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# Three-dimensional evaluation of retinal ganglion cell axon regeneration and pathfinding in whole mouse tissue after injury

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ABSTRACT

Injured retinal ganglion cell (RGC) axons do not regenerate spontaneously, causing loss of vision in glaucoma and after trauma. Recent studies have identified several strategies that induce long distance regeneration in the optic nerve. Thus, a pressing question now is whether regenerating RGC axons can find their appropriate targets. Traditional methods of assessing RGC axon regeneration use histological sectioning. However, tissue sections provide fragmentary information about axonal trajectory and termination. To unequivocally evaluate regenerating RGC axons, here we apply tissue clearance and light sheet fluorescence microscopy (LSFM) to image whole optic nerve and brain without physical sectioning. In mice with PTEN/SOCS3 deletion, a condition known to promote robust regeneration, axon growth followed tortuous paths through the optic nerve, with many axons reversing course and extending towards the eye. Such aberrant growth was prevalent in the proximal region of the optic nerve where strong astroglial activation is present. In the optic chiasms of PTEN/SOCS3 deletion mice and PTEN deletion/Zymosan/cAMP mice, many axons project to the opposite optic nerve or to the ipsilateral optic tract. Following bilateral optic nerve crush, similar divergent trajectory is seen at the optic chiasm compared to unilateral crush. Centrally, axonal projection is limited predominantly to the hypothalamus. Together, we demonstrate the applicability of LSFM for comprehensive assessment of optic nerve regeneration, providing in-depth analysis of the axonal trajectory and pathfinding. Our study indicates significant axon misguidance in the optic nerve and brain, and underscores the need for investigation of axon guidance mechanisms during optic nerve regeneration in adults.



## Introduction

Retinal ganglion cells (RGCs) do not regenerate their axons, leading to loss of visual functions in glaucoma and after trauma or stroke. Studies on the limited regenerative capacity of RGCs have identified several strategies that stimulate axon regeneration. These include enhancing the intrinsic growth capacity as well as neutralizing repulsive cues in the environment (Cho et al., 2005; Duffy et al., 2012; Leaver et al., 2006; Lingor et al., 2008; Liu et al., 2006; Muller et al., 2007; Qiu et al., 2002; Su et al., 2008; Winzeler et al., 2011; Wong et al., 2003). We and others have used knockout (KO) mice to demonstrate that RGC-specific deletion of PTEN (phosphatase and tensin homolog), SOCS3 (suppressor of cytokine signaling 3) or KLF4 (Krüppel like factor 4) induces RGC axon regeneration (Moore et al., 2009; Park et al., 2008; Smith et al., 2009). More recent studies have shown that combining PTEN KO mice with either SOCS3 deletion, or Zymosan and a cAMP analogue leads to substantially more regeneration than targeting them individually (de Lima et al., 2012; Kurimoto et al., 2010; Sun et al., 2011), pointing to the importance of targeting multiple factors to induce extensive regeneration.

With the recent progress made in promoting RGC axon regeneration, it is becoming increasingly important to investigate whether regenerating RGC axons can find their targets in the brain. While appropriate pathfinding of RGC axons has been documented comprehensively in regeneration-competent species including fish and amphibians (Beazley et al., 1997; Becker and Becker, 2007; Stelzner et al., 1986), the degree to which RGC axons in adult mammals correctly reinnervate their targets is unclear.

A common method for assessing optic nerve regeneration is histological sectioning. However, tissue sections provide incomplete spatial information. For instance, it is difficult to determine the precise trajectory of axons or their final destinations. In this study, we applied a tetrahydrofuran (THF) based-clearing method that renders tissues relatively transparent (Becker et al., 2012; Erturk et al., 2012), and combined it with LSFM, allowing deep tissue fluorescence imaging in unsectioned optic nerve and brain. Using these methods, we found in PTEN/SOCS3 KO mice that regenerating axons follow circuitous paths, with many axons

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making multiple turns and extending back to the eye. Axon turning was prevalent in nerve regions with strong astroglial activation. Many RGC axons generated branches in the optic nerve and brain as they re-grow. In the optic chiasm, a major decision point en route to visual targets, high numbers of regenerating axons in PTEN/SOCS3 KO mice or PTEN KO/Zymosan/cAMP analogue-treated mice diverge into the ipsilateral optic tract or to the opposite optic nerve. Following bilateral optic nerve crush, a similar growth pattern is seen at the optic chiasm compared to unilateral crush. Centrally, axonal projection is limited primarily to the hypothalamus. In summary, we demonstrated the combined application of tissue clearance and LSFM for comprehensive analysis of optic nerve regeneration, providing in-depth assessment of the axonal trajectory. Our study shows substantial misdirection of RGC axon growth in adult mice, and underscores the need for investigation into the mechanisms that underlie misguidance during regeneration in adults.

## Materials and methods

All experimental procedures were performed in compliance with animal protocols approved by the IACUC at the University of Miami Miller School of Medicine. For all surgical procedures, mice were anaesthetized with ketamine and xylazine. Eye ointment containing atropine sulfate was applied preoperatively to protect the cornea during surgery, and Buprenorphine (0.05 mg/kg, Bedford Lab) was administrated as post-operative analgesic.

