REFEYN
WEIGHING MOLECULES WITH LIGHT

# *Refeyn Two$^{MP}$*

# *Mass Photometer*


***User Manual***

***Version 1.0***

# User Manual

## Contents

# Warranty

Your Refeyn Two$^{MP}$ instrument comes with a 12-month parts and labour warranty and has been thoroughly tested against Refeyn's published specification.

The warranty covers the repair or replacement of the instrument or a discretionary refund of the purchase price.

Please keep the supplied shipping crate and packing material for the duration of the warranty period, so that in the unlikely event of needing to return the instrument, it can be done so safely.

To qualify under the terms of the warranty, the system must be used in accordance with this guide and any training provided by Refeyn Ltd.

Failure to do so may invalidate the warranty and no liability is accepted for loss or damage arising from the incorrect use of the Two$^{MP}$ System.

Refeyn Ltd reserves the right without prior notice to alter the specification of the Two$^{MP}$ System to improve performance and benefits for the user.

# Safety Information

## 1. Product Identification

| | |
|---|---|
| Refeyn Two$^{MP}$ is manufactured by: | Refeyn Ltd • 1 Electric Avenue • Oxford • OX2 0BY • UK |
| Model Number: | Two$^{MP}$ |
| Product Description: | Research Grade Mass Photometer |
| Technical Assistance: | support@refeyn.com<br>+44 (0) 1865 203956 |

## 2. Electrical Ratings

Power supply for Refeyn Two$^{MP}$:

Model No.: AHM180PS12
Input Rating: 100-240 Vac • 50/60 Hz • ~2.2A
Output Rating: 12V DC • 13.75A

## 3. Embedded Laser System Ratings

Refeyn Two$^{MP}$ Mass Photometer:

| | |
|---|---|
| Primary laser: | 488 nm (± 5 nm) • 2 W Max • Class 4 |
| Autofocus laser: | 638 nm (± 5 nm) • 700 mW Max • Class 4 |

## 4. Laser Safety Information

Caution – Use of controls or adjustments or performance of procedures other than those specified herein may result in hazardous radiation exposure.


**The Refeyn Two$^{MP}$ Mass Spectrometer is a Class 1 laser system**, making it safe to use in an open laboratory environment. The safety interlocking designed into the Two$^{MP}$ Mass Photometer eliminates the risk of exposure to the high-power, Class 4 laser system within the instrument.

Users should not attempt to remove screws securing either the Mass Photometer enclosure, or the Electronics Controller Housing. All screws in these housings are security type and should not be removed, except by qualified Refeyn Ltd engineers.

Care should be taken when using the instrument. If there is any indication of a fault resulting in the escape of laser radiation from the unit, switch off the unit immediately and contact Refeyn Ltd Technical Support at support@refeyn.com.

## 5. Environmental Operating Conditions

- Refeyn Two$^{MP}$ instruments are intended for indoor, laboratory use only.

- The Two$^{MP}$ instrument should be operated on a secure, flat surface in a dedicated area.
- Make sure there is enough space around the instrument to allow air to circulate and avoid overheating. Cables other than specified for use by Refeyn should be kept tidily away from the instrument and Electronics Controller.
- For optimum performance, Two$^{MP}$ Photometers should be operated in an environment with stable temperature 20°C +/- 2°C (with a temperature variation of less than 1 °C per hour) with relative humidity < 70% at 20°C, at an altitude of < 5000 m above sea level.
- As with any analytical instrument, standard precautions must be taken to prevent environmental or cross contamination when collecting/handling samples and loading the coverslip onto the Two$^{MP}$ instrument.

For full details of optimal placement of your Two$^{MP}$ in your laboratory, see Site Preparation Guidance, below.

## 6. Transporting the Two$^{MP}$ Mass Photometer

Refeyn Two$^{MP}$ Mass Photometers are intended for static use, in the laboratory position where they have been initially installed. If the system is to be moved to a different location, please contact Refeyn Ltd Technical Support (support@refeyn.com) for advice.
The Mass Photometer optics unit weighs 30 kg, and the electronics controller weighs 10 kg. It is therefore recommended that local policies on handling such weights are adhered to, should the unit need to be moved.
In all circumstances, the Mass Photometer should be lifted by two or more individuals, and anyone with an incapacity to lifting heavy objects should avoid doing so.
As the unit is not intended to be moved, the lifting positions are not indicated ergonomically, however, the unit is robust if lifting from the base.

## 7. Servicing and Maintenance

There are no user serviceable parts inside Refeyn Two$^{MP}$ Mass Photometers.
These systems must only be installed by trained service personnel from Refeyn Ltd. To avoid any personal risk, users are prohibited from accessing the interior of the Two$^{MP}$ system. All external screws are purposely selected to have security features requiring specific tools to unscrew them. This is intentional, and these screws should not be accessed by users or anyone not fully trained by Refeyn Ltd.

