

Investigating the Role of Endothelial Connexin-43 in Alzheimer's Disease: An
End to End Framework for Immunofluorescence Visualization and Quantitative
Image Analysis

By

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*Abstract of Investigating the Role of Endothelial Connexin-43 in Alzheimer's Disease:
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Objective: Despite extensive evidence of APOE4-driven vascular dysfunction, cerebral endothelial connexins that play a critical role in endothelial communication and vasodilation remain poorly characterized in Alzheimer's disease (AD). Connexin expression changes in the hippocampal vasculature in early stages of AD may be the missing link that can bind systemic APOE dysfunction to impaired hippocampal neurovascular coupling. This study aims to (i) establish and validate a reproducible dual-label immunofluorescence and Slide Scanner pipeline to visualize endothelial Connexin-43 (Cx43) in coronal sections as a first step and (ii) develop a preliminary channel specific quantitative image analysis pipeline capable of processing high throughput whole section images in a single batch.

Methods: Brain tissue was extracted from 10 ApoE $\epsilon 3/\epsilon 3$ and 10 ApoE $\epsilon 4/\epsilon 4$ mice, cryostat sectioned, immunofluorescent stained to generate 4 coronal cross sections per brain along with representative middle cerebral artery (MCA) cross section samples. These were imaged using Olympus VS200 Slide Scanner at 40 $\times$, following high-resolution validation of the protocol on Olympus FV 3000 confocal microscope at 60 $\times$. Preliminary images were analyzed using VS200 ASW, and Fiji/ImageJ. A parameter-locked Fiji/ImageJ macro was developed for all three channels to (i) compute hippocampal vessel area fraction from CD31 masks, (ii) quantify Cx43 puncta counts within atlas-anchored ROIs, and (iii) estimate nuclear density from DAPI (nuclei

per mm²); the batch routine exports overlays and CSVs, with full-cohort execution deferred until validation.

Results: The finalized protocol yielded continuous CD31-positive microvasculature and discrete AF594-positive puncta consistent with Cx43 along vessel walls, while secondary-only controls showed no structured signal. Slide scanning achieved complete hippocampal coverage suitable for cohort throughput, and confocal resolved submicron puncta and verified morphology. An image set of 80 hippocampal whole coronal sections with exposure records and focus-point maps was curated. The batch code produced correct overlays and tabular outputs on small ROIs of test sections.

Conclusions: This study delivers a scalable staining and acquisition protocol, a modality comparison tailored to endothelial Cx43 mapping, and a ready-to-run customizable analysis pipeline. Although further refinement in image analysis pipeline are necessary for standardization, and cohort-level metrics and group comparisons are deferred, feasibility, specificity, and analysis-readiness are demonstrated, laying the groundwork to interrogate endothelial Connexin-43 as a potential mechanistic link between APOE4, disrupted neurovascular coupling, and cognitive decline.

CHAPTER 1: INTRODUCTION

The clinical manifestations of Alzheimer's disease (AD) surface as the endpoint of an integration of decades-long cascades of systemic pathologies. Vascular dysregulation within the brain has emerged as one of the earliest pathological events in late-onset Alzheimer's disease.^{1,2} Vascular morphological and degenerative changes in affected brain regions are also among the most prominent anatomical features postmortem.³ The CA1 subfield of the hippocampus has been shown to undergo focal atrophy even in preclinical stages compared to healthy aging.⁴

The hippocampus is a principal locus of early structural and vascular pathology in Alzheimer's disease. Structural imaging demonstrates that hippocampal atrophy begins in the prodromal phase, particularly in CA1 and subicular regions, well before the onset of dementia.⁵ Stereologic autopsy studies extend these findings, showing AD brains to have ~32% smaller hippocampal formation volume and ~23% fewer neurons, largely confined to the subiculum and fascia dentata, underscoring a regional selective vulnerability beyond normal aging.^{6,7} This structural fragility is compounded by vascular dysfunction. In cognitively unimpaired ApoE ϵ 4 carriers, dynamic contrast enhanced MRI reveals hippocampal blood-brain barrier (BBB) breakdown, a pathology independent of amyloid and tau that precedes atrophy and marks preclinical hippocampal vascular impairment.⁸ Even in normal aging, the hippocampus is the first site to exhibit BBB breakdown, and patients with mild cognitive impairment show increased BBB permeability in CA1 and dentate gyrus compared with cognitively normal individuals.⁹ Together, this convergent evidence such as atrophy, selective neuron loss, and early BBB disruption identifies the hippocampus as a structurally and functionally disadvantaged region, rendering it highly susceptible to early cerebrovascular injury in AD¹⁰.

Functional hyperemia is a key mechanism of neurovascular coupling, and is critically dependent on endothelial gap junctional signaling among endothelial cells within the arterial wall, particularly through connexin mediated communication [11,12](#).

Gap junctions are intercellular channels that permit direct electrical and metabolic coupling between adjacent cells, and are highly conserved across species, representing a fundamental principle of vascular signaling (see Figure 1). In the cerebral vasculature, connexin isoforms such as Cx37, Cx40, Cx43, and Cx45 form these junctions enabling rapid conduction of hyperpolarization along the endothelium. This spread of electrical signals coordinates the propagation of vasodilation during neurovascular coupling. [13](#)

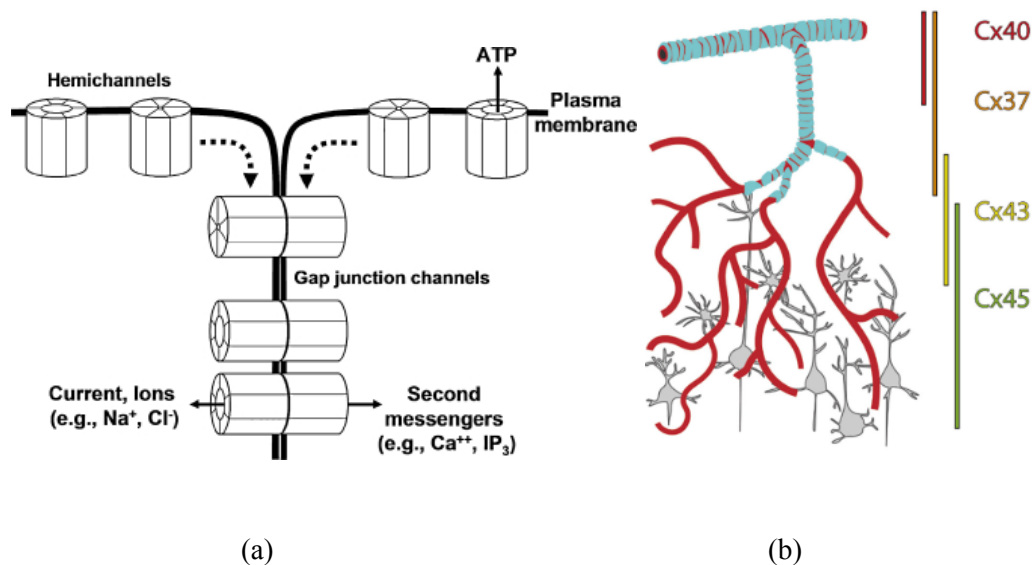


Figure 1.(a) Hemichannels and Gap Junctions; (b) Microvascular distribution of Cx37, Cx40, Cx43, Cx45 [12](#) [13](#)

Connexins have been widely studied in the brain, with most work focused on neuronal Cx36 in electrical synapses, astrocytic Cx43 and Cx30 in glial networks and hemichannels, and oligodendrocytic Cx47 and Cx32 in panglial coupling that sustains myelin [14,15,16](#). These studies have

established that connexins influence network synchronization, glial homeostasis, and responses to injury, and that their dysregulation contributes to conditions such as epilepsy, ischemia, demyelination, and AD, where increased astrocytic Cx43 around amyloid plaques has been repeatedly described.¹⁵ In contrast, connexins expressed by brain endothelial cells, particularly those governing vascular conduction such as Cx37, Cx40, and Cx43, have received little direct investigation in the context of pathologies such as Alzheimer's disease ¹².

Apolipoprotein E (ApoE) is the principal cholesterol and lipid carrier in the central nervous system, where it plays a critical role in membrane turnover, lipid transport, and injury repair ¹⁷. The three primary ApoE isoforms— $\epsilon 2$, $\epsilon 3$, and $\epsilon 4$ exhibit distinct functional properties and disease associations. The $\epsilon 3$ allele is the most common and is considered the canonical isoform, while the $\epsilon 2$ allele confers a protective effect against Alzheimer's disease (AD) ¹⁸. Conversely, the $\epsilon 4$ isoform is the strongest genetic risk factor for sporadic late-onset AD ¹⁷.

The pathological effects of ApoE $\epsilon 4$ manifest early in the disease course. Structural and functional changes, including reduced hippocampal volume, impaired connectivity, and altered metabolism, are detectable in $\epsilon 4$ carriers before the onset of clinical AD ¹⁷. Postmortem studies reveal that $\epsilon 4$ carriers with AD exhibit a greater burden of hippocampal neuritic plaques, cortical and hippocampal neurofibrillary tangles, and a heightened risk of cerebral amyloid angiopathy compared to non-carriers ¹⁹. Furthermore, ApoE $\epsilon 4$ modulates the impact of systemic risk factors. For example, the combination of ApoE $\epsilon 4$ and type 2 diabetes results in a synergistic increase in AD risk ¹⁷.

The influence of ApoE $\epsilon 4$ extends beyond amyloid and tau to the cerebrovasculature. Both human and animal studies have identified blood-brain barrier (BBB) dysfunction, pericyte degeneration, and impaired cerebrovascular regulation in $\epsilon 4$ carriers, independently of amyloid deposition ^{20,21}. The recapitulation of these vascular abnormalities in humanized ApoE4 knock-in mouse models validates their use as a preclinical model for investigating cerebrovascular changes ^{8,20}.

Apolipoprotein E shuttles cholesterol and phospholipids between astrocytes, neurons, and vascular cells to support membrane turnover, synaptic plasticity, and injury repair ²². Although most ApoE in the central nervous system is produced locally, peripheral ApoE also interfaces with the cerebrovascular endothelium. Under normal physiology, these pools are largely separated by the blood-brain barrier (BBB), but in states of neuropathology, lipoprotein transport mechanisms (particularly receptor-mediated transcytosis through endothelial LRP1/LDLR pathways) link peripheral lipid metabolism to CNS homeostasis ²³. Recent experimental work demonstrates that peripheral ApoE isoforms differentially influence brain function, with ApoE4 in particular impairing BBB integrity and promoting cerebrovascular dysfunction ²⁴. Together, these findings suggest that isoform-dependent peripheral ApoE-lipid interactions can act as upstream modulators of endothelial vulnerability, providing a systemic pathway through which ApoE4 accelerates cerebrovascular dysfunction in Alzheimer's disease.

Despite extensive evidence for APOE4-driven vascular dysfunction, endothelial connexins remain unexplored in Alzheimer's disease. Understanding this gap is critical, as alterations in vascular connexin signaling may precede neuronal degeneration and represent a missing mechanistic link between ApoE4, impaired hippocampal neurovascular coupling, and subsequent cognitive decline. To address this, the present study focuses on Connexin 43 (Cx43) in hippocampal microvessels of 60-week-old ApoE3 and ApoE4 mice, an age at which vascular pathology is established, but also before end-stage neurodegeneration, allowing detection of connexin remodeling as an intermediate phenotype. The central aim is to visualize and quantify endothelial Cx43 alongside CD31 using optimized immunofluorescence high-resolution slide-scanning, and image analysis, establishing a standardized pipeline for quantification of the same in whole coronal sections.

CHAPTER 2: METHODOLOGY

All procedures were approved by Brown University Institutional Animal Care and Use Committee IACUC (protocol #23-01-0003) and conducted in accordance with institutional guidelines. Two groups of adult mice aged 60 weeks, of genotypes ApoE $\epsilon 3/\epsilon 3$ (female n=5 and male n=5) and ApoE $\epsilon 4/\epsilon 4$ (male n=5, female n=5) were exposed to isoflurane to overdosage levels. All mice were procured from Taconic Biosciences Inc at five weeks of age and were housed in standard cages under a 12-h light/dark cycle and provided ad libitum access to autoclaved LabDiet 5010 rodent chow.

The mice were transported from the animal facilities in a closed dark breathable enclosure. Prior to the experiments, it was noticed that the female mice in both ApoE $\epsilon 3/\epsilon 3$ and ApoE $\epsilon 4/\epsilon 4$ genotypes were visibly sharper, quicker, leaner and had a healthier coat than their age-matched male counterparts. The body weights of each animal were recorded for health screening purposes and they were subsequently transferred to the covered induction chamber of the isoflurane vaporizer. ([Appendix A: Animal Body Weights](#)) Prior to the euthanasia, they were allowed to rest and de-stress in the chamber's dim lighting for a minimum 15 minutes to a maximum 45 minutes, away from any abrupt changes in lighting and vibration. Mice were checked for normal grooming behavior at least 5 minutes before the euthanasia procedure. These stress reduction measures were in place as Connexin 43 is a dynamic protein whose expression levels are sensitive to stress. Interestingly, it is also noted that anesthetics affect different genotypes differently.²⁵.

The primary method of euthanasia was overdosage of isoflurane and the secondary method performed was cervical dislocation. Oxygen flow rate was set to 2L/min and isoflurane was set at 3%. After 2 minutes, isoflurane was increased to 5% to prevent hypoxia induction before respiratory depression onset, and maintained until animals were unresponsive, followed by cervical dislocation as the confirmatory

euthanasia method, in compliance with institutional animal care protocols. Subsequently, decapitation and brain tissue extraction were performed (in under 2 minutes) and the tissue was placed in ice cold Hibernate-A Medium (*Thermo Fisher Scientific Cat#A1247501*) for 1 minute. Experimental cohort information is summarized below:

Genotype	Number of Animals (n)	Female	Male
ApoE $\epsilon 3/\epsilon 3$	<u>10</u>	<u>5</u>	<u>5</u>
ApoE $\epsilon 4/\epsilon 4$	<u>10</u>	<u>5</u>	<u>5</u>

Table 1. Mice Demographics

2.1 Tissue Dissection

Following euthanasia, rapid tissue extraction was prioritized to minimize degradation induced pre-analytic variability in membrane-associated target Cx43. Isoflurane concentration and timing were standardized to avoid hypoxia prior to respiratory depression, and net time for extracted tissue transfer to ice-cold Hibernate-A medium was minimized to ~1 to 2 min. This controlled sequence reduced handling-related confounds before fixation and downstream immunolabeling.

