Integrated Multimodal Optical and Magnetic Resonance Imaging (IMAGO)

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ABSTRACT

IMAGO has the ambitious objective to develop *in-vivo* histological MRI with a resolution down to the micron scale by exploiting transient Anomalous Diffusion MRI validated and integrated with optical microscopy. Calibration phantoms with multiscale ad hoc and controlled features have been used together with new fluorinated diffusing probes. As proof of concept, IMAGO images on a fixed spinal cord were obtained showing their superior sensitivity to detect sub-microstructures compared to conventional diffusion MRI. In a long-term vision, IMAGO opening up new possibilities in *Health* in terms of early diagnostic, therapeutic strategies and patients handling.

Keywords: diffusion MRI; anomalous diffusion; optical microscopy; imaging resolution; multiscale systems; complex systems.

1. INTRODUCTION

In recent years, Nuclear Magnetic Resonance (NMR) Imaging (MRI) thanks to the introduction of molecular diffusion MRI (DMRI) has greatly enhanced medical diagnostics [1]. However, the fact of not providing information on sub-microscopic scales or on parameters describing a complex system behaviour makes DMRI still insensitive in detecting early structural changes due to diseases. On the other hand, optical imaging is able to visualize in vitro cellular and sub-cellular structures determining their size, shape and dynamics. Therefore, tissue histology is used to confirm pathologies and optical single-particle-tracking (SPT) can support specific dynamics models from which extract parameters describing structural-disorder and phase-transitions in complex systems. However, optical imaging suffers of scarce light penetration in tissues. By taking advantage of transient-Anomalous Diffusion (tAD) NMR [2,3], IMAGO goal is to significantly increase DMRI resolution and diagnostic performance with improved sensitive and specific detection already in the early or pre-symptomatic phase of numerous human pathologies.

DMRI currently used in clinical applications has an intrinsic resolution around 10-20μm that is still insufficient for detecting early structural changes due to diseases [1]. In some neuro-pathologies, for example, the injury of astrocytes and glia can induce sub-micrometric degenerations as swelling of the cell body, proliferation, shape modification, neurons atrophy, membrane-surface structural changes with permeability modification. In particular, in multiple sclerosis (MS) disease, before macroscopic axonal degeneration currently detected by DMRI, an axonal shape modulation, myelin nanoscopic degeneration with increased roughness of the axonal surface occurs. Current DMRI approaches based on Normal diffusion of bulk water is not able to detect none of the above characteristic, sub-microscopic changes and collective rearrangement. Being able to probe media with a length scale (l_D) below the micron and quantify new local parameters, tAD would dramatically increase DMRI potential diagnostic in detecting collective, micro and sub-micro architectural changes of human tissues due to pathological damage.

To this aim, in the IMAGO phase-I:

- a prototype methodology validated in experiments on calibration phantoms with multiscale ad hoc and controlled features has been developed and validated to establish reliable tAD parameters and their information content
- The integration of the theoretical and experimental tAD MRI results in a systematic and coherent view was obtained, assessing potential and limits of tAD by MRI.
- As proof of concept, tAD MRI on fixed spinal cord was obtained. The extracted information, validated with light-microscopy histology and compared to conventional DMRI show the superior diagnostic power of tAD MRI.

2. STATE OF THE ART

Molecular diffusion in biological systems is a key process to probe micro-architectural and topological characteristics of tissues in which diffusion occurs. By using DMRI, the l_D through which diffusion phenomena are explored and, consequently, tissue structures are investigated is approximately $l_D = MSD^{1/2}$, where in case of Normal diffusion $MSD=6D\Delta$ is the mean squared distance travelled by molecules in a given interval of time Δ and D is the diffusion coefficient. Tissues are therefore probed on the scale of l_D (around 10-20μm), which is two orders of magnitude smaller than the MRI resolution (typically 1mm for clinical scanners). This property of DMRI and the non-ionizing nature of MRI explains why DMRI methods have revolutionized the clinical diagnostics over the last 20 years [1].

Current DMRI approaches are based on Normal diffusion models of experimental data analysis. However, the presence of heterogeneous, complex, multi-scale, or disordered structures may drastically alter well established results for Normal diffusion in bulk water, leading to new phenomena such as tAD that can be exploited to investigate submicroscopic organization [4].

