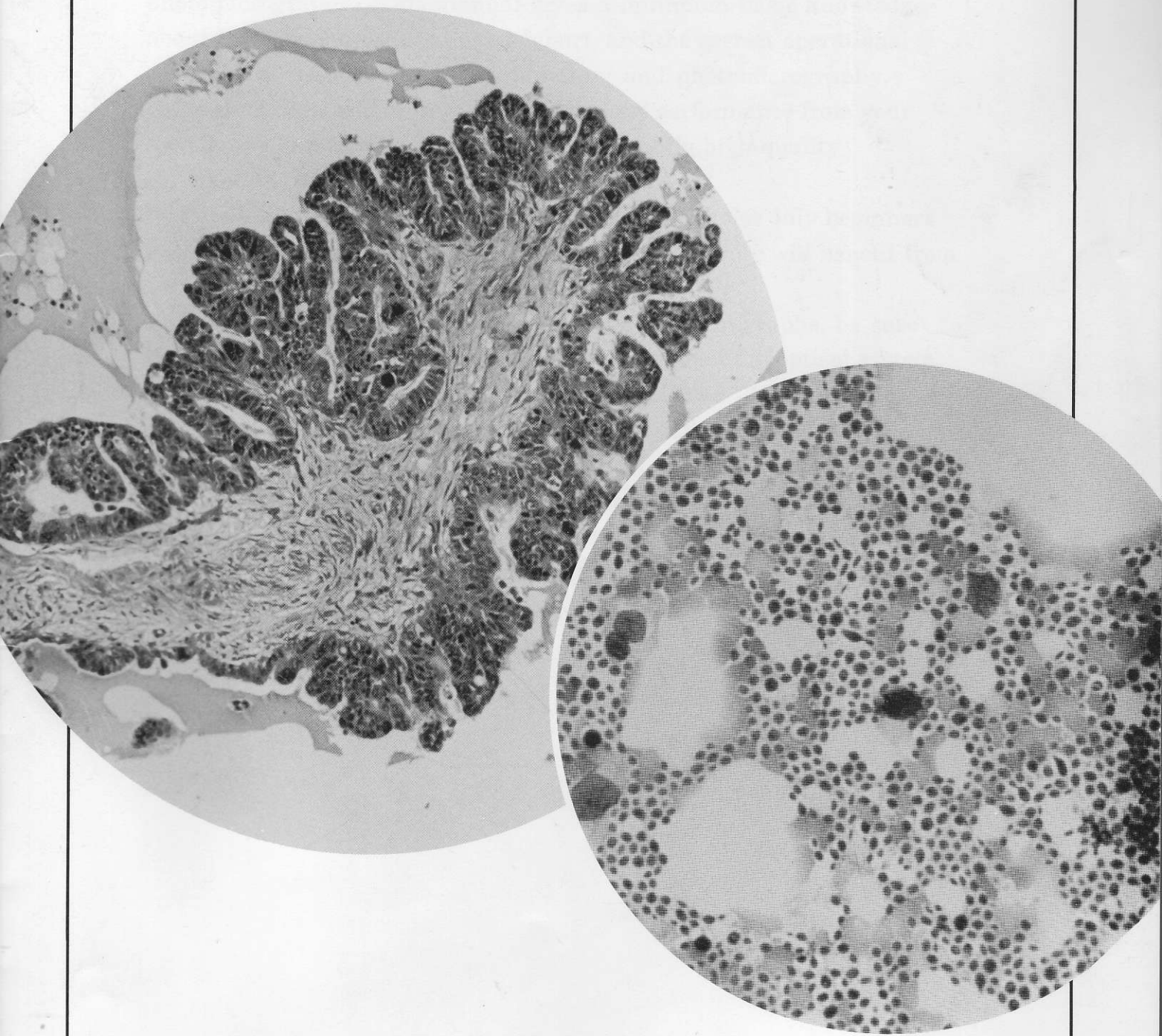
Nikon

HOW TO USE A MICROSCOPE AND TAKE A PHOTOMICROGRAPH



Introduction

This manual is designed to provide basic and useful information for a variety of microscope users: for beginners who are just learning how to handle microscopes, for those who now use microscopes in their work, and those who take photomicrographs for recording data and making presentations to academic societies and professional groups. It is also intended for those who wonder why there are so many diaphragms and controls on a microscope, and for those who want to take better photomicrographs. This manual gives a minimum basic knowledge about the role and function of each part, and the correct operational procedures, mainly microscope handling and photomicrography.

Explanations will help you to get the best performance from your microscope and so that you can take consistently high-quality photomicrographs.

This manual is composed of Chapters I to VII. Not only beginners but also those who wish to take photomicrographs only will benefit from reading this manual from the beginning.

Before using a microscope and taking photomicrographs, be sure that each part is clean, especially the seven parts of the optical system, as described in Chapter V (1) "Points to Be Cleaned". If the microscope is used every day, this is especially true. In addition, this manual explains the system in which a NIKON OPTIPHOT microscope is used in combination with the UFX-IIA photographic attachment.

In this manual, "F diaphragm" means "field diaphragm" and "A diaphragm" means "aperture diaphragm."

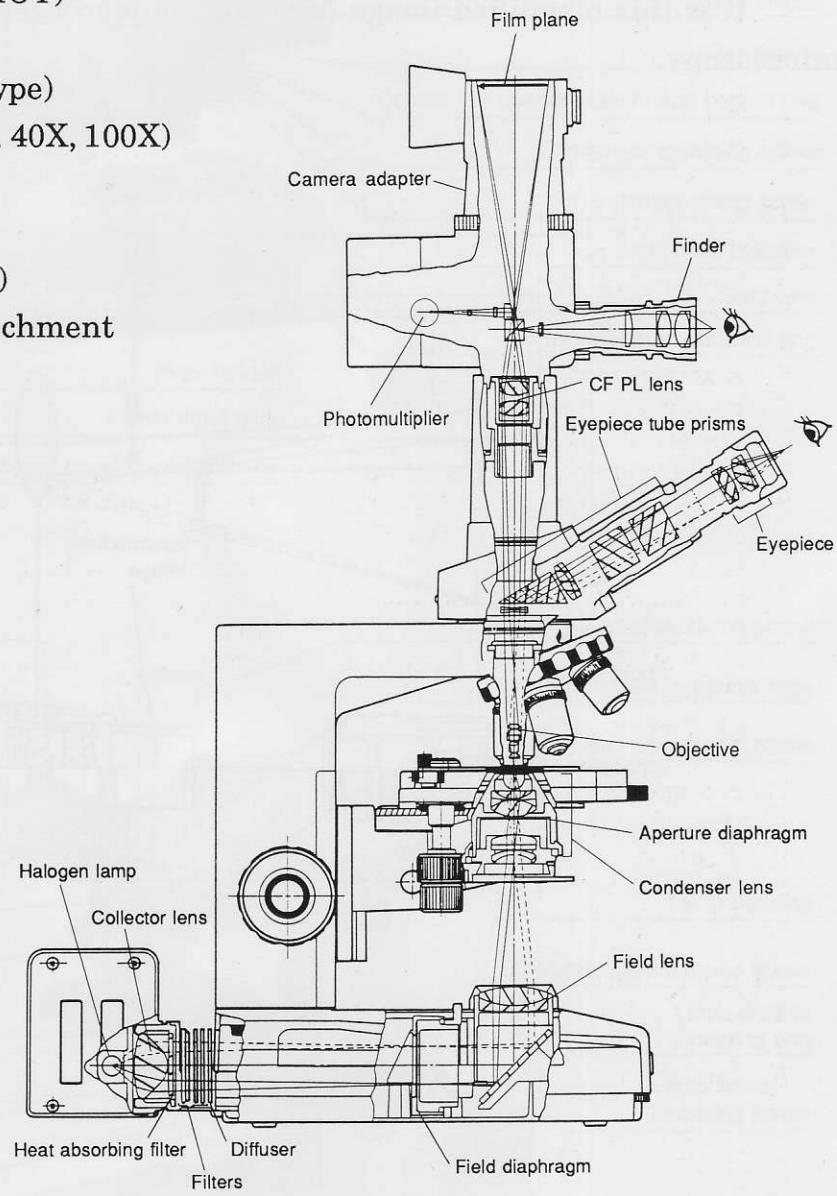
Contents

I. Optical and Mechanical System Configuration and Nomenclature

1. Optical path diagram

Introduced here is an optical path in the optical microscope with a photomicrographic attachment.

<Configuration>
Lamp housing (OPTIPHOT)
Stand (OPTIPHOT)
Condenser (Swing-out type)
Objectives (4X, 10X, 20X, 40X, 100X)
Eyepiece tube (F tube)
Eyepiece (CFW 10X)
Projection lens (PL 2.5X)
Photomicrographic attachment (UFX-IIA)



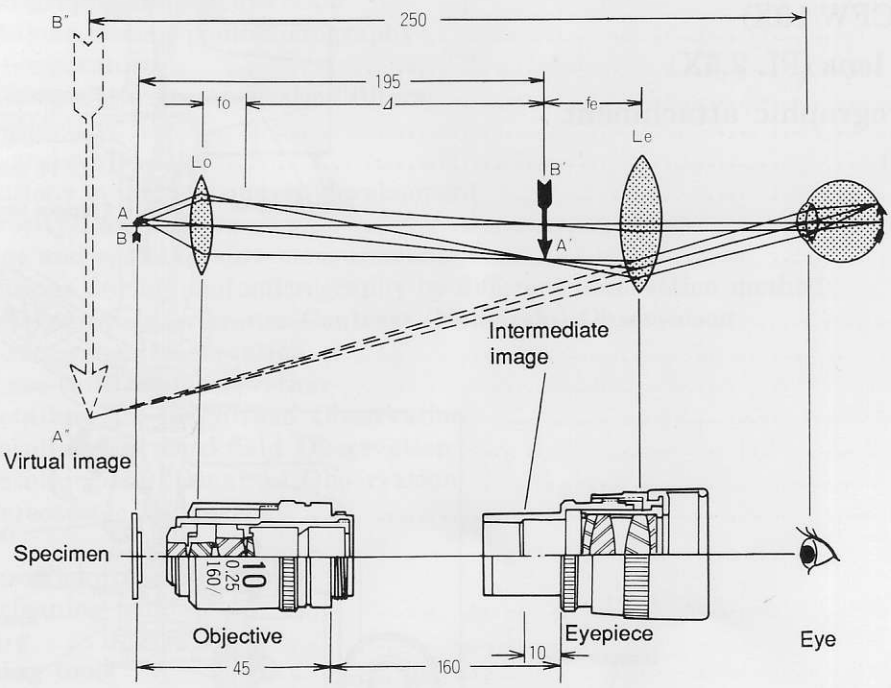
Optical system in a photomicrographic system
(Composed of the Nikon OPTIPHOT microscope and the UFX-IIA photomicrographic attachment)

