Tomocube

Operating Guide for HT Systems

TomoStudio 3.0

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Table of Contents

Sample Preparation 3

1.1 Overview of Specimen Requirements for Qualified Holotomography 3

1.2 TomoDish 3

1.3 Cell seeding 4

Initialization 5

2.1 Microscope Initialization 5

2.2 Sample Mounting 6

Adjusting Lens Position and Calibration 9

3.1 Adjusting 9

3.2 Set/Working 12

3.3 Calibration 13

Imaging and Maintenance 14

4.1 Holotomogram(HT) Imaging 14

4.2 HT + Bright-field (BF) Imaging 18

4.3 Fluorescence (FL) Imaging 19

4.4 HT + FL Imaging 22

4.5 Multi-Point (MP) Imaging 24

4.6 Display Setting Options 27

4.7 Clean Up 28

Coloring 29

5.1 Overlay 29

5.2 Matrix 30

5.3 Nucleus 32

5.4 Organelle 33

5.5 Edge 34

5.6 Color Combination 35

Sample Preparation 3

1.1 Overview of Specimen Requirements for Qualified Holotomography 3

1.2 TomoDish 3

1.3 Cell seeding 4

Initialization 5

2.1 Microscope Initialization 5

2.2 Sample Mounting 6

Adjusting Lens Position and Calibration 9

3.1 Adjusting 9

3.2 Set/Working 12

3.3 Calibration 13

Imaging and Maintenance 14

4.1 Holotomogram(HT) Imaging 14

4.2 HT + Bright-field (BF) Imaging 18

4.3 Fluorescence (FL) Imaging 19

4.4 HT + FL Imaging 22

4.5 Multi-Point (MP) Imaging 24

4.6 Display Setting Options 27

4.7 Clean Up 28

Coloring 29

5.1 Overlay 29

5.2 Matrix 30

5.3 Nucleus 32

5.4 Organelle 33

5.5 Edge 34

5.6 Color Combination 35

Sample Preparation

1.1 Overview of Specimen Requirements for Qualified Holotomography

	Specification
Bottom material	Glass
Glass-bottom thickness	#1.5 coverslip (0.12 mm – 0.17 mm) #1.5H coverslip (high precision, thickness: 0.17 0.005 mm) is strongly recommended.
Depth of field (DOF)	40 μm
Recommended sample thickness	< 50 μm Dependent on the optical density of the sample
Diameter of culture dishes	> 50 mm

1.2 TomoDish

Preparing your sample on a glass-bottom dish is strongly recommended. TomoDish is a glass-bottom dish which is specially designed for the HT series. The glass window area of TomoDish is 20 mm by 20 mm, and the glass thickness is #1.5H.

You can place a #1.5H coverslip on top of the sample grown on the TomoDish and visualize the sample using both S and H models. Also, when using the H model, you can directly observe the cultured cells grown on the TomoDish by immersing the condenser lens (upper water immersion lens) in the medium.

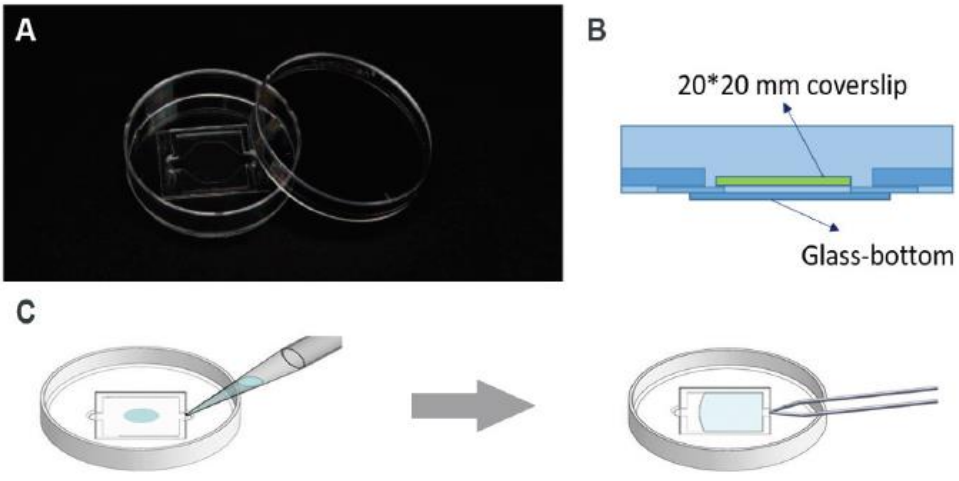


Figure 1. TomoDish (A) Photo (B) Side view diagram (C) Sample mounting procedure

Start the cell culture around 24 hours in advance to allow the cells to settle. A good layer of cells for HT imaging should have formed around 12 hours after seeding cells onto the TomoDish. If you need to transfect cells with plasmids, we recommend starting transfection around 18 hours after cell seeding.

For more detailed information, refer to our sample preparation protocol and TomoDish manual

1.3 Cell seeding

1) Adherent cells

Seed 2 105 cells onto a TomoDish. The confluency should be around 50% - 70% after incubating for around 18 hours. This range of confluency is the most suitable condition for the quantitative analysis of a single cell. If the confluency is higher than 70%, finding a blank area next to the imaging target for calibration may be difficult. This protocol is optimized for commonly used adherent cells such as HeLa, NIH3T3, A549, etc. The cell seeding number and appropriate confluency depend on the characteristics of your cells, therefore you will need to adjust the cell number accordingly.

2) Suspension cells

You can simply observe the suspension cells by taking 20 μL of the culture and adding to a TomoDish. If you want to start the cell culture using TomoDish, seed 4 x 10^5 cells onto a TomoDish and incubate for 24 hours. Gently place a 20 20 mm #1.5H coverslip on top of the TomoDish glass bottom and check if the coverslip is attached to all four of the adhesive corners and the suspension is evenly dispersed inside the square bottom.

3) Microorganisms

In the case of liquid cultures such as bacteria or microalgae, they can be directly observed without specific pre-treatment. Take 20 μL of the suspension and add it to the bottom of a TomoDish. Gently place a 20 20 mm #1.5H coverslip on top of the TomoDish and confirm that the coverslip is attached to all four of the adhesive corners and the suspension is evenly dispersed inside the square. 10^6 – 10^7 cells/mL is the optimal range of the concentration for easily finding a target in a field-of-view (FOV).

Initialization

2.1 Microscope Initialization

Warming up the laser for at least 30 minutes before use is recommended to stabilize.

The recommended order of initialization:

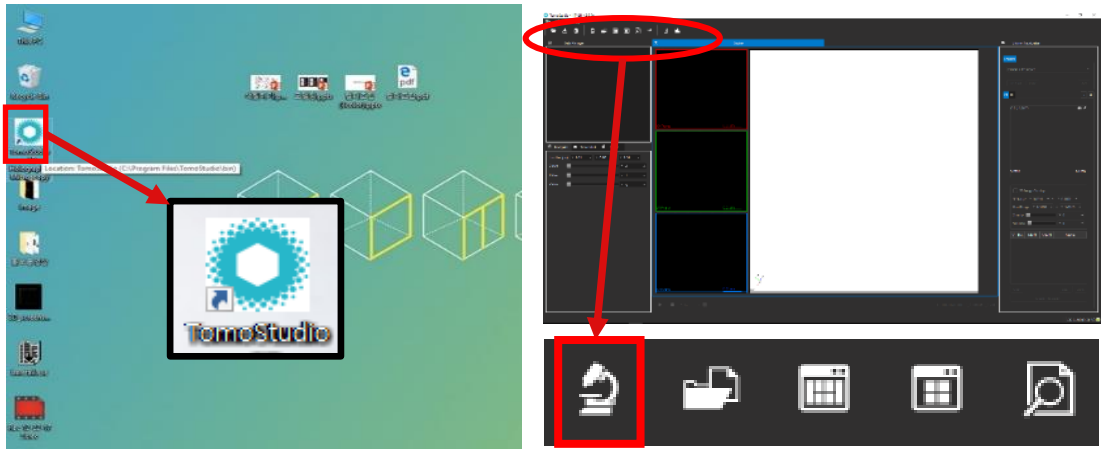
1) *Turning on the microscope*
2) *Preparing samples*
3) *Turning on TomoStudio.*

To initialize TomoStudio and the HT system, please follow the directions below:

1. Turn on the microscope by rotating the power knob clockwise. While waiting for the laser to warm up, prepare and mount the sample on the stage.

2. Double click the TomoStudio icon to launch the software and wait for initialization. If a microscope-shaped icon appears, the microscope has been connected to your computer successfully. Click the icon to start imaging.

   If the microscope icon does not appear, turn off the microscope and TomoStudio software. Then, restart from the beginning.



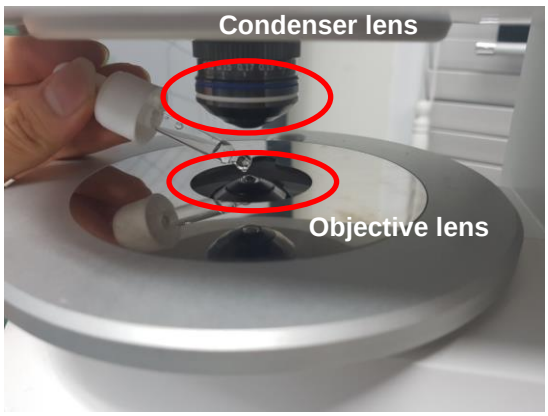
2.2 Sample Mounting

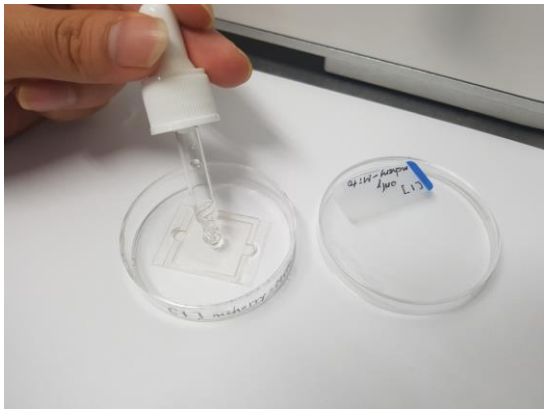
2.2.1 Sample Mounting

HT series microscope is manufactured in two versions: S and H models. The S model is equipped with an air lens and therefore the next step is not required. For the H model, both the objective lens and the condenser lens are water immersion.

1. Using a dropper, apply purified water on top of the objective lens.

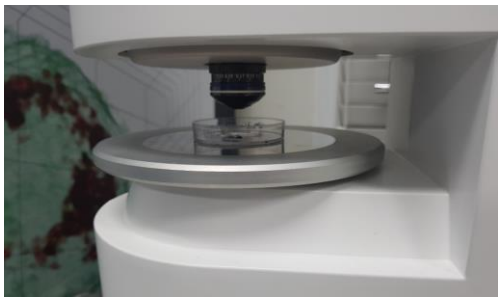
> *For long-term imaging (> 1 hour), apply Immersol W [1] instead of water to prevent drying.*





2. If the sample is covered with a coverslip, apply water on top of the coverslip as well. If the dish does not have a top coverslip and is filled with medium, you can direcrly immerse the condenser lens into the medium.

3. Mount the sample on the stage.



[1] Immersol W is Carl Zeiss' artificial non-evaporating, low-viscosity oil with a RI of 1.334, similar to water's RI (1.333)

2.2.2 Sample Mounting with TomoChamber

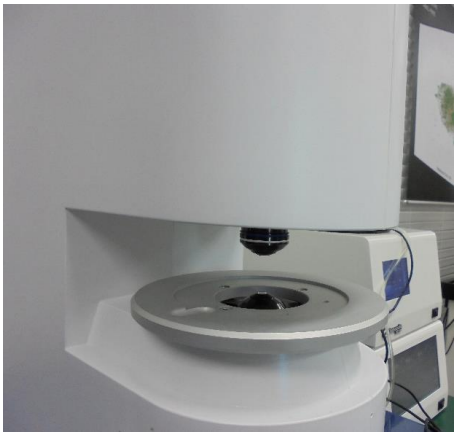
For long-term live cell imaging, using a TomoChamber is recommended. TomoChamber is a custom incubator designed to be equipped to the HT instrument which maintains optimal temperature and CO_2 level.

