AWARD NUMBER: CDMRPL-21-0-NF210068P1

TITLE: Investigate and Target an Inflammatory Response During NF1-OPG Formation

PRINCIPAL INVESTIGATOR: Wei Li

CONTRACTING ORGANIZATION: National Eye Institute, Bethesda, MD

REPORT DATE: July 2023

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. **PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.**

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
July 2023	Annual	01Jul2022-30Jun2023

4. TITLE AND SUBTITLE

Investigate and Target an Inflammatory Response During NF1-OPG Formation

5a. CONTRACT NUMBER
CDMRPL-21-0-NF210068P1

5b. GRANT NUMBER
NF210068P1

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Dr. Wei Li, Dr. Fransico Nadal-Nicolas, Dr. Wenxin Ma, Dr. Kiyoharu Miyagishima

E-Mail: liwei2@nei.nih.gov

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

National Institutes of Health/National Eye Institute
31 Center Dr. 31/6A16
Bethesda, MD20892

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Pediatric low-grade glioma (pLGG) is the most common brain tumor in children, but rarely occurs in adults. About 14% of pLGGs arise from individuals with neurofibromatosis type 1 (NF1), which frequently develop along the optic pathway and thus are also known as NF1-associated optic pathway gliomas (NF1-OPGs). As the most common tumor in the central nervous system (CNS), OPGs develop in nearly 20% of children with NF1 and are predominately diagnosed in patients younger than 7 years of age with few, if any, adult cases. Thus, both sporadic and NF1-associated pLGGs appear to arise from the transient vulnerability to hyperactive RAS-mediated ERK/MAPK signaling in neural stem and progenitor cells in the developing CNS. While most NF1-OPG patients have excellent survival, approximately 30%, despite conventional chemotherapies, will experience vision loss, highlighting an unmet clinical need for novel therapies. Recent imaging studies have shown that loss of retinal ganglion cells (RGCs) – the only neuronal population connecting the eye to the brain – is associated with vision loss in NF1-OPG patients.

15. SUBJECT TERMS
None listed.

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRDC
U	U	U	UU	20	19b. TELEPHONE NUMBER *(include area code)*

TABLE OF CONTENTS

1. INTRODUCTION

Pediatric low-grade glioma (pLGG) is the most common brain tumor in children, but rarely occurs in adults. About 14% of pLGGs arise from individuals with neurofibromatosis type 1 (NF1), which frequently develop along the optic pathway and thus are also known as NF1-associated optic pathway gliomas (NF1-OPGs). As the most common tumor in the central nervous system (CNS), OPGs develop in nearly 20% of children with NF1 and are predominately diagnosed in patients younger than 7 years of age with few, if any, adult cases. Thus, both sporadic and NF1-associated pLGGs appear to arise from the transient vulnerability to hyperactive RAS-mediated ERK/MAPK signaling in neural stem and progenitor cells in the developing CNS. While most NF1-OPG patients have excellent survival, approximately 30%, despite conventional chemotherapies, will experience vision loss, highlighting an unmet clinical need for novel therapies. Recent imaging studies have shown that loss of retinal ganglion cells (RGCs) – the only neuronal population connecting the eye to the brain – is associated with vision loss in NF1-OPG patients. However, **the mechanism(s) underlying OPG-associated loss of RGCs and vision remains largely unknown**. Using a newly developed NF1-OPG model, we found that Nf1-deficient ($Nf1^{-/-}$) migrating glial progenitors (GPs) abnormally maintained stem/progenitor-like characteristics and were associated with an inflammatory response of increased numbers of $Iba1^{+}$ microglia, and subsequent axonal/myelin degeneration and Bax-mediated apoptosis of RGCs. Accordingly, **we hypothesize that abnormally differentiated $Nf1^{-/-}$ migrating GPs induces abnormal infiltration of microglia, triggering axonal/myelin degeneration and RGC death. Thus, targeting OPG-associated immune responses may provide a novel therapy for NF1-OPG to prevent and treat vision loss**. Specifically, we propose three specific aims to investigate this hypothesis.

Aim 1: To determine the source(s) and phenotypic abnormalities of increased infiltrating $Iba1^{+}$ microglia by $Nf1^{-/-}$ migrating GPs.

Aim 2: To transiently eliminate or immunometabolically modulate microglia as a means to prevent or alleviate OPG-associated nerve injury and RGC death.

Aim 3: To determine the mechanism inducing abnormal microglial infiltration by using the Mosaic Analysis with Double Markers (MADM) model.

2. Keywords

Neurofibromatosis type 1 (NF1), Optic pathway glioma (OPG), glial progenitors (GPs), microglia, macrophage, retinal ganglion cells (RGCs), visual impairment, RAS, MEK, ERK/MAPK signaling pathway, MADM (Mosaic Analysis with Double Markers).

3. Accomplishments

- **What were the major goals of the project?**
    - *List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project identify these dates and show actual completion dates or the percentage of completion.*

Aim 1: To determine the source(s) and phenotypic abnormalities of increased infiltrating Iba1^{+} microglia by *Nf1*$^{-/-}$ migrating GPs.

Major Task 1: Characterize microglia during OPG initiation and progression.

Subtask 1: To submit for local institutional animal care and use committee (IACUC) and USAMRDC Animal Care and Use Review Office (ACURO) approval (Months 1-3)**.**

Subtask 2: Develop and expand the hGFAP-cre;*Nf1*flox/flox;Rosa26td-TOM;CX3CR1-eGFP (*Nf1*hGFAPCKO) mouse model (Months 4-12)**.**

Subtask 3: Detection of abnormal microglia during NF1-OPG initiation (Months 7-12)**.**

Subtask 4: Investigation of immune cell abnormalities in NF1-OPG models (Months 7-18)**.**

Aim 2: To transiently eliminate or immunometabolically modulate microglia as a means to prevent or alleviate OPG-associated nerve injury and RGC death.

Major Task 2: Modulating microglia to prevent and inhibit OPG.

Subtask 1: To transiently ablate microglia populations during the postnatal stages to inhibit NF1-OPG formation and vision loss (Month 18-30).

Subtask 2: To immunometabolically modulate microglia during the postnatal stages for inhibiting NF1-OPG formation (Month 24-36).

Aim 3: To determine the mechanism inducing abnormal microglial infiltration by using the Mosaic Analysis with Double Markers (MADM) model.

Major Task 3: Understanding induction of microglia.

Subtask 1: To generate and expand the MADM-WT and MADM-Nf1 colonies (Month 6-12).

Subtask 2: To correlate the stem/progenitor status of *Nf1*$^{-/-}$ cells with OPG phenotypes (Month 13-24).

Subtask 3: To correlate the stem/progenitor status of *Nf1*-/- cells with OPG phenotypes (Month 25-36).

- **What was accomplished under these goals?**
    - *For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.*

Major Task 1: Characterize microglia during OPG initiation and progression.

Subtask 1 by the labs of Yuan Zhu and Wei Li (100% of completion):

To submit for local institutional animal care and use committee (IACUC) and USAMRDC Animal Care and Use Review Office (ACURO) approval (Months 1-3)**.**

Zhu Lab

Zhu lab revised its animal protocol, submitted to the Institutional Animal Care and Use Committee (IACUC) and got approved for the proposed experiments on April 12, 2022.

