Feasibility study

For projects within the Operational Programme Research, Development and Education

Priority axis 1, investment priority 1, specific objective 1,

Call identification: **Excellent research**

EVROPSKÁ UNIE
Evropské strukturální a investiční fondy
Operační program Výzkum, vývoj a vzdělávání

MINISTERSTVO ŠKOLSTVÍ, MLÁDEŽE A TĚLOVÝCHOVY

1. BASIC DATA

Item	
Project title	Advanced imaging and analytical methods for studies of cellular dynamics
Applicant	*Institute of Experimental Botany AS CR*
Primary and secondary project discipline according to the expertise and disciplines tree of the OP RDE	1AB5 Biosciences 1AB3 Chemistry

2. SHORT DESCRIPTION OF THE PROJECT - ABSTRACT

Rapid technological progress in recent years contributed significantly to the accumulation of large amounts of information on the composition of living organisms. These are formed by a mixture of low molecular weight substances, biopolymers and lipid molecules. However, sequence and structure information alone is not enough to understand the mechanisms that contribute to the physiology and metabolism of plant and animal organisms. Therefore, it is important to study the relationships between the individual components of living organisms and to spatially localize these processes. Methods of optical, electron and magnetic resonance microscopy are currently used for these purposes. The great advantage of optical fluorescence microscopy using specific fluorescence markers is the opportunity to study cellular processes in native conditions. However, the spatial resolution and depth of field of optical microscopy are limited. In contrast, very high spatial resolution can be achieved by conventional electron microscopy, which is on the other not suitable for studies of unfixed native, wet or live samples. Imaging with magnetic resonance is characterized by lower resolution, but it offers to follow processes in living organisms on the functional level.

By significant improvements of a combination of advanced imaging methods and environmental electron microscopy in particular, the aim of this project is to push forward the knowledge in the field of in vivo and dynamic in-situ studies of biological samples, including specific immunological labelling of cell structures using synthetic nanoparticles. New technologies and procedures will be used in studies of the extracellular space and the surfaces of plant cells and their signalling and transport systems at the plasma membrane and endomembrane system (IEB CAS). In parallel, monitoring and analysis of nuclear architecture, gene expression regulation and transport of macromolecules between the nucleus and cytoplasm will be performed in animal cells (IKEM). All participating R&D centres represent high quality scientific research and for the purpose of this project they form interdisciplinary excellent research team, supporting each other through their individual research activities. They have long experience in the preparation of specific probes for detection in microscope and their testing using in *in vitro* liposome systems (IMC CAS), implementation of advanced fixation techniques for animal and plant cells (IEB CAS, IKEM) and research and development of the new physical methods and technologies for electron microscopes (ISI CAS).

The understanding dynamic processes at biomembranes at the ultrastructural level is crucial in both basic and applied research. Therefore, in all three proposed research programs, the project contains a number of microscopy application procedures for the preparation and subsequent detection of nanoparticles for their use in medicine and agriculture. They include the optimization of the transport of biologically active substances within the organism to prevent side effects in medicine, minimizing the ecological load by the enhancement of

chemical protection in plants and nanotechnology-based, genetic modification-free improvements of plant cell walls for textile industry.

3. DESCRIPTION OF APPLICANT AND PARTNERS OF THE RESEARCH CENTRE

3.1. Brief characterization of the project applicant

Institute od Experimental Botany CAS, v. v .i. (IEB CAS), VAT n. 61389030, is a legal entity established for indefinite period of time, with its registered office at Rozvojová 263, 165 02 Prague 6. The founder of IMC CAS is the Academy of Sciences of the Czech Republic, a state organizational unit. It is headed by its director RNDr. Martin Vágner, CSc., IEB Board and the Supervisory Board. The director is a statutory body authorised to act on behalf of IEB CAS.

IEB CAS was founded in 1962. Its 14 laboratories are located in two cities, Prague and Olomouc. IEB mission is to conduct basic research in the field of plant experimental biology, namely in plant genetics, cell biology, physiology, phytopathology and biotechnology. It is also active in the field of plant applied research. Main research topics include mechanism of action of plant hormones, their crosstalk during the signalling from the environment, functional genomics, molecular mechanisms of growth and development and plant pathophysiology. Biotechnological topics include design and preparation of edible vaccines from plants and mechanisms of phytoremediation. Using molecular biology procedures in breeding, a spectrum of apple trees resistant to fungal diseases has been obtained. Studies of plant hormones resulted in the synthesis of compounds, which delay skin aging or show promising cytostatic effects. Detailed information about research activities of IEB CAS in 2010-2014 could be found in the information brochure on the following web address: http://www.ueb.cas.cz/cs/system/files/users/public/ueb_scirep_2010-2014_small.pdf.

IEB CAS is equipped with high end instrumentation for three main research strategies of modern experimental plant biology. These include advanced chemical analysis of plant hormones, genetic analysis and mapping of complex genomes and advanced microscopy analysis, which has been dramatically developed in IEB during past decade and is crucial for this project.

Besides scientific monographies and chapters in monographies, in 2014-2015 there were 277 papers in scientific impacted journals, including the most prestigious multidisciplinary journal Nature, Science and PNAS. More than one quarter of IEB CAS contributions has been published in journals belonging to the best quintile of the discipline and more than a half of IEB CAS contributions has been published in journals form the best quintile. Only less than 15% papers has been published in journals with impact factors below the median of the discipline. The majority of papers have been published in collaboration with reputable foreign institutions. Besides a plenty of informal collaborations and contacts, the co-operation with foreign institutions has been supported in 2014-2015 by co-operative projects that included 7th framework program (2 projects) and COST program (20 projects). High quality of researchers from IEB CAS is well illustrated by a number of their lectures presented on international conferences (132 in 2014-2015) and numerous memberships in editorial boards of prestigious scientific journals (37 in 2015) as well as in the governmental and non-governmental organizations. Internationally leading role of IEB ASCR in the area of hormonal regulations is well documented also by the fact that it organizes on regular basis prestigious meeting of experts in this field named ACPD (Auxins and Cytokinins in Plant Development). Last meeting has been organized in 2014 and the next is going to be held in 2018

(http://acpd.cas.cz/). International excellence of IEB CAS is also reflected by around 100 lectures presented by foreign researchers in 2014-2015. For the same period, IEB CAS researchers produced around 250 peer reviews for impacted scientific journals and also more than 700 reviews for local and foreign organizations including grant proposals for Czech Science Foundation (GA ČR), Technological Agency of the Czech Republic (TA ČR), Grant Agency of Charles University (GA UK), Ministry of Agriculture of the Czech Republic, Ministry of Education, Youth and Sports, DFG (Germany), FWO (Belgium), ERC (EU), NSF (USA) and BARD (Izrael). In past two years researchers and students from IEB CAS have been awarded by a number of local and international awards that could be found listed in annual reports on the following link: http://www.ueb.cas.cz/en/category/internet-site/ustav/vyrocni-zpravy. IEB CAS publishes two impacted scientific international journal, Biologia Plantarum (ISI IF_{2015} 1.665) and Photosynthetica (ISI IF_{2015} 1.558).

More information about IEB CAS, institutional organizational scheme, members of IEB board and Supervisory board are available online at following links: http://www.ueb.cas.cz/en/content/organisational-scheme and http://www.ueb.cas.cz/en.

IEB CAS's financial participation in the proposed project is 79 493 730.40 CZK.

3.2. Brief characterization of the project partners

Institute of Macromolecular Chemistry of the AS CR v. v. i.

The Institute of Macromolecular Chemistry of the CAS, v. v. i. (IMC CAS), VAT N. 61389013, is a legal entity established for indefinite period of time, with its registered office at Prague 6, Heyrovského náměstí 2, postal code 162 06. The founder of IMC CAS is the Academy of Sciences of the Czech Republic, a state organizational unit. It is headed by its director, Ing. Jiří Kotek, Dr., IMC Board and the Supervisory Board. The director is a statutory body authorised to act on behalf of IMC CAS.

IMC CAS is the Czech Republic's largest institution active in the field of research of polymer materials. In this field, it ranks among the most important centres of academic (basic) research not only in the Czech Republic, but also in the world. IMC CAS enjoys the knowledge potential of more than one hundred scientists in the key areas of macromolecular chemistry, physical chemistry, and polymer physics, but also in the fields that overlap with biochemistry, biophysical chemistry, tissue engineering, medicine, pharmacy and biotechnology, particularly in developing new polymers for medical, pharmaceutical and diagnostic use and examining elementary principles of the interaction between synthetic polymers and organisms. The staff of the IMC CAS includes several dozen world-renowned scientists.

Since its founding in the late 1950s, the Institute of Macromolecular Chemistry has been known by Czech and world scientists for its activities focused on the research of polymers for medical use. The Institute was founded by Professor Otto Wichterle, the inventor of a polymeric material and technology for the production of soft contact lenses - one of the most important Czech inventions commonly used and respected throughout the world. The programme of research of polymers for medicine is still one of the key research activities of IMC CAS.

The major contribution of IMC CAS to the research and development of polymers for pharmaceutical and medical applications, specifically in the field of new polymeric forms of drugs and polymers used in regeneration medicine is demonstrated by the prestigious Czech Head prize that was awarded to IMC CAS's scientists in recent years (2002 National Prize, 2005 & 2008 Invention Prize). In 2008, the Institute ranked among the 'Czech Top 100' in the

category "Education-Science -Health-Humanity" as the only institute of the Czech Academy of Sciences.

Information about IMC CAS is available at the website http://www.imc.cas.cz/.

IMC CAS's financial participation in the proposed project is 59 974 292.20 CZK

Institute of Clinical and Experimental Medicine (IKEM)

For forty-five years Institute for Clinical and Experimental Medicine (IKEM) accounts for an integral part of the Czech medical science, clinical medicine and education of medical students and doctors. It is the largest specialized clinical and scientific research center in the Czech Republic. IKEM is an organization directly managed by the Czech Ministry of Health and consists of 4 specialized centers (Cardiology Centre, Transplant Centre, Diabetes Centre and Centre for Experimental Medicine), Complement of the above-mentioned centers, 8 clinics, 15 professional workplaces, bases and laboratories and over 1600 employees.

IKEM is focused on the area of cardiovascular diseases, organ transplants, diabetes and metabolic disorders. Among its main functions, besides specialized clinical activities in selected areas, belongs also scientific research, education of researchers and training of physicians.

Scientific research activities of Institute for Clinical and Experimental Medicine strive to solve medically and socially serious problems. The scientific research uses a multi-sectoral task team approach and the latest technology and long-term cooperation with a wide range of medical facilities at home and abroad. The duration and nature of the research varies according to its orientation. There is generally performed short-term and long-term research with the character of mostly basic and applied medical research.

In 2014-2015 there have been 68 research projects financially supported from Ministry of Health of the Czech Republic, Grant Agency of the Czech Republic, Ministry of Education, Youth and Sports, Ministry of Interior of the Czech Republic, Technological Agency of the Czech Republic and Ministry of Industry and Trade of the Czech Republic. 7 project have been supported by European commission through the 7th framework program. 27 projects were performed with the IKEM institutional financial support in 2014-2015.

Further information about IKEM is available at the website http://www2.ikem.cz/

IKEM's financial participation in the proposed project is 59 917 050.08 CZK.

Institute of Scientific Instruments CAS, v. v. i.

Institute of Scientific Instruments, Academy of Sciences of the Czech Republic, v. v. i. (ISI CAS), VAT n. 680811731, is a legal entity established for an indefinite period with its registered office in Brno, Královopolská 62/147, Zip Code 612 64 The founder of ISI CAS is the Academy of Sciences of the Czech Republic, a state organizational unit. The authorities of ISI CAS are Director Ing. Ilona Müllerová, DrSc., ISI board and the Supervisory board. The Director is the statutory authority and is authorized to act on behalf of ISI CAS.

ISI was founded in 1957 as an institution providing instrumentation for other institutes of the Academy of Sciences in the fields of electron microscopy, magnetic resonance imaging and later lasers. After 1989 the process of transformation occurred and only the most promising areas of research remain at ISI CAS. The structure of scientific departments and research groups is set so that it is built on the research focus of the projects, which are addressed at ISI CAS. At ISI CAS, theoretical, experimental and applied research is connected with the involvement of following scientific departments: Special technology, Electron microscopy, Magnetic resonance and Cryogenics, Medical signals, Optical micromanipulation

techniques and Coherent optics. Since 2013, the Application laboratories of advanced microtechnologies and nanotechnologies (ALISI) have been a part of ISI, created with the support of the R&D project. With its activities, ISI contributes to raising the level of knowledge and education and to utilizing the results of scientific research in practice. In collaboration with the universities, it carries out doctoral study programs, thereby contributing to the education of young scientists.

Information about ISI CAS is available at websites https://www.isibrno.cz/ and http://alisi.isibrno.cz/.

ISI CAS's financial participation in the proposed project is 79 834 636 CZK.

3.3. Characterization of R&D centres entering the project

Starting situation:

Proposed project will be realized by R&D centres of three CAS institutions, i.e. IEB ASCR (main applicant), IMC ASCR and ISI ASCR and R&D centre at IKEM Ministry of Health of the Czech Republic.

In all these centres, mutually complementary topics that are going to be addressed within this proposal are studied on top level for decades. All centres entering the project are primarily oriented to research and development and they are either accredited (IKEM) or active in teaching and education activities within accredited bachelor, master and doctoral programs, primarily at Charles University in Prague, but also at many other universities. Research strategy of these centres are focused on advanced optical, electron and magnetic resonance microscopy studies of cellular structures of plants (IEB CAS), animals and humans (IKEM). These technologies are tightly in many aspects to the chemical synthesis of nanoparticle systems, which are for a long time in the centre of attention of R&D centre of IMC CAS. Research strategy of R&D centre of ISI ASCR includes primarily the development of new instrumentation and techniques in the area of environmental electron microscopy. By joining all these strategies we plan to create centre of excellent research that is going to be focused on the main objective, which could be defined as follows. To push forward the understanding of cellular processes in vivo by substantial improvement of environmental electron microscopy and associated techniques that include specific labelling with synthetic nanoparticles. This aim is accompanied by a number of associated, more specific objectives, which include also other methods and techniques for sample preparation.

The main applicant IEB CAS enters the project with the **R&D centre focused on advanced microscopy analysis of plant cells.** This centre is composed of researchers from 5 laboratories and for the purpose of this centre of excellence it is going to be leaded by the head of Imaging Facility of IEB CAS, RNDr. Jan Petrášek, Ph.D. (http://www.ueb.cas.cz/if). Research strategy of laboratories within the R&D centre IEB includes studies of cytoskeleton dynamics, endomembrane transport, integral and peripheral membrane proteins, membrane lipids and extracellular material. In all these areas, the instrumentation of the centre is supported by the institution, CAS and a number of research projects from various resources. The development of advanced imaging methods has started in 2006 within the Framework of Research centre of MSMT REMOROST, project n. LC 06034 (http://remorost.ueb.cas.cz/; 2006-2010) with the installation of confocal laser scanning microscope Zeiss LSM 5 Duo equipped with spectral detection and fast line scanner. Using this microscope, in 2006-2010 there have been published numerous original contributions using multichannel and spectral imaging as well as photomanipulation techniques like FRAP and FRET. The unit was equipped with automated

station for *in situ* hybridization and immunohistochemistry on whole mounts and sections (The Operational Programme Prague - Competitiveness, project CZ.2.16/3.1.00/21159; 2009), which allowed improvement of immunofluorescence methods. Thanks to the success in the CAS competition for expensive instruments, non-invasive *in vivo* methods of optical fluorescence microscopy were further extended in 2012 by the installation of spinning disk confocal microscope Nikon Eclipse Ti-E with Yokogawa CSU-X1. Studies of cellular processes in higher time and spatial resolution continued thanks to the investment into highly sensitive and spectral confocal laser scanning inverted microscope Zeiss LSM 880 (The Operational Programme Prague-Competitiveness, project CZ.2.16/3.1.00/21519; 2015). Together with new upright microscope with structured illumination, Zeiss Apotome 2 (investment of IEB CAS, 2015) the imaging facility is now equipped with high end instrumentation for in vivo fluorescence microscopy. Imaging facility of the IEB CAS R&D centre unit is also equipped with transmission electron microscope, which was installed in 2012 (The Operational Programme Prague-Competitiveness, project CZ.2.16/3.1.00/24014). Imaging facility of IEB CAS (http://www.ueb.cas.cz/if) has been officially classified as independent unit within the organization structure of IEB ASCR at the end of 2015. Scientific publications published by R&D centre focused on microscopy techniques are listed in Annex 1. With respect to the proposed project, there should be emphasized publications in top journals that contributed very significantly to the understanding of targeted exocytosis in plants, understanding of dynamics of auxin carriers in plant development and definition of the role of membrane lipids for signal transduction in plants.

In the proposed project, the **R&D centre of the IMC CAS partner institute** is represented by the **SUPRAMOL centre**, led by RNDr. Petr Štěpánek, DrSc. This team has long been engaged in a systematic physicochemical research of the processes of self-organization of polymer systems and their application for the controlled preparation of nanoparticle systems that are used primarily for biomedical applications. The research pursued by the team members resulted in detailed understanding of the differences in the formation of nanoparticles depending on the type of external changes, particular changes in temperature, pH, thermodynamic quality of a solvent, ionic strength or the presence of a surfactant. The high-level control of the process of the formation of nanoparticles and their properties has been achieved through systematic physical and physicochemical approach to the processes of phase separation and subsequent self-organization, including the study of model systems, which requires a high level of professionalism in the use of relevant experimental techniques, primarily the static, dynamic and electrophoretic light scattering , small-angle scattering of X-rays and neutrons, isothermal titration calorimetry and radionuclide studies. Furthermore, the team has extensive experience in the field of polymers specifically degradable by oxygen reactive forms and polymeric antioxidants, either in nanoparticle systems for targeted transport and controlled release of pharmaceuticals, or with antioxidant gels for topical application - wound healing (clinically used HEMAGEL® was developed at this site).

More information about R&D partner centre SUPRAMOL are available on the following link: http://www.imc.cas.cz/en/umch/c_supra.htm.

R&D centre at IKEM enter into a project through the participation of its **Department of Diagnostic and Interventional Radiology (ZRIR)**, which provides all radio diagnostic examinations within IKEM and additionally specializes in the development and research in the

fields of interventional radiology and magnetic resonance imaging. ZRIR provides special services to healthcare and scientific-research facilities in the Czech Republic.

Within ZRIR the proposed project will be realised primarily by the group of experimental MR spectroscopy and imaging, which has the longest tradition in the application of research in the field of magnetic resonance (MR) in the Czech Republic. This group is equipped with appropriate instrumentation, including an 1.5T Magnetom Avanto Tim system, a 3T MagnetomTrio Tim multinuclear imager accomplished with various hardware and software (especially for ^{1}H, ^{19}F, ^{31}P nuclei and imaging of small animals), an experimental 4.7T Bruker Biospec 4.7/20 scanner, two relaxometers (operating at 0.5T and 1T) for relaxometry studies, optical microscopes, and a fluorescence imaging facility for small animals. This group provides the special examinations including visualization of transplanted cells and in this area is clearly leading institute in the country.

In a research group at ZRIR IKEM working 14 researchers and Ph.D. students. In the field of clinical research group working on the issue of metabolic syndrome and related issues in the area of diabetes, obesity and cardiovascular disease. It developed a series of investigative algorithms for MR spectroscopy, functional MR imaging and cardiac investigations on clinical tomographs. The experimental research group is focused on molecular imaging, including the testing of new contrast agents for cell labelling.

ZRIR staff is actively involved in research projects both within the individual grants, as well as collaborators in research projects of other departments IKEM. The department is intensively involved in research in the field of cell imaging, in which it cooperates with many Czech and foreign laboratories. One of the important results of the group and other workers of IKEM is imaging of transplanted pancreatic islets labelled with specific contrast agents using MR imaging in experimental models and in clinical experiment. This model of transplantation and treatment of diabetes can be regarded as an example of successful theranostics (method using imaging for therapy control). As a significant achievement can be seen also a study of labeling of bone marrow cells (and other kinds of cells) with iron nanoparticles and monitoring their migration after transplantation. Workplace demonstrated the suitability of the commercial contrast agents Resovist and Endorem as the first in the world, and not long after installing experimental MR tomography in 1999. These experiments were conducted and carried out in cooperation with other departments IKEM and the Institute of Experimental Medicine CAS (IEM CAS).

Institute cooperates on research projects especially with the National Institute of Mental Health; Faculty of Science, 1st Faculty of Medicine, Faculty of Mathematics and Physics at Charles University; Institute of Macromolecular Chemistry CAS, Department of Organic Chemistry of the CAS, IEM CAS, and a number of others. From foreign institutions is very well developed cooperation with the MR Center of Excellence Medical University in Vienna, Molecular Small Animal Imaging Centre (moSAIC) in Leuven, Preclinical MR imaging center, Radboud University Nijmegen Medical Centre (RUNMC), and the University of Bergen.

Sources of information about ZRIR are available on the website http://www2.ikem.cz/www/en?telpra=165.

The R&D centre of the partner ISI CAS enters the project through the **Centre of Environmental Electron Microscopy (EEM R&D Centre**), whose base is the scientific group Environmental electron microscopy led by Ing. et Ing. Vilém Neděla, Ph.D, accompanied by top scientists from ISI and other Czech and foreign research institutions. The EEM group is firmly embedded in the structure of the ISI and the OP RDI project entitled Application and

Development Laboratory of Advanced Micro-technologies and Nanotechnologies (ALISI). The activities of ISI and ALISI are financially secured by the institutional contribution from ASCR, targeted financing through domestic (e.g. TACR Competence Centres TE01020118, TE01020233, GACR Centre of Excellence GB14-36681G) and international research grants (e.g. FP7 606988 SIMDALEE2, FP7-PEOPLE 316679 Transact), the National Programme for Sustainability (LO1212) and partly through contract research for a variety of partners. The basic mission of the EEM R&D centre in the project is to link theoretical, experimental and applied research in the fields of electron optics and microscopy, interferometry and coherent optics, optical micromanipulation techniques, technological use of electron and laser beams, cryogenics and superconductivity. The main efforts of the EEM R&D centre in the project will be directed towards discovering and developing new experimental methods for studying the properties and microstructures of predominantly animate and inanimate matter, respectively new approaches in the field of high technology. Other sources of information can be found on the links http://www.isibrno.cz/, http://alisi.isibrno.cz/ and http://eem.isibrno.cz/.

One of the pioneers of environmental scanning electron microscopy (ESEM) in the Czech Republic is Prof. Rudolf Autrata from the Institute of Scientific Instruments CAS, v.v.i. in Brno. Professor Autrata was also the author of numerous patents and articles in the field of research and development of detection systems for SEM and ESEM. He first introduced the monocrystalline scintillation materials YAG: Ce3 + and YAG: Ce3 + as the essential element of signal electron detectors. The R&D Centre of Environmental electron microscopy (EEM R&D Centre) led by the former student of Prof. Autrata, Ing. Vilém Neděla, Ph.D., continues in this over thirty-year-old tradition of research and development of ESEM and detection systems. It deals with the applied, but in particular basic research focused on the simulation of interaction of electrons with gas, water and solids in ESEM, the design and development of detection systems working on the principle of the impact ionization of gas molecules by signal electrons, through the development of scintillation photomultiplier detectors to detect secondary and backscattered electrons in an environment of high pressure gas. The EEM R&D Centre has also long been involved in the simulation of gas flow in ESEM, specifically optimizing the design of electron-optical components of ESEM with respect to the pumping of gas. In cooperation with a number of academic partners the R&D centre carries out research of a broad spectrum of hard to observe phenomena in particular radiation damage of sensitive samples, often in the scope of dynamic in-situ experiments. The centre also has significant activities in the research and development of special microscopic methods and the instrumentation and integration of new technologies into the ESEM. The EEM R&D Centre is currently one of the few centres in the world, which comprehensively deals with the issue of environmental scanning electron microscopy.

In 2014, the EEM R&D Centre succeeded in an internal ASCR competition to promote the purchase of expensive equipment. With this financial support a uniquely equipped high resolution electron microscope QUANTA 650 FEG was purchased in 2015 and so the European laboratory of environmental electron microscopy began its activities (Fig. 3.1). Besides the wide range of conventional detection systems, the new microscope is equipped with a custom-modified sample chamber of large dimensions, a heated and cooled sample holder (from -20°C to 1000°C), a system of two precision micromanipulators with extensive accessories, e.g. the system of the controlled injection of fluids and gases for the sample and the EBIC system. The microscope also enables performing an energy dispersive elemental X-ray analysis of non-conductive samples without having to cover their surfaces with other layers in an environment of high pressure gases and, in the "deceleration" mode, the study of

samples in conditions of a very low energy primary electron beam. The study of thin slices and nano-particles in liquids is possible thanks to the conventional STEM and the special wet-STEM mode. The new microscope is a great tool for interdisciplinary scientific cooperation and, after a series of planned adjustments and after adding a unique system developed at ISI ASCR in Brno, it will be a unique device on a global scale. The equipment of the EEM R&D Centre is on an elite level, given the topic of the project.

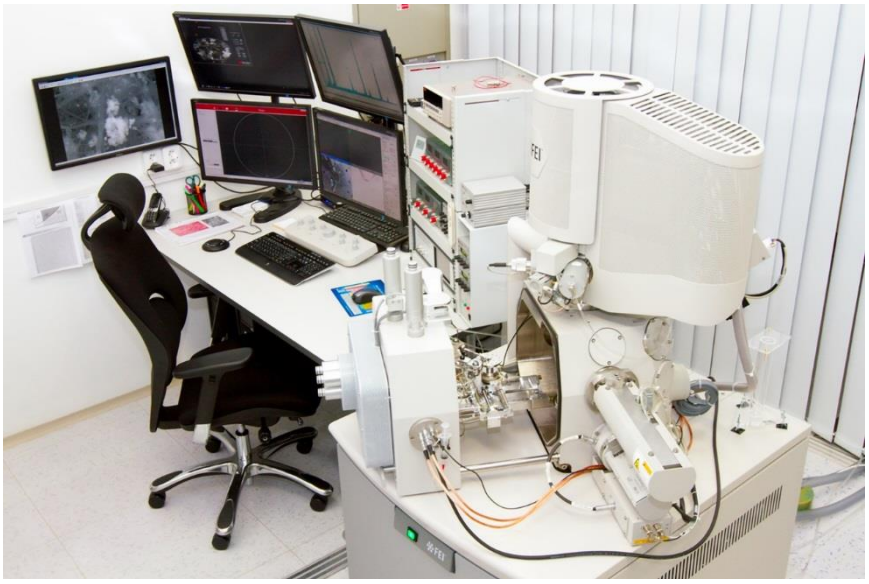

Figure 3.1: The ESEM Quanta 650 FEG workplace, ISI CAS.

Proposed organisational structure of the project

Both applicant and partner R&D centres are aware of the fact that the management and organisational structure of the proposed project (Fig. 3.2) is crucial, taking into account the interdisciplinary character of the research and number of participating researchers and administrative workers. The effective adjustment of the structure will be contributing very significantly to reach project objectives. It will also reduce possible risks and uncover pitfalls that might be faced during the project implementation.

Project administration council (Fig. 3.3) will be constituted of project director, who will be responsible for the project (Jan Petrášek, principal investigator of the applicant R&D centre of IEB CAS), deputy director (Vilém Neděla, main investigator of partner EEM ISI CAS), heads of individual research R&D centres, i.e. Petr Štěpánek (main investigator of partner SUPRAMOL IMC CAS), Daniel Jirák (main investigator of partner ZRIR IKEM) and project manager that will be nominated.

The role of project administration council will be to supervise to the fulfilment of project monitoring indicators, to control financial management of the project, discuss and approve any modifications in the project budget, nominate the director of the project to negotiate with MSMT for change management process or any other issues, to keep control and to evaluate results, to solve possible conflicts, to define conditions for tendering procedures and take part in the evaluation of tender offers. The council will meet at least 4 times a year. The meeting could be organized also in case of dealing with unforeseen issues (financial, personal, and administrative). Results of council meetings will be obligatory for every members of the project. In case of conflict resolution, the council will follow the wording of Partnership

agreement, which is already provisionally approved by the applicant and all partners and ready for ratification in the second round of the evaluation procedure. Minutes of meetings will be supplemented to progress reports and final report. Council meetings will be hosting personally all members.

Figure 3.2: The management and organisational structure of the proposed centre of excellent research.

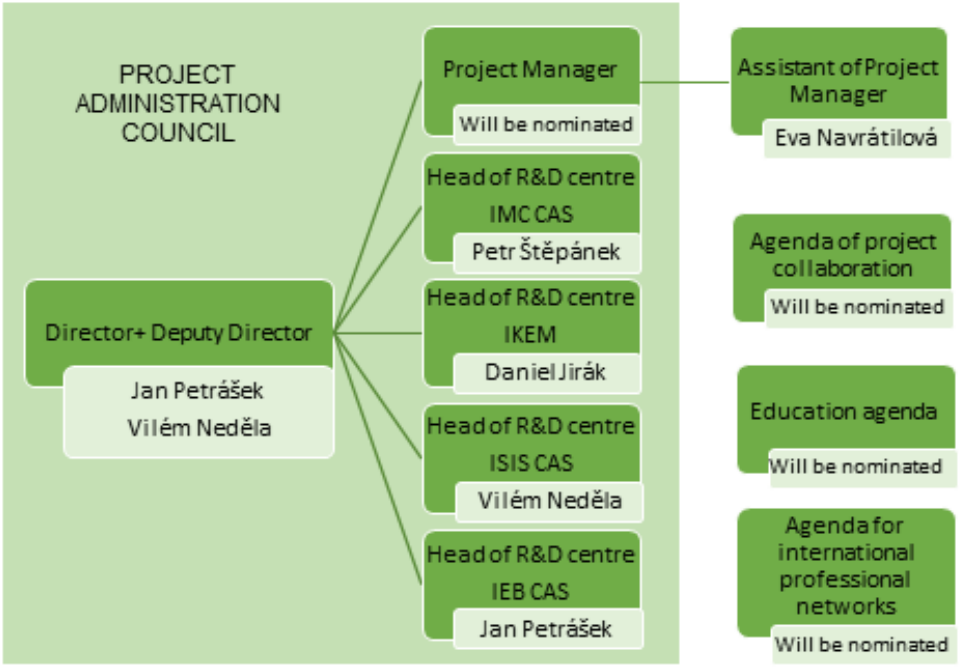

Figure 3.3: Project administration council of the proposed centre of excellent research.

Director of the project, Jan Petrášek, will be responsible for the successful project implementation. He will be leading the project in its entirety. Based on the previous negotiations in the project administration council, he will make decisions about main topics of the project and possible changes and conflicts, he will approve budgets of individual parts of the project, timetables of individual project parts, together with deputy director he will approve and confirm finish of individual project milestones.

Deputy director/main investigator of partner EEM ISI CAS, Vilém Neděla, will be a member of a project administration council, together with the project director he will be co-responsible for the successful project implementation. He will have authority to nominate project manager. He will guarantee principles of information flow between individual members of project administration council and will manage supplies from third parties. He

will be answerable to the project director having authority to depute individual tasks to other members of the project administration council. Seen from outside, he will have authority to negotiate in the framework of a given schedule of individual project phases and about their budget.

Heads of R&D centres/main investigators of partners R&D centre IEB CAS (Jan Petrášek), SUPRAMOL IMC CAS (Petr Štěpánek), EEM ISI CAS (Vilém Neděla) and ZRIR IKEM (Daniel Jirák) will be members of project administration council. Within the project team, their task will be to supervise and lead administrative management of partners. They will be further responsible for guarantee principles of information flow between individual members of project administration council. Seen from outside, they will have authority to negotiate in the framework of a given schedule of individual project phases and about their budget.

Project manager, will be nominated, he/she will be a member of project administration council. Will be responsible for the project implementation management in relation to the grant provider and individual members of the project. He/she will be responsible for complying the terms of the funding obligatory for this type of project and possible change management processes within the project. He/she will control administrative outputs of the project and wages specifications in particular. He/she will give notice to the heads of R&D centres on the possible discrepancies and inconsistencies in relation to funding rules and also will support project team with valuable feedback. He/she will responsible for the economic assessment of the project (profitability assessment) and will control spending of budget. He/she will be subordinate to the director of the project.

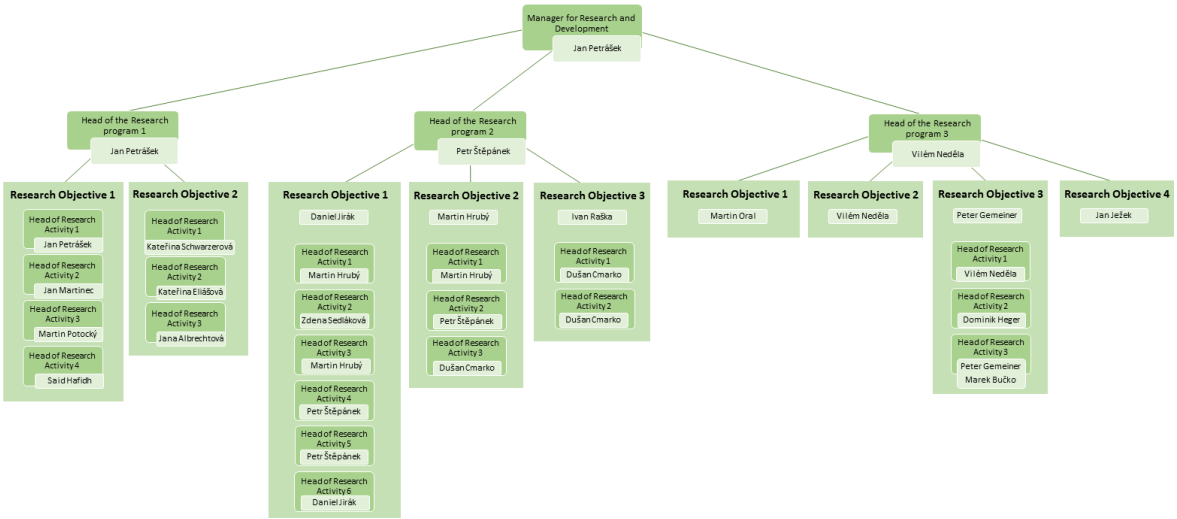

Figure 3.4. Executive board for research and development.

Executive board for research and development (Fig. 3.4) will be composed of project manager for research and development, who will be responsible for the substance and structure of the project (Jan Petrášek), heads of individual research programs of the project (see chapter 5), i.e. 1. (Jan Petrášek), 2. (Petr Štěpánek) and 3. (Vilém Neděla) and senior researchers of individual research programs. The mission of the board for research and development will be to supervise the fulfilment of medium-term and long-term implementation plans with respect to mutual linkage of R&D centres of applicant and partners and with respect to the fulfilment of planned project outcomes, including milestones of individual research activities. The council will meet at least 4 times a year. The meeting could be organized also in case of dealing with unforeseen issues (delay in the particular activities,

technical issues). Results of council meetings will be obligatory for every members of the project. Minutes of meetings will be supplemented to progress reports and final report. Council meetings will be hosting personally all members.

Heads of individual research programs will be responsible for individual research programs and research supervision of their teams, they will be supervising the fulfilment of the schedule and particular objectives, record individual results, inform the executive board for research and development about the fulfilment of particular objectives (bound to planned date), identify possible issues that may arise during the investigation of the project and inform the board immediately about these issues. They will prepare materials for progress reports and final reports as well as situation reports on request of the board. They will be organizing regular team talks of their particular teams, where technical and administrative issues will be solved. As the outcome of planned coordination meetings of the board, individual tasks will be assigned in a way that their investigation will produce clear outcomes that will allow clear decision on further progression. Minutes of meetings will be containing information on the tasks fulfilment, new tasks, and in case of need critical points will be specified and solution suggested. Every member of the team will be obligated to keep laboratory notebook in good shape and make good documentation for potential replacement or more importantly for archiving purposes.

The incorporation of the structure of the proposed centre of excellent research into the current institutional organisational scheme of IEB CAS will be implemented by the establishment of independent accounting entity, which will be established as independent unit within the structure of IEB CAS (see organigram in Annex 2). This unit will have relation to 5 laboratories and 1 additional unit (imaging facility) of the current R&D centre of IEB CAS. Annex 2 is also highlighting the link to three partner's R&D centres SUPRAMOL IMC CAs, IKEM and ISI CAS.

3.4. Current Research activities of R&D centres related to activities of the project

R&D centre of the applicant IEB CAS implements in past two years (2014-2015) several research activities in the area of microscopy investigations of structures of plant cells that are directly related to the development of advanced confocal microscopy (see chapter 3.3.). As it is clear from the list of published original contributions (Annex 1), there were revealed fundamental information about the mobility of membrane transporters of plant hormone auxin, about involvement of membrane lipids into signalling pathways associated with membranes, about protein complex for the targeted delivery of cell wall components, about the role of cytoskeleton in the intracellular transport and arrangement of cell surfaces and most importantly about implications of all these processes for plant development. Following the results of these high quality papers, which include number of papers with citations in between 100x-500x, there were several projects investigated in 2014-2015 devoted to integral membrane proteins, membrane lipids and microdomain membrane organisation (Annex 3).

Above mentioned activities have not been supported by projects PO VaVpI and OP VK. R&D centre of IEB CAS is however the partner of the project in the framework of "National Infrastructure for Biological and Medical Imaging" (Czech-BioImaging, CzBi; http://www.czech-bioimaging.cz/), which has been approved for funding by the Ministry of Education, Youth and Sports for the period 2016 - 2019 (Large Infrastructures for Research, Experimental Development and Innovations, LM2015062). Through this project, R&D centre

of IEB CAS is integrated within Prague's node into large pan-European research infrastructure Euro-BioImaging (http://www.eurobioimaging.eu/). On the European level, the CzechBioimaging has been evaluated positively and is thus validated for future applications within the ESFRI (European Strategy Forum on Research Infrastructures) network. In 2016, R&D centre of IEB CAS through CzBi takes part in the call 02_16_017 (Research Infrastructures for educational purposes), where it is primarily focused on investment into improvement of instrumentation and only very limited budget is planned for research activities, which is the main point of this proposal for the centre of excellent research.

Proposed project is based on the development of several research programs and activities that are directly bound to present activities of R&D centre of IEB CAS in the area of studies of intercellular material, cell surfaces, cell walls, plasma membrane and endomembrane system of plant cells. The list of projects with the brief description of their research agenda, which have been investigated by members of R&D centre of IEB CAS in 2014-2015 and which are going to be further developed by proposed project could be found in Annex 3. Through the proposed project the R&D centre of IEB CAS plans to extend further the new horizons that have been opened by the activities of imaging facility and to complement these techniques with the newly investigated instrumentation and techniques of advanced environmental electron microscopy.

The quality of current research programs and activities in the area of light microscopy investigations of plant cells is documented by numerous original contributions, which are based on advanced microscopy analysis. The best 10 publications are listed in Annex 4. Publications that are based on the advanced fluorescence microscopy represent roughly half of publication output of IEB CAS in the scientific journals with impact factor higher than 5 and authors of these papers belong to the best in IEB CAS.

Between 2014 and 2015, the **R&D centre SUPRAMOL of the IMC CAS partner institute** implemented a number of projects (see Annex 3) in order to develop procedures that would make it possible to control very precisely the particle size in the desired range usable for biological applications, i.e. between 10 and 20 microns for gastrointestinal applications, between 100 and 200 nm for the selective passive accumulation in solid tumours, 50 nm for optimal internalization into cells, and between 5 and 20 nm for overcoming the haematoencephalic barrier in the brain. It also succeeded in optimizing the preparation processes in the manner that allows for the formation of particles with a narrow dimension distribution. For therapeutic purposes, the nanoparticles were tested using anticancer medicines that, unlike other "drug delivery" systems, can be embedded in a non-covalent manner. This means that the resulting product is a new formulation of an existing drug, not a new molecule (new drug), which substantially shortens the approval process while retaining all the advantages of this approach. Nanoparticles with radionuclides suitable for non-invasive imaging PET and SPECT methods were also prepared and tested for diagnostic and radiotherapeutic purposes.

A substantial portion of the path to a practically applicable system is a proper choice of its chemical structure and the biodegradability. For that reason, the team members have developed, among other things, biodegradable nanoparticles that are mainly based on polyesters or micellar systems, decaying by erosion or by changing the pH and, moreover, having extremely low (almost equal to zero) adsorption of proteins on the surface of the nanoparticle due to a protective layer composed of biocompatible PEO / HPMA polymers or currently of poly (2-alkyl-2-oxazoline)-type polymers. Furthermore, the team has extensive

experience in the field of polymers specifically degradable by oxygen reactive forms and polymeric antioxidants, either in nanoparticle systems for targeted transport and controlled release of pharmaceuticals, or with antioxidant gels for topical application - wound healing (clinically used HEMAGEL® was developed at this site).

The targeted development of team activities in the field of nanoparticle systems during the last five years is represented by more than thirty publications in prestigious international journals such as Langmuir (IF = 4.38), Macromolecular Rapid Communications (IF = 4.929) Polymer Chemistry (IF 5.368) Journal of Controlled Release (IF = 7.261), Biomacromolecules (IF = 5.78) Nanoscale (IF = 6.23). International response to the team's scientific results has also been reflected in invitations to the publishing of referative articles and chapters in books and plenary lectures at international conferences. The list of top 10 publications with citation counts and other relevant publications are listed in Annex 4. The attractiveness of the research activities performed by the team members has resulted in high interest in cooperation from international partners through joint projects, internships, and PhD or postdoctoral stays with team members.

R&D centre of partner IKEM (ZRIR) is currently addressing in field of molecular and cellular imaging several research projects that are funded from different sources (CSF, AZV Ministry of Health, GA UK). During the implementation it cooperates with the above named scientific partners (see chapter 3.3) and listed in Annex 3.

None of the existing research activities of IKEM has been supported under OP RDI and OP Education for Competitiveness. Through OP Education for Competitiveness program the Institute implemented in the past, only one project, and a project called "Longevity without drugs: Popularization and promotion of innovations in research of non-pharmacological possibilities of influencing health status" (registration number CZ.1.07 / 2 March 00 / 35.0039).

The results of the research activities of IKEM workers are traditionally published in the form of scientific publications. Among the most important 10 results of MR group in the years 2009-2015 are publications listed in Annex 4. Publications of workers and the entire ZRIR are quite extensive, including publications in international journals with IF. Workers of ZRIR are members in the editorial boards of domestic and foreign journals (Cardiovascular and Interventional Radiology, Contrast Media and Molecular Imaging, Cor et Vasa, Czech radiology, Practical radiology). Prof. Peregrin (Head of ZRIR) is the president of the Foundation CIRSE (Cardiovascular and Interventional Radiological Society of Europe) for education.

IKEM at the end of 2015 employed 73 scientists, 10 best workers are listed with the relevant scientometric data in Annex 4.

The most important results of the **R&D Centre of the partner ISI CAS (EEM)** in the years 2014-2015 included the implementation of the reconstruction of the unique experimental ESEM AQUASEM II and the opening of a new European laboratory for a high resolution environmental electron microscope equipped with a QUANTA 650 FEG microscope, whose description is provided in chapter 3.3. The R&D Centre has long been participating in the research and development of custom solutions for scintillation detectors for electron microscopy. The results are tens of realized backscattered electron detectors for the companies Hitachi, Jeol and FEI and for example also in collaboration with the company Tecpa s.r.o. and AutraDet unique Edge free fiber optics for detectors supplied by Hitachi. On the basis of the cooperation agreement, the EEM R&D Centre has been cooperating with the company Crytur for a long time as a global manufacturer of scintillation monocrystal. Together

with this company and the company Tescan, the new scintillation monocrystal CRY18 [1] was introduced. In the area of the research and development of ionization detectors for ESEM, the EEM R&D Centre is a world leader. In recent years, the centre developed a unique scintillation ionization detector for a secondary electron detector for ESEM [2, 3], enabling the implementation of dynamic in-situ experiments in a wide range of gas pressures from 0.0001Pa to 1000Pa in the ESEM sample chamber. The as of now only detector in the world capable of detecting an energy-separated and considerably amplified signal in an environment of high pressure gases in the ESEM was patented by the centre in 2011 [4]. Within a series of applied research projects of the agency TACR and MPO (projects) the EEM R&D Centre worked with a number of companies on research of various materials in the field of energy, electrochemical sensors, differential pressure gauges etc. The EEM R&D Centre also developed and published a number of unique methods, for example for the study of the morphology of human stem cells in REM and ESEM [5, 6], for the study of live and experiment surviving small animals in the high pressure ESEM environment [7, 8, 9] for the study of native surfaces of plant samples, in particular of early somatic embryos (Low temperature method for ESEM) [10, 11], and vegetable waxes [12], a method for the repeated observation of biological samples in ESEM [13] and a method for studying the morphology of very sensitive biological and polymer samples [14, 15, 16]. The EEM R&D Centre is currently the only one in the world, owning the technology and know-how for the high resolution morphological characterization of completely native and humid polyelectrolyte capsules and particles [17, 18] and for the dynamic in-situ studies of ice [19, 20] using the ESEM.

