Measuring Molar Mass for Fluorescing Samples

Summary

This technical note describes how to perform light scattering measurements of fluorescing samples. Wyatt Technology allows users to correct for the effects of fluorescence by using narrow bandwidth interference filters or a non-standard laser wavelength. Once the contribution from fluorescence is removed, ASTRA will subsequently determine the correct molar mass and RMS radius for fluorescing samples.

Related References

M1000	ASTRA 6 User's Guide
M3200	DAWN HELEOS II Manual (Appendix E Interference Filter Option)
M1500	Optilab rEX Manual
M1510	Optilab T-rEX and UT-rEX Manual
TN1007	ASTRA Quick Guide
TN1010	Correcting for Absorbance at the Laser Wavelength

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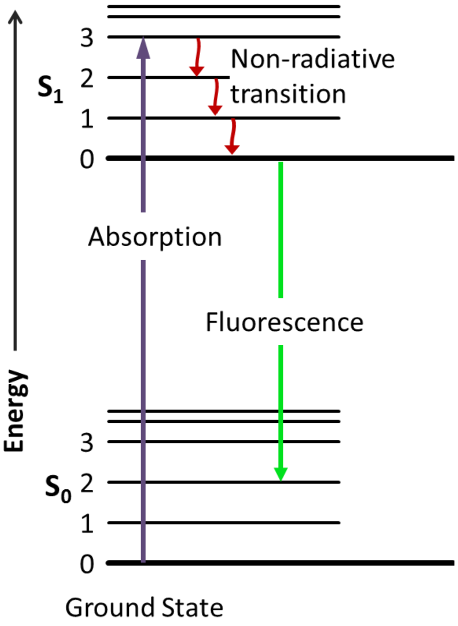


Jablonski diagram illustrating the electronic states of a molecule and the allowed transitions. After an electron absorbs a photon, the system is excited electronically and vibrationally. The system relaxes, and eventually fluoresces at a longer wavelength.

Introduction

In a typical light scattering experiment, a single frequency, polarized light source (i.e, a laser) is used to illuminate a solution containing a suspension of the macromolecules or nanoparticles of interest. Rayleigh scattering represents the simplest and most common light scattering experiment whereby particles much smaller than the incident wavelength scatter light elastically (i.e., at the same wavelength as the incident light). Wyatt Multi-Angle Light Scattering (MALS) detectors have a laser with a default nominal wavelength of 658 nm. If the macromolecule of interest contains a fluorophore that absorbs at this incident wavelength, the light that reaches the photodiode detectors is the summation of the photons from Rayleigh scattering and the photons from fluorescence. If the correct properties such as molar mass of a sample are to be measured, we must account for known variations in the amount of light reaching the detector so that we do not incorrectly attribute increased intensity from fluorescence to an increase in molar mass.

Note: Generally speaking, fluorescence will only affect the determination of molar mass via light scattering; the root-mean square radius is typically not affected.

Wyatt Technology allows HELEOS users to correct for intensity differences due to fluorescence via two methods:

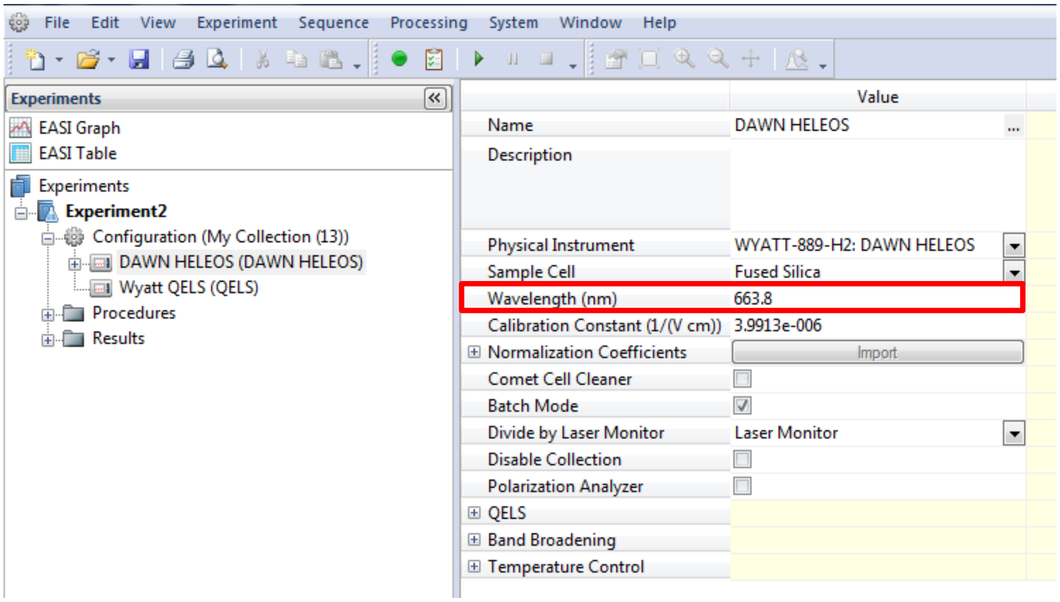
(1) Installing narrow bandwidth interference filters such that only elastically scattered light will reach photodiode detectors.

(2) Using a laser with an incident wavelength that does not overlap with the fluorophore’s excitation spectrum.

Note: For instructions on installing interference filters, please refer to the *DAWN HELEOS II User’s Guide, Appendix E: Interference Filter Option*.

Diagnosing Data and Configuring ASTRA

To assess whether or not the light scattering measurements are affected by fluorescence, first determine the wavelength of the laser in the DAWN HELEOS (I or II). The standard laser is a linearly polarized GaAs laser with a nominal wavelength of 658 nm. To determine the laser wavelength in your instrument, open an ASTRA experiment file and navigate to the HELEOS configuration window as shown in Figure 1. The laser wavelength will auto-populate when the physical instrument is selected in ASTRA 6.



	Value
Name	DAWN HELEOS
Description	
Physical Instrument	WYATT-889-H2: DAWN HELEOS
Sample Cell	Fused Silica
Wavelength (nm)	663.8
Calibration Constant (1/(V cm))	3.9913e-006
Normalization Coefficients	Import
Comet Cell Cleaner	☐
Batch Mode	☑
Divide by Laser Monitor	Laser Monitor
Disable Collection	☐
Polarization Analyzer	☐
QELS	
Band Broadening	
Temperature Control	

Figure 1: A screenshot of the HELEOS configuration window. The laser wavelength will autopopulate when the Physical Instrument is selected.

Samples that do absorb at the laser wavelength will result in a significant decrease in the forward monitor signal (transmitted light). As shown in Figure 2, a batch injection of a fluorescent sample into a HELEOS instrument caused a significant drop in the forward monitor signal (higher concentrations will result in a larger decrease). However, if it is not known whether the sample of interest contains fluorophores that are excited at the laser wavelength, it is recommended to measure excitation and emission spectra for the sample in question using a standard spectrofluorimeter. Common fluorophores that absorb at 658 nm include lignin and Cyanine-5 dyes.

