PRINCIPAL INVESTIGATOR COVER LETTER AND TERMS AGREEMENT

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Re: **Biofuel cells based on microbial cells, oxidoreductases and nanocomposite materials**

I, Principal Investigator (PI) Prof. **Evgeny Katz**, hereby acknowledge that I have submitted a proposal to *2020 U.S.-Ukraine Alternative Energy Research Competition RFP.* I will be collaborating with Prof. **Mykhailo Gonchar** of Institute of Cell Biology, NAS of Ukraine.

If awarded, I undertake this research in good faith and will uphold my portion of the collaborative work as proposed in the submission.

I attest that the information contained in this proposal is truthful and that it has been prepared with the full knowledge and consent of Ms. Shannon Robinson, leadership representative of Clarkson University.

I affirm that I have read and understand CRDF Global's policies and standards outlined within the *2020 U.S.-Ukraine Alternative Energy Research Competition RFP*, including CRDF Global's Plagiarism Policy[1]. I agree to adhere to CRDF Global's Plagiarism Policy, and understand that CRDF Global will not provide funding to an application in which plagiarism exists. If plagiarism is detected, penalties may be imposed up to and including my exclusion from this funding opportunity and barring my participation in future funding opportunities.

February 13, 2020

Principal Investigator Signature: professor Evgeny Katz — Date

February 14, 2020

Institution Leadership Representative Signature: — Date
Shannon Robinson
Associate Vice President for Research & Technology Transfer
/ Executive Director of Institutional Planning

[1] For more information, please see CRDF Global's Plagiarism Policy

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Re: **Biofuel cells based on microbial cells, oxidoreductases and nanocomposite materials**

I, Principal Investigator (PI) Prof. **Mykhailo Gonchar**, hereby acknowledge that I have submitted a proposal to *2020 U.S.-Ukraine Alternative Energy Research Competition RFP.* I will be collaborating with Prof. **Evgeny Katz** from Clarkson University (Potsdam, NY, USA).

If awarded, I undertake this research in good faith and will uphold my portion of the collaborative work as proposed in the submission.

I attest that the information contained in this proposal is truthful and that it has been prepared with the full knowledge and consent of Pof. **Andriy Sibirny**, leadership representative of Institute of Cell Biology, NAS of Ukraine.

I affirm that I have read and understand CRDF Global's policies and standards outlined within the *2020 U.S.-Ukraine Alternative Energy Research Competition RFP*, including CRDF Global's Plagiarism Policy[2]. I agree to adhere to CRDF Global's Plagiarism Policy, and understand that CRDF Global will not provide funding to an application in which plagiarism exists. If plagiarism is detected, penalties may be imposed up to and including my exclusion from this funding opportunity and barring my participation in future funding opportunities.

Head of Department of Analytical Biotechnology,

Prof., Dr, DrSci _____ **Mykhailo Gonchar** 20.02.20

Principal Investigator Signature Date

The Director of Institute of Cell Biology, NAS of Ukraine,

Prof., Dr, DrSci _____ **Andriy Sibirny** 20.02.20.

Institution Leadership Representative Signature Date

ІНСТИТУТ БІОЛОГІЇ КЛІТИНИ НАН УКРАЇНИ 25255758 ЛЬВІВ

[2] For more information, please see CRDF Global's Plagiarism Policy

CRDF GLOBAL COVER SHEET

GENERAL PROJECT INFORMATION				
Project Title (*not to exceed 25 words*)	Biofuel cells based on microbial cells, oxidoreductases and nanocomposite materials			
Amount Requested *(excludes cost-shares)*	Total	U.S. Sub-Team	Ukrainian Sub-Team	
	$72,000	$15,000	$57,000	
Research Categorization[3]	Research Area	Sub-Research Area	Research Focus	
	1. Natural Sciences	1.6 Biological Sciences; 1.4 Chemical Sciences.	1.6.4 Biochemistry and Molecular Biology; 1.4.5 Electrochemistry.	
Research Involves use of Human/Animal Subjects	None	Project Duration	12	

UKRANIAN SUB-TEAM					
INSTITUTION INFORMATION					
Institute Name	Institute of Cell Biology, NAS of Ukraine			Institution Type	Research Institute
Mailing Address	14/16, Drahomanov str.				
	Lviv		79005		Ukraine
PRINCIPAL INVESTIGATOR INFORMATION					
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Highest Degree	DrSci		Biochemistry		2001
Residency Status	Legal Citizen				
INSTITUTION LEADERSHIP REPRESENTATIVE INFORMATION					
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E-mail	sibirny@cellbiol.lviv.ua		Telephone #	+380322612108	
Total number of Ukrainian sub-team members, including Ukrainian PI, graduate students, secondary collaborators					8

[3] Please reference the CRDF Global Research Areas document found here: http://www.crdfglobal.org/sites/default/files/crdf-global-research-areas_updated-june-2015.pdf

U.S. SUB-TEAM					
INSTITUTION INFORMATION					
Institute Name	Clarkson University			Institution Type	University/Academic
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	Potsdam	NY	13699		
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Highest Degree	PhD		Electrochemistry		1983
U.S. Residency Status	Legal Citizen		Gender	Male	
INSTITUTION LEADERSHIP REPRESENTATIVE INFORMATION					
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Have you received a grant under a previous CRDF Global program or been a participant in a CRDF Global-funded workshop?				No	
If yes, please list program and grant number or workshop title in the following text box			N/A		
Total number of U.S. team members, including PI, students, and secondary collaborators				3	

[4] If you do not know your congressional district, please click on this link and search for your representative using your Institution's zip code.

PROJECT ABSTRACT

Should not exceed 350 words

Biofuel cells (BFCs) are the novel bioelectrochemical devices that use living microorganisms or their enzymes to generate electricity via enzymatic oxidation of organic substrates, including industrial waste products. The aim of the project is a development of new microbial, enzymatic and combined BFSs based on cells of mutant or recombinant strains of the yeasts and the selected fungi (overproducers of oxidoreductases) as well as purified enzymes from these sources, immobilized onto electroconductive nanocomposite materials. As substrates of enzymatic reactions and primary energy sources, there will be used low-cost and often toxic technogenic by-products for which there is a problem of their utilization, in particular: methanol and formaldehyde – for the mutant producers of alcohol oxidase (AOX); methylamine – for the recombinant overproducer of methylamine oxidase (MAOX), L-lactate – for the recombinant producer of flavocytochrome b_2 (FC b_2) and phenol derivates – for the selected fungal producers of laccase.

The scientific and technical objectives of the project are defined as follows: 1) cultivation of microbial and fungal cells; isolation and purification of oxidoreductases; 2) synthesis and structural-morphological characterization of nanocomposite materials; 3) development of laboratory prototypes of microbial BFCs and their functional characterization; 4) construction and characterization of laboratory prototypes of enzymatic BFCs based on purified oxidoreductases and nanocomposites.

The novelty and innovative power of the project are based on idea of combining the mutant/recombinant microbial or fungal cells and their enzymes with the mediator systems (synthetic dyes, ferrocene, cyanoferrate derivatives, etc.) coupled with nanocomposite materials. The obtained nanocomposites will be used for various aims: as electron transfer mediators, as matrices for immobilization of biocatalysts and as nanozymes (such as artificial peroxidases) for biocathode construction.

This project is based on collaboration between scientists of the Department of Analytical Biotechnology of the Institute of Cell Biology, NAS of Ukraine and the Department of Chemistry and Biomolecular Science of Clarkson University in Potsdam (NY,USA). Scientific partners (UA and US) are carrying out innovative scientific research in the complementary fields. Both teams are high-level professionals in various fields, including microbiology, enzymology, nanotechnology, biotechnology, analytical chemistry, and bioelectrochemistry.

PROJECT NARRATIVE

Should not exceed ten (10) pages. Text should be Arial font size 10 within 1-inch margins

The search for new renewable energy technologies and approaches for the utilization of technogenic by-products (often toxic) are the main problems of modern science. In this regard, the development of biofuel cells (BFCs) based on microbial cells or enzymes capable of utilizing the by-products as substrates with the simultaneous generation of electricity can help to solve these two actual problems – cheap renewable energetics and environmental protection [Beretta *et al*., 2019; Das *et al*., 2014].

The aim of the project is a development of new microbial, enzymatic and combined BFSs based on cells of mutant or recombinant strains of yeasts and the selected fungi (overproducers of oxidoreductases) and purified enzymes from these sources, immobilized onto electroconductive nanocomposite materials. As substrates of enzymatic reactions and a primary energy sources, it is planned to use low-cost and often toxic technogenic by-products for which there is a problem of their utilization, in particular: methanol and formaldehyde – for the mutant producers of alcohol oxidase (AOX); methylamine – for the recombinant overproducer of methylamine oxidase (MAOX), L-lactate – for the recombinant producer of flavocytochrome b_2 (FC b_2) and phenol derivates – for the selected fungal producers of laccase. All the proposed mutant, recombinant and classically selected producers of the corresponding oxidoreductases were constructed/selected at the Department of Analytical Biotechnology, Institute of Cell Biology, NAS of Ukraine. The mentioned above enzymes have useful biotechnological characteristics (high thermostability, high affinity for target substrates) but are currently commercially unavailable.

Recent progress in nanobiotechnology [Katz (Ed.) 2014] opens the possibility of creating biocatalytic elements based on nanoparticles, which have unique properties not inherent to macroparticles. Nanosized materials have a high sorption capacity, ability of self-assembly and, in some cases, unique catalytic properties. Using nanoparticles in fuel cell technology is extremely actual [Zhao *et al*., 2017; Li *et al*., 2019; Kwon *et al*., 2018; Hasan *et al*., 2018; Holade *et al*., 2015], especially in combination with natural or recombinant biocatalysts (enzymes) created on the base of gene engineering technology.

