Basics of Inverted Microscope
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Optical microscopes can be broadly categorized into two types, upright and inverted, based on the positions of the light source and the objective.

**Upright Microscope**
- Upright microscopes are constructed with the tip of the objective pointing downward so as to view the specimen from above.
- Light is directed on the specimen from below.
- This type of microscope is suitable for viewing prepared slides.

**Inverted Microscope**
- Inverted microscopes are constructed with the tip of the objective pointing upward so as to view the specimen from below.
- The objective is underneath the stage and light is directed on the specimen from above.
- This type of microscope is suitable for viewing culture vessels such as Petri dishes.

Stereo microscopes enable magnified images to be viewed in three dimensions.
- Stereo microscopes are convenient for directly viewing specimens such as insects.
- They are constructed like binoculars with separate left and right light paths, enabling specimens to be simultaneously viewed with both eyes in three dimensions.
- The distance between the objective and the stage is long compared with that of other types of microscope, enabling activities such as dissection of tiny insects placed under the objective to be performed during specimen microscopy.
Basics of Microscopes

1-1-2 Classification by Type of Microscopy

When observing a specimen using a microscope, the type of microscope and observation method used will differ depending on the specimen to be observed and the observation conditions. It is important to select an observation method that is suitable for the conditions of the specimen to be observed and the purpose of the microscopy observations.

◆ Bright Field (BF) Microscopy
The most common optical observation method is to observe color and brightness information from a stained specimen. The entire field of view is illuminated and appears bright. However, as the specimen is stained prior to observation, it is important to remember that the observed colors are not those of the actual specimen.

◆ Phase Contrast Microscopy (PC, PH)
Phase contrast microscopy is suitable for viewing colorless and transparent specimens and live cells. It utilizes the difference between light rays propagating directly from the light source and light rays refracted by the specimen when light passes through it to add bright/dark contrast to images of transparent specimens. The microscope is fitted with a phase-contrast objective and a condenser for observations. Specimens may be made to appear dark against a bright background (positive contrast) or bright against a dark background (negative contrast). The borders of images are surrounded by a characteristic bright "halo."

◆ Differential Interference Contrast (DIC) Microscopy
Differential interference contrast (DIC) microscopy is suitable for viewing colorless and transparent specimens and live cells. It utilizes the phase difference generated in regions of the specimen where a gradient is present when light passes through it to add bright/dark contrast to images of transparent specimens. The microscope is fitted with a DIC prism and a polarizing plate for DIC observations. In DIC microscopy, the contours of objects appear shadowed as if they are illuminated obliquely from above, giving a three-dimensional appearance. However, some areas may be difficult to view due to the orientations of gradients in the object and the actual specimen may not have the three-dimensional form observed by microscopy. In addition, because DIC utilizes polarized light, plastic Petri dishes cannot be used.
Fluorescence Microscopy

Fluorescence microscopy is a technique for viewing specimens stained with fluorescent dye and obtaining images from fluorescence generated by fluorescent proteins in a specimen. A high-intensity ultrahigh-pressure mercury lamp is generally used as the light source for generating fluorescence in the specimen (excitation light). Because fluorescence is emitted from a dark field of view, this method offers high detectability and can specifically distinguish the detection site. A major difference between this and other methods is that the specimen itself emits light.

Detection of cell nuclei and cytoskeleton under fluorescence microscopy (left: cell nuclei; right: cytoskeleton)

<table>
<thead>
<tr>
<th>Name of Method</th>
<th>Features</th>
<th>Main Areas of Use</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bright Field Microscopy</td>
<td>• Commonest observation method</td>
<td>• Observation of stained specimens</td>
</tr>
<tr>
<td></td>
<td>• Entire field of view illuminated by light source</td>
<td></td>
</tr>
<tr>
<td>Dark Field Microscopy</td>
<td>• Transparent specimens appear bright against a dark field of view</td>
<td>• Observation of colorless specimens such as bacteria</td>
</tr>
<tr>
<td>Phase Contrast Microscopy</td>
<td>• Observation by converting differences in light paths due to refraction into bright/dark contrast</td>
<td>• Observation of colorless, transparent specimens</td>
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<tr>
<td></td>
<td></td>
<td>• Observation of live cells</td>
</tr>
<tr>
<td>Differential Interference Contrast Microscopy</td>
<td>• Observation by utilizing gradients in specimens to add colors and bright/dark contrast</td>
<td>• Observation of colorless, transparent specimens</td>
</tr>
<tr>
<td></td>
<td>• Specimens appear three dimensional</td>
<td>• Observation of live cells</td>
</tr>
<tr>
<td>Polarizing Microscopy</td>
<td>• Observation by converting areas of birefringence into bright/dark or color contrast</td>
<td>• Observations of stone or mineral crystals</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Detection and measurement of birefringence</td>
</tr>
<tr>
<td>Fluorescence Microscopy</td>
<td>• Observation utilizing fluorescence generated by the specimen itself</td>
<td>• Observation of cells or tissues stained or labeled with fluorescent dye</td>
</tr>
<tr>
<td></td>
<td>• High detectability</td>
<td>• Observation of specimens via autofluorescence</td>
</tr>
<tr>
<td>Relief Contrast Microscopy</td>
<td>• Observation by conversion of irregularities on the specimen into bright/dark contrast</td>
<td>• Observation of cells in plastic containers</td>
</tr>
<tr>
<td></td>
<td>• Specimens appear three dimensional</td>
<td>• Observation of sperm and eggs</td>
</tr>
<tr>
<td>Dispersion Staining Microscopy</td>
<td>• Observations utilizing changes in dispersion color due to differences in the refractive index of the immersion liquid</td>
<td>• Asbestos count and qualitative analysis</td>
</tr>
</tbody>
</table>
1 Basics of Microscopes