The ventral side of the collected tissue was exposed immediately post-tissue collection, and a partial coronal slab containing the middle cerebral artery (MCA) was isolated within 2 min, while maintaining the tissue on ice cold Hibernate-A medium. The slab was divided as: the larger portion (Type A; whole coronal tissue including hippocampus) was immersion-fixed in 4% PFA at 4 °C for 14 to 15 h; the smaller superficial portion enriched for MCA (Type B) was trimmed and immersion-fixed for 15 min to avoid over-fixation of surface vessels. After fixation, tissues entered graded sucrose (15% then 30% in PBS) until sinking, then proceeded to OCT embedding and snap-freezing as detailed in the outline in Figure 2 on the next page.



Figure 2. Experimental Workflow

2.2 Tissue Fixation

Reliable detection of Cx43 and CD31 is dependent on fixation strategy and thus represented a key parameter in developing the protocol. Transcardial perfusion fixation was initially employed at 6 mL/minute (20 mL PBS, followed by 10 mL of PFA) which resulted in poor antigen labeling for CD31 although it is widely used for preserving morphology of brain tissue. It is plausible that this is either due to the shear stress of the perfusion process or prolonged exposure to PFA interfering with the CD31 epitope of endothelial cells lining the blood vessels, masking their epitopes (see Figure 3).

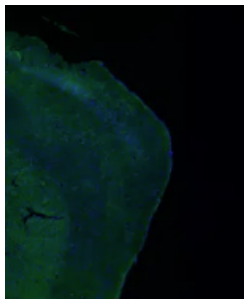


Figure 3. Staining results of perfused tissue.

The finalized staining protocol thus required omission of perfusion, and immersion fixation was employed to produce observable labeling. The Type A tissues extracted for this study were immersion-fixed for 14 hours in 4% Paraformaldehyde at 4°C and Type B tissue (MCA enriched), being smaller in volume and prone to overfixation due to the location of the MCA on its surface, required a minimal fixation period. This fixation period was optimized to be 15 minutes in 4% PFA based on level of shredding during cryostat sectioning. This produced consistent reproducible labeling ([see Figure 10](#)).

Formaldehyde diffuses into tissue at about 1 mm per hour at room temperature (slightly faster if previously perfused, slower if immersion-fixed and kept cold). The size of the tissue directly determines how long fixation must last for uniform preservation. The deeper regions within a larger tissue, such as

for rat brains, may remain under-fixed if not cut into smaller pieces. Although overnight fixation(12-24 hours) is preferred for many protocols, a 14 hour duration provides sufficient cross linking without epitope loss unlike prolonged fixation.

Alternative fixatives were evaluated conceptually and were excluded as they carried limitations for immunofluorescence of brain tissue:

- Bouin's solution is effective for histology , especially H&E staining, but its picric acid component imparts a yellow color to the tissue and interferes with fluorescence imaging.
- Methanol is commonly used in cell culture to preserve microtubules, but in brain tissue it causes dehydration and poor structural preservation.
- Acetone is rapid and suitable for cytology but induces shrinkage and brittleness, compromising section quality.

Due to these limitations, and since antibody validation protocols are almost universally optimized for 4% Paraformaldehyde (PFA), this was selected as the most appropriate choice. Concentrations below 3% PFA risk incomplete fixation and tissue degradation, whereas >6% leads to over-fixation and epitope masking. Given that brain tissue is ~80% water, 4% PFA provided the optimal balance between stabilizing protein structure and maintaining epitope accessibility.

During over-fixation, formaldehyde continues to form methylene bridges between proteins, creating a dense, cross-linked network. This leads to epitope loss through three primary mechanisms: (i) steric hindrance, in which excessive cross-linking physically blocks antibody access; (ii) chemical modification of amino acid residues such as lysine, arginine, and tryptophan, altering antigenic sites; and (iii) conformational distortion, in which proteins are locked into non-native structures that no longer display the correct epitope geometry. Of these, steric hindrance is the most common cause of epitope masking, while chemical modification and conformational distortion are more epitope-specific. These

considerations reinforced the selection of carefully timed PFA immersion fixation as the optimal approach for this study.

2.3 Cryoprotection Strategy

For this study, graded sucrose infiltration was performed at 4 °C for both Type A and Type B tissues. Samples were equilibrated sequentially in 15% sucrose followed by 30% sucrose until they sank, indicating complete infiltration (typically 24-48 h, depending on tissue volume). Interestingly, these immersion-fixed mice brain tissues appeared to equilibrate immediately upon introduction to 10% sucrose.

Cryoprotection prevents ice crystal formation that otherwise occurs when fixed brain tissue is frozen without equilibration, even under rapid freezing. In the absence of a cryoprotectant, extracellular water nucleates first, producing hypertonic brine that extracts intracellular water, leading to osmotic shrinkage of the cell, membrane stress, and protein denaturation. Together with ice crystal growth in both intra- and extracellular compartments (higher damage), these processes damage the morphology and generate sectioning artifacts such as chatter, tearing, vacuoles in white matter, and irregular vascular contours. By replacing freezable water with a cryoprotectant, and increasing extracellular viscosity, cryoprotection minimizes ice crystal formation and preserves fine morphology and antigenicity.

Sucrose is a non-permeating cryoprotectant, remains extracellular and gradually dehydrates tissue, producing a viscous matrix that favors glass-like solidification on freezing. This ensures smooth vascular profiles, preserved neuropil architecture, and reduced background in downstream immunostaining. Other cryoprotectants that were considered were ..

- Glycerol, which is a partially permeating agent and is widely used for thin, free-floating sections but infiltrates large brain tissue unevenly and decreases block rigidity at normal cryostat temperatures (-18 C to -22C), leading to compression folds and chatter during cryostat sectioning.

- DMSO is a highly permeating cryoprotectant, promotes vitrification in cell culture applications but alters lipid bilayers, extracts lipids, and can denature proteins, making it unsuitable for preserving antigenicity in whole brain sections.

For these reasons, sucrose infiltration was adopted as the optimal cryoprotection strategy for both Type A and Type B tissues in this study.

2.4 OCT Embedding and Orientation

Following fixation and cryoprotection, tissues were embedded in OCT compound to provide mechanical stability during cryostat sectioning and to preserve orientation of coronal sections. Rapid freezing was essential to suppress ice crystal formation, which otherwise distorts membranes and neuropil. OCT was chosen over alternative embedding media such as gelatin or agar as it is compatible with immunofluorescence, permits more ease of use, and ability to yield artifact-free cryostat sections.

Brains were oriented coronally within embedding molds to ensure consistent anatomical registration across samples. For Type A tissues (whole brain), the cerebellum was removed in the coronal plane, and the exposed surface served as the base for sectioning. This cut was performed in a single motion with a microtome blade to ensure a flat, even surface as incomplete or angled cuts risked embedding the sample at a tilt and prevented acquisition of true coronal sections. For Type B tissues (MCA), sufficient space was maintained between the tissue and the base of the mold to prevent immediate sectioning upon block trimming, since the MCA lies at the tissue surface and is encountered in the initial slices.

This two-step OCT equilibration minimized bubble formation within the block, which can severely impair sectioning quality, and reduced the likelihood of boundary layer separation between tissue and OCT. Such detachment commonly leads to loss of sections during cutting. Gentle swirling of the tissue in the first OCT further improved adhesion prior to transfer into the final embedding mold.

2.5 Snap Freezing Protocol

For this study, OCT embedding followed by cryostat sectioning was chosen for maximum antigen preservation. Isopentane pre-cooled with LN₂ was used for snap-freezing as it wets the cryomold and eliminates the vapor jacket, enabling rapid, uniform heat extraction and consistently finer ice formation within the tissue than direct LN₂ immersion. A metal beaker was filled to two-thirds with isopentane and nested in a Dewar such that LN₂ rose to one-third of the beaker height; the bath equilibrated for ≥ 15 min before use. Bath temperature was verified qualitatively by dipping a chilled metal spatula and confirming immediate vapor on withdrawal. To prevent vertical thermal stratification and to disrupt any solid isopentane layer formation at the bottom, the bath was gently stirred about once per minute. Solid crust formation of isopentane at the base creates a cold bottom and warm surface, yielding non-uniform freezing.

The mold containing the OCT-embedded specimen (bubble-free, final cutting face down) was grasped with long forceps and lowered into the clear isopentane without fully submerging the mold, until the OCT turned uniformly opaque. Half-immersion is avoided as it enforces different cooling rates between submerged and exposed faces; full submersion is also avoided as contact of the still-liquid top-layer OCT with isopentane can pit or craze the block surface. The mold was withdrawn while a thin cap of OCT at the center on top remained unfrozen and placed on powdered dry-ice to complete hardening or tempering, which equalizes residual thermal gradients without surface damage (see Figure 4). As OCT becomes opaque on freezing, tissue orientation was marked on the mold/block before quenching (e.g., dorsal notch, anterior arrow).

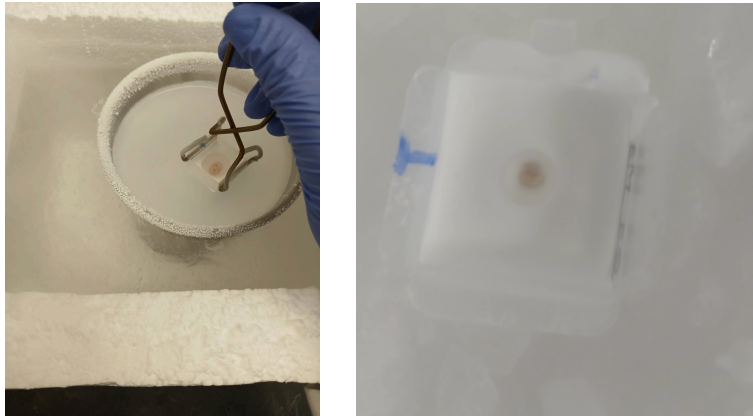


Figure 4. Mold is withdrawn, leaving a thin liquid OCT on the top center (left) and placed on dry ice (Right)

After tempering, blocks were wrapped in aluminum foil (to buffer thermal transients during transfer and to limit frost deposition). They were sealed in labeled bags, and stored at -80°C to prevent long-term dehydration or freezer burn from freezer air. For fixed and sucrose-equilibrated tissue, powdered dry ice or a dry ice-alcohol slurry is an acceptable alternative when ultra-rapid quenching is unnecessary; however, LN_2 -cooled isopentane provides the most reproducible snap-freeze for morphology-critical brain specimens and was therefore used here.

Slow freezing of any specimen produces large ice crystals and the characteristic “Swiss-cheese” distortion of architecture; thus the goal is to cool fast enough that water remains vitreous. Direct immersion in liquid nitrogen (LN_2) is discouraged due to vigorous surface boiling that creates a vapor barrier that insulates the block, causing slower, unpredictable initial cooling and frequent OCT-tissue cracking that is usually detectable only during sectioning and not by the naked eye. Direct immersion in LN_2 has also been shown to explode the resulting OCT-tissue block. By contrast, sucrose-cryoprotected, fixed tissue can be frozen in powdered dry ice, dry ice-alcohol slurries, or using LN_2 but indirectly in isopentane without incurring artifact, but true snap-freezing remains preferred for delicate morphology. Hence, LN_2 -cooled isopentane was used to wet the mold and avoid a vapor jacket, yielding uniform quenching and fewer ice-crystal artifacts.

Three freezing strategies are widely used: (i) flash-freezing of fresh tissue in OCT, which is rapid and preserves antigenicity but is highly prone to artifact; (ii) OCT embedding of 4% PFA-fixed, sucrose-cryoprotected tissue, as employed here, which yields superior morphology and tolerates either snap-freezing or slower coolants; and (iii) enzyme-preservation methods, typically used for muscle, in which tissue is supported in tragacanth without OCT, but which are technically demanding and highly susceptible to freeze-thaw injury.

2.6 Cryostat Sectioning Protocol

2.6.1 Hippocampal Sectioning Technique I

Prior to tissue sectioning, the OCT blocks stored at -80 were transferred to and left overnight in the -20 freezer for a gradual thawing to -20. The blocks were then transferred to the cryostat (Leica CM 3050 S) and allowed to thermally equilibrate for at least two hours. Immediately prior to sectioning, the foils were unwrapped, the molds were removed and the OCT-tissue blocks were mounted on the chuck using liquid OCT such that the surface of the block touching the base of the mold faces upwards on the chuck. The chuck was then placed in the cryostat to cool and solidify.

The chamber (CT) was held at -18 °C and the object temperature (OT) at -22 °C, which produced a firm, non-compressible block suitable for vascular morphology. The block was oriented to the coronal plane, faced with 20-50 µm trimming cuts to expose a flat surface, and the anti-roll glass plate was cleaned and set parallel to the blade edge with a fine gap so the tissue lay flat beneath it throughout the stroke. Section thickness was 25 µm (recommended for blood-vessel analysis). Using a sharp low-profile disposable blade (clearance angle ~5-7°) and a steady, slow advance, the ribbons were guided so that each section remained planar under the anti-roll plate with no edge curl. Tinkering with the positioning of the roll plate relative to the blade helps so that the sample sections lay flat on the stage or metal plate.

Positively charged slides (electrostatically adhesive) were pre-cooled in the chamber and used to thaw-mount sections by touching the slide to the leading edge and lowering smoothly to avoid folds or compression lines. Six sections containing different sections of the hippocampus cross section were obtained at -1.5mm to -2.5mm Bregma per brain tissue (Type A). Section quality was monitored continuously; if compression or chatter appeared, the feed rate was reduced or Object Temperature (OT) was lowered slightly, and if brittle fracture occurred, the chuck/chamber temperature was raised by 1-2 °C with the clearance angle marginally increased. Mounted sections were air-dried for 5 minutes in a dust-free tray and then stored at -80 °C in air tight sealed slide boxes to prevent dehydration.

