Neuroinflammation Levels Measured by Microglial Cell Activation

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Neuroinflammation Levels Measured by Microglial Cell Activation

A Capstone Submitted to the

University Honors Program

In Partial Fulfillment of the

Requirements of the Baccalaureate Degree

With Honors

Department Of

Biological Sciences

By

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DeKalb, Illinois

May 2025
Capstone Title (print or type): Neuroinflammation Levels Measured by Microglial Cell Activation

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Faculty Approval Signature: [Signature]

Department of (print or type): Biological Sciences

Date of Approval (print or type): May 5, 2024

Date and Venue of Presentation: Conference on Undergraduate Research and Engagement (April 30th)

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**Abstract**

Neuroinflammation is an inflammatory response in the brain that can be caused by different stressors such as diseases and/or external factors such as traumatic brain injuries. It is important to note duration and intensity of neuroinflammation levels when determining the impacts of these stressors to the brain environment. During neuroinflammation, a type of immune cell that becomes activated in the brain is called microglial cells. Microglial cells play a role in progression of the pathophysiological effects from the brain stressor. Studying changes in microglial cell shape provides evidence of the degree of neuroinflammation in the brain.

Researchers can quantify neuroinflammation based on the visualization of microglial shape changes from highly branched, or ramified, shapes (inactivated microglial cells) to more spherical microglial cell shapes (activated microglial cells). The degree of microglial cell activation elucidates the degree of neuroinflammation in different brain regions after exposure to brain stressors. The goal of this study is to develop an image analysis workflow for microglial cell activation found in 40 µm rodent brain cryosections that were stained using the antibody to microglial cells, iba1, and a secondary antibody linked to Alexafluor 568 to stain microglial cells red. A Zeiss LSM 900 with Airyscan 2 confocal laser scanning microscope was used to acquire image data, and Imaris 9.1.1 was used to perform 3D rendering and microglial cell segmentation to identify and quantify microglial activation. The workflow developed will be used to determine the degree of neuroinflammation from stress in rodent model systems.
**Introduction**

Neuroinflammation is a pathophysiological response resulting from negative stimuli to the brain environment such as traumatic brain injury (TBI) and/or radiation therapy; additionally, it is becoming an increasingly important process to study in order to understand the sequelae that occur years after initial injury (Shao et al., 2022). Neuroinflammation involves all cells in the central nervous system such as neurons, macroglia, and microglia (Shabab et al., 2017). Intensity of negative stimuli, age, genetics, environmental factors, and past neuroinflammatory events are some of the many factors that can lead to varying levels of neuroinflammation (Shabab et al., 2017). Neuroinflammation is a key factor in many neurodegenerative diseases such as Alzheimer's disease and multiple sclerosis as well in cancer since radiation therapy is one of the primary methods used to treat this disease (Shabab et al., 2017; Turnquist et al., 2020). So, investigating the pathway of neuroinflammation and having ways to characterize levels of neuroinflammation is of crucial importance to understanding many brain-related illnesses.

Radiation-induced neuroinflammation and brain injury are prevalent in both adults and children who have been diagnosed with brain cancer. Radiation therapy is a standard treatment method for primary and metastatic brain tumors where estimates of about 60% of diagnosed cancer cases receive radiation therapy during the course of their treatment (Turnquist et al., 2020). However, in the process of treating cancerous tissues with radiation, healthy brain tissue is affected and damaged leading to acute, early-delayed, and/or late-delayed cognitive side effects (Turnquist et al., 2020). Cognitive side effects can include learning and memory effects, increased intracranial pressure, and key neurological impacts (Turnquist et al., 2020). Accurate estimates of patients that experience cognitive decline due to radiation are hard to obtain due to different tumor types and radiation treatment plans for each patient; additionally, they are likely
underreported due to the need for a long-term follow-up with the patients and bias towards patients that exhibit higher neurological functioning (Turnquist et al., 2020).