1-2 Components of an Inverted Microscope

- Sites of Operation of an Inverted Microscope

![Diagram of an inverted microscope with labeled components]

- Filter Pocket
- Lamp Housing for Transmitted Illumination
- Field Iris Diaphragm Lever
- Transmitted Illuminator
- Condenser Height Adjustment Knob
- Insert Plate
- Aperture Iris Diaphragm Lever
- Stage
- Condenser
- Reflected Fluorescence Illuminator
- Eyepiece
- Lamp Housing for Reflected Light Illumination
- Observation Tube
- Transmitted Light ON-OFF Button
- Microscope Frame
- Light Intensity Control Knob
- Objective
- Transmitted Illuminator
- Focusing Knob
- Revolving Nosepiece
- Stage Knob
- Power Supply Unit for Transmitted Light Observation
- Power Supply Unit for Fluorescence Observation
“Optical systems” is the general term for systems that produce images of objects by using the properties of light. They consist of combinations of components such as lenses, lamps, and reflecting mirrors. The basic functions and components of a microscope are listed below. There are four optical systems in a microscope that are particularly basic to its function: the objective, the eyepiece, the illumination system (condenser, diaphragm, light source, filters), and the observation tube.

<table>
<thead>
<tr>
<th>Basic properties and configuration of a microscope</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basic Function</td>
</tr>
<tr>
<td>Illuminate specimen</td>
</tr>
<tr>
<td>Mount the specimen and determine the location to be observed</td>
</tr>
<tr>
<td>Bring the specimen into focus</td>
</tr>
<tr>
<td>Create a magnified image of the specimen</td>
</tr>
<tr>
<td>Observe and record</td>
</tr>
</tbody>
</table>
1-3 Adjustment of Microscope Illumination

1-3-1 Adjusting Köhler Illumination

Centering the Condenser

Condenser
1. Rotate the turret (either manually or electrically) to select the “BF” brightfield observation (with which no optical element is engaged in the light path).
2. Move the aperture iris diaphragm lever to open the diaphragm.
3. Move the field iris diaphragm lever to the fully open position (Ø → Ø).
4. Engage the 10x objective and bring the specimen into focus.
5. Using the field iris diaphragm lever, stop down the field iris diaphragm until its image is just inside the field of view.
6. Rotate the condenser height adjustment knob to bring the field iris diaphragm image into focus.
7. While gradually opening the field iris diaphragm lever, install the Allen screwdriver provided with the microscope in the adjustment hole and rotate it so that the field iris diaphragm image is centered in the field of view of the eyepieces.
8. To check centration, open the field iris diaphragm lever until its image inscribes the field of view. Now the condenser is centered.

* In actual observation, open the field iris diaphragm until its image circumscribes the field of view.

Effect of Field Iris Diaphragm
This is the iris diaphragm to adjust the area to be illuminated. By narrowing down to the level circumscribing the field of view depending on objectives, the excess light can be shielded to acquire the image with good contrast.

* Depending on the condenser to be used, the field iris diaphragm may not be viewed by the objective of 40x or higher magnification.
1. What is Phase Contrast Observation?

Phase contrast observation is suitable for observing colorless and transparent samples and live cells. Because unstained samples are colorless and transparent under conventional bright field observation, no information on contrast or color is obtained so that they appear invisible. In contrast, phase contrast observations utilize the difference in light paths (phase shift) between refracted light rays that pass through the sample and direct rays from the light source. This adds bright/dark contrast to samples, enabling even transparent samples to be observed.

2. Components of a Phase Contrast Microscope

- Field Iris Diaphragm
- Aperture Iris Diaphragm
- Eyepiece
- Stage
- Light Intensity Control Knob
- Condenser Centering Knob
- Condenser for Phase Contrast Observation
- Ring Slit Centering Screw
- Objectives for Phase Contrast Observation
- Revolving Nosepiece
- Focusing Knob
- Centering Telescope U-CT30-2
- Long Working Distance Universal Condenser IX2-LWUCD
- Phase Contrast Ring Slit
- Motorized Long Working Distance Universal Condenser IX3-LWUCDA
- Phase Contrast Ring Slit
- Ultra Long Working Distance Condenser IX-ULWCD
- Phase Contrast Ring Slit
2-3 Preparation for Phase Contrast Observations

2-3-1 Centering the Phase Contrast Ring Slit

Centering the Phase Contrast Ring Slit

1. Open the aperture iris diaphragm during phase contrast observation.
2. Engage the phase contrast objective in the light path and bring the specimen into focus.
3. Remove an eyepiece and attach the centering telescope in place.
4. Engage the ring slit of the condenser matching the phase contrast objective in the light path.
5. Rotate the knurled section of the centering telescope to focus on the ring slit \(a\) and the phase plate \(b\) of the objective.
6. Pushing the optical element centering knobs, turn the phase contrast ring slit centering screws (in positions marked) so that the ring slit image overlaps with the phase plate of the objective.

※ Do not release the hand suddenly while the optical element centering knobs are being pushed in. The optical element centering knobs may be popped out.

7. Remove the centering telescope and attach an eyepiece in place.

※ If the vessel is not completely flat, it may become necessary to adjust the centering again to obtain the optimum contrast. Adjust the centering in each objective power.

