Perkin-Elmer (Nasmyth) Spinning Disk Confocal User Guide

Room 00-030

Micron Advanced Bioimaging Unit

Department of Biochemistry

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Micron OXFORD Advanced Bioimaging Unit

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I. Instrument summary

The Spinning disk UltraVIEW confocal microscope from PerkinElmer provides **optical sectioning** while still permitting **rapid imaging**, on the order of 20 frames/s. It is therefore particularly useful for **live cell imaging** as the high imaging speed and low background provide crisp images of even fast moving objects in live cells.

The UltraView system is mounted on an IX81 Olympus inverted microscope with environmental chamber for temperature and CO_2 control. It is equipped with an electron-multiplying charge-coupled device camera (EM-CCD Hamamatsu Photonics) and it has 6 laser lines, 405, 440, 488, 514, 561 and 640 nm. This allows a very wide range of fluorescent probes to be used on the system. The system is run by Volocity software.

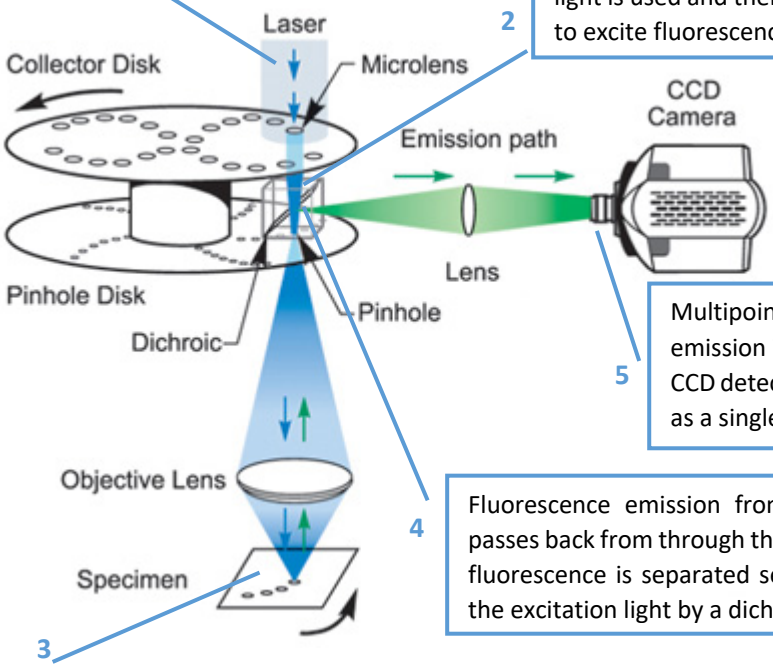
The instrument is also fitted with a photo-bleach/activation unit, which can be used to provide either single spots or arbitrary shapes via line or raster scans, suitable for FRAP, photodamage and photoactivation experiments. The automated XYZ stage can be programmed for image stitching of large samples and time-lapse imaging at multiple positions.

It is located in room 00-030 in the basement of New Biochemistry and is maintained by Micron staff. It is open to all researchers and access should be arranged via Micron staff.

II. How does spinning disk illumination works?

Light from the UltraView's lasers, delivered by fiber optics into the scanning unit, illuminates a window of ~1000 of the 20000 microlenses on the collector disk.

1



2

Microlenses focus the laser through the pinholes, increasing the transmission of light onto the sample and ensuring that a point-source of light is used and there is enough light to excite fluorescence in the sample.

5

Multipoint fluorescence emission is focused onto a CCD detector and read out as a single image.

4

Fluorescence emission from the sample passes back from through the pinholes and fluorescence is separated separated from the excitation light by a dichroic mirror.

3

The objective lens focuses laser light onto the sample. The spinning of the disc causes the sample to be scanned by 1000 points of light at once, i.e. multipoint illumination.

IV. Objectives

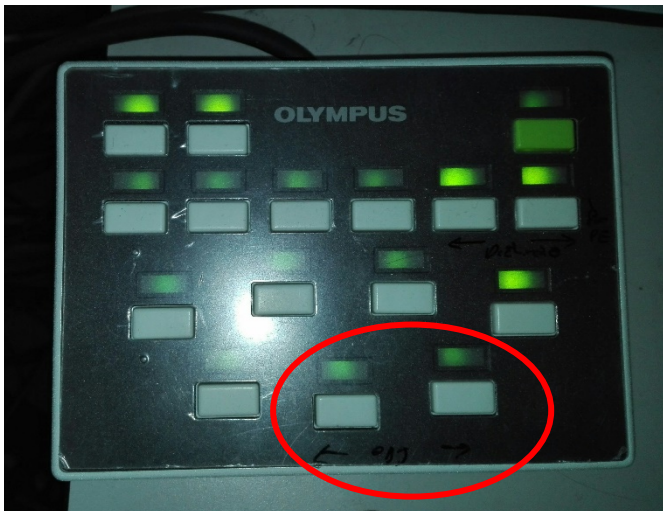
Magnification	Numerical Aperture (NA)	Immersion Medium	Description
10x	0.40	Air	Olympus UPlanSApo
20x	0.75	Air	Olympus UApo
40x	1.30	Oil	Olympus UPlanFL
60x	1.35	Oil	UPlanSApo
100x	1.35 - 0.5	Oil	UPlanApo

