アポディゼーション位相差顕微鏡法と生物医学応用

大瀧達朗

Apodized Phase Contrast Microscopy and Its Biomedical Applications

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人間の目や普通のカメラは物体の像を明暗のコントラストとして捉える．色は光の波長に応じた明暗を表現している．顕微鏡は小さな物体を観察する重要な器械で，細胞や微生物の発見は今日の医学や生物学の基礎となった．無色透明な細胞などは染色して観察するが，細胞毒性の問題がある．位相差法は無色透明な位相物体を透過した直接光と回折光に光学系で位相差を与え像面で干渉させて像にコントラストを与える．無染色で観察する優れた方法だが従来法では像に現れるハロという現象で微細部分がつぶれる問題があった．この問題の解決のためアポディゼーション位相差顕微鏡法を発案し開発した．細胞などの位相物体で光波に生じる回折角と位相差に関係があることを見出し，大きな物体で生じるハロを減らし微小物体を高コントラストで観察可能にした．最近我々はコントラストの異なる検出力の高い ABH（apodized bright contrast high）法を開発した．応用として肺疾患の原因のアスベスト繊維の検出に用いた．また生細胞の分析に細胞内小器官の屈折率分散が異なることに着目し，波長選択による像コントラストの違いからミトコンドリアと油滴の区別を示した．生殖補助医療分野では，初期胚のタイムラプス撮像で微細な顆粒状構造や繊維状構造を確認した．無染色観察は臨床分野で重要であり，アポディゼーション位相差顕微鏡法は広く応用が可能であろう．その原理と応用について報告する．

Human eyes and general imaging devices detect images as the contrast in brightness or intensity. A colour appears as a contrast depending on differences in wavelengths of light. Microscopes are important instruments for observing small objects, and have contributed to the progress in bacteriology, biology and medical science. Cells are typically colourless and transparent phase objects. Conventional phase-contrast microscopes are suitable for observing phase objects, but large phase-object images lose detailed structures because of halo artifacts. Other than for thin specimens, they are also often used for finding or checking cultured cells. Apodized phase-contrast microscopy was developed to reduce the halos when imaging fine anatomical structures. A relationship exists between the angle of diffraction and phase difference of objects in cells. Apodized phase-contrast microscopy weakens the diffracted light produced by large objects to lower their relative image contrast and increases the contrast of small objects. An apodized phase plate provides an optical filtering. In this study, a bright contrast method for apodized phase-contrast microscopy, called apodized bright contrast high (ABH), was developed. Various biomedical applications, such as analysis of asbestos, cellular organelles and early embryos, were experimented. Apodized phase-contrast microscopy provides images of fine structures. Its principles and biomedical applications are described in this paper.

Key words アポディゼーション位相差顕微鏡法，コントラスト，分散，屈折率，細胞内小器官
apodized phase contrast microscopy, apodization, contrast, dispersion, refractive index, organelle

1 Introduction

Human eyes and general imaging devices detect images as the contrast in brightness or intensity. Colours are expressed as contrasts depending on differences of wavelengths of light. An optical microscope is a useful instrument for observing small objects. Robert Hooke discovered the cell using early microscopes with eyepieces, and he published *Micrographia* in 1665. Robert Koch identified microorganisms, such as various bacteria, in the late 19th century, and supported his postulates describing relations between a disease and a microorganism. Progress in bacteriology, biology and medical science has been achieved as a result of many microscopic observations. Modern optical microscopes incorporate several important optical principles.

Microscopes and optical principles

Ernst Abbe introduced "numerical aperture", $n \sin \alpha$, and defined the limit of the delineating power d of the microscope (1881)[1]. It is expressed by

$$d = \lambda / (2n \sin \alpha),$$

where λ is the wavelength of light, n is the refractive index of the medium and α is the semi-angle of the maximum incident ray to the objective. This limit of the delineating power is known as under coherent illumination. The aperture radius, r, of the objective is calculated by $r = f n \sin \alpha$, where f is the focal length of the objective.