2. Magnification principles

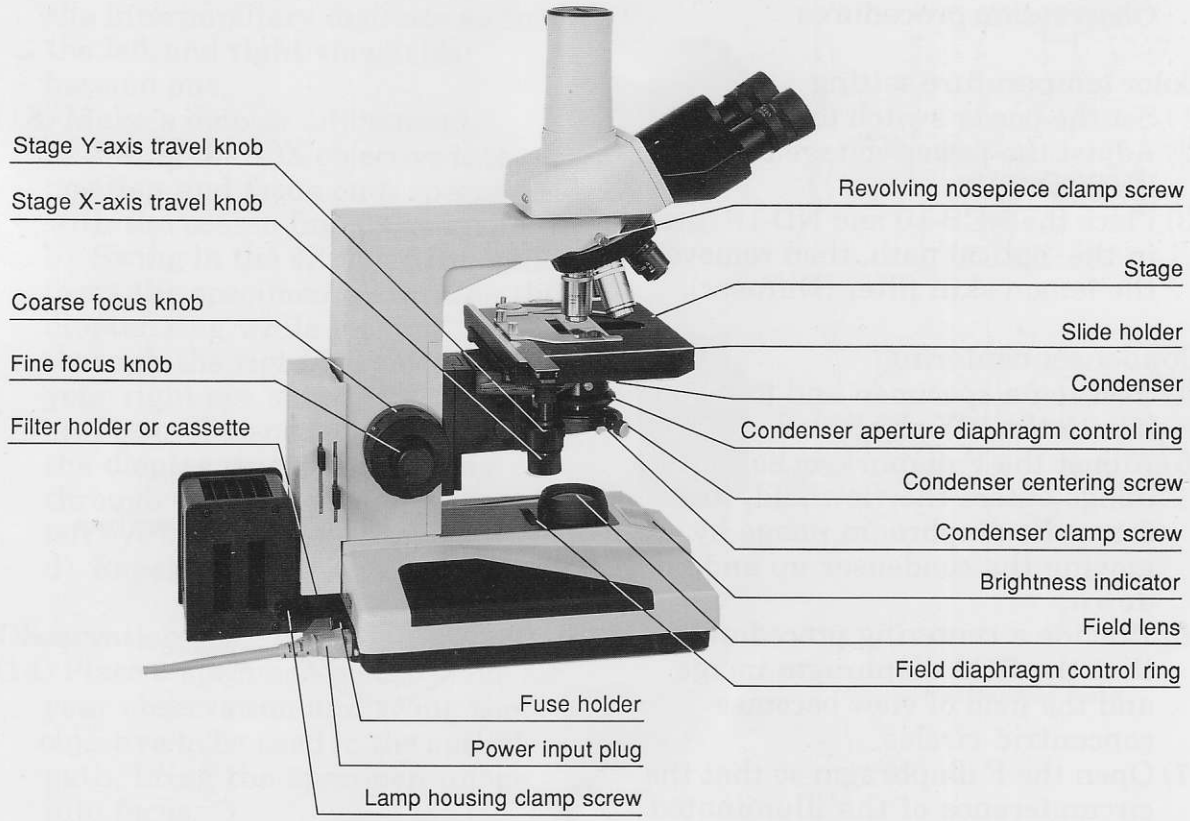
The principle of a microscope is that two convex lens systems are appropriately combined to magnify specimens.

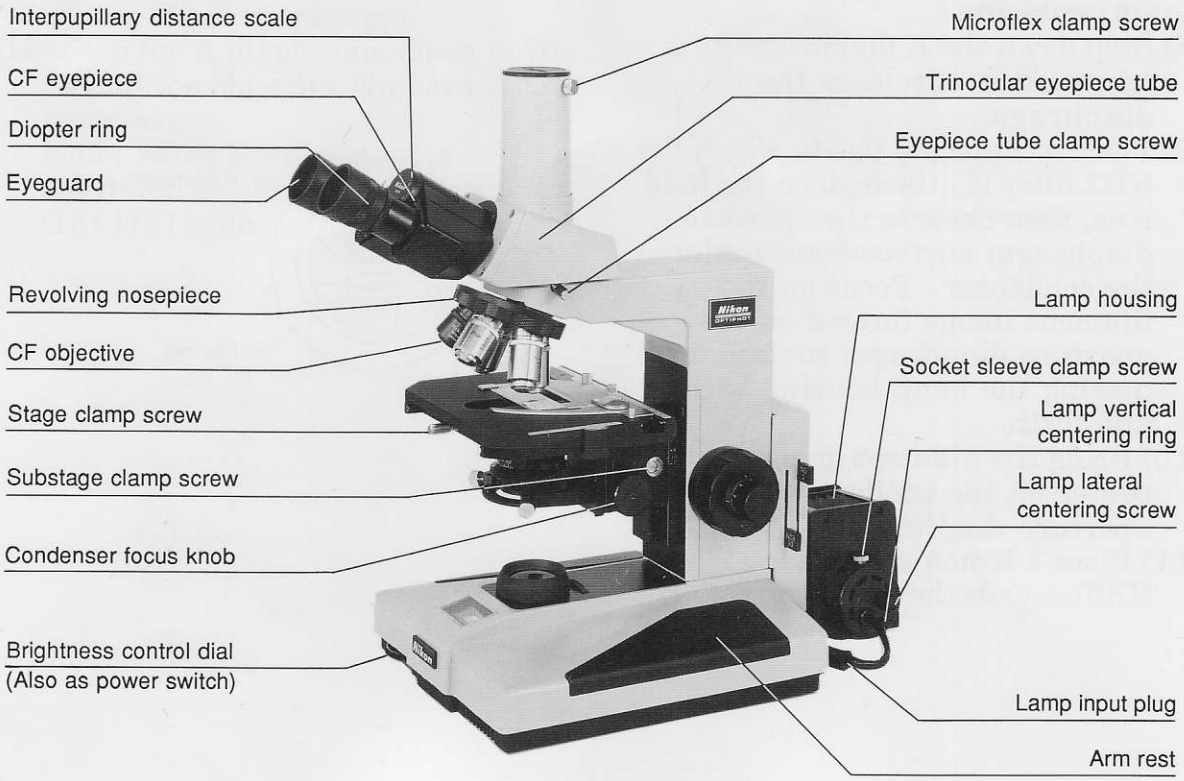
Close to a specimen, a convex lens system Lo called an objective is used for 1-100 times magnification to create a real image A'B'. Close to the eye, a lens system called an eyepiece is used for 5-20 times magnification to create a virtual image A"B" at the distance of distinct vision (about 250mm from the eye).

It is this magnified image A"B" that human eyes observe during microscopy.



3. External view and nomenclature





II. Observation and Photomicrography Procedures

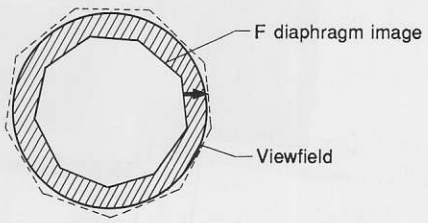
1. Observation procedures

Color temperature setting

(1) Set the power switch to ON.
(2) Adjust the power voltage at PHOTO (9V).
(3) Place the NCB-10 and ND-16 filters in the optical path, then remove the lemon skin filter (Diffuser).

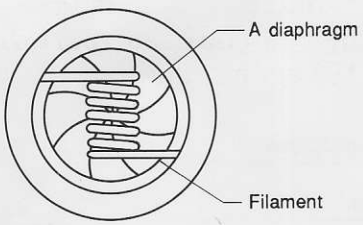
Condenser centering

(4) Position a specimen and focus on it with the 10X objective.
(5) Adjust the F diaphragm so that its image enters the viewfield, then focus the diaphragm image by moving the condenser up and down.
(6) Provide a centering procedure so that the field diaphragm image and the field of view becomes concentric circles.
(7) Open the F diaphragm so that the circumference of the illuminated area circumscribes the eyepiece field of view.



Lamp centering

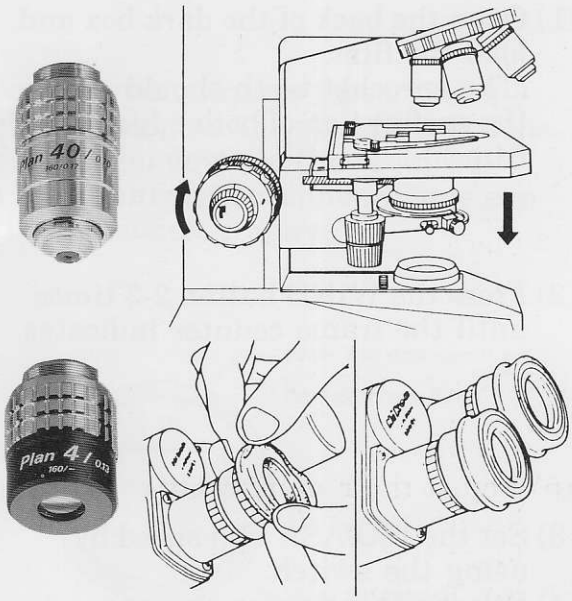
(8) Stop down the A diaphragm to a minimum and release the F diaphragm.
(9) Place a centering tool to which the ND2 filter is attached, on the field lens, then observe the aperture diaphragm surface from under the condenser. Focus on the filament image formed on the aperture diaphragm surface, by moving the lamp housing back and forth.
(10) Bring the filament image to the center on the condenser's aperture diaphragm surface.
(11) Place a lemon skin filter (Diffuser) in the optical path.



Interpupillary distance and diopter correction adjustment

(12) Observe the specimen and adjust the interpupillary distance so that the left and right viewfields become one.

(13) Make a diopter adjustment.
a) Swing the 40X objective into position and focus on a specimen with the coarse-fine focus knob.
b) Swing in the 4X objective, and focus the specimen by turning the diopter ring while looking through the right eyepiece with your right eye only.
c) Focus the specimen by turning the diopter ring while looking through the left eyepiece with your left eye only.
d) Repeat twice.

