FLARIM v2.0, an improved method to quantify transcript-ribosome interactions *in vivo* in the adult *Drosophila* brain

Petra Richer¹, Sean D. Speese¹, Mary A. Logan^{1*}

¹Jungers Center for Neurosciences Research, Department of Neurology, Oregon Health and Science University, Portland, United States

*Corresponding author

Abstract

Neural injury triggers striking immune reactions from glial cells, including significant transcriptional and morphological changes, but it is unclear how these events are coordinated to mount an effective immune response. Here, we present a new variant of the Fluorescence assay to detect ribosome interactions with mRNA (FLARIM), which we term FLARIM v2.0, to visualize single immune gene transcripts and association with ribosomes in glia responding to neurodegeneration. Specifically, using an *in vivo* axotomy assay in *Drosophila*, we show that matrix metalloproteinase-1 (Mmp-1) mRNAs and associated ribosomes are detected in distal processes of reactive glia where they are actively engulfing degenerating axonal material, suggesting that local translation is an important component of the glial immune response to axotomy. This work also validates our enhanced FLARIM assay as a promising tool to investigate mechanisms of mRNA transport and translation in a wide range of *in vitro* and *in vivo* paradigms.

Introduction

Glia are the resident immune cells of the brain and respond swiftly to neuronal trauma, pathogenic insult, and degeneration(1-3). Following neuronal damage, activated glia undergo distinct transcriptional, morphological, and functional changes(4-6). In many cases, reactive glia are neuroprotective, releasing pro-survival factors and clearing damaged neurons through phagocytic engulfment(7, 8). Thus, understanding how glial immune responses are activated and carried out will offer new insight into approaches that could delay the onset or progression of a range of neurodegenerative disorders and injury conditions.

The fruit fly, *Drosophila melanogaster*, offers a powerful genetic *in vivo* model to explore evolutionarily conserved glia-neuron signaling events after neural injury(9-11). For example, after axotomy, adult *Drosophila* axons undergo a classic Wallerian degeneration (WD) program, and local glial cells display striking immune responses to invade injury sites and rapidly clear degenerating axonal material(12-14). Notably, nerve injury triggers robust transcriptional changes, including upregulation of the conserved glial engulfment receptor Draper and the secreted protease matrix metalloproteinase-1 (Mmp-1)(12, 15-22).

Our lab has recently shown that upregulation of Mmp-1 is necessary for timely glial clearance of degenerating axonal projections in the olfactory system of adult flies(22). Mmp-1 is secreted from local ensheathing glial cells, likely to facilitate extracellular matrix remodeling, allowing glia to extend membrane processes into neuropil regions and clear degenerating olfactory receptor neuron axons(22, 23). Notably, the cell bodies of ensheathing glial cells responding to degenerating axons in the olfactory system do not enter neuropil regions, which raises interesting questions about how key immune molecules are released within the neuropil at injury sites(12, 24). Previous work in other model systems has indeed demonstrated that directed mRNA transport and local translation are important for glia to carry out normal functions. For

example, in oligodendrocytes, myelin basic protein transcripts are localized to oligodendrocyte processes to adequately myelinate axons in an activity-dependent manner, while astrocytes have been shown to influence interactions at tripartite synapses and the gliovascular interface through a subset of discrete localized and locally translated transcripts(25-29).

In order to further explore the transcriptional and translational changes that are essential for proper glial responses to damaged axons, our lab has utilized various single molecule fluorescence *in situ* hybridization (smFISH) techniques for the detection of individual transcripts and a new variation of Fluorescence assay to detect ribosome interactions with mRNA (FLARIM), referred to as FLARIM v2.0, to detect ribosome association with *mmp-1* transcripts in glial cells following nerve injury(30). smFISH has been employed in glial cells and various *Drosophila* tissues to detect individual mRNAs; however, previous studies have not yet employed mRNA and ribosome detection together in the context of neuronal injury *in vivo*(31-38). Here, we have utilized single molecule inexpensive FISH (smiFISH), hybridization chain reaction (HCR), and FLARIM, in order to visualize *mmp-1* transcript localization and translation in reactive glia following axotomy(30, 39-41). Our findings reveal that *mmp-1* transcripts are localized to and associate with ribosomes at distal glial processes at injury sites, suggesting that local translation of *Mmp-1* may be an important mechanism by which glia access and phagocytically clear neuronal debris.

Results

Ensheathing glia respond to olfactory receptor neuron injury in *Drosophila*

Axotomy in the adult *Drosophila* antennal system is a well-characterized model to investigate the molecular and cellular underpinnings of axon degeneration and glial immune responses (Fig. 1A)(12, 42). The olfactory receptor neuron (ORN) cell bodies reside in the external olfactory organs of the fly, the antennae and maxillary palps, and project to the antennal lobes of the central brain, where they synapse onto second order neurons(43, 44). Surgical removal of the antennae and maxillary palps results in axotomy of the ORNs, which undergo typical WD over the course of days, after which they are cleared by the surrounding glial cells(12, 45). In the antennal lobes, glial cell bodies (ensheathing glia and astrocytes) are located at the very edges of the antennal lobes and extend projections to closely associate with neuropil regions (axons, dendrites, and synapses) and demarcate olfactory glomeruli (Fig. 1B)(9, 46). In context of ORN injury, ensheathing glia have been demonstrated to phagocytose and clear degenerating projections(24).

Individual ensheathing glial cells have highly complex morphologies, with extensive fine processes that interact with multiple glomeruli within the antennal lobe (Fig. 1B)(46-48). In the event of an injury, the cell bodies of ensheathing glia remain positioned at the outskirts of the antennal lobes, while the processes further invade glomeruli to clear degenerating ORNs(12, 22, 24). This raises the question of how intracellular signaling is mediated within ensheathing glial cells from the cell body to the distal processes after an injury event. In order to explore this question, we utilized multiple single molecule FISH (smFISH) methods to study how a well characterized immune gene, *mmp-1*, is localized and regulated at the transcriptional and translational level following ORN axotomy.

smiFISH reveals upregulation and localization of *mmp-1* transcripts following neuronal injury

Our previous RNAseq and qPCR studies have demonstrated that the *mmp-1* transcript is acutely upregulated in the central nervous system (CNS) and ventral nerve cord (VNC) of the fly following neuronal injury. As prior research has shown that *mmp* transcripts can be localized to subcellular compartments and locally translated in an activity dependent manner, we wanted to determine the localization of *mmp-1* mRNA in ensheathing glia following ORN axotomy(49, 50). In order to accomplish this, we utilized smiFISH to detect the *mmp-1* transcript following injury

Figure 1: *mmp-1* transcripts are upregulated in ensheathing glia following neuronal injury

(A) Schematic representation of the olfactory receptor neuron (ORN) injury, demonstrating the removal of the 3rd antennal segments and maxillary palps. ORN cell bodies in the aforementioned structures extend their projections to the antennal lobes (ALs) of the central fly brain (red box). Created with BioRender.com (B) Representative image of ensheathing glia surrounding the ALs in an uninjured brain. White arrows indicate ensheathing glial fine processes within ALs. AL: antennal lobe, AN: antennal nerve. Single slice (1µm). Scale bar: 20µm. Genotype: ;UAS-mCD8::GFP/UAS-mCD8::GFP;TIFR-Gal4/UAS-LacZ::NLS. (C) smiFISH schematic. Created with BioRender.com (D'-D''') Representative images of *mmp-1* smiFISH in uninjured and injured (4.5hpi) single ALs (yellow dotted line). Images are MIPs: 3.75µm. Scale bar: 10µm. Control genotype: ;tub-Gal80^{ts}/+;repo-Gal4/+. Mmp-1^{RNAi} genotype: ;tub-Gal80^{ts}/UAS-Mmp-1^{RNAi};repo-Gal4/+. (d'-d''') High magnification images of *mmp-1* smiFISH (white box), representing single transcripts and transcription start sites (yellow arrows). MIP: 3.75µm. Scale bar: 2µm. (E) High magnification image of *mmp-1* transcripts in ensheathing glial processes (white arrows) 4hpi. Single slice: 0.43µm. Scale bar: 2µm. Genotype: ;UAS-mCD8::GFP/UAS-mCD8::GFP;TIFR-Gal4/UAS-LacZ::NLS.

