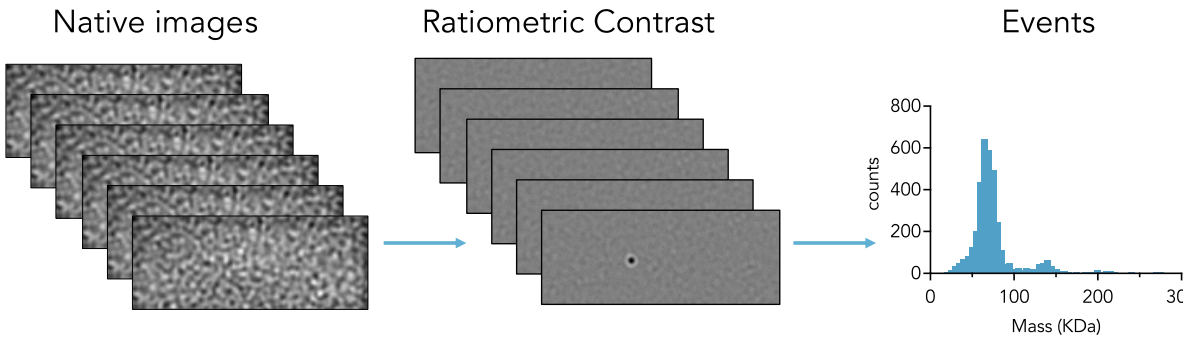
Refeyn TwoMP: CMI Getting Started Guide to Mass Photometry

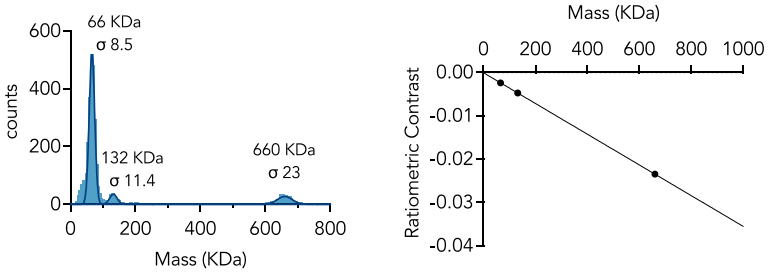
Sample Preparation and Experimental Design | Getting Started | Data Collection | Data Analysis | Shutdown | Data Management

Introduction

Mass Photometry (MP) measures light scattering of single particles as they adsorb onto a glass microscope slide, and is used to rapidly determine mass, oligomeric state, and heterogeneity of a wide range of macromolecules and their complexes in solution, under equilibrium conditions and without the need for labels. Mass photometry can be used to determine masses of diverse macromolecules from 30 KDa to 5 MDa, including proteins, nucleic acids, lipids, and small viruses, such as AAV. Built on the principles of interferometric scattering microscopy, Mass photometry measures the interference between the light scattered by a molecule in contact with the measurement surface and the light reflected by the surface. Data is collected as a short movie (1 minute) and then processed using ratiometric imaging, allowing weakly scattering single macromolecules to be distinguished from the high background signal. For scattering particles greater than 30 KDa and smaller than 100 nm, the Ratiometric Contrast of each particle (or point spread function representing each molecule that touches the surface) has a signal intensity that is directly proportional to mass and refractive index. To correlate ratiometric contrast to mass, the Refeyn TwoMP instrument is calibrated using molecular standards of known mass with refractive index similar to the analyte of interest (e.g., protein, RNA, *or* DNA). Calculated masses of single particles are plotted as a histogram and can be counted or fit to Gaussian distribution.



Calibration with Protein Standards



Instrument Overview

The CMI has a **Refeyn TwoMP** mass photometry instrument from Refeyn, Ltd, with Accurion vibration-isolation bench. Data collection is performed using Refeyn AcquireMP software and data analysis is performed using Refeyn DiscoverMP software.