One type of cell present in the central nervous system that plays a crucial role in neuroinflammation caused by radiation is microglial cells. Microglial cells are brain macrophages that play a key function in the brain’s immune system for tissue repair and overall brain defense (Shabab et al., 2017; Turnquist et al., 2020). Microglial cells go through transitions between activation and inactivation to fulfill their immune system roles. Inactivated microglia are found in a highly branched, or ramified, form under physiological conditions where they investigate the brain environment for inflammatory cues, promote the function of neurons and synaptic remodeling, and remove brain debris by phagocytosis (Shao et al., 2022). Activated microglia are more spherical in shape under pathological conditions where they play a role in the pathophysiological effects associated with neuroinflammation (Shao et al., 2022). Since there are these shape changes during microglia activation and inactivation, neuroinflammation can be identified and quantified based on counts of activated (spherical) and inactivated (ramified) microglia.

A good model organism to study microglia cell activation in radiation-induced neuroinflammation is Mus musculus. M. musculus is known colloquially as a mouse, and it is common model organism to study mammalian cancers as it is genetically similar to humans and shares developmental pathways of disease with humans (Müller & Grossniklaus, 2010; Vanhooren & Libert, 2013). M. musculus reproduce quickly, are easy to care for, and are not expensive to maintain compared to other model organisms (Müller & Grossniklaus, 2010; Vanhooren & Libert, 2013). Ethical considerations are taken into account when using M.
Musculus in research practices in accordance with Institutional Animal Care and Use Committee (IACUC) which reviews and approves studies involving animals.

This research study focuses on utilizing adult female *M. musculus* to induce brain injury and neuroinflammation via radiation exposure to a $^{137}$Cs source. The control group was exposed to 0 Gy gamma radiation, and the experimental group was exposed to 8 Gy gamma radiation. Three months after radiation exposure, the mice brains were fixed and extracted from the mice. The extracted brains were thinly sectioned by a cryostat into 40 µm slices. These mice cryosections subsequently underwent immunohistochemistry staining so that microscopy image analysis could be completed with Imaris 9.1.1. A refined workflow for microglial cell analysis was created to standardize image analysis. The data obtained from the analyzed unirradiated mice brain samples and the analyzed irradiated mice brain samples were compared to gain more insight into the levels of microglial cell activation depending on the level of radiation exposure.

**Materials and Methodology**

*Mice and Irradiation*

Animals were maintained and treated according to institutional procedures at NIU (IACUC protocol # LA22-007). The adult female mice, *M. musculus*, (C57BL6J line) used for these experiments were housed in the Psychology animal facility and maintained by graduate students and staff supporting the Neuroscience and Behavior Program in the Psychology Department at NIU. These mice are housed under standard temperature, humidity, and light-dark periods per day, with food and water available at all times.

Adult female mice were pair housed in a single cage where one mouse was exposed to 8 Gy to the head (body shielded with lead) from a $^{137}$Cs source, and the other mouse was sham
treated (0 Gy). After radiation exposure, the mice were then pair-housed in the Psychology Department animal facility for three months on a 12:12 light/dark cycle and given ad libitum access to Teklad 2014 chow and water. Prior to irradiation exposures, mice were sedated using isoflorene gassing (0.5-1.5% in oxygen) for 30 minutes. Mouse bodies were shielded with 1/32” lead sheeting so that only the mouse head was irradiated at a dose rate of 1.55 Gy/minute at room temperature. All experimental animals were adults of similar age.

**Immunohistochemistry and Imaging/Image Processing**

Three months after treatment, the mice were deeply sedated and perfused with 4% paraformaldehyde by heart puncture; additionally, the brains were quickly removed and placed into 4% paraformaldehyde in phosphate buffer (PB) overnight (IACUC protocol Brain Fog: Effects of whole brain irradiation $^{137}$Cs on motor and cognitive function #LA22-0007). Subsequently, the brains were placed in a 30% sucrose solution in PB for approximately 24 hours until the brains sank. These cryopreserved brains were then prepared for cryosectioning into 40 um sections, and the coronal cryosections were collected in a microfuge tube containing PB buffer.

