Chapter 3

Imaging MS of Rodent Ocular Tissues and the Optic Nerve

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Abstract

The visual system is comprised of many specialized cell types that are essential for relaying sensory information about an animal's surroundings to the brain. The cells present in ocular tissue are notoriously delicate, making it particularly challenging to section thin slices of unfixed tissue. Maintaining the morphology of the native tissue is crucial for accurate observations by either conventional staining techniques or in this instance matrix-assisted laser desorption ionization (MALDI IMS) or imaging using mass spectrometry. As vision loss is a significantly debilitating condition, studying molecular mechanisms involved in the process of vision loss is a critically important area of research.

Key words Matrix-assisted laser desorption ionization, Imaging mass spectrometry, Rodent ocular tissue, Optic nerve, Ocular pathology

1 Introduction

Matrix-assisted laser desorption ionization imaging mass spectrometry (MALDI IMS) or imaging using mass spectrometry methodologies has seen significant advances in recent years, with improvements in both instrumentation and sample preparation techniques allowing for high-quality, high spatial resolution data to be acquired from single cells and small anatomical structures [1–3]. This high spatial resolution molecular information allows for the mapping of molecular spatial distributions occurring in fine structures, such as the cells that comprise ocular tissues [4–8]. The highly organized cellular structure of the neural retina is made up of multiple cell types of varying dimensions (~10–80 μm) in close proximity. The main function of these cells is to relay information gathered by the light-sensitive photoreceptors to the brain through the optic nerve. Disruption of cellular processes in this region of the body can result in a variety of blinding diseases. Due to the small size and varying densities of the tissues present in the eye globe, special considerations need to be made to prevent

Laura M. Cole (ed.), *Imaging Mass Spectrometry: Methods and Protocols*, Methods in Molecular Biology, vol. 1618,
DOI 10.1007/978-1-4939-7051-3_3,

compression and stretching of these regions during the sectioning process. Obtaining sections from fresh frozen tissues, while preserving the morphology, is crucial so that they are suitable for high spatial resolution MALDI IMS, in order to reveal the native distribution of molecular information.

Ice crystal formation during freezing has long been known to affect the resulting morphology of fresh frozen tissues. Rapid freezing prevents ice crystal growth and subsequent damage to the tissue. Liquid nitrogen provides the lowest temperature to freeze tissue in a laboratory environment, although immersing tissue directly into liquid nitrogen results in the outside of the tissue freezing much faster than the central regions. This causes expansion of the central regions and resulting in a pressure differential in the tissue and freeze fracturing of the tissues during sectioning. Rapid freezing of ocular tissues using liquid nitrogen indirectly (liquid nitrogen cooled environment) preserves the morphology of the native tissue and prevents freeze fracturing of the tissue.

The rodent lens occupies a large proportion of the ocular globe and therefore it presents several problems for the analysis of the neural retina or cornea from rodent ocular tissue. The density of the lens affects section quality, notably around the central region of the ocular globe, with its density being greatest at the central core region. As a result, stretching during sectioning of the surrounding neural retina and cornea occurs and gives poor quality sections. Another problem posed by the lens tissue is the vast quantities of protein (up to 300 mg/mL) present in the fiber cells of the tissue. This high concentration of protein prevents successful washing of the tissue, as the proteins are difficult to completely remove and often contaminate the surrounding tissue regions. This contamination may result in ion suppression or high background signals, producing misleading results. Removal of the rodent ocular lens without disruption to the morphology of the surrounding tissue from a freshly dissected or unfrozen tissue is challenging, and this is addressed in the following paragraphs.

Analysis of molecular information from the optic nerve of rodent tissue using high spatial resolution imaging mass spectrometry also requires specific considerations based on the size of this tissue. Many animal models of ocular disease have been developed in mice. The diameter of a mouse optic nerve is approximately 400 μm and its length can vary depending on the dissection. Cutting sections of optic nerve tissue, with small dimensions from distal to proximal at the region of the optic nerve, requires alignment in the same axis along the length of this tissue. Alignment is important because, an un-straightened nerve is free to travel in and out of the sectioning plane, requiring analysis of many sections to gather information from all regions distal to proximal.

