**OpenHiCAMM Microscope Automation User Guide**

This guide contains step-by-step instructions for running Fiji, Micro-Manager, and OpenHiCAMM using a Zeiss Axioplan 2 microscope, and Prior Slide Loader and Stage hardware. It contains many instructions specific to the BDGP Insitu project’s automated embryo imaging workflow and setup.

1. Verify available space

The first step is to make sure there is enough available space for the run. A 96-slide run requires about 4 TB of disk space. The workflow volume is located at /data/insitu\_acq, and has about 36 TB of total storage capacity.

Click on the Finder icon in the dock, then open “Macintosh HD”, and double click on the “data” volume, then on the “insitu\_acq” folder. You should now see the contents of the /data/insitu\_acq folder. The status bar on the bottom should show the amount of available space in the /data/insitu\_acq volume. Make sure there is enough space for the run. If there is not enough space, clear out one of the older runs to make space.

2. Set up the microscope and slide loader

The slide loader on switch is located all the way on the back side of the loader. The microscope on switch is the green switch on the lower right side toward the rear.

Make sure all USB cables are plugged in. All the USB ports on the iMac should be filled. Make sure there is a piece of adhesive tape used to secure the lens filter slider on the lower right side of the Microscope. That slider is a little loose and can become misaligned during the run if it’s not secured.

Make sure the scope is set to use the green 20x lens. That is the only lens that is used during the run. Make sure the lower lens is up and the light intensity set set between 6 and 7.

The Nikon camera should be set on top of the microscope and plugged in to power and to the iMac via USB. Make sure the camera is set to the “On” position.

3. Load slides and cartridges

There are four cartridges that can be connected to the slide loader. Each cartridge holds up to 50 slides. The cartridges are numbered 1 to 4, from left to right. The slides are numbered 1 to 50, from bottom to top of the cartridge. Each slide should be labeled, and the frosted label edge of the slide should be facing outward. You should keep track of all of the slide names using a spreadsheet. Later on, you will copy-paste the contents of the spreadsheet to register the slide information into OpenHiCAMM.

The format of the spreadsheet should be:

<Cartridge Position> <Slide Position> <Experiment ID>

where the Cartridge Position is a number from 1-4, the Slide Position is a number from 1-50, and the experiment ID is a short, unique text identifier for each slide in the workflow.

3. Start Fiji, Micro-Manager, and OpenHiCAMM

Click on the Fiji icon in the dock. If you see a console window with an error message “Module threw exception, IllegalStateException” it’s OK to ignore it. Just close the Console window. Now make sure the Fiji toolbar is in focus, then go to the Fiji menu and select Plugins → Micro-Manager → Micro-Manager Studio. In the “Micro-Manager startup configuration” window, make sure “/Users/insitu/fruitfly\_reference\_5\_nikon af-obj.cfg” is selected as the configuration file, then press OK.

If the slide loader, camera, and microscope are all on and working properly, you should hear the camera shutter noise, then the Micro-Manager window should be visible. If you get a device-releated error message, try turning off then turning back on the loader, camera, and/or microscope, then re-start Fiji. If that doesn’t work, try re-starting the iMac.

In the Micro-Manager window, locate the “Configuration settings” section. There should be a row with Group label “Nikon”. Click on the empty space in the “Preset” column in the “Nikon” row. Then select the “D5100” preset. That is the preset for the D5100 camera, which is the one we will be using. You should hear the camera shutter noise again.

To test the camera, you can try clicking the “Snap” button. You should hear the camera shutter noise, then an image window pop up. If no slide is loaded, it will probably be all black, that’s OK.

Now, make sure the Micro-Manager window is still focused, then go to the Micro-Manager Menu and select Plugins → OpenHiCAMM. This will load the OpenHiCAMM plugin module.

4. Create and configure a new workflow

At this point we need to create a new workflow directory. Click the “Choose” button next to the “Workflow Directory” row. You should now be able to view the contents of the insitu user’s home directory. Scroll down to the bottom to find the “workflows” directory. This is actually a link to the /data/insitu\_acq/workflows directory. Double-click the workflows directory to view its contents.

