Contrast techniques in microscopic observation

Hervé SAUER - Joëlle SURREL

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Table des matières

I.Presentation

Module :

Optique instrumentale, microscopie

Author(s):

Hervé SAUER[1]&*Joëlle SURREL*[2] - Institut Optique Graduate School & IUT St Étienne

Abstract:

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Keywords:

Microscope polarisant, éclairage orthoscopique, éclairage conoscopique, optique de Fourier, contraste de phase, strioscopie, fond noir, objectif de Mirau, contraste interférentiel différentiel (DIC), prisme de Wollaston, prisme de Nomarski, épifluorescence

Prerequisites:

Fonctionnement de base du microscope et de son éclairage comme donné par le grain M11G1 ainsi que des bases avancées d'optique (optique géométrique, optique physique, interférences, polarisation, biréfringence, optique de Fourier).

Learning outcomes:

Appréhender les techniques qui améliorent le contraste des images d'objets microscopiques usuels en utilisant la polarisation, le filtrage des fréquences spatiales, les interférences ou encore la fluorescence. (Principes physiques et mise en œuvre pratique sur les microscopes commerciaux actuels).

Course overview:

- Introduction
- Le microscope polarisant
- Fond noir et contraste de phase
- Techniques interférométriques
- Microscopie de fluorescence
- Conclusion

Design & production:

Le Mans Université

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1 - herve.sauer@institutoptique.fr
2 - joelle.surrel@univ-st-etienne.fr
3 - http://www.gnu.org/licenses/fdl.txt

II.Cours

Many microscopic specimens of biological or technological interest are transparent or of similar reflectivity as their environment; so when they are observed by means of microscopes, they form invaluable quasi-smooth images. It is possible to use stain methods or « *chemical* » developments, but these techniques are difficult to perform and unavoidably modify the specimens observed(alteration of the metabolism of the living cells, irreversible corrosion of surfaces, etc.)

But those « *imperceptible* » raw objects, if they do not really modify the illumination light intensity they transmit or reflect, usually change other properties of illumination light such as phase or polarization. Suitable systems placed in microscopes can then transform these variations, undetectable by the human eye and the usual detectors, into visible modulations of light intensity. Some methods are now very « *common* » and the required systems are offered by all large microscope manufacturers, for transmitted light observation (*diascopy*, or « *biological microscopes* ») and for reflection light observation (*episcopy*, or « *metallographic microscopes* »).

Contrast techniques are frequently performed for several types of observations; they are even essential in several scientist, technical and industrial fields, from routine medical analysis to controls of cutting edge technology materials. Therefore, it is very important to know these techniques and to understand the different and interesting inherent optical methods to which they refer. The goal of unit 2 in module 11 of "*Optical microscopy*" is to present the main elements of that field. It follows unit 1 "Principles and basic utilization of a microscope" in the same module, and refers to it concerning definitions and basic concepts in microscopy which will not be recalled here.

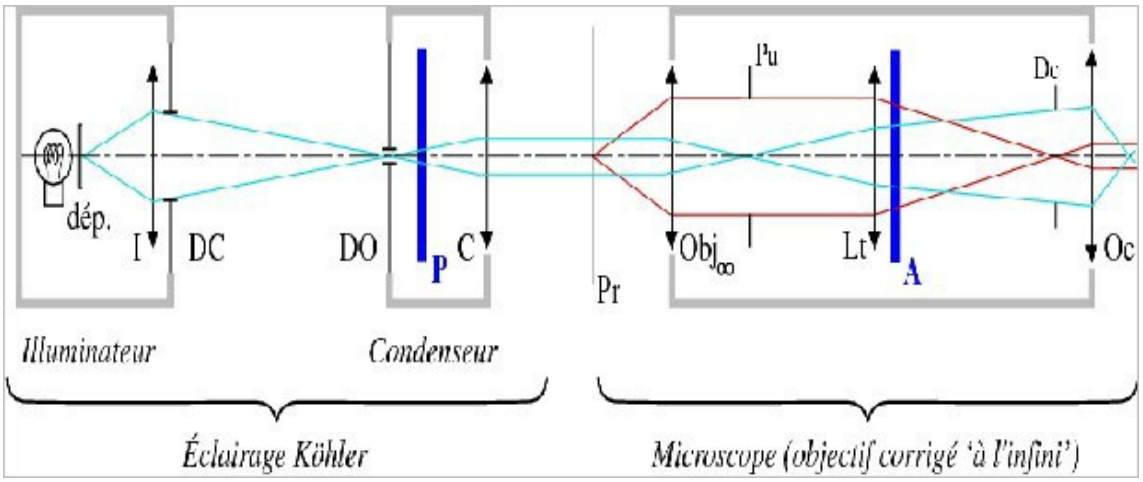
1. Polarized light microscopes

Numerous specimens can modify, by birefringence, rotative power, dichroism, diffusion, etc, the polarization of the incident, transmitted or reflected light. Then, underlining and differentiating, and even identifying the different parts of a heterogeneous specimen by precisely measuring their optical properties can be very useful. For example, thin crystal sections would often be observed to identify chemical or geological materials, and metals or polycrystalline materials polished surfaces would be observed for metallographic characterization. Several polarization methods, of different levels of complexity, can be performed depending on the aim of the experimentation: visualization or identification.

1.1. Transmission light observations

a) Orthoscopic light microscopy

This easy-performing method permits underlining and differentiating birefringent transparent materials. The light beam, which is collimated and at a perpendicular angle to the specimen surface (so called orthoscopic light), is polarized, in main cases linearly, by a polarizer inserted in the light unit; an analyzer is placed in the microscope, above the preparation. For all practical purposes, the collimated illumination would be carried out by Köhler illumination with a very small aperture diaphragm. *(see unit UNIT M11G1). See Figure 1.*



dép.: *Dépoli* / frosted glass

I: *Optique de l'illuminateur* / *optics of the illuminator.*

Pr: *Préparation (transparente) observée, sur porte échantillon orientable* / Observed preparation (transparent), on adjustable stage.

DC: *Diaphragme de Champ (très fermé)* Field diaphragm (very closed)

Obj.: *Objectif du microscope (corrigé à l'∞)* Infinity-corrected objective of the microscope

DO: *Diaphragme d'Ouverture* Aperture diaphragm

Pu: *Pupille de l'objectif* Objective pupil

Dc: *Diaphragme de champ de l'oc.* Eyepiece field diaphragm

P: ***Polariseur (orientable)*** **Polarizer (adjustable)**

Lt: *'lentille' de tube* Tube lens

Oc: *Oculaire* Eyepiece

C: *Optique du condenseur* optics of the condenser.

A: ***Analyseur (orientable)*** **(Adjustable analyzer)**

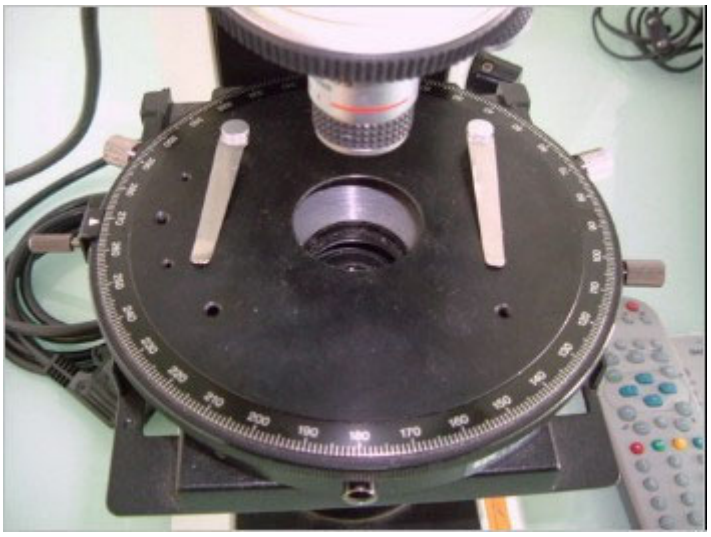
Illuminateur: Illumination system

Condenseur: Condenser

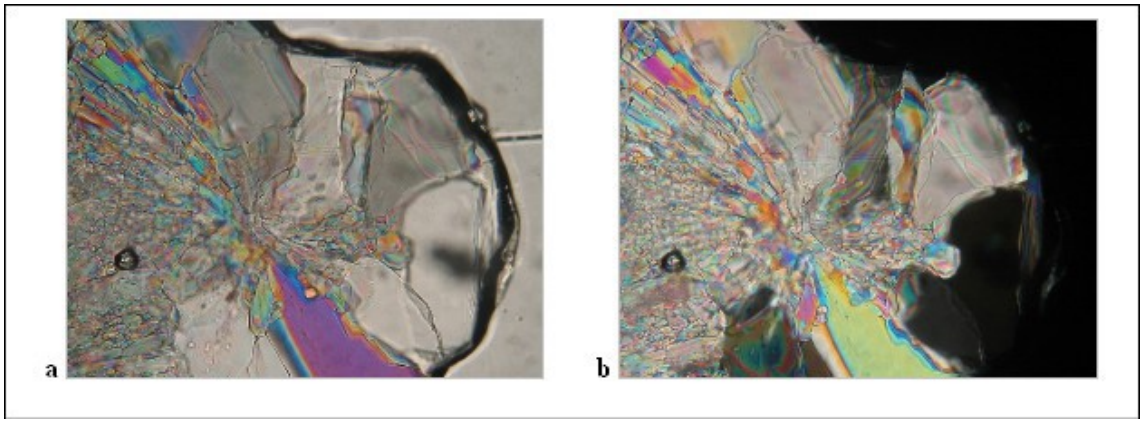
Microscope (*objectif corrigé "à l'infini"*): Infinity-corrected microscope

Eclairage köhler : Köhler illumination

The polarizer, analyzer, and preparation would be precisely and independently adjustable around the microscope optic axis by means of appropriate mechanisms on the stand. (see Figure 2).



Most of the time, birefringence of the objects, which are observed with polarized light microscope, would form, in white light, some distinct colors permitting to distinguish and locate the various components of the specimen (Figure 3). The colors observed are due to the appearance of fluted spectra that interfere with polarized light. When neutral lines of the specimen's homogeneous regions neutral lines are oriented to $\pm 45°$ to the polarizer, and the analyzer is crossed or parallel to the polarizer, then the visible color would be one of Newton shades 1. (Refer to lessons about physical optics and birefringence and polarization, for example [Born 1999 [Principles of Optics]]).



b) Materials identifications (‘quantitative polarization with orthoscopic illumination')

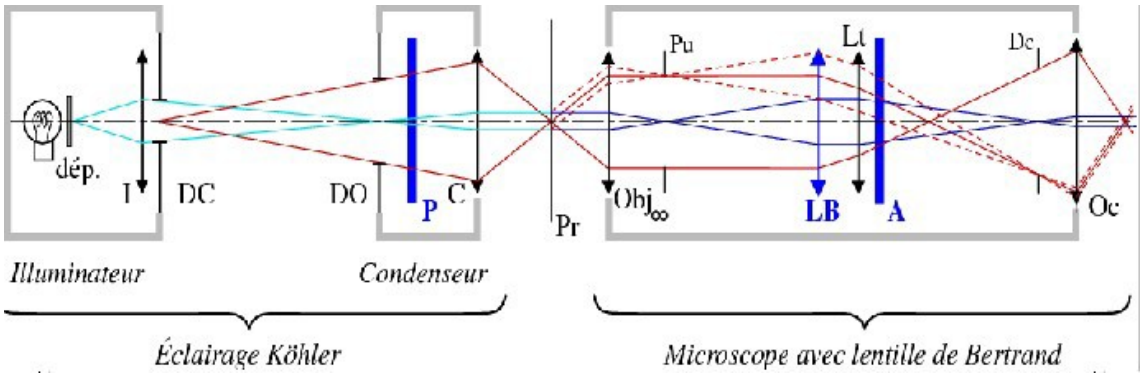
When the observed specimen is a thin slide with polished parallel faces of a known thickness, you can describe it more in detail. Indeed, all homogeneous anisotropic material slides of parallel faces, whatever their uniaxial or biaxial natures and their main axis orientations are, act as retardation slides, of which characteristics (neutral lines orientations and optical path differences between both linearly polarized plane waves to every neutral lines) depend on material birefringences and orientations (see [Born 1999 [Principles of Optics]]). With orthoscopic illumination, polarized light microscopes are used as polariscopes and all standard methods of polarization analysis can be performed. So you can use a **de Senarmont** compensator (which is constituted by a $\lambda/4$ slide and a polarizer), a **Soleil** compensator, a **Babinet** compensator, etc. The measurement obtained can be very useful to analyze or even to identify the specimen's nature. However, quantitative polarization microscopy is very

delicate work and requires highly specialized equipment (middle or top-of-the-range microscopes *with suitable optional accessories*). Above all, to make good quality measurements, it is necessary to work with particular objectives, called "strain free"objectives, whose lenses, made by specific methods, are mounted on particular opto-mechanical barrels which avoid stress, so as not to have inferred birefringence which would give false results

Among these methods, the one known as ***« Newton shades »***is the easiest to perform and it is actually used very often in geology to identify rocks by means of a polished section of known thickness. This method will be described more in details in the *Case study* section of this unit M11G2.

c) Conoscopic light microscopy

Conoscopic light microscopy is performed as a complement to the orthoscopic light method to characterize birefringent materials on thin slides with parallel faces, because knowing the neutral axis orientations and path difference numerical values between both related polarized rays would not be sufficient to identify materials without any uncertainty. This method consists in illuminating a uniform region of the specimen with a cone-shaped polarized light (so-called conoscopy) and then in observing through an analyzer the interferences said to be "in convergent light" which are formed at infinity or at the focus of the objective [Born 1999 [Principles of Optics]]. For practical purposes, the cone-shaped light beam should be made by **Köhler** illumination with a very open aperture diaphragm; the field diaphragm should be closed enough to limit the illumination to the chosen region in the middle of the field. The interferences figure in convergent light will appear on the image focus of the objective of the microscope. Observing the interferences figure through the eyepiece of the microscope requires adding a convergent objective, called "Bertrand lens", to the body of the microscope, which will project the interferences figure at infinity (Figure 4). It is important to highlight that this method does not give an image of the preparation but a complex figure (Figure 5cd) which will permit the characterization of birefringent properties of the specimen's uniform region which had been chosen with orthoscopic illumination.



dép.: *Dépoli* / **Frosted glass**

Pr: *Préparation (transparente) observée* **Observed preparation (transparent)**

I: *Optique de l'illuminateur* **Optics of the illuminator**

Obj.: *Objectif du microscope (corrigé à l'∞)* **Microscope infinity-corrected objective**

DC: *Diaphragme de Champ (très fermé)* **Field diaphragm (very closed)**

Pu: *Pupille de l'objectif (plan focal image)* **Objective pupil (focal image plane)**

DO: *Diaphragme d'Ouverture* **Aperture diaphragm**

LB: ***'lentille' de BERTRAND (amovible)***, son foyer objet coïncide avec Pu. **Removable Bertrand lens; its focus coincides with Pu.**

P: ***Polariseur*** **Polarizer**

Lt: *'lentille' de tube* **Tube lens**

Dc: *Diaphragme de champ de l'oc.* **Field diaphragm of the eyepiece**

C: *Optique du condenseur* **Optics of the condenser**

A: ***Analyseur*** **Analyzer**

Oc: *Oculaire* **Eyepiece**

Illuminateur: illumination source

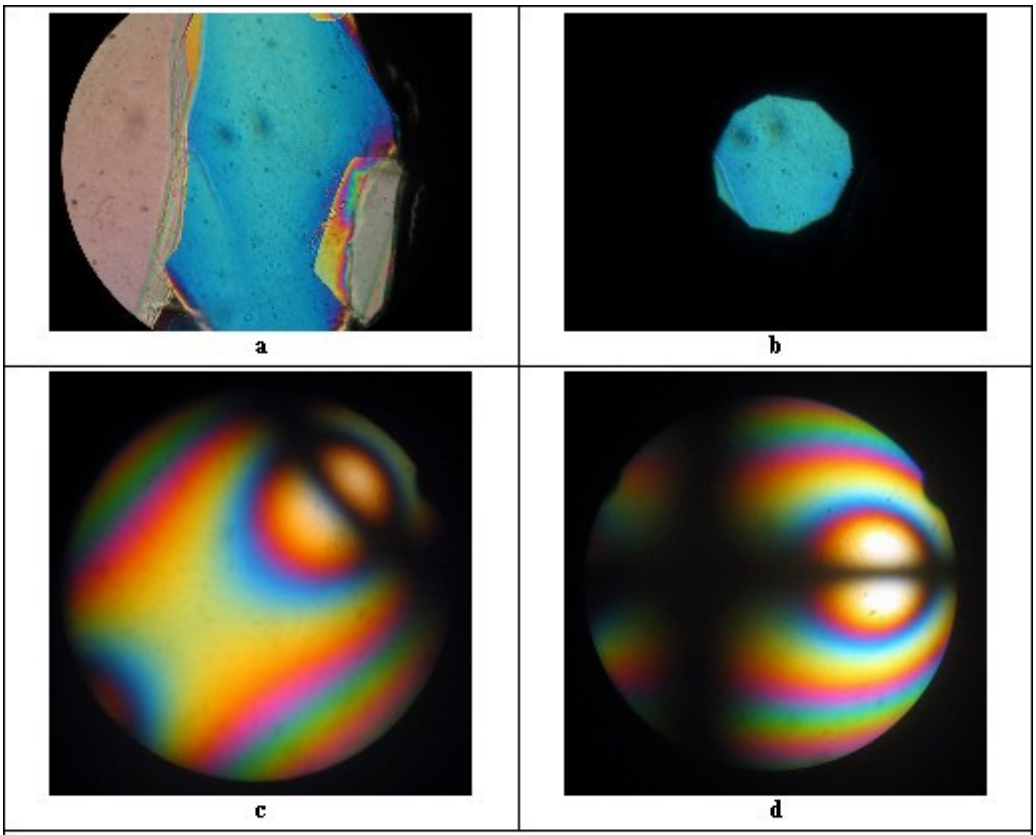
Condenseur: condenser

Microscope avec lentille de Bertrand: Microscope with Bertrand lens.

