PNC
Piano nazionale per gli investimenti complementari al PNRR
Ministero dell'Università e della Ricerca

FIT4MEDROB

D10.3.1

RTB3 - METHODS AND PROTOTYPES OF BIOHYBRID INTERFACES FOR EXO-PROSTHESIS INTEGRATION/ADOPTION#1

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Duration: 44 months
Website: www.fit4medrob.it

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DISSEMINATION LEVEL OF DELIVERABLE		
PU	Public, fully open, e.g. web	**X**
CO	Confidential, restricted under conditions set out in Partners Agreement	

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History of Changes

VERSION	SUBMISSION DATE	CHANGES
1.0	30/11/2023	First version
1.1	20/09/2024	Renaming of the Deliverable in light of the upcoming reorganization of the Deliverables/Objectives Introduction modified following reviewers' suggestions.

TABLE OF CONTENTS

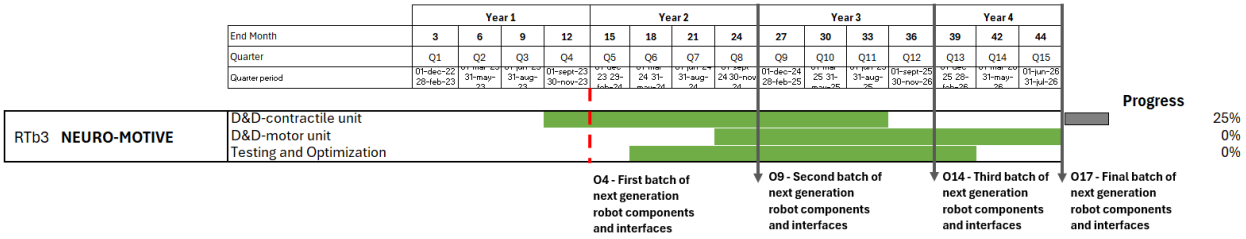
1 EXECUTIVE SUMMARY

Mission 3 is devoted to support **frontier research topics** pertaining to physical and computational aspects of robot *bodies*, robot intelligence, and interfaces with the patient. Seven research topics (RTa1…RTa4, RTb1..RTb3) are articulated in 19 sub-projects, running in parallel and covering complementary enabling technologies in the field of robotics and biorobotics.

In view of laying the foundations for the next wave of healthcare and personal care robots, this piece of research aims at gaining significant breakthroughs in the fields of (bio)materials interacting with human tissues. In this context, **Research Topic b3,** targets the clinical need of **Exoprosthesis Integration/Adoption** and comprises of two complementary subprojects**: NEURO-MOTIVE** and **IMPLAMUSCLE.** More in detail the aim of RTb3 is to create instruments designed to facilitate and regulate the integration of muscles and nerves while capturing signals from the muscle tissue. The resulting scaffolds and lab-on-a-chip will be tested in advanced 3D human in vitro models.

NEUROMOTIVE progress

The timeline of NEUROMOTIVE is represented in the Gantt below.

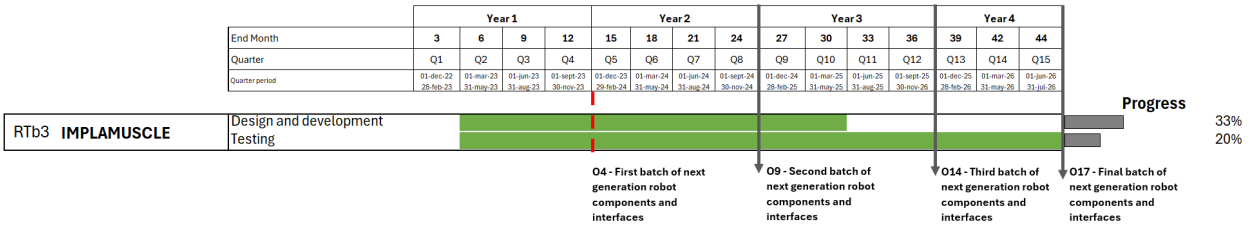


The "**Design and Development-contractile unit**" phase includes the design of the structure and the process for the fabrication of contractile units (tasks 5.1); the progress is currently **25%**. Key accomplishments during this phase include the definition of the critical steps to be implemented to build up an in vitro contractile neuromuscular unit. The "**Design and Development-motor unit**" (task 5.2) and the "**Testing and Optimization**" phases (task 5.3, 5.4) have **not yet began** as reported in the Gantt chart.

There are no deviations on the original plan and the research is progressing as originally foreseen.

IMPLAMUSCLE progress

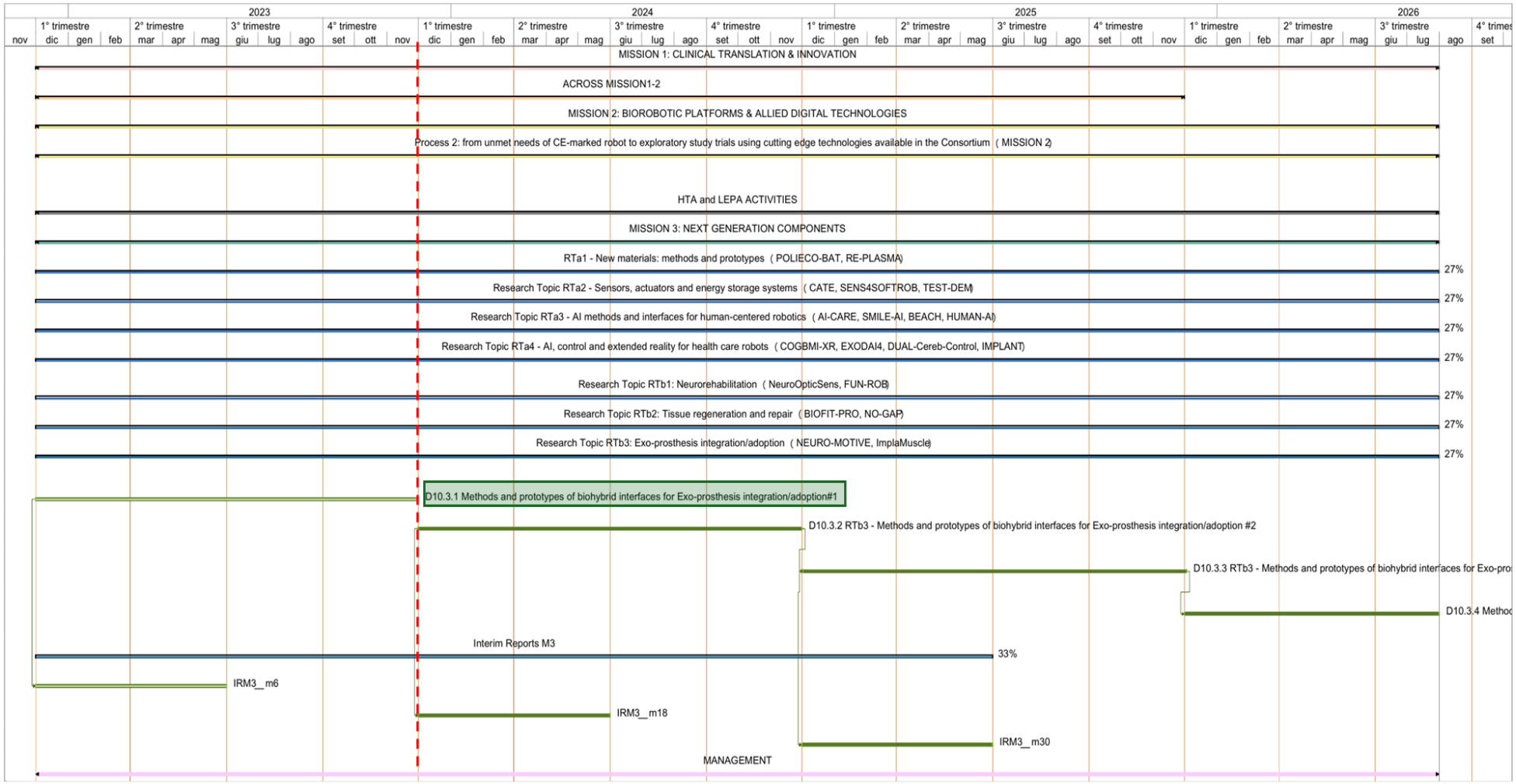
The timeline of IMPLAMUSCLE is represented in the Gantt below.



The "**Design and Development**" phase includes the development and characterization of the implantable microelectrode arrays for remote stimulation and recording (tasks 6.1, 6.2, 6.3, 6.4.); the progress is currently **33%**. Key accomplishments during this phase include the *definition of the design of an implantable multi-electrode array for electrical stimulation and signal collection from muscle tissue.* The "**Testing**" (tasks 6.4, 6.5, 6.6) phase includes the development and characterization of the lab-on-a-chip device for the in vitro stimulation and recording of electrophysiological signals, and its progress is currently **20%**. Key accomplishments during this phase include the *definition of the microfabrication approach and of the materials to be used*).

There are no deviations on the original plan and the research is progressing as originally foreseen.

Overall, NEURO-MOTIVE and IMPLAMUSCLE will make substantial contributions to Fit4MedRob Project by pioneering a new class of exo-prostheses controlled directly by the central nervous system, eliminating the need for external electromechanical devices. If successful, these advancements could extend broadly across the field of actuation in soft medical robotics, significantly enhancing patient acceptance and compliance.


2023
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MISSION 1: CLINICAL TRANSLATION & INNOVATION
ACROSS MISSION1-2
MISSION 2: BIOROBOTIC PLATFORMS & ALLIED DIGITAL TECHNOLOGIES
Process 2: from unmet needs of CE-marked robot to exploratory study trials using cutting edge technologies available in the Consortium (MISSION 2)
HTA and LEPA ACTIVITIES
MISSION 3: NEXT GENERATION COMPONENTS
RTa1 - New materials: methods and prototypes (POLIECO-BAT, RE-PLASMA)
27%
Research Topic RTa2 - Sensors, actuators and energy storage systems (CATE, SENS4SOFTROB, TEST-DEM)
27%
Research Topic RTa3 - AI methods and interfaces for human-centered robotics (AI-CARE, SMILE-AI, BEACH, HUMAN-AI)
27%
Research Topic RTa4 - AI, control and extended reality for health care robots (COGBMI-XR, EXODAI4, DUAL-Cereb-Control, IMPLANT)
27%
Research Topic RTb1: Neurorehabilitation (NeuroOpticSens, FUN-ROB)
27%
Research Topic RTb2: Tissue regeneration and repair (BIOFIT-PRO, NO-GAP)
27%
Research Topic RTb3: Exo-prosthesis integration/adoption (NEURO-MOTIVE, ImplaMuscle)
27%
D10.3.1 Methods and prototypes of biohybrid interfaces for Exo-prosthesis integration/adoption#1
D10.3.2 RTb3 - Methods and prototypes of biohybrid interfaces for Exo-prosthesis integration/adoption #2
D10.3.3 RTb3 - Methods and prototypes of biohybrid interfaces for Exo-pro
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IRM3__m18
IRM3__m30
MANAGEMENT

2 NEURO-MOTIVE: FABRICATION OF HYBRID MULTIFUNCTIONAL CONSTRUCTS FOR MUSCULAR AND NEURAL INTEGRATION AND CONTROL

In this Section the founding idea, the work program, the identified sub tasks and the involved groups in the NEURO-MOTIVE subproject (Fabrication of hybrid multifunctional constructs for muscular and neural integration and control), as detailed in **Annex E,** are summarized. Both one-to-one and groups meetings have been carried out to:

- conceptualize the device "Bioengineered Neuromuscular Actuator" (BNA).
- define the methodologies to evoke, modulate and control the BNA.
- identify the strategies to couple BNA with peripheral nervous systems (PNS).
- define of work plan and sub activities.

Rationale. Being positioned under the clinical need "Exo-prosthesis integration/adoption", SubActivity#5 aims at producing a new generation of actuators for advanced prosthetics. In this area the actuation is a critical step and, nowadays, different working principles can be exploited to mimic the actuation capability of skeletal muscles such as hydraulic, pneumatic, piezoelectric, electromagnetic, shape memory alloys, twisted and coiled polymer muscles. Such systems allow both miniaturization, integration and actuation but still possess different drawbacks [26]. SubActivity#5 will follow a change in the design paradigm of the actuation systems for medical robotics by developing a BNA based on the logic of the muscoloskeletal motion (see **Annex E** for details): signals from the brain are transformed in controlled movements and force generation. The development of such BNA will contribute to (i) the achievement of a new generation of actuators for medical robotics based on the working principle of living muscles and to (ii) the increase of knowledge in the field of neural control of artificial machines.

