APPLICATION NOTE

A1R MP/AX R MP Multiphoton Confocal Microscope

The Effects of Refractive Index Mismatch Between Immersion Liquid and Tissue Clearing Reagent on Image Resolution in Deep Areas

Introduction

Purkinje cells, which have a characteristic shape, are difficult to capture with a confocal system using visible light excitation, from the surface to the deep regions of the cerebellum, even if they are subjected to tissue clearing treatment, so observation with a multiphoton confocal system by IR light excitation is more suitable. Nevertheless, especially during detailed observations in deep areas, the difference between the refractive index of the immersion liquid of the objective lens and that of the tissue affects brightness and resolution. This article focuses on the effect this difference in refractive index has at different observation depths. In addition, we captured the state of the PSF along the optical axis for each depth using fluorescent beads, conducted nonlinear deconvolution processing using this result as a parameter, and verified its beneficial effect on image elongation along the optical axis.

Experiment overview

Purkinje cells (Fig. 1, 2 and 4): Sagittal brain slices (1 mm thickness) cut from LC3GFP mouse were fixed overnight at 4°C with 4% formaldehyde. After washing with PBS, they were prepared at room temperature for 3 days in PBS containing 2% Triton X100. The tissue clearing process was performed over several days with RapiClear 1.47 (SunJin Lab, RC147001).

Fluorescence-bead sample (Bead Cake) for PSF measurement (Fig. 3): TetraSpeck beads (Thermo Fisher Scientific, T7280) of 200 nm diameter were adjusted to a density of 6.0 x 10^{10} particles/ml and then mixed in a RapiClear 1.47 gel. Mixed in 4% agarose solution and embedded in 1mm thick i-Spacer (SunJin Lab, IS009).

Microscope: A1R MP
Objective: CFI Plan Apochromat Lambda 10X/0.45 (Fig. 1), Silicone immersion objective CFI Plan Apochromat Lambda S 25XC Sil/1.05 (Fig. 2), Water immersion objective CFI Apochtomat LWD Lambda S 40XC WI/1.15, and Silicon immersion objective CFI Plan Apochromat Lambda S 40XC Sil/1.25 (Fig. 3 and 4).

Results

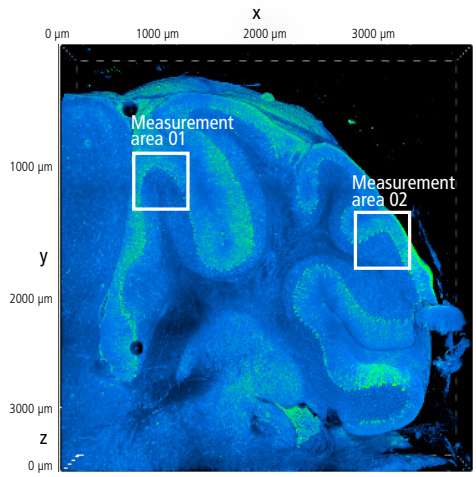


Fig. 1: 3D image of cerebellum captured by image stitching and Z-stacking

Pixel size: 1.23 µm
A comparison of images of a measurement area 01 where Purkinje cells are located, excited at 488 nm and 920 nm respectively and obtained using the same objective, is shown in Fig. 2. A comparison of images resulting from the difference in the refractive index when a measurement area 02 is acquired using a water immersion objective and a silicon immersion objective is shown in Fig. 4.