One way to improve section quality and reproducibility is embedding of the tissue; however, conventional embedding compounds

such as optimal cutting temperature media (OCT) or paraffin wax are not suitable for the MALDI ionization process without special sample preparation techniques. Carboxyoxymethyl cellulose (CMC) has been demonstrated on a number of tissue types to help in the sectioning process [4, 9]. Orientation of the sample in an embedding medium so that sections can be obtained along the optic nerve tissue from proximal to distal in the same section also poses a challenge.

In this chapter, we will discuss sample preparation methods for ocular tissue from rodents to help preserve the morphology of the neural retina and prevent contamination from proteins present in the ocular lens. An example is provided that demonstrates alignment of the optic nerve tissue allowing for longitudinal sections along the optic nerve to the optic nerve head with the neuronal tissue passing into the neural retina.

2 Materials

2.1 Sample Preparation

1. Carboxymethyl cellulose sodium salt.
2. Hypodermic needle, gauge 19–21.
3. Cryostat.
4. Large weighing boat $5^1/_2'' \times 7/8''$ H.
5. 100 mM ammonium acetate.
6. 2,5-Dihydroxybenzoic acid (DHB).
7. Nikon Eclipse 90i microscope.
8. Craftman 8 in. drill press.

2.2 Data Acquisition and Processing

1. IMS data was acquired using a Bruker Ultraflextreme II TOF/TOF mass spectrometer.
2. Data acquisition and image processing was performed using Fleximaging 4.1.

3 Methods

3.1 CMC Embedding Methods for Rodent Ocular Tissues

Embedding has long been the method of choice to improve quality of sections for pathological interpretation or immunohistochemistry techniques, although many of these techniques are not suitable for analysis by mass spectrometry without special sample preparation methodologies. OCT is a compound embedding medium comprised of polymeric molecules such as polyethylene glycol (PEG). This medium provides a dense resilient solid material once frozen, and provides sections with limited stretching or compression of the tissue as it passes across the blade. Due to the chemical composition of OCT ions, suppression of tissue-derived

signals is commonly observed without prior washing of the section. CMC is also polymeric in nature, but unlike OCT, does not suppress MALDI signal and was first demonstrated by the analysis of whole-body animal sectioning [9]. CMC also provides a dense medium with resilient properties once frozen.

3.2 Preparation of Carboxymethyl Cellulose Solution

Dissolving CMC powder in deionized water can take many hours, depending on the concentration. The concentration should be optimized based on the sample type, with higher concentrated solutions (up to 5%) better suited for denser tissues (*see* **Note 1**). Practical concentrations for rodent and human ocular tissue are 1–2.7%

1. Weigh out the desired amount of CMC and place in a suitable bottle along with a magnetic stir bar, and shake the solution to ensure the powder becomes immersed.
2. Place the bottle onto a hot plate at 70 °C and initiate the magnetic stirrer at a moderate speed.
3. Leave the solution on the hot plate until CMC power is completely dissolved, checking periodically to ensure the stir bar is still cycling and shaking to dislodge large clumps of undissolved material.
4. Once dissolved, allow to cool to room temperature. Degas the solution prior to use, using a sonication bath (*see* **Note 2**).
5. The solution should be stored at 4 °C to prevent microbial growth and sonicated to degas before each use.

3.3 Embedding of Eye Tissue for Neuronal Retina Analysis Using CMC

Because the neural retina is a very delicate tissue, rapid freezing of the tissue is essential to retain the morphology in its native form. Since immersing room or body temperature tissue directly into liquid nitrogen results in freeze fracturing of tissue, freezing in a liquid nitrogen cooled environment provides the most optimal freezing times. CMC is also affected by freezing time, affecting the resulting structural integrity of the support material; better results are observed with faster freezing times.

