MICROSCOPE 4

Nikon Eclipse Ti-U Inverted Compound Microscope

TRAINING GUIDE

Instruction for use of Microscope 4. Detailing the techniques available, location of supplies, and requirements for use of this shared resource.

3364G KHS Advanced Microscope Suite

Prepared by Ashley Vanhouten

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Introduction

Training requirements:

- Training on the use of this instrument is required prior to keycard access.
- First come, first served – if you are planning any time-sensitive analysis remember to reserve your time on the shared calendar to prevent any scheduling delays.
- Please remember to log your time, even retroactively, to provide data of usage.

Calendar information:

- Viewing of the shared calendar is accessible on the Microscopy Suite GVSU page

  https://www.gvsu.edu/clas/labresource/microscopy-facility-13

- Access to the calendar is automatically added with Keycard request
- To add the calendar to your account please follow the steps outlined in "Advanced Microscope Suite Calendar Access"

Supplies available:

- Drawer 11 includes a ready supply of cleaning agents for the microscopes. If low, please email Ashley Vanhouten.
- Sparkle, IPA 70%, and Ethanol are the only cleaning agents approved for use in the suite.
- If there is an advanced issue please contact your PI, Aaron Perry, or Ashley Vanhouten for additional support.

Please report issues:

If you encounter a situation where the microscope has become damaged or is malfunctioning in any way, please communicate this issue with your PI.

PIs, please communicate issues to Aaron Perry, Ashley Vanhouten (Equipment Repair), or Zach Hancock (IT Support) so we may provide support for this space. Examples of when to reach out include, but are not limited to:

- Bulb outages
- Software calibration issues
- Mechanical focus issues
- Error messages
- Initialization issues

Microscopy Techniques

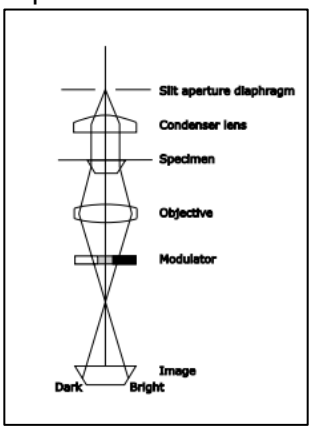
Bright-field is the most basic microscopy technique and uses the illumination that generates a path of light that will pass through your specimen. How the light path is modified (reflected or absorbed) as it passes through your specimen generates the image.

NAMC (Nikon Advanced Modulation Contrast) microscopy: NAMC is used to visualize changes in phase across a sample creating a pseudo 3D image. This technique is similar to Differential Interference contrast (DIC), but NAMC does not require polarizing light. NAMC works through plastic and glass (petri dishes/slides with covers) which often depolarizes light creating halo effect and/or shade-off artifacts with large samples features.

See figure below for the principle of the NAMC technique. There are 2 key components for this technique:

1. Slit diaphragm on the condenser aperture (slide)
2. Modulator inside the NAMC objective.

The modulator is a density filter placed at the exit pupil of the NAMC objective, it divides the exit pupil into 3 regions, dark, half-dark and transparent. In NAMC the image appears in relief just like in DIC (differential interference contrast). There is no influence of double refraction, thus enabling viewing of specimens without the need of a polarizer.