(Fig. 1C)(40). The Mmp-1 smiFISH probeset included 41 probes and was designed utilizing Oligostan (Supplementary Table 1)(40). Our previous RNAseq and qPCR data suggested that there is a strong transcriptional response in the ensheathing glia 3 hours post ORN axotomy(22). As a result, we used this as a starting point for our smiFISH studies.

In accordance with our qPCR and RNAseq data, we found that smiFISH reveals a low level of Mmp-1 transcript present in the uninjured brain, with the highest amount of transcript being expressed in cells surrounding the antennal lobe, likely ensheathing glia (Fig. 1D). In response to injury, we observe a significant increase in the amount of *mmp-1* transcript detected at 4.5hpi (Fig. 1D"). Interestingly, when labeling ensheathing glial membranes with GFP, we also observed the localization of the *mmp-1* smiFISH signal to fine processes following injury (Fig. 1E).

To determine if the Mmp-1 probeset is specific to the *mmp-1* transcript, we performed an injury experiment where Mmp-1 was knocked down in all glia using RNAi. As expected, the RNAi knockdown eliminated most of the smiFISH signal (Fig. 1D""-D"""). We did however detect large spots of Mmp-1 transcript right outside the antennal lobe, where the nuclei of ensheathing glia are located (Fig. 1d'-d'"""). We posit that these large intense puncta represent the site of Mmp-1 transcription in the nucleus, as the transcript would not be subject to RNAi mediated degradation until it was transported to the cytoplasm(40, 51).

The FLARIM v2.0 method can be used to detect mRNAs and associated ribosomes

While the smiFISH approach allowed us to localize the *mmp-1* transcript to distal glial processes following neuronal injury, it does not give us insight into whether there may be local translation of the transcript at distal sites. Currently, the most accepted method for investigating local translation is the use of photoconvertible fluorescent timer proteins, however this approach requires tagging the protein of interest in the context of native mRNA with all its regulatory 5' and 3' elements(52, 53). More recently, a non-transgenic approach was developed to assess the ribosome load on a transcript of interest while maintaining spatial localization within the cell. This method, Fluorescence assay to detect ribosome interactions with mRNA (FLARIM), utilizes pairs of oligonucleotide probes that bind separately to ribosomes and to the mRNA of interest (Fig. 2A)(30). When these probes are in close proximity they form a full sequence for a linker probe carrying a complete Hybridization Chain Reaction (HCR) initiator (Fig. 2A)(39, 41).

While the original method was able to robustly assess the ribosome load on transcripts of interest, it has never been used *in vivo* on whole mount tissue. Moreover, the use of the linker probe requires a 2nd round of hybridization before initiating the HCR reaction. Lastly, the original method made use of separate pools of gene specific probes for transcript localization and ribosome association, which can be problematic on short transcripts where probe binding sites may be limiting (Fig. 2A). To address these drawbacks, we have utilized split initiator technology to include half an HCR initiator, split B3 initiator, for reporting ribosome association on the 3' end of the gene specific probe and the other half of the B3 initiator on the 3' end of a set of 25 probes that will hybridize to the 18S rRNA (Supplementary Tables 2 and 3)(39, 41). Finally, we tagged the gene specific probes with a different full HCR initiator (B2) on the 5' end to allow for transcript localization (Supplementary Table 3)(39, 41). By taking advantage of new developments in oligonucleotide synthesis, Integrated DNA Technologies' oPools Oligo Pools service, we are able to synthesize a probeset for a gene of interest for ~\$100 USD. We are calling this modified version FLARIM v2.0, and we herein demonstrate that this relatively inexpensive method is able to achieve transcript localization and ribosome association at depth in whole mount tissue (Fig. 2B).

In FLARIM v2.0, probes are first hybridized to the transcript of interest and to 18S ribosomes. Then, during the detection stage, fluorescent metastable hairpins are used to generate signals via HCR (Supplementary Fig. 1). On the gene-specific probe, the full initiator sequence opens up a set of hairpins to create a fluorescent signal indicating the presence and localization of the mRNA transcript. Moreover, when the split initiators of the 18S ribosome

A

FLARIM (Burke *et al.*, 2017)

B

FLARIM v2.0

Figure 2: Novel FLARIM v2.0 probeset

(A) FLARIM (Burke *et al.*, 2017): Utilizes 4 probes to achieve distinct mRNA and ribosome-specific signals by HCR amplification and allows for assessment of ribosome load on transcripts of interest. **(B)** FLARIM v2.0: Utilizes 2 probesets: 1) an 18S ribosome-specific probe with the first half of a split B3 initiator and an mRNA-specific probe with the second half of the split B3 initiator and 2) a full B2 initiator on the 5' end of the gene specific probe. This B2 initiator allows for transcript localization and aids in quantification by creating a normalization channel for the FLARIM signal. Hybridization with these probesets and amplification using metastable hairpins generates a separate fluorescent signal for mRNA and mRNA-ribosome association detection *in vivo*. Created using Biorender.com.

probes and the gene-specific probes are reconstituted, a second set of hairpins are opened, creating a distinct fluorescent signal to allow for mRNA-ribosome association detection (Fig. 2B).

The dual detection method produces diffraction limited spots, if amplification is carried out for shorter periods of time and allows for transcript and ribosome detection in a cell-type specific manner, providing spatiotemporal resolution. The fluorescent tags on hairpins can also be varied to accommodate multiplexing within the same sample. Additionally, FLARIM v2.0 can be used to investigate mRNA localization and local translation in response to various stimuli, such as neuronal injury. We decided to assess the regulation of *mmp-1* following axotomy using the FLARIM v2.0 method.

Application of the FLARIM v2.0 method in the *Drosophila* ORN injury model

In order to validate our Mmp-1 probeset and explore how an immune gene is regulated following axotomy, the FLARIM v2.0 method was tested in the context of the ORN injury model (Fig. 3A). In uninjured brains, transcripts are sparse and localized to the edges of the antennal lobes, where ensheathing glial cell bodies are located. Some transcripts are also associated with ribosomes, generating a FLARIM signal and indicating that some translation may be occurring under basal conditions. In brains that have undergone an ORN injury, there is a robust upregulation of the *mmp-1* transcript (magenta) and ribosome-association (cyan) signal both around the antennal lobes and within them (Fig. 3A). These results mirror the time course of Mmp-1 protein upregulation following antennal and maxillary palp injury, which is robust 1-day post-injury (dpi)(22). Moreover, labeling of ensheathing glial cell membranes with GFP allows for the localization of *mmp-1* mRNAs and associated ribosomes within the antennal lobes 20hpi. To quantify these results, a total fluorescence intensity analysis of each signal was completed within the antennal lobes. Spot detection was not possible, as many transcripts and ribosomes were located close to, or even overlapping, each other. The total intensity of both *mmp-1* transcript and ribosome-association signals significantly increased within antennal lobes 20hpi (Fig. 3B).

