

WP2900: Innovations in FFF – Eclipse NEON

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Summary

The Eclipse™ NEON is the latest generation of Wyatt Technology's instrumentation for field-flow fractionation (FFF). Introduced in late 2020, it represents a major breakthrough in FFF technology.

The primary aim of the Eclipse NEON program was to improve usability, robustness, and performance of the Eclipse controller and fractionation channels, while streamlining user interaction with the system via the instrument's front panel interface and VISION™ software. New features include a large multitouch display providing complete status at a glance and intelligent guidance, the Dilution Control Module™ which increases the detector signals by up to a factor of 10, and the Eclipse Mobility™ which calculates electrophoretic mobility.

This white paper describes the technical innovations of the new Eclipse system and how they help the FFF user achieve new levels of performance with less effort. Additional information can be found by following the links to our website, and from the references.



Introduction

Field-flow fractionation (FFF) is a method for separating macromolecules and nanoparticles in solution or liquid suspension by size. A complete system incorporates a pump, autosampler, FFF flow controller, and the channels wherein separation takes place. FFF has become a powerful and popular characterization method thanks to the ability to couple the output seamlessly to online light scattering for absolute characterization—foremost static light scattering (FFF-MALS) but dynamic light scattering as well (FFF-MALS-DLS).

The developments described herein enable users of Wyatt Technology's Eclipse system to productively utilize the essential properties of FFF-MALS with enhanced ease:

- **Range of separation:** FFF-MALS can analyze sample sizes from a few nanometers up to several micrometers.
- **Tunability:** Simply by changing flow rates, a single channel can explore complex samples comprising macromolecules, particles, and emulsions.
- **Flexibility:** A selection of channels accepts sample loads from a fraction of a microgram up to milligram quantities; the entire system is compatible with aqueous and organic solvents.
- **Absolute characterization:** Size and molar mass are determined by online MALS and DLS detection. Additional detectors analyze composition and drug loading efficiency as a function of size.

FFF is a perfect front-end for light scattering detectors because it converts polydisperse samples to a series of homogeneous size fractions which, upon elution from the FFF channel, are measured sequentially by MALS, DLS and

other detectors to determine molar mass, size, conformation and composition. Whereas SEC-MALS is the workhorse for macromolecular characterization, FFF-MALS can deal with ultra-high-molar-mass macromolecules, nanoparticles and combined samples that are not amenable to separation on an SEC column. FFF-MALS is a versatile technique that accommodates many different applications from high-molar-mass polymers, nanoparticle products and environmental tracer nanoparticles to proteins, vaccines, gene vectors and nano-drug delivery vehicles.

Wyatt's complete FFF-MALS system includes a DAWN® online multi-angle light scattering instrument together with a WyattQELS™ embedded DLS module, an Optilab differential refractometer and a UV/VIS absorption detector to analyze:

- Size
- Molar mass
- Mass concentration
- Particle concentration
- Conformation
- Composition and particle payload

How field-flow fractionation works

Field-flow fractionation (FFF) is a separation method that works without a stationary phase. Separation is achieved by applying a force field perpendicular to the longitudinal transport. In this discussion, 'FFF' is taken to mean the asymmetrical-flow field-flow fractionation variant (AF4), which is the variation of FFF with the broadest range of applications and the widest user base.

In AF4 (Figure 1), the bottom wall of the channel is composed of an ultrafiltration membrane supported by a frit. The upper wall is a solid block. Flow is introduced to the channel from the inlet port and is split in two different paths: one along the channel, towards the outlet and detectors, and the other permeates through the membrane, creating a cross flow which is the force field that drives separation. The sample is introduced to the channel and concentrated against the membrane by the cross flow. Brownian motion causes the particles to diffuse up away from the membrane, creating a diffusive force which balances the cross flow.

