PROMIS project proposal:

Science Fund
of the Republic of Serbia

HEMOGLOBIN-BASED SPECTROSCOPY AND NONLINEAR IMAGING OF ERYTHROCYTES AND THEIR MEMBRANES AS EMERGING DIAGNOSTIC TOOL

HEMMAGINERO | **Institute of Physics Belgrade and Institute for Medical Research** |

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Research field of the Project: Biophotonics (1st), Biomedicine (2nd)

Keywords: multiphoton microscopy; imaging; non-linear, fluorescence correlation spectroscopy; hemoglobin

STATE OF THE ART & MOTIVATION

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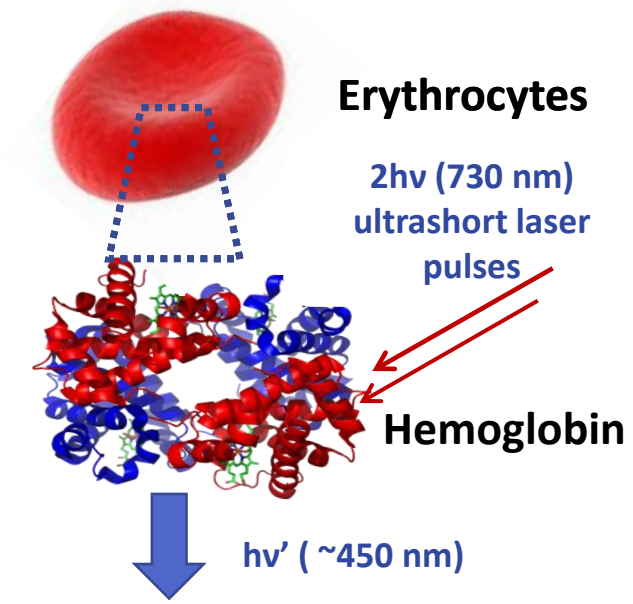
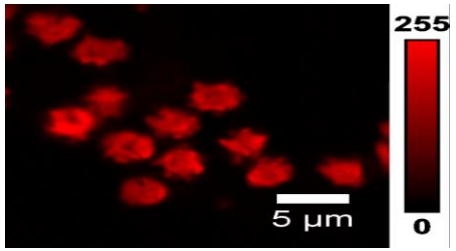


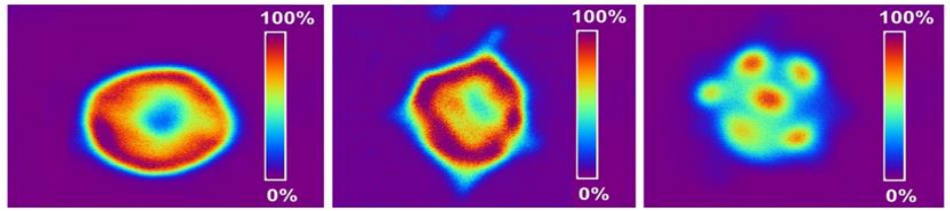


Erythrocytes by TPEF microscopy, our study [1]

Hemoglobin (Hb) becomes a fluorescent molecular target upon two-photon excitation by ultra-short (femtosecond) laser pulses [2]
Two – photon excitation fluorescence (TPEF) microscopy [3,4] enables label- and fixation- free imaging of erythrocytes

- **tracking blood vessels** within the tissues [5]
- **imaging of hemoglobin in mouse retina** [6]
- **imaging of hemoglobin pathologies** (i.e. sickle cell disease) [7]
- **mapping of hemoglobin distribution on single cell level (our result [1])**

changes in erythrocytes morphology follows hemoglobin spatial distribution by TPEF



Our studies [1,9]

erythrocyte morphology is good "biomarker" in progression of modern age disorders [8]

