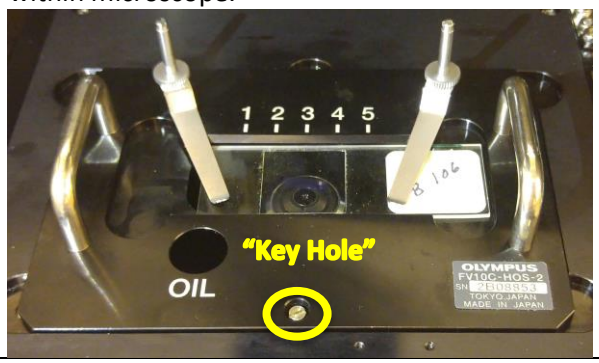
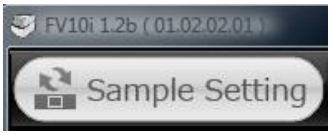
Number: 11

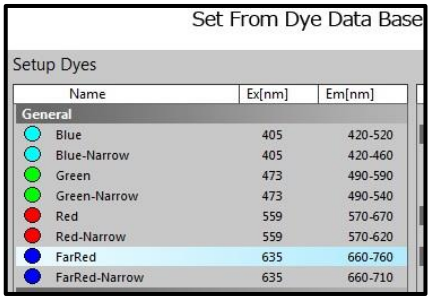
WORK INSTRUCTION BREAKDOWN SHEET

Operation: Laser Scanning Confocal Microscope (LSCM) Operations
Instrument: Olympus Fluoview FV10i

IMPORTANT STEPS	KEY POINTS	REASONS WHY
A logical segment of the operation when something happens to advance the work.	**Anything in a step that might:** 1. **Make or break the job** 2. **Injure the worker** 3. **Be a Cultural Consideration** 4. **Make the work easier to do (i.e., "knack", "trick", special timing, or bit of special information).**	**Reasons for each key point.**
Sample Stage Preparation		
Put on clean disposable gloves.		Minimizes contamination.
Gather necessary resources.	• Use the sample holder shown in the image to the right. • Note the following distinguishing features of this sample holder: ▪ Two cantilevered metal sample lock arms, ▪ Centrally-located rounded rectangular sample site with "Container" numbers (1-5) above, ▪ Oil deposition site (optional use), ▪ Small round "key hole" at bottom.	**Glass Slide Sample Holder** 
Place prepared sample on sample holder and "lock" in place.	• **NOTE**: Verify cover slip has a height equal to or less than a #1.5 cover slip. • Ensure the glass slide is oriented upside down with the cover slip underneath; then place it in the rounded rectangular sample site. • Center the sample below the #3 "container" slot in the slide holder. • Rotate both cantilevered metal sample lock arms to hold glass slide in place; ensure they do not cover any portion of the sample. • Press down on the pivot end of sample lock arms until they touch the base of the sample holder.	**Glass Slide Sample Holder**  • Cover slips thicker than #1.5 can damage the objective lenses of the confocal microscope. • "Container" #3 is treated as the default container as it is located in the middle of the slide holder; it is the simplest position to align visually when inspecting a slide with a single sample.

Open microscope hood.	With one hand placed on top of the microscope hood, push the "Hood Open" button with a finger from the other hand to gently open the hood.	Use one hand to guide the hood open to prevent it from "slamming" open, jarring the microscope, and fatiguing the hood hinge assembly.
Carefully clean perimeter of microscope stage with tissue wiper.		Minimizes contamination and frequency of deep cleaning and maintenance of confocal microscope. 
Place prepared sample holder on confocal microscope stage.	• First, carefully wipe top and bottom of sample holder with tissue wiper. • Then place it on microscope stage by aligning the sample holder "key hole" over the short cylindrical metal rod.	Wiping sample holder minimizes contamination within microscope. 
Gently close the hood.	• Using two hands on the upper front corners of the hood, gently but firmly, close hood completely.	This is done to insure that the lid latches completely closed on both sides, to avoid incidental contact with its lasers.
Remove disposable gloves.		Minimizes contamination.
Software Startup and Dye Selection		
Start Olympus software and log in.	• Double click confocal microscope icon "FV10i-SE.exe" on the desktop. • User ID: Administrator • Password: Administrator • NOTE: software will not start if sample holder is not in place on confocal microscope stage.	 
If necessary, click and drag unneeded software windows out of the way to bring dark/black window (titled: "FV10i 1.2b (01.02.02.01)") to front.		Opening confocal microscope software simultaneously opens image manipulation/editing software.

