

Microscopy : Fundamentals

HERVÉ SAUER- JOËLLE SURREL

Table des matières

I. Présentation	3
II. Lesson	4
1. Microscope structure.....	4
1.1. Microscope structure.....	4
2. Illumination.....	6
2.1. Role and properties.....	6
2.2. Köhler illumination.....	6
3. Imaging properties of a microscope.....	8
3.1. Constraints while imaging with very high numerical aperture objectives.....	8
3.2. Illumination coherence and resolution limit.....	14
3.3. Other properties and characteristics of a microscope.....	16
4. Supplement.....	17
4.1. Supplement 1.....	17
4.2. Supplement 2.....	18
III. Case study	20
1. Implementation of a transmitted light microscope.....	20
1.1. Adjustment of the microscope for visual observations.....	20
1.2. Determining several microscope characteristics.....	24
2. Utilization of a metallographic microscope.....	29
2.1. Presentation.....	29
2.2. Adjustments.....	30
2.3. Resolution limit.....	33
3. Digital photomicrography.....	34
3.1. Digital Photomicrography.....	34
IV. Exercises	35
1. Exercises.....	35
Solution des exercices	38
Glossaire	42
Bibliographie	43
Webographie	44

I.Présentation

Module :

Instrumental optics, microscopy

Auteur(s) :

Hervé SAUER¹&Joëlle SURREL² - Institut Optique Graduate School & IUT St Étienne

Résumé :

Presentation of the structure of the optical microscope (objective, eyepiece, pupil and field diaphragm) and the specificities of image formation under high numerical aperture. Köhler illumination is also detailed.

Mots-clés :

Microscopy, Köhler illumination, High numerical aperture imaging

Pré-requis :

Geometric optics. (Knowledge of physical optics on the resolution limit of lenses can be useful, but is not absolutely essential)

Objectif(s) pédagogique(s) :

To provide an understanding of the practical operation of a microscope and to enable students who have completed this module to knowingly handle a simple modern microscope (understanding the marking of the objectives, choosing them according to the preparation to be observed, basic settings of the Köhler lighting and the microscope, etc.)

Plan du cours :

- Introduction
- Structure of the microscope
- Lighting
- Imaging properties of the microscope
- Supplements
- Conclusion

Conception & production :

Le Mans Université

Licence :

Licence GNU³

1 - herve.sauer@institutoptique.fr

2 - joelle.surrel@univ-st-etienne.fr

3 - <http://www.gnu.org/licenses/fdl.txt>

II. Lesson

As indicated by its etymology (micro- [*μικρός* *small*] and -scope [*σκοπεῖν* *observe*]), a **microscope** is an instrument allowing a visual observation of very tiny objects or object details located in a close vicinity of the observer, that would be indistinguishable to the naked eye even when placed at the punctum remotum. Hence, similarly to a magnifying glass, an essential property of a microscope is its **magnification**, which represents its aptitude to deliver an image angularly magnified of the observed object. However, this parameter is not sufficient to fully characterize the instrument performances. Indeed, this property has to apply to all objects, including the smallest ones. Therefore, the second key property of a microscope is its separating or resolving power². In practice, this latter point has very important consequences, since it requires work with **diffraction-limited optics of very large numerical apertures** (*i.e. without aberrations*).

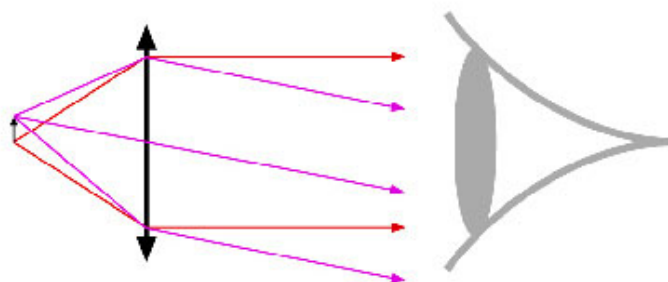
Even though *optical microscopy* is a very ancient science that took off in the XVIth century, it still remains an advanced technology, widely used in numerous research and industry areas, with a lot of technical applications such as the extension to the UV range, or the availability of digital photomicrography easy-to-use systems.

This first unit of the microscopy course aims at presenting in details the basic concepts of instrumental optics (geometrical optics and diffraction), on which are based all microscopy systems. A second unit more specifically presents the numerous techniques dedicated to improve the contrast of the observed objects.

1. Microscope structure

1.1. Microscope structure

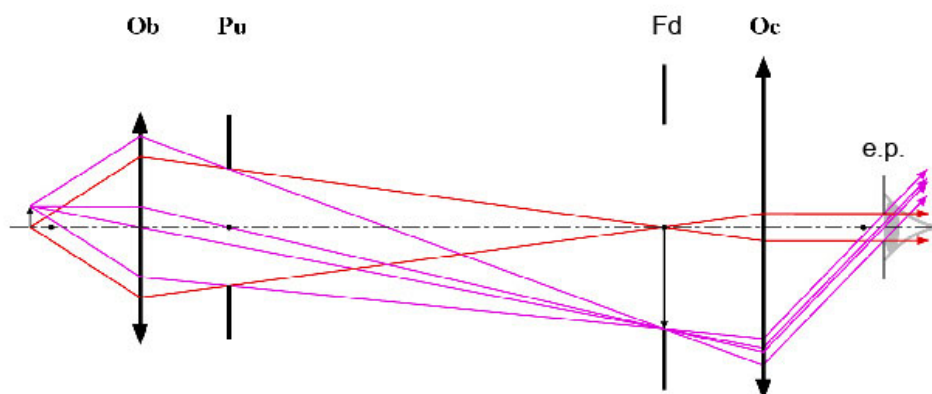
As a tool to visually observe small objects located very near the optical system, a microscope must transform a real object located at a finite distance of the system into a virtual image, located at infinity for a perfect eye, and with an angular size larger than the one seen by the naked eye when the object is placed at the punctum proximum. The simplest system verifying those criteria is represented on figure 1. It is composed of a unique thin lens whose focal plane coincides with the observed object. With this configuration, the magnification is larger than 1 if the lens focal length is shorter than the distance object-eye when the object is located at the punctum proximum, and, in fact, the shorter the focal lens and the larger the magnification. The **magnifying glass** works on this principle.



This system can be improved by replacing the simple lens by a singlet or a doublet with aspherical surfaces. But even with this improvement, it will exhibit strong field limitations due to the field aberrations that can't be corrected, and to the vignetting introduced by the eye pupil which is the system field diaphragm. In practice, the magnification and numerical aperture that can be used remain rather small.

One solution to those problems is shown on figure 2. This compound system, composed of an objective_o and an ocular_e, allows resolving the problem of pupils conjugations (the eye pupil vignetting is suppressed, since the eye pupil can be placed at the exit pupil of the microscope), and of field aberrations (the full system is composed of many lenses to compensate for those aberrations). This structure has been universally used in microscopy for more than a century.

Let's describe more precisely how this works. The objective 'Ob' transforms the object into a real and magnified image. This intermediate image is in turn transformed by the eyepiece or ocular lens 'Oc' into a virtual image located at infinity and finally observed by the eye. The magnifying power of the entire microscope is the product of the transverse linear *magnification of the objective* and the angular *magnification of the eyepiece*. The eye pupil must imperatively be placed at the instrument exit pupil, called eyepoint or Ramsden disc_e, to avoid any limitation of the field of view by the iris. Consequently, the distance from this exit pupil to the last surface of the eyepiece, called "eye relief_e", must be at least equal to a minimum value allowing for a comfortable observation (15 to 20 mm for a naked eye, 20 to 25 mm if the user is wearing eyeglasses). The eyepoint is the image by the ocular of the objective exit pupil. The objective exit pupil is defined by the aperture 'Pu' and is always located in the back focal plane of the objective. This aperture, the objective pupil, is also the microscope global pupil, and by definition controls the amount of light that penetrates through the microscope and therefore the object-side numerical aperture of the system. Consequently, it is sometimes called "aperture diaphragm". It is interesting to note that the objective entrance pupil is located at infinity (**object-space telecentric** system); this is a very important property of all microscope objectives, which makes every point in the object field equivalent in terms of the cone of rays used to make the image. This is also a very important point when analyzing the microscope associated with its illumination (see the « Illumination » section). Finally, the diaphragm 'Fd' (called 'field diaphragm'), located in the intermediate space at the eyepiece front focal plane (for an observer with an optically perfect eye), limits the field of view in a clearcut fashion.



2. Illumination

2.1. Role and properties

An object can be properly observed only when properly illuminated.

The properties of a good illumination system are the following:

- Uniformity (to observe the object and not the illumination inhomogeneities)
- An illumination area limited to the microscope field of view {To avoid parasitic light}
- A good control of the numerical aperture (or in other words of the partial coherence of the illumination) {see the subsection « Illumination coherence and resolution limit »}

The illumination system must also be able to work in two very different observation modes:

- In the first mode, mostly transparent objects are illuminated in **transmission**; we speak of *transmitted* (diascopic[☺]) light microscopy (from the Greek 'dia-', through).
- In the second mode, opaque objects are illuminated by reflection, in general through the observation objective which plays a double role; we speak of *reflected* (episcopic[☺]) light microscopy (from the Greek 'épi-', above).

Those conditions can be fulfilled using various methods, that differ from each other in terms of simplicity and degrees of perfection. The most efficient technique, universally used on professional microscopes, is called Köhler illumination [[Ein neues Beleuchtungsverfahren für mikrophotographische Zwecke]]. This method is presented in the next paragraph in the case of transmitted light microscopy. Its implementation for reflected light microscopy is shown on Figure C2, in the supplements at the end of the course sequence.

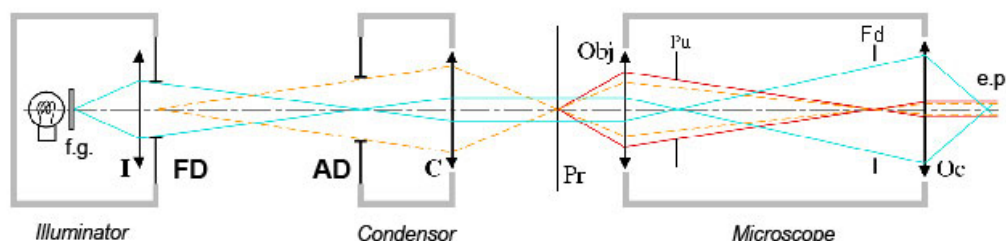
2.2. Köhler illumination

Invented by A. Köhler at the end of XIXth century [[Ein neues Beleuchtungsverfahren für mikrophotographische Zwecke]], it is based on the three following optical conjugations:

- Illumination field diaphragm ↔ Specimen, via the condenser optics
- Illumination aperture diaphragm ↔ Objective entrance pupil (**located at infinity**), via the condenser optics

- Frosted glass in front of the lamp \leftrightarrow Aperture diaphragm, via the illumination system optics.

A schematic view of this illumination system, with its elements and optical conjugations, is represented on figure 3 (see below). The aperture diaphragm (AD) is - by mechanical construction - located in the front focal plane of the condenser optics C . **The second optical conjugation is therefore always achieved.**



The illumination optic I is very uniformly illuminated by the lamp and its frosted glass, located a few centimeters behind. The preparation (Pr), located at the image of lens I (limited by the field diaphragm FD) by the condenser C , is therefore uniformly illuminated. However, the frosted glass (whose texture and illumination are less uniform) is imaged at the aperture diaphragm AD . Since this latter element is by construction in the front focal plane of the condenser optics C , the frosted glass is imaged at infinity, as far as possible from the sample.

This configuration has several remarkable properties. Since the aperture diaphragm is located at the condenser focal plane, the system (AD, C) exit pupil is located at infinity (image-space telecentric system). This means that, in addition to being exposed to the same illumination, all the points within the specimen are illuminated by the **same light cone**, which, considering that the objective is an object-space telecentric system, warrants that all the points in the microscope field of view exhibit the same photometric response. This property is very important in practice and would be very hard to obtain using other configurations... In addition, Köhler illumination allows controlling two important parameters in a totally independent manner:

- The illumination field at the specimen, which is controlled by the field diaphragm FD . It is often useful, for parasitic light issues, to limit the illuminated area to the area seen and studied by the microscopist.
- The angle of the light cone reaching each point of the preparation, or, in other words, the illumination numerical aperture, controlled by the aperture diaphragm AD . This controls how much light reaches the sample but also, more importantly, how the final image of the observed object is formed; see the sub-section « Illumination coherence and resolution limit », about image formation under partially coherent illumination.

By design, Köhler illumination also ensures that the aperture diaphragm AD is imaged onto the objective pupil Pu . In a similar fashion, the illumination field diaphragm (FD) is imaged onto the ocular field diaphragm Fd . Consequently, with Köhler illumination, no vignetting is introduced, and the field of view is perfectly uniformly illuminated.

Therefore, to the exception of very low-budget systems, all microscopes now include a Köhler illumination system, which is most of the time pre-aligned, and in particular pre-centered. The optical conjugation field diaphragm/sample (obtained by translation of the condenser) is however left to the user responsibility, therefore allowing the observation of preparations on slides of various thickness.

3. Imaging properties of a microscope

As was mentioned in the introduction, the first role of a microscope is to improve the visualization of very small objects by a human observer. Two essential characteristics of a microscope are therefore its magnification and **resolution limit**. For a 'diffraction-limited' objective (meaning an objective **limited by diffraction and not by its defects or aberrations**), the resolution limit r is proportional to the (mean) wavelength λ and inversely proportional to the object-side numerical aperture NA_{obj} , i.e. $r = Cte \cdot \lambda / NA_{obj}$. The precise value of this constant Cte is somewhat arbitrary; when using the Rayleigh criteria with incoherent illumination, $Cte \sim 0.61$ (see basic optics course or [[Principles of Optics]]). The object-side numerical aperture of the objective is defined by $NA_{obj} = n \cdot |\sin(\alpha_{obj,max})|$ where n is the index of refraction of the material located between the object and the objective, and $\alpha_{obj,max}$ is the angle formed by the optical axis and the most marginal ray penetrating through the microscope objective. Therefore, microscope objectives must have a **very large numerical aperture and be well corrected for optical aberrations**. Those objectives are made of numerous lenses that are very precisely positioned with respect to one another, and therefore are rather fragile and expensive. On another note, to obtain a better spatial resolution, it is interesting to work in a medium of refractive index larger than 1, such as water ($n \simeq 1.33$) or an immersion oil (cedar or synthetic oil, $n \simeq 1.52$), to obtain numerical apertures larger than 1. Thus, the objectives of largest magnification ($\times 100$) are usually (oil) immersion objectives with numerical apertures often larger than 1.25.