1. Bukara et al. (2017) Journal of Biomedical Optics 22 (2), 026003
2. Zheng et al. (2011) Biomedical optics express 2 (1), 71-79G
3. Larson (2010) Nature Photonics 5 (1), 1
4. Denk et al. (1990) Science 248 (4951), 73-76
5. Garret et al. (2012) Journal of Biophotonics **5** (5-6), 458-468
6. He et al. (2015) Biomedical optics express 6 (3), 1055-1066
7. Vigil and Howard (2015) Biomedical optics express 6 (10), 4098-4104
8. Maulucci et al. (2017) PloS one 12 (9), e0184109
9. Stančić et al. (2017) 13th Multinational Congress on Microscopy

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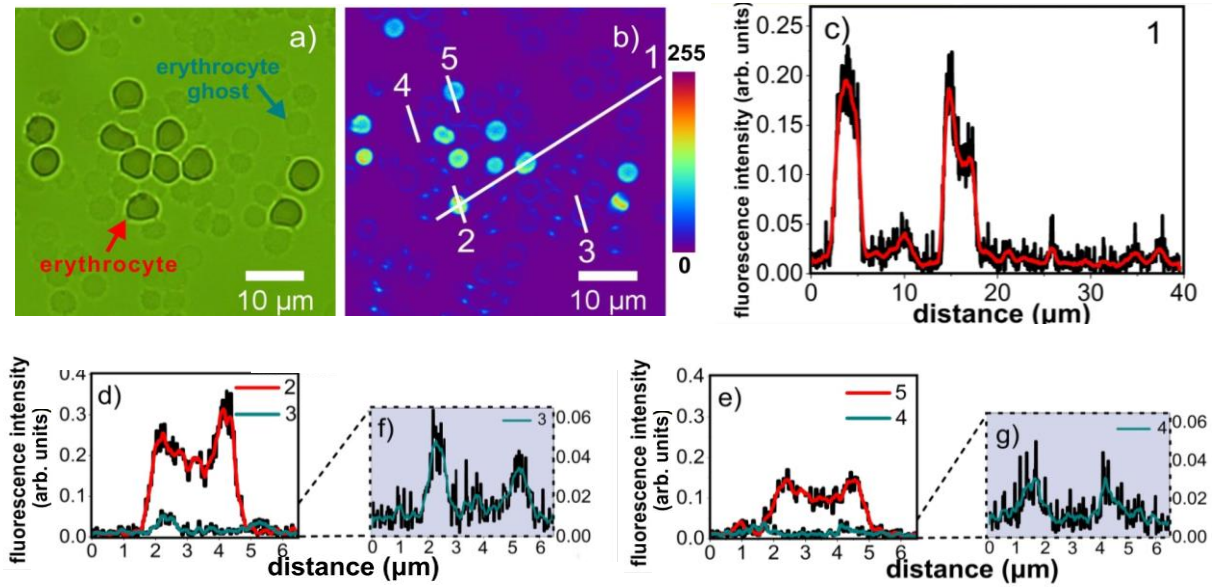

Results from our work [1]

For the first time, we employed an advanced imaging technique, TPEF microscopy, for visualization of residual hemoglobin in erythrocyte membranes (ghosts) [1], which is predictive parameter of oxidative stress [2,3]

Mechanism of interaction of hemoglobin with ultrashort laser pulses and thereby origin of TPEF signal are still not completely understood: the detected signal increases by exposure time [4]

erythrocyte morphology as a result of the complex interaction of lipids and proteins can be additionally quantified using **fluorescence correlation spectroscopy (FCS)** [5]

Label-free modality of nonlinear microscopy **third- (THG) harmonic generation microscopy** allows examination of morphology of stored erythrocyte *in situ* [6]

1. *Bukara et al. (2017) Journal of Biomedical Optics* 22 (2), 026003
2. *Kostić et al. (2014) Colloids and Surfaces B: Biointerfaces 122, 250-259*
3. *Welbourn et al. (2018) Free Radical Biology and Medicine* **103**, 95-106
4. *Shirshin et al. (2018)Laser Physics Letters* **15** (7), 075604
5. *Conrard et al. (2018) Cellular Physiology and Biochemistry* **51** (4), 1544-1565
6. *Saytashev et al. (2016) Biomedical optics express* **7** (9), 3449-3460

