Bringing CLARITY to Neuromuscular Connectome of Jaw Muscles: Effects of Sex and Genetics in Mice

2025 Research Aid Awards (RAA)

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FollowUp Form

Award Information

In an attempt to make things a little easier for the reviewer who will read this report, please consider these two questions before this is sent for review:

- *Is this an example of your very best work, in that it provides sufficient explanation and justification, and is something otherwise worthy of publication? (We do publish the Final Report on our website, so this does need to be complete and polished.)*
- *Does this Final Report provide the level of detail, etc. that you would expect, if you were the reviewer?*

Title of Project:*
Bringing CLARITY to Neuromuscular Connectome of Jaw Muscles: Effects of Sex and Genetics in Mice

Award Type
Research Aid Award (RAA)

Period of AAOF Support
July 1, 2025 through June 30, 2026

Institution
University of Toronto

Names of principal advisor(s) / mentor(s), co-investigator(s) and consultant(s)
Dr Limor Avivi-Arber; Dr Barry Sessle; Dr James Posluns

Amount of Funding
$5,000.00

Abstract

(add specific directions for each type here)

Uploaded

Respond to the following questions:

Detailed results and inferences:*

If the work has been published, please attach a pdf of manuscript below by clicking "Upload a file".

OR

Use the text box below to describe in detail the results of your study. The intent is to share the knowledge you have generated with the AAOF and orthodontic community specifically and other who may benefit from your study. Table, Figures, Statistical Analysis, and interpretation of results should also be attached by clicking "Upload a file".

AAOF Results.pdf

The objective of this project was to develop and apply an optical tissue clearing, immunolabelling, and three-dimensional imaging workflow for the mouse orofacial neuromuscular connectome. The original proposal aimed to quantify structural differences in peripheral neuromuscular connectome components of the anterior digastric and masseter muscles in male and female A/J and C57BL/6J mice. During the project, it became apparent that reliable whole-mount clearing, immunolabelling, and imaging of orofacial skeletal muscle required substantial protocol optimization before the originally planned quantitative biological comparisons could be performed.

The completed study therefore focused on developing a reproducible CLARITY-based workflow for whole-mount 3D visualization of mouse orofacial skeletal muscle architecture. Eighteen male and female mice from the A/J and C57BL/6J strains were included. Muscle samples from nine mice were used in the protocol optimization phase, during which different tissue types, labels, incubation durations, dilutions, and imaging conditions were tested. Muscle samples from the remaining nine mice were used in confirmatory experiments to assess reproducibility across age, sex, and strain groups.

Digastric, masseter, and tongue muscles were optically cleared using a passive CLARITY workflow. Clearing duration differed substantially between muscle types. Digastric muscle cleared most rapidly, with a mean clearing duration of 31.37 days (n = 30; range 17–69 days). Masseter samples required longer clearing, with a mean of 87.24 days (n = 33; range 63–101 days), and tongue samples required the longest clearing, with a mean of 104.09 days (n = 43; range 55–131 days). These results supported the anterior digastric muscle as the principal tissue for protocol optimization, as it was smaller, more uniform, and more consistently cleared than larger or more irregularly shaped orofacial muscles.

A series of fluorescent labelling optimization experiments evaluated multiple structural, neural, neuromuscular junction, myofiber-type, and nuclear labels. These included Wheat Germ Agglutinin, laminin, pan-neurofilament, neurofilament light chain, PGP9.5, synaptophysin, choline acetyltransferase, alpha-bungarotoxin, myosin heavy chain IIa, myosin heavy chain I, and DAPI. Of the markers tested, Laminin-AF647 was the most successful. It provided the clearest and most reproducible visualization of the myofiber basal lamina in anterior digastric muscle. The optimized protocol used Laminin-AF647 incubation at 1:50 dilution, with weekly refreshing of the labelling solution, followed by washing, refractive-index matching in thiodiethanol, and light-sheet fluorescence microscopy.