<table>
<tr>
<td colspan="2">If not already selected on the “Sample Setting” main toolbar tab, select the “Set from Dye Data Base” tab near the top center of the program window.</td>
<td> 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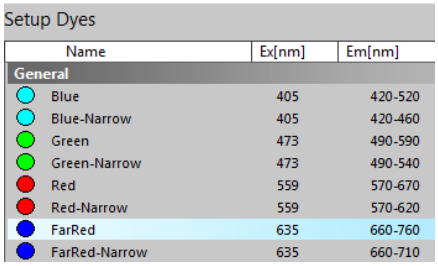
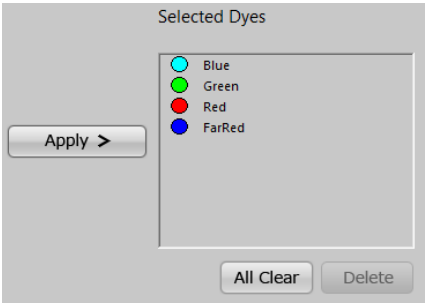
<tr>
<td>Select two to four fluorescent dyes for use in observation.</td>
<td>• No more than four and at least two dyes must be selected before imaging can begin.
• To select a dye, either:
○ double-click, or
○ single-click and then click “Apply”.
• <u>If either it is unknown whether actual dyes were used, or no actual dye was used</u> to prepare sample, Operator should first test/select four dyes from the “General” section (e.g., Blue, Green, Red, and FarRed).
• <u>If no dye was used</u> to prepare sample, but the fluorophore excitation wavelengths of the sample are known, Operator should test/select the dyes corresponding to those wavelengths.
• <u>If dye was used</u> to prepare sample, Operator should test/select those corresponding dyes.</td>
<td>• A fluorescence “dye” in this context can be thought of as a fluorophore excitation wavelength.
• The wavelengths of the microscope’s four diode lasers are: 405, 473, 559, and 635nm.
 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<tr>
<td colspan="2">Click the “Start” button near the bottom center of the screen.</td>
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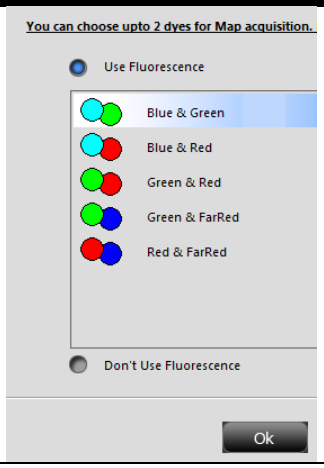
<tr>
<td>When more than 3 kinds of fluorescence dyes have been chosen, select a combination to be used in the Map Image Acquisition.</td>
<td>Click on the desired fluorescence dye combination and then click “OK” at the bottom of the combination selection window.</td>
<td> 
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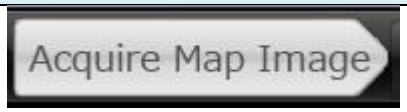
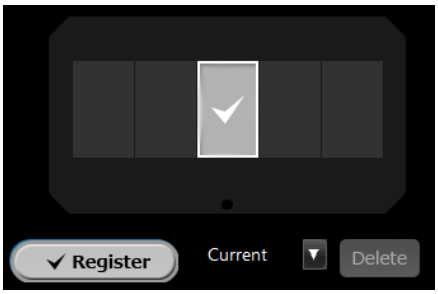
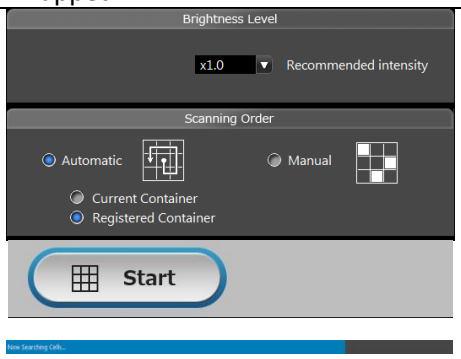
Acquisition of Map Image		
On the "Acquire Map Image" main toolbar tab, select and "Register" the "containers" that span the sample area on the glass slide.	• Click the middle box of the ***Container Selection Tool*** to select **Container #3.** ○ NOTE: select additional containers if sample spans more than just Container #3. • Click **Register** each time after selecting a container(s). • If necessary, click "Delete" to deselect registered containers.	  • For glass slides, "container" refers to the numbered location (1-5) printed on the sample holder above the glass slide sample site. • Other parts of the glass slide can be looked at by switching "containers"; only registered "containers" will be mapped.
Initiate map acquisition.	• Select the **Brightness Level** of "x1.0" • Select "**Automatic**" scanning order. • Select "**Registered Container**". • Click **"Start"** button below this area. • The blue search progress bar (i.e., "Now Searching Cells…" located at bottom of software window), will appear and show the scan progress. • The ***Map Image Acquisition*** scan automatically begins once the blue search progress bar disappears and the grid will begin to populate with data/images.	 Other brightness levels or quantities of grid squares are not relevant to basic / certification operation of the microscope.