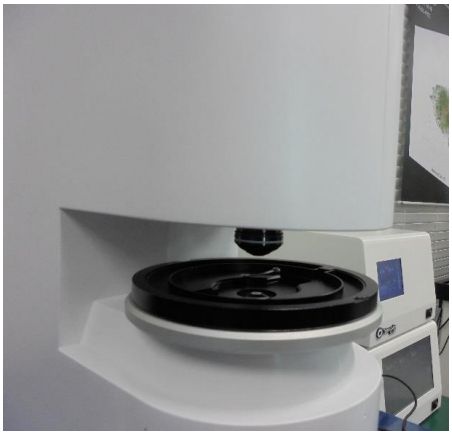
> *For detailed information of TomoChamber, refer to TomoChamber manual.*

1. First, pour water into the water reservoir in TomoChamber's main-body. Then, mount the TomoDish onto the main body and secure it with the spring clip.

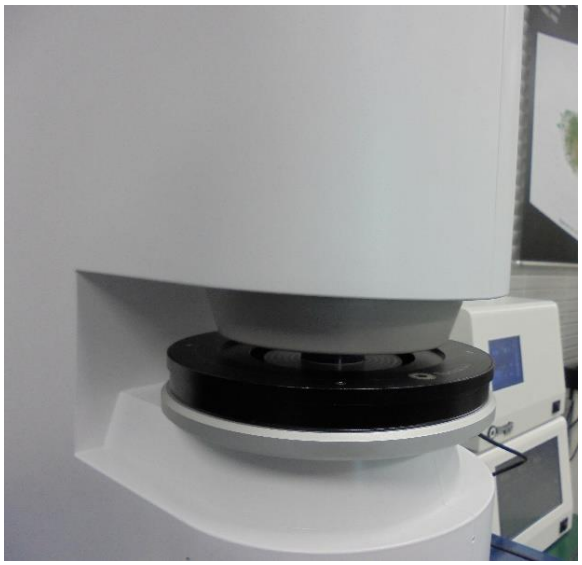
2. Remove the stage plate from the microscope sample stage.

3. Apply Immersol W onto the objective lens and mount the TomoChamber on the microscope stage.





4. Close the TomoChamber with its cover and directly immerse the condenser lens into the sample. After adjusting the lens focus, assemble the lens warmer.



5. Turn on the temperature controller and automatic gas mixer using the on/off switch at the front of the device. Then, turn the CO_2 regulator knob 360 degrees counterclockwise.

Adjusting Lens Position and Calibration

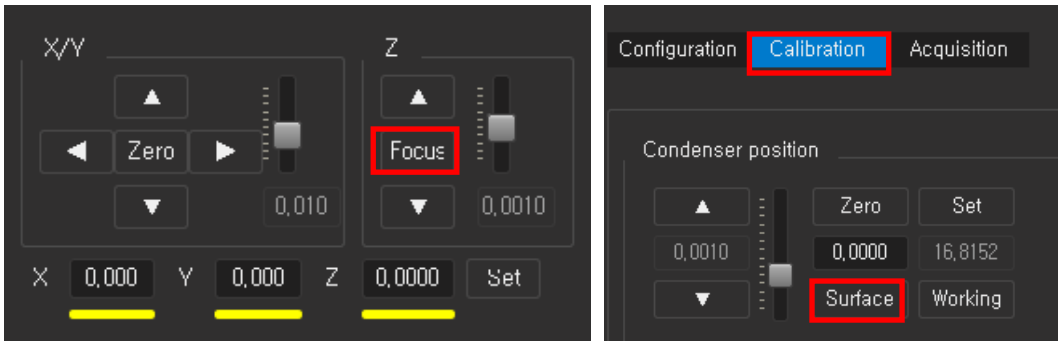
3.1 Adjusting

3.1.1. Preset lens position

Click the **[Calibration]** tab in the control panel.

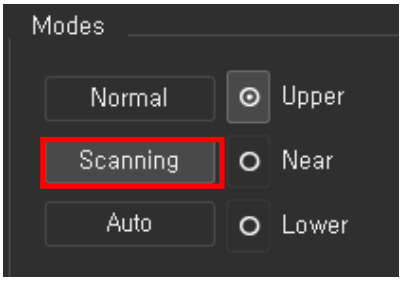
Click the **[Focus]** and then **[Surface]** buttons. These two buttons bring the objective lens and condenser lens to the initial positions according to the value in **Preference**, respectively.

> *Hover mouse over the button in TomoStudio to see an explanation for each button's function*



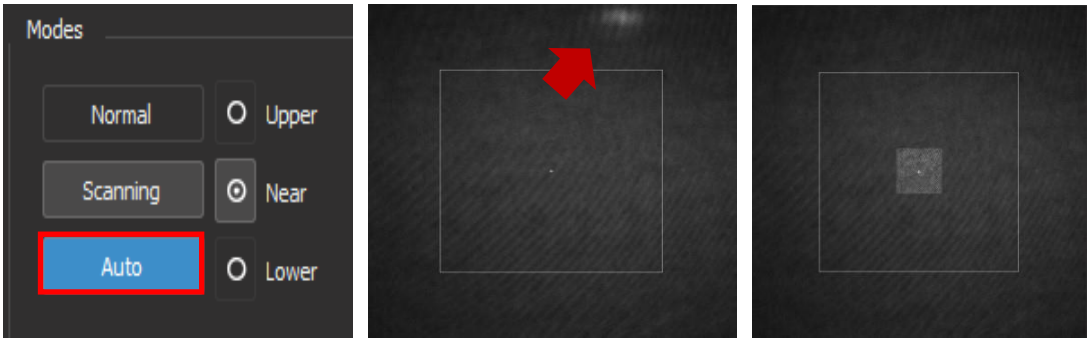
3.1.2 Adjusting Condenser Lens Position

1. To adjust the position of the condenser lens, click **[Scanning]**.



2. A white box circulating the screen will be displayed. The focus status will be indicated as **'Upper,' 'Near'** or **'Lower'**. This sign indicates the current position of the condenser lens in comparison with the ideal focus position; for example, if the 'Upper' sign is checked, the position of the condenser lens is higher than the ideal position.

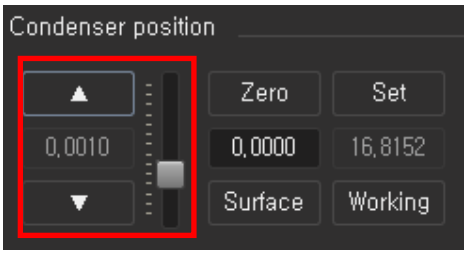
3. Click on the **[Auto]** function to automatically adjust the condenser lens position. If the white box comes into the center and **'Near'** sign is checked, it means the focus has been correctly adjusted.



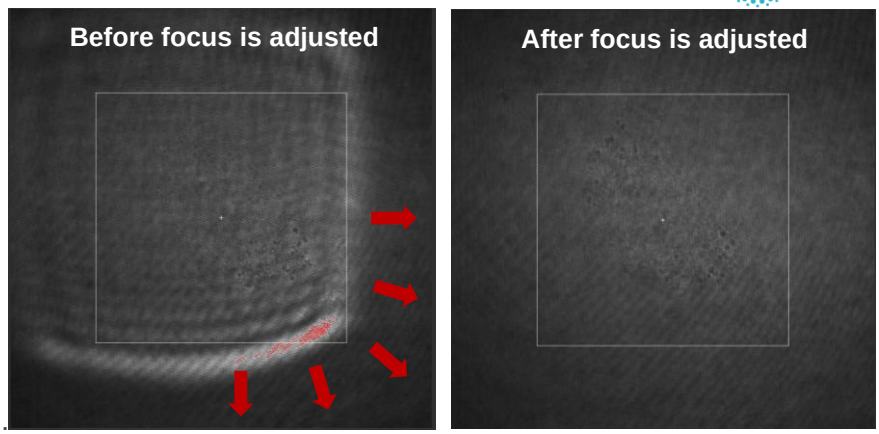
4. Click the **[Normal]** button to finish scanning.

Alternatively, you can further adjust the condenser lens position manually by using the condenser position controller.

1. To manually adjust the position of condenser lens, click the up/down buttons of the controller. Its scale is in mm (millimeter), and you can change the moving distance per click by adjusting the slider on the right.

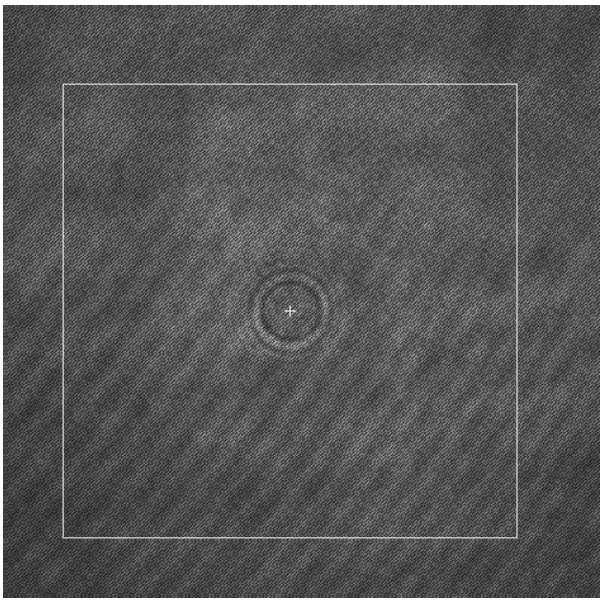


2. Adjust focus of the image by using the condenser position controller. After adjusting, the screen should appear as below.



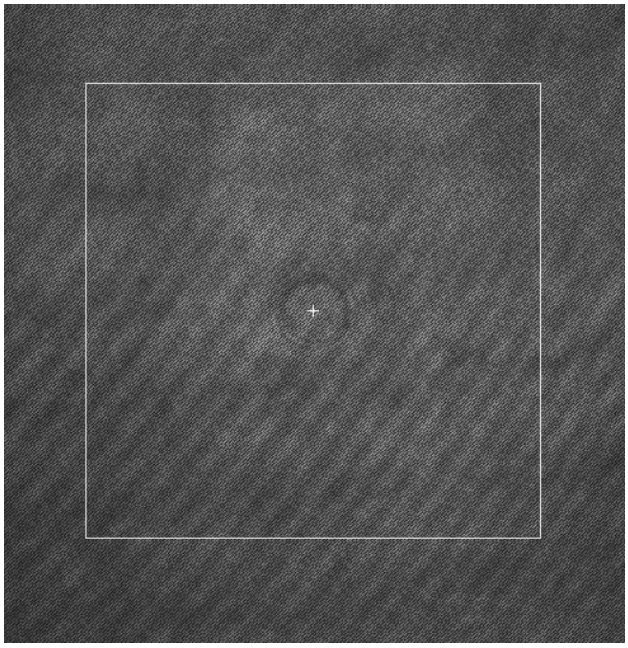
3.1.3 Adjusting Objective Lens Position

1. Find a cell to observe using the X/Y arrow buttons in TomoStudio, X/Y knobs on the microscope or 3D explorer joystick. Move the position of the lens until the targeted cell appears within FOV.