1. Care should be taken to gently enucleate the ocular globe from the sacrificed animal including as much of the optic nerve if required. Freeze enucleated eye immediately on a large plastic weighing boat floating on top of liquid nitrogen in a desired orientation.
2. Once the tissue is frozen throughout (freezing time depends on sample size) CMC solution can then be conservatively applied to the sample, ensuring it does not defrost the tissue, using a 1 mL or Pasteur pipette, coating the tissue surface first on one side, the weigh boat can be removed briefly from the liquid nitrogen to better observe the sample.

3. Once the CMC from the first application has frozen more can be added and frozen if needed. The sample can be inverted using precooled forceps before adding more CMC to the opposite side to encase the tissue.

3.4 CMC Embedding for Optic Nerve Analysis with Alignment Using a Hypodermic Needle

1. Once enucleated the eye can be mounted onto a medium gauge (19–21G) needle, slightly piercing the globe around the region of the optic nerve head. The remaining optic nerve tissue should be straightened along the needle, marking the Luer lock region in the place aligned with the tissue as seen in Fig. 1a.
2. The tissue should then be frozen by immersing the base of the needle into liquid nitrogen without immersing the actual tissue, while holding with long forceps as seen in Fig. 1b, after 1–2 min, the pupil will take on an opaque appearance once the lens has frozen.
3. The tissue on the end of the needle can then be quickly immersed into the CMC solution contained in a weighing boat with suitable depth and the sample can now be immersed in liquid nitrogen to encase the tissue. This process can be repeated a number of times until sufficient material is surrounding the sample.
4. The sample should be handled with care, as any twisting of the sample or the needle could result in the mark on the Luer lock used to indicate the tissue region becoming displaced.

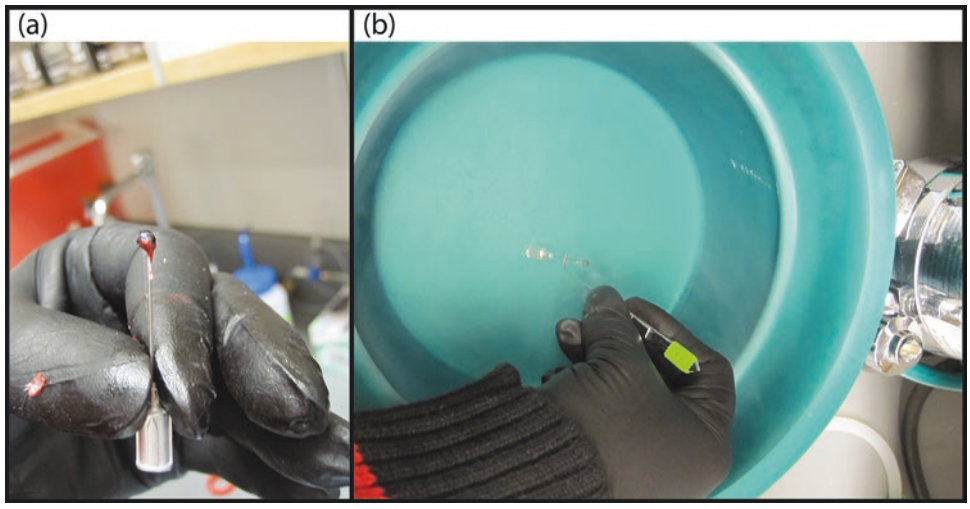

Fig. 1 Image displaying (**a**) alignment of mouse optic nerve tissue along hypodermic needle after dissection, (**b**) displaying initial freezing process where the lower portion of the hypodermic needle is immersed into liquid nitrogen using long handled forceps before immersing in CMC solution