A series of control experiments were also conducted to ascertain the specificity of the probesets and our signal. In the absence of hybridized probes, the hairpins amplified alone do not generate a fluorescent signal (Fig. 3A, B). Additionally, hybridization of only the 18S FLARIM probes does not result in a signal, as only half of the initiator sequence is present, and hairpins are unable to open and generate a fluorescent signal (Fig. 3A, B). We also determined that the *mmp-1* probeset was specific to our transcript of interest. Upon Mmp-1 RNAi knockdown in glial cells, *mmp-1* mRNA and ribosome-association signals were significantly diminished in the injured condition (Fig. 4A, B, C).

Translation machinery is present in ensheathing glial processes

We observe that *mmp-1* transcripts and associated ribosomes are localized to ensheathing glial processes following ORN injury. As a result, we hypothesize that transcript localization and local translation of immune genes may be mechanisms employed by glial cells to mediate responses after a neuronal injury event. This is of particular interest in relation to Mmp-1, as it is a secreted molecule employed by ensheathing glia to remodel the surrounding extracellular matrix and provide access to sites of damage(22, 23). However, in order for this to occur, translation and secretory machinery must also be present in ensheathing glial processes to properly modify and secrete Mmp-1(27). Evidence for translation machinery in glial processes has been demonstrated by previous groups. A study in mouse astrocytes has shown that along with a local pool of transcripts, endoplasmic reticulum (ER) and Golgi apparatus (GA) are also present in perivascular processes and endfeet(27). Therefore, we performed immunohistochemistry in uninjured and injured brains to assess whether the aforementioned organelles are present in glial projections of the ALs. Super-resolution microscopy allowed for the visualization of ER and GA within ensheathing glial fine processes (Fig. 5A-D). We were able to

Figure 3: FLARIM v2.0 to detect *mmp-1* in the *Drosophila* ORN injury model

(A) Representative images of *mmp-1* FLARIM v2.0 probeset in uninjured and injured ALs with ROIs quantified (dotted circle). Injured: 20hpi. Hairpins only: 20hpi (no probes, only amplification with fluorescent hairpins). 18S Probes only: 20hpi (18S FLARIM probes with one half of the split initiator sequence and amplification with fluorescent hairpins). Single slice: 0.5µm. Scale bar: 20µm. Genotype: ;UAS-*mCD8::GFP*;TIFR-Gal4. (B) Quantification of *mmp-1* FLARIM v2.0 total intensity (a.u.) in ALs. *mmp-1* mRNA Uninjured (n=22), *mmp-1* mRNA Injured (n=24), Ribosome-association Uninjured (n=22), Ribosome-association Injured (n=24), *mmp-1* mRNA 18S Probes Only (n=16), Ribosome-association 18S Probes Only (n=14), *mmp-1* mRNA Hairpins Only (n=8), Ribosome-association Hairpins Only (n=7). Mean ± SEM; Uninjured *mmp-1* mRNA and Injured *mmp-1* mRNA: t-test ****(p<0.0001); Uninjured Ribosome-association and Injured Ribosome-association: Mann-Whitney test ****(p<0.0001); non-significant (ns). (C) Representative image of *mmp-1* FLARIM v2.0 probeset in an injured AL (20hpi). Single slice: 0.5µm. Scale bar: 10µm. Genotype: ;UAS-*mCD8::GFP*;TIFR-Gal4. (c') *mmp-1* transcripts are associated with ribosomes in ensheathing glial processes (white arrows). Single slice: 0.5µm. Scale bar: 2µm.

Figure 4: FLARIM v2.0 is specific to the *mmp-1* transcript and its associated ribosomes

(A) Representative images of *mmp-1* FLARIM v2.0 in control uninjured and injured (20hpi) brains. MIP: 15.5μm. Scale bar: 20μm. Genotype: *tub-Gal80^{ts}/+;repo-Gal4/+*. (B) Representative images of *mmp-1* FLARIM v2.0 in brains expressing Mmp-1 RNAi in all glial cells (*repo>Mmp-1^{RNAi}*). MIP: 15.5μm. Scale bar: 20μm. Genotype: *tub-Gal80^{ts}/UAS-Mmp-1^{RNAi};repo-Gal4/+*. (C) Quantification of *mmp-1* FLARIM v2.0 total intensity (a.u.) in ALs. Control: Uninjured *mmp-1* mRNA (n=10), Injured *mmp-1* mRNA (n=14), Uninjured Ribosome-association (n=11), Injured Ribosome-association (n=14). *repo>Mmp-1^{RNAi}*: Uninjured *mmp-1* mRNA (n=8), Injured *mmp-1* mRNA (n=12), Uninjured Ribosome-association (n=8), Injured Ribosome-association (n=10). Mean ± SEM; Mann-Whitney test ****(p<0.0001).

observe that ER and GA are present under both basal conditions and following ORN injury (Fig. 5A-D). These results further suggest that transcript localization and local translation of immune genes could be supported in glial processes, and could play a functional role in mediating rapid glial responses following neuronal injury

Discussion

In this study, we have modified HCR and FLARIM methods to develop a novel dual probeset strategy to visualize single mRNA transcripts and their associated ribosomes *in vivo* (30, 39, 41). Specifically, we have applied this approach to visualize Mmp-1 transcripts in ensheathing glial cells responding to acute axon injury in adult *Drosophila*. It is becoming increasingly clear that directed transport of select mRNAs and local translation are essential for a wide range of cellular responses, including glial cell function in both the developing and mature brain (54-57). Thus, we propose that this novel methodology to visualize and quantify transcripts and, notably, ribosome association in whole tissue will be broadly valuable.

The mechanisms of mRNA localization and local translation are highly conserved and provide cells with the ability to restrict responses to subcellular compartments, conserve energy, and localize responses to specific stimuli [49]. Directed transport/translation of transcripts has been well described in neurons (58-62). More recently, the importance of local translation has been explored in glial cells. Local transcriptomes and translomes have been characterized within astrocyte peripheral processes at the tripartite synapse, as well as astrocytic endfeet at the gliovascular interface (27, 28). In oligodendrocytes, myelin basic protein (*mbp*) mRNA is localized to oligodendrocyte sheaths and is required for proper myelin and axon development, while radial glia transport and locally translate mRNAs in their distal processes during development (25, 55). Research has also focused on how specific stimuli activate such mechanisms. Peripheral processes in astrocytes induce local translation in response to fear conditioning in mice, while neuronal activity may control myelination by oligodendrocytes through the induction of local protein synthesis (26, 29).

