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From injury to recovery: Spatiotemporal dynamics of the visual pathway during spontaneous structural and functional regeneration after optic nerve transection in zebrafish

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ABSTRACT

In adult mammals, optic nerve injury leads to irreversible vision loss due to its extremely limited regenerative capacity. In contrast, adult zebrafish possess a robust capacity for spontaneous visual system regeneration, although the spatiotemporal coordination of recovery across the retina, optic nerve, and brain remains poorly understood. In the present study, the regenerative dynamics following optic nerve transection were systematically characterized in adult zebrafish over a 5 week period using hematoxylin-eosin staining, immunohistochemistry, transmission electron microscopy, single-cell RNA sequencing, and optokinetic response (OKR) behavioral assessments. At 1 week post-injury (1 wpi), retinal ganglion cell depletion was evident but showed significant recovery by 2 wpi. Concurrently, the injured optic nerve displayed a marked increase in diameter and cell number at 2 wpi, including widespread expression of proliferating cell nuclear antigen, consistent with heightened proliferative activity. Single-cell transcriptomic profiling at 2 wpi revealed five principal cell populations: fibroblasts, mural cells, immune cells, mature oligodendrocytes, and myelin-forming oligodendrocytes. By 4–5 wpi, remyelination within the optic nerve and re-establishment of synaptic architecture in the optic tectum were strongly correlated with functional restoration of OKR behavior. These findings provide a comprehensive spatiotemporal framework of visual pathway regeneration in zebrafish, establishing a valuable model for elucidating

conserved mechanisms of neural repair with translational potential for human vision restoration.

Keywords: Zebrafish; Visual pathway; Optic nerve transection; Regeneration

INTRODUCTION

The regenerative capacity of the vertebrate visual system exhibits profound interspecies variation, with a striking contrast between adult mammalian and fish species. In mammals, damage to the optic nerve, a crucial component of the central nervous system (CNS), typically results in permanent vision loss due to the limited regenerative capacity of the mammalian CNS (Laha et al., 2017; Williams et al., 2020). In contrast, adult zebrafish possess an extraordinary regenerative ability after optic nerve injury, enabling restoration of retinal ganglion cells (RGCs), regeneration of axons, re-establishment of synaptic connections, and recovery of visual function (Baier & Scott, 2024; Becker & Becker, 2008, 2014; Chen et al., 2022; Diekmann et al., 2015; Goldman, 2014; Gorsuch & Hyde, 2014). While previous studies have focused on individual components of this process (Bastmeyer et al., 1993; Beckers et al., 2019; Chen et al., 2022; Diekmann et al., 2015; Münzel et al., 2014), comprehensive resolution of regeneration dynamics across the entire visual pathway has remained elusive.

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(ARPA-H) launched the Total Human Eye Allograft (THEA) program, aiming to restore vision through whole-eye transplantation by combining advanced microsurgical methods with genetic interventions, with the successful completion of a full-eye and partial facial transplant representing a pivotal advancement in the field. This procedure achieved long-term graft survival without immunological rejection, and electroretinogram measurements confirmed that the transplanted retina remained responsive to light stimulation (Ceraadini et al., 2024). However, despite these promising outcomes, light perception was not recovered, due to the failure to reconnect the donor optic nerve with central visual pathways. This limitation underscores a critical barrier to vision restoration in humans—the inability to regenerate damaged optic nerve tissue.

Unlike mammals, adult zebrafish can regenerate the entire visual system after optic nerve injury, including retinal ganglion cell replacement, axonal regrowth, remyelination, and synaptic reconstruction in visual processing centers. To elucidate the mechanisms underlying this capacity, the present study systematically investigated structural and functional regeneration following optic nerve damage in adult zebrafish. An integrative approach combining histological analysis, immunohistochemistry, transmission electron microscopy, single-cell RNA sequencing, and optokinetic response (OKR) testing was used to map regenerative progression across the retina, optic nerve, and brain at multiple post-injury time points. Results revealed a sequential and precisely coordinated regenerative program, characterized by distinct phases of RGC restoration, re-establishment of optic nerve continuity, remyelination of regenerated axons, and synaptic remodeling within the brain. OKR analysis further demonstrated a strong correlation between structural regeneration and the progressive recovery of visual function. These findings provide a comprehensive framework for understanding vertebrate visual system regeneration and highlight the zebrafish as a powerful model for identifying therapeutic targets to promote optic nerve repair in non-regenerating species.

MATERIALS AND METHODS

Zebrafish husbandry

Adult male and female zebrafish (3–6 months old), including wild-type AB and transgenic (*Tg(gfap:GFP)*) lines, were obtained from the National Aquatic Biological Resource Center (China) and Nanjing Hunter Biotechnology Co., Ltd. (China). Fish were maintained in a standardized housing system (Haisheng Biotechnology, China) under a 14-h light/10-h dark cycle at 28.5°C and were fed brine shrimp twice daily. All maintenance and experimental procedures were conducted in accordance with the Ethical Review Committee of the Affiliated Eye Hospital of Wenzhou Medical University (Experimental approval number: YSG20230901).

Reagents and instruments

All reagents and materials are listed in Supplementary Table S1.

Optic nerve transection (ONT) in adult zebrafish

ONT was performed following established protocols (Elsaeidi et al., 2014; Zou et al., 2013). Adult zebrafish were anesthetized in 0.03% MS-222 (w/v) (Sigma-Aldrich, USA) until complete sedation. A paper towel placed on the lid of a

Petri dish immersed in anesthetic solution was used to prevent movement during surgery. All surgical instruments were sterilized in 70% ethanol prior to use. The corneal dermis was carefully removed using sterile forceps with an upward circular motion. To expose the optic nerve, forceps (spaced 0.5 mm apart) were inserted beneath the ventral side of the eye and directed downward to elevate the dorsal side. While stabilizing the eye with curved forceps, the dorsal orbital cavity was accessed, and surrounding fatty tissue was meticulously removed. The optic nerve was then transected approximately 0.5 mm distal to the optic nerve head, taking care to avoid injury to the ophthalmic artery. Fish exhibiting intraoperative bleeding were excluded. Following ONT, fish were transferred to clean system water and monitored until normal swimming behavior resumed.

Hematoxylin-eosin (H&E) staining and data processing

For histological analysis, adult zebrafish were euthanized with 0.1% MS-222 (w/v). The eyeball, optic nerve, and surrounding bone tissue were carefully excised to preserve anatomical integrity. Tissues were fixed in Bouin's solution (Phygene, China) at 4°C for 12 hours, rinsed thoroughly, dehydrated through a graded ethanol-xylene-paraffin series using a HistoCore PEARL processor (Leica, Germany), and embedded ventral side up. Sections were cut at 5 µm with a HistoCore BIOCUT microtome (Leica, Germany) and dried at 65°C for 1 h. H&E staining and dewaxing were completed using an Autostainer XL (Leica, Germany). Paraffin sections were deparaffinized in xylene (Aladdin, China) (8 min), rehydrated through graded ethanol (Aladdin, China) (100% for 6 min, 70% for 2 min), and rinsed in distilled water (2 min). Hematoxylin staining was performed (3 min), followed by differentiation (45 s), bluing (1 min), and counterstaining with eosin (Biosharp, China) (1 min). Slides were dehydrated in ethanol (95% for 2 min, 100% for 4 min) and cleared in xylene (4 min). Images were acquired and analyzed using 3D Panoramic scanner software (3DHISTECH, Hungary).

Retinal and optic nerve histomorphometric analysis

Retinal integrity was assessed using H&E-stained sections (5 µm). For each specimen, three cross-sections were collected at a standardized location 400 µm distal to the optic nerve head. Retinal thickness was measured across the nerve fiber layer (NFL), RGC layer (RGCL), and inner plexiform layer (IPL), with triplicate measurements averaged for each biological replicate ($n=6-7$ fish). RGC density was quantified by counting morphologically intact RGCs in three retinal sections per fish, with means calculated for each biological replicate ($n=6-7$ fish).

Optic nerve morphometry was performed by measuring post-injury diameter at three cross-sectional planes spanning the lesion site. Mean values were calculated per individual ($n=7-9$ fish). Relative optic nerve diameter was determined by normalizing post-injury measurements to pre-injury measurements (µm). Cell density at the injury site was assessed within a standardized 0.2 mm long×0.2 mm wide region across three sections, with means representing single biological replicates ($n=7-9$ fish).

Tissue preparation for cryosections

Adult zebrafish were euthanized with 0.1% MS-222 (w/v), and eyeballs were carefully dissected and fixed in 4% paraformaldehyde (PFA) (Biosharp, China) at 4°C overnight. Fixed tissues were rinsed in 1× phosphate-buffered saline

(PBS), followed by sequential dehydration in 15% and 30% sucrose solutions in PBS at room temperature. Samples were embedded in optimal cutting temperature (OCT) compound (SAKURA, USA), snap-frozen on dry ice, and stored at -80°C . Cryosections ($12\ \mu\text{m}$) were prepared using a cryostat (Leica, Germany) and subsequently processed for immunohistochemistry.

Immunohistochemistry

Cryosections were permeabilized in 0.3% Triton X-100 (Yeasen, China) in PBS for 10 min (three times), then blocked with 10% donkey serum in 0.3% Triton X-100 /PBS for 1 h. Slides were incubated overnight at 4°C with primary antibodies diluted in PBS containing 0.3% Triton X-100 and 10% normal donkey serum (Yeasen, China). Following incubation, sections were washed in PBS containing 0.3% Triton X-100 (PBST) and incubated at room temperature for 5 h with secondary antibodies (1:500 dilution in PBST). After secondary antibody incubation, the slides were stained with $1\ \mu\text{g}/\text{ml}$ 4',6-diamidino-2-phenylindole (DAPI) (Phygene, China) at room temperature for 12 min, then washed in $1\times$ PBS and mounted using Fluoromount™ Aqueous Mounting Medium (Sigma-Aldrich, USA). Fluorescence images were acquired using a Zeiss Cell Observer SD microscope (Carl Zeiss, Germany). Primary and secondary antibodies are provided in Supplementary Table S1. Proliferating cell nuclear antigen (PCNA)- and L-plastin-positive cells were assessed within a standardized $0.2\ \text{mm}\ \text{long}\times 0.2\ \text{mm}\ \text{wide}$ region from three biological replicates.