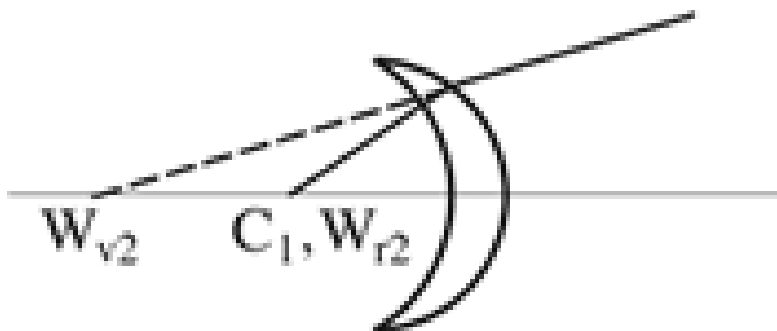
However, using those objectives of very high numerical aperture and corrected for most optical aberrations is not without certain problems, that are described in this section.

3.1. Constraints while imaging with very high numerical aperture objectives

a) Introduction

The main problem with image formation under very high numerical apertures is the very high inclination of the involved optical rays. Numerical apertures of 0.70 ; 0.95 (in air) or 1,30 (in immersion oil) lead to extreme rays making with the optical axis angles of (respectively) 45, 72 and 59 ! Therefore, we are far from the Gauss conditions and the domain where approximate stigmatism can be used. To obtain high quality images, one needs to work with optical conjugations that are rigorously stigmatic and aplanetic. Thus, when designing microscope objectives, curvature centers and Weierstrass points of the involved spherical surfaces are of great use.

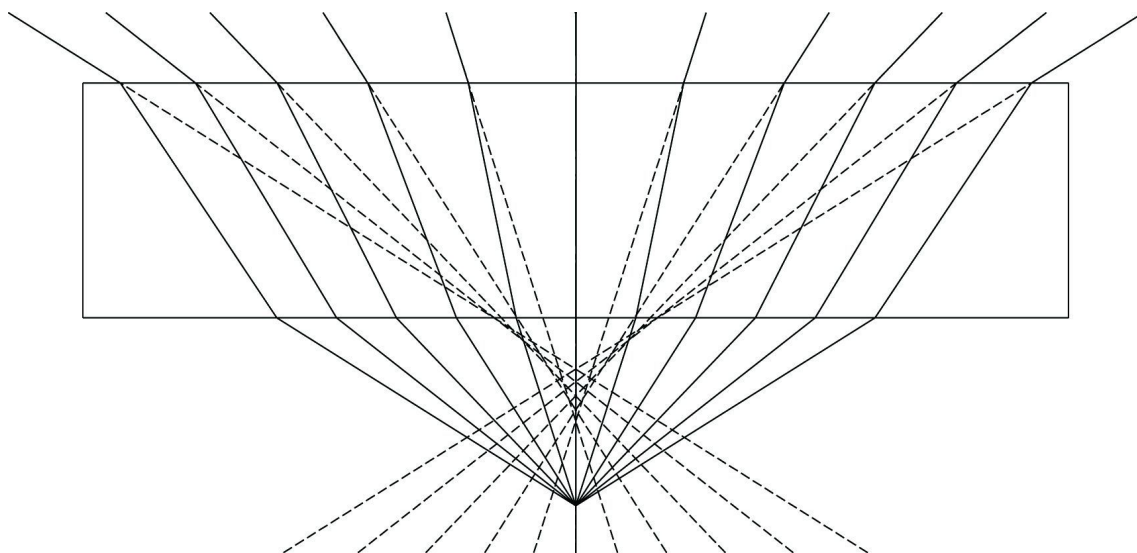
Remark : These objectives are based on the **aplanetic meniscus of Amici** (see fig. below). The object is located at the curvature center of the first surface. Its image (superimposed to the object) is located at the real Weierstrass point of the second surface, which creates an expanded virtual image of the object seen under a smaller numerical aperture.



Therefore, for each particular microscope objective, the pair of points object/image is fixed. Moreover, simple planar interfaces may have a significant influence on the imaging quality, as shown in the following paragraph.

b) Influence of the coverslip

Most microscopic samples observed in transmission are protected by a thin glass slide with planar and parallel surfaces called coverslip². This coverslip has an optical power equal to zero but still affects the oblique rays that are going through it (lateral displacement). This phenomenon increases when the inclination of the rays increases, which translates into a loss of stigmatism, as shown on figure 4 (introduction of spherical aberrations).



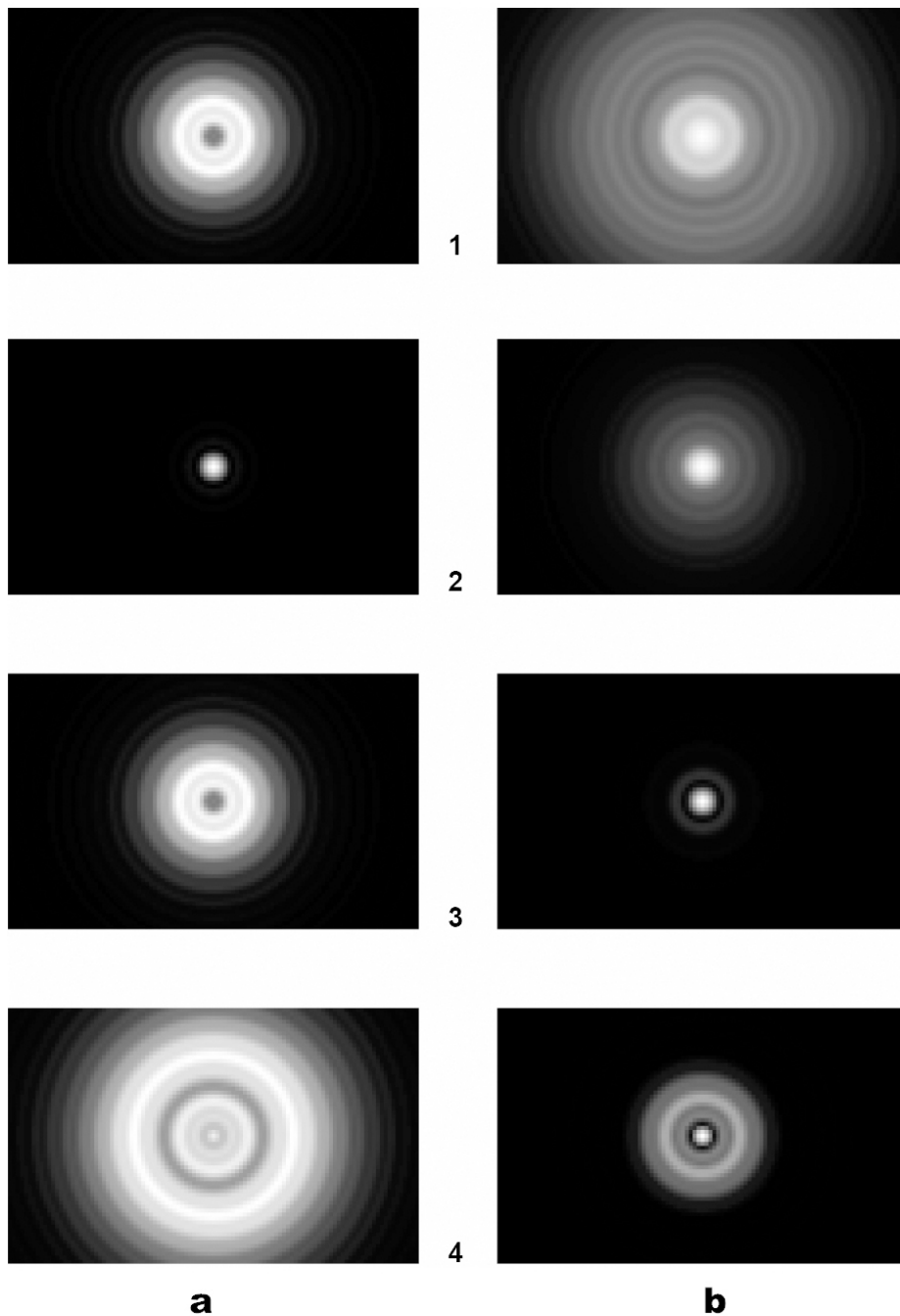
We usually consider that the coverslip significantly affects the observed image quality as soon as the objective numerical aperture exceeds 0.30. For numerical apertures larger than 0.70, image deterioration is obvious and sometimes a serious problem.

However, it is possible to solve this problem by introducing within the objective (by a judicious design) an aberration that will exactly compensate to the one introduced by the coverslip. One must realize, however, that such an objective will give a bad image of a sample with **no coverslip**. Manufacturers generally offer two versions of all their objectives of numerical aperture larger than 0.30, one to work with a coverslip and one to work without a coverslip. This latter version is usually dedicated to the so called metallographic microscopes, where opaque objects are observed using back illumination through the objective (see Fig.12, in the supplements, at the end of this course). The particular version intended is identified by an inscription on the objective mount (see the subsection « objectives labeling »).

Remarque

The aberration introduced by the coverslip depends on its thickness and index of refraction. The correction introduced by the objective is therefore valid only for a specific kind of coverslips. A standard type of coverslip was defined (norm ISO TC172-8255), with a thickness of 0.170 mm , a refractive index of 1.517 at 588 nm and a Abbe number_D of 64 (standard glass N-BK7 of Schott or S-BSL7 of Ohara). The higher the numerical aperture, and the more drastic the tolerances on the first two parameters. The thickness should be, for example, accurate within $\pm 5\text{ m}$ to work without significant loss of quality under a numerical aperture of 0.85. It is however extremely difficult to buy such coverslips (commonly available coverslips are usually accurate within $\pm 0,030\text{ mm!}$). This explains why some very high-quality objectives of very high numerical aperture have a rotating ring allowing to adjust the correction to the real thickness of each particular coverslip.

It is possible to qualitatively observe this phenomenon by using as the object a slide covered with a thin aluminum layer, with small holes in this layer. These holes, illuminated using Köhler illumination, will serve as point-like light sources on a dark background, and will allow observing the objective point-spread function. For a perfect objectif and perfect focusing, the point-spread function is an Airy pattern. Otherwise, the observed pattern and most of all its evolution with focusing give information about the system quality. This (classical) method for measuring the aberrations of an optical system is called "bright point method". The point-spread functions of a perfect system (column a) and of a system with spherical aberration (column b) are shown on figure 5 for several focus up and below the paraxial image.



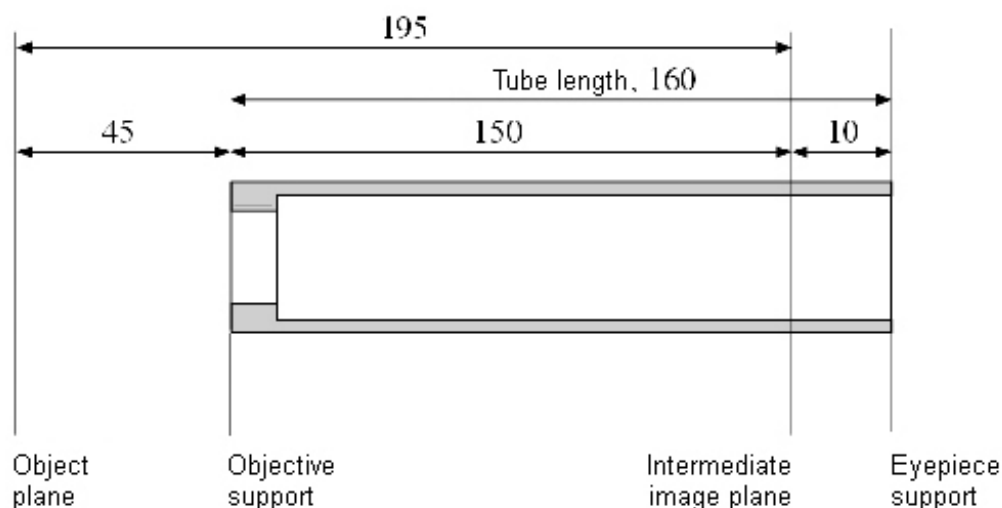
From one row to the next, the focusing is varied of the same distance. The paraxial image corresponds, for both columns, to the second row. The image a2 therefore represents the Airy pattern. In presence of spherical aberrations, the best focusing does not correspond to the paraxial image. The best point-spread function obtained in this case (image b3) exhibits secondary rings of intensity far superior to the ones of the Airy pattern, which explains the image blurring obtained with such an aberrating system. Please note the point-spread function symmetry with respect to the plane of best focusing obtained in the case of an aberration-free system (column a). With spherical aberration (column b) this symmetry is completely lost, and the point-spread functions obtained before and after the plane of best focusing (b3) greatly differ in shapes.

Remarque

The dynamic of an image reproduced on a screen (8 bits) is very much lower than the eye dynamic, which would observe very dim secondary rings, invisible here.

c) Tube length and other dimensional standards of a microscope

Using rigorously stigmatic optical conjugations, such as the Weierstrass points, when designing the microscope objectives, requires that the intermediate image observed with the eyepiece to be at a precise location, fixed with respect to the objective. Focusing should therefore be obtained by varying the distance object/objective, keeping the distance objective/eyepiece fixed. A microscope should thus work like an optical viewfinder with a fixed working distance. To allow for a certain interchangeability of the microscope components (objectives, eyepieces...) between various manufacturers, several precise dimensional standards have been defined for the microscope (ISO TC172 [8036 à 8040 + 8255 + 8578...]). The main ones are shown on Fig. 6 below [[Encyclopedia of Engineering techniques]].



The intermediate image plane must therefore be 10 mm below the eyepiece support and the object plane 45 mm below the objective support¹. The tube length (i.e. the distance between the eyepiece and objective supports) has a normalized value of 160 mm. However, it is possible to find other tube lengths: for example, older metallographic microscopes have a tube length of 230 mm. But more importantly, a certain type of objective **corrected for an infinite tube length** (or, in short, *corrected for ∞*), is offered by most manufacturers for high quality equipment. These objectives are designed to image the object at infinity without aberration. This distance is brought back to a finite distance by an additional converging optical system, called **tube lens²**, located in the microscope stand. A real image is therefore formed, equivalent to the intermediate image of "conventional microscopes", and observed with the eyepiece. With this set-up, it is easier to design and insert additional components in the optical path, which is useful for the techniques using contrast or for reflected light illumination.