The novelty and innovative power of the project are based on idea of combining the mutant/recombinant microbial or fungal cells and their enzymes with the new mediator systems (based on synthetic dyes, ferrocene, cyanoferrate derivatives, etc.), and nanocomposite materials coupled with mediator systems. The obtained nanocomposites will be used for various aims: as electron transfer mediators, as matrices for immobilization of biocatalysts and as nanozymes (such as artificial peroxidases) for biocathode construction.

The aim of the proposed scientific project is a combination of fundamental science with applied biotechnology using advanced interdisciplinary approaches.

The scientific and technical objectives of the project are defined as follows:

1. Cultivation of microbial and fungal cells; isolation and purification of oxidoreductases – promising biocatalysts for the construction of biofuel cells (BFCs);
2. Synthesis and structural-morphological characterization of nanocomposite materials;
3. Development of laboratory prototypes of microbial BFCs and study of their functional characteristics;
4. Construction of laboratory prototypes of enzymatic BFCs based on purified oxidoreductases and nanocomposites, and studying their operational parameters.

This project is based on collaboration between scientists of the Department of Analytical Biotechnology of the Institute of Cell Biology, NAS of Ukraine and the Department of Chemistry and Biomolecular Science of Clarkson University in Potsdam (NY,USA). Scientific partners (UA and US) are carrying out innovative scientific research in the complementary fields. Both teams are high-level professionals in various fields, including microbiology, enzymology, nanotechnology, biotechnology, analytical chemistry, and bioelectrochemistry.

Biofuel cells (BFCs) are the novel bioelectrochemical devices [Gamella *et. al*., 2018] that use living microorganisms or their enzymes to generate electricity through enzymatic oxidation of organic substrates. This innovative technology has a number of potential applications: generation of renewable energy, production of bio-hydrogen, wastewater treatment, water desalination, etc. [Logan *et al*., 2019]. The BFCs principle is based on the catalytic oxidation of organic substrates by a biocatalyst, which produces the electrons that are transferred to the anode, generating an electric current. A decisive influence on the BFCs performance is the efficiency of electron transfer from the substrate (fuel) through biocatalysts to the electrode surface (anode and cathode). There are two main types of BFCs – microbial and enzymatic. Microbial BFCs are characterized by high stability (up to five years); they are cheaper than enzymatic BFCs and capable of simultaneous utilization of different substrates, but their widespread use is limited by a low current density caused by a slow electron transport through cell membranes to the anode [Gunawardena & Fernando, 2008; Sayed & Abdelkareem, 2017]. The enzymatic BFCs typically have a much higher current density, but capable

only of partial oxidation of the fuel and have a limited shelf life (7-10 days) due to a low stability of the enzymes [Bullen *et al*., 2006].

The main aim of the current project is a construction of new microbial, enzymatic and combined BFCs on the basis of cells of recombinant, mutant strains of yeast and selected fungi – overproducers of oxidoreductases, as well as using the purified enzymes from these sources, immobilized onto electroconductive nanocomposites. Low-cost and often toxic technogenical by-products, for which utilization is actually a problem, will be used as substrates of enzymatic reactions (energy sources). Therefore, the project intends to combine two scientific goals – cheap renewable energetics and environmental protection.

The new BFCs on the basis of mutant cells of thermotolerant methylotrophic yeast *Ogataea polymorpha* – producer of alcohol oxidase (AOX) and purified enzyme preparations of AOX will be used for generation of electrical energy due to oxidation of methanol or/and formaldehyde – the most toxic technogenic pollutants of wood, paint and the other industries. A peculiarity of cells of the mutant yeast strain of *O. polymorpha* C-105 (*gcr1 catX*) is an impairemet of glucose catabolite repression and catalase deficiency [Gonchar *et al*., 1998], as well as the thermostability of the enzymes (including AOX). AOX is a FAD-containing octameric protein with a wide substrate specificity (oxidation of primary alcohols and formaldehyde), which uses oxygen as an electron acceptor. The oxidation of methanol catalyzed by AOX and electrochemical current generation looks as follows:

1) CH_3OH + **AOX-**FAD → **AOX-**$FADH_2$ + CH_2O (enzymatic reaction);
2) **AOX-** $FADH_2$ + 2 $mediator_{ox}$ → **AOX-**FAD + 2 $mediator_{red}$ + $2H^+$ (mediator reaction);
3) 2 $mediator_{red}$ → 2 $mediator_{ox}$ + 2 e^- (electrochemical reaction).

In general, this enzyme-electrochemical scheme is typical for the other BFCs based on oxidases with a difference in the nature of their cofactor, substrate, enzymatic product or mediator type.

For BFCs based on cells of a recombinant strain of *Saccharomyces cerevisiae* C13ABYS86 or purified preparation of recombinant $(His)_6$-tagged methylamine oxidase (MAOX) [Stasyuk *et al*., 2014], as a fuel will be used methylamine – a harmful by-product of chemical and pharmaceutical technologies.

On the basis of the cells of a recombinant strain *O. polymorpha* tr1 (*gcr1 catX CYB2*) [Dmitruk *et al*., 2008] or purified recombinant flavocytochrome b_2 (FC b_2), we are going to construct BFCs which will be used as a fuel L-lactate – cheap biotechnological product. FC b_2 is heme- and FMN-containing mitochondrial protein capable of unspecific electron transfer to a number of inorganic acceptors (phenazine methosulfate, potassium ferricyanide, ferrocene, osmium complexes, Methylene Blue, etc.) [Smutok *et al*., 2005]. The oxidation of L-lactate catalyzed by FC b_2 and electrochemical current generation can be illustrated as follows:

1) L-lactate + **FC*b*$_2$**$^{(ox)}$ → **FC*b*$_2$**$^{(red)}$ + pyruvate (enzymatic reaction);
2) **FC*b*$_2$**$^{(red)}$ + 2 $mediator_{ox}$ → **FC*b*$_2$**$^{(ox)}$ + 2 $mediator_{red}$ (mediator reaction);
3) 2 $mediator_{red}$ → 2 $mediator_{ox}$ + 2 e^- (electrochemical reaction).

Despite the high prospect of using FC b_2 in biofuel cells, today there is virtually no information related to this aspect. Only one research has been demonstrated that *H. anomala* yeast cells containing redox enzymes (including FC b_2) are capable of mediating electron transfer to the electrode surface by generating the current [Prasad *et al*., 2007]. However, the efficiency of electron transfer between yeast cells and the electrode was poor. The low efficiency of microbial BFC, based on *H. anomala* cells, can be explained by a low content of redox enzymes and, probably, by their low stability, related with non-thermotolerance of the used yeast species. The enzyme from thermotolerant yeast *O. polymorpha* is characterized by increased thermal and chemical stability. Important peculiarity of FC b_2 for the BFC technology is a low redox potential, which ranges from -81 mV to -28 mV [Cenas *et al*., 2007; Capeillere-blandin *et al*., 1986; Christgen *et al*., 2019]. In addition to the gene-engineered enrichment of the yeast cells eight-fold overexpression of the corresponding *HpCYB2* gene, we used a nanotechnological approach – additional enrichment of the cells by the enzyme immobilized onto gold nanoparticles. We have shown that the combination of both approaches for enrichment of yeast cells with the target enzyme significantly increases the local concentration of the enzyme inside the cells and, respectively, improves the efficiency of the bioelement by increasing the current of microbial amperometric biosensor [Karkovska *et al*., 2015]. We will use these approaches also for designing the new BFCs.

For BFCs based on the cells of selected fungi (laccase producers) or purified preparations of this enzyme, phenol derivatives (a toxic waste product from the chemical and pharmacological industries) will be used as a fuel source. Laccase is a copper-containing enzyme with a wide range of substrate specificity and can use oxygen as an electron acceptor releasing water as a by-product. Laccase is widely used for biofuel cell technology [Mano *et al*., 2018; Ghosh *et al*., 2019]. This enzyme has a high redox potential (from 400 mV to 800 mV), making it a promising cathode biocatalyst [Tominaga *et al*., 2014]. The ability of laccase and FC b_2 for direct electron transfer to electrode modified with gold nanoparticles [Kavetskyy *et al*., 2019; Smutok *et al*., 2017], is the basis for the design of mediator-free enzymatic BFCs.

In the current project, electroconductive nanomaterials based on noble and transition metals or their hybrid forms [Holade *et al*., 2014], as well as carbon materials (graphene derivatives [Koushanpour *et al*., 2016] and carbonated cellulose fibers) will be used for increasing the current density at the electrode. To

improve the efficiency of electron transfer in the BFCs, we plan to use nanocomposites – synthesized nanomaterials, additionaly modified by inorganic and organic electron-transfer mediators (synthetic dyes, cyanoferrate derivatives, etc.). For better permeability of the mediator through the cell wall, the permeabilization of the cells will be used. Carbon-based materials, modified with noble and transition metals (electrodeposited Pt, Pt nanoparticles, hybrid metal nanoparticles), including peroxidase-like nanozymes, are planned to be used to improve the BFCs performance. The main operational parameters (current and power density, maximum operating time, etc.) for the constructed laboratory prototypes of microbial and enzymatic BFCs will be investigated.