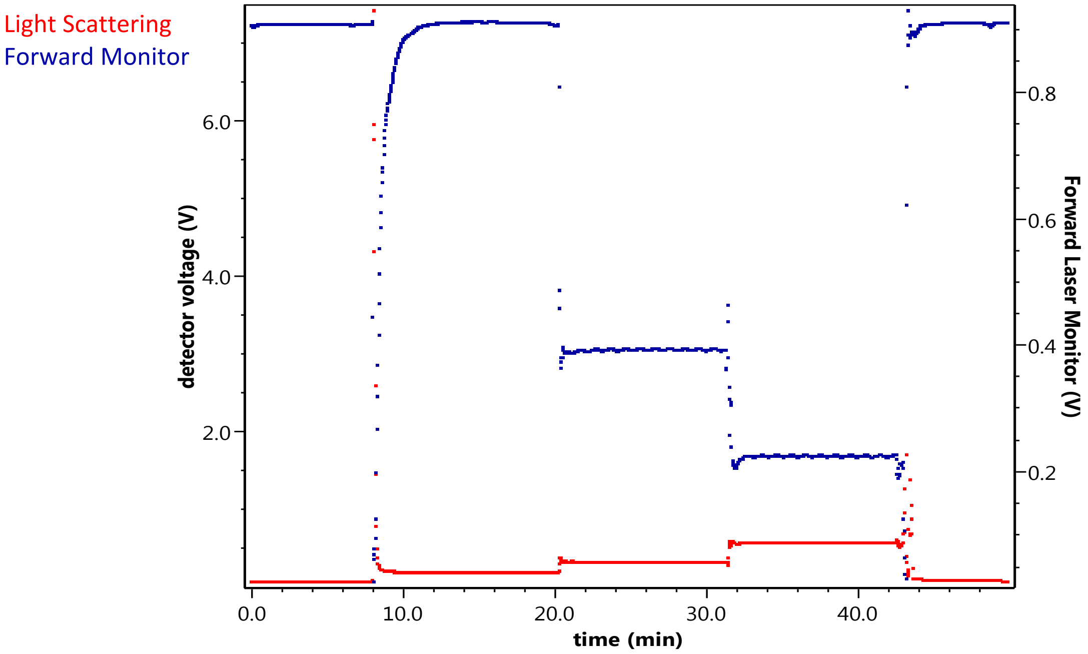


Figure 2: Light scattering signal at angle 90° (in red) and forward monitor signal (transmitted light, in blue) from a batch experiment of a sample that is excited at 665 nm. The forward monitor decreases significantly when the sample is injected indicating that the sample absorbs at the laser wavelength.

If the sample is excited at the laser wavelength, interference filters must be installed and/or the HELEOS laser must be upgraded to the longer wavelength of 785 nm to avoid the excitation wavelengths. Wyatt Technology offers customized orders of a 785 nm laser option for the HELEOS or as an upgrade option for instruments operating at 658 nm. Interference filters with a 20 nm bandwidth are used to filter out light of a different wavelength than that of the laser wavelength and prevent it from reaching the photodiode detectors. Without interference detectors the molecular weight measurement of a fluorescing sample will be inaccurate due to the additional contribution of fluorescence to the scattered light of the sample.

When an instrument is outfitted with a 785 nm laser:

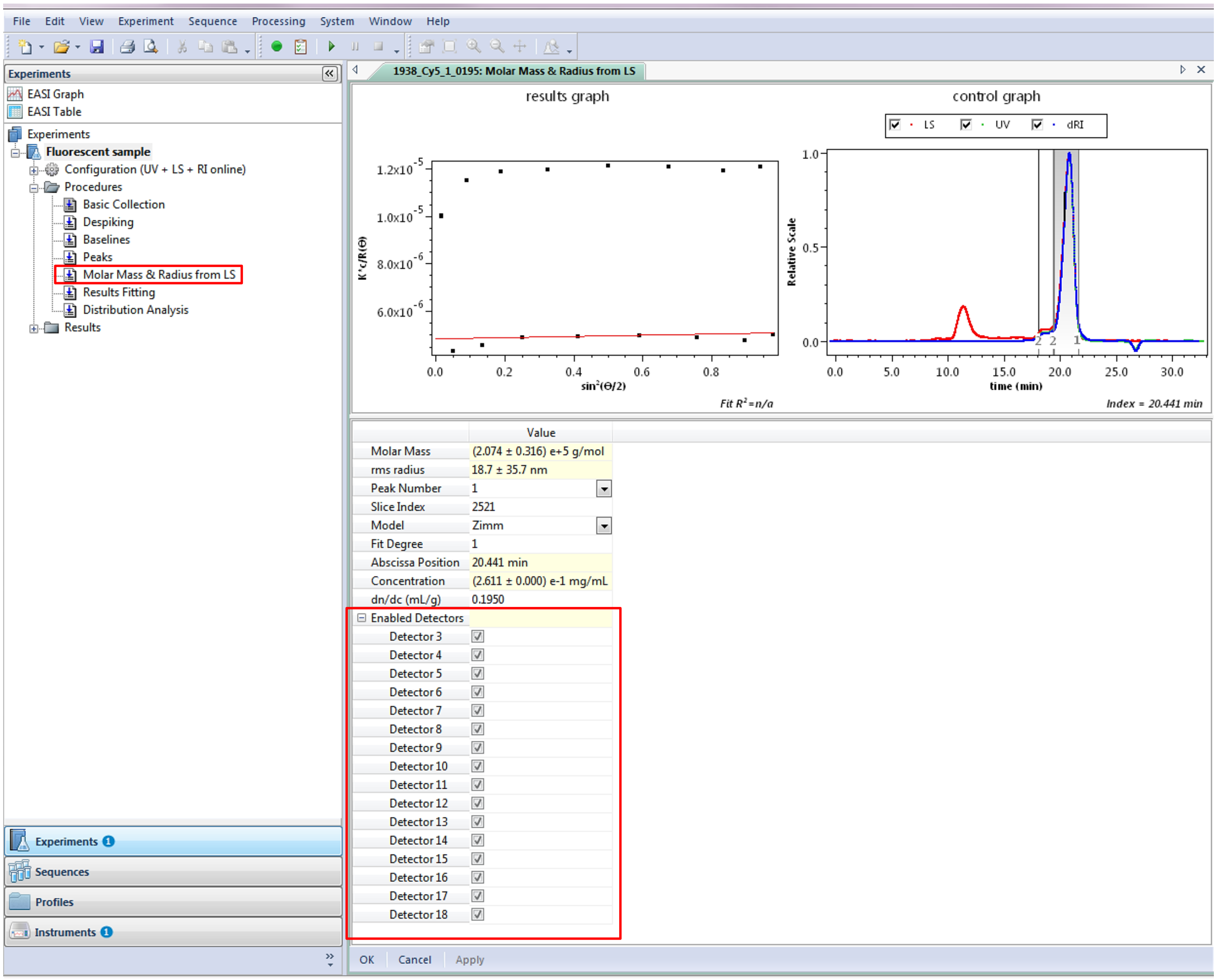
- Ensure that the **Wavelength (nm)** field in the configuration window of the HELEOS instrument has been updated to reflect the longer wavelength (the wavelength autopopulates when a new method is created in ASTRA 6).
- Ensure that a calibration constant is measured in toluene at the new laser wavelength and enter the new value in the HELEOS configuration window (Figure 1:Figure 1).
- The dn/dc entered in the **Peak** procedure should correspond to the dn/dc of the sample at the longer wavelength. If a dRI detector is used as a concentration source, then ensure that the wavelength of the detector matches the longer wavelength of the light scattering detector. ASTRA uses a single dn/dc value to calculate the concentration from the dRI signal and to calculate the molar mass from the scattered intensity ($I \alpha M c (dn/dc)^2$). A mismatch in the laser wavelength and the wavelength of the dRI detector LED might result in error in the molar mass measurement due to the difference in dn/dc at those two wavelengths.

Note: Wyatt Technology offers LEDs of various wavelengths other than the standard 658 nm wavelength that can be installed in the Optilab (U)T-rEX instruments. Please contact support@wyatt.com for more information.