2.6.2 Hippocampal Sectioning Technique II

Cryostat sectioning was performed at -19 °C with OCT-embedded mouse brains mounted on specimen chucks. To minimize folding and improve recovery of delicate sections, a reverse pick-up approach (see Figure 5) was employed. Rather than completing the full forward cutting stroke, the advance was stopped just short of complete release from the blade, leaving the section partially attached to the block face. The handwheel was then reversed, drawing the specimen block backward so that the section was laid flat against the cold chuck surface. Sections were subsequently collected directly from the block face using a warm glass slide. Any movement while picking up the slices can create artifacts. For sample collection, place the coated surface (up side) of a slide onto the tissue, such that it "melts" onto the slide. This approach stabilized the section, prevented curling at the anti-roll plate, and facilitated intact recovery of MCA block slices.

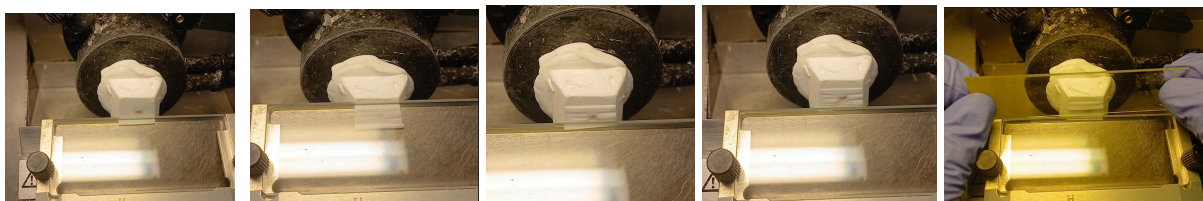


Figure 5. Reverse wheeling process to directly collect tissue-OCT section from the chuck

2.6.3 Techniques For Sectioning Type B Tissue

The cryostat sectioning protocol for the middle cerebral artery included using OCT-tissue blocks that required warmer chamber temperature of -19°C and object temperature -19°C to produce clean slices. These were significantly effortless to melt onto the slides using the above mentioned reverse wheel method (see Figure 5 on previous page), due to their small size.

2.7 Immunofluorescence Staining

All bulk reagents (buffers, rinse solutions) were prepared the day prior to staining and equilibrated to room temperature (RT) before use. Blocking solution and antibody dilutions were prepared fresh on the day of staining within 2 hours before staining. The blocking solution consisted of 0.5% PBT (PBS containing 0.5% Triton X-100) supplemented with 10% Normal Donkey Serum and 0.5% Cold-Water Fish Skin Gelatin. The primary antibody (anti-CD31, rat IgG2 κ , BD Pharmingen, Cat. 553370, clone MEC13.3) was diluted 1:200 in fresh blocking solution and anti-Cx43 (rabbit, MilliporeSigma, Cat. C6219) at 1:400 immediately before application. The secondary antibodies (donkey anti-rat IgG (H+L), Alexa Fluor 488, Invitrogen/Thermo Fisher, Cat. A-21208, and donkey anti-rabbit IgG (H+L), Alexa Fluor 594, Invitrogen/Thermo Fisher, Cat. A-21207) were both prepared at 1:300 in fresh blocking solution immediately before use. Nuclei were counterstained with DAPI (Invitrogen/Thermo Fisher, D3571; powder stock diluted per manufacturer's instructions) following secondary incubation.

Primary Antibody	BD Pharmingen 553370, clone MEC13.3
Primary Host	Rat IgG2a, κ
Working Dilution	1:200
Matched Secondary	Donkey anti-Rat IgG (H+L), Alexa Fluor 488, Thermo Fisher A-21208

Secondary Host	Donkey
Working Dilution	1:300
Cross-Reactivity Mitigation	Highly cross-adsorbed to reduce off-target binding to mouse and rabbit Ig; rat primary avoids anti-mouse secondaries on mouse tissue.

Table 2. CD31 (PECAM-1) immunostain: antibodies, hosts, and working dilutions

Primary Antibody	MilliporeSigma C6219 (polyclonal)
Primary Host	Rabbit IgG
Working Dilution	1:400
Matched Secondary	Donkey anti-Rabbit IgG (H+L), Alexa Fluor 594, Thermo Fisher A-21207
Secondary Host	Donkey
Working Dilution	1:300
Cross-Reactivity Mitigation	Highly cross-adsorbed to minimize cross-binding to mouse and rat; rabbit primary avoids anti-mouse secondaries on mouse tissue.

Table 3. Connexin-43 (Cx43) immunostain: antibodies, hosts, and working dilutions

Nuclear Counterstain	DAPI (4',6-diamidino-2-phenylindole, dilactate), Invitrogen D3571, 10 mg powder
Primary Host	—

Working Dilution	(counterstain; vendor typical 1 µg/mL working from a 5 mg/mL stock)
Matched Secondary	—
Secondary Host	—
Working Dilution	1:20,000 in final PB wash
Cross-Reactivity Mitigation	Chemical dye (no antibody); not subject to Ig cross-reactivity. Ex/Em ~358/461 nm

Table 4. Nuclear counterstain (DAPI): reagent and working conditions

Day 1: Primary Immunolabeling

Slides (positively charged, electrostatic) were warmed to RT for 15 min and then thawed on the bench for an additional ~20 min in a slide rack. Sections were rinsed in PBS (three changes, 5 min each; ~225 mL per vessel) with ~10 gentle dips per change to remove residual OCT and salts. Slides were then transferred to 0.5% PBT for permeabilization/wetting (two changes; 5 min each), leaving the rack in the final vessel while setting up the blocking step. Individual slides were removed with forceps, briefly wicked at the edges, and a hydrophobic barrier was drawn around each tissue section (PAP pen). After brief air-drying for 5 seconds, 200 µL of fresh blocking solution was applied per tissue section, and slides were incubated for 1.5 hours at room temperature in a humidified chamber. Blocking solution was aspirated and replaced with 200 µL of primary antibody (diluted in blocking solution) per slide; sections were incubated overnight at 4 °C in a sealed humid chamber.

Day 2: Secondary Immunolabeling and Mounting

The next day, 12 hours postprimary antibody incubation, slides were equilibrated to RT and rinsed in 0.5% PBT (three changes, 5 min each) to remove unbound primary. 200 µL of secondary antibody

solution (donkey anti-rat Alexa Fluor 488, 1:300 and donkey anti-rabbit Alexa Fluor 594, 1:300 in blocking) was applied per slide, and slides were incubated for 2 hours at room temperature in a light-protected humid chamber (wrapped in aluminum foil). Subsequent steps were performed under normal room light. Slides were rinsed in 0.5% PBT (three changes, ~5 min each), then in 0.1 M phosphate buffer (PB; two changes, 5 min each); for nuclear counterstaining, DAPI was included in the second PB rinse (working solution prepared by adding 10 μ L of 1 mg/mL DAPI stock to 200 mL PB; 1:20,000). Sections received a final brief distilled water dip (≤ 3 quick dips) to remove residual phosphate prior to mounting. Slides were mounted with ProLong Gold antifade and coverslipped carefully to avoid bubble formation, then cured for 24 hours in the dark at room temperature. The prepared slides were placed in sealed slide boxes at 4 °C and imaged the following day.

Mechanism of Action of Reagents

Rinses in phosphate-buffered saline (PBS; ~50 mM phosphate at near-physiological ionic strength and pH 7.2-7.4) maintain osmotic balance while removing OCT, fixative residues, and unbound reagents; preserving ionic strength prevents tissue swelling or extraction that can occur in hypotonic media. Subsequent washes in 0.5% PBT (PBS + 0.5% Triton X-100) reduce surface tension and, in fixed tissue, gently solubilize lipid barriers to improve antibody access to intracellular and membrane-adjacent epitopes; the detergent also enhances wetting so solutions exchange uniformly across the section. A hydrophobic PAP-pen barrier raises the contact angle at the tissue edge, confining small reagent volumes to the section and maintaining effective antibody concentration without edge wicking. Incubations were performed in a humidified chamber for overnight primary, to minimize evaporative concentration drift and ensure consistent microenvironment across slides.

The blocking solution (PBS/Triton with 10% normal donkey serum and 0.5% fish-skin gelatin) saturates non-specific protein-binding sites and Fc-like interactions: serum proteins competitively occupy hydrophobic and ionic sites and match the host species of the secondary antibody, while gelatin provides a

collagen-derived film that further suppresses low-affinity adsorption, lowering background and stabilizing antibody distribution during incubation. DAPI was introduced at low concentration during a phosphate-buffer rinse to label nuclei via minor-groove binding of dsDNA while the buffer continued to remove unbound secondary; shielding from strong light limited premature photobleaching of Alexa-fluorophores. The brief distilled-water dip immediately before mounting removed residual phosphate that can crystallize under coverslips; the dip was kept short to avoid osmotic swelling of the section. ProLong Gold antifade provided a curing, high-refractive-index matrix that limits free-radical mediated fluorophore loss, yielding stable, low-background preparations for imaging.

2.8 Image Analysis Pipeline

Olympus VS200 Slide Scanner scans slides and outputs the data in a proprietary Virtual Slide Image (.VSI) format. The resulting imaging output files are complex, pyramidal structures presented as a “series” of 2D images resulting from a single scan of a slide. Further, each series has three planes corresponding to the three channels. The series differ in resolution. VSI files come with rich metadata (similar to OME-TIF formats).

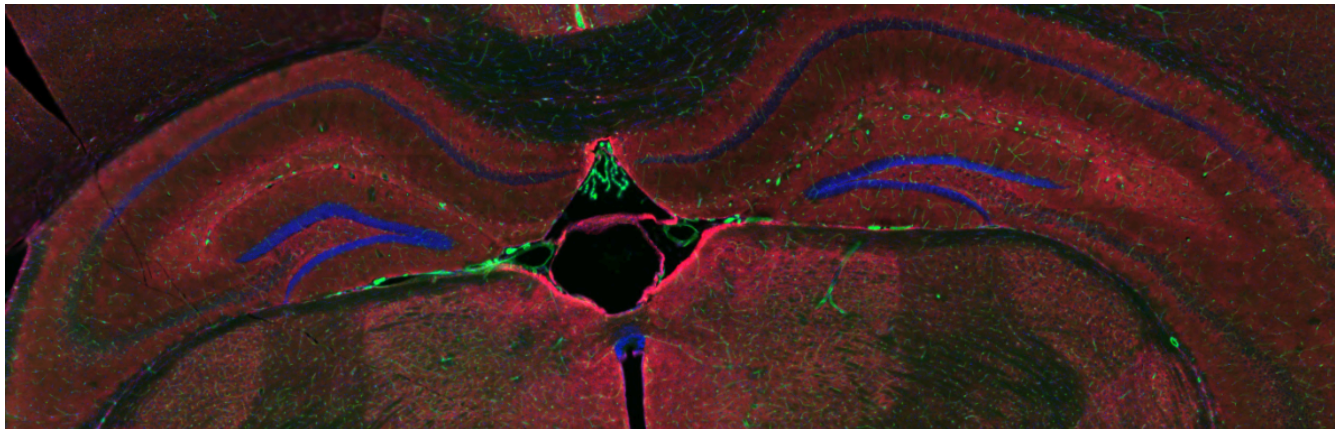


Figure 6. Sample Image of Analyzed Hippocampal Region

At 40 $\times$ magnification, each VSI image file was approximately 7 GB corresponding to a resolution of roughly 65K x 65K for the entire coronal section. For a cropped ROI of the hippocampus, the average file size was found to be 2 GB corresponding to a rough 45K x 45K resolution (see Figure 6).

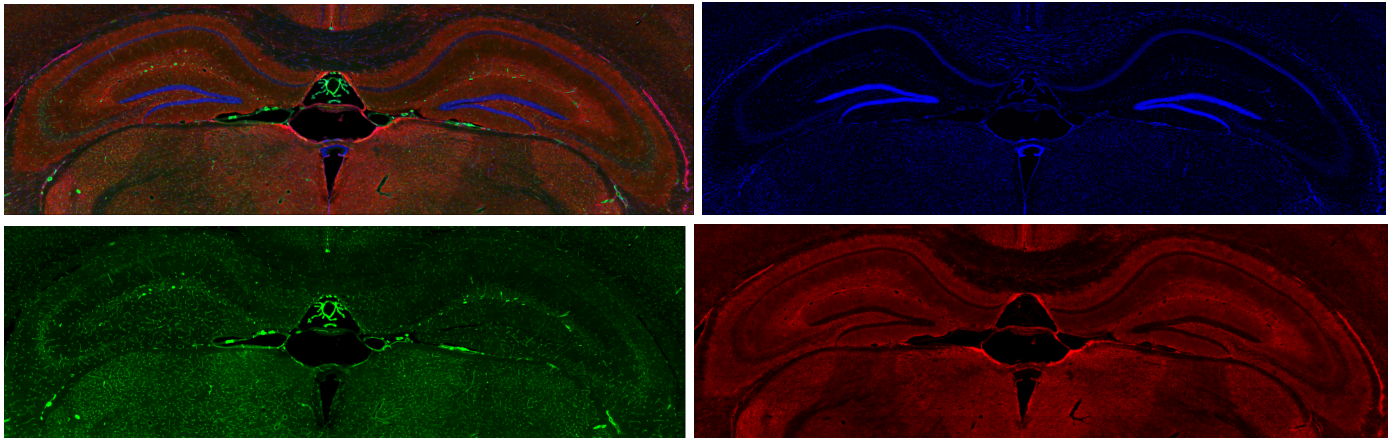


Figure 7. Sample Hippocampal image, split into 3 channel specific images. Top Right shows cell nuclei- DAPI(blue); Bottom Left- CD31- FITC(Green); Bottom right- Cx43-mCherry (Red)

These were cropped to rectangular regions containing the hippocampal area using Olympus VS200 ASW software and saved in their original .vsi format to prevent any loss in metadata. All subsequent image analysis testing was performed in the original .vsi format. The additional 46 images of the MCA cross section, acquired from ten selected MCA samples (ApoE3 female n= 5, ApoE4 female n=5) were not subjected to quantitative analysis as they consistently failed quality control, and required re-imaging which was outside the scope of the timeline of the project.

From a total of 104 images, 4 images per brain sample were chosen, yielding 80- images. These were cropped to rectangular regions containing the hippocampal area using Olympus VS200 ASW software and saved in their original .vsi format to prevent any loss in metadata. All subsequent image analysis testing was performed in the original .vsi format. The additional 46 images of the MCA cross section, acquired from ten selected MCA samples (ApoE3 female n= 5, ApoE4 female n=5) were not subjected to quantitative analysis as they consistently failed quality control, and required re-imaging which was outside the scope of the timeline of the project.