3.5 Cryostat Sectioning of Ocular Tissue

3.5.1 Obtaining Sections for Neural Retina and Cornea Analysis

1. Samples should be mounted onto the cryostat chuck using OCT in the desired orientation; a flat surface can be shaved onto the mounting CMC surface using a razor blade to ensure correct orientation and good adherence.
2. The angle of the stage should be adjusted to allow for a less acute passage of the tissue section over the blade as seen in Fig. 2, as acute angles cause the surrounding CMC to fragment and lose structural integrity.
3. CMC should be trimmed using larger section increments of 30–50 μm until the tissue becomes visible.
4. Before section procurement, the blade should be replaced as trimming can cause blade dulling that will diminish section quality. Once the tissue is visible, excess CMC can now be removed from around the sample using a precooled razor blade if necessary.
5. Once a desired region for sectioning is reached, the section can be manipulated into position on the target surface using a fine-tipped paint brush on the surrounding CMC material, preventing contamination or disruption of the tissue.
6. Flat sections thaw mount better than wrinkled sections as wrinkled sections thaw at different rates across the tissue. This can cause tissues to stretch and fold, affecting the morphology and resulting in artifacts in the MALDI IMS data. One way to prevent wrinkled and stretched sections is to remove the lens, *see* Subheading 3.6.

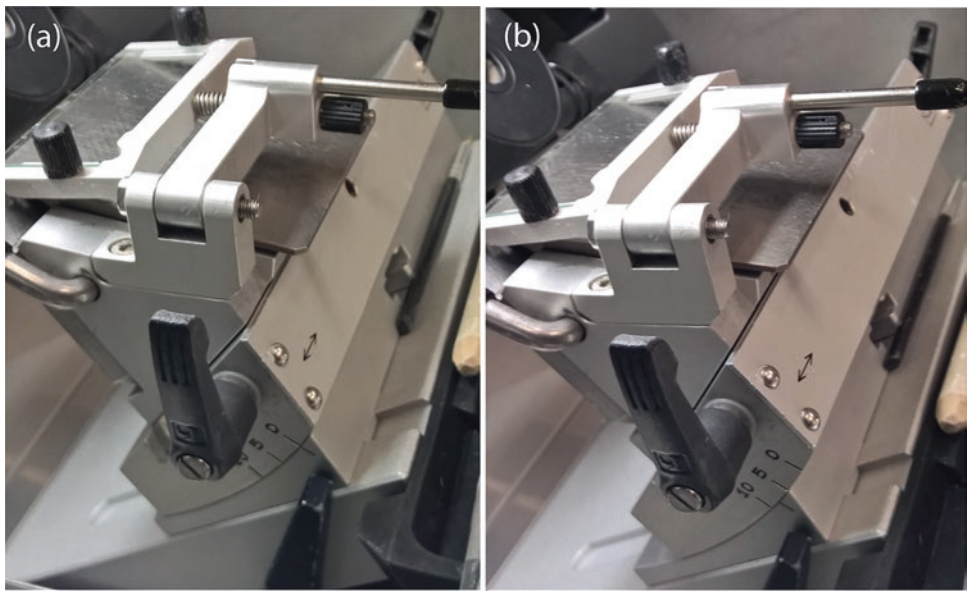

Fig. 2 (**a**) Image showing less acute angle on a Leica CM 3050 S sample stage. (**b**) Image showing more acute angle on a Leica CM 3050 S sample stage

7. Once thaw mounted onto a target plate or slide, the section should be air dried immediately, while providing heat from a gloved hand before returning the sample to the cryostat or to a vacuum desiccator.

3.5.2 Obtaining Sections from Aligned Optic Nerves

1. Attach the sample to the sample chuck, with the mark on the Luer lock indicating the position of the tissue on the needle facing downward toward the sample chuck. The sample should be frozen to the sample chuck using minimal OCT, keeping the needle as parallel to the chuck as possible as seen in Fig. 3.
2. Once attached the chuck the sample can be placed in the sample chuck holder. The sample can be advanced toward the blade using the manual control adjustment on the cryostat to bring the sample closer to the blade and sample stage. Make adjustments to the sample holder until the needle is as visually parallel to the blade as possible.
3. The sample can now be trimmed to allow the needle to get closer to the blade to make adjustment more accurate, taking care not to let the luer lock collide with the blade, Fig. 4. Once satisfied with the adjustment the needle can be pulled from the embedded sample using a gentle twisting motion. Continue with trimming until the impression left behind by the needle is visible on the block face as seen in Fig. 5, initially the widest region of the impression indicates closest region to the blade, fine adjustments can be made to ensure the sample alignment is optimal. The cryostat blade should be replaced at this point as trimming of the

