BME SENIOR CAPSTONE PROJECT

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

TEAM MEMBERS: Arrietty Bui, Kerry Chen, Varshini Ramanathan, Ash Sze

PRINCIPAL INVESTIGATOR: Irene Georgakoudi

ABSTRACT: Traumatic brain injury (TBI) is a leading cause of death and disability worldwide. However, TBI remains difficult to identify and treat in the clinic due to a lack of known biomarkers that can be used as diagnostic and therapeutic targets. To this end, 3D-engineered brain tissues seeded with human-induced neuronal stem cells (hINSCs) are assessed using multimodal label-free two-photon excited fluorescence (TPEF). TPEF generates endogenous fluorescence from several metabolic co-enzymes and stress-associated cellular products, which are measured by spectral intensity and fluorescence lifetime imaging. We aim to correlate optical measurements with biochemical and metabolomic assays in the context of two major aspects of TBI, glutamate excitotoxicity and oxidative stress. This work will ultimately be used to develop a metabolic model that will use optical measurements to identify biomarkers that are implicated in TBI-associated pathways.

KEYWORDS: Traumatic brain injury, two-photon imaging, metabolic pathways, mass spectrometry, 3D-engineered brain tissue, metabolic computational model

ELEMENTS OF ENGINEERING DESIGN:

The *design of this project* is the characterization of optical readouts by mapping them to specific metabolic pathways that are affected by injury. This can be achieved with a 3-compartments system: cell culture, imaging, and computational model. The *objectives are set* based on each compartment as follows. First, a specific injury will be induced in mono-culture and 3D co-culture and its impact will be examined via microscopy, metabolic assays, and mass spectrometry. Then, computational models will be created to identify affected metabolic pathways from biochemical data. These *objectives can be tested and evaluated* as follows. Once a comprehensive cell culture protocol has been developed; we will only consider our cultures viable if they remain stable at passage 3 and are healthy as observed in baseline imaging readouts, which will be compared to imaging readouts from validated cultures in the lab. Mass spectrometry will follow the validated protocols from the Lee lab and therefore does not require evaluation. Similarly, the acquisition of optical images will be adapted from the imaging protocols of the Georgakoudi lab. We will evaluate the successful induction of our injury conditions by asserting that trends of optical readouts and mass spectral data converge, indicating that the experimental treatment successfully induced a consistent metabolic shift. As an additional safety net, results from metabolic assays should agree with the data from mass spectrometry given that both methods measure metabolomics concentrations.

Multiple *engineering principles* are applied in this project with the most prominent one being two-photon microscopy (TPEF) - an imaging modality for injury assessment. Compared to standard fluorescence microscopy, TPEF utilizes a pulsed, non-linear excitation process where 2 photons are used to excite the fluorophore. By lowering the amount of energy needed per photon, TPEF uses a longer wavelength, which generates less tissue damage and penetrates deeper. Notably, sufficient laser intensity for this excitation is only achievable in the focal plane. This restricts the volume of the signal generation as out-of-focus signals from the planes above and below the focal plane of the sample are removed. These characteristics make TPEF depth-resolved, facilitating the imaging of thick and highly scattering specimens like engineered brain tissue (EBT) without the need for slicing or biopsy (non-invasive). For this project, endogenous fluorophores such as FAD and NADH will be used so that the imaging process is label-free where samples can be dynamically examined over time (live imaging).

There are 2 major *realistic constraints*: ethical concerns and translatability of the computational model. Specifically, there are ethical concerns about incurring TBI in human brains or using postmortem samples. As a *solution*, we will use 3D-engineered brain tissues which show pathophysiology observed in an *in-vivo* model [15]. 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, we plan to use a model of brain metabolism at baseline derived from literature because there is no existing TBI metabolic model to our knowledge. Metabolic model source code is difficult to obtain, and models may be designed based on assumptions and conditions specific to the institution which published that model. That is, it may be a non-trivial task to adapt existing models in a way that is relevant to our data. A solution to this problem would be to write our own model based on the key differential equations governing the metabolic processes of interest to us (central metabolism, glutamate-glutamine conversion, and oxidative stress). This would be outside the scope of our capstone but would be doable by future students.

DESIGN FLOWCHART

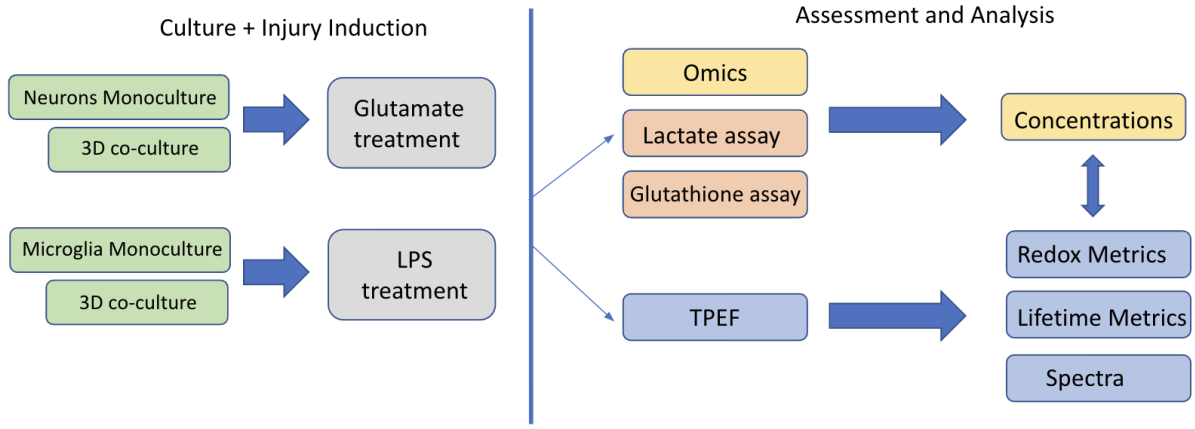


Figure 1. Schematic overview of experimental plan

DESIGN ELEMENT TABLE

Design Elements	Success Measures
LPS - microglia monoculture Glutamate - neuronal monoculture	Study 1: Optimize injury conditions - Optical readouts indicate a decrease in free NADH and glycolysis shift a. Phasor shifts to bottom right b. Redox ratio increases c. Spectral constituents have an increased NADH concentration
LPS - 3D neuron-microglia co-culture Glutamate injury - neuron-astrocyte 3D co-culture	Study 1: Optimize culture conditions - No significant difference in optical readouts between Kaplan Lab's and our scaffolds Study 2: Optimize injury conditions - Verify that, using statistical analysis, injury occurs a. Cell viability decreases after injury b. Glutathione assay indicates that glutathione is down-regulated in glutamate excitotoxicity c. Glutathione assay indicates an increased oxidized-reduced glutathione ratio for LPS condition (oxidative stress) Study 3: Induce and asses injury - The results of mass spectra and metabolic assays should be consistent with each other - Student's T-Test shows significantly different peak heights in glutamate and glutamine at 0h and 24h for glutamate excitotoxicity - The trend of ptical readouts is similar to that of monoculture
Computational Model Differential Equations	1. Investigate the outline and syntax required to create differential equations and their associated graphs 2. Establish a set of differential equations to model the concentration of

MATLAB Functions	upstream, TBI-related molecules using results acquired from mass spec 3. Create functions using the differential equations on MATLAB to simulate specific concentrations at specific times a. Analyze the simulated upstream pattern for TBI indicators b. Verify the output of our function with the experimental results

Table 1. Table describing design elements and success measures to validate and verify them.

