

## APPLICATION NOTE

## AX/AX R with NSPARC Confocal-based Super Resolution Microscope

## 3D visualization of Paneth cell granule secretion: verification of distortion correction along optical axis using objective correction collar

Confocal microscopy enables the visualization of the three-dimensional structures of biological samples with high spatial resolution, making it an indispensable tool in life science research. However, when observing thick biological samples, spherical aberration occurs due to the sample's thickness and the refractive index mismatch between the sample and the medium. Therefore, proper knowledge and techniques are required to eliminate distortion along the optical axis and accurately image the true 3D structure. In this application note, we demonstrate a method for achieving high-resolution 3D time-lapse imaging with minimized distortion deep within thick organoids.

Some objectives are equipped with a correction collar to compensate for variations in coverslip thickness as well as spherical aberration caused by sample thickness. This produces sharper images, particularly in the deeper sections of thicker samples, by minimizing spherical aberration. Standard usage involves focusing on the target in sample while rotating the correction collar to a position where the target appears brightest and at the highest resolution.

Paneth cells, located at the base of small intestinal crypts, secrete cytoplasmic granules rich in an antimicrobial peptide,  $\alpha$ -defensin, in response to stimulus from luminal bacteria and food, and contribute to innate enteric immunity, as well as to regulating the composition of the gut microbiota. To elucidate the dynamics of granule secretion by Paneth cells, it is necessary to track three-dimensional granule dynamics with high temporal and spatial resolution. We attempted high-resolution 3D time-lapse imaging of the spatial dynamics of secretory granules from Paneth cells located deep within a small intestinal organoid (a 3D culture system of small intestinal epithelial cells). Light from the objective passes through various media of differing refractive indices, including Matrigel (the extracellular matrix surrounding small intestinal organoids) and spatially layered cells, before reaching the Paneth cell granules. Therefore, spherical aberration correction using a correction collar is necessary. We demonstrated that the images with the least distortion along the optical axis can be obtained when the correction collar is set to a position derived by calculation. Under these optimized conditions, we performed high-speed XYZ time-lapse imaging using the highly sensitive NSPARC detector to capture the 3D dynamics of Paneth cell granules secreted into the lumen of small intestinal organoids.

Keywords: correction collar, working distance (WD), refractive index (RI), spherical aberration, confocal microscope, 3D image

### Sample preparation

Small intestinal epithelial organoids were prepared by isolating crypts from the small intestines of mice and embedding them in Matrigel (Corning Inc., NY, RI = 1.34). Organoid cell membranes on day 3 of culture were stained with CellMask™ Deep Red (Thermo Fisher Scientific, MA), while the granules secreted by the Paneth cell were stained with Zinpyr-1 (Santa Cruz Biotechnology, TX). These were seeded in an 8-well chamber (Matsunami Glass Ind., Ltd., Japan, coverslip thickness: No. 1S; 0.16–0.19 mm). The observation target was Paneth cells, in which both granules at the apical end and lumens were visible in the same X-Y plane.

### Experiment 1: Optimization of the correction collar position for imaging Paneth cell granules within organoids

Small intestinal organoids are highly sensitive to changes in temperature and humidity, and as such must be embedded in Matrigel (a holding medium with an RI of 1.34, similar to water) in order to remain active on the microscope stage. The Paneth cells and secreted granules in the organoids exhibit high contrast under diascopic bright-field microscopy, and can therefore be assumed to have a refractive index of over 1.34.

CFI SR Plan Apochromat IR 60XC/1.27 WI (WD=0.18 mm) and CFI Plan Apochromat Lambda S 60XC/1.30 Sil (WD=0.3 mm) objectives were used to observe an identical small intestinal epithelial organoid prior to granule secretion via stimulation. The thickness of each layer along the optical axis was estimated from the objective displacement for each position of the correction collar (Figure 1). Sequential Z-stack images were acquired to compare distortion along the optical axis of each lens (Figure 2).