2.9 Analysis Software and Tools

Given the large file sizes, multiple image visualization tools were evaluated - OlyVIA 4.2 and QuPath 0.6.0, which were found to be quick in rendering the high-resolution images along with the image metadata. Fiji/ImageJ 1.54p was slow to render high-resolution series. By way of illustration, when using the same computer, OlyVIA and QuPath would render an image in about 5 seconds while Fiji/ImageJ required 5 minutes for the highest resolution (65K x 65K) series. Fiji/ImageJ is not optimized for high-resolution whole-slide data; operations are slow. However, it provides flexibility in selecting ROI, applying filters, adjustments, and several other features (Figure 8 shows the image import options). The test analyses were done programmatically using Fiji/ImageJ macros. A comparison of software packages available for analyzing biological images was also reviewed [28](#). Fiji/ImageJ Version 1.54p was used for testing. Images were imported for visual inspection using the following settings:

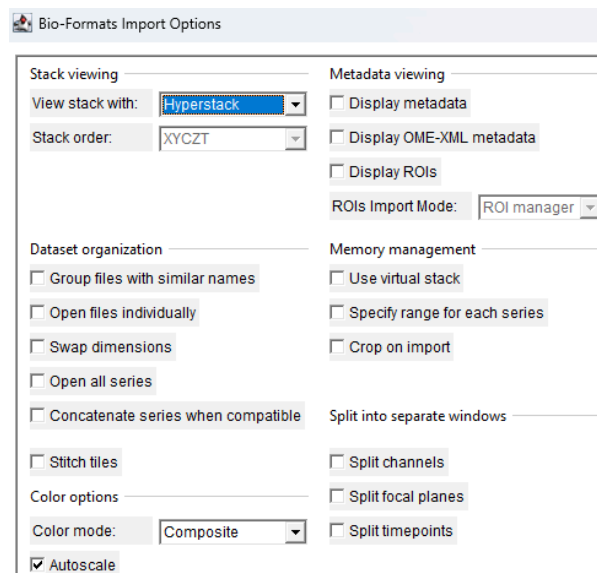


Figure 8. Fiji/ImageJ Image Import Options

In some cases particularly for complex image formats such as .vsi, the ImageJ Bio-Formats Importer performs image series selection by using the image series number-name match, especially while opening the image through the GUI. It must be noted that, when the image is processed through a program, the

series index is a more preferable choice. Series with index value zero does not have the number zero in the series name and the image series has to be accurately retrieved and confirmed prior to processing.

Given the slow processing speed encountered with ImageJ, an AWS Elastic Compute (EC2) server in USA Virginia Region was used for a faster data analysis pipeline. Two different servers were tested - one for fast single-threading, and one for multi-threading. Both versions yielded similar processing times. The specifications for the AWS computers that were used are provided below. ImageJ does not take advantage of parallel processing features or loading by tiles (like QuPath).

Machine	“Z1” Series	“G5” Series
Purpose	Ideal for high speed single threaded computing	Ideal for parallel computations like image processing (with GPUs)
Virtual Processors	12 vCPU	8 vCPU
Memory	96 GB	32 GB
Architecture	x86_64	x86_64
Storage	450 GB	450 GB
Storage Type	SSD	SSD
Network	Up to 10 Gbps	Up to 10 Gbps

Table 5. Server configurations for the image-analysis data pipeline

Sample performance metrics from the “Z” series machine when analyzing the images are in Figure 9. The memory allocation is further managed by having regular Java garbage collection calls in the ImageJ image analyses program.

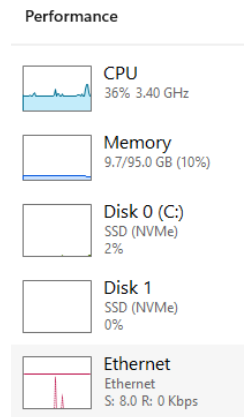


Figure 9. Compute Server Performance when processing a sample image

The data transfer to the AWS computers had to be done without compressing the files as it would result in loss of image data fidelity. Files were transferred without compression using WinSCP and enabling the binary transfer mode. The OpenSSH services were launched on these servers to receive SFTP port 22 data transfers through WinSCP.

2.10 Image Analysis Algorithm

- Processes all .vsi files in a specified input folder in batch mode
- Supports downscaling of image series/ resolution for faster testing
- Handles 3 channels:
 - C1 = DAPI (nuclei)
 - C2 = FITC (vasculature)
 - C3 = mCherry (connexins)
- Detects poor image quality conditions
- Applies filters and adjustments like CLAHE, Gaussian Blur, Background Subtraction, Thresholding, Binarization, etc.
- Allows for per channel customization of filters and adjustments
- Detects and measures particles per channel

- Optional logic to measure overlap between C3 and C2
- Saves results to disk

The Fiji/ImageJ macro is provided in [Appendix C - Image Analyses Program](#).

2.11 Freehand Cropping of ROIs

Freehand cropping of hippocampus area, to mark a Region of Interest (ROI), is a challenge as Olympus provides the scanned file in a proprietary VSI format and the corresponding file viewing software, OlyVIA, does not have freehand cropping features. This resulted in the hippocampus being cropped to a rectangular box but using an accurately scaled image.

Software	Freehand Cropping Challenges
Olympus OlyVIA (software from the Slide Scanner vendor) and QuPath	ROI selection can be performed only in fixed shapes. Freehand cropping is not a supported feature.

Table 6. ROI selection using OlyVIA and QuPath

However, when attempting freehand cropping using third party software products (listed in the table that follows), the key challenge to be noted was when marking an ROI on a computer screen using a downsampled or preview version of a high-resolution image (e.g., 65,000 × 65,000 pixels for a 40× mice brain image)- the ROI coordinates correspond to the lower-resolution data. These lower-resolution coordinates do not automatically map to the full-resolution image, which can result in spatial misalignment or shifts when the ROI is upscaled programmatically for processing as a cropped image. Olympus VS200 ASW appears to run this without errors, albeit on a rectangular ROI, as it has access to the proprietary .vsi image file format.

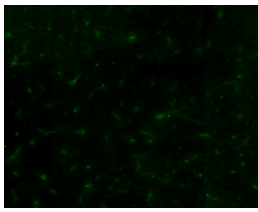
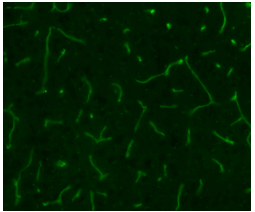
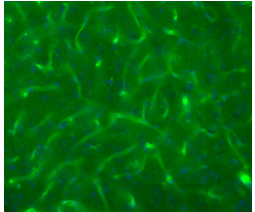
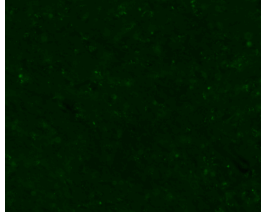
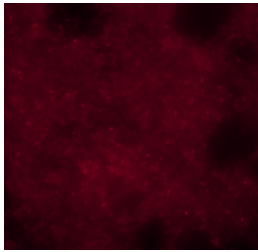
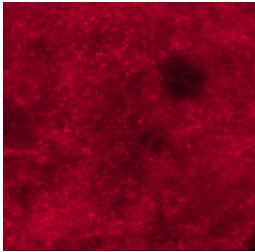
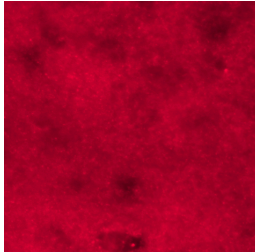
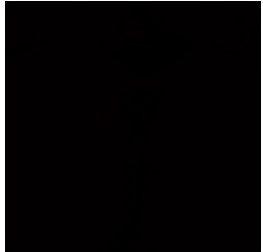
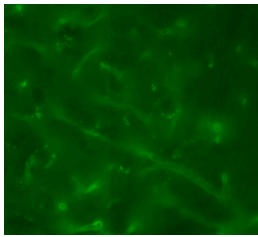
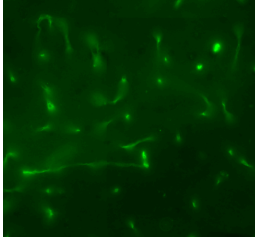
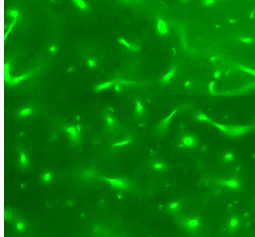
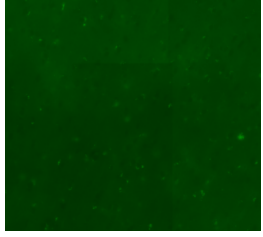
Software	Freehand Cropping Problems
Fiji ImageJ (freeware)	Freehand cropping of non-contiguous areas leads to multiple ROIs, duplicate in-memory images, and the system running out of memory. Example: A 1.5 GB image file took up 24 GB RAM when hippocampus was marked out without “undo” capability.
Napari (freeware)	The export to OME-TIF does not offer to save only the cropped area.
QuPath (freeware)	The export to OME-TIF does not offer to save only the cropped area.

Table 7. Comparison of Freehand Cropping Tools

CHAPTER 3: RESULTS

3.1 Protocol Validation

Numerous protocols were tested before arriving at a protocol feasible for the objectives of this study for multiplex immunofluorescent labeling both CD31 and Cx43. The antibody dilutions listed in the ensuing table were used to arrive at the optimal dilutions of the primary and secondary antibodies:

Antibody	Low Concentration	Mid Concentration	High Concentration	Secondary-only control (no primary)
Primary Antibody- anti CD31	 1:1000	 1:200	 1:50	
Primary Antibody- anti Cx43	 1:800	 1:400	 1:100	
Secondary antibody - donkey anti-rat IgG (H+L), Alexa Fluor 488,	 1:600	 1:300	 1:100	

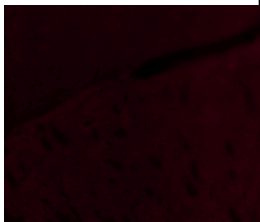
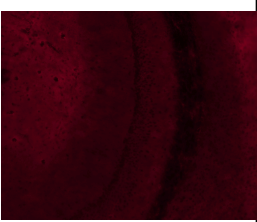
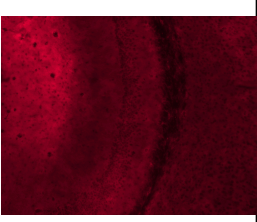
Antibody	Low Concentration	Mid Concentration	High Concentration	Secondary-only control (no primary)
Secondary antibody - donkey anti-rabbit IgG (H+L), Alexa Fluor 594,	 1:800	 1:400	 1:100	

Table 8. Optimization of Dilutions of Primary and Secondary Antibodies

Optimal antibody and secondary dilutions were established empirically because working concentrations for anti-Cx43, anti-CD31, and Alexa Fluor 488/594 secondaries were not defined for the need of the study. With methodological input from a collaborating laboratory at The Ottawa Hospital Research Institute, a four-point dilution series for each reagent (four dilutions × four reagents; 16 conditions) was prepared. Adjacent sections were processed in parallel and imaged under identical acquisition settings; conditions were compared using overview mosaics for signal-to-background ratio, preservation of expected morphology (endothelial labeling for CD31; discrete puncta for Cx43), uniformity across regions, and absence of off-target staining, with no-primary and secondary-only controls included. The selected working dilutions were those that maximized specific signal while minimizing background and variability, and these settings were used for all subsequent experiments (see Figure 10 on the next page for the end result of the multiplex immunofluorescent staining).

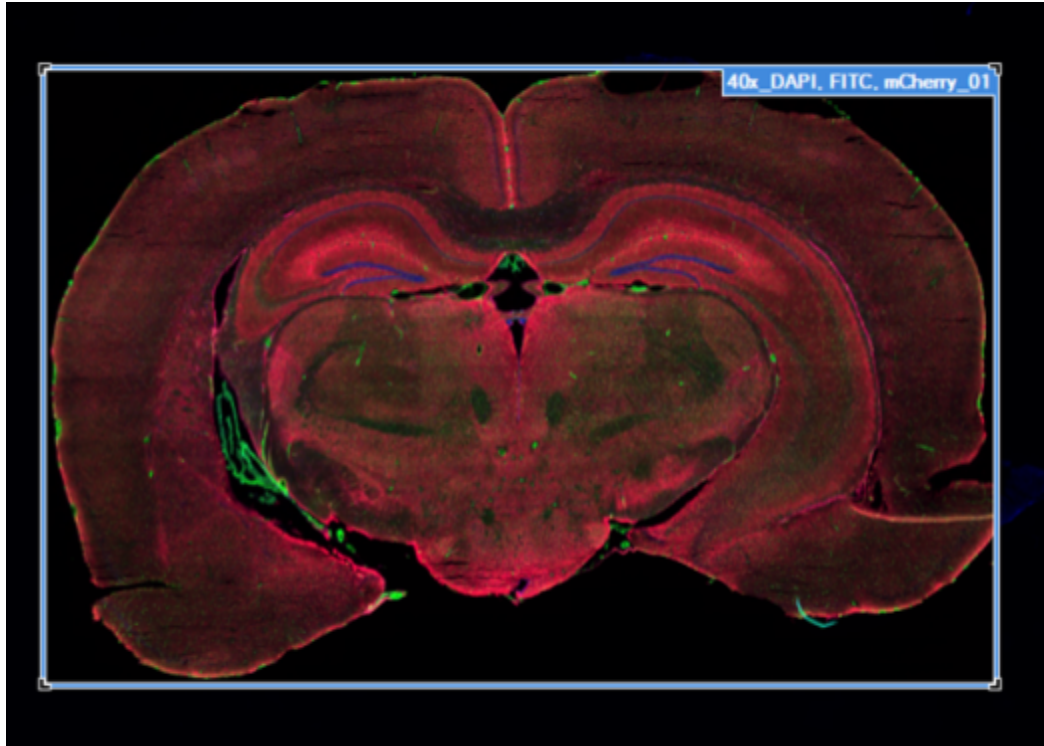


Figure 10. Olympus VS200 Slide Scanner Image of a Whole Coronal Section following the established protocol.

