**AMDCC Pilot and Feasibility Program: Progress Report**

**PROJECT TITLE:** Detecting Diabetic Neuropathy in Rodents by Imaging Corneal Nerves

**TIMELINE:** AWARD START DATE: 30th September 2009
NOTICE OF EXECUTION OF ARRA SUB-CONTRACT: 26th January 2010
FUNDS AVAILABLE: 10th February 2010
CORNEAL CONFOCAL MICROSCOPE INSTALLED: 22nd April 2010
PROGRESS REPORT: 30th September 2010

**SPECIFIC AIMS:**

1. To evaluate the viability of using corneal confocal microscopy (CCM) to visualize sensory nerves in the corneal stroma of rats and mice both *in vivo* and *ex vivo.*

2. To determine whether CCM can detect structural neuropathy in mouse models of diabetic complications currently being developed by the AMDCC and to compare CCM against the current indices of structural pathology being used to phenotype neuropathy in these models.

3. To establish whether CCM can detect onset and progression of structural neuropathy in a longitudinal study of cohorts of diabetic mice and to compare CCM against currently accepted indices

   of peripheral neuropathy in these animals.

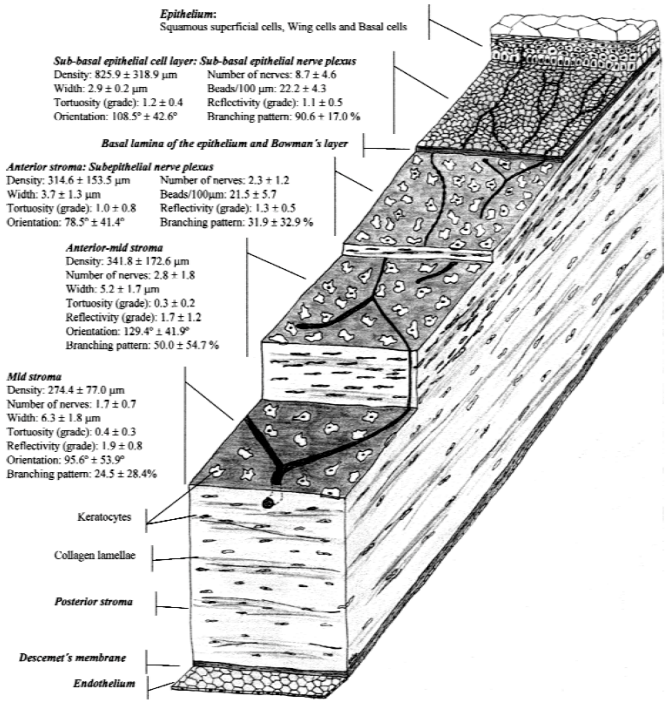


Figure 1. Corneal anatomy. From Oliveira-Soto and Efron, 201

**PROGRESS REPORT**

**General Observations**
Delays in gaining access to award funds and the delivery and installation of the corneal confocal microscope (CMM) have meant that work on the project began only in May 2009 (see timeline above). Consequently, data in this report reflects only studies performed over the last 5 months.

**Specific Aim 1**
*CCM calibration:* As the CCM is designed for human use, we validated our machine and technique by imaging nerves from human volunteers and comparing images with those in the published literature.
Structures corresponding to those identified as corneal nerves were identified in the corneal stroma between Descemet's membrane and Bowman's layer. Other structures noted as the scan passed through the cornea were epithelial cells, Bowman's layer and resident dendritic cells of the local immune system (see Fig. 2).

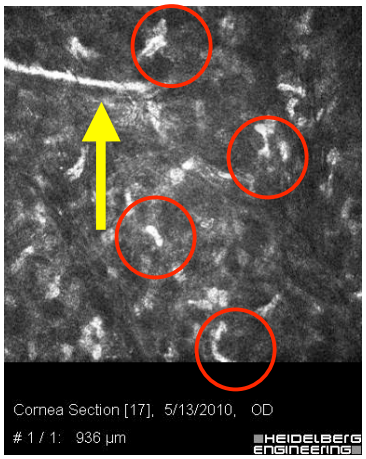


Figure 2. Nerve fiber (arrow) and dendritic cells (circled) in the corneal stroma of a human subject.

*In vivo imaging*: We constructed a restraint and anesthesia system that allows mice and rats to undergo CCM imaging under transient inhalable anesthesia, so that they can recover after the procedure and be used for repeated studies over time. Using this system, we identified corneal nerve fibers in live rats and mice (Fig. 3.).

