



The Nikon TE2000 Inverted Microscope

Fluorescence image acquisition using NIS-Elements [basic research] and the B&W Hamamatsu Orca C4742-95 camera

INSTRUCTIONS

PLEASE HANDLE CAREFULLY!

Handle the microscope gently, taking care to avoid sharp impacts.

1) TO SWITCH-ON THE MICROSCOPE AND LOOK AT YOUR SAMPLE:

Bright-field Microscopy Imaging :

- Turn on the transmitted light by turning on the switch on the Nikon power supply box on the bench to the left side of the microscope.
- Press “lamp on/off” button at the base of the microscope on the left.
- Adjust the light intensity by rotating the control dial situated on the left side of the microscope, below the on/off switch.
- For bright-field [non-fluorescence] transmission imaging: Check that the “**D**” (Diffuser) and “**HA**” (Heat Absorber) filters at the top left of the microscope are pushed in. Select the optional neutral density **ND filters** as necessary to dim the light intensity [top right]: **ND16** and **ND2** – push both in for maximum light attenuation, typically the best setting.
- The “eyepiece turret” dial should be set to **O** (open), when set to **C** it is closed.
- The "port/optical path" dial on the right side of the base should be set to **1**. All the light now goes to the microscope eyepieces and none to the cameras.
- The "reticule in/out" lever at front of the base to the right should be turned clockwise and set to a 2 o'clock position. This lever is under the colour camera.
- The filter wheel located underneath the objective wheel should be on the empty position indicated by the double arrow symbol (↔).

- Place the culture vessel or glass slide on the stage.
- Move a low power objective (e.g. **4x** or **10x**) into the light path.
- Focus on the specimen. Adjust the condenser to the correct height for Koehler illumination¹. Ask core staff for help with this if necessary.

Please take extra care to avoid sharp impacts when switching between objectives. You should drop the height of the objective and then refocus.

- Have the condenser dial set to A for stained cells/tissue, or to Ph1 for phase contrast when using the 4x, 10x and 20x objectives or to Ph2 for phase when using the 40x and 60x objectives.
- If microscope slides are to be viewed, they can be placed with the cover-slip side either up or down [with the **60x** objective place slides cover-slip down]. Focus with the coarse focusing wheel and revolve to objective **20x** or **40x** if required.
- Adjust the 20x, 40x or 60x objective **correction collar** for the optimal resolution².
- A 60x oil objective and a 1x air objective are also available if required [see Appendix One]. These must be fitted by the Core staff. *If you need to use either objective, please ask a member of core staff to set up the microscope correctly for you.*

¹ With multi-well plates Koehler illumination may not be possible as the condenser might have to be moved higher to prevent it hitting the top of the plate. There are two white arrow markers on the condenser to show the approximate height position for Koehler illumination.

² With the 20x to 60x objectives the correction collar should be set to the thickness of the support material: e.g. approx. 2mm for slides (cover-slip up) or 0.17mm for the thickness of a 0.17mm cover-slip (cover-slip facing down). The correction collars compensate for differences in vessel base or slide/cover-slip thickness. The objective collars can be adjusted by rotating them, and used in conjunction with the normal focus, an optimal image can be obtained. Please ask Core staff for help with adjusting correction collars [details of the correction collar settings are given in the 'objectives' info sheet and on-line].

The 60x oil objective with DIC [Differential Interference Contrast] Microscopy:

- Ask a member of core staff to attach the **60x oil objective** and set up the transmitted light DIC optics [if required].
- Have the condenser dial set to **DIC H**.
- Slide in the **polariser P**→ filter [located above the condenser turret] to the left.
- Push in the black **T-A analyser slider** [located under the objectives & filter wheel, to the left of the microscope base].
- Place microscope slide cover-slip side down. Move a low power objective (4x or 10x) into the light path. When you have found an area of interest carefully revolve to the 60x oil objective.
- Place a drop of oil on the objective. *Make sure you use the correct oil. Only Cargille oil should be used with this objective.*
- For correct **DIC** you should have set the condenser height correctly for Koehler illumination. You then rotate the analyser and slide in/out the DIC prism [using the mini-slider under the **60x objective**] to give the best contrast enhanced image.

