

Very short introduction to light microscopy and digital imaging

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1 Light Microscopy Basics

In this section we will briefly describe the basic principles of operation and alignment of a microscope. These notes are based on [1], [4] and the microscopy introductory lectures by Rudi Rottenfusser at the 2005 MBL Physiology Course.

In figure 1 we present the basic elements required for magnification. The specimen lies on the focal plane of the objective lens, which focuses it to infinity. The tube lens then focuses the parallel rays on the intermediate image plane. The obtained real image is now a magnified version of the sample. A second round of magnification, which gets multiplied to the previously described one, is obtained by focusing this real image to infinity once again using the eyepiece. This creates a new image focused to infinity, a condition to which the eye is adapted for viewing.

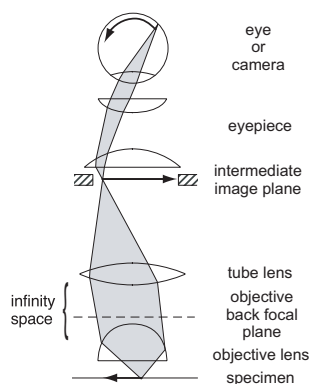


Figure 1: Basic diagram of the imaging light path [4]

One of the keys to obtaining sharp images is correct alignment. In the imaging path we are able to move the objective around to make the plane of the specimen we are interested in coincide with the objective focal plane. However, this is not the only required alignment: the transmitted illumination has also to be set up correctly to obtain a homogeneous specimen illumination. In figure 2 we present both light paths. In the illumination path the light coming out from the lamp is focused on the condenser diaphragm by the collector lens. Previously to arriving at that plane

it can be filtered spatially (its edges can be cut) by the field diaphragm, the reason for this will become clear when we describe the alignment protocol. The condenser diaphragm lies on the focal plane of the condenser lens, which makes sure that the light coming from that plane is collimated and goes through the specimen as parallel beams, which ensures the most possible homogeneous illumination. Finally, we see the illumination is focused once more on the objective back focal plane, the conjugate plane to the specimen plane.

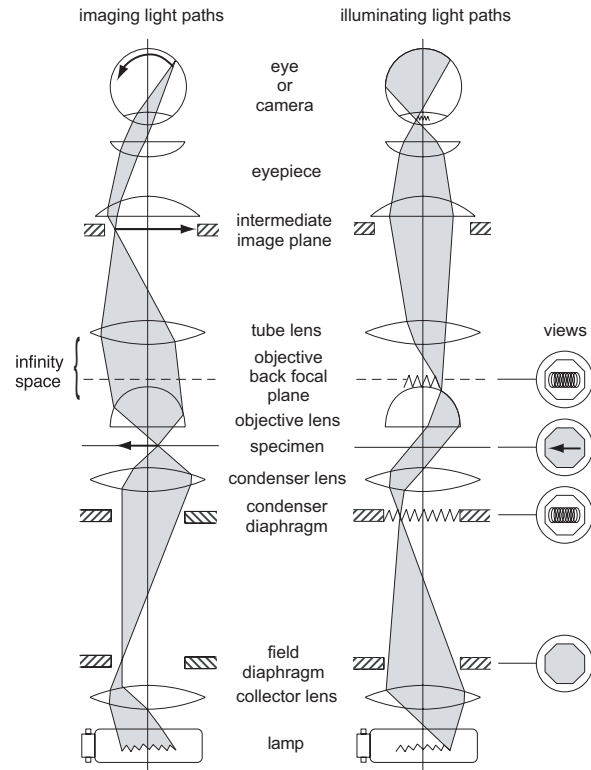


Figure 2: Imaging and illuminating light paths [4]

The key parameter in all optical elements of the microscope is the numerical aperture (NA). It is defined as $n \sin(\alpha)$, where n is the index of refraction of the medium and α is the maximum angle of light compatible with the optical element (fig. 3). Since light rays corresponding to bigger angles give the highest spatial resolution (they are the ones corresponding to higher wave numbers in fourier space), this magnitude is just telling us what the maximum achievable resolution is. We see that if the objective is in air ($n \simeq 1$) the maximum NA will be of order one. Therefore, by immersing it into a different medium like oil or water a higher resolution can be achieved because more light rays can be collected, as can be seen in figure 4.

