Please sign on the log sheet before switching on system.

**Turn on system**
- Turn on **A** only if confocal mode or laser is needed. Widefield application can omit this step.
- Switch on main power control ①
- Switch on microscope controller ②
- Turn on computer power ③

- Click to log into USER at the startup screen
- Start the MetaMorph software
  - For widefield users, please click MetaMorph WF icon
  - For Confocal/Laser users, please click MetaMorph CSU icon

**Set the temperature and CO2 control for live cell imaging**
(Only applicable for live cell imaging, please skip this step if it is not needed):
- Switch on "Incubator" for temperature and CO2 control.
- Switch on the Power of Tokai Hit incubation system controller. Temperature can be altered via pressing the green button of each heating parts on the touch screen.
- Make sure the CO2 sliding button is turned ON.
- Turn on CO2 tank by turning the main switch anticlockwise.
- Turn on CO2 regulator by turning regulator clockwise to set output pressure at 100kPa.
- Turn on tube switch for TIRF ➔ Put on objective heater on objective if oil objective is used.
- Metal ball floats is an indication of the presence of CO2 gas.
- MilliQ water has to be added into the water chamber and covered if overnight(s) acquisition is required.

Key in the temperature of interest ➔ Enter

Make sure the metal ball is floating
Sample locating and focusing

- Select **objective lens**. Apply a drop of immersion oil if **60x** oil and **100x** oil objective lenses are used.
- Place your sample, make sure the coverslip bottom is facing down (slide/dish/chamber slide)
- To view under the microscope, go to **Eyepieces → select fluorescence channel → Current Shutter**.
- The intensity of each channel can be adjusted by clicking one channel and inserting a value of interest.
- The brightness of the image and the amount of stray light can be adjusted via the **aperture diaphragm lever**.

![Image showing microscope settings](image)

- For Brightfield, click **Trans → Current shutter** (can press on Bright Field LED button as an alternative) → **brightfield brightness** can be adjusted using the knob.

![Image showing brightness adjustment](image)

- Press the right arrow of the **DISP** button to display the XY coordinates and Z position.
- Move the **Stage Controller** to adjust XY position (**XY speed** can be adjusted: [↑↓←→])
- Focus the sample with the **focusing knob** → Clockwise_Down; Anti-clockwise_Up (Focusing speed can be adjusted: [↑↓←→])
- Switch on the “**PFS**” and adjust the focus to lock the focal plane of interest.
Switching to Acquisition mode

For Widefield imaging:
- Click on **WF Camera** → Click **Live** in the “Multi Dimensional Acquisition” → Select a **channel** and click **Current Shutter** to view on the monitor screen.

For Confocal/laser imaging:
- Click on **CSU Camera** → Click **Live** in “Multi Dimensional Acquisition” → Select a **channel** and click **Current Shutter** to view on the monitor screen.

Image Acquisition
- Click **Multi Dimensional Acquisition** on the task bar
- Go to **Main** tab to set up acquisition configuration step by step. Check the box(es) of the application(s) as required.
- Click **Saving** → **Select Directory** (all data should be saved in E drive/USER under your name)
  - Type in the base name of your file (experiment or date or etc.) in **Base Name**.
  - Do not use digits at the end of the base name, a digit will be added according to the acquisition sequence.
  - Another suffix will be added for record time series image (t1, t2…) or multi-stage-position image (s1, s2…).
• If multiple fluorescence channels are required,
  – Check the box of "Multiple wavelengths" in the main menu
  – Click Wavelengths
  – Key in the number of channels in "Number of Wavelengths"

![Image of Multi Dimensional Acquisition settings]

• Select each wavelength to set the required “Illumination”.
For Widefield Imaging:
  - Select "WF DAPI Single" for Blue emission (such as DAPI)
  - Select "WF GFP Single" for Green emission (such as GFP)
  - Select "WF RFP Single" for Red emission (such as mCherry)
  - Select "WF Cy5 Single" for Far-Red emission (such as mCherry)
  - Select "Trans" for brightfield channel
  - Select "DAPI/GFP/RFP/Cy5 Quad" channel(s) only when stream application is required.