Here, we show that that our *mmp-1* FLARIM v2.0 approach can reliably detect significant *mmp-1* upregulation in glia following olfactory nerve injury and that this strategy allows us to monitor changes in the spatial distribution of *mmp-1* transcripts, as well as association with ribosomes within one day after axotomy. We propose that our detection of transcript/ribosome association indicates that local translation of secreted Mmp-1 protein occurs at the distal process of glial cells as they invade injury sites (Fig. 3). We observe markers for both ER and GA in fine distal processes of ensheathing glia under both basal and injury conditions, suggesting that this glial subtype is equipped with organelles to locally translate and secrete a released factor such as Mmp-1 (Fig. 5). Given the ramified morphology of ensheathing glia, and the well-known role of Mmp-1 in ECM remodeling, rapid, local production of Mmp-1 is likely important for proper extension of glial processes within the deeper regions of the antennal lobes. As a result, this would aid phagocytic engulfment of degenerating olfactory neurons.

From a technical standpoint, FLARIM v2.0 offers a number of important features and, notably, some key advantages over the original FLARIM approach (Fig. 2). Fewer probes are required to monitor association of ribosomes and transcripts of interest. Transcripts and ribosomes are visualized as diffraction-limited spots and can be quantified to assess relative changes in gene expression and ribosome association in discrete cell types. Moreover, this method allows for flexible fluorescent labeling within samples. Finally, the generation of probes ordered as oPools is very cost effective. This and related protocols also offer the obvious benefit of greater spatial resolution to quantify gene upregulation in discrete cell types in heterogeneous tissue, as opposed to cruder approaches (e.g., quantitative-PCR on crushed tissue).

Figure 5: Endoplasmic reticulum and Golgi apparatus are present in ensheathing glial fine processes

(A) Representative super-resolution images of ER immunostaining in an uninjured AL. MIP (1.75µm). Scale bar: 10µm. Genotype: ;UAS-mCD8::GFP/UAS-mCD8::GFP;TIFR-Gal4/TIFR-Gal4. (a', a'') ER is present within ensheathing glial fine processes of an uninjured AL. MIP (1.75µm). Scale bar: 3µm. (a''') Colocalization of ensheathing glia and ER signal. (B) Representative super-resolution images of ER immunostaining in an injured (20hpi) AL. MIP (1.75µm). Scale bar: 10µm. Genotype: ;UAS-mCD8::GFP/UAS-mCD8::GFP;TIFR-Gal4/TIFR-Gal4. (b', b'') ER is present within ensheathing glial fine processes of an injured AL. MIP (1.75µm). Scale bar: 2µm. (b''') Colocalization of ensheathing glia and ER signal. (C) Representative super-resolution images of GA immunostaining in an uninjured AL. MIP (1.75µm). Scale bar: 10µm. Genotype: ;UAS-mCD8::GFP/UAS-mCD8::GFP;TIFR-Gal4/TIFR-Gal4. (c', c'') GA is present within ensheathing glial fine processes of an uninjured AL. MIP (1.75µm). Scale bar: 2µm. (c''') Colocalization of ensheathing glia and GA signal. (D) Representative super-resolution images of GA immunostaining in an injured (20hpi) AL. MIP (1.75µm). Scale bar: 10µm. Genotype: ;UAS-mCD8::GFP/UAS-mCD8::GFP;TIFR-Gal4/TIFR-Gal4. (d', d'') GA is present within ensheathing glial fine processes of an injured AL. MIP (1.75µm). Scale bar: 2µm. (d''') Colocalization of ensheathing glia and GA signal.

More broadly, this approach offers a novel experimental readout for a component of glial immunity that has not yet been explored in flies and minimally in other model organisms, namely local translation of immune genes at injury sites. Our results now offer a new platform to explore future questions to investigate how mRNA transcripts are transported, translated, and eventually degraded once an immune response is no longer required. Previous work from our lab has shown that insulin-like signaling (ILS) pathways are acutely activated in ensheathing glial cells responding to olfactory nerve axotomy(63). Because ILS cascades are known to promote protein translation via mammalian target of rapamycin (mTOR), this offers an intriguing candidate by which immune gene translation is locally enhanced within glia in response to neurodegeneration(64). Future screening efforts will reveal how select immune gene transcripts are shuttled throughout glial processes to support proper innate immune reactions to acute trauma and perhaps also chronic neurodegenerative conditions.

Methods

Probe design and synthesis

Probes to the transcript of interest were designed using Oligostan in R Studio(40, 65). The probes were compared to a database of noncoding RNAs ([http://flybase.org\(66\):http://ftp.flybase.net/genomes/Drosophila_melanogaster/dmel_r6.37_FB2020_06/fasta/](http://flybase.org(66):http://ftp.flybase.net/genomes/Drosophila_melanogaster/dmel_r6.37_FB2020_06/fasta/); dmel-all-miRNA-r6.37.fasta.gz, dmel-all-miscRNA-r6.37.fasta.gz, dmel-all-ncRNA-r6.37.fasta.gz) for sequence similarity using Geneious (Biomatters). Any probes similar to ncRNAs (miRNA, snoRNA, lncRNA) were discarded from the probeset. The remaining probes were used to make up the gene-specific probesets. B2 and split B3 initiator sequences were added to the probes to allow for HCR amplification and FLARIM detection, which have been previously described(30, 39). Probes were synthesized in 96-well plates or as 50 pmol oPools (Integrated DNA Technologies). Probe sequences can be found in Supplementary tables 1-3.

ORN Injury Assay and Dissection

Third antennal segments and maxillary palps were removed bilaterally using forceps, as previously described(45). Flies were raised at 25°C and returned to this temperature following injury, until dissection at 20hpi. Fly lines containing the tubulin-Gal80ts transgene were raised at 18°C and moved to 30°C for 7 days to induce Mmp-1 RNAi expression. These flies were then injured and moved back to 30°C until dissection. Fly heads were pulled and fixed in 4% paraformaldehyde (PFA) + 0.1% Triton X-100 for 20min at room temperature (RT) on a rocker. Then, heads were washed in 1X PBS-TX (0.01% Triton X-100) on a rocker at RT (3x2min). Brains were dissected in 1X PBS-TX (0.01% Triton X-100). Brains were then fixed in 4% PFA + 0.1% Triton X-100 for 20min at RT on a rocker and washed with 1X PBS-TX (0.1% Triton X-100) on a rocker at RT (3x2min). To increase probe penetration, brains were also permeabilized for 20min in 1X PBS-TX (0.5% Triton X-100), while rocking at RT. Brains for immunostaining were not additionally permeabilized.

smiFISH

The smiFISH reagents, flap hybridization, and protocol have been described previously(37, 40). The smiFISH protocol has been adapted as follows: After dissection, fixation, and permeabilization, brains were placed in hybridization buffer(40) with the Mmp-1 smiFISH ATTO 550 (Integrated DNA Technologies) probeset and hybridized overnight in a thermal cycler at 37°C. The following day, brains were washed in 1X SSC/10% formamide/0.1% Triton X-100/0.1%

Tween-20 at 50°C (2x30min) in a thermal cycler. Brains were then mounted in Vectashield (Vector Labs) under #1.5 coverslips and imaged.