Finally, in figure 5 we present the different parameters of an objective. Each detail might have relevance when it comes down to not only resolution and magnification power, but also to spherical or color aberrations.

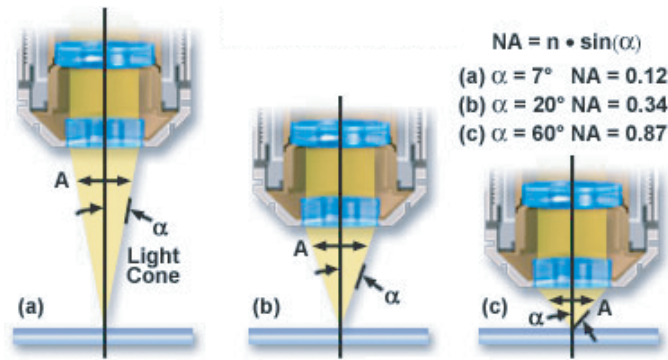


Figure 3: Numerical apertures of different objectives [2]

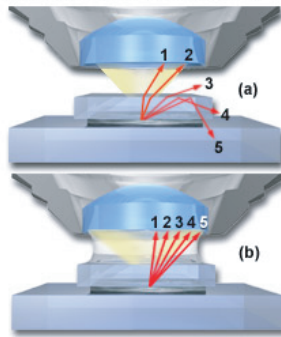


Figure 4: Light rays coming from the specimen and collected by the objective in the case of *a*) a dry (or air) objective and *b*) an oil immersion objective [2]

1.1 Aligning a Light Microscope: Köhler Illumination

Obtaining a homogeneous illumination is key to ensure that the imaging is being taken at the highest possible resolution supported by the microscope and to optimize the contrast. In the following paragraph we describe the protocol to obtain Köhler illumination.

1. Open field and condenser diaphragms.
2. Focus on specimen. This ensures that the optimization will be done for that particular position of the objective along the optical axis.
3. Correct for proper color temperature. A yellowish color might be present if the direct output of the lamp is used without any neutral density filters.
4. Close field diaphragm.



Figure 5: Specifications of an objective. [2]

5. Focus field diaphragm by moving the condenser up and down.
6. Center the field diaphragm. This will center the illumination and focus it on the sample.
7. Open the field diaphragm to fill the view of the observer. If the diaphragm is opened beyond the field of view, that light will just contribute to the background due to scattering with the sample outside the field.
8. Look at the objective back focal plane. This can be done either by using a Bertrand lens or by removing the ocular and looking at the tube.
9. Center an in-focus image of the lamp and mirror images at the objective back focal plane using the focus and adjustment screws on the lamp housing. Close the condenser aperture to $0.3 \sim 0.9$ the NA of the objective. This is a compromise between having a higher NA, which increases the resolution, and not illuminating on a bigger sample area, which will increase the background.
10. Done!

1.2 Different techniques

The site www.microscopyu.com hosted by Nikon is a great source of information for different imaging techniques, alignment protocols and various other information on microscopy.

1.2.1 Darkfield

The basic concept of this microscopy is to illuminate the sample with high spatial frequencies, filtering the direct light. This can be done by shining a hollow cone of light on the sample. This way a black background is obtained where outlines of cells, for example, can be easily seen. This technique, however, has some disadvantages. The resolution is usually not very high, thick samples are hard to image and the depth of focus is low. Additionally, internal cellular structure cannot be easily interpreted because of the lack of information given by the direct beams of light. More information on darkfield can be obtained from <http://www.microscopyu.com/articles/stereomicroscopy/stereodarkfield.html>.

