HKU Med LKS Faculty of Medicine
Centre for PanorOmic Sciences
香港大學泛組學科研中心
Imaging and Flow Cytometry Core

Cytiva IN Cell Analyzer 6500HS

Standard Operating Procedure

Contents

Imaging and Flow Cytometry Core

A. Overview

IN Cell Analyzer 6500HS is specialized in performing repetitive imaging tasks on a microplate format. Data generated by the instrument is grouped into a folder containing TIFF images and an ".xdce" file directory storing positional metadata of the TIFF images acquired. Users can choose to view the images one by one but if one wish to analyse the images together, please keep both the .tiff and .xdce files together.

Before an imaging session, please bring along the following:

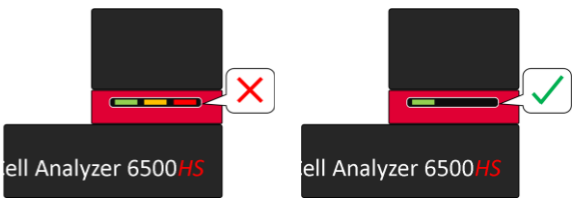
1. ***Manufacturer, catalogue number and Dimension chart*** of the plate/slide the cells were seeded on.
2. ***Plate map*** you wish to image. Including positive and negative labelling control. Positive and negative treatment control.
3. ***Excitation and emission spectra of fluorophores*** that have been used to label the cells. Otherwise, name of the dyes. You will need a nuclear stain in order to count cells with automated procedure.
4. ***Portable data storage device*** of >100 GB of space.

B. System start up

1. Press the black button ①beside gas inlet tubes to turn the machine on.

2. Turn on the computer ②.

3. Wait until both orange and red indicator lights disappeared.



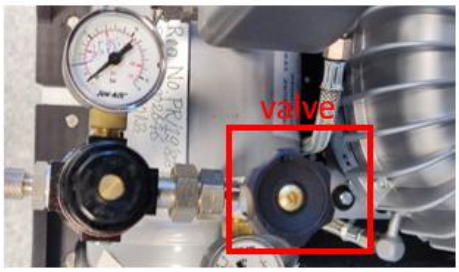
4. Double-click the ***IN Cell Analyzer*** icon on desktop.

C. Setup of live cell imaging module (Optional)

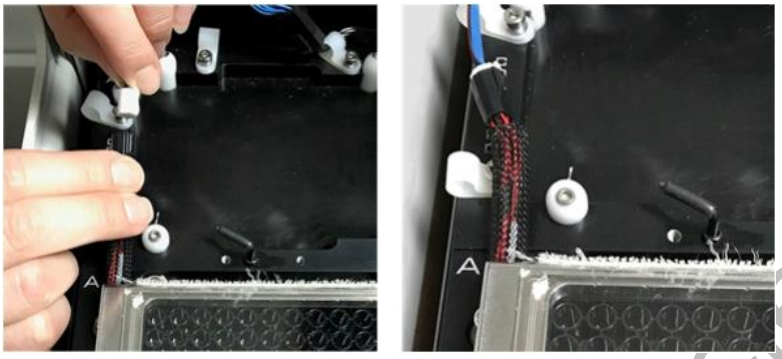
1. If the water level in the humidifier bottle (next to the power button of IN Cell Analyzer) is between 1/4 and 1/2 full, skip to step 6.

2. To fill water, remove the insulation sleeve from the humidifier by sliding it down the bottle.
3. Open the bottle by turning the glass bottle instead of turning the lid. This helps ensure that the cables do not become tangled and/or break.
   Note: Do not touch the diffuser. Oil and particulates from your hands can damage the diffuser.

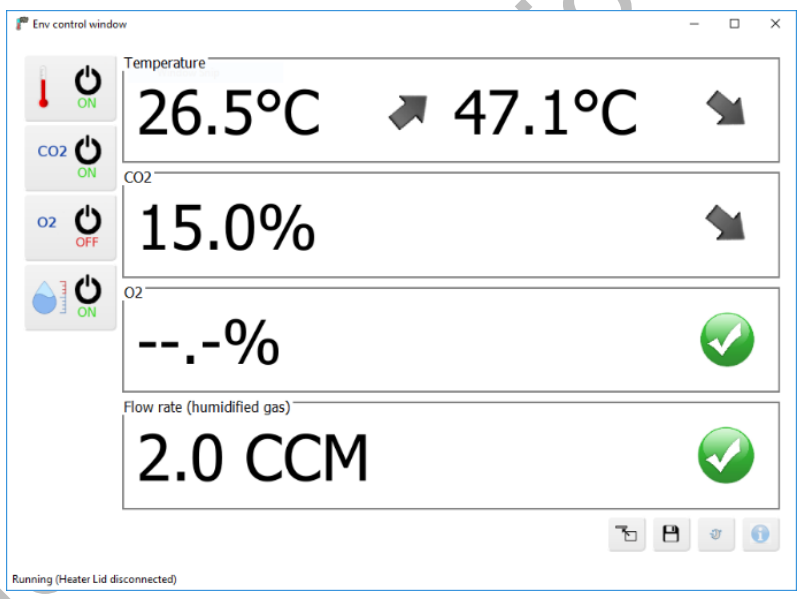
4. Fill the bottle about 1/2 full with sterile, deionized water.
5. Replace the lid by turning the glass bottle instead of turning the lid.
6. Replace the insulation sleeve and place the humidifier back into the compartment on the right side of the IN Cell Analyzer system.
7. Open on the valves CO_2, N_2 gas tank and air compressor and regulate their pressure to 25 psi.
   If fail to reach 25 psi, contact technical staff.