The issue of the characterization of the surface of ice using ESEM is addressed with the University of Cambridge in a joint project. In the area of the Monte Carlo simulation of the interaction of signal electrons with gas, the EEM R&D Centre is one of the world leaders. On the basis of the activities of several GACR projects, the first version of special software for the simulation of trajectories of signal electrons in gas was created and the quantification of the amount of signal electrons of specific energy hitting the ionization detector was created [21, 22].

This software is a unique tool for the development of highly specific detectors for ESEM. The EEM R&D Centre has also long been involved in the simulation of gas flow in ESEM, and optimizing the design of electron-optical components of ESEM with respect to the pumping of gas [23, 24]. As part of the integration of new technologies of laser micromanipulation techniques, optical microscopy and Raman spectroscopy in the ESEM, the EEM R&D Centre within ISI ASCR cooperates with a group of optical micromanipulation techniques led by Prof. Zemánek [25].

Existing research activities were supported within the ALISI centre, therefore in the framework of the OP RDI project. As part of the proposed project, their further development is planned, as described in Chapter 5.3. A list of references mentioned in this text could be found in Annex 3 as the evidence for the work supported by ALISI centre.

3.5. Connections of R&D and education

The applicant IEB CAS is active in both pre-gradual and post-gradual university education. At the end of 2015, there were 62 PhD students working in IEB CAS (13 from abroad) and 151 pre-gradual students. Researchers of IEB CAS are very active in teaching in regular accredited programs at several universities including Faculty of Science, Charles University in Prague, University of Chemistry and Technology in Prague and Palacký University in Olomouc. In 2013-

2015 altogether 5000 h in bachelor, magister and doctoral stage of the study has been lectured by members of research teams form IEB CAS. At the end of 2015, there were 24 collaborative projects with universities implemented, 32 researchers from IEB CAS had partial contract at the university and 21 university workers had partial contract in IEB CAS. For numerous universities IEB CAS researchers elaborated opponent's reviews for their bachelor, master and PhD thesis (CU, UP, CULS, UCT, CTU, MU and others). The up to date list of universities and their accredited doctoral programs with the participation of IEB CAS could be found in Annex 5.

R&D centre of IEB CAS is involved in teaching as well as in tutoring of many students ranging from talented secondary school students, bachelor and diploma to PhD Students, who work on their theses within the laboratories of R&D centre of IEB CAS. For the centre, the most frequent collaboration is with the Faculty of Science, Charles University, in particular with department of experimental plant biology, department of genetics and microbiology and department of biochemistry. Researchers of the R&D centre supervise and consult PhD thesis, their list is briefly mentioned in Annex 5 and in detail in CVs of team members in Annex 7. Student of accredited doctoral programs are directly involved in the investigation of the particular research programs.

The R&D centre SUPRAMOL IMC CAS collaborates Faculty of Science, Charles University in Prague, Palacký University in Olomouc, Institute of Clinical and Experimental Medicine, Institute of Organic Chemistry and Biochemistry AS CR, v.v.i., 1st Medical Faculty of the Charles University in Prague and Faculty of Science of the Masaryk University in Brno. **R&D centre SUPRAMOL** is in active in the framework of accreditations with Faculty of Science, Charles University for the discipline macromolecular chemistry (student Loukotová), for physical chemistry (students Rabyk, Holubová, Kaberov, Sincari) and for analytical chemistry (student Trousil), accreditation with Faculty of Mathematics and Physics Charles University in Prague for discipline biophysics (student Babuka) and accreditation with University of Chemistry and Technology Prague, discipline chemistry of macromolecular compounds (student Kolouchová). Members of SUPRAMOL centre are active in teaching and organizing seminars (Štěpánek, Hrubý), they serve as tutors of bachelor, diploma and dissertation theses (studenti Trousil, Brezaniová, Pospíšilová, Groborz).

Partner IKEM has traditionally been concerned with education and postgraduate courses for scientists and practitioners through extensive teaching activities at the level of undergraduate, postgraduate and continuous education. IKEM is involved in teaching of Czech as well as foreign students from all three Prague medical faculties of Charles University, through extensive lectures. It also provides training and attestation internships and organizes specialized seminars for students. Selected IKEM clinics are direct teaching faculties workplace and many doctors of IKEM are active academics in all three Prague medical faculties of Charles University.

Apart from the medical faculties of Charles University IKEM is deeply involved in teaching graduate students at the Institute of Postgraduate Medical Education (IPVZ) and participates in the education and training of undergraduate, graduate and Ph.D. students at the Institute of Health Studies, Technical university of Liberec, Faculty of Science, University of South Bohemia, the Faculty of Science Charles University, Faculty of Nuclear Sciences and Physical Engineering CTU, Faculty of Food and Biochemical Technology UCT, and the Department of Medical Biochemistry and Laboratory Diagnostics, 1st Faculty of Medicine Charles University.

At the IKEM there is also carried out training of paramedical staff (in collaboration with IDPVZ) and doctors and nurses significantly contribute to the lifelong education of nurses and doctors outside IKEM.

Overview of current accredited doctoral programs (accreditation granted by the Ministry of Education Youth and Sports to IKEM) is given in Annex 5.

R&D centre ZRIR at IKEM is in the education of scientists and physicians involved mainly through extensive lectures. Workers lectured or participated in several subjects at CTU and the Charles University. In the field of postgraduate training is ZRIR sub-department of interventional radiology IPVZ. It organizes regular courses in the field of interventional methods and organizes certification in the field of interventional radiology. Many ZRIR workers are involved in postgraduate training IPVZ and undergraduate teaching in other departments (Faculty of Medicine, Charles University, University Hospital in Hradec Kralove, The University Hospital Brno). Workplace radiologists and interventional radiology is an accredited institution for post-secondary education in the field of interventional radiology. The workplace has further accreditation of Ministry of Health for postgraduate teaching for radiology assistants - Certified course - Magnetic resonance imaging, as well as professional experience - Magnetic resonance imaging and professional experience - Imaging procedures in interventional radiology and cardiology. Workers of MR group also organized the project Clinical MR spectroscopy within the educational event of the European Society for Magnetic Resonance in Medicine and Biology "Lectures on MRI."

For more information about education in the individual departments of the Institute are available on the website IKEM: http://www.ikem.cz/cs/vzdelavani/a-6/

ISI CAS including its R&D Centre (EEM) has many years of experience in cooperation with universities in the field of study programs and continuing education. Through joint accreditation at selected study programs, ISI cooperates particularly with the Brno University of Technology and Masaryk University, but also e.g. with the Faculty of Science of Palacký University in Olomouc. In 2015, four professors and four associate professors worked at the Institute. ISI staff lectured a total of 495 lesson hours in bachelor, master and doctoral programs and guided 30 dissertations. List of accredited doctoral programs, to whose ISI contributed to is provided in Annex 5.

Currently the head of the EEM research group, Dr. Neděla, is leading two doctoral students of Electrical Engineering and Communication. Their dissertation is devoted to the following topics: The study of the effect of magnetic and electrostatic fields on the efficiency of the detection of secondary electrons in the high pressure gas environment of the ESEM and the Detection of photons in the high pressure gas environment of the environmental scanning electron microscope. These topics fit entirely into the research programs and activities of ISI, in which the students in the framework of their doctoral program contribute their research work. Dr. Neděla is also involved in teaching the subject Electrotechnical Materials, Material Systems and Manufacturing Processes, through which the aforementioned doctoral program students pass.

ISI is also dedicated to supporting young researchers. In the last two years, it gained two young researchers working within the EEM research group "Support Programme for Perspective Human Resources – Wage support of postdocs at ASCR worksites". The area of their research falls under the topic of the study of biological samples in conditions of low energy environmental scanning electron microscopy and the research of new methods for sensitive water containing samples in the native state and the research of nanostructural

modifications of construction binding by the combination of the methods of environmental electron microscopy and physico-chemical analyzes. These topics are fully in line with the research focus of the EEM research group.

The EEM research group organizes the autumn school of the foundations of electron microscopy every two years with the support of other ISI research groups and co-organizing companies and partners. This is a five-day theoretical course with practical demonstrations designed especially for doctoral students, postgraduate students and young professionals in the field of electron microscopy.

4. Project Proposal General Objectives

Project activities	
Activities that are going to be carried out to fulfil general objectives of the project	Development of the centre: a), b), d), e) a f).

5. Research programs, International cooperation, Teams, Infrastructure

5.1. Research program 1: Functional microscopy of plant biomembranes and surfaces

Abstract

Biomembranes are complex structures composed of lipids and proteins. Their main role lies in the compartmentation of organelles that form the endomembrane system of all eukaryotic cells. In addition, in the form of a plasma membrane (PM) they form an important communication barrier between the internal and external environment of cells and organisms. Biomembranes fulfil several basic functions associated with the exchange of substances and signal transduction. In plant cells, PM represents the barrier that in the outward direction is involved in the formation of cell walls and surfaces. In the opposite direction, PM regulates the transport of wide spectrum of substances and determines the positioning of components of PM-associated signal transduction machinery.

The understanding of the dynamic structure of PM as well as endomembranes and their phospholipid and protein composition would not have been possible without significant advances in the microscopy techniques used in their studies. These techniques have been for a long time limited to studies of chemically fixed preparations that did not reflect well the native structure of biomembranes, cell walls, and surface structures, or they used rather unspecific labelling of lipid and protein membrane components. For this reason it is necessary to develop non-invasive, *in vivo* techniques of microscopy analysis, which would allow both specific labelling of protein and lipid structures as well as their observation in high resolution. By the substantial improvement of *in vivo* microscopy techniques and specific labelling in collaboration with partners from EEM ISI CAS, ZRIR IKEM and SUPRAMOL IMC CAS, the aim of

this research program is to contribute to the understanding of the structure of the PM, endomembrane system, and surfaces of plant cells. Two objectives that will be addressed within this research program will be 1) The elucidation of the significance of biomembrane dynamics for plant development and 2) The identification of composition of cell surfaces and intercellular space.

5.1.1. Relation to research programs of the centre, further centre development

This research program is proposed as direct continuation of several recent research activities of R&D centre of IEB CAS, which are specified in detail in chapter 3.4. All of these activities represent high quality research, which has been in past decade performed in R&D centre of IEB CAS with the emphasis on the advanced fluorescence microscopy techniques of plant biomembranes, cytoskeleton, cell wall and cell surfaces. The main idea of this research program is to further improve and technically support research activities that already yielded high quality original contributions in high quality scientific journals. Advanced imaging techniques of confocal laser scanning microscopy (FRAP, FRET, RICS, FCS, spectral analysis, co-localization analysis) spinning disk microscopy (kymograph analysis) and structured illumination microscopy that has been all optimized by research teams of R&D centre of IEB CAS will be shifted to the qualitatively new level by adding the expertize and instrumentation of non-invasive electron microscopy together with dramatic improvements in the specific labelling of components of plant membranes and surfaces. Altogether, although for IEB CAS this research program represents primarily curiosity-driven activity, the involvement of three other partners into this centre of excellence is going to open several new horizons for oriented research in the area of nanotechnologies.

5.1.2. State of the art

The aim of this research program is to contribute to the understanding of the structure of the PM, endomembrane system, and surfaces of plant cells. In agreement with the strategy of this proposal for excellent research centre, it will be primarily oriented towards advanced imaging techniques combining both light and electron microscopy. Such combination of techniques represents very unique opportunity to achieve significant advance in both biology of eukaryotic cells on one side and advanced instrumentation on the other side.

The plasma membrane (PM) is the defining feature of all living cells, which serves as the interface with the surrounding environment. Current models postulate that PM is laterally organized in various domains with distinct protein and lipid composition and diverse spatial and temporal characteristics (Jarsch et al., 2014; Sekeres et al., 2015). Existence of such multiple domain types have been proposed to be crucial for the efficient channelling of cellular signalling, membrane traffic and communication (Malínský et al., 2013). The dynamics of these domains and their protein and lipid constituents is tightly connected to the cortical cytoplasm through cytoskeletal network, actin filaments (AFs) and microtubules (MTs). The role of highly dynamic plant AFs (Henty-Ridilla et al., 2013) in the endosomal trafficking is well-established through the activity of myosins (Brandizzi and Wasteneys, 2013; Ueda et al., 2015). The dynamics of both AFs and MTs is differentially regulated by Rop (Rho of Plants) GTPases, which act as common upstream factors signalling to both cytoskeletons (Chen and Friml, 2014).

Thanks to their intensive research, auxin carriers might exemplify the membrane cargo on which the mechanisms of intracellular trafficking of membrane proteins could be easily

studied. Although clathrin has been established as the important element of the recycling machinery for some auxin carriers (PINs), it is not known how the clathrin-coated vesicle might be attached to acto-myosin and which myosins might influence the localization of auxin carriers in the plasma membrane. Similarly, the role of dynamins that perform membrane scission activity during the formation of endocytic vesicle is unclear. The trafficking of PIN proteins has been followed by confocal microscopy in our laboratory in several studies. We have shown by FRET that dynamin DRP1A (Mravec et al., 2011) interacts with PIN2 on the growing ends of the cell plate and that the PM deposition of this auxin carrier is regulated by its ubiquitylation (Leitner et al., 2012) and also that endocytosis of tobacco PIN proteins is cytoskeleton-dependent and involves the activity of NtGNL1a guanine GTP exchange factor (Jelínková et al., 2015). Our recent fluorescence recovery after photobleaching and fluorescence spectroscopy data indicate that auxin influx and efflux carrier mobility within the plasma membrane are differentially dependent on cytoskeleton (Laňková et al., 2016). Preliminary microscopy data further show that even single mutation in the individual alpha- and beta-tubulin genes triggered changes in the membrane distribution of PIN1 and PIN2 in Arabidopsis roots. The connection of PIN dynamics to MTs was recently shown to be assisted by MTs plus end binding protein CLASP (Ambrose et al., 2013). Auxin carriers are maintained in the plasma membrane in the association with cell wall (Martinière et al., 2012; Feraru et al., 2011), which creates another constraint that affects membrane dynamics.

Cell wall is a unique plant structure, which connects plant cells within tissues providing them with a mechanical support. Cell walls are able to increase their volume while retaining mechanical properties at the same time. Numerous metabolic processes are located within cell walls, and some products may be stored there. Cell walls are modified through the deposition of various organic or inorganic compounds, which leads to further fortification of the cell wall against mechanical stress, decrease of permeability for various compounds including water, and pathogens attack. Besides above mentioned fascinating properties of cell walls, the cell wall is economically important structure. The main load-bearing component of the cell wall, the cellulose, is used extensively in the industry or everyday life of man. Since an effective method of in vitro cellulose synthesis is still not available, the only source of cellulose are cell walls.

Models that are used these days for studies of biomembranes and surfaces range from artificial membrane vesicles through plant protoplasts isolated from various cell types, cultured cells, seedlings of various age to intact plants represented by model organism *Arabidopsis thaliana*. The most effective is to study the dynamics of PM and cell wall synthesis in polarized, elongating cells that are represented in this project by individual tissues of root and shoot, including both diffusely growing root and hypocotyl cell and tip-growing root hairs as well as pollen tubes (male gametophyte). In addition, cell wall synthesis and composition of surface layers is often studied on cells with more complex morphology, e.g. epidermal lobed cells of plant leaves. In all these systems, microscopy techniques that are being used in past decades are largely invasive and they do not allow to study structures of PM, cell wall and cell surfaces non-invasively in high detail. This is addressed in this research program by combining expertize of three partners supporting the research centre of IEB CAS (see Fig. 5.1.1). Besides techniques optimized in IEB CAS, i.e. advanced fluorescence microscopy, this program will utilize environmental electron microscopy techniques of ISI CAS and newly designed nanoparticles of IMC CAS to bring the microscopy on the qualitatively new level.

Identification and observation of structures in high resolution under native conditions and ideally with specific labelling represents stimulus that will shift both basic research in the field

of plant cell biology, but it will also potentially be important for practical applications, e.g. targeting of specific nanoparticles into specific compartments, which in case of cell wall will potentially open new possibilities for the modification of textiles.

The topic of this research program represents highly interdisciplinary research activity, where the success depends on the high quality research in the area of plant cell biology (IEB CAS), human and animal cell biology (IKEM), macromolecular chemistry (IMC CAS) and development of new instrumentation (EEM ISI CAS). Each of two main research aims that are specified below contain several experimental activities and their milestones. These milestones represent achievements that are firstly, scientifically excellent with very high publication significance and secondly, they are ambitious on the international level so that proposed centre of excellence will further increase its international reputation. Although the primary goal of this research program is rather basic interdisciplinary research, the character of this excellent research centre has capacity to produce information that could potentially lead to the applications, mainly in the field of plant nanotechnologies and more importantly dramatic improvement of functional microscopy.

5.1.3. Research objectives, activities and results

This research program is primarily affiliated to the R&D center of IEB CAS. Interdisciplinary character of this research is represented by numerous co-operations within the framework of 2 research objectives and 7 activities. Their depiction is schematically represented in Fig. 5.1.1. Detailed description of each research objective and all research activities, including their milestones is described in the following text.

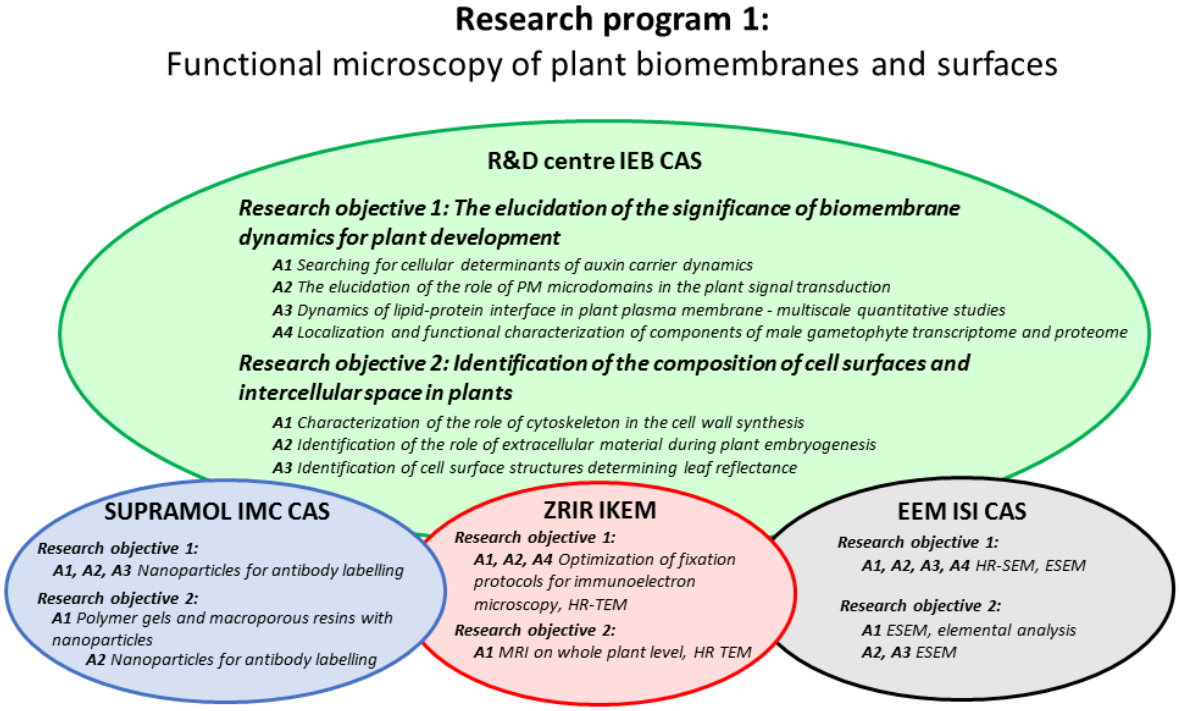

Figure 5.1.1: Schematic depiction of research program 1. This research program is primarily affiliated to the R&D centre IEB CAS (green). Involvement of partner's R&D centres, SUPRAMOL IMC CAS (blue), ZRIR IKEM (red) and EEM ISI CAS (grey), is specified for individual research activities of IEB CAS.

Research Objective 1: The elucidation of the significance of biomembrane dynamics for plant development

As it has been introduced in the chapter 5.1.2, biomembranes and PM in particular are composed of dynamic mixture of phospholipids and proteins, which undergoes continuous rearrangements. Since PM represents an important nexus between the extra and intracellular space, this dynamics necessarily determines a plenty of signalling events. These include positioning of integral plasma membrane proteins and protein complexes with various functions such as harvesting light energy, channelling and pumping various ions, transporting a wide spectrum of low molecular weight molecules, receiving of ligands with subsequent signal transduction and remodelling the membrane for exo- and endocytosis events. Transport of membrane vesicles is responsible for the turnover of membranes between PM and endomembranes and this process is regulated on multiple levels for fine tuning of the lipid and protein composition of the membrane.

This research objective is focused on 4 activities that address 3 aspects of the membrane dynamics, i.e. how is the distribution of integral membrane proteins regulated (activity 1 and 4), how are membrane proteins assembled into microdomains (activity 2) and how the interaction between lipids and proteins defines membrane dynamics (activity 3). The research will be performed by the applicant research centre and three collaborating partners (See Fig. 5.1.1). 4 research activities include 1) Searching for cellular determinants of auxin carrier dynamics, 2) The elucidation of the role of PM microdomains in the plant signal transduction 3) Dynamics of lipid-protein interface in plant plasma membrane - multiscale quantitative studies and 4) Localization and functional characterization of components of male gametophyte transcriptome and proteome.

Activity 1: Searching for cellular determinants of auxin carrier dynamics

Head: *RNDr. Jan Petrášek, Ph.D.*

Plant hormone auxin plays an important morphoregulatory role during the development of sessile plant body (Vanneste and Friml, 2009). The setting of auxin concentration gradients in the plant tissues depends largely on the activity and localization of its influx and efflux plasma membrane transporters from AUX1/LIKE AUX1 (AUX1/LAX), PIN and ABCB protein families (Petrášek and Friml, 2009). To the various, developmentally regulated extents, all of these carriers are recycled between plasma membrane and endomembrane compartments by endosomal recycling pathways (Luschnig and Vert, 2014). The process of recycling that includes both exocytosis and endocytosis (Richter et al., 2009) determines the ability of particular carrier to be redistributed to another plasma membrane domain without *de novo* protein synthesis. Although the molecular machinery that regulate plant vesicle trafficking processes and the role of auxin in this process is intensively studied (Offringa and Huang, 2013), the trafficking of individual auxin carriers is described only fragmentary. The best characterized is the trafficking of PIN auxin efflux carriers that includes small GTPases, clathrin-dependent endocytosis (Kleine-Vehn and Friml, 2008), exocyst-dependent exocytosis (Drdová et al., 2013) and depends on the sterol composition of the plasma membrane (Men et al., 2008). Phosphorylations and de-phosphorylations of PINs regulate their recruitment into the various membrane domains (Habets and Offringa, 2014). The trafficking of AUX1/LAX and ABCB carriers also involve the activity of small GTPases and is dependent on the sterol composition of the plasma membrane, but details are mostly missing.

One of the major information that is lacking these days is the information on the localization of individual auxin carriers on the sub-cellular and ultra-structural levels. Within this activity we will concentrate our effort on the testing of “ultra-structural phenotypes” i.e. the localization of individual carriers in the set of *Arabidopsis thaliana* lines carrying mutations in genes coding for cytoskeletal and associated proteins and enzymes for cell wall biogenesis and sterol biosynthesis. Methods of high resolution fluorescence microscopy, advanced confocal microscopy, fluorescence correlation techniques as well as high resolution scanning electron microscopy and environmental scanning electron microscopy will be used in collaboration with partners from EEM ISI CAS (Fig. 5.1.1).

Major hypothesis that will be experimentally challenged is that individual types of auxin carriers differ in their association with cytoskeleton and that this determines their behaviour. As the extra value that will come out of this project will be the creation of web-based database of ultra-structural images and values characterizing dynamics and function of auxin carriers with respect to structures of cytoskeleton, plasma membrane and cell wall. This database could be further extended in future and could serve as an invaluable source of information for the community of experimental plant biologists.

This activity contains 3 research goals.

Firstly, cell structure determinants of the localization and dynamics of auxin carriers will be characterized. As the first step, molecular toolbox for indirect immunofluorescence staining and *in vivo* localizations of auxin carriers and screening by several high-end microscopy approaches for the localizations of auxin carriers in the collection of insertional mutant lines of *Arabidopsis thaliana* will be generated. This collection is already available in the laboratory. For MTs, the collection includes insertions in genes for *Arabidopsis thaliana* tubulin genes, microtubule-bundling proteins, plus-end binding proteins, microtubule stabilizing and severing proteins, nucleators and some other regulators of polymerization. For AFs, we have collected mutations in genes for individual actins, actin nucleation factors and actin binding proteins. The ultimate goal is to assemble the collection of mutants that would be complete in terms of up to date knowledge. At the moment we have around 40 individual lines ready and plan to genotype and verify with qRT-PCR other 50 lines, including cell wall biogenesis mutants and sterol biosynthesis deposition mutants.

To search and screen for the localization of auxin carriers, specific antibodies for indirect immunofluorescence staining or translation fusions of genes coding for auxin carriers with fluorescent proteins are needed. As the first step, we will start to generate primary antibodies against auxin carriers. At the moment, we already have functional, mostly commercial (Agrisera, NASC) polyclonal antibodies against Arabidopsis PIN1, PIN2, PIN3 and PIN4. They are used regularly in the laboratory for the immunostainings in Arabidopsis seedlings using automated immunostaining station (Intavis Pro VSi). The protocol allows simultaneous labelling of two auxin carriers using green and red Alexa-labelled secondary antibodies (Invitrogen). The antibodies against AUX1/LAXes and ABCBs will be produced commercially (not all are available at the moment). We will also generate new gene constructs carrying genes for auxin carriers in translational fusions with photo-activable GFP (PA-GFP) for the photo-activated localization microscopy (PALM) as well as with other spectral variants of fluorescent proteins for co-localizations. It will be also necessary to perform genotyping and qRT-PCR verifications of all mutants used in this work. For *in vivo* approaches, we have already started crossing and have collection of mutants in individual tubulin and actin genes crossed with all members of PIN family in fusion with fluorescent protein under natural promotor. In principle, there should not be any pitfalls during the generation of the set of antibodies and

marker lines, but it could be that we will need to test more antibody versions generated using oligopeptides synthesized from various regions of the carrier.
Based on our preliminary data that shows changed patterns in the root distribution of individual PINs, we will screen for the localization of all auxin carriers using indirect immunofluorescence staining in wild type and mutant seedlings of *Arabidopsis thaliana*. We will concentrate on root, hypocotyl, cotyledons and eventually first young leaves to follow the expression domains for individual auxin carriers. This primary screen will be performed by confocal laser scanning microscopy, the attention will be concentrated on the cellular and subcellular level using immersion objectives with high magnification and numerical aperture. In parallel, *in vivo* data will be grabbed from crossings of mutants with fluorescent marker lines for all individual auxin carriers. This mostly classical confocal fluorescence screen will be complemented with spinning disk confocal microscopy, where individual PM microdomains containing auxin carriers are easily observable (Fig. 5.1.2). We will also perform correlative spectroscopy determinations combined with FRAP in the roots of Arabidopsis thaliana mutants according to procedure described recently in our laboratory (Laňková et al., 2016).

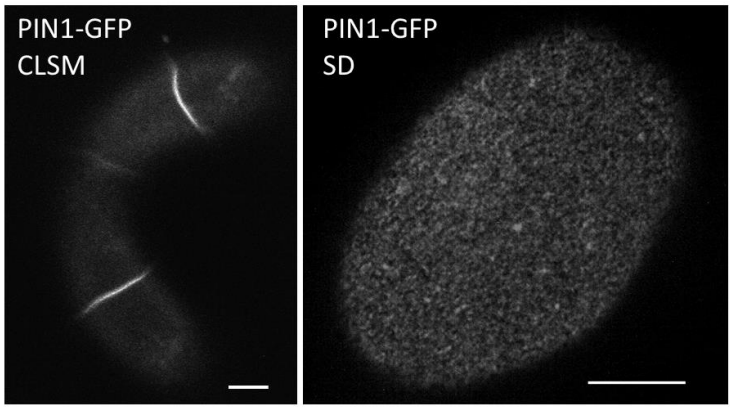

Figure 5.1.2: **Distribution of PIN proteins in the plasma membrane and cortical cytoplasm *in vivo*.** PIN1::PIN1:GFP in tobacco BY-2 cells observed by CLSM (left) or spinning disk (SD) microscope (right). In contrast to CLSM imaging spinning disk allows the visualization of plasma membrane heterogeneity that might be tracked *in vivo* or their distribution quantified after fixation. Scale bars 10µm.

In addition to above mentioned fluorescence-based microscopy, high-resolution scanning electron microscopy on fixed samples (HR-SEM) will be employed in the collaboration with partner from EEM ISI CAS. As shown in Fig. 5.1.3, we have optimized protocol for the preparation of samples for high quality HR-SEM in PM and cortical cytoplasm. The technique uses protoplasts isolated from the plant tissue (tobacco, Arabidopsis) and preparation of "membrane ghosts" (Krtková et al., 2012b), the fraction of PM containing many associated proteins. Subsequent exposition of this fraction to the fixation procedure (Fišerová and Goldberg, 2014) give very good results that allow clear identification of many structures within the region below plasma membrane that could be easily identified based on their morphology (Fig. 5.1.3A, B). Moreover, this technique will be combined with specific immunostainings using nanogold-labelled secondary antibodies (Fig. 5.1.3C) or fluoronanogold-labelled secondary antibodies for correlative approaches combining fluorescence and electron microscopy (Goldberg and Fišerová, 2010). Other nanoparticles for this technique will be

prepared by partner R&D centre SUPRAMOL IMC CAS. Improvements of the instrumentation of partner at ISI CAS will also allow screening our candidates *in vivo* with ESEM to understand how these interactions define the localization under *in vivo* conditions. We plan to apply immunocytochemistry also in ESEM according to Rosso et al (2013) and in collaboration with partner from IKEM to further improve electron microscopy fixation protocols for cultured cells of tobacco and Arabidopsis as well as for whole mounts of root, cotyledons and hypocotyls of Arabidopsis (Fig. 5.1.1).

Secondly, new determinants of auxin carrier dynamics will be identified. The same procedure starting from membrane ghosts as in HR-SEM will be used here to obtain fractions (protoplasts ghost) that will be used to isolate all proteins that are in association with plasma membrane within the cortical cytoplasm. Protoplasts will be isolated from the roots of *Arabidopsis thaliana* wild type seedlings using enzymatic digestion. The technique is already established in the laboratory (Krtková et al., 2012b). We will also try to perform the same procedure with our set of mutants. By immunoprecipitations using specific antibodies against auxin carriers it will be possible to get fractions that will potentially contain interacting partners. Depending on the mutant, some of these interaction might be found to be missing or appearing. We will identify interaction partners using outsourced matrix-assisted laser desorption/ionization spectra (MALDI) service. We will also take opposite approach using antibodies against proteins that appeared to be important for the localization of auxin carriers and identify these spectra as well. Finally, co-localization studies of the identified candidates with auxin carriers will be performed.

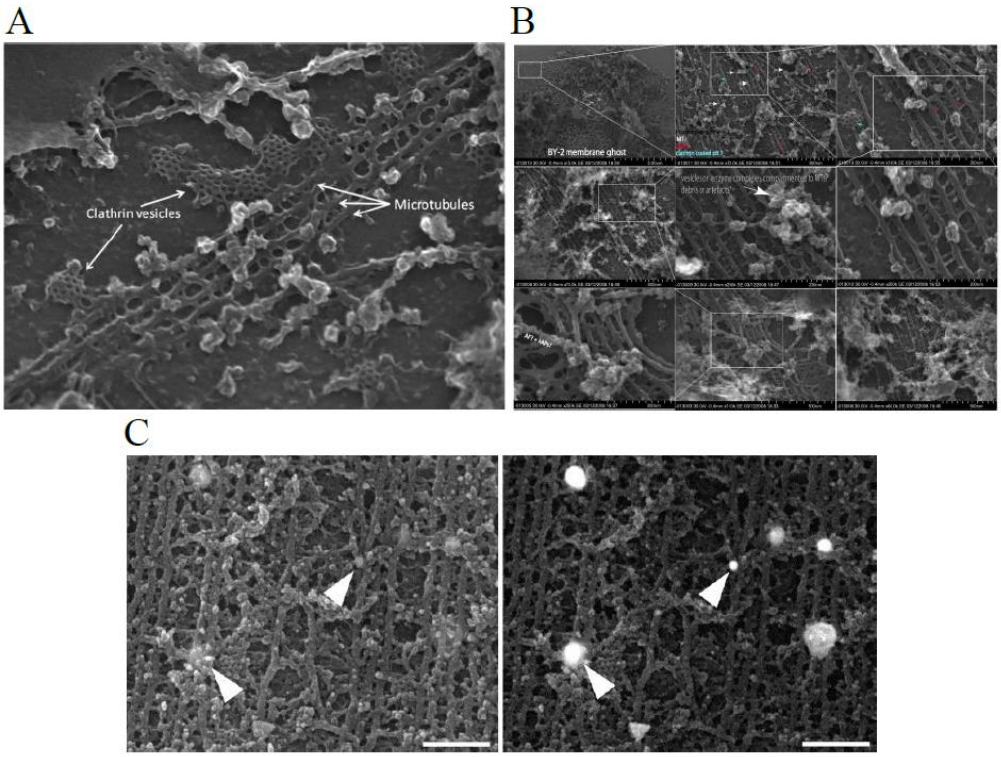

Figure 5.1.3: High resolution scanning electron microscopy (HR-SEM). (A, B) Samples from membrane ghosts isolated from tobacco BY-2 cells. Structures of cytoskeleton are well observable, including microtubules and associated clathrin vesicles **(A)**. The complexity of structures observable with HR-SEM **(B)**. (Fišerová, J., Goldberg, M. unpublished). **(C)** Immunogold staining of microtubule plus

end binding protein EB1 on HR-SEM image from Tradescantia epidermal peels. Secondary electrons (left) and back-scattered electrons (right) (Barton et al., 2008).

Thirdly, web-based catalogue of localizations, dynamics, function and interactions of auxin carriers will be generated. Since the project will generate huge amount of images, the aim will be to improve our techniques for image analysis so that we will be able to quantify even subtle changes in the distributions of auxin carriers. We plan to integrate data from CLSM, SD, HR-SEM and EREM to perform, where possible, correlative measurements.

Milestones: Web-based catalogue containing novel determinants of dynamics of auxin carriers obtained by advanced fluorescence and electron microscopy approaches.

Activity 2: The elucidation of the role of PM microdomains in the plant signal transduction
Head: *RNDr. Jan Martinec, CSc.*

Using primarily molecular biology approaches and advanced light and electron microscopy the primary goal of this activity is to elucidate the role of PM microdomains and proteins participating in their formation in the perception and processing of the signal from the environment. This activity is the continuation of our long-lasting activities in the field of PM-associated signalling activities (Krtková et al., 2012a; Krčková et al., 2015; Matoušková et al., 2014; Pejchar et al., 2015) and in particular, the project focused on the flotillin proteins and their homologs from plants (see chapter 3.4). Both flotillins and HIRs (Hypersensitive Induced Reaction) proteins are peripheral membrane proteins, which have been located in discrete PM locations with variable dynamics in our laboratory (Fig. 5.1.4) and which correspond to previously published punctate patterns of other proteins (Jarsch et al., 2014). These localizations reflect heterogeneity in the PM organization and correspond very likely to lipid ordered phase (Lo phase), in which the aggregation of proteins with various function (e.g. receptors, ion channels, enzymes, etc.) is facilitated. These domain thus serve as important hubs for various signalling events (Konrad and Ott, 2015; Tapken and Murphy, 2015). However, the role of flotillins and their homologs in the formation and maintenance of microdomains is not yet know, they very probably act as scaffolding proteins for various kinases, receptors, transporters and cytoskeletal proteins. It is still not fully understood, whether plant flotillins play similar role in endocytosis of membrane receptors or carriers as described for their animal homologs (Fan et al., 2015).

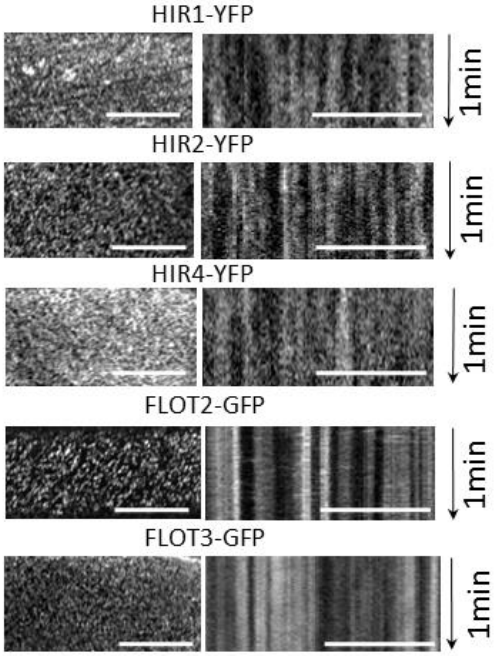

Figure 5.1.4: Localization and dynamics of HIR proteins and flotillins (HIR1-YFP, HIR2-YFP, HIR4-YFP, FLOT2-GFP, FLOT3-YFP) in elongating root epidermal cells of *Arabidopsis thaliana*. Spinning disk confocal microscopy. Scale bar 10µm (left) and 5 µm (right). Left, PM microdomains of variable dimensions for all tested proteins. Right, kymographic analysis (1 min) showing stable microdomains for both flotillins in contrast to HIRs.

There are three interconnected goals of this activity.

Firstly, we will determine how flotillins and HIRs contribute to the integrity of Lo PM phase. Fluorescent probes with differential emission in Lo and Ld (lipid-disordered) phase (Laurdan, di-4-ANEPPDHQ) will be used in plants with modified expression of flotillins and HIRs. Both overexpressors (35S) and conditional single and multiple mutants (CRISPR-Cas9) will be studied with confocal laser scanning microscopy, structured illumination microscopy and spinning disk microscopy. Using this approach, the identity of microdomains after chemical modification of sterol composition of PM will be also followed using filipin or methyl-β-cyklodextrin.

Secondly, interaction of flotillins, HIRs and their interactors will be studied in vivo. One of the most important result of the previous project was the immunoaffinity purification followed by LC-MS/MS identification of a set of interacting proteins (Junková et al., in preparation). Genes coding for selected candidates will be expressed in translational fusions with fluorescent proteins and the interaction tested with confocal microscopy by co-localization analysis and FRET. In addition, inhibitors of endocytosis and intracellular trafficking will be used to test the endomembrane-PM dynamics of microdomain components. Immunofluorescence staining using specific antibodies will be utilized to further test dimerization of flotillins and their homologs. In the collaboration with partners from IKEM and EEM IMC CAS (see Fig. 5.1.1.), gold and nanoparticle-labelled antibodies will be prepared and plant samples observed using TEM and HR-SEM and ESEM (EEM IMC CAS). Electron microscopy approach is expected to provide details in the composition of microdomains and will be further complemented with super-resolution microscopy on these samples (instrumentation within the CzBioimaging node).

Thirdly, signalling role of flotillin and HIR microdomains in reaction to changes in the environmental conditions will be studied. Besides changed transcription of flotillins and HIRs after various stress conditions, changes in the localization and physical interactions of HIRs within their microdomains are expected (Qi et al., 2011). Therefore, a spectrum of biotic and abiotic stressors (cold, heat, hypoxia, bacterial and fungal pathogens) will be applied and microdomain characteristics observed, namely their aggregation or disassembly and oligomerization or dissociation. Methods for assessing protein dynamics and interactions with confocal microscopy (FRAP and FRET) will be performed. Qualitatively new information will be obtained from ESEM in collaboration with partners from (ISI CAS) using specific antibodies prepared in the previous part of this activity.

Milestones: Collection of data on the microdomain composition of plant plasma membrane.

Activity 3: Dynamics of lipid-protein interface in plant plasma membrane - multiscale quantitative studies

Head: *Ing. Martin Potocký, Ph.D.*

As it has been mentioned in state of art of this research program, the co-operation of lipid and protein components of PM is not well understood and therefore this research program is focused on the mechanisms defining this dynamic interplay.

In past 15 years, minor acidic membrane phospholipids such as phosphatidylinositol 4,5-bisphosphate (PIP2) or phosphatidic acid (PA) have been implicated in many regulatory processes that occur at the PM. PA and PIP2 (together with other minor phosphoinositides) are now recognized as more than just precursors or intermediates in classical signaling pathways and their role in targeting of effector proteins to distinct PM domains or in modulating of PM curvature is indisputable (Sekeres et al., 2015). Interestingly, with the exception of sterols, the role of lipids in the lateral organization of the PM is not well understood and we know very little about the involvement of acidic phospholipids despite their relative abundance in the detergent-insoluble membranes (Furt et al., 2010). In particular, the mechanistic details about the interaction, dynamics and mutual regulation between membrane proteins and their membrane microenvironment are scarce even for animal/yeast models, and virtually unknown in plant cells.

Highly polarized plant cells like root hairs or pollen tubes represent particularly convenient model system for studies on membrane organization due to their prominent protein and lipid segregation in the plasma membrane resulting in functionally specialized domains. These are formed by properly balanced exocytosis, endocytosis, endomembrane trafficking and two-dimensional lateral mobility within plasma membrane. Furthermore, these cell types are amenable to genetic, molecular, pharmacological, biochemical, microscopic and systems approaches.

Molecular simulations and especially molecular dynamics (MD) have become an extremely valuable tool to study membrane and protein systems, since they provide molecular resolution that is still unreachable through experimental approaches. Recently, with the increase in computational power and better algorithms, MD computations have reached time and length scales directly comparable with experiments and they thus represent a complementary tool to the experimental methods. Moreover, they enable us to probe the system of interest at the single-molecule level (Bennett and Tieleman, 2013). Typical simulated times for all-atom simulations are up to microseconds, but this can be further

increased even to milliseconds using a coarse-grained (CG) force field, in which groups of atoms are represented as one particle (Ingolfsson et al., 2016). In the past 4 years we have made significant contribution in establishing MD-based research in plant science community (Pleskot et al., 2012; Potocký et al., 2014; Pleskot et al., 2015).

The main question that is going to be addressed in this activity is: **What are the roles of distinct classes of membrane lipids in modulating protein lateral mobility and partitioning in the plasma membrane?**

Based on our previous work and recent progress in the field, we aim to characterize in detail the dynamics behaviour of plant plasma membrane using state-of-art live imaging, spectroscopy, and molecular dynamics computations. Rather than examining the artificial membranes detached from the native biological context, our approach will be to examine the lateral dynamics of phospholipids and proteins in the intact plasma membrane of living plant cells, with the full biological context present.

This activity contains 4 interconnected research goals.

Firstly, **genetically-encoded probes for quantitative microscopic assessment of lateral phospholipid mobility in living cells will be developed and optimized.** We will continue our work on the fluorescent biomarkers detecting PA (Potocký et al., 2014) as well as other acidic phospholipids (Simon et al., 2014). Constructs bearing tandem duplicate or triplicate lipid-binding domains will be prepared to achieve highly specific and selective binding necessary for quantitative studies. We will design and test genetically-encoded markers specific for common structural phospholipids such as phosphatidylcholine (PC) and phosphatidylethanolamine (PE). These will be based on PC-binding domains of BSP-A/PDC-109 proteins (Anbazhagan et al., 2011) and PE-binding Maximin H5 peptide (Dennison et al., 2013), respectively. We will test the constructs in transient assays and stably transformed Arabidopsis and tobacco lines expressing optimized constructs will be prepared and characterised as well. This part of the work is going to be connected with the research program of partner institutions IMC CAS and ISI CAS, with the aim to test new nanoparticles for simultaneous specific labelling for fluorescence and electron microscopy (see Fig. 5.1.1).

Secondly, **quantitative assessment of phospholipid dynamics in plant plasma membrane will be performed.** The mobility PM phospholipids will be assessed by a combination of advanced optical methods, including fluorescence recovery after photobleaching (FRAP), single-particle tracking (SPT) and raster image correlation spectroscopy (RICS). In addition to phospholipid markers based on genetically encoded lipid-binding domains fused to XFPs (first goal), we will also use commercially available synthetic fluorescent phospholipid analogs. This will allow us to analyze the behaviour of both free and occupied fraction of given phospholipid type (Kay et al., 2012). In order to explore the lateral heterogeneity of plasma membrane compartments, the detailed analysis of phospholipid dynamics will be also performed in Arabidopsis and tobacco cells co-expressing membrane proteins, that are known to segregate into various mesoscopic domains, like PIP2;1, KAT1, NRT1.1, SLAC1 and PIN2 (Jarsch et al., 2014; Kleine-Vehn et al., 2011; Li et al., 2011). We will particularly focus on the comparison of distinct membrane domains.