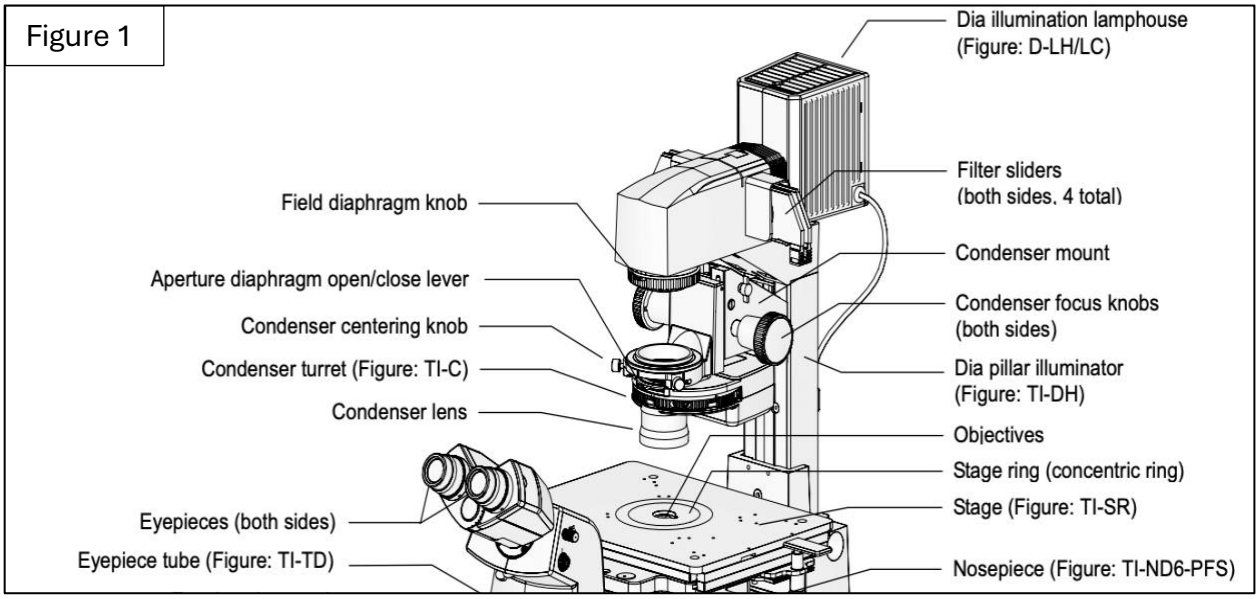


Epi-fluorescence microscopy: The use of fluorescent molecules in microscopy has revolutionized our ability to study many biological specimens. Fluorescent molecules (or fluorophores) can be used to tag otherwise transparent cellular structures to determine information such as cellular location. Increased sensitivity possible with fluorophores allows the observation of organelles and even molecule-specific signals. Light that illuminates the samples from above is used (thus the term epi-fluorescence) and the objective lens acts as both the illumination condenser and the fluorescent light collector. Electrons of a fluorescent molecule can absorb a photon of excitation light and then emit

some of the gained energy as a photon with less energy and therefore a longer wavelength. It is this emitted light that is detected during Epi-fluorescence microscopy.

Microscope Images

Figure 1



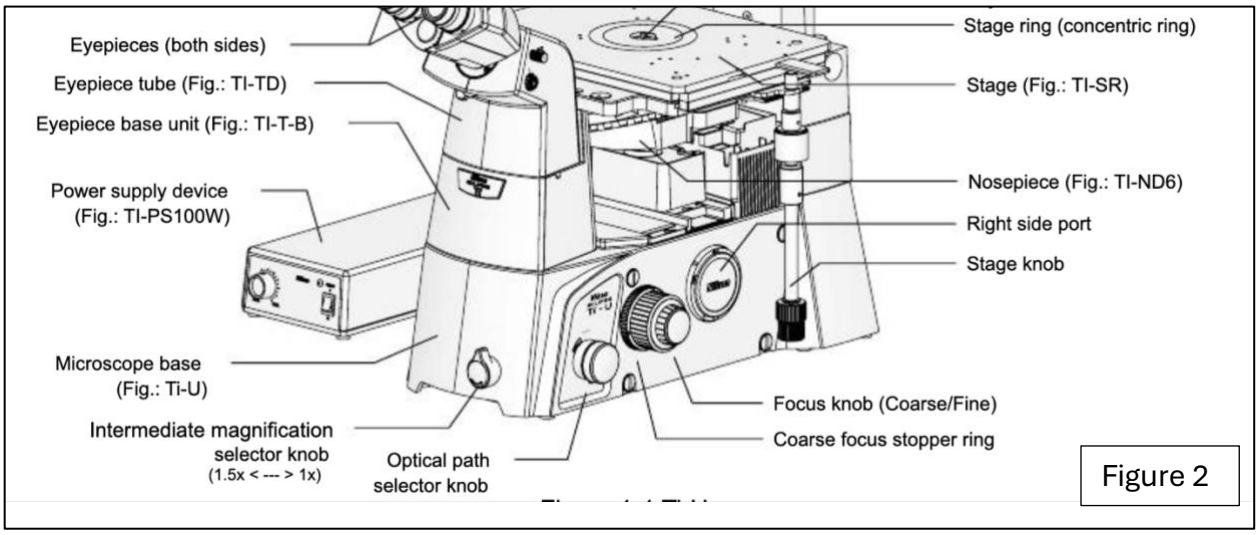


Figure 2

Figure 3

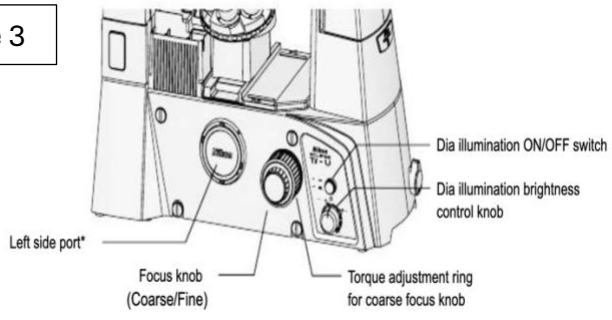


Figure 1-2 Ti-U (Left View)