Li Lab

The protocol (NEI-606) for Li lab was granted a three-month extension when it was set to expire on 7-13-2023. It has been reviewed and revised, currently pending approval from IACUC. The experiments for this grant have been ceased since the ACURO did not recognize the extension and resume once the new protocol is approved.

Subtask 2 by the labs of Yuan Zhu and Wei Li (20% of completion): Develop and expand the hGFAP-cre;*Nf1*flox/flox;Rosa26td-TOM;CX3CR1-eGFP (*Nf1*^{hGFAP}CKO) mouse model (Months 4-12)**.**

Zhu and Li Lab

Zhu lab encountered several unexpected challenges. Dr. Wang Zheng and Mr. Matt Krause, the two main researchers on this grant, left the lab in August 2022 and January 2023, respectively. Immediately after Matt Krause left, our mouse colonies had pinworm infection and were required for treatment for three months. From January to March 2023, no breeding was allowed, as all newborn pups were required for euthanasia during pinworm treatment.

Experimentally, Zhu lab took two approaches to overcome the challenges. First, we replaced all the existing breeding cages with young breeders after the completion of pinworm treatment at the beginning of April 2023. Second, we established a protocol for a commercial company, Transnetycs, to outsource the genotyping of our NF1-OPG related colonies. Using the Transnetycs service, Li lab has expanded the NF1-OPG-related colonies at the NIH/NEI campus. Now both Zhu and Li labs are in the process of generating and monitoring a large number of control and mutant mice for the NF1-OPG colonies. We are ready to introduce the CX3CR1-eGFP reporter and perform the proposed experiments in Year 2.

Subtask 3 by the labs of Yuan Zhu and Wei Li (100% of completion): Detection of abnormal microglia during NF1-OPG initiation (Months 7-12)**.**

Li and Zhu Labs

Li lab analyzed multiple cohorts of control and NF1-OPG model mice by THREE levels of investigation.

1. Visual/behavioral assay:

Littermate control and NF1-OPG model mice were subjected to a visual and behavioral assay, known as optomotor response (OMR), which estimates visual acuity by measuring the correct versus incorrect head movement in response to light stimulus.

2. Imaging/anatomical assay:

A non-invasive imaging method or optical coherence tomography (OCT) was employed to measure the thickness of the retinal nerve fiber layer (RNFL) and ganglion cell complex (GCC) as an indirect way to infer tumor-induced loss of axons of retinal ganglion cells (RGCs).

3. Histological assay:

After the completion of these two non-invasive assays, control and NF1-OPG model mice were sacrificed and processed for histological analysis of the optic nerves and retinas.

Results

Using multiple statistical methods, Zhu lab was able to divide NF1-OPG model mice into two groups, visually normal and abnormal. As compared with the control littermates, 30 of 48 (62.5%) NF1-OPG model mice exhibited a significant reduction of OMR-estimated visual activities ($p < 0.0001$), while the remaining 37.5% of

mutant mice had almost normal vision ($p = 0.8634$) (Figure 1A). Of note, a subset of our NF1-OPG model mice had more severe neurological deficits required for euthanasia at earlier ages, such as 1 month. All age groups analyzed ranging from 1 month to 5 months exhibited similar OMR-estimated visual activities with two functionally distinct, normal and impaired groups. This observation suggests that visual abnormalities in our NF1-OPG model are independent of other neurological abnormalities and premature lethality, but instead a tumor-dependent phenotype.

When the same groups of control and mutant mice were subjected to OCT, all NF1-OPG model mice exhibited a significant reduction of OCT-inferred retinal thickness in the center and medial, but not peripheral region, which is consistent with centrally and medially tumors in this model (Figure 1B and 1C). As a positive control for this imaging assay, mice with the retinal degeneration 1 (rd1) mutation showed a uniform reduction of OCT-inferred retinal thickness due to degeneration of photoreceptors (Figure 1B and 1C). Surprisingly, the OCT-inferred retinal thickness was equally reduced between OMR-estimated normal and impaired groups of the NF1-OPG model mice (Figure 1C). These results suggest that OPG-induced axonal loss of RGCs is not sufficient to impair OMR-estimated visual activities, and thus other factors, such as immune cells as hypothesized by our central hypothesis, can play an active role in visual impairment.

We next investigated how histopathological abnormalities in the optic nerves (ONs) impact on the OCT-inferred axonal loss and OMR-estimated visual impairment among these NF1-OPG model mice. We used three criteria, (1) affected ON volume (Figure 3A), (2) total cell number and (3) cellular density in the affected areas (Figure 1D). Although the volume of affected nerves was only marginally increased in the NF1-OPG model mice compared to control mice ($p = 0.045$) (left panel, Figure 1D), total cell number and cellular density were both markedly increased in mutant nerves (mid and right panels, Figure 1D). Importantly, the abnormalities in the affected optic nerves are indistinguishable between the two visually and functionally distinct groups (Figure 1D). These results are consistent with our recent publication (Jecrois et al. Developmental Cell 2021) that this NF1-OPG model has a complete tumor penetrance, which consequently damages and causes loss of RGC axons (inferred by OCT here).

Despite all developing OPGs and exhibiting RGC axonal loss, then why only 67.5% of mutant mice exhibit OMR-estimated visual and behavioral abnormalities. We quantified the number of Iba1+ microglia and CD68+ reactivated microglia. Consistent with the fact that CD68 is not expressed in the normal optic nerve, little or no Iba1+CD68+ microglia were observed in the control mice (Figure 1E). In contrast, up to 100-200-fold increases of reactivated microglia were observed in the optic nerves of the NF1-OPG model mice, though a subset of mice did show a relatively mild increase (Figure 1E). Of note, the levels and variations of increased reactivated microglia were not significantly different between visually/functionally normal and impaired mutant mice (Figure 1E). We further used two additional ways by quantifying the density and frequency of reactivated microglia in all microglia (Figure 1E) but failed to identify the difference between these two visually/functionally distinct groups.

Finally, we used a flat-mount preparation of retinas and measured the number of RGCs using Brn3 immunofluorescent staining. Using two criteria, including Brn3 signal and RGC density, we detected a reduction in all NF1-OPG model mice. Most importantly, the visually and functionally impaired mutant groups exhibited a significantly more severe loss of RGCs than those with normal vision (Figure 1F). These results suggest that the severity of OPG-induced RGC loss is a potential mechanism underlying visually/functionally normal and impaired groups.

Subtask 4 by Shih, Li and Zhu labs (10% of completion): Investigation of immune cell abnormalities in NF1-OPG models (Months 7-18)**.**

Shih, Li and Zhu labs

Zhu and Shih labs started pilot experiments to isolate single cells from normal and OPG-bearing optic nerves. Unfortunately, these experiments failed to produce sufficient single-cell-derived RNAs for genomic studies. To test the single-cell RNA-sequencing (sc-RNA-seq) protocol, we used FACS to isolate CD45-negative tumor cells and CD45-positive immune cells from a mouse malignant peripheral nerve sheath tumor (MPNST), which provided sufficient materials for sc-RNA-seq. We isolated 8,433 and 3,082 CD45-negative and CD45-positive cells, which respectively produced 12,464 mean reads and 1,545 median genes per cell versus 46,867 mean reads and 2,940 median genes per cell. Notably, nearly 73% of CD45-positive immune cells are tumor-

associated macrophages, which is consistent with what we observed using a flow cytometry (FACS)-based immune profiling analysis.