- **Air lenses do not require immersion media** and you should be careful not to get any oil or water on them

- Use **standard immersion oil** (small brown dropper bottle 1.514) with the **oil objective lenses**

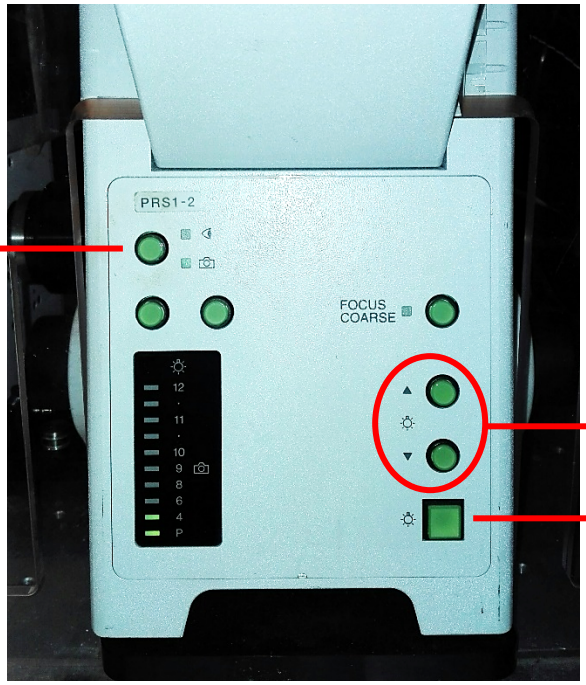
- Make sure the iris adjustment ring on the 100x is positioned at 1.35 to ensure high NA/resolution. If you aim imaging deeper into the sample at the cost of lower NA/resolution, turn the ring accordingly. Please set it back at maximum to keep 1.35 as default.

- Switch objectives via Volocity software under the command Olympus Microscope (see further details on sections V and VI). Alternatively use the control pad.



V. Microscope Considerations

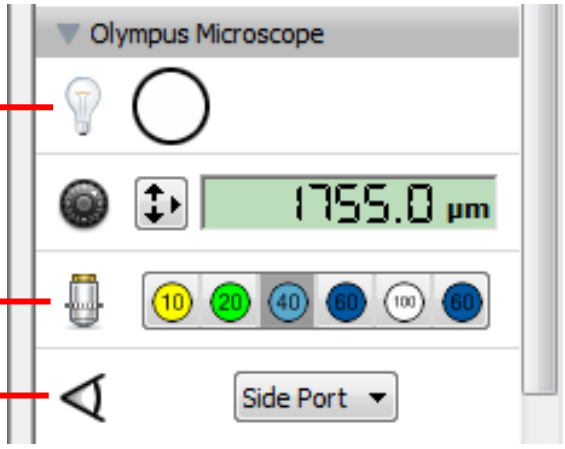
- This microscope is **not equipped with a LED light source**, suitable to locate your sample using fluorescence through the ocular (eyepieces). This implies that the only light source available to use with the ocular is the Halogen lamp for brightfield imaging.
- **Brightfield illumination** is activated with the software by selecting **DIC channel** (Section VII)
- An interlock switches off laser emission when the ocular is selected. To find your sample with laser excitation and fluorescence emission use the CCD detector. Switch between **ocular** and **detector (side port)** in the main microscope body or via Volocity software (described in section VI).



Switch between Ocular/ detector

change halogen lamp intensity (brightfield)

Halogen lamp switch



Halogen lamp switch

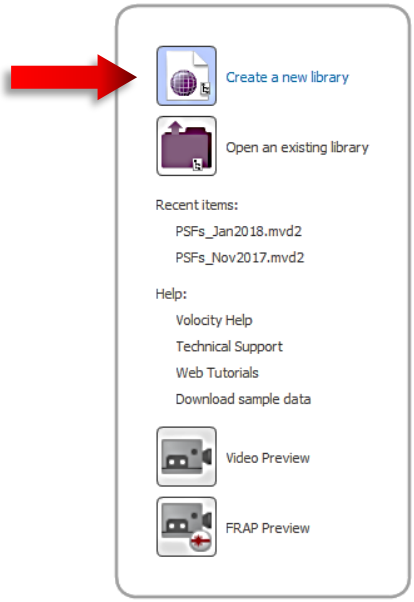
Swap objectives

Switch between Ocular/ detector (side port)