The contents of the workflows directory lists all the currently stored automated image workflow directories. We need to create a new one. Click the “New Folder” button. Then enter a name for the workflow directory. Once you have created the directory, you will automatically enter into the directory to view its contents. This is not what we want, because we need to select the directory itself. Click on the drop-down select box below the “File:” field at the top of the dialog box, then select the “workflows” directory (the second option from the top of the list of options). We are now viewing the contents of the workflows directory again. Now, select the directory you just created (do NOT double-click! Just a single-click will do). You should still be viewing the contents of the “workflows” folder, but with the folder you created selected. Now, make sure that the folder name **also** appears in the “File:” box at the top of the dialog. If the folder name you created does **not** appear in the “File:” field at the top, single-click the folder **again**. The “File:” field should now show the folder you created. Now you can click on the “Choose Workflow Directory” button. Do **not** click the “Choose Workflow Directory” button unless and until the “File:” field correctly shows the directory you created! This is an unfortunate bug in the Java Swing file selection interface, so there’s not much to be done about it.

Now wait patiently for about 10 seconds while a new database is created. Once it is created, the “Edit Workflow...” button will become enabled. Click on the “Edit workflow” button.

You should now see the “Slide imaging workflow” window. We want to create a workflow that looks like this:

* Start
* SlideLoader
  + SlideSurveyor
    - CustomMacroROIFinder
      * SlideImager
        + ImageStitcher

A brief explanation of the workflow: the SlideLoader loads the slides, then the SlideSurveyor uses the camera’s “live” mode to make a quick low-res scan of the entire slide. Next, the CustomMacroROIFinder searches the low-res scan for embryos and marks their location in the database as a position list. Then, the position list is fed to the SlideImager, which takes high-quality images of every ROI location. Finally, the Image Stitcher stitches together multiple images into a single ROI image when a single image is too small to capture the entire ROI.

You can create this workflow by first selecting the “Start” folder. Then select “org.bdgp.OpenHiCAMM.Modules.SlideLoader” in the “Module Type” drop-down, and click the “+” button. Then select the “SlideLoader” folder, select the SlideSurveyor drop-down item, and click “+” again. Keep following this procedure until all the workflow modules have been created. Each module should be indented and show up as a sub-folder of its parent module. If any of your modules are lining up vertically then you have done it wrong. If you make a mistake, just select the module you need to remove and click the “-” button. Once finished, press the “Done” button.

5. Configure the Workflow Modules

Now click on the “Configure...” button in the “OpenHiCAMM” window. You should see the “Module configuration” window pop up. There should be 5 tabs in the window corresponding to each of the 5 modules we created: SlideLoader, SlideSurveyor, CustomMacroROIFinder, SlideImager, and ImageStitcher. We need to configure each of these modules one-by-one.

For the most part, the default configuration for these modules should not need to be changed. However, any configuration related to stage coordinates will usually need to be recalibrated, as any time the stage’s limit switches are hit (by the workflow due to a bug or a user with the joystick by accident), it will throw off the stage’ entire coordinate system, and any stage positions will need to be manually recalibrated. We will now go over configuration for each module, including manual stage recalibration.