INTRODUCTION AND BACKGROUND

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. Beyond fatality, TBI results in severe long-term disabilities, both mentally and physically. [3] Pathophysiologically, Traumatic brain injury can be divided into 2 phases: a primary mechanical impact on the brain followed by secondary biochemical and inflammatory cascades of different types of brain cells. The two major biochemical cascades that we plan to characterize are oxidative stress and glutamate excitotoxicity [9]. Following the injury, an influx of excess calcium ions into the mitochondria triggers the production of reactive oxygen species (ROS) and free radicals. These molecules depolarize the mitochondrial inner membrane, disrupting the electron transport chain and inhibiting the oxidative phosphorylation process. This deprives the nerve cells of ATP and facilitates apoptosis. In junction with oxidative stress, glutamate and aspartate neurotransmitters accumulate at the synapses as the impaired glutamate transporters fail to recycle excess glutamate from injured neurons. These molecules bind to NMDA and AMPA receptors that promote calcium, potassium, and sodium uptake. Cell depolarization triggers downstream cascades that prolong the effect of oxidative stress. From these observations, it can be said that the biochemical pathways involved in secondary injury are highly complex and intertwined. 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 symptoms and, therefore, are often undiagnosed, preventing early treatment. Consequently, the *long-term objective* is to develop 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 (EBT) of neurons and glial cells (astrocytes and microglia) are injured using the controlled cortical impactor (CCI), mimicking a mild blast TBI. The EBT model, 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, with CCI, 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, we *propose* to examine TBI via its constituents by introducing a specific secondary injury to 2D brain cell cultures and studying the cellular metabolic interactions and environments in a controlled manner.

Two-photon excited fluorescence (TPEF) can then be used to assess the functional and morphological changes of the injured brain cells by obtaining the metrics of cellular metabolic function. TPEF detects autofluorescent signals from several key biomolecules, namely FAD, NADH, LipDH, and lipofuscin. The former three are metabolic coenzymes which are implicated in most metabolic perturbations, and lipofuscin is a complex of fluorescent proteins and lipids that accumulates under cellular stress conditions. These endogenous fluorophores can be analyzed using computational techniques that reveal underlying concentration-based and metabolic shifts in the samples: redox ratio, mitochondrial clustering, phasor analysis, and spectral deconvolution.

The redox ratio is the relative ratio of glycolytic to oxidative metabolism. In brief, it is computed by obtaining a “NADH image” (755ex/460em) and a “FAD image” (860ex/525em) and dividing them

according to the formula (NADH/(NADH+FAD)). *Mitochondrial clustering* is the extent of mitochondrial fractionation, which occurs in response to ROS accumulation. It is computed by segmenting and cloning mitochondrial regions in an image. Then, the power spectral density of the cloned image is computed, which determines the image frequency. Highly fractionated mitochondria will have a high frequency, and vice versa. *Phasor analysis* is a technique to obtain fit-free visualizations of FLIM images with overlapping concentrations of lifetimes over different pixels.

In brief, time-series fluorescence lifetime data is sine and cosine transformed, giving two coordinates *g* and *s* that correspond to the lifetime, *tau*, of the fluorescent decay. Any one *tau* localizes on a circular plot (see Fig. 2). The localization of the (*g, s*) coordinate pair for any given pixel is determined by the linear combination of different *tau* values constituent in the pixel. A fluorophore's binding environment affects its lifetime, but its concentration does not (i.e. higher concentrations of a single fluorophore simply cause a shift in the phasor distribution towards that fluorophore's lifetime). As such, by assessing the overall phasor distribution, conditions such as shifts in relative concentrations of fluorophores and shifts in fluorophore binding configuration can be observed. *Spectral constituents* are obtained from the overall spectral intensity curve via non-negative matrix factorization. In this method of spectral deconvolution, the user specifies the number of total constituents and the model computes optimal concentrations of non-negative vectors and weights that minimize the error (residual) from the overall spectrum. In this way, concentrations and emission spectra of constituent fluorophores are determined.

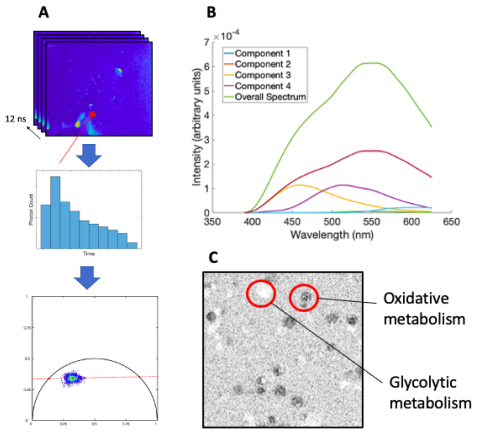


Figure 2. Description of lifetime phasor analysis (a), redox ratio (b), and spectral deconvolution (c).

Compared to state-of-the-art diagnosis procedures like MRI, TPEF is more sensitive to cellular-level metabolic shifts. However, TPEF fails to detect non-fluorescence metabolites such as lactate. Additionally, while it is known that an increase in redox ratio correlates to an increase in glycolytic metabolism and vice versa, conducting redox ratio studies in conjunction with exact biochemical measurements will allow us to quantify how shifts in oxidative and glycolytic metabolism affect our optical readouts. This lack of specificity is a *critical roadblock* for using TPEF to study injured brain metabolism. Consequently, the *specific goal* is to characterize and map optical metrics to specific altered metabolic pathways predicted by a metabolic computational model. The *central hypothesis* for identifying the pathways is to input relevant biochemical metrics from assays and mass spectrometry to the computational model.

Two-photon imaging is a 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. Additionally, biological assay and mass spectrometric methods are well-investigated in the context of TBI [1-4]. Therefore, **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. 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.

In general, we propose to formulate a relationship between output molecular concentrations from assays and optical readouts via a computational model. 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 and from the computational mode, specific altered metabolic pathways. 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 EBT model 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.*

SPECIFIC AIMS, METHODS, AND RESULTS

Specific Aim 1 (SA1): *Assessment of controlled secondary injury in monoculture*

Thus far, injury to the 3D engineering brain tissue is induced in a non-specific manner via a stereotaxic impactor. In order to study a targeted metabolic cascade (secondary injury) of TBI such as glutamate excitotoxicity, specific metabolic perturbations must be introduced and changes to relevant metabolic pathways can be detected. However, since this study has not been done previously, it's important that we perform a preliminary study on the monoculture to optimize treatment concentrations and refine detection methods.

Study 1: Induce secondary injuries to monoculture of microglia

Excess lipopolysaccharide (LPS) can be added to the microglia monoculture to trigger oxidative stress. The rationale for using microglia as a primary cell line is because LPS interacts with transmembrane signaling receptor toll-like receptor 4 which is expressed primarily on microglia. Our postdoc, Maria, has determined an optimal exposure time and concentration (1 ug/mL at 100 uL which successfully induces LPS uptake in microglia. We will use 3 well replicates for each concentration at each time point, and one control set. To assess the cultures, we will use multimodal TPEF (see Data Acquisition) at 12 and 24 hours. At the last time point, mass spectrometry will be performed on the injured condition and the control condition.