Aim. By developing new materials and structures for medical robotics, and by using advanced control strategies in terms of interfaces with human body, NEUROMOTIVE **5** will realize a prototype represented by an engineered muscle, interfaced with the PNS, which will be able to induce an index/thumb-like "opposition" kinematic. This product, although characterized by low TRL value, will represent the first attempt in the use of a bioengineered muscle to control a robotic hand by using stimuli generated by the brain.

Definition of the group involved. Paolo Netti (UNINA), Francesco Urciuolo (UNINA), Valeria Panzetta (UNINA), Donatella Di Lisa (UNIGE), Alberto Rainer (UCBM), Riccardo Di Corato (CNR-IMM), Enrico Binetti (CNR-IMM), Laura Blasi (CNR-IMM), Emanuele Gruppioni (INAIL).

Definition of the work plan. Participants have been working on the definition of 4 sub-tasks:

- **ST1 Exogeneous engineered neuromuscular motor.** In this ST, strategies to fabricate a BNA that works as an "external" living actuator that receive signals from PNS will be developed. ST1 has been divided into two activities (ST 1.1 and ST 1.2) as described below.
- **ST2 Control and modulation of the bioengineered actuator.** In this ST strategies and techniques to evoke and to modulate the contraction of engineered muscle will be implemented. ST2 has been divided into two activities (ST2.1 and ST2.2) as described below.
- **ST3 Design of neuromorphic interface (Open Question).** Development of an implantable interface in order to transfer the signal from the PNS to the CUs/BNA. **At this regard a new partner should be identified.**
- **ST4 Endogenous engineered muscular tissue (Open Question).** Development of an implantable version of the BNA able to capture and integrate the host's neurostimulation.

The final BNA will be constituted by an assembly of different contractile units (CU) that will be bio-fabricated starting with skeletal muscle cells. Each CUs will be able to generate contraction and force along a specific direction. By assembling different CUs, it will be possible to generate movements along multiple directions to drive kinematics elements mimicking an index/thumb-like "opposition" movement. The contraction of the BNA (or CUs) will be obtained by realizing either a neuro-muscular junction or by inserting in the CUs electroactive nanoparticles (eNP) able to generate local electrical potentials once activated by external fields. Finally, the BNA will be connected to the PNS by means of a neuromorphic interface which will capture electrical signals from the PNS. Such signals will be transduced by the interface, into signals which stimulate either the innervated or the not-innervated BNA activating the contraction.

2.1.1 Sub-task 5.1.1 - Design of the structure and the process for the fabrication of neuromuscular motor

Based on their expertise, participants (UniNa/UCBM) have defined the critical steps to be implemented to build up functional CUs: in the next months it will be established and implemented the bio-fabrication method, the CU dimensions and the culture conditions to maintain CU viable. Here, by starting from skeletal muscle cells (either primary or IPSc-derived) CUs (0.5-1mm in diameter) will be fabricated and arranged as linear fascicles or ring-like tissues. The diameter of 0.5-1 mm has been chosen to facilitate the nutrient supply avoiding in this way the implementation of complex culture conditions. The CU will be obtained by means of bioprinting-derived approach to induce the alignment of muscle cells in the CU as previously demonstrated [27].
Confinement of muscle cells into (hollow) fiber structures will be achieved via ad hoc optimized microfluidic-enabled printing strategies. A library of biomaterial hydrogels (both synthetic and ECM-derived) will be tested with the aim to find the optimal conditions for in vitro muscle maturation. The bioprinted bundle will be inserted into stretchable/actuable framework (obtained by lithographic and/or microextrusion techniques to provide the construct with a sequence of electro-mechanical stimuli during the in vitro conditioning phase and to measure the achievable contraction force/strain. Multiphysics in silico modelling will be exploited to achieve the computational optimization of the CU design.
For preliminary evaluation of both contraction force and strain, the muscle bundles will be bio-printed as ring-like structures at different cell densities and placed around two stiff posts. It is well known that under this configuration muscle cells are able to align and fuse, forming a contractile fascicle-like structure. At this point the ring-like bundle will be transferred in a similar structure formed by flexible posts with known mechanical properties. The defection of the posts will provide information of the passive contractile force at different cell densities. It will be chosen the cell density that maximize the contraction force without compromising the nutrient supply. This configuration will be also used to evaluate the active force (force under stimulation) generated by the signals defined in the ST2 and ST3. One limitation of this approach consists in the limited deflection of the flexible posts which can hinder the evaluation of the full stroke of the contractile units (or the BNA). Techniques for the evaluation of the full contraction of the muscle bundle is currently under evaluation.

Goal: fabrication of a contractile unit, driven by signals defined in the ST2 and ST3, capable of generating a linear movement of kinematic elements connected to the CU.

2.1.2 Sub-task 5.1.2 Design and optimization of motor device

After the fabrication of the CU and the establishment of its properties in terms of cellular organization and actuation capabilities, CUs will be spatially arranged according to different configurations to obtain the final BNA. To carry out this activity different issues will be addressed:
- how to increase the force/power generation;
- how to induce different kinematic schemes and movement transmission.
- to explore different motion mechanisms (e.g., monoaxial *vs*. agonist/antagonist scheme)

Goal: fabrication of BNA, driven by signals defined in the ST2 and ST3, capable of generating a linear movement of kinematic elements connected to the CU.

2.1.3 Sub-task 5.2.1 Innervated neuromuscular actuator

The CUs will be coupled with motoneurons to create neuro-muscular junctions (NMJs). One of the goals of this activity is the generation of contraction of the CU by stimulating the motoneurons with electrical signals. To this end in this activity, motoneurons and skeletal muscle cells will be characterized to obtain their electrophysiological parameters by using multielectrode arrays (MEAs) provided by UniGE. After the electrophysiological characterization of the single cell lines, measurements will be performed in presence of NMJ on either 2D or 3D systems. The 3D system is represented by the CUs developed in the ST1. Also, in ST2.1, the CU will be subjected to different electrical signals that will be varied in terms of frequency and to characterize the electromechanical response of the innervated CU under different stimulation conditions. The recording of the spontaneous electrophysiological activity will be performed using two types of commercial MEA60 devices: the standard MEA60 and the 3D MEA60. The standard MEAs consisted of 60 flat microelectrodes (made of TiN/SiN) with a 30 µm electrode diameter, spaced 200 µm apart, arranged in an 8 × 8 square grid (excluding the four corner electrodes). These MEAs will be provided by Multi Channel Systems (MCS) in Reutlingen, Germany. The 3D MEA60 have the same spatial organization of the electrodes, but in this case the electrodes are pyramidal (they are 100 µm high and have a tip with a diameter of 12

µm) and are 250 µm spaced among them. The electrophysiological activity will be recorded using the MEA 2100 System from MCS. These recordings will be conducted outside of the incubator, maintaining a temperature of 37 °C. To prevent evaporation and maintain the pH of the medium, a continuous flow of humidified gas (comprising 5% CO2, 20% O2, and 75% N2) was supplied into a small plastic enclosure that covered the experimental MEA setup during the measurement sessions.
Goal: definition of the stimulation parameters for the innervated-CU

2.1.4 Sub-task 5.2.2 Non-Innervated neuromuscular actuator

In this subtask, electro active nanoparticles (eNP), developed at CNR_IMM, will be used to activate the contraction of sarcomeres in CUs. In detail, different nanoparticle architectures have been considered and discussed within the subtask team. The goal is to synthetize nanoparticles able to convert an external remote trigger, such as an ultrasound stimulus and/or a focused laser irradiation, into an electric signal suitable for cellular stimulation. For this purpose, several nanoparticle architectures will be tested during the project. For achieving such multifunctional nanostructures, the idea is to combine a piezoelectric nanocrystal with stimuli-responsive and/or conducting/passivating materials. The best-performing nanostructures will be stabilized in suspension by an organic layer that allows the dispersion of the nanoparticles into an implantable hydrogel. Moreover, the size of the systems will be kept in the range of 300-500 nm, to avoid cellular internalization by the actuator sarcomeres. The choice of the piezoelectric material is still an open point since the group is exploring different strategies, by favoring the materials (organic or inorganic) with low or negligible toxicity. The biocompatibility and the stability of the nanoparticles will be tested by in vitro study on 2D cell cultures and by testing the ion leakage after mid-long storage. The functional characterization of the material will be achieved at IMM, involving other researchers with expertise in the field of piezoelectric materials. The integration of the external trigger(s) into the characterization setup is, at the moment, the main limitation, but different strategies have been considered and will be tested as soon as the first samples are obtained. eNP will be inserted in the CU developed in the ST1 at a concentration and distribution able to generate the desired contraction. To do this, the non-innervated contractile units will be subjected to different external fields able to activate the eNPs. Here both electromechanical (**UNINA, UCBM, CNR-NANOTEC**) and electro-physiological properties (UNIGE) of the non-innervated CU will be assessed. In this ST the possibility to use the eNP also in the innervated CU will be explored to increase and/or modulate the electromechanical performance of the CU.
Goal: definition of the stimulation parameters for the non-innervated-CU
Open questions: effective response of the sarcomeres to the electric signal generated by eNP in the CU.

2.1.5 Sub-task 5.3 Design of neuromorphic interface

An implantable interface will be developed to transfer the signal from the PNS to the CUs/BNA. The interface will be able to capture signals from the resident PNS transforming them into input signals for either motoneurons or the eNPs which drive the innervated CUs or the non-innervated CU, respectively. **At this regard a new partner should be identified.**
Goal: engineering neuromorphic interface.
Open questions: identification of a new partner.

2.1.6 Sub-task 5.4 Endogenous engineered muscular tissue

During the definition of the activities the possibility to implement a bio-fabrication method to generate an implantable version of the BNA has been discussed. This should be able to capture and integrate the host's neurostimulation.
Goal: fabrication of an implantable BNA.
Open questions: size of the BNA; how to integrate the BNA in the host; identification of a new partner.
This activity is still under discussion.

3 IMPLAMUSCLE: IMPLANTABLE MICROELECTRODES ARRAY FOR ELECTRICAL STIMULATION AND SIGNAL COLLECTION FROM MUSCLE TISSUE

The aim of IMPLAMUSCLE is the development of implantable microelectrode array for electrical stimulation and signal collection from muscle tissue (**Figure 1**), through two different approaches:

a) Design and implementation of an implantable electrode system on flexible biocompatible substrates, activated by RFID (Radio Frequency Identification Device) coupling: the main advantage in using a wireless system lies in the reduction of infection and/or skin inflammation by avoiding the use of cables coming out of the epidermis.

b) Design and manufacture of graphene electrodes for the stimulation and recording of electrophysiological signals in 'passive' devices using "mechanochemical approach" and 3D printing.

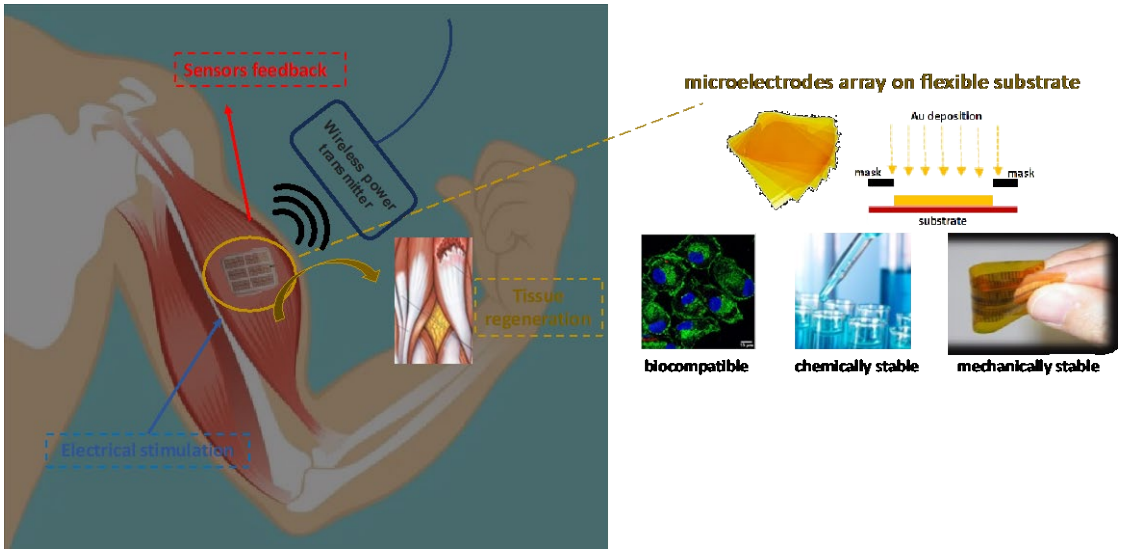

Fig. 1 - Graphical abstract of SubActivity#6.