VI. Starting Volocity Software

1. Start Volocity.

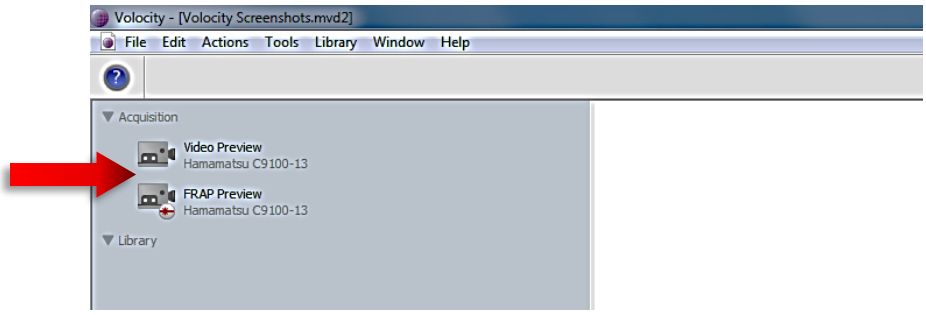
2. Volocity saves data automatically into libraries. You can create a new library or open an existing library. Micron recommends **creating a new library** for each set of experiments, because the program can crash when the library gets too big.



3. Save the library locally, into your User Documents folder. Do not forget to **TRANSFER** the library at the end of your booking into your **Micron server space** (\\micron1\abcd1234) or into **OMERO**. (abcd1234 represents your university SSO)

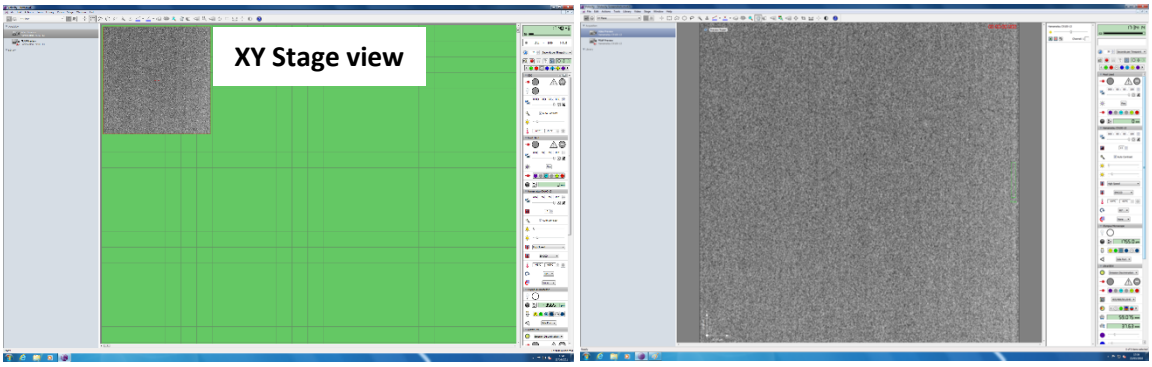
4. Select one option from Acquisition (left panel)

   - **Video Preview** for standard XYZ, time –lapse imaging, point visiting, stitching
   - **FRAP Preview** for Photodamage and Photoactivation experiments



VII. Adjusting the settings

1. Select Video Preview under Acquisition (Left panel)
2. Choose your preferred view among **XY stage** and **Live video** on the Video tab or with shortcuts Ctrl+Shift+X and Ctrl+Shift+V, respectively.



3. Find your sample using the focusing wheel. Bear in mind Microscope Considerations (PAGE X) if you opt to use the Binocular, otherwise use Live video **preview** (through Side port – detector).

*You need to select a **channel** and **Resume Preview** to turn on excitation source.*