Thirdly, **computational study on mutual interplay between charged lipids and membrane proteins will be performed.** Our focus will be to investigate detailed nanoscale spatiotemporal dynamics of phospholipid/protein interactions using MD simulations. We will capitalize on the results from research goal and the availability of three-dimensional structures for PIP2;1, NRT1.1 and SLAH3 (as a homology model). This will reveal patterns of lipid interaction together with the preferences for particular lipid type and thus add to the

information available directly from the crystal structure. Moreover, the diffusive nature of each component of the membrane will be analysed with the emphasis on how proteins change the mobility of adjacent phospholipids and vice versa - how the presence of particular lipid affects protein behaviour in terms of mobility and coalescence into higher-order structures.

Lastly, **membrane organization dynamics of membrane proteins in cells with altered levels of acidic phospholipids will be studied.** Insights into acidic phospholipid functions in lateral plasma membrane organization will be gained by disrupting the levels of individual lipids at the PM (these will be selected based on results of experiments from the second and third goal). We will resort to achieving this using localized expression of enzymes that either up- or downregulate phospholipids (i.e. phospholipases, lipid kinases and phosphatases) at the PM using a targeting vector. The functional effects of PM phospholipid modulation on membrane heterogeneity and protein partitioning into microdomains will be tested by advanced fluorescent microscopy (refer to second goal).

Milestones: Set of in vivo data on the dynamics and interactions of membrane lipids and proteins.

Activity 4: Localization and functional characterization of components of male gametophyte transcriptome and proteome.

Head: *Said Hafidh, Ph.D.*

This activity is focused on the utilization of advanced microscopy techniques, including high resolution environmental microscopy of partner EEM ISI CAS for the characterization and understanding of the function of newly identified components of male gametophyte transcriptome and proteome. In particular, the main attention will be paid on transcripts and proteins assisting in the cytoskeleton dynamics, synthesis of PM and cell wall.

There are two research goals within this activity.

Firstly, the role of mRNA transport and localization of the translation machinery in maintaining cellular polarity in asymmetrically growing plant cells will be studied. We will exploit current -omic and advanced microscopy resources available for Arabidopsis and tobacco as model species to identify and characterize the role of mRNA transport and localization of the translation machinery in growing pollen tubes and during fertilization. Transcriptomics, translatomics and sequestromics following the subcellular fractionation of transcripts according to their translation status will be used to identify transcripts with discontinuous expression patterns. Of them, both known asymmetrically distributed transcripts (CSK and membrane-associated transport-related) and new potential targets will be highlighted. These newly identified transcripts will supplement already known translationally repressed mRNAs, which e.g. include cell wall protein NTP303 (Honys et al. 2000, Honys et al. 2009) and its orthologue NTP805, TUA6 (tubulin α-6-chain), FAD2 (fatty-acid desaturase 2), AHA9 (plasma membrane H+-ATPase 9), AtFH5 (formin homology 5), AVP1 (vacuolar- type H+-pumping pyrophosphatase 1, possibly auxin-related) and PUP11 (purine permease 11 transmembrane transporter; Hafidh et al. unpublished). The identity of translationally repressed transcripts will be verified by their tagged-tail sequencing.

Discontinuously expressed transcripts and their associated RNA-binding proteins will be identified and their localization dynamics will be assayed using fluorescent and electron microscopy, in the collaboration with partners at SUPRAMOL IMC CAS and EEM ISI CAS (see Fig. 5.1.1) including two-component λN22 RNA visualization system (Schönberger et al. 2012) and F-WISH (whole-mount RNA in situ hybridization; Bleckmann et al. 2015). The localization

dynamics of transcripts of interest and co-localization with number of subcellular markers in relation to their translation status during pollen tube growth will be described. The characterization of the regulatory mechanism of the mRNA translational silencing and localization will be achieved by the identification of RNA-binding proteins and other proteins comprising the EPP granules (Honys et al. 2009). For this, RNA-interacting proteins will be identified by RNA pull-down assay, immunoprecipitation and Y3H screen. RNA-binding proteins will be identified by MS analysis that will also enable us to characterize the dynamics of their posttranslational modifications.

Selected targets will be characterized from both functional and regulatory point of view. For the functional characterization, we will exploit the mutant phenotyping facilities available (Reňák et al. 2012). Gene-specific mutants will be either ordered as T-DNA lines or, more likely, will be generated using the established robust global and tissue-specific amiRNA knock-down strategy. We will further verify the effect of the ectopic overexpression of genes of interest and the subcellular localisation dynamics of analysed fusion proteins. We will also investigate the regulatory role of individual RNA sequence elements in the control of RNA localization, translation status and protein-binding. For this, we will analyze their leaders, trailers and eventually ORFs, binding proteins and to integrate the RNA-editing mutants.

Secondly, **the role of GPI-anchored transient receptors during pollen tube-pistil interaction will be addressed.** We will implement a combination of microscopy techniques including FRET and BiFC together with immunopreciptation approaches to establish ligand-receptor interaction modules during pollen tube guidance through the pistil prior to fertilization. We will specifically focus on the role of GPI-anchored proteins expressed on the surface of pollen tube plasma membrane. We have previously identified 14% of the pollen tube secretome grown through the pistils to constitute of GPI-anchored proteins (Hafidh et al., 2016). To isolate and enrich these predicted pollen tube GPI-anchored cell surface receptors, pollen tubes will be first grown by semi in vivo approach for 24 h (Hafidh et al., 2014, Hafidh et al., 2016). Prior to the secretome collection, pollen tubes will be treated with recombinant Pi-phospholipase C (Pi-PLC) and ultrafiltration will be performed to concentrate secreted proteins further. Identified GPI-anchored candidate proteins together with potential bound ligands will be tagged with split YFP to demonstrate their interaction in planta. In parallel, TRICEPS couple affinity purification will be used to tag ligands and probe pollen tube membrane receptors. To establish affinity and enthalpy of interaction, two biophysical approaches will be exploited, Microscale thermophoresis (MST) and Isothermal titration calorimetry (ITC).

Milestones: ***Set of in vivo data on the localization and dynamics of male gametophyte transcriptome and proteome components.***

As the outcome of Research objective 1 we plan to publish 50 papers in impacted scientific journals, 25 in collaboration with foreign collaborators (indicator 2 02 16) and 25 with authorship of members of the research team of the centre of excellent research (indicator 2 02 11). The proportion (indicator 2 02 14) will be 50%.

Research Objective 2: Identification of the composition of cell surfaces and intercellular space in plants

As it has been mentioned in the state of art of this research program, the cell wall participates in the formation of plant cell body. It is of a special significance in epidermal cell

layer of aerial plant organs. Here, epidermal cells deposit a specific set of compounds, hydrophobic polymers. Their main role is to limit the water loss, to prevent external factors to enter the plant cell body, and to prevent pathogens penetration into cells. This specialized structure is called the plant cuticle (Yeats and Rose, 2013). The cuticle is usually formed by hydrophobic polymer cutin combined with waxes. Besides the importance of the hydrophobic character of the cuticle, its micro- and nanostructure has also an important role in the plant life. This structure is formed by epicuticular waxes, which crystallize on the surface of the cuticle. They are further increasing the water-impermeability of the cuticle, contribute to self-cleaning character, and influence the reflectance of plant surfaces (Koch and Barthlott, 2009). Above-mentioned properties of the cell wall and specialized cell walls of epidermal cells indicate the importance of these structures for plants. Besides this, self-cleaning surfaces or reflectance may be used also in the development of new techniques in the industry or remote sensing and monitoring of plant ecosystems. In addition, it seems that complex structure of extracellular material is informative for the very early development of plant embryos, suggesting signal transduction events between extra and intracellular environment. Although both scanning electron microscopy and fluorescence microscopy provided important data on the composition of surface structures and intercellular structures, there is still significant lack of methods and instrumentation optimized for non-invasive imaging in high resolution coupled to elemental analysis as well as probing of these structures with nanoparticles. These stimuli are challenged in this research objective by joint effort of applicant research centre and three collaborating partners (See Fig. 5.1.1). It contains three main activities, 1) Characterization of the role of cytoskeleton in the cell wall synthesis, 2) Identification of the role of extracellular matrix during plant embryogenesis and 3) Determination of vegetation status by leaf surface structures.

Activity 1: Characterization of the role of cytoskeleton in the cell wall synthesis
Head: *RNDr. Kateřina Schwarzerová, Ph.D.*

The cell wall has been investigated intensively during past years with numerous important findings published recently, especially in the field of the cellulose synthesis. However, the research of the cell wall, a complex extracellular composite structure consisting of carbohydrate, lipid and phenol polymers, still remains problematic. The cellulose is known to be synthesized by membrane multienzyme complexes (Kumar a Turner 2015). Their movement is controlled by a microtubular cytoskeleton (Paredez et al. 2006). The synthesis of other carbohydrate polymers is confined to the Golgi apparatus and is deposited to the cell wall through vesicle transport processes that are controlled by actin cytoskeleton. The synthesis of cutin and waxes is located to the endoplasmic reticulum and its transport pathways to the cell wall are is understood only fragmentary (Domínguez et al. 2015, Lee and Suh 2015). Therefore, any progress in the understanding of the cell wall function and synthesis may contribute significantly in many fields of experimental plant biology, but also to the industry, agriculture or environment protection.

This activity contains three research goals.

Firstly, new factors and mechanisms participating in the synthesis of plant cell wall will be identified. This work is based on the experience of the laboratory with identifying cytoskeleton-based mechanisms for intracellular trafficking processes and the synthesis of cell wall. Through the integration of both actin-based trafficking and microtubule-based positioning of cellulose synthase complexes (Sampathkumar et al., 2013; Yanagisawa et al.,

2015), the synthesis of cell wall represents a very complex process. In our previous work, we have suggested several "meeting points" for the interaction of actin and microtubular cytoskeleton (Petrášek and Schwarzerová, 2009; Šlajcherová et al., 2012; Krtková et al., 2016) and some of them are now being under investigation in our laboratory. These include connection between actin nucleating complex Arp2/3 and microtubules (Havelková et al., 2015) and newly discovered role of this complex in the cell wall synthesis (Sahi et al., in preparation). Non-invasive observation of mutant plants in the laboratory of partner institution EEM ISI CAS on environmental raster (scanning) electron microscopy (ESEM or ESEM) already showed changes in the surface composition of their cuticle and cell walls. Using high-resolution microscopy ESEM, cell walls of Arabidopsis wild-type and mutant plants exhibiting changes of the cell wall will be studied. There are no similar techniques like ESEM, which enable the observation of native plant cell surfaces with high magnification and without fixation and further sample processing. These procedures change or even degrade lipid surface structures of cuticle and waxes. ESEM combined with qualitative analysis or micromanipulation is of great potential for the research of native plant surfaces, cell walls and their properties. High resolution of this techniques enables the imaging of micro- and nanostructures of cuticle (crystals of waxes) or cell walls (cellulose microfibrils or other polymers). Mutant lines with disrupted cytoplasmic signalling pathways involved potentially in the cell wall synthesis will be used for screening and characteristic changes in the cell wall structure and cell surface will be then experimentally associated with particular signalling pathways by molecular biology approaches. These will include studies of plants with rescued mutations, determination of lipid and polysaccharide composition, lignin composition, visualization of cellulose synthase complexes, lignin transporters and other components of the signalling machinery by fluorescence microscopy. Antibodies against actin nucleation machinery that has been generated in our laboratory (Havelková et al., 2015) will be used in combination with secondary antibodies coupled to nanoparticles in the collaboration with partners from SUPRAMOL IMC CAS. Moreover, since mutant plants are more fragile, this trait will be studied with MRI microscopy on the whole plant level in the collaboration with R&D centre of IKEM (Fig. 5.1.1).

Secondly, the plant surfaces will be screened with microscopy approaches to study pathogen penetration into the cell. The interaction between the cuticle and waxes deposition in epidermal cells of apple leaves and the infection by *Venturia inaequalis* fungal pathogen causing apple scab will be studied. Only youngest leaves are susceptible to the infection, whereas older leaves acquire an ontogeny resistance. Our preliminary experiments suggested that the cuticle of apple leaves has a specific structure undergoing a specific developmentally-regulated changes during leave maturation. The goal is to verify a hypothesis that specific organization of apple cuticle represents a barrier for the pathogen. Similar as in the previous part of this activity, EREM microscopy will be combined with fluorescence microscopy and molecular biology toolbox to identify mainly cytoskeleton-dependent processes for the recycling of the plasma membrane and synthesis of cell wall during infection process.

Thirdly, synthesis of newly identified cell wall-associated structure on the basis of plant trichomes will be studied. Another cell type with complex shape reflected by very complex processes of cell wall synthesis and rearrangements are trichomes, emergences on the leaf surface. As it has been shown in our laboratory, secondary cell walls of these cells accumulates metals like zinc, copper or cadmium and these might are deposited in the callose-rich ring structure at the bottom of trichomes (Kulich et al., 2015). We have also showed here that this deposition is dependent on polarized exocytosis, a cytoskeleton-dependent process. In the

collaboration with partner institution ISI CAS, the composition of metal deposits in these structures will be observed and analysed by electron microscopy coupled to elemental analysis (see Fig. 5.1.1.). This approach will also include electron microscopy observations of TiO_2 nanoparticles that have been shown to be deposited in these callose-rich rings in our preliminary experiments upon their addition into the substrate. The aim is to uncover the mechanism of their uptake and deposition within the plant. To distinguish whether TiO_2 nanoparticles are absorbed into plants directly or they are firstly forming low molecular weight complexes in the rhizosphere and absorbed in this form, polymer gel with TiO_2 nanoparticles will be prepared in the collaboration with partner from SUPRAMOL IMC CAS (see Fig 5.1.1) and plants cultured in this gel. Low molecular weight complexes of Ti (i.e. dissolved TiO_2 nanoparticles) will be freely diffusing through the gel, while TiO_2 nanoparticles will be trapped. The opposite design will be performed in nutrient solution (Hoagland type) containing macroporous resin (8-HQ type), which will bind dissolved TiO_2 particles. The presence of Ti will be assessed by electron microscopy. This technique will allow distinguishing clearly the uptake of TiO_2 nanoparticles and their complexes.

Since the results obtained with TiO_2 could be generalized also for other nanoparticles, this activity has very promising expectation for possible application of nanotechnologies in plants. Moreover, thanks to the similarities in the composition of trichome cell wall of Arabidopsis and cotton plants, our results also contribute to the application of nanotechnology-based improvements of cotton textiles.

Milestones: Set of data characterizing the role cytoskeleton and associated proteins in the synthesis of cell wall and cell surfaces

Activity 2: Identification of the role of extracellular material during plant embryogenesis.
Head: *Mgr. Kateřina Eliášová, Ph.D.*

Individual phases of somatic embryogenesis in conifers are studied in our laboratory on both biochemical and histological levels. The role of endogenous phytohormones, polyamines, phenolic compounds, saccharides and storage compounds are informative for in individual phases of somatic embryogenesis (Gemperlová et al., 2009), but the understanding of the co-operation between intracellular structures in the formation of somatic embryo is still only fragmentary. Our previous studies showed how individual actin isoforms co-operate during early phases of the somatic embryo development with specific isoforms expressed specifically only in suspensor cells with decreasing expression during maturation phase (Schwarzerová et al., 2010; Vondráková et al., 2014).

Firstly, we will focus on the cytoskeletal changes in suspensor and meristematic cells during embryo maturation with respect to possible the role of cytoskeleton in the synthesis of cell wall and formation of extracellular material. The main attention will be paid to the interconnection between suspensor and meristematic cells and to the role of suspensor cells at the onset of maturation phase followed by disintegration of suspensor. **Secondly, we will concentrate on changes that are induced by abiotic stresses** (Cvikrová et al., 2016; Eliášová et al., 2016) on the surface of young somatic embryos of spruce. In both these goals, surface layers will be studied *in vivo*, i.e. without any fixation step. Advanced techniques of environmental scanning electron microscopy (AQUASEM II) and unique sample handling will be performed in co-operation with partner from ISI CAS (Fig. 5.1.1) and will allow studying somatic embryos in almost native state. This technique has been already used for non-invasive observation in plant cells (Zheng et al., 2009; Stabentheiner et al., 2010; McGregor et al., 2013). From our preliminary observations and results of partner's laboratory (Neděla et al.,

2016) it seems that young somatic embryos are covered with the layer of material, sometimes called "extracellular matrix", which is very probably composed from pectin polysaccharides, proteins and lipophilic substances. In the framework of this project proposal, it is planned to analyse both composition of the extracellular material as well as its changes during somatic embryogenesis.

Milestones: Set of functional data characterizing the role of extracellular material for the plant embryogenesis.

Activity 3: Identification of cellsurface structures determining leaf reflectance

Head: *Prof. RNDr. Jana Albrechtová, Ph.D.*

Leaf optical properties (reflectance, absorption, and transmittance) in the visible part of electromagnetic spectrum (400-700 nm) are determined by biochemical leaf composition, primarily the content of photosynthetic pigments. Leaf optical properties in near infrared (NIR, 700-2300 nm) bring also information about biochemical composition (e.g. lignin, cellulose), water content and leaf internal structure. The relationship between leaf structure and reflectance has not been yet studied intensively, the study focused on leaf surface and reflectance in visible spectrum (Buschman et al. 2012) could serve as one of the sporadic examples. The relation between leaf structure, biochemical composition and optical properties is important for interpretation of hyperspectral data acquired by remote sensing techniques, which are used for monitoring of vegetation physiological status or phenology (Mišurec et al. 2012).

We propose to study impact of epidermal surface properties and internal structure of leaves with different extent of xeromorphic adaptation on their reflectance in NIR. Leaf structural parameters serve as input to various radiative transfer models (e.g. PROSPECT, LIBERTY) that can be scaled up for modeling vegetation properties on large spatial scale and used for interpretation of hyperspectral data from remote sensing. New DLM radiative transfer model (Dorsiventral Leaf radiative transfer Model; Stuckens et al. 2009) will be used for scaling up of leaf level reflectance to higher hierarchical levels (e.g. canopy). DLM model considers non-uniform distributions of pigments, water and dry matter within a dorsiventral leaf. The significance of effect of this non-uniformity is not known in case of scaling up for the canopy level.

Application of stereological methods on images acquired by light, confocal or electron microscopy (Albrechtová et al. 2007, Lhotáková et al. 2008, Kubínová et al. 2014) serve as a suitable tool for quantification of leaf anatomical parameters that later serve as input to radiative transfer models.

Leaf optical properties will be measured using spectral radiometer (FieldSpec 4-ASD) in combination with a contact probe or integrating sphere. Up to now there can be hardly find a detailed study on relationship between leaf NIR reflectance and its surface structure. The main breakthrough in the topic will be simultaneous measurement of NIR reflectance and leaf structure using environmental electron microscopy that will be performed in collaboration with the partner institution ISI CAS (Fig. 5.1.1.).

Milestones: Spectral library from leaves with different internal structure and xeromorphic adaptations

As the outcome of Research objective 2 we plan to publish 20 papers in impacted scientific journals, 10 in collaboration with foreign collaborators (indicator 2 02 16) and 10 with

authorship of members of the research team of the centre of excellent research (indicator 2 02 11). The proportion (indicator 2 02 14) will be 50%.

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5.1.4. International co-operation and integration of foreign strategic partner

Research team of R&D center of IEB CAS has long-lasting interactions with teams from leading foreign institutions in all areas that are proposed to be supported by this project.

One of the most fruitful and long-lasting co-operation is maintained with the laboratory of Prof. Jiří Friml, leading scientist in plant cell and developmental biology (H-index 73, 19 500 citations). During past decade (2005-2015), 16 joint original contributions and reviews were published in collaboration between R&D center of IEB CAS that have been co-authored by Jan Petrášek and Jiří Friml (see CV of Jan Petrášek in Annex 7). It is important to mention that this co-operation has been always exclusively based on mutually supporting and complementary expertize between R&D centre of IEB and Prof. Friml laboratory. Prof. Friml shifted from University Tuebingen to leading plant biotechnology institute PSB VIB Ghent, Belgium and after successful period in Belgium, he is now firmly established in Institute of Science and Technology (IST Austria). Prof. Friml agreed to continue in this long lasting and successful collaboration by signing letter of intent for Research partnership (Annex 6 and see obligatory supplement) and will represent the main "strategic" partner of this proposed center of excellent research. He is ready to invite students and scientists to visit his laboratory, prepare joint research proposals as well as organize meetings of both laboratories. Cell biology-oriented research of Prof. Friml thematically overlaps with the majority of research activities proposed in this research program. Other currently running collaborations that are already supported by joint publications with Jan Petrášek and members of R&D team of IEB CAS include laboratories of Prof. Eva Benková (IST Austria), Assoc. Prof. Christian Luschnig (BOKU Vienna, Austria), see his letter of support (Annex 6), Assoc. Prof. Jürgen Kleine-Vehn (BOKU Vienna, Austria), Prof. Dominique Van Der Straeten (Ghent University, Department of Physiology, Belgium), Prof. Peter Nick (KIT Karlsruhe, Germany) and Prof. Angus Murphy (University of Maryland, USA).

Research objective 1 of this research program is further extending running collaboration between laboratory of Dr. Eric Ruelland (UPEC, Paris, France), which is also supported by the letter of intent for a co-operative research by the Dr. Jacques Moscovici (dean of UPEC) for the activity 2 of the research objective 1 (Annex 6). Laboratory of Assoc. Prof. David Honys, which is through the Dr. Said Hafidh responsible for the activity 4 of the research objective 1 has long-lasting co-operation with Prof. Enrico Schleiff, director of the Buchmann Institute of Molecular Life Sciences and Professor at the Goethe University Frankfurt, Germany. Prof. Schleiff offered in his letter of intent (Annex 6) wide collaboration including exchange of researchers and students as well as application for common projects. Importantly, he is ready to involve Frankfurt Center of Advanced Light Microscopy and Frankfurt Center of Electron Microscopy into collaborative activities.

Activities of research objective 2 of this research program also further extend collaborations with several leading laboratories in the field. Besides already mentioned laboratories of Prof. Eva Benková (IST Austria) and Prof. Peter Nick (KIT Karlsruhe, Germany), research activity 1 of this objective will profit from the collaboration with Prof. Daniel Szymanski (Purdue University, USA), Dr. Sabine Müller (University of Tübingen, Germany), Dr. Camille Laroue (CNRS/ECOLAB, Toulouse, France). Long-lasting collaboration with Dr. Grégory Mouille, leader of Analytical Chemistry Plant Observatory Laboratory at Institut Jean-Pierre Bourgin (INRA Versailles, France) will provide for the proposed project the analytical expertize complementing electron microscopy approaches for studies of cell wall (see letter of support in (Annex 6). Activity 2 of this research objective devoted to the extracellular material will greatly profit from the running collaboration with Dr. Lelu-Walter, director of research at National Institute of Agriculture Research (INRA, Val de Loire Orléans, France), who offered based on the running collaboration to serve as "strategic partner" for this topic, see details in the letter of intent (Annex 6). Finally, running collaborations in the framework of activity 3 of this objective with Prof. Roeland Samson (University of Antwerpen, Belgium) and Dr. Petya Campbell (Joint Center for Earth Systems Technology, University of Maryland Baltimore County, NASA Goddard Space Flight Center, Biospheric Sciences Branch, USA) are expected to continue within proposed project.

5.1.5. Research team

Composition of research team, description of organization for individual research activities

Research team of this research program 1 is composed of members of 6 laboratories of R&D centre of IEB CAS (see Annex 2) and several newly hired researchers. As depicted in a form of organization chart for this research program in Fig. 5.1.5, 7 research activities described in detail in the chapter 5.1.3 are assigned to individual responsible researchers. Every research activity will be performed with direct responsibility to the head of the research program. The summarization of nominated members of the team as well as members that will be nominated for all years of the project is given in Table 5.1.1 together with their role in the project, H index and FTEs (full time equivalents). In addition, curriculum vitae for all nominated members of the team are given in Annex 7. These CVs summarize expertize of the team members, their best 8 publications in the research field of the proposed project and other relevant information (see Annex 7).

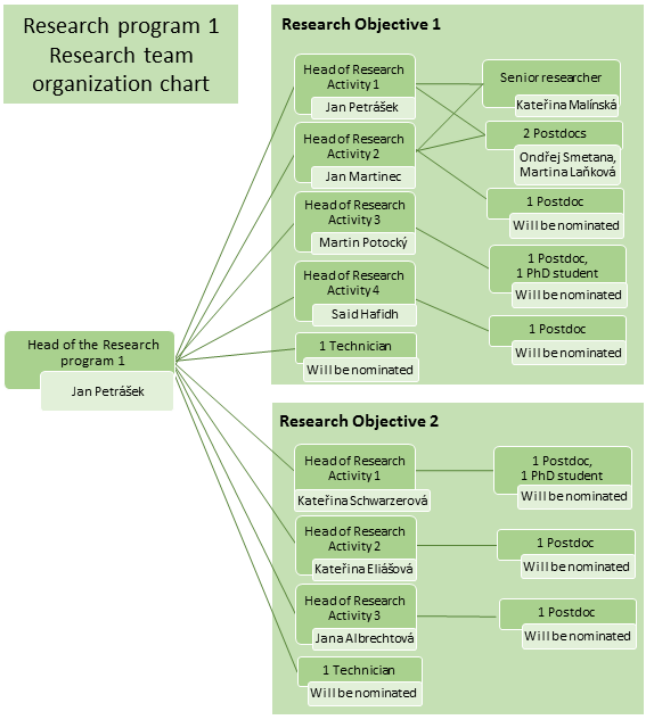

Figure 5.1.5: Organization chart of the research team of research program 1. The head of the research program will be responsible for co-ordination of activities in both research objectives. Researchers responsible for individual research activities will be directly answerable to the head of the program. Head of the research program will be also responsible to assign and manage work of technicians.

RNDr. Jan Petrášek, Ph.D., **excellent researcher** (2017-2022), will be heading the research program 1and within this program he will be responsible for activity 1 of the first objective, but he will also take supervision in research objective 2. He will serve as the main coordinator of the whole centre. Besides coordination and publication activities he will perform the advanced fluorescence microscopy, take part in electron microscopy (HR-SEM and ESEM) on membrane proteins performed at ISI AS CR partner department. As shown in his CV (Annex 7), his expertise is in the field of plant hormones, hormonal crosstalk, cell polarity, cytoskeleton dynamics, auxin action, auxin transport, regulation of auxin carrier's activity and localization and auxin transport inhibitors.

Ing. Martin Potocký, Ph.D., **key researcher** (2017-2022)**,** will be heading the activity 3 of research objective 1, but he will also take part in activities 1 and 2 of this research objective. As shown in his CV (Annex 7), he is the expert in the biochemistry of plasma membrane lipids and proteins, tracking their mobility *in vivo* by means of advanced fluorescence microscopy and *in silico* methods for the simulation of molecular dynamics.

Prof. RNDr. Jana Albrechtová, Ph.D., **key researcher** (2017-2022), will be heading the activity 3 of research objective 2. She will co-ordinate research on leaf surfaces studied by ESEM done at ISI CAS partner department and she will correlate the outputs of these results with reflectance measurements. She will be hired for this research program to provide her expertise in the field of spectral studies of foliage in remote sensing in combination with her deep knowledge of plant physiology and anatomy that she guaranties as Professor at Department of Experimental Plant Biology at Faculty of Science, Charles University, see her CV in Annex 7.

RNDr. Jan Martinec, CSc., **senior researcher** (2017-2022), will be heading the activity 2 of research objective 1. He will take part in advanced fluorescence microscopy of membrane

microdomains and in the sample preparation for electron microscopy performed at ISI CAS partner department. He is the expert in the field of plant phospholipid signalling and the spatial organization of plant plasma membrane, see his CV in Annex 7.

Mgr. Kateřina Eliášová, Ph.D., **senior researcher** (2017-2022) will be heading the activity 2 of research objective 2. She will take part in advanced fluorescence microscopy of somatic embryos and in the sample preparation for electron microscopy performed at ISI CAS partner department. She is the expert in plant anatomy and preparation of plant tissues for both light and electron microscopy, see her CV (Annex 7).

RNDr. Kateřina Schwarzerová, Ph.D., senior researcher (2017-2022) will be heading the activity 1 of research objective 2. She will be hired to this research program to co-ordinate advanced fluorescence microscopy of *Arabidopsis thaliana* cytoskeletal mutants as well as the sample preparation for the electron microscopy and elemental analysis performed in ISI CAS partner laboratory. She is the expert in the field of cytoskeleton dynamics, cellular morphogenesis and formation of cell wall, see her CV in Annex 7.

Ing. Kateřina Malínská, Ph.D., senior researcher (2017-2022) will be involved in activities 1 and 2 of research objective 1. She will perform advanced confocal fluorescence microscopy on integral and peripheral membrane proteins. She is guarantor of cloning of new DNA constructs in the co-operation with research program 2 (IMC and IKEM). Her expertise is mainly in the field of advanced confocal microscopy of membrane proteins and their functional testing using molecular biology approach, see CV (Annex 7).

Mgr. Ondřej Smetana, Ph.D., junior researcher (2017-2022) will be hired to this research program for his involvement in activities 1 and 2 of research objective 1. As shown in his CV (Annex 7), he has spent 5 years as postdoc in leading laboratory in plant developmental biology. He is the expert in advanced confocal microscopy and will be participating in the confocal and electron microscopy of auxin transporters and will take part in the preparation of web localization database.

Mgr. Martina Laňková, Ph.D., junior researcher (2018-2022) will be involved in activities 1 and 2 of research objective 1 as expert for FCS and RICS methods. She will participate on DNA cloning, biochemical analyses and antibody preparation, for her expertise see CV in Annex 7.

Said Hafidh, Ph.D., junior researcher (2017-2022), will be heading the activity 4 of research objective 1. He is the expert in advanced confocal microscopy on newly identified members of pollen transcriptome that he will study within the project including the ESEM analysis in partner ISI CAS. As documented in his CV (Annex 7), his expertize includes many aspects of cell biology of plant sexual reproduction.

As shown in table 5.1.1 and figure 5.1.5, there will be another **8 researchers nominated (postdoctoral researchers and PhD students) for their half capacities and 2 technicians for full capacity**. The recruitment of these team members, mostly postdocs and PhD students, is already being under way, they will be mostly selected from laboratory team members of groups affiliated to the project.

New postdoc (2017-2022), will be nominated to the team involved in activity 1 of research objective 2. In the collaboration with teams from IMC CAS and ISI CAS he/she will analyse element composition of surface structures of plant trichomes and study transport of material for the synthesis of cell wall using nanoparticles.

New postdoc (2018-2022), will be nominated to the team involved in activity 2 of research objective 1 and will take part in the cloning of DNA constructs for membrane domain identification and their advanced fluorescence microscopy and ESEM microscopy at ISI CAS.

New postdoc (2018-2022), will be nominated to the team involved in activity 3 of research objective 1 and will take part in the determination of plasma membrane lipids and proteins mobility *in vivo* by means of advanced fluorescence microscopy and *in silico* predictions and modelling of plasma membrane dynamics.

New postdoc (2017-2022), will be nominated to the team involved in the activity 2 of research objective 2. He/she will take part in ESEM and confocal fluorescence microscopy of intercellular materials and surfaces of somatic embryos.

New postdoc (2018-2022), will be nominated to the team involved in the activity 4 of research objective 1, where he/she will take part in confocal fluorescence microscopy and electron microscopy of pollen transcriptome and proteome.

New postdoc (2017-2022), will be nominated to the team involved in the activity 3 of research objective 2. He/she will analyse leaf surfaces by ESEM done at ISI CAS partner department and he will correlate the outputs from these results with reflectance measurements and data from earth remote sensing.

New PhD student (2017-2022), will be nominated to the team involved in the activity 1 of research objective 2. He/she will perform electron microscopy under native conditions (ESEM) in cooperation with partner ISI CAS on the collection of plant cytoskeletal mutants.

New PhD student (2017-2022), will be nominated to the team involved in the activity 3 of research objective 1. He/she will study mobility of PM lipids and proteins with the means of advanced fluorescence microscopy and optimize protocols for *in vivo* electron microscopy (ESEM).

2 new technicians (2017-2022), will be nominated to support teams working on research objectives 1 and 2. These technicians will provide laboratory services including cultivation of experimental plant material, preparation of media and buffers and maintenance of laboratory databases and other necessary service.

Table 5.1.1: Research team of the research program 1. See text for the description of the role for each researcher.

First Name and Surname	Working position	Role in the team, affiliation to research activities	H-index	Year 1	Year 2	Year 3	Year 4	Year 5	Year 6
				FTE during the project					
Jan Petrášek	Excellent Senior Researcher	Head RP1; Head Activity 1, Objective 1	23	0.7	0.7	0.7	0.7	0.7	0.7
Martin Potocký	Key Senior Researcher	Member RP1; Head Activity 3, Objective 1	14	0.7	0.7	0.7	0.7	0.7	0.7
Jana Albrechtová	Key Senior Researcher	Member RP1, Head Activity 3, Objective 2	17	0.45	0.45	0.45	0.45	0.45	0.45
Jan Martinec	Senior Researcher	Member RP1, Head Activity 2, Objective 1	13	0.5	0.5	0.5	0.5	0.5	0.5
Kateřina Eliášová	Senior Researcher	Member RP1, Head Activity 2, Objective 2	4	0.5	0.5	0.5	0.5	0.5	0.5
Kateřina Schwarzerová	Senior Researcher	Member RP1, Head Activity 1, Objective 2	11	0.5	0.5	0.5	0.5	0.5	0.5
Kateřina Malínská	Senior Researcher	Member RP1, Member Activity 1,2, Objective 1	7	0.5	0.5	0.5	0.5	0.5	0.5
Ondřej Smetana	Junior Researcher	Member RP1, Member Activity 1,2, Objective 1	4	1.0	1.0	1.0	1.0	1.0	1.0
Martina Laňková	Junior Researcher	Member RP1, Member Activity 1,2, Objective 1	5	0.0	1.0	1.0	1.0	1.0	1.0
Said Hafidh	Junior Researcher	Member RP1, Head Activity 4, Objective 1	8	0.5	0.5	0.5	0.5	0.5	0.5
To be nominated	Junior Researcher, postdoc	Member RP1, Member Activity 1, Objective 2	-	0.5	0.5	0.5	0.5	0.5	0.5
To be nominated	Junior Researcher, postdoc	Member RP1, Member Activity 2, Objective 1	-	0.0	0.5	0.5	0.5	0.5	0.5
To be nominated	Junior Researcher, postdoc	Member RP1, Member Activity 3, Objective 1	-	0.0	0.5	0.5	0.5	0.5	0.5
To be nominated	Junior Researcher, postdoc	Member RP1, Member Activity 2, Objective 2	-	0.5	0.5	0.5	0.5	0.5	0.5
To be nominated	Junior Researcher, postdoc	Member RP1, Member Activity 4, Objective 1	-	0.0	0.5	0.5	0.5	0.5	0.5
To be nominated	Junior Researcher, postdoc	Member RP1, Member Activity 3, Objective 2	-	0.5	0.5	0.5	0.5	0.5	0.5
To be nominated	Junior Researcher, PhD student	Member RP1, Member Activity 1, Objective 2	-	0.5	0.5	0.5	0.5	0.5	0.5
To be nominated	Junior Researcher, PhD student	Member RP1, Member Activity 3, Objective 1	-	0.5	0.5	0.5	0.5	0.5	0.5
To be nominated	Technician	Member RP1, Member Activity 1-4, Objective 1	-	1.0	1.0	1.0	1.0	1.0	1.0
To be nominated	Technician	Member RP1, Member Activity 1-3, Objective 2	-	1.0	1.0	1.0	1.0	1.0	1.0

Results of key and excellent members of the research team in 2011-2015

RNDr. Jan Petrášek, Ph.D., **excellent researcher**

Selected 5 research publications related to the proposed project with citations specified:

1. Marhavý, P., Bielach, A., Abas, M., Abuzeineh, A., Duclercq, J., Tanaka, H., Pařezová, M., Petrášek, J., Friml, J., Kleine-Vehn, J., Benková, E.: Cytokinin modulates endocytic trafficking of PIN1 auxin efflux carrier to control plant organogenesis. Developmental Cell 21, 796-804, 2011.
   ISI IF_{2011} 14.030, number of citations: 104 (Scopus without auto-citations), 105 (WOS), 137 (Google Scholar)
2. Mravec, J., Petrášek, J., Li, N., Boeren, S., Karlova, R., Kitakura, S., Pařezová, M., Naramoto, S., Nodzynski, T., Dhonukshe, P., Bednarek, S.Y., Zažímalová, E., de Vries, S., Friml, J.: Cell Plate Restricted Association of DRP1A and PIN Proteins Is Required for Cell Polarity Establishment in Arabidopsis. Current Biology 21, 1055-1060, 2011.
   ISI IF_{2011} 9.647, number of citations: 32 (Scopus without auto-citations), 27 (WOS), 43 (Google Scholar)
3. Leitner, J., Petrášek, J., Tomanov, K., Retzer, K., Pařezová, M., Korbei, B., Bachmair, A., Zažímalová, E., Luschnig, C.: Lysine63-linked ubiquitylation of PIN2 auxin carrier governs hormonally controlled adaptation in *Arabidopsis* root growth. Proceedings of the National Academy of Sciences 109, 8322-8327, 2012.
   ISI IF_{2012} 9.737, number of citations: 54 (Scopus without auto-citations), 48 (WOS), 64 (Google Scholar)
4. Havelková, L., Nanda, G., Martinek, J., Bellinvia, E., Sikorová, L., Šlajcherová, K., Seifertová, D., Fischer, L., Fišerová, J., Petrášek., J., Schwarzerová, K.: Arp2/3 complex subunit ARPC2 binds to microtubules. Plant Science 241, 96-108, 2015.
   ISI IF_{2015} 3.362, number of citations: 1 (Scopus without auto-citations), 1 (WOS), 1 (Google Scholar)
5. Jelínková, A., Müller, K., Fílová-Pařezová, M., Petrášek, J.: NtGNL1a ARF-GEF acts in endocytosis in tobacco cells. BMC Plant Biology 15, 272, 2015.
   ISI IF_{2015} 3.631, number of citations: 1 (Scopus without auto-citations), 1 (WOS), 1 (Google Scholar)

5 research projects related to the research program 1 with financial support specified (only PI or co-PI):

1. 2011-2014, Principal Investigator, Czech Science Foundation (GACR), project GAP305/11/2476, Auxin transport and cytoskeleton in the morphogenesis of plant cells, 7 240 000 CZK
2. 2012-2015, Principal Investigator, Program of internal support of projects of international collaboration of CAS with BOKU Vienna, Austria, project M200381203, Molecular mechanisms of regulation of membrane auxin carriers, 731 000 CZK
3. 2016-2019, Institutional partner of co-operative project, National Infrastructure for Biological and Medical Imaging (Czech Bioimaging), Ministry of Education, Youth and Sports, project code LM 2015062 1 924 000 CZK

4. 2011-2012, Principal Investigator, competition for financial support of expensive instrument, confocal spinning disk microscope Nikon Eclipse Ti-E, Yokogawa CSU-X1, 8 000 000 CZK

5 patents and commercial applications related to the proposed project:
No patents or commercial applications in 2011-2015.

Ing. Martin Potocký, Ph.D., **key researcher**

Selected 5 research publications related to the proposed project with citations specified:

1. Pleskot R, Pejchar P, Žárský V, Staiger CJ, Potocký M. (2012) Structural insights into the inhibition of actin-capping protein by interactions with phosphatidic acid and phosphatidylinositol (4,5)-bisphosphate. PLoS Computational Biology 8(11):e1002765.
   ISI IF_{2012} 4.867, number of citations: 13 (Scopus without auto-citations), 18 (WOS), 21 (Google Scholar)
2. Potocký M, Pejchar P, Gutkowska M, Jimenéz MJ, Potocká A, Alché JD, Kost B, Žárský V. (2012) NADPH oxidase activity in pollen tubes is affected by calcium ions, signaling phospholipids and Rac/Rop GTPases. Journal of Plant Physiology, 169(16):1654-63.
   ISI IF_{2012} 2.699, number of citations: 23 (Scopus without auto-citations), 24 (WOS), 31 (Google Scholar)
3. Pleskot R, Li J, Žárský V, Potocký M, Staiger CJ. (2013) Regulation of cytoskeletal dynamics by phospholipase D and phosphatidic acid. Trends in Plant Science 18(9):496-504.
   ISI IF_{2013} 13.479, number of citations: 25 (Scopus without auto-citations), 26 (WOS), 31 (Google Scholar)
4. Potocký M, Pleskot R, Pejchar P, Vitale N, Kost B, Žárský V. (2014) Live-cell imaging of phosphatidic acid dynamics in pollen tubes visualized by Spo20p-derived biosensor. New Phytologist 203(2):483-94.
   ISI IF_{2014} 7.672, number of citations: 11 (Scopus without auto-citations), 11 (WOS), 13 (Google Scholar)
5. Pleskot R, Cwiklik L, Jungwirth P, Žárský V, Potocký M (2015) Membrane targeting of the yeast exocyst complex. Biochimica et Biophysica Acta-Biomembranes 1848(7):1481-1489.
   ISI IF_{2015} 3.687, number of citations: 1 (Scopus without auto-citations), 1 (WOS), 4 (Google Scholar)

5 research projects related to the research program 1 with financial support specified (only PI or co-PI):

1. 2013-2016, Principal Investigator, Czech Science Foundation (GACR), project 13-19073S, Multiscale analysis of signalling phospholipids and their interaction protein partners in the regulation of plant tip growth, 7 800 000 CZK
2. 2012, EMBO short-term fellowship grant, Regulation of pollen NADPH oxidase by Rac/Rop GTPases, 8 000 EUR
3. 2009-2012, Co-principal investigator, Grant Agency of the Academy of Sciences of the Czech Republic, project IAA601110916, Phosphatidic acid and diacylglycerol-mediated signalling in the polar growth of plant cells, 4 000 000 CZK

4. 2009-2011, Principal Investigator, Czech Science Foundation (GACR), project 522/09/P299, Characterisation of NADPH oxidase from tobacco pollen and its role in regulation of polar cell expansion, 1 050 000 CZK

5 patents and commercial applications related to the proposed project:
No patents or commercial applications in 2011-2015

Prof. RNDr. Jana Albrechtová, Ph.D., **key researcher**

Selected 5 research publications related to the proposed project with citations specified:

1. Mišurec J; Kopačková V; Lhotáková Z; Hanuš J; Weyermann J; Entcheva-Campbell P, Albrechtová, J (2012) Utilization of hyperspectral image optical indices to assess the Norway spruce forest health status. Journal of Applied Remote Sensing 6, Article Number: 063545
   ISI IF_{2012} 0.876, number of citations: 0 (Scopus without auto-citations), 3 (WOS), 7 (Google Scholar)
2. Kopačková, V; Mišurec, J; Lhotáková, Z; Oulehle, F, Albrechtová, J (2014) Using multi-date high spectral resolution data to assess the physiological status of macroscopically undamaged foliage on a regional scale. International Journal of Applied Earth Observation and Geoinformation, 27: 169-186
   ISI IF_{2014} 3.470, number of citations: 5 (Scopus without auto-citations), 1 (WOS), 6 (Google Scholar)
3. Kubínová, Z; Janáček, J; Lhotáková, Z; Kubínová, L, Albrechtová, J (2014). Unbiased estimation of chloroplast number in mesophyll cells: advantage of a genuine three-dimensional approach. Journal of Experimental Botany. 2014, 65(2), 609–620.
   ISI IF_{2014} 5.526, number of citations: 2 (Scopus without auto-citations), 1 (WOS), 4 (Google Scholar)
4. Kopačková V; Lhotáková Z; Oulehle F; Albrechtová J (2015). Assessing forest health via linking the geochemical properties of a soil profile with the biochemical parameters of vegetation. International Journal of Environmental Science and Technology 12 (6): 1987-2002
   ISI IF_{2015} 2.344, number of citations: 0 (Scopus without auto-citations), 1 (WOS), 2 (Google Scholar)
5. Mišurec, J; Kopačková, V; Lhotáková, Z; Campbell, P; Albrechtová, J (2016). Detection of Spatio-Temporal Changes of Norway Spruce Forest Stands in Ore Mountains Using Landsat Time Series and Airborne Hyperspectral Imagery. Remote Sensing 8 (2) Article Number: 92.
   ISI IF_{2015} 3.036, number of citations: 0 (Scopus without auto-citations), 0 (WOS), 0 (Google Scholar)

5 research projects related to the research program 1 with financial support specified (only PI or co-PI):

1. 2012-2016, Principal Investigator, KONTAKT II, project n. LH12097, Innovation of monitoring methods for the determination of health of Norway spruce stands in Ore mountains. Foreign partner Dr. P. Campbell, NASA Goddard Space Flight Center and University of Maryland, USA, 3 438 000 CZK

2. 2010-2014, Principal Investigator, Czech Science Foundation (GACR), project P501-10-0340, The influence of increased concentration of CO_2 and irradiance on the structure and function of photosynthetic apparatus in wood plants, 3 464 000 CZK.

