**September 19-29, 2025, SUSTech, Shenzhen, China**

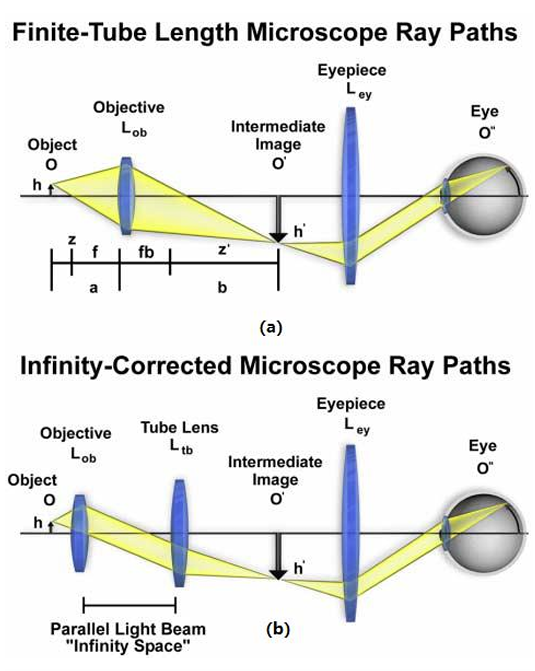
Biomedical Engineering Laboratory I (Fall 2025)

Lab 1 introduction to microscopy

**Group 5**

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**Figure 1**: Schematic illustrations[1] of (a) finite tube length and (b) infinite tube length microscopy ray paths.

Abstract

*The optical microscope, a cornerstone of scientific discovery, allows human to observe the object beyond the limit of the naked eyes. Its performance is governed by the wavelength of light and the objective’s numerical aperture (NA). Here, we present four practical investigations to test the core principles and imaging techniques of optical microscope. During these experiments, we verified our microscope’s infinity-corrected design by measuring its true magnification, observed the consequent loss of resolution by altering the objective’s NA using different pinhole apertures, demonstrated the effect of phase contrast microscope against the bright-field mode, and applied fluorescence microscope to visualize the specific components within stained cells (BPAE). This lab successfully integrated theoretical optics with hands-on validation, helping us get a further understanding of modern light microscopy in biomedical research.*

INTRODUCTION

The microscope is one of the greatest inventions of mankind. Before its invention, our understanding of the world was limited to what they could see with the naked eye, or with the aid of simple lenses. And in this lab, we mainly focus on optical microscope, which consists of eyepiece, objectives, brightness adjustment knob, coarse and fine focusing knob, stage, light bulb, phase contrast slider, etc.

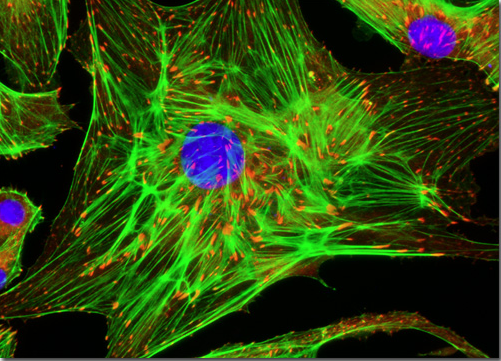
Microscope resolution is the smallest distance between two distinguishable points. It is limited by the Abbe diffraction limit (d = 1.22λ / 2NA). This means resolution depends on the light's wavelength (λ) and the objective's Numerical Aperture (NA). The NA (NA = nsinθ) measures an objective's light-gathering ability. Higher NA leads to a sharper image.

Modern optical microscopes use a two-stage magnification process, which utilizes lenses in the scope’s objective and eyepiece. One design is the finite tube length microscope (Fig. 1a), while another more modern design uses infinity-corrected optics (Fig. 1b).

The phase contrast microscope is designed for viewing transparent specimens. It makes invisible phase shifts in light waves visible, which involves separating the direct light from light that is diffracted and making them destructively interfere in order to form a biomedical image[2].