Retina whole-mount preparation

Retina whole-mounts were prepared as described previously (Nagashima & Hitchcock, 2020). Zebrafish were acclimated in darkness for 2 h prior to euthanasia with 0.1% MS-222 (w/v). Eyes were dissected with a portion of the optic nerve retained for manipulation and fixed in 4% PFA for 10 min. The cornea was removed to generate an eye cup, and the sclera was peeled away. Retinas were rinsed with pre-cooled PBS to remove residual pigment, excised at the optic disc, and immersed in 5% sucrose/4% PFA for overnight fixation at 4°C . After fixation, tissues were washed three times (20 min each) in 5% sucrose/0.1 mol/L PBS, incubated in blocking solution (10% donkey serum/1% Triton X-100/1% Tween-20 (Yeasen, China)/1% DMSO (Sigma-Aldrich, USA)) for 2 h, and stained with primary antibodies overnight at 4°C . Secondary antibodies were applied the following day and incubated for 12 hours. After washing, the retinas were trimmed into a four-leaf clover shape and mounted with the RGCL facing upward. Images were captured using a $40\times$ objective lens, with the retina divided into four regions. Within each region, three distinct areas were imaged: peripheral (P), middle (M), and central (C) retina. A total of 12 images per retina ($n=3\text{--}4$ fish) were taken across all Z-axes. RGCs were counted using ImageJ.

Transmission electron microscopy

Transmission electron microscopy was performed following previously described protocols (Tian et al., 2016; Zou et al., 2013). Zebrafish were euthanized with 0.1% MS-222 (w/v), and a 1 mm segment of the optic nerve adjacent to the lesion site was carefully excised and immersed in 2.5% glutaraldehyde (Phygene, China) in 0.1 mol/L phosphate buffer (pH 7.4) at 4°C overnight. Subsequent preparation followed standard electron microscopy protocols (Mi et al.,

2007). Transverse sections of the lesion site were imaged in two randomly selected regions using transmission electron microscopy (Hitachi, Japan) by the Wuhan Servicebio Technology Company (China). Images were captured at $5\ 000\times$ magnification for 1 wpi and 2 wpi; $15\ 000\times$ magnification for normal, 3 wpi, and 5 wpi; and $50\ 000\times$ magnification for all post-injury time points for detailed enlargement. A total of 55–58 axons per group were assessed. Myelin thickness was calculated as the difference between the outer and inner axonal diameters. The G-ratio of axons ($n=120$ axons per group) was calculated according to established methods (Münzel et al., 2014). All data were obtained from three to four zebrafish.

Optokinetic response (OKR) assay

For behavioral assessment of visual function, adult zebrafish were anesthetized with 0.03% MS-222 (w/v), immobilized in a sponge holder with the head and gills exposed to maintain respiration, and placed in a Petri dish containing system water. To eliminate visual input from the contralateral (left) eye, it was covered with a sponge and wrapped in tin foil to block light exposure. The fish were then positioned on the OKR apparatus (ViewPoint, France), with software-controlled parameters for contrast, color, spatial frequency, and angular velocity. A 5 min adaptation period using a 100% contrast stimulus (black and white) was applied to ensure recovery from anesthesia and reliable tracking. Subsequently, visual stimuli of increasing spatial frequency were introduced, and eye movements were manually counted over a 1 min period. After testing, the fish were carefully returned to fresh system water. Eye rotations were measured by video observation, expressed as number of rotations per minute.

Mirror aggression assay

Zebrafish were acclimated for 1 h at $28\pm 0.5^{\circ}\text{C}$ before being individually transferred to 3D-printed behavioral chambers filled with 150 mL of ultrapure water (conductivity: $400\text{--}700\ \mu\text{S}/\text{cm}$) within a ZebraCube video tracking system (ViewPoint, France). Following 5 min of acclimation, a 6 min aggression trial was conducted under 50% illumination. Automated tracking was performed with a background threshold of 20–25 pixels and speed range of $1.5\text{--}3\ \text{mm}/\text{s}$ to quantify mirror-zone entries (frequency and duration). For quantification of mirror-directed aggression, the average number of attack events and percentage of time spent within the mirror zone ($\leq 1.0\ \text{cm}$ from the mirror surface) during 5 min were measured according to previously established protocols (Lin et al., 2022).

Social behavior assay

Pairs of zebrafish were placed in 3D-printed behavioral chambers containing 200 mL of ultrapure water (conductivity: $400\text{--}700\ \mu\text{S}/\text{cm}$) and acclimated for 5 min under 50% illumination. Social interactions were recorded over a 12 min trial across three predefined zones using ZebraCube video tracking system (ViewPoint, France) (sampling interval: 30 s; velocity filter: $1.5\text{--}3\ \text{mm}/\text{s}$). Spatiotemporal interaction dynamics were subsequently evaluated via cluster-based analysis.

Shoaling assay

Groups of four zebrafish were monitored in 400 mL of ultrapure water (conductivity: $400\text{--}700\ \mu\text{S}/\text{cm}$) under 100% illumination. The shoaling-cube module was used to track collective metrics, including nearest neighbor distance, polarization, and mean speed, over 8 min (15 s intervals;

20–25 pixel threshold). Four biological replicates were analyzed to assess inter-individual spatial organization.

Locomotor behavior assay

Individual zebrafish ($n=10$ fish per group) were placed in 24-well plates filled with 150 mL of ultrapure water and subjected to a 4 min trial consisting of alternating 2 min light and dark phases (50% intensity). Locomotor activity, including velocity and cumulative distance traveled (measured in 2-min intervals; speed filter: 1.5–3 mm/s), was recorded using the ZebraCube video tracking system (ViewPoint, France).

Optic nerve dissociation for single-cell suspension

Optic nerves from 18 zebrafish (balanced by sex) at 2 wpi were pooled to generate a single-cell suspension, ensuring sufficient cell capture while minimizing inter-individual variability. Pooling was necessary due to the limited tissue volume and low mRNA expression in the zebrafish optic nerves (Yu et al., 2024), and thus no biological replicates were included. Dissected nerves were minced into 1 mm fragments with a sterile scalpel and suspended in 5 mL of digestion buffer containing 2 mg/mL collagenase type II (Sigma-Aldrich, USA) and 200 U/mL DNase I (Worthington, USA) in RPMI medium (Corning, USA). Samples were then incubated at 37°C with shaking for 30 min. The resulting suspension was passed through a 100 μ m filter (Falcon, USA) and centrifuged (400 $\times$ g, 10 min, 4°C). Pelleted cells were resuspended in red blood cell lysis buffer (Solarbio, China), incubated for 2 min, passed through a 40 μ m filter (Falcon, USA), collected by centrifugation (400 $\times$ g, 10 min, 4°C), and resuspended in PBS containing 0.04% bovine serum albumin (BSA) (Sigma-Aldrich, USA). Cells were manually counted using Trypan blue (Thermo Fisher Scientific, USA) and AO-PI staining (Logos Biosystems, Korea) after centrifugation (400 $\times$ g, 10 min, 4°C) and resuspended. Single cells were processed using a Chromium Controller (10X Genomics, USA) according to the manufacturer's protocols.

Single-cell RNA sequencing (scRNA-seq)

Single-cell transcriptomic profiling was performed using a Chromium Next GEM Single Cell 3' Kit v.3.1 (10X Genomics, USA) and Chromium Next GEM Chip G Single Cell Kit (10X Genomics, USA). Cell suspensions were loaded onto the Chromium Controller (10X Genomics, USA) to generate single-cell gel bead-in-emulsions (GEMS) according to the manufacturer's protocols. Captured cells were lysed, and the released RNA was barcoded during reverse transcription in individual GEMs. Cell-barcoded 3' gene expression libraries were sequenced on the Illumina NovaSeq6000 system (Illumina, USA) by Shanghai Biochip Co., Ltd. (China).

ScRNA-seq and data analysis

Raw scRNA-seq data were processed as previously described (Yu et al., 2024). Low-quality cells were excluded based on stringent filtering criteria: RNA feature counts between 300 and 5 000, total RNA counts between 500–8 000, mitochondrial gene percentage below 20%, hemoglobin- and red blood cell-related gene percentages below 10%, and total RNA counts not exceeding the 95th percentile. A total of 3 359 cells were sequenced, with 1 341 high-quality cells retained for downstream analyses. Cell clusters not associated with optic nerve tissue were excluded. Detailed quality control procedures are presented in Supplementary Text S1 and Supplementary Table S2.

Data integration was performed using Seurat v.5.1, followed

by normalization, scaling, dimensionality reduction, and unsupervised clustering. Visualization was conducted using t-distributed stochastic neighbor embedding (t-SNE) based on the first 30 principal components. Cell types were annotated using canonical marker gene expression profiles, including mature oligodendrocytes (*tspan2a*, *mag*, *mbpa*), myelin-forming oligodendrocytes (*egr2b*, *gldn*, *mbpb*, *s100b*, *si:ch211-234p6.5*, *ndrg1a*), fibroblasts (*col1a1a*, *col1a1b*, *dcn*), immune cells (*nr4a1*, *srgn*), and mural cells (*rgs5a*, *rbpms2a*, *myh11a*). The immune cell cluster lacked expression of microglial or macrophage markers and instead showed high expression of generalized immune function-related genes. Consequently, this population was designated broadly as "immune cells" without further subclassification. Gene Ontology (GO) enrichment analysis was conducted to identify functional pathways and biological processes associated with each cell population. Proliferation scores were computed based on curated gene sets representing proliferation-related pathways (Supplementary Text S2).

Optic tectum reinnervation analysis

Optic nerve regeneration was assessed via biocytin-based axonal tracing, as described previously (Hu et al., 2024; Münzel et al., 2014). Adult zebrafish were anesthetized (MS-222; 0.03% w/v), and the optic nerve was transected proximally to the optic nerve head. A gelatin sponge saturated with 0.5% biocytin (Sigma-Aldrich, USA) was applied to the lesion site for anterograde tracer uptake. Fish were returned to system water for 3 h to permit biocytin transport before euthanasia with 0.1% MS-222 (w/v). Brains were dissected, fixed overnight at 4°C in 4% PFA, rinsed with PBS, and dehydrated by sequential immersion in 15% and 30% sucrose solutions at room temperature. Tissues were embedded in OCT compound and cryosectioned into 50- μ m-thick slices. Sections were stained using a VECTASTAIN ABC Kit (Vector Laboratories, USA) and developed with 3,3'-diaminobenzidine (DAB) chromogen (Boster, China). All samples were stained simultaneously to minimize technical variability, with stringent controls to prevent overstaining. In uninjured controls, the ratio of biocytin-labeled area to total optic tectum area was quantified and normalized to 100% as a baseline reference. Following optic nerve injury, reinnervation percentages (compared to baseline) served as the primary metric for assessing regenerative capacity.