1.2.2 Phase Contrast

A microscope in phase contrast mode detects slight differences in the light's phase induced by the sample. As can be seen in figure 6, the illumination goes through a condenser annulus, where only certain spatial frequencies are selected. When the phase in the illumination light gets changed by the different features of the sample it will get diffracted and, therefore bent. By filtering out the original components using the phase plate we just get an image of the sample's effect on the light. More information on phase contrast microscopy can be found at <http://www.microscopyu.com/articles/phasecontrast/phasemicroscopy>

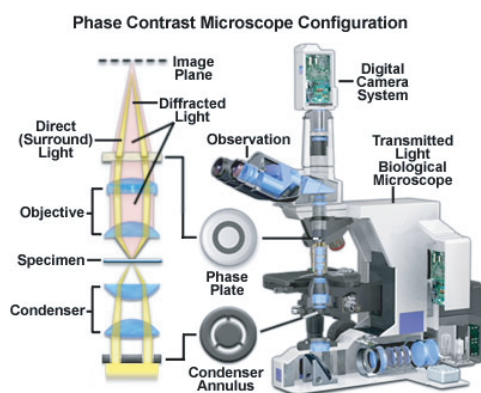


Figure 6: Diagram of a phase contrast microscope [2]

1.2.3 DIC

In DIC microscopy the illumination is polarized and then divided into two rays with different polarization, the ordinary and extraordinary beams, by the Condenser Nomarski Prism. The polarized rays will also be separated spatially by an amount smaller than the optical resolution limit. These two beams will go through the specimen and will accumulate different phases, which upon recombination by the Objective Nomarski Prism, will interfere to give a signal related to the local properties of the sample. Before acquisition the original light beam is filtered out by another polarizer, the analyzer, with its axis oriented perpendicularly to the axis of the original polarizer. This way, even though the linear component of the light does not get imaged, the resulting elliptical and circular components will still be detected. A diagram of a DIC microscope can be found in figure 7. A more detailed description of the DIC microscope can be found at <http://www.microscopyu.com/articles/dic/desenarmontdicintro.html>.

2 Digital Imaging

This section is based on the microscopy lectures by Jennifer Waters at the 2005 MBL Physiology course.

Light can be detected and quantified in a variety of ways. In microscopy systems the most commonly used detection device is the charged-coupled device (CCD), a solid-stated based detector. As we will see CCDs allow for light detection with spatial discrimination. On the other hand, if one

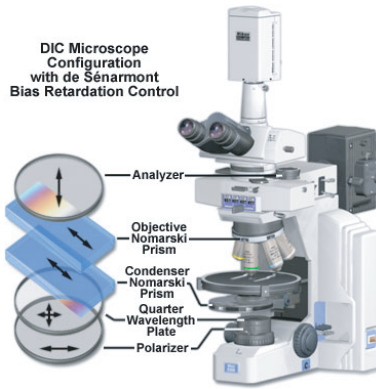


Figure 7: Diagram of a DIC microscope [2]

is interested in obtaining light from only one source, point detectors with no spatial discrimination such as photomultiplier tubes (PMTs) might be more appropriate.

Charged-coupled devices consist of a dense matrix of photodiodes, each of them with a charge storage region. As photons reach each pixel electrons will be accumulated in the well. This process will go on for as long as the integration or exposure time lasts or until the well is saturated, which happens when a density of $\sim 1000\text{electrons}/\mu\text{m}^2$ is reached. There are many parameters that are key to a camera's performance. The compromise between all of them will usually depend on the type of experiment.

Ideally, the *CCD spatial resolution* has to be matched to the resolution of the microscope. The pixel size should be at least $1/2$ - $1/3$ of the Airy disk size. In figure 8 we show the relation between pixel size and spatial resolution.

