Objectives

10x/0.3NA HC PL Fluotar
20x/0.7NA HC PL Apo
40x/0.85NA HCX PL Apo (correction collar)

63x/1.4-0.6 NA oil HCX PL Apo (correction collar)
100x/1.4-0.7NA oil PL Apo (correction collar)

Standard coverslip thickness 0.17 (#1.5 coverslip)

Light Source & Filter Sets

Sutter Lambda LS – xenon arc lamp

<table>
<thead>
<tr>
<th>Label</th>
<th>Excitation</th>
<th>Dichroic</th>
<th>Emission</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAPI</td>
<td>Chroma 31000 v2 (325-375 ex / 400 / 435-485 em)</td>
<td></td>
<td></td>
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<tr>
<td>FITC/GFP</td>
<td>Chroma 31001 (465-495 ex / 505 / 515-555 em)</td>
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<tr>
<td>Cy3</td>
<td>Chroma 41007a (530-560 ex / 570 / 572.5-657.5 em)</td>
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<tr>
<td>Quad - DAPI</td>
<td>394.5-409.5</td>
<td>Sedat Quad</td>
<td>430-480</td>
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<tr>
<td>Quad – FITC</td>
<td>480-55</td>
<td>Sedat Quad</td>
<td>507-543</td>
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<tr>
<td>Quad - Cy3</td>
<td>542.5-567.5</td>
<td>Sedat Quad</td>
<td>579-631</td>
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<td>Quad – Cy5</td>
<td>630-660</td>
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<td>669-741</td>
</tr>
<tr>
<td>Brightfield</td>
<td>open</td>
<td>Sedat Quad</td>
<td>open</td>
</tr>
</tbody>
</table>

Note: You cannot use the Sedat quad pass set to view signal through the eyepieces – only to the camera.

Camera

QImaging Retiga 2000R CCD-Camera (7.4 x 7.4 μm pixels)
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Quick Guide:

1) Turn on components in order.
2) Log into Windows with your IdentiKey and start Slidebook 6 software.
3) Put sample on scope coverslip up. If using oil immersion, do not switch back to air objective. **No oil on air objectives!**
4) Find and focus your sample through the oculars.
5) Acquire images, timelapses, Z-series, etc.
6) Save and copy your data. The LMCF is not responsible for long-term data storage. See Appendix A for information on how to transfer data to MCDB Dept Server.
7) Lower stage away from sample, and remove sample from stage. Properly clean oil off of any immersion objectives you may have used with lens paper. If you are at all unsure of this process, ask for help!
8) Leave the microscope on a low power objective for next user.
9) Close Slidebook.
10) Log use in Excel sheet, save and close.

⇒ **If someone is signed up within the next hour (Check the MCDBCal!):**
11) Log off Windows and leave the system on.

⇒ **If no one is signed up within an hour or you are the last of the day:**
11) Shut down system in reverse numerical order: #3 PC, #2 xenon lamp, #1 power bar.
**Starting Up the System:**

1) Turn on the main power strip.

2) Turn on the Sutter epi-illumination light source. If this was turned off within the last 30 minutes – WAIT until the lamp has cooled before turning it back on.

3) Turn on the PC and login with your IdentiKey.
Finding your sample through the oculars:

Once the components are on, open Slidebook 6 from the shortcut on the Desktop.

Make sure the stage is in the lowered position and that the 10x objective is in place. To rapidly lower the stage if needed, press the down arrow “rapid stage motion” button on the right side of the microscope. To change the objective, open the Focus Window and choose the 10x objective.

Place slide on the stage, coverslip up. It is recommended that you first find and focus your sample at the 10x low magnification (as this objective has a long working distance). If you are going to higher magnification right away, please ensure you do not run the sample into the objective while trying to focus. If you go to oil immersion, DO NOT GO BACK TO AIR! Do not get oil on the air objectives! Please do not use the objective changing buttons on the left side of the microscope as these frequently get stuck.

The first time the microscope is turned on, you will need to open the fluorescence shutter. Click the “Shutter Closed” button located near the red light on the right side of the microscope. If the red light is not on, you should not need to click this button. You will only need to do this once at the start.
Ensure the light path is directed to “Vis” on the left side of the microscope by pushing in on the knob. To send the light to the camera, pull all the way out to “Photo”. Do not confuse this knob with the analyzer located below it – the analyzer should remain out about 1 in.