In the confirmatory experiments, the optimized Laminin-AF647 protocol was applied to eight anterior digastric bellies representing different age, sex, and strain combinations. This produced full-length and full-thickness labelling of the myofiber basal lamina in anterior digastric samples. Cross-sectional and 3D reconstructed images showed myofiber boundary delineation consistent with expected skeletal muscle organization. However, some samples showed regional reductions in signal-to-noise ratio and reduced central myofiber delineation, indicating that even the optimized protocol requires further refinement for consistently uniform whole-volume labelling.

The optimized protocol was also trialled on larger samples, including a complete digastric muscle, a tongue segment, and a masseter segment. These larger samples demonstrated antibody penetration and detectable Laminin-AF647 signal across substantial tissue volumes, but image quality was more variable than in isolated anterior digastric bellies. In particular, larger samples showed greater regional variability in signal-to-noise ratio, optical clarity, and structural definition. These findings indicate that muscle size, thickness, morphology, and tissue-specific optical properties strongly influence clearing and immunolabelling performance.

The study did not generate inferential statistical comparisons between sex or strain groups because the primary endpoint became methodological optimization rather than biological hypothesis testing. Time-to-clear data were analyzed descriptively because the samples were heterogeneous in tissue type, size, thickness, age, sex, and strain. Imaging outcomes were assessed qualitatively using predefined criteria, including tissue structure preservation, optical transparency, labelling specificity, penetration depth, signal detectability, signal-to-noise ratio, morphological consistency, and reproducibility.

The principal inference from this study is that CLARITY-based 3D imaging is feasible for mouse orofacial skeletal muscle, especially the anterior digastric muscle, but each tissue, marker, fluorophore, incubation schedule, and imaging configuration requires careful optimization. The study established a practical methodological foundation for future studies of the peripheral orofacial neuromuscular system. However, reliable labelling of axonal and neuromuscular junction markers was not achieved under the tested conditions, and automated whole-volume segmentation of individual myofibers was not yet reliable using currently available automated image segmentation systems. These limitations prevented the originally planned quantitative connectome reconstruction and sex/genetics comparisons, but they also define the next key technical steps for the research program.

Overall, this work generated a reproducible CLARITY-compatible workflow for whole-mount 3D visualization of anterior digastric myofiber architecture, identified Laminin-AF647 as an effective conjugated label for CLARITY applications to the digastric muscle, demonstrated the increased difficulty of clearing and labelling larger orofacial muscles, and established a foundation for future hypothesis-driven investigations of sex, genetic, age-related, and treatment-induced variation in orofacial neuromuscular plasticity.

Were the original, specific aims of the proposal realized?*

The original specific aims were partially realized.

The original AAOF proposal aimed to use CLARITY-based optical tissue clearing and immunohistochemistry to visualize and quantify structural features of the peripheral neuromuscular connectome in the anterior digastric and masseter muscles of male and female A/J and C57BL/6J mice. The intended outcomes included quantitative assessment of neuromuscular structures across sex and genetic background.

The full quantitative biological comparison was not completed because reliable whole-mount immunolabelling of neuronal and neuromuscular junction markers in CLARITY-prepared orofacial skeletal muscle proved more technically challenging than anticipated. Although tissue clearing and myofiber basal lamina labelling were successful, the neural, neuromuscular junction, myofiber-type, and nuclear labels tested did not produce sufficiently consistent, specific, and interpretable signal to allow axonal tracing,

neuromuscular junction mapping, or quantitative comparison of neuromuscular architecture between sex or strain groups. In addition, automated 3D segmentation of individual myofibers was not reliable enough for whole-volume quantitative analysis.

However, the project did achieve an essential prerequisite aim. It established and optimized a CLARITY-based workflow for whole-mount 3D visualization of mouse anterior digastric muscle architecture. The optimized protocol preserved tissue structure and produced full-thickness Laminin-AF647 labelling of myofiber basal lamina in anterior digastric samples. The project also identified important technical limitations of candidate labels and imaging/segmentation approaches, and clarified the additional optimization required for larger muscles such as the masseter and tongue.

Therefore, while the original sex- and genetics-based quantitative comparison was not fully realized, the project successfully generated the methodological foundation needed to support future studies of sex, genetic, age-related, and treatment-induced variation in the orofacial neuromuscular connectome.