8. Adjust the field iris diaphragm so that its image circumscribes the field of view and observe the phase contrast.

※ Engaging the green filter in the light path will improve the contrast.
When light passes through a sample, differences in the refractive index and the thickness of the sample along the light path give rise to differences in the distance traveled by the light. These differences in distance are known as “light path differences” and are utilized by differential interference contrast (DIC) observations to enable the observation of colorless, transparent samples.

The images below compare neural cells (NG108-15) photographed by DIC and phase contrast observations. In DIC microscopy, the peripheries of the cells are shadowed as they are illuminated obliquely from above, making them appear three dimensional (Figure A).

The ways in which contrast is added and the characteristics of the resultant images thus vary between DIC and phase contrast observation. The method most suited to the purpose of microscopy should be chosen.

### Differential Interference Contrast Observation

**Comparison of DIC and Phase Contrast Observations**

<table>
<thead>
<tr>
<th>How Contrast is Added</th>
<th>DIC Observation</th>
<th>Phase Contrast Observation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Contrast added by gradients in sample thickness</td>
<td>Contrast added at sample borders or points</td>
</tr>
<tr>
<td>Image Features</td>
<td>Bright/dark or color contrast added, conveying a three-dimensional appearance; Shadows added depending on orientation</td>
<td>Bright/dark contrast added; Pronounced halo around thick samples</td>
</tr>
<tr>
<td>Contrast Adjustment and Selection</td>
<td>Fine adjustment of three-dimensional contrast possible</td>
<td>Choice of negative or positive contrast</td>
</tr>
<tr>
<td>Resolution</td>
<td>High</td>
<td>Poor compared with DIC*</td>
</tr>
<tr>
<td>Suitable Samples</td>
<td>Capable of observing structures with sizes ranging from minute to large; Sample thicknesses up to several 100 µm</td>
<td>Useful for observing minute structures; Sample thickness up to 10 µm</td>
</tr>
<tr>
<td>Use of Plastic Containers</td>
<td>No</td>
<td>Yes</td>
</tr>
</tbody>
</table>

*In phase contrast observations, illumination is limited by the ring slit, and consequently its resolution is poor compared with DIC.
Performing differential interface contrast observation requires:

1. In place of the condenser used for bright field observation, a “universal condenser” fitted with a built-in polarizer and a DIC prism are required.
2. A “DIC prism (DIC slider)” and an “analyzer” are required below the objective.

Components of a Differential Interference Contrast Microscope

- Analyzer for Transmitted Light U-ANT
- DIC Slider for Transmitted Light U-DICT
- Shift DIC Slider for Transmitted Light U-DICTS
- High Resolution DIC Slider for Transmitted Light U-DICTHR
- High Contrast DIC Slider for Transmitted Light U-DICTHC
3-3 Preparing for Differential Interference Contrast Observations

3-3-1 Cross-Nicol Adjustment

1. Rotate the condenser’s turret for the BF (brightfield) light path (with no optical element engaged in the light path).
2. When IX3-LWUCDA is used, push the button \( \text{a} \) of IX3-LWUCDA to engage the polarizer in the light path.
   When IX-LWPO is used, move the polarizer detaching lever \( \text{b} \) on the IX-LWPO polarizer to engage the polarizer in the light path.
3. Engage the 10× objective in the light path, place an optimum specimen for brightfield observation on the stage, bring the specimen into approximate and remove the specimen out of the light path.
4. Drop the U-ANT into the analyzer mount of the U-DICT. Hold the U-DICT so that the side with indication faces down, insert it into the revolving nosepiece.
5. Remove the eyepieces from the observation tube, look into the observation tube.
6. Move the prism movement knob \( \text{f} \) of the DIC slider in the clockwise direction around the axis until the knob is stopped. First a black interference stripe then a rainbow-colored interference stripe will be observed. Stop the knob at the position in which the black interference stripe can be seen. (State of \( \text{g} \))
7. When IX3-LWUCDA is used, loosen the polarizer rotation knob \( \text{d} \) by rotating the clamping knob \( \text{c} \) in the clockwise direction until the knob stops.
   When IX-LWPO is used, loosen the clamping of the polarizer rotation/clamping knob by rotating slightly the polarizer rotation/clamping knob \( \text{d} \) in a counter-clockwise direction.
8. While looking into the observation tube, rotate the polarizer rotation/clamping knob \( \text{d} \) on the polarizer unit horizontally until the black interference stripe becomes darkest. This is the cross-nicol position.
9. After determining the position, clamp the polarizer.
   When the IX3-LWUCDA is used, rotate the clamping knob \( \text{c} \) in the counterclockwise direction around the axis until the knob is stopped.
   When the IX-LWPO is used, rotate the polarizer rotation/clamping knob \( \text{d} \) in a clockwise direction until the knob stops.
Prepare for Differential Interference Contrast Observations

Observation Process

1. Rotate the condenser turret to engage the suitable optical element for the objective in use in the light path.
2. Engage the objective to be used in the light path.
3. Place the specimen on the stage and bring the specimen into focus by moving the objective up or down.
4. Adjust the field iris diaphragm so that its image circumscribes the field of view.
5. Adjust the aperture iris diaphragm to enhance the contrast.
6. Move the prism movement knob of the DIC slider to select the interference color that can provide the optimum contrast in accordance with the specimen.

U-DICT: The background interference color is continuously variable from the gray sensitive color to purple sensitive color.

U-DICTS: The background interference color is continuously variable from black to light gray.

U-DICTHC: The background interference color is continuously variable from black to light gray.

U-DICTHR: The background interference color is continuously variable from black to light gray.
What is Relief Contrast?

