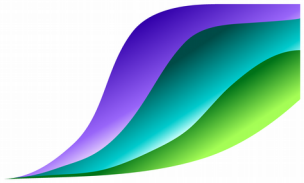


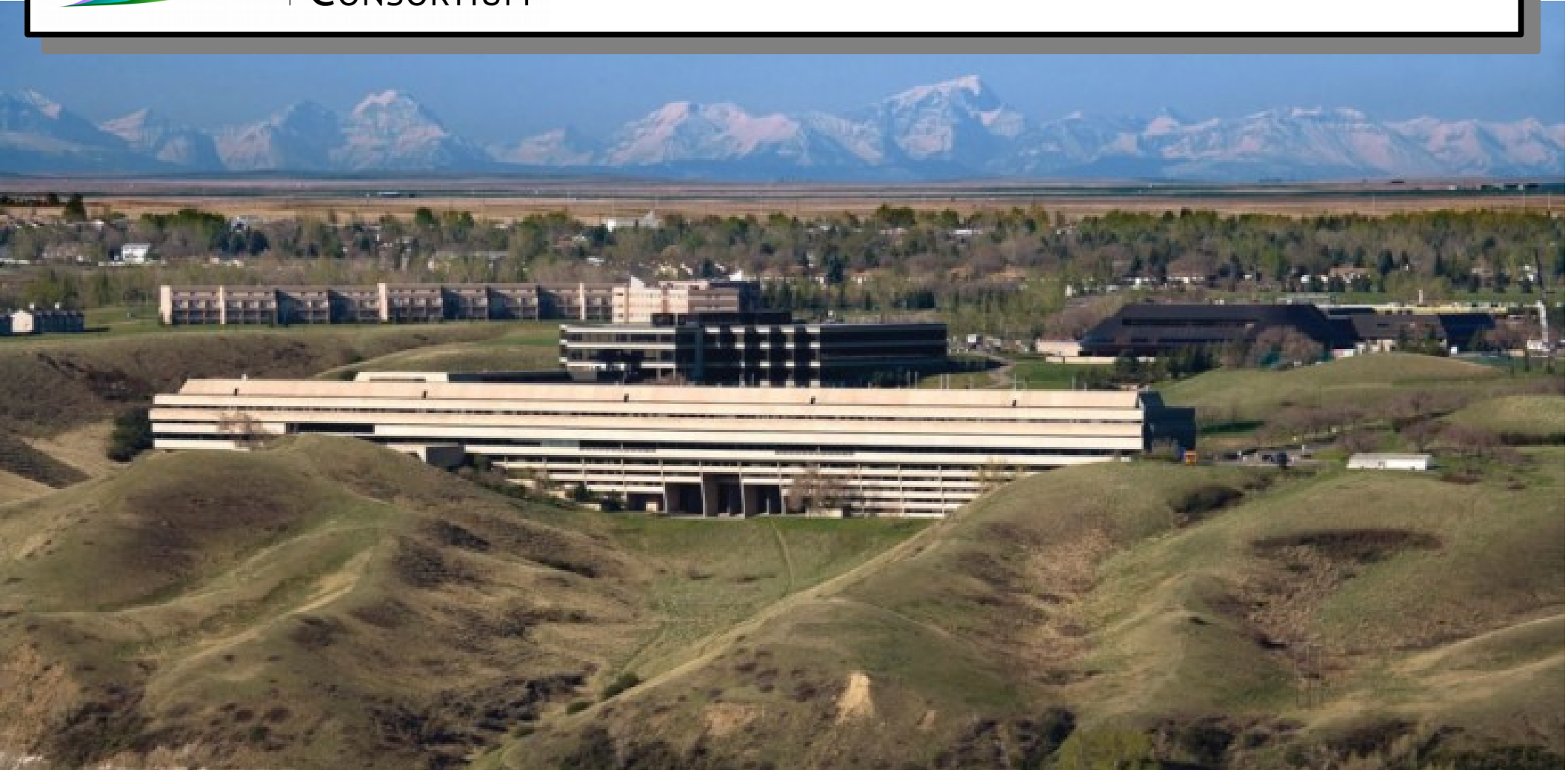


Borries Demeler, Ph.D.
Dept. of Chemistry and Biochemistry
Canada 150 Research Chair
in Biophysics



**NORTHWEST
BIOPHYSICS
CONSORTIUM**

Experimental Design



Experimental Design and Data Collection

- A. Checks to run to make sure the instrument is in good working condition**
- B. Decide which optical system is most appropriate**
- C. Buffer considerations**
- D. Speed selection and length of an experiment, resolution considerations**
- E. Column height**
- F. Concentration selection**
- G. Temperature considerations**
- I. Instrument settings**

Experimental Design and Data Collection

A. Checks to run to make sure the instrument is in good working condition:

Absorbance optics:

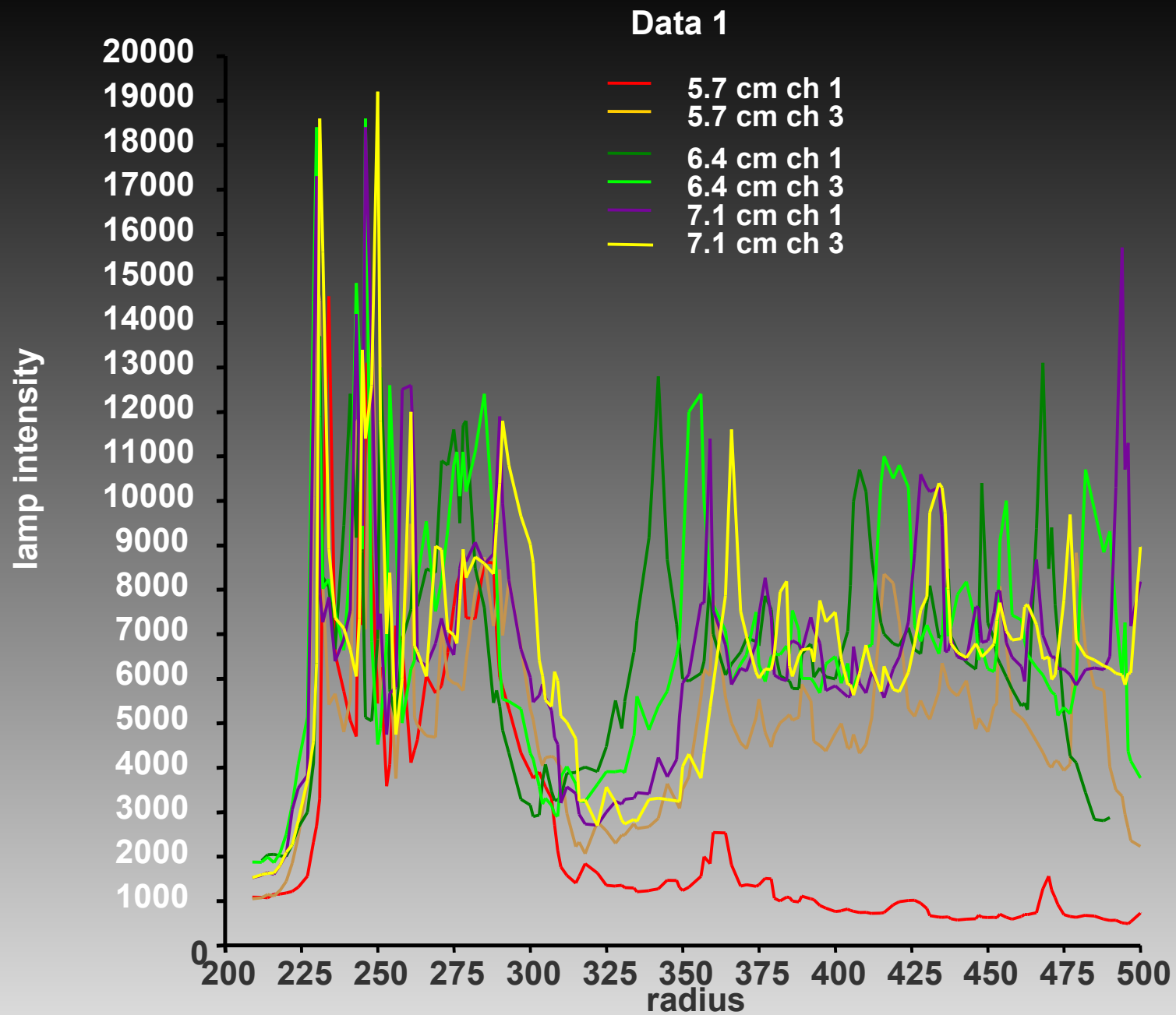
Run an intensity wavelength scan with a windowless cell at 5.9 cm, at 6.5 and 7.0 cm. Overlay the scans and make sure that a) the peak intensity at 230 nm is at least 15,000 (or clean the lamp) and make sure that intensity plots from all three positions overlay (guarantees that the optical system is properly aligned).

Perform a rotor calibration and check radial alignment, centerpiece alignment

Check the overlaid menisci of 40-50 scans of the same sample – they should form a single sharp peak, if the meniscus shifts back and forth, the slit assembly may need to be serviced.

Take advantage of unique absorption peaks (i.e., heme, metal in the visible) to extend concentration range with absorbance optics. Match absorbance and emission peaks.

Inspect cell housings and centerpieces for scratches, deformation, etc.



Experimental Design and Data Collection

A. Checks to run to make sure the instrument is in good working condition:

Interference optics:

Check to make sure the radial calibration is correct and matches the absorbance results from identical cells.

Attempt to adjust the laser timing to produce a well-balanced contrast throughout both channels of a cell

Make sure the interference optics are properly focused. A run of 1 mg/ml BSA should produce random residuals. Check sharpness of centerpiece walls. Beckman service can properly focus the optics.

Experimental Design and Data Collection

B. Optical system considerations

Considerations for using absorbance optics:

Advantages:

- **Great for low concentration of proteins or nucleic acids**
- **Use absorbance maximum**
- **Flexible wavelength selection**
- **Second channel can be used for sample in intensity mode**

Downsides:

- **Scanning speed is slow**
- **Monochromator does not reset properly – only can use single wavelength**
- **Intensity variation as function of wavelength (maximum at 230 nm)**
- **Sensitive to refractive artifacts**
- **Buffers should be non-absorbing**

B. Optical system considerations

Considerations for using Rayleigh Interference optics:

Advantages:

- **Great for samples with high refractive index**
- **Fast scanning speed**
- **No temporal distortion**
- **High radial resolution, low stochastic noise**
- **No buffer interference – arbitrary buffers are possible**

Downsides:

- **Sensitive to refractive index artifacts (Wiener skewing, lens effect)**
- **Exact meniscus match required**
- **Sample must be dialyzed or column purified**
- **Second channel can't be used for sample, meniscus match necessary**

B. Optical system considerations

Considerations for using Aviv fluorescence optics:

Advantages:

- **Exquisite selectivity – can be used against impure background**
- **Very high sensitivity (down to pM concentrations)**
- **No refractive artifacts (no lens effect)**
- **Fast scanning speed**
- **great for hetero-interactions, in-vivo studies, GFP fusion proteins**
- **No buffer interference – arbitrary buffers are possible**

Downsides:

- **Relatively high stochastic noise**
- **Most samples must be labeled**

Experimental Design and Data Collection

B. Optical system considerations

Absorbance optics:

use 230 nm for optimal signal to noise ratio (largest emission peak), use lower wavelengths for small protein concentration.

Protein extinction is usually 3-10 fold better when using 230 nm instead of 280 nm. Do not measure at concentrations above 0.9-1.0 OD, regardless of wavelength. Check extinction profile to pick a high extinction wavelength:

http://www.uslims.uthscsa.edu/emission_list.php

In velocity experiments it is important *not* to change the wavelength mid-run. When measuring multiple concentrations at different wavelengths, perform multiple runs.

Buffers *may not* absorb, or only very little. Always check absorbance against a water blank to confirm the level of baseline absorbance. Most of the signal should come from the analyte, not from the buffer. Together, both analyte and buffer should not exceed the dynamic range of the detector.

Experimental Design and Data Collection

B. Optical system considerations

Intensity Measurements:

Can be used to measure samples in the reference channel as long as the total optical density is less than 0.5 OD to avoid resetting of photomultiplier gain setting – doubles capacity!

