Near-infrared biosensors and optogenetics to advance preclinical studies in neurobiology

1. Research idea. Preclinical studies, including target selection and validation in living animals, are key early steps in the drug discovery process that can save companies millions of dollars in clinical trials on unsuccessful targets. In neurobiology, testing drug candidates in the brain of living animals can clarify drug action and reveal potential side effects. Our technology will allow faster and cheaper preclinical studies, and new experimental setups for studies of biological processes in their native environment.

The technology originates in the lab of our co-founder Dr. Verkhusha at Albert Einstein College of Medicine. It includes a set of research tools for non-invasive *(i) optical imaging of biological processes (biosensors)* and *(ii) optical activation of biological processes (optogenetic system)*. These tools are the engineered genes coding for sensor proteins derived from bacterial photoreceptors naturally sensing near-infrared (NIR) light. Mammalian tissues are relatively transparent to NIR light, which penetrates one-inch deep inside living tissue, i.e. through the whole mouse. NIR light is non-toxic to cells and organisms, in contrast to short-wavelength visible light used by alternative technologies. Our sensor proteins are controlled by NIR light, genetically encoded, and do not require any supply of chemicals. The technology offers faster ways to screen and validate targets because the same research tools are applicable in both cell and animal models. Since no supply of chemicals or installation of invasive light guides are required, it makes experiments less expensive and more robust.

Our technology is modular. The NIR fluorescent proteins (FPs) (Nature Communications, 7:12405 (2016)) and the NIR optogenetic system (Nature Methods, 13:591-7 (2016)) can be used as building blocks to create biosensors and optogenetic tools for specific purposes. Using NIR FPs, we engineered probes for cell and protein labeling, for studies of protein-protein interactions, for IκBα reporter for NF-κB pathway, and for cell-cycle reporter. Recently, we have generated a functional NIR calcium biosensor. Using NIR optogenetic system, we developed a system for NIR-light induced expression of the gene of interest and NIR system for re-localization of desired proteins between cellular compartments.

The NIR biosensors and optogenetic tools will be most useful in neurobiology, where they will allow visualizing complex dynamic processes in health and disease, including substance use disorders (SUDs). Modern calcium sensors provide a robust reliable readout of neuronal activity. NIR calcium sensor will allow large-scale recording of functional neural circuits in the brain. There is an immediate application of NIR biosensors that makes them commercially attractive: a combination with other probes controllable by light of the visible spectrum. A combination of NIR calcium sensor with light-gated opsins will allow precise all-optical control and readout in neuronal circuits, without any interference between probes. NIR calcium sensor will also be an ideal tool for a combination with other sensors, including voltage, cAMP and DAG. Simultaneous visualization of dynamics of several processes will help to identify the mechanism of action of selected compounds and to minimize a chance of selecting a drug with off-target effects.

The NIR optogenetic system will aid in identification of active neuronal circuits in the brain of a living animal. Repeated neuronal activity results in induction of the immediate-early genes (IEG) controlled by c-fos and other promoters. These promoters are widely used for neuronal activity mapping and also in SUD research. A use of drug-induced gene expression systems to control of the activity dependent promoters with drug-induced gene expression systems enabled chronic labeling of active ensembles of neurons and a possibility to artificially retrieve memories using optogenetics (Nature 484:381-5 (2012)). A use of NIR light-induced gene expression for this purpose will enable a substantially more precise temporal resolution of neuronal activity. In addition, this approach should allow a development of light induced models of SUD, because development of drug addiction involves persistent neurobiological changes in active neurons, including activation of IEGs.

Based on NIR calcium biosensor and NIR optogenetic system for gene expression, we plan to develop commercial products for (i) *all-optical electrophysiology* based on a combination of NIR calcium sensor with opsins, (ii) reporters for monitoring of neurotransmitter release based on *engineered cells* containing NIR calcium sensor, (iii) *assays for non-invasive labeling* of active neurons with reporter genes or opsins, and (iv) *actuators for* precisely controllable *release of neurotransmitters* based on engineered cells, containing NIR optogenetic system.