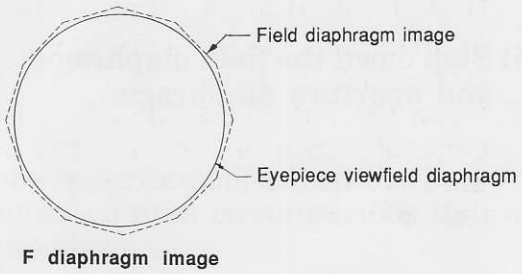


Observation adjustment

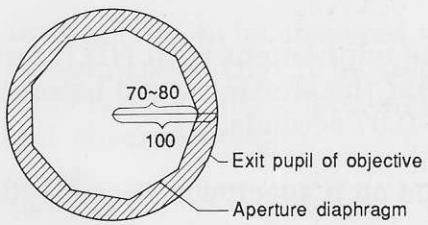
(14) Place a specimen on the stage for your observation and swing the objective to be used in the optical path, bring the specimen image into focus.

(15) Focus the F diaphragm image within the viewfield by moving the condenser up and down, then center the F diaphragm.

(16) Stop the A diaphragm down to 70-80% of the objective Numerical Aperture.
Note: When the objective or specimen is changed, perform (15), (16) again.



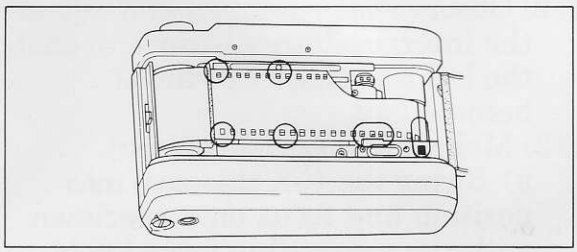
F diaphragm image



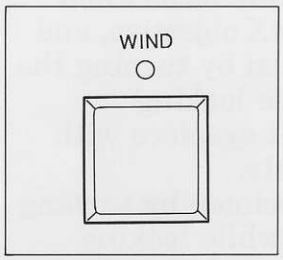
A diaphragm image

2. Photomicrography Procedures

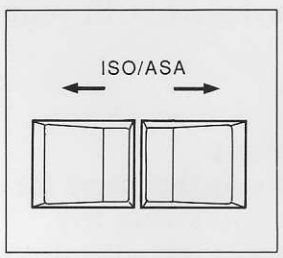
(1) Open the back of the dark box and load the film.
(The sprocket teeth should engage the perforation of both edges of the film.)

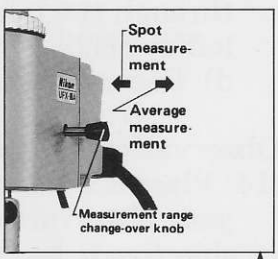


(2) Press the WIND button 2-3 times until the frame counter indicates "1".

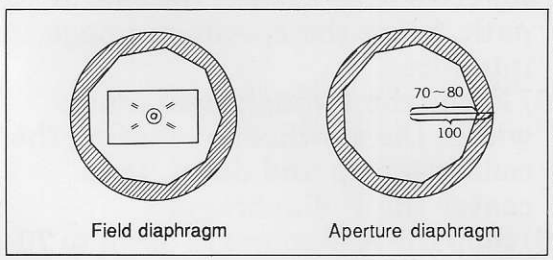


(3) Set the ISO/ASA film speed by using the switch.
(4) Select either spot or average exposure measurement.
(See IV (8))



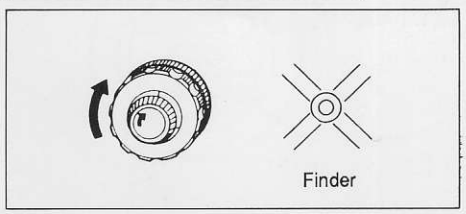


(5) Stop down the field diaphragm and aperture diaphragm.

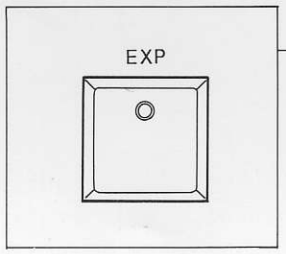


(6) Make adjustment with ND filters so that the shutter speed becomes 0.25-0.07 seconds.

(7) Focus on a specimen. (See III (5))



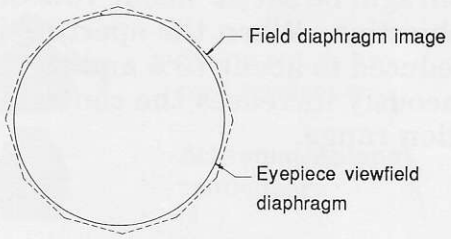
(8) Press the exposure switch.
Note: When an objective is changed, start with (4), or start with (6) if the specimen is changed.

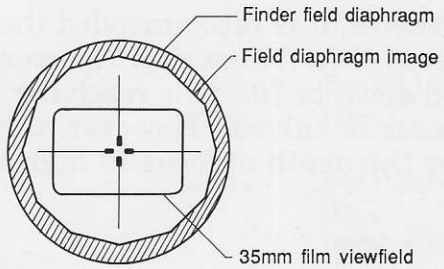


III. Optical Microscopes and Their Operation

1. Field diaphragm

The field diaphragm is used to restrict the illumination range (observation range). The residual diffused reflected light occuring on the specimen or lens surface is restricted by stopping the field diaphragm down to the required range. As a result, flare is reduced and a high contrast specimen image can be observed. This is especially important in photomicrography.





Operating tips

(1) When observation through the eyepiece tube is performed, adjust the F diaphragm so that the circumference of illuminated area circumscribes that of the eyepiece field of view as shown in the figure above.

(2) However, when only a certain part of the viewfield has to be observed with high contrast and high resolving power, it is recommended that it be stopped down by just leaving the part to be observed. This is especially effective during fluorescence or differential interference contrast observations.

(3) To prevent fading in an unnecessary part of a specimen during epi-fluorescence observation or photomicrography, stop down the F diaphragm, excite only the necessary part. Then, the specimen's fading area will be minimized.

(4) During photomicrography, it is recommended that the diaphragm be stopped down as shown in the above right figure so that the diameter of the illuminated area be set slightly larger than the diagonal of the film format.

(5) It is important to focus the field diaphragm image by moving the condenser up and down.

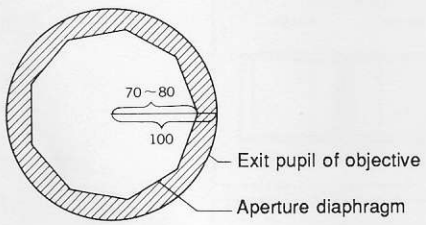
(6) Especially when oil-immersion objectives (more than 40X) are used with an AA condenser, it is recommended to apply oil between the condenser and the glass slide. As a result, the edge of the F diaphragm image and the image itself becomes dark, exhibiting the effect of the field diaphragm to the fullest.

2. Aperture diaphragm

The aperture diaphragm is attached to the condenser lens and used to control resolving power, contrast and depth of focus. However, these factors cannot be controlled independently.

	Resolving power	Contrast	Depth of focus	Brightness
Aperture diaphragm open 100%	High	Low	Shallow	Bright
Aperture diaphragm stopped down	Low	High	Deep	Dark

* In general, it is recommended that the diaphragm be set at about 70%-80% of the stated N.A. (Numerical Aperture) of the objective. When the aperture is stopped down to 70%, the resolving power is reduced to about 70% and the brightness is halved. However, this simultaneously increases the contrast and doubles the depth of focus in high magnification range.



Aperture diaphragm size

Operating tips

(1) To obtain an appropriate level of resolving power, contrast and depth of focus, it is recommended to stop down the aperture to 70% -80% of the stated value. However when a specimen with a light dye and low contrast is observed and photomicrographed, it is better to stop down further. When a specimen has a dark color, aperture should be opened.

(2) If the aperture is stopped down too much, a diffraction image will appear on the specimen image and a white fringe appears which makes it look as if there is a clear minute structure. (This is called "false resolution".)

(3) When an AA condenser is used with a powerful objective such as100X, be sure to apply oil to the AA condenser in order to obtain a maximum N.A. of 1.35.
Oil immersion is not necessary when using a swing-out condenser. It should be noted, however, that the N.A. is 0.9 at the maximum.

(4) When high resolving power is required to see a minute structure, it is necessary to apply oil to the condenser. It is also necessary, for maximum utilization of the objective N.A., that the user stop down the field diaphragm as much as possible, while opening the aperture at 100%.

3. Condensers

The condenser is used to obtain a bright, even viewfield. It also has considerable influence on resolution, contrast, depth of focus and brightness, all of which affect the basic quality of a microscopic image. Therefore, its utilization is very important.

	Numerical aperture (N.A.)	Illumination field	Remarks
 Abbe condenser	1.25	4.6ø	For 4X-100X (Oil-immersion)
 Swing-out Achromat condenser	0.90	3.4ø (12)	For 2X-100X (Dry)
 Achromat/Aplanat condenser	1.35	2.8ø	For 10X-100X (Oil-immersion)

How to choose

The Abbe condenser is suitable to use for observations in practice and inspection applications. However, the swing-out or AA condenser is the best choices for photomicrography (especially in color). The AA condenser is especially suited for critical observation at high magnifications (to observe minute structures).

Operating tips

(Abbe condensers)
To be used at the point where focusing is provided to F diaphragm image within the field of view.

(Swing-out condensers)
2X-4X objective: To be used at the point where focusing is provided to F diaphragm image at 10X magnification and the top lens is swung out.
10X-100X objective: To be used at the point where focusing is achieved at F diaphragm image within the field of view.

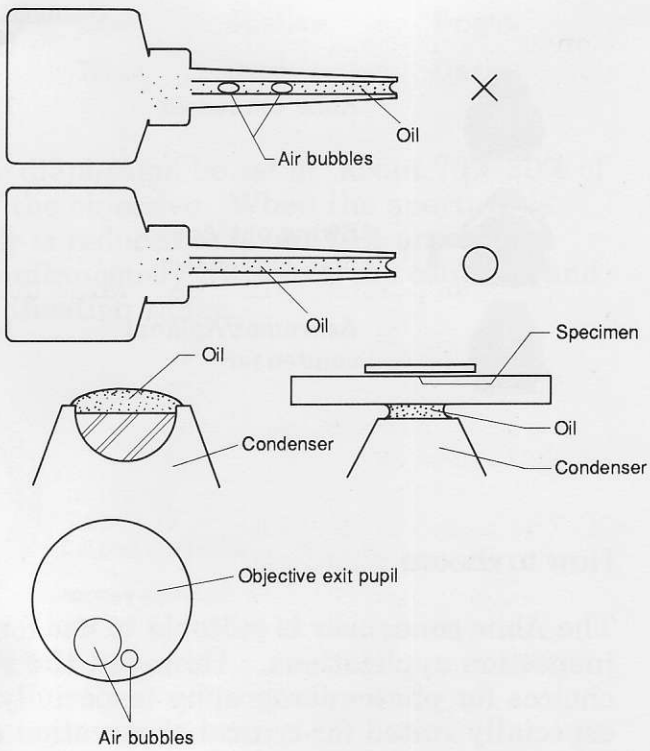
(AA condensers)
10X-40X objective: To be used at the point where focusing is provided to F diaphragm image within the viewfield, without using oil.
40X-100X objective: To be used at the point where focusing is provided to F diaphragm image within the viewfield, using oil.