Further, Li and Shih labs have performed optic nerve crush (ONC) experiments to develop FACS and single-cell-based RNA and ATAC sequencing (Multiome) strategies, particularly focusing on immune cells including microglia ($CD45^{intermediate}$ $CD11b^{high}$) and other myeloid ($CD45^{hi}CD11b^{high}$) and lymphoid ($CD45^{hi}CD11b^{intermediate/low}$) lineages (Figure 2A-B). In the ONC model, we have successfully identified infiltrated immune cells at different time point after surgery (Figure 2C). The changes in cellular proportion and their functionality were further validated and determined by computational analysis of sc-RNAseq results (Figure 2D). In Year 2, we will try to isolate Nf1-/- red and sibling Nf1+/+ green cells as well as CD45+ immune cells for bulk and single-cell based RNA and ATAC sequencing (see Major Task 3). Additionally, we have acquired MERSCOPE technology from Vizgen Inc., enabling us to investigate spatial transcriptomic alterations during disease progression and to monitor treatment outcomes. Li Lab has conducted preliminary tests on this equipment, as detailed in his report. We have also developed a set of probes specifically designed for MERSCOPE, utilizing the RNAseq data previously established in our laboratory for studying neurodegenerative diseases. This method will be implemented in our research involving NF1-OPG models to assess the extent of neuroinflammation and cellular interactions.

In summary, we have validated our recent findings (Jecrois et al. Developmental Cell 2021) using completely different cohorts of our NF1-OPG model mice housed at the NIH/NEI campus. Of note, 100% of the NF1-OPG model mice analyzed, regardless of their ages and health conditions, exhibited robust tumor formation in the optic nerves, loss of RGC axons (inferred by OCT). Most importantly, immune responses by reactivated microglia – this phenotype serves as the foundation of this project. It should be also noted that we made some unexpected observations -- only 67.5% of mutant mice exhibited impaired vision estimated by the OMR assay. The more severe loss of RGCs observed in the visually impaired group than those with normal vision suggest a potential threshold of RGC loss leading to visual impairment (see more discussions below).

Major Task 2: Modulating microglia to prevent and inhibit OPG.

Subtask 1 by the labs of Wei Li and Yuan Zhu (5% of completion): To transiently ablate microglia populations during the postnatal stages to inhibit NF1-OPG formation and vision loss (Month 18-30).

Subtask 2 by the labs of Wei Li and Yuan Zhu (5% of completion): To immunometabolically modulate microglia during the postnatal stages for inhibiting NF1-OPG formation (Month 24-36).

This Aim will be mainly carried out in from Year 2. We have expanded the NF1-OPG colonies and ready for the proposed experiments. As a preliminary study, Li lab translated the ground squirrel results to the mouse model of optic nerve crush, demonstrating that manipulating succinate dehydrogenase (SDH) activity using SDH inhibitor, we can improve RGC survival even when the drug was applied post-injury (Figure. 3).

Aim 3: To determine the mechanism inducing abnormal microglial infiltration by using the Mosaic Analysis with Double Markers (MADM) model.

Major Task 3: Understanding induction of microglia.

Subtask 1 by Zhu lab (50% of completion): To generate and expand the MADM-WT and MADM-Nf1 colonies (Month 6-12).

Zhu lab

Due to the unexpected challenges described above, we did not start to expand our MADM colonies until the beginning of April 2023. However, we analyzed a cohort of hGFAP-cre-driven MADM-WT and MADM-Nf1 mice at advanced ages (> 6 months). Specifically, we used FACS to investigate the number of green and red fluorescent cells from multiple regions of the central nervous system (CNS), particularly, the two regions associated with OPG formation, optic nerve and hypothalamus. In our recent publication, we found that the conventional NF1-OPG model mice with the genotype of hGFAP-cre;$Nf1^{flox/flox}$ underwent initial cellular expansion in the neonatal stages after migrating GPs arrive at the distal region of the optic nerve (Jecrois et al. Developmental Cell 2021). When this model was constructed into the MADM background, cellular expansion measured by the ratio of red to green cells (R/G) was observed for nearly 10 folds and became plateau after development (postnatal 30 or P30) in both optic nerve and hypothalamic regions, respectively (Figure 4A and 4B). As a critical control, the R/G ratio in the MADM-WT mice remained relatively constant at 1.0 (Figure 4A

and 4B). Importantly, we validated that hGFAP-cre was not expressed in the immune system, as no Cre activity was detected in the spleen (Figure 4C). Of note, hGFAP-cre activity was detected in the peripheral nervous system, particularly trigeminal nerves, though minimally in sciatic nerves (Figure 4D). Together, both conventional and MADM-based hGFAP-cre driven NF1-OPG models are of developmental origin and undergo tumorigenesis in the developing optic nerve, recapitulating the pediatric nature of human NF1-OPGs.

Subtask 2 by Zhu and Shih labs (5% of completion): To correlate the stem/progenitor status of *Nf1*$^{-/-}$ cells with OPG phenotypes (Month 13-24).

Subtask 3 by Zhu and Shih labs (5% of completion): To correlate the stem/progenitor status of *Nf1*-/- cells with OPG phenotypes (Month 25-36).

Three labs will work together to establish the protocols using FACS to isolate Nf1-/- red and sibling Nf1+/+ green cells as well as CD45+ immune cells for bulk and single-cell based RNA and ATAC sequencing.

- **What opportunities for training and professional development has the project provided?**
    - *If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report."*
    - *Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. "Training" activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. "Professional development" activities result in increased knowledge or skill in one's area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.*

Zhu Lab

Although this project was not designed to focus on training and professional developmental opportunities for the researchers, we have tried to explore the unique opportunity to train new members in the lab. From January 2023, Zhu lab started to have undergraduate students working in the lab and prepare them for medical and graduate school application in the future. Over the past summer, we hosted a total of five undergraduate students as research interns from the University of Maryland at College Park, Johns Hopkins University, Georgetown University and Georgia Institute of Technology. Zhu lab used our weekly lab meeting as a platform to introduce and discuss our disease studies, particularly in the context of NF1.

Li Lab

Li lab has recruited a new post-bac student, Maxell Foote and will train him to work on this project.

Shih Lab

A new postbac student, Michael Liew, who possesses a strong background in computational skills, will receive training in data analysis under the guidance of our current postdoctoral researcher, Dr. Jaanam Gopalakrishnan, who will be conducting the scRNAseq experiment.

- **How were the results disseminated to communities of interest?**
    - *If there is nothing significant to report during this reporting period, state "Nothing to Report."*
    - *Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest* ***in learning and careers in science, technology, and the humanities.***

Dr. Zhu recently joined the Visual Restoration Initiative (VRI) sponsored by the Gilbert Family Foundation (GFF), which is comprised of over 10 labs from leading universities, including Harvard, Stanford, Johns Hopkins, Washington University at St Louis, University of Michigan and University of Washington at Seattle. Most of the VRI investigators are not in the NF1 field but have the expertise in retinal biology and blinding diseases. We will work with VRI investigators to develop novel therapies to protect neuronal loss and enhance neuronal functions using our NF1-OPG model. We are also working with the GFF to disseminate our results to the NF communities and improve the awareness of detecting visual impairment in young children and developing preventive and early interventional therapies.

