# Two$^{MP}$ and MassFluidix HC Standard Operation Protocol

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PPE (BSL-1)

1. Laboratory Coat
2. Nitrile Gloves
3. Goggles / Safety Glasses

Materials to bring

1. Calibrant
2. Buffer
3. Diluted sample
4. Gasket, glass slide or HC chip (if already bought 1 set from us)

## *1. Initialization*


- Turn **ON** the Isolation switch which is located at the front of your active vibration isolation table. For that press the buttons “Power” and then “Isolation”.
- Sign in the logbook.

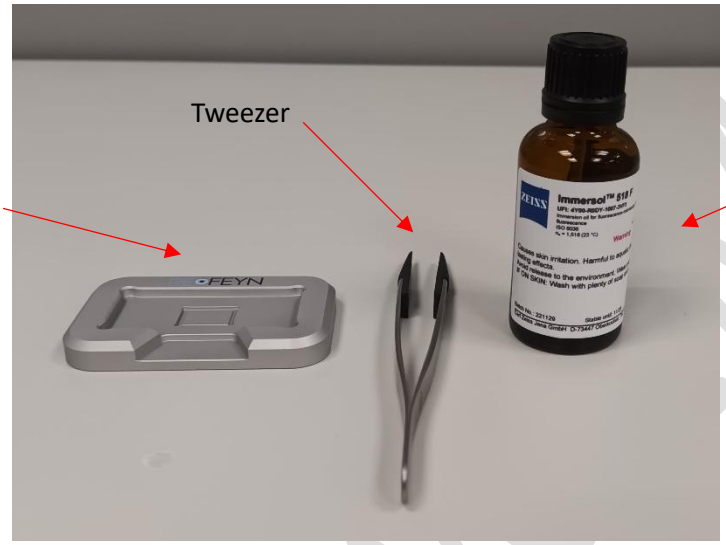
Note: The electronics unit and Acquire$^{MP}$ (AMP) software should be turned **ON** by staff at least one hour before starting the experiment.

- Go to the Acquire$^{MP}$ (AMP) software.

- Press “Open project folder” to create or locate your file (you only need to do this at the start of your experiments).

Note: The file should be saved to your designated folder and the naming structure would be: PI_Username.

- Take out the box with an Alignment Tool, Tweezer and Immersion oil in the drawer labeled with TwoMP.

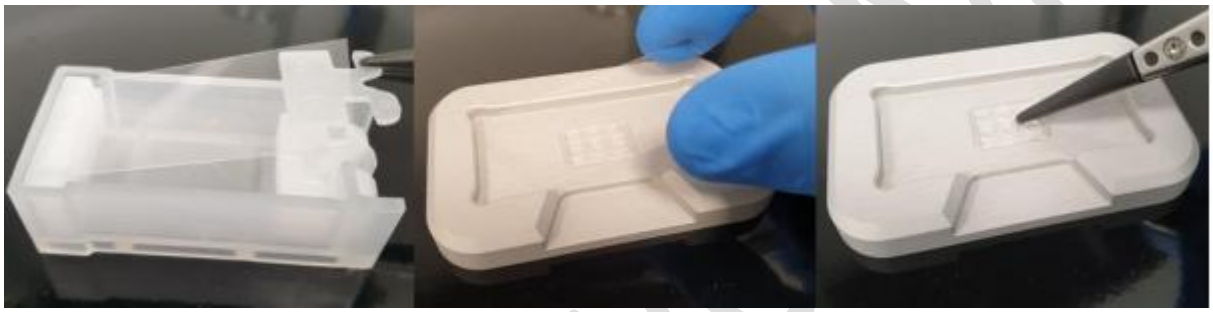



- Take out the Sample Carrier Slide (glass slide) and Sample Well Cassette (gasket). If you need to purchase a new set, please contact our staff.


Note: You should buy a whole set of them from CryoEM staff and bring them to the lab.

- Using tweezers and gloves, take a gasket and place it on the Alignment Tool in the middle.

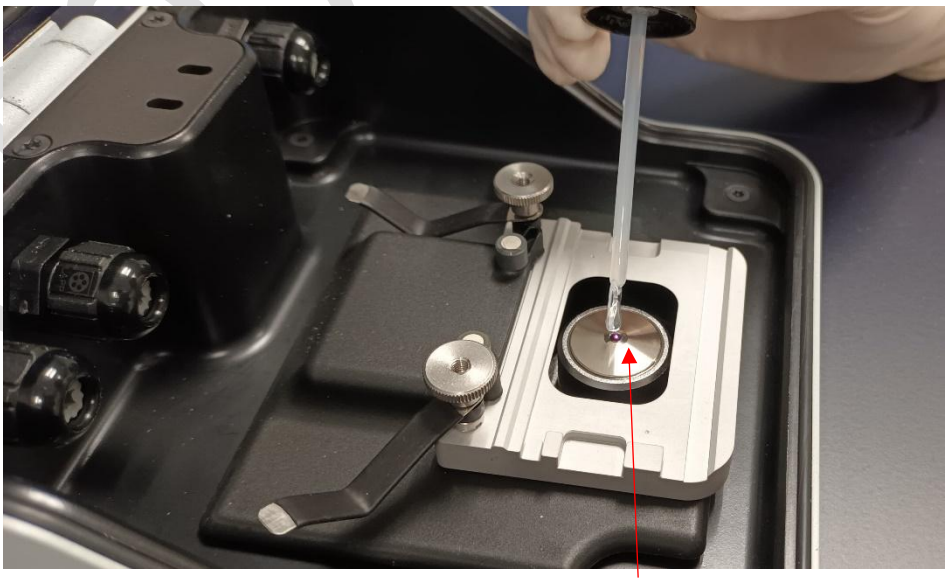
- Using tweezers and gloves, place glass slide on top. Assure the gasket is fixed onto the glass slide by applying light pressure on the slide with the back of the tweezer.