Epi-Fluorescence Microscopy [with the Hamamatsu Camera³]

- You can use IP Labs software with the B&W Hamamatsu Orca camera as well for fluorescence imaging. IP Labs already has a large user base in the Centre and you may find it an easier program to use. However NIS Elements can also capture fluorescence images using the sensitive [and low noise] B&W Hamamatsu Orca camera, and recently there have been many useful updates to NISElements.
- Use this guide if you intend to use NIS Elements for fluorescence imaging. There are separate in-house guides for fluorescence imaging using IP labs software with the Hamamatsu camera and for full colour imaging using NIS-Elements [Basic research] with the Nikon DS-5M colour camera.

³ You can use the Nikon Colour camera for fluorescence image capture, but the image quality may be very poor if the fluorescence signal is weak. Only the Hamamatsu B&W camera can capture fluorescence images with low noise (no speckles) and it's the recommended choice this – fluorescence 'colour' is applied by look-up tables [LUTs].

The mercury fluorescence arc lamp

- Note the last switch-off time in the notebook beside the microscope. *The arc lamp must be left off for at least 30 minutes to cool down before it is switched on again.*
- Switch on the illuminated power switch on the front panel of the mercury lamp power supply box (on the bench on the right side of the microscope) and make sure the "power" monitor light (green) is on. **Always switch the mercury lamp on and off with the PC, LCD display, microscope and camera switched OFF.**
- Press the ignition button for 2-3 seconds to light the lamp. Hold it down until the orange "lamp ready" light stays on. Leave the lamp to warm up for few minutes. *Leave the mercury lamp on for at least 30 minutes before switching it off.*
- Unless you also capturing phase contrast or transmission images as well, the height and settings of the condenser above the stage are irrelevant for epi-fluorescence imaging.

Fluorescence Filter Sets

Optical filters are required in the microscope to ensure appropriate excitation of the fluorophore. These dichroic filter sets are moved into the fluorescent light path by rotating the fluorescence filter wheel, which is located underneath the objective wheel [nosepiece], on the right side of the microscope base.

The following filter sets are available:

- | | | |
|----------------|------------------------|--|
| • DAPI | Hoechst | - Blue emission colour [violet excitation] |
| • FITC | GFP, Alexa-488 | - Green emission colour [blue excitation] |
| • TRITC | Rhodamine , Alexa-546 | - Red emission colour [green excitation] |
| • CY5 | CY5, Alexa-633 | - Far Red emission colour [red excitation] |
| • ↔ | Transmitted light only | - Fluorescence off [Phase Contrast] |

For “transmitted light” the filter wheel is normally set to the empty position indicated by the double arrow symbol (↔). Note: the excitation light colour is seen at the specimen and the emission light colour is seen down the microscope via the eye-pieces , although CY5 emission can only seen by the microscope’s Hamamatsu fluorescence camera.

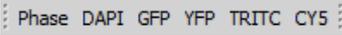
- During fluorescence microscopy, **turn off the bright-field** halogen light by using either the switch on the left side of the microscope main body, or by rotating the transmitted light intensity knob anti-clockwise to zero.
- Rotate the fluorescence **filter wheel** to select the desired excitation filter set [**DAPI, FITC, TRITC or CY5**].
- There is a fluorescence light shutter underneath the filter wheel (at the right hand side) that needs to be opened. Set to the **O** [open] position for the **fluorescence light** to pass to the objectives. In the **C** position the shutter is closed. The shutter should be kept closed [**C**] during transmitted light microscopy.

- Adjust the fluorescence light brightness by inserting the ND filters (**ND4** and **ND8**) into the optical path. These are located near the rear **UV lamp housing**.

