	SKILLS CENTER **STANDARD OPERATING PROCEDURE**	**A BIOFIZZ** **PRODUCTON**
Introduction to Fluorescence Microscopy **Module Hours: 3.0**	**Effective Date: 02/24** **PRQs:** - **Skills Center Safety** - **Light Microscopy (BF)**	**Revision 1.0** **Author: Beiyi Xu** **Checked by Editor: M. Stowell**

Background

Fluorescence microscopy is a widely used imaging technique that allows researchers to visualize specific cellular structures with high contrast and molecular specificity. Unlike brightfield microscopy, which relies on light absorption, fluorescence microscopy takes advantage of fluorophores, which are molecules that absorb light at a specific wavelength and emit it at a longer wavelength. This technique enables precise tracking of cellular components, making it essential in cell biology, neuroscience, and pathology.

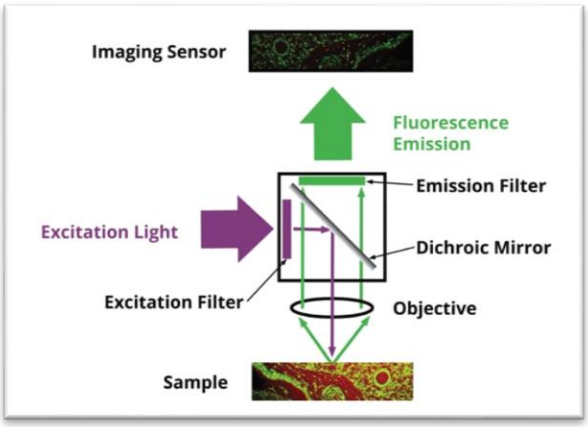

Figure 1. An illustration of how the filter cube functions to excite a sample and detect the emitted fluorescence. (DOVERMOTION, n.d)

The Nikon Eclipse TE200, an inverted widefield fluorescence microscope, is used in this experiment to visualize biological specimens labeled with three distinct fluorophores. These fluorophores selectively bind to cellular structures, allowing targeted visualization through fluorescence emission. The excitation-emission process is governed by Stokes' shift, where an excited fluorophore absorbs light at a specific wavelength and then emits it at a longer wavelength. Excitation is necessary because fluorophores must first absorb energy to reach an excited state before they can relax and emit fluorescence, making the process highly dependent on selecting the appropriate excitation wavelength. To selectively detect this emitted fluorescence, the microscope employs fluorescence filter cubes, which contain three critical optical elements: an excitation filter, a dichroic mirror, and an emission filter as seen in **Figure 1**. The excitation filter ensures that only the desired excitation wavelength reaches the specimen. The dichroic mirror reflects this excitation light toward the sample while allowing longer-wavelength emitted fluorescence to pass through. Finally, the emission filter blocks any residual excitation light, ensuring that only the emitted fluorescence reaches the detector.

This SOP involves imaging different FluoCells™ Prepared Slides. Each fluorophore will be imaged separately using the appropriate filter cube.

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Powered by SPOT 5.1, a digital camera is used to capture fluorescence images. This software requires careful optimization of camera settings to achieve the best picture. Once images are captured, FIJI (ImageJ) will be used for image processing, including contrast adjustments, background subtraction, channel merging, and analysis to create a single capture that contains all three fluorophores.

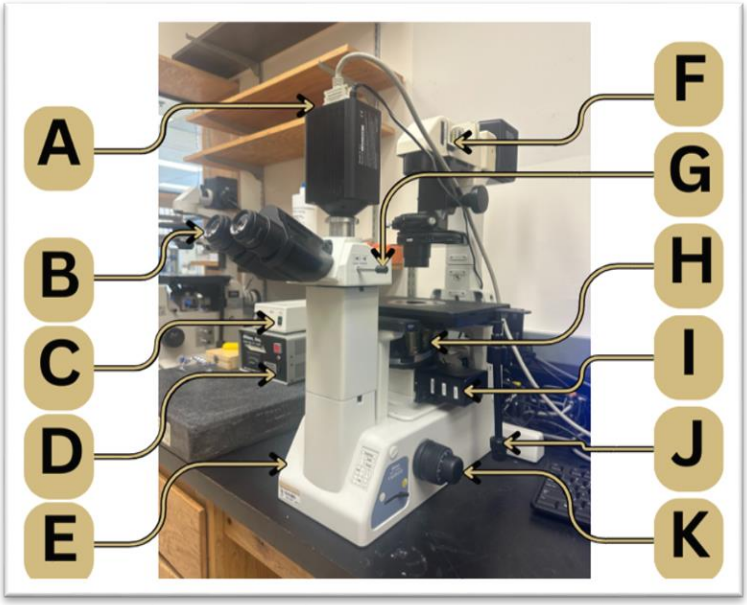
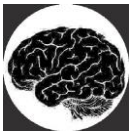