## 8. Electrical Supply to the Instrumentation

Two$^{MP}$ systems must utilize the power supply and cables supplied with each instrument. Under no circumstance should these cables be replaced with similar cables. If in any doubt about the mains cables feeding electricity to the units, contact Refeyn Ltd Technical Support (support@refeyn.com).

Power supply:

XP Power AHM180PS12
INPUT: 100 – 240V • 50/60 Hz • ~2.2A

OUTPUT: 12V DC • 13.75A

Any deviation from the factory-provided electrical connection and power supply parts may affect the safety characteristics of the instrumentation. If there is any doubt, please contact Refeyn Ltd Technical Support (support@refeyn.com).

## 9. Intended use

The Refeyn Two$^{MP}$ Mass Photometer is intended for research use only. It is intended to be used for measuring molecular mass distributions of biomolecules in solution and is to be operated in a laboratory setting in alignment with the environmental requirements specified in this manual.

# Site Preparation Guide

## 1. Transport Requirements

The instrument is delivered in a single palletised wooden crate (80 cm x 120 cm x 95 cm, W x D x H) weighing approximately 100 kg. A horizontal clearance of approximately 1 metre is recommended for delivery of the instrument to its intended installation room. A pallet truck will be required to move the crate. The crate should be stored at 10-25°C without major or rapid temperature changes and at lower than 75% humidity prior to unpacking and installation.

## 2. Shipping Crate Contents

- Refeyn Two$^{MP}$ Mass Photometer and Electronic Controller
- Instrument Computer with mouse and keyboard.
- PC Monitor
- Instrument shipping kit including mains cables, USB cable, microscope objective, immersion oil and sample stage power supply and sample top plate.

## 3. Room/Site Requirements

The Mass Photometer optics unit has a footprint of 504 mm x 314 mm x 135 mm (W x D x H) and weighs approximately 30 kg. It is connected to the electronics control unit via a braided stainless-steel conduit of approximately 1.5 metres in length. We recommend that the Mass Photometer unit is placed on a heavy, sturdy workbench which can support the instrument and electronics control unit. Internet connectivity is essential for the duration of the installation and occasional availability may be necessary for remote access and updates.

The electronics control unit has a footprint of 430 x 330 x 133 (W x D x H) and weighs approximately 10 kg. The electronics control unit can be installed next to the Mass Photometer or separately, under the bench or on a separate bench, as needed. Please note the conduit connecting the electronics control unit to the mass photometer has a minimum inside bend radius of 70 mm. A possible layout is shown below.

The instrument and PC require three power outlets in total (one for the electronics control unit and two for the PC and monitor,100-250 V, 50/60 Hz). The instrument is fitted with 2 m (6.5 ft) power cables (a possible layout is shown below). Power consumption is less than 1 A at 230 V AC.

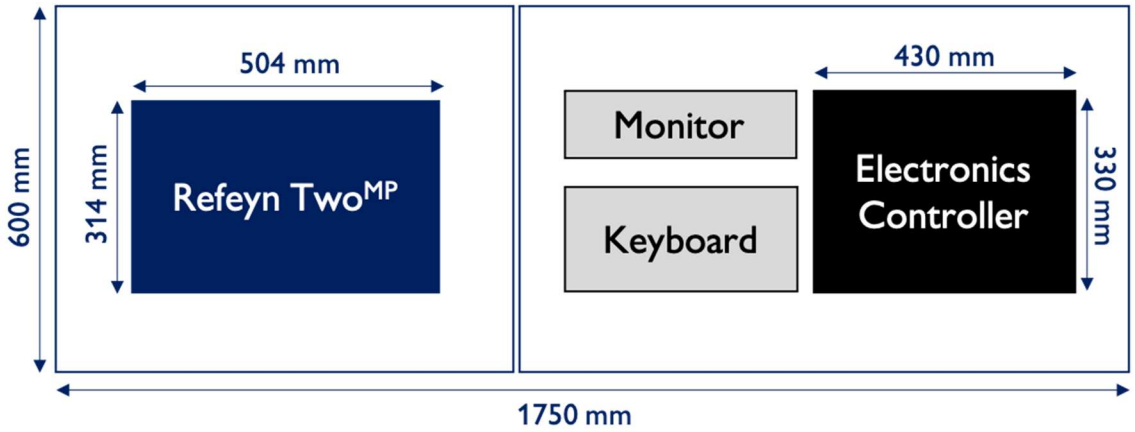



**Figure 1:** Measurements of the Refeyn Two$^{MP}$ Mass Photometer and required space for the setup

The instrument should not be near air-conditioning units or similar, to minimise exposure to direct airflows.

We recommend that the supplied workstation and peripherals (keyboard, mouse etc) are placed on a table that is not coupled to the instrument to avoid vibrations caused by operating the computer corrupting measurements (Fig. 1). The instrument is vibration sensitive, so it is advised that for the most challenging of experiments the environment should be as free from disturbance as possible; i.e. no excessive vibrations caused by noisy equipment or air currents.

The installation room should have a **stable temperature 20°C +/- 3°C** (with a temperature variation of less than 1 °C per hour) with relative humidity < 70% at 21°C.