- **What do you plan to do during the next reporting period to accomplish the goals?**
  - *If this is the final report, state "Nothing to Report."*
  - *Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.*

Mouse colonies:

1. We will continue to optimize the breeding strategies and expand the NF1-OPG colonies in both Children's National and NIH/NEI campuses. Particularly, we will focus on introducing the CX3CR1-eGFP reporter into the NF1-OPG model background and accelerate the progress for the experiments proposed in Task 1.

2. We will monitor the output of the NF1-OPG colonies at the NIH/NEI and start the experiments to target microglia for the treatment experiments proposed in Task 2.

3. We will try to expand the colonies for the new MADM-Nf1 model and prepare them for the experiments proposed in Task 3.

Assay development:

1. The OCT used for our preliminary studies is designed for humans and thus does not have sufficient resolution to measure the retinal nerve fiber layer (RNFL), which is comprised of the axons of RGCs. We had to measure the thickness of combined RNFL and ganglion cell complex (GCC) in order to detect the difference between control and mutant retinas. Li lab purchased a visible-light OCT, which will provide sufficient resolution to detect the reduction only in the RNFL and to infer loss of RGC axons.

2. Li lab purchased MERSCOPE (Vizgen Inc.) which will allow probing spatial transcriptomic changes during the disease progress and monitoring treatment outcomes. We went through several rounds of testing experiments to optimize detection efficiency and resolution (Figure. 5).

3. Three labs will work together to optimize the protocols to perform single-cell RNA and ATAC sequencing from normal and OPG-bearing optic nerves.

4. IMPACT: *Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:*

- **What was the impact on the development of the principal discipline(s) of the project?**
    - *If there is nothing significant to report during this reporting period, state "Nothing to Report."*
    - *Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).*

NF1-OPG is a benign tumor both in mouse models and human patients. One of the challenges in the field is that a widely used NF1-OPG model has little or no loss of RGCs, leading to a disconnection between tumorigenesis in the optic nerve and retinal abnormalities. Given that visual impairment is the major clinical outcome of this benign tumors in humans, it is of particular significance that our NF1-OPG model exhibits robust loss of RGCs in two different animal facilities (Children's National Hospital and NIH). The consistent retinal abnormalities in our NF1-OPG model will establish its utility as a model for more common blinding diseases caused by degeneration of RGCs.

Vision loss is the major clinical outcome for patients with NF1-OPG. Nearly 70% of our NF1-OPG model mice exhibit OMR-estimated visual impairment, providing a valuable platform to test novel therapies for preventing and treating this tumor-associated blinding disease.

- **What was the impact on other disciplines?**
    - *If there is nothing significant to report during this reporting period, state "Nothing to Report."*
    - *Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.*

    Many blinding diseases, such as glaucoma, are caused by degeneration of RGCs, though the underlying mechanisms are often not well understood. We expect that this collaborative team, particularly two retina disease experts at the NIH/NEI campus, will extend our NF1-OPG disease model into more common eye diseases.

- **What was the impact on technology transfer?**
    - *If there is nothing significant to report during this reporting period, state "Nothing to Report."*

- *Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:*
    - *transfer of results to entities in government or industry;*
    - *instances where the research has led to the initiation of a start-up company; or*
    - *adoption of new practices.*

“Nothing to Report”.

- **What was the impact on society beyond science and technology?**
    - *If there is nothing significant to report during this reporting period, state "Nothing to Report."*
    - *Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:*
        - *improving public knowledge, attitudes, skills, and abilities;*
        - *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
        - *improving social, economic, civic, or environmental conditions.*

“Nothing to Report”.

5. CHANGES/PROBLEMS: *The Project Director/Principal Investigator (PD/PI) is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:*

- **Changes in approach and reasons for change**
    - *Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.*

"Nothing to Report"

- **Actual or anticipated problems or delays and actions or plans to resolve them**
    - *Describe problems or delays encountered during the reporting period and actions or plans to resolve them.*

Zhu Lab

Personal changes have been a major challenge during this reporting period. Two main researchers initially assigned to work on the grant, Dr. Wang Zheng and Mr. Matt Krause, left the lab two and six months after the grant started, respectively. We had to mobilized two senior researchers to train several new lab members as well as a few undergraduate researchers to work on the project.

Li Lab

Recruiting proper scientists to join the project remains a major challenge, but we have a new postbac student just joined us.

Shih Lab

Two main researchers initially assigned to work on the grant, Dr. Chunhong Liu and Dr. Nilisha Fernando, left the lab last year. However, we have a postdoc Dr. Jaanam Gopalakrishnan who joined last year and a new postbac student Michael Liew who just joined us. These two scientists will be trained to perform single-cell RNA-seq and ATAC-seq analysis.

- **Changes that had a significant impact on expenditures**
    - *Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.*

“Nothing to Report”.

- **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**
    - *Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.*
- **Significant changes in use or care of human subjects**
- **Significant changes in use or care of vertebrate animals.**
- **Significant changes in use of biohazards and/or select agents**

Our mouse colonies were treated for pinworm infection from January to March 2023. As the treatment was delivered via mouse food, all newborn pups would not receive any drugs for three weeks before weaning. We were required to euthanize all newborn pups for three months, which significantly delayed our experiments. We are in the process of recovering mouse colonies at the Children’s National Hospital as well as establish a backup colony in the Dr. Wei Li’s lab at the NIH/NEI campus.

6. PRODUCTS: *List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."*

- **Publications, conference papers, and presentations**

  Report only the major publication(s) resulting from the work under this award.

  - **Journal publications.** *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).* Yes

Publications:

1. Milde, T., Fangusaro, J., Fisher, M.J., Hawkins, C., Rodriguez, F.J., Tabori, U., Witt, O., **Zhu, Y**. and Gutmann, D.H. (2023). Optimizing preclinical pediatric low-grade glioma models for meaningful clinical translation, *Neuro-Oncology*. 2023 Nov 2;25(11):1920-1931. doi: 10.1093/neuonc/noad125. PMID: 37738646.

  - **Books or other non-periodical, one-time publications.** *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: Author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

  "Nothing to Report"

  - **Other publications, conference papers, and presentations.** *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national,*

local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.

Meeting presentations by Yuan Zhu:

Seminar presentation:

International:

1. "Investigate Pathogenesis of and Develop Novel Therapies for NF1-associated Tumors", Shanghai Neurofibromatosis Forum, the 22nd Shanghai International Congress on Plastic & Aesthetic Surgery and the 12th Forum on Plastic & Aesthetic Surgery, Shanghai, China, June 10, 2023.
2. Investigating Novel Therapeutic Strategies for Brain Tumors in Children and Adults, Key Laboratory of Systems Biomedicine, Ministry of Education, Shanghai Center for Systems Biomedicine, Shanghai JiaoTong University, Shanghai, China, June 12, 2023.

Notes: I only presented the data from the published work, while trying to use the cartoon to illustrate unpublished observations for international presentations.

National:

1. "Investigating MEK inhibition and resistance in the treatment of nervous system tumors". Georgetown-Lombardi Oncology Grand Rounds/Visiting Professor Program, Georgetown-Lombardi Comprehensive Cancer Center, Georgetown University, Washington DC, February 17, 2023.
2. "Investigating and Targeting Tumors in the Nervous System". Greehey Children's Cancer Research Institute, University of Texas Health San Antonio. March 24, 2023.

- **Website(s) or other Internet site(s)**

  List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

"Nothing to Report".