The incident light diaphragm must be open to allow light from the xenon lamp to the sample. The position of the lever marked by a blue arrow can be adjusted to adjust intensity of the light. The two levers should be in the down or “0” position. These are located on the right side of the microscope.

Unless you have a deliberate reason to change it, ensure that the optivar is set to 1x. If this is changed, your pixel sizes will change accordingly. If you change this, please ensure that you return it to the 1x position when you are done. This wheel is useful if you want a magnification for example that is greater than 4x but smaller than 10x. It can be 1.0x, 1.25x, or 1.60x. Make a note of the optivar position as this information is not in the image metadata.

Select the appropriate fluorescence channel. You cannot use the Sedat channels to look through the eyepieces (since you won’t be looking through the emission filters). Click “Open Fluor” to turn on the fluorescence light. Click it again to turn the light back off when not looking to avoid photobleaching your sample.
Look through the eyepieces and focus on your sample. To quickly move the stage up, press the up arrow on the Rapid Stage Motion on the right side of the microscope. Be very careful not to run the sample into the objective – you will crack your slide and damage the objective. Use the focus knobs on either side to change focus. Change the speed of the focus knobs on either side of the microscope by using the STEP buttons labelled “Focus Speed” until the front display reads the desired focus speed. S3 is coarse focus and S0 is fine focus.

Control the X,Y stage with the Prior joystick. To change the speed of motion, click the button shown by the red arrow. The column on the right of the display indicates the relative speed of motion.

Change objectives and/or channels until you have found and centered on your sample. Turn off the fluorescence light and pull out the knob on the left to “Photo” when you’re ready to take a picture.
Operating Slidebook 6:

Make a directory for your own work in the “SAVE YOUR FILES HERE” shortcut on the Desktop. Do not save your data anywhere else and be sure to copy it when you finish. The LMCF is NOT responsible for data storage.

If at any point while using Slidebook you need help/explanation of a particular window or option press F1 with that window open and the Help will open to the page pertaining to that function or window.

Slidebook will open with a new “slide”, which is the sub-directory in which your work from the current session will be saved. Save this slide to your own directory. It is necessary to have a “slide” open to allow you to acquire images.
In the **Home** menu at the top, you will find the Focus and Capture buttons. These are the two major menus you will use to control the microscope.

**The Focus Window:**

As previously mentioned, the Focus window controls the choice of **objective** and **filter**. The switching of whether the light from the scope **should go to the camera or the eyepieces** will be done on the microscope stand and so the option is greyed out here, as is the magnification changer (optivar). It also controls the **shutters** that protect the sample from either the trans-illumination (Open/ Shut Bright) or epi-illumination (Open/ Shut Fluor). Note that when the tab says, “Open”, it means that the shutter is closed and clicking it will accomplish the actions shown in the tab. Vice versa for shutting. The little lamp icon will appear as a yellow light when the shutter is open/light is on.

There is a **slider, called “Lamp”** that controls the brightness of the trans-illuminator. Click on the “Brightfield” filter option and “Open Bright”.

If there were a choice of cameras, you would make it here, and you can set both the **exposure time for the camera** and the binning of the image. Choose the conditions that will minimize photobleaching. For dim samples, or where resolution is not so important, one can also increase the binning. Snap will show a view of the image one is getting. At the lower left of the window are arrow-heads that will move the stage, just the way the joystick does, and there are windows that allow a choice of the size of the steps that clicking the arrows will achieve. There are also controls for **Z and for auto-focus**; more on that below.

The tabs for “condenser”, “laser power”, and “neutral density” are currently not set up, as these cannot be controlled electronically or are not available on this system.

Click “Live” in the camera control area to display the image on the screen. Note: you are continuously exposing your sample to illumination and thus photobleaching it. Clicking “Snap” will take a picture of it, opening the shutter for the pre-set amount of time (the exposure time), and save it to the “Slide” file.

The color shown in the display is a pseudo-color and it not part of a save image file. You can adjust the pseudo-coloring and look-up table (LUT) scaling as desired.
Focus Window – Z Tab:

This is the place from which you can set up a Z-stack for 3D (or 4D) imaging. After pushing the “Z” tab at the top of the Focus widow, one can set up a Z-stack. There are a two ways to implement this: Set focus by hand and use it to “set top” (click that button) and “set bottom” and then set the desired step size. The fewer the steps, the less photobleaching, but more steps yields higher resolution in Z up to a point. The point spread in Z is about 1.5 – 3x the resolution in XY, thus very small step sizes will not yield additional information.