Abbe set up an arrangement of illumination from a point light source (coherent illumination) to the diffraction grating, and it was used as the starting point of imaging theory. For microscopic imaging, he used an arrangement, which illuminates equidistant parallel gratings with point light sources. When a plane wave generated from a point light source illuminates the diffraction grating with an interval d, the wave is diffracted in a direction u given by $\sin \alpha = m \lambda / d$, where m is an order of 0, ± 1, ± 2, ... This type of image is caused by Fraunhofer diffraction. In a lens with a focal length f, a point image is formed at a distance of $f \sin \alpha$ from the optical axis according to the angle α. These diffraction images represent spectra in the frequency domain of the diffraction grating. An object having an arbitrary amplitude distribution and spectrum related by the Fourier transform. Further, the light beam advances and forms an image on the optical conjugate plane of the object. This spectrum and image are also related through a Fourier transform. Whether the diffraction grating is an amplitude grating or a phase grating, a conjugate image is formed. This idea was applied to the imaging theory of the microscope.

Lord Rayleigh proposed the resolution limit of two optically independent points through an optical system (1896, 1903)[2)3)]. He defined a criterion when the distance between two neighbouring images formed through an optical system becomes the distance to the first dark circle of the Airy pattern or radius of the Airy disc. When a plane wave illuminates a pinhole object, the wave is diffracted and imaged through an objective (convex lens). The image forms an Airy pattern on the image plane. The lens shall be ideal and it produces no aberration. Then the wavefront forms spherical waves to the image.

The Rayleigh criterion at the diffraction limit having a circular aperture is expressed by

$$d = 0.61 \lambda / (n \sin \alpha).$$

This shows that two neighbours cannot be clearly distinguished if the distance between them is less than roughly half wavelength of light. It is important to note that this resolution holds for human eyes when recognizing sufficiently bright point images. In many cases, single points may still be visible.

August Köhler developed an illumination method in 1893[4)], which is called Köhler illumination. The illumination requires that the field is uniformly bright. The Köhler illumination system consists of, from a light source to the object plane, a collector lens, a field stop, an aperture stop, and a condenser. The light source is focused by the collector lens on the front focal plane of the condenser (which has the aperture stop) as an air image of the light source. Then, all diverging light through the aperture stop of the condenser uniformly illuminates the object plane if the light source does not have uniform distribution. The field stop and object plane (field) are in optically conjugate planes. In addition, the aperture stop and the exit pupil of the objective lens are in optically conjugate planes. They control the illumination field and the illuminating angles to the object plane, respectively. Most microscope illumination systems are based on Köhler illumination. Further, the field is conjugate to the image plane and projected on the retina by the eyepiece, and the aperture stop is conjugate to the objective back focal plane.

Microscopic observation of phase objects

Microscopic observations of unstained living cells are important for biomedical applications. Cells are typically phase objects. Many microscope systems are used for observing phase objects, with phase-contrast microscopy by Zernike (1935, 1953)[5)6)], differential interference contrast (DIC) microscopy by Smith (1950)[7)], and by Nomarski (1953)[8)], and modulation contrast microscopy by Hoffman and Gross (1975)[9)], which are mostly commercialized for observing unstained cells. Differential interference contrast microscopy uses the polarization and interference of light waves. Modulation contrast microscopy uses a kind of oblique illumination, which shades the image according to changes in the refractive index. It is suitable for observing large structures, and is widely used in fertility treatment such as in vitro fertilization (IVF). Many other methods have been proposed for observing phase objects, such as dark field illumination and optical staining method.

2 Phase-contrast microscopy

Incident light wave, $\phi 0 = \sin \omega t$, transmits through a phase object such as a colourless transparent cell. The transmitted light has a phase difference δ due to a difference in refractive index. Then the light transmitted is described as $\phi 1 = \sin (\omega t + \delta)$. If the phase object produces a small phase

difference as compared to the wavelength, $\phi 1$ can be described as $\phi 1 \approx \sin \omega t + \delta \cos \omega t$. This shows that a phase difference of 1/4 wavelength (or $\pi/2$ rad) is generated between the direct light, $\sin \omega t$, and the diffracted light, $\delta \cos \omega t$. Through bright field optical systems, the image cannot be seen because the direct light and the diffracted light do not interfere at the image plane. If the focus is changed slightly, an image contrast appears because the optical path difference between the direct light and the diffracted light is changed, which causes interference.

