LMU MÜNCHEN
DEPARTMENT OF CELLULAR PHYSIOLOGY
BIOMEDICAL CENTER MUNICH – BMC
VEIGEL LAB

Lab Course V2b

Sizing Proteins with Mass Photometry

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1. Introduction

To understand a biological process at a fundamental level, it is often necessary to dissect its mechanism down to the molecular components driving it. Triggered by specific cellular signals, biomolecules (such as proteins or nucleic acids) dynamically interact with each other to form larger scale assemblies, and later disassemble again, or reassemble in a different way.

Atomic structures of biomolecular complexes provide detailed snapshots of intermediates within such pathways but represent only puzzle pieces of a greater mechanistic context. Observing the fast dynamics of biomolecular assembly in real time usually involves lower-resolution light microscopy, which provides "only" single-molecule sensitivity, but micro- to millisecond time resolution. Most single-molecule microscopy methods use fluorescence as a contrast mechanism due to its superior signal-to-background ratio. Although fluorescence has dominated the single-molecule toolkit for decades, it bears several disadvantages that limit its utility in some applications. Since most biomolecules are poorly fluorescent themselves, a fluorophore label needs to be attached, which may change the chemical properties of the biomolecule or interfere with the process of interest. Photobleaching of fluorophores limits the ultimate observation time and photon yield of a fluorophore. For very fast imaging, the fluorescence cycle time itself limits the collectable photon yield, which determines how well an object can be followed. Finally, an extrinsic label does not provide any physical information about the biomolecule itself, except for its approximate location.

These issues can be overcome by directly imaging molecules without relying on external markers. For instance, light scattering does not suffer from the above-mentioned limitations. In the recent years, technical breakthroughs in scattering-based microscopy have enabled single-molecule sensitivity. This course will introduce you to the concept of interferometric scattering (iSCAT) microscopy, how it enables single-protein sensitivity, and how it can be used to size proteins and their assemblies in solution. This recently developed application of iSCAT microscopy is called mass photometry.

1.1.Principle of iSCAT microscopy

Any particle scatters light as long as its optical properties are different from its surrounding. In the extreme case, this holds true down to single molecules. Hence, light scattering provides a general visualisation mechanism. In practice, however, there are two major obstacles that make it very difficult to detect the scattering signature of a single molecule.

Firstly, the pure scattering intensity of a particle, e.g. as it would be detected in a dark-field microscope, depends strongly on its size and optical properties. According to Rayleigh scattering theory, the detected intensity I_{det} of an individual nanoparticle on a camera is related to the square of its volume V and refractive index n_p:

$$I_{det} = I_{inc}|s|^2 \propto V^2 \left|\frac{n_p^2 - n_m^2}{n_p^2 + 2n_m^2}\right|^2 \quad (1)$$

I_{inc} is the incident light intensity used for illumination, $|s|^2$ is the scattered fraction, and n_m is the refractive index of the medium surrounding the particle. To put **Eqn. 1** into relation, 20 nm gold particles are about the smallest objects that are detectable with modern dark-field microscopes, when taking advantage of the enhanced emission of light stimulated by plasmon resonance. The scattering signal of a 5 nm protein would be about a factor 250,000 smaller than that of a 20 nm gold particle (with n_{AuNP}, 543nm ≈ 18.2, $n_{protein}$, average = 1.59, n_{water} = 1.33).
The second hurdle to detecting single molecules with light scattering is the overwhelming background scattering that any surrounding matter creates and that obscures the tiny scattering signature of a molecule.

The extreme size and refractive index dependence of the scattering intensity may be overcome by an interferometric detection scheme. An iSCAT microscope (**Fig. 1**) collects light that is reflected at the interface between a glass coverslip and an aqueous solution along with light scattered by particles near the interface. In this scenario, the detected intensity on the camera is a combination of the two interfering electric fields of reflected and scattered light:

$$I_{det} = I_{inc}(r^2 + |s|^2 + 2r|s|\cos\varphi) \quad (2)$$

r^2 is the reflected fraction of the incident light at the interface and φ the phase difference between reflected and scattered light. As explained above, the pure scattering term $|s|^2$ becomes negligibly small for weak scatterers. Hence, the iSCAT signal for weak scatterers is determined by the combination of reflected light and the interferometric signal $2r|s|\cos\varphi$, which has a linear dependence on particle volume and refractive index. Note that this scaling is beneficial for the detection of weakly scattering particles. Comparing the signals of a 20 nm gold particle and a 5 nm protein in the example above using this linear scaling yields only a 500-fold difference. This is the critical foundation for detection of single biomolecules with iSCAT microscopy.

