Karlsruhe Institute of Technology

KSOP
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Biological Fluorescence Microscopy Laboratory

Report

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1 Introduction

The Biological Fluorescence Microscopy lab provided us with the basics of light microscopy and showed us its applications in modern biology. The invention of the light microscope gave way to observing cells. This microscope made magnification of cells possible up to 1000 times and with a resolution of 0.2µm. Some pre-requisites are present in order to view the cells. Firstly, the specimen is illuminated with a bright light; secondly the specimen is prepared for light to pass effectively through it and lastly usage of proper lenses for precise focusing on to the eye. The fluorescence microscope is an optical microscope that uses fluorescence and phosphorescence instead of, or in addition to, reflection and absorption to study properties of organic or inorganic substances.Two techniques were used in the lab to observe the cellular structures, the Differential Interference Contrast and Phase contrast. The lab was divided into two days between which, on the first day the solutions were prepared and the specimen kept for latter microscopic investigation in the following day.

2 Materials and Methods

In this part we will discuss the materials, methods and microscopy techniques used in the laboratory.

2.1 Pipette Usage and Calibration

Before starting the staining of cells, we first needed to learn the usage of the microliter pipettes. These precise micropipette systems have a dial with which the desired volume of liquid can be adjusted. The plunger has two stop positions, the first is calibrated to be the desired volume while the second stop is there for complete discharge of fluid. The second stop should not be used while taking in fluid as this will result in a wrong volume (around 10-20% more).
We did a small calibration step before going on to staining the cells to measure the error from the pipette. We transferred a certain volume of water with a known density of 1 g/mL to a precise scale. This way we can measure the calibration error of the pipette. In figure 2.1, a plot of the measured values is shown. The errors are rather small, with 0.6% for 1000 µL, 2.3% for 100 µL and 10% for the 10 µL. However, we did not take into account the fact that the temperature influences the density of water or that the scale introduces a rounding error. Especially for 10 µL, the scale did not display any values smaller than 0.001g.

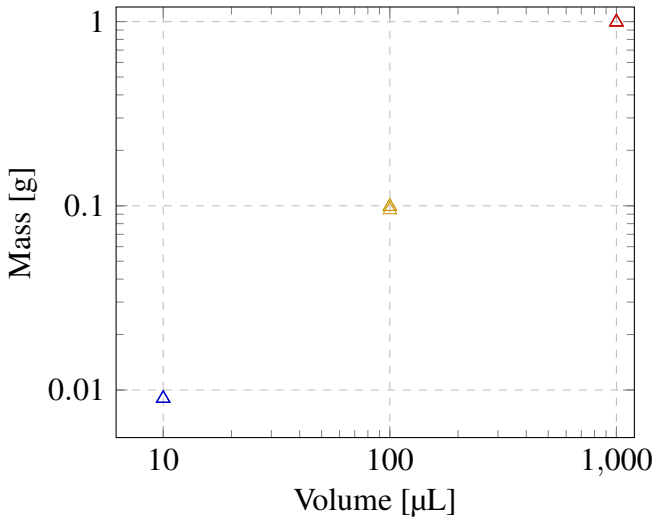


Figure 2.1: Pipette calibration by measuring intended volume vs. the mass of water.

2.2 Fixation and Staining of Cells

In the next step, we did the fixation and fluorescent staining of living mouse embryonic fibroblasts. We stained actin filaments and thus the cytosceleton of the cells with Phalloidin Alexa Fluor 488. This dye is an affinity marker for F-actin that is derived from a toxic fungus with a fluorescent molecule attached. Its excitation peak lies at 495nm and its emission peak at 519nm. For the staining of DNA molecules we use DAPI (4′,6-Diamidin-2-phenylindol), also an affinity marker, but in contrast to the aforementioned dye, DAPI possesses autofluorescence. It has its excitation and emission peaks at 358nm and 461nm respectively.
The cells have been prepared beforehand on glass cover slips in a nourishing solution and incubated overnight at 37°C. To begin, we removed the four glass cover slips with the cells from the culture medium and put them on a glass carrier, that has been rendered hydrophobic with Parafilm. After washing the cover slips with phosphate buffered saline (PBS), a commonly used buffer solution, the cells were fixed with paraformaldehyde (PFA) and incubated. The fixation of the cells with PFA is based on its ability to crosslink proteins inside cells. The fluorescent staining solution can only enter the cells if the cell walls are permeabilized by a detergent like Triton X-100. Thus another washing and incubation step with PBS + Triton X-100 is done. We prepared a 1000 µL staining solution with Phalloidin Alexa Fluor 488 and DAPI, with concentrations of 1:200 and 1:800, respectively. The dilution medium was 1% bovine serum albumin (BSA) in PBS. BSA acts as a blocking solution, that blocks unspecific binding sites, so that only actin and DNA molecules will be stained. We add staining solution to our cells and incubate for an hour. After an additional washing process, we embed the stained cells in Mowiol and n-propyl-gallate onto microscope slides. This embedding medium hardens and ensures longterm stability of the cell samples.