The detailed description of the activity executed are reported in the **Annex F** and summarised below. In the first year of activity different partners with specific expertise in the consortium have been identified.

Rationale. The driving idea of approach (a) is to utilise the effect of electric stimulation to explore regeneration, re-inhabitation and revascularisation of innested, de-innervated and/or de-vascularised muscular tissue. In fact, it is known from recent work the benefits attributed to electrical stimulation of muscles: speed up of the re-innervation process, improvement of the vascularisation process, reduction of the motor rehabilitation time, and promotion of the production of more intense electromyographic signals by the grafted muscle. The most challenging part of this proposal is the realisation of the (microelectrode array + signal generation/collection) system, fully biocompatible, implantable and working wirelessly. The proposal provides a novelty compared to the state of the art (see **Annex F** for details): the use of a wireless system via RFID (Radio Frequency Identification Device) offers various advantages such as the reduction of infections and/or skin inflammation and the non-use of cables coming out of the epidermis. With regard to (b) approach, the true potential of microfluidic lab-on-chip technology lies in its capacity to design synthetic culture systems where various control parameters (e.g., cell types and positioning, transcellular chemical gradients, molecular and oxygen gradients, flow levels, and patterns, as well as mechanical forcing regimens) can be precisely regulated. Moreover, this platform bridges current gaps in the capabilities of existing in vivo and in vitro approaches by providing an integrated perspective on complex physiological systems at a cellular resolution. During the initial six-month period, our primary focus was on conceptualizing and designing a microfluidic culture Lab-on-a-Chip (LoC) platform. The platform mimics organ functionality by faithfully recreating multicellular architectures, vascular-parenchymal tissue interfaces, chemical gradients, mechanical cues, and vascular perfusion. The compartmentalization of microfluidic systems allows individual cell populations to be cultured and sampled separately while still enabling interactions between them. This approach represents the only existing method for

experimentally co-culturing fundamentally different cell populations with distinct nutrient and microenvironment requirements.

People involved: Laura Pastorino (UNIGE), Francesco Ferrara (CNR-NANOTEC), Antonio Turco (CNR-NANOTEC), Michela Chiappalone (UNIGE), Donatella Di Lisa (UNIGE), Pietro Siciliano (CNR-IMM), Maria Assunta Signore (CNR_IMM), Luca Francioso (CNR-IMM), Luciano Velardi (CNR-IMM) and Laura Blasi (CNR-IMM).

Methods. Participants identified several horizontal Sub-Tasks (STs), for each of the two approaches, dedicated to the implementation of the subactivity 6, using the specific competencies of one or more of the identified groups.

Concerning the (a) approach:

- **ST1 Deposition and characterization of metallic films on flexible biocompatible substrates.** In this ST, strategies to fabricate metallic films on flexible polymers will be developed, with a particular attention towards the optimization of adhesion and mechanical properties of the metal layers.
- **ST2 Design, fabrication and characterization of the microelectrode array on flexible biocompatible substrates.** In this ST, strategies to choose the right microelectrode array configuration will be implemented to maximize the electrical stimulus transfer efficiency.
- **ST3 Development and realization of the signal generation/data acquisition system.** In this ST, strategies to develop and realize the wireless system via RFID (Radio Frequency Identification Device) will be investigated.
- **ST4 Tests and Validation.** In this ST, validation tests of the designed and realized system will be scheduled to verify the performance.

3.1.1 Sub-task 1.1 - Deposition of metallic films via physical and chemical methods

Based on our expertise, metallic films of gold or platinum will be deposited by thermal evaporation and/or RF magnetron sputtering techniques. The deposition parameters and thicknesses will be modified in order to explore the adhesion and mechanical stability of the deposited films. Substrates based on biocompatible polymers such as polyimide (PI), C-parylene and polydimethylsiloxane (PDMS) will be used. Possible chemical/physical treatment on polymeric substrates will be investigated to improve the adhesion of the metallic layers.

3.1.2 Sub-task 1.2 Characterization of metallic films

The deposited metallic films will be characterized by different point of view. X-ray diffraction (XRD), Raman and Atomic Force Microscopy (AFM) will be performed to investigate the crystalline structure and the morphology, Scanning Electron (SEM) and Transmission Electron (TEM) Microscopies to analyse the substrate-film adhesion, also based on the different types of polymeric substrates chosen. Finally, MTT tests will be done to study the biocompatibility and nanoindentation tests to investigate on mechanical (applying deformations such as compression, stretching, torsion and rotation) and chemical stability (in aqueous solutions).

3.1.3 Sub-task 2.1 Design and fabrication of the microelectrode array

The design of the electrodes geometry will be performed by finite element software simulation (Comsol Multiphisics) in order to identify the configuration which maximizes the stimulus transfer efficiency. Starting from the serpentine structure of the electrodes, known to be the most utilized structure for stretchable electronic systems, other possible geometries will be considered with the aim of miniaturization and signal coupling maximization. Different microelectrode arrays will be deposited on flexible polymeric substrates the basis of the results obtained in the ST1.

3.1.4 Sub-task 2.2 Characterization of the microelectrode array

The fabricated microelectrode arrays will be characterized as described in ST1.2 for metallic films. Moreover, electrical characterization by 4-point probe technique will be performed to investigate on electrical behaviour to repetitive bending or permanent curvature of the substrate.

3.1.5 Sub-task 3 Development and realization of the signal generation/data acquisition system

After the fabrication of microelectrode arrays, the signal generation system to be used will be realized at IMM laboratories, while data acquisition hardware will be a commercial product; this aspect in order to guarantee compact dimensions, low power and high performance.

3.1.6 Sub-task 4 Tests and Validation

Tests and validation will be done on different devices by a partner to be identified.
Concerning the (b) approach:
We will focus on the development of passive lab-on-chip systems utilizing cutting-edge carbon-based microelectrodes (CBMs) synthesized through 3D printing. These innovative microelectrodes will serve as invaluable tools for investigating the electrophysiological properties of individual cells as well as for exploring the intricate interactions between diverse cellular populations. The GANTT chart of the approach b activities is reported above.

3.1.7 Sub-task 4 Tests and Validation

3.1.7.1 Sub-task 4.1 Design of a lab-on-chip with passive microfluidics fabricated by soft litography and CBMs multi electrode array (MEA).

This task involves the design of an innovative lab-on-chip system with passive microfluidics, created through soft lithography, and a multi-electrode array (MEA) featuring Carbon-Based Materials (CBMs) fabricated by 3D printing. Our efforts during this period (months 7 – 12) centered on devising a system with integrated 3D-printed carbon-Based MEA (CBMs MEA) that facilitates on-demand communication between diverse cellular populations, eliminating the need for microfluidic pumps and enabling the recording of electrophysiological cell activity with unprecedented resolution and sensitivity. A multi-compartment device interconnected through one or more microchannels will be initially fabricated using polydimethylsiloxane (PDMS) and placed on a polymeric substrate produced by 3D printing, which incorporates the CBMs-based MEA on its surface, as depicted in **Figure 2**.

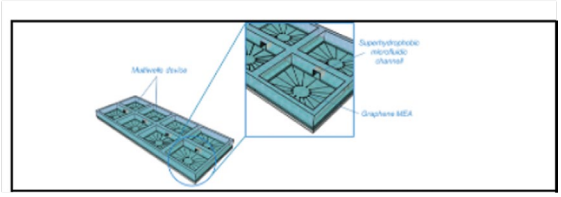

Fig. 2 - Scheme of conveiced device.

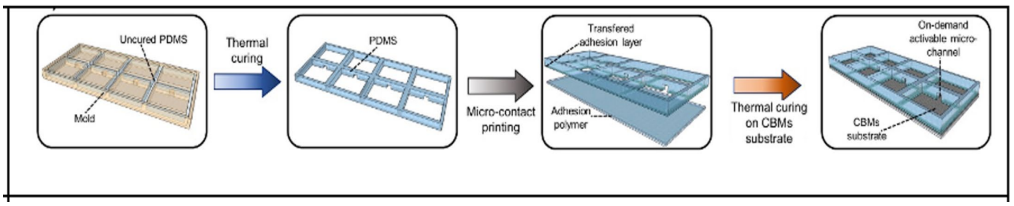

Fig. 3 - Assembly of the device.

The PDMS component of the chip will be manufactured using specific molds. PDMS replicas will be produced through soft lithography. The plastic component, integrating the CBMs-based MEAs, will be manufactured through an innovative 3D printing strategy. Pristine CBMs will be non-covalently bonded to plastic pellets via mechanochemistry to preserve the electronic properties of the nanomaterial. The fabrication process will be complete controlled by open 3D system, enabling to fine-tune and optimize parameters. This system allows to seamlessly transition from research settings to high-throughput fabrication. The process Is simplified, making it easier to move from a research context to large-scale production. This approach ensures a homogeneous dispersion of CBMs on the electronic surface without necessitating chemical functionalization steps (e.g., oxidation), which can affect the final MEA performance. To attach the PDMS component to the plastic substrate integrating the MEA, a thin layer of uncured, diluted PDMS will be applied to the bottom part of the PDMS component using microcontact printing. The device will then be placed onto the part of the device that includes the MEA and cured. In this configuration, the hydrophobicity of PDMS will prevent the passage of culture media during biological tests. Applying pressure to the top of the microchannels between two compartments, entrapped air can be removed, hydrophobic forces overcome, and the channels filled with culture media, thereby facilitating communication between the selected

compartments (**Figure 3**). This system creates a decoupled cellular population system allowing for detailed characterizations of interactions between different cell populations.

3.1.7.2 Sub-task 4.2 Functionalization of polymeric microparticles with Carbon Based materials through mechanochemical approaches.

The task focuses on functionalizing polymeric microparticles for 3D printing using Carbon-Based Materials (CBMs) through innovative mechanochemical methods**.**
This subtask will involve two different activities:

1. Functionalization of polymeric pellets through mechanochemical approaches
2. Stabilization of nanocomposites pellets Through thermal treatments

3.1.7.3 Sub-task 4.3 Fabrication and characterization of 3D printed Carbon based materials (CBMs) multi electrode array (MEA) integrating passive microfluidics for stimulation and recording of electrophysiological activity.

Task entails the fabrication and characterization of a 3D printed multi-electrode array (MEA) with integrated passive microfluidics for stimulating and recording electrophysiological activity, utilizing Carbon-Based Materials (CBMs).
This subtask will involve two different activities:

1. Fabrication and characterization of 3D printed Carbon based materials CBMs MEA and passive microfluidics.
2. Device assembly.

3.1.7.4 Sub-task 4.4 Test on biological systems.

The task involves testing the developed technologies and systems on biological samples, conducted in collaboration between CNR Nanotec and UniGE.
This subtask will involve two different activities:

1. Lab-on-chip fabrication.
2. Biological tests.

The main achievements of this period are:

I) Designed strategies for producing CBMs-based MEAs to enhance neuronal electrophysiological recording.
II) Designed a passive microfluidic platform that enables the decoupling of cell populations while preserving cellular crosstalk.
III) Designed of the integration of CBMs MEA with a multicompartment device featuring on-demand activable communication systems.
IV) Identified specific characteristics required for the 3D printer to produce CBMs-based MEAs, through the printing of pellets functionalized with CBMs using mechanochemical approaches.

