial for the present project, has a value *per se*. The planned developments in microscopy techniques, indeed, are relevant to other IIT-projects and, more generally, of high technological impact.

Task 1. ***Development of new in-situ, intracellular, single molecule detection.***
The project needs to develop a technique for single molecule, in situ, recognitions, This is important to detect mobility of macromolecules (RNA/protein) in response to different stimuli or genetic conditions. Sensitive and innovative methodologies for the in situ detection of specific macromolecules (RNA/proteins) along the MN axon or at the NMJ in different in vitro ALS-reconstituted systems (or to follow muscle regeneration/degeneration) will be set-up and optimized according to the strategy here outlined. A new technique able to detect the presence of a specific, label free, macromolecule –identified via its Raman signature- in small (order 100 nm^3) regions of space, below the cell membrane or in the membrane itself will be developed. The probe is a photonic-plasmonic device (conic pillar, with the as-low-as-possible curvature radius in the tip, nanofabricated on top of a photonic crystal). Once the exciting laser radiation is focused on the photonic crystal, the electric field intensity is amplified in a small region around the tip of the pillar by a large factor (up to date results indicate about four orders of magnitude) . The fluorescence or Raman signal of the molecule, arising from molecules present in this small region of space is thus detected back from the same photonic-plasmonic device. The conic pillar (diameter from 100 to 5 nm) –mounted on a standard AFM stage- crosses the cell membrane and is placed in the desired position.

The plasmonic-photonic device already exists at the lab-level and has been successfully tested on both fluorescence and Raman mode (De Angelis, 2008; De Angelis, 2009). Within the project we aim to:
1 - Optimize the design and the materials of the device (both photonic crystal and metallic pillar) via a fully *ab-initio* FTDT numerical simulation (Conti, 2008) (years 1-2)
2 - Realize and test –either on model molecules and in the final intra-cellular environment- an optimize device. Compare standard Raman mode (with CW laser) and stimulated or CARS mode (pulsed laser). (years 1-3).
3 – Test the effectiveness (on both simple molecules and miRNA) of the capability of molecules recognition in a intra-cellular environment building on a Raman-spectra database (years 2-3)
4 – Realize a 2D array of pillars, with a parallel or multiplexed read-out of the signal, and realize a lab-level easy-to-use device for biological application (years 4-5).

Task 2. Development of newly conceived optical set-up for stained samples
The frontiers in sub diffraction microscopy on labeled objects are based on not yet fully exploited physical phenomena as the non-linear optical excitations (e.g. STED) or the use of the so called "structured illumination". Development on this field needs to i) Select new

classes of fluorophores and ii) Develop newly conceived optical set-ups. Specifically we plan to:

1) develop a new class of fluorophores (the rare earth doped $NaYF_4$ crystals- or the PbS-based nano-phosphors) that do not suffer from bleaching or blinking phenomena and are excited by two NIR photons (up-conversion) (year 1-3).
2) develop fast, electro-optic based, scanning systems for confocal and/or STED and/or two photons microscopy (year 1-3);
3) exploit the Bessel-like light structuring of the light to implement the axial and lateral resolution of linear and non-linear Structured Illumination microscopes (year 3-5);

Task 3. Development of newly conceived optical set-up for label-free samples using coherent illumination

Exploit new microscopy technique based on the coherence of the incoming light (DHM, Digital Holographic Microscopy). DHM combines holography with state of the art digital image acquisition and processing technology. The recorded hologram is a complex interference pattern produced by the propagation of a coherent laser beam through a thick sample. Numerical processing allows to reconstruct full three dimensional images from a single shot hologram with no need for scanning. Moreover DHM produces high contrast images with no need for sample staining. As a consequence DHM promises to be a unique tool for dynamic studies in live cell imaging such as fast 3D tracking or the analysis of 3D shape deformations.

DHM is an emerging and rapidly evolving technique and many different implementation are being explored. However imaging with coherent light poses three major drawbacks: a poor spatial resolution, weak depth sectioning and fixed pattern noise due to unwanted diffraction. It has been recently shown that using structured light fields, such as speckle patterns, can overcome those drawbacks maintaining the advantages of phase microscopy: high contrast live cell imaging and one shot 3D imaging.

We have recently developed highly efficient techniques and algorithms for wavefront modulation with Spatial Light Modulators (SLM) and fast hologram reconstruction with modern Graphic Processing Units. Building up on such a background we will setup a Digital Holographic Microscope using wavefront modulated coherent light. Our microscope will be capable of dynamic 3D imaging with sub diffraction limited spatial resolution and a framerate that could be real-time for interactive applications or even as high as 1 kHz for batch analysis (year 1-3).

Task 4. Development of a time-resolved Infrared micro-spectroscopy beyond the diffraction limit

Mid-Infrared (MIR) spectroscopy (in the wavelength range 2.5 to 16 mm) is a fast, non-destructive analytical technique, which allows one to map the chemical structure of biological system. A MIR spectrum of a bio-system provides the fingerprints of the all constituents, namely proteins, carbohydrates, nucleic acids and lipids, in one single experiment without any injure for the samples. (Liu, 2006) Moreover, the kinetic of a bio-systems, as protein aggregation and/or denaturing, can be easily exploited within a millisecond time-resolution.(Sot, 2010)

By coupling MIR spectroscopy with an infrared microscope one may obtain the mapping of a given molecular frequency in any biological system – and therefore a mapping of its different chemical components - on a few-micron space scale. The possibility to work at a single-cell or even at a sub-cellular level, offers the opportunity to reveal changes caused by pharmaceutical drugs, toxic particulate matter or simply different cell-cycle stages, and to follow them as a function of time. Presently, however, MIR micro-spectroscopy is limited by poor space resolution if compared with other microscopies, as the result of the combined effects of diffraction and of the low brightness of conventional MIR sources. (Petibois, 2009) The task of our project is to extend MIR micro-spectroscopy beyond the diffraction-limited resolution. In this perspective, the imaging capabilities could provide a map of the biochemical functions of the cellular compartments (nuclear, perinuclear and cytoplasmic matrix) and possibly cellular organelles (mitochondria). An effective path towards this task requires several steps.
1) Development of brilliant table-top quantum-cascade lasers (QCL) sources for the MIR radiation in order to improve the signal-to-noise ratio (year 1-2).
2) Use of a nanoantenna, (metallic nanostructure), to focus the incident electromagnetic radiation on a region smaller than the wavelength. Such a device, once coupled to metamaterial superlenses to preserve the subwavelength information, will represent an easy-to-handle spectroscopic set-up (Mattioli, 2009) (year 3-4).
3) Coupling of the MIR radiation from lasers to an Atomic Force Microscope (AFM) metalized tip. The radiation scattered by the tip provides the absorption coefficient of the sample within a lateral resolution of tens of nanometers (Knoll, 1999) (year 5).

Objectives
- *Deliver a table top, ready-to-use, plasmonic antenna combined with Raman analysis for intracellular single molecule detection.*
- *Deliver new generation of sub-diffraction microscopy techniques and protocols, either in labeled (development of new fluorophores, of new fast scan techniques, of Bessel function based structured illumination) or label-free (digital holographic microscopy, mid Infrared chemical sensitive absorption microscopy) samples.*
- *Use the previous techniques to follow the macromolecules (RNA/proteins) traffic along the MN axon or at the NMJ in different in vitro ALS-reconstituted systems to follow muscle regeneration/degeneration.*

Workpackage 5. Functional aspects

Task 1. Electrophysiological tests

By electrophysiological recordings and dynamic calcium imaging, we plan to investigate whether modulating the expression of ALS-associated mutant proteins has any effect on passive and active membrane properties, basal spontaneous activity and Ca2+ buffering capacity of MN. Furthermore, we shall measure both electric rhythmic activity and Ca2+ transients frequency, which have been demonstrated to regulate maturation of these cells in early spinal cord development and that persist as burst activity in hypoglossal MN. If alterations are found, a specific study will be initiated to identify the ion channels affected, since it has been demonstrated that Ca2+ oscillations during development have different cellular sources, being due to the activity of different voltage-dependent Ca2+-channels, Na+-

channels and neurotransmitter receptor activation. We plan to perform such analyses initially in the in vitro reconstituted systems described above and subsequently, when available, in mice model systems.

MN responsiveness to different neurotransmitters, including glutamate, GABA, acetylcholine and glycine will be also investigated to identify possible alterations in terms of receptor function and ion permeability.
In muscle or nerve-muscle cultures, we plan to analyze the effects of altered expression of FUS, TDP-43 or mSOD on NMJ function in terms of spontaneous and evoked synaptic activity, to identify alterations in excitation-contraction coupling as well as AChR responsiveness like Ca2+ permeability and current which could reveal alteration in subunit composition or regulatory interacting proteins. To this aim, intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) will be monitored by mean of time-resolved fluorescence-based digital Ca^{2+} imaging, and the Ca^{2+} permeability of cation-selective channels will be measured by simultaneously recording transmembrane currents and $[Ca^{2+}]_i$ variations.

Task 2. Bio-mechanical measurements
In vivo - To detect early subtle abnormalities of neuro-motor function in transgenic mouse models of neuro-muscular diseases, general behavioural test will be integrated and correlated with biomechanical motor tests. By developing appropriate instrumentation and/or experimental protocols, the codification of strain and stress measured signals will lead to detect aberrant performances in specific motor tasks. Therefore, we intend to design and realize novel experimental protocols and instrumentation to perform a morphological and functional analysis on different neuromuscular junction (NMJ) districts (motoneuron – skeletal muscle, phrenic nerve – diaphragm). Tissue contraction strain will be measured contactless by a high-resolution and a high-speed "Digital Image Correlation" system realized ad-hoc.
In vitro - Measurements of the same biomechanical and functional properties will then be carried out for single muscle fibers/myotubes in a biological system of co-cultures between muscle and neuronal cells (2 Dimensional system) and in a system of *ex vivo* muscle engineered tissue ("X-MET" 3 Dimensional bio-structure), obtained from a muscle primary culture without the use of any scaffold and innervated with neuronal cells isolated from wt or pathological mouse models (i.e. ALS mouse models).