2. Adjust focus until the edge of the target cell becomes sharp and clear.

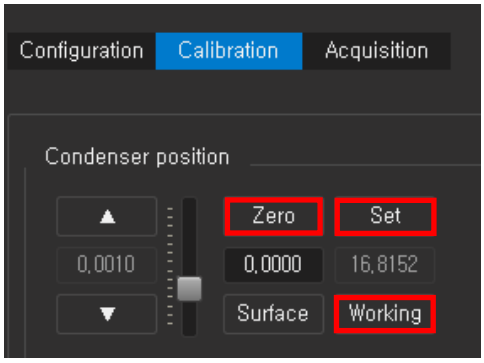
> *For more explanations on how to find the focus, refer to our instruction videos.*



3.2 Set/Working

If you have more samples that are composed of the same types of cells and are plated on the same specifications of coverslips or glass-bottom culture dishes such as TomoDish, the **[Working]** button provides an easier and quicker option to go back to the previous settings.

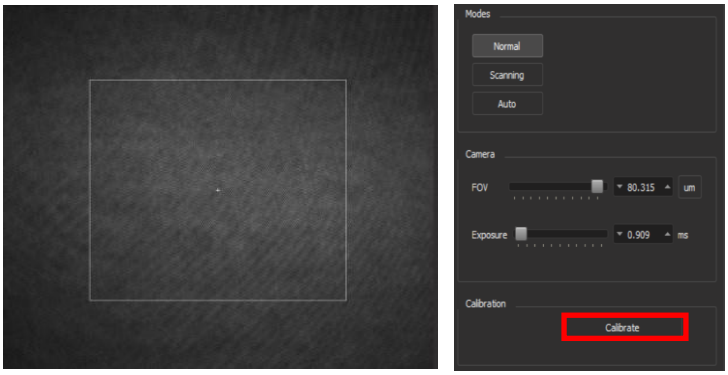
After adjusting the focus and before changing the sample, click the **[Set]** button to save the current fine-tuned lens position. Clicking the **[Zero]** button will return the condenser to its retracted position. Place a new sample on the stage and click the **[Working]** button, then the program will load the saved settings.



3.3 Calibration

Calibration is required to acquire a clear and accurate RI measurement from a sample. A background image is captured during the calibration process, which is used to compute the tomogram. The quality of the background image determines the clarity of the cell image. Therefore, calibration is an important process.

1. Adjust the focus on a cell in the field of view, then move to a blank spot. Ensure that there is nothing in the field of view, then click **[Calibrate]**.



2. Multiple images can then be taken after a single calibration step. However, you must re-calibrate when you change the FOV or when the acquired images show aberrations.

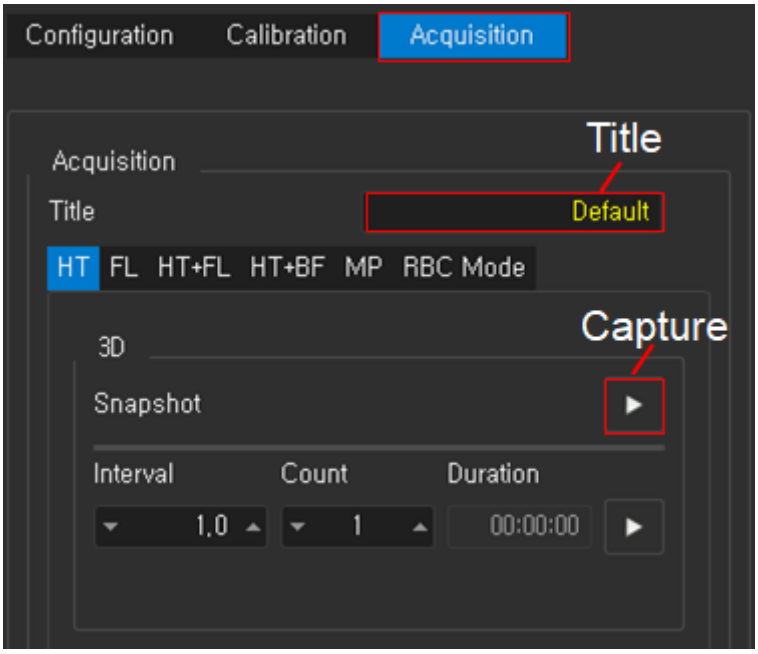
3. To check if calibration has been done correctly, click the **[Acquisition]** tab, and the image should be shown in real-time Phase mode. If calibration is successful, the image should be a uniform green, with as little yellow or ripple patterns in the background as possible. If this is not the case, move to a blank space and try again.

4.1 Holotomogram(HT) Imaging

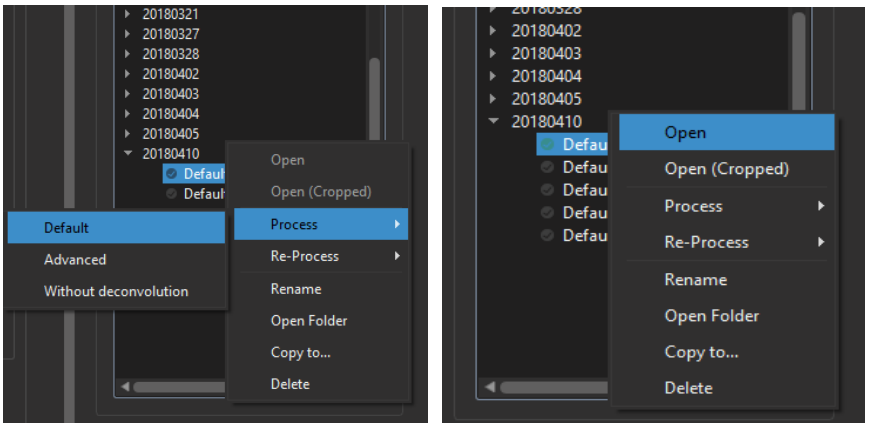
4.1.1 HT Snapshot

1. Find the target sample to image and adjust the focus on the objective lens using the X/Y knobs or 3D joystick. The target is in focus when the outline becomes transparent and blends in with the background. Then, move to a nearby blank area to acquire a background image for calibration (refer to 3.3).

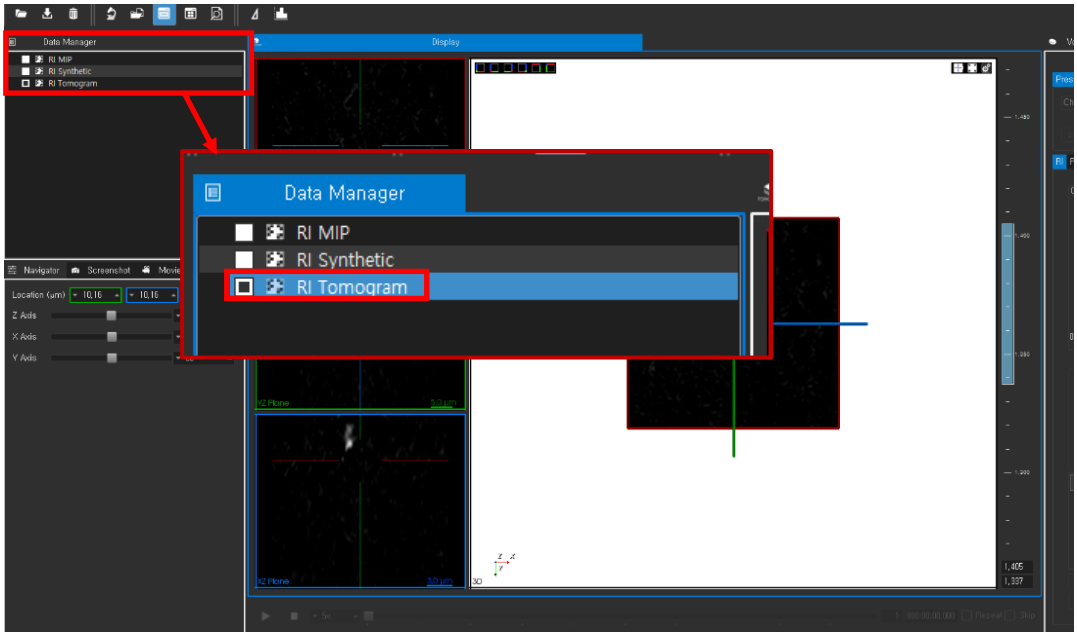
2. After calibration, focus on the cell again and click the **[Acquisition]** tab. Type in the title and click the **[Capture]** ▶ button. When acquisition is finished, a 3D image file will be automatically saved, and it will appear in the data table on the right.



3. After capturing your data, it needs to be processed. Double-click the selected file or right-click and then select **[Process]** - **[Default]** to start processing. After processing is completed, the grey dot icon to the left of the filename will become green. To view your images, double-click the processed files or right-click and select **[Open]**.



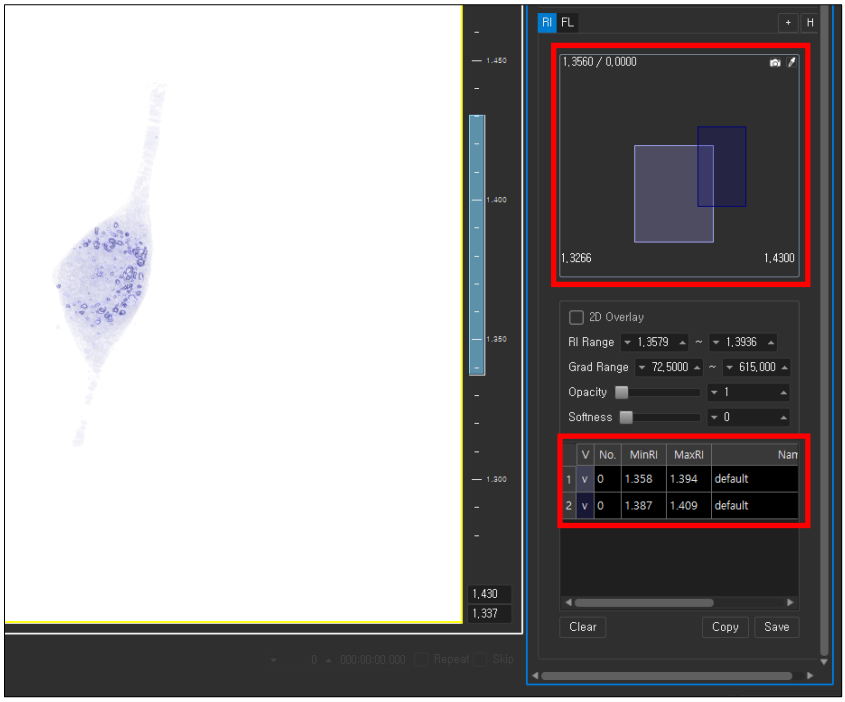
4. After clicking **[Open]**, a new screen for coloring the image will appear. The three images on the left in the red, green, and blue boxes are 2D slice images of the XY, YZ, and ZX planes. Check the **[RI Tomogram]** box in the [**Data Manager**] tab.



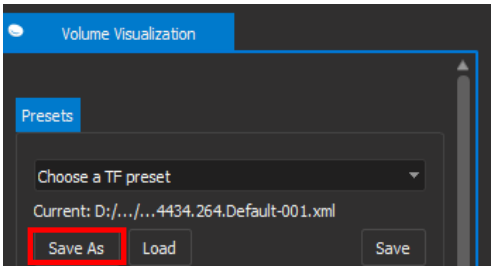
5. Left-click and drag the mouse cursor to draw a transfer-function box (TF box) on the RI canvas to the right of the main image window. The x-axis indicates refractive index range and y-axis is gradient. You can draw multiple TF boxes in different sizes that reflect the refractive index range and gradient values, and the objects with those values will appear in the display panel.