Fluorescence microscopy relies on the Stokes shift. It applies specific light on the sample, which leads to associated excitation of the fluorophore. With the emission filter, the emitted light is project as a fluorescent image to the CCD camera. The combination of images that are processed by different light shows the overall information of fluorescently areas of the sample.

We conducted four complementary experiments in this lab to invoke these principles. Firstly, the actual magnification of an objectives lens was compared to theoretical values to confirm the infinity-corrected nature of the microscope. Secondly, the contribution of numerical aperture was explored by limiting the objective with the pinhole apertures and monitoring the resulting resolution changes. Thirdly, phase contrast microscopy was used to image unstained MC3T3 cells to review the capability to see otherwise unseen structure. Lastly, fluorescence microscopy was utilised to image bovine pulmonary artery endothelial (BPAE, Fig. 2) cells, a line of cells harvested after excision of the main stem of the pulmonary artery from a young bovine, achieved by P. Del Vecchio during the latter part of the 1970s[5]. (Nathan S. et al., Olympus Confocal, 2008) stained with three different fluorophores, allowing identification, simultaneously, of the nuclei, cytoskeleton, and mitochondria. Together, these exercises united the theoretical optics to the hands-on practice of the microscopy, as well as presented the benefit that advanced imaging modalities contribute to biomedical research.



**Figure 2:** BPAE in fluorescence microscopy[8].

MATERIALS AND METHODS

An inverted optical microscope (Olympus CKX53) equipped with 4×, 10×, 20×, and 40× objectives, including phase-contrast capabilities and a broadband mercury lamp, was employed throughout the experiments. A digital imaging system was used for recording data. The following specimens and accessories were utilized: transparent rulers, diffraction gratings (70 and 110 lines/mm, Edmund Optics, transmission diffraction grating), pinhole apertures of three diameters (200 μm, 500 μm, and 1000 μm, Thorlabs P200K, P500K and P1000K), microscope slides, MC3T3 cell samples, and fluorescently labeled slides (Invitrogen FluoCells #1 BPAE Cells, with MitoTrackerTM Red CMXRos, Alexa FluorTM 488 phalloidin and DAPI). Two regular ruler (minimum scale 1mm, total length 15cm and 50cm) were used to measure the image distance and the projection. A 90° bent steel ruler was used to measure the object distance (Fig. 3).

Four experimental modules were carried out. First, under the 4x objective, the true magnification of the optical system was determined by removing the eyepiece, integrating maximum brightness, directly imaging a transparent ruler on the eyepiece tube, measuring the projected scale and comparing it against the scales on the ruler, while the theoretical magnification was calculated by measuring the object distance (o) and image distance (i) of the microscope system and substitute the figures into the formula (1).

(1)

Two results were compared statistically. Second, the numerical aperture of the 10× objective was modified by sequentially placing pinhole apertures of varying sizes above the lens while observing gratings of different line densities, allowing evaluation of diffraction effects and resolution changes. Third, 20x phase contrast imaging was performed by adjusting the microscope’s phase slider, enabling comparison of MC3T3 cell samples under bright-field and phase-contrast conditions. Finally, 40x fluorescence microscopy was conducted by exciting stained slides with specific illumination channels (UV/blue/green from filtered broadband mercury lamp) and capturing images at different emission wavelengths, followed by staining and merging the channels into an RGB composite. The emission colors were identified through eyepieces, including cyan (nucleus excited by UV), green (cytoskeleton excited by blue) and red (mitochondrion excited by green).

图示

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**Figure 3:** Assumed object and image distance in our microscope based on the basic ray path of an inverted microscope[1].

All measurements and image acquisitions were conducted under correct focal distance and repeated by multiple group members to ensure consistency, and scale calibration was applied to microscopic images under different magnification (4x, 10x, 20x, 40x) to convert pixel dimensions into physical units.