The phase-contrast method was discovered by Zernike[5)6)]. It alters a phase difference of $\pi/2$ between the direct and the diffracted light from the phase object, and the image is seen by an enhanced contrast caused by the interference of the lights. The phase-contrast method was described in detail in the literature, such as by Bennett et al. (1951)[10)] and Pluta (1975, 1989)[11)]. In Japan, phase-contrast method was described by Tojo (1942)[12)]. The phase-contrast microscope is now widely used by doctors and biologists. It is used to visualize almost transparent non-staining cells and microorganisms. When the phase difference is small, the transmitted light consists of two components, the direct light (zero order diffracted light) and weak diffracted light having a phase shift of 1/4 wavelength according to the phase difference of the specimen. The phase-contrast objective lens changes the phase shift between the incident direct light and the diffracted light to 0 (same phase) or 1/2 wavelength by an internal phase plate (phase ring). Then, the direct light (background light) and the diffracted light interfere with each other on the imaging plane, and the phase object appears as a contrasted image of bright and dark. Two contrast types of phase-contrast microscopy are bright contrast and dark contrast. Dark contrast refers to an object which phase is shifted 1/2 wavelength and an object which refractive index is higher than the surroundings looks dark relative to the background.

Conventional phase-contrast microscopes are suitable for observing phase objects; however, large phase-object images lose detailed structures because of halo artifacts. Other than for thin specimens, they are often used for finding or checking cultured cells. However, when the direct light is weakened to increase the contrast to observe fine structures, the halo phenomenon of light becomes large, and the fine portion collapses. Thus it was considered not suitable for observing fine parts and thick cells. If the halo can be reduced, it will be easy to observe inner details with no staining.

3 Apodized phase-contrast microscopy

To reduce halo artifacts and to enhance details, apodized phase-contrast microscopy was developed to image fine anatomical structures in 1998–2001[13)~15)]. It achieved reducing halos and enhancing details by applying an apodization method to the conventional phase-contrast method. Apodization is an optical filtering method[16)17)]. The halos are seen as either bright areas or dark areas on phase object images, such as thick cells, which give large optical path differences (*OPD*s), or phase differences (*OPD*/λ), to the light wave. The unwanted halos hide small phase differences behind large phase ones and prevent high resolution. We developed and confirmed the performance of 10×, 20× and 40× magnification objectives with apodized dark contrast low, or low absorption (ADL)[18)]. Compared to off-axis illumination, the effect of apodized phase-contrast microscopy using leaf replica specimens, a common phase object specimen, was reported[19)]. At the same period, the apodized method was applied to high magnification objectives with high numerical aperture (NA) for the purpose of obtaining high-resolution images. The magnification was 100× and NA was 1.30 with oil immersion. We reported its detection capability by imaging actin bundles in living cells[20)21)]. The diameters of actin bundles ranged from 20 nm to 60 nm[22)]. Furthermore, the image formation of isolated phase objects with a very small phase difference below the diffraction limit was considered, and estimations of the size of an observable phase object was reported in detail[23)]. Brief introductions to phase contrast microscopy and apodized phase-contrast microscopy were described in *Experimental Medicine*, *Jikken-Igaku*[24)25)].

4 Principle and experiment

Apodized phase-contrast microscopy has been developed to reduce halos and to enhance details[13)]. Figure 1 shows an optical layout of apodized phase-contrast microscopy with additional bandpass filter(s). The apodized phase plate sets on the transform plane, which is usually at the objective's back focal plane. The principle of conventional phase-contrast method is shown in Fig. 2. It shows that illuminating light (incident light) is diffracted by the phase object and forms (Fraunhofer) diffraction image. The phase plate alters phase and weakens the direct light, and then enhances image contrast at the image plane (not shown). Figure 3 shows the principle of apodized phase-contrast method. Apodization areas weaken selected diffracted lights caused by large pattern or objects. Therefore, large object images have

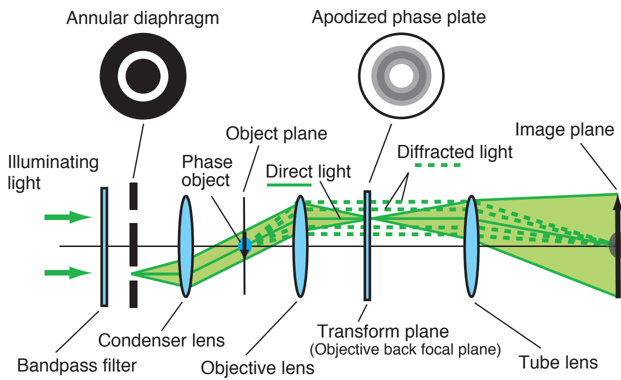
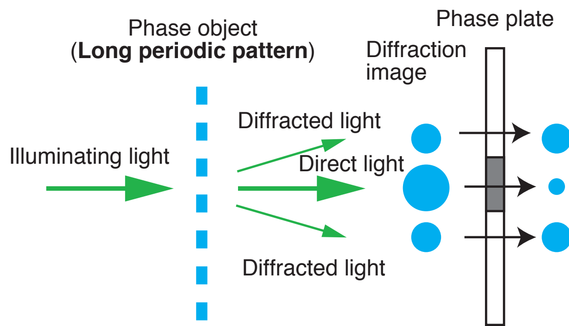


Fig. 1 Optical layout of apodized phase contrast microscopy with bandpass filters. The apodized phase plate was placed in the transform plane. It alters phase between direct and diffracted light to interfere at the image plane. One of the bandpass filters was added to provide selected wavelengths of illuminating light.



