Biweekly Report 2 - 10/13/2022 **Senior Design Group 9**

PROJECT TITLE: Characterization of Brain Metabolic State under Injury using Two-Photon Microscopy

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PI/MENTOR: Irene Georgakoudi

PROJECT DESCRIPTION: [written by Ash]

The objective of the project is to characterize brain metabolism after traumatic brain injury (TBI). To achieve this goal, we will be identifying pathways related to the injury in co-cultured cells. Members of the team will be doing cell culturing, imaging, assays, and image analysis to understand how optical readouts (imaging and analysis) relate to biochemical changes (measured by assays and metabolomics). These results will then be used to build a computational metabolic model to correlate optical readouts to changes in metabolic pathways. This fulfills the clinical need for better TBI diagnostics and treatments. Due to limited translational relevance between rodents and humans, we will be using engineered brain tissues derived from human neuronal stem cells. Cost of computational analysis is low. The main cost will come from culture materials and the engineered tissue. In the case that the metabolic computational model is too difficult to achieve, we will directly correlate optical readouts with biochemical assay results using statistical methods. TBI diagnosis and treatment is a highly researched field, so there is a risk that other research groups will be competing for similar results.

ENGINEERING DESIGN ELEMENTS: [written by Kerry]

Traumatic brain injury is the leading cause of death among individuals under the age of 45 in the US, with an incidence of 1.5 million each year. On top of fatality, TBI results in severe long-term disabilities, both mentally and physically. This makes developing a diagnostic method and treatments to TBI highly necessary for the overall improvement of the younger generation. Our work will also benefit the clinical research area and serve as a foundation for further treatment development for TBI.

The goals of this project include co-culturing cells on a 2D platform, imaging them, and analyzing the data in order to characterize brain metabolism after blast injury. These objectives will interplay to identify the pathways related to the injury in co-cultured 2D cells. We are going to design a system with three main components: protocols for 2D co-culture and associated wet lab work, imaging workflow, and analysis code for the model. The scientific methods that we will apply include 2-dimensional *in vitro* co-culture and metabolic biochemical assays. We will use multimodal two-photon microscopy for imaging and quantitative image analysis in MATLAB, which include redox ratio and phasor distributions. Additional engineering methods include computational modeling in MATLAB, cellular segmentation methods applied in Python and the use of a LEICA TCS SP8 Microscope with its associated processing software, LAS X.

The use of engineered brain tissues is precisely to avoid ethical concerns of incurring TBI to human brains or using postmortem samples. While there are ethical concerns due to the use of human neuronal stem cells, this is necessary to accurately determine if our results are clinically translatable. Additionally, while this in-vitro model is a simplified human brain, previous research has shown that it mimics that damage that is observed in in-vivo model. Regarding the cost, a great portion of this project involves computational

analysis, which is relatively inexpensive. However, the purchase of cell culture materials (such as cell media, feeder cells, and nutrients), relevant biochemical assays and treatments, such as L-glutamate and lipopolysaccharides, can contribute to a large part of the total cost of the project. The social impact of this project is to improve TBI treatment outcomes by facilitating early diagnosis. Specifically, compared to current state-of-the-art techniques, two-photon microscopy is entirely label-free and highly sensitive. By quantifying optical readouts, two-photon microscopy can be used to assess TBI biomarkers, offering novel therapeutic solutions. A key limitation is the difficulties and complexities in implementing the metabolic computation model, regarding which we decide to examine only the optical readouts.

Given the high prevalence of TBI, it is likely that there are other experiments or research plans with similar project ideas. Two-photon imaging is the most commonly used neuroimaging technique due to its high depth penetration and potential for metabolic sensitivity, so many groups researching the impact of TBI or other neurodegenerative diseases choose to use two-photon imaging. As such, the novelty of this study depends on the identification of TBI biomarkers, not just the development of a two-photon platform to study brain injury.