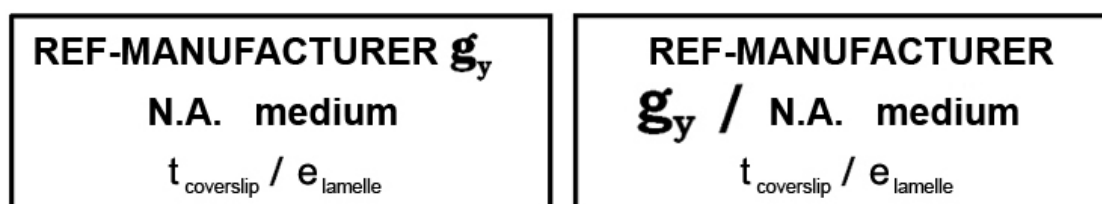
The key point is that a slide with parallel surfaces, even curved, does not introduce aberration for (and only for) objects located at infinity (see the two supplement pages at the end of that course grain).

Considering the multiplicity of existing configurations, the user must be very careful in using the right objectives with the right microscope³. To help him in this task, the length of the matching tube lens is usually engraved in the objective mount.

d) Objective labeling

As was just mentioned, there are plenty of available objectives, even within a single manufacturer. To help the user, several useful characteristics are always engraved on the objective mounts, naming: the objective magnification⁴, numerical aperture, immersion medium (if different from air), the coverslip thickness and the length of the tube lens (in mm) for which the objective is corrected. This information is usually complemented with a reference, specific for each manufacturer, which identifies the objective "class" (see the next subsection about "microscope objective classes").

This information is usually mentioned in the following way, together with eventual additional indications specific to each manufacturer and objective series:



Here are some examples extracted from objective catalogs of several manufacturers:

Achromat 63 / 0.80 160 / 0.17 ZEISS	NCSPlan40 0.70 160 / 0 OLYMPUS	N PLAN 20x/0.40 ∞ / 0.17 LEICA	PlanFI 100x/0.95 ∞ / 0.14 - 0.20 OLYMPUS	Plan 100x/1.25 Oil ∞ / 0.17 WD 0.15 NIKON	M Plan Apo 20 0.42 ∞ / 0 f = 200 MITUTOYO
---	---	--	--	---	---

Immersion medium are usually listed as follows: *Oil* for immersion oil ($n \simeq 1,517$ à 588 nm), *W* for water and *Gly* for glycerin. On very old -french- objectives it is possible to find the label *ih* (for *homogen immersion*), also referring to oil immersion objectives.

We also remind the reader that the indication ($\times Z$) engraved on the eyepiece mount is its **angular magnification factor**. Sometimes this number is followed by a second number, called *field number*, which gives the diameter, in millimeters, of the effective observed area of the intermediate image (this number ranges from 18 for a low-cost eyepiece to 26 for a very high-quality eyepiece, and is usually around 22; of course, *the objective must also be matched with the eyepiece*). On another note, a symbol representing eyeglasses or the letter *L* means that the eyepiece has an exit pupil located far enough to be comfortably used with correction eyeglasses.

Attention

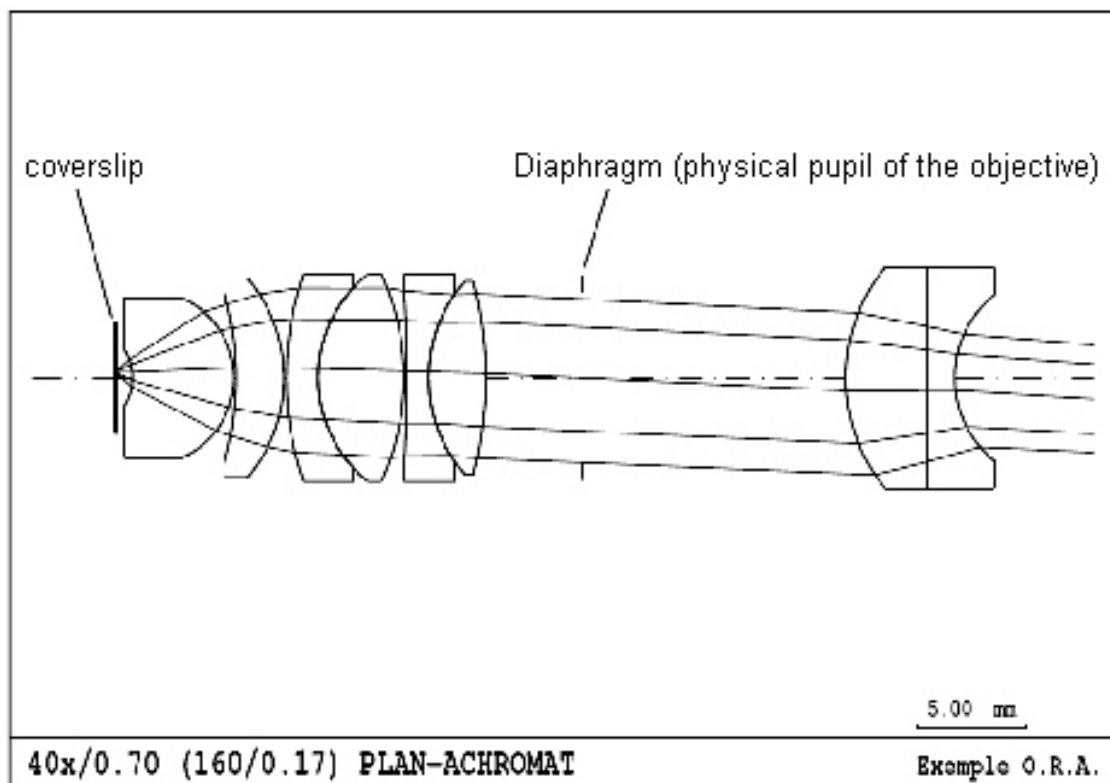
Do not forget to use the rubber eyecup if not wearing eyeglasses.

e) Microscope objective classes

Ideally, a microscope objective should give a diffraction limited image everywhere within the observed field. Such a property of course. Comes at a cost and is not always necessary for all microscopy applications. This explains why most manufacturers offer three or four "classes" of objectives, achieving various performances and at various prices. Those classes can slightly vary depending on the manufacturer and are usually listed as follows:

Class	Correction	Main aberrations	Use	Price (depending on Gy)
Achromat	Achromatisation (simple folding)	Large field curvature (in focus at the center and blurred on the outside, or vice-versa). Chromatic aberration of apparent width. (Astigmatism).	Eyepiece of field number ~18 – School, recreational use	~ 100 € to ~ 400 €
Apochromat	Apochromatisation (double folding)	Reduced chromatic aberration compared to Achromat objectives.	—	(Obsolete)
Plan-Achromat (or "Plan") (See figure 9)	Achromatisation Flat-field	Objectifs of high quality, quasi-diffraction limited over a large (central) part of the field of view. Some manufacturers offer two levels of quality for this class (objectives "semi-apochromatic").	Eyepiece of field number ranging between 20 and 22 <i>Usual professional observations</i>	~ 250 € to ~ 2000 €
Plan-Apochromat (or "Plan-Apo")	Apochromatisation Flat field	Objectives of highest quality, quasi-diffraction limited over the full field of view.	Eyepiece of field number ranging between 22 and 26 Microphotography	~ 1000 € to ~ 4000 €

On figure 9, we show an example of optical configuration for an objective 40 of medium quality (type "Plan-Achromat").

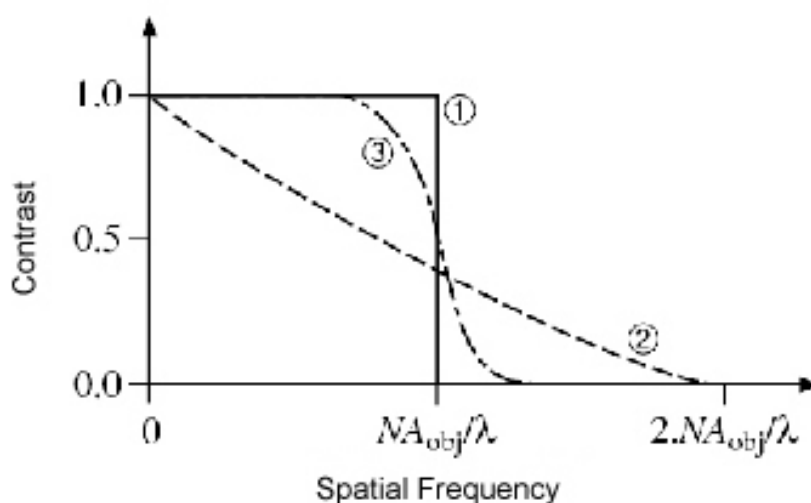


3.2. Illumination coherence and resolution limit

Diffraction imposes a resolution limit on the order of the wavelength, when observing objects with an optical system such as a microscope. To quantitatively determine how an image deteriorates due to diffraction, it is interesting to use the object spatial frequency space and to consider the system *modulation transfer function* (MTF). (See the course on Image Formation in the module Fourier Optics, or the references [[Introduction to Fourier Optics and

holography]], [[Principles of Optics]] .) The theory shows that image degradation does not only depend on the objective properties (numerical aperture, aberrations) but also on the illumination *spatial coherence*, a property directly related to its numerical aperture.

When the aperture diaphragm is almost closed (point-like source), the object is illuminated with a quasi planar wave, and the illumination is said to be spatially coherent. Fourier formalism can then be applied to the complex amplitudes of waves. In that case, an optical system with no aberration (diffraction-limited) is a low-pass filter for the spatial frequencies: it leaves unaltered all frequencies smaller than NA_{obj}/λ where NA_{obj} represents the object-side **numerical aperture** of the objective and perfectly cuts all larger frequencies (see. Fig. 10, trace 1). In those conditions, images are well contrasted but image elements are circled with fringes related to the Gibbs phenomenon.



Conversely, if the aperture diaphragm is wide open so that the illumination numerical aperture is larger than the objective numerical aperture, then the illumination is said to be totally incoherent. The Fourier formalism can then be applied to the wave intensity (modulus squared of the complex amplitude). An optical system without aberration is then a low-pass filter whose transfer function gradually decays from 1 to 0 when the frequency increases from 0 to $2 \cdot NA_{obj}/\lambda$ (see Fig. 10, trace 2). The cutoff frequency is twice as large as the one obtained in the case of a coherent illumination. However, for a periodic object, the higher the spatial frequency and the smaller the contrast after transmission through the optical system.

For intermediate cases, the illumination is said to be partially coherent (See reference [[Principles of Optics]]), and it is not possible anymore to rigorously define a transfer function, since the system is no longer linear. However, one can obtain a good idea of the effect that will have the optical system on the image by determining the contrast of a sinusoidal test pattern as a function of its spatial frequency (See Fig. 10, trace 3). One can notice that the contrast remains equal to 1 for all frequencies smaller than $(NA_{obj} - NA_{ill})/\lambda$ and then gradually decays to reach 0 at frequency $(NA_{obj} + NA_{ill})/\lambda$, where NA_{ill} is the illumination numerical aperture, determined by the setting of the condenser aperture diaphragm; reference [[Encyclopedia of Engineering techniques]] presents a clever demonstration of that property. Almost all microscopy observations are made in those illumination conditions. Indeed, they give an image containing high spatial frequencies, and with very well contrasted low and medium frequency components, which satisfies the observer subjective sense of vision. The optimal value of the illumination numerical aperture has to be

adjusted for every object (and observer), and is empirically on the order of two thirds of the objective numerical aperture.

To verify the influence of the illumination coherence on image formation, it is interesting to observe *diatoms* preparation tests. Diatoms form a very vast and diverse family of microscopic aquatic animals, and their skeletons are made of very fine periodical structures with patterns of dimensions ranging between $\sim 2 \mu\text{m}$ and $\sim 0,25 \mu\text{m}$ (depending on the species). These 'objects' are therefore particularly well adapted to directly discover the microscope objective resolution limit.

3.3. Other properties and characteristics of a microscope

a) Working distance – Parfocality

Since a microscope is a fixed-working-distance viewfinder, the distance object/frontal lens of a given objective is fixed when the focus is realized. This distance, called 'Working Distance_≡', usually varies from several millimeters for low magnification objectives (10) to several tens of millimeters for higher magnification objectives (100, immersion). A large working distance facilitates the observation by limiting the risk of collision between the preparation and the objective, and also allows observing specific objects surrounded with raised patterns, such as integrated circuits with their connection slots. However, it is very difficult (but possible at very high cost) to conciliate a large working distance with a high numerical aperture. Thus, manufacturers usually offer, in addition to a standard series of objectives, specific objectives with high working distance for users interested by this property¹. Recent standard objectives of low working distance usually have a mount with a spring to limit the unfortunate consequences of a collision objective/preparation.

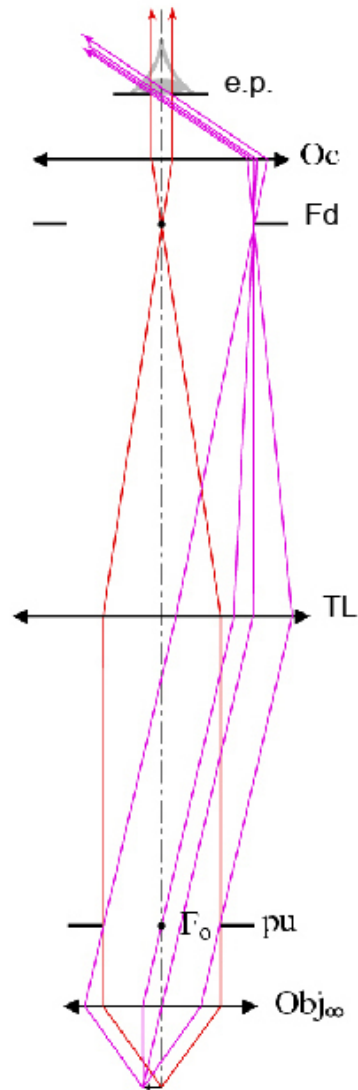
In addition, following a dimensional standard imposing a precise distance (usually 45 mm) between the object plane and the objective support (see Fig. 6 of the subsection "Tube lens and other dimensional standard of a microscope"), the focus must normally be preserved when interchanging objectives. One should however realize that the microscope depth of focus is usually very small, since it is on the order of $\lambda/(NA_{obj})^2$ where NA_{obj} is the object-side numerical aperture of the objective (axial focusing error – see the basic optical course). This usually leads to an exceptionally low tolerance on the objective mechanical mount and on the nosepiece. The effective quality with which the focus can be preserved while interchanging objective is called parfocality.

b) Photomicrography and video

A microscopist often needs to keep recordings of his or her observations. Photomicrography is a great way of achieving this filing. It consists in making a **real image** of the object under observation on a photographic film, or nowadays more often on a CCD or CMOS camera. For practical reasons (and sometimes for image quality reasons, *as the field may not be flat enough*) it is not possible on current microscopes to place the camera in the intermediate image plane; in addition, we advise against modifying the eyepiece focus to obtain an real image at the output of the eyepiece. The solution is rather to replace the standard eyepiece with another optical system called *projection eyepiece*, which is specifically designed to give on the camera an image of required quality (See reference [[Encyclopedia of Engineering techniques]]). In any case, one should make sure to match the camera resolution with the image resolution in order to obtain optimum results (see the third case study). To facilitate the image acquisition, it is possible to equip the microscope with a trinocular head, allowing binocular viewing by the observer simultaneously with image recording (of the full image or a part of it) on the (video) camera.