Eclairage Köhler : Köhler illumination

Rappel

On microscopes without removable BERTRAND lens, it is often possible to make that observation using an auxiliar viewfinder (in order to adjust phase contrast) which would be inserted in place of the eyepiece, but then you could not use the camera exit or the microscope.



a : Image obtained with orthoscopic light (**without** Bertrand lens) of a citric acid crystal specimen ($P \perp A$, the neutral lines of the sky blue crystal at 45° to P, 40×/0.75 objective).

b :Same specimen and same conditions of observation as **a,** but here the field diaphragm of Köhler illumination is closed just enough to illuminate a pretty much homogeneous area (monocrystal of pretty constant thickness).

c :Conoscopic image obtained with **b** using a Bertrand lens and an highest aperture diaphragm in Kohler illumination (crystal neutral line at 45° to P). We can note that we can still see the sky blue color of the crystal in orthoscopic observation.

d :Same as **c** but adjusting the sample at 45° to the previous one. (Crystal neutral line // P).

Complément

The figures c and d are typical of a biaxial crystal, a property that we could not determine on the images a and b with orthoscopic light. (On the figures c and d, the center of the image corresponds to a light beam which is the normal to the preparation and the edges correspond to a beam inclined by $\arcsin(0.75) \approx 49°$ in the air where 0.75 is the numerical aperture of the $40\times$ objective.

Remarque

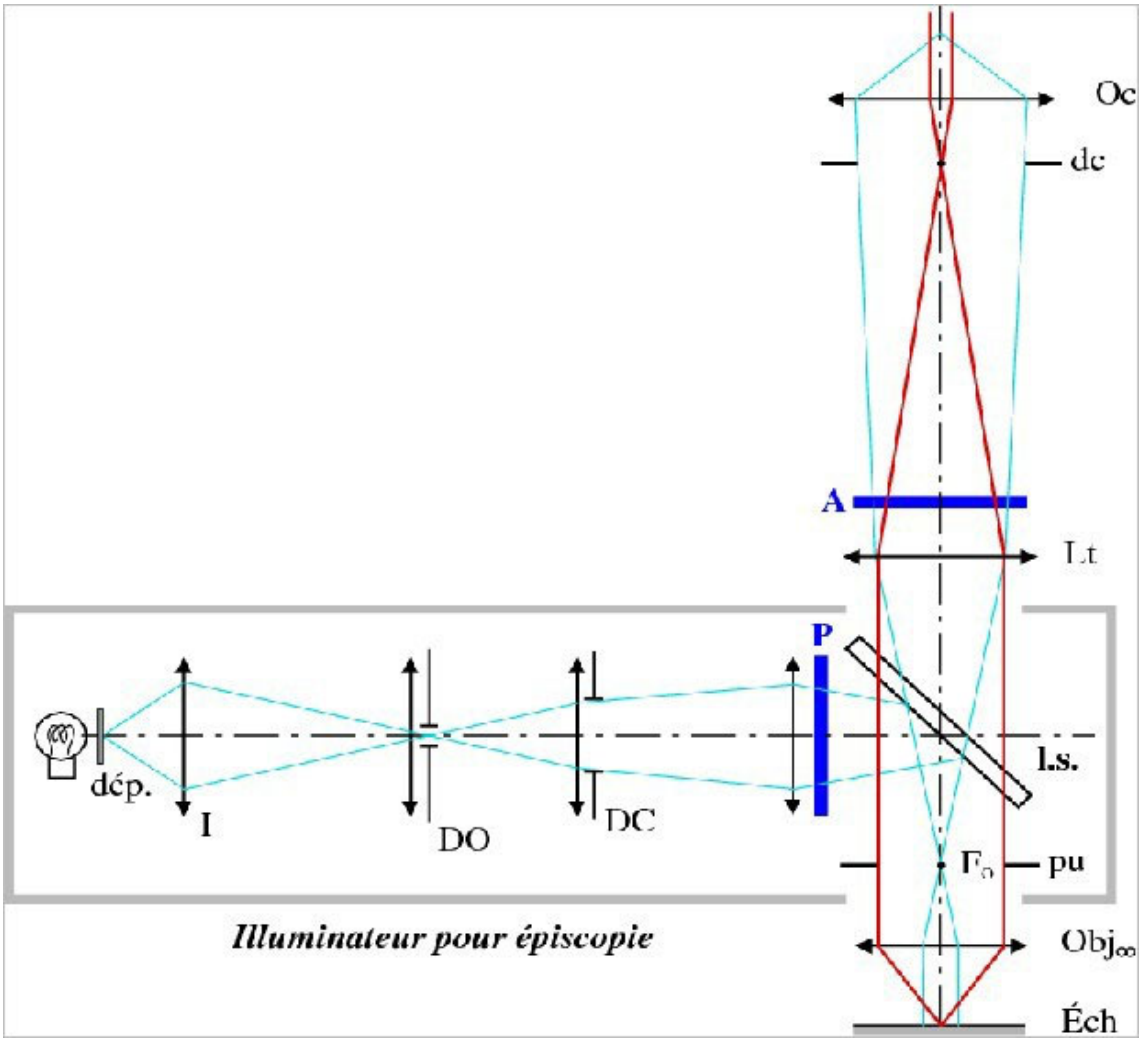
Because birefringent crystals optic axes can be oriented in any direction to the microscope axis, it could be useful to work with theodolite stages which allow adjusting in any directions the normal to the preparation which has to be inserted between two half-spheres of glass. See, for instance, the reference [Roblin 99 [Encyclopédie des Techniques de l'Ingénieur (tome R7)]] or the article *http://www.zeiss.com/C1256D18002CC306/0/F2BA0A81B5929487C1256D59003351AA/$file/*

46-0014_e.pdf[4] and geology units and experiments about rock identification with polarized light microscopes to learn more about the topic.

1.2. Reflection light observation

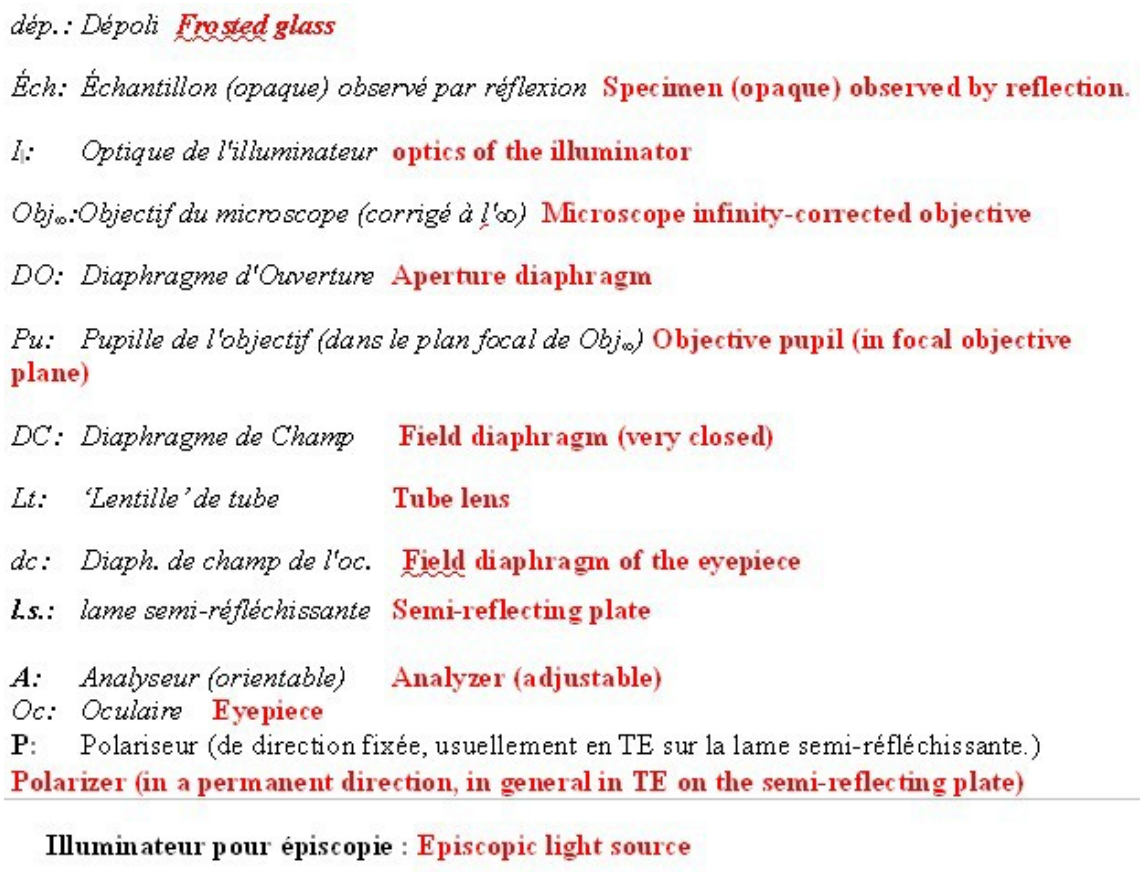
a) Orthoscopic reflection light

During reflection light observation with orthoscopic illumination, it is required, as in diascopy, to add a polarizer in **Kohler** illumination and an analizer to the body of the microscope (Figure 6). It has to be noted that in episcopy, the polarizer has a **fixed** orientation which permits to always make a linear TE (or TM) polarization possible on the semi-reflecting slide of the reflection illuminator. This is necessary because a processed semi-reflecting slide transforms all incident polarization except TE and TM polarization into elliptical polarization (*See the lesson about polarization or about ellipsometry*).



Illuminateur pour épiscopie

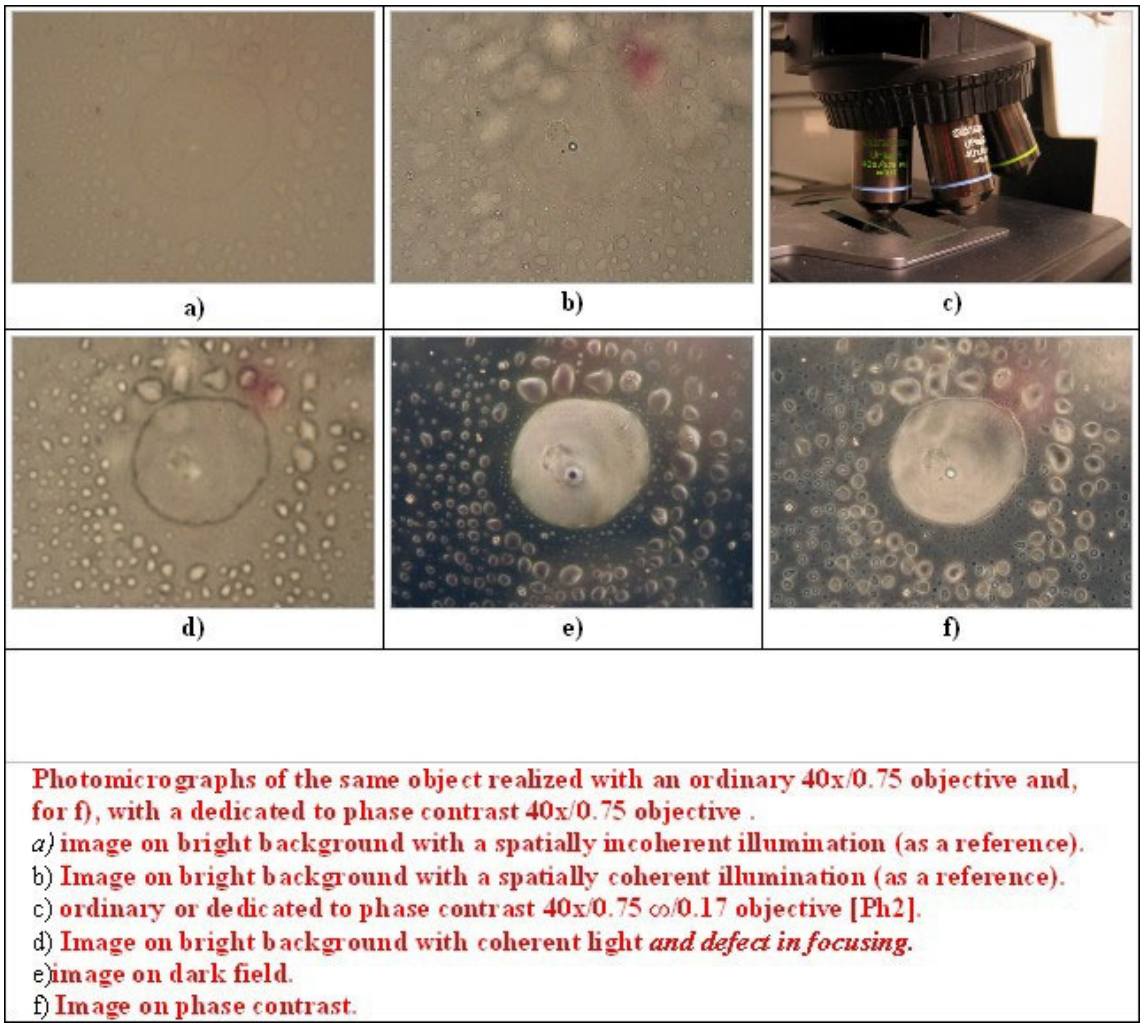
4 - http://www.zeiss.com/C1256D18002CC306/0/F2BA0A81B5929487C1256D59003351AA/$file/46-0014_e.pdf



The physical origin of a contrast in the specimen in episcopic polarized illumination can be due to diffusion phenomena (or depolarization) or to reflection on birefringent materials or on stacked layers, some of them being birefringent (ellipsometry of the stack of thin anisotropic layers [Yeh 1980 [Optics of anisotropic layered media: a new 4×4 matrix algebra]][Yeh 2005 [Application à la métallographie des méthodes interférentielles à deux ondes polarisées]]. The Study Case section of this unit M11G2 will present some examples of episcopic orthoscopic light observations.

2. Black field and phase contrast

Phase objects, such as a living cell's organelles and a polished material's surface defects, are also a very important class of almost uniformly transparent or reflecting microscopic objects, which must be observed with great precision. There are several methods to transform these phase variations which are invisible to the human eye into observable illumination variations. We are going to present the methods which perform *spatial-frequency filtering*. Figure 7 shows several pictures obtained on the same preparation by means of different methods, which will be described later.



Photomicrographs of the same object realized with an ordinary 40x/0.75 objective and, for f), with a dedicated to phase contrast 40x/0.75 objective .
a) image on bright background with a spatially incoherent illumination (as a reference).
b) Image on bright background with a spatially coherent illumination (as a reference).
c) ordinary or dedicated to phase contrast 40x/0.75 ∞/0.17 objective [Ph2].
d) Image on bright background with coherent light *and defect in focusing.*
e)image on dark field.
f) Image on phase contrast.

2.1. Recall about spatial-frequency filtering

We will only recall here the main features of spatial-frequency filtering methods used in microscopy. The reader is expected to already know about the principle of spatial-frequency filtering and the standard **double diffraction** assembly. For any question about this basis's method and details, please refer to unit UNIT M9G1 and to the references [Lowenthal 67 [Progrès récents en optique cohérente: filtrage des fréquences spatiales, holographie]], [Goodman 72 [Introduction à l'optique de Fourier et à l'holographie] (ou 2006)] and [Born 99 [Principles of Optics]].

The theory, which results from linear filtering on complex amplitude, will be presented supposing a **monochromatic and spatially coherent** illumination. Note that human eye and usual detectors are sensitive to brightness, which is proportional to the square modulus of the complex amplitude.

Concerning usual observations in microscopy **with white light**, you just have to keep in mind that the global observation corresponds to the incoherent superpositions (i.e in illumination) of different monochromatic images obtained by filtering; the enhancement of the objects contrasts is then conserved with polychromatic light. Some comments will be made in some cases about spatial coherence.

Fondamental

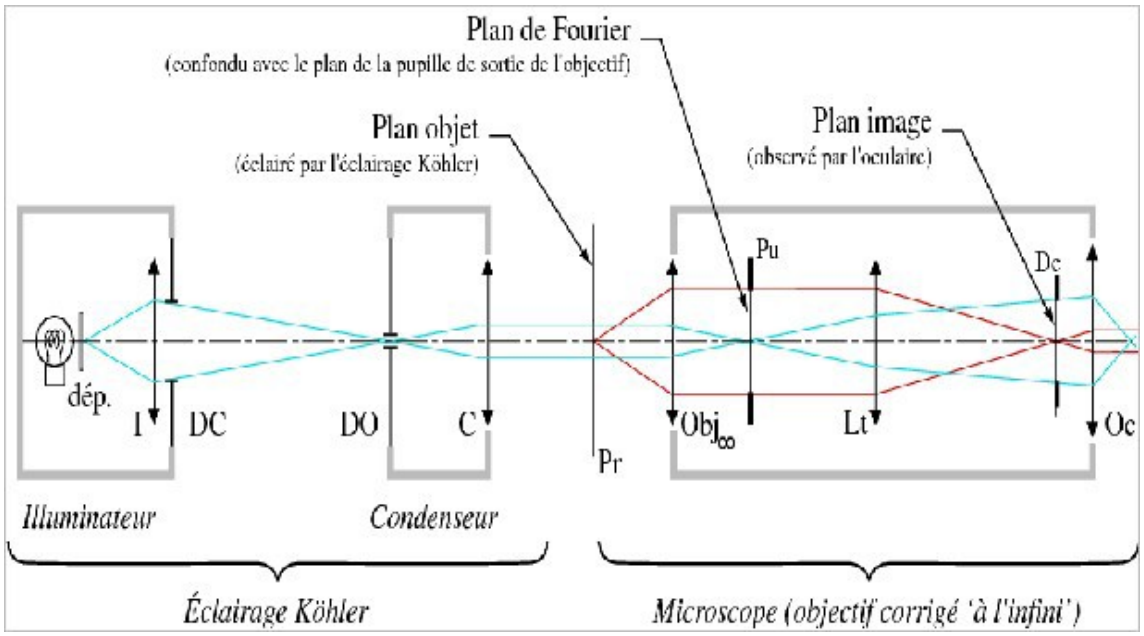
Double diffraction will be represented by the formulation:

$$\text{object} \xrightarrow{TF\ 1} \text{Fourier plan (filtering)} \xrightarrow{TF\ 2} \text{filtered image}$$

(complex amplitude) *(complex amplitude)*

Let a microscopic preparation be a thin object **characterized by its complex transmittance** $t(M)$ where M represents a dot on the preparation plane.

