Final Internship Report

Project Title: Histological Analysis and Quantitative Assessment of Brain Cells Using ImageJ and Brain Atlas Tools

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Date: April 15 2025

1. Introduction

Background

The mammalian brain is a complex organ made up of diverse cell types and specialized regions, each critical for cognitive and physiological functions. Studying their spatial organization and how they change in disease, is key to neuroscience research. The central nervous system (CNS) contains many distinct neuroanatomical regions, and recognizing normal and abnormal cellular variations helps pathologists interpret changes accurately. It's also important to distinguish true pathology from common histological artifacts in CNS tissue, which can obscure or mimic disease.

The major cell categories in the CNS include cells of neuroectodermal origin (neurons, astrocytes, oligodendrocytes, and ependymocytes) and cells of mesenchymal origin (meninges, blood vessels, adipose tissue, and microglia).

- **Neurons** of neuroectodermal origin are characterized by variations in size and shape. They can be classified as small or large neurons, with further anatomic subtypes. They can also be classified according to the neurotransmitters they release. Most neurons have multiple dendrites but only one axon. Large neurons have large cell bodies, nuclei with prominent nucleoli, and Nissl substance, while these features may not be apparent in small neurons.
- **Astrocytes** of neuroectodermal origin play multiple roles within the CNS, including maintaining the blood-brain barrier, recycling glutamate and GABA, maintaining extracellular ionic balance, and providing neuronal metabolic support. Astrocytes have cytoplasmic extensions that contact neuron surfaces and extend to the pial surface, forming the glia limitans.
- **Oligodendrocytes** of neuroectodermal origin are responsible for forming and maintaining myelin sheaths in the CNS. They ensheath multiple axons, in contrast to Schwann cells in the peripheral nervous system. In white matter, oligodendrocytes are arranged in linear rows between nerve fibers.
- **Microglia** of mesenchymal origin, comprise the reticuloendothelial system of the CNS and constitute 5-20% of the brain's glial cell population. They are functionally heterogeneous. In normal brain regions, only small numbers of microglia are typically recognized.

Staining is a critical process used to highlight key tissue features and enhance contrast for microscopic examination. Hematoxylin, a basic dye, stains acidic structures such as cell nuclei, imparting a bluish-purple hue to basophilic components like DNA, RNA, and the rough endoplasmic reticulum. Conversely, eosin, an acidic dye, acts as a counterstain, targeting basic structures such as cytoplasmic proteins and extracellular matrix components, which appear pinkish-red (eosinophilic). Together, hematoxylin and eosin (H&E) staining form the gold standard in histopathology, providing a clear and detailed view of tissue morphology for diagnostic and research purposes.

While H&E staining offers a broad overview of tissue architecture, specialized techniques are required to investigate specific cellular processes. One important method for assessing neuronal degeneration and

apoptosis is the TUNEL (Terminal deoxynucleotidyl transferase dUTP Nick-End Labeling) assay, which detects DNA fragmentation. This technique relies on the enzyme terminal deoxynucleotidyl transferase (TdT), which catalyzes the addition of labeled nucleotides to the 3'-hydroxyl termini of fragmented DNA. The labeled nucleotides can be detected using fluorescence microscopy, light microscopy, or flow cytometry, making TUNEL staining a powerful tool for assessing apoptotic cell death in histological samples.

In research and clinical diagnostics, TUNEL staining is essential for studying neurodegeneration, cancer, and other pathological conditions involving programmed cell death. Different TUNEL assay kits use distinct labeling methods, including fluorescent dyes (FITC), chromogenic substrates (HRP-DAB), and bromodeoxyuridine (BrdU-Red), each with specific advantages in sensitivity, speed, and signal amplification.

Aim of the Study

This internship focused on two complementary histological objectives aimed at improving the analysis of cellular changes in the central nervous system following irradiation. The first involved classifying cells and mapping anatomical regions in brain sections using ImageJ, guided by the Allen Brain Atlas and classical histology texts such as Garman RH's Histology of the Central Nervous System. Cells were manually identified as neurons or glia using ImageJ's Cell Counter plugin, while stained sections were annotated for regional anatomy based on atlas references.