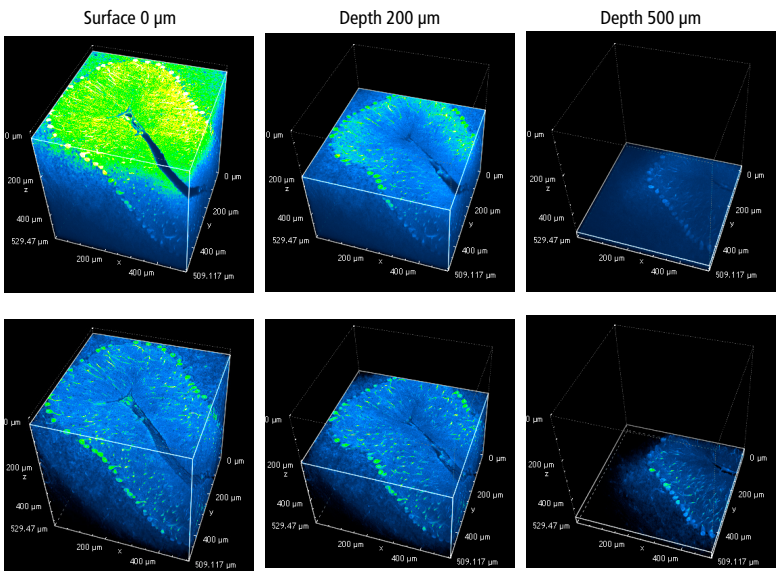


Fig. 2: Purkinje cells at various depths, obtained at different wavelength excitations

From the surface to a deep part of the measurement area 01 in Fig. 1 was captured at 488 nm (upper) and 920 nm (lower). In all images, the GFP fluorescence detection range was 500 to 550 nm, and all acquisition conditions other than the excitation wavelength, including the objective used and LUT adjustment for pseudo color display, are the same. To capture purkinje cells using 488 nm excitation, it is difficult to maintain the brightness from surface to deep areas even in clearing processed tissue.
Pixel size: 0.5 µm, Z-step: 0.37 µm