This object is illuminated by a flat wave from **Köhler** illumination with a spatially coherent light, i.e. with a quasi-punctual aperture diaphragm. *See Figure 8 (See unit M11G1 for more information about Köhler illumination*).



dép.: Dépoli **Frosted glass**

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DC: Diaphragme de Champ (très fermé) **Field diaphragm (very closed)**

Obj.: Objectif du microscope (corrigé à l'∞) **Microscope infinity-corrected Objective**

DO: Diaphragme d'Ouverture **Aperture diaphragm**

Pu: Pupille de sortie de l'objectif **Exit pupil of the objective**

Dc: Diaphragme de champ de l'oc. **Field diaphragm of the eyepiece**

C: Optique du condenseur **Optics of the condenser**

Lt: 'lentille' de tube **Tube lens**

Oc: Oculaire **Eyepiece**

C **The components for spatial-frequency filtering will be inserted in Fourier plan.**

Plan de Fourier (confondu avec le plan de la pupille de sortie de l'objectif): Fourier plan (at the same position than the exit pupil of the objective)

Plan objet (éclairé par l'éclairage Köhler): Object plane (Illuminated by Köhler illumination).

Plan image (observé par l'occulaire): Image plane (observed through the Eyepiece)

Illuminateur: Light source

Condenseurs: Condenser

Eclairage Köhler: Köhler illumination

Microscope (objectif corrigé à l'infini): Microscope (infinity-corrected objective)

In the *Fourier plan* , which corresponds in microscopy to the focal image plane of the objective of the microscope (in the same position as the exit pupil of this objective), we have a complex amplitude which is proportional to the Fourier transform of *t* by one homothety and on a phase factor:

$t \overset{TF\ 1}{\rightarrow} \tilde{t}$

In the present case, the object is usually only a phase object, which means:

$t(M) = exp(i \cdot \Phi(M))$

where Φ is a real value function representing the phase difference introduced by the object at each point of the object. The global phase of the wave having no importance, we choose Φ so that its mean value is 0. If the phase variations are weak ($|\Phi| << 1$),we can estimate the exponential function with its limited development (first order) and the complex amplitude at the object level is such as:

$t(M) \approx 1 + i \cdot \Phi(M) \quad (\phi \text{ real}, |\Phi| << 1)$

That is to say at the Fourier plan:

$1 + i \cdot \Phi(M) \overset{TF\ 1}{\rightarrow} \delta + i \cdot \tilde{\Phi}$

where $\delta$is the Dirac distribution, and $\tilde{\Phi}$ the Fourier transform of Φ. In practice, it means there is a very luminous dot in the center of the Fourier plan (δ) and diffracted light in all the plane ($\tilde{\Phi}$).

For double diffraction assembly **without filtering** in the Fourier plan, we have:

$t(M) \approx 1 + i \cdot \Phi(M) \overset{TF\ 1}{\rightarrow} (\delta + i \cdot \tilde{\Phi}) \overset{TF\ 2}{\rightarrow} 1 + i \cdot \tilde{\tilde{\Phi}} = 1 + i \cdot \check{\Phi}$

where $\check{\Phi}(M) = \Phi(-M)$ (*The reversal of the image inferred by the double Fourier transform of the double diffraction assembly simply expresses the combination of geometrical optics and negative enlargement*). The brightness seen by the human eye is proportional to $|1 + i \cdot \check{\Phi}|^2 = 1 + \check{\Phi}^2$, which is equal to 1 for a first-order calculation, because the second-order term is not significant ($|\Phi| << 1$, $\check{\Phi}^2$is negligible in front of 1); usually only a phase object can be seen as a uniformly enlightened zone.

Actually, this result is not completely right, because the finite numerical aperture of the objective produces a limited light spread in the Fourier plan (this limit corresponds to the exit pupil of the objective). Thus the complex amplitude of the final image is therefore the complex amplitude of the object which has been convolved by the coherent percussional response of the objective (the complex amplitude of an Airy disk if the objective is *« diffraction-limited »*). Consequently, the image can show some variations of the modulus of the complex amplitude (Gibbs phenomenon due to the brutal cuts of high spatial frequencies, marked by the presence of fringes where the phase objects are quickly changing). See Figure 7b.

2.2. Defect in focusing with spatially coherent illumination

A defect in focusing the microscope on the preparation can be seen as the introduction of a coherent transfer function which would be different from the pupil function in the exit pupil of the objective (See for instance [Goodman 72 [Introduction à l'optique de Fourier et à l'holographie]] [Born 99 [Principles of Optics]]). We will not try to achieve here the precise mathematical formalization of this problem for double diffraction. All you need to know is that apart from flat or spherical wavefronts, the modulus of complex amplitudes of waves do not remain spatially uniform when waves are propagating in free space. Then, except on phase objects conjugate planes, there is no reason for illumination to be spatially constant, and the eye can then see "something" in the image (with monochromatic and polychromatic illumination) when there is a defect in focusing microscopes. However, the image is not directly related to the original phase object and therefore this method is not very useful, practically speaking. But it can be used to locate phase objects on a preparation before using more efficient contrast techniques. See Figure 7d.

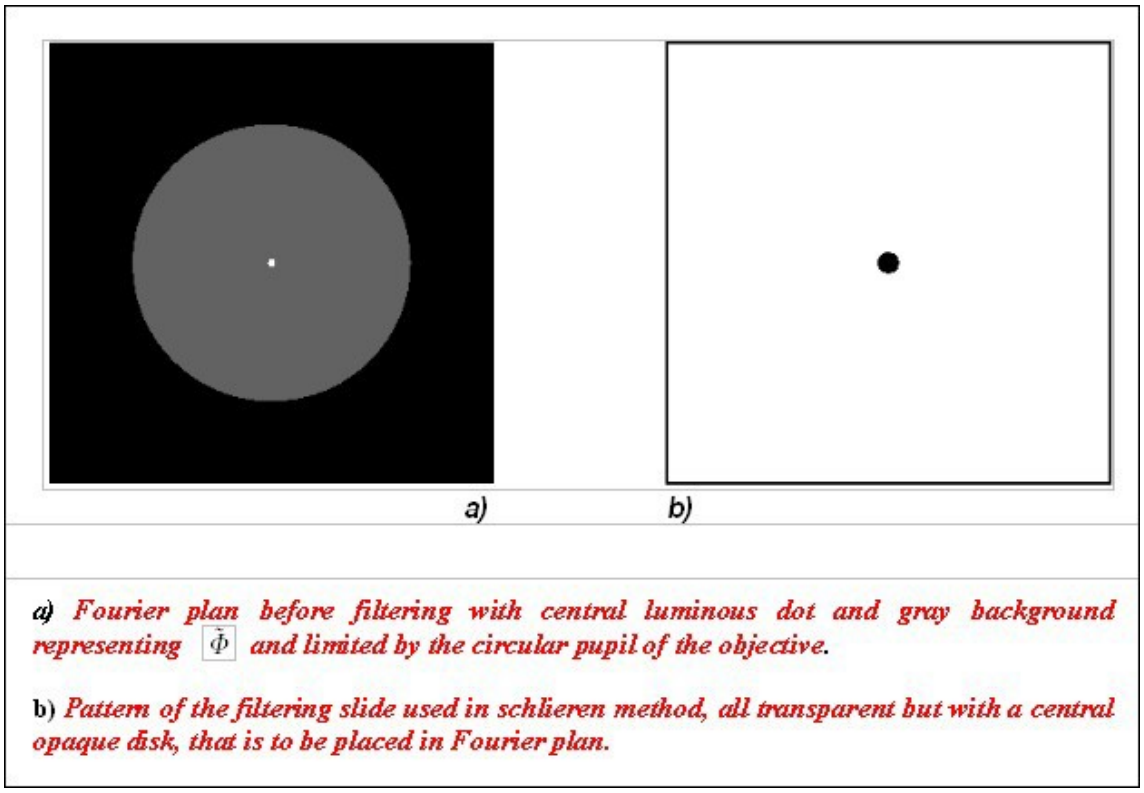
Remarque

N.B: With spatially incoherent illumination, a defect in focusing leads to a very different result because the linear filtering formalism has to be applied to illumination. Since the original object is uniform in illumination, so does the image, whatever the defect in focusing is.

2.3. Schlieren method and dark field

a) Description of schlieren method filtering

Concerning the schlieren method, filtering in the Fourier plan allows the suppression of the luminous central dot (see again Equ. 5 and see Figure 9a). For practical purposes, that can be performed introducing a high quality glass slide with flat and parallel faces in the Fourier plan ; we would have put in the center of one of the faces of the slide a tiny opaque disk. See Figure 9b. The slide is positioned so that the luminous dot is covered by the disk.



a) Fourier plan before filtering with central luminous dot and gray background representing $\tilde{\Phi}$ *and limited by the circular pupil of the objective.*

b) *Pattern of the filtering slide used in schlieren method, all transparent but with a central opaque disk, that is to be placed in Fourier plan.*

Ignoring the size of the disk, the double diffraction assembly gives:

t \approx 1 + i \cdot \Phi \overset{TF~1}{\rightarrow} \theta \times \delta + i \cdot \tilde{\Phi} = i \cdot \tilde{\Phi} \overset{TF~2}{\rightarrow} i \cdot \tilde{\tilde{\Phi}} = i \cdot \check{\Phi}

The illumination, which is proportional to the square modulus of the complex amplitude, is then expressed by $\check{\Phi}^2$.

Remarque

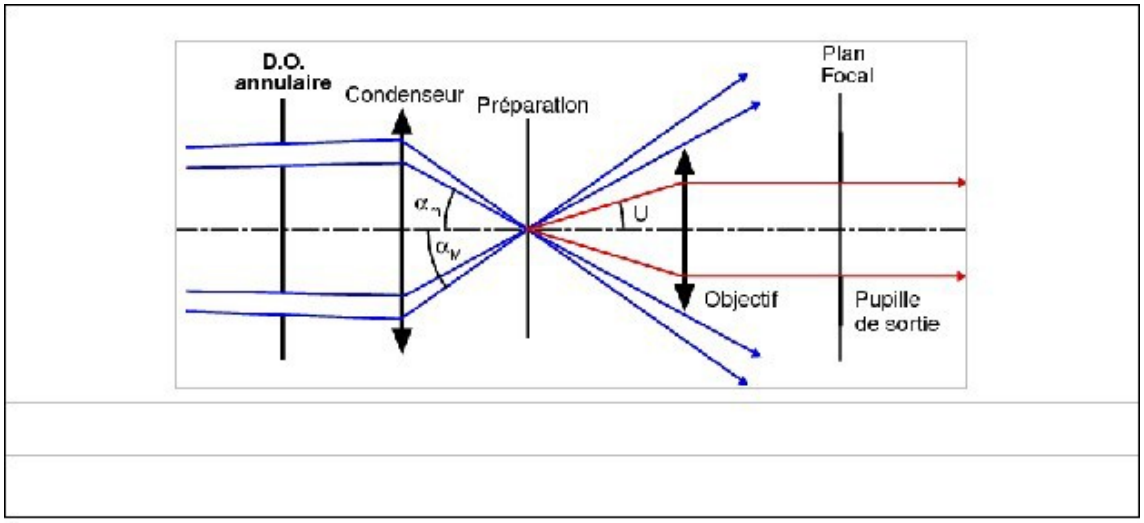
The phase differences introduced by the elements which are present on the observed preparation are seen as luminous regions on a dark background.

Quadratic dependence of light on phase differences introduced by various objects (with very-low phase differences $|\Phi| << 1$) and the disappearance of the very-low spatial frequencies due to the finite size of the disk placed in the center of the Fourier plan, give an image that can be very different from the one of the initial phase object. That image mainly permits to detect very small objects and the limits of bigger objects, but does not give any precise information about weak phase variations which can be present inside the objects.

b) How to perform the dark field method in diascopy.

The method explained above can be performed with spatially coherent illumination. In microscopy, contrary to the assembly on bench where a laser can be used if necessary, the light source is ordinarily not intrinsically spatially coherent since it just consists of standard KÖHLER illumination with a very closed aperture diaphragm. The brightness of the image is then very low because only a very small part of the light source's power actually illuminates the preparation; moreover, most of the useful power is absorbed by the central disk present in the Fourier plan. So it is necessary to first use a powerful illumination ($\sim 100\ W$ halogen lamp, or $\sim 1500\ lumen$) and then to use a spatially partially coherent source, and not a strictly coherent one, to increase the useful flux. From a theoretical point of view, each point of an incoherent spread light source is coherent with itself, and the Fourier formalism can be applied

to the complex amplitudes of the wave which comes from this point of the source; then, you just have to sum up in an incoherent way, i.e. with illumination, every image formed by each point of the source to reform the global image you are actually observing. The schlieren method, also called "**dark field**", is therefore performed on commercial microscopes, using annular sources and the objectives pupils to cut the nil spatial frequency (Figure 10 and Figure 11).



Annulaire: Annular

Condenseur: Condenser

Préparation: Preparation

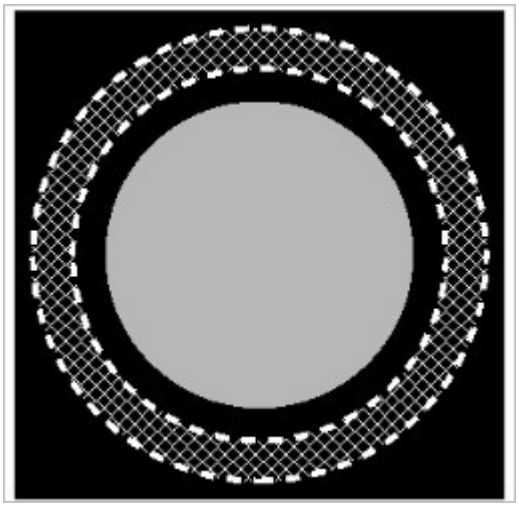
Objectif: Objective

Plan focal: Focal plane

Pupille de sortie: Exit pupil

The preparation's illumination is only carried out with rays which are highly oblique to the axis of the optical system and which can vary between the limits of the two angles α_m and α_M ($0 < \alpha_m < \alpha_M$). Concerning the directions, these rays make a sort of cone-shaped sheet. This is done by stopping up the center of the aperture diaphragm plane of a KÖHLER illumination. The angle α_m has to exceed the maximum acceptance angle of the microscope objective, which means that $n \cdot \sin(\alpha_m) > NA_{obj} = n \cdot \sin(U)$ where n is the refraction index of the medium observed through the objective (see Figure 10). If the observed preparation is uniform, there is no light going through the objective: the background is then black. By contrast, if some small objects are present, those will diffract light in all directions and they will be seen in the final image as luminous marks on a very dark background. This method can be used for phase objects, amplitude objects and mixed objects

Figure 11 shows the high pass spatial frequencies filtering (rigorously band pass spatial frequencies if we take into account the finite size of the pupil) done by means of this method. As we have already mentioned for the schlieren method, this sort of filtering can sometimes markedly change the object's appearances. This way of visualization is mainly used to underline the presence of certain objects or of very small details (sometimes, it is possible to detect very small objects which are below the limit of resolution of the objectives by means of a standard bright background). See Figure 7e.



Complément

Visualization of the Fourier plan with dark field illumination: The image of the annular aperture diaphragm of Köhler illumination, represented by hatching and the white dotted line, is, by construction, out of the exit pupil (represented by the gray disk) of the objective of the microscope, and hidden. The seen light in the pupil comes from the light diffracted by the preparation; you can consider this light as the uncoherent superposition of the $\tilde{\Phi}$ centered on each point of the annular source and limited by the circular pupil of the objective, which still does not admit any nil spatial-frequencies and other close low frequencies. The global observed image is then the uncoherent superposition of a set of complex amplitude images which have experimented an high pass filtering (rigorously called a band pass).

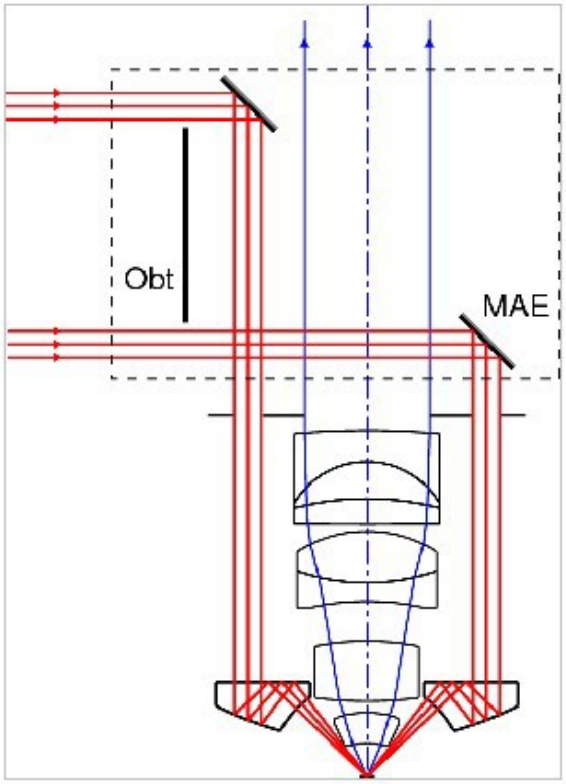
Attention

N.B: For immersion objectives, whose numerical apertures usually exceed 1.25, the dark field illumination condenser has to be particularly efficient since the illumination has to be carried out through numerical apertures between 1.3 and 1.4. For such characteristics, you will also need an immersion condenser.

c) Dark field in episcopy

It is also possible to work with a dark field on opaque objects observed by reflexion. This method is very often performed for metallurgical checkings and microelectronic integrated circuits observations. But the illumination through the objective makes it much harder to perform this method. In fact, a metallographic objective for dark field is constituted of a standard metallographic objective, rounded by an annular optics which permits an oblique illumination. The semireflecting slide in the light unit is replaced by an elliptical annular mirror with holes. Figure 12 is a diagram of the principle of such an objective summoned by a mirror unit of light injection from episcopic KÖHLER illumination.

Such objectives, whose diameters largely exceed standard objectives' ones, are sold by the larger manufacturers with their middle or top-of the range metallographic microscopes. They need a specific “mirror light unit” and a particular revolving turret with threaded holes whose diameters will be superior to the usual value, in order to enable annular light to go through.

