REFEYN
WEIGHING MOLECULES WITH LIGHT

Refeyn Discover^{MP} User Manual

Version 2024 R2

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Getting started

Compatibility

- This version of Discover^{MP} can analyse files from Acquire^{MP} 2024R2 or earlier and open saved results from Discover^{MP} 2024R2 or earlier.
- TDMS files are no longer supported. Refeyn's tdms_to_mp_converter application can be used to convert TDMS files into **.mp** files. To download this application, visit https://refeyn.filecamp.com.

Installing/updating

If installing Discover^{MP} for the first time, jump to step 4.

1. Make sure all instances of Discover^{MP} are closed.
2. Uninstall the old version of Discover^{MP} by going to Control Panel > Programs > Programs and Features and then selecting Discover^{MP} to uninstall.
3. Check the uninstall has been successful by going to C:\Program Files (x86) in the Windows file explorer and checking that there is no folder called DiscoverMP. If there is, manually delete the folder.
4. Execute the Discover^{MP} setup executable file (the filename will be like: DiscoverMP_v0.0.0_setup.exe). Double clicking on the file will bring up an install wizard.
5. Follow the steps of the installer, making sure to read the terms and conditions before accepting.
6. Once the installer has been completed, the software is ready to use.

Multiple displays

When using multiple displays, the settings must be correct to avoid issues with the application's layout. The most common issue comes from having different zoom factors for each display. To make sure this does not happen, each display must be set to the same zoom factor. This can be done by going to **Settings** > **Display** and selecting the same scale factor (ideally 100%) under **Scale and layout** for each of the displays found under **Rearrange your displays**.

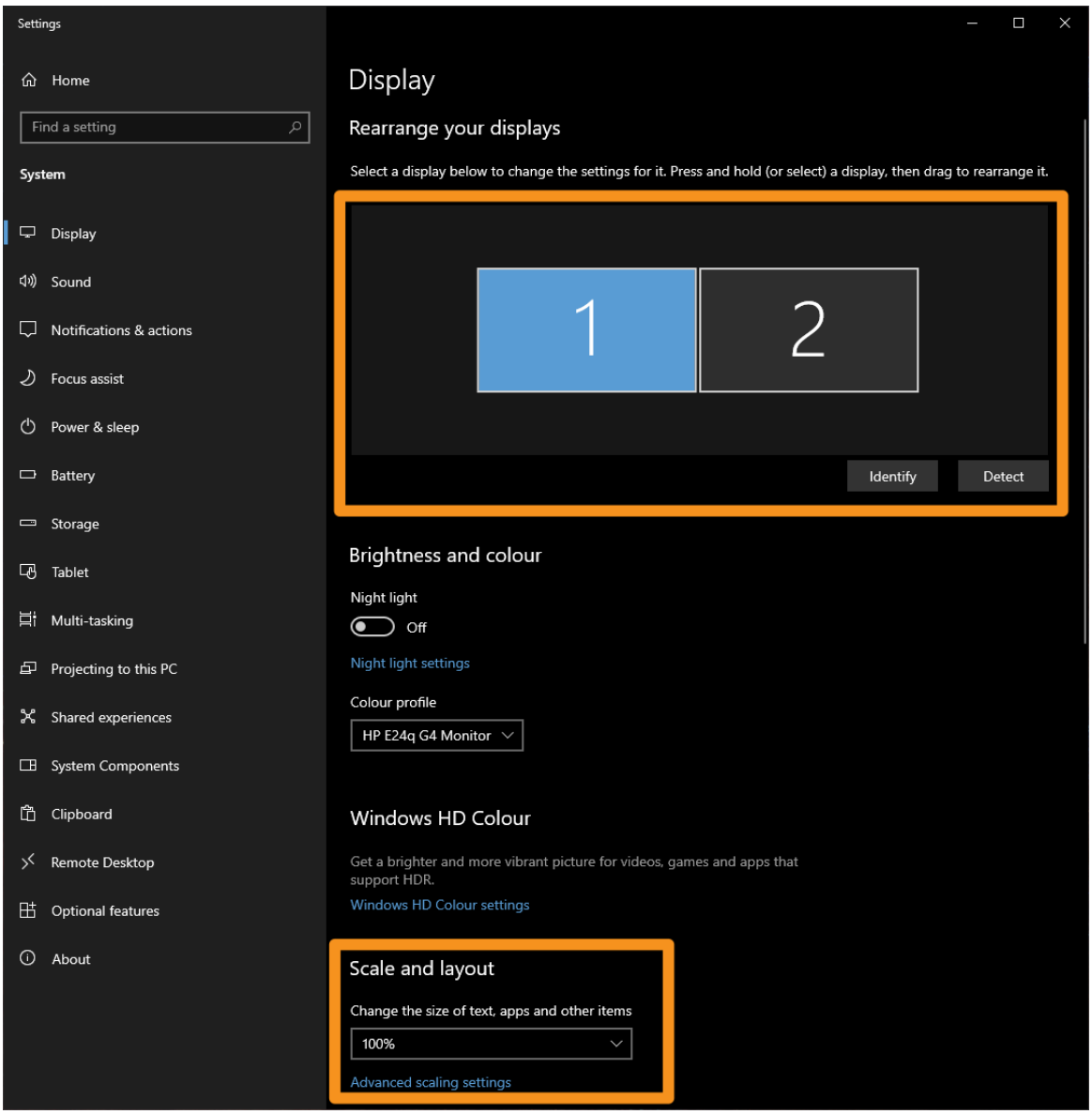


Figure 1: Selecting display scale for multiple monitors

End User License Agreement (EULA)

When Discover^{MP} is run for the first time, it will display Refeyn's End User License Agreement. Make sure to read the terms and conditions before accepting. In addition, a choice for Discover^{MP}'s telemetry mode is shown with the following options:

- Feature preview mode
- Normal mode
- Offline mode

The details of each mode are listed to their right. Accepting will also confirm the current selected telemetry mode, this can be changed in Preferences.

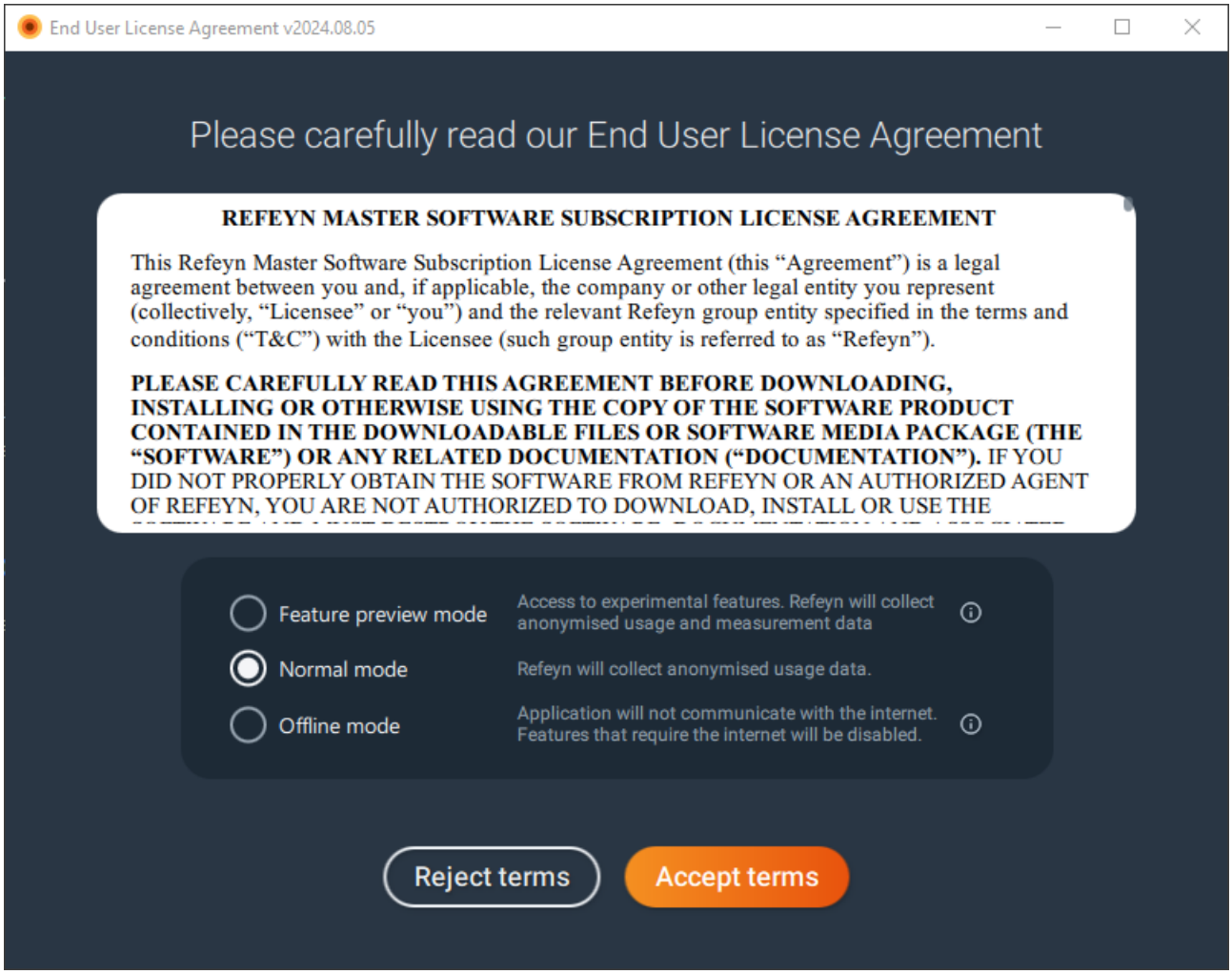


Figure 2: End User License Agreement and mode selection

Licensing

When you open the application for the first time the program will prompt you to activate your licence with your licence key. The licence key is normally provided with the software, and looks like ABCDE-FGHIJ-KLMNO-PQRST. If you do not have a licence key, one can be obtained by contacting Refeyn using support@refeyn.com.

If the PC on which you are installing Discover^{MP} has an internet connection, make sure **Use this PC's internet connection** is selected, then simply enter the licence key into the box and click **Finish**.

If the PC does not have an internet connection or the connection fails, choose **Use another PC's internet connection**, then enter the licence key into the box, click **Next** and note down the *machine code* shown in the dialog. Next, on a different PC with an internet connection, open the webpage shown in the dialog. This will load a form in which you should enter the machine code and your licence key. On clicking **Activate**, an .skm activation file will be downloaded. Copy this file onto the PC on which you wish to activate Discover^{MP}. Enter your licence key into the Discover^{MP} dialog and use the **Browse** button to select the .skm activation file you copied over, then click **OK**. Discover^{MP} can now be run.

Deactivating the licence

If you wish to move your licence for Discover^{MP} to another PC, you should deactivate the licence on the PC you no longer wish to use. To do this, open Discover^{MP} and go to **Help** > **Deactivate licence**. A warning message will be shown. Clicking **Yes** will close Discover^{MP} and deactivate the licence on that PC. You can then activate your licence key on another PC. To reactivate a licence after deactivating it, simply open Discover^{MP} again and re-register the licence, following the instructions above.