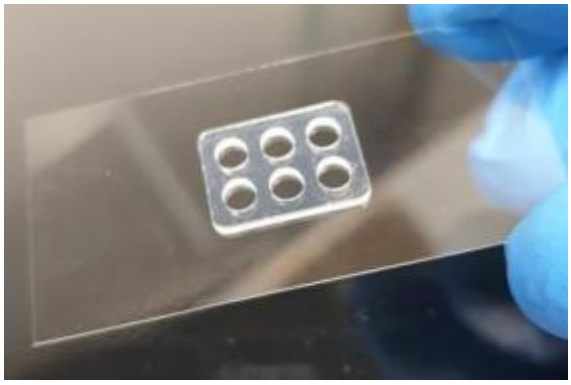
- Open the lid on the Two$^{MP}$ unit. Ensure that the objective and sample carrier are clean (no dried-out oil left on it by the last user).

Note: It should be cleaned by the previous user. If in doubt, please follow the steps of cleaning the objective using lens cleaning tissue.

- Place a drop of immersion oil in the middle of the objective lens. The drop size is sufficient to cover the center of objective but must not flow to the edges of your objective.




- Mount the prepared glass slide with gasket facing up to the Two$^{MP}$ unit.

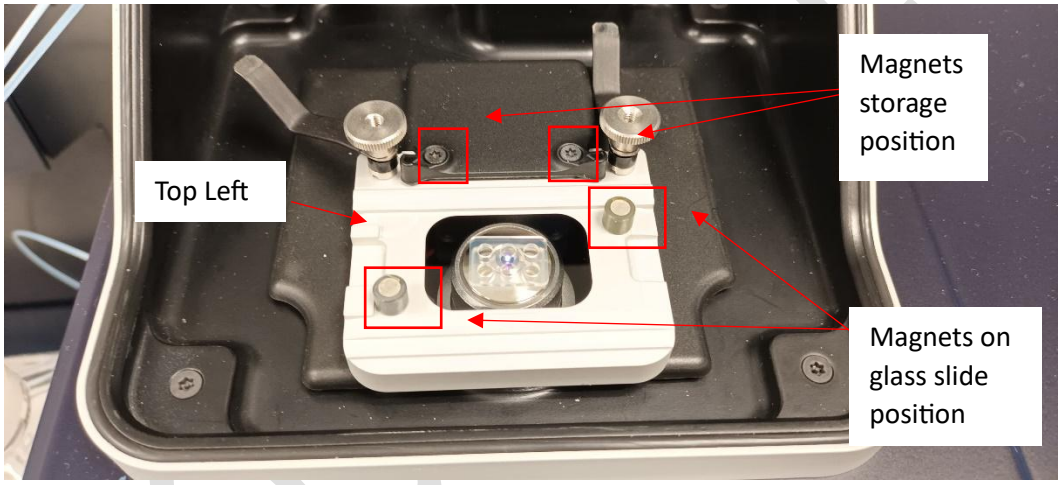


- Ensure there are no bubbles in the immersion oil to ensure integrity of the Auto-Focus ring. Hold the glass slide with the small magnets provided.

Note: The magnets should be stored and placed in the marked position.

Note: The magnets side with red dots should be facing downwards.

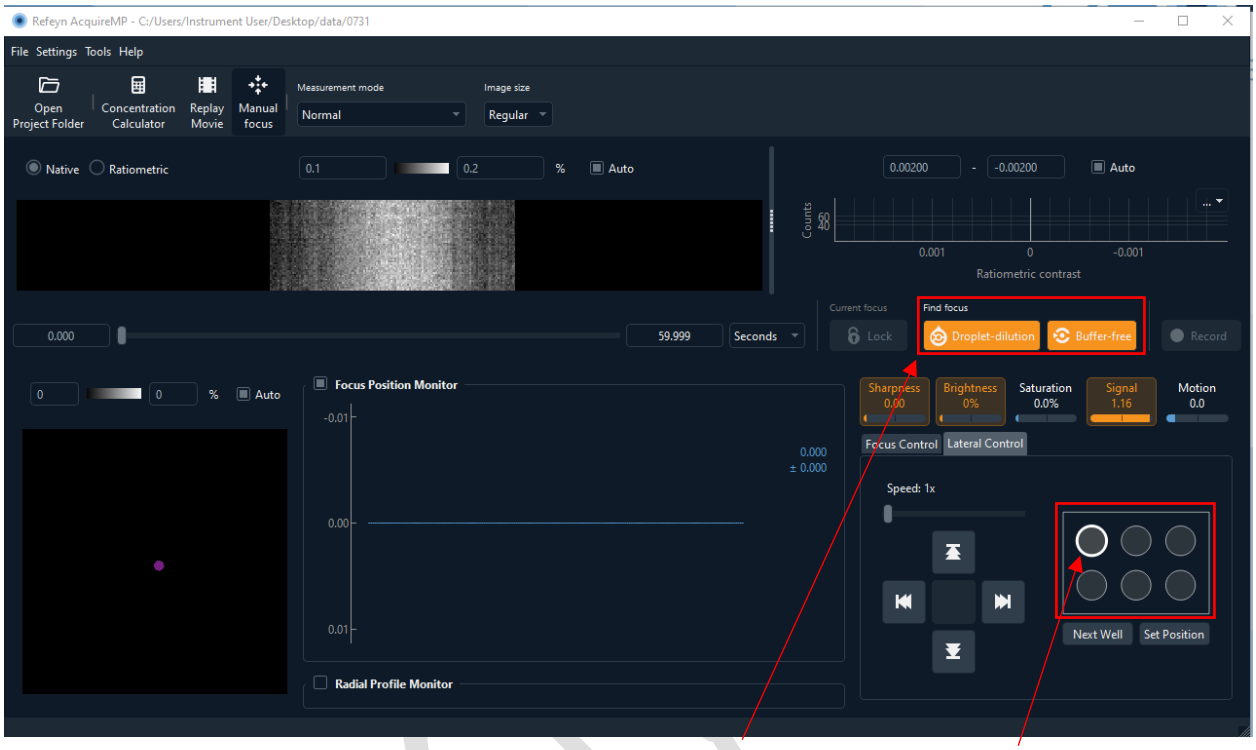
Note: Make sure the glass slide touches the top left corner.




