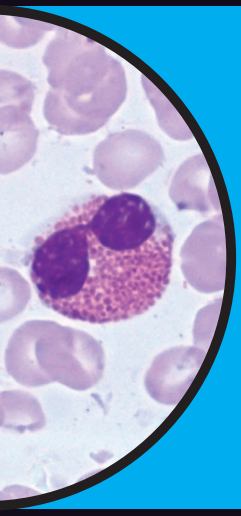
## Learn microscopy the easy way

This book is intended for all who are interested in microscopes and microscopy. You will find basic information on microscopy, how microscopes work, how best to prepare a sample, the role of contrasting techniques and what a perfect illumination is good for. Some easy experiments invite you to get used to microscopic preparation and to play around. You don't need to study this book from first to last page – well, except that we would love if you did.



### You will learn

- How to use a microscope
- The technology behind microscopy
- How to carry out first simple experiments

During her 10 years in microscopy **Anke Koenen** dealt with different materials as well as different microscope types. **Dr. Michael Zölffel** is a microscopist by passion since he received his first ZEISS microscope at the age of 14. He works in optical industry as a teacher for light microscopy since more than 20 years.

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# Microscopy

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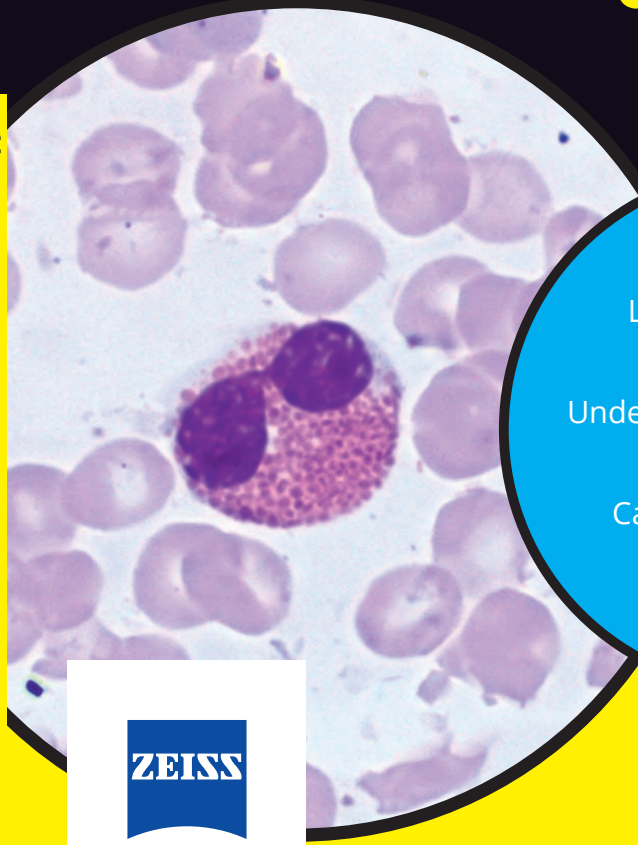

Learn how to use a microscope

Understand the technology behind it

Carry out first simple experiments

ZEISS

Seeing beyond

**Anke Koenen**
**Dr. Michael Zölffel**

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Microscopy

Koenen, Zölffel

*Anke Koenen and Dr. Michael Zölffel*

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## Microscopy for Dummies

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# Brief Contents

# Contents

# About the Authors

**Anke Koenen** has dealt with all manner of materials and many different instrument types during her 10 years in microscopy.

**Dr. Michael Zölffel** is a passionate microscopist. Since receiving his first ZEISS microscope at the age of 14, he has devoted himself to microscopy, both privately and professionally. He has worked in the optical industry as a teacher of light microscopy and its countless applications for more than 20 years.

# Special Thanks

A special thanks to Dr. Annette Bergter, Jan Birkenbeil and Dr. Markus Cappellaro for content support and critical proofreading.

# Introduction

Our world is full of little wonders which you can already see with the naked eye. Think about the colorful wings of a butterfly, mold growing on forgotten bread, fleas doing jumping exercises on your dog. Imagine that you could increase the power of your eye by a factor of, let's say, 100 – how exciting that would be. Now you could also see that a drop of water is crowded with little life, that there is a bunch of dust mites sleeping together with you in the bed and that bacteria can look like rods or spheres.

Whatever your reason for wanting to learn more about microscopes and their use might be – congratulations that you have decided to step into this interesting hidden world.

# About This Book

This book is intended for all who are interested in microscopes and microscopy. You will find basic information on microscopy, how microscopes work, how best to prepare a sample, the role of contrasting techniques and what a perfect illumination is good for. Some easy experiments invite you to get used to microscopic preparation and to play around. You don't need to study this book from first to last page – well, except that we would love if you did.

# Conventions Used in This Book

To make this book easy for you to navigate, we've set up a few conventions:

- ✔ New terms are printed in **bold** to make them stand out.
- ✔ Species names are printed in *italic* letters, as is common in science.
- ✔ Symbols will guide you through important facts and figures. If you are patient enough to read the introduction to the end, you will find all of them explained.
- ✔ Some background information is hidden in boxes, which are easy to skip if you choose.

# Foolish Assumptions

In writing this book, we have given some thought about who you are and what you might expect from a book called *Microscopy for Dummies*. These are the possibilities we assumed:

- ✔ Until now, you have not had a microscope but you are fascinated to learn about the universe it may open up to you.
- ✔ You are already the proud owner of a microscope, but you struggle with how to use it.
- ✔ You use a microscope in a laboratory, in a classroom or at home. You sit in front of this tool with all the knobs, sliders and objectives. And at the end of the day you need to give a microscopic image to your boss. The best image, which exactly shows what he or she wants to see. With the right contrast, the perfect illumination, details perfectly in focus … but you have no clue how to achieve this.
- ✔ You're not an expert in optical physics and you don't want to be one. You're not interested in complex calculations of refraction index, optical aberrations or interference. You simply want to learn how a microscope works, what all the knobs are good for and how best to use them.

If one of these statements applies to you, you've found the right book.

# How This Book Is Organized

This book is divided by topic into parts and then into chapters. The following sections tell you what types of information you can find in each part.

## Part I: Basics of Microscopy

Learn about the different types of microscopes and how they work. You will read about the physical terms connected to microscopy and their meaning.

## Part II: A Deeper Look into a Light Microscope

Here you get an overview of the components of a light microscope and their function.

### Part III: Sample Preparation in Light Microscopy

Whether fixed or living samples, transparent or solid – you will get an idea of which preparation and staining technique is best suited to which specimen.

### Part IV: Digital Documentation

If you need to capture and save your image, you need microscope cameras and a piece of software. Here is an overview of what to keep in mind when deciding on a camera for digital documentation.

### Part V: The Part of Tens

If you're looking for small, easily digestible pieces of information around microscopes and microscopy, this part is for you. You can find ten basic experiments and more. For example, you will learn how to prepare an onion and have a look into a droplet of water.

## Icons Used in This Book

You may be looking for particular information while reading this book. To make certain types of information easier for you to find, we've placed the following icons in the left-hand margins throughout the book:

This icon highlights tips that can make using a microscope easier.

These are facts important to mention to avoid misuse or damage to your equipment.

Here you will find technical details that are not complicated, but no basics anymore.

To ensure that you don't forget important information, this icon serves as a reminder.

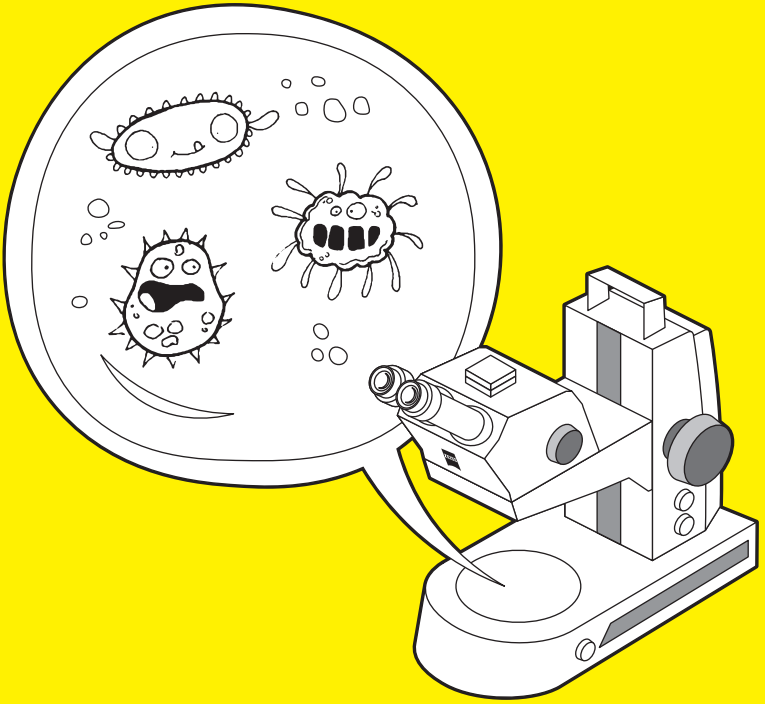
Special technical terms are explained here.


This symbol shows practical examples.

## Where to Go from Here

Let's get ready to microscope! Start from the beginning, or pick a chapter that interests you. Try out the little experiments. Skip over the parts that distract you and simply enjoy making things visible.

# Part I
# Basics of Microscopy



**IN THIS PART ...**

We introduce a wonderful device that enables you to enter a world invisible to the naked eye ...

... and explain the general purpose of light microscopes

You'll get a brief introduction to physical things that are important to know ...

... and find an overview of the different types of microscopes and what they are particularly suitable for

IN THIS CHAPTER

- About the eye, magnification glasses and the microscope
- Looking at the purpose of a microscope
- Learning how a microscope works

# Chapter 1
# The Microscope and How It Works

Your eyes are fascinating things! And you can also see fascinating things – colors, shapes, movements. But how are image created in your brain, and how do you see colors? Or not, if you think of the percentage of people suffering from a red-green color vision deficiency (which, by the way, applies to about 9 percent of men). And what is the smallest thing a human eye can see? You can easily recognize sesame seeds, which are about 1 mm in size. You can detect even a human hair, which is between 17 to 181 µm – especially if it's a stranger's hair in a hotel sink. And no problem at all to see (and hear!) the 8 mm typical house fly. But how about *E. coli* bacteria with a length of 2 to 6 µm, the influenza virus with its 100 nm, red blood or sperm cells? Difficult, isn't it?! There must be a way to magnify these tiny things – and of course there is!

## How the Eye Creates an Image

All images we see are the result of reflected light from the objects we look at. The light enters the eye through the transparent, dome-shaped cornea. The cornea bends the light rays so that they are able to pass through the pupil. This iris controls how much light passes through the pupil. The iris has the ability to change the pupil size from 2 mm up to 8 mm, allowing for less or more light into the eye depending on the situation. After passing the iris, the light passes the

crystalline lens. This lens focuses light on the retina, which is the final destination of the entering light. Because the front part of the eye is curved, it bends the light, creating an upside-down image on the retina. The retina receives the image that the cornea focuses through the eye's internal lens and transforms this image into electrical impulses that are carried to the brain by the optic nerves. The optic nerves from the two eyes join inside the brain. The brain uses the information from each optic nerve to combine the vision from the two eyes, creating one image and allowing you to see it the right way up (Figure 1.1).

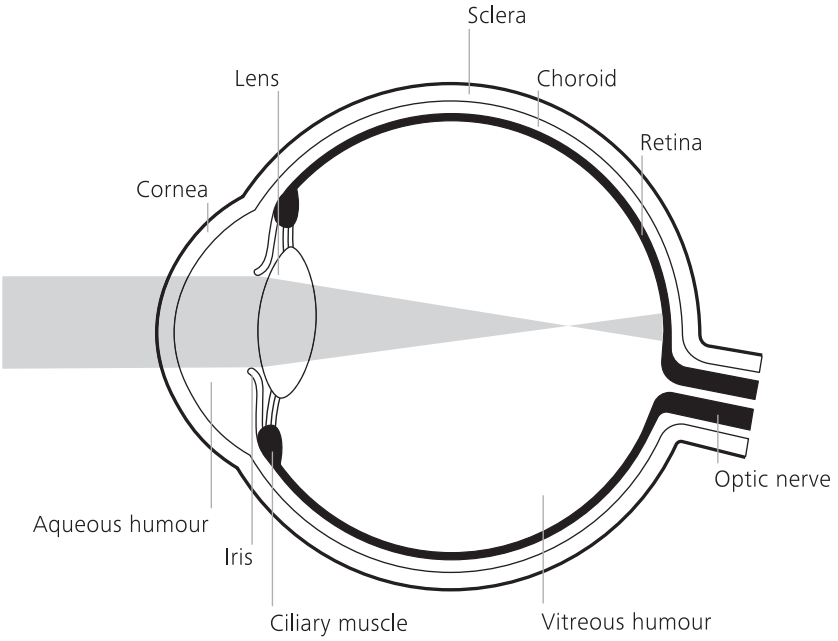



**Figure 1.1:** Eye and vison

# Life Is Full of Colors

The retina consists of special light-sensitive cells called rods and cones. Both types of cells work as photoreceptors and absorb light. The majority of the approximately 120 million rods are located in the outer layer of the retina. **Rods** allow us to see in poor lighting conditions and give us our black-and-white vision. **Cones**, on the other hand, have the primary function of seeing details such as wrinkles and freckles as well as colors. The roughly six million cones are primarily arranged in the macula, the central area of the retina. Cones have preferences; each

type receiving a narrow band of light which largely corresponds to the single colors red, green, or blue. The signals received by the cones are transmitted via the optic nerves to the brain where they are interpreted as color. **Color-blind** people are either missing or deficient in one (or more) of these types of cones.

### Funny side fact

Plenty of animals beat us out in the color vision department. Many birds, fish or insects have four types of cones; for example, they can also see ultraviolet light. To a bumblebee roses may not be so red after all, but full of patterns which we can't see.

| Structure | Function |
|---|---|
| Cornea | Clear window of the eye, covering iris and pupil |
| Sclera | White part of the eye protecting the eyeball |
| Pupil | Opening that allows light to enter into the eye |
| Iris | Colored part, surrounding the pupil, controlling how much light enters the eye by changing the size of the pupil |
| Lens | Jelly-like structure behind the pupil helping to focus the light onto the retina |
| Retina | Over 100 million light-sensitive cells detecting light, turning it into electrical impulses and transmitting messages to the brain |
| Optic nerves | Carry information to the brain for processing |

**Table 1.1:** Having an eye on the eye

## Seeing a Human Hair With a Human Eye

When we talk about microscopes and their function we need to keep some size relations in mind. According to different sources, the smallest object that an average human can see with the naked eye close up is about 0.1 mm, providing that your eyes are in a good shape and you have enough light. This is about the size of a human ovum and definitely smaller than some of the largest protists like amoebas.

$$0.001 \text{ mm} = 1 \text{ } \mu\text{m} = 1{,}000 \text{ nm}$$

Table 1.2 gives a small impression of the size relations we're talking about.

| Sample | Size |
|---|---|
| Virus | 30–50 nm |
| Bacteria | 1 µm |
| Spider web silk thread | 1–3 µm |
| Human red blood cell | 5–10 µm |
| Human hair (diameter) | 20–40 µm |

**Table 1.2:** How big is one micrometer?

# Let's Have a Look Through a Magnifying Glass

To visualize objects that are too small for the human eye, you need to enlarge them somehow. Let's try a magnifying glass. A magnifying glass consists of a curved, so-called **convex** piece of glass which works like a lens. When light travels from the medium air to the medium glass, the light rays are bent. This effect is called refraction. Lenses can bend multiple light rays to a specific focal point, creating a clear image of an object (Figure 1.2). Often, the object appears to be larger. The magnifying glass needs the object to be positioned within its front focal plane to produce an enlarged image with a defined magnification. The more convex a lens is, the higher the magnification. Usually, simple lenses cannot exceed a 15-fold magnification.