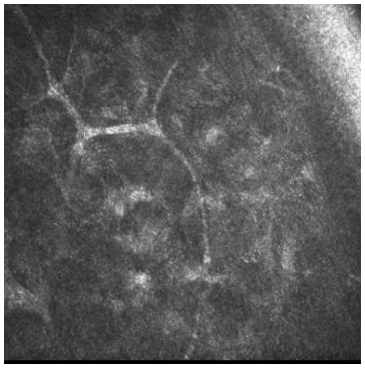
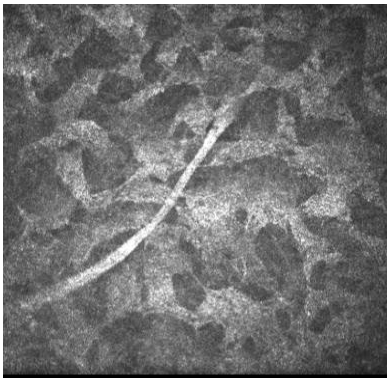


Figure 3. Corneal nerve fibers in the stroma of mouse (left) and rat (right).



Consultation with Dr. Rayaz Malik (University of Manchester, UK) confirmed our interpretation of the images. However, Dr. Malik noted that the clinical studies using CCM that have quantified corneal nerve fiber loss in diabetic patients (Quatrinni et al. 2007) made their measurements using the distal tips of the corneal nerves that are located within or adjacent to Bowman's layer as a sub-epithelial nerve plexus. This would equate to measurements of C fiber terminal branches in the epidermis, whereas the images of Figure 3 could be equated to more proximal portions of nerve in the dermis. As diabetic neuropathy is considered a dying back neuropathy, examining the most distal portions of nerves offers the greatest likelihood of detecting early disorders. Bowman's layer also offers a convenient anatomic reference point so that all measurements can be made in the same plane. Unfortunately, it is a little known fact (amongst us mere peripheral nerve dudes at least) that mice and rats have a poorly defined Bowman's layer, which explains why we were not seeing one in our scans (Labbe et al. 2006). We have now refined our image searching and can identify distal corneal nerve fibers in Bowman's layer of rats and mice (Figure 4)

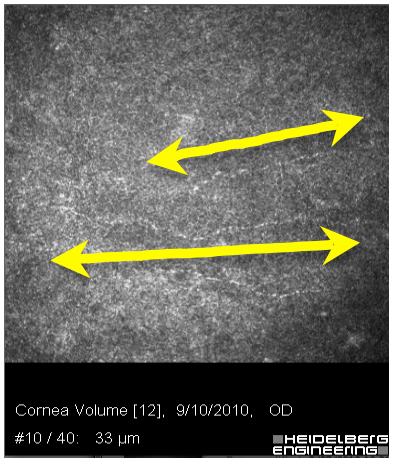
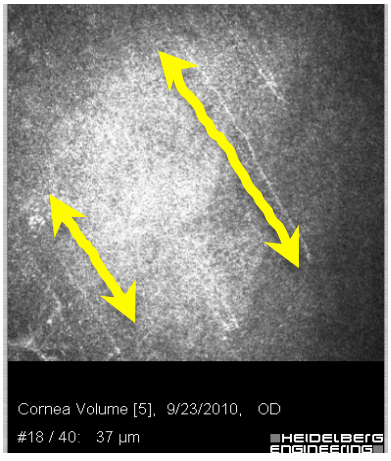



Figure 4. Distal corneal nerve fibers in Bowman's layer of mouse (left) and rat (right). Nerve fibers run adjacent to the yellow illustration lines. Note that the diameter of these fibers is significantly smaller than those of Figure 3, despite all images being at the same magnification. Each image also shows an image depth (33 or 37 µm), although at present this represents only depth after the first image is captured and not necessarily the distance from the corneal surface.




*ex vivo imaging:* The purpose of these studies was to determine whether corneal nerves could be preserved after removal of the eye so that eyes could be removed, stored and shipped to UCSD from AMDCC sites that are generating mouse models. We therefore imaged corneal nerves in live mice prior to removing the eyes and placing them in assorted solutions at 4°C, where they were maintained for up to 7 days before repeat imaging (Fig. 5). We scored the integrity of the deep corneal nerves at each time on a 3 point scale: + = nerve structure equivalent to in vivo image, +/X = nerves viable, X = Nerves structurally damaged or absent. As shown in Table 1, both 0.9% saline and 0.1M phosphate buffer allowed storage at 4°C for at least 3 days, with reasonable preservation of structure for up to 7 days. Sadly, immersion fixation in 10% formalin disrupted corneal structure such the no viable nerves were identified. This is unfortunate, as eyes collected by the AMDCC to date have been routinely stored in this fixative.

| **TABLE 1** | Day 1 | Day 2 | Day 3 | Day 4 | Day 7 |
|---|---|---|---|---|---|
| 0.9% saline | + | + | + | + | +/X |
| 0.1M phosphate buffer | + | + | + | + | +/X |
| 10% formalin | X | X | X | X | X |