You can change the regions and colors of the drawn TF boxes by double clicking them. Right-clicking a TF box will make the color disappear from the 3D display. Furthermore, you can draw another TF box to overlay over the previous TF boxes. We recommend that you start by coloring a wide area, such as the cell matrix, and work down to small details like organelles.

> *For more information on how to color the images, refer to Chapter 5 (Coloring).*

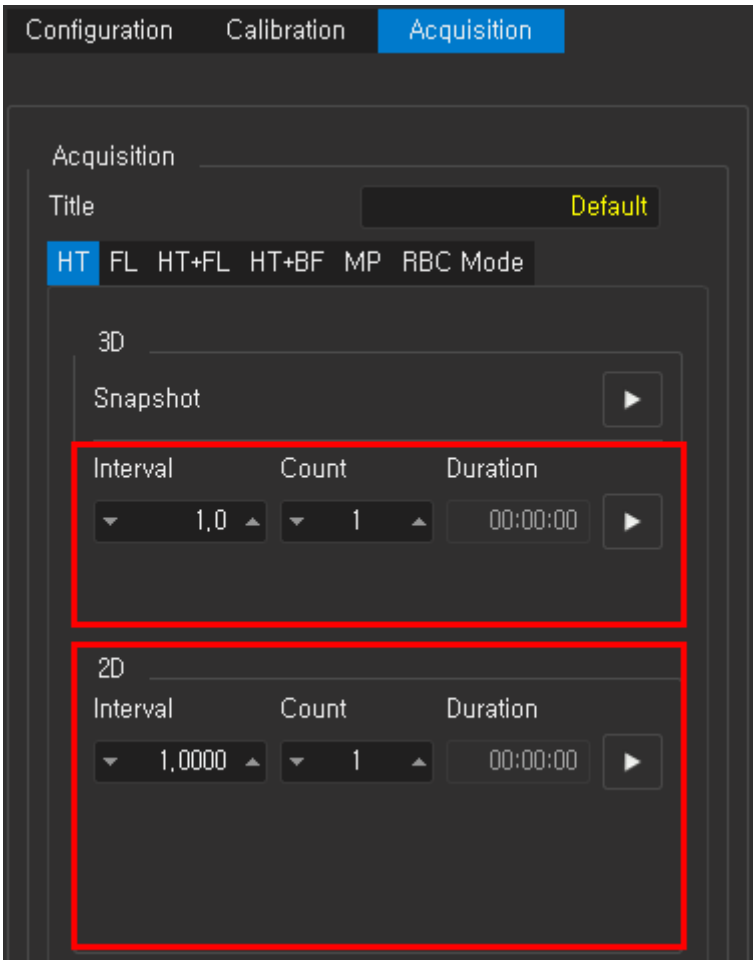


6. After finishing the coloring process, you can save the current coloring information and apply it to future samples (preset file: .xml format) with the **[Save As]** button in the [**Presets**] tab. When saving the preset file, there are three options: **[Data]**, **[Preset]**, and **[Other]**; these ask you where you want to save the file. **[Data]** saves the file in each data folder. **[Preset]** saves the file in the 'presets' folder (system default). **[Other]** allows you to choose a folder to save the preset file.



4.1.2 HT Time-Lapse

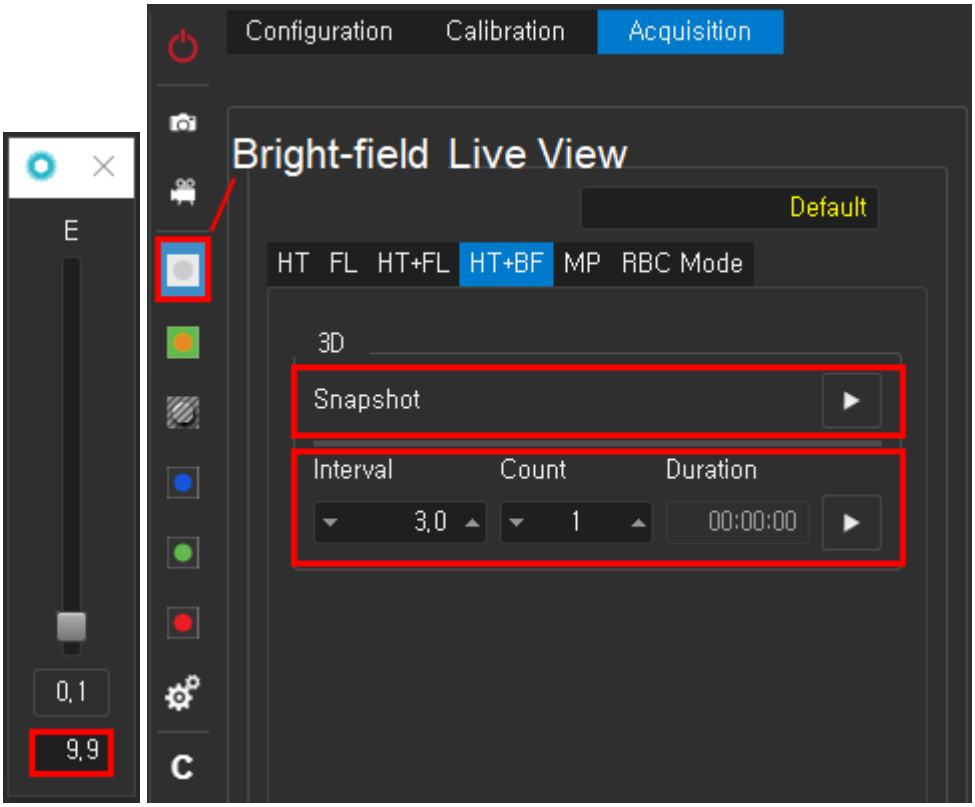
2D/3D time-lapse images can also be acquired using TomoStudio.



The red box shows the control panel for capturing 3D/2D time-lapse images. **'Interval'** refers to the time interval (unit: seconds) between two sequential captures. **'Count'** is the number of images to be taken. **'Duration'** shows the estimated time required for completing the time-lapse imaging. Click the **[Capture]** ▶ button to start 3D time-lapse imaging.

4.2 HT + Bright-field (BF) Imaging

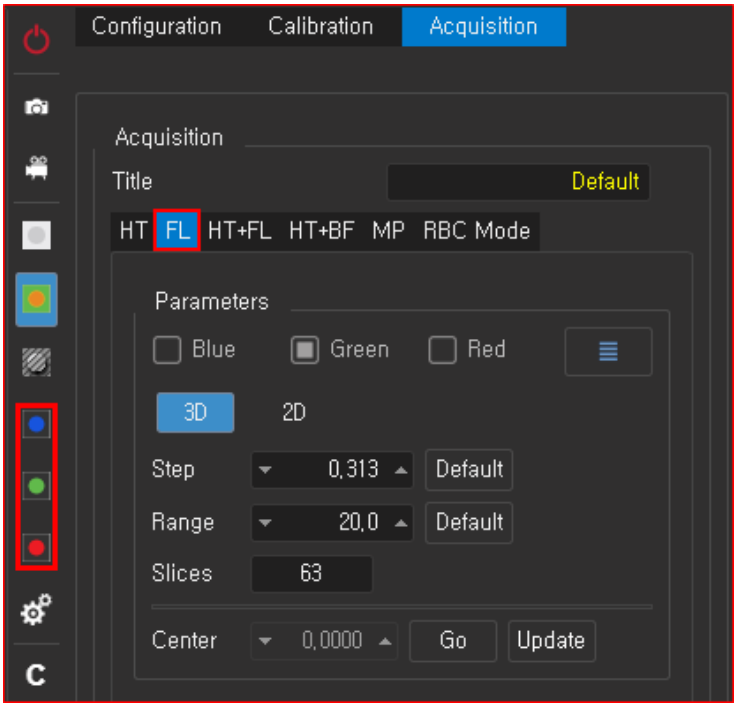
1. You can also view and capture images in the traditional Bright-field mode. To view your sample in real-time Bright-field, click the **Bright-field** icon in the live view tab. You can adjust the exposure by moving the slider or entering a value in the exposure settings next to the display. The bottom box (shown in red) represents the actual exposure value.



2. To capture HT and BF images simultaneously, click the **[HT+BF]** tab. Click the **[Capture]** button next to **Snapshot** for a single 3D HT+BF image each, or the **[Capture]** button after setting the **'Interval'** and **'Count'** below for a time-lapse image.

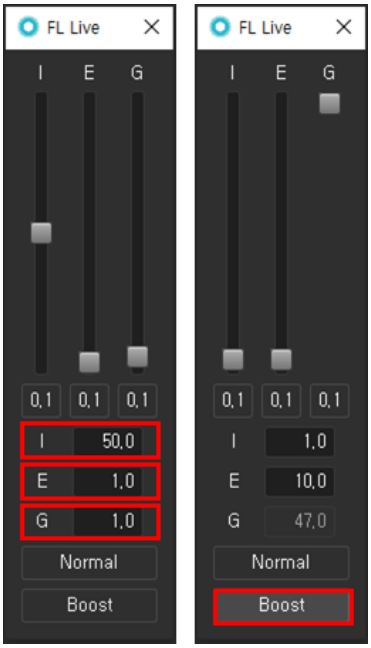
4.3 Fluorescence (FL) Imaging

1. Click the **[Acquisition]- [FL]** tab. Then, click the **Fluorescence** icon and the FL color channel you wish to view in the live view tab.



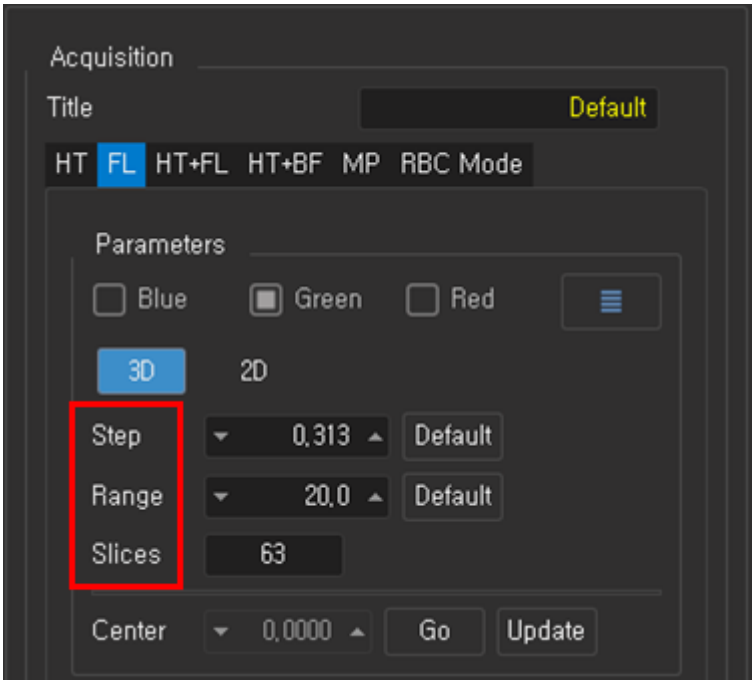
2. In the FL Live window, **'I'** represents Intensity, **'E'** represents Exposure, and **'G'** represents Gain. Move the sliders to set the values for each setting. Set the values for each fluorescence channel (Blue, Green, Red) you wish to image.