Main user interface panels

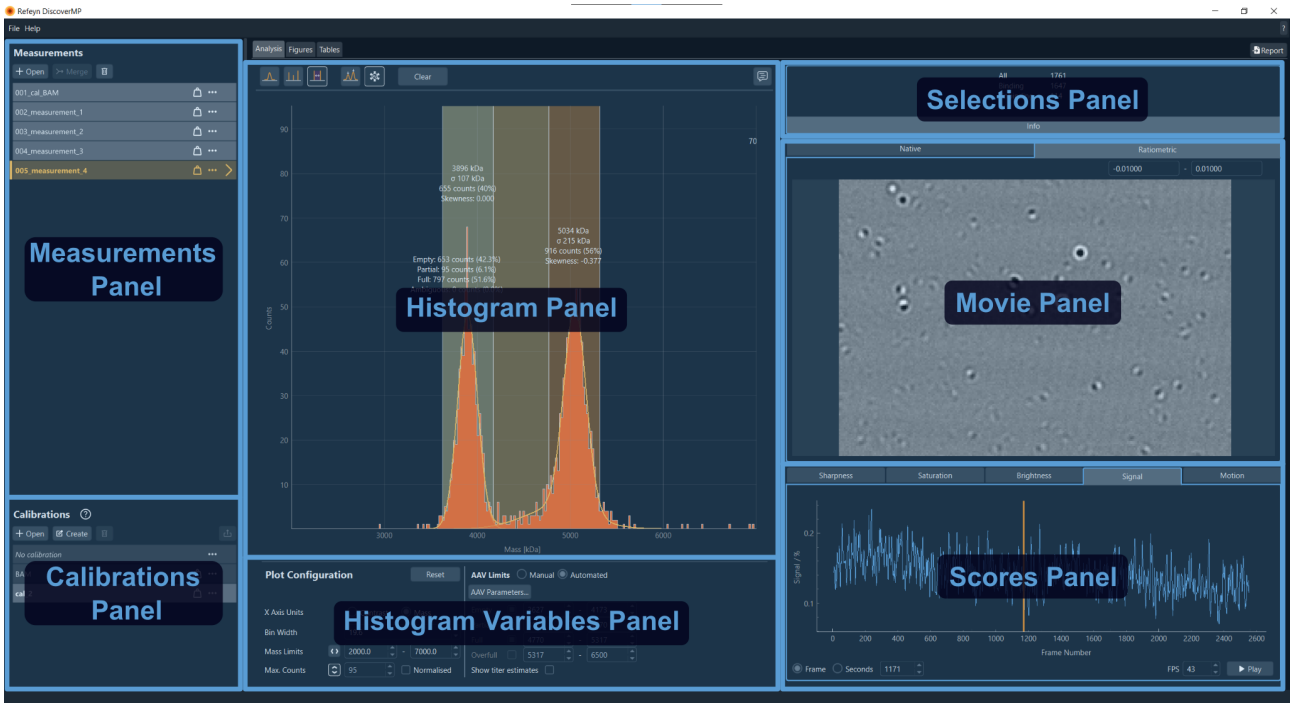


Figure 3: Main panels for data analysis

The main Discover^{MP} window by default displays Measurements Panel and Calibrations Panel on the left-hand side, and Analysis Panel on the right which, in turn, consists of the Histogram Panel, Histogram Variables Panel, Selections Panel, Movie Panel, and Scores Panel. Further analysis features can be accessed in Figures Panel (see Figure 16) and Tables Panel (see Figure 18) by switching from the *Analysis* tab to the *Figures* tab and the *Tables* tab, respectively.

Managing a workspace and measurement files

Opening and closing a workspace

When opening Discover^{MP}, the user will have access to a **workspace** where multiple files can be visualised and analysed. To analyse a measurement file, just add it to the workspace and the analysis will start automatically. There are three main types of measurement files that can be added to a workspace:

- A mass photometry results file (**.mpr**) or a recording file (**.mp**) saved in Acquire^{MP}
- A mass photometry results file (**.mpr**) saved (or exported) in Discover^{MP} v2.5 or later

To open a previously saved workspace, go to **File** > **Open Workspace** (or press **Ctrl+O**) and select the **workspace file** (**.dmp**) of interest. This will open all measurement files, analysis and data associated with this workspace.

To close a workspace, go to **File** > **Close Workspace**.

Legacy results folders

Legacy results folders were generated by Discover^{MP} v2.4 or earlier. As of Discover^{MP} 2024R2, loading these folders is no longer supported.

There are 2 options for loading legacy results folders into Discover^{MP}:

- In one of the following versions of Discover^{MP}, go to **File** > **Open Legacy Results**, or press **Ctrl+Shift+O**. This opens a file explorer dialog, where the results folder can be selected.
  - v2.5
  - 2022R1
  - 2023R1
  - 2024R1
- Load the original **.mp** file of each measurement to be converted into the latest version of Discover^{MP}, which will then be re-analyzed.

After following either method, the loaded measurements can be converted into **.mpr** files.

- To create **.mpr** files for all measurements, save the workspace (**File** > **Save Workspace**).
- To export indiviual measurements as **.mpr** files, right click on the measurement in the **Measurements Panel**, then click **Export** > **Results**.

Managing files in a workspace

Adding files

To add a mass photometry results file (**.mpr**) or a recording file (**.mp**) to a workspace, press the **+ Open** button on the top-left corner of the **Measurements Panel**. Alternatively, when Discover^{MP} is first opened, there is also a **+ button** in the central panel (see Figure 4). This opens a file explorer dialog. It is also possible to simply drag a measurement file from filesystem onto the Discover^{MP} window to add it to the workspace.

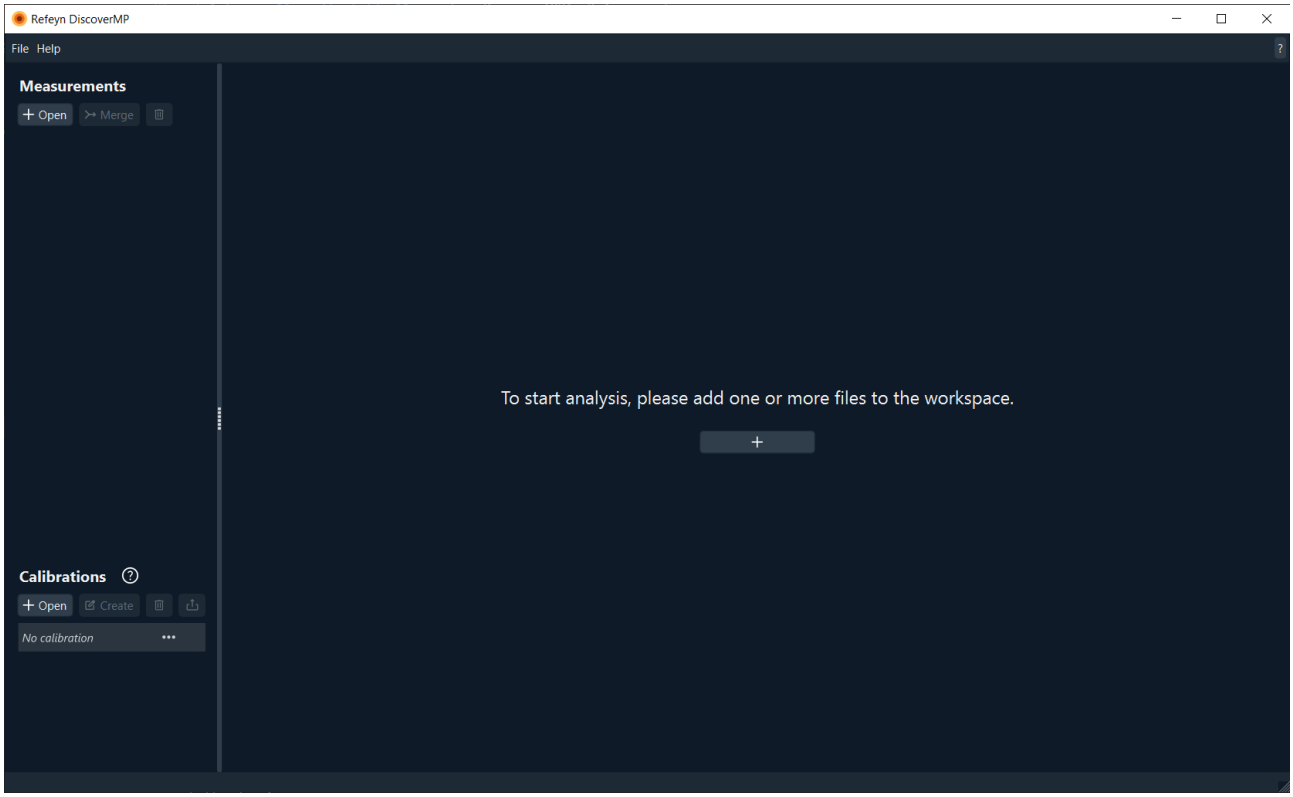


Figure 4: Main window when first opening application

Once a measurement file has been selected, the data is loaded into the application. If the measurement file is large, this may take a while to load.

Mass photometry results files (**.mpr**) are available immediately after loading. On the other hand, recording files (**.mp**) have to first be analysed by Discover^{MP}. The recordings are analysed one at a time, in the order in which the files have been added to the workspace. While the file is being analysed, the background for that measurement in the files panel turns into a progress bar.

The maximum number of measurements that can be added to a workspace is dependent on the computer's virtual memory. The more files added, the larger virtual memory required. If your computer runs out of memory, some data might be lost. We suggest no more than 24 files to be added to a workspace.

Selecting and visualising measurement files

To select a measurement file, click on its name in the Measurements Panel. When selected, the measurement file's name will be highlighted in yellow. To visualise the data associated with a measurement file, double-click on the file's name and the data will be shown in the **Analysis Panel**.

The measurement file displayed on the Analysis Panel will be highlighted in bold and have an arrow pointing to the Analysis Panel next to the filename.

Removing measurement files

Measurement files can be removed from a workspace by selecting them and clicking on the **Bin** icon at the top-left of the Measurements Panel. Alternatively, a measurement can be removed by either right-clicking on it, or clicking the **More** button (three dots) and choosing **Delete**. Note that this only deletes the measurement file from the workspace folder, and not the original measurement file that had been initially added to the workspace.

Merging measurement files

Measurement files can be merged in a workspace by selecting the measurements of interest and clicking on the **Merge** icon at the top-left of the Measurements Panel. This will automatically generate a new measurement containing the merged data. Merged files will not have movie or movie quality score data available. For files to be merged, the device, measurement mode, and image size need to be the same.

To enable mass mode for a merged measurement file, a calibration needs to be applied to **each individual measurement** before the merge.

Data analysis

Mass photometry results files (**.mpr**) are ready to be worked with as soon as they are loaded into the workspace. Files recorded with Acquire^{MP} 2023R2 or earlier, or in the case live analysis has been explicitly disabled, are of the recording file (**.mp**) type. When such a file is added to a workspace, it is automatically loaded and queued for analysis. To stop the analysis, click on the **Stop** icon at the top of the Measurements Panel. The analysis can be started again by clicking on the **Start** icon at the top of the Measurements Panel.

Analysis modes

When recording movies in Acquire^{MP} there are four modes available, **Normal mode**, **AAV mode**, **Antibody Stability mode** and **Samux mode**; and one experimental mode, **Fast detection mode**. Depending on the mode used, the functionality available in the Analysis Panel associated with that measurement file will be different.

- **Normal mode**: This is the default mode in Acquire^{MP} for acquiring and analysing biomolecules when using a One^{MP}, Two^{MP}, One^{MP} Auto or Two^{MP} Auto
- **AAV mode**: This mode should be used for measurements of AAV samples when using a One^{MP}, Two^{MP}, One^{MP} Auto or Two^{MP} Auto.
- **Antibody Stability mode**: This mode is recommended for measurements which are to be analysed with the Antibody Stability module in Streamline^{MP}. It is available when using a One^{MP}, Two^{MP}, One^{MP} Auto or Two^{MP} Auto.
- **Samux mode**: This is the default and only mode in Acquire^{MP} for measurements of AAV samples when using a Samux^{MP}.

The following table shows which Discover^{MP} features are available in which modes.

Feature	Normal Mode & Antibody Stability Mode	AAV Mode	Samux Mode
Acquisition movie	X	X	X
Data quality scores	X	X	X
Histogram	X	X	X
KDE	X	X	
Contrast	X	X	X
Calibrated value (mass, bases, or base pairs)	X	X	X
Acquisition plot setup	X	X	X
Vertical lines	X	X	X
Interval regions	X	X	X
Automated peak fitting	X	X	
Manual peak fitting	X	X	X
AAV limits		X	X
Automated AAV fitting			X
Spatial and temporal masking	X	X	
Virus titer			X

The **Fast detection mode** [EXPERIMENTAL] is available only on Two^{MP} manual instruments, and is intended for use in the MassFluidix HC system, with samples that are particularly unstable.

Acquisition visualisation and editing tools

Acquisition movies and scores

In the Analysis Panel, there are two different types of movies available: native and ratiometric.

Native movies: Native data acquired using Acquire^{MP}.