Applications

- Mass range: 30 kDa to 5000 kDa
    - Proteins/complexes
    - Nucleotides of up to ~5000 bp
    - Empty vs Full ratio for AAV and small viruses
- Oligomeric state and heterogeneity

Key Features

- Small amount of sample required: typically, a few µl at ~100 nM, measured at 10-20 nM
- Single particle counting
- Fast and easy to use

Limitations

- Limited mass and concentration range
- Difficult/impossible to work with membrane proteins and detergents
- No good standards for protein conjugates and mixed-typed complexes

Required Supplies

Provided by the CMI:

- Reusable Sample Well Cassettes (6-well silicone gaskets)
- Immersion oil: Zeiss Immersol 518 F
- Whatman® lens cleaning tissue, Grade 105
- Isopropanol and ultra-pure water, for cleaning
- No. 1.5H high precision glass coverslips (24x50 mm), Thorlabs CG15KH (or Refeyn Pre-cleaned slides)
- 1000x Protein Calibration Mix (10 µl aliquots of 3 µM Thyroglobulin, 10 µM BSA or beta-amylase in PBS with 5% glycerol)
    - Sigma beta-amylase from sweet potato (A8781)
    - Sigma Bovine Thyroglobulin (609310)
    - ThermoFisher Bovine Serum Albumin Standard (23209)

Purchased by the User:

- Optional Pre-cleaned Sample slides: Refeyn Sample Carrier Slides
    - Academic users may order this item in PPMS.
- Optional Supplies (varies by experiment):
    - Poly-lysine: Sigma P4832 (for measuring nucleic acids)
    - Calibration standards for non-protein samples:
        - Invitrogen Low DNA Mass Ladder: Invitrogen 10068013
        - Millennium RNA Marker: ThermoFisher AM7150
        - *Empty AAVs, high mass protein standard*

Sample Preparation and Experimental Design

Assay Buffers

- Buffers should be filtered to remove dust and particulates which will scatter light.
- Mass photometry is compatible with a range of buffers, but should avoid scattering particles when possible, including carrier proteins and detergents.
- Detergents will cause background and at concentrations above the CMC, detergent micelles will scatter light. This makes measurement of membrane proteins very difficult.
    - Use the lowest concentration of detergent possible, preferably below the CMC.
    - Under limited circumstances, membrane proteins in detergent have been analyzed by mass photometry, but scattering of the empty micelles may complicate interpretation.

Samples

- The ideal concentration range for sample measurement is 10-20 nM. Samples should be prepared at 100-200 nM for best results, as you will typically perform an ~8-10x dilution when mixing your sample into the buffer droplet.
    - The Concentration Calculator in the AcquireMP Tools menu can help with this.
- You may need to try multiple concentrations to find the optimal conditions for your protein.
    - If the concentration is too high, peaks will broaden, and particles may not be counted if they land too close to each other.
    - If the concentration is too low, you may not have enough events to fit a Gaussian curve and oligomers may dissociate.
- Calibration standards should be measured in sample assay buffer.

Calibration Standards

- Calibrate mass about every 1 hour.
    - Calibrate in assay/sample buffer, as the refractive index of the solvent impacts the ratiometric contrast.
    - Mass standards must have a similar refractive index to your sample particles.
    - Use protein standards for protein samples, a DNA standard or ladder for DNA samples, etc.
- The CMI provides two different 1000x protein calibration mixes stored in 10 µl aliquots in the -20˚C freezer below the instrument. Perform two 1:10 dilutions of the calibration mix with your buffer. The first dilution (1:10) can be stored at 8˚C for ~7 days. The second dilution (1:100) is the working stock which you will dilute into the buffer droplet.
    - BSA-TG 1000x Protein Calibration Mix: 10 µl aliquots of 3 µM Thyroglobulin 10 µM BSA in PBS and 5% glycerol
    - BAM-TG 1000x Protein Calibration Mix: 10 µl aliquots of 3 µM Thyroglobulin 10 µM beta-amylase in PBS and 5% glycerol
- For, specific instructions on preparing DNA and RNA calibrants see the CMI's Guide to Measuring Nucleic Acids.

