Optimizing tissue preparation and storage for analysis of polyunsaturated fatty acids using Agilent's FTIR imaging systems

Application note

Biomedical

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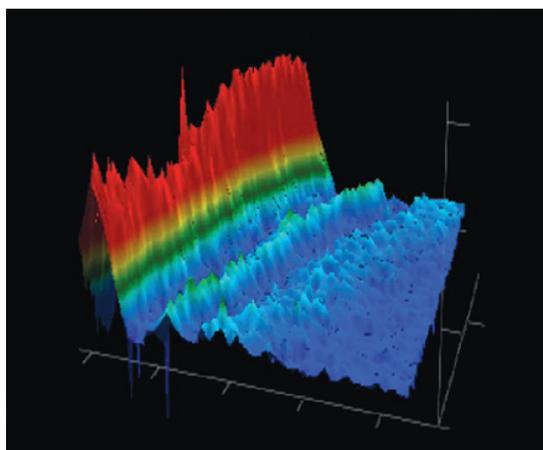
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Introduction

Mammals require dietary-based polyunsaturated fatty acids (PUFA) such as docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA) and arachidonic acid (AA) for many biological processes, including normal functioning of the retina and brain. For example, oxidation of DHA in brain tissue due to enzymatic or free radical action in vivo is thought to contribute to complications of Alzheimer's disease [1, 2]. Fourier transform infrared (FTIR) imaging with high spatial resolution is a suitable tool to investigate the PUFA distribution in tissue sections from animal models of disease, providing comprehensive information of the biochemical components on the subcellular scale.

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It has long been assumed that tissue components should be stable under typical storage conditions (dark, dry, room temperature). Recently, we observed that some PUFA absorption bands, namely, the olefinic functional group (C=C-H, imaged by integration of the 3012 cm^{-1} band) and, to a lesser extent, the lipid carbonyl (C=O, at 1735 cm^{-1}) decrease over time. The aim of this study was to explore the factors contributing to this oxidative degradation and to determine protocols required to minimize the rate of loss, in order to ensure an accurate spectroscopic assessment of PUFA in biological tissues.

Methodology

Tissue acquisition
Tissue was acquired from a 13 month-old wild type mouse (C57BL/6). All experimental protocols for animal studies were approved by the Protocol Management Review committees following the guidelines established by the Canadian Council for Animal Care.

Sample preparation
Retina samples coated with optimal cutting temperature compound (OCT, Sakura Finetek Inc., USA) were flash frozen in a beaker of isopentane cooled in liquid nitrogen. They were then cryosectioned to 7 µm thickness at -20 °C, and mounted on MirrIR substrates (Kevley Technologies, Chesterland, OH) or on BaF_2 windows. Each sample was stored in a sealed plastic case at -70 °C to preserve the biochemical content. Before imaging, an individual slide was brought to room temperature in a dark slide box with silica bead packets, until dry.

Confirmation that bright room light and room temperature could cause oxidation of PUFA was obtained by monitoring the 3012 cm^{-1} band in a BaF_2-mounted sample left on the lab bench near a window. Retina was imaged initially, and again after 24 and 90 hours of natural day/night light cycles.

The effects of typical slide storage were then monitored over several months in order to assess the approximate amount of degradation that might be encountered under standard conditions, that is, dark, room temperature storage. The first FTIR image was acquired 12 hours after removal from the freezer. Subsequent images were acquired at 3, 7, 15, 31 and 272 days after the start of the dry/thaw process. Sample exposure to light was limited to the short time required to set up the FTIR microscope before data collection. Room lights and microscope illumination were switched off during the imaging measurement.

Instrumentation
Images were obtained using an Agilent Cary 620 FTIR spectrochemical microscope with a 64 × 64 pixel focal plane array (FPA) detector at 5.5 micron pixel resolution interfaced to an Agilent Cary 670 FTIR spectrometer. All MirrIR data was collected using reflection mode (MirrIR) or transmission mode (BaF_2) mosaic imaging analysis and processed using an in-house software routine and Agilent Resolutions Pro software. The spectra were obtained over a spectral range of 4000–900 cm^{-1} with 4 cm^{-1} spectral resolution. All spectra of retina samples were obtained by co-addition of 256 scans, ratioed to a background with a matching number of scans. Instrument operating parameters are given in Table 1.