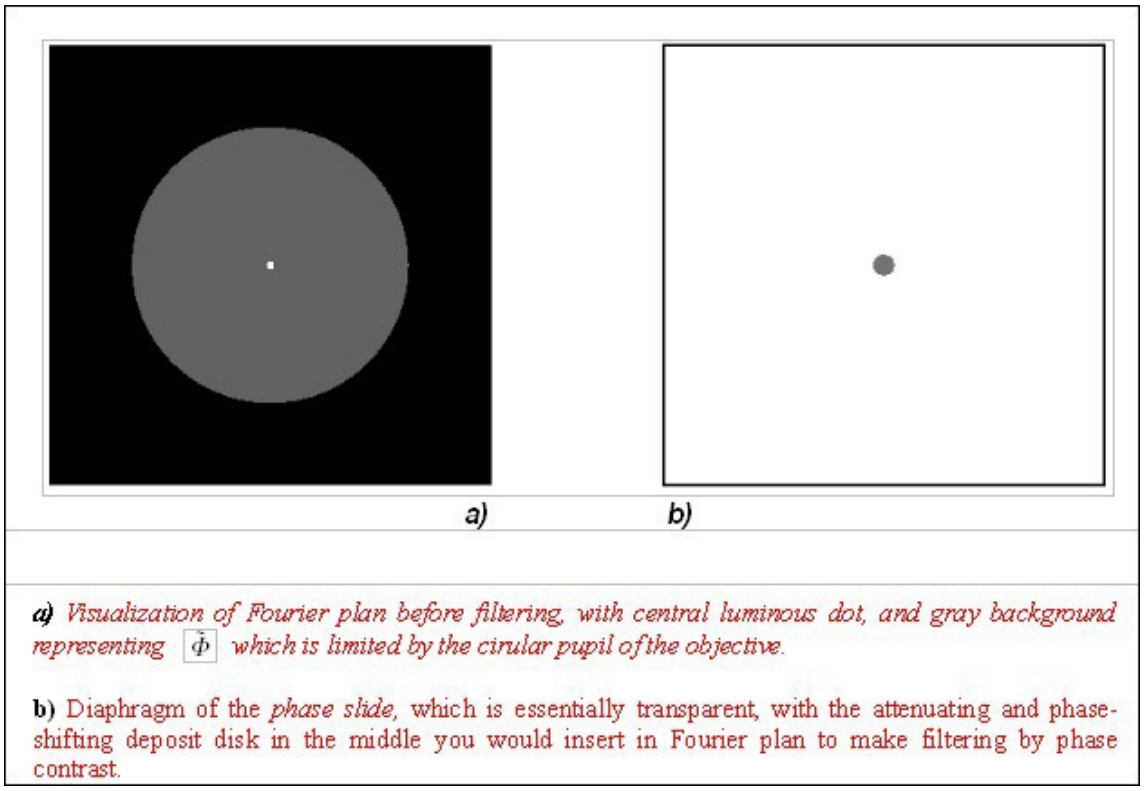


On this diaphragm, the objective has a numerical aperture of 0.4 [20×/0.40 ∞/0] and the cone-shaped illumination has a numerical aperture between ~0.6 et ~0.7.
MAE: elliptical annular mirror plane at 45° reflecting light from episcopic Köhler illumination.
Obt: Opaque stop (to limit stray light).

2.4. Phase contrast

a) Filtering description

In the phase contrast method, filtering in the Fourier plan consists in making a phase shift of $\pm\pi/2$ radians and in dimming the luminous central dot. See Figure 13a. In practice, this can be done introducing in the Fourier plan a good quality glass slide with flat and parallel faces; you should put a very small disk of an appropriate thickness of an absorbing dielectric material (often aluminum) on the center of one of its faces. See Figure 13b. We call this slide "*phase slide*". It has to be placed so that the disk covers the luminous dot.



a) Visualization of Fourier plan before filtering, with central luminous dot, and gray background representing $\check{\Phi}$ *which is limited by the cirular pupil of the objective.*

b) Diaphragm of the *phase slide*, which is essentially transparent, with the attenuating and phase-shifting deposit disk in the middle you would insert in Fourier plan to make filtering by phase contrast.

Without taking into account the limited size of the disk, the double-diffraction assembly is, if γ represents the (amplitude) attenuation coefficient of the central luminous dot ($\gamma \in]0.1]$) :

t \approx 1 + i \cdot \Phi \overset{TF~1}{\rightarrow} \pm i \cdot \gamma \cdot \delta + i \cdot \tilde{\Phi} \overset{TF~2}{\rightarrow} \pm i \cdot \gamma + i \cdot \tilde{\tilde{\Phi}} = i (\pm \gamma + \check{\Phi})

The factor $\pm i = e^{\pm i \cdot \pi/2}$ placed before the term $\gamma \cdot \delta$ represents the phase difference equal to $\pm\pi/2$ introduced by the phase slide.

The illumination, which is proportional to the square modulus of the complex amplitude, can be expressed as :

$$(\pm\gamma + \check{\Phi})^2 \approx \gamma^2 \pm 2\gamma\check{\Phi} \approx \gamma^2\left(1 \pm \frac{2\check{\Phi}}{\gamma}\right)$$

without taking into account the insignificant second-order term $\check{\Phi}^2$ compared to the other terms in this first-order calculation.

Fondamental

The phase variations of the object are seen as luminosity variations on a more or less bright background. (See Figure 7f.)

The image illumination variations are proportional to the object phase variations.

Remarque

Depending on the phase of the central luminous dot in the Fourier plan being delayed or respectively advanced, (phase difference respectively $+\pi/2(i.e. \times +i)$ or $-\pi/2(i.e. \times -i)$ expressed as a rule $e^{-i\omega t}$), a phase delay in the wavefront (optical path in the biggest object; Φ positive as a rule $e^{-i\omega t}$) will be seen respectively as more or less "luminous" than the background, and the same will occur concerning advanced phases. In the first case (more luminous details with a phase delay), we talk of bright phase contrast and in the second case,

of dark phase contrast (see.[Françon 50 [Le contraste de phase en optique et en microscopie]] and [Born 99 [Principles of Optics]]).

We can notice that, **without** being dimmed, or with $\gamma = 1$, the illumination observed by the eye or through a camera will be proportional to $1 \pm 2\check{\Phi}$ and then will have a very low contrast since .

The contrast C of the resulting image, ratio of the main luminosity variations by the mean value of that luminosity, can be expressed as:

$$C = \frac{\Delta\Phi}{\gamma}$$

where $\Delta\Phi$ represents the typical phase variations inside the object.

The smaller γ is (or the more substantial the dimming is), the better the contrast. (For a strictly nil γ, the preceding results cannot be applied; actually, it is a schlieren method case, as explained in the paragraph above.

For practical purposes, the finite size of the attenuating and phase shifting disk also adulterates (dims) $\tilde{\Phi}(P)$ for all points P which are very near to the center, and which correspond to very-low spatial frequencies. Thus the phase contrast method is not adequate to highlight spatial variations of slow phases whose variation lengths are, for instance, a half or a quarter of the field observed. In addition, you can notice that the finite size of the exit pupil of the objective of the microscope, which has not been explained in the above formalism, also means that transfer functions of spatial frequencies will be cut off starkly; consequently, a Gibbs phenomenon produces artificial oscillations (or fringes) near the fastest phase variations of the object.

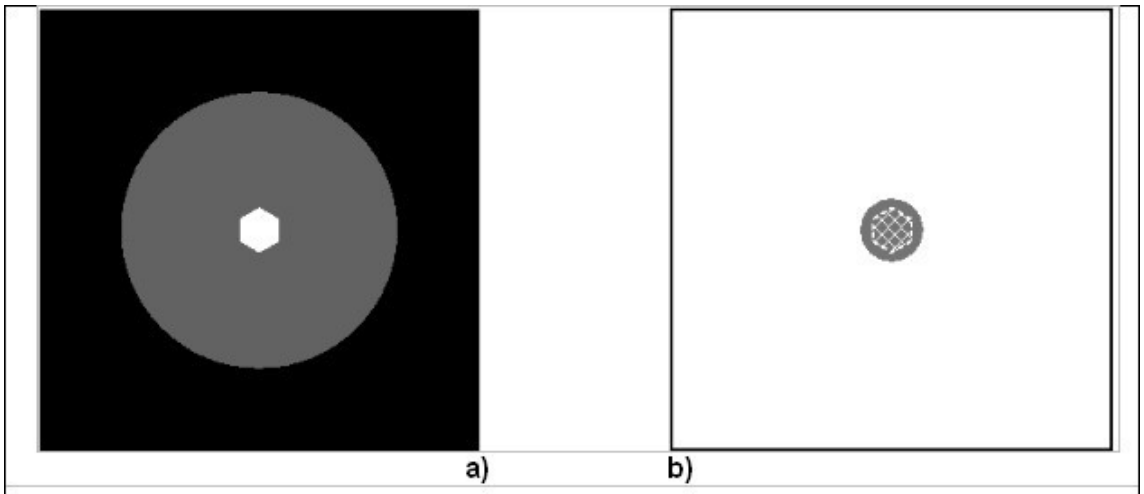
Remarque

From an experimental point of view, it is hard to perform a precise $\pm\pi/2$ phase shift, especially at the same time for all the wavelengths you can see with a standard white light illumination. In fact, the transformation of the phase variations into illumination variations still occurs, but with less efficiency if the phase shifting ideal value is not respected. For example, a $\pm\pi/4$ mistake compared to the $\pm\pi/2$ ideal value would only produce a contrast fall by ~30%. Moreover, if the object observed generates simultaneously small variations of phase and transmission, which can happen rather frequently in biological objects, the ideal phase shift will no longer be exactly of $\pm\pi/2$, but will depend on the object (See [Françon 50 [Le contraste de phase en optique et en microscopie]] and [Françon 54 [Le microscope à contraste de phase et le microscope interférentiel.]]). For practical purposes, the precise value of the introduced phase shift is not essential for qualitative observations of the image details. Conversely, the phase contrast method is never used to perform phases *measurements*, but only to highlight the presence of spatial variations of phases in bi-dimensional objects.

b) Practical performance

Just as in the above-mentioned schlieren method, the phase contrast method is performed with spatially coherent illumination, or in other terms, in standard microscopy with KÖHLER illumination with a very closed aperture diaphragm. Consequently, the image's luminosity is very low as the useful flux to illuminate the preparation is very weak and a large part of it is absorbed by the phase slide. In practice it is then necessary, as with a dark field, to use a powerful illumination and a spatially coherent source.

First, we could decide to work with a more open aperture diaphragm and to perform filtering on a larger zone (it would cover the D.O. image in the pupil plane of the objective. See Figure 14).

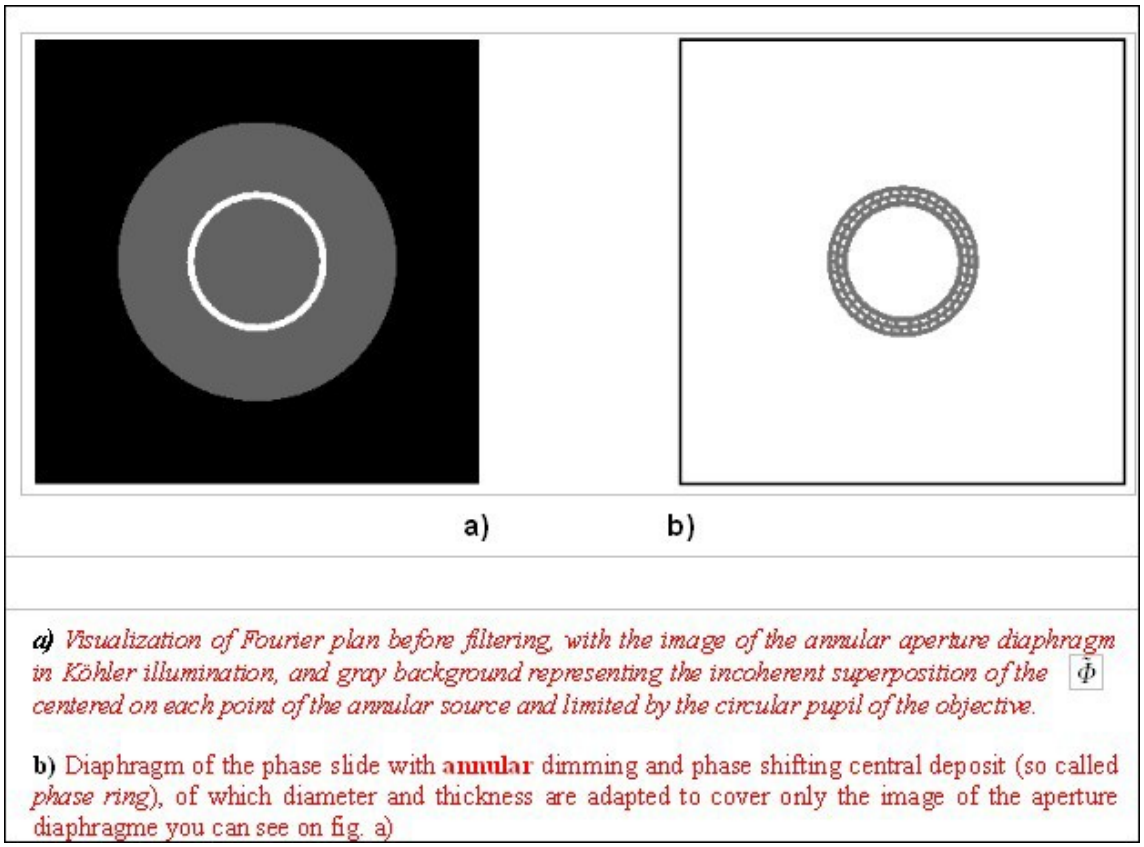


a) Visualization of the exit pupil plane of the objective before filtering. We can notice in the center, the image of the aperture diaphragm (usually polygonal) of Köhler illumination, formed by construction in that plane (See again Fig. 8)

b) Diagram of the phase slide with dimming and phase shifting deposit in the middle; the disk diameter has to be large enough to entirely cover the image of the aperture diaphragm of Köhler illumination; all points of that image have to be dimmed and phase shifted.

Every luminous dot of the image of the aperture diaphragm (Figure 14a), being the center of the spatial frequencies space for this particular coherent wave, represents the Dirac peak in formula 8. The gray background roughly shows the incoherent superposition of all the $\tilde{\Phi}$ which are centered on each point of the image of the aperture diaphragm and limited by the circular pupil of the objective. Then, we can see on Figure 14b that on average, the filtering spreads far into the low and medium spatial frequencies around each Dirac peak δ because of the important needed diameter of the central deposit on the phase slide. As a consequence, increasing the luminosity by means of a sufficiently open aperture diaphragm will generate an **unacceptable deterioration of low and medium spatial frequencies** of the global image which is formed by the incoherent superposition of the different filtered images of complex amplitudes; this method is therefore not appropriate for practical purposes.

In fact, the most common method consist in using an **annular** aperture diaphragm (replacing in **Köhler** illumination the adjustable iris diaphragm by an annular aperture diaphragm) and a annular-shaped filter in the Fourier plan, commonly called *phase ring*. See Figure 15. Both can be rather thin in order to preserve low spatial frequencies whereas the surface of the annular aperture diaphragm can be wide to give the image a good illumination. Moreover, this configuration maintains a revolution symmetry so the method does not depend on the object's orientation.



a) Visualization of Fourier plan before filtering, with the image of the annular aperture diaphragm in Köhler illumination, and gray background representing the incoherent superposition of the $\tilde{\Phi}$ *centered on each point of the annular source and limited by the circular pupil of the objective.*

b) Diaphragm of the phase slide with **annular** dimming and phase shifting central deposit (so called *phase ring*), of which diameter and thickness are adapted to cover only the image of the aperture diaphragme you can see on fig. a)

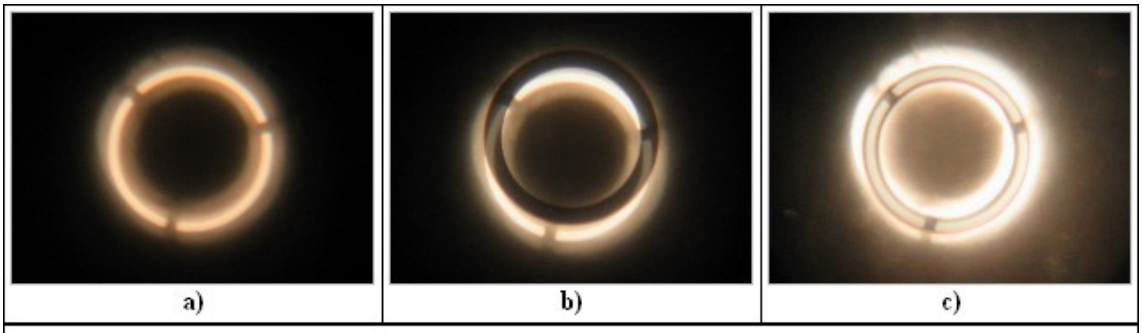
The phase slide has to be introduced in the Fourier plan which, as said before, coincides with the exit pupil of the objective plane (See again Figure 8). However, on ordinary objectives, this plane is often inaccessible (for instance, it can be at the level of the thread of the mechanical mount of the objective). Microscopes manufacturers provide two solutions. The first and the most commonly used one consists in inserting the phase slide in the objective when manufacturing, and then producing a range of special objectives dedicated to phase contrasts. But this method cannot be used in episcopy, because illumination, passing through the objective, would be spoiled by the phase slide. (See again **Köhler** illumination in episcopy, M11G1, or Figure 6, setting aside the polarizer and analyzer). The second one, available on very top-of-the range microscopes (called « *research microscopes* »), consists in producing, thanks to another optics, an image of the Fourier plan in a plane sufficiently far from the mount in order to allow the insertion of the phase slide, which is then generally removable without any difficulty. That second method, which can be performed in episcopy simply inserting that optics below the semi-reflecting slide of the illumination, has the advantage of enabling observations in phase contrast with all sorts of objectives, and not only with some specialized ones. However, producing that optics which will not spoil the quality of the microscope images is very difficult and thus very costly.

Conseil

You also have to remember that the rings diameters and the widths of annular illumination and of phase rings have to be adapted to one another so that the system functions propperly. This is obviously only possible if the condenser and the objective are adapted to each other.

From a practical point of view, in order to make an observation in phase contrast with a standard microscope, it is necessary to set up the objective in which the phase slide is integrated by means of the objective turret (or, on a research microscope, to set up the needed objective and to turn on the phase slide with the appropriate control); the annular aperture diaphragm is adapted to the condenser and objective must then be inserted into the condenser of the Köhler light unit thanks to the provided mechanism. In most cases, a device

which permits centering the diaphragm is provided. To center the diaphragm, the Fourier plan must be observed. This can be done inserting the BERTRAND lens in the optical path if the microscope stand is fitted with one (see again Figure 4) or replacing the eyepiece by a particular viewfinder provided by the manufacturer. You can see on Figure 16 some pictures of real observation of the Fourier plan with this particular viewfinder.



a) Image of the annular aperture diaphragm of Köhler illumination with a normal objective **without** phase slide. (That corresponds to figure 15a. The microscope pupil, of which the outline is on the outside of the picture, cannot be seen because the light, diffracted by the observed object, is concentrated in low spatial frequencies and only formed the halo around the ring. The three seen «cuts» at 120° in the ring are due to technical necessities when manufacturing the annular diaphragm.

b) Image of the annular aperture diaphragm of Köhler illumination superimposed to the phase ring of the phase slide (objective dedicated to phase contrast); it has be badly adjusted. (We can notice that the phase slide has dimmed the light).

c) the same than b), but with a correct adjustment. The direct image of the annular aperture diaphragm is totally hidden by the phase plate.