DISSEMINATION

1. 18.09.23-21.09.2023. European Materials Research Society (EMRS) Fall Meeting, Warsaw, Poland. Grasso Giuliana, Onesto Valentina, Silvestri Niccolò, Camargo de Oliveira Carolina, Pellegrino Teresa, Loretta L. del Mercato. Multifunctional stimuli-responsive bioengineered systems for cancer therapy: towards precision medicines. Session I01 – symposium I: Synthesis and characterization of functional nanocomposite materials (oral presentation).
2. 18.09.23-21.09.2023. European Materials Research Society (EMRS) Fall Meeting, Warsaw, Poland. Helena Iuele, Onesto Valentina, Forciniti Stefania, Colella Francesco, Loretta L. del Mercato. Design and Application of pH-sensing Hybrid systems for noninvasive metabolism monitoring in 3D tumour models. Session I02 – symposium I: Synthesis and characterization of functional nanocomposite materials (oral presentation).
3. 10-13.09.2023. EUROSENSORS, XXXV Conference, Lecce, Italy. Anna Chiara Siciliano, Stefania Forciniti, Valentina Onesto, Helena Iuele, Giuseppe Gigli, Loretta L. del Mercato. 4D Optical Mapping of pH in 3D Cell Systems (oral presentation).
4. 04-08.09.2023. 33rd Annual Conference of the European Society for Biomaterials (ESB 2023), Davos, Switzerland.Stefania Forciniti, Valentina Onesto, Niccolò Silvestri, Sabrina Hochheim, Carolina Camargo de Oliveira, Teresa Pellegrino, Loretta del Mercato. Biomimetic platforms for in vitro cell growth and biomedical applications: towards precision medicine. Session S7.2: SSB+RM meets ESB: Programmable Biomaterials (oral presentation).
5. 04-08.09.2023. 33rd Annual Conference of the European Society for Biomaterials (ESB 2023), Davos, Switzerland. Onesto Valentina, Stefania Forciniti, Helena Iuele, Francesco Colella, Daniele De Martino, Loretta L. del Mercato. Hybrid pH-sensing systems for precisely probing single-cell acidification in in vitro tumor models. Session S2.2: Sensing cells and their microenvironments (oral presentation).
6. 04-08.09.2023. 24th Conference on Material Science (YUCOMAT 2023 - MRS Serbia), Herceg Novi-Montenegro. Francesco Colella, Valentina Onesto, Giuliana Grasso, Stefania Forciniti, Loretta L. del Mercato. Design and synthesis of a fluorescent ratiometric microsensor for potassium cations tracking (oral presentation).
7. 18 – 23 June 2023. BioEM 2023, Oxford, UK. G. Suarato, A. Marrella, S. Fiocchi, E. Chiaramello, M. Bonato, M. Parazzini, P. Ravazzani, “Shape effect on the electrical output of magneto-electric nanoparticles”.
8. 18 – 23 June 2023. BioEM 2023, Oxford, UK. S. Fiocchi, E. Chiaramello, V. Galletta, A. Marrella, G. Suarato, M. Bonato, M. Parazzini, P. Ravazzani, “A computational framework for magnetoelectric nanoparticles application as neural interfaces”.
9. 18 – 23 June 2023. BioEM 2023, Oxford, UK. V. Galletta, E. Chiaramello, S. Fiocchi, A. Marrella, G. Suarato, M. Bonato, M. Parazzini, P. Ravazzani, “A new promising approach for motor nerve stimulation: magnetoelectric nanoparticles”.
10. 21 – 23 June 2023, GNB2023, Padova, Italy V. Galletta, E. Chiaramello, S. Fiocchi, A. Marrella, G. Suarato, M. Bonato, M. Parazzini, P. Ravazzani, “Magnetoelectric nanoparticles as promising tools for nerve stimulation”,
11. 27.05.23-02.06.2023. European Materials Research Society (EMRS) Spring Meeting, Strasbourg, France. Onesto Valentina, Stefania Forciniti, Helena Iuele, Francesco Colella, Daniele De Martino, Loretta L. del Mercato. Smart functional pH-sensing scaffolds for extracellular pH mapping in in vitro tumour models. Session H04 – symposium H: Advanced strategies for smart functional and multifunctional biomaterials and biointerfaces (oral presentation).
12. 27.05.23-02.06.2023. European Materials Research Society (EMRS) Spring Meeting, Strasbourg, France. Stefania Forciniti, Valentina Onesto, Francesca Serio, Niccolò Silvestri, Carolina Camargo de Oliveira, Teresa Pellegrino, Loretta del Mercato. Stimuli-responsive platforms for in vitro cell growth and cancer therapy: towards precision medicine. Session J06 – symposium J: Design and scaling up of theranostic nanoplatforms for health: towards translational studies (oral presentation).
13. 04.09.2023 CMD30 FisMat 2023– Milan (Italy). Antonio Turco et al, “Mechanochemical approach for the fabrication of Carbon based porous elastomeric (nano)composites: from environmental remediation to piezoresistive devices”. Oral presentation.
14. 04.09.2023 CMD30 FisMat 2023– Milan (Italy). Giulia Siciliano et al., “Development of a MIP based electrochemical sensor for TGF1 detection and its application in liquid biopsy”. Oral presentation.
15. 10.09.2023. EUROSENSORS, XXXV Conference, Lecce, Italy. Antonio Turco et al., “Mechanochemical Approach for Carbon Nanotubes Based Piezoresistive Sensors Fabrication”. Poster.

16. 29.09.2023. European Researchers' Night 2023, Monastero degli Olivetani, Lecce, Italy. Federica Carnevali, Francesco Colella, Eliana D'Amone, Stefania Forciniti, Giuliana Grasso, Helena Iuele, Valentina Onesto, Ilaria Serra, Anna Chiara Siciliano, Loretta L. del Mercato. Optical sensors and multifunctional biomaterials for applications in precision medicine and biorobotics (poster).
17. 29.09.2023. Notte dei ricercatori STREETS Federico II, c/o Complesso di Monte Sant'Angelo, University of Naples Federico II, Naples.

Meetings in this period

List of in person and online meetings, including dates, place, participants.

1. 1/02/2023 (14:00-16:00): KOM of A10. Participants: CNR-IEIIT, CNR-IMM, CNR-NANOTEC, UNINA, UCBM, INAIL, UNIPI.
2. 7/02/2023 (14:00-16:00): Tissue regeneration. Partecipants: CNR-IEIIT, CNR-IMM, CNR-NANOTEC, UNINA, UCBM, INAIL, UNIPI.
3. 9/02/2023 (12:00-13:00): Neuroreabilitation. Partecipants: CNR-IEIIT, CNR-IMM, CNR-NANOTEC, UNINA, UCBM, INAIL, UNIPI.
4. 9/02/2023 (15:00-14:00): Exo-prosthesis integration / adoption. Partecipants: CNR-IEIIT, CNR-IMM, CNR-NANOTEC, UNINA, UCBM, INAIL, UNIPI.
5. 28/02/2023 (09:00-10:00): Meeting Nanotec-Cnr (del Mercato) e UNINA (Netti).
6. 28/02/2023 (10:40-12:00): Meeting Nanotec-Cnr (del Mercato) e UNIGENOVA (Pastorino).
7. 8/03/2023 (10:00-12:00): Meeting Nanotec-Cnr (F. Scalera, F. Gervaso, A. Polini, L. L. delMercato) e INAIL (Gruppioni, Buganè).
8. 15/03/2023: Meeting in person at CNR NANOTEC Lecce with CNR NANOTEC Lecce researchers on research activity proposals of Fit4MedRob.
9. 17/03/2023 (09:00-10:00): Meeting Nanotec-Cnr (Quarta, del Mercato, Carbone, Scarfiello) e IEIIT-Cnr (Ravazzani, Chiaramello, Parazzini, Marrella, Suarato).
10. 27/03/2023 (09:00-10:00): Meeting Nanotec-Cnr (Quarta, del Mercato) e UniPisa (Chisari, Dalise).
11. 12/04/2023 (09:30-17:00): Meeting in person Nanotec-Cnr (Quarta, del Mercato, Carbone, Scarfiello) e IEIIT-Cnr (Ravazzani, Chiaramello, Parazzini, Marrella, Suarato, Fiocchi, Dossi, Bonato, Gallucci, Benini).
12. 20/04/2023 (11:00-13:00): Meeting AL10 with CNR-IEIIT (Emma Chiaramello on behalf of Paolo Ravazzani), CNR-IMM (Pietro A. Siciliano); UCBM (Alberto Rainer) to examine the list of subactivities received in A10 following interactions among researchers in A10.
13. February 28 2023: Andolfi (UNIGE), Del Mercato (CNR), Di Lisa (Unige), Pastorino (UNIGE) via TEAMS
14. 15/05/2023: Online meeting with all partecipants involved in Activity 10.
15. May 30 2023: Andolfi (UNIGE), Di Lisa (Unige), Iuele (CNR) via TEAMS.
16. June 21-23 2023: Ferrara (CNR), Turco (CNR), Di Lisa (UniGE), Pastorino (UniGE), Chiappalone (UniGE) – via TEAMS
17. July 17 2023: Urciuolo (UNINA), Netti (UNINA), Panzetta (UNINA), Di Corato (IMM), Binetti (IMM) via MSTEAMS
18. July 18 2023: Urciuolo (UNINA), Grupioni (INAIL), Netti (UNINA) via MSTEAMS
19. July 19 2023: Urciuolo (UNINA), Di Lisa (UNIGE) via MSTEAMS
20. July 20 2023: Urciuolo (UNINA), Rainer (UCBM/CNR_NANOTEC) via TEAMS
21. 24/07/2023: Online meeting. Partecipants: F. Scalera (CNR NANOTEC Lecce) and F. Urciuolo (UNINA) on collaborations on SubActivity#3.
22. August 7 2023: Di Lisa (UNIGE), Onesto (CNR) via TEAMS
23. August 7 2023: Urciuolo, Netti, Di Lisa, Rainer, Gruppioni, Di Corato, Binetti
24. September 3 2023: Di Lisa (UNIGE), Onesto (CNR) via telephone
25. September 11 2023: Di Lisa (UNIGE), Onesto (CNR) via TEAMS
26. September 16 2023: Di Lisa (Unige), Onesto (CNR) via TEAMS
27. 21-22/09/2023: Meeting of Activity 10: Biohybrid Interfaces and Biomaterials (Mission 3), c/o CNR NANOTEC, Seminar Room "Rita Levi Montalcini", Lecce; participants: PI from each subactivity and other researchers related to activity 10.
28. October 3 2023: Di Lisa (UNIGE), Onesto (CNR) via telephone.
29. October 4, 2023- Online meeting. Participants: F. Scalera (CNR NANOTEC Lecce) and F. Urciuolo (UNINA) SubActivity3.