   Click '**Boost**' to find your fluorescence-stained samples quickly.
   (Boost mode decreases intensity and exposure and increases gain to find samples with the least photobleaching and phototoxicity effects)



3. After finding the sample, click Normal to return to the previous imaging settings.

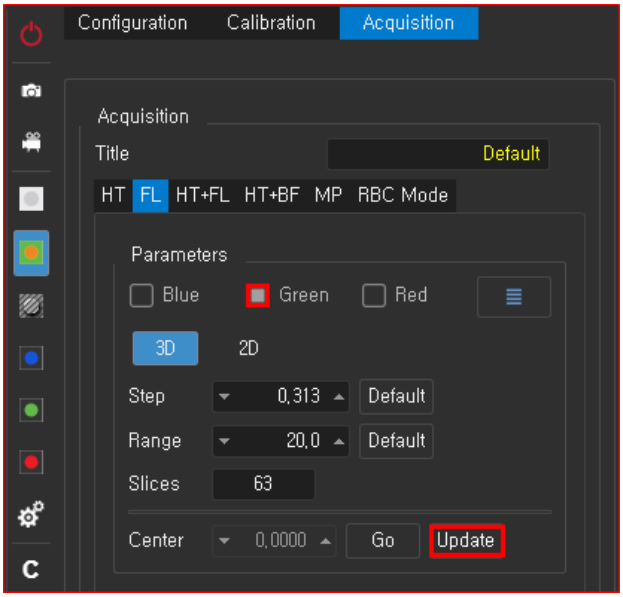
4. To acquire a 3D fluorescence image, adjust '**Step**' and '**Range**'. The scale of 'Step' and 'Range' is in μm (micrometer). '**Step**' is the interval distance between two sequential slices. '**Range**' is the vertical distance between the first picture and the last one. '**Slice**' is the number of pictures to be taken.



For adherent cells, the recommended settings are 0.313 μm for **'Step'** and 30 μm for '**Range**'. Generally, these settings will give suitable oversampling for subsequent deconvolution. Alternatively, the user can find appropriate settings for their own experiments.

Note: we do not recommend setting **'Step'** below 0.15 μm or above 0.45 μm, which will most likely result prolonged exposure which might cause cell death or a deconvolution error, respectively.

5. After adjusting focus, the '**Update**' button will turn yellow. Click to update z-axis focus for the fluorescence imaging. Check the box next to the fluorescence channel color you wish to image.



6. To capture a 3D FL image, click the '3D' tab and **[Capture]** button.

   To capture a 2D FL image, click the '2D' tab and **[Capture]** button.

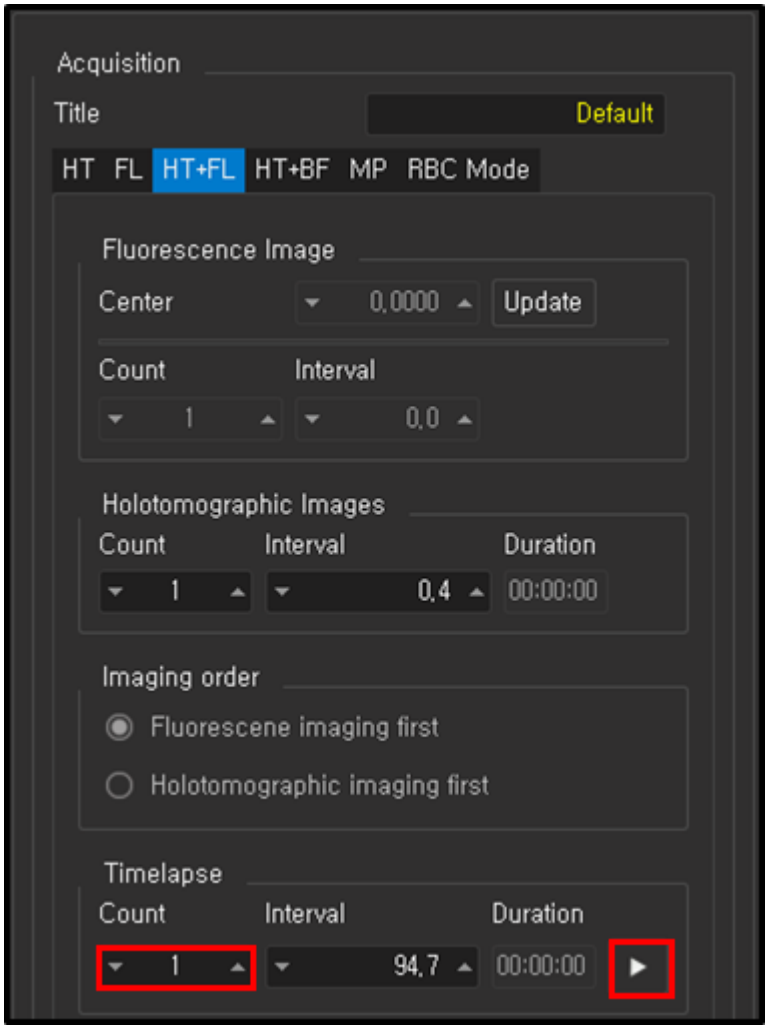
   To capture time-lapse images, first set the **'Interval'** for the number of seconds between each cycle and set **'Count'** as the number of cycles to be captured. **'Duration'** shows the estimated time required for completing the time-lapse imaging. Click the **[Capture]** button to begin imaging.

4.4 HT + FL Imaging

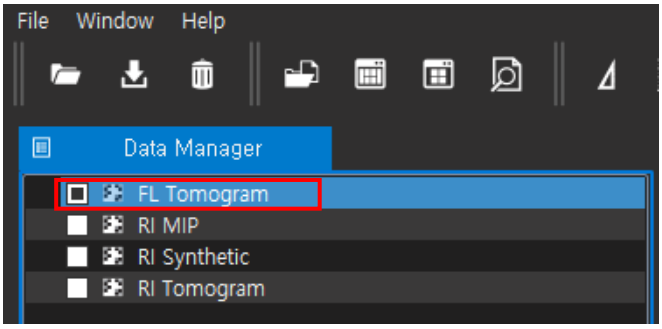
1. To capture HT and FL images simultaneously, proceed with the FL setup as explained in (4.3), then click the **[HT+FL]** tab. To capture a single 3D snapshot image, set '**Count**' as 1 and click the **[Capture]** ▶ button.

   For capturing HT+FL time-lapse images, set the **'Count'** as the number of images you would like to acquire and set the **'Interval'** as the number of seconds between each image. Then begin imaging by clicking the **[Capture]** ▶ button. Finally, process the image (4.1.1) and save the file.

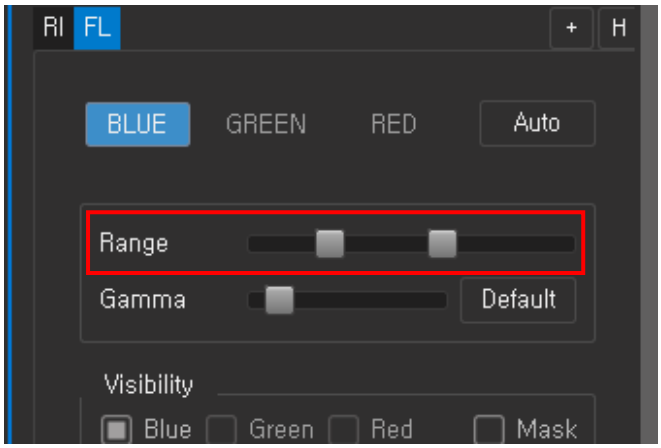
> ***To ensure that the focus of fluorescence signals match well with HT signals, click 'Update' to update the z-axis focus of fluorescence signals.***



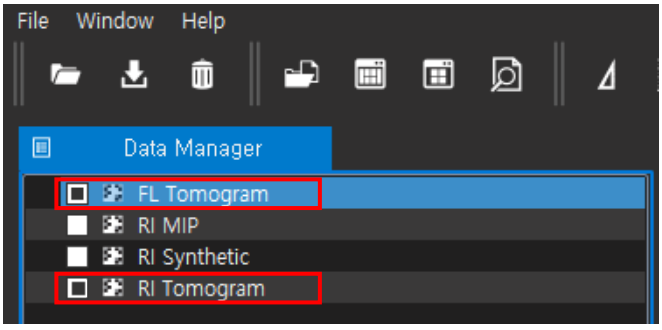
2. Click the processed image file with the right mouse button and then press **[Open]**. The processing screen will appear on the left panel. Choose **[FL Tomogram]** to adjust the fluorescence images.



You can adjust the **'Range'** and **'Gamma'** in the **[FL]** tab. There are two buttons on both sides of the **'Range'** scale bar. The distance between these two buttons determines the fluorescence brightness.



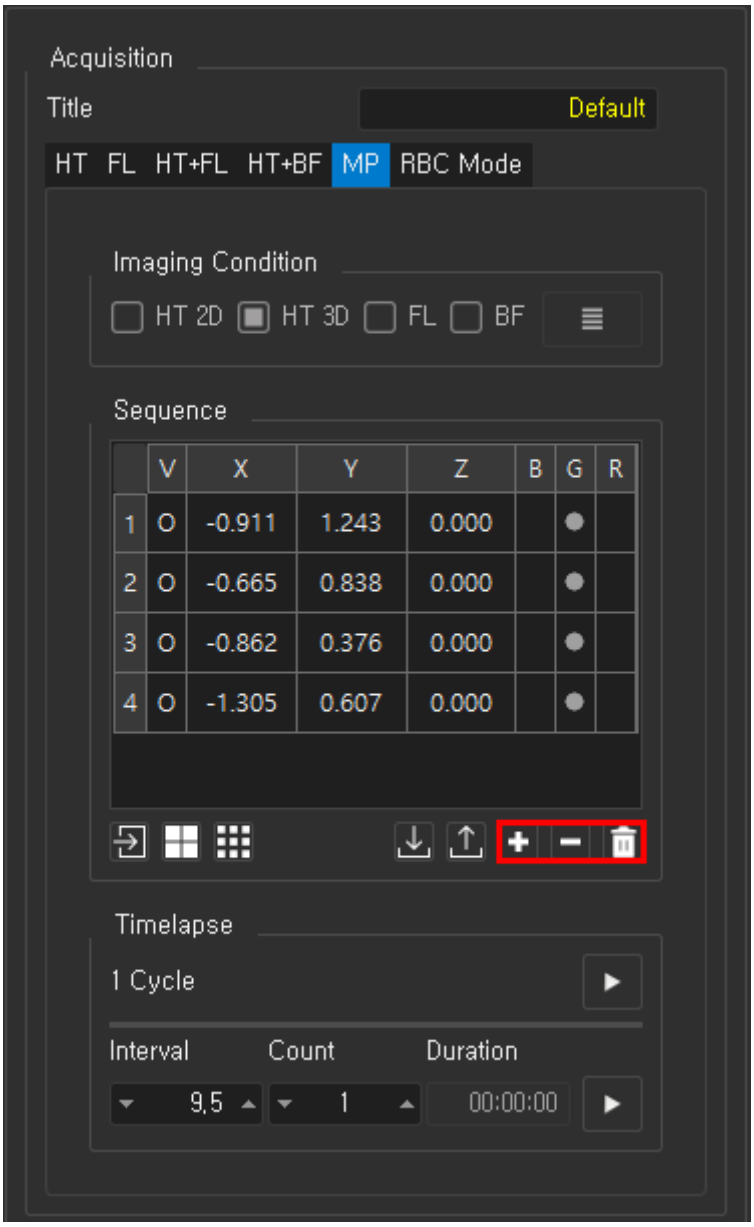
3. After adjusting the settings, check both the **[RI Tomogram]** and the **[FL Tomogram]** on the left panel; this allows you to simultaneously visualize both fluorescence and 3D-holotomogram images. Note that if the 3D-holotomogram does not appear, you may need to overlay colors first (refer to 4.1.1).