Ratiometric movies: During analysis the ratiometric images are calculated. This involves dividing future (time-averaged) movie frames by past (time-averaged) movie frames. During each of these operations, the data that is common to both images is removed, leaving only the difference between each of the pairs of frames. This is the ratiometric image which in turn comprises the ratiometric movie.

To see an overlay of the spatial distribution of all detected events, right-click on the Movie Panel and then checking **Distribution**. A high quality measurement should give a roughly uniform spread across the movie.

In the **Scores Panel**, there are five options to choose from: **Sharpness, Saturation, Brightness, Signal** and **Motion**. Swapping between these modes will display the corresponding value for each frame in the movie.

The **Sharpness** value shows how precisely the sample was positioned and held in focus during the measurement. The minimum recommended **Sharpness** value for the One^{MP} and Two^{MP} is 4%, and 1.6% for Samux^{MP}. Please note that the value can be exaggerated by a dirty glass surface, giving the false impression that the sample is in focus. In this case, the user is advised to move the coverslip to a clean region before starting the recording in Acquire^{MP}. 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.

The **Saturation** value 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%.

The **Brightness** value shows how much light the camera has detected in relation to its saturation level. This indicates how efficiently the dynamic range of the camera has been used. A high brightness value (at least 25% for One^{MP}, 50% for Two^{MP}, and 70% for Samux^{MP}) is desirable to minimise shot-noise in the data.

The **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.

The **Motion** value 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.

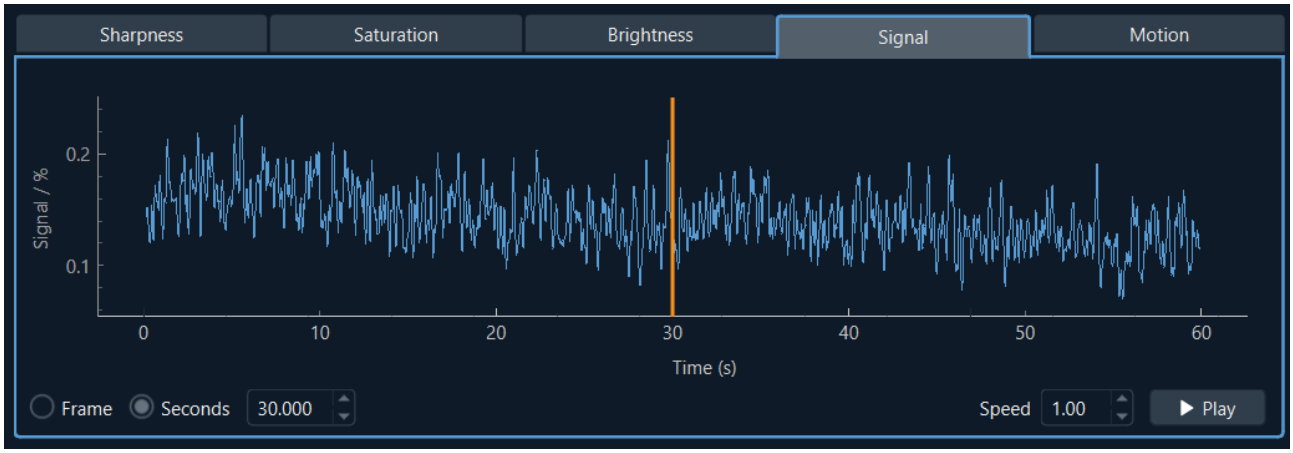


Figure 5: Score Panel

The frame shown in the Movie Panel can be changed by moving the vertical orange cursor along the x-axis of the Scores Panel graph. To display a frame of a specific frame number or time value, select the desired unit (**Frame** or **Seconds**) by clicking on the relevant option button under the score graph, and entering the value in the field next to it. The movie can be played using the **Play** button. To change the playback speed, change the value in the field next to the **FPS** or **Speed** label, depending on which units have been chosen.

You can move forward and backward through the movie by hovering over the Score graph and using the mouse wheel.

Analysis plot types

Histogram vs KDE

For Normal, AAV and Antibody Stability modes, the main plot can be toggled between Histogram mode and Kernel Density Estimate (KDE) mode. The buttons to switch the mode are found in the **Histogram Variables Panel**, labelled *Histogram* and *KDE*. If you want the KDE mode to show an estimate of a Probability Density Function, then check the *Normalise* button (the y-axis will be labelled "Normalised density"). For Samux mode, only histogram mode is available.

Contrast vs calibrated value

The measurements can be calibrated into units of mass (kDa, MDa) or nucleic acid length (bases, base pairs) (see the section in this manual on calibration). The histogram may then be alternated between showing contrast and the calibrated value on the X axis by selecting the relevant plot in the Histogram Variables Panel. If no calibration has been applied, only the contrast mode is available.

Analysis settings

Discover^{MP} gives the option to change the analysis settings for measurements loaded into the workspace. This will affect the behaviour of the event finding and fitting algorithm.

The default settings are tested on a wide range of conditions and samples and are known to produce reliable results. Therefore, it is not recommended to deviate from the default settings. The ability to change settings is initially limited to One^{MP} measurements only, however this can be changed to allow settings to be altered for any measurement in Preferences.

When changing the settings for a measurement, right-click on the measurement in the Measurements Panel and click **Settings**.

The settings dialog allows the following settings to change:

- Number of Averaged Frames: number of (binned) frames that are used for signal averaging.
- Reflectance Correction: if enabled analysis compensates for the spatial contrast bias due to uneven reflectance of the glass surface.
- Motion Correction: if enabled analysis compensates for contrast errors due to transverse translation of the glass with respect to the optical system.
- Event Finding - Threshold 1: sensitivity adjustment for picking up event signatures in the temporal domain (high values mean low sensitivity).
- Event Finding - Threshold 2: sensitivity adjustment for picking up event signatures in the spatial domain (high values mean low sensitivity).
- Event Fitting - Use AAV (contrast-dependent) fit error metric: if enabled a metric is used to rescale the fit error to avoid false AAV detections.

If new settings are confirmed for a given measurement, the measurement will be automatically re-analysed. The default settings can always be reverted to by clicking **Restore Defaults** in the settings dialog.

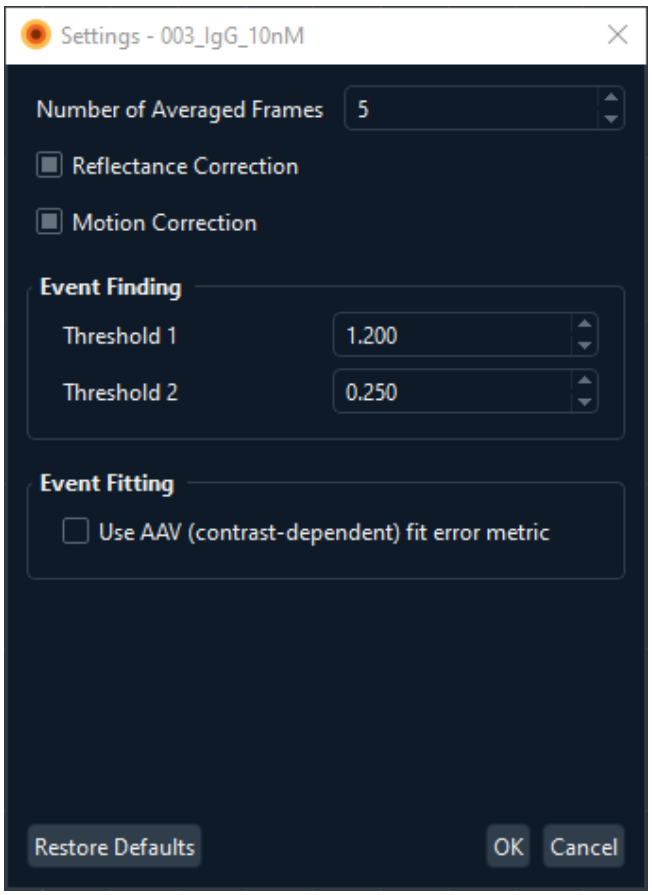


Figure 6: The settings dialog

Acquisition plot setup

Changing the limits

The plot axis limits may be altered in three ways:

- By manually changing the values in the Histogram Variables Panel
- By hovering over the histogram and using the mouse wheel:
  - Scrolling changes the y-axis maximum
  - Ctrl-scrolling changes the x-axis minimum
  - Shift-scrolling changes the x-axis maximum
  - Alt-scrolling translates the histogram along the X axis
- By clicking the two buttons next to the axis limit boxes to automatically set the axes to the maximum values found in the data.

The Y axis maximisation button locks the Y value to always show the maximum value. So, if other settings are changed, the counts/density axis will adjust to show all the data. The X axis does not update in this way.

Bin width and bandwidth

In histogram mode, contrast values are binned into ranges of values (bins) and then summed within these bins to calculate the number of counts shown on the histogram. The width of the bins can be varied by changing the **Bin width** in the Histogram Variables Panel. The histogram is always split into equal regions with the size of the bin width. The bin width units are contrast value (in contrast mode), or kDa, bases or bp (in calibrated mode).

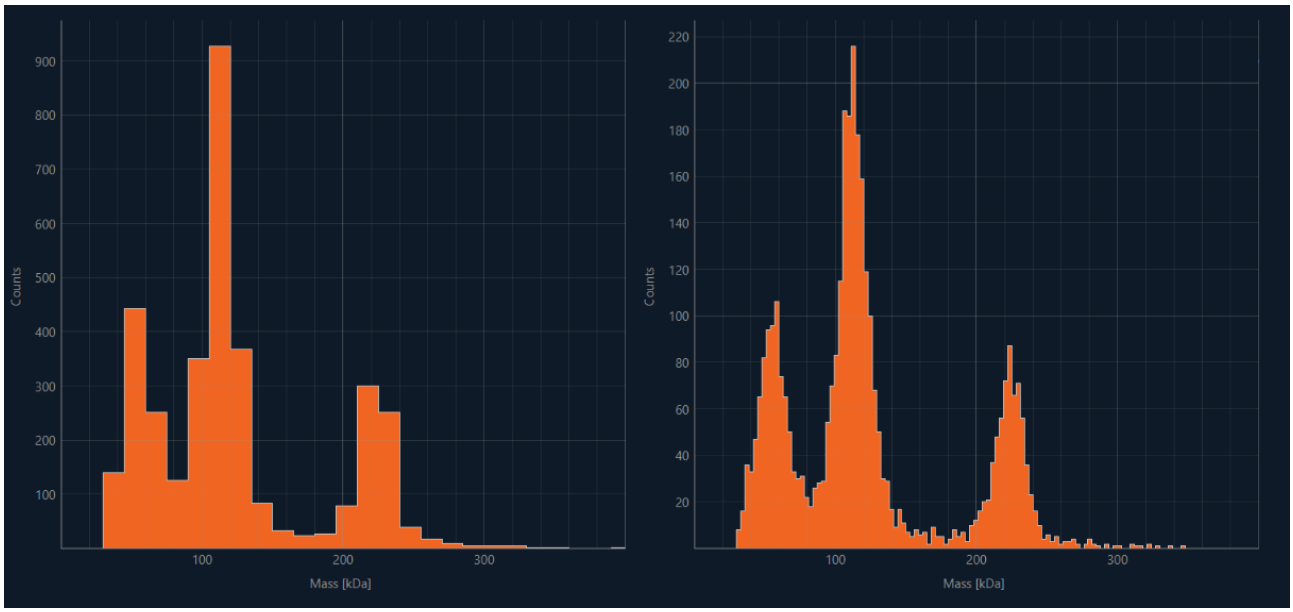


Figure 7: Two histograms of the same data with bin widths of 15kDa (left) and 3kDa (right)

In KDE mode, bandwidth is analogous to the bin width and can be changed in the Histogram Variables Panel. The bandwidth units are contrast value (in contrast mode), or kDa, bases or bp (in calibrated mode).

Normalise

The counts data can also be normalised by selecting the **Normalised** check box. This will divide the counts in each bin by the total number of events. This facilitates easier comparison between histograms with differing total numbers of events. In KDE mode, this button switches between plotting counts density (where the area under the graph equals the number of counts) and plotting normalised density (where the area under the graph equals 1, giving a probability density function).