Fig. 3 Image of CMC embedded mouse eye attached to a cryostat chuck with the mark on the luer lock of the needle oriented toward the chuck and mounted with the needle parallel to the chuck surface

Fig. 4 Image of partially sectioned CMC embedded mouse eye with the hypodermic needle still in place, so it can be aligned parallel to the cryostat blade

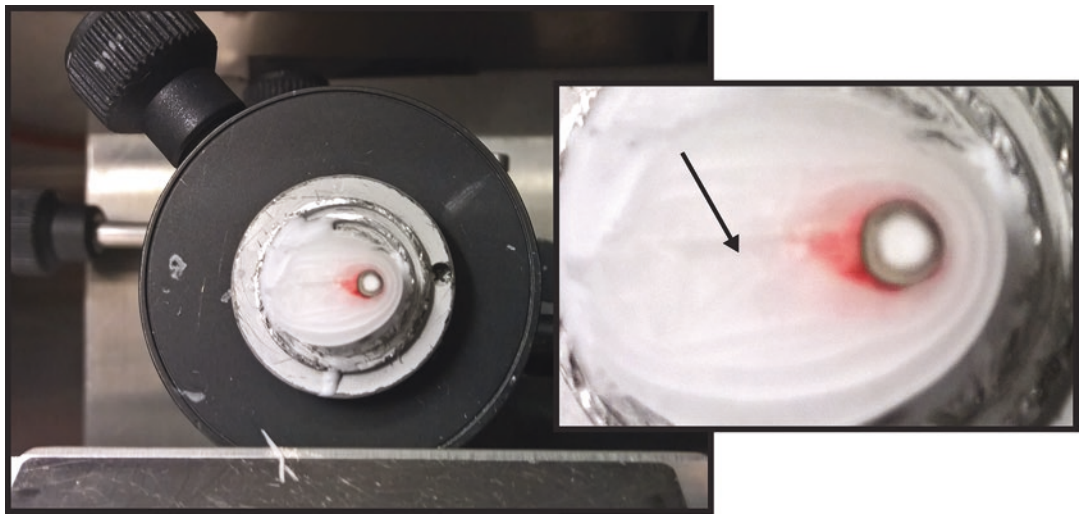

Fig. 5 Image of CMC embedded mouse eye once the hypodermic needle removed and partially trimmed to the point where the needle impression is visible, indicated by the arrow in the zoomed inlay

CMC results in premature dulling of the blade. The position of the anti-roll bar can also be optimized at this point. Section procurement can begin after the impression is no longer visible.

4. The surrounding CMC material around the tissue should have sufficient structural integrity to manipulate the section into position on the sample surface (if the material has poor structural integrity, *see* **Note 3**).
5. The CMC should prevent tissue curling although wrinkles may still be observed in larger sections. These wrinkles can affect

section quality, wrinkles turn into folds during the thaw mounting process. Adjusting the anti-roll bar position may help to prevent wrinkles and gently flatting the tissue before thaw mounting will prevent folds.

3.6 Removal of the Lens from Frozen Tissue

The lens is a very large, dense region of the rodent eye tissue in comparison to the neural retina or cornea. Sectioning of the mid region of the eye to obtain imaging mass spectrometry data from the neural retina or cornea, free from sectioning artifacts such as wrinkles or folds, can be challenging. The lens from rodent eyes can be gently removed using a fine-tipped paint brush, although the success rate of thaw mounting such a section and retaining good morphology is low. One method to overcome these issues is to physically remove the lens from an embedded, and partially sectioned, eye using a drill press and dry ice, ensuring the tissue remains frozen. This will improve both section quality and reproducibility.