Probe Hybridization and Signal Amplification

The HCR v3.0 hybridization and amplification protocol has been described previously for whole-mount fruit fly embryos(41, 67). The protocol was utilized in adult fruit fly brains as follows: After permeabilization, brains were pre-hybridized for 20min at 37°C in probe hybridization buffer (Molecular Instruments), which had already been heated to 37°C. Brains were then hybridized overnight (16-18hrs) with the gene-specific and 18S FLARIM probes in a thermal cycler at 37°C. The following day, brains were washed in probe wash buffer (Molecular Instruments) at 37°C in the thermal cycler (4x15min). During the washes, HCR hairpins (Molecular Instruments) were snap-cooled, and the amplification buffer (Molecular Instruments) was moved to RT. Brains were washed again in 5X SSCT (0.1% Tween-20) at RT (2x5min). Brains were pre-amplified with amplification buffer at RT for 10min. Then, hairpins were added to the amplification buffer and the HCR and FLARIM signals were amplified. Amplification times were empirically determined for each probeset in this paper (*mmp-1* HCR B2 FLARIM split B3 Alexa Fluor 546: 1hr; 18S FLARIM B3 Alexa Fluor 647: 6hrs). Following amplification, brains were washed in 5X SSCT (0.1% Tween-20) at RT (5min, 2x30min, 5min), and then mounted in Vectashield (Vector Labs) under #1.5 coverslips and imaged.

Immunostaining

Antibodies were diluted in 1X PBS-TX (0.1% Triton X-100) and brains were incubated in primary antibodies overnight on a shaker at 4°C. Brains were washed in PBS-TX (0.1% Triton X-100) at RT (3x30min) and incubated in secondary antibodies in 1X PBS-TX (0.1% Triton X-100) for 2hrs at RT on a shaker. Brains were then washed again in 1X PBS-TX (0.1% Triton X-100) at RT (3x30min) and mounted in Vectashield (Vector Labs) under #1.5 coverslips and imaged.

Antibodies

The following primary antibodies were used: mouse beta-galactosidase 40-1a (Developmental Studies Hybridoma Bank) at 1:100, chicken anti-GFP (ThermoFisher, #A10262) at 1:1000, mouse anti-nc82 (Bruchpilot; Developmental Studies Hybridoma Bank) at 1:50, goat anti-GMAP (Developmental Studies Hybridoma Bank) at 1:800, mouse anti-Cnx99A 6-2-1 (Developmental Studies Hybridoma Bank) at 1:400. All secondary antibodies (Jackson ImmunoResearch 703-545-155, 715-295-150, and 705-295-147) were used at a 1:400 dilution.

Microscopy and Analysis

Samples were imaged on a Zeiss LSM 700 with a Zeiss 40X 1.4 NA oil immersion plan-apochromatic lens. Samples with Golgi and ER staining were imaged using a Zeiss Elyra 7 with lattice SIM with a Zeiss 63X 1.4 NA oil immersion plan-apochromatic lens at the OHSU Advanced Light Microscopy Core (ALMC). Brains within the same experiment were imaged on the same day, using the same microscope settings. smiFISH and FLARIM v2.0 images were pre-processed by smooth filtering in Zen, while Golgi and ER images were SIM processed in Zen. Volocity 3D Image Analysis Software (Quorum Technologies) was used for fluorescence quantification, while Imaris Cell Imaging Software (Andor Technology) at the OHSU ALMC was used for super-resolution image processing and colocalization analysis. GraphPad Prism 8 (Graphpad Software) was used for statistical analysis: Student's t-test, Mann-Whitney test. Normality was tested using the D'Agostino-Pearson normality test. Outliers were identified using the ROUT method. Experiments were not blinded. For *mmp-1* FLARIM v2.0 quantifications, 5um was removed from the top of each z-stack to exclude ensheathing glial cell bodies from the analysis, and only quantify *mmp-1* mRNA

and ribosome-association signals within the ensheathing glial processes of the ALs. A circular ROI was defined in each AL, where the total fluorescence for each signal was calculated.

Drosophila Stocks

Adult flies between 4-14 days old were used for experiments. The following lines were used: w1118 (BDSC 5905), UAS-mCD8::GFP (BDSC 5137), UAS-LacZ::NLS (BDSC 3956), TIFR-Gal4(68), repo-Gal4(12), tubulin-Gal80ts (BDSC 7108), UAS-Mmp-1 RNAi(69).

Acknowledgements

We acknowledge expert technical assistance by staff in the Advanced Light Microscopy Core, supported by grant P30NS061800. We thank Alexandra Houser for help with smiFISH experiments. We would also like to thank Dirk Bohmann, Bloomington Drosophila Stock Center at Indiana University, and the Developmental Studies Hybridoma Bank at the University of Iowa for flies and antibodies.

Funding

This work was supported by National Institutes of Health grants (R21 NS084112, R21 NS107771, and R01 NS117934), the Ken and Ginger Term Professor Award (M.A.L.), and the Tartar Trust Fellowship at Oregon Health and Sciences University (P.R.).

Conflict of Interest

The authors declare that no competing interests exist.

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Probeset Design

Probesets

Probeset	Concentration	Probe Volume in Hybridization Buffer	Hybridization Buffer Volume
Mmp-1 smiFISH	0.867μM	2μL	100μL
Mmp-1 HCR B2 FLARIM split B3	2.0μM	2μL	100μL
18S FLARIM split B3	1.6μM	2μL	100μL

FLARIM v2.0 Protocol

1 Dissection and Hybridization

2 Washes and Amplification

Supplementary Figure 1: Probe design and protocol workflow

Probeset design: Probesets to the transcript of interest were designed using Oligostan (Tsanov et al., 2016) and sequence similarity was compared to noncoding RNAs.

Probesets: Probeset sequences are listed in in Supplementary tables 1, 2, and 3. The smiFISH probeset assembly, protocol, and reagents are described in Tsanov et al., 2016. For the Mmp-1 HCR B2 FLARIM split B3 and 18S FLARIM split B3 probesets, the FLARIM v2.0 protocol was used (see Methods for details)

FLARIM v2.0 Protocol: Dissection and hybridization: fly brains are dissected and hybridized overnight with gene-specific and 18S ribosome probes in a thermal cycler. Washes and amplification: the following day, brains are washed and amplified with fluorescent hairpins to generate mRNA-specific and ribosome-association signals (see Methods for details).