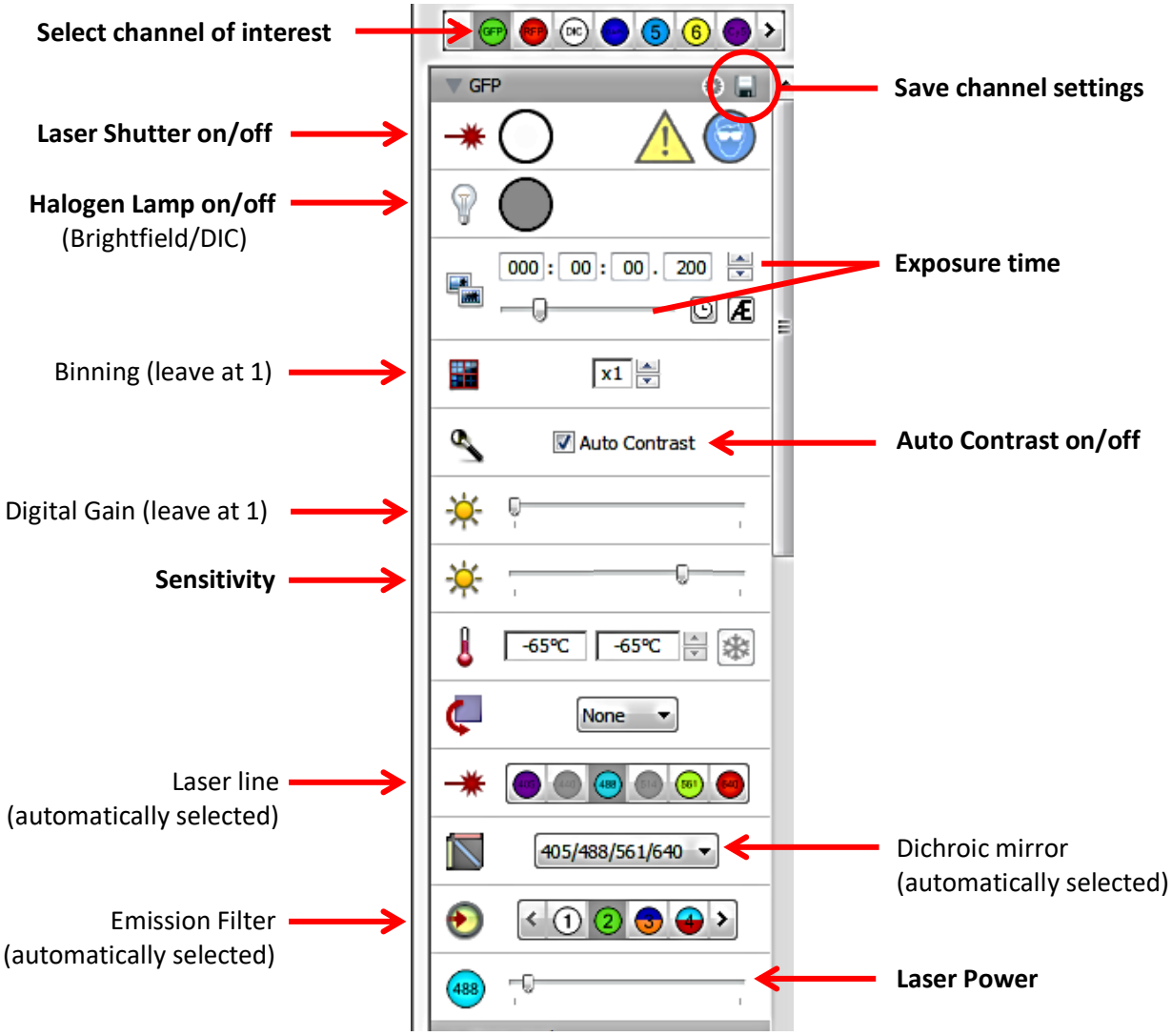
Channel	Light Source	Emission/ Bandwidth
GFP	488 nm laser	527/55
RFP	568 nm laser	615/70
DIC	Halogen lamp (transmitted)	N/A
DAPI	405 nm laser	445/60
CFP ⑤	440 nm laser	477/45
YPF ⑥	514 nm laser	587/125
Cy5	640 nm laser	705/90

Note: The symbols for live **Preview** or acquisition (**Experiment**) can be misleading. They are independent and each of the buttons is used to **start *and* stop** live video or acquisition, respectively.

4. Resume Preview and adjust the **settings for each channel** of interest. Do not forget to **Save**, otherwise the system automatically picks last used/saved settings.