During measurement, the correction collar was rotated incrementally in only one direction to eliminate mechanical hysteresis. We used the water immersion objective first, since water is easier to remove. Although there were concerns about photobleaching, no photobleaching was confirmed by observing other cells in the same chamber after the experiment.

### Experiment 2: Live imaging of Paneth cell granule secretion

After inducing granule secretion from Paneth cells by adding 10  $\mu$ M of carbachol (CCh) to the culture medium, high-speed XYZ time-lapse imaging was performed using a resonant scanner and a piezo Z drive. Figure 3 shows images illustrating Paneth cell granule secretion toward the lumen as three-dimensional dynamics.

30 Z-stacked images were acquired sequentially at 0.25  $\mu$ m intervals within 0.083 seconds, achieving continuous data acquisition with a time resolution of 2.625 seconds/frame in the acquired 3D movie. We used an NSPARC detector with higher sensitivity to minimize potential damage from laser irradiation.

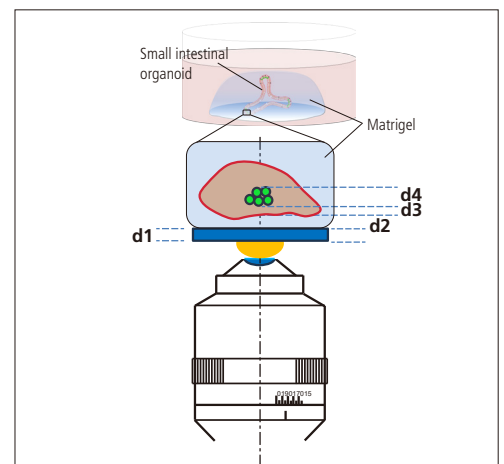


Figure 1. Calculation of the equivalent Coverslip thickness

		Refractive index
d1	Coverslip thickness (fixed at 0.17 mm in this study)	1.52
d2	Average thickness of Matrigel from coverslip to bottom of organoid	1.34
d3	Average intracellular distance from bottom of organoid to bottom of secretory granule	1.52
d4	Z-stack width of secretory granule (intracellular average value)	1.52

Equivalent coverslip thicknesses (d2<sub>CG</sub>) (d3<sub>CG</sub>) (d4<sub>CG</sub>) were calculated by multiplying d2, d3, and d4 by the coefficients x1.34/1.52, x1.52/1.52, and x1.52/1.52, respectively.

The effective coverslip thickness (d1 +  $\sum \Delta d^*_{CG}$ ) was calculated from the difference ( $\Delta d^*_{CG}$ ) between the equivalent coverslip thickness (d<sup>\*</sup><sub>CG</sub>) and objective displacement. This was used as a guideline to determine how far the correction collar should be operated from the position (0.17) derived based on the standard coverslip thickness of 0.17 mm (Table 1).

We confirmed that by adjusting the correction collar according to these guidelines, distortion along the optical axis is minimized, resulting in the brightest Z-stack image (Figure 2).

Figure 2: XY, XZ, and YZ images of secretory granules from the same Paneth cell within the same organoid. The brightest images with the least distortion along the optical axis, as denoted by the red dotted lines, were obtained with the correction collar positioned at 0.17 for the IR 60XC/1.27 WI (upper row) and at 0.16–0.15 for the Lambda S 60XC/1.30 Sil (lower row).