Results

Procedure 1 is to measure the True Magnification of an objective. Each group member observed projection of the ruler using the eyepiece tube once. The length on ruler is obtained by directly counting the number of minimum scales(Table 1).

The average measured magnification is 4.2858, standard deviation σ = 0.010897247.

Then we measured two distances: the distance between the objective lens and the sample(o), the distance between the objective and eyepieces(i) (Table 2). The average theoretical magnification is 32.73, standard deviation σ = 0.442379927.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Length on Eyepiece(mm) | 21.50 | 17.12 | 17.11 | 17.11 |
| Length on Ruler(mm) | 5.00 | 4.00 | 4.00 | 4.00 |
| mmeas | 4.3 | 4.28 | 4.2775 | 4.2775 |

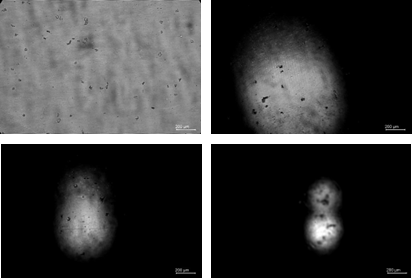
**Table 1:** Length on eyepiece and on ruler, and the calculated measured magnification.

|  |  |  |  |
| --- | --- | --- | --- |
| o(cm) | 1.652 | 1.650 | 1.599 |
| i(cm) | 53.46 | 53.80 | 53.12 |
| mTheo | 32.36 | 32.61 | 33.22 |

**Table 2:** Measured object and image distance, and the calculated theoretical magnification.

There is a significant difference between true magnification and the theoretical value. The discrepancy comes from the infinity-corrected design of our microscope. The method we used to measure the object distance is incorrect since the existence of the tube lens (Fig. 1b). The true object distance is impossible for us to acquire given that we are unable to find the accurate position of the tube lens. It proved that our microscope is an infinite-tube length microscope.

Procedure 2 is to alter the numerical aperture of an objective lens. In this procedure we kept the position unchanged, placed pinholes of different sizes on the objective lens and took photos of two different diffraction gratings with digital camera.The smaller the pinhole, the smaller the angle, and the smaller the resolution becomes. (Fig. 4)



**d**

**c**

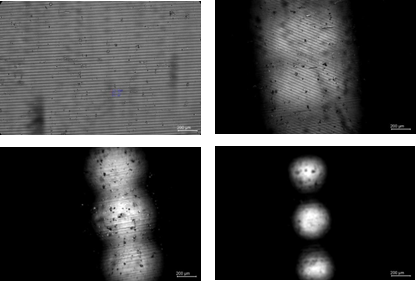
**a**

**b**

**Figure 4:** (a)70 lines/mm grating, (b) pinhole aperture 200μm, (c) pinhole aperture 500μm, (d) pinhole aperture 1000μm

It is apparent that the bright spot is getting smaller when the pinhole aperture is shrinked. Moreover, the resolution is also reduced. Stripes are observed in figure 2 to figure 4, but not in figure 5. The view split into two under 200μm pinhole.

Then, we replaced the grating to an 110 lines/mm grating. (Fig. 5.)

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**d**

**c**

**b**

**a**

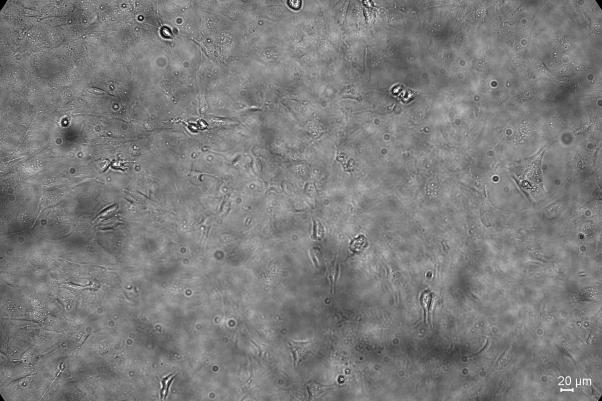
**Figure 5:** (a)110 lines/mm grating, (b) pinhole aperture 200μm, (c) pinhole aperture 500μm, (d) pinhole aperture 1000μm.