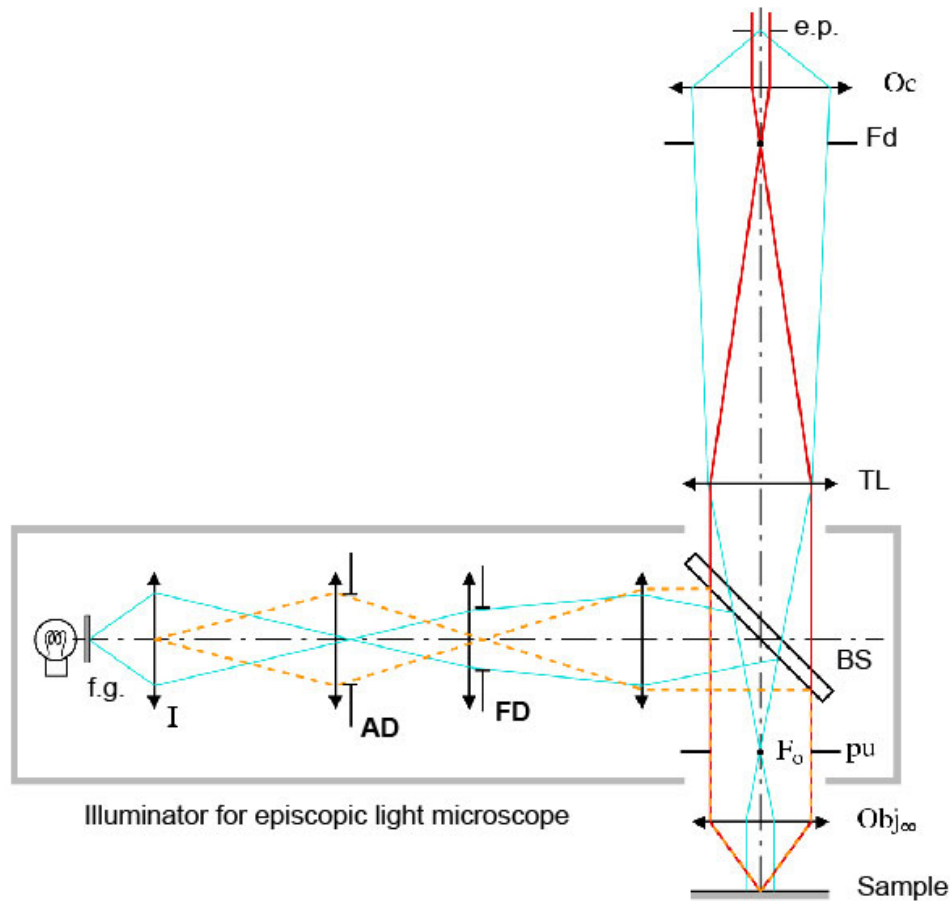
4. Supplement

4.1. Supplement 1



Compare to figure 2

4.2. Supplement 2



Compare to figure 3

* *
*

In this course sequence, we have detailed the basic principles and keys specificities of the architecture of the optical instrument "microscope" and its illumination. The three following case studies show practical and illustrated examples, based on real and typical situations and equipment, in order to clarify and make more practical the basic knowledge and descriptions that were previously introduced.

Books [[Optics]], [[Geometric and wave optics]] and [[Instrumental optics, Fourier optics]] are a great source of supplementary information in general optics. Books [[Modern optical microscopy]], [[Introduction to the microscope : basic principles and utilisation]] or [[Encyclopedia of Engineering techniques]] provide very useful supplementary information in

the microscopy area; the paper [[Confocal microscopy]] is a great introduction to confocal microscopy, that will not be treated in this module. The websites <http://www.molecularexpressions.com/>⁴, <http://www.microscopyu.com/>⁵ or <http://www.olympusmicro.com/>⁶ also present some elements of microscopy in a very imaged and educational mode.

The following unit (#2) of this *optical microscopy* module largely uses the elements presented in this unit to address complementary techniques such as phase contrast and differential interference contrast. These such techniques are very important for example in biology where they allow observing living biological systems in great details without perturbing them by adding toxic dyes. In other technical areas, they also allow highlighting raised patterns of opaque objects, that would be totally invisible to classical microscopes...

4 - <http://www.molecularexpressions.com/>

5 - <http://www.microscopyu.com/>

6 - <http://www.olympusmicro.com/>

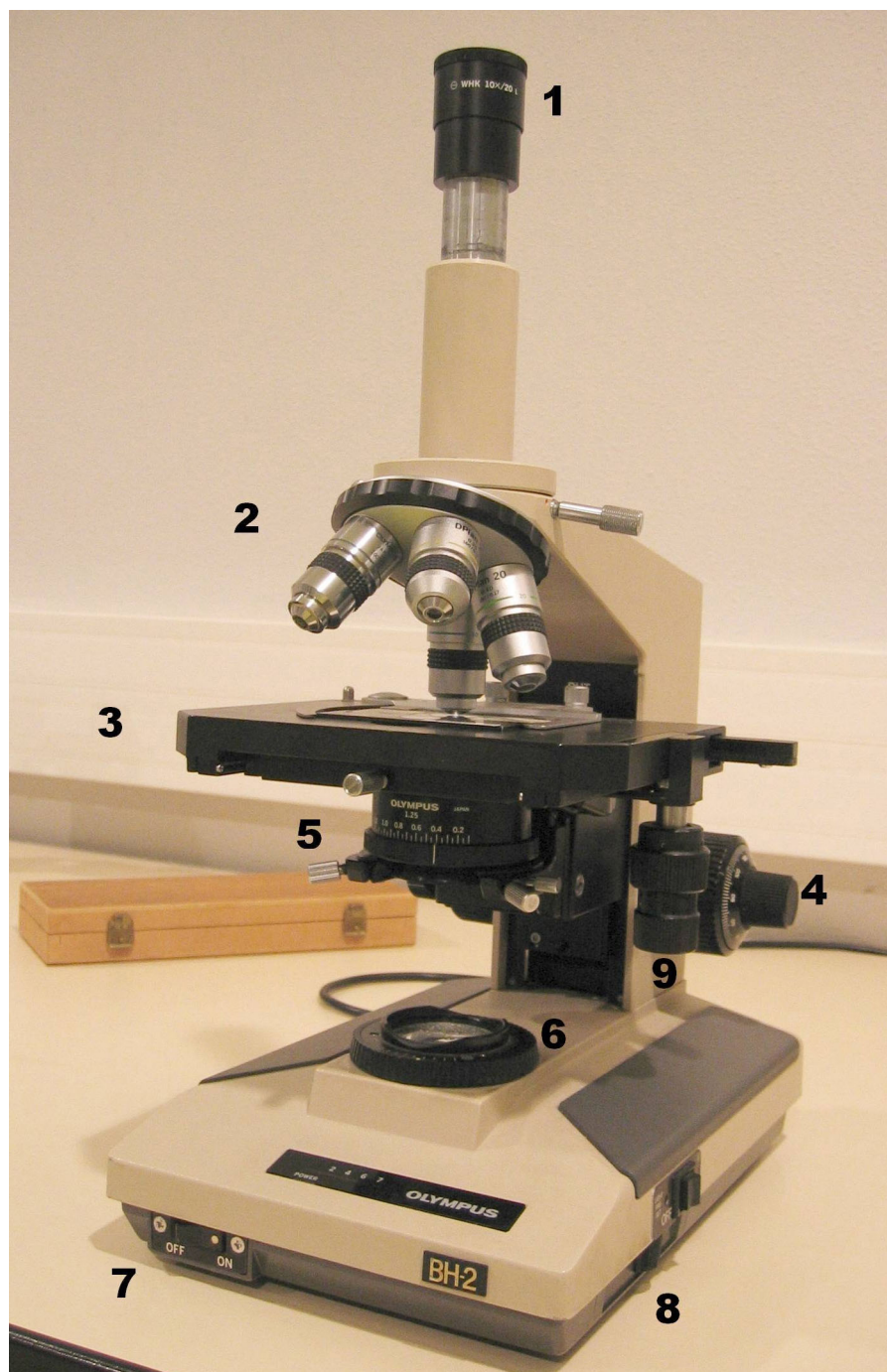
III. Case study

1. Implementation of a transmitted light microscope

1.1. Adjustment of the microscope for visual observations

a) Introduction

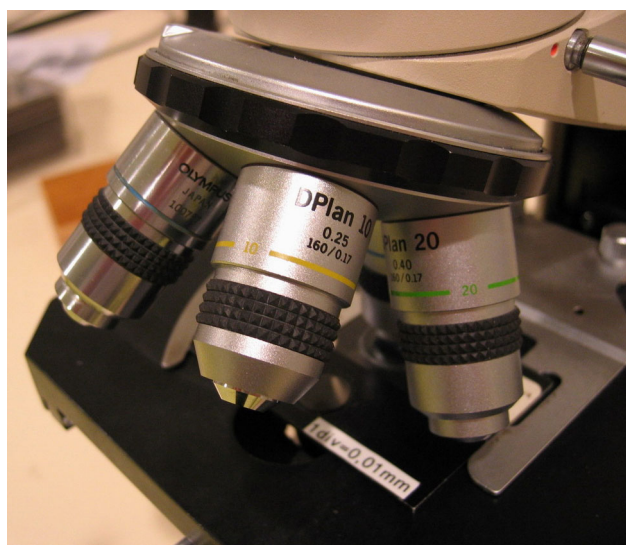
In this paragraph, we will study in greater detail the use and practical characterization of a **simple** 'biological' microscope with a Köhler illumination system integrated into its stand (see Fig.13).



b) Getting started

The user first switches on the integrated illumination system (using the proper switch (7) located on the stand), and sets the illumination power to a low value using the dimmer (8). Then, he lowers the microscope stage using the large lateral focus knob (4) and places the transparent preparation (here, a colored earthworm section) with a coverslip on the microscope stage. He fully opens the field diaphragm using the knob (6) at the bottom of the stand and the aperture diaphragm with the knob (5) on the condenser block below the microscope stage. By rotating the nosepiece (2) (see also Fig. 14), he places an objective of low magnification on the tube axis, for example a 10 objective designed to work with a

coverslip (label 160/0,17) ; normally, there should be enough room above the sample for the objective when the microscope stage is in low position.



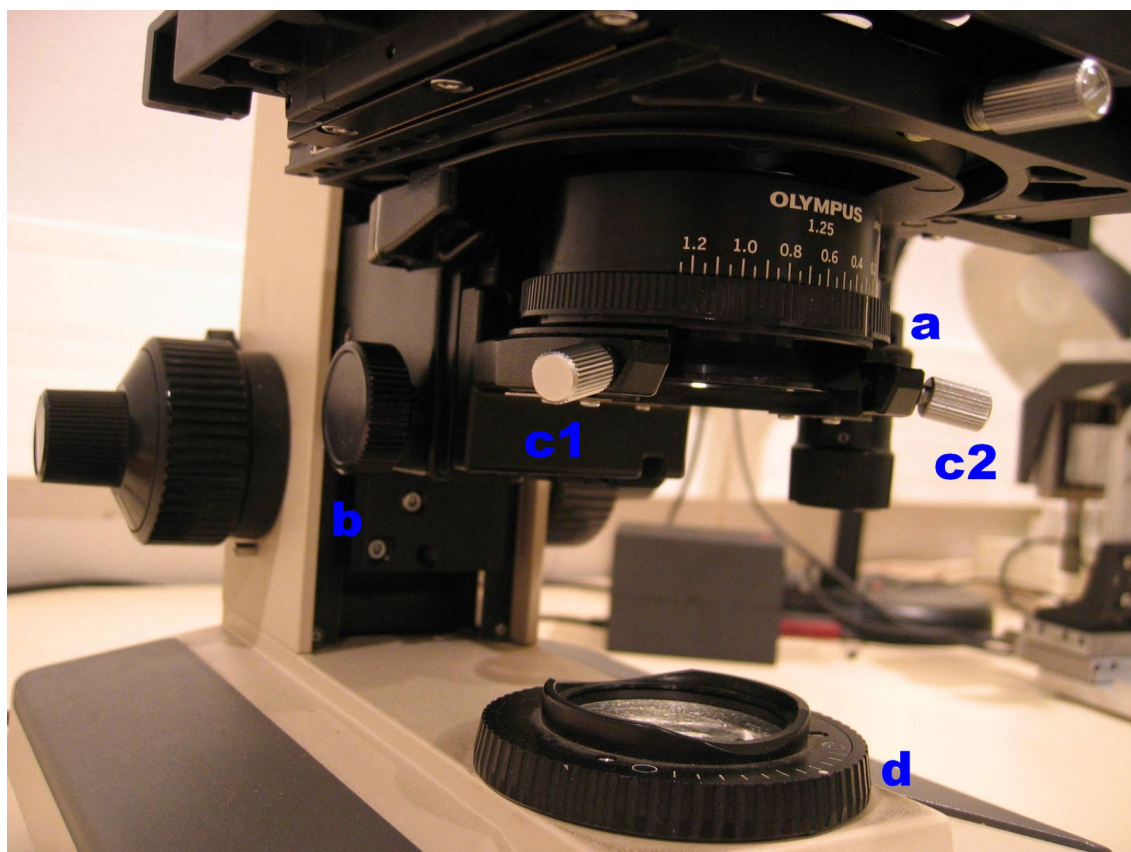
The user then roughly centers the sample below the objective using the horizontal translational control knobs of the stage (9), and, **while being careful to avoid any collision between the preparation and the microscope objective** [which could damage both the preparation and the objective!!], he pulls up the sample stage using the focus knobs (4) to bring the preparation a few tens of millimeter below the objective frontal lens. Then, he brings one eye to the eyepiece¹ and **by slowly moving the preparation away from the objective** using the focus knob, he seeks to see several elements of the sample in focus

Remarque

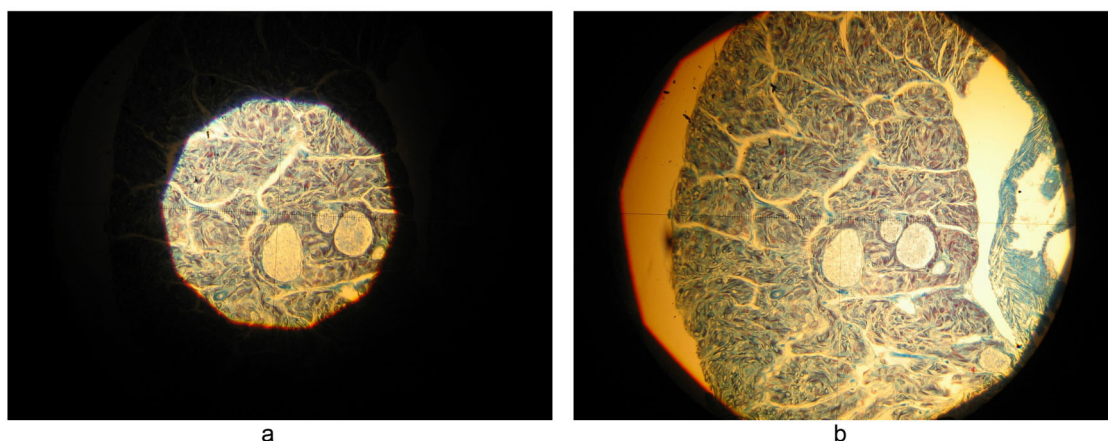
At this stage, the observation is usually of bad quality, because the Köhler illumination system is not well adjusted.

c) Adjustment of the Köhler illumination system

A large part of the Köhler illumination system is integrated in the microscope stand and is preadjusted. However, some adjustments are accessible and need to be optimized for each observation (see Fig. 15).



