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INFORMATION FROM *IN VITRO* BIOMEDICAL DIAGNOSTICS — A ROAD TO PERSONALIZED MEDICINE

Abstract: In a number of neurodegenerative diseases (NDs) the production of the anti-neuronal immunoglobulin G (IgG) is a significant feature of the inflammatory process. It was shown that human IgGs may induce diverse physiological effects on neurons and glial cells of animal origin. In an ongoing project we propose to use IgGs for *in vitro* diagnostics of NDs. Based on already known cellular signaling responses recorded by fluorescent markers robust multipurpose processing of a single patient's IgG sample effect on seeded normal cells can give a complex physiological information. This is achieved with microfluidics and automated microscopy towards experimental/clinical personalized diagnostics. Such a medical device is based on 1) the development of procedures based on a lab-on-a-chip microfluidic system with intracellular light sensors; 2) defining the standardized *in vitro* personalized diagnostic protocols; 3) design of a small-scale pilot platform based on automated/miniaturized fluorescence microscopy. Most of these principles have already been partly tested though a EC-H2020 project and a national Innovation Fund project. The interdisciplinarity of our research comprises of the following approaches: Cellular neurophysiology, Biophysics of intracellular fluorescent indicators, Video microscopy of intracellular molecular signaling, Microfluidics and biochip design, Advanced custom-made optics for automated microscopy and Machine learning for signal analysis. The designed personalized diagnostics technology will be applicable for a variety of NDs for a sustained healthcare system.

INTRODUCTION

Although a vast literature exists on successful preclinical and even on some clinical studies of amyotrophic lateral sclerosis (ALS) therapy, this is still a fatal disease without a reliable cure. Often patients being conscious

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throughout the illness are demanding euthanasia. It is a consensus among researchers and clinicians alike that such a poor prognosis is caused because there is a lack of information or its organization for a robust diagnostic tool that can primarily predict but also follow the therapeutic procedure. Since ALS is in 90% of cases of sporadic nature the main problem is to recruit patients for screening early enough. This can be achieved first by screening patients for comorbidities (e. g. FTD or muscle cachexia) or recruiting them based on genetic or proteomic markers (e. g. TDP-43 and FUS/TLS genes) in order to subject them to a robust point-of care diagnostics platform for prediction of disease and/or its stage and later for the follow-up of therapy. Such a multifactorial information — rich diagnostic tool is expected to offer a personalized approach to the disease and better patient stratification. The diagnostic process of ALS immunoglobulin G (IgG) application on stained cell cultures for an automated recording system is of a highly innovative potential. Although experimentally confirmed and used in describing ALS pathogenesis, such phenomena were never used previously in practical terms. Our approach towards standardizing the procedure for a market pilot follows on an innovative path that could be linked to the published [1, 2] and patented technology [3, 4] of automated microscopy for multidimensional cell profiling for personalized diagnostics. This approach led us to the pilot platform for high-throughput multidimensional cell profile analysis upon ALS IgG challenge. In such a setup the multivariate information-rich single cell analysis is a starting point for identifying relationships among ALS IgG effects at a systems level and a step toward phenotypic/physiological profiling at the single-cell level.

BACKGROUND (BASIC) RESEARCH

In a number of neurodegenerative diseases (NDs) the production of the anti-neuronal immunoglobulin G (IgG) is a significant feature of the immunoinflammatory process. It was shown by us and others that, compared to control, IgG from NDs patients may induce diverse physiological effects not only on neurons but also on glial cells of animal origin. Thus, for example, studies on amyotrophic lateral sclerosis (ALS) have shown that human IgG increased intracellular Ca^{2+} in motor neurons, enhanced glutamate release from synapses residing on lower motor neurons and enhanced the release of acetylcholine from the axon terminal at the neuromuscular junction [5–7]. These crucial events in our own early experiments were shown not only on neurons (facilitation of synaptic activity and Ca^{2+} transient change) but also on astroglia (Ca^{2+} transients, vesicle trafficking) [8–10].

The current state of the art findings obtained with ALS IgG can be focused on the following discoveries:

a) Ca^{2+} imaging. Ca^{2+} imaging with fluorescent dyes provided information on intracellular calcium mobilization in response to ALS IgGs on several cultured cell types (neurons, glia, and cell lines [11]);

b) Reactive Oxygen Species (ROS) imaging. Pilot experiments [12] on microglial cell lines (BV-2) transfected with ROS-sensitive fluorescent constructs have shown that ALS IgGs induce acute ROS signaling.

c) Synaptic activity. The observed effect on hippocampal neurons in culture was a rise in frequency of post-synaptic currents not seen with IgGs from healthy or disease controls [8,9].

d) Vesicle trafficking. We have previously shown that ALS IgGs increase the mobility of acidic vesicles (mostly endosomes and lysosomes) in primary cortical astrocytes [10].