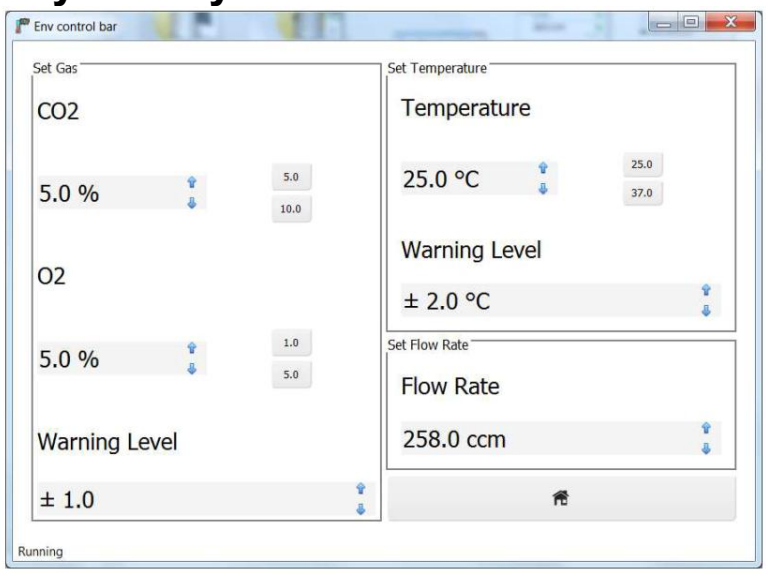
8. Place a dummy plate onto the stage. Place the Live Cell Sample Lid on top of the dummy plate.
9. Connect the electrical connector for the humidifier heater to the Live Cell Sample Lid, as shown in the following figure.



10. Click ***Load*** on the main toolbar of the IN Cell acquisition software to close the plate access door.
11. In the IN Cell acquisition software, click ***Environmental Control*** to open the Environmental Control Software.



12. In the ***Main Status Window***, use the Component Toggle bar on the left side of the window to turn the appropriate controls on.
13. Click any of the values in the ***Main Status Window*** to open the ***Settings Window***.



14. Ensure that the CO_2, O_2 (optional), Temperature, and Flow Rate set points are defined appropriately for your experiment. If required, use the following information to change the set point values.

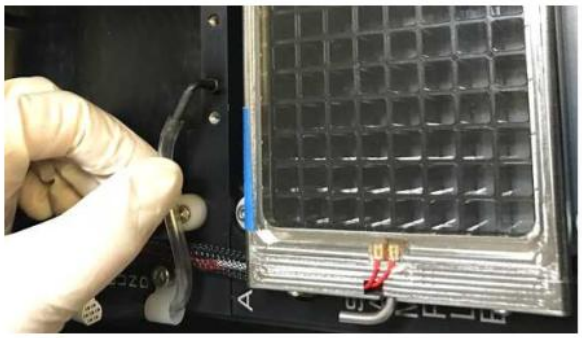
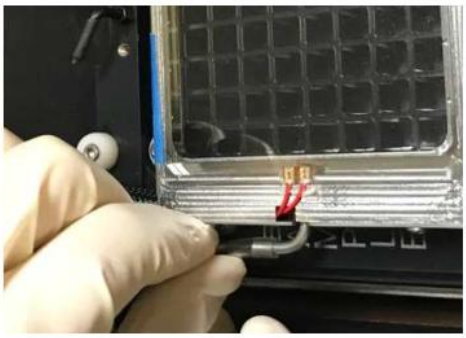
- **CO_2.** Type a value into the set point field. Use the up/down arrows or the 5.0/10.0% quick set buttons to change CO_2 concentration. CO_2 concentration can be controlled from 0% to 20%.
- **O_2.** Type a value into the set point field. Use the up/down arrows or the 1.0/5.0% quick set buttons to change O_2 concentration. O_2 concentration can be controlled from 0% to 20%.
- **Temperature**. Type a value into the set point field. Use the up/down arrows or the 25.0/37.0°C quick set buttons to change the temperature. Temperature can be controlled from room temperature to 42°C.
- **Flow Rate.** Type a value into the set point field or use the up/down arrows to set the flow rate in cubic centimeters per minute (ccm). Recommended flow rate is 50 ccm for all applications except hypoxic conditions that require <5% O_2. *To achieve <5% O_2 at the sample, flow rate must be reduced to 30 ccm*.