Objectives
- Derive the most suitable electro-physiological tests and Ca2+ imaging measurements in order to calibrate MN as well as NMJ activities in wild type versus ALS conditions. The set-up of standardized measurements should allow to analyze in reconstituted in vitro systems as well as in mice models the correlation with disease onset and progression and to evaluate the beneficial effect of putative therapeutic treatments.
- In line with this, the setting up of experimental procedures and instrumentation to study the biomechanical performances of in vitro reconstituted NMJ (co-culture of the ex-vivo Muscle Engineered Tissue "X-MET" with motoneurons) should provide new strategies for implementing the types of outcome measurements.

Workpackage 6. Strategies of gene delivery into muscle and/or MN cells

Based on our preliminary studies, we plan to use two alternative approaches of gene delivery: a) viral and b) non-viral vectors.

Task 1. Virus-mediated gene delivery

The advantage of using viral vectors in a gene therapy approach is their natural ability to enter the cells and to guarantee high transfection rate and a rapid transcription of the foreign material inserted in the viral genome.

Adeno Associated Virus (AAV, serotype 6) vectors will be used to deliver genes with potential therapeutic effects into both muscle and MN cells. In particular, this project aims at: i) following the movement of the retrograde activity of the virus and to determine the MN transduction efficiency, and ii) defining and characterizing the specific effect of relevant genes to counteract both muscle wasting and neuron degeneration. It has been previously demonstrated that AAV can successfully transduce spinal MNs after a single unilateral intramuscular injection in muscles of adult mice (Pirozzi et al., 2006; Towne et al., 2010). To this end, we want to exploit the ability of AAV viruses to transduce genes, identified in WP2, from one specific muscle to the lower (spinal cord) and upper (motor cortex) motor neurons in different MN districts via retrograde movement. In order to be able to track the virus in vivo, we will produce particles containing fluorinated capsid proteins and we will develop new 19F-MRI and/or iron oxide nanoparticle-labeled proton MRI procedures to elucidate the migratory behavior of specific AAV-6 in vivo. Recently a partner of this project demonstrated the potential use of 19F MRI and MRS to monitor in vivo at 7T fluorinated drugs delivery (Porcari et al., 2008).

Suitable reporter constructs with MN-specific gene expression will be utilized as markers of tissue-specific delivery. Finally, we will test the effects in the pathogenesis of ALS of potential therapeutic genes including miRNAs, identified in the course of the project, and mIGF-1. In a second phase, we will correlate molecular information (identified in other sections of this project) with physiopathological features of the animals extracted from specific MR measured parameters.

Task 2. Non-viral delivery

Synthetic non-viral materials, such as cationic biopolymers, lipid assemblies and modular protein domains are rapidly gaining popularity as vectors for delivering genes and other macromolecules to target organs and cells. Although currently less efficient than their viral counterparts, non-viral vectors are under intense investigation as a safer alternative. These vectors have several potential advantages compared to viral systems, including improved safety profiles, versatility in application, relative ease in production and lower limitation in the size of the material to be transported. In particular, in the framework of the present proposal we will face many different conditions where efficient delivery of RNA is wanted and where no viral vectors can be utilized (see in WP1. the methodology, using synthetic mRNA, for efficiently generating iPSCs or even for their trans-differentiation - Warren et al., 2010). Therefore, we propose to develop, customize and test different nanoparticles as non viral systems for *in vitro* manipulation of cells (WP1) as well as for *in vivo* delivery of DNA and RNA molecules. Development of such technologies will include the functionalization of nanoparticles in order to confer to the carrier the desired trophism towards MN or muscular cells.

Polyelectrolyte DNA/RNA complexes and nanostructured biopolymers

The supramolecular complexes of different cationic biopolymers (chitosan, polyethylenimine, polylysine) with DNA or RNA molecules are currently extensively investigated mainly due to their simplicity and stability as well as the reduced size favouring their prolonged persistence in blood. Among these polymers, chitosan shows the most promising potential, since its structure allows an easy functionalization with different residues that by mimicking different ligands of cell receptors (dopamine-based molecules, monosaccharides, membrane or nuclear translocation signals, cationic lipids, etc.) could promote the specific targeting of these carriers. Moreover, as it has been recently shown, nanostructured chitosan particles can provide a long-term release of incorporated nucleic acids (Palocci et al. 2008). Besides the traditional approaches to the preparation of polymeric nanoparticles, based on the ionotropic gelation, participants of this projects developed a novel proprietary strategy [PCT n° RM2004A000555] to fabricate nanostructured polymers conjugated with nucleic acids in one step process (Chronopoulou et al., 2009). The methodology is based on an osmotic process. Depending on the physico-chemical conditions different morphologies are easily obtained (spheres, sponges, disks, and fibers) also tuning the particles size (ranging from 30 to 500 nm).
The DNA/RNA entrapment efficiency (adsorbed quantity/initial quantity in the solution) inside the nanoparticles is currently under investigation for different nanostructures by using spectroscopic techniques. The final goal is the optimization of all the process parameters (nucleic acid and polymer molecular weights, NA/P charge ratio, size, shape and surface properties of the nanoparticles, etc.) for specific applications.

Cationic lipid DNA/RNA complexes (lipoplexes) and vesicular nanostructures (liposomes and niosomes)

Cationic lipid-DNA complexes (lipoplexes) were first used to introduce plasmid DNA into cells in 1987; since then many cationic lipid formulations have been exploited for the delivery of nucleic acids into cells in cultures, in animals and even in patients enrolled in phase I and II clinical trials. In comparison to other gene delivery modes, such as viral vectors, cationic liposomes are technically simple and easy to formulate, are not biologically hazardous and may easily be tailored for specific applications. However, the transfection efficacy of cationic liposome/DNA complexes (lipoplexes) is still unsatisfactory, especially when compared with viral vectors. Further, the structure-activity relationships of lipoplexes and of the mechanisms involved in the process of intracellular gene delivery are still scarcely known. Such knowledge is crucial to improve the biological performance of lipoplexes and to design tailored cationic liposomes (Mancini et al., 2005).
In fact, the development of strategies to increase the liposome ability to mediate intracellular delivery of both DNA and RNA molecules will the subject of intensive research activity.

We will develop new cationic/anionic mixed liposomes and/or niosomes (formulated with phospholipids and/or synthetic surfactants) capable of complexing nucleic acid and delivering them to cells *in vitro* and *in vivo.*
Relying on our expertise in the synthesis of novel lipid molecules (Mancini et al., 2008) and on designing vesicular delivery systems, we will systematically vary the structure of the components correlating the structural changes to the capability of complexing nucleic acids and to the physicochemical and biological (toxicity, pH-sensitivity, *in vitro* and *in vivo* stability, efficiency of transfection, mechanism of internalization, uptake, intracellular

trafficking) features of lipoplexes. The knowledge of the structure-activity relationship and theoretical simulations will help us to design and develop new efficient formulations.

Modular protein domains

Modular protein engineering enables the construction of chimeric polypeptides in which selected domains provide the required activities for non-viral nucleic acid delivery. These multidomain protein structures must be able to perform relevant functions that mimic those of viruses, namely, nucleic acid condensation, targeted cell attachment and internalization, endosomal escape and nuclear transfer. A properly designed combination and spatial distribution of such partner elements must be able to deliver expressible DNA or RNAs to specific cell-types in whole organisms.
In this context, nucleic acid binding proteins such as Dps (Chiancone et al., 2010) or HVJ envelope protein can be genetically engineered by fusion to the non-toxic fragment C of Clostridium (TTC) or Botulinum (BoNT) toxins or to neurotrophic derived peptides and act as biological carriers to motoneurons. These constructs represent the minimal unit scaffold (about 250 aminoacids long) able to target and efficiently transfect MN cells. Each fragment C can also be engineered to include/exclude the translocation domain, useful for prompt internalization of the construct. Further functionalization can be achieved on the N-terminal region by appropriate conjugation with endosomal escape peptides (Collins et al., 2007), with nuclear targeting sequences such as High mobility group box 1, HMGB1 domain (Frost et al., 2007) or with 19F labelled iron oxide nanoparticles currently manufactured in the PI lab using ferritin proteins as a template (Prastaro et al., 2010).
The array of synaptic selector domains thus obtained can be conveniently modified by innovative protein chemistry strategies ("click chemistry") in order to obtain covalent coupling to the nanostructured biopolymers outlined in the previous paragraphs.

Objectives

- Set-up appropriate strategies enabling the delivery of specific molecular constructs to MN or muscle cells. The comparison between viral vectors and nanocarriers will allow to define the most effective strategy for tissue-specific delivery. Moreover, the development of 19F-labelling and MRI analysis will allow to track in vivo the body distribution of the different macromolecular compounds under analysis.

- Specific effort will be devoted to the development of nanocarriers for RNA delivery to cells and to their functionalization by selector peptides or modular protein domains in order to provide cell-type specific delivery.

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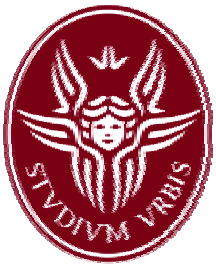
Total Summary of financial requests of the proposal (k€)

	WP1	WP2	WP3	WP4	WP5	WP6	TOTAL
Personnel	300	950	830	700	460	520	3760
Equipment	180	400	110	550	200	150	1590
Consumables	470	520	150	100	190	480	1910
Travels	20	40	40	25	20	20	165
Other	30	40	20	25	30	30	175
Total	1000	1950	1150	1400	900	1200	7600

Project A2



Novel strategies for the imaging and treatment of brain tumours through targeting cancer stem cell-specific signalling pathways.