Notes: Laser *shutter* is *on* by default every time you switch Channel under Preview. Some *settings repeat* in different sections, for example under Hamamatsu C9100-13 (Detector), UltraView, Most Used. They are interconnected, so no matter each one you decide to tweak, changes will be applied to all.

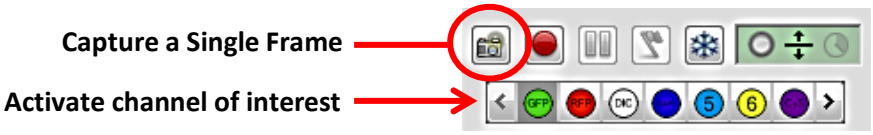
Tool options (necessary ones in **bold**):



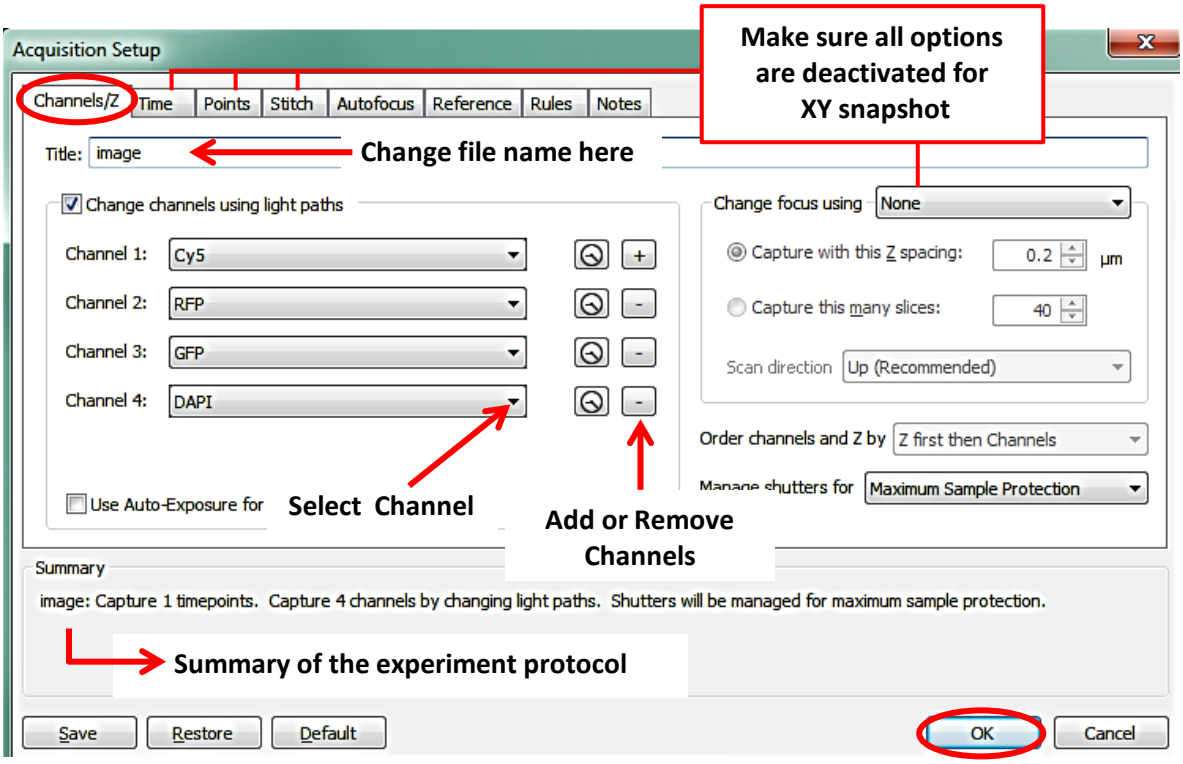
Note: The **Voxel inspector tool**  can be useful to adjust settings and measure pixel intensities. Ensure you stretch the dynamic range of the 16-bit detector without saturating the brightest pixels. Saturation occurs at intensities equal to 65535.