15. Click ***Eject*** on the main toolbar of the IN Cell acquisition software to open the plate access door.

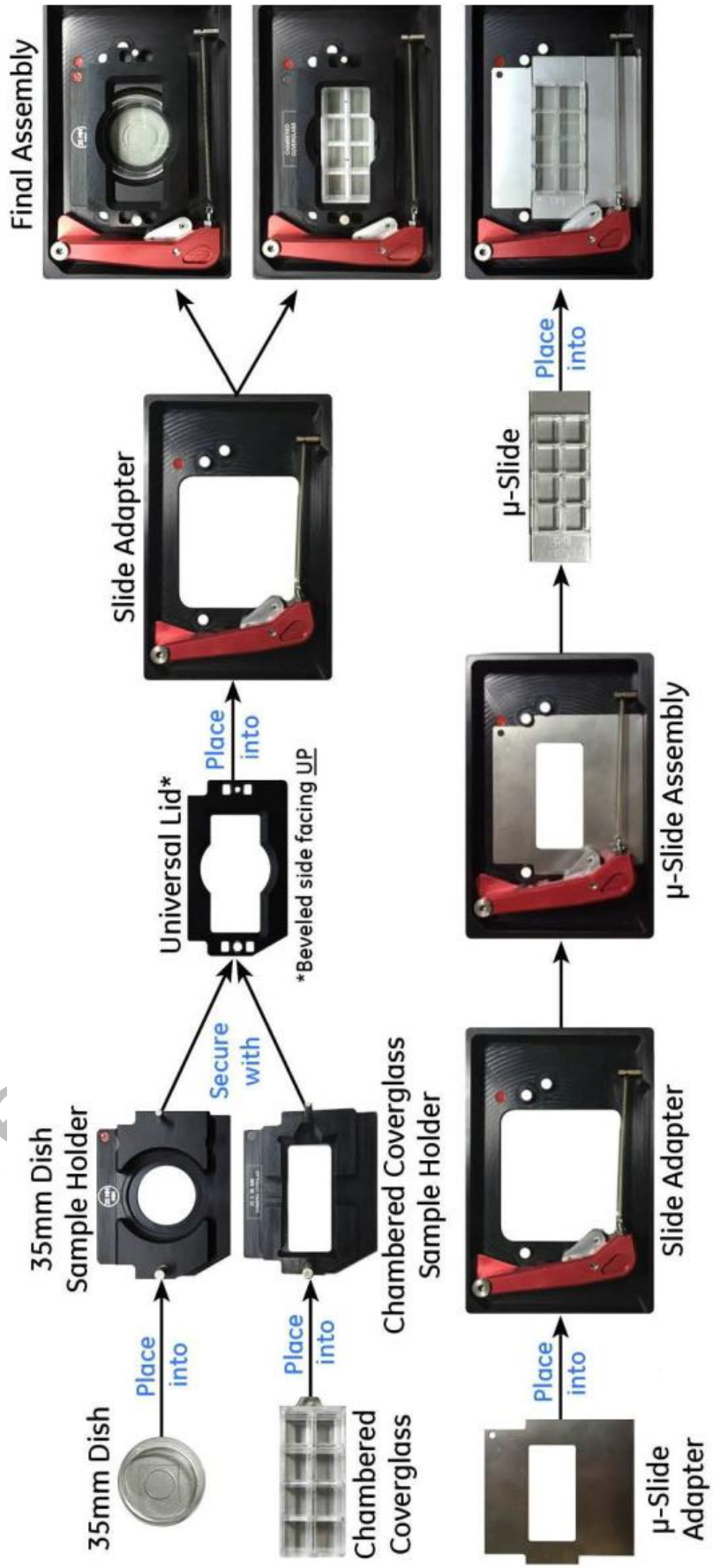
16. Remove the dummy plate and load your sample by choosing one of the following.
    - ***If using a 96-well plate:***
      Place the plate in the stage opening. If desired, seal the plate with a breathable membrane.

    - ***If using a 35mm dish, chambered coverglass, or µ-slide adapter (refer to the picture on the next page):***
      a. Load the sample into the appropriate sample holder. If using a 35mm dish or chambered coverglass, secure with the Universal Lid.
      b. Place the loaded sample holder into the slide adapter.
      c. Place the slide adapter into the stage opening.