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Synopsis of the scientific proposal

Background and rationale

Brain tumors are the most life-threatening diseases of adulthood and childhood. Most of brain tumors arise from aberrant development of stem/progenitor cells belonging to two cell lineages (glial and neuronal) leading to I to IV grade gliomas (up to glioblastoma multiforme, GBM) or medulloblastoma (MB). GBM is mainly occurring in adulthood when it is the most frequent and most aggressive primary brain malignancy with a very poor survival rate (5 year survival less than 3%). Conversely, MB is the most frequent aggressive and poor prognosis brain malignancy of childhood, arising from oncogenic transformation of cerebellar neural progenitors. MB multimodal treatments (surgical resection, chemotherapy, and/or radiotherapy) have improved survival, nevertheless it is still incurable in about a third of cases and survivors commonly have severe treatment-induced long-term side-effects, such as developmental, cognitive and endocrinological defects, due to the young age of patients. MB prognosis is determined by patient classification in two risk classes being average risk the patients aged more than 3 years at diagnosis, non-metastatic and totally or nearly totally resected, while patients not fulfilling these criteria are regarded as high risk. Similarly, the extent of surgical removal is the most significant prognostic factor influencing progression free and overall survival in GBM patients, despite combined chemotherapy and radiotherapy regimens. In addition, GBM and MB are composed of an heterogeneous family of distinct tumours based on different genetic/epigenetic features, whose impact on prognosis and therapy sensitivity is poorly understood.

Improving technologies addressing the major risk factor of GBM and MB by achieving nearly complete surgical tumor resection through more accurate intraoperative imaging delineation of the tumor and better genetic/epigenetic characterization of selected tumor cell populations are thus needed. Furthermore, novel and risk-adapted therapeutic strategies are also needed based on specific targeting of post-surgical residual tumor cells without toxic side affects on normal cells.

Cancer stem cells and the concept of the "niche". To this regard, an important issue deals with the presence of **cancer stem cells (CSC)** populating brain tumors, which represent the reservoir of tumor initiating cells sustaining tumor maintenance and progression. These CSCs are also believed to represent the cells of origin of the tumor from untrasformed tissue stem/progenitor cells where a number of genetic and epigenetic oncogenic hits is reponsible for their aberrant proliferation and differentiation leading to malignancy. CSCs are resistant to conventional chemo/radiotherapy and may remain "dormant" untill their reactivation in order to re-initiate tumor. These cells need to be unmasked and specifically eradicated by therapies targeting their stemness properties, in order to prevent tumor recurrence. To this end, signals that regulate both individual CSCs and their assembly as a cell population together with their cell progeny, have to be identified, in order to envisage innovative and targeted therapeutic strategies aimed at CSC eradication and progression towards the growth of bulk tumor cell population. Inded, CSCs reside in a tissue "niche" assuring stemness maintenance, where stem cells i) sustain and are sustained by endothelial cells and tumoral vasculogenesis, ii) exchange intercellular signals with neighboring cells to maintain the "niche" architecture and iii) eventually generate a progeny of differentiated cells composing the bulk tumor cell population.

Cancer stem cells and the "neoangiogenic niche". GBM CSCs have been described to closely interact with the vascular niche which plays a critical role for their maintenance (Calabrese et al 2007; Gilbertson & Rich, 2007). The connection between CSCs and the endothelial compartment appears critical in several cancers: indeed, in GBM, neuroblastoma and lymphoma tumor vasculature has been suggested to derive from cancer cells (Bao et al 2006; Pezzolo et al 2007; Folkins et al 2009). Importantly, normal neural stem cells and more recently GBM CSCs have been shown to be able to differentiate into functional endothelial cells and promote angiogenesis through the release of VEGF and stromal-derived factor 1, in *in vitro* culture of GSCs and in *in vivo* orthotopic transplants, indicating the neoplastic origin of a portion of the vascular endothelium (Wurmster et al 2004; Ricci-Vitiani et al 2010). Therefore, the ability of cancer stem-like cells, endowed with stem cell plasticity, to directly contribute to the tumor vasculature by endothelial cell differentiation, represents a new mechanism of angiogenesis that may have considerable therapeutic implications, since they may be targeted by drugs hitting the same genomic alteration as cancer cells, in addition to conventional anti-angiogenic treatments (e.g. VEGF/VEGFR therapy). On the other hand, the targeting of the process of GBM CSCs differentiation into endothelial cells might offer new therapeutic options for cancer treatment. Therefore, the identification of the signals involved in this process represents a very important challenge to be investigated.
Such a challenge deals with i) the features of the cell of origin driving the process and ii) the underlying mechanistic signals. The proponents of this project have undertaken this endeavor in the past few years, obtaining evidence for a general view of the nature of "mesenchymal stem cells" based on an identical set of expressed antigens (first and foremost CD146), with ability to self-renew and the capacity to form clonal colonies *in vitro*, and an *in situ* identity as subendothelial microvascular cells (Cossu & Bianco, 2003; Bianco et al. 20038; Sacchetti et al. 2007). Of note, all of these populations share the newly recognized property of directing the formation of functional blood vessels in which they integrate as subendothelial mural cells.
Stem cells and neoangiogenetic niche signals. Among the cellular signals, Notch and Hedgehog pathways play a critical role in stem cells and neoangiogenic response. Both of them sustain stem cells and brain CSCs and neoangiogenesis (see below). Most importantly, crucial roles of Notch in the neurovascular unit has been described, where the four Notch receptor paralogues and their ligands are expressed in endothelial cells (EC), in vascular smooth muscle cells and pericytes, with a role for Notch signaling in maintaining the integrity and homeostasis of vessels and in the remodeling of the primordial vascular plexus (Hofmann & Iruela-Arispe, 2007; Carlson et al, 2005; Swift & Weinstein, 2009). Most interestingly, the accumulated knowledge regarding the biology of the Notch3 receptor emphasizes its central role in the vasculature (e.g. CADASIL disease, where a key feature is the degeneration of vascular smooth muscle cells thus resembling common forms of small vessel disease). To this regard, The proponents of this project have unraveled several mechanisms of Notch signaling, specifically including Notch3 and provided novel insights through the development of Notch transgenic mouse models (reviewed in Talora et al, 2008; Bellavia et al. 2008) as well as the study of the role of Notch3 in the interactions between tumor and endothelial cells (Indraccolo et al, 2009).

Targeting pathogenic signaling pathways. A number of signaling pathways has been reported to be responsible for subversion of developmental processes of stem/progenitor cells leading to malignancy and for sustaining stemness properties of CSCs. Among them, the Hedgehog (Hh) and Notch pathways are the most crucial players during development and

their deregulation is a leading cause of a wide variety of tumors, including MB and GBM, where Hh and Notch promote several tumorigenic steps including cell proliferation, maintenance of CSCs, survival, tissue spreading and metastatic potential, angiogenesis.

The PI and other proponents of this project have identified cancer related genes underlying a number of novel Hh regulatory mechanisns, involving ubiquitin-, acetylation- and miRNA-dependent control as well as the tumorigenic role of Notch through the development of transgenic mouse tumor models (Di Marcotullio et al, 2004; Di Marcotullio et al, 2006; Ferretti et al, 2008; Canettieri et al, 2010; Po et al, 2010; Talora et al. 2008). The PI is also using and generating several genetically modified mice which provide useful brain tumor model systems, suitable to study the behavior of CSCs. Furthemore, the unique ability of the proponents of this project have and their prior success in numerical simulation and modeling of complex biological events (Cavagna et al 2008, Ballerini et al 2008; Cavagna et al. 2010), will allow to approach numerical simulation of the complex events underlying the nature and range of action of signals involved in dictating rules of the behavior of CSCs as a whole population. The chemists participating to this proposal have also developed a number of molecules that target Hh pathway and may by exploited as drugs specifically affecting CSCs. They include a series of histone deacetylase inhibitors as well as novel lead compounds obtained from screening of chemical libraries from natural extracts derived from medicinal plants.

Indeed, in the last few years Hh and Notch pathways has been identified as druggable therapeutic targets in cancer, providing the proof-of-principle of the efficacy of their antagonism for *in vitro* or *in vivo* suppression of the growth of MB and GBM and of brain CSCs, in preclinical models and in clinical trials. The rationale for the development of drugs targeting the multiple levels of regulation of Hh and Notch pathways is based on i) the heterogeneity of molecular defects sustaining the pathway activation in cancer (see above) as well as on ii) the need to overcome the reported resistance occurring during single-agent therapy. Although the heterogeneity of the above described regulatory mechanisms provides a rationale for the search of additional molecules specifically targeting Hh and Notch signaling at various misregulated levels, this issue is still largely not understood and unexplored. Furthermore, the role of targeting Hh and Notch pathways in the control of CSCs is also not understood. Therefore, screening of small molecule libraries are required to search for novel Hh and Notch inhibitors acting upon bulk tumor cell population or specifically CSCs.

Targeting and drug delivery into tumor cells and CSCs. Although reducing the side toxic effects on normal cells, therapeutic strategies targeting specific molecular events misregulated in cancer does not rule out unwanted consequences. For instance, inhibition of Hh patwahy, while efficiently killing brain cancer cells, is also impairing skeletal formation in developing young animals, because of the general role of Hh signaling in tissue development and in normal stem cell population. Therefore, selective targeting of cancer cells is needed, through development of innovative and efficient delivery methods. An efficient drug delivery system to be employed for *in vivo* brain targeting must show: i) ability to circulate in the bloodstream for a prolonged period of time; ii) ability to overcome the blood-brain-barrier (BBB) iii) ability to overcome intracellular membrane barriers such as the endosomal membrane and the nuclear membrane via step-wise membrane. To this regard, functionalized nanoparticles could provide precision detection, targeted treatment, and real-time tracking that conventional technology lacks. There are several approaches using different non-invasive delivery systems, that represent promising although challenging

strategies, such as liposomes and niosomes, polymeric nanoparticles and N-linked peptido-resorcarenes, which have been all developed by chemists and physicists participating to this proposal.

Immunotherapeutic strategies against brain tumors. Glioma development and progression is influenced by intrinsic properties of the glioma cells, as well as by microenvironmental factors and a variety of leukocytes subsets. A number of experimental evidences support a role of the innate and adaptive immune system in the control of glial and neuronal brain tumor development and progression (Friese et al 2004; Dunn et al 2007).