This time the fringe spacing is reduced. As a result, the stripes are unclear from 500μm pinhole. The diffraction effect became stronger, and we saw three spots in view at most.

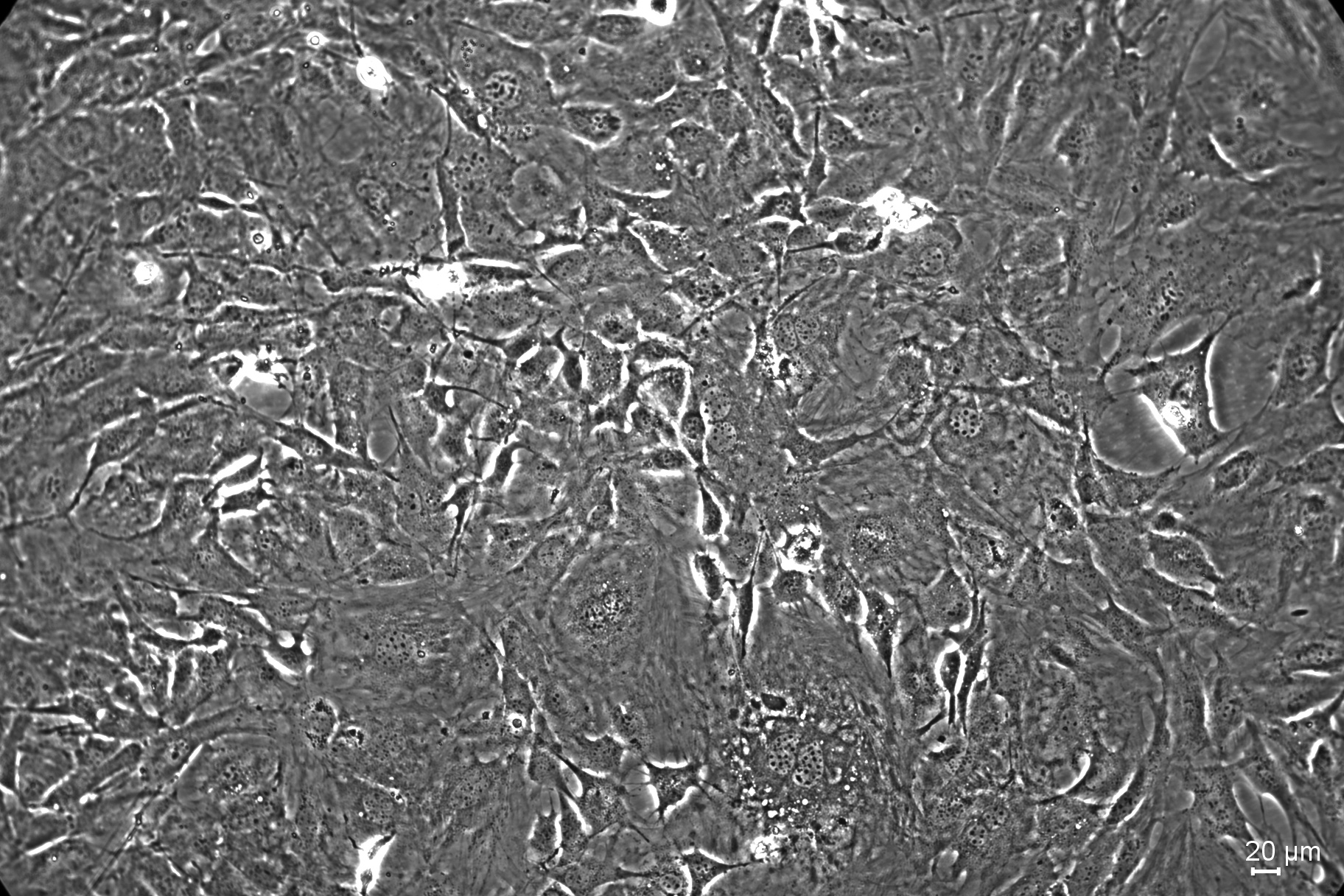
Procedure 3 is trying phase microscopy. We used the regular bright field and the phase contrast mode to observe MC3T3 cells in the same place. The 20x objective used in this procedure is examined in advance (Fig. 6.). On the barrel, “LCAch” stands for aberration correction, where LC means long working distance for thick samples like petri dishes, and Ach means achromat objective. “0.40” is the NA. “iPC” stands for integrated phase contrast, which implies that this objective is suitable for this experiment. “∞” means infinity corrected. “1” means the recommended coverslip thickness is 1mm. “FN22” means the field number is 22mm.