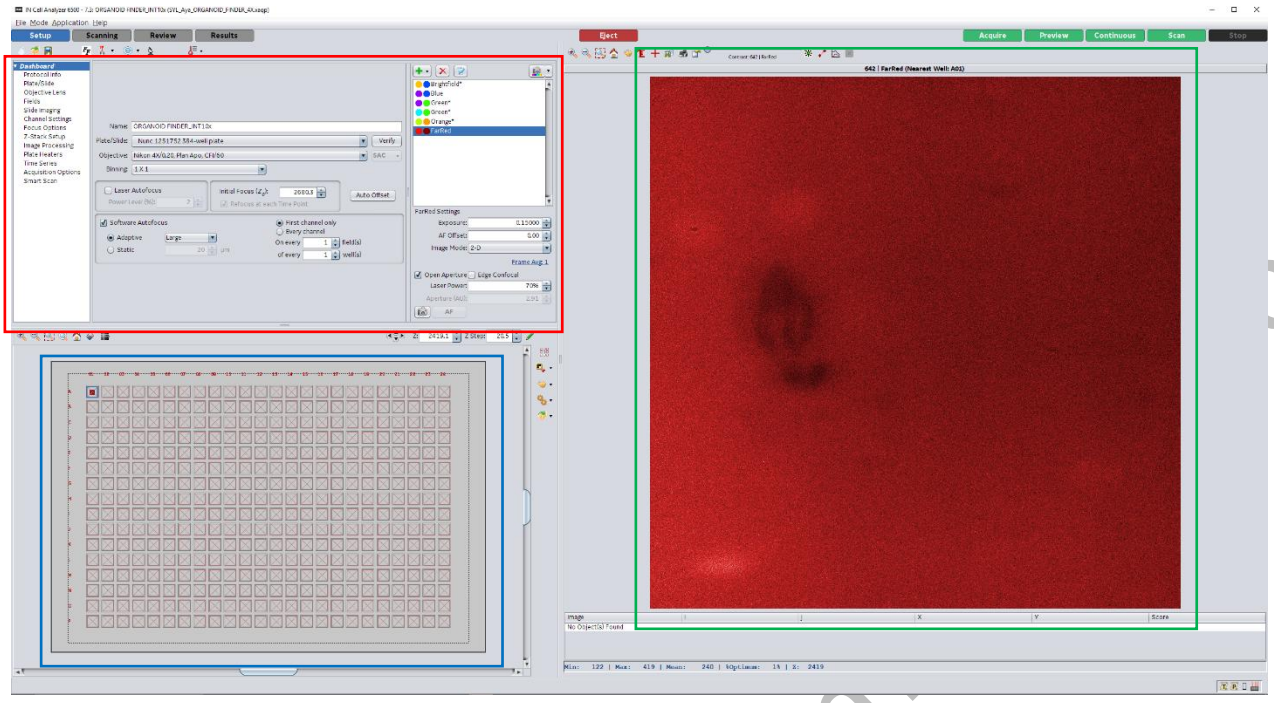
17. Install the Live Cell Sample Lid on top of the plate or slide adapter. Ensure that the gasket on the lid is positioned squarely over the rim of the plate or adapter so that the live cell chamber is securely sealed.
18. Disconnect the gas input tubing from its docking location and connect it to the gas input on the Live Cell Sample Lid.

19. Click ***Load*** on the main toolbar of the IN Cell acquisition software to close the plate access door.



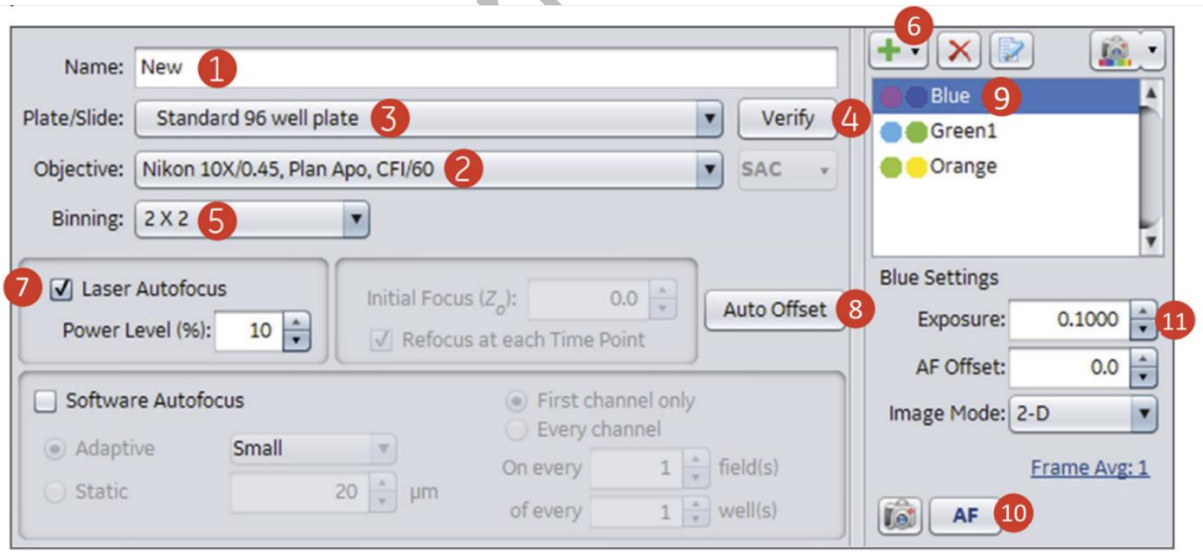
D. Protocol setup



Top Left panel: for setting up protocol.

Right panel: for image preview. Scroll to zoom.

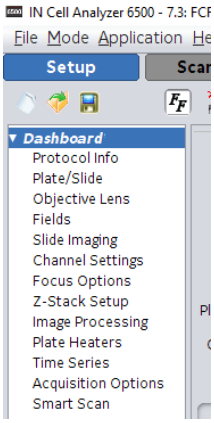
Lower left panel: for plate map. Scroll to zoom and double-click a spot WITHIN plate area to move the lens to that position.



