Tips for Proper Microscope Use:

1. Clean the objective **before** and after you use it.

2. For a given slide, clean the oil off the coverslip before switching from an oil to an air objective.

3. When using an oil objective and switching to a new slide:
   
   A. Press and hold the ‘Escape’ button until you hear a ‘beep’ to escape the objective.
   B. Remove the current slide.
   C. Add the new slide. (Add more oil to the objective if needed)
   D. Press and hold ‘refocus’ button until you hear a ‘beep’ to refocus the objective.

4. If twisting the focus knob doesn’t move the objective up and the microscope ‘beeps,’ then you can hold the ‘refocus’ button while moving the knob to override this safety. **Only do this if you know it is safe to move the sample closer to the stage!**

5. If handling DIC sliders, please wear gloves.

6. Always be aware of the position of the objective relative to the stage and the sample. Bumping the objective into the stage can instantly cause major damage to the objective or stage. The objective can also break samples in half.

7. Do not save data anywhere on the C: drive, as it will be immediately deleted.

Thank you for following these guidelines! If you are ever unsure what to do, please ask any Microscopy Core staff for help any time!
Quick start guide for Widefield Fluorescence Imaging using AxioVision software

1. **If device is off:** Power on the device by turning on the orange labeled switches in ascending order and log in with your level 1 password. **Otherwise,** just log in with your level 1 password.

2. Click on the AxioVision software.

3. The halogen light turns on with device start up. Turn it off by pressing the ‘HAL’ button.


5. Manually move the objective down until you hear a ‘beep.’

6. Choose the objective using the software (see *Tips for Choosing the Right Objective* on pg. 6).

7. Clean the objective(s) you will use. (see *Cleaning the Objectives* on pg. 7).

8. Slide your sample all-the-way back, coverslip up, underneath the metal holders on the stage.

9. Move the sample directly below the objective using the stage joystick.
10. If using the oil immersion objective, add immersion droplet onto the sample coverslip.

11. Adjust the Xenon lamp power by putting the wheel to the first click up from the lowest position.

12. Choose the filter cube. Use the color that is easiest to see with your eye to focus on the sample.

13. Push the ‘eyepiece lever’ to the eyepiece position.

14. With the room light off, carefully focus on your sample. (see *Tips for Safely and Quickly Focusing on your Sample* on pg. 8).

15. Close the fluorescence shutter and push the ‘eyepiece lever’ to camera position.

16. Turn on the live imaging with AxioCam open by pressing the ‘live’ and ‘AxioCam’ buttons on the left of the AxioVision window. Move the AxioCam window to the rightmost side of the 1st monitor.
17. OPTIONAL: If you are doing near infrared (NIR) detection, turn on ‘NIR mode’ in the ‘General’ tab in the AxioCam window.

18. Open the fluorescence shutter (see step 15) and focus the image on the camera. You may need to adjust the exposure in the AxioCam window to reduce pixel saturation.

19. Close the fluorescence shutter to avoid sample photobleaching (see step 15).

20. In the workspace area, click on the plus sign next to ‘Multidimensional acquisition.’

21. Click on the lower ‘Multidimensional acquisition.’

22. Click on the ‘C’ tab.

23. Click on ‘Extended Parameters.’

24. In the newly opened Extended Parameters window, choose ‘Current Focus Position’ for every channel by clicking the dropdown arrow, **even** for the channels you aren’t using.

   **Important:** Failure to do this step can result in sample damage.
25. Narrow the width of the DAPI and FITC columns, and move the ‘Extended Parameters’ window to the other monitor.

26. Uncheck the channels you are not using.

27. Make sure the histogram in AxioCam ‘Adjust’ tab is in ‘log’ mode by clicking the ‘log’ button.

28. Increase the Xenon lamp brightness wheel to maximum (see step 10).

29. Adjust the exposure for each channel by pressing the ‘…’ button in the ‘exposure’ row for each column in the Extended Parameters window.

   **Note:** For standard imaging and tiling, the brightest pixel should be 50% saturated. For Optical Sectioning, the brightest pixel should be 90-95% saturated.

30. **OPTIONAL:** If Channel specific focus is needed, adjust the channel specific focus for some channels by clicking the ‘…’ next in the ‘Focusing’ row for each column.

31. **OPTIONAL:** Setup tiling or z-stacking, if appropriate (see [Large Image Acquisition](#) on pg. 9 or [Z-stack acquisition](#) on pg. 12).

32. Click ‘Start’ in the Extended Parameters window to begin collecting an image.

33. Save any images in the default zvi format. Large images can be stitched and registered (see [Z-stack acquisition](#) on pg. 12).