INTRODUCTION [written by Arri]

Traumatic brain injury can be divided into 2 phases: a primary mechanical impact to the brain followed by secondary biochemical and inflammatory cascades of different types of brain cells. However, the biochemical pathways involved in secondary injury are highly complex and intertwined, and thus, despite ongoing research, the understanding of its mechanisms and consequences remains incomplete. Notably, secondary injury can develop over a long period of time, increasing the severity of the original injury. In other words, patients with mild TBI can suffer neurological problems and long-term disability months after the injury. For this reason, patients with mild TBI have no initial symptom and, therefore, are often undiagnosed, preventing early treatment. Consequently, there is an urgent need for a technique that can diagnose TBI on a molecular level, which is the biochemical cascade of secondary injury.

To examine the long-term cellular effects of mild TBI, the 3D engineered brain tissues of neurons and glial cells (astrocytes and microglia) are injured using the controlled cortical impactor (CCI), mimicking a mild blast TBI. A non-invasive imaging technique can then be used to assess the injured engineered brain by obtaining the metrics of cellular metabolic function and relative metabolite concentrations within cells. The engineered brain tissue, while a simplified human brain, still undergoes most of the complex secondary response following the impact and, thus, can be used to develop diagnostic and treatment frameworks for TBI. However, it is challenging to completely characterize this model due to the evolving complex cellular environment and unpredictable changes arising from the interactions between multiple cell types. Thus, the optical readouts of the brain cellular interactions and environments must first be examined under precise and controlled conditions that represent all facets of TBI. This will provide the foundation for an imaging platform that can study the cellular response of complex 3D models post-injury.

We propose to formulate a relationship between output molecular concentrations from assays and optical readouts. To achieve this, the brain tissue model will be imaged with two-photon microscopy in parallel with assay analysis under the same secondary injury conditions at multiple time points. At the last time point, metabolomics will be performed. Optical readouts such as redox ratio, spectral deconvolution, and

fluorescence lifetime, can be extracted by analyzing the micrographs with MATLAB. We hypothesize that, under different perturbed or injured conditions, the trend in optical readouts and molecular concentration will be different since different metabolic mechanisms are involved. Therefore, we can say that a specific trend in optical readouts will be characteristic of a set of output concentrations. This will allow us to characterize the optical readouts for a specific injury condition.

This work will fit within the research being performed by Ph.D. candidate Yang Zhang and postdoctoral scholar Maria Savvidou in the ODDET Lab. So far, they have acquired multimodal (spectral, fluorescence lifetime, and intensity) two-photon images of the engineered brain tissues (EBT) under injury and control conditions. **Our work will focus on the specific characterization of aspects of TBI in simpler, controlled culture and injury settings in order to better understand the correlation between cellular shifts and optical readouts in the EBT data.**

While biological assay methods are well-investigated in the context of TBI [1-4], the novelty of this project lies in correlating a non-invasive, label-free method (TPEF) with these invasive methods for the eventual use of optical methods alone for a diagnostic TBI model. A non-invasive, label-free platform for the assessment of TBI does not exist to our knowledge.

EXPERIMENTAL METHODS [By Varshini and Kerry]

a. Cell Culture Protocol

We have established a technique for microglial monoculture and LPS induction already, and we have a confluent culture which we will begin experimentation on next week. We also have observed Kaplan Lab researchers executing a protocol of hINSC differentiation into neuronal monoculture, so we should be able to begin work on the monocultures as well. However, what we do not yet know are the specific aspects of our co-culture models, including finding an alternative plate for cell culture since it can be hard to perform co-cultures in the glass bottom well plates that are required for imaging.

In addition, we still have to determine the time duration for cell exposure to chemicals, as well as the number of trials needed to correlate measurements of metabolites.