### *Mice, optic nerve crush injury and intravitreal injection*

SOCS3$^{f/f}$/PTEN$^{f/f}$ (Sun et al., 2011) mice (female; 4–6 week old) were intravitreally injected with 1–2 μl volume of AAV2-Cre in the left eyes at 2 weeks prior to crush injury. 1 μl (1 μg/μl) ciliary neurotrophic factor (CNTF; Pepro Tech) was intravitreally injected immediately after injury and at 3 days post-injury, and bi-weekly thereafter. PTEN KO/ZYM/cAMP mice, PTEN$^{f/f}$ (Groszer et al., 2001) (female; 8 week old) received intravitreal AAV2-Cre injection followed by optic nerve crush 2 weeks later. Zymosan (Sigma-Aldrich; 12.5 μg/μL) along with the cAMP analogue CPT-cAMP (Sigma; 50 μM, 3 μL) was injected as described (de Lima et al., 2012). Zymosan and CPT-cAMP were injected intravitreally immediately after crush injury, and additional Zymosan at half the original dose plus CPT-cAMP at the original dose again 3 and 6 week later. For each intravitreal injection, a glass micropipette was inserted into the peripheral retina, just behind the ora serrata, and was deliberately angled to avoid damage to the lens. For optic nerve crush injury, the optic nerve was exposed intraorbitally and crushed with jeweler's forceps (Dumont #5; tip dimension, 0.1 × 0.06 mm) for 5 s approximately 1 mm behind the optic disc. Using retrograde and anterograde experiments, completeness of axotomy has been confirmed in our previous study (Park et al., 2008). Two to 7 days before sacrifice, l–2 μl of cholera toxin β subunit (CTB)-Alexa 555 (2 μg/μl, Invitrogen) was injected into the vitreous with a Hamilton syringe (Hamilton) to anterogradely label regenerating RGC axons. *ALDH1L1-EGFP* (Doyle et al., 2008; Yang et al., 2011), *PLP-EGFP* (Mallon et al., 2002) and *CX3CR1-EGFP* mice (Jung et al., 2000) received unilateral optic nerve crush. At 14–17 days later, optic nerves from these transgenic mice were treated for tissue clearance and analyzed for the distribution of glial cells in the injured optic nerve. Uninjured mice (C57BL/6 at 5 week old) with bilateral CTB tracing received intravitreal CTB-555 injection in the right eye and CTB-488 injection in the left eye.

### *AAVs*

cDNA of Cre was inserted downstream of the CMV promoter/β-globin intron enhancer in the plasmid pAAV-MCS (Stratagene), containing the AAV2 inverted terminal repeats and a human growth hormone polyA signal. pAAV-RC (Stratagene) that encodes the AAV2 genes (rep and cap) and the helper plasmid (Stratagene) that encodes E2A, E4 and VA were used for co-transfection of 293T cells to generate recombinant AAV. AAV2 viral particles were prepared by the University of Miami Viral Vector Core using an FPLC method to produce titers of approximately $4 \times 10^{13}$ particles/mL.

### *Tissue preparation and clearing*

Mice were perfused transcardially with PBS followed by 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) at 5 ml/min. The optic nerve and the brain were dissected and post-fixed with 4% PFA in PBS overnight. For histological sectioning, samples were cryoprotected by incubating in 30% sucrose overnight. For tissue clearing, samples were rinsed with PBS and stored at 4 °C until needed. Tissue clearing was performed as described (Becker et al., 2012; Erturk et al., 2012), with minor modifications. Samples underwent dehydration by incubation in increasing concentration of THF (Sigma-Aldrich) solutions under constant rocking. Optic nerves were incubated in 50% THF (diluted in water v/v), 80% THF (v/v) and 100% THF for 15 min each. Dehydrated optic nerve was rendered clear by incubating in BABB (a mixture of benzyl alcohol and benzyl benzoate (Sigma-Aldrich) at a ratio of 1:2) for 20 min. Adult mouse brain was incubated in 50% THF for 12 h, 80% THF for 12 h, 100% THF for 3 × 12 h, and BABB for 12 h before imaging.

### *LSFM (ultramicroscopy)*

Ultramicroscope illuminates specimen with a thin sheet of light formed by two lenses, allowing imaging of large tissues, yet with cellular resolution (Fig. 1C). Ultramicroscopy was performed as previously described (Erturk et al., 2012). Between 100 and 500 optical slices were imaged. The scan speed was 0.5–1.5 s per section, which was about 2–3 min for the optic nerve and 5–10 min for the brain for a complete scan of the tissue. Images were collected at 2 to 5 μm increment in Z axis.

### *Image processing, neurite tracing and statistical analysis*

Images, videos and 3D volume rendering were prepared using Imaris software v7.6.1 (Bitplane). CTB-labeled RGC axons in the optic nerve and brain were traced using Imaris Filament Tracer Module. To quantify the number of axons that regenerated long distances in the optic nerve, we counted the CTB-labeled fibers that crossed different distances from the lesion site after scanning through 100 to 200 individual horizontal optical slices. To quantify the number of axons that extended into different regions beyond the optic chiasm, we counted the CTB-labeled fibers that were found in the optic tracts, opposite optic nerve and hypothalamus after scanning through 100 to 300 individual horizontal optical slices. We used Student's *t* test for two group comparisons using SPSS statistics software.

## Results

### *3D visualization of RGC axonal projections in uninjured mouse*

In this study, we set out to apply tissue clearance and LSFM to evaluate regeneration of RGC axons without histological sectioning. Initially we visualized RGC axonal projections in the whole brain of uninjured animal. Adult mice received intravitreal injection of fluorophore-conjugated CTB, a lipophilic tracer used routinely to label CNS axons in regeneration studies. Seven days later, samples were processed for tissue clearance. THF-based clearing methods rendered mouse optic nerve and brain transparent to a large extent (Figs. 1A and B). A total of 1 h incubation in THF/BABB solutions was sufficient to clear the optic nerve, while it took approximately 3 days for the brain. Normally, RGCs send axons to central targets including the lateral geniculate nucleus (LGN) and superior colliculus (SC), which are