However, the following fundamental unresolved theoretical and experimental questions prevent the take-off of diagnostic MRI, based on the tAD principles:

- What mechanism makes the diffusion anomalous in soft condensed matter, and which AD parameters are mostly affected by the underpinning microstructural feature?
- How the different diffusion mechanisms can be distinguished experimentally?
- How to relate at a mesoscopic scale (accessible by DMRI methodologies), stochastic transport processes, weak ergodicity breaking, and aging in heterogeneous complex systems that occur at a nano/microscopic scale?
- Can we use tAD regime to obtain sub-microstructural information in living tissues increasing the resolution of DMRI?

3. BREAKTHROUGH CHARACTER OF THE PROJECT

IMAGO Phase-I project aims to provide advances beyond the state-of-the-art, by successfully answering the basic questions of foundational nature. Towards this goal, new scientific and technological breakthroughs were developed and used to pave the way for improving the intrinsic resolution of DMRI investigations to go down to the micron resolution scale. We used modern methods of statistical physics and diffusion theory, as well as efficient geometry-adapted Monte Carlo (MC) simulators together with selected geometrical and topological characteristics of samples where tAD occurs. Results were validated by complementary techniques of optical-microscopy and molecular-dynamics simulation [5].

Tab. 1. Principal scientific breakthrough obtained by IMAGO

	Conventional DMRI	tAD MRI from IMAGO
Intrinsic resolution	10-20 μm	below 1μm
Nanostructured surface differentiation	no	yes
Local measurement	no	yes
Disorder Degree quantification	no	yes
Axonal diameter standard deviation (SD)	no	yes

Specifically, we focused on three main different kinds of controlled phantoms mimicking diffusion in tissues: 1) Water mono- and poly-disperse polistyrene packedbeads each fitting cells diameter (6, 10, 20 μm) were used to mimic extracellular space. 2) Sephadex hydrogel characterized by multiscale pores for mimicking a generic multi-compartmentalized tissue. 3) Highly porous polymeric matrices of polyvinyl alcohol (PVA) with differently nanostructured pore walls that can mimic nanoscopic degeneration related to the roughness of axonal surfaces as occurs in MS.

Four different types of diffusing probes were used to investigate different dynamic ranges and to obtain different kind of diffusion: hindered, confined, tAD due to diffusion times spreads in different compartments and tAD due to entrapped probe in a space for longer times: a) water, b) $B_{12}F_{12}^{--}$, c) 100nm liposome d) fluorinated 4kD dextran.

The aforementioned diffusing probes allow investigating a large range of dynamics up to those studied by SPT techniques [6]. To be sure of acquiring the NMR signal of diffusing probes without contamination from background proton signal, we developed new methods of tracers fluorination and optimized NMR techniques to quantify the diffusion by refocusing the 19-fluorine signal. This scientific breakthrough born from the needs of the IMAGO has great potential and a highly innovative character in the MRI field. Thus, it is currently also part of the Neptune project funded by INFN focused on increase the sensibility of 19-fluorine MRI. In the phase-II IMAGO project, we would also obtain dual 19-fluorine-MRI and fluorescence detection probes.

To validate tAD MRI information obtained in a fixed mouse spinal cord, a specific software for the extraction of morpho-topologic parameters from 2D-optical microscopy images was developed. Tab. 1 resumes the breakthrough character of Phase-I IMAGO comparing features in conventional and tAD DMRI highlighted in the results section.

4. PROJECT RESULTS

IMAGO results suggesting a strong advance beyond the state-of-the-art are schematically highlighted in the following three

figures. In all cases, diffusion NMR measurements were performed on a Bruker 9.4T Avance scanner (10mm internal diameter bore) equipped with a gradient unit (maximum gradient-strength =1200mT/m) for collecting S(Δ) data to fit with suitable functions representing diffusion models.