## 4. Instrument Supporting Bench

While the Refeyn Two$^{MP}$ Mass Photometer will function when placed on a robust, solid laboratory bench (capable of supporting 50kg), for best results we recommend use of a vibration-isolation bench, such as a bench-top active anti-vibration unit, such as the **'Accurion i4 for Refeyn Two$^{MP}$'** made by **Accurion** will allow you to work on a robust laboratory bench.

## 5. Additional requirements for experiment preparation

High-purity solvents (HPLC or better), reagents and clean glassware must be used throughout all preparations for best performance of the instrument (see *Experimental Guidelines*).

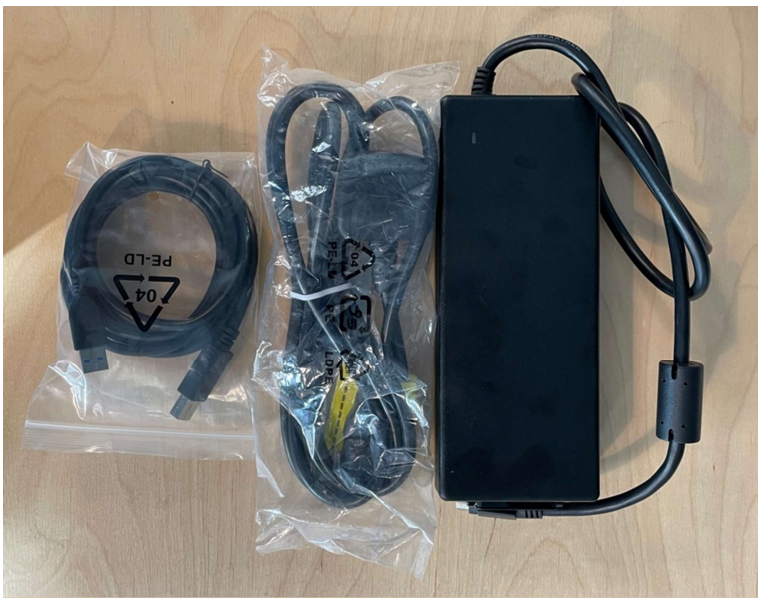
# Getting Started

## 1. What's in the box?

- Mass Photometer and Electronic Controller

- Instrument PC and Monitor

- Mains Cable (UK versions shown) and USB communications cable.
- XP 12V DC Power Supply



## 2. Switching on the Mass Photometer

The Refeyn Two$^{MP}$ Mass Photometer must be installed and verified by a trained engineer from Refeyn Ltd. Users must receive training in the functionality and use of the Mass Photometer before using the instrument.

Make sure the USB 3.0 cable is connected between the PC and the Electronics Controller.

Ensure the instrument power switch is in the "OFF" position (denoted "0" on the switch). Make sure the instrument mains cable is securely plugged into XP power supply and that the 4 pin DC Plug supplying 12V to the electronics unit is plugged into the inlet next to the on/off switch. Switch on the instrument at the power inlet switch. As the instrument controller receives power, the cooling fans in the Electronics Controller may be heard.

## 3. Launching the Mass Photometer Software

The Two$^{MP}$ Mass Photometer is controlled by two applications, the acquisition software Acquire$^{MP}$ (AMP) and the analysis software Discover$^{MP}$ (DMP). For detailed information on how to use the software package, please read the related user manuals.

# Experimental Guidelines

## 1. Coverslip and gasket cleaning

For precise Mass Photometry experiments, Refeyn Ltd. recommends Marienfeld high precision glass coverslips. These coverslips have been found to exhibit better performance in Two$^{MP}$ measurements than standard quality variants. As with other coverslips, these items need to be cleaned before use to remove any residual particles that would interfere with the Mass Photometry measurement.
There are two procedures for cleaning standard No. 1.5H glass coverslips (24x50 mm$^2$) before performing Mass Photometry measurements: one based on individual sequential rinsing of coverslips and an alternate batch procedure using a bath sonicator. Either procedure can be followed, depending on the user requirements. High-purity solvents (HPLC or better), reagents and clean glassware must be used throughout all preparations for best performance of the instrument.

The following is required:

- Purified water (e.g. Milli-Q®)
- Isopropanol (HPLC grade or higher)
- A dry nitrogen source or compressed air with in-line filter (regulated to 0.5-2 bar)
- Solvent squirt bottles (i.e. Thermo Scientific Nalgene 2423-0500)
- Marienfeld high precision glass coverslips, 24x50 mm$^2$, No. 1.5H, Marienfeld cat no, 0107222
- Self-adhesive silicone culture wells (e.g. Sigma Aldrich GBL103250, Grace Bio-Labs reusable CultureWell™ gaskets)
- Batch cleaning procedure: Adjustable Cover-Slip Rack, Electron Microscopy Sciences (EMS) cat no. 72243

### Procedure 1: Individual rinsing

Hold a coverslip by a corner with soft-tipped tweezers (soft-tipped tweezers reduce the risk of breaking a coverslip compared to metal tips) or by hand (wearing gloves). Rinse the coverslip sequentially with purified $H_2O$, then isopropanol, $H_2O$, isopropanol and $H_2O$ in turn. Then dry the coverslip under a clean stream of dry nitrogen. Take care in all steps to hold the coverslip from the bottom and rinse/blow downwards – this ensures that any residual particulate matter that might be on the tweezer tips or gloves are not swept over the coverslip surface.