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Supplementary Table 1: smiFISH *mmp-1* probe sequences

Probe	Sequence (5' → 3')
Mmp-1 smiFISH-1	CTAAGGTTACAAAATGCACTGCCCGTAGGTTACACTCGGACCTCGTCGACATGCATT
Mmp-1 smiFISH-2	AATCGGGCGTCATGGAATCTGTAGTACTTGTTTACACTCGGACCTCGTCGACATGCATT
Mmp-1 smiFISH-3	AGCCGAAC TGGGACAGGTAGATCTCTGTTACACTCGGACCTCGTCGACATGCATT
Mmp-1 smiFISH-4	CGACTATGAACACTGAACTTTGGCAGTTTGCTTACACTCGGACCTCGTCGACATGCATT
Mmp-1 smiFISH-5	GATCTTGGTGGTTAGATAAGGCGGGCAAGTCTTACACTCGGACCTCGTCGACATGCATT
Mmp-1 smiFISH-6	TATATAGTCACTATCACTTGGGCGAATTTGGCTTACACTCGGACCTCGTCGACATGCATT
Mmp-1 smiFISH-7	CAGGGCACTATCCACACTCTACACTCTTACACTCGGACCTCGTCGACATGCATT
Mmp-1 smiFISH-8	GGCTAGCACAGCTCCCAGTTTCCAAGTTACACTCGGACCTCGTCGACATGCATT
Mmp-1 smiFISH-9	GAGCTCCTTTCCCATGTGCCGACATTTTACACTCGGACCTCGTCGACATGCATT
Mmp-1 smiFISH-10	TTTGGAACTTTACTCGAGTGGCTAGCGTTGATTACACTCGGACCTCGTCGACATGCATT
Mmp-1 smiFISH-11	GCTGGTATCTCTTAGCTCTTTAGCTAACGTTACACTCGGACCTCGTCGACATGCATT
Mmp-1 smiFISH-12	GGCTGCGTCAAAGTCATCACCGTTGCTTACACTCGGACCTCGTCGACATGCATT
Mmp-1 smiFISH-13	GTTCGAACTCGTTGTGTCGAGCCTTCGACTTACACTCGGACCTCGTCGACATGCATT
Mmp-1 smiFISH-14	TATTACCTGTGGACGAGGGCGTGTTCTTGTACACTCGGACCTCGTCGACATGCATT
Mmp-1 smiFISH-15	GAATGCCCGTGAAGCCCTCGCTGATTTACACTCGGACCTCGTCGACATGCATT
Mmp-1 smiFISH-16	CTTAGGGTAGACGCCGTCATCTGACGTTACACTCGGACCTCGTCGACATGCATT
Mmp-1 smiFISH-17	GAAGAAGTACGCTCTTGCCGTTCTGTAGGTGATTACACTCGGACCTCGTCGACATGCATT
Mmp-1 smiFISH-18	CTTGGAGTCCTTGACAGATGGAGTCGTCTATTACACTCGGACCTCGTCGACATGCATT
Mmp-1 smiFISH-19	TCAGCTGGTTTGTTTTCGCCCATACAGTTACACTCGGACCTCGTCGACATGCATT
Mmp-1 smiFISH-20	CGCCTTGTCTGCTCGTCCAAC TTGAATTACACTCGGACCTCGTCGACATGCATT
Mmp-1 smiFISH-21	TCAGAGCGGAGCTCTGATCTGAGTGGGTACACTCGGACCTCGTCGACATGCATT
Mmp-1 smiFISH-22	CCACCTGGAACAGATTGGTGCCCTCGAGTTACACTCGGACCTCGTCGACATGCATT
Mmp-1 smiFISH-23	CCATGCTCGCTCTCCACGAAC TTGATTTACACTCGGACCTCGTCGACATGCATT
Mmp-1 smiFISH-24	GATCTTGTAGGTTAGGTTCTTCACACGCC TTACACTCGGACCTCGTCGACATGCATT
Mmp-1 smiFISH-25	CGCGAAGCTTTGGAAC TCTCGATGGCTTACACTCGGACCTCGTCGACATGCATT
Mmp-1 smiFISH-26	TGTGTGGTGGTGGAACGGGTGCCGATTGATTACACTCGGACCTCGTCGACATGCATT
Mmp-1 smiFISH-27	AGGCTCGCTATTTGTGTCATGTGTTCCACTTACACTCGGACCTCGTCGACATGCATT
Mmp-1 smiFISH-28	GCCGCTGGCACTTTGAACTTTATATCACTTACACTCGGACCTCGTCGACATGCATT
Mmp-1 smiFISH-29	TCTTTAGTTTCTTCACAGGGCTGTTTCGAAC TTACACTCGGACCTCGTCGACATGCATT
Mmp-1 smiFISH-30	TGCTGCCCTTGAAGAAGTATATCTTGCC TTACACTCGGACCTCGTCGACATGCATT
Mmp-1 smiFISH-31	CCCTGCGCCGAGTTGAAGAGTGTGCTTTACACTCGGACCTCGTCGACATGCATT
Mmp-1 smiFISH-32	GAAGGGGGGCGTGCCGAGTCAACAGTTACACTCGGACCTCGTCGACATGCATT
Mmp-1 smiFISH-33	CCCTTGAAGAAGTACGTGTAGCCATTGTTGTTTACACTCGGACCTCGTCGACATGCATT
Mmp-1 smiFISH-34	CTCTTGGCCGGATCGAAGCGCCAGAA TTACACTCGGACCTCGTCGACATGCATT
Mmp-1 smiFISH-35	CCCAAACCATTTGCGGCGTCCAGATGGTTACACTCGGACCTCGTCGACATGCATT
Mmp-1 smiFISH-36	CGGGTTCAAAGCCGCGATAGAAGGGCGCTTACACTCGGACCTCGTCGACATGCATT
Mmp-1 smiFISH-37	AAATGCGCGACCGATCTCCGCGTCGATTACACTCGGACCTCGTCGACATGCATT
Mmp-1 smiFISH-38	CAGCGGGCAGGGACATCAGCTTCATTTACACTCGGACCTCGTCGACATGCATT
Mmp-1 smiFISH-39	TATTTTTGTTTGGTTCTCTCCAATTCCTTGCA TTACACTCGGACCTCGTCGACATGCATT
Mmp-1 smiFISH-40	TTTGATTTCCTCTCATCGTATTCGATATGATTACACTCGGACCTCGTCGACATGCATT
Mmp-1 smiFISH-41	GCTGGTATCTCTTAGCTCTTTAGCTAACGTTACACTCGGACCTCGTCGACATGCATT

Supplementary Table 1: *mmp-1* smiFISH probeset
mmp-1 gene-specific sequences (magenta), FLAP sequence (blue; Tsanov, *et al.*, 2016)