Imaging and Flow Cytometry Core

Sample loading

1. Select ***Dashboard*** for setting up the scanning protocol.

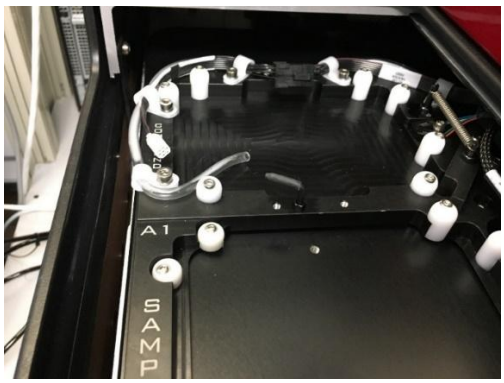


2. Select ***Eject*** to open the machine door for loading a plate.

3. load your sample by choosing one of the following.
   - ***If using a 96-well plate:***
     Place the plate in the stage opening. If desired, seal the plate with a breathable membrane.

   - ***If using a 35mm dish, chambered coverglass, or µ-slide adapter (refer to the picture on P.7):***
     d. Load the sample into the appropriate sample holder. If using a 35mm dish or chambered coverglass, secure with the Universal Lid.
     e. Place the loaded sample holder into the slide adapter.
     f. Place the slide adapter into the stage opening.

4. Insert the plate with A1 at top left corner of the sample frame.



5. Select ***Load*** to close the machine door.

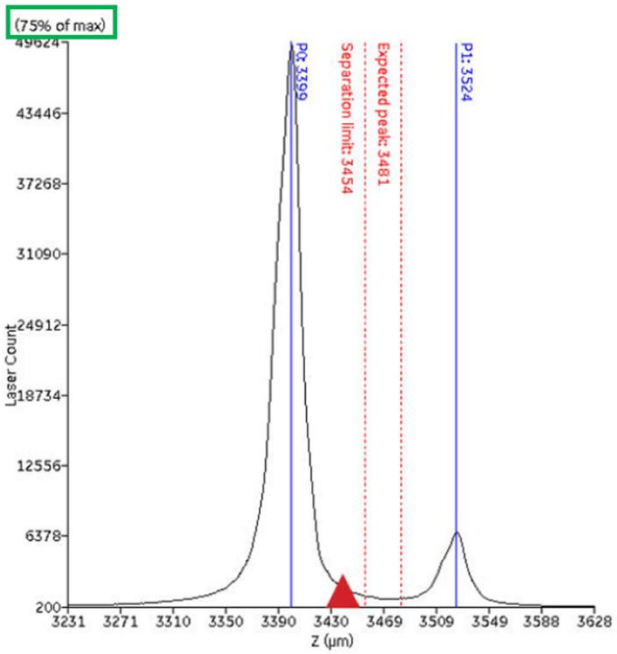
6. Click the ***Start a new protocol icon***  , then enter a unique protocol name ❶ . Click ***Save***  .
   Note: Name should be 16 characters or less. Do not use special characters.

Plate/Slide verification

7. In the Objective drop-down ❷, select 10× objective.

In the ***Plate/Side*** ❸ drop-down, select correct plate map (manufacturer and catalogue number).
*If you cannot find your correct plate/slide map, please contact technical staff for assistance.

8. Click ***Verify*** ❹ to confirm that your plate/slide type matches the map selected.
9. If the laser count is **100% of max,** decrease laser power by clicking  , adjusting the slider position and then clicking  . Repeat step 8.

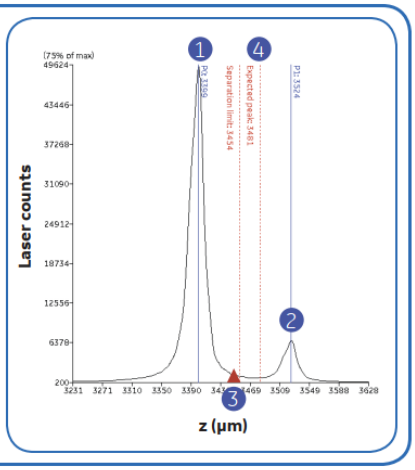


10. Confirm that the z value of Peak0 (P0) is within 10% of red arrowhead and that Peak 1 (P1) is within 10% of Expected Peak.
    - If the expected values differ from the measured value by ***less than*** 10%, no changes are required. Close **Laser Autofocus** (LAF) tool. For more information, see "Interpreting LAF traces" below.
    - If the expected values differ from the measured values by ***more than*** 10%, please ask technical staff to create a new plate map for you.