3.2 Validation of Antibody Binding (Confocal Microscope)

To verify that the granular red signal on whole-slide images represented Connexin-43 (Cx43) puncta rather than background, selected fields in middle cerebral artery (MCA) and hippocampus were imaged on an Olympus FV3000 confocal microscope in resonant mode using a 60× oil objective (10× overview for orientation). Z-stacks were acquired across the section thickness (~25 μm) at 512 × 512 pixels, with 16-bit capture and fixed detector settings according to the protocol. Excitation lines were 405/488/561 nm for DAPI/Alexa Fluor 488/Alexa Fluor 594, respectively, with emission detection windows 430 to 470 nm (SD1), 500 to 540 nm (SD2) and 570 to 670 nm (HSD3); laser power, HV, gain and offset were kept constant across fields. For presentation, stacks were rendered as maximum-intensity 2D projections, and individual optical sections were examined to assess morphology.

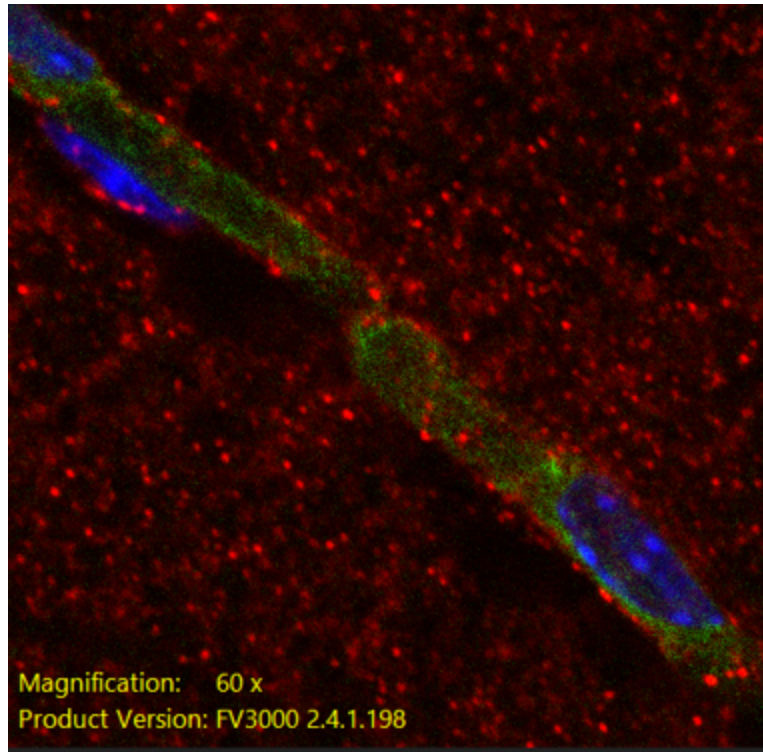


Figure 11. Confocal validation of antibody specificity for Connexin-43 (Red); Cell Nuclei- DAPI(blue);
CD31- FITC(green)

Confocal planes resolved the red channel into discrete submicron puncta at or near the diffraction limit that persisted across adjacent z-slices, in contrast to the diffuse appearance on the Slide Scanner(see Figure 11 and Figure 12). In MCA, puncta localized along FITC-labeled vascular profiles; in the vessels within the coronal sections, they appeared as distinct membrane-adjacent foci within the neuropil. These observations indicate that the signal initially interpreted as “background” corresponds to bona fide Cx43 puncta, supporting the use of confocal-validated puncta counts in the downstream analysis.

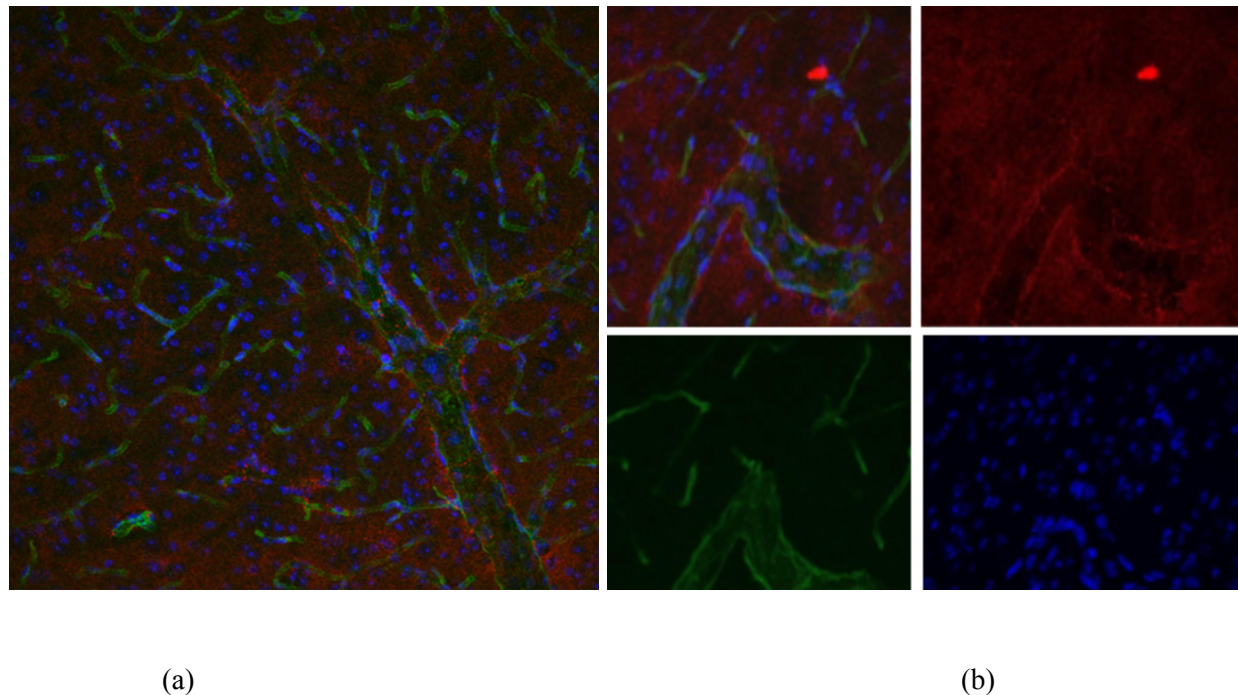


Figure 12. Confocal validation of antibody labeling in vasculature. (a). Punctate Connexin-43 immunofluorescence observed in close association with the vasculature. Composite image showing nuclei (DAPI, blue), endothelial marker (green) and Connexin-43 (red). (b) Second representative field, shown as a composite (right) and separated into individual channels: green- endothelial CD31 marker, Connexin-43 (red) and nuclei- DAPI (blue)

In both middle cerebral artery (MCA) and hippocampal fields, the confocal single planes resolved the mCherry signal into discrete, high-contrast puncta with lateral extents (see Figures 11 and 13), at or near the diffraction limit (apparent diameters typically ~ 0.3 to $1.0 \mu\text{m}$), rather than the diffuse haze seen on the Slide Scanner. Puncta persisted across adjacent z-planes and were absent from no-primary controls, consistent with specific Cx43 labeling. In MCA, puncta were enriched along vascular profiles (co-registered with FITC), and in the hippocampus they appeared as distinct foci against low background. These observations confirm that the signal initially judged as “background” corresponds to bona fide Cx43 puncta, supporting the downstream puncta analysis on the batch-standardized datasets.

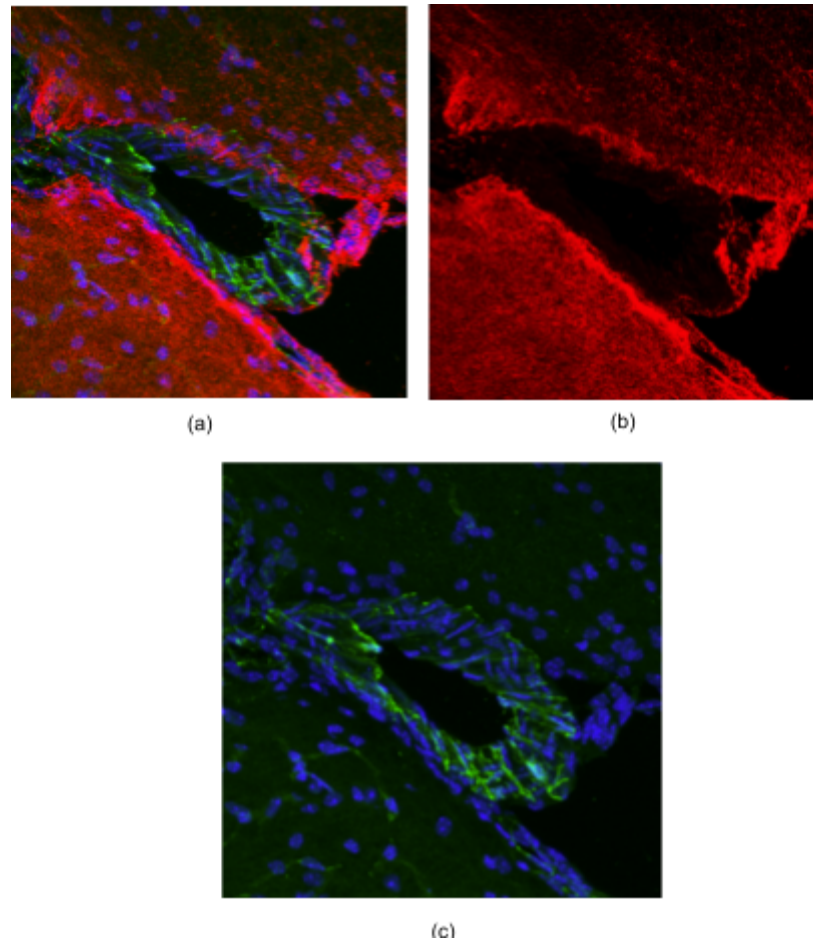


Figure 13. Confocal immunofluorescence imaging of middle cerebral artery(MCA). (a) Composite image showing DAPI(blue), endothelial marker (green), and Connexin-43(red) around the vessel wall. (b) Connexin-43 channel separately(red), highlighting vascular distribution. (c) Endothelial marker (green) with DAPI demonstrating vascular localization of endothelial structures.

3.3 Imaging Strategy and Modality Selection

Following establishment of working dilutions for anti-Cx43, anti-CD31, and Alexa Fluor 488/594 secondaries, the study required high-throughput acquisition across hundreds of coronal sections under uniform conditions suitable for quantitative comparisons. Because target regions (e.g., hippocampus) extend over millimetre-scale areas that exceed a single confocal field, and because per-slide z-stacking and mosaicking would impose substantial design changes which were outside the scope of the project, a

whole-slide scanning with fixed, clip-safe exposures as the primary modality was adopted and confocal microscopy was used only for targeted validation of puncta morphology.

3.4 Optical Compatibility and Mounting

The Slide Scanner was hence chosen as it captures entire coronal sections or large ROIs in a single tiled run, supports batch imaging with identical exposure settings across the cohort, and achieves the required throughput at manageable operating time in alignment with project scope. Maintaining identical settings across many slides is impractical using a confocal microscope (see Figure 14) due to per-ROI adjustments and instrument drift, large areas that require mosaics and multi-plane z-stacks with long acquisition and stitching times; and full 3D analysis at cohort scale was outside the scope of the project.

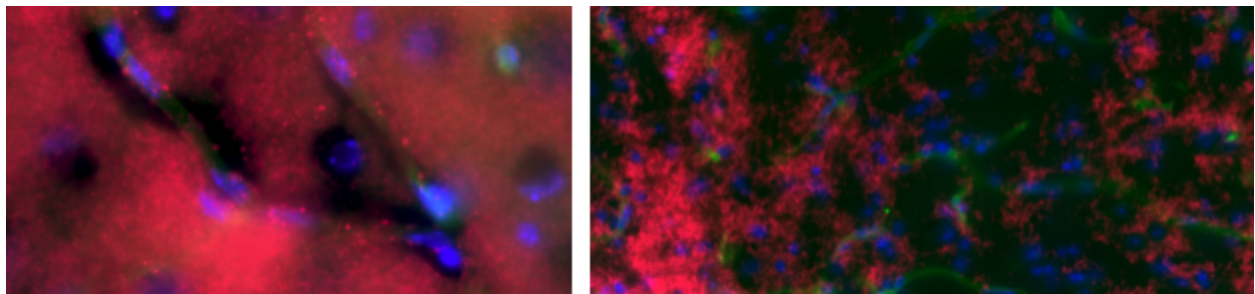


Figure 14. Confocal (60× magnification) vs Slide Scanner (40× magnification, dry)

The study targeted endothelial CD31 and Connexin-43 (Cx43) and therefore immunolabeled with Alexa Fluor 488 and Alexa Fluor 594, adding DAPI for nuclei to provide a blue- green- red triad with wide spectral separation for sequential whole-slide acquisition. As slide scanning has long exposure trains, a hard-curing antifade mountant (ProLong Gold/Diamond) was selected to suppress photobleaching and to stabilize the specimen. After overnight cure, ProLong reaches a glycerol-like refractive index ($n \approx 1.47$) and forms a rigid matrix that preserves AF488/AF594/DAPI brightness through 25- μm sections.

Given the mounting medium's refractive index ($n \approx 1.47$), imaging was performed with the Olympus VS200 40× dry objective. This lens is corrected for air above a #1.5 coverslip and therefore minimizes

wavefront error under the conditions employed. By contrast, use of a 40× oil-immersion lens (designed for immersion oil of $n \approx 1.515$) would have imposed a refractive-index mismatch beneath the coverslip due to the lower-index mountant. The resulting layered refractive index discontinuity generates depth-dependent spherical aberration, manifesting as axial blur and progressive loss of high-frequency contrast with section depth.

The #1.5 coverslips of 0.17 mm thickness were selected, as most high-NA (Numerical Aperture) slide-scanner objectives are designed to be corrected for a 170 μm glass layer. At this thickness, primary spherical aberration from the glass/air interface is minimized. Deviations from this design thickness, whether thinner or thicker, introduces path length differences: marginal rays are either retarded or advanced relative to paraxial rays, broadening the point-spread function and reducing contrast. This effect is compounded by specimen thickness. In 25 μm sections, rays traverse additional mountant, and any refractive-index mismatch between the specimen medium and the objective design index produces proportionally larger wavefront errors.

Standard oil-immersion 40× objectives are engineered to assume a near-uniform optical path of $n \approx 1.515$ (oil) through a coverslip of $n \approx 1.52$ into the specimen. With ProLong Gold or Diamond ($n \approx 1.47$) beneath the glass, the optical system instead encounters a layered index stack for which the objective is not corrected. This produces depth-dependent spherical aberration, perceived as axial blur and attenuation of high spatial frequencies as focus moves deeper into the section.