Statistical analysis

All statistical analyses were conducted using Prism v.9.5.0. (GraphPad Prism, USA). One-way analysis of variance (ANOVA), followed by Tukey's *post hoc* test, was employed for comparisons among three or more groups. Unpaired *t*-test was used to compare data from two independent groups. Data are expressed as mean $\pm$ standard error of the mean (SEM), with $P < 0.05$ indicating statistical significance.

RESULTS

Structural alterations in the retina and optic nerve following ONT in adult zebrafish

To characterize morphological responses to optic nerve injury, ONT was performed in adult zebrafish (Figure 1A). H&E staining of the retina and optic nerve was used to assess structural alterations across time. At 3 days post-injury (dpi), both the nerve fiber layer (NFL) and the ganglion cell layer (GCL) showed significant thickening (Supplementary Figure

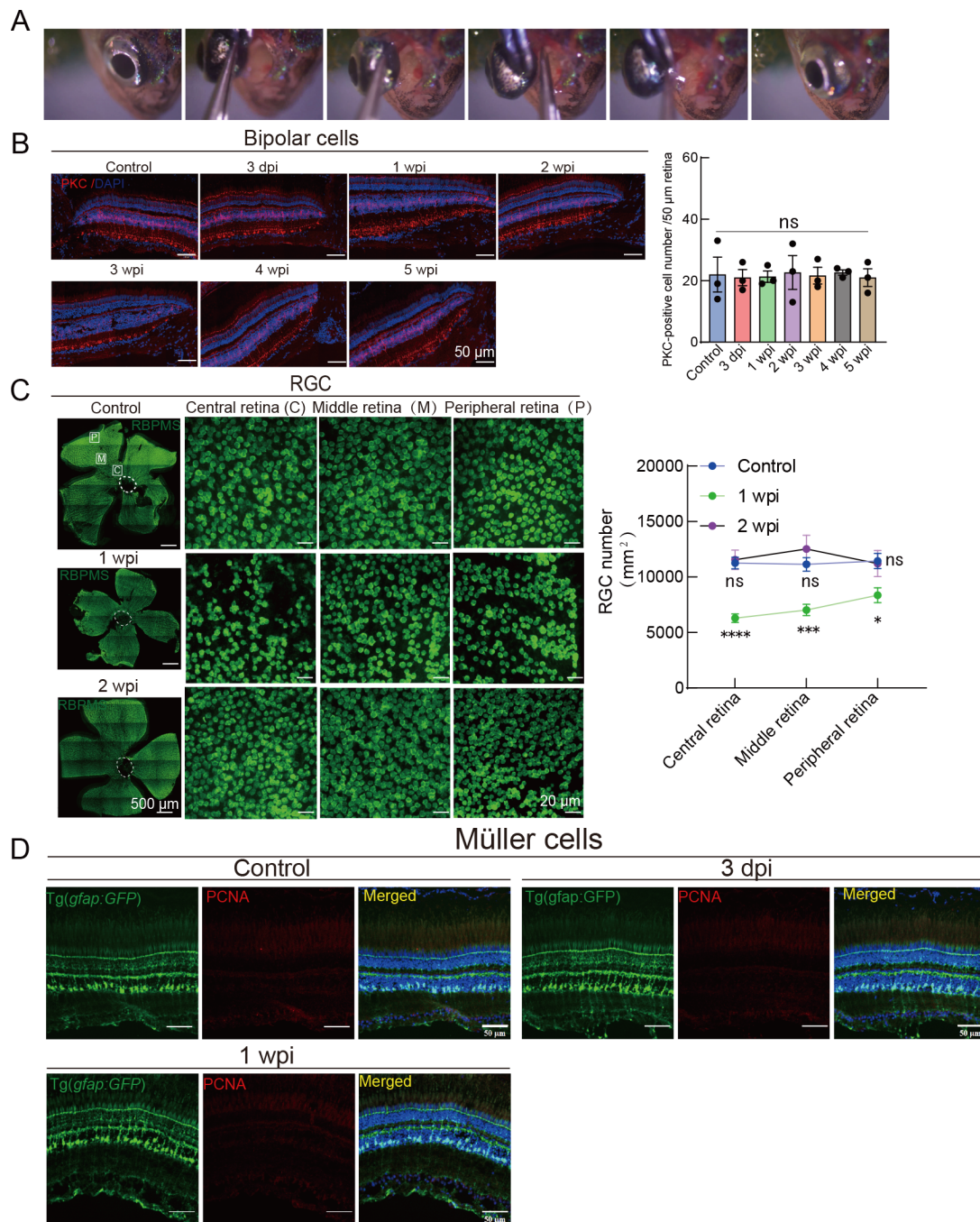


Figure 1 Cellular changes in zebrafish retina following optic nerve transection (ONT)

A: Detailed depiction of the surgical method used for ONT. B: Retinal protein kinase C (PKC) and DAPI co-immunostaining: Left: Representative images of retinal sections showing co-immunostaining for PKC and DAPI at different time points post-transection. Scale bar: 50 μ m. Right: Quantitative analysis of PKC-positive cells ($n=3$ fish). C: Flat-mounted retinal ganglion cell (RGC) analysis: Left: Flat-mounted retinal images showing changes in RGC layer at different time points post-injury. White square represents measurement area, covering the central (C), middle (M), and peripheral (P) retina. Scale bar: 500 μ m for full retina; 20 μ m for magnified region. Right: Statistical analysis of RGC number at various time points post-transection ($n=3-4$ fish). D: Co-immunostaining of Müller cells (GFP) and proliferating cell nuclear antigen (PCNA) in transgenic zebrafish (*Tg(gfap:GFP)*) at different time points. Scale bar: 50 μ m. Statistical significance was assessed using one-way ANOVA followed by Tukey's multiple comparisons test. ns: Not significant; *, $P<0.05$; ***, $P<0.001$; ****, $P<0.0001$.

S1A–C). While GCL thickness gradually returned to baseline, the NFL continued to thicken at 1, 2, 3, and 5 wpi, with transient normalization observed at 4 wpi (Supplementary Figure S1A, B). In contrast, the IPL exhibited a downward trend from 3 dpi to 4 wpi, although this reduction was not statistically significant (Supplementary Figure S1A, D). Quantification of RGC density revealed no significant

differences among the control, 1 wpi, and 2 wpi groups in cross-sectional analyses (Supplementary Figure S1E).

To further examine retinal structural dynamics, immunostaining was performed to assess potential changes in cellular composition. Staining with anti-PKC α antibodies, which specifically label bipolar cells (Yazulla & Studholme, 2001; Haug et al., 2019), showed no significant changes in

bipolar cell organization following ONT (Figure 1B). In contrast, substantial changes were observed in the RGC population. At 1 wpi, RGC numbers were markedly reduced, but returned to baseline by 2 wpi, indicating rapid recovery (Figure 1C). To assess the involvement of Müller glia, transgenic *gfap:GFP* zebrafish were used to visualize glial cells (Bernardos et al., 2007). PCNA staining revealed no evidence of Müller glial proliferation at either 3 dpi or 1 wpi, suggesting a lack of Müller glia-derived neurogenic response during early regeneration (Figure 1D).

The optic nerve, a crucial pathway that transmits visual information from the retina to the CNS, was also examined at different time points post-injury using H&E staining. In uninjured zebrafish, the optic nerve presented with a uniform diameter and low cellular density. By 3 dpi, the nerve was fully transected, with complete discontinuity at the injury site (Figure 2A). Partial reconnection of the optic nerve was observed at 2 wpi (Figure 2A). By 5 wpi, the connection between the optic nerve and the lesion was significantly enhanced, with most optic nerve fibers exhibiting partial reconnection to the injury site (Figure 2A).

Compared to uninjured zebrafish, the optic nerve of injured zebrafish exhibited marked thickening, especially at the lesion site, with the most prominent changes observed at 2 wpi (Figure 2B). This thickening progressively decreased over time, approaching pre-injury dimensions (Figure 2B). In parallel, cell density at the lesion site increased significantly at 1, 2, 3, 4, and 5 wpi, with the most pronounced changes detected at 1 wpi (Figure 2C).

To determine whether these accumulating cells were proliferative, immunostaining for PCNA-positive cells was performed. PCNA-positive cells were enriched at the lesion site, exhibiting a distribution pattern largely consistent with the H&E staining results (Figure 2C, D). These findings indicate that optic nerve thickening following ONT is driven by robust cell proliferation and is accompanied by gradual reconnection of nerve fibers to the injury site.

While H&E staining effectively captured morphological changes and cellular accumulation, it failed to capture the organizational and structural alterations in microtubules, a key component of the optic nerve cytoskeleton (Jang et al., 2024; Logan & Menko, 2019). To assess cytoskeletal organization, tubulin immunostaining was performed. At 3 dpi, the optic nerve was completely severed, with a near absence of fluorescence signals at the proximal lesion site near the brain (Figure 2E). By 1 wpi, fluorescence signals extended outward from the injury site, and by 2 wpi partial reconnection between the severed optic nerve segments and surrounding tissues was evident (Figure 2E). By 5 wpi, tubulin staining revealed substantially improved continuity, indicating structural reconnection of the optic nerve and associated pathways (Figure 2E). Collectively, H&E and immunostaining analyses revealed substantial thickening of the optic nerve following injury, accompanied by an increase in both total and proliferating cell numbers. To further explore the cellular dynamics underlying optic nerve regeneration, scRNA-seq was performed at 2 wpi. Injured optic nerves were dissected and processed into single-cell suspensions, followed by sequencing with stringent quality control (Figure 3A, B). Five major cell populations were identified, including fibroblasts, mural cells, immune cells, mature oligodendrocytes, and myelin-forming oligodendrocytes (Figure 3C). Among these, mature oligodendrocytes exhibited the lowest proliferation

scores, while immune cells displayed significantly higher scores (Figure 3D). The myelin-forming oligodendrocyte cluster showed a significantly elevated score compared to the immune cell cluster. Notably, the mural cell and fibroblast clusters achieved significantly higher scores than the myelin-forming oligodendrocyte cluster (Figure 3D). GO enrichment analysis demonstrated that the mature oligodendrocytes were enriched in myelination-associated pathways (Figure 3E), while myelin-forming oligodendrocytes were enriched in material preparation processes (such as “protein refolding” and “mRNA stabilization”). Gene set enrichment analysis (GSEA) targeting the “myelination” term confirmed these findings (Figure 3E; Supplementary Figure S2). Fibroblasts were significantly enriched in extracellular matrix remodeling, connective tissue development, cell proliferation, and response to injury, supporting their key role in tissue repair and regeneration. In contrast, mural cells were enriched in pathways related to blood vessel morphogenesis and developmental growth. Immune cells were primarily enriched in extracellular matrix organization and inflammatory response pathways (Figure 3E). Despite the identification of an immune cell cluster, microglia and macrophages were not detected in the single-cell dataset (Supplementary Text S1). This likely reflects temporal dynamics in immune cell populations, with microglia and macrophages having already receded by 2 wpi. To investigate this, immunohistochemical analysis was performed at earlier time points using L-plastin, a pan-immune marker that labels microglia and other immune cells (Herbomel et al., 1999; Le Guyader et al., 2008; Mitchell et al., 2018; Mitchell et al., 2019). Co-staining with Iba1 (a microglial marker) and L-plastin revealed a robust increase in immune cell presence at 1 wpi, followed by a gradual decline at 2 and 3 wpi (Figure 4A). Many of these immune cells were proliferative, as indicated by co-labeling with PCNA (Figure 4B).