**Figure 6:** 20x objective used in this experiment.

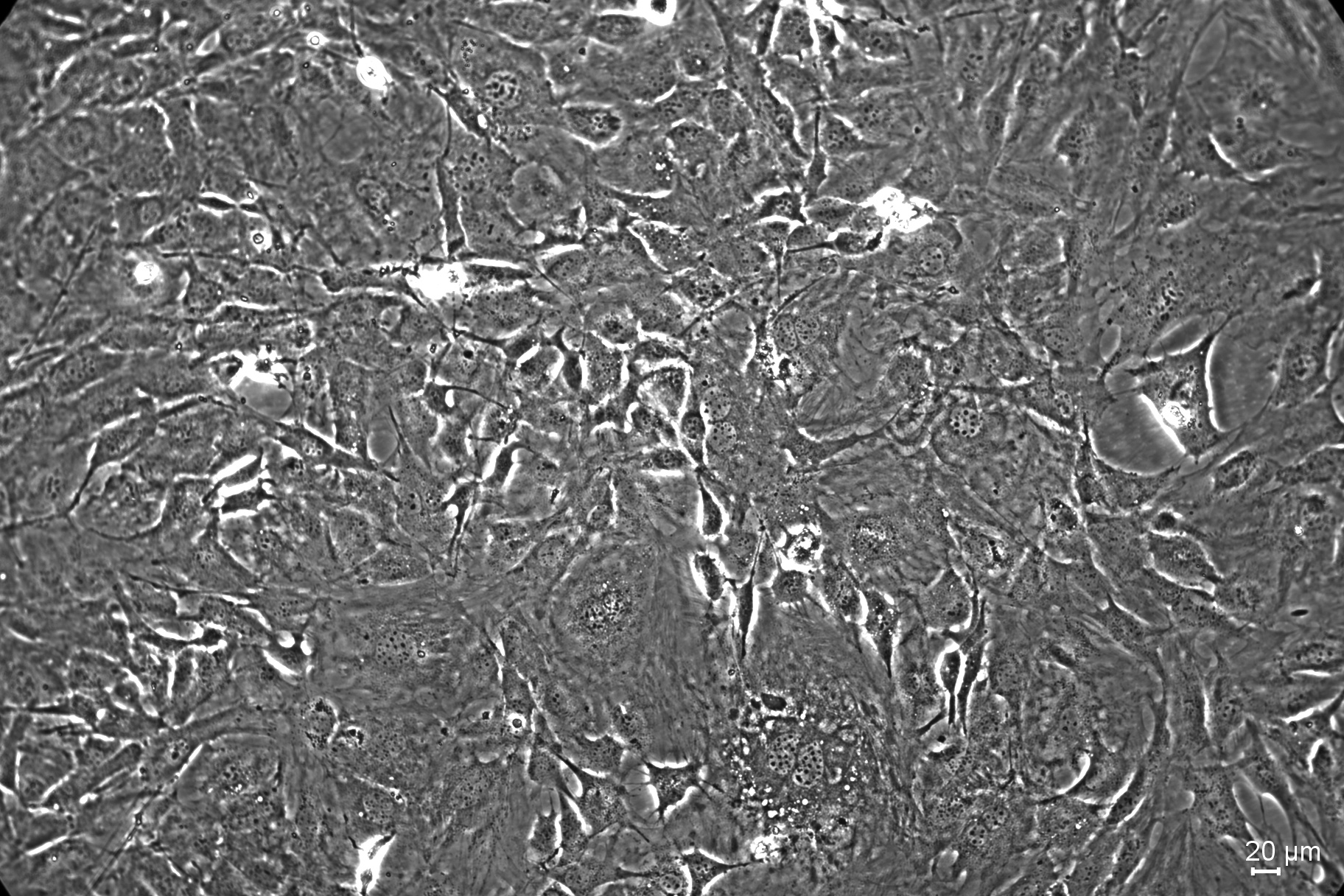


**Figure 7:** Cell image under regular bright field.



**Figure 8:** Cell image under phase contrast mode.

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**Apoptotic bodies**

**Figure 9:** Part of figure 11, the structure that we assumed to be apoptotic bodies is labelled[9].

The cells were observed under phase contrast mode first and adjusted to suitable focal distance before observed under bright field. The focal distance remained unchanged. Under bright field, no clear cell structure is visible (Fig. 7). Under phase contrast mode, cells with bright edges could be found(Fig. 8). Some possible subcellular structure can be identified (Fig. 9).

Procedure 4 is trying fluorescence microscopy. In this procedure, a mercury lamp is used as broadband light source to excite different fluorescent stains. (MitoTrackerTM Red CMXRos, Alexa FluorTM 488 phalloidin and DAPI)

nucleus_b255

**a**

cytoskeleton

