APPLICATION NOTE

Phase contrast objective (dark contrast)
ECLIPSE Ti2-E inverted research microscope

Method for selecting a phase-contrast objective for label-free visualization of spatio-temporal dynamics of cell division and organelles

Cells contain various organelles such as nuclei, nucleoli, mitochondria, actin filaments, lysosomes, and lipid droplets. During mitosis, the localization and morphology of organelles change dynamically. Abnormalities in organelles are known to cause many diseases such as metabolic disorders, neurological diseases, cancer, and aging. Live-cell imaging using a microscope is an excellent technique for elucidating life phenomena and diseases, because it provides information on spatio-temporal localization and dynamics. Phase-contrast microscopy allows label-free visualization of cells, making it suitable for long-term live-cell observation. Contrast in the phase-contrast image is determined by the type of phase plate. This application note introduces differences in images due to phase plates and how to select a phase contrast objective.

What is phase contrast observation?

Phase contrast observation is a method for visualizing areas with different refractive indices and thicknesses in a transparent object. There is a dark contrast method that visualizes areas with a higher refractive index than their surroundings in dark (black) contrast against the background, and a bright contrast method that visualizes them in bright (white) contrast against the background.

When light reaches an object, it splits into direct light, which is transmitted through the object, and diffracted light with a variety of angles that depend on the size and structure of the organelles (Fig.1). The phase contrast objective has a phase plate with a phase ring (Fig. 2) that has the following two functions.

1) A function that visualizes the phase change as a light/dark contrast by shifting the phase of the direct light by one quarter wavelength with the phase ring, causing the direct light and diffracted light to interfere with each other.
2) A function that improves phase difference detection sensitivity by reducing the intensity of direct light with a neutral density filter.