2) TO START THE HAMMATSU CAMERA AND CAPTURE IMAGES:

- **Switch on** the Hamamatsu camera by pressing the switch on the large **camera power supply box** next the 24" computer monitor. Turn on the computer (PC on the floor under the bench) and its 24" LCD monitor (button top right edge).
- If you are a "Well" user **log on to the system and network** with your usual username and password. Alternatively, log on directly to the PC as "User" with the password "cyto02" or as 'generic'.
- Double-click on the NIS Elements [Basic Research]  icon on the desktop:
- Scroll down the pop-up camera driver window and choose the "**Hamamatsu**" for fluorescence B&W image capture. You can also select the '**Nikon DS-U1**' for capturing colour images, or '**none**' just to view saved images. Click OK [any camera selected must be switched on].
- Once you have found an image down the microscope that you wish to take a picture of, the port/optical path dial (on the right side of the base of the microscope) needs to be moved to **Port 5** for the **Hamamatsu** camera [**Port 5 is camera only**, with no light to the microscope eye-pieces].
- To view the sample via the microscope **eye-pieces** again, return the **Port** to **1**.
- You should see the **Orca-285 Settings** control window (camera controls differ depending on the camera selected⁴). If that window isn't there, right click over the non-image area and select '**Acquisition Controls, Orca-285 Settings** [Ctrl_Alt_C] to activate it. Or you can also use **View, Acquisition Controls, Orca-285 Settings** within the main menu.
- From the main menu, click the **Live (+)**  button, or select **Acquire, Live Fast** or **Live Quality** to open up a live image window on the screen. The "live image" window appears. This live video window is intended to allow you to set the photo exposure time and put the image in focus while viewing it on the computer monitor.
- You can modify the live image by altering the **Orca-285 Settings** options (image acquisition parameters), e.g. hardware gain, exposure, command and advanced setup.

⁴ Note, if you use the **Nikon DS-U1 colour camera** with NIS Elements, you will get a very different set of options under **Camera Settings**. See the specific Nikon colour camera in-house Core guide for more details.

- Select the correct nosepiece objective  via NISElements software using the **Manual Microscope Pad** [cntrl-alt-M]. Set the microscope zoom to 1.0x or 1.5x using the  tab in the top menu. This will calibrate the image in $\mu\text{m}/\text{pixels}$ allowing a scale bar to be added - using the **Show Scale**  button on the left of the live image window.
- The Phase, DAPI GFP, YFP, TRITC and CY5 buttons  below the main menu bar can select and add the correct colour overlay [LUT] to the B&W image, e.g. green for GFP and grey for Phase.

In addition the LUT colour can be switched to grey or back to colour via the **LUTs** panel tab [Cntrl-Alt-L].

- The bright grey colour scheme of the NISElements software can be changed to a dark graphite colour if wished, using the sun/moon tab  at the top right of the screen.

Exposure settings

- Adjust the camera Manual exposure time as required via the drop-down. The time is listed in milliseconds to seconds. A **slower** exposure time will **brighten** the image. You can set the exposure using the **Auto Exposure** button.
- With Manual exposure, set the camera hardware **Gain**⁵ to **1.0**.
- Clicking the “**Pixel Saturation Indication** (Cntrl-Shift-S)” button  will enable you to see pixels that are saturated [over-exposed & overlaid bright red]. Click the **View, Visualization Controls, LUTs** to bring up the LUTs panel.
- Use a consistent manual exposure time if you want to compare samples (or the software might automatically brighten/darken inherently dark/light images). Also ensure other settings (e.g. Gain, Binning) are consistent [Orca-285 Settings]. Make a note of your settings – the camera settings will be saved with the image if you save in formats such as **tiff** or **nd2**, but not with **jpg**.

Capturing a bright-field image

- View a live image using the **Live (+)** button  below the top menu bar. Capture the image using the **Capture** button  (cntrl+ or -).

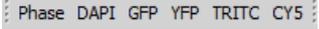
⁵ Avoid increasing the camera gain too high as it will introduce excessive noise. With bright-field images you can increase the halogen lamp brightness instead. Higher camera Gain can help with dim fluorescence samples by reducing camera exposure time (faster video refresh). You can also ‘average frames’ to reduce noise using **Acquire, Average**.