The *success measure* is to validate that the optical readouts trend consistently toward glycolysis, as it is well-known that oxidative stress causes a shift to glycolytic metabolism. The phasor distribution should move to the bottom right to indicate increased free NADH, the redox ratio should increase, and spectral constituents should have an increased NADH concentration; all of these validations are based on previous work on oxidative stress in the ODDET lab.

Former Study 2: Induce secondary injuries to monoculture of neurons

Because neuron-astrocyte (NA) co-culture models are mostly neurons with few astrocytes, we decided not to pilot on neuronal monocultures because there is not a significant added degree of complexity between the neuronal monocultures and the NA co-cultures.

Data acquisition and analysis: The effect of the induced perturbations will be examined via two-photon excitation microscopy. We will perform multimodal (spectral intensity and fluorescence lifetime) TPEF acquisition according to standard imaging protocols in the Georgakoudi lab. We will measure spectral intensity at 755nm, 860 nm and 910nm excitation, detecting on a multi-wavelength PMT every 10 nm from 490 to 630 nm. We will measure fluorescence lifetime at the same excitations but detect only at 460 and 525 nm using a hybrid detector and a PicoQuant TCPSC module which allows for the high temporal resolution needed for fluorescence lifetime imaging. Redox ratio, mitochondrial clustering, lifetime phasor distributions, and spectral constituents are obtained by custom-written MATLAB code.

Potential Pitfalls and Alternatives:

Glutamate excitotoxicity is a fast-acting and sensitive process; also, unlike LPS induction, we have not previously performed this protocol in the lab. Therefore, it is possible that we will encounter difficulties inducing sufficient glutamate uptake or dealing with unprecedented reactions to the addition and removal

of exogenous glutamate. Alternatively, a previous study has shown that 200 μM of DL-TBOA is sufficient to inhibit the NMDA glutamate receptor and, thus, induce glutamate excitotoxicity in human neurons derived from embryonic cells without extraneous glutamate [14].

Specific Aim 2 (SA2): *Assessment of glutamate injury in neuron-astrocyte 3D co-cultures.*

Study 1: Optimize culture conditions for 3D neuron-astrocyte and neuron-microglia co-cultures

We will begin by using neuron-astrocyte scaffolds prepared by the Kaplan Lab's brain group, which are available to us through a related collaborative project on neurodegeneration. In parallel, as we conduct Study 2 onwards on these scaffolds, we will work on mastering the cell differentiation and seeding protocol so that we can continue to prepare our own scaffolds as per the Kaplan Lab's established protocol. Our *success measure* will be a repetition of an imaging experiment under baseline conditions on both scaffolds seeded by the Kaplan Lab and by us. Our *success measure* will be no significant difference in imaging readouts (TPEF redox ratio, FLIM phasor distributions, spectral emission) in our scaffolds and the Kaplan lab's scaffolds.

Study 2: Test glutamate and LPS conditions for 3D neuron-astrocyte co-cultures using glutathione assays and imaging

Glutamate:

As noted above, we won't pilot on the monocultures and thus, we have changed the aims of this study to be more comprehensive. We will test glutamate concentrations from 100-300 uM at exposure times of 15 and 30 minutes as well as a long exposure (6 hours), as per a range of papers that are summarized in the Appendix. Note that the upper range of the glutamate concentration is higher than that mentioned in the papers because, unlike the paper, we will use 3D culture and 3D diffusion is worse than 2D diffusion. Consequently, to achieve sufficient cell death caused by glutamate excitotoxicity, we will have to examine higher glutamate concentration We will replace the cell media with a magnesium-free minimal medium for one day prior to injury because magnesium can occupy AMPA receptors and prevent glutamate activation. After glutamate exposure, we will rinse the scaffolds with minimal medium and replace them with the normal neurobasal medium.

LPS: We will test those conditions (100 ug/mL at 100 ul, 24 hours exposure) as well as two auxiliary conditions (75 and 150 ug/mL) on the 3D scaffolds and assess as in SA 1, study 2.

We will image these cultures at 0, 6, 12, and 24 hours using two-photon spectral and FLIM imaging as described in SA 1. We will conduct 3 imaging replicates on one scaffold for three concentrations and time points, yielding 9 total datasets. We will *assess cell viability* visually, via manual image assessment, as dead cell bodies in scaffolds can be identified as round, bright debris in two-photon images. We will *assess success* by comparing imaging signal-to-noise ratio and cell death numbers to those acquired under optimal conditions in the previously-assessed 2D monoculture.

Regarding success measure, we will *assess glutamate uptake* via a glutathione assay (Sigma Aldrich), as we expect glutathione to be down-regulated under glutamate excitotoxicity. We will *assess oxidative stress* as successful if we observe an increased oxidized-reduced glutathione ratio, as determined through the glutathione redox assay, as glutathione's antioxidant activity will be induced by the LPS activation.

Study 3: Induce glutamate and LPS injury and assess using imaging, mass spectrometry, and glutathione assay.

We will use our optimized conditions from Study 2 and induce glutamate excitotoxicity and LPS stress at one concentration and one exposure time with the media protocol described above. We will conduct a glutathione assay conducted at 12 and 24 hours and mass spectrometry conducted at 24 hours. We will image as described in SA1 for each injury condition.

Glutamate: We will use three imaging replicates on three injury scaffold replicates, with three imaging replicates on one control scaffold (medium change and rinse only) and one non-treated scaffold (no rinse and no glutamate). We will assess glutamate uptake as described in Study 2, and we will assess

the effectiveness of the mass spectrometry protocol by observing significantly different peak heights in glutamate and glutamine at 0h and 24h (Student's T-Test).

LPS: We will use three imaging replicates on three injury scaffold replicates, with three imaging replicates on one control scaffold (LPS solvent medium only) and one non-treated scaffold. We expect to see a significant difference between *at least two* of the metabolite peak heights; unlike glutamate, we do not do the direct metabolic consequences of oxidative stress, so we cannot obtain a precise benchmark of success for the mass spectrometry peaks.

Data acquisition: As in SA 1 and:

Mass Spectrometry: Relevant metabolites (based on previous research) – glutamate, glutamine, creatinine, and decanoic acid – will be tagged so that their concentration changes can be detected with mass spectrometry [13]. The *goal* of using mass spectrometry in both studies is to identify metabolites that are heavily upregulated or downregulated after injury induction and verify that the detection method is suitable. To achieve this, we will conduct mass spectrometry at the start and end time points.

Data Analysis

Imaging: As in SA 1.

Mass Spectrometry: ANOVA with a post-hoc Student's t-test will be used to determine treatment concentration, for each study, that results in significant concentration change of desired metabolites.

Potential Pitfalls and Alternatives:

If no peaks are significantly different between baseline and injured mass spectrometry results, we will have to consider tagging different metabolites. We will have *verified* the LPS uptake by comparison with the previous benchmark set by Maria, so we should not have to consider the lack of LPS uptake as a reason for failed results. However, we can perform Nile Blue staining of our scaffolds, which localizes to lysosomes and will stain the lipofuscin produced by oxidative stress, in order to ascertain that LPS uptake was successful.