In contrast to immune cells, astrocytes—major glial components of the CNS—are essential for maintaining brain homeostasis, providing neural support, and facilitating repair, and may exhibit different temporal dynamics from microglia during the regeneration process (Sofroniew & Vinters, 2010). However, the existence of astrocytes in zebrafish remains debated (Grupp et al., 2010; Lyons & Talbot, 2014).

Although glial fibrillary acidic protein (GFAP) is widely used as an astrocyte marker in both fish and mammals (Alunni et al., 2005; Hol & Pekny, 2015; Nielsen & Jørgensen, 2003), its expression in the zebrafish optic nerve is unconfirmed. To assess astrocyte presence, GFAP expression in the optic nerve was examined using *Tg(gfap:GFP)* zebrafish. Consistent with prior reports (Koke et al., 2010), no GFP signal was observed in the optic nerve before or after injury (3 dpi and 1 wpi) (Supplementary Figure S3).

Some studies have proposed cytokeratin as an alternative biomarker for zebrafish astrocytes (Koke et al., 2010). However, our scRNA-seq data at 2 wpi revealed cytokeratin expression only in musculature and myocardial cell populations (Supplementary Text S1). As these cell types are not present in the optic nerve, their presence likely reflects contamination from adjacent tissues during optic nerve dissection.

Temporal progression of demyelination and remyelination in the optic nerve following ONT

The optic nerve consists of axons tightly ensheathed in

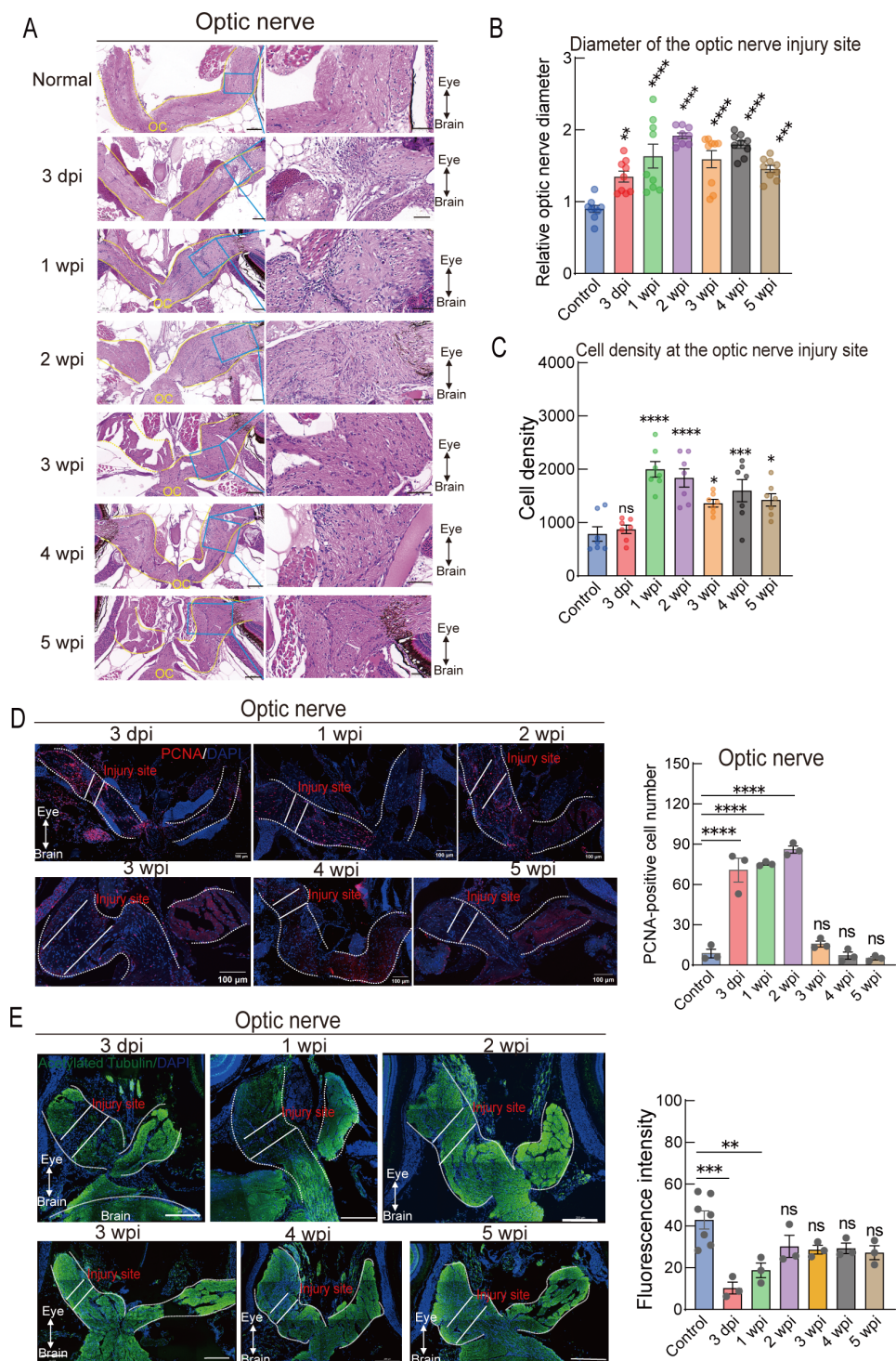


Figure 2 Time-dependent changes in zebrafish optic nerve following optic nerve transection (ONT)

A: Histological analysis of optic nerve at various time points following ONT (Normal, 3 dpi, 1 wpi, 2 wpi, 3 wpi, 4 wpi, and 5 wpi). Optic nerve is outlined with a yellow dashed line. Regions enclosed by sky-blue frames highlight the injury site at higher magnification. OC: optic chiasm. Scale bar: whole optic nerve: 200 μ m; magnified region: 50 μ m. B, C: Quantitative analysis of optic nerve diameter (B) and cell density (C) at various time points post-injury. In (B), optic nerve diameter is expressed relative to pre-injury baseline (normalized length, μ m). D: Temporal changes in PCNA expression and distribution at the ONT site. Left: Representative images showing PCNA expression at injury site. Optic nerve boundary is delineated by a white dashed line, with white line indicating injury site. Scale bar: 100 μ m. Right: Quantitative analysis of PCNA fluorescence intensity at transection site across time points ($n=3$ fish). Error bars represent standard error of the mean (SEM). E: Temporal changes in acetylated tubulin expression and spatial distribution at the ONT site. Left: Representative images showing acetylated tubulin expression at the injury site. Optic nerve boundary is indicated by a white dashed line, with white line indicating injury site. Scale bar: 200 μ m. Right: Quantitative analysis of acetylated tubulin fluorescence intensity at transection site over time. Error bars represent SEM. Control group, $n=7$ fish, other post-injury time points, $n=3$ fish per group. Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparisons test. ns: Not significant; *: $P<0.05$; **: $P<0.01$; ***: $P<0.001$; ****: $P<0.0001$.