Adoptive transfer of cytotoxic lymphocytes such as $CD8^+$ T cells and NK cells alone or in combination with conventional therapeutic agents is under investigation in patients with CNS tumors, but well-defined protocols for the *in vitro* selection and expansion of cytotoxic lymphocyte populations with efficient anti-tumor activity and preferential ability to migrate to the CNS, the main site of tumor growth, are still largely undefined. The proponents of this project have a long-standing experience in studying the molecular mechanisms controlling the functions and trafficking of cytotoxic effector cells under patho-physiological conditions (Accapezzato et al., 2004; Rawson 2007). In addition, the proponents of this project have recently identified the chemokine CX3CL1, one of the principal chemoattractant for the NK cells, as a potent inhibitor of glioma cell invasiveness (Sciumè et al 2010). This is of particular interest since recent evidence indicates that activated CXCR4 is expressed by both tumor cells and vascular endothelial cells in all grades of astrocytoma (Woerner et al., 2005), that Hh may regulate CXCR4 gene expression (Schuller et al 2005) and that CXCR4-dependent glioma growth was inhibited in vivo by systemic administration of CXCR4 antagonist AMD3100 (Rubin et al., 2003).

Imaging brain tumors. Nuclear Magnetic Resonance Imaging (MRI) and Positron Emission Tomography (PET) are well established techniques for brain tumors anatomical and functional imaging. However, there is space for technological improvements, that can crucially increase the performances of these techniques. Ultra-low field MRI (i.e. MRI using precession fields of a few μT) is emerging as a promising development, fully compatible with intraoperative applications and other diagnostic tools. It can acquire images with enhanced contrast, that offer better tumor delineation. PET imaging is complementary to MRI, offering easy functional contrast. Current PET spatial resolution is however poor, due to both the energy and temporal resolution of the sensors. Including information about the time elapsed between the emission and the detection of the photon allows better spatial resolution and reduced background noise.

On the other hand, both techniques can exploit functionalized contrast agents; in this regard, magnetonanoparticles (MNP) can play an important role, because are easily matched to subcellular sizes and properties, an can be easily functionalized. Functionalization with biomolecules allows interaction with biological structures, potentially specific to a target or a function. MNP are inherently good MRI contrats agents, because of their usually superparamagnetic properties. Depending on the particle size, from hundreds to a single cell can be imaged by MRI. Electronic/magnetic properties of nanostructured systems depend on structure and aggregation, thus low level structural characterization of these materials is needed in order to gain full understanding about their action.

In order to fully exploit the molecular imaging power, the imaging technique and a smart contrast agent are obviously essential. However, acting on the biological system can enhance specific features of the signal. The action of strong pulsed electric fields can be used to

trigger and control the delivery of substances from artificial systems (micelles and/or liposomes), or to induce specific responses directly on the living cells. This procedure can be fundamental in enhancing the ability of imaging of biological systems acting in a controlled manner.

Therefore the **aims** of this proposal are:

1. Providing either *in vitro* cell culture and *in vivo* mouse models of brain tumors (MB, GBM) as well as target molecules featuring bulk tumor and CSCs, suitable for being exploited in imaging technologies and/or therapeutic targeting.
2. Understanding the interplay between CSCs and neoangiogenesis in the "tumor stem cell niche" and bulk tumor cell population and involved signaling molecules, suitable for being exploited in imaging technologies and/or therapeutic targeting (**supported by the "Integrative Genomic & Bioinformatic" Platform and "Lab-on-chip" Laboratory**).
3. Understanding the dynamics of CSC population and cells of their progeny as well as unraveling the underlying intercellular signals through design and validation of mathematical models (**supported by the "Integrative Genomic & Bioinformatic" and "Imaging & Microscopy" Platforms**).
4. Pharmacological screening of available small drug and natural products libraries to identify drugs able to suppress the growth of MB and/or GBM stem cells, mainly focusing to the Hh and Notch pathways and neoangiogenesis (**supported by the "Nanotechnologies for drug delivery" Laboratory**).
5. Developing molecular imaging technologies (MRI and PET) *in vivo* to improve tumor detection during intraoperative brain tumor delineation (to achieve nearly complete tumor resection) and in diagnostic and follow-up procedures, with specific emphasis to the imaging of CSCs based on multifunctional nanoparticle-dependent targeting of specific markers (**supported by the "Imaging & Microscopy" Platform**).
6. Developing multifunctional therapeutic nanoparticle-based delivery systems targeting CSC-specific signaling pathways and neoangiogenesis to provide proof-of-principle evidence of tumor treatment *in vivo* (**supported by the "Nanotechnologies for drug delivery" Laboratory)**.
6. Development of innovative immunotherapeutic strategies against glial and neuronal brain tumors based on the infusion of cytotoxic effector cells and haematopoietic progenitor cells (**supported by the "Imaging & Microscopy" Platform).**

The **experimental plan** is composed of 4 Workpackages.

WP1. Cancer stem cells and *in vivo* mouse models of brain tumors.

The workpackage is subdivided in 6 Tasks.

Task 1. Providing either in vitro cell culture and in vivo mouse models of brain tumors (MB, GBM) suitable for being exploited in imaging technologies and/or therapeutic targeting. The activities include: i) CSC *in vitro* studies (using *ex vivo* MB and GBM stem cells); ii) development of *in vivo* mouse models of brain tumors (using orthotopic transplant of GBM and MB stem cells/neurospheres and genetic models of MB targeting the genes mainly involved in Hh signaling control in order to provide the proof-of-principle that the

selective CSC misregulated mechanisms can be effectively exploited for curative nanoparticle-driven targeted therapy.
In vivo mouse tumor models:
- MB models: GFAP or Math1-Cre/LoxP-Ptch1 or SmoA1 transgenics, Numb defective or over expressing mutants;
- GBM models: GFAP-Cre/LoxP- p16(Ink4a)/p19(Arf)/K-Ras(v12)-expressing; Bmi1-Ink4a/Arf doubly null – De Vries et al. 2010, Bruggeman et al. 2007)

Task 2. Analysing and manipulating tumor stem cell "niche" and tumor cell population (using spatially distributed *silicon nanowires*, single cell resolution microscopy and mathematical models of intercellular signal exchange and communication within the whole cell population). **These studies will be carried out with the support and collaboration of "Integrative Genomics & Bioinformatics" and "Imaging & Microscopy" Platforms**. These studies are expected to shed light on the manner in which signals are exchanged among cells within a single progeny, and how these manners of signal exchange may influence size and composition of the whole population.
QDs, NIR illuminating radiation (as used in TP microscopy, but also in Optical Coherence Tomography, OCT), and upconversion fluorescent nanoparticles will be seen as a basic toolkit for envisioning novel modes of in vivo 3-D microscopy at single cell resolution and imaging, live and in vivo or *in vitro*, discrete 4-dimensional events such homing of stem cells to their niches, population kinetics, pattern generation and change, cell differentiation and tissue morphogenesis. We plan to devise fluorescent tracers that would allow to monitor the pattern of symmetric or asymmetric kinetics, the kinetics of cell growth from a single stem cell, discrete 4-dimensional events and key aspects of cell-cell interaction and pattern formation, using multiplex labeling. Finally, generating suitable timed 3-D images of stem cell behavior in vivo, we will be generating the material required to approach numerical simulation of the complex events being imaged. The unique ability of the proponents of this project and their prior success in modeling complex biological events, will make this possible (Cavagna et al 2008, Ballerini et al 2008; Cavagna et al. 2010). This approach is pursued for its inherent potential to define the nature and range of action of signals involved in dictating rules of complex behavior. This has obvious applicative facets, besides promising to provide novel angles for interpreting observed biological phenomena. To mention one, rules dictating the self-renewing behavior of stem cells are highly speculative, and postulate either local cues derived from the extracellular environment (the "niche") or events defined as "stochastic" and yet, in essence, not understood. At least in principle, additional mechanisms could be involved, such as, direct communication among a small number of cells in a defined space, or topological sensing. Of note, similar mechanisms do operate in the biological world, from bacteria to bird flocks, and elucidating their involvement could set the stage for direct analysis of the individual signals involved, and perhaps for redefining key questions of stem cell biology.

Task 3. Targeting brain tumor neoangiogenesis. The activities will be carried out using *in vitro* cell culture systems using brain CSCs and *in vivo* models using conditional transgenc mice (including Notch3 and Notch4 knock-out and overexpressing animals and the above described Hedgehog mouse models) and will include: i) analysis of the role of CD146 in CSCs and microvasculature development; ii) identification of the intracellular signals and of the role of Notch and Hedgehog pathways in the formation of the niche and of microvasculature development in CSC-derived (orthotopic transplants) brain tumors; iii)

investigating the role of Hedgehog or Notch/VEGF interplay, playing a critical role in sprouting angiogenesis; iv) targeting of the above signals (by loss-of-finction/siRNA or gain-of-function/agonist/lentiviral overexpression) in the control of CSC-derived tumor microvasculature development.

Task 4. Providing target molecules featuring bulk tumor and CSCs for imaging technologies and/or therapeutic targeting. **These studies will be carried out with the support and collaboration of "Integrative Genomic & Bioinformatic" Platform.** The activities include: i) Selection of tumor and/or cancer stem cell markers to be used for production of multifunctional nanoparticles (WP2 and WP3) (small molecule Hh or Notch inhibitors, miRNAs, antibodies against markers specifically expressed in brain tumors and CSCs, neoangiogenesis, CXCR4 antagonists; ii) discovery of novel markers or molecular targets specific for brain tumor stem cells (including miRNA and/or protein-encoding mRNAs, by Illumina Genome Analyzer-based high-throughput genomic and epigenomic deep sequencing technology, supported by "Integrative Genomic & Bioinformatic Platform) or other chemokine/chemokine receptor axis (e.g. under the control of Hh inhibitors which can regulate CXCR4 expression by glioma thus contributing to their anti-tumor effects).

Task 5. Development of a very compact and high sensitivity system for PCR-based detection of nucleic acids in selected CSCs. This Task will be carried out with support of the "Lab-on-chip" Laboratory, a modified total analysis system approach by downsizing and integrating its multiple steps (injection, reaction, separation, and detection) onto a single device, yielding a sensor-like system with a fast response time, low sample consumption, on-site operation, and high stability.
The system integrates the main operations of the molecular analysis:

a. the pre-treatment unit for sample manipulation and preparation;
b. the handling unit to dispense, mix and move the samples
c. the detection unit where the molecules are quantitatively detected by an amorphous silicon sensor array.

