

Darkfield Microscopy for Native-Blood Analysis

Technical Background

Introduction

What makes a darkfield microscope a truly suitable diagnostic instrument? Which parameters are decisive? Hardly any other microscope approaches the physical limits as close as the darkfield microscope for native-blood analysis. The experienced user expects a large magnification and high resolution, high image contrast and ease of use. In addition, not only shapes, numbers and arrangement of the blood cells must be discernible, but also certain structures in the blood plasma that help find a comprehensive diagnosis.

This article is intended to give users of darkfield microscopes a guideline to understand the basic optical principles of the instrument, and also to assist the alternative practitioner in the decision which microscope to buy. But do not fear: The explanations in this paper are limited to an absolute minimum of physical/technical terms. First, we explain all parameters that are of basic importance for the imaging quality of a microscope. Then, we describe the peculiarities of darkfield microscopy and demonstrate achievable image qualities by means of examples.

A little bit of theory: Basics of microscope optics

A microscope is an optical instrument with an extremely large optical magnification which makes – in the true sense of the word – microscopically small objects observable for the human eye. The object is imaged in a two-step process via objective and eyepiece: The objective produces the so-called intermediate image of the object. According to international standards, the microscope objectives are designed to project this image to a plane situated 10 mm below the upper edge of the eyepiece tubes. With a ground-glass screen in that plane, the intermediate image could be observed, it is, as the optician calls it, a real image. Its diameter measures, e. g. 18 mm and is denoted the field number (FN). The intermediate image is then further magnified with the eyepiece like with a magnifying glass and observed with the eye. The total magnification M_{tot} of the microscope results from the product of objective magnification M_{obj} and eyepiece magnification M_{eye} :

$$M_{tot} = M_{obj} \cdot M_{eye}$$

Example: With a 10x eyepiece and a 40x objective, the total magnification amounts to $10 \cdot 40 = 400$.

In addition to the magnification, the numerical aperture (NA) is the decisive parameter for the quality of the microscopic image. The numerical aperture describes the light collection efficiency of an objective, and the corresponding mathematical expression contains (half) the aperture angle of the light cone that the objective can receive (Fig. 1):

$$NA = n \cdot \sin\alpha$$

The symbol n is the refractive index of the medium between specimen and objective. As the sine cannot exceed a value of 1, the maximum for the NA in air ($n = 1$) also amounts to 1. This value, however, cannot be reached because then, the marginal rays of the light cone would propagate at right angles to the optical axis ($\alpha = 90^\circ$).

Example: With air between objective and specimen ($n = 1$), and with an aperture angle of the light cone of 60° , i. e. $\alpha = 30^\circ$, $NA = 0.5$.

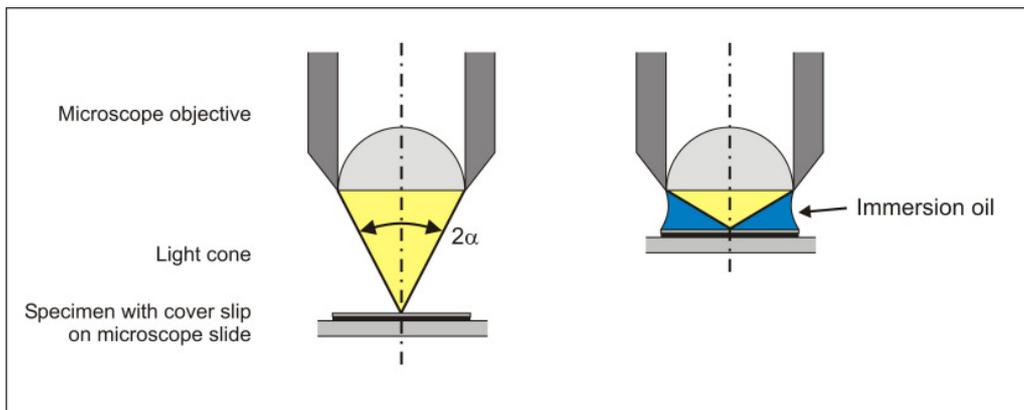


Fig. 1: The numerical aperture describes the light cone that an objective can receive. Increasing apertures (right) result in increasing resolutions. High numerical apertures demand the use of immersion oil.

