Summary

Bio-NMR was an FP7 project of the CP&CSA type of the Capacities Program. It started September 1st 2010 and ended August 31st 2014. It consisted in Transnational Access (TA), Joint Research Activities (JRA) and Networking Activities (NA). The Bio-NMR project has:

- Provided the whole European biological and structural community access to top NMR instruments and expertise;
- Contributed to move the frontiers of, and remove limitations to, the applications of biological NMR;
- Spread good practices within the consortium and the entire community;
- Significantly increased the awareness of the potentialities of Biological NMR in the whole scientific and biomedical communities, a key aspect of close future sustainability.

The Bio-NMR project stems on a long tradition of European NMR infrastructures offering Transnational Access since 1994. During these two decades of service, the Bio-NMR infrastructures offered well above 10000 days of access to over than 500 projects. Around 900 scientists from 21 European countries visited one or more of the Bio-NMR Consortium infrastructures. Our activity has contributed significantly to the European biological NMR being at the forefront in the world as it is now.

In BioNMR, alone 11 infrastructures provided 5610 days of instrument time (vis-a-vis the stipulated 4222 over a period of 4 years. Users have accessed top state-of-the-art instruments consisting in one 1000 MHz (the only one available worldwide), 4 x 950 MHz, 6 x 900 MHz, 1 x 850 MHz, 6 x 800 MHz, 2 x 750 MHz, 6 x 700 MHz, 14 x 600 MHz, 8 x 500 MHz, 2 x 400 MHz, 3 x 850 MHz WB, 3 x 700 MHz WB, 3 x 600 MHz WB, 3 x 500 MHz WB, 5 x 400 MHz WB (one of them equipped for DNP), for a total of 67 NMR spectrometers, plus a relaxometer and 4 EPR instruments. The replacement cost of the above listed NMR instrumentation with the flanking facilities can be estimated in circa 200 M€, where the cost of a 900 MHz is about 4.5 M€, of a 950 about 9 M€, and of a 1000 MHz about 12 M€.

The total EC contribution to the Bio-NMR project was 9 M€, i.e. less than the cost of the 1000 MHz instrument alone. For 9 M€, European researchers enjoyed 1400 days/year of TA (240 projects from users from 27 different European and associated countries) for 4 years to, not only the 1000 MHz, but to the whole range of instruments listed above. For the same 9 M€, we performed JRA to keep our infrastructures at the forefront in experimental techniques. For the same 9 M€, we run a large and ambitious NA program to i) disseminate the NMR culture ii) attract new scientific communities and iii) liase with ESFRI infrastructures in BioMedical Sciences (in primis with the Integrated Structural Biology ESFRI infrastructure Instruct). Bio-NMR proposes itself as a model of how access to a large and diversified collection of instruments and expertise can be successfully provided at the European level.

Publications confirm that the goal of increasing quality and quantity of access has been met. Until October 2014, 300 peer reviewed publications specifically resulting from both JRA and TA activities and acknowledging Bio-NMR have appeared, with a mean Impact Factor of 6.056. Out of the total Bio-NMR publications, 167 are Open Access, a very high share that makes Bio-NMR listed among the FP7 "top 30 projects in publications" (font: OpenAIRE).

JRAs activities have advanced the technology enormously: from in cell NMR to fast methods and solid state NMR, from membrane proteins and fibrils to new methodologies. Among the latter, the methodological advancements to overcome the molecular size limit of NMR, which have allowed the study of high molecular weight biomolecules via NMR. For instance, the structural model of Hsp90 in complex with the Tau protein (*Karagöz et al.*, ***Cell 2014***, *156(5), 963–974*), showed the power of

Bio-NMR in the new fields of structural/cellular biology. In the last year of the project dissemination and implementation of the advancements in the various access-providing facilities was completed.

A survey carried out in the third year of the project finalized to evaluate the socio-economic impacts of Bio-NMR, highlighted a Bio-NMR community even bigger than the expectations. This was also reflected in a general growth of access request. Furthermore, it has become increasingly clear to all the Bio-NMR consortium partners that an assessment of the socio-economic impact of BioNMR should not be limited to its role in the European structural biology community, but it goes beyond, impacting several other fields of Science. To remain within the Biomedical Sciences, metabolomics and Drug Discovery communities have been identified as research fields with relevant sizes, in which NMR is having, an increasingly significant impact, which will be reflected on the European Health system and Pharmaceutical industries.

Bio-NMR context and objective

Determining the structural basis of biological components is key to understanding any biological process. In the last decades the potentialities of the Nuclear Magnetic Resonance (NMR) spectroscopy has improved dramatically and nowadays reveals essential information on the structure and dynamics of complex biomolecular systems, addressing the needs of many fundamental fields of research in the Life Sciences. Beside the great contribution in base research, applications to more and more concrete health issues turned to be available. The power of NMR lies in its capability to elucidate structure, dynamics, and interactions of biomolecules in solution, in immobilized states and in the cell, at the atomic level, and to provide information on the kinetics and thermodynamics of their molecular interactions at atomic resolution. NMR allows scientists to study biomolecules in solution, in immobilized states and even within living cells. Therefore, NMR is suited to provide answers to the increasingly difficult biomedical challenges of the future. The Bio-NMR project has endorsed NMR-based biological and biomedical research in Europe to further structural biology research in the European Union and beyond.

The objective of the NMR for Structural Biology project was to significantly contribute to answer the complex questions posed by research in the Life Sciences through a system of coordinated Biological NMR infrastructures providing access and expert guidance to biomedical researchers and scientists. Bio-NMR also aimed at contributing to demonstrate the potential of NMR technology applied in structural biology. The Bio-NMR project was carried out on the basis of three overarching activities:

- **Transnational Access (TA) activities:** To strengthen, increase and optimise the access to biological NMR for researchers in the European Research Area (ERA) through the coordination of a strong and comprehensive group of NMR Research Infrastructures. To provide geographically balanced and coordinated access to excellent biomedical research projects requiring top/unique NMR instrumentation and expertise, provided by major facilities in the field of biological NMR in Europe.

- **Joint Research Activities (JRA):** To address present bottlenecks and limitations in the capability of NMR to solve structures. JRAs will include studies aimed at improving sensitivity per unit time and per single experiment; developing non-invasive in-cell-NMR methodologies for structural systems biology; methods for structure determination by solid-state NMR; and NMR technology towards large macromolecular complexes or aggregates, both in solid and liquid states. To integrate, in a pro-active and timely manner, the results of such research activities into the portfolio of the infrastructures to improve quality and quantity of the access during the project.