5 patents and commercial applications related to the proposed project:

No patents or commercial applications in 2011-2015

5.1.6. Description of key equipment/investments

In tables presented below, descriptions of key equipment/investments and functional modules are given together with their purchase costs and technical specification. All instruments, machines and software (with purchase costs not being less than 1 mil CZK) that are planned to be used in the RP 1 are listed individually. Items with lower prize are assembled into functional modules, according to their characteristics and linkage to research activities of RP 1, but also RP 2 and RP 3.

Required investments are described further in the project budget (see Annex 8 and obligatory attachment in the online application MS2014+), commentary on budget (see Annex 9). Their pricing is based on quotations, which are all supplemented in Annex 10.

Key equipment / functional module	No. of items	Planned total price without VAT (thousands CZK)
1. Functional module for the upgrade of image acquisition on spinning disk microscope and stereomicroscope	1	1 961
Typical features: The module consists of 7 items in total. It includes ultrasensitive EMCCD camera for fluorescence microscopy on spinning disk microscope (Andor iXon ULTRA), high resolution CMOS camera (Nikon) for fluorescence stereomicroscope, image acquisition and analysis software, two control PCs, one PC for image evaluation, and a software multilicence for image analysis (NIS elements). This upgrading of existing microscopes in the form of improved image acquisition and subsequent evaluations will be used in all activities of both research objectives of the research program 1. Purpose of the acquired equipment: Highly sensitive EMCCD camera will be used to upgrade the image acquisition on an existing fluorescence spinning disk microscope. In particular, it allows to capture weak fluorescence signals with improved spatial resolution in contrast to the existing camera. Therefore, it will be possible to capture both the spatial distribution of lipids and proteins in the area of the plasma membrane, as well as their dynamics. This equipment is therefore crucial for success of activities 1-3 of research objective 1 and activity 1 of research objective 2. A high resolution CMOS camera will be used to upgrade the image acquisition on fluorescence stereomicroscope Nikon SDZ25, where the existing camera does not suit and cannot be operated under a new operating system. The system contains also PC with NIS-Elements image analysis software for image acquisition and analysis as well as EDF module		

for 3D image reconstruction from somatic embryo surfaces (activity 2 of research objective 2).
Two other PCs will be used for the post-processing of images from both microscopes. The set will be supplemented by a new version of the multilicence for NIS-Elements image analysis system, which has been used for a long time by teams of the IEB CAS R&D centre, but it needs to be upgraded to a new version that is needed for new operating systems Win10.

Infrastructure readiness:
The infrastructure of IEB CAS is ready to accommodate planned instruments, no additional costs are necessary. Spinning disk microscope is located in the laboratory space of imaging facility of IEB CAS in the building B1, the stereomicroscope is located in the laboratory of team that will be involved in the activity 2 of research objective 2, in the building B1.

2. Functional module of instruments for the preparation and maintenance of experimental material and plant molecular biology	1	1 630

Typical features:
This module consists of several instruments for plant molecular biology, namely 2 incubation orbital shakers, 2 automatic autoclaves and 4 PCR thermocyclers. These instruments will be used in all activities of both research aims of the RP 1.

Purpose of the acquired equipment:
Air-conditioned shaker incubation devices and automatic autoclaves will be used for the standardized preparation of tobacco transgenic lines and lines of *Arabidopsis thaliana* as well as for the preparation of sterile culture media and other material. In a number of cases, it will replace outdated equipment that is already in use for a long time, and the cost of maintaining it is too high. These machines will be used by members of teams belonging to research objective 1 (activities 1, 2 and 3) and research objective 2 (activities 1 and 2), as specified in Fig. 5.1.1. Due to the high sensitivity of the plasma membrane associated processes, it is urgent to check the temperature ratios of incubation for subsequent microscopic, biochemical and molecular biology analysis of the cultured material.
PCR thermocyclers in two modifications with silver blocks and aluminum blocks will serve for routine PCR procedures in a number of modifications, especially in activities 1, 2 and 4 of research objective 1 and activities 1 and 2 of research objective 2.

Infrastructure readiness:
The infrastructure of IEB CAS is ready to accommodate planned instruments, no additional costs are necessary. Instruments will be placed in laboratories and culture rooms of building B1 of IEB CAS.

3. Automated station for histochemistry	1	1 621

Typical features:
This station will be purchased for automated immunohistochemical and histochemical staining in biological material, including plant tissues. It allows specific labelling of both proteins and nucleic acids (RNA, DNA). The system will contain module for staining in plant organs or whole seedlings, i.e. in whole mounts.

Purpose of the acquired equipment:
The purpose of the purchase of this instrument is to extend and standardize the protocols for immunofluorescence imaging of proteins in plant material, which is carried out at a workplace of the IEB R&D centre at a similar station installed in 2009 and is expected not to have sufficient capacity for the proposed project. By acquiring the intended device (in 2019), new immunocytochemical methods will be made available for all activities of the two research objectives of RP 1. However, new nanoparticle-labeled antibodies prepared in the RP2 will be tested on this device to prepare labeled samples for electron microscopy within the framework of RD3 (see fig. 5.1.1).

Infrastructure readiness:
The infrastructure of IEB CAS is ready to accommodate planned instruments, no additional costs are necessary. The machine will be located at the second floor of building B1 of IEB CAS.

5.1.7. Research program budget - relation to the overall project proposal budget

The budget of this RP1 is attached in Annex 8 together with detailed comments for all individual items (see Annex 9). The budget and comments on budget could be also found in obligatory attachments online in MS2014+ system.

5.2. Research program 2: Dynamics of Nano- and Microparticle Systems in Living Cells

Abstract

Gradual introduction of nanoparticle and microparticle systems in medical and technical fields caused a revolution in numerous technologies. Detailed understanding of the interaction of these systems with living organisms, especially animals, is the key not only to a medical use of these systems, but also to minimize or completely eliminate health and environmental risks of uses of such systems. The research program builds on years of experience, knowledge, cooperation and synergy established R & D centres at the Institute of Clinical and Experimental Medicine (IKEM) and the Institute of Macromolecular Chemistry of the Academy of Sciences of the Czech Republic (IMC ASCR). Research centre in IKEM has experience in tissue cultures of animal cells, cell transplantation, animal models, confocal microscopy, imaging and radionuclide techniques, nuclear magnetic resonance and optical imaging. Research Center for IMC ASCR (SUPRAMOL) has experience with the preparation, physico-chemical characterization, radiation stability and instrumental studies on polymers and supramolecular polymer systems for biomedical applications including multimodal imaging probes, conjugates, polymers, antibodies and other proteins, micro- and nanoparticles. The intent of the research program is to explore the dynamics of nano- and microparticles in cell systems from three main perspectives represented by three research goals: 1) Polymer systems and as a tool for expanding the possibilities of studying cell microscopy techniques; 2) Soluble and supramolecular systems sensitive to oxidative stress and the presence of reactive oxygen species for the transport of biologically active substances into target cells and cellular compartments and 3) The dynamics of the cell nucleus and transport of biologically active substances into and from it.

5.2.1. Relation to research programs of the centre, further centre development

This research program is proposed as direct continuation of several recent research activities of R&D centre of IMC ASCR “SUPRAMOL”, which are specified in detail in chapter 3.4. All of these activities represent high quality research, which has been in past decade performed in R&D centre of IMC ASCR with the emphasis on self-assembled nanosystems such as micelles, polymerosomes and nanoparticles both as curiosity-driven fundamental research and as targeted applied research for biomedical and technical applications. This allows us to efficiently implement findings from fundamental research into practical outputs also due to experience and equipment for both organic and polymer synthesis and detailed physico-chemical characterization of nanosystems, therefore in-house covering all the needed areas.

Recently studied projects include polymer micelles as theranostic radiopharmaceuticals, stimuli-responsive polymer nanosystems assembled by changes in pH, temperature, and redox potential, lipase-biodegradable and reactive oxygen species-biodegradable polyester nanoparticles as drug delivery systems, solid lipid nanoparticles for photodynamic therapy, polyester nanoparticles for the delivery of immunomodulators for cancer immunotherapy, metal nanoparticles with defined shape and size as protein-conjugable labels for electron microscopy , contrast agents for ^{19}F-magnetic resonance imaging based on fluorinated polymers and biometal ions-assembled nanoparticles. With help of the newly implemented experimental techniques, the effect of the contrast agent will be examined also on the cellular

level by assessment of functionality of the labeled cells, agent cytotoxicity, intracellular localization and the degradation pathways.

Part of the research is devoted to scavengers of copper for oral therapy of Wilson´s disease and reactive oxygen species-scavenging gels for wound healing (currently commercialized as Hemagel®). The well-established radionuclide laboratory also included in this project is mostly working on radiolabelled polymers where the radiolabel serves as the active theranostic component, as tracker to follow fate of the system in complex biological environment or to quantify ultratrace amounts of bioactive components in polymer implants. We also study radiolysis of biologically relevant polymers by ionizing radiation. Therefore, the R&D centre of IMC ASCR "SUPRAMOL" represents an excellent platform with synergic scope of interest and experience with other partners to fulfil the goals of the proposed project.

5.2.2. State of the art

The interaction of an electron beam (beta radiation) used in electron microscopy with the matrix leads to the damage of the latter, which is particularly undesirable in case of native samples.[1-3] As for living cells, the DNA in the cell nucleus is the main vital structure damaged by the radiation, which results in mutations or cell death, depending on the intensity of the damage.[4, 5]At higher doses of irradiation, the damage of cell membranes also plays its role. The damage due to radiation is only partially caused by a direct interaction of flying electrons with the target structure; in most cases, it is caused indirectly by the fact that the ionizing radiation generates reactive oxygen species (ROS) in water that constitutes most of the mass of living organisms, which subsequently damages the cellular structures chemically. The most important reactive oxygen species thus generated in living systems and contributing to the oxidative stress are hydrogen peroxide, superoxide, and hydroxyl / hydroperoxyl radicals. All these phenomena contribute to the fact that, when electron microscopy is employed, the sample is damaged by electrons; hence, electron microscopy in its current form offers very limited possibilities for studying native (living) samples.

The damage is first to be detected and then minimized. Detection at the macroscopic scale uses different dosimeters of various sorts (mostly based on ionization of gas, scintillation, or radiochemical reaction – film dosimeters, chemical radiochromic dosimeters based on radiolysis with subsequent spectrophotometric evaluation) [6]. However, options of the in situ detection during the electron microscopy proper are very limited[7-9].

There are many known and described antioxidant quenchers of reactive radicals, including reactive oxygen species; both low molecular ones (e.g. ascorbic acid, tocopherol, polyphenols) and polymeric ones [10, 11]. The vast majority of them are stoichiometric, i.e., the antioxidant is exhausted after the reaction with radicals. This is, of course, a great disadvantage, especially at higher radiation doses. In living systems, there are also catalytic antioxidants that catalyse the degradation of reactive oxygen species, e.g. by disproportionation, namely such enzymes as superoxide dismutase or catalase [12-15]. The stability of these enzymes is limited, though, e.g. due to changes in pH.

The effect of radiation on the polymer structure of natural and synthetic origin can, however, be also utilised, in particular for radiation crosslinking, due to which we can prepare gels and insoluble nanofibers without adding any chemical crosslinkers [16, 17].

The spatial distribution of membrane proteins plays a vital role in the membrane processes, since, e.g., protein aggregation caused by the ligand binding often triggers intracellular signalling responses. Although this area is currently being intensively studied, many questions

still remain unanswered. Imaging of this distribution can be done in particular by fluorescent [18] microscopy or electron [19] microscopy, using appropriate labels (tags), such as antibodies and other proteins labelled by fluorescents or metallic nanoparticles [18, 20]. However, it would be much more advantageous to have in place some multimodal labels viewable by both electron microscopy and optical microscopy at the same time due to the complementarity of the two imaging modalities, in particular such labels that would be homogeneous in terms of their composition. Magnetic resonance imaging (MRI) has a lower resolution than the optical / electron microscopy, but it allows for imaging biological processes in native biological entities at the functional level, especially when using ^{19}F-MRI with its minimum natural background occurring due to the small content of fluorine in the tissue [21-24].

Disruption of the complex mechanism of important signalling pathways results in misregulation of their target genes, which is a phenomenon frequently associated with various diseases and developmental defects [25, 26]. Wnt signaling and Shh signalling represent signalling pathways with essential roles also in pluripotency and differentiation of embryonic stem (ES) cells [27-29]. Although our understanding of the molecular mechanism behind these two signalling pathways has significantly improved, almost no attention has been paid to long noncoding RNA (lncRNA) molecules, which has recently been identified as an important factor affecting protein function and gene expression [30, 31]. Canonical Wnt signalling is activated by interaction of the Wnt molecules with their receptors Lrp5/6 and Frizzled, which further leads to transport of beta-catenin from the cytoplasm into the nucleus. In the nucleus beta-catenin creates a complex with the Tcf/Lef transcription factors and activates them, which triggers transcription of their target genes [26]. Shh signalling is activated by the interaction of the Shh ligand with its receptor Smo, which leads to activation of the nuclear transcription factors Gli and to transcription of their target genes [26]. Potential interactions of various lncRNA molecules with the nuclear mediators of Wnt signalling (beta-catenin and Tcf/Lef proteins) and Shh signalling (Gli proteins) would have a considerable effect on their function and on the transcriptional activity of their target genes, and in turn on their biological roles including those in pluripotency and differentiation of ES cells.

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5.2.3. Research objectives, activities and results

This research program is primarily affiliated to the R&D centre of SUPRAMOL IMC CAS and ZRIR IKEM. Interdisciplinary character of this research is represented by numerous co-operations within the framework of 3 research objectives and 11 activities. Their depiction is schematically represented in Fig. 5.2.1. Detailed description of each research objective and all research activities, including their milestones is described in the following text.

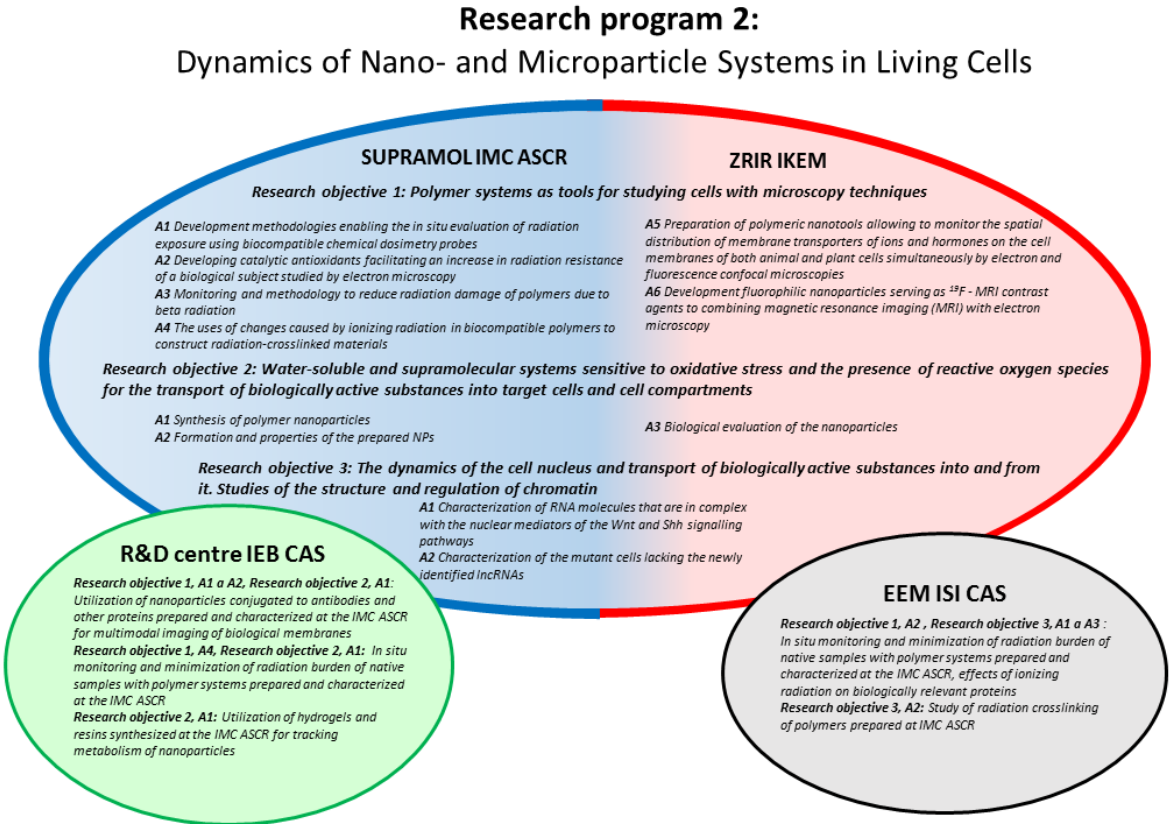

Figure 5.2.1: Schematic depiction of research program 2. This research program is primarily affiliated to the R&D centres SUPRAMOL IMC CAS (blue) and ZRIR IKEM (red). Involvement of proposer's R&D centre IEB ASCR (green) and partner's R&D centre EEM ISI CAS (grey), is specified for individual research activities of SUPRAMOL IMC CAS and ZRIR IKEM.

Research objective 1: Polymer systems as tools for studying cells with microscopy techniques

Interaction of electron beam (beta radiation) used in electron microscopy with the biological matrix leads to damage, which is particularly problematic for native samples. For live cells the main vital structure which is deteriorated by radiation is DNA in the cell nucleus, resulting accordingly to intensity of damage to mutations or cell death. At higher doses of irradiation also damage of cell membranes plays a role. Radiation damage is only partially caused by direct interaction of electrons with the target structure, but from the vast majority

it is caused indirectly by the reactive oxygen species generated from water forming overwhelming majority of the mass of living tissues. The most important reactive oxygen species thus generated in the living systems, and contributing to the oxidative stress, are hydrogen peroxide, superoxide, hydroxyl and hydroperoxyl radicals.

The Research objective 1 therefore comprises a total of 6 activities, **which all point to a significant improvement of opportunities to study cells using fluorescence and electron microscopies and magnetic resonance imaging.** Activities are focused on developing methodologies for in situ monitoring of radiation burden to native samples, on developing catalytic antioxidants to reduce their radiation damage, on methodology to reduce radiation damage of polymers due to beta radiation and on the uses of changes caused by ionizing radiation in biocompatible polymers to construct the radiation crosslinked materials. Another focus will be on the preparation of polymer nano tools to monitor the spatial distribution of membrane transporters of ions and hormones within the cell membranes of both the animal and plant cells simultaneously by electron and fluorescence confocal microscopies, on preparation and uses of fluorophilic nanoparticles that will serve as ^{19}F-MRI contrast agents as complementary imaging modality with electron microscopy (inherent contrast due to the presence of fluorine atoms) and fluorescence (after incorporation of fluorescent labels).

Activity 1: Development methodologies enabling the in situ evaluation of radiation exposure using biocompatible chemical dosimetry probes.

Head: *Mgr. Martin Hrubý, Ph.D.*

Biocompatible chemical dosimetric probes will be based on polymers bearing two spectrally distinct fluorescent labels, one easily degradable by reactive oxygen species generated by radiation and the second label which is radiation-stable serving as an internal calibration of the probe concentration . Evaluation of radiation exposure will be evaluated by comparing the fluorescence intensity of the two fluorophores at the site.

Alternatively, the polymer will be prepared carrying radiation-stable fluorophore and a FRET (fluorescence resonance energy transfer) quencher sensitive to radiolysis. Radiolysis of the quencher will then switch on fluorescence. Because the employed polymers are biocompatible and do not contain toxic metals, such probes do not damage cells. The evaluation of radiation exposure will serve as a feedback in the development of instrumentation friendly to living object. Further evaluation of the effects of radiation damage will be done using electron paramagnetic resonance spin labeled polymers and in situ electrochemical methods. On the same principle (the combination of pH -responsive and pH -insensitive fluorescent label on one of the polymer) will be used for exploring probe radiation and photochemically induced pH changes in target cells.

Milestones: Fluorescent probes for the evaluation of radiation exposure and to evaluate radiation caused by local changes in pH.

Activity 2: Developing catalytic antioxidants facilitating an increase in radiation resistance of a biological subject studied by electron microscopy.

Head: *Ing. Zdena Sedláková, CSc.*

Firstly, the antioxidants will be based on polymer conjugates of proteins with enzymatically nonstoichiometric high antioxidant activity (superoxide dismutase and more stable manganic salen complexes emulating its activity, catalase and more stable porphyrin and phthalocyanine complexes emulating its activity, etc.). Unlike the stoichiometrically active antioxidants (ascorbic acid, tocopherol, etc.), these enzymes are “inexhaustible” and much

more active. Their polymer component protects the enzyme against chemical degradation caused by a cell and facilitates the penetration into cellular structures, which should be thus protected against radiation. These polymeric antioxidants will be compared with antioxidants based on sterically hindered amines of the 1,1,6,6,-tetramethylpiperidine type.

Milestones: Antioxidant decreasing the radiation burden of a native sample.

Activity 3: Monitoring and methodology to reduce radiation damage of polymers due to beta radiation.

Head: *Mgr. Martin Hrubý, Ph.D.*

Biocompatible polymers and their defined supramolecular aggregates (micelles, nanoparticles and liposomes) are key building blocks of modern nanopharmaceuticals, but their radiation lability/resistance is still largely overlooked, which can be very significant for radiation sterilization of such systems or if the polymer is used to construct polymer diagnostic or therapeutic radiopharmaceuticals.

Milestones: *Set of data on the radiation stability of polymers in biological environment.*

Activity 4: The uses of changes caused by ionizing radiation in biocompatible polymers to construct radiation-crosslinked materials.

Head: *RNDr. Petr Štěpánek, DrSc*.

We will develop conceptually new, microfibrous, biodegradable functional material prepared from a modified storage polysaccharide present in humans (glycogen) as direct-contact dressing/interface material for wound healing. Double bonds will be introduced into glycogen via allylation and will be further exploited for crosslinking of the microfibers. Triple bonds will be introduced by propargylation and will serve for further click functionalization of the microfibers with bioactive peptide. A simple solvent-free method involving controlled freezing and following freeye-drying allowing the preparation of thick layers will be used to produce nano/microfibers from allylated and/or propargylated glycogen. Crosslinking of the samples will performed by microtron beta-irradiation.

Milestones*: Radiation-crosslinked material suitable for wound healing..*

Activity 5: Preparation of polymeric nanotools allowing to monitor the spatial distribution of membrane transporters of ions and hormones on the cell membranes of both animal and plant cells simultaneously by electron and fluorescence confocal microscopies.

Head: *RNDr. Petr Štěpánek, DrSc*.

For this purpose, antibodies against these membrane proteins will be conjugated to polymer-biocompatibilized CdSe/CdTe quantum dots. The quantum dots are optically viewable due to fluorescent properties and are also visible by electron microscopy on the basis of electron density due to elemental composition. Conjugation of the antibodies will be performed by oxidative cleavage with periodate followed by reductive amination on the glycosylation site or, if appropriate, *via* thiol groups generated from disulphide bridges by tris (carboxymethyl)phosphine (in both cases the bond is fully preserving the antibody binding site intact). This conjugation will be combined with labeling the antibodies with nanoparticles of gold, silver and palladium of different sizes and shapes thus enabling to distinguish in electron microscopy more tags simultaneously in one sample.

Elegant alternative will be the use of chimeric proteins, the protein will combine the antibody with the appropriate binding activity with protein strongly chelating relevant metal serving as a marker for electron microscopy (transferrin chelating Fe, Ga, Zr or Hf,

phytochelatins or metallothioneins chelating Cd) and fluorescent protein GFP, allowing simultaneous monitoring of fluorescence.

Milestones: Set of polymer nanotools allowing to monitor the spatial distribution of membrane transporters and ion hormones on the cell membranes of all animal and plant cells simultaneously by electron and fluorescence confocal microscopies.

Activity 6: Development fluorophilic nanoparticles serving as ^{19}F - MRI contrast agents to combining magnetic resonance imaging (MRI) with electron microscopy.

Head: *Ing. Daniel Jirák, Ph.D.*

For precise spatial imaging of soft tissue, magnetic resonance imaging (MRI) is extremely suitable. This modern non-invasive diagnostic imaging method is based on measurement of signal coming from nuclear spin relaxation of proton, usually hydrogen protons of water in tissue are exploited. Paramagnetic agents, which accelerate the spin relaxation of surrounding water protons enhancing their signal intensity are often (appx. 40 % of all examinations) used in MRI. However, generally all tissues contain water so usually inhomogeneous background deteriorates the detection accuracy. Using a non-^{1}H MRI may solve this difficulty. As a suitable agents seem to be probes based on ^{19}F. Fluorine, a natural ^{19}F monoisotope, has resonance frequency close to hydrogen one, therefore standard commercial scanners can be used for fluorine probe detection after only relatively minor hardware and software changes (usually an RF coil tuned to fluorine frequency and simple sequence modification is sufficient). The ^{19}F nucleus shows comparably high sensitivity as the hydrogen. In addition, due to nearly zero concentration of fluorine in the living organisms, there is no background in fluorine-based images or spectra. Although the local concentration of the fluorinated labels may be in many cases high (numerous perfluorinated compounds are biologically inert), it will be probably necessary to search ways how to improve the sensitivity of fluorine detection. Low, but highly specific fluorine signal obtained at lower resolution may in addition to it be supplemented by and merged with highly spatially resolved anatomical proton ^{1}H MR images or other methods such as fluorescence. An example of a ^{19}F MR imaging with a fluorescein-bound probe from our laboratory can be seen in Fig. 5.2.2.

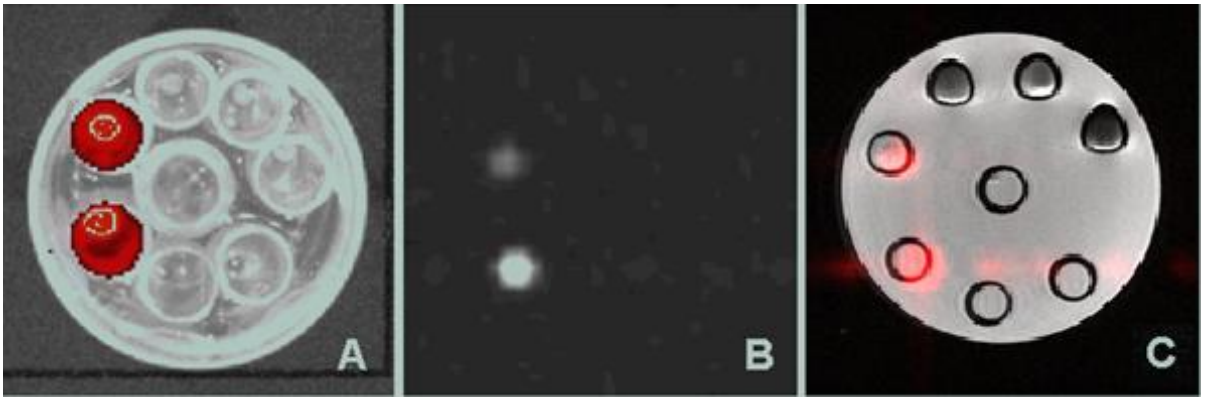

Figure 5.2.2: Imaging of 300 pancreatic islets. *In vitro* optical imaging (A), ^{19}F MRI (B), and merged ^{19}F and ^{1}H MRI (C) of two samples with a fluorescent-bound fluorine probe (two test tubes on the left – 1: 300 pancreatic islets labeled with fluorine contrast agent, 2: reference (incubation medium with 163mM concentration of fluorine) and control ones.

The development of self-assembling block copolymers with different blocks being able to segregate in immiscible phases under appropriate conditions has proven their enormous

potential to obtain different nanostructures of interest, both in 2 and 3 dimensions. This naturally leads to investigation of physical and biophysical aspects of self-assembled polymer materials since self-organization is the governing principle of many biophysical activities and structures such as biological membranes, multienzyme complexes etc.

When one of the blocks is water-soluble, such macromolecules are called amphiphilic. Amphiphilic block copolymers are widely studied due to their ability to form organized aggregates in water solution and various microphase segregated morphologies in bulk. Such aggregates may be used as, e.g., drug delivery systems, nanoreactors (micellar catalysis) etc.

Recently, new copolymers called “polyphiles” were developed that are more complex than conventional amphiphiles – they contain an additional perfluorinated block with number of fluorine atoms, and such copolymer therefore bears the third immiscible macromolecular domain: the fluorophilic (perfluoroalkyl) phase which is immiscible with both hydrophilic and hydrophobic (lipophilic) blocks.

Self-organized polyphile aggregates enable, e.g., to combine biocompatibilizing and colloidally stabilizing effects of hydrophilic blocks of the copolymer with possibility to incorporate a drug or fluorescent label into the hydrophobic block while enabling to visualize the whole system with ^{19}F-MRI or electron microscopy due to the presence of fluorophilic blocks.

All the fluorophilic nanoparticles will serve as ^{19}F-MRI contrast agents combining this modality with electron microscopy (inherent contrast due to the presence of fluorine atoms) and fluorescence (after incorporation of fluorescent labels).

Milestones: Fluorophilic nanoparticles serving for multimodal imaging in biological systems.

Research objective 2: Water-soluble and supramolecular systems sensitive to oxidative stress and the presence of reactive oxygen species for the transport of biologically active substances into target cells and cell compartments.

This issue is thematically linked to the Research objective 1 concerning oxidative damage by radiation, which is largely just that reactive oxygen species generated during irradiation. The goal is the development of a nanoparticulate system for the targeted transport and controlled release using particles sensitive to external concentration of reactive oxygen species. Nanoparticles responsive to changes in the environment are very suitable as carriers for applications in nanomedicine in the diagnosis and therapy of numerous diseases. This research will contribute to the creation of new transport concept "drug delivery" platform based on biodegradable polymeric nanoparticles react to the specific characteristics of the external environment directed at the inflammatory and malignant tissue. These two types of tissue typically produce much higher concentrations of reactive oxygen species such as superoxide or hydrogen peroxide. The newly prepared and studied nanoparticles are designed to degrade and release the biologically active ingredient in the presence of reactive oxygen species at biologically relevant concentrations. This system will also be used as a carrier antioxidants closed with oxidative stress caused by irradiation with an electron beam (protection of the biological sample during irradiation).

Among the aforementioned oxidation responsive polymers, the preparation of polymeric systems sensitive to H_2O_2 through the cleavage of boronic ester compounds or by the formation of oxalate bounds is a straightforward approach. Boronic ester compounds and oxalate bounds can be introduced to the motifs of polymeric nanoparticles design and the

cleavage leads to polymer backbone degradation followed by cargo release. With these strategies small hydrophobic chemotherapeutic agents can be released upon exposure to biologically relevant oxidative conditions, *e.g.*; from μM to mM concentrations of H_2O_2. ROS tissue-specific delivery of therapeutics bearing boronic acid groups can be achieved by using metabolite monomers that are normally present in the human metabolism to create ROS-responsive biodegradable/biocompatible NPs. ROS-responsive NPs could also be prepared by using curcumin as a polymer backbone under reaction with oxalyl chloride forming ROS-responsive oxalate linkages. Furthermore, curcumin in presence of bortezomib form a complex that can be exploited for drug delivery applications that have been shown *in vivo* synergistic effects.

Research Objective 2 includes altogether 3 activities focused on the development of functional tests of nanoparticles sensitive to reactive oxygen species.

Activity 1: Synthesis of polymer nanoparticles

Head: *Mgr. Martin Hrubý, Ph.D.*

The polymers and nanoparticles (NPs) thereof will be prepared by nanoprecipitation. One example is shown herein: using the nanoprecipitation the polymers (and the cargo: Nile Red dye (NR) or chemotherapeutic drugs – paclitaxel, others) will be firstly dissolved in appropriate solvent miscible with water, loaded into syringes and injected into water/PBS by a programmable pump. The polymer undergoes controlled precipitation in water leading to NPs formation. The organic solvents will be further removed by evaporation under reduced pressure and/or dialysis. Counterpart NPs with equivalent NR-loaded amounts will be prepared as ROS un-responsive NPs from the well-known FDA-approved polyester PLGA with similar molecular weight of the synthesized polymers. The NR will be choice because it is a hydrophobic dye largely used as model of hydrophobic drugs with the advantage to be fluorescent only if in contact with hydrophobic environments (particles core) being the NR fluorescence quenched at hydrophilic environment (cells and surroundings). Briefly, NR is used as a model of drug and simultaneously as a fluorescent probe.

Milestones: Polymer nanoparticles.

Activity 2: Formation and properties of the prepared NPs.

Head: *RNDr. Petr Štěpánek, DrSc*.

The NPs self-assembly in aqueous and simulated physiological conditions will be studied by static, dynamic and electrophoretic light scattering methods, as well as, SAXS technique. Investigations will be focused on the ability of the polymer molecular structures response/or not to the external H_2O_2 concentrations (relevant physiological and pathological levels). The stability/degradability of the NPs in bioactive environments will be also tested. The following characteristics of the NPs will be evaluated: weight-average molecular weight [to determine NPs aggregation number and density - NPs porosity (important for drug delivery)]; second virial coefficient (characteristics of NPs-solvent interactions - particles stability); radius of gyration and hydrodynamic radius from which the structure ratio ($\rho = R_G/R_H$) characteristic for the inner structure (architecture) will be calculated (NPs inner structure); ζ-potential (NPs stability through surface charge); polydispersity index of NPs (the width of size distribution of the NPs); degradation behaviour (studies of the NPs degradation and their degradation products *in vitro)*; The use of scattering methods will provide a set of data suitable for full characterization of the NPs on supramolecular and molecular levels. For characterization of

individual NPs, imaging techniques electron microscopy (TEM and cryo-TEM) and atomic force microscopy (AFM) will be used.

The NPs degradation and degradation products will be evaluated by SEC and NMR experiments to validate the NPs degradation mechanism upon contact with biological relevant levels of ROS. The studies will be performed based on previously published methodology from the authors. The encapsulation efficiency and drug release will be determined by fluorescence correlation spectroscopy and HPLC methods.

Milestones: The full set of physico-chemical characterization data on the polymer nanoparticles.

Activity 3: **Biological evaluation of the nanoparticles.**

Head: Doc. RNDr. Dušan Cmarko, CSc.

The cytotoxicity of the new prepared NPs will be evaluated by using tetrazolium (MTT) based colorimetric assay for cell growth and chemosensitivity. The NPs will be tested after incubation with normal cells (macrophages and human fibroblasts) and after incubation with cancer (HeLa and PC-3) cells.

To determine the capability of the prepared NPs to release bioactive agents in response to a physiologically different relevant source of ROS (cancer or inflammation), we will compare the amount of intracellular NR dye released from NR-loaded NPs in activated (ROS overproducing) *vs* non-activated macrophages based on the disappearance of the NR fluorescence in presence of H_2O_2 or in presence of a ROS-generated molecule in inflammation, in cancer cells (PC-3 and HeLa cells) or in both cells, following the previously described methodologies.

The NR-loaded NPs will be exposed to a range of concentrations (0 to 3.3 vol.%) of H_2O_2. Fluorescence intensity of NR will be monitored in a 96 well plate using a micro plate reader. Release of the dye due to oxidation and destabilization of the investigated systems will be assessed over time based on disappearance of NR fluorescence.

The experiments will be performed utilizing a molecule known to generate superoxide, nitric oxide and peroxynitrite, SIN-1 (Sigma-Aldrich). NR loaded NPs will be treated with a range of SIN-1 concentrations (1 to 100 mM). NR release will be quantified as described for H_2O_2 experiments above. From the NR release the degradation rate of the polymeric NP will be quantified.

The NR-loaded NPs will be incubated with PC-3 and HeLa cancer cell lines (ROS rich cell producer environment) at different incubation times. Fluorescence intensity of NR will be monitored in a 96 well plate using a micro plate reader. Release of the dye due to oxidation and destabilization of the investigated systems will be assessed over time based on disappearance of NR fluorescence. The loss of fluorescence for each sample at each incubation time will be compared with the fluorescent value from the samples incubated with the macrophages cell lines as negative controls according to the aforementioned method.

To follow intracellular trafficking of NPs, the cells (cancer and non-cancer cells) will be incubated with Lysotracker dye to label lysosomes and then incubated with the NR-loaded NPs. The co-localization of NPs within Lysotracker-labeled cellular compartments will be determined. The results will be evaluated by labeling lysosomes with Texas Red dextran (10,000 kDa).

All the series of the experiments aforementioned will be performed by using as a reference the ROS unresponsive counterpart with equivalent amounts of NR-loaded PLGA NPs.

The NPs cytotoxicity will be tested after incubation with normal cells (macrophages and human fibroblasts) and after incubation with cancer (HeLa and PC-3) cells and activated macrophages (model of inflammation). Boronic acid-based NPs loading the chemotherapeutic paclitaxel (cancer model) or the anti-inflammatory drug dexamethasone (inflammation model, new approach) will be tested. Curcumin oxalate-based NPs complexed with bortezomib will be tested on cancer model, as well as, the curcumin oxalate-based NPs loading dexamethasone in inflammation model.

Paclitaxel will be utilized because is a drug clinically used to treat ovarian, breast, lung and pancreatic cancers. Furthermore, paclitaxel loaded into micelles was already approved in Korea for the first-line treatment of metastatic or recurrent breast cancer, second-line treatment of metastatic breast cancer after failure of standard chemotherapy and for first-line treatment of locally advanced or metastatic non-small cell lung cancer. Bortezomib (Velcade®) it is approved for treating relapsed multiple myeloma and mantle cell lymphomas being a highly potent chemotherapeutic drug (dose in humans 1.3 mg/m^2). Bortezomib can be complexed with curcumin for synergistic effect, however, this approach has not been shown as a curcumin-based polymer, which could potentially increase the chemotherapeutic effects. Dexamethasone is used to treat many inflammatory conditions, moreover, is employed in cancer patients undergoing chemotherapy to counteract certain side effects of their antitumor treatments.

Polymeric nanoparticles will be provided for biological testing in *in vivo* animal models both within IKEM and workplace strategic partner (1st Faculty of Medicine of the Charles University in Prague), these tests are however not direct part of this project.

Milestones: The full set of biological characterization data on the polymer nanoparticles.

Research objective 3: The dynamics of the cell nucleus and transport of biologically active substances into and from it. Studies of the structure and regulation of chromatin.

The aim of the proposed project is to identify and characterize new factors involved in the regulation and structural-functional organization of chromatin during the process of differentiation.

Activity 1: Characterization of RNA molecules that are in complex with the nuclear mediators of the Wnt and Shh signalling pathways

Head: *Doc. RNDr. Dušan Cmarko, CSc.*

Using the RNA immunoprecipitation RIP-seq method and antibodies against the nuclear mediators of Wnt signaling (beta-catenin and Tcf/Lef proteins) and Shh signaling (Gli proteins) we will identify RNA-interacting partners of these proteins in embryonic stem (ES) cells and we will focus on long noncoding RNAs (lncRNA). The newly identified interactions between lncRNAs and the nuclear mediators of Wnt and Shh signaling will be confirmed by the RIP and RNA pull down methods. The nuclear localization pattern of selected lncRNAs will be identified using light and electron microscopy. Using known agonists and antagonists of canonical Wnt signaling and Shh signaling we will analyze the influence of the activity of the Wnt signaling and Shh signaling pathways on co-localization of lncRNAs and their protein partners in the nucleus. The agonist and antagonists of the pathways will be used directly, but also as part of various nanoparticles (SUPRAMOL ÚMCH). We will analyze the transcription level of selected lncRNAs and also selected interactions during the process of differentiation of ES cells into

neuronal progenitors. We will also focus on identification of other proteins that are in complex with the identified lncRNAs and we will study their role in canonical Wnt signaling and Shh signaling.

Milestones: Identification and characterization of new factors that regulate chromatin structure.

Activity 2: Characterization of the mutant cells lacking the newly identified lncRNAs

Head: *Doc. RNDr. Dušan Cmarko, CSc.*

This activity 2 is directly dependent on activity 1 of this aim. Using the CRISPR/Cas9 method we will create mutant embryonic stem cells lacking selected lncRNAs newly identified in activity 1 as partners of the nuclear mediators of canonical Wnt signalling and Shh signalling. We will perform a phenotypic analysis of the mutant cells and we will study the effect the lack of the selected lncRNA will have on the expression of target genes of canonical Wnt signalling and Shh signaling by: a. performing a Q-RTPCR analysis for selected known target genes of both signalling pathways in wild type and mutant cells, b. creating a global transcription profile of wild type and mutant cells using the RNA-seq technique. We will also study the effect the lack of the newly identified regulatory factor, lncRNA, will have on the process of differentiation of ES cells and on their ultrastructure.

Milestones: Identification and characterization of new factors that regulate chromatin structure.

5.2.4. International Cooperation

Within this research program, ZRIR IKEM will cooperate with the foreign teams in the frame of collaborations of newly hired group of researchers from 1st Faculty of Science. This concerns especially the Technical University of Darmstadt, where the main goal of the cooperation up to now has been to show a high-resolution structure of replication regions in the nucleus using a wide range of microscopic methods, see letter of support from Prof. C. Cardoso in Annex 6. We will also cooperate with Prof. G. Griffiths from University of Oslo, as stated in his letter of support in Annex 6. Prof. Griffiths has extensive background in different biological models for testing various systems including nanoparticles delivery systems and molecular probes in vivo. The expertise and equipment of the group of Prof. Cardoso and Griffiths are complementary to those of the other members of the consortium.

The workplace at IKEM has established collaboration with a series of foreign laboratories in the framework of international projects such as the recent European Network for Cell Imaging and Tracking Expertise project (ENCITE). Currently this workplace is actively cooperating with Dr. O. Bieri, University of Basel Hospital, since 2004 with UoB (Prof. Thorsen). MR group also closely collaborates with Center of MR Excellence in Vienna (Dr. S. Trattnig), E. Moser). The collaborations are documented by joint publications.

Researchers affiliated with R&D team from SUPRAMOL IMC ASCR are part of running collaborations with centres from the University of Minnesota, Minneapolis, USA; Commissariat a l´Energie Atomique, Saclay, France; University of Science and Technology, Hefei, China; University of Oslo, Norway; University of Ghent, Belgium (see letter of support from Prof. R. Hoogenboom, Annex 6); University of Antwerp, Belgium; Helmholtz zentrum Rossendorf, Germany (see letter of support from Dr. Holger Stephan, Annex 6); Technical University of Munich, Germany; CNRS Bordeaux, France; Institut Pluridisciplinaire de Recherches sur les Materiaux, Pau, France; University of Bahia Blanca, Argentina.

The team's international reputation is also evident from the fact that it has been chosen by nine foreign students for their doctoral studies or an annual post-doctoral internship (two of them as self-payers) and the team also hosted Professor T. Seery from the University of Connecticut, USA during the part of his sabbatical leave.

5.2.5. Research team

Composition of research team, description of organization for individual research activities

Research team of this research program 2 is composed of members of laboratories of R&D centres IMC CAS and ZRIR IKEM and several newly hired researchers. As depicted in a form of organization chart for this research program in Fig. 5.2.3, the research activities described in detail in the chapter 5.2.3 are assigned to individual responsible researchers. Every research activity will be performed with direct responsibility to the head of the research program. The summarization of nominated members of the team as well as members that will be nominated for all years of the project is given in Table 5.2.1 together with their role in the project, H index and FTEs (full time equivalents). In addition, curriculum vitae for all nominated members of the team are given in Annex 7. These CVs summarize expertize of the team members, their best 8 publications in the research field of the proposed project and other relevant information (Annex 7).

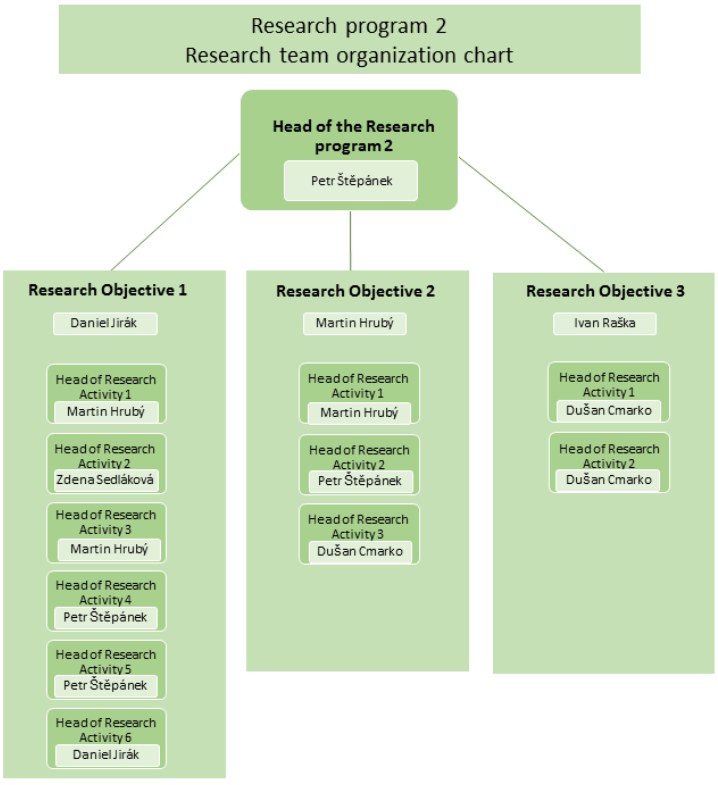

Figure 5.2.3: Organization chart of the research team of research program 2. The head of the research program will be responsible for co-ordination of activities in three research objectives. Researchers responsible for individual research activities will be directly answerable to the head of the program.