Bright-field light power supply

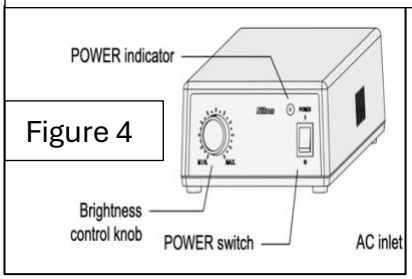


Figure 4

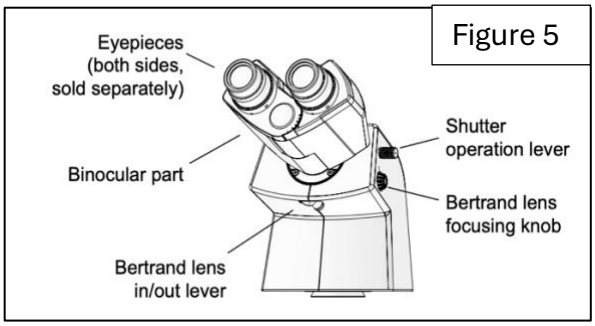


Figure 5

Epifluorescence Components figure 6, 7 & 8

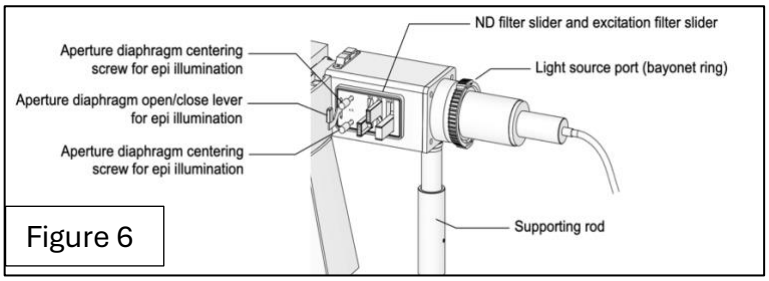


Figure 6

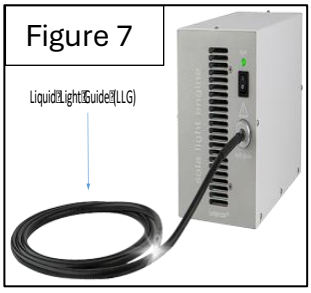


Figure 7

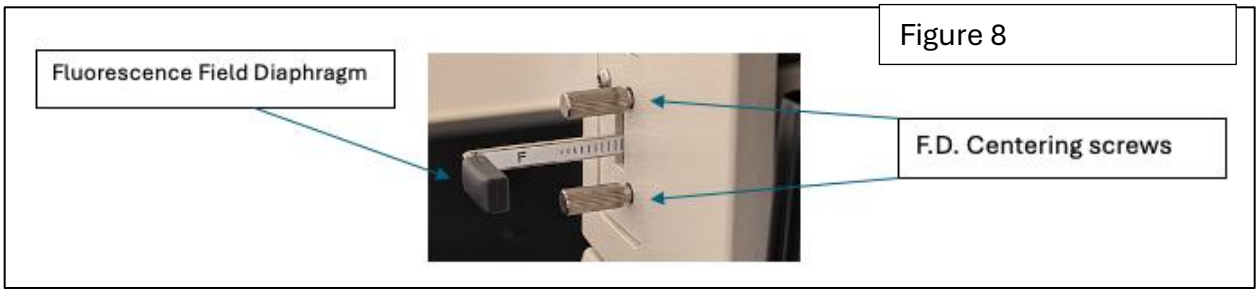


Figure 8

Bright-Field Microscopy Adjustment

1. Turn on the light source power supply by pressing the rocker switch to the “I” position. Unit is located to the left of the microscope. See Figure 4 in Microscope Images for location.

2. Turn on the Dia-illuminator, by pressing the ON/OFF push button switch on the left side of the microscope. See Figure 3 in Microscope Images for location.

   Note that there are 2 constant voltage settings marked, this is to assist with color reproduction and image analysis.

3. Select Filter Sliders (see figure 1 for location). For optimal color reproduction Push in Filter ND4 & Filter NCB11

   - Fully push in to insert filter into optical path
   - Fully pull out to remove filter from optical path
   - Partially inserted will disrupt the optical path

   ND filter (Neutral Density)– These act like sunglasses for your microscope. Each ND filter reduces the light through the optical path by a specific percentage.

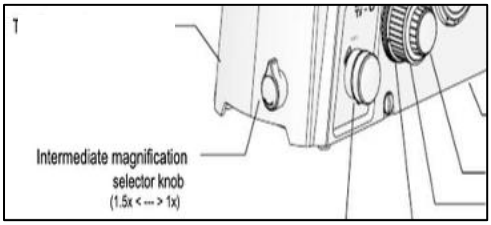
   For example, ND4 reduces the light by 75% allowing only ¼ of the light to pass the filter. This can reduce light intensity uniformly without altering color temperature, prevent specimen phototoxicity (especially in live-cell imaging) and manage exposure times (slower/faster) to help obtain the best image of your specimen.

   NCB11 filter (Neutral Color Balance)– Is essential for Bright-field microscopy. This filter provides color correction to the yellow hue of the light. In image acquisition, this ensures accurate color rendering in color photomicrography. This also alleviates eyestrain over time.

4. Check that the Optical Path Selection is switched to the EYE position

    - [EYE] – 100% Light to eyepieces
    - [L] – 100% light to camera
    - [L80] – 80% Light to camera 20% light to eyepieces
    - [R] – Empty port

5. Rotate the intermediate magnification selector dial on the front of the microscope to the "1x" position. See image below:



-Rotate Right will bring the 1.5x into position
-Rotate Left will bring the 1x into position

6. Bring the 10x objective into the light path

    Rotate the objectives by gripping the nosepiece. Rotating by gripping the objectives can cause cross-threading and lead to damage. See figure 2 in Microscope Images for location.

7. Bring the "A" setting into position on the condenser turret. See figure 1 in Microscope Images for location.

8. Place your sample on the microscope stage.

9. Bring your sample into focus using the coarse and fine focus knobs

    Remember that this unit is an inverted compound microscope, so instead of the stage moving up and down this means that the objective turret does.