We varied the sample microstructural features in a controlled way and observed the consequent modifications of the theoretical, simulated and experimental signal. This approach allows us to understand and reveal the fundamental relation between tAD parameters and the underlying structure. Fig. 1 displays results obtained by taking into account the subdiffusion parameters α of the tAD relation: MSD $\propto \Delta^{\alpha}$, with $\alpha < 1$ [2] to investigate pores and mesh in Sephadex hydrogel. Pressure scanning electron microscopy (VP-SEM, Hitachi SU-3500)) combined with Peltier cool stage control showed beads with surface pores (from 10 μm up to 1μm) and mesh of about 3nm (see inserted A and B of Fig.1). Water, $(B_{12}F_{12})^{--}$ and liposomes, characterized by a diameter of about 0.2nm, 2-3nm and 100nm, respectively, were used as diffusion probes. Both water and liposomes in G50-Sephadex are characterized by α closed to 1, because their dimension does not match with peculiar sizes of the Sephadex matrix. Conversely, diffusion of $B_{12}F_{12}^{--}$ shows subdiffusion because its dimension is closed to the mesh dimension of Sephadex surface beads. Therefore $B_{12}F_{12}^{--}$ is entrapped and resides close to mesh for a longer time showing $\alpha=0.76\pm0.03$.

Unfortunately, due to covid-19 restrictions, we did not carry out SPT experiments, as the instrumentation is located in the Applied Physics Department of a hospital (Policlinico Universitario Fondazione Agostino Gemelli). However, to mitigate this drawback we have enhanced the validations of the tAD by increasing the computational effort of simulations. To describe the molecular diffusion in confining systems we performed MC simulations of random trajectories of particles, known as reflected Brownian motion. In particular to take into account the multi-scale diffusion in porous media, we are using a fast random walk (FRW) algorithm [5], able to adapt to different local confining structures. Different comparison tests to verify the non-Normal diffusion were used.

Fig. 2 shows water subdiffusion results deriving from multiscale diffusion in two highly porous polymeric matrices (polyvinyl alcohol, PVA) with randomly oriented interconnected pores of size in the range of 0.5-10μm. A close inspection of the wall performed with field-emission scanning-electron microscopy (FE–SEM) images obtained by using a HR FESEM AURIGA (Zeiss) reveals a different degree of surface roughness. The surface topography is a manifestation of the inner structure of the materials. Sample PV2 is characterized by a more roughness nanostructured surface compared to PV1 specimen. tAD-derived α values allows to discriminate the two different surfaces whereas the mean diffusivity parameter (MD) of conventional DMRI does not. The more roughness wall pores show a lower value of α [7]. Fig. 3 displays the proof of concept of phase-I IMAGO. tAD MRI images on an excised mouse spinal cord were performed using optimized protocols

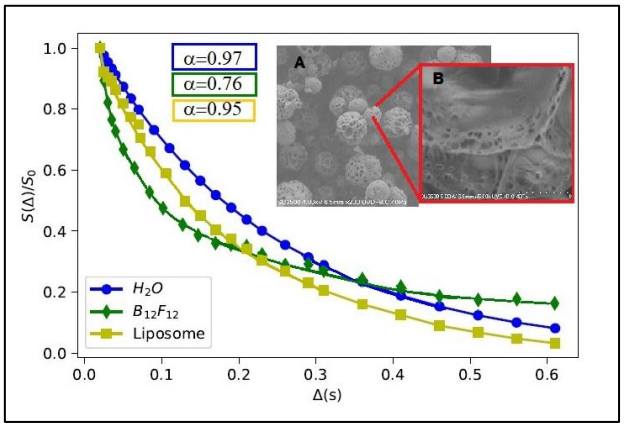

Fig. 1. tAD NMR-signal of three different diffusing probes (water, $B_{12}F_{12}^{--}$, liposomes) in Sephadex-G50. Only $B_{12}F_{12}^{--}$ shows a marked subdiffusion with $\alpha=0.76\pm0.03$ because its size fits well with the mesh size of Sephadex beads-surface of 3nm.

developed and tested by IMAGO. Results were validated with optical microscopy and compared to conventional DMRI.

A specific software for extraction of morphological and topologic parameters from 2D-optical images was developed in MATLAB. The script takes in input selected 2D-light microscopies, performs pre-processing (Wiener filter of width 0.2μm), segmentation, object recognition, applies selection rules, providing the desired quantitative measures. As expected DMRI well differentiates white- and gray-matter.

Conversely, α-maps discriminate white-matter regions characterized by different axons sizes (5 - 0.5μm) and spatial arrangement that are not discriminated using conventional MD values [8].