Basically, the objective can receive more light when the numerical aperture increases. High numerical apertures, however, implicate one problem: When the angle of incidence into the objective becomes too large, strongly inclined marginal rays will experience total reflexion on the boundary surface of the first objective lens. This light is then completely reflected out of the beam path and will thus not contribute to the microscopic image. This is prevented by bringing a medium with an index of refraction larger than 1 between objective and specimen. Then, the refractive index difference is smaller, and the marginal rays can propagate into the optical system. This procedure is called 'immersion' of the objective, which is usually carried out with immersion oil with an index of refraction of $n \approx 1.5$.

Example: With immersion oil between objective and specimen, $n = 1.5$, a numerical aperture of $NA = 1.25$ corresponds to an angle α of 56° , so that the light cone has a geometrical aperture of 112° .

The larger the light cone is that forms the microscopic image, the more information is contained in it. Thus, the resolution of the image increases with increasing NA of the objective. In addition, the condenser determines the properties of the light delivered by the microscope light source. As the condenser forms the light cone that is received by the objective, its numerical aperture will also determine the resolving power of the microscope. The well-known formula for this resolving power describes the smallest distance Δx between two objects points that still can be resolved, i. e. in which they can be observed as two separated points. If λ is the median wavelength of the light source, NA_{Obj} and NA_{Cond} the numerical apertures of objective and condenser, respectively, we get:

$$\Delta x = \lambda / (NA_{Obj} + NA_{Cond})$$

This is the famous formula for a microscope's resolving power as found by Ernst Abbe (1840 - 1905). It is with this formula that industrial mass production of microscopes had become possible. Like with the objective, the condenser can reach numerical apertures > 1 only when immersed with oil.

Example: With (white) light from a tungsten halogen lamp, the median wavelength is about $\lambda = 0.55 \mu\text{m}$. With a 100x objective with $NA_{Obj} = 1.25$ and with a (darkfield) condenser with $NA_{Cond} = 1.4$, $\Delta x = 0.21 \mu\text{m}$.

But how do these properties depend on each other? Would it, e. g., be possible to observe more object detail by simply increasing the total magnification? This situation is comparable with a TV monitor: its image is composed of single picture elements (pixels). Their structure is fine enough not to disturb the image when observed from some distance. With decreasing distance to the screen, more image detail becomes visible, but below some distance, the pixel structure will completely disturb the image impression. The visible details increase in size, but not more detail becomes visible. For a microscope, this means that magnifications larger than the so-called 'useful' magnification will not lead to a higher image resolution. These magnifications are so-called 'empty' magnifications.

There is a rule of thumb for the useful magnification range of a microscope. With NA_{Obj} once again as the objective's numerical aperture, this range, M_{use} , is:

$$M_{use} = (500 \dots 1000) \cdot NA_{Obj}$$

Example: For a 100x objective with $NA_{Obj} = 1.25$, the maximal useful magnification amounts to $1000 \cdot 1.25 = 1250$. The maximal magnification of the

eyepieces must then not exceed $1250 / 100 = 12.5$ when the total magnification shall remain in the useful magnification range.

Some manufacturers offer microscopes with total magnifications of up to 18.000. These magnifications can only be empty and are achieved by, e. g., displaying the microscopic image on a large monitor.

The above-mentioned parameters can be found – among others – as printed markings on the barrel of a microscope objective (Fig. 2).



Fig. 2: Planachromatic objective set.

The first term denotes the optical correction. Usually, all objectives are achromats which means that chromatic aberrations between the red and the blue parts of the optical spectrum are optically corrected for. When even the green part of the spectrum is incorporated in this correction, the objectives are apochromatic. These (very expensive) objectives guarantee high color fidelity of microscopic images. The imprint 'Plan' means that the image field curvature is corrected to yield an image that is flat over its entire diameter. Achromats usually produce a sharp image across a limited area around the center of the field while its edges are blurred. Although this may seem a disadvantage, the microscopist often observes interesting specimen sections in the center of the field so that achromats are often sufficient. When a completely sharp image is desired, additional optical elements are required and result in so-called planachromatic objectives. These are usually more expensive than achromats with comparable optical parameters. In addition, there are also semiplan-achromatic objectives. Here, the area of maximal sharpness is larger than for achromats, but smaller than for planachromats.

