

Nasmyth Ultraview Vox User Protocol

Switch on all wall sockets labelled Nasmyth, switch camera on (power supply located on table behind monitor), switch on laser switch in laser rack, switch computer on if off. The incubator heater is on by default. It is best to leave the heating on at 25 °C even if you don't need it. Change the temperature to 37 °C if necessary. Log in with your Biochemistry login.

When you have finished imaging: log off but leave the computer on. Switch off laser switch in laser rack, switch camera off, Leave heating on at 25 °C.

Start Volocity (currently 6.0). Create a new library or open an existing library. (It is better to create a new library for each session as they become very big and have to be moved as one entity.) Libraries have to be saved in My Documents and then moved onto an external hard drive.

Open Video Preview. If no preset channels appear, close Volocity, double-click Default-volocity-setup.reg on the desktop and restart Volocity. This registry file can be used whenever you want to get back to the default settings.

In the top menu under Video click on Live Video instead of XY Stage to switch to large camera view.

Change objective in the side menu under **Olympus Microscope**, make sure turret is in its lowest position before you do so. The turret can be moved manually on the microscope. XY positions are controlled through the joystick.

In Volocity the z-drive is controlled in three locations:

1. **Olympus Microscope**, microscope z-drive (Olympus Focus Drive), which is useless for most applications except for microinjections where the piezo z-drive cannot be used
2. **Ultraview**, piezo z-drive, which has to be used for acquisition. (In Acquisition Setup the z-drive has to be set to Ultraview in Change focus using...). Click on arrows icon, move arrow and Set Top, Set Bottom. Make sure your zero position (Set Zero) is somewhere around the centre, otherwise correct it by hand on the microscope and set it again in UltraView.
3. **Most Used**, piezo z-drive as above

You have to find your cells either in Brightfield/DIC (see below under DIC) where you can use the eyepieces or by using the lasers and the camera because there is no fluorescence light source on this system.

There are **pre-set channels** for (Fig. 1):

GFP: 488 nm laser line

RFP: 561 nm laser line

DIC: Brightfield light is controlled in Volocity, DIC channel has to be chosen and bulb icon (1 in Fig. 1) clicked (there is no good DIC on this system because the addition of the required analyser would in the current set-up remove half the fluorescence signal)

DAPI: 405 nm laser line
 CFP: 440 nm laser line
 YFP: 514 nm laser line
 (There is a 640 nm laser line, but currently there is no pre-set channel for it)

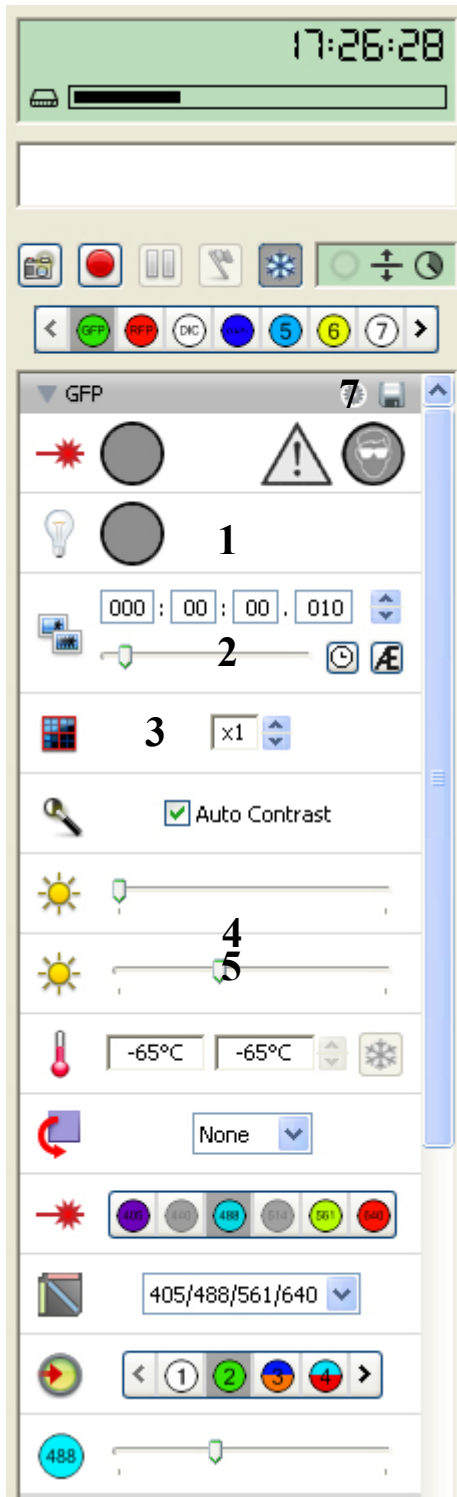


Figure 1.