### Procedure 2: Batch cleaning

This procedure requires an ultra-sonic cleaning bath, a glass beaker and a rack to hold several coverslips apart in the beaker. Ensure that both the beaker and the rack are clean to begin with – putting the rack in the beaker with a 50:50 mix of purified $H_2O$ and isopropanol and sonicating for 5 minutes can be a useful precaution. Populate the (clean) rack with coverslips, place in the beaker, add enough purified $H_2O$ to submerge the coverslips and sonicate for 5 minutes. When sonication is complete, dispose of the water, place the rack back in the beaker and cover with a 50:50 mix of purified $H_2O$ and isopropanol, and sonicate for a further 5 minutes. Again, when that time has elapsed, dispose of the solution mix and sonicate one more time in purified $H_2O$. Dry the cleaned coverslips under a clean stream of nitrogen (as before, holding with soft-tipped tweezers or gloved hand and always blowing downwards towards the tweezers is helpful here).

### Gasket Cleaning

Measurements may be made utilising a clean gasket to retain the sample. Before use, gaskets need to be cut into segments with 4 wells. Gaskets taken fresh from the packaging do not require any cleaning and can be used right away. However, used gaskets can be reused after proper cleaning. Directly after the measurement store the gaskets in a falcon tube with 100% isopropanol. Before re-use clean the gaskets either by sequential rinsing with purified $H_2O$, isopropanol and $H_2O$, or by applying a batch cleaning procedure comparable to the coverslip batch cleaning procedure (see above). Therefore, gaskets can be put into a fresh falcon tube and cleaned in an ultra-sonic cleaning bath. Ensure the gaskets are well separated not sticking to each other to get them cleaned properly. Finally, to ensure proper adhesion to the coverslip it is important to dry the gaskets very well, again using a stream of dry nitrogen.

## 2. General Experimental Procedure

Important: Be aware that Mass Photometry measurements are temperature sensitive. To ensure most accurate results, Refeyn Ltd. recommends keeping buffers and samples at room temperature

### Preparing the sample carrier

In order to keep the sample droplet in shape Refeyn Ltd recommends reusable self-adhesive silicone culture wells. The CultureWell gaskets have wells of 3 mm diameter, 1 mm deep. 4 wells cover enough area of the coverslip to be used in a single experimental session, before replacing the coverslip/gasket combination.

Place a clean gasket in the centre of the clean coverslip and assure it is fixed tightly by applying light pressure with a clean item such as the back of a pipette tip.

### Mounting the sample carrier

Apply a droplet of immersion oil on the objective and mount the prepared coverslip so that the red autofocus laser is roughly centred. Make sure there are no bubbles in the immersion oil to ensure integrity of the Auto-Focus ring. Hold the coverslip down with the small magnets provided.

### Finding focus

Add a 15 - 18 µl droplet of buffer and adjust the focus in two steps – make sure to display the live image in native format.

1) Move coarsely until you see the autofocus ring which indicates that you are close to the focus position
2) Then adjust the focus position in fine steps to find accurate focus using the sharpness monitor. In order to get into correct focus, maximise the yellow line representing the standard deviation of the native image (sharpness). Check by ensuring that the native image shows the coverslip surface roughness with high contrast.

Important: Be aware to choose the correct maximum. Aside from the global maximum, which you should aim for, there is also a local maximum close to focus.

After finding the global maximum, set and lock the focus.

Record a short movie of the buffer to determine the background in the later analysis.

### Calibration

For calibration we recommend the NativeMark™ Unstained Protein Standard from ThermoFisher Scientific (NM). A final dilution of 1:500 is recommended for the NM Standard.
Another option is running experiments with proteins of known molecular mass, i.e. standard proteins like BSA (66 kDa), apoferritin (480 kDa) and thyroglobulin (670 kDa), separately and combine the resulting ratiometric contrasts in one calibration file.

### Sample addition

The method of applying a sample to perform a measurement will be explained using a NM sample:

Prepare a 1:50 dilution of the NM protein standard, mix well and add 2 µl of this pre-dilution into the 18 µl buffer droplet in the gasket used for focussing. Mix the two solutions thoroughly using a pipette with 10-20 µl volume to avoid low counts of protein landing events due to slow diffusion. While loading the sample, make sure to not touch the stage or coverslip to maintain a stable focus.

## 3. Further Information

For detailed information on how to acquire and analyse data, please see the manuals for Acquire$^{MP}$ and Discover$^{MP}$.