VIII. Imaging in XY

1. For a quick XY snapshot from a **single channel**, activate the channel of interest with previously saved settings and click on the bottom **Capture a Single Frame**.



2. For XY snapshots of multiple channels, set up an experiment protocol on **Video/ Acquisition setup** or right click to select Acquisition setup. Then press **Ok** and **Start the experiment protocol**.

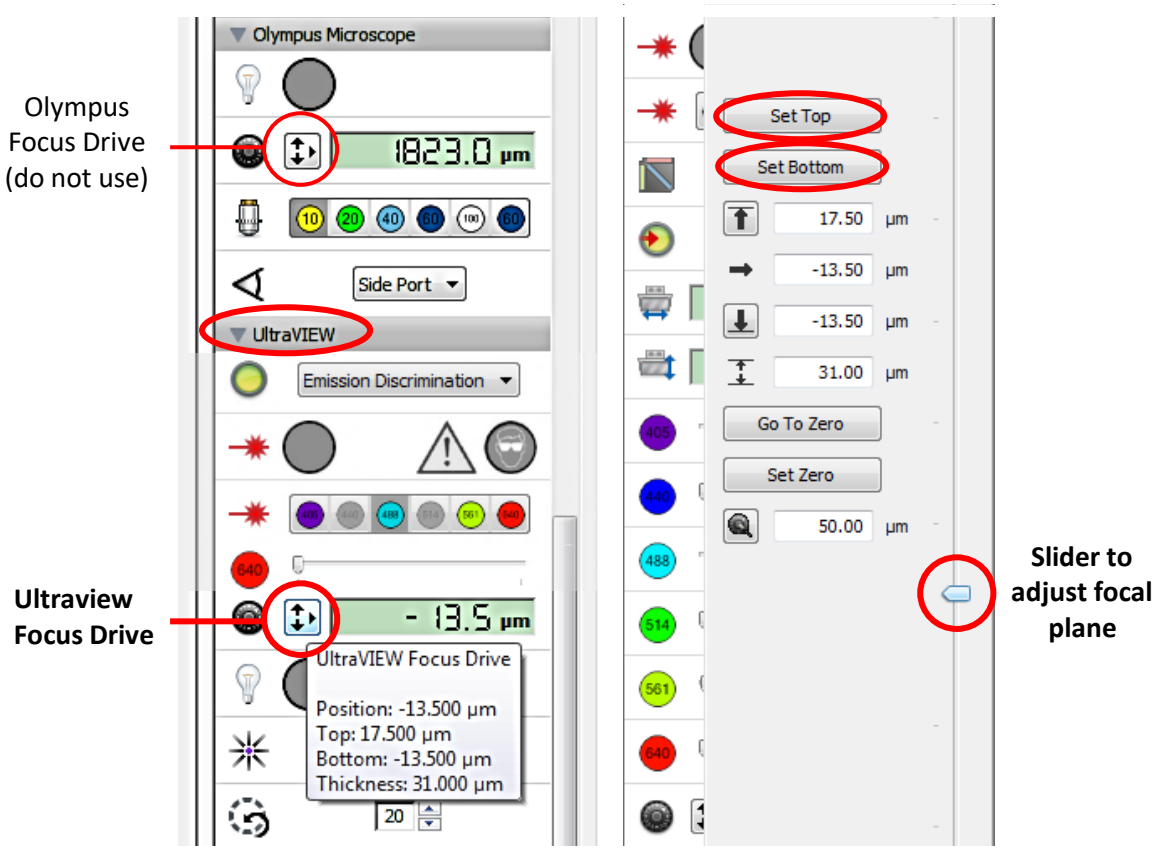


IX. Imaging in XYZ

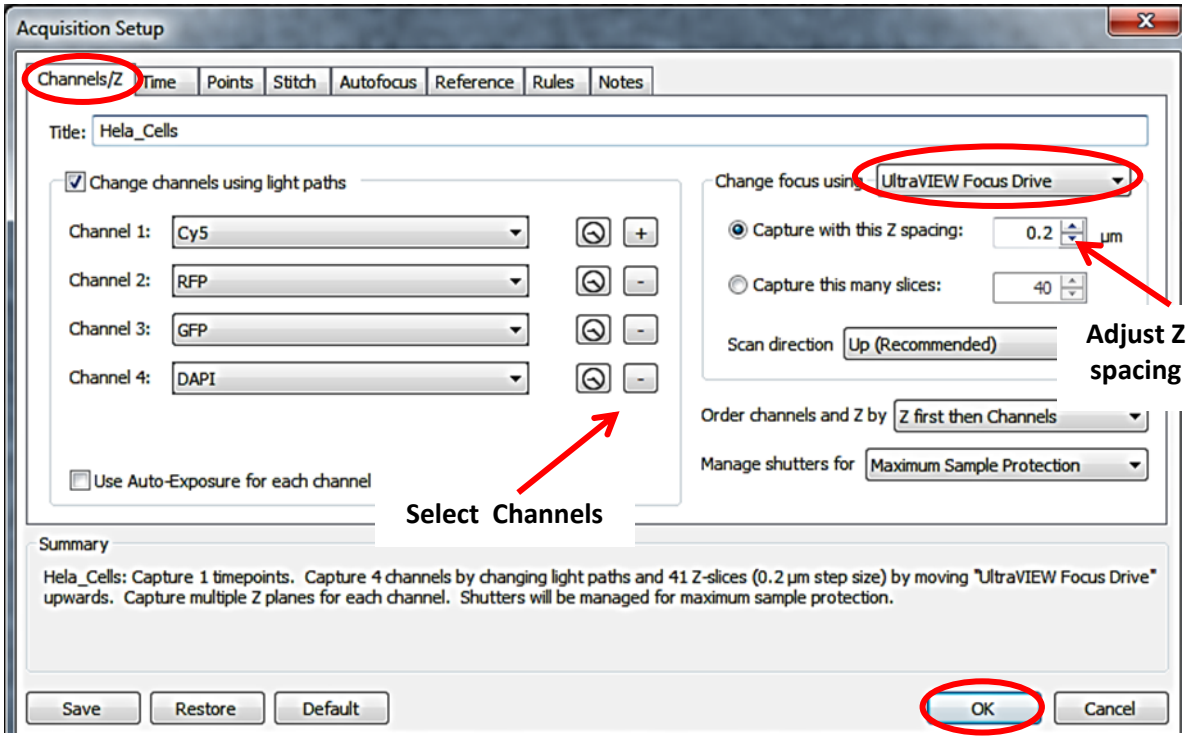
1. Resume video preview and set Z planes under the **Ultraview Focus Drive**. Click on the knob to display Z position, and set Top and Bottom Z planes by **sliding the adjuster**. Do not adjust the focus with the microscope focus wheels.

Note: The system has two different and independent focus drives: Olympus Focus drive (microscope focus wheel) and **Ultraview Focus drive** (Ultraview stage insert). We recommend using the later due to stability precision.

Tool options (necessary ones in **bold**):