**b**

mito

**c**

**Figure 10:** Image under mercury lamp, original and tinted image, in (a) UV excitation/blue emission (b) blue excitation/green emission (c) green excitation/red emission channel.

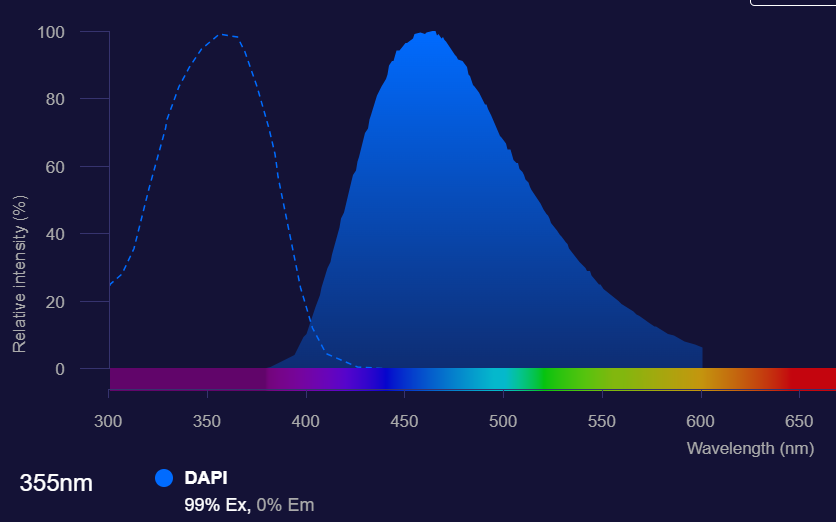
merged_2

**Figure 11:** Merged image of three channels.

DAPI has a excitation peak at 357nm and a emission peak at 461nm, therefore it emits blue light under UV(Fig. 12a). It is located at the nucleus, leading to a demonstration of nucleus position in blue emission channel. We found that nucleus are in the center of the cells. The figure we got from Capture software is in monochrome, and we stained it in pure blue (Fig. 10a).

Alexa FluorTM 488 phalloidin has a excitation peak at 488nm and a emission peak at 520nm (Fig. 12b) Therefore it emits green light under blue light. The stain binds F-actin[4] and show its structure. We were able to see cytoskeleton one by one. The figure we got from the Capture is in monochrome, and we stained it green(Fig. 10b).