In particular:

1) the pre-treatment step involves thermal cycling of a solution for DNA amplification by Polymerase Chain Reaction (PCR). A thermal cycle (90-50-70 °C) on 0.1ml water has been successfully implemented by a PolyDiMethylSiloxane (PDMS) chamber to confine the sample and to avoid evaporation, a thin metal film heater optimized for uniform temperature distribution on a $1cm^2$ area, and an a-Si:H p-i-n junction for temperature monitoring.

2) the droplet-handling unit, relying on the electrowetting method, is designed to dispense and move the sample along a microfluidic path and in particular from the heating chamber to the sensor array. The unit includes a set of metal pads beneath a layer of PDMS that provides the hydrophobic surface needed by the electrowetting technique.

3) the detection and quantification of the biomolecules under analysis is performed measuring fluorescence of labeled molecules or the DNA absorbance under UV excitation, by using a matrix of amorphous silicon photodiodes. In experiments on labeled DNA, Alexa Fluor 350 and Cy5 have been utilized as fluorescent dyes, whose emitted light is revealed by a photosensor able to detect different spectral regions. The minimal detectable surface density of Alexa Fluor 350 can be estimated to be around 50 $fmol/cm^2$, while a detection limit around 0.4 pmol/μl has been measured for the Cy5 dye.

Task 6. Pharmacological screening of available small drug and natural products libraries to identify drugs able to suppress the growth of MB and/or GBM stem cell growth, mainly focusing to the Hh and Notch pathway-dependent control and *in vivo* proof-of-concept/efficacy studies in whole-animal model systems. The compounds will be prepared in WP3 and will be screened and tested as described in WP1/Task 1-4. Active compunds will be distributed to WP2 and WP3 investigators for preparing nanoparticles suitable for imaging technologies and therapeutic drug delivery.

Workpackage 2. Molecular Imaging of brain tumors.
The workpackage is subdivided in 3 Tasks (**These studies will be carried out with the support and collaboration of "Imaging & Microscopy" Platform**).
Task 1 & 2. This project aims at developing and exploiting molecular imaging technologies. We will develop advanced imaging instrumentation (Ultra low field MRI and Time of Flight PET) for macroscopic imaging and microimaging applications in combination with the use of functionalized MNP. A Magnetic Resonance at Ultra-Low Magnetic Fields (ULF) and an NMR-compatible PET with Time-Of-Flight capabilities will be built and tested on small animals. Activities will include the development of Ultra-low field technologies and the exploitation of a new kind of scintillating crystals and new solid-state photodetectors for a Time Of Flight PET prototype. PET-MRI compatibility for combined imaging will be ensured.
A new class of nanoparticles functionalized with monoclonal antibodies, recombinant scFv, micro RNA and Hh pathway inhibitors, to improve specificity of MRI and PET imaging of tumors will be developed and produced. The effectiveness of a magnetic field to confine MNPs will be studied. MNP will be optimized also through an experimental study of the electronic and magnetic properties of the more relevant magnetic containing particles/clusters, appropriately functionalized for tailoring their magnetic properties to the final applications.
Suitable Ultra low field and high field MRI techniques for exploiting functionalized MNP contrast *in vitro* and *in vivo* will be developed. The minimal detection limit of MNP, both in vitro and in vivo, will be assessed. Studied parameters will include number of MNP per cell, minimal size of cell population, effect of MNP size, effect of MR imaging parameters. Validation of MR results will be obtained by means of combined fluorescent imaging and postmortem histological analyses (staining). Cancer stem cells will be the target of the *in vivo* imaging.
Task 3. We will investigate with experimental studies and theoretical modelling the feasibility of a controlled delivery of physical-chemical substances in proximity of target cells through the use of electromagnetic (EM) fields. This will be used to improve the resolution and the contrast in the images acquisition and to study the responses of cells to specific bio-molecules. The effectiveness of such a technique will be studied in function of the size of nanoparticles included in the carriers. Finally, with the aim of widen the electroporation techniques to enhancement of imaging for in vivo applications, devices in the nanometric scale able to record electrical signals from intra- and extra-cellular environment will be developed.

Workpackage 3. Design and production of multifunctional nanoparticles for targeted drug delivery. (**These studies will be carried out with the support and collaboration of "Nanotechnologies for drug delivery" Laboratory**).

Multifunctional therapeutic nanoparticle-based delivery (liposomes, niosomes, polymeric nanoparticles and N-linked peptido-resorcarenes) of conventional antitumoral drugs, de novo synthesized class I-selective HDAC inhibitors, small molecule Hh inhibitors, natural miRNA targeting Smo and/or Gli1 (see above), or novel drugs identified following screening procedures (WP1) will be designed and produced. Overall, the key objectives of this WP consist in the design and assembly of intelligent nano-carriers for drug delivery to specific brain neoplastic cells or tracking tumor cell niches by magnetic nanoparticles. The strategy entails the functionalization of synthetic carriers, in order to render them specific for the malignant cell receptors or tissue specific recognition motifs. To this end, the WP will develop synthetic routes for the contruction of biomimetic scaffolds. The design and synthesis of novel multifunctional nanostructures will be accompanied by dedicated high resolution imaging instruments able to detect, direct and follow the effective pathway of these devices up to the final molecular target, available in platforms of the core facility (Platforms 1 and 2). In particular target molecules featuring brain tumors and guided delivery of nucleic acids to genetically impaired cells, will be exploited for both imaging and delivery strategies. Molecular surface recognition motifs for specific cell receptors will be generated either by selection of peptide aptamers or by coupling with specific peptidic receptor ligands. In particular, efficient synthetic strategies will be developed in order to generate multifunctional supramolecular structures based on peptide derivatives of resorcin-arenes scaffolds, a technology that allows for the generation of a wide array of bio-inspired mechanically-interlocked molecular architectures. The final objective of the WP is to bring novel synthetic "theragnostics" devices to the stage of *in vivo* studies.

The workpackage is subdivided in 2 Tasks:

Task 1) design and preparation of nanoparticles. The strategies at the basis of R&D are based on the synthesis of high affinity peptide ligands for receptor targeting. Synthetic supramolecular devices, based on a resorcin-arene scaffolds with peptide branches, will be produced according to well established technologies. These macromolecular assemblies are endowed with great flexibility (foldamers) and their size can be adjusted by combining building blocks of appropriate symmetry. Again, the design and chemical synthesis of appropriate selectors is a compelling target for the delivery of these supramolecular devices towards specific receptors. Chemotrophism towards selected tissues will be approached by the computer-aided design of peptido-mimetic ligands, able to generate a host-guest interaction and hence biological interfacing with the desired target. Dendrimers motifs can also be integrated into these supramolecular systems in order to increase functionality, thus allowing multiple functions such as drug conjugation or nucleic acid transport within common building blocks.

In the advanced stage of the project, drug loaded/decorated nanoparticles will be tested *in vitro* and *in vivo* for their delivery capabilities and therapeutic/diagnostic efficacy.

Task 2) identification of small molecule drugs targeting brain tumor and CSCs, through i) screening of chemical libraries of novel compounds (variably substituted flavonoids, benzophenones, xanthones, anthraquinones, alkaloids, steroids, and terpenoids) from natural extracts derived from medicinal plants, submitted to a very innovative computational technique (virtual library design), or ii) the preparation of additional compounds, including HDAC inhibitors, in order to target Hh pathway and/or apoptosis and differentiation processes.

Workpackage 4. Development of innovative immunotherapeutic strategies against glial and neuronal brain tumors based on the infusion of cytotoxic effector cells and haematopoietic progenitor cells.

Task 1. We propose to analyze the anti-tumor effector functions of distinct NK cell subsets, and the preferential CNS homing and trafficking of expanded cytotoxic effector populations used for cell therapy. In particular: we will identify both in mouse and humans, the anti-tumor NK cell populations with a preferential CNS homing and monitor the trafficking and effector functions of expanded NK cell populations used for cell therapy using *in vivo* tumor models of brain malignancies; expression of ligands recognized by innate immunity receptors (such as NKG2D and DNAM1) on human and mouse GBM and MB cells to predict tumor cell sensitivity to NK cell killing, and thus the efficacy of NK cell-mediated immunotherapy. In addition, we will assess the therapeutic efficacy of nanoparticle-mediated drug delivery in *in vivo* tumor models, and the *in vitro* and *in vivo* immunomodulatory properties of drug-containing nanoparticles. Since haematopoietic progenitors are efficently recruited by glioma tumors *in vivo* in response to CXCL12, they are considered as promising candidates for cell-based delivery of therapeutic molecules to experimental glioma (Tabatabai et al 2005). Thus we will also use these haematopoietic progenitors as a vheicle to drive drug-containing nanoparticle to the GBM tumor site.

Task 2. An additional aim is to develop new protocols to identify tumor antigens for diagnostic and therapeutic purposes, as well as new proteins and their isoforms expressed by tumor cells following activation, neoplastic transformation, or metastatic progression. A major focus will be on the improvement of the pre-fractionation and separation techniques required for the set-up of reproducible and efficient high-throughput immuno-proteomic protocols. The originality of this approach is to identify these protein biomarkers by using patients' T cell libraries. This strategy enables to characterize, among the multitude of proteins belonging to the proteome of a given cell line, only those previously selected on the basis of their immunogenicity. The immunogenicity is defined as the capacity of non-molecularly-identified proteins, expressed by the proteome of tumor cells (i.e., brain tumors), to be recognized by memory T cells, which have been derived from individuals sensitized to the tumor. Memory T cells are known to represent the most specific probes in terms of recognition capacity. Then, only the selected immunogenic spots will be molecularly characterized by MALDI-TOF. This strategy of *reverse tumor immunology* will allow to identify with high efficiency tumor associated antigens (TAA) to be included in the design of antitumor vaccines.
Our project is also to apply the nano-phosphorous technology for detecting a wide repertoire of CD8 T cell each specific to a single epitope (viral, tumor, etc). A combinatorial encoding strategy that allows the multiplexed detection of a several T cell populations within a single sample will be set up. This technology is based on the concept that each peptide-HLA multimer will be encoded by nano-phosphorous with fluorescent intensities unique to a specific MHC/peptide complex. Direct detection of antigen-specific T cells from peripheral blood using this combinatorial approach will results in a significantly increased sensitivity, and most importantly, will allow comprehensive screenings to be performed on limited patient material.