- **Networking Activities (NA):** To spread good practices and standardization within the Bio-NMR users to continuously improve the quality and quantity of access and to increase the role of the Bio-

NMR project and its positive impact on the ERA of Life Sciences and, through it, on society. To raise public awareness about the Bio-NMR consortium as a major and important integrated European Research Infrastructure (RI). To liaise with public authorities, structural biologists, biologists at large, the health sector, industries and funding authorities, in collaboration with other European Structural Biology projects and RIs, in order to explore the long-term sustainability of the Bio-NMR European RI.

NMR spectroscopy has evolved from a "home-grown" technique towards a mature and powerful structural tool in a relatively short time through close interactions between academic and industrial research. The evolution of its impact in Life Sciences has occurred in parallel with the development of increasingly powerful NMR instruments. This is accompanied by a rise in costs of NMR investments from a few hundred thousand € to several million €. The estimated cost of next-generation instruments with a 1.2 GHz magnet is 15-20 M€. Several specialised NMR spectrometers, optimised to meet the complexities and particularities of life science research, are needed to tackle one complex problem. The multiplication and complexity of the know-how required for biological NMR poses a second major challenge. Given these structural, technical and economic challenges, Europe's NMR community has seen the growth of several large-scale NMR infrastructures where economies of scale have been optimised by competitively acquiring funds for several high-end instruments and specialised experts in one physical place. The Bio-NMR project stemmed on a long tradition of NMR infrastructures which started to provide coordinated NMR Transnational Access to the biomedical and other communities since under FP3 in 1994, and continuing through the years, being the FP6 EU-NMR and the EAST-NMR FP7 RI the two more recent preceding the Bio-NMR project. This brought to the build-up of a European Biological NMR community consisting of hundreds of research groups which are including an organised NMR RI users' group. Access has continuously and greatly improved side-by-side with the increase in the strength of the user community and this has contributed to the increased impact of European research in the life sciences.

The Bio-NMR project was specifically addressing Structural Biology. To strengthen and increase the visibility of Bio-NMR in this area of Science all networking activities were conceived and carried out in collaboration with the ESFRI Instruct - Integrated Structural Biology, a pan-European Research Infrastructure providing expertise and access to high quality instruments for structural cell biology researchers, and that was in its preparatory phase at the time of the Bio-NMR proposal preparation. One of the key technologies included in the ESFRI Instruct is NMR, since it is the only biophysical technique that can be used to detect and quantify weak molecular interactions in solution and simultaneously provide detailed structural information at atomic resolution.

The Bio-NMR project aimed at completing the structuring of the Biological NMR infrastructures, their user community and biological NMR researchers in Europe into a coherent research community prepared to tackle scientific and biomedical challenges of increasing complexity at the forefront of research worldwide. The structuring effect is achieved by the provision of transnational access, the solicitation and expansion of the pre-existing strong user group, the development of new research tools (in JRAs) towards increasing the quality and quantity of access and, importantly, the stimulation of a broad range of networking activities linking stakeholders to clearly define the needs of the ERA and to develop long term sustainability of NMR RIs.

The Bio-NMR aimed at overcoming the following current bottlenecks and limitations in NMR:

(i) NMR access: through the continuous integration of results from the four JRAs in order to improve the quality, rapidity, and quantity of data acquisition.

(ii) Solid-state NMR methodology: setting up a data collection methodology to contribute to obtain high quality data more rapidly and in a more standardised way.

(iii) Automated and standardized NMR structure calculation protocols: Bio-NMR strived to make structure determination more automated, through a strategic mechanism which exploited the close link between the Bio-NMR partners and the sustainable grid architecture created by the e-infrastructure eNMR first and the following WeNMR project, which provided grid computing for structure calculation and validation using both NMR and SAXS data.

Eleven NMR research infrastructures offered TA to European research groups**,** with the aim of providing more than 4000 days of instrument time over the project life time. A total of 67 NMR spectrometers, plus a relaxometer and 4 EPR instruments, were available to the users. These instruments included the highest field NMR world-wide: one 1000 MHz, two 950 MHz, five 900 MHz and three solid-state 850 MHz. The different specific expertises of the eleven research infrastructures part of the Bio-NMR consortium ensured the beneficial exploitation of the full potential in human resources of scientists in the broad European geographical distribution (Figure 1).

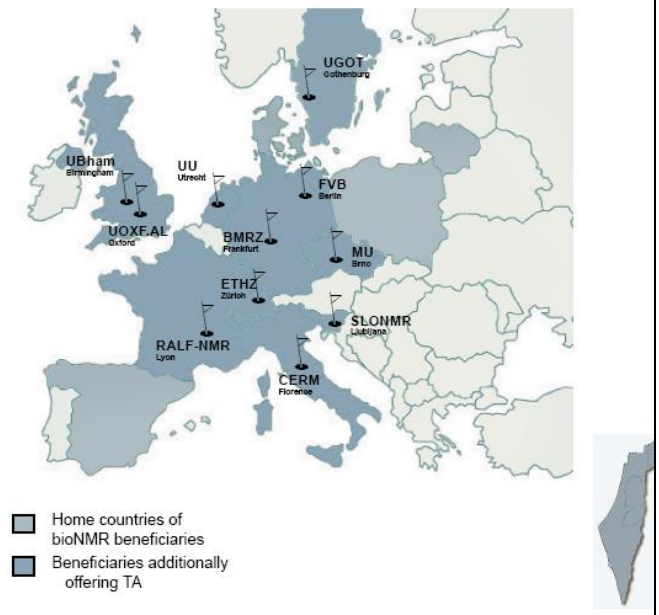

Figure 1. Geographical distribution of access providers and partners in Bio-NMR

The determination of the structure and dynamics of proteins was provided at all eleven sites. Furthermore, users could rely on the following specialities at the different TA sites:

CERM: Metalloproteins, their complexes, and their functional aspects in LS and SS-NMR. Paramagnetism-based structural restraints.

BMRZ: RNA and complexes, in-cell NMR, membrane proteins, DNP and computational aspects of Structural Biology in LS and SS-NMR

UU: Nucleic acids and protein-nucleic acid interactions, computational aspects of interactions, LS and SS-NMR methodology

RALF-NMR: SS-NMR, and automated, rapid, structure determination.

