Београд
03.03.2025.

Изјава

У вези са руковођењем др Станка Николића пројектним задатком

„T5 – FCS development“

на пројекту “Hemoglobin-Based Spectroscopy and Nonlinear Imaging of Erythrocytes and Their Membranes as Emerging Diagnostic Tool” у периоду 01.09.2020-31.08.2022.

Потврђујем да је др Станко Николић, виши научни сарадник запослен у Институту за физику у Београду, био учесник пројекта “Hemoglobin-Based Spectroscopy and Nonlinear Imaging of Erythrocytes and Their Membranes as Emerging Diagnostic Tool” – HEMMAGINERO, финансираном од стране Фонда за науку Републике Србије кроз програм ПРОМИС. Др Николић је успешно руководио пројектним задатком T5 – FCS development (Fluorescence Correlation Spectroscopy experimental setup development) и био је кључни истраживач за све модификације и развој аквизиционог система и софтвера који су неопходни како за FCS експерименталну поставку у задатку T5 тако и за поставку за THG development (Third Harmonic Generation imaging modality development) у заадатку T4. Др Николић је својим ускуством допринео значајним унапређењима аквизиционих система и сифтвера за стварање слике, а такође и успешно обучио и водио млађе истраживаче у овим активностима.

Прилог: релевантне странице из описа пројекта HEMMAGINERO у којем се виде улога и експертиза др Николића (засенчено жуто)

Руководилац пројекта HEMMAGINERO

Др Александар Крмпот, научни саветник

Институт за физику у Београду

25. Rabasovic, D. M. *et al.*, Nonlinear microscopy of chitin and chitinous structures: a case study of two cave-dwelling insects. *Journal of Biomedical Optics* **20** (1), 016010 (2015).
26. Drvenica, I. *et al.*, Maltose-mediated long-term stabilization of freeze-and spray-dried forms of bovine and porcine hemoglobin. *Journal of the Serbian Chemical Society* (2019).
27. Tserevelakis, G. J. *et al.*, Imaging Caenorhabditis elegans embryogenesis by third-harmonic generation microscopy. *Micron* **41** (5), 444-447 (2010).
28. Vitali, M. *et al.*, A single-photon avalanche camera for fluorescence lifetime imaging microscopy and correlation spectroscopy. *IEEE Journal of Selected Topics in Quantum Electronics* **20** (6), 344-353 (2014).
29. Krmpot, A. J. *et al.*, Functional Fluorescence Microscopy Imaging (fFMI). Quantitative Scanning-Free Confocal Fluorescence Microscopy for the Characterization of Fast Dynamic Processes in Live Cells. *Analytical chemistry* (2019).
30. Zipfel, W. R., Williams, R. M. & Webb, W. W., Nonlinear magic: multiphoton microscopy in the biosciences. *Nature biotechnology* **21** (11), 1369 (2003).
31. Coates, C. J. & Decker, H., Immunological properties of oxygen-transport proteins: hemoglobin, hemocyanin and hemerythrin. *Cellular and Molecular Life Sciences* **74** (2), 293-317 (2017).
32. Kuang, L. *et al.*, A novel cross-linked haemoglobin-based oxygen carrier is beneficial to sepsis in rats. *Artificial cells, nanomedicine, and biotechnology* **47** (1), 1496-1504 (2019).
33. Hoppe, M., Brün, B., Larsson, M. P., Moraeus, L. & Hulthén, L., Heme iron-based dietary intervention for improvement of iron status in young women. *Nutrition* **29** (1), 89-95 (2013).
34. Chakraborty, D., Sarkar, S. & Das, P. K., Blood Dots: Hemoglobin-Derived Carbon Dots as Hydrogen Peroxide Sensors and Pro-Drug Activators. *ACS Sustainable Chemistry & Engineering* **6** (4), 4661-4670 (2018).
35. Bukara, K. *et al.*, Comparative studies on osmosis based encapsulation of sodium diclofenac in porcine and outdated human erythrocyte ghosts. *Journal of biotechnology* **240**, 14-22 (2016).