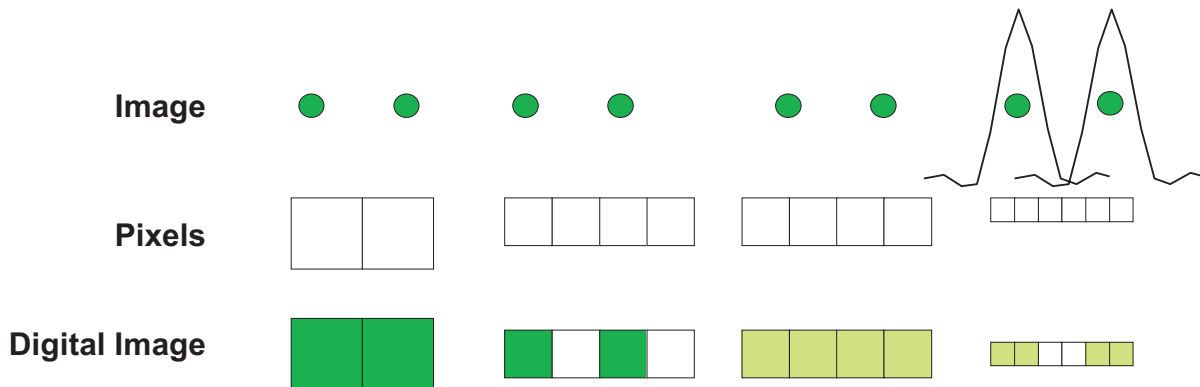


Figure 8: Resolution limitations of a CCD camera (Jennifer Waters, Nikon Imaging Center, Harvard Medical).

There are different *noise sources* in these systems. First, we can divide them into signal, optical

and camera noise. Any fluctuations in the object we are imaging falls into the category of *signal noise*. The fluctuations of photon counting statistics are included here. *Optical noise* consists of any photons detected by the camera that come from sources other than the area of interest in our field of view. By *camera noise* we understand all sources of change in the detector output that are not produced by light photons.

The camera noise has two different components: dark and readout noise. *Dark (or thermal) noise* is caused by electrons that accumulate in the chip due to thermal fluctuations. This type of noise accumulates with exposure time and can be decreased by cooling the chip. *Readout noise* is related to errors in the digitalization of the signal. This noise is independent of the exposure time and can be reduced, for example, by binning the CCD. *Binning* consists in averaging the signal over a certain number of pixels. This will obviously lead to a loss in resolution, but since the signal is now digitalized only once for that set of pixels, the readout noise is decreased.

Now we can turn to the analysis and visualization of acquired digital images. The first lesson here is that we should never judge an image by the way it looks on the screen. As an example, in figure 9 we present the same picture with different gray levels. The lesson to be learned here is that the differences are very subtle to the naked eye, which means that our eyes should not always be trusted when processing digital images.

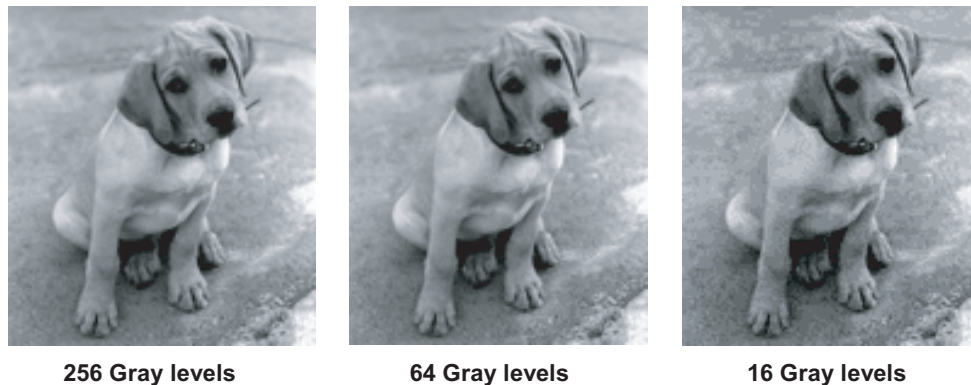


Figure 9: Same picture displayed with different gray levels (Butch Moomaw, Hamamatsu)

Since a computer can only display $2^8 = 256$ different gray levels, and most cameras can generate images of higher bit depth, images will have to be rescaled for proper viewing on a computer screen.