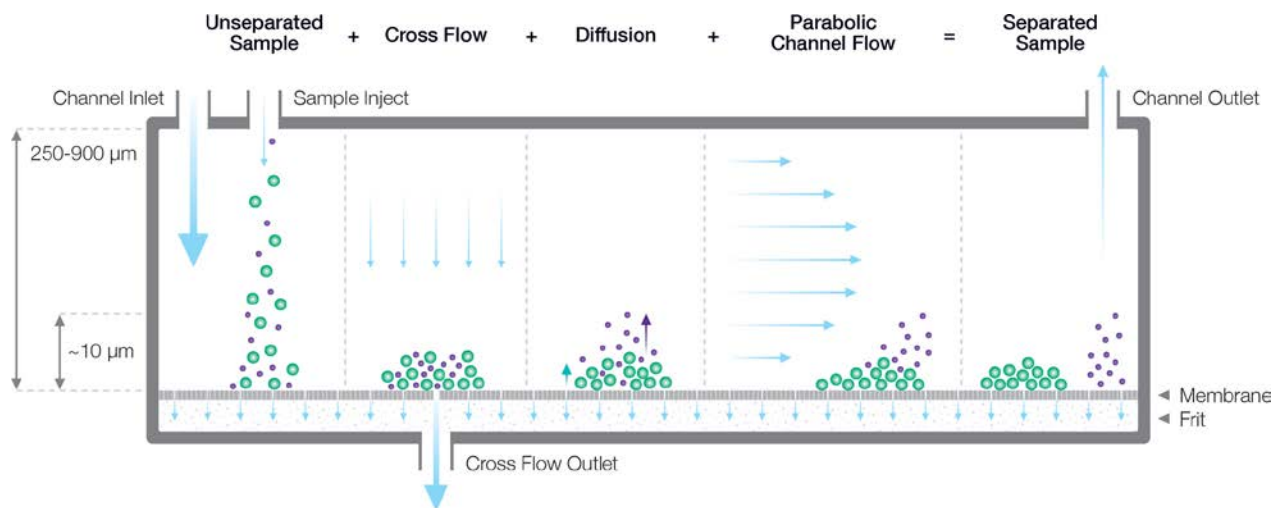


Figure 1. Principle of FFF separation. The average height of particles above the membrane results from the balance of cross flow and diffusion. Separation is achieved in a laminar flow with a height-dependent parabolical velocity profile which transports the sample through the channel.

After some time, a stationary distribution is established. Going away from the membrane, up into the channel, the concentration decreases over a length scale which is given by the ratio of the diffusion coefficient and the cross-flow velocity. Particles will, therefore, extend into the channel to a different average distance that depends on their diffusion coefficient, or size.

Once the elution process is started, the longitudinal flow transports the particle cloud towards the channel outlet. There is a parabolical flow profile, in which flow velocity increases with distance from the membrane. This leads to different particle velocities depending on their diffusion coefficient. Smaller particles, which are higher up, will

travel faster and elute earlier compared to bigger particles.

The relationship between the retention time t_r , the hydrodynamic radius R_h , the channel height w , the volumetric cross-flow rate F_x and channel-flow rate F_{ch} is expressed in Equation (1)¹ which is valid for constant cross flow and approximates the exact form, which can be found in the literature².

$$t_r = \frac{\pi\eta}{kT} \cdot R_h \cdot w^2 \cdot \ln\left(1 + \frac{F_x}{F_{ch}}\right) \quad (1)$$

The sample generally proceeds down the channel within a few micrometers from the membrane and is diluted by the solvent volume above the sample layer which—due to the parabolic velocity-profile—travels at much higher speed. This is the reason that Wyatt Technology developed the Dilution Control Module (DCM) which reduces dilution by a significant factor. It is described later in this white paper.

Making FFF more accessible

Given its unique capabilities, why has FFF not arrived in every laboratory working with nanoparticles and high-molar-mass macromolecules? The reason is that FFF is seen as more complex compared to column chromatography or batch particle characterization methods like dynamic light scattering or particle tracking analysis. The complexity arises from the nature of FFF, which requires multiple flow paths to be combined in a precise and controlled way. Channel flow, injection flow, cross flow, and the detector flow each play an important role in the experiment and need to be well controlled. Deviations will lead to lack of reproducibility in retention time, and in more extreme cases might even cause a complete failure of the separation.

Wyatt Technology's new FFF instrument, the Eclipse NEON, relieves the need for the user to proactively check the various flow rates to detect any deviations. The system is monitored continuously during a run, and deviations are detected immediately—preventing an unsuccessful experiment and thereby saving time and precious sample.

Another pain point that has been addressed is method development. VISION DESIGN software helps find the optimum parameters for separation. *In silico* simulations

enable the versatility of FFF to be exploited without time-consuming trial-and-error method development.

Productivity enhancements with the Eclipse NEON

The Eclipse offers several new features to increase productivity and robustness:

- A 10" multi-touch display on the front panel shows system information and status. Relevant system parameters and status indicators are monitored in real time, and deviations are communicated to the user in clear messages, both on the front panel and in VISION.