Vertical lines

The **Vertical lines** button can be found in the **Histogram Toolbar**. When it is selected, vertical lines can be added to the plot by double clicking with the left mouse button on the plot or by dragging with the left mouse button. In addition to the vertical line, a label will be added displaying the contrast/calibrated value.

Dragging will draw a line at the median of the selected values (no line is drawn if there are no counts in the region).

The labels generated from vertical lines can be moved to more favourable positions by dragging them with the mouse. To delete a label and its corresponding vertical line, either select the label with the mouse and press delete, or click the label while holding the shift key.

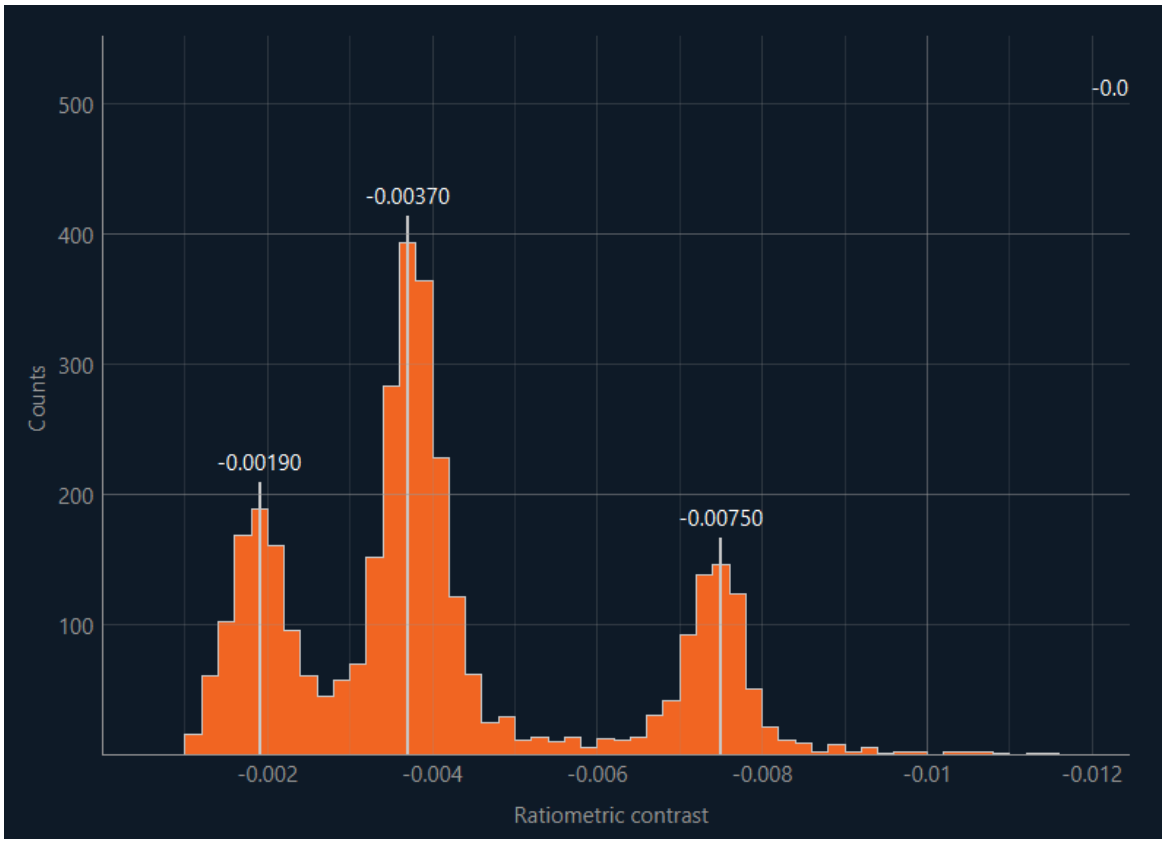


Figure 8: Vertical lines set at rough positions of histogram peaks

Interval regions

Adding interval regions to the plots provides information about events in the selected contrast/calibrated unit interval. To add an interval region, click the **Interval region** button and drag with the left mouse button over the desired interval. The selected region will then be highlighted and bound by pale yellow lines. Alternatively, double-click on a peak to automatically fit a normal distribution and add an interval region about the detected peak. The fitted region will be centred about the peak with bounds of +/- 2σ. A label will also be displayed which gives the median value of the region, the contrast/calibrated value bounds for the region and the total number of counts for the region displayed as a an absolute number and as a percentage value compared to the total count of binding/unbinding events (depending on whether the interval covers binding or unbinding events).

The labels generated from interval regions can be moved and deleted in the same way as for vertical lines.

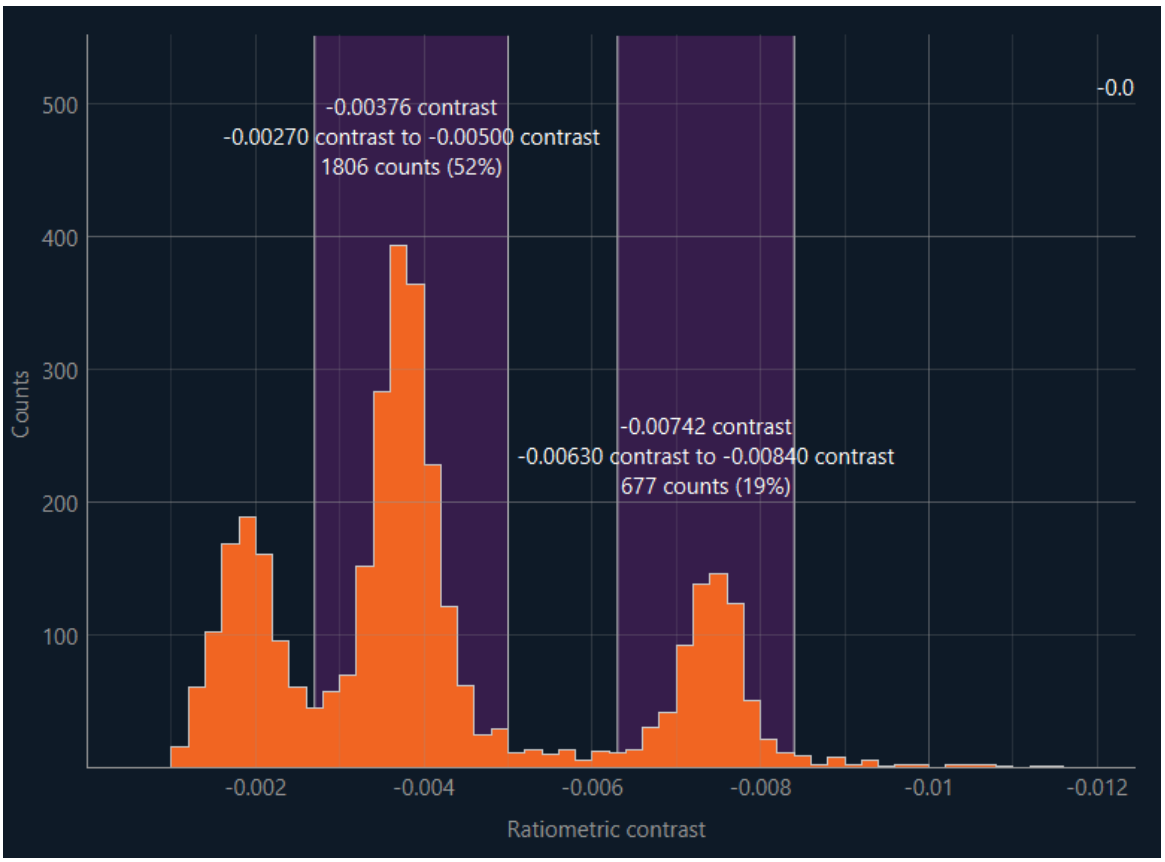


Figure 9: Interval regions set over a couple of histogram peaks

Peak fitting

In histogram mode

In histogram mode, fitting a peak to the data provides information about peak position, peak width, integrated counts, and skewness. Peaks can be fit automatically or manually.

Information about the fit is displayed above the curve in either contrast or calibrated value (mass or nucleic acid length). The information displayed is:

- Mean peak value
- Width of the fitted peak (σ represents the standard deviation)
- Sum of the number of counts under the fitted peak expressed as the number of counts and the proportion of counts. (The proportion is defined as the number of counts compared to the total number of binding/unbinding events depending on whether the peak is located in the binding or unbinding domain of the histogram)
- Pearson median skewness of the curve;
  - Skewness = 0: the mean and median are the same; there is a normal distribution
  - Skewness < 0: the mean is smaller than the median; the outliers of the distribution curve are further out towards the left
  - Skewness > 0: the mean is greater than the median; the outliers of the distribution curve are further out towards the right

In KDE mode

In KDE mode, pressing the Peak fitting button adds vertical lines with labels at each local maximum of the KDE plot.

Automatic fitting

Once analysis of a measurement is complete, peaks will be added automatically to the data (except for Samux^{MP} measurements). The peaks can be manually deleted as with all other peaks. If you have removed your peaks, or you have a Samux^{MP} file, you can add the automatically generated peaks by pressing the **Automatic peak fitting** button located in the **Histogram Toolbar**.

The automatic peak fitting can produce non-normally distributed peaks as it tries to fit to all of the data present. The non-normally distributed peaks are easy to distinguish as they will have a non-zero skewness value.

Manual fitting

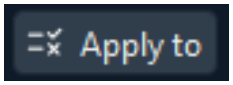
To fit a Gaussian peak, first select the **Guassian Fit** button from the **Histogram Toolbar**, then either drag with the left mouse button over a contrast/calibrated value interval or double click near the centre of the desired peak.

More than one peak may be fitted at the same time by selecting multiple regions in the graph sequentially. If a new region impinges on an existing Gaussian fit, the new peak fit will replace the previous one.

The labels generated from gaussians can be moved and deleted in the same way as for vertical lines.

Avoid dragging the peak fit range across an adjacent peak as the fitting routine will only fit to the newly selected peak.

Applying overlays to other measurements



Use this button to apply the gaussian fits, vertical lines and interval regions to other measurements in the workspace. Before the overlays are applied, plots are cleared and the plot settings are changed to match the settings of the currently opened measurement.

This feature is only available when Feature Preview mode is selected in Preferences.

AAV regions

There are two modes to apply AAV limits to measurements. In **Manual** mode, the user determines where to place the empty, partial, full, and overfull bounds. In **Automatic** mode, an algorithm locates the regions, based upon the expected mass of the capsid and the transgene length.

There are potentially four AAV regions; empty, partial, full and overfull. The empty region is the lower mass peak representing binding events from empty capsids. The partial region can represent partially filled capsids between the empty and full regions. The full region is the higher mass peak representing binding events from full capsids. Finally, the overfull region can be used to accumulate all counts with a higher mass than the full region. Overfull is not used in **Automatic** mode.

Common Procedure

To enable the AAV limits, load a calibration (which must also come from a movie recorded in AAV mode) and analyse the AAV movie in Discover^{MP}. Once the movie is analysed, the histogram will automatically switch to mass mode.

Add the AAV overlay by toggling the AAV overlay button.

The AAV overlay has a label describing the details from the AAV overlay, which can be moved and deleted in the same way as for vertical lines.

Manual Mode

Select the **Manual** mode radio button.

Each of the four regions is activated and deactivated with a check box in the **AAV Limits** panel.

The AAV overlays can be edited in multiple ways:

- By dragging either edge of the region you want to adjust
- By dragging the entire region that you want to move
- By manually adjusting the values that are visible beneath the histogram in the **AAV Limits** panel

The AAV limits can be saved and used in later sessions selecting the **Save** button on the AAV limits section. AAV limits saved will be available on the drop-down menu above the AAV limits section. When selecting **Apply to all**, the AAV limits selected will be applied to all measurements opened in the current workspace.

In manual mode, the AAV limits can range between 2,000 and 10,000 kDa. The mass range of particles that can be measured varies depending on the mass photometer. Consult the specifications of the mass photometer used for data acquisition in the respective user manual.