5.1. SlideLoader

5.1.1. Loading

* Make sure the SlideLoader module’s “Loading” sub-tab is selected (not “Pool).
* Make sure “Slide loader” radio button is selected, not “Manual”.
* Make sure the path to slide loader device is set. It should read “/dev/tty.usbserial-FTEKUITV”.
* Now, using the joystick connected to the stage, move the stage all the way to the left and away from you, as far as it can go, until the limit switches are pressed. (This is the only time we will deliberately hit the limit switches). You should hear a slick “click” when the horizontal and vertical limit switches are pressed. Now, very gently, use the joystick to move the stage toward you and to the right, **just** until the limit switches are no longer being pressed down. You should be able to hear another “click” sound when the horizontal and vertical limit switches are released. Once you hear the click leave the stage at its current position and leave the joystick alone. This will be the stage’s slide loading position.
* Click the “Set X/Y Stage Origin” button. This will set the stage’s coordinate system so that the loading position is set to (0,0).
* Next, hit the “Read From Stage” button directly below the “Enter ***Loading*** Stage X/Y Coordinates” label.
* Now, using the joystick, move the stage to the middle of the coordinate system, about where it should be if you were viewing a slide. Then hit the “Read From Stage” button directly below the “Enter ***Initial*** Stage X/Y Coordinates” label. This will be the “initial” stage position. This position is used to move the stage out of the way when the slide loader performs its calibration step, otherwise the slide loader arm would collide with the stage.
* **Optional**: At a later step, we will need to put a sample slide on the stage for stage calibration. If you have cartridge(s) and slide(s) loaded, you may use the Manual Slide Loading section to have the Slide Loader manually place a slide on the stage:
  + Click the “Scan for Slides” button. The Slide Loader will typically run through its initialization phase, it takes about 1-2 minutes. Then it will scan both cartridges for slides.
  + Once the scan is complete, you may select a slide to load, then click the “Load Slide” button. The slide loader will then place the slide on the stage for you.
  + Later on when stage calibration has completed, you may return to this configuration tab and click the “Return Slide” button to put the slide back in the cartridge in the correct slot.
  + Alternatively, you may skip this step and manually pull the slide out and place it on the stage using your hand.

5.1.2. Pool

* Now click the Slide Loader’s “Pool” sub-tab.
* Make sure “Or Manually Enter Slide Layout” is selected, not “Automatically Scan For Slides”.
* Now, open the spreadsheet with the slide information you created earlier. Remember, the format should be: <Cartridge Position> <Slide Position> <Experiment ID>
* Select all cells in the spreadsheet and hit CMD+c to copy all cells to the clipboard
* Now, Click into the text area field at the bottom of the “Pool” configuration, and hit CMD+v to paste the contents of the spreadsheet into the text area field. If the contents pasted correctly, each row should be on its own line, and each cell should be separated with a tab field.
* Once you have verified that the information in the pool configuration text area is accurate, click the “Create New Pool” button. You should see a new Pool identifier e.g. “P00002” in the “Select Pool:” list. Make sure the newly created pool is selected.

5.2. SlideSurveyor

5.2.1. Set up XY Grid Position List

* The SlideSurveyor needs an X/Y-grid position list in order to know how to build up the low-res image of the entire slide. The selected default position list should be “/Users/insitu/PositionList-D5100-SlideSurveyor.pos”, but it will need to be recalibrated. Click the “Show XY Position List Dialog”.
* You should now see the Micro-Manager “Stage Position List” window. Click on the “Create Grid” button near the bottom.
* Next, manually load a sample slide. Move the stage to the top of the slide, and press the “Set” button. Do the same for the bottom, left, and right sides. We are defining the area that should be surveyed. This will usually not be the entire slide, just the area that could have potential ROIs on it.
* Enter the Pixel Size [um] value. This should be the same as the “HiRes Pixel Size” from the SlideSurveyor configuration dialog. The default value for the Nikon D5100 and the 20x lens is 0.125 um.
* The overlap should be set to 25%. Set the overlap metric to “%”, then set the value to 25.
* Now press OK. You should see a list of grid positions in the Stage Position List window.
* Hit the “Save as...” button, and save the file as “PositionList-D5100-SlideSurveyor.pos” in the insitu user’s home directory.
* Hit the “Close” button.
* **Optional**: The pixel size value can be discovered manually by centering an ROI from the slide, snapping an image using the “Snap” button in the Micro-Manager window, then moving the ROI so that it is off center, then snapping another image. Next find the ratio of the difference in stage coordinates and the offset in pixels of the ROI between the two snapped images. The stage coordinates can be found in the Micro-Manager window below the Group/Preset buttons, listed as “XY=(xxxx.xxx, yyyyyy.yyy)um”. Three significant digits should be sufficient.