The second objective centered on quantifying degenerative changes in TUNEL-stained brain samples to assess apoptosis. ImageJ was used to detect and count TUNEL-positive cells, allowing for the comparison of control and irradiated samples. These two tasks were designed to complement each other, providing a balanced approach that integrated traditional histological interpretation with digital analysis methods, and contributing to a more systematic workflow for studying CNS pathology.

2. Materials and methods

2.1 Tools & Resources

- ImageJ with Cell Counter plugin / ICY / Fiji
- Brain Atlas (Nissl and sagittal settings)
- Histological Stainings:
    - Hematoxylin & Eosin (H&E)
    - Cresyl violet
    - GFAP staining
    - TUNEL staining

2.2 Task 1: Brain Cell Recognition and Quantification

Objective A: Brain Cell Identification Using ImageJ

- Manual classification of cells into neurons and glia was performed using morphological markers and ImageJ's Cell Counter plugin using morphological reference was taken from Garman RH's "Histology of the CNS." [1, 2]

Procedure

- Open histological images in ImageJ.
- Convert the image: Image > Type > 8-bit or 16-bit.
- Apply thresholding: Image > Adjust > Threshold → check Dark background → click Auto → then Apply.

- Convert to binary: Process > Binary > Make Binary.
- Convert to mask: Process > Binary > Convert to Mask.
- Separate overlapping cells using: Process > Binary > Watershed.
- Count cells manually using: Plugins > Analyze > Cell Counter.
- Save marked images and summary files for documentation.

Objective B: Anatomical Brain Region Identification

- Brain structures were localized by comparing experimental sections with reference plates from the Allen Brain Atlas [3].
- Emphasis was placed on locating the hippocampus and subventricular zone, cerebellum, cortex Figures 4–7.

Procedure

- Open stained brain images (Figures 4-7).
- Compare observed regions with the Nissl and sagittal views of the Allen Brain Atlas.
- Use cortical shape, cellular density, and layer organization as anatomical clues.
- Annotate and label specific regions (hippocampus, cerebellum) accordingly.

2.3 Task 2: Quantitative Analysis of brain cells

Objective: Detection of 1) neurons and glia cells via H&E or cresyl violet; 2) astrocytes via GFAP; 3) apoptotic cells via TUNEL Staining

- **Open the photograph of histological sample in ImageJ.**
- **Enhance image contrast (if necessary):** Image > Process > Enhance Contrast.
- **Initialize cell types using the Cell Counter plugin:**
  Plugins > Cell Counter > Initialize.
  Select **Type 1** for the first cell type and add markers.
  Similarly, select **Type 5** for the other cell types and add markers.
- **Save the markers:** Save the markers as .XML files for future use.
- **Load markers for future use:**Use the **Load** option in the Cell Counter plugin to apply the saved markers to the same or different images.
- TUNEL-stained images were analyzed to detect and count apoptotic cells .

Procedure

- Open histological sample in ImageJ.
- Convert the image type: Image > Type > 8-bit or 16-bit.
- Apply auto thresholding: Image > Adjust > Auto Threshold.
- Further refine: Image > Adjust > Threshold > Dark background > Auto > Apply.
- Convert the image to binary: Process > Binary > Make Binary.
- Generate a mask: Process > Binary > Convert to Mask.
- Perform particle analysis: Analyze > Analyze Particles, ensuring options for *Display Results* and *Summarize* are selected.
- Save the watershed images, result tables, and binary masks for analysis.