In general, fill centerpiece channel as full as possible (~ 0.45 ml), but leave some room above meniscus in at least one channel to provide air region for intensity referencing

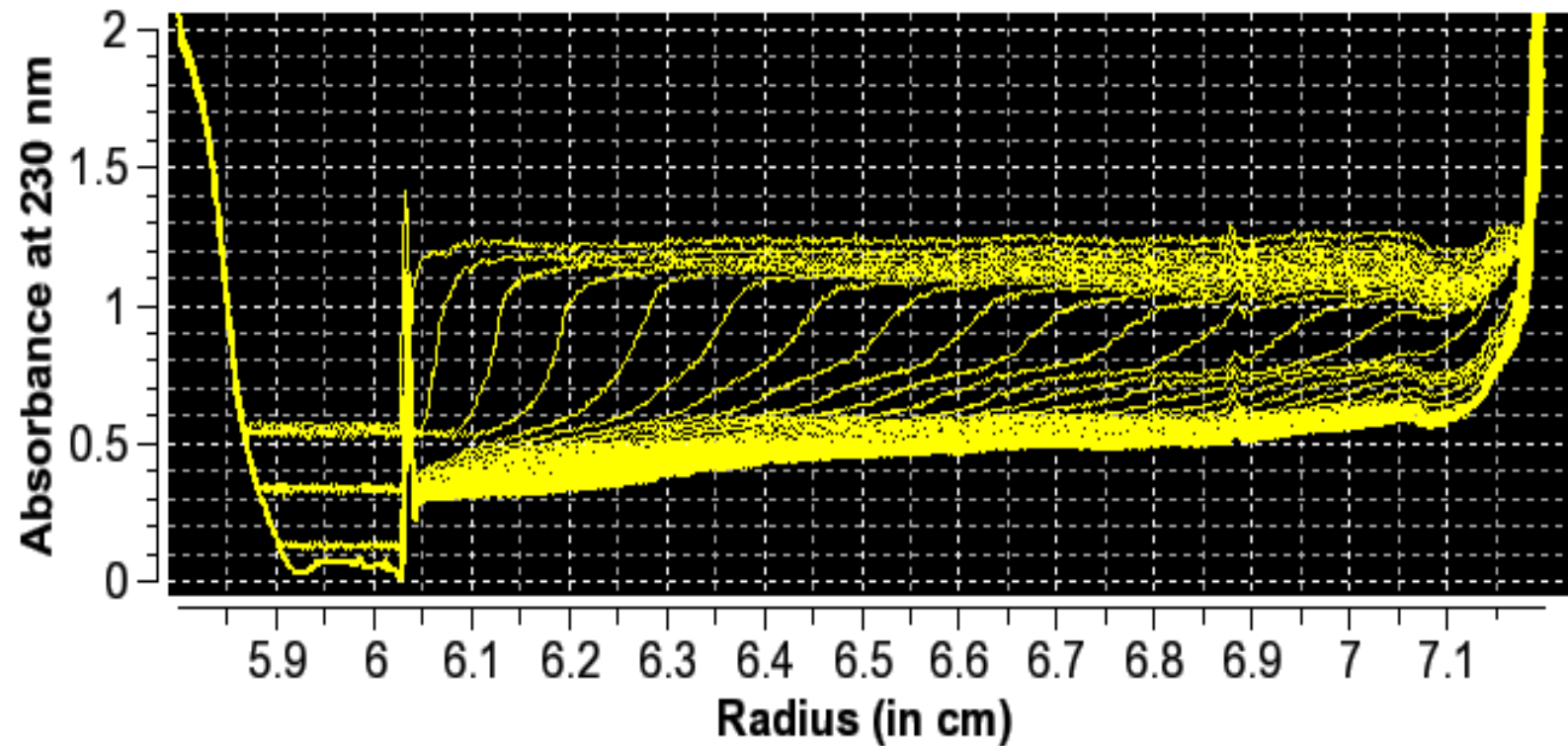
Requires time/radially invariant noise removal by fitting with the 2-dimensional spectrum analysis

Reduces stochastic noise by a factor of square root of 2

Provides absolute intensity data to check instrument performance

Experimental Design and Data Collection

Gain setting change of the photomultiplier tube when run in intensity mode and absorbance is both channels is too high. Solution: load water in reference channel or lower OD in the reference channel.



Experimental Design and Data Collection

B. Optical system considerations

Interference optics:

Use for high protein concentrations (1 mg/ml and above), non-absorbing molecules and molecules with high refractive index