Figure 2. Nikon Eclipse TE-200 Microscope. (A) labels the camera. (B) labels the eyepiece. (C) labels the on/off for brightfield lamp. (D) labels the on/off for UV fluorescence lamp. (E) labels the on/off for brightfield. (F) labels the on/off for brightfield illumination. (G) labels the switch for eyepiece/camera viewing. (H) labels the objective lens carousel. (I) labels the fluorescence filter selector. (J) labels the stage controls. (K) labels the focus knob.

While fluorescence microscopy provides excellent molecular specificity, it has limitations. The diffraction limit restricts resolution to ~200 nm, preventing visualization of structures below this scale. Photobleaching, the irreversible degradation of fluorophores due to prolonged exposure to excitation light, can reduce fluorescence intensity over time. Autofluorescence, the natural emission from biological samples, can interfere with signal detection but can be minimized using appropriate filters and sample preparation.

In summary, fluorescence microscopy is a powerful imaging technique that enables the visualization of specific cellular structures with high contrast and molecular specificity. Understanding the principles of excitation-emission, filter cube selection, and image acquisition ensures accurate fluorescence detection and high-quality imaging. By learning to operate the Nikon Eclipse TE200, optimize settings on the digital camera, and process images using FIJI, students will gain hands-on experience in fluorescence microscopy. Mastering these techniques will enhance your ability to conduct precise and reproducible imaging in biomedical and life sciences research (日本語で読む, n.d.).

	SKILLS CENTER **STANDARD OPERATING PROCEDURE**	**A BIOFIZZ**  **PRODUCTON**
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1. **Purpose**
   1.1. This SOP guides users in preparing, imaging, and analyzing fluorescently labeled biological samples using the Nikon Eclipse TE200 microscope and SPOT 5.1 software, with FIJI for post-processing.
2. **Scope:**
   2.1. This procedure applies to research and educational settings where fluorescence microscopy is used to study cellular structures through multi-color fluorescent staining.
3. **Responsibility**
   3.1. It is the responsibility of the user to understand and perform the procedure described in this document.
   3.2. It is the responsibility of the user performing the procedure to fully document any deviations from the written procedure.
   3.3. It is the responsibility of the user to become trained on the procedure.
4. **Definitions**
   4.1. Fluorophore: A molecule that absorbs light at a specific wavelength and emits it at a longer wavelength.
   4.2. Excitation/Emission Spectrum: The range of wavelengths at which a fluorophore absorbs and emits light.
   4.3. Stokes Shift: The difference between the excitation and emission wavelengths of a fluorophore.
   4.4. Photobleaching: The irreversible loss of fluorescence due to prolonged light exposure.:
   4.5. Multi-Channel Imaging: Capturing images at different wavelengths to visualize multiple fluorophores in a single sample.
   4.6. Image Stacking: The process of overlaying multi-channel images into a composite image to represent different stains in a sample.
5. **Materials/Equipment**
   5.1. Nikon Eclipse TE200 with halogen and fluorescent lamp
   5.2. Invitrogen FluoCells™ Prepared Microscope Slides (In 4°C or -20°C fridge) (Invitrogen, 2025)
   5.3. SPOT compatible camera
   5.4. Computer with SPOT 5.1 software
   5.5. Kimwipes
   5.6. Gloves

5.7. FIJI

6. Procedure

6.1. Confirm the fluorescence lamp is ventilated, darken the workspace for optimal imaging, and **never look directly into the light source** to avoid eye damage.

