Tomochamber media change Protocol

H_2O_2 treatment and ROS detection in live cells

Purpose: Exchange media without removing TomoDish from TomoChamber.

This method will be useful for when the researcher wants to image the same cell, while changing the media condition (i.e. media change in long-term imaging, adding fluorescent dyes or washing out drugs).

This protocol illustrates a step-by-step guide to the multi-point (MP) imaging of live cells before and after H_2O_2 treatment. Reactive oxygen species (ROS) in cells were detected using ROS fluorescent dye.

Setting up Multi-point imaging

For calibration and setting up TomoChamber, refer to 'Time-lapse protocol'.

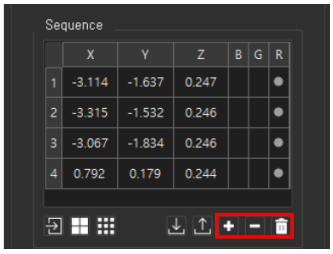
1. Calibrate HT-2H and set-up TomoChamber with live cells cultured on TomoDish.

   30 minutes prior to imaging, incubate cells in media with Image-iT™ Green Hypoxia Reagents (Invitrogen, I14834) diluted to a final concentration of 10 μM.

2. In **[Acquisition]** panel, under **[MP]**, you may find **[Imaging Condition]** tab. For acquisition of 3D RI tomogram of multiple cells, click checkbox next to **[HT 3D]**.
3. There are two ways to record multi-points of cells which you want to take images:

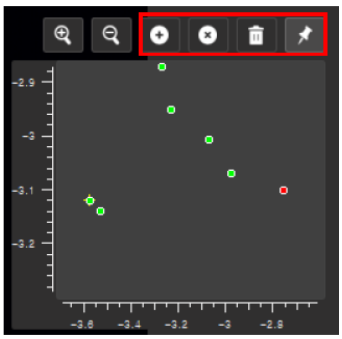
   a. Use **[+]** button under **[Sequence]** tab to add the present position of the objective lens to the list.

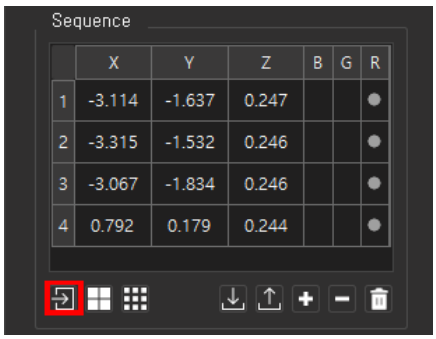
You can also delete a selected position on the list using [-] button. Clicking the [Trash can] icon clears all saved positions.



   b. Alternatively, use the mini-map to import positions. Click **[+]** button on the mini-map to add the present position of the objective lens to the mini-map. After saving all the positions which you want to acquire images on the mini-map, click the **'Import icon'** under **[Sequence]** tab to import the list of point from the mini-map into the MP list.

For optimal image quality, adjust z-axis focus for each point you add.





To check the calibration status, we recommend taking one snapshot of multiple points and reviewing image quality before beginning time-lapse imaging.

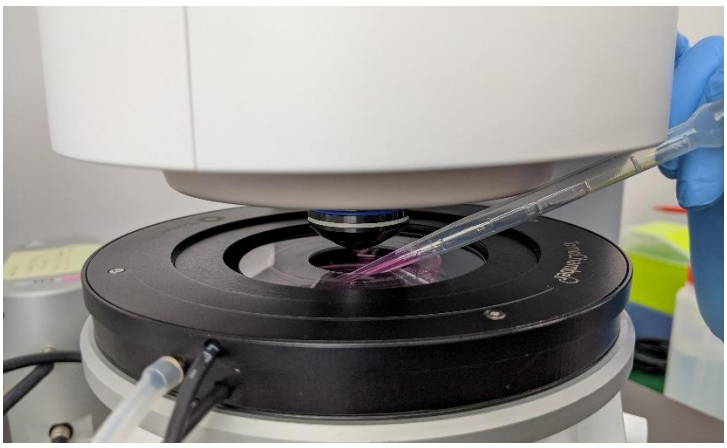
4. After finalizing the list of MP positions, click **'Capture icon'** next to **[1 Cycle]** to acquire one snapshot of multiple points.
    a. To capture time-lapse images, Set **[Interval]** and **[Count]** before clicking **'Capture icon'** to begin time-lapse imaging.

H_2O_2 treatment by media change

You may click [Zero] to initialize the condenser lens position. However, we recommend moving condenser lens to 10 mm to minimize the movement as much as possible.

1. Prepare warmed-up media added with H_2O_2, diluted to a final concentration of 250 μM (Sigma Aldrich, H1009).
2. Click **[Set]** button under **[Calibration]** tab to save the current position of the condenser lens.
3. Disconnect lens-warmer from condenser lens. Change condenser position to 10 mm by typing in '10.0000' in the input space under **[Zero] button**.
4. Carefully remove the media from the TomoDish using a sterile plastic 3 mL pasteur pipette.

    Be careful not to touch the bottom of the dish with the pipette-tip, which will cause displacement of the TomoDish.

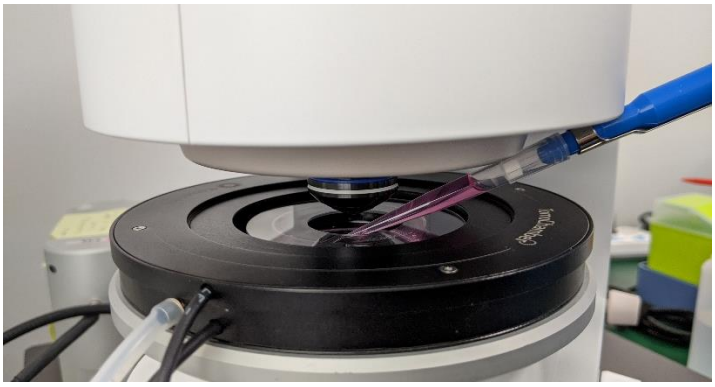


Either one of plastic Pasteur pipette or pipette-tip can be used according to the preference.