The original idea of usage of the catalase-deficient mutant cells of the yeast *O. polymorpha* C-105 as a cathode biocatalyst in microbial BFCs will be tested. The hydrogen peroxide secreted outside the cells could be reduced at the cathode. This allows replacing the catalase heat-producing reaction of H_2O_2 decomposition by a useful, current-generating process of hydrogen peroxide reduction at the cathode. As a catalyst for the reduction of H_2O_2 at the cathode, nanocomposites that exhibited nanozyme properties (artificial peroxidases) will be used.

The realization of the current project will allow combining original ideas, the novel technological approaches and experimental skills of the UA-team (specialists in the field of microbiology, enzymology, analytical biotechnology, nanotechnology) and US-group (specialists in the field of electrochemistry, analytical chemistry, nanochemistry). The unique properties of mutant, recombinant and selected yeast cells and fungi as well as of bionanocomposites will allow the construction of new microbial and enzymatic BFCs with improved characteristics. The expected results of the project are beyond the current level of technology and therefore are innovative.

Milestones

Task 1. ***Cultivation of microbial and fungal cells; isolation and purification of oxidoreductases – promising biocatalysts for the construction of biofuel cells (BFCs)***

1.1 Screening of molds and mushroom fungi by their ability to overproduce extracellular laccase in liquid cultures.

Screening of some mold fungi and more than 25 strains of the mushrooms belonging to the genera *Trametes* and *Phallus*, available in the collection of the Institute, for their synthesis of extracellular laccase in liquid cultures *in vitro* will be performed. The best enzyme's overproducers will be selected for further investigation.

1.2 Cultivation of mutant and recombinant yeast strains and selected fungi, overproducing the target oxidoreductases.

The optimal conditions for the cultivation of the producers will be determined, ensuring the maximum yield of the target enzymes (composition of the culture medium, intensity of aeration, temperature, time of cultivation, *etc.*). To maintain the highest activity of the enzymes inside the cells, the biomass of the organisms will be frozen or lyophilized under optimal conditions.

1.3 Isolation, purification, enzymatic and electrochemical characterization of the enzymes: alcohol oxidase (AO), methylamine oxidase (MAO), flavocytochrome b_2 (FC b_2) and laccase.

Enzymes, isolated from the mutant, recombinant yeast cells and the culture medium of the selected fungi will be concentrated and/or purified using ion exchange and/or affinity chromatography. Purified preparations of AO, MAO, FC b_2 and laccase will be investigated using different approaches: electrophoretic (to test their purity), spectrophotometric (to determine kinetic parameters – K_M and k_{cat}) and electrochemical (activity toward electron-transfer mediators, determination of redox potential).

Task 2. ***Synthesis and structural-morphological characterization of nanocomposite materials***

2.1. Synthesis of nanomaterials based on noble and transition metal nanoparticles by chemical reduction.

The nanosized materials of noble and transition metals or their hybrid forms (which will consist of 2-3 different noble or transition metals) will be obtained using chemical reduction from the corresponding ions. Citrate, ascorbate or sodium borohydride will be used as reducing agents in optimal ratios of reactants.

2.2. Carbon-based nanomaterial screening.

The different carbon materials (graphene derivatives and carbonized delignocellulose fibers and their derivatives) will be analyzed by cyclic voltammetry for testing their electrical conductivity.

2.3. Synthesis of nanocomposite materials with high efficiency of electron transfer from biocatalyst to electrode and characterization of their electrono-mediator activity.

To improve communication between the biocatalyst and the electrode, screening and additional modification of the synthesized nanomaterials by inorganic electron transfer mediators (synthetic dyes, electrically conductive films, ferrocene, cyanoferrate derivatives, etc.) will be carried out. The electrochemical characteristics of the obtained nanocomposite materials and their compatibility with

BFC biocomponents will be investigated. For enzymatic BFCs, based on FC b_2 and laccase, optimization of the methods for direct mediator-less transfer of electrons from reduced oxidoreductases to electrodes via the nanocomposites will be carried out.

2.4. Screening and synthesis of new nanocomposite materials possessing nanozyme (nanoperoxidase) activity.

In order to create the novel BFC cathodes, the screening and synthesis of new nanomaterials with nanzyme (peroxidase) activity will be performed. The best selected nanozymes will be tested for their ability to reduce hydrogen peroxide at the electrode.

2.5. Structural and morphological characterization of the obtained nanomaterials.

The structure and morphology of new nanomaterials will be investigated by scanning electron microscopy (SEM), atomic force microscopy (AFM), energy dispersive X-ray (EDRS) spectroscopy, atomic absorption spectroscopy (AAS), and dynamic light scattering approach (DLS).

Task 3. ***Design of laboratory prototypes of microbial BFCs and study of their functional characteristics***

3.1. Design of BFC laboratory models based on mutant, recombinant yeast and selected fungal cells producing oxidoreductases.

The anode of microbial BFC will be modified with selected nanocomposite materials with the best electron-mediator activity. Carbon electrodes modified with different types of Pt (electrodeposited Pt, Pt nanoparticles, platinum black) are planned to be used as the cathode material. The electrodes with immobilized laccase or nanozymes will also be used as cathodes. Selection of the optimum composition of the anolyte (fuel substrate, buffer, cell concentration, *etc.*) will be performed. To provide more efficient communication between cellular redox systems and the electrode surface, yeast and fungal cells will be used in a permeabilized state. The optimal type of separator membrane will be selected and the possibility of using single chamber BFC will be studied.

3.2. Investigation of the functional characteristics of microbial BFCs.

The functional characteristics of engineered microbial BFCs will be evaluated by the use of inexpensive organic substrates and some by-products, for which there is a problem of remediation: methanol, formaldehyde – for the AO-overproducing mutant yeast strain; methylamine – for the recombinant yeast producer of MAO, L-lactate – for the recombinant producer of FC $b_2$and phenolic compounds– for the fungal producers of laccase. The enzymatic kits, developed in the laboratory, will be used to study the kinetics and utilization level of the relevant substrates in the BFC system. The possible advantages of the constructed laboratory models of new microbial BFCs based on nanocomposites and enzyme-producing cells will be investigated (high current and power density, long operating time).

When creating microbial BFCs, the original idea of using catalase-free mutant cells of the yeast *O. polymorpha* C-105 as a cathode biomaterial will be tested; in this case, secreted by the yeast cells hydrogen peroxide will be reduced at the cathode. This allows to replace the catalase-mediated heat-producing reaction of H_2O_2 decomposition by a useful, current-generating process of cathodic reduction of hydrogen peroxide. As a catalyst for the reduction of H_2O_2 at the cathode, nanocomposites will be used that exhibit the nanozyme (artificial peroxidase) activity.

Task 4. ***Design of laboratory prototypes of enzyme BFCs on the basis of purified oxidoreductases and nanocomposites and study of their operational parameters***

4.1. Investigation of the possibility of using selected nanomaterials as an immobilization matrix for covalent or physical binding of biocatalysts on the electrode surface.

In order to ensure a high local concentration of the BFC biocatalyst and to increase its stability, screening for covalent and physical immobilization of the purified preparations of AO, MAO, FC b_2 and laccase on the electrode surface will be carried out. The nanosized carriers will be functionalized by carboxyl and amino groups using appropriate reagents (ω-mercaptohexadecanoic acid, cysteamine, hydrazine or carbodiimide– depending on chemical nature of nanomaterial) followed by covalent immobilization of the corresponding enzymes.

4.2. Screening of fixing polymer membranes of different nature.

Screening of various polymeric membranes (electrodeposited, photopolymeric, dialysis) will be carried out for their ability to reliably retain enzymes on the surface of the electrodes, a good electrical conductivity and a high permeability for enzymes' substrates.

4.3. Investigation of the basic operational parameters of enzyme-based BFCs.

The basic operational parameters will be investigated for the designed laboratory prototypes of enzymatic BFCs, based on AO, MAO, FC b_2 and laccase: current density, power density and maximum operating time. The most effective BFC variants will be selected and compared with existing enzyme-based analogues.

4.3. Development of hybrid cell-enzyme BFCs.

The possibility of creating hybrid cell-enzyme-based BFCs will be evaluated. In such novel devices, as the anode material will be used immobilized oxidoreductase; hydrogen peroxide-generating yeast cells will be placed in the biocathode compartment, and nanozyme (artificial peroxidase) will be used as a cathode to catalyze the reduction of hydrogen peroxide.