30. October 7 2023: Di Lisa (UNIGE), Onesto (CNR) via telephone.

Publications

1. Magnetoelectric nanoparticles shape modulates their electrical output. A. Marrella, G. Suarato, S. Fiocchi, E. Chiaramello, M. Bonato, M. Parazzini, & P. Ravazzani. Frontiers in Bioengineering and Biotechnology, 2023, 11. https://doi.org/10.3389/fbioe.2023.1219777
2. Preparation, characterisation and applications of bone char, a food waste-derived sustainable material: A review. Piccirillo C. Journal of Environmental Management, 339, 117896.
3. Enhanced Delivery of 5-Aminolevulinic Acid by Lecithin Invasomes in 3D Melanoma Cancer Model. Antonio Gaballo, Andrea Ragusa, Concetta Nobile, Nunzia Gallo, Luca Salvatore, Clara Piccirillo, Alessia Nito, Annalisa Caputo, Gabriella Guida, Alfredo Zito, Raffaele Filotico, and Alessandra Quarta. ACS Molecular Pharmaceutics, 2023, https://doi.org/10.1021/acs.molpharmaceut.3c00494.
4. Multilayer Polyelectrolyte Capsules for Sensing and Drug Delivery: Fundamentals and Applications. L. L. del Mercato, S. Leporatti, M. M. Ferraro, N. A. Hanafy, R. Rinaldi, W.J.Parak and S. Carregal-Romero. In "Bio-Nano Interfaces". Chapter 57. Pag. 1385-1429. Ed. Wolfgang J. Parak, Jenny Stanford Publishing, www.jennystanford.com, in Press 2024.
5. In Vitro and In Vivo Biocompatibility Assessment of a Thermosensitive Injectable Chitosan-Based Hydrogel for Musculoskeletal Tissue Engineering. Canciani, B.; Semeraro, F.; Herrera Millar, V.R.; Gervaso, F.; Polini, A.; Stanzione, A.; Peretti, G.M.; Di Giancamillo, A.; Mangiavini, L. Int. J. Mol. Sci. 2023, 24, 10446.
6. Fluorescent nano- and microparticles for sensing cellular microenvironment: past, present and future applications (2023) Grasso G., Colella F., Forciniti S., Onesto V., Iuele H., Siciliano A.C., Carnevali F., Chandra A., Gigli G., del Mercato L.L. Nanoscale Advances 2023, 2023,5, 4311-4336. Front cover.
7. pH-Sensing Hybrid Hydrogels for Non-Invasive Metabolism Monitoring in Tumor Spheroids (2023) Rizzo R., Onesto V., Morello G., Iuele H., Scalera F., S. Forciniti, Gigli G., Polini A., Gervaso F., del Mercato L.L. Materials Today Bio, 2023, 20, 100655.
8. Visible Light-Near-Infrared Dual-Band Electrochromic Device. M. Pugliese, R. Scarfiello, C.T. Prontera, R. Giannuzzi, G.V. Bianco, G. Bruno, S. Carallo, F. Mariano, A. Maggiore, L. Carbone, G. Gigli, V. Maiorano. ACS Sustain Chem Eng, 2023, 11(26) 9601.
9. Ultrasensitive qPCR platform for rapid detection of bacterial contamination of raw biological samples at the point of care. V. Garzarelli, M. S. Chiriacò, M. Cereda, G. Gigli, F. Ferrara. Heliyon, 9, 5, 2023, e16229.
10. Colour tunability by optically induced electron transfer in diarylamine-dibenzothiophene derivatives. Francesco Ruighi, Eduardo Fabiano, Lorenzo Franco, Alessandro Agostini, Samuel Zatta, Giuseppina Anna Corrente, Amerigo Beneduci, Antonio Cardone, Gianluca Accorsi, Agostina Lina Capodilupo. Dyes and Pigments, 219, 2023, 111582.
11. Thermochromic Printable and Multicolor Polymeric Composite Based on Hybrid Organic-Inorganic Perovskite. Marco Cinquino, Carmela Tania Prontera, Antonella Giuri, Marco Pugliese, Roberto Giannuzzi, Antonio Maggiore, Davide Altamura, Fabrizio Mariano, Giuseppe Gigli, Carola Esposito Corcione, Cinzia Giannini, Aurora Rizzo, Luisa De Marco, Vincenzo Maiorano. Advanced Materials, 2307564 (2023).
12. Enhanced Durability of an All-Solid-State WO3 Based Electrochromic Device on a Single Substrate by Using a Complementary Anodically Coloring Poly(o-ethoxyaniline). Vitantonio Primiceri, Marco Pugliese, Roberto Giannuzzi, Marco Esposito, Giuseppe Gigli, Vincenzo Maiorano, Pierluigi Cossari. ACS Appl. Electron. Mater., 2023.
13. Collagen Membrane as Water-Based Gel Electrolyte for Electrochromic Devices. Carmela Tania Prontera, Nunzia Gallo, Roberto Giannuzzi, Marco Pugliese, Vitantonio Primiceri, Fabrizio Mariano, Antonio Maggiore, Giuseppe Gigli, Alessandro Sannino, Luca Salvatore, Vincenzo Maiorano. Gels 2023, 9(4), 310.
14. Hybrid electrochromic device with transparent electrolyte. Roberto Giannuzzi, Carmela Tania Prontera, Vitantonio Primiceri, Agostina Lina Capodilupo, Marco Pugliese, Fabrizio Mariano, Antonio Maggiore, Giuseppe Gigli, Vincenzo Maiorano. Solar Energy Materials & Solar Cells 257 (2023) 112346.
15. Strongly enhanced light–matter coupling of monolayer WS2 from a bound state in the continuum. E. Maggiolini, L. Polimeno, F. Todisco, A. Di Renzo, B. Han, M. De Giorgi, V. Ardizzone, C. Schneider, R. Mastria, A. Cannavale, M. Pugliese, L. De Marco, A. Rizzo, V. Maiorano, G. Gigli, D. Gerace, D. Sanvitto, D. Ballarini. Nature Materials, 22, 964-969 (2023).

16. Toward highly efficient solution-processable OLEDs: inkjet printing of TADF emissive layer. Marco Cinquino, Carmela Tania Prontera, Antonio Maggiore, Alessandra Zizzari, Marco Pugliese, Fabrizio Mariano, Vitantonio Valenzano, Ilaria Elena Palamà, Riccardo Manfredi, Giuseppe Gigli, Vincenzo Maiorano. Advanced Electronic Materials, 2023, accepted.

LIST OF ABBREVIATIONS

BNA: bioengineered neuromuscular actuator
CU: contractile unit
eNP: electro-active nanoparticles
NMJ: neuromuscular junction

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Piano Nazionale Complementare (PNC) – Decreto Direttoriale n. 931 del 6 giugno 2022 – Avviso per la concessione di finanziamenti destinati ad iniziative di ricerca per tecnologie e percorsi innovativi in ambito sanitario e assistenziale

Project identifier: PNC0000007

Start date: 01/12/2022

Duration: 44 months

Website: www.fit4medrob.it

ACTIVITY 10 – BIOHYBRID INTERFACES AND BIOMATERIALS
SUB ACTIVITY#5
RESEARCH TEAMS PERIODIC REPORT, M1-M12

PI: Francesco Urciuolo
Partner Acronym: UNINA
Date: 15/10/2023

TABLE OF CONTENTS

1 DELIVERABLES WORKED ON DURING THIS PERIOD

- D 10.1.1 – SubActivity#5: Fabrication of hybrid multifunctional constructs for muscular neural integration and control

2 SUBACTIVITY#5: FABRICATION OF HYBRID MULTIFUNCTIONAL CONSTRUCTS FOR MUSCULAR AND NEURAL INTEGRATION AND CONTROL

Overview. In this section the driving idea, the work program, the identified sub tasks and the involved groups in the SubActivity#5 (**Fabrication of hybrid multifunctional constructs for muscular and neural integration and control)**, will be summarized. Different one-to-one meetings have been carried out in order to better figure out both the expertise and the contribution of each group. Moreover, different group meetings have been made in order to define the collaborations and to define the sub activities.

Rationale. Being positioned under the clinical need "Exo-prosthesis integration/adoption", SubActivity#5 aims at producing a new generation of actuators for advanced prosthetics. In this area the actuation is a critical step and, nowadays, different working principles can be exploited to mimic the actuation capability of skeletal muscles such as hydraulic, pneumatic, piezoelectric, electromagnetic, shape memory alloys, twisted and coiled polymer muscles. Such systems allow both miniaturization, integration and actuation but still possess different drawbacks. Electromagnetic-based systems, for instance, are heavy and do not guarantee large contractile strokes; pneumatic and hydraulic actuators require cumbersome actuation systems, while electroactive polymers need large voltages [**1**]. SubActivity#5 will bring a paradigm shift in the design of the actuation systems for medical robotics by developing an actuator based on the logic of the animal motion: signals from the brain are transformed in controlled movements and force generation. To date, efforts have already been made in the fabrication of bioengineered neuromuscular actuators, activated by light or electrical signals, that are able to generate movements such as swimming and/or walking [**2, 3**]. Nevertheless, no attempts have been made in the development of bioengineered neuromuscular actuators (BNAs) that, once interfaced with signals coming from the brain, are able to guide the movements of finger-like systems. The development of such BNA will contribute to (i) the achievement of a new generation of actuators for medical robotics based on the working principle of living muscles and to (ii) the increase of knowledge in the field of neural control of artificial machines. The driving idea of the Sub Activity #5 is schematized in Figure 1. Briefly, by developing new materials and structures for medical robotics, and by using advanced control strategies in terms of interfaces with human body, the SubActivity#5 aims at developing a BNA by means of emerging tissue engineering strategies (e.g. bioprinting). The BNA will act as an external living actuator able to receive stimuli from the resident peripheral nervous system (PNS) of amputees. After contraction, the bioengineered actuator will be able to control the movement of kinematic elements connected to the robotic prosthesis.

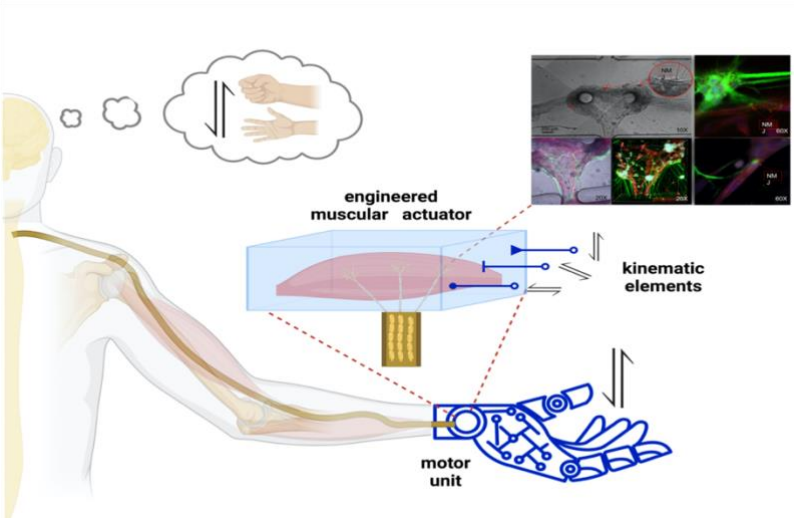

Figure 1 Graphical abstract of the SubActivity #5

Aim. The final goal of such activity has been identified in the in the realization of a prototype represented by an engineered muscle, interfaced with the PNS, which will be able to induce an index/thumb-like "opposition" kinematic. This product, although characterised by low TRL value, will represent the first attempt in the use of a bioengineered muscle to control a robotic hand by using stimuli generated by the brain.

To reach such an ambitious goal, in the first six months of activity different partners with specific expertise in the consortium have been identified. Peoples involved in the definition of the sub tasks are listed below:

- Paolo Netti (UNINA)
- Francesco Urciuolo (UNINA)
- Valeria Panzetta (UNINA)
- Donatella Di Lisa (UNIGE)
- Alberto Rainer (UCBM)
- Riccardo Di Corato (CNR_IMM)
- Enrico Binetti (CNR_IMM)
- Laura Blasi (CNR_IMM)
- Emanuele Gruppioni (INAIL)

The participants have identified two main questions to address (represented by the vertical pillars P1 and P2 in Figure 2). Moreover, from M7-M12 partners have worked to conceptualize the final BNA device, together with the strategies for its fabrication, control and modulation. Four horizontal Sub Tasks (ST1-ST4) have been identified each of one devoted in solving specific issues and related to specific expertises of one or more identified groups. Also, the distribution of the activities in Figure 2 has been conceived in order to maximize the interactions and the collaborations between different groups.

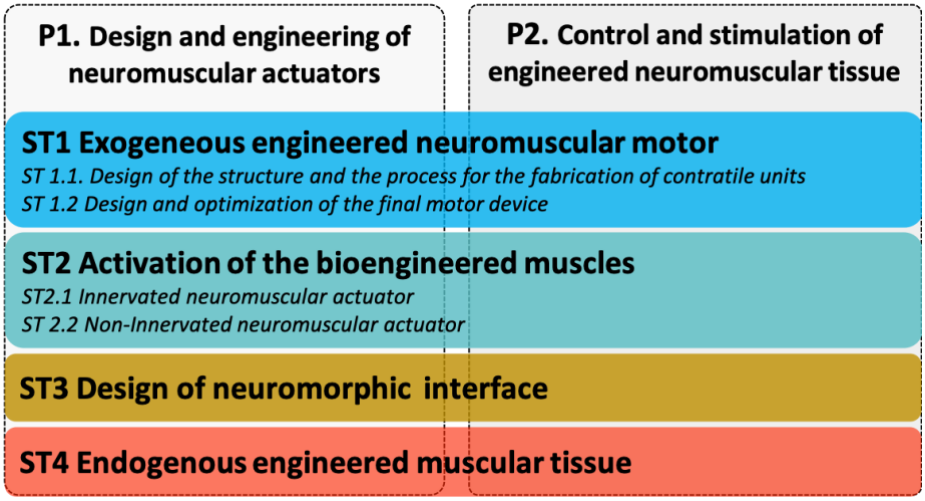

Figure 2 Identification of pillars and sub tasks characterizing the SubAtvitiy#5

2.1 ACHIEVEMENTS

The main achievements of this period are:

(i) definition of the scientific and technological issues and partners identifications
(ii) device conceptualization and control, definition of work plan and sub tasks identification

From M1 to M6 the identified partners have been involved in the problem definition by identifying two main issues to be addressed as synthesized by the pillars following pillars, P1 and P1, reported in the section 2 and Figure 2.

- P1 Design and engineering of neuromuscular actuators: definition of the techniques and methodologies to build up (i) living contractile units (CU), (ii) their spatial arrangement for the obtainment of the BNA and (iii) the connection to kinematics elements able to perform desired movements.

- P2 Control and stimulation of engineered neuromuscular tissues: definition of the techniques and methodologies to control and to modulate the contraction of CUs and their interface with the PNS.