For confocal/laser Imaging:
  - Select “CSU DAPI” for Blue emission (e.g. BFP) channel
  - Select “CSU GFP” for Green emission (e.g. GFP) channel
  - Select “CSU RFP” for Red emission (e.g. mCherry) channel
  - Select “CSU CY5” for Far-red emission (e.g. Cy5) channel
  - Select “Trans” for brightfield channel

Channel Settings
For Widefield Imaging:
  - Select "W1" to adjust the first channel
  - Click Live at the bottom of “multi-dimensional acquisition” panel to have real time image
  - Adjust Gain and Exposure time to have optimum signal intensity.
  - Adjust Gain if necessary (1x, 2x or 4x)
  - Higher Digitizer value gives a higher camera readout speed.
  - Select "W2" and repeat the same procedure to adjust the second channel.
Timelapse

- Set up “Time interval” between each acquisition time point → Set the Duration of the entire experiment or Number of time points, either one will do → Click Acquire to start the acquisition.

Perfect Focus System (PFS)

The allowable PFS focusing range refers to the range defined for each objective (where PFS is usable).

- For glass bottom dish, focus on the sample near to the bottom surface of the sample vessel
- For plastic dish, focus on the sample near to the bottom surface of the sample vessel, and then move the objective down by about 1000um.
- The status of the PFS is displayed on the LCD of the controller or the front panel of PFS indicator.

<table>
<thead>
<tr>
<th>PFS indicator</th>
<th>PFS on/off</th>
<th>Shown on the display</th>
<th>PFS operating status</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>On</td>
<td>PFS on</td>
<td>PFS: ON</td>
<td>Perfect focusing is in progress</td>
<td>The PFS will maintain at the focal point if the location of interest is marked.</td>
</tr>
<tr>
<td>Blinking at slow intervals</td>
<td>PFS on</td>
<td>PFS: DIS</td>
<td>Waiting for interface detection</td>
<td>When the interface is detected within the allowable focusing range by moving the focusing position, the PFS is automatically turned on to start perfect focusing.</td>
</tr>
<tr>
<td>Blinking at fast intervals</td>
<td>PFS off</td>
<td>PFS: OFF</td>
<td>Perfect focusing is off</td>
<td>The interface is detected within the allowable focusing range. Turn on the PFS to start perfect focusing.</td>
</tr>
<tr>
<td>Off</td>
<td>PFS off</td>
<td>PFS: OFF</td>
<td>Perfect focusing is off</td>
<td>The interface is not detected within the allowable focusing range. In this case, turning on the PFS places it in an interface detection waiting state.</td>
</tr>
</tbody>
</table>
Multi stage positions

- Label the position(s) of interest. Label should be ended with digit “1” so that the number will be automatically updated to record the subsequent position.
- Click “Live” to find the right position (x, y) and focus level (z)
- Click “+” to add the position (x, y, z) in position list
- To overwrite recorded stage position, highlight the one to be replaced and click “→”.
- Make sure the PFS status is “ON” to ensure every position is in focus.
- Click Acquire at the bottom to start acquisition.

Adjust Focus during Time Lapse Acquisition

- If amendment is needed halfway through the acquisition process, click “Pause” → “Live” → choose a Position of interest → select wavelength → click “Go to”.
- Adjust the position and focus followed by clicking “Set to current” → click “Stop” (initially it is “Live”) and then “Resume” to continue the acquisition.
Z Series

a. Select “Z Series” in main menu

For Spherical object, use “Range around current” mode:
- Tick “Range around current”
- Focus the center of your object
- Set up “Step Size” for distance between each focus plane
- Set up “Number of Steps” for the total number of planes

Otherwise, use “Top” and “Bottom” mode:
- Tick off “Range around current”
- Find any one end of your sample with fine focus, click “Set Top To Current”
- Find the other end of your sample with fine focus, click “Set Bottom To Current”
- Set up “Step Size” or “Number of Steps” for distance between each focus plane
Slide Scanning

