APPLICATION NOTE

**AX R MP Multiphoton Confocal Microscope**

# High-speed, deep, high-resolution multiphoton imaging using expansion microscopy

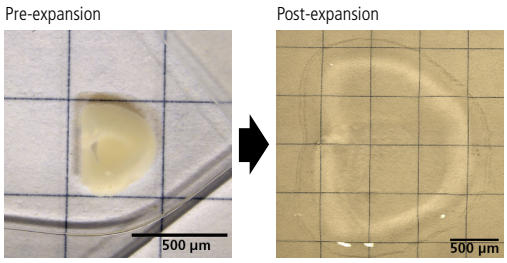
Microglia, the brain immune cells, monitor their territories using their ramified processes to contribute to the formation and maintenance of neural circuits. A recent study showed that microglial processes have thin filopodia that sense their surroundings with different dynamics from the thicker microglial processes (Bernier et al., 2019). Because it has been assumed that microglia exhibit diversity in gene expression, morphology, and cell density across distinct brain regions (Masuda et al., 2020), there is a growing interest in the correlation of fine morphology with the heterogeneity of microglia. To address this issue, an imaging technology that can capture large-volume images with the subcellular resolution is required. This application note introduces an efficient imaging technique that combines a high-speed multiphoton system and expansion microscopy, a recently developed super-resolution microscopy technique.

Keywords: Expansion microscopy, Microglia

## Methods

Expansion microscopy (ExM) was proposed to overcome the diffraction limit of optical microscopy by physically expanding tissue specimens (Chen et al., 2015). In ExM, amine groups of proteins in biological specimens are cross-linked by a chemical agent (Acryloyl-X, SE, etc.), followed by gelation using hydrogels based on polyacrylamide and a water-absorbing polymer. Gelled samples are physically homogenized by enzymatic digestion or thermal denaturation and isotopically expanded by adding water. The extent of expansion (up to 4.5x in ExM) enables nanoscale resolution beyond conventional optical microscopy. Since the expanded specimen is about 99% water, acquiring high-resolution images within large volumes is possible using a water immersion objective with a long working distance, even at deep layers.

In this study, CX3CR1-EGFP heterozygous mice that express EGFP in brain microglia were perfused with 4% paraformaldehyde, and 300 µm-thick coronal brain slices were prepared by a vibratome. The brain slices were permeabilized and immunostained with rabbit anti-GFP antibody and Alexa 488-labeled goat anti-rabbit IgG secondary antibody. After tissue expansion, Z-stack images were obtained using AX R MP multiphoton microscope with a 20x water immersion objective.




Stereo microscope images (SHR Plan Apo 1x, zoom 0.63x)

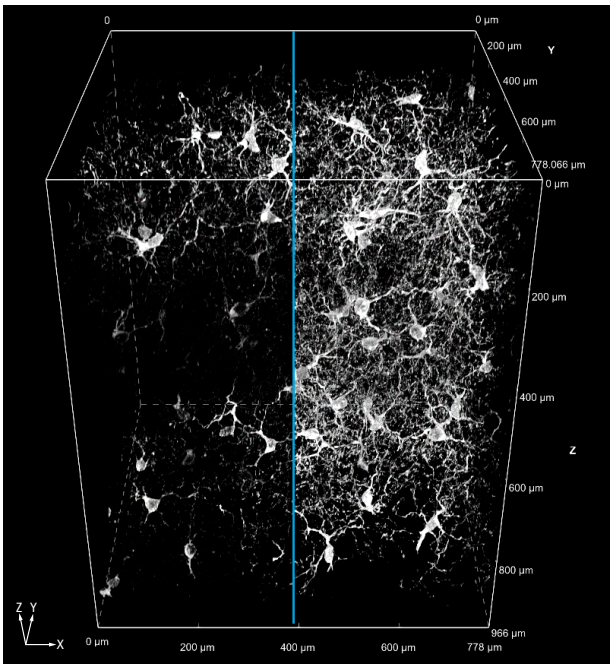



**Figure 1. Advantages of Z-intensity correction**

Two-photon excitation microscopic image by AX R MP (excitation wavelength: 860 nm, detection wavelength: 500-550 nm)
Left: Without intensity adjustment by Z-stack depth, Right: With adjustment
Poor penetration of dye-conjugated secondary antibodies causes non-homogeneous staining of large tissue samples. Nonlinear intensity adjustment of the excitation laser controlled by the microscope system significantly improves the intensity nonuniformity across different imaging depths.