*** When using a 1X objective:**

Remove the condenser and install a white frosted filter onto the field lens then perform lamp centering when necessary.
Pay special attention during photomicrography because uneven lighting is apt to occur.

*** Condenser oil immersing**

(1) Remove any air bubbles in the nozzle of the oil container.
(2) Bring the condenser down, then apply a sufficient amount of oil to the condenser top lens taking special care that air bubbles do not enter the oil.
(3) Slowly bring the condenser up to the extent that the oil touches the slide glass, paying attention so that air bubbles do not exist between the slide glass and condenser.
(4) Remove the eyepiece and make sure no air bubbles exist by looking at the exit pupil of the objective. If there are no bubbles, this completes oil immersing.



<Note 1> If air bubbles are observed, lower the condenser and wipe up the oil, then try immersing again. In this case, always clean off the oil on the slide glass too, because air bubbles can be found here too.
<Note 2> When oil immersing is being provided on condensers, it is often the case for objectives. However, often it is impossible to judge whether the air bubbles confirmed to exist in the exit pupil of Step (4) are present on the objective side or condenser side. To check:

Move the condenser with the condenser centering screw.

If air bubbles move: Air bubbles exist on the condenser side.

If air bubbles do not move: Air bubbles exist on the objective side.

4. Koehler Illumination

This illumination method was devised by a German named Koehler in 1893 as the one nearing the ideal and has been generally used as the accepted standard. Nikon also adopted this method in its microscopes after improving the ease of operation.

How to adjust

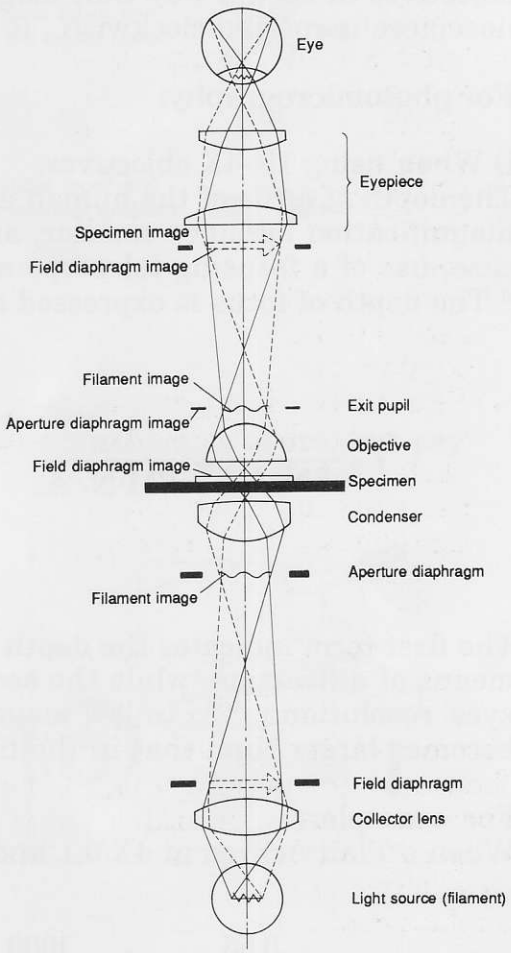
Concrete procedures are based on (4) to (11) in "Microscopic Observation Procedures". The basic requirements can be summarized as follows:

(1) The lamp's filament image should be formed on the aperture diaphragm surface of the condenser.
--> **Lamp housing adjustment must be done correctly**

(2) A field diaphragm image should be formed on the specimen side.
--> **Condenser vertical centering must be done correctly**

(3) The aperture diaphragm and field diaphragm should work independently.
-->Stopping down the aperture to a minimum makes the viewfield dark but does not produce vignetting. Conversely, stopping down the field does not affect the numerical aperture in the least.

(4) The field diaphragm and the aperture diaphragm should be properly adjusted to match the objective each time the objective is changed.
--> **Set the microscope so that the field diaphragm image is properly formed within the viewfield, and close the aperture diaphragm to the proper level.**



Koehler illumination diagram

5. Focusing

For observation:

Focus the specimen with a 10X objective in the first place.
Then, change the magnification to 4X, 40X, and 100X, etc. by rotating the revolving nosepiece. If focusing is attempted with a high magnification objective from the beginning, it may break the specimen slide.

<Reference>
When attaching objectives to the nosepiece, accepted practice is to attach objectives in such a way that magnification increases when the revolving nosepiece is rotated clockwise. It is recommended that you follow this practice.

For photomicrography:

i) When using 1X-4X objectives:
The depth of focus of the human eye becomes larger as objective's magnification becomes smaller, and this makes focusing difficult. In this case, use of a focusing telescope makes focusing easier.
* The depth of focus is expressed as follows:

$$t = \frac{n \cdot \lambda}{2N.A.^2} + \frac{\lambda \cdot n}{7M \cdot N.A.}$$

where,
λ: Wavelength used
M: Total magnification
N.A.: Objective numerical aperture
n: Refractive index

The first term indicates the depth by physical expansion in the optical image by means of diffraction, while the second term shows the depth when the human eyes' resolution is 2'. In low magnifications, the depth in the second term becomes larger than that in the first term, which means difficulty in focusing.

For example:
When a Plan Achromat 4X/0.1 and a CFW 10X eyepiece are used,

$$t_o = \frac{0.55}{2 \times 0.1^2} + \frac{1000}{7 \times (4 \times 10) \times 0.1} = 27.5 + 35.7 = 63.2\ \mu m$$

the second term becomes larger than the first term, making focusing hard to achieve. If a focusing telescope (4X) is used,

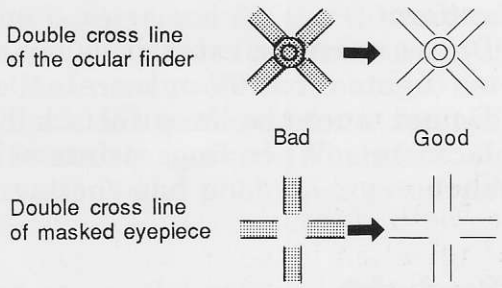
$$\frac{1000}{7 \times (4 \times 10 \times 4) \times 0.1} = 8.9\ \mu m$$

the visible depth of focus of the human eye becomes smaller by 26.8μm (35.7 — 8.9 = 26.8 μm), which indicates that focusing becomes easier.

<Note> Since only a portion of the finder's center can be seen when using a focusing telescope, determine the composition first, then position the telescope and focus.

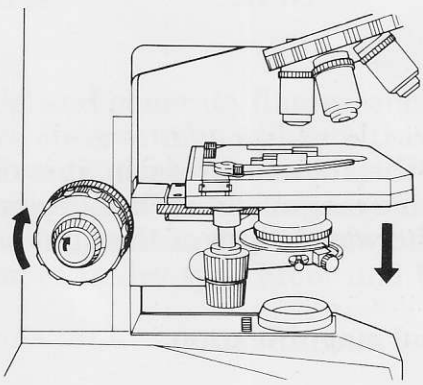
[Focusing]

(1) Turn the diopter ring of the finder or the focusing telescope to focus the double cross line .
(2) Turn the fine focus knob so that both the double cross line and the specimen image come into clear focus.
(3) When using a low power objective, focus it by turning the fine focus knob 5-6 times and use the average value of the scale readings.



ii) When using 10X-100X objectives:
A focusing telescope is not necessarily a must. Focus them as above, but pay attention to the following points:

• Turn the fine focus knob in the direction shown on the right to focus. If it was turned too far, turn it back and refocus (the direction which moves the stage from down to up) to eliminate the influence of play due to gear slack.



6. Halogen Lamp

The halogen lamp used as a light source in the microscope has higher luminance and color temperature than conventional tungsten lamps. Its luminance is about four times greater.

As long as the lamp voltage is constant, the halogen lamp maintains almost the same level of brightness and color temperature whether it is a new one or one nearing the end of the life. This light source is especially suitable for color photomicrography.

<Caution>
(1) Do not use at an excessively low voltage. Use it within the green range of the meter (lights up at 6V or more).
(2) Do not touch the lamp surface directly with your bare hands during lamp replacement. When fingerprints or oil adhere to the lamp, clean it with alcohol.

7. Resolution

The minimum distance between two dots which can be identified as they are is known as the resolution of a microscope.

$$\mathbf{d = 0.61 \times \frac{\lambda}{N.A.}}$$ (λ: Light source wavelength N. A.: Numerical aperture)

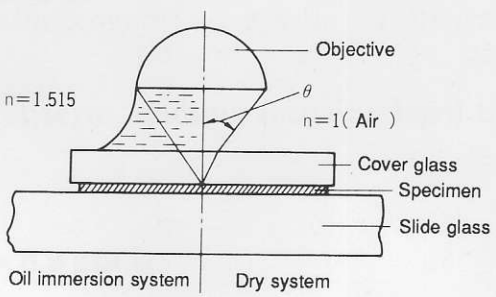
From the above expression, it is obvious that resolution does not depend on objective magnification but depends on the numerical aperture of the objective and the wavelength of the light source in use.

8. Total magnification

M = Mo x Me (x Mi)
M: Total magnification
Mo: Objective magnification
Me: Eyepiece magnification
Me indicates Projection Lens (PL) magnification when photomicrography is performed.
Mi: Intermediate magnification
Mi=1 with ordinary diascopic illumination

[Numerical Aperture]

$N.A. = n \cdot \sin\theta$



9. Objectives

Objective types

(1) CF Achromat
In this type of objective , correction of the axial chromatic aberration is applied to the C line (red) and F line (blue) based on the standard wavelength. Since aberrations at the center of the viewfield is fully corrected, resolution and contrast at the center are excellent, making it ideal for general observations.

(2) CF Plan Achromat (Plan)
Like CF Achromat, axial chromatic aberration is corrected for the C line (red) and F line (blue). However, corrections are also fully made for curvature of the field and other aberrations, resulting in excellent resolution and contrast not only at the center but at the periphery of the field. When the center is focused, the periphery of the ultra-wide field is simultaneously focused. Therefore, this objective is suitable for ultra-wide field observations and photomicrography.