MitoTrackerTM Red CMXRos has a excitation peak at 579nm and a emission peak at 599nm(Fig. 12c). It binds mitochondria[5] and emits red light under green light. Mitochondria are in the cell and arrange around the nucleus. The figure we got from the Capture is in monochrome, and we stained it red(Fig. 10c).

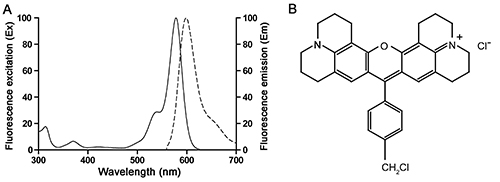
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**b**

**a**

**c**



**Figure 12:** Absorption and fluorescent spectrum of (a)DAPI[6], (b) Alexa FluorTM 488 phalloidin[6], (c) MitoTrackerTM Red CMXRos[7].

The three figures are merged in software(Fig. 11). We took the photos in the same position and observed cellular structures. We found nucleus, cytoskeleton and mitochondria in one cell and understood their arrangement.

discussion

This set of experiments presented the important principles of optical microscopy in a systematic fashion, together with their applications to biological imaging. The results of the magnification experiment indicated a significant difference between measured and theoretically obtained values. This discrepancy was caused by the fact that the simple thin-lens model cannot be applied to infinity-corrected instruments. In these, the tube lens participates in the ultimate forming of images, so that the measurement of the distances between the object as well as the image cannot be directly performed. Then, the obtained result gave experimental support to the infinity-corrected construction of the Olympus CKX53 microscope.

In the numerical aperture experiment, the positioning of the pinholes of various diameters above the 10× objective directly restricted the cone of diffracted light entering the lens. Smaller apertures decreased the effective NA, leading to decreased light gathering capability as well as deteriorated resolution. As a result, details as fine as the 110 lines/mm grating could no longer be resolved using smaller apertures, as observed in Fig. 5. Moreover, the effect that the image sometimes separated into two as well as several circular fields beneath the smallest pinhole can be explained due to diffraction as well as imperfect alignment: the aperture introduced multiple diffraction orders, as well as small misalignments of the pinhole along the optical axis resulted in the partial passage merely of the partial cone of diffraction, where separated sub-images appearing amongst the eyepiece appeared[10]. These observations indicated the significance of the NA, as this determined the resolution as well as image fidelity.

The phase contrast experiment verified the value of this technique as used to visualize nearly transparent biological cells. Elements that were unseen beneath bright-field became distinctly outlined under phase contrast, as phase shifts brought about through the results of the intracellular composition were transformed into noticeable intensity differences. This indicated why phase contrast nevertheless maintains a paramount role as used to execute live-cell imaging where staining is undesirable.

Fluorescence microscopy further expanded the range used to identify specific subcellular constituents. In exciting three various fluorophores, we were capable of simultaneously imaging the nuclei, cytoskeleton, as well as the mitochondria. In the merged RGB overlay, the relationships amongst them were revealed, demonstrating the capability that the multiplex fluorescence labeling possesses. While so doing, the process also reflected the considerable problems that the procedure also posed, including photobleaching, optical channel misalignment, as well as the accurate scale bar calibration required to quantify the cellular structure. In all experiments, the common principle is that the image is formed based on diffracted light being captured and reconstructed. Whether by restricting the limitation of NA, increasing phase difference, or selectively measuring fluorescent emission, the task of the microscope is to control light paths to extract the most useful information.

These laboratory procedures underscored the theoretical principles and underscored why contemporary microscopy combines numerous complementary modes of imaging.

Acknowledgments

I would like to express my sincere gratitude to the members of the research team who contributed to the successful completion of this study. Their dedication, expertise, and commitment were instrumental in the realization of our research objectives. I am thankful for their valuable insights, collaborative spirit, and unwavering support throughout the project.

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