Without a correction collar simultaneously tuned for both coverslip thickness and specimen index, an oil-immersion 40× lens cannot compensate for these errors. For this reason, the 40× dry objective, although lower in theoretical NA, produced superior effective resolution and contrast for these preparations.

The causal sequence follows:

Targeting of (CD31/Cx43) → Use of dyes (AF488/AF594 + DAPI) → Use of antifade mountant (ProLong, $n \approx 1.47$) → 40× dry objective (air-corrected) → #1.5 coverslip—yields better field-wide sharpness and stable signal during long, multi-tile scans.

Slide Scanner Specifications and Throughput

Slides were initially scanned on an Olympus Slideview VS200 with a UPLXAPO 40×/0.95 air objective (WD = 180 μm) and 1.0× camera adapter coupled to a Hamamatsu ORCA-Fusion sCMOS (1×1 binning), yielding 0.1625 $\mu\text{m}/\text{px}$ sampling (X,Y), and checked for image sharpness under both 40× dry and 40× oil objective for confirmation of the above.

Acquisition Workflow and Focus Policy

For the trial, slides were loaded into the Olympus Slideview VS200 and, per the prompt, confirmed to be free of immersion medium. Trays were exchanged and scans were configured in Single Scan, Fast FL mode. In the scan settings, only the fluorescence channels required for this study were enabled (DAPI, FITC, mCherry), with all others disabled. A 4× DAPI overview was first acquired to delineate tissue, after which regions of interest (ROIs) and the focus map were defined (focus-point density set to High; no Z-stack). For exposure calibration, Live mode was used on each channel to adjust exposure in three representative ROIs per slide, using the brightest field to ensure headroom and <10% saturated pixels while watching for bleaching or artifacts. The finalized exposure values were then entered into the scan settings, and the 40× scan was initiated with sequential acquisition of DAPI → FITC → mCherry over the defined ROIs and focus points. Initial optimal exposure times were determined to be in the following ranges for the three channels as DAPI: ~17 ms, FITC : ~454 ms, mCherry : ~99.80 ms.

3.5 Exposure Time Optimization

Due to the variability in exposure times produced by the auto-exposure mode across slides, an optimization protocol was implemented using eight representative sample slides (four per group).

Standardizing exposures is necessary to separate acquisition conditions from tissue context and enable valid between-group comparisons. Auto-exposure targets a brightness criteria that varies with local anatomy and staining, and provides slide-specific intensity scaling and potential clipping in bright regions. Fixing exposures from the upper-intensity tail (brightest ROIs) prevents saturation while preserving 16-bit dynamic range for dim structures, and measured differences reflect biology more than acquisition conditions. This improves reproducibility of puncta counts and vessel area estimates and permits direct quantitative comparison between ApoE3 and ApoE4 datasets. [Appendix B - Scan Times](#) has additional information on slides used to arrive at optimal exposure times.

For fluorescence exposure standardization across coronal sections, an unvarying clip-safe exposure times per channel using a brightest-region sampling strategy was first established. On the Slide Scanner (16-bit acquisition; fixed illumination and camera gain/offset; all automatic contrast/gamma disabled), three brightest ROIs per slide per channel (FITC vessels, mCherry puncta, DAPI nuclei) were selected on an initial set of eight representative slides (four ApoE3, four ApoE4); two additional slides were then added to validate stability (total candidates per channel: up to 21 ROIs). At each ROI the software's auto-exposure was invoked once and the exposure time (ms) recorded, while keeping all other parameters constant (See [Appendix B - Scan Times](#)). For FITC and mCherry, one slide with acquisition failure was excluded; DAPI values from that slide were retained as they were valid. For each channel, auto-exposure times (lower time = brighter ROI) were ranked and the fixed exposure was defined as 90% of the 3rd-lowest value (a robust approximation to the ~10th percentile), providing a $\geq 10\%$ safety margin against saturation at true intensity maxima. Then the resulting settings were validated on one ApoE3 and one ApoE4 slide using the scanner's saturated-pixel overlay/histogram, targeting $\leq 0.1\%$ saturated pixels per channel; if exceeded, exposure was reduced by an additional 5% and rechecked. The finalized fixed exposures applied to the entire batch were: DAPI 6.5 ms, mCherry 37 ms, and FITC 310 ms. All subsequent imaging was performed with auto-exposure off, unchanged illumination and gain/offset, and identical acquisition settings across slides to preserve quantitative comparability.

Final Acquisition Parameters - Fixed Exposure Times (Slide Scanner)

Final values used for batch scan	Exposure time in ms
DAPI	6.5 ms
FITC	310 ms
mCherry	38 ms

Table 9. Slide Scanner Exposure Times

Fluorescence was acquired sequentially with the instrument’s DAPI, FITC (collecting AF488- CD31), and mCherry (collecting AF594- Cx43) filter sets and images were saved as 16-bit channel planes. Scan parameters were balanced against specimen size: Type-1 tissue (larger area) required ~13 min per slide, whereas MCA blocks (small, surface-proximal ROIs) completed in ~3-4 min with the same optical settings since there are fewer tiles and little refocusing is required. Sampling at 0.1625 $\mu\text{m}/\text{px}$ was retained for all tissues to keep vessel-edge sharpness and puncta detectability consistent across datasets.

3.6 Qualitative Data Analysis Results

(i) Hippocampal region shows decreased vascular branching compared to rest of the brain regions in both ApoE $\epsilon 3/\epsilon 3$ and ApoE $\epsilon 4/\epsilon 4$

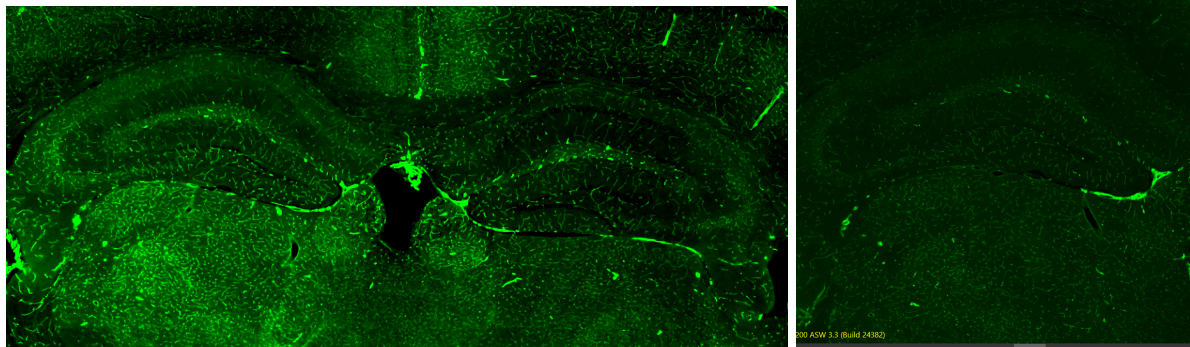


Figure 15. Decreased Vascular Branching in Hippocampus

FITC/CD31 staining delineated a continuous microvascular network across hippocampal strata with occasional medium-caliber vessels along the hippocampal fissure (see Figure 15). Under identical acquisition and display settings (see Methods), the hippocampal parenchyma showed visually lower fine-capillary density and branching complexity relative to adjacent cortex within the same section, with larger inter-capillary spacing in strata radiatum/oriens. This pattern was observed across the aged sections imaged in this batch, consistent with age-associated microvascular rarefaction; formal quantification of vessel area fraction, length density, and branch-point density will follow.

(ii)(a) Cx43 shows a consistent spatial pattern across coronal sections, with enrichment in hippocampal subregions

Across four independent brains (one coronal section per animal), Cx43 immunofluorescence displayed a reproducible, non-uniform distribution. Signal was enriched in the hippocampal formation with laminar bands (dentate gyrus molecular layer and CA1 strata radiatum/lacunosum-moleculare), whereas surrounding neocortex was more diffuse and white-matter tracts were relatively low (see Figure 16).

Despite modest inter-animal differences in overall intensity, the spatial pattern was conserved. Occasional linear, vessel-like profiles with punctate Cx43 were seen along the hippocampal fissure. Formal subfield quantification will follow.

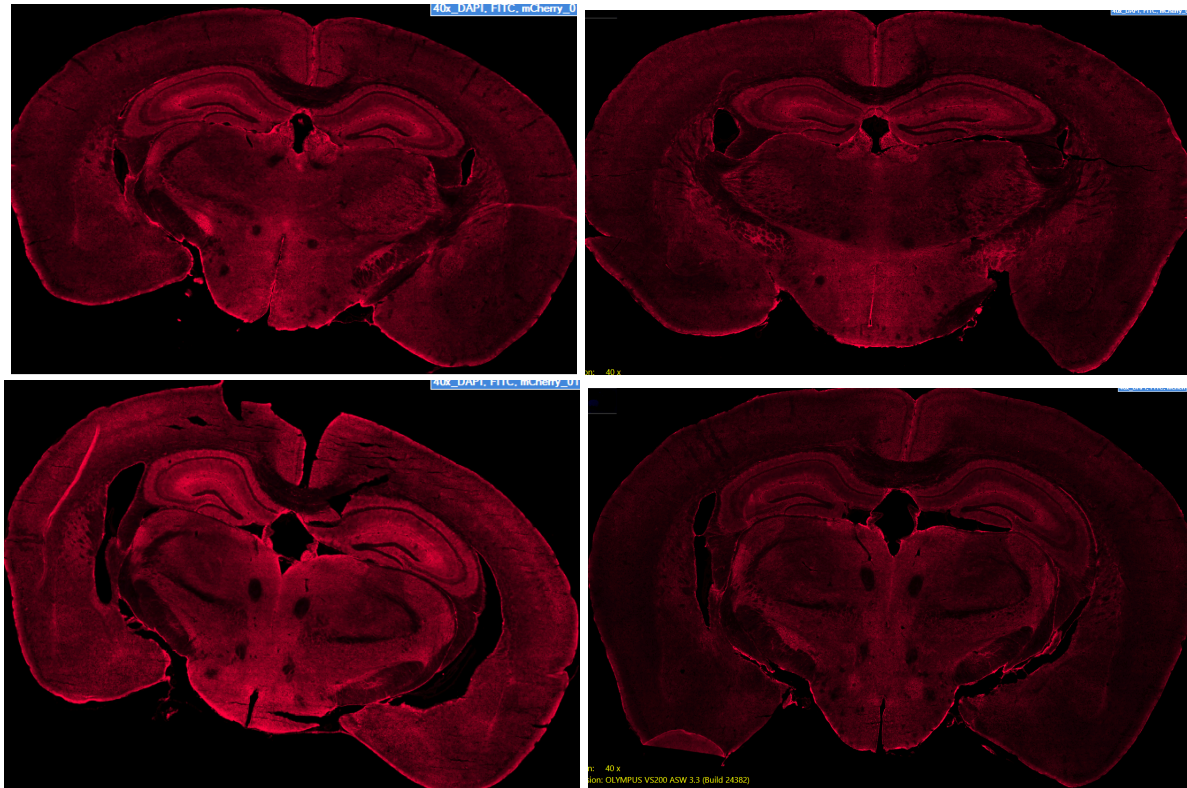


Figure 16. Cx43 non uniform expression patterns in whole coronal section across four different brains(ApoE3 n =2, ApoE4 n=2)

(ii)(b) Differential expression of Cx43 in DG, CA1, CA2 within Hippocampus

Using the adult mouse coronal atlas (P56) [26](#) to guide ROI boundaries, Cx43 immunofluorescence exhibited a laminar, subfield-specific pattern across the hippocampal formation. Signal was most evident in the dentate gyrus (molecular layer > granule cell layer) and along CA1 strata radiatum/lacunosum-moleculare, with comparatively lower intensity across the narrow CA2 band (see Figure 17 on the next page).

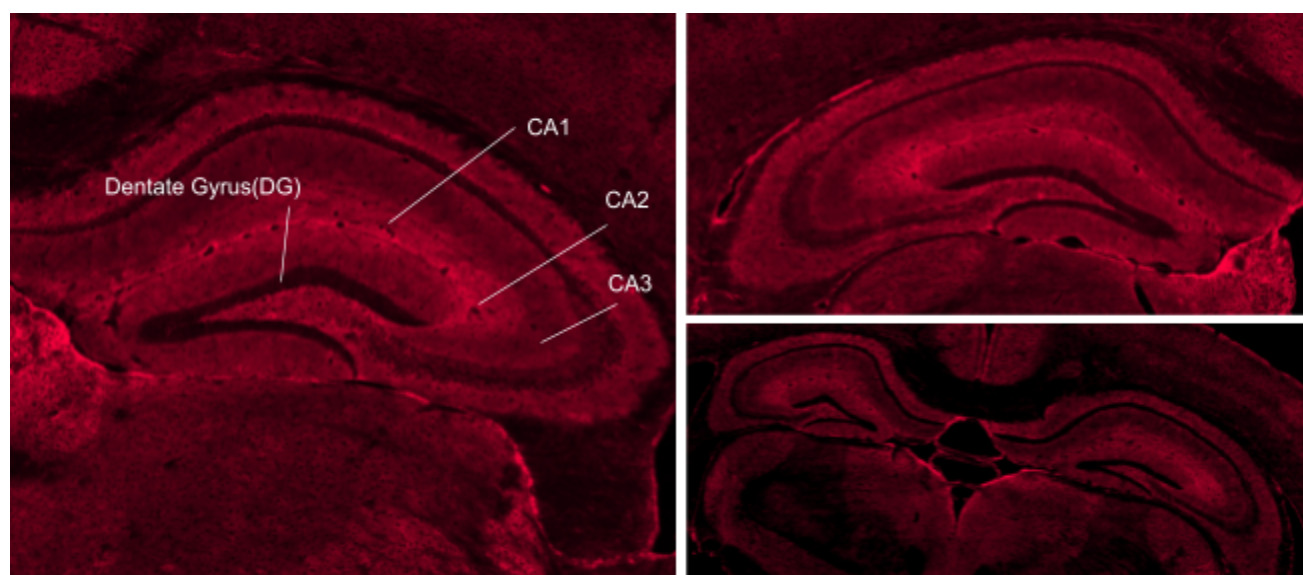


Figure 17. Representative Cx43 immunofluorescence in the hippocampus with subfield boundaries (DG, CA1, CA2) drawn by reference to an adult mouse coronal atlas.

