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3-D imaging mass spectrometry of protein distributions in mouse Neurofibromatosis 1 (NF1)-associated optic glioma

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ABSTRACT

Neurofibromatosis type 1 (NF1) is a common neurogenetic disorder, in which affected individuals develop tumors of the nervous system. Children with NF1 are particularly prone to brain tumors (gliomas) involving the optic pathway that can result in impaired vision. Since tumor formation and expansion requires a cooperative tumor microenvironment, it is important to identify the cellular and acellular components associated with glioma development and growth. In this study, we used 3-D matrix assisted laser desorption ionization imaging mass spectrometry (MALDI IMS) to measure the distributions of multiple molecular species throughout optic nerve tissue in mice with and without glioma, and to explore their spatial relationships within the 3-D volume of the optic nerve and chiasm. 3-D IMS studies often involve extensive workflows due to the high volume of sections required to generate high quality 3-D images. Herein, we present a workflow for 3-D data acquisition and volume reconstruction using mouse optic nerve tissue. The resulting 3-D IMS data yield both molecular similarities and differences between glioma-bearing and wild-type (WT) tissues, including protein distributions localizing to different anatomical subregions.

Biological significance: The current work addresses a number of challenges in 3-D MALDI IMS, driven by the small size of the mouse optic nerve and the need to maintain consistency across multiple 2-D IMS experiments. The 3-D IMS data yield both molecular similarities and differences between glioma-bearing and wild-type (WT) tissues, including protein distributions localizing to different anatomical subregions, which could then be targeted for identification and related back to the biology observed in gliomas of the optic nerve.

1. Introduction

Optic nerve function is vital for delivering visual information from light sensitive cells of the retina to the visual cortex of the brain. As the optic nerve travels from the eye, the left and right nerves cross at the chiasm before entering into the brain, coursing through the optic tracts and radiations, and ultimately terminating in the visual cortex. Any pathological process that disrupts these nerve fibers can result in visual loss, especially tumors of the optic nerve and chiasm. One of the most common histological tumor types affecting the optic nerve and chiasm is the optic pathway glioma (OPG). These brain tumors are over-represented in people with the Neurofibromatosis type 1 (NF1) cancer predisposition syndrome [1].

In the context of NF1, OPGs typically arise in young children [2], where they are composed of glia-like cells (astrocytomas or gliomas). While only 15–20% of children with NF1 develop these tumors, approximately 30–50% of children with NF1-OPGs will experience visual impairment and require chemotherapy. Since treatment is most often instituted without a prior tissue diagnosis and pathological specimens are uncommon, much of our understanding of the molecular and cellular pathogenesis of these brain tumors derives from the use of genetically-engineered mice [3]. In contrast to their human counterpart, the murine optic nerve is very small (4 mm in length, 1 mm across the main body, and 300 μm for the diameter of the optic nerves, with a total of around 500 μm in depth at the chiasm). This poses significant challenges for proteomic discovery efforts, which is further amplified for studies in which mass spectrometry is combined with MALDI IMS to assess the spatial distributions of identified proteins in 2-D [4–6] and 3-D spaces [7–11].

MALDI IMS is an invasive technique that requires surface sampling of sectioned tissues, and thus necessitates disruption of the native 3-D tissue structure. Consequently, reconstruction of a 3-D volume from separately-measured 2-D experiments is an essential step in the process. One of the common ways for accomplishing this is by matching

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landmarks or fiducials from one 2-D image onto a neighboring 2-D image. The use of fiducials for image registration has been incorporated into a number of imaging modalities including MRI, CT, PET and MALDI IMS. Methods for incorporating the reference points within the sample vary from one technique to another, and a number of approaches have been previously reported for MALDI IMS [7,10,12]. As such, some investigators have utilized printed fiducials to register optical images to images generated by signals observed through MALDI IMS analysis [7], while others have used fiducial markers to help align breast cancer explants grown in mice [10]. These fiducials were produced by injecting dyes into gelatin surrounding the sample tissue. Multimodal image alignment has also been achieved using gold sputtered fiducial markers to combine Secondary Ion Mass Spectrometry (SIMS) and MALDI IMS of tissue samples [12].

In this report, we introduce a novel and practical way of introducing fiducials directly into the cutting block. Our approach simplifies an often time-consuming and error-prone part of the 3-D MALDI IMS workflow, and significantly reduces the effort involved in 3-D reconstruction. The fiducials introduced are not dyes or physical markers added after sectioning, but instead are rigid rods that are visible both under optical microscopy as well as through MALDI IMS, and require minimal human effort to introduce into the sample. The fiducial-producing rods are embedded into the block around the sample tissue in a triangular configuration. These triangular regions describe the tissue space well in both the x and y directions and provide good anchors for translation, rotation, and other registration transformations of the 2-D measured planes. By acquiring data points around fiducials for each section, registering both optical and MALDI IMS data becomes more accurate and efficient.