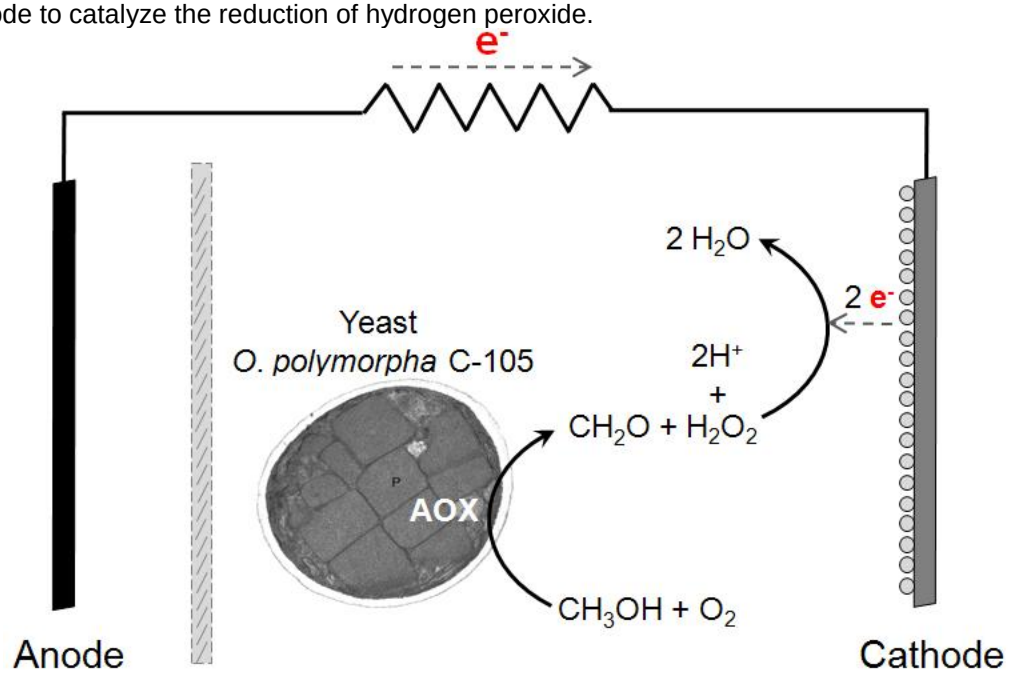

Fig. The scheme illustrating the cathode process of reduction of H_2O_2, produced by mutant yeast cells.

Ukrainian and USA partners (UA and US) have all the necessary specialized equipment to carry out the planned research under the current project.

UA has the material and hardware support for the cultivation of the yeast and fungal cells (culture media, sterile boxes, autoclaves, thermostats, thermostatic shakers, disintegrators, centrifuges "Sorvall", "Eppendorf", lyophilizer "Christ alpha 1-2 LDplus"; the equipment for isolation, purification of enzymes and study of their physico-chemical and kinetic characteristics(chromatographic columns, chromatographic carriers, apparatus for vertical electrophoresis of proteins VE-2M Helicon, peristaltic pumps, fraction collectors, refrigerators, spectrophotometers "SHIMADZU UV-1650", fluorimeter "Quantech filter"); equipment for synthesis and characterization of micro- and nanoparticles (atomic force microscope "Solver P47-PRO (NT-MDT)", scanning electron microscope "SEM-microanalyser REMMA-102-02", transmission electron microscope "REM-100", fluorescence microscope "Axio Lab. A1 ", optical microscopes); apparatus for conducting electrochemical studies (commercial electrodes of different configuration and different surface area values, electrochemical cells, potentiostats "CHI 1200A" and "PGstat16"; enzymatic kits for methanol, formaldehyde, methylamine, L-lactate and phenols assay to evaluate the fuel substrate consumption in BFCs.

US has a high-tech modern equipment for the design and operation of BFCs: Electrochemical equipment: Potentiostat/Galvanostat (Autolab, ECO Chemie) PGSTAT12 (two sets) – with additional modules (FRA2 impedance measurements module; BIPOT bipotentiostat module; ADC750 fast sampling module for fast chronoamperometric measurements; SCAN-GEN fast potential scanning module for ultra-fast cyclic voltammetry; ECD module for low current measurements on nanoelectrodes; FI20 filter and integrator module for chronocoulometric measurements; Rotating disk-ring electrode setup); Surface plasmon resonance instrument (Autolab, ECO Chemie) SPRINGLE connected with the potentiostat/galvanostat for in situ electrochemical-SPR measurements; Quartz crystal microbalance (QCM) (Autolab, ECO Chemie) connected with the potentiostat/galvanostat for in situ electrochemical-QCM measurements; Optical Contact Angle and Surface Tension Meter (KSV Instrument Ltd) Modular CAM 200 connected with the potentiostat/galvanostat for in situ electrochemical-contact angle measurements; Orion 3-Star pH Meter; Keithley 236 Source-Measure Unit; ELISA instrument; SpectraMax i3x Microplate Reader (Molecular Devices, LLC., CA, USA). Optical / biochemical equipment: Shimadzu Spectrophotometer UV-2401PC; Barnstead Nanopure Diamond Lab Water System; Refrigerated Universal Centrifuge (Labnet) Z300K; Clinical and Educational Incubator (Barnstead); Excellence XS Analytical Balance (Mettler). Available shared equipment (University facilities): AFM, STM, SAXS and WAXS techniques, HPC, SLS, DLS, particle size analyzers, high resolution SEM, TEM, NMR, Perkin Elmer LS 50B Fluorescence spectrometer, and mass spectrometer for proteomic analysis, Leica TCS SP5 II Laser Scanning Confocal Microscope, dynamic light scattering instrument (Malvern Zetasizer Nano), etc.

The list of project authors/participants:

US-Team:

Prof. Evgeny Katz;
PhD Paolo Bollella;
PhD Student Madeline Masi.

UA-Team:

Prof. Mykhailo Gonchar;
DrSci Oleh Smutok;
PhD Galina Gayda;
PhD Andriy Zakalskiy;
PhD Tetyana Prokopiv;
PhD Nataliya Stasyuk;
PhD Olha Demkiv;
MS Student Bohdan Pshoniuk.

REFERENCES CITED

This section must only include bibliographic citations and not be used to provide parenthetical information outside of the Project Narrative

1. Beretta G., Daghio M., Espinoza Tofalos A. et al. (2019) Progress Towards Bioelectrochemical Remediation of Hexavalent Chromium. *Water*, *11*, 2336. https://doi.org/10.3390/w11112336
2. Bullen R.A., Arnot T.C., Lakeman J.B., Walsh F.C. (2006) Biofuel cells and their development. *Biosens. & Bioelectron., 21*, 2015–2045. https://doi.org/10.1016/j.bios.2006.01.030
3. Capeillere-blandin C., Barber M., Bray R. (1986) Comparison of the processes involved in reduction by the substrate for two homologous flavocytochromes b_2 from different species of yeast. *Biochem. J.*, *238*, 745-756. DOI:10.1042/bj2380745
4. Cenas N., Le K.H., Terrier M., Lederer F. (2007) Potentiometric and further kinetic characterization of the flavin-binding domain of Saccharomyces cerevisiae flavocytochrome b_2. Inhibition by anions binding in the active site. *Biochemistry*, *46*, 4661–4670. DOI:10.1021/bi602634y
5. Christgen S.L., Becker S.M., Becker D.F. (2019) Methods for determining the reduction potentials of flavin enzymes. *Methods in Enzymology*, *620*, 1-25 https://doi.org/10.1016/bs.mie.2019.03.004
6. Das M., Barbora L., Das P., Goswami P. (2014) Biofuel cell for generating power from methanol substrate using alcohol oxidase bioanode and air-breathed laccase biocathode. *Biosens. & Bioelectron.*, *59*, 184-91. https://doi.org/10.1016/j.bios.2014.03.016
7. Dmitruk K.V., Smutok O.V., Gonchar M.V., Sibirnyĭ A.A. (2008) Construction of flavocytochrome b_2-overproducing strains of the thermotolerant methylotrophic yeast *Hansenula polymorpha* (*Pichia angusta*). *Microbiology* (Moscow), *77*, 213-218. https://doi.org/10.1134/S0026261708020100
8. Gamella M., Koushanpour A., Katz E. (2018) Biofuel Cells – Activation of micro- and macro-electronic devices. *Bioelectrochemistry, 119*, 33-42. https://doi.org/10.1016/j.bioelechem.2017.09.002
9. Ghosh B., Saha R., Bhattacharya D., Mukhopadhyay M. (2019) Laccase and its source of sustainability in an enzymatic biofuel cell. *Bioresource Technology Reports*, *6*, 268-278. https://doi.org/10.1016/j.biteb.2019.03.013
10. Gonchar M.V., Maidan M.M., Moroz, O.M., Woodward J.R., Sibirny A.A. (1998) Microbial O_2 - and H_2O_2-electrode sensors for alcohol assays based on the use of permeabilized mutant yeast cells as the sensitive bioelements. *Biosens. & Bioelectron.*, *13*, 945–952. https://doi.org/10.1016/S0956-5663(98)00034-7
11. Gunawardena A., Fernando S., To F. (2008) Performance of a Yeast-mediated Biological Fuel Cell. *Int J Mol Sci*., *9*, 1893–1907. https://doi.org/10.3390/ijms9101893
12. Hasan M.Q., Kuis R., Narayanan J.S., Slaughter G. (2018) Fabrication of highly effective hybrid biofuel cell based on integral colloidal platinum and bilirubin oxidase on gold support. *Sci Rep*., *8*, Article ID 16351. DOI:10.1038/s41598-018-34740-w
13. Holade Y., MacVittie K., Conlon T., Guz N., Servat K., Napporn T.W., Kokoh K.B., Katz E. (2014) Pacemaker activated by an abiotic biofuel cell operated in human serum solution. *Electroanalysis, 26*, 2445-2457. https://doi.org/10.1002/elan.201400440
14. Holade Y., MacVittie K., Conlon T., Guz N., Servat K., Napporn T.W., Kokoh K.B., Katz E. (2015) Wireless information transmission system powered by an abiotic biofuel cell implanted in an orange. *Electroanalysis, 27*, 276-280. https://doi.org/10.1002/elan.201400653
15. Karkovska M., Smutok O., Stasyuk N., Gonchar M. (2015) L-lactate-selective microbial sensor based on flavocytochrome b_2-enriched yeast cells using recombinant and nanotechnology approaches. *Talanta*, *144*, 1195-1200. DOI:10.1016/j.talanta.2015.07.081
16. Katz E. (Ed.). (2014) *Implantable Bioelectronics - Devices, Materials and Applications*, Wiley-VCH, Weinheim, Germany, ISBN: 978-3-527-33525-1 (book)
17. Kavetskyy T., Stasyuk N., Smutok O. et al. (2019) Improvement of amperometric laccase biosensor using enzyme-immobilized gold nanoparticles coupling with ureasil polymer as a host matrix. *Gold Bulletin*, *52*, 79-85. https://doi.org/10.1007/s13404-019-00255-z
18. Koushanpour A., Guz N., Gamella M., Katz E. (2016) Graphene-functionalized 3D-carbon fiber electrodes – Preparation and electrochemical characterization. *Electroanalysis, 28*, 1943-1946. https://doi.org/10.1002/elan.201600110
19. Kwon CH.,Ko Yo., Shin D. et al. (2018) High-power hybrid biofuel cells using layer-by-layer assembled glucose oxidase-coated metallic cotton fibers. *Nature Commun.*, *9*, Article ID 4479. https://doi.org/10.1038/s41467-018-06994-5
20. Li Y., Xiong W., Zhang C., Yang X. (2019) Research on Flexible Thin-Disk Glucose Biofuel Cells Based on Single-Walled Carbon Nanotube Electrodes. *Journal of Nanomaterials*, *2019*, Article ID 1609579, 7 p. https://doi.org/10.1155/2019/1609579
21. Logan B.E., Rossi R., Ragab A., Saikaly P.E. (2019) Electroactive microorganisms in bioelectrochemical systems. *Nature Reviews Microbiology*, *17*, 307-319. https://doi.org/10.1038/s41579-019-0173-x

