SPPC アレイ検出器を用いた共焦点超解像イメージング

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Confocal Super-Resolution Imaging using SPPC Array Detector

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細胞は生き物の体を作る基本的な構成要素である．この数十マイクロメートル程度の大きさの細胞の中には，核，ミトコンドリア，小胞体，ゴルジ体などのたくさんのオルガネラ（細胞内小器官）や，無数の蛋白質が存在し，生命活動を維持するために機能している．オルガネラや蛋白質は蛍光染色が可能で，長年蛍光顕微鏡でイメージングされてきた．近年では，様々な細胞機能や病態生理のメカニズムをより正確に理解するために，より高い空間分解能での観察が求められるようになってきている．

共焦点レーザー顕微鏡システム AX/AX R は，焦点外の不要な光を取り除いた断層像を取得することが出来る蛍光顕微鏡であり，細胞の観察に使用されている．我々は，AX/AX R に超解像検出器ユニット NSPARC（Nikon SPatial ARray Confocal）を新たに搭載した．NSPARC は SPPC アレイという特殊な検出器を用いることで，従来の共焦点顕微鏡の長所を損なうことなく，空間分解能を向上させることが出来る．本稿では，まず NSPARC の空間分解能向上の光学的原理を説明する．続いて，検出器に使用している SPPC アレイ検出器について説明する．最後に，生物サンプルでのアプリケーション例を紹介する．

Cells are the basic elements of living organisms. Cells, which are several tens of micrometers in size, contain numerous organelles such as the nucleus, mitochondria, endoplasmic reticulum, and Golgi apparatus, including countless proteins that function to maintain life activities. Organelles and proteins can be fluorescently stained and have been imaged with a fluorescence microscope for several years. To understand the physiological mechanism more accurately, higher spatial resolution imaging is required.

The confocal laser microscope system AX/AX R is a fluorescence microscope that can acquire thin optical sectioning images without unnecessary out-of-focus light, and is utilized to image cells. We installed a new super-resolution detector unit NSPARC (Nikon SPatial ARray Confocal) on the AX/AX R. NSPARC employs a special detector called an SPPC array to improve spatial resolution without sacrificing the advantages of conventional confocal microscopes. Here, we first explain the optical principle of improving the spatial resolution of the NSPARC, and then explain the SPPC array detector. Finally, we introduce an application model for biological samples.

Key words 共焦点顕微鏡，超解像イメージング
confocal microscopy, super resolution

1 Introduction

Confocal microscopy [1] targets tissue sections and cell clusters with a size of several hundred micrometers to several millimeters, and cells of tens of micrometers (Fig. 1(a)). The smallest target is a microstructure within cells called organelles, which have a size of several hundred nanometers. These organelles can be labeled with different colors with fluorescent proteins or fluorescent dyes, and their intracellular dynamics can be imaged with a confocal microscope (Fig. 1(b-e)). The structures and subcellular localizations of organelles are closely related to the cell function and pathophysiology and are subject to imaging in fields such as life science and drug discovery.

Although conventional confocal microscopes can capture the external shapes of organelles, it is difficult to accurately capture the fine internal structure. There were problems in terms of large trade-offs, such as a significant decrease in temporal resolution and an increase in photo-damage, as described below. Therefore, we developed a super-resolution detector unit called NSPARC that can improve the spatial resolution of conventional confocal microscopy applications without these trade-offs.