Table 1. Agilent Cary 670 FTIR and 620 FTIR microscope collection parameters

Parameter	Value
Speed	5 kHz
Spectral resolution	4 cm^{-1}
Scans	256
Spatial resolution	5.5 µm
Mosaic	7 x 4 tiles (~2.5 x 1.4 mm)

Results and discussion

PUFA loss was dramatic under the accelerated exposure conditions. The 3D image of the PUFA distribution on day 1 (Figures 1 and 2) shows that, as expected in a retina sample, the distribution is not uniform. The highest concentration of PUFA is found in the ROS, which forms an arc along the outer region of the retina. The square pixels selected to best follow this arc do not contain identical PUFA load, even at the start, but do represent the region of greatest PUFA concentration. A section of the retina of a control mouse was imaged to assess gradual loss of PUFA over time under normal storage conditions. Figure 2 shows that the loss of olefinic CH intensity was less obvious in the first two weeks compared to longer storage times under dark, dry, room temperature conditions.

The complex organization of cells in the retina creates layered patterns in the unstained section, under white light illumination (Figure 1A). PUFA content is highest in the discs of the ROS (Figure 1B, C), while lesser amounts are detected along the axons. All trace of PUFA vanished within 48 hours under the accelerated exposure conditions (data not shown).

After one month, the loss was more apparent; after eight months, little PUFA remained. These observations imply that, in a typical laboratory setting, a sample measured three or four days after thawing would still contain a measurable amount of PUFA, and even after a few weeks of storage, elevated PUFA could be detected in, for example, the rod cell outer segment (ROS) of retina.
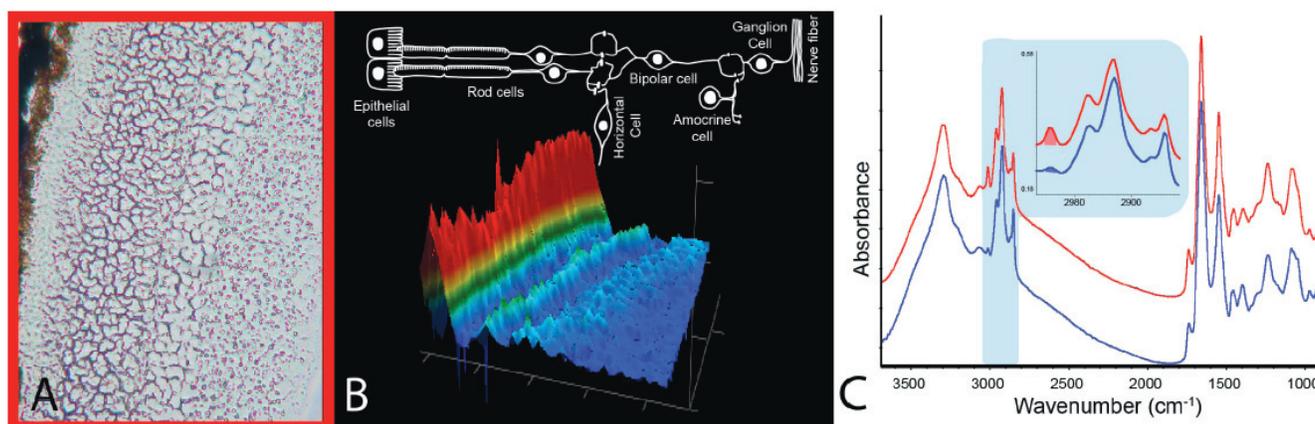