To adjust the Köhler illumination system, one first needs to almost completely close the field diaphragm with the collar (**d**) at the bottom of the microscope stand and then to axially move the condenser below the microscope stage using the knob (**b**) so as to see the image of the field diaphragm and the preparation simultaneously in focus – the preparation is then only illuminated on a very small area of polygonal shape (see Fig. 16a). If necessary, one can center the field diaphragm on the microscope field using the two screws positioned at 120° (**c1-2**), allowing the condenser to be centered on the microscope axis.



d) Finalization of the adjustment

To finish adjusting the microscope, the user has to open the field diaphragm to illuminate **only** the observed field (and maybe only the region of interest, if it is smaller) (see Fig. 16b) and to optimize the focus on the sample. Then, he can play around with the illumination coherence

via the condenser aperture diaphragm (**a**) to optimize the contrast of the objects of interest in the preparation, and adjust the illumination power via the electronic dimmer (**8**) to achieve a comfortable observing condition.

1.2. Determining several microscope characteristics

a) Introduction

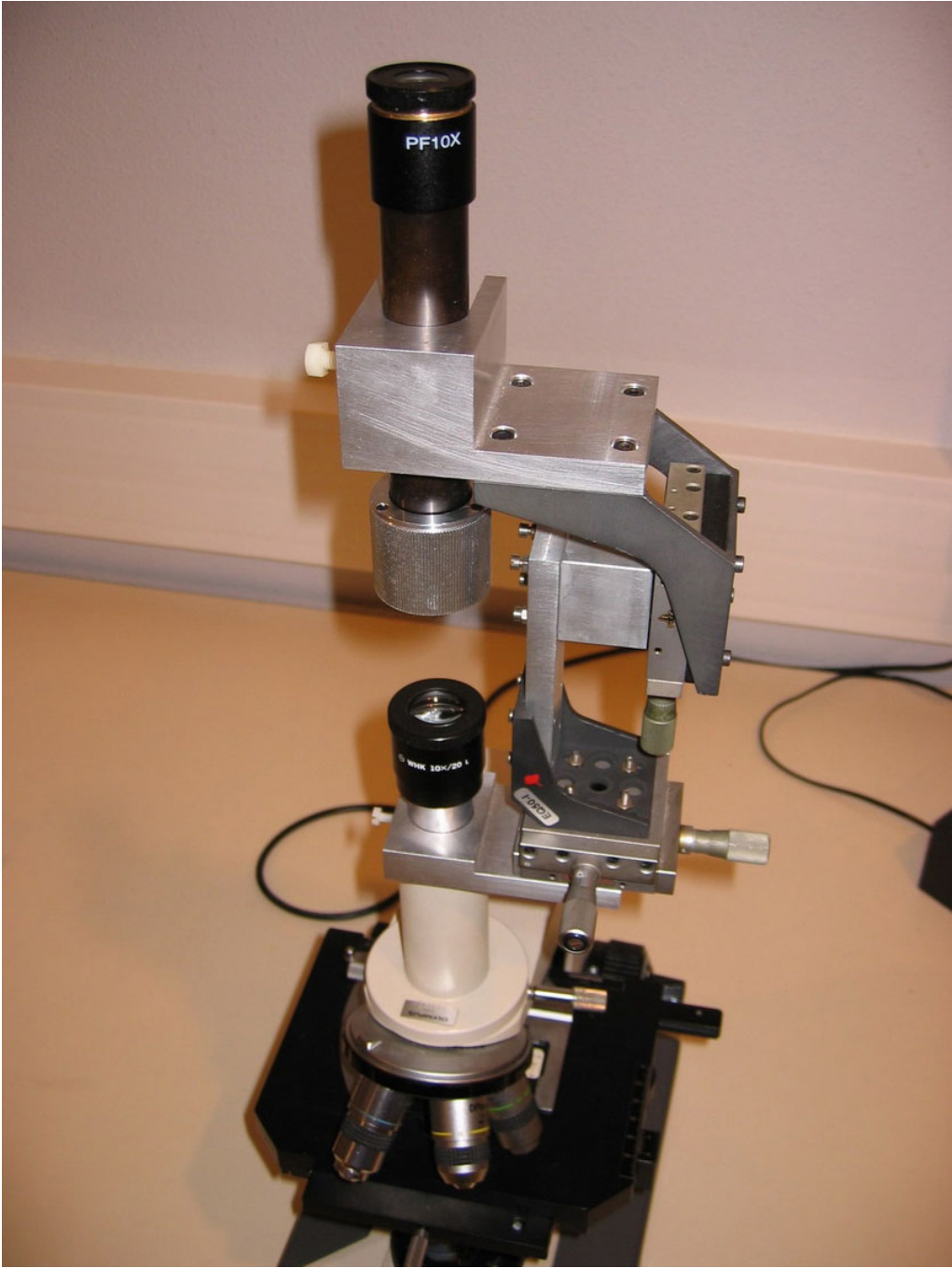
It is easy to determine or verify several optical characteristics of a given objective/eyepiece configuration. To do so, one has to first adjust the microscope (as detailed previously) on a particular preparation called "**objective micrometer**" which is made of a calibrated scale, usually to the 1/100th of millimeter, engraved on a transparent slide and covered with a standard coverslip of thickness 0.17 *mm*.

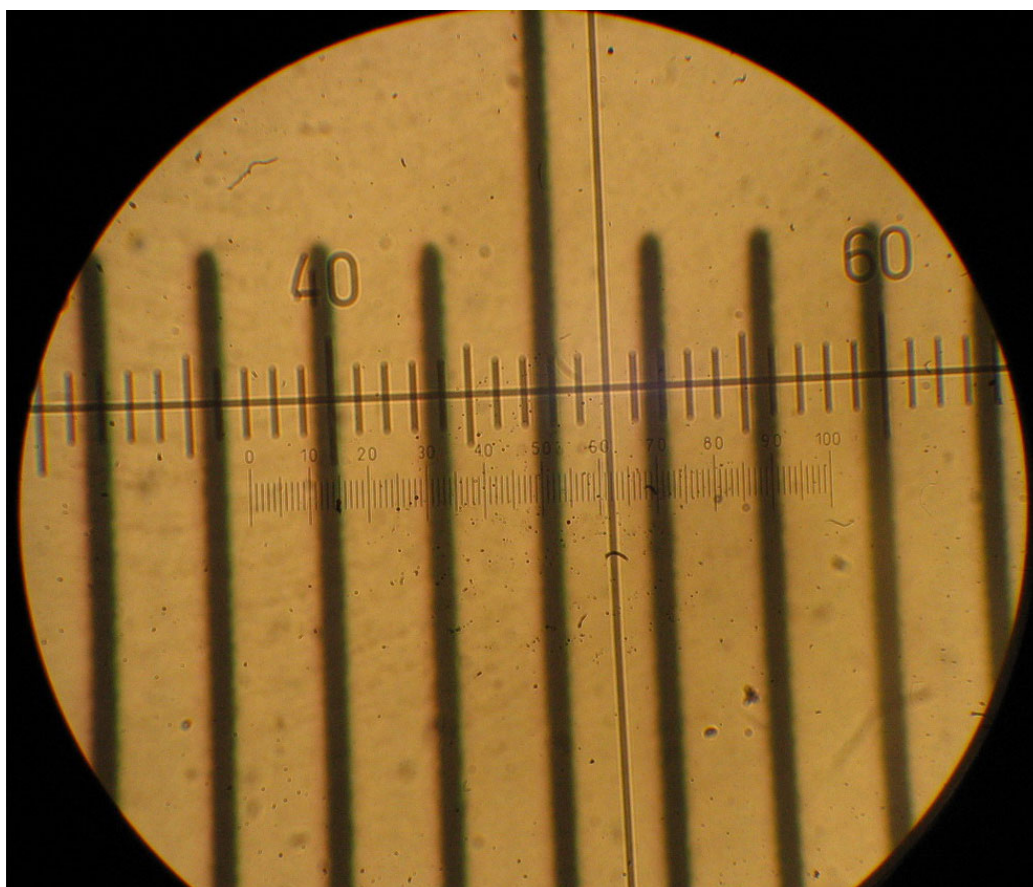
b) Measurement of the total optical power of a microscope

After aligning the microscope, the user places one eye behind the eyepiece and observes the micrometer scale highly magnified. By looking at this same image located at infinity through a telescope with a micrometric ocular previously angularly calibrated, it is possible to determine the angular size $\Delta\theta$ of the image of N objective micrometer intervals (see Fig.17 and Fig.18). If the distance between two successive scales of objective micrometer is equal to Δx , then we immediately deduce that the total optical power (dioptric power) of the microscope is:

$$P_{micro} = \frac{\Delta\theta}{N \cdot \Delta x}$$

$\Delta\theta$, Δx and P_{micro} being respectively expressed in radian, meters and dioptres.





In figure 18, we can see that 4 intervals of the micrometer scale (at $1/100 \text{ mm}$), i.e. 0.040 mm , are covering the telescope field of view between scale $11(\pm \sim 1/2)$ and scale $87(\pm \sim 1/2)$ (by taking in the two cases the left side of the micrometer line), i.e. therefore $76 \pm \sim 1$ basic angular intervals of the telescope. Using a preliminary calibration of the telescope with a calibrated collimator, we could determine that an interval of the telescope micrometer corresponds to $0.0482 \pm 1\%$. Therefore, we deduce that $\Delta\theta = 3.66 \pm \sim 2.3\%$ and the total microscope power is:

$$P_{micro} = \frac{3,66 \times \pi / 180}{0,040 \times 10^{-3}} = 1598 \delta \pm \sim 2,3\% \approx 1600 \delta \pm 40 \delta$$

By definition the microscope angular magnification factor is:

$$G_{c, micro} \stackrel{\text{def}}{=} \frac{P_{micro}}{(1/d_{norm})}$$

where d_{norm} represents the **normalised** value of the minimum distance for a in-focus visualization through a standard optically perfect eye; it is fixed to 0.25 m .

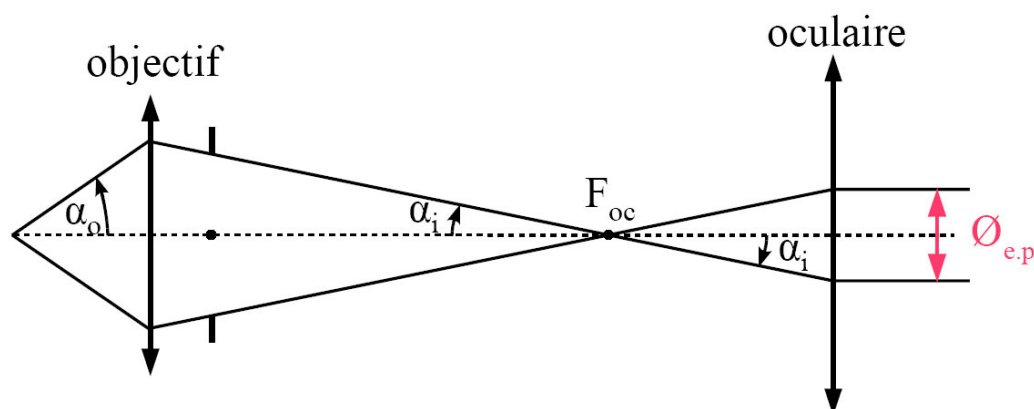
Using equations 2 et 3, we deduce that the microscope angular magnification factor is 400 ± 10 . This result is in perfect agreement with the value $G_{c, micro} = |g_{y, obj}| \cdot G_{c, oc}$ expected for an objective 40 and an eyepiece 10.

It is therefore possible to easily check (within the measurement uncertainty) the manufacturers labels engraved on the optics. One can note that when the eyepiece is micrometric, as it is the case here, the objective magnification $|g_y|$ can be independently determined by directly comparing the scale of the micrometer objective image with the scale of the eyepiece micrometer (for which each interval corresponds to $1/10^{\text{th}}$ of mm). One can also determine the ocular power P_{oc} by measuring with the auxiliary telescope the angle $\Delta\theta_{oc}$ corresponding to N_{oc} intervals Δx_{oc} of the scales of the ocular micrometer, and using the

equation $P_{oc} = \Delta\theta_{oc}/(N_{oc} \cdot \Delta x_{oc})$. The corresponding measurements and verifications using figure 18 are left to the reader as an exercise.

c) Numerical aperture of the objective and microscope exit pupil (or eyepoint)

The relationship between the object-side numerical aperture NA_{obj} and the diameter of the eyepoint $diameter_{e.p.}$ can be seen on the aperture ray tracing for an object located on the optical axis, (see figure 2 in the course section). The schematic view below show the main features:



Since the objective and ocular are assumed to be aplanetic for their respective optical conjugations, it is possible to apply the Abbe sine condition (see the basic course on geometric optics). For the objective, this implies that the numerical apertures in the object and image spaces (the object and image medium being air, of refraction index 1), $NA_{obj} = |\sin\alpha_0|$ and $NA_{im} = |\sin\alpha_i|$, are linked to the objective magnification, g_y , by :

$$NA_{im} = \frac{NA_{obj}}{|g_{y,obj}|}$$

In addition, the Abbe sine condition for the conjugation **focal point/infinity** (see basic course of geometric optics or reference [[Principles of Optics]]) of the eyepiece can be written (noting that the object-side numerical aperture of the eyepiece is identical to the image-side numerical aperture of the objective):

$$\frac{\phi_{e.p.}}{2} = f'_{oc} \cdot |\sin\alpha_i| = f'_{oc} \cdot NA_{im}$$

where f'_{ep} is the ocular focal distance.