From M7 to 12 participants have

- conceptualized the BNA device
- defined the methodologies to evoke, modulate and control its contraction
- identified possible strategies to couple BNA with PNS
- implemented a work plan with subtasks and timing

Work plan. Participants have been working on the definition of the pillars and sub-tasks reported in Figure 2. Briefly, the final BNA will be constituted by an assembly of different contractile units (CU) that will be bio-fabricated starting from skeletal muscle cells (either primary or IPSc-derived). Each CUs will be able to generate contraction and force along a specific direction. By assembling different CUs, it will be possible to increase the output force and generate movements along multiple directions in order to drive kinematics elements mimicking an index/thumb-like "opposition" movement (Figure 3). The final BNA will be composed by different CUs properly assembled. The contraction of the BNA (or CUs) will be obtained by realizing either a neuro-muscular junction or by inserting in the CUs electroactive nanoparticles (eNP) able to generate local electrical potentials once activated by external fields. Finally, the BNA will be connected to the PNS (boundary 1 in Figure 3) by means of a neuromorphic interface which will capture electrical signals from the PNS. Such signals will be transduced by the interface, into signals which stimulate either the innervated or the not-innervated BNA finally activating the contraction. At boundary 2 of the BNA, kinematic elements will be organized in a way to transform to contraction to mimic the index/thumb-like "opposition" movement.

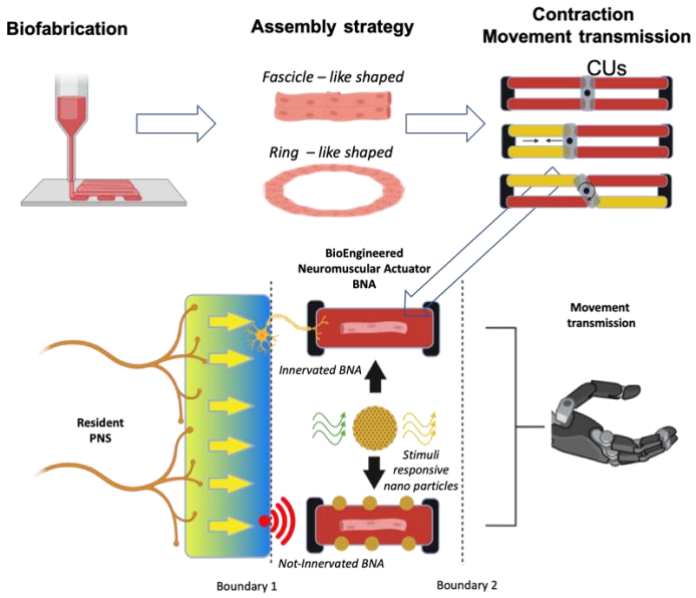

Figure 3 Conceptualization of the BNA, control, modulation and integration

In order to address the questions posed in P1 and P2, four sub tasks have been identified as schematized in figure 2 while their temporal distribution is reported in figure 4.

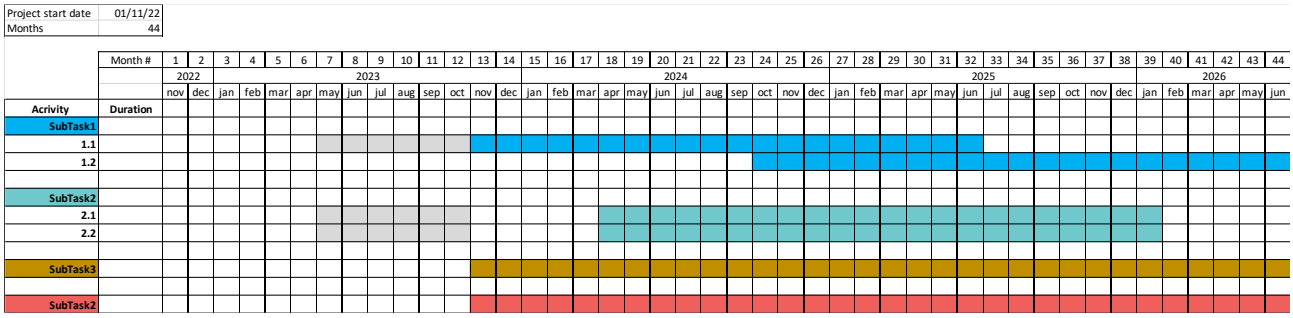

Figure 4 Temporal distribution of sub-tasks.

ST1 Exogeneous engineered neuromuscular motor

In this ST, strategies to fabricate a BNA that works as an "external" living actuator that receive signals from PNS will be developed. ST1 has been divided into two activities (ST 1.1 and ST 1.2) as described below.

ST 1.1. Design of the structure and the process for the fabrication of neuromuscular motor: 20 months

UniNa has a strong experience in the in vitro fabrication of thick an viable engineered tissues [4, 5, 6] as well as organ chip technology and design of macroscopic and miniaturized bioreactors [7, 8]. Coupled with the expertise of UCBM in the field of bio fabrication [9] participants (UniNa/UCBM) have defined the critical steps to be implemented in order to build up functional CUs: in the next months it will be established and implemented the bio-fabrication method, the CU dimensions and the culture conditions to maintain CU viable. Here, by starting from skeletal muscle cells (either primary or IPSc-derived) CUs (0.5-1mm in diameter) will be fabricated and arranged as linear fascicles or ring-like tissues. The diameter of 0.5-1 mm has been chosen in order to facilitate the nutrient supply avoiding in this way the implementation of complex culture conditions. The CU will be obtained by means of bioprinting-derived approach in order to induce the alignment of muscle cells in the CU as previously demonstrated [**9**]. Confinement of muscle cells into (hollow) fiber structures will be achieved via ad hoc optimized microfluidic-enabled printing strategies. A library of biomaterial hydrogels (both synthetic and ECM-derived will be tested with the aim to find the optimal conditions for in vitro muscle maturation. The bioprinted bundle will be inserted into stretchable/actuable framework (obtained by lithographic and/or microextrusion techniques) in order to provide the construct with a sequence of electro-mechanical stimuli during the in vitro conditioning phase and to measure the achievable contraction force/strain. Multiphysics in silico modelling will be exploited to achieve the computational optimization of the CU design.

For preliminary evaluation of both contraction force and strain, the muscle bundles will be bio-printed as ring-like structures at different cell densities and placed around two stiff posts. It is well known that under this configuration muscle cells are able to align and fuse, forming a contractile fascicle-like structure. At this point the ring-like bundle will be transferred in a similar structure formed by flexible posts with known mechanical properties. The defection of the posts will provide information of the passive contractile force at different cell densities. It will be chosen the cell density that maximize the contraction force without compromising the nutrient supply. This configuration will be also used to evaluate the active force (force under stimulation) generated by the signals defined in the ST2 and ST3. One limitation of this approach consists in the limited deflection of the flexible posts which can hinder the evaluation of the full stroke of the contractile units (or the BNA). Techniques for the evaluation of the full contraction of the muscle bundle is currently under evaluation.

- **Goal**: fabrication of a contractile unit, driven by signals defined in the ST2 and ST3, capable of generating a linear movement of kinematic elements connected to the CU.

ST 1.2 Design and optimization of motor device: 21 months

After the fabrication of the CU and the establishment of its properties in terms of cellular organization and actuation capabilities, CUs will be spatially arranged according to different configurations to obtain the final BNA. To carry out this activity different issues will be addressed:

- how to increase the force/power generation;

- how to induce different kinematic schemes and movement transmission;

- to explore different motion mechanisms (e.g. monoaxial *vs*. agonist/antagonist scheme)

- **Goal**: fabrication of BNA, driven by signals defined in the ST2 and ST3, capable of generating a linear movement of kinematic elements connected to the CU.

ST2 Control and modulation of the bioengineered actuator.

In this ST strategies and techniques to evoke and to modulate the contraction of engineered muscle will be implemented. ST2 has been divided into two activities (ST2.1 and ST2.2) as described below.

ST2.1 Innervated neuromuscular actuator: 22 months.

Based on the preliminary activities, the ST2.1 activity will try to address the issue described in the P2. The CUs will be coupled with motoneurons in order to create neuro-muscular junctions (NMJs). One of the goals of this activity is the generation of contraction of the CU by stimulating the motoneurons with electrical signals. To this end in this activity, motoneurons and skeletal muscle cells will be characterized to obtain their electrophysiological parameters by using multielectrode arrays (MEAs) provided by UniGE. After the electrophysiological characterization of the single cell lines, measurements will be performed in presence of NMJ on either 2D or 3D systems. The 3D system is represented by the CUs developed in the ST1. Also, in ST2.1, the CU will be subjected to different electrical signals that will be varied in terms of frequency and amplitude in order to characterize the electromechanical response of the innervated CU under different stimulation conditions. The recording of the spontaneous electrophysiological activity will be performed using two types of commercial MEA60 devices: the standard MEA60 and the 3D MEA60. The standard MEAs consisted of 60 flat microelectrodes (made of TiN/SiN) with a 30 µm electrode diameter, spaced 200 µm apart, arranged in an 8 × 8 square grid (excluding the four corner electrodes). These MEAs will be provided by Multi Channel Systems (MCS) in Reutlingen, Germany. The 3D MEA60 have the same spatial organization of the electrodes, but in this case the electrodes are pyramidal (they are 100 µm high and have a tip with a diameter of 12 µm) and are 250 µm spaced among them. The electrophysiological activity will be recorded using the MEA 2100 System from MCS. These recordings will be conducted outside of the incubator, maintaining a temperature of 37 °C. To prevent evaporation and maintain the pH of the medium, a continuous flow of humidified gas (comprising 5% CO_2, 20% O_2, and 75% N_2) was supplied into a small plastic enclosure that covered the experimental MEA setup during the measurement sessions.

- Goal: definition of the stimulation parameters for the innervated-CU

ST2.2 Non-Innervated neuromuscular actuator: 22 months

In this subtask, electro active nanoparticles (eNP), developed at CNR_IMM, will be used to activate the contraction of sarcomeres in CUs. In detail, different nanoparticle architectures have been considered and discussed within the subtask team. The goal is to synthetize nanoparticles able to convert an external remote trigger, such as an ultrasound stimulus and/or a focused laser irradiation, into an electric signal suitable for cellular stimulation. For this purpose, several nanoparticle architectures will be tested during the project. For achieving such multifunctional nanostructures, the idea is to combine a piezoelectric nanocrystal with stimuli-responsive and/or conducting/passivating materials.

The best-performing nanostructures will be stabilized in suspension by an organic layer that allows the dispersion of the nanoparticles into an implantable hydrogel. Moreover, the size of the systems will be kept in the range of 300-500 nm, to avoid cellular internalization by the actuator sarcomeres. The choice of the piezoelectric material is still an open point since the group is exploring different strategies, by favoring the materials (organic or inorganic) with low or negligible

toxicity. The biocompatibility and the stability of the nanoparticles will be tested by in vitro study on 2D cell cultures and by testing the ion leakage after mid-long storage. The functional characterization of the material will be achieved at IMM, involving other researchers with expertise in the field of piezoelectric materials. The integration of the external trigger(s) into the characterization setup is, at the moment, the main limitation, but different strategies have been considered and will be tested as soon as the first samples are obtained. eNP will be inserted in the CU developed in the ST1 at a concentration and distribution able to generate the desired contraction. To do this, the non-innervated contractile units will be subjected to different external fields able to activate the eNPs. Here both electromechanical (**UNINA, UCBM, CNR_NANOTEC**) and electro-physiological properties (UNIGE) of the non-innervated CU will be assessed.

In this ST the possibility to use the eNP also in the innervated CU will be explored in order to increase and/or modulate the electromechanical performance of the CU.

- **Goal**: definition of the stimulation parameters for the non-innervated-CU
- **Open questions:** effective response of the sarcomeres to the electric signal generated by eNP in the CU.

ST3 Design of neuromorphic interface (Open Question)

An implantable interface should be developed in order to transfer the signal from the PNS to the CUs/BNA. The interface should be able to capture signals from the resident PNS transforming them into input signals for either motoneurons or the eNPs which drive the innervated CUs or the non-innervated CU, respectively. **At this regard a new partner should be identified.**

- **Goal**: engineering of neuromorphic interface.
- **Open questions:** identification of a new partner.

ST4 Endogenous engineered muscular tissue (Open Question)

During the definition of the activities the possibility to implement a bio-fabrication method to generate an implantable version of the BNA has been discussed. This should be able to capture and integrate the host's neurostimulation.