For each channel choose exposure time (2 in Fig. 1), binning (3, has to be the same for all channels), gain (4) OR sensitivity (5) (see below under Hamamatsu C9100-13) and laser power (6). Save each channel before moving to the next one (7, disk icon next to channel tab).

Under [Hamamatsu C9100-13](#) (Fig. 2) the camera settings can be changed from the default EMCCD to Conventional mode (1). High Speed, Moderate Speed/Quality, High Quality (2) settings can be chosen. In EMCCD mode, use Sensitivity (3) to turn the multiplier up and increase your signal, do not use Gain (4) as this increases the background. In Conventional mode, Sensitivity does not work, use Gain to increase signal.

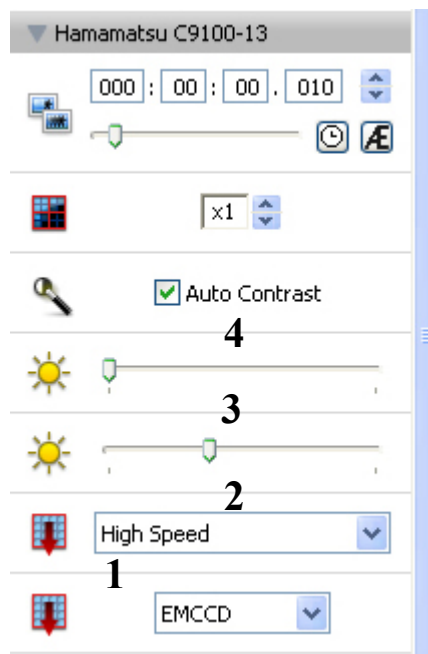


Figure 2.

If you want to acquire **6** several stage positions do the following:
 Calibrate the stage:

- In the Edit menu choose:
 - Preferences
 - X-Y Stage
 - X-Y Stage Device: Ultraview XY Stage

Focus Device: Olympus Focus Drive or Ultraview Focus Drive depending on what you are using for acquisition

Under the Stage menu click on Calibrate Stage (after removing your slide and making sure the **objective** is in its **lowest position**: press escape button on left hand focus drive or turn focus away from you until warning noise). Under the Video menu click on XY Stage. Choose your first position using the joystick, click on Update Point in the Stage menu. Choose your next position using the joystick, click on Add point. When you have chosen all your points you want to define the z positions. In the Stage menu click on Review Points. In Ultraview Focus Drive set top and bottom of your sample, then click Next to continue with the next position or Finish as instructed by the dialogue box.

[Set up your experiment in Acquisition Setup](#) in the Video menu:

Channels/Z

Add or remove channels (different channels can be acquired at different timepoints), choose z-spacing and REMEMBER to set Change Focus Using to Ultraview Focus Drive. Channels first then Z is best if you want to make sure that all channels are captured at exactly the same Z. Don't choose Don't Manage shutters, as this leaves the shutters open even between time points. Maximum Sample Protection closes the shutter after each exposure and Maximum Speed closes the shutter between time points.

Time

This should be self-explanatory.

Points

Change XY Using: None if you are imaging one stage position

Ultraview XY Stage if you are imaging several points/stage positions

FRAP

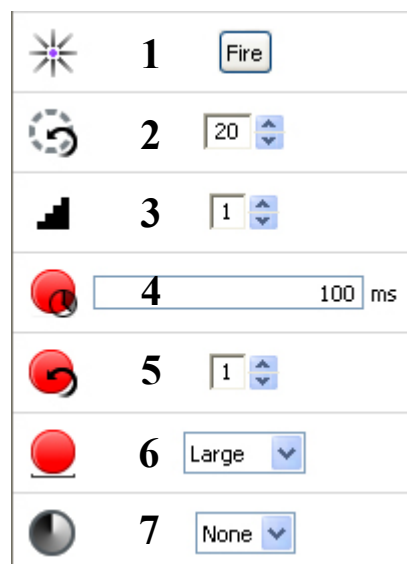


Figure 3.