- On capture the “**Freeze**” button  below the main menu will freeze the video preview without closing the window. Clicking the **Live(+)**  button again will return the window to a live image for focussing and exposure adjustment.
- To capture a transmitted light [bright-field] picture, click on the **camera** icon [Capture  (Ctrl+)] in the **Live** window. The ‘Phase’ button  should be selected.
- This will acquire a high resolution image at the **Orca-285 Settings, Format for Capture** resolution⁶ e.g. 1344x1025 pixels [no binning] or 672x512 pixels with 2x2 binning. The live image is may be set to a lower resolution, i.e. 672x512 pixels [2x2 binning] to make the live image more responsive during focussing [**Format for Live** under **Orca-285 Settings**].
- Capturing an image freezes the ‘Live-Fast’ window, so click the video camera **Live(+)** button  to restore the live video image.
- If both the Nikon colour and Hamamatsu B&W cameras are switched on, you can swap between them via the main menu **Acquire, Select Driver**.

TO CAPTURE AND SAVE B&W FLUORESCENCE CAMERA IMAGES:

- Ensure the [phase contrast] bright-field halogen lamp is switched off.
- Right side, under the microscope objectives: Move to the correct fluorescence filter set: DAPI (**uV** - Blue), **GFP** (Green), TRITC (**Red**) or **Cy5** (Far Red). Move the mercury lamp shutter to O [Open]. Focus on the specimen [Port set to “**1**”].
- Switch to the Hamamatsu Orca B&W camera at **Port “5”**, which sends all the light to the camera.
- Click the Live  button (+), or select **Acquire, Live Fast** to open up a live image window on the screen. The “Live - Fast” dialog box appears. Set the photo exposure time [**Orca-285 Settings**] and put the image in focus while viewing it on the computer monitor.
- You can use **Acquire, Capture Multichannel Image, Multichannel setup** to set the software for the sequence of fluorescence images your wish to acquire, e.g. tick DAPI [blue], FITC [green], YFP [yellow], TRITC [red] or CY5 [purple] as required – you only need to do this once.

⁶ The Hamamatsu camera captures **12-bit B&W** (4,096 grey levels) images by default, with a maximum resolution of **1344 x 1025** pixels [with binning set to **1x1**, i.e. **no binning**]. This can be reduced to **672 x 512** pixels with binning set to **2x2** – where every four pixels of the CCD are combined, doubling camera sensitivity and halving resolution. **Camera binning** is adjusted in the **Orca 285** window, under the **Format for capture** tab. Generally leave binning to 1x [the camera’s maximum resolution]

- Acquire a fluorescence image sequence using the **Acquire, Capture Multichannel Image, Capture Manually** menu option. The Live-Fast window will open. Click the **Camera** [Capture (cntrl +)]  icon. The software will acquire a phase and fluorescence image sequence, pausing at each channel [fluorochrome] to allow you adjust the camera settings [exposure] and focus. Use the ‘Next multichannel capture button’ to capture the next image. A **multichannel image** will be manually captured, e.g. a colour combined Phase, DAPI, FITC, and TRITC fluorescence image [**All**].
- Select the ‘**In colour**’ button under **LUTs** can apply the correct fluorescence colour overlay (e.g. green for GFP) or to view the image in B&W grey.
- You can alternatively capture a single channel fluorescence B&W image using the method outlined on the previous page for capturing a bright-field image. Simply click the Phase, DAPI, GFP, YFP, TRITC or CY5  button below the main menu bar and the live view will be set for that fluorochrome and add the correct colour overlay [**LUTs**] to the B&W image, e.g. green for GFP and grey for Phase. Use the **Camera** icon  [Capture (Ctrl+)] to capture an image. Capturing an image freezes the ‘Live-Fast’ window, so click the video camera **Live(+)** button  to restore the live video image.
- The colour look-up-tables [**LUTs**] are applied to a 12-bit 4,096 grey levels B&W image. If you save it as a Tiff file the image will still be 12-bit and appear black in Photoshop – saving a jpg will convert the image to 8-bit [256 grey levels] and overcome the problem. Alternatively you can use freebie NISViewer, ImageJ or MetaMorph to view to the 12-bit B&W image. You can convert the image from a 12-bit B&W to an 8-bit B&W or RGB colour image within NISElements using the ‘Image, Convert’ menu options (e.g. Change bit depth, Convert to RGB). *Ask if you need assistance.*
- You can adjust the image contrast by grabbing the grey level histogram sliders in the LUTs panel . This can remove background fluorescence in the dark regions of the image by pushing dark grey pixels to black. To make the contrast adjustment permanent click on the LUTs ‘Modify Image Through LUTs’  button. Load the LUTs panel using Cntrl-Alt-L.