Phase-contrast and differential interface contrast (DIC) observations have already been described as techniques for observing clear and transparent samples and live cells. Similar to DIC, relief contrast observation enables samples to be viewed in three dimensions. However, unlike DIC, which does not permit the use of plastic containers, plastic containers can be used for relief contrast observation. Relief contrast observation can thus be used to obtain three-dimensional images, similar to those of DIC observation, of cells in plastic containers.

For example, when viewing egg cells and sperm cells, plastic containers are often more convenient for performing operations than glass. However, DIC cannot be used with plastic containers. Phase contrast observation is also unsuitable for viewing thick cells such as egg cells. In such cases, relief contrast observation can be used.

<table>
<thead>
<tr>
<th>Comparison of Relief Contrast Observation and DIC</th>
</tr>
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<tbody>
<tr>
<td>Relief Contrast Observation</td>
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<tr>
<td>How Contrast is Added</td>
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<tr>
<td>Image Features</td>
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<td>Contrast Adjustment and Selection</td>
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<tr>
<td>Resolution</td>
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<td></td>
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<tr>
<td>Suitable Samples</td>
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<td></td>
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<tr>
<td>Use of Plastic Containers?</td>
</tr>
</tbody>
</table>

*In relief contrast observation, illumination is limited by the ring slit and consequently resolution is poor compared with DIC.
4-2 Components of Relief Contrast System

A relief contrast microscope consists of an inverted microscope and following units.

- **Mid Long Working Distance Condenser**
  This condenser not only incorporates a rectangular slit modulator for relief contrast imaging, but it can also be used for phase contrast or DIC observation by inserting a phase contrast ring slit or a DIC prism element.

- **Relief Contrast Objectives**
  Relief contrast objectives contain a special objective modulator. The Olympus lineup includes two series, Achromat and Plan-semi-apochromat, each having magnifications of 10×, 20×, and 40×.
Basic Adjustment Methods for Relief Contrast Observation

Use an OLRC-dedicated objective.

**Adjusting the Modulator**

- In the adjustment stage, it is recommended to use a general dyed specimen in order to facilitate the focus adjustment. If such a specimen is not available, use dirt or scratch on the petri dish for focusing.

- Open the field iris diaphragm (FS lever).

  1. Rotate the turret to engage the desired modulator in the light path.
  2. Engage the objective with the same magnification as the above modulator in the light path and bring the specimen into focus.
  3. After obtaining the focus, remove the specimen, remove an eyepiece and look into the eyepiece sleeve.

- When the U-BI90 or U-TBI90 binocular observation tube or U-TR30H-2 trinocular observation tube is used, remove an eyepiece and attach the U-CT30-2 centering telescope.

  Bring the objective’s exit pupil into focus.
  
  - U-CT30-2: Turn the upper adjustment ring.

  The objective’s exit pupil looks as shown. Overlap “A” of the modulator with the gray area of the objective using the centering knobs.

  - A: Area where light is transmitted permanently
  - B: Area where the transmittance varies according to the rotation of the dedicated polarizer.

  - Circumferential-direction movement:
    Insert a centering knob directly into the modulator’s groove through the modulator rotation groove and turn the knob.

  - XY-direction movement:
    Insert the two centering knobs into the two insertion holes and turn the knobs.

- Also adjust the modulators with other magnifications.

- During actual observation, expand them depending on the degree of external contact with the field of view.
5-1 Features of Fluorescence Observation

**Features of a Fluorescence Microscope**

Fluorescence microscopes were invented in the early 1900s and have been further improved with the development of fluorescent antibody techniques (for observing fluorescent immunostained specimens). In recent years, their use has expanded to the fields of cellular and molecular biology, utilizing their advantages as optical microscopes capable of observing live cells. Fluorescence observation involves viewing fluorescent signals emitted by samples labeled with fluorescent dyes or by fluorescent proteins themselves. It has the following features and advantages.

1. Capable of detecting and visualizing objects (substances/structures to be detected) even if they are much smaller than the resolution determined by the wavelength and the numerical aperture → detection of single proteins
2. Capable of specifically detecting and visualizing objects, as well as their location and movement → highly sensitive
3. Capable of detecting changes in brightness and color of fluorescence (fluorescence wavelength) → quantitative analysis, such as measurement of intracellular ion concentrations
4. Capable of utilizing differences in the color (wavelength) of fluorescence to observe specimens stained with multiple dyes → simultaneous detection/visualization of multiple objects

5-2 Composition of a Fluorescence Microscope

A fluorescence microscope is a combination of a conventional optical microscope and a reflected fluorescence illuminator. The most commonly operated devices during fluorescence observation are the focusing knob for bringing the sample into focus, the X–Y stage handle for positioning specimens, the revolving nosepiece for switching the objective, the mirror unit turret for switching the excitation method, the shutter for preventing fading when the sample is not under observation, the ND filter for adjusting the excitation light intensity, and the aperture iris diaphragm.
5-3 Choice of Fluorescence Mirror Unit

5-3-1 What is the Mirror Unit?

You may not immediately be able to picture the “mirror unit.” Even with the microscope in front of you, you may not be sure which part this refers to. It is located between the objective and the eyepiece on the light path from the light source (lamp housing). In fluorescence imaging, the sample is only illuminated with excitation light of the target wavelength, with the aim of observing only the fluorescent signal emitted by the sample. The mirror unit is used to select light in a specific wavelength range.

Composition of the Mirror Unit

The mirror unit has a cubic structure consisting of the following three filters.