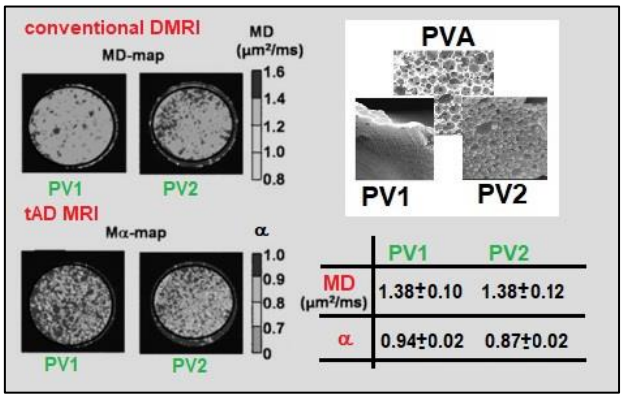

Fig. 2. Conventional DMRI and tAD maps of two PVA samples with different pore surface roughness. MD of DMRI does not discriminate the samples while mean α value is significantly lower in PV2 sample constituted of more roughness pore wall.

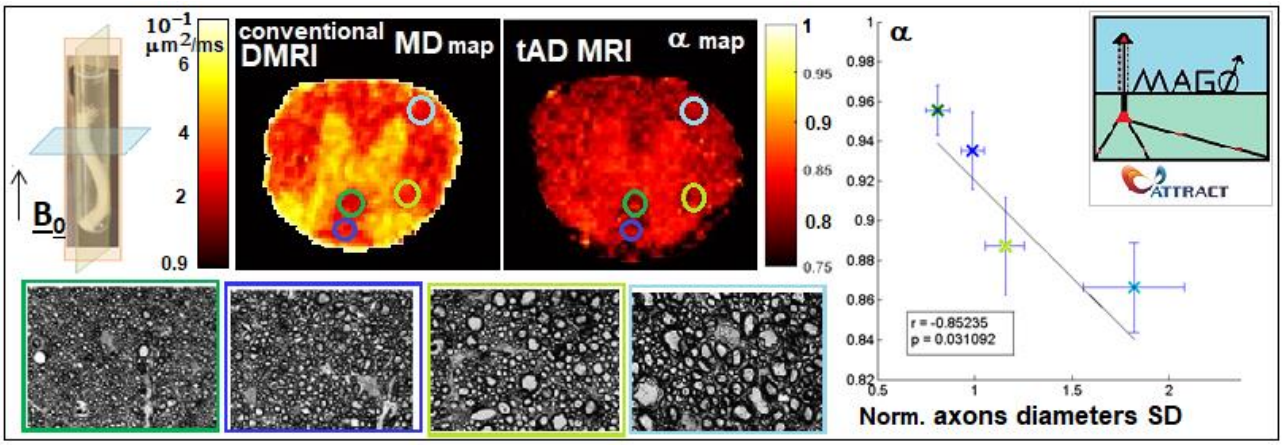

Fig. 3. Proof of concept of phase-I IMAGO. tAD MRI images of an excised mouse spinal cord were obtained using methodologies developed and tested by IMAGO. Results obtained at B_0=9.4T were validated with 2D-optical-microscopy histology and compared to conventional DMRI. α mean values (see values in graph) obtained in specific white-matter regions discriminate among different white-matter axons size distribution highlighted with different colours (green, blue, light green, light blue). No conventional DMRI parameter (such as MD, for example) is able to differentiate the different axons distribution displayed [8]. In the top right insert, the IMAGO logo.

5. FUTURE PROJECT VISION

IMAGO Attract phase-II has the ambitious objective to develop *in-vivo* histological MRI with a resolution down to the micron scale, exploiting the developments and results of tAD obtained in IMAGO Attract phase-I.