22. Mano N., de Poulpiquet A. (2018) O_2 Reduction in Enzymatic Biofuel Cells. *Chemical Reviews*, *118*, 2392-2468. https://doi.org/10.1021/acs.chemrev.7b00220
23. Prasad D., Arun S., Murugesan M., Padmanaban S. et al. (2007) Direct electron transfer with yeast cells and construction of a mediatorless microbial fuel cell. *Biosens. & Bioelectron.*, *22*, 2604-10. https://doi.org/10.1016/j.bios.2006.10.028
24. Sayed E.T., Abdelkareem M.A. (2017) Yeast as a Biocatalyst in Microbial Fuel Cell. In book: Old Yeasts - New Questions, Candida Lucas and Celia Pais, IntechOpen, http://dx.doi.org/10.5772/intechopen.70402
25. Smutok O., Gayda G., Gonchar M., Schuhmann W. (2005) A novel L-lactate-selective biosensor based on flavocytochrome b_2 from methylotrophic yeast Hansenula polymorpha. *Biosens. & Bioelectron.*, *20*, 1285-1290. https://doi.org/10.1016/j.bios.2004.04.020
26. Smutok O., Karkovska M., Serkiz Ya., Vus B., Čenas N., Gonchar M. (2017) Development of a new mediatorless biosensor based on flavocytochrome b_2 immobilized onto gold nanolayer for non-invasive *L*-lactate analysis of human liquids. *Sensor & Actuators B*, *250*, 469-475. https://doi.org/10.1016/j.snb.2017.04.192
27. Stasyuk N., Smutok O., Zakalskiy A., Zakalska O., Gonchar M. (2014) Methylamine-sensitive amperometric biosensor based on $(His)_6$-tagged *Hansenula polymorpha* methylamine oxidase immobilized on the gold nanoparticles. *BioMed Research International*, *2014*, Article ID 480498, 8 pp. http://dx.doi.org/10.1155/2014/480498
28. Tominaga M., Togami M., Tsushida M., Kawai D. (2014) Effect of N-Doping of singlewalled carbon nanotubes on bioelectrolysis of laccase. *Anal. Chem.*, *86*, 5053–5060. https://doi.org/10.1021/ac500700h.
29. Zhao C.E., Gai P., Song R., Chen Y., Zhang J., Zhu J.J. (2017) Nanostructured material-based biofuel cells: recent advances and future prospects. *Chem Soc Rev., 46,* 1545-1564. DOI:10.1039/c6cs00044d

STUDENT/POST-DOCTORAL RESEARCHER MENTORING PLAN
This should not be used to circumvent the Project Narrative page limit

Ukrainian Sub-Team Participant

Student Bohdan Pshoniuk received a Bachelor's Degree from the Stepan Gzhytskyi National University of Veterinary Medicine and Biotechnologies. We plan to expand his level of education in related fields, namely, molecular biology and analytical biotechnology. We encouraged Bohdan to attend lectures at the Ivan Franko National University of Lviv and the Institute of Cell Biology, NAS of Ukraine for additional training courses: Genetic Engineering, Bioinformatics, Molecular Biology, and a special training course on Microbial Protein Production. Attending by the student new disciplines will ensure his personal and professional growth. He will receive a thorough knowledge in modern protein bioengineering: selection of protein overproducers, design of expression cassettes for overproduction and secretion of protein products, technologies of obtaining hybrid (fusion) proteins, features of post-translational modification, immobilization of the enzymes to create bioreactors, acquaintance with modern fundamentals of protein engineering, approaches to obtaining artificial, semi-synthetic proteins. All this will help him in preparation of Master's degree, participation in research projects of the department of analytical biotechnology Institute of Cell Biology, and will be a prerequisite for continuing his studies as PhD for his future career.

It is planned to attract Bohdan to participate in scientific seminars of the Department and the Institute, in national and international scientific conferences, scientific competitions for students, as well as in various social and cultural events of the institution. It will promote his social activity and formation of high efficiency and a responsible attitude to the educational and scientific process. The student will receive appropriate social and psychological counseling and support from the Department staff if necessary.

STUDENT/POST-DOCTORAL RESEARCHER MENTORING PLAN
This should not be used to circumvent the Project Narrative page limit

US Sub-Team Participants

As a group, we have been very successful in the mentorship of junior researchers, as indicated by their successful placement. Dr. Marcos Pita, the first postdoc in Dr. Katz's group at Clarkson University, has received a tenure-track position in Spain, after being rated No.1 on the national competition list, later he received tenure and promotion to the next academic level. Another postdoc, Dr. Vera Bocharova, received the prestigious Wigner scholarship in Oak Ridge National Laboratory; she is presently a head of a research group. Dr. Jan Halámek received a tenure-track Assistant Professor position at SUNY Albany, where he is performing very successful research in forensic science, expecting soon tenure and promotion. Dr. Roberto Luz returned back to Brazil where he received a tenure-track professor position. The most recent postdoc, Dr. Maria Gamella, returned back to her home country (Spain) where she received an academic position. A few more postdocs found jobs in industry in the USA. Dr. Nataliia Guz is in the process of getting an academic position after 2-year maternity leave. In the last five years, five Ph.D. graduates from Dr. Katz's group received postdoc positions in universities and government labs, or industry jobs (more information about the alumni is available on-line: https://webspace.clarkson.edu/~ekatz/Alumni.html).

Our mentorship of junior researchers has included research guidance, and emphasizing independent creativity balanced in the framework of a group effort. In addition, all our students and postdocs receive support and advisement in preparing their CVs, web sites, job searches, career stages, job market environment and networking. This is accomplished in group and individual discussions. Presentations are scheduled in seminar/interview formats and then critiqued. We even discuss interview behavior and dress code.

Junior researchers are also exposed to our network of colleagues and seminar visitors, as well as attend and present at conferences. The former research networking has included joint work and co-authorship with leading researchers and their groups locally and at other institutions: most recently, with Prof. Joseph Wang (UCSD; http://joewang.ucsd.edu/), Prof. Sergiy Minko (University of Georgia; http://nsmlab.com/), Prof. M.J. Schöning (Aachen University of Applied Sciences, Germany; https://www.fh-aachen.de/menschen/schoening/), Prof. D.M. Kolpashchikov (University of Central Florida; https://sciences.ucf.edu/chemistry/people/kolpashchikov-dmitry/), and many others (https://webspace.clarkson.edu/~ekatz/Collaborators.html). Generally, our publication record is in top journals, and high citation of articles (https://webspace.clarkson.edu/~ekatz/publications_most_cited.htm) has facilitated career development of graduate students and postdocs. Particularly, numerous highlights on our research on implantable bioelectronics and biosensors, including highlights in *Nature*, *Science*, *Scientific American*, *New Scientist*, *New York Times*, as well as in various magazines, radio and TV programs (https://webspace.clarkson.edu/~ekatz/Mass%20Medium.html) have encouraged students and postdoctoral scholars.


Dr. Nataliia Guz (from the Katz group) received an award for the best poster presented at the conference: "Soft Magnetic Materials and Systems",

Postdoctoral researchers and graduate students of Dr. Katz have received numerous awards (including those from ACS, ECS, NYS and others) for research and presentation quality.

Specifically with postdoctoral researchers: we have also encouraged development of communication and management skills by mentoring graduate and undergraduate students, and sharing leadership roles in the team, as well as preparing presentations, publications, and occasionally helping write grant proposals.

KEY PARTICIPANT INFORMATION FORM
Complete ONE for each participant involved

TEAM MEMBER INFORMATION				Ukrainian Sub-Team Participant	
Last Name (surname)	Gonchar	First Name (Given)	Mykhailo	Middle	Vasylyovych
Current Position	Head of Department of Analytical Biotechnology		Classification on Project		Researcher/Engineer
Institute Name	Institute of Cell Biology, NAS of Ukraine				
Complete Mailing Address	14/16, Drahomanov str.		Lviv	79005	Ukraine
E-mail Address	mykhailo1952@gmail.com		Telephone #		+380322612144
Highest Degree/ Year Awarded	DrSci		Biochemistry		2001
Gender	Male				
Description of project role (responsibilities, expertise, level of effort on project):					
Project management, supervision of the UA-team research, preparation of scientific reports and international publications. Representation of the results on the international conferences and forums.					