RNDr. Petr Štěpánek, DrSc., **excellent researcher** (2017-2022)**,** he will responsible for the Research Objective 1 (supervising Activities 4 and 5) and Research Objective 2 (supervising Activity 2) and will be also member of team working on other activities of Research Objectives 1 and 2. In addition to coordination activities and writing publications he will perform physico-chemical characterization of nanoparticle systems using advanced scattering techniques at the partner institution IMC. He will also be in charge of the cooperation with IKEM in the development of MRI contrast agents. His expertise and publication record are given in detail in his CV (Annex 7).

Prof. RNDr. Ivan Raška, DrSc., key researcher (2017-2022), will be newly hired for the project, he will be supervising the activity of 3 in objective 2 and activities 1 and 2 of objective 3. He will supervise experiments, analysis of data, manuscript writing or recruitment of new collaborators. Moreover, he will consolidate cooperation between partner laboratories in this project as well as between international partners as C. Cardoso and G. Griffiths. His expertise and publication record are given in Annex 7.

Mgr. Martin Hrubý, Ph.D., key researcher (2017-2022), he will be responsible for the Research Objective 1 (supervising Activities 1 and 3) and Research Objective 2 (supervising Activity 1) and is also member of team working on other activities of Research Objectives 1 and 2. He will be responsible for the organic and polymer synthesis and will coordinate the experiments within these activities at the partner institution IMC. His expertise and publication record are given in detail in his CV (Annex 7).

Ing. Daniel Jirák, Ph.D., key researcher (2017-2022), he will responsible for the Research Objective 1, heading the activity 6 of objective 1 and being active in other activities of objectives 1, 2. Besides coordination and literature activities he will be responsible for visualization of cells by magnetic resonance and optical imaging. His expertise and publication record are given in detail in his CV (Annex 7).

RNDr. Zdena Sedláková, CSc., **key researcher** (2017-2022)**.** She will be responsible for the Research Objective 1 (supervising Activity 2) and is also member of team working on other activities of Research Objectives 1 and 2. She will be responsible for studies of antioxidation properties of the materials and their properties in quenching by radicals and for degradation studies of materials due to oxidative stress at the partner institution IMC. Her expertise and publication record are given in detail in his CV (Annex 7).

Doc. RNDr. Dušan Cmarko, Ph.D., senior researcher (2017-2022). Under the guidance of Prof. Raška, he will be responsible for the activity 3 of research goal 2 and for the activities 1 and 2 of research goal 3. He will be focused on dynamics of nano- and microparticle systems and elucidating the factors involved in function chromatin organization in animal/mammalian cells. He will be responsible for planning and supervising the work, performing the in vivo experiments, electron microscopy experiments and will write manuscripts. DC has profound experience in the field of cellular biology acquired in different laboratories in Europe. He received long-term postdoctoral position in University of Lausanne. He was PI for three grants founded by the Grant Agency of Czech Republic and participated in several other national and international grants. His expertise and publication record are given in detail in his CV (Annex 7).

RNDr. Jan Kučka, Ph.D., **senior researcher** (2017-2022). He is radiochemist, who will perform experiments on radiation damage and evaluation of in situ dosimetry at the partner institution IMC within activities of Research Objectives 1 and 2. His expertise and publication record are given in detail in his CV (Annex 7).

Ing. Jiří Pánek, Ph.D., **senior researcher** (2017-2022). He will perform fluorescence microscopy studies of the prepared materials at the partner institution IMC within activities of Research Objectives 1 and 2. His expertise and publication record are given in detail in his CV (Annex 7).

Ing. Lenka Poláková, Ph.D., junior researcher (2017-2022). She will perform studies of antioxidation properties of polymers and part of the polymer synthesis at the partner institution IMC within activities of Research Objectives 1 and 2.

Mgr. Rafal Poreba, Ph.D., junior researcher (2017-2022). He will perform part of the study of antioxidation properties of polymersas and characterization of polymer materials at the partner institution IMC within activities of Research Objectives 1 and 2.

Mgr. Zdeňka Syrová, Ph.D., **junior researcher** (2017-2022)**.** She will be responsible for the activity 3 of research goal 2 and for the activities 1 and 2 of research goal 3. She will perform imaging of the samples by light microscopy. She will be co-responsible for experiments on interactions of nanoparticles with live mammalian cells. She will be co-responsible for experiments on interactions of nanoparticles with live mammalian cells. Her research is focused on the nanomedicine and regenerative medicine where nanomaterials are applied as carriers of therapeutic agents or cells. She has undergone training in foreign laboratories in the field of regenerative medicine and stem cells. For several years she worked in this scientific field. As a leader of the scientific group she used her expertise to develop the study of the interaction of cells with nanofibre structures. Her expertise and publication record are given in detail in his CV (Annex 7).

Ing. Tomáš Vacík, Ph.D., **junior researcher** (2017-2022)**.** He will be responsible for the activity 3 of research goal 2 and for the activities 1 and 2 of research goal 3. He will be responsible for planning and performing mainly molecular biology experiments, analyses and presentations of the acquired data and also for writing publications. His research is focused on elucidating the molecular mechanisms behind various biological processes involved in regulation of gene expression and chromatin structure. His expertise and publication record are given in detail in his CV (Annex 7).

Mgr. Ondřej Sedláček, Ph.D., junior researcher (2017-2022). He will perform organic and polymer synthesis at the partner institution IMC within activities of Research Objectives 1 and 2.

Mgr. Alessando Jager, Ph.D., junior researcher (2017-2022). He will prepare polymer nanoparticles and other nanocarriers and be responsible for their characterization at the partner institution IMC within activities of Research Objectives 1 and 2.

Mgr. Eliezer Jager, Ph.D., junior researcher (2017-2022). He will be in charge of preparation of nanoparticles and their characterization at the partner institution IMC within activities of Research Objectives 1 and 2.

Mgr. Mariia Rabyk, Ph.D., junior researcher (2017-2022). She will perform chemical modification and instrumental characterization of polymer materials at the partner institution IMC within activities of Research Objectives 1 and 2.

Mgr. Andrea Gálisová, junior researcher (2017-2022). She will be responsible for the activity 6 of research goal 1. She will perform imaging of the samples by magnetic resonance.

Mgr. Markéta Jirátová, junior researcher (2017-2022). She will be responsible for the activity 6 of research goal 1. She will be preparing cell lines and performing in vitro experiments (microscopy, cytotoxicity assays etc.).

Mgr. Miloslav Drobný, junior researcher (2017-2022). He will be responsible for the activity 6 of research goal 1. He will be a radiofrequency coil constructor for magnetic resonance experiments and will be responsible for measurements on 3T clinical MR scanner.

Junior researcher, PhD student (2017-2022), will be involved in activity 3 of research goal 2 and for the activities 1 and 2 of research goal 3. She/He will perform imaging of the samples by light microscopy.

Junior researcher, PhD student (2017-2022), will be involved in activity 3 of research goal 2 and for the activities 1 and 2 of research goal 3. She/He will perform molecular biology experiments.

Junior researcher, PhD student (2017-2022), will be involved in the activity 3 of research goal 2 and for the activities 1 and 2 of research goal 3. She/He will focus on function chromatin organization in animal/mammalian cells.

Junior researcher, PhD student (2017-2022), will be involved in the activity 6 of research goal 1. She/He will construct a radiofrequency coils for experimental 4.7 T scanner.

3 PhD students (2017-2022) will take part in the experimental work and data analysis at the partner institution IMC within activities of Research Objectives 1 and 2.

2 technicians (2017-2022) will contribute routine work for preparation of the experiments and general technical support at the partner institution IMC CAS within activities of Research Objectives 1 and 2.

Technician (2017-2022) for the teams working on research in activity 6 of objective 1, she/he will ensure laboratory services.

Table 5.2.1: Research team of the research program 2. See text for the description of the role for each researcher.

First Name and Surname	Working position	Role in the team, affiliation to research activities	H-index	Year 1	Year 2	Year 3	Year 4	Year 5	Year 6
				FTE during the project					
Petr Štěpánek	Excellent Researcher	Head RP2, head activities 4, 5 objective 1; head activity 2 objective 2; supervision other activities objectives 1 and 2	30	0.45	0.45	0.45	0.45	0.45	0.45
Ivan Raška	Key Researcher	Member RP2, supervision activity 3 objective 2; supervision activities 1, 2 objective 3	31	0.4	0.4	0.4	0.4	0.4	0.4
Martin Hrubý	Key Researcher	Member RP2, head activities 1, 3 objective 1; Head activity 1 objective 2; supervision other activities objectives 1 and 2	17	0.45	0.45	0.45	0.45	0.45	0.45
Daniel Jirák	Key Researcher	Member RP2, head activity 6 objective 1; member other activities objectives 1, 2	16	0.45	0.45	0.45	0.45	0.45	0.45
Zdena Sedláková	Key Researcher	Member RP2, head activities 2 objective 1; member other activities objectives 1 and 2	16	0.45	0.45	0.45	0.45	0.45	0.45
Dušan Cmarko	Senior Researcher	Member RP2, head activity 3 objective 2; head activities 1, 2 objective 3	14	0.4	0.4	0.4	0.4	0.4	0.4
Jan Kučka	Senior Researcher	Member RP2, activities of objectives 1, 2	12	0.4	0.4	0.4	0.4	0.4	0.4
Jiří Pánek	Senior Researcher	Member RP2, activities of objectives 1, 2	4	0.4	0.4	0.4	0.4	0.4	0.4
Lenka Poláková	Junior Researcher	Member RP2, activities of objectives 1, 2	2	0.5	0.5	0.5	0.5	0.5	0.5
Rafal Poreba	Junior Researcher	Member RP2, activities of objectives 1, 2	7	0.5	0.5	0.5	0.5	0.5	0.5
Zdeňka Syrová	Junior Researcher	Member RP2, member activity 3 objective 2; member activities 1, 2 objective 3	7	0.4	0.4	0.4	0.4	0.4	0.4
Tomáš Vacík	Junior Researcher	Member RP2, member activity 3 objective 2; member activities 1, 2 objective 3	8	0.4	0.4	0.4	0.4	0.4	0.4
Ondřej Sedláček	Junior Researcher	Member RP2, activities of objectives 1, 2	6	0.5	0.5	0.5	0.5	0.5	0.5
Alessandro Jager	Junior Researcher	Member RP2, activities of objectives 1, 2, 3	10	0.5	0.5	0.5	0.5	0.5	0.5
Eliezer Jager	Junior Researcher	Member RP2, activities of objectives 1, 2, 3	14	0.5	0.5	0.5	0.5	0.5	0.5
Mariia Rabyk	Junior Researcher	Member RP2, activities of objectives 1, 2	2	0.5	0.5	0.5	0.5	0.5	0.5

Table 5.2.1 - continuation

First Name and Surname	Working position	Role in the team, affiliation to research activities	H-index	Year 1	Year 2	Year 3	Year 4	Year 5	Year 6
				FTE during the project					
Andrea Gálisová	Junior Researcher	Member RP2, member activity 6 objective 1	2	0.4	0.4	0.4	0.4	0.4	0.4
Markéta Jirátová	Junior Researcher	Member RP2, member activity 6 objective 1	0	0.4	0.4	0.4	0.4	0.4	0.4
Miloslav Drobný	Junior Researcher	Member RP2, member activity 6 objective 1	1	0.2	0.2	0.2	0.2	0.2	0.2
To be nominated	Junior Researcher, PhD student	Member RP2, member activity 6 objective 1	-	0.6	0.6	0.6	0.6	0.6	0.6
To be nominated	Junior Researcher, PhD student	Member RP2, member activity 3 objective 2; member activities 1, 2 objective 3	-	0.6	0.6	0.6	0.6	0.6	0.6
To be nominated	Junior Researcher, PhD student	Member RP2, member activity 3 objective 2; member activities 1, 2 objective 3	-	1.0	1.0	1.0	1.0	1.0	1.0
To be nominated	Junior Researcher, PhD student	Member RP2, member activity 3 objective 2; member activities 1, 2 objective 3	-	1.0	1.0	1.0	1.0	1.0	1.0
Will be nominated	PhD student	Member RP2, activities of objectives 1, 2	-	0.5	0.5	0.5	0.5	0.5	0.5
Will be nominated	PhD student	Member RP2, activities of objectives 1, 2	-	0.5	0.5	0.5	0.5	0.5	0.5
Will be nominated	PhD student	Member RP2, activities of objectives 1, 2	-	0.5	0.5	0.5	0.5	0.5	0.5
Will be nominated	Technician	Member RP2, activities of objectives 1, 2	-	0.5	0.5	0.5	0.5	0.5	0.5
Will be nominated	Technician	Member RP2, activities of objectives 1, 2	-	0.5	0.5	0.5	0.5	0.5	0.5
To be nominated	Technician	Member RP2, member activity 6 objective1	-	0.4	0.4	0.4	0.4	0.4	0.4

Results of key and excellent members of the research team in 2011-2015

RNDr. Petr Štěpánek, DrSc., excellent researcher

Selected 5 research publications related to the proposed project with citations specified:

1. Giacomelli, Fernando C.; Stepanek, Petr; Giacomelli, Cristiano; Schmidt, Vanessa; Jaeger, Eliezer; Jaeger, Alessandro; Ulbrich, Karel, , pH-triggered block copolymer micelles based on a pH-responsive PDPA (poly[2-(diisopropylamino)ethyl methacrylate])inner core and a PEO (poly(ethylene oxide)) outer shell as a potential tool for the cancer therapy, SOFT MATTER 2011, 7, 9316-9325, Times Cited: 33
2. Angelov, Borislav; Angelova, Angelina ; Filippov, Sergey K.; Narayanan, Theyencheri; Drechsler, Markus; Stepanek, Petr; Couvreur, Patrick ; Lesieur, Sylviane, DNA/Fusogenic Lipid Nanocarrier Assembly: Millisecond Structural Dynamics, JOURNAL OF PHYSICAL CHEMISTRY LETTERS 2013, 4, 1959-1964 Times Cited: 31
3. Borisova, O. ; Billon, L. ; Zaremski, M. ; Grassl, B. ; Bakaeva, Z. ; Lapp, A.; Stepanek, P.; Borisov, O., pH-triggered reversible sol-gel transition in aqueous solutions of amphiphilic gradient copolymers, SOFT MATTER, 2011, 7, 10824-10833 Times Cited: 29
4. Rodriguez-Emmenegger, C.; Jaeger, A.; Jaeger, E.; Stepanek, P.; Bologna Alles, A. ; Guterres, S. S.; Pohlmann, A. R. ; Brynda, E., Polymeric nanocapsules ultra stable in complex biological media, COLLOIDS AND SURFACES B-BIOINTERFACES, 2011, 83, 376-381 Times Cited: 25
5. Angelov, Borislav ; Angelova, Angelina ; Filippov, Sergey K.; Drechsler, Markus ; Stepanek, Petr ; Lesieur, Sylviane, Multicompartment Lipid Cubic Nanoparticles with High Protein Upload: Millisecond Dynamics of Formation, ACS NANO, 2014, 8, 5216-5226, Times Cited: 25

5 research projects related to the research program 1 with financial support specified (only PI or co-PI):

1. GA ČR, 16-02870S, principal investigator, „What can polysaccharides and peptide-like structures make together? Self-assembling biomimetic hybrid polymer architectures.**“** 2016-2018, 5 296 000 CZK
2. Norway funds, MŠMT 7F14009, co-investigator (with 3 partners), „Macromolecular toolbox for biomedical applications“, 2014-2016, 25 548 000 CZK in total
3. GA ČR, P304/12/0950, principal investigator (with 1 partner), „Chelating polymers as therapeuticals for Wilson´s disease“, 11 411 000 CZK
4. GA ČR, P208/10/1600, principal investigator, "Polymeric particles and nanostructured materials stabilized by surface active molecules“, 2010-2014, 7 421 000 CZK
5. MŠMT Kontakt, LH14079, principal investigator, "Termoresponsive polymeric catalytical depots: injectable reactors shaped in situ for biomedicine applications“, 2014-2016, 1 859 000 CZK.

5 patents and commercial applications related to the proposed project:

1. Deutsche Institut fur Kautschuktechnologie, projekt „Characterization and analysis of india rubber emulsions“, principal investigator, 2013-2014, 204 000 CZK
2. Patent application, Mikroparticles of chemically modified biopolymers for the prevention and decresing of symptoms of Wilson's diasease, 2015

Prof. RNDr. Ivan Raška, DrSc., key researcher

Selected 5 research publications related to the proposed project with citations specified:

1. Juda P, Smigová J, Kováčik L, Bártová E, Raška I. Ultrastructure of cytoplasmic and nuclear inosine-5'-monophosphate dehydrogenase 2 "rods and rings" inclusions. J Histochem Cytochem. 2014 Oct;62(10):739-50. (IF 1.959, number of citations WOS: 2)
2. Smirnov E, Borkovec J, Kováčik L, Svidenská S, Schröfel A, Skalníková M, Švindrych Z, Křížek P, Ovesný M, Hagen GM, Juda P, Michalová K, Cardoso MC, Cmarko D, Raška I. Separation of replication and transcription domains in nucleoli. J Struct Biol. 2014 Dec;188(3):259-66. (IF 3.231, number of citations WOS: 2)
3. Popov A, Smirnov E, Kováčik L, Raška O, Hagen G, Stixová L, Raška I. Duration of the first steps of the human rRNA processing. Nucleus. 2013 Mar-Apr;4(2):134-41. (IF 3.033, number of citations WOS: 5)
4. Křížek P, Raška I, Hagen GM. Flexible structured illumination microscope with a programmable illumination array. Opt Express. 2012 Oct 22;20(22):24585-99. (IF 3.14, number of citations WOS: 10)
5. Smigová J, Juda P, Cmarko D, Raška I. Fine structure of the "PcG body" in human U-2 OS cells established by correlative light-electron microscopy. Nucleus. 2011 May-Jun;2(3):219-28. (IF 3.033, number of citations WOS:14)

5 research projects related to the research program 1 with financial support specified (only PI or co-PI):

1. 2014-2017, co-PI, Projekt MSMT no. 7F14009, Czech - norwegian research programme, Macromolecular toolbox for biomedical applications, 5 552 000 Kc.
2. 2012-2018, co-PI, GACR č. P302/12/G157, Projects supporting excellence in basic research, project name: Dynamics and organization of chromosomes during cell cycle and during differentiation in norm and in pathology, 30 942 000 Kc.

5 patents and commercial applications related to the proposed project:

No patents or commercial applications in 2011-2015.

Mgr. Martin Hrubý, Ph.D., key researcher

Selected 5 research publications related to the proposed project with citations specified:

1. Sedláček, O.,Monnery B., D.,Filippov, S.,Hoogenboom, R., Hrubý, M.: Poly(2-oxazoline)s - are they more advantageous for biomedical applications than other polymers? Macromolecular Rapid Communications (2012), 33(19), 1648-1662, IF = 4.929, Citation WOS: 69
2. Jäger, E., Höcherl, A., Janoušková, O., Jäger, A., Hrubý, M., Konefal, R., Netopilík, M., Pánek, J., Slouf, M., Ulbrich, K., Štěpánek, P.: Fluorescent boronate-based polymer nanoparticles with reactive oxygen species (ROS)-triggered cargo release for drug-delivery applications. Nanoscale (2016), 8(13), 6958-6963, IF = 7.760, Citation WOS: 0
3. Sedláček, O.,Hrubý, M.,Studenovský, M.,Větvička, D.,Svoboda, J.,Kaňková, D.,Kovář, J.,Ulbrich, K.: Polymer conjugates of acridine-type anticancer drugs with pH-controlled activation Bioorganic &Medicinal Chemistry (2012), 20(13), 4056-4063, IF = 2.903, Citation WOS: 18

4. V. V. Philimonenko, A. A. Philimonenko, I. Šloufová, M. Hrubý, F. Novotný, Z. Halbhuber, M. Krivjanská, J. Nebesářová, M. Šlouf: Simultaneous detection of multiple targets for ultrastructural immunocytochemistry. Histochemistry and Cell Biology (2014), 141(3), 229-239, IF = 2.780, Citation WOS: 7
5. M. Hrubý, S. Filippov, P. Štěpánek: Smart polymers in drug delivery systems on crossroads: which way deserves following? European Polymer Journal (2015), 65, 82-97, IF = 3.485, Citation WOS: 11

5 research projects related to the research program 1 with financial support specified (only PI or co-PI):

1. New multistage nanodiagnostics for cancer imaging and prediction of antiangiogenic therapy efficacy, AZV, 16-30544A, principal investigator, 15 972 000 CZK, IMC 5806 000 CZK
2. Novel cancer diagnostics based on glycogen as body´s own nanosized carrier, AZV, 15-25781A, co-investigator, 10 601 000 CZK, IMC 5093 000 CZK
3. Hybrid materials based on macrocyclic ligands for medical applications, GAČR, 13-08336S, co-investigator, 11 928000 CZK, IMC 2852 000 CZK
4. Self-assembled polymeric nanostructures as bimodal magnetic resonance ultrasound contrast agents for imaging. GAČR 16-03156S, principal investigator, 8461 000 CZK, IMC 2913 000 CZK
5. Scaling-up biodegradable nanomedicines for multimodal precision cancer immunotherapy (PRECIOUS), Horizon 2020, H2020-NMP-2015-686089, co-investigator, 7000 000 EUR, IMC 592 000 EUR

5 patents and commercial applications related to the proposed project:

1. Czech patent PV 2012-739: Podešva, J., Hrubý, M., Kovářová, J., Spěváček, J. Antioxidants forautostabilised elastomeric polyurethans
2. Kučka, J., Hrubý, M., Studenovský, M., Větvička, D. Plastická hmota pro mobilní stínění zdrojů ionizujícího záření. Utility model, CZ 24789 U1, submitted 6. 10. 2011 (2011-24932), registered 10. 1. 2013. prodej licence
3. Patent application PV 2015-770: Hrubý, M., Vetrík, M., Kučka, J., Macková, H., Štěpánek, P., Mattová, J., Poučková, P., Urbánek, P. (2015) Polymer on the basis of polysaccharide and its usage in the prevention and treatment of Wilson's diasease.
4. Patent application PV 2016-117: Švec, P., Hrubý, M., Kučka, J., Sedláček, O., Štěpánek, P., Petřík, M., Nový, Z., Hajdúch, M. (2016) Iodined radiolabelled analogs of choline, their preparation and usage as medication.
5. Cígler, P., Hrubý, M., Kužel, S.: Composition for inducing increase of formation of biologically active compounds in plants and use thereof. Patent WO2005/118508 A2, 2005, prodej licence

Ing. Daniel Jirák, Ph.D., key researcher

Selected 5 research publications related to the proposed project with citations specified:

1. Rehoř I, Vilímová V, Jendelová P, Kubícek V, Jirák D, Herynek V, Kapcalová M, Kotek J, Cerný J, Hermann P, Lukeš I. Phosphonate-Titanium Dioxide Assemblies: Platform for Multimodal

Diagnostic-Therapeutic Nanoprobes. J Med Chem. 2011 Jul 28; 54(14):5185-5194 IF_{2011} 5.207, number of citations: 23 (WOS)

2. Kacenka M, Kaman O, Kotek J, Falteisek L, Cerny J, Jirak D, Herynek V, Zacharovova K, Berkova Z, Jendelova P, Kupcik J, Pollert E, Veverka P, Lukes I. Dual imaging probes for magnetic resonance imaging and fluorescence microscopy based on perovskite manganite nanoparticles. J Mater Chem. 2011, 21(1):157-164 IF_{2011} 5.207, number of citations: 21 (WOS)
3. Deligianni X, Jirák D, Berková Z, Hájek M, Scheffler K, Bieri O. In vivo visualization of cells labeled with superparamagnetic iron oxides by a sub-millisecond gradient echo sequence. MAGMA. 2014 Aug;27(4):329-37 IF_{2014} 2.869, number of citations: 3 (WOS)
4. Kačenka M, Kaman O, Kikerlová S, Pavlů B, Jirák Z, Jirák D, Herynek V, Černý J, Chaput F, Laurent S, Lukeš I. Fluorescent magnetic nanoparticles for cell labeling: Flux synthesis of manganite particles and novel functionalization of silica shell. J Colloid Interface Sci. 2015 Jun 1;447:97-106 IF_{2015} 3.368, number of citations: 4 (WOS)
5. Aasen SN, Pospisilova A, Eichler TW, Panek J, Hruby M, Stepanek P, Spriet E, Jirak D, Skaftnesmo KO, Thorsen F. A Novel Nanoprobe for Multimodal Imaging Is Effectively Incorporated into Human Melanoma Metastatic Cell Lines. Int J Mol Sci. 2015 Sep 8;16(9):21658-80 IF_{2015} 2.862, number of citations: 0 (WOS)

5 research projects related to the research program 1 with financial support specified (only PI or co-PI):

1. Self-assembled polymeric nanostructures as bimodal magnetic resonance ultrasound contrast agents for Imaging, 16-03156S 2016-2018, 8 461 000 CZK

5 patents and commercial applications related to the proposed project:

No patents or commercial applications in 2011-2015

RNDr. Zdena Sedláková, CSc., **key researcher**

Selected 5 research publications related to the proposed project with citations specified:

1. L. Poláková, V. Raus, L. Kostka, A. Braunová, J. Pilař, V. Lobaz, J. Pánek, Z. Sedláková: Antioxidant properties of 2-hydroxyethyl methacrylate-based copolymers with incorporated sterically hindered amine, Biomacromolecules (2015), 16(9), 2726-2734, IF = 5.750, Citation WOS: 0
2. F. Surman, T. Riedel, M. Bruns, N. Yu. Kostina, Z. Sedláková, C. Rodriguez-Emmenegger: Polymer brushes interfacing blood as a route toward high performance blood contacting devices. Macromolecular Bioscience. (2015) 15(5), 636-646, IF = 3.851, Citation WOS: 11
3. Rodríguez Emmenegger, C., Brynda, E., Riedel, T., Sedláková, Z., Houska, M., Bologna Alles, A.: Interaction of blood plasma with antifouling surfaces, Langmuir (2009)11, 6328-33, IF = 3.898, Citation WOS: 80
4. Nguyen N., H., Rodríguez Emmenegger, C., Brynda, E.,Sedláková, Z., Percec, V.: SET-LRP of N-(2-hydroxypropyl)methacrylamide in H2O, Polymer Chemistry (2013) 4, (8), 2424-2427, IF = 5.368, Citation WOS: 25
5. Rodríguez Emmenegger, C., Schmidt B. V. K., J., Sedláková, Z., Šubr, V., Bologna Alles, A., Brynda, E., Barner-Kowollik, C.:Low temperature aqueous living/controlled (RAFT)

polymerization of carboxybetaine methacrylamide up to high molecular weights, Macromolecular Rapid Communications. (2011) 32(13), 958-965, IF = 4.596, Citation WOS: 24

5 research projects related to the research program 1 with financial support specified (only PI or co-PI):

1. Hemagel - further improvement of healing properties of commercially viable product, 2012-2015, Co-investigator, TA ČR, Project number: TA02010141, ÚMCH: 4 331 000 CZK, Project: 10 967 000 CZK
2. Synthesis and characterization of biologically active surfaces prepared via controlled radical polymerization techniques and "click" chemistry, 2012-2014, Principal investigator, GA ČR, Project number: P106/12/1451, 6 564 000 CZK
3. Non-fouling polymers for biomedical applications prepared via controlled radical polymerization, 2013-2015, Principal investigator, MŠMT, Project number: LH13178, 1 666 000 CZK

5 patents and commercial applications related to the proposed project:

1. Preparation for the oral application CZ305391 (B6), 2015, Sedláková Z., Poláková L.
2. Oral preparation PCT/CZ2015/050011, 2015, Sedláková Z., Poláková L.
3. A method of displaying of alphanumeric information KZ29766, 2015, Suleimenov I., Mun G., Sedlakova Z., Shaltykova D, Iglikov I.,
4. A method for reproducing images KZ29768, 2015, Suleimenov I., Sedlakova Z., Mun G., Shaltykova D., Semenyakin N.
5. A method of introducing of symbol information KZ29310, 2013, Suleimenov I., Z. Sedlakova, Mun G., Semenyakin N., Shaltykova D., Obukhova P., Panchenko S.

5.2.6. Description of key equipment/investments

In tables presented below, descriptions of key equipment/investments and functional modules are given together with their purchase costs and technical specification. All instruments, machines and software (with purchase costs not being less than 1 mil CZK) that are planned to be used in the RP 1 are listed individually. Items with lower prize are assembled into functional modules, according to their characteristics and linkage to research activities of RP 1, but also RP 2 and RP 3.

Required investments are described further in the project budget (see Annex 8 and obligatory attachment in the online application MS2014+), commentary on budget (see Annex 9). Their pricing is based on quotations, which are all supplemented in Annex 10.

Key equipment / functional module	No. of items	Planned total price without VAT (thousands CZK)
1. Functional module for automated high pressure freezing and substitution of biological samples	1	8 450
Typical features:		

2 instruments for automated high pressure freezing and freeze substitution. High pressure freezing is the preferred method for preserving samples (up to 5 mm) in their close-to-native state. Freeze substitution is a common follow-on procedure to high pressure freezing suitable for immune-electron microscopy.

Purpose of the acquired equipment:
It will allow to visualize highly dynamic processes or the structural changes of samples at a nanometre resolution and with millisecond precision. Freeze substitution is time-consuming method and freeze substitution processor will speed up and make more effective process of sample preparation mainly for immune-electron microscopy. It will be combined with our existing Leica EM AFS2 instrument and will serve for the activities of research objective 3 of this RP and activities of activities of RP1 utilizing nanoparticle systems in plant cells and activities of RP3 that will utilize prepared nanoparticles for testing in EREM setup (Fig. 5.2.1).

Infrastructure readiness:
Instruments will be installed in the standard laboratory space, the requirement for the electricity supply is fulfilled in the planned room of ZRIR IKEM.

2. Functional module for synthesis of monomers, polymers and nanoparticles	1	5 210

Typical features:
The functional setup for synthesis of monomers, polymers and nanoparticles consists of the following equipment: microwave synthesizer with controllable power output, possibility of monitoring the cell temperature and sample changer; rotary evaporator with automatic vacuum station for evaporation of solvents; mercury UV lamp for fotochemical reactions; preparative HPLC setup for purification of prepared polymers; FT-IR spectrometer for monitoring chemical reactions and basic characterization of monomers and polymers; microfluidic setup including a plasma oven for preparation of polymeric nanoparticles and a sonicator for their dispersion.

Purpose of the acquired equipment:
This functional setup is intended for a well-controlled preparation of monomers, polymers and nanoparticles. It will be therefore crucial for activities of RP2, but also for activities of RP1 utilizing nanoparticle systems in plant cells and activities of RP3 that will utilize prepared nanoparticles for testing in EREM setup (Fig. 5.2.1).

Infrastructure readiness:
All components of this setup can be installed in a standard chemical laboratory; we anticipate installation in a newly reconstructed lab room 808 at IMC that is appropriate without additional modifications. Necessary supplies in the form of standard electricity supply are available in the lab room.

3. GPC system	1	5 040

Typical features:

Gel permeation chromatography setup consisting of a HPLC setup with thermo-regulated autosampler, UV-VIS detector with diode array (DAD), refractometer and multi angle laser light scattering detector (MALLS).

Purpose of the acquired equipment:
Since the biological behaviour of polymers is critically dependent on their molecular weight, the determination of distribution of molecular weights of polymers is essential for their usage in *in vivo* systems applications. This setup will be thus crucial for activities of RP2, but also for activities of RP1 utilizing nanoparticle systems in plant cells and activities of RP3 that will utilize prepared nanoparticles for testing in EREM setup (Fig. 5.2.1).

Infrastructure readiness:
The setup can be installed in a common chemical laboratory, we anticipate installation in a newly reconstructed lab room 501 at IMC that is appropriate without additional modifications. Necessary supplies in the form of standard electricity supply are available in the lab room.

4. CMOS camera for transmission electron microscope	1	4 266

Typical features:
High resolution 16 Mp camera for transmission electron microscope FEI Tecnai G2 20 Sphera. It reaches high speeds, strong dynamic performance and improved noise performance, allowing both effective low dose scanning of cryo-samples and direct high intensity scanning of difraction images.

Purpose of the acquired equipment:
With this camera, it will be possible to improve existing microscope for crystallographic reconstructions of thin protein crystals and structural characterization of nanoparticles.
This setup is crucial for activities of research objective 3 of this RP, but also for activities of RP1 utilizing nanoparticle and immunology systems in plant cells and also activities of RP3 that utilizes prepared nanoparticles for testing in EREM setup (Fig. 5.2.1).

Infrastructure readiness:
The camera will be inserted into our already installed transmission microscope.

5. Functional module for advanced imaging	1	2 603

Typical features:
This functional setup consists of components for microscopy, i.e. thermo-controlled microscopy stage (based on a Peltier element, temperature range -20 to +100 °C, aperture 5 mm), pulsed laser diodes (wavelength 405 and 450 nm, tunable power 20 mW, frequency up to 40 MHz, including collimation optics) and data analysis software for fluorescence confocal microscopy (for methods FLIM, FLIM-FRET, FLCS) as well as components for fluorescence techniques including lasers for the autoradiography-fluorescence scanner Typhoon (wavelengths 473, 532, 635 nm) with appropriate filters for 2D scanned images and the necessary sample holders, and a fluorimeter for measurements of excitation and absorption spectra in the range 200-900 nm, variable slit size 0,5-20 nm, for characterization of fluorescence properties of conjugates prepared for imaging techniques.

Purpose of the acquired equipment:
Advanced imaging of morphology and functional properties by specialized microscopy and autoradiography techniques of samples prepared during the project. This setup is crucial for

activities of RP2, but also for activities of RP1 utilizing nanoparticle systems in plant cells and activities of RP3 that will utilize prepared nanoparticles for testing in EREM setup (Fig. 5.2.1).

Infrastructure readiness:
A modern confocal microscope and autoradiography setup Typhoon are installed at IMC and all conditions are met for their flawless operation, such as air-conditioned dust-free laboratory, anti-vibration optical bench and other equipment. Planned equipment is anticipated to be installed in the laboratory for confocal microscopy (room 816) without additional modifications and imaging setup Typhoon will be installed in the laboratory 501. For the fluorimeter we anticipate installation in a newly reconstructed lab room 812 at IMC that is appropriate without additional modifications. Necessary supplies in the form of standard electricity supply are available in all lab rooms involved.

6. GC system	1	1 977

Typical features:
Gas chromatography setup with auto-sampler, temperature conductivity detector (TCD), and flame ionization detector (FID).

Purpose of the acquired equipment:
Gas chromatography is a necessary method of characterization of mixtures for monitoring chemical reactions and the purity of chemical compounds. This setup will be thus crucial for activities of RP2, but also for activities of RP1 utilizing nanoparticle systems in plant cells and activities of RP3 that will utilize prepared nanoparticles for testing in EREM setup (Fig. 5.2.1).

Infrastructure readiness:

The setup can be installed in a normal chemical laboratory, we anticipate installation in lab room 508 at IMC that is appropriate without additional modifications. Necessary supplies, i.e. standard electricity supply, technical gases (nitrogen, hydrogen, oxygen) are available, including space in the room 508 for pressure containers complying with regulatory requirements.

7. Instrument for automated freezing of thin liquid samples	1	1 500

Typical features:
The instrument for the preparation of vitrified fluid samples or extremely thin samples for cryo-TEM. For technical specification, see Annex 10 of this feasibility study.

Purpose of the acquired equipment:
In this instrument, the sample is maintained in a temperature and humidity controlled environmental chamber prior to freezing. This allows to increase efficiency and reproducibility of cryo-TEM. It is important for activities of research objective 3 of RP 2. It is also needed for the optimization of combinations of immunological and nanoparticle approaches in electron microscopy of plant and animal cells (Fig. 5.2.1).

Infrastructure readiness:
Instruments will be installed in the standard laboratory space, the requirement for the electricity supply is fulfilled in the planned room of ZRIR IKEM.

8. Melting temperature instrument	1	1 425

Typical features:

Melting temperature instrument with automatic recording for at least 6 samples, optical monitoring of not only the melting temperature but also additional temperature sensitive processes in polymers and their solutions.

Purpose of the acquired equipment:
Determination of the melting temperature of polymers as a basic characteristic property of both low- and high-molecular weight compounds, determination of temperature dependence of phase transitions in polymers and their solutions. It is crucial primarily for activities of RP 2.

Infrastructure readiness:
The setup can be installed in a normal chemical laboratory, we anticipate installation in a newly reconstructed lab room 808 at IMC that is appropriate without additional modifications. Necessary supplies in the form of standard electricity supply are available in the lab room.

9. Functional module for MR/CT image analysis	1	1 169

Typical features:
Functinal module consisting of powerful computer system (iMac) combined with software for digital imaging. The main characteristics are powerful graphics allowing the usage of high resolution screen, software Osirix for the analysis of MR/Ct images and VGStudio Max 3.0 software for automated segmentation and 3D animations.

Purpose of the acquired equipment:

The computer systems together with high-end software OsiriX and/or VGStudio Max 3.0 will be used for advanced post-processing techniques in 2D and 3D navigations in bio-medical images analysis. This module is crucial primarily for the activity 6 of research objective 1 in RP 2, i.e. for the development of new contrasting compounds for MR.

Infrastructure readiness:
Instruments will be installed in the standard laboratory space, the requirement for the electricity supply is fulfilled in the planned room of ZRIR IKEM.

5.2.7. Research program budget - relation to the overall project proposal budget

The budget of this RP2 is attached in Annex 8 together with detailed comments for all individual items (see Annex 9). The budget and comments on budget could be also found in obligatory attachments online in MS2014+ system.

5.3. Research program 3: New methods and instruments for high-resolution microscopy and chemical analysis of highly susceptible biological samples in native state.

Abstract

Research and development of top-class analytical and imaging instruments and methods are crucial in the fundamental exploration of both living and inanimate nature. Our research program is built on long-term experience, knowledge and the tradition of the R&D centre of the Environmental Electron Microscopy at the Institute of Scientific Instruments of the Czech Academy of Sciences (EEM R&D centre of ISI CAS), which has been pioneering methods of environmental electron microscopy in the Czech Republic. In cooperation with partners, the objective of this research program is to research and develop novel, combined environmental electron-microscopic, optical and chemical methods and instruments with the intention to provide both imaging quality and sample preservation significantly beyond the current commercial methods. Highly sensitive samples of plant and animal tissues, polymer samples and ice will be explored using newly developed methods in their native state. Within this research program, we propose to assemble of new low-dose high-resolution environmental electron and optical fluorescence microscope with sensitive X-ray and Raman microanalysis, together with the possibility of mechanical and laser micromanipulations optimized for living of wet samples. Thanks to the integration of all above mentioned methods into a single instrument and the possibility of their simultaneous application, this project opens new dimensions of correlative functional imaging of living and inanimate samples. The entire range of the application methods will be supplemented with high-resolution transmission electron cryo-microscopy, namely due to the limited resolution of optical, environmental scanning and transmission scanning electron microscopy of wet samples.

5.3.1. Relation to research programs of the centre, further centre development

This research program is a direct continuation of long-term activities of the R&D centre EEM CAS described in detail in chapter 3.4. This program reflects present needs for new methods and instrumentation for research and development in the field of *in vivo* studies of highly sensitive, mostly biological and polymer samples. The main goal of the program is to support the research activities of the R&D centre IEB CAS, the main applicant of the project. The research represented by numerous activities within this research program is focused to further support development of applicant and partner centres. It will be oriented on advanced microscopy in high detail on minimally-treated native samples or samples labelled with special nanoparticles (SUPRAMOL, IMC CAS) using a high resolution, analytical ESEM of new concept. This instrument will be equipped with multidimensional correlative microscopy, based on the combination of light imaging and manipulation methods and electron microscopy methods. Further analysis of samples will be performed in the cooperation with R&D centre ZRIR IKEM using magnetic resonance imaging (MRI). All activities planned within individual research objectives are highly ambitious, but realistic, mainly thanks to direct continuity to the current research of the R&D centre EEM CAS and the long-term experience in the field of electron microscopy.

Research program is divided into 4 research objectives: 1) Simulation of the interaction of the electron beam with gas, liquid and solid matter in ESEM, 2) New highly sensitive detectors and detector principles for ESEM, 3) New methods for the characterization of highly

susceptible samples, dynamic in-situ experiments and 4) The unique analytical ESEM with high resolution – integration of new systems and methods, correlative microscopy.

5.3.2. State of the art

Simulation of electron beam interaction with gas, liquid and solid matter in ESEM.

The advantage of the environmental scanning electron microscope (ESEM) [1,2] is the possibility to directly observe non-conductive specimens without charging artefacts [3,4], fully hydrated wet samples [5,6,7], plants [8,9,10,11] microgel particles [12] and the possibility to perform dynamic „in-situ“ experiments [13,14,15], all under pressure environment ranging from units to thousands of Pa. A significant disadvantage of ESEM is the scattering of the primary electron (PE) beam in the gaseous environment [16,17]. Danilatos has claimed [18,19] that the image resolution at a high pressure can be comparable with that of the SEM. However, the “skirt effect” causes a decrease in the probe current in the focussed spot so that the signal-to-noise ratio (SNR) is lowered. This fundamental problem is currently mitigated by decreasing the distance in the gaseous environment which (PE) pass through, increasing the PE energy and the probe current, using a lower scanning speed and choosing a suitable gas type and its pressure [20,21]. Especially high beam current (tens to hundreds pA) and low scanning speed are responsible for extensive radiation damage of susceptible biological and polymer samples and avoid study of this samples in their native state [22]. In consideration of mentioned background, the gas scattering phenomena are important to study, as it has been shown by Berre et al. [23] and Mansour et al. [24]. Monte Carlo (MC) simulated results are utilized for optimizations of the ESEM resulting in increasing the detection efficiency and minimizing the “skirt effect” [25,26,27]. An open source platform providing an infrastructure for tracking particles through complex geometries and detectors with possibility to adding physical processes is Geant4 [29]. The toolkit can be extended with modules for the simulation of charged particle-water interaction [30] and modelling of early biological damage induced by ionising radiation at the DNA scale [31] which has potential to be applied to study of radiation damage in ESEM.

This topic is going to be studied in the frame of research objective 1. In terms of difficulty the considerably ambitious result will be the world complex software for the simulation of primary electron beam interaction with gas, liquid and solid. Results of research and development activities within this objective are expected to be very unique and will be very important both for basic research (complex simulation of physical phenomena accompanied by the interaction of electrons with different state of matter) and potentially also for applied research. Results of MC simulations of electron beam scattering in gas environment would allow to optimize the electron optical column of the ESEM and to develop a new design of differentially pumped chambers, including detectors.

New highly sensitive detectors and detection principles for the ESEM

To study sensitive samples with low emissivity of signal electrons in the ESEM, research and development of new highly sensitive detection systems is crucial. The most efficient secondary electron (SE) detector for ESEM uses the principle of gas ionisation. It makes use of an amplification through an ionization cascade between a grounded specimen holder and a positively biased signal electrode placed under the pole piece of the objective lens [32,33,34]. The amplification of the SEs depends mainly on the intensity of the electrostatic field between the detection electrode and the grounded sample holder as well as on the pressure and the

type of gas [35,36,37]. The first commercial ESEM detector used a single electrode and was called the environmental secondary detector (ESD) [38]. It was later improved into the so-called gaseous secondary electron detector (GSED), patented by FEI. The GSED detects a cleaner SE signal but not completely pure [39,40,41]. Meredith et al. [42] simulated contribution of the SEs, PEs and BSEs on the total amplification by the ionisation phenomena. Fletcher [37] published curves showing lower BSE contribution to the gas-amplified signal collected in the GSED. The newest detection system is the Helix, patented by FEI, represents recent research in the field of the SEs detection in ESEMs. This system uses the magnetic field of an ultrahigh resolution magnetic immersion objective lens and the electrostatic field of an annular detection electrode placed at the bottom part of the objective [43,44].

Some methods for the detection of the signal electrons have been examined by our EEM group. Autrata et al. [45] designed a very sensitive BSE detector using the original YAG (yttrium aluminium garnet) and YAP (yttrium aluminium perovskite) scintillators for the study of the material contrast. This scintillator-photomultiplier detector is commonly used in SEMs but also in the Hitachi VP-SEM. The YAG scintillator with a small hole in its centre was used as the second pressure limiting aperture (PLA) in our ESEM AQUASEM II for studying wet biological samples [46,47]. The YAG BSE detector has been adapted for the topographical SE contrast detection by gas ionization and finally designed as the so-called combined detector [48,49,50]. According to our experimental results, the combined detector used in the ESEM allows recording the pure material contrast in high gas pressure.