3. Interferometric techniques

3.1. General points

Interferometry is a common method performed in optics to reveal and measure the phase of a luminous wave. Most of the 'macroscopic' configurations made with an imaging interferometer, performing in *normal interferometry* (comparing a wavefront of interest to a reference wavefront) or in *differential interferometry* (comparing a wavefront of interest to the same translated or sometimes inclined wavefront) can be transposed to microscopy.

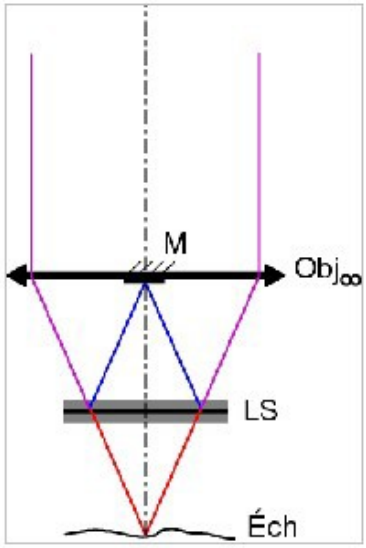
Complément

That concerns particularly the interferometer of MACH-ZEHNDER, MICHELSON, FABRY-PEROT, etc. Microscopists often call these interferometers by the name of the physician who adapted them to microscopy, which is why we talk about the systems of "MICHELSON-LINNIK" (MICHELSON), of "MIRAU" (MICHELSON), of "WATSON" (MICHELSON), of "TOLANSKY" (FABRY-PEROT), etc. See the reference [Roblin 99 [Encyclopédie des Techniques de l'Ingénieur (tome R7)]] for more information.

But most of these transpositions of interferometry to microscopy require very specific equipment which are not commonly found. Only two systems, easily adjustable to ordinary commercial microscopes (the MIRAU objective and the NOMARSKI differential interference contrast), are commonly available and used, and we will only describe those two.

3.2. Mirau objective

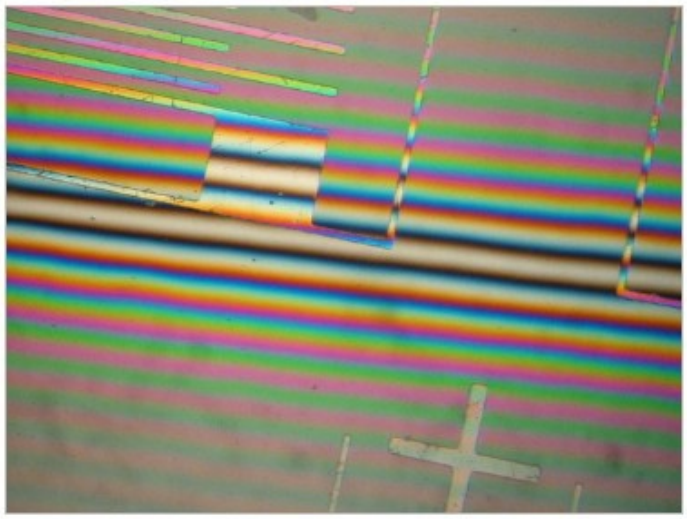
It is an adaptation of MICHELSON interferometer to microscopy. Its main principle is shown on Figure 17.



Obj$_\infty$: Objectif de microscope (corrigé à l'infini) à grande frontale / Infinity-corrected objective for wide working distance.
LS: Lame semi-réfléchissante 50% avec compensatrice symétrique / 50% semi-reflecting plate with symmetrical compensator
M: Miroir plan de référence / Reference mirror plane
Éch: Échantillon à observer / Specimen to observe.

The mirror induces a central stopping up in the optical system.

Such objectives are used in **episcopy**. Light coming from **KÖHLER** illumination is focused by the objective (see Figure17) and then separated into two waves by the semi-reflecting slide. Both beams are reflecting, one of them on the specimen, and the other on the reference mirror; then they are recombined by the semi-reflecting slide. Fringes of equal thickness can therefore be observed on the image of the object (see Figure18). Because of the high cost due to the wide working distance needed to mechanically set up the interferometer and also because of more fundamental reasons which will be explained later, MIRAU objectives have in general small numerical apertures ($NA <\sim 0.30$). As a consequence, the focusing axial latitude (around λ/NA^2) is very superior to the shift which is needed to sensibly modify the fringes (an axial shift of $\lambda/2$ has a consequence of 2π radians, on the phase shifting, so of ± 1 on the interference order). A thin movement in focusing the microscope generates a big shift of the fringes in the field, while the object sharpness barely changes.



Photomicrograph of aluminium wire on a substrate of silicon plane. a 10×/0,30∞/0 MIRAU objective . These linear fringes are mainly due to a very slight angle between the substrate and the reference mirror of MIRAU objective. The patterns reliefs are characterized by fringes shift compared to those present on the substrate.

Those fringes represent iso-shifting curbs which to a certain extent are typical features of the relief of the specimen. Such interferogram can be analyzed by all the ordinary techniques used for the interferometric control of macroscopic objects. It is especially possible, and even very common, to perform the *phase-shift* technique [Malacara 2007 [Optical Shop Testing]-ch14] inserting a MIRAU objective on a piezoelectric block, in order to induce a controlled axial movement of the block: objective + semi-reflecting slide + mirror of reference to the observed specimen surface (see Figure 19); this provides a very precise (around $\lambda/20$) relief cartography which lateral resolution corresponds to the resolution power of a microscope objective.

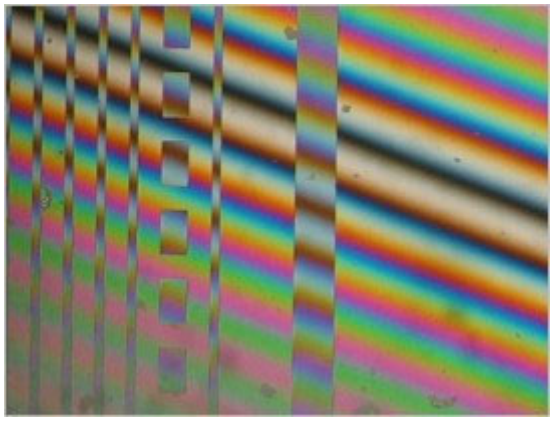
Commercial Mirau objective and piezoelectric block mounted up on a standard turret of a metallographic microscope. The 10×/0.30∞/0 objective has a 7.4 mm work distance between the semi-reflecting slide and the specimen. You can notice the particular stage where a micrometric adjustment setting is available and which permit swinging the sample along two axis and adjusting precisely the parallelism between the specimen and the reference mirror of the MIRAU objective (control of the number of seen fringes in the field).

Attention

Fringes made by Mirau objective are essentially related to the phase of the light after its reflection on the specimen (compared to the phase of the light reflected on the reference mirror). That can have some practical consequences and cause some interpretation difficulties:

- **First of all, for a defined wavelength λ the phase shift between both waves in the air slide of a thickness *e* in the MICHELSON interferometer is well defined only for an angle of a given incidence θ [well known expression $\Delta\phi = (2 \cdot e \cdot \cos(\theta)) \times (2\pi/\lambda)$]. So the objective numerical aperture has to be limited in order to avoid a fall of the fringes' contrast when moving away from the fringes of zeroth order, and to make the link between the phase shift and the local thickness of the to-be characterized air slide clear.**
- **So the objective numerical aperture has to be limited in order to avoid a fall of the fringes' contrast when moving away from the fringes of zeroth order, and to make the link between the phase shift and the local thickness of the to-be characterized air slide clear. $\lambda/2$,it is impossible to determine the steps heights because of the phase modulus determination 2π. But to solve this problem, we can work with white light (see again Figure 18 where we can see, for instance, a shift of two interfringes on the rectangular pattern on the top left corner of the picture). Actually, it is possible to perform the phase-shift technique with low temporal coherence illumination to combine nanometric precision with high dynamic range measurement.**
- **For an inhomogeneous specimen, consisting for instance of a silicon substrate on which precise patterns of transparent dielectric materials such as resin or**

precise layers of various materials (like oxide, nitride, resin, etc) are deposed, the wave phase shift when reflecting on various points of the specimens will depend on the local structure of the specimen (refraction index, thickness), on the wavelength, on the incidence angle and on the polarization (*electromagnetism problems*, see for instance [Yeh 2005 [Application à la métallographie des méthodes interférentielles à deux ondes polarisées]]). The phase difference which occurs in a wave reflected between two close layers will then not be directly related to the height difference between both layers. Moreover, the dependence on the incidence angle and on the wavelength of the phase shift by reflection on a pile/stack of material can be different, sometimes very different, to the one on the reference mirror, which can result in a variation of the fringes' contrast, depending on the local structure of the object (See Figure 20). These phenomena make the interpretation of raw data more difficult when the object does not present surface simple height variations and when it is not constituted of a thick homogeneous material [Feke 1998 [Interferometric back focal plane microellipsometry]] [deGroot 2004 [Determination of fringe order in white-light interference microscopy]].



Photomicrograph of transparent resin patterns on a silicon substrate with white light with Mirau objective 10×/0.30. Fringes contrast difference (especially the difference of the black fringe of zeroth order) between the different regions can be well observed here.

3.3. 'Normarski' Differential interference contrast

a) Lateral shearing interferometry and differential interference contrast

The Differential Interference Contrast (DIC) is a variant of the lateral shearing interferometry, where a wavefront interferes with the same one sheared transversely. When the shear is wide (Figure 21a), interference fringes usually appear. These provide information about the wavefront form (in one direction)[Malacara 2007-ch4&ch3] [Optical Shop Testing]. But the interpretation is only easy when the interesting details of the wavefront are precisely determined and separated from each other by a distance which is superior to the shear or if the wavefront has a smooth form without high spatial frequencies (i.e low order Zernike polynomial). For a wavefront formed by a complex and large object with numerous details of high spatial frequencies, the interpretation is more difficult, or even impossible; this is why this interferometry method is not commonly performed to observe microscopic objects. It is better to perform a technique where the lateral shear between both wavefronts is smaller than the objective resolution of the microscope. Every point of the image (whose resolution is preserved) are then modulated by a two waves interference term $\frac{1}{2}(1+\cos(\Delta\varphi+\varphi_0))$ where the local phase shift $\Delta\varphi$ is actually directly proportional to the local slope (gradient) of the

wavefront in the direction of the shear (Figure 21b). So we transform phase gradients into illumination variations observable by the human eye or by means of a detector. That is why we call this method *differential interference contrast*. The term φ_0 eis a parameter, in general unchanged for all points of the observed field, which will defined performing this method. This parameter can often be adjusted by means of a setting available on the microscope which allows the observer to vary the image appearance being observed. A value of $\pm\pi/2$ (modulo π) is particularly interesting because it permits directly determining the sign of the object phase gradient in the direction of the shear, thanks to a higher or lower brightness on the intermediate illumination background of the image; that artificially gives the impression of seeing the relief of the object with a lateral grazing illumination.

- Wavefronts which have been split to perform a wide shear
- Wavefronts which have been split to perform a small shear

Complément

The local distance of altitude δ between both surfaces is related to the local slop in the splitting direction.

δ is the (signed) local path difference between both wavefronts. Its local phase shift is $\Delta\varphi = 2\pi\delta/\lambda$.

With white light illumination instead of monochromatic illumination as it was implicitly described before, it is important to take into account the incoherent superimposition of different monochromatic contributions. Because both $\Delta\varphi$ and φ_0 generally depend on λ, the image is colored because of the presence of fluted spectra (Newton shades, usually of low order and bright colors). Color contrasts underline the phase variations produced by the object, and then, in a certain way, the 'relief' of the object observed (see Figure 22).

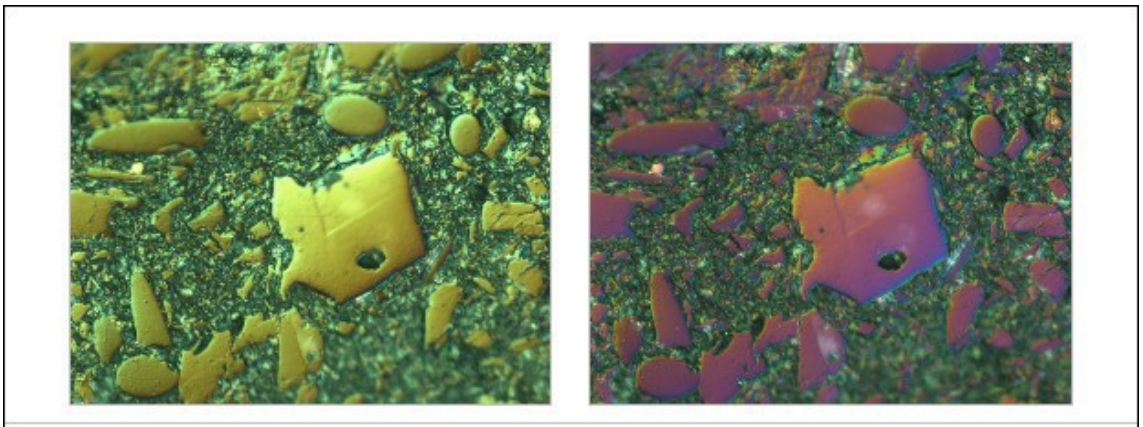


Figure 22: Photomicrographs in differential interference contrast.

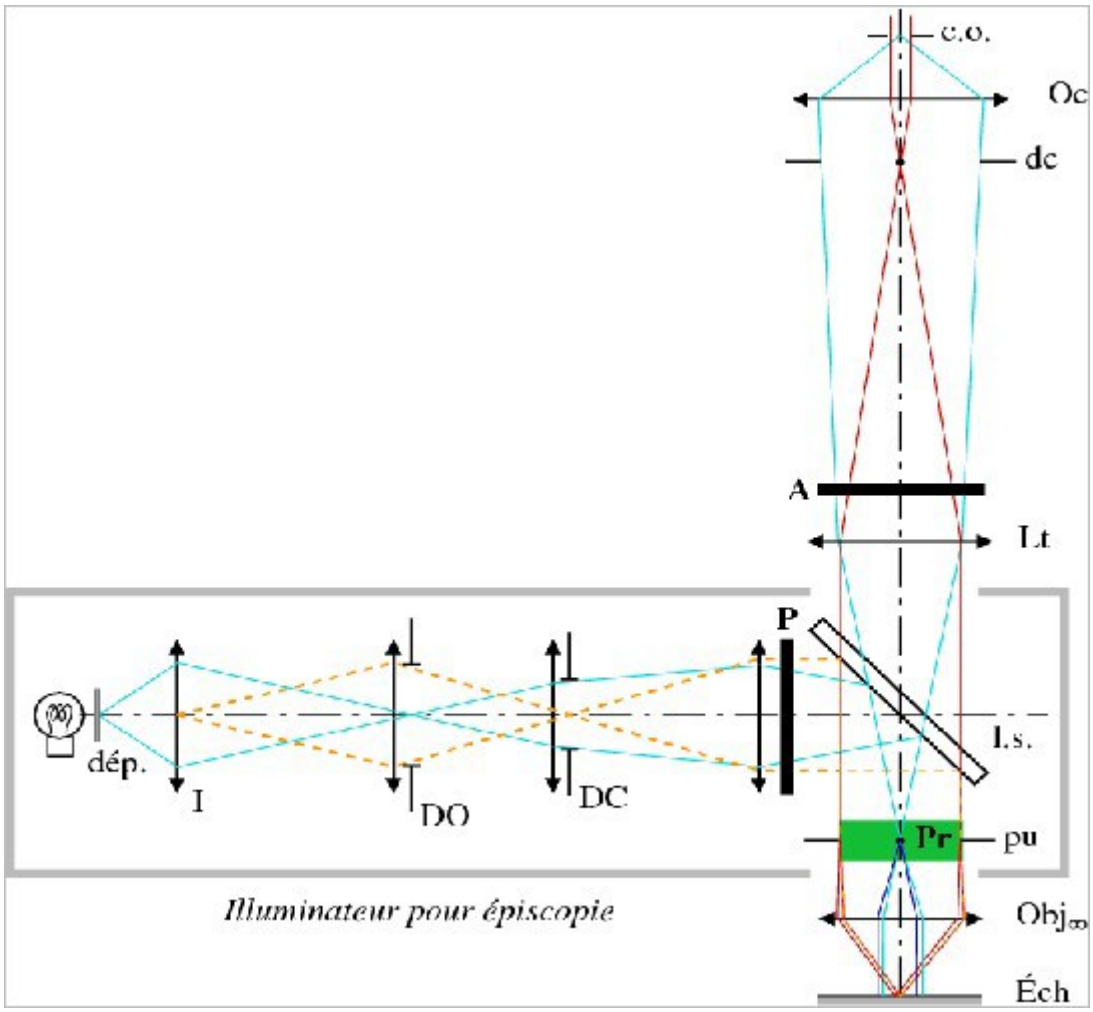
Photomicrographs with white light and with a 50×/0.80 objective in differential interference contrast, for two different adjustments of the parameter φ_0. Shearing direction is 45° to the horizontal *(NW-SE)*.

Standard lateral shearing interferometry in microscopy is performed with birefringent prisms and polarized light. The key device of the method is a WOLLASTON (bi)prism inserted in the image focus of the objective or a NOMARSKI (bi)prism inserted below the image focus of the

objective. Both prisms can split an incident beam to two rays separated by a small angle $\Delta\alpha$ In the objective of the microscope, that angular splitting is transformed into a lateral shear $|f| \cdot \Delta\alpha$ where f is the object focal of the objective. To determine rough estimates, with an objective $50/0,80$ with a focal of $\sim 4mm$ and a resolution $r \sim 0.4\mu m$. An Airy disk radius in that which is visible], rays shifts, which would be less than the resolution, in the object plane, in order to be in interferential contrast mode, produces an angle $\Delta\alpha < r/|f| \approx 10^{-4} rad \approx 0.3'$, which is really inferior to the minute of arc. The next paragraph presents the most important points for performance in episcopy, which would be the easiest to do and to understand.

b) Practical performance in reflection microscopy

It is essential to analyze the equipment and the microscope illumination to correctly take into account the problems of the source spatial coherence. Figure 23 shows a diaphragm of D.I.C. microscope with episcopic KÖHLER illumination.