The position of the magnifying glass depends on its type. A magnifying glass used by a dentist is close to the eye and delivers a higher magnification than a reading glass that is held close to the object.

## Build your own little microscope

By the way: assuming, that you're not a specialist in precision optics, grinding your own lenses, you can nevertheless build and try out a simple magnifying glass. A drop of water has a similar refraction index to glass and you can see letters enlarged through it, for example.

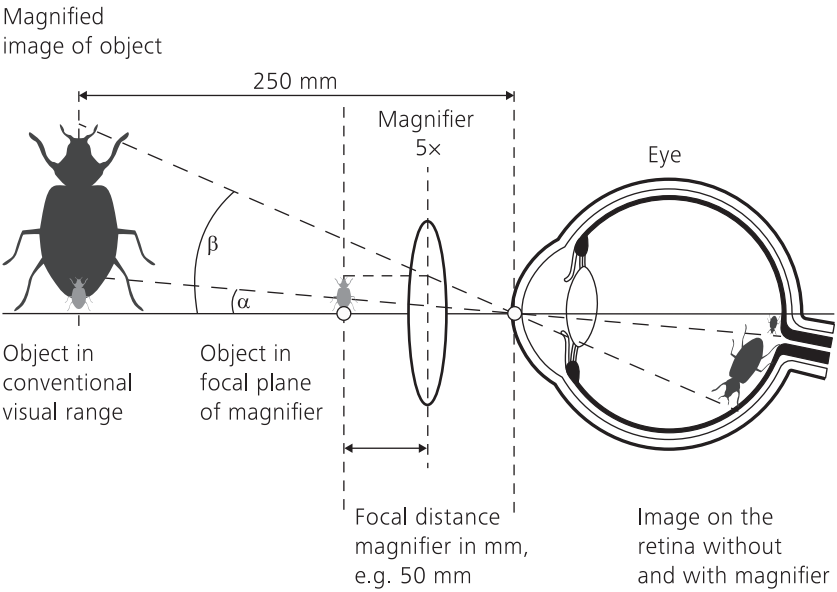



**Figure 1.2:** Magnification

IN THIS CHAPTER

- Terms to know about microscopy
- Visualizing structural details
- A matter of magnification and resolution
- Why contrast is important

# Chapter 2
# What Microscopes Do – Physically

You've already seen that it's more or less all about lenses and light, and that a microscope is a clever combination of these two major functional components – objective and eyepiece – to produce an enlarged image of a minute object. But that's only part of the job description … The job of a microscope is to magnify, resolve, and contrast tiny details such as bacteria, cells or viruses so that they become recognizable to the human eye.

## What is a Microscope?

If you are wondering what exactly a microscope is, the word itself already tells you the secret. »Scope« comes from the Greek word »skopein« and means to observe. »Micro« comes from the Greek word »mikros« and means extremely small. Together, you can see that a microscope is a tool that allows you to observe small objects.

| Term | Description |
|---|---|
| Magnification | The purpose of magnification is to ensure that a detail is displayed large enough for the human eye to see it. |
| Resolution | The purpose of resolution is to make it possible to differentiate specific details from neighboring details. |
| Contrast | The purpose of contrast is to ensure that a detail exhibits a significant difference in brightness or color to its surrounding environment. |
| Sharpness | Sharpness is best described as the line contrast of resolved structures. |
| Depth of field | This is the thickness of the optical section of an object through which a sharp image is observed through the entire stack. |

**Table 2.1:** Important physical terms

# The Microscopic Magnification

To be recognized, details must be displayed at a sufficient size. In other words, magnification is the ability to make small objects seem larger, such as making a microscopic organism visible to the human eye. A microscope's total magnification is a combination of the eyepieces and the objective lens.

The objective magnification ranges from low to medium to high and is indicated on each objective lens.

| | Object magnification | Good for | Challenge |
|---|---|---|---|
| Low | 1× to 5× | Large overview images for samples sizes up to 25 mm | It can be difficult to illuminate large object fields homogenously |
| Medium | 10× to 40× | Suitable for most applications | Incorrect color reproduction, spherical aberration due to incorrect sample conditions may occur |
| High | 60× to 100× | Small samples and fine structures | Stray light reduces the contrast in finely structured details; insufficient resolution, color reproduction, spherical aberration, image brightness may occur |

**Table 2.2:** Low, medium and high magnifiction

By simple multiplication with the magnification of the eyepieces you get the total magnification performance of your microscope.


$$20 \times \text{objective} \times 10 \times \text{eyepiece} = 200 \times \text{total visual microscope magnification}$$

## The Useful Magnification

The highest magnification is not always the best for visualizing objects. The optimum magnification depends on the structures you want to see. When selecting a combination of eyepieces and objective lenses for an optimum magnification, you need to take the **numerical aperture** (**NA)** of the objective into account. The NA of an objective defines the objective's resolution. A simple formula helps you to calculate the useful magnification range:

$$\text{Useful magnification} = (500 \text{ to } 1000) \times \text{numerical aperture of objective}$$

In other words: with a numerical objective aperture of 1.4, the useful magnification of the entire system is between 700× and 1400×. A magnification exceeding this range results in a so called empty magnification. **Empty magnification** occurs when the image continues to be enlarged, but no finer details are resolved and no additional information can be read out of the image. This is often the case when higher magnification eyepieces are used.

## Resolution in Terms of a Microscope

Resolution in microscopy is the ability to distinguish two objects from each other. It is the minimum distance at which two distinct points of a specimen can still be discriminated from each other. In a conventional light microscope, the smallest **resolvable distance** between neighboring structures is approximately 0.2 µm. In other words, the maximum resolution is 200 nm, which is due to the wavelength (λ) of light. This dependency was first observed by **Ernst Abbe** in 1873 and the diffraction formula for lateral resolution thus named after him:

$$\text{Abbe resolution} = \lambda/2\text{NA}$$

This value of 200 nm, however, is a quite theoretical one, which can only be reached under perfect conditions.

Beside the wavelength of light used to examine a specimen, other influencing factors are also quite important. One main factor in determining resolution is the numerical aperture (NA) of the objective as explained above. The higher the numerical aperture, the better the resolution. But also the type of the specimen as

well as the **refractive index** of the medium between objective's front lens and sample surface can influence the resolution.

## About Stick Insects and Contrast

Some animals are really masters of camouflage. Think about a stick insect hanging in the branches of a tree, or a chameleon. They use the effect of – let's call it anti-contrast – for their survival. What does that mean for microscopy? The answer is the contrast! The contrast is what allows the human eye to distinguish patterns and shapes from a background relative to the light intensity of the background. Without contrast in intensity and/or colors, the object is invisible for your eyes even under adequate resolution and magnification. In general, a minimum contrast value of 2% is needed by the human eye to distinguish differences between the image and its background. In microscopy, you can improve the **contrast** by proper sample preparation and using contrasting techniques (read more about this in Parts II and III).

**IN THIS CHAPTER**

- Upright and inverted microscopes
- Stereo and zoom microscopes
- Light, electron, and X-ray microscopes

# Chapter 3
# Different Types of Microscopes

There are many different types of microscopes and many ways to group them (see Figure 3.1). One way is to have a look at how the image is created. This can be by sending a beam of light or electrons to the sample, by scanning across, or even by using X-rays. The most common microscope is the optical microscope, which uses visual light passing through a sample to produce an image (which is accordingly called a light microscope). Other types of microscopes are confocal, electron or X-ray microscopes.

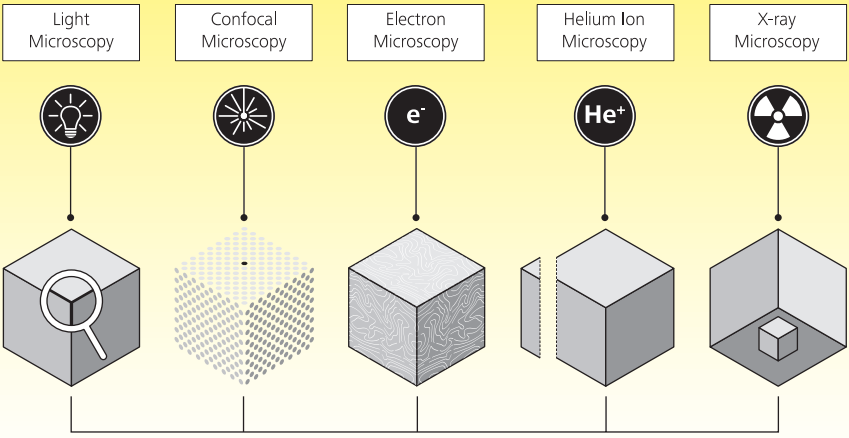



**Figure 3.1:** Types of microscopes

# Compound Microscopes – Clever Combination of Lenses and Light

The simplest version of a microscope has only a single optical system and basically works like a magnifying glass, which collects and focuses the light. To get higher magnifications than 25-fold you can either increase the curvature of your lens (which is limited) or increase the number of lenses.

And that's exactly the principle of the most commonly used compound microscope: a magnifying optical system containing several lenses, a light source and a mechanical mount to hold the object at a defined position in front of the objective. **Compound microscopes** allow for a much higher magnification and create the image in two steps: The first lens close to the object is called the objective. The objective creates an enlarged image of the object. This image is further enlarged by the second lens, the eyepiece, which acts like a simple magnifying glass and magnifies the image of the first lens. To keep away distracting light from the two lenses and to keep their spacing in an exact position, both are mounted in a tube. To further improve the resulting image and correct chromatic and geometric image aberration, objectives and eyepieces may consist of multiple lenses of different glass types.

Compound microscopes are built of different components such as stand, stage and objectives, to name just a few. The components are modular and can be exchanged between different microscopes. Which components are used in each case to assemble the microscope depends on the application. Depending on where the objectives are positioned in relation to the sample, you can classify them as **upright** and **inverted microscopes**.

# The Location of the Sample Defines the Microscope Type

**Upright microscopes** were the first microscopes invented and are still the classic form of a compound microscope. Here, the sample is positioned below the objectives. Simply said, you look down to see your sample. Upright microscopes are usually used to study fixed samples, slides or coverslips.

On **inverted microscopes**, the sample is positioned above the objectives. Basically, you look up to see the sample. Inverted microscopes are the instruments of choice for work with cell cultures in live cell imaging. Biological samples in a fluid tend to sink in the fluid, so you need to look from the bottom up through the petri

dish or a multiwell plate. Inverted microscopes are also common in metallography or if you work with large and heavy samples.

You can use both microscope types in transmitted or reflected light. Again, the type of sample decides the applicable illumination technique.

## Two Eyes – One Image

Compound microscopes, no matter if upright or inverted, use a single beam path to create the image. Even if you look with both eyes into the eyepieces, they are unable to provide stereoscopic images. In contrary, microscopes with two beam paths can produce stereoscopic and hence three-dimensional images. That's why they are called **stereo microscopes**. The two beam paths are tilted at an angle of 11–17° and supply your eyes with slightly different image information. Your brain calculates this image. Stereo microscopes are often used for sample preparation or to manipulate objects, because they offer large working distances and do not require a specific sample preparation. The limiting factor of stereo microscopes is their relatively low magnification as well as a quite low resolution.

## Zoom Microscopes: Experts for Large Fields

Zoom microscopes or **macro systems** all mean the same. They work with only one of the two stereo beam paths and expand the remaining channel. That is why such one-channel systems have a high numerical aperture, allowing for high resolution, and are extremely light sensitive. Even at low and medium magnifications you will achieve resolutions up to twice as high as with conventional stereo microscopes. This makes them perfectly suited for the microscopy of larger samples or whole sample microscopy.

## There Is More than Light

Whether using stereo, zoom or compound microscopes, in each case the image is created by using light. These microscopes can be your perfect choice if you want to use them at home, have a limited budget, or just need a smaller magnification. Light microscopes have their limitations in magnification, which are due

to the optical properties of light. To study smaller structures, you probably need other, more powerful methods of image creation: laser beams, electron beams or X-rays.

## Confocal Microscopes Deliver Optical Sections

Unlike stereo, zoom and compound microscopes, confocal microscopes use **laser light** as a light source. The laser scans the sample using different patterns, and the image is assembled with a computer. The laser can penetrate the sample deeper than light from a bulb. The result is a three-dimensional image of controlled depth of field. You can examine interior structures of cells, model organisms and tissue by stacking several images from different optical planes.

## Electron Microscopes

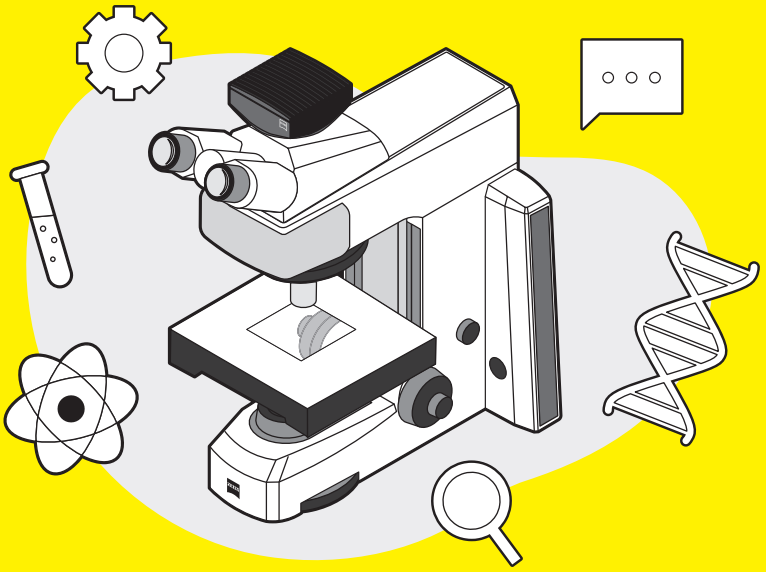
Put simply, electron microscopes use electrons rather than visible light to create an image. To use electrons for microscopy, you need to focus the electron beam and you need a vacuum. The beam is sent onto the sample, bounces off and creates a three-dimensional image of the surface. The wavelength of the electrons is much smaller than the wavelength of light from a bulb or laser, resulting in higher resolution. When you want to use an electron microscope, your sample must be electrically conductive, so the electrons bounce off. That's why samples are often coated in a thin layer of gold or other metal. As you can imagine, this process doesn't fit very well with living biological samples.

## X-ray Microscopes

X-ray microscopes use **electromagnetic radiation** (X-rays) to produce images of tiny objects. Unlike an electron microscope, you can use them to generate an image of living cells. X-rays penetrate easier than visible light into the depth of a sample. X-ray microscopes can mirror the inside of samples non-destructively and create a three-dimensional image of the inner structure of an object with higher spatial resolution.