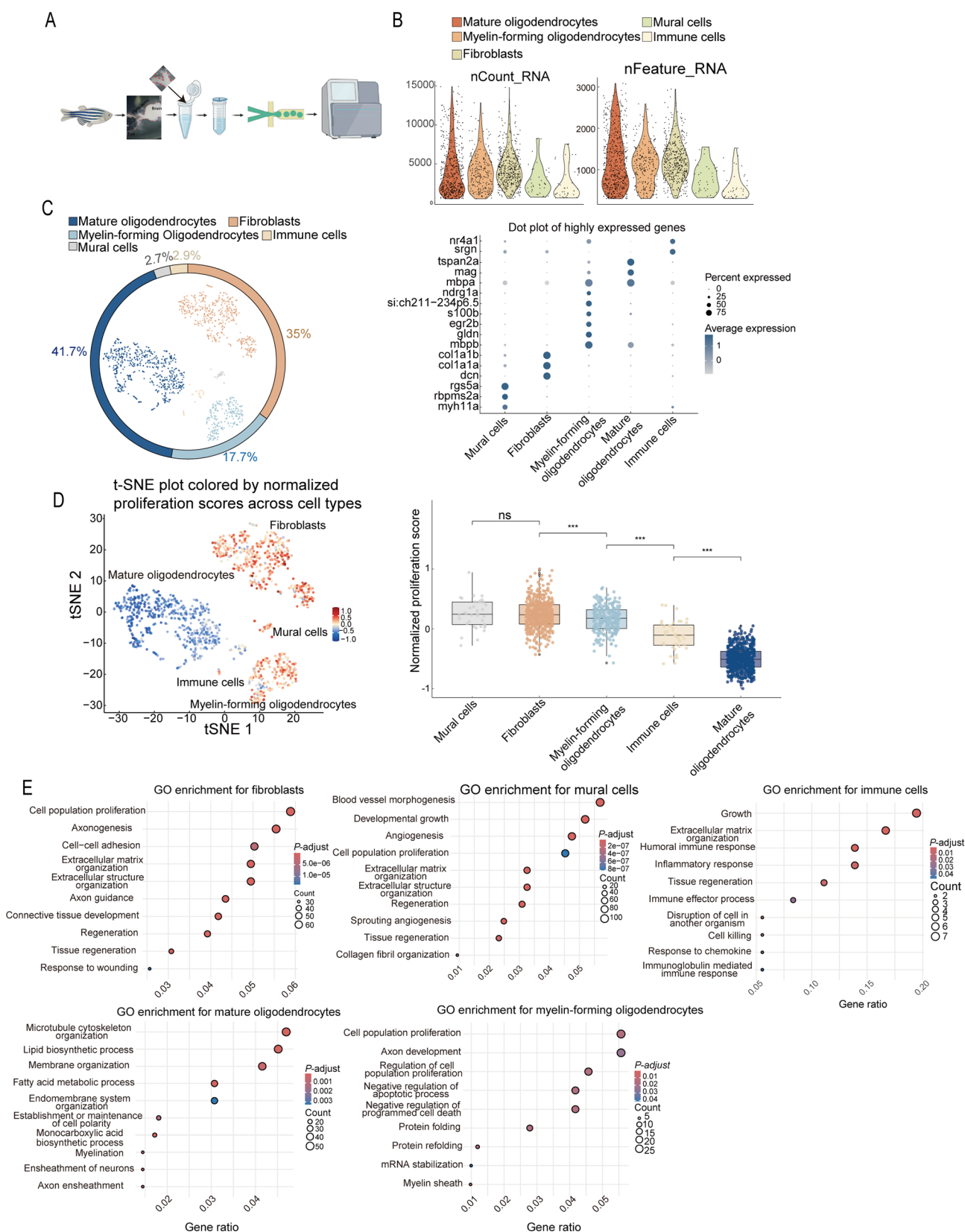


Figure 3 Single-cell analysis of the optic nerve at 2 wpi

A: Schematic representation showing single-cell sequencing workflow (designed with BioRender.com). B: Violin plots showing quality control metrics, including number of detected features (nFeature_RNA) and RNA counts (nCount_RNA), across five identified cell clusters. C: Left: Inner t-SNE plot visualizing zebrafish optic nerve cells, with outer ring depicting proportion of each cell population. Right: Dot plot showing marker genes specific to each cell type. D: Left: t-SNE plot highlighting different cell type colored by proliferation scores. Right: Boxplot comparing proliferation scores between different cell types. ***: $P < 0.001$; ns: Not significant. E: Gene Ontology (GO) enrichment analysis for mural cells, fibroblasts, myelin-forming oligodendrocytes, immune cells, and mature oligodendrocytes.

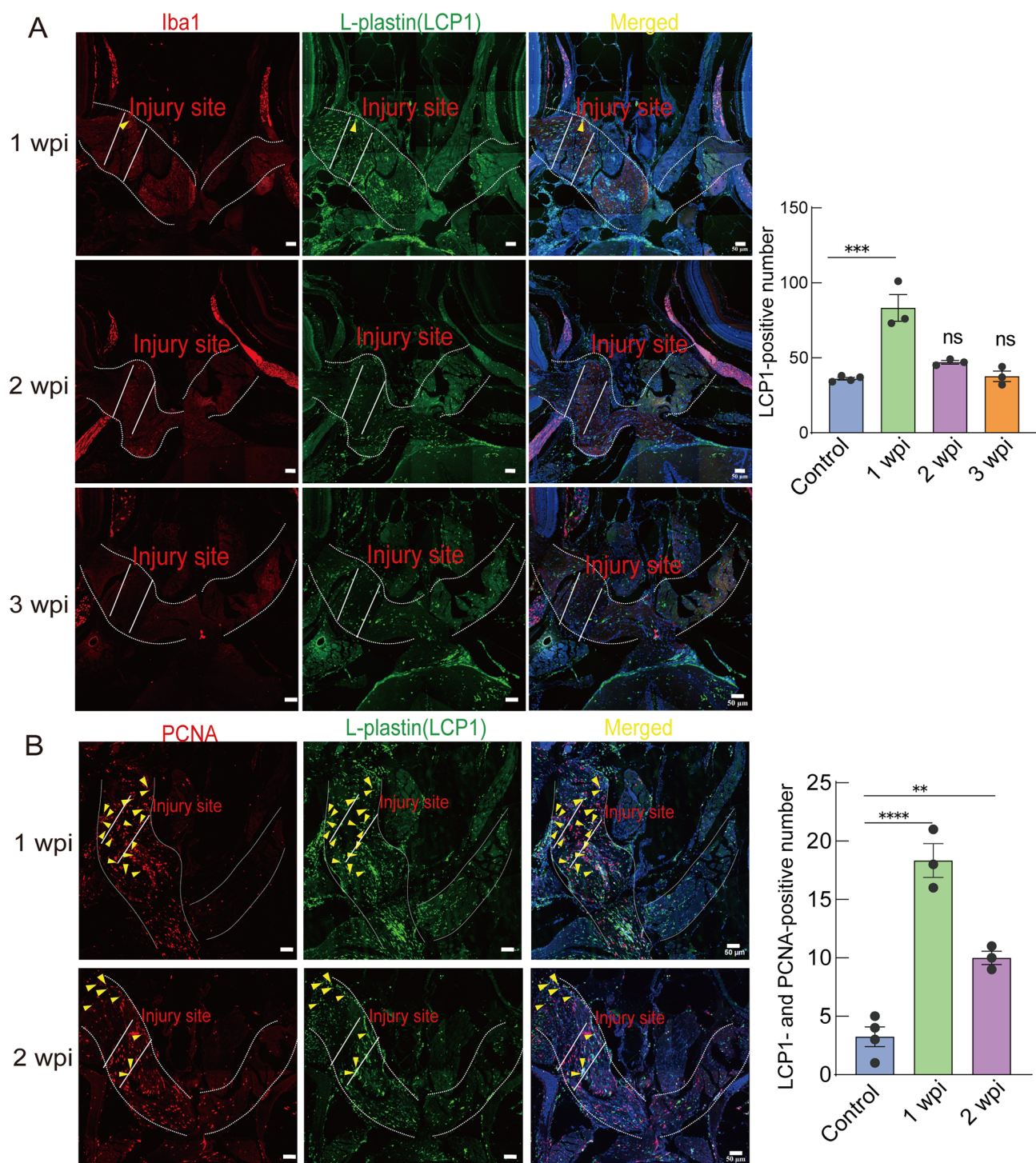


Figure 4 Immune cell dynamics in the optic nerve at different post-injury time points

A: Left: Co-staining of Iba1 and L-plastin at different post-injury time points. Scale bar: 50 μ m. Yellow arrows highlight colocalization of Iba1 and L-plastin. Optic nerve boundary is indicated by a white dashed line, with white line indicating injury site. Right: Statistical analysis of L-plastin-positive cells at different post-injury time points ($n=3-4$ fish). B: Left: Co-staining of L-plastin and PCNA at different post-injury time points. Scale bar: 50 μ m. Yellow arrows highlight colocalization of PCNA and L-plastin. Optic nerve boundary is indicated by a white dashed line, with white line indicating injury site. Right: Statistical analysis of L-plastin- and PCNA-positive cells at different post-injury time points ($n=3-4$ fish). Statistical analysis was conducted using one-way ANOVA followed by Tukey's multiple comparisons test. ns: Not significant; *: $P<0.01$; **: $P<0.001$; ***: $P<0.0001$.

myelin, a lipid-rich structure essential for rapid conduction of action potentials and overall neural function (Laha et al., 2017; Saab & Nave, 2017). Zebrafish possess a remarkable ability to regenerate compact myelin following injury, enabling full restoration of optic nerve integrity following demyelination (Münzel et al., 2014; Zou & Hu, 2021). Given the critical role of

myelin in axonal conduction, remyelination is essential for the restoration of axonal function following optic nerve transection. To evaluate this process, remyelination of the optic nerve was investigated at multiple post-injury stages. Prior to injury, the myelin sheaths of individual axons looked normal (Figure 5A). At early time points (1–2 wpi), the optic nerve exhibited

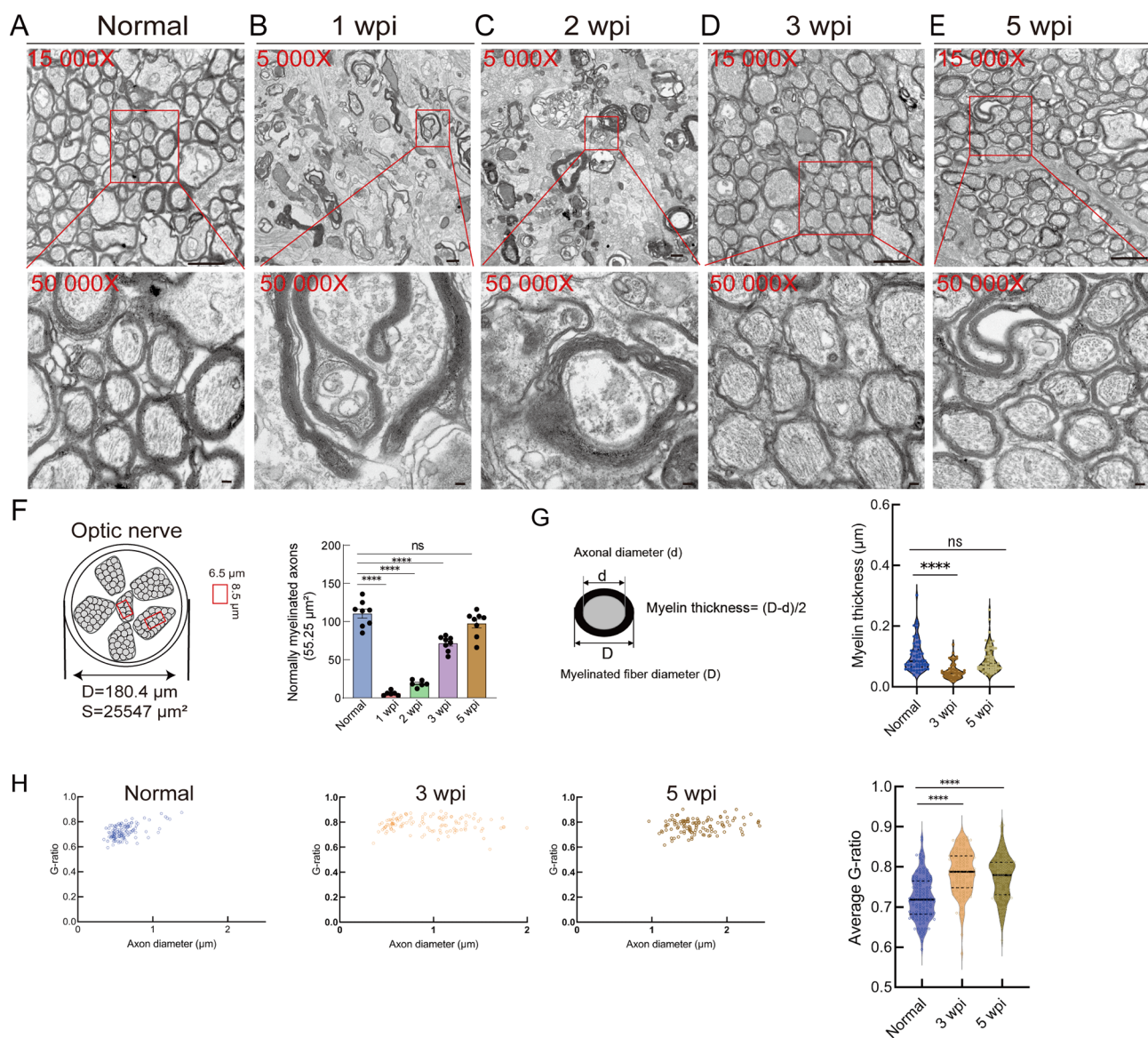


Figure 5 Demyelination and remyelination of zebrafish optic nerve at different post-injury time points