Experimental Design Tips

- PROTECT THE OBJECTIVE, by cleaning carefully and regularly.
- You will find focus using a buffer droplet of 15-18 µl and then mix your sample in the droplet.

  - You may need to optimize the buffer and the dilution factor.
  - Filter buffers and samples to remove particulates and aggregates that may scatter.
- If measuring samples with mass close to the lower limit of 30 kDa, position the objective slightly off center to limit interference from back reflection.
- DNA and RNA will not adhere to glass. Carrier slides must be coated with poly-L-lysine.
- Large proteins (>1 MDa) may not adhere to glass due to their size). This may be remediated with poly-L-lysine.
- In normal measurement mode (used for proteins and nucleic acids), the Refeyn TwoMP has three pre-defined image sizes. They are small, regular, and large. Choose your image size according to the expected sample mass. Always calibrate with the same image size with which you plan to collect data.
  - Regular is the default image size and is recommended for most samples with particle masses below 1 MDa.
  - Large is recommended for samples with high particle mass (>1 MDa), as it allows for the detection of more events in a single frame. While increasing the image size improves statistics, it also decreases the signal-to-noise ratio, thereby decreasing mass sensitivity for smaller particles.
  - The recommended maximum event counts for each image size are as follows:
    - Small: 3,000 events
    - Regular: 6,000 events
    - Large: 30,000 events

General Care and Maintenance

- The Refeyn Two^{MP} instrument will generally be powered off.
  - To avoid overheating, the instrument ***should not be powered on for more than 12 continuous hours.***
- Allow 45-60 min to equilibrate the temperature after turning on the power. This is a good time to prepare samples.
- Keep the lid to the optics compartment (located on the top of the instrument) closed to minimize dust in the instrument.
- **The objective must be cleaned thoroughly between coverslips, or any time you pause for more than 30 minutes, and very thoroughly after use.** DO NOT LEAVE IMMERSION OIL ON THE OBJECTIVE OR YOU WILL DAMAGE IT PERMANANTLY!

Getting Started

Resources

Additional resources are available at the instrument, including: Refeyn TwoMP Mass Photometer User Manual, Refeyn AcquireMP User manual, and Refeyn DiscoverMP User Manual for additional information about data collection and analysis, as well as Refeyn Technical notes, Application notes and procedures for measuring AAVs and nucleic acids.

Startup

1. Book time on the PPMS calendar before you start.
2. Login to the computer using your PPMS credentials (HMS ID and password).
3. Warm Up Instrument
   a. Before turning on instrument power, open the optics compartment and examine the objective. If there are any visible marks, clean with a Whatman lens tissue and isopropanol.
   b. Use the power strip located under the computer monitor to turn on the instrument power. ***Allow at least 45 minutes*** for instrument temperature to stabilize prior to data collection.
   c. Open the Refeyn AcquireMP software.
      i) AcquireMP should be on during warm up to allow the objective to warm up.
      ii) The software will not open if the instrument is not turned on.
4. Turn on the vibration-isolation unit (located below the Refeyn TwoMP) and press the Isolation button. The light will blink repeatedly, then remain on.
5. Create a new project or open an existing project.
   a. Select "Open Project Folder."
   b. Navigate to your data folder.
      i) You will need to create a new folder if this is your first time using the instrument.
   c. Select an existing project folder, or right-click and create a new folder for your project.
      i) Provide a project folder name.
      ii) Click "Select Folder."

Preparing and Mounting Coverslips

1. Use Alignment Tool to setup coverslip.
   a. Using soft-tipped tweezers, place the gasket in the small rectangular indent
   b. Place a ***clean coverslip*** on top of the gasket in the larger rectangle. (See below if not using pre-cleaned coverslip.)
   c. Press gently on the coverslip between the wells with tweezers to adhere the gasket to the coverslip. Do not put pressure directly on the wells, as this may damage the coverslip. The bottom is now face-up.
2. Place a small drop of Zeiss Immersol immersion oil on the objective lens. Wipe any excess oil with a Whatman lens tissue.
3. Flip and mount the prepared coverslip so that the red autofocus laser is roughly centered. Secure the coverslip with the provided magnets.