- **Goal**: fabrication of an implantable BNA
- **Open questions:** size of the BNA; how to integrate the BNA in the host; identification of a new partner

This activity is still under discussion.

3 DISSEMINATION ACTIVITIES (SCIENTIFIC PAPERS, POP MAGAZINES, TV, ETC.)

List of dissemination activities organized in categories. Add dates and venues in case of public events.

- Notte dei ricercatori STREETS Federico II, September 29 2023, Complesso di Monte Sant'Angelo, University of Naples Federico II, Naples.

4 MEETINGS IN THIS PERIOD

- July 17 2023: Urciuolo (UNINA), Netti (UNINA), Panzetta (UNINA), Di Corato (IMM), Binetti (IMM) via MSTEAMS
- July 18 2023: Urciuolo (UNINA), Grupioni (INAIL), Netti (UNINA) via MSTEAMS
- July 19 2023: Urciuolo (UNINA), Di Lisa (UNIGE) via MSTEAMS
- July 20 2023: Urciuolo (UNINA), Rainer (UCBM/CNR_NANOTEC) via TEAMS
- July 24 2023: Urciuolo (UNINA), Scalera (CNR_NANOTEC), Polini (CNR_NANOTEC) via TEAMS
- August 7 2023: Urciuolo, Netti, Di Lisa, Rainer, Gruppioni, Di Corato, Binetti.
- September 21-22 2023: Lecce, CNR_NANOTEC: meeting with the participation of all partners.

5 RECRUITMENT IN THIS PERIOD

- October 1, 2024, Sara Sibilio, Female, (post-doc fellowship - UNINA) working on ST1, enrolled for 24 months.

6 APPROXIMATE EXPENDITURE IN THIS PERIOD

Personnel

- 48 K€: 1 post-doc fellowship (UNINA)

Equipment

- € 102350,68 2PP microprinter (UCBM)

Other Expenses

Building

LIST OF ABBREVIATIONS

BNA: bioengineered neuromuscular actuator

CU: contractile unit

eNP: electro-active nanoparticles

NMJ: neuromuscular junction

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Piano Nazionale Complementare (PNC) – Decreto Direttoriale n. 931 del 6 giugno 2022 – Avviso per la concessione di finanziamenti destinati ad iniziative di ricerca per tecnologie e percorsi innovativi in ambito sanitario e assistenziale

Project identifier: PNC0000007

Start date: 01/12/2022

Duration: 44 months

Website: www.fit4medrob.it

ACTIVITY 10 – BIOHYBRID INTERFACES AND BIOMATERIALS

SUB ACTIVITY#6

RESEARCH TEAMS PERIODIC REPORT, M1-M12

PI: Francesco Ferrara (CNR), Luciano Velardi (CNR)
Partner Acronym: CNR NANOTEC, CNR IMM
Date: 14/10/2023

TABLE OF CONTENTS

1 DELIVERABLES WORKED ON DURING THIS PERIOD

- D 10.1.1 – SubActivity#6: Implantable microelectrodes array for electrical stimulation and signal collection from muscle tissue

2 SUBACTIVITY#6: IMPLANTABLE MICROELECTRODES ARRAY FOR ELECTRICAL STIMULATION AND SIGNAL COLLECTION FROM MUSCLE TISSUE

Overview: The planned goals for contributing to the preparation of the deliverable D10.1.1. ”Methods and prototypes of biohybrid interfaces” of the sub-activity 6 entitled “Implantable microelectrode array for electrical stimulation and signal collection from muscle tissue”, can be summarized as follows:

- definition of a detailed structure of the work program;
- consolidation of an integrated, interactive and collaborative team made of different researchers belonging to different working groups;
- identification of the materials and methods to achieve the objectives of the activity;
- schedule of planned activities.

Aim: The aim of the sub-activity 6 is the development of implantable microelectrode array for electrical stimulation and signal collection from muscle tissue, through two different approaches:

a) design and implementation of an implantable electrode system on flexible biocompatible substrates, activated by RFID (Radio Frequency Identification Device) coupling: the main advantage in using a wireless system lies in the reduction of infection and/or skin inflammation by avoiding the use of cables coming out of the epidermis;

b) design and manufacture of graphene electrodes for the stimulation and recording of electrophysiological signals in 'passive' devices using “mechanochemical approach” and 3D printing.

To reach our goals, in the first year of activity different partners with specific expertise in the consortium have been identified. Peoples involved in the definition of the sub tasks are listed below:

- Laura Pastorino (UNIGE)
- Francesco Ferrara (CNR_NANOTEC)
- Antonio Turco (CNR_NANOTEC)
- Michela Chiappalone (UNIGE)
- Donatella Di Lisa (UNIGE)
- Pietro Siciliano (CNR_IMM)
- Maria Assunta Signore (CNR_IMM)
- Luca Francioso (CNR_IMM)
- Luciano Velardi (CNR_IMM)
- Laura Blasi (CNR_IMM)

Rationale. The driving idea of approach (a) is to utilise the effect of electric stimulation to explore regeneration, re-inhabitation and revascularisation of innested, de-innervated and/or de-vascularised muscular tissue. In fact, it is known from recent work the benefits attributed to electrical stimulation of muscles: speed up of the re-innervation process, improvement of the vascularisation process, reduction of the motor rehabilitation time, and promotion of the production of more intense electromyographic signals by the grafted muscle. The most challenging part of this proposal is the realisation of the (microelectrode array + signal generation/collection) system, fully biocompatible, implantable and working wirelessly. The proposal provides a novelty compared to the state of the art: the use of a wireless system via RFID (Radio Frequency Identification Device) offers various advantages such as the reduction of infections and/or skin inflammation and the non-use of cables coming out of the epidermis.

It is well known that the adoption of interdigitated electrodes array for stimulating muscle tissues guarantees superior performance compared with conventional setup (for example through metal wires in close proximity to the muscle tissue) in terms of stimulus efficiency [1, 2]. The identification of the substrate, as well as the material and the geometry of the electrodes [3], are of great importance. A desirable electrode system would include a flexible material as substrate that offers low modulus responses to elongation, resulting in more elastic deformations and better contact between the moving tissue and the electrode.

With regard to (b) approach, the true potential of microfluidic lab-on-chip technology lies in its capacity to design synthetic culture systems where various control parameters (e.g., cell types and positioning, transcellular chemical gradients, molecular and oxygen gradients, flow levels, and patterns, as well as mechanical forcing regimens) can be precisely regulated [4, 5]. Moreover, this platform bridges current gaps in the capabilities of existing in vivo and in vitro approaches by providing an integrated perspective on complex physiological systems at a cellular resolution [5-7]. During the initial six-month period, our primary focus was on conceptualizing and designing a microfluidic culture chip, often referred to as a Lab-on-a-Chip (LoC) platform. This platform mimics organ functionality by faithfully recreating multicellular architectures, vascular-parenchymal tissue interfaces, chemical gradients, mechanical cues, and vascular perfusion [4, 5].

The compartmentalization of microfluidic systems allows individual cell populations to be cultured and sampled separately while still enabling interactions between them. To the best of our knowledge, this approach represents the only existing method for experimentally co-culturing fundamentally different cell populations with distinct nutrient and microenvironment requirements [5, 7-9].

2.1 ACHIEVEMENTS

The aim of the sub-activity 6 is the development of implantable microelectrode array for electrical stimulation and signal collection from muscle tissue, through two different approaches:

a) design and implementation of an implantable electrode system on flexible biocompatible substrates, activated by RFID (Radio Frequency Identification Device) coupling and avoiding the use of cables crossing the epidermis, thus reducing the onset of infection and/or skin inflammation;
b) design and manufacture of graphene electrodes for the stimulation and recording of electrophysiological signals in 'passive' devices using “mechanochemical approach” and 3D printing.

Concerning the (a) approach, the work carried out during this period covered the following points:

1. Adoption of executive block diagram of the system;
2. Programming in work packages (WPs) and exact timing;
3. Identification of substrate and commercial integrals to be used.

Methods. Participants identified several horizontal Sub-Tasks (STs), for each of the two approaches, dedicated to the implementation of the sub-activity 6, using the specific competencies of one or more of the identified groups.

Concerning the (a) approach:

- **ST1 Deposition and characterization of metallic films on flexible biocompatible substrates.** In this ST, strategies to fabricate metallic films on flexible polymers will be developed, with a particular attention towards the optimization of adhesion and mechanical properties of the metal layers.
- **ST2 Design, fabrication and characterization of the microelectrode array on flexible biocompatible substrates.** In this ST, strategies to choose the right microelectrode array configuration will be implemented to maximize the electrical stimulus transfer efficiency.
- **ST3 Development and realization of the signal generation/data acquisition system.** In this ST, strategies to develop and realize the wireless system via RFID (Radio Frequency Identification Device) will be investigated.
- **ST4 Tests and Validation.** In this ST, validation tests of the designed and realized system will be scheduled to verify the performance.

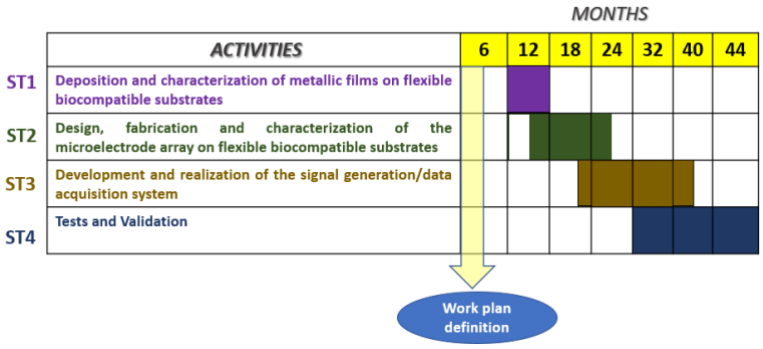

Figure 1 Gantt chart of (a) approach

Work plan. For the purpose of the (a) approach, substrates based on biocompatible polymers, such as polyimide (PI), C-parylene and polydimethylsiloxane (PDMS) (lower Young's modulus than Si, thus less harmful to tissue), were initially selected. Furthermore, the use of gold or platinum materials will ensure high conductivity, mechanical and chemical stability and biocompatibility of the system. The serpentine structure of the electrodes will provide flexible and stretchable properties for application in muscle tissue. Due to its high tensile properties, the serpentine configuration is the most studied and adopted structure for stretchable electronic systems [3].

Concerning the external data collection system to be used, commercial devices were identified as offering compact dimensions, low power and high performance.

ST1 Deposition and characterization of metallic films on flexible biocompatible substrates (6 months).

- **ST 1.1 Deposition of metallic films via physical and chemical methods.**

  Based on our expertise, metallic films of gold or platinum will be deposited by thermal evaporation and/or RF magnetron sputtering techniques. The deposition parameters and thicknesses will be modified in order to explore the adhesion and mechanical stability of the deposited films. Substrates based on biocompatible polymers such as polyimide (PI), C-parylene and polydimethylsiloxane (PDMS) will be used. Possible chemical/physical treatment on polymeric substrates will be investigated to improve the adhesion of the metallic layers.

  Goal: definition of substrate surface treatment to improve the adhesion of metallic layers; fabrication of metallic films on flexible substrate with good uniformity and adhesion.

- **ST 1.2 Characterization of metallic films.**

  The deposited metallic films will be characterized by different point of view. X-ray diffraction (XRD), Raman and Atomic Force Microscopy (AFM) will be performed to investigate the crystalline structure and the morphology, Scanning Electron (SEM) and Transmission Electron (TEM) Microscopies to analyse the substrate-film adhesion, also based on the different types of polymeric substrates chosen. Finally, MTT tests will be done to study the biocompatibility and nanoindentation tests to investigate on mechanical (applying deformations such as compression, stretching, torsion and rotation) and chemical stability (in aqueous solutions).

  Goal: characterization of physical, biocompatible, and mechanical characteristics of the metallic thin films deposited on flexible substrates, in order to be implantable in muscle tissue.

ST2 Design, fabrication and characterization of the microelectrode array on flexible biocompatible substrates (1 year).

- **ST 2.1 Design and fabrication of the microelectrode array.**

  The design of the electrodes geometry will be performed by finite element software simulation (Comsol Multiphisics) in order to identify the configuration which maximizes the stimulus transfer efficiency. Starting from the serpentine structure of the electrodes, known to be the most utilized structure for stretchable electronic systems, other possible geometries will be considered with the aim of miniaturization and signal coupling maximization. Different microelectrode arrays will be deposited on flexible polymeric substrates the basis of the results obtained in the ST 1.