## Part II

# A Deeper Look into a Light Microscope



**IN THIS PART ...**

We explain the structural and optical components of a light microscope and why a good alignment is essential to achieve image quality

You'll learn more about the major components of a light microscope and their function

Take a glance at the most important parts of different objective lenses and different light sources ...

... and understand contrasting techniques and their favorite applications

**IN THIS CHAPTER**

- Main optical components of a light microscope
- Monocular, binocular or digital tubes

# Chapter 4
# Components of Light Microscopes

Light microscopes are complex tools with many adjustable components. This chapter provides an overview of all those components. A better understanding of their function and how they influence the image quality is crucial for microscopic imaging. The most important components are shown in Figure 4.1.

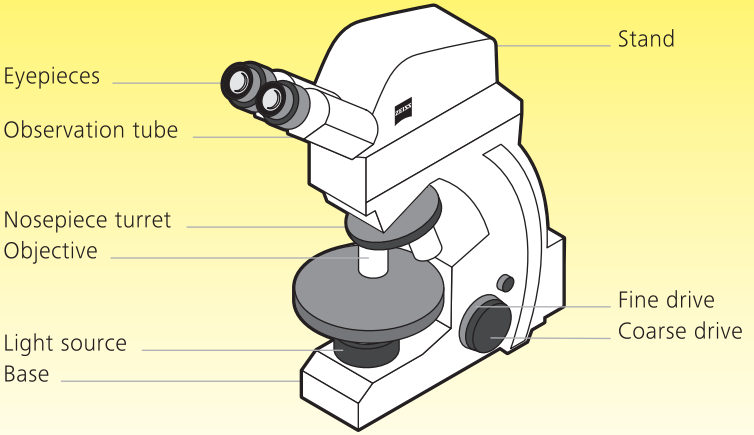



**Figure 4.1:** Parts and components at a glance

| Term | Description |
|---|---|
| Stand | The stand is the main body of the microscope and houses all components for illumination and image acquisition. |
| Tube | The tube is connected to the stand and holds the eyepieces. Binocular tubes hold two eyepieces; monocular tubes hold one eyepiece. Cameras can be mounted to photo tubes (trinocular tubes). Ergo tubes have adjustable viewing angles and/or heights. |
| Eyepieces | Eyepieces are inserted into the top end of the tube and make the magnified image available to the human eye. Typically, the eyepiece gives a 10× magnification of the image produced by the objective lens and has a visual field number of 16 to 25. This number is the diameter of the intermediate image given in mm. Eyepieces are interchangeable. Special eyepieces for wearers of glasses are available, too. |
| Nosepiece turret | The nosepiece turret houses the objective lenses and allows the user to switch between the different magnifications or objective types. |
| Objective lenses | Objective lenses collect the light from the sample and generate the enlarged, intermediate image. They consist of several lenses. You can differentiate types referring to e.g. their color correction performance and image flatness or the suitable contrasting technique. |
| Stage | The stage holds the sample with the help of simple clamps or specimen holders. It is attached to the stage carrier mounted to the stand. Stages can be moved up and down as well as on two horizontal axes to position the sample into focus. |
| Focus drive | The focus drive usually moves the stage up and down with separate adjustment for coarse and fine focusing. |
| Condenser | The condenser focuses the light coming from the light source to illuminate the specimen. It may contain diaphragms to modulate the image contrast and filters to adjust the spectrum or intensity of the illumination. |
| Light source | There are many options for light sources, ranging from simple mirrors to halogen or LED lights. The famous Köhler illumination is a process that provides homogeneous illumination without stray light and optimal image contrast. |

**Table 4.1:** Parts and components

IN THIS CHAPTER

- Achromat, semi-apochromat and apochromat objectives
- Decipher the hieroglyphs on an objective

# Chapter 5
# Types of Objectives

Objective lenses are the optical elements closest to the sample. They gather light from the sample, which is focused to produce the real image that is seen through the eyepiece. Objective lenses are the most complex part of the microscope due to their multi-element design.

## Objective Lenses and Color Correction

Light transmitted through a lens generates a certain color aberrations (color fringes), due to the different refractive behavior of different wavelenghts in glass. Different types of objective lenses show a different degree of color correction. Depending on their ability of chromatic or color correction, objectives are assigned into three different classes:

- ✔ **Achromat**: »Achro« is Greek and means »colorless«. Chromatic objectives are axially corrected for two colors, typically red and blue. Physically spoken, these objectives bring the two wavelengths for red and blue into the focus on the same plane. Achromatic objectives are furthermore corrected for spherical aberration for green. They don't produce strong color fringes around the details of the image (**chromatic aberration**). The letter »A« on objectives indicates that this objective is achromatic. These objectives are most common and widely used on laboratory microscopes, providing lower apertures, excellent working distances and a strong contrast.

- ✔ **Semi-apochromat**: Like achromat objectives, semi-apochromats are corrected chromatically for red and blue light. Additionally, they are corrected spherically for two or three colors. Due to this fact these objectives come up with a higher numerical aperture, resulting in brighter images. Plus,

they provide better resolution and contrast. These objectives are also called **fluorites**, referring to the calcium fluorite, which is used for their construction.

- ✔ **Apochromat:** If you use apochromatic objectives, your images are free of traces of color fringes. This is achieved by a clever combination of different materials with opposite refraction behaviors. Apochromatic objectives bring three wavelengths, typically red, green, and blue, into the focus on the same plane. Additionally, they are corrected spherically for either two or three wavelengths. It's no surprise that apochromatic objectives offer the highest level of color correction.

## Oil or Water? Increasing the Resolution

Already in 1847, the Italian physicist Giovanni Battista Amici demonstrated that image sharpness and brightness increase dramatically upon direct contact between the front lens of the objective and the sample by means of a liquid. In other words, if you fill a media between the objective lens and the specimen, you can increase the numerical aperture to achieve higher resolution. These media are referred to as **immersion liquids**. Typical immersion liquids are synthetic immersion oil, glycerole, water or silicone oil. The indication »Oil«, »Glyc«, »W« or »LCI« on the objective itself tells you which immersion fluid you should use.

In contrary, **dry objectives** are designed for use without an immersion fluid. They are typically of low to mid magnification and you can use them with or without cover glass.

## The Thickness of the Coverslip Glass

To visualize samples such as bacteria, cell cultures or blood, you normally use a coverslip glass to protect your little fellows from contamination. But physics and especially optics is a tricky thing. The use of a glass coverslip changes the way light refracts from the object into the objective. As a result, the objective needs to make proper optical corrections to produce the best quality image. This is the reason why some objectives can be adapted to a range of coverslip thicknesses for which they are optimized.

# Planar Objectives Provide Flat Images

Objective lenses are curved – convex or concave, but either way curved. This design results in field curvature, showing images that are accordingly also curved

and not flat. The higher the magnification you're using, the worse this effect gets. But how to make an image flat again? Clever optical designers came up with objectives that are calculated to eliminate the field curvature. You get a flat image, sharp to the edges. Such objectives are indicated by the word »**plan**« or »**planar**«. Flatness of an image is particularly important in reflected light applications. All objectives for reflected light work (**Epi objectives**) are flat-field corrected all the way to the edge.

# Markings on the Objective that Reveal Secrets

There is a lot of written stuff or otherwise visualized information on an objective lens. Just like a short user manual – so let's have a quick look at what you can get from this (Figure 5.1).

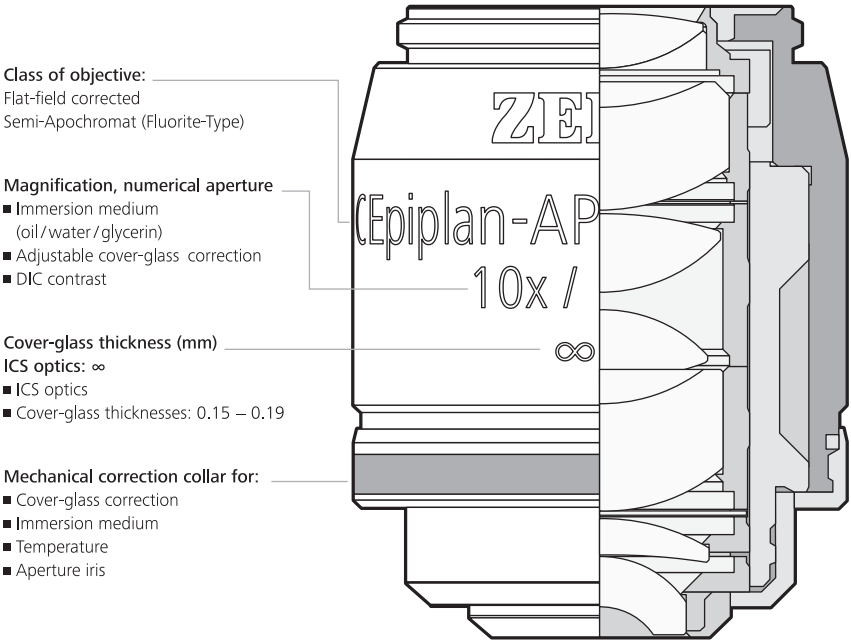



**Figure 5.1:** What objectives can tell you

| Example Term | Description |
|---|---|
| ZEISS | Producer of the objective |
| Plan-APOCHROMAT | This line indicates the class of the objective, whether it is color corrected and flat-field corrected. An »LD« before the name is a sign for an objective with a long working distance, normally corresponding to a slightly lower resolution. »Epi« means objectives for reflected light illumination. |
| 63×/1,4 Oil | This objective comes with a magnification of 63×. The numerical aperture is 1.4. »Oil« tells you that this is an objective to be used with the matching oil as immersion fluid. |
| Korr | The objective comes with adjustable cover glass correction. |
| ∞/ 0,19–0,15 | Lens image distance is infinite. The cover-glass thickness can be adapted between 0.19 and 0.15. |
| DIC, Ph | This objective is built for use with a certain contrasting technique. |
| Correction collar | Already the Korr tells you that you can use the objective with cover glasses of varying thickness. With the correction collar, you adjust the lens position to the cover glass thickness and also the immersion fluid. |
| Color coding | The color code tells you at one glance which magnification the objective provides:<br>✔ black: 1×/1.25×<br>✔ brown: 2.5×<br>✔ red: 4×/5×<br>✔ orange: 6.3×<br>✔ yellow: 10×<br>✔ green: 20×<br>✔ light blue: 40×<br>✔ dark blue: 63×<br>✔ white: 100× |

**Table 5.1:** Markings on an objective

IN THIS CHAPTER

- Light is reflected or passes the sample
- What Köhler has to do with it
- Comparison of different light sources

# Chapter 6
# Illumination

Illumination of the sample is one important controllable variable in achieving high-quality images in microscopy. The design of a light microscope must ensure that the light rays are organized and precisely guided through the instrument to the sample.

## Köhler Knows How to Adjust Your Illumination

To image your samples with optimized contrast and resolution, you need to apply **Köhler illumination**. This concept was introduced in 1893 by the ZEISS scientist August Köhler. It describes a routine that brings illumination and imaging beam paths into a defined, aligned state, providing homogeneous illumination without stray light. You achieve this by projecting an image with a small iris diaphragm called a **field diaphragm** into the plane of the sample already in focus. When doing so, the image from the light source cannot appear in focus together with the object plane. In Köhler illumination you homogeneously illuminate the object field diameter which is imaged by the objective. To set up Köhler illumination, you need to follow several alignment steps. When you change the objective and therefore the magnification, the alignment process must be repeated (see »The Part of Tens«).

# Illumination and the Microscope Optical Train

The condenser with the aperture diaphragm and the illuminating beam path with the luminous-field diaphragm are the critical elements in achieving Köhler illumination. Image forming light rays passed through the specimen are captured by the microscope objective and directed into the eyepieces. Throughout the optical train of the microscope, illumination is directed and focused through a series of diaphragms and lenses as it travels from the light source to illuminate the specimen and then into the eyepieces. By closing or opening the **condenser diaphragm**, you control the angle of the light rays emerging from the condenser and reaching the sample. Along with the numerical aperture of the objective, this setting also determines the **numerical aperture** of the entire microscope system. With the luminous-field diaphragm you determine which area and size of the specimen is illuminated. The aperture diaphragm of the condenser is imaged into the pupil of the objective and regulates the illumination of this pupil. The entire optical system is designed in such a manner that aperture angles of the light cones are correctly set together with the aperture diaphragm.

# Condensers Help to Adjust the Illumination

When properly adjusted, light from the condenser will fill the back focal plane of the objective with image-forming light by projecting a cone of light to illuminate the field of view. The condenser aperture diaphragm is responsible for controlling the angle of the illuminating light cone and, consequently, the numerical aperture of the condenser. Another reason for the existence of adjustable diaphragms and filters in the microscope is that the illumination should often be readjusted after each change of the objective. This is, in part, because the size of the observed object field changes with every objective magnification. Example: an objective with a low magnification (for example 4×) provides a large object field. If a switch is made to the 40× objective, the diameter of the imaged field of view shrinks by a factor of 10.

# The Sample Decides the Illumination Beam Path

You want to observe glass slides with tissue, dishes or multi-well plates with cell culture, thin sections or crystalline components? Then go for transmitted light microscopes. Simply said, all transparent samples are usually observed in transmitted light. **Opaque samples** are visualized in reflected light. The range of specimens falling into this category is enormous and includes most metals, ores, ceramics, many polymers, semiconductors, and many more materials. In fluorescence microscopy, you typically use reflected light, too.

## Transmitted Light Microscopy for Transparent Samples

In transmitted light microscopy the light is transmitted from the light source through the sample via the objectives. Samples are required to allow light to pass through them. A transmitted light microscope uses light that passes through a condenser into an adjustable aperture, then through the sample into the objective and to the eyepiece. The condenser and condenser aperture control the light for the right balance of resolution and contrast. This type of microscopy is used to distinguish morphological characteristics and optical properties of samples.

## Reflected Light Microscopy for Opaque Samples and Fluorescence

A transmitted light microscope will typically be of little use to anyone wanting to examine the structure of metallic samples, the surface of ceramics, integrated circuits, or printed paper documents. Because light is unable to pass through these specimens, it must be directed onto the surface and eventually returned to the microscope objective. Reflected light microscopy is the method of choice for fluorescence and for imaging specimens that remain opaque. Much like the fluorescence microscope, in reflected brightfield microscopy the sample is illuminated through the objective.

# Let There Be Light – A Glance at Microscope Light Sources

The variety of available light sources is as rich and overwhelming as deciding on a type of pasta. There is no best or worst. It also applies that the application and the type of result that you want determines your light source. In transmitted light microscopy, either brightfield or phase contrast, both halogen bulbs or LED arrays are widely used. Both have advantages and limitations – it's a matter of preference. And money …

## Stay Traditional with Halogen

Halogen bulbs are the traditional light source with a tungsten filament inside a glass casing. The casing is filled with halogen gas such as iodine or bromine, simply to increase the life span of the filament. They are widely used in transmitted light microscopes for brightfield and phase contrast, and may even be bright enough for most applications in polarized light. They are a good choice for examination of stained cells and tissue sections, as well as numerous reflected light applications. Halogen lamps are relatively inexpensive, easy and cheap to replace. Being full spectrum lamps, halogen lights give off great color integrity, which basically means that the sample is going to show up as a true-to-color life image. Due to their warmer color tint many pathologists for instance use halogen bulbs as the samples appear to be richer in color. For true to daylight color impression the halogen light source is combined with a blue conversion filter. There must be a »but«, for sure. Most of the energy emitted by these lamps is dissipated as heat. Over their life span halogen bulb glass starts to darken and intensity drops down.