6.2. Ensure the microscope is clean and free of dust. Use a Kimwipe and lens cleaning solution if necessary to wipe the objectives.

6.3. Setup the microscope (Use Figure 2 for guidance)

6.3.1. Turn on the **brightfield halogen lamp** if brightfield imaging is required.

6.3.2. Turn on the **fluorescence lamp** for fluorescence imaging.

6.3.3. Switch on the **microscope main power**. The halogen lamp should illuminate.

6.3.4. Power on the **SPOT camera** and check that it is connected to the computer.

6.3.5. Log into the computer using the credentials provided under the monitor.

6.3.6. Open SPOT 5.1 Software to initialize the camera.

6.3.7. Navigate to **Camera > Initialize** to start the camera.

a. For a live view, go to **Camera > Display Live Images**.

b. To capture a single image, select **Camera > Single Image**.

i. Adjust image settings via **Setup > Image Settings**.

ii. Under Appearance, adjust **gamma** (recommended range: 0.45-1 for fluorescence and 0.8-1.2 for brightfield). Leave Correct Colors off.

iii. Under Exposure, select the appropriate image type (recommended: Brightfield - Transmitted Light or Fluorescence Microscopy).

1. Adjust brightness to the highest level possible while avoiding photobleaching and phototoxicity (Nikon's MicroscopyU, n.d.).
2. Modify gain to optimize image acquisition:
    a. Increase gain to aid live image focusing for speed.
    b. Decrease gain for single images to improve quality.

iv. After changing exposure settings, press **Recompute Exposure** to apply changes in real-time.

