APPLICATION NOTE

A1R MP+/AX R MP Multiphoton Confocal Microscope

Digital pathology based on intrinsic fluorescence spectra of liver fibrosis using a multiphoton microscope

In recent years, an increase in metabolic syndrome caused by the accumulation of visceral fat has become a widespread problem. Nonalcoholic fatty liver disease (NAFLD) and its severe form, nonalcoholic steatohepatitis (NASH), are metabolic syndromes in the liver that progress to liver cirrhosis and hepatocellular carcinoma. The development of accurate and early diagnostic techniques for pathological conditions with respect to NAFLD/NASH is one society's most significant issues.

This application note introduces a quantitative evaluation technique for liver fibrosis developed by Dr. Takashi Saitou and Dr. Takeshi Imamura of the Department of Molecular Pathogenesis, and Dr. Yoichi Hiasa of the Department of Gastroenterology and Metabology, Graduate School of Medicine, Ehime University.

Keywords: multiphoton microscopy, digital pathology, liver fibrosis, autofluorescence

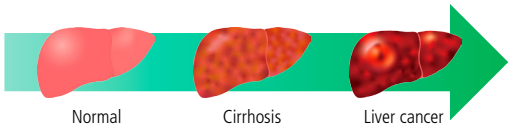
Outline of Research

Cells and tissues of multicellular organisms intrinsically contain many kinds of chemical compounds that emit fluorescence, including nicotinamide adenine dinucleotides (NADH), flavins, lipofuscin, melanin, porphyrin, collagen, elastin, and vitamins. Since these molecules play important roles in tissue development, homeostasis, and disease progression, the spatial distribution of endogenous fluorophores can be used to determine the state of cells and tissues.

Since the multiphoton microscope (MP) enables fluorescent excitation over a wide wavelength range by inducing simultaneous absorption of multiple photons and excitation of fluorescent material having an emission peak in the ultraviolet range, it is suitable for imaging autofluorescent substances consisting of multiple components. However, the autofluorescence obtained by tissue observation generally shows broad spectral profiles derived from a mixture of multiple distinct fluorophores, making it difficult to separate individual fluorescence sources.

Dr. Saitou et al. have developed a liver fibrosis evaluation technique that, instead of separating distinct emission sources of multiphoton excitation spectra images, identifies spectral profiles characteristic of biological tissues, and applies them to the image segmentation method, and also executes quantitative morphological measurement.

Here, we introduce their report on spectral image analysis and mathematical morphology measurement of hepatocyte metabolites, vitamins, lipid droplets and inflammatory cells in autofluorescence within a mouse model of carbon tetrachloride (CCl_4) induced liver fibrosis.

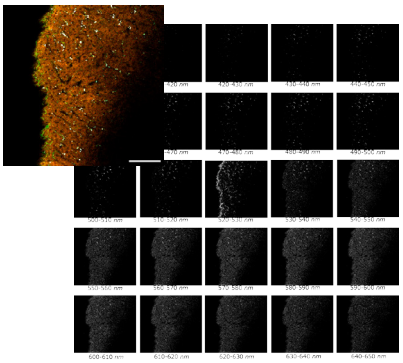


It is necessary to develop accurate and early diagnostic technology for hepatitis pathology in order to suppress the onset of liver cirrhosis and hepatocellular carcinoma.

Endogenous fluorescent materials to be subject to multiphoton excitation

Molecule	Process	λ_{ex} (nm)
Tyrosine	3PE	<700
Tryptophan	3PE	700-740
Serotonin	3PE	700-720
Melatonin	3PE	700-720
5-HIAA	3PE	700-720
5-HTOL	3PE	700-720
Retinol	2PE	700-830
Flavins	2PE	700-730
NADH	2PE	690-730
Pyridoxine	2PE	690-710
Folic acid	2PE	700-770
Cholecalciferol	2PE	<700
Elastin	2PE	700-740
NFTs	2PE	700-780
Lipofuscin	2PE	700-850
Collagen	2PE	700-740
Microtubules	2PE	
Skeltal muscle	2PE	

Reference: Zipfel, et al, *Proc Natl Acad Sci U S A, 100:7075-7080, 2003*



MP excitation images of each spectrum band

Multidimensional biological information of multiphoton excitation spectrum images is extracted by image analysis