## Fluorescence Microscopy Is Sensitive in its Favorite Light Source

The most basic requirement of a fluorescence microscopy light source is closely matching the excitation wavelength of the fluorochrome to achieve a high-contrast image – that is, an image with a high **signal-to-noise ratio**. Wavelengths that match the fluorochrome strengthen the signal, but any peripheral wavelengths produce background noise that can overshadow the signal emitted by the object of interest. Another related requirement is the illumination intensity, i.e. the number of photons specific to the excitation wavelength that reach the specimen. Lower intensity is necessary to protect against **photobleaching**, which

results in loss of contrast and therefore information. Especially for live cell imaging applications you need to protect living cells against phototoxicity and to reduce photobleaching. The cells also need to be protected from the heat generated by the light source and from the vibrations caused by mechanical filtering and switching devices. If you consider this, you will enjoy happy living cells.

### LED Light Sources Are a Major Advance for Fluorescence Microscopy

LEDs are the more up-to-date light source and are becoming more and more commonplace. LEDs are more expensive and you normally can't replace them by yourself. But they are energy efficient: LED arrays have good longevity and are quite reliable – the average life of an LED bulb is around 50,000 hours. They produce a brilliant white light with a slight blue tint color temperature or a specific narrow wavelength peak. They are low in heat emission. You can run an LED bulb for hours to days and it will not heat up as much as some of the now rarely used high pressure mercury arc light sources. You may use your microscope equipped with LED in exactly the same way as with a halogen bulb, but they are especially useful for fluorescence microscopy. Fluorescence microscopy requires an intense light source at the specific wavelength that will excite fluorescent dyes and proteins. LEDs are available in a variety of colors that match the excitation bandwidth of many commonly used fluorescent dyes and proteins.

## Mercury Arc (HBO) Lamps

Mercury arc lamps – HBO – are still the ideal light sources for applications that require high intensity spectral lines emitted in the deep UV to visible light regions. They are traditionally used in fluorescence microscopy. They can generate ample light at desired wavelengths, but only a small percentage of the projected light is useful in any particular application. The other wavelengths need to be suppressed to avoid background noise that reduces image contrast and obscures the fluorescent light emissions. This process of suppressing extraneous light is complex, expensive and only partially effective: even after decades of refinements, the best filters are not 100% percent successful at blocking the bleed through of non-specific photons. Nevertheless, mercury arc lamp remains a workhorse in fluorescence microscopy and is still considered one of the best illumination sources, especially for those fluorophores whose excitation maxima coincide with the spectral lines emitted by the hot mercury plasma. Mercury arc lamps are tedious to align within the lamp housing.

## Xenon Arc (XBO) Lamps

Xenon arc lamps feature a largely continuous and uniform spectrum across the entire visible spectral region. The luminous arc burns in an atmosphere of pure xenon gas at high pressure. It produces a bright white light that closely mimics natural sunlight. XBO is suited to stringent applications requiring the simultaneous excitation of multiple fluorophores over a wide wavelength range still sometimes asked for analytical fluorescence microscopy. They are now very difficult to obtain.

## Metal Halide (HXP) Lamps

Metal halide illumination sources are rapidly emerging as a serious challenger to the application of mercury and xenon arc lamps for investigations in fluorescence microscopy. These light sources feature a high-performance arc discharge lamp housed in an elliptical reflector that focuses the output into a liquid light guide for delivery to the microscope. The lamps are designed to generate a concentrated spot of light at a specified distance. One advantage of pre-assembled reflector lamps is that each time the lamp is replaced, the reflector is also replaced into a fixed position within the lamphouse, thus eliminating the stringent and cumbersome alignment requirements of mercury and xenon arc lamps. The reflectors on metal halide lamps are coated with multiple dichromatic interference filter layers that enable much of the thermal radiation to pass through the reflector while the ultraviolet and visible wavelengths are concentrated at the focused spot.

IN THIS CHAPTER

- Transmitted and reflected light contrasting techniques
- Brightfield, darkfield, phase contrast, and others
- Fluorescence

# Chapter 7
# Contrasting Techniques

To see something clearly with the naked eye or distinguish certain structures with your microscope, there must be a difference in brightness between a structure and the surrounding or background. This is what is called **contrast**. In general, only a minimum contrast value of 2% is needed by the human eye to distinguish differences between the image and its background. The better the contrast, the easier it is to see differences in an image. This necessity has led microscopists to experiment with contrast enhancing techniques for more than two hundred years in an attempt to improve specimen visibility and to bring more detail to the image without altering the specimen itself. It is a common practice to close the condenser aperture diaphragm below the recommended size or to wrongly lower the substage condenser to increase specimen contrast. But also different contrasting techniques with well-known characteristics help out.

## Brightfield

Some microscopic samples exhibit a natural contrast – such as chlorophyll-containing plants, metals, and pigments – or can be synthetically stained using special histological staining techniques, for example. Brightfield illumination is recommended for such types of specimens. The designation is derived from the bright background against which a high-contrast image of the sample is created. Transparent specimens are viewed with transmitted light brightfield and opaque specimens with reflected light brightfield. Inherently low-contrast specimens, such as unstained bacteria, thin tissue slices, and adherent live cells, rely on specialized contrast-enhancing techniques to assist with imaging these virtually transparent samples.

## Darkfield

Fine dendritic or minute point-shaped objects as well as minute and unstained structures are contrasted using darkfield transmitted or reflected light illumination. In darkfield microscopy, no light from the illuminator can pass into the imaging system; only light diffracted by the structure is captured by the objective. This is why the structures appear extremely rich in contrast – bright against a background as dark as possible. This method is especially useful to detect tiny and isolated structural details, such as bacteria. When working with reflected light, darkfield illumination is used to identify grain boundaries in polished and etched metal sections, for example, as well as to detect contaminants and flaws in surfaces.

## Phase Contrast

Transparent and thin objects appear with very low contrast in brightfield and are consequently often barely visible. Here, phase contrast is the method of choice for thin, unstained samples such as culture cells on glass. Remember that light has the characteristics of a wave. When passing through a structure, the wave is slowed down compared to the light passing through air. This generates a shift of the formerly perfectly aligned waves – a **phase shift**. This can only happen when there are density differences and thus works best for live or non-embedded samples. Histological tissue sections will not show any contrast due to the homogenization by the embedding medium. Phase contrast is an excellent method to increase contrast when viewing or imaging living cells in culture, but typically results in halos surrounding the outlines of edge features. These halos are optical artifacts that often reduce the visibility of boundary details. The technique is not useful for thick specimens such as plant or animal tissue sections.

## Oblique Illumination

Oblique illumination is recommended to contrast objects that are too thick for phase contrast. The term refers to using diaphragms to direct the illuminating light beam onto the specimen at an acute angle. Due to the highly directed, one-sided illumination, one side of a structure boundary appears bright and the other darker, which results in a shadow-casting relief image showing fine structural details. In thick tissue, longer wavelength (red) light can be used – this increases the penetration. However this light is not easily detected by our eyes; a camera is thus required to capture the images.

## Improved Hoffman Modulation Contrast (iHMC)

Hoffman modulation contrast is a form of oblique transmitted light illumination in combination with a grayscale modulator comprising neutral gray absorbing layers mounted in the objective's back focal plane. The modulator consists of three grayscale strips that run vertically through the pupil of the objective. This results in this method's middle gray image background and modulates a relief contrast. iHMC is used in the live-cell microscopy of sperm and egg cells when conducting artificial insemination in human and veterinary medicine. It works with plastic vessels. So you might be familiar with this kind of contrast from news reports, movies or TV shows.

## Differential Interference Contrast (DIC)

In interference contrast, previously split partial beams are combined as in phase contrast, interfering with each other as a result. Due to the interference of the two twin images in the intermediate image plane, a light-dark contrast is generated at the edges of objects. By laterally shifting the objective-sided DIC prism, also known as **DIC slider**, the contrast can be adjusted in such a way that the light and dark object edges appear on a gray background. As the illumination appears darker on one side and brighter on the other side, a pseudo-relief image is perceived by our brain. This can – but does not have to – match the actual topography of the sample.

## Polarization Contrast

Many materials, like most crystals and some biological structures such as muscle cells, are birefringent. This phenomenon fulfills an important diagnostic function in mineralogy, forensic microscopy, polymer research, or the quality control of textile fibers. In polarized light microscopy, transmitted illumination is typically used. But also reflected light is applied to visualize the contrasts in the grain structure of opaque metals such as aluminum, zirconium and others. For simple polarization, the microscope must be equipped with two crossed **polarizers**. In most cases, at least one polarizer and an analyzer are used for a polarization microscope.

# Fluorescence

Fluorescence is a low-energy form of radiation (emission) that results from a previous high energy illumination (excitation). The energy difference between the excitation and the emission is referred to as the **Stokes' shift**. As soon as the excitation stops, the fluorescence emission stops almost immediately. The particular advantages of fluorescence microscopy include a strong image contrast and its specificity; that is, its ability to specifically detect individual structures down to individual molecules. For the purpose of fluorescence excitation, the specimen is illuminated through the objective using reflected light.

Gas discharge lamps such as HBO, XBO, HXP or long-life LED light sources are used as light sources. LEDs make it possible to change the excitation wavelengths extremely quickly. The downside to LED light sources is that they still exhibit rather low excitation intensity in certain spectral ranges.

Part III

# Sample Preparation in Light Microscopy

**IN THIS PART ...**

We explain some general requirements for a sample to get the interesting information out of it with a light microscope

When structures need to be preserved and fixed: an overview of preparation techniques and the equipment you need

You'll learn how to prepare histological slides and how to stain your sample

IN THIS CHAPTER

- Preparation options for light microscopy
- Wet, dry, smeared or squashed specimen
- Buying prepared slides

# Chapter 8
# Sample Preparation Techniques and Equipment

Microscopy is used in many fields and microscopes are useful for different types of imaging. Samples may be cells – living or dead, tissue, fluids like blood, microorganisms including parasites and viruses, as well as little fellows from the area of biology, zoology and botany – or solid specimens such as different materials. If you need to choose a suitable microscopy and therefore preparation technique, you need to know what details you want to see from your sample, how large it is, and in what condition.

## Requirements for Transmitted Light Brightfield Microscopy

There are many different forms of microscopy techniques. One of the key techniques is transmitted light brightfield microscopy. Here, the specimen is illuminated with a beam of light that passes through it. There are certain requirements for those samples:

- ✔ Cells are kept in living conditions.
- ✔ Samples need to be transparent, otherwise the brightest illumination will keep you in the dark.

- ✔ Samples must be thin and flat, otherwise you will have too many out-of-focus regions.
- ✔ To distinguish different structures, a nice colorization helps.

Samples for transmitted light brightfield are normally put on glass slides. The main methods of placing samples onto microscope slides are wet mount, dry mount or sectioning, smear, squash and staining.

## Dry Mounts

A dry mount is the most basic technique: simply put the complete sample or a thinly sliced section of your sample on the center of the slide and place a coverslip over the sample. Dry mounts are ideal for observing hair, feathers, airborne particles such as pollens and dust as well as dead matter such as insect and aphid legs or antennae. Opaque specimens require very fine slices for adequate illumination. Since they are used for primarily inorganic and dead matter, dry mounts can theoretically last indefinitely.

## Wet Mounts

Used for aquatic samples, living organisms and natural observations, wet mounts suspend specimens in fluids such as water, brine, glycerol and immersion oil. A wet mount requires a liquid, tweezers, pipette and paper towels for preparation. Although wet mounts can be used to prepare a significantly wide range of microscope slides, they provide a transitory window as the liquid will dehydrate and living specimens will die. Organisms such as protozoa may only live 30 minutes under a wet mount slide. In addition, protozoa like *Paramecium* may be too large and/or move too quickly under the wet mount (see Figure 8.1).

# Smear Slides

In smears, the sample is spread thinly across one slide's surface using a second slide as the spreader. The resultant smear is dried, sometimes additionally fixed and stained. In medicine, samples of bodily fluids such as blood, cerebrospinal fluid or semen are smeared. The angle of the smearing slide determines the length of the smear; a steeper angle creates a shorter smear. For samples like blood, begin by backing the smearing slide into the sample and then push across the slide, pulling the blood in the opposite direction to create a smooth layer (Figure 8.2).

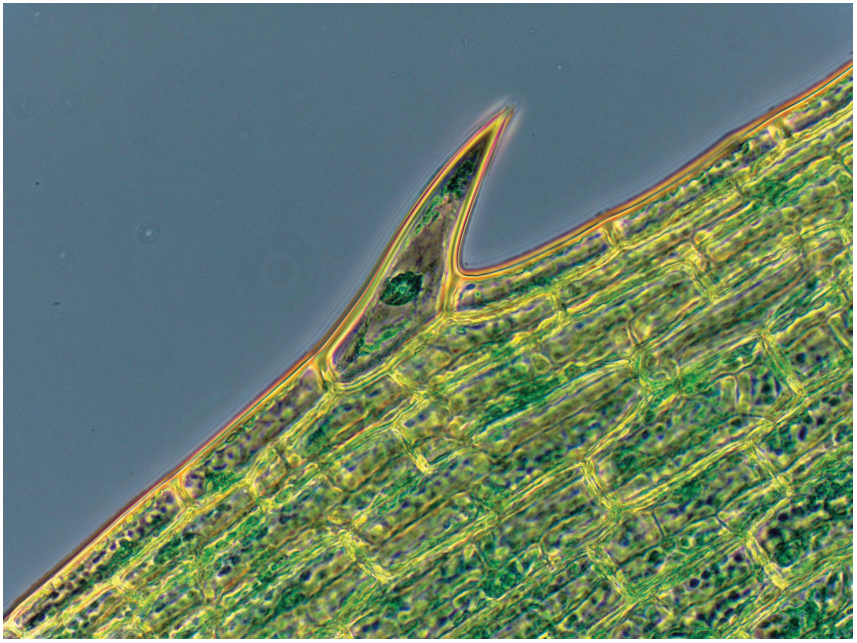


**Figure 8.1:** Wet mount sample

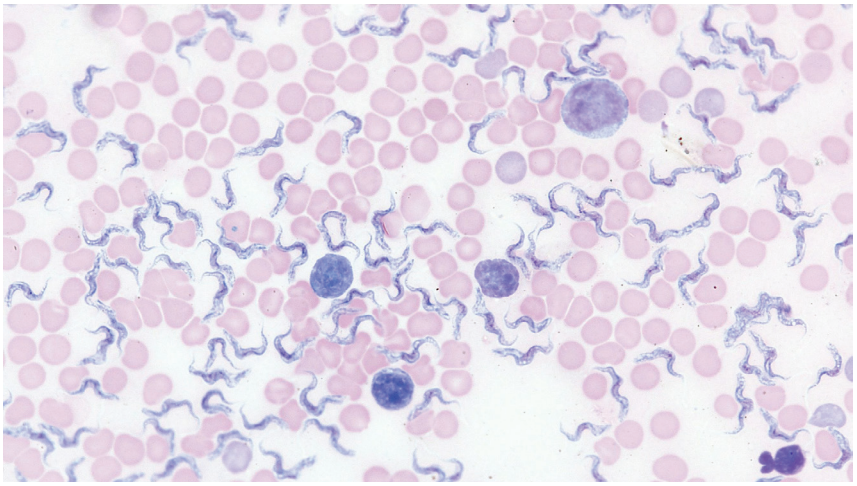


**Figure 8.2:** Blood smear sample

## Squash Slides

A simple way to prepare a thin sample is to squash or flatten a small piece of tissue under the coverslip. Designed for soft samples, squash slides begin by preparing a wet mount; place the lens tissue over the cover glass; gently press down, careful not to destroy the sample or break the cover glass, and squash the sample, then remove excess water.