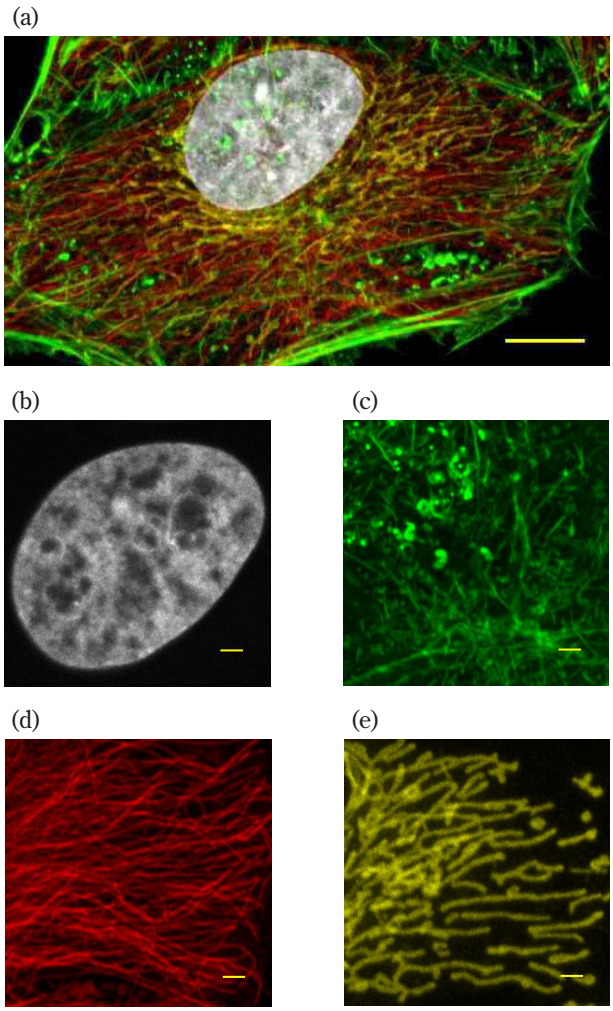

Fig. 1 Multicolor images captured with a confocal microscope
(a) Four types of organelles of HeLa cells were fluorescently labeled and captured with a confocal microscope (AX).
White: Nucleus
Green: Actin (cytoskeleton)
Red: Microtubules (cytoskeleton)
Yellow: Mitochondria
(b-e) Enlarged monochrome image of each organelle
Scale bar (a) 10 μm, (b-e) 2 μm

2 Optical principles of NSPARC

First, an outline of the principles of a confocal microscope is given. A confocal microscope is a type of laser scanning microscope that restricts out of focus light by passing the fluorescence signals from a sample through a confocal pinhole and extracting only the fluorescence signals near the focal plane. In the confocal optical system laser scans the sample surface in a state in which the pinhole and the laser light source always maintain a conjugate relationship. A sample image can be captured by measuring the intensity of fluorescent light that has passed through the pinhole with a photodetector such as a photomultiplier tube (PMT) and creating a light intensity map that associates the position of the scanning mirror with the measured light intensity (Fig. 2). A confocal microscope image is characterized by a high signal-to-noise ratio (S/N) because of the suppression of out of focus light by the pinhole.

To obtain images with a high spatial resolution while ensuring a high S/N, it is necessary to narrow the pinhole diameter to the Airy diameter, which is the spot size calculated from the theoretical calculation of the point spread function (PSF) of the fluorescence signal. In confocal microscopes, it is common to use the values converted to the Airy unit (AU) to express the pinhole diameter, and this expression is also used in this paper.

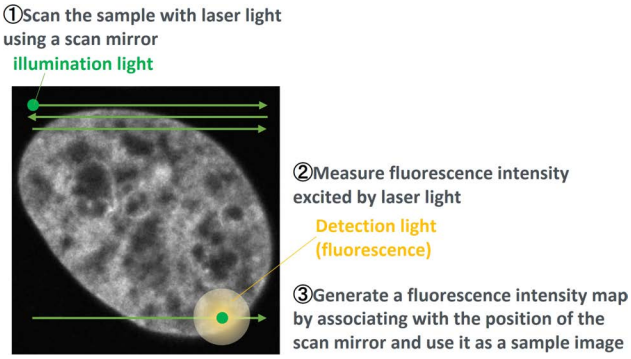

Fig. 2 Fluorescence signal image acquisition scheme of confocal microscope

For general observation, the pinhole diameter is set to a value close to 1 AU (= 1.22 λ/NA), which balances the spatial resolution and S/N. However, when spatial resolution is prioritized, the pinhole may be narrowed to less than 1 AU. If the pinhole diameter is reduced, the spatial resolution improves, but when the pinhole diameter is less than 1 AU, the fluorescence signal is also cut off significantly, resulting in the deterioration of S/N. Comparing the relationship between the spatial resolution and fluorescence intensity with respect to the change in pinhole diameter, it can be seen that the decrease in fluorescence intensity is steeper than the improvement rate of the spatial resolution under the condition of a narrow pinhole with less than 1 AU (Fig. 3). The deterioration of the S/N also adversely affects the results of deconvolution (inverse convolution calculation) image processing. Therefore, if the pinhole size is reduce, it is not always possible to capture a high spatial resolution images. In order to capture high-resolution images, it is necessary to improve the resolution while maintaining the S/N. Therefore, it is necessary to decrease the operation speed or increase the laser power to improve the S/N. However, these measures are accompanied by large trade-offs such as a decrease in temporal resolution and an increase in photodamage to the sample. NSPARC is a microscope device that

has overcome this trade-off relationship between spatial resolution and S/N, and adopts an optical system based on image scanning microscopy [2]–[5] (hereinafter referred to as ISM), enabling image acquisition with both high S/N and high spatial resolution.