5.2.2. Other SlideSurveyor Settings

* Make sure HiRes Pixel Size is set to the same value you used to produce the XY Position list in the previous section. The default value for the Nikon D5100 and 20x lens is 0.125 um.
* Make sure Invert X axis, Invert Y axis are both set to “Yes”. This is required for the Prior stage driver.
* **Optional: Recalibrate Z-axis.** *(You may lose your settings if you do this, so you should consider doing it after you’ve completed all of the other configuration settings first. Then press “Finish” to save the settings before you attempt this step)*. Find a ROI in the sample slide you still have loaded, and manually focus the ROI. Then turn off the microscope and turn it back on. This sets the Z-axis origin to the current Z-axis position. Turning off the microscope could cause Micro-Manager to stop working. If so, you will need to quit OpenHiCAMM, Micro-Manager, and Fiji, restart everything, and re-load the workflow directory in OpenHiCAMM. Then go back into the configuration and set the Z-axis as described above. This will calibrate the autofocus engine, and only needs to be done once in a while.
* Make sure the image scale factor is set to 0.3. This is an optimization that shrinks the low-res entire slide image to 1/3 size before post-processing and searching for ROIs. This helps speed up ROI finding.
* The Post-processing macro script should already be pre-set. It should contain the following text:

run("Bandpass Filter...", "filter\_large=25 filter\_small=15 suppress=Vertical tolerance=5 autoscale saturate");

run("Bandpass Filter...", "filter\_large=25 filter\_small=15 suppress=Horizontal tolerance=5 autoscale saturate");

5.3. CustomMacroROIFinder

* Click on the CustomMacroROIFinder tab.
* Make sure Task Type is set to SERIAL
* Make sure Tile Overlap Percentage is set to 25. This should be the same value you used when generating the XY grid position list in the SlideSurveyor configuration section.
* Make sure the HiRes Pixel Size is set to 0.125. This should be the same value you used when generating the XY grid position list in the SlideSurveyor configuration section.
* Make sure ROI Margin Percentage is set to 2.
* Make sure the ROI Image Scale Factor is set to 0.5. This is another optimization that shrinks the input low-res entire slide image to make processing faster.
* Make sure the HiRes Image Width and Height are set to the camera’s image dimensions. The default for the Nikon D5100 is 4928 (width) x 3264 (height). You can automatically set the dimensions for another camera by pressing the “Set Image Dimensions” button.
* The “Custom ROI Finding Macro” script should already be pre-set. It should contain the following text:

run("8-bit");

setAutoThreshold("IsoData");

setOption("BlackBackground", false);

run("Convert to Mask");

run("Gray Morphology", "radius=7 type=circle operator=dilate");

run("Analyze Particles...", "size=500-5000 exclude clear add in\_situ");

5.4. SlideImager

* Open the “SlideImager” configuration tab.
* Make sure the “Load Acquisition Settings File” field is set to “/Users/insitu/AcqSettings-phase2.xml”. If not, click the “Select File” button and select it. The AcqSettings-phase2.xml file sets the acquisition engine to use OpenHiCAMM’s FastFFT autofocus driver.
* **Optional:** If you need to re-create an acquisition engine settings XML file, click the “Show Acquisition Dialog” button, then make sure the “Autofocus” check box is checked. Then click the “Options” button in the autofocus section, and make sure the following options are set:
  + FastFFT-1st step size: 10.0
  + FastFFT-1st step number: 20
  + FastFFT-2nd step size: 1
  + FastFFT-2nd step number: 10
  + FastFFT-Threshold: 0.02
  + FastFFT-Crop ratio: 0.2
  + FastFFT-Channel: <blank>
  + FastFFT-minAutoFocus: -300
  + FastFFT-maxAutoFocus: 1000
  + FastFFT-autofocusDuration: 0
  + FastFFT-liveMode: yes
  + Then hit the “Close” button, then hit the “Save as...” button, and save the file as “AcqSettings-phase2.xml” in the insitu user’s home directory. Then hit the “Close” button in the Acquisition Dialog window to close it.
  + Make sure the “Load Position List From File” field is blank. Instead, the “Or choose the module that will generate the position list” field should be set to “CustomMacroROIFinder”. This will ensure that the SlideImager gets the set of positions discovered by the ROI finder module.
  + Make sure the “How many dummy images to take” field is set to 3. This ensures the camera is ready to acquire images before an acquisition run is started.
  + Make sure the Pixel Size is set to the same as all the other pixel size values. The default is 0.125 um for the Nikon D5100 and the 20x lens.
  + Make sure the Invert X axis and Invert Y axis are both set to “Yes”. This is required for the Prior stage to interpret the coordinates correctly.
  + Make sure the “Set Initial Z Axis Position” is set to “Yes” and the value is set to 0. The Z (focus) axis was already calibrated in the previous step, so there’s no need to repeat that step here.