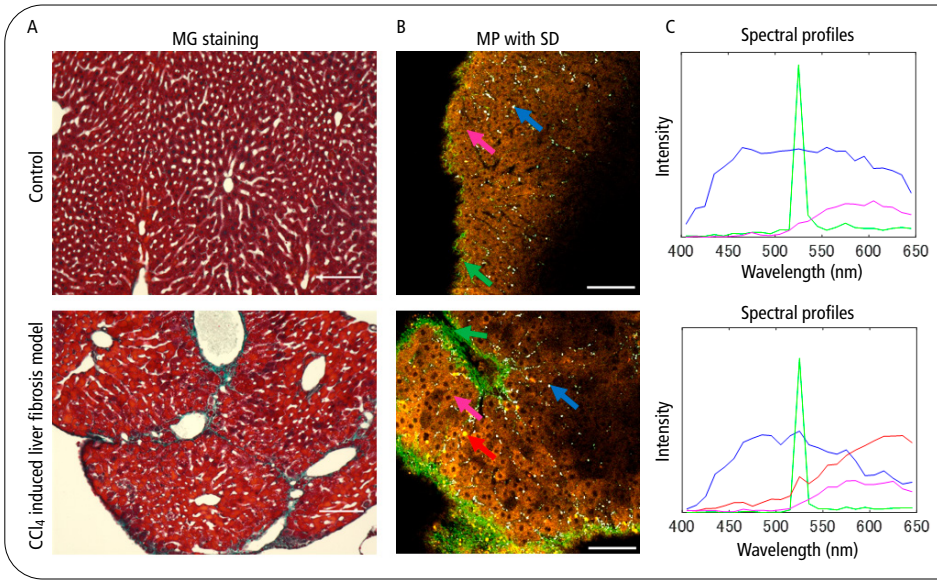


Figure 1: MP excitation spectral imaging of liver tissue in a CCl_4-induced liver fibrosis model

(A) Masson Goldner stained images of the liver tissue of a normal liver and a CCl_4 hepatitis model
(B) Multiphoton excitation spectral images of a normal liver and CCl_4 liver. The spectra were detected at a wavelength range of 400-650 nm and with a bandwidth of 10 nm (recorded as a total of 25 channels), using an SD unit. The InSight DeepSee Ti:Sapphire laser oscillator (Spectra-Physics) was used at a wavelength of 1050 nm as an excitation light source
(C) Extracted spectra of characteristic regions (indicated by arrows in (B))

Scale bar: 100 µm
Field of view: 0.5 mm x 0.5 mm
Resolution: 512 x 512 pixels
Z-stacks were obtained with a step size of 2 µm from the deepest part of the liver tissue (depth 100 to 200 µm) to the surface.
Objective: CFI75 Apochromat 25XC W 1300 (NA: 1.10)

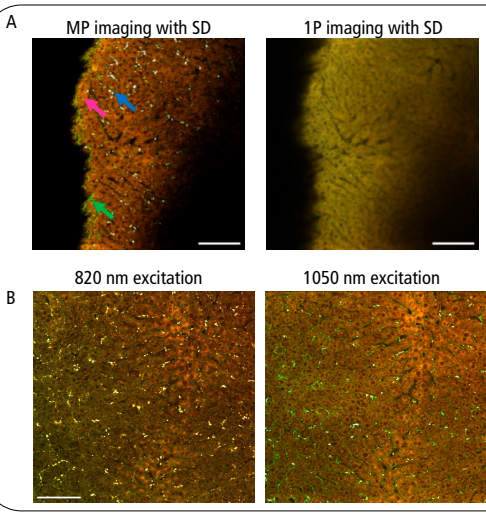


Figure 2: Examination of autofluorescence measurement conditions of liver tissue

(A) Comparison of MP and confocal images of autofluorescent spectra of intact mouse liver
Ti: Sapphire laser oscillator (1050 nm) was used for multiphoton excitation, and four visible wavelength laser lines (405, 488, 561, and 640 nm) were used for single photon excitation.
The blue arrow indicates white-colored dot-like objects, the green arrow indicates a region of strong SHG signal emission, and the magenta arrow indicates a region of hepatic parenchyma, primarily composed of hepatocytes.
(B) Comparison of multiphoton excitation autofluorescent images at three different excitation wavelengths (820 nm, 1050 nm, 1200 nm).
Detected via an NDD unit with the following emission filter sets:
(1) 450 nm DM (dichroic mirror) and 417/60 nm (center wavelength/band width) BF (bandpass filter), (2) 560 nm DM and 525/50 nm BF, (3) 662 nm DM and 617/73 nm BF.
Scale bar: 100 µm