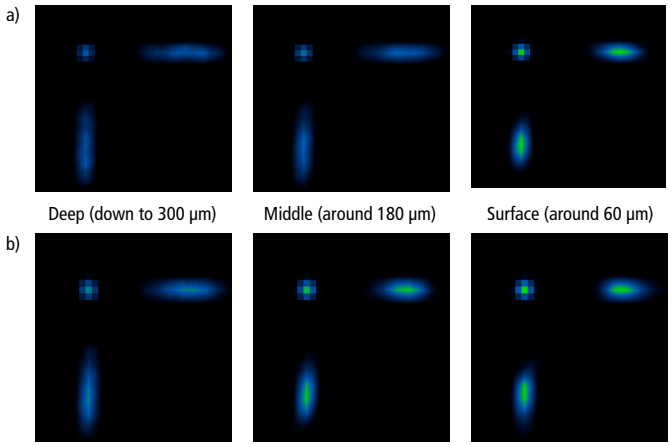


Fig. 3: PSF comparison by difference in immersion liquid

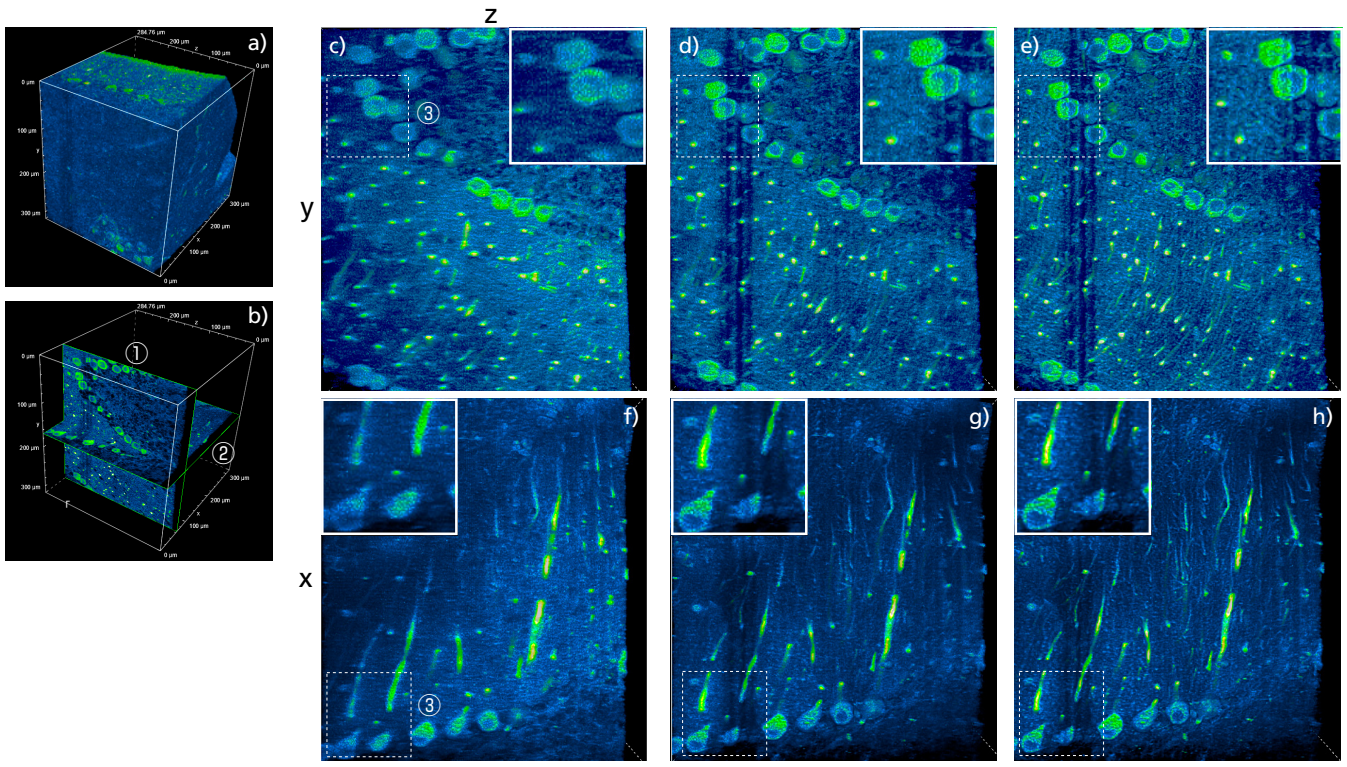
PSF images of bead cake averaged at each depth (XY/YZ/XZ orthogonal view).

a) Captured using a water immersion objective
Pixel size: 0.32 µm, Z-step: 0.33 µm (Nyquist)
b) Captured using a silicone immersion objective
Pixel size: 0.31 µm, Z-step: 0.21 µm (Nyquist)

The PSF along the optical axis was compared under the condition of refractive index of 1.47. For a silicone immersion objective using an immersion liquid having a refractive index closer to that of the bead cake even when the correction ring position is fixed to the cover glass thickness, it can be seen that sufficient brightness and the PSF can be maintained to a depth of around 180 µm.

Fig. 4: Improved image quality with silicone immersion objectives and nonlinear deconvolution processing

From 3D images (a, b) of the measurement area 02 in Fig. 1, the YZ cross section image ① is shown in the upper row (c, d, e) and the XZ cross section image ② is shown in the lower row (f, g, h). In images c to h, the right side of each image is the surface layer and left side is at a depth of about 300 µm.
c) and f): Obtained using a water immersion objective. ③ indicates image elongation along the optical axis and decreased brightness in the deep area.
d) and g): Obtained using a silicon immersion objective. Compared with c) and f), brightness in deep areas is improved and image elongation along the optical axis is reduced.
e) and h): Using the PSF along the optical axis, measured using beads (Fig. 3 b), as a parameter, a nonlinear deconvolution process was performed on the data of d) and g). This resulted in further improvement of the PSF and reduction of image elongation compared to c) and f).



Summary

Under the condition of a refractive index of 1.47, it was revealed that brightness and the condition of the PSF along the optical axis are optimally maintained from the cover glass surface down to the deep regions when using a silicone immersion objective, which has a closer refractive index to that of the bead cake than a water immersion objective (Fig. 3). Also, when observing Purkinje cells in cerebellum tissues that were clearing processed using a reagent with a refractive index of 1.47, brightness was better maintained and image elongation along the optical axis was more suppressed to a depth of around 180 µm when a silicon immersion objective was used (Fig.4 d and g), compared to when a water immersion objective (Fig.4 c and f) was used. In addition, the improvement of the PSF along the optical axis up to the deeper regions could be observed when nonlinear deconvolution processing was applied using the PSF parameters from bead cake (Fig. 4 e and h).

Acknowledgement

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