For topographical contrast observation, the combined detector is equipped with a thin electrode deposited on the bottom of the YAG. If the electrode is positively biased [51], this detector works similarly to the ESD.

Suitable methods for suppressing the BSE background in the detected signal by the ionisation detector have been investigated by our team [52]. Similarly to Danilatos [38], multiple circular electrodes are used for detection in the ionisation detector.

Our recent work in the field of detection systems for the ESEM presents a patented ionisation SE detector with the electrostatic separator (ISEDS) [53,54]. This detector allows recording a strongly amplified signal of SEs with a minimal influence of the BSEs and, as the world's first detector, allows an energy separated detection of signal electrons for the gas pressure from 50 Pa to 300 Pa [55]. This project was supported by the grant GAAV KJB 200650602, supervised by the applicant. The detector is currently being improved with a magnetic circuit to increase the signal amplification. Our ISEDS, , was integrated into a combined SE and BSE detection system see Fig. 5.3.1, where BSEs are detected using a new generation of highly-efficient scintillation single crystal CRY 18. This work was supported by the Grant Agency of the Czech Republic, GAP 102/10/1410, which was supervised by head of Research Program 3 (RP3). The last of our new detectors, to be patented in the near future, is a scintillation SE detector for SEM and ESEM [56,57].

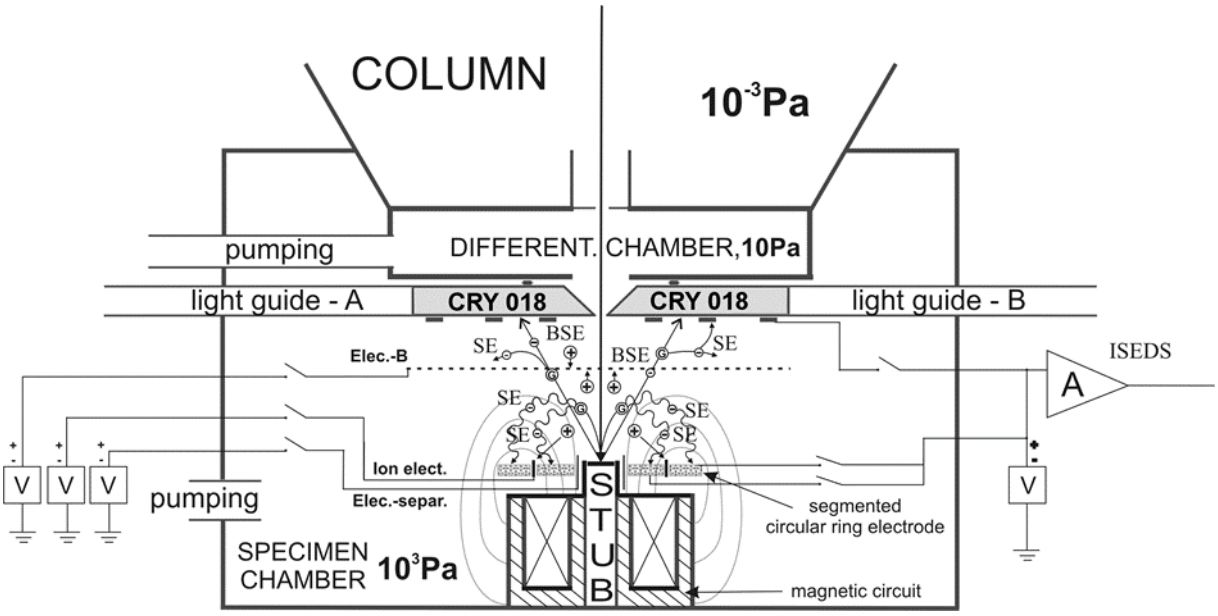

Figure 5.3.1: System for combined detection of SE and BSE in ESEM.

In order to understand the signal generation in the gaseous environment, the program EOD [58], used for the design of imaging, scanning and detection systems in electron microscopes, has been extended with a MC module to include the collision phenomena of electrons with the gases in the specimen chamber of the ESEM. This module was built made and has been improved several times by applicant's team [59,60].

Our scintillation SE detector for SEM and ESEM are able to continuously and seamlessly work within the pressure range from 0.001 Pa to 1000 Pa and image very susceptible wet samples under beam current 1 pA. Also detectors specialized for imaging during dynamic in-situ experiments completely missing. Our patented ISEDS is currently sole detector capable of signal energy filtration together with high resolution detection in the ESEM. VaV centre EEM is the only one in the Czech Republic equipped with a new Wet-STEM detector from the FEI Company for study of nanoparticles in liquids. The Wet-STEM detector is highly specialized equipment whose use is rather sporadic. The detector was introduced in 2005 by Borger et al. [61]. It's using in the ESEM for study of magnetic nano-vectors was published by Maraloiu et al. [62]. Better alternative, functional improvements or entirely new concept of nano-objects detection in liquids in the ESEM are not yet known and therefore they will be researched and developed within the objective 2 of this research program.

The detectors are one of the most important parts of the microscope. Their quality and technical parameters determine resolution and range of detectable information. VaV centre EEM is world leader in the research and development of detectors for the ESEM.

It is one of the few sites, which is equipped with its own MC software capable of relatively high precise simulation of signal electrons interaction with gas and developed several unique detectors. Ambition and overall contribution to basic and applied research is based on a series of new detectors, which will be within the framework of this project developed and used for biology and chemistry.

New methods for susceptible sample characterization, dynamic in-situ experiments.

Basic methods for setting the appropriate thermo-dynamic conditions in the ESEM specimen chamber [63,64] are insufficient for observation of susceptible wet samples.

Temperature and pressure close to or at the sample surface has not yet been possible to satisfactorily measure. Integration of the micro sensors for temperature and pressure measurement into the sample holder was described only by Lary et al. [65]. According to the information of EEM group, only two groups in the world are currently focused on gas flow simulation in ESEM specimen chamber. Danilatos et al. [66] introduced the design of structural modifications of differentially pumped chamber of ESEM and compared commercial microscopes from FEI and ZEISS companies in terms of pumping efficiency of the space and the scattering of the primary beam in it [67]. Simulation results of Danilatos [66] were validated using different counting method and software ANSIS by VaV centre EEM [68]. Furthermore, the EEM group published the results of simulation of gas flow in the differentially pumped chamber of ESEM AQUASEM II [69] and a special detector for ESEM [70].

New method for long-duration observation of biological samples in the ESEM was introduced [71] by VaV centre EEM as well as new methods for Dynamical in-situ experiments, such as the study of dehydration of biological specimens [72], observation of water droplets and salt crystallization in the ESEM, were published [73]. Our last work introduces also a new method for observation of the natural structure of somatic embryos and a study of the "extracellular matrix" [74,75], see Fig. 5.3.2. Low-temperature scanning electron microscopy (LTSEM) [76] has already been used for the observation of natural [77] and laboratory-generated ice surfaces, grain boundaries [78] as well as solid impurities at the grain boundaries or on the grain surfaces of dust particles [79]. Unfortunately, LTSEM requires low pressure in the specimen chamber (usually below 10^{-4} Pa) which causes potentially undesirable ice sublimation, called (thermal) etching, revealing impurities that would otherwise remain buried under the ice surface. Only a very few studies have examined non-contaminated ice (Fig. 5.3.3) at high pressures using the ESEM [80]. VaV centre EEM is specialised on development and testing of new methods for the study of natural or contaminated ice morphology and in-situ dynamical experiments with ice using our non-commercial ESEM AQUASEM II [81,82]. New methods for study of very susceptible bio-polymers like spherical polyelectrolyte complex beads with or without live cells was recently developed by our VaV centre EEM. Actually, we are only one in the worlds capable to study this type of samples in native and fully wet state [83,84,85].

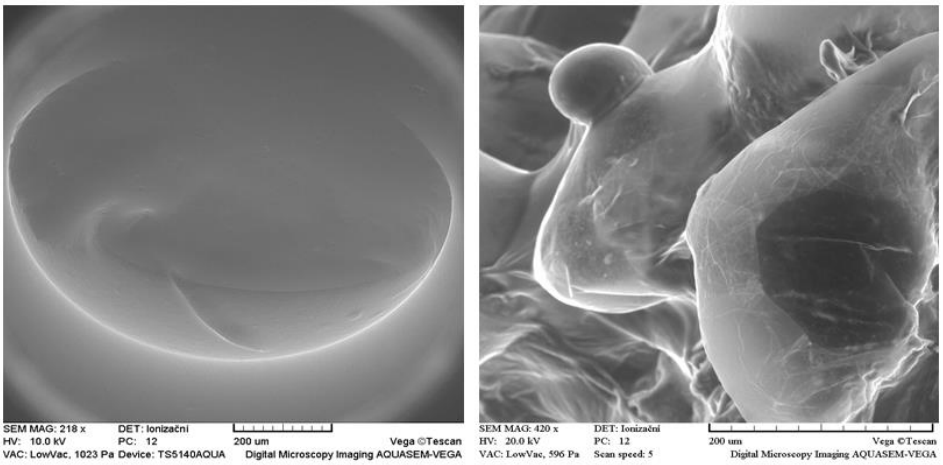

Figure 5.3.2: ESEM images of native samples. Left, native and completely wet polyelectrolyte capsules containing living cells in a semi-liquid core. Right, "extracellular matrix" on the surface of early somatic embryos in the native state shown by the Low Temperature methods for ESEM.

An ability to precisely adjust and control thermodynamic conditions in the PE beam area and its surroundings on the sample surface is crucial to most applications in ESEM, due to the problem complexity and difficulty is still impossible. Solving of this problem is therefore globally rather ambitious, especially in case of observation of very susceptible samples. VaV centre EEM is currently one of the world leaders in the field of new methods for the ESEM, especially methods for study of ice in environmental conditions and morphological characterisation of wet polyelectrolyte capsules in their native states (Fig. 5.3.2). This issue addressed in research objective 3 of this research program is essential for research and development of unique methods for the study of biological and polymer samples and logically fits into the project context. In terms of basic research, new contrasts on the biological sample surfaces in their native state will be investigated, which can bring a breakthrough possibilities in ESEM applications to biological sciences. High potential for applied research is supported by a demonstrable interest of companies producing electron microscopes and their components.

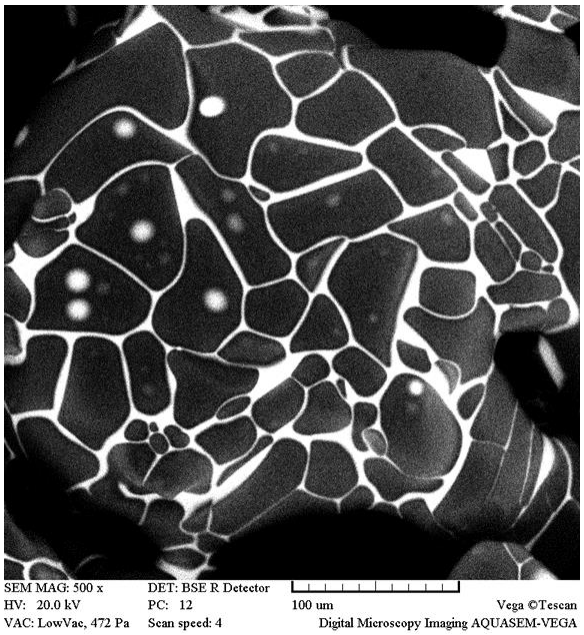

Figure 5.3.3: Contamination of ice by high concentration uranyl solution. The contamination was achieved by evaporation of water during dynamic in-situ experiment in ESEM.

Unique analytical high resolution ESEM - integration of new systems and methods and correlative microscopy

Few realized integrations of advanced technologies and methods to the SEM and ESEM is known. Jensen et al, [86] published results of in-situ study of electrochemical processes in the ESEM using cycling voltammetry integrated into a sealed electrochemical cell. Integration of commercially available Raman spectrometer to ESEM equipped with EDS, cathodoluminescence and STEM published Guillaume et al. [87]. Indisputable benefits of the combination of an optical microscope with SEM and using special staining techniques of biological samples with synthetic chemicals published Perkovic et. al [88] and the study of cells and tissues Shannon et al [89].

Research, development and integration of new technologies into electron microscopes and electron microscopes development itself is very complex, costly and in terms of possible damage of the microscope risky activity. However, it is crucial for basic research and

development in many fields of science. There are only few scientific groups worldwide, which would have know-how and scientific and technical facilities at adequate level to be able to successfully implement these activities. Our proposed technical solution of integration of ESEM, Raman spectrometer and an optical microscope is world unique. In combination with other systems, such as systems optical and mechanical micromanipulation, targeted injection of gases and liquids with a new hydration system for ESEM and other systems to provide worldwide scientific excellence workplace focused on the study of susceptible native and live specimens in the ESEM and realization of special dynamic in-situ experiments. High ambition of the research objective 4 is undisputable as well as the importance for basic and applied research.

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5.3.3. Research objectives, activities and results

Research program 3 is focused at research and development of a conventional environmental scanning electron microscope (ESEM) with high resolution into a new generation of a complex analytical microscopic tool capable of providing new information on a wide variety of native and wet biological and polymer samples that are difficult to study with current techniques. All will be in a correlation with a range of new specialized and classical methods. The key part will be the study of the interaction of electrons with matter (Objective 1), which is essential for the development of new detection principles and detector types (Objective 2), methods and procedures for the characterization of sensitive samples or dynamic in-situ experiments (Objective 3) leading to a construction of a new-generation ESEM (Objective 4). There are numerous collaborations planned for this research program with the involvement of applicant's R&D centre IEB CAS and partners SUPRAMOL IMC CAS and ZRIR IKEM (Fig. 5.3.4).

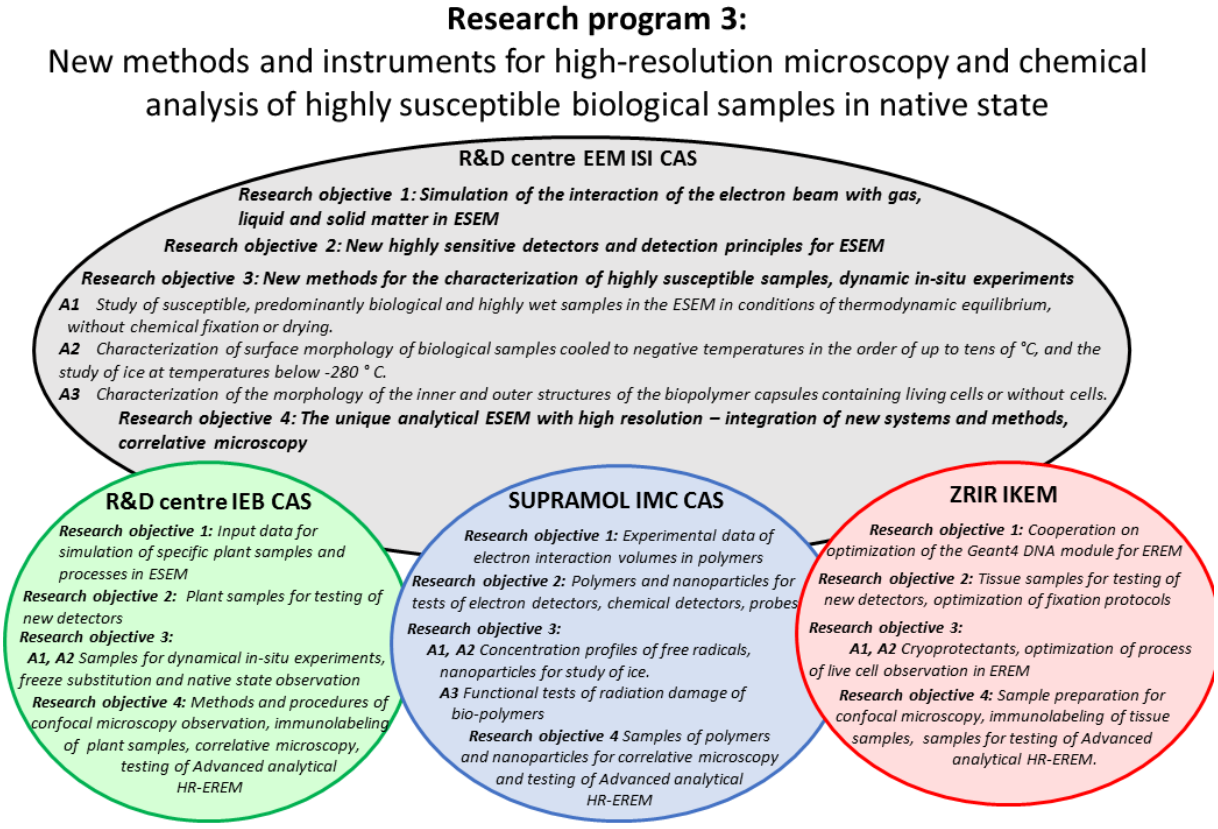


Figure 5.3.4: Schematic depiction of research program 3. This research program is primarily affiliated to the partner R&D centre EEM ISI CAS (grey). The involvement of applicant's R&D centre of IEB CAS (green) and partner's R&D centres, SUPRAMOL IMC CAS (blue), ZRIR IKEM (red) is specified for individual research activities of EEM ISI CAS.

Research Objective 1: Simulation of the interaction of the electron beam with gas, liquid and solid matter in ESEM.

Head: *Mgr. Martin Oral, Ph.D.*

Depending on the specimen type and the observation conditions, the primary electrons (PE) in the ESEM may pass through and interact with substances in different forms states of matter, such as a gas (an environment in differentially pumped chambers and in a specimen chamber), an aqueous solution or a liquid in general (a layer on the sample surface or a completely liquid sample) and solid state (sample). Passing through gas the PE beam becomes increasingly spread and a so-called “skirt” is formed which encompasses electrons travelling under increasingly diverging angles and with increasingly varying energies. A layer of water on the sample surface causes more similar spread of the PE beam, which is unfavourable with respect to detection of signal electrons emitted from the sample. The spread of the PE beam in such multi-layered system ultimately causes a lower achievable resolution, which is the main drawback of ESEM in comparison with standard SEM. Similar problems are encountered in the case of a microanalysis in ESEM, where spatial resolution is suppressed, signal intensity is determined erroneously, or phantom materials are detected.

Electron-matter interactions can be simulated by Monte-Carlo (CM) methods. The simulations need to take the various environments into account, including the processes on interfaces between those environments. Speaking globally, there is no software capable of simulating the scattering of electrons in the mentioned multi-layered environments that are common in ESEM in the both standard and transmission modes.

This activity contains 2 research goals:

Firstly, creation of a comprehensive program code for MC simulations of PE propagation in the high pressure environment of ESEM, the primary electron beam focusing on the sample, and subsequent interaction with the sample.

At the core of the intended MC simulation code, there will be an accurate integrator of electron trajectories in electric and magnetic fields of optical components and detectors. That is the deterministic part of the simulation. For tens of keV the relativistic dynamics starts to make a difference, but for low energies, it has a negligible influence (and it is better to use the non-relativistic formulation).

In a comparison of well-known algorithms, a greater case will be given to use the correct probability density distributions. An initial literature research revealed that ignoring these function often leads developers to substitute them with breaking up the given deterministic part of the trajectory into overly small steps, and in each of them the probability of an interaction is evaluated using uniformly distributed random number generator (which can be suitably parameterized). That may lead to statistically equivalent results if the random numbers are generated correctly (the mentioned density distributions are generated somewhat implicitly), on the other hand, this way of calculation tends to be slow and it may lead to cumulative round-off errors. We found out that most of the random physical processes can be modelled using an analytic probability density function of it can be determined to be an auxiliary MC simulation prior to the actual time-consuming simulation. Using this procedure we expect a great speed-up of the simulation.

The interaction of the electrons with solid matter will be realized using a specialized external simulation package or library, and it will eventually be integrated with the rest of the MC code. Based on long term testing the program Geant4 will be used including its specialized modules. This part of the simulation is quite complex and its solution would be beyond the scope of the project. Similarly, the environmental gas flow in the vacuum (low pressure) system will be simulated using a specialized program and the resulting data will be possible to import into the planned program. However, the use of an external simulation code does not enforce any simplification or loss of generality to the intended calculation procedure.The first

step will be the creation of a complex program code for MC simulation of PE propagation in the high-pressure environment of the ESEM including the focusing of the PE beam onto the sample and the subsequent interaction with the sample. The simulation code will also account for potential signal electron propagating from the sample trough the gas towards the detector. The program will be gradually extended, starting with the modelling of an unaffected focused PE beam up to the interactions of the PE:

- with gas of a spatially variable pressure which is due to the flow dynamics caused by the differential pumping through the pressure limiting apertures, in subsonic and supersonic regimes,
- with water or an aqueous solution covering biological samples (also covers condensation), or a sample in a liquid form,
- with solid matter of the sample itself, including the generation of signal electrons.

Secondly, **the basic code will be further extended with modules specialized to specific problems in ESEM or the project partners**.

In collaboration with partners ZRIR IKEM and applicant´s R &D centre IEB CAS, models for simulation of plant and tissue samples and processes in ESEM will be created, see figure 5.4.3. The "Geant4-DNA chemistry module“ for the study of radiation damage of biological samples in ESEM and the study of chemical processes in polymer systems will be applied and tested, together with partner SUPRAMOL IMC CAS. For study of degradation processes in ESEM, the base code will be extended with modules for simulation of heat or electric charge accumulation as a result of the interaction with the PE. Study of new detection contrast and principles will be allowed using modules for simulation a specific crystalline structure of solid substances, for example, a module for a study of PE interaction with ice.

Milestones:

The results of the above tasks will provide key input data for the objectives 2 and 3. Results will be in at least 4 scientific journals (output type 2 02 11):

1) A complex program code capable of simulation the transport of PE through individual parts of a microscope - from its travel through the optical column until its interaction in the sample, followed by the description of a potential signal electron emission. *The output will provide a detailed insight into the processes in the microscope for the following design of detectors and observation methods.*

2) Optimization of the MC software for the simulation of PE skirts in the gas using imported velocity and pressure distributions in the presence of differential pumping and pressure limiting apertures in an ESEM. *The output will provide so far the most accurate data on interactions involving electron in a realistic environment of an ESEM. Currently, the pressure and speed distributions are assumed to be constant in simulations.*

3) Simulation of signal electron emission in ESEM, as a basis for the development of new detection systems. *The output will provide input data for the activities concerning the research and the development of new detectors for ESEM.*

4) More extensions of the MC software for the study of long-term effects of the PE beam on the sample, such as heat and electric charging. *The output will enable the research and the development of methods for special experiments with aim of the most conserving handling of sensitive sample surfaces, possibly extending towards correlative microscopy.*

5) The description of electrochemical processes in water upon the impact of the PE beam. A study of radiation damage, the identification of the main mechanisms of the creation of hydroxyl radicals, their distribution of concentration and their propagation through the

system. *The output enables to reveal the extent and the intensity of damage of samples by electrochemical processes, and to minimize their effect.*

Research Objective 2: New highly sensitive detectors and detection principles for ESEM

Head: *Ing. Vilém Neděla, Ph.D.*

The development of new detectors, detection principles and strategies is essential for further advances of ESEM as a tool for susceptible, often wet samples in their native state and polymer samples under conditions minimizing degrading effects of the PE.

The EEM R&D centre of ISI CAS is currently on of the world leaders in this area. If the detection efficiency is increased by at least 50 %, it is possible to obtain a signal with a significantly higher signal-to-noise ratio, or at a higher image resolution. Alternatively, it is possible to use less intensive PE beam that is gentle to susceptible samples (roughly a halved PE beam current) with preserving the signal-to-noise ratio. If the new sensitive detectors are assisted by high-performance electronics capable of transmitting signal in a large bandwidth without distortion, it will be possible to image sensitive samples at high scanning speeds with minimal beam current, exposing them to lower energy dose. Furthermore, the new detectors must be able to detect signals according to their energies and emission angles in high gas pressure in ESEM. In connection with the possibility to achieve new contrast types from samples in their native state, it will be possible to obtain new information on the dynamics of the processes in biological, chemical samples and ice.

This activity contains 2 research goals.

Firstly, an optimization of MC software for the possibility of simulating new detection systems on the principle of gas ionization.

In the initial phase, the activities will be a continuation of the results of research objective 1. The MC software will be utilized in the physical description of the processes in the interaction of the signal electrons emitted from the sample with the gas in the chamber and the material of the detection electrodes in the ESEM.

A series of experimental measurements of the dependence of the detected signal from the ionization detector on the gas pressure, the voltage on the detection electrodes, etc., will be performed. The results will be used to calibrate the constants of the MC program, such as the relaxation speed of the ionization and excitation processes, and to align the simulated and the experimental data for known configurations of already tested detector geometries. Based on the calculation of the positive ion concentration between the sample and the detector, the position of the detection electrodes will be modified, and the recombination processes in partially ionized plasma will be better understood and the method of their control in the specimen chamber of the ESEM will be designed.

The presence of the positive ions makes it possible to neutralize the negative charge on sample surfaces from the PE beam; on the other hand, it decreases the efficiency of signal multiplication through an ionization avalanche. Utilizing the above results, the function of the MC program for simulation of gas molecule impact ionization by signal electrons and ionization cascades to maximize signal multiplication and optimization of the ESEM parameters between the sample and the detector will be adapted, which will be used for the research and the development of detectors. The EEM R&D centre of ISI CAS is currently the only scientific institution in the world where simulations of this kind are performed.

Secondly, development of various detectors working on the principle of gas ionization.

. Such detectors will be developed with the prospect of detecting very weak signals from specimens with low emission coefficients or detecting signals emitted with a given energy range. Electron trajectories in a combined electrostatic and magnetic field and in gas, an optimization of the geometry of the detection electrodes, computation of intensity of electrostatic and magnetic fields of the detector, a design of detection electrodes geometry will be simulated as well as gas molecule impact ionization by signal electrons and ionization cascades to maximize signal multiplication and optimization of the ESEM parameters between the sample and the detector. Based on the previous work, we aim to research, develop and finalization of the third generation of the patented ISEDS detector and its integration into the HR-ESEM QUANTA 650 FEG. Finally, a multi-mode use of the detector, such as the in-lens or specimen chamber position, energy filtration of the detected electrons, MC simulations of the information depth of the detected electrons with a specific energy will be designed.

Another contribution will be the research and development of specialized signal amplifiers that will enable the connection of several detection electrodes simultaneously. Spatially dependent signal from different locations around the sample will be then detected by a simple switching.

Milestones:

Due to the new detectors, the ESEM will no longer be a method allowing just high resolution imaging of the morphology and the material contrast. A realization of 3D sample surface imaging and the discovery of new contrast types are expected. The outputs will be published in at least 4 scientific papers (result type 2 02 11):

1) The third generation of the detector in the sample plane, the ISEDS III. *One of the most efficient secondary electron detector for the ESEM, completely independent of the microscope type, allowing energy filtration of the detected electrons.*

2) A new energy selective detector allowing to distinguish not only new contrast types but also an approximate location of their emission under the surface in the matter. *The new detection concept based on electron detection according to their energy and location of emission.*

3) A new multi-positional ionization detector for ESEM. *3D tomography by the detection of electrons emitted under specific angles from the sample.*

4) A new ionization detector located under the sample for STEM and Wet-STEM regimes in ESEM. *The acquisition of new information on thin wet samples with the extension into correlative techniques of imaging the inner and the outer structure of samples.*

5) A new ionization detector utilizing purposely a designed gas flow for the improvement in avalanche ionization of signal electrons in ESEM. *A completely new detector concept for a highly-efficient detection in ESEM*.

Research Objective 3: New methods for the characterization of highly susceptible samples, dynamic in-situ experiments

One of the most prospective areas of research from the point of view of future applications of ESEM is the study of living, very susceptible samples in the native state and naturally wet samples, or special samples, such as ice. Samples can be studied statically or in dynamically variable conditions, during phase transitions, or under the in-situ influence of various physical and chemical phenomena in the chamber of an ESEM.

This research area constitutes a complementary activity to the research of the project partners which will enable completely unique results, not attainable by the methods those partners routinely use. Owing to the wide range of the problems and the degree of the integration of the partners, the division into the following activities will be used:

Activity 1: Study of susceptible, predominantly biological and highly wet samples in the ESEM in conditions of thermodynamic equilibrium, without chemical fixation or drying

Head: *Ing. Vilém Neděla, Ph.D.*

The concepts of the novel methods for the study of native biological and wet polymer samples in ESEM are based on the recent results of the EEM R&D centre of ISI CAS and they show that the absence of chemical treatments of metal coatings enables observation of new contrast types. These contrast types correspond to the local concentration of chemical substances in the sub-surface layers of biological samples, but also to the biopolymer, a few nanometres thick layers on the sample surface. The observation of the mentioned samples with a high resolution in ESEM encounters significant problems caused by the necessity to set and maintain a long-time thermodynamic equilibrium of the environment on the surface of different samples.

The long-term stabilisation of the thermodynamic equilibrium relies on a precise setting of the conditions on the surface of different samples (varying specific heat, dimensions, material density, electric and thermal conductivity and the relief of the surface) even during sharp changes of the gas pressure before and after the observation in the microscope. The heating by the PE beam as well as cooling the sample by the holder has a significant influence. The efficiency of cooling depends on the sample area and its contact with the cooled holder, thermal conductivity, the heat removal by the water layer, etc. All these processes will be described in detail and quantified in the dependence of the PE beam parameters, specimen type and the other mentioned parameters. Using the ANSIS SW for simulation of gas flow and heat transfer, the distribution of gas pressure and its flow velocity in the vicinity of the sample surface will be accurately described.

Further activity will be aimed at the realization of the real sample surface temperature measurement. The simulation of that quantity is too complex owing to the different sizes, forms, chemical compositions and physical properties of the various samples. Using the simulation results from above mentioned SW, the relevant mechanisms of sample heat conductivity, heat conduction through water layer and by contact with cooled specimen holder will be accurately described. In collaboration with partners SUPRAMOL IMC CAS, ZRIR IKEM and applicant´s R&D centre IEB CAS, the temperature dependence of the selected group of biological and polymer samples in the "real" environment the ESEM specimen chamber and during the observation of the sample with the PE beam will be simulated and accurately described.

As the sample - objective lens distance is in the order of units of millimetres, the temperature measurement is quite difficult. An integration of temperature, pressure and humidity micro-sensors will be proposed on the cooled specimen holder. For precise temperature measurements, methods for direct measurement of the temperature of the sample will be proposed and implemented into the specimen chamber of the microscope, including an infra-red camera. The combination of these methods will enable direct measurements of key experimental parameters and it will provide information on the thermal influence of the PE beam and heat dissipation on the sample.

New methods for observing the surface of native specific samples in the ESEM and new methods for applications of micromanipulation and microinjection techniques for the study of morphology and dynamic in-situ experiments of biological samples and polymers will be developed in collaboration with partners SUPRAMOL IMC CAS, ZRIR IKEM and applicant´s R&D centre IEB CAS, see Figure 5.3.4. The results of the research on the thermodynamic equilibrium in ESEM in the connection with the new sample holder will allow the study of a wide range of samples by the project partners - plant samples (applicant´s R&D centre IEB CAS), animal samples (ZRIR IKEM), polymers (SUPRAMOL IMC CAS).

Milestones:

1) Implementation of measuring of sample temperature, pressure and gas humidity near the sample surface in the ESEM. *The result will enable the development of new methods for study of sensitive biological samples and unique experiments in exploring of ice*.

2) The physical description of gas pressure distribution and rate of flow near the sample surface and its influence on changes of sample temperature, further description of the relevant mechanisms of the heat conduction through the sample, water layer and by contact with a cooled substrate in conditions of the ESEM. *The result will allow developing new methods for studying sensitive biological and polymer samples.*

3) Description of the temperature dependence of the selected group of biological and polymer samples in "real" environment in the ESEM specimen chamber and during sample observation with the PE beam. *Results allow to develop new methods for observing the temperature changing samples in the context of dynamic in-situ experiments.*

Activity 2: Characterization of surface morphology of biological samples cooled to negative temperatures in the order of up to tens of °C, and the study of ice at temperatures below - 280°C.

Head: *Mgr. Dominik Heger, Ph.D.*

A key activity in this research area will be the development of a new cooled specimen holder capable of cooling samples down to -70 °C and a new sophisticated system for cooling ice samples down to the nitrogen or helium boiling point. The commercially available holders by far do not meet the requirements for the experiments that will be described in the following text. A critical parameter is not just the target temperature, it is also its stability, the possibility to set the rate of temperature change, a high cooling capacity ensuring a thorough inner cooling or even more voluminous samples and the repeatability of the settings. A part of the activity will also be the development of the control electronics and the installation of the system into the specimen chamber.

A very precise temperature regulation under -20 °C is essential for further development of the method for studying the morphology plant and other samples in the conditions of low relative humidity in the chamber, called Low Temperature method (LTM). On the basis of the new equipment, it will be possible to advance this method further towards minimizing damage of the inner structure of the sample and towards the study of ultrastructure. The development of new extensions to existing micro-manipulators in the specimen chamber a new sample preparation technique of biologic samples by in-situ methods of freeze sublimation and freeze fracturing with the use of micro-manipulators directly in the chamber, so called LT-ESEM.

The system for cooling ice samples down to the nitrogen and helium boiling points of will be subsequently used for a direct study of frozen ice samples under conditions very close to those in nature. For this purpose, the current technique of ice observation will be enhanced

and dynamic in-situ experiments with ice, ice contamination and monitoring of ice metamorphosis will be realized. The processes of solidification, metamorphosis and thawing of ice samples to get an insight into them in natural ice and snow; natural ice will be compared to that from the Arctic and from sea-bound regions, the behaviour of frost flowers during their sublimation will be characterized. Finally still unexplored opportunity to examine impurities in the frozen state at the edges of ice grains and supplement them with an X-ray elemental analysis and fluorescence analysis will be provided.

The methods for the examination of heavily under-cooled samples will be applied in studies of the project partners – freeze damage of plants (applicant´s R&D centre IEB CAS), the stabilization of pharmaceutical biomacromolecules and the dynamics of phase transitions of miomimetic systems (SUPRAMOL IMC CAS). In collaboration with ZRIR IKEM, possibility of using cryoprotectants and optimization studies of animal cells in so called LT-ESEM will be studies.

Milestones:

1) Cooled sample holder with a very precise temperature control up to -70°C.

2) Cooled sample holder optimized for the study of ice up to -280°C. (It allows advanced experiments focused on study of pure and contaminated ice structures)

3) Improvement of methods for observation of ice, description of solidification processes, metamorphic and melting ice samples, characterizing the behaviour of ice blossoms at their sublimation.

4) Developing of a new method for the dating of ice samples from the glacial wells using correlative X-ray and optical fluorescent spectroscopy.

5) New tools for micromanipulation and microinjection techniques specifically developed for the study of external and internal structure, and dynamic in-situ experiments of susceptible biological samples and polymers. Special injectors, micro-knife etc. created for the specific needs of the project partners will enable new perspectives on current issues.

6) New techniques for preparation of biological samples using in-situ lyophilization and freeze-fracturing in the specimen chamber of microscope (LT-ESEM).The method allows the study of the structure of deep-frozen sample prepared in the microscope chamber, the sample will be stabilized as close as possible to native state without the need for expensive and complicated cryo-device.

Activity 3: Characterization of the morphology of the inner and outer structures of the biopolymer capsules containing living cells or without cells.

Head: *Ing. Peter Gemeiner, DrSc and Ing. Marek Bučko, Ph.D.*

Currently, the EEM R&D centre of ISI CAS is the only one in the world capable of studying the surface morphology of naturally wet biopolymer capsules containing a semi-liquid core with living cells in ESEM without damage.

The possibility to observe the nano-morphology and the inner arrangement of polymer capsules and particles with immobilized cells at high resolutions in the native state without invasive modifications will be used in further research and studies of the properties of new immobilized biocatalysts containing living cells. Morphologically and functionally new discoveries are expected from the development of innovative immobilisation procedures using ESEM. The ESEM technique will be used for the characterization of sample surfaces, the determination of the geometry of the particles and for the study of samples at high resolutions. These studies will be followed by the research of the morphology of the inner in

the outer capsule wall surfaces and particles and the status of the cells after opening with a micro-manipulator. Another follow-up research will be the in-situ observation of reactions of the particle material with selected chemical substances and the study of pores using WetSTEM. The correlative microscopy will enable studying samples of particles and capsules using ESEM and fluorescence optical microscopy with a very high resolution and the depth of field, while data specific to optical microscopy are recorded simultaneously. The realization of dynamic in-situ experiments aimed at the morphological characterization of polymers in the dependence on outside influences (pressure, temperature, radiation) is expected.

Functional tests of radiation damage of the bio-polymer and study of capability to free radicals compensation will be realized in collaboration with SUPRAMOL IMC CAS.

The ESEM technique will be utilized in the development of advanced and high-performance biochips and biosensors for the examination of the changes in glycolysation state of cell surfaces, which is closely correlated with most biological (physiologic and pathogenic) processes that a cell takes part in. The area of research, preparation and utilization of biochips and biosensors for (glyco)biosensing is globally very progressive. Thus, sample glycoprofiling will become a significant supplemental method for the characterization of samples analysed in the project, including highly susceptible samples, which will also widen the potential application impact. The main effort will be dedicated to the development of such biochip devices that will enable a fast, high-performance and sensitive analysis of biological samples with an applicability in biology, biomedicine, biotechnology, in the development of biomarkers (such as in cancer research) and in examination and detection of pathogen micro-organisms, in combination with advanced imaging methods. The microscopic methods will be utilized for the characterization, functional monitoring and testing of biochips and biosensors.

Milestones:

1) Characterization of surface morphology of biopolymer capsules with a semi-liquid core containing live cells in native wet state.

2) Advanced surface characterization of samples to determine the geometry of the particles and study of samples with very high resolution. Description of the morphology of the outer and inner capsule wall, the particles and the state of the cells after opening particles by micromanipulator, in-situ reactions of the material particles with selected chemicals and studies of pores in the semipermeable membrane.

Owing to strong interdisciplinary collaboration, publishing of at least 10 articles (results type 2 02 11) and 8 articles with foreign co-authorship (results type 2 02 11) is assumed.

Research Objective 4: The unique analytical ESEM with high resolution - integration of new systems and methods, correlative microscopy

Head: *Ing. Jan Ježek, Ph.D.*

The ESEM is a highly versatile tool for the study of nano-morphology and material composition of a wide range of samples. A keynote activity of this section is to improve functional utilities of the ESEM in terms of resolution in high-pressure gas conditions and its extension with other advanced technologies and methods. The fundamental vision of this activity is to create a nano-lab in the specimen chamber of the advanced analytical HR-ESEM, where preparation, observation and chemical analysis of live or partially treated biological samples and polymers will be implemented. The combination of electron microscopy, light microscopy and spectroscopy allow parallel analysis of samples and provide well correlated and complementary data. Integration of the ESEM with optical fluorescent microscopy and

Raman spectroscopy with the possibility to a non-contact micromanipulator utilizing an optical trap is supposed. The above mentioned will give rise to a unique analytical instrument whose usage will be guaranteed by the project partners in the field of plant and animal biology.

This activity contains 3 interconnected research goals.

Optimization of the design of a lower part of the microscope objective lens (in terms of gas pumping) and integration of new detectors. This aim allows suppressing scattering of the primary electron beam before entering the sample chamber. Based on the results of MC simulations (research objectiveI) design modifications of differentially pumped chambers and the lower part of the lens of the ESEM will be designed and implemented; integration of new detectors (research objective 2).

The signal to noise ratio and microscope resolution will be substantially increased. Simulation results from previous goals will be utilized in this part. New highly sensitive detectors developed within the research objective 2 will be integrated into the ESEM column. For manipulation with the specimen, micromanipulation technique based on an optical trap will be integrated into the specimen chamber of the ESEM.

New methods for selective hydration, targeted charge compensation and advanced studies of chemically active substances as well as biochemical reactions closely relate to the need for local application of liquids and gases or their pumping from the sample during chemical reactions or observing particularly complicated samples in the ESEM. Methods will be based on advanced instrumentation developed using results of simulations of gas flow in the vicinity of the sample, or a controlled and precisely directed flow of gases around samples. This will make possible not only selective moisturizing of sensitive sample sites but also increasing the concentration of the gas ionized by the PE beam and the better local compensation of charge on sharp edges or spits of electrically nonconductive specimens at low gas pressure in the ESEM. On the contrary, targeted exhausting of hazardous gases or increasing the efficiency of the avalanche signal multiplication of ionization detectors in ESEM will also be possible. These methods are applicable beyond the conventional investigation of samples in the ESEM and allow to achieve unique results with high resolution and with minimal damage of susceptible samples.

Integration of the optical fluorescent microscopy and the Raman spectroscopy into the ESEM is a technological challenge due to the need to coordinate the conflicting requirements for space and ports on the sample chamber. The aim of this activity is design of a new technical solution and implementation of integration of an optical fluorescent microscope and the Raman micro-spectrometer into the specimen chamber of the ESEM. Simultaneously the system for cooling of an objective of the optical microscope for ensuring the presence of immersion water layer between the lens and the glass slide in the ESEM will need to be developed. With respect to internal configuration, there is a collision with STEM and Wet-STEM detector and therefore a new detector design is needed. Integration of these detectors into the objective of a light microscope will be newly developed, but must ensure the possibility of simultaneous use of optical microscopy and ESEM working in STEM and Wet-STEM modes. At the same time, a new ionisation detector for detection of signal electrons in ESEM will be developed. It will work together with the mentioned systems. Implementation of these systems is part of the know-how of the EEM ISI ASCR.

The above mentioned analytical tool has great application potential for project partners. Using correlative ESEM fluorescent microscopy and Raman spectroscopy, ice samples will be studied; the different crystal modifications and amorphous ice will be studied using cryo-

holder; the phase changes and in particular their effect on impurities in the ice will be described. Characterization of the extent and manner of changes in the concentration of solutes during ice freezing, cells or biomimetic systems with the following description and minimization of frost damage will be possible. Direct monitoring of dynamics of the molecules adsorption on the ice surface, phase transformations, solidification and crystallization of the present impurities, and in-situ measurement of pH in the ESEM, polymer defectoscopy etc. will be allowed.

In collaboration with partners R&D centre, special technique for in-situ experiments in correlative electron and light microscopy for the study of biological samples and susceptible polymers will be developed (SUPRAMOL IMC CAS), methods and procedures correlative microscopy will be optimized (R&D centre IEB CAS) as well as preparation of tissue samples and immunolabeling for correlative microscopy (ZRIR IKEM).

Milestones:

Owing to strong interdisciplinary collaboration, publishing of at least 6 articles (results type 2 02 11) and 3 articles with foreign co-authorship (results type 2 02 11) is assumed.

1) A new design of a differentially pumped chamber and a bottom part of ESEM objective allowing integration of new detectors as well as an optical microscope. *It allows increasing of ESEM resolution, detection of new contrasts, weak signal and energy separated signals in pressure conditions up to 500 Pa; preparation for integrating of the optical microscope.*

2) Instrumentation for high effective selective hydration of samples, local charge compensation necessary for the study of chemically active samples and chemical reactions inside of the ESEM specimen chamber. *It allows to observ*e strongly drying and inhomogeneously drying samples, strongly charged samples or to study chemical reactions on the basis of local injection of liquids and gases.

3) Integration of the optical fluorescent microscope and the Raman micro-spectrometer into the specimen chamber of the ESEM.

4) Realization of cooling of an objective of the optical microscope for ensuring the presence of immersion water layer between the lens and the glass slide in the ESEM. *The system allows working with high resolution in both modes – immersion and non-immersion, the optical microscope will be will mechanically independent therefore well adjustable for accurate image correlation.*

5) A detector of signal electron capable of simultaneous recording of the optical signal and the signal from backscattered electrons. *Parallel imaging of samples using different microscopic techniques will be allowed.*

6) A special detector of signal electrons capable of simultaneous recording of the optical signal and the signal from transmitted electrons (Wet-STEM mode). *Parallel imaging of samples using different microscopic techniques will be allowed.*

7) Realization of series of dynamic in-situ experiments in the mode of combined electron and light microscopy and Raman spectroscopy.

