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Since microscope had been invented, microscopists showed various structures of biological specimens from tissue sections to proteins and microscopy became one of the most general methods in biology. Microscopy is categorized to light microscopy and electron microscopy. Various types of light microscopes have been developed by using the characteristics of photon such as fluorescent, phase contrast, evanescent wave, and multi-photon excitation. The biggest advantage of light microscopy is an *in vivo* imaging. Because specimens for electron microscopy must be set in vacuum condition, it is difficult to keep the specimen alive in an electron microscope by current technique. Just as described, light microscopy is useful for various observations but the resolution of light microscopy is limited by diffraction to about 200 nm. In contrast, because the wavelength of electron is very short (0.0037 nm for 100 kV), the resolution of standard TEM (100 kV) attains to 0.3 nm. For that reason, to observe small structures that can not be observable by light microscopy, transmission electron microscope (TEM) is used. However, specimens for TEM are needed to be processed with several treatments which could alter intact structures *in vivo*. These treatments inevitably cause various artifacts such as shrinkage of tissues and deformation of ultrastructures of cells.

In common with both microscopies, observation of living specimens with high resolution is an ideal. As a one of the problems for light microscopy, two-photon microscopy is possible for *in vivo* imaging but, observable depth is limited to ~ several hundred μm from surface by light scattering. On the other hand, electron microscopy also has another problem that cryo-electron microscopy can manage ultrastructures of vitreous specimens in the close-to-physiological state but, image contrast of micrographs is obscure, because major components of biological specimens are light

elements.

As a microscopic method for biological research, *in vivo* imaging of deep portion and high contrast imaging of vitreous specimens are thought of as adequately important. Therefore, in order to dissolve these problems, he tried to two approaches; one is *in vivo* imaging of living animals with a stick-type objective lens, another is tuning of Zernike phase plate for vitreous complex specimens.

First, he tried to establish the *in vivo* imaging for deeper organs. In the field of neuroscience, low-invasive *in vivo* imaging would be a very useful method for monitoring the morphological dynamics of intact neurons in living animals. At present, there are two widely used *in vivo* imaging methods; one is the two-photon microscope method, and the other is the fiber optics method. However, these methods are not suitable for the *in vivo* imaging of deeper subcortical structures. In his study, he has developed a novel method for the *in vivo* imaging of pyramidal neurons in layer V of the cerebral cortex, utilizing a MicroLSM system and a stick-type objective lens that can be directly inserted into the target tissue.

In the MicroLSM system, the approaches to Thy1-YFP-expressing cerebral cortex layer V pyramidal neurons are approached by inserting the stick-type objective lens into the brain. Entire basal dendrites, cell bodies, and axonal branches in a layer V pyramidal neuron were imaged *in vivo* by the MicroLSM system. The morphology of the pyramidal neuron imaged by the MicroLSM system closely resembled that of the neuron in the fixed horizontal slice preparation observed by the CLSM. By using this method, we succeeded in obtaining clear images of pyramidal neurons in layer V of the cerebral cortex under a low-invasive condition.

In this study, he did not try a neuroscientific functional experiment using the MicroLSM system and show any data for such an experiment. However, the stick-type

objective lens is able to equip a catheter to irrigate internal tissue with saline or to drain excess saline. The catheter is considered to be applicable to the staining of internal tissue. This method would be useful for the analysis of brain dynamic morphology regulated by posttranslational modification of cytoskeletal proteins, such as tubulin polyglutamylation, tau protein phosphorylation and tau protein dephosphorylation. This *in vivo* imaging technique for deeper organs, tissues and cells may be useful not only in the field of neuroscience, but also in other fields of biology.

Second, he tried to tune the phase plate for the application of Zernike phase contrast TEM (ZPC-TEM) to vitreous complex biological specimens. Cryo-electron microscopy of vitreous specimens is ideal for observation of native biological ultrastructures. However, it requires a large amount of defocus to gain adequate contrast with conventional TEM and the defocusing selectively visualize low frequency component of objects by attenuation of the contrast of high frequency components. In contrast, ZPC-TEM can recover the contrast of low frequency components of objects without defocus. However, the ZPC-TEM with a film type quarter wave plate (FTQW plate) having a 700 nm diameter central hole has another problem especially in imaging of complex biological specimens such as cultured cells and tissue sections. Strong halos occurring around specimen structures hinder the interpretation of images. Therefore, this problem has restricted application of the ZPC-TEM only to purified small particles. In order to overcome this problem, a new FTQW plate with a smaller central hole was fabricated and tested it on vitreous biological specimens.