## Stained Tissue Section

A more complicated sectioning procedure occurs when the structure and organization of a whole small organism or a tissue piece needs study. For most samples, first the tissue is preserved and hardened and the water is removed. Then the sample is embedded in a rigid medium such as wax or plastic and sliced into very thin sections only several microns thick using a precision machine called a **microtome**. The sample is oriented to give cross-sections or longitudinal sections when sliced. The sections are adhered onto microscope slides, the embedding medium removed – except when you use plastic – and the tissues stained to differentiate structures and cells. If fast working is essential, as is often the case in surgical biopsies for cancer, the samples are frozen, sliced with a freezing microtome, stained and examined.

## Pre-Prepared Slides

Especially useful for educational purposes and for those who do not want to undertake the laborious process of creating their own slides, prepared microscope slides are available in all areas of science, including plants, animals, single-cell organisms, organ or tissue cells, blood, bacteria and so on. Prepared microscope slides also offer access to infectious bacteria, animal tissue, marine life, diseased cells and other specimens that may not be easily available to students or hobbyists. In addition, they can be used as controls for students and researchers to compare their own samples.

IN THIS CHAPTER



# Chapter 9
# Sample Preparation of Histological Tissue

Histology is the study of cells and tissues, which is typically done with a transmitted light microscope. The preparation of histological samples can vary greatly based on the inherent properties of the samples such as size and hardness as well as expected post-processing, which includes planned staining techniques or other down-stream applications. Samples for histological observation may be obtained from different sources. These are, for example, large samples, whole kidneys or tiny pieces of a gut. Typical samples for histology are:

- ✔ extracted organs or parts of organs after biopsy
- ✔ incisional biopsy tissue
- ✔ punch biopsy pieces, which are normally used to examine skin
- ✔ shave biopsy, also used for skin
- ✔ curettings, normally taken from uterus or cervix
- ✔ core biopsy extracted with a needle

## Fix Histological Samples to Keep Them Fit for Purpose

To prevent degeneration or drying-out, the specimen should be fixed as soon as possible after the biopsy. For this primary fixation you put the fresh sample in a

buffered formaldehyde or glutardialdehyde fixative for up to 12 hours. You probably know this as **formalin**. Fixative agent and the duration of fixation is crucial and mainly depends on the size of the sample. Fixation is crucial as it prevents decay and preserve cells and tissues in a »life-like« state by stopping enzyme activities. It stabilizes the chemical components and hardens the specimen in anticipation of further microscopic examination. An alternative approach for animal tissues is to pass the fixative through the blood stream of the animal before removing the organs. This technique called **perfusion** may help reduce artifacts, false or inaccurate representations of the specimen that result from chemical treatment or handling of the cells or tissues.

## Cut Slices Thin Like Parma Ham

Once fixed, samples are placed into an embedding medium that will hold it rigidly in position while sections are cut. Usually this is **paraffin wax**, but other materials such as a glycerol-based freezing medium are also used to surround the sample during sectioning. Sectioning then takes place on a **microtome** – a tool comparable to a butcher's meat slicer, which shaves the sample into thin slices usually a few micrometers thick. As paraffin is insoluble in water, any water in the specimen must first be removed by dehydration in alcohol and replaced by an organic solvent such as xylene. Once cut, sections are mounted on a glass slide and stained to bring out specific features and color characteristics of the sample before being imaged on a microscope.

## Bringing More Color into Life

Cells and other structures are colorless. If you want to reveal structural details with brightfield microscopes you need to make them somehow visible. How? Try some form of staining … Generally, you mount the fixed tissue on a microscope slide and then treat it with any of a variety of dyes and stains that have been adapted for this purpose. Sometimes the tissue is treated with a single stain, but more often a series of stains is used, each with an affinity for a different kind of cellular component. Read more about this in the next chapter!

IN THIS CHAPTER

- Staining techniques for histological samples
- How to stain blood
- Differentiating bacteria with a Gram stain

# Chapter 10
# Important Staining Techniques

Apart from a few natural pigments such as melanin, the cells and other elements making up most specimens are colorless. In order to reveal structural details and produce contrast, some form of staining is required.

## Hematoxylin and Eosin: The Major Stain in Histology

Hematoxylin and eosin (H&E) stain is one of the routine tissue stains used in histology. It highlights various structures, organelles and other features and delivers good overview images. Certainly it is no surprise to hear that it combines the two histological stains hematoxylin and eosin … H&E stain is quite easy and fast to apply to distinguish between cell nuclei and cytoplasm. As a result, you get cell nuclei appearing blue, and cytoplasm and many extra-cellular components in shades of pink. In histopathology, many conditions can be diagnosed alone by examining an H&E.

## If H&E Is Not Enough

Sometimes additional information is required. Some of the »special stains« use dyes or metallic impregnations to define particular structures or microorganisms or use immuno-histochemical methods (IHC) to locate diagnostically important proteins with labeled antibodies. Molecular methods such as ***in-situ*** **hybridization (ISH)** may also be required to detect specific DNA or RNA sequences. These

methods can all be applied to paraffin sections. In most cases, the produced slides are completely stable and can be kept for many years.

Just in case you are a fan of Campari – the alcoholic liqueur contains **carminic acid**. The intensely red dye is extracted from dried, pulverized bodies of certain female scale insects, *Dactylopius coccus*, which are cactus-eating insects native to tropical and subtropical America. You can use this natural dye also for different stainings. Cheers!

## Papanicolaou Staining

Probably you know this method as pap-staining or **pap-smear**. It was developed in 1928 by George N. Papanicolaou and is one of the standard techniques in gynecology to detect precancerous and cancerous cells in the cervix. **Pap-staining** is used for fixed wet mount samples. Typical is the transparent appearance of the cytoplasm, which also allows the visualization of cells in overlaying parts. To stain the nuclei, you use **hematoxylin**. To stain the cytoplasm, you use dye mixtures consisting of **eosin**, **azure** and the azo dye **orange G**. These mixtures are available as ready-to-use stain and you don't need to play around by yourself.

## Giemsa and Wright Staining to Reveal Blood Components

When staining blood, either Pappenheim, Giemsa, Wright or Romanowsky stain are used, or a combination thereof. In most cases, simply Wright's stain is used. Red blood cells will stain pink, platelets appear blue/purple and cytoplasmic granules stain pink to violet. White blood cells include granulocytes (neutrophils, eosinophils and basophils) and agranulocytes (lymphocytes and monocytes). Neutrophils are displayed as a deep blue/purple nucleus under the microscope.

## Gram Stain for Bacteria

The Gram stain is a method for staining bacteria that was developed in 1884 by the Danish bacteriologist **Hans C. Gram** and differentiates between **Gram-positive** and **Gram-negative** bacteria. This process makes it possible to identify the different types of bacteria and visualize their morphology with an optical microscope. Gram staining is an important part of the diagnosis of infectious diseases, as Gram-positive and Gram-negative bacteria have a different cell wall composition and thus require treatment with different antibiotics.

# Part IV
# Digital Documentation

**IN THIS PART ...**

We have a look at the rapid development in processing and digital documentation of microscopic images in recent time

Present the principle of operation and important characteristics of digital cameras and ...

... give an overview of the different software options for microscopy and what they are good for

IN THIS CHAPTER

- Working principle of CCD and CMOS sensors
- Mosaic filters in front of the sensor
- Pixels, resolution and sensors
- Collect more photons by choosing the right camera adapter

# Chapter 11
# Digital Microscope Cameras

Seeing is believing … but sometimes that's not enough. Sometimes there are norms and regulations, telling you that you need to document your findings properly. Or perhaps you need proof for a criminal investigation that must be shown at court to help decide if somebody is guilty or not. Or you are just so proud of your microscopic image that you want to show it to all your friends and colleagues. Maybe even apply for the Nobel Prize with a nice little project? In all these cases, you must somehow take a picture of your findings, keep it and archive it.

## What You Expect From a Microscope Camera

Camera technology is as diverse as your imaging and documentation tasks. Which microscope camera you choose for your job depends heavily on the application you want to document. That's easy if you have the one and only application, day in, day out. But if you need a camera for multiple applications, then your choice will be different. Thinking about what you want, you may come up with a small footprint, high megapixel count, high read-out speed, and low noise. Sorry to disappoint you. You will never find all that, together, unless someone comes up with a way to break the laws of physics. Unfortunately, you will always have to

make compromises. For sure! But here are some general questions that can help you decide on the right microscope camera:

- ✔ Are you working with fluorescent, colored or stained samples?
- ✔ Do you need a certain resolution and a specific field of view for your application?
- ✔ Do you need a sensitive low noise camera for work with sensitive living cells?
- ✔ Is your sample alive and kicking? Moving fast? Very bright?

## CCD, CMOS – What the Hell? Physical Things You Should Know

This question is aimed more or less at the technical fans among you. Whether you go for one or the other sensor type may influence the resolution and speed of the camera. And that's why this section is a little technical.

**CCD** means *Charge Coupled Device*. So far, so good … The image acquisition principle is based on the conversion of light (photons) into an electrical charge signal (electrons). We are really lucky that silicon is a perfectly suited material to do this conversion in the visible light spectrum as silicon can be processed nowadays at high quantities and qualities.

By the way, this "inner photoelectric process" was explained by Albert Einstein, who received his Nobel Prize for explaining this effect in 1921. And in 1970, the first working CCD sensor was presented. Willard Boyle and George E. Smith were awarded the Nobel Prize for the invention of the Charge Coupled Device in 2009. The CCD sensor consists of a lot of small, light-sensitive individual segments or buckets, also known as **pixels** (picture elements), which are arranged in a rectangular matrix (the image area of the sensor). When you acquire an image, it is projected onto the sensor image area. During the exposure time, the sensor converts the amount of light arriving at each pixel into a number of electrons (charge) per each little image spot and holds them in the pixel. You can imagine these photons as raindrops collected in buckets. The efficiency - how many incoming raindrops (photons) are caught in these buckets - is called quantum efficiency. After the exposure time is over, these charge packets

are moved by electrical signals out to an electron voltage conversion station. This architecture is the reason for the name: the charges are moved from one bucked to the next, and they are coupled to each other - thus, a Charge Coupled Device. The conversion station sits in one or multiple corners of the sensor area. The collected raindrops (the size of electron packets) get measured per pixel in this converter and translated by an electronic analog-to-digital converter into digital numbers that are sent to your computer for storage in an image file, for display by your graphics adapter on your computer screen or for further processing. This exposure / readout / transmission / storage procedure happens for each single image and needs to be executed really quickly to be ready for the next image. If your sensor holds a lot of pixels, it needs to be very fast to be ready with no noticeable delay for the next exposure. Modern digital cameras are no longer restricted to the speed values of the old video standards, which produced 25 frames per second (PAL-Standard) or 30 frames per second (NTSC-Standard). Modern cameras can go much faster, or go slower at higher pixel counts.

So if you require high frame rates, all these readout steps must be very short, and the same goes for the exposure time. Therefore, at high frame rates the time to collect photons must be short, and that means the signal will get dimmer since fewer photons can be collected. To counter this, increase the light intensity of your lamp – but be aware – there are limits. For example, if you are looking at living cells, it might get so hot in the focus point, that the cells get cooked and die. Or you might have a camera with large pixel buckets, that can collect more photons. Or a highly noise-free camera, which can see the least number of photons over the noise. Or you could just go slower and give the camera more time to collect the photons.

The digital numbers represent the intensity signal of the photons: the more photons, the higher the digital number. Dark parts of the image show a signal close to zero, whereas bright intensities show a number up to 256, which fits to 8 bit. More sophisticated cameras can resolve even more signal intensities and represent these in 14 bit or more. The **dynamic range** refers to the darkest part and the brightest part in a single image which can be handled by a digital camera device. This is an important quality parameter since it enables you to see very dim structures in your samples, while bright parts are still visible. The sensitivity of a camera is limited visually by the signal intensity, which is still detectable in the background noise. This is unavoidable in technical systems. Minimizing these noise sources requires intense

engineering and, combined with speed performance and sensor size, drives up the price of a scientific camera.

**CMOS** is the abbreviation for *Complementary Metal Oxide Semiconductor*. Wow! Basically, this is the technical term for a manufacturing processes for electronic circuitries on a silicon substrate and does not describe any function of an image sensor itself. Originally it was used to build analog and digital integrated circuits. But as this technology is really capable of defining a whole lot of functionality on a small area of silicon and is also using the silicon base material, which is sensitive to visual light, engineers worked hard to combine the functionality of a light detector and its support electronics in one device. This was a complicated process as digital electronics cause a lot of noise and a lot of unwanted side effects. Light detecting structures can pick it all up as disturbing image artefacts. But finally, we have produced fast, large and sensitive CMOS image sensors. The basic principle of a CMOS image sensor is the same as with a CCD: it offers a pixel matrix structure, where the pixels are responsible for converting the light into an electron-based charge package. However, no charge packages are moved around, because the conversion from photo-generated charge into a voltage signal is done directly in each single pixel and not in the corners of the sensor. And below each column of pixels, the voltage signals are converted into digital numbers in parallel with many on-chip A/D converters, which can now be implemented on the same chip. So the conversion can be much faster and at short exposure times, higher frame rates can be reached. In addition, even all the support electronics can be implemented on the same chip. That means the cameras can be built much cheaper and the whole camera needs less power overall. As soon as the noise performance was exceeding the levels of CCD cameras, CMOS took over and is now the main technology used in digital camera systems.

## Color or Monochrome Cameras?

Before talking about the relative advantages and compromises of color and monochrome cameras, we must first ask how cameras based on these types of sensors can detect color signals at all! To answer that question, remember that light can be described as an electromagnetic wave with a given wavelength. And don't forget that our eyes can see specific ranges of wavelengths as colors. So the trick now is to get a CCD or CMOS sensor (same principles apply to both) to filter out some of the wavelength bands and assign them color information such as red, green and blue, which can be used to describe the universe of colors.

There are several methods for enabling a camera to pick up colors. Because the sensors can not separate colors by their own working principles (they pick all the photons in their range of wavelengths where silicon is working as a detector - from about 400 nm up to 1,000 nm), some additional technical means are needed. One initial method is to use colored illumination. Just shine red, green and blue light onto your sample and take three shots in a sequence. Next, combine the images into an RGB image, for example, on your computer. In a similar method, put a three-color filter wheel in front of the sensor with a white light illumination, then do the same sequence. This can be done automatically in a modern microscope where controllable filter wheels and software acquisition control can be used. Another method might be using a camera that has three built-in sensors which are looking into the scene through a prism. Each sensor looks at the same image through a different color RGB-filter at the back of the specially-formed prism. You can achieve a color image in one shot as all three channels are acquired simultaneously. However, it might be more difficult to match the camera to the microscope, due to the optical side effects of such types of beamsplitter prisms.