2.3 Microscopy Techniques

A short overview of the used microscopy techniques is given in the following section.

Phase Contrast Microscopy Phase contrast microscopy enhances contrast by making phase differences that occur between light that passes through refractive media visible. In our case, light is retarded by passing through different parts of the cell with different optical pathlengths and it interferes with light that is not retarded.

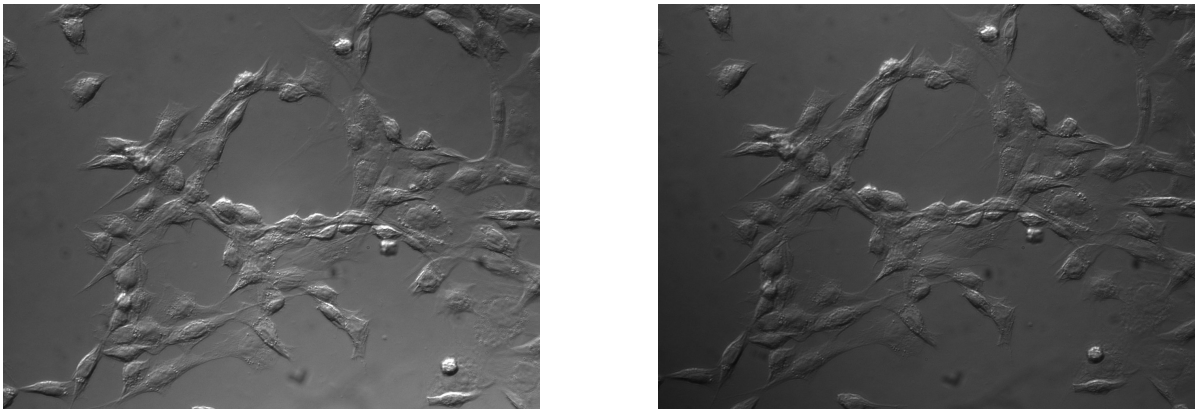
Differential Interference Microscopy Differential interference microscopy works similar to phase contrast microscopy by also making differences in optical pathlengths visible, but the images do not show diffraction halos at edges. It creates pseudo three dimensional images, by emphasizing edges and lines.

Fluorescence Microscopy Fluorescence microscopy is a microscopy technique that can visualize cell structures effectively by staining of proteins with fluorescent markers. The fluorophores emit light at longer wavelengths than excitation, so that by using appropriate bandpass filters only fluorescent light and thus the desired structures are visualized.

3 Results and Discussion

After proper processing of cells and leaving them overnight we acquired images of the cells using the three microscopy methods aforementioned.

3.1 DIC and Köhler Illumination

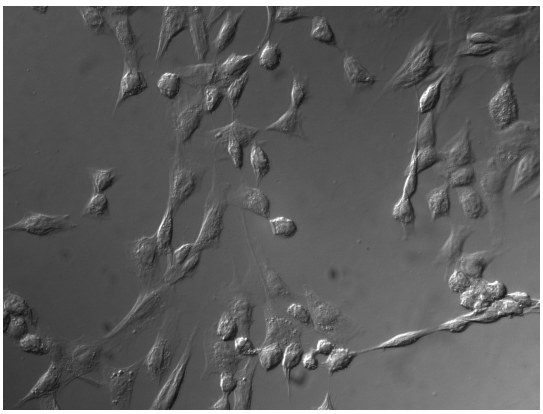


(a) Image of Cells with Köhler Illumination

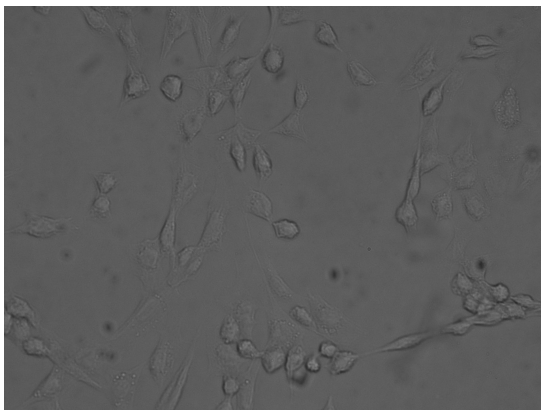
(b) Image of Cells without Köhler Illumination

Figure 3.1: Analyzing the Köhler Illumination.