- **Technologies or techniques**

  Identify technologies or techniques that resulted from the research activities. In addition to a description of the technologies or techniques, describe how they will be shared.

  "Nothing to Report".

- **Inventions, patent applications, and/or licenses**

  Identify inventions, patent applications with date, and/or licenses that have resulted from the research. State whether an application is provisional or non-provisional and indicate the application number. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

"Nothing to Report".

- **Other Products**

  Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment, and/or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:

  - *data or databases;*
  - *biospecimen collections;*
  - *audio or video products;*
  - *software;*
  - *models;*
  - *educational aids or curricula;*
  - *instruments or equipment;*
  - *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
  - *clinical interventions;*
  - *new business creation; and*
  - *other.*

-

1. We have developed a series of genetically engineered mouse (GEM) models for NF1-OPG and are working with the Jackson Lab to distribute into the research community.
2. Biospecimen from different stages of NF1-OPG have been collected for future research.
3. We have established behavioral, anatomical and histological assays to measure OPG-induced axonal degeneration, RGC loss, and visual impairments.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

- **What individuals have worked on the project?**
    - *Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change."*

Zhu Lab

1. Yuan Zhu (PD/PI), no change.
2. Yiwei Wang (researcher), no change. Of note, Dr. Wang was absent from May 29 to July 31, 2023 for vacation and visa application-related delay in China.
3. Tingying Jia (researcher), no change.
4. Heather Gordish -Dressman (collaborator, statistician), no change.

Personnel changes:
Wang Zheng (researcher) left the lab on August 26, 2022.
Matt Krause (researcher) left the lab on January 6, 2023.

Li Lab

1. Wei Li (PD/PI), no change.

Personnel changes: Maxwell Foote (postbac) joined the lab in March, 2023.

Shih Lab

1. Han-Yu Shih (PI) – no change
2. Michael Liew (postbac) joined the lab in June, 2023.
3. Dr. Jaanam Gopalakrishnan joined the lab in Jan, 2022.
4. Dr. Chunhong Liu left the lab in Jan, 2022.
5. Dr. Nilisha Fernando left the lab in Sep, 2022.

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**
    - *If there is nothing significant to report during this reporting period, state "Nothing to Report."*
    - *If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported*

previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

PI/PD, Dr. Yuan Zhu has received grant funding from a Gilbert Family Foundation grant in 2023.

The Gilbert Family Foundation Award 2023 (PI: Zhu). 2/1/2023 – 1/31/2026
Level of Effort: 10%
Source: The Gilbert Family Foundation
Annual Direct Costs: $350,000
Title of Project: Development and characterization of genetically engineered mouse models for NF1-associated OPG
Project Goal: The main objective of this proposal is to develop and characterize genetically engineered mouse models for NF1-associated optic pathway glioma and provide research support for the Gilbert Family Foundation-sponsored Visual Restoration Initiative.

- **What other organizations were involved as partners?**
    - *If there is nothing significant to report during this reporting period, state "Nothing to Report."*
    - *Describe partner organizations - academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) - that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.*

      Provide the following information for each partnership:
        - **Organization Name: National Institutes of Health**
        - **Location of Organization:** (Bethesda, Maryland)
        - **Partner's contribution to the project** *(identify one or more)*
            - **Financial support (No);**
            - **In-kind support** *(e.g., partner makes software, computers, equipment, etc., available to project staff)*; **(Yes)**
            - **Facilities** *(e.g., project staff use the partner's facilities for project activities)*; **(Yes)**
            - **Collaboration** *(e.g., partner's staff work with project staff on the project)*; **(Yes)**
            - **Personnel exchanges** *(e.g., project staff and/or partner's staff use each other's facilities, work at each other's site)*; and **(Yes)**
            - **Other.**

- **Notes:** The collaborative details with the NEI/NIH are provided by Drs. Wei Li and Han-Yu Shih in their support letters.

8. SPECIAL REPORTING REQUIREMENTS

- **COLLABORATIVE AWARDS**: *For collaborative awards, independent reports are required from BOTH the Initiating PI and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to https://ebrap.org for each unique award.*
- **QUAD CHARTS:** *If applicable, the Quad Chart (available on https://www.usamraa.army.mil) should be updated and submitted with attachments.*

Collaborating/Partnering PIs, Drs. Wei Li and Han-Yu Shih will submit their progress reports via https://ebrap.org.

9. APPENDICES: *Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc. Reminder: Pages shall be consecutively numbered throughout the report.* ***DO NOT RENUMBER PAGES IN THE APPENDICES.***