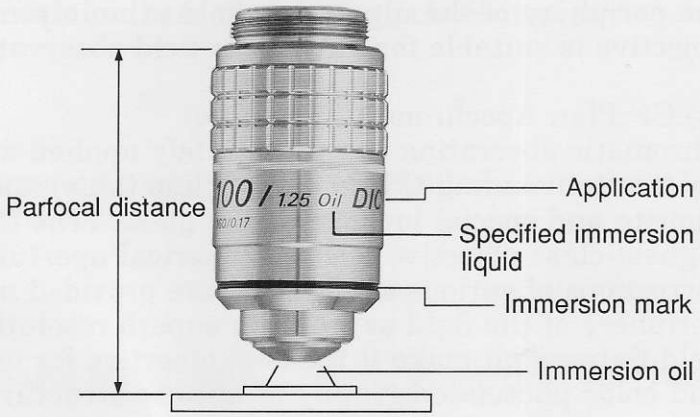
(3) CF Plan Apochromat (Plan Apo)
Chromatic aberration is appropriately applied across the entire visible spectrum including C line (red), F line (blue) and G line (violet) by using a fluorite and special low dispersion glass. The CF Plan Apochromat is the highest-class objective whose numerical aperture is large and to which corrections of various aberrations are provided not only at the center but the periphery of the field as well. Its superb resolution color reproducibility and field flatness all make it the best objective for most microscopic examinations and color photomicrography of minute structures.

(4) Epi-fluorescence Objectives (Fluor/UV-F)
Since ordinary objectives absorb ultraviolet light and generate fluorescence, lens is subject to deterioration. Epi-fluorescence observations utilize ultraviolet light for observing the fluorescence of the specimens. Epi-fluorescence objectives are designed for this purpose and feature excellent transmission of ultraviolet light, no generation of fluorescence and less chance of lens deterioration. The epi-fluorescence objective comes in dry type Fluor and UV-F type that utilizes glycerin.

(5) CF Polarizing Objectives (P)
A polarizing microscope is used to examine the polarization characteristics of a specimen. Since even a small distortion in the optical system influences polarization and causes image quality deterioration, distortion is minimized in designing polarizing objectives. The differential interference contrast (DIC) objective also features less distortion. However, in terms of the degree of distortion, DIC objectives are rated between ordinary objectives and polarizing objectives.

(6) CF Phase-Contrast Objectives
CF Phase-Contrast objectives are used for phase contrast observations of colorless, unstained specimens. Phase contrast processing has been applied to ordinary objectives.

Objective Indications



Objectives with cover glass correction ring

When dry objectives with large numerical apertures (more than 0.75) are used, it is desirable that the thickness from the cover glass surface to the specimen surface is 0.17mm. However, since the thickness of a specimen, mounting medium, cover glass, etc. is not even, it is often a difficult task to maintain the 0.17mm. To fully display the performance of the objective, objectives with a correction ring are designed so that the thickness between the cover glass surface to the specimen can be corrected within a 0.11-0.23 mm range (in case of CF Plan Achromat 60X) at the objective side.

<Adjustment procedures>

(1) Open the aperture.
(2) Set the correction ring at 0.17mm, then focus the specimen. (Remember how the image was observed.)
(3) Rotate the correction ring by 2-3 scales toward the 0.23mm setting and refocus. Then compare the image with that before. If the contrast is better, turn the ring in the same direction by 2-3 scales again.
(4) If the image becomes worse, turn the ring in the opposite direction by 1-2 scales to obtain the best position.
(5) This procedure is necessary every time a specimen is changed.

No-cover-glass objectives

No-cover-glass objectives are used for observing specimens without using a cover glass in such cases as smeared specimens such as blood, with objectives with large numerical aperture (N.A. 0.4 or more). ("NCG" or "No Cover Glass" is engraved on this type of objectives.)
Examples: CF Plan NCG 40X, CF Plan Apo NCG 100X, etc.

[Reference]

The following color codes are used to show the objective magnification.

Magnification	1X	2X	4X	10X	20X	40X
Color code	Black	Brown	Red	Yellow	Green	Light blue

50X	60X	100X
Light blue	Cobalt blue	White

IV. Photomicrography and Its Operation

1. Precautions during photomicrography.

(1) Place the microscope on a vibration-free table, preferably in a semi-dark room.
(2) Check for dust and foreign matter in the optical system before taking pictures (Important).
(3) Set the aperture (70%-80%) and field diaphragms.
--> See the Field Diaphragm/Aperture Diaphragm section.
(4) Use a bright optical system.

When projection lenses are changed, exposures are:

PL 2.5X	PL 4X	PL 5X
1 sec.	2.4 sec.	4.0 sec.

When using the same total magnification, exposures are:

Total magnification	100X	100X	80X
Eyepiece x objective	2.5 x 40	5 x 20	4 x 20
Exposure	1.02	1.77	1.11

(5) Use objectives with less chromatic aberration and excellent flatness. (Plan Achromat or Plan Apochromat type)
(6) Use No-Cover-Glass objectives for no-cover specimens.
(7) When using 1X, 2X objectives, take precautions to prevent uneven illumination.
--> See "6. Precautions To Prevent Unevenness."
(Provide centering with care — use a white frosted filter for 1X magnification.)

2. Color temperature

The color temperature is used to indicate the color of the light source or its difference when color photomicrography is performed. When a black iron bar is heated or charcoal is burnt, it becomes red as the temperature increases. Then it turns to yellow, white and blue, in accordance with further increases in temperature. Instead of the iron bar or charcoal, the color temperature uses a fictitious, ideal substance called a "full radiator", and its radiation energy is used as a standard. In other words, the equivalent absolute temperature of the radiating full radiator is used to indicate the color temperature of a certain light source. The color temperature is indicated in units of Kelvin (K).

<Example>
Blue sky - - - - - - - - - - 8000 - 10000 K
Sun at noon - - - - - - - - 5500 - 6500 K
Halogen lamp (12V-50W) - - 3400 K (at 12V)
Tungsten lamp (6V-30W) - - 2850 K (at 7.5V)

- Proper color temperature of film

  Daylight type ---------- 5500 - 6000 K
  Tungsten type ---------- 3200 or 3400 K

- Relationship between microscope's light source and filter

$$\mathbf{M(Mired)} = \frac{10^6}{\mathbf{T(K)}} \qquad \text{(at 9V)}$$

Halogen lamp (through microscope) - - 3460K = 289M
Film ---------- -5500K = 181M

For Nikon OPTIPHOT microscope, the above correction value can be obtained by inserting an NCB-10 filter when the halogen lamp is lit at 9V.

Perform photomicrography at 9V and with the NCB-10 --> **In order to match the color temperature of the microscope's light source with that of the film used.**

3. Color temperature compensation filters

When performing color photomicrography, it is necessary to check the finished film and apply tonal compensation if color temperature compensation is considered to be necessary. The color temperature compensation filter is used for this purpose. Generally, the Nikon microscope is designed to produce excellent photomicrographs by the following procedures: set the lamp voltage at 9V, insert an NCB-10 filter in the optical path, then, by using ND filters, etc., adjust the shutter speed so that it becomes 0.25-0.07 seconds.

To check the finished film, set the photomicrographed specimen on the microscope and check if the film has been reproduced in the same color tone as that through the binocular eyepieces. It can also be checked by making an inspection to see if the non-photographed portion has been reproduced in gray or not. The film needs to be checked on the illuminator (light box) with correct color temperature. However, it should be noted that results may vary depending on the difference in film emulsion number and the developing solution used by the film laboratory. External factors, such as heat and moisture can also influence color reproduction.

Filters that are designed to eliminate the background color (white portion where no specimen is photographed) are shown below.

Background colors and appropriate color temperature compensation filters

Background colors to be absorbed	Color temperature compensation filters
Blue	CCY series (yellow)
Green	CCM series (magenta)
Red	CCC series (cyan)
Blue and green (cyan)	CCR series (red)
Blue and red (magenta)	CCG series (green)
Red and green (yellow)	CCB series (blue)

Basically, the microscope's color temperature can be matched to the film's proper color temperature if photomicrography is performed with a 9V lamp voltage and by using an NCB-10 filter. Since the ratio of short wavelength (blue) and long wavelength (red) can be made constant, color temperature compensation is generally necessary for middle range wavelength only. In other words, it is only necessary that the user prepares a total of eight color temperature compensation filters—one each of 5, 10, 15, 20 for both CCM (Magenta) and CCG (Green) (available at any camera retailer). For various other situations, refer to the above table.

Utilization of color temperature compensation filters when the background is colored.
Place the reversal film on the illuminator with the correct color temperature and overlay the proper CC filter on top of it, to make the background gray. Place this CC filter on the field lens and take a photomicrograph again. This makes the background color gray.

4. Color film
Since medical photomicrography requires excellent color reproduction, high resolution, and superb color contrast, etc., daylight reversal color film is generally used for this purpose.

For general tissue specimens, priority of importance is given to resolution and color reproduction, so it is not recommended to use film with excessively high sensitivity: **Reversal film with ISO/ASA 100 or less is recommended.**

Film properties

Type of film	ISO/ASA	Properties
Kodachrome 25 (KM)	25	Good resolution, color balance and background tone.
Kodachrome 64 (KR)	64	Almost identical to Kodachrome 25 (KM), but has a slight yellow tinge in the background.
Ektachrome 64 (ER)/ Ektachrome 64 Professional (EPR)	64	Good color balance and easy to use.
Fujichrome 100 Professional D (RDP)	100	Good color balance and easy to use.
Konica Film SR100 Chrome	100	Good contrast.

(Note) Since film is constantly under improvement, this table is only for reference.

5. Black-and-white film

Since interference colors peculiar to certain parts of specimen structures must be reproduced in tones of black and white, it is recommended that you use film with appropriate contrast.

Also, because minute details of the specimen must be reproduced on film, film with fine grain should be used.

There are many different kinds of black-and-white film available on the market. Therefore, it is important to select the exact film depending on the status of a specimen (For example: Specimen staining is light; Specimen has sufficient contrast, but details need to be expressed), or its purpose.

Generally, "Panatomic-X" by Kodak is recommended. When using black-and-white film, please pay attention to the following points:

a) ISO/ASA film speed - - Susceptible to film developing solution and processing time.
b) Uneven development - Becomes a problem with high-contrast film

For a) ISO/ASA film speed, refer to "Film Developing Solution and Processing Time" shown below.
For b) Uneven development, refer to "Precautions to Avoid Uneven Development."

