FSEC: CMI Getting Started Guide to Fluorescent Size-Exclusion Chromatography

Sample Preparation | Getting Started | Data Collection | Data Analysis | Shutdown

Introduction

Size-exclusion chromatography (SEC) separates molecules based on hydrodynamic volume. Fluorescent Size-Exclusion Chromatography (fSEC) in an analytical SEC method that uses fluorescence detection to monitor eluting fluorescent protein peaks. It can be used to analyze purified or unpurified samples as a rapid test of monodispersity and aggregation state of a protein of interest.

Instrument Overview

The CMI has a SEC-MALS system with light scattering (LS) detectors and refractive index (RI) detector from Wyatt and liquid chromatography system and UV detector from Agilent. The system can be replumbed for fSEC by directing flow through an Agilent fluorescence detector and bypassing the RI and LS detectors. There is no fraction collector on this system, as it is intended for analytical chromatography.

Chromatography System

- Agilent 1260 Infinity Isocratic Liquid Chromatography System
- Agilent 1260 Infinity Autosampler
- Agilent variable wavelength UV detector
- Agilent 1100 HPLC G1321A Fluorescence Detector
- Inline solvent degasser

Applications

- Aggregation
- Mass estimate from comparison to protein standards
- Mondispersity
- Purify (if combined with UV)

Required Supplies

- Sample filters, 0.02-0.2 µm filters (recommended)
- Autosampler vials (provided by the CMI)
- Analytical SEC Column:
    - A range of analytical SEC columns can be used including silica and agarose columns
    - Analytical columns
        - e.g. SEPAX SRT SEC-300, 7.8 mm x 50 mm, guard column size
        - maximum pH of 7.5
    - Make sure that the column is compatible with your sample and buffer. Some detergents and membrane proteins will require special columns designed for hydrophobic samples and buffers.

Sample Preparation

Assay Buffers

Running buffer should always be chosen to be compatible with both the SEC column and the protein sample. **Make sure you know the buffer compatibility of the SEC column you are using.**

- **Prepare sufficient buffer** (1L is recommended)
- Recommended Buffer: PBS or HBS (25 mM HEPES pH 7-7.5, 150 mM NaCl), filtered.
- Most silica columns will not tolerate pH above 7.5.

Samples

- Know the UV extinction coefficient (in ml/mg•cm) and concentration (in mg/ml) of your protein.
  - Concentration should be accurately measured to assess column recovery.
- Filter or centrifuge samples before use.
  - Protein aggregates can damage the column.
- Recommended protein concentration varies depending on fluorescence intensity.

Getting Started

Resources

Additional resources are available at the instrument, including instrument and software manuals. Videos regarding advanced uses of OpenLab software can be found on the Agilent website.

Experimental Design Tips

- Choose a column such that your major peak of interest is in the center of the column, neither near the void peak nor near the solvent peak.
- You may need to optimize the fluorescence excitation and emission wavelengths.

General Care and Maintenance

- Treat the SEC columns and chromatography system with care.
  - Avoid rapid pressure changes when a column is attached by changing flow rates gradually (using a maximum flow gradient to 0.1 ml/min^2).
  - Always **filter** or centrifuge samples before loading.
  - Only load well-behaved protein samples.
  - Use filtered buffer with at least 100 mM salt and a pH appropriate to the column.
- Store columns and system in water with 0.02% NaN_3 when not in use.
- Follow recommended start-up and shut-down sequences.

OpenLab Notes

- Loading a method will automatically execute it! So, make sure to open methods with a Zero Flow AND/OR use STANDBY in OpenLab to open files without executing them.

Startup

1. Before you start, book time on the PPMS calendar.
2. Login to the computer using your PPMS credentials (HMS ID/eCommons).
3. The Agilent chromatography system will usually be on, but if not, then:
   a. Turn on the pump.
   b. Turn on everything else.
4. Disconnect the MALS detector from the line out of the UV detector.
   a. Cap the inlet to the MALS detector.
5. Attach the Fluorescence detector to the line out of the UV detector.
6. Disconnect Wyatt HPLCManager software.
   a. Right click HPLCManager Icon in bottom TaskBar (looks like a green or yellow molecule)
   b. Click Disconnect HPLC