1. **Five figures described in the text.**
2. **Figure legends.**

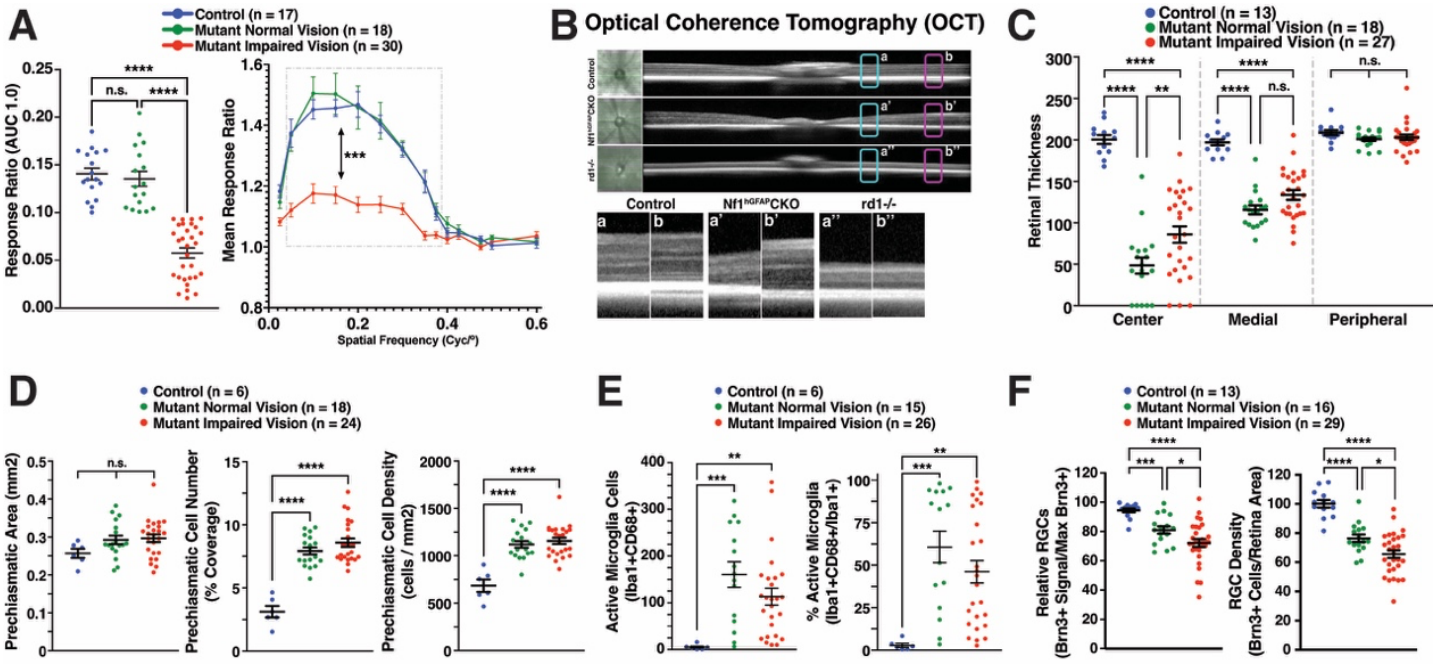


Figure 1. Optomotor response (OMR) of NF1-OPG mice identifies mutants with impaired vision and corresponding the severity of RGC loss. (A) Mouse vision was assessed by optomotor response (OMR) testing platform, and the ratio of the correct versus incorrect head movement plotted over stimulus spatial frequency in control and Nf1^{hGFAP}CKO (*Nf1*$^{-/-}$) mice. The average ratio of the correct vs the incorrect head movement was computed, and mutants were assigned to either normal or impaired vision groups. **(B-C)** Optic coherence tomography (OCT) images (**B**) of a control, Nf1^{hGFAP}CKO, and retinal degeneration 1 (*rd1*$^{-/-}$, as a positive control for axon loss) mouse show retinal thickness in parapapillary and peripheral regions, and analysis of retinal thickness in mutants with normal and impaired vision (**C**) develop similar reduction at the center and medial retina. **(D-E)** Investigation of the prechiasmatic region for enlargement (**D**, left), cell number (**D**, center), and cell density (**D**, right) found comparable outcomes in mutants independent of visual impairment. Likewise, axonal loss-associated activation of microglia (**E**) was observed in both mutants with normal and impaired vision. **(F)** Quantification of Brn3a-positive RGCs in whole-mount retina of control and Nf1^{hGFAP}CKO mice demonstrated a direct association between RGC loss and visual impairment. Charts show mean ± SEM and analysis by unpaired, two-tailed Student's t-test. Significance: * (<0.05), ** (<0.01), *** (<0.001) ****(<0.0001).

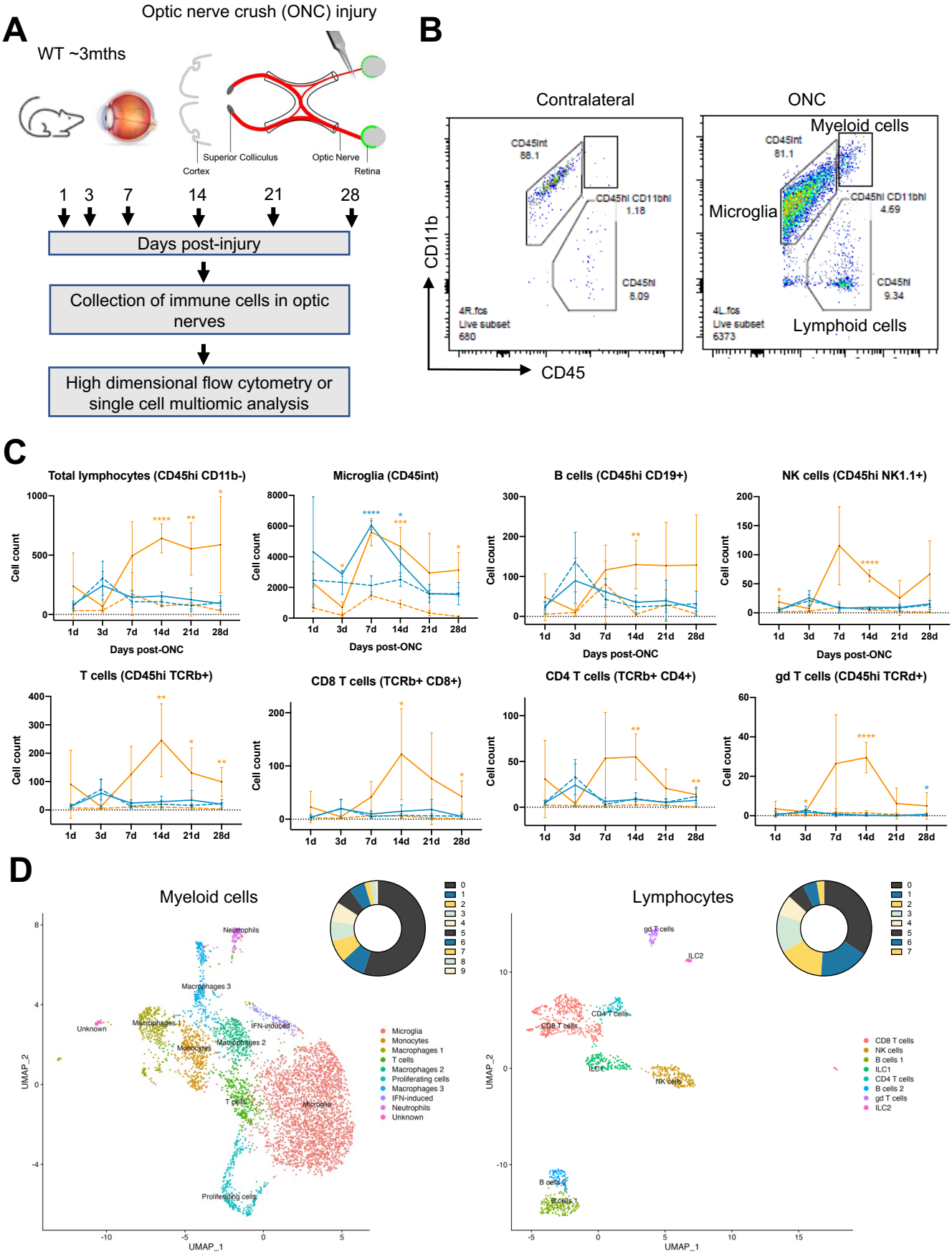

A
Optic nerve crush (ONC) injury
WT ~3mths
Superior Colliculus
Optic Nerve
Cortex
Retina
1 3 7 14 21 28
Days post-injury
Collection of immune cells in optic nerves
High dimensional flow cytometry or single cell multiomic analysis
B
Contralateral
ONC
Myeloid cells
Microglia
Lymphoid cells
CD45int 88.1
CD45hi CD11bhi 1.18
CD45hi 8.09
CD45int 81.1
CD45hi CD11bhi 4.69
CD45hi 9.34
4R.fcs Live subset 680
4L.fcs Live subset 6373
CD11b
CD45
C
Total lymphocytes (CD45hi CD11b-)
Microglia (CD45int)
B cells (CD45hi CD19+)
NK cells (CD45hi NK1.1+)
T cells (CD45hi TCRb+)
CD8 T cells (TCRb+ CD8+)
CD4 T cells (TCRb+ CD4+)
gd T cells (CD45hi TCRd+)
Cell count
Days post-ONC
1d 3d 7d 14d 21d 28d
D
Myeloid cells
Lymphocytes
UMAP_1
UMAP_2
Neutrophils
Macrophages 3
Macrophages 1
Unknown
IFN-induced
Monocytes
Macrophages 2
T cells
Microglia
Proliferating cells
ILC2
CD4 T cells
CD8 T cells
NK cells
ILC1
B cells 2
B cells 1
Microglia
Monocytes
Macrophages 1
T cells
Macrophages 2
Proliferating cells
Macrophages 3
IFN-induced
Neutrophils
Unknown
CD8 T cells
NK cells
B cells 1
ILC1
CD4 T cells
B cells 2
gd T cells
ILC2