Interpreting LAF traces

The ***Laser Autofocus*** tool uses a near infrared laser to locate areas of significant refractive index difference between the objective and the sample. When looking at the trace:

- Peak 0 ❶ represents the interface between air and the bottom of the plate/sample
- Peak 1 ❷ represents the interface between the bottom of the plate and the sample
- The x-axis represents distance in the z dimension, the y-axis represents laser counts
- The red arrowhead ❸ indicates the ***Expected Bottom Height*** of the plate
- The red dotted line ❹ indicates the ***Expected Peak*** from the plate/sample interface



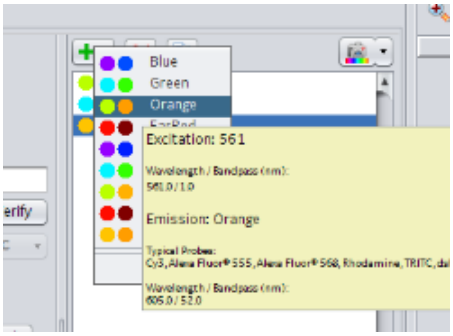
11. In the Objective drop-down, select the objective needed for your assay. Below is the guide for reference.
    - 4X cell cluster counting
    - 10X individual cell counting (large amount)
    - 20X single cell morphology quantification
    - 40X sub-cellular morphology quantification

Channel selection and focus adjustment

1. In the ***Binning*** ❺ drop-down, set binning to 2 × 2.
   Note: Binning reduces image file sizes dramatically, that is, at 1 × 1 binning a single channel image is 8 MB, at 2 × 2 binning it is 2 MB.

2. Set binning to 1 × 1 only when maximal image resolution is required.

3. Click the ***Add New Channel*** icon ❻ and add channels required for your experiment. Hover the cursor on the channels to read filter specifications. Fluorescent channels available:
   "BLUE"
   "GREEN"
   "ORANGE"
   "FarRed"



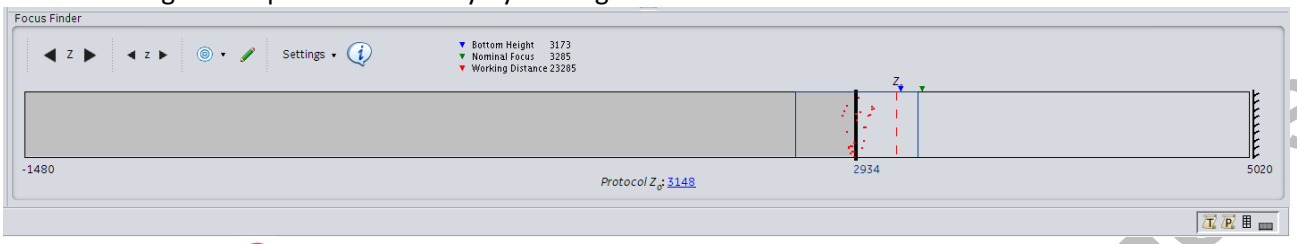
4. Check ***Laser Autofocus (LAF)*** ❼ box or select .

5. If your specimen has multi-colour labels, please find a site with all of the colours and click ***Auto-offset*** ❽ or . This will use both LAF and ***Software Autofocus*** to find the focal plane for each channel. Click ***OK*** to apply the offsets. Here are the guidelines on which focus strategies to use:
   - Adherent cell: use laser autofocus + auto-offset
   - Z-stack with fixed positions: deactivate both laser autofocus + auto-offset. Check ***initial focus*** to define starting position.
   - Unpredictable heights (e.g. spheroids in 3D matrix): use software autofocus.

Laser Autofocus uses a near infrared laser to locate areas of significant refractive index difference between the objective and sample.

Software Autofocus is to take a focal stack of images, calculate some metric of image sharpness over the stack, and find the peak.

6. You can change focus position manually by clicking ***Focus Finder*** .



7. Highlight a channel 9 and click ***AF*** 10.
8. Adjust ***Exposure*** 11 to target 10000 to 20000 counts for fixed cells or approximately five times the background for live cells. Repeat for all additional channels.
   Note: Use the ***Min/Max*** values at the bottom of the image panel or hover over the image to optimize ***Exposure***.
   Keep ***Max*** value below 45000 counts to prevent saturation (65535 counts).
9. Select ***imaging modes***.
   Fluorescent imaging modes: 2D, 3D and max intensity projection
   Brightfield imaging modes: 2D, DIC, PH (Phase contrast) and 3D

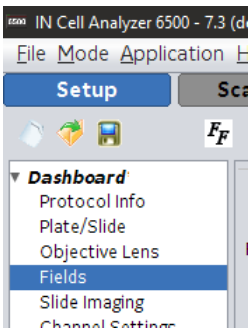
10. If your sample is monolayer cell culture, select ***open aperture.***

    If your sample is thick or has high background, try imaging in ***IRIS*** or ***EDGE confocal mode***. Compared to IRIS confocal mode, EDGE confocal mode has greater improvement in image contrast and resolution in all three dimensions by quantitatively removes out-of-focus light to improve visualization and segmentation of structures in thicker samples.

11. For confocal mode, uncheck the ***Open Aperture*** box and adjust ***Aperture*** (AU) as needed (recommend 1 to 2 AU, AU= airy units) on a channel-by-channel basis. Smaller values are more confocal while larger values are closer to Open Aperture mode.
    Note: You might need to refine exposure time after adjusting aperture settings.