1. The embedded sample should first be trimmed on a cryostat to expose the majority of the lens on the block face of the embedded tissue. A suitable sized drill bit should be precooled on dry ice before mounting into the drill press (Fig. 6a).
2. The embedded eye should be accurately placed in the path of the drill bit which can be checked before turning on the drill. The majority of the lens tissue can then be drilled out, taking care not to damage the surrounding neural retina or cornea, *see* Fig. 6a.

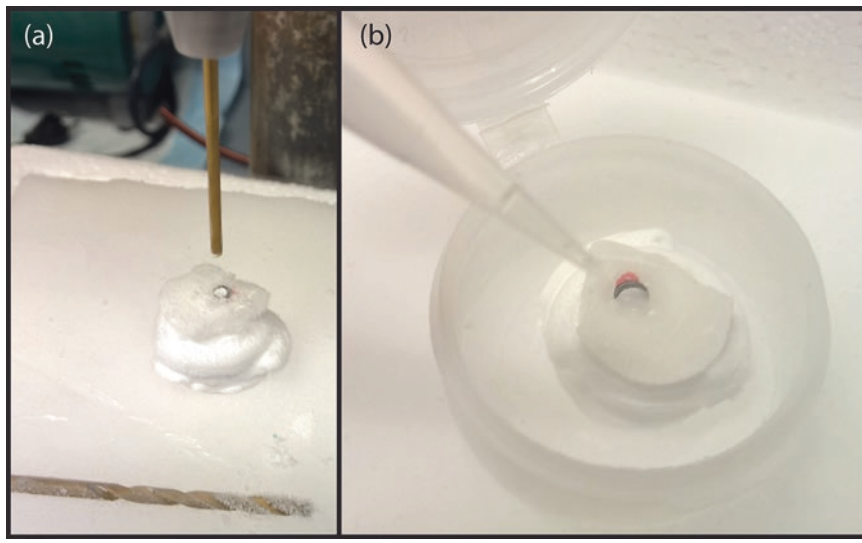

Fig. 6 (**a**) Image of CMC embedded mouse eye placed on a block of dry ice below a 7/64″ drill bit mounted into a pillar drill used to remove lens tissue from a partially sectioned mouse eye. (**b**) Image of mouse eye with the lens tissue removed and the void backfilled with CMC solution

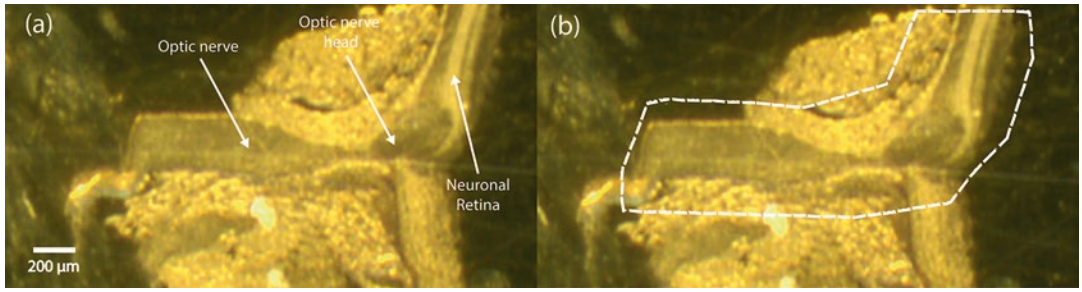

Fig. 7 (**a**) Optical image of a section mounted onto a gold-coated MALDI sample plate taken from mouse eye indicating the hypodermic needle aligned optic nerve, optic nerve head, and neural retina regions. (**b**) Optical image of the same section indicating region where data were acquired

3. The hole should then be back filled with CMC solution using a 1 mL pipette while keeping the tissue on dry ice, Fig. 6b. Trim the protruding CMC from the hole before continuing sectioning.