The following number describes the magnification of the objective. It is also coded by a ring whose color is standardized – like the entire marking of the objective - according to DIN ISO 8578. The number behind the slash is the numerical aperture. In the bottom line, the first number is the mechanical tube length which, according to DIN ISO 9345-1, amounts to 160 mm. This is the distance between the mounting surface of the objectives at the nosepiece and the mounting surface of the eyepieces at the top of the eyepiece tubes.

DIN ISO 9345-2 also describes microscopes with infinite tube lengths. They are marked with the symbol „∞“. In their tubes, the light rays propagate (almost) in parallel so that the intermediate image forms (almost) in an infinite image distance. In order to observe it with the eyepieces, however, the image must be transferred back from infinity with a tube lens. One advantage of infinity optics is its insensitivity against additional optical elements in the beam path, the image quality, however, is comparable to that of a microscope with 160 mm tube length. The figure behind the tube length is the cover-slip correction. Specimens are usually covered with glass slips with thicknesses of 0.17 mm. These cover slips must be included into the optical design to avoid optical distortions. The influence of the cover slips increases with increasing numerical apertures, i. e. the steeper the light rays pass the specimen. While 4x and 10x objectives may also be used without cover slips, objectives with higher magnifications require special optical designs when they shall be used without cover slips.

Contrasting techniques: From brightfield to darkfield

As in all imaging techniques, the illumination of the specimen plays an important role in the formation of the microscopic image. For every particular kind of specimen, a dedicated contrasting technique is required by which the instrument yields optimal image contrast.

Transmitted-light brightfield microscopy closely resembles the way that a slide is illuminated in a projector: Different specimen areas attenuate the light differently and, in addition, absorb different spectral ranges. This, however, requires specimens with sufficient absorbance which is in particular quite difficult when blood cells shall be observed: It would be necessary to apply – usually toxic – staining reagents, which would regularly lead to the death of the cells under investigation. This method is thus basically unsuitable for native specimens.

One idea to increase the visibility of structures of low contrast is phase-contrast microscope, as invented by Frits Zernike in 1930. The microscope contains additional optical components that transform the different refractive indices of cells and surrounding medium into brightness contrasts.

Highest contrast, however, is achieved with a darkfield microscope. This technique utilizes dedicated condensers that prevent the light that has passed the specimen from propagating to the objective directly. The light that comes from the condenser has the form of a hollow cone. Without a specimen, nothing can be observed through the eyepieces, the image field is – in the true sense of the word – dark. The optical construction of the condenser therefore has to direct light towards the specimen in an extremely flat angle. For objective magnifications of up to 40x, this can be realized with an annular diaphragm in the rear focal plane of the condenser (Fig. 3). For highest apertures ($NA > 1$), darkfield condensers are usually designed as mirror systems, so-called cardioid-type condensers, according to their geometrical form (Fig. 3). These condensers can thus only be used for darkfield microscopy, brightfield observations are impossible. A cardioid-type condenser can reach numerical apertures of as high as $NA = 1.4$.