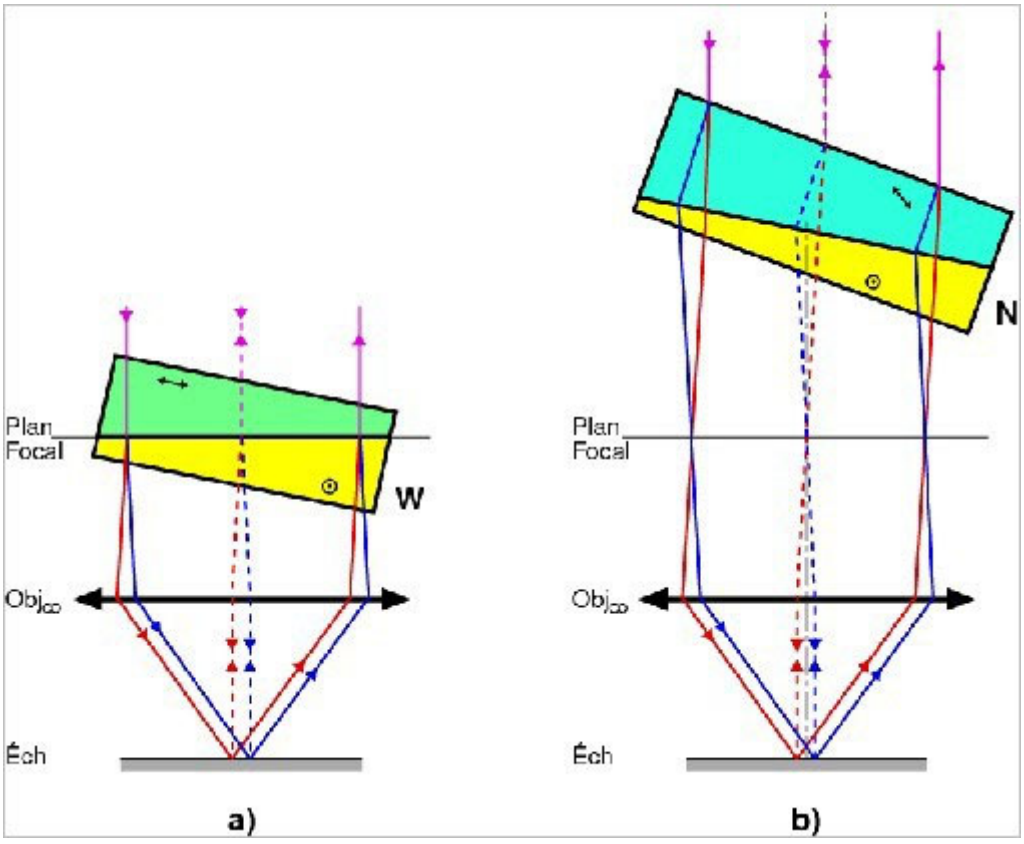


P: *Polariseur Polarizer*
A: *Analyseur Analyzer*
Pr: *Prisme biréfringent (WOLLASTON ou NOMARSKI); voir détails Fig.24 et 25. Birefringent prism (WOLLASTON OR NOMARSKI); for more details, see Fig. 24 and 25.*

(Other elements of the diaphragm have been described in the lesson M11G1 and also in Fig. 6)

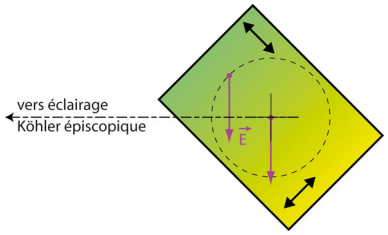
Illuminateur pour épiscopie : Episcopic light source.

Figure 24a is a diaphragm showing the principles of the functioning of D.I.C. with a WOLLASTON (bi)prism. Such a component consists of two prisms of a uniaxial birefringent material (quartz most of the time), assembled together with their axis crossed to $90°$. This component splits in an angular way all the beams that hit it. Splitting happens in the image focal plane of the objective of the microscope, so that a transverse splitting is obtained in the object plane. After specular reflection on the specimen, the split beams are recombined by the same prism. An optimal interference contrast is obtained, as described Figure 25, when neutral lines of the WOLLASTON prism are oriented to $45°$ to the incident polarization and when the analyzer is crossed or parallel to the polarizer (with fixed direction to remain in TE on the semireflecting slide in Köhler illumination *-refer to the paragraph about polarized microscope with episcopic light*.)



Designed rays within prism are simplified to the essential [*complete quantitative calculation of the deviation for a particular beam is proposed as an exercise at the end of this lesson*]. Designed rays are limited to the main beam and to the marginal beam for the central point of the field. (Swinging the prisms permits a correct identification of the plane where beams are splitted. Moreover, that presents the interest of avoiding that stray reflections of episcopic illumination light on the component faces spoiled directly the observed image in the eyepiece).

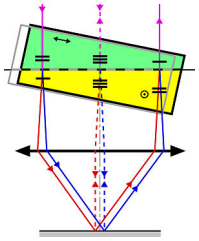
However, as it was already highlighted in the paragraph about phase contrast, the focal plane image of the objective is a mechanically unreachable place since it coincides with the thread of the objective of current commercial microscopes. Georges Nomarski had the idea of modifying the WOLLASTON prism as indicated on Figure 24b [NOMARSKI 55 [Application à la métallographie des méthodes interférentielles à deux ondes polarisées]] with the optical axis of the uniaxial material of one the prisms swung off the interface plane (we may consider in some way that this configuration is the contraction of a SAVART plate, which splits rays by pure translation and of a standard WOLLASTON prism). This new configuration allows moving the birefrigent prism away from the focus of the objective conserving at the same time the same functioning. Then, the prism can be mounted on a slider and be easily inserted or removed. This dispositive, known as *D.I.C. NOMARSKI*, has been patented and exploited by all the microscopes manufacturers.



View from the top of the microscope in D.I.C. (Fig. 23). The incident light is polarized linearly in TE on the semi-reflecting plate of the Köhler illumination of the microscope (*See § about polarized light microscopes in episcopy*). For an optimal contrast of the interferences, the neutral lines of the birefringent prism are placed at $45°$ from this polarization. In practice, this orientation is imposed by the way the prism is inserted in the stand. The analyzer, located below, will also optimally be oriented at $45°$ from the neutral lines of the prism (i.e. crossed or parallel to the polarizer).

Performing the NOMARSKI differential interference contrast (D.I.C.) by reflection by means of a recent episcopic microscope is very simple. All you have to do is to turn the polarizer and the analyzer on, to cross the analyzer to the polarizer moving the orientation knurl of the analyzer and then turning the NOMARSKI prism on inserting it in the slider made for that purpose.

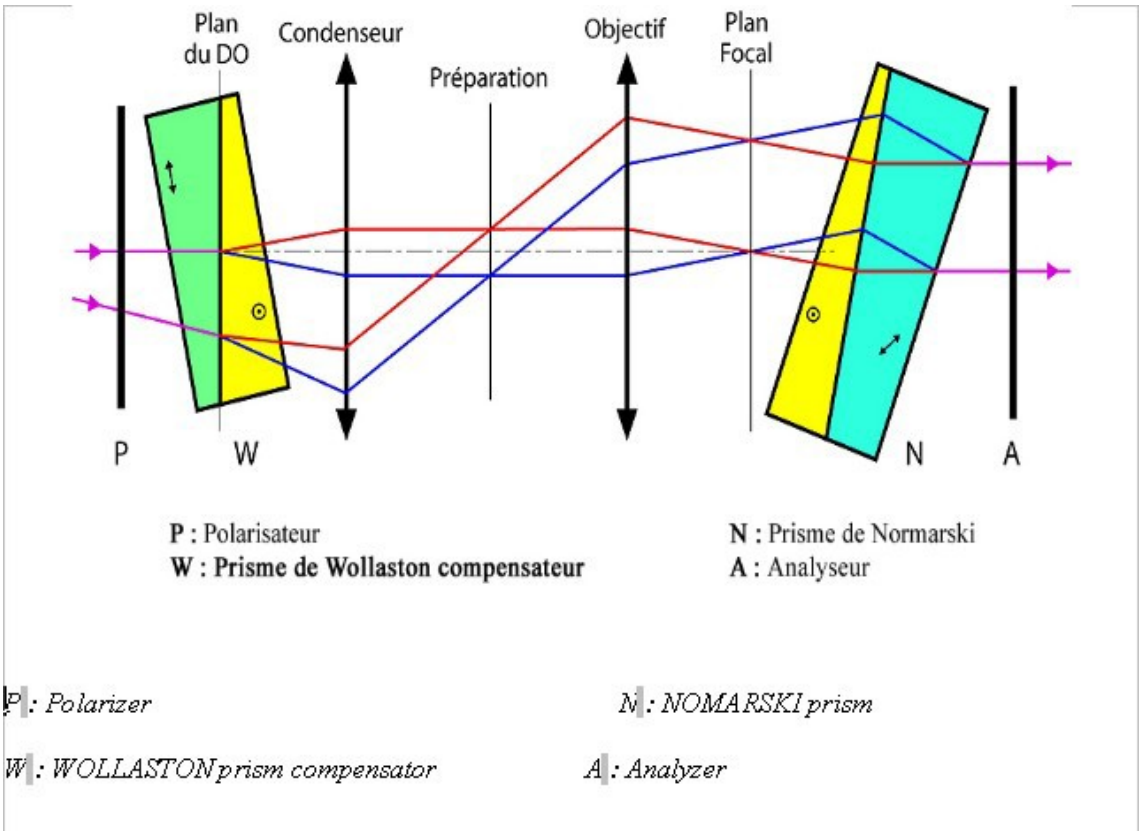
Usually, a knurled thumbscrew is used to translate the prism into its plane in the direction of the rays shearing of an adjustable distance ξ Such a shift leads to a breakage of the automatic self-compensation of optical path of both waves which interfere when the birefringent prism is passing (in both ways) (*see Figure 26 and refer to the unit about the interferences in polarized light*). Therefore, we introduce in the optical path the equivalent of a birefringent plate of an adjustable thickness $e = \kappa \cdot \xi$ where κ depends on the internal angle of the prism. Then appears a term $\varphi_0 \approx 2\pi \cdot \Delta n \cdot \kappa \cdot \xi / \lambda$ (where Δn is the difference between the indexes of the slow axis and the fast axis of the equivalent retardation plate) depending more or less significantly on λ according to the adjustement ξ. With this term of adjustable chromatic phase difference appear adjustable shades when we work with white light. For instance, if we observe the image background of sensitive shades (purple of first order, extinction of green in the fluted spectrum), then it is possible to make an observation in colored contrast with a high sensitivity the variations of gradient on the wave front (see Figure.22).



Regarding the scheme of the prism with back lines and a colored background (bi-prism "centered" or "equilibrated"), the horizontal lines (–, = or ≡) indicate the optical paths which are (almost) equal between each other. There is an exchange of ordinary and extraordinary indexes for a same given polarization ray between the prism represented in yellow and the one represented in green. Therefore, it is easy to notice that, after the light has passed through the bi-prism (in both ways), there is compensation of the path differences between both polarizations for all the incident rays (for a horizontal plane specimen which does not produce any differential interferometric effect). If the component is translated (scheme with grey lines), the compensation does not happen and there is a non-null path difference between both polarizations after the light has passed twice through the component. Nevertheless, this path difference is similar for all the considered incident rays.

c) NOMARSKI D.I.C. in transmission

It is also possible to use the differential interferential contrast in diascopy. However, as the light passes through the prism only once, it is necessary to introduce an external compensation. This is usually performed inserting a WOLLASTON prism with appropriate characteristics into the aperture diaphragm plane of the KÖHLER illumination (See fig. 27). Obviously, the WOLLASTON prism to insert depends on the condenser, on the microscope's objective and on the NOMARSKI prism used. In order to ensure their range of objectives and condensers, the microscopes' manufacturers commercialize for that purpose an appropriate set of WOLLASTON and NOMARSKI prisms.



4. Fluorescence microscopy

Some microscopic objects, little highlighted or differentiated by means of the contrast techniques previously described, are *fluorescent*, which means they can re-emit, on a band with specific wave length, the luminous energy of shorter wave lengths received. Highlighting emissions by fluorescence turns out to be often of high interest because it enables to differentiate identical objects in appearance for any other visualization technique. Some preparations are fluorescent by nature. It is often the case of vegetal objects (for instance the chlorophyll is fluorescent in the IR when it is excited in the red; the human eye can see other natural components which are fluorescent when they are excited in the UV or in the violet/blue). Other materials, such as some polymers, also have this characteristic.

In the other cases, the adjunction of special products (the *« fluorochromes »*) makes it possible to *« spot »* very selectively some specific parties of the preparation on which they rather fix. Many scientific and high technological fields, such as the immunobiology or plastics industry, use intensively this visualization technique.

However, the observation of microscopic objects fluorescence causes delicate problems that can only be solved with the implementation of very specialized methods such as the epifluorescence, which will be described in the next paragraph.

4.1. Principe de l'épifluorescence

The first problem comes from the wave lengths of the excitation source. Indeed, they must always be short if we want to use substances fluorescent for the human eye. Therefore, using those sources, which provide a great quantity of blue, of violet and even very often of ultraviolet is required. Then, it is generally recommended to replace the simple incandescence lamp (halogen lamp) of the KÖHLER illumination by a Xenon burner or by a HBO burner with mercury vapor. Moreover, those short wave lengths require the use of optics made of special materials such as fluorine or quartz due to the fact that standard optical glasses highly absorb in this spectral domain or most are even opaque in the ultraviolet. All the manufacturers offer such optics and include elements made of fluorine, a material which, in addition, makes it possible to improve the chromatic correction of objectives thanks to its very low dispersion. Some manufacturers offer additional objectives made with materials of high purity which do not present any fluorescence that could trouble the images.

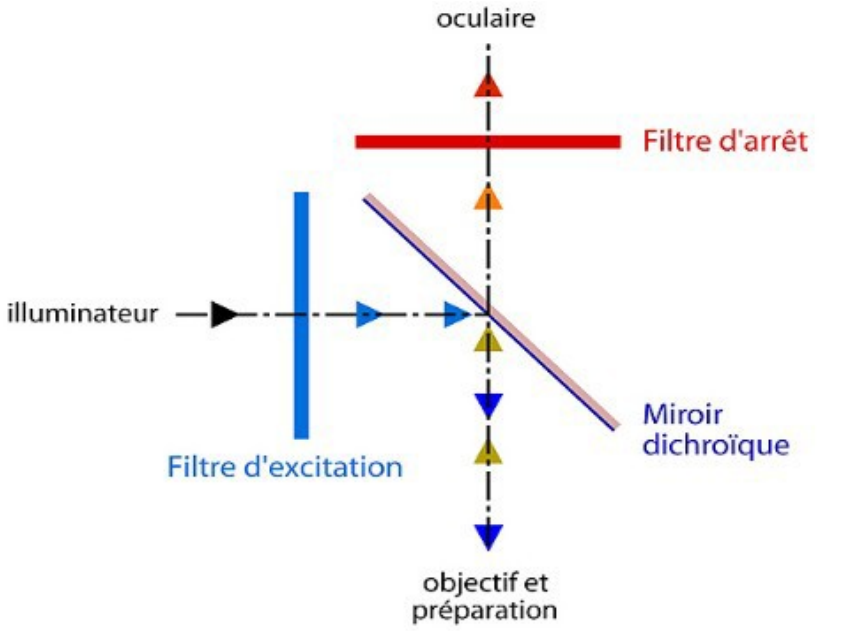
The second and main problem of the detection of microscopic objects fluorescence is due to the very low energetic efficiency of this conversion mechanism of luminous flux wavelength. The fluxes we are trying to detect are extremely low whereas the illumination and its standard diffusion by preparation are violent. To make the observation possible, we need to keep the signal-to-noise ratio to an acceptable level. To do so, we usually make the most of the spectral separation of the excitation and of the useful signal which we can separate by appropriate filtering.

The *epifluorescence* is a method enabling overcoming the difficulties previously mentioned: the preparation is illuminated from above by means of an episcopic illuminator. The observation of fluorescence is performed in a microscopy-type configuration by reflection.

One of the advantages of this *« backlight »* is due to the fact that the observed preparations are usually more transparent than reflective. The reflected and backscattered excitation light, which constitutes the parasitic luminous flux to eliminate, is therefore weaker than the transmitted and diffused forward light, which would constitute the parasitic flux in a line illumination configuration. As the fluorescent emission is an isotropic phenomenon, the useful signal does not depend on the direction of the illumination and the signal-to-noise ratio is therefore definitely better in episcopy. This advantage is even strengthened if we take the preparation for the light to pass through into account. In that case, the excitation must pass only through the cover slip ($0.17mm$), which can be highly useful if made of quartz (or even absent) if we work with UV, whereas in the other case it must pass through the object slide ($\sim 1mm$) which is almost always made of glass.

The second advantage of the episcopic configuration lies in the possibility to perform an efficient spectral filtering of the excitation and of the useful signal in the illumination unit. In

order to work in epifluorescence, we replace the semi-reflecting slide of the KÖHLER illumination by reflection (See Figure 6 or 23) by a « cube » composed of two filters and one dichroic mirror whose configuration is represented on Figure 28.



Ce "cube" remplace la lame semi-réfléchissante standard de l'éclairage Köhler par réflexion.

This "cube" replaces the standard semi-reflecting slide of the KÖHLER illumination by reflection.

The *excitation filter* selects the only wavelengths which are appropriate to the fluorescence excitation of the specimen in the luminous flux coming from the source. The *dichroic mirror* reflects the short wavelengths corresponding to the excitation and transmits the wider wavelengths. Therefore, it strengthens, in the first place, the effect of the excitation filter. The light coming from the specimen, which contains the fluorescence light and the parasitic light of the backscattering excitation, hits the dichroic mirror. The fluorescence is transmitted with very little losse, whereas the main part of the parasitic light is reflected. The luminous beam then reaches the rejection filter which, by definition, stops the residual parasitic excitation light and lets the fluorescence pass through. With high quality filters and mirrors, this disposition enables excellent results with a very simple implementation which is very safe for the user. In general, the manufacturers offer a large range of combinations for filters and dichroic mirrors that are adapted to the substances and fluorochromes commonly used.

The epifluorescence also has the advantage of enabling the conjoint use of the light transmitted. The images obtained in fluorescence are generally composed of small emissive zones spread in a dark background. It is very hard, under these conditions, to identify the objects which originate the fluorescence. Since then, it is very useful to be able to simultaneously observe the preparation in diascopy. That does not cause any practical problem on recent microscopes which are composed of two separate illumination units, one diascopic and the other episcopic. The power of each unit can be adjusted separately. Besides, we generally use diascopic phase contrast to improve the visualization and the identification of objects.