Data Collection

1. Launch OpenLab Software using the HPLC (online) desktop shortcut.
   a. It's generally safest to download latest method to instrument (should be the last method run and should open with pump in standby and lamps off).
   b. General Procedures
      i) To Enable a module: Right-click→Control (and turn on, off, or Standby).
      ii) To Edit a module: Right-click→Method (and change parameters).
      iii) When loading a new method, you will often be asked if you'd like to save changes to the previous. **If you are using a CMI default method, always continue without saving.**
2. Purge Pump:
   a. Replace storage buffer (water + 0.02% NaN_3) with your running buffer
   b. Open purge valve on pump (by turning knob ¼ turn to the left)
   c. Load Purge Method (e.g. 0_Purge_OPEN_PURGE_VALVE…)
      i) Max flow gradient should be set to 100 ml/min^2 for purge steps to allow rapid flow change. Only use when Pump Purge Valve is manually opened, and column is bypassed.
   d. Right-click Iso.Pump and select Method.
   e. Change flow to 5 ml/min.
      i) **Purge at 5 ml/min for at least 5 minutes.** There should be *no backpressure* if the purge valve is open.
   f. Change flow to 0 ml/min.
   g. Stop flow.
   h. **Close the purge valve.**
3. Equilibrate Column
   a. Right-click Iso.Pump and select Control.
      i) Click on Standby.
   b. Load Equilibration Method (e.g. 1_Equilibration_0ml_min…)

c. Set a low flow rate for the column attachment.
   i) Right-click Iso.Pump and select Method.
   ii) **Make sure the Max Flow Gradient is 0.1 ml/min².**
      - Expand Advanced settings.
      - Enter Max Flow Gradient (e.g. 0.1ml/min²).
   iii) Set a low flow rate to attach column with a wet connection (e.g. 0.1 ml/min).
      - Right-click Iso.Pump and select Method.
      - Enter flow rate.
      - Click apply.

d. Attach your FSEC column under low flow.

e. Ramp up to the experiment flow rate.
   i) 0.4 ml/min is the recommended flow rate for Sepax SRT SEC columns.

4. Before injecting samples, equilibrate column with at least 2 column volumes buffer.

5. **Edit/Write the method for data collection offline.** You may already see a method that suits your needs. It is always best to check the existing parameters before loading a method.
   a. Launch OpenLab Software using the HPLC (offline) desktop shortcut.
   b. Select an existing method to edit.
      i) There will be a list of files on the left-hand side. Click "Methods."
      ii) Expand "1_CMI_FSEC_Methods." Double-click to select a method.
      iii) At the top of the screen select "Method > Edit Entire Method…"
         - Deselect Data analysis and Run time Checklist. Click "OK."
      iv) Method Information:
         - Add any relevant comments. Click "OK."
      v) Select Injection Source:
         - Als. Click "OK."
      vi) Iso. Pump
         - Set flow rate (0.400 ml/min for CMI shared columns).
         - Set pressure limit to 20 bar or the maximum pressure of your column, whichever is *lower pressure*.
         - Under "Advanced," ensure maximum flow gradient is 0.100 ml/min².
      vii) Sampler
         - set default injection volume (can be overwritten in the sample list).
         - (optional) Enable Needle Wash and enter wash vial location (e.g. vial 100)
         - Set Stoptime (10.00 min for CMI shared columns). This is the point at which data collection will end for a given injection. The flow remains unchanged at the stoptime.
      viii) Fluorescence detector
         - set excitation and emission wavelengths (e.g. ex280 and em330 for Trp/Tyr)
      ix) VWD (Variable Wavelength Detector)
         - set Wavelength (e.g. 280 for protein).
   c. If you have changed any parameters, save as a new method.
      i) File > Save As… > Method.
      ii) Navigate to the "2_User_Methods" folder.
      iii) Name your method. Include the flow rate, stop time, excitation/emission wavelengths, and your initials.

6. Load the experiment method.
7. **Prepare Sample Queue**
   a. Go to Run Queue (optional)
      i) Pause Queue to delay start of sample injections until Queue is resumed.
      ii) Resume Queue (play button) to start sample injections as soon as sample list is added to queue.
   b. Go the Sample Entry Tab.
      i) Double-click sample position or [+] button to add to the sample list.
      ii) Enter/check:
         - Sample Location (vial position)
         - Sample Name
         - Method Name (click the … to select method from CMI_FSEC_Methods folder)
         - Inj Volume, Injection Volume (maximum 100 µl)
      iii) Select a destination for your data files.
         - Click the Settings icon (⚙).
         - Select the path: "C:\Chem32\1\Data\"
         - Select the subdirectory: Your lab folder (if your lab does not have a folder, you'll need to add one in File Explorer, as you cannot edit file structures in Open Lab)
8. Add sample to Queue.
   a. If Queue is paused, go to Run Queue and click Resume Queue to start the first injection.
   b. If the Queue is active, submitting the sample list will run the first sample immediately.