3.7 Tissue Washing and Matrix Application for Lipid Analysis

In the example provided for this method, the optical image in Fig. 7 displays a section taken from a mouse optic nerve, optic nerve head, and neural retina. The section was thaw mounted onto a gold-coated MALDI target plate and air dried immediately prior to vacuum desiccation. The section was then dehydrated in a vacuum desiccator overnight before washing three times for 30 s each with 100 mM ammonium acetate. The sample was then dried again for another 2 h in a vacuum desiccator. 2,5-Dihydroxybenzoic acid (DHB) was deposited by sublimation onto the samples using a custom sublimation apparatus for 20 min at 54 mTorr pressure at 120 °C.

3.8 Data Acquisition

Data were acquired in positive ion mode using a MALDI-TOF mass spectrometer (UltrafleXtreme II, Bruker Daltonics, Billerica, MA) equipped with a Smartbeam II 1 kHz Nd:YAG frequency tripled to 355 nm wavelength, and the laser was set to the minimum spot size with 8% laser power. Optical measurements were taken using a Nikon Eclipse 90i microscope of burn patterns in sublimed DHB surfaces to ensure laser power setting and shot counts provided a sub 10 µm burn pattern.

4 Notes

1. A variety of CMC solution concentrations have been reported in the past from 1 to 5%. This should be optimized on a tissue-to-tissue basis with higher concentrations better suited to denser tissues. One disadvantage to using higher concentrations of CMC is premature dulling of the blade.

2. Liquid water and CMC solutions contain a small amount of air that forms bubbles upon freezing. These bubbles affect the quality of the frozen CMC substrate. The gas dissolved in the liquid can be removed by degassing prior to use. This can be either performed with a vacuum degasser or via sonication.
3. Poor structural integrity or striations observed in the embedding medium can be a result of poor anti roll bar position or angle of the sample stage having been incorrect. Anti-roll bar placement, too close to the sample results in "chattering" thicker sections are also more prone to this effect.

5 Results

The optical image Fig. 7 displays a section taken from a mouse optic nerve and retinal tissue where the nerve was aligned along a hypodermic needle thaw mounted onto a gold-coated MALDI target plate. The transition of the optic nerve into the ocular globe at the optic nerve head indicated by the arrows is clearly visible. The image in Fig. 7b displays the region where data were acquired and includes the optic nerve region with some of the surrounding connective tissue, the optic nerve head, and a small portion of the neural retinal layers. The data in Fig. 8 display lipid distributions from multiple anatomical regions of a mouse optic nerve and neural retina. The signal at *m/z* 592.5 in Fig. 8a observed in red in the central overlay, previously identified as A2E [4], defines the retinal pigment epithelium in the neural retina, above the point where the optic nerve meets the ocular globe at the optic nerve head. The optic nerve and optic nerve head regions are clearly defined by a signal at *m/z* 760.6 in Fig. 8b (white in central overlay) previously identified as PC(16:0_18:1) [5]. The distribution displayed by this signal is highly abundant in the optic nerve and traverses the optic nerve head and passes through into the inner and outer plexiform, inner nuclear and ganglion cell layers of the neural retina. A signal observed at *m/z* 734.5 in Fig. 8c displays high abundance in the outer nuclear layer of the neuronal retina, with lower intensity signal for this lipid observed in the inner plexiform and nuclear layers of the neural retina. The signal observed for an unidentified ion at *m/z* 762.6 displays high abundance in the inner plexiform and nuclear layers of the neural retina, displayed in green in the central overlay, this signal spans up to the optic nerve head region of the tissue. This distribution observed in green in the central overlay suggests an association with the neuronal tissues of the neural retina, but not the optic nerve. The signal at *m/z* 834.7, displayed as yellow in the central overlay, is present in high abundance in the photoreceptor layer of the tissue, with lower intensity spanning into the inner layers of the neural retina. The spatial resolution of