ASTRA Analysis when using Interference Filters

Interference filters should be installed on at least three detectors. Typically, the interference filters are all installed either on odd detectors or even detectors. When analyzing results in ASTRA, detectors with no interference filters should be disabled. In the **Molar Mass & Radius from LS** procedure, expand the **Enabled Detectors** node and uncheck all detectors that do not have interference filters installed (Figure 3). The Debye plot in Figure 4 shows the difference in the intensity measured by detectors with and without interference filters for a fluorescent sample. The intensity is plotted as $K^*c/R(\theta)$ (where K^* is an optical constant, c is concentration, and $R(\theta)$ is the Rayleigh scattering ratio) against scattering angle (expressed as $\sin^2(\theta/2)$). The $K^*c/R(\theta)$ ratio for detectors with interference filters is greater than that of detectors with no interference filters (i.e. a lower intensity reaches the detectors with filters and therefore, the inverse intensity $1/R(\theta)$ is greater).

The calculated weight-average molar mass when detectors with no interference filters are enabled is 216.0 kDa (Figure 5Figure 1). However, when only detectors with interference filters are enabled, the weight-average molar mass calculated is 83.7 kDa. The light emitted by this fluorescent sample causes the molar mass measured to be overestimated by more than a factor of two if interference filters are not installed.



	Value
Molar Mass	(2.074 ± 0.316) e+5 g/mol
rms radius	18.7 ± 35.7 nm
Peak Number	1
Slice Index	2521
Model	Zimm
Fit Degree	1
Abscissa Position	20.441 min
Concentration	(2.611 ± 0.000) e-1 mg/mL
dn/dc (mL/g)	0.1950
Enabled Detectors	
Detector 3	☑
Detector 4	☑
Detector 5	☑
Detector 6	☑
Detector 7	☑
Detector 8	☑
Detector 9	☑
Detector 10	☑
Detector 11	☑
Detector 12	☑
Detector 13	☑
Detector 14	☑
Detector 15	☑
Detector 16	☑
Detector 17	☑
Detector 18	☑

*Figure 3: Screenshot of the **Molar Mass & Radius from LS** procedure in the ASTRA software. Detectors used for the analysis can be enabled from this screen.*

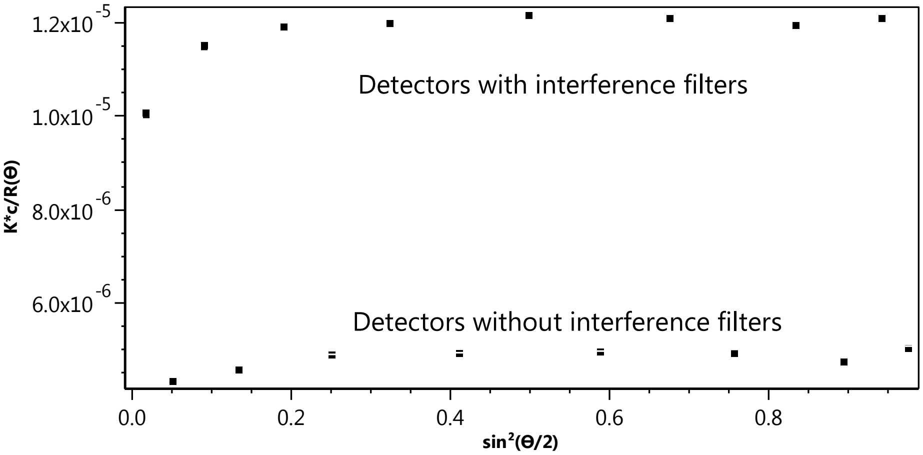


Figure 4: Results graph (Debye plot) of fluorescent sample showing two sets of detectors with and without interference filters.