Specific Aim 3 (SA3): *Develop a computational metabolic model that predicts injury pathway activation based on biochemical readouts.*

Study 1: Develop a basic computational metabolic model for TBI cultures

We will obtain relevant brain metabolism computational models from the literature (neuron/astrocyte/microglia metabolism, oxidative stress models, and injury models) and modify them for the purposes of our study by adjusting concentration conditions based on our mass spectrometry results from SA 1 and SA 2. Molecules involved in the model that are not present in our spectrometric results will be treated as assumed constants based on literature values. The completed model will include central metabolism, detoxification of reactive oxygen species, and the glutamate-glutamine cycle. It will be able to predict the relative level of pathway activation (ex. glycolytic vs. oxidative metabolism) based on the input concentrations of downstream metabolites, as obtained from mass spectrometry. We will validate the accuracy of our metabolic model by performing mass spectrometry on baseline cultures and assessing the similarity of our predicted pathway results with results obtained from the metabolic models from which our model was derived. This is to ensure that in integrating multiple models, we preserved the integrity of each individual model.

Study 2: Use the metabolic model to predict injury pathway activation

From SA 1 and SA 2, we will have imaging data from secondary injury at multiple time points and corresponding mass spectrometry data from the final time point. We can use the metabolite concentrations and the metabolic model to predict levels of pathway activation under injury conditions, and then correlate those pathway activations with the optical readouts from those same conditions. While the metabolic model does not output pathway activations, it predicts concentrations of upstream effectors

that would cause observed downstream metabolite concentrations. By associating upstream effectors with particular injury pathways, we can estimate the pathway activations under different injury conditions.

There are a number of published models investigating brain metabolic injury pathways, including ODE-based modeling, flux balance analysis, metabolic diffusion analysis, multi-domain spatially distributed brain energy metabolism models, etc. Regarding microglia, a paper uses a flow cytometry-based analysis using a controlled cortical impact model after TBI injury on mouse microglial cells, where isolated microglia undergo morphological changes and expression of activation markers are examined [16]. Another paper discussed using cytokine assays on microglial cells to see their regulatory mechanisms of microglia-mediated neuroinflammation. Specifically, a cytokine signaling network is established for the regulation of TNFα, IL-6, IL-10, TGFβ, and CCL5 after introducing bacterial lipopolysaccharides (LPS) [17]. A 5-compartment model uses flux balance analysis to estimate the kinetic model parameters using the basis of glutamate concentration in the synaptic cleft and ATP hydrolysis. This model is governed by Michaelis-Menten equations on kinetic mass balance to see reaction rates and transport of the biomarkers [18].

Currently, there are no models that precisely cover our specific aims. However, the papers have a detailed outline of the differential equation sets that they used to derive their model, which we can replicate and modify. Our goal is to develop our own models for brain metabolite analysis using the existing models and mathematical equations.

Data Acquisition: This SA uses data obtained in SA 2.

Data Analysis: We will correlate optical readouts with pathway activations in LPS and glutamate conditions using a linear regression across time points. We will train on 4 out of the 5 replicates from SA 1 and test on the other using k-fold cross-validation. The regression will be validated based on a 0.85 Pearson correlation, and a statistically significant improvement for testing data from the wrong pathway to the correct pathway (i.e. glutamate optical readouts correlated against LPS concentrations should correlate significantly worse than glutamate readouts correlated against glutamate concentrations).

Potential Pitfalls and Alternatives: If the linear regression cannot separate the injury pathways, we will consider machine learning approaches. This will require much larger-scale data acquisition and would likely go beyond this project's scope. However, future students could improve replicates of this data across more concentrations. Then, they could use a logistic regression based on imaging and mass spectrometry raw data to directly classify imaging data as either glutamate-resultant or LPS-resultant.

PRELIMINARY RESULTS

We are using this space to describe the majority of our experimental progress over the past few weeks. We have not directly addressed our Specific Aims in these experiments, but these experiments and analyses are providing the foundation which we will eventually use to conduct our experiments and provide a baseline for our specific aims' results.

Cell culture Workflow

Arri and Kerry started cell culture training, specifically developing a cell passaging protocol, with HMC3 cell line with our postdoc Maria. Using this protocol, we will be able to work independently in the biohood moving forward. We discussed sterile techniques for biosafety hood operations and the rationale for cell splitting.

Protocol for passaging cell:

Materials needed: DMEM cell culture medium, trypsin, HMC3 cells in cell culture dish.

1. Warm the DMEM and trypsin in a 37˚C water bath for 15~20 minutes.

2. Spray the biohood workspace with 10% bleach and 75% ethanol and wipe surface with tissue paper.
    a. This also applicable to any bottle or items that will be brought into the hood such as DPBS, new and old cell culture dish, centrifuge tubes, DMEM, Trypsin
3. Aspirate old media from the cell culture dish
    a. Avoid opening disk completely and tilt disk to 1 side to facilitate aspiration
4. Wash twice with 5 mL PBS
    a. Aspiration of the PBS should be done gently and not be directly in contact with the cells attached to the bottom surface of the dish
    b. After adding PPBS, shake disk gently to distribute PBS
5. Add 1.5 mL of 0.25% trypsin-EDTA and put the dish in the 37˚C incubator for 2-3 minutes
    a. The enzyme Trypsin cleaves the adhesion complexes formed between the cells and the bottom surface of the dish, detaching the adherent cells
    b. After 2-3 minutes, use microscope to check confluency / cell suspension
6. Add 8.5 mL of media to the cell culture dish
    a. The added media serves to deactivate the enzymatic reaction
7. Transfer the cells (with trypsin and cell culture media) into a 15 mL conical centrifuge tube
8. Centrifuge at 1500 rmp for 3 minutes to pellet the cells
    a. This allows the cells to be free from trypsin
9. Aspirate cell culture media in conical centrifuge tube and re-suspend the cell pellet in 5 mL of new cell culture media
    a. Make sure the cells are well-mixed by gently pipetting
    b. Avoid bubbles formation
10. Calculate the desired seeding ratio and add the corresponding amount of cell suspension and media to the new cell culture dish
    a. This depends on when the cells will be used
        i. We plan to independently practice cell passaging in 5 days so we picked a ratio of 1:5 so that sufficient confluency is reached. This means that ⅕ of the cell suspension (0.5mL) and 9.5 mL of new medium are needed for a 10 mL dish
        ii. Extract 0.5 mL of the cell suspension from conical centrifuge tube and gently inject into the new cell culture dish with 9.5 mL of fresh media
    b. Tilt the dish in north-west-south-east direction to distribute the cells
11. Aspirate and discard the old cell suspension in the centrifuge tube to biohazard bin
12. Put the new cell culture dish in the 37˚C incubator overnight