Automatic Mode

In Automatic mode the regions are automatically assigned based upon:

- The empty capsid mass in kDa
- The transgene size in kb

These values can be specified in Acquire^{MP} when the movie is recorded, in which case they are automatically read by Discover^{MP}. If these values were not provided by Acquire^{MP}, or are they are incorrect, they can also be specified in Discover^{MP} by pressing **AAV Parameters…**, which brings up a dialog in which new values can be entered. The user can choose to directly specify the full capsid mass instead of specifying a transgene length. On clicking **OK** the AAV limits will be automatically re-calculated.

Automatic calculation of the AAV regions is done as follows:

First, the range surrounding the provided empty capsid mass is analysed to detect the presence of a peak. If a peak is successfully fitted and the fit is deemed good enough, it serves as the basis for drawing the region centred on the peak. The width of this region depends on the peak's standard deviation and the Auto AAV region width setting in Preferences. In the absence of a successfully fitted peak, the region is centered on the provided empty capsid mass, with a fixed width of ±120 kDa multiplied by the Auto AAV region width.

Next, a peak is sought around the expected full mass, calculated using the provided transgene length if applicable. This peak is forced to have the same standard deviation as the empty peak, provided an acceptable one was found. The full peak is used in a similar way to define a region, based on the standard deviation and the Preferences setting. If no peak can be fitted, a region is drawn around the provided full capsid mass, with a fixed width of ±120 kDa multiplied by the Auto AAV region width.

Particles between the empty and full regions are classified as partial.

The method used to fit peaks to the histogram in this algorithm is specially suited to AAV data and is therefore not the same as the method used for Peak Fitting on the Discover^{MP} histogram.

Virus titer estimate

The titer estimation feature can be accessed by pressing the designated "Show titer estimates" checkbox within the software. This is only available with movies from a Samux^{MP}.

Instructions for best results:

- The software is most accurate when used with samples that have a particle count between 500 and 1500 for a 1 minute movie
- Use a clean buffer without any surfactants or other inclusions to prepare the sample for analysis
- Use (partially) purified samples
- It is recommended to repeat measurements to obtain a more accurate titer estimate

Refining the data

The user can manually exclude movie frames and events that should clearly be omitted from the data analysis. These may include frames where there has been a physical disturbance to the instrument, areas of the movie image which display excessive underlying noise, and events that are clearly outside the expected range of protein masses within an experiment. This functionality is only available for Normal, AAV and Antibody Stability modes.

Temporal selection

This feature is not available for measurements taken on Samux^{MP}. If there is a physical disturbance during movie recording, the movie may show high noise images. An example of a noisy ratiometric frame is shown in Figure 10.

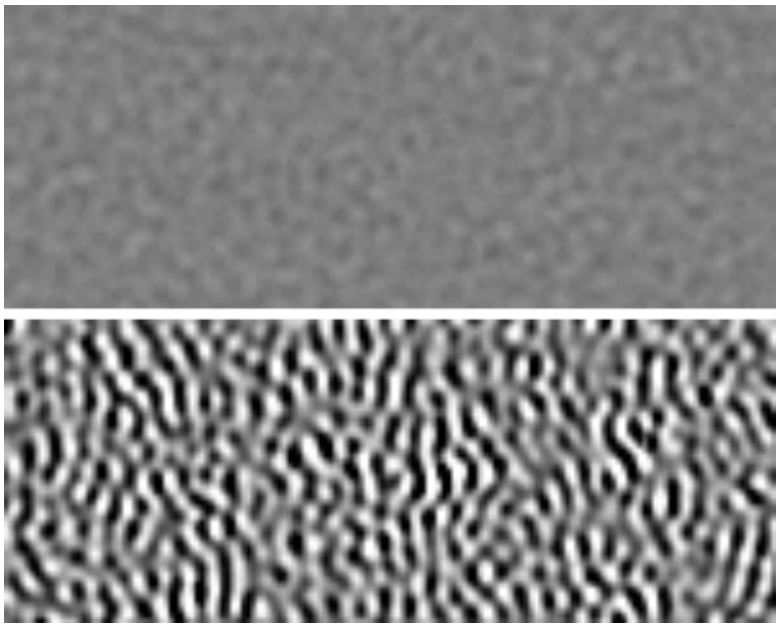


Figure 10: A comparison of a regular (above) and a noisy (below) ratiometric frame

To exclude a number of frames, hold down the left mouse button and drag over the region to be excluded in the score graph. This action produces a purple overlay for the frames that are excluded and updates the **Selections Panel** to reflect the excluded events.

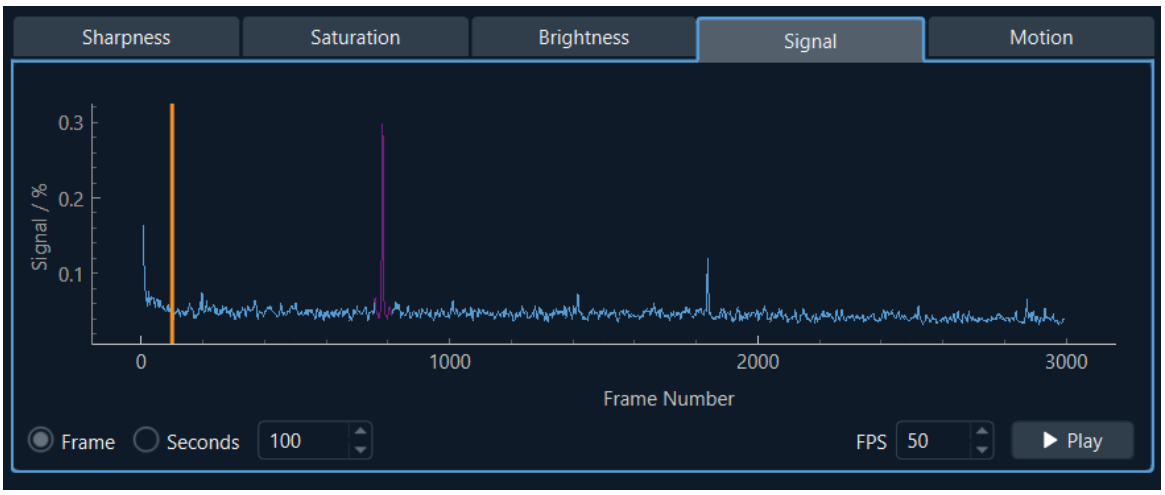


Figure 11: Signal score graph with removed frames indicated in purple

As frames are excluded, the related event counts are removed from the histogram. It is a good idea to watch the histogram to observe how much the plot changes.

The temporal mask can be deleted, either by right-clicking on the **Score** graph and clicking **Clear Selection** or by selecting **Clear** in the Selections Panel in the time mask tab (see Figure Figure 12). The mask can be inverted (using the **Invert** button) so as to include *only* events in the selected region(s) in the data analysis. The mask can be disabled (i.e. shown but not applied) by unticking the **Apply** button.

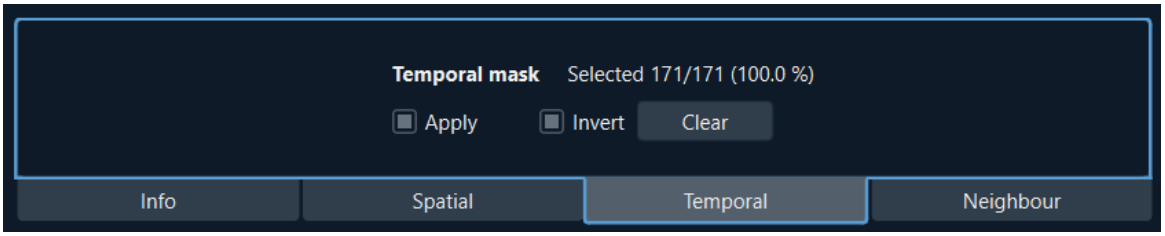


Figure 12: Selections Panel contains information about total binding and unbinding counts, as well as spatial, temporal, and nearest neighbour masking

Spatial selection

Feature not available for measurements taken on Samux^{MP}. The aim of mass photometry experiments is to deliver meaningful movies for data analysis. However, on occasion, there may be a region or small area of the movie image which shows high noise or is in some way visibly erratic. Such an area can be removed using a spatial mask.

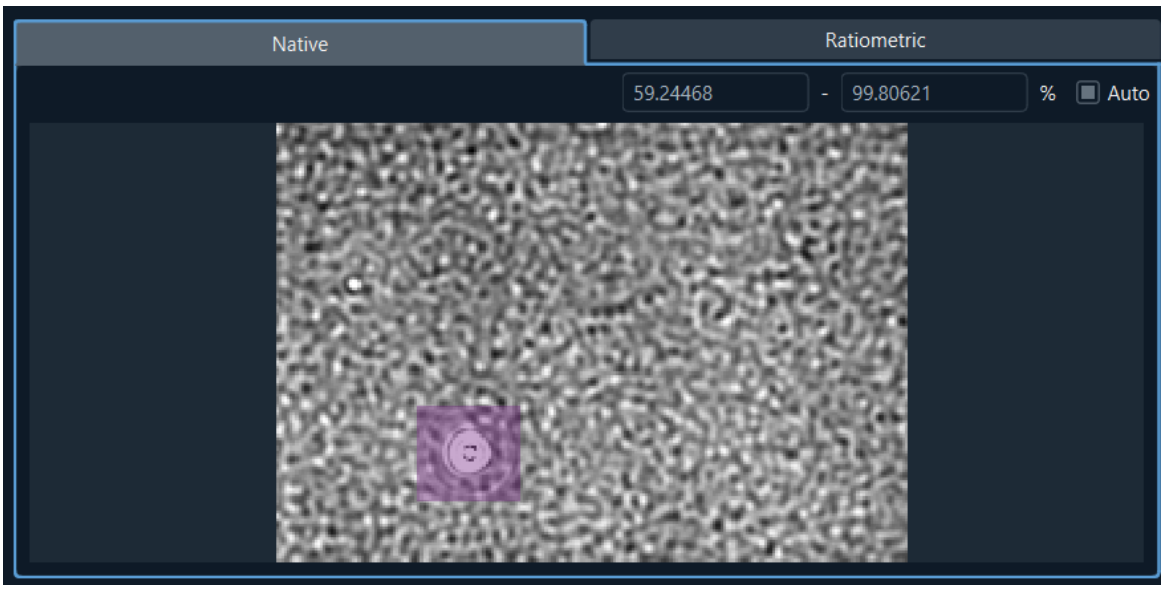


Figure 13: Image area selected for spatial masking shown in purple

The spatial mask selection may be made on any frame by pressing and holding the left mouse button and dragging over the region to be removed. This region will then be removed for all frames in the movie. Multiple regions may be selected for spatial masking.

Similar to the temporal mask, multiple regions may be selected. The mask may be cleared, inverted and disabled using the buttons in the Selections Panel.

The spatial mask tab of the Selections Panel holds information, including an indication of the number of events that are currently selected and used for the histogram plot.

When applying a spatial mask, the data points arising from that region of the movie are removed from the histogram. If the spatial mask is inverted, the related data points are included and all other events (outside the mask) are removed.

The event distribution can be used to show potential regions to remove (found by right-clicking on the Movie Panel and then checking **Distribution**). If the orange overlay shows clear hotspots, these regions may be a candidate to be removed. Ideally, the orange overlay should be fairly homogeneous.

Nearest neighbour selection

The **Nearest Neighbour** tab allows you to adjust the minimum allowable distance (in pixels) between neighbouring events. Events that are closer together than the set value will be removed from the histogram.

Calibration

Precise calibration of the contrast value and mass of a protein or length of a nucleic chain is required for accurate calibrated value determination. This procedure is performed during instrument installation and is repeated periodically at a frequency to suit the needs of each user. It is recommended that a calibration is performed before measurements requiring high calibrated value accuracy.

The calibration relies on measurements of several proteins of known mass/nucleic acid length, or a standardised protein mixture. Full details of the laboratory procedure can be found in the user manual for the mass

photometer. This section of this manual assumes that the correct steps have been taken to measure a set of proteins on one of Refeyn's mass photometers.

After analysing a calibrant measurement in Discover^{MP}, histogram peaks are observed at the expected positions on the contrast axis. The peaks can be labelled with either vertical lines, interval regions or Gaussian fits (Gaussian fits in histogram mode only).