The same pattern was observed across all 20 brain sample sections acquired with fixed settings. These observations support subfield-specific distribution of Cx43 in DG and CA fields; formal quantification will follow.

3.7 Pipeline for Preliminary Quantitative Image Analysis

To verify end-to-end execution rather than to estimate biological effects, four coronal image samples of low resolution were processed with the parameter-locked Fiji/ImageJ macros. The pipeline returned (i) DAPI nuclear counts, (ii) CD31 vessel-area fraction, and (iii) Cx43 puncta counts within atlas-anchored hippocampal ROIs. As expected with downsampled images and non-final filters/ROIs, absolute values are biased (nuclei and vessels are under-segmented; puncta are partially merged or lost), and only descriptive outputs are reported. Across sections, relative rankings were internally consistent, and magnitudes were stable enough to confirm pipeline feasibility. Parameter stability requires additional customization to ROI and other modifications. Definitive quantification will be repeated on full-resolution data with finalized ROIs, filter cutoffs, and calibration.

Sample ID	Count	Total Area	Average Size	%Area	Perim.	Circ.	Solidity
E3_F1_HC_S53_C1	8,646	1,577,316	182.433	15.183	58.903	0.594	0.789
E3_F1_HC_S53_C2	17	1,743,712	102571.294	16.784	666.116	0.575	0.897
E3_F1_HC_S53_C3	12,675	6,011,399	474.272	57.863	66.651	0.591	0.791
E3_M1_HC_S011_C1	8,217	1,458,453	177.492	12.346	57.703	0.594	0.788
E3_M1_HC_S011_C2	36	7,469,473	207485.361	63.232	1345.976	0.539	0.889
E3_M1_HC_S011_C3	23,677	6,072,457	256.471	51.405	56.758	0.595	0.787
E4_F3_HC_S171_C1	8,961	1,439,402	160.63	13.312	55.33	0.594	0.784
E4_F3_HC_S171_C2	13	2,169,969	166920.692	20.069	729.591	0.612	0.907
E4_F3_HC_S171_C3	8,526	7,197,173	844.144	66.562	81.409	0.605	0.808
E4_M4_HC_S131_C1	9,038	1,694,543	187.491	15.816	60.069	0.595	0.792
E4_M4_HC_S131_C2	10	430,432	43043.2	4.017	498.691	0.634	0.899
E4_M4_HC_S131_C3	5,705	6,794,227	1190.925	63.415	93.29	0.612	0.817

Figure 18. Sample Fiji/ImageJ Analysis Data - Reformatted Output showing channel-specific summary per slide: DAPI nuclei count, FITC microvasculature fractional area, and mCherry–Connexin-43 puncta count.

In Figure 18, which is an image of a formatted Microsoft Excel table, the naming convention for “Sample ID” is as follows: Genotype_Sex_TissueType_SlideNumber_ChannelNumber. Blue color is DAPI, green color is FITC, and red color is mCherry.

Slide/Image Number	Parameters and Channel		
	Channel 1: DAPI - Cell Nuclei Count	Channel 2: FITC-Fractional Area/ % microvasculature occupied area	Channel 3: mCherry-Connexin-43 Count
S53	113	14.65%	249
S03	115	36.80%	283
S181	132	43.80%	224
S131	172	4.07%	180

Table 10. Representative Fiji/ImageJ particle-analysis metrics across hippocampal ROIs (count, total area, average size, % area, perimeter, circularity, solidity).

Pilot was run on lower-resolution images showing end-to-end outputs: DAPI counts, CD31 vessel-area masks, and Cx43 puncta detections. Values are illustrative and not used for inference; downsampling and non-final filters bias absolute magnitudes. The divergence is expected at this downsampled scale given puncta loss/merging, threshold sensitivity in the CD31 mask, and absence of manual or machine-learned ROI markings.

A definitive run will use full-resolution VSI files, optimized thresholding, automatic pixel calibration, marked ROI areas upscaled for high resolution processing,, and report nuclei/mm², % vessel area, and Cx43 puncta normalized to vessel area (puncta per mm² of vessel). These outputs confirm end-to-end feasibility and stable ordering across channels.

3.7.1. Channels - Color Mapping

The image colors/ stains, and mapping to channels, presented in the Fiji ImageJ Graphical User Interface (GUI) when rendering an image may not correspond to the image metadata. ImageJ uses a LUT (lookup table) to map colors to grayscale when processing for GUI rendering and can arbitrarily assign a color (like red for mCherry) to any channel number, regardless of what the metadata says. Hence, the actual channels - color mapping, to be used for programmatic analyses, must be obtained from the metadata (screenshots are on the next page). Navigation for lookup of metadata is illustrated in Figure 19.

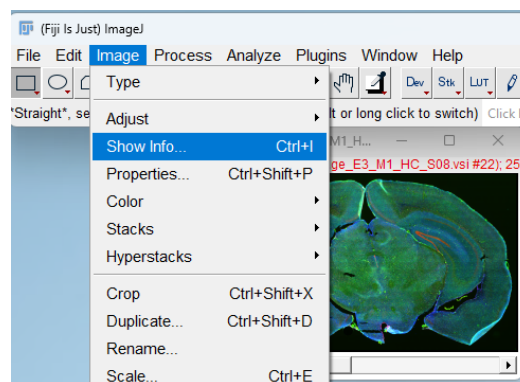


Figure 19. Fiji/ImageJ Lookup of Image Properties

The staining dyes to channel mapping was also confirmed by checking the excitation wavelengths for each channel (Figure 20). The mapping for FITC was always channel 2 across all GUI and metadata representations while it varied for DAPI and mCherry leading to the extra checks.

Fluorophore	Excitation Peak (nm)	Emission Peak (nm)	Visible Color
DAPI	358	461	Blue 
mCherry	587	610	Red 

Figure 20. Wavelengths - Channel Mapping Confirmation

3.7.2. ImageJ and Delayed Response with Pyramidal Image Analysis

(i) The ImageJ software, while great at providing flexibility for analysis, does not work well on images over 1.5 GB. Adequate time must be budgeted for data analyses and for investigating plugins such as Big Data Viewer or OlympusImageJPlugin. ImageJ uses Java and it is important to run garbage collection operations at regular intervals in the program to free up memory. Code should also close all open windows when a particular image, and its temporary copies, have been processed.

(ii) ImageJ does not appear to take advantage of multiple cores and threads to speed up execution i.e., the degree of parallelism appears to be low. However, on a Windows application, multiple instances of ImageJ could run which allowed for parallel execution of the analyses program by splitting up the 80 images to be analyzed across multiple instances of ImageJ on the same computer.

(iii) ImageJ also has limitations on parsing through multi-channel metadata when file sizes are large, despite the system RAM size. In this instance, conversion to ome.tiff files are preferred widely, although this limits the original .vsi file having images at different resolutions to a single highest resolution ome.tiff image.

(iv) Certain basic file management functions, like recursively scanning a Windows directory, resulted in ImageJ errors. A basic Windows BAT script was tested to feed the image names to the ImageJ program.

CHAPTER 4: DISCUSSION

Although quantitative analysis was not performed, qualitative assessment suggested Connexin-43 puncta were visible in association with vascular structures across hippocampal regions (DG, CA1, CA2). The density/intensity seemed variable, with DG showing more prominent puncta than other regions. In MCA images, labeling along endothelial borders was also apparent. Staining consistency was sufficient to validate antibody specificity by confocal microscopy, though scanner images showed some variability in signal/background.

The reason for regional differences may be because DG has high plasticity and metabolic demand and may need stronger gap junctional coupling. CA1 is highly vulnerable in AD and might show altered connexin expression as part of vascular compromise. CA2 is often relatively resistant but has unique signaling features; comparing puncta here could be informative. As the mice were older and displayed lower vascular staining, it is possible that age-associated vascular rarefaction may underlie reduced labeling, which fits with literature on hippocampal vessel loss and impaired neurovascular coupling.

All apparent blur predominantly reflected acquisition constraints rather than biology. First, focus-map sparsity or suboptimal placement across the curved hippocampal laminae permitted local defocus between tiles. Second, use of a global exposure policy (rather than per-slide, clip-safe tuning) depressed signal-to-noise on dim sections and drove near-clipping on bright ones, both perceived as softness. Third, 25- μm sections at 40 $\times$ air with refractive-index mismatch among tissue, ProLong, coverslip (#1.5), and objective correction introduced slight spherical aberration, most evident in the mCherry and FITC channels. Fourth, spectral mismatch between mCherry and a Texas Red/mCherry filter set reduced excitation/emission efficiency and contrast. Fifth, staining variability and batch effects elevated diffuse background. Sixth, instrumental factors (stage tilt, thermal drift, illumination non-uniformity) produced tile-to-tile differences; variability within slides from the same brain implicates the scanner rather than

biological heterogeneity. Finally, widefield slide scanning lacks confocal pinhole rejection, and out-of-plane fluorescence and autofluorescence contribute to more noise.

Findings from this work may help run high throughput images collected and define whether endothelial connexin changes are present in advanced preclinical stages; in future studies, the same approach can be extended to younger cohorts (10-12 weeks) to test whether early connexin alterations arise prior to amyloid pathology, and further linked to peripheral ApoE-lipid interactions as upstream drivers of cerebrovascular injury.

Future Directions

Generative pre-trained transformers (GPT), using large language models (LLM), were used to speed up engineering data analysis code for ImageJ. Applications used were Open AI ChatGPT, Google Gemini, and Microsoft Copilot. While the AI applications generally produced similar code, which was helpful and accelerated code development, all of them erred in providing code snippets that included commands invalid for ImageJ. While these errors were easier to identify, the AI generated code also had logic workflow errors that resulted in erroneous processing of the wrong image window. Identifying logic workflow issues was crucial and time consuming. Establishing the veracity of the code prior to analyses would be useful. Ground truth [27](#). For processing high throughput images in a single batch, it is imperative that the program detect biological regions automatically. Machine learning applications/ plugins like Weka Segmentation can be used for segmenting the exact region of the brain i.e., hippocampus in this case, to narrow down the analyses. The ROI Manager in Fiji ImageJ, QuPath, etc. can also be used to save ROIs and apply to other images if the ROIs have unvarying coordinates across samples.

CHAPTER 5: CONCLUSIONS

This study established a preliminary framework for visualizing and quantifying endothelial Connexin-43 in murine models of Alzheimer's disease, focusing on the hippocampus and middle cerebral artery. Through the optimization of immunofluorescence protocols, antibody dilutions, and imaging modalities, reproducible staining was generated and vascular-associated puncta was captured consistent with connexin expression. Confocal microscopy provided validation of antibody binding, while slide-scanner imaging enabled broader tissue coverage and the potential for large-scale quantitative analysis. A custom image analysis pipeline was developed to detect puncta and vascular area, though further refinements are needed for robust, high-throughput quantification aiming for ground truth.

Although the dataset requires further analysis, the results demonstrate that Connexin-43 puncta can be observed in association with hippocampal and vascular structures, supporting the feasibility of investigating endothelial gap junctions in Alzheimer's disease tissue. Importantly, this work highlights the technical and analytical considerations required for studying endothelial connexins at scale, including the influence of staining variability, imaging platforms, and anesthetic protocols.

Taken together, the study provides a methodological foundation for future work exploring the contribution of vascular connexins to Alzheimer's disease pathophysiology. By refining the imaging and analysis pipeline developed here, subsequent studies can more rigorously evaluate whether changes in endothelial connexin expression precede or accompany neurovascular dysfunction in Alzheimer's disease. Ultimately, the approaches piloted in this project open the door to systematically characterizing vascular gap junctions in the brain, an area of research that remains underexplored yet may be critical to understanding the vascular contributions to dementia.

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Appendix A - Animal Body Weights

Male		Female	
Genotype and ID	Body Weight (g)	Genotype	Body Weight(g)
ApoE $\epsilon 3/\epsilon 3$ - M1	37.538	ApoE $\epsilon 3/\epsilon 3$ - F1	29.02
ApoE $\epsilon 3/\epsilon 3$ - M2	44.381	ApoE $\epsilon 3/\epsilon 3$ - F2	26.24
ApoE $\epsilon 3/\epsilon 3$ - M3	50.24	ApoE $\epsilon 3/\epsilon 3$ - F3	37.71
ApoE $\epsilon 3/\epsilon 3$ - M4	43.104	ApoE $\epsilon 3/\epsilon 3$ - F4	29.65
ApoE $\epsilon 3/\epsilon 3$ - M5	50.545	ApoE $\epsilon 3/\epsilon 3$ - F5	37.71
ApoE $\epsilon 4/\epsilon 4$ - M1	41.416	ApoE $\epsilon 4/\epsilon 4$ - F1	44.12
ApoE $\epsilon 4/\epsilon 4$ - M2	44.315	ApoE $\epsilon 4/\epsilon 4$ - F2	32.53
ApoE $\epsilon 4/\epsilon 4$ - M3	37.229	ApoE $\epsilon 4/\epsilon 4$ - F3	26.80
ApoE $\epsilon 4/\epsilon 4$ - M4	44.579	ApoE $\epsilon 4/\epsilon 4$ - F4	27.7
ApoE $\epsilon 4/\epsilon 4$ - M5	32.857	ApoE $\epsilon 4/\epsilon 4$ - F5	36.08

Table 11. Animal Body Weights

Appendix B - Scan Times

Optimal exposure time for scanning was tested across 8 slides.