Were the results published?*

No

Have the results of this proposal been presented?*

Yes

To what extent have you used, or how do you intend to use, AAOF funding to further your career?*

AAOF funding was instrumental in allowing me to undertake a technically demanding research project at the interface of orthodontics, neuroscience, optical tissue clearing, immunolabelling, and three-dimensional microscopy. This support enabled me to gain experience with animal tissue preparation, CLARITY-based optical tissue clearing, whole-mount immunolabelling, refractive-index matching, light-sheet fluorescence microscopy, and 3D image reconstruction. These are skills I would not otherwise have developed through conventional clinical orthodontic training.

Although the originally planned sex- and genetics-based quantitative comparisons could not be completed within the experimental timeframe, this work established a methodological framework that can now support future studies. Examples of these potential studies include investigations of orthodontic tooth movement, skeletal anchorage functional appliances, botulinum toxin therapy, orthognathic surgery, and other interventions that may alter orofacial muscle structure and neuromuscular function.

This funding has therefore contributed directly to my development as an academic orthodontist. It supported my research training, helped me engage with a ongoing wide-scope research program and provided a foundation for future academic work combining orthodontic patient care, teaching, and clinically relevant basic science research. I intend to use the experience and preliminary data generated through this project to write a future methodology based publication; and have presented the findings of this project at several research conferences.

Accounting: Were there any leftover funds?*

If "yes", enter your best estimate and work with your grants manager to finalize financial reports and send refund payable to: AAOF
Attn: George
401 N. Lindbergh Blvd.
St. Louis, MO. 63141-7839

If "no", enter zero.

$0.00

Not Published

Are there plans to publish? If not, why not?*

The results have not yet been published in a peer-reviewed manuscript. A manuscript is planned based on the completed thesis, focusing on optimization of CLARITY-based optical tissue clearing, immunolabelling, and 3D light-sheet imaging for whole-mount mouse orofacial skeletal muscle.

The thesis developed from this project has been successfully defended, and will be published by the School of Graduate Studies, University of Toronto.

Presented

Please list titles, author or co-authors of these presentation/s, year and locations:*

1. Poster Presentation
Brinding CLARITY to orofacial muscles: 3D Imaging of Murine Digastric Muscle Architecture
- Canadian Association of Orthodontics Meeting September 2025 (Quebec City)
- Authors: Jarlath Mc Donnell, James Posluns, Barry Sessle, Limor Avivi-Arber

2. Poster Presentation
Application of CLARITY for three-dimensional laminin-based visualization of myofibre architecture in the mouse anterior digastric muscle
- University of Toronto, Faculty of Dentistry Research Day (February 2026 - 1st Place Graduate Student Clinical Sciences Category)
- Authors: Jarlath Mc Donnell, James Posluns, Barry Sessle, Limor Avivi-Arber

3. Poster Presentation
Development of a Hydrogel-Based Optical Tissue Clearing and Immunolabelling Protocol for Three-Dimensional Imaging of Whole-Mount Murine Anterior Digastric Muscles
- European Orthodontics Society Meeting, Dublin (Ireland) June 2026
- Authors: Jarlath Mc Donnell, James Posluns, Barry Sessle, Limor Avivi-Arber

Was AAOF support acknowledged?

If so, please describe:

- AAOF logo placed on each poster
- Statement in acknowledgements in each project poster, acknowledging the AAOF for financially supporting project
- During oral presentations, statement of AAOF financial support.

Internal Review

Reviewer comments

Original specific aims were partially achieved. The findings from the study were presented at regional and international meetings. A manuscript is currently being prepared. Overall, the project has been successfully completed.