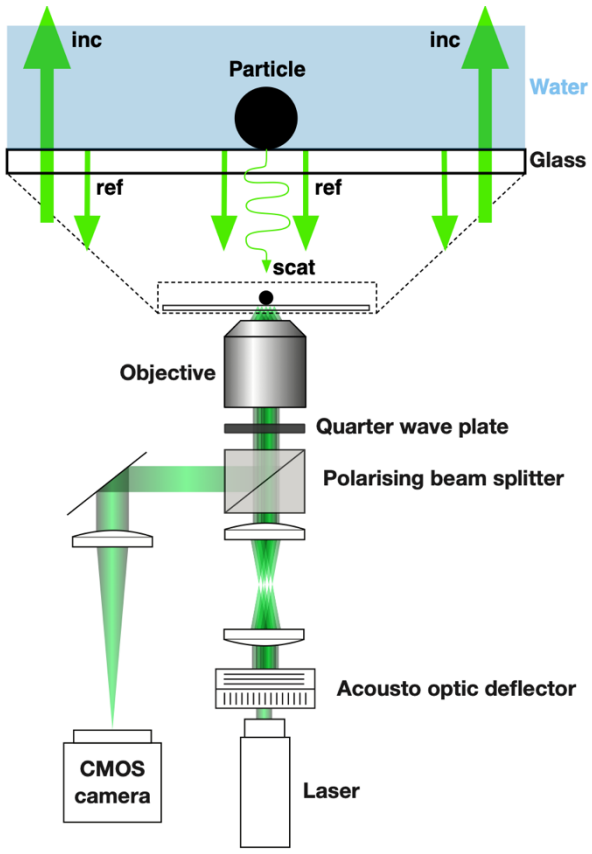

Figure 1: Schematic illustration of an iSCAT microscope.

1.2.Background suppression in iSCAT microscopy

Although interferometric scattering is more sensitive to small objects than pure scattering, unspecific scattering of larger structures obscuring a molecule's signal remains a major challenge for its detection. The non-specificity of light scattering introduces an important experimental constraint. The approximate content of a sample should be known in advance, otherwise it becomes difficult to interpret the observed signals.

For a simple aqueous solution of proteins added on a glass cover slip, however, it turns out that the only major source of background scattering is the surface roughness of the coverslip. Hence, the raw iSCAT image of a glass coverslip is dominated by a static pattern of varying dark and bright areas depicted in **Fig. 2a**.

When taking a video of the same field of view (FOV), one can take advantage of the static nature of this background using image post-processing. Light scattering signals are perfectly additive. To visualise the fine, varying details in an image one can take the first frame in a video as a pixelwise estimate of the glass roughness signal and make a pixelwise subtraction from all

subsequent frames. This will create a background suppressed video highlighting only the changing features compared to the first video frame (**Fig. 2b**).