The last and most common method is a color pattern filter on top of the pixels. This means that a specific R, G, or B color filter sits within each single pixel so a single pixel can only detect one color. These color filters are arranged in certain regular alternating patterns on the sensor. The best known one is the so-called **Bayer pattern**, which was invented by a Kodak scientist of this name many years ago. The pattern implies a certain, small loss of spatial resolution, as, for example, a green pixel only detects spatial information in the green channel and misses the blue and red information at that same spot. However, with the help of special computer interpolation algorithms and the huge number of small pixels and careful adaption to the microscope, these effects can be minimized and are negligible most of the time. But at certain cases, when you go to the limits of your instrument, it might be worth giving it a little more thought.

And one more thing: color is only defined in the spectral range in which the human vision system is working. It is just defined by our physiological capabilities, working roughly in a range from 400 nm (deep blue) up to 720 nm (deep red). However, these silicon-based sensors can see and convert photons in a broader wavelength spectrum up to 1,000 nm, as mentioned previously. So every color camera in the world needs an infrared blocking filter to cut off the invisible light from 720 nm up to 1,000 nm since no color can be assigned to this signal. Indeed, the color reproduction of a color camera would be really very poor if you operated such a system without the IR filter in place.

All of these color acquisition methods do have one common effect: they are reducing the camera sensitivity compared to a monochrome camera. They reduce the signal intensity depending of the wavelength and cut off the near infrared spectrum.

Coming back to the question of whether you are working with fluorescent samples … if you answered yes, well done! Here is an easy rule. Use monochrome cameras for fluorescence and color cameras for brightfield acquisition of, for example, your stained histological tissue. Monochrome sensors do not detect any color. Each pixel of the sensor simply measures how much light hits it. Monochrome microscope cameras can achieve higher **spatial resolution** than color cameras. And because transmitted light is not diminished by the filter required for color acquisition, they give you increased sensitivity and acquisition speed.

## You Can Use Color Cameras for Fluorescent Imaging, But …

Of course you can also use a color camera to acquire images of fluorescent samples, but it won't be perfect. Remember: to create a color image, there is a mosaic filter (the so-called **Bayer filter**) in front of the monochrome sensor. It takes four pixels together – one red, one blue and two green – to form one color pixel. With this filter in place, only one in four pixels detect red light while the other three pixels in a quadrant see nothing. The same goes for blue, respectively. In the case of green, however, two pixels out of four collect green light. The camera generates the colored image by interpolating the values for the pixels that contain no light information. So, putting it the other way around: the Bayer color mask throws away three-quarters of your red, three-quarters of blue and half of the green fluorescence information. Based on this, a color camera does not work well for dimmer samples and will not deliver high-quality fluorescent images even for bright samples. Plus, you lose sensitivity for fluorescence by using a color mask.

## How to Get Colorful Images with a Monochrome Camera

So why on earth, you ask, would I use a monochrome camera that produces black and white images for my fluorescence samples? Give me the color, right? Wrong. But how can that be? It's actually quite simple. In fluorescence imaging, you use different filter sets to collect the different color channels. Probably you've already heard of DAPI, Cy5 or FITC? All of these dyes have known wavelength and matching filter sets. They are precise examples of fluorescence filter sets. Each dye is captured individually one after the other into individual images. You can choose a color by using software for each image. Either close to the real wavelength or completely different colors. Either to improve contrast or to ensure that red -green deficiency is not a problem. You get a fantastic multichannel pseudo-color image, all the while enjoying the better sensitivity of the monochrome camera.

# Fluorescence and Brightfield on the Same Microscope

So what if you have both brightfield and fluorescence applications, but only one microscope? Should you buy a color camera because of the brightfield application? And also use it for the fluorescence imaging? Well yes, you can do that, but keep in mind the loss of sensitivity. The best advice: if your microscope is equipped with two camera ports, then install two cameras.

# Sensor Size, Pixel Size and Differences in Resolution

Remember the Abbe resolution mentioned in Chapter 2? The resolution of your complete microscope system depends on the numerical aperture of the objective you use and the wavelength. Following this formula, lower wavelengths and higher numerical apertures of your objective give you better resolution.

As the sensor is supposed to convert a full image, it measures the intensity distribution of the microscopic image over the image area. Therefore the pixels are arranged as a matrix to cover the area. So you get two new qualities: pixel count and sensor area. And if you divide the sensor area by the pixel count, you get the pixel size.

Now it becomes clear: the smaller the pixels, the finer the image content can be resolved. So the pixel size can be defined in such a way that it can pick up the resolved microscopic image without losing any image content.

The scientists who defined this condition were Harry Nyquist in 1928 and later on Claude Shannon. They found out that a given dimension can be only measured correctly if the measurement tool can resolve at least half the distance it needs to measure. In other words, it tells you that your pixels should – at most – be half the size of the minimum resolvable distance of your microscope setup (as we said before, the smaller the pixel, the better your camera resolution). To be on the safe side, scientists have calculated a factor of 2.3 by which the camera pixels should be smaller than the minimum resolution of the microscope.

$$\text{Camera resolution in sample area} = (\text{sensor pixel size} \times 2.3) \,/\, (\text{objective power} \times \text{camera adapter power})$$

But now we have a conflict: This works against the sensitivity requirement: large pixels are more sensitive as they collect more photons, less noisy and ultimately

offer a higher signal quality than small pixels. So for best resolution, you need enough light to get a decent signal from your pixels, or else your camera needs a sufficiently low noise level to work well at lower light levels. That's the point: finding the right compromise is always key in microscopy.

To match Abbe's formula with Nyquist's theorem for a given objective, you can use a camera adapter.


If you have microscope camera with a pixel size of 6.45 µm, a 20× objective and a 1.0 camera adapter, the resulting pixel size in your sample area is:

$$6.45\ \mu m : (20 \times 1.0) = 0.32\ \mu m \text{ size of pixel in sample area}$$

Now, using the Nyquist criterion:

6,45 µm x 2.3 / (20 x 1.0) = 0,74 µm size of resolvable structures in sample. This number must match the optical resolution of the lens.

Binning is the process of combining a cluster of pixels into a single pixel.

## Binning

Camera sensitivity can be increased by combining photo-generated signal charges from neighboring pixels. With CCD cameras, this can be done during readout of the charge so this also increases the camera frame rate. Sensitivity is increased, as the readout noise is added only once per binned pixel information and the signal amplitude is enlarged according to the squared binning number, as compared to multiple single readouts.

With CMOS cameras, this can be done only after readout by digitally adding the values of neighboring pixels so that no speed improvement can be reached. Sensitivity is increased since the readout noise is reduced by the binning number and the signal amplitude is increased.

One severe side effect is the loss of image resolution as the effective pixel area is enlarged. Binning factors can range from 1 × 1 (no binning) up to multiple pixels such as 5 × 5. Binning reduces the amount of image data produced as the resulting pixel count is decreased. As the sensitivity is increased, you can increase the acquisition speed due to possible shorter exposure times.

## Optimum Resolution or Optimum Field of View (FOV)

The eyepiece is designed to visualize the full area of the intermediate image presented by the optical system of the microscope. The size of this intermediate image area depends on the microscope: which is to say, it can vary from 18 mm in diagonal up to 23 or 25 mm. A digital camera on a microscope looks directly into the intermediate image.

If the camera you are considering has a small sensor and you use a camera adapter of 1.0, you will get quite a small FOV. To maximize the percentage of FOV you capture or to enlarge the FOV, you could use a **demagnifying adaptor** of e.g. 0.5× or 0.63×. It projects a smaller version of the sensor area of the camera so the sensor can see a larger field. But this comes at the expense of the resolution as more information is projected into a single pixel and you will need to recalculate the pixel size to make sure you still match your camera to the resolution of your imaging system. If you want to have optimal resolution and optimal FOV, you need larger chip sizes with smaller pixels and more of them.

## Collecting More Photons with the Right Camera Adapter

Different types of experiments may require different types of microscope cameras – but you already knew that! And there is another tiny but very important thing to decide on. Choosing the right camera adapter for your widefield fluorescence microscope can increase sensitivity and enlarge the field of view at the same time.

A camera adapter is an optomechanical element that connects the microscope camera with the microscope's body. The simplest version of the adapter is just a hollow, threaded metal tube. Such an adapter does not change the magnification of the microscope system. The magnification factor of this adapter is therefore 1× and the adapter is referred to as a 1.0× adapter. On the contrary, a 0.5× adapter includes a lens system that reduces the effective magnification of the microscope system by twofold. This in turn reduces the size of the image on the camera sensor. The effective size of the image pixels therefore increases by twofold and the area of each image pixel increases by fourfold. Each of the pixels now collects four times more light on average, resulting in a significantly increased SNR (Figure 11.1). The effect of the 0.5× camera adapter is similar to 2×2 sensor binning.

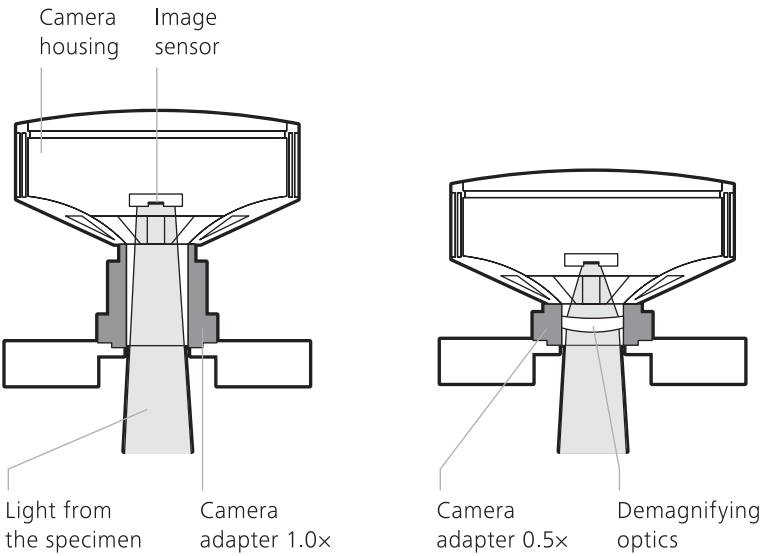



**Figure 11.1:** Different camera adapters

# Sensitivity

The term "sensitivity" comes into play when very week signals are being observed. Usually this happens in fluorescence microscopy or other contrasting methods which either limit the amount of available light or require you to protect living samples from light-induced damage. So the question is, what is the minimum amount of signal required to be able to produce a usable digital image?

Several factors influence this:

- ✔ the wavelength-dependent capability of the sensor to convert photons into an electrical signal, known as quantum efficiency
- ✔ the size of the pixel area that is collecting the photons
- ✔ the inherent multiple noise sources of the sensor and the camera
- ✔ the available exposure time to catch a detectable and unblurred image of your sample

Initially, the easiest way to determine the sensitivity of your camera is to take a glance at the **quantum efficiency curve**. This curve tells you how much of the light hitting the camera from the sample is used to produce the final image. Silicon-based sensors (CCD or CMOS) work from around 350 nm up to the range of 1,000 nm. Peak sensitivity is usually reached around 500 to 550 nm. The peak quantum efficiency is in the range of 70 % for standard sensors and up to 98 % for special back-illuminated devices.

Alongside quantum efficiency, the pixel size of the camera also influences sensitivity: the larger a pixel, the more photons can be caught and subsequently the more signal will be generated. This parameter is very dominant: a 6.5 µm pixel offers double the area compared to a 4.5 µm pixel and so can be twice as sensitive. Sometimes you might rather give up resolution than sacrifice sensitivity. In that case you would be better off choosing a microscope camera with a large pixel size. These collect more light so are the better choice for low-light applications. The high NA of the objective lens is then used to collect light rather than increase resolution in this combination. And remember: pixel size can always be enlarged by applying the binning method.

Imagine this: you are standing in your kitchen and the range hood is turned on and making noise. Someone outside the kitchen is trying to ask you a question. You know this won't work because the noise of the fan overlays and distorts the voice of the person asking the question in such a way that it becomes incomprehensible. Something similar can happen in an electronic camera. Several noise sources exist and can obstruct the electron signal you want to record.

Some dynamic examples are: readout noise, dark current noise, photon shot noise. radio telegraph noise …

Some static examples are: photo response non-uniformity, dark current non-uniformity …

Some of these unwanted signals can be compensated for or minimized, some not. For example, the dark current noise can be minimized by careful sensor design and production, and by sensor cooling. Cooling reduces this type of noise especially for longer exposure times. However, it makes a camera more expensive. The static noise can be removed by doing a black reference image, which will be subtracted from the final image automatically after exposure. This requires the careful acquisition of a black image in complete darkness and can be done just once at the beginning of the work.

# Signal-to-Noise Ratio (SNR or S/N)

To acquire images with detectable content requires the signal from the sample to be distinguished from the background noise. If you need a measure for the detection quality or low light sensitivity of your camera, you can use the signal-to-noise ratio as a quality parameter. If your signal-to-noise ratio is larger than 1, you will start getting a detectable signal, but if it is smaller or equal to 1, noise is dominant and it will obstruct your signal. The higher the SNR, the better your image will appear.

SNR becomes especially crucial when you work with low light samples. In this case you cannot optimize your signal so you have to minimize the noise. Cameras with minimized noise are usually more sophisticated and specialized and therefore more expensive.

The maximum SNR of a camera is a measure of the so-called available dynamic range. This describes the relation between the smallest detectable signal and the brigthest portion in a single image that can be recorded by a digital camera system. For example, if you look at a printed circuit board with a macroscope in a quality analysis workflow, you want to check the quality of the solder spots in the dark shadows next to an electronic component as well as the quality of the reflective solder spot surface.

But how can you influence and reduce the noise?

- ✔ Use purpose-built microscope cameras with good quality sensors.
- ✔ Allow free airflow to your camera and do not heat it further by hot surroundings.

Increasing the gain setting doesn't improve the SNR. When you increase the gain, both the signal and the noise will increase. As a result, the SNR does not change. Gain only changes the contrast of your image.

