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Super-Resolution Ultrasound Imaging Reveals Spatiotemporal Vascular and Glial Remodelling Following Spinal Cord Injury

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Abstract

Study Design:Animal study.

Objectives:To quantify temporal changes in spinal cord microvascular density and flow after spinal cord injury using ultrasound localization microscopy (ULM), and to correlate these alterations with astrocytic reactivity measured by GFAP expression.

Setting:Preclinical laboratory study in a rat model of thoracic spinal cord injury.

Methods:Eleven Sprague-Dawley rats underwent T9–T11 laminectomy. Nine received a standardized 200 kdyn contusion injury and were assigned to acute (24 hours), subacute (7 days), and chronic (21 days) groups (n=3/group); two served as uninjured controls. ULM was performed using a Vevo 3100 system with a 40 MHz transducer after intravenous Definity microbubble injection. Vessel density and flow speed were quantified at the lesion epicenter and adjacent rostral and caudal regions. Arterial and venous compartments were differentiated by flow direction. Histologic sections were stained for GFAP, and percent area staining was quantified. One-way ANOVA with Tukey post hoc testing and Pearson correlation were used for analysis.

Results: ULM generated super-resolved spinal microvascular imaging at 5–10 μm resolution. Venous vessel density declined significantly from 0.92 ± 0.18 in the acute phase to 0.42 ± 0.23 in the subacute phase and 0.11 ± 0.07 in the chronic phase especially dorsally. Arterial density remained stable. Flow speed transiently decreased during the subacute phase,

with partial venous recovery by Day 21. Chronic GFAP area increased significantly versus earlier phases and correlated with early venous loss and chronic arterial flow changes.

Conclusion: ULM enables high-resolution longitudinal monitoring of SCI microvascular remodeling and identifies vascular biomarkers linked to later astrogliosis.

Introduction

Spinal cord injury (SCI) is a common traumatic injury that can result in complete or incomplete neurological deficits and trigger a cascade of pathological processes, including vascular disruption, neuroinflammation, glial activation, and scar formation.¹ While the initial mechanical insult causes direct damage, secondary injury mechanisms occurring over hours to weeks drive much of the irreversible functional loss. Among these, microvascular dysfunction plays a pivotal role by impairing tissue perfusion, disrupting the blood-spinal cord barrier (BSCB), and amplifying inflammatory and neurodegenerative responses.^{2,3}

Importantly, the pathological process consists of distinct phases, acute, subacute, and chronic, each characterized by unique cellular and vascular changes along the trajectory of injury. Understanding these phase-specific and spatially distributed alterations is critical for identifying therapeutic targets and optimizing the timing of interventions. Current imaging modalities used in SCI, including magnetic resonance angiography (MRA), contrast-enhanced ultrasound (CEUS), and Doppler ultrasound, provide important anatomical, perfusion, and hemodynamic information, but they do not adequately capture the fine microvascular architecture and slow-flow dynamics needed for precise longitudinal assessment of spinal cord vascular remodeling. MRA offers valuable structural and large-vessel perfusion detail but lacks sufficient spatial and temporal resolution for capillary-level evaluation. Doppler ultrasound enables real-time flow assessment but is limited in sensitivity to low-velocity microvascular flow. CEUS improves visualization of tissue perfusion, yet it

does not provide detailed mapping of the microvascular network or precise tracking of regional vascular changes over time. Despite its clinical relevance, our knowledge of the temporal and spatial dynamics of microvascular remodelling in SCI remains limited, largely due to the lack of high-resolution, quantitative in vivo imaging tools.⁴

Ultrasound localization microscopy (ULM) is an emerging imaging technique that achieves super-resolution by localizing and tracking the trajectories of intravenously injected microbubbles (MBs).⁴⁻⁶ Unlike conventional Doppler or CEUS, ULM enables the reconstruction of microvascular networks with spatial resolution below the acoustic diffraction limit and can resolve flow within small-caliber vessels, including capillaries and venules.^{4,7} These capabilities make ULM uniquely suited for quantifying microvascular structure and function in vivo. Accordingly, ULM may address an important gap in SCI imaging by enabling high-resolution, longitudinal evaluation of spatiotemporal vascular remodeling that is not readily achievable with existing modalities.^{8,9}

Our study aimed to quantify SCI-induced alterations in spinal vessel density, flow velocity, and vascular morphology in a rat contusion SCI model. Assessments were conducted at specific post-injury time points to capture acute, subacute, and chronic vascular changes. By using ULM imaging, we sought to establish a comprehensive imaging framework for high-resolution, longitudinal evaluation of vascular remodelling in vivo. This platform may improve understanding of SCI pathophysiology, support biomarker discovery, and inform injury phase- and type-specific therapeutic strategies for spinal cord repair.

Materials and Methods

All experiments in this study were conducted in strict compliance with the ethical guidelines of the Mayo Clinic Institutional Animal Care and Use Committee (IACUC) and adhered to the National Institutes of Health (NIH) regulations for the care and use of

laboratory animals. All methods followed the ARRIVE guidelines to ensure ethical and transparent reporting of animal research. Rats were housed in pairs under standard laboratory conditions, including a 12-hour light/dark cycle, with unrestricted access to food and water.

Animal Preparation and Operative Procedure

To minimize stress and allow for environmental adaptation, rats underwent a five-day acclimation period prior to surgery. Anesthesia was induced with an intraperitoneal injection of a Ketamine-Xylazine cocktail (80 mg/kg Ketamine and 5 mg/kg Xylazine), selected for its rapid induction of immobility and effective analgesia. The depth of anesthesia was continuously monitored using reflex assessment (e.g., toe pinch) and respiratory observation. Preoperative analgesia was administered via a subcutaneous injection of Buprenorphine (0.1 mg/kg) to ensure adequate pain management. During the surgical procedure, the rats were placed on heating pads set to 37°C to prevent hypothermia, a common complication during prolonged anesthesia and surgical exposure. A midline incision extending from T8 to T12 was made through the skin, and underlying tissues were carefully dissected to expose the surgical site. The muscles were retracted to create a clear surgical field, and a two-level laminectomy at T9-T11 was performed under a surgical microscope to expose the thoracic spinal cord.

For rats assigned to the SCI groups, a contusion injury was induced using the Infinite Horizons Impactor, calibrated to deliver a force of 200 kdyn. This procedure replicates the severity of human SCI and creates a reproducible injury model. Post-surgical incisions were sutured, and rats were returned to heating pads for three days to support recovery and maintain normal body temperature.

Manual bladder compressions were performed three times daily until normal bladder function was restored to prevent urinary tract infections and renal damage resulting from

temporary loss of neural bladder control. Postoperative analgesia was administered 72 hours post-surgery with a single subcutaneous injection of Buprenorphine (0.1 mg/kg). Rats were closely monitored for signs of infection, abnormal behavior, or distress. Identification numbers were marked on their tails using non-toxic markers, which were reapplied every 3-4 days to ensure accurate identification throughout the study. Postoperative care and monitoring were conducted consistently across all experimental groups by AD and AKG to ensure uniformity and reliability. **(Figure 1)**

Animal Groups and Experimental Design

A total of 11 Sprague-Dawley rats were used in this study. Of these, nine rats underwent a T9-T11 laminectomy followed by a 200 kdyn contusion injury using the Infinite Horizons Impactor to replicate the pathophysiology of spinal cord injury (SCI) in humans. These injured rats were randomly divided into three experimental groups based on the timeline of post-injury ULM evaluations. The acute group (N=3) was evaluated 24 hours post-injury to assess early hemodynamic changes and initial vascular disruptions. The subacute group (N=3) was evaluated seven days post-injury to investigate intermediate recovery dynamics and ongoing microvascular changes. The chronic group (N=3) was evaluated three weeks post-injury to study long-term vascular remodeling, spinal cord recovery, and functional restoration. Additionally, two rats underwent a T9-T11 laminectomy without contusion injury and served as uninjured controls, providing baseline data for imaging, histological, and immunohistochemical analyses. This ensured that the observed changes in injured groups could be attributed specifically to SCI rather than the surgical procedure itself. All animals received rigorous postoperative care to ensure consistent recovery and reduce complications.