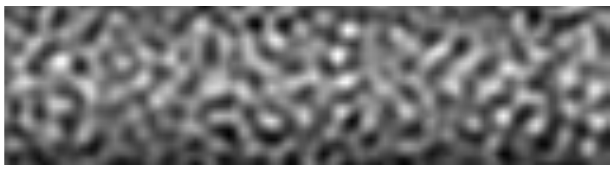
Clean Coverslip (required for standard No. 1.5H high precision glass cover slides provided by CMI).

- Hold a coverslip by a corner with soft-tipped tweezers or by hand, wearing gloves.
- Using the provided squirt bottles, rinse the coverslip sequentially with MilliQ water, then isopropanol, MilliQ water, isopropanol and MilliQ water.
- Dry the coverslip with filtered air from the valve directly to the left of the MP. Hold the coverslip from the bottom and rinse/blow downwards to ensure that residual particulate from tweezer tips or gloves are not swept over the coverslip surface.
- When working with nucleic acids, treat the coverslip surface with poly-L-lysine to increase adsorption. See CMI Refeyn TwoMP Guide to Measuring Nucleic Acids.

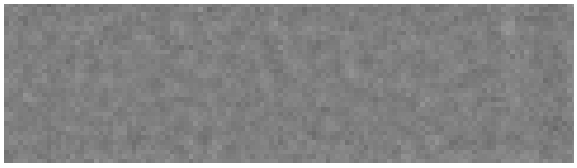
Data Collection

The **first sample you measure** should be a calibration standard, appropriate to your experiment type (measured in your sample buffer and containing calibrants with refractive index similar to your sample).

1. Find Focus.
   a. Check and adjust the image size (located in the tool bar) according to your estimated sample mass. **Most protein samples will be measured with the Regular image size**.
   b. Leaving optical compartment lid open, use the lateral control arrows at 50x speed to move the stage so the red laser is somewhat centered to the first well you plan to use.
      i) Choose gasket well and click "set position."
   c. **Add 15-18 µl of buffer to the well**. Do not let your pipet tip touch the carrier slide.
   d. Lower the lid of the optical compartment and click "droplet dilution" to find focus. The focus settings will lock automatically.
   e. AcquireMP will detect air bubbles in the immersion oil and dirt on the slide, but you may also want to manually examine key quality parameters.
      i) Select "Manual Focus" from top Menu.
         (1) Examine the auto-focus ring. It should be a thin, clean outline with no interruptions.
            (a) Interruptions in the autofocus ring indicate bubbles in the immersion oil.
            (b) Use the lateral control to move the stage and/or lift the carrier slide slightly to move the bubbles away from the ring reflection.
         (2) Examine quality metrics.
            (a) Quality metrics will turn orange if they are outside the optimal range.
            (b) Signal should be less than 0.06 if there is no detergent in your buffer.
            (c) Sharpness should be between 4.5-6.5%
      ii) Switch between the native and ratiometric views to assess image quality. A clean aqueous buffer and slide should look similar to the image below. If there are particularly bright or dark spots that could be dirt or dust, use the lateral control arrows to adjust the image on 1x speed. With only small adjustments, you will not need to refocus.