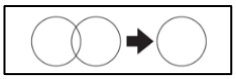
    Turn the **Course Focus knob** to raise the objective turret to its upper most limit (look at the microscope from the side while doing so to prevent damaging the optical components). The objective turret can come into contact with your specimen and damage the objective in place.

    Look into the eyepiece and lower the objective turret using the **Coarse Focus knob** until you can see your specimen.

Use the **Fine focus knob** to get image in acceptable focus. You may need to adjust Light Intensity**.**

- View the *microscope* from the side when you raise the objective turret – so you don't accidentally damage the objectives.
- View the specimen through the eyepiece when you raise the objective turret to focus.
- Never rotate the right and left focus knobs in opposite directions.

10. Adjust the interpupillary distance



When you have the specimen in focus, get the best image possible by looking into the eyepieces using both eyes. Adjust the binocular head such that the distance between your eyes allows the field of view from the left eye and the right eye to coincide. It helps to pretend you are looking into the distance.

11. Adjust the diopters

The **diopter adjustment ring** on each eyepiece can be adjusted to match the vision in each eye.

Follow these steps:

- Turn the nosepiece to bring the 40X objective in place and focus on your specimen (use the **Coarse focus knob** if needed then the **Fine focus knob**).

- Return to the 10X objective and look into the right eyepiece with your right eye.

  (To avoid using your left eye it is a good idea to cover it with a piece of paper *and* do not press on your eye as this changes the shape of your lens!).

  Focus on your specimen by turning the **right Diopter adjustment ring**.

- Look into the left eyepiece with your left eye (cover your right eye with a piece of paper). Focus on the sample by turning the **left Diopter adjustment ring**.

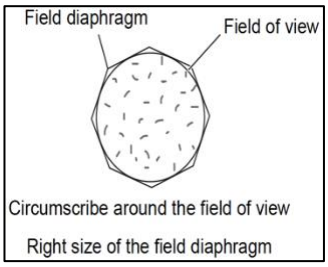
- Repeat the above steps until focus is perfect!

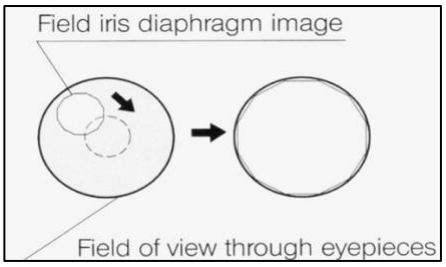
12. Fully focus and center the condenser

Köhler illumination acts to generate an even illumination of the sample to produce a sharper image and will increase contrast between areas of staining or between

regions of tissue in sections. This technique is particularly useful when utilizing DIC or Epifluorescence.

a. Rotate to close the field diaphragm. See figure 1 in Microscope Images for location.





You should see your specimen narrowed into an octagonal shape within the field of view through the eyepieces.

b. Adjust the focus onto the field diaphragm image by rotating the condenser focus knob. See figure 1 in Microscope Images for location.

At this point, the specimen in this view should be in focus. What you are looking to accomplish in this step is for the octagonal edges presented to become crisp and clear by moving the condenser up or down.

c. Center the field diaphragm image by utilizing the Condenser Centering Knobs. There are two, one on each side of the condenser. See Figure 1 in Microscope Images for location.

d. Adjust field diaphragm to bring these edges just outside your field of vision

13. Choose your objective Adjust the aperture Diaphragm. See figure 1 in Microscope Images for location.

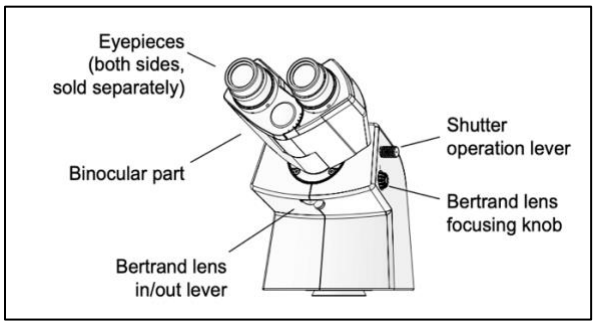
It is important that you adjust the Aperture diaphragm for each objective used as this affects resolution, contrast, focal depth and brightness of your image.

This is accomplished using the **Aperture diaphragm lever** on the top of the condenser (clockwise opens the diaphragm). The **Aperture diaphragm** setting will be dependent upon the Numerical Aperture (NA) of the Objective chosen.

Note: It is recommended that the proper size of the **Aperture diaphragm** is 70-80% of the Numerical Aperture of the objective. Thus, an objective with an NA of 0.75 should be set to a position indicated by 0.525-0.6.

A small **Aperture diaphragm** reduces resolution and brightness but increases depth of focus and contrast whereas a large **Aperture diaphragm** increases resolution and brightness but reduces depth of focus and contrast.

14. Adjust the Bertrand Lens. Bring the Bertrand Lens into the optical path by switching the Bertrand lens in/out lever from “O” position to “B” position.



15. Open the Shutter operation lever to bring the focal plane into view in the eyepieces

16. Use the Bertrand lens focusing knob (white rotating knob) to bring the octagonal shape into focus.

17. Once satisfied with the image, close the shutter lever and remove the Bertrand lens from the optical path “B” position to “O” position.