Supplementary Table 2: 18S FLARIM split B3 probe sequences

Probe	Sequence (5' → 3')
18S FLARIM-sB3-1	CTTTGAGACAAGCATATAACTACTGGCAAAAAAAAAAAAAAGTCCCTGCCTCTATATCT
18S FLARIM-sB3-2	GCCTTTTGCGGTTTCACCTTTTAATTGCAAAAAAAAAAAAAAGTCCCTGCCTCTATATCT
18S FLARIM-sB3-3	TAGAATTACCACAGTTATCCAAGTAAAAAAAAAAAAAGTCCCTGCCTCTATATCT
18S FLARIM-sB3-4	CGCTTGCGTTTTAGCCTAATAAAAGCAAAAAAAAAAAAAAGTCCCTGCCTCTATATCT
18S FLARIM-sB3-5	TTGTTATTTTCGCTCACTACCTCCCCAAAAAAAAAAAAAGTCCCTGCCTCTATATCT
18S FLARIM-sB3-6	AAGTGTA CTACATTCCAATTACAGGGCAAAAAAAAAAAAAAGTCCCTGCCTCTATATCT
18S FLARIM-sB3-7	TATACGCTATTGGAGCTGGAATTACCGAAAAAAAAAAAAAGTCCCTGCCTCTATATCT
18S FLARIM-sB3-8	CGGTCCAAGAATTTACCTCTCAAAAAAAAAAAAAAGTCCCTGCCTCTATATCT
18S FLARIM-sB3-9	GAACCTCTA ACTTTCTGTTCTTGATTAA TGAAAAAAAAAAAAAGTCCCTGCCTCTATATCT
18S FLARIM-sB3-10	GCATCGTTTATGGTTAGAACTAGGGCGAAAAAAAAAAAAAGTCCCTGCCTCTATATCT
18S FLARIM-sB3-11	AAAGTTTCAGCTTTGCAACCATACTTAAAAAAAAAAAAAGTCCCTGCCTCTATATCT
18S FLARIM-sB3-12	GGTAAGTTTTCGCGTTTGAGTCAAATTAAAAAAAAAAAAAAGTCCCTGCCTCTATATCT
18S FLARIM-sB3-13	CACCCATAGATTCGAGAAAGAGCTAAAAAAAAAAAAAGTCCCTGCCTCTATATCT
18S FLARIM-sB3-14	GGAATTAACCAGACAAATCACTCCAAAAAAAAAAAAAAGTCCCTGCCTCTATATCT
18S FLARIM-sB3-15	CTGTTATTGCTCAATCTCATTATTGCTAAAAAAAAAAAAAGTCCCTGCCTCTATATCT
18S FLARIM-sB3-16	AGCATGAAAGTGGTTCAGCGGTTTACAAAAAAAAAAAAAGTCCCTGCCTCTATATCT
18S FLARIM-sB3-17	CACACTTACTGGGAATTCCAAGTTCATAAAAAAAAAAAAAAGTCCCTGCCTCTATATCT
18S FLARIM-sB3-18	GGTGTGTACAAAGGGCAGGGACGTAAAAAAAAAAAAAGTCCCTGCCTCTATATCT
18S FLARIM-sB3-19	TGATCCTTCCGCAGGTTACCTACGAAAAAAAAAAAAAGTCCCTGCCTCTATATCT
18S FLARIM-sB3-20	CAATTTGTCCATTTAAGAAGCTAGTGTCAAAAAAAAAAAAAAGTCCCTGCCTCTATATCT
18S FLARIM-sB3-21	GTTTCCCGGGAAGCGACTGAGAGAAAAAAAAAAAAAGTCCCTGCCTCTATATCT
18S FLARIM-sB3-22	CCAGACTTGCCCTCCAATTGGTCCTTAAAAAAAAAAAAAGTCCCTGCCTCTATATCT
18S FLARIM-sB3-23	GTTACCGTTGCAACCATGGTAGTCAAAAAAAAAAAAAAGTCCCTGCCTCTATATCT
18S FLARIM-sB3-24	CTGCCTTCCTTAGATGTGGTAGCCGAAAAAAAAAAAAAGTCCCTGCCTCTATATCT
18S FLARIM-sB3-25	TCCCTCTCCGGAATCGAACCCCTGATAAAAAAAAAAAAAGTCCCTGCCTCTATATCT

Supplementary Table 2: 18S FLARIM split B3 probe sequences

18S ribosome-specific probe sequences (blue) and B3 split initiator sequence (green; Choi *et al.*, 2014).