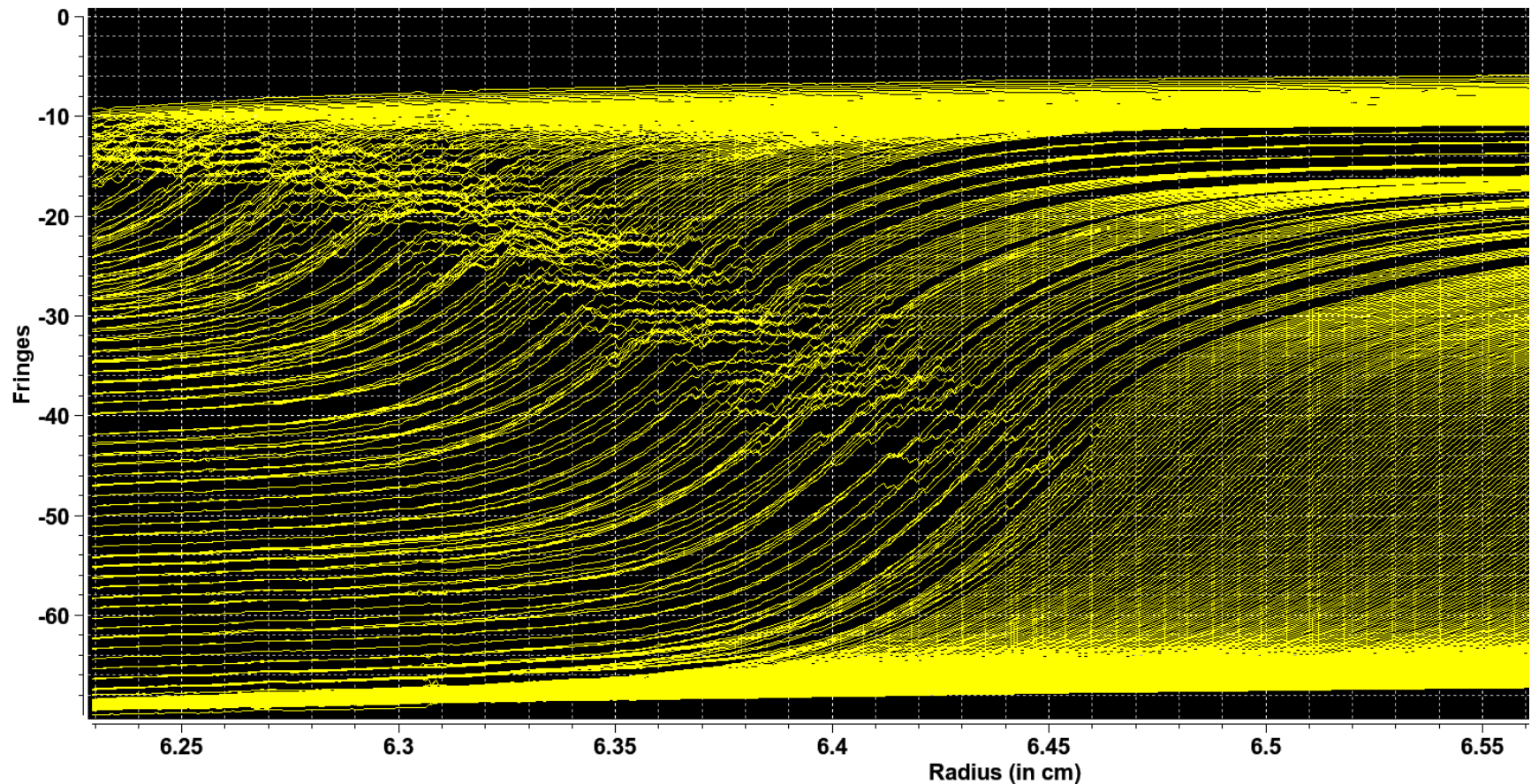
Measure either against water if buffer concentration is low – if any buffer components sediment they can be fit with 2DSA as separate species, or measure using meniscus matched dialysate.

Use for systems containing buffers that absorb too strongly in the UV (nucleotides, reductants, small organic molecules). In this case, use meniscus matching centerpieces and use column eluate or dialysate for reference.

Avoid steep gradients that produce refractive index artifacts

Experimental Design and Data Collection

Too steep gradient in early velocity scans causes optical (refractive) artifacts. Use 3-mm centerpieces, reduce concentration and/or speed.



Experimental Design and Data Collection

C. Buffer Considerations

Interference optics allows the use of absorbing buffers, since only concentration differences are measured, not absorbance. Use interference optics for experiments that involve nucleotides, reductants, absorbing buffers such as TRIS and other absorbing buffer components. If in doubt, scan the buffer against water in the desired wavelength range.

Minimize or eliminate gradient forming materials such as glycerol, sucrose, etc. since they will introduce hard-to-model density and viscosity variations throughout the cell

Absorbance experiments require use of non-absorbing buffers. TCEP may be used above 260 nm, other reductants can change extinction based on their oxidation state, causing shifts in the baseline absorbance during the run. Always run a buffer wavelength scan against water for an unknown buffer.

Experimental Design – General Principles

D. Speed selection and length of an experiment, resolution considerations

Important Considerations:

In principle, AUC experiments provide the sedimentation and diffusion of an analyte. The accuracy of this determination, in addition to a well-functioning Instrument, is entirely dependent on the **available signal**.

If the experiment is run too slow, the sedimentation resolution will be obscured by the diffusion signal, and it will become difficult to resolve separate species in the mixture. If the experiment is run too fast, sedimentation will proceed too rapidly to collect a sufficient number of scans, and the diffusion signal will be too small, and shape and molecular weight information will not be available or be very unreliable. Goldilocks principle...

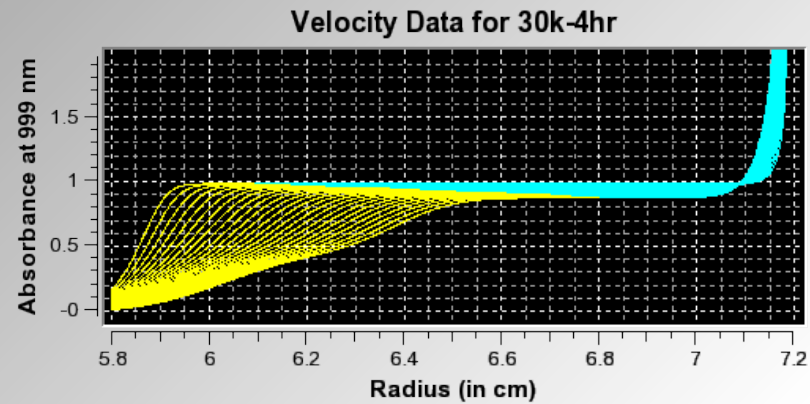
Experimental Design – General Principles