TEAM MEMBER INFORMATION				Ukrainian Sub-Team Participant	
Last Name (surname)	Smutok	First Name (Given)	Oleh	Middle	Volodymyrovych
Current Position	Senior Researcher		Classification on Project		Researcher/Engineer
Institute Name	Institute of Cell Biology, NAS of Ukraine				
Complete Mailing Address	14/16, Drahomanov str.		Lviv	79005	Ukraine
E-mail Address	smutok.oleg.2015@gmail.com		Telephone #		+380322612144
Highest Degree/ Year Awarded	DrSci		Microbiology		2019
Gender	Male				
Description of project role (responsibilities, expertise, level of effort on project):					
Planning, coordination, and realization of experiments related with enzymology (isolation, purification and characterization of the enzymes), nanotechnology (synthesis and characterization of metallic, as well as carbon-based nanoparticles), and electrochemistry (construction and characterization of microbial, enzymatic biofuel cells on the base of microbial cells, oxidoreductases, and nanocomposite materials). Preparation of scientific reports and international publications.					

TEAM MEMBER INFORMATION				Ukrainian Sub-Team Participant	
Last Name (surname)	Gayda	First Name (Given)	Galina	Middle	Zufarivna
Current Position	Senior Researcher		Classification on Project		Researcher/Engineer
Institute Name	Institute of Cell Biology, NAS of Ukraine				
Complete Mailing Address	14/16, Drahomanov str.		Lviv	79005	Ukraine
E-mail Address	galina.gayda@gmail.com		Telephone #		+380322612144
Highest Degree/ Year Awarded	Dr		Bioorganic chemistry		1983
Gender	Female				

Description of project role (responsibilities, expertise, level of effort on project):

Coordination and realization of experiments focused on microbiology (screening the microbial and fungal producers of oxidoreductases) and enzymology (isolation, purification and enzymatic characterization of the enzymes: alcohol oxidase (AO), methylamine oxidase (MAO) and laccase. Biofunctionalyzation of the nanocarriers by purified enzymes for the construction of biocatalysts for biofuel cells. Screening of new nanocomposite materials possessing nanozyme (nanoperoxidase) activity. Participation in preparing scientific reports and international publications.

TEAM MEMBER INFORMATION				Ukrainian Sub-Team Participant	
Last Name (surname)	Zakalskiy	First Name (Given)	Andriy	Middle	Yevstakhovych
Current Position	Researcher		Classification on Project		Researcher/Engineer
Institute Name	Institute of Cell Biology, NAS of Ukraine				
Complete Mailing Address	14/16, Drahomanov str.		Lviv	79005	Ukraine
E-mail Address	andriy.zakalskiy@yahoo.com		Telephone #		+380322612144
Highest Degree/ Year Awarded	Dr		Biochemistry		1985
Gender	Male				

Description of project role (responsibilities, expertise, level of effort on project):

Cultivation of mutant and recombinant yeast cells and selected fungi, overproducing the target oxidoreductases. Affinity chromatography of (His)6-tagged methylamine oxidase and enzymatic characterization of the enzyme. Participation in construction of laboratory prototypes of microbial biofuel cells and studying their functional characteristics.

TEAM MEMBER INFORMATION					Ukrainian Sub-Team Participant
Last Name (surname)	Prokopiv	First Name (Given)	Tetyana	Middle	Markyanivna
Current Position	Researcher		Classification on Project		Researcher/Engineer
Institute Name	Institute of Cell Biology, NAS of Ukraine				
Complete Mailing Address	14/16, Drahomanov str.		Lviv	79005	Ukraine
E-mail Address	tetyanaprokopiv@gmail.com		Telephone #		+380322612144
Highest Degree/ Year Awarded	Dr		Microbiology		2013
Gender	Female				

Description of project role (responsibilities, expertise, level of effort on project):

Screening and modification of the synthesized nanomaterials by inorganic electron transfer mediators (synthetic dyes, electroconductive films, ferrocene, cyanoferrate derivatives, etc.). Screening of various polymeric membranes (electrodeposited, photopolymeric, dialysis) with an ability to reliably retain enzymes on the surface of the electrodes, possessing a good electrical conductivity and a high permeability for enzymes' substrates. Participation in the design of new enzymatic biofuel cells and studying their operational parameters.

TEAM MEMBER INFORMATION					Ukrainian Sub-Team Participant
Last Name (surname)	Stasyuk	First Name (Given)	Nataliya	Middle	Yevgenivna
Current Position	Young Researcher		Classification on Project		Researcher/Engineer
Institute Name	Institute of Cell Biology, NAS of Ukraine				
Complete Mailing Address	14/16, Drahomanov str.		Lviv	79005	Ukraine
E-mail Address	stasuk_natalia@ukr.net		Telephone #		+380322612144
Highest Degree/ Year Awarded	Dr		Physical Chemistry		2013
Gender	Female				

Description of project role (responsibilities, expertise, level of effort on project):

Synthesis of nanocomposite materials with a high efficiency of electron transfer from biocatalyst to electrode and characterization of their electron mediator activity. Structural and morphological characterization of the obtained nanomaterials. Testing the possibility of using selected nanomaterials as an immobilization matrix for covalent or physical binding of biocatalysts on the electrode surface. Participation in the design of laboratory prototypes of enzymatic and hybrid biofuel cells and studying their operational parameters.

TEAM MEMBER INFORMATION					Ukrainian Sub-Team Participant
Last Name (surname)	Demkiv	First Name (Given)	Olha	Middle	Mykhailyvna
Current Position	Young Researcher		Classification on Project		Researcher/Engineer
Institute Name	Institute of Cell Biology, NAS of Ukraine				
Complete Mailing Address	14/16, Drahomanov str.		Lviv	79005	Ukraine
E-mail Address	demkiv@yahoo.com		Telephone #		+380322612144
Highest Degree/ Year Awarded	Dr		Microbiology		2013
Gender	Female				

Description of project role (responsibilities, expertise, level of effort on project):

Screening of some mold fungi and more than 25 strains of the mushrooms for their synthesis of extracellular laccase in liquid cultures in vitro. Isolation, purification, enzymatic and electrochemical characterization of the laccase from the best enzyme producers. Synthesis of nanocomposite materials with a high efficiency of electron transfer from biocatalyst to electrode and characterization of their electron mediator activity. Participation in construction of laboratory prototypes of microbial biofuel cells and studying their functional characteristics.

TEAM MEMBER INFORMATION					Ukrainian Sub-Team Participant
Last Name (surname)	Pshoniuk	First Name (Given)	Bohdan	Middle	Vasylyovych
Current Position	Engineer		Classification on Project		Student
Institute Name	Institute of Cell Biology, NAS of Ukraine				
Complete Mailing Address	14/16, Drahomanov str.		Lviv	79005	Ukraine
E-mail Address	psoniukbohdan@ukr.net		Telephone #		+380322612144
Highest Degree/ Year Awarded	BS		Biotechnology		2019
Gender	Male				

Description of project role (responsibilities, expertise, level of effort on project):

Technical support in screening, cultivation, and stabilization of mutant, recombinant yeast strains and selected fungi - overproducers of the target oxidoreductase. Participation in the synthesis of nanocomposite materials for application in the construction of biofuel cells.

TEAM MEMBER INFORMATION				US Sub-Team Participant	
Last Name (surname)	Katz	First Name (Given)	Evgeny	Middle	N/A
Current Position	Full Professor		Classification on Project		Researcher/Engineer
Institute Name	Clarkson University				
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E-mail Address	ekatz@clarkson.edu		Telephone #		1-315-268-4421
Highest Degree/ Year Awarded	PhD		Electrochemistry		1983
Gender	Male				

Description of project role (responsibilities, expertise, level of effort on project):

Project management, supervision of the US-team research, preparation of scientific reports and international publications. Representation of the results on the international conferences and forums.

TEAM MEMBER INFORMATION				US Sub-Team Participant	
Last Name (surname)	Bollella	First Name (Given)	Paolo	Middle	N/A
Current Position	Research Assistant Professor		Classification on Project		Post-Doc
Institute Name	Clarkson University				
Complete Mailing Address	Science Center, Department of Chemistry & Biomolecular Science, 8 Clarkson Ave.		Potsdam, NY	13699	USA
E-mail Address	pbollell@clarkson.edu		Telephone #		1-315-268-2389
Highest Degree/ Year Awarded	PhD		Chemistry		2017
Gender	Male				

Description of project role (responsibilities, expertise, level of effort on project):

Experimental work on electrochemical systems, advising a PhD student

TEAM MEMBER INFORMATION					US Sub-Team Participant
Last Name (surname)	Masi	First Name (Given)	Madeline	Middle	N/A
Current Position	PhD student		Classification on Project		Student
Institute Name	Clarkson University				
Complete Mailing Address	Science Center, Department of Chemistry & Biomolecular Science, 8 Clarkson Ave.		Potsdam, NY	13699	USA
E-mail Address	masim@clarkson.edu		Telephone #		1-347-524-3426
Highest Degree/ Year Awarded	BA		Chemistry		2017
Gender	Female				
Description of project role (responsibilities, expertise, level of effort on project):					
Performing electrochemical experiments on modified electrodes.					