By using the relation between the eyepiece angular magnification factor and its focal length ($G_{c,oc} \stackrel{def}{=} d_{norm}/f'_{oc}$), we obtain :

$$\phi_{e.p.} = (19,5 \pm 5,1\%) \times (42,9 \pm 0,5\%) = 837 \mu\text{m} \pm 5,6\% \approx 0,84 \text{ mm} \pm \sim 0,05 \text{ mm}$$

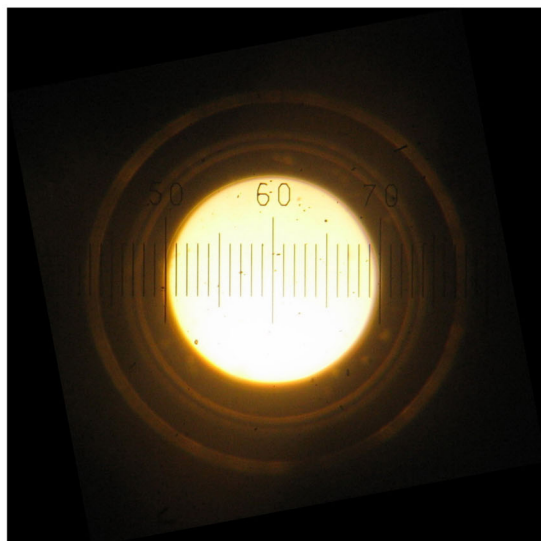
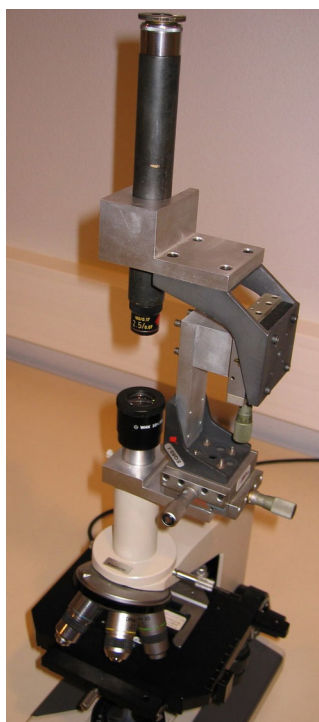
We note that the diameter of the microscope exit pupil is linearly proportional to the object-side numerical aperture of the objective and inversely proportional to the microscope angular magnification factor.

The eyepoint diameter can be measured using an additional calibrated viewfinder (see Fig.20 and Fig. 21a).

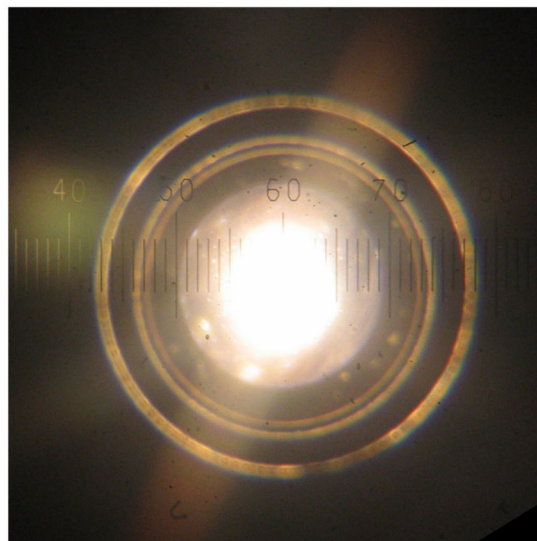
Attention

To make a correct measurement of the eyepoint diameter using an additional viewfinder, one has to open the aperture diaphragm of the Köhler illumination

condenser wide enough, otherwise it is the image of this diaphragm (which is formed in the same plane as the microscope exit pupil) that can be seen and measured with the viewfinder (see also figure 3 in the course section, where the various optical conjugations are indicated)



a



b

On figure 21a, it can be seen that the eyepoint diameter represents $19,5 \pm \sim 1$ intervals of the viewfinder scales. A preliminary calibration of the viewfinder using a micrometer shows that one interval on the viewfinder scale corresponds to $42,9 \mu\text{m} \pm \sim 0,5\%$. Consequently, the diameter of the microscope exit pupil is:

$$\phi_{c.o.} = (19,5 \pm 5,1\%) \times (42,9 \pm 0,5\%) = 837 \mu\text{m} \pm 5,6\% \approx 0,84 \text{ mm} \pm \sim 0,05 \text{ mm}$$

By using equation 6, one can obtain a value for the objective numerical aperture. As seen in the previous paragraph, the microscope angular magnification factor $G_{c, ep} \cdot |g_{y, obj}|$ is equal to $400(\pm 10)$ for the same objective/ocular pair, which allows us to obtain:

$$NA_{obj} = \frac{\phi_{e.p.} \cdot G_{c, oc} \cdot |g_{y, obj}|}{2 \cdot d_{norm}} \approx 0,67 \pm \sim 8\% \approx 0,67 \pm 0,05$$

with $d_{norm} = 250 \text{ mm}$.

This value is perfectly compatible with the indication 0.65 engraved in the mount of the 40x objective used here.

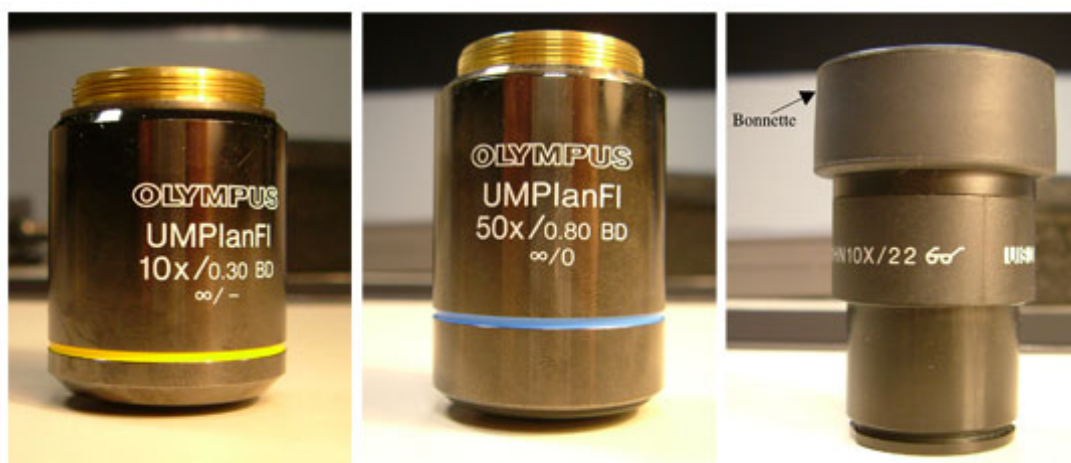
2. Utilization of a metallographic microscope

2.1. Presentation

The photography on figure 22 represents a professional microscope allowing the observation of opaque objects by reflexion using its episcopic Köhler illumination system. This high quality microscope works with *infinity-corrected* objectives and has a "trinocular" head allowing for a comfortable binocular observation together with high-quality photomicrography using the dedicated output equipped with a projective ocular.



Figure 23 shows photographs of two objectives and one eyepiece of this system:

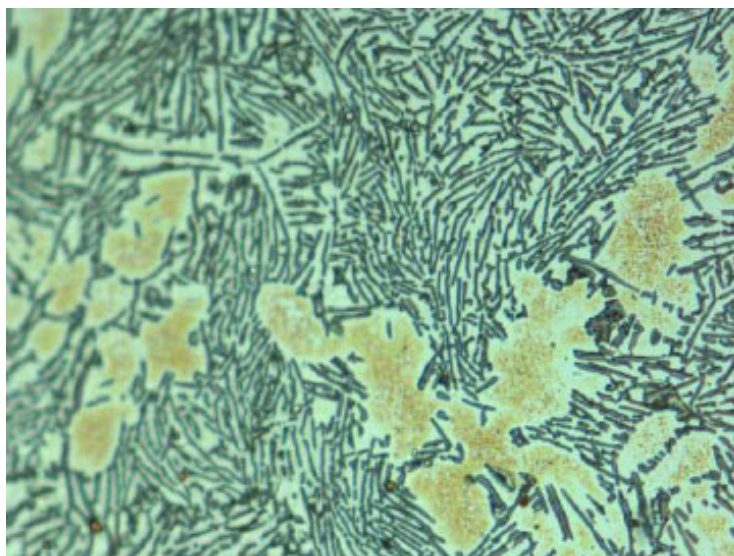
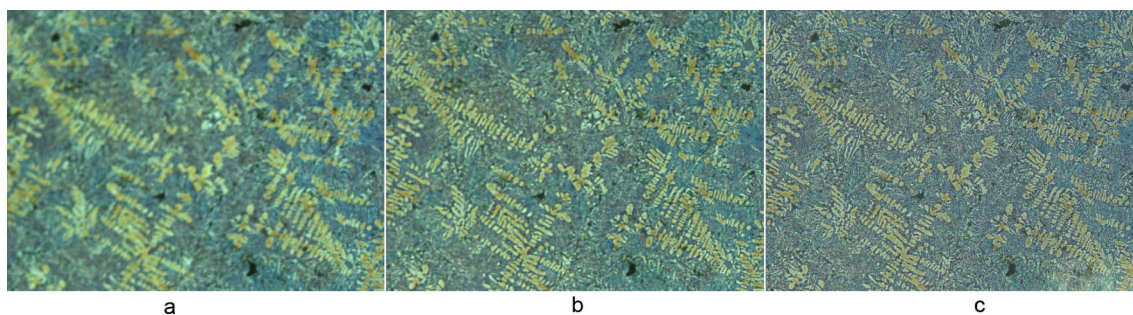


One can note the correction $\infty/0$ for the objective $50\times/0.80$, indicating that it is designed to work with a tube lens (∞) and without a coverslip (0), which is classical in episcopic microscopy. The indication $50\times$ represents the total magnification for the ensemble objective/tube lens. The indication $\infty/-$ for the objective $10\times/0.30$ means that it is also corrected for a 'infinite tube length'; nevertheless, its relatively small object-side numerical aperture (of 0.3) allows it to be used with or without coverslip. The two objectives, of model UM Plan FI, belong to the class *Plan-Achromatic* designed for a universal metallographic (UM) use; the mention FI indicates that they are specifically designed to be used in the near-ultraviolet thanks to the use of specific glasses (such as fluorine) transparent in this wavelength range and of very small intrinsic fluorescent (autofluorescence). The eyepiece $10\times/22$ has an angular magnification factor of 10, and is designed to observe an intermediate image of 22 mm. It is specifically designed to be used comfortably by an observer wearing eyeglasses (after folding the rubber eyecup), which means that the *eye relief* (the distance between the last ocular surface and the microscope exit pupil plane) is on the order of 25 mm.

2.2. Adjustments

The episcopic observation needs only small adjustments, because the Köhler illumination focus is preadjusted by the manufacturer to coincide with the objective focal plane. Therefore, the user only needs to turn on the illumination block, to adjust the micrometric ocular to its own sight, and to focus on the sample while being careful, as always, to avoid any collision between the objective and the sample (one should start with a sample several tens of millimeters below the objective frontal lens and then should move the sample away from the objective using the focus knobs). The user should then adjust the field diaphragm of the Köhler illumination system to illuminate only the observed region or the region of interest, if the latter is smaller, in order to limit the parasitic light. The last step is to optimize the focus on the studied objects.

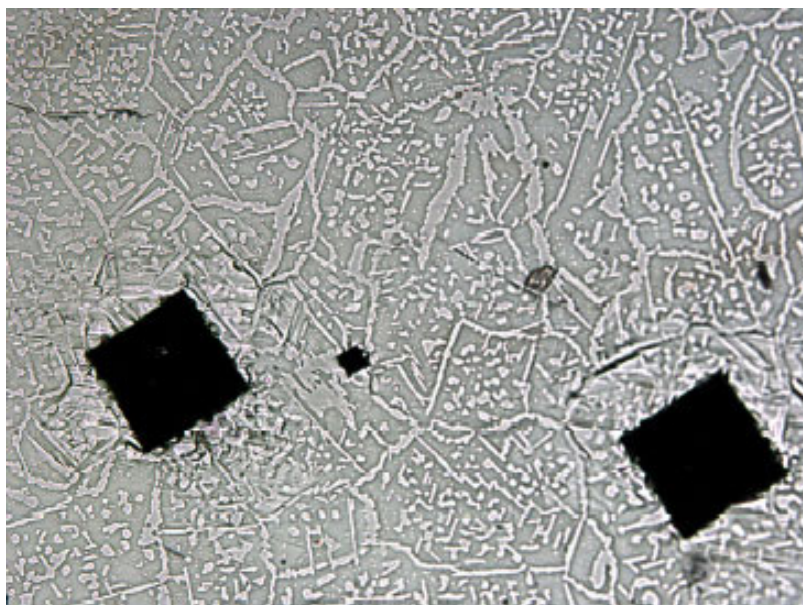
The user should also adjust the aperture diaphragm: indeed, if it open to its maximum the contrast is not optimum. For example, the contrast on photography 24 b) is much better than the contrast on photography a). In the case c), the aperture diaphragm is too closed.



On photography 25, it can be noted that during the slow primary cooling phase, there was formation of large grains corresponding to a solid Al(Si) solution, and then, the temperature keeping decreasing, the eutectic point (577°C) was reached with formation of silicon needles.

This analyzing process is used to observe polished planes of materials whose phases show differences in reflexion. The applications mainly relate to the analysis of metal alloy or corroded structures, and to hardness measurements.

To measure Vickers hardness, an imprint is made with a indenter under a given load during 15 s. The applied force range between 1 and 120 N. The diamond indenter has a pyramidal shape with a square basis and with opposite faces making a 136° angle.