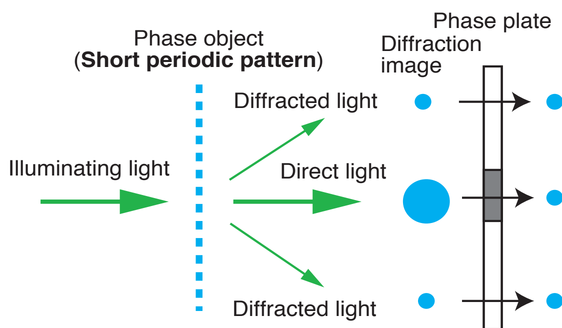
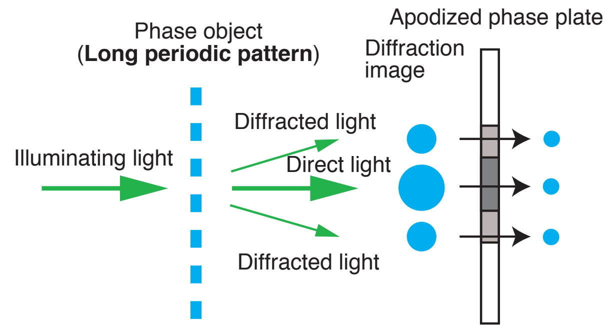


Fig. 2 Principle of conventional phase contrast [adapted from (23)]. To enhance the contrast, the direct light is weakened by the phase plate. (a) Halos appear because strong diffracted light is produced when phase difference is large. (b) Short periodic pattern diffracts light with large angle of diffraction.

weak contrast and small object images have relatively strong contrast.

Newly developed apodized phase plate has a phase ring for bright contrast, which has 1/4 wavelength phase shift throughout the most of visible region with 2% transmittances. In addition, it has two apodization areas with 8% transmittances[26)27)]. Apodized bright contrast high (ABH) performs that the direct light is especially weakened by the



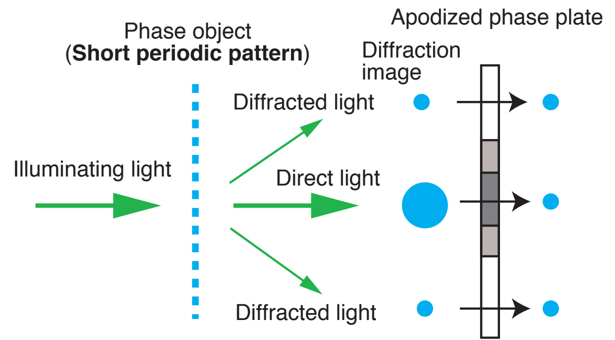


Fig. 3 Principle of apodized phase contrast [adapted from (23)], (a) Apodization areas (half tones) weaken selected diffracted lights caused by large pattern or objects. (b) Short periodic pattern diffracts light with large angle of diffraction, and passes no attenuation areas.

phase ring and selected diffracted light is weakened by apodization areas. We reported that the apodized phase-contrast microscopy (ABH) worked well for observing biological objects such as organelles in living cells. In addition, a new test method for identification of asbestos was reported[24)]. The principle of the test method was based on analysis of refractive indices. We applied this test method to distinguish organelles in unstained living cells[27)].

Microscope and specimens

Figure 4 shows an experimental set up of apodized phase-contrast microscopy. An inverted microscope was used for observing cultured cells.