FVB: Protein structure determination by SS-NMR

ETHZ: SS-NMR of proteins and their aggregates

UOXF.AL: LS-NMR of macromolecular assemblies and membrane proteins

MU: RNA and complexes, transient interactions

SLONMR: DNA structure determination

UBham: Metabolomics, in-cell NMR

UGOT: Fast NMR methods

JRAs were driven by the need for increasing quality and quantity of TA. As mentioned, the efforts were directed to identifying the major current limitations (mainly addressing quality) and bottlenecks (mainly addressing quantity) of Biological NMR, that were still encountered even at the cutting-edge research infrastructures that constituted the Bio-NMR access-providing team. To this goal, further partners were selected on the basis of their scientific experience in overcoming both bottlenecks and limitations and on their excellent research records in the technology development area. Four topics were chosen met these criteria and were consistent with the total budgetary restraints for JRA activities (see below). All topics were identified as reachable within the first three years of the project, and results implemented at one or more of the TA-providing infrastructures during the fourth year of the project, thus contributing to increase the quality and quantity of access:

- High-sensitivity DNP and fast NMR in solids and solution
- In cell-NMR towards structural systems biology

- Improving biomolecular structure determination by solid-state NMR
- Improving biomolecular structure determination of large, functional systems

Networking activities within the Bio-NMR project were crucial for its success. Planned activities involved several "circles" of stakeholders (Figure 2).

The inner circle is represented by the consortium itself, its users, and other scientists developing NMR methodologies. Tight networking among the Bio-NMR consortium partners was considered crucial to allow the optimisation of exploitation of the synergies between TA and JRA. Networking with European researchers active in developing NMR techniques for Life Sciences who, together with the consortium partners, constitute ERA's scientific excellence in the field were identified as key activities, as well as the networking of Extra-European NMR researchers, in order to increase the visibility of the consortium outside Europe and to keep abreast of any new development.

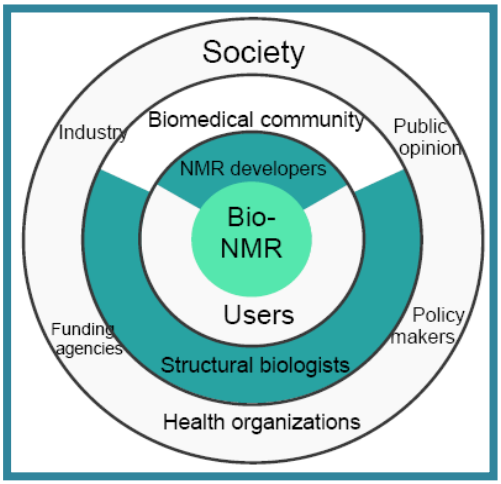

Figure 2. The three circles of Bio-NMR stakeholders

The intermediate circle includes the biomedical community and the structural biologists and Bio-NMR partners presented the potentialities and the available services to this community at their meetings, as past experience suggested it was an extremely effective activity. Particularly important the activities conceived for the reinforcement and the establishment of new interactions with ESFRI BMS infrastructures (including Instruct) for increasing and strengthening the role of NMR within the European Research Area.

The outer circle includes health communities, industries, public authorities and society as a whole. European taxpayers and decision makers deserve justification for investments made to RIs. Therefore, Bio-NMR had the double task of raising awareness within the general public of the importance of the research in biological NMR and of the advantages and economies of scale that derive from organized, networked RIs. Particularly, Bio-NMR addressed the impact that research in biological NMR has on health. Structural Biology addresses major health challenges, such as cardiovascular diseases, cancer, and neurological diseases. Networking activities had also the goal of addressing the issue of the sustainability of the RIs beyond the duration of the Bio-NMR project and to individuate the possible evolution of Bio-NMR into a more formal organization.

A description of the main S&T results

The research activities carried out through the Bio-NMR project have a tremendous impact on the scientific community, demonstrated by the 300 publications directly related to Bio-NMR project activities, with a mean Impact Factor exceeding 6. Out of these Bio-NMR publications, over 50% are Open Access, a very high share that makes Bio-NMR listed among the FP7 "top 30 projects in publications". The impact is also witnessed by the many well-received conference talks and posters.

During the last project period, results of the Joint Research Activities were disseminated and implemented at the Infrastructures offering Transnational Access. Thus, European NMR researchers directly benefitted from the improved NMR methodology.

The main achievements of each of the four JRAs are summarized below.

JRA1: High-sensitivity DNP and fast NMR in solids and solution

The purpose of this JRA was to improve NMR sensitivity. Activities exploited emerging methods for achieving nuclear hyperpolarization within structural and cell biology contexts, and furthering new fast and ultra-fast methods of NMR data acquisition in the liquid and solid states. In accordance with the objectives of this activity, we have been able:

- To expand the capabilities of high-resolution ssNMR using microwaves generated by gyrotrons for signal enhancement.
- To explore the potential of liquid-state DNP approaches in contemporary structural biology NMR research and to translate it into technologies for RI users.
- To enhance NMR's sensitivity by developing new fast acquisition schemes for liquid-state multidimensional Bio-NMR spectra.
- To develop a new suite of experiments and of data processing algorithms, capable of bypassing Nyquist stringent sampling criteria and developing the most efficient way of very rapidly collecting and efficiently processing hyperdimensional (~4-6D) solution NMR spectra of high quality and structural biology impact.

The value of DNP for the enhancement of NMR spectra of solid protein preparations was explored. Apart from signal enhancements, detrimental effects such as line broadening were observed, while low temperatures reduced side chain mobility. Approaches were tested how to obtain well-resolved spectra of proteins and decent improvements of signal–to-noise at the same time. For sample preparation a method was found that yielded excellent spectra. There is quite a bit of enhancement still observed when deuterated proteins are investigated, even at temperatures up to 180 K. Two-dimensional spectra recorded at this temperature showed much better resolution than at 100 K. This investigation represents a starting point for further studies on high-temperature DNP.

The use of hyperpolarized water was explored to enhance the sensitivity of nuclei in biomolecules using the rapid exchange that amide groups undergo by exchanging their protons with the hyperpolarized species coming from the water, upon dissolution within the NMR spectrometer tube. A method was developed for preparing water solutions with greatly enhanced ^{1}H NMR signal relative to pure water. A hyperpolarized water sample with a combination of organic and aqueous solvents and underivatized TEMPO that partitions into the organic phase were used to provide concentrated hyperpolarized water with relatively long T_1. Dilution of H_2O with D_2O improves the enhancement and its lifetime. Also glycerol increased the quantity of hyperpolarized exchangeable protons better than DMSO. Exchangeable amide and amine protons and heteronuclei directly bound to exchangeable protons are easily polarized by injection of hyperpolarized water. Also, a single pulse ^{15}N NMR spectrum of Alanine's amine after dissolving it with hyperpolarized water yielded a polarization enhancement of $\geq$250x over its single-pulse thermal counterpart. For exchangeable protons with slow exchange rates, polarization could be transferred from protons to heteronuclei, and back to protons for final observation. Further developments in this field include protocols and techniques for a shuttle DNP spectrometer. This allows data averaging by shuttling a solution NMR sample back and forth between a low field and a high field position for polarization and detection, respectively.