5.1. Technology Scaling

Two major critical issues need to be successfully addressed and resolved to scale our technology up to TRL5-7: A) Prove that the IMAGO protocol is scalable *in-vivo*, B) scaling in humans using clinical scanners.
A) In *in-vivo* DMRI, there are two main problems to be addressed: perfusion that overlaps the diffusive processes and respiration and involuntary movements of living systems that compromise the resolution obtained in vitro. The phase-II of the scale-up is a demonstration of the IMAGO protocol focused on the brain tissue of animal models *in-vivo* made with an MRI scanner dedicated to the animal studies. It serves to minimize the risk of capital investment and the time invested in the production of the IMAGO protocol for clinical scanners, further validating the process. In practice, in phase-II, we will consider *in-vivo* mouse brains of healthy animals and affected by MS. At this stage validation and integration with light microscopy able to investigate dynamic processes deep inside tissues is again necessary. Diffusing probe molecules will be more elaborate compared to those used in phase-I. They must be no-toxic for the animal and at the same time be fluorescent and contain 19-fluorine for NMR detection. All last generation technological and engineering developments will be used to compensate for the effect of the breath and involuntary animal movements. Any changes in the acquisition sequences and/or corrections in the functions to fit data will be introduced to consider the effect of perfusion.
B) Scale-up the technology from animal-models to human investigation on clinical MRI-scanners. Based on results obtained in step A) and on realistic assumptions concerted with the industrial and clinical team, the technology will be transferred using endogenous diffusing biological-water or commercially available molecules already approved for clinical use in humans. The prototype will concern the IMAGO protocol for MS early diagnosis thanks to IMAGO's subcellular sensitivity will be demonstrated.

5.2. Project Synergies and Outreach

The additional organizations needed for achieving TRL5-7 are: Politecnico di Milano (IT), where pharmaceutical-chemists developed dual 19-fluorine and fluorophores probes to be used *in-vivo* [9], and two top leading companies in the MRI field: Bruker (the preclinical group) and Siemens Italia (Siemens develops and markets clinical MRI scanners and accessories). Moreover, we include in the consortium the IRCCS Santa Lucia foundation (IT) dedicated to neurological research where a Siemens MRI scanner (that will be dedicated to the Attract phase-II) is located. The IMAGO Principal-Investigator has a longstanding collaboration with these organizations. They were informed about the development of phase-I IMAGO and have already suggested strategies about technological scaling to TRL5-7.
The relevant added value of phase-II IMAGO is the close interaction between scientists and experts of different

background who will debate and collaborate for paving the way to the generation of a new technology. IMAGO 's goal was very ambitious and therefore the commitment was immediately intense. This made it impossible to take care of the disclosure aspect with the same dedication. Therefore, we will include in the consortium a company dedicated to the communications activities and dissemination of the project, that carefully will consider intellectual property right and patents, and compatibly adopt an Open-Access policy. EU-founded projects led by SMEs will be identified to further develop and convert all IMAGO outcomes in fully available industrial technology and in clinical diagnostics field.

5.3. Technology application and demonstration cases

The evolution of MS, related to axonal damage, is well known. Therefore, we have chosen MS to demonstrate the properties of the new diagnostics offered by IMAGO based on subcellular resolution and on parameters quantifying new tissue properties. Through longitudinal studies carried out in subjects with different MS-stages, we will demonstrate the ability of IMAGO to perform an early-diagnosis compared to other imaging diagnostics. In a long-term vision, phase-II IMAGO opening up new possibilities in *Health* in terms of early diagnostic, therapeutic strategies and patients handling.
As the project developed and will develop new technological breakthroughs to pave the way for improving the intrinsic resolution of DMRI *in-vivo*, it has the concrete possibility of going on the market by offering different products, both in preclinical and in clinical MRI.

5.4. Technology commercialization

As already highlighted, going towards the final goal there is the concrete possibility of marketing different products with impact in the preclinical and clinical field. The interaction with Bruker and Siemens has already identified some of these. The next steps are: a) patents of diagnostic methodology and software; b) evaluate the real interest of Bruker and Siemens in purchasing this technology or we will contact potential investors interested in the development of the final products and their marketing.

5.5. Envisioned risks

In the envisaged ATTRACT Phase-II the IMAGO core risk is related to respiration and involuntary movements that can compromise the resolution obtained *in-vivo* compared to that in vitro. The mitigation strategy makes use of Machine-Learning algorithms that will be instructed about all the sources of disturbance, allowing cleaned from motion-artifacts images.

5.6. Liaison with Student Teams and Socio-Economic Study

The IMAGO Phase-II consortium, without gender prejudice, wishes to inspire young people to consider IMAGO-related science subjects at university level and beyond, promoting careers in this field and in related companies. To support the training of researchers of the Master-in-Science, we propose: 1) special mentoring programs; 2) special training and orientation programs 3) including them as active part of the scientific-economic debate.

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