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Total Summary of financial requests of the proposal (k€)

	WP1	WP2	WP3	WP4	TOTAL (k€)
Personnel	2.500	1.370	500	750	**5.120**
Equipment	400	310	100	50	**860**
Consumables	2.100	240	350	700	**3.390**
Travels	50	35	25	50	**160**
Other	50	45	25	50	**170**
Total	**5.100**	**2.000**	**1000**	**1.600**	**9.700**

Principal Investigators Curricula

Irene Bozzoni

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Academic degrees

- 1979: Specialization in General Pathology (70/70) - Sapienza University of Rome
- 1975: Laurea in Biology (110/110 cum laude) - Sapienza University of Rome

Academic career

Since 1993: Full Professor of Molecular Biology – Sapienza, University of Rome
1986-93: Associate Professor of Molecular Biology – Sapienza University of Rome
1980-86: Researcher at "Centro Acidi Nucleici" of CN.R.

Research experience

May-July 2002: Visiting Professor at the University of Brandeis, Boston
April-June 1996: Visiting Professor at the University Boulder Colorado
May-June 1986: Visiting Professor at the National Institute of Health di Bethesda
January-March 1985: Visiting Scientist at the University of Zurich
February-April 1979: EMBO fellow at the European Molecular Biology Laboratory
1978-80: Research fellow at the Centro Acidi Nucleici of C.N.R., Rome
1977-78: Post-doc at the Institute of Molecular Biology II, University of Zurich

Academic appointments

She is Director of the PhD Programme in Genetics and Molecular Biology and Coordinator of the "Laurea Magistrale" in Genetics and Molecular Biology at Sapienza, University of Rome

-Since 2010: Member of the CEPR (Committee of Experts for Research Policy) of the Italian Ministry of University
-Since 2010: Member of the EMBO (European Molecular Organization) "Membership Committee"
-Since 2008: Member of the "Sapienza" University committee on "Research and Technology Innovation"
-Since 2007: Member of the Technical and Scientific Committee of the Consorzio Sapienza Innovazione of the "Sapienza" University
-Since 1996: Representative of the "Sapienza" University at the "Consorzio Interuniversitario per le Biotecnologie" and member of its scientific committee.
- 2005-2008: Member of the Advisory Committee of the Armenise-Harvard Foundation
-Since 2003: Scientific appointment at the "Istituto di Biologia e Patologia Molecolari" of C.N.R.

-2002-2003: Member of the evaluation committee of the Faculty of Sciences of Sapienza Univ.
-2000-2008: Member of the Scientific Committee of the Chiara D'Onofrio Foundation.
-Since 1994: Member of the European Molecular Biology Organization (EMBO). She has been part of the "Membership Committee".

Academic awards
- **2003**: Price "Giovanna Jucci" awarded by **Accademia Nazionale dei Lincei.**
- **2006**: Price "Prof.Tartufari" awarded by **Accademia Nazionale dei Lincei**.

Memberships
- European Molecular Biology Organization
- Academia Europaea
- SIBBM and SIB

She has taken part in committees for national competitions of: Directors of C.N.R. Institutes, University full- and associate-professors, University and C.N.R. researchers. She serves as referee for the: French "Agence d'evaluation de la Recherche et de l'enseignement" (AERES), Wellcome Trust, European Molecular Biology Organization (EMBO), Human Frontier Science program (HFSP), Association Francaises contre les Myopathies (AFM) and several international scientific journals.
She is author of more than **100 scientific publications** on international journals, chapters on books and several popular articles. **Eight patents** have been deposited; two of them have been licensed to the Industry. She coordinates a research group of 24 people including university and CNR researchers, as well as post-doc, PhD and undergraduate students and technicians. One of the patent products has recently obtained the "Orphan drug" designation by EMEA (European Medicines Agency).

Research projects:
The work of Prof. Bozzoni has been financed with continuity from early '80s, when she became a Principal Investigator, by several national and international agencies such as:
- European Union (Integrated Projects of the VII Research Framework - RIGHT and SIROCCO), European Science Foundation (ESF),
- Italian Ministry of University (PRIN e FIRB),
- National Research Council (CNR),
- Ministry of Health (AIDS project),
- Telethon,
- Italian Cancer Research Association (AIRC),
- Istituto Pasteur, Fondazione Cenci-Bolognetti,
- Parent Project ONLUS,
- Italian Institute of Technology (IIT) –SEED projects

Scientific fields of interest
The main research interests of Irene Bozzoni have been on one side the study of post-transcriptional control in eukaryotes, with specific emphasis to the role of small non coding RNAs in different regulatory mechanisms, and on the other the study of non-canonical

functions of RNA and their exploitation for the gene therapy of inherited and acquired genetic disorders.

Major scientific accomplishments:

1) She set up as first in Italy (in the late '70s) several recombinant DNA technologies (**Cell**, 1982, 30, 163; **Cell**, 1985, 42, 317).
2) She discovered that small nucleolar RNAs (snoRNAs) are encoded inside the introns of protein coding genes (**EMBO J**., 1987, 6, 3493; **Genes and Dev**., 1988, 2, 23; **EMBO J.,** 1996, 15, 1121).
3) She described an interesting autoregulatory loop controlling the expression of r-protein genes in yeast (**EMBO J**., 1991, 10, 2215; **EMBO J.** , 1995, 14, 4022)
4) She described the existence of a specific "RNA factory" required for the expression of a class of RNA polymerase II transcripts different from mRNAs, the snoRNAs and the miRNAs (**EMBO J.**, 2000, 19, 6218; **EMBO J.**, 2004, 23:2392; **Nat. Struct. Mol. Biol.,** 2008**,** 15: 902; ***Mol Cell Biol***., 29:5632-8).
5) She exploited several different activities of RNA, such as decoy, ribozymes and more recently antisense and RNAi in order to interfere with gene expression in a sequence-specific way. (**PNAS**, 1996, 93, 7219; **Human Gene Therapy**, 1998, 9, 621; **PNAS**, 2002, 99:9456).
6) One important success in designing "therapeutic RNAs" was accomplished in the Duchenne Muscular Dystrophy disease with a procedure that is now entering pre-clinical experimentation. (**PNAS** 2002, 99:9456; **PNAS** 2006, 103: 3758; **Hum. Gene Ther**., 2006, 17: 565; **Hum. Gene Ther**., 2008, 19: 601; **Mol Cell Biol**., 2009, 29:5632)
7) She was among the first to identify important functions for microRNAs, a new class of non coding RNAs, (**Cell,** 2005, 123: 819; **PNAS**, 2007, 104:7957; **PNAS**, 2007, 104:19849).

Bibliometric data (from ISI Web-of-Science, since 1985)

# Publications -	102
# Total IF -	731.98
# Citations -	2330
# H-factor -	30

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Curriculum viate

since 1997 Professor of General Pathology, I Medical Faculty, Sapienza University of Rome
since 2006 Director, "Sapienza Innovation" Joint Lab on "Genomics and Cell Therapy"
since 2001 Director, PhD School in Molecular Medicine, Sapienza University
since 1997 Director, Laboratory of Molecular Oncology, Dept. of Mol. Medicine, Sapienza University, Rome.
1994-97 Dean of the Faculty of Medicine and Surgery, University of L'Aquila.
1991-94 Director, Department of Experimental Medicine, University of L'Aquila.
1990-97 Director, Post-graduate School of Oncology, University of L'Aquila.
1990-97 Director, PhD Program in Experimental Medicine, University of L'Aquila.
1990-97 Professor of General Pathology, Medical School, University of L'Aquila, Italy.
1987-90 Associate professor of General Pathololgy, Medical School, University of L'Aquila, Italy
1985-86 Visiting scientist, Lab. of Pathology, NCI, NIH, Bethesda MD USA.
1984-87 Assistant Professor, Dept. of Exp. Med, University La Sapienza, Rome, Italy.
1979-84 Senior Investigator, CNRS, Foundation for Hormone Research, Paris, France.
1980-84 PhD in Biochemistry & Developmental Biology, University of Paris VI, France.
1977-81 Board certification in Endocrinology, Medical School, Catholic University, Rome.
1971-77 MD degree, Medical School, Catholic University, Rome, Italy

Memberships

since 2010 member of the Directive Council of the Italian Socitey of Pathology

Other activities

since 2009 Member, Innovation Commitee, Sapienza University
since 2007 Member, "Sapienza Innovation" Technical and Scientific Committee, Sapienza University
since 1998 member, University PhD Program Commitee, Sapienza University
2006-09 member, Patent & Technology Transfer Committee, Sapienza University.
2000-09 Chairman, Research Committee Medical School, Sapienza University
2000-03 member of the National Committee for Biomedical Research, Ministry of Health.
1998 member, CNR National Committee for Oncology Research Program
1995-97 member, National Committee for Biomedical Research, Ministry of Health.

Major fundings (recent)

- AIRC (Italian Association for Cancer Research) 2008-10: “Role of Hedgehog regulatory pathways in human and experimental medulloblastoma” (320 k€, PI).
- Telethon Foundation 2008-10 Regulation of the Hedgehog signaling pathway in neural development and stem cells #GGP07118 (250 k€, PI).
- EU Commission FP7 Marie Curie Program Initial Training Network – Call ID “FP7-PEOPLE-ITN-2008” (2010-13) “Hedgehog/Gli signaling and its pharmacological modulation for regenerative medicine” (421 k€, Partner).
- EU Commission FP6 Marie Curie Program 2005-09 #022238 “The physiological role of E2F1 acetylation: a physiological approach” (300 k€, PI).
- Mariani Foundation 2009-10 “Identification of molecular targets for the control of medulloblastoma stem cells” (180 k€, PI).
- MIUR-FIRB (Italian Fund for Basic Research, Ministry of Research) International Italy-Israel Cooperation Program 2007-09 #RBIN04P4ET “Genetic control of normal cerebellar development and of medulloblastoma tumorigenesis”.
- MIUR-FIRB (Italian Fund for Basic Research, Ministry of Research) 2007-09 “Molecular Imaging Program” #RBIP06293N.
- MIUR-FIRB (Italian Fund for Basic Research, Ministry of Research) 2009-11 “Network for Proteomic Research”.
- MIUR-PRIN 2010-11 “Bifunctional role of Rb/E2F1 in the choice of cell proliferation and apoptosis (48 k€)
- MIUR FAR 2008-10 “Identification and production of cancer stem cells as a tool to study antitumor therapies” (200 k€).
- Ministry of Health “Stem Cells Program” 2011-2012 “Cancer Stem Cells: Molecular Targets and Therapeutic Implications” (100 k€).