Ultrasound localization microscopy imaging Contrast Agent Preparation

In our study, we used a contrast agent, Definity (Lantheus Medical Imaging, Billerica, MA, USA), which comprises lipid microspheres. Following mechanical activation, a 2 ml vial of Definity contains 0.75 mg of lipid blend and 1.1 mg/ml of octafluoropropane.¹⁰ Definity MBs were diluted with normal saline solution (NSS) to yield an initial concentration of 2×10^9 bubbles per ml. All 11 rats were intravenously injected with a 0.2 ml diluted MB solution and flushed with another 0.2 ml of NSS through the tail vein.

Ultrasound localization microscopy (ULM)

All animals were assessed by ULM under isoflurane anesthesia (induction at 3-5% and maintenance at 1-3%). Their skin and muscles were dissected with a previous incision line to expose the spinal cord. A Vevo 3100 high-frequency US system (FUJIFILM VisualSonics Inc., Toronto, ON, Canada) equipped with an MX550S linear array transducer was applied for all imaging procedures. Operating at 40 MHz, this system provided high spatial resolution, allowing us to visualize the detailed anatomy of the rat spinal cord., the transmit power was set at 25% of the maximum to minimize the destruction of IV-MBs. Following the injection of IV-MBs, 2 mL of NSS was applied directly to the exposed spinal cord at the open skin site, and ultrasound gel was then applied to ensure optimal contact with the transducer. The ULM imaging was conducted to assess microvascular dynamics within the spinal cord. All rats were perfused immediately, and spinal cords were collected. (**Figure 2)**

Image Acquisition

A line-by-line focused imaging approach was employed, improving resolution by focusing on specific areas within the field of view, covering an 8 mm × 5 mm (axial × lateral)

195 area of the rat spinal cord. The system's high frame rate of 395 Hz was critical for capturing
196 fast physiological processes, including blood flow and vascular dynamics. For each imaging
197 session, 15 beamformed IQ (in-phase and quadrature) datasets were collected, each
198 consisting of 1000 frames captured continuously. A spatiotemporal singular value
199 decomposition (SVD)-based clutter filter was applied to the IQ datasets to eliminate
200 stationary tissue signals.¹¹ After clutter removal, the MB data were further divided into seven
201 subsets based on different speed ranges and movement directions, allowing for a
202 comprehensive analysis of MB dynamics and blood flow patterns.¹² MB localization and
203 tracking algorithms were then applied to each MB subset, yielding high-resolution images of
204 the microvascular structures.¹¹ These individual ULM images were combined to create a
205 detailed map of blood flow within the spinal cord. To ensure uniform noise intensity across
206 the field of view, a noise equalization method was applied before processing the MB data.¹³
207 Motion correction was achieved using phase-correlation-based subpixel registration on tissue
208 images to generate motion maps, which were applied to correct MB datasets. The noise-
209 equalized and motion-corrected IQ data were then interpolated to achieve a resolution of 6
210 $\mu\text{m} \times 5.5 \mu\text{m}$ (axial $\times$ lateral) using 2D spline interpolation. Following interpolation, a 2D
211 normalized cross-correlation between each frame and the point spread function (PSF) was
212 used to localize the MBs. Pixels with correlation coefficients below 0.5 were excluded,
213 ensuring only high-confidence MB signals were retained. The MB centroids were localized
214 using a regional maximum search on the cross-correlation maps, and their paths were tracked
215 using a bipartite graph-based algorithm, with a minimum persistence threshold of 15 frames.
216 A Kalman filter was applied to refine the MB tracks, improving accuracy by predicting and
217 smoothing the MB trajectories based on speed and acceleration. B-mode US imaging was
218 used to identify regions of interest (ROIs) at the injury site, as well as rostral and caudal to
219 the site, for further analysis of vessel density (VD) and blood flow speed (FS).

Region of Interest (ROIs)

ROIs were anatomically defined relative to the injury epicenter identified via pre- and post-injury B-mode ultrasound landmarks (vertebral level and lesion contour). The epicenter ROI encompassed the central 0.5 mm × 0.5 mm area at the injury site, centered along the rostrocaudal axis and extending 0.5 mm rostrally and caudally, with additional segmentation at 0.25 mm and 0.5 mm increments to cover symmetric peri-lesional zones (total span: 2.5 mm transverse). Each ROI was configured as a rectangular field (0.5 mm transverse × 0.5 mm dorsoventral) to standardize VD and FS measurements. Sub-segmentation into gray matter (ventral/dorsal horns) and white matter was performed based on intensity thresholding from co-registered B-mode images. Arterial and venous VD and FS were averaged across symmetric ROIs, with differentiation by microbubble trajectory direction (centripetal for arteries entering the cord, centrifugal for veins exiting), as validated in prior ULM-SCI studies. The injury epicenter was localized, and perpendicular lines were drawn 0.25 mm from the epicenter to establish the injured surface and ROI boundaries. Rostral and caudal ROIs were defined 0.5 mm horizontally from the epicenter to assess blood flow upstream and downstream of the injury. VD was quantified by calculating the vessel area within the ROI relative to the background area, using an adaptive noise threshold to distinguish between vessel signals and background noise. FS was determined by tracking the movement of MBs through the vessels, creating a comprehensive flow velocity map. Average FS values were calculated for each rat and compared across experimental groups, including normal, acute, subacute, and chronic SCI groups. The direction of flow was used to differentiate arteries (vessels entering the spinal cord) from veins (vessels exiting the spinal cord), and the arterial and venous VD and FS were averaged at the injury site, rostrally, and caudally. FS was

directly obtained by tracking the movement of MBs such as in the previous paper published by Tang et al.¹⁴

The objective was to compare the mVD and mFS at specific injury sites across various SCI stages (Acute, Subacute, Chronic). Bar graphs were generated to show the mean values and SD for mVD and mFS across the different SCI stages at each injury site. **Figure 2** shows the principle of MBs separation based super-resolution imaging. Also, **Figure 3** shows the SCI regions in sagittal and coronal views.

Perfusion and immunohistochemistry

All rats were deeply anesthetized using a combination of Ketamine (80 mg/kg) and Xylazine (10 mg/kg) and then perfused transcardially with 0.01 M PBS (pH 7.4), followed by 4% paraformaldehyde (PFA) in 0.01 M PBS. After perfusion, the vertebral column was carefully removed and post-fixed in 4% PFA for 24 hours to further stabilize the tissues.¹⁵ The spinal cords were then carefully extracted then embedded in paraffin to facilitate cutting into thin slices. The paraffin-embedded spinal cord segments were then cut into 10 µm-thick transverse sections using a microtome. All tissue sections were evaluated blindly by two independent examiners, AK and AD.