Creating a calibration

Clicking the **Create** button found in the **Calibrations Panel** will open the calibration dialog. The user is initially prompted to choose the calibration type from the following options:

- mass calibration
- single-stranded nucleic acid calibration
- double-stranded nucleic acid calibration

Next, the user is asked to choose which measurements to use for the calibration. Any measurement which has Gaussian fits, vertical lines or intervals drawn on its histogram can be used.

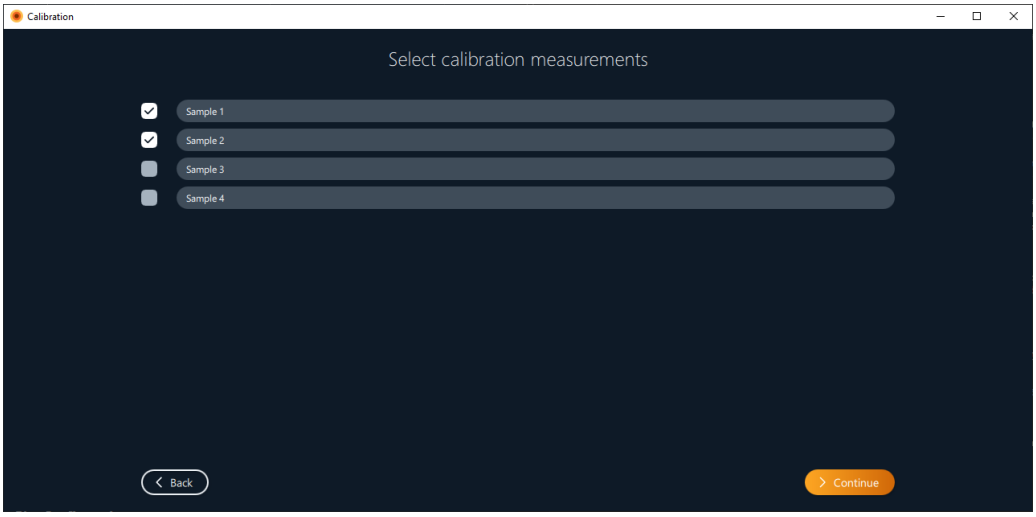
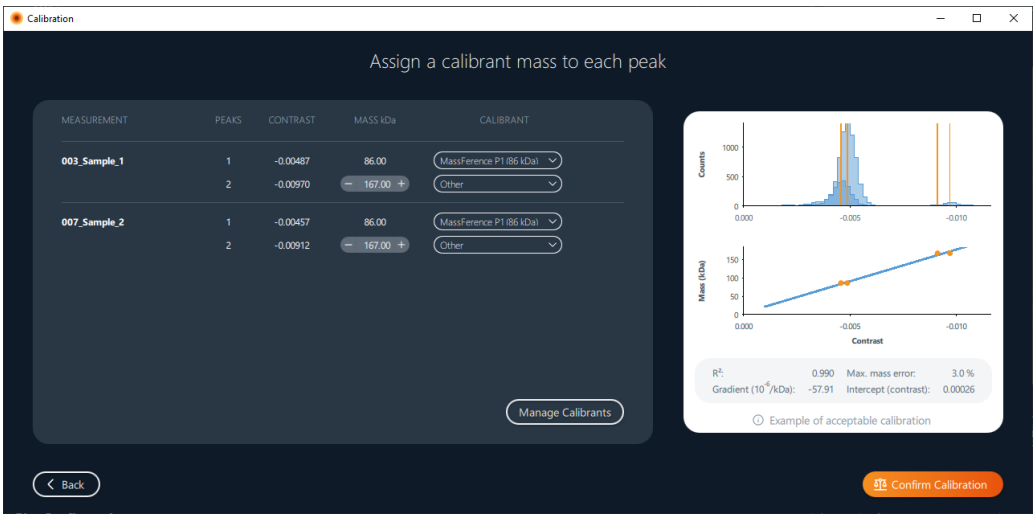


Figure 14: Select calibrant measurements

In the next step, the contrast values for each of the peaks, interval regions, and vertical lines in each chosen measurement are shown in a table. Each peak has a corresponding drop-down box, from which the corresponding known calibrant value can be chosen. If the calibrant used is not listed, select **Other** from the drop-down and enter its value manually. To omit a peak from the calibration, select **Unassigned** instead.



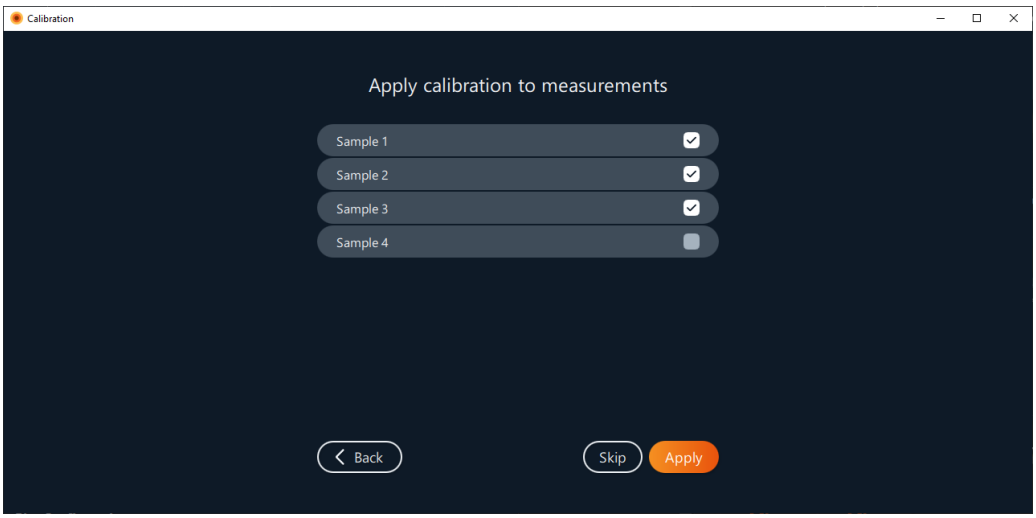
To edit the available list of calibrants, click **Manage Calibrants**.

Discover^{MP} will fit a straight-line calibration using the points entered in the table. Two measures are used to characterise the match between the data points and the obtained calibration:

1. Maximum error: Maximum percentage difference between the given value and the value calculated from the calibration
2. r^2 coefficient of determination for the fitted straight line

TIP Ensure that the fitted calibration curve (yellow line) is visibly a good fit to the calibration data. If it is not, check for entry errors in the table, or repeat the calibration measurement(s).

Next, the user is asked to name the calibration. Finally, the user is shown a list of all measurements to which the calibration can be applied. The user should tick all measurements to which the calibration should be applied.



After a calibration has been created it will be automatically added to the Calibrations Panel.

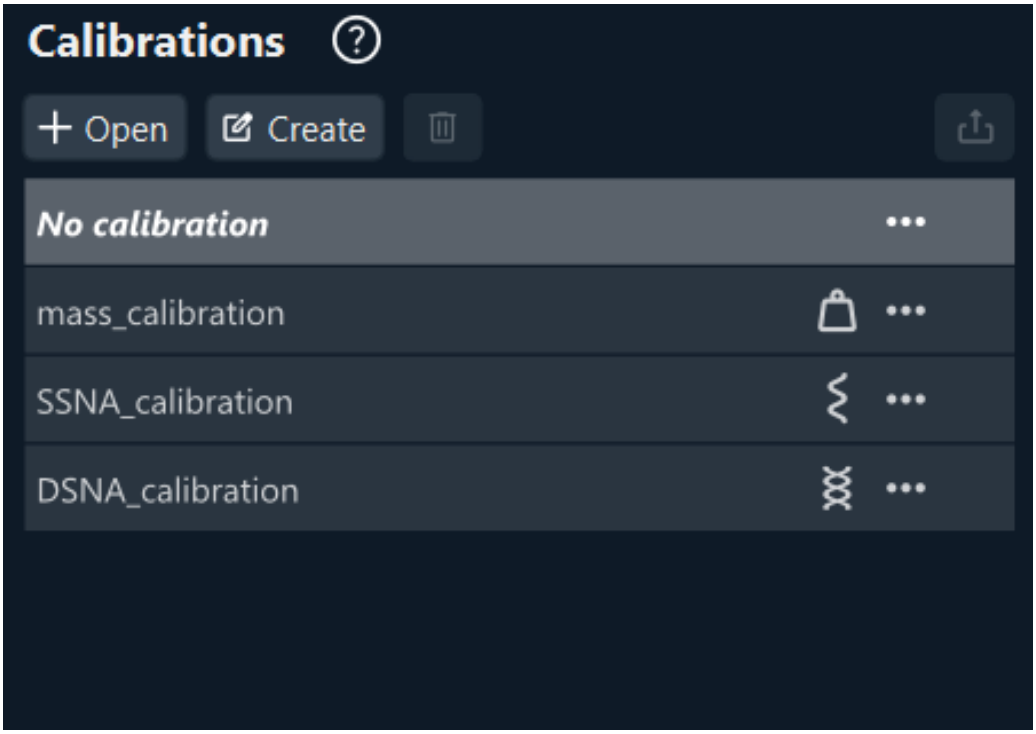


Figure 15: Calibration Panel: calibration type (mass, single-stranded nucleic acid or double-stranded nucleic acid) is indicated by the attached icon

Applying a calibration

To apply a calibration to one or more measurements after it has already been created, first select the measurement file(s) to which you wish to apply the calibration and then double-click on the calibration listed in the Calibrations Panel.

To remove a calibration from one or more measurements, first select the measurement file(s) from which you wish to remove the calibration and then double-click on *No calibration* in the Calibrations Panel.

You should not use a calibration derived from a movie with one image size for a sample movie with a different image size. For example, if the calibration was created from a movie recorded with the Regular image size in Acquire^{MP}, it should not be used for a sample movie that was recorded using the Large image size.

TIP When clicking on a measurement file, the applied calibration will be highlighted in bold. If no calibration is applied, *No calibration* in the Calibrations Panel will become bold.

Managing the Calibrations Panel

To add a calibration, you can generate a new calibration or press the **+ Open** button to open a previously saved calibration file (**.mc**).

To remove a calibration from the panel, select the calibration and click the **Bin** icon in the Calibrations Panel.

TIP All calibrations in the Calibrations Panel will be automatically saved as calibration files (**.mc**) when saving the workspace.

Figures

Figure Types

There are 3 types of figure you can use to visualize your data:

- 2D Histogram - Displays measurement results on top of each other on one 2D graph.
- 3D Histogram - Displays measurement results spaced out on the Z axis of one 3D graph.
- Vertical histogram series - Displays measurement results, each on their own 2D graph.

Figure 16: Figures Panel

Generating a new figure

Figures can be generated using one or multiple measurement files opened in the workspace. To generate a new figure, go to the **Figures Panel** > **Add Figure** > **2D histograms**, **3D histograms** or **Vertical histogram series**. More than one figure can be created in the same workspace.

Once a figure is generated, the **Setup** section is selected by default. Here you can define the figure title and the limits and units of its axes. Changing the figure title under **Setup** will automatically change the name on the figure tab. Figure setup can be accessed at any time by pressing **Setup**.

- **Title**: Histogram title
- **X axis units**: Ratiometric Contrast, Mass ([kDa] or [MDa]), Bases, or Base Pairs
- **X axis limits**: Minimum and Maximum
- **Y axis units**: Counts, Normalised Counts, Density (KDE), or Normalised Density (KDE)
- **Y axis limits**: Minimum and Maximum

Please note that, upon choosing *X axis units* other than Ratiometric Contrast, only those measurements calibrated with the appropriate calibration type will be displayed in the figure.

Defining a figure

Under **Style**, you can define which elements will be displayed in the figure and can edit its formatting. After selecting an item under **Style**, formatting options will be displayed in the panel below. You can also remove elements (such as the title) from the figure by ticking or unticking them.

Formatting options available for figures:

- **Figure**: Background colour; figure size
- **Title**: Font type, colour and size
- **Axes**: Font type, colour and size; Major and minor ticks; Histogram depth and Histogram distance (3D histograms)
- **Labels**: Font type, colour and size, label content, Rotation (2D histograms and vertical histogram series)
- **Grid**: Axis of grid, thickness, position and colour of grid lines
- **Legend (only stacked histogram)**: Font type and size; legend title

Formatting of labels will apply to labels for *all* overlays: vertical lines, interval regions, Gaussian fits and AAV limits.