Perspectives of parahydrogen-induced hyperpolarization of solutions using the SABRE ("signal amplification by reversible exchange") technique were also explored and seem promising for

molecules that interact with the respective catalyst. Work to convert this into a technique for repetitive experiments is described. Using this approach, polarizations up to 1500 could be observed.

For fast acquisition schemes, the BEST-TROSY pulse program was developed and its benefits were shown for a number of H-N-C correlation experiments required for sequential resonance assignment and spin-coupling measurements in proteins and nucleic acids. Other ultrafast 2D NMR experiments were developed that deal with sensitivity limitations by a new compressed sensing algorithm or by monitoring multiple spectral regions of interest simultaneously. Also, a new approach was demonstrated to achieve optimal sampling in terms of resolution and bandwidth, and it was shown that Fourier Transformation can be used for processing of sparsely sampled multidimensional data. A set of new 4-6D pulse sequences for the assignment of backbone and side-chain resonances of particularly Intrinsically Disordered Proteins was developed and applied. Progress in processing of non-uniformly sampled NMR data was achieved as well as a method for reconstructing high-resolution NMR spectra from incomplete, non-uniformly sampled (NUS) data.

A suite of macros was written enabling the acquisition of basic ultrafast 2D NMR spectra (TOCSY, HMQC, HSQC, sofastHMQC, BEST HSQC) on both Agilent and Bruker platforms to automate their setup and acquisition. These macros enable a general user to set these experiments up for organic, biomolecular and biologically-oriented (*in vivo*) efforts. At this time, their most general implementation consists of a stand-alone Matlab package.

JRA2: In cell-NMR towards structural systems biology

The purpose of this JRA was to exploit the non-invasive character of NMR spectroscopy, which enables the investigation of structure, function and dynamics of proteins and other biological macromolecules in intact biological entities such as living cells. In accordance with the objectives of this activity, we have been able:

- To establish different cellular systems for in-cell NMR spectroscopy such as mammalian cells and plant cells.
- To enhance the sensitivity of in-cell NMR experiments.
- To establish in-cell NMR protocols for the investigation of different biological macromolecules such as nucleic acids and intrinsically unstructured proteins.
- To develop NMR techniques for in-cell NMR.
- To develop protocols for solid state in-cell NMR.

To study the behavior of proteins and other biological macromolecules in their natural environment a larger variety of cell types is necessary. Different delivery procedures of isotope-labeled proteins into eukaryotic cell types for the efficient creation of in-cell NMR samples and high-resolution in-cell NMR measurements have been described in detail. One of these protocols additionally describes the use of time-resolved NMR spectroscopy to monitor post-translational protein phosphorylation reactions by endogenous cellular kinases in mammalian cell extracts. Another protocol describes a ssNMR study on an overexpressed membrane protein in *E. coli* bacteria. The protocols entail detailed, step-by-step descriptions of how to prepare in-cell NMR samples, to check their quality and to ensure their suitability for in-cell NMR measurements. They further outline specific NMR experiments to perform successful in-cell NMR experiments in different eukaryotic cell types.

In some cases, full three-dimensional structure determination inside living cells might be necessary to study the *in vivo* state of the macromolecules of interest. For this, NMR spectra have to be collected and structural parameters obtained while the cells are alive. Then, the cellular life-span as well as the life-span of the macromolecule of interest in the cellular environment becomes a limiting

factor. It was demonstrated that a full three-dimensional structure determination of a protein in living *E. coli* cells can be performed. Since the environment inside an NMR tube is not stable and nutrients and oxygen are consumed, the time of the experiments was shortened as much as possible. While the structure calculation itself follows standard protocols, NMR experiments were adjusted to ensure that cells are alive during the entire time of the experiments.

The intrinsic sensitivity limit of NMR is overcome by developing a protocol which enables high resolution solution NMR to be performed on living human cultured cells, in which the protein of interest is overexpressed and isotopically labelled. For this, cells were transiently transfected with the DNA vector in high copy number per cell, and the culture medium exchanged with a labelled medium at the time of transfection. In a relatively short time high levels of isotopically labelled protein were reached, providing good S/N ratio in a short time, so that high cell viability is maintained.

G-quadruplex topologies of telomeric repeat sequences from vertebrates were investigated in the presence of molecular crowding (MC) mimetics, namely polyethylene glycol 200 (PEG), Ficoll 70 as well as *Xenopus laevis* egg extract. While the behavior of the telomeric repeat in *X. laevis* egg extract or in Ficoll resembles results obtained under dilute conditions, PEG promotes the formation of high-order parallel topologies. The data suggest that PEG should not be used as a MC mimetic.

Heteronuclear ^{13}C direct detection 2D NMR experiments have been successfully performed on the ^{13}C,^{15}N enriched proteins yeast Atx1, yeast Cox17, and human □-synuclein in *E. coli* cells. Unfolded proteins or protein fragments provide a well-resolved carbonyl detected experiments whereas folded proteins or structured motifs did not give detectable signal intensity. The results have been compared with information obtained through ^{1}H-^{15}N correlation 2D NMR experiments and interpreted on the basis of effective correlation times.

JRA3: Improving biomolecular structure determination by solid-state NMR

The purpose of this JRA was to address the development of experiments needed to make structure determination by ssNMR routine within structural biology. In accordance with the objectives of this activity, we have been able:

- To extend current solid-state structure determination methods to larger systems, involving the design of multi-dimensional sequential resonance assignment and distance measurement experiments and apply them to non-crystalline solids such as membrane proteins.
- To develop optimized protocols and techniques for obtaining multidimensional ssNMR spectra involving proton chemical shifts from fully protonated or partially deuterated proteins at increased resolution and sensitivity, taking also into account new opportunities like ultra-fast MAS.
- To propose robust experimental and analytical procedures for the study of fast and slow motions in the solid state.
- To obtain more reliable distance measurements, and particular long distance measurements that are critical to determining structure by minimising dipolar truncation and relayed polarization transfer through the design of new ssNMR pulse sequences and labelling schemes.
- To explore the potential of paramagnetic effects, and in particular their orientational aspects as a source of long-distance constraints in the structural investigation of metalloproteins.

Experimental methods have been developed to enable the routine study of proteins with more than 100 amino acids. Multidimensional correlation experiments have been extended to make assignment of proteins with 200-400 residues feasible. Transfer schemes were optimized using recent advances in analytical and numerical experiment design principles. Proton chemical shifts were exploited in

experiments with three independent dimensions (^{1}H, ^{13}C, ^{15}N) which turn out to be of great advantage for robust structure determination procedures. Recent progress has been made by applying homonuclear dipolar decoupling and partial deuteration.

Fractional deuteration was investigated as a means of spectral simplification and to identify structural constraints. A suitable balance of deuteration level at the exchangeable sites was tested in combination with various spinning regimes. Additional labeling schemes designed to facilitate assignments were also investigated. Investigations were designed to test labeling schemes that enable proton detected triple resonance spectroscopy for spectral assignment of biologically relevant ssNMR samples.