Film Developing Solution and Processing Time

Type	Film name	Manufacturer	ISO/ASA	Characteristics	Remarks
Low sensitivity, high resolution	Panatomic-X	Kodak	100	Low sensitivity, but fine grained	Tissue specimens, ICs
General use	Neopan SS Plus-X Pan	Fuji Kodak	100 100	Moderate gradations	Universal type
Copy use		Fuji Kodak	20 64	High resolution, high contrast	ICs
High sensitivity	Neopan 400 Professional Tri-X Pan	Fuji Kodak	400 400	High resolution	Fluorescent specimens
Scientific use	Technical Pan Film 2415	Kodak	50	Variable contrast with developing solution; high resolution	DIC specimens; fluorescent specimen

(Note) ISO/ASA values are based on the following developing solutions:
D-76 developing solution: 20° C, 8 minutes. High contrast development.
Finedol developing solution: 20° C, 10 minutes. Fine-grain development.

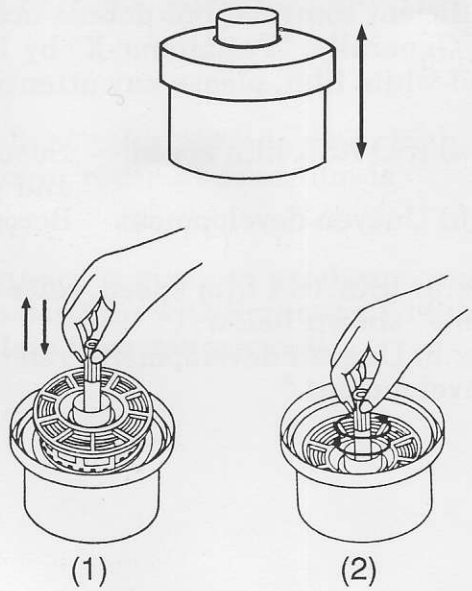
6. Precautions to prevent uneven development
Unevenness prevention during black-and-white film development

* There are two different reel developing tanks. The stainless steel, direct-reel tank has less chance of uneven development. With this tank, rolling in of film is somewhat difficult, however, it becomes easier with practice.

* With a plastic, belt-reel tank, rolling in of film is easier. However, because unevenness is liable to occur, handle it with care by following these directions:

(1) Take the roll out of the tank, insert it again, immediately. Repeat it twice.
(2) Rotate the roll three times in both directions.
(3) Wait for 30 seconds.
(4) Repeat (1) to (3).

(Stainless steel tank)
Shake it for 10 seconds, rest for 30 seconds.



Prevention of uneven illumination with low power objectives
There is almost no danger of uneven illumination with 10X-100X objectives. However, special care should be taken when using low-power objectives such as 1X, 2X, 4X.

1X objectives: Remove the condenser lens and place a diffuser on the field lens. Field diaphragm fully open.
Make sure that there is no difference in brightness between the center and periphery of the viewfield, by looking through the binocular eyepiece tube. If any difference is observed, adjust for it by moving the lamp housing back and forth or by lamp centering.

2X, 4X objectives: Swing out the top lens by using a swing-out condenser. After confirming Koehler illumination (Field diaphragm image must be formed on the specimen) by using a 10X objective, swing out the top lens. If any unevenness is observed through the binocular eyepiece tube, adjust for it by moving the lamp housing back and forth or by lamp centering.

7. Reciprocity Law Failure
In accordance with the law of reciprocity, the amount of exposure is the product of luminance applied to the film surface and exposure time. Based on this law, the same exposure is obtained either with f5.6 and 1/60 sec., or f8 and 1/30 sec.

However, a discrepancy occurs under prolonged exposure time. In photomicrography, especially, exposure time is apt to become lengthy. This is attributable to the special characteristics of microscopy—it requires high magnification, makes use of special properties of light, and uses only a specified wavelength. This results in low level light and long exposure time.

Because of the failure of the reciprocity law, the prolonged exposure time results in under exposure and loses color balance. In this case, mere exposure compensation is not enough.

During photomicrography, it is important that shutter speed be within 0.25-0.07 seconds. This is adjusted by using ND filters if the level of light is high, or if it is insufficient, by changing the combination of the objective and projection lenses, or by using highly sensitive film.

<Film data examples>

Unit: in second

	1/1000~1/10	1	10	100
Kodachrome 25 (KM)	Exposure compensation not required	$+\frac{1}{2}$ step	+2 steps	+3 steps
	Compensation filter not required	Not required	CC10B	CC20B

	1/4000~1	8	16	32
Fujichrome 100 Professional D (RDP)	Exposure compensation not required	$+\frac{1}{2}$ step	$+\frac{2}{3}$ step	+1 step
	Compensation filter not required	CC5R	CC5R	CC10R

8. Average and spot exposure measurement

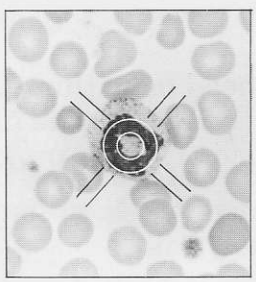
The proper exposure time can be usually obtained with a photomicrographic attachment featuring automatic exposure. However, it is not always true depending on the specimen color and the distribution of tissue. This is due to the use of average exposure measurement, or the fact that the light detector has a characteristic which is more sensitive to red. (Sensitivity to red is slightly low in a photomicrographic attachment that uses a photomultiplier as an optical detector.)

Especially when a specimen with only blue or only red is used, special care should be taken for exposure compensation. Considering the congeniality between the specimen and the photomicrographic attachment, test shooting is recommended for safety. In this case, try several shots by changing exposures by ±3 steps per 2/3 step, based on the automatically set shutter speed. (During general brightfield observation)

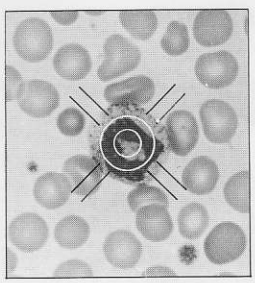
Test photomicrography ---> $-2, -1\frac{1}{3}, -\frac{2}{3}, 0, +\frac{2}{3}, +1\frac{1}{3}, +2$ (step)

For a small specimen which has relatively high contrast when compared with its background, 1% spot exposure measurement is effectively used. In this case, a portion of the small specimen is the target. For photomicrography of both bright and dark portions, and whose ratio is 50 : 50 (for ICs, etc.), 30% average exposure is recommended.

Example of spot exposure measurement and average exposure measurement



Spot exposure measurement



Average exposure measurement

Table below indicates proper utilization of average/spot exposure measurement.

General, stained tissue specimens ---> Average exposure measurement
(HE stain, etc.)
IC specimens

Blood specimens ---> Spot exposure measurement
Chromosome specimens
Fluorescent specimens with
dark background

9. Precautions during photomicrography by different observation methods

a) Differential Interference Contrast (Nomarski) Observation

(1) Black-and-white film - Use GIF (exposure compensation not required)
Film: Kodak, Technical Pan Film 2415 (ISO/ASA 100)
* Developing conditions: D-76, 20°C, 8 minutes

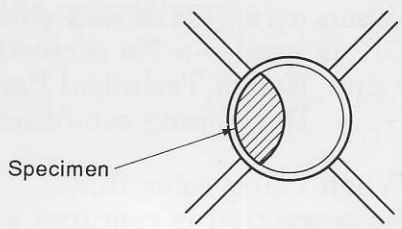
(2) Color film
Interference color: Gray sensitive - NCB-10, 9V, CC5M compensation
(To eliminate green in a polarizing plate)
Interference color: First order red (530nm) - No NCB-10, 12V, max.
Interference color: Blue - NCB-10, 9V

(3) Precautions during photomicrography
(a) Since shutter speed is usually slow, take precautions against external vibrations, especially when magnification is high. The use of a vibration-free table is recommended.
(b) Since dust in the optical system causes decreased EF values, which lowers the contrast, complete cleaning must be performed. Remove the eyepiece, remove the Nomarski prism from the optical path and look at the aperture, then you can see the dust shining.
(c) Air bubbles often enter during oil immersion of the condenser. Since air bubbles cause decreased EF values and lowered contrast, they must be removed before microscopy. To check for air bubbles, remove the eyepiece and look at the aperture.
(d) When especially high resolution is required, the aperture should be opened 100%.
(e) Since the use of the field diaphragm is very effective for removal of flare during DIC observation, it is recommended that the user stops down the field diaphragm to the furthest point within the photographic area. This makes photomicrography with higher contrast possible.

b) Fluorescence Observation
(1) It is recommended that you use highly sensitive film, ISO/ASA 400 for example. However, films with higher sensitivity (more than ISO/ASA 400) will cause grainy images.

(2) Exposure compensation
If the specimen is smaller than the 1% spot exposure measurement area, exposure compensation is necessary to match the size of the specimen.

For example:
If the specimen size is about half of the 1% measurement area, make under exposure compensation by 1 step.



Generally, with U. V. B. excitation, photomicrography can be done properly without exposure compensation. However, sharper coloring may be obtainable by applying approx. -1 step exposure compensation, which is particularly effective for projection purposes using a reversal film.

For photomicrography with G excitation, however, underexposure compensation of one step more than other excitations is recommended. In other words, compensation will be 1 to 2 steps under normal exposure.

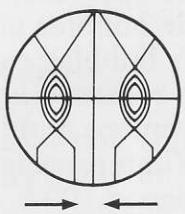
In fluorescent photomicrography, the status of the specimen and the staining greatly influences the success of photomicrography. For safety, it is recommended that the user makes a few trial shots by making under exposure compensation in one step. (For example, 0, -1, -2)

3) To increase contrast, stop down the field diaphragm to the extent where vignetting does not occur.

4) To achieve photomicrography without uneven illumination, it is necessary to provide centering of the light source (Hg) correctly.

<Hg lamp centering procedures>

i) Arrange the lamp image and mirror image side by side using a centering tool as shown in the right-hand figure. At that time, the field diaphragm is stopped down to the minimum. This allows you to confirm the lamp's luminance point.



ii) Then overlap the lamp and mirror image each other at the center of the window as indicated by the arrows, with the lamp vertical centering screw.

iii) Next, swing in the 10X objective, and, while observing a slide glass marked with a glass pencil through the eyepiece tube, make the final adjustment to eliminate unevenness. Do this by moving the collector lens back and forth, or by fine adjustment using the centering knob.

c) Phase-contrast Observation

(1) When using black-and-white film:
GIF is used. --> No correction is required.
Film: Kodak Technical Pan Film 2415 (ISO/ASA 100)
Developing conditions: D-76, 20° C, 8 minutes.

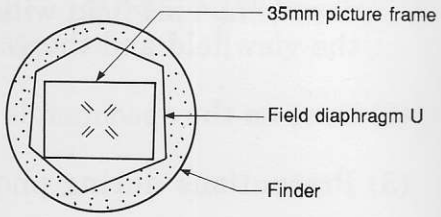
(2) When using color film:
No correction is required even when GIF is used.

d) Metallurgical Brightfield Observation

(1) Diaphragm

(a) Stopping down the field diaphragm as much as possible will remove flare effectively.