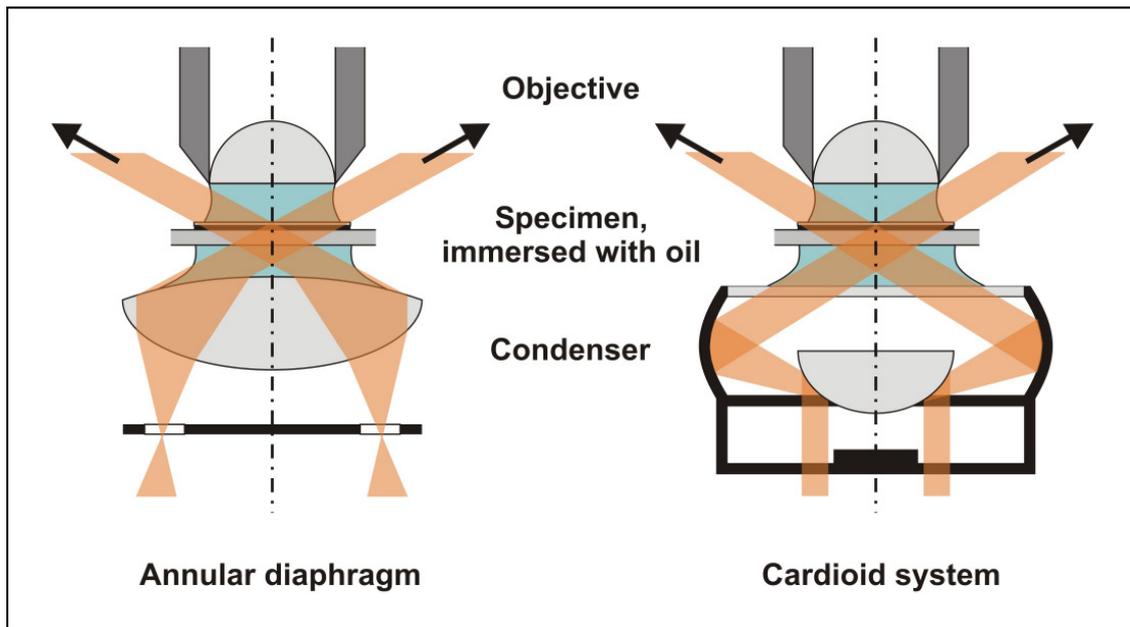


Fig. 3: Darkfield condenser with annular diaphragm (left) and cardioid-type condenser (right).

Every object within the specimen directs light to the objective through the mechanisms of refraction, scattering and diffraction. Thus, only object structures that differ from a flat plane become visible, e. g. edges, angles, borders etc.

Even the objective must meet special demands. As the contrasting technique relies on the observation of non-direct light, the NA of the objective must be smaller than that of the condenser. This makes it necessary to equip the objective with an iris diaphragm that allows an optimal alignment of the darkfield image (Fig. 4). Upon closing it, the scattered-light background fades away, and the image contrast increases.



Fig. 4: Objective Plan 100/1.25 – 0.60 Oil with iris diaphragm.

It must be pointed out that all low-priced darkfield microscopes come with condensers with numerical apertures of not more than $NA = 1.25$. Thus, it is only as high as the NA of the objective, usually a 100/1.25 – 0.60 Oil with iris diaphragm. This has the consequence that the NA of the objective must strongly be reduced to yield sufficient image contrast. This, however, wastes the greatest part of the resolving power of this high- NA objective – fine structures in the blood plasma can hardly be observed. It shall also be noted that one microscope manufacturer even offers a darkfield microscope with a 100x objective completely without iris diaphragm. Allegedly, this would make the handling of the microscope easier and ensures high image quality. With the technical background described above, this statement is doubtful – to say the least...

Comparison: An oil-immersed condenser with $NA = 1.25$ emits a light cone with a total geometrical aperture of 112° . In comparison, with a condenser with $NA = 1.4$, the geometrical aperture already amounts to 138° .

Figure 5 shows a darkfield microscope with dedicated equipment for native-blood analyses. The optical system contains the described cardioid-type darkfield condenser with $NA = 1.4$ and two objectives: an achromat 10/0.25 serves as centering aid for the condenser, the Plan 100/1.25 – 0.60 Oil for the diagnostic observation of the native-blood specimen. An eyepiece pair with 10x magnification contributes to the total magnification of 1000x.

Another important technical detail is the cold-light source which directs the complete visible part of the spectrum of a 100-W tungsten halogen lamp via the collector to the condenser, but absorbs the infrared part of the spectrum. Thus, the specimen does not heat up and can be observed over a long time. The illumination beam path contains a variable grey filter with which the light intensity can be attenuated without changing the color temperature of the light source.



Fig. 5: Darkfield microscope Hund H 600 LL HP 100.

In addition, the observation tube of the depicted microscope has a camera port so that the practitioner can also show the microscopic image with ease to the patient via a connected camera.