Ultra-fast MAS enables an increase in resolution by allowing longer acquisition times, and in sensitivity, by shortening the interscan delays, without heating and sample deterioration. At present the fastest MAS probes commercially available reach 70 kHz for rotors with an external diameter of 1.3 mm. A probe is being developed for MAS frequencies up to 100 kHz, which allows broadband low-power rf methods that are especially important for biological samples with high salt. For when MAS frequencies exceed the amplitude of spin interactions and the applied Rabi nutation frequencies, novel NMR methods, for instance with more efficient polarization transfer or heteronuclear decoupling, have been developed and applied. A simple and reversible way to expand a commercial 1.3 mm HCN MAS probe with a ^{2}H channel with sufficient field strength for *J*-decoupling of deuterium was presented. A simplified model of the DREAM scheme was developed to semi-quantitatively describe polarization-transfer patterns for relevant spin systems to provide practical guidelines for optimized Cα/Cβ transfer. In order to increase the resolution, a variety of *J*-decoupling techniques have been proposed. A new experiment was introduced which simultaneously improves the performance of through-bond transfers and accomplishes virtual homonuclear *J*-decoupling. An INADEQUATE scheme was presented where double quantum and zero quantum coherences are combined to improve the sensitivity and resolution of 2D J-based ^{13}C-^{13}C correlations under 60 kHz MAS.

The use of high magnetic fields, ultra-fast MAS, and 100% H^N re-protonation in perdeuterated samples allow complete assignment and structure determination of medium-sized proteins using resolved ^{1}H resonances. The ^{1}H dipolar network can be reduced employing the RAP (Reduced Adjoining Protonation) labeling scheme, to yield random protonation at non-exchangeable sites. Experiments for assignment of the backbone ^{1}H, ^{15}N, ^{13}CA and ^{13}CO resonances under ultra-fast MAS were developed. The fold of ZnII-SOD was determined using a 3D (H)NHHRFDR experiment and automated assignment procedures. Experiments to determine heteronuclear relaxation rates (^{15}N-R_1, ^{15}N-R_{1rho}, ^{13}C-R_1) were also designed to achieve dynamic information as well as distance constraints from paramagnetic relaxation rate enhancements.

NMR is used extensively to investigate protein dynamics. To date, little work has been done to study dynamics of membrane proteins although ssNMR is ideally suited to study these in a close to native environment. The lack of overall tumbling makes timescales accessible that cannot be investigated by solution NMR. Site resolved information could be obtained by combining the dynamic experiments with those encoding the chemical shifts to yield pseudo 3D spectra. Methods were set up to characterize internal dynamics and residual global or domain motions in microcrystalline samples, membrane proteins and biomolecular complexes. A further step in the characterization of protein motions in solids was to complement ^{15}N relaxation data with measurements such as backbone dipolar or CSA order parameters and side-chain methyl dynamics complementing spin relaxation and providing information on slow, functionally important motions. Small sample volumes of highly deuterated samples in combination with ^{1}H back-exchange were used, together

with very high MAS spin rates, which is ideally suited for crystalline protein preparations. For reorientation processes without changes in the isotropic chemical shift, CODEX spectroscopy is promising, but it is important to use conditions that avoid spin diffusion. In order to interpret dynamics data, first steps toward a motional model were taken.\

PCSs can be useful for protein structure determination because they provide information on the relative position of the observed nuclei in a common frame defined by the paramagnetic metal ion, and can also determine the structure of the crystal. A protocol for their use and a program for performing the calculations needed to determine the protein structure has been developed. The routine was implemented for the use of PCSs in the program CYANA.

JRA4: Improving biomolecular structure determination of large, functional systems

The purpose of this JRA was to improve structure calculation strategies and to develop/improve methods, for generating structures of biomolecular complexes or large oligomeric assemblies. This addresses both the quality and quantity of access. In accordance with the objectives of this activity, we have been able:

- To improve the precision and accuracy of solution and ssNMR structure calculations of large systems and complexes, also by assessing the value of chemical shifts and paramagnetic restraints for NMR structure determination and validation.
- To improve the capabilities for achieving sequence-specific assignments in large systems, also by methods of automation.
- To widen the methods portfolio for determining biomolecular interactions in large assemblies and determining relative orientations of components.
- To explore and enhance the potential of NMR in an integrated structural biology context.

Despite advances in modeling of protein-protein complexes by docking, additional information is often required to identify best solutions. NMR data deliver restraints that can be used at sampling and/or scoring stages, like in the HADDOCK approach. Quantitative use of chemical shifts (CS) in the scoring stage can help to resolve ambiguities. It has been implemented in a new docking protocol, CS-HADDOCK. To enhance the structure determination process of macromolecular assemblies by NMR, long-range pseudocontact shift (PCS) restraints were implemented into the data-driven protein docking package HADDOCK. The efficiency of the method was demonstrated on a synthetic, yet realistic case.

An estimated 60% of genes in every genome code for homo-oligomeric proteins. The symmetry ambiguity to distinguish intra- and intermolecular correlations is a major challenge in NMR spectroscopic structure determination of oligomeric symmetric proteins. A general method has been developed that is valid for non-crystallographic and crystallographic symmetries and implemented in CNS. The method relies on an optimization scheme using experimental distance restraints, starting from a random conformation. The method has been validated and has been distributed in the ARIA package.

A program (MaxOcc) was produced to analyze the conformational variability of flexible multidomain proteins composed of rigid domains by integrating data from different sources. Pseudocontact shifts (PCSs) and residual dipolar couplings (RDCs) arising from the presence of a paramagnetic metal ion rigidly attached to the C-terminal domain of the protein were considered together with PCSs and RDCs arising in the presence of metal ions placed in the N-terminal domain of the protein, and with SAXS data. The MaxOcc web portal allows users to compute maximum

occurrence values using any combination of restraints given the 3D structure of the two domains as input. MaxOcc is embedded within the NMR grid services of the WeNMR project.

A series of multidimensional NMR experiments that exploit ^{13}C direct detection, high dimensionality and acquisition of sparsely sampled data has been introduced to investigate intrinsically disordered proteins of large size and complexity. In addition, a set of amino-acid selective NMR experiments based on ^{13}C direct detection (aasNMR) have been developed as an aid for the resonance assignment of the intrinsically disordered proteins. Further, a novel NMR experiment for the determination of $^{3}J_{(H\alpha,H\beta)}$-coupling constants has been designed.