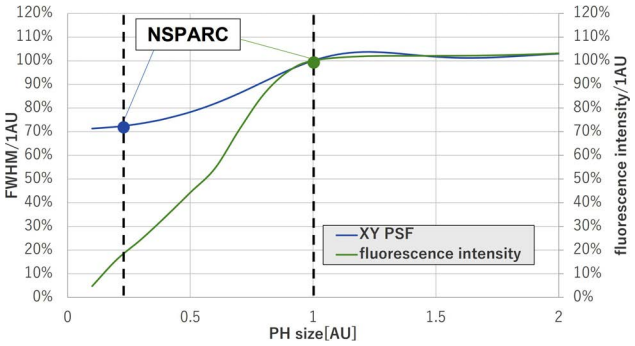

Fig. 3 Relationship between pinhole diameter and fluorescence -spatial resolution
FWHM: Full Width at Half Maximum

Next, ISM, which is the imaging technology used in NSPARC, is explained. In ISM, instead of a pinhole, a two-dimensional array detector is placed at a position at which it can be conjugated with a laser light source. While this detector functions as a pinhole, it also performs light intensity measurements at the same time. In addition, by using an array detector, the fluorescence projected onto the detection surface can be divided and captured by each pixel, and the spatial resolution can be improved by the image processing described below. Under standard observation conditions, projection was performed under the condition that 1 AU is inscribed onto a 5 × 5 pixel array detector, and fluorescence signal for the size of 0.2 AU per pixel is captured (Fig. 4).

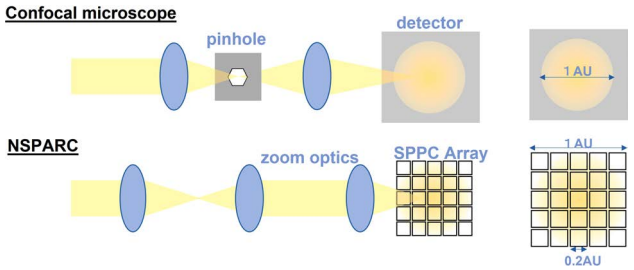

Fig. 4 Comparison of the optical systems of a confocal microscope and NSPARC

Each pixel of an array detector generates a light intensity map from the results of the light intensity measurements, and outputs it as an image. These output images have an improved spatial resolution because each pixel corresponds to a pinhole with a diameter of 0.2 AU, but they are dark and have a poor S/N because these are images captured with small pinhole. However, as a fluorescent signal equivalent to 1 AU can be secured if the images obtained from each pixel are summed up, the S/N of the images can be increased. However, image shifts occur between the images obtained by each pixel, and the spatial resolution then becomes equivalent to 1 AU just by summing up. In ISM, images with both a high resolution and high S/N, which were difficult to obtain with conventional confocal microscopes, can be obtained by performing reassignment processing. This is a process during which images are shifted and summed up while a spatial resolution of 0.2 AU is maintained (Fig. 5).

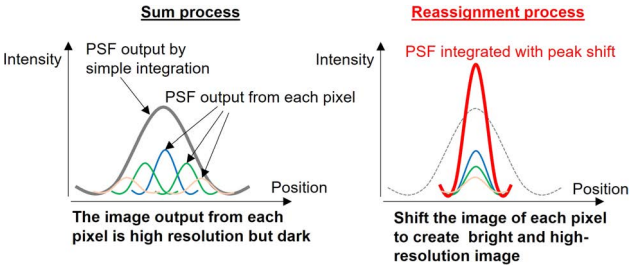

Fig. 5 Relationship between reassignment processing and PSF

The basic principle of reassignment processing, which is the image processing used in ISM, is explained. Effective PSF_{eff}, which represents the imaging performance of a confocal microscope, can be obtained by calculation, and the effective PSF_{eff} of each pixel of an array detector can also be estimated. In reassignment processing, the amount of PSF shift due to each pixel arrangement of the array detector is calculated by a theoretical calculation and is corrected to match the PSF peak position of each pixel by shifting it in the reverse direction during summing up.