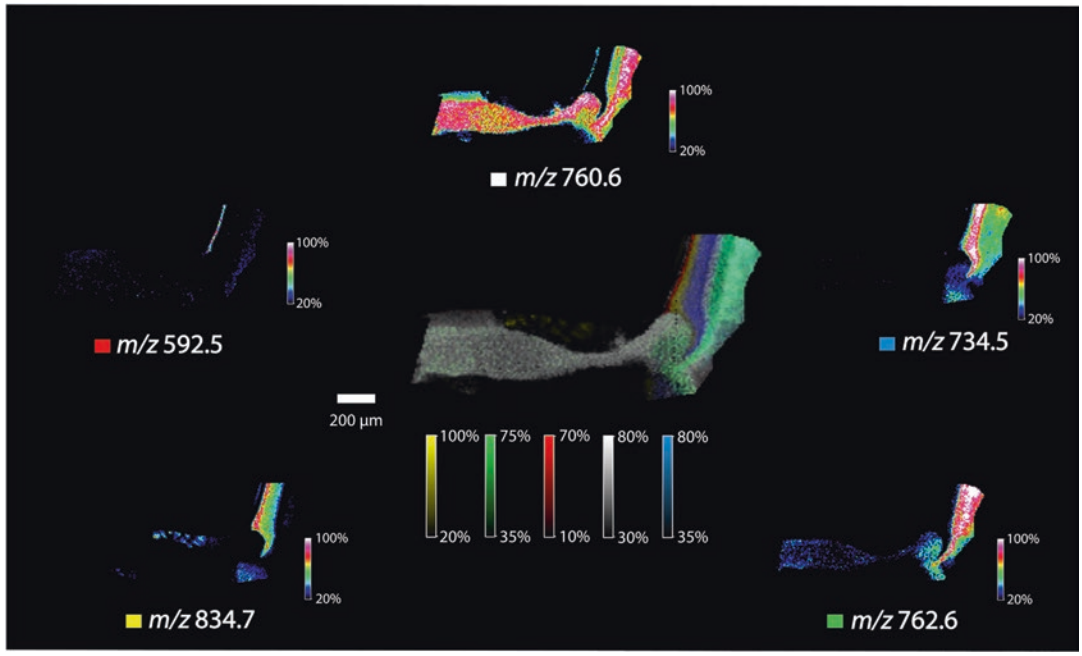

Fig. 8 MALDI IMS data acquired in positive ion mode of mouse optic nerve and neural retina. Ion images display the distributions of (**a**) A2E, *m/z* 592.5, (**b**) PC(16:0_18:1), *m/z* 760.6, (**c**) unidentified ion, *m/z* 734.6, (**d**) unidentified ion, *m/z* 762.6, and (**e**) unidentified ion, *m/z* 834.7. The central image is an overlay of the individual ion maps shown

10 µm, in this example, provides high signal intensity from small anatomical regions of mouse ocular tissue, thus providing MALDI images that display clear definition of multiple cell layers in the neuronal retina and clear definition of the optic nerve and optic nerve head.

6 Conclusions

Embedding ocular tissues in frozen degassed CMC greatly improves section quality and reproducibility from section to section preserving the morphology of the native tissue, which improves the quality of MALDI IMS data obtained. This embedding method allows for successful and reproducible sections from the neural retina and cornea regions of the tissue. Further manipulation by removing the ocular lens and refilling the space further improves section quality from more central regions of the ocular globe and prevents proteins from lens tissue contaminating the neural retina or cornea regions during washing protocols. Aligning the optic nerve along a hypodermic needle allows data to be acquired along the full length of the tissue in one section allowing for comparisons of molecular abundances in distal and proximal

regions, including the optic nerve head, to be compared in the same data set. The methods described here can be easily adapted to be suitable for other tissues analyzed by MALDI IMS, which are problematic in similar areas, such as section quality or alignment of small features.

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