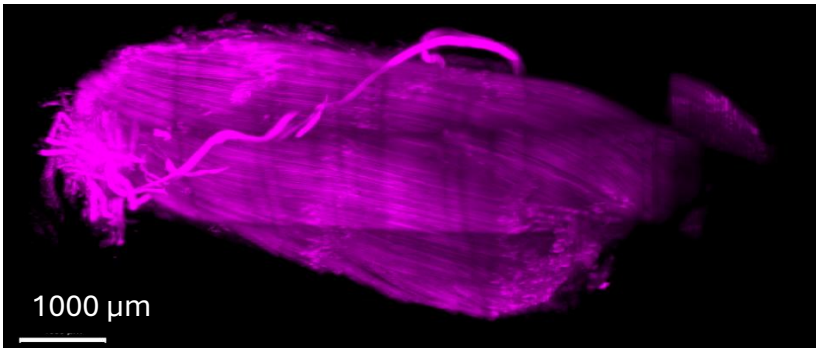
Reviewer Status*

Approved

File Attachment Summary

Applicant File Uploads

- AAOF Results.pdf



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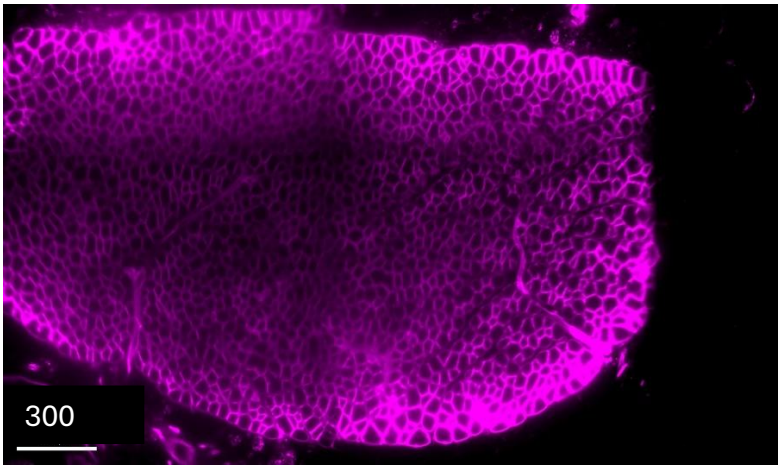

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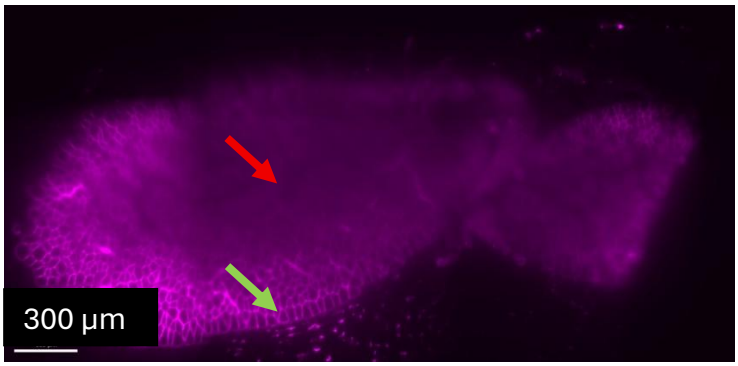

C

Figure 1: Results from Confirmatory Experiments applied to anterior digastric bellies by using the optimized immunolabelling protocol

A A 3D reconstruction of an anterior digastric belly from an old male A/J (ADG46) mouse showing Laminin-AF647 (pink) labelling of myofibre basal lamina throughout the full length of the sample. B. Cross-sectional view of an anterior digastric belly from an old female A/J mouse (ADG48) showing Laminin-AF647 (pink) delineation of myofibre basal lamina with generally high SNR ratio throughout most of the section, although signal intensity is slightly reduced in the central region. However, for some samples, as shown in C. from an old, male C57/BL/6J (ADG6) while peripheral regions showed clear Laminin-AF647 (pink) delineation of myofibre sarcolemma with generally high SNR (green arrow), central regions showed reduced delineation and lower SNR (red arrow). Images were acquired using the Zeiss Lightsheet Z.1 light-sheet microscope with a 20x objective at 0.36x zoom.