1. **Excitation Filter**
   This filter restricts the illumination from the light source. Out of a broad wavelength band, it permits only light in the optimum excitation wavelength range (excitation light) for the fluorescent dye to pass through.

2. **Dichromatic Mirror**
   This mirror reflects light with wavelengths shorter than a specified wavelength, while allowing longer wavelengths to pass through, transmitting the excitation light toward the objective. It also permits fluorescence from the sample to pass through, transmitting it to the eyepiece.

3. **Emission Filter**
   This filter completely blocks excitation light, permitting the passage of only fluorescence from the sample.

Optical System Paths

Light emitted by a light source such as a mercury or xenon lamp has an extremely broad wavelength range, from ultraviolet and visible light through to near-infrared. By passing this through an excitation filter, light of the optimum wavelength for exciting the fluorescent dye (excitation light) can be selected.

The light that passes through the excitation filter is next reflected by a dichromatic mirror, which rotates its direction of travel by 90°. It then exits the mirror unit and passes through the objective, before illuminating the sample. When the sample is illuminated with excitation light with wavelengths specific to each fluorescent dye, the dye in the sample is excited and emits fluorescence. This fluorescence passes through the objective and re-enters the mirror unit, reaching the dichromatic mirror. The dichromatic mirror has the property that it reflects light at short wavelengths (such as the excitation light), but allows longer wavelength light to pass through. The long-wavelength fluorescence emitted by the sample passes through the dichromatic mirror and reaches the third component, the emission filter. This selects only light of the specific fluorescence wavelength of the fluorescent dye and this reaches the observer as the signal.
Observing Multiple Fluorescent Dyes

If only one fluorescent dye is to be observed, choose a suitable excitation and emission filters for that dye. Detailed information is published by filter manufacturers. You should select the recommended appropriate mirror unit. However, care is required if two or more fluorescent dyes are to be observed simultaneously. We consider how to choose a mirror unit in this case. As an example, we assume that two fluorescent dyes are to be used: Alexa Fluor 488 and Alexa Fluor 546.

First, look up the excitation and emission spectra of each dye (Figure 1).

Of the two fluorescent dyes in Fig. 1, a “dye separation” bandpass filter with a narrow transmission range must be selected for observing Alexa Fluor 488 fluorescence. This is because Alexa Fluor excitation light may excite not only Alexa Fluor 488 but also Alexa Fluor 546, and if an Alexa Fluor 488 emission filter with a transmission range that permits the passage of long wavelengths is used, fluorescence from Alexa Fluor 546 may also be observed (Figures 2 and 3).

It is also possible to use several mirror units suitable for different fluorescence wavelengths together to observe only the target signal, simplifying subsequent image processing. After obtaining an image with the Alexa Fluor 488 mirror unit (U-FBWA), replace it with the Alexa Fluor 546 mirror unit (U-FGW) and acquire another image. Then, superimpose the two images on a computer.
5-4 Preparing for Fluorescence Imaging

5-4-1 Centering the Mercury Burner

※ Set the main switch of the power supply unit for mercury burner to “I” (ON) and wait for 5 to 10 minutes until the arc image stabilizes before proceeding with the mercury burner centering.

1. Close the shutter of the mirror turret.
2. Engage the B excitation fluorescence mirror unit (Ex: U-FBW) in the light path. When the XL fluorescence mirror unit is in use, engage the IX3-FGFPXL in the light path. (If these mirror units are not available, engage another fluorescence mirror unit in the light path.) If you use a fluorescence mirror unit for U-excitation, you must always view the specimen through an antiglare plate.
3. Engage the 10× objective in the light path, and place the centering target U-CST on the stage by facing the crossline side down.
4. Turn ON the transmitted illumination lamp, and focus on the crossline containing the double circle while viewing through the eyepiece.
5. Move the stage until the crosslines are overlaid on the center of field.
6. Rotate the revolving nosepiece to engage the empty position (the objective cap should be removed) in the light path.
7. Turn OFF the transmitted illumination lamp, and open the shutter of the mirror turret.
8. Turn the collector lens focusing knob to project the arc image on the U-CST. (Fig. -A)
   If the arc image is not projected, adjust the burner centering knobs.
9. Turn the burner centering knobs to bring the arc image on the center of the right (left) half of the field. (Fig. -B)
10. Fit the Allen screwdriver in the mirror focusing screw on the rear of the lamp housing and adjust it to bring the mirror arc image in focus. (Fig. -C)
11. Turn the burner centering knobs to overlay the arc image with the mirror arc image. (Fig. -D)

※ During observation, adjust the collector lens focusing knob so that the observed field is uniform.
Fluorescence Observation

5-5 Fluorescence Observation Procedure

Preparation
- Attach the fluorescence mirror unit and objective matching the observation method.
- Center the mercury burner (refer to page 20).
- Set the main switch of related control boxes to "I" (ON).

1. Set the main switch of the power supply unit to "I" (ON) and wait for the lamp brightness to stabilize (5 to 10 minutes after ignition).

2. Place the specimen on the stage.

3. Engage the fluorescence mirror unit matching the specimen in the light path.

4. Engage the objective in the light path, open the shutter and focus on the specimen.

5. If necessary, engage the ND filter in the light path to adjust the brightness.

6. Adjust the field iris diaphragm.

7. Adjust the aperture iris diaphragm.
   IX3-RFAL only

8. Start observation.

* Engage the shutter if you take a short break during the observation.
6-1 Principles of the Confocal Microscope

The principle of the confocal microscope and its greatest advantage is the use of a confocal optical system. Figure 1 shows a confocal optical system and a conventional optical system. In the confocal optical system, pinhole 1 (a point light source) is projected onto the specimen, while pinhole 2 and a detector (usually a photomultiplier tube) are placed at the position of the specimen image. Because pinhole 1 (the point light source), the specimen, and pinhole 2 (image position) are all in conjugate positions, this type of configuration is known as a confocal optical system. As pinhole 1 is not always required; recent confocal laser microscopes use the core of a single-mode fiber or a laser diode (LD) as a light source, which also doubles as the pinhole.