Finally, we might want to perform some basic image processing on our images. The most commonly used parameters are brightness, contrast and gamma. All of these transformations consist of changing the mapping between image and displayed intensity. In figure 11, for example, we see how the *brightness* shifts the mapping up and down, saturating some of the pixels. The slope of this mapping can be changed by adjusting the *contrast* (fig. 12). Finally, it can also be useful not to use a linear mapping. We can obtain log-scale-like mapping by adjusting the image's *gamma value*. This allows us, for example, to give pixels with lower intensity a comparable importance with those of a higher intensity, as can be seen in figure 13.

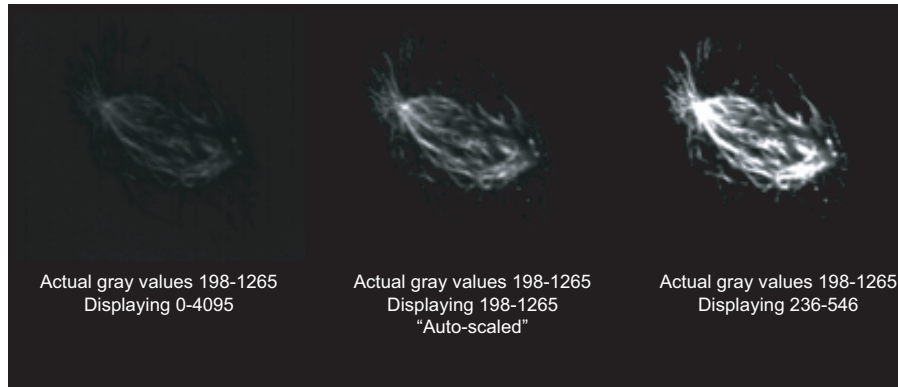


Figure 10: Same image displayed with different rescalings (Jennifer Waters, Nikon Imaging Center, Harvard Medical)

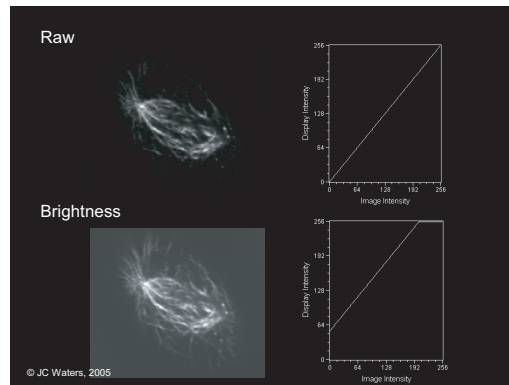


Figure 11: Adjusting the brightness of a digital image (Jennifer Waters, Nikon Imaging Center, Harvard Medical).

3 Calibrating a Microscope Using a Resolution Target

One of the first and easiest ways one can start being quantitative when approaching biology problems is to include scale bars in every image taken. As we will see throughout the first weeks of the course this will allow us to make all kinds of measurements and estimations not only about numbers and sizes, but also about composition and rates (once we include time in the picture, of course).

In the lab we will use a standard resolution target [3], which can be used for both testing the quality of an optical system or calibrating it. In fig. 14 we present a picture of the resolution target and a table that shows how to read it. The target is separated into groups and each group is divided into different elements. For example, in the lower right corner you have group 0, element 1. Group 0 continues in the upper left corner with element 2 and so on. The table gives the density of lines in lines per millimeter, therefore the periodicity of the lines in each element is $1/\text{density}$.

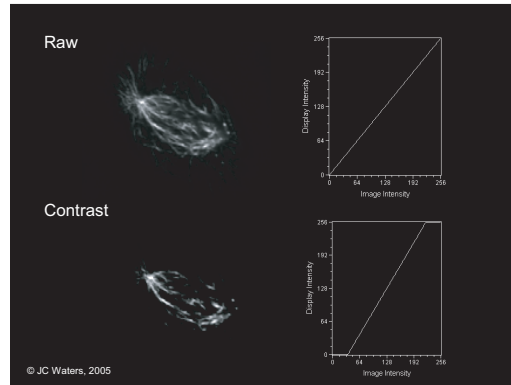


Figure 12: Adjusting the contrast of a digital image (Jennifer Waters, Nikon Imaging Center, Harvard Medical).