GFAP Expression Post-SCI: Immunofluorescence and Quantification

To evaluate astrogliosis following SCI, immunofluorescence staining for glial fibrillary acidic protein (GFAP) was performed on 10-µm-thick coronal sections obtained from the lesion epicenter and adjacent regions. After deparaffinization and rehydration, antigen retrieval was carried out using 1 mM EDTA, followed by blocking in 10% normal donkey serum with 0.1% Triton-X in PBS. Sections were incubated overnight at 4°C with rabbit anti-GFAP (1:1000, DAKO), and then with donkey anti-rabbit Iso 647 (1:500, Jackson

Immunoresearch). Nuclei were counterstained with DAPI, and sections were mounted in SlowFade medium. Fluorescent images were acquired using a Zeiss Axio Zoom V16 microscope (excitation 550 nm, emission 570 nm) under identical exposure settings. Quantitative analysis of GFAP-positive area (%Area) was performed using ImageJ, where signal intensity thresholds were applied to isolate astrocytic labeling, and the %Area was calculated relative to the entire cross-sectional area of the spinal cord. All image processing and quantification were conducted under blinded conditions by two independent observers (AK and AD). Data from acute, subacute, and chronic SCI groups were analyzed via one-way ANOVA with Tukey's post-hoc test,

Statistical analysis

To assess longitudinal changes in spinal cord microvascular structure and function following SCI and explore correlations with chronic astrocytic reactivity, quantitative analyses were performed across three groups: acute (Day 1 post-injury), subacute (Day 7), and chronic (Day 21). For each group, mean values and standard deviations (SD) were calculated for two primary ULM-derived metrics: mean vessel density (mVD) and mean flow speed (mFS). Data normality was evaluated using the Shapiro-Wilk test. For datasets meeting parametric assumptions, one-way analysis of variance (ANOVA) was conducted to identify significant differences in mVD and mFS among the groups, followed by Tukey's honestly significant difference (HSD) test for pairwise comparisons. Similarly, GFAP-positive area (%Area), derived from immunofluorescent images, was compared across the acute, subacute, and chronic stages using ANOVA with Tukey's HSD post-hoc tests. Due to observed differences in arterial density across timepoints, a region-stratified ANOVA with Tukey's HSD post-hoc analysis was performed on arterial mVD data. To evaluate inter-compartmental and inter-phase relationships in vascular remodeling, Pearson correlation

analyses were conducted across arterial and venous mVD and mFS, stratified by spinal cord region (dorsal vs. ventral) and timepoint (acute, subacute, chronic). GFAP %Area values were included in the correlation matrix to assess associations between early vascular changes and long-term astrocytic activation. Pearson correlation coefficients (r) and two-tailed p-values were calculated using the SciPy statistics module in Python (v3.11), with statistical significance set at $p < 0.05$. Results were visualized in a color-coded correlation matrix to highlight positive (r approaching +1) and negative (r approaching -1) relationships. All statistical analyses were performed using MATLAB R2023a, Python (SciPy, NumPy, pandas, statsmodels), and RStudio (ggplot2, corrplot) for data visualization. No data points were excluded unless explicitly stated, and analyses were conducted under blinded conditions.

Results

ULM imaging revealed important differences in vascular structure and hemodynamics across the segmented ROIs. **Figure 3B** illustrates representative imaging results acquired from a normal (uninjured) spinal cord, in the coronal plane from conventional B-mode ultrasound and ULM-derived maps. As shown in **Figure 3B1**, the B-mode image shows the gross anatomical boundaries of the spinal cord but lacks sufficient resolution to resolve capillary-level structures. Dorsal (D), ventral (V), rostral (R), and caudal (C) orientations are labeled for reference. In contrast, the ULM-derived microvascular density and speed maps (**Figure 3B2, 3B3**) provides a super-resolution reconstruction of the spinal microvasculature, visualizing fine vessel structures and their spatial organization. In the rostral and caudal ROIs, vascular networks appear dense and well-organized, while within the injury epicenter, especially in the dorsal region, a clear reduction in vessel density and continuity is observed.

Blood Flow Density Changes Following SCI

Figure 4 shows B-mode ultrasound images (**Figure 4.A1-A3**) and corresponding ULM-derived blood flow density maps (**Figure 4.A4-A6**) acquired at acute (Day 1), subacute (Day 7), and chronic (Day 21) timepoints following SCI. While B-mode images show the gross anatomical structure of the spinal cord, they lack the resolution to resolve microvascular-level perfusion. In contrast, ULM-derived blood flow density maps provide high-resolution spatial quantification of microvascular perfusion dynamics over time. In the acute SCI phase (**Figure 4.A4**), blood flow density is markedly reduced and spatially heterogeneous, with minimal perfusion observed within the dorsal lesion epicenter (circled). By the subacute phase (**Figure 4.A5**), there is a moderate restoration of perfusion, with increased density observed across both dorsal and ventral compartments. In the chronic phase (**Figure 4.A6**), blood flow density increases substantially, with extensive and tortuous vascular networks emerging throughout the imaged field.

Arterial Blood Flow Density Across SCI Phases

To assess structural integrity of the arterial vasculature during SCI progression, we quantified arterial blood flow density using ULM. The metric, expressed as the mean density ratio, was evaluated across acute, subacute and chronic SCI timepoints. In the overall spinal cord compartment, arterial density showed no significant differences among groups ($p > 0.05$, one-way ANOVA). The mean density ratios were 0.55 ± 0.19 for the acute group, 0.66 ± 0.18 for subacute, and 0.71 ± 0.20 for chronic animals (**Figure 4B.1.a**), suggesting stable arterial perfusion over time. When stratified by region, arterial density remained unchanged in both the dorsal and ventral compartments. Dorsal values were 0.47 ± 0.14 (acute), 0.53 ± 0.05 (subacute), and 0.64 ± 0.22 (chronic), with no statistically significant differences

observed (**Figure 4B.1.b**). Similarly, ventral arterial densities were 0.68 ± 0.13 (acute), 0.79 ± 0.16 (subacute), and 0.77 ± 0.21 (chronic), also showing no significant variation between timepoints (**Figure 4B.1.c**).

Venous Blood Flow Density Across SCI Phases

To evaluate structural remodelling in the venous microvasculature during SCI progression, we quantified venous blood flow density using ULM and expressed it as mean density ratio. Compared to arteries, venous vessels showed a pronounced and time-dependent decline in density, particularly between acute and chronic stages. In the overall spinal cord compartment (**Figure 4B.2.d**), venous density significantly decreased from 0.92 ± 0.18 in the acute group to 0.42 ± 0.23 in the subacute group ($**p < 0.01$), and further to 0.11 ± 0.07 in the chronic group ($***p < 0.001$). Pairwise comparisons also revealed a significant reduction from subacute to chronic ($*p < 0.05$), indicating a progressive loss of venous microstructure over time. In the dorsal region (**Figure 4B.2.e**), venous density declined from 0.97 ± 0.16 (acute) to 0.35 ± 0.26 (subacute, $p < 0.05$), and to 0.16 ± 0.06 (chronic, $**p < 0.01$). However, the difference between subacute and chronic was not statistically significant ($p > 0.05$), suggesting that most dorsal venous loss occurs early, between acute and subacute stages. In the ventral region (**Figure 4B.2.f**), venous density dropped from 0.87 ± 0.21 (acute) to 0.48 ± 0.23 (subacute) and further to 0.06 ± 0.05 (chronic), with a significant difference between acute and chronic groups ($**p < 0.01$), but not between acute vs. subacute or subacute vs. chronic ($p > 0.05$ for both).

Blood Flow Speed Changes Following SCI

Figure 5 presents representative B-mode ultrasound images (**Figure 5 A1-A3**) alongside corresponding ULM-derived flow speed maps (**Figure 5 A4-A6**) acquired in acute,

subacute and chronic SCI. During the acute phase (Panel A4), blood flow speed is markedly diminished across the dorsal spinal cord, particularly within the injury epicenter (circled). In the subacute phase (**Figure 5.A5**), microvascular flow becomes more prominent and organized, with increased speeds and directionally coherent flow across both dorsal and ventral compartments. By the chronic phase (**Figure 5.A6**), flow speed is elevated and widespread throughout the spinal cord.