# Troubleshooting Guide

## Auto focus (AF) ring related

| | |
|---|---|
| AF ring very blurry or super broad | Please compare your ring to images in Figure 2a, b and c, and make sure there is a liquid column (droplet) on top of the glass surface, which is required for focussing. No liquid added will lead to an oversaturation of the camera in native and ratiometric mode when lid is closed (Fig. 3a and e).<br><br>Representative images of native and ratiometric mode with clean coverslip mounted and buffer column added are depicted in Figure 3b and f. |
| AF ring is broken or not complete | In case there are bubbles in the immersion oil, the AF ring will be disrupted (Fig. 2d). Make sure to remove the bubbles for the autofocus to function properly. Lifting the coverslip slightly might move the bubbles away from the ring reflection. |

Auto Focus ring

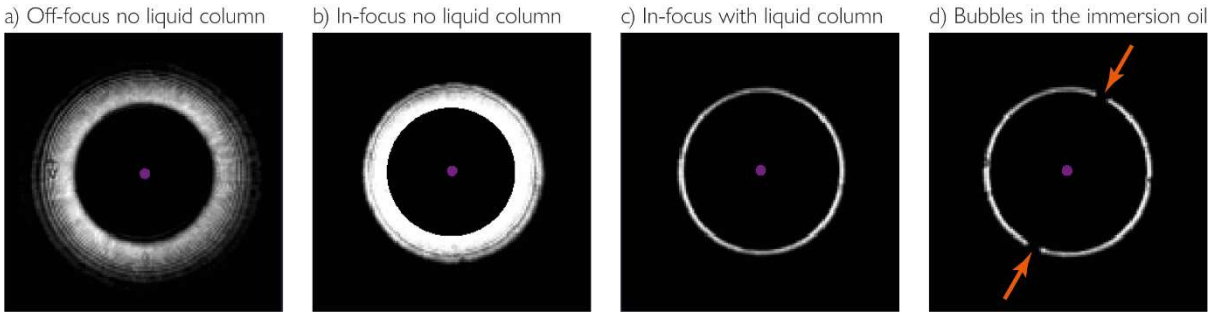



**Figure 2:** Representative images of the auto focus (AF) reflection ring under certain circumstances. a) Faint and blurry ring indicating that there is no liquid column on top of the coverslip and the instrument is not in focus. b) Bright AF reflection, with a digital "beam stop" mask lets the reflection look like a broad ring. Missing liquid column on the coverslip will prevent the ring from looking as wanted (c). d) A broken ring indicates that there are bubbles in the immersion oil. Bigger bubbles will result in greater disruptions in the ring (orange arrows). Integrity of the ring is essential for a stable function of the auto focus. Purple dot represents the centre of the ring, required for the calculation of the ring radius.

## Sharpness value is lower than usually at global maximum

| | |
|---|---|
| Not enough immersion oil on the objective | Check if there is enough immersion oil on the objective. If the value is still too low after adding extra oil, there might be another issue. |

### Signal is above 0.07% when loading buffer

| | |
|---|---|
| Coverslips are not clean | Coverslips are not clean, because they have been directly taken from the box without cleaning, have not been cleaned properly, or have been stored for some time after cleaning. Check glass surface in native mode and compare to images in Figure 3b and c, which show a clean surface and buffer.<br><br>Repeatedly clean coverslips with clean Milli-Q and isopropanol. If coverslips are still dirty after washing, check if solvents are dirty. Add a washing step with different alcohol, e.g. ethanol. |
| The buffer contains particles e.g. dust or big undissolved components | Filter the buffer to 0.2 µm. Be aware that old or not correctly stored filters can increase the number of fine particles in the buffer due to filter debris.<br><br>Prepare fresh buffer and make sure all components are well dissolved. Filter the buffer to 0.2 µm. |
| The buffer contains detergent (unintentionally) | Signal is increased in case there is detergent in the buffer (Fig. 4d, g, h, i, and j). If buffer is not supposed to contain detergent, make sure it was not introduced during the filtering process. |

### Buffer blank shows wavy noise floor (varyingly strong) in ratiometric mode

| | |
|---|---|
| Coverslips are dirty or not well cleaned | Check glass surface in native mode and compare to Figure 3b. A dirt layer on the coverslip will not completely be averaged out by the rolling background correction in ratiometric mode. A stronger background signal will be detected, which resembles Figure 3g with low concentrations of detergent. The signal value will also be increased (see above). |
| Buffer contains detergent (un)intentionally | Record and analyse a buffer blank movie. Detergents have a mass on their own and detergent-specific peaks will be detected. Histograms will show a peak at a detergent-specific (negative) contrast or (positive) mass value with a mirroring peak in the same regime at the opposite (positive) contrast or (negative) mass. If particles of interest (e.g. proteins/ complexes) are expected to result in a higher contrast or mass compared to the noise peak, |

| | |
|---|---|
| | they might still be analysable. Be aware that the resolution will be affected. If possible, reduce or remove the detergent. |
| Buffer contains high concentrations (mM) of a small molecule | Although small molecules are below the detection limit, they can still give strong background signal when at high concentrations. Record and analyse a buffer blank movie and test if high concentrations of small molecules will interfere with analysis of the protein of interest. If possible lower the concentration of small molecules. |
| Glass surface has been coated | Residual coating material might cause increased signal values. Perform additional washing steps or optimise the coating by lowering the concentrations of coating material and test different coating conditions e.g. shorten the incubation time. |