The monoculture provides a baseline for co-cultures, which investigates the cell-to-cell interaction. Both the monoculture and the coculture will receive two injury inductions. Glutamate treatment will be performed on neurons for monoculture and on neuron-astrocyte co culture; LPS treatment will be performed on microglial monoculture and microglia-astrocyte coculture. LPS stimulates immune responses by interacting with the transmembrane signaling receptor toll-like receptor 4, which is preferentially expressed on microglia. Glutamate is a biomarker for brain injury, because an abnormal amount of glutamate over excites nerve cells, triggering injury cascades associated with excitotoxicity. Glutamate uptake is primarily mediated by astrocytes, and excess amounts affect both astrocyte and neuronal metabolism [dorsett].

After the injury treatments, the samples will undergo a series of assays and be taken for TPEF imaging. Seahorse assay measures and quantifies the rate of ATP production in cells using oxygen consumption rate at different time points. Mitochondria assay is cell-based and multiplexed, which will predict potential

mitochondrial dysfunction. TPEF is capable of showing high-resolution and depth-resolving images. These post-culture work would provide data for concentration, lifetime, redox metrics, etc.

Constraints and Alternatives for Cell Culture

Inverted microscopy is only compatible with glass-bottom well plates. However, co-cultures are mostly performed on trans-well plates since it's challenging to perform co-culture on glass-bottom well plates. Currently, we are considering culturing on trans-well inserts and cutting them out to place in glass-bottom well plates for imaging, but this may cause trauma to the cells. We may have to pilot this technique on our existing microglia and compare cell death rates to those not cultured and cut from trans-well inserts.

In the case where the TBI cell cultures are not viable, alternatives include using purely mathematical models based on existing data. This would complicate analyses as the existing data is 3D scaffold data which contains silk fluorescence contributions and red-stained microglia. However, all our objectives would still be possible with additional image processing steps.

b. Imaging Specification

Imaging will be performed on a Leica TCS SP8 multiphoton microscope with a tunable, pulsed femtosecond Ti:sapphire laser. This laser allows for the high peak powers necessary for two-photon absorption. A tunable laser is needed because we will use multiple excitation wavelengths to excite different fluorophores.

Our major TPEF endogenous fluorophores known thus far are NADH, FAD, LipDH, and lipofuscin. The former three are metabolic co-enzymes, and lipofuscin is a stress-associated complex of lipids and proteins. Two-photon excitation will be performed at 755nm for ideal excitation of NADH and 860nm for ideal excitation of FAD/LipDH. Lipofuscin has broad excitation and emission characteristics so it will be excited at both wavelengths.

Spectral detection will occur using a PMT with an adjustable detection bandpass. This way, the bandpass window will be shifted to collect data from spectral locations from 390-630nm at a 10nm step size.

Fluorescence lifetime imaging (FLIM) detection will occur using two hybrid PMTs (HyDs) at 460 +/- 20 nm and 525 +/- 25 nm bandwidth detection, for ideal emission of NADH and FAD/LipDH, respectively. More precisely, a PicoQuant time-correlated single photon counting (TCPSC) module will be used to acquire FLIM photon decay curves.

c. Assays

We chose our assays to give us relevant information that can be well correlated with our functional imaging techniques.

The Seahorse metabolic assay measures ATP production from glycolytic and metabolic processes. This is critically important because TPEF imaging is a metabolic imaging technique; at our excitation and emission wavelengths, we aim to preferentially detect NADH at 755ex/460em and FAD at 860ex/525em. However, recent work in our lab has shown that there may be fluorescent crosstalk between detection channels. So, using the assay will help us quantify to what extent our metabolic imaging actually does provide a ratiometric of oxidative to glycolytic metabolism, and help us correct for any inconsistencies.

A glutathione or mitochondrial Oxyblot will allow us to quantify the effect of glutamate excitotoxicity on mitochondrial function. This is another metric that we can quantify optically with the lab's algorithms to identify oxidative/glycolytic ratio and mitochondrial clustering. Again, we hope to more precisely correlate optical readouts with a specific molecular readout with the use of this assay.