6.4. Place the FluoCells™ Slide from the 4°C or -20°C fridge on the stage.

6.5. Focus the sample in **brightfield mode** with the live camera while ensuring the **empty filter cube** is selected.

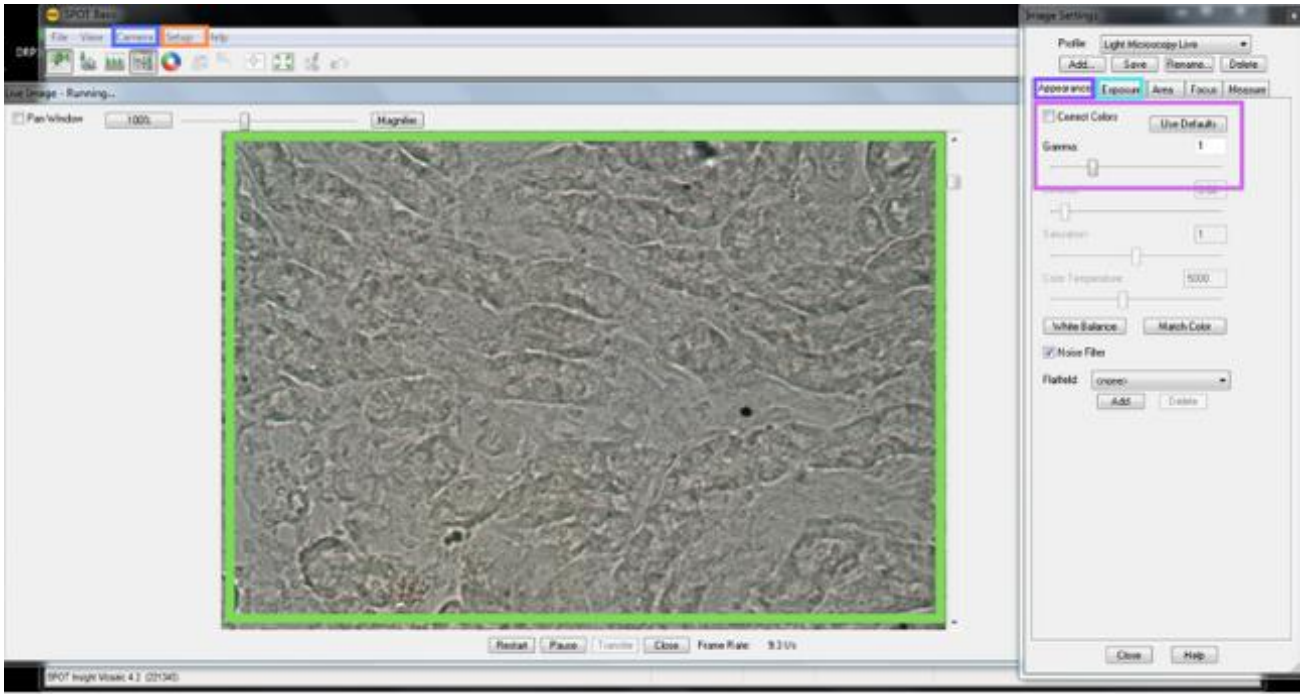

Figure 3. Mouse kidney cells are focused on brightfield mode at 20x. Camera options tab. Setup > Image Settings. Image Settings Apperance tab. Image Settings Exposure tab. The Gamma is set to 1 and correct colors is off.

6.7. Find the excitation and emission wavelengths of each filter cube.
  6.7.1. Choose the correct filter set for each fluorophore.
  6.7.2. *Important:* Using an incorrect filter will result in loss of signal or improper image acquisition.
6.8. Using **Live Camera Mode**, adjust **focus and exposure** in fluorescence mode.
6.9. Capture single images of each stain at different magnifications required.

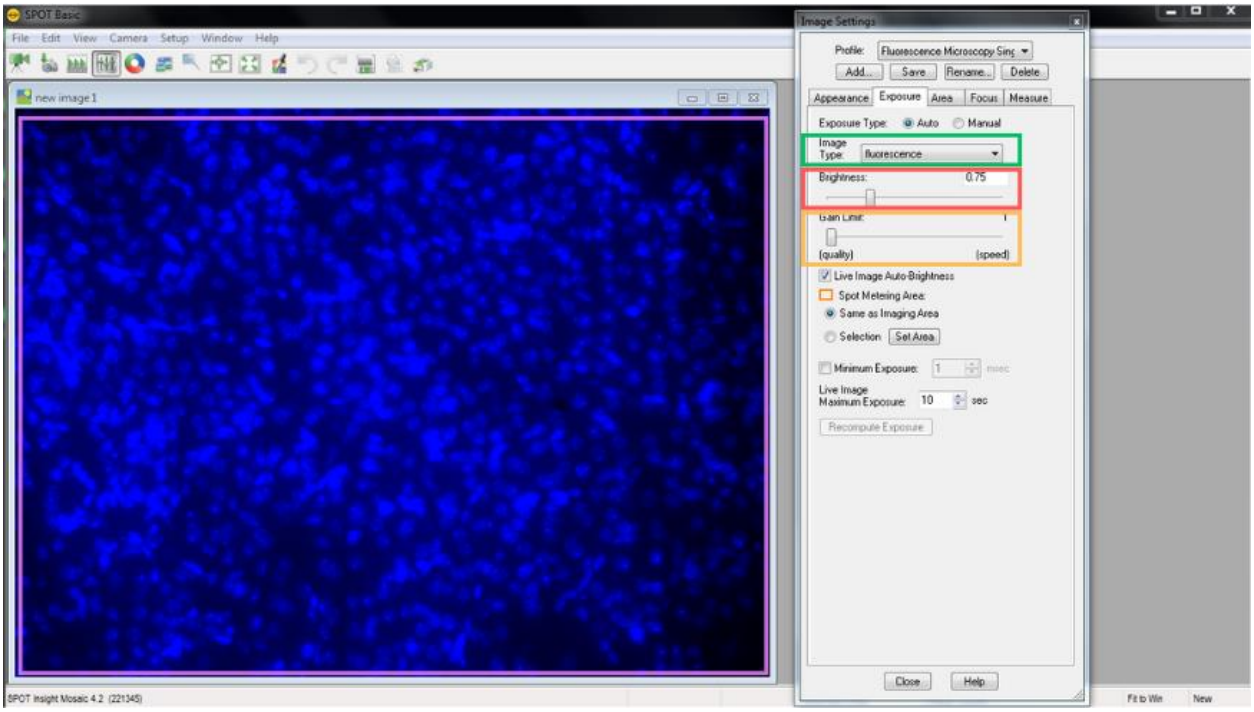

Figure 4. A single image of Mouse Kidney Cells Captured using the DAPI filter at 20x. Under the exposure tab, the image type is set to fluorescence, brightness is set to 0.75, and gain limit is set to 1.