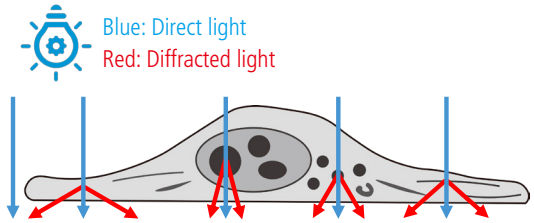


Fig. 1: Light transmitted through a cell

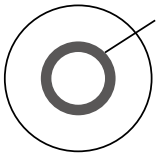


Fig. 2: Functions of a phase plate

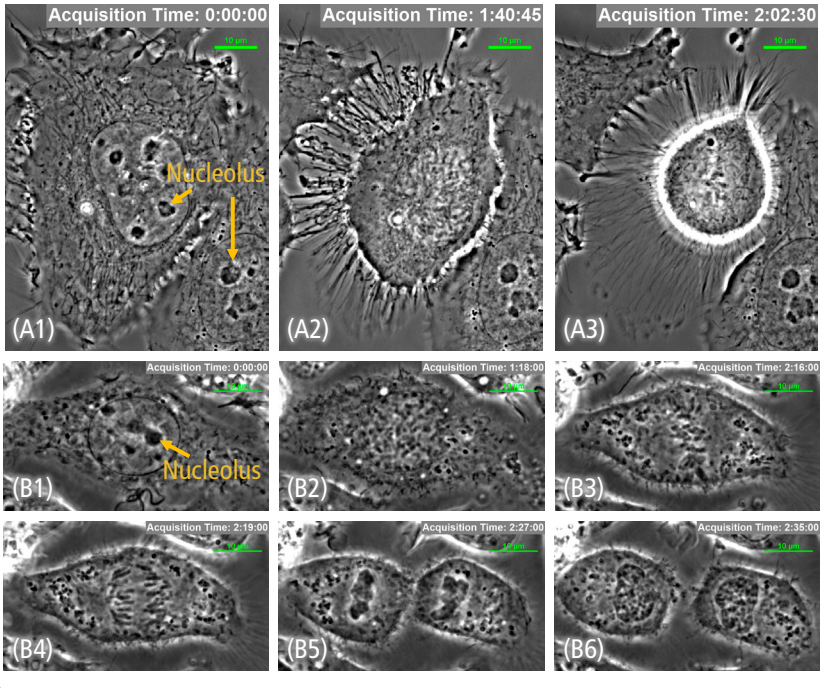


Fig. 3: Time-lapse images of mitosis by apodized phase contrast method
(A1-A3) HeLa Cells
(B1-B6) Mouse macrophages (J774.1)
In interphase (A1, B1), nucleoli are observed. In prometaphase (A2, B2), the nucleoli disappear and chromosome aggregation occurs. In metaphase (A3, B3), the chromosomes are aligned in the equatorial plane. In anaphase (B4), chromatids migrate to both poles. At telophase (B5, B6), the cell divides and the nuclear membrane and nucleolus are remodeled.
Objective: CFI Plan Fluor ADH 100X (NA 1.3),
Inverted microscope: Ti2-E, Camera: DS-Qi2, Scale bar: 10 µm

Time-lapse Video
HeLa Cells
Interval: 15 sec, Duration: 2h
Click to view the video

Time-lapse Video
Macrophages
Interval: 1 min, Duration: 9h
Click to view the video

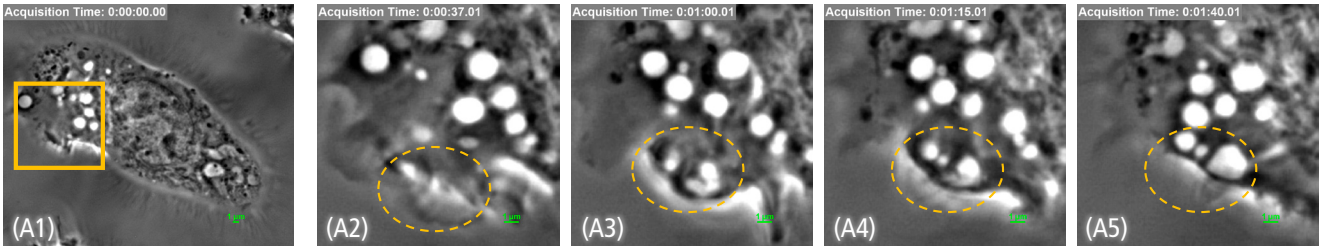


Fig. 4: Apodized phase contrast images of macropinocytosis by activated macrophages

(A1) Macrophage (J774.1 cell line) activated by LPS stimulation

(A2-A5) Enlarged images of the square area in A1. Extracellular fluid taken up by macropinocytosis is observed as bright (white) vesicles due to a lower refractive index than the surrounding area.

Objective: CFI Plan Fluor ADH 100X (NA1.3), Scale bar: 1 µm

Time-lapse Video
Macrophages
Interval: 250 msec
Duration: 5 min

Click to view the video

Apodized phase contrast method

The phase plate of an apodized phase contrast objective has two neutral density filters (apodization areas) surrounding a phase ring (Fig. 5), which can selectively reduce the intensity of light diffracted from a phase object. The diffraction angle produced by an object depends on the size of the object (Fig. 6). The apodized phase plate selectively attenuates diffracted light from large specimens by means of the apodization areas (Fig. 6 (A)), and allows high-contrast visualization of the microstructure of small specimens (Fig. 6 (B)).

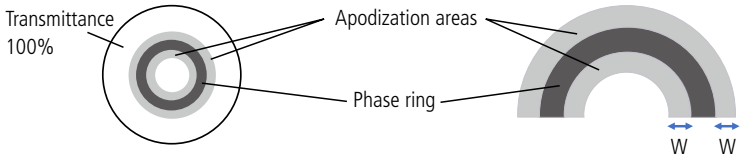


Fig. 5: Schematic diagram of phase ring and apodization areas

Differences in the appearance of the image are produced by changing the transmittance of the phase ring and the apodization areas, and the width (W) of the apodization areas.