Mean Arterial Flow Velocity Across SCI Phases

Quantitative analysis of arterial flow velocity across SCI phases revealed a significant, transient suppression during the subacute stage. In the overall spinal cord, the acute group exhibited a significantly lower mean speed ratio (0.91 ± 0.11) compared to the subacute (1.1 ± 0.1 , $p < 0.05$) and chronic (1.1 ± 0.1 , $p < 0.05$) groups. No significant difference was observed between the subacute and chronic timepoints (**Figure 5B.1.a**). In the dorsal compartment, a similar trend was observed: acute models showed a lower mean arterial speed ratio (0.41 ± 0.14) compared to chronic animals (0.64 ± 0.22 , $p < 0.05$), while differences between acute and subacute (0.53 ± 0.05) and subacute vs. chronic were not statistically significant ($p > 0.05$) (**Figure 5B.1.b**). In contrast, ventral arterial flow velocity remained stable across all timepoints, with no significant differences observed between acute (0.68 ± 0.13), subacute (0.79 ± 0.16), and chronic (0.77 ± 0.21) groups ($p > 0.05$ for all comparisons) (**Figure 5B.1.c**).

Mean Venous Flow Velocity Across SCI Phases

Venous flow velocity, represented as mean speed ratio, showed a distinct temporal profile compared to arterial flow across SCI progression. In the overall spinal cord compartment, venous velocity was significantly decreased in the subacute group ($0.86 \pm$

0.12) compared to both the acute (1.07 ± 0.13 , $p < 0.05$) and chronic (1.04 ± 0.09 , $p < 0.05$) groups. No significant difference was observed between acute and chronic timepoints ($p > 0.05$), indicating a transient suppression of venous perfusion that was partially resolved by the chronic stage (**Figure 5B.1.d**). When analyzed by region, neither the dorsal compartment (**Figure 5B.1.e**) nor the ventral compartment (**Figure 5B.1.f**) exhibited statistically significant changes in venous velocity across timepoints. In the dorsal region, mean speed ratios were 1.08 ± 0.17 (acute), 0.88 ± 0.10 (subacute), and 1.09 ± 0.06 (chronic), with all pairwise comparisons yielding $p > 0.05$. Similarly, ventral flow speeds were 1.05 ± 0.11 (acute), 0.85 ± 0.15 (subacute), and 0.98 ± 0.09 (chronic), with no significant differences detected.

Immunohistochemistry and GFAP Expression

Figure 6 illustrates the temporal dynamics of astrocyte reactivity following SCI assessed through GFAP immunofluorescence staining. Representative coronal spinal cord sections at acute, subacute and chronic SCI are shown in **Figure 6 A1-A9**. DAPI nuclear staining (**Figure 6 A1-A3**) marks the overall cellular architecture, while GFAP staining (**Figure 6 A4-A6**) shows the astrocytic activation. Merged images (**Figure 6 A7-A9**) demonstrate the spatial overlap between cellular localization and astroglial response. Quantitative analysis of GFAP-positive areas is shown in Panel B. While no statistically significant difference was detected between acute ($1.52 \pm 0.42\%$) and subacute ($1.40 \pm 0.05\%$) groups ($p > 0.05$), a marked and statistically significant increase was evident in the chronic group ($3.3 \pm 0.87\%$) compared to both acute and subacute phases ($*p < 0.05$).

Heatmap Correlation

Heat map analysis identified significant correlations between chronic GFP expression and regional vascular remodelling across different injury phases. Specifically, chronic GFP levels were inversely correlated with subacute artery ventral density ($r=-1, p=0.01$) and acute vein ventral density ($r=-1, p=0.02$), suggesting that lower vessel density in these early phases may contribute to - or reflect - the extent of chronic pathological changes captured by GFP expression. Conversely, chronic GFP was positively associated with chronic artery ventral speed ($r=1, p=0.01$), indicating that increased arterial flow dynamics may accompany, or drive sustained pathological activity. Subacute artery ventral density was positively correlated with both chronic vein dorsal density ($r=1, p=0.01$) and acute vein ventral density ($r=1, p=0.03$), implying coordinated vascular remodelling between arterial and venous systems across phases. However, it negatively correlated with chronic artery ventral speed ($r=-1, p=0.02$), possibly reflecting a trade-off between vessel structural integrity and flow capacity over time. Additionally, acute vein dorsal density was inversely associated with acute artery dorsal speed ($r=-1, p=0.02$), suggesting early mismatches in vascular perfusion dynamics. Chronic vein dorsal density was positively linked to acute vein ventral density ($r=1, p=0.03$) and negatively to chronic artery ventral speed ($r=-1, p=0.01$), highlighting potential phase-dependent interactions between venous architecture and arterial hemodynamic. Together, these results suggest that specific vascular changes during acute and subacute stages are tightly coupled to chronic outcomes and that spatially distinct vessel compartments may differentially contribute to long-term tissue remodelling or dysfunction.

In the acute phase of spinal cord injury (SCI), one significant correlation ($p<0.05$) emerged, highlighting early disruptions in vascular dynamics. Specifically, vein dorsal density was significantly negatively correlated with artery dorsal speed ($r=-1.00, p=0.02$), suggesting that reduced arterial perfusion may be accompanied by compensatory or maladaptive changes in dorsal venous architecture. This finding implies that even in the early

phase post-injury, dorsal vascular compartments are functionally interdependent, with arterial flow limitations potentially driving venous congestion or hypoperfusion.

In the subacute phase, significant intravascular coordination was observed between venous compartments. A strong inverse correlation was detected between vein ventral speed and vein dorsal speed ($r=-1.00$, $p<0.001$), indicating a redistribution of venous flow along the ventral-dorsal axis. This divergence may reflect evolving mechanical or metabolic demands in the injured spinal cord, where compensatory adjustments in venous drainage arise as structural remodeling progresses. In the chronic phase, multiple significant correlations ($p<0.05$) showed persistent neurovascular alterations. Chronic GFP expression, a marker of long-term pathological remodeling, was strongly negatively correlated with vein dorsal density ($r=-1.00$, $p<0.001$), reinforcing the notion that venous rarefaction is tightly linked to chronic injury progression. Conversely, GFP showed a significant positive correlation with artery ventral speed ($r=1.00$, $p=0.01$), suggesting that increased arterial perfusion may be sustained in chronically reactive regions. Moreover, vein dorsal density was inversely associated with artery ventral speed ($r=-1.00$, $p=0.01$), while artery ventral density was positively correlated with vein dorsal speed ($r=1.00$, $p<0.001$). These inter-compartmental relationships point to a chronic imbalance between structural arterial integrity and venous outflow, wherein elevated arterial flow may coincide with impaired venous clearance. Collectively, these findings highlight the spatial and temporal complexity of vascular remodeling in SCI, with chronic GFP expression serving as an integrated marker of persistent arterial-venous dysregulation.

Discussion

Super-resolution ULM has provided unprecedented insights into microvascular remodeling following SCI in a rat model, capturing both structural and functional changes

with sub-100 μm resolution across anatomically defined regions of interest. Our longitudinal analysis from the acute phase (Day 1) to the subacute phase (Day 7) and chronic phase (Day 21) revealed dynamic, compartment-specific vascular alterations, highlighting dorsal vulnerability and a decoupling between vessel density and blood flow speed.

Arterial density remained statistically stable across phases, indicating preserved arterial integrity. In contrast, venous density exhibited progressive rarefaction, with the most pronounced declines observed in the dorsal regions between the early post-injury period and the intermediate recovery stage. Functionally, acute dorsal hypoperfusion showed partial arterial recovery chronically, but venous speeds remained unaltered, evolving from epicenter deficits to tortuous networks. These vascular patterns paralleled delayed astrocytic reactivity, with GFAP expression peaking chronically. Heatmap correlations showed mechanistic ties: chronic GFAP inversely linked to subacute arterial ventral density and acute venous ventral density but positively to chronic arterial ventral speed; acute dorsal mismatches and subacute redistributions further predicted outcomes. By establishing ULM metrics as early biomarkers of glial scarring, our findings reposition microvascular dysfunction as a central driver of SCI progression, informing targeted therapies to restore perfusion and mitigate chronic pathology. Monitoring of spinal cord perfusion pressure (SCPP) via lumbar catheters or direct probes following traumatic SCI provide a useful solution to mitigate secondary injury. Real-time ULM assessment of mVD and mFS could guide individualized hemodynamic management by optimizing SCPP.