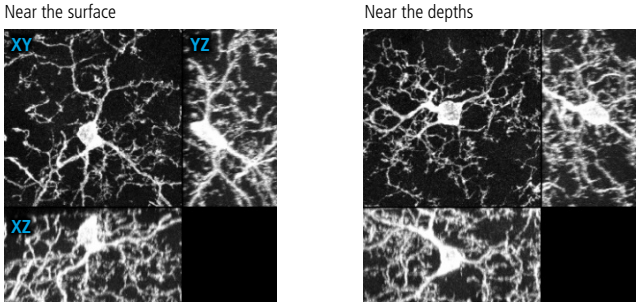



**Figure 2. Advantages of water immersion objectives**

In ExM, the sample refractive index of 1.33, which is similar to the value of water, eliminates the problem of refractive index mismatch. As a result, the spread of the confocal volume with increasing optical depth due to the spherical aberration can be significantly reduced.

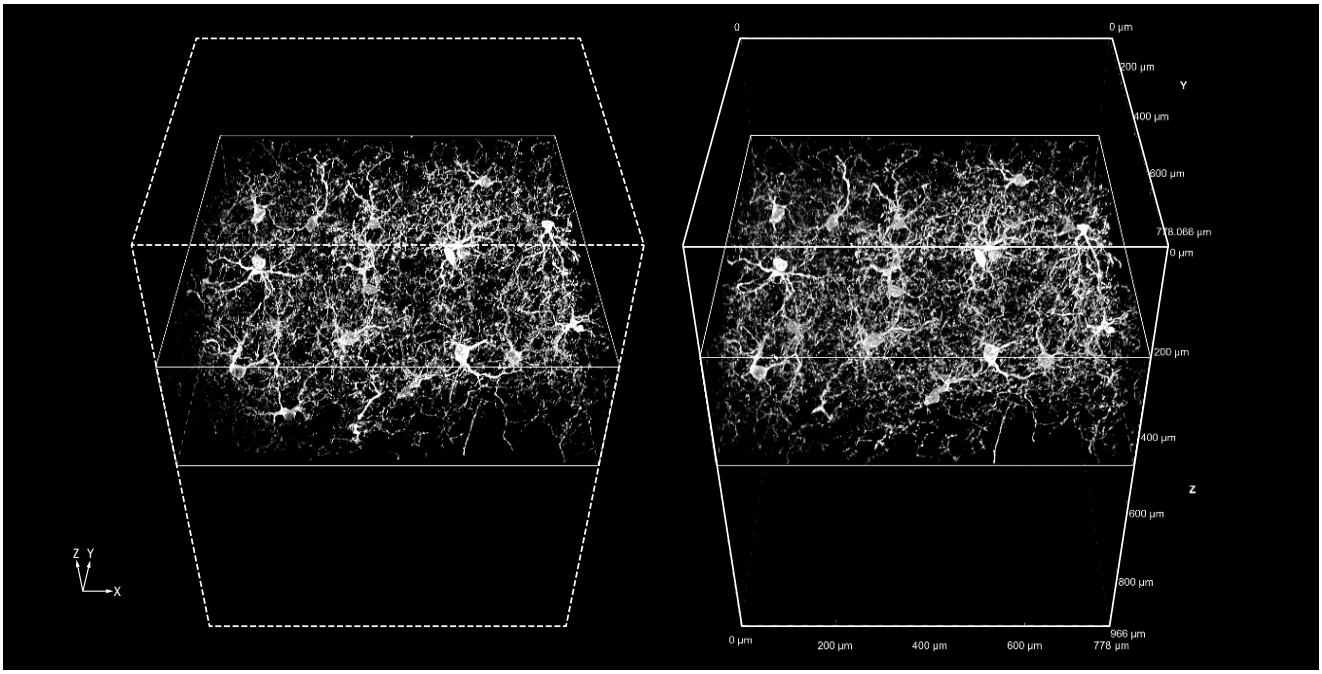
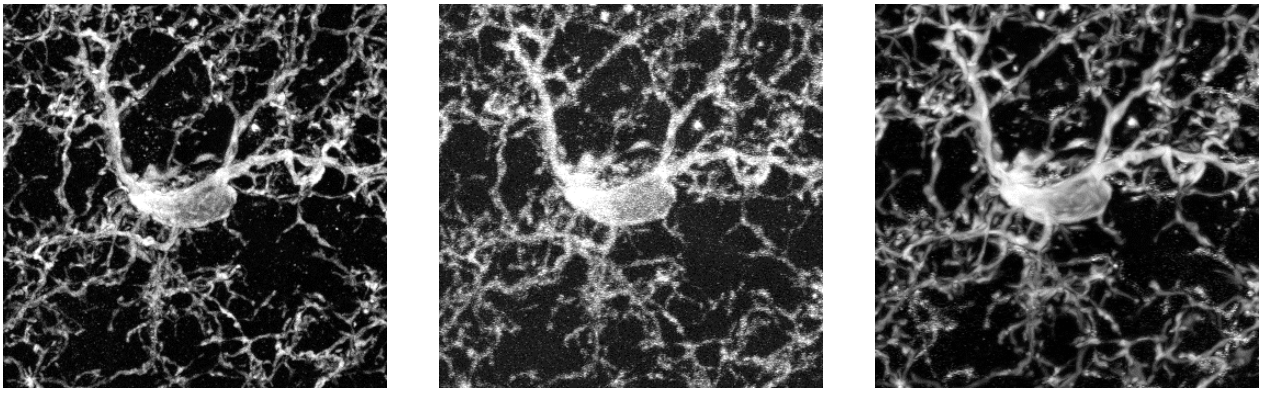