18. Adjust the brightness of the image.

19. Observe specimen

NAMC (Nikon Advanced Modulation Contrast) Adjustment

After setting up the microscope for Brightfield and focusing on your specimen, continue through the following steps:

1. Rotate the desired objective into the light path

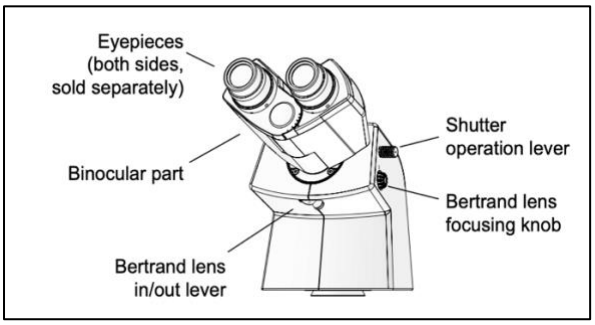
    a. 10x = NAMC1
    b. 20X = NAMC2
    c. 40X = NAMC3

2. Rotate condenser turret position into optical path that corresponds to the objective selected.

    a. NAMC 1
    b. NAMC 2
    c. NAMC 3

3. Fully open the Aperture Diaphragm so that the pathway is unobstructed

4. Rotate the Bertrand lens into the optical path by switching the lever under the eyepieces from “O “ to “B”



5. Move the shutter on the right-hand side of the microscope to bring the back focal plane into view

6. Rotate the Bertrand lens focusing knob (white knob) to bring focal plane (octagonal shape) into focus.

    This process is similar in nature to bringing the condenser into focus. What you are looking for here is the outer edges of the octagon shape to become crisp clear in-focus lines.

7. When finished, close the shutter and remove the Bertrand lens from the optical path Position “B” to Position “O”.

8. Rotate the NAMC collar of the objective to obtain the contrast you are looking for on your specimen.

   This collar acts as a “modulator” to control image brightness and contrast. It helps to grip the nosepiece of the objective turret with one hand and adjust the modulator with the other hand while viewing.

9. Rotate the 360-degree polarizer located above the condenser.

   This polarizer allows for the adjustment of the direction of shading contrast in 360 degrees.

10. View your specimen

Epi-Fluorescence Adjustment

*Safety precaution – fluorescence lighting presents a risk to your eyes. Looking directly at the light source can cause damage to your eyes. While utilizing fluorescence lighting, it is recommended to be positioned behind the orange shield and to not look directly at the light source outside of the microscope.

It is safe to look at fluorescence lighting through the instrument because it is designed with a system of filters and mirrors that block harmful excitation light from reaching the users eyes.

Photo-bleaching – fluorescence microscopy requires image setup and analysis quickly as photo-bleaching can occur if specimen is exposed for extended periods. Be mindful of this and remember to shutter the light source when not viewing/capturing images.

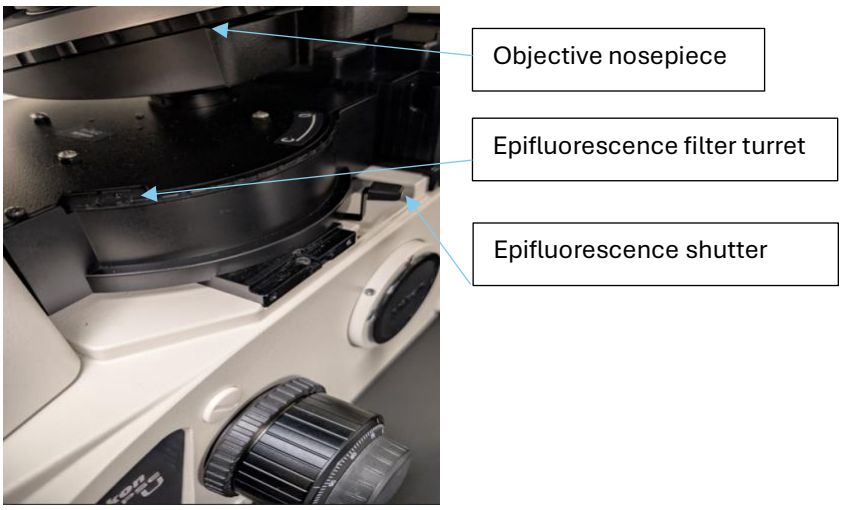
1. After setting up and focusing on your specimen in Brightfield, shutdown the bright-field light source.

   To do so, you will need to press the push button On/Off switch on the left-hand side of the microscope. (Figure 3)

   Then power off the power supply (Figure 4) rocker switch.