Goal: design and fabrication of an optimized implantable microelectrode array configuration such to maximize the electrical signal transfer efficiency.

- **ST 2.2 Characterization of the microelectrode array.**

The fabricated microelectrode arrays will be characterized as described in ST1.2 for metallic films. Moreover, electrical characterization by 4-point probe technique will be performed to investigate on electrical behaviour to repetitive bending or permanent curvature of the substrate.

Goal: implantable microelectrode arrays with good mechanical and chemical stability, and electrical performance.

ST3 Development and realization of the signal generation/data acquisition system (1,5 years, Open question).

After the fabrication of microelectrode arrays, the signal generation system to be used will be realized at IMM laboratories, while data acquisition hardware will be a commercial product; this aspect in order to guarantee compact dimensions, low power and high performance.

Goal: Signal generation/data acquisition system working at low energy power.

Open question: identification of commercial devices to be implemented.

ST4 Tests and Validation (1 year, Open question)

Tests and validation will be done on different devices by a partner to be identified.

Goal: realization of a working implantable microelectrode array system for electrical stimulation and signal collection from muscle tissue.

Open question: identification of a partner for tests and validation on the implantable microelectrode array system.

Concerning the (b) approach:

We will focus on the development of passive lab-on-chip systems utilizing cutting-edge carbon-based microelectrodes (CBMs) synthesized through 3D printing. These innovative microelectrodes will serve as invaluable tools for investigating the electrophysiological properties of individual cells as well as for exploring the intricate interactions between diverse cellular populations.
By leveraging their exceptional sensitivity and precision, we aim to gain profound insights into the electrical activities of single cells. Moreover, these systems will enable us to unravel the complexities of cell-to-cell communication and cooperation, shedding light on the dynamic interactions that underpin various biological processes.

- **ST1 Design of a lab-on-chip with passive microfluidics fabricated by soft litography and CBMs multi electrode array (MEA). (CNR Nanotec) (month 6 – 12):** This task involves the design of an innovative lab-on-chip system with passive microfluidics, created through soft lithography, and a multi-electrode array (MEA) featuring Carbon-Based Materials (CBMs) fabricated by 3D printing.
- **ST2 Functionalization of polymeric microparticles with Carbon Based materials through mechanochemical approaches. (CNR Nanotec) (months 13 – 44):** the task focuses on functionalizing polymeric microparticles for 3D printing using Carbon-Based Materials (CBMs) through innovative mechanochemical methods**.**
- **ST3 Fabrication and characterization of 3D printed Carbon based materials (CBMs) multi electrode array (MEA) integrating passive microfluidics for stimulation and recording of electrophysiological activity**; **(CNR Nanotec) (13 – 44):** Task entails the fabrication and characterization of a 3D printed multi-electrode array (MEA) with integrated passive microfluidics for stimulating and recording electrophysiological activity, utilizing Carbon-Based Materials (CBMs).
- **ST4 Test on biological systems. (CNR Nanotec – UniGE) (months 33 – 44):** the task involves testing the developed technologies and systems on biological samples, conducted in collaboration between CNR Nanotec and UniGE.

Figure 2 Gantt chart of (b) approach

The main achievements of this period are:

(i) Conceptualized and designed innovative strategies for producing CBMs-based MEAs to enhance neuronal electrophysiological recording.

(ii) Designed a passive microfluidic platform that enables the decoupling of cell populations while preserving cellular crosstalk.

(iii) Designed of the integration of CBMs MEA with a multicompartment device featuring on-demand activable communication systems.

(iv) Identified specific characteristics required for the 3D printer to produce CBMs-based MEAs, through the printing of pellets functionalized with CBMs using mechanochemical approaches.

ST1 Design of a lab-on-chip with passive microfluidics fabricated by soft litography and CBMs multi electrode array (MEA)

Our efforts during this period (months 6 – 12) centered on devising and creating a system that facilitates on-demand communication between diverse cellular populations, eliminating the need for microfluidic pumps, which can complicate device management during laboratory analysis. This system will also be integrated with a 3D-printed carbon-Based MEA (CBMs MEA), enabling the recording of electrophysiological cell activity with unprecedented resolution and sensitivity. More specifically, a multi-compartment device interconnected through one or more microchannels will be initially fabricated using polydimethylsiloxane (PDMS) and placed on a polymeric substrate produced by 3D printing, which incorporates the CBMs-based MEA on its surface, as depicted in Figure 3.

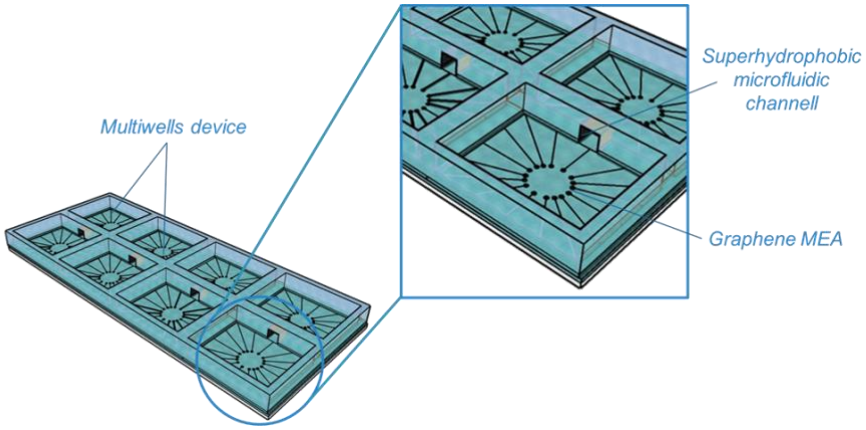

Figure 3 Scheme of the conceived device

The PDMS component of the chip, featuring microfluidic channels, will be manufactured using specific molds created with a 3D printer or micromilling machine available at CNR-Nanotec. PDMS replicas will be produced through soft lithography using a prepolymer solution, with the cured PDMS layer removed from the mold.

The plastic component, integrating the CBMs-based MEAs, will be manufactured through an innovative 3D printing strategy. Pristine CBMs will be non-covalently bonded to plastic pellets via mechanochemistry to preserve the electronic properties of the nanomaterial [10]. These prepared nanocomposites will be employed in 3D printing.

With open 3D system, the fabrication process will be complete controlled, enabling to fine-tune and optimize parameters such as layer separation, droplet size, and other critical factors.

This system allows to seamlessly transition from research settings to high-throughput fabrication by using the same plastic granules typically employed in injection molding. The process Is simplified, making it easier to move from a research context to large-scale production. While a wide range of certified original materials is available, there will

be the capability to utilize new materials specifically developed for the Fit4Med project. The flexible nature of our fabrication process, combined with the ability to create complex geometries and integrate various functions, opens up exciting possibilities for producing components that feature both hard and soft materials.
This approach ensures a homogeneous dispersion of CBMs on the electronic surface without necessitating chemical functionalization steps (e.g., oxidation), which could adversely affect the nanomaterial's mechanical and electronic properties, consequently affecting the final MEA performance. Furthermore, the quantity and distribution of graphene can be finely tuned by varying the initial dimensions of the plastic pellets, allowing for precise control of the resulting electrodes.
To attach the PDMS component to the plastic substrate integrating the MEA, a thin layer of uncured, diluted PDMS will be applied to the bottom part of the PDMS component using microcontact printing, thus preserving the microfluidic channel surfaces. The device will then be placed onto the part of the device that includes the MEA and cured. In this configuration, the hydrophobicity of PDMS will prevent the passage of culture media from one compartment to another during biological tests. However, by applying pressure to the top of the microchannels between two compartments, entrapped air can be removed, hydrophobic forces overcome, and the channels filled with culture media, thereby facilitating communication between the selected compartments. The assembly procedure for the device is illustrated in Figure 4.

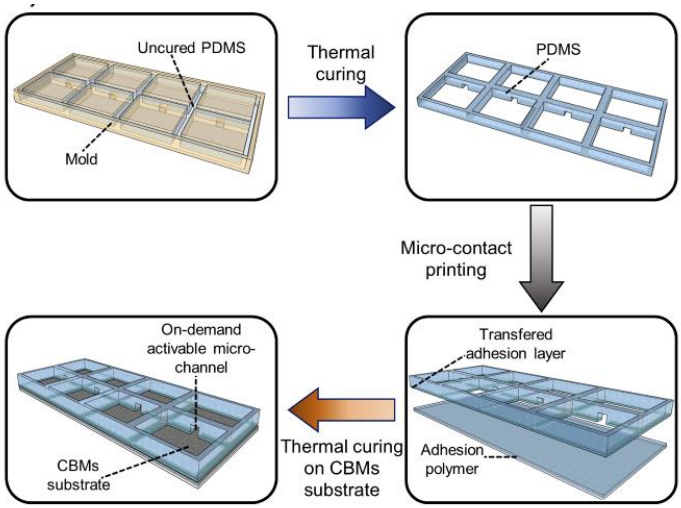

Figure 4 Assembly of the device

Depending on the specific application requirements, the channel's hydrophobicity may be further enhanced to prevent solvent diffusion within the channels before activation. This can be achieved through solvent pre-treatment of the mold, which increases the PDMS surface roughness and hydrophobicity [11].
An essential feature of this system is its ability to facilitate, on demand, cell-cell interactions between cells plated in each compartment, thanks to the presence of microchannels. This system creates a decoupled cellular population system that is both metabolically and functionally connected, allowing for detailed characterizations of the metabolic and functional interactions between different cell populations.

ST2 Functionalization of polymeric microparticles with Carbon Based materials through mechanochemical approaches
This subtask will involve two different activities:

1. Functionalization of polymeric pellets through mechanochemical approaches
2. Stabilization of nanocomposites pellets Through thermal treatments

ST3 Fabrication and characterization of 3D printed Carbon based materials (CBMs) multi electrode array (MEA) integrating passive microfluidics for stimulation and recording of electrophysiological activity
This subtask will involve three different activities:

1. Fabrication and characterization of 3D printed Carbon based materials (CBMs) multi electrode array (MEA)

2. Fabrication and characterization of passive microfluidics by soft litography
3. Device assembly

ST4 Test on biological systems
This subtask will involve two different activities:

1. Lab-on-chip fabrication
2. Biological tests

3 DISSEMINATION ACTIVITIES (SCIENTIFIC PAPERS, POP MAGAZINES, TV, ETC.)

- 04.09.2023 CMD30 FisMat 2023– Milan (Italy)
- Antonio Turco et al, "Mechanochemical approach for the fabrication of Carbon based porous elastomeric (nano)composites: from environmental remediation to piezoresistive devices". Oral presentation.
- 04.09.2023 CMD30 FisMat 2023– Milan (Italy)
- Giulia Siciliano et al., "Development of a MIP based electrochemical sensor for TGF1 detection and its application in liquid biopsy". Oral presentation.
- 10.09.2023. EUROSENSORS, XXXV Conference, Lecce, Italy.
- Antonio Turco et al., "Mechanochemical Approach for Carbon Nanotubes Based Piezoresistive Sensors Fabrication". Poster.

Scientific Papers

- Garzarelli, Valeria; Chiriacò, Maria Serena; Cereda, Marco; Gigli, Giuseppe; Ferrara, Francesco. "Ultrasensitive qPCR platform for rapid detection of bacterial contamination of raw biological samples at the point of care". Heliyon Volume 9, Issue 5. DOI: 10.1016/j.heliyon.2023.e16229
- Turco Antonio; Elisabetta Primiceri; Maria Serena Chiriacò; Velia La Pesa; Francesco Ferrara; Nilo Riva; Angelo Quattrini; Alessandro Romano; Giuseppe Maruccio. "Advancing Amyotrophic Lateral Sclerosis Disease Diagnosis: A Lab-on-Chip Electrochemical Immunosensor for Ultra-Sensitive TDP-43 Protein Detection and monitoring in serum patients". Talanta *Under Revision*

4 MEETINGS IN THIS PERIOD

- June 21-23 2023: Ferrara (CNR), Turco (CNR), Di Lisa (UniGE), Pastorino (UniGE), Chiappalone (UniGE) – via TEAMS
- Meeting of Activity 10: Biohybrid Interfaces and Biomaterials (Mission 3), 21-22 September 2023, Lecce - CNR NANOTEC Seminar Room "Rita Levi Montalcini"; participants: PI from each sub-activity and other researchers related to activity 10.

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