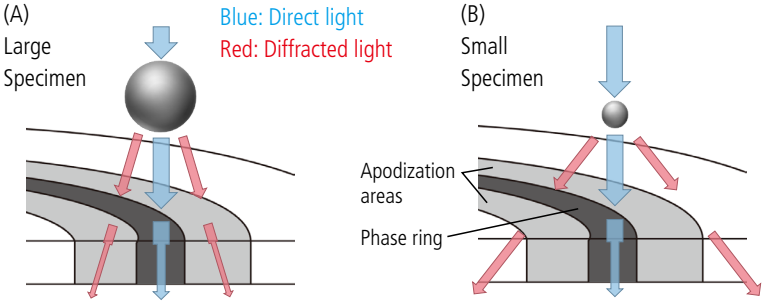
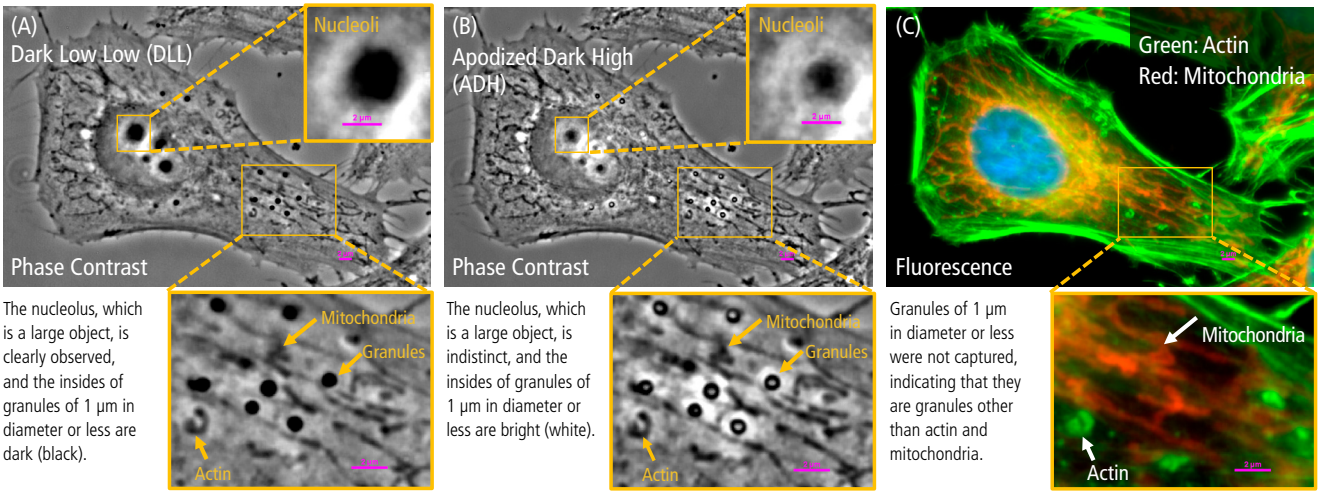


Fig. 6: Angle of diffracted light through an apodized phase plate due to specimen size

(A) Since large specimens have small diffraction angles, much of the diffracted light (low spatial frequencies) passes through the apodization areas and is attenuated, reducing contrast. (B) Since small specimens have large diffraction angles, much of the diffracted light (high spatial frequencies) passes through the transparent portion of the phase plate, resulting in high contrast without light attenuation.

Image comparison of phase contrast and apodized phase contrast

Large nucleoli of 3 µm in diameter or more were clearly observed in the DLL phase-contrast image (Fig. 7 (A)). Since apodized phase contrast enhances the contrast of small specimens, spherical granules of 1 µm in diameter or less were observed with high contrast, showing bright (white) interiors with bright and dark areas reversed. Therefore, granules and actin were easily distinguished visually (Fig. 7 (B)).



The nucleolus, which is a large object, is clearly observed, and the insides of granules of 1 µm in diameter or less are dark (black).

The nucleolus, which is a large object, is indistinct, and the insides of granules of 1 µm in diameter or less are bright (white).

Granules of 1 µm in diameter or less were not captured, indicating that they are granules other than actin and mitochondria.

Fig. 7: Image comparison of DLL and ADH phase contrast in flat HeLa cells

(A) DLL phase contrast image (objective: CFI Plan Fluor DLL 100X), Nucleoli larger than 3 µm in diameter were observed with high contrast.