D. Speed selection and length of an experiment

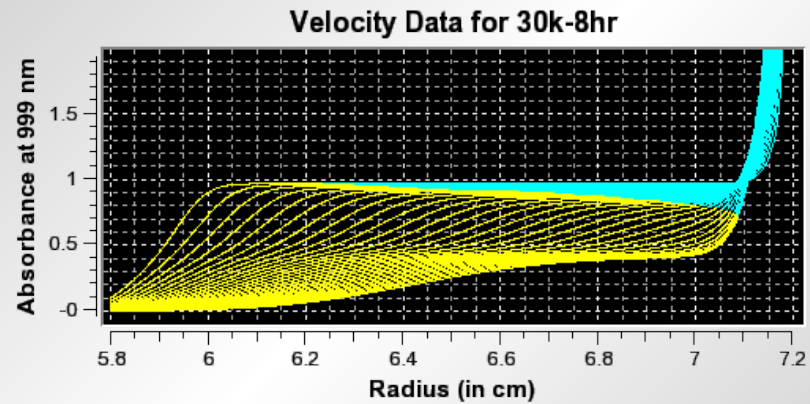
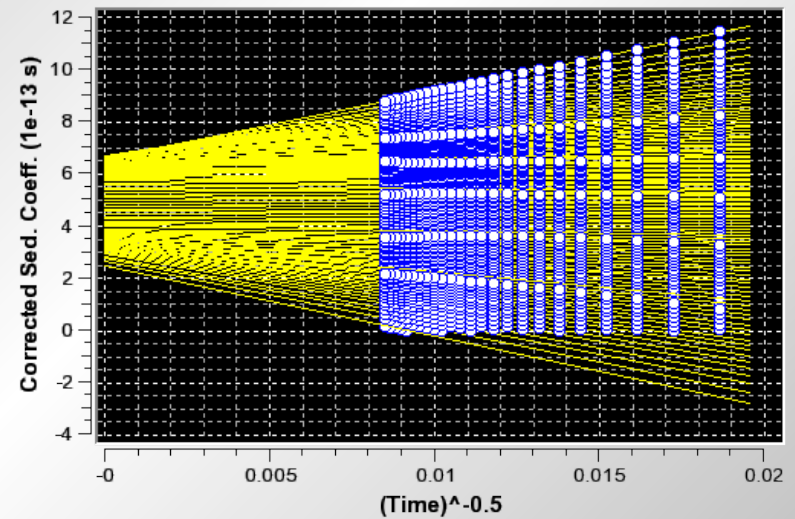
The optimal speed for an AUC experiment depends on the following parameters:

- **Analyte properties:**
 - **Sedimentation coefficient**
 - **Diffusion coefficient**
 - **Partial specific volume (density + solvation)**
- **Buffer properties**
 - **Viscosity**
 - **Density**
- **Desired Resolution**
 - **Slow speed: optimal diffusion signal**
 - **High speed: optimal sedimentation signal**

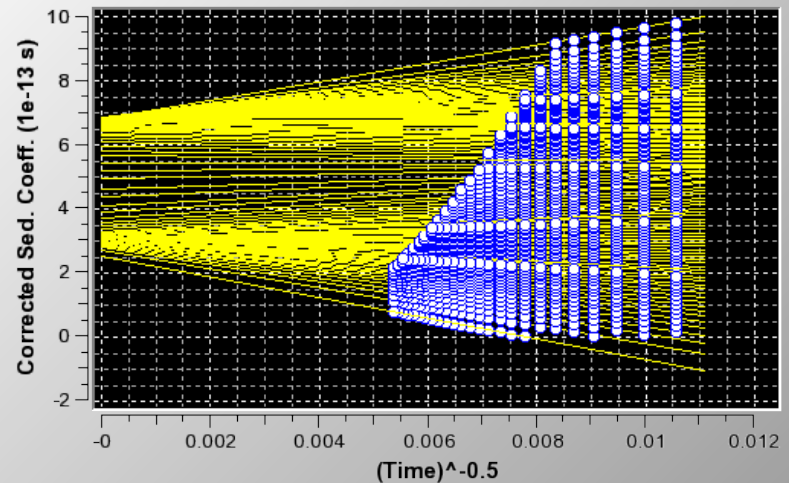
Effect of Time on Resolution



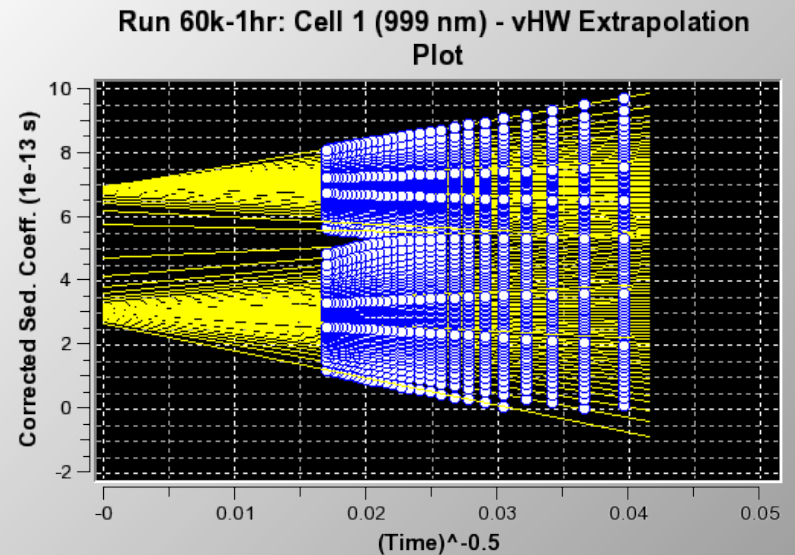
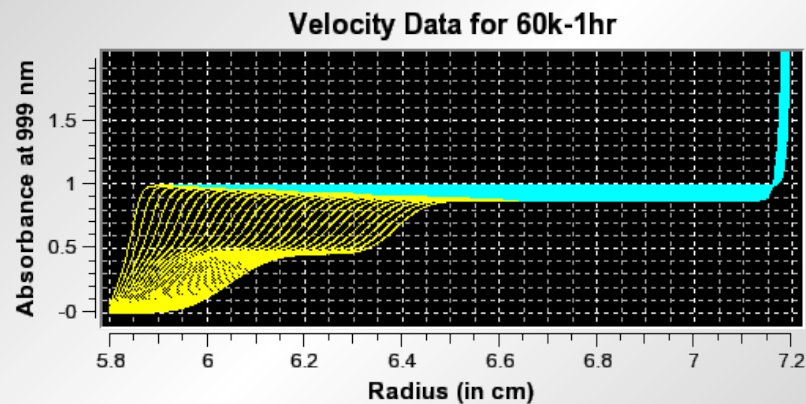
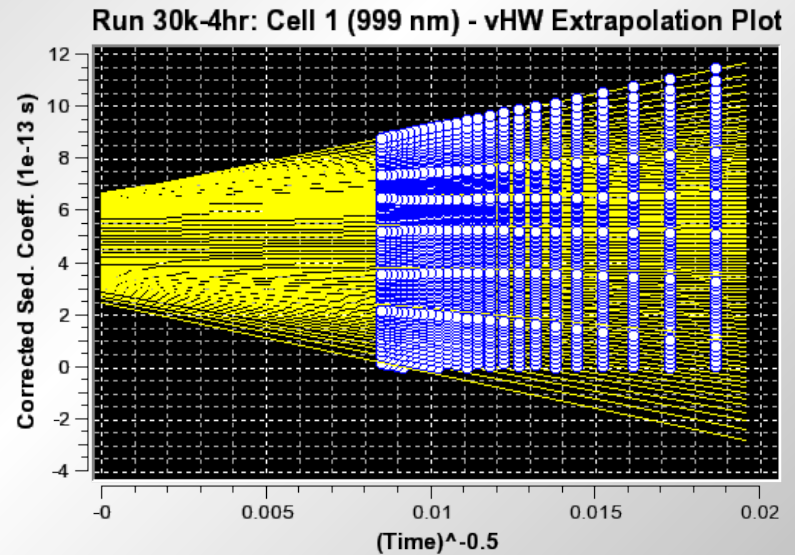
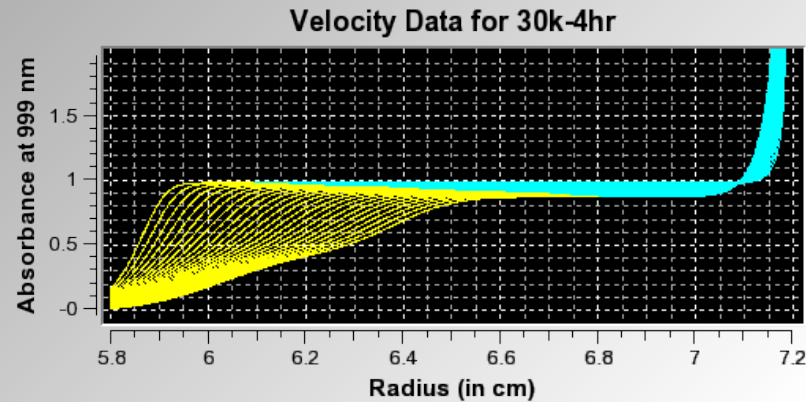
Run 30k-4hr: Cell 1 (999 nm) - vHW Extrapolation Plot