# Frame Rate

Frame rate is the inverse of the time the camera needs to acquire and read out an image. This depends on a number of factors:

- ✔ the readout and data transmission speed of the sensor and interface technology

- ✔ the number of pixels
- ✔ bit depth
- ✔ whether binning is used or not (CCD sensors only)
- ✔ whether a sensor sub region (Region of Interest Readout) is active (CCD and CMOS sensors)
- ✔ exposure time

More pixels and higher bit depth mean that more data needs to be acquired per frame, resulting in longer readout times and lower frame rates. CMOS sensors can be read out much faster due to a more parallelized readout structure and therefore they can reach much higher frame rates compared to CCD cameras. This faster readout only kicks in if the readout time is sufficiently short and negligible compared to the exposure time. Just ask yourself: how many frames per second can you get out of a camera if it is exposing for one second and the readout time is 10 ms? Exactly: no more than one frame per second because the exposure time is larger by orders of magnitude compared to the readout time. On the other hand, the maximum achievable frame rate at a minimum exposure time would be 100 frames per second as the sensor can be read out 1 s / 10 ms = 100 times per second.

| Example term | Description |
|---|---|
| CCD | A Charge Coupled Device. Proven reliable technology with a long history of optimization offers high sensitivity, good dynamic range, very homogenous image quality, low number of image artefacts; usable for long exposure times with cooling. |
| CMOS | A Complementary Metal Oxide Semiconductor. Successor to CCD technology, recent breakthrough for mass production of quality products, currently high innovation rate, fastest image readout due to massive parallel readout architecture, highest dynamic range, high light sensitivity, rolling and global shutter technology available, high-quality mass production technology. |
| Resolution | The spatial resolution of a digital camera is related to the pixel density, which is defined by the pixel count per sensor area. The smaller the pixel aperture, the finer the sampling of the presented structure. The reproduction of fine structures (lines) requires at least two pixels per structure sequence (line pair). |

**Table 11.1:** Good-to-know terms for microscope cameras

| Example term | Description |
|---|---|
| Pixel size | One pixel is the smallest effective area on the sensor, which becomes one image picture element. Smaller pixels are good for higher resolution, lower in dynamic range, less light sensitive and noisier. Larger pixels are good for better light sensitivity, less noise, higher in dynamic range, reducing the spatial resolution. The best pixel size is a balance between sensitivity (larger pixel) and resolution (smaller pixel). |
| Signal-ro-noise ratio | Noise can come from various physical sources and it limits the low light detection capability of a microscope camera. Post-processing algorithms can be used to minimize noise, but this sacrifices other image factors such as resolution. |
| Cooling | Cooling is used to minimize the thermal generation of electrons (dark current) in the sensor silicon material and the resulting dark current noise, and therefore enables longer usable exposure times. |
| Binning | Camera sensitivity can be increased by combining photo-generated signal charges from neighboring pixels during readout in CCD sensors. CMOS sensors convert the photo-generated charge into a voltage signal inside the pixel and therefore cannot do charge binning. However, binning can be emulated on the digital domain by adding neighbored pixel values with a similar effect. |
| Frame rate | The frame rate of a digital camera denotes the number of images that can be delivered per second (fps = frames per second). It is limited by sensor readout speed, digital data transmission and exposure time. |
| Spectral sensitivity | All kinds of light detectors show a wavelength dependent light sensitivity. The conversion efficiency is the ratio of incoming photons to generated signal electrons stated as a percentage. |
| Camera adapter | A c-mount camera adapter is used to mount a camera onto the microscope. Depending on the magnification factor of the adapter, the camera's sensor may cover more (lower magnification) or less (higher magnification) of the image coming out of the microscope (intermediate image). Using a lower adapter magnification causes demagnification of the intermediate image, resulting in a larger field of view for the final image and enlargement of pixel size, thus increasing light intensity detected by the sensor as well as reducing effective camera resolution. |

**Table 11.1:** (*Continued*)

IN THIS CHAPTER



# Chapter 12
# Microscope Software

Only with the right microscope software can you unleash all features of your microscope system. The range spans from free-of-charge image viewers and simple image acquisition tools to scientific analysis software allowing you to set up and document advanced and time-consuming experiments. Specific modules and levels allow you to configure your microscope system for nearly every application.

## Microscopy with a Finger Tip

With apps you can use your smartphone or tablet as a microscope or install microscopic software on your mobile device. Both ways are welcomed. Some apps turn your smartphone into a magnifier. You can use it as a macro camera for close-up images of insects, for example. You can also download apps that offer a collection of microscopic images to learn more about histology or zoology. More advanced apps can be connected to your microscope and used as software to acquire images, measure and annotate, share and create reports.

## Image Viewer and Image Acquisition

A selection of mostly cost-free software is available that you can use to acquire and view images. Normally they allow you to control the microscope camera, acquire images and videos. Often simple features such as annotations, measurements

and simple report functions are included. Such software is recommended if you are not yet sure which software you like and what modules you really need. Of if it's just enough to acquire an image here and there.

## Advanced Research Microscope Software for Experts

This software is recommended for more experienced users who want to manage everything from microscope control to image processing and data analysis. In most cases the software is modular – for different experiments, samples and applications you can have a dedicated software module. This can range from simple autofocus to determine the optimal focus position over multichannel fluorescence acquisition, where you combine any number of fluorescence channels with images in transmitted light, to stack image acquisition or deconvolution. Also, you can set up time lapse experiments with different parameters with the respective module to document dynamic processes in your specimens.

## Open Application Developments

If you have an application that goes beyond the modules offered with available software, or want to simplify, customize or automate your workflows, you can access so-called open application development software. With scripts you can create your own applications and interfaces, and exchange your data with external applications.

IN THIS CHAPTER

- Antonie van Leeuwenhoek, the first microscopist
- Robert Hooke and his book *Micrographia*
- Chester Moor Hall invents the achromat lens system
- The triumvirate of Carl Zeiss, Ernst Abbe and Otto Schott

# Chapter 13
# A Brief Look Back

## Visualizing Bacteria With Up to 270× Magnification

The history of early microscopes already dates back to the 13th century. Single-lens magnifying glasses were used to enlarge objects visibly. While working in a store using such magnifying glasses to count the number of threads in cloth, Antonie van Leeuwenhoek taught himself methods for grinding and polishing small, curved lenses and therefore became the »father« of microscopy. He reached magnifications up to 270×. In the 1670s, he started to explore microbial life with this microscope. He was the first to observe and describe bacteria after viewing the very little living animalcules in the mouth of an old man who had never cleaned his teeth, the circulation of blood corpuscles in capillaries, and living sperm cells, among a host of other unique (at the time) specimens.

## The Leeuwenhoek Microscope

Leeuwenhoek designed and built several hundred small microscopes. The main body of these microscopes consisted of two flat and thin metal (usually brass)

plates riveted together. Sandwiched between the plates was a small bi-convex lens capable of magnifications ranging from 70× to over 250×, depending upon the lens quality. The sample was placed on a pin that was moved with two screws. With one screw Leeuwenhoek adjusted the distance between the sample and lens and with the other one he adjusted the height of the specimen. Looking against the light, he was able to observe even tiny animals swimming about in a drop of water.

## Seeing Cells with the First Compound Microscope

**Robert Hooke** built the first compound microscope, that is still preserved consisting of an objective and an eyepiece. He used this microscope to study all kind of nature, especially the world of fossils and geology. In 1665, he published his famous book *Micrographia*, which shows impressive drawings of fleas with proboscis, eyes and claws. He was also the first to visualize the structure of cork. Since the structure reminded him of chambers in a monastery, he called them »cells«.

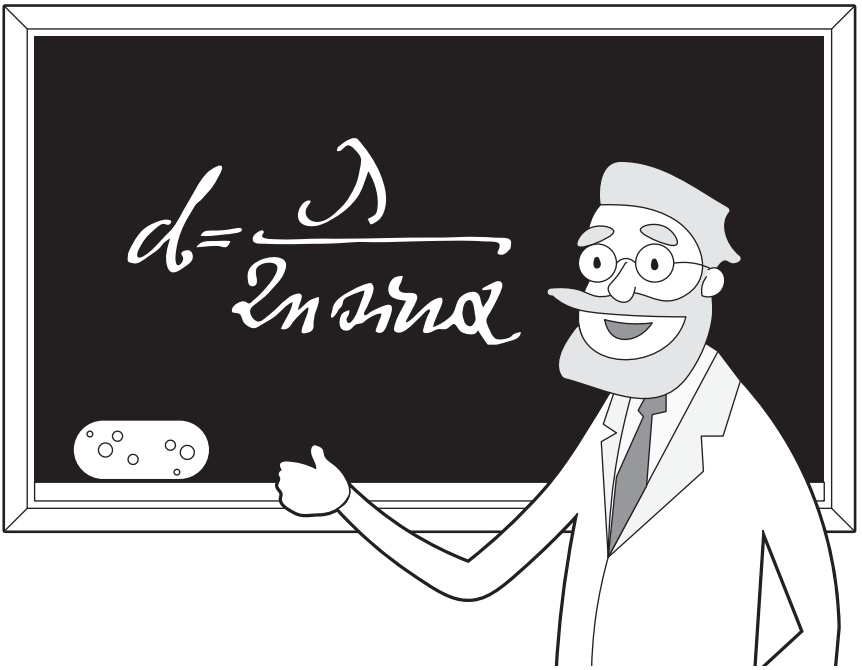
## Better Lenses – Better Quality

Being able to build microscopes with more than one lens and to produce lenses at a better quality are leading to a higher image quality. Early microscopists were hampered by optical aberration, blurred images, and poor lens design, which impeded high-resolution observations until late in the 1800s. **Chester Moor Hall** started to experiment with manual polished lenses of different glass types to increase the microscopic image quality. In Germany, **Joseph von Fraunhofer** also worked on these achromatic lenses. The quality of the microscope depended significantly on the know-how of the polisher.

Light with different wavelengths and therefore different color is refracted and distracted differently while passing through a lens. This physical phenomenon results in **chromatic aberrations** on the edges of observed objects. If you use systems with only one lens, this aberration can be neglected. But if a microscope with more than one lens is used, the aberration multiplies.

## Calculated Lens Quality – The Breakthrough of Commercial Microscopy

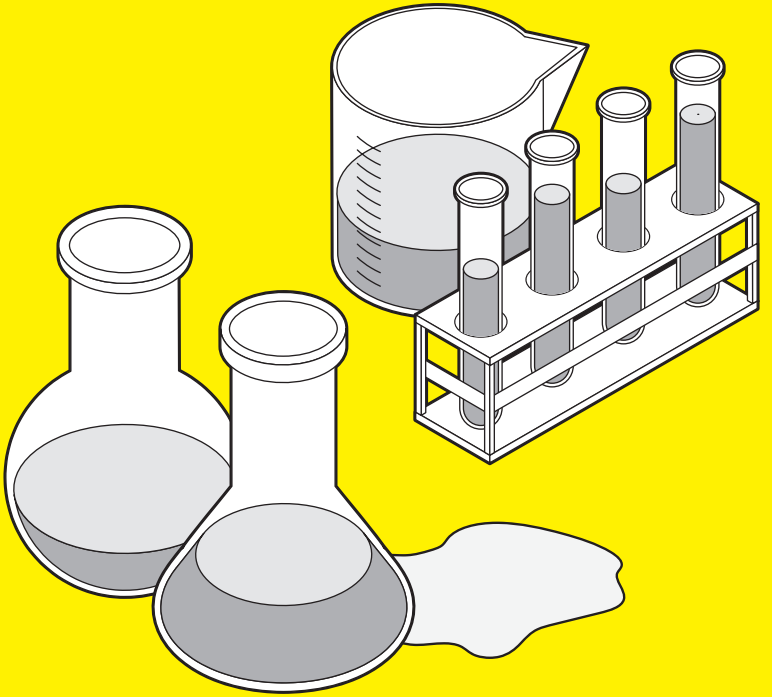
In the mid-19th century, the German mechanic **Carl Zeiss** began his own business in the German university town of Jena, Thuringia. In 1857, the Zeiss workshop produced the first genuine compound microscope equipped with an eyepiece and an objective. Carl Zeiss refused to accept the trial-and-error method used at the time for the production of optics. He acknowledged that his manufacturing procedure had to be based on precise rules and strict guidelines. For assistance in this endeavor, Carl Zeiss formed a partnership with **Prof. Ernst Abbe**, a brilliant physicist and mathematician. In 1872, Abbe formulated his wave theory of microscopic imaging and defined what would become known as the »**Abbe resolution formula**« that must be fulfilled to produce sharp images. The construction of microscopes on a sound theoretical basis was possible, and still is today.



## Development of New Optical Glasses

Unfortunately the quality of optical glass produced during that period was not sufficiently high to provide the theoretical resolution that was dictated by Abbe's »resolution formula«. There was definitely the need for high-quality glass. Then Abbe met **Otto Schott**, a glass chemist, in 1881. Together they developed several new glass formulas and made adjustments to the mixing and annealing process to eliminate internal defects and produce optical-grade glass with a uniform refractive index. Finally, in 1886, they introduced a new type of objective, the **apochromat**. The creation of apochromat objectives eliminated color aberrations, which greatly assists bacteriologists in identifying infectious bacteria, and brought the resolving power of the microscope to the limit we know today.

# Part V
# The Part of Tens



**IN THIS PART ...**

Read about interesting things in connection with microscopy

Or maybe you're interested in how microscopy evolved?

If you'd like to get started with some nice experiments, some easy-to-follow step-by-step procedures are explained

And, very important! We explain how to set up Koehler illumination

And, last but not least, we give some tips on how your microscope and you will stay friends forever

IN THIS CHAPTER

- Preparing cork, onion and yeast cells, and more
- Basic equipment you need for your experiments

# Chapter 14
# Ten Basic Experiments

**Johann Heinrich Pestalozzi**, the great Swiss educator, transformed 19th-century thinking with his belief that learning requires one to develop the powers of the »head, heart and hands«. The hands-on approach sparks a greater curiosity. And it's also curiosity that accelerates your learning. Dive now into microscope experiments that are easy and fun, providing endless opportunity for microscopy.

## Cork Cells

Robert Hooke was the first to take a look at the honeycomb structure of cork under a microscope. Now open a bottle of wine and step into the findings of Hooke yourself.


You need:

- ✔ A piece of cork
- ✔ A glass slide with coverslip
- ✔ A sharp knife or razor blade
- ✔ Tweezers
- ✔ A pipette
- ✔ Water

Carefully cut a very thin slice of cork using the razor blade or sharp knife from the end of the cork. The thinner your slice, the better is the outcome. Drop a few drops of water onto the slide. The drop of water

has to be bigger in diameter than your cork slice. Place the cork slice on the slide right into the water droplet. Hold the coverslip at an angle to the slide so that one edge of it touches the water drop. Carefully lower the coverslip and ensure no air bubbles are trapped beneath it. Place the slide on your microscope and view it using the lowest magnification, first. What you can see are:

- ✔ Rectangular cells
- ✔ Cell walls surrounding the cells

## Onion Tissue Cells

An onion is a perfect simple example to demonstrate the cellular structure of plants and to investigate the most important cell organelles: cell wall, cytoplasm, mitochondria (organelles for energy production), and the cell vacuole. And the fresh raw material for this experiment is quite likely already in your kitchen.


You need:

- ✔ An onion
- ✔ A glass slide with coverslip
- ✔ A sharp knife or razor blade
- ✔ Tweezers
- ✔ A pipette
- ✔ Water
- ✔ Methylene blue (optional, for staining)
- ✔ Gloves (optional)

You know that thin membrane between the layers of an onion? This is exactly what you need for your sample. First peel the onion and cut it into four pieces. Remove one of the layers and peel off a piece of the clear membrane from the inside layers with the tweezers. This is the thin, transparent membrane in between the layers. Now, drop a few drops of water onto the slide. Place the removed membrane flat on the surface of the slide. The drop of water prevents the onion from wilting and drying. If you have methylene blue, it's now the time to

add a drop of it to the slide. It helps to visualize the cell structures. Better wear gloves to do so, as methylene blue is toxic if ingested. Place the coverslip onto the slide. There should be no air or bubbles! And that's it – have fun investigating the structures under your microscope. Start with lowest magnification, and you will see:

- ✔ Rectangular cells
- ✔ Cell walls surrounding the cells
- ✔ Dark stained nuclei containing the genetic material (DNA)
- ✔ Vacuoles (cell organelles filled with water and many organic or inorganic substances) in the center
- ✔ Small granules inside the cells within the cytoplasm (containing reserve substances to be stored like starch)

## Cheek Cell Swabs

Have a look at your own body tissue by investigating your cheek cells. Scary, isn't it?! By the way, the correct name for these cheek cells is **squamous epithelium cells** and they are used, for example, to test whether you have multi-resistant bacteria before you undergo surgery.