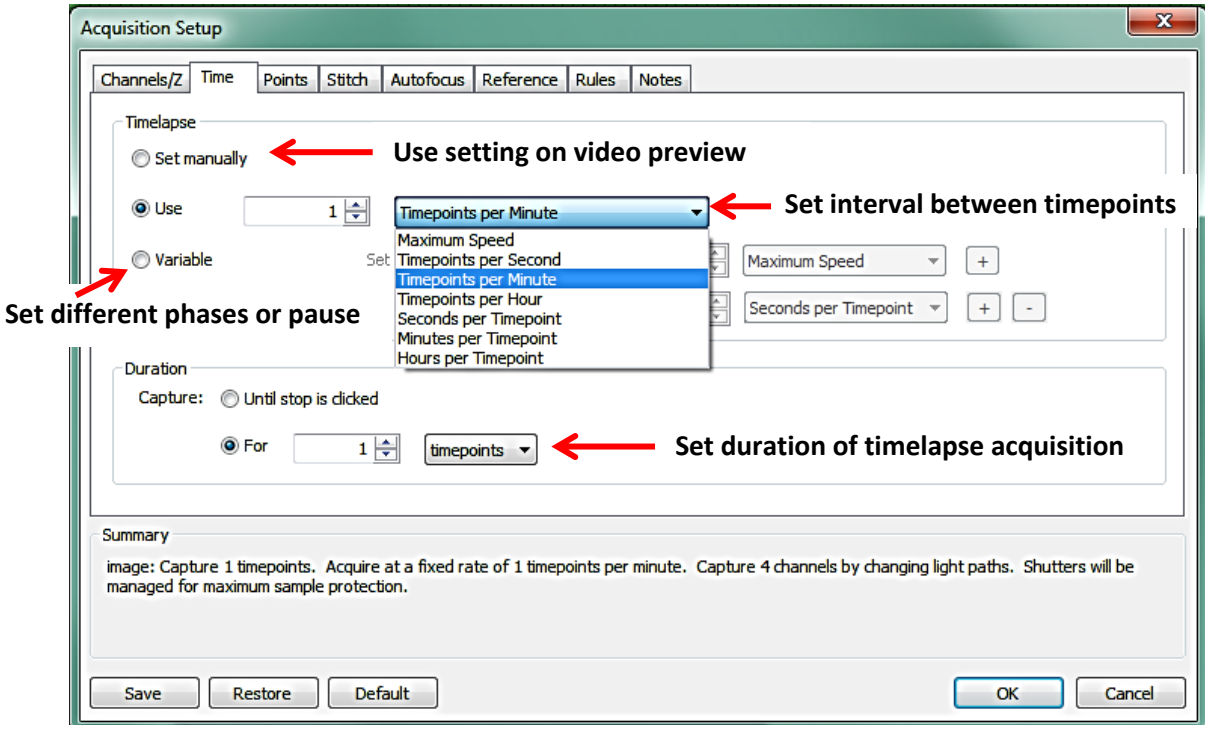


2. Update experiment protocol in **Acquisition setup** to include Z- stack with Ultraview Focus Drive. Start the experiment protocol.



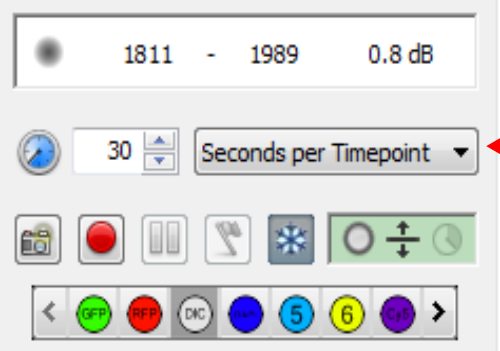
X. Set up time lapse experiment

1. Resume video preview to adjust the settings for each channel. Set Z planes if required.
2. Go to **Video/ Acquisition setup** to adjust settings under the **Time** tab. Alternatively, select "Set manually" and use the appropriate knob on the video preview (see below).



Examples:

- 1 timepoint every 10 seconds: select **Seconds per Timepoint** from the drop-down menu and enter 10 into the field.
- Add a **pause**: click on the clock icon or add a different phase in the "+" menu. Select 0 timepoints per second and define the length interval of that phase.
- **Maximum speed:** Volocity acquires images at the fastest rate supported by the hardware.

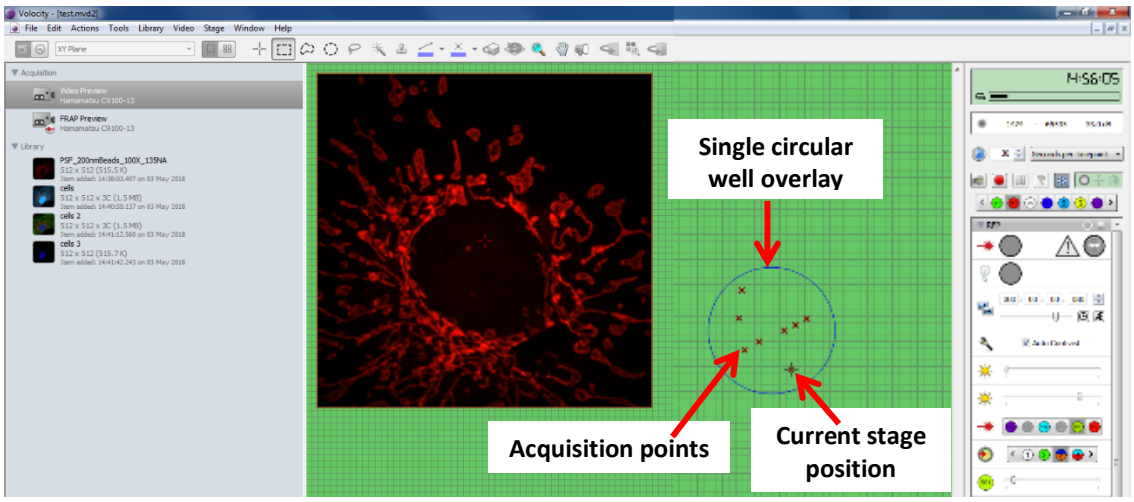


Alternative way to set timelapse experiment

XI. Set up multipoint imaging

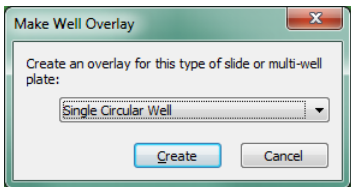
1. Select **XY stage view** under Video menu or with shortcut Ctrl+Shift+X.
    - green area represents the full working area of the stage
    - each square represents one field of view
    - grey crosshair represents the current position of the stage
    - move the stage with joystick or by double clicking on the point to which you want the stage to move

2. Add **Overlays** (blue shape) and acquisition **Points** (red crosses) in the stage area to configure the area or fields of view you want to image. **Overlays** allow you to easily define the boundaries of your chamber, whether you have your sample mounted on a multi-well plate, petri dish or coverslip.