Moreover, in addition to ALS, in a number of other NDs such as, Lambert-Eaton — myasthenic syndrome, Guillain-Barré syndrome, Rasmussen's encephalitis, Systemic Lupus Erythematosus, Multiple sclerosis and Neuromyelitis Optica, production of anti-neuronal IgGs is a significant feature of the immune-inflammatory process.

We want to use this knowledge to design a microfluidic device in order to obtain a lab-on-chip for innovative and disruptive diagnostics for better segregation of patients of NDs. Further on we will present the main directions of this ongoing project (already funded by EU-H2020 program and the Innovation Fund of Republic of Serbia and the EIT Jumpstarter program).

APPLIED RESEARCH

The objectives addressed are: 1) Development of procedures based on the lab-on-a- chip microfluidic systems with light sensor probes within living cells reacting to IgG or total sera from patients as a diagnostic and prognostic technology related to diverse NDs. 2) Defining mark-up characteristics of the standardized in vitro approach for personalized diagnostic protocols. 3) Design of a small-scale pilot platform based on automated/miniaturized fluorescence microscopy.

The first step will be thorough standardization of already investigated and published data on biophysical processes monitoring of Ca^{2+} — transients and ROS generation by fluorescent dye imaging (on ALS IgGs [8–11] on Lupus erythematosus CSF and brain autoantibodies [13]). As an outlook of these studies IgGs or sera from different ND patients will be assessed on animal cell models (cultures of neurons, astrocytes, cell lines) throughout these biophysical processes. Further development should go

towards the same tests on human cells derived from inducible pluripotent stem cells (iPSC). Using this combination of sensitive readouts, the goal is to correlate quantitative parameters of these physiological biomarkers (unlike existing molecular or biochemical) with disease specific parameters for the disruptive personalized *in vitro* diagnostics using machine learning protocols. This approach will thus introduce a novel and unforeseen diagnostic approach with physiological biomarkers as opposed to the existing „static" molecular or biochemical markers. To accomplish this in a robust clinical/lab setup an innovative automated integrated microchip microscopy system is being designed as the first prototype.

The interdisciplinary approach will add an integrated added value to a compound solution that combines advanced biochip technology, microfluidics, cellular biophysics, custom-made micro-optics and software analytics into a genuinely novel area of theragnostic research and technology. The multivariate single-cell robust physiological analysis offered by the proposed in vitro diagnostic technology will provide the adequate approach for the personalized care of patients with a multifocal disease (such as NDs generally are).

NOVEL DESIGN OF THE OPTICS-ON-CHIP

The integration of optics within the microfluidic device towards optics-on-a-chip serves as a genuinely new technical idea. Namely, the following solutions have never been put in place in one device: 1) coded-aperture imaging and scanning holography — techniques requiring minimum amount of optical components, while providing 3D imaging and software-based autofocusing; 2) stabilized LED or diode laser for fluorescence-excitation illumination; 3) mobile phone computing power used for hologram reconstruction. As a contingency measure alternative designs are also envisaged: a stand-alone, miniaturized, microscope optical system or attachment to a standard microscope with an add-on mount.

MACHINE LEARNING PROTOCOLS

A specific software and novel data acquisition and processing capabilities have been developed. The software will acquire fluorescence image series and will discriminate the response pattern of cells treated with IgG or sera. Supervised machine learning will be employed to classify the traces. The extracted trace features will be fed into unsupervised machine learning-based software to identify the diseases itself as well as its progression phenotypes.