- Before you start running your samples, please ensure that your buffer, samples and calibration standards are ready and at room temperature.

## 2. *Focus*

- Select a well (on the gasket) you want to start your experiment on by double clicking the circle in the software.




<u>Droplet-dilution Method</u>

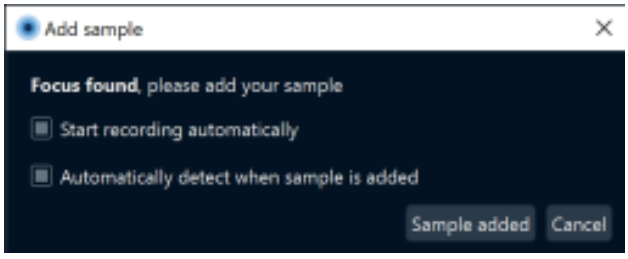
- Take 10 µl of very clean buffer in a pipette and add it to the selected well.

Note: Never touch the bottom of the glass with pipette tip.

- Close the lid. Press “droplet dilution” button. A window will pop up confirming that the software is now finding focus. Once the window disappears and you see the lock sign turns orange, it means that it has found focus.

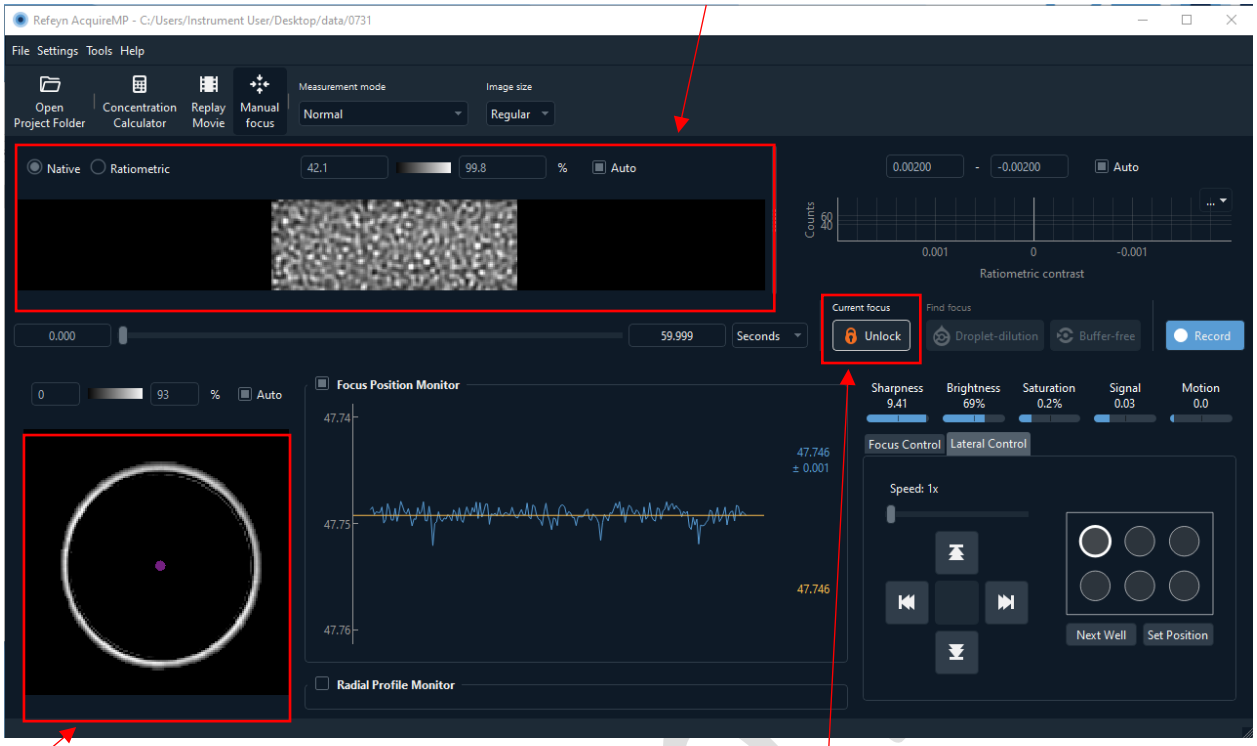
<u>Buffer-free Method</u>

- Close the lid. Press “buffer-free” button.
- A window will pop up after the auto-focus ring is formed. Make sure both “start recording automatically” and “automatically detect when sample is added” are selected.




- Pipette 20 µl of sample into the well, and close the lid. The system will record automatically.