5.3.4. International Cooperation

The EEM R&D Centre, which in the project will ensure the implementation of Research h Program 3, has established close contacts with the world's elite scientists in the field of environmental electron microscopy, e.g. Dr. Debbie Stokes (University of Cambridge), Prof. Brandon Griffin (Oak Ridge Natl Lab, USA), Prof. Miloš Toth (University of Technology, Sydney,

AUS), Prof. Brandley Thiel (University at Albany, College of Nanoscale Science and Engineering), as well as elite scientists from the fields of the application of special electron microscopic techniques, such as. Prof. Takanori Koshikawa (Fundamental Electronics Research Institute Osaka Electro-Communication University Osaka, Japan), Prof. Makoto Shiojiri (Kyoto Institute of Technology, Japan), and Prof. Witold Słówko (Faculty of Microsystem Electronics and Photonics, Wroclaw University of Science Technology). These prominent personalities have expressed their support and have also promised scientific cooperation on the proposed project, see Annex 6. On the basis of the cooperation agreement, the EEM R&D Centre also cooperated with the ESEM founder, Dr. Danilatos (ESEM Research Laboratory, Sydney, AUS). The theme of cooperation was the development and research of new technologies in the area of ESEM. The result of the above cooperation was several impacted articles. The EEM R&D Centre has extensive experience with dealing with the grant projects of most Czech grant agencies and ministries (GACR, TACR, MoE, MIT) and EU funded projects. In addressing these projects, the aforementioned foreign scientists representing world leaders in SEM and ESEM research and development were involved. Under the project of the MoE, ECOP, CZ.1.07 / 2 March 00 / 20.0103, of which the centre was the investigator, a foreign scientist from Germany, Dr. Krzyžánek, was reintegrated into ISI ASCR. Support of the presented project has also been declared by the company FEI Czech Republic through the General Manager Jiří Očadlík, the company JEOL USA inc. through Dr. Masahiro Kawasaki, Director of TEM applications and Business Solutions Americas, the company AutraDet Company through the CEO Ing. Roman, MBA and the company TECPA through the business owner Vladimír Palupa, see Annex 6.

In this research program 3 the international cooperation will be expanded through the participation of Ing. Peter Gemeiner, DrSc, who has long worked on the topic of the study of morphology and properties of polyelectrolyte capsules and particles for the development and characterization of immobilized biocatalysts with world leading experts, who expressed support for the presented project, such as. Prof. Thomas Heinze (Institut für Organische Chemie und Makro-molekulare Chemie, Jena, Germany), Prof. Roberto Fernandez Lafuente (Instituto De Catálisis y Petroleoquímica, Madrid, Spain), Prof. Salvatore Desantis University of Bari Aldo Moro, Italy) and Prof. Marko D. Mihovilovic (Institute for Applied Synthetic Chemistry, TU Vienna), see Annex 6. In addition, the participation of Mgr. Dominik Heger Ph.D. in the research program New Methods and devices for high resolution microscopy and the chemical analysis of highly sensitive native biological samples will enhance international cooperation. Mgr. Dominik Heger, Ph.D. has long worked on the topic of study of ice with Kiate Kim (Senior Research Scientist, Korea Polar Research Institute, Korea), Prof. Thomas Loerting (Institute of Physical Chemistry, University of Innsbruck) and Dr. Xin Yang (British Antarctic Survey). Likewise, these world experts also promised scientific participation on studies of ice in the proposed project, see Annex 6.

5.3.5. Research team

Composition of research team, description of organization for individual research activities

The summarization of nominated members of the team as well as members that will be nominated for all years of the project is given in Table 5.3.3, together with their role in the project, H index and FTEs (full time equivalents). In addition, curriculum vitae for all nominated members of the team are given in Annex 7. These CVs summarize expertize of the team

members, their best 8 publications in the research field of the proposed project and other relevant information (see Annex 7).

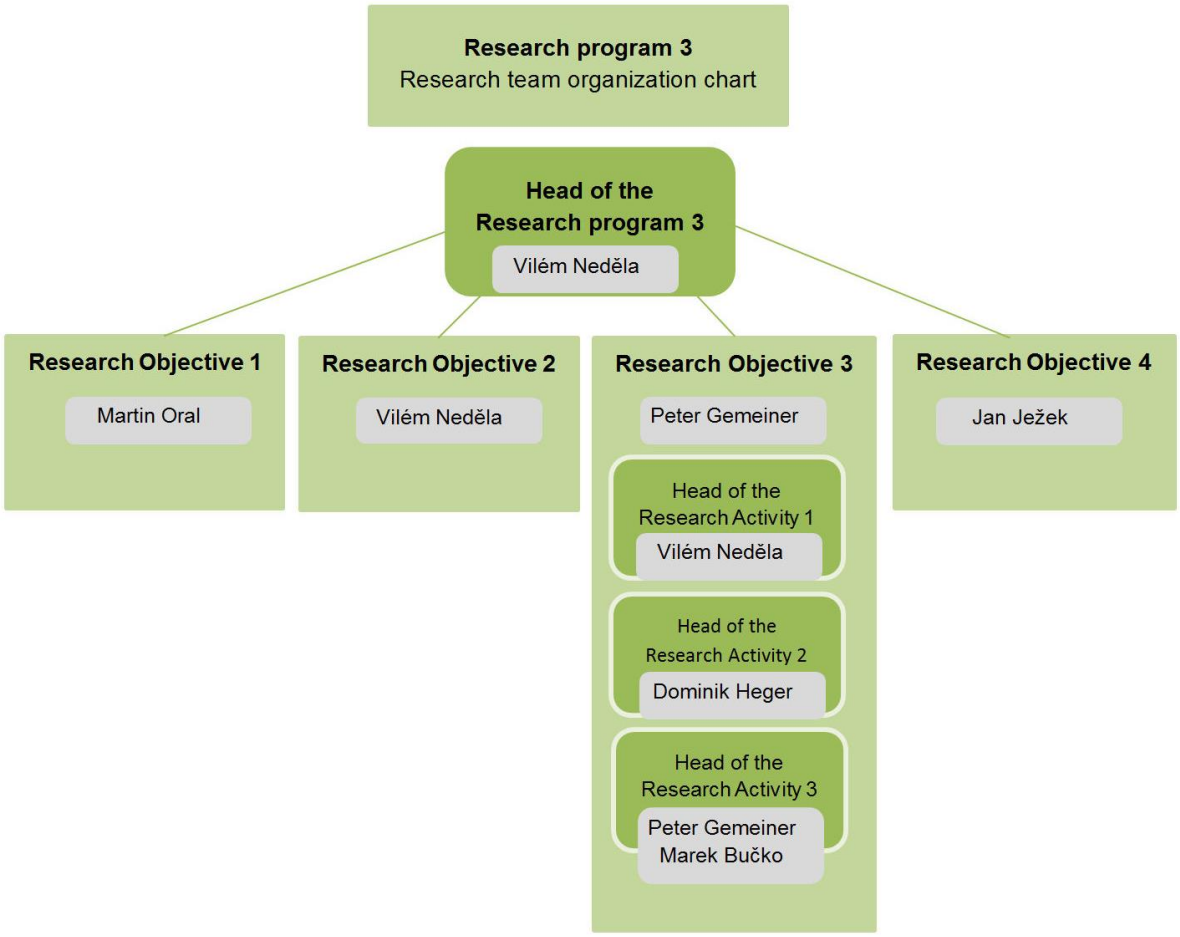


Figure 5.3.5: Organization chart of the research team of research program 3. The head of the research program will be responsible for co-ordination of activities of senior researchers.

Ing. et Ing. Vilém Neděla, Ph.D., key researcher (2017-2022), is the founder and head of the research group Detection Systems and later Environmental electron microscopy at ISI ASCR. Nearly twenty years of experience in the field of electron microscopy. Specialist in the research and development of detection systems for scanning electron microscopy (SEM) and environmental scanning electron microscopy (ESEM). Experience in the area of Monte Carlo simulations, simulations of gas flow in the ESEM, special methods and techniques for the ESEM, integration of new technologies into the ESEM. The head of two laboratories in environmental electron microscopy at ISI ASCR. He has published more than 30 articles with IF. He has participated in or developed 9 types of detectors for electron microscopes. The creator of numerous new methods for studying sensitive samples in ESEM. The originator of the rebuilding of the Vega (Tescan) electron microscope on the experimental ESEM AQUASEM II. The author of numerous prototypes and 2 patents. The investigator of numerous scientific and research projects. For a detailed description of activities and results, see the CV (Annex 7). In the project, overall responsibility for the activities of the Research program 3. He will be responsible for Research Objective 2 and Activity 1 in Objective 3. Partly responsible for Objective 4.

Ing. Peter Gemeiner, DrSc., excellent researcher (2017-2022), will be a newly hired excellent scientist. Ing. Peter Gemeiner, DrSc., is an excellent research scientist, has 35 years of experience in various areas of immobilized biotechnology and biosensors for bio-recognition techniques and lectinology. He is the co-author of 2 monographs, 7 book chapters, about 210 publications in CC journals, 30 patents and copyright certificates, he has about 2,800 citations, in the Hirsch index his work is a 29. He trained 16 doctoral students and led 25 theses. He is the trainer-specialist for 1 doctoral student. He participated as the head of the Slovak group of international projects FP4 EC Brussels (Programs PECO 1992 Copernicus 1994, INCO Copernicus 1997/98 FP5 (QoL), COST, 1998-2016 (5). He was head of the national projects GAV, VEGA, APVT / APVV. He participated in the solving of SF OP R&D projects He was the responsible investigator for the contractual cooperation with the companies Whatman plc, Biotika a.s., LIKO a.s., Biocel a.s., Iontosorb s.r.o., BCS Engineering a.s, Ing. Stanislav Krčmař, CSc. (Moravské Bránice) Biorealis s.r.o and Axxence Slovakia s.r.o. For a detailed description of activities and results, see the CV (Annex 7). Responsible for Objective 3 and Activity 3, partial responsibility for developing immobilized whole cell biocatalysts and preparing samples for the characterization of environmental electron microscopy and other imaging techniques.

Ing. Marek Bučko, Ph.D., key researcher (2017-2022), the newly hired key researcher. Ing. Marek Bučko, PhD. is an independent researcher. He defended his dissertation in the field of biotechnology, "Immobilized biosystems: potential and limitations of immobilization of biocatalysts in polymeric microcapsules" in 2008. He is the co-author of 22 CC works cited 327 times, in the Hirsch index of works he has an 11. He led 1 project of the agency APVV, he is the leader of 1 VEGA project, he participated in the projects SF OP R&D, APVT, APVV, VEGA, COST, Block Grant SAV. He trained 1 doctoral student, he is the supervisor of 1 doctoral student. For a detailed description of activities and results, see the CV (Annex 7). Under Objective 3, Activity 3 he is responsible for the development of immobilized whole cell biocatalysts and preparing samples for the characterization of environmental electron microscopy, and other imaging techniques.

Ing. Jaroslav Katrlík, Ph.D., researcher - senior (2017-2022), will be the newly hired researcher. Ing. Jaroslav Katrlík, PhD. is a senior researcher with extensive experience in the field of analytical and bioanalytical chemistry and glycomics focusing on research, development and application of biosensors and biochips. He is the co-author of 30 CC works cited more than 400 times, 3 book chapters, in the Hirsch index of works he has a 14. He trained 1 doctoral student, he was the supervisor-specialist of 1 doctoral student, and he is the supervisor of 2 doctoral students and led 12 dissertations. He is the Deputy Head of the Slovak group in the international projects FP7-PEOPLE-2012-ITN and QNRF NPRP, he participated in the projects FP5 and COST. He led / leads 3 APVV agency projects, 1 VEGA project, he participated in the solving of the projects SF OP R&D, APVT, APVV and VEGA. He successfully participated in international competitions of business plans aimed at research applications of nanotechnology and biotechnology ("NANOCHALLENGE 2006 ', Padova, Italy – member of the winning team;" From research to enterprise ", the Central European Initiative, Trieste, Italy, 2006 – head of the winning team). For a detailed description of activities and results, see the CV (Annex 7). Under Objective 3 responsible for the development, characterization of the use of advanced high-performance biochips and biosensors for glyco-recognizing proteins and cells.

Mgr. Dominik Heger, Ph.D., key researcher (2017-2022), will be the newly hired key researcher. Mgr. Dominik Heger, Ph.D. is the head of the Ice Photochemistry team dealing

with the (photo) chemistry and (photo) physics and transformation mechanisms of (organic) substances. Expert in optical spectroscopy: UV-Vis absorption and diffuse reflectance, fluorescence, transient spectroscopy and on the processing of experimental data. He has broad scientific interests ranging from mechanistic organic chemistry, photochemistry and spectroscopy to the physics of ice. Together with others he built the laboratory of time-resolved spectroscopy covering from femtoseconds to hours: pump-probe spectroscopy, nanosecond flash photolysis, time-resolved fluorescence. Author or coauthor of more than 30 peer-reviewed articles, with an h-index of 14 and with average citation counts on articles of 16.2. For a detailed description of activities and results, see the CV (Annex 7). Responsible for Objective 3 Activity 2. The guarantor of the theme of study of ice using microscopic and optical methods in relation to applications for environmental chemistry and pharmacy.

Ing. Jan Ježek, Ph.D., researcher - senior (2017-2022), is a member of a group of Optical micromanipulation techniques dealing with the application of the mechanical effects of laser radiation during its interaction with dielectric objects, Raman spectroscopy, two-photon polymerization and microfluidic systems. An expert in the design and construction of optical and optomechanical systems, Raman microspectroscopy and microfluidic systems. Together with others he designed and built several functional optical assemblies for laser micromanipulation techniques, assemblies for Raman spectroscopy, a highly stable microscope for measuring forces in optical traps, a Lightsheet fluorescence microscope and other systems. Author or co-author of 15 peer-reviewed articles with an h-index of 8, 1 utility model and 19 functional samples. For a detailed description of activities and results, see the CV (Annex 7). Responsible for Objective 4. Expert in the integration of optical fluorescence microscopy, Raman spectrometer and laser micromanipulation techniques in the ESEM. Responsible for the research and development of new methods integrated into the ESEM during the use of the above techniques.

Ing. Ivo Konvalina, Ph.D., researcher - junior (2017-2022), is a researcher with extensive experience in the design and study of electron-optical systems with a particular focus on detection systems. She deals with calculations of the distribution of electrostatic and magnetic fields in high resolution, low voltage and environmental electron microscopes. She applied her experience with the Monte Carlo simulations and particle tracing in the design of new detection systems and in the study of the properties of existing systems with regard to the contrast mechanisms of image formation. She is the author of dozens of publications in high-impact and professional journals. Under Objective 1 co-responsible for the simulation of interaction of the electron beam with gas liquids and solids. MC simulations of the trajectories of signal electrons in gas, simulation of processes of signal multiplication in gas, cooperation in the design and optimization of new detectors in direct response to simulation results. Cooperation in the development of new comprehensive MC software for ESEM.

Mgr. Martin Oral, Ph.D., researcher - senior (2017-2022), is a member of the group Electron optics, dealing with theory and simulations in charged particle optics and the design of particle-optical devices. Expert on accurate and efficient computing of trajectories in electric and magnetic fields and focusing systems for the processing of charged particle beams. He designed by way of correction the distortion of ion tracks on an inclined sample in the ToF spectrometer, a deflection system with a shifted middle deflection for the environmental scanning electron microscope and participated on a number of other device designs. He is the author of methods for calculating aberration coefficients of general particle optical systems and for the correct calculation of current density distribution in clusters of particles. He developed a program library for fast and parallelizable calculations of exact

trajectories of particles. He is the author or co-author of 16 peer-reviewed articles with an h-index of 2. He will be responsible for Objective 1 – Simulation of the interaction of the electron beam with gas liquids and solids. Creating a program code for the comprehensive Monte Carlo software ESEM, the application of the resulting code on the addressed issues, integrating data from other special programs, especially for the simulation of the speed and pressure of the gas, heat transfer, etc. For a detailed description of activities and results, see the CV (Annex 7).

Doc. Ing. Mgr. Jiří Maxa, Ph.D., researcher - junior (2017-2022), is a researcher, has 28 years of experience in equipment design, including 3D solid modelling and subsequent mathematical-physical analysis. He participated in the design and analysis work in the development of the differentially pumped chamber and detectors for ESEM, hydration device for the sample chamber. He trained 1 doctoral student, he is the supervisor of 5 doctoral students and led 30 master and bachelor theses. He participated in the grants: GACR – GA101/95/0074-Methodology of teaching CAD at technical universities. GACR – GA102 / 01/1271 – Study of detection methods and systems in the extreme conditions of environmental scanning electron microscopy. GA ASCR, KJB 200650602 - Detection system for recording signals of pure secondary or backscattered electrons in ESEM. GA 102/05/0886 – Research of detection systems of true secondary electrons in the newly conceived environmental scanning electron microscope. ECOP-CZ.1.07/2.2.00/28.0193 In the framework of Objective 1 and 3 co-responsible for the simulation of flow velocities and pressures of gases in ESEM, for the simulation of heat transfer in the vicinity of the sample of the ESEM. Responsible for designing new systems for special micro-manipulation, micro-injection of liquids and gases and new detectors for ESEM, as well as for the new construction modifications of ESEM using CAD systems and specialized software modules.

Ing. Eva Navrátilová, Ph.D., researcher-– junior (2018-2022), is a member of the EEM research group at ISI ASCR. Actively participated in several projects: 3 GACR projects and 1 MPO project. In 2015 she received salary support from the Support Program for Perspective Human Resources – Wage support of postdocs at ASCR worksites. Under objective 1 and 4 co-responsible for the development and testing of the development of the new MC software, and methods for the study of bio-polymer capsules and particles. Cooperation in the implementation of special and dynamic experiments in ESEM and applications in correlation microscopy techniques. Cooperation with project partners in the area of integrating chemical methods and probes into the ESEM.

Ing. Eva Tihlaříková, researcher - junior (2017-2022), is a member of the EEM research group at ISI ASCR. Expert on Monte Carlo simulation and related themes. She has extensive experience with the testing of detectors and the development of special purpose applications. She is the co-author of the new methods for the study of living organisms in ESEM and the author of two articles with impact factor on the topic of ESEM and several articles without an IF She actively participated in solving a number of research and development projects and was a member of the research team of the projects of the GACR, Ministry of Education and MIT. Under Objective 1 co-responsible for the simulation of interaction of the electron beam with gas liquids and solids. Creating a program code for the Monte Carlo simulation of an electron beam with water and partially also solid matter in the ESEM. Research and development modification of the program Geant, cooperation in research and development of new detectors, integration of new technologies into the ESEM.

New researcher - junior will be nominated (2017-2022). Under Objectives 1, 2 and 3 co-responsible for the simulation of radiation damage to the samples, simulation of thermal

conduction, new methods and devices for the chemical analysis of highly sensitive native biological samples. The study of electrochemical processes in water after the impact of the electron beam. Investigation of the phase transition under the effect of various physical and chemical influences in-situ in the sample chamber and pH measurement in the ESEM, the new methods. Cooperation in the development of detectors for dynamic in-situ examination of polyelectrolyte capsules and particles, mapping the effect of chemicals and other factors on these samples.

Ing. Jiří Hudec, researcher - junior (2017-2022), is a member of the EEM research group at ISI ASCR. Currently, he is a Ph.D. student at FEEC at the Brno University of Technology. Co-author of two articles with an impact factor on the topic of detection and several articles without an IF. He was a member of the research and investigative team of ISI ASCR of the GACR project and two projects of the Ministry of Education. Under Objective 2 and 4 co-responsible for the research and development of new highly sensitive detectors and their integration into the ESEM. Specializing in STEM and Wet-STEM detector. Experimental verification of the results of MC simulations in ESEM. Dynamic in-situ experiments focused on the morphological characterization of polymers and optical and mechanical micromanipulation. Cooperation in the characterization and optimization of the conditions for polymers in the ESEM chamber. The preparation of biological and polymer samples for SEM and ESEM.

RNDr. Jiří Runštuk, worker - junior researcher (2017-2022), is a member of the EEM research group at ISI ASCR, has worked in the field of electron microscopy for a long time (from the year 1983). He designed and implemented several construction solutions of differentially pumped systems, a number of modifications and improvements. Designed and implemented an ionization detector for an environmental microscope, contributed to further development and modification. He contributed to the development of a cooling (Peltier) holder for a greater range of temperatures. Deals with the possibilities of the monitoring of difficult (in REM) to observe samples and dynamic experiments in ESEM. Under Objective 2 and 3 and 4 co-responsible responsible for the implementation of special and dynamic in-situ experiments in the ESEM especially the study of ice. Cooperation in the development of all electronics, the construction of new detectors and the integration of new methods and equipment. Servicing microscopes and their calibration.

Pavel Vitámvás, worker - research technician (2017-2022), is a member of the EEM research group at ISI ASCR. Fourteen-years of experience in the field of electronics and four year experience in the engineering field. Author of multichannel ionizing amplifiers on the electronics side and software for the control unit. Experience with CAD programs, Eagle (electro) and Solidworks (the engineering part of work). He participated in the implementation of several special detection systems for ESEM, for example a detector in the plane of the sample. Participation in the development of a cooling (Peltier) holder for a greater range of temperatures. Development and integration of an enhanced camera system for observing samples and micromanipulation in the microscope and QUANTA AQUASEM II. Under Objective 2 and 4 co-responsible for the development of all electronics, PCB design, manufacture, installation, recovery and implementation into the system. The design of electronic circuits. Creation of drawing documentation, management of production components, implementation into assemblies. Servicing microscopes and their calibration. Cooperation in integrating new methods of microscopy, and chemical sensors into the ESEM.

New worker - research technician will be nominated (2017-2022). Under Objective 2 and 4 co-responsible for the development of all electronics, PCB design, manufacture, installation,

recovery and implementation into the system. The design of electronic circuits. Creation of drawing documentation, management of production components, implementation into assemblies. Servicing microscopes and their calibration. Cooperation in integrating new methods of microscopy, and chemical sensors into the ESEM.

New worker - research technician will be nominated (2017-2022). Under Objective 2 and 4 co-responsible for the development of all electronics, PCB design, manufacture, installation, recovery and implementation into the system. The design of electronic circuits. Creation of drawing documentation, management of production components, implementation into assemblies. Servicing microscopes and their calibration. Cooperation in integrating new methods of microscopy, and chemical sensors into the ESEM.

Table 5.3.1: Research team of the research program 3. See text for the description of the role for each researcher.

Name and surname	Workers position	Role in team, affiliation to research activity	H-index	1st year	2nd year	3rd year	4th year	5th year	6th year
				FTE during project implementation					
Vilém Neděla	Key researcher	Head, RP 3, head Objective 2, head Activity 1 Objective 3	5	1.0	1.0	1.0	1.0	1.0	1.0
Peter Gemeiner	Excellent researcher	Member RP 3, Supervision - head Objective 3, head Activity 3 Objective 3	29	0.3	0.3	0.3	0.3	0.3	0.3
Marek Bučko	Key researcher	Member, RP 3, head Activity 3 Objective 3	11	0.4	0.4	0.4	0.4	0.4	0.4
Jaroslav Katrlík	Senior researcher	Member, RP 3, Activity 3, Objective 3	14	0.2	0.2	0.2	0.2	0.2	0.2
Dominik Heger	Key researcher	Member, RP 3, head Activity 2 Objective 3	14	0.4	0.4	0.4	0.4	0.4	0.4
Jan Ježek	Senior researcher	Member, RP 3, head Objective 4	8	1.0	1.0	1.0	1.0	1.0	1.0
Ivo Konvalina	Junior researcher	Member, RP 3, Objective 1	5	0.3	0.3	0.3	0.3	0.3	0.3
Martin Oral	Senior researcher	Member, RP 3, Head Objective 1	2	0.5	0.5	0.5	0.5	0.5	0.5
Eva Navrátilová	Junior researcher	Member, RP 3, Objective 1, 4	0		1.0	1.0	1.0	1.0	1.0
Jiří Maxa	Junior researcher	Member, RP 3, Objective 1, 3	1	0.2	0.2	0.2	0.2	0.2	0.2
Eva Tihlaříková	Junior researcher	Member, RP 3, Objective 1	2	1.0	1.0	1.0	1.0	1.0	1.0
Jiří Hudec	Junior researcher	Member, RP 3, Objective 2, 4	0	1.0	1.0	1.0	1.0	1.0	1.0
Will be nominated	Junior researcher	Member, RP 3, Objective 1, 2, 3		1.0	1.0	1.0	1.0	1.0	1.0
Jiří Runštuk	Junior researcher	Member, RP 3, Objective 2, 3, 4	0	1.0	1.0	1.0	1.0	1.0	1.0
Pavel Vitámvás	Technician	Member, RP 3, Objective 2, 4	0	1.0	1.0	1.0	1.0	1.0	1.0
Will be nominated	Technician	Member, RP 3, Objective 2, 4	0	1.0	1.0	1.0	1.0	1.0	1.0
Will be nominated	Technician	Member, RP 3, Objective 2, 4	0	1.0	1.0	1.0	1.0	1.0	1.0

Results of key and excellent members of the research team in 2011-2015

Ing. Peter Gemeiner, DrSc, **excellent researcher**

Selected 5 research publications related to the proposed project with citations specified:

1 . M. Bučko, A. Schenkmayerová, P. Gemeiner, A. Vikartovská, M.D. Mihovilovič, I. Lacík: Continuous testing system for Baeyer-Villiger biooxidation using recombinant *Escherichia coli* expressing cyclohexanone monooxygenase encapsulated in polyelectrolyte complex capsules. *Enzyme & Microbial. Technology. 49, 284-288, 2011.* IF: 2,367. The number of citation without auto citation: 14

2. M. Bučko, D. Mislovičová, J. Nahálka, A. Vikartovská, J. Šefčovicová, J. Katrlík, J. Tkáč, P. Gemeiner, I. Lacík, V. Štefuca, M., Polakovič, M. Rosenberg, M. Rebroš, D. Šmogrovièová, J. Švitel: Immobilization in biotechnology and biorecognition: from macro- to nanoscale systems. *Chemical Papers, 66, 983-998*, 2012. IF: 0,879. The number of citation without auto citation: 17

3. A. Schenkmayerová, M. Bučko, P. Gemeiner, D. Chorvát, I. Lacík: Viability of free and encapsulated *Escherichia coli* overexpressing cyclopentanone monooxygenase monitored during model Baeyer-Villiger biooxidation by confocal laser scanning microscopy. *Biotechnology Letters 34, 309-314, 2012.* IF: 1,583. The number of citation without auto citation: 7

4. A. Schenkmayerová, M. Bučko, P. Gemeiner, D. Trelová, I. Lacík, D. Chorvát, Jr, P. Ačai, M. Polakovič, M. Rebroš, M. Rosenberg, V. Štefuca, V. Nedela, E. Tihláriková: Immobilisation of a whole-cell biocatalyst overexpressing Baeyer-Villiger monooxygenase: Physical and biocatalytic properties of polyvinyl alcohol lens-shaped particles versus spherical polyelectrolyte complex microcapsules. *Applied Biochemistry & Biotechnology 174, 1834-1849,* 2014. IF: 1,735. The number of citation without auto citation: 2

5. M. Šunderić; A. Šedivá, D. Robajac, G. Miljuš, P. Gemeiner, O. Nedić, J. Katrlík: Lectin-based protein microarray analysis of differences in serum alpha-2-macroglobulin glycosylation between patients with colorectal-cancer and persons without cancer. *Biotechnology and Applied Biochemistry* 2016*, DOI: 10.1002/bab.1407, in press.* IF: 1,429. The number of citation without auto citation: 1

5 research projects related to the research program 3 with financial support specified (only PI or co-PI):

1. Project of SF EU OP R&D, Projects of Structural Funds of EU, Research and Development Operational Programme “Applied research in the field of industrial biocatalysis” (2012-2014): ICh SAS 439 094 EUR (total: 1 153 663,99 EUR), position: co-Principal Investigator
2. Project of SF EU OP R&D, Projects of Structural Funds of EU, Research and Development Operational Programme “Centre for materials, layers and systems for aplications and chemical processes under extreme conditions”, acronym: MACHINA (2009-2011): ICh SAS 69 323,45 EUR (total: 1 327 735,83 EUR), position: co-Principal Investigator
3. Scientific Grant Agency of the Ministry of Education, science, research ans sport of the Slovak Republic and the Slovak Academy of Sciences VEGA 1/0229/12 „Novel, more efficient immobilization technologies for biocatalysts of oxido-reductive reactions and construction of biosensors and biobatteries“ (2012-2015): ICh SAS 54 960 EUR, position: co-Principal Investigator

4. Scientific Grant Agency of the Ministry of Education, science, research ans sport of the Slovak Republic and the Slovak Academy of Sciences VEGA 2/0127/10 „Deciphering the glycocode using tools of lectinomics: Nanoscale-controlled immobilisation of lectins with an microarray-based format of glycoform detection“ (2010-2013): ICh SAS 49 102 EUR + capital costs 5023 EUR, , position: Principal Investigator

5 patents and commercial applications related to the proposed project:

1. Active collaboration with Axxence s.r.o. Bratislava.

Ing. Marek Bučko, Ph.D., key researcher

Selected 5 research publications related to the proposed project with citations specified:

1. M. Bučko, A. Schenkmayerová, P. Gemeiner, A. Vikartovská, M.D. Mihovilovič, I. Lacík: Continuous testing system for Baeyer-Villiger biooxidation using recombinant *Escherichia coli* expressing cyclohexanone monooxygenase encapsulated in polyelectrolyte complex capsules. *Enzyme & Microbial. Technology. 49, 284-288, 2011.* IF: 2,367. The number of citation without auto citation: 14
2. M. Bučko, D. Mislovičová, J. Nahálka, A. Vikartovská, J. Šefčovicová, J. Katrlík, J. Tkáč, P. Gemeiner, I. Lacík, V. Štefuca, M., Polakovič, M. Rosenberg, M. Rebroš, D. Šmogrovièová, J. Švitel: Immobilization in biotechnology and biorecognition: from macro- to nanoscale systems. *Chemical Papers, 66, 983-998*, 2012. IF: 0,879. The number of citation without auto citation: 17
3. A. Schenkmayerová, M. Bučko, P. Gemeiner, D. Chorvát, I. Lacík: Viability of free and encapsulated *Escherichia coli* overexpressing cyclopentanone monooxygenase monitored during model Baeyer-Villiger biooxidation by confocal laser scanning microscopy. *Biotechnology Letters 34, 309-314, 2012.* IF: 1,583. The number of citation without auto citation: 7
4. A. Schenkmayerová, M. Bučko, P. Gemeiner, D. Trelová, I. Lacík, D. Chorvát, Jr, P. Ačai, M. Polakovič, M. Rebroš, M. Rosenberg, V. Štefuca, V. Nedela, E. Tihláriková: Immobilisation of a whole-cell biocatalyst overexpressing Baeyer-Villiger monooxygenase: Physical and biocatalytic properties of polyvinyl alcohol lens-shaped particles versus spherical polyelectrolyte complex microcapsules. *Applied Biochemistry & Biotechnology 174, 1834-1849,* 2014. IF: 1,735. The number of citation without auto citation: 2
5. A. Schenkmayerová, M. Bučko, P. Gemeiner, Katrlík, J. Microbial monooxygenase amperometric biosensor for monitoring of Baeyer-Villiger biotransformation. *Biosensors & Bioelectronics 50, 235-238,* 2013. IF: 6,451. The number of citation without auto citation: 1

5 research projects related to the research program 3 with financial support specified (only PI or co-PI):

1. Slovak Research and Development Agency APVV-0302-10 „Immobilization techniques for preparation of biocatalysts for industrial production of natural flavours“ (2011-2014): ICh SAS 65 000 EUR (total 249 968 EUR), position: Principal Investigator
2. Slovak Research and Development Agency APVV-15-0227 “Immobilized recombinant microorganisms for the biotechnological production of chemical specialties using biocatalytic cascade reactions” (2016-2020), total 249 509 EUR, position: Principal Investigator

3. Scientific Grant Agency of the Ministry of Education, science, research ans sport of the Slovak Republic and the Slovak Academy of Sciences VEGA 2/0090/16 „Development of novel immobilized biocatalysts utilizing recombinant microorganisms for biocatalytic cascade reactions“ (2016-2019): ICh SAS 54 740 EUR, position: Principal Investigator

5 patents and commercial applications related to the proposed project:

1. Acctive collaboration with Axxence s.r.o. Bratislava.

Mgr. Dominik Heger, Ph.D., key researcher

Selected 5 research publications related to the proposed project with citations specified:

1. Solomek, T.; Heger, D.; Ngoy, B. P.; Givens, R. S.; Klan, P., The Pivotal Role of Oxyallyl Diradicals in Photo-Favorskii Rearrangements: Transient Spectroscopic and Computational Studies. *J. Am. Chem. Soc.* **2013,** 135, 15209-15215. IF: *11.444.* Number of citations without auto citations: 8
2. Krausko, J.; Runštuk, J.; Neděla, V.; Klán, P.; Heger, D., Observation of a Brine Layer on an Ice Surface with an Environmental Scanning Electron Microscope at Higher Pressures and Temperatures. *Langmuir* 2014**,** 30, 5441-5447. IF: *4.45.* Number of citations without auto citations: 3
3. Bartels-Rausch, T.; Jacobi, H. W.; Kahan, T. F.; Thomas, J. L.; Thomson, E. S.; Abbatt, J. P. D.; Ammann, M.; Blackford, J. R.; Bluhm, H.; Boxe, C.; Domine, F.; Frey, M. M.; Gladich, I.; Guzmán, M. I.; Heger, D.; Huthwelker, T.; Klán, P.; Kuhs, W. F.; Kuo, M. H.; Maus, S.; Moussa, S. G.; McNeill, V. F.; Newberg, J. T.; Pettersson, J. B. C.; Roeselová, M.; Sodeau, J. R., A review of air–ice chemical and physical interactions (AICI): liquids, quasi-liquids, and solids in snow. *Atmos. Chem. Phys.* 2014, 14, 1587-1633. IF: *5.053.* Number of citations without auto citations: 26
4. McNeill, V. F.; Grannas, A. M.; Abbatt, J. P. D.; Ammann, M.; Ariya, P.; Bartels-Rausch, T.; Domine, F.; Donaldson, D. J.; Guzman, M. I.; Heger, D.; Kahan, T. F.; Klán, P.; Masclin, S.; Toubin, C.; Voisin, D., Organics in environmental ices: sources, chemistry, and impacts. *Atmos. Chem. Phys.* 2012, 12, 9653-9678. IF: *5.510.* Number of citations without auto citations: 33
5. Heger, D.; Nachtigallova, D.; Surman, F.; Krausko, J.; Magyarova, B.; Brumovsky, M.; Rubes, M.; Gladich, I.; Klan, P., Self-Organization of 1-Methylnaphthalene on the Surface of Artificial Snow Grains: A Combined Experimental-Computational Approach. *Journal of Physical Chemistry A* 2011, 115, 11412-11422. IF: *2.946.* Number of citations without auto citations: 16

5 research projects related to the research program 3 with financial support specified (only PI or co-PI):

1. Grant agency of the Czech GAČR: GP203/09/P445 Luminescence and electrochemical studies of the compound in ice, (2009-2011), Budget: 55 000 Euro. Main investigator.
2. Agreement on cooperation, University of Cambridge, (2015), 6 000 EUR.

5 patents and commercial applications related to the proposed project:

1. Heger, D.; Krausková, Ľ., Patent pending, Czech priority application 2015, PV 2015-55.
2. Proof of Concept; Optical technology for reading the pH from acid-base papers. (CZ.1.05/3.1.00/10.0216).

Ing. et Ing. Vilém Neděla, Ph.D., key researcher

Selected 5 research publications related to the proposed project with citations specified:

1. Neděla, V; Tihlaříková, E; Hřib, J. The Low-Temperature Method for Study of Coniferous Tissues in the Environmental Scanning Electron Microscope. Microscopy Research Technique 2015, 78, No. 1, 13-21. IF: 1.130. The number of citation without auto citation: 1
2. Neděla, V.; Hřib, J.; Vooková, B. Imaging of early conifer embryogenic tissues with the environmental scanning electron microscope. Biologia Plantarum, 2012, 56, No. 3, 595-598. IF: 1.692. The number of citation without auto citation: 1
3. Krejčí, J. ; Sajdlová, Z. ; Neděla, V ; Flodrová, E; Šejnohová, R. ; Vránová, H. ; Plička, R. Effective Surface Area of Electrochemical Sensors. Journal of the Electrochemical Society. 2014, 161, No. 6, b147-b150. IF: 3.266. The number of citation without auto citation: 1
4. Krausko, J. ; Runštuk, Jiří ; Neděla, Vilém ; Klán, P. ; Heger, D. Observation of a Brine Layer on an Ice Surface with an Environmental Scanning Electron Microscope at Higher Pressures and Temperatures. Langmuir. 2014, 30, No. 19, 5441-5447. IF: 4.457. The number of citation without auto citation: 3
5. Schenkmayerová, A. ; Bučko, M. ; Gemeiner, P. ; Treľová, D. ; Lacík, I. ; Chorvát Jr., D. ; Ačai, P. ; Polakovič, M. ; Lipták, L. ; Rebroš, M. ; Rosenberg, M. ; Štefuca, V. ; Neděla, Vilém ; Tihlaříková, Eva. Physical and Bioengineering Properties of Polyvinyl Alcohol Lens-Shaped Particles Versus Spherical Polyelectrolyte Complex Microcapsules as Immobilisation Matrices for a Whole-Cell Baeyer–Villiger Monooxygenase. Applied Biochemistry and Biotechnology. 2014, roč. 174, č. 5, s. 1834-1849. ISSN 0273-2289. IF: 1.735. The number of citation without auto citation: 2

5 research projects related to the research program 3 with financial support specified (only PI or co-PI):

1. Czech Science Foundation GAP102/10/1410 The study of the influence of magnetic and electric fields for amplification of secondary electron signals detected by a novel detector in VP-SEM (2010-2013). The volume of funds received: 375 000 CZK.
2. Czech Science Foundation GA ČR GA14-22777S The study of electron-gas interactions in conditions of pressure gradient of low energy environmental scanning electron microscope (2014-2016). The volume of funds received 7 744 000 CZK.
3. Ministry of education youth and sports MŠMT CZ.1.07/2.3.00/20.0103 Support for human resources and transfer of knowledge in conditions of international cooperation of research teams (2011-2014). The volume of funds received: 25 180 000 CZK.
4. Ministry of industry and trade MPO FR-TI1/118 New generation of electrochemical sensors and biosensors using modified thin DLC layers (2009-2013). The volume of funds received: 8 026 000 CZK.

5. Ministry of industry and trade MPO FR-TI1/305 Application of Laser Technologies into the Process of Crystalline Silicon Solar Cells Production (2009-2013). The volume of funds received: 2 830 000 CZK.

5 patents and commercial applications related to the proposed project:

1. Neděla, V.; Jirák, J. Patent No. EP2195822, Ionization detector for environmental scanning electron microscopy. 2011.
2. Fořt, T; Sobota, J; Neděla, V; Dupák, L; Kapounek, P; Dupák, J. New sputtering equipment for deposition of B-DLC layers. 2012.
3. Sobota, Jaroslav; Kučerová, R.; Neděla, Vilém; Rek, Antonín; Krejčí, J. Functional sample of electrochemical sensor with deposited thick carbon layer. 2012.
4. Fořt, T; Dupák, L; Sobota, J; Neděla, V; Kapounek, P; Dupák, J; Krejčí, J. Replica of the original sputtering apparatus with improved functionality for the deposition of B-DLC layers. 2013.
5. Fořt, T; Kučerová, R.; Neděla, V; Krejčí, J. Functional sample of electrochemical sensor with deposited 885nm thin B-DLC layer containing 24 wt% of boron. 2013.

5.3.6. Description of key equipment/investments

In tables presented below, descriptions of key equipment/investments and functional modules are given together with their purchase costs and technical specification. All instruments, machines and software (with purchase costs not being less than 1 mil CZK) that are planned to be used in the RP 1 are listed individually. Items with lower prize are assembled into functional modules, according to their characteristics and linkage to research activities of RP 1, but also RP 2 and RP 3.

Required investments are described further in the project budget (see Annex 8 and obligatory attachment in the online application MS2014+), commentary on budget (see Annex 9). Their pricing is based on quotations, which are all supplemented in Annex 10.

Key equipment / functional module	No. of items	Planned total price without VAT (thousands CZK)
1. Confocal laser scanning microscope	1	11 983
Typical features: A confocal laser scanning microscope with a wide range of accessory options including special filters, coupling of lasers with different wavelengths and other components (see Annex 10). For further description see the feasibility study, chapter 6.2. Purpose of the acquired equipment: Confocal laser scanning microscope will be acquired for the purpose of implementation of correlative microscopy involving ESEM (Environmental Scanning Electron Microscope), confocal microscope and Raman spectroscopy with subsequent extension of the application range for these methods. The new fluorescence microscope will be installed in close proximity to the ESEM to achieve precise correlative microscopy of highly sensitive specimens. The ESEM is already equipped with software for correlative microscopy providing the needed interface for the individual microscope synchronisation. This setup will be		

primarily used for research objective 4 of RP 3 and it will serve also for all other applications in plant and animal cells (Fig. 5.3.4).

Infrastructure readiness:
The infrastructure of the ISI CAS is available without need for further investment. ISI CAS uses a building adapted to house laboratories of electron microscopy, providing sufficient space needed for confocal microscope, as further specified in chapter 6.1.

2. Functional module of optical components for the integration of optical and laser technologies into ESEM	1	3 496

Typical features:
A set of 9 independent optical components needed for development, implementation and further integration of the confocal laser microscope, Raman spectrometer and the laser micromanipulators into a QUANTA 650 FEG ESEM. For a detailed list of components see the feasibility study chapter 6.2 and Annex 10.

Purpose of the acquired equipment:
The purpose of the acquisition will be research, development and implementation of a special functional unit of optical systems designed for integration into the specimen chamber of the ESEM. This unit represents a special customised solution allowing the combination of methods of optical and electron microscopy and other analytical methods for studies of biological and polymeric specimens without the necessity to transport the specimens, all with a defined position and in a defined environment. Benefits of this solution include the possibility of simultaneous imaging and analyses by all methods available in the ESEM after the integration and high-accuracy correlative microscopy. This setup will be primarily used for research objective 4 of RP 3 and it will serve also for all other applications in plant and animal cells (Fig. 5.3.4).

Infrastructure readiness:
The complete infrastructure is at the site without the need for further investment.

3. Functional module of the software and PC for simulation of gas flow and heat transfer in ESEM and software for the X-Ray EDS analyser ESEM Quanta 650 FEG	1	3 058

Typical features:
A system using the finite volume method to address continuum mechanics by the accurate description of the continuity by derivation from the forces acting on the individual parts of the liquid: gravitation, pressure, friction between adjacent liquid parts, turbulences. The liquid status is described through its flow rate and pressures in all points where the liquid is present. For a detailed description see the feasibility study, chapter 6.2.

Purpose of the acquired equipment:

Analyses of gas flow in the chambers of the Environmental Scanning Electron Microscope (ESEM) and heat transfer in the specimen area for specification of the appropriate structural shape of the developed device in the design stage. This will save time and money in new device development. The experimental measurement should merely prove correctness of the choice. It will also provide information about gas behaviour in places inaccessible for experiments. The software purchase will allow for full use of the options offered by the existing EDS X-Ray analyzer in the QUANTA 650 FEG microscope and further extension of

these options for elemental characterisation of native biological and polymeric specimens in the environment of high-pressure gases in ESEM. The purpose of the software is described in detail in chapter 6.2 and will be used in all activities of this RP.

Infrastructure readiness:
The software for simulations of gas flow and heat transfer in ESEM will be installed in a server that will form part of the supply. The server will be installed in a prepared room together with the other computation stations of the ISI CAS. The infrastructure is ready without the need for further investment. Also the infrastructure for purchase of the auxiliary software to the X-Ray analyzer ESEM QUANTA 650 FEG is available without the need for further investment.

5.3.7. Research program budget - relation to the overall project proposal budget

The budget of this RP3 is attached in Annex 8 together with detailed comments for all individual items (see Annex 9). The budget and comments on budget could be also found in obligatory attachments online in MS2014+ system.

6. INFRASTRUCTURE

6.1. Utilization of existing infrastructure

The proposed centre of excellence is multidisciplinary, combining a biological, physicochemical and technological viewpoint for the imaging and analysing the dynamics of cellular processes in plant and animal models. Infrastructure and equipment of the proposed R&D centre of advanced microscopic analysis of plant cells of IEB CAS are built on a long-term basis with the aim of studying the processes in plant cells using a combination of molecular biological and microscopy approaches (Chapter 3.3). With respect to microscopy approaches, the IEB R&D centre is well-equipped for fluorescence microscopy, with a major focus on *in vivo* approaches. However, the proposed project will also allow the development of research and the use of the infrastructure and equipment of the project partners involved, i.e. R&D SUPRAMOL IMC CAS, ZRIR IKEM and ISI CAS. The main purpose of the individual partner engagement is to create a multidisciplinary unit that brings a qualitative shift to the field of microscopy-oriented studies of cells through the development of new technologies and procedures for the detection of cellular structures. These include new nanoparticle systems (SUPRAMOL IMC CAS), new procedures for cryo-fixation of cells (ZRIR IKEM) and new detectors for non-invasive electron microscopy (EEM ISI CAS). As the added value, the project includes in all three research programs the use of microscopy techniques for the targeted preparation and subsequent detection of nanoparticles used in medicine and agriculture. Linking the applicant investigator and partner's infrastructures to solve the proposed project thus offers the potential for a number of application outputs.