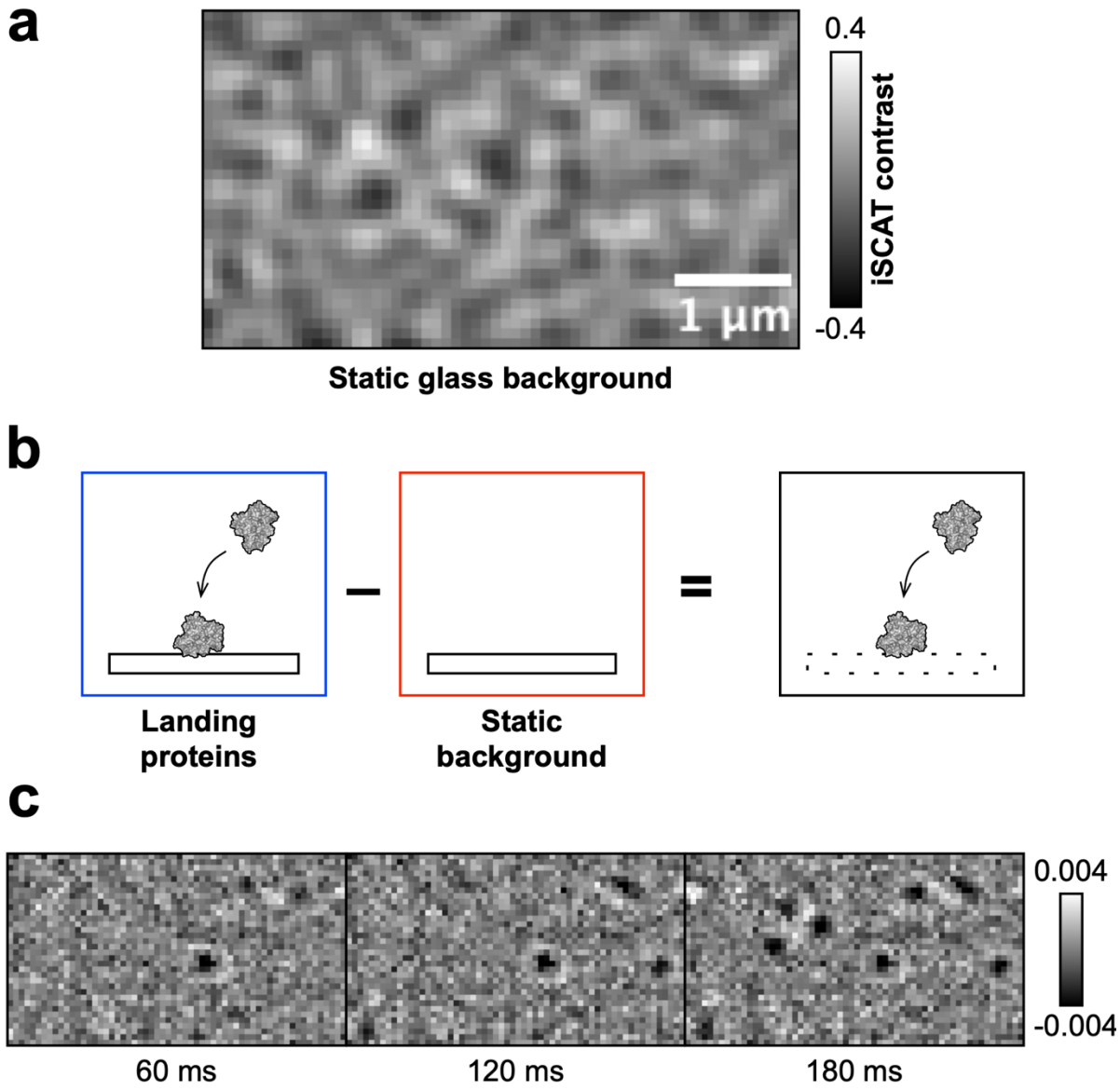

Figure 2: Background suppression strategy for imaging of single proteins with iSCAT microscopy. (a) Raw iSCAT image of a glass surface. (b) Simple background subtraction removes static features, such as the glass roughness from a video and revealing dynamic events. (c) Time lapse of the same coverslip area as above with the frame at 0 s subtracted, revealing landing protein molecules (dark spots).

The example in **Fig. 2c** shows a background suppressed time lapse revealing single proteins landing on a glass coverslip recorded on an iSCAT microscope.

1.3.Mass photometry

While label-free visualisation of biomolecule dynamics already has powerful implications on its own, the signal scaling of biomolecules in iSCAT microscopy has proven very useful for the specific application of mass determination. For the same type of biomolecule, be it a protein or nucleic acid, the specific volume and refractive index vary by only a small percentage. Therefore, the linear volume scaling makes the iSCAT signal of biomolecules also directly linear to their molecular mass *M*:

$$|s| \propto V \left| \frac{n_p^2 - n_m^2}{n_p^2 + 2n_m^2} \right| \propto M \tag{3}$$

This means that determining the amplitude of the iSCAT signal for a single molecule provides an indirect estimate of its molecular mass M. If compared to a set of standards with known molecular mass, the mass of an unknown sample can be determined.