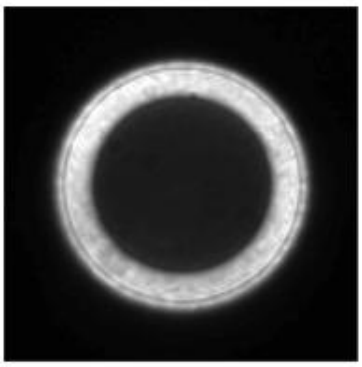
Camera views (native / ratiometric)



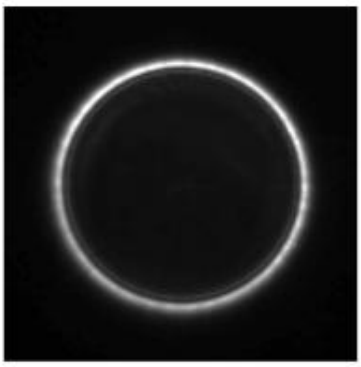

Auto-focus ring

Focus found

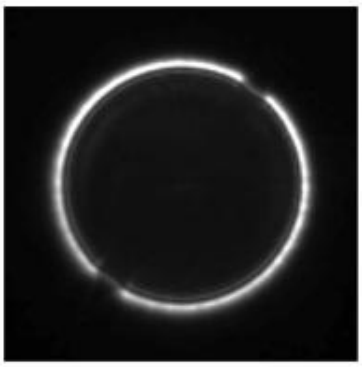
Note: Check that the auto-focus ring is continuous and camera native views are good without unusual large black or white dots.



Good focus with Buffer-free find focus before samples has been added

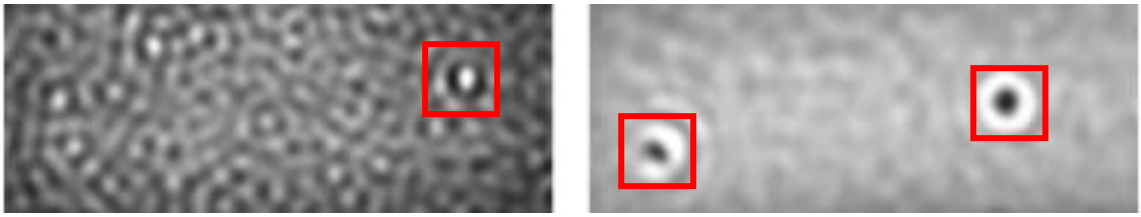


Good focus following the completion of either find focus method

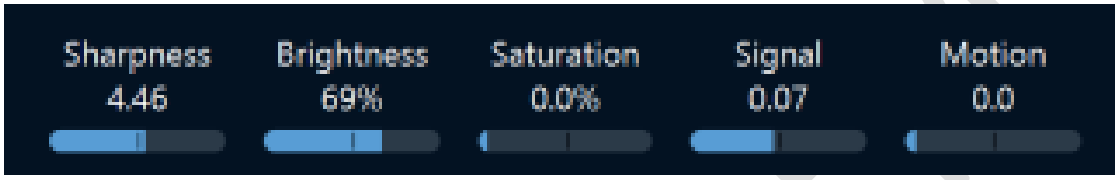


Bad focus due to a bubble in the immersion oil

- Check the cleanness of the glass slide. If particles are found, unlock the focus and move stage until the particles are outside of the view. Repeat the focusing procedure.



- The quality indicators are positioned in the centre right of the main window. After focus, these should all be **blue**. Once sample has been added, the signal indicator may move into the **orange** and this is completely normal.




**Sharpness:** shows how precisely the sample was positioned and held in focus during the measurement. The minimum recommended sharpness is 4%. The value can be exaggerated by a dirty glass surface, giving the false impression that the sample is in focus. In this case, users are advised to move the coverslip to a clean region before starting the recording. Additionally, the sharpness value tends to decrease slightly with image size because the illumination profile is more uniform as the light is distributed over a larger area. During the recording of a movie, sharpness tends to increase as particles bind to the surface. That is expected and no reason for concern. However, a significant drop of the sharpness value usually indicates that the sample moved out of focus, which may impact data quality.

Note: For sharpness, it should be around 7%-8%.

**Brightness:** shows how much light the camera has detected in relation to its saturation level. This indicated how efficiently the dynamic range of the camera has been used. A high brightness value (at least 50%) is desirable to minimize shot-noise in the data.

**Saturation:** shows the percentage of pixels in the movie that are saturated (i.e. overexposed). Ideally, this graph will be a flat line sitting at 0%. A small percentage of saturated pixels is tolerable but the saturation value should not exceed 0.5%.

**Signal:** value is indicative of the amount of change in the data. Frames with higher signal usually show particle binding/unbinding events. For a movie containing only a buffer measurement, this value should be 0.06% or lower. Please note that the choice of image size influences the signal level. As the image size is increased the same amount of light is distributed over a larger area and therefore per-pixel noise increases. Hence, the signal level for a clean buffer will tend to be higher for larger image sizes.

Note: For signal, it should be around 0.04% for 30-50kDa sample and around 0.06% for larger particles.

**Motion:** gives an indication of the amount of lateral motion detected during the acquisition of a frame. The value is calculated from the frame data by determining the amplitude of the motion signature in the ratiometric frame. Small-scale motions are tolerable, but values exceeding 5% are considered to be considerable motion and may impact data quality significantly.