A–E: Transmission electron microscopy (TEM) images of optic nerve at various post-injury time points. Red frame highlights locally magnified area. (A, C, E) Top panel: 15 000 $\times$ magnification. (B, D) Top panel: 5 000 $\times$ magnification. Bottom panel: 50 000 $\times$ magnification. Scale bar: 1 μm for top panel, 0.25 μm for bottom panel. (A) Normal optic nerve ($n=4$ fish); (B) 1 wpi ($n=3$ fish); (C) 2 wpi ($n=3$ fish); (D) 3 wpi ($n=4$ fish); (E) 5 wpi ($n=4$ fish). F: Quantitative analysis of myelin sheath number. Left panel: Schematic representation of zebrafish optic nerve, highlighting regions analyzed. Right panel: Quantitative analysis of number of myelin sheaths in zebrafish optic nerve at different post-injury time points. Error bars indicate standard error of the mean (SEM). G: Quantitative analysis of myelin thickness. Left panel: Schematic showing calculation of myelin thickness, defined as half the difference between the outer and inner diameters of the myelin sheath. Right panel: Quantitative analysis of myelin sheath thickness at different post-injury time points ($n=55$ – 58 axons per group). Error bars indicate standard error of the mean (SEM). H: Left: G-ratio of different post-injury time points (Normal, 3 wpi, and 5 wpi), Right: Average G-ratio of different post-injury time points. Statistical analysis was conducted using one-way ANOVA followed by Tukey's multiple comparisons test. ns: Not significant; ****: $P<0.0001$.

pronounced demyelination, characterized by a significant reduction in both the number and thickness of myelin sheaths (Figure 5B, C, F–H). By 3 wpi, remyelination had initiated, as evidenced by an increase in myelinated axons and progressive thickening of myelin sheaths, although a subset of axons remained unmyelinated and overall sheath thickness had not yet fully returned to baseline levels (Figure 5B, C, F–H). At 5 wpi, both the number and thickness of myelin sheaths closely resembled those observed prior to injury, suggesting substantial structural recovery (Figure 5E, F–H). However, G-ratio analysis indicated that myelin compaction remained incomplete, even at 5 wpi, implying that full

functional restoration of the myelin architecture may require a longer period of recovery (Figure 5H).

Temporal dynamics of synaptic loss and recovery in the optic tectum and retina following ONT

Although regeneration in the zebrafish retina and optic nerve is well characterized, the corresponding responses within the brain, particularly in synaptic architecture, remain less defined. The optic tectum (TEO), a key brain region involved in visual processing, is organized into distinct laminar structures, including the stratum marginale (SM), stratum opticum (SO), stratum fibrosum et griseum superficiale (SFGS), stratum

griseum centrale (SGC), stratum album centrale (SAC), and stratum periventriculare (SPV) (Vanegas, 1975). To assess RGC axonal regeneration and target reinnervation following ONT, anterograde labeling was performed using biocytin. Representative images illustrated progressive optic nerve reinnervation of the optic tectum at various post-injury time points (Figure 6A). In uninjured zebrafish, 100% of the optic tectum was innervated by the optic nerve (Figure 6A, B). At 1 wpi, the labeled area was reduced to approximately 64%, indicating substantial denervation (Figure 6B). By 4 wpi, biocytin labeling had returned to baseline levels, indicating successful optic nerve regeneration following ONT in adult zebrafish (Figure 6B).

Synaptic connectivity between RGCs and target neurons in the brain is essential for visual processing (Baier & Scott, 2024). Synaptic loss following ONT is a major contributor to visual dysfunction (Baier & Scott, 2024). To assess synaptic recovery, immunostaining for vesicular glutamate transporter 1 (Vglut1), a presynaptic marker, was performed on brain and retinal tissues at various time points post-injury. In the brain, Vglut1 expression was primarily localized to the SM and SFGS layers of the optic tectum (Figure 6C). At 3 dpi, Vglut1 labeling in the SFGS was markedly reduced, indicating acute synaptic loss. However, this signal progressively recovered and was fully restored by 4 wpi (Figure 6C, E). In contrast, presynaptic structures within the SM remained largely unchanged before and after injury (Figure 6C). In the retina, Vglut1-positive presynaptic terminals were observed in both the IPL and outer plexiform layer (OPL) of the retina (Figure 6D). Notably, the density of presynaptic terminals in the retinal IPL progressively decreased at 3 dpi, although this reduction did not reach statistical significance ($P > 0.05$) (Figure 6D, F).

Visual function recovery following ONT in zebrafish

To determine whether structural regeneration in the retina, optic nerve, and brain is accompanied by restored visual function, OKR assays were conducted at multiple post-injury time points in adult zebrafish. While preceding analyses confirmed the recovery of retinal ganglion cells, axonal continuity, myelination, and synaptic structure, the extent of functional visual restoration remained uncertain. The OKR assay—originally developed for larval zebrafish—has been adapted for use in adults, enabling quantitative evaluation of visual tracking behavior (Huang & Neuhauss, 2008; Zou et al., 2013).

To ensure reliable measurement, adult zebrafish were anesthetized and immobilized in sponge holders to minimize movement during testing (Figure 7A). The contralateral (left) eye was completely occluded with aluminum foil to eliminate compensatory visual input, allowing for specific assessment of the injured visual pathway (Beckers et al., 2023) (Figure 7A). A sham-operated group was included to control for nonspecific effects of surgery. In both injury and sham groups, eye movement frequency increased in response to the number of visual grating stimuli, with the highest frequency observed at “Strip20” (Figure 7B). At 3 wpi, OKR performance to low spatial frequency stimuli (e.g., “Strip10”) indicated partial recovery of visual tracking (Figure 7C). Higher spatial frequencies (e.g., “Strip20,” “Strip40,” and “Strip60”) required longer recovery intervals, with full restoration occurring after approximately one month (Figure 7C).

Although OKR provides a robust measure of primary visual function, it does not fully capture the complexity of visually

mediated behaviors. Therefore, additional behavioral experiments were conducted to assess higher-order visual responses. Despite partial OKR recovery at 3 wpi, impairments persisted in other visual behavioral domains (Figure 7D–G). Mirror aggression assays showed increased duration spent in the mirror zone (Figure 7D); shoaling tests revealed increased nearest neighbor and inter-individual distances (Figure 7E); social interaction was reduced (Figure 7F); and dark-phase locomotor activity was diminished (Figure 7G). These findings indicate that assessing functional recovery after ONT requires evaluation of complex, visually guided behaviors beyond basic reflexive responses.

An integrative approach combining histology, immunohistochemistry, electron microscopy, scRNA-seq, OKR testing, and complex behavioral assays revealed the temporal progression of neural injury and repair following ONT. In the early post-injury phase, RGCs began to recover, followed by the assembly of tubulin-based proteins in the optic nerve, accompanied by gradual myelin sheath regeneration (Figure 8A). As neural connections and myelin integrity were restored, synaptic activities in the optic tectum gradually recovered, culminating in OKR functional recovery observed at the behavioral level.

Correlation analysis demonstrated a strong association between OKR functional recovery and key regenerative indices, including synaptic repair, optic tectum reinnervation, and the number of regenerated axons within the optic nerve (Figure 8B, C).

DISCUSSION

Previous investigations have largely focused on isolated components of the visual system, such as the retina, optic nerve, or brain, without integrating spatial and temporal dynamics across the entire pathway. This study addresses this limitation by providing a comprehensive spatiotemporal framework of optic nerve regeneration in adult zebrafish, establishing a foundation for future multi-omics analyses at defined anatomical sites and regenerative phases.

Histological assessment via H&E staining revealed no significant reduction in RGCs at 1 wpi. However, immunohistochemical analysis with the RBPMS marker identified a transient decrease in RGC density at this time point, followed by full recovery to baseline levels at 2 wpi. These findings contrast with those of Zou et al. (2013), who reported an initial 90% RGC survival within the first two weeks but observed a subsequent attrition of 75% by 7 wpi. Similarly, Chen et al. (2022) employed *isl2b:GFP* transgenic zebrafish to assess RGC dynamics post-ONT and observed approximately 73% RGC survival, proposing that reduced GFP intensity reflected cellular dysfunction rather than neuronal loss. These findings suggest that RBPMS-based quantification may be insufficient to definitively determine RGC loss. To investigate the potential involvement of Müller gliosis after ONC (Goldman, 2014; Hoang et al., 2020), transgenic zebrafish (*Tg(gfap:GFP)*) were employed to visualize Müller glial cells. No evidence of Müller glial cell activation was detected. Our results suggested that 70%–100% of RGCs remained viable at 1 wpi.