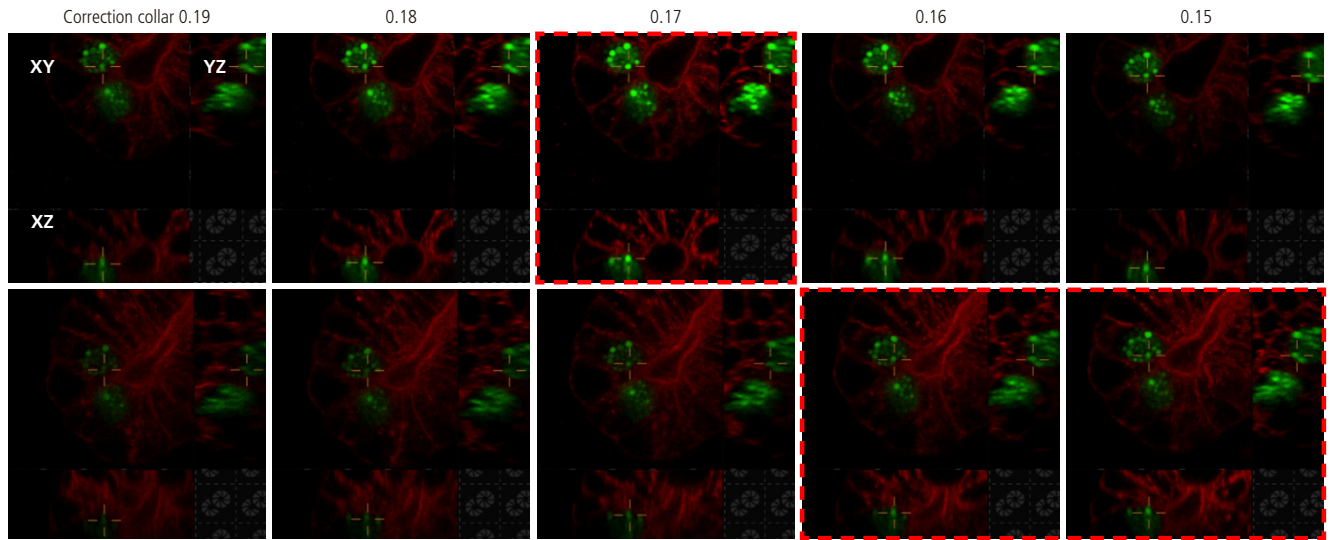
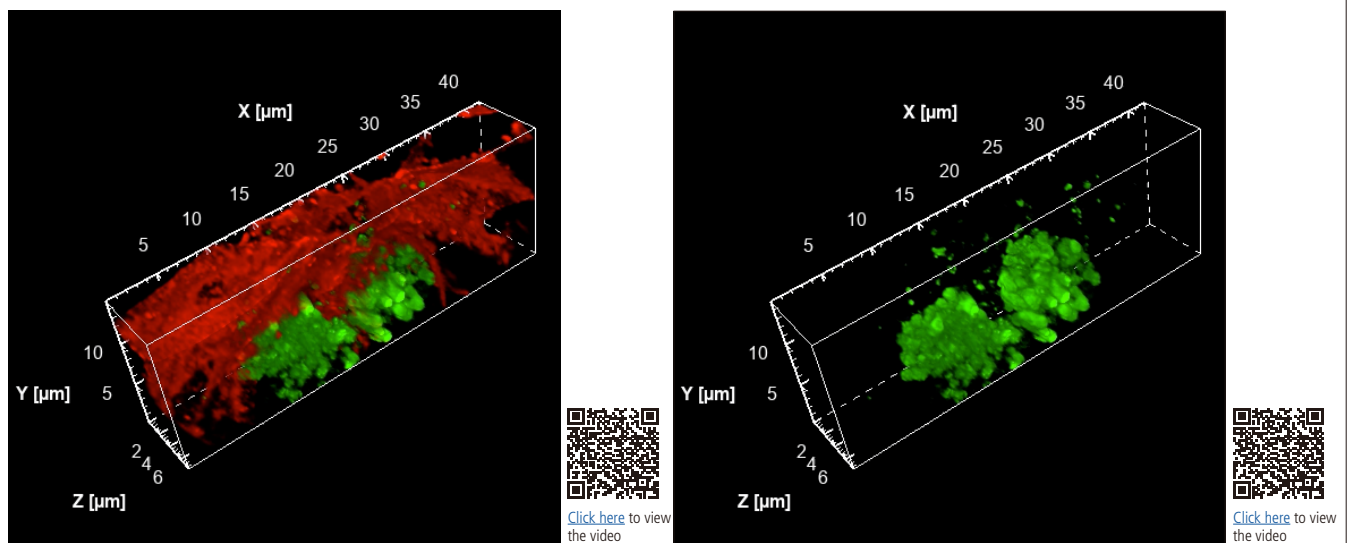