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<td>Complete map grid acquisition.</td>
<td>
• The Map Image Acquisition area will form an expanding grid of image squares until either the entire “container(s)” has been scanned, or the Operator clicks “Stop” once area of interest is satisfactorily captured.
• This SOP proceeds with the Operator having clicked “Stop” after 9 squares were acquired.
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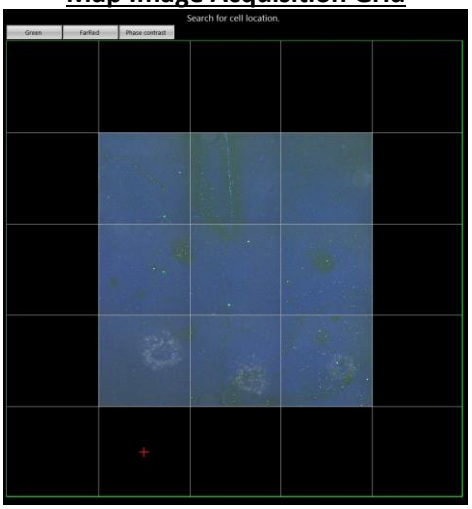
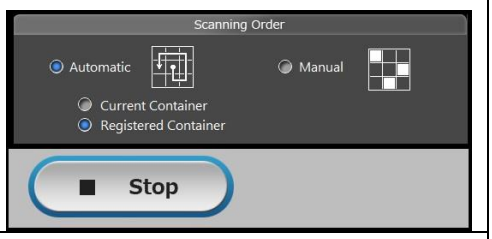
<td>
<u>Map Image Acquisition Grid</u>




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<tr>
<td>Evaluate effectiveness of the selected fluorescence channels.</td>
<td>
Click the Channel Buttons above the Map Image Acquisition Grid to toggle them on/off.
• First toggle the “Phase contrast”.
○ If most of the image is only observable when the “Phase contrast” channel is active, a new set of dyes should be selected that will cause the sample to fluoresce.
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• The two fluorescence Channel Buttons are set automatically according to the characteristics of the fluorescence dyes selected earlier.
• Unless desired, DO NOT CLICK “Start” again or the time-consuming acquisition process will restart.
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<td colspan="2">When Operator has judged that the chosen “channels” adequately interact with the fluorescence dyes and wishes to continue with sample analysis, click “NEXT”.</td>
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Basic Image Observation, Capture, and Saving

The **Observation Window** has 3 main areas with different functions:

- The **Left Area** is for approximate navigation within a single sample container or among several sample containers.
- The **Middle Area** is for specific navigation within the sample area, and for selection of active fluorescence channels. It corresponds with the location of the red crosshairs on the ***Map Image Acquisition Grid*** in the **Left Area**.
- The **Right Area** is for focus adjustments and fine-tuning of laser properties; its density of features can make acclimation difficult.