Native image of clean slide and buffer



Ratiometric image of clean slide and buffer

2. Collect Sample Data (first sample is typically a calibration standard).
   a. Inject 2 µl of diluted sample into the buffer droplet used to find focus. Mix thoroughly to ensure even sample distribution.
   b. Click "record". The default video length is 60 seconds.
   c. When the video is complete, you will be prompted to name and save the file.

   d. Data analysis is enable by default for all movies, generating .mpr files for faster data analysis in DiscoverMP analysis software.
   e. The total counts for your video should be between 1500 and 3000 (visible in DiscoverMP analysis software). If this is not the case, adjust the concentration and measure the standards again. Data will be flagged if it exceeds the recommended total counts.
3. Continue data collection (or Go to Data Analysis).
   a. Click "Next well" to automatically move to the next well OR unlock the focus and use lateral control arrows to manually select the well.
   b. Repeat Find Focus and Collect Sample Data.
   c. You can apply an exported calibration curve to the analysis preview by clicking on the three dots in the upper righthand corner and selecting "Load calibration."
   d. Each carrier slide can accommodate six sample measurements.
      i) When you have used all six wells, set up the next carrier slide using the alignment tool and continue sample measurements.
   e. Clean objective and replace oil between every slide.
   f. Calibration standards do not need to be measured for each carrier slide. Plan to remeasure calibrants every hour.
4. Clean the objective when data collection is complete or if pausing for more than 30 minutes (see cleaning protocol in the Shutdown section below).

Buffer-Free Focus for dilute samples

Buffer-free focus is designed for use with samples at low concentrations that cannot be diluted into the buffer droplet (i.e. a sample that is already between 10-20 nM).

1. Use the lateral control arrows to center a well over the objective.
2. Click "Buffer-free." The software will begin focusing. This will take 2-3 minutes.
3. When prompted, pipet 15 µl of your sample to the well.
4. (Optional) Select both "Start Recording Automatically" and "Automatically detect when sample added" to begin data collection as soon as lid is closed. OR click after sample is added.

Data Analysis

1. Open the Refeyn DiscoverMP software.
2. Upload your data files.
   a. Click the "+open" button in the Files panel. Select all files you would like to analyze.
      i) Data collected in the current version of AcquireMP will be analyzed .mpr files and will load quickly.
      ii) Older files (.mp) will take a few seconds to upload. Each file will have a file upload progress bar. *Do not record a new movie in the data acquisition software while files are uploading in the analysis software, as the software may crash*.
3. Basic navigation
   a. To select a file, double click on the file name once it has loaded completely.

b. Adjust the Contrast range to focus on a particular region of the data.
   i) The acquisition software measures both binding and unbinding events.
   ii) Set the upper contrast to 0, to hide unbinding events.
   iii) Shorten the lower contrast limit to focus on a particular region of the data, and to hide rare aggregates. (-0.04 is good for protein standards with thyroglobulin)
c. Play the movie to check for any events that may be dirt or large aggregates.
   i) You can exclude events from being counted by clicking and dragging on the movie to select the problem area. Events in the selected area will be excluded for the entirety of the video.
d. Use the info tab to assess if the number of counts is within the optimal range for your sample.

4. Create a calibration curve.
   a. Double click on the file with your mass calibration standards (collected within ~1 hour of your samples and having similar refractive index).
   b. Fit Gaussian Peaks
      i) Auto-peak calling is enable.
         (1) You may wish to delete some (or all) of the auto-detected peaks.
      ii) To create a Gaussian fit, double-click near each population or click and drag over a peak.
   c. In Mass Calibration Section (bottom left), click "Create"
      i) Choose Mass Calibration to calibrate in KDa (or select a nucleic acid calibration)
      ii) Select the file(s) to be used for calibration (the first file is auto-selected)
      iii) Select your calibrants from the calibrant pull-down (or create your own).
         (1) Use the ratiometric contrast to identify the correct standard for each data point (larger proteins will more negative contrast value).
         (2) The R^2 value for the CMIs 1000x Protein Calibration Mix is typically > 0.9995.
      iv) Saving the calibration file will automatically add it to mass calibration panel. You can rename individual calibration curves by right-clicking on the file in the calibration panel.
      v) Export your calibration curve to add it to the project folder.
5. Apply calibration curves to other data files.
   a. Double click the file of interest. Adjust the contrast limits and selected peaks as necessary.
   b. Double click the calibration curve you would like to apply.
   c. The x-axis will change from ratiometric contrast to mass in Daltons.
6. Create Figures in the DiscoverMP software.
   a. Click the "Figures" tab and select Add Figure
   b. Choose 2D Histogram, 3D Histogram, or Vertical Histogram Series.
   c. Drag the measurement file(s) from the list on the left and drop it in the figure set-up box.
   d. Deselect any aspects of the data (e.g., Gaussian curves for certain peaks) you do not wish to include.
   e. Use the formatting options to change the graph title, axes, units, and style.
7. Create Tables in DiscoverMP software.
   a. Tables of relevant mass photometry parameters can be generated for any/all data files.
      i) CSV format for large numbers of samples
      ii) PDF format for up to 24 samples