Experimentally, a solution of biomolecules is pipetted onto a glass coverslip and their unspecific attachment to the glass surface is recorded. To retain minimal background and observe isolated molecular landing events, a slightly modified image processing scheme from the one in **Fig. 2b** is applied. The static background estimate is continuously renewed by pixel-wise calculation of

$$ratiometric\ frame_i = \frac{mean(frame_{i+1}{:}frame_{i+n})}{mean(frame_{i-n}{:}frame_{i-1})} - 1 \tag{4}$$

, where i designates the frame index, and n is an averaging window size that can be used to enhance the signal-to-noise ratio of the resulting frames. **Fig. 3** visualises the output of the image processing scheme established by **Eqn. 4**. The resulting ratiometric frames highlight landing molecules as spots that gradually fade in and out of the image, reaching their maximal contrast when i is the moment of the molecular landing event.

The maximal contrast of a molecule is then determined by fitting a model point spread function (PSF), which is often approximated by a two-dimensional Gaussian, to the spot image. The amplitude of the fitted PSF represents the molecule's iSCAT signal.

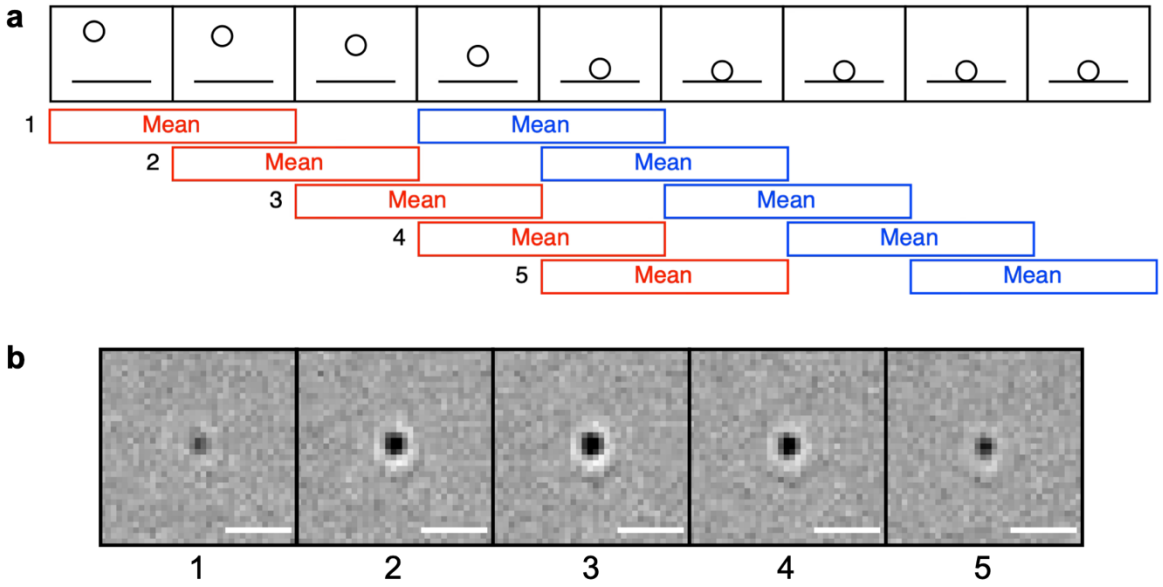

Figure 3: Image processing scheme used for mass photometry. A molecular landing event (a, top row) is highlighted using a continuous background removal strategy. The respective ratiometric frame number in b is generated by dividing the mean of the blue frames by the mean of the red frames in a. This image processing scheme makes a landing particle fade in and out of the ratiometric video. At the moment of landing (frame 3), the particle appears at its maximum contrast, which is quantified for the mass analysis.

1.4.Quantification of molecular size distributions and equilibrium constants

It is a plausible assumption that the landing events on the glass coverslip randomly sample the population of molecules in solution (disregarding differences in diffusion and glass attachment probabilities between molecule species). Hence, it is possible to get a quantitative estimate of the size distribution of proteins or nucleic acids by plotting the masses recorded for all molecular landing events in a histogram. A molecular species with a dedicated mass will be represented by a peak in the mass histogram. The relative abundance of multiple species in a sample will be represented by the relative areas under each peak. This way, it is possible to get a full picture of equilibrium states during molecular assembly, such as during homo- or hetero-oligomerisation of proteins.