Figure 1. Comparison of conventional microscope and confocal optical systems
6.2 Imaging Properties of Confocal Microscopes

Confocal microscopes are based on the confocal optical system illustrated in Fig. 1. They are characterized by a higher contrast and resolution than conventional microscopes, as shown below.

1. Point illumination eliminates stray light from horizontal directions adjoining the specimen.
2. Only information from the focal point passes through the pinhole and reaches the detector; light from outside the focal point is blocked by the pinhole, providing depth resolution (Z-axis) and enabling optical cross-sectional images to be obtained. This is impossible with a conventional microscope.
3. In particular, in fluorescence confocal microscopes, incoherence between light from the light source and fluorescence from the specimen means that the point spread function (PSF) that can be achieved is determined by the product of the PSFs of the illumination and detector systems (the so-called “square law”). Consequently, the PSF is sharper than that of conventional microscopes and the XYZ resolution is higher (however, in reflective confocal microscopes, the PSF and resolution differ in terms of the argument from the amplitude, including the specimen phase, when coherence is taken into account).

Figure 2 shows a schematic diagram of the confocal effect. Figure 3 compares actual images obtained using conventional and confocal microscopes.

The images show cultured cells (PtK2) with a thickness of approximately 5 µm. With a conventional microscope, overlapping information from above and below the focal plane is also transmitted, making the details unclear. In contrast, with a confocal microscope, only information from the focal plane is acquired, providing images with better resolution and contrast.
Image Formation in a Confocal Microscope

Although confocal microscopes have many features, the confocal optical system that forms its core basically comprises a single point of illumination and a single point of detection, which is not involved in image formation. By fixing the illumination and scanning the specimen (stage) two dimensionally in the XY plane or by fixing the specimen and scanning the illumination (a laser beam in this case), information can be obtained from different points and can be converted into an image. An easy way to understand this is to compare it with the way in which images are generated on a TV monitor by scanning lines.

The schematic diagram in Fig. 4 shows how images are formed by scanning. However, it is difficult to scan the specimen (stage) two dimensionally in practice for various reasons such as the scanning speed and mechanical stability. Consequently, the illuminating laser beam is generally scanned using an element capable of deflecting light at a high speed, such as a galvano mirror. A stepping motor or piezo element can be used to provide gradual movement of the objective or stage in the Z direction at the end of each two-dimensional scan.

It is important to realize that images from confocal microscopes are an amalgamation of individual points, and as such they are digital in nature. Recently, there have been efforts to digitalize microscopy; confocal microscopes can be regarded as pioneering digital microscopes. A range of image processing techniques can be used for highly accurate quantitative analysis or Fourier analysis, and three-dimensional structures can easily be reproduced from multiple images.

Figure 4. Schematic Diagram of Image Formation by Laser Beam Scanning
Digital Cameras

7-1 Color and Monochrome Cameras

Fluorescence microscopy must be capable of capturing extremely faint light (signals). The intensity of fluorescence signals is only about 1/1,000,000th that of light shone on the sample. Cooled CCD cameras are used as cameras in fluorescence microscopes. They can generate bright images even from very faint light. Out of the following cameras, select the most suitable one for your purposes.

7-1-1 Color Cameras

Color cameras are often used as a simple way of acquiring fluorescence images to present in papers or in conference presentations. The color filters above all the individual CCD elements enable fluorescence images to be obtained that display the same colors as visible fluorescence images. If an appropriate mirror unit is used, images of samples multi-stained with red, blue, and green dyes can easily be obtained with the same appearance as that seen in visual observation.

However, when fluorescence entering a color camera passes through any of the RGB color filters above the elements, depending on the color of the fluorescence, some elements will sense the light whereas others will not. Consequently, this method is unsuitable for quantitatively evaluating proteins expressed in terms of the intensity and location of fluorescence. A monochrome camera should thus be used for analyzing very faint fluorescence.

7-1-2 Monochrome Cameras

Monochrome cameras are suitable for acquiring images of live cells and analyzing (quantitating) the fluorescence intensity. Unlike color cameras, these cameras have no color filters above their CCD elements, so that they are capable of extremely high-sensitivity imaging with no light loss due to filters. For example, from the viewpoint of phototoxicity, light should be as faint as possible when observing live cells and an extremely high sensitivity is required to capture the very faint fluorescence emitted by the sample. Monochrome cameras also have superior cooling functions to color cameras. Within CCDs, a signal called a “dark current” is generated when no light is input. As this dark current is associated with heat, cooling CCDs can suppress it to extremely low levels. Many color cameras can only be cooled to around 10°C, but some monochrome cameras can be cooled to –80°C. However, as all fluorescence is shown as black and white images, imaging must be performed separately for each dye. For multi-staining, software must be used after imaging to add pseudo-color for each dye.
The use of parfocal compensation with a C-mount camera adaptor allows matching of the focus of the observed image with that of the image on the monitor.

- The hexagonal screwdriver supplied with the microscope is used in parfocal compensation.