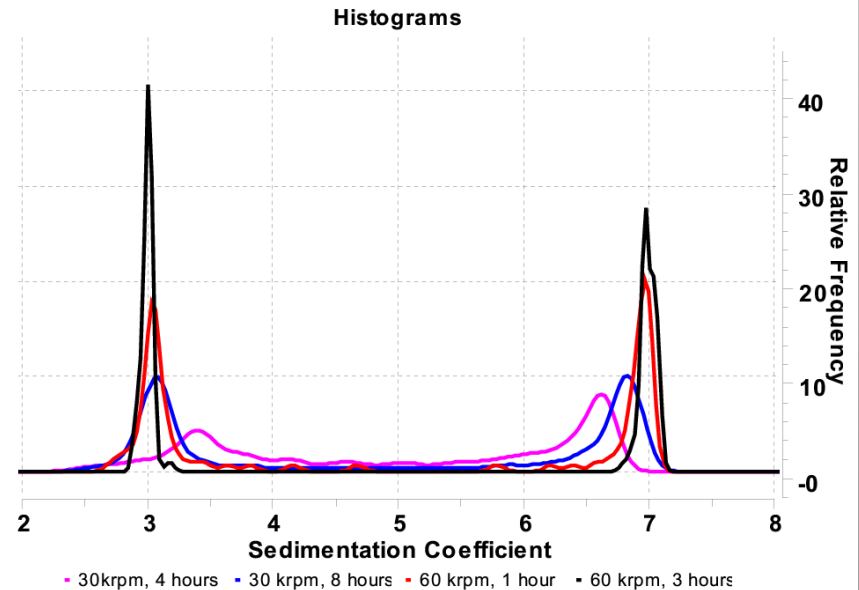
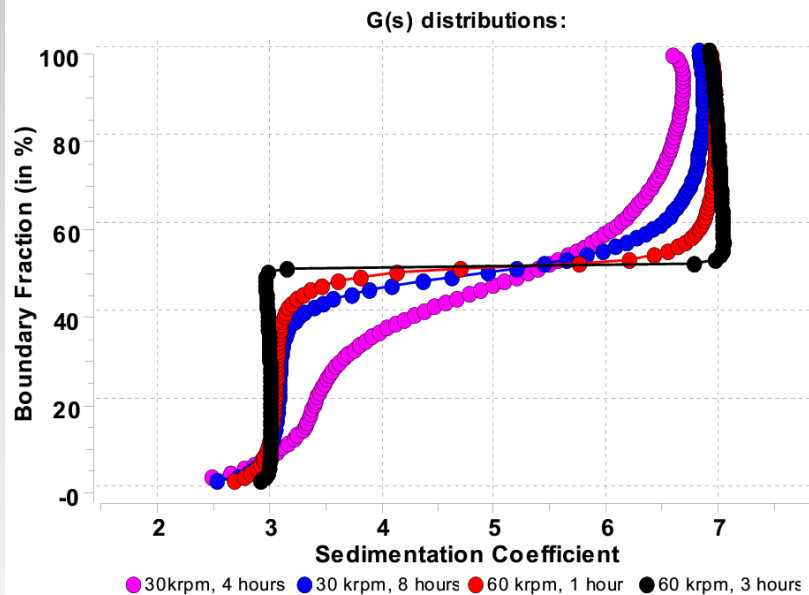
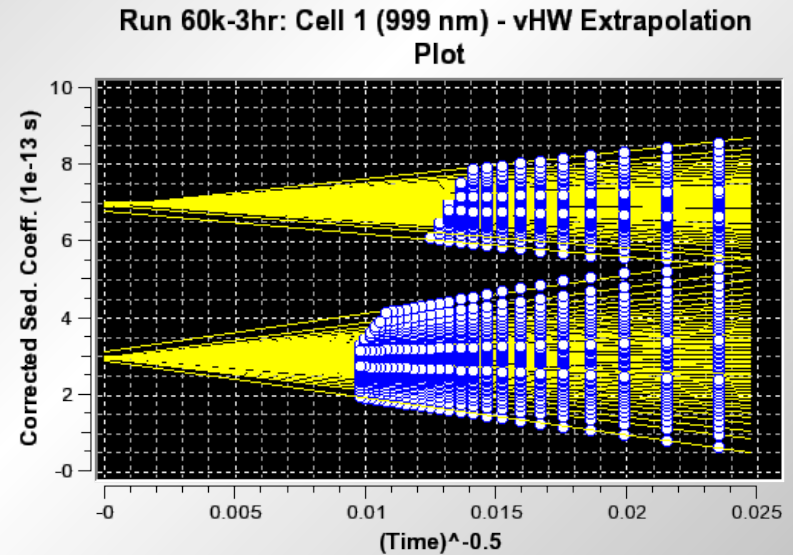
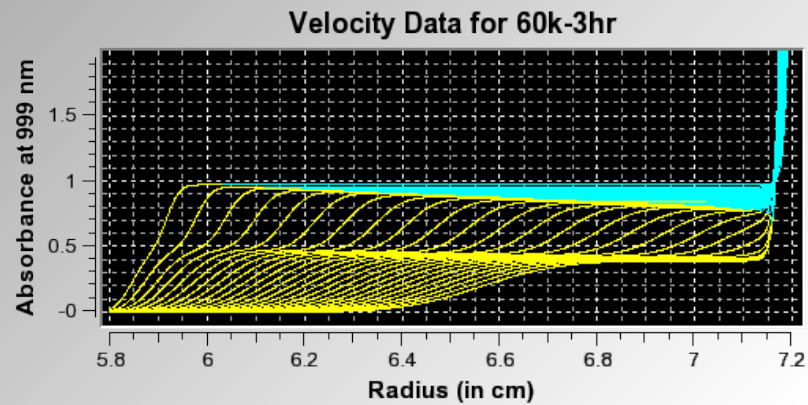
Run 30k-8hr: Cell 1 (999 nm) - vHW Extrapolation Plot