Spatiotemporal Heterogeneity in SCI Vascular Recovery

The microvascular response to SCI is inherently heterogeneous, with our ULM data revealing stark dorsal-ventral disparities that reflect differential anatomical susceptibilities to contusive forces.² In acute stage, dorsal regions at the injury epicenter displayed marked

hypoperfusion, characterized by reduced vessel continuity, heterogeneous blood flow density, and diminished speeds, extending rostrally and caudally in segmented ROIs.¹⁵ This evolved subacutely to moderate reperfusion, with increased density and coherent flows, culminating chronically in widespread, tortuous networks indicative of adaptive yet potentially maladaptive angiogenesis.^{15,16} Ventral compartments, conversely, demonstrated greater resilience, maintaining stable arterial density and flow speeds throughout, though sharing the progressive venous decline.¹⁷ This asymmetry aligns with biomechanical models where dorsal columns bear higher compressive loads, perpetuating hypoperfusion and exacerbating secondary cascades like edema and inflammation.¹⁸⁻²⁰

Our study advances the field by providing the first comprehensive, longitudinal integration of structural and functional ULM metrics across spinal compartments, surpassing prior efforts that focused on isolated phases or modalities. For instance, Beliard et al. investigated chronic vascular alterations using ultrafast Doppler and ULM in a rat contusion SCI model, documenting reduced blood velocity, asymmetrical hypoperfusion, and dorsal horn changes at 4 and 8 weeks post-injury, which shares similarities with our chronic tortuosity findings but differs in lacking acute-to-subacute temporal tracking and full longitudinal compartmental analysis.⁹ Similarly, Xu et al. investigated super-resolution ULM for penumbra imaging in rat compression SCI models of varying severities, correlating microvascular saturation and flow with locomotor scores and histology from acute to early chronic stages, akin to our hemodynamic quantification but distinct in not emphasizing dorsal-ventral stratification or extended chronic outcomes with gliosis correlations.⁸ Harmon et al. applied nonlinear ULM to quantify perfusion in rat contusion SCI, resolving smaller branching vasculature and tissue flow segmentation post-injury, which parallels our structural-functional decoupling but contrasts with our work by focusing on methodological advancements without histopathological ties or long-term dorsal-ventral dynamics.²¹

Histological validations corroborate our findings, such as those by Smith et al., who histologically quantified microvascular loss post-contusive SCI in rats using stereology and TEM, revealing acute vessel regression and dorsal grey matter rarefaction persisting up to three segments from the epicenter, similar to our dorsal venous decline and its link to chronic pathology, though their ex vivo approach lacks the in vivo hemodynamic insights provided here.² Clinically, this dorsal vulnerability underpins persistent sensory impairments in SCI patients, including proprioceptive deficits from dorsal column disruption. By capturing the structure-function decoupling, stable arterial density enabling flow recovery amid venous attrition, ULM offers a nuanced diagnostic framework, facilitating spatially targeted therapies like pro-angiogenic agents to enhance dorsal revascularization and improve neurological outcomes.

Vascular Predictors of Gliotic Remodelling

A cornerstone of our investigation is the predictive linkage between early vascular dysfunction and chronic glial reactivity, elucidated through robust correlations that highlight hypoperfusion as a pivotal trigger for astrogliosis. Chronic GFAP expression, marking scar formation, inversely correlated with early density deficits and positively with sustained arterial speeds implying that initial perfusion mismatches fuel maladaptive remodelling. Dorsal-specific patterns amplified this: acute venous-arterial imbalances and chronic venous rarefaction inversely tied to GFAP with subacute flow redistributions signalling evolving compensatory mechanisms. Additional associations included subacute arterial ventral density positively correlating with chronic venous dorsal density and acute venous ventral density, but negatively with chronic arterial ventral speed; chronic venous dorsal density positively linked to acute venous ventral density and negatively to chronic arterial ventral speed. These patterns suggest phase-dependent arterial-venous interactions, where early dorsal mismatches

and subacute redistributions contribute to chronic dysregulation, potentially perpetuating hypoxia and glial activation.

Fassbender et al. investigated microvascular dysfunction following SCI, demonstrating that hypoperfusion leads to ischemia, which triggers oligodendrocyte apoptosis through oxidative stress and activation of the FAS/p75 pathway. Similarly, Huang et al. examined astrocyte signaling within the neurovascular unit (NVU) after CNS injury, including SCI, and described how reactive gliosis and GFAP upregulation contribute to glial scar formation. These scars provide initial protection but may also hinder regeneration. Together, these findings highlight the importance of targeting microvascular pathology for neuroprotection. Therefore, ULM metrics may serve as valuable biomarkers for stratifying injury severity and monitoring therapeutic efficacy.

ULM's Technical Superiority and Translational Horizon

ULM's technical superiority lies in its ability to overcome limitations of conventional imaging, delivering microvascular-level resolution and quantitative hemodynamic at depths unattainable by optical methods. Unlike Doppler's insensitivity to slow flows or MRI's >100 μm vessel constraints, ULM's microbubble-based approach resolved fine networks and enabled ROI-specific analyses, correlating vascular parameters with GFAP to uncover biomarker potential.

Translationally, ULM's non-invasive, repeatable format, demonstrated in human spinal cord functional imaging, holds promise for clinical adoption. It could enable bedside perfusion monitoring post-decompression, guide regenerative therapies (e.g., stem cells targeting venous rarefaction), and evaluate interventions in real time.

Limitations and Future Directions

Despite its strengths, our study has several limitations. First, it was conducted in a rodent model, which, while useful for mechanistic insights, does not fully recapitulate human spinal anatomy or injury heterogeneity. Translation to large-animal models is essential. Second, imaging was limited to three timepoints; denser longitudinal sampling, especially in the acute-to-subacute window, could help identify precise therapeutic windows. Third, GFAP was used as a primary marker of tissue response, and future analyses should expand to include microglial activation, neuronal viability, and BSCB integrity. Finally, spinal imaging in humans faces technical challenges including acoustic access and motion artifacts. Overcoming these will require continued engineering optimization and clinical protocol development to realize the full translational potential of ULM.

Conclusion

Super-resolution ULM in a rat spinal cord injury (SCI) model reveals microvascular dysfunction, with stable arterial density, venous rarefaction, and evolving dorsal hypoperfusion mirroring human SCI pathology. These changes, linked to chronic glial scarring, position ULM as a tool for early risk stratification and guiding interventions like perfusion stabilization and regenerative therapies, enhancing functional outcomes in SCI patients via noninvasive monitoring.

Data Availability Statement: The data generated or analyzed during this study are available from the corresponding author upon reasonable request.

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Author Contributions: All authors contributed to the study conception, design and review of the final manuscript.

Conflict of Interest:

The Mayo Clinic and some of the authors (J.Z., C.H., and S.C.) have a potential financial interest related to the technology referenced in this research.

Ethical Approval

All experimental procedures involving animals were approved by the Institutional Animal Care and Use Committee (IACUC) at Mayo Clinic and were conducted in accordance with institutional guidelines for the care and use of laboratory animals.