You need:

- ✔ A glass slide with coverslip
- ✔ A Q-tip or toothpick
- ✔ Methylene blue
- ✔ Gloves

Scrape the inside of your cheek with the Q-tip or toothpick. Attention – do not hurt yourself. This is not about preparing a blood smear. Wipe the Q-tip or toothpick on to the center of the slide. Gently wipe the edge of the coverslip over the scrapings. What you're doing is called a smear. A smear helps to get a sample layer thin enough to view it clearly under a microscope. Drink a pot of tea and leave the smear to dry. When the smear is dry, add a drop of methylene blue. Better wear gloves to do so, as methylene blue is toxic if ingested. This stain helps

to visualize the nuclei of cells. Place the coverslip on the slide and have a look with your microscope using lowest magnification first. What you can see is:

- ✔ Individual cells
- ✔ The cell membrane surrounding the cells
- ✔ The nucleus
- ✔ Cytoplasm (which is the more or less liquid stuff inside the cells and contains all the small structures cells need to survive)

## Elodea

*Elodea* or waterweed is a genus of submerged aquatic plants used in aquariums. You should be able to buy this from pet shops. *Elodea canadensis* is a species that works best for this experiment because it has thin, straight leaves. But all other species do their job as well.


You need:

- ✔ A glass slide with coverslip
- ✔ A dropper
- ✔ An *Elodea* leaf
- ✔ Water
- ✔ Salt solution (2 teaspoons of salt in a cup of water)

Pick off an entire *Elodea* leaf and place it on the microscope slide. Add a drop of water and observe the cell walls and the green chloroplasts. If you add a drop of salt solution afterwards you can witness a process called osmosis. The cells are shrinking because the salt forces the intracellular water molecules to leave the cell. What you can see is:

- ✔ Individual cells
- ✔ Cell wall surrounding the cells
- ✔ Chloroplasts (the green plant structures in which photosynthesis takes place)

## Ouch! – Blood Smear

This experiment is not good for weak nerves. But you also don't have to be Dracula's little brother ;o). Human blood appears to be a red liquid to the naked eye, but under a microscope you can see that it contains several elements. The plasma is the liquid part of blood, and is actually colorless. The red blood cells give blood its red color. White blood cells are interspersed in the sea of red blood cells and help fight infection. The smaller platelets are fragments of red blood cells and function in clotting.


You need:

- ✔ A glass slide with coverslip
- ✔ A pin
- ✔ Blood

Sterilize the pin by running it through a flame. Poke your little finger, squeeze it, place a drop of blood on a slide and cover with the coverslip. Gently wipe the edge of the coverslip over the blood and produce a smear. Now cover with the coverslip. Place the slide on the microscope stage and increase magnification until you see the components of blood:

- ✔ Plasma
- ✔ Red blood cells
- ✔ White blood cells
- ✔ Platelets

Due to safety issues people from laboratories normally don't use their own blood. But maybe you can find a volunteer?

## Yogurt Bacteria

Yogurt is produced by bacterial fermentation of milk. Such bacteria are beneficial, keeping us healthy and helping us to digest our food. Why not have a look to see if some of these yogurt cultures are visible?

It's quite amazing! Did you know that there are 10 times more bacteria in our guts than cells in our entire body?


You need:

- ✔ Yogurt with active cultures
- ✔ A glass slide with coverslip
- ✔ A Q-tip or toothpick
- ✔ Methylene blue (optional)
- ✔ Gloves (optional)

You only need a small amount of yogurt, so enjoy the rest. Smear a tiny probe with the toothpick on the slide. If you have methylene blue available, place a small drop of the solution on the slide. Wear gloves and do not allow children to handle methylene blue solution because of its toxicity. Place the coverslip on the slide and have a look with your microscope using the lowest magnification first. Bacteria will appear small even at the highest magnification, and you can see some of the typical species used for yoghurt cultures:

- ✔ *Lactobacillus delbrueckii* (rod shaped)
- ✔ *Lactobacillus acidophilus* or *casei*
- ✔ *Streptococcus salivarius* (sphere shaped)
- ✔ *Bifidobacterium bifidus* (rod shaped, often branched bacteria)

## Pond Water Habitats

Looking for a nice swim? Be aware that there are a lot of little fellows living in ponds and puddles. The dirtier the water appears, the more fascinating it becomes under the microscope. You can determine the water quality of a lake by having a look at the planktonic organisms present.


You need:

- ✔ A glass jar or similar
- ✔ A Petri dish or glass slide with coverslip
- ✔ A dropper

Take your glass jars and make a little excursion to the next pond or puddle to collect some water with some mud and wrotten leaves. If there are no ponds nearby, water from the banks of a lake, river or stream will suffice. You need to settle the dirt to the bottom before you use the dropper to take a sample. Drop some liquid into your petri dish or on the glass slide. By the way – if you have a concave slide it's much easier to hold the water in place. Carefully place the coverslip on the slide to avoid harming any organisms. Have a look with your microscope using the lowest magnification first. Do you see anything? Be patient, as some creatures may take a while to calm down and hold still. You will find a lot of planktonic microorganisms including:

- ✔ Bacteria
- ✔ Algae
- ✔ Protozoa (which are mostly larger than bacteria)
- ✔ Small animals like arthropods
- ✔ Hydras (small animals with tentacles)

## Yeast Cells

Yeasts are fungi that are used to make bread, beer and wine by fermentation. Bread yeast is a nice sample to study cellular processes. The yeast uses sugars in the flour or from cereals, producing alcohol ethanol and bubbles of the gas carbon dioxide. In case of bread the gas makes the dough rise.


You need:

- ✔ A glass jar or bowl
- ✔ Warm water
- ✔ Active yeast

- ✔ Sugar
- ✔ A Petri dish or glass slide with coverslip
- ✔ A dropper or pipette

Put the yeast together with warm water and two tablespoons of sugar in a bowl and stir it. Let the solution sit for 45 minutes. Place a drop of this mixture on a glass slide, cover it with the coverslip and observe it with your microscope. To visualize yeast cells, you need higher magnifications. What you can see is:

- ✔ Yeast cells (which are more or less round-shaped)
- ✔ Reproducing (budding) yeast cells
- ✔ Gas bubbles (carbon dioxide) that yeasts produce during the fermentation of sugar

## Potato Starch

Potato starch grains are birefringent. To visualize the structures, you need a light microscope that is equipped with polarization contrast and/or darkfield illumination.


You need:

- ✔ A potato
- ✔ Water
- ✔ A glass slide with coverslip
- ✔ A dropper or pipette
- ✔ A knife

Cut the potato and scrape a little amount on the glass slide. Try to avoid large pieces of potato, the thinner the better. Drop a bit of water over your smeared potato and cover it with the coverslip. You can see the oval starch grains best in darkfield and/or with polarized light. You see:

- ✔ Starch grains with a cross-like appearance in polarized light or with grain walls in darkfield
- ✔ Eccentric layering of the grains, which is typical for potato starch

# Find the Needle in the Haystack

Now, let's enrich microorganisms such as ciliates by making a hay infusion. The bacterial concentration can become very high. You can recognize a high amount of bacteria when your hay infusion looks somewhat cloudy.


You need:

- ✔ Hay
- ✔ Pond water
- ✔ A petri dish or glass slide with coverslip
- ✔ A dropper
- ✔ Milk
- ✔ A glass bowl or beaker

Cut the hay into smaller pieces, place it in the bowl and add pond water. Adding 2 drops of milk to feed the bacteria inside and animate them to reproduce. The ciliates feed on the bacteria and reproduce, too. Let this mixture stay for several days or weeks. The infusion will turn turbid over the next couple of days, which is a sign of microorganism growth. Drop some liquid from the surface of the water for microscopic investigation into your petri dish or onto the glass slide. Carefully place the coverslip on the slide to avoid harming any organisms. Have a look with your microscope using the lowest magnification first. What you can find is a large variety of protists:

- ✔ Flagellates
- ✔ Ciliates
- ✔ Amoebae
- ✔ Heliozoa (»sun animalcules«, due to the arms radiating from the body)

The bacterial concentration will be very high and you do not know which bacteria or protists you are growing. Disease-causing organisms can also proliferate in a hay infusion. It is essential to ensure strict hygiene when handling the infusion; for example, wash hands thoroughly after handling the samples.

IN THIS CHAPTER

How to align the illumination according to Köhler

# Chapter 15
# Ten Steps to Set up Köhler Illumination

August Köhler introduces this concept in 1893. This process brings illumination and imaging beam paths into a defined, aligned state, providing homogeneous illumination without stray light.

Here's how to set up Köhler illumination on your upright transmitted light microscope:

1. Choose the 10× objective as it is the easiest to start with.
2. Set the condenser to brightfield position.
3. Move the condenser to its top position.
4. Fully open both the luminous field stop and aperture stop.
5. Bring your specimen into focus.
6. Close the luminous field stop until you see it in the field of view.
7. Focus the luminous field stop image by lowering the condenser.
8. Use the condenser centering screws to center the luminous field stop image.
9. Open the luminous field stop until its image disappears, just outside the field of view.
10. Remove an eyepiece and look into the tube. Close the condenser aperture stop diaphragm until the visible objective opening is illuminated by at least two-thirds of its diameter, then replace the eyepiece.

IN THIS CHAPTER

- Why the right cleaning is important
- Dirt is not equal to dirt
- How to clean in a proper way

# Chapter 16
# Ten Tips and Tricks to Handle and Clean Your Microscope

A microscope often represents a significant investment, so you'll want to take good care of it. They are sophisticated optical instruments that require periodic maintenance and cleaning to guarantee successful microscopy and perfect images. Over the years, a variety of cleaning procedures have been recommended. The choice of the best cleaning method depends on the nature of the optical surface and the type of substance to be removed.

## Handle with Care

Your microscope is best kept in a quiet workplace, not too small and vibration-free. Try to avoid a place directly in front of the window. The incoming light may lead to stray light and badly influence your imaging results. If you need to carry your microscope, you should always use two hands. Do not grab the objective in the nosepiece turret to have a better grip. Carry it carefully, ensuring that you do not knock it against anything. Even if you have a solid, sturdy-looking instrument, it contains complex optics from diverse lenses, which hate shaking. When you have finished your work, switch the microscope off and cover it with a dust cover. Dust is an enemy to your lenses!

## The Effect of Dirt on the Image

Some optical surfaces are more sensitive to dirt than others. In particular, the objective lenses are critical, but anything close to the object or to a camera sensor has a great impact on the image quality. You can easily recognize dirt on optical surfaces if you have an idea of the best result you can expect from your application. If you compare your expectation with the given image, you will immediately recognize whether or not your microscope is soiled anywhere. If the sharpness or contrast of the image is less than optimum, there is a high probability that your microscope optics are not clean.

## How to Locate Dirt

If you have the impression that you have some dirt in your microscope you can proceed with the following steps as an easy check:

- ✔ Carefully rotate the objectives by a small amount within their thread.
- ✔ Check the glass slide and coverslip by moving the specimen.
- ✔ Check the condenser by moving it up and down and, if possible, slightly swiveling or turning the front lens.

The affected optical surface is identified when the dirt follows the movement of the suspected optical component. The camera is the only exception to this rule: dirt on the protection glass of the camera sensor will not move when you move the camera.

## Types of Soiling

You may say that dirt is dirt – but that would be too simple. To decide on the cleaning method, you need to know what you want to wipe away. There can be dust, glass particles from broken sample slides, tiny parts of your skin, clothing fibers, pollen, liquid or dried-up embedding or immersion media, culture solutions residue from improper cleaning attempts, fingerprints, grease. As you can see, this is a long list.

## How to Avoid Dirt

First rule to keep your microscope clean is to use it with clean hands only. Avoiding eating and drinking is not just a critical thing to do in safety laboratories, but it also helps to avoid a dirty microscope. Always keep your microscope covered with the dust cover when it is not in use.

## Things You Need to Clean your Microscope

The goal is the complete removal of dust and dirt without leaving any residue of the cleaning agent and without damaging the surfaces. You need the following utensils and agents:

- ✔ Long, thin wooden sticks, preferably of bamboo. Go out for a Chinese dinner and take the chopsticks home with you.
- ✔ High-purity cotton
- ✔ Absorbent polyester swabs for optical cleaning, or cotton swabs.
- ✔ Soft facial tissues
- ✔ Microfiber cloth
- ✔ Dust blower
- ✔ Distilled water
- ✔ Diluted dishwashing liquid
- ✔ Solvent for the removal of greasy or oily dirt such as analytical grade n-hexane

Cleaner‘s naphtha, white spirit, petroleum ether and others are trivial names for petroleum fractions containing n-hexane.

- ✔ Only for coverslips: pure acetone.

## And now – Cleaning Procedure Time

- ✔ Blow all loose dust particles away with a dust blower.
- ✔ The painted surfaces of microscopes can be cleaned with a microfiber cloth.
- ✔ Remove all water-soluble dirt with distilled water. If this is not successful, repeat using diluted dishwashing liquid.
- ✔ Remove any remaining residue with a dry cotton swab.
- ✔ To remove oily dirt, first use diluted dishwashing liquid. If the result is not satisfactory, repeat using a solvent.
- ✔ Greasy dirt must always be removed with a solvent.
- ✔ Place the objectives, eyepieces and cameras on a dust-free surface.

## Cleaning Optical Components

To clean the optical components, you need to remove them from the microscope and place them on a dust-free surface. These are the eyepieces, the objective lenses and the microscope camera. To clean, dip the cotton swab into the cleaning solution and shake off excess liquid. Move the utensil in a spiral motion from the center to the rim of your optical surface. Never wipe in a zigzag pattern, as this will merely spread the dirt.

Not all solvents can be recommended for cleaning microscope optics. Among those that clean very efficiently, some are toxic, e.g. chloroform or acetone. Acetone can be recommended when oil and grease are to be removed from coverslips only. Acetone attacks most types of plastic as well as rubber. Other solvents are unfriendly to the environment, still others will leave residues on the surface.

## It's Not All About Dirt

A blurred image may not always be due to dirt. If you use an objective with a large numerical aperture in conjunction with a coverslip of the wrong thickness you can get blurred images. Dry objectives of this type normally have a correction collar, which permits compensation for spherical aberration. Many highly corrected

immersion objectives also require specially selected coverslips of a thickness of 0.17 mm, if the best image is required. Immersion objectives should only be used in conjunction with a suitable, bubble-free immersion liquid.

## Five Goodies for Optimum Results

Optimum results in microscopy depend not only on the cleanness of the microscope optics, but also on perfect specimen preparation:

- ✔ Thickness of the specimen
- ✔ Staining method and intensity
- ✔ Refractive index and dispersion of the embedding or immersion medium
- ✔ In high-resolution microscopy: close distance of a living cell from the cover slip
- ✔ Correct coverslip thickness (for example 0.17 ± 0.01 mm) to avoid spherical aberration