Pasteur pipette has the advantage that it can bend easily to access the TomoDish and draw in 3 mL of media at once, and the pipette-tip has the advantage that it can add the exact amount of a solution.

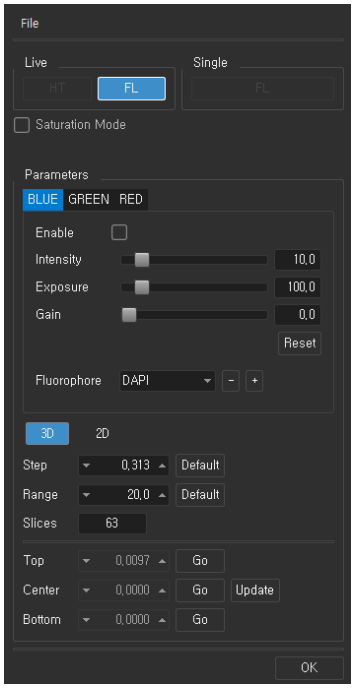
5. Gently add 3 mL of H_2O_2 containing media to the Tomodish using 1000p pipette-tip.

   If washing step is required, repeat removing and adding media.



6. Click **[Working]** under **[Calibration]** tab to move the condenser lens to its previous working position. Carefully mount the lens-warmer on the condenser lens.
7. Incubate the cells for 2-4 hours at 37°C, 5% CO_2 to allow ROS generation to occur.

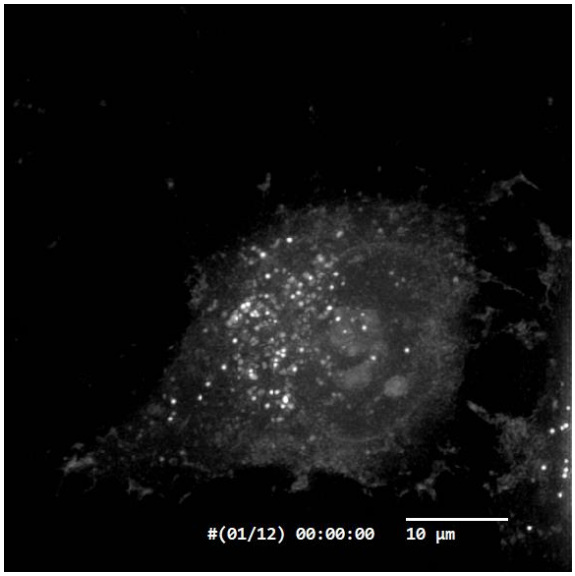
Acquiring Multi-point HT and FL images

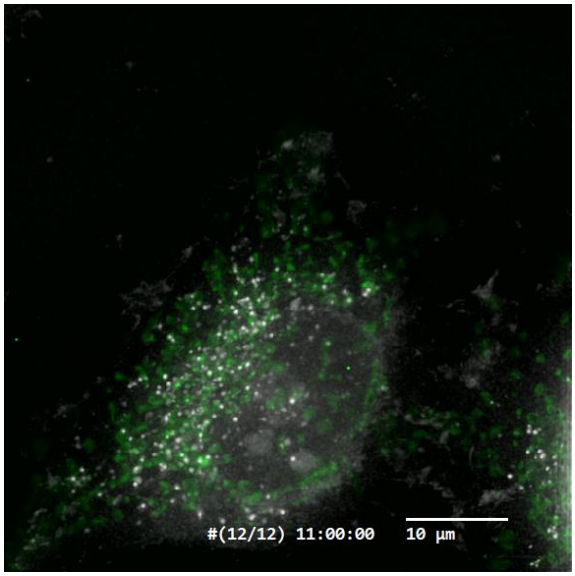


1. For 3D RI tomogram and 3D fluorescence imaging, check boxes next to **[HT 3D]** and **[FL]** in **[Imaging Condition]** tab under **[MP]** in **[Acquisition]** Panel.
2. Adjust 3D fluorescence image settings by clicking the icon. A new window will pop up where you can adjust parameters for each of the Blue, Green and Red channels. Check box next to **[Enable]** under the fluorescence channel you want to activate. For Green Hypoxia Reagent stained cells, enable the green channel.
    a. Click **[FL]** under **[Live]** menu to view live fluorescence images. Adjust **[Intensity]** and **[Exposure]** so that the fluorescence signal is strong, but not too saturated.
    b. Then adjust **[Step]** and **[Range]** of 3D fluorescence image. For imaging of a typical mammalian cell, 0.313 µm steps and 30 µm range is recommended.
    c. Finally, adjust z-axis focus until the fluorescence image is in sharp focus. Then update the center of 3D fluorescence image by clicking **[Update]**. Click **[OK]** to close the window.
3. Back in **[MP]** tab, re-adjust z-axis focus of each of the MP positions, if necessary. You can do this by double-clicking each position under **[Sequence]** tab to move objective lens position to the area. Adjust z-axis focus and right-click the same position and click **[Update]**.

Appendix A. Maximum intensity projection image of live HepG2 cell before and after H_2O_2 treatment. 3D holotomography and fluorescence images were acquired using HT-2H. Green fluorescence indicates the presence of ROS within cells.

Cell #1. At 0 and 11 hours after H_2O_2 treatment.





Cell #2. At 6, 7, 8 hours after H_2O_2 treatment.