The effective PSF_{eff} of a confocal microscope is obtained from Eq. (1) from PSF_{ex}, as determined by the excitation optical system, PSF_{em}, as determined by the detection optical system, and the pinhole function, PH.

$$PSF_{eff}(r) = PSF_{ex}(r)\{PSF_{em}(r) \otimes PH(r)\} \quad (1)$$

In the ISM optical system using an array detector, each pixel acts as a tiny pinhole and moreover, it is at a position shifted from the optical axis by the arrangement of each pixel. Therefore, the effective PSF_{eff} of each pixel is obtained from Eq. (2) when the pinhole is positioned at the position with the same axis shift as the arrangement of the array detector.

$$PSF_{eff}(r) = PSF_{ex}(r)\{PSF_{em}(r) \otimes PH(r-d)\} \quad (2)$$

It can be seen that PSF_{eff} in this case has a peak position

at a position shifted from the optical axis (Fig. 6). As the shift amount and the shift direction also change depending on the position of the pixel, the correction amount of the shift is calculated for each pixel, and the output images from each pixel are moved and summed up. The process of shifting and summing up these images is called reassignment processing, and the images captured at each pixel can be summed up while maintaining a spatial resolution of 0.2 AU. The reassigned image can haven an improved spatial resolution by about 1.3 times while the S/N can also be maintained with respect to the standard 1 AU image.

In addition, it is possible to perform deconvolution of the reassigned image by using the effective PSF_{eff} after reassignment processing. This image processing can remove background light blurring and improve the spatial resolution.

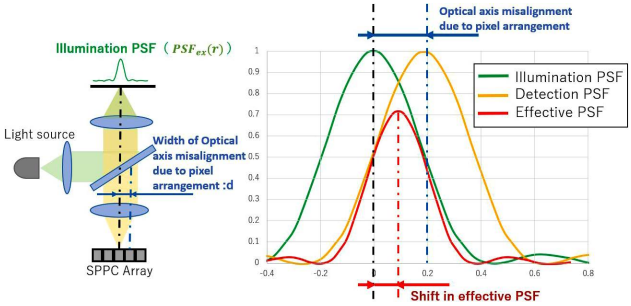

Fig. 6 PSF of ISM

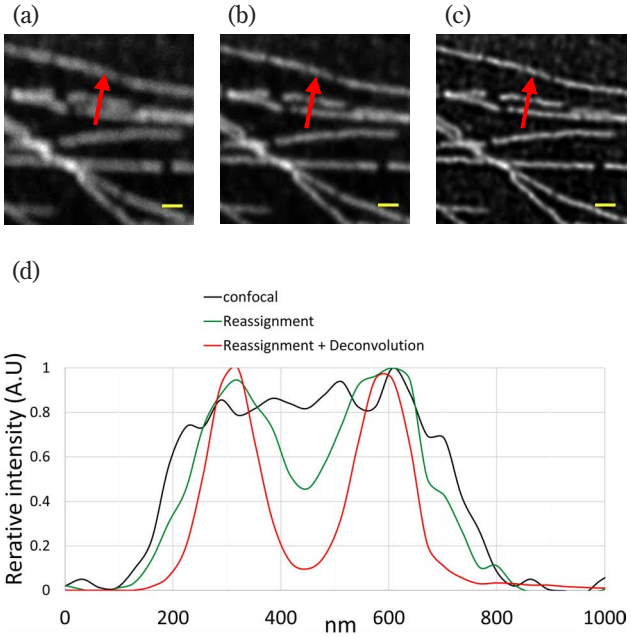

Fig. 7 Spatial resolution in biological samples
Microtubules of HeLa cells captured with PlanAPOλD 60x 1.42
(a) Confocal image
(b) Reassigned image
(c) Reassigned image + Deconvolution processing
Scale bar is 500 nm.
(d) Line profile shown in (a) to (c)

Fig. 7 shows (a) a confocal image, (b) a reassigned image, and (c) a deconvoluted image of the reassigned image. It can be seen that the two linear structures that could not be resolved in the confocal image can be confirmed as the cleavage after the reassignment processing and can be further clearly resolved into two by deconvolution.

3 SPPC Array detector

Next, we explain the single pixel photon counter (SPPC) array detector newly adopted by NSPARC. The detector used in NSPARC is a newly developed SPPC array. The SPPC array is a photon counting device in which avalanche photo diodes (APDs) are arranged in array form. It is sometimes called a single photon avalanche diode (SPAD) array. A two-dimensional array detector was required for ISM. There was also the idea of using a conventional PMT in the early stages of development, but it is difficult to arrange the detection surface in an array in addition to its large size. Therefore, we adopted an SPPC array in which 25-pixel APDs are installed in array form.