3. Implementation

3.1. Credentials of PI and members of Project team

Aleksandar Krmpot (PI), Ph.D. in Physics is experienced researcher in development and applications of advanced optical microscopic techniques, both qualitative (i.e. imaging) and quantitative. The main achievements of Aleksandar Krmpot that qualify him to be PI of this project are:
-experience in laser development and maintenance, setting up experiments in optics, optical system design
-developed experimental set up for nonlinear laser scanning microscopy (NLSM) that utilizes ultrashort laser pulses
-experience in TPEF and SHG imaging
-experience in THG microscopy development and applications.
-experience in FCS microscopy development and applications
-strong collaboration with most relevant local biomedical institutions and research experience in TPEF and SHG imaging applications for addressing various problems (please refer to the list of publications and patents)
-strong international collaboration and working experience abroad (Aleksandar Krmpot is a guest researcher at Karolinska Insitutet, Stockholm, Sweden)
-excellent communication and organization skills (please refer to the list of organized events-conferences, competition in physics for high school students provided in CV)

Ivana Drvenica (P1), MSc. in Pharmacy, Ph.D. in Biochemical Engineering and Biotechnology is an early career investigator and competent participant for this project proposal owing to her experience in (please refer to the list of references):
-characterization of the functional status of both animal and human erythrocytes by testing their mechanical and osmotic fragility
-development and optimization of process for preserved erythrocyte membranes isolation and their biochemical (antioxidant status, cholesterol content, residual hemoglobin content) and morphological characterization (flow cytometry, photon correlation spectroscopy, scanning electron microscopy, atomic force microscopy and two photon excitation fluorescence microscopy).

Stanko Nikolić (P2), Ph.D. in Physics is qualified as participant in this project proposal because of his work in the field of functional fluorescence microscopy, as well as experience in (please refer to the list of refernces):
- experience in setting-up experiments in optics, atomic physics, and optical system design
-programming of the Field Programmable Gate Array digital circuits for potential control of experiment in nonlinear microscopy, fluorescence correlation spectroscopy and data acquisition
-strong international collaboration and working experience abroad (Stanko is a visiting researcher at Karolinska Institute, Stockholm, Sweden. He was also a full-time postdoctoral researcher at Texas A&M University at Qatar)

Ana Staničić (P3) is a Ph.D. student in Biology at final stage, who works on testing the effects of isolated xenogeneic hemoglobin on the functional activities of different cells. Through this research, she gained the needed knowledge and experience for this project implementation in terms of skills in preparing the erythrocyte and hemoglobin samples, and assessing their morphological and biochemical characteristics, respectively.

Danica Pavlović (P4), Ph.D. in Biology is an early stage investigator qualified as participant in this project proposal owing to her experience in (please refer to the list of references):

-preparation of biological samples of different origin
-experience in TPEF and SHG imaging
-experience in optical-morphological characterization of biological samples using different microscopic and spectroscopic techniques (scanning electron microscopy, nonlinear fluorescent microscopy, fourier transform infrared spectroscopy), microtomography and infrared cameras

Mihajlo Radmilović (P5) is a Ph.D. student in Biophotonics from 2018, having fundamental knowledge of cell cultures, protein extraction and nonlinear optics and light-biological matter interaction needed for this project implementation.

Complementarity and synergy of the team members. The main research of the IMR part of the team (ID and AS) is focused on erythrocytes and hemoglobin bio-functionality on the cellular and molecular level, with an aim of various application including diagnostics and drug delivery (in the case of erythrocytes) and cell culture or dietary supplement (in the case of hemoglobin). From the other hand the expertise and research activities of the IPB team members (AK, SN, DP and MR) is development and applications of advanced microscopic and imaging techniques. For the particular research proposed here, the expertise of the both sub-teams is crucial, and their synergy would lead to the novel experiences on hemoglobin based imaging, deeper understanding of hemoglobin-laser interaction and its potential application for biodiagnostics and biotechnology. IMR sub-team will be in charge for the samples preparation, ethical issues and biochemical analysis, while IPB sub-team will upgrade the existing and develop new microscopic techniques, perform demanding spectroscopic measurements and other optics related issues. All together, they will perform the imaging and measurements, data processing and manuscript preparation.
The two sub-teams have already established collaboration through current national projects and it is recognized by one joint publication [3] in Journal of Biomedical Optics and number of abstracts at the conferences (Photonica 2015, RBC 2016, Multinational Congress on Microscopy (MCM 2017), Photonica 2017), among which the noteworthy is the invited lecture at Regional Biophysics Conference http://www.rbc2018.si/ given by PI Aleksandar Krmpot.
The team members (AK, SN and DP) already have experience with social media advertisement and the organization of the rather large scientific events such as PHOTONICA 2017 (http://www.photonica.ac.rs/2017/committees.php and https://www.facebook.com/pg/photonica2019/about/?ref=page_internal

3.2. Implementation plan

Table 3.1.Members of Project team.

ID	Name and family name	Scientific institution	Person-months
PI	Aleksandar Krmpot	Institute of Physics Belgrade - IPB	24
P1	Ivana Drvenica	Institute for Medical Research - IMR	24
P2	Stanko Nikolić	Institute of Physics Belgrade - IPB	24
P3	Ana Stančić	Institute for Medical Research - IMR	24
P4	Danica Pavlović	Institute of Physics Belgrade - IPB	24
P5	Mihajlo Radmilović	Institute of Physics Belgrade - IPB	24
		Total person-months	144

Table 3.2.Tasks.