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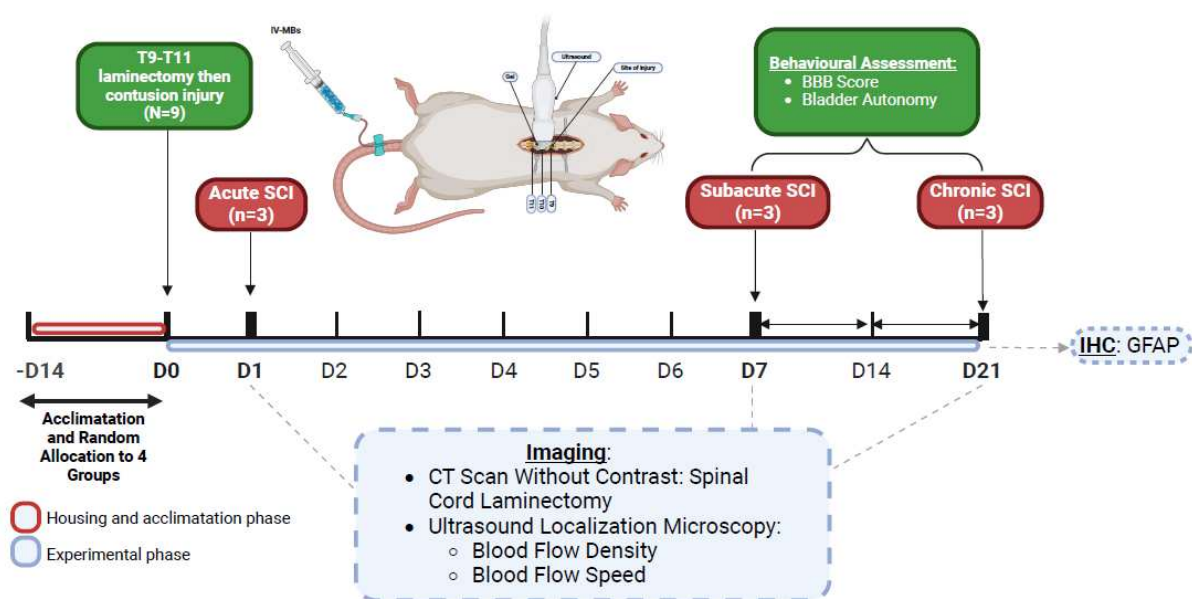


Figure 1: Schematic outline of the study design for evaluating spinal cord injury (SCI) recovery in a rat model: Nine rats undergo T9-T11 laminectomy followed by contusion injury, and they are categorized into acute (n=3), subacute (n=3), and chronic (n=3) SCI groups. Behavioral assessments, including the Basso, Beattie, and Bresnahan (BBB) locomotor scale and bladder autonomy tests, are performed to track functional recovery. Imaging techniques, such as CT scans for spinal cord visualization and ultrasound localization microscopy (ULM) for blood flow density and speed analysis, provide structural and hemodynamic insights. At the study endpoint, immunohistochemistry (IHC) with GFAP staining is used to evaluate gliosis and spinal tissue integrity. This timeline-based approach integrates behavioral, imaging, and histological analyses to assess SCI progression and recovery. The panel was created using [Biorender.com](https://biorender.com).

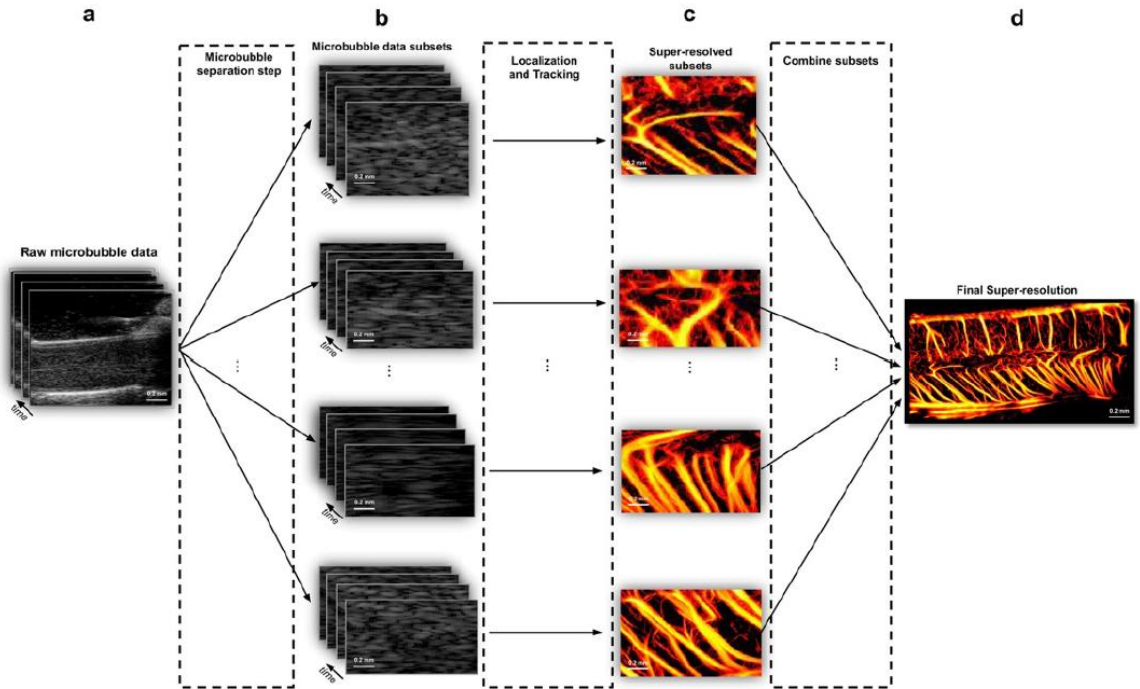


Figure 2: Principle of Microbubble Separation-Based Super-Resolution Imaging (a) The raw spatiotemporal microbubble data is obtained after tissue clutter filtering to remove background noise. (b) Microbubbles were separated based on their flow speed and direction, generating datasets with a reduced microbubble concentration for improved localization. (c) Each subset undergoes independent microbubble localization and tracking to reconstruct precise vascular flow trajectories. (d) Finally, all subset signals are combined to generate the super-resolved ultrasound localization microscopy (ULM) image, providing a detailed visualization of the microvascular network.