3. Results And Discussion

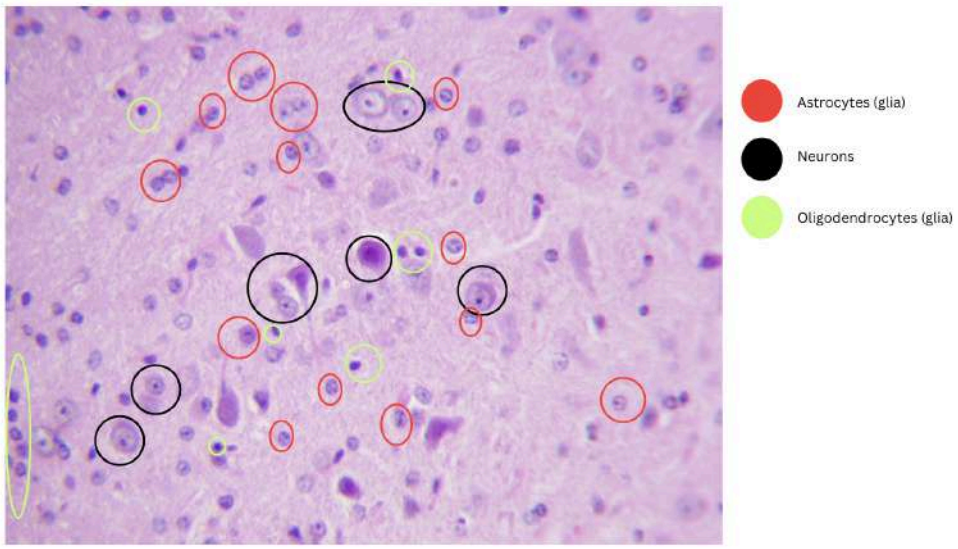


Fig 1a: Nissl method, H&E staining, zoom 10x40. Rat brain (Raw)

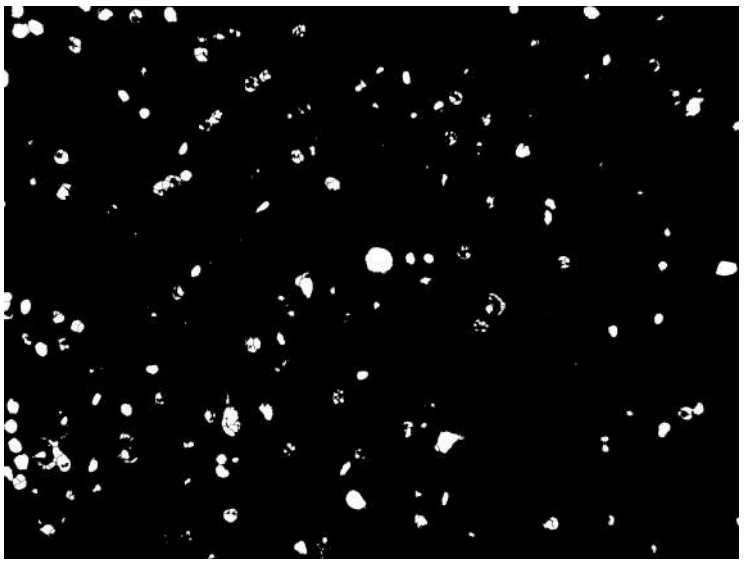


Fig 1b: Nissl method, H&E staining, zoom 10x40. Rat brain (Watershed)

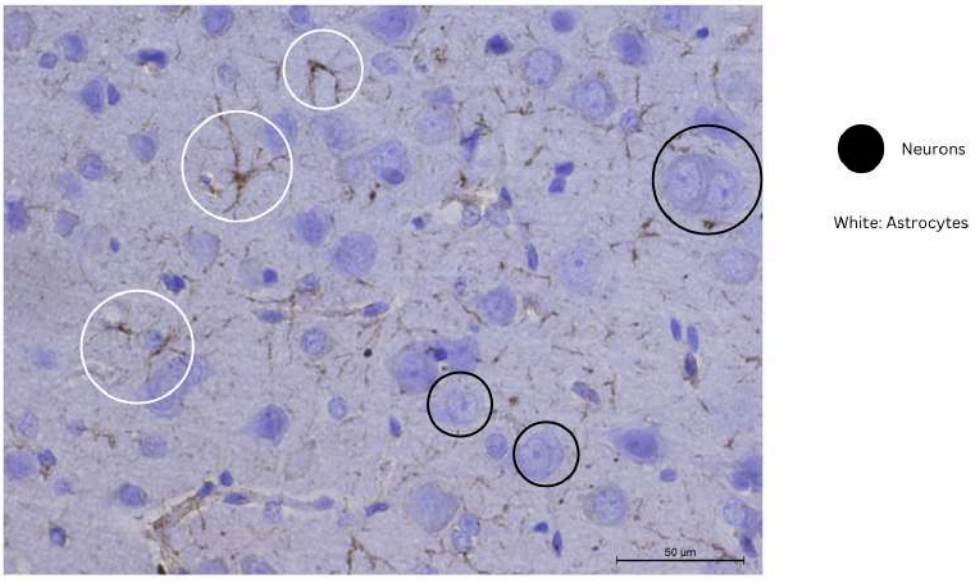


Fig 2a: GFAP staining, zoom 10x40. Rat brain (Raw)