For an equilibrium between two binding partners A and B, the so-called dissociation constant K_d is used as an important measure of affinity between the two species:

$$K_d = \frac{[A][B]}{[AB]} \tag{5}$$

, where [A], [B] and [AB] designate the equilibrium concentrations of the respective species. For a monomer-dimer equilibrium of the same molecule A, the relation simplifies to

$$K_d = \frac{[A]^2}{[A_2]} \quad (6).$$

In an MP measurement, the total concentration of a protein is usually known. And since the mass histogram provides a direct estimate of the relative equilibrium abundances of the two species, it is possible to calculate the dissociation constant of a complex from a single MP measurement.

2. Experimental procedure

In this course, your goal is to determine the dissociation constant of the yeast alcohol dehydrogenase dimer-tetramer equilibrium using mass photometry. For this, you will first learn how to prepare coverslips for MP. Then, you will learn how to perform an MP measurement on a commercial *Refeyn OneMP* mass photometer and record movies of a set of standard proteins as well as alcohol dehydrogenase. With the help of the analysis software *DiscoverMP*, you will generate ratiometric movies and extract iSCAT signals from all recorded molecular landing events. With this dataset, you can derive the conversion between iSCAT signal and molecular mass using the standard protein dataset. And finally, you will be able to determine the dissociation constant of alcohol dehydrogenase.

2.1.Preparation of coverslips

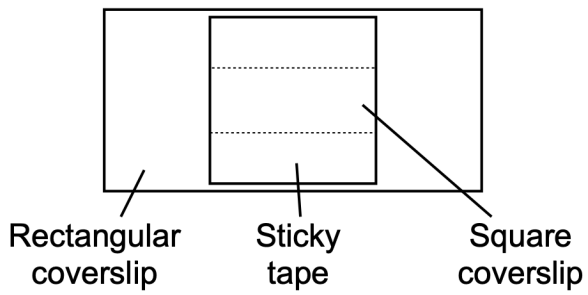

Figure 4: Scheme of an MP flow chamber

Coverslips need to be cleaned from manufacturing residues before they can be used for mass photometry. An MP chamber consists of two coverslips (a large rectangular and a smaller square one), which are attached to each other with two parallel strips of double-sided sticky tape, forming a channel used to insert and exchange solutions (**Fig. 4**). As a first step, you need to prepare these so-called flow chambers.

Coverslip cleaning protocol:

- Stack 10-14 coverslips of each type (large: Menzel-Gläser, 50 mm × 24 mm, #1.5, small: Brand, 22 mm × 22 mm) in their respective cleaning racks
  (Please note that the large coverslips have a "more homogeneous" side with less scattering background in MP. The coverslip box has a label at the bottom that says "DV2", where the "2" points towards the preferable coverslip side. You can mark the orientation of your coverslip by breaking off one corner using a diamond cutter.)
- Submerge the racks in Millipore water and put into bath sonicator for 5 min
- Exchange water for isopropanol and repeat bath sonication for 5 min
- Exchange isopropanol for Millipore water and repeat bath sonication for 5 min
- Blow-dry coverslips individually with compressed air and store in a box protected from dust

Flow chamber assembly protocol:

- Wrap a cutting board with aluminium foil
- Place a small coverslip on the foil
- Attach two parallel strips of double-sided sticky tape along the edges
- Remove the separation lining from the sticky tape
- Cut out the coverslip with a scalpel, removing the protruding ends of the sticky tape
- Attach the small coverslip with sticky tape to the "good" side of a large coverslip
- Store assembled flow chamber in storage box protected from dust
- Repeat procedure with next flow chamber
  (Please note that assembling too many flow chambers at the same time increases the chance of dust landing on the cleaned coverslips over time, which may create unwanted background scattering in MP measurements.)

2.2.Setting up the mass photometer

We will work on a commercial *Refeyn OneMP* mass photometer. Please wait for an introduction by your tutor. After a couple of measurement rounds in supervision, you can measure the remaining samples by yourself.

Instrument setup:

- Launch computer and Windows
- Switch on *OneMP* at the hardware unit
- Open the acquisition software *AcquireMP* using the desktop shortcut
- The panel depicted in **Fig. 5** should open