### Buffer blank shows particles in native mode and/or landing events in ratiometric mode

| | |
|---|---|
| Buffer contains detergent close or above the critical micelle concentration (CMC) | Although lower concentrations of detergent only generate a wavy noise floor (Fig. 4g and h), concentrations close or above CMC will generate signal that resembles actual landing events in ratiometric mode (Fig. 4i). These events originate from detergent micelles and, depending on their size, might even be seen as big particles in native mode (Fig. 4c).<br><br>Be aware that the CMC of detergents varies not only due to concentration, but also due to different buffer conditions (e.g. salt) depending on the detergent's nature. |
| Glass surface has been coated | Check if coating material has been applied properly. Not uniformly distributed coating material might be visible as huge dirt particles in native mode (Fig. 4c). Residual coating material in ratiometric mode might resemble actual landing events depending on the nature of the material (resembling Fig. 4i).<br><br>Optimise the coating by lowering the concentrations of coating material and test different coating conditions e.g. shorten the incubation time or perform additional washing steps. |

### Ratiometric shows strong signal and no single events detected after loading the sample

| | |
|---|---|
| Detergent in the sample buffer | If the sample buffer contains high concentrations of detergent, noise produced by this detergent might partially or totally mask landing events of the molecules of interest (Fig. 4j). |

| | |
|---|---|
| Sample concentration is too high | If there is no detergent in the sample, very likely the sample concentration too high. Particles landing very close to each other will not be distinguished as single events (Fig. 4j), and thus the software will not detect them. Be aware: The higher the concentration, the closer the particles, the fewer events will be detected by the analysis software.<br><br>Dilute sample with a higher dilution factor (e.g. 100 times) prior to loading in order to obtain a concentration where single protein events can be detected (Fig. 4k). |

## Point-spread function (PSF) of landing events is deformed

| | |
|---|---|
| Shape of PSF is not symmetric and round | The PSFs of landing events indicate whether the sample carrier is in correct focus. Asymmetric, egg-shaped PSFs most likely mean the glass surface is close to focus, but not in focus. This effect may not be as obvious for smaller particles as it is for larger particles (e.g. particle size > 200 kDa).<br><br>Ensure that you are in the correct focal plane by setting the sharpness value to its highest global maximum.<br><br>Important to note, that particles larger than 150 – 200 nm in size may show deformed PSFs, because they are larger than the upper detection limit of the instrument. |
| Shape of PSF becomes asymmetric during the measurement | Most likely the glass surface moves out of focus during the recording time. Check if the focus is locked. Other possible causes may be:<br><br>• *Drastic temperature change:*<br>Make sure all buffers and samples are at room temperature prior to loading.<br>• *Relaxation of immersion oil:*<br>After mounting a new coverslip on the stage or moving the sample carrier to a new gasket well, make sure the immersion oil had enough time to settle.<br>• *Not enough immersion oil:*<br>Ensure there is enough immersion oil. Therefore, check the value of the global maximum in the sharpness monitor.<br>• *Gasket is leaking:*<br>Check whether the buffer drop in the gasket well is still intact and the liquid did not vanish due to |

| | |
|---|---|
| | leakage. Make sure the silicon gaskets are properly sealed next time. |

### Many events detected are white spots with positive ratiometric contrast

| | |
|---|---|
| Focal plane is not correct | Make sure the focus is set such, that the sharpness monitor is at the highest global maximum peak. Focus set to a lower, local maximum causes destructive interference, which results in inverted ratiometric contrasts. |
| Particles do not bind well to glass surface | There are various reasons why particles are not adhering well to bare glass. Net charge of particles plays an important role. Optimise buffer conditions by testing different pH's and/or salt concentrations.<br><br>If changing the buffer conditions does not improve binding, the glass surface can be coated with a material to change its properties (e.g. negatively charged nucleic acids adhere better to positively charged surfaces generated by coating the coverslip with APTES or poly-lysine). |
| Glass surface is close to saturation | When the glass surface is crowded with proteins, freshly landing particles might not adhere to the glass itself, but rather collide with a protein already attached to the surface. Consequently, the new protein particle may not land, or the protein already bound to the surface may detach. Both incidences generate a void/ unbinding event.<br><br>Dilute sample to reduce particle concentration or reduce movie recording time. |