Supplementary Table 3: *mmp-1* HCR B2 FLARIM split B3 probe sequences

Probe	Sequence (5' → 3')
Mmp1 HCR-B2/FLARIM-sB3-1	CCTCGTAAATCCTCATCAATCATCCAGTAAACCGCCAAAAAATAAGGTTCCAAATGCACGTGCCGCTAGGAAAAAAAAAAACCACTCAACTTTAACC
Mmp1 HCR-B2/FLARIM-sB3-2	CCTCGTAAATCCTCATCAATCATCCAGTAAACCGCCAAAAAATCGGGCGTCATGGAATCTGTAGTACTTGTAAAAAAAAAAACCACTCAACTTTAACC
Mmp1 HCR-B2/FLARIM-sB3-3	CCTCGTAAATCCTCATCAATCATCCAGTAAACCGCCAAAAAGCCGAACGGGACAGGTAGATCTCTGAAAAAAAAAAACCACTCAACTTTAACC
Mmp1 HCR-B2/FLARIM-sB3-4	CCTCGTAAATCCTCATCAATCATCCAGTAAACCGCCAAAAAGACTATGAACACTGAACTTTGGCAGTTTGTCAAAAAAAAAAAACCACTCAACTTTAACC
Mmp1 HCR-B2/FLARIM-sB3-5	CCTCGTAAATCCTCATCAATCATCCAGTAAACCGCCAAAAAGATCTTGGTGGTTAGATAAGGCGGGCAAGTCAAAAAAAAAAAACCACTCAACTTTAACC
Mmp1 HCR-B2/FLARIM-sB3-6	CCTCGTAAATCCTCATCAATCATCCAGTAAACCGCCAAAAATATATAGTCACTATCACTTGGGCGAATTTGGCAAAAAAAAAAAACCACTCAACTTTAACC
Mmp1 HCR-B2/FLARIM-sB3-7	CCTCGTAAATCCTCATCAATCATCCAGTAAACCGCCAAAAAGGGGCACTATCCACACTCTACACTCAAAAAAAAAAAACCACTCAACTTTAACC
Mmp1 HCR-B2/FLARIM-sB3-8	CCTCGTAAATCCTCATCAATCATCCAGTAAACCGCCAAAAAGGCTAGCACAGCTCCAGTTTCCAAGAAAAAAAAAAACCACTCAACTTTAACC
Mmp1 HCR-B2/FLARIM-sB3-9	CCTCGTAAATCCTCATCAATCATCCAGTAAACCGCCAAAAAGAGCTCCTTTCCCATGTGCCGACATTAAAAAAAAAAACCACTCAACTTTAACC
Mmp1 HCR-B2/FLARIM-sB3-10	CCTCGTAAATCCTCATCAATCATCCAGTAAACCGCCAAAAATTGGAACCTTTACTCGAGTGCGCTAGCGTTGAAAAAAAAAAAACCACTCAACTTTAACC
Mmp1 HCR-B2/FLARIM-sB3-11	CCTCGTAAATCCTCATCAATCATCCAGTAAACCGCCAAAAAGCTGGTATCTCTTAGCTCTTTTAGCTAACGAAAAAAAAAAACCACTCAACTTTAACC
Mmp1 HCR-B2/FLARIM-sB3-12	CCTCGTAAATCCTCATCAATCATCCAGTAAACCGCCAAAAAGGCTGCGTCAAAGTCATCACCGTTGCAAAAAAAAAAAACCACTCAACTTTAACC
Mmp1 HCR-B2/FLARIM-sB3-13	CCTCGTAAATCCTCATCAATCATCCAGTAAACCGCCAAAAAGTTGCAACTGTTGTGCGAGCCTTCGACAAAAAAAAAAACCACTCAACTTTAACC
Mmp1 HCR-B2/FLARIM-sB3-14	CCTCGTAAATCCTCATCAATCATCCAGTAAACCGCCAAAAATATTACCTGTGAACGAGGGCGTGTCTTGAAAAAAAAAAACCACTCAACTTTAACC
Mmp1 HCR-B2/FLARIM-sB3-15	CCTCGTAAATCCTCATCAATCATCCAGTAAACCGCCAAAAAGAATGCCCGTGAAGCCCTCGCTGATTAAAAAAAAAAACCACTCAACTTTAACC
Mmp1 HCR-B2/FLARIM-sB3-16	CCTCGTAAATCCTCATCAATCATCCAGTAAACCGCCAAAAATTAGGGTAGACGCCGTCATCTGACGAAAAAAAAAAACCACTCAACTTTAACC
Mmp1 HCR-B2/FLARIM-sB3-17	CCTCGTAAATCCTCATCAATCATCCAGTAAACCGCCAAAAAGAAGAAGTAGTCTTGCCGTTCTTTAGTGGTGAAAAAAAAAAAACCACTCAACTTTAACC
Mmp1 HCR-B2/FLARIM-sB3-18	CCTCGTAAATCCTCATCAATCATCCAGTAAACCGCCAAAACTTGGAGTCCTTGCAGATGGAGTCGCTTAAAAAAAAAAAACCACTCAACTTTAACC
Mmp1 HCR-B2/FLARIM-sB3-19	CCTCGTAAATCCTCATCAATCATCCAGTAAACCGCCAAAAACAGCTGGTTTGTGTCGCCATACAGAAAAAAAAAAACCACTCAACTTTAACC
Mmp1 HCR-B2/FLARIM-sB3-20	CCTCGTAAATCCTCATCAATCATCCAGTAAACCGCCAAAAAGCCTTGTGCTCCTCGTCCAACCTTGAAAAAAAAAAACCACTCAACTTTAACC
Mmp1 HCR-B2/FLARIM-sB3-21	CCTCGTAAATCCTCATCAATCATCCAGTAAACCGCCAAAAATCAGAGCGGAGCTCTGATCTGAGTGGGAAAAAAAAAAACCACTCAACTTTAACC
Mmp1 HCR-B2/FLARIM-sB3-22	CCTCGTAAATCCTCATCAATCATCCAGTAAACCGCCAAAAACACCTGGAAACAGATTGGTGCCTCGAGAAAAAAAAAAACCACTCAACTTTAACC
Mmp1 HCR-B2/FLARIM-sB3-23	CCTCGTAAATCCTCATCAATCATCCAGTAAACCGCCAAAAACCATGCTCGCTCTCCACGAACTTGATAAAAAAAAAAACCACTCAACTTTAACC
Mmp1 HCR-B2/FLARIM-sB3-24	CCTCGTAAATCCTCATCAATCATCCAGTAAACCGCCAAAAAGATCTTGTAGGTGAGGTTCTTCCACAGCCAAAAAAAAAAACCACTCAACTTTAACC
Mmp1 HCR-B2/FLARIM-sB3-25	CCTCGTAAATCCTCATCAATCATCCAGTAAACCGCCAAAAAGCGGAAGCTTTGGAACCTCCTCGATGGCAAAAAAAAAAAACCACTCAACTTTAACC
Mmp1 HCR-B2/FLARIM-sB3-26	CCTCGTAAATCCTCATCAATCATCCAGTAAACCGCCAAAAATGTGTGGTGGTGGAAACGGGTGCCGATTGAAAAAAAAAAAACCACTCAACTTTAACC
Mmp1 HCR-B2/FLARIM-sB3-27	CCTCGTAAATCCTCATCAATCATCCAGTAAACCGCCAAAAAGGCTCGCTATTTGTGTCATGTGGTTCACAAAAAAAAAAACCACTCAACTTTAACC
Mmp1 HCR-B2/FLARIM-sB3-28	CCTCGTAAATCCTCATCAATCATCCAGTAAACCGCCAAAAAGCCGCTGGCACTTTGAACCTTTATATCACAAAAAAAAAAACCACTCAACTTTAACC
Mmp1 HCR-B2/FLARIM-sB3-29	CCTCGTAAATCCTCATCAATCATCCAGTAAACCGCCAAAAATCTTTAGTTTCTTCCAGGGCTGTTTGAACATAAAAAAAAAAAACCACTCAACTTTAACC
Mmp1 HCR-B2/FLARIM-sB3-30	CCTCGTAAATCCTCATCAATCATCCAGTAAACCGCCAAAAAGCTGCCTTGAAGAAGTATATCTTGCCAAAAAAAAAAACCACTCAACTTTAACC
Mmp1 HCR-B2/FLARIM-sB3-31	CCTCGTAAATCCTCATCAATCATCCAGTAAACCGCCAAAAACCTGCGCGAGTTGAAGAGTGTGTCTAAAAAAAAAAACCACTCAACTTTAACC
Mmp1 HCR-B2/FLARIM-sB3-32	CCTCGTAAATCCTCATCAATCATCCAGTAAACCGCCAAAAAGAAGGGGGCGTGGCCGAGTCAACAGAAAAAAAAAAACCACTCAACTTTAACC
Mmp1 HCR-B2/FLARIM-sB3-33	CCTCGTAAATCCTCATCAATCATCCAGTAAACCGCCAAAAACCTTGAAGAAGTACGTGTAGCCATTGGTGTAAAAAAAAAAACCACTCAACTTTAACC
Mmp1 HCR-B2/FLARIM-sB3-34	CCTCGTAAATCCTCATCAATCATCCAGTAAACCGCCAAAAACTTTGGCGGATCGAAGCGCCAGAAAAAAAAAAAAACCACTCAACTTTAACC
Mmp1 HCR-B2/FLARIM-sB3-35	CCTCGTAAATCCTCATCAATCATCCAGTAAACCGCCAAAAACCAAAACCATTTGCGGCTCCAGATGGAAAAAAAAAAACCACTCAACTTTAACC
Mmp1 HCR-B2/FLARIM-sB3-36	CCTCGTAAATCCTCATCAATCATCCAGTAAACCGCCAAAAAGGGTTCAAAGCCGCGATAGAAGGGCGCAAAAAAAAAAAACCACTCAACTTTAACC
Mmp1 HCR-B2/FLARIM-sB3-37	CCTCGTAAATCCTCATCAATCATCCAGTAAACCGCCAAAAAATGCGCGACCGATCTCCGCTCGAAAAAAAAAAACCACTCAACTTTAACC
Mmp1 HCR-B2/FLARIM-sB3-38	CCTCGTAAATCCTCATCAATCATCCAGTAAACCGCCAAAAAGCGGGGGCAGGGACATCAGCTTCATAAAAAAAAAAAACCACTCAACTTTAACC
Mmp1 HCR-B2/FLARIM-sB3-39	CCTCGTAAATCCTCATCAATCATCCAGTAAACCGCCAAAAATATTTTGTGTTGTTCTCTCCAATTCCTTGCAAAAAAAAAAAACCACTCAACTTTAACC
Mmp1 HCR-B2/FLARIM-sB3-40	CCTCGTAAATCCTCATCAATCATCCAGTAAACCGCCAAAAATTGGATTTCCTCTCATCGTATTCGATATGATAAAAAAAAAAAACCACTCAACTTTAACC
Mmp1 HCR-B2/FLARIM-sB3-41	CCTCGTAAATCCTCATCAATCATCCAGTAAACCGCCAAAAAGCTGGTATCTCTTAGCTCTTTTAGCTAACGAAAAAAAAAAACCACTCAACTTTAACC

Supplementary Table 3: *mmp-1* HCR B2 FLARIM split B3 probe sequences
mmp-1 gene-specific sequences (magenta), B3 split initiator sequence (green; Choi *et al.*, 2014), and full B2 initiator sequence (orange; Choi *et al.*, 2014).