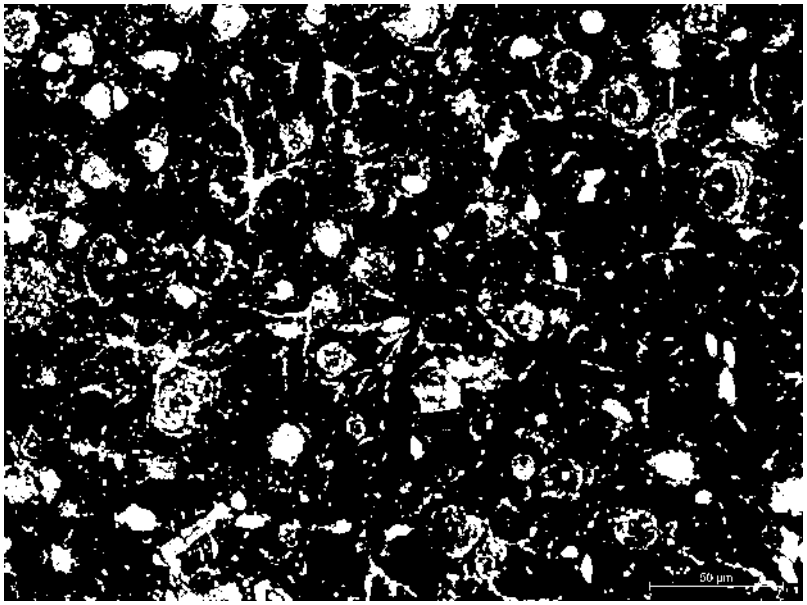


Fig 2b: GFAP staining, zoom 10x40. Rat brain (Watershed)