Table 1: This table shows interlayer objective displacement, sample thickness, equivalent coverslip thickness, difference from objective displacement, and the position of the correction collar according to the guidelines, when using the IR 60XC/1.27 WI (left) and Lambda S 60XC/1.30 Sil (right). The guideline correction collar position was 0.17 for the IR 60XC/1.27 WI and slightly lower than standard at 0.16–0.15 for the Lambda S 60XC/1.30 Sil.

	Design value	Objective displacement	Average sample thickness ( $d^*$ )	Equivalent coverslip thickness ( $d^*_CG$ )	Difference from objective displacement ( $\Delta d^*_CG$ )	Effective coverslip thickness ( $d^*_CG + \Delta d^*_CG$ )		Design value	Objective displacement	Average sample thickness ( $d^*$ )	Equivalent coverslip thickness ( $d^*_CG$ )	Difference from objective displacement ( $\Delta d^*_CG$ )	Effective coverslip thickness ( $d^*_CG + \Delta d^*_CG$ )
WD	0.18						WD	0.3					
Distance from objective tip to coverslip	0.18	0.126				0.17	Distance from objective tip to coverslip	0.3	0.247				
Coverslip thickness	0.17						Coverslip thickness	0.17					0.17
Distance from coverslip to bottom of organoid	0	0.025	0.025	0.022	-0.003	-0.003	Distance from coverslip to bottom of organoid	0	0.034	0.032	0.028	-0.006	-0.006
Distance from bottom of organoid to bottom of secretory granule	0	0.008	0.009	0.009	0.001	0.001	Distance from bottom of organoid to bottom of secretory granule	0	0.004	0.004	0.004	0	0
Z-stack width of secretory granule	0	0.017	0.02	0.02	0.002	0.002	Z-stack width of secretory granule	0	0.016	0.017	0.017	0.001	0.001
Guideline correction collar position						0.17	Guideline correction collar position						0.165

Unit: mm

Figure 3: From Figure 2, the brightest images with the least distortion were obtained from the IR 60XC/1.27 WI with correction collar position at 0.17. Using this configuration, CCh stimulus was applied and high-speed 4D (XYZ-t) images were obtained. These show the granules (green) released by the stimulus moving from the cell membranes (red) toward the lumen (upwards). The image on the right shows only the secretory granules of Paneth cells, which are of interest.



## Results and discussion

As demonstrated in this experiment, spherical aberrations must be considered when the subject is not in contact with the coverslip and multiple media with different refractive indices are interposed. Although this was a simplified examination of the optical path length along the optical axis, it was found that the brightest and least distorted images could actually be captured at a guideline correction collar position calculated using measured values and an equivalent coverslip thickness (Figure 2).

While silicon immersion objectives with a larger numerical aperture (NA) were expected to produce brighter images, water immersion objectives ultimately outperformed under the present refractive index conditions. This is presumed to be because there was a large difference in refractive index between the silicone (RI=1.406) and the Matrigel (RI=1.34) holding the organoid, resulting in a greater spherical aberration effect than that of a water immersion objective.

Ultimately, the three-dimensional dynamic behavior of Paneth cell granules secreted into the lumen of small intestinal organoids could be captured with minimal distortion along the optical axis using high-speed XYZ time-lapse imaging with a water-immersion objective (Figure 3).

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Authors: Ryu Nakamura, Yumiko Ohuchi and Shingo Nagawa at Nikon Corporation

## Product information

### AX/AX R with NSPARC Confocal based Super Resolution Microscope

The super-resolution detector NSPARC, featuring a 25-detector array, achieves even higher resolution with a high S/N ratio, without impairing the functions of the conventional AX/AX R confocal microscope.

Product information is [here](#)