Figure 3. High-throughput imaging with resonant scanner

Left: Z-stack/3D image by galvano scanner. It took about 55 minutes to acquire 251 Z-stack images at a pitch of 1 µm.
Right: A part of the Z-stack/3D image from surface to deep layer acquired at 1 µm pitch with a resonant scanner was extracted for comparison. A Z-stack of 966 images was acquired in only about 20 minutes, and the cellular-level quality of the microglia image is comparable when compared with the left image obtained by the galvano scanner in the same depth range.



Galvano scanner | Resonant scanner (Z-intensity correction) | Resonant scanner (Z-intensity correction + Denoise.ai)

Figure 4. Advantages of Denoise.ai

Resonant scanners allow for an extremely short pixel dwell time (laser irradiation time per pixel in point scan), so a deterioration in the S/N of images can be an obstacle when observing fine structures such as microglial processes and filopodia. In this case, the Denoise.ai function (image processing for acquired images) can automatically calculate the S/N for each pixel to reduce noise.

## Summary

Expansion microscopy is very effective for macroscopic and microscopic imaging and analyzing microglia widely distributed in the brain. To support scientists to acquire information-rich imaging data, AX R MP high-speed multiphoton system is equipped with a Z-intensity correction function that allows uniform sample excitation regardless of depth, a water immersion objective with low magnification, high NA, and long working distance, a resonant scanner for rapid image acquisition, and a denoise function for preventing image deterioration due to shot noise. AX R MP has boosted the possibility of more multifaceted observation and analysis by expansion microscopy.

## Product Information

### CFI75 Apochromat LWD 20XC W

Supporting large FOV imaging of 22 mm, this objective provides bright images even at the edge of its large field of view at low magnifications. It achieves both a high NA (1.00) and a long working distance (2.8 mm), enabling crisp imaging deep within the sample.

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## Reference

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