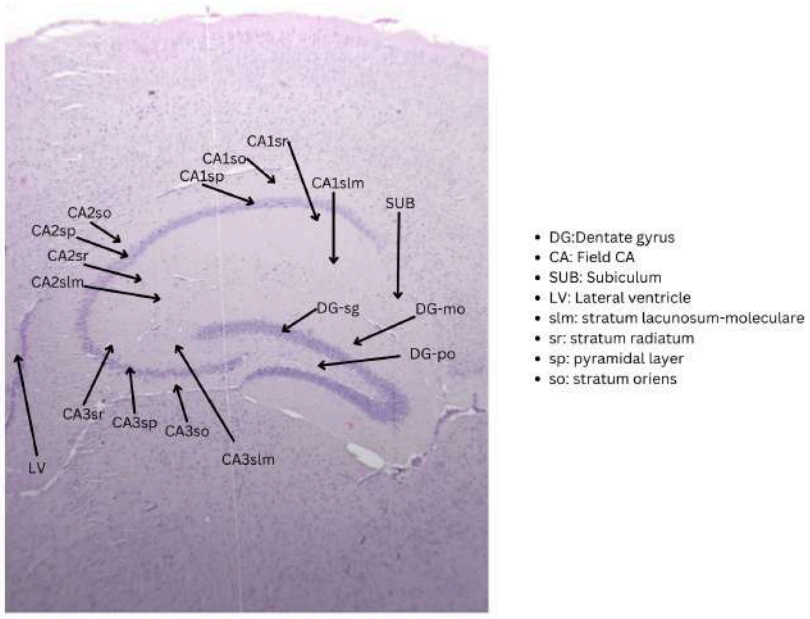


Fig 3: H&E staining, Rat brain (Raw). Hippocampus

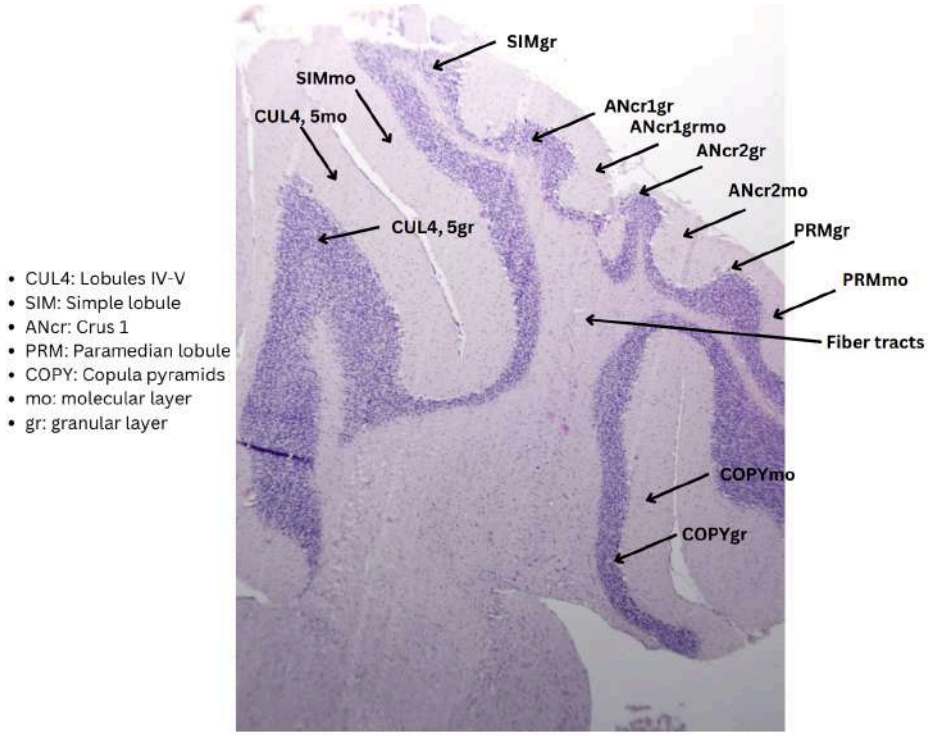


Fig 4: H&E staining, Rat brain (Raw). Cerebellum

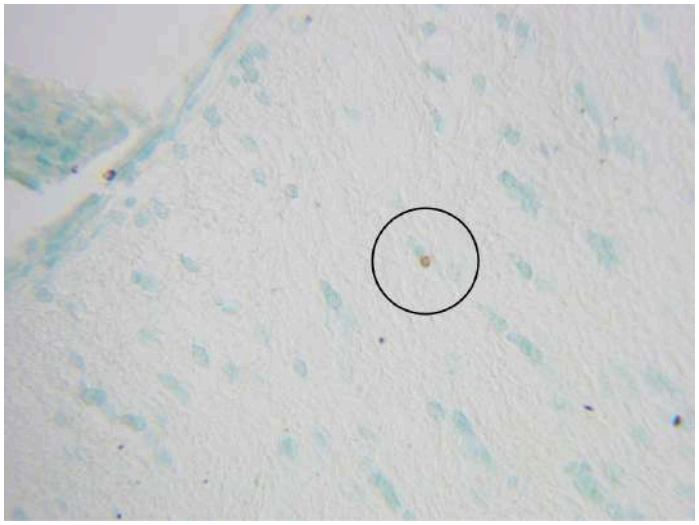