Proteomics and Metabolomics

This portion of the project will be conducted in conjunction with the Lee Lab (Tufts ChBE). The Georgakoudi and Lee labs are already collaborating on a similar project for a different pathological scheme, so it is feasible to extend the collaboration to the TBI project. Assays alone will not be sufficient to develop a metabolic model of injury, as a wide breadth of measurements is needed to fully understand the impacts of intersecting metabolic pathways. We will begin with a targeted mass spectrometry measurement to quantify proteins that we know do not autofluoresce (and therefore would not be detectable optically) such as cytokines and glutamate, and see if we can still draw a correlation between those measurements and our optical readouts. While we may not be able to optically quantify specific protein effectors of injury cascades, we can understand how their levels relate to downstream metabolic shifts that we can quantify using our imaging techniques.

Overall method

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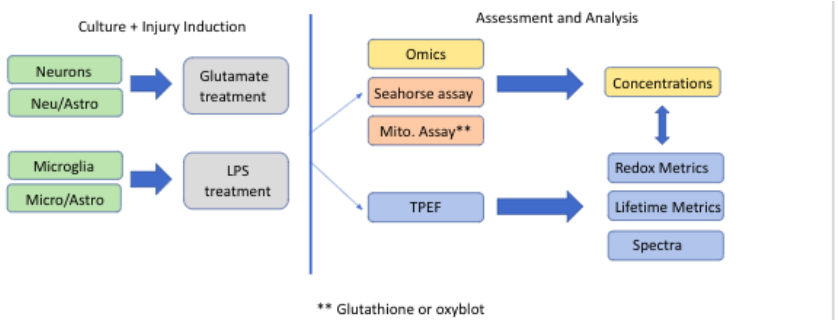

1. Establish 2D neuronal-astrocyte and neuronal-microglia co-cultures. We will differentiate hiNSCs into these cell types using a protocol developed by the Kaplan Lab.
2. Induce specific aspects of secondary injury into the 2D cultures. For each treatment, we will have 4 well replicates for imaging and analysis (so in total, 8 replicates). In particular, we aim to assess the impact of the following treatments:

a. Lipopolysaccharide (LPS), which causes systemic inflammation, oxidative stress, and the consequent production of lipofuscin (a fluorophore we are interested in quantifying as a TBI biomarker)
b. Glutamate, an amino acid and neurotransmitter that mediates much of the neuroinflammatory response
c. Peroxide, which alters the oxidative-reductive state of the cell to simulate the impaired metabolism caused by injury
d. TGF-alpha, which mediates the neuro-inflammatory response in glial cells

3. At defined time points, which are outlined in the table below, we will image half of the cultures using multimodal TPEF. The other half will be subject to metabolic biochemical assays which will give us standard readings for metabolic outcomes of different types of injury.

	Glutamate	LPS
Omics	24	48
Imaging	6, 24	24, 48
Cytokine	2, 6, 12, 24	24, 48
Mitochondrial	24	24, 48
ATP (Seahorse)	6, 12, 24	24

Table 1. Time points for imaging and assays. The unit is in hours.

a. We know the relevant timepoints for certain treatments: glutamate treatment should be studied within a few hours, while LPS generation of oxidative stress can take days to develop. However, we are still refining a formal protocol.
b. We are still researching the assays that will be most relevant for us to study cellular metabolic outputs. For now, we are thinking of using the Seahorse Assay (Agilent Technologies) which quantifies ATP production.
c. We could also have an oxygen consumption assay, cytokine assay, etc.