1) DAPI Channel

S/NO	Genotype	Slide Number	Exp time for ROI- 1 (ms)	Exp time for ROI-2 (ms)	Exp time for ROI-3 (ms)
1	ApoE ε3/ε3	S08	11.36	14.05	24.1
2	ApoE ε3/ε3	S57	29	10.15	16.29
3	ApoE ε3/ε3	S016	21.3	19.26	16.55
4	ApoE ε4/ε4	S98	6.58	7.02	10.02
5	ApoE ε4/ε4	S117	8.43	6.09	12.18
6	ApoE ε4/ε4	S135	8.63	11.44	6.86
7	ApoE ε4/ε4	S170	3.72	4.08	7.12
8	ApoE ε3/ε3	S49	11	13	16.64

Table 12. DAPI Exposure Times

2) FITC Channel

S/NO	Genotype	Slide Number	Exp time for ROI- 1 (ms)	Exp time for ROI-2 (ms)	Exp time for ROI-3 (ms)
1	Apoε3/ε3	S08	806	667	509
2	Apoε3/ε3	S57	590	494	454
3	Apoε3/ε3	S016	337	300	310

S/NO	Genotype	Slide Number	Exp time for ROI- 1 (ms)	Exp time for ROI-2 (ms)	Exp time for ROI-3 (ms)
4	ApoE4	S98	N/A	N/A	N/A
5	ApoE4	S117	375	547	423
6	ApoE4	S135	193.96	345.45	384.09
7	ApoE4	S170	576.93	324.75	350
8	ApoE3	S49	528	569	457

Table 13. FITC Exposure Times**3) mCherry Channel**

S/NO	Genotype	Slide Number	Exp time for ROI- 1 (ms)	Exp time for ROI-2 (ms)	Exp time for ROI-3 (ms)
1	ApoE3	S08	68	39.77	43.89
2	ApoE3	S57	67.2	38.5	33.48
3	ApoE3	S016	40.96	43.16	35.55
4	ApoE4	S98	72.7	51.93	73.24
5	ApoE4	S117	38	53	25.7
6	ApoE4	S135	50.64	68.86	81.9
7	ApoE4	S170	66.29	61.6	89.53
8	ApoE3	S49	45.19	41.53	27.68

Table 14. mCherry Exposure Times

Appendix C - Image Analyses Program

1. Fiji ImageJ Macro

```

/////////////////////////////////////////////////////////////////

// Fiji ImageJ macro to analyze .vsi images of mice hippocampus on a Windows computer.

// Update variables as needed. Launch from Fiji/ImageJ Plugin GUI.

// Nayana Harilal * August 2025

/////////////////////////////////////////////////////////////////

// >> Off to the races!

timestamp = call("java.time.LocalDateTime.now");

print("Started image analyses macro at " + timestamp + "\n");

// Set folders and files for input and output

sourceImagesFolder = "C:\\specify\\input\\image\\folder\\";

outputFolder = "C:\\specify\\output\\folder\\";

overlayOutputFile = "overlayData.csv"; // holds c3 - c2 overlap info

imageChannelOutputFile = "imageChannelData.csv"; // holds count, etc. One row for each image - channel
combo

// Set some processing variables

pixelWidth = pixelHeight = 0.1625; // true for final images; use metadata calls if needed

pixelUnit = "micron";

poorQualityCount = 0; // number of low quality images

setBatchMode(true); // true or false. true suppresses GUI windows

enableOverlayCalc = true; // set to true to compute c3 & c2 overlap

downScaleSeriesBy = 5; // import the series having this index value. speeds up testing if > 0

print("Downscaling from highest resolution by a total of " + downScaleSeriesBy + " series.");

```

```
overlayOutputHeader = "IMAGE" + "," + "AREA" + "," + "PIXELS" + "," + "WIDTH" + "," + "HEIGHT" +
"," + "UNIT";
```

```
File.append(overlayOutputHeader, outputFolder + overlayOutputFile);
```

```
// Extensions plug-in needed for ext metadata and other commands
```

```
run("Bio-Formats Macro Extensions");
```

```
// Get list of source objects
```

```
listOfSourceObjects = getFileList(sourceImagesFolder); // not a recursive scan
```

```
numberOfSourceObjects = listOfSourceObjects.length; // can be image & non-image objects
```

```
vsiImageCounter = ometifImageCounter = imgCounter = 0;
```

```
// Count the number of .vsi and .ome.tif objects in the source folder
```

```
for (i = 0; i < numberOfSourceObjects; i++) {
```

```
    if (endsWith(listOfSourceObjects[i], ".vsi")) {
```

```
        vsiImageCounter = vsiImageCounter + 1;
```

```
    }
```

```
    if (endsWith(listOfSourceObjects[i], ".ome.tif")) {
```

```
        ometifImageCounter = ometifImageCounter + 1;
```

```
    }
```

```
}
```

```
numberOfImageFiles = vsiImageCounter + ometifImageCounter;
```

```
print("Found " + numberOfSourceObjects + " object/s in " + sourceImagesFolder + ". This includes " +
```

```
vsiImageCounter + " .vsi images/s and " + ometifImageCounter + " .ome.tif image/s.");
```

```
//////
```

```
////// >>>>> START OF MAIN "FOR" CODE BLOCK FOR EACH SOURCE OBJECT <<<<< ////
```

```
//////
```

```
for (i = 0; i < numberOfSourceObjects; i++) {
```

```

/////

///// >>>>> START OF MAIN "IF" BLOCK. RUNS FOR EACH IMAGE FILE <<<<<< /////

/////

if (endsWith(listOfSourceObjects[i], ".vsi") || endsWith(listOfSourceObjects[i], ".ome.tif")) {

    imageName = listOfSourceObjects[i];

    inputImageFile = sourceImagesFolder + imageName;

    imgCounter = imgCounter + 1;

    // block for .vsi processing config

    if (endsWith(listOfSourceObjects[i], ".vsi")) {

        SeriesCount = 0;

        maxResolution = 0;

        maxResIndex= 0;

        timestamp = call("java.time.LocalDateTime.now");

        print("\n" + timestamp + " : Processing " + imgCounter + " out of " + numberOfImageFiles + " images. File
name is " + inputImageFile);

        // >> Get the total number of series for the image & the index value of highest res series

        Ext.setId(inputImageFile); // loads the metadata w/o opening image

        Ext.getCurrentFile(inputImageFile); // Required for importer to access internal temp file

        Ext.getSeriesCount(SeriesCount);

        print("Total number of series is " + SeriesCount + " for " + imageName);

        // Get the index value of the series/ datasets having highest res

        // Note: Internal index value is the visible title series number less 1.

        for (j = 0; j < SeriesCount; j=j+1) { // get the max res index

            Ext.setSeries(j);

            Ext.getSizeX(width);

            Ext.getSizeY(height);

```

```

    resolution = width * height;

    if (resolution > maxResolution) {
        maxResolution = resolution;
        maxResIndex = j;
    }
}

print("Highest resolution series has index value of " + maxResIndex);

openSeriesIndex = maxResIndex + downScaleSeriesBy;

Ext.setSeries(openSeriesIndex); // series index to be imported

Ext.getSeriesName(openSeriesName);

print("Importing series index " + openSeriesIndex + " having series name " + openSeriesName);

run("Bio-Formats Importer", "open=[" + inputImageFile + "] autoscale color_mode=Composite
rois_import=[ROI manager] view=Hyperstack stack_order=XYCZT series_" + openSeriesIndex);

Ext.setSeries(openSeriesIndex); // set it to the internal index value for the opened series. importer does not
seem to refresh, so the explicit re-update

Ext.getSeriesName(openSeriesName);

print("Imported. Now processing active series index " + openSeriesIndex + " having series name " +
openSeriesName);

} // end .vsi config block

// block for .ome.tif processing config

if (endsWith(listOfSourceObjects[i], ".ome.tif")) {

    // placeholder for .ome.tif images

    ometifFileName = listOfSourceObjects[i];

    print("WARNING: Program ended. Encountered .ome.tif file: " + ometifFileName + ". Code needs .ome.tif
processing logic.");

    exit();

```

```

    } // end .ome.tif config block

    ///// Quality checks

    getStatistics(area, mean, min, max, stdDev);

    contrast = max - min;

    getHistogram(values, counts, 256);

    darkPixels = counts[0];

    brightPixels = counts[255];

    skipReason = "";

    if (contrast < 15) skipReason += "low contrast;";

    if (stdDev < 5) skipReason += "low stdDev;";

    if (darkPixels > 0.8 * area) skipReason += "underexposed;";

    if (brightPixels > 0.8 * area) skipReason += "overexposed;";

    if (skipReason != "") {

        poorQualityCount = poorQualityCount + 1;

        print("WARNING - LOW QUALITY: " + inputImageFile + ": " + skipReason);

        //File.append(inputImageFile + ", " + skipReason + "\n", skippedCSV);

        //close();

        //continue;

    }

    // Calibration metadata placeholder

    ///// >>> PER SLICE/ CHANNEL ANALYSES STARTS HERE <<<<<

    ///// >>> Split channels, analyze the image series, and save the results

    run("Split Channels"); // convert composite image to three windows

    channelNames = newArray("DAPI", "FITC", "mCherry");

    sliceTitle = ""; fitcMask = ""; mcherryMask = ""; overlayCount = 0; overlayArea = 0;

    for (c = 1; c <= 3; c=c+1) {

```

```

selectImage(c); // select the right image window ; assumes 1 is dapi, 2 fitc, 3 mcherry

sliceTitle = imageName + "_C" + toString(c);

sliceTitle = replace(sliceTitle, ".vsi", "");

sliceTitle = replace(sliceTitle, "Image_", "");

sliceTitle = replace(sliceTitle, "_cropped", "");

rename(sliceTitle); // column 1 of report

selectImage(c); // insurance!

sliceTitle = getTitle();

// ADJUST, FILTER, & MEASURE, per channel

run("Set Measurements...", "area mean min centroid integrated median skewness kurtosis perimeter shape
redirect=None decimal=3"); // grab all available data because who knows...!

// run("Clear Results");

if (c == 1) { // nuclei (blue)

    // C1 - DAPI - Adjust, filter

    run("Gaussian Blur...", "sigma=1.50");

    run("Enhance Contrast", "saturated=0.35");

    run("Enhance Local Contrast (CLAHE)", "blocksize=127 histogram=256 maximum=3 mask=*None*");

    run("Subtract Background...", "rolling=95 sliding");

    setAutoThreshold("Otsu dark no-reset");

    setOption("BlackBackground", true);

    run("Convert to Mask"); // binarization

    run("Watershed"); // segment

    // run("Close"); //close fills small black holes in galaxies far away. but sadly also closes the active
window :(

    // run("Grays"); // <-- resets color display to grayscale else ImageJ will display per LUT of the ImageJ

```

UI


```

// C1 - DAPI - Analyze/ measure

run("Analyze Particles...", "size=20-Infinity circularity=0.2-0.7 show=Overlay exclude summarize
add");

} else if (c == 2) {    // vasculature (green)

// C2 - FITC - Adjust, filter

run("Subtract Background...", "rolling=300 sliding");

setAutoThreshold("Triangle dark 16-bit no-reset");

setOption("BlackBackground", true);

run("Convert to Mask");

run("Remove Outliers...", "radius=8 threshold=50 which=Bright");

run("Fill Holes");

run("Watershed");

// C2 - FITC - Analyze/ measure

run("Analyze Particles...", "size=200-Infinity circularity=0.1-0.7 show=Overlay exclude summarize
add");

selectImage(c);

run("Duplicate...", "title=fitc_binary"); // used in overlap logic

rename("fitc_binary");

} else if (c == 3) {    // proteins/ connexins (red)

// C3 - mCHERRY - Adjust, filter

run("Subtract Background...", "rolling=50 sliding");

run("Enhance Local Contrast (CLAHE)", "blocksize=19 histogram=255 maximum=3 mask=*None*");

setAutoThreshold("MaxEntropy");

setOption("BlackBackground", true);

run("Convert to Mask");

```

```

// C3 - mCHERRY - Analyze/ measure

run("Watershed");

run("Analyze Particles...", "size=10-Infinity circularity=0.3-1.0 show=Overlay exclude summarize
add");

run("Duplicate...", "title=mcherry_binary"); // used in overlap logic

rename("mcherry_binary");

} // end IF block for C3


if (enableOverlayCalc && c == 3) { // execution of C3 - C2 overlay count

    // Detect presence of connexins on vasculature (using temp image of mCherry overlapping FITC)

    overlay_mask = imageName + "_Overlay";

    imageCalculator("AND create", "mcherry_binary", "fitc_binary");

    rename(overlay_mask);

    selectImage(overlay_mask); // C3 atop C2

    run("Set Measurements...", "area limit display redirect=None decimal=3");

    run("Analyze Particles...", "size=5-Infinity show=Nothing display");

    overlayCount = nResults;

    totalPixelCount = 0;

    for (p = 0; p < overlayCount; p=p+1) {

        totalPixelCount += getResult("Area", p);

    }

    overlayArea = totalPixelCount * pixelWidth * pixelHeight; // convert to microns

    overlayOutputRow = imageName + "," + overlayArea + "," + totalPixelCount + "," + pixelWidth + "," +
pixelHeight + "," + pixelUnit;

    File.append(overlayOutputRow, outputFolder + overlayOutputFile);

    close("overlay_mask");

```

```

    } // overlap count code block

} // end of FOR loop for an image series. all three slicez/ channel were processed

// all slices/ channels are processed for the chosen image file (& series), move on to next image file

run("Close All"); // close open *image* windows before processing next image

run("Clear Results"); // clear detailed results

call("java.lang.System.gc"); // run garbage collection, free unused memory

} //>>>>> END OF MAIN "IF" BLOCK. RAN FOR EACH IMAGE FILE IN SOURCE FOLDER

<<<<<< //>>>>> END MAIN "FOR" CODE BLOCK. RAN FOR ALL OBJECTS IN SOURCE FOLDER <<<<<<

//>>>>>

// Log the end of macro

run("Close All"); // closes image windows, doesn't close results windows

close("Results"); // closes detailed results window

close("ROI Manager");

selectWindow("Summary"); // holds the results from Analyze Particles. One row for each image - channel

combo

saveAs("Results", outputFolder + imageChannelOutputFile); // don't clear/ reset sumamry window until this

save-all-rows occurs

close(imageChannelOutputFile);

print("\nNumber of low quality images: " + poorQualityCount);

timestamp = call("java.time.LocalDateTime.now");

print("\nCompleted image analyses macro at " + timestamp);

exit();

```

2. Windows BAT Program

rem Optional program to recursively scan images folder to generate list of .vsi images

```

rem Output file can be used to feed a modified version of the Fiji/ImageJ macro

@echo off

set "root_dir=C:\high-level-directory\where\images\reside"

set "OUTPUT_FILE="C:\directory\for\saving\the\output-file\vsi_image_list.txt"

echo Scanning folder: %root_dir%

echo.

rem Check if folder exists

if not exist "%root_dir%" (

    echo The directory %root_dir% does not exist.

    exit /b

)

rem Recursively scan and list .vsi files

for /r "%root_dir%" %%f in (*.vsi) do (

    echo %%f>> "%OUTPUT_FILE%"

)

pause

```