UltraView PhotoKinesis Device (1): double-click to adjust laser power during bleaching, specify an ROI and click Fire to bleach for testing

UltraView PK Cycles (2): define the number of bleach cycles for ROI

UltraView PK Step Size (3): If set to 1 every line is bleached, if set to 2 every other line is bleached and so on

UltraView PK Spot Period (4): Set ms bleach time for spot bleaching

UltraView PK Spot Cycles (5): Set the number of times the spot is bleached for set number of ms

UltraView PK Spot Size (6): Choose between small (700 nm using 100x objective is the smallest

option), medium and large

UltraView PK Attenuation (7): For laser powers from 1 to 100% choose None. If 1% laser power is too strong (for photoconversion and photoactivation experiments), Low will result in 0.1% and High will result in 0.01% laser power.

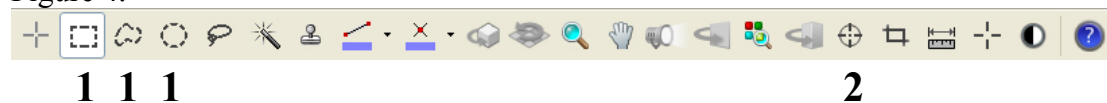
First [calibrate the system](#) using a fluorescent slide (marker pen) located in a small orange container in the top drawer of the drawer unit.

Open FRAP preview, right mouse click for drop down list or look in FRAP drop-down menu: Calibrate UltraView Photokinesis Device

Set exposure to 100 ms, set gain to 1, set 488 nm laser power to 2% and save settings

Start calibration as instructed by the calibration wizard. When the coarse calibration is complete (the wizard will tell you) mount the marker pen slide on the microscope and focus in FRAP preview through the camera with Auto Contrast clicked. It does not matter if the slide is already mounted during the coarse calibration. Adjust the PhotoKinesis Device laser power by double clicking the Fire button and moving the 488 nm slider to 100%. 10 PK cycles should be set. Define an ROI (see below) and click Fire to check if the bleaching is strong enough. Click to the left or right of the camera window to lose the ROI and bring the bleached area properly into focus (sharp edges). Move the bleached area to the left or right third of the window. Now follow the wizard, which will bleach an area and mark it with an ROI. Both have to overlap perfectly. If they do, click Finish. Otherwise fine-tune the calibration by adjusting the position of the ROI and clicking Update. Follow the wizard to Finish. When you save the updated calibration you will get an error message (Command could not be completed because access is denied), which you can ignore. Test whether your bleached area overlaps the ROI in different parts of the window and position your ROIs for the experiment where you achieve the best overlap.

Figure 4.



[Set up the conditions](#) you want to use for the bleach using the PhotoKinesis buttons explained above and the conditions for pre-bleach and post-bleach imaging as you would for any live-imaging. Use the ROI tools (1 in Fig.4) or the Spot Tool (2) to define the area(s) to be bleached. The bleaching step is only done in one section. Bleaching of several ROIs and one spot at the same z-position is possible. Z-stacks are possible for pre- and post-bleaching imaging. In UltraView piezo-drive Set Zero to define your Bleach section, Set Top, Set Bottom to define stack size.

[Set up a FRAP experiment](#) using Acquisition Setup the FRAP menu:

FRAP:

Bleaching device to use: UltraView PK Device

Bleaching device light path: choose your channel

Acquire pre-bleach images for: ideally often enough to a) get rid of initial strong loss of fluorescence and b) to see linear fluorescence loss over time

Acquire recovery images: to suit your experiment (the first post-bleach image is taken directly after bleaching and the following at your chosen intervals)

Number of bleach/recovery cycles: for FLIP several cycles are used

Channels/Z:

Change focus using: UltraView Focus Drive

Use F6 to start recording a PhotoKinesis experiment.

Preset objectives and pixel sizes with the currently installed 2x magnifier:

10x objective: 0.0837 μm

60x NA1.4 objective: 0.139 μm

60x NA SI objective: 0.138 μm

100x objective: 0.0826 μm