The following text provides an overview of the existing infrastructure for each partner. The text is the most detailed for the EEM ISIS CAS, whose mission is to upgrade scientific instruments.

The infrastructure of the applicant investigator **R&D centre of the IEB CAS** is integrated into the structure of laboratories and common workplaces of IEB CAS (See Annex 2), which are equipped with all facilities for advanced molecular biology-oriented research. With regard

to the proposed project, the infrastructure equipment of the Imaging facility will be used (http://www.ueb.cas.cz/if). In addition to all basic equipment for plant molecular biology, Zeiss LSM 5 Duo and Zeiss LSM 880 laser confocal microscopes with spectral detection, spinning disk confocal microscope Nikon Eclipse Ti, Yokogawa CSU-X1, Zeiss Apotome 2 structured illumination microscope and automated station for immunohistochemical methods, will be used in all activities of RP 1 and related activities (Fig. 5.1.1). In 2018, the spatial resolution of Zeiss LSM 880 will be considerably improved by installing the AiryScan detection unit within the framework of OPVVV project "Research Infrastructures for Educational Purposes - Building and Upgrading" (Reg. No. CZ.02.1.01/0.0/0.0/16_013/0001775; 2017-2020).

Mainly within the RP 2, the **R&D centre SUPRAMOL IMC CAS**, will be using mainly existing equipment of the IMC CAS. In particular, following technologies will be used: static, dynamic and electrophoretic light scattering, synthetic and radiochemical laboratories, analytical services, X-ray scattering, nuclear magnetic resonance, UV-VIS spectroscopy, atomic force microscopy and X-ray fluorescence. All other equipment of IMC CAS will be available in case of need without any additional costs, the same applies for activities in RP 1 and RP 3 (Figure 5.2.1).

R&D centre ZRIR IKEM is equipped with microscopy infrastructure available for the project, preferably for activities of RP 2, with all overlapping activities of RP1 and RP 3 (Figure 5.2.1). In particular, within the scope of activity 6 of research objective 1 (RP 2), this group will use the instrumentation, including an 1.5T Magnetom Avanto Tim system, a 3T MagnetomTrio Tim multinuclear imager accomplished with various hardware and software (especially for ^{1}H, ^{19}F, ^{31}P nuclei and imaging of small animals), an experimental 4.7T Bruker Biospec 4.7/20 scanner, two relaxometers (operating at 0.5T and 1T) for relaxometry studies, optical microscopes, and a fluorescence imaging facility for small animals.

R&D centre EEM ISI CAS is equipped with both software and instruments that will be used within the project. Infrastructure of ISI CAS involves mechanical workshops, modern CNC machine tools, laser welding labs, arc welding workshops, steam and sputtering equipment for the production of precise sub-nanometre thin films, electron welding, magnetic resonance imaging equipment, radio signal processing laboratory up to 10 GHz, digital signal processing lab, etc. These laboratories will enable the preparation of unique components as well as larger units for special devices developed within the project.

The electron microscopy area is represented by five scanning electron microscopes (REM) and two EREMs (Fig. 6.1 and 6.2) and sample preparation laboratories. Two of these microscopes (FEI QUANTA FEG 650 and EREM AQUASEM II non-commercial experimental microscope) are part of EEM laboratory (environmental electron microscopy). Other laboratories are equipped with REM VEGA microscopes from Tescan, HR-REM Jeol 6700F from Jeol and UHR-REM MAGELLAN 400 from the FEI. Most microscopes are also equipped with sophisticated EDS, EBSD and cathodoluminescence detectors for material analysis.

Above mentioned laboratories and microscopes are designed for the development and testing of detectors and will be used for experimental measurements based on the results of MC simulations (RP 3, research objective 1), the optimization of new highly sensitive detectors and detection principles in EREM (RP 3, research objective 2), the verification of new methods for characterization of highly sensitive samples, dynamic *in situ* experiments, correlative microscopy (RP 3, research objective 3), and finally the integration of new systems and methods into a microscope (VP 4, research objective 4).

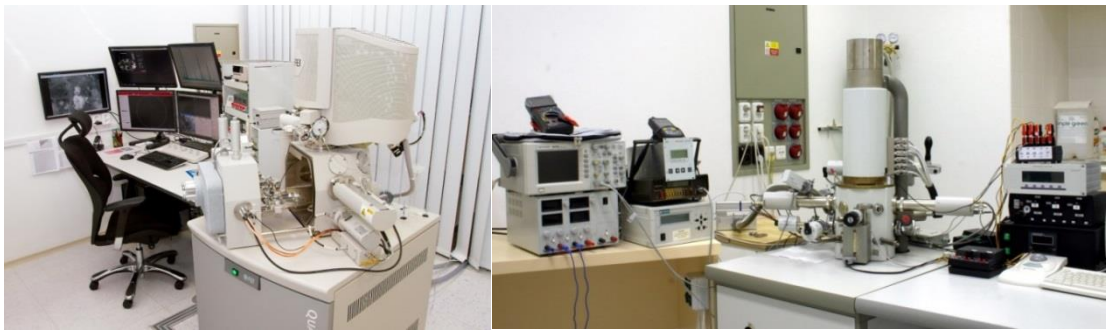


Figure 6.1: New QUANTA 650 FEG environmental scanning electron microscope, equipped with micromanipulators and other unique systems (left) and experimental scanning electron microscope AQUASEM II, rebuilt by the EEM team at ISI CAS in Brno (right).

Figure 6.2: The Jeol JSM-6700 high-resolution scanning electron microscope (left), the Vegas robotic scanning electron microscope from Tescan with thermo-emissive cathode (center) and the high-resolution scanning electron microscope MAGELLAN 400 from FEI at ISI CAS in Brno.

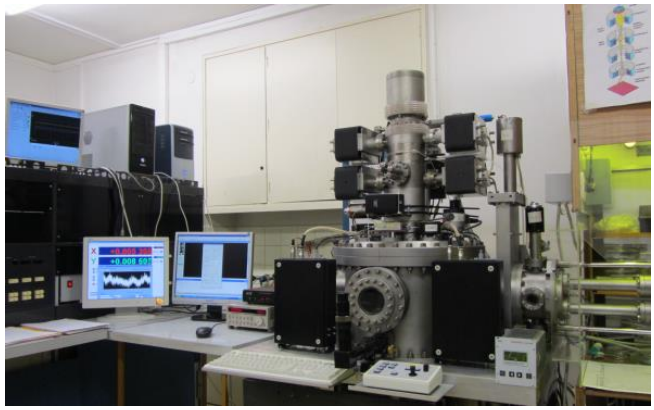
Within the EEM R&D centre part of the group of Electron Optics of ISI CAS, which deals with the study of electron-optical elements and systems both theoretically and practically. It has its own executive programs for electromagnetic fields and their optical properties (Geant and EOD), with interface developed in cooperation with TU Delft and programs from University Manchester. Thanks to the specialization of individual members (e.g. particle optics, vacuum physics, and fine mechanics), performing both theoretical plan and design including experimental realization can be carried out by the group. Furthermore, the group focuses on the development of methods of electron-optical modelling. The software will be used in the present project to simulate electron beam, gas and solid beam interactions in EREM (RP 3, research objective 1).

The Department of Special technologies at ISI CAS is engaged in the development of technologies and constructions of technological equipment, which constitute the necessary background for the construction of electron-optical devices operating in a vacuum environment. These technologies include electron beam welding and micro-machining, vacuum soldering, vacuum bore development and production, magnetron sputtering, thin film characterization and coating. The lab is equipped with modernized Tesla PZ 810 vertical vacuum furnace allows soldering with silver, copper or nickel solder and annealing in vacuum, and a small laboratory vacuum furnace suitable for soldering and heat treatment of smaller components (Fig. 6.3). Olympus LEXT OLS3100 optical microscope is available for routine sample preparation and routine viewing of surfaces (Fig. 6.3). The experience of members of the team and the equipment will be used in the design of differentially pumped chambers and detectors with newly designed design (VP 3, research objectives 1 and 2). Special sample

holders, brazed vacuum components, flanges, and many small parts and larger devices will be used to implement VP 3, research objectives 3 and 4.

Figure 6.3: Modernized Tesla PZ 810 vertical vacuum furnace (left), small laboratory vacuum furnace (center) and Olympus LEXT OLS3100 optical microscope (right).

The department of special technologies also includes group of electron lithography ISI CASR, which deals with the research of micro-litography technology using an electron lithograph (Fig. 6.4). Its activity is mainly focused on large-area microstructures for diffraction optical elements for laser beam forming, submicron diffraction holographic structures, metallic thin-film structures and silicon dielectrics for biosensors and conductivity chemical sensors and lateral resolution structures under 100 nanometers produced by engraving using the AFM microscope. It runs off-line and on-line software to manage exposure, modeling and simulation of optimizing the parameters of the structures created and to facilitate their design. The electronic lithograph, AFM microscope and associated software will be used in the design of new detectors and sample holders (RP 3, research objective 2) and research and development of new methods and chemical sensors (RP 3, research objective 3).



Obr. 6.4: Electron beam lithograph with 15 kV accelerating beam and 100 nm resolution.

Department of optical micromanipulation techniques ISI CAS deals with the use of mechanical effects of laser radiation in its interaction with dielectric objects and its own Raman spectrometer. An experimental device (the only one in the Czech Republic) has been designed and implemented that allows spatial gripping of micro-objects and nano-objects into light fields and their controlled displacement in space. The device is expanded with the ability to generate several optical traps and is independently spatially positioned using a computer. An original method using standing Gaussian waves to design objects was proposed. Also included is an optical scalpel that utilizes the absorption of a focused laser beam to evaporate

a very small volume (about 1 µm x 1 µm x 1 µm) of material on the surface of the object or inside the object. The manipulation system was enriched with a pulsed laser capable of performing laser micro-ablation, which is experimentally used for changes in the optics of optically trapped objects, the perforation of the outer and inner membranes of living organisms, and the fusion of living cells. The device enables optical sorting, using appropriately spatially spaced laser beam intensities to sort micro-objects or sub-micrometer objects by size or refractive index. Thanks to photo-polymerization, it is possible to create microstructures with a focussed laser beam. The enlightened area solidifies and becomes the part of the microstructure generated

Raman spectrometer is planned to be utilized for the *ex vivo* and *in vivo* biological determinations and as a supportive non-destructive technique for electron and optical microscopes to study the molecular structure of biological samples and sensitive polymers. It will also be used for dynamic in-situ experiments in combined electron and light microscopy, Raman spectroscopy (RP 3, research objectives 3 and 4). Experimental device for gripping objects in light fields will be used within the project for working with biological samples and polymers (RP 3, research objectives 3 and 4). The above-mentioned EREM-optimized technology integration will represent main focus of R&D activities in RP 3, research objective 4.

6.2. Utilization of new infrastructure and equipment

Within the proposed project, no building activity is planned, the project proposal is focused on the utilization of existing laboratory infrastructure as much as possible, bringing some new instruments into it. 3 research programs (RP) of the proposed centre contain in their description the specifications of the instrumentation, their characteristics, purpose and readiness of the existing infrastructure, for RP 1 in chapter 5.1.6, for RP 2 in chapter 5.2.6 and for RP 3 in chapter 5.3.6. Technical and pricing specifications for new instruments are presented in the form of quotations (see Annex 10). As stated in the obligatory annex describing the project budget, planned investments will be purchased in tenders during year 2018, only one machine in 2019. Tendering procedures will be conducted according to the rules currently in force.

The following text summarizes and provides further details describing the usefulness of acquiring equipment and devices not yet mentioned in chapters 5.1.6, 5.2.6 and 5.3.6.

R&D centre of the IEB CAS plans to modernize instrumentation in three areas.

In 2018, modernization of the acquisition of weak signals by installing a highly sensitive EMCCD camera to an existing spinning disk microscope will be performed, which will be key to the successful achievement of RP 1, particularly in activities 1-3 and research objective 2, activity 1. The image acquisition at the existing fluorescence Nikon SDZ25 stereo microscope will be improved by installing a CMOS camera, including PC and software (Activity 2, research objective 2). In addition, a set of image analysers, which are already obsolete at the IEB R&D centre, will be modernized.

In 2018, cultivation and molecular biology facilities of the R&D IEB will be upgraded and strengthened by acquisition of 2 incubation shakers, 2 automated autoclaves and 4 PCR thermocyclers. These facilities will be used in all activities of the two research objectives of the RP 1. For all these devices, the purpose of replacing old non-conforming devices and increasing the capacity given by recruiting new team members.

In 2019, the equipment of automated histochemical stainings of plant material will be modernized and expanded. By purchasing a new station, new immunocytochemical methods will be made available for all activities of the two research objectives of RP 1. Also, new nanoparticle-labelled antibodies prepared in RP 2 will be tested on this device and labelled samples for electron microscopy will be prepared within the framework of RP 3.

R&D centre SUPRAMOL IMC CAS plans to modernize the equipment in five areas. Acquisition of all instruments is essential to solving the project, replacing obsolete, inadequate or insufficiently sinking devices in a number of cases. In 2018, a set of devices for the synthesis of monomers, polymers and nanoparticles, a GPC system, a functional module for advanced imaging equipment, a GC system, and meting temperature machine will be obtained. The purpose of the purchase and how to add existing infrastructure is given in chapter 5.2.6. All facilities will be preferably used by members of teams form activities of RP 2.

R&D centre ZRIR IKEM plans to modernize instrumentation in 4 areas. As stated in chapter 6.1, ZRIR IKEM is focused on MRI technology, but within VP 2, in collaboration with partners, the ^{19}F MRI contrast agents are planning to combine MRI imaging with electron and fluorescence microscopy in activity 6 of research objective 1. That is why ZRIR IKEM, through its involvement in VP 2, nominates a group of electron microscopy led by Prof. Ivan Raška (Table 5.2.3) and intends to equip its laboratories with equipment for cry-fixation to perform electron microscopy in high detail and to modernize existing electron microscopes that will be used within the project.

In 2018, Leica EM ICE, high pressure freezer (Leica EM FSP) and automatic plunge freezer with bare grid technique (Leica EM GP) will be purchased to serve primarily for activities of RP 2. The technical specification is given in annex 10.

The electron microscopy will include a FEI Morgagni transmission electron microscope. Routine microscope equipped with Mega View III CCD camera with the iTEM software (Olympus SIS) and Tecnai G2 Sphera 20 tomographic electron microscope with an LaB6 cathode. The microscope is equipped with 4MPi “slow scan” Gatan USC 1000 CCD camera (Model 894) and with Gatan 626 and Gatan 914 cryo-transfer holders with an option of high-resolution analysis and 3D reconstruction of vitrified samples. In 2018, High resolution 16 megapixels CMOS camera for existing FEI Tecnai G2 20 Sphera is planned. With such camera, we will be able to make microscope improvements for crystallographic reconstructions and structural characterization of isolated particles.

In 2018, MR/CT imaging analysis will also be improved by purchasing a powerful iMac computer and multi-volume software VGStudio Max 3.0 for processing and analyzing medical images and automatic or semi-automatic segmentation and 3D animation. This device is key for activity 6 of research objective 1 of RP 2 (Figure 5.2.1), i.e. for the development of new contrasting compounds for MR.

R&D centre EEM ISI CAS plans to modernize instrumentation and software equipment in 3 areas.

In 2018, the software needed to simulate gas flow and to characterize gas pressure distribution in the EREM sample chamber will be purchased. The software should allow Computational Fluid Dynamics (CFD) simulation and should be usable for a wide range of research applications. The software should have a wide range of physical and chemical models or their combinations, allowing modelling a wide range of tasks (flow, convection heat, conduction and radiation, chemical reaction and multiphase flow with heat sharing). It is also important to quickly build a model, efficiently create a computing network, perform accurate

calculations, parallelize tasks on multiple processors or kernels, and implement own models, pause the calculation, change or correct the settings, and then continue the calculation. In addition, the software or software adds-on for modifying CAD models used for simulations, will be acquired. It should allow modification of CAD models or to complete creation and modification of 2D and 3D geometries for CFD simulations. The software should be capable of producing solid, shell and beam models of fixed parts for structural and thermal calculations, and should allow the storing of individual operations and their retroactive editing, thus allowing for rapid implementation of all design changes and updates. In addition, the creation of 2D sketches by using simple curves, 3D operations and creating 3D solids from 2D sketches, parametric modelling (dimensional parameters), and importing models created in external CAD programs, modification of existing and imported models, and model preparation for volume networking.

The above-mentioned software complex is indispensable for the calculation of gas velocities and pressures in the space of the differentially pumped chamber and EREM sample chamber, more precisely to characterize the boundary conditions for the simulation of primary electron beam interactions with gas, solved as a major research topic within VP 3, research objective 1 and signal interactions with gas and the development of new detectors for EREM, addressed within VP 3, objective 2. The software is also very important for the complex characterization of environmental influences affecting sensitive biological and polymeric samples in the EREM sample chamber and the development of special methods for these samples within RP3, research objective 3.

In addition, software will be purchased for EDS analyzer QUANTA FEG 650, which will be used in the characterization of highly sensitive samples. Within RP 3, objectives 1, 2 and 3, it will be necessary to purchase additional EDS analyzer software. The software will allow the detection of the primary electron beam scatter by means of detected X-ray spectra, depending on operating parameters (EREM pressure and type, energy and current, working distance), and development of methods to suppress these parasitic effects on the accuracy of detected spectra within research objective 1 and 3 of RP 3. It will allow obtaining high-precision quantitative results of energy-dispersive elemental X-Ray microanalysis from samples composed mainly of light elements under low electron beam conditions, mainly thanks to standardized quantification and a large CCD chip area. This characteristic will be used for EDS analysis of biological and polymeric samples in research objectives 3 and 4 of the RP 3. It will also enable the measurement of thin electrically conductive layers with thicknesses permitting the passage through the high energies of the primary electron beam. The Cliff-Lorimer method (TEM, STEM and SEM) will be used to measure thickness. The above-mentioned possibility of measuring thin film thicknesses will be used in the research and development of new detectors for REM and EREM realized under research objective 2. A new option for the software package will also be a search for a similar spectrum and the possibility of distinguishing overlapping X-ray spectra, automatic particle analysis and other possibilities for automatic processing of sample specimens with light elements in a high pressure gas environment in EREM.

Within the research objectives 3 and 4 of RP 3 and in relation to RP1 and RP2 the purchase of confocal laser scanning microscope for correlative microscopy is planned. One of the main objectives of the proposed project is the correlation microscopy of EREM techniques, optical fluorescence microscopy and Raman spectroscopy, supplemented with techniques for mechanical and non-contact laser micro-manipulation with living biological samples (e.g. cells) and their subsequent integration into EREM sample chamber. This will create a unique

analytical tool, which will be represent, after the equipment with new detectors from RP 3, aim 2, very unique setup. Newly purchased light fluorescence microscope will be used not only for the study of stained tissues in fixed specimens, but also for live samples and other highly sensitive materials and polymers. For this reason, the microscope will be equipped with lasers with wavelengths in the range of 400-800 nm. The microscope will also be equipped with a Raman spectrometer, which will be used, among other things, for testing and calibrating equipment integrated into EREM. By placing a fluorescence microscope in close proximity to EREM, a complex of devices, technically and positively configured, will be created to minimize sample transfer times and avoid artifacts and degradation of living samples. Also, due to the scope of planned work in the project, the use of another similarly equipped optical microscope would be inefficient in time and financial terms, unprofitable and unrealistic due to planned modifications and interconnections.

As part of the activities of RP 3, research objective 4, the purchase of several components for their usage for the integration of fluorescence microscope, Raman spectrometer and laser micromanipulation system into the new EREM QUANTA 650 FEG. The properties of each component depend on the purpose of their use. Raman spectroscopy requires a laser with a very narrow spectral width. The optical micro-manipulation laser must have a well-defined transverse profile and its wavelength must not damage the objects under investigation. The Raman spectroscopy camera requires very high sensitivity and very low noise levels. List and characterization of purchased components is following:

Raman's laser designed to excite Raman's wavelength of 785 nm and a very narrow spectral line. This property is important for the high resolution of the detected spectra.

Spectrograph for Raman scattering detection. Several types of grids will need to be fitted for detection in both a wide spectrum band and for detailed sample scans.

Spectroscopic camera for Raman signal detection behind a spectrograph. Because of the very low intensity of the detected light, a camera with high sensitivity and high quality noise suppression is needed. These features have cameras cooled to temperatures below -100 °C.

Fluorescent lamp to excite fluorescence on a sample. Thanks to the different dyes used, it is necessary to change the emitted wave length, for example by changing the filters or switching the laser diodes.

Fluorescence camera designed to detect fluorescence emitted from the sample. The fluorescent signal is weak and the camera needs increased sensitivity and noise. This is achieved by cooling using the Peltier article. For high-quality video capture, a minimum VGA resolution is required.

Trapping laser for optical manipulation with a wavelength of 1064 nm and a power of at least 1 W. The wavelength is designed so as to avoid radiation absorption by the biological preparation while minimizing water absorption.

Microscope lenses with high numerical aperture intended to be incorporated into the microscope chamber will need to be usable in vacuum. The magnification of the lenses will be 60X or 100X.

Spinning system for laser beam spatial positioning intended for three-axis positioning of the laser beam in the plane of the sample. It will be placed in the microscope chamber.

Camera lens located in front of a fluorescent camera. Designed to sharpen the image to other planes.

Computer and control cards for control of experimental apparatus.

Optics, mechanics, filters, vacuum components, i.e. components designed for optical assembly. Components will need to be selected for incorporation into the microscope vacuum chamber.

Optical fibers, optics, mechanics, i.e. components designed to introduce laser radiation into the microscope chamber. They will be located outside the vacuum chamber.

Chemistry and laboratory equipment for cleaning of optical components and for sample preparation.

7. ADMINISTRATION OF THE PROJECT

The administrative team is composed of the project deputy director (0.2 FTE), project manager (1 FTE), the assistant of project manager (0.1 FTE), administrative worker of ISI CAS (1 FTE) and two more administrative workers for partial capacity (0.2 IKEM and 0.4 FTE IMC) in research program 2 that will be implemented in the coordination of two partners and therefore it calls for more intensive coordination. The team is proposed very efficiently and it is described in detail in chapter 3 in the paragraph "Proposed organisational structure of the project". The specification of members is mentioned in the obligatory supplement "Implementation team" and justification of salaries is specified in budget comments (Annex 9).

7.1. Risk analysis

7.1.1. Risk analysis - activities a, d, e, f

During the project preparation, activities a), d), e) and f) were assessed with respect to the incidence of possible risks. Following text specifies the list of identified risks and all measures for their prevention and elimination of their impact. These preventions will be implemented in the proposed organisation structure of the centre of excellence, which is given in detail in chapter 3.3 and in the description of the composition of research teams for individual research programs, in chapters 5.1.5, 5.2.5 and 5.3.5. The risk elimination measures in all activities will be guided by the contingency principle, i.e. by the continuous assessment of the coincidence of the individual risks with the proposal for their solving by the project council as outlined below.

Main risks identified for the activity a) i.e. support of research which, by its quality and originality, shall achieve international excellence, which shall result in an increase in research performance by the research centres, are listed below, indicating the probability of occurrence (P) and impact on the overall success of the project (I):

1) Insufficient quality of work of individual members of research teams of particular research program, low quality laboratory book handling, bad protocols or even the absence of protocols, improper procedures, etc. P 60%, I 60%.

2) Bad date observance of planned activities of individual team members, P 60%, I 40%.

3) Non-compliance with the planned procedures by individual team members, P 20%, I 60%.

4) Hiding of failure during experiments or even data fabrication, cheating, P 5%, I 80%.

5) Impossibility to reach the goal by chosen method, P 60%, I 20%.

6) Insufficient activity of involved laboratories towards the objectives of the project, P 5%, I 80%.

7) Insufficient quality of the outcomes of involved laboratories that would prevent the publication of these results in top scientific journals, P 5%, I 80%.

8) Diversion of laboratories from the pre-arranged topics of the project, P 5%, I 80%.

9) Insufficient activity of R&D centres towards the activities of the project, P 5%, I 60%.

10) Diversion of R&D centres from the pre-arranged topics of the project, P 5%, I 80%.

The measures for prevention and elimination of all risks in activity a) are planned in the following way:

With respect to the character of the work in the area of basic research in biology, chemistry and instrumentation development, the measures are based on the contingency management approach, which is characterized by continuous searching for the most suitable and effective ways of management suitable for the particular situation, i.e. for the particular phase of the research project. This approach requires continuous effort of leaders on all levels for the timely efficient elimination of risks as well as their consequences. Very important is high consistency in the reporting of scientific results, suitable structure of the team and flexible approach of group leaders. The basis for the application of this approach will be regular meetings of involved laboratories, which will be organized by leaders of research activities or research program (see Fig. 3.4 and 5.1.5, 5.2.3 a 5.3.5). Periodicity of these meetings is suggested once a week for teams of individual research activities, once a month for all teams within one objective, 4 times a year for the research program and once a year for the whole centre of excellent research.

The risk prevention and elimination measures are divided into three groups depending on the severity of the risk, from the smallest to the highest. For each group, strategy for solving the particular risk are proposed including multiple risks:

Risks 1-5 will be eliminated in the most consistent way on the lowest level possible, i.e. on the level of individual teams for particular research activities. Leaders of these team will monitor the incidence of these risks through regular meetings as well as through face to face discussions with all members of the team at least twice a week. Based on these meetings, the leader will urgently suggest alternative procedures, technique or methods or stimulate members of the team by providing direct help increasing their motivation. In case of failure with the application of these measures, the leader of the team will consult with the head of the research program on their regular monthly meetings. From these meetings, the solution for the elimination of the impact of these risks will be suggested and applied immediately, including possible personal changes.

Risks 5-8 will be eliminated by heads of research programs 1-3, who will apply the measures to solve these issues based on their regular meetings (4 times a year). Minutes of meetings will be containing information on the identified risks and measures for their elimination.

Risks 9 and 10 will be monitored and solved by the director of the centre of excellent research, who will eliminate these risks on the regular meetings of the executive board for research and development (Fig. 3.4), which will meet 4 times a year. The meeting could be organized also in case of dealing with unforeseen issues (delay in the particular activities, technical issues). Results of council meetings will be obligatory for every members of the project. Minutes of meetings will be supplemented to progress reports and final report. Council meetings will be hosting personally all members.

Main risks identified for the activity d) i.e. development of research teams - support for research via support for, or an increase in the number of, research teams, including their long-term expansion to include domestic or foreign research or technical workers, are listed below, indicating the probability of occurrence (P) and impact on the overall success of the project (I):

1) Inefficient division of labour within teams performing research in certain research activity, P 20%, I 40%.
2) Inefficient division of labour in between teams within one research objective, P 40%, I 40%.
3) The lack of capacity or sufficiently qualified members of the team, P 20%, I 60%.
4) Inability to secure the future existence of the team, mainly during the period of project sustainability, P 20%, I 60%.
5) Inefficient division of labour within the research program. Lack of involvement of foreign researchers, P 20%, I 60%.
6) Change in the team composition within the research program harming the sustainability of the whole centre of excellent research, P 5%, I 60%.
7) Inefficient division of labour within centre of excellent research teams performing, lack of foreign partner collaboration, P 40%, I 60%.

The measures for prevention and elimination of all risks in activity d) are planned in the following way:

One of the most serious risk within the scientific wok in collectives is their ineffective leadership. Therefore, the main attention will be paid to eliminate this risk and to increase the working efficiency within teams and in between teams. The risk prevention and elimination measures are divided into three groups depending on the severity of the risk, from the smallest to the highest. For each group, strategy for solving the particular risk are proposed including multiple risks:

Risks 1-2 will be monitored by leaders of research activities by their continuous supervision over the progression of the work as by their interaction with all members of the team, including technicians and students. Leaders of working teams will be obliged to perform every week personal consultations with all members of the team including students and technicians. The measures for the increasing of the efficiency of the work will be applied immediately, without delay through the proposal for the improvement of the situation.

Risks 3-5 will be will be monitored and eliminated on regular meetings of leaders of research activities with the head of the research program. The authority of head of the program will be to suggest personal and technical measures to reduce the risk consequences. The head of the research program will also continuously monitor options for recruitment of foreign researchers and options for the applications to international grant competitions, including the active stimulation of potent members of the team for grant applications.

Risks 6-7 will be will be monitored and eliminated primarily by the director of the centre, who will be obliged not only to take active part on planned meetings, but also to actively communicate with heads of all three research programs with the aim to propose the solution without delay.

Main risks identified for the activity e), i.e. development of internationalization - strengthening of the international dimension and intensive scientific co-operation with at least one leading organization for research and knowledge sharing abroad in relation to the

supported activity a), are listed below, indicating the probability of occurrence (P) and impact on the overall success of the project (I):

1) Inconsistency in the implementation of pre-arranged activities with foreign institutions, P 20%, I 40%.
2) Low activity of members of research teams in the collaboration with foreign partners, P 20%, I 60%.
3) Inability to create suitable conditions for the implementation of collaborative activities with foreign partners, P 20%, I 60%.

The measures for prevention and elimination of risks in activity e) are planned in the following way:

As it is mentioned in the chapter 5.1.4, the project proposal contains the description of collaboration with the main strategic partner of the centre of excellent research, Prof. Jiří Friml from IST Austria. There are also numerous other collaborators for every research activity and research objective. Risks 1-3 will be monitored and eliminated with respect to the successful implementation of these collaborations by the director of the centre of excellent research and heads of research programs, within their regular meeting, 4 times a year. Minutes of these meetings will be containing suggestions for measures to decrease possible impact of lower intensity of the collaboration.

Main risks identified for the activity f), i.e. management of the project, are listed below, indicating the probability of occurrence (P) and impact on the overall success of the project (I):

1) Non-compliance with project indicators, P 20%, I 80%.
2) Indiscipline in the financial management of the project, P 20%, I 80%.
3) Inappropriate institutional support of R&D centres including conditions for tendering procedures and acquisition of equipment, personal politics and management of intellectual property, P 20%, I 60%.
4) Formal conflicts within the implementation team of the proposed centre, including also conflicts in the area of intellectual property of research results P 20%, I 80%.

The measures for prevention and elimination of risks in activity e) are planned in the following way:

Main executive power for application of measures to prevent risks of this activity is the project administration council (Fig. 3.3), which is described in chapter 3.3. The council will meet at least 4 times a year. The meeting could be organized also in case of dealing with unforeseen issues, i.e. delay in the particular activities or technical issues. The outcome of the council's meetings, which will be guided by the principles of contingency, will be binding on all project participants. In case of conflict solving, the council will follow the wording of Partnership agreement, which is already provisionally approved by the applicant and all partners and ready for ratification in the second round of the evaluation procedure. In the field of intellectual property treatments, the project will be assisted by specialized patent department at the IMC CAS.

As shown in Table 7.1, most of the risks of activities a), d), e) and f) are in the medium risk zone, so there is a good guarantee that the project will not face any major problems.

Probability	**Impact**	Very low 5%	Low 20%	Medium 40%	High 60%	Very high 80%

Very high	80%					
High	60%		a5	a2	a1	
Medium	40%			d2	d7	
Low	20%			d1, e1	a3, d3, d4, d5, e2, e3, f3	f1, f2, f4
Very low	5%				a9, d6	a4, a6, a7, a8, a10

Table 7.1: Risk contingency table summarizing the probabilities and impacts of the individual risks associated with the activities a), d), e) and f). The degree of danger for the project is expressed in color as low (white), medium (orange) and high (red).

7.1.2. Risk analysis - activity b

During the project preparation, the activity b) has been assessed with respect to the incidence of possible risks. Following text specifies the list of identified risks and all measures for their prevention and elimination of their impact. These preventions will be implemented in the proposed organisation structure of the centre of excellence, which is given in detail in chapter 3.3 and in the description of the composition of research teams for individual research programs, in chapters 5.1.5, 5.2.5 and 5.3.5.

Main risks identified for the activity b) i.e. completion, reconstruction or upgrade of infrastructure to materially, technically and informationally support and facilitate research activities in relation to the supported activity a), are listed below, indicating the probability of occurrence (P) and impact on the overall success of the project (I):

1) Problems in tendering procedures and correct installation of the purchased equipment that would raise from inconsistencies in fulfilling their time schedule and administration, P 20%, I 40%.

2) Problems associated with bad service after new instruments failure or malfunctions, P 40%, I 40%.

3) Problems related to training of new workers on new devices, P 5%, I 40%.

4) Problems related to dramatic change of the planned technology and its availability. P 20%, I 40%.

5) Problems with investments installed in premises not owned by the applicant or partner. P 5%, I 60%

6) Problems with the equipment financing. P 40%, I 40%.

The measures for prevention and elimination of risks in activity b) are planned in the following way:

For **risks 1 and 5**, the most crucial is the competence of the director of the centre of excellent research, who will eliminate these risks on the regular meetings of the executive board for research and development (Fig. 3.4), which will meet 4 times a year. The meeting could be organized also in case of dealing with unforeseen issues (delay in the particular activities, technical issues). Results of council meetings will be obligatory for every members of the project. To prevent the risk 1, co-operation with the relevant tendering procedure administrative support that is available in applicant's and partners's institutions. For tendering process and final selection of the best combination, it is urgent to prepare high-quality documentation. Tendering procedure calls are scheduled at the onset of the project period and it is expected their smooth course. In many cases, the upgrade of existing devices is planned, where the control over the procedure is much easier. To eliminate risk 5, contract or

agreement on the dismantling and removal of equipment will be prepared and signed. However, the probability of this risk is very low and it applies only for the usage of some electron microscopes in partner ZRIR IKEM.

To address situations related to the prevention and elimination of the potential impact of risks **2-4 and 6**, the key competency of the relevant RP (1-3) is to address these risks at regular meetings 4 times a year. The outcome of these meetings will be a list of measures to remedy the identified risks. In the risk 2, the most important will be to speed this process as much as possible in the form of an immediate complaint or exchange for another piece, project administration will strictly keep the agenda, ensuring that the guarantee conditions are strictly fulfilled. For the elimination of risk 3, key researchers will have part of their working time allocated to train new workers. Prevention of risk 4 is secured by the quality of nominated members and by their constant contact with developments in the field of instrumentation, where it is also not possible to assume that the technology will dramatically change to the beginning of the project in 2018. Prevention of risk 6 will be realized through regular contact between the project administration and the grant provider, and eventual delays will be eliminated through temporary funding from the applicant's or partner's resources, with the subsequent transfer of funds upon their receipt from the provider.

As shown in Table 7.2, most of the risks of activity b) are in the medium or low risk zone, so there is a good guarantee that the project will not face any major problems.

Probability	**Impact**	Very low 5%	Low 20%	Medium 40%	High 60%	Very high 80%
Very high	80%					
High	60%					
Medium	40%			**b2, b6**		
Low	20%			**b1, b4**		
Very low	5%			**b3**	**b5**	

Table 7.2: Risk contingency table summarizing the probabilities and impacts of the individual risks associated with the activity b). The degree of danger for the project is expressed in color as low (white), medium (orange) and high (red).

8. BUDGET

Detailed specification of project budget for individual years is attached in the corresponding part of the application support and in Annex 8 of this feasibility study. Description of adequacy and economy of all costs for salaries of research and administrative members of the team, for services and other costs is specified in detail in Annex 9.

8.1. Co-financing during the project implementation

The applicant IEB CAS (VVI) and two partner VVI institutions (IMC CAS and ISI CAS) are ready to co-finance with 5%, partner IKEM (OSS) has 0% level of co-financing based on law. The co-financing is specified for all years of project implementation in the corresponding part of the application support and also in Annex 8. The main source of co-financing will be represented by the institutional support of the Academy of Sciences of the Czech Republic and, to a lesser extent, resources from the economic activities of individual partners (patents, utility models, recognized varieties, etc.).

9. SUSTAINABILITY

9.1. Sustainability of activities a, d, e, f

This project is proposed based on the informal analysis of existing scientific performance of all laboratories involved, which has been carefully made in advance by leaders of involved laboratories and R&D centres. In this analysis, the potential of leading members to perform scientific activities on long term basis, based on the continuous acquiring of financial support from local and international grant competitions, has been taken into account. All R&D centres entering the project, i.e. the applicant R&D microscopy centre IEB CAS and partners from SUPRAMOL IMC CAS, ZRIR IKEM and EEM ISI CAS fulfil very well all parameters of long-term sustainable research activities development, which was in their case never dependent on the massive, one-shot subvention of national or international character. As it is documented in chapter 3 (including mentioned Annexes), in the CVs of excellent and key members of the team (Annex 7) and in their publication lists, grant projects holdings and patents awarded (chapters 5.1.5, 5.2.5 and 5.3.5), the essential parameter , i.e. cost/benefit balance, is fulfilled very well for all subjects entering the project. This balance is even shifted towards the benefit, all partners represent very crucial corner stones of their institutions. This praxis, very decent to the public money resources, will be further supported by the proposed project, in which we are going to continue with the same philosophy. Moreover, we will logically continue in this also after the project implementation, during the period of its sustainability. The activities of involved laboratories could be easily continuing even without financial resources of the proposed project, but the project itself would not be possible to exist in its mission, i.e. to reach strong interdisciplinary extension for the applicant as well as all participating partners.

To follow the above mentioned logic of the proposed project, high attention will be paid to the scientific and financial post-project sustainability already during the project implementation period. Based on the evaluation indicators adjustment, there are 4 active participations in the projects of international collaboration planned, which will be implemented in the framework EU program Horizon 2020, program INTER-EXCELENCE, which replaces several activities of international collaboration terminating this or next year. Members of the applicant research team have been successful in these programs, as documented in chapter 3 and Annex 3.

Financial resources from these projects will constitute only part of the overall budget. Other money will be brought by researchers of the centre of excellence through their applications to the local and international grant competitions. There is one former ERC applicant in the team (Jan Petrášek), who plan to apply for the ERC consolidator grant and there is also good expectation that other members of the team will be applying to this prestigious grant agency, namely Martin Hrubý (IMC CAS) and Dominik Heger (ISI CAS). Proposed project would create very good conditions for such activities. As documented in CVs of project implementation team, the majority of involved researchers is able to regularly obtain grant projects from other grant agencies like Czech Grant Agency, but also form programs of international collaboration from MSMT. Imaging unit of R&D centre of IEB CAS is also the partner of the project in the framework of "National Infrastructure for Biological and Medical Imaging" (Czech-BioImaging, CzBI), which has been approved for funding by the Ministry of Education, Youth and Sports for the period 2016 - 2019 (Large Infrastructures for Research, Experimental Development and Innovations, LM2015062). Through this project, R&D centre imaging unit is integrated into the road map of large infrastructures with

subsequent access to large pan-European research infrastructure Euro-BioImaging within the ESFRI (European Strategy Forum on Research Infrastructures) network. This situation offers very good opportunity to get additional resources in projects planned within the pan-European infrastructures ESFRI.

Above mentioned possibilities are essential for the fulfilment of parameters of planned performance during the sustainability period, as they are specified in the table 9.1. Another important financial resource for this period will be generated form patenting some solutions that are going to be optimized for the potential applications within the proposed project, e.g. instrumentation development and nanoparticle systems for plant biotechnologies and some clinical applications.

Description of cost-benefit (CBA) analysis results

A complete CBA analysis is a part of the obligatory attachments in MS2014+ system. Here, basic information is given about its structure and parameters of individual items.

Investments

The table of Investment costs at the tab Investments and resources reflects the project budget. Both investments and other items are inserted into this table.

Resources

Financial resources mentioned at the tab Investments and resources are in agreement with the project call and it is expected 5% co-financing form institutional resources of the applicant and partners, as mentioned in chapter 8.1.

Operating and financial costs

Expected cash flow related to the project after the project implementation period is specified at the tab Operating costs and revenues. The balance of operating costs is sufficient for the project financial sustainability, fulfilling declared aims, including socio-economic impacts.

Operating revenues

Financing of operating and financial costs is separated into operating revenues and financing of the operating loss. The item operating revenues represents primarily economical activities that is carried out thanks to the project (contractual research, patents and rights, etc.). Here we expect that within the short period after the project implementation (2 years), part of the costs might be covered form the contractual research, which will be based on two international patents.

However, operating and financial costs are higher than operating revenues and therefore, they will be financed from other resources in the form of financing of operating loss. The resources of financial sustainability, which will be used to cover the operating loss of the project, are resources from MŠMT, GAČR, TAČR and other public institutions, revenues from international grants (FP7, Horizon 2020, FP9), institutional resources of CAS, or others.

Residual value

With respect to the fact that the project is not generating a financial profit, this value is equal to 0.

Financial analysis

For the purpose of verification of financial sustainability, the result at the tab Sustainability for FA (sustainability= yes). This criterion is fulfilled.

Socioeconomic impacts

Number of newly created jobs (FTE) corresponds to the number of created jobs directly generated by the project. Number of FTEs is specified for all year, when these FTEs will be staffed, with the separation to Praha and Brno. With respect to the quality of the team, it is expected that FTEs will be maintained on the same level in the first year after the project implementation good with subsequent slight decrease, which is rather rough estimate, taking in to consideration the aging of the team and too far future. The measures that will contribute to the sustainability of the project, will include in the first place strategies described in the chapter 7.1, i.e. in the analysis of risks and more importantly in the description of their monitoring and solving. In securing sustainability the activities of the centre will conform to the principles of contingency management with the aim to secure the sustainability of research activities, structures of individual teams, international co-operations and effective management of the project. Based on the project outcomes, it is possible that some teams will be extended and other reduced, but this should not influence the overall performance negatively. With respect to wide involvement of students and young research workers, it is expected that in future these workers will naturally revitalize individual teams of the project. This is very frequent praxis in the basic research and there is no alternative to that.

The contractual research scope and patents have been added into socio-economic impacts (250 000 CZK from 2024), in agreement with planned revenues in the financial analysis. The project has, thanks to its nature, also very good potential to obtain even more benefits to keep its own sustainability from these resources, but these are not possible to precisely calculate and therefore they are not mentioned in CBA analysis.

The values of socio-economic impacts reflecting the number of results of research activities of the project (publications and patents) is inserted into CBA in accordance with the RIV definition and it is also in agreement with planned levels of indicators of the project. Number of reviewed professional articles impacted in databases Web of Science and Scopus corresponds to indicators 2 02 16 (Scientific publications with a foreign co-authorship created by the supported entities) and 2 02 11 (International Patent Application formed by the supported entities).

Economic analysis

To verify the positive impact of the project the tab financial costs of return on investment for EA, where the value should be higher than 0. This requirement is fulfilled by the proposed project.

9.2. Sustainability of activity b

The plan of sustainability of the activity b) is forming the part of the above described CBA analysis. The investments that were purchased within the project implementation should be modernized in future and this is reflected in the CBA.

9.3. Plan of the development of results and outcomes during the sustainability period

As it is specified in the table 9.1, the plan for outcomes and results for the period of sustainability in years 2023-2027 follows the logic of project performance and productivity after the end of the centre of excellent research project. Both FTE capacities and results in the form of research publications and patents are expected to correspond the project capacity in

this respect at the end of the implementation period. The reasoning for this expectation is based on the idea that the performance of the centre of excellence at the date of its termination is adjusted so high that there will very good expectation for the acceptance of results in wide interdisciplinary range of scientific fields with subsequent opportunities for grant applications and good hearing in the scientific community. It is of one of the most important characteristics of this proposed project to extend it in future. Both applicant and partners have long tradition in top quality research and this tradition is more than good guarantee of future sustainability of project-associated research activities.

Table 9.1. Planned results and outcomes within the period of sustainability, i.e. between years 2023-2027.

	Indicator specification	Final value at the end of project implementation	Values planned for the sustainability period				
			Year 1	Year 2	Year 3	Year 4	Year 5
Outcome	CO 25 The number of researchers working in the modernised research infrastructures	190,03	40	38	35	35	35
Result	2 03 12 Number of participations of supported research teams implemented within the international cooperation programmes.	4	1	1	0	1	1
	2 02 11 Scientific publications (selected types of documents) created by the supported entities	170	40	34	34	34	34
	2 02 16 Scientific publications (selected type of documents) with a foreign co-authorship created by the supported entities	85	20	17	17	17	17
	2 20 11 International Patent Application (PCT) formed by the supported entities	2	0	0	1	0	0

10. ANNEXES

List of Annexes:

Annex 1: List of publications of R&D centre IEB CAS.

Annex 2: Organigram of IEB CAS highlighting the inclusion of organizational structures of proposed project into the organization structure of IEB CAS.

Annex 3: List of projects implemented in 2014-2015 for all R&D centres of this project proposal

Annex 4: List of 10 best publications of researchers of involved R&D centres

Annex 5: List of universities and their accredited doctoral programs with the participation of involved R&D centres

Annex 6: Letters of intent from foreign collaborators and companies stating their readiness for research partnership with proposed centre of excellent research

Annex 7: CVs of all nominated senior members of research team of the proposed project

Annex 8: Detailed budget according to budget chapters, articles and items for individual years of the project implementation.

Annex 9: Comments to budget.

Annex 10: Quotations for key equipment and investments.