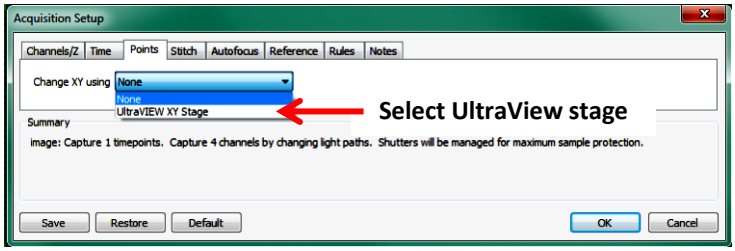
- Go to **Stage/Make Well Overlay** to create an overlay. Select your type of dish/slide from the drop-down menu and click **Create**. A user-friendly wizard will guide you through the steps to create the overlay. Move the stage accordingly and click **Next**.
  i. You can **Save** or **Restore** Overlays from the stage menu. Select individual wells or group of wells with the ROI tool (Shortcut key R) and select **Disable Selected wells/Enable Selected wells** from the Stage menu.

Note: We recommend you to use a *smaller overlay* than your actual sample to avoid problems with imaging on the borders.



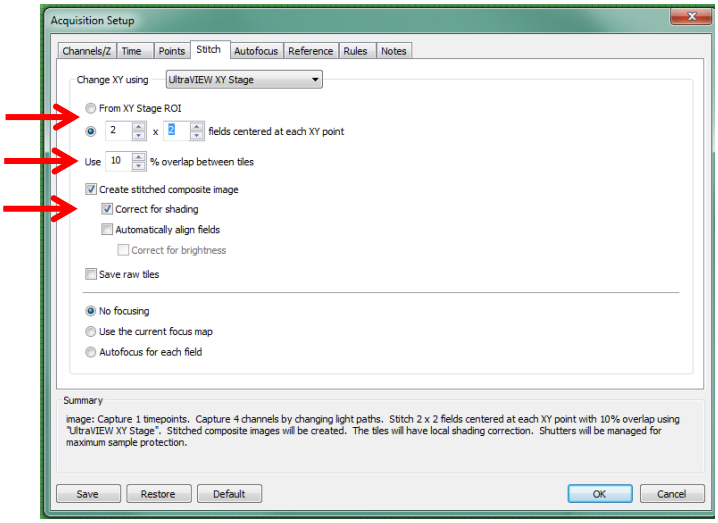
- To add **acquisition points**, you should first focus on your specimen and **Set Zero** on the Ultraview Focus Drive.
  i. You can add points *manually* by moving the stage and clicking **Add point** on the Stage menu with the shortcut **Ctrl+Shift+A**. Each point is shown as a red cross on the XY Stage view.
  ii. Volocity can generate *random* points for you with the option **Create Points from well overlay** from the Stage menu. Select from the drop-down menu **Multiple Random Points** for single acquisition points or **Multiple tiles points** to add a matrix of multiple points at the centre of each well.
  iii. You can Inspect and Edit points. To navigate through individual points use the commands **Go to Next Point** or **Go to Previous Point** on the Stage menu. You can *change the focus of a point* and save on **Update Point** from the stage menu. Alternatively use the Wizzard **Review Points**; you can review and *adjust the focus* at each point, and the current focal position is stored with each point when you select **Next**.
  iv. You can **Save** or **Restore points** from the Stage menu.

3. Go to Video/ Acquisition Setup/ Points and select Change XY using Ultraview XY Stage to include point visiting in your Acquisition experiment protocol.



XII. Stitching

1. Create a large image made up of multiple fields of view (tiles) stitched together in **Acquisition Setup/Stitch**.
2. You can previously set a **XY Stage ROI** with the ROI tools (dashed square, circle, or free shape; shortcut key R) or simply select number of rows and columns to automatically **draw a matrix** centred in your current position or at each XY point.
    - Keep 10% overlap between tiles
    - Tick the option **Create a stitched composite image** and **correct for shading** to remove borders between tiles.



XIII. Data handling and storage

The PE spinning disc saves the Library in the .mdv2 format which can be opened directly by ImageJ FIJI via Bioformats plugin.

No files should be kept on the local machine for longer than is strictly necessary (the hard drives are liable to be wiped of data without warning).

The preferred option is to upload files to OMERO using the OMERO Insight client installed on the machine. You will already have an OMERO account with your Biochemistry account.

You may alternatively transfer files to your Micron space, which can be connected to by mapping a network drive to: \\Micron1\username , where username is your University single sign on (SSO).

You should keep enough time at the end of your booking to transfer your data.