2. Verify the shutter for Epifluorescence is closed.
   a. C = Closed
   b. O = Open

   This lever is located on the right-hand side facing the microscope, below the epifluorescence filter turret



3. Rotate the fluorescence cube desired into the optical path by rotating the filter turret under the stage on the right-hand side

    - Position 1 – Empty
    - Position 2 – UV
        - Excitation wavelength: 375 nm
        - Emission wavelength: 460 nm
        - Beamsplitter: 415
    - Position 3 – B
        - Excitation wavelength: 480 nm
        - Emission wavelength: 535 nm
        - Beamsplitter: 505
    - Position 4 – G
        - Excitation Wavelength: 560 nm
        - Emission wavelength: 635 nm
        - Beamsplitter: 600
    - Position 5 – Empty
    - Position 6 - Empty

4. Check filter selection in optical path

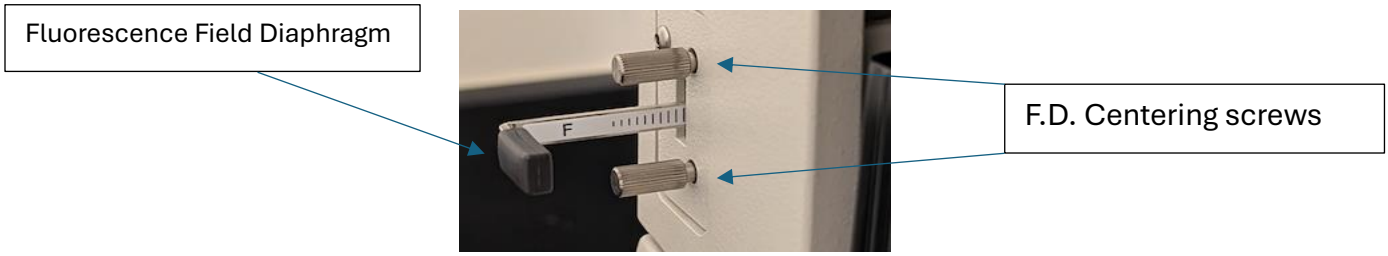
    a. ND4 (Neutral Density) cut light to ¼
    b. ND8 (Neutral Density) cut light to 1/8
    c. FILTER (Excitation Filter Slider) – NOTE that this instrument utilizes fluorescence filter cubes that have a large range – this will help with excitation. See technical specification page for additional information.

5. It is recommended for easier viewing to shut off the overhead lights and utilize the lower lights located under the upper cabinets in the room. This should reduce the interference of fluorescence with your specimen.

6. Open the shutter for epifluorescence. Located under the epifluorescence filter turret. Black lever on the right-hand side of the microscope.

7. Close the epifluorescence field diaphragm. Lever pictured below, located at the left-hand side of the microscope towards the wall.

    a. Fully pushed IN = Closed
    b. Fully pulled OUT = Open



8. Open the Aperture Diaphragm to allow more light onto the sample.

    a. Located on the epifluorescence arm (Figure 6)
    b. Fully pushed IN : Closed
    c. Fully pulled OUT: Open

9. Center the field of view by using the centering screws on the field diaphragm, then open the field of view (octagonal shape) just past the field of view

    a. This procedure is the same as in brightfield – for additional instruction on process see step 12 in Brightfield Adjustment.

10. Adjust the aperture diaphragm to correspond to the objective requirements

    a. This procedure is similar to brightfield adjustment step 13.

11. View Specimen

Remember to shutter the fluorescence light source when not needed to preserve fluorescence of specimen.

Image Acquisition

All Microscopes in the Advanced Microscope Suite operate on the same acquisition software: NIS Elements BR (Basic Research)

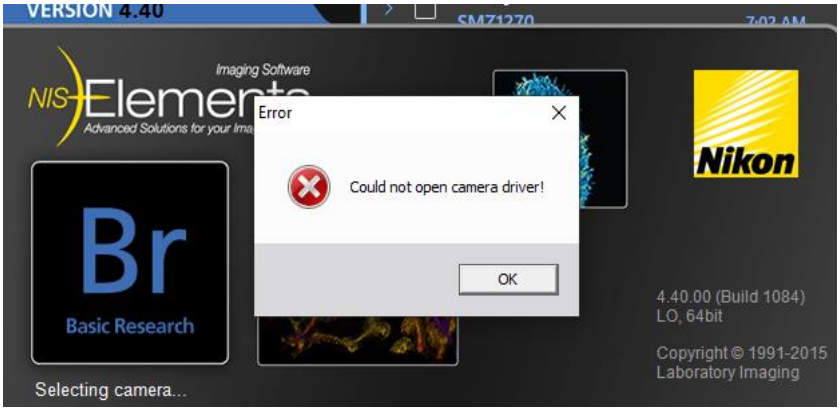
Acquisition Software:

NIS ELEMENTS - BR

Follow the following steps to acquire an image for analysis:

1. Turn on the computer
2. Power on camera (CoolSNAP MYO)
    a. Located on left port of microscope
    b. Power switch is at top of unit. Black push button switch (fan will initialize at startup)

    If you do not power on the camera before launching the software, the following error code will pop up.



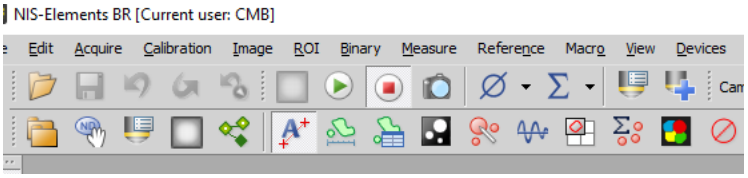
    If this pops up, close NIS-Elements and verify camera(s) are on and then relaunch NIS-Elements