Adding and removing measurement files

To add one or more measurements to a figure, select the measurement files in the Measurements Panel, then drag and drop the files into the setup section on the right-hand side of the Figures panel. To remove a measurement file, right click and select **Remove** or press the delete key. Measurement files removed from the workspace will automatically be removed from the Figures Panel.

The relative position of the plots in the figure can be changed by changing the order of the listed measurement files in the Figures Panel. The order of measurement files can be changed by dragging and dropping the files or by right-clicking and choosing the relevant option.

Formatting individual measurements

Each measurement file added to a figure will show a list of its associated overlays (vertical lines, Gaussian fits, intervals and AAV limits). By default, all overlays will automatically be shown in the figure. Please note that overlays must first be created in the Analysis Panel. Removing an overlay from the Analysis Panel will automatically remove it from the Figures Panel as well. An overlay can be removed from the figure by unticking it in the list. This will have no impact on the Analysis Panel.

Formatting options available for individual datasets:

- **Histogram:** fill colour, line pattern, line colour, line thickness
- **Gaussians:** peak line colour, peak line thickness, combined line colour, combined line thickness, label colour
- **Vertical lines:** line colour, line thickness, label colour
- **Intervals & AAV limits:** fill colour, label colour

Figure themes

Figure settings can be saved as a theme and edited in the **Histogram Themes Editor** which can be accessed by pressing **Edit themes** in Figures Panel. The editable features are the same ones listed in Defining a figure. If you want to generate a theme from an already made figure, you can select **Generate theme from figure**. This will automatically populate all the settings of a new theme using those explicitly used for the current figure. The themes can be shared by using export and import theme. When setting a theme as default, every new figure will automatically have those settings applied. To apply a previously saved theme you can select a theme under **Choose theme**.

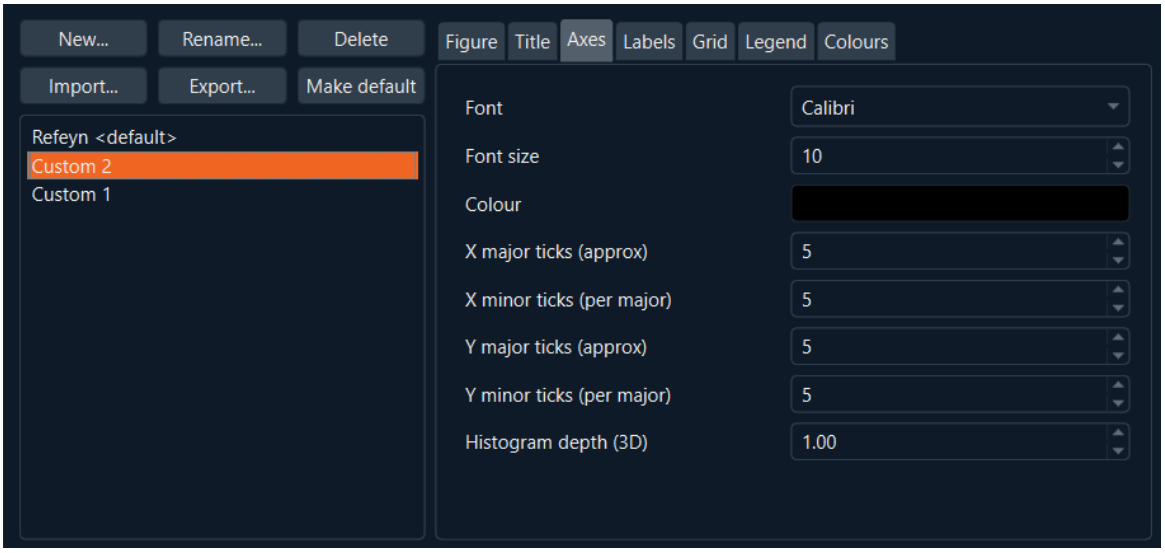


Figure 17: Histogram Themes Editor dialog

Tables

Generating a new table

Tables can be generated using one or multiple measurement files opened in the workspace. To generate a new table, go to the **Tables Panel** > **Add Table** > **pdf - limited to 24 measurements** or **csv - unlimited measurements**. More than one table can be created in the same workspace.

The columns of both the pdf and csv table are customisable and can be saved as a template. The column options contain all the histogram overlay information as well as additional information for making decisions about the data.

Both the column and the row order of the tables can be changed by changing the order in the **Added columns** panel and the **Added measurements** panel respectively.

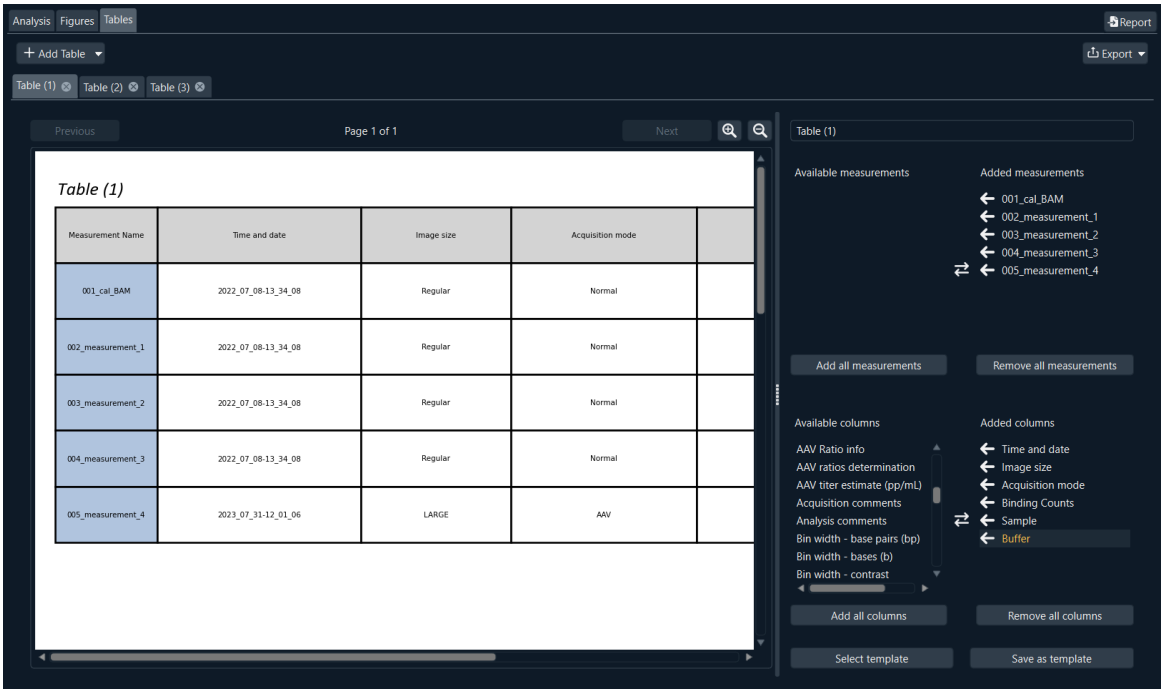


Figure 18: Tables Panel

Saving and exporting

Saving workspaces

After running the analysis, a full record of the measurement files, analysis results, figures and tables generated can be saved in the form of a **Workspace**. To save a workspace go to **File** > **Save Workspace**. This will open a dialog where the save location can be chosen. The workspace consists of a folder containing:

- A workspace file (**.dmp**)
- A calibration folder, containing all the calibration files (**.mc**)
- One or more mass photometry results files (**.mpr**), one for each measurement file opened in the workspace

Exporting a figure or table

To export a figure from the Figures Panel or table from the Tables panel, press the **Export** button located at the top-right of the panel. This will open a dialog where you can choose to export the current figure or table or all figures or tables and a file format. You can choose to save figures as PNG, SVG, PDF or EPS and tables as CSV or PDF.

Exporting Summary report

To export a PDF report combining PDF tables and figures press the **Report** button found at the top right of the Discover^{MP} window. In the Summary report dialog there is the option to choose which tables and figures to include and in which order. There is as well an option to include comments in the **Notes** field. When finished, press **Generate PDF**. This will open a dialog where you can choose the save location and filename.

Exporting results and analysis data

To export data related to individual measurement files, **right-click** on the measurement file, then press **Export** and select one of the following export options:

- **Results**
- **Events**
- **Report**
- **Movie**
- **Figure**
- **Scores**
- **Raw frames**; **Native** or **Ratiometric**

Batch export is also possible by selecting multiple measurements, instead of a single measurement, before right-clicking to access the export option.

Export options

Results - Exports individual measurement results files (**.mpr**). This will open a dialog where the save location can be chosen.

Mass photometry results files (**.mpr**) will automatically be generated when saving the entire workspace.

Events - The mathematical algorithm for finding binding and unbinding events is a two-stage process that first detects step changes of the glass reflectivity and then assesses whether the respective peak in the ratiometric image matches the expected signature. The original data generated by this algorithm can be exported as a **.csv** or **.h5** file.

Report - A single measurement report detailing the acquisition and analysis settings for a given set of results can be exported. This will bring up a window showing the value of every setting. The right-hand "Description" column can be removed by unticking the box at the top of the dialog labelled **Include descriptions**. To save the report, press the **Export** button and choose a file format. Reports can be saved as PDF, CSV or JSON.

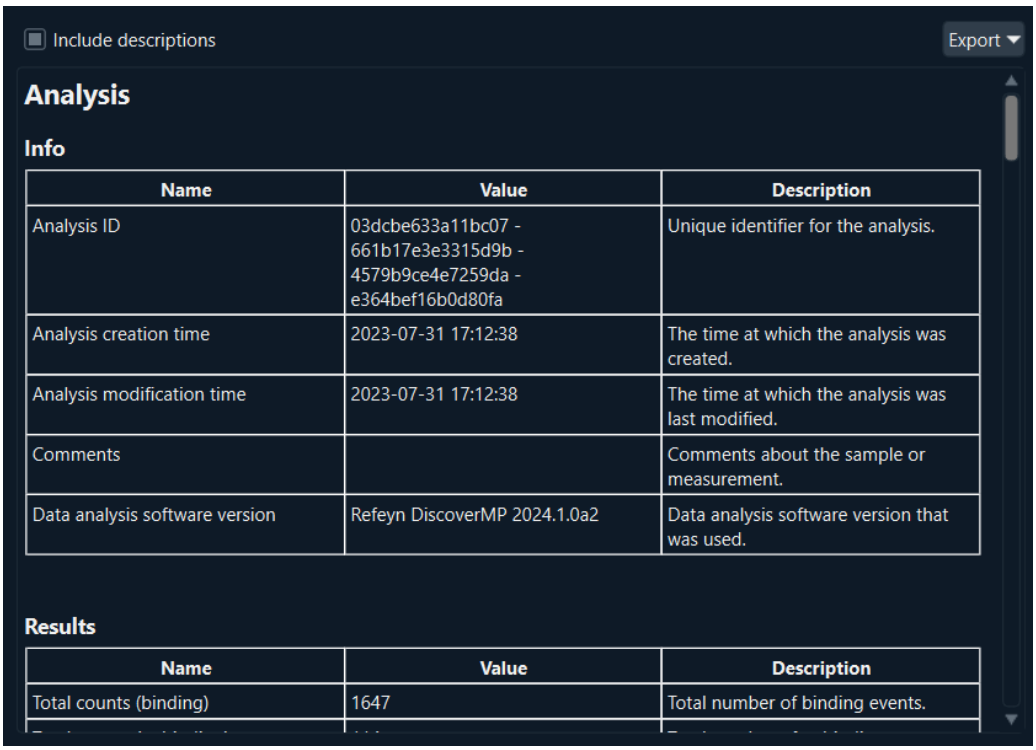


Figure 19: An example of a report

Movie - Movies generated can be exported as a MP4, MOV, AVI or TIFF. When exporting a movie, an additional dialog will open with the following options:

- Movie: Which movie to export (native or ratiometric).
- Start/Stop Frame: The range of frames to export.
- Upscale: this causes the movie to be saved at a higher resolution (without interpolation).
- Frame Rate: the number of frames played per second.
- Colormap: this shows the colours that will be used to represent high and low values.
- Colorbar: when ticked this adds a colorbar to every frame of the movie to show the contrast scale.
- Annotate Frames: when ticked, this adds a label to each frame indicating the frame number.