OPTIONAL: At this point, Operator can select the bright green "**Navigation**" button in the upper right of the **Left Area** to receive software-assisted guidance through parameter adjustment and image observation, capture, and saving. Alternatively, the Operator can continue on with following steps.

The **Left Area** enables *larger*-scale, quicker, and *less*-detailed inspection of areas of interest.

- Click on the desired containers in the ***Container Selection Tool*** to observe corresponding sections of the glass slide.
- Click on the ***Channel Buttons*** above the ***Map Image Acquisition Grid*** to toggle on/off the phase contrast and/or the dominant light wavelengths.
- Double click anywhere on the ***Map Image Acquisition Grid*** to navigate around areas of interest; the red crosshairs indicate the position of the objective lens.
- Use the "***Zoom***" slider bar to the right of the ***Map Image Acquisition Grid*** to magnify areas of interest.
- Return to the **Left Area** as needed during sample inspection.

The majority of the **Middle Area** displays image squares corresponding to the colors of the Channel Buttons above the group of squares. These image squares enable *smaller*-scale and *more*-detailed inspection of the areas of interest.

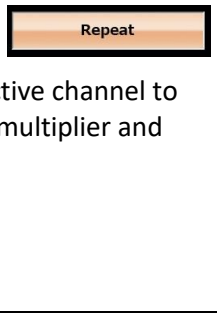
- Click on the ***Repeat Button*** to toggle on/off scanning of the selected containers.
  - When active, ***Repeat Button*** is a coppery color; black when inactive.
  - *Always* activate the ***Repeat Button*** before adjusting any parameters, or immediately afterward, to refresh the images.

- Click on the dropdown arrow immediately to the right of the ***Repeat Button*** and select **X2** rastering speed.
- Click on the ***Channel Buttons*** above the image squares to toggle on/off the phase contrast and/or the dominant light wavelengths.
- Double click, or click-and-drag, anywhere on any of the image squares to navigate around areas of interest; the red crosshairs on the ***Map Image Acquisition Grid*** in the **Left Area** will move accordingly.
- Use the "***Zoom***" slider bar to the right of the group of image squares to magnify areas of interest; slider range is 1.0X to 6.0X which equates with 10X to 60X "Total" magnification.
  - **DO NOT CLICK THE "10x" BUTTON** unless Operator fully understands how to safely use the "Oil Immersion" feature.

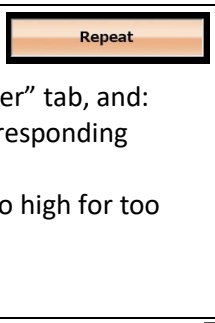
Adapted from Graupp, P. & Wrona, R. (2006) The TWI Workbook: Essential Skills for Supervisors. New York, NY. Productivity Press.

The **Right Area** enables adjustment of several parameters and settings. It is also used to specify the quality of images and the location where they will be saved once the final scan is initiated.

- **OPTIONAL**: With ***Repeat*** button activated at **X2** raster speed, click on the "Sensitivity" tab, then:
  - Click and drag the horizontal slider bar of each active channel to adjust the sensitivity of the corresponding photomultiplier and detection of photoelectrons.