CRDF GLOBAL BUDGET NARRATIVE FORM

Describe and justify the expenses included in each budget line item. If a category doesn't apply to your budget, please insert N/A for "not applicable" in the space provided.

Institution Name:	Institute of Cell Biology, NAS of Ukraine	Applicant type:	Primary Institution

Individual Financial Support (IFS)
Describe the level of effort projected for the PI and other team participants. Provide justification for pay rate and any fringe benefits included.

Gonchar Mykhailo Vasylyovych will work as a Principal Investigator/Project Director for this project and will commit 12 calendar months and dedicate 38 % of his working time during this period;
Smutok Oleh Volodymyrovych will work as a Researcher for this project and will commit 12 calendar months and dedicate 38 % of his working time during this period;
Gayda Galina Zufarivna will work as a Researcher for this project and will commit 12 calendar months and dedicate 38 % of her working time during this period;
Zakalskiy Andriy Yevstakhovych will work as a Researcher for this project and will commit 12 calendar months and dedicate 38 % of his working time during this period;
Prokopiv Tetyana Markyanivna will work as a Researcher for this project and will commit 12 calendar months and dedicate 33 % of her working time during this period;
Stasyuk Nataliya Yevgenivna will work as a Researcher for this project and will commit 12 calendar months and dedicate 38 % of her working time during this period;
Demkiv Olha Mykhailyvna will work as a Researcher for this project and will commit 12 calendar months and dedicate 38 % of her working time during this period;
Pshoniuk Bohdan Vasylyovych will work as a graduate research assistant for this project and will commit 5 calendar months and dedicate 13% of his time during this period.

Equipment, Supplies and Services (ESS)
Justify the purpose and cost rationale of each ESS line item included in the budget. General or non-descript line items such as "supplies" or "services" are not acceptable. Please itemize.

We request an amount of $2,450 for the General Supplies and Expendable Materials.
Planar "DropSens" electrodes (4 mm gold - $ 500.0, and 4 mm platinum - $ 550.0), glass reference electrodes ($ 350.0) and glass electrochemical cells ($ 250.0) will be used for construction of model biofuel cells. Noble metal salts (Gold(III) chloride hydrate - $ 250.0; Chloroplatinic acid hexahydrate - $ 250.0; Palladium (II) chloride - $ 300.0) are required for synthesis of nanomaterials for electrodes modification. The price of each position of supplies and materials is calculated as the producer's catalog price including the costs for transportation to Institute.

Travel
Explain the need for travel - how the travel will benefit the project's aims - and your calculations of travel costs for domestic and foreign travel. Break down by airfare, hotel, per diem, etc.

We request $ 310.0 for domestic travel as Conference Registration Fees and Other Expenses for attendance at the International Scientific Conference in Kyiv (Ukraine).
Foreign travels from Lviv to the Clarkson University (Potsdam, NY, USA) are planned ($17,235). DrSci. Smutok O., Dr. Stasyuk N., and Dr. Demkiv O. are suggested to attend U.S. PI laboratory for collaborative work. The financial calculation is based on $850 Amount airfare per person, $151 Amount International lodging/Per Diem rates per person for 31 nights, $160 Amount visa fee per person and $60 Amount medical insurance per person based on the U.S. Government allowances in effect at the time of travel.

Indirect Costs (IDCs)
Justify indirect costs % of the total sub-team direct expenses requested. Indicate if a NICRA or other institutional IDC certification is applicable.

Indirect costs (10 %) will be used for the cover of communal payments, communication items (internet, telephone and mail), office and accounting expenses.

Cost Share (optional)
If applicable, describe the cost share that is being undertaken. Describe what items the cost-share will be applied to.

Not applicable

CRDF GLOBAL BUDGET NARRATIVE FORM

Describe and justify the expenses included in each budget line item. If a category doesn't apply to your budget, please insert N/A for "not applicable" in the space provided.

Institution Name:	Clarkson University	Applicant type:	Primary Institution

Individual Financial Support (IFS)
Describe the level of effort projected for the PI and other team participants. Provide justification for pay rate and any fringe benefits included.

Funding is requested for one Postdoctoral Research Associate to devote approximately 10% effort toward this project.

Funding is also requested for one part-time graduate student who will also devote approximately 1% effort toward this project.

Fringe benefits are charged on the Research Associate's salary at the anticipated rate of 34%.

Equipment, Supplies and Services (ESS)
Justify the purpose and cost rationale of each ESS line item included in the budget. General or non-descript line items such as "supplies" or "services" are not acceptable. Please itemize.

$951 is requested for the purchase of chemicals, biochemical, solvents and other lab consumables required for the completion of the proposed project.

Travel
Explain the need for travel - how the travel will benefit the project's aims - and your calculations of travel costs for domestic and foreign travel. Break down by airfare, hotel, per diem, etc.

Not applicable

Indirect Costs (IDCs)
Justify indirect costs % of the total sub-team direct expenses requested. Indicate if a NICRA or other institutional IDC certification is applicable.

Indirect Costs are calculated in accordance with Clarkson University's Indirect Cost Rate Agreement with the Department of Health and Human Services. Currently, Clarkson is using a rate of 53% of modified total direct costs (MTDC), per DHHS Agreement dated 06/14/19. If any rate other than the appropriate negotiated rate is applied to a project due to published program restrictions or sponsor policy, all unrecovered indirect costs are designated as cost sharing by Clarkson University. Clarkson's threshold for equipment is $5,000 as allowed by the Uniform Administrative Requirements, Cost Principles, and Audit Requirements for Federal Awards (UG 2 CFR § 200).

Cost Share (optional)
If applicable, describe the cost share that is being undertaken. Describe what items the cost-share will be applied to.

Not applicable

CRDF Global PROJECT MILESTONE PLAN (TEMPLATE/ SAMPLE)

Copy template to complete. Information should match the proposal Project Narrative and Project Budget

Reporting Period (Complete for each quarterly segment applicable top project duration.)			**Responsible Team**	
First Quarterly Reporting Period			***Mark all that apply***	
Milestone:	Description:	Associated Deliverable(s):	U.S. Sub-Team	Ukrainian Sub-Team
Cultivation of microbial and fungal cells; isolation and purification of oxidoreductases – promising biocatalysts for the construction of biofuel cells (BFCs)	Over 25 strains of fungi will be screened for their ability to produce laccase, and prospective producers of this enzyme will be selected. For cells of recombinant and mutant strains of the yeasts and selected fungi (overproducers of oxidoreductases), the cultivation conditions will be optimized. The produced biomass will be stabilized (using permeabalisation, lyophilization). Purified preparations of oxidoreductases (AOX, MAOX, FC b_2, and laccase) will be obtained using ion-exchange and affinity chromatography.	Scientific report	$3,750	$8,500
Total Amount Requested for this Reporting Period:		$12,250	$3,750	$8,500
Second Quarterly Reporting Period			***Mark all that apply***	
Milestone:	Description:	Associated Deliverable(s)	U.S. Sub-Team	Ukrainian Sub-Team
Synthesis and structural-morphological characterization of nanocomposite materials	The methods of synthesis and functionalization of metal nanoparticles will be optimized and more than 20 variants of mono- and bimetallic nanoparticles hybrids (based on noble and/or transition metals) will be obtained. Among newly synthesized nanomaterials, artificial peroxidases (nanoperoxidases) will be selected. Conductive carbon materials – derivatives of graphene and carbonated cellulose fibers will be tested and characterized. For the obtained nanomaterials, their physico-chemical, structural-morphological, catalytic and electrochemical characteristics will be investigated. Screening and additional modification of the synthesized nanomaterials by inorganic electron transfer mediators will be carried out and the electrochemical characteristics of the obtained nanocomposite materials and their compatibility with the BFCs biocomponents will be investigated.	Scientific report	$3,750	$12,300

Total Amount Requested for this Reporting Period:		$ 16,050	$ 3,750	$ 12,300
Third Quarterly Reporting Period			***Mark all that apply***	
Milestone:	Description:	Associated Deliverable(s)	U.S. Sub-Team	Ukrainian Sub-Team
Development of laboratory prototypes of microbial BFCs and study of their functional characteristics	New laboratory prototypes of microbial BFCs will be constructed on the basis of mutant, recombinant yeast and selected fungi cells, producing oxidoreductases, and their functional and operational characteristics will be investigated.		$3,750	$8,200
Analysis of Experimental Data	Both teams will conduct analysis on data collected by two institutions to combine them for the next researching stages	Scientific report	-	$1,500
Total Amount Requested for this Reporting Period:		$ 13,450	$ 3,750	$ 9,700
Fourth Quarterly Reporting Period			***Mark all that apply***	
Milestone:	Description:	Associated Deliverable(s)	U.S. Sub-Team	Ukrainian Sub-Team
Construction of laboratory prototypes of enzymatic BFCs based on purified oxidoreductases and nanocomposites, and studying their operational parameters	New laboratory prototypes of enzymatic BFCs based on purified preparations of AOX, MAOX, FC b_2, laccase as well as nanoperoxidases and electron-mediated nanocomposites will be obtained and characterized. For BFCs, the possibility of creating the third-generation mediator-free enzyme bioelectrodes based on FC b_2 and laccase in combination with nanomaterials will be investigated.	Scientific report	$3,750	$8,500
Travel to US Institution	Members of the Ukrainian team will visit their partner institution in the USA. The main objective is face-to-face interaction in providing joint experiments and future publications	Trip Report Travel documents (copies of boarding passes, visa, other receipts) Photos	-	$16,245
Completion of manuscript to be submitted for publication and final CRDF-report	The teams will work to prepare a final manuscript to be submitted for publication in the peer-reviewed journal and Final CRDF-report	Copy of the manuscript Final CRDF-report	-	$1,755
Total Amount Requested for this Reporting Period:		$ 30,250	$ 3,750	$ 26,500