Fig 5: slide 35_3_tunel staining, control

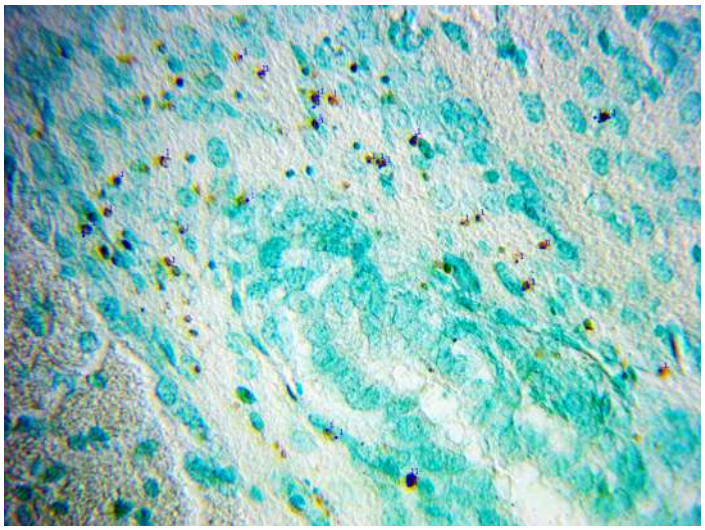


Fig 6: slide 21_12_1_tunel staining, irradiated

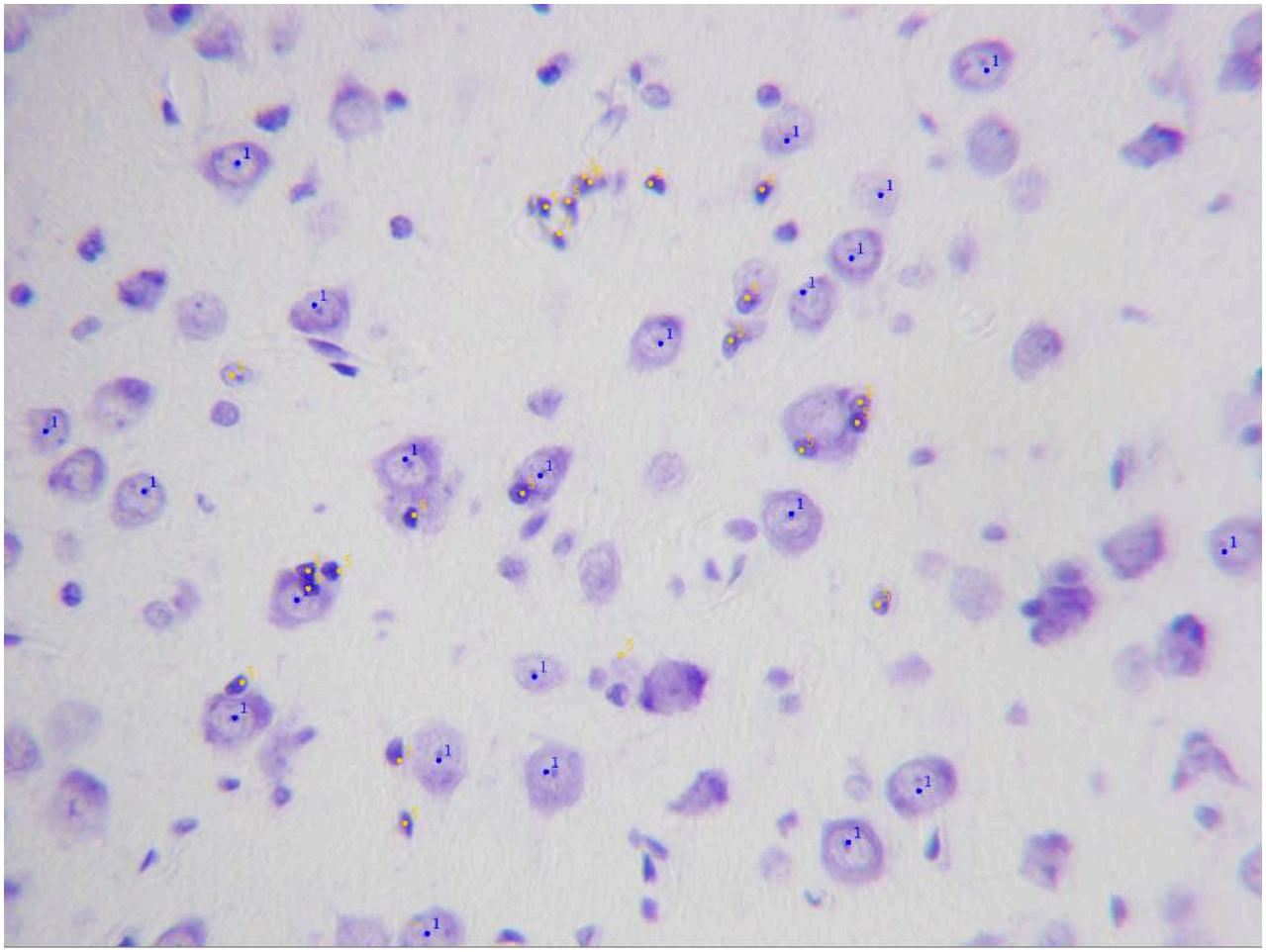


Fig 7: Cresyl Violet staining, Rat brain. Cortex: 1 - neurons, 5 - glia cells. Zoom 40x10