OBJECTIVES

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GENERAL OBJECTIVE

Utilization of state of the art optical microscopic techniques and expanding their applicability as a tool for erythrocytes/erythrocyte derivatives imaging under various physiological and pathophysiological conditions

- Better understanding of the underlying biological basis of specific disorders
- development of novel methods for diagnosis
- disease risk stratification, using erythrocytes as evolving sensitive markers.

- ***Particular objective 1*** **(PO1).** **Deep insight into photo-physical/chemical processes in the interaction of the hemoglobin with ultra-short laser pulses *in vitro* and *ex vivo*.**

- ***Particular objective 2*** **(PO2).** **Hemoglobin-based label- and fixation- free TPEF imaging of erythrocytes/erythrocyte membranes under physiological/pathophysiological conditions (diabetes mellitus type 1 and inflammatory bowel disease)**

- ***Particular objective 3*** **(PO3).** **Upgrading of the existing hemoglobin-based non-linear imaging of erythrocytes/erythrocyte membranes by additional modality (THG) and combination with complementary quantitative microscopic methods (FCS)**

  THG upgrade (PO3a) and FCS development (PO3b)

OVERVIEW OF THE METHODOLOGY AND OUTCOMES

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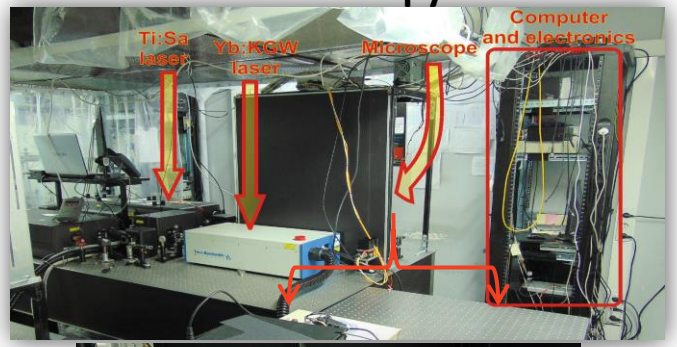
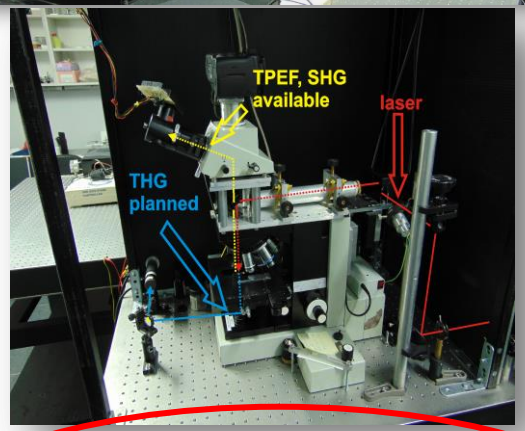
Our, in-house developed, experimental setup for TPEF/SHG microscopy





plus consent for open facilities at Karolinska Institutet, Stockholm (FCS) and and DESY, Hamburg (THz spectroscopy)

Inputs: methods and "know-how"

Outputs: potential applications and significance

- Hemoglobin isolation and sample preparation
- TPEF pattern writing and imaging
- Spectroscopic measurements
- THz measurements

PO1

- Erythrocytes tracing
- Material for optical memories and/or document security
- Biosensing

Hemoglobin photoactivation enables TPEF imaging

- Erythrocytes isolation and preparation
- Examination of morphology, osmotic and mechanical erythrocytes fragility
- TPEF imaging of healthy and/or altered human and animal erythrocytes
- Residual hemoglobin analysis in erythrocyte membranes