2. The team. Our team includes two faculty members of Albert Einstein College of Medicine, Drs. Shcherbakova and Verkhusha. The core technology originates in the lab of Dr. Verkhusha, who is an internationally renowned leader in the field of protein engineering and development of fluorescent proteins (FPs) and biosensors. He pioneered development of several photoactivatable FPs, probes for super-resolution photoactivated localization microscopy (PALM). For example, PAmCherry developed in his lab is now provided by Takara Bio (former Clontech). Also, photoswitchable FP, Dendra2, is a widely applied tool for protein and cell tracking experiments, which is currently offered by Takara Bio (former Clontech) and Evrogen. Verkhusha lab also engineered bright blue FP, mTagBFP, that is currently distributed by Evrogen. In recent years Verkhusha lab has focused on a development of near-infrared (NIR) FPs. iRFP series of proteins engineered on the basis of bacterial phytochromes (Nature Biotechnology, 29:757-61 (2011), Nature Methods, 10:751-4 (2013)) are currently the most widely used NIR FPs. They found applications in neurobiology, cancer research, regenerative medicine, and other areas of biology and biomedicine. His lab also recently reported the first NIR optogenetic system, which does not require exogenous chemicals to function. This system was successfully applied to non-invasive gene activation *in vivo*. Published this year (Nature Methods, 13:591-7 (2016)), it already generated the interest of researchers in different areas.

Dr. Shcherbakova is a senior scientist in Verkhusha lab. Her research, focused on development of sensor proteins controlled by NIR light, has resulted in a series of high profile publications. In (Nature Methods, 10:751-4 (2013)) the team reported the first generation of NIR FPs, named iRFPs, for *in vivo* imaging of several processes. iRFPs became widely used tools in research and generated substantial interest in industry. Dr. Shcherbakova is the leading author in the recent paper that reports the second generation of NIR FPs, named miRFPs (Nature Communications, 7:12405 (2016)). miRFPs are monomeric and can be used as easy as proteins from green fluorescent protein (GFP) family. Moreover, miRFPs serve as building blocks to engineer NIR biosensors for studies of dynamic processes in cells and in animals.

Encouraged by a feedback from adopters of the technology in academia and an interest from companies that contacted Einstein to evaluate the technology, the team conceived a start-up company to explore commercial potential of NIR optical tools. In December 2015 the team was selected to participate in the highly competitive ELabNYC program designed for aspiring entrepreneurs and successfully completed it in June 2016.

3. The products. There are many possibilities to develop our platform technology into ready-to-use products. Our NIR FPs, calcium biosensor and optogenetic tool for gene expression can be delivered to customers in the following forms: regular mammalian plasmids, BacMam vectors for efficient gene delivery in cells, AAV and lentiviruses for gene delivery *in vivo*, functional cells lines, transgenic animals. Our goal is to develop products that are in high demand and are readily applicable to neurobiology. Thus, *a NIR calcium biosensor* and *a NIR optogenetic system for gene expression* would be best received in the form of ready-to-use AAV and lentiviruses. Our other future products are engineered cell lines that contain our NIR tools and can “sense” neurotransmitters /neuromodulators and provide optical fluorescence readout, or produce specific neurotransmitter /neuromodulator under the control of NIR light illumination. The proposed products create value for the customer. It is also a good choice for an early stage company. Once DNA constructs are created, AAV and lentivirus preparations can be ordered

from different suppliers as needed. Once reporter and actuator cells are created, they can be stored, maintained and delivered to customers on request. Below we will discuss the use of the four types of our products based on NIR calcium sensor and NIR optogenetic system.

(i) AAVs coding for NIR calcium sensor can be created using FLEX technology and applied in mice with tissue-specific Cre expression. We will also prepare AAVs with cell-type specific promoters upon request. AAVs coding for NIR calcium sensor can be combined with opsin-based tools and other biosensors for all-optical electrophysiology experiments and studies of signal pathways activation. In animals, NIR calcium sensor can be visualized in large-scale recording setups or can be studied in localized populations with a single-cell resolution. Regular microscopy will provide information on calcium changes in cell-based assays.

(ii) We will apply NIR calcium sensor to develop cell-based reporters for neurotransmitters that express specific G protein-coupled receptors (GPCRs) and a calcium sensor. When GPCR binds the neurotransmitter, it triggers a signaling pathway that lead to increase in intracellular calcium concentration, which is detected by the calcium sensor. These cells can be used for drug screening in cells cultures, similar to other cell-based research tools, including Cell-Based GPCR Reporter Assays provided by ThermoFisher Scientific. The unique advantage of our engineered cells is their applicability for non-invasive in vivo imaging in animals. The cells can be implanted to specific areas of the brain and work as sensors in vivo. Recently, such cells were developed to reveal dynamics of dopamine, norepinephrine, and acetylcholine using common cyan-yellow calcium sensor (Nature Methods, 11:1245-52 (2014); Nature Neuroscience, 13:127-32 (2010)). Cell-based reporters expressing NIR calcium sensor will be compatible with opsin-based optogenetics technology and will enable simultaneous use with reporters based on cyan-yellow sensors for studies of dynamics of several neurotransmitters/neuromodulators.