4.5 Multi-Point (MP) Imaging

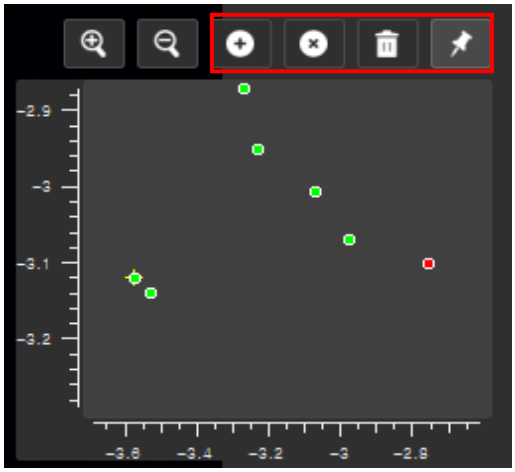
MP mode allows for the acquisition of images at multiple-points in the sample with only one click. The **'Imaging Condition'** tab allows you to select whether you will capture HT 2D, HT 3D and/or FL images.

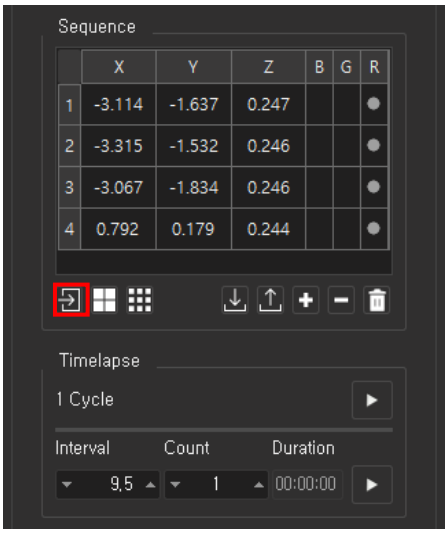
1. Click the **[+]** button to save the current position of the sample to the list. For optimal image quality, adjust the z-axis focus for each point. You can also delete a previously selected position in the list using the **[-]** button. Clicking the **[Trash can]** icon clears all saved positions.



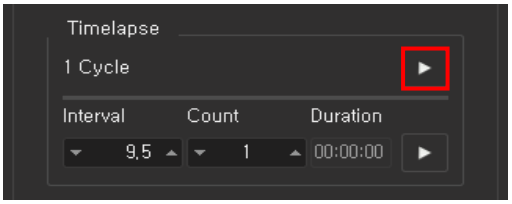
2. You can also use the mini-map to temporarily save positions. This function will be useful when you want to go back to a certain position without using the MP mode. A mini-map will appear when you place the mouse cursor on the bottom right corner of the display.

   [+] and **[x]** buttons add and remove a position, respectively. Clicking the **[Trash can]** icon clears all saved positions. The **[Pin]** icon locks up the mini-map on the display screen. You can also move to a specific position by double clicking the corresponding location in the map. The **[Import]** icon imports the list of points from the mini-map into the MP list.

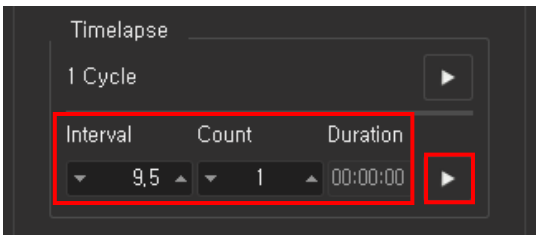




3. To acquire snapshots of multiple points, click **[Capture]** next to **'1 Cycle'**.

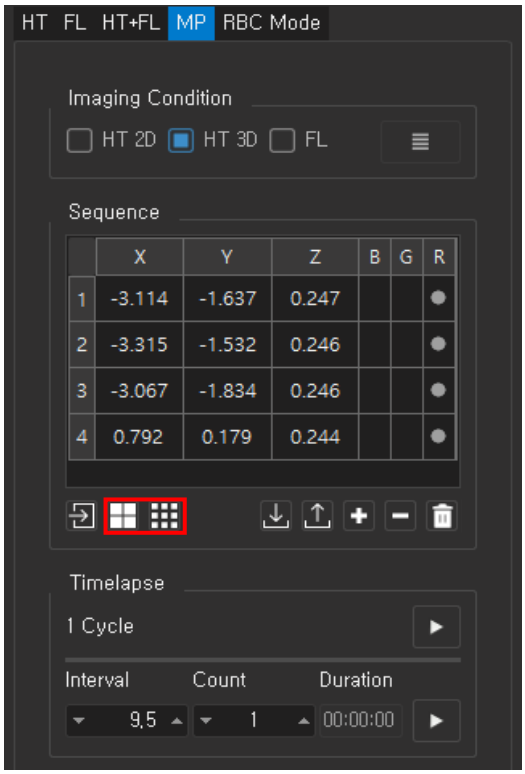


4. To capture time-lapse images, first set the **'Interval'** for the time in seconds between each cycle and set the **'Count'** for the number of cycles to be captured. **'Duration'** shows the estimated time required for completing the time-lapse MP imaging. Click the **[Capture]** button to begin time-lapse imaging.



5. MP mode also offers tile scanning and matrix scanning options. **Tile scanning** is useful for capturing areas larger than the single FOV, and **matrix scanning** can be used for cell or particle counting when it is required to sample large areas.

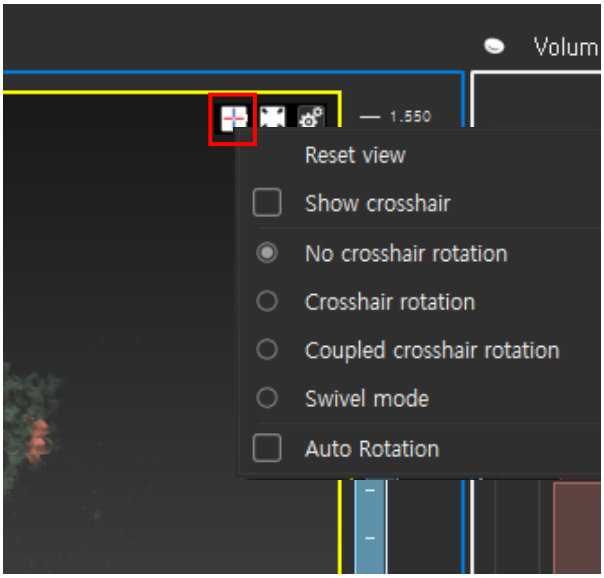
> *For detailed information, refer to TomoStudio SW manual.*



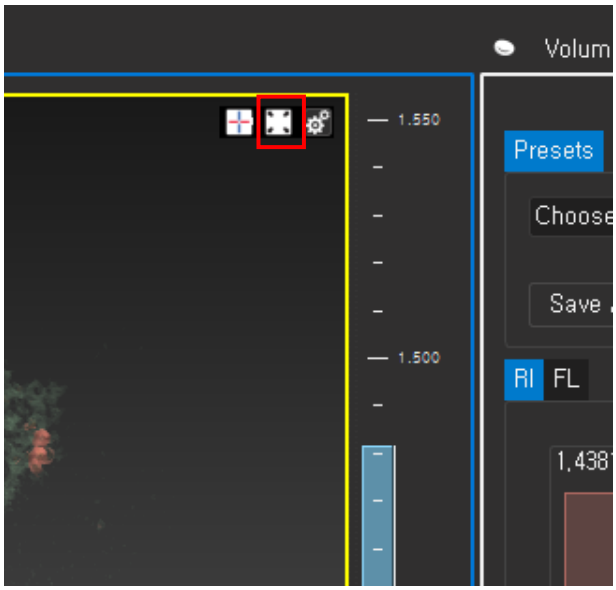
4.6 Display Setting Options

There are display setting options on the upper-right corner of each image. These options appear when you place the mouse cursor on the display area.

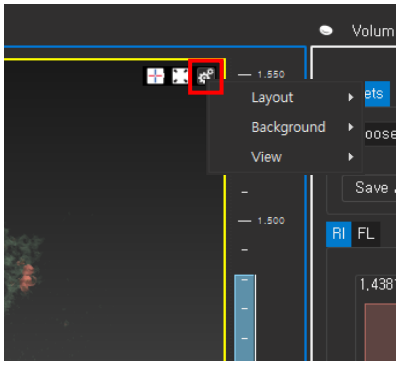
1. The first icon is for setting view options. **'Reset view'** restores the 3D image back to the initial XY position. The user can also choose to rotate the image automatically and whether to show or hide crosshair lines.



2. The second icon enlarges or minimizes the image in the display panel.

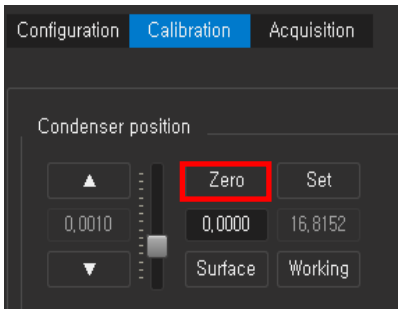


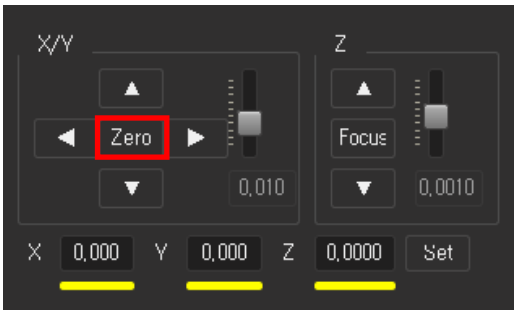
3. The last icon consists of **'Layout'**, **'Background'**, **'View'** options. **'Layout'** is an option for setting layouts of the images. **'Background'** allows you to choose the color of background. There are black, white and gradient, which is the default setting. Finally, you can also decide whether to show or hide the boundary box, axis grid, time-stamp, and title on an image using **'View'** setting.



4.7 Clean Up

1. Click the **[Zero]** button in the condenser position controller and in the X/Y control box. The condenser lens and objective lens will move to the initial position. Remove the sample, then exit the TomoStudio and turn off the microscope.





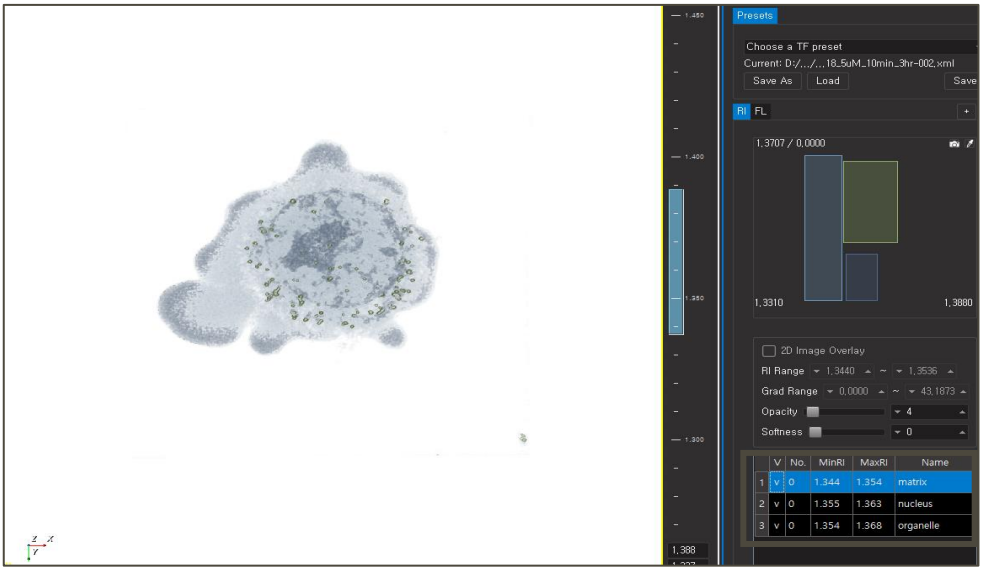
2. Carefully clean the objective and condenser lenses with the lens cleaning tissue or Texwipe swab. In the case of water-immersion lens, disinfect using 70% ethanol and then wipe with isopropanol to clean the lens.