1. Looking through the eyepiece, bring the specimen into focus.
2. Switch to the monitor image on the video light path.
3. Using the hexagonal screwdriver, undo the parfocal adjustment lock screw (LOCK) on the C-mount camera adaptor.
4. While viewing the monitor image, slowly turn the parfocal adjustment screw with the hexagonal screwdriver to bring the image into focus.
5. When the image is in focus, tighten the lock screw with the hexagonal screwdriver.

### Types of Camera Adapter and Imaging Area

<table>
<thead>
<tr>
<th>Camera Adapter (Projection Lens)</th>
<th>Projection Magnification</th>
<th>Projection Area (FN)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>2/3” CCD</td>
</tr>
<tr>
<td>U-TVO.35x-C-2</td>
<td>0.35x</td>
<td>—</td>
</tr>
<tr>
<td>U-TVO.5x-C-3</td>
<td>0.5x</td>
<td>22</td>
</tr>
<tr>
<td>U-TVO.63x-C</td>
<td>0.63x</td>
<td>17.5</td>
</tr>
<tr>
<td>U-TV1x-2+U-CMAD3</td>
<td>1x</td>
<td>11</td>
</tr>
<tr>
<td>U-TV1x-C</td>
<td>1x</td>
<td>11</td>
</tr>
</tbody>
</table>

* Depending upon the type of CCD.
Objectives

1 Selecting an Objective

1-1 Basic Specifications

The objective is the component that produces the initial image of the object under observation. It is the most important device in determining a microscope’s optical properties. It must be designed and manufactured with the utmost precision.

It is important to learn about the basic specifications of objectives and understand something about the most suitable objectives for various specimens and purposes.

 Numerical Aperture (NA)
This parameter determines the resolution, focal depth, and image brightness. Increasing the numerical aperture, increases the resolution and the brightness of images. The numerical aperture is greater for higher magnification objectives.

 Magnification (M)
This is the magnification of the intermediate image (actual inverted image) of the specimen. In addition to low (4×–10×), medium (20×–50×), and high (over 100×) magnifications, other magnifications such as ultralow (less than 2.5×) are also available.

 Working Distance (W.D.)
This is the distance between the tip of the objective and the focal plane when the latter is in focus. Increasing the numerical aperture of an objective, reduces the working distance.

 Cover Glass Thickness
The thickness of the cover glass is indicated as a number on the objective.
There are three types of objectives: those designed for use with specimens under a cover glass, those designed for use with uncovered specimens, and those designed for use with both covered and uncovered specimens.

 Immersion and Dry Systems
In one observation method, a liquid is used to fill the space between the specimen and the objective. Objectives for use in this type of microscopy are known as “immersion objectives”. The immersion liquid may be immersion oil, water, glycerin, or some other liquid. The lens barrel of the objective is marked “Oil,” “W,” or “Gly,” indicating the type of immersion to be used. A “dry objective” is used to perform observations without an immersion liquid.

 Numerical Apertures of Objectives
To ensure that a high-magnification objective has a sufficiently high numerical aperture for magnification, the space between the specimen and the objective is filled with liquid to increase the numerical aperture. The refractive index depends on the immersion liquid used.
8-1-2 Types and magnifications of objectives

The top part of this objective is labeled with the large letters UPlanSapo, indicating the type of objective. Below this, the label “60 ×/1.35 Oil” indicates the magnification, the numerical aperture, and the immersion type. Underneath this, the label “∞/0.17/ FN 26.5” indicates the mechanical tube length, the cover glass thickness, and the field number. Of the two lines below this, the top one indicates the objective magnification and the lower one the type of immersion. These are shown in the figure below. (These labels are prescribed as international standards by the ISO and JIS.)

<table>
<thead>
<tr>
<th>Color Code (magnification)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.25x</td>
</tr>
<tr>
<td>Black</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Color Code (immersion)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oil</td>
</tr>
<tr>
<td>Black</td>
</tr>
</tbody>
</table>

8-2 Adjustment of Objectives with Correction Collar

- **Objectives with Correction Collar**
  
  If the thickness of a specimen cover glass differs from the design value of the objective, the spherical aberration increases, causing resolution and contrast to deteriorate. Objectives with a large NA are particularly affected; they are fitted with a correction collar to compensate for this.
  1. Bring the specimen into focus.
  2. Turn the correction collar and refocus
  3. If resolution and contrast have been improved as a result of turning the collar, continue turning it in the same direction and repeatedly adjust the focus to achieve the best resolution and contrast.
  4. If resolution and contrast have deteriorated as a result of turning the collar, turn it in the opposite direction and repeat from step 2 above.
8-3 Types of Objective