Data Analysis

1. If data collection is incomplete, Launch OpenLab Software using shortcut on Desktop called HPLC (offline).
   a. If data collection is complete, you can analyze in HPLC (online).
2. Select your data file.
   a. Navigate to your file in the Data Analysis File Organizer (left panel).
   b. Double-click to load single file or sequence file.
3. Sequence Navigator (top right panel).
   a. Double-click on a run in the sequence to load it.
   b. Select all and right-click to choose multi-run overlay options.
4. Signals are automatically displayed as an overlay in Full (normalized) scale (up arrow)
   a. Click the down arrow to display each signal in the same scale.
5. Align the x-axes.
   a. Use the dropdown menu to select an individual signal.
   b. Click the "set time reference points to chromatogram" icon.
      i) Located to the right of the selection arrow. Hover over icons to see names/functions.
   c. Select the center of the most prominent shared peak.
   d. Repeat for all signals.
   e. Use the dropdown menu to view all loaded signals.
   f. Click the "Align the time axis of the signals" icon.
6. If applicable, calculate the area under the peaks.

a. Navigate to the Integration tab.
b. Select a signal. You will see a calculated peak area, height, and width based on automatically selected baselines.
c. If necessary, use the toolbar to adjust baselines and/or peak ranges and recalculate.

7. Export a report of the data.
   a. Navigate to the integration tab. Click the printer icon to identify peaks, calculate results, and print a pdf report.
      i) The report will include sample, method, and file information, as well as chromatograms and peak results for each signal.
8. Export the raw data.
   a. File > Export file > CSV File…
   b. Select "signal" and update the file name. Click "OK."
      i) Type, "C:\Chem32\1\Data***labname\yourname\filename.csv***"
      ii) The "Browse…" button in this window allows users to select a file to replace, but does not allow users to select a file location.
   c. Select the signal to export. Click "OK."

Shutdown

Shutdown Sequence

1. Purge Pump:
   a. Replace running buffer with storage buffer (water + 0.02% NaN_3).
   b. Open purge valve on pump by turning knob ¼ turn to the left.
   c. Load Purge Method (e.g. 0_Purge_OPEN_PURGE_VALVE…)
      i. Max flow gradient should be set to 100 ml/min^2 for purge steps to allow rapid flow change. Only use when Pump Purge Valve is manually opened, and column is bypassed.
   d. Right-click Iso.Pump and select Method.
   e. Change flow to 5 ml/min.
      i. **Purge at 5 ml/min for at least 5 minutes.** There should be *no backpressure* if the purge valve is open.
   f. Change flow to 0 ml/min.
   g. Stop flow.
   h. **Close the purge valve.**
2. Equilibrate Column
   a. Right-click Iso.Pump and select Control.
      i. Click on Standby.
   b. Load Equilibration Method (e.g. 1_Equilibration_0ml_min…)
      i. **Make sure the Max Flow Gradient is 0.1 ml/min^2.**
         1. Expand Advanced settings.
         2. Enter Max Flow Gradient (e.g. 0.1ml/min^2).
      ii. Set flow rate to 0.4 ml/min. Equilibrate system in several column volumes Milli-Q Water with 0.02% Sodium Azide.

   c. Your column may be stored in 20% ethanol or the buffer of your choice, but *the system should be returned to Water+NaN_3 after you remove your column (run for at least 5 minutes at 1 ml/min).*
3. Change the flow rate to 0.1 ml/min. Detach your FSEC column and cap both ends.
4. Set the flow rate to 0 ml/min.
5. Reconnect the inlet and outlet tubing to the spacer.
6. Disconnect the inlet tubing that goes from the UV detector to the fluorescence detector and reattach it to the MALS detector.
7. Cap the inlet valve on the fluorescence detector.
8. Logoff from PPMS!

CMI Users must *bring all their own materials and supplies* (including pipettes and tips!).

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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