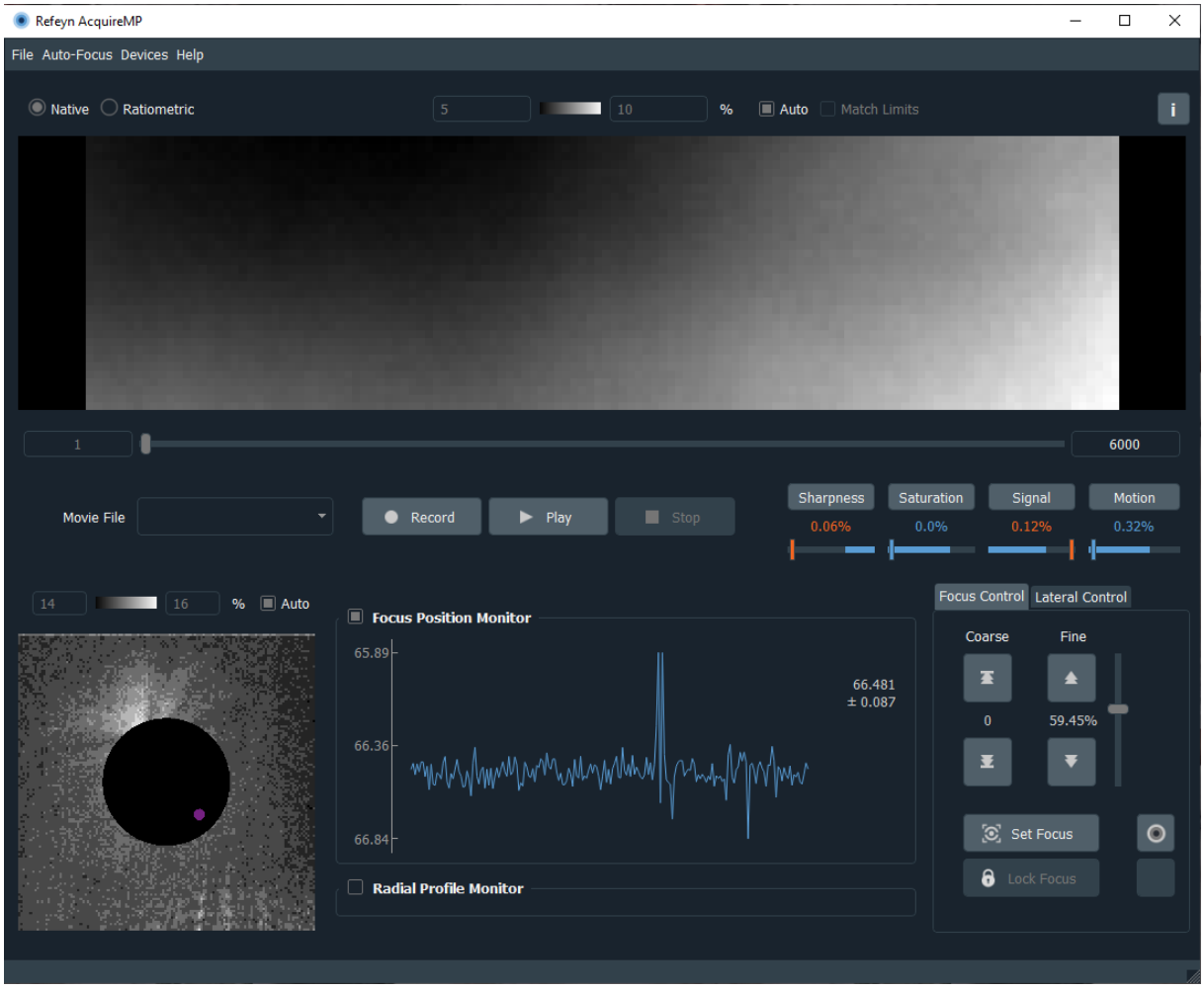

Figure 5: Main panel *AcquireMP*

- Go to *Devices* → *Acquisition camera…*
- In the appearing panel click on *Advanced…* and confirm with *Yes*

- For our measurement, adjust the camera settings to those displayed in **Fig. 6**
    - ROI width: 512; height: 138; horizontal offset: 894; vertical offset: 328
    - Frame binning: 5; Pixel binning: 4
    - Frame rate / Hz: 1000 (falls back to 999)
    - Exposure time / ms: 0.95
- Click *Save*