Effect of Rotorspeed with constant $\omega^2 t$ on Resolution:



Resolution Comparison:



Experimental Design and Data Collection

D. Speed selection and length of an experiment

Velocity experiments:

Attempt to obtain a minimum of 40 scans for each cell measured

The faster the rotor speed, the better the s-value resolution. Whenever possible, measure at the maximum speed supported by the instrument to characterize composition. Slower speeds emphasize diffusion signal needed for better anisotropy and molecular weight determinations.

For small and slow sedimenting samples, it is often possible to scan multiple samples (cells) and still obtain a sufficient number of scans on all cells

For very large samples, diffusion will be small and will have less of an effect on the s-value resolution, and good resolution can be obtained even at lower speeds

Use of interference or fluorescence optics is preferred when 7 cells are used, since it allows for faster scanning (seconds rather than minutes), and allows even multiple samples to be scanned at high speed before the sample is pelleted

Experimental Design and Data Collection

D. Speed selection and length of an experiment

Velocity experiments:

ALWAYS collect scans from the first seconds of the early experiment all the way until most material is pelleted. Remember, you can always discard scans later, but repeating the experiment to obtain missed data is not desirable

Use the finite element simulation routine to simulate all expected components in a system. You should model all components by shape, MW, s and D using the “Simulation:Model s , D and f from Molecular Weight for 4 basic shapes” and then use the “Simulation:Finite Element Simulation” module to predict how long to run the experiment and what speed should be selected. In order to guarantee that you will obtain enough scans, keep in mind that at the preferred setting for velocity experiments, you need to expect about 1.5 minutes for each absorbance scan of a properly filled cell (i.e, all the way full), and about 5 seconds for each interference scan.

Experimental Design – General Principles

Example: Spherical Gold Nanoparticle in aqueous solvent with known density and viscosity:

Let's assume a spherical 2 nm (core diameter) gold particle which is decorated by an organic functionalization layer with a 1 nm shell thickness, and let's further assume the PSV is 0.2 ml/g.

For spherical particles we can use the Stokes - Einstein relationship to estimate the frictional coefficient:

$$f = 6\pi\eta r$$

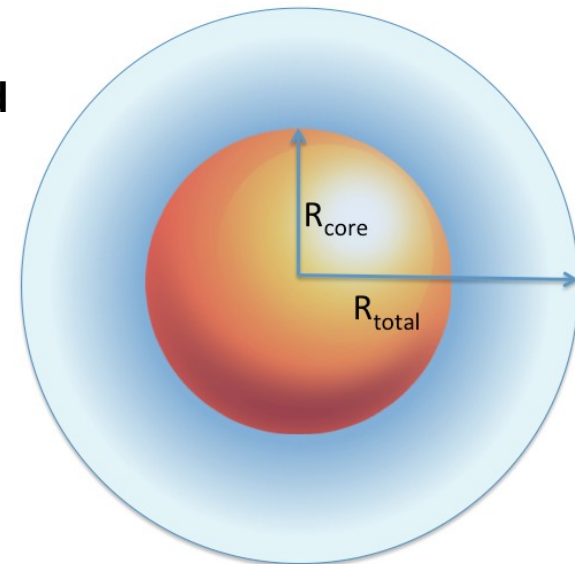
Given the PSV, we can further estimate the MW:

$$V = \frac{4}{3}\pi r^3 \quad M = \frac{\bar{v}N}{V}$$

Knowing density and viscosity of our buffer we can now predict sedimentation and diffusion properties of this particle:

$$s = \frac{M(1 - \bar{v}\rho)}{Nf} \quad D = \frac{RT}{Nf}$$

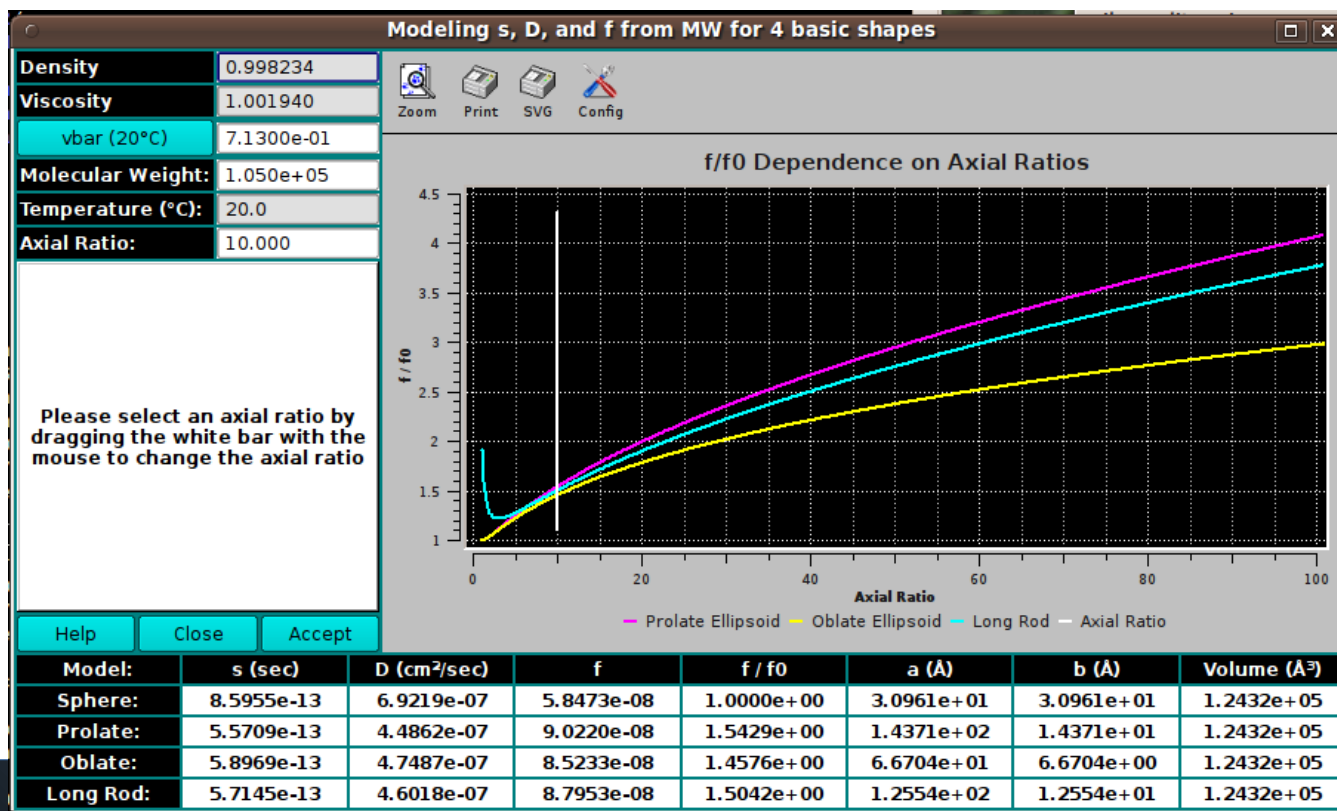
$$\begin{aligned} \eta &= 0.01 \text{ p} & \rho &= 1.0 \text{ g/ml} & r &= 2 \times 10^{-7} \text{ cm} \\ f &= 3.77 \times 10^{-8} & M &= 20,853 \text{ g/mol} \\ s &= 7.11 \text{ s} & D &= 1.03 \times 10^{-6} \text{ cm}^2/\text{s} \end{aligned}$$



Experimental Design – General Principles

Example: Anisotropic Particles

For anisotropic particles, use the estimated frictional ratio (f/f_0) to adjust the frictional coefficient using the frictional coefficient from the equivalent sphere, which can be estimated from the molecular weight and the partial specific volume or the density of the analyte, or use UltraScan to predict the values:



Self -Associating Equilibrium Experimental Design:

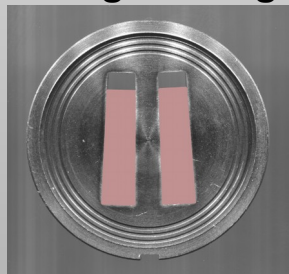
E. Column Height

For all experiments fill both channels as full as possible, but leave enough room for a small air-to-air region, which is required to align the scans and correct integral fringe shifts during editing.

Longer columns provide more data points, later scans have increased resolution.

If there is limited amount of sample, increase volume by dilution. A longer column is more important than higher sample signal.

To reduce stress on centerpieces, try to closely match the fill volumes on both sides of the septum. Leave a small airbubble in each channel to generate a meniscus. 0.45 ml volume is optimal to get a large enough column. Make column as large as possible.



Self -Associating Equilibrium Experimental Design:

Always run several concentrations of your sample!

Use 2 different loading concentrations at the same wavelength

Increase concentration range by measuring at different wavelengths such as 280 nm, 230 nm and ~210 nm, check absorbance spectrum!

If interference optics are available, use them to extend concentration range.

Maximizing Resolution of the Sedimentation Coefficient:

In order to maximize the Resolution of your Analysis:

Run at the fastest speed possible (simulate!)

Always collect data until the end of the Run

Collect early scans for best estimate of C_0

Recommended OD: 0.1 – 0.9

Fill cells as high as possible to get a long column

Later scans provide better resolution than earlier scans

F. Concentration selection

For velocity experiments, a loading concentration between 0.2-0.9 OD is recommended.

For interference experiments, at least 1 mg/ml is desirable for good signal-to-noise ratio.

In order to maximize the signal from all species in a reversibly self-associating sample, it is recommended that the concentration range be made as large as possible to assure that sufficient signal from each species is present in the data. It is recommended to measure data from 210, 230 and 280 nm to detect mass action.

G. Temperature considerations

Velocity experiments require a constant velocity and constant temperature. Therefore it is critically important to temperature-equilibrate the rotor before acceleration. This is best accomplished by letting the rotor with loaded sample cells sit in vacuum at the temperature at which the run is to be performed for at least 1 hour before the experiment is started.

Experimental Design and Data Collection

I. Instrument settings

For UV absorbance experiments it is important that optimal data acquisition settings are selected. Options include radial resolution, the number of repeat measurements, and for wavelength measurements the wavelength resolution. Settings for each experiment:

Wavelength measurements: Wavelength measurements should be performed as follows: measure 3 scans of each concentration with 1 nm resolution and zero averages, continuous mode. Measurement should include 20 nm above and below the desired wavelengths.

Velocity experiments: 0.003 cm resolution, no averaging, no scan delays, continuous mode

Interference data collection for velocity experiments should be performed in continuous mode without delay between scans. Superfluous scans can be discarded later.