The first task with microscope was to analyze the Köhler illumination. Using a 40x objective we watched the sample in the coverslip which was put upside down on the stage of the microscope. We opened the condenser (DIC II) and the field diaphragms all the way and focused the sample. Then we closed the diaphragm to a small spot of light in the eyepiece and focused it on the centre of the sample and then reopened the diaphragm. An image was acquired thus with Köhler illumination. The procedure was repeated with the light spot focused at a corner of the sample instead of centre. The result is illustrated in Figure 3.1. The one with proper Köhler illumination is brighter and more pronounced. However, this is not that big issue in modern microscopes these days. With Köhler and DIC illumination the images were taken again with and without the polarizer. As illustrated in Figure 3.2, DIC illumination does not work without the polarizer since this plane polarized light source is an essential feature in this type of microscopy. With no polarizer, the rays are not able to combine to one vibrational plane. Hence, no significant interference takes place and thereby there is no path difference between the rays. This leads to negligible contrast between the cells and the surrounding.



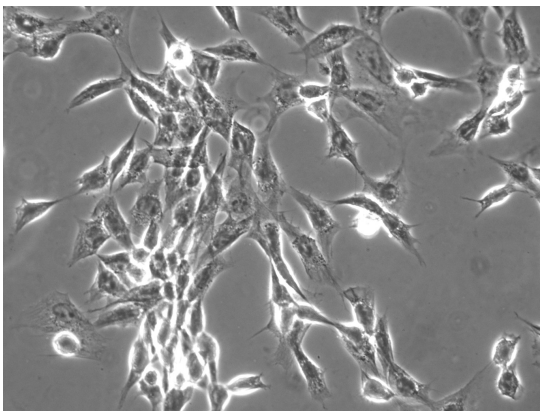
(a) Image of Cells with Polarizer in DIC Illumination



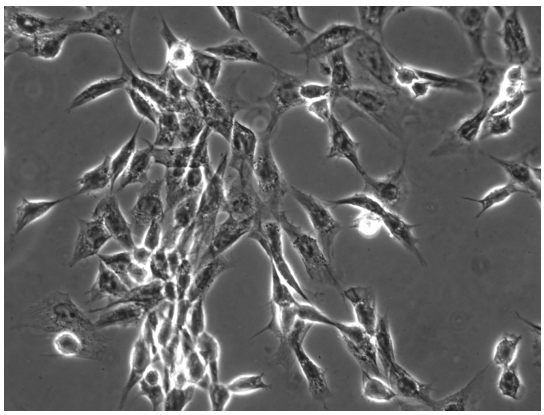
(b) Image of Cells without Polarizer in DIC Illumination

Figure 3.2: Analyzing Impact of Polarizer in DIC Illumination.

3.2 Phase Contrast



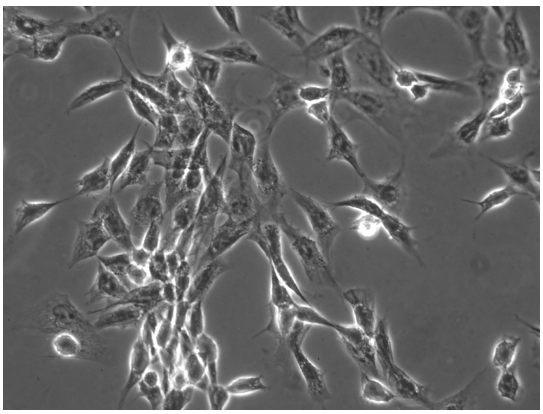
(a) Image of Cells with Aperture Diaphragm Fully Open.



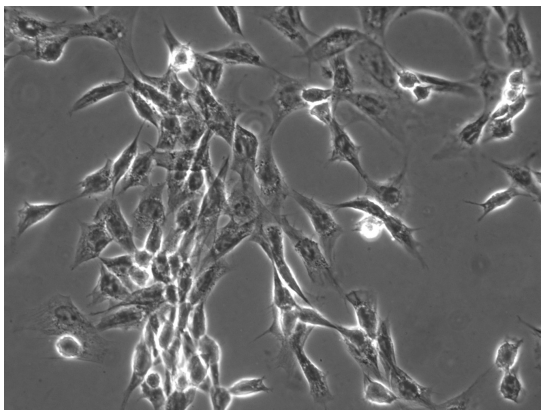
(b) Image of Cells with Aperture Diaphragm Nearly Closed.

Figure 3.3: Analyzing Aperture Effect in Phase Contrast.