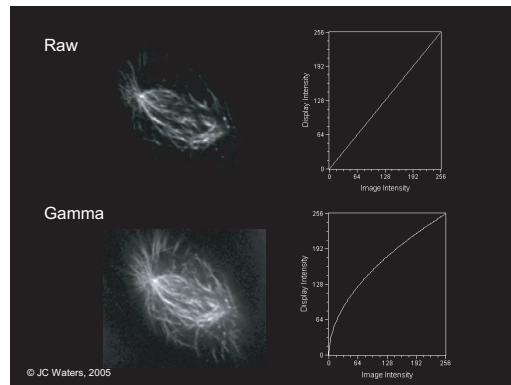
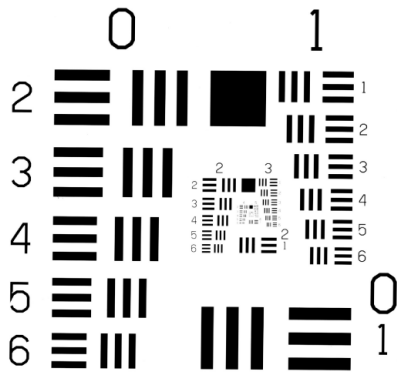


Figure 13: Adjusting the gamma value of a digital image (Jennifer Waters, Nikon Imaging Center, Harvard Medical).

When used for testing the quality of an optical system the idea is that diffraction (light getting diffracted from the feature's edges) and aberrations (mainly due to imperfections in the mirrors and lenses that are part of the system) are the ultimate limitations. The presence of simple periodic features makes it easy to determine when the pattern is starting to be affected by these effects.

This resolution target has a range from 1mm to 4 μm , which is going to be reasonable for most applications in this lab. When using regular objectives one has to make sure that the focus is not made through the target's glass, the side with the features should be facing the objective in order to reduce possible aberrations. When using an immersion oil objective a large cover slip should be put in between making sure that no oil gets on the target. Each magnification will have a suitable group to look at.

A good check is to test the linearity of the microscope: is there a linear change in magnification when one changes objectives? Additionally, one might want to play with the focus and take different



Element Number	Line Pairs per millimeter							
	Group Number							
1	1.00	2.00	4.00	8.00	16.00	32.00	64.00	128.00
2	1.12	2.24	4.49	8.98	17.96	35.92	71.84	143.70
3	1.26	2.52	5.04	10.08	20.16	40.32	80.63	161.30
4	1.41	2.83	5.66	11.31	22.63	45.25	90.51	181.00
5	1.59	3.17	6.35	12.70	25.40	50.80	101.60	203.20
6	1.78	3.56	7.13	14.25	28.51	57.02	114.00	228.10
Line pairs/mm = LP				Line width (mm) = 1/(2LP)				
Space width (mm) = 1/(2LP)				Line length = 5(line width)				

Figure 14: Picture of the resolution target and table that shows how to read it [3]

pictures for different focus settings which are close to what appears to be the right one. In that way, when looking at the pictures later with a program like Photoshop, one can have an idea of the sensitivity of the system to small displacements of the focal plane.

References

- [1] Eugene Hecht. *Optics*. Addison-Wesley, Reading, Mass., 4th edition, 2002. Eugene Hecht. ill.; 24 cm.
- [2] MicroscopyU. Nikon microscopyu (www.microscopyu.com).
- [3] Newport. Usaf-1951 test targets (www.newport.com).
- [4] E. D. Salmon and J. C. Canman. Proper alignment and adjustment of the light microscope. In *Current Protocols in Immunology*, pages 21.1.1–21.1.26. John Wiley & Sons, Inc., 2002.