   b. Choose parameters to include in data table.
8. Save and export the data.
   a. Save the workspace to preserve your analysis. This will automatically export all calibration curves as mass calibration (.mc) files. It will also re-save mass photometry results files with all user changes, including peak selection and applied calibrations curve in .mpr format.
   b. Right click individual files or multiple selected files to export the data in the following formats:
      i) A list of events in CSV format.
      ii) A DiscoverMP-generated report in PDF, CSV, or JSON formats.
      iii) The movie in MP4 format.
      iv) Scores, or data on brightness, motion, sharpness, saturation, and signal, can be exported as an .h5 file.
      v) Raw frames in .h5 format.

Shutdown

Note on Cleaning the Objective:

- **Residual oil will burn onto the objective and permanently damage it**.
- The objective is not covered under the service agreement for the instrument. Clean it well!

1. Close the acquisition software. The stage will immediately return to the center position.
   a. File > Quit
   b. Failure to properly close the acquisition software can lead to difficulties upon startup for the next user.
2. **Turn off the instrument power and vibration isolation bench.**
   a. Instrument should not be on for more than 12 hours and MUST BE SHUT DOWN EVERY DAY.
   b. Do not leave the instrument powered on unless the next user is standing next to you.
3. Remove the coverslip from the instrument and return the magnets to their original position.
   a. Coverslips should be discarded in a sharps container.
   b. Place used gaskets in the designated falcon tube (filled with isopropanol) on the shelf above the instrument. These will be cleaned for reuse by CMI staff.
4. **Clean the objective with isopropanol and Whatman lens tissue.**
   a. It is vital that the objective is thoroughly cleaned after each use and when pausing for > 30 min.
   b. Remove excess immersion oil with a dry sheet of the Whatman lens tissue (located by instrument).
   c. Fold a new sheet of lens tissue and wet with isopropanol.
   d. Using soft-tipped tweezers or your fingers to rub as much of the Immersol off the objective with the lens tissue.
   e. Repeat this step at least four times, until the objective is shiny and there is no visible oil left.
   f. Close the optics compartment to avoid dust accumulation.
5. Dispose of Isopropanol waste in the labeled bottle in the Hazardous Waste Collection bin.
6. Logoff from PPMS!

Data Management

Technology	Mass Photometry		
Instrument	Refeyn TwoMP		
Recommended Repository	Generalist Repository		

Data Collection Software

Current Version	AcquireMP, version 2024 R1.1		
Data Files (Type, ~size)	Processed data file (regular)	.mpr	~30 MB/measurement
	Processed data file (large)	.mpr	~125 MB/measurement

Data Analysis Software

Current Version	DiscoverMP, version 2024 R1		
Data Files (Type, ~size)	processed results file (regular)	.mpr	~30 MB/measurement
Readable Exports	processed results file (large)	.mpr	~125 MB/measurement
	mass calibration	.mc	~1 KB/measurement
	workspace file	.dmp	~500 bytes/experiment
	histogram	.png	~200 KB/measurement
	events file	.csv	~0.5-2 MB/measurement

Book time and Report Problems through the PPMS system: https://ppms.us/hms-cmi

- rates are based on *booked and real-time usage.*

Contact cmi@hms.harvard.edu with questions.

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