Figure 5 shows comparison images between using (a) conventional bright medium (BM) and (b) apodized bright contrast high (ABH). Apodized bright contrast image provides wide latitude, for instance, nucleolus can be seen in details. The experiments were performed using the following: an inverted microscope Eclipse Ti-E with a 40× objective lens (CFI Plan Fuor 40X, 0.75 NA, developed ABH), a magnification 2.5× lens (VM 2.5X, Nikon, Japan), and a digital camera (iXon3, EMCCD 2/3", 512 × 512 pixels, Andor, USA). Bandpass filters (OD4 full width half maxi-

mum 50 nm, Edmund Optics Japan) were also used when the proposed method was performed. Specimens were cultured Cos-7 cells (African green monkey kidney fibroblast-like cells). To determine observing organelles, we compared apodized phase-contrast images with fluorescent images. Mitochondria, lipid droplets and nucleus were fluorescently labeled[27]. It was confirmed that mitochondria formed variety of shapes. Round shaped objects were difficult to distinguish mitochondria with lipid droplets. Lipid droplets were enhanced at 400 nm of wavelength, and mitochondria were relatively enhanced at 700 nm of wavelength. It was assumed that the dispersions of refractive index of lipids were larger than that of surrounding cytoplasm. By selecting wavelengths illuminations, the contrast of intracellular organelles changed. In addition, it was observed that image contrast of some dots in the nucleoli was changed by illuminating light wavelengths.

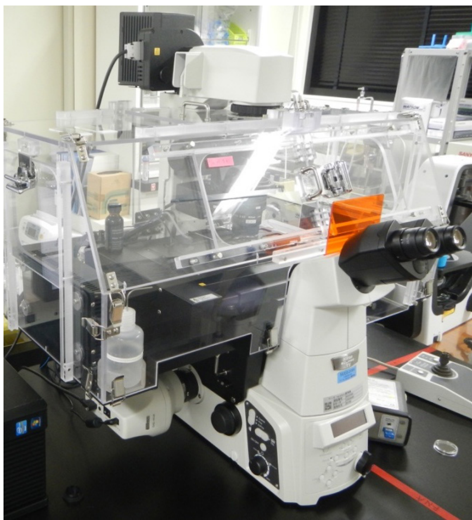


Fig. 4 Experimental set up of apodized phase-contrast microscopy with an inverted microscope and an incubation system for observing cultured cells.

Differentiation is one of the suitable applications of apodized phase-contrast microscopy due to thickness of specimens. Thick specimen (phase object) produces unwanted halos. Early embryos of mice are around 80 μm thick, while those of human are approximately 100 μm to 130 μm thick. Time-lapse imaging for observing early embryos using differential interference contrast microscopy has been proposed and used in assisted reproductive technology (ART)[28]. Apodized phase-contrast microscopy is also a good method for ART application because of resolution. In application for differentiation, early embryos were observed using apodized phase-contrast microscopy. We compared images among with conventional phase-contrast objectives and an apodized phase-contrast objective for capturing cultured mouse early embryos. Halo images were seen in inner early embryos using the conventional phase-contrast objectives. Their images obstructed detailed structure images. On the contrary, clear images were obtained using the apodized phase-contrast objective. Small granules were observed using ABH microscopy[29]. Figure 6 shows a micrograph of cultured mouse early embryos. Finer details appeared using ABH than any conventional type. Fine granules in the perivitelline space and fibrous structures in the cell body were observed.

In apodized phase-contrast microscopy, fibrous structures in mouse and human embryos and oocytes, as well as fine granules were observed[29]. Fine granules in the perivitelline

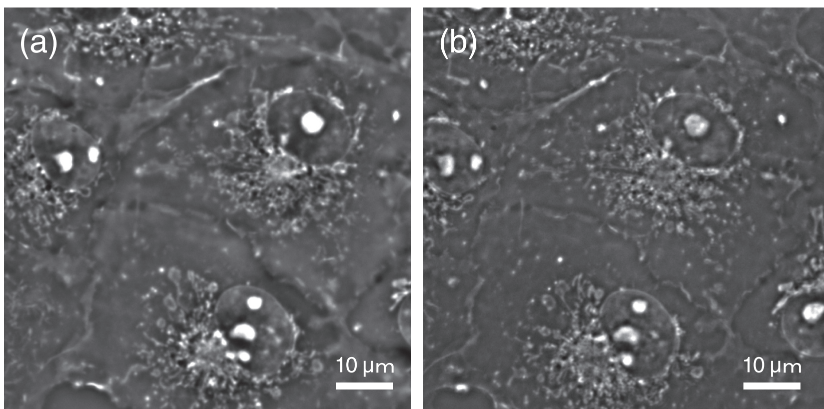


Fig. 5 Micrographs of Cos-7 cells using (a) conventional bright contrast medium (BM) and (b) apodized bright contrast high (ABH) [adapted from (23)]. Halo is useful for finding, but it obstructs image details, e.g. nucleoli's inner detail. Specimen: Cos-7, monkey kidney tissue origin cells. Objective lenses: magnification 40×, numerical aperture 0.75 (CFI Plan Fluor 40X, 0.75 NA).