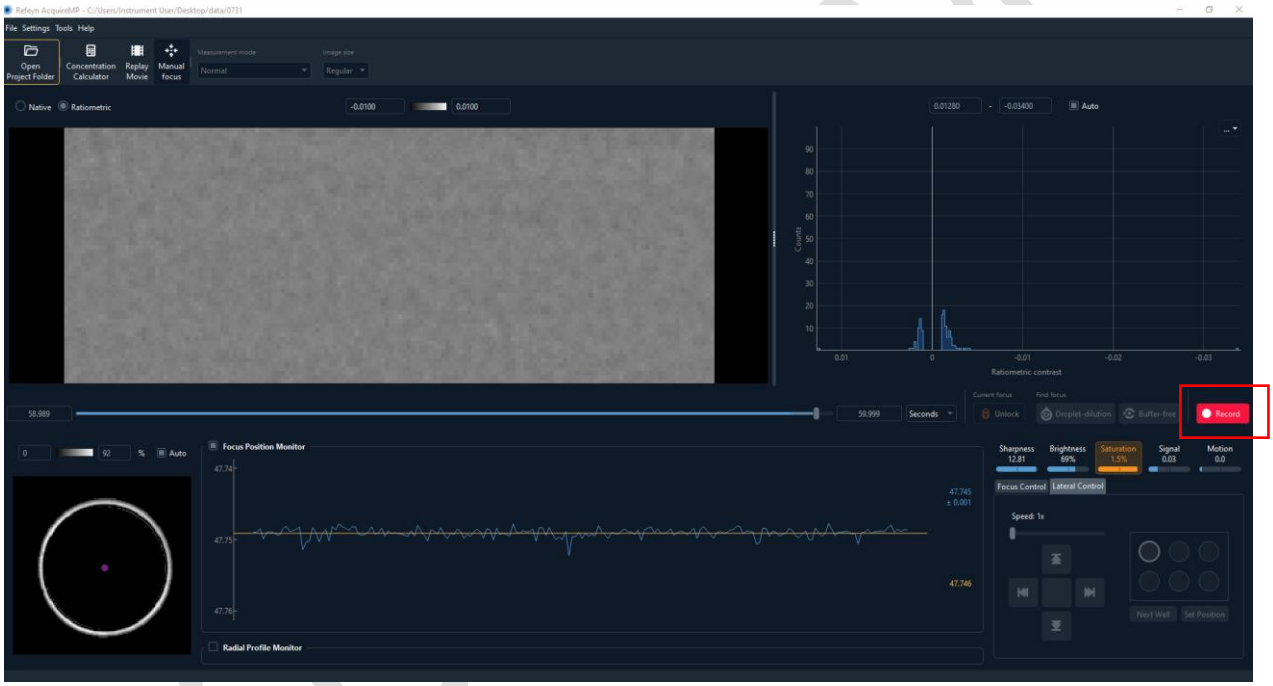
# 3. Measure

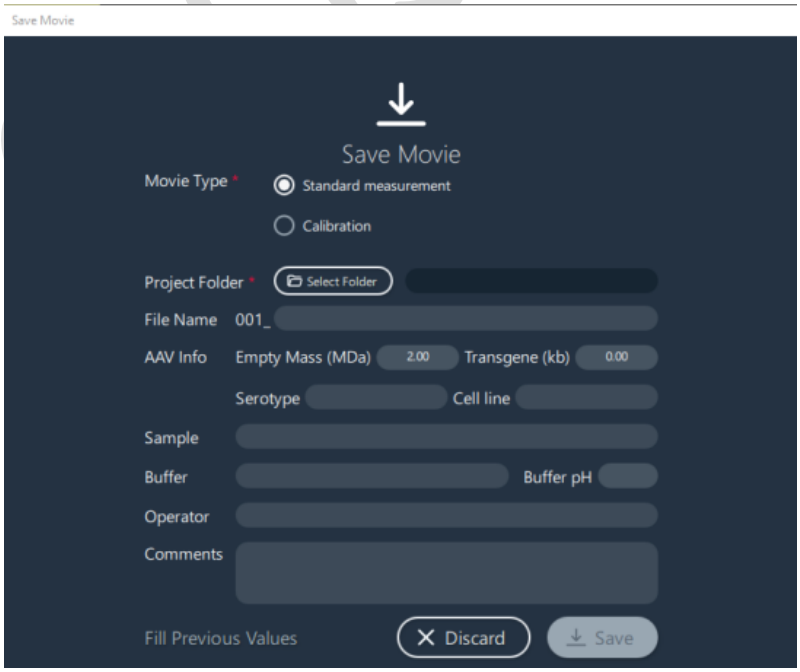
- Add 5-10 µl of your diluted sample (100pM- 100nM) and add to it the same well you found focus on. Mix 3-4 times by pipetting up and down.

Note: Never touch the bottom of the glass with pipette tip.

Note: The total volume of buffer and your diluted sample to a well must be less than 20 µl.

- Close the lid. Press “record” button.
- The software will now record data for 1min. Once the recording is finished, it will ask you to save the video into the project that you created at the start of the experiment. The data will be saved as .mp file.



Note: The file should be saved to your designated folder and the naming structure would be: PI_Username.