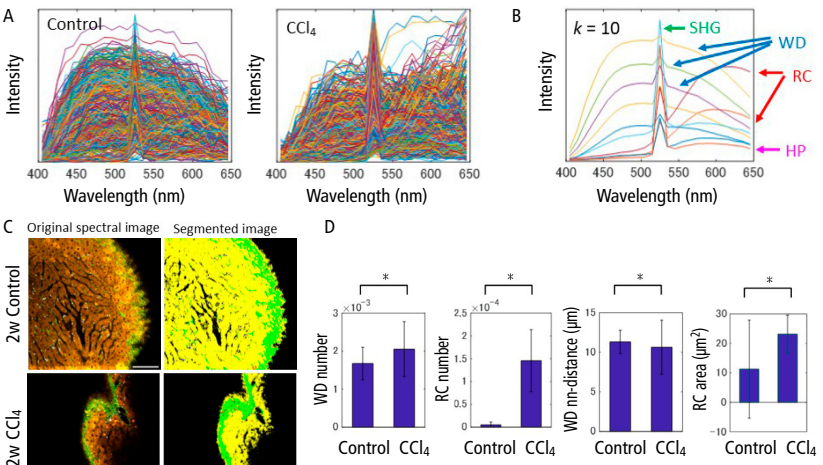


Figure 3: Image segmentation and mathematical morphology analysis of spectral image data

(A) Spectral profiles collected at each key point detected in the image sets by application of the FAST feature detection algorithm.
(B) Internal markers divided into 10 classifications. Based on the spectra collected from the image, 10 typical spectra were constructed using the k-means clustering method.
SHG: Second harmonic generation, WD: White dots, RC: Red cells, HP: Hepatic parenchyma
(C) Comparison of original and segmented images. Segmented images representing HP, SHG, WD, and RC are in yellow, green, white and red, respectively. Scale bar: 100 µm
(D) Mathematical morphology analyses of the segmented images. From the left, comparisons between the control and CCl_4 models with respect to the number of WD and RC sites, the nearest neighbor distance between WD sites, and the RC area.

Results

1. Identification of imaging conditions that effectively capture autofluorescence in liver tissue

Autofluorescence images of liver tissue obtained under each excitation condition for confocal (405, 488, 561 and 640 nm) and multiphoton (820, 1050 and 1200 nm) microscopy were compared. The result was that multiphoton excitation imaging by near-infrared 1050 nm light most efficiently captured hepatocellular hypermetabolism, fat-soluble vitamins stored in hepatic stellate cells, and inflammatory cells associated with liver damage. Increased collagen fibers (SHG), the appearance of inflammatory cells (red cells; RC), and changes in the localization of fat-soluble vitamins (white dots; WD) were observed in the liver tissue of the CCl_4 hepatitis model when compared to normal liver tissue. This shows the effectiveness of autofluorescence spectral imaging with respect to fibrotic livers.

2. Development of a quantitative analysis method for autofluorescence spectral imaging of hepatic tissue

In order to quantitatively analyze the multidimensional information in autofluorescence spectral images of a liver containing characteristic molecular composition and morphological information, internal markers were constructed based on the spectrum clustering method. Each internal marker corresponds to SHG, WD, RC, and hepatocytes (HP), and by applying these to image segmentation and mathematical morphology analysis, a quantifying method for molecular and morphological information accompanying the progression of pathological conditions was developed.

Summary

The ability of multiphoton excitation at the near-infrared 1050 nm wavelength to effectively visualize hepatocellular hypermetabolism, fat-soluble vitamins stored in hepatic stellate cells, and inflammatory cells associated with liver damage was clarified. This result can be achieved only by the combination of a multiphoton microscope capable of long wavelength excitation and a spectrum measuring device. It was also revealed that spectral imaging of native fluorescence from the liver tissue of a CCl_4-induced liver fibrosis mouse can differentiate not only between normal and diseased states, but also between progressive disease states. This approach provides a basis for spectroscopy-based digital histopathology of chronic liver diseases, and can be applied to a range of diseases associated with autofluorescence alterations.

Multiphoton microscopy has been widely applied to *in vivo* imaging due to such advantages as low phototoxicity and high penetrability into deep areas, and it is expected that real-time digital diagnosis using multiphoton microscopy and computational methods will be realized in the future.

Reference

Tissue Intrinsic Fluorescence Spectra-Based Digital Pathology of Liver Fibrosis by Marker-Controlled Segmentation
Saitou et al
Front Med (Lausanne). 2018 Dec 11;5:350. eCollection 2018
https://www.frontiersin.org/articles/10.3389/fmed.2018.00350/full

Product information

AX R MP Multiphoton Confocal Microscope

A high-speed resonant scanner captures rapidly changing biological reactions at high resolutions of up to 2K x 2K (2048 x 2048 pixels) over a large field of view of 22 mm. The AX R MP efficiently obtains a wide range of information, and supports research of life phenomena.