6.10. Save each image in **TIFF format** for further processing (**File > Save Image**).

6.10.1. To export these images from the computer, you will need a USB drive to export the images to, then transfer them locally to your own device.

6.11. Turn off the microscope

6.11.1. Turn off the fluorescence lamp to prevent unnecessary photobleaching.

6.11.2. Power down the microscope, halogen lamp, and SPOT camera.

6.11.3. Log out and shut down the computer.

6.11.4. Store FluoCells™ back in the 4°C or 20°C fridge.

6.11.5. Ensure the workspace is left clean and all materials are returned to their original state.

6.12. Analyze the images in FIJI.

6.12.1. Open each TIFF image (**File > Open**).
6.12.2. Convert to grayscale (**Image > Type > 8-bit**).
6.12.3. Merge channels (**Image > Color > Merge Channels**), selecting which TIFF corresponds to blue, green, and red.
6.12.4. Adjust brightness/contrast if needed and save the final composite image (**Image > Adjust > Brightness/Contrast**).

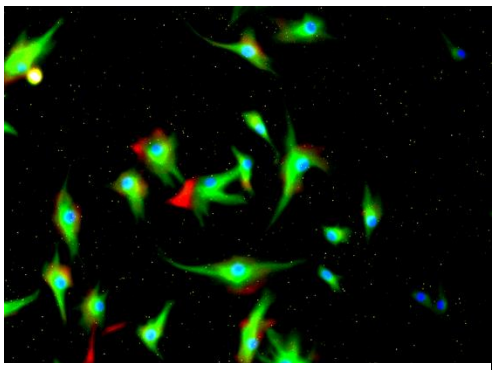

Figure 5. Edited composite of fluorescent BPAE cells at 20x after FIJI processing.

7. Troubleshooting

7.1. If the fluorescence signal is weak or not visible, check that the correct filter cube is selected, and that the fluorescence lamp is on.
7.2. If images appear blurry, ensure the sample is in focus in brightfield mode before switching to fluorescence mode.
7.3. If overexposure occurs, reduce the brightness and gain settings in the Exposure tab.
7.4. If the camera is not displaying an image, confirm that the SPOT camera is powered on and properly connected to the computer.
7.5. If TIFF images do not open in FIJI, verify the file format and try converting to 8-bit grayscale before merging channels.

8. References

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9. Module Methods Task

9.1. Explain the difference between **excitation and emission wavelengths**.
9.2. Describe why **specific filter sets** are necessary for each fluorophore. Discuss the consequences of using the **wrong filter** for a fluorophore.
9.3. Define **photobleaching** and describe strategies to minimize it.
9.4. Research the DAPI-50LP-A-000 Filter, 31004 Texas Red® / Cy3.5™, and 41001 FITC/ EGFP/ Bodipy Fl/ Fluo3/DiOComplete filters. Research the fluorophores DAPI, Alexa Fluor™ 488, and Alexa Fluor™ 568. Write in the table below to identify which filter cube excites which dye.

Fluorophore	**Excitation Wavelength (nm)**	**Emission Wavelength (nm)**	**Filter Cube Used**
DAPI			
Alexa Fluor™ 488			
Alexa Fluor™ 568			

9.5. Capture images of the FluoCells™ Prepared Slide #3 (Mouse kidney: Alexa Fluor™ 488 WGA, Alexa Fluor™ 568 Phalloidin, DAPI) at **10x and 40x**, ensuring all three fluorophores are visible.
  9.5.1. Save images in **TIFF format** for further processing.
9.6. Open and process images in **FIJI** following the outlined procedure.
  9.6.1. Merge the **blue, green, and red channels** into a composite image.
  9.6.2. Adjust **brightness/contrast** as necessary.

9.6.3. Include one composite the 10x and one composite 40x image in your MMT. Label the figure.

9.7. What does each fluorescent dye label, and do the observed results match expectations?

9.8. What unexpected variations, if any, were observed? What were the benefits of higher magnification? Were there any limitations of higher magnification?