Note: TIFF format only uses the start and stop frame values. The other settings have no impact on the TIFF format.

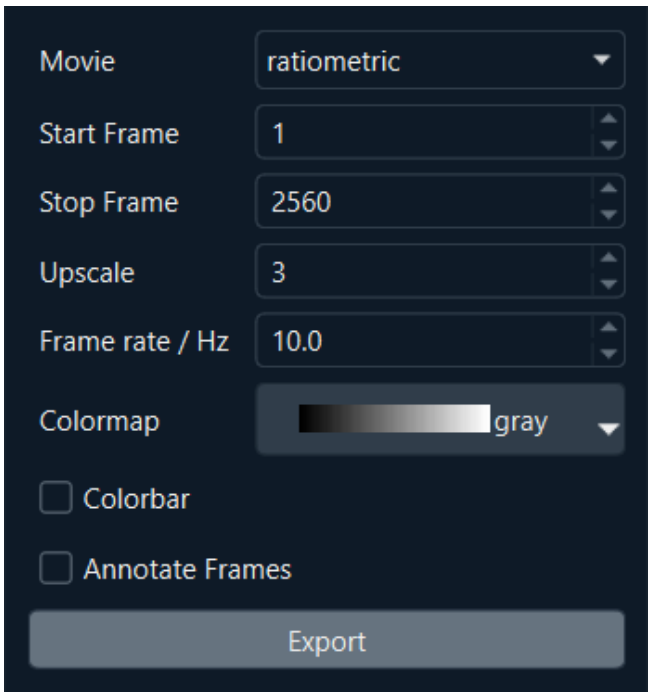


Figure 20: Export movie dialog

Making the movie larger using third party software (e.g. Windows Media Player), may result in interpolation that reduces accuracy and blurs images. It is preferable to perform this operation at the time of exporting the movie. The recommended value for upscaling is 3. The larger the upscale value, the larger the exported file will be and the longer it will take to export.

Figure - Exports an automatically-generated histogram of an individual measurement results file (as a PNG, SVG, PDF, or EPS file). This will open a dialog where the save location can be chosen.

Scores - Data on brightness, motion, sharpness, saturation and signal can be exported as a **.h5** file.

Frames - This options allows for the export of all frames from a movie. Users can choose between exporting ratiometric or native moves by pressing **Export** > **Raw Frames** > **Ratiometric** or **Native** as desired. This will generate a **.h5** file. Additionally, it is possible to export a single frame from a movie. For this, a users is required to go to the Analysis Panel, right-click on the movie and press **Export frame**. This will export the currently displayed frame as a PNG or PDF file.

Exporting calibrations

After a calibration is added to the Calibrations Panel, it can be exported as a calibration file (**.mc**) by selecting the calibration and clicking on the **Export icon**, found at the top right of the Calibrations Panel.

Additional functionality

Analysis comments

It is possible to save comments to a set of Discover^{MP} results. When a movie has been loaded, the comments button located in the top right corner of the Analysis Panel can be pressed. This opens a dialog with two tabs. The first tab is **Analysis**. These are comments that should relate to the information from Discover^{MP}. The second tab is **Acquisition**, which displays the sample information and comments provided in Acquire^{MP} when the movie was recorded. These comments will be included in a generated report.

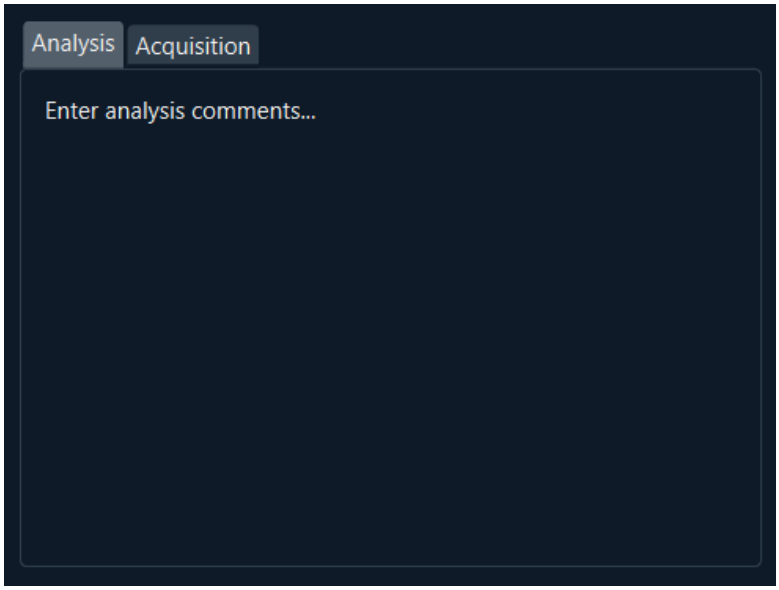


Figure 21: Comments dialog displaying an example comment

Tooltips

Discover^{MP} includes tooltips which may be displayed by hovering over buttons. These explain the use and purpose of each button.

What's this

Discover^{MP} application includes What'sThis boxes which may be displayed by clicking the **Question Mark button** (found at the top right of the window) and then clicking on a UI element, such as a button or tickbox. These boxes will contain detailed information about the element in question.

Analysis settings for One^{MP} users

One^{MP} users have access to an additional settings dialog which enables changes to the analysis pipeline. Settings apply to individual measurement files. To access settings, go to the File Panel, right-click on the desired measurement file and select **Settings**

Analysis can be split up into two stages: the finding of events and the fitting of events.

Finding events: The mathematical algorithm for Finding Events is a two-stage methodology that first detects step changes of the glass reflectivity (Filter 1) and then assesses whether the respective peak in the ratiometric image matches the expected signature (Filter 2).

Fitting events: This takes the found events and fits a Point Spread Function to the events. The position of the best fit will become the center of the event.

The settings used for threshold 1 and 2 in the analysis have been described above. The default values should be appropriate for most cases, but expert users may wish to adjust them. To return to the default settings at any time, select **Reset** in the Settings dialog.

The value for **Number of Binned Frames** defines the number of native frames that are averaged during the calculation of a single ratiometric frame. Increasing this value from 5 to 6 or 7 usually decreases noise and enhances the sensitivity for the detection of low-mass particles. However, increasing it also leads to more crowded ratiometric frames, especially for concentrated samples. The associated signal overlap may lower sensitivity and mass precision.

The **Reflectance Correction** when ticked will apply Refeyn's algorithm to reduce signals that can be produced by the reflection of the glass surface.

The **Motion Correction** when ticked will apply Refeyn's algorithm to reduce artefacts in the ratiometric contrast images that are an effect of the transverse motion of the microscope slide, either due to drift or an external vibration source. The algorithm estimates the amplitude of the motion signature in the ratiometric frame and subtracts it.

An advanced settings feature is available when selecting **Advanced** in the Settings dialog. We advise users to not change advanced settings unless instructed to do so by Refeyn.

Preferences

Discover^{MP} has a small set of preferences that the user can interact with via **File** > **Preferences**.

Telemetry mode

In the Preferences dialog, the current telemetry mode can be updated.

- **Feature preview mode** - Allows access to experimental features. Measurement data, and basic data about the user's use of the software will be anonymously reported to Refeyn, including error reports.
- **Normal mode** - Basic data about the user's use of the software will be anonymously reported to Refeyn, including error reports.
- **Offline mode** - Discover^{MP} will not communicate with the internet, or allow any feature that requires the internet.

Other settings

With **Show calibration help** checked, Discover^{MP} will show a dialog explaining how to apply a calibration when a calibration file is opened for the first time.

With **Enable auto fitting for non-Samux^{MP} movies** checked, after a non-Samux^{MP} measurement is analysed, Discover^{MP} will attempt to automatically fit its peaks.

With **Edit settings for all movies** checked, Discover^{MP} allows the user to edit analysis settings for any measurement in the workspace. It is recommended to keep this un-checked.

The **Auto AAV region width**, specified in units of standard deviation σ of the peak Gaussian, guides the auto AAV limits algorithm in defining the empty/full region boundaries.

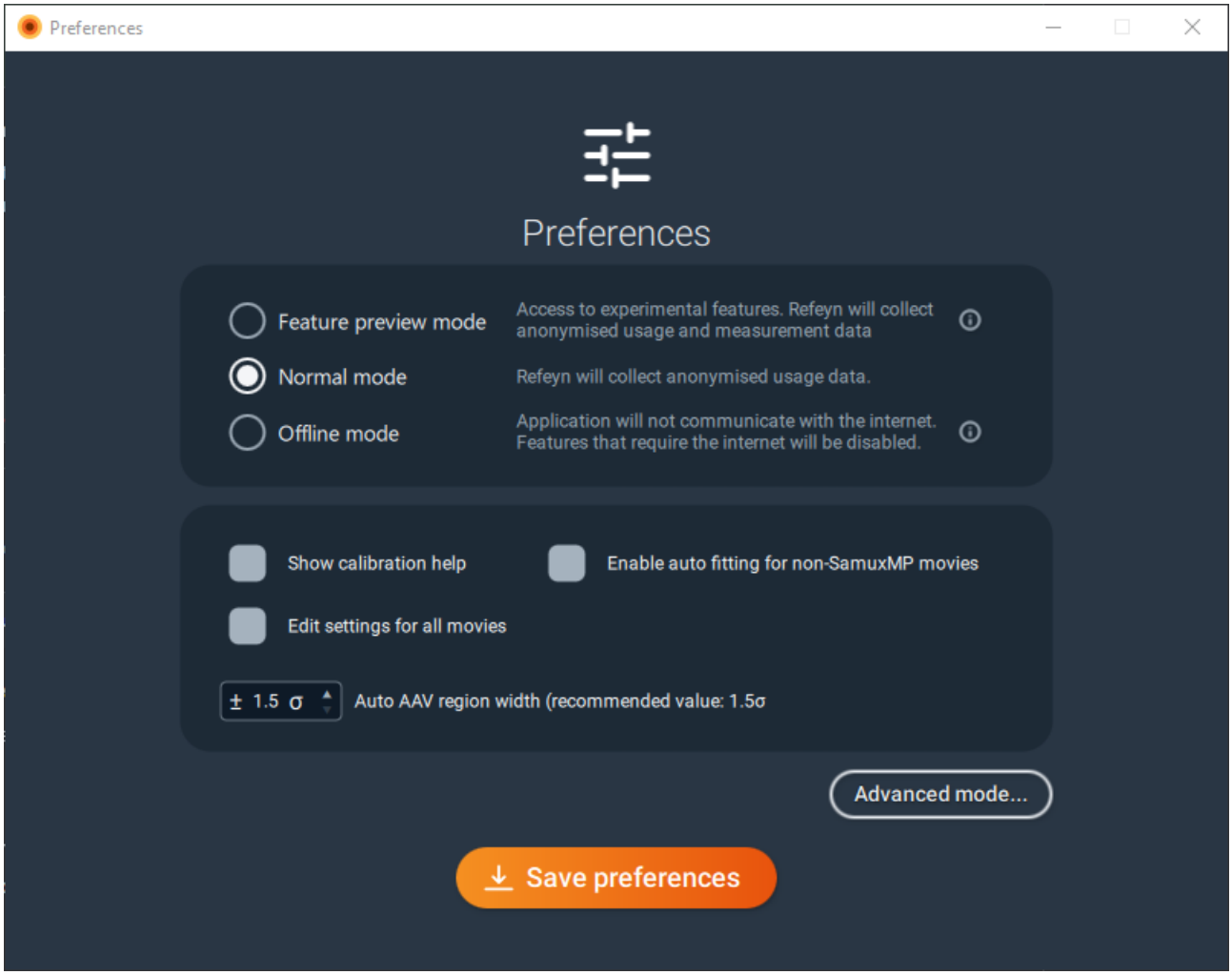


Figure 22: Preferences dialog

General information

Refeyn Ltd service and support

Refeyn Ltd offer service and technical support for our mass photometry systems, and the Refeyn software applications:

Refeyn Ltd
Unit 9, Trade City
Sandy Lane West
Oxford
OX4 6FF
Email: support@refeyn.com
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