The SPPC array used in NSPARC is paved with 25 pixels with a 50 μm × 50 μm square being one pixel (Fig. 8). The light receiving surface of the detector is only 250 μm × 250 μm, even if 25 pixels are combined. It is possible to increase the size of the detector, but increasing the size of the detector would lengthen the optical system required for focusing, which would increase the size of the device. In contrast, in the case of a small detector, a high accuracy is required for alignment adjustments. The pixel size of the detector also affects the sensitivity and noise. After various considerations, it was judged that a pixel size of 50 μm was optimal.

Although the size is smaller than that of a PMT, the parameters directly related to image quality, such as detection efficiency and wavelength range sensitivity have the

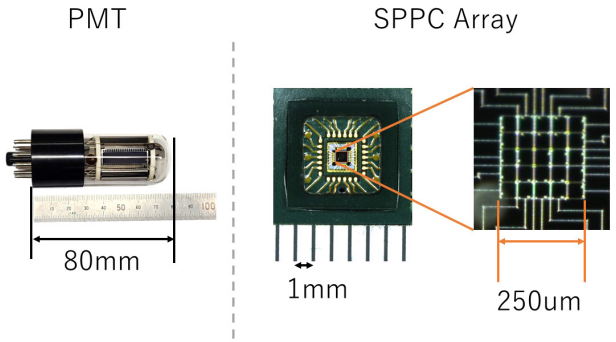

Fig. 8 PMT and SPPC array size comparison
The PMT is approximately 80 mm × 28 mm, while the SPPC array is approximately 7 mm × 7 mm.

same performance as for a PMT. From the images actually captured, it can be seen that the SPPC array images are comparable to those of a PMT (Fig. 9).

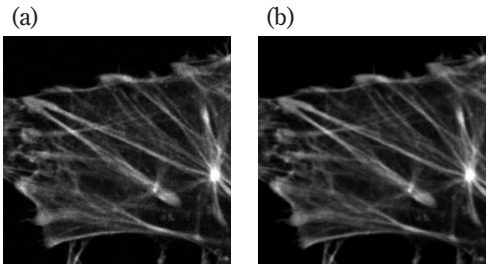

Fig. 9 Comparison between the GaAsP PMT and SPPC array
(a) Confocal image captured by the GaAsP PMT
(b) Confocal image captured by the SPPC array detector

The SPPC array also represents an improvement in terms of usability, although this does not directly affect the image quality. The sensitivity of the PMT may deteriorate when receiving strong incident light. Therefore, it is necessary to be careful to not use intense light during use. The confocal microscope AX was equipped with a safety function to stop imaging when strong light was incident. Compared with the PMT, the SPPC array receives no damage due to incident light and can be used with ease. Therefore, there is no hassle of imaging potentially being interrupted by the safety mechanism.

The process of constructing an image from the signal output of the SPPC array is described. The SPPC array is an array of APDs that multiplies a single incident photon and outputs a single electrical pulse signal. Many detectors, such as a PMT, acquire the voltage value by converting the light to the strength of the voltage and doing an AD conversion. Then, the AD-converted numerical value becomes the brightness value of the image (Fig. 10 top). However, the SPPC array outputs a unique voltage pulse regardless of the magnitude of the light intensity when the photon hits the light receiving surface. It is possible to count the number of incident photons by counting these pulses with an electric circuit. In the SPPC array, this count number becomes the brightness value of the image (Fig. 10 bottom). Thus, the SPPC array is also significantly different from the PMT in terms of the signal output method, and can realize a higher S/N and more stable measurements than a PMT in feeble light measurement.

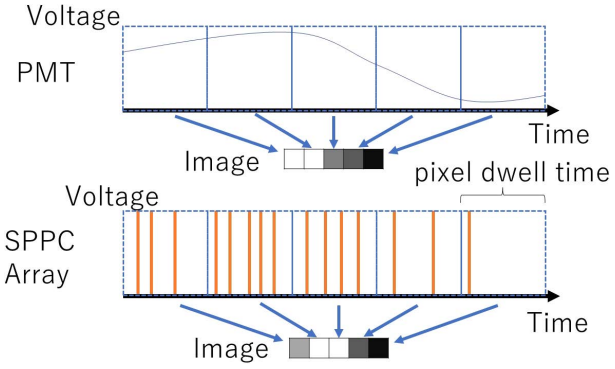

Fig. 10 Difference in image construction methods between the PMT and SPPC array
In the PMT, the strength of the analog voltage becomes the brightness value of the image, but in the SPPC array, the number of pulses counted within the time of one pixel becomes the brightness value of the image.