3. Verify optical path is set to the [L] position on the optical path selector

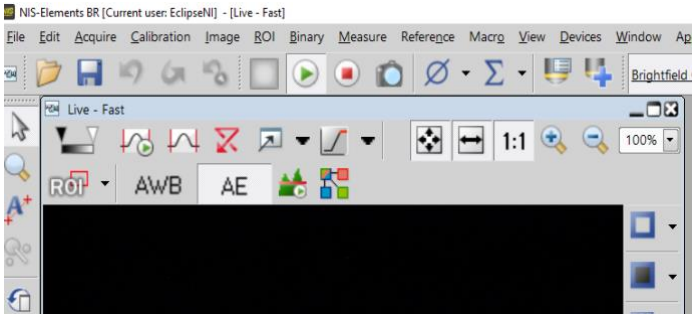
    - [EYE] – 100% Light to eyepieces
    - [L] – 100% light to camera
    - [L80] – 80% Light to camera 20% light to eyepieces

- [R] – Empty port

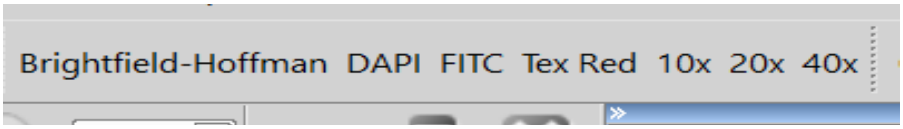
4. Open NIS-Elements Software (located on desktop)
5. Once software is initialized, to capture an image, you will select the Play  button located in the top tool bar.



1. This will populate an interior window within the NIS Elements software. The top left of that window will state "Live-Fast" to indicate active camera/live feed of your specimen.

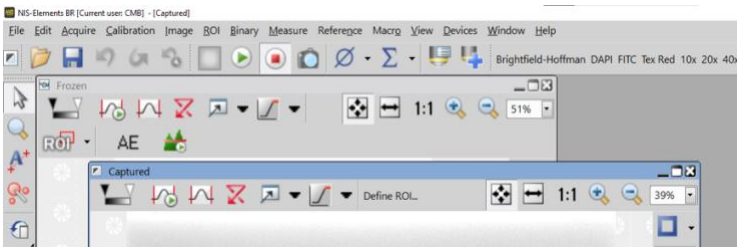


2. Verify settings prior to image capture. There are calibrated options provided at the top of the page for each objective and filter options. Correct selection will make image analysis later (i.e. measurements) easier.

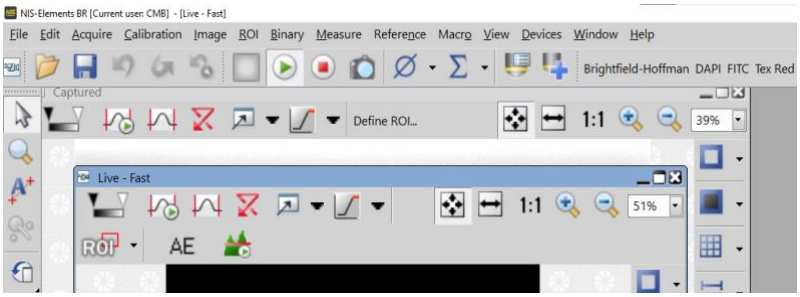


There are many options to improve image quality – consult the NIS-ELEMENTS guide located at the desktop of each computer for more detailed information.

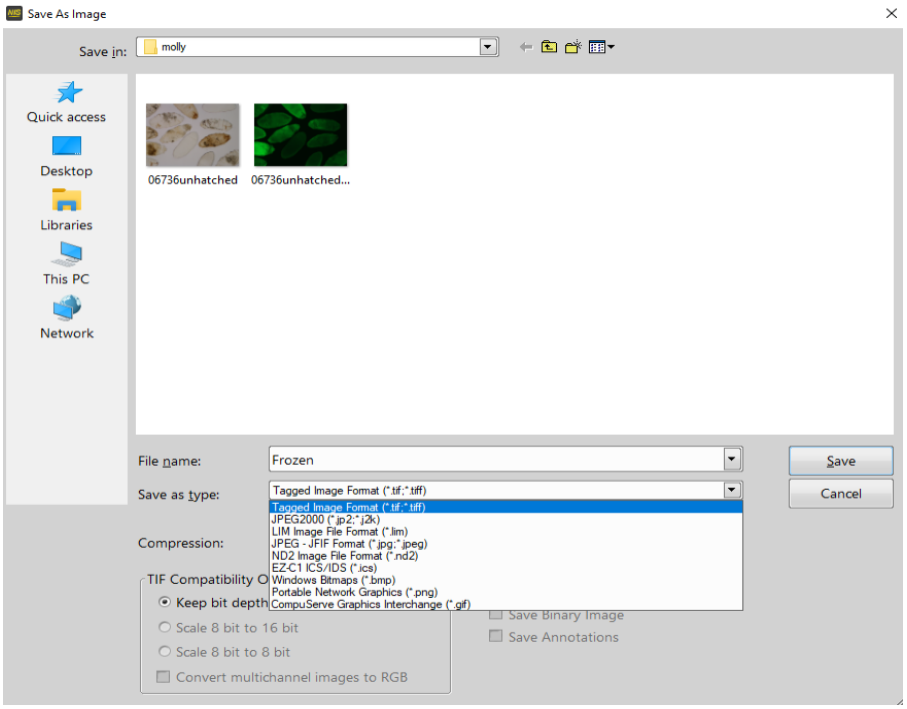
6. To capture an image from "Live-Fast" select the "capture" button  . This action will open a second interior window within NIS – Elements (as pictured below). Viewing the top left corner of each window:
    a. "Frozen" – this is the camera window, the stop icon  is highlighted.
    b. "Captured" – this is your image.