### Less events detected than expected for this sample concentration

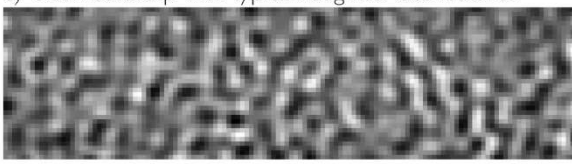
| | |
|---|---|
| Sample is not mixed properly | After adding the sample to the buffer, mix well by pipetting up and down three to four times. We generally recommend using a pipette with a larger volume (10-20 µl) comparable to the volume of the drop used for focussing. |
| Glycerol in the buffer | Due to higher viscosity of buffers containing glycerol, it is recommended to mix the sample very well to achieve sufficient counts. |

| | |
|---|---|
| Particles may form higher order assemblies | Consider that the formation of higher order assemblies reduces the effective particle concentration. In this case increase particle concentration. |
| Sample is aggregated | Detecting only a few events might indicate aggregation of your sample. Optimise buffer conditions to decrease aggregation propensity (e.g. pH, salt, additives, …). |

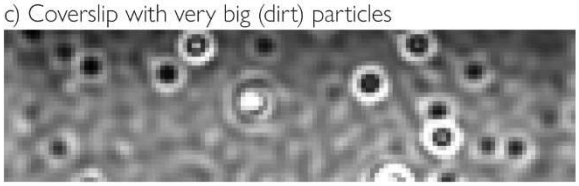
### Native mode (glass surface)

a) No liquid column - camera oversaturated

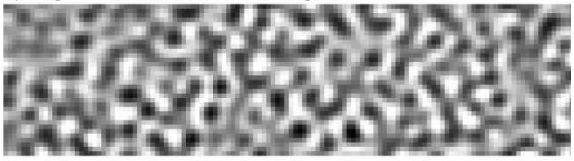
b) Clean coverslip with typical rough surface features



c) Coverslip with very big (dirt) particles



d) High concentration of detergent



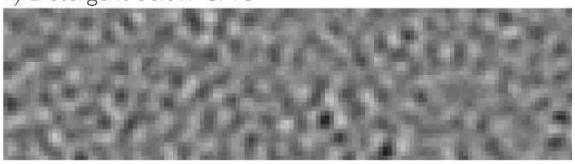
### Ratiometric mode

e) No liquid column - camera oversaturated

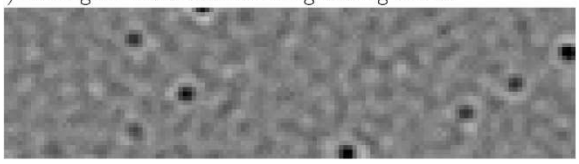
f) Clean buffer/ no background

g) Low concentration of detergent

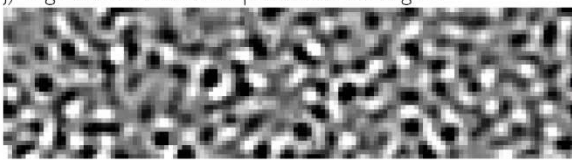
h) Detergent below CMC



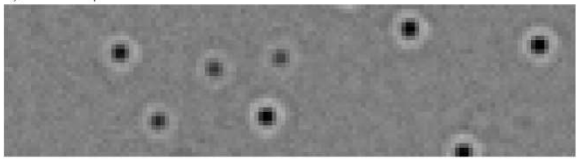
i) Detergent micelles resembling landing events



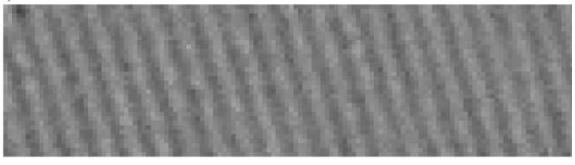
j) High concentration of protein or detergent



k) Good particle concentration



l) Interference waves



m) Protein aggregate

n) Rolling or wobbling particles

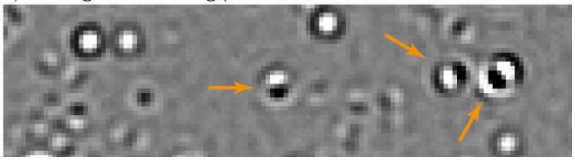


Figure 3: Representative images in native (glass surface) and ratiometric (rolling background correction) mode to exemplify common challenges in mass photometry.

| | |
|---|---|
| Pre-diluted sample has lower concentration than expected | Particles could have been depleted from the stock solution due to adhesion to the Eppendorf tube. Try to bring the protein in solution again by vortexing or mixing with the pipette. In case this does not help, prepare a fresh pre-dilution of the sample. We also generally recommend protein low binding tubes. |
| Particles already depleted from the solution before starting the recording | Follow the recommended loading procedure: First load buffer blank and adjust focus, then add sample to the buffer droplet, mix well and quickly close the lid. Start the recording immediately after loading the sample. |
| Particles might not adhere to bare glass | Consider coating the glass surface to reverse charge effects (see section "Particles do not bind well to glass surface"). |
| Expected large particles are not detected well | Larger particles (>1 MDa) in the sample might be underrepresented in recordings using the default field of view (FOV). In order to reduce the tendency for larger particles in a mix not being detected well, it is advisable to record movies in Acquire$^{MP}$ using a larger FOV. Also keep in mind that the abundance of larger particles might be lower than for smaller particles in solution. |