4 Applications using biological samples

Three applications using NSPARC are introduced. First, the images of multi-color imaging are shown in Fig. 11. The fixed HeLa cell nucleus was labeled with a fluorescent probe called DAPI, the microtubule with Alexa Fluor™ 488 (Thermo Fisher Scientific Inc-JP. Tokyo, Japan), and the mitochondrial outer membrane with Alexa Fluor™ 568 (Thermo Fisher Scientific Inc-JP.) (Fig. 11(a)). Excitation was done sequentially at three excitation wavelengths of 405, 488, and 561 nm, and imaging was captured with NSPARC. Figs. 11(b-d) show reassignment + deconvolution images, and Figs. 11(e-g) show confocal images. In NSPRAC images, the contrast and S/N are remarkably improved, and the microstructure of each organelle is clearly captured. NSPARC can improve spatial resolution in multi-color imaging, which is a conventional application of confocal microscopy, and is expected to play an active role in the study of interactions between organelles and the localization of protein molecules.

Next, images of live cell mitochondria are shown in Fig. 12. Mitochondria are composed of a double membrane consisting of the outer and inner membranes; the inner membrane has a stripe-like structure called the cristae invaginated toward the interior. MitoTracker™ Green FM (Thermo Fisher Scientific Inc-JP.) was used as a fluorescent probe to observe the cristae structure. Fig. 12(a) shows the reassignment + deconvolution image, and Fig. 12(b) shows the confocal image. The cristae, which were blurred in the latter image, could be clearly visualized in the first image with increased contrast. Next, the morphological changes of mitochondria was observed by time-lapse imaging. The morphology of mitochondria is known to change dynamically within the cell, with repeated division and fusion on a second-by-second basis. In Fig. 12(c), the time-lapse images at 1.9 fps in 3-s intervals captured the moment when minute

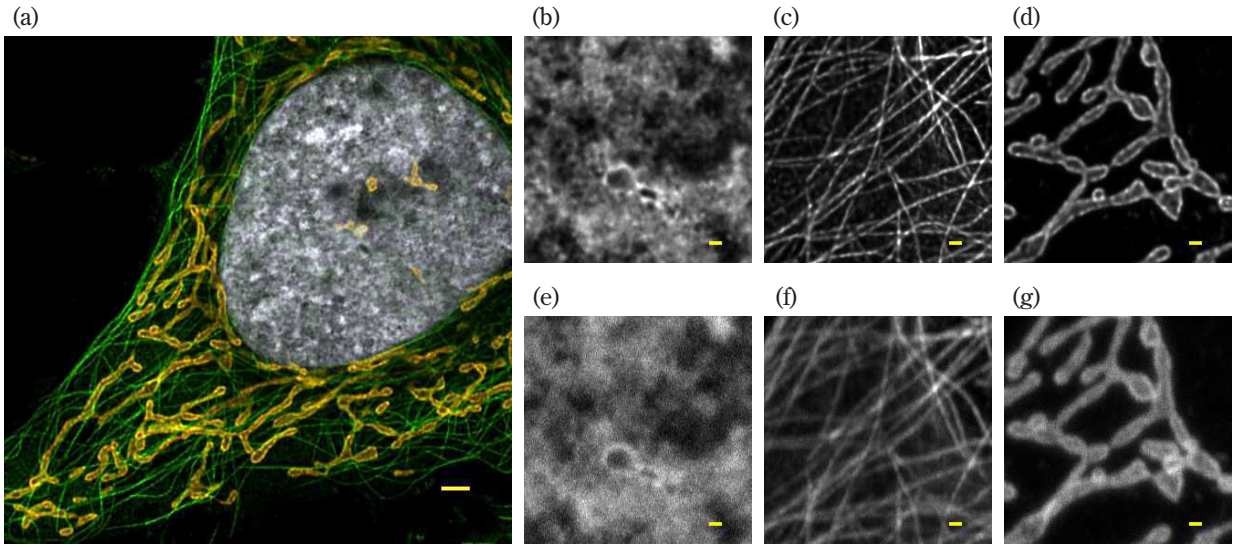

Fig. 11 Multi-color imaging images

(a) Reassignment + deconvolution image of fixed HeLa cell
White: nucleus (405 nm excitation), green: microtubules (488 nm excitation), orange: mitochondria (561 nm excitation)
Captured with a PlanAPOλD 60x 1.42 oil objective lens.
(b-d) Reassignment + deconvolution images. b) nucleus, c) microtubules, d) mitochondria
(e-g) Confocal images e) nucleus, f) microtubules, g) mitochondria
The scale bar is (a) 2 μm, (b-g) 500 nm.