TO SAVE THE FLUORESCENCE CAMERA IMAGES:

- Go to File, Save As. Save in either TIFF [uncompressed for archiving, editing, image analysis] or jpg [compressed for email, PowerPoint]⁷. If you save in JPEG2000 or ND2 probably only NIS Elements can read the format.
- Save images in your own folder in “**User Images**” on the **E:** [User Data] drive.

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Images are **12-bit** black and white (4,096 grey levels) for this Hamamatsu camera. You can use ImageJ: Image, Type, to convert these **12-bit** images to **8-bit** [256 grey levels] B&W, if you want to view them correctly in PhotoShop or in PowerPoint. **JPEG2000** is a new [more efficient compression] licensed jpg format that may not be read by some programs - Photohop CS4 requires a free plug-in.

TO FINISH (...and go home):

- Close NIS Elements. Switch off the camera power supplies. Press the halogen transmission lamp on/off button at the base of the microscope on the left and turn off the switch on the lamp power box.
- Switch off the computer *before you switch off the mercury lamp*. Leave the 24" LCD monitor in standby.
- Before turning off the mercury lamp *allow the fluorescence mercury bulb to run for a minimum of 30 minutes* and check with the on-line booking that nobody else will be using the microscope in the next few hours.
- Switch off the power switch on the mercury lamp power box and note your name, the time off, date and indicated hours, in the notebook provided.
- If you used the **60x oil objective** please remember to wipe off the oil and inform a member of core staff when you have finished so that the objective can be removed.

The 60x microscope oil objective should only be cleaned with the lens tissues provided. The standard Nikon air objectives don't need cleaning after use.

If you have any microscope/imaging problems or queries, just ask a member of the Microscopy Core staff for help. There is additional help and advice on our Core web-pages: <http://www.well.ox.ac.uk/microscopy>.

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TROUBLESHOOTING

If you get imaging problems, check the following:

Is there a shadow in the image with transmitted light illumination?

If you are using bright-field microscopy the condenser must be adjusted correctly to Kohler illumination [otherwise you might see the dark shadow of the phase rings in the centre of the image and get poor image quality]. Ask a core member how to set up Kohler illumination on the microscope.

Alternatively an incorrectly set filter slider or other component may be obscuring the light path.

Is the mercury bulb or transmission bulb turned on?

Check the orange and green lights on the power boxes and the microscope white button/brightness control. Check light can be seen at the specimen.

Can you see a coloured light passing through the bright field [phase-contrast] sample?

Move the filter wheel to the bright field double arrow (\leftrightarrow) position.

Is the fluorescence filter block in place? Are you using the correct filter block – do you need it ?

Rotate the filter wheel and see if any light of the right colour appears.

Have you checked the dial below the eyepiece?

It should be set to O.

Is the objective correctly positioned?

The objective wheel should gently click into position.

Is the port/optical path in the correct position?

Port 1 is for observation down the microscope. Port 5 is for the Hamamatsu B&W camera only. Port 4 is split 50/50 between the microscope and the Nikon colour camera.

Is the transmission light too faint or too bright?

Remove/Add the neutral density filters. Ensure the top 'p \leftrightarrow ' polariser and the black T-A polariser slider [left side, under the objectives] aren't pushed into the light path unless you need them.

**FOR ANY QUERIES OR DOUBTS, JUST ASK
A MEMBER OF THE CORE STAFF**