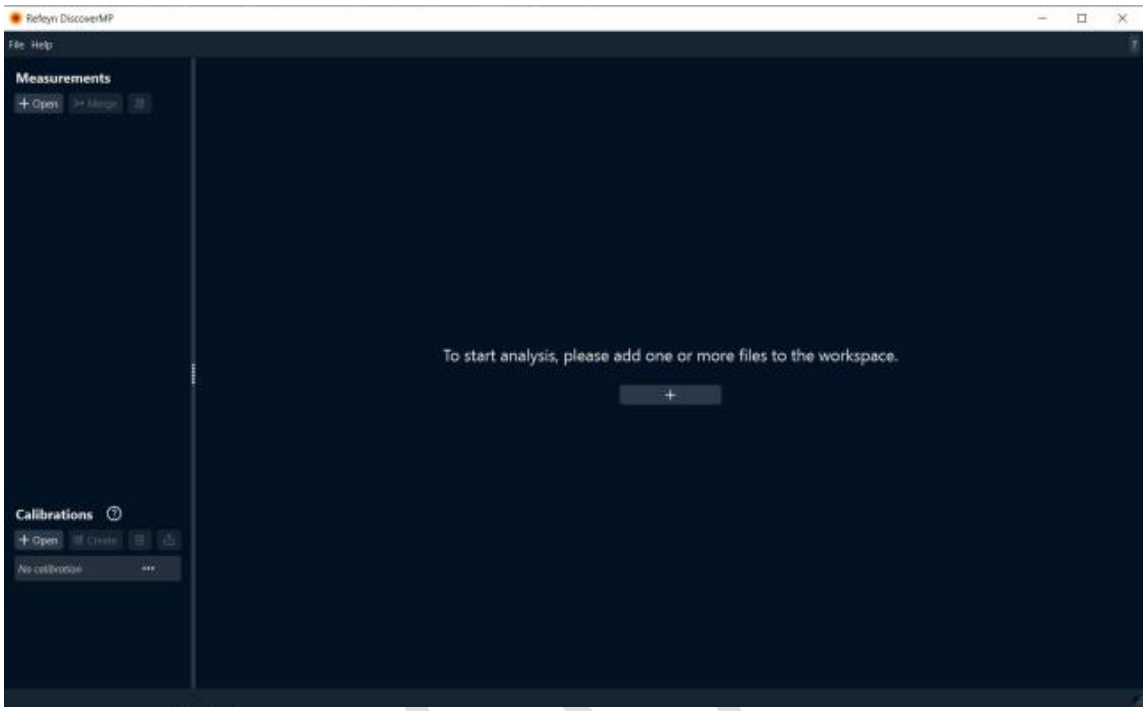
- Unlock the focus control and select next well you want to use for the new measurements.
- Repeat the steps from **2. Focus** until you have used all 6 wells in the gasket.
- Once you have used all 6 wells, throw it into the sharp box and use a new glass slide with the new gasket.

# *4. Clean up and shutting down Two$^{MP}$*

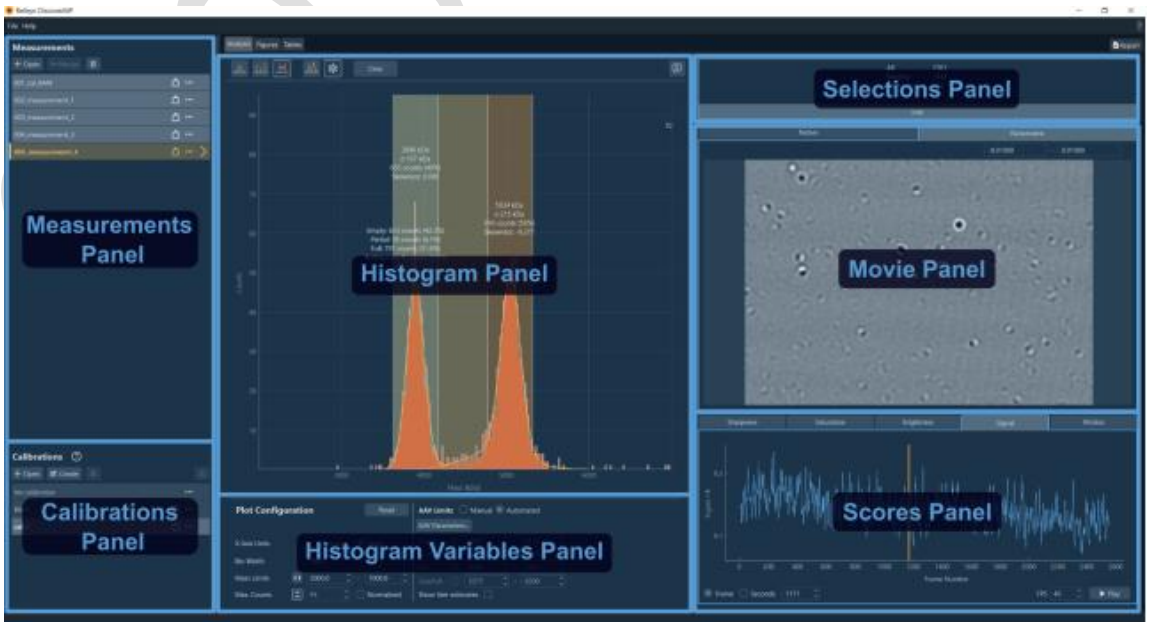
- Close the Acquire$^{MP}$ (AMP) and Discover$^{MP}$ (DMP) software.
- Open the lid on Two$^{MP}$ Unit and place the magnets on storage position.
- Discard the glass slide to sharp box.
- Wear gloves to clean the objective with lens cleaning tissue.
- Take out a half piece of tissue, fold it well and wipe the excess oil on objective.
- Then take another tissue, fold it well, add some drops of 100% IPA on one end and start cleaning the center moving outwards to the edges. Repeat this process until the objective is clean. Ensure that the stage plate is also clean and oil free.
- Turn **OFF** the Isolation switch which is located at the front of your active vibration isolation table.
- Turn **OFF** the electronics unit if our staff told you there are no users afterwards.
- Sign in logbook.