Optic nerve regeneration proceeded in a sequential manner, progressing from complete severance at 3 dpi to partial reconnection by 2 wpi and near-complete structural restoration by 5 wpi, reflecting the temporally ordered nature of neural repair. In a parallel study currently under review, full-length

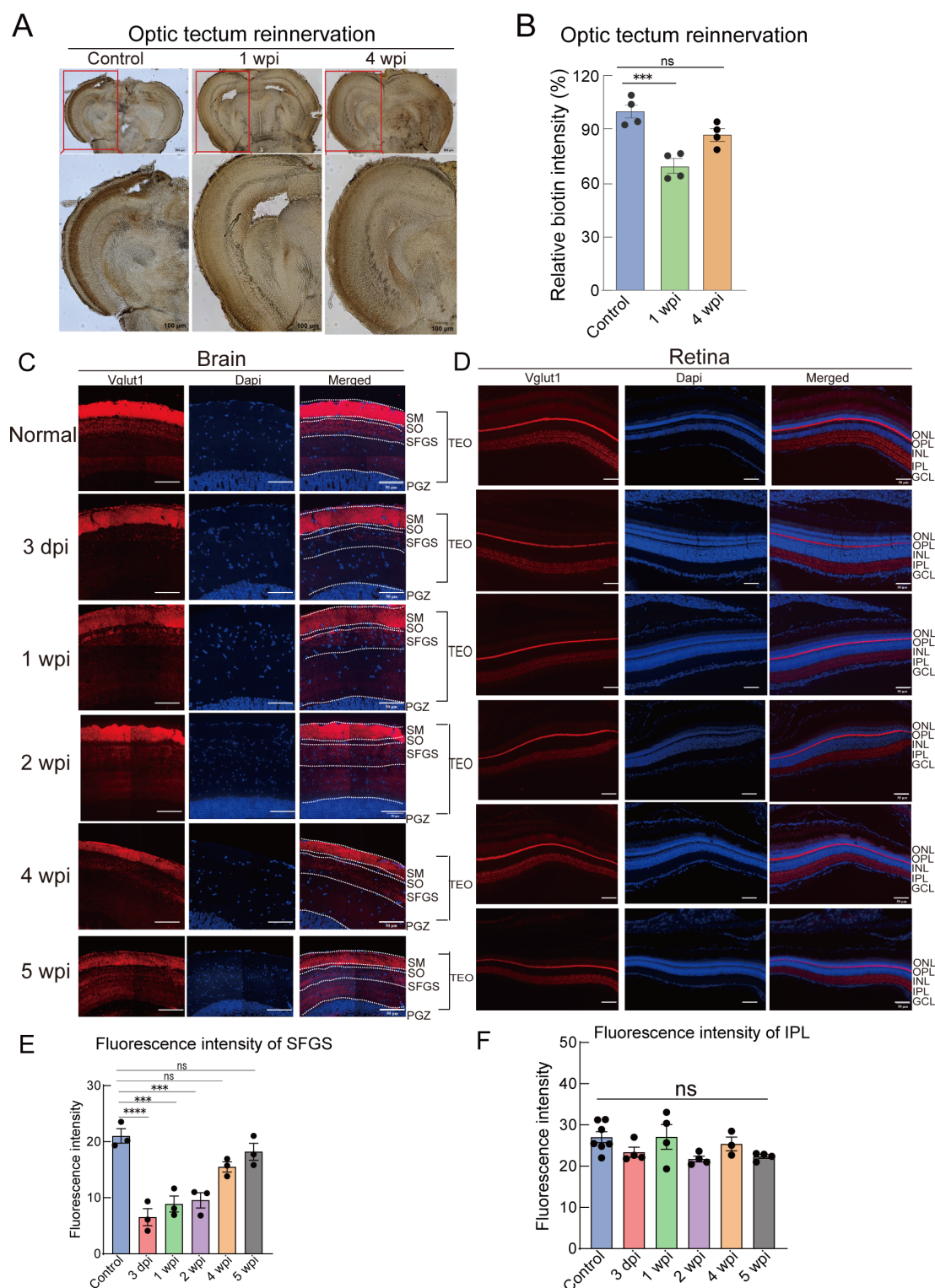


Figure 6 Synaptic remodeling in the eye and brain of zebrafish following ONT

A, B: Optic tectum reinnervation following ONT in zebrafish. A: Representative image showing optic tectum reinnervation. Top panel: optic tectum; bottom panel: magnified view of optic tectum. Scale bar: 200 μ m (top panels) and 100 μ m (bottom panels). B: Quantitative analysis of optic tectum reinnervation in zebrafish following ONT ($n=4$ fish). C: Distribution of synapses at different time points post-injury in the brain. Distribution of synaptic precursors (Vglut1) in various brain regions and their changes at different time points post-injury. Scale bar: 50 μ m. D: Distribution of synapses at different time points after optic nerve injury in the retina. Scale bar: 50 μ m. GCL: Ganglion cell layer, IPL: Inner plexiform layer, INL: Inner nuclear layer, OPL: Outer plexiform layer, ONL: Outer nuclear layer. E: Quantitative analysis of fluorescence intensity in the SFGS region of the optic tectum ($n=4$ fish). Error bars indicate standard error of the mean (SEM). F: Quantitative analysis of fluorescence intensity in the IPL region of the retina. Error bars indicate standard error of the mean (SEM). Control group, $n=7$ fish, other post-injury time points, $n=4$ fish per group. Statistical analysis was conducted using one-way ANOVA followed by Tukey's multiple comparisons test. ns: Not significant; ***: $P<0.001$; ****: $P<0.0001$.

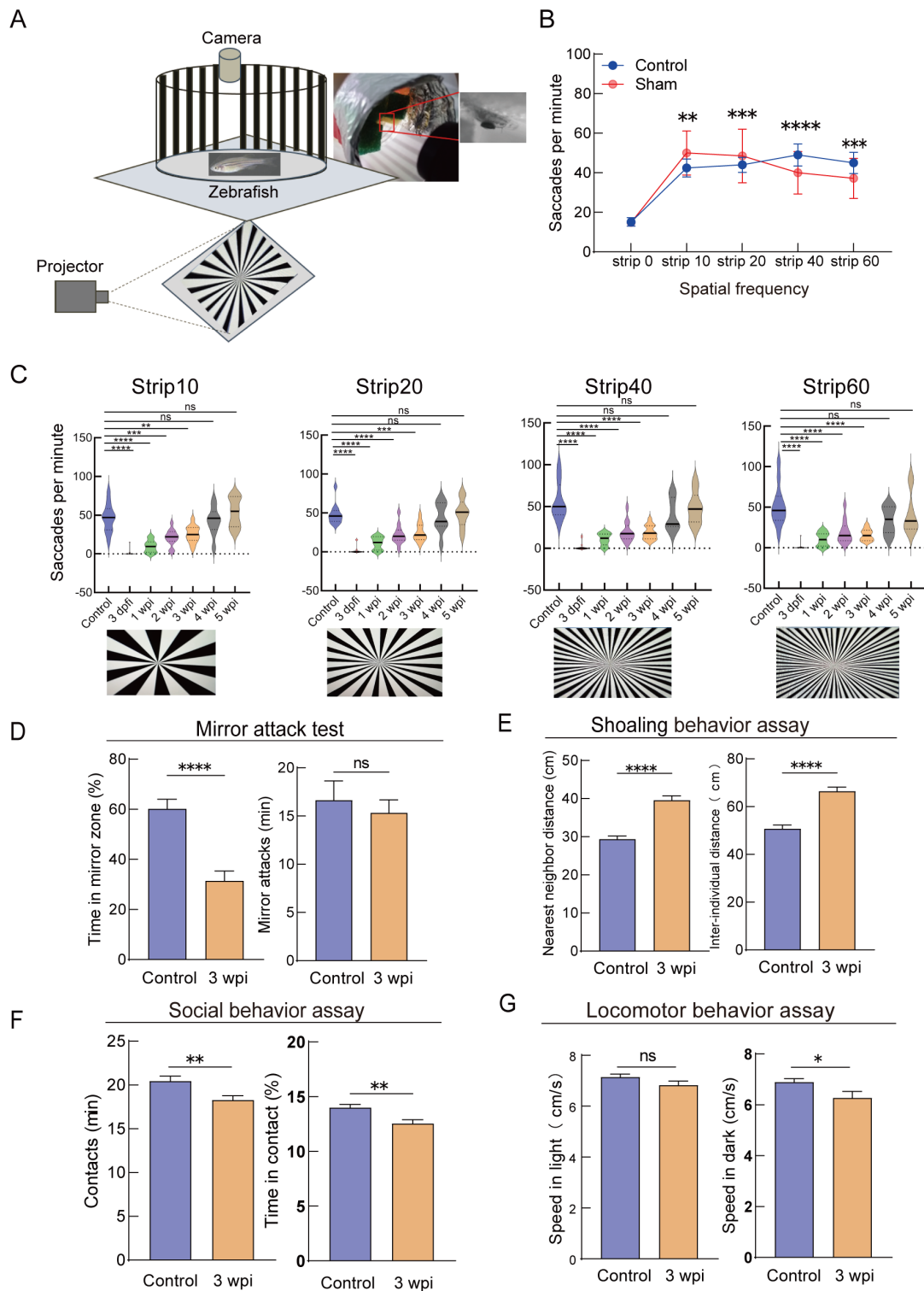


Figure 7 Behavioral assays in adult zebrafish following ONT

A: Experimental apparatus used for OKR measurement in adult zebrafish. During the experiment, the left eye was covered with aluminum foil to block external light interference, and zebrafish were immobilized using a sponge to ensure head stability. A grating pattern was projected onto a cylindrical screen to stimulate the right eye, with eye movements recorded. B: OKR response in uninjured and sham group adult zebrafish. OKR responses of uninjured zebrafish and sham group were evaluated at various grating frequencies (10, 20, 40, and 60 strips). $n=19$ fish for each group. $n=6$ fish for sham group. Error bars indicate standard error of the mean (SEM). C: OKR responses at various time points following ONT: normal fish (uninjured), 3 dpi, 1 wpi, 2 wpi, 3 wpi ($n=12$ fish per group), 4 wpi, and 5 wpi ($n=9$ fish per group). Error bars indicate standard error of the mean (SEM). Statistical analysis was conducted using one-way ANOVA followed by Tukey's multiple comparisons test. ns: Not significant; *: $P<0.05$; **: $P<0.01$; ***: $P<0.001$; ****: $P<0.0001$. D–G: Behavioral assessment at 3 wpi following ONT. D: Mirror attacks ($n=10$ fish) E: Shoaling behavior assay, nearest neighbor distance and inter-individual distance in a group of four fish ($n=2$ trials). F: Social behavior assay, percentage of time in contact ($n=10$ fish for each group) G: Locomotor behavior assay ($n=10$ fish for each group). Statistical analysis was conducted using unpaired t -test. ns: Not significant; *: $P<0.05$; **: $P<0.01$; ***: $P<0.001$.