34. Make sure to clean the objective, log out, and manually lower the stage when you are done.
Tips for Choosing the Right Objective

There are two main properties of your sample used to determine the best objective for your purpose. Match the properties of your sample with the corresponding objective properties (see ).

Table 1: Corresponding Properties of Samples and Objectives

<table>
<thead>
<tr>
<th>Sample Properties</th>
<th>Objective Properties</th>
</tr>
</thead>
<tbody>
<tr>
<td>The refractive index (RI) of your mounting media</td>
<td>The RI of the immersion droplet</td>
</tr>
<tr>
<td>The depth you need to image</td>
<td>The working distance of the objective</td>
</tr>
<tr>
<td>The size of the field and level of detail you need</td>
<td>The magnification of the objective</td>
</tr>
</tbody>
</table>

Match Refractive Index of Mounting Media and Immersion Media

1. Check the RI of the solution between the coverslip and the glass slide.
   
   Example: This can be water or buffer, glycerol, or a commercially available mount like ProLong Gold. This is called the ‘mounting media.’

2. Use an objective for which the immersion media’s refractive index matches the mounting media’s.
   
   Note: It is always good practice to match RI of the mounting media and the immersion media, but RI matching is more important the larger the objective magnification and the deeper you are imaging. Therefore, the air objectives are sufficient for low magnification (≤ 20x) imaging and the 40x air is sufficient for some thin samples.

Choose the Right Working distance (WD) for the Depth You Need

3. WD is how far you can, in principal, image into your tissue, before the objective hits the glass coverslip. Choose an objective with a WD that will reach your target regions.
   
   Note: Light scatter and aberrations may make image quality very poor before the working distance is reached. If this occurs, consider tissue clearing protocols.

Choose the Right Magnification for the Appropriate Detail and Field of View

Higher magnification allows for the resolving of smaller features, and low magnification allows for larger fields of view.

4. Choose the objective with the magnification that gives the best balance between detail and field of view.
   
   Note: Low magnification objectives speed up tiling quite a bit due to large fields. But be conscious of the trade off with brightness and detail with field of view.
Cleaning the Objectives

This is a broad overview of best practices while cleaning objectives on upright microscopes. Finger oil or Nikon oil left on the objective can lead to poor image quality for you or any user following you.

1. Keep the lens paper sheets in the book clean from finger oil and dust:
   - **Example:** Use the center of a sheet for cleaning, and the outer edges for holding.

2. Hold the paper just under the objective.
   - **Note:** Make sure the stage is down low enough to give room to clean the objective.

3. Using an unused, clean spot, press the paper firmly to the center of the objective lens and scrub in a spiral. Look at the paper after a spiral scrub and note the amount of oil on the spot.
   - **Note:** This pushes grime outward, while never allowing it to retreat back to the center.

4. Repeat steps 1-3 until two successive spots are dry.

5. If oil persists after 6 or more spots, then:
   - A. Put gloves on.
   - B. Apply some ethanol on the spot on the lens paper you will use to scrub the objective.
   - C. Follow steps 1-2 with a new, unused lens paper sheet.
   - D. Follow step 3.
   - E. Clean once more with a dry, unused spot on the lens paper.

6. If oil still persists after 6 or more spots, then contact a core member for help.
Tips for Safely and Quickly Focusing on your Sample

1. **Manually** lower the objective at least a few centimeters from the objective.
2. Put your sample on the stage, coverslip up (see [Quick start guide for Widefield Fluorescence Imaging using AxioVision software](#) on pg. 2 step 8)
3. If using an oil objective, add Zeiss oil.
4. Use the stage control joystick to position the sample directly below the objective.
   
   **Note:** You can use the light from the Xenon bulb or the Halogen light to aim the objective.
5. If using an immersion: move the stage up until the immersion droplet touches the coverslip.
6. While the microscope is in eyepiece mode and the room is light off, look through the eye-piece and turn on the fluorescence light source on and open the fluorescence shutter.
7. Move the objective with the focus knob at moderate speed (~1 turn for ~5 s) with the coarse knob until the sample is in focus.
Large Image Acquisition

This is for taking a large image of a whole tissue slice by taking sequential pictures with different stage positions to reconstruct into a larger image.

1. First follow steps 1-31 of the *Quick start guide for Widefield Fluorescence Imaging using AxioVision software* on pg. 2.
2. Select the ‘Mosaic’ tab under the workspace.
3. Check the ‘Mosaic’ checkbox.
4. In the ‘Mosaic’ tab, select ‘Rectangle’ for acquisition mode.
5. Under ‘Region,’ type ‘1’ for columns and rows, ‘10’ for Overlap, and select ‘Rectangle.’
6. Click ‘Setup.’