(B) Apodized (ADH) phase contrast image (objective: CFI Plan Fluor ADH 100X), Granules of 1 µm in diameter or less were observed with high contrast. Granules with a high refractive index were observed as bright (white) images in which light and dark are reversed.

(C) Fluorescence image (objective: CFI Plan Fluor ADH 100X), Blue: Nuclei, Green: Actin, Red: Mitochondria

Inverted microscope: Ti2-E, Camera: DS-Qi2, Scale bar: 2 µm

	Contrast Low→High		
Phase Contrast	DLL (Dark Low Low)	DL (Dark Low)	DM (Dark Middle)
Apodized phase contrast	ADL (Apodized Dark Low)	ADM (Apodized Dark Middle)	ADH (Apodized Dark High)

Table. 1: Types and contrasts of phase plates

- If contrast is high, the light and dark areas of the object will be reversed.
- The higher the contrast, the greater the halo.
- Apodized phase contrast reduces halos.

Halo reduction and microstructural contrast enhancement by means of apodization area width

The width of the apodization areas, W (Fig. 5) can control which objects are contrast-enhanced. The apodization area width of apodized phase contrast objectives with magnifications of 20x to 40x is designed to allow high-contrast observation of objects of 10 µm or less. Furthermore, by attenuating the diffracted light from objects larger than 10 µm, halos that appear at the edges of cells can be reduced, enabling observation of organelles. The 100x high-magnification apodized phase contrast objective is designed for high-contrast observation of small organelles of 3 µm or less. The halos of mitotic large spherical HeLa cells were reduced more by an ADH objective than by a DM objective (Fig. 8 (B2), Fig. 8 (C2)). Halos in a small, round J774.1 cell line (mouse macrophage-like cells) were reduced more by the ADH objective (Fig. 9 (B)) than by the DM objective (Fig. 9 (C)), allowing high-contrast observation of the cell interior. In addition, the ADH objective (Fig. 9 (B)) can observe nucleoli and details with higher contrast than the DLL objective (Fig. 9 (A)).

Phase contrast and apodized phase contrast image comparison (100x) and depth of focus on the object plane

In general, fluorescence imaging requires a high-NA objective that can capture a large amount of light, while the contrast in a phase contrast images is determined by the type of phase plate (Fig. 8, Fig. 9). Also, high NA objectives have a shallow depth of focus, resulting in a narrow range of focus within the specimen.

- Depth of focus of NA1.3 lens = 0.25 µm
- Depth of focus of NA1.45 lens = 0.20 µm

Therefore, a low NA objective with a wide focal range is useful for phase contrast imaging when observing the entire cell.