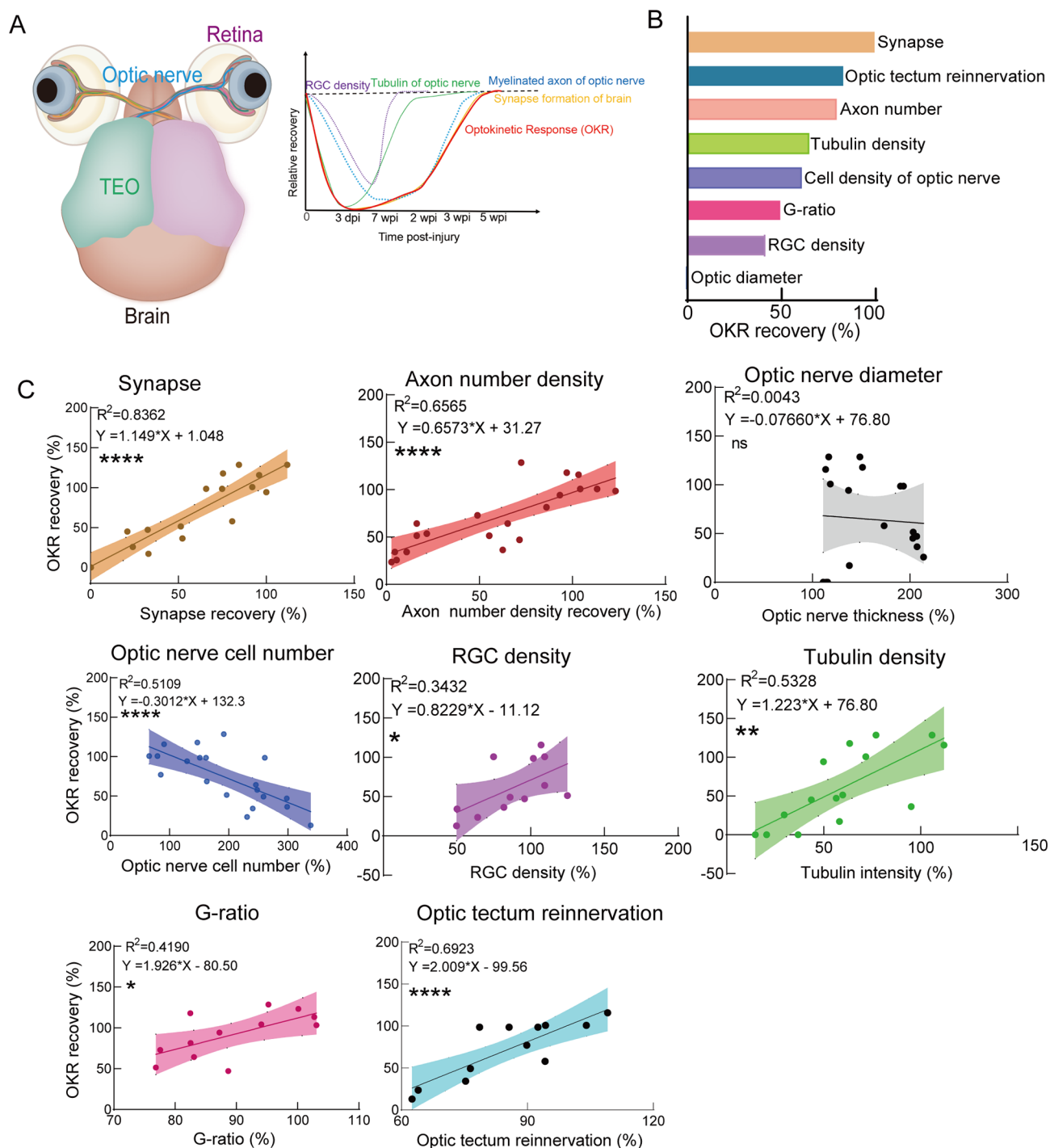


Figure 8 Overview of regenerative process in zebrafish retina, optic nerve, and brain following ONT

A: Left panel: Schematic showing anatomical layout of zebrafish eye, optic nerve, and brain, providing a structural overview of the visual pathway and its components. Right panel: Schematic showing temporal and spatial coordination of regeneration across the retina, optic nerve, and brain post-transection. Diagram highlights sequential recovery process, starting with RGC regeneration in the retina (purple line), followed by optic nerve reconnection (green line) and remyelination (sky blue line) to restore signal conduction, concluding with synaptic restoration in the brain (yellow line) to re-establish visual system connectivity and recover OKR behavior (red line). B: Schematic of correlations between OKR recovery and key factors. X-axis represents percentage recovery of the OKR, y-axis represents contributing factors. Bar height corresponds to each factor's relative contribution to functional recovery, with greater height reflecting larger effect size, as determined by R^2 value from nonlinear regression. C: Correlation analysis of various factors and functional recovery (OKR) during zebrafish visual pathway regeneration. For correlation analysis, all data were normalized to percentage values relative to pre-injury levels. Each parameter was derived from normalized data pooled across at least three zebrafish per group. Post-injury time points were directly aligned with corresponding OKR behavioral measurements to assess recovery dynamics. Correlation analysis was performed using nonlinear regression (curve fitting) in GraphPad Prism. ns: Not significant; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$; ****: $P < 0.0001$.

axonal regrowth was observed, with regenerating axons extending continuously from the retina to the brain, rather than rejoining distal segments of the severed nerve. Following injury, the optic nerve exhibited a significant increase in both diameter and cellular density, with peak changes occurring at 2 wpi. Based on these observations, single-cell sequencing was conducted on the optic nerve at 2 wpi. Single-cell transcriptomic profiling of the optic nerve at this stage showed substantial fibroblast populations. Given prior evidence implicating fibroblasts in tissue regeneration (Hu et al., 2022), their abundance in the injured optic nerve suggests a contributory role in structural restoration. Single-cell sequencing also revealed a low abundance of immune cells, with neither microglia nor macrophages definitely identified; given their scarcity and the ambiguity of marker expression, these cells were collectively categorized as a general immune population. To better evaluate their presence and temporal dynamics, earlier time points were analyzed using immunohistochemistry with Iba1 and L-plastin, markers for microglia and pan-immune cells, respectively. Co-staining revealed a significant increase in microglia and other immune cells during early post-injury time points, with many in a proliferative state. These findings highlight the involvement of immune cells, including microglia, in the early optic nerve regenerative process in zebrafish.

In previous work, Lyons & Talbot (2014) noted a key distinction between CNS of anamniotes (including fish) and amniotes (including mammals), highlighting the apparent absence of stellate astrocytes in the former. Although a few studies have suggested the possible presence of stellate or rounded astrocytes in fish (Alunni et al., 2005; Kawai et al., 2001), the evidence remains inconclusive. To further investigate the presence of astrocytes in zebrafish, the expression of *GFAP*, a classic astrocyte marker, was examined in the optic nerve before and after injury using transgenic zebrafish (*Tg(gfap:GFP)*). Consistent with previous research (Koke et al., 2010), no detectable GFAP signal was observed in the optic nerve at any time point, including 3 dpi and 1 wpi. In conjunction with our immunohistochemical and single-cell transcriptomic data, these results support the conclusion that astrocytes are absent in the zebrafish optic nerve.

Additionally, remyelination was first evident by 3 wpi, likely driven by the proliferative oligodendrocyte populations identified at 2 wpi, including myelin-forming and mature oligodendrocytes. The emergence of newly formed sheaths by 3 wpi indicated active remyelination, although the precise initiation of myelin wrapping could not be captured within the temporal resolution of the current dataset. Higher-resolution temporal analyses, including higher-frequency sampling or live imaging, will be necessary to determine the earliest stages of myelin reformation with greater precision.

Myelin regeneration was similarly dynamic, with significant demyelination occurring in the early stages (1–2 wpi) but near-complete recovery of myelin sheath number and thickness by 5 wpi. These findings emphasize the critical role of remyelination in restoring neural conductivity and functional recovery, consistent with previous research (Münzel et al., 2014). In parallel, synaptic remodeling followed a temporally coordinated trajectory. In the optic tectum, acute synaptic loss at 3 dpi was followed by a gradual recovery, culminating in full restoration by 4–5 wpi. These findings are consistent with those reported in previous research demonstrating synaptic

depletion in the IPL shortly after optic nerve crush (Beckers et al., 2019). Although IPL synaptic density showed a decreasing trend at 3 dpi, this reduction did not reach statistical significance.

Implications for regenerative biology and therapeutic applications

The THEA program aims to enable successful whole-eye transplantation by integrating advanced microsurgical techniques with cutting-edge genetic and cell-based therapies to preserve and regenerate the neural circuits connecting the retina to the brain. Owing to their robust regenerative capacity, zebrafish represent a valuable model for studying optic nerve repair mechanisms that remain limited in mammals. The present findings provide direct evidence for post-transplantation synaptogenesis within the brain, a process critical for the re-establishment of functional visual pathways. These results underscore the importance of synaptic reconnection as a prerequisite for the restoration of primary visual function, offering mechanistic insights for future therapeutic applications.

Limitations and outlook

This study systematically characterized pathological changes and functional restoration across various time points following ONT in adult zebrafish. The OKR assay effectively captured primary visual function, particularly visual acuity. However, while reflexes such as the optokinetic response and dorsal light reflex are useful for an initial evaluation of functional recovery, more advanced techniques, such as electrophysiological recordings (Torres et al., 2024) and complex visual behavior tests (e.g., chasing behavior) (Loring et al., 2020), are necessary for a more comprehensive assessment of visual function.

The present study establishes a detailed spatiotemporal atlas of zebrafish optic nerve regeneration, providing a robust foundation for future multi-omics studies of the entire visual pathway. Integration of transcriptomic data with proteomic and metabolomic analyses may provide a more complete understanding of the molecular pathways involved in neural repair.

CONCLUSION

This study presents a comprehensive spatiotemporal overview of the regenerative process in adult zebrafish following ONT, integrating histological, immunohistochemical, ultrastructural, single-cell transcriptomic, and behavioral analyses. Functional restoration was tightly coupled to axonal reinnervation and synaptic reassembly within the optic tectum. Together, these findings define a coherent temporal cascade of regeneration and establish a foundational framework for future multi-omics exploration of visual pathway repair.

DATA AVAILABILITY

All RNA sequencing data from this project have been deposited in the NCBI database (BioProjectID PRJNA1264696), National Genomics Data Center (CRA025814), and Science Data Bank (<https://doi.org/10.57760/sciencedb.j00139.00220>). All data that support the findings of this study are available from the corresponding author upon reasonable request.

SUPPLEMENTARY DATA

Supplementary data to this article can be found online.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

B.G.S., Y.K.Z., and W.C.W. designed the research; B.G.S., Y.W., H.Y.W., S.J.L., and S.R.H. performed the research; B.G.S., Y.W., S.J.L., S.R.H., G.M.Z., W.T.Y., W.C.W., and Y.K.Z. analyzed the data; B.G.S., Y.W., Y.K.Z., and W.C.W. wrote the paper. All authors read and approved the final version of the manuscript.

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