4. We will then analyze the images using the associated techniques for each imaging mode.
   a. Intensity: redox analysis (compute ratio of intensities at different detector channels)
   b. Lifetime: phasor analysis (transform lifetime data to frequency space)
   c. Spectral: spectral deconvolution (identify constituent spectra from overall spectrum)
      i. It is possible that the deconvolution will prove difficult given the potential number of fluorophores. In this case, we will focus on intensity and lifetime.
5. Given the results of the imaging and the results of the assays, we will establish a correlation between different optical readouts and a molecular readout from the assays.
   a. We could do this using a multiple regression, to see if combinations of optical data as input can predict assay readouts as output
   b. We would also begin working on developing a computational metabolic model which could do this optics-molecular correlation in a more flexible and translatable manner than a regression model.
6. Finally, we will apply this method to the EBT data - for which we have no assay ground truth - to understand the molecular basis of the optical readouts.

a. It is very likely that it will be difficult to directly apply the co-culture regression model to the EBT data due to inherent biological differences in the 2D and 3D models. Even if we can only do a qualitative assessment of the EBT data using our findings from the 2D computational model, we can work towards modifying the co-culture modeling approach to work for the EBT data.
b. Potentially, a PCA that can classify different types of injury in the 2D cultures can then be applied to understand the most important components of the optical data. This would be a more flexible way of transferring knowledge from the 2D to 3D cultures.

DISCUSSION

Development of A Metabolic Computational Model (CMM)

While there's no comprehensive computational model for the brain's metabolism, published research has indicated that the astrocyte computational model can be representative of brain metabolism. This is because, out of all the cells in the brain, astrocytes are the key regulators for metabolism and neurovascular coupling. [5] Additionally, astrocytes modulate most of the mechanisms that protects the central nervous system (CNS) post-injury such as uptake of extracellular glutamate from glutamate toxicity and regulation of antioxidant gene following oxidative stress. [6] From these findings, we propose to first construct a brain metabolic model from existing astrocyte models from published literature. Then, experimental data that we obtained from the assays and imaging can be included in the metabolic functions as variables so that the model mimics TBI conditions.

The dynamical model of astrocytes can be a promising function of the CMM as it describes how metabolic processes change over time, detailing all of the equilibrium and transient states. Previous research has used this model to study the brain metabolism under normal versus perturbed physiological states. [7]

The computational model for astrocyte-neuron interaction is another promising function when studying the metabolism of astrocyte-neuron co-culture. This model examines the mechanism underlying neurotransmitter transport and analyzes the metabolic changes following brain injury. We propose to employ this model with studying glutamate excitotoxicity. [8]

(Updated) TIMELINE [By Arri]