In any photomicrographic situation, stopping down the F diaphragm up to the picture frame, while looking through the finder, will make better results.

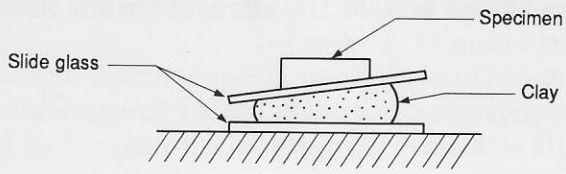


When 35mm film is used.

(b) When performing photomicrography of specimens with uneven surface such as IC patterns, stopping down the diaphragm as much as possible will create large depth of focus.

Although stopping down to 80% is generally recommended, the diaphragm can be stopped down to 50%, if there is no problem with regard to resolution.

(2) The specimen must be placed parallel to the stage surface. Otherwise, the entire viewdfield can not be focused perfectly, especially during high magnification. If the specimen cannot be placed parallel for some reason, use of clay is also effective as shown in the figure below. However, for more precision, use a special inclination stage.



(3) When using a universal illuminator, be sure to remove the polarizing plate and Nomarski prism. (For prevention of inferior view due to double imaging caused by the use of the Nomarski prism, and staining due to the polarizing plate.) Also, be sure to insert an ND 32 filter slider for compensating the optical path length and focusing of the F diaphragm image.

(4) When an M Plan 1X objective is used, be sure to use a polarizing plate in order to remove flare. During color photomicrography, it is better to use a CC5M filter to correct the color tone of the polarizing plate.

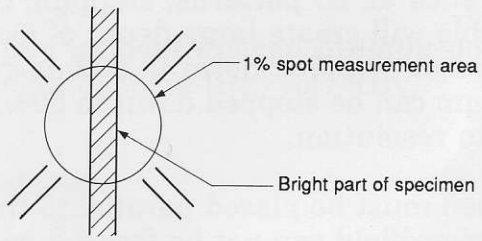
e) Metallurgical Darkfield Observation

(1) Aperture diaphragm and field diaphragm
Both diaphragms should be opened. If both diaphragms are stopped down, the viewfield will be dark because the illumination does not cover the viewfield and there will be a shortage of light.

(2) Dust on the specimen is detected by its shine; it should be removed.

(3) Precautions during photomicrography

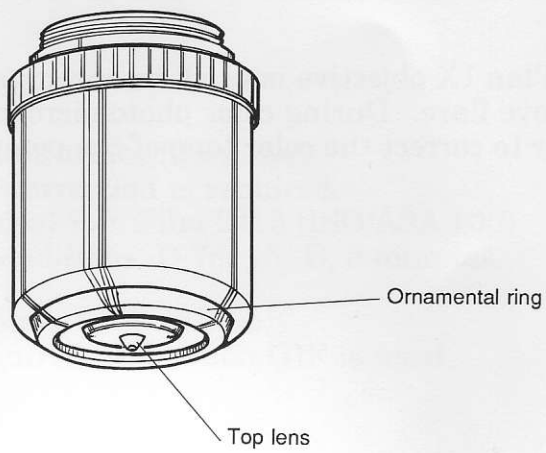
(a) Under this type of microscopy, shiny objects such as scratches are seen in a darkfield. Therefore, exposure compensation is usually necessary.



For the specimen as described above, it is necessary to set the 1% measurement mode and make a -1 step or -2 steps exposure compensation. (The degree of correction is based on the ratio of the specimen size to the size of the measurement area. If the ratio is 0.5, the correction value is -1 step.)

(b) Since the viewfield is dark, it is better to use film with a high ISO/ASA value.

(4) Although the BD Plan Achromat 60X, 100X objectives are provided with a safety scheme, their top lenses edge project out of the metal ornamental ring. Therefore, it will come in contact with the specimen if the stage is lifted too far. Take care so that the lens is not broken.



f) Metallurgical Nomarski Observation

(1) Photomicrographic conditions
(a) Interference color: Gray sensitive - 9V, NCB-10, CC5M
(b) Interference color: First order red (530nm) - 12V, No NCB-10, CC5M
or 9V; NCB-10, CC5M
(c) When black and white film is used:
- GIF, 12V (Max.)
- Film: Kodak's Technical Pan Film 2415
(To increase contrast)

g) Stereoscopic Microscopes

(1) Halogen lamp - - 5.5V, NCB-10
(2) Fiber illuminator - - Maximum voltage, with NCB-10
(3) Fluorescent illuminator - - No filter
Since the viewfield becomes light blue under (2) and (3), a compensation filter is required.

Others

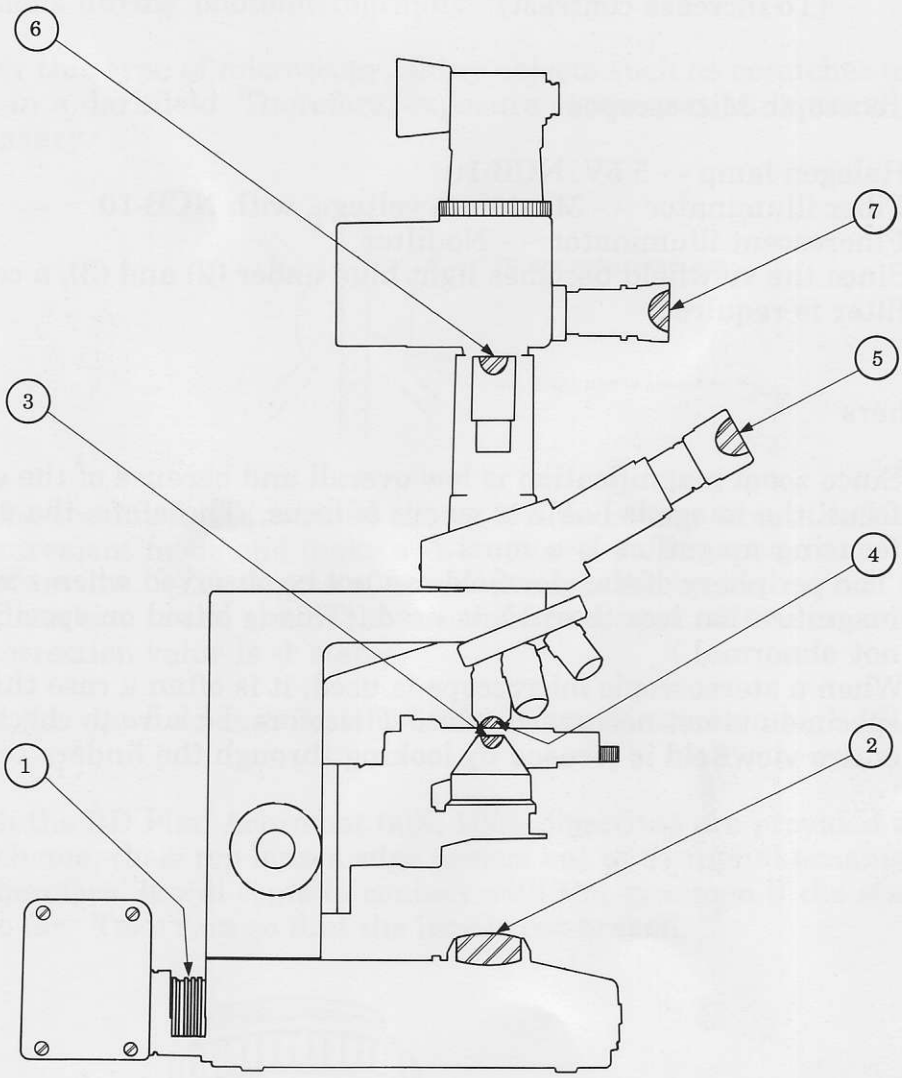
(a) Since zoom magnification is low overall and because of the eye's depth of focus, the image is liable to go out of focus. Therefore, the use of a focusing magnifier is a must.
(b) The periphery of the viewfield can not be observed when zoom magnification less than 1X is used. (This is based on specifications and not abnormal.)
(c) When a stereoscopic microscope is used, it is often a case that the specimen is not necessarily flat. Therefore, be sure to check whether the entire viewfield is focused by looking through the finder.

V. Microscope Cleaning and Storage

Inspection and cleaning of the optical system is particularly important, for both photomicrography and observation.

When using a microscope, always start with removing dust in the optical system.

1. Dust cleaning points



It is recommended that, as a rule, you always clean the seven points indicated above before using a microscope.

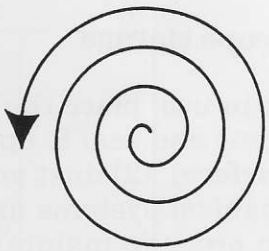
Before working on these points, using a blower, blow off dust and other foreign matter attached to the microscope, specifically:
(1) binocular eyepiece tube, (2) stage surface, and (3) base surface.

2. Wiping

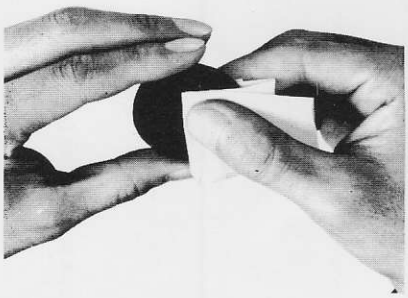
Wiping without care will smear the equipment. Wiping the lens surface requires skill and attention.

Until you are accustomed to this job, it is not recommended that you provide intensive wiping from the beginning. Instead, begin with rough wiping over the entire equipment and repeat it until the equipment is totally clean.

For wiping lenses and filters, the basic procedure is to wipe the object from the center, winding a spiral to the periphery.

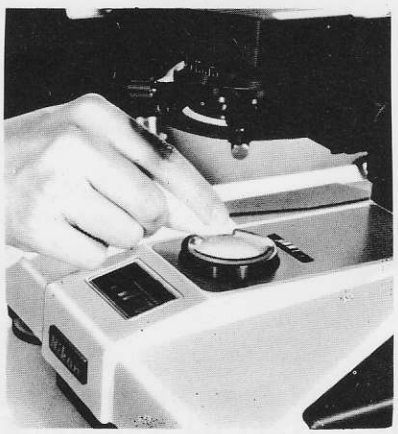


How to wipe each part



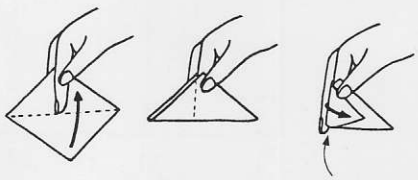
Filter

Objective



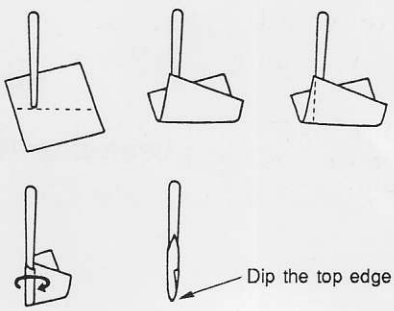
Field lens

How to roll the lens paper around your finger



Dip the top edge in alcohol.