Task/subtask number	Task/subtask title	Start month	End month	Members of Project team[2]	Person-months	Description
T1	Sample preparation	1	22	P1, P3, P4, P5	88	**Related objectives: PO1, PO2, and PO3**
ST1.1	Material processing	1	22	P1, P3, P4, P5	88	All activities from blood collection (including ethical committee's approvals) till getting the final samples of erythrocytes and/or ghosts and/or pure hemoglobin and/or its derivatives ready for imaging/microscopic measurements.

ST1.2	Basic analysis	1	22	P1, P3, P4, P5	88	Basic physico-chemical analysis of samples
T2	Hemoglobin photoproduct characterization	1	10	PI, P1, P3, P4, P5	50	**Related objective: PO1**
ST2.1	Formation of the photoproduct	1	10	PI, P1, P3, P4, P5	50	Hemoglobin and its derivatives obtained in T1, irradiation in order to obtain the photoproduct. , along with use of various patterns and uniform (raster) scanning
ST2.2	Spectroscopic measurements	1	10	PI, P1, P3, P4, P5	50	One photon absorption/excitation/emissin and emission spectra measurements. Two photon excitation/emission spectra measurements. Applied to both, hemoglobin photoproduct and methemoglobin.
T3	TPEF imaging and erythrocytes morphology studies	1	14	PI, P1, P3, P4, P5, P6	84	**Related objective: PO2**
ST3.1	TPEF imaging of animal erythrocytes	1	12	P1, P3, P4, P5	72	Imaging of healthy (as reference)/altered porcine/bovine erythrocytes for morphology studies. Image processing
ST3.2	TPEF imaging of human erythrocytes	1	12	P1, P3, P4, P5	72	Imaging of healthy (as reference)/altered human erythrocytes for morphology studies. Image processing
ST3.3	Morphology examination	8	14	PI, P1, P3, P4, P5	36	Examination of various erythrocytes morphologies using SEM and AFM and ektacytometry. Comparison with TPEF data. Relation assessments with suspected pathologies.
T4	THG development	1	15	PI, P1, P2, P3, P4, P5	90	**Related objective: PO3a and PO2**
ST4.1	THG upgrade	1	6	PI, P2, P5	18	Modification of existing TPEF experimental setup for THG detection. Additional equipment purchasing. Optical and mechanical design and upgrade of existing software for data acquisition
ST4.2	Testing THG imaging	4	7	PI, P1, P2, P3, P4, P5	18	Imaging of test sample (starch), erythrocytes and ghosts. Identifying and fixing the problems. Training of less experienced members.
ST4.3	THG imaging of erythrocytes	6	15	P3, P4, P5	27	THG imaging of erythrocytes of normal/altered morphology Simultaneous THG and TPEF imaging.
ST4.4	Complementation with TPEF	12	15	PI, P1, P3, P4, P5	15	Comparison of THG and TPEF images. Identifying, extracting and understanding additional data obtained by THG
T5	FCS development	6	22	PI, P1, P2,P3, P4, P5,	96	**Related objective: PO3b and PO2**
ST5.1	FCS experimental setup development	6	16	PI, P2, P5	30	Purchasing the equipment. Optical and mechanical design. Electronic equipment outsourcing. Modification of software for data acquisition and processing already developed in Karolinska Institutet (KI).
ST5.2	Testing FCS measurements	14	17	PI, P1, P2, P3, P4, P5	18	Recording autocorrelation curve (ACC) for common test samples (dye solutions). ACC for fluorescently labeled structural lipid/protein in erythrocyte membrane. FCS test measurements at KI and results comparison. Identifying and fixing the problems. Training of less experienced members.
ST5.3	FCS measurements on the erythrocytes' membrane	16	22	P3, P4, P5	18	FCS measurements on erythrocyte membrane of normal/altered morphology. Comparison of the ACC. Studying the mobility and concentration of fluorescently labeled structural lipid/protein on membrane.
ST5.4	Complementation with TPEF and THG	19	22	PI, P1, P3, P4, P5	15	Relating the data on diffusion and concentration obtained by FCS with morphology alterations imaged by TPEF and/or THG for healthy erythrocytes and various pathophysiological conditions
T6	Dissemination	1	24	PI, P1, P2, P3, P4, P5,	144	**Related objectives: PO1, PO2 and PO3**
ST6.1	Results publishing	8	24	PI, P1, P2 P3, P4, P5	96	Preparation and submission of manuscripts. Presentation at conferences.
ST6.2	Website and social media	1	24	P1, P3, P4, P5	96	Presentation of the activities, news, results and available experimental setup on the Project website and Facebook group