* *
*

This unit M11G2 presented the main lines regarding the principles of the most standard techniques to improve the contrast and the visualization of objects used in microscopy nowadays. Reading of the reference [Roblin 99 [Encyclopédie des Techniques de l'Ingénieur (tome R7)]] as well as consulting websites such as *http://www.molecularexpressions.com/*[5], *http://www.microscopyu.com/*[6] or, *http://www.olympusmicro.com/*[7] (all three in English) may provide additional and interesting information on this subject.

5 - http://www.molecularexpressions.com/
6 - http://www.microscopyu.com/
7 - http://www.olympusmicro.com/

III.Case study

1. Technique using polarisation

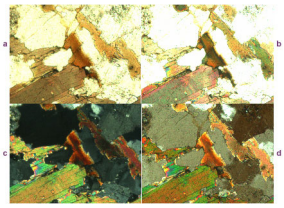
1.1. Diascopy

This type of observation enables us to know the optical properties of a material, and is mainly used in geology in order to determine the different types of crystals which compose a rock.

Figure 1 is the image of a $30\mu m$ thick granite plate observed in transmission in day light (a) and then in polarized light.

Birefringent crystals show some Newton shades which are distinctive features of their birefringence and of the plate's thickness.

Granite is composed of three crystals: quartz, mica and feldspar. The different varieties of feldspar are very similar and can only be distinguished from one another thanks to optical methods.

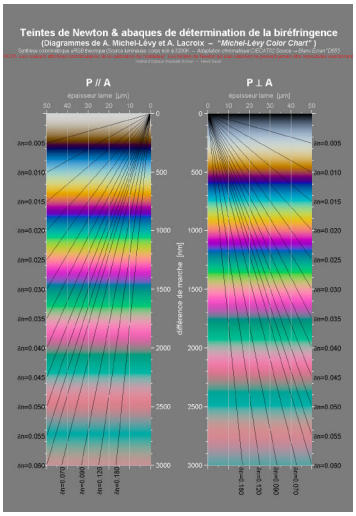


(**a**) : day light, (**b**) : plate between parallel polarizers , (**c**) plate between crossed polarizers , (**d**) any position of the two polarizers.

Remarque

Geologists usually stand between the crossed polarizer and analyzer, then have the plate spin on its plane and observe the variations of the crystals' shades. Thanks to Michel-Lévy's table, they can determine the order of magnitude of the crystals' birefringence. Knowing the plate's thickness, they can deduce from it the nature of the crystals amongst possible materials selected from purely geological knowledge.

Figure 2 is a simplified representation of Michel-Lévy's table.



In the left part of Figure 2 shows the Newton shades when observed between a parallel polarizer and an analyzer; the right part shows these shades between crossed a polarizer and an analyzer. The upper scale indicates the thickness e of the plate, which grows from 0 to $50\mu m$.

The figures in white, in the center, indicate the path difference introduced between the plane wave due to the slow axis and the plane wave due to the fast axis of the retardation plate. Beyond the interference order 3 (around $\delta = 1700nm$) shades become very pale, they are washed-out, whiter.

Attention

Here is explained how geologists can use this diagram:

- **By comparison they can determine the diagram shade which matches the one observed on the specimen. To avoid any ambiguity, they spin the analyzer by $90°$ in order to see if the complementary color is matching correctly.**
- **Then they determine the black straight line that passes at the intersection of the horizontal determined by the color and of the vertical white straight line given by the plate's thickness.**
- **Finally they can read the corresponding value of $|\delta n|$.**

In the presented case (Figure 1), we can observe in the top left corner of the picture a large quartz crystal which appears white between parallel polarizers and black between crossed polarizers. We can give the following interpretation: one of the neutral axes of the quartz plate happens to be on the plane of the plate and parallel to the polarizer. For another relative orientation of the polarizer P and the crystal, the crystal being always cut parallel to the axis, we would observe more pronounced colors.

Conseil

Indeed, in order to observe Newton shades, the directions of the neutral axes of the crystal must be at $\pm 45°$ to those of the polarizer.

In the latter case, given that the thickness of the plate is $30\mu m$ and the birefringence of quartz is 9.10^{-3}, the path difference equals $270nm$, which would correspond to a straw-yellow shade between crossed polarizer and analyzer, and to a dark red between parallel polarizer and analyzer. Another possibility would be that the crystal could have been cut perpendicularly to the optical axis, the latter then being parallel to the microscope axis. In this case, rotating the plate would not bring up any particular colored shade.

On Figure 1d, P remains unchanged and only A has rotated. There is still no Newton shade, and the intensity decreases according to Malus law.

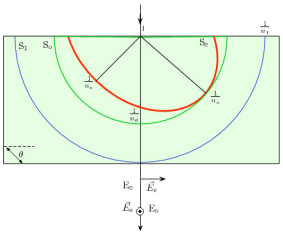
In the bottom left corner of the picture we can see a *« black mica »*, a biotite which appears brown between parallel polarizers and yellow rimmed with Newton shades between crossed polarizers. These shades must come from the fact that the direction of the polycrystals' optical axes differ. Finally feldspar corresponds to the right corner crystals, which appear white between parallel polarizers and gray-blue between crossed polarizers.

Remarque

Note that when observing, we have no direct access to the absolute value of the birefringence ($n_e - n_o$) in the case of a uniaxial medium, but only to a minorant of this value, because there is no reason that the optical axis should be on the plane of the rock's cut. Actually, we evaluate the quantity $n_\theta - n_o$, where n_θ represents crystal's index according to the propagation direction of the extraordinary ray.

For a normal incidence (perpendicular to the input diopter), the ordinary and extraordinary rays seem to merge when they come out of the plate of thickness e, but there is actually a path difference $\delta = (n_\theta - n_0) \cdot e$ between them, since according to the propagation axis the extraordinary ray *« sees »* the index n_θ as illustrated on Figure ... .

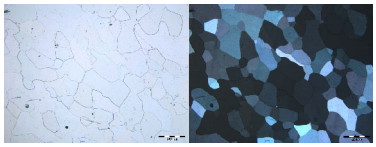
The same goes for a biaxial crystal where we only have access to the difference of two indexes lying between the biggest and the smallest of the indexes peculiar to the material.



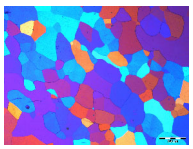
This technique makes it possible, for instance, to control polyamide or polyoxymethylene castings which crystallisation must be even on the whole casting. It can also be used to control cellulose acetate membranes of dialyzers.

1.2. Episcopy

The picture on the left side of Figure 4 shows grains in an aluminum structure. These grains barely differ from one another. We can observe them better with a polarized light microscope, because the shades of gray differ from one grain to another. This shade difference comes from the fact that the crystallographic orientations differ from one grain to another.



In order to increase this differentiation, the Metallography Department of the Ecole des Mines in Saint-Etienne lays a $150nm$ thick alumina layer and a wave plate for $\lambda = 530nm$ on the specimen. The grains appear then with different colors (Figure 5).



For more details, please refer to the following website: *http://www.olympusmicro.com/primer/java/polarizedlight/crystal/index.html*[8]

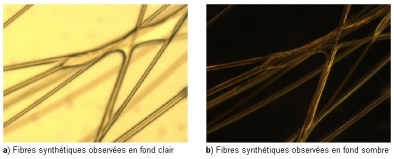
2. Black field or Schlieren method

This technique is based on filtering spatial frequencies. It reveals the presence but not the structure of flaws or irregularities that can not be observed against a light background. It is also used to observe poorly reflecting objects, such as paper, fibres, plastic or composite materials.

8 - http://www.olympusmicro.com/primer/java/polarizedlight/crystal/index.html

2.1. Diascopy

Observing thin synthetic fibres on a black field is more pleasant than on a light field, because the contrast is accentuated, and the fibres perfectly stand out on a black field.



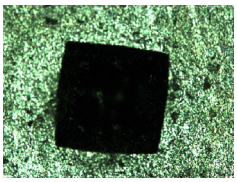
a) Fibres synthétiques observées en fond clair b) Fibres synthétiques observées en fond sombre

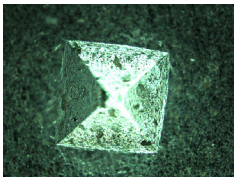
2.2. Episcopy

This technique is used to observe semi-opaque structures with internal reflections or plane structures where light diffusion can be observed on the edges of the grains.

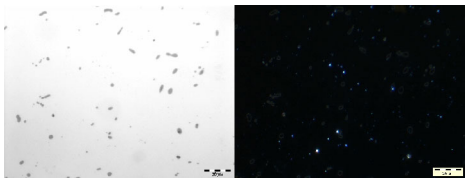
Remarque

For instance, the picture of a Vickers print for a hardness measurement does not enable us to see the bottom of the print when the picture has been taken on a light field. Conversely, when the picture is taken on a black field, the inside of the print is clearly observable.





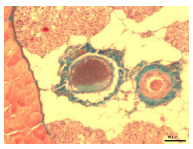
This technique also reveals the aluminum oxides of the manganese sulphides in steel. On picture **a)** of Figure 9, we can barely distinguish the manganese sulphides from the aluminum oxides. Conversely, on picture **b)**, we can easily spot the aluminum oxides: they are the shiny dots on the picture.



3. Technique of phase contrast

3.1. Diascopy

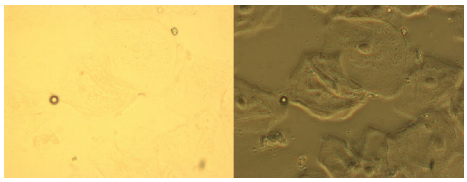
The absorption differences of the structures of cells or micro-organisms are not different enough from one another to make a contrasted observation possible. In order to increase the contrast, we can resort to the technique of coloring the specimen, but this will have the major downside of killing the cell, as in the example of Figure 10.



On the left, we can see the muscle fibres, and in the center of the picture, a vein with a blue colored wall that is less thick than the arterial wall on its right.

The technique of phase contrast avoids this downside and enables us to observe living cells. Compared to other techniques, its advantage is its insensitivity to polarization and birefringence.

On Figure 11, the cell is observed on a light background, and we can barely see its limit in the centre of the top half of the picture. Conversely, in phase contrast the cell and its nucleus can be easily spotted. A downside of this technique is the presence of a white halo around each cell, which makes it very difficult to precisely see its edge.



This technique also enables us to highlight the flaws that occur when transforming amorphous plastic material, such as modified polystyrene, through injection moulding, when the mould and the plastic material temperatures are not correct or when there is a change in the injection pressure.

4. Differential interference contrast (DIC) in episcopy

4.1. General points

Interferometry is a common method performed in optics to reveal and measure the phase of a luminous wave. Most of the 'macroscopic' configurations made with an imaging interferometer, performing in *normal interferometry* (comparing a wavefront of interest to a reference wavefront) or in *differential interferometry* (comparing a wavefront of interest to the same translated or sometimes inclined wavefront) can be transposed to microscopy.

Complément

That concerns particularly the interferometer of MACH-ZEHNDER, MICHELSON, FABRY-PEROT, etc. Microscopists often call these interferometers by the name of the physician who adapted them to microscopy, which is why we talk about the systems of "MICHELSON-LINNIK" (MICHELSON), of "MIRAU" (MICHELSON), of "WATSON" (MICHELSON), of "TOLANSKY"

(FABRY-PEROT), etc. See the reference [Roblin 99 [Encyclopédie des Techniques de l'Ingénieur (tome R7)]] for more information.

But most of these transpositions of interferometry to microscopy require very specific equipment which are not commonly found. Only two systems, easily adjustable to ordinary commercial microscopes (the MIRAU objective and the NOMARSKI differential interference contrast), are commonly available and used, and we will only describe those two.

IV.Exercises

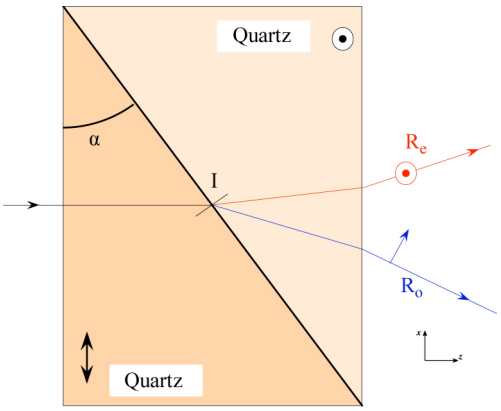
1. Ray paths in a Wollaston prism

As a reminder, we can recall that a **Wollaston** prism is made of two right-angle prisms cut in the same uniaxial birefringent crystal, generally a quartz crystal. The two prisms have parallel cuts and the two optical axes are perpendicular. To make the exercise simpler, we will only consider the rays for which the plane (x, z) perpendicular to the edges of the Wollaston prism is the plane of incidence.

Question 1

[*Solution n°1 p 45*]

Using Huygens' construction, explain the ordinary and extraordinary ray paths in Wollaston prism for a null angle of incidence.



Question 2

[*Solution n°2 p 48*]

Show that the angle formed between the ordinary and the extraordinary rays is roughly worth : $2(n_e - n_o)\tan\alpha$

Question 3

[*Solution n°3 p 49*]

This Wollaston prism is used with a infinity-corrected objective of a $4\,mm$ focal ($\times 50$ magnification associated with a lens of a $200mm$ tube) and a 0.8 numerical aperture. What must the value of angle α be if we want the ordinary and extraordinary rays to be separated from one another by an angle equal to $2/3$ of the resolution of the objective in its image space, that is to say, according to Rayleigh criterion for an objective limited by the diffraction, to $2/3$ of the angular radius of the Airy disk? (the ordinary and extraordinary indexes of the quartz in the visible are $n_o \approx 1.544$ and $n_e \approx 1.553$).

To solve this problem, the lesson on birefringent medium must be referred to ([Surrel [Optique instrumentale Optique de Fourier]] for a simplified approach and [Huard [Polarisation de la lumière]] to take it further .

2. Interferential contrast in the case of a transmission microscope

Of two Wollaston prisms identical to the one studied previously, one is positioned at the level of the object focal point of the condenser, and the other one at the level of the image focal point of the objective. These two prisms are placed between crossed polarizers, and at $\pm 45^\circ$ to the prisms' axes.

Question 1

[*Solution n°4 p 49*]

Express the lateral shift d between the extraordinary and the ordinary ray.

Question 2

[*Solution n°5 p 50*]

Show that the system visualizes the gradient of the phase variations of the specimen. To make things simpler, we will consider that the source is monochromatic.

Solution des exercices

>Solution n°1 *(exercice p. 43)*

The direction z corresponds to the direction of propagation of the light and (x,z) is the plane of incidence. On the following animation, the first prism reached by the light is green with a purple checkerboard, and the second is blue. In I three wave surfaces are represented: the first one is purple and of spherical shape, and corresponds to the ordinary wave surface of the two prisms; the second one is of a gray-green color and is relative to the extraordinary wave surface of the first prism; finally the third one is red and shows the extraordinary wave surface of the second prism. Quartz being a positive crystal, the ellipsoidal wave surfaces are inside the spherical wave surface.

The wave surface corresponding to the ordinary ray in the first prism is tangent to the gray-green ellipsoid relative to the extraordinary wave surface, the tangent direction being parallel to the direction of the optical axis, which direction is the same as the axis x. This ordinary wave surface is also tangent to the red ellipsoid relative to the extraordinary wave surface in the second prism, the tangent direction being now parallel to the axis y.

The animation shows these wave surfaces under different angles of view. The first angle shot corresponds to Figure 2, then the camera moves to face the plane (y,z) and finally it moves upward to face the plane of incidence (x,z). Figure 3 corresponds to the animation's last angle shot.