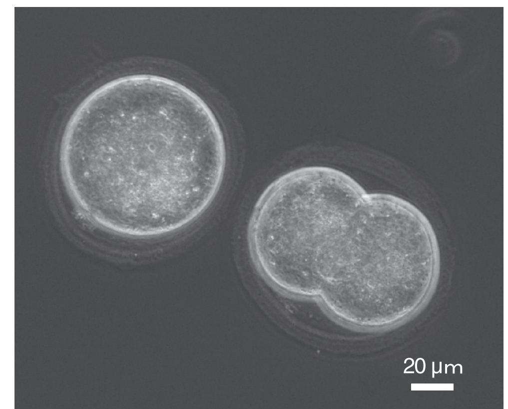


Fig. 6 Micrograph of mouse early embryos [adapted from (23)]. Enhanced contrast image. Minute granules or droplet were observed in the perivitelline space between cell membrane and zona pellucida.

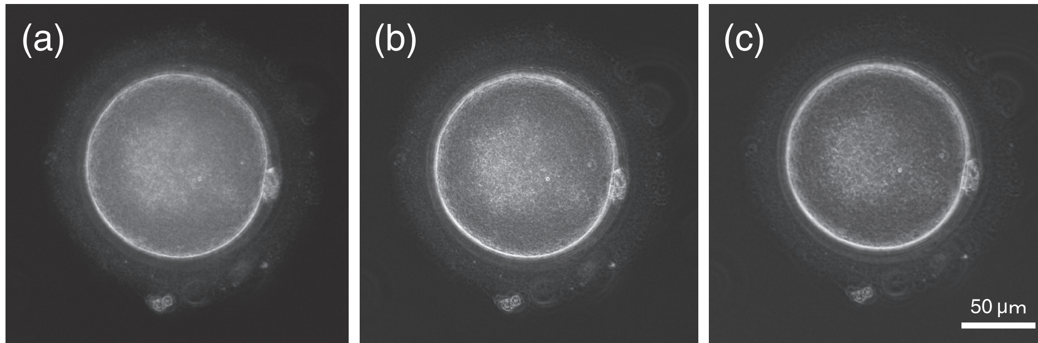


Fig. 7 Granularity images in human oocyte without staining, using selected wavelength illuminations. Centre wavelengths of illumination were (a) 450 nm, (b) 550 nm and (c) 650 nm. More contrast images of granularity (centre part) were obtained using 650 nm illumination than using 450 nm. Dispersion seems different between granularity and fibrous structures in the surrounding area. Objective lens: magnification 40×, numerical aperture 0.75 (CFI Plan Fluor 40X, 0.75 NA, ABH).

space were observed in some human embryos. The granularity was observed not as granular structures but as fibrous structures with higher contrast than surrounding area. Embryo images without staining, that imaged using bandpass filters. Higher contrast images of granularity were obtained using 650 nm illumination than with 450 nm (Fig. 7). Dispersion seems different between granularity and fibrous structures in the surrounding area. It was showed that this microscopy improves the visualization of human embryos and oocyte for clinical use. These experiments were performed using the following: an inverted microscope Eclipse Ti-E with a 40× objective lens (CFI Plan Fluor 40X, 0.75 NA, developed ABH, Nikon, Japan), and a digital camera (DS-Ri2, 24 mm × 35 mm, Nikon, Japan). A stage-top incubator (Tokai Hit, Japan) was also used.

5 Conclusion

Apodized phase-contrast microscopy and its biomedical applications were discussed. Structures were distinguished using apodized phase-contrast microscopy with bandpass filters. For living cell observation, attempts to identify intracellular organelles by forming phase-contrast images at specific wavelengths were described. In ART, culturing for several days by time-lapse imaging was performed. Very small granules in the cell were observed by time-lapse imaging at high speed. Intracellular organelles without staining and several intracellular organelles can be observed and distinguished using apodized phase-contrast microscopy at specific wavelengths of light. This method can be used for observing cellular organelles without staining. Apodized phase-contrast microscopy will be widely used for time-lapse and live-cell imaging. Analysis of intact intracellular organelles will elucidate the structure and function of living organisms. (Most of this review was presented at OPTIC 2019 in Taiwan[30]).

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