ST6.3	Local popular science events	6	24	PI, P1, P2 P3, P4, P5	108	Participation in the local popular science events, conventional media (TV, radio, press), educational/lecturing activities. Organization of the workshop as a satellite even at PHOTONICA 2021 conference.
T7	Management	1	24	PI, P1, P2 P3, P4, P5	144	**Related objectives: PO1, PO2 and PO3**
ST7.1	Meetings	1	24	PI, P1, P2 P3, P4, P5	144	Kick off (contract ratification) and regular meetings (tasks assignment), progress monitoring (results discussion) and reports writing.
ST7.2	Budget realization.	1	22	PI, P1	44	Equipment purchasing and procurement according to the rules. Administration of business trips. Outsourcing.
ST7.3	New project preparation	22	24	PI, P1, P2 P3, P4, P5	12	Searching for open calls. New proposal drafting.

Involvement of members of the Project team:

PI (AK) will manage the project, organize and supervise all the activities/tasks. AK will be mostly responsible for T7 (all activities), ST6.1 (writing the manuscripts) and ST6.3. His expertise in designing optical systems and developing the microscopic techniques will be used mostly in ST 4.1, 4.2, 5.1, and 5.2. In T2, T3, ST 4.3, 4.4, 5.3, 5.4 AK will supervise the measurements and give the training to the less experienced team members (AS, DP, MR). AK will also be responsible for maintenance of the equipment and prompt reaction and solving the issues (risk mitigation).

P1 (ID) will help to PI with project management and will organize all activities at IMR. Her expertise will particularly be used for blood collection and selection and for sample preparation (hemoglobin isolation, erythrocyte preparation, gradual hypotonic hemolysis). In addition to T7 and T6 (all subtasks), ID will mostly organize activities in T1 and T3 and supervise and train less experienced colleagues (AS, DP, MR) in highly demanding sample preparation and imaging protocols of erythrocytes under pathophysiological conditions. ID will be involved in T2, ST 4.3, 4.4, 5.3, 5.4 through planning the measurements/imaging and data discussions.

P2 (SN) has an expertise in setting up and the systems of data acquisition as well as in data/image processing and calculations which will be extensively used in ST 4.1, 4.2, 5.1, and 5.2. In T2, T3, ST 4.3, 4.4, 5.3, 5.4 SN will be in charge for custom solutions (modification of the software according to the current requirements), malfunctioning of the acquisition system mitigation. SN will train less experienced team members in image and data processing.

P3 (AS) will be in charge for material preparation and all measurements/imaging in T2, T3, ST 4.3, 4.4, 5.3, and 5.4 mostly through the sample manipulation on the site. AS will process and prepare the images for dissemination and also coordinate the website and Facebook group updating.

P4 (DP) will be in charge for conducting the TPEF imaging and image processing in T2 and T3. In T2 DP will utilize TPEF setup for photoproduct formation and arbitrary patterns inscription. DP will be trained (by ID) within T1 at IMR. DP will be involved in results dissemination (scientific events) and organization of the project workshop.

P5 (MR) will be in charge, primarily, for the imaging and measurements in ST 4.3, 4.4, 5.3, 5.4 upon proper training given by AK and SN. He will be partially involved in TPEF imaging within T2 and T3. MR will be trained (by ID) within T1 at IMR. MR will prepare material for the website and Facebook group update.

All the team members will examine and discuss the results together preparing them for the manuscripts and writing the manuscripts. Also, all the team members will participate in the science events presenting and disseminating the results of the project. All the team members will participate at the meetings helping in management and decision making, as well as be included in the preparation of the new project proposal.

It is worth noting that the already initiated research on erythrocyte imaging [3] was funded through the ongoing national projects III45016, OI171038 and III 46010 on which the team members are engaged. Activities on HEMMAGINERO will be supported by the aforementioned projects through: work space, available equipment and personnel engagement (person-months) sharing.

Table 3.3. Milestones. Only directly related (sub)tasks are given in the milestones table. T6 and T7.2 assumed to be related the milestones M02-07.

Milestone ID	Milestone name	Task/subtask number	Due month	Means of verification
M01	Project visible	ST6.2	1	Web site launched and Facebook group created
M02	Ethics	T1	2	Ethical committee approval
M03	Hemoglobin photoproduct characterized	T2, T1	10	Measured spectra. Drafted manuscript. Presentation on a conference.
M04	Erythrocytes morphology related to specific pathophysiological	T3, T1	14	TPEF images of healthy and altered morphology erythrocytes (human and animal). Drafted manuscript. Presentation on a