Research activity

Dr A. Gulino has made important contributions to our understanding in the field of cancer genetics and molecular oncology and more recently on the functional role of the relationships between developmental process and tumorigenesis. Gulino’s expertise is mainly in developmental biology and molecular oncology fields, addressing several types of cancers (brain tumors, neuroblastoma, breast, colon and ovarian cancers, T cell leukemia) as well as several underlying oncogenic and oncosuppressor signaling pathways. Dr Gulino’s main research fields are: 1) Cancer genetics (functions and identification of novel mutations), molecular basis of genomic instability and DNA repair (BRCA1-2, MRE11, Nbs). 2) Molecular mechanisms of cellular responses to DNA damage (cell cycle checkpoints). 3) Stem/progenitor cell biology and signal transduction. 4) Transcriptional regulation mechanisms of main signaling pathways involved in developmental biology and cancer (Hedgehog/Gli1, Notch, NFkB, AP1, N-Myc, retinoid receptors, bHLH, TrkA). 5) Development of transgenic mouse models for human diseases. 6) miRNA expression in brain tumors and their functional role in the relationships between developmental process and tumorigenesis.

Publications

Total publications: 235
Sum of the Times Cited: 6.502
Average citations per item: 20
H index: 40

Massimo Levrero

NAME	Massimo Levrero
Place and date of birth	Naples, 28-08-1957
Address	Department of Internal Medicine Sapienza University of Rome Viale del Policlinico 155 00161 Rome - Italy
Phone	+39 06 49970892
e-mail	massimo.levrero@uniroma1.it
POSITION TITLE	Professor of Medicine – Sapienza University - Rome Head Laboratory of Gene Expression – Rome Oncogenomic Center

CURRICULUM VITAE

Education - Training

INSTITUTION	*DEGREE*	*YEAR(s)*	*FIELD OF STUDY*
University of Rome La Sapienza - Rome - Italy	MD	1975-1981	Medicine (6-yr program)
University of Rome La Sapienza - Rome - Italy	Residency	1981-1984	Internal Medicine
University Paris VI - Paris - France	DEA	1985	Molecular Virology
Institut Gustave Roussy – Paris - France	Visit Scient	1986-1988	Molecular Biology- Molecular Virology
Freie Universitat Berlin - Berlin	Visit Scient	1997	Cell Biology Molecular Biology
University of California San Diego	Visit. Scient.	1999	Molecular Biology

Positions held

1981 - 1984	Medical Residency, Internal Medicine, University of Rome La Sapienza
1985 - 1987	Staff scientist at the Institut Gustave Roussy in Villejuif (France)
1987	Assistant Professor of Biochemistry - University Paris VI
1988	Director Laboratory of Gene Expression – A. Cesalpino Foundation
1988 - 1992	Assistant Professor of Medicine - University of Rome "La Sapienza"
1992 - 2004	Associate Professor of Medicine - University of Cagliari (Italy)
2002 - 2006	Coordinator of the EC network HepCResist
2004 -	Associate Professor of Medicine – University of Rome La Sapienza
2004 -	Member of the EC VIRGIL Network of Excellence on viral resistance
2005 - 2008	Scientific Board of the Rome Oncogenomic Center
2007 -	Head European Associated Laboratory (EAL) INSERM U785 - Sapienza University

Concurrent Positions

1998 - 2000	Member of the Editorial Board of Journal of Hepatology
1999 - 2001	Associate Editor of Digestive and Liver Diseases
2003 – 2006	Associate Editor American Journal of Gastroenterology
2010 -	Associate Editor Journal of Hepatology
1998 - 2000	Board Member and Secretary - Associazione Italiana Studio Fegato
2000 - 2005	Council Member - EASL (European Association for the Study of the Liver)
2003 - 2005	Scientific Secretary of EASL

2005 – 2007	Administrative (Past) Secretary of EASL
2002 – 2007	Member of the Council of the UEGF (United European Gastroenterology Federation)
2007 – 2010	Member of the ANRS CCS4 Scientific Committee
2010 -	Member of the International Scientific Board of ANRS

HONORS AND AWARDS

1982	Italian Heath Ministry Prize "Role of the hepatitis B virus in the development og hepatocellular carcinoma".
1984	Fondazione Cenci Bolognetti / Istituto Pasteur Prize “Regulation of the immune response to the hepatitis B virus".
1990	Fondazione Carlo Erba – Emilia Ciafrese Prize on Molecular Virology

MEMBERSHIPS

Italian Association for the Study of the Liver
European Association for the Study of the Liver
International Liver Cancer Association
American Association for Cancer Research
Societa’ Italiana di Medicina Interna

RESEARCH SUPPORT

Past

Network Coordinator and PI

EC grant (5th FW) QLRT-2001-00954 - HepCresist
MIUR - FIRB RBAU01CH2M – p53 family in cancer

PI

EC 6th FW - Virgil (Network of Excellence) – HBV in vitro models
MIUR – FIRB - EpiChrom – Modulation of p73 transcriptional activity by acetylation
MIUR – FIRB - Liveromics – IFN-dependent transcription
AIRC ROC Grant – ChIP-Chip and Chromatin immunoprecipitation related techniques

Active

Network Coordinator and PI

MIUR - FIRB RBAP10XKNC- Epigenetic and metabolic alterations in the molecular pathogenesis of cancer: the impact of calorie restriction (CR)

PI

EC 6th FW – Active p53 (Integrated Project) – Regulation of P73 gene expression
MIUR COFIN - Analysis of the p53-p73 transcriptome using ChIP on Chips and Chromatin immunoprecipitation related techniques
AIRC Investigator Grant #3077 – Epigenetic regulation of p73 proteins function
AIFA – A multicenter observational study to evaluate factors influencing efficacy, tolerance and compliance to antiviral treatment with interferon and ribavirin in chronic hepatitis C patients in daily clinical practice
ANRS - HBV-induced antiviral innate responses in infected hepatocytes

Submitted

AIRC - Investigator Grant renewal
CAPIPLO - CSC identification and evaluation of molecular pathway deregulation in hepatocellular carcinoma
CARIPLO - Epigenetic control of viral and host transcriptome in chronic HBV infection and HBV-related hepatocellular carcinoma: from chromatin dynamics to biomarkers identification and clinical decisions

PUBBLICATIONS

153 publications in peer reviewed journals that include Nature, Science, Nat Cell Biol, Mol Cell, J Exp Med, EMBO J, PNAS USA, Mol Cell Biol, J Clin Invest, Cancer Res, Oncogene, J Biol Chem, J Cell Biochem, Cell Growth Diff, Gene, Human Gene Ther, J. of Virology, Virology, J Gen Virol, Immunology, Clin. Exp. immunol., Clin. Immunol. Immunopathol, Lancet, Gastroenterology, Hepatology, J of Hepatology, J. Viral Hepatitis, J Infect Dis, Blood, Int J Cancer,

Sum of the Times Cited: 6,873
Average Citations per Item: 30.28
h-index: 46
IF 854.62

Giancarlo Ruocco

Giancarlo Ruocco was born in Rome, Italy, on November 7th, 1959. He took the Degree in Physics, full mark "cum laude", at the Department of Physics, "Sapienza" University of Rome, in 1981 discussing a thesis on the “Raman scattering study of the vibrational properties of liquid water”.

From 1984 to 1992, he has been Researcher at the University of L'Aquila. From 1992 to 2000 he has been Associate Professor at the University of L'Aquila. From 2000 he is Full Professor at the Department of Physics, "Sapienza" University of Rome.

His research activity ha been devoted to the study, using different specifically developed and implemented experimental (Brillouin scattering, Raman scattering, Inelastic x-ray scattering, Impulse stimulated scattering, photon correlation spectroscopy, non-linear optics, ..) and numerical (MD) techniques, of the dynamics of disordered matter (liquid, supercooled liquids, glasses, gels, colloids,...). In the period 1981-1985 he participated to the development of the first four-pass grating based Brillouin spectrometer. In the period 1990-2000 he participated to the development of the meV resolution Inelastic X-ray Scattering technique, a novel and yet state-of-the-art technique to investigate the THz dynamics in disordered materials. He also co-proposed and designed the first synchrotron based UV scattering beamline (IUVS, Elettera, Trieste) and a beamline (still under construction) for x-ray transient grating experiments at the Free Electron Laser facility FERMI (Elettra, Trieste). In the last few years, following the development of the "Soft"-INFM research center, he focus his activity on studying and manipulating soft- and bio-matter. More recent activities -carried on in the on-campus laboratories- span from time (fs)- and energy-resolved Raman scattering to Holographic Optical Manipulation, from Macro- to micro-rheology, from state of the art non linear optics to photon correlation spectroscopy. In the last period he has been involved in the development of new microscopy-based set-up (fast scan for confocal-like microscopes, exploitation of structured illumination microscopes).

He has been invited speakers at about 50 international conferences and he is co-author of more than 250 publications on international referred journals (among them about 60 publications are on Physical Review Letters, about 60 on Physical Review A/B/E, and 6 on high impact journals as Science, Nature, Review of Modern Physics, Nature family).

He has been and is Principal Investigator in different International and National projects.

He is Referee for the major journals in the field, as Science, Nature, Nature Materials, Physical Review Letters, Physical Review E/B, Europhysics Letters, Journal of Chemical Physics and many others. He is member of the editorial board of “Condensed Matter”journal.

He served and serves in different committees of the University of Rome "La Sapienza", the Istituto Nazionale per la Fisica della Materia (INFM, then INFM-CNR), the European Synchrotron Radiation Facility (member of the Scientific Advisor Committee from 1999 to

2007, presently member of the Council), the “Laboratorio Europeo Spettroscopie non Lineari”, LENS (former member of the Directive Council), and the European Community (former member of the ESFRI panel on “Soft X-ray Free Electron Lasers”, referee for FP7 and ERC projects).