PO2

- Diagnostic and disease risk stratification based on hemoglobin distribution and erythrocytes morphology

THG and FCS provide additional info for diagnostics

- Opto-mechanical design and setting up the transmission arm at the existing TPEF setup
- Upgrade of existing software for two channel imaging
- Opto-mechanical design and development of FCS experimental set up

PO3

- FCS measurements of diffusion properties of structural proteins/lipids in erythrocytes membrane;
- THG and FCS setups available to Serbian and broader scientific community

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IMPACT

- Production of the critical scientific data to understand the nature complex processes, such as erythrocytes change of morphology and hemoglobin spatial distribution
- Introduction of cutting-edge microscopic techniques to the essential infrastructure for Serbian scientists
- Protocols for erythrocytes bioimaging and outcomes in terms of emerging diagnostics

DISSEMINATION

Data publication in the open-access journals

→ Contribution to the *Open Science* as one of the goal of EU and Republic of Serbia Strategy plan for Research and Innovation 2016-2020

Scientific seminars and workshop organized

→ Reaching the level of excellence and innovation/research capacities of leading EU research institutes

Brochures
Layman's website
Social media
Newsletters

→ Contribution to the *Open Innovation* (EU strategy for 2016-2020) through dialogue with the general public (stakeholders, medical stuff, patients…)

IMPLEMENTATION

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Task	Related objectives
Sample preparation	PO1, PO2, and PO3
Hemoglobin photoproduct characterization	PO1
TPEF imaging and erythrocytes morphology studies	PO2
THG development	PO3a and PO2
FCS development	PO3b and PO2
Dissemination	PO1, PO2 and PO3
Management	PO1, PO2 and PO3

(supported officially from most of the local biomedical institutions)

The most important deliverables and outcomes:

10 milestones
13 deliverables

- Hemoglobin to ultrashort laser pulses interaction characterized
- Imaging protocols, morphology assessment, potential diagnostic
- Developed and operational THG and FCS setups

COMPETENCE & MULTIDISCIPLINARITY

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Institute of Physics-Belgrade

Institute of Medical Research

www.imi.bg.ac.rs

Team members	Competence
Aleksandar Krmpot (PI), Ph.D. in Physics (2010)	-Experience in TPEF, SHG and THG imaging; collaboration with most relevant biomedical institutions . -Experience in FCS and TPEF microscopy setup development; strong international collaboration and working experience. -Organizational skills (project leading, conference organization)
Ivana Drvenica (P1) , Ph.D. in Biochemical Engineering and Biotechnology (2015); M.Sc. in Pharmacy (2009)	-Experience in: erythrocytes functional status characterization by testing their mechanical and osmotic fragility; development and optimization of process for preserved erythrocyte membranes isolation and their biochemical and morphological characterization -erythrocytes imaging using advanced microscopic techniques
Stanko Nikolić (P2), Ph.D. in Physics (2015)	-experience in the field of functional fluorescence microscopy and optical system design. - Experience in programing and data acquisition systems
Milica Matić (P3), Ph.D. student, Biophotonics (since 2019)	-experience in preparing the erythrocyte and hemoglobin samples. Experience in blood and blood-related diseases.
Danica Pavlović (P4), Ph.D. in Biology (2019)	-experience in field of morphological and photonic characteristics of biological samples (cuticular structures of insects and their possible biomimetic application) -experience in advanced imaging techniques
Mihajlo Radmilović (P5), Ph.D. student, Biophotonics (since 2018)	fundamental knowledge of cell cultures, protein extraction and nonlinear optics and light-biological matter interaction needed for the project implementation.

Already established mutual collaboration (IPB to IMR) through current national projects:
-one joint publication (Bukara et al. 2017)
-number of abstracts at the conferences (Photonica 2015 and 2017, RBC 2016, Multinational Congress on Microscopy (MCM 2017))
and
an invited lecture (RBC 2018).