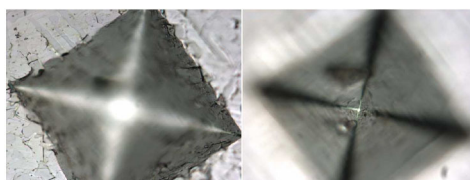


In the example presented on figure 26, a force of 49 N is applied during 15 s . The measurement of the imprints diagonals allows calculating the Vickers hardness of the welding bead. The mean value of the diagonals measurements give $185\ \mu\text{m}$, which corresponds to a Vickers hardness of :

$$HV_{49} = 0,1854 \frac{F}{d^2} = 265$$

The force F is expressed in newton, and the imprint diagonal d in millimeters. In this particular case, the obtained value corresponds to a semi-hard material.

With an objective of magnification 50 and of numerical aperture 0.80 , the sample surface and the imprint bottom cannot be simultaneously in focus (figure 27).



A microscope equipped with an objective $\times 50$ and an eyepiece of angular magnification 10 has a angular magnifying factor of 500 , which corresponds to a power of $2000\ \delta$, i.e. a focal distance of 0.5 mm :

$$f'_{micro} = \frac{1}{P_{micro}} = \frac{d_{norm}}{G_{norm}}$$

Where d_{norm} represents the minimum normalized distance of distinct viewing for a normal eye; it is equal to 25 cm .

From the value of the microscope focal length, we deduce that the depth of field for a normal eye of dioptric amplitude $a = 4\delta$ is on the order of $1\ \mu\text{m}$:

$$|A_{remotum} A_{proximum}| = |f'_{micro}| a = \frac{a}{P_{micro}^2}$$

A fine tuning of the microscope stage position allows measuring the order of magnitude of the imprint depth, which is of $\sim 20 \mu\text{m}$. We note that this order of magnitude is in very good agreement with the calculated value, since the penetration depth is linked to the diagonal measurement by the relation:

$$H = \frac{d}{7} = \frac{185}{7} = 26 \mu\text{m}$$

$$H = \frac{d}{7} = \frac{185}{7} = 26 \mu\text{m}$$

2.3. Resolution limit

With the objective $50\times/0.80$ (i.e. of object-side numerical aperture 0.80), the object-space spatial frequency cutoff for a coherent illumination and a wavelength of $0.55 \mu\text{m}$ (in the middle of the green spectrum) is $0.80/(0.55 \mu\text{m}) \simeq 1450 \text{mm}^{-1}$. For a completely incoherent illumination it reaches the double, i.e. $\sim 2900 \text{mm}^{-1}$. Sinusoidal test patterns that would allow to directly test those limits should therefore have periods of respectively $\sim 0.70 \mu\text{m}$ et $\sim 0.35 \mu\text{m}$, which is totally unfeasible. For a completely incoherent illumination, the resolution limit in the sense of Rayleigh criterion is, by definition, equal to the radius of the Airy pattern in the intermediate image given by the objective and the tube lens (since the eyepiece doesn't bring any additional limitation):

$$r' = \frac{1,22\lambda}{(2NA_{im})}$$

where NA_{im} is the **image-side** numerical aperture of the system objective + tube lens. To bring this value back to the object space, one needs to use the Abbe sine condition applied to the optical conjugation *object/intermediate image* of the system objective + tube lens, which is supposed to be aplanetic; if NA_{obj} is the object-side numerical aperture of this system:

$$\frac{NA_{obj}}{NA_{im}} = |g_y|$$

and we obtain in the object space:

$$r = \frac{1,22\lambda}{2NA_{obj}}$$

The numerical application gives $0.42 \mu\text{m}$ for the objective $50/0.80$ in the visible, in good agreement with the indication in the manufacturer datasheet.

3. Digital photomicrography

3.1. Digital Photomicrography

We wish to digitally record with a CCD or CMOS sensor the images obtained with a microscope, for example equipped with an objective $10\times/0.25$ or $40\times/0.70$ designed to work with an eyepiece of field number 22. We have to form a real image of the observed object at a finite distance, on the sensor. In that goal, we can either directly use the intermediate image formed by the objective (and the tube lens, if there is a tube lens), or use an additional optic called **projective ocular** which will make another image of this intermediate image. The first solution is rarely chosen on the current microscopes for various practical reasons: it can be mechanically difficult to access the intermediate image, it can be preferable to *simultaneously* perform standard visual observation and digital acquisition, the intermediate image might have some residual aberrations,...; therefore we will only consider the second solution, and we will look at the required characteristics for the projective ocular. The problem is to find a good compromise between resolution and observed field. Indeed, the image formed by the sensor must, rigorously, follow the Shannon sampling theorem to be correctly registered without artifacts ('aliasing'). This means that the image spatial cutoff frequency must be smaller than the sensor Nyquist frequency, i.e. to half its sampling frequency. Noting $g_{y, ocProj}$ the magnification of the projective ocular in the conjugation : *intermediate image* \rightarrow *sensor*, one must have :

$$\frac{2 \cdot NA_{obj}}{\lambda} \times \frac{1}{|g_y| \cdot |g_{y, ocProj}|} \leq F_{NyquistSensor} = \frac{F_{Sampl.Sensor}}{2}$$

For a standard 'video' sensor of format $\frac{1}{2}$ inch (i.e. of dimensions $6.4mm \times 4.8mm$), with 752×582 pixels, and of pixel pitch $\sim 9\mu m$, the sensor spatial sampling frequency is $\sim 110\text{ mm}^{-1}$ and its Nyquist frequency is $\sim 55\text{ mm}^{-1}$. Noting that the cutoff frequency of the intermediate image, $2 \cdot NA_{obj}/(\lambda \cdot |g_y|)$, is $\sim 90\text{ mm}^{-1}$ for the objective $10\times/0.25$ and 65 mm^{-1} for the objective $40\times/0.70$, we therefore deduce that $|g_{y, ocProj}|$ must be close to (or larger than) ~ 1.6 in the first case, and ~ 1.2 in the second case. In the first case, for the sensor to cover the same field of view than the one seen with the ocular of field number 22, it must have a width of $1,6 \times 22\text{ mm} \sim 35\text{ mm}$! Thus, with a video sensor $1/2''$ of width 6.4 mm , the observed image linear dimension cannot exceed, respectively, a fifth or a fourth of the image observed with the eye ($1/25$ or $1/16$ if we speak about surfaces) in order to not undersample the image and to benefit from the objective full resolution.

For a digital camera sensor of 8 Mpixels (e.g. 2448×3264) of dimensions $\sim 10.8\text{ mm} \sim 8.1\text{ mm}$ ($\sim 13.5\text{ mm}$ on the diagonal) and of pixel pitch $\sim 3.3\mu m$, the spatial sampling frequency is $\sim 300\text{ mm}^{-1}$ and the Nyquist frequency $\sim 150\text{ mm}^{-1}$. The projective ocular magnification, $|g_{y, ocProj}|$, must therefore be on the order of (or larger than) ~ 0.61 for the objective $10\times/0.25$, and ~ 0.43 for the objective $40\times/0.70$ to respect Shannon sampling theorem. The field-of-view of diameter 22 mm in the intermediate image space would therefore be projected onto dimensions of 13.4 mm and 9.5 mm (respectively), which are smaller than the sensor dimensions. In that case, the magnification will be increased so that the circular image covers the rectangular sensor, which means that the intermediate image field will be matched with the diagonal of the sensor; a projective ocular magnification of $13.5/22 \sim 0.62$ would therefore be well-adapted for the two objectives considered here.

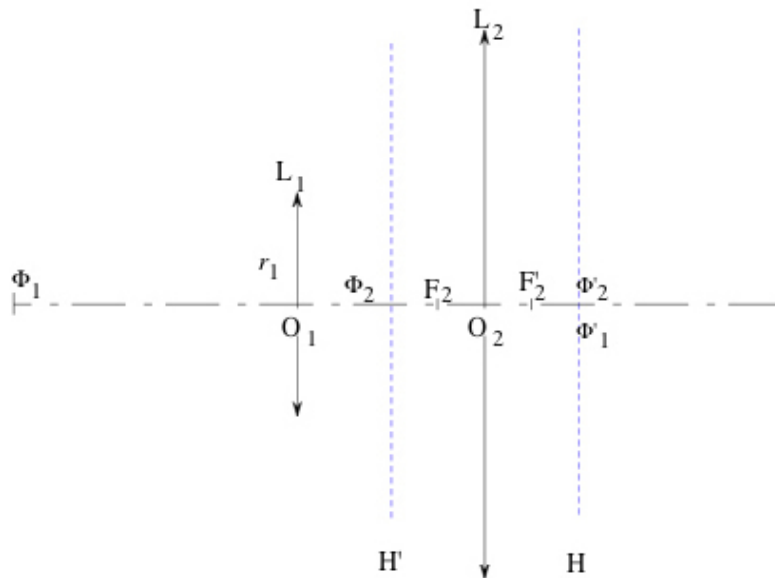
IV. Exercises

1. Exercises

A microscope is made of an objective that we assume to be a thin lens of focal length $f' = 5.25 \text{ mm}$ and a Huygens eyepiece (3,2,1) of focal length $f'_{oc} = 16.5 \text{ mm}$.

The characteristics of such an eyepiece are summarized on figure 28.

- ϕ_1 and ϕ'_1 are the object and image focal points of the field lens L_1 , of focal length f'_1 .
- ϕ_2 and ϕ'_2 are the object and image focal points of the eye lens L_2 , of focal length f'_2 .
- F_2 and F'_2 are the object and image focal points of the eyepiece.



We note that the 'symbol' (m, p, q) characterizes an ocular with two thin lenses by $\frac{f'_1}{m} = \frac{e}{p} = \frac{f'_2}{q} = \epsilon$ where e is the interstice of the doublet $\overline{O_1O_2}$. For a Huygens ocular (3,2,1),

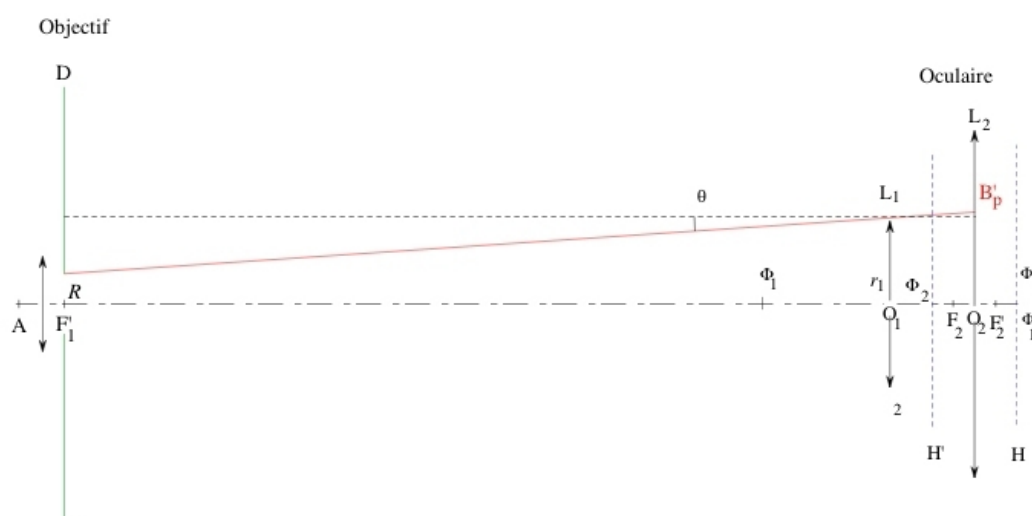
we have the particular properties: $f'_{oc} = \frac{3}{2}\epsilon$, $\overline{O_1O_2} = 2\epsilon = \frac{4}{3}f'_{oc}$, $\overline{O_1F_2} = \frac{3}{2}\epsilon = f'_{oc}$ and $\overline{O_2F'_2} = \frac{\epsilon}{2} = \frac{1}{3}f'_{oc}$,

with, in this case $\epsilon = \frac{2}{3} \times 16.5 \text{ mm} = 11.0 \text{ mm}$.

The schematic view of the whole microscope is represented on figure 29.

The radius of the eyepiece lens L_1 is $r_1 = 6 \text{ mm}$, and the radius r_2 of lens L_2 is large enough so that no ray passing through L_1 is stopped by L_2 . The image of an object point A by the objective is a point A' that coincides with the object focal point F_2 of the eyepiece. In the air, the objective is aplanetic for the couple of points (A, A') located on the optical axis. The numerical aperture is limited by the circular diaphragm D located at the image focal plane of the objective, and is equal to 0.65.

The microscope optical interval, i.e. the distance between the objective image focal point F'_1 and the eyepiece object focal point F_2 , is $\Delta = 210 \text{ mm}$. This value is used in some metallographic microscopes of tube length 230 mm .



Question 1

[Solution n°1 p 38]

Why are we using the configuration where the objective image is located at the eyepiece object focal point F_2 ?

Question 2

[Solution n°2 p 38]

What does the sentence « the objective is aplanetic for the couple of points (A, A') » mean?

Question 3

[Solution n°3 p 38]

Calculate the objective magnification.

Question 4

[Solution n°4 p 38]

What is the value of the intrinsic optical power of the full microscope?

Question 5

[Solution n°5 p 38]

Calculate the microscope angular magnifying factor; express it as a function of the absolute value of the objective transverse magnification $\mathcal{G}_{y, obj}$ and the eyepiece angular magnification $G_{c, oc}$

Question 6

[Solution n°6 p 39]

Determine the radius R of the aperture diaphragm D ; deduce the position and radius R' of the exit pupil. What is the value of the eye relief? Is it large enough to allow for an observation with eyeglasses?

Question 7

[Solution n°7 p 40]

Determine the "full-light" field in the object space (i.e. the field for which the whole cone of light rays entering the pupil gets through the microscope without being stopped), the

corresponding angular field in the image space, the location and radius of the diaphragm that could be inserted to suppress the vignetting effect at the periphery of the total field.

Question 8

[Solution n°8 p 41]

Evaluate the focusing range.

Solution des exercices

>Solution n°1 (exercice p. 36)

In order to not tire the eye, the observer adjusts the eyepiece position so that the image given by the whole microscope is at infinity. The intermediate image given by the objective is therefore located at the front focal plane of the eyepiece.

>Solution n°2 (exercice p. 36)

This means that the two frontal planes passing through points A and A' are conjugated, i.e. that the Abbe sine condition is verified for this pair of planes.

>Solution n°3 (exercice p. 36)

The system studied here is the objective, with a pair of conjugate planes passing through points A and A' chosen as origins; the transverse magnification of this system corresponds to the nominal magnification of the objective $g_{y, obj}$:

$$\overline{A'F'_1} = g_{y, obj} f'_1$$

i.e. :

$$g_{y, obj} = \frac{\overline{A'F'_1}}{f'_1} = \frac{\overline{F_2F'_1}}{f'_1} = \frac{-\Delta}{f'_1} = \frac{-210}{5,25} = -40$$

The "minus" sign means that the image located at A' is inverted.