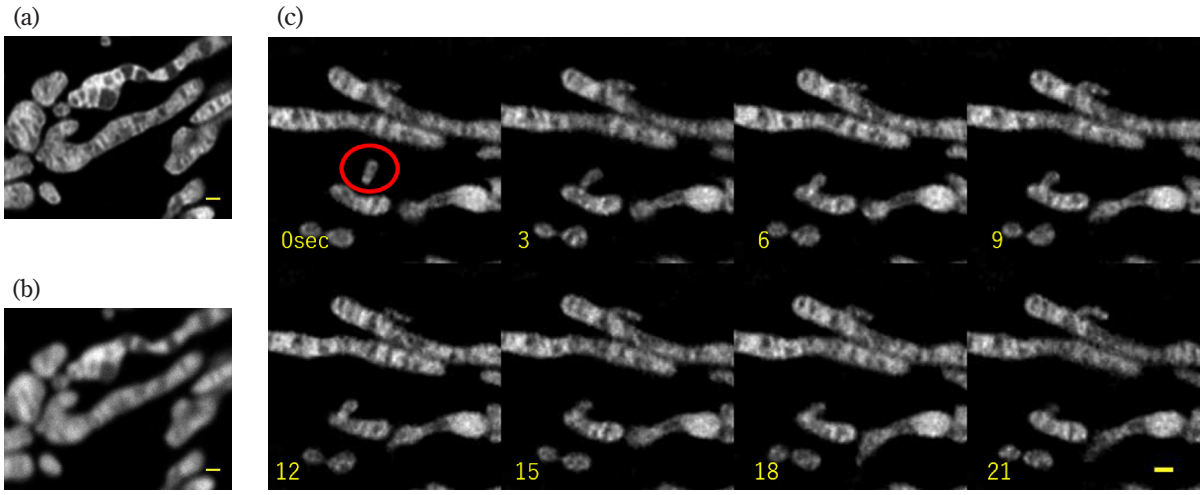

Fig. 12 Live cell imaging of mitochondria

Image of mitochondria labeled with MitoTracker™ Green FM. Captured with a PlanAPOλD 60x 1.42 oil objective lens.
(a) Reassignment + Deconvolution image and (b) Confocal image
(c) Time-lapse image of assignment + deconvolution image
Frame rate of 1.9 fps, captured in 3-s intervals.
The scale bar is 500 nm.

mitochondria of about 500 nm moved around violently and fused into large mitochondria. It is known that there is a correlation between mitochondrial morphology and cell viability, and NSPARC is expected to play an active role in the research focusing on the relationship between mitochondrial morphology and disease.

Finally, the *z*-stack images of the cleared mouse brain slice are shown in Fig. 13. A 5 × 5 tiling image (Fig. 13(a)) captured with a 10 × objective lens and a partially enlarged image (Fig. 13(b)) are shown. It can be seen that the neurons expressing a fluorescent protein called eYFP are densely packed. Further, the objective lens is switched to a 60x oil immersion objective lens and a *z*-stack image of the light blue frame part of Fig. 13(b) is captured. The three-dimensional image is shown in Fig. 13(c). The dendrites were observed to form the neural circuits and run in various directions. In addition, spiny structures called spines with a diameter of about several hundred nm could also be con-