## Disturbances appear in the field of view (FOV) in ratiometric mode

| | |
|---|---|
| Uniform straight waves appear in the FOV | Interference waves (Fig. 4l) are caused by back reflections of the laser from the surface of the buffer droplet. They typically appear when not enough liquid volume is used. Thus, we recommend using a final volume of >15 µl. Provided that sufficient volume was applied, and interference waves are still present, moving the coverslip slightly can do the trick. |
| Big tornado-like disturbances traverse FOV | The sample contains aggregates. Very big particle assemblies can be observed when floating in close vicinity to the glass surface even if they do not land (Fig. 4m).<br><br>Filter the sample and test if there are still sufficient landing events detectable. Otherwise find buffer conditions that reduce the aggregation propensity of the sample. Affected frames in the recording can be excluded in the analysis software Discover$^{MP}$. |

| | |
|---|---|
| Wave-like disturbances appear and disappear | Huge aggregates floating near the FOV (not yet visible in the FOV) generate these wave-like disturbances. They disappear when aggregates float further away from the FOV.<br><br>Filter the sample and test if there are still sufficient landing events detectable. Otherwise find buffer conditions that reduce the aggregation propensity of the sample. Affected frames in the recording can be excluded in the analysis software. |

### Measured particles do not show the expected mass

| | |
|---|---|
| Calibration and/ or sample measurement were not in focus | Repeat the calibration measurement and double check its accuracy with standard proteins e.g. BSA. Repeat the measurement of your sample in triplicate. |
| Calibration was performed under different conditions | Various factors (e.g. buffer composition, temperature) have an impact on the ratiometric contrast. It is not recommended to use a calibration from a different day. Make sure buffer composition matches for calibration standard and sample, especially when using high concentrations of glycerol (> 5%), which has an impact on the refractive index of the solution. |
| Calibration was performed with particles of different refractive index | Different biological material (e.g. proteins, DNA, RNA, lipid micelles) possess different refractive indices, which has an impact on the ratiometric contrast. Use alike biomolecules for calibration and sample. |
| Measured mass still differs from expected mass | Repeat the measurement of your sample in triplicate.<br><br>If mass continuously differs from expected mass, consider other factors e.g. binding of proteins, DNA, RNA or glycosylation. |

### Many small events detected, though the sample should contain larger assemblies

| | |
|---|---|
| Big particles wobble | Very large particles (> 1 MDa) sticking to the glass surface are sometimes continuously visible. Due to their enormous size and wobbling movement, the rolling background correction is not able to compensate, and as a consequence these so-called wobblers tend to generate alternating negative and positive ratiometric contrast (Fig. 4n). This, in turn, can generate signal that mimics smaller binding and unbinding events.<br><br>Ignore small events if not relevant for the study. |

| | |
|---|---|
| Big particles loose parts or disassemble | Consider that big particles might lose subunits in the process of landing on the glass surface. At measuring concentration, big protein assemblies could also disassemble.<br><br>To stabilise bigger assemblies, we recommend cross-linking. |

# Care and maintenance

## 1. Cleaning

- The objective must be regularly cleaned to avoid immersion oil spilling over the whole objective. This cleaning should be performed using lens cleaning tissue and isopropanol. Using a clean lens cleaning tissue, remove as much oil as possible from around the front lens of the objective. Dampen a fresh lens cleaning tissue with isopropanol and use this to thoroughly clean the residual oil from the objective.

- Unless otherwise required by local procedures, there is no need to clean the Two$^{MP}$ instrument more than once a week.
- Dampen a sheet of absorbent tissue with purified water. Wipe all exterior surfaces of the instrument. The absorbent tissue must be moist enough to ensure water is in contact with the instrument. Use further sheets of tissue as required.
- Use absorbent tissue dampened with water to wipe the surfaces under the sample lid (but avoid touching the microscope objective).
- The working area around the instrument can be cleaned with standard laboratory materials and methods.

# Key Specifications[1]

## Refeyn Two$^{MP}$ Mass Photometer

| | |
|---|---|
| Mass range | 30 kDa – 5 MDa |
| Resolution (FWHM) | 25 kDa @ 66 kDa |
| | 60 kDa @ 660 kDa |
| Mass precision | ± 2% |
| Mass error | ± 5% (single measurement) |
| Concentration range | 100 pM – 100 nM |
| Sensitivity: | << 1 ng of protein |
| Wavelength: | 488 nm |
| Field of view: | 4 x 11 µm (@ 500 Hz) up to |
| | 12 x 17 µm (@ 135 Hz) |
| Pixel size: | 12 nm |

[1] All specifications subject to meeting the installation requirements

# General information

## 1. Refeyn Ltd Service and Support

Refeyn Ltd offer service and technical support for Two$^{MP}$ Systems:

Applications and Service Support

Refeyn Ltd
1 Electric Avenue
Oxford
OX2 0BY

Email: support@refeyn.com
Call: +44 (0) 1865 203 956

## 2. Disposal and recycling

- Before disposing of this equipment, you must:
    - Check with the appropriate local organisation to obtain advice on local rules and regulations about disposal and recycling.
    - Contact Refeyn Ltd before disposal begins.

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