Optimized protocols and techniques for obtaining automatic resonance assignment of protein spectra and determining protein structures were developed in the context of ssNMR, taking into account both ^{13}C-based 2D and 3D correlation spectra under moderate MAS, and new opportunities such as ^{1}H detection and ultra-fast MAS. These methods to achieve rapid sequential assignments and structure calculations open up the possibility of routinely characterizing solid samples in by NMR, which induces a marked shift for the structural biology of large, poorly soluble and non-crystalline systems.

While keeping large soluble proteins in a sedimented phase, through MAS well-resolved spectra may be obtained, applying either solid-state or solution-like measurement strategies. Pulse sequences for assignments applied to sedimented proteins provide high-quality ss-like NMR spectra suitable for structural investigation. The techniques developed may be applied to large soluble systems and include the application of MAS to concentrated solutions of proteins. The approach has been applied to ferredoxin and to soluble beta-amyloid assemblies obtained by ultracentrifugation of free monomers in solution.

Besides keeping the research at the forefront of scientific developments, the outcomes of these JRAs were aimed at improving quality and quantity of Transnational Access. Bio-NMR partners providing transnational access were committed to give a total of 4222 TA days during the 4 years of the project. The goal has been plenty reached, since 5610 access days have been provided.
Access provision has supported 357 users from 27 different European countries for a total of 240 projects. These projects, addressing various aspects of Structural Biology, spanned from protein-structure determination to protein-protein and protein-nucleic acids interaction studies; from membrane proteins characterization to cellular chemical biology, etc., providing a tremendous contribution for the understanding of chemistry of life. The outcomes of these researches have important fall-outs in all areas of life sciences, with particular impact in biomedical sciences. Results already appeared in peer-reviewed journals, some of them in high impact journal. For example the analysis carried out in the frame of the project “HMGB1 binds to CXCL12 and increases its cellular activity: a structural investigation” (Bio-NMR 00066) contributed to the characterization of the heterocomplex between two key protein that promotes the recruitment of inflammatory cells to damaged tissues (Schiraldi et al. JEM 209 (3): 551, 2012). Another brilliant example is the work performed under the project “Structural studies of the Sup35 prion”(Bio-NMR 00003) that gives a molecular-level explanation for the different conformation and infectivity of the Sup35pNM and Sup35p prion proteins. These results stress the paramount importance of solid state NMR applied to such large molecular systems which allows a molecular-level structural characterization of the aggregates employed in functional studies (Luckgei et al. Angew. Chem. Int. Ed., 52: 12741, 2013). The integration of different techniques (ITC, SPR, Absorption Spectroscopy, *P. pastoris* expression system and NMR) has been at the basis of the “Structure determination of heme-binding CFEM proteins” (Bio-NMR 00203). The project, initially approved by Instruct and supported for the NMR analysis by Bio-NMR, concerns Candida albicans, a commensal fungus of human mucosal surfaces

that is a common cause of superficial infections. In particular the researchers provided a paradigm for how receptors embedded in the cell wall matrix can mediate nutrient (heme) uptake across the fungal cell envelope (Kuznets et al., PLOS Pathogens, 10(10):e1004407, 2014).

Bio-NMR potential impact

The whole Networking activities plan was aimed at maximizing the impact – and the perceived impact – of the Bio-NMR project on Biological NMR researchers, on the Biomedical community in general, and on Society as a whole. It was therefore very ambitious, as a result of the necessity to networking at three different levels.

The "inner circle" of activities addressed Bio-NMR partners, Bio-NMR users and NMR researchers in Europe. Links with the partners have been strengthened and the biological NMR community, which includes the partners and the users, further structured. Among the various activities that took place in the project life time, it is worth to be mentioned the organization of yearly internal meetings with local operators (LOM) of the 11 facilities providing access. These LOMs have been precious opportunities to train, discuss and define common standards and protocols.

The interaction among the consortium's local operators was paralleled by the dissemination of knowledge to the TA user community. This was possible thanks to the fact that the users were performing experimental set ups together with the local operators, thus directly benefiting of hands-on experience. Moreover, the synergy with the latest improvements made through JRA which were installed at the TA Infrastructures added to the value for the scientific community.

The Annual User Meetings were organized with a format aiming at maximizing the interactions of the consortium with its growing user group: through user lectures mixed with talks by invited key-note speakers and poster sessions to make the scientific sessions as vivid as possible; through the lively discussions at the General Assemblies to which users were invited to present ideas for the improvement of the service.

Outreach activities have been carried out by all partners, aiming both at the expansion of the European user group to the larger Structural Biology and biological communities and towards extra European NMR RIs. Among the others, particularly successful has been the assignment of six awards to excellent NMR students working outside Europe for spending fully covered visits at one of the Bio-NMR Infrastructures.

Other initiatives included the organization of training activities for RI staff on specific techniques, training courses for new users, an on-line consultancy service, scientific knowledge transfer within Bio-NMR and to users through visits at their labs, twinning and networking of young researchers.

The "intermediate circle" addressed key stakeholders belonging to the biomedical community and the structural biologists. Aiming at strengthening the ERA, and in collaboration with Instruct, these activities focused on promoting structural and cellular biology in the broad biomedical ERA, in creating an integrated Structural Biology network and in expanding the broader biomedical communities.

Several different kind of events were organized in order to reinforce the links with the other Structural Biology platforms, including the virtual community, and to promote the integration and correlation of NMR with other Structural Biology techniques: i) two "brainstorming" meetings which resulted in two high impact reviews; ii) three meetings on how NMR spectroscopy can be used in combination with other biophysical methods; iii) Two joint meetings with computational biology experts.

Aiming at the expansion of the user group to the larger Structural Biology and biological communities, four meetings "NMR in Biology" were organized as satellites at annual FEBS meetings (these meetings feature an average attendance of several thousands researchers). These meetings played an important role in outreaching outside our community and in disseminating the NMR potentialities to a non specialized audience. The event is becoming one of the key symposia of the main program of the FEBS annual meetings. For this reason, even though only 3 events were foreseen to be organized during the project life-time, we participated also to the 2014 edition and we hope that our community will be able to participate also in the future editions.

Networking activities also addressed other Structural Biology platforms, establishing links with several ESFRI BMS Infrastructures and organizing three foresight meetings.

Also thanks to the networking activities carried out in the first part of the Bio-NMR project, the importance of biological NMR in Structural Biology has become unquestionable. A user survey performed as a starting point for an analysis of the Bio-NMR impact confirmed that NMR plays an essential role in many areas of modern Life Sciences. NMR spectroscopy can grow and adapt as new challenges appear and the Bio-NMR consortium partners could give a significant contribution to other European frontiers research fields in the Life Science besides Structural Biology, for instance "drug discovery" and metabolomics. Consequently, raising the awareness of possible biological NMR support to biomedical- and health-related research turned out to be a key action.