<table>
<thead>
<tr>
<th>Series</th>
<th>Features</th>
</tr>
</thead>
<tbody>
<tr>
<td>UPLSAPO</td>
<td>Thanks to the application of Olympus’ original UW multi-coating, these Super Apochromat objectives fully compensate for both spherical and chromatic aberrations from the UV to the near infrared region. Their sensitivity to fluorescence emissions ensures the acquisition of sharp, clear images, without color shift, even in brightfield and Nomarski DIC observations.</td>
</tr>
<tr>
<td>PLAPON</td>
<td>These Apochromat objectives feature UW multi-coating to provide flat images from high transmission factors up to the near infrared region of the spectrum. They are also fully suitable for low (1.25× and 2×) magnification observations.</td>
</tr>
<tr>
<td>UPLFLN</td>
<td>These objectives also provide flat images from high transmission factors up to the near infrared region of the spectrum through the employment of UW multi-coating. With their high S/N ratio, excellent resolution and high contrast imaging, they are especially effective in brightfield and Nomarski DIC observations.</td>
</tr>
<tr>
<td>UPLFLN-PH</td>
<td>Universal phase-contrast objectives that provide the same critical optical performance as the UPLFLN series. The phase membrane allows a high-contrast image to be produced without using an interference filter.</td>
</tr>
<tr>
<td>UPLFLN-P</td>
<td>These strain-free Semi-Apochromat universal objectives reduce internal strain to an absolute minimum and are best suited for polarizing and Nomarski DIC microscopies.</td>
</tr>
<tr>
<td>PLFLN</td>
<td>Despite its high magnifying power, the PLFLN100X is an easy-to-use, non-oil immersion type objective. This objective offers exceptional flatness in all fields of view.</td>
</tr>
<tr>
<td>PLN</td>
<td>These standard objectives are suited to clinical laboratory and examination work. They ensure superb field flatness up to FN 22 with brightfield observation in transmitted light.</td>
</tr>
<tr>
<td>PLN-PH</td>
<td>These general objectives were developed for transmitted phase-contrast light observation and are used for clinical inspections, providing consistent image flatness up to FN 22.</td>
</tr>
<tr>
<td>PLN-CY / PLFLN-CY</td>
<td>This CY objective series equipped with ND filter provides the same level of brightness even if the magnification is changed. No brightness adjustment is required.</td>
</tr>
<tr>
<td>PLN-P / ACHN-P</td>
<td>Primarily used for clinical inspection and student training, these highly cost-efficient objectives enable transmitted polarized light observations and are compliant with FN 22.</td>
</tr>
<tr>
<td>UCLPLFLN</td>
<td>These universal Semi-Apochromat objectives are dedicated to tissue culture observations through bottles and dishes, offering excellent contrast and resolution in brightfield, DIC and fluorescence observations.</td>
</tr>
<tr>
<td>CPLFLN-PH / LUCPLFLN-PH</td>
<td>These objectives are exclusively designed for culture specimens. An excellent phase-contrast image is assured regardless of the thickness and material of the vessel.</td>
</tr>
<tr>
<td>CPLFLN-RC / LUCPLFLN-RC</td>
<td>These objectives are designed for observation of live cells including oocyte. Plastic vessels applicable for Relief Contrast observations.</td>
</tr>
<tr>
<td>CPLN-PH / LCAHN-PH</td>
<td>Combining easy focusing with excellent cost efficiency, these high-quality phase-contrast objectives are especially suitable for routine inspections involving many specimens.</td>
</tr>
<tr>
<td>CPLN-RC / LCAHN-RC</td>
<td>These objectives are designed for observation of live cells including oocyte. Plastic vessels applicable for Relief Contrast observations.</td>
</tr>
<tr>
<td>UPLFLN-PHP / CACHN-PHP / LCACHN-PHP</td>
<td>This series is used in combination with the pre-center type phasecontrast slider IX2-SLP. When changing the objective magnification in phase-contrast observation, no centering adjustment is necessary.</td>
</tr>
<tr>
<td>LUMPLFLN-W</td>
<td>This is a series of water immersion objective lenses developed for experiments in electrophysiology. The UW multi-coating displays flat images with a high transmission factor up to the near infrared region, while also achieving excellent DIC and fluorescence from the visible range to infrared. Also available is a 60× objective with NA 1.1 and 1.5 mm working distance, making it ideal for fluorescence imaging of brain tissue as well as other tissue and specimens.</td>
</tr>
<tr>
<td>XLUMPLFLN-W</td>
<td>This XLUMPLFLN-W objective allows the measurement of cell membrane electric potential.</td>
</tr>
<tr>
<td>No cover objectives</td>
<td>These no cover objectives are specially designed for microscopy without a cover slip such as for blood smear specimens.</td>
</tr>
<tr>
<td>UAPON340 Series</td>
<td>These objectives feature highest transmission of 340 nm wavelength light, ensuring maximum performance in fluorescence microscope through UV excitation.</td>
</tr>
<tr>
<td>TIRF objectives</td>
<td>These objectives make it easy to produce an evanescent wave field. So little light is leaked that a high-contrast image can be obtained against a dark background.</td>
</tr>
</tbody>
</table>

[Classification]
Cleaning the Objective

Technique for Wiping Lenses: Describe Widening Circles from the Inside Out …

◆ Using the Eyepiece as a Magnifying Glass

Ensure that the microscope is turned off before starting to clean the lens. The cleaning solution used to wipe the lenses is flammable, so if the power is turned on, heat from the light source may cause a fire. Start by removing the lenses from the microscope and check the eyepiece, the objective, the condenser, and the filter for dirt and contamination. The surface of the lens to be cleaned can be easily viewed by turning it face up and using a magnifying glass. If a magnifying glass is not available, the eyepiece can be inverted and used instead.

Next, use a blower or brush (e.g., a small paintbrush) to remove dirt and dust. This reduces the risk of damage to the lens surface when it is wiped with paper soaked in cleaning solution. Now wrap a piece of lens tissue around the end of a pointed chopstick or tweezers as shown in the diagram and soak it in cleaning solution.

Be careful not to allow metal areas of tweezers or sharp points to protrude from the tissue, as this may damage the lens surface.

◆ Preparations for wiping with a stick

When cleaning the lens surface, wipe it in widening circles from the inside out, tracing a spiral path. This gathers dirt and contamination dissolved in the cleaning solution at the frame around the lens, ensuring its complete removal. This method is used to wipe eyepieces and other components with comparatively large lenses, but high-magnification objectives and other very small lenses should be wiped by holding the tweezers still and rotating the lens around them.