5.5. ImageStitcher

* The ImageStitcher currently has no configuration options, so there’s nothing required to do here.

5.6. Saving the Configuration

* Hit the “Finish” button to store the configuration and return to the “OpenHiCAMM” main window.
* If you see any configuration error messages, follow the instructions to correct the errors, then hit the “Finish” button again.

6. Starting the Workflow Runner

* Make sure the “Start Task” module is set to “SlideLoader”. This should be the default and only option for this workflow, but it’s good to check.
* Now hit the “Start” button to start the workflow. You should see the Workflow Runner dialog pop up with a log window describing each of the steps as they occur.
* The first step is usually the Slide loader calibration step. It takes about 1-2 minutes to complete, and only happens once per run at the very beginning. The stage should automatically move to the initial position you configured in the SlideLoader configuration to stay out of the way of the loader arm. The arm will circle all the way back, then all the way forward, all the way up, and all the way down. Just wait patiently with you hand next to the slide loader off switch in case anything goes wrong.
  + **WARNING:** On the very last step of the Slide loader calibration step, when the slide arm is facing towards you and is just about to sweep the arm down, occasionally the slide loader locks up before sweeping down and make a series of very loud clinking noises like it’s stuck. If that happens, consult the troubleshooting section 7.1.
* The next step is usually the slide scanning step. The slide loader will scan all the slides in the loaded cartridges and verify that the slide count matches what you entered in the SlideLoader Pool section. If the loader mis-counts the slides (which does occasionally happen), it will prompt the user to re-scan the slides. You should choose “Yes” to re-scan. Usually the loader counts correctly after a re-scan.

7. Troubleshooting

7.1. Slide Loader getting stuck during initialization

* On the very last step of the Slide loader calibration step, when the slide arm is facing towards you and is just about to sweep the arm down, occasionally the slide loader locks up before sweeping down and make a series of very loud clinking noises like it’s stuck. If that happens, turn off the loader right away. In fact, you should have your hand hovering over the loader on/off switch during this step just to be safe. If the loader gets stuck, first try turning the loader off then on, then try again. If it happens again, you need to reset the Slide Loader by doing the following:
  + Close and exit the OpenHiCAMM, Micro-Manager, and Fiji entirely. Make sure there’s no black dot underneath the Fiji icon in the dock, if so, right click it and select “Quit”.
  + Click the VirtualBox icon in the dock, then select the “mmwin” virtual machine and click the “Start” button. This will start a Windows virtual machine with the Prior slide loader vendor software.
  + Once the windows virtual machine has finished startup up, select the VirtualBox VM’s window to focus it, then in the menu select Devices → USB. You should see a list of devices to patch through to the virtual machine. There should be two devices that begin with “FTDI”. One is the microscope, the other is the loader/stage. Select each of them one-by-one.
  + Then in the menu select Devices → USB again, and ensure that both devices are checked. If either or both are not checked, you need to unplug and re-plug each of the USB devices on the back of the iMac, then try selecting them again.
  + Now in the windows machine, double-click on the “SlideLoader Demo” icon.
  + Click the “Connect” button. For the loader port number, enter “3” then press OK. For the stage port number, enter “1” and press OK. If either does not work, try 2, 1, then 4, 5, 6 until you get a working configuration. Once the correct port number has been entered, you should hear a “click” from the slide loader. Exit the window with the “Arm In/Out” buttons. Then the slide loader will perform its calibration setup. That should be all you need to do to fix the slide loader. Just exit out of all of the windows, then power off the windows virtual machine.
  + The “SlideLoader Demo” software can also be used to recalibrate the loader coordinates.

8. Viewing the Workflow Report