In this frame, the organization of the second meeting with representatives of the BMS RIs on "Strengthening links of Bio-NMR with other BMS Infrastructures" was very fruitful. At this meeting, which was conceived as a round table, took part the representatives of most BMS Infrastructures (Instruct, ELIXIR, INFRAFRONTIERS, EATRIS, ECRIN, BBMRI, EuroBioImaging, EU-OPENSCREEN, BioMedBridges and ISBE). Being the Bio-NMR community known primarily for the biological NMR contribution to structural biology, the meeting was also an important occasion to bring to the attention of the other BMS that biological NMR can address the needs also of many other fundamental fields of research in the Life Sciences, and on how the user community of each BMS RI can exploit them.

Networking in the "outer circle" included activities addressing health communities, industries, public authorities and society as a whole, with the double task of raising awareness within the general public of the importance of the research in biological NMR and of the advantages and economies of scale that derive from organized, networked RIs.

For what concerns the general public, during the project life time several information campaigns for the European general public were organized, three of them specifically addressing New Member States in order to promote NMR-based research in those countries. For this purpose, advertising material (posters, leaflets, videos, etc) was specifically realized. With the same goal of dissemination we organized press releases and presented the Bio-NMR project with booths at major conferences. Strictly related to these dissemination activities the participation of the Bio-NMR consortium to the 2nd International Conference on Research Infrastructures - ICRI 2014 (Athens, 2-4 April 2014). This event has been an important occasion to demonstrate that the BioNMR community it is not just a temporary consortium, but a reality of the European scenario of research infrastructure.

The outcomes of the events which were organized to specifically address health organization representatives or industry of pharmaceutical and biotech industry representatives have been fundamental for focusing on the perspectives of our biological NMR community. Among these, of particular relevance the two events organized in the last year ("NMR for Health in Europe" addressing health representatives and "Bio-NMR Industry meeting"), which have been precious for the final definition of the "collaborative position papers with stakeholders" document. Moreover,

they provided a very positive feedback and support to the actual idea of maintaining the bioNMR community with a formal organization after the end of the Bio-NMR project.

As mentioned, a total of 300 peer reviewed publications resulting from both JRA and TA activities and acknowledging Bio-NMR have been published at the time of the writing of this document, with a mean Impact Factor of 6.056. At least 20 further publications could be added to this list, since they are strictly related to the project even though not acknowledging Bio-NMR (and the authors indicated that this was an omission). Such figures are undoubtedly outstanding, taking into account that Bio-NMR has seen a consistent increase in the number of publications with respect to the previous projects Eu-NMR and East-NMR (see Figure 4, source: Web of Science).

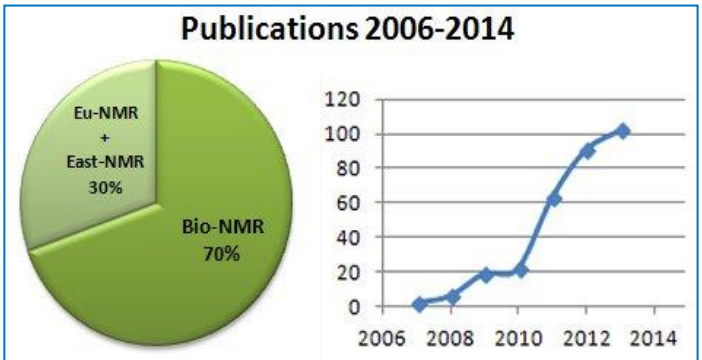

Figure 3. Increase of publication linked to Bio-NMR community

Out of the total Bio-NMR publications, 167 are Open Access, a very high share that makes Bio-NMR listed among the FP7 "top 30 projects in publications" (OpenAIRE).

Noteworthy, there is a considerable lapse of time between the experimental phase and data publication. Several articles resulting from Transnational Access are in preparation, other already submitted, so we do expect that several tens of publication will result from the Bio-NMR project (articles acknowledging Eu-NMR, ended over 4 years ago, have been published also in the last few months!).

A rough (under)estimate indicate that besides the researchers co-authoring the abovementioned articles (ca 1000), the scientific outcome of Bio-NMR has, up to today, impacted at least further 4500 additional researcher (counted as the authors of articles citing Bio-NMR publications, excluding self-citations).

The European biological NMR community is a large, well-structured community, with the Bio-NMR consortium as the most organized entity, and with its own capacity to impact on Science. The mere replacement value of only the NMR instrumentation of the Bio-NMR consortium, estimated in roughly 200 M€, indicates that the structured biological NMR community in Europe is able to attract and mobilize resources that exceed by almost two orders of magnitude the EC contribution to Bio-NMR. However, EC recognition has been a strong element in support of the policy of national/regional governments towards the development of NMR infrastructures. The Bio-NMR community, being conscious of its power in many fields of biological sciences, in some of which being the unique technology, such as in-cell NMR or fibrils/membrane proteins structural determination, looks ahead with the trust that also the contiguous fields of research will increasingly appreciate and exploit the potency of the biological NMR approach.

The analysis of the potential impact of this biological NMR community structured through the Bio-NMR project and its predecessors Eu-NMR and East-NMR has been defined thanks to the activities carried out in the frame of networking with the various stakeholders. The strategy plan oriented to the self sustainability of the NMR research infrastructure that we developed is obviously independent

of the research policy of the EC for the next years, but undoubtedly the whole European research strategy and therefore also the research area of perspective BioNMR users is highly influenced by it.

The outcome of the numerous interactions with different categories of stakeholders (industry, BMS ESFRI RIs, Health Organizations, as well as EC and National representatives) can be summarized in the following three points:
1) The European biological NMR community is recognized to be a large, well-structured community, and with its own capacity to impact science within and beyond Structural Biology.
2) In order to ensure Europe's forefront position in biological NMR research, an appropriately co-ordinated European infrastructure is required. Bio-NMR proposes itself as a model of how access to technological platforms such as NMR can be successfully provided at the European level. For this purpose, the partners of the Bio-NMR project are establishing EuroBioNMR, a coordinated pan-European infrastructure for biological NMR. Further details are given in the Sustainability Plan document, a deliverable of the Bio-NMR project.

It is widely accepted, and underlined continuously in EC communications and documents, that the creation of pan-European Infrastructures, either single sited or distributed, is one of the means for the optimization of resources, since it maximizes the value for money of investments. Maintaining, and possibly further developing, cross border cooperation in research, is key for strengthening the ERA. Bio-NMR has contributed to further structuring the existing biological NMR community and to provide transnational access in an optimized way. The improvement in quality and quantity of access obtained during the four years of the project demonstrated that the model adopted is successful.