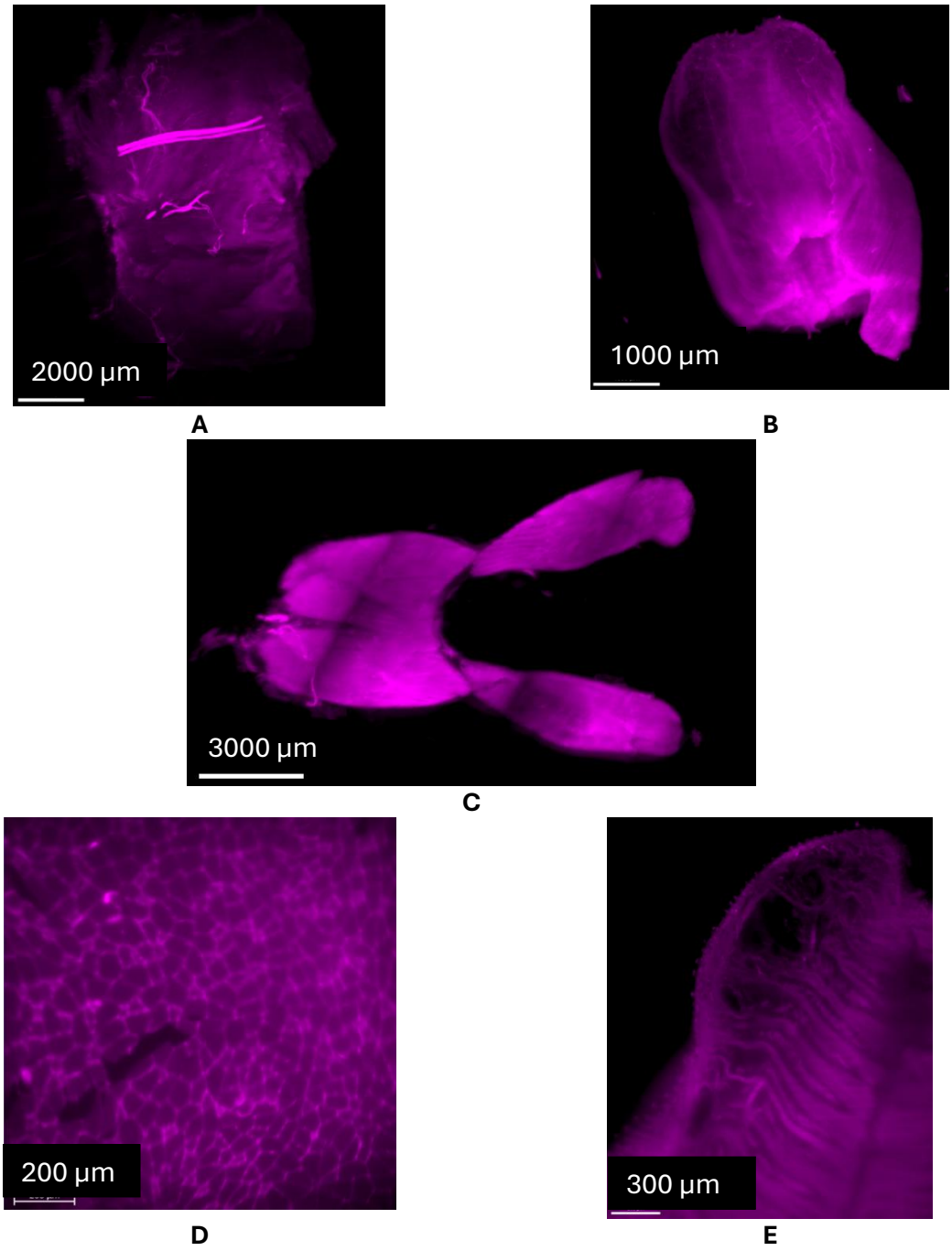

Figure 2: Results from Large-Sample Application applied to masseter muscle segment, tongue segment, and complete digastric muscle using the optimized immunolabelling protocol

A-C. A 3D reconstructions showing penetration of Laminin-AF647 (pink) throughout the full tissue volumes of a masseter segment (A), a tongue segment (B), and a complete digastric muscle (C). Images were acquired using UltraMicroscope Blaze light-sheet microscope with a 4x objective at 1x zoom. **D-E**. Sectional views showing limited delineation of myofibre basal lamina and low SNR in masseter (D) and tongue (E) segments. Sectional views were acquired using a Zeiss Lightsheet Z.1 light-sheet microscope with a 20x objective at 0.36x zoom.