These brain sections were processed for immunohistochemistry in suspension in a microfuge tube. After blocking in 3% BSA, 0.1% Triton X-100 in PBS, the brain tissue sections were stained with a rabbit anti-ibal polyclonal antibody (Wako Fujifilm, cat. # 019-19741) at a 1:1000 dilution in 0.2% Tween 20, 5% goat serum in PBS overnight at 4°C. Secondary antibody staining with goat anti-rabbit AlexaFluor 568 at a 1:500 dilution in 0.2% Tween 20 in PBS for two hours at room temperature was performed to stain microglial cells red. The brain tissue sections were then mounted onto a subbed slide with IBIDI mounting medium containing DAPI.
for a nuclear counterstain using a Corning no. 1.5 coverslip sealed to the glass slide using nail polish.

A Zeiss Plan apochromat 20X oil immersion lens (NA = 0.8) was used to sample the dentate gyrus of the cortex of the mouse brain section. The image data was collected at 3424 X 3424 pixels with a voxel size of 0.0618 um and a 16-bit data depth. Also, 1 µm z-stack data was collected to enable three-dimensional (3D) rendering of the data. No averaging was needed because of the high signal-to-noise ratio of the Zeiss LSM 900 with Airyscan 2 confocal laser scanning microscope. Image data were pre-processed by Imaris 9.1.1 that utilizes a GPU accelerated computer to render the image data into 3D as a maximal intensity projection (MIP). Further digital processing (surface processing) using Imaris also partitioned the image data into discrete groups of pixels - image segments - to inform object detection and related tasks to represent the morphology of the objects and detect boundaries. The number of identified objects then were determined, along with features of cell shape such as sphericity.

**Imaris 9.1.1 Microglial Workflow for Standardized Image Acquisition**

A workflow based on the five steps of the creation wizard in the Imaris 9.1.1 program for microglial cells was created. Each of the five creation wizard steps were analyzed based on the images acquired from the microscopy analysis, and the acquired image data was then run through Imaris to gain quantitative data relating to the levels of microglial activation and neuroinflammation in our rodent brain tissues. When creating this creation wizard procedure, one parameter or setting was changed at a time to see how that change would affect the final acquired image in terms of aspects like shape, clarity, and smoothness. Qualitative analysis of these final images was completed to see how well the features were reflected based on the changes in the
program’s creation wizard. This was completed to have a final standardized procedure for future image acquisition so that when future acquired images are ran through the Imaris software, there will be a standard mode of comparison across all samples. The method used here is supported by an Imaris specialist.

Results

After the immunohistochemistry staining was finalized, image collection using Zeiss LSM 900 with Airyscan 2 confocal laser scanning microscope began where four images of the cortical microglial were collected (two images from unirradiated samples and two images from irradiated samples). Once image collection was completed, the acquired images were imported into the Imaris 9.1.1 software to be pre-processed into 3D renderings for improved resolution of cell shapes and for further analysis. The pre-processed images (Figure 1a and 1b) were examined for microglia and suggested that there were different amounts of activation based on the shape changes in the unirradiated samples and the irradiated samples. Figure 1a displays the microglia present in the unirradiated mice, and Figure 1b shows the microglia present in the irradiated mice. However, in these pre-processed images, there are some background signals and additional structures that hinder the ability to analyze the microglia present.
Figure 1. **Imaris 9.1.1 pre-processed 3D images of rodent brain tissue sections into maximal intensity projections (MIPs).** The images show sections through the cortex from an unirradiated (0 Gy) mouse brain (a). In panel (b), a section through the cortex of an irradiated (8 Gy) female mouse brain is shown. Microglial cells are stained red, and the nuclei were counterstained blue with DAPI for reference. These were the images that were first obtained after imaging, and further processing in Imaris began with these images.