![Setup button]

7. On the toolbar on the bottom of the screen, remove the current saved fields by clicking the red ‘X’.

![Toolbar with 'X' highlighted]

Note: The current saved field is a blue-bordered box, and the current position of the stage is marked by a green crosshair.

8. Open the Fluorescence Shutter in the color you want to scan the sample with. You may need to adjust the exposure or the Xenon lamp power wheel.

![Fluorescence Shutter]

Note: To get the whole sample in the large image, the easiest way is to add the leftmost, rightmost, lowest, and topmost regions into the large image, and the computer will include the whole sample in the large image. The next few steps walks you through this process.

9. Look for the leftmost region of your sample you wish to be in the large image using the joystick.

![Joystick]

10. Add this region to your large image using the ‘Add’ button on the toolbar at the bottom of the screen.

![Toolbar with 'Add' button highlighted]

11. Repeat steps 9 and 10 for the rightmost region, topmost region, and lowest region in any order.

12. When done, your large image should look like a square, blue grid of fields-of-view.
13. Select ‘ok’ at the bottom of the screen to save this tiled region.

14. Click ‘Start’ in the Extended Parameters window to begin collecting an image.

15. Remember to save images in the default file format, .zvi.

16. Make sure to clean the objective, log out, and manually lower the stage when you are done.
**Z-stack acquisition**

This section discusses how to use the Apotome for sectioning and setting up a Z-stack. You can use the Apotome’s optical sectioning without Z-stacking. You can Z-stack without the optical sectioning, but is not usually recommended.

**Note:** Make sure to tell Core staff which objectives and colors you’ll need for optical sectioning, since calibration may have to be done prior to you using it. A Core staff member will make sure it is done before you arrive.

1. First follow steps 1-31 of the *Quick start guide for Widefield Fluorescence Imaging using AxioVision software* on pg. 2.

2. If setting up the Apotome, continue with step 3. If setting up Z-stacking, skip to step 14.

**Setting up the Apotome**

3. Push in the Apotome to ‘second click,’ which is just putting it all the way in. You will hear a beep.

**Note:** Sometimes the fluorescence turns on right away. Close the fluorescence shutter whenever you are not immediately using it to save your sample from photobleaching.

4. Make sure the correct grid is in place in Acquisition->ApoTome->Grid. HL is for the 40x, 63x, or 100x. The PL is for the other objectives.
5. You may want to check the slice thickness with the current grid/objective/color settings in Acquisition->ApoTome->Depth Info.

![ApoTome Depth Info](image)

6. Click ‘Apotome’ in the workspace area.

![Workspace](image)

7. Select the ‘Settings’ tab under the workspace.

![Settings](image)

8. Choose if you want the grid visible or optical sectioning during live capture.

![Live Mode](image)

9. Choose whether to take snapshots without processing (grid visible) or with optical sectioning.

![Acquisition Mode](image)
10. If ready to take images, continue with steps 11-13. If setting up a Z-stack, skip to step 14.

11. Click ‘Start’ in the Extended Parameters window to begin collecting an image.

12. Remember to save images in the default file format, .zvi.

13. Make sure to clean the objective, log out, and manually lower the stage when you are done.

**Setting up a Z-stack.**

14. Select the ‘Z’ tab under the workspace.

15. Check the ‘Z-stack’ checkbox.

16. In the Z-stack options, select the number of slices and slice distance. The slice distance should be a third of the Depth in the Depth info window (see step 5).

17. Choose ‘center’ or ‘Start/Stop’ mode.

**Note:** ‘Center’ does a Z-stack with a total height selected with the current focus as the center of the stack. ‘Start/Stop’ does a stack that starts at one height and ends at another. I recommend ‘Start/Stop’ for most applications.
18. If using center mode, just specify a center focus height by clicking the ‘…’ button. Change the focus and click ‘OK’ when done. Skip to step 21.

19. If using ‘Start/Stop’ mode, select the start height by clicking the ‘…’ button next to ‘Start.’ Change the focus and click ‘OK’ when done.

20. Select the stop height by clicking the ‘…’ button next to ‘Stop.’ Change the focus and click ‘OK’ when done.

21. Click ‘Start’ in the Extended Parameters window to begin collecting an image.

22. Remember to save images in the default file format, .zvi.

Make sure to clean the objective, log out, and manually lower the stage when you are done.