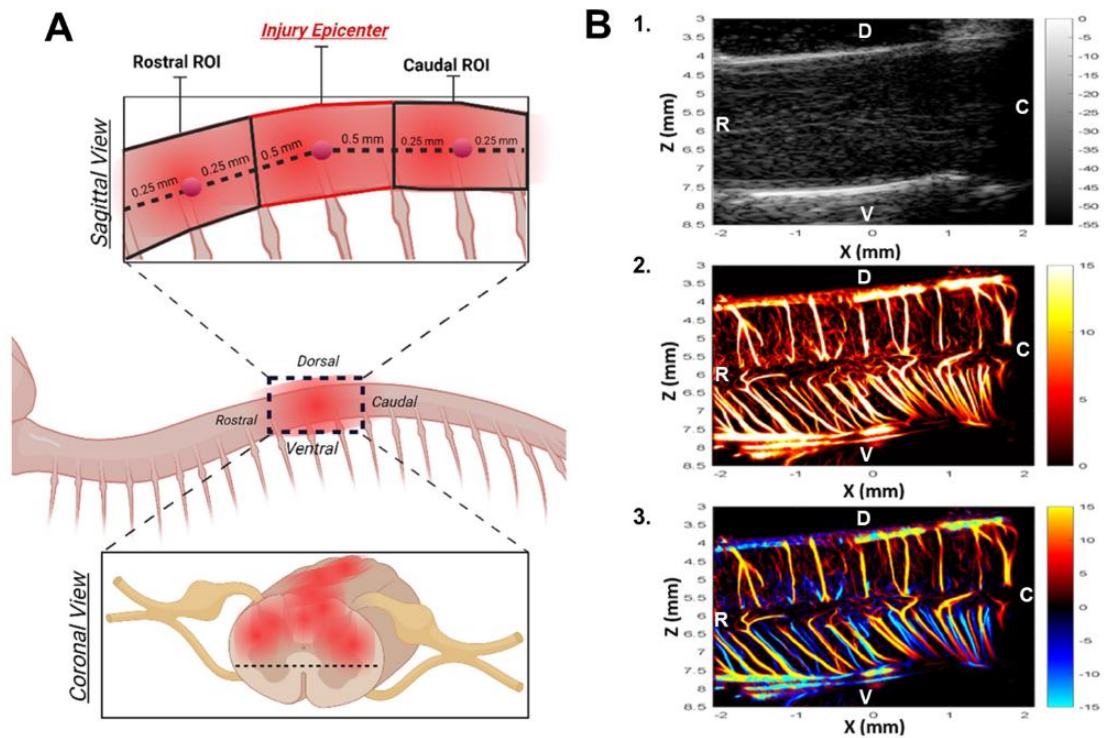


Figure 3: Ultrasound Localization Microscopy (ULM) in an Injured Spinal Cord
Anatomy: (A) Schematic representation of the spinal cord injury (SCI) epicenter, highlighting regions of interest (ROIs) in the sagittal and coronal views. The injury site is divided into rostral and caudal ROIs, with incremental distances from the epicenter to evaluate vascular changes along the spinal cord. (B) Ultrasound-based imaging analysis: (1) Conventional B-mode ultrasound image showing the anatomical structure of the normal uninjured spinal cord. (2) Super-resolution ULM image revealing the spinal microvascular network with high spatial resolution. (3) ULM-derived velocity map, displaying blood flow speed and direction within the spinal cord vasculature.

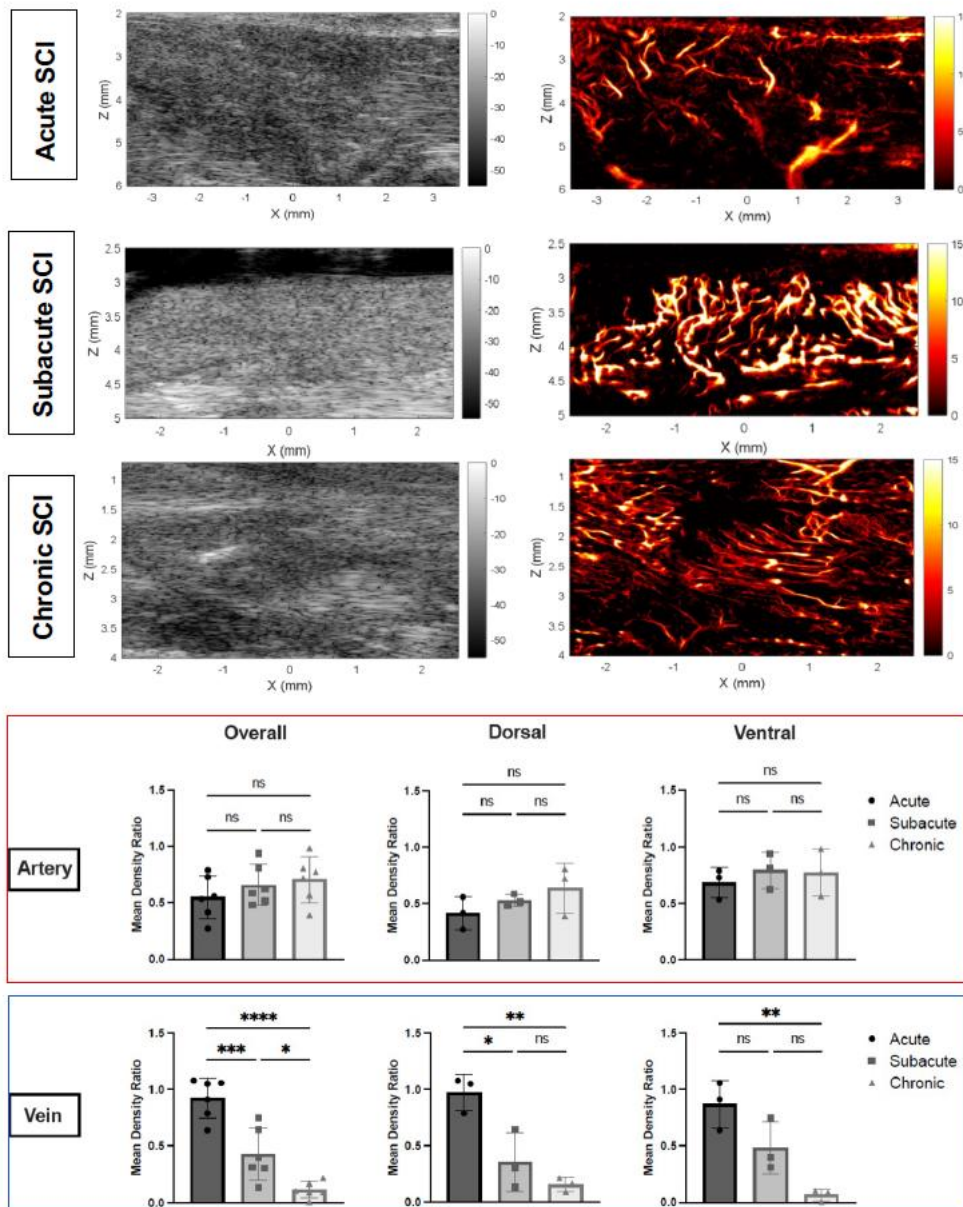


Figure 4: Vascular Changes in the Spinal Cord Across Acute, Subacute, and Chronic Phases of SCI: (A) Super-resolution ultrasound imaging of microvascular structures in SCI progression. (1-3) B-mode ultrasound images of the spinal cord in the acute (1), subacute (2), and chronic (3) SCI stages. (4-6) Corresponding super-resolution ULM images reveal vascular structures at each time point, with circled regions highlighting notable vascular remodeling. (B) Quantification of arterial and venous density ratios across SCI stages. (1) Arterial density analysis shows no significant changes in mean density ratio across SCI phases in the overall (a), dorsal (b), and ventral (c) spinal regions (ns: not significant). (2) Venous density analysis reveals a significant reduction in venous density in the chronic phase, compared to the acute and subacute phases in overall (d), dorsal (e), and ventral (f) regions (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). These findings suggest progressive venous vascular decline over time, while arterial density remains unchanged.