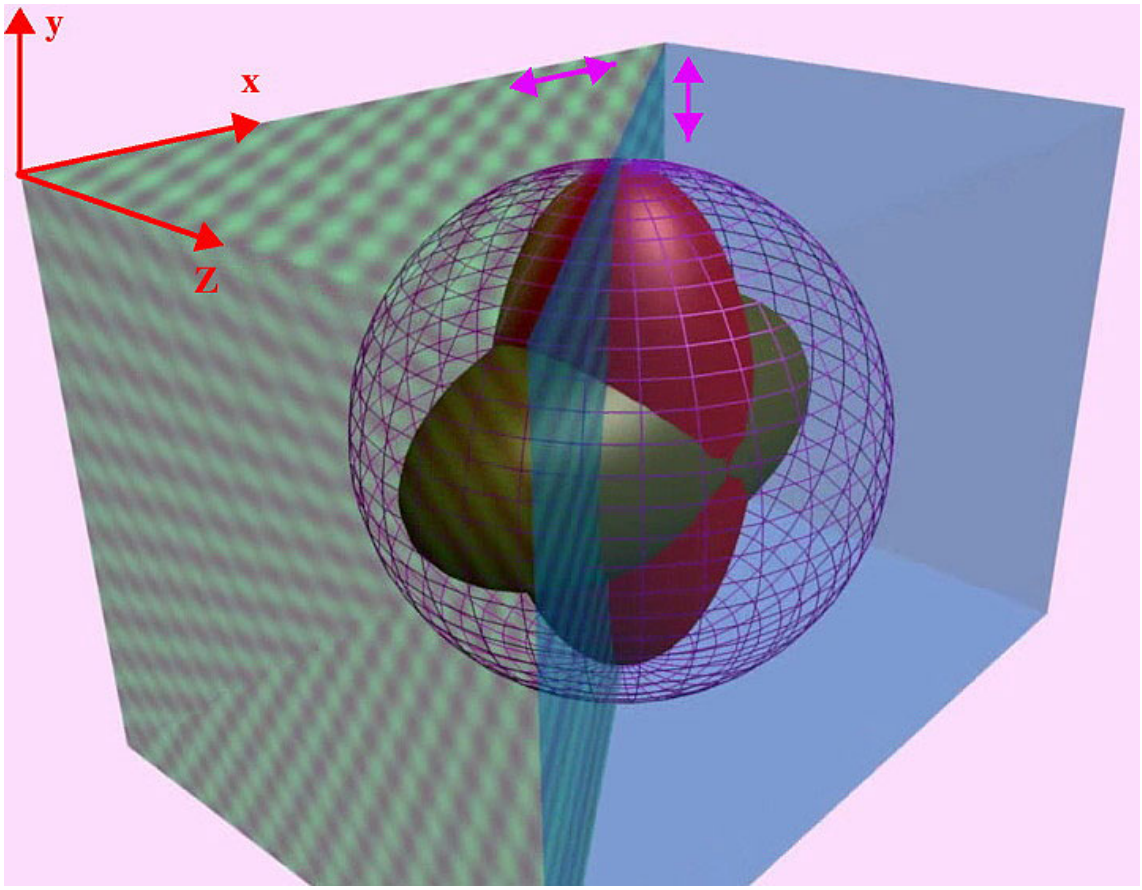
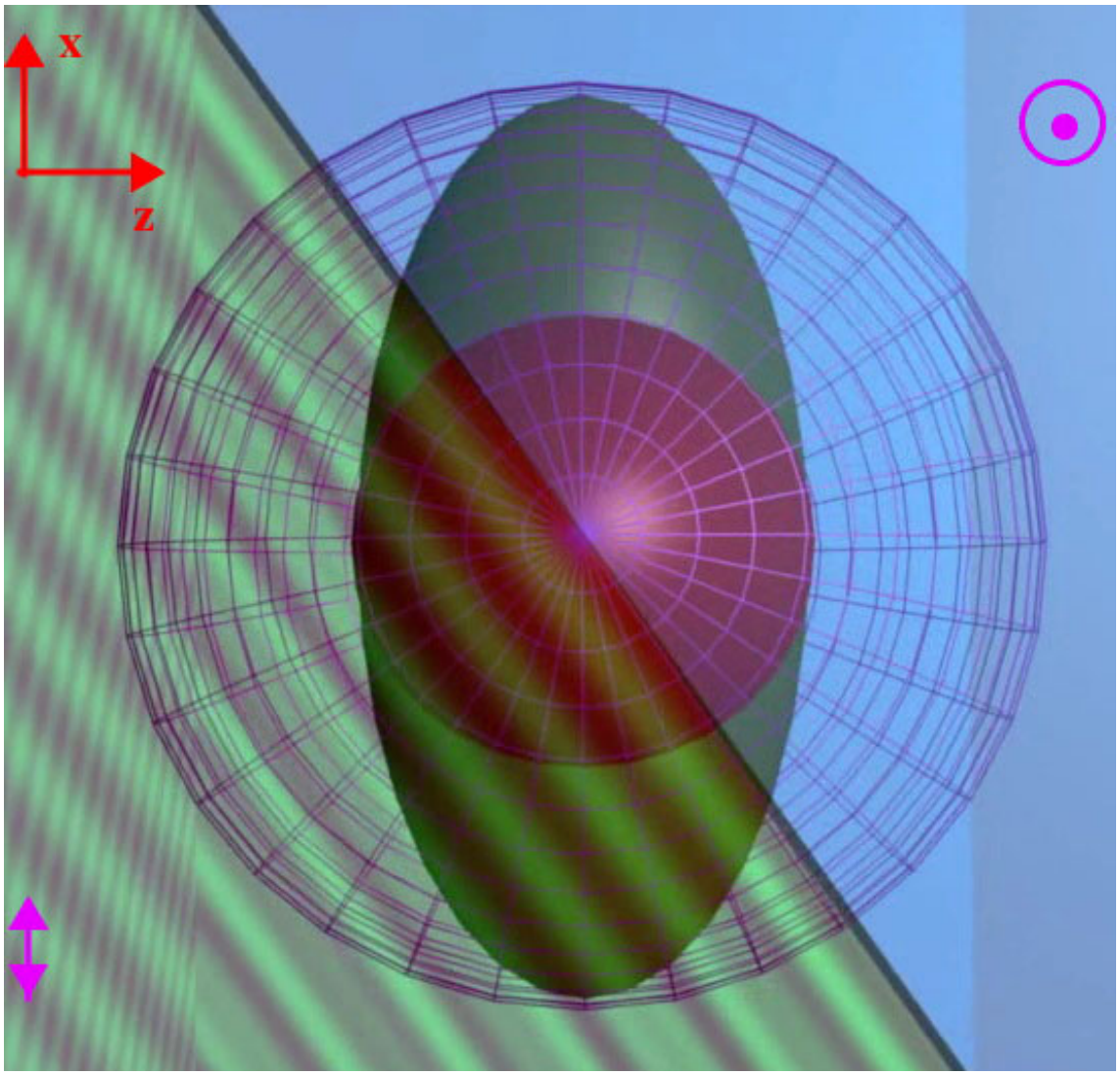


Figure 3 shows these wave surfaces, the prisms being seen from above and following direction y. See Figure 4 for a configuration of Figure 3.



From now, we will be thinking from Figure 4. Since the angle of incidence is nil at the level of the first diopter, the angle of refraction has a zero value as well, and in the first prism the ordinary and extraordinary rays are not separated, but they propagate at different speeds. The thickness of the glue between the two prisms acts as a parallel faces plate of very little thickness. We decide to ignore the slight translation of the ray due to this plate.

The axes of the two prisms being crossed, the ordinary ray in the first prism becomes extraordinary in the second and vice versa. The second animation shows the propagation of the electric field vector. The incident light is polarized with a linear polarization and a direction at a $45°$ angle to the directions of the neutral lines in the two prisms' quartz crystals. Both components of the electric field vector are directed following these neutral lines. These two components are depicted in black when they are in the air.

When going through a diopter, concentric circles have been depicted, in order to get a visual representation of the phenomenon, but they do not correspond to any physical phenomenon.

In the first prism, the component depicted in red is relative to the extraordinary ray, and the blue one to the ordinary ray. The latter propagates faster, because the crystal is positive.

When passing through the second prism, the ordinary and extraordinary rays exchange their functions for the crossed crystals' axes.

In the air the two components go back to propagating at the same speed.

For the first prism, the main plane is contained in the plane of incidence which is the same as the plane of Figure 4. The wave surfaces relative to the ordinary and extraordinary rays are noted $S_{o,1}$ and $S_{e,1}$. For the second prism the spherical wave surface $S_{o,2}$ relative to the ordinary beam is not separated from $S_{o,1}$ since both prisms are made from the same material. Conversely for the extraordinary wave surface $S_{e,2}$,the main axis of the ellipse is perpendicular to the figure's plane, since the optical axis is perpendicular to the plane of incidence.

The prolongation of the ordinary incident ray touches the ordinary wave surface at point O. The wave plane $P_{o,1}$, tangent in O, touches the separating diopter of the prisms in K. During the refraction, no phase difference is introduced, the refracted wave plane also passes through point K. This wave plane $P_{o,2}$ is relative to the extraordinary ray, since as we have already mentioned it, the axes of both prisms being crossed, the ordinary ray in the first prisms becomes extraordinary; this plane is then tangent in a point A at the extraordinary wave surface $S_{o,2}$ of the second prism. The extraordinary ray $R_e$passes by the point A. At the level of the diopter between the second prism and the air, Descartes Laws simply apply :

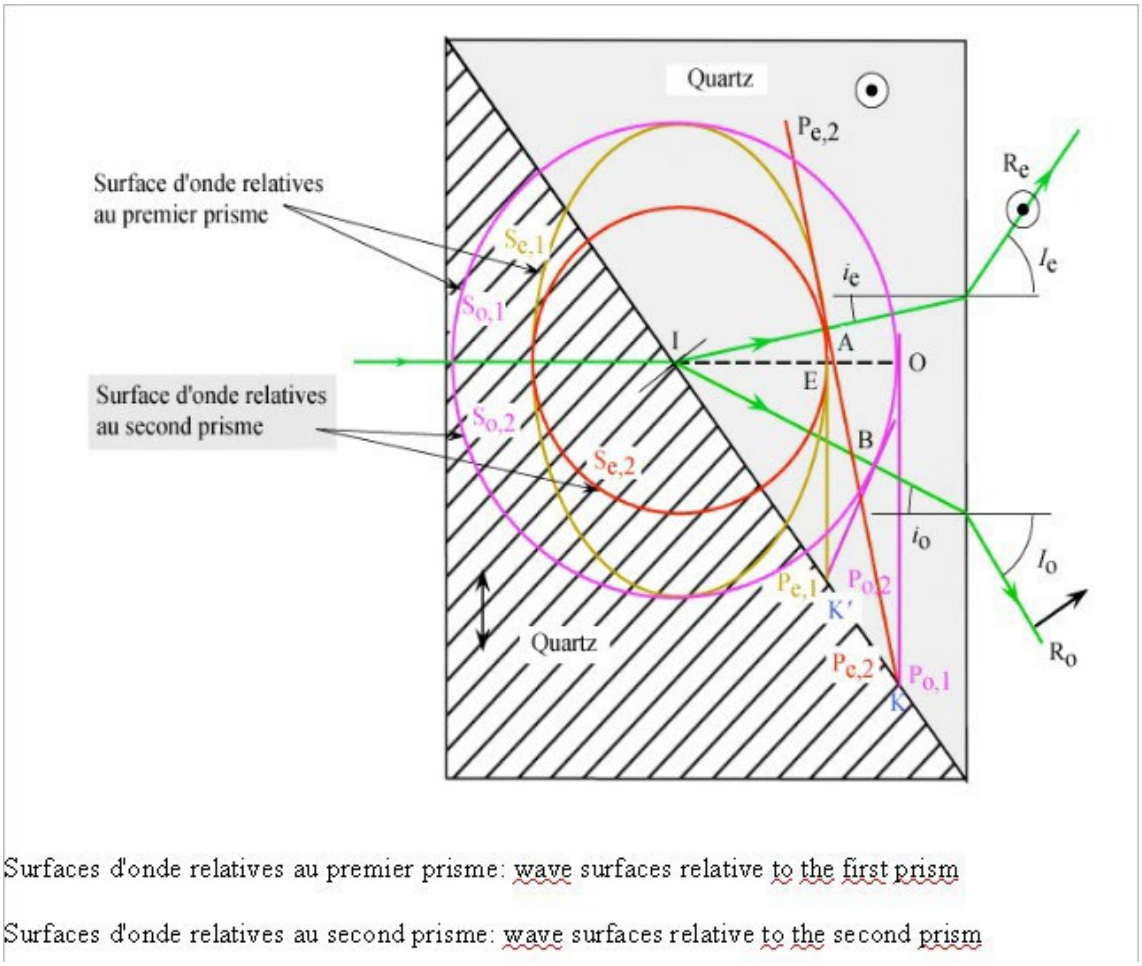
$n_e \sin i_e = n \sin I_e$

n being the index of the outside environment, which is generally air.

In the same way, the extraordinary incident ray's prolongation touches the extraordinary wave's surface in a point E. The wave plane $P_{e,1}$,tangent in E, touches the prisms' separating diopter in K'. During the refraction, no phase difference is introduced, the refracted wave plane passes through the point K'. This wave plane $P_{o,2}$ is relative to the ordinary ray; the axes of both prisms being crossed (the extraordinary ray in the first prism becomes ordinary), this plane is tangent in a point B to the ordinary wave surface $S_{o,2}$ of the second prism. The ordinary ray R_o passes by point B. Out of the second prism, Descartes Laws apply again:

$n_o \sin i_o = n \sin I_o$

n being the air index.



Remarque

This type of construction gives access to the rays direction, that is to say to the energy flows (direction of Poynting vectors), but not to the wave vectors' direction. It corresponds to the geometrical optical classic constructions which indicate the energy's path. However, in this particular case - the incidence being normal and the Wollaston prism being crossed in this direction - there is no difference between the Poynting vectors' directions and the wave vectors' directions in both prisms and in the air (one of the reasons for this is that the cut of the ellipsoid with the plane of incidence happens to be a circle in the second prism).

>Solution n°2 *(exercice p. 43)*

At the level of the separating diopter between the two prisms, the angle of incidence of the ordinary and extraordinary rays is equal to the angle of the prism α. Applying Descartes Law at a point I:

$n_o \sin(\alpha) = n_e \sin(\alpha - i_e) = n_e[\sin(\alpha) \cdot \cos(i_e) - \cos(\alpha) \cdot \sin(i_e)]$

$n_e \sin(\alpha) = n_o \sin(\alpha + i_o) = n_o[\sin(\alpha) \cdot \cos(i_o) + \cos(\alpha) \cdot \sin(i_o)]$

Angles i_e and i_o being small:

$n_o \sin(\alpha) \approx n_e \cdot \sin(\alpha) - \cos(\alpha) \cdot n_e i_e$

$n_e \sin(\alpha) \approx n_o \cdot \sin(\alpha) + \cos(\alpha) \cdot n_o i_o$

Hence :

$\sin(\alpha)[n_e - n_o] = n_e i_e \cdot \cos(\alpha)$

$\sin(\alpha)[n_e - n_o] = n_o i_o \cdot \cos(\alpha)$

And :
$n_e i_e = (n_e - n_o) \cdot \tan(\alpha)$
$n_o i_o = (n_e - n_o) \cdot \tan(\alpha)$
Out of the second prism in the air, at the level of the third diopter and considering that the index n of the air is equal to 1:
$n_e \sin(i_e) = \sin(I_e)$
$n_o \sin(i_o) = \sin(I_o)$
Considering the approximation of the small angles:
$\sin(I_e) \approx n_e i_e = (n_e - n_o) \cdot \tan(\alpha) \approx I_e$
$\sin(I_o) \approx n_o i_o = (n_e - n_o) \cdot \tan(\alpha) \approx I_o$
The value of the angle between the two rays is equal to:
$I_e + I_o = 2(n_e - n_o) \cdot \tan(\alpha) = \in$

>Solution n°3 *(exercice p. 43)*

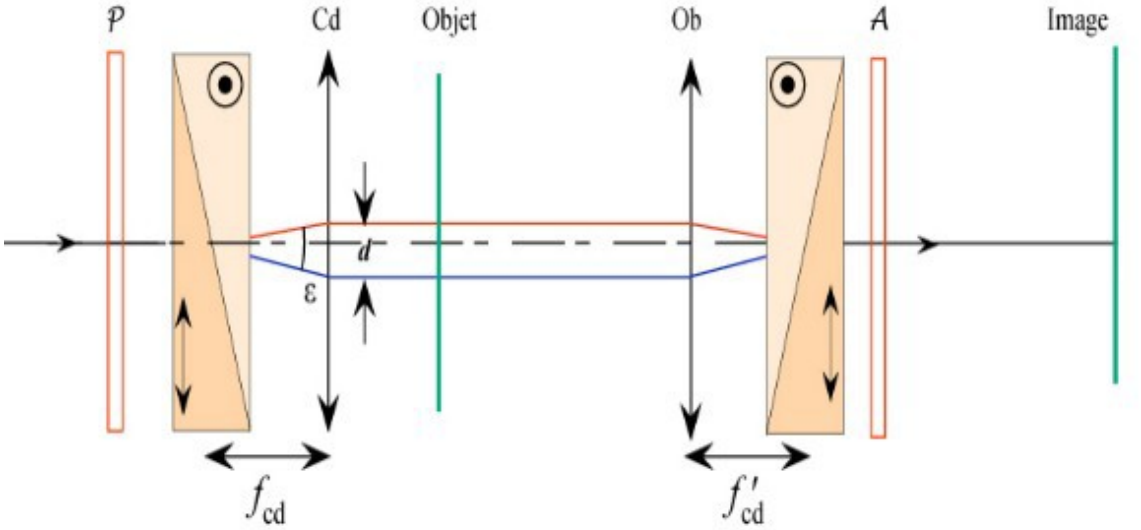
The value of the radius of the Airy disk due to the objective in its object space is equal to:
$$r = 1.22\frac{\lambda}{2 \cdot NA} = 1.22 \cdot \frac{0.55}{2 \cdot 0.80} = 0.42\mu m$$
which corresponds to an angular radius in its image space of:
$$\frac{r}{f'} \approx 10^{-4} rad$$
which therefore leads to a necessary angular deviation between the two ordinary and extraordinary rays of:
$$\epsilon = \frac{2}{3}\frac{r}{f'} \approx 0.7 \cdot 10^{-4} rad$$
hence an angle for the prism of:
$$\alpha = \arctan\{\frac{\epsilon}{2(n_e - n_o)}\} \approx 55 \cdot 10^{-4} rad \approx 0.2°$$

>Solution n°4 *(exercice p. 44)*

The angle ϵ being small, the distance d between the two rays is roughly equal to: $f'_{obj} \cdot \epsilon$,

Attention

f'_{obj} being the condenser's focal distance. Distance d is always very small, it remains less than inferior to the resolution limit of the objective in order not to perceive the shearing of the image, which is of the order of a fraction of a micrometer.



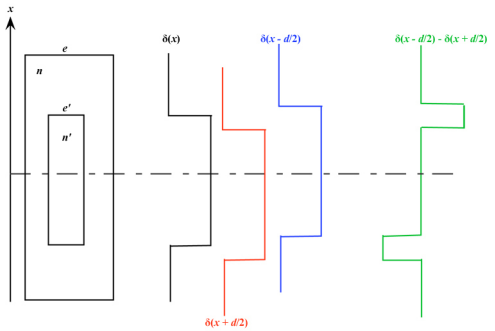
Objet: object
image: Image

>Solution n°5 *(exercice p. 44)*

At any point x of the object where a path difference exists, the variation of the path difference between the ordinary ray and the extraordinary ray shifted from d has a value of:

$$\Delta(x) = \delta(x + \frac{d}{2}) - \delta(x - \frac{d}{2}) = \delta'(x) \cdot d$$

where $\delta'(x)$ is the derivative of the path difference. To illustrate this, we consider a homogeneous medium of index n, of constant thickness e , except on an area of thickness e' where the index is equal to$n' > n$. Figure 6 shows the path difference in function of the position x at the level of the object. The same path difference for the extraordinary ray is depicted in red and in blue for the ordinary ray, shifted of $\pm d/2$; the difference of the two path differences corresponding to the two rays that interfere at the level of the image plane is depicted in green.



Attention

Therefore, the path difference highlighted by the interferences is a linear function of the slope of the path difference introduced by the object. This means that we do not visualize the phase variations, but its gradient variations.

Glossaire

Georges Nomarski

1919-1997: Former researcher at the French Optics Institute (of Paris, now Palaiseau).

Illuminations

The correct mathematical demonstration of this result requires starting from the second-order limited development. The reader can verify it as an exercise.

Manufacturers

It has to be said that those systems are usually set up on middle or top-of-the-range microscopes which are currently equiped with infinity-corrected objectives . This unit diaphragms would always take this fact into account; the tube lens is included in the stand.

Newton shades

Material dispersion (wavelength-dependent refractive index) rigorously produces a fluted spectrum slightly different from Newton' shades' one. Howerer, this dispersion is sufficiently weak in most practical cases to make this effect imperceptible on the seen colors.

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