EuroBioNMR proposes to maintain the structure of the Bio-NMR for centralized access, with the aim of not restricting the users to national access. On the basis of a criterion of reciprocity, each NMR infrastructure will make available a certain percentage of their national access for the EuroBioNMR centralized access system, ensuring the users for continuing to have the opportunity to choose the more European appropriate facility for their specific research. Of course, no travel and accommodation will be reimbursed in the absence of ad-hoc funding.

The first available 1200 MHz spectrometers are expected to be delivered in 2016. The cost of such an instrument is expected to be in the order of 14 MEUR (plus VAT); it is thus unlikely that many single laboratories will be able to acquire it. Up to today, out of the 9 infrastructures that already committed with Bruker for the acquisition of the forthcoming 1.2 GHz NMR spectrometer, 7 are European and most of these are Bio-NMR partners.
During the years, it has been highlighted that for the biomedical research the highest available fields are a must for NMR research. In this context, the 1.2 GHz NMR spectrometer will predominantly serve the needs of biological NMR applications, where in terms of resolution and sensitivity most benefits will be realized, allowing to add new elements for the comprehension of the complex mechanisms of life.
In this scenario, keeping alive the Bio-NMR scheme will turn even more important. Thanks to the experience maturated through Bio-NMR and previous projects, EuroBioNMR will be a warranty for high quality research for the instrument optimization. EuroBioNMR will thus represent an added value for Europe since it will be key in supporting the provision of access to this forthcoming instrumentation to a number of research/excellent project as wide as possible.

3) The Bio-NMR users' community, the potential users from other scientific communities in the Life Sciences, and the industry stakeholders, are willing to consider, when appropriate, the inclusion of NMR infrastructure access costs in projects' budgets, for instance as a leverage effect within public-funded projects (H2020 projects in particular). It is to be hoped that in all major public money-

funded calls, particularly in calls issued by the EC or by other international organizations, a suggestion be made to explicitly allocate budget to access to Research Infrastructures.

Our analysis of the future sustainability and impact of EuroBioNMR is based on:

Demand from all stakeholders:

- EuroBioNMR should be able to continue coordinating the provision of access to cutting edge technology and expertise, and would represent a warranty of quality and high standards.
- EuroBioNMR should offer the expertise and instrumentation necessary to exploit the potency of the biological NMR approach in several fields of research within health and life sciences, and not excluding possible applications in food science, as well as agricultural sciences and even other fields outside life sciences, such as material sciences.

Demand from the Bio-NMR consortium (including users and NMR researchers)

- EuroBioNMR will be able to maintain the established fruitful relationship with the biological NMR users' community built up through the access provision in the last 20 years and significantly grown thanks to the Bio-NMR project. This users' community is a well-established and lively group which has expressed the firm intent to coordinate themselves in order to maintain and improve the interaction with the EuroBioNMR from one side, and with the Industry (in particular Bruker) from the other side, via the use of innovative funding scheme such as PCP and PPI within H2020.

Demand from (mainly) the structural biologists, the biomedical community and related platforms.

- Not only EuroBioNMR could give the opportunity to the various research groups to get in contact with each other and/or with members of the organization to establish at best new collaborations, but it could also elaborate a more ambitious roadmap of activities, based on the achievements and successes of Bio-NMR.
- EuroBioNMR could also foster the development of NMR technology for the broad life science community including chemical, pharmaceutical and biotech, food industries and agricultural sciences, thus contributing to ensure Europe's forefront position in biological NMR research.

Demand from Industry:

- EuroBioNMR will facilitate the cooperation with industries since:
    - Companies are increasingly using high cost equipments but do not have necessarily the economic robustness to acquire for their laboratories top-notch high field NMR instruments and maintain them at the top, including hiring high level NMR spectroscopists, so that they prefer to outsource these investments.
    - Industries can rely on EuroBioNMR to identify the best scientific partner/approach/technology for their specific needs, either in the form of service provision or collaboration.
    - There is a need to "speak the same language", which is not obvious between Industry and the Scientific Community (for instance when considering the IP management). In this context, EuroBioNMR could facilitate the set-up of bilateral agreements between EuroBioNMR partners and industry, by providing a basic legal framework. The NMR infrastructures can provide to industrial users affordable warranted service coupled with data security, confidentiality and secrecy.
    - The NMR infrastructures can also provide to industrial partners consultancy upon signature of confidentiality agreements

- There is mutual interest in establishing Industry-Academy collaborations, either in the form of partnership, or with the academy providing specific services as third party.

Demand from the European and national authorities

- EuroBioNMR should coordinate activities to increase the strength of the biological NMR community at the European level, and represent its members at the EU level.

Scientific Impact

Through EuroBioNMR it will be possible to maintain the top level scientific collaboration among the partners. Most important, to maintain the sharing of best practices/protocols for the optimization of experimental procedures and results as successfully achieved through the Local Operator Meetings during the Bio-NMR project and previous I3 project (Eu-NMR, East-NMR). This will turn particularly important as the new 1200 MHz spectrometers will become available, allowing the development of new possible applications. Participation of Bruker in the Bio-NMR project has been strategic in this respect, and its involvement in EuroBioNMR will be just as important, since research linked to the instrument optimization is carried out with Bruker collaboration.

Economic impact

The expected economic impact of EuroBioNMR is twofold:

- Direct impact: we can estimate that the optimization of the use of existing / future equipments would increase up to a factor of two in terms of number of users, compared to a non-connected community. This means, that the optimization of instrumentation usage is directly affecting the quality and the quantity of scientific throughput.
- Indirect impact: by creating a critical mass of scientific & private users, EuroBioNMR should provide additional sustainable market opportunities to the European Industry in terms of procurement of high-class equipment (such as it was the case for the 1,2 GHz spectrometer).

Strategic Impact

EuroBioNMR will contribute to the European strategic impacts since it will support the NMR EU-players (Scientific and Industry) to stay connected and innovative (by creating an active community at the EU level, establishing closed and long-term communication with the public authorities both at the Regional, national and EU level). As such, Europe will be able to maintain and even strenghten its leading position in the field of biological NMR, and the competitiveness of the European Industry will also be reinforced, as already mentioned in the section “Economic Impact”.

In conclusion, we can highlight the following elements:

- the Bio-NMR community is now active and well-connected at the EU-level
- the consortium has granted access to existing equipment by optimising the available resources and has supported high-quality research with outstanding S&T impacts
- There is a need for maintaining the existing services, setting-up new added-value activities, which could be done via a new structure (EuroBioNMR) that is expected to be sustainable but,
- In terms of scientific activities, the incomes individuated in the developed Business model cannot cover the financial needs for continuing the provision of transnational access at the same level as it has been through Bio-NMR. As such, additional support from EU funding (directly or indirectly) will be necessary to provide the Community with the possibility to stay at the forefront of the research and development in Europe.