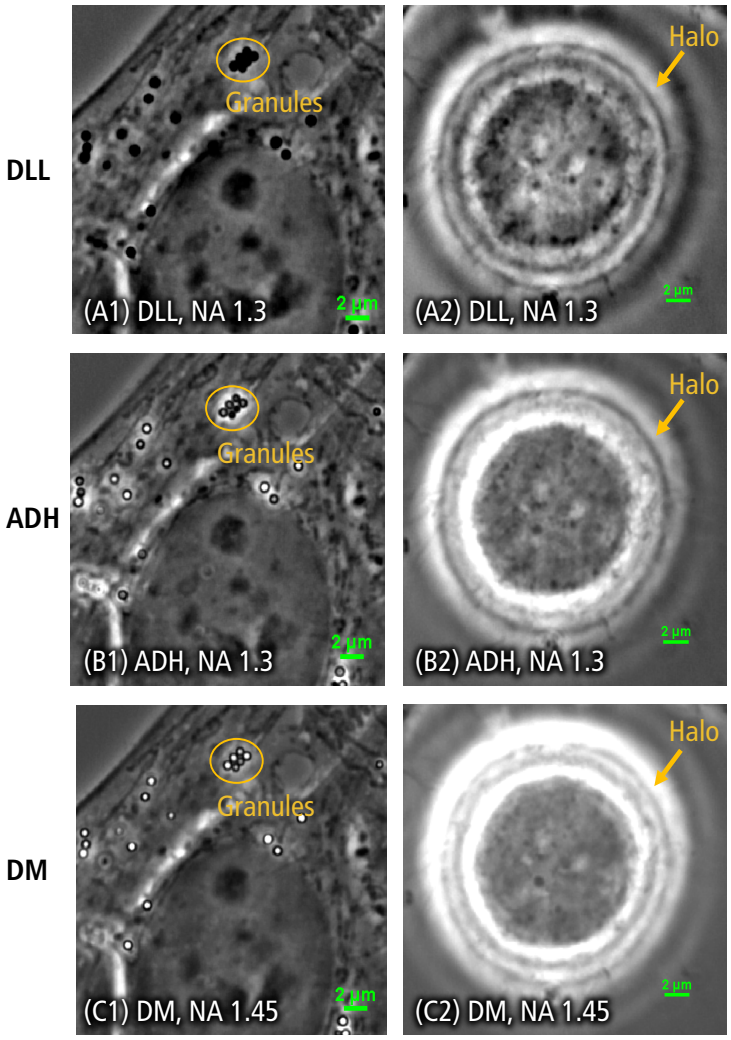


Fig. 8: Phase contrast and apodized phase contrast image comparison (magnification: 100x)
Left: Flat HeLa cells
Right: Round, thick HeLa cells in mitosis

Objectives:
(A) CFI Plan Fluor DLL 100X (NA 1.3)
(B) CFI Plan Fluor ADH 100X (NA 1.3)
(C) CFI Plan Apo DM Lambda 100X (NA 1.45)
Inverted microscope: Ti2-E, Camera: DS-Qi2,
Scale bar: 2 µm

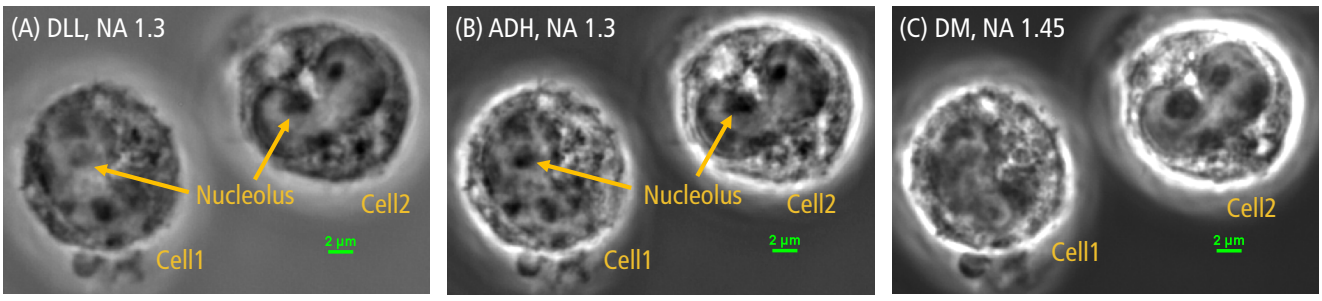


Fig. 9: Phase contrast and apodized phase contrast image comparison of small round cells (magnification: 100x)
Specimen: Mouse macrophage-like cell line (J774.1), Inverted microscope: Ti2-E, Camera: DS-Qi2, Scale bar: 2 µm
Objectives: (A) CFI Plan Fluor DLL 100X (NA 1.3), (B) CFI Plan Fluor ADH 100X (NA 1.3), (C) CFI Plan Apo DM Lambda 100X (NA 1.45)

Phase contrast objective CFI Plan Fluor DLL 100X Oil (NA 1.3)

- For imaging flat HeLa cells
- Reduces halos in mitotic cells
- For observation focusing on the entire cell
- High contrast observation of large organelles
- Clear observation of nucleoli larger than 3 µm in diameter
- Fig. 7 (A), Fig. 8 (A2)