# *5. Sample analysis*

- Open the Discover$^{MP}$ (DMP) software.
- Click on + sign to load all your files you saved in AMP software. For mass calibration, use the measurements run with calibrants (highly purified biomolecules of known mass).




- Once it loads, it will automatically start processing your movie and generate a graph.
- You can start working with the first file once it is processed (while the processing for other ones are running in the background).



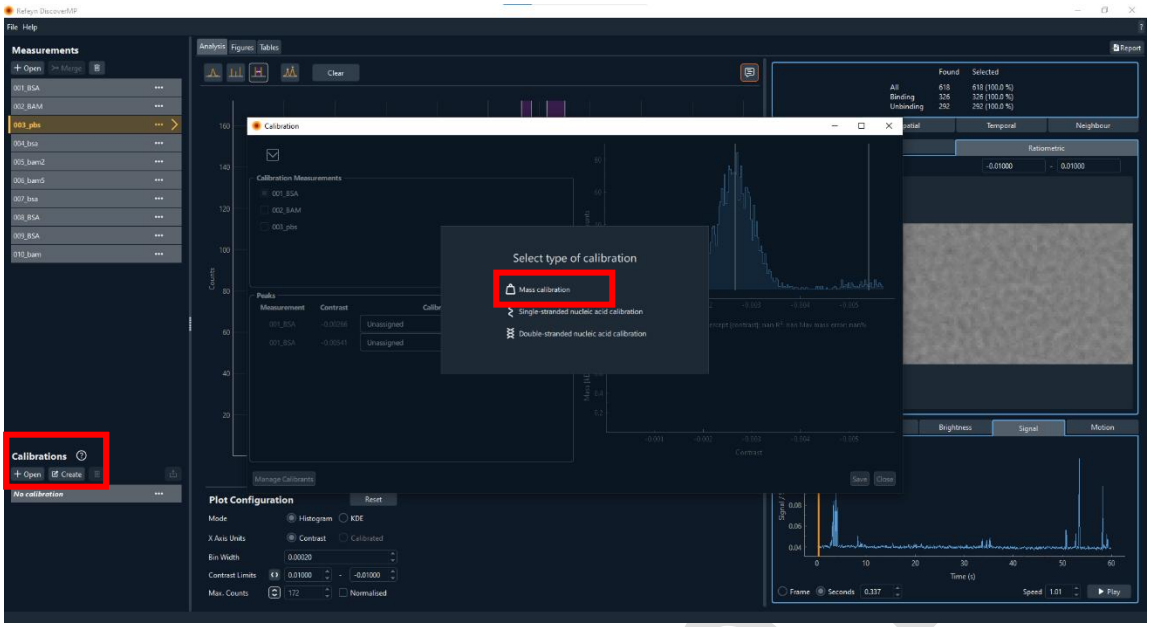

**Left panel:** has all the files you have obtained from your measurements and your mass calibrations that are saved.

**Middle panel:** represents the movie/each frame in the movie the software is using to process your data.

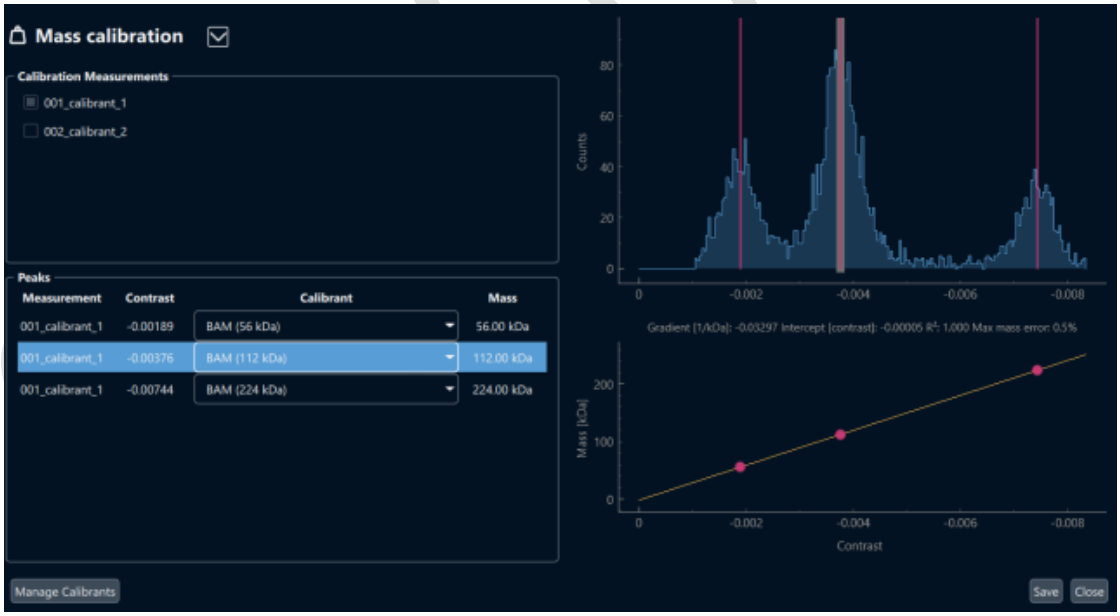
**Right panel:** is the histogram of all events that are captured from the movie. The histogram that you see will appear with ratiometric contrast. In order to convert it to mass, a mass calibration will be needed and applied to your measurements.