How to roll the lens paper around a stick.



Dip the top edge in alcohol.

3. Cleaning tools

a) Pure alcohol - Lens, filter surface, glycerin cleaning
b) Petroleum benzine - Oil immersed objectives and condensers
c) Blower - Removal of dust and foreign matter
d) Lens paper
e) Willow or cedar sticks - Used to wipe objectives by sharpening the edge.

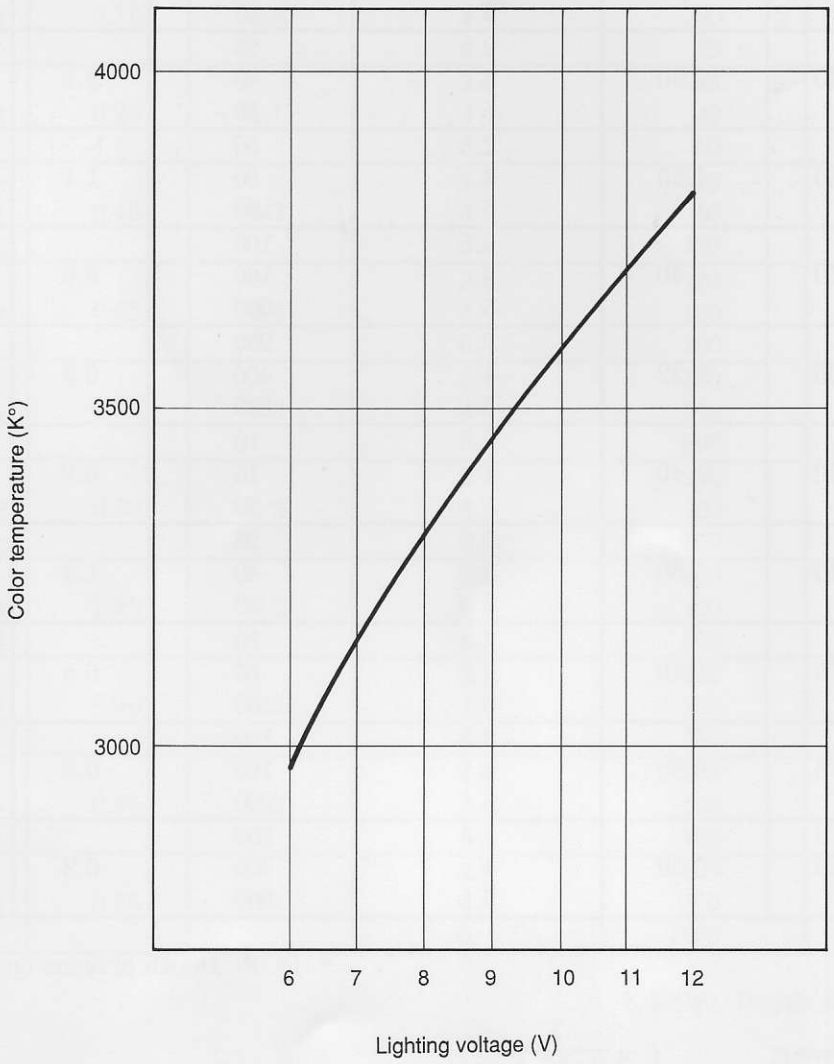
4. Microscope storage

When not in use, place the microscope in a polyethylene bag together with a drying agent and seal it up. This is to protect it from (1) mold (which generates on lens surface), (2) dust and foreign matter, and (3) rust. Since both optical and mechanical systems are precisely adjusted, avoid vibration even during storage in order to maintain the initial high performance.

VI. Reference Data

1. Lamp Voltage and Color Temperature Curve (Measurement results)

Lighting Voltage and Color Temperature of OPTIPHOT's Halogen Lamp (12V-50W)



(Measurement conditions)
-- Microscope: OPTIPHOT and PFX-35
-- Filter: Heat absorption filter
-- Condenser: Swing-out Achromat condenser
-- Projection lens: PL 5X
-- Lamp: OSRAM 12V-50W halogen lamp (Brand-new)

2. 35mm Camera Optical Data

(Biological microscopes)

Objectives		Numerical aperture N.A.	Resolution ε(μm)	CF PL projection lens magnification (X)	Total magnification β(X)	DOF* t(μm)	Actual viewfield φ(mm)
CF N Plan Achromat	4x	0.13	2.10	2.5 4 5	10 16 20	16.3	4.3 2.7 2.2
	10x	0.30	0.90	2.5 4 5	25 40 50	3.1	1.7 1.1 0.9
	20x	0.50	0.55	2.5 4 5	50 80 100	1.1	0.87 0.54 0.43
	40x	0.70	0.40	2.5 4 5	100 160 200	0.6	0.43 0.27 0.22
	100x (Oil)	1.25	0.22	2.5 4 5	250 400 500	0.3	0.17 0.11 0.09
CF N Plan Apochromat	4x	0.20	1.40	2.5 4 5	10 16 20	6.9	4.3 2.7 2.2
	10x	0.45	0.60	2.5 4 5	25 40 50	1.4	1.7 1.1 0.9
	20x	0.75	0.40	2.5 4 5	50 80 100	0.5	0.87 0.54 0.43
	40x	0.95	0.30	2.5 4 5	100 160 200	0.3	0.43 0.27 0.22
	100x (Oil)	1.40	0.20	2.5 4 5	250 400 500	0.2	0.17 0.11 0.09

* DOF: Depth of focus on film plane

Resolving power: $\varepsilon = \frac{\lambda}{2 \times N.A.}$

Depth of focus: $t = \frac{n\lambda}{2 \times N.A^2}$

Real field of view: $\Phi = \frac{\sqrt{24^2+36^2}}{\beta}$

($\lambda = 0.55\mu m$)

$\delta = 0.05$ Resolution on film 20/mm

n: Refractive index of objective side medium

n = 1.0 Dry objective

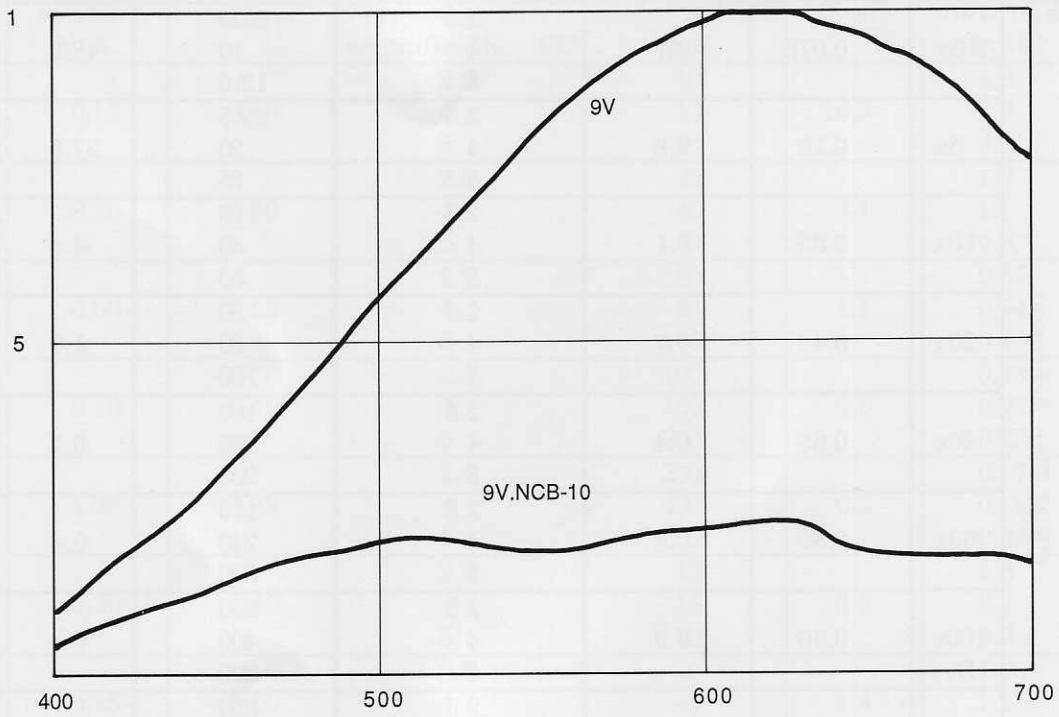
n = 1.52 Immersion objective

(Metallurgical microscopes)

Objectives		Numerical aperture N.A.	Resolution ε(μm)	CF PL projection lens magnification (X)	Total magnification β(X)	DOF* t(μm)	Actual viewfield φ(mm)
CF M Plan Achromat	2.5x	0.075	3.7	2.5	6.25	48.9	6.9
				4	10		4.3
				5	12.5		3.5
	5x	0.10	2.8	2.5	12.5	27.5	3.5
				4	20		2.2
				5	25		1.7
	10x	0.25	1.1	2.5	25	4.4	1.7
				4	40		1.1
				5	50		0.9
	20x	0.40	0.7	2.5	50	1.7	0.87
				4	80		0.54
				5	100		0.43
	40x	0.65	0.4	2.5	100	0.7	0.43
				4	160		0.27
				5	200		0.22
	60x	0.80	0.3	2.5	150	0.4	0.29
				4	240		0.18
				5	300		0.14
	100x (Dry)	0.90	0.3	2.5	250	0.3	0.17
				4	400		0.11
				5	500		0.09
	100x (Oil)	1.25	0.2	2.5	250	0.3	0.17
				4	400		0.11
				5	500		0.09
CF M Plan Apochromat	50x	0.90	0.3	2.5	125	0.3	0.35
				4	200		0.28
				5	250		0.19
	150x	0.95	0.3	2.5	375	0.3	0.11
				4	600		0.07
				5	750		0.06
	200x	0.95	0.3	2.5	500	0.3	0.09
				4	800		0.05
				5	1000		0.04

* DOF: Depth of focus on film plane

3. Microscope Illumination
Light Spectrum Distribution on Film Surface



(Conditions)
OPTIPHOT
PL 2.5X
Swing-out condenser

NIKON CORPORATION

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