The result: darkfield images of native-blood specimens

After the native-blood specimen is ready to be examined, the condenser should be centered according to the manual by observing the specimen through the 10x objective. Afterwards, a drop of oil is applied to the specimen's cover slip, the 100x objective is rotated into the beam path and brought in contact with the immersion oil (Fig. 4). After a suitable section of the specimen is chosen and focused, the objective's iris diaphragm is closed far enough to yield optimal contrast between bright structures and dark background.

Figure 6 shows a darkfield image obtained with a properly aligned microscope of the type shown in Fig. 5. Figure 7 shows another section of the same specimen. It was taken with the same (video) camera system, but with a darkfield microscope the condenser of which has an NA of only 1.2. This microscope also has the light from a cold-light source fed directly into the condenser via a curved light guide.

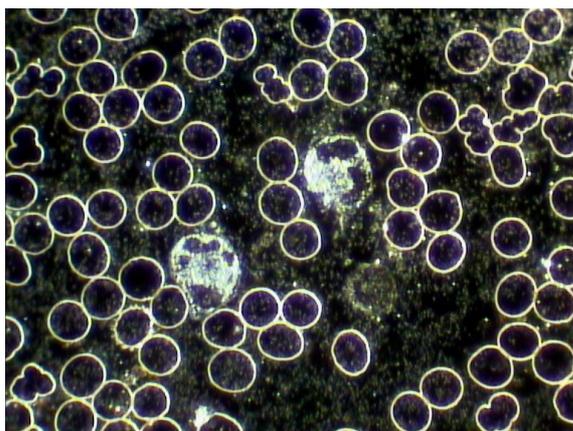


Fig. 6: Native-blood specimen, darkfield image. Objective: SPL 100/1.25 – 0.60 Oil. Microscope: Hund H 600 LL HP 100.

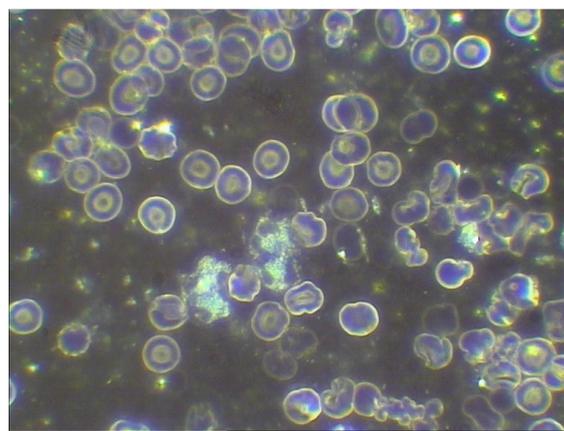


Fig. 7: Native-blood specimen, darkfield image. Objective: SPL 100/1.25 – 0.60 Oil. Darkfield microscope with condenser NA = 1.2.

The image of the second microscope with the smaller condenser aperture is significantly poorer in contrast. The areas between the blood cells are not truly dark, and a slight haze covers the entire image. Thus, the overall contrast is significantly smaller than in Fig. 6, and it is almost impossible to observe fine image structures.

Figure 8 shows a set of microscopic images taken with the same microscope as in Fig. 7, but with the objective's iris diaphragm closed in several steps. This experiment was intended to find the ideal alignment for darkfield.

It showed very quickly that upon closing the iris diaphragm further, the image contrast increased due to the more and more effective blocking of disturbing stray light. On the other hand, however, the overall image brightness decreased rapidly. The third image thus reveals a higher noise level, the fourth a significant decrease of the brightness in image structures which the camera cannot compensate any more. In addition, none of these images reaches the resolution of Fig. 6 which was taken with a condenser of higher aperture and with the Hund darkfield microscope.