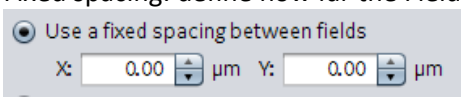
E. Field selection

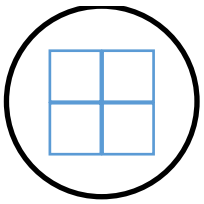
1. On left menu area, select ***Fields*** to set number and placement of fields.



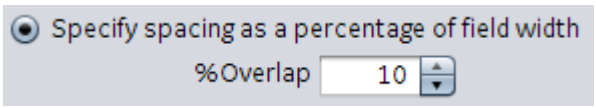
2. Select between fixed spacing, randomized placement, customized location or point list.

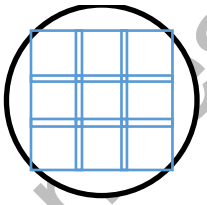
- Fixed spacing: define how far the Field-of-view (FOV)s should be apart.



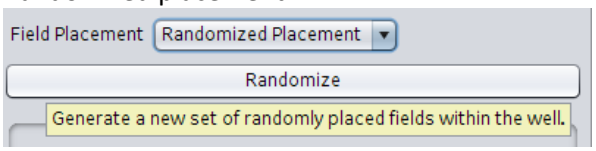


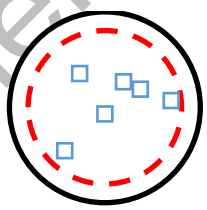
- Tiling: define % overlap between FOVs (for stitching, set 10-20%).



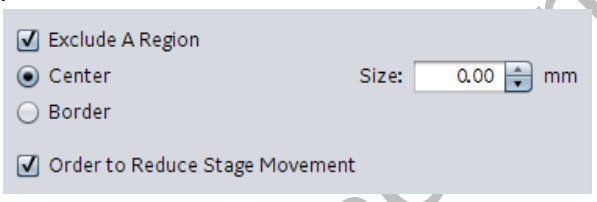


- Randomized placement:

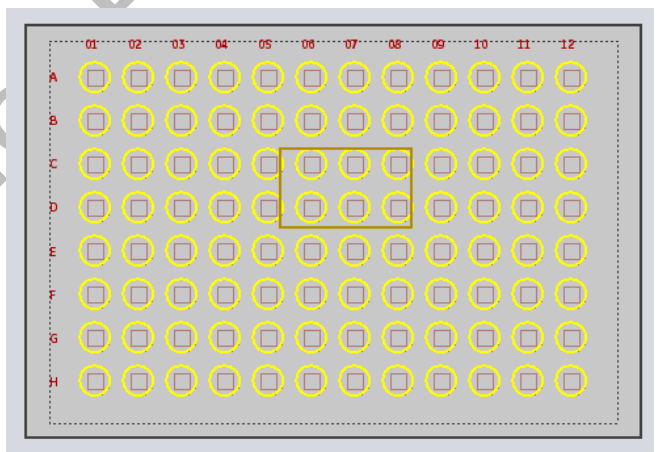




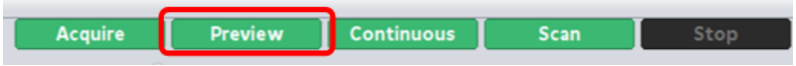
You may programme the randomizer to exclude centre or border part by defining appropriate size (per mm).
For randomized placement, check ***order to reduce stage movement*** for shorter capture time.



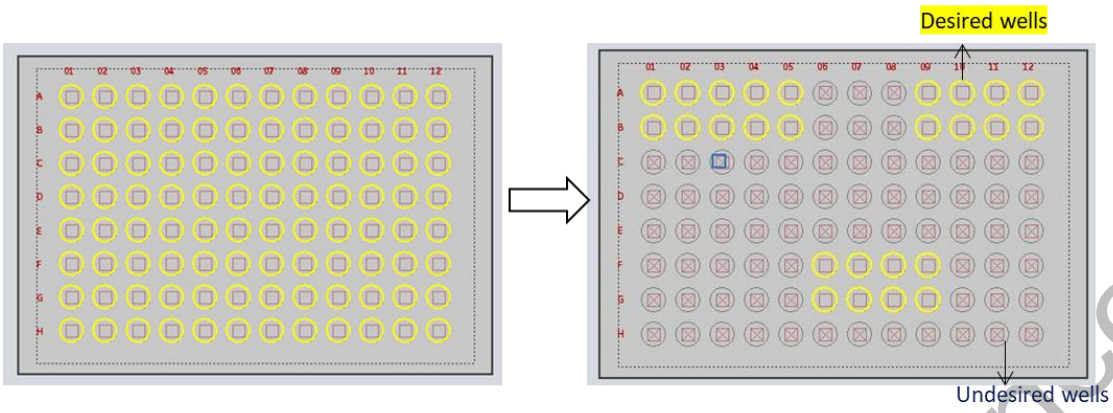
3. Click  and draw on plate map to define an area for preview scan (optional). It is better to see where the fields of view should be located before setting up fields.