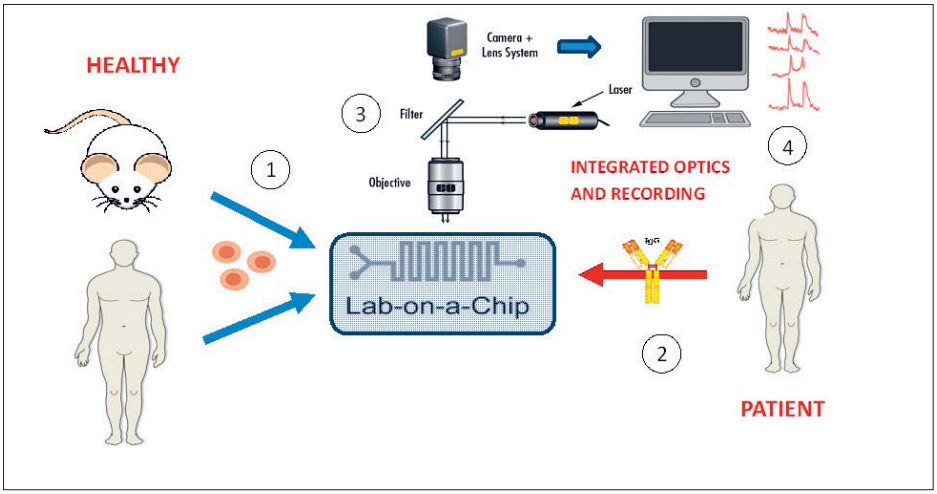

Figure 1. Proposed medical device workflow. 1) Seeding cells (either cultured animal cells or human iPSCSs — derived cells or cell lines) in the microfluidic lab-on-a-chip; 2) treatment with patient IgGs through the microfluidic flow-control system; 3) fluorescence recording system (laser — objective — lens system — camera). In the final system development the laser light source will be substituted with a LED system); 4) signal analysis using machine learning protocols for personalized medicine.

CONCLUSION

Although the effects of ALS IgGs are well studied and documented in the Belgrade laboratory and elsewhere the process of IgG application for *in vitro* diagnostics of ALS is completely novel and offers a practical multidimensional functional analysis for personalized medicine. Thus, based on a standard clinical blood sample and upon routine serum separation and IgG purification one can obtain under an automated microscope actually a pattern of activities in the form of fluorescence signaling in time and space thus establishing a personalized signature of the disease for the individual patient (Fig. 1). The combining of several recording modes raises the reliability of the obtained diagnostic pattern. On the other hand, this procedure can give early diagnosis of ALS since it has been shown that inflammation markers appear early in the disease model [14]. The complex multifactorial nature of ALS underlines the need for a personalized treatment and patient stratification. It is strongly believed that the multivariate single-cell analysis offered by the developed *in vitro* diagnostic technology may present exactly the adequate approach for the personalized patient care of such a multifocal disease. At the same time the pattern analysis of the multivariate single-cell response allows for a robust point-of-care diagnostics necessary for

improved and efficient clinical decisions. This approach will contribute to the sustainability of the health care of ALS patients by drafting and planning a large-scale prototype in an operational environment. In addition, the designed personalized diagnostics technology of *in vitro* testing of IgGs from patient sera can be proposed for other motoneuron and neuroinflammatory neurodegenerative processes, thus allowing for sustainability of the health care system in the particular area of neuroinflammation as the common mechanism of neurodegenerative diseases.

Finally, the ongoing project will rise an opportunity for partnership with relevant SMEs that could contribute to the upgrade and strengthening of the designed technology in its many aspects from standardized cell culturing to the hardware and software design for automated microscopy.

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INFORMACIJA U *IN VITRO* BIOMEDICINSKOJ DIJAGNOSTICI — PUT KA PERSONALIZOVANOJ MEDICINI

Sažetak

Kod brojnih neurodegenerativnih bolesti (ND) proizvodnja antineuronskog imunoglobulina G (IgG) je značajna karakteristika inflamatornog procesa. Pokazalo se da humani IgG mogu izazvati različite fiziološke efekte na neurone i glijalne ćelije životinjskog porekla. U projektu koji je u toku predlažemo da se IgG koriste za *in vitro* dijagnostiku ND robusnom multifunkcionalnom obradom pojedinačnog uzorka (IgG pacijenta), na zasejanim normalnim ćelijama, koje mogu dati složene fiziološke odgovore zasnovane na već poznatim intraćelijskim signalima, snimljenim pomoću fluorescentnih markera u mikrofluidičkom čipu sa automatizovanom mikroskopijom, sve u pravcu kliničke personalizovane dijagnostike. Ovakav medicinski uređaj zasnovan je na: 1) razvoju procedura zasnovanih na mikrofluidičkom sistemu laboratorije na čipu sa intracelularnim svetlosnim senzorima; 2) definisanju standardizovanih *in vitro* personalizovanih dijagnostičkih protokola; 3) dizajnu male pilot platforme zasnovane na automatizovanoj/minijaturizovanoj fluorescentnoj mikroskopiji. Većina ovih principa je već delimično testirana kroz projekat EC-H2020 i projekat nacionalnog Fonda za inovacije. Interdisciplinarnost našeg istraživanja obuhvataju sledeće pristupe: ćelijska neurofiziologija, biofizika intracelularnih fluorescentnih indikatora, video-mikroskopija intracelularne molekularne signalizacije, mikrofluidika i dizajn biočipa, napredna optika po meri za automatizovanu mikroskopiju i mašinsko učenje za analizu signala. Predložena personalizovana tehnologija dijagnostike biće primenjiva na različite NB u održivom zdravstvenom sistemu.