Brain sections and brain cells of CNS laboratory rodents were analyzed using H&E and cresyl violet, TUNEL, and GFAP staining to assess nervous tissue structure, recognizing neurons and glia cells, neuronal degeneration, and glial activity, respectively. ImageJ was used for quantitative analysis of stained samples. H&E staining (Fig. 1) revealed general tissue architecture, with 526 distinct regions (covering 3.124% of the total area), allowing clear visualization of neuronal and glial morphology. GFAP staining (Fig. 2) highlighted 3,756 reactive astrocyte regions (17.54% area), aiding in the identification of astrocytes and neurons. Figures 3 and 4 depict H&E staining of hippocampus and cerebellum. Figures 5 and 6 compare TUNEL staining between control and irradiated brain sections, demonstrating increased apoptosis in irradiated samples. Next step was to analyze TUNEL stained samples of 2 experimental groups: irradiated and control. A total of 8 control and irradiated samples are considered for further statistical studies. Figure 7 illustrates the identification of neurons and glia cells in cortex (cresyl violet staining), where the blue-stained structures (labeled 1) represent neurons, and the yellow-stained regions (labeled 5) indicate glia cells.

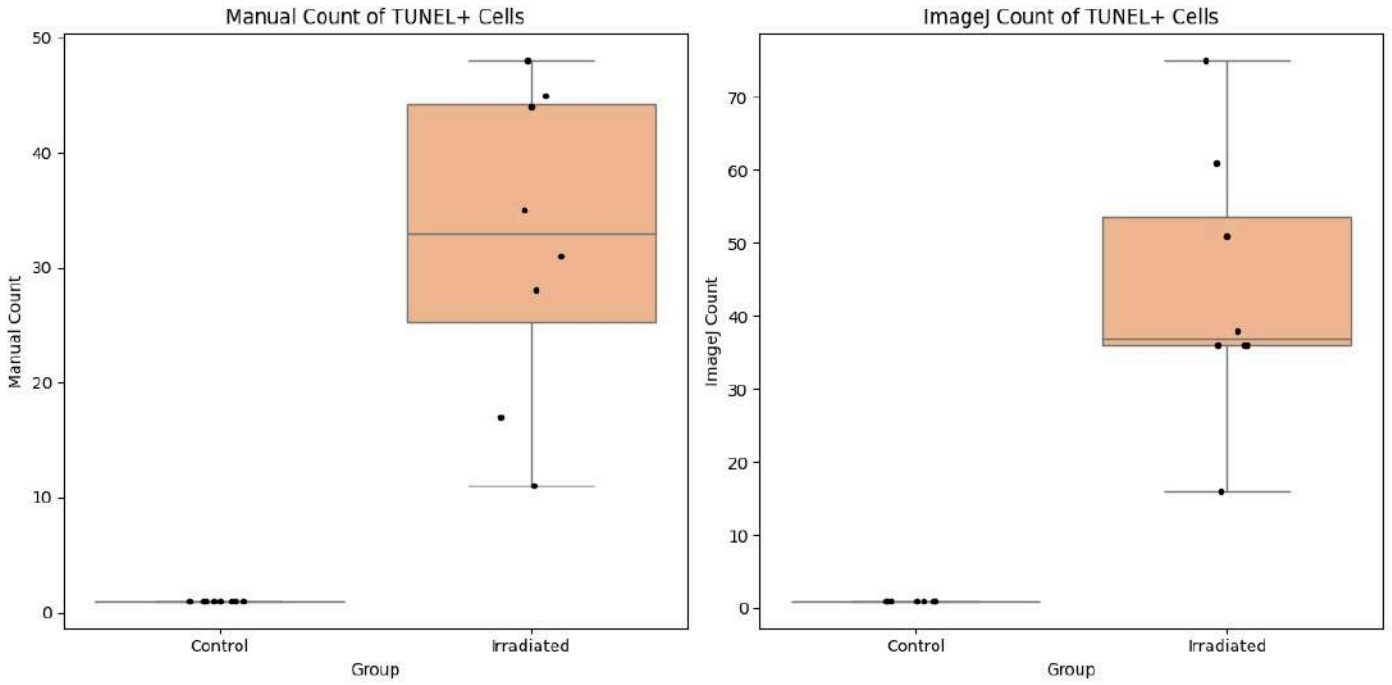
Summary of Statistical Test Results

Table 1: Number of Tunel positive cells in control vs irradiated group

Group	Slide	The number of Tunel positive cells (obtained manually)	The number of Tunel positive cells (obtained using ImageJ)
Control	Slide 35_3 - 1	1	1
	Slide 35_3 - 2	1	1
	Slide 35_3 - 3	1	1
	Slide 35_5 -1	1	1