Figure 1. Photomicrograph and FTIR imaging results from freshly thawed retinal tissue section (A) Photomicrograph of tissue, from outer pigmented layer to ganglion region. (B) FTIR images (1 × 3 tiles) processed for integrated area of olefinic CH stretch at 3012 cm^{-1}; images acquired at 12 hours (immediately after thaw and dry in the dark). Schematic of cell distribution across section; filled white circles denote nuclei. (C) Spectra extracted from disc region of rod cells with high PUFA content (red) and from axonal region between rod and bipolar cells (lower PUFA content, blue), highlighting 3012 cm^{-1} band for integration.
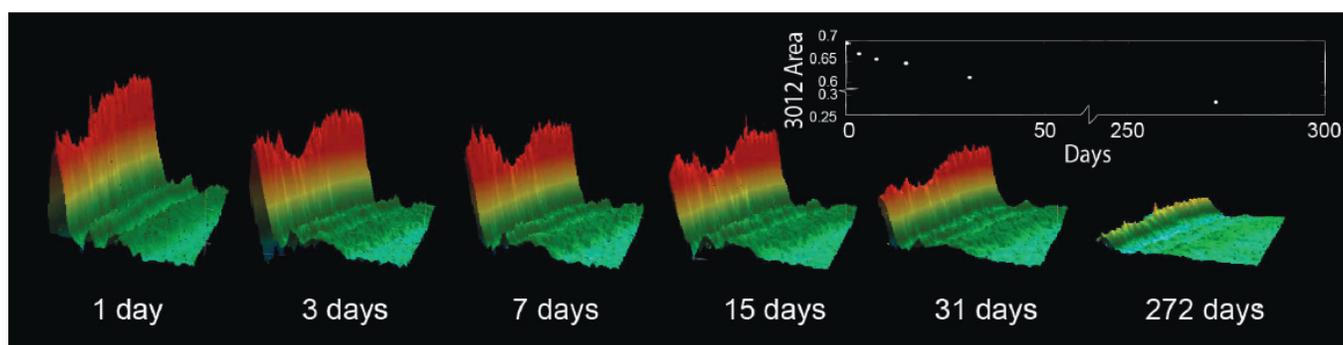

Figure 2. FTIR imaging results show steady loss of PUFA during long-term storage of retinal tissue sections, in dark, dry room temperature conditions. FTIR images (1 × 3 tiles) have been processed for integrated area of olefinic CH stretch at 3012 cm^{-1}. The first image was acquired at 12 hours (immediately after thaw and dry in the dark), and the remainder at intervals noted, during 8 months of storage under same conditions. Graph inserted shows decrease of PUFA band intensity, averaged from 36 pixels representing the region of highest intensity on Day 1.

Data courtesy of Dr. Kathleen Gough, University of Manitoba, Winnipeg, Canada.

The FTIR images shown in Figure 2 provide qualitative information about the distribution of PUFA in retina tissues, while integrated peak area measurements of a set of 36 pixels from each image provide quantification of C=C–H loss through oxidation.

From the plot of the mean value of the integrated olefinic CH band area for each time point (Figure 2, top right), it can be seen that the loss of PUFA is immediate, steady, and significant after only a few days. The mean value has decreased noticeably after the first measurement, and is reduced by ~8% in the first two weeks. It continues to decline steadily; after one month, some 15% has vanished; after eight months, less than one third of the original PUFA content can be detected.

Smaller decreases were observed in the carbonyl band at 1735 cm^{-1}, usually ascribed to lipids. The fact that this band did not completely vanish, along with the observation that the profile in the OH stretch region also changed over time, was interpreted to mean that new, unknown oxidation products were likely now present.

Conclusions

The Agilent 670 and 620 FTIR imaging microscopy system has been used successfully to analyze PUFA levels in freshly thawed tissue sections of mouse retina, and to follow the loss of the spectral biomarkers over time, under standard storage conditions. The results have shown that in order to obtain a reasonably accurate representation of the olefin functional group (C=C–H), freshly cryosectioned samples should be imaged as soon as possible, following thawing and drying, while maintained under dark conditions. Failure to do so will likely lead to an underestimation of the quantity of PUFA in a tissue sample.

These findings will inform other research groups looking into the analysis of PUFA in biological tissue samples using FTIR.

References

1. Fraser, T., Taylor, H. & Love, S. (2010). *Neurochem. Res.*, *35*, 503–513.

2. Hartmann, T., Kuchenbecker, J. & Grimm, M. O. W. (2007). *J. Neurochem.*, *103*, 159–170.

For more information

Stitt, D. M., Kastyak-Ibrahim, M. Z., Liao, C. R., Morrison, J., Albensi, B. C. & Gough, K. M. (2012). Tissue acquisition and storage associated oxidation considerations for FTIR microspectroscopic imaging of polyunsaturated fatty acids. *Vib. Spectrosc.*, *60,* 16-22

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