Imaging Workflow

We have spent time over the last few weeks observing and practicing with our postdoc Maria as she acquires imaging datasets from neuron-astrocyte (NA) scaffolds for a different project on the relationship between the HSV virus and Alzheimer's. As such, the brain model is relevant but the outcome is not relevant to our project. We are being trained and supervised on this data to save time, as Maria has 10-day acquisition periods for this project which prevent her from being able work on the TBI project during the data acquisition periods. Arri and Varshini have compiled an imaging protocol from this observation which will be used for our NA scaffold imaging for glutamate excitotoxicity. The protocol reads as follows:

1. Maintain Excel spreadsheet from centralized code base (on Georgakoudi network drive) to auto-generate file names for control and injury scaffolds.
2. Maintain plastic well plate with labeled control and injured scaffold sections and numbered wells to track each ROI throughout timepoints.
3. For each scaffold, remove from its place in the well and secure it with a metal harp to a glass bottom dish. Place the smooth (non-cut) side up.
4. Set the 40x water objective and add one drop of DI water. Lower the objective z stage fully and secure the dish in the sample stage.
5. Search for cells using brightfield viewing through the eyepiece. Cells are transparent in this mode but the edges can be seen when adjusting the Z height using the tuning knobs. Once cells are found, turn off the microscope internal light, and lower all coverings, then confirm the presence of cells in Live imaging mode (sometimes identifying cells in the above manner leads to silk identification, so it's important to do this confirmation step).
6. Set the depth range based on the range of visible cells. Z slices are 4 microns apart.
7. Begin with intensity acquisition mode (xyz). All settings (line average, frame average) are set to 1, except for 8 frame accumulation. Speed is 60, bidirectional. The pinhole is at 1 AU (airy units).
8. Turn on the transmission PMT and both PMTs and HyDs.
9. Set and tune the wavelength for 755 nm.
10. Begin acquisition. Save name according to spreadsheet.
11. Repeat for 860, changing the depth by +1 micron to adjust for laser co-registration.
12. Change to FLIM acquisition mode. Set CFD (voltage) to 40 for 755, 60 for 860. Acquire and ensure that decay traces are smooth with no interrupting signal. FLIM data is saved separately and must be renamed individually.
13. Change to spectral acquisition mode (xy-lambda). Change detector to the wavelength-adjusting PMT. Open the pinhole to 7.77 AU (full opening).
14. After spectral acquisition, change the pinhole back to 1 AU immediately as the open pinhole causes photobleaching.
15. Repeat for as many ROIs are acquired per scaffold.
16. Retrieve the plastic well plate from the incubator. Return to the hood. Spray a tweezer with ethanol and dry in the hood. Pick up the scaffold from the edge and return to its original well. Retrieve the next scaffold and continue.

LPS Microglial Optimization

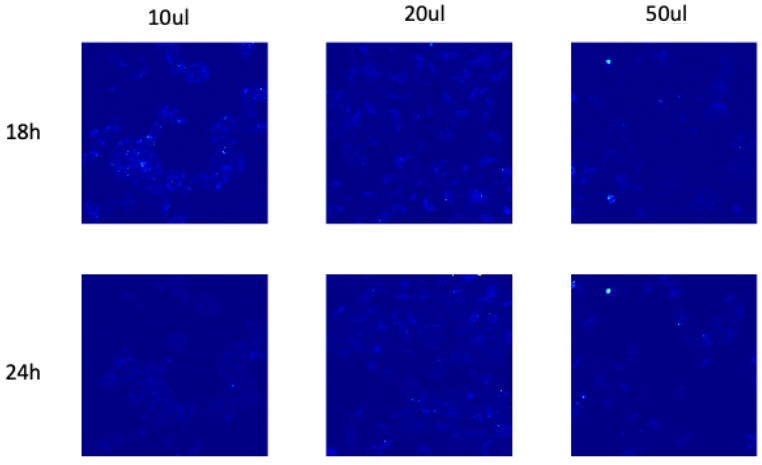


Figure 3. Optimization parameters for LPS induction in microglial monocultures.

Images of increasing concentrations of LPS in microglia at 755 nm excitation and summed spectral emission (490-630 nm) are shown in Figure 3. The same regions were imaged at 18 hours and 24 hours post-induction. Images are normalized to maximum intensity. LPS localization, indicated by the saturated bright spots, is present at all concentrations and time points but is most noticeable at 50 ul at 18h, and at 20 and 50 ul at 24h. The same regions, particularly at 50ul, accumulate more LPS as a

function of time. Additionally, at high concentrations, particular regions seem to preferentially uptake LPS, which is undesirable for studies of multiple cell regions. However, due to a lack of replicates – this pilot was done at 6 concentrations (3 concentrations are not shown) for 2 timepoints with only 1 sample per condition – no conclusions can be made about optimal concentrations, and optical readouts cannot be analyzed with statistical significance.

FLIM Phasor Preliminary Analysis

While the experimental process gets off the ground, Varshini has been working on FLIM analysis of primary injury (which are old datasets acquired by Maria and Yang) and is trying to simulate the role of different fluorophores in causing a phasor distribution. The goal of this analysis is to develop an understanding of how simultaneous activation of multiple injury pathways (via primary injury) affects FLIM readouts, which will help us understand if pathway-specific injury can be determined via imaging readouts by comparing pathway-specific and primary injury results. These findings are summarized below.

I (Varshini) did a comprehensive analysis of phasor distributions from neurons, astrocytes, and microglia cultured on 3D scaffolds and subject to CCI. They were imaged at 8h, 24h, and 48h. I show here the major phasor distribution phenotypes present in the 8h data. 24h and 48h data are complicated by signal issues due to cell death and system issues that cause one detection channel to be unusable.