7. To return to your live view, press the play button. The two windows status will be as follows (pictured below):
    a. "Captured" – image captured
    b. "Live-fast" – live view of specimen



8. Once you've captured your image and you want to save select the  or go to File =>Save As



Remember this is a shared resource, so be mindful of where/how you store your data so you can easily find your data again later. There are multiple login options, make sure you know which profile you are logged in under.

Also, note the formatting you save your image as - .TIFF and .JPEG are popular file formatting options for later annotation.

9. Additional annotation/measurements and image capture instructions can be found within the NIS-Elements user guide. A copy can be found on the desktop of any computer in the Advanced Microscope Suite.

Ending your Microscopy Session

It is a courtesy to all to return settings to our "DEFAULT SETTING" to allow the next user to efficiently and easily examine their specimens. It is also setup to prevent any accidental damage to the microscopes, so please follow these instructions.

1. Remove specimen and clean work area if required
    - Use Ethanol or IPA to disinfect
    - Sparkle to clean any spills/ non-hazardous
2. Turn off accessory equipment (camera & computer) if no longer in use.
3. Set the optical path switching knob to "EYE" so that 100% of light is directed to the eyepieces.
4. The microscope stage should be in its most centered position
5. Rotate epifluorescence turret to position 1 (or any empty position)
6. Remove epifluorescence filters
    - Pull out ND filters
    - Pull out Sliding filter
7. Power OFF epifluorescence light source (SOLA Light Engine) located to the left of the microscope.
    - Power switch at front should be OFF (Power LED will go dark)
    - Power switch at rear of power source needs to always be left ON (runs fan even after light is off)
8. Remove unnecessary brightfield/NAMC filters
    - Recommended to leave NCB and D filters in
    - Pull OUT GIF and ND filters completely from light path
9. Return the Condenser turret to Brightfield setting – Position A
10. Return the lowest objective to the optical path (10x)
11. Return the diopter adjustment rings to their reference position (zero position)
12. Turn the Dia-illumination brightness control knob to the lowest illumination.
13. Power off the Dia-illumination to the microscope
    - via the push button located on the left-hand side of the base of the microscope.
14. Turn off Dia-illumination power source
    - Via the rocker switch located on the power source to the left of the microscope.
15. Cover microscope

Technical Specifications

- Microscope Make: Nikon
- Microscope Model: Eclipse Ti-U
- Objectives
    - 10x
        - 10x/0.25 ∞/1.2 WD 6.2 NAMC 1
    - 20x
        - S Plan Fluor (with clamp) ELWD 20x/0.45 NAMC 2
        - ∞/0-2 WD 8.2 -6.9 OFN22
        - Correction collar 0-2
    - 40x
        - S Plan Fluor (with clamp) ELWD 40x/0.60 NAMC 3
        - ∞/0-2 WD 3.6-2.8 OFN22
        - Correction collar 0-2
- Condenser 0.4 NAMC
    - Condenser turret positions
        - A – Brightfield
        - NAMC 1
        - NAMC 2
        - NAMC 3
- Eyepieces
- Bertrand Lens
- Intermediate selector knob
    - 1x
    - 1.5x
- Camera
    - Monochromatic
    - Make: Photometrics CoolSNAP
    - Model: MYO
    - 1940 x 1460 imaging array
    - 4.54 x 4.54 μm pixels
    - 20 MHz read out – high speed readout to maximize temporal resolution
    - Thermoelectric cooling
    - Fan disable option for vibration-sensitive applications
- Light source -Bright field
    - Make: Nikon
    - Model: TI-PS100W/A
- Light source – Epifluorescence
    - Sola Illuminator Light Engine
    - Lumencor

- Bright-field/ NAMC filters
    - ND : Neutral Density
    - NCB: Neutral Color
    - D: Diffuser Filter
    - GIF: Green Interference Filter

- Epi-fluorescence filters
    - ND4
    - ND8
    - FILTER – Excitation Filter Slider

- Epifluorescence cubes: Chroma's AT filter sets create 50% greater signal intensity than spectrally similar soft-coated filters, sputtered coatings, AT Bandpass sets
    - Position 1 – Empty
    - Position 2 – UV (39000 Fluorochrome: DAPI/Hoechst/AlexaFluor 350 and similar)
        - Excitation wavelength: 375 nm
        - Emission wavelength: 460 nm
        - Beamsplitter: 415
    - Position 3 – B (39002 Fluorochrome: GFP/FITC/Cy2/AlexaFluor 488 and similar)
        - Excitation wavelength: 480 nm
        - Emission wavelength: 535 nm
        - Beamsplitter: 505
    - Position 4 – G (39010 Fluorochrome: Texas Red/mCherry/AlexaFluor594 and similar)
        - Excitation Wavelength: 560 nm
        - Emission wavelength: 635 nm
        - Beamsplitter: 600
    - Position 5 – Empty
    - Position 6 - Empty

- Software: NIS Elements
    - Manual available on desktop of units for image acquisition tutorials.