Figure 2. Abnormal immune cell infiltration in optic nerve upon injury. (A) The schematic outlines the experimental design. In brief, optic nerve crush surgery was performed on 3-month-old wild-type B6 mice. Immune cell profiles in optic nerve were then assessed using flow cytometry (FACS) or single cell RNAseq at various time points, including 1, 3, 7, 14, 21, and 28 days post-treatment. **(B)** FACS analysis revealed increased myeloid ($CD45^{hi}CD11b^{high}$) and lymphoid ($CD45^{hi}CD11b^{intermediate/low}$) immune cells in the crushed optic nerve as compared to contralateral optic nerve. **(C)** Dynamics of immune cell infiltration following optic nerve crush. Microglia, $CD45^{intermediate}$. B cells, $CD45^{hi}$CD19+. NK cells, $CD45^{hi}$NK1.1+. ab T cells, $CD45^{hi}$TCRb+. CD8+ T cells, $CD45^{hi}$TCRb+CD8+. CD4+ T cells, $CD45^{hi}$TCRb+CD4+. gd T cells, $CD45^{hi}$TCRd+. **(D)** UMAPs revealed distinct clusters of myeloid (left) and lymphoid (right) immune cells in the brains after 14 days of optic nerve crush injury, defined by their transcriptomes through single cell RNA-seq analysis.

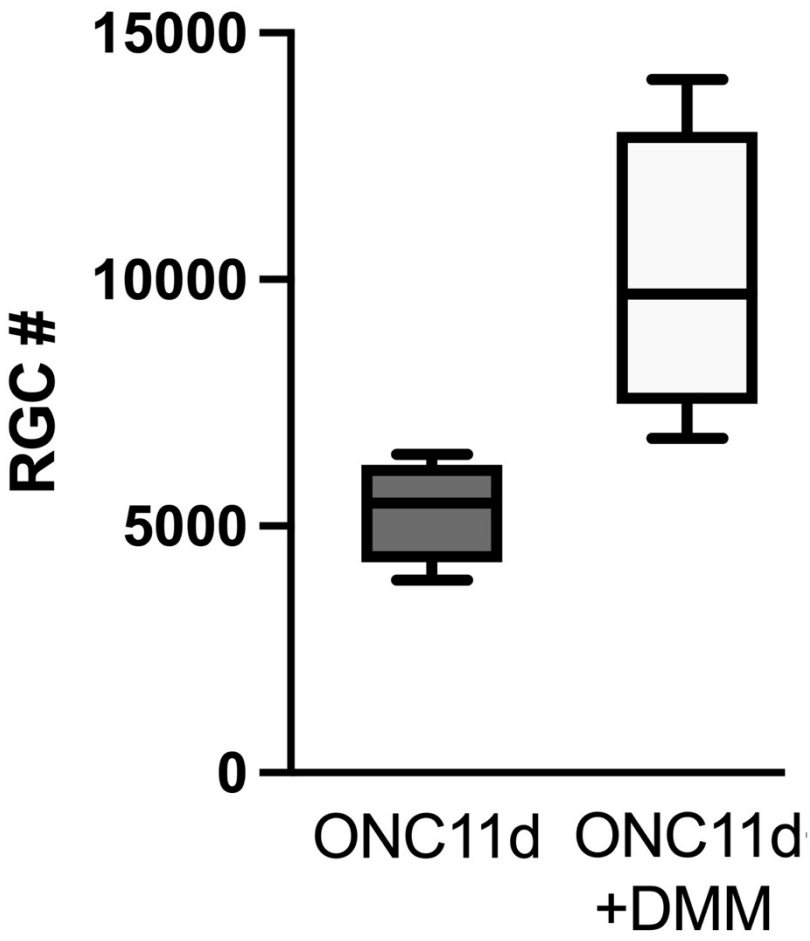


Figure 3. SDH inhibitor DMM improves RGC survival after optic nerve crush.

Intraocular injection (every other day, 6 injection total, ~175mM) of DMM significantly improved the number of RGCs survived at 11 days after ONC.

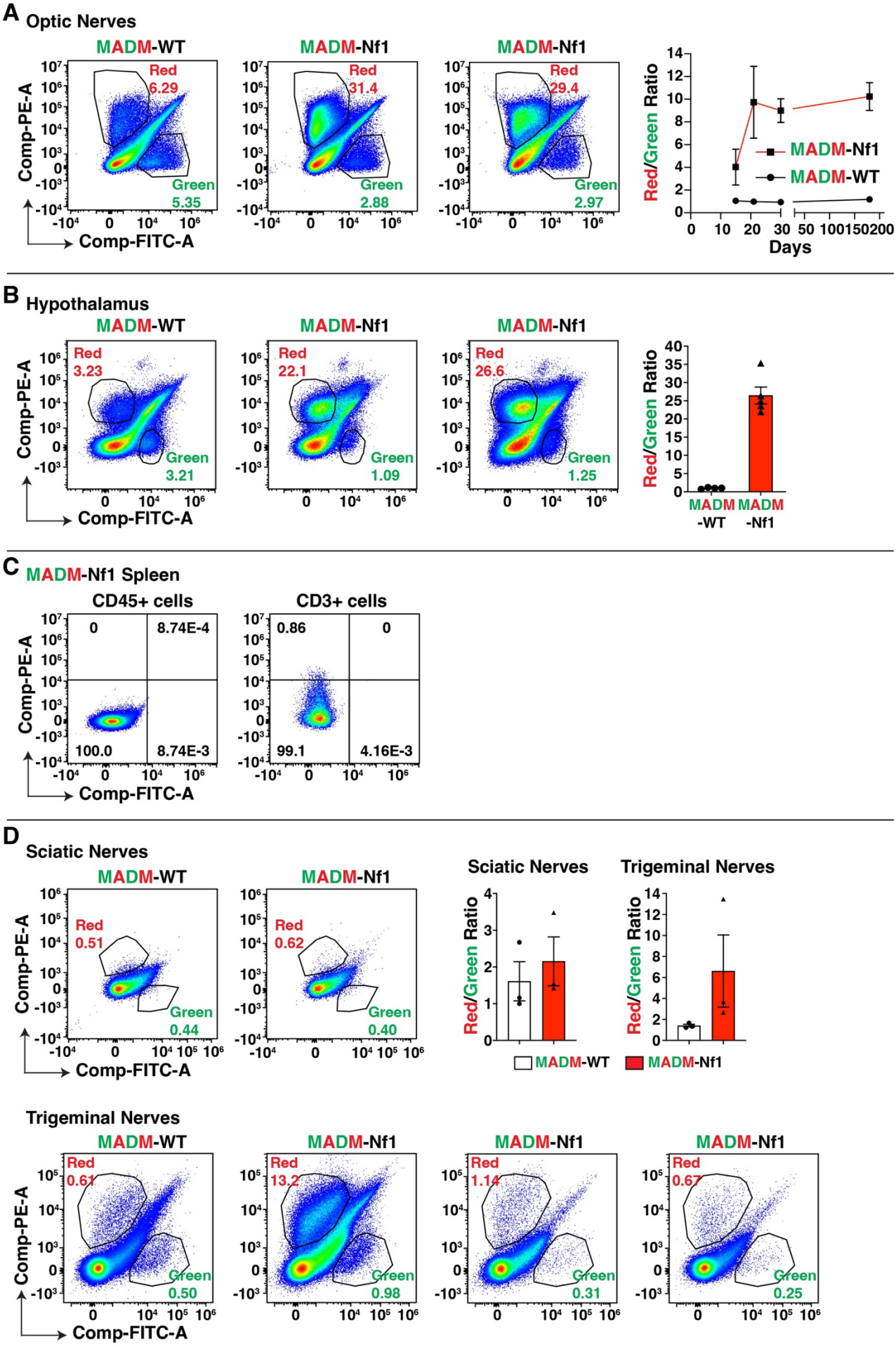
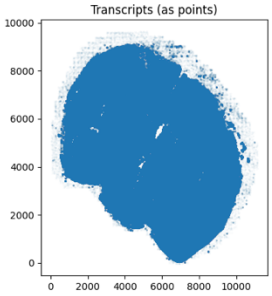
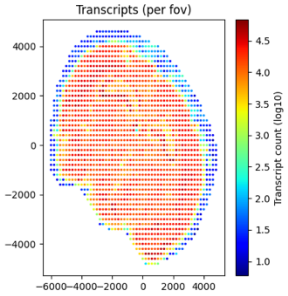
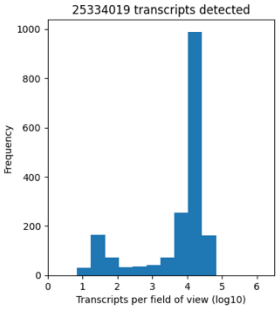