	Slide 35_5 - 2	1	1
	Slide 35_11 - 1	1	1
	Slide 35_11 - 2	1	1
	Side 35_11 - 3	1	1
Irradiated	Slide 21_9 -1	45	75
	Slide 21_9 - 2	35	61
	Slide 21_9 - 3	11	36
	Slide 21_11 - 1	31	36
	Slide 21_11 - 2	28	16
	Slide 21_12 - 1	44	36
	Slide 21_12 - 2	48	54
	Slide 21_12 - 3	17	38

Sample	**Count**	**Missing**	**Mean**	**Median**	**SD**	**Minimum**	**Maximum**	**W**	**P**
Control	8	0	1.00	1.00	0.00	1	1	NaN	NaN
Irradiated	8	0	32.38	33.00	13.42	11	48	0.93	0.54



To evaluate the effects of irradiation on cellular populations, we compared manual and ImageJ-based cell counts between control and irradiated samples. Initial assessment of data normality using **Shapiro-Wilk tests** confirmed that both Manual_Count (Control: W=1.000, p=1.000; Irradiated: W=0.930, p=0.515) and ImageJ_Count (Control: W=1.000, p=1.000; Irradiated: W=0.932, p=0.537) **followed normal distributions**. However, **Levene's test** revealed **significant heteroscedasticity (unequal variances)** for both measures

(Manual_Count: W=6.441, p=0.024; ImageJ_Count: W=17.567, p=0.001), necessitating the use of **Welch's t-test** and **Mann-Whitney's U-Test** for group comparisons. This analysis demonstrated **highly significant** irradiation-induced increases in cell counts (Manual_Count: t=-6.637, $p=2.94\times10^{-4}$; ImageJ_Count: t=-6.615, $p=3.00\times10^{-4}$), with both quantification methods showing consistent results. These findings validate our experimental approach while highlighting the importance of accounting for variance heterogeneity in comparative analyses of radiation effects on cellular populations.

Analysis of TUNEL-positive cells in the subventricular zone (SVZ) of the hippocampus revealed a statistically significant increase in the irradiated group compared to controls ($p < 0.05$, Mann-Whitney U-test). Quantification of apoptotic cells using both manual and ImageJ-based methods showed a consistent and robust elevation in the number of TUNEL-positive cells following irradiation. In control samples, only one TUNEL-positive cell was typically observed per slide, whereas irradiated tissues exhibited markedly higher counts. These findings corroborate the induction of apoptosis by irradiation and emphasize the need for non-parametric statistical approaches when variance heterogeneity is present.

4. Conclusion

Overall, this internship provided valuable hands-on experience at the intersection of classical histology and digital pathology. The project's aims were met through a structured approach that combined manual expertise with open-source computational tools. Cell classification and regional mapping not only improved my familiarity with CNS microanatomy but also highlighted the importance of spatial context in interpreting histological data. The quantitative analysis of TUNEL-stained sections revealed a clear and statistically significant increase in apoptotic cells following irradiation ($p < 0.001$), with strong agreement between manual and ImageJ-based counts. This finding reinforced the importance of cross-validating results using both human judgment and automated methods. More broadly, the experience underscored that while digital tools like ImageJ can greatly enhance analytical efficiency, they are most powerful when it is under control of a specialist. Integrating standard staining techniques with computational analysis deepened my understanding of CNS structure and pathology, and emphasized the importance of methodological rigor in producing reliable, reproducible histological data. This experience has equipped me with both the technical skills and critical perspective necessary for future work with histological samples.

5. References

1. Garman RH. Histology of the Central Nervous System. Toxicologic Pathology. 2011;39(1):22-35. doi:10.1177/0192623310389621
2. Gurina TS, Simms L. Histology, Staining. [Updated 2023 May 1]. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2025 Jan-. Available from: http://www.ncbi.nlm.nih.gov/books/NBK557663/
3. Brain atlas https://atlas.brain-map.org/atlas?atlas=2#atlas=2&structure=10703&resolution=5.23&x=7446.156616210 9375&y=2515.7501220703125&zoom=-2&plate=100884125&z=6
4. **https://www.abcam.com/kits/tunel-staining#:~:text=TUNEL%20**
5. **https://www.sciencedirect.com/topics/engineering/histological-analysis**