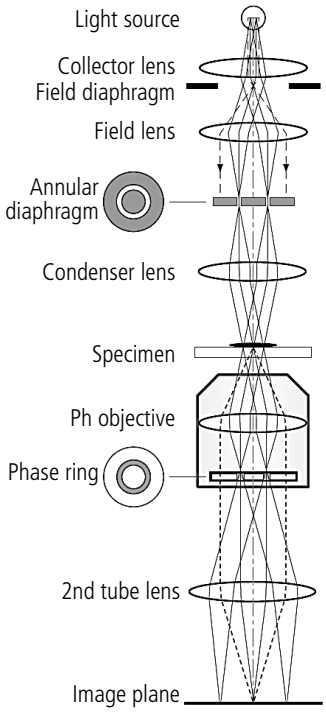
Apodized Phase contrast objective CFI Plan Fluor ADH 100X Oil (NA 1.3)

- For imaging cells with thick cytoplasm
- For imaging macrophage endocytosis
- High contrast observation of mitochondria, lysosomes, and lipid droplets under 1 µm
- Spherical granules and actin can be distinguished in unstained cells
- Fig. 7 (B), Fig. 9 (B)

Phase contrast objective CFI Plan Apo DM Lambda 100X Oil (NA 1.45)

- High NA objective
- For acquisition of merged fluorescence + phase contrast images
- High-contrast observation of specific organelles with shallow depth of focus
- Fig. 8 (C1)

Fig. 10: Features of phase contrast (DLL, DM) and apodized phase contrast (ADH) objectives (magnification: 100x) and specimens suitable for observation



Illumination light that reaches the specimen is divided into bright direct light that has passed through phase objects (solid lines) and diffracted light generated by the phase objects (dotted lines). In phase contrast observation (dark contrast), the amplitude of the direct light is attenuated by an ND filter, and at the same time, the phase of the direct light is advanced by one quarter wavelength by a phase ring. The phase change can be observed as light and dark due to interference between the direct light and diffracted light.

Fig. 11: Optical path diagram of phase contrast microscope

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For a more detailed explanation of the principles of phase contrast microscopy, please refer to "Introduction to Phase Contrast Microscopy" in MICROSCOPYU.
https://www.microscopyu.com/techniques/phase-contrast/introduction-to-phase-contrast-microscopy

Summary

- Phase contrast imaging is an important tool for label-free visualization of cellular changes and organelle dynamics over a long period of time, and for advancing the understanding of biological phenomena and diseases.
- The DLL phase contrast objective is suitable for observing entire cells.
- The apodized phase contrast (ADH) objective (100x) can observe granules of 1 µm in diameter or less with high contrast.
- Apodized phase contrast (ADH) can enhance the contrast of small organelles and simultaneously reduce halos when visualizing intracellular structures.
- The high NA phase contrast objective (100x) is suitable for acquisition of merged fluorescence + phase contrast images.

References

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- Tatsuro Otaki, Artifact halo reduction in phase contrast microscopy using apodization. *Optical Review* 7: 119-122 (2000).
- Tatsuro Otaki et al. Apodized phase contrast microscopy yields refined dynamic images of organelles in living cultured cells. Proceedings of the 29th Kogaku Symposium (Japan): 43-46 (2004).
- Kaoru Katoh et al. *Biophysics* 44 (6), 260-264 (2004).
- Nikon Research Report 24-30, Vol.2 2020
- Nikon MICROSCOPYU Apodized Phase Contrast (https://www.microscopyu.com/tutorials/apodized-phase-contrast)

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Product Information

ECLIPSE Ti2-E inverted research microscope

The automatic focus maintenance system (PFS) corrects focus drift in real time with high accuracy. This enables the acquisition of in-focus images even during long-term time-lapse imaging.

Phase contrast objectives (dark contrast)

- CFI Plan Fluor DLL 100X Oil (NA 1.3)
- CFI Plan Fluor ADH 100X Oil (NA 1.3)
- CFI Plan Apo DM Lambda 100X Oil (NA 1.45)

Colorless and transparent living cells can be visualized with high contrast. Localization and dynamics of organelles can be observed over a long period of time.