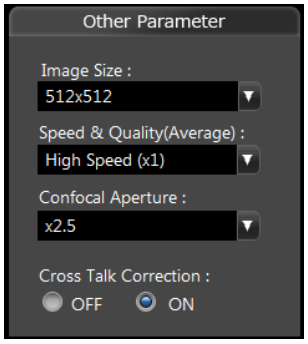


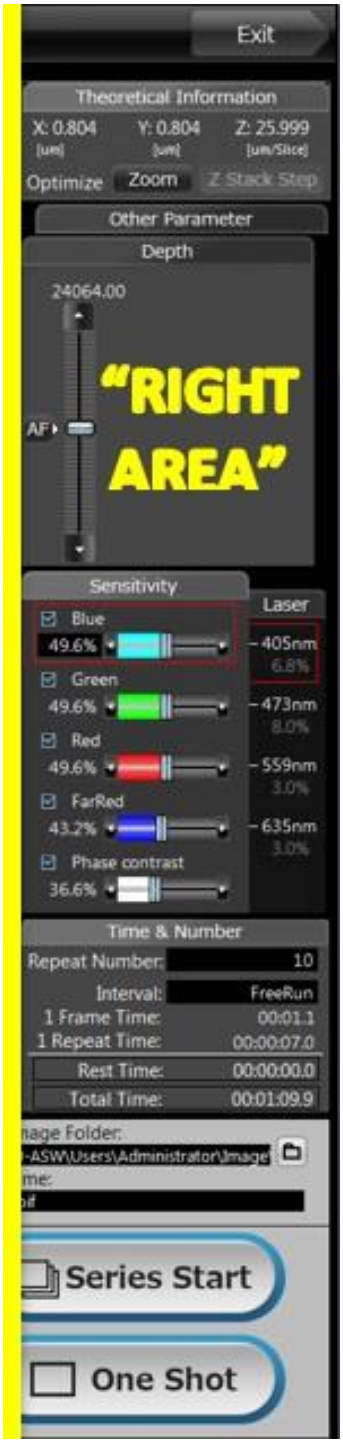
- **OPTIONAL**: With ***Repeat*** button activated at **X2** raster speed, first click on the desired channel while on the "Sensitivity" tab; then click on the "Laser" tab, and:
  - Click and drag the horizontal slider bar of the corresponding channel to adjust its laser output/intensity.
  - **CAUTION**: setting any laser output percentage too high for too long can severely damage a sample.



- **FOR 3D IMAGES ONLY** [for a single 2D image, skip this step/row and proceed to following step/row]:
  - First, ensure "Z Stack" is selected from the dropdown menu near the top left of the **Left Area** (left of green "Navigation" button).
  - Second, with ***Repeat*** button activated at **X2** raster speed, click on the "Depth" tab, then click and drag the left slider bar (located closest to the "AF" button) as follows:
    - **Drag Up** until the area of interest is just barely visible; then click the "Set" button below the "End" field, and
    - **Drag Down** until the area of interest is just barely visible; then click the "Set" button below the "Start" field.
  - Finally, adjust the "Step" field to specify the desired number of image "Slices", or cross-sections, between the End and Start positions.
    - NOTE: A lower "Step" value increases the number of "Slices", resulting in both better resolution and longer scanning/processing time; the converse also applies.

- When the composite image displayed in the lower right image square in the **Middle Area** meets Operator's needs and is ready to be saved, click on the "Other Parameter" tab, then:
  - Click "Image Size" dropdown menu and select desired size/resolution to save images (typically set to 1024x1024).
  - Click "Speed & Quality" dropdown menu and select one of five settings depending on Operator's need for quick scanning/processing (i.e., "High Speed (x1)") or better image quality (i.e., "High Quality (x16)").
    - Click "Confocal Aperture" dropdown menu and select one of seven settings that specify how much light interacts with the sample (typically set to x1.0 for saving); [Aperture range: x1.0 = least light; x5.0 = most light].





With ***Repeat*** button *inactivated/off*:

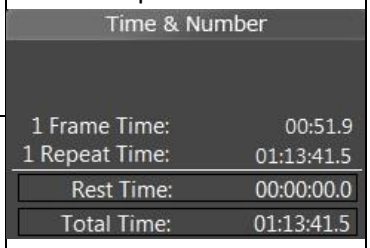
- Click on the tiny folder icon to the right of "Save Image Folder" field and within the popup dialogue box:
  - Select an appropriate file path;
  - Enter an appropriate file name for the image;
  - Click on "Save".

<u>TO SAVE SINGLE 2D IMAGE ONLY</u>:

With ***Repeat*** button *inactivated/off*:

- Click on "One Shot" button.

An estimate of the image processing time is displayed in the "Time & Number" panel.



<u>TO SAVE 3D IMAGE "SLICES" ONLY (i.e., "Z Stack")</u>:

With ***Repeat*** button *inactivated/off*:

- Click on "Series Start" button.

<u>OPTIONAL</u>: Although beyond the scope of this "Operations" SOP, Operator can choose to proceed with the image "Review" process using the proprietary image editing software (FV10-ASW 3.1). Alternatively, Operator can postpone or skip the image review/editing process and proceed to the Shutdown Work Instructions.

Proceed to "Laser Scanning Confocal Microscope (LSCM) Shutdown" Work Instructions