Coloring

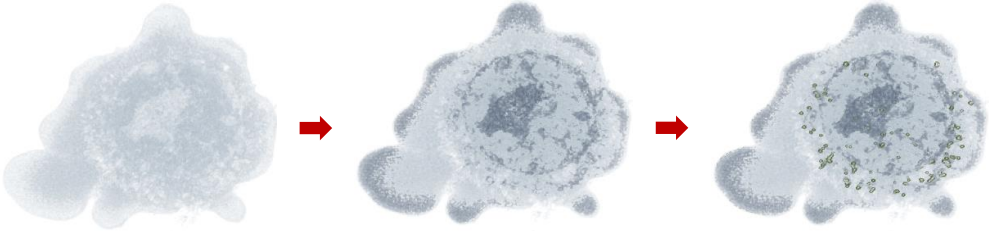
5.1 Overlay

After processing, the captured holotomogram can be colored manually. Select the data by right-clicking and click **[Open]** which will automatically open the analysis tab. Under the **'Data 'Manager'** panel on the left, click **[Tomogram]** and you will see the **'Presets'** panel on the right of the screen. In the RI canvas, you can draw the color boxes for different RI components of the cell.

Start by creating a color box, and you will see that the color box information automatically appears on the list below the RI canvas. All color boxes can be overlaid on top of each other, with the box with the highest RI value on the very top. The overlaid color boxes may not look different at the front view; however, they may appear different when viewed from the side after rotating the imaged object. Therefore, how the color boxes are laid out is important.



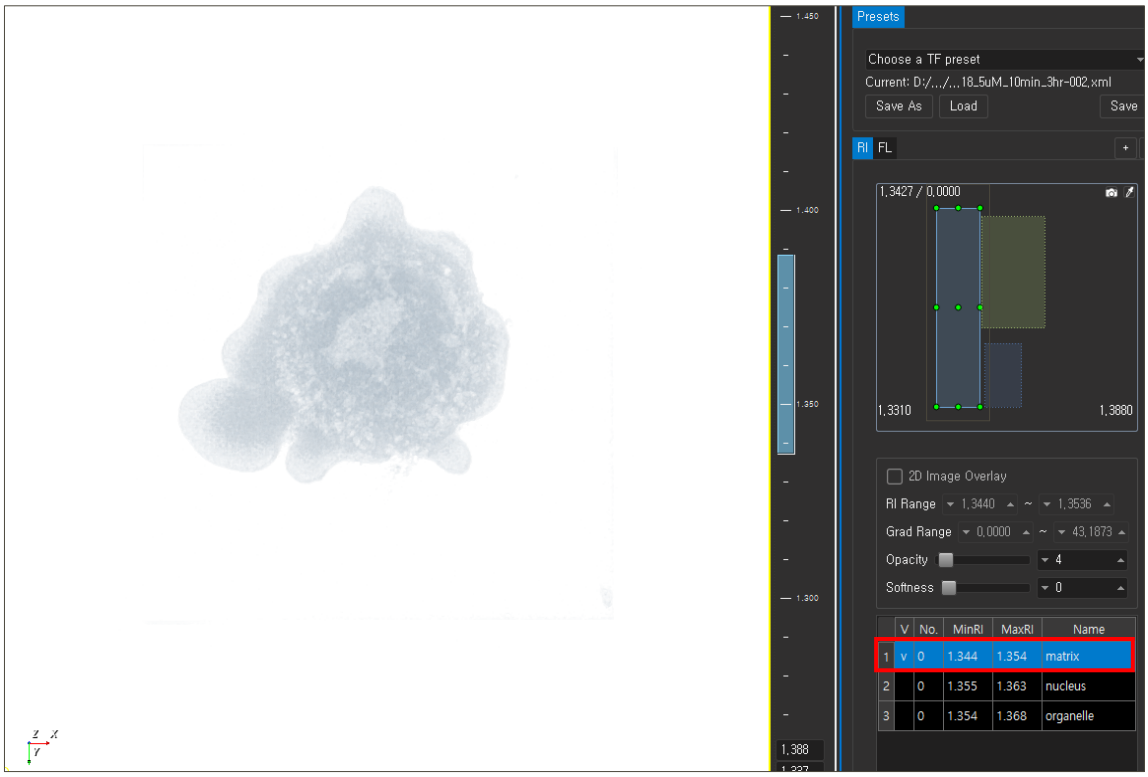
We recommend coloring from the wider area and narrow down to the smaller details later. It is recommended to color the cellular matrix first, and the organelles later. However, you can also color the details which you would like to emphasize at the last time. Note that the RI values for the cellular components are similar for most cell types, thus saving the color box pallets as presets and re-using them can be useful.



5.2 Matrix

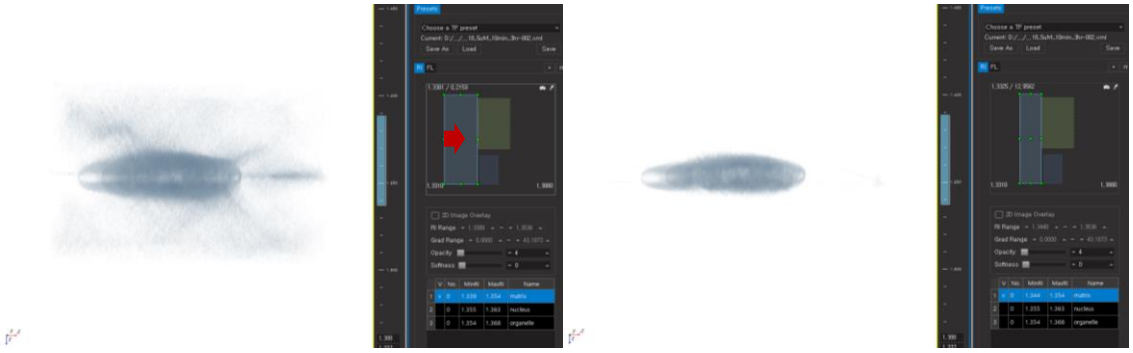
To color the matrix, refer to the values in the table and adjust the color box's width and height to find the optimal coloring for your image.

If the opacity of the matrix is too high, the cell image may look too dark making it difficult to distinguish the organelles. The recommended opacity for matrix is between 4-9.



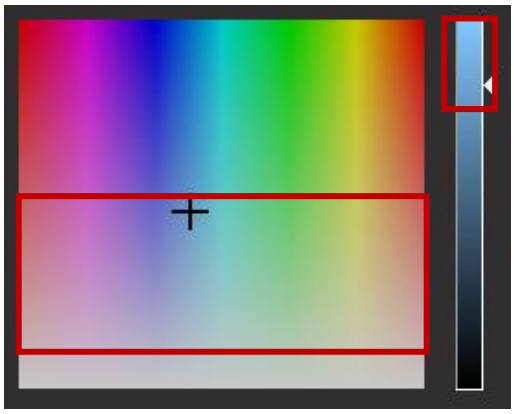
Reference value for the matrix color box:

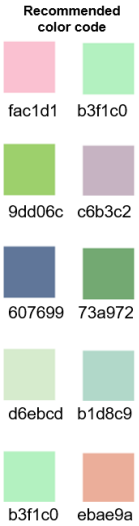
No.	MinRI	MaxRI	MinGrad	MaxGrad	Opacity	Softness	Name
0	1.344	1.354	0.00	43.19	4.5	0.0	matrix



Increasing the size of the box by dragging too far to the left will color the background noise too, which appears as the smear in the first image. To remove the noise, decrease the box size by dragging the left side of the box to the right (arrow). This will increase the minimum RI value, and the smear will disappear after the correction as shown in the right image.

If the inside of the matrix looks empty, you can also increase the maximum RI value by dragging the right side of the box to the right. However, if the color of the matrix is too dark, it can be difficult to see the details of intracellular organelles. For this reason, we recommend using bright colors for the matrix.



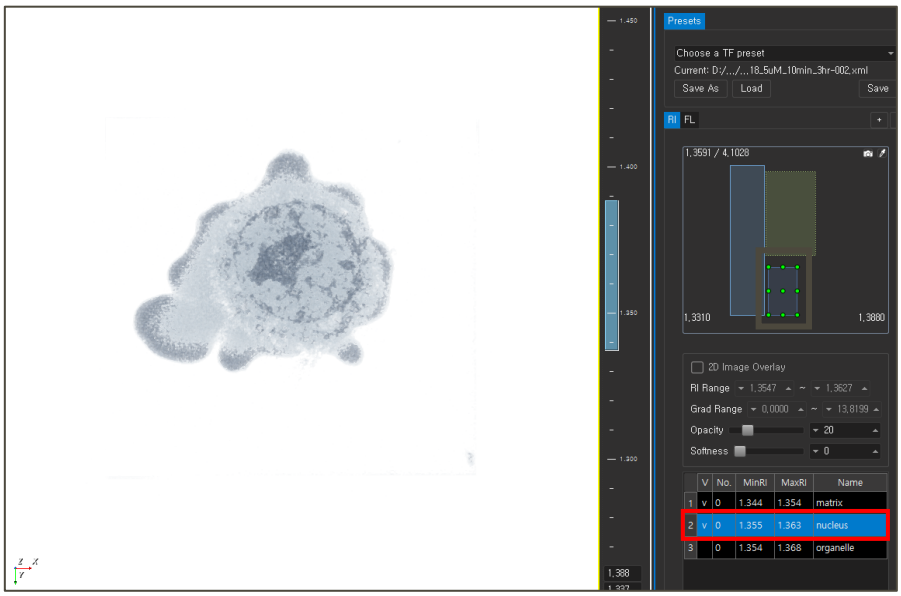


We recommend a few color codes for the matrix. Alternatively, you can also choose a color of your preference. We recommend the colors within the red box, which are bright and have low chroma. You can also choose a color with high chroma but adjusting the opacity will be required then.

5.3 Nucleus

To color the nucleus, draw a box near the bottom-center of the canvas. Usually, the nuclear membrane and some of the cellular components are colored together for their similar RI values. Therefore, it is highly recommended to emphasize the nucleus distinctively by optimizing the RI of its color box.

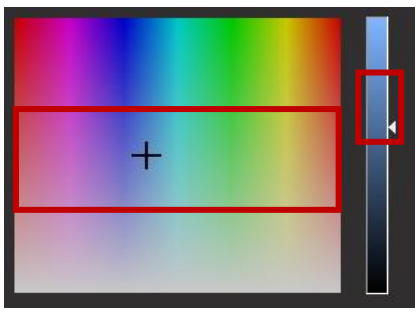
The nucleus is likely to obscure the organelles; therefore it is required to avoid setting the gradient of the nucleus color box too high. The recommended opacity is between 10-25.



Reference value for the nucleus color box:

No.	MinRI	MaxRI	MinGrad	MaxGrad	Opacity	Softness	Name
0	1.355	1.363	0.00	13.82	20.5	0.0	nucleus

Avoid using colors complementary to the matrix color pallet. We recommend using vivid colors that are not too dark.