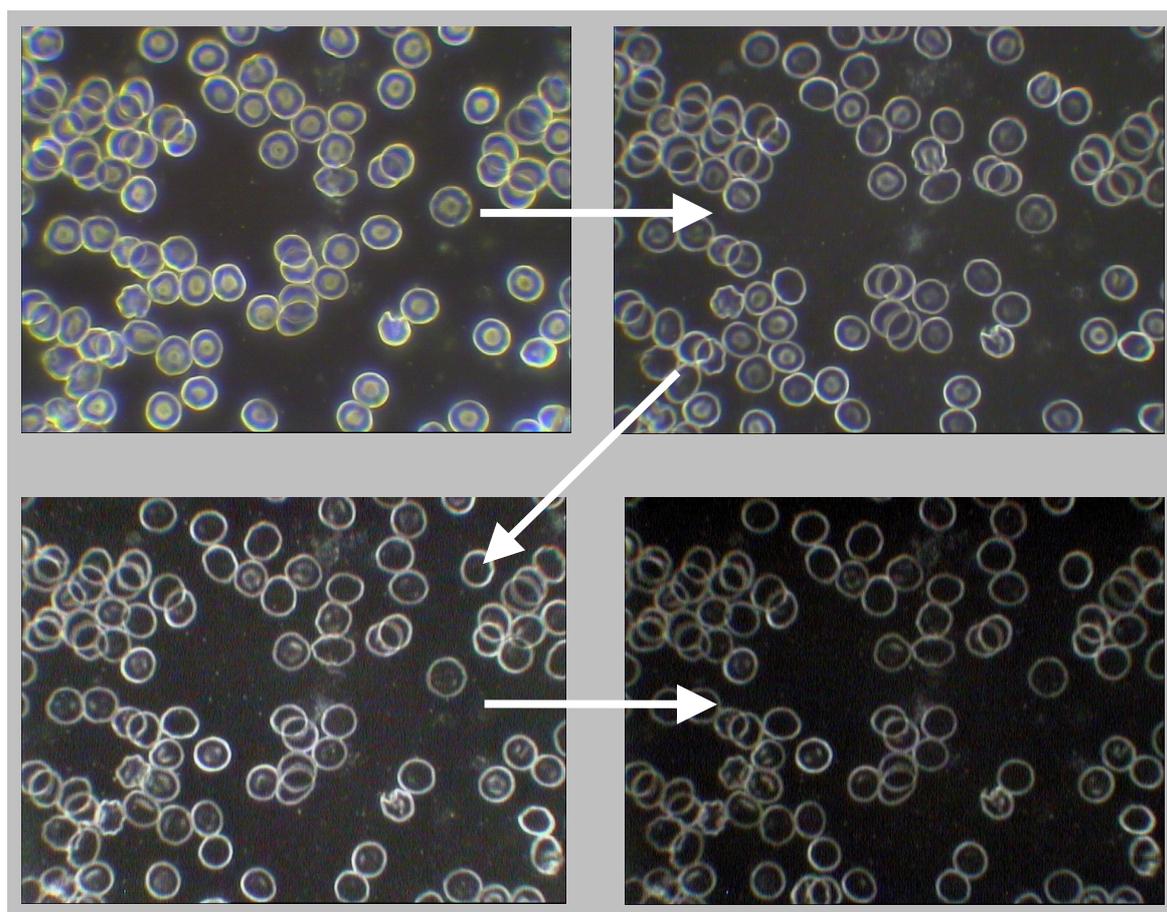


Fig. 8: As in Fig. 7, but with iris diaphragm closed between consecutive images.

Summary: High optical quality = good darkfield!

There are only few parameters that determine the imaging quality of a microscope. The most central of these is the numerical aperture that not only determines the accessible amount of light, but also the resolving power. Even minute differences in the apertures lead to large differences in the geometrical apertures of the light cones that leave the condenser and enter the objective. The combination of contrasting technique, magnification and numerical aperture determines if a microscope is suitable for a certain application or not. A darkfield microscope always requires high condenser and objective apertures in order to yield maximal contrast at highest resolution in native-blood analyses.

To illustrate these interrelations, two darkfield microscopes with different condenser apertures and illumination concepts were compared. Although the difference in the numerical apertures does not seem very large, the microscopic images show significant disparity. It is quite obvious that the manufacturing costs of the low-priced microscopes do not allow the design of an instrument that gives the practitioner a high-contrast, high-resolution image of a native-blood specimen. The images are of poor contrast, and by attempting to close the objective's iris diaphragm further, resolving power gets lost.

It is true that a practitioner 'observes something' with both instruments. The practitioner should, however, take care that everything which is of decisive importance for

a safe diagnosis can be observed – to the good of the patient. High optical quality, with specifications appropriate for the respective application, are still the key factor for this.

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