By applying new FTQW plate (central hole diameter is 300 nm), even though micrifying the central hole increased the intensity of halos, the frequency of halos became lower. Furthermore, the comparison of phase contrast images acquired with a

700 nm or a 300 nm diameter central hole FTQW plate showed that though intensity of halos was stronger but, the image graininess was improved in the phase contrast image acquired with the 300 nm diameter central hole. This improvement of image graininess would be caused by the recovery of the contrast of lower frequency components that was not recovered by FTQW plate with the 700 nm diameter central hole. As a result, recovery of lower frequency component improves the fidelity of phase contrast images. Actually, phase contrast images of quickly frozen cultured cells acquired with FTQW plate with the 300 nm diameter central hole successfully showed various fine cytoskeletal filaments. Furthermore, phase contrast images of vitreous brain sections acquired with FTQW plate with the 300 nm diameter central hole successfully showed the bridging structures and short filaments binding vesicles. ZPC-TEM with the new plate successfully visualized the intracellular fine features of cultured cells and brain tissues in in-focus images. This result indicated that reduction of the central hole diameter makes ZPC-TEM applicable from protein particles to tissue sections.

Taken together, ZPC zero-loss images shown in this study evidently indicated that ZPC method gives us opportunity to observe the vitreous specimen with higher fidelity than the conventional method. As like the CEMOVIS (cryo-electron microscopy of vitreous sections) and TOVIS (tomography of vitreous sections), he named the combination of ZPC-TEM and vitreous sections as PEMOVIS (phase contrast electron microscopy of vitreous sections). The PEMOVIS will be a powerful imaging method, and advance the new field of imaging science for close-to-physiological ultrastructures.

論文の審査結果の要旨

生物試料を対象とした光学顕微鏡、電子顕微鏡において“生きている” 試料、あるいは生きた状態にできるだけ近い試料を高分解能で観察することは理想である。しかし、現在よく行われている光学顕微鏡による in vivo 観察では外部より入射した光が生体試料により散乱、吸収されて減衰するために観察可能な領域は表層から数 100 μm 程度の深部までに限られている。一方、電子顕微鏡観察では生理状態に近いといわれるガラス化凍結試料の観察像はコントラストが弱く、微細構造をはっきり観察することができない。

本研究ではこれらの問題の解決のため、2 つの新規観察法を試みている。一つは光学顕微鏡においてスティック型対物レンズを用いた in vivo 観察法、もう一つは電子顕微鏡用ガラス化凍結生物試料の Zernike 位相差電子顕微鏡を用いた高コントラスト観察法である。

スティック型対物レンズを用いた in vivo 観察法において、スティック型対物レンズを麻酔下のマウス脳組織に刺入して、深部構造の観察を行った結果、麻酔下のマウス Thy-1 YFP H 系統の大脳皮質第 5 層錐体細胞が観察された。このスティック型対物レンズを使用することによって脳だけでなく、その他の組織においても、その深部構造の観察が可能であると考えられる。

Zernike 位相差電子顕微鏡法を用いた複雑な生物試料の観察法の開発においては、まず通常使用している中心孔径 700 nm の位相板を用いてマウス初代培養神経細胞急速凍結試料を観察した。この方法により細胞骨格などが高コントラストで観察されたが、観察像の粒状性が悪く、細胞膜近傍にハロの出現が見られ、像が乱されていた。そこで位相差法の改良のため、位相板の中心孔径を縮小したときの位相差像のシミュレーションを行ったところ、コントラストの強度と出現するハロの周期が位相板の中心孔径と相関していることが分かった。中心孔径を小さくする程、コントラストの強調が強くなり、出現するハロの周期が長くなることが示された。この現象が、実際の観察においても生じることを確認するために、中心孔径 300 nm の位相板を用いて非晶質炭素薄膜及び、培養細胞急速凍結試料を観察した。その結果、シミュレーションと同じような像が得られたとともに、急速凍結試料位相差像において像の粒状性が改善された。さらにマウス脳組織ガラス質凍結超薄切片位相差像では樹脂包埋マウス脳組織超薄切片電子染色試料と同程度のコントラストで微細構造を観察できることが示された。

位相板を用いない従来の電子顕微鏡では電子染色に用いる重金属との親和性に依存した微細構造の観察、もしくは、ガラス化凍結試料の生体分子分布に基づく微細構造をぼんやりとしか観察することができなかったが、中心孔径 300 nm の位相板を用いた Zernike 位相差電子顕微鏡法により、生物試料本来の分子分布に基づく微細構造を明瞭に観察することが可能になった。本方法により、従来の試料調整法と電子顕微鏡観察で問題となって

いた多くの点を解決することができる。例えば従来法では種々の形態変形や生体分子の流出が起こることが知られている。また、電子染色という選択的コントラスト強調のために観察が困難となる微細構造が存在すると考えられる。本研究で新たに開発された Zernike 位相差電子顕微鏡法では、これらの問題点の改善により、生理的微細構造の新規発見に繋がることが期待される。