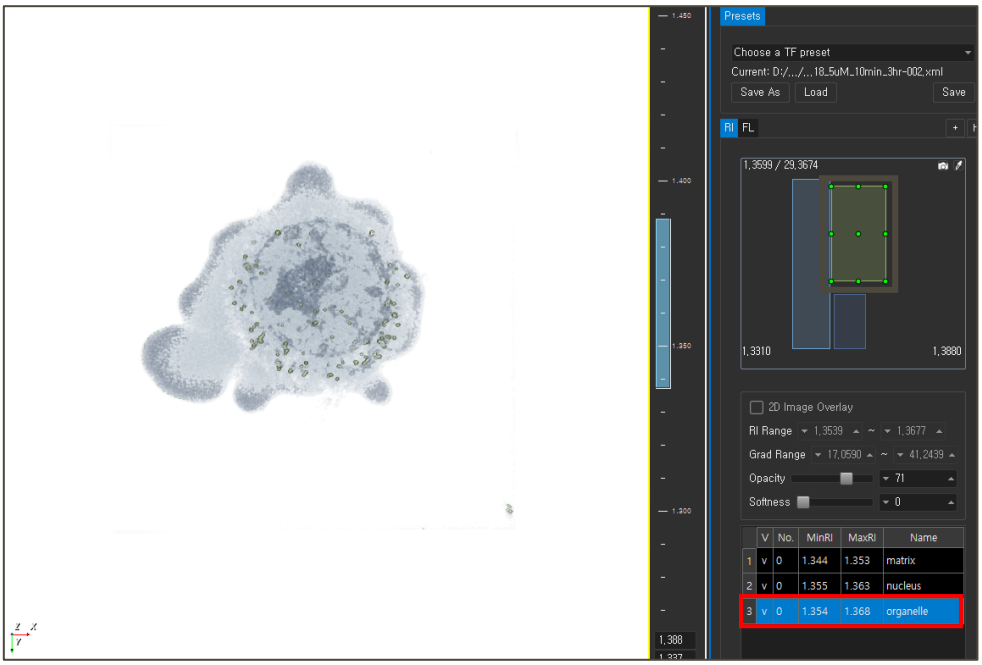


5.4 Organelle

If the min RI value of the color box is too low, unnecessary details such as the edge of the cell may also be highlighted. Adjust the min RI values to avoid it.

The recommended opacity is between 40-75. Increase the opacity just enough to see the organelles from the side view. Avoid increasing opacity too much because lower opacity makes images more natural.

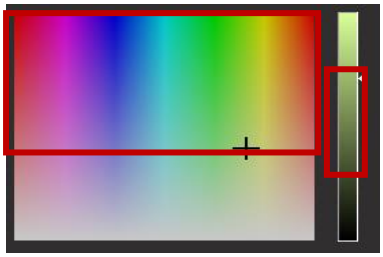
Choose a color which is different from the matrix/nucleus color pallet and avoid using complementary colors.

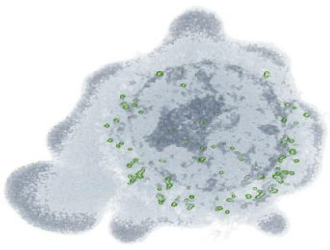


Reference value for the organelle color box:

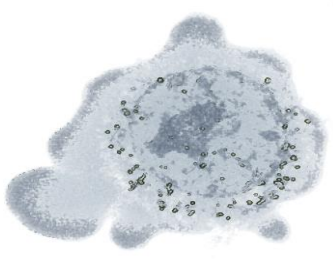
No.	MinRI	MaxRI	MinGrad	MaxGrad	Opacity	Softness	Name
0	1.354	1.368	17.06	41.24	71.5	0.0	organelle

If the chroma of the color is too high, the organelles may not look natural. On the other hand, if the brightness is too low, the organelles may appear too dark.





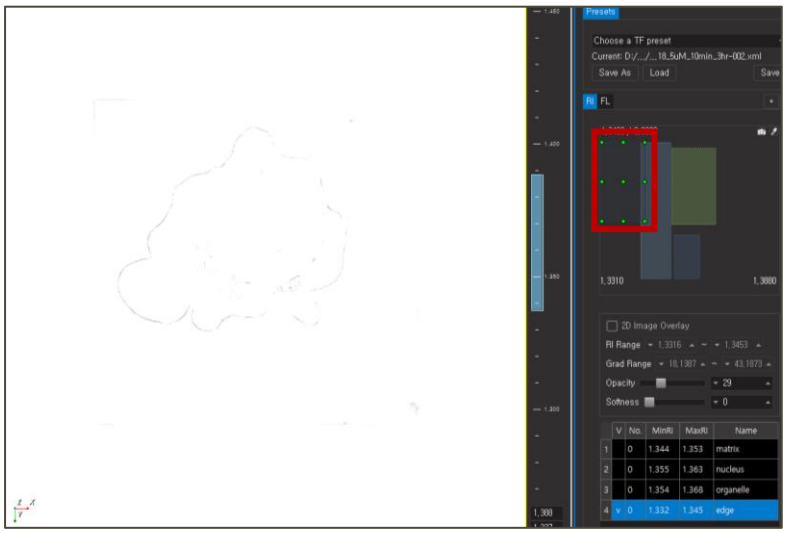
▲ When the chroma is too high

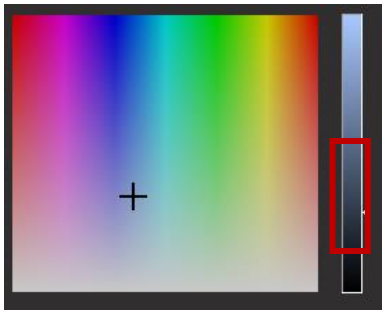


▲ When the brightness is too low

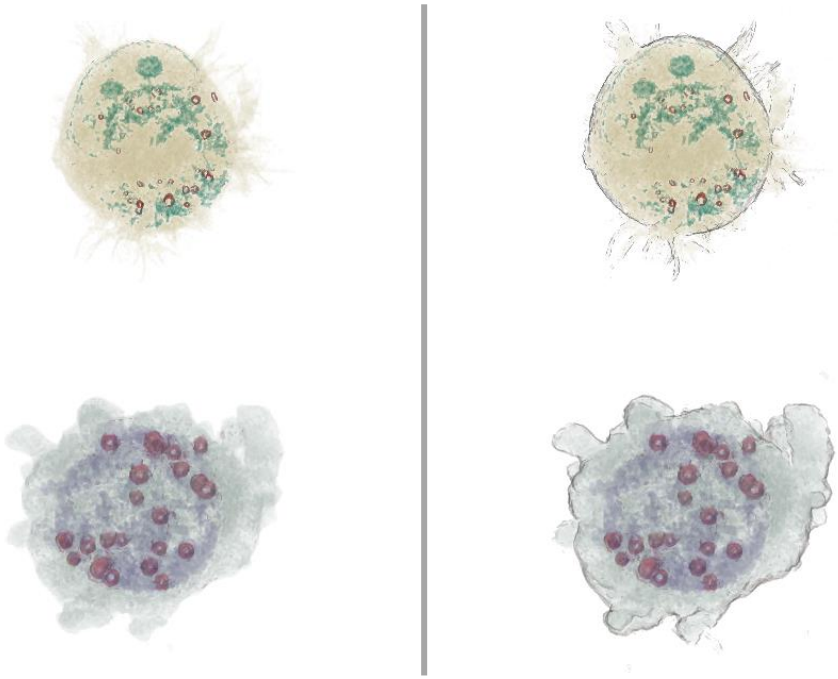
5.5 Edge

You can also selectively color the edge of the cell. It is recommended to color a minimal portion of the cell edge and set the opacity between 10-30.





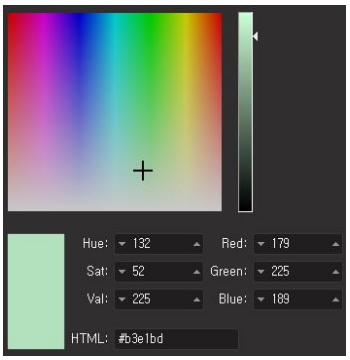
We recommend using a color similar with that of the matrix but with reduced brightness.



5.6 Color Combination

5.6.1 Coloring the matrix

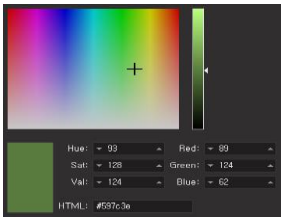
This is an example of a cell undergoing mitosis. Color the matrix first. The opacity of the current color box is 4. Choose a color near the bottom of the palette and increase its brightness.

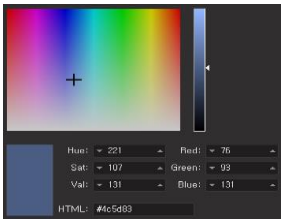


5.6.2 Coloring the organelles

In order to avoid colors which are complementary to green, we used red. The opacity of the current color box is 34.

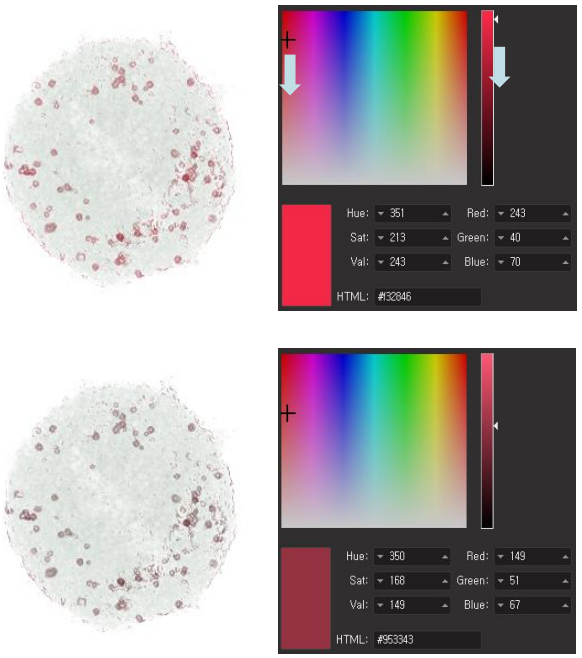
If you would like to use complementary colors, reducing the chroma and the brightness will make the colors match well. (This tip also applies to all other colors.)





5.6.3 Adjusting chroma and brightness

In the first image, the organelles are colored with high chroma and brightness. Because green and red are opposing colors, the colors do not harmonize.



In this case, reducing the chroma and the brightness can help. As shown in the figure, choose a color near the bottom of the color palette. Also, reduce the brightness by adjusting the arrow on the brightness bar to the right of the color palette.

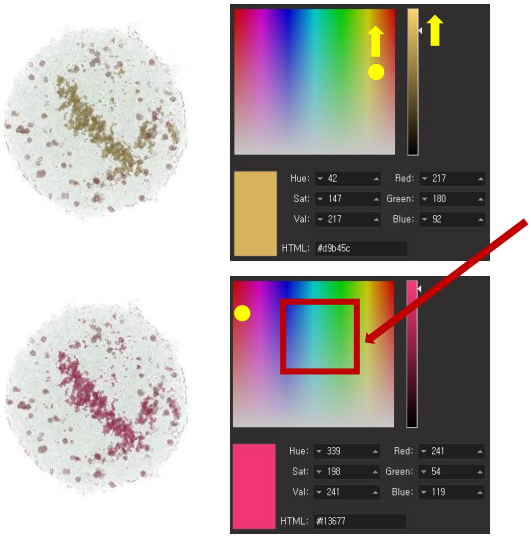
Keep adjusting the chroma and the brightness until you find a natural color for your image. Or you can also use non-opposing colors.

5.6.4 Choosing colors to emphasize details

The yellow dot in this figure shows the position of the color in the palette used for the organelles.

In this image showing mitosis, we would like to emphasize the chromosomes. However, the picture above is using the colors with the same chroma for both the matrix and the chromosomes, and therefore the chromosomes do not look emphasized.

We recommend using a color with a different chroma to emphasize the organelles. For this image, we recommend dark green/ bluish green/ blue. Using low opacity for the matrix will allow it to well match organelles with other colors.



5.6.5 Adjusting opacity

Usually the last cellular component needs to be colored with high opacity. However, the component might become too dark when you make its opacity high as shown in the first image.

Therefore, using a lighter color by reducing the chroma or by increasing the brightness is recommended. You can also make a similar result by reducing the opacity. However, with the latter method, the organelles may become difficult to observe when viewed from the side.