Please follow the procedures below strictly in order to have a smooth scanning of your sample:

1. Click “Scan Slide” application located at the top of the menu bars.
2. Under the "Main" tab, select the objective to be used for slide scanning, e.g. 4x, 10x, 20x and etc.
3. Go to “Acquisition” tab → Select the “Number of wavelengths” required → click on “Shading correction” if needed → Make sure the “Hardware Auto Focus” is turned OFF when setting the area to be scanned.
4. Select a desired fluorescent channel from the drop down window → click “Live” to view the selected channel → Adjust the “Exposure (ms)” and “Target intensity”, ignore the Real-time auto focus offset first. Adjust the parameters for the next channel according to the same procedures.

5. Go to “Slide Area” tab → based on the “Live” window → use joystick to set the Upper left of the area to be scanned and then click “Set to Current” → repeat this for the Lower right area.
6. Remains the “Live” window → turn on the **PFS button** on the front panel controller (focus point will be changed once turning on PFS) → go to “Acquisition” tab and turn on the “**Hardware Auto Focus**”.

![Image of Nikon microscope control panel with PFS button highlighted and software interface showing acquisition settings]

7. Remains the “Live” window → go to a **fluorescent channel** → Adjust to a desired focal plane (the Real-time auto focus offset will change in corresponding to the focal plane being adjusted) → this offset value can be similarly applied to the other channel(s) if needed → close the “Live” window.

![Image of microscope control panel with fluorescent channel selected and software interface showing acquisition settings]

8. Turn OFF the “**PFS**” button on the front control panel → Click “**Scan**”.

![Image of microscope control panel with PFS button off and software interface showing scanning options]
9. To stitch the images, go to “Data Review” tab → click on “Current scan” if you would like to process the images immediately. Alternatively, you can open any scanned images via “File” button → select channels of interest → key in the size (according to user’s preference) and image overlap (usually 10% is adequate) → click “Show Image”.

10. Go to “File” on the menu bar → save the stitched image in a designated folder.
Review Acquired Images

- Click **Review Multi Dimensional Data** in the Task Bar after Images Acquisition
- Choose your folder in **Select Directory** and select an image **Data set (base name + suffix. nd)** and then click **View**
- Select the **Wavelength** acquired to be displayed.
- Display a single image by clicking **any single grid**.
- Select Stage position in the pull down menu.
- To review series images, left click the header number of the **Row** or **Column** for displaying images of **Time series** or **Z-series** respectively. Then click **Load Image (s)**
- To export series images as movie, please refer to MetaMorph analysis software protocol.

- To Overlay images of different channels, check the **Color Composite** box in the **Display tab** and then assign corresponding channel to the RGB color to composite a overlay image.
- To stack all plans in a z-series to create a single 2D image, choose **Maximum projection** in **Z Projection** tab and check the **Z Projection** box.
**Turn off system**

Please check if the equipment will be used by other users. Please switch off system if no one books equipment over two sessions (1h) after you.

- **IF 100x/60x objective lens(es) is/are USED, it must be cleaned thoroughly with the LENS PAPER instead of Kimwipes.**
  - Oil residue from the objective lens should firstly be removed using a DRY lens tissue.
  - Repeat this step with a new area/piece of the lens cleaning tissue until no oil streaks are seen on the tissue.
  - Switch objective to lowest magnification in the software and press "ESC" to reach the Lower Z-limit.
  - a. Exit MetaMorph software
  - b. Transfer data to Faculty Core Facility storage server
  - c. Shut down the computer
  - d. Switch off microscope controller
  - e. Switch off main power control
  - f. Switch off laser power A if confocal mode is used.
  - g. Switch off the Power of Tokai Hit incubation system controller.
  - h. Turn off CO₂ regulator by turning regulator clockwise to the end
  - i. Turn off CO₂ tank by turning the main switch clockwise
  - j. Take off objective heater on objective
  - k. Release the valve and remove the water from the chamber by plugging a 50ml syringe (located in the tool box) to the tube.