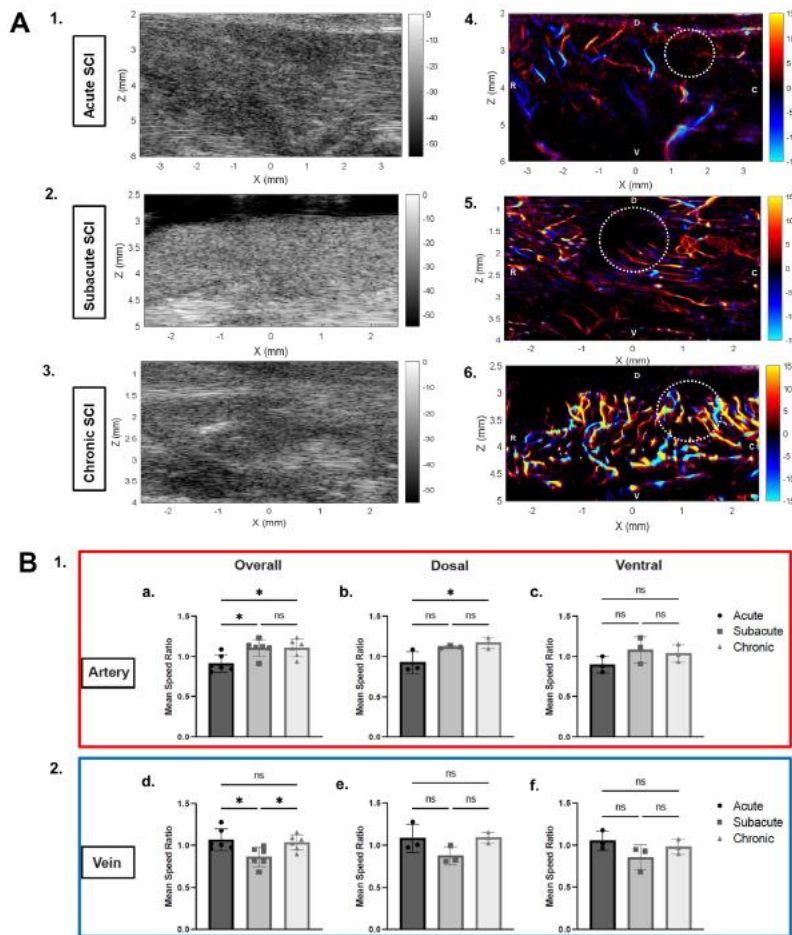


Figure 5: Blood Flow Speed Changes in the Spinal Cord Across Acute, Subacute, and Chronic Phases of SCI: (A) Super-resolution ultrasound imaging of blood flow speed in SCI progression. (1-3) B-mode ultrasound images of the spinal cord at different SCI stages: acute (1), subacute (2), and chronic (3). (4-6) Corresponding super-resolution ULM velocity maps display blood flow speed variations, with circled regions highlighting alterations in vascular flow dynamics across different phases of injury. (B) Quantification of arterial and venous blood flow speed across SCI stages. (1) Arterial flow speed analysis shows a significant decrease in the acute phase compared to the subacute and chronic phases in the overall (a) spinal region, while no significant differences are observed in the dorsal (b) or ventral (c) regions (* $p < 0.05$, ns: not significant). (2) Venous flow speed analysis shows a significant reduction in the acute phase compared to later stages in the overall (d) region, whereas no significant changes are detected in the dorsal (e) and ventral (f) spinal regions (* $p < 0.05$, ns: not significant). These findings indicate a dynamic regulation of vascular flow following SCI, with early-stage impairments recovering over time.

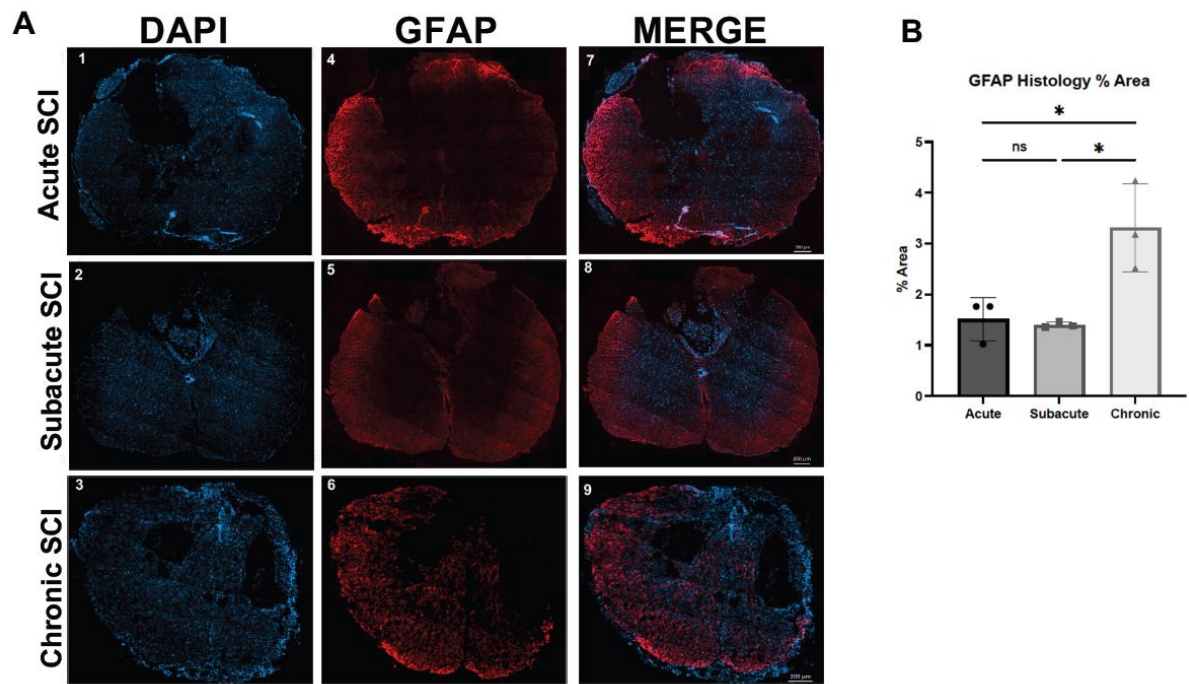
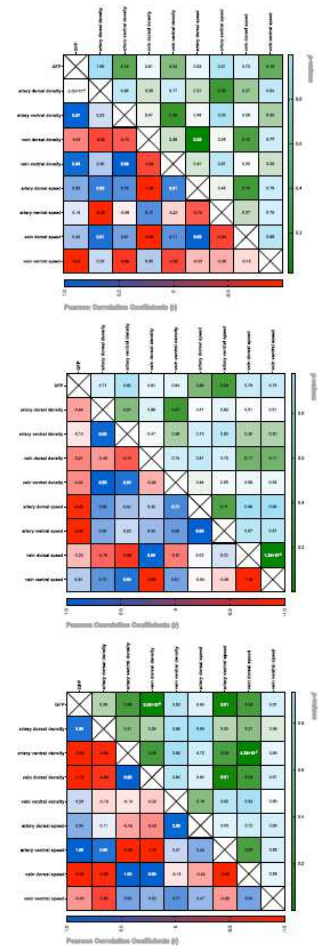
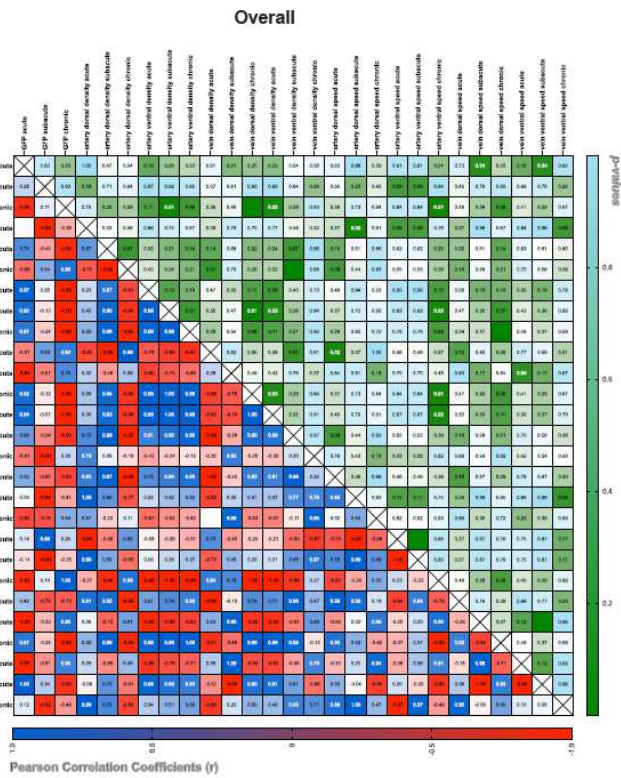


Figure 6



Acute

Subacute

Chronic

Figure 7