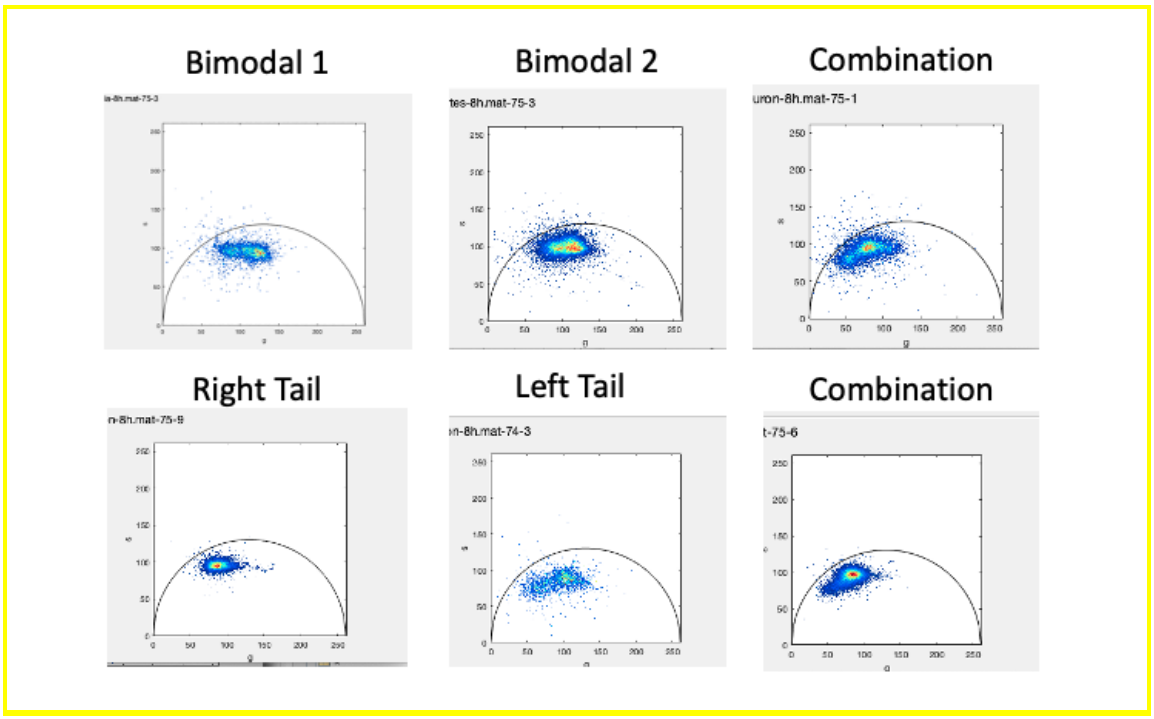


Figure 2. Characterization of Phasor Distributions in 8h Post-CCI Monocultures. The 4 major phenotypes observable in the injured phasor distributions are shown, each with a representation of that phenotype. Two distributions with combinations of multiple distribution types are also shown.

Modality of a phasor distribution determines how many distinct cellular populations there are in an image. A specific combination of fluorophores will localize as an elliptical, unimodal distribution (see Background figure). Non-injured phasors tend to be largely unimodal except for the occasional presence of the right tail. Therefore, I suspect that the multi-modality of these distributions is a result of the metabolic response of certain cell types to injury. To this end, I used the phasor simulation I developed

last summer to test some possibilities of what fluorophores could be causing these multi-modal phenotypes. For now, I focused on the "Bimodal 1" phenotypes, which has a slight negative slope to the distribution with two distinct distributions localized on the left of the phasor distribution. I tested two possibilities, an increase in bound NADH and an increase in FAD, which are shown in Figure 3. I create a simplified variant FLIM image by splitting an image into "patches" (here, there are two patches to simulate a bimodal distribution). Because FLIM analysis is in the frequency space, it is location-invariant, so it is scientifically sound to simulate cellular population as distinct halves in an image rather than actually simulating cellular morphologies.

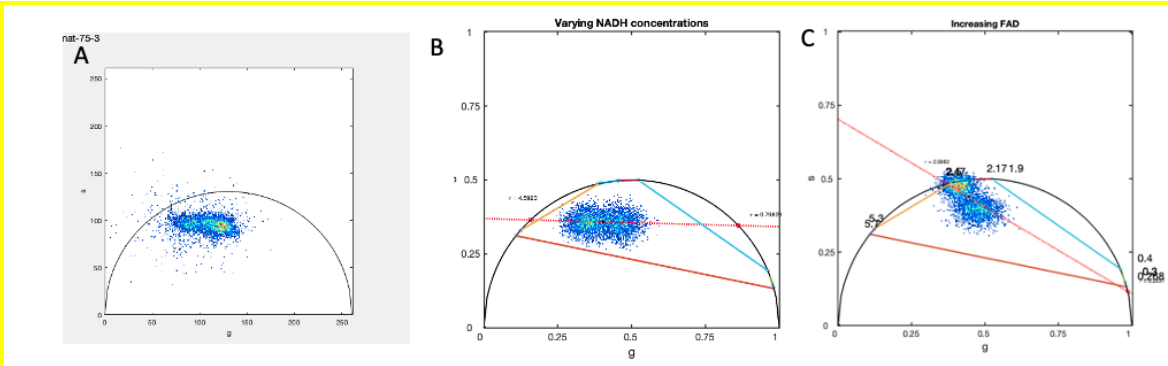


Figure 3. Simulation of 8h Post-CCI Phasor Distributions (a) An experimental distribution from 8h neurons which follows the "bimodal 1" phenotype. (b) Simulation of increasing bound NADH (NAD(P)H) in one half of the image, all other factors constant. © Simulation of increasing FAD in one half of the image, all other factors constant.

The bound NADH simulation correctly recapitulated the overall distribution location, but did not capture the negative slope of the distribution (compare the slope of the red line in (b) to the tilt of the distribution in (a)). To mitigate this, I tried simulating an FAD increase next, which did make the slope too negative but at an overshoot, even with very minute concentration variations (0.1 difference between the two halves of the image). Therefore, I suspect that the "bimodal 1" phenotype may be a combination of increased NAD(P)H as well as increased FAD. Again, although this does not directly impact our specific aims, this is important foundational knowledge to understanding the composition of the CCI fluorophore composition so that we can compare it to the data we plan to acquire of secondary injury.

Computational model framework and progress

The computational model will be built in MATLAB and relies on the differential equations provided by papers modeling metabolic pathways relevant to TBI. Blood flow equations will not be used because it does not apply to our engineered tissue models.

Our goal is to create a system of first order differential equations which takes experimental results from our project and outputs downstream values. Our results may not cover all inputs required by the equations given by the papers we read. Therefore, certain constants and inputs will need to be inferred from papers or just be set to a constant value.

Varshini had the chance to use ODE's in MATLAB in her bioinformatics class. Using that prior knowledge, she instructed Ash on the basics of the process. He investigated Varshini's existing code along with the equations provided by the metabolic modeling papers. In order to implement an ordinary differential equation, We first need to set rate parameters which cannot be modified. These will be values provided by the papers we read. Second, we need to set initial concentrations which would depend on experimental results. The ODE implementation puts the variables together such that they can be solved by

an ODE and plotted using multiple conditions (varied initial concentrations). The example below uses S0 = 0.07 and E0 = 1.0 but the values can be changed, or written as vectors to model multiple conditions. The example ODE below is not specific to any metabolic pathway. It is a test run of using ode23s in order to better understand how to implement more complicated equations in the future.

```
%% ODE Implementation
y0 = [E0; S0; ES0; P0];
[t, y] = ode23s(@(t,y) write_enzyme_ode(t, y, KCAT, K1, k1m), tspan, y0);
plot(t, y(:,4)); % plot different columns to see different variable concs
title('Product Concentration via Analytical ODE Solution');
xlabel('Time (s)');
ylabel('Concentration of Product (mM)');

% if you want to test multiple conditions, this will plot them all on one
% graph (you need to write E0 the initial concetrations as vectors tho
P_ode = cell(num_cond,1);
legendText = cell(1,num_cond);
subplot(1,2,1); hold on
for i = 1:num_cond
    y0 = [E0(i); S0(i); 0; 0];
    [t, y] = ode23s(@(t,y) write_enzyme_ode(t, y, KCAT, K1, k1m), tspan, y0);
    plot(t, y(:,4));
    P_ode{i} = y(:,4);
    legendText{i} = strcat("S0 = ",num2str(S0(i)), ", E0 = ",num2str(E0(i)));
end
legend(legendText);
title('Product Concentration via Analytical ODE Solution');
xlabel('Time (s)');
ylabel('Concentration of Product (mM)');
```