- The software will automatically fit peaks. If all peaks look relevant to you, move to the next step. Otherwise, click clear at the top, and all the peaks will disappear. You can then double click on the peak that seems relevant to your sample.
- You could also set contrast limits as you wish.

- Once the peaks are fit, press “create” and then “mass calibration” button. You can also press “Open” to choose a .mc file as calibration.

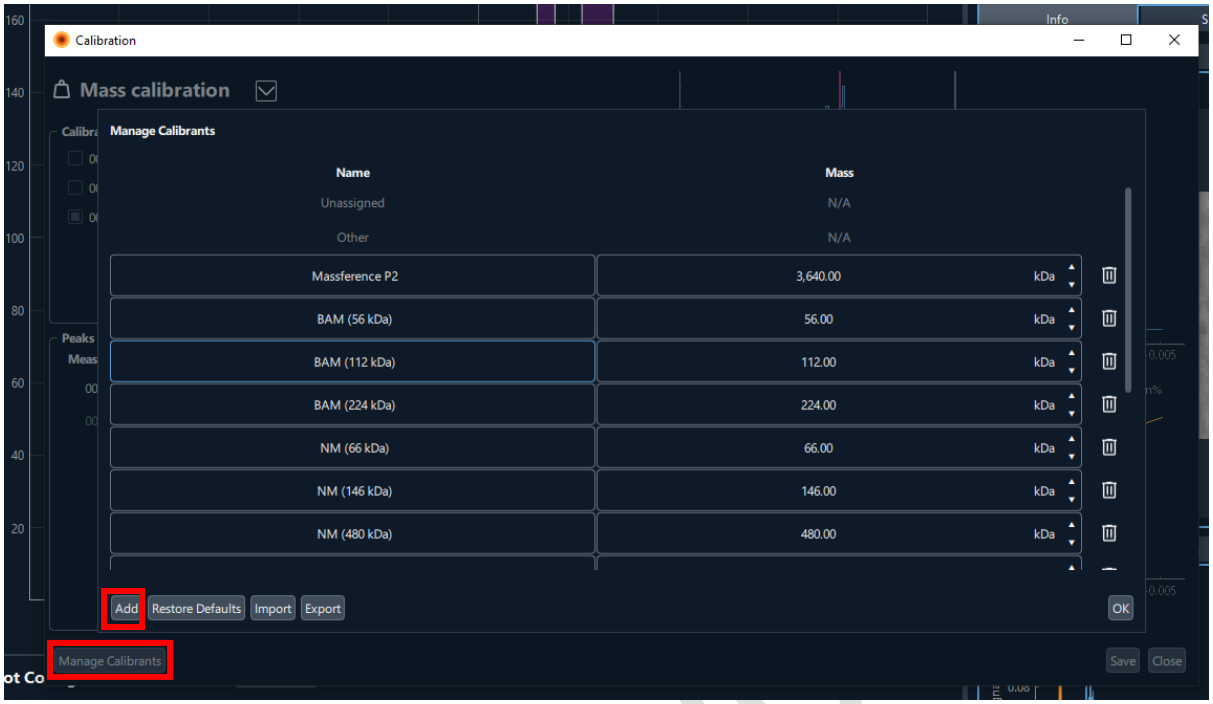



- A window will pop up with contrast values for all peaks already added. Set mass for each value and save. If there is only one peak/point, add another point and set mass and contrast as 0, which will then allow you to save the calibration.
- This will then create a calibration file at the button pane, which you can rename, and then export using the arrow sign at the top of this pane. This will be saved as .mc file.




- To add saved calibrations points on the current calibration, click append from file and load the exported calibration file. This will then show you a straight line with a mass error. Mass error shouldn’t be more than 5%. If it is, check if the MW molecular weights are well attributed to the contrast, if the gaussian fitted in the peaks captures the particle distribution measured. If there is no obvious mistake on the previous you might need to repeat your experiments.

- If your calibrant and mass are not on the list, press “Manage Calibrants” at the bottom left corner. Type in the name and mass of your calibrant and then press "Add”.




- In total you may want to get three points for mass calibration, it could be from one protein complex giving three points, or two independent measurements, one giving two points and the other one giving one point or so on.
- Right click on the calibration file and click apply to all. Or select the measurement you want to apply it to and double click on it. You can now see your graphs in mass.
- You can also generate figures by simply clicking on Add figure, drag your files to the figures pane. You could overlap multiple files or work on them separate simply by adding figure.

# *6. Data Transfer*

- Open FileZilla software.
- Follow “CryoEM Microscope Data Management User Guide” to transfer your data using FileZilla.
- In your own computer, use FileZilla or other similar SFTP client software to download the data from the SFTP server.

- CMSFTP
  Host: sftp://cmsftp01.cpos.hku.hk
  Username: your CMSFTP account
  Password: your CMSFTP account
  Port:

- HPC
  Host: cryoem@cmsftp01.cpos.hku.hk
  Username: your HPC account
  Password: your HPC account
  Port: 22

Note: Files uploaded to FileZilla are just temporary. All data residing in the SFTP server will be removed after 14 days automatically. There will be no backup of the data. Please make sure you have successfully downloaded the data to your local computer within 14 days.

Note: The local data stored in the computer of Two$^{MP}$ will be removed on the first working day of each calendar month.

## *Checklist before leaving the lab:*

- Clean the objective following the proper procedures.
- Shut down the Two$^{MP}$ following the proper procedures.
- Discard the glass slide with gasket into the sharp box.
- Tidy and clean the bench area.
- Sign the logbook.
- Remember to take all your personal belongings with you when you leave.
- Remember to transfer your data to your personal computer if you need it.