We then switched the condenser to Phase 2 (Ph2) after removing the polarizer. The images were taken then with aperture diaphragm fully opened and nearly closed. The Figure 3.3 illustrates the result. The image with fully open aperture has brighter background compared to that with nearly closed aperture.
The procedure of taking images were repeated with and without polarizer. Even though it is expected to have significant difference in imaging, Figure 3.4 shows that it does not. We conclude that the contrast enhancement is mainly due to the dark field effect because there has been no significant interference in the first place.



(a) Image with Polarizer.

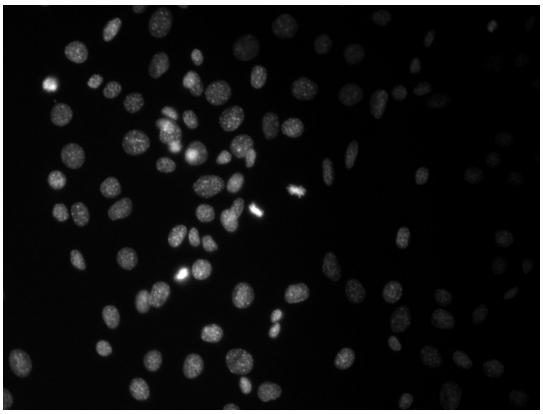


(b) Image without Polarizer.

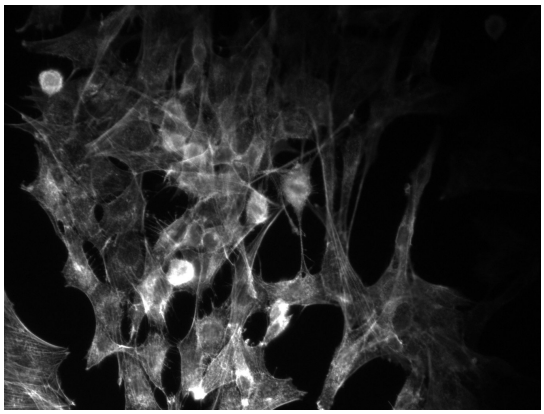
Figure 3.4: Analyzing Impact of Polarizer in Phase Contrast.

3.3 Fluorescence Microscopy

Lastly, we switched to DIC objective and condenser to DIC II. A proper filter was set to acquire images of cells as shown in Figure 3.5. Depending on which filter is used, we can see fluorescent images of the binding sites of DAPI and Phalloidin Alexa Fluor 488, i.e. DNA and action filaments. In figure 3.5b, we can see the DNA and thus the nucleus of the cells, while in figure 3.5b, we can observe actin filaments. Figure 3.6 illustrates fluorescent images of another sample cells with longer exposure.

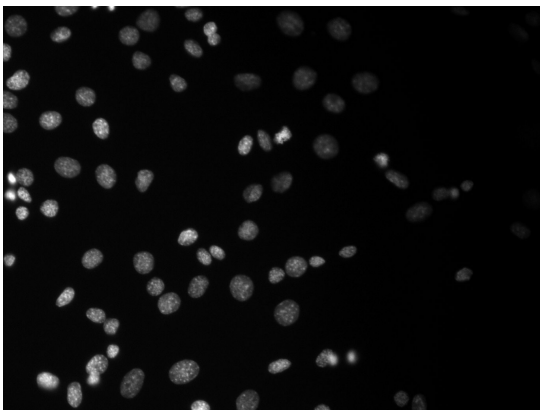


(a) Fluorescent Image of Cells.

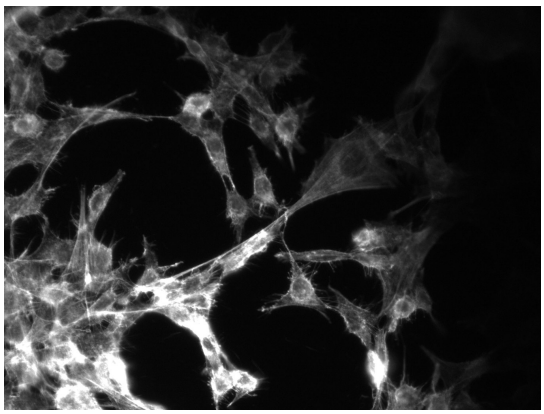


(b) Fluorescent Image of Actin.

Figure 3.5: Analyzing Fluorescence.



(a) Fluorescent Image of Cells.



(b) Fluorescent Image of Actin.

Figure 3.6: Analyzing Fluorescence.

4 Conclusion

In conclusion imaging of cells can be done using various microscopic technique. Each of the methods have their own advantages and disadvantages. The Fluorescence Microscopy approach has allowed cells to be studied in great details and produce stunning images that could be studied rigorously. It is a pioneering tool for modern biology and has provided us with some valuable experience with microscopy techniques and cell understanding. However, it is also very crucial to be very precise with the chemicals and handling of the cells to obtain results through microscope.

5 Bibliography

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