PI OTHER SOURCES OF SUPPORT FORM

PI Name	Mykhailo Gonchar		
If no other sources of support, check "None." Otherwise, complete table below for each source (duplicate as needed).			☐ **"None"**
Project/Proposal Title	Development of biosensors and biofuel cells based on redox enzymes	**Location of Research**	Ukraine/Lithuania
Support	√ Current ☐ Pending Submission Planned in Near Future		
Source of Support	Ministry of Education and Science of Ukraine	**Level of Effort (%)**	10 %
Award Amount	$ 8,000	**Period Covered**	03/20 – 12/22

PI OTHER SOURCES OF SUPPORT FORM

PI Name	Evgeny Katz		
If no other sources of support, check "None." Otherwise, complete table below for each source (duplicate as needed).			☐ **"None"**
Project/Proposal Title	HFSP Collaborative Research Grant	**Location of Research**	Clarkson University
Support	√ Current ☐ Pending Submission Planned in Near Future		
Source of Support	Human Frontier Science Program	**Level of Effort (%)**	0%
Award Amount	$420,000	**Period Covered**	10/18 – 09/21
Project/Proposal Title	EAGER: NSF-BSF: Quorum Biosensing using Magnetic Field-Activated Molecular Machines	**Location of Research**	Clarkson University
Support	√ Current ☐ Pending Submission Planned in Near Future		
Source of Support	National Science Foundation	**Level of Effort (%)**	0%
Award Amount	$150,000	**Period Covered**	08/19 – 01/21
Project/Proposal Title	Wearable-implantable Sensors for Continuous Monitoring of Biomarkers in Animals and Humans	**Location of Research**	Clarkson University
Support	☐Current √ Pending ☐ Pending Submission Planned in Near Future		
Source of Support	Queensland University of Technology (DOD-prime)	**Level of Effort (%)**	0%
Award Amount	$200,000	**Period Covered**	09/20 – 02/22

PREVIOUS CRDF GLOBAL AWARD FORM
Individual forms should not exceed one page.

CRDF Global Award Number:	UKB2-9044-LV-10		
Title of Previous Project:	Production of trial series and preparation of technical documentation for bioanalytical kit for enzymatic analysis of L-lactate in clinical diagnostics and beverage industries		
Start Date	05/2011	End Date	05/2012

Please briefly describe the previous research project. Be sure to provide specific information regarding results and objectives. Were all objectives of the research plan achieved? If not, what prevented you from doing so? Please list scientific publications and conference reports that were published as a result of CRDF Global award.

As a result of this CRDF-project, the optimization of a process of L-lactate cytochrome *c*-oxidoreductase (flavocytochrome b_2, FC b_2) production and its isolation have been performed. It was obtained preparation with 70 units of enzyme activity that allows produce 3 000 "Lactatest" kits. Optimization of composition parameters of the "Lactatest" (concentration of enzyme, chromogen, and buffer), storage form of the kit's components and optimization of reaction conditions have been done.

The manual (instruction) for using the proposed kit and description of laboratory technology to produce "Lactatest" were prepared according to the standards of Ukraine.

The trial series of the kit "Lactatest" was prepared and its testing on the real samples of food products (dairy products, juices, ketchups etc.) as well as human liquids have been done. The analysis of L-lactate by the use of "Lactatest" in sweat samples obtained from the trained athletes was also performed. The correlation level for obtained results with the reference methods has been done.

Several international publications with a high impact factor were published and acknowledgment of CRDF has been indicated.

The list of publications with affiliated CRDF-grant # UKB2-9044-LV-10:

1. Smutok O., Broda D., Smutok H., Dmytruk K., Gonchar M. (2011) Chromate reducing activity of the *Hansenula polymorpha* recombinant cells overproducing flavocytochrome b_2. ***Chemosphere***, *83*(4), 449-454 (IF - **3.613**). https://doi.org/10.1016/j.chemosphere.2010.12.078;

2. Smutok O., Gayda G., Dmytruk K., Klepach H., Nisnevich M., Sibirny A., Puchalski C., Gonchar M., Sibirny V. Amperometric Biosensors for Lactate, Alcohols, and Glycerol Assays in Clinical Diagnostics / in epy "**Biosensors - Emerging Materials and Applications**", (2011), ISBN 978-953-307-328-6, pp. 401-446.

How will the work accomplished during this project contribute to the proposed research? Please address specific project results (data, models, methods) that the proposed project will further develop and/or build upon.

The developed methods for isolation of flavocytochrome b_2 allowed obtaining highly purified preparations of the enzyme which will be used for the construction of mediated and/or mediatorless enzymatic biofuel cells.

Moreover, developed in the frames of previous CRDF-project enzymatic kit "Lactatest" for L-lactate assay will be used to evaluate the fuel substrate consumption in biofuel cells.

Due to the realization of the previous CRDF-project, a more close relationship between the scientific group of the Institute of Cell Biology, NAS of Ukraine and the company "Engineering Laboratory Ltd." was established. On the base of acquired experience, it will be easier to introduce our innovations into the practice, especially, in the fields of amperometric biosensors' and biofuel cell construction.

PREVIOUS CRDF GLOBAL AWARD FORM
Individual forms should not exceed one page.

CRDF Global Award Number:	UKB1-9048-KV-10		
Title of Previous Project:	Development of technology for production of recombinant human arginase as anticancer enzyme and element of biosensor for arginine		
Start Date	05/2011	End Date	05/2012

Please briefly describe the previous research project. Be sure to provide specific information regarding results and objectives. Were all objectives of the research plan achieved? If not, what prevented you from doing so? Please list scientific publications and conference reports that were published as a result of CRDF Global award.

The objective of the previous CRDF-project was to develop an innovative technology for expression of recombinant human arginase in yeast host and its purification to homogenous state and to make this product commercially available. This enzyme is currently not commercially available but is of great interest for anticancer enzymotherapy (medicine) and as an element of novel biosensor for arginine (analytical biotechnology).

As a result of the project, we optimized the purification protocol for recombinant human arginase expressed in two species of yeasts, bakers' yeast *Saccharomyces cerevisiae* and methylotrophic yest *Hansenula polymorpha* to make it cost-efficient. We achieved production of the purified preparations of the recombinant arginase sufficient for the use in bioanalytical devices and for further manipulations to be used in animal studies and clinical trials (in PEGylated form).

Obtained in the frame of previous CRDF-project enzyme was used also for the other scientific investigations. As a result, some international publications with a high impact factor were published and acknowledgment of CRDF has been indicated.

The list of publications with affiliated CRDF-grant # UKB1-9048-KV-10:

1. Stasyuk N., Smutok O., Gayda G., Gonchar M., Koval'chuk. Y. (2011) A new bi-enzyme potentiometric sensor for arginine analysis based on recombinant human arginase i and commercial urease. ***Journal of Materials Science and Engineering: A***, *1*, 819-827;

2. Stasyuk N., Smutok O., Gayda G., Koval'chuk. Y., Vus B., Gonchar M. (2012) Bi-enzyme L-arginine-selective amperometric biosensor based on ammonium-sensing polyaniline-modified electrode. ***Biosens. & Bioelectron.***, *37*(1), 46-52 (IF - **5.63**) https://doi.org/10.1016/j.bios.2012.04.031

Zakalskiy A,, Zakalska O., Rzhepetskyy Y., Potocka N., Stasyk O., Horak D., Gonchar M. (2012) Overexpression of (His)(6)-tagged human arginase I in *Saccharomyces cerevisiae* and enzyme purification using metal affinity chromatography. ***Protein Expr Purif***., *81*, 63-68. (**IF – 1.64)** DOI:10.1016/j.pep.2011.09.001

How will the work accomplished during this project contribute to the proposed research? Please address specific project results (data, models, methods) that the proposed project will further develop and/or build upon.

The methods developed during this project will be valuable for the proposed project, namely, for isolation of the recombinant enzymes from cell extracts and their purification using affinity chromatography. There will be also used methods for stabilization of the enzymes and their immobilization on the electrodes, proposed in the earlier project.

SUGGESTED REVIEWERS AND REVIEWERS NOT TO INCLUDE
(Optional)

Suggested Reviewers				
#	Name	Affiliation	Email	Brief Justification
1.	**Jan Halamek**	Department of Chemistry, SUNY Albany, USA	jhalamek@albany.edu	Expert in bioelectrochemistry, bioelectronics and biofuel cells
2.	**Marcos Pita**	Instituto de Catálisis y Petroleoquímica, CSIC, Spain	marcospita@icp.csic.es	Expert in bioelectrochemistry, bioelectronics and biofuel cells
3.	**Antonio López**	Instituto de Catálisis y Petroleoquímica, CSIC, Spain	alopez@icp.csic.es	Expert in bioelectrochemistry, bioelectronics and biofuel cells
4.	**Arunas Ramanavicius**	[1]Department of Physical Chemistry, Vilnius University; [2]Laboratory of NanoTechnology State Research Institute Center for Physical Sciences and Technologies	arunas.ramanavicius@chf.vu.lt	Expert in construction and characterization of novel biosensors, as well as biofuel cells
5.				
6.				
7.				

Suggested Reviewers Not to Include				
#	Name	Affiliation	Email	Brief Justification
1.				
2.				
3.				
4.				
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10.				