Alternatively, one can set the reference position and step size. More on this in the Capture window.

Focus Window – XY Tab:

This is the place from which you can choose multiple locations on your sample to visit in sequence. After pushing the “XY” tab at the top of the Focus window, one can choose multiple locations. To make these choices, one can use the joystick to motor around the sample, choosing regions of interest. When you have one, and it is in good focus, click “Set Point” and the computer then knows to go to just that point. Almost any number of locations can be picked, but be aware that the number of images taken (in Z, over time, and at multiple XY locations) will soon add up to a big file! When at a location, you can adjust X,Y, or Z and then click “Update Z” or “XY” and the new addresses will be stored. Stored addresses can be erased, one by one or all together, and replaced if desired.

One can also set up the acquisition of a montage image by either defining the corners of the area to be acquired (go to that position and then click the corner with “Set Edge” selected). You must define at least two diagonal corners that use the same Z position, or three corners for slope interpolation. Slope interpolation will adjust the Z position along a Z plane that fits through all three positions. To acquire a montage around your current position, choose “Set Extent”, choose how many fields of view to acquire (e.g., 2x2 will acquire 4 images total) and click the center dot. If you want your current position to be the top left of the montage, click the top left corner button instead of the dot.
The Capture Window:

The next tab across the top of the screen in Home is Capture. This opens a window that does what its name suggests. Here you can set up montages, 3D, and multiple locations for imaging.

Upper left: Capture Settings: leave it at Default.

**Extent, Offset and Binning (pixels):** Have “Image” clicked, leave Bin Factor at 1, unless you want to get more light into a single pixel, in which case, choose 2. Note that this will result in larger pixels and loss of spatial resolution (do you need to make very precise localization measurements?). Click Full Chip to take images as large as the camera chip can accommodate.

**Capture Type:** select either 3D, time lapse, or both. When these boxes are ticked, the windows just to the right for setting up each function will become active.

The Timelapse capture window allows you to set conditions for that round of image acquisition. Do click on Display, Renormalize to t = 0.

The 3D Capture Window will display the information you input in the Z Tab of the Focus Window (see above). Select use current position or use reference position to take a range around that position. Click Use top and bottom positions to use the positions you define in the Focus Window. Set the range, number of planes (aka steps) to acquire, and/or step size. These will all update as you change any one.

**Filter Set:** something must be clicked here, or the scope won’t operate. Make sure you click the filters you want to use. The images will be taken in order from the top, so if you have clicked several and want a particular order (e.g., DAPI last to minimize bleaching of the other fluors), you can order them by moving specific filters up or down. Do not change the “Filter Set” - the microscope setup only contains one configuration of filters (all available). For each filter selected, you will need to set the exposure time.

**Exposure:** Here, you can also set the exposure or click “find best”. Under manual one can set a different exposure for each filter set, which can be very important. Find best may not always yield optimal results. The options to the right of this (Laser power, etc.) are not operational. You can also choose your objective from here. (Magnification Changer doesn’t automatically change the optivar, as it is not motorized). You can also name your file and enter metadata as comment. “Start” will initiate the program you have specified.
The Capture Window – Advanced Options:

The Advanced button (top left) allows you to specify details of capture preference. In the “General” tab you can choose the order in which two channels are used: ABABAB, etc., or ABBAABBA, etc. This will affect the number of moves the scope has to make to follow your instructions. Camera image updates will control what you see on the monitor while the program is in action: updating when possible is often the best. This is a good balance between forcing the system to always update (even when memory may be maxed out) or never updating.

The “4D” tab again offers specific choices for a 4D acquisition.
The “Focus” tab allows you to choose when autofocus is done. Note, as mentioned above: THIS MUST BE CLICKED TO HAVE AUTOFOCUS WORK. Its conditions will be the ones set in the Focus window, but this tab is an essential part of having it work. The other tabs are somewhat self-explanatory, allowing you to add metadata, etc.

See Dante’s user guide for more extensive information regarding timelapse experiments and autofocus in Slidebook 6.

**FINALLY taking the picture:**

Two ways to take a picture: if you only need a single color and single focal plane, click SNAP in the camera control area of the Focus window.

If you have set up a multi-color, multi-focal plane, and/or multi-position experiment, click Start the Acquisition in the Capture window. After setting all the parameters and saving the slide in your lab folder, click the Start button in the bottom right to start the 2D/3D/4D image acquisition.

All files are saved to the “Slide” you saved at the beginning in a Slidebook proprietary format.