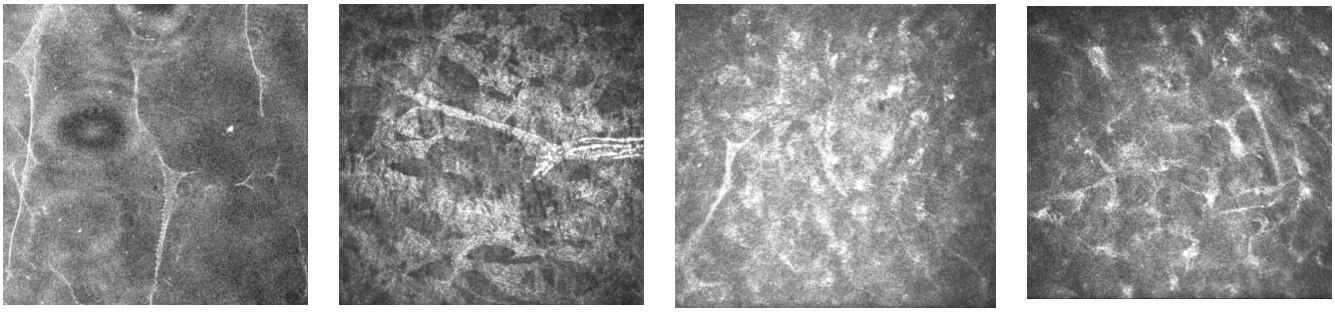
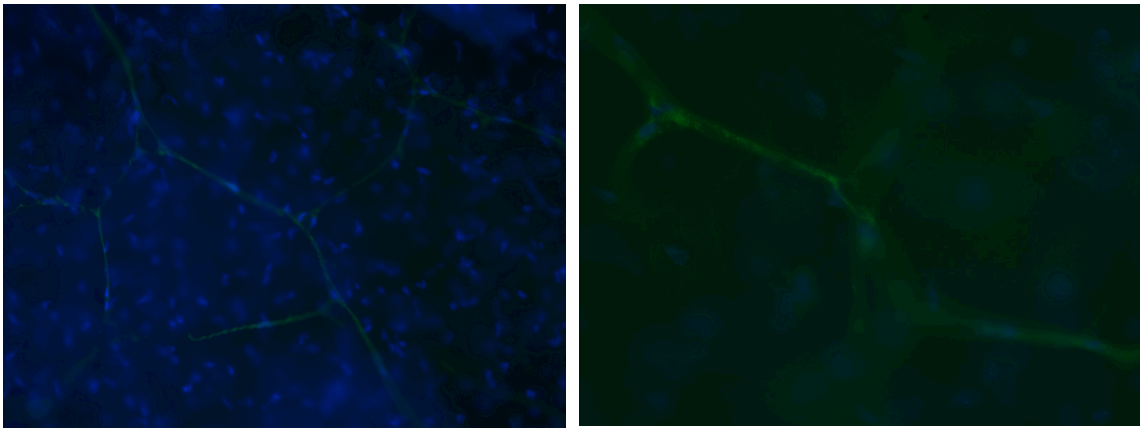


Figure 5: Corneal nerve fibers detected in the eye of a live mouse (left) and on days 1, 4 and 7 (left to right) after removal and storage of the eye in 0.9% sterile saline at 4°C.

*Image Validation*: To confirm that the structures being viewed represent corneal nerve fibers, we immunostained unfixed corneas using an antibody to the pan-neuronal marker PGP9.5, as used routinely to identify epidermal nerve fibers (Quatrinni et al. 2007; Beiswenger et al. 2008).

Figure 6.

PGP9.5 +ve nerves (green) in the mouse cornea. Cell nuclei are counterstained (blue).



*Ongoing and follow-up studies:* We are currently working on the following refinements:

1. We have purchased 3D image reconstruction software that we hope to interface with the Heidelberg software so that the larger nerve fibers easily detected in deep stroma can be traced distally to the sub-epidermal plexus. I have recently appointed a post-doctoral fellow in bioengineering who has experience designing imaging systems who will develop this enhancement of the system.
2. We are developing a novel quantification system based on the particular needs of rodent CCM images.
3. Now that we have gained experience detecting the distal branches of corneal nerve fibers, we will re-visit the *ex vivo* studies to determine the preservation of these fibers in saline and phosphate buffer. We are also trying a few alternative fixatives in the hope that we can find something that allows us to receive material from AMDCC sites.
4. We are expanding the immunostaining of corneal nerves to characterize their phenotype as either peptidegic or non-peptidergic (Jones and Marfurt, 1998).

**SPECIFIC AIM 2**

This aim is on hold until we can work out a better fixative than 10% formalin that would also be acceptable to other members of AMDCC that are working with the eyes.

**SPECIFIC AIM 3**

This aim is dependent on completing development of a quantification system for rodents (see above). We have a number of studies involving diabetic rats or mice in progress and CCM images are being collected to provide cross-sectional evaluations of any quantitative differences induced by diabetes. Once the quantification system is verified, we will start longitudinal studies to evaluate the progression of changes to corneal nerve fibers relative to those in epidermal nerve fibers.

**REFERENCES**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