4. Click preview on top-right corner.



5. Customise your wells to be imaged. Yellow circle means your wells are selected to place the fields. In Plate View, click and drag while holding down ***Shift*** to deselect wells. To reselect, click and drag while holding down ***Ctrl***.

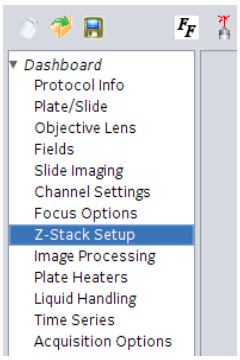


6. Click ***Save***.

F. Z-stack setup (optional)

This function would be the most useful if used together with EDGE or IRIS confocal fluorescent imaging mode.

1. In the ***Channel Settings*** area of the ***Dashboard***, click on a channel to show settings for that channel.
2. Set ***Image Mode*** to ***3D*** for every channel you wish to acquire z-stack.
3. Select ***Z-Stack Setup*** on menu.

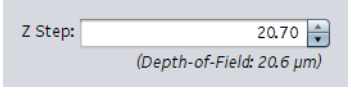


4. Check ***Link 3D Parameters*** box.
   Note: Link 3D Parameters will invalidate channel offsets, increasing acquisition speed and ensuring that the Z stack is collected through the same volume for all channels.

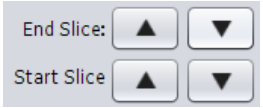
5. Select **FF** focus finder tool  to focus onto the structure you wish to capture.

6. Click  to set ***current z-position*** as 3D focus height (mid-point of z-stack).

7. decide on z-stack optical section interval. The software will provide "Depth-of-field" in µm for each objective. Maximum z-stack interval available is 50 µm.



8. Use the ***End Slice*** and ***Start Slice*** arrows to add or remove Z slices from the scan range.
   Note: The scan range may be asymmetrical and does not have to include the 3D Focus position.



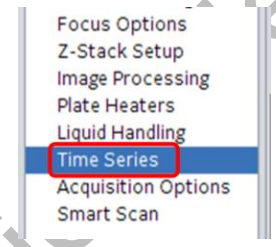
9. Set the ***Z Step*** size. Below are the typical Z step sizes for various structures of interest.

Structure of interest	Typical Z Step
Foci/Puncta	1-3 µm
Organelles	3-6 µm
Spheroids	10-15 µm

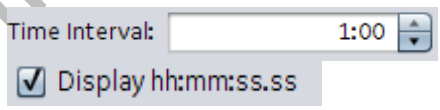
10. Finalise other protocol parameters and click ***run*** at the top right corner to start scanning.

G. Time series setup (optional)

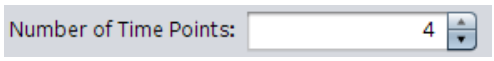
1. Select ***Time Series*** on menu.



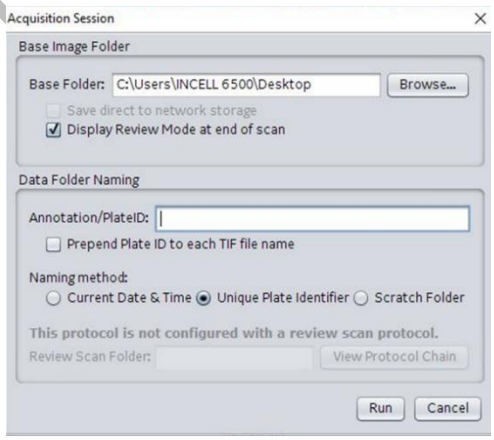
2. Check ***acquire time series*** button.
3. Set ***time interval***. Check ***Display hh:mm:ss.ss*** for clearer unit display.



4. Set the ***number of time points*** required.



5. If more than one well needs to be imaged. Please switch ***Mode*** to ***Multi-well***.
6. Click ***Add***.
7. Finalise other protocol parameters and click ***run*** at the top right corner to start scanning.
8. In the ***Acquisition Session*** window, click ***Browse*** to locate your folder in "DataStorage (E:)\Your PI's lab\Your name". Input ***Annotation/Plate ID*** with your experiment's name, and click ***Run***.



H. System shutdown

1. (for live cell imaging) Turn off the valve of CO_2, O_2 and compressed dry air.
2. (for live cell imaging) Turn off all functions in ***Env control window***.
3. (for live cell imaging) Close ***Env control window***.

4. Click ***Eject*** on IN Cell software.
5. (for live cell imaging) Unplug gas tubing and heating connector.
6. Take out you plate and click ***load*** to close the sliding door.
7. Select ***Application-> Hardware-> Shutdown Instrument***.
8. Turn off computer. (keep computer on for data transfer on IN Carta offline station)

I. Data transfer

1. After scanning is finished, there will have (a) one .xdce file; (b) tiff images and (c) one folder named "thumb". Make sure (a) to (c) files are copied during transfer.
2. Go to computer (file explorer) and select ***IN-CARTA*** under ***Network***.
3. Transfer your folder to one of the IN Carta drives.
   Folder naming rule: IN_Carta drive# / [Your lab PI's initials + "Lab"] / [Your name] / [Your image folder]