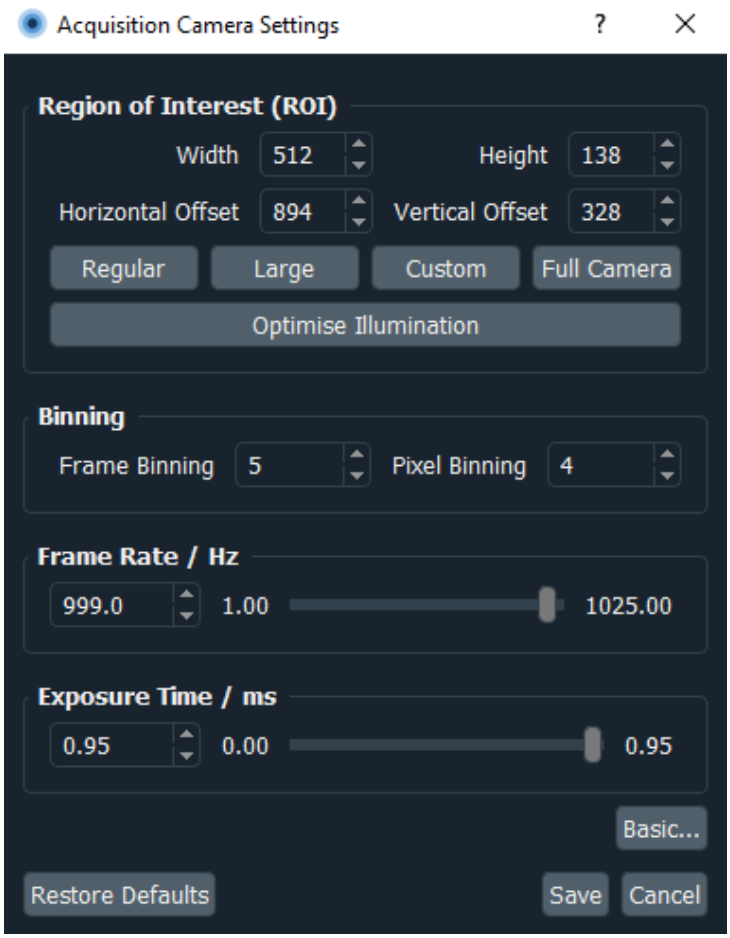

Figure 6: Acquisition camera settings

- Go to *Devices* → *AODs…*
- Make sure the settings are as displayed in **Fig. 7**
    - Amplitude (V) X: 0.21; Y: 0.70
    - Run Time (msec) X: 0.999; Y: 0.999
- Click *Save*

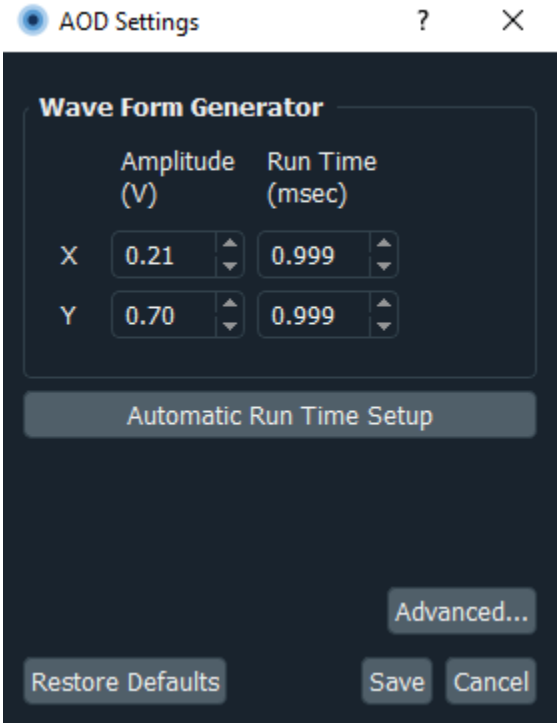

Figure 7: AOD settings

2.3.Protein preparation

To later convert the iSCAT contrast of a protein into its molecular mass, we need to measure a set of proteins with known mass, so-called mass standards. Several proteins, which are normally used as size standards for size exclusion chromatography have proven useful as MP standards. It is important to measure these standard proteins in the same buffer as the sample protein, because the refractive index of the buffering medium may vary with the buffer's salt composition (remember n_m in **Eqn. 3**). Hence, we will dilute the standard protein stocks into the working buffer. MP requires a very low sample concentration, typically in the range of 5 to 50 nM.