Leveraging these refinements, we measured optic nerve tissues from control and glioma-bearing genetically-engineered mice using a multitude of 2-D MALDI IMS experiments at different cutting depths. Subsequently, we registered these 2-D experiments in 3-D space and reconstructed the optic nerve/chiasm in 3-D. Since molecular concentrations can vary significantly in tissue volumes, the true molecular distribution cannot be adequately assessed when only one or a few sections are analyzed. In a 3-D projection, the continuity of the signals observed throughout the 3-D space shows the true variation in distribution and abundance more clearly in this volume. The resulting 3-D ion distributions provided a number of protein signals that varied in both abundance and spatial localization. These signals were then targeted for identification to provide a deeper understanding of the proteins present in the optic glioma microenvironment than would have been possible with 2-D IMS alone. In the process, we also developed a novel workflow that improves the efficacy and accuracy of 3-D ion images from 2-D measurements of mouse optic nerve tissue.

2. Materials and methods

Mice. *Nf1*$^{flox/flox}$ (WT) and *Nf1*$^{flox/mut}$; GFAP-Cre mice (OPG mice) [13] were generated as previously described [14]. All mice were maintained on a C57BL/6 background and used in accordance with an approved Animal Studies protocol at the Washington University School of Medicine. Mice were euthanized at three months of age, and optic nerves/chiasms collected from anesthetized and Ringer's solution-perfused mice. Tissues were frozen on dry ice at Washington University and shipped overnight to Vanderbilt University. Tissues were stored at −80 °C until required. Tissues from one OPG and WT mice were used for 3-D MALDI IMS experiments, 2 separate OPG mice tissues were used for protein identification methods. Immunohistochemistry was also performed on tissues from representative OPG and WT mice.

2.1. Sample preparation.

Frozen OPG and WT tissues were embedded in a desired orientation, with the tissues in close proximity to one another, along with fiducial registration points in 2.7% Carboxymethyl cellulose solution (CMC) (Sigma Aldrich, St Louis, MO, USA) within a custom-made mold. Further details and images of this process are provided in the Supplement Information section and Supplemental Fig. 1.

Sections of 10 μm thickness were obtained with a cryostat (Leica CM3050S, IL, USA) at −20 °C from OPG and WT tissues including fiducial markers and placed onto poly-lysine coated ITO coated glass slides (45 × 45 mm) (Delta Technologies Ltd., Loveland, CO, USA). The ITO glass was poly-lysine coated, using poly-L-lysine solution (Sigma Aldrich). A Teflon-coated slide (Electron Microscopy Science, Hatfield, PA, USA) was used to flatten each section, including the surrounding CMC so that the fiducial markers ended up in reproducible locations from section to section. Four sections were thaw mounted onto separate ITO coated 25 × 75 mm poly-lysine coated slides for microextraction protein identification purposes, leaving 33 sections for IMS analyses. For each slide, the section number and depth position along the z-axis were recorded. Tissue washes were comprised of 70% and 100% ethanol for 30 s each, then in a Carnoy's wash (60:30:10 ethanol:chloroform:acetic acid) for 2 min, followed by 100% ethanol, deionized water, and 100% ethanol for 30 s each [15]. Sample plates were then air dried before high resolution Differential Interference Contrast (DIC) microscopy scans were taken. The tissue sections were measured at 2.13 μm/pixel, collecting a DIC microscopy image for each individual tissue section along with its associated rod-based fiducial markers on a Nikon Eclipse 90i microscope (Nikon instruments Inc., Melville, NY, USA).

2.2. Matrix application

Sinapinic acid (Sigma Aldrich) matrix solution (5 mg/mL in 90:9.7:0.3 acetonitrile (ACN):H_2O:trifluroacetic acid (ACN:H_2O:TFA)) was applied by spray coating with a HTX Technologies TM sprayer (HTX Technologies, Carrboro, NC, USA), employing eight passes with a flow rate of 0.1 mL/min at a 120 °C temperature. The spray head path was alternated at each pass by 90° while traveling at 1300 mm/min during application. Each sample plate was placed into individual petri dishes inside zip lock bags flushed with dry nitrogen before being placed in the −80 °C freezer. Sample plates were then brought to room temperature and subjected to a rehydration procedure [15] before IMS measurement.

2.3. MALDI IMS data

MALDI IMS experiments were performed on a Bruker AutoFlex III mass spectrometer (Bruker Daltonics Billerica, MA, USA) using a SmartBeam 1 kHz laser with a mass range of 4000–26,000 Da and 40 μm spatial resolution. Although the HTX-TM sprayer is capable of coating samples so data can be acquired at very high spatial resolution (10–20 μm, depending on matrix, solvents and spray conditions) the spatial resolution used in this study (40 μm) was chosen based on spectral quality and number of pixels that could be practically managed.

IMS measurements were acquired throughout regions of interest that were defined around each tissue section, with margins of around 4–5 pixels beyond the tissue edge. IMS data were also acquired in regions around each of the rod-introduced fiducials for every section. Mass calibration was performed prior to image acquisition using a mixture of insulin, cytochrome C, apomyoglobin, and trypsinogen. The collected IMS data consisted of 33 individual 2-D IMS experiments, all acquired with the same instrumental parameters described above, each data set reporting a different tissue depth. The 2-D IMS data sets were collected using FlexImaging 4.0 (Bruker Daltonics), amounting to a total of about 51 GB of raw IMS data. The 2-D ion images were normalized using total ion current (TIC) in FlexImaging.

2.4. 3-D reconstruction of 2-D MALDI IMS

The OPG and WT optic nerve samples, mounted next to each other in the preceding workflow steps, were sectioned throughout as 10-μm

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