To also save images you can open in software other than Slidebook, one will want to export the images as TIFFs. It is highly recommended that you save both the proprietary “Slide” file as well as exporting as TIFFs, because the “Slide” file represents your raw data with all the associated metadata (info on acquisition parameters). Avoid saving files with lossy compression types (e.g., jpeg).
Other Features:

- When building a montage, acquire and apply a flat field correction to reduce the effect of uneven illumination in your final image.

Create a projection of your 3D Z-series data.

- Deconvolution
When you’re finished:

1) Save and copy your data in the “SAVE YOUR FILES HERE” file path. The LMCF is not responsible for long-term data storage. See Appendix A for information on how to transfer data to Collie.

2) Lower stage away from sample, change to 10x objective, and remove sample from stage. Return optivar to 1x if you changed it. Properly clean oil off of any immersion objectives you may have used with lens paper. If you are at all unsure of this process, ask for help!

3) Close Slidebook.

4) Log use in Excel sheet, save and close.

➔ If someone is signed up within the next hour (Check the MCDBCal!):
5) Log off Windows and leave the system on.

➔ If no one is signed up within an hour or you are the last of the day:
6) Shut down system in reverse numerical order: PC, Xenon light source, and power strip on the wall.
APPENDIX A – Saving to your lab’s folder on Collie (MCDB Dept Server):

➤ Questions regarding MCDB Server should be directed to Erik Hedl.

1) Map the network drive. Right click on Computer icon on desktop or Computer in Start menu. Click on “Map network drive...”

2) Drive letter should be Z: and the folder should be \collie.int.colorado.edu\<your lab name> (for example \collie.int.colorado.edu\OlwinLab). If your login for Collie is not your IdentiKey, check the box “connect using different credentials.”

3) Click finish.

4) If you selected “connect using different credentials,” enter your username and password.

5) Click Ok and the drive should now appear. Copy over your files. If you have a lot of files, be sure to allow time for the transfer. Under ideal conditions you will be able to copy close to 42 gigabytes per hour, don’t count on ideal conditions. Also, it is safer to copy (not move) your files, then delete after the copy has completed.

➤ You cannot, must not, should not reserve time on the microscopes in MCDB Cal to copy data – reservations are for imaging only.

➤ The LMCF PCs are not long-term data storage places and we are not responsible for lost data.

➤ Do not forget to always safely store and backup your raw data – this represents the ground truth of what you acquired that day and has all the associated metadata. Many journals are now requiring that you submit raw data with your manuscript.
APPENDIX B – Parts of the microscope:

(not all these components are present on our microscope)
APPENDIX C – Factors that Affect Quality of Digital Images:

The goal of image acquisition is to acquire a quantitative image that is as close to the fundamental limits of resolution as you can achieve. This requires good signal to noise ratio (SNR). You want your signal to be visible above the background, which is affected by the noise level. If your signal of interest is close to the background level and you have high noise, you will not be able to accurately resolve your signal within the noise variations.

To increase signal: use a #1.5 coverslip, clean coverslip and slide, mount as close to coverslip as possible, use camera binning

To decrease noise: optimize sample prep to maximize signal, use shorter exposure times

To decrease background: clean coverslips, optimize sample prep, dark room, close field diaphragm

1) **Exposure time**

The exposure time should be as fast/short as possible to obtain a good SNR image. Longer exposure times will lead to increased photobleaching, and if you want to acquire a thick Z-series (many planes) or are imaging live cells, don’t set exposure time too high. For live cells, photobleaching and phototoxicity are more important considerations and exposure times should be carefully considered.

2) **Bit Depth / Autoscaling**

Slidebook by default will autoscale the image you acquire to show min-max range. It is imperative that your image not contain under- or over-saturated pixels since you will lose information regarding intensity distributions (e.g., can’t tell the difference between a spot with intensity 5,000 or 10,000 – they both show up as 4,096 in an 12-bit image) or subtleties in structure (e.g., thin or thick membrane might look the same). You can adjust the LUT of the image to display a defined range or the full dynamic range.

3) **Binning**

Binning is the combining together or pooling or adjacent pixels into a single, larger pixel. For example, 2x2 binning will combine a total of 4 pixels (2 per side) into a single bigger square pixel. This has the benefit of producing a brighter pixel (better SNR) at the same exposure time at the cost of reducing resolution. For live samples binning can be especially useful to allow for acquisition of a greater number of images (Z-series or timelapse) with less photobleaching and phototoxicity.