(iii) NIR optogenetic system for gene expression will allow light control of activity dependent promoters for labeling of active ensembles of neurons precisely at a defined time. Instead of fluorescence or luminescence reporters, the neurons can be labelled with opsins to enable light-induced activation of neuronal circuits involved in memory formation or in persistent biological changes involved in SUD (Nature, 484:381-5 (2012)). We plan to create a lentivirus-based system enabling NIR light-dependent labeling of active neurons in the brain with passive reporters or active opsins.

(iv) NIR optogenetic system will allow us to create engineered cells that produce and release neurotrasmitters/neuromodulators under the control of NIR light illumination. It can be achieved through light-induced gene expression of enzymes/molecules responsible for synthesis and release of neuroactive molecules or through development of specific NIR optogenetic tools, i.e. NIR light-induced calcium channel. These engineered cells can be implanted in the desired regions of the brain to controllably produce neuro-active molecules upon light illumination. This can be used for easy modeling of different pathological conditions in the brain to test effect of candidate drugs. In other words, these cells should provide an inexpensive, easy to use method to create *in vivo* models of various neurological disease states, including SUD.

To explore commercial potential of the proposed products and establish interactions with our future customers and partners, we will start distributing minimal viable products (MVPs), which are standard cell lines expressing NIR FPs, NIR calcium sensor, and NIR optogenetic system for light-induced gene expression. Using these MVPs, potential customers and partners will be able to evaluate the research tools. Specifically, they can combine NIR sensors with opsins and other sensors. They can also try the cells in in vivo models by using implanted cells and xenograft tumors in the brain and other organs for their experiments.

4. Operation plan. Industry end users of our research tools are drug development companies that use cell-based assays and already use or would like to use animals at early drug screening

and validation stages. It can be also contact research organizations that perform in vivo studies for a large company that ordered this service.

We already started contacting drug development companies that use animal models in their pipeline. We got interest and started a communication with Regeneron. We plan to directly approach R&D scientist at other companies, including Roche, Merck, Eli Lilly and others. Companies that expressed interest in the first generation of our NIR fluorescent probes, including Novartis and Xactagen, are in our priority list. Many of these companies are developing treatments for neurological and psychiatric disorders and should benefit from new possibilities to screen drug candidates in the native environment of a living cell and animal.

We are also targeting companies providing research tools. First, we focus on established companies that currently offer technologies for in vivo imaging of biological processes, such as Perkin Elmer, Promega, and Li-COR Biosciences. We already got interest from Perkin Elmer. Second, we try to engage large life science companies that provide state-of-the-art research tools for various areas of research and drug development, such as ThermoFisher Scientific and Sigma Aldrich, both of which currently provide cell-based assays. Life science companies providing research tools are particularly important future partners for us, as our exit strategy is an acquisition by one of these companies. We already contacted and got interest from Molecular probes division of ThermoFisher Scientific. Third, we plan to target medium-size life science companies that work as distributors of reagents, in addition to selling their own products, including Sapphire North America, Mo Bi Tec, and Tebu Bio. To increase visibility of our technology, we plan to participate in biotech events and conferences and further develop our web site www.ilightbio.com.

To test the market for our desired products, learn about our customers' needs, and inspire their interest in our technology, we will provide minimal viable products (MVPs) to our potential customers and partners. We plan to provide cell lines expressing NIR FPs, NIR calcium sensor, and NIR optogenetic system for gene expression as MVPs for validation and testing by our clients under MTA agreement. Drug discovery companies will be able to test the technology in action and provide us a useful feedback that will be helpful in the future development of the ready-to-market products. Life science companies will get interest and also will be able to test our technology and provide their feedback on its development.

Our short-term plans also include IP protection for our technologies. For our NIR optogenetic technology, a provisional patent application (PPA) was submitted in October 2015 and will be converted into PCT before October 2016. For our NIR imaging technology, a PPA was submitted in April 2016. We plan to submit a US patent application for this technology within the next six months. We are also discussing a possibility to establish an option license agreement Einstein office of Biotechnology. In case of our success in this Challenge program, the funding will be spent for IP protection. IP protection and option agreement will secure our position and should lead to a more productive dialogue with potential clients and partners. Next we plan to apply for SBIR grants to fund a development of the ready-to-use products described here. We are currently looking for a scientist interested in business who will be able to work full time at the company and participate in the SBIR application.

To summarize, our short-term goals are (i) to secure IP protection, (ii) to receive feedback from potential clients and partners and to inspire their interest in our research tools, (iii) to apply for funding in the form of SBIR grants for development of our four ready-to-use products, such as (1) AAVs and lentiviruses coding for NIR calcium sensor, (2) NIR cell-based reporters for neurotransmitters, (3) lentivirus-based system for NIR light-dependent labeling of active neurons in the brain, and (4) NIR cell-based actuators releasing neurotransmitters upon light illumination.