Thus, after the pre-processed Imaris images were examined, further Imaris analysis was completed to segment the microglial cells and exclude any background signal or unnecessary structures. Figures 2a and 2b display the final processed Imaris images after completing the creation wizard steps with segmentation and object detection as well as with the post-creation wizard modifications. Specifically, Figure 2a displays the processed microglia from the unirradiated mice brains, and Figure 2B displays the processed microglia from the irradiated mice brains. Completing this further Imaris processing allows the user to extract additional information that is specific to the microglia present, creates a better visual representation of the data, quantifies the microglia based on how many are present, and quantifies the microglia in what levels of sphericity they are present.
The Imaris software is able to color-code each microglial cell as an individual object, so it was able to provide quantitative numbers of the microglia present in the brain sections exposed to radiation and not exposed to radiation. The cell counts present in the control and experimental samples were comparable (43 in the control and 54 in the experimental) even though it appears that there were more microglia in the control sample. Figure 2a and 2b reveal that there are different levels of microglia activation between the control and experimental samples, with more branched, or ramified, microglia in Figure 2a (unirradiated sample) and more spherical microglia in Figure 2b (irradiated sample).

Figure 2. Segmentation of the microglial cells illustrates the shape change in microglial cells from unirradiated and irradiated cells. Pre-processed, 3D rendered image data (Figure 1) was further processed using Imaris 9.1.1 to classify surfaces using absolute intensity thresholding for object detection. The images show individual microglial cells identified by different colors for an unirradiated cortical brain section (a). This is compared with data from a cortical brain section from an irradiated mouse (b). Although it appears that there are more microglial cells in the untreated brain section, the object ID counts for microglial cells in each treatment were about the same. However, a noticeable shift towards more spherical microglial cell shape was noted in the irradiated sample.
To see the different levels of microglial cell activation clearly, Imaris is able to display the data present in Figures 2a and 2b into a gallery view as seen in Figure 3. The gallery view in Imaris takes all the objects in the image file and allows the user to order them based on different categories like sphericity, volume, surface area, etc. For this data, the sphericity ordering was utilized to better quantify the microglia counts. The gallery represents a visual sorting of the most ramified to most spherical cells. The histograms were created from numerical values for sphericity (0-1) that were calculated by Imaris. A calculated value of 1 indicated a perfectly spherical object. Three categories for spherical microglial cells (least spherical, moderately spherical, and most spherical) were established by us. The least spherical microglial cells had sphericity values between 0.0-0.28, moderately spherical microglia had sphericity values between 0.281 - 0.43, and most spherical microglia had values of 0.43 and greater. Figure 3a and the corresponding histogram associated with it displays data from the unirradiated brain samples; additionally, they exhibit that there was more branched, or ramified, microglia present in these samples. Figure 3b and the corresponding histogram associated with it displays data from the irradiated brain samples; additionally, they exhibit that there was more spherical microglia present in these samples.
Figure 3. Gallery view and graphical representation of processed Imaris control and experimental microglial cells. The segmented microglial cells (see Figure 2) were ordered by Imaris into the gallery views shown above, and each microglial cell was color coded according to the Sphericity heat map from the most ramified (inactivated) to the most spherical (activated). Further, the sphericity of the microglial cells was placed into categories: least spherical (0.0-0.28), moderately spherical (0.281 - 0.43) and most spherical (0.43+). The unirradiated microglial sample displays more microglial cells in the least spherical category (a). The irradiated microglial sample shows more microglial in the moderately and most spherical categories (b). The corresponding histograms show the number of cells in each category. Both methods show more spherical microglial cells in the irradiated samples and less spherical microglial cells in the unirradiated samples.
The data shown in Figure 3 shows two gallery views that order the present microglial cells based on sphericity level from least spherical in the top left corner to most spherical in the bottom row all the way to the right. The gallery views show a broad range of sphericities in microglial cells in the unirradiated and irradiated samples, but more spherical, or activated, cells were found in the irradiated sample. The heat map color-codes the microglial on a color gradient from least spherical (purple) to most spherical (red), where the unirradiated mice brains had the lowest levels of sphericity (more purple cells) and the irradiated mice brains had higher levels of sphericity (more red cells). The values for the control and experimental heat maps vary where the untreated mice brains had a lower range of 0.144-0.539, and the treated mice brains had a higher range of 0.146-0.678. The data in the gallery view in Figure 3a presents the same results that the histogram to its right has, and the data in the gallery view in Figure 3b presents the same results that the histogram to its right presents.