Since 2000 he is leader of the "GLAS" (Liquids and Amorphous Solid Group) at the Department of Physics, "Sapienza" University of Rome. The group is constituted by six staff researchers and different PostDocs, PhD students and undergraduate students. In the first call of the prestigious and highly selective Ideas Starting grant of the European Research Council (ERC), two staff members of the group (C. Conti and T. Scopigno) have been granted.

In 2004, he founded the Research Center "SOFT" of the INFM. Since then (Apr 2004) to Dec 2008 he has been the director of "SOFT”. The center was devoted to the study of the microscopic dynamics in soft matter and disordered materials. At present the research center coordinates the activity of about 50 university scientists and employ a staff of about 25 researchers. After the restructuring of the INFM-CNR, the research center “SOFT” became part of the CNR institute named “Istituto per I Processi Chimici-Fisici” (IPCF).

Since Nov 2007 he is the director of the Physics Department at "Sapienza" Universita' di Roma. Belongs to the Department about 150 staff professors and about 350 non staff researchers (from PhD students to researchers of other institutions operating at the Physics Department).

Bibliometric data (from ISI Web-of-Science, Nov 2010)

# Publications -	275
# Total IF -	1200
# Citations -	5300
# H-factor -	40

Angela Santoni

Name: Angela Santoni
Born : Nereto (TE) , February 21 ,1950
Citizenship: Italy
Address: home - 00184 ROMA, Via Baccina 32,
work- Department Experimental Medicine,
University of Rome "La Sapienza"
phone 39-6-44340632; fax 39-6-44340632

EDUCATION AND DEGREES

1972 Ph.D. in Biology, University of Perugia, Perugia, Italy
Graduation Score: 110/110 cum laude, July 27,1972

ACADEMIC POSITIONS

1972-73 Acting Assistant Professor, Institute of Hygiene, Medical School, University of Perugia, Perugia, Italy.
1974-75 Research Associate, Institute of Pharmacology, University of Perugia.
1975-77 Fogarty Fellow, Laboratory of Experimental Chemotherapy and Immunodiagnosis, NCI-NIH, Bethesda, MD.
1978-81 University Researcher, Chair of Virology, University of Perugia.
1982-83 Research Expert, Laboratory of Immunodiagnosis, NCI, NIH, Bethesda, MD, and Biological Response Modifiers Program, Biological Therapeutics Branch, Section of Natural Immunity, NCI, CRF, Frederick, MD.
1983-1986 University Researcher, Department of Experimental Medicine, University "La Sapienza", Rome.
1986-1989 Full Professor, Chair of General Pathology, Medical School, University of L'Aquila, L' Aquila.
1988-1993 Head, Ph.D. Course of Biotechnology, University of L'Aquila, L'Aquila.
1990-present Full Professor, Chair of Immunology, Medical School, University of Rome " La Sapienza", Rome, Italy.
1991-2000 Head of Pathophysiology Laboratory, "Regina Elena" Cancer Institute, Rome.
1993- present Head, Ph.D. Course of Immunology, University of Rome "La Sapienza", Rome.
1994-1997 Director of Biomedical Technologies Institute, CNR, Rome.
1994-2002 Vice President and President of the International Society for Natural Immunity
1994-2009 Member, Scientific Council "Istituto Pasteur - Fondazione Cenci Bolognetti", University of Rome "La Sapienza".
1996-2001 Member, Scientific Council "Center for the Study of Immunogenetics and Experimental Oncology", C.N.R., Turin.
1997-1999 Member of the National Committee for A.I.D.S. (Department of Health).
1998-2001 President Scientific Council of Biomedical Technologies Institute, CNR, Rome.
2000-2003 Member of AIRC (Italian Association for Cancer Research) Grant Committee.
2001-present Member of " European Molecular Biology Organization (EMBO).
2001-present Principal Investigator of the Centre of Excellence "Molecular Biology. and Medicine" (BEMM) of University of Rome "La Sapienza".
2002-2008 Council Officer of the Italian Society for Immunology.
2003-present Director of the Specialty School in Clinical Pathology.

2004-2007 Member of EMBO Long term Fellowship Committee.
2005-present Member of AIRC (Italian Association for Cancer Research) Advisory Board.
2006-present Coordinator of PhD program in Molecular Biology and Medicine.
2007-present Member of the National Committee for Health Research
2009-present Member of the Patent Committee, “Sapienza” University of Rome
2009-present Scientific Director "Istituto Pasteur - Fondazione Cenci Bolognetti", University of Rome "La Sapienza"

SCIENTIFIC SOCIETIES

- European Molecular Biology Organization (EMBO)
- American Association of Immunologists (A.A.I.)
- Società Italiana di Immunologia, Immunologia Clinica ed Allergologia (S.I.I.C.A.)
- Società Italiana di Patologia (S.I.P.)

Dr. Santoni is Editor in Chief of Immunology Letters, Member of the Editorial Board of the European Journal of Immunology and the American Journal of Reproductive Immunology, referee of several international journals such as Journal of Immunology, Blood, Cancer Research, Clinical Cancer Research, Immunology, Journal of Leukocyte Biology.

Dr. Santoni is recipient of grants from several research agencies including MIUR (Coordinator of a national program from 1987), CNR, Ministry of Health, AIRC and the European Union.

SCIENTIFIC INTERESTS

Over the last 30 years, the main interests of Dr. Santoni's have been focused on many aspects of NK cell biology and their role in the resistance against tumors. More recently she focused her attention on understanding the role of the intracellular signaling pathways triggered by several surface activation/adhesion receptors in NK cell functions and first demonstrated that integrins function as signaling receptors in NK cells. More recently, she has been interested in analysing the molecular mechanisms regulating the expression of NK cell activating receptor ligands on both normal and neoplastic cells, and she demonstrated that NK cells represent an immunosurveillance mechanisms capable of eliminating drug-induced senescent tumor cells.

Author of more than 250 publications in highly qualified international journals and invited speaker to many national and international scientific congresses.

H-index 41;
Number of total citations 5881

Anna Tramontano

Place and date of birth: Napoli, 14/7/1957
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Department of Biochemical Sciences
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P.le A. Moro, 5 00185 Rome
Tel: +39 06 49910556

Curriculum vitae

since 2001	Professor of Biochemistry, I Medical Faculty , University of Rome "La Sapienza"
since 2004	Director of the Bioinformatics Program, CRS4, Pula (CA)
1996 - 2001	Director of the Computational Biology and Chemistry Department at IRBM (Merck Research Laboratories in Pomezia, Rome) and Director of Information Technology and Research Information Systems at IRBM and Professor of Chimica Computazionale II at the University of Naples
in 1994/95	Director of Biocomputing Unit of IRBM
in 1990/93	Biocomputing Group Leader at IRBM and Professor of Bioinformatics at the University of Milan
in 1988/90	Staff member of the European Laboratory for Molecular Biology in Heidelberg (D) in the Biocomputing Programme.
in 1987	Research fellow at International Institute of Genetics and Biophysics in Naples
in 1985/86	Consultant for Biosym Technologies Inc., San Diego, CA, USA
in 1984/85	Post-doctoral research fellow at the Department of Biochemistry and Biophysics of the University of California, San Francisco (USA)
in 1981/84	Research fellow at the International Institute of Genetics and Biophysics in Naples
in 1980	*Laurea in Physics, summa cum laude, at the University of Naples*

Awards

- Tartufari Prize (2010)
- KAUST Global Research Partnership award 2008
- Premio Minerva per la ricerca scientifica 2005
- Premio speciale della cultura per le Scienze Naturali della Presidenza del Consiglio dei Ministri 2002
- Marotta Prize 2001 - National Academy of Science
- EMBO member (1993 -)
- Academia Europaea (2009 -)

- Accademia Medica Romana
- International Society for Computational Biology (ISCB) Board of directors (2001-)
- ISCB Vice president (2002-2005)

Memberships

- EMBO member (1993 -)
- Academia Europaea (2009 -)
- Accademia Medica Romana
- International Society for Computational Biology (ISCB) Board of directors (2001-)
- ISCB Vice president (2002-2005)

Other activities

- Chair of the ERC LS2 panel for Advanced Grants
- Independent expert reviewer for the evaluation of FP7 projects
- Evaluator the FP7 Health Theme in the areas of GENOMICS and SYSTEMS BIOLOGY
- European Molecular Biology Laboratory Scientific Advisory Committee (2006-2011)
- Max Planck Institute for Molecular Genetics, Berlin Advisory Board (2008 -)
- European Boinformatics Institute (EBI) Advisory Committee chair
- EMBO fellowship committee (2008 -)
- Swiss Institute for Bioinformatics Advisory Board (2009 -)
- Scientific Advisory Board of the Centro Nacional de Biotecnología (2009 -)
- Scientific Council of Institute Pasteur - Fondazione Cenci Bolognetti (2002 -)
- Scientific Advisory Board of the University of Zurich Research Priority Program "Systems biology/Functional Genomics"
- Member of Commissione Innovazione of Sapienza University
- Member of the ESFRI Elixir project steering committee and Italian Ministry delegate
- Co-organizer Critical Assessment of Techniques for Protein Structure Prediction Experiment(CASP)
- Associate Editor of Bioinformatics
- Associate Editor of Proteins
- Advisory Editorial Board of EMBO Journal and EMBO Reports (2007-2008)
- Editorial Board The FEBS Journal
- BioSapiens Network of Excellence Steering Committee (2003 - 2009)
- FEBS Publication Committee (2006 -2008)

Major funding

- King Abdullah University for Science and Technology (KAUST) Award (2008-2013) 5,500 K$ (PI)
- FIRB Italbionet (2004-2011) 500 K€ (PI)
- Fondazione Banca di Roma (2010-2012) 500 K€ (PI)
- AIRC (2005-2010) 200 K€ (PI)

- Italian Institute of Technology (IIT) Seed Projects (2010-2012) ~1,000 K€ (Co-PI)
- EU: BioSapiens NoE (2005-2009) 500 K€ (PI)

Publications (source = ISI since 1985)

Total Articles in Publication List:	133
Articles With Citation Data:	133
Sum of the Times Cited:	4310
h-index:	32