Working buffer

20 mM MOPS

100 mM NaCl

Adjusted to pH 7.3 with NaOH

Filtered through 0.2 µm syringe filter

Protein stocks (stored as aliquots at -80 °C)

151 µM bovine serum albumin (BSA) → Monomer mass: 66 kDa

56.4 µM apoferritin 24mer from horse spleen → 24mer mass: 479 kDa

16.5 µM thyroglobulin (TG) dimer from bovine thyroid → dimer mass: 660 kDa

68 µM alcohol dehydrogenase (ADH) tetramer from yeast

Preparation:

- Make serial dilutions of the stocks in working buffer immediately before measuring the respective sample on the mass photometer
- Required working concentration are:
    - 20 nM BSA monomer
    - 20 nM apoferritin 24 mer
    - 30 nM thyroglobulin dimer
    - 5-10 nM ADH tetramer

2.4.Performing an MP measurement

- Dilute your protein sample to the required working concentration

- Open microscope lid
- Add a drop of immersion oil onto the microscope objective
- Place a flow chamber onto the microscope stage and fill with working buffer
  (Make sure there is no air bubble in the centre of the illumination path between coverslip and objective.)
- To select a new saving folder, go to *File* → *New project folder…* and choose a suitable working folder path
  (Make sure you know at the end, which movie belongs to which sample. Taking notes might help for later.)
- Choose an acquisition frame number (box on the right side of the slider beneath the camera live preview) that corresponds to 45-60 s in real time.
  (According to our camera setting above – 1000 Hz frame rate and 5-fold temporal averaging – our effective acquisition speed is 200 frames per second)

- Close microscope lid and adjust the focus using the coarse and fine control, maximising the *Sharpness* value of the image.
  (Image sharpness is measured by calculating the root mean square deviation of the pixel values for the native camera image. There are multiple local maxima for this value at different focus positions corresponding to the multiple maxima of interference according to the phase difference cosine term in **Eqn. 2**. Make sure you find the global maximum for the microscope to achieve maximum contrast and sharpness of your images. Landing molecules should appear as sharp, dark spots in this focus position.)
- Click *Set Focus* to save this focus position and then *Lock Focus* to let the stage actively hold the saved focus position
- If the FOV contains a large scattering defect or dirt particle, find a better area using the lateral control elements. You might need to re-adjust the focus.
  (You can toggle between *Focus Control* and *Lateral Control* in the lower right of the panel.)

- Open microscope lid
- Carefully inject your protein sample into the flow chamber by placing a drop at one end of the channel and sucking it in with a tissue at the opposite end
- Close lid and wait shortly until the active focus control has reached the saved position
- Click *Record* to acquire a movie
  (You can watch your molecules land in real time by switching the acquisition camera live preview from *native* to *ratiometric*. Note that whatever you see is just for live preview. The software will always record the raw video.)
- Wait for recording to finish, choose the sample type, e.g. sample or mass standard (does not really matter what you choose though), and click *OK* for saving.
  (Clicking *Cancel* here will exit without saving the video to the drive!)

3. Data analysis

We will use the MP analysis software *DiscoverMP* to analyse the landing molecules in your recorded movies. The program computes ratiometric movies, detects candidate particles and fits a model PSF to each of them determining the iSCAT signal. Your tutor will perform the video analysis with you and provide you with a list of contrast values for each video as CSV-files.

Your report should contain the following analysis steps to obtain a K_d for ADH:

1) Generate contrast histograms for your standard proteins.
2) Determine the peak centres using your method of choice.
3) Make a plot of contrast as a function of molecular mass from the determined peak centres and protein species sequence masses (your tutor may help you assign the contrast peaks).
4) Determine a linear conversion function between contrast and mass.
5) Convert the experimental contrast values for your ADH sample into molecular mass.
6) Plot an ADH mass histogram.
7) Determine the dimer and tetramer masses.
8) Determine the dimer-tetramer K_d from the respective histogram counts of each species.

4. Further reading

Young *et al.* 2018, Quantitative mass imaging of single biological macromolecules. *Science* **360**, 423-427. *DOI:10.1126/science.aar5839*

Soltermann, et al. 2020, Quantifying protein-protein interactions by molecular counting with mass photometry, *Angew. Chem. Int. Ed.* **59**, 10774. *https://doi.org/10.1002/anie.202001578*

Young, Kukura 2019. Interferometric scattering microscopy. *Annual Review Physical Chemistry* **70**, 301-322. *https://doi.org/10.1146/annurev-physchem-050317-021247*