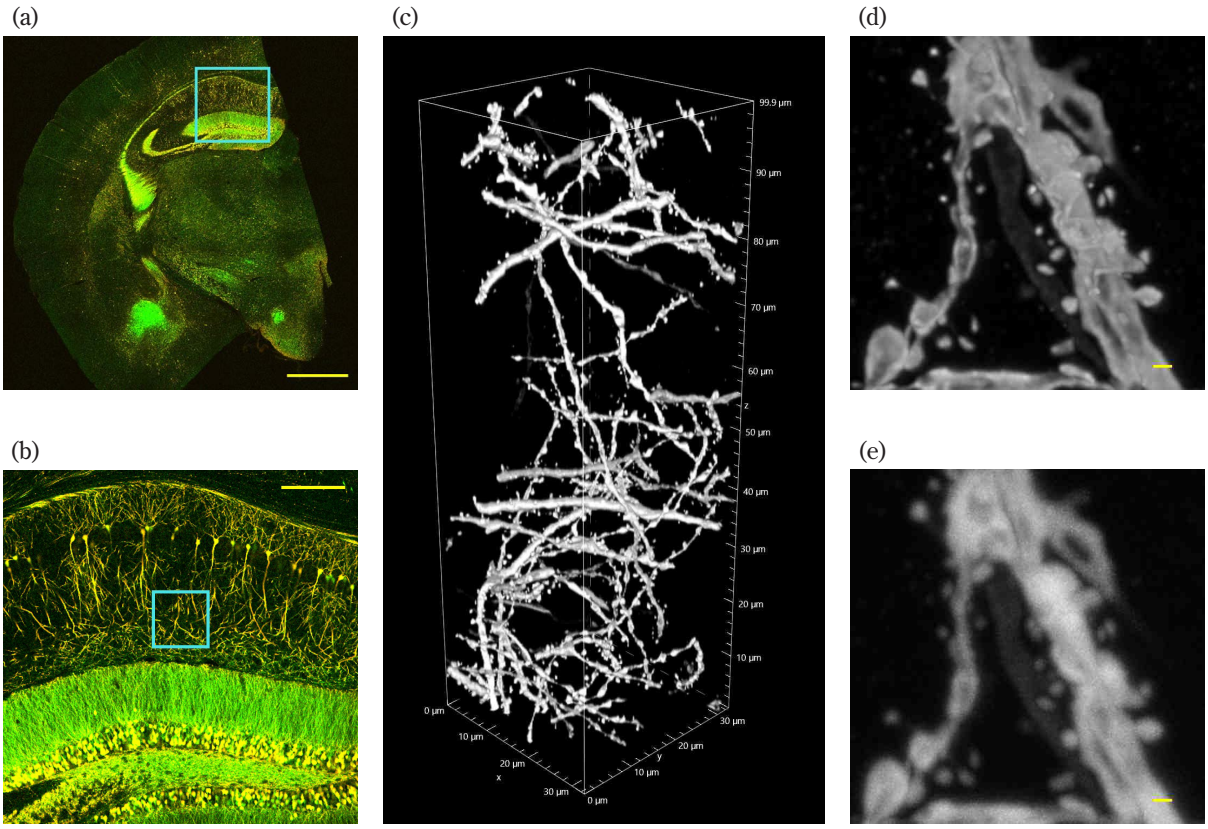

Fig. 13　z-stack images of cleared mouse brain slice

(a) Entire image of a cleared mouse brain slice. A 5 × 5 tiling confocal image captured with PlanApo10x/0.45.

(b) Enlarged confocal image of part of (a)

(c) Three-dimensional image of reassignment + deconvolution image of the light blue frame part of (b)
Captured using PlanApo λD60x 1.42 oil.

(d) Enlargement of max projection of 80–100 µm deep part of (c) (reassignment + deconvolution image)

(e) Enlargement of max projection of 80–100 µm deep part of (c) (confocal image)

Scale bar (a) 1000 µm, (b) 200 µm, (d, e) 500 nm

Sample courtesy: Lin Daniel, PhD. SunJin Lab Co.

firmed on the surface of the dendrites. Fig. 13(d) shows an enlarged part of the max projection image of the 80~100 µm deep part of the z-stack in Fig. 13(c). Compared with the confocal image in Fig. 13(e), it can be seen that the spines are not just spherical, but have various shapes. Thus, NSPARC is also useful in deep z-stack imaging.

As shown in the above three examples, NSPARC can improve the spatial resolution for general applications, such as multi-color imaging, live cell imaging , and deep imaging of confocal microscopy without trade-offs.

5 Conclusion

We believe that NSPARC equipped with ISM technology, which has no trade-offs compared with conventional confocal imaging, will become the standard for confocal microscopy in the future. In addition to the results shown in Section 4, it is possible to greatly improve spatial resolution in many confocal microscope applications. Furthermore, this technique can be expected to be widely used not only in bioimaging in academia, but also in industrial fields, such as in drug efficacy evaluation and toxicity testing in drug discovery. For example, we can image cancer invasion in 3D cultured cells by utilizing the deep imaging capability of NSPARC, and image morphological changes due to drug responses in mitochondria and nerve cells sensitive to light damage by utilizing low phototoxicity. It is expected that new evaluation indices such as morphological changes of organelles and protein localization, which have been unobservable or overlooked so far, will be discovered, leading to an improved efficiency and accuracy of drug efficacy evaluation.

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