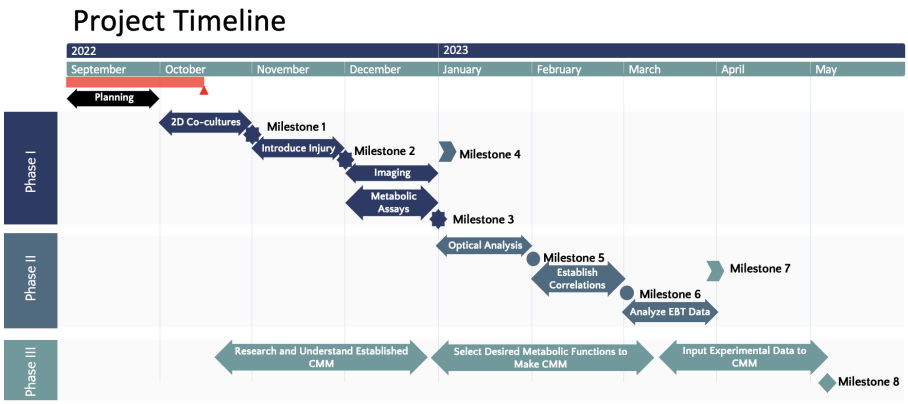

Figure 1. Timeline for project

Milestone 1: Successfully create a suitable protocol for the 2D co-cultures where the cells have high viability, which can be determined via cell viability assays.
Milestone 2: Induce the correct secondary perturbation to 2D co-cultures. This can be validated by using mass spectrometry to detect and measure the concentration of the main metabolite involved in the secondary injury to confirm that the level is high.
Milestone 3: Image cultures with multimodal two-photon microscopy and perform metabolic biochemical assays with no problem.
Milestone 4: Obtain sufficient data to perform analysis and start Phase II
Milestone 5: Analyze all obtained images with the appropriate method for each imaging modality to obtain relevant optical readouts. The obtained values should be statistically significant and, if possible, will be validated with values published in research papers. Differences between hypothesized and actual outcomes at this stage will drive the rest of Phase II and may lead to repeats of experiments performed in Phase I.
Milestone 6: Establish the relationship between optical readouts to the corresponding results from the assays. Statistical analysis will be used to confirm that there exists a strong correlation between the two variables. Additionally, the most suited regression model will correctly predict the obtained assay readouts from the optical inputs.
Milestone 7: Characterize the molecular basis of the optical readouts for the EBT data using the established regression model.
Milestone 8: Create a computation metabolic model using the optics-molecular data. Performance of the model will be compared to the published model. While there's no established computational metabolic model for TBI, the computational metabolic model under normal conditions can be modified using TBI-specific variables to mimic TBI conditions.

Timing Constraints:

Setting up an injury experiment takes a large amount of time. We may not be able to complete this project in the designated time period especially if we run into issues with our cell culture protocol. Additionally,

the computational part of the project requires data first. If we are not able to acquire this data, the computational timeline will be pushed back further.

In order to account for these challenges, the computational model outline will be established in conjunction with imaging. This way, if we run out of time, we still have a scaffold for future groups to pick up from.

PARTICIPATION [written by Ash]

The individual contribution of each group member to the project will be as follows:

1. Computational modeling includes writing code in MATLAB to analyze patterns in data found through co-cultures and imaging. Examples of results are an algorithm that simulates metabolic pathways and graphs which use the simulation to present data.
2. Imaging includes using multi-modal two-photon microscopy to observe engineered brain cell activity after simulated concussions.
3. Image analysis includes ratiometric analysis of different detector channels, phasor analysis, data processing, cellular segmentation and automatic annotation.
4. Cell culturing includes developing co-cultures and writing the secondary injury protocol

Ash will be responsible for computational modeling and assisting with image analysis under guidance from Varshini.

The first task in computational modeling is to compile existing code that models glutamate excitotoxicity, lipofuscin, general brain metabolics and any other pathways relevant post TBI. These models will likely be used on MATLAB in the form of differential equations. Ideally, the models can successfully model chemical outputs given optical readouts.

Varshini will be responsible for guiding team members through higher-level computational work and imaging/image analysis. She will be working primarily on imaging/image analysis as well as coding for the computational model.

As the one most experienced with the Georgakoudi lab, Varshini will be the main point of contact with the post-doc, Maria, who will further instruct Kerry and Arri in how to culture engineered brain tissue cells. Additionally, she will be able to connect relevant resources from the Georgakoudi lab to our project such as existing protocols and models.

Kerry will be responsible for imaging and cell culturing. She will be familiarizing herself with two-photon microscopy and using her experience in cell culturing to guide Arriety.

A part of cell culturing is developing the protocol. Kerry will be receiving Microglia cell culturing instruction alongside Arri by post-doc Maria from the Georgakoudi lab. In the meantime, she used available protocols and online papers to include well numbers, replicates, time points, duration of chemical exposure, correlating measurements and number of trials into the draft protocol.

Arrietty will be responsible for developing co-cultures and writing the secondary injury protocol. She will also be using her experience in the lab to help with image analysis.

Arrietty has been using her existing knowledge of computational modeling to find models for TBI chemical pathways. This includes compiling external resources, using her summer research and reading her BME6 paper.

Overall, we are slightly off-track because the computational models are harder to implement than we anticipated. Additionally, TBI cell cultures require additional training from the post-doc who has a busy schedule. After the training, we will be able to better define challenges, alternatives and specifics (timestamps, replicates, etc.). Additionally, Varshini is attending BMES and will be returning next week from Texas.

References

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