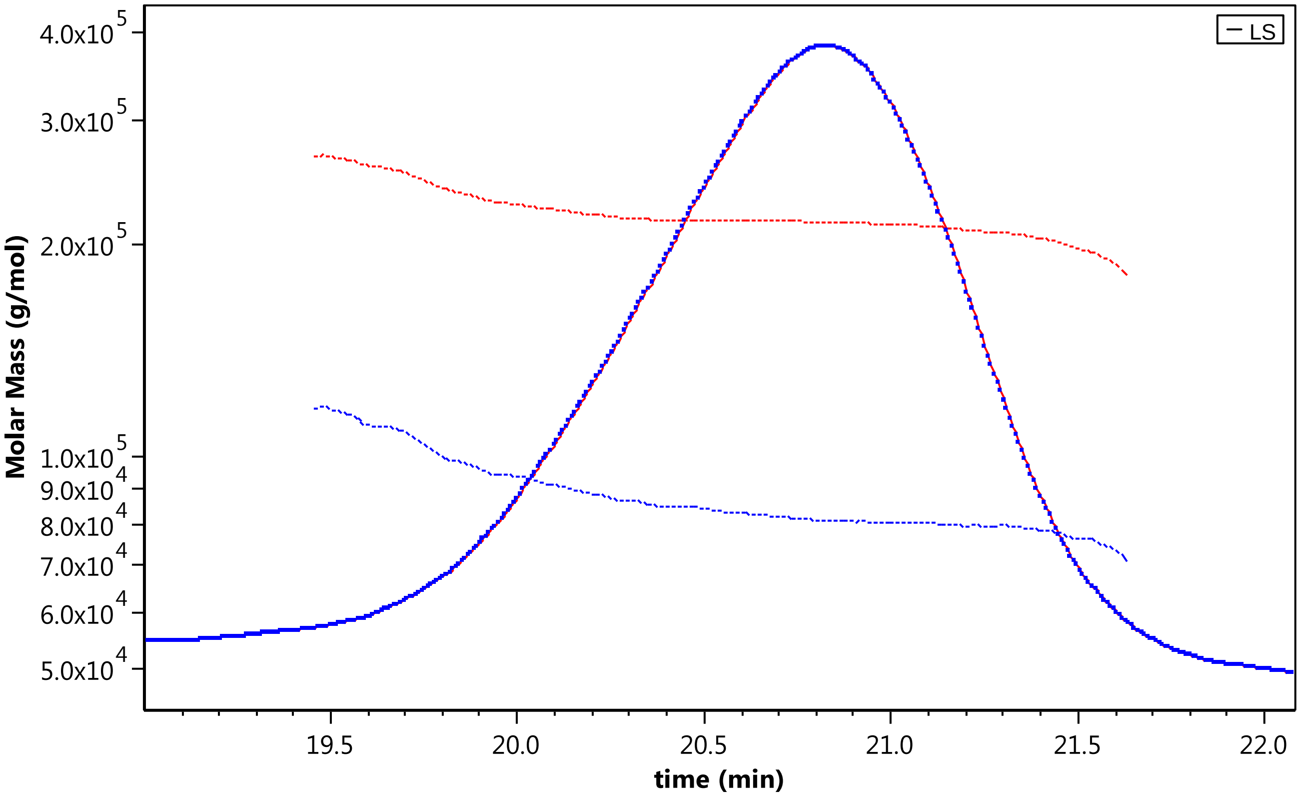


Figure 5: Molar mass versus elution time of a fluorescent sample calculated when detectors with interference filters are enabled (blue line) and when detectors with no interference filters are enabled (red line). The LS signal chromatogram is displayed in blue and red (overlaying traces). The molar mass is overestimated when no interference filters are used.

Typically the ASTRA software divides each photodiode's signal by the laser monitor to normalize the scattering signals relative to incident laser beam power. The photodiode of the laser monitor will measure the laser light before it enters the flow cell of the HELEOS. However, as the sample will absorb light in a wavelength range which includes the wavelength of the laser being used. Changes through the flow cell in the incident intensity due to absorbance can be accounted for by dividing each photodiode's signal by the "forward" monitor signal rather than the "rear" laser monitor signal. To correct for a sample which absorbs the laser light, open the ASTRA experiment file and open the HELEOS Configuration (Figure 1). Select **Forward Monitor** from the **Divide by Laser Monitor** drop down menu and click **OK** to save your changes. Please see the Technical Note *TN1010 - Correcting for Absorbance at the Laser Wavelength* for more information on how to measure samples that absorb at the wavelength of the MALS laser.