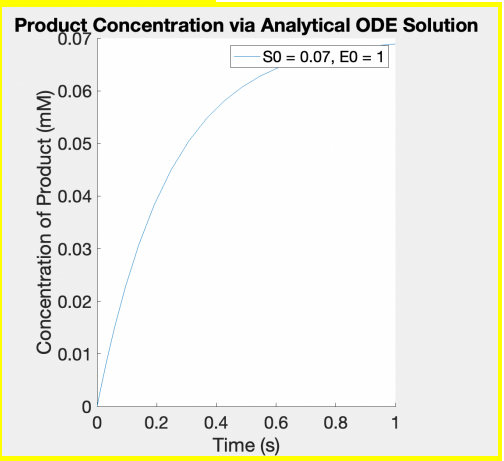


Figure 4. ODE implementation on MATLAB for a sample graph of product concentration over time via an Analytical ODE solution

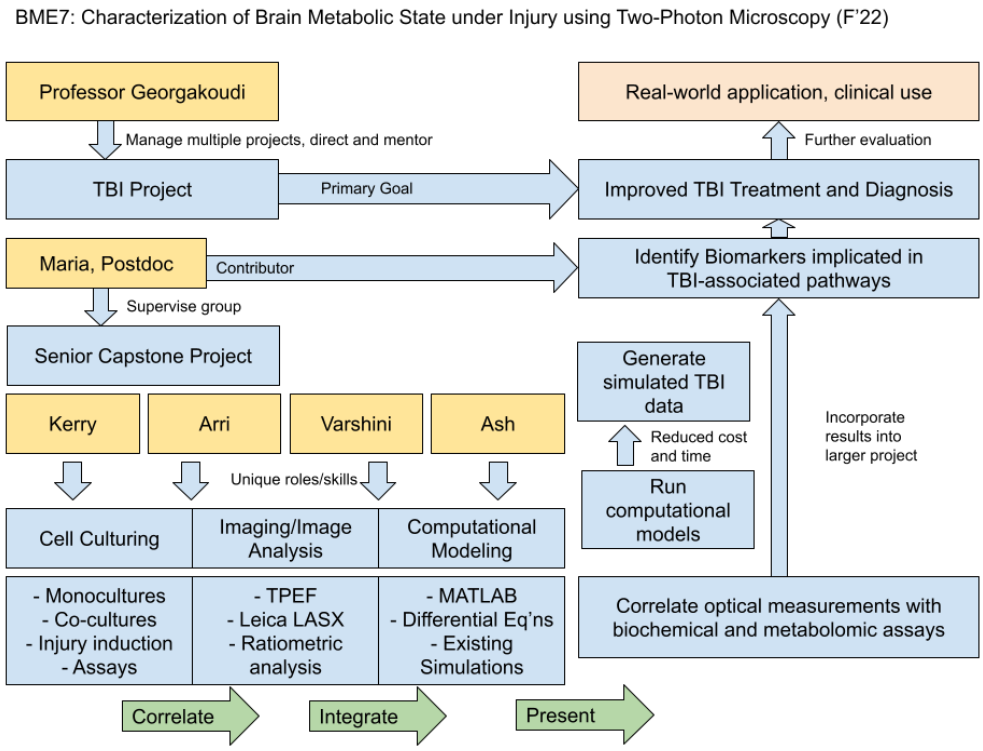
DISCUSSION AND FUTURE WORK

In the first semester of the project, we hope to successfully develop monocultures for microglia, then neurons. We may run into issues with the protocol, which would push back the computational part of the project. In order to account for that, we will start working on an outline for the computational model ahead of time using external resources.

In the second semester of the project, we aim to develop co-cultures for further imaging and metabolic analysis. With monoculture data from the first semester, we will be able to link the optical readouts with the metabolic data using statistical tools on MATLAB. We can compare this correlation in data to our computational model framework and adjust it as needed. Ideally, the researched functions we collected will accurately reflect our results.

The goal of our project is to correlate optical measurements with biochemical and metabolomic assays. Understanding this correlation will allow us to develop algorithms to predict biochemical and metabolomic data purely from optical readouts. This is valuable because it reduces the time and cost required to see the metabolic effects of TBI on brain cells. Culturing cells and completing assays can cost thousands of dollars over the span of many months. The development of this algorithm will make it possible to do TBI research in a shorter time span and a smaller budget. This broadens the availability of TBI research to labs that do not have the resources to do cell culturing long-term. This also helps address the morality of using engineered brain tissue because it reduces the need for handling it.

UNIFYING FIGURE



Supp. Figure 1. The unifying figure describes a simplified hierarchy of the Georgakoudi lab, the roles and objectives of our project, the contribution of our project to the main project objective, and real world implications.

INDIVIDUAL CONTRIBUTIONS

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

1. Computational modeling includes compiling code on brain metabolic pathways affected by TBI, extracting valuable functions from external sources and writing code in MATLAB to analyze patterns in data found through co-cultures and imaging. Examples of results are an algorithm that simulates neuron-astrocyte interaction and graphs the simulated data.
2. Imaging includes using multi-modal two-photon microscopy to observe engineered brain cell activity after simulated concussions. Imaging will be done on monocultures first(microglia, neurons, astrocytes), then co-cultures in the second semester if there is enough time.
3. Image analysis includes ratiometric analysis of different detector channels, phasor analysis, data processing, cellular segmentation and automatic annotation. This data will be correlated with cell culturing data.
4. Cell culturing includes developing co-cultures and writing the secondary injury protocol. We are starting by culturing microglia because there exists an established protocol. The goal is to culture neurons and co-cultures as well and collect metabolic data for correlation to image analysis results.

Ash will be responsible for computational modeling and assisting with image analysis under guidance from Varshini. The guidance comes in the form of experience from systems biology, example code and how to extract differential equations from papers. 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. The previous senior capstone project from the Georgakoudi lab tried to tackle the creation of a machine learning algorithm. Therefore, it would be valuable to communicate with previous students as to what limitations and challenges they have encountered

.

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. She will be able to connect relevant resources from the Georgakoudi lab to our project such as existing protocols and models. Varshini has been doing research on alternate assay techniques for measuring glycolysis in neurons because the seahorse assay is located in Grafton. There may be additional issues with using assays and cell culturing because the timespan and cost extends beyond both BME7 and 8. We settled on an alternative over time by selecting fewer assays and spending more time on existing ones. Varshini has also been instructing Ash on using differential equations for MATLAB and imaging for all project members.

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. Kerry has compiled many computational modeling papers, many of which contain useful differential equations for the code.

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. Arri 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. Working closely with Varshini, Arri is also communicating with Maria and the professor about possible alternatives for our project and training for the use of new assays. She has also been further assisting with finding computational models that are relevant to the pathways we are studying. Alongside Kerry, Arri has been undergoing cell culture training in order to prepare the astrocytes and microglia required for mass spec and assays.

All members of the project have completed the training required to work in the lab. This includes BSL2, baseline eye exam, laser training, microscopy training, etc. Overall, we were able to catch up with our schedule. TBI cell culture training has been scheduled and will be done over the next few weeks. After the training, we will be able to better define challenges, alternatives and specifics (timestamps, replicates, etc.). We need to optimize the concentrations before culturing. Due to the scale of our experiments and the inexperience of our lab, we will be consulting our supervisor, professor Georgakoudi and the professors for BME7 for advice on getting results in a timely manner. Currently, we are focusing on completing hands-on training, identifying the rationale and key information of our papers, and establishing the framework for our computational model.