A
Optic Nerves
MADM-WT
MADM-Nf1
MADM-Nf1
Red
6.29
Green
5.35
Red
31.4
Green
2.88
Red
29.4
Green
2.97
Comp-PE-A
Comp-FITC-A
Red/Green Ratio
MADM-Nf1
MADM-WT
Days
B
Hypothalamus
MADM-WT
MADM-Nf1
MADM-Nf1
Red
3.23
Green
3.21
Red
22.1
Green
1.09
Red
26.6
Green
1.25
Red/Green Ratio
MADM -WT
MADM -Nf1
C
MADM-Nf1 Spleen
CD45+ cells
CD3+ cells
0
8.74E-4
100.0
8.74E-3
0.86
0
99.1
4.16E-3
D
Sciatic Nerves
MADM-WT
MADM-Nf1
Red
0.51
Green
0.44
Red
0.62
Green
0.40
Sciatic Nerves
Trigeminal Nerves
MADM-WT
MADM-Nf1
Trigeminal Nerves
MADM-WT
MADM-Nf1
MADM-Nf1
MADM-Nf1
Red
0.61
Green
0.50
Red
13.2
Green
0.98
Red
1.14
Green
0.31
Red
0.67
Green
0.25

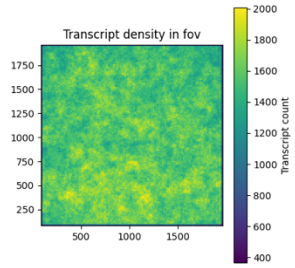
Figure 4. Analysis of Nf1-/- and Nf1+/+ sibling cells using the MADM system.

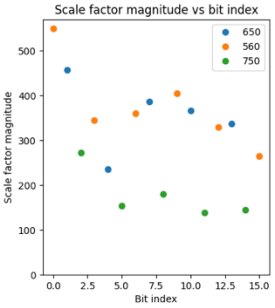
(A-C) FACS analysis of GFP+ and RFP+ cells in the aged (> 6m) Optic Nerves (A), Hypothalamus (B) and Spleen (C). Representative FACS images show the distribution of red and green cells in ONs (A) and Hypothalamus (B) from one MADM-WT and two MADM-Nf1 mice. The FACS images in (C) is to show that there is no Red and Green cells in the immune cells (CD45+; left panel) and T cells (CD3+; right panel) from one MADM-Nf1 mouse. The R/G ratio were quantified in Optic Nerves (A) and Hypothalamus (B). The R/G ratio quantification data at P15; P21 and P30 in (A) was adapted from Emmanuelle S. Jecrois et al (2021 Developmental Cell). (D) As a control experiment, the R/G ratio was determined in the peripheral nerves of the same MADM-WT and MADM-Nf1 model mice, including sciatic nerves and trigeminal nerves shown in the top and bottom panels, respectively.

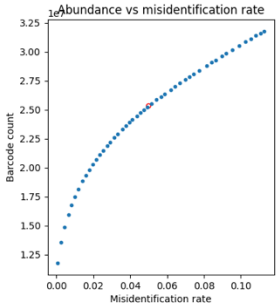

Transcripts (as points)

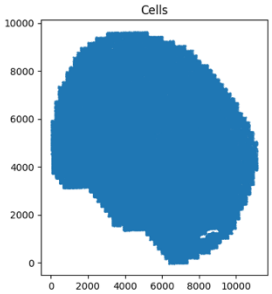

Transcripts (per fov)
Transcript count (log10)

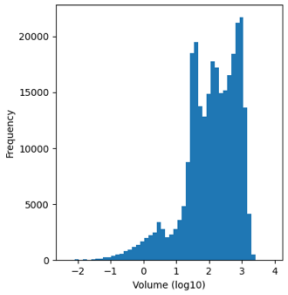

25334019 transcripts detected
Frequency
Transcripts per field of view (log10)

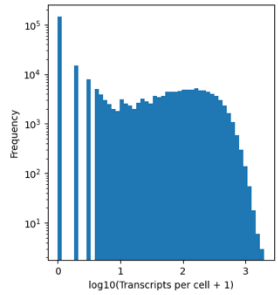

Transcript density in fov
Transcript count

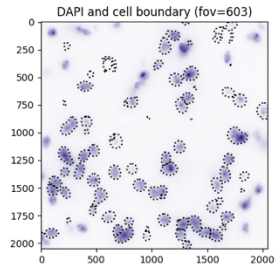

Scale factor magnitude vs bit index
650
560
750
Scale factor magnitude
Bit index

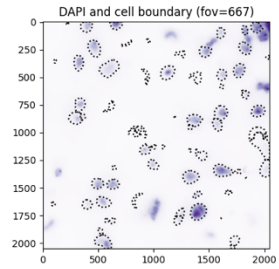

Abundance vs misidentification rate
Barcode count
Misidentification rate


Cells


Frequency
Volume (log10)


Frequency
log10(Transcripts per cell + 1)


DAPI and cell boundary (fov=603)


DAPI and cell boundary (fov=667)

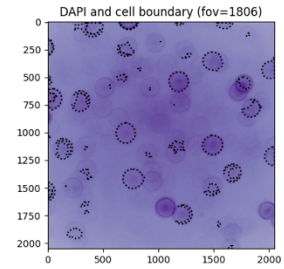

DAPI and cell boundary (fov=1806)

Figure 5. Spatial transcriptomics – feasibility study

We tested 150 genes in developing mouse brain tissue to demonstrate capability of the instrument. 1st row, left: visualization of individual transcripts within detection area; middle: transcript count per area; right: amount of transcripts per field of view. 2nd row, left: transcript density in one field of view (example); middle: scale factor magnitude vs. bit index; right: abundance vs misidentification rate demonstrates low error rate. 3rd row left: detected cells across total imaging area; middle: detected cell volumes; right: amount of transcripts per cell. 4th row: three panels showing sample fields of view to demonstrate automated cell boundary detection based on DAPI staining and RNA dye.