>Solution n°4 (exercice p. 36)

Applying the relations defining the power and intrinsic power, the microscope optical power can be expressed in the following way:

$$P_{i, micro} = \frac{\Theta''}{AB} = \frac{1}{f'_{micro}}$$

where f'_{micro} represents the image focal distance of the microscope, that is to say of the ensemble eyepiece-objective; θ'' is the angle through which the final image is seen, and AB represents the object size. Introducing the intermediate image size $A'B'$, we obtain:

$$P_{i, micro} = \frac{\Theta''}{AB} \cdot \frac{A'B'}{AB} = P_{i, oc} \cdot g_{y, obj}$$

$P_{i, oc}$ represents the ocular intrinsic power. The microscope intrinsic power $P_{i, micro}$ is equal to the product of the objective magnification and the ocular intrinsic power.

Expressing the ocular intrinsic power as a function of its focal length f'_2 , we obtain:

$$P_{i, micro} = g_{y, obj} \cdot \frac{1}{f'_2} = 40 \cdot \frac{1}{16,5 \cdot 10^{-3}} = 2424\delta$$

>Solution n°5 (exercice p. 36)

The angular magnifying factor is given by:

$$G_{c, micro} = P_{i, micro} \cdot d_{norm} = 2424 \times 0,25 = 606$$

or

$$G_{c, micro} = g_{y, obj} \cdot d_{norm} P_{i, oc} = g_{y, obj} \cdot G_{c, oc}$$

The microscope angular magnifying factor $G_{c, micro}$ is equal to the product of the objective transverse magnification and the ocular angular magnifying factor. Those two values are indicated on the objective and ocular mounts.

>Solution n°6 (exercice p. 36)

By applying the Abbe sine condition to the couple of points A and A' :

$$n \cdot \overline{AB} \cdot \sin \alpha_m = n' \cdot \overline{A'B'} \cdot \sin \alpha_i$$

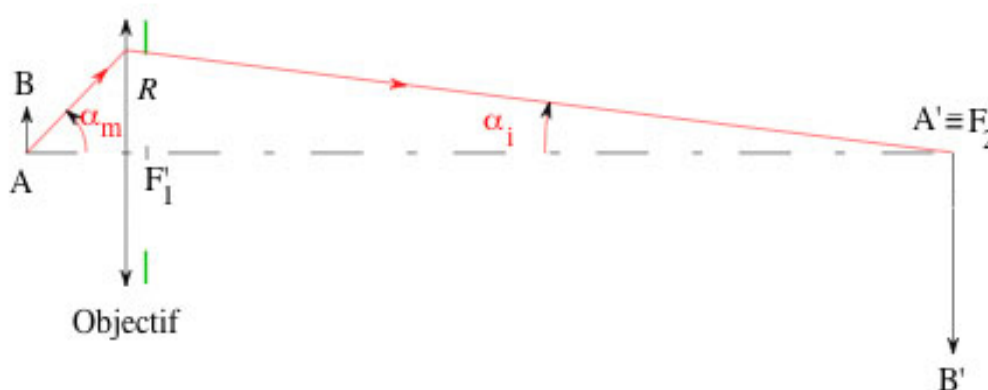
we obtain:

$$\sin \alpha_i = \frac{n}{n'} \frac{\overline{AB}}{\overline{A'B'}} \sin \alpha_m = -\frac{\sin \alpha_m}{g_{y, obj}}$$

since, here, $n = n' = 1$.

On figure 30, we note that:

$$\tan \alpha_i = \frac{-R}{F'_1 F_2} = \frac{-R}{\Delta}$$



For a microscope, the value of the angle α_m is large, since the numerical aperture of an objective is generally as large as possible in order to see the object details with a great resolution. By contrast, the angle α_i is small (in absolute value) according to formula 1, because of the division by the absolute value of the objective magnification (40, in this case); consequently, we can make the approximation $\tan \alpha_i \approx \sin \alpha_i$, from which we deduce :

$$\frac{R}{\Delta} \approx \frac{\sin \alpha_m}{g_{y, obj}} = \frac{NA_{obj}}{g_{y, obj}}$$

i.e.:

$$R = \frac{\Delta \cdot NA_{obj}}{g_{y, obj}} = \frac{210 \times 0,65}{40} \approx 3,4 \text{ mm}$$

The eyepoint (i.e. the microscope exit pupil) is the image D' of diaphragm D given by the eyepiece. We can apply Newton's equation to the pair of points (D, D') :

$$\overline{DF_2} \cdot \overline{D'F'_2} = f f' = - (f'_{oc})^2$$

We therefore obtain, noting that $\overline{DF_2} = \Delta$:

$$\overline{F'_2 D'} = \frac{(16,5)^2}{210} = +1,3 \text{ mm}$$

The eyepoint and the image focal plane of the eyepiece are always very close to each other.

Since the eye pupil must be placed on the eyepoint for an optimal observation without vignetting, it is interesting to determine the *eye relief*, that is to say the distance between the eyepoint and the ocular eye lens. By using a property of a Huygens ocular (3,2,1) : $O_2F'_2 = f'_{oc}/3$ we deduce the value of the eye relief:

$$\overline{O_2D'_2} = \overline{O_2F'_2} + \overline{F'_2D'} = \frac{16,5}{3} + 1,3 = 6,8 \text{ mm}$$

This value is **very small, not large enough** to allow an observation with eyeglasses. This is one of the drawbacks of Huygens oculars, which are replaced on current high-quality microscopes with more complex combinations of 4 to 5 lenses, with an eye relief far larger.

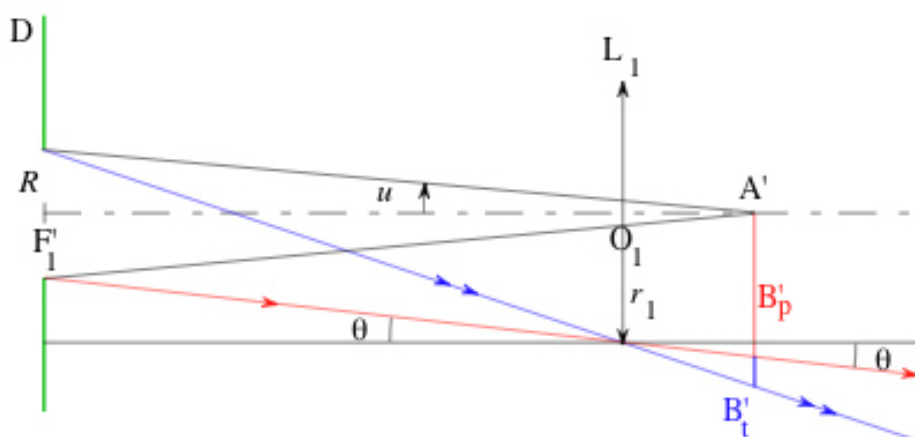
The exit pupil radius can be inferred by the magnification formula applied to the pair of points D, D' :

$$g_{y,oc}(D,D') = -\frac{\overline{F'_2D'}}{f'_{oc}} \approx -\frac{1,3}{16,5} \approx -0,079$$

from which we deduce a radius of 0.27 mm for the eyepoint. One can note that the microscope exit pupil is smaller than the eye pupil (diameter $\sim 2 \text{ mm}$ for an observation by bright daylight); consequently the eye doesn't introduce any additional limitation, and the system aperture is really determined by the objective numerical aperture.

>Solution n°7 (exercice p. 36)

The microscope intermediate space containing the diaphragm D , the intermediate image $A'B'$ and the eyepiece field lens L_1 is represented in figure 31. An object A located on the optical axis gets imaged in A' . If the object moves away from the optical axis, its images moves towards B'_t . A ray that would make a higher angle with the optical axis than the blue ray (on the figure) would be stopped by the eyepiece field lens. The lens L_1 is therefore the field diaphragm since by hypothesis no ray going through L_1 can be stopped by L_2 . Since the aperture diaphragm and the only field diaphragm are located between the objective and the eyepiece field lens, it's easy and natural to choose this intermediate space to determine the "full-light" field .



On figure 31, the rays traced in red and blue correspond, respectively, to the limit of the "full-light" field and the total field. We can apply the trigonometric relations:

$$\tan \Theta = \frac{A'B'_p - r_1}{O_1A'} = \frac{r_1 - R}{F'_1O_1}$$

Recalling that $\overline{F_1 F_2} \stackrel{def}{=} \overline{F_1 A'} \stackrel{def}{=} \Delta$ and using the Huygens ocular properties $\overline{O_1 A'} = \overline{O_1 F_2} = f'_{oc}$ (figure 29), we obtain:

$$A'B'_p = \frac{r_1 - R}{F'_1 O_1} \cdot O_1 A' + r_1 = \frac{r_1 - R}{\Delta - f'_{oc}} \cdot +r_1 \approx \frac{6 - 3,4}{210 - 16,5} \cdot 16,5 + 6 \approx 6,22\text{mm}$$

In the object space:

$$|AB_p| = \frac{A'B'_p}{g_{y,obj}} = \frac{6,22}{40} = 0,155\text{mm}$$

The diameter of the object-side "full-light" field is therefore equal to the double of this value, i.e. roughly 0.31 mm . To determine the "full-light" field in the microscope image space, we can calculate the half-angle field for the ocular, corresponding to $A'B'_p$:

$$\Theta''_{1/2} = \arctan\left(\frac{A'B'_p}{f'_{oc}}\right) \approx 20,6^\circ$$

We can deduce that the "full-light" field in the image space, θ' , is roughly equal to 41.2 .

To suppress the contour field (i.e. the darkening region at the periphery of the full field), a diaphragm must be inserted at a real image location; it is not possible to insert it at the image given by the objective since this is a virtual image located behind the ocular field lens. Therefore, the diaphragm is inserted at the image A' given by the field lens. The thin lens conjugation equation applied to the field lens L_1 gives:

$$\frac{1}{\overline{O_1 A''}} = \frac{1}{\overline{O_1 A'}} + \frac{1}{O_1 \Phi'_1}$$

where A'' is the image of A' given by L_1 ; using the properties of a Huygens ocular that were mentioned in the introduction to the exercises, and by noting that A' is superimposed with F_2 , we calculate a distance of $+11\text{ mm}$ for $\overline{O_1 A''}$.

The transverse magnification associated with the couple of planes going through A' and A'' is equal to:

$$g_{y, L_1 (A', A'')} = \frac{\overline{O_1 A''}}{\overline{O_1 A'}} \approx 0,67$$

The diaphragm must therefore have a radius r corresponding to the "full-light" field calculated in the intermediate space located between the field lens and the eye lens of the ocular:

$$r = g_{y, L_1 (A', A'')} \cdot A'B'_p \approx 4,17\text{mm}$$

>Solution n°8 (exercice p. 37)

The focusing range represents the distance from which the object can be axially displaced without significantly damaging the image. It depends on the observer dioptric amplitude (i.e. its own focusing range), and of the focal distance of the considered system:

$$|\overline{A_{\text{remotum}} A_{\text{proximum}}}| = |f f'| a = \frac{a}{(P_{\text{micro}})^2} \approx 0,68\mu\text{m}$$

for a user "accommodation" power (focusing range) of 4δ .

This focusing range decreases when the microscope optical power increases.

Glossaire

Abbe number

The Abbe number, also known as the V-number or constringence evaluates the material dispersion. It is measured with the Abbe number, which can vary between 20 and 90. The smaller this number and the more dispersive the material.

Coverslip

Thin glass slide placed on top of a preparation in order to protect it. It has a normalized thickness of 0.170mm.

Diascopic light microscopy

Transmitted light microscopy, used to observe transparent object (with transmission illumination).

Episcopic light microscopy

Reflected light microscopy, used to observe opaque objects (with a reflected illumination).

Eyepoint or Ramsden disc

Exit pupil of the microscope, located at one or two centimeters behind the last surface of the eyepiece, and where the eye pupil has to be placed.

Eye relief

Distance between the microscope exit pupil and the ocular surface closest to the eye.

Objective

Converging optical system forming a magnified image of the object under observation. It usually has a large numerical aperture (in the object space) so that the microscope can resolve details of size close to the wavelength.

Ocular or Eyepiece

Converging optical system that transforms the intermediate image formed by the objective into a virtual image located at the infinity to be observed by the eye.

Resolution Power

Capacity of an optical instrument to distinguish between two objects that are really close to each other. The higher the resolution power, and the smaller the resolution limit (the distance between the two objects).

Working Distance

Distance between the object under observation and the frontal lens of the objective. It is usually of several tenths of millimeters for the current high magnification objectives.

Bibliographie

[Confocal microscopy] LICHTMAN J., *La microscopie confocale*, POUR LA SCIENCE, 1994octobre-, n° n° 204.

[Ein neues Beleuchtungsverfahren für mikrographische Zwecke] KÖHLER A., *Ein neues Beleuchtungsverfahren für mikrographische Zwecke* (p.p.244++), Z. Wiss. Mikr, 1893--, n° 67H4, .

[Encyclopedia of Engineering techniques] ROBLIN G., *Encyclopédie des Techniques de l'Ingénieur*, -, -, 1999, *Microscopie optique* (p.tome R7 R6712 (+ R6710 + R6714)), .

[Geometric and wave optics] PÉREZ JOSÉ-PHILIPPE, *Optique géométrique et ondulatoire*, Masson, Paris, 1994.

[Instrumental optics, Fourier optics] SURREL JOËLLE, *Optique instrumentale Optique de Fourier*, Ellipses, Paris, 1996.

[Introduction to Fourier Optics and holography] GOODMAN JOSEPH W., *Introduction à l'optique de Fourier et à l'holographie*, Traduction française de "Introduction to Fourier Optics", MASSON & Cie, Paris, 1972 (2006 3ème éd. anglaise).

[Introduction to the microscope : basic principles and utilisation] WASTIAUX G., *Initiation au microscope: bases pratiques et utilisation*, Lavoisier, -, 2001.

[Modern optical microscopy] WASTIAUX G., *La microscopie optique moderne*, Lavoisier, -, 1994.

[Optics] HEICHT EUGÈNE, *Optique*, Pearson, Paris X, 2005.

[Principles of Optics] BORN MAX, WOLF EMIL, *Principles of Optics*, 7th Ed., PERGAMON PRESS, Oxford - New York - Toronto - Sydney - Paris - Frankfurt, 1999.

Webographie

[] <http://www.molecularexpressions.com/> (consultation - - 2007).

[] <http://www.olympusmicro.com/> (consultation - - 2007).