REFERENCES

1. Haenseler, W. *et al.* A Highly Efficient Human Pluripotent Stem Cell Microglia Model Displays a Neuronal-Co-culture-Specific Expression Profile and Inflammatory Response. *Stem Cell Reports* **8**, 1727–1742 (2017).
2. Chapman, G. A. *et al.* Fractalkine Cleavage from Neuronal Membranes Represents an Acute Event in the Inflammatory Response to Excitotoxic Brain Damage. *J. Neurosci.* **20**, RC87–RC87 (2000).
3. Dorsett, C. R. *et al.* Glutamate Neurotransmission in Rodent Models of Traumatic Brain Injury. *J Neurotrauma* **34**, 263–272 (2017).
4. Wu, Y.-H. *et al.* In Vitro Models of Traumatic Brain Injury: A Systematic Review. *Journal of Neurotrauma* **38**, 2336–2372 (2021).
5. Robertson, J. M. Astrocyte domains and the three-dimensional and seamless expression of consciousness and explicit memories. *Medical Hypotheses* **81**, 1017–1024 (2013).
6. Bylicky, M. A., Mueller, G. P. & Day, R. M. Mechanisms of Endogenous Neuroprotective Effects of Astrocytes in Brain Injury. *Oxidative Medicine and Cellular Longevity* **2018**, 6501031 (2018).
7. Kim, O. D., Rocha, M. & Maia, P. A Review of Dynamic Modeling Approaches and Their Application in Computational Strain Optimization for Metabolic Engineering. *Frontiers in Microbiology* **9**, (2018).
8. Oschmann, F., Berry, H., Obermayer, K. & Lenk, K. From in silico astrocyte cell models to neuron-astrocyte network models: A review. *Brain Research Bulletin* **136**, 76–84 (2018).
9. Ng, S. Y. and A. Y. W. Lee. "Traumatic Brain Injuries: Pathophysiology and Potential Therapeutic Targets." Front Cell Neurosci **13**: 528, (2019).
10. Shi, H., et al. "The in vitro effect of lipopolysaccharide on proliferation, inflammatory factors and antioxidant enzyme activity in bovine mammary epithelial cells." Anim Nutr **2**(2): 99-104, (2016).
11. Mark, L. P., et al. "Pictorial review of glutamate excitotoxicity: fundamental concepts for neuroimaging." AJNR Am J Neuroradiol **22**(10): 1813-1824, (2001).
12. Chapman, G. A., et al. "Fractalkine cleavage from neuronal membranes represents an acute event in the inflammatory response to excitotoxic brain damage." J Neurosci **20**(15): RC87, (2000).
13. Posti JP, Dickens AM, Orešič M, Hyötyläinen T, Tenovuo O. Metabolomics Profiling As a Diagnostic Tool in Severe Traumatic Brain Injury. Front Neurol. doi: 10.3389/fneur.2017.00398. (2017)
14. Gupta, K., et al. "NMDA receptor-dependent glutamate excitotoxicity in human embryonic stem cell-derived neurons." Neurosci Lett 543: 95-100. (2013).
15. Liaudanskaya, Volha et al. “Modeling Controlled Cortical Impact Injury in 3D Brain-Like Tissue Cultures.” Advanced healthcare materials vol. 9,12 (2020).
16. Toledano Furman, N., Gottlieb, A., Prabhakara, K.S. et al. “High-resolution and differential analysis of rat microglial markers in traumatic brain injury: conventional flow cytometric and bioinformatics analysis.” *Sci Rep* **10**, 11991 (2020).
17. Anderson, W. D. et al. “Computational modeling of cytokine signaling in microglia.” *Royal Society of Chemistry* **12**. (2015).
18. Calvetti, D. et al. “Dynamic activation model for a glutamatergic neurovascular unit”. *Journal of Theoretical Biology*, Volume 274, Issue 1, 12-29 (2011).

APPENDIX

1. Transwell 2D co-cultures are not compatible for confocal/two-photon imaging:
    a. https://www.corning.com/catalog/cls/documents/application-notes/CLS-AN-521-A4.pdf
    b. https://www.researchgate.net/post/Can_transwell-cultured_Caco-2_monolayers_be_imaged_with_BD_Pathway_confocal_microscopy_while_keeping_them_in_transwell
2. Glutamate Protocol Table

Arri has reviewed the relevant papers to identify different media compositions that were used. Using this information, we are working on refining the protocol for the glutamate excitotoxicity study. Additionally, given that most of the paper that we found were using 2D cultures, we plan to look at other papers that use 3D cultures to determine if a change of medium is required prior to the injury induction.

Ref	Relevance	Glutamate Concentration	Exposure	Media/rinse	Notes
https://www.sciencedirect.com/science/article/pii/S03043940130021 52	HESCs treated with glutamate at physiological concentrations	200 uM	24h	DMEM (with supplements) first and then changed to Neurobasal-A (with supplements) 50uM DAPT was included in first medium change	Changed to trophically deprived, glutamate-free mimal medium (90% salt-glucose-glycine medium and 10% MEM) 1 day prior to injury
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6773069/	NMDA and glutamate on rat cortical culture	100 and 300 uM	30 minutes	Control media = MEM + 0.01% BSA + 25mm Hepes + 10 um glycine Rinse: MEM 200:1 dilution	Younger culture is less sensitive so use higher conc. No change of medium prior to experiment After toxicity assay: cells are exposed to control solution, NMDA, or glutamate for 30 mins and then rinse
https://www.nature.com/articles/cddis2012194	rat derived hippocampal, cortical, midbrain neurons	100 uM glutamate in 10 uM glycine, Mg2+ free medium	15 minutes	HBSS (hippocampi) Neurobasal medium (Gibco-Invitrogen) supplemented with B-27 (Gibco-Invitrogen) and 2 mM L-glutamine (culture)	No change of medium prior to experiment

https://onlinelibrary.wiley.com/doi/full/10.1046/j.1471-4159.2000.0751045.x	mouse cortical cultures	100 to 500 uM	Didn't remove, glutamate media because they were testing enzyme degradation	Medium: Neurobasal medium, 2% B27, 0.5 m*M* L-alanyl-L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 0.25 µg/ml amphotericin (Life Technologies) + 1% horse serum	No change of medium prior to experiment
https://www.nature.com/articles/s41419-018-0351-1	Rat hippocampal neurons from Wistar rat E18 embryos	30 and 100 uM (100 is most sig) Also test at 1 and 10 uM but not sig	30 mins	For low-astrocyte cultures: cytosine with AraC (Sigma-Aldrich) at conc. of 2 uM at DIV2-5	No change of medium prior to experiment
https://journals.biologists.com/jcs/article/131/22/jcs214684/56996/Axonal-degeneration-induced-by-glutamate	(E18 Sprague-Dawley) Rat embryonic hippocampal neurons	20uM	6 hours	After 3 h, the plating media was changed to Neurobasal™ medium supplemented with 2% B27, 0.5 mM GlutaMAX™-I and P/S After 3 days, a third of medium was replaced and treated with 5 uM AraC to inhibit glial cell proliferation	No change of medium prior to experiment