Within the unirradiated microglial sample’s histogram associated with Figure 3a, there are the categories of least spherical, moderately spherical, and most spherical. The majority of the microglial cells present in this sample were categorized to be in the least spherical category. This indicates that there was little microglial activation. Within the irradiated microglial sample’s histogram associated with Figure 3b, there are the categories of least spherical, moderately spherical, and most spherical. The majority of the microglial cells present in this sample were categorized in the moderately and most spherical categories. This conveys that there was an increased level of microglial activation in this sample. Thus, when comparing the two samples, the experimental brain displayed more microglial activation than the control brain did.
Discussion

The data from this image analysis comparing a tissue sample from an irradiated mouse with a tissue sample from an unirradiated mouse reveals a wide range of sphericity of microglial cells with the treated brains having more spherical (activated) microglia and the untreated brains having less spherical (inactivated) microglia. These findings agree with what is known about radiation-induced brain injury and neuroinflammation since microglia become activated from brain stressors such as radiation. In this case, the mice with the activated and more spherical microglia were the mice that were exposed to the radiation stimuli, whereas the mice that did not have any radiation exposure had mostly inactivated and less spherical microglia.

These results contribute novel information to the knowledge base of radiation-induced brain injury and neuroinflammation as this study elucidates quantifiable data based on microglial activation in the categories of least spherical, moderately spherical, and most spherical microglial shapes. The image analysis and Imaris software were able to provide numerical categories for microglial activation based on creation wizard thresholds to give further support of differing levels of activated microglia between the control and experimental brains. This supports that there is a gradient, or range, between complete activation and inactivation of microglia. The Imaris software was also able to make object detection easier by color-coding each microglial cell different for distinction thereby facilitating the data collection process.

This project only scratched the surface as to what the Imaris software is capable of competing in terms of image analysis, quantification, and data collection for microglial cells and determining levels of neuroinflammation based on microglia activation. If developed further, the usage and analysis of microglial cell activation in humans could provide a useful way of determining neuroinflammation in the brain following radiation exposure. This could then help
to predict the short- and long-term effects of radiation exposure and help anticipate possible neurological side-effects to be experienced by the individual. Additionally, this research focused on two groups of mice, one with 0 Gy exposure and one with 8 Gy exposure. It would be interesting to complete additional research with more mice groups that have different dosages of radiation exposure to elucidate how varying levels of exposure affect microglia activation.

Going through this data analysis with the images acquired, a refined workflow has now been developed for the continued analysis of microglial cell activation as an indicator of radiation-induced neuroinflammation in the brain. This workflow will help to facilitate future research with microglial cells and provide a standardization as to how the Imaris 9.1.1 image analysis works with the possibility of being able to apply this workflow to subsequent, future versions of Imaris.

Lastly, this project provided a research experience that allowed for hands-on access to state-of-the-art microscopy approaches and analysis tools. Being able to use these technologies and complete these experiments opens exciting new avenues for more opportunities in an ever more competitive STEM workforce.

Acknowledgements
I would like to thank my mentor, Dr. Linda S. Yasui, for all the support and guidance provided to me throughout this research project. I would like to thank Dr. Yasui for allowing me to be a part of her research laboratory and providing me with the training necessary to take part in this research. Additionally, I would like to thank the members of the Wallace Laboratory in the Psychology Department at Northern Illinois University for their help and contributions to this research. The Wallace Laboratory members contributed greatly to this project as they carried out
the handling of the mice, mice care, mice brain perfusions, mice brain extractions, and
cryosectioning of the extracted mice brains for immunohistochemistry staining. Thank you to
NIU’s Office of Engagement and Experiential Learning, the Honors Program, and the McKearn
Fellowship Program for providing support on my research endeavors. The Departments of
Biology and Psychology, the College of Liberal Arts and Sciences, and NIU Research and
Innovative Partnerships provided funds for the purchase of Imaris software and the computer.
The Zeiss LSM 900 with Airyscan 2 confocal microscope was acquired using funds provided by
an NSF MRI grant (Award # 2018748).
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