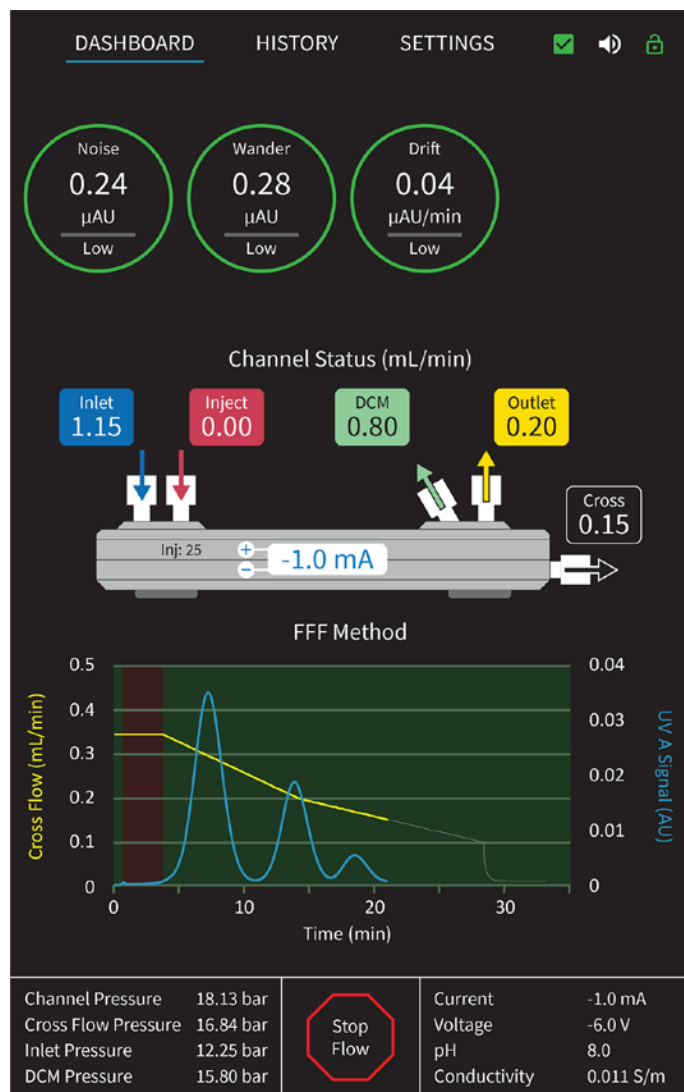
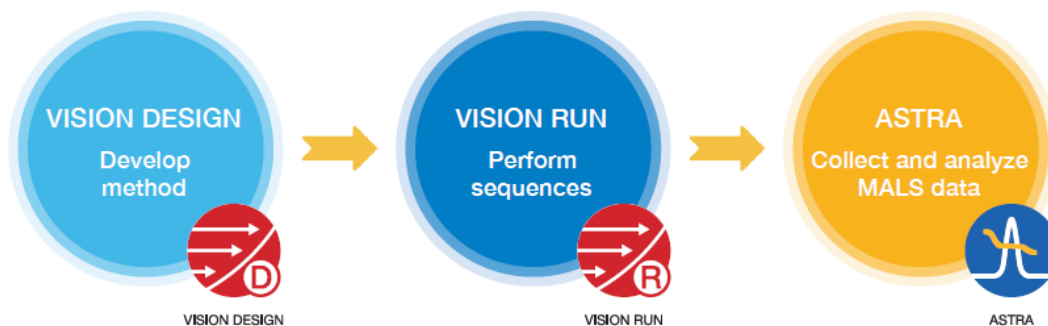


Figure 2. The Eclipse NEON front panel displays a wide range of useful information across the tabs. Important instrumental parameters are shown along with progress of the experiment. Any alarms appear on the DASHBOARD, and are saved in the HISTORY tab for review.

- Flow control is implemented via sophisticated Coriolis flow meters, which measure and regulate the cross flow, injection flow and focus flow. The corresponding data traces are stored and can be retrieved for inspection, which is useful to validate results from unintended sample runs. Deviations like a pressure jump or incorrect injection volume can be identified immediately in order to make an informed decision about the validity of the result.
- Pressure sensors at different locations in the system enable sophisticated system diagnostics and maintenance recommendations, such as changing the post-pump filter membrane or the channel membrane.
- Five leak sensors are strategically positioned to catch every possible spill before it can cause damage. A vapor sensor alerts users to escaping volatile solvents.



VISION software

The Eclipse system is operated by VISION software which provides a powerful and easy-to-use interface for method design, system control, sample runs and data analysis.

VISION consists of two modules:

- VISION DESIGN- supports method design, data review and certain analyses
- VISION RUN- controls the system, collects and stores system data.

Computer-aided method development

In combination, VISION DESIGN and VISION RUN support an integrated workflow for method development and optimization:

1. Develop an optimal separation method for an unknown sample based on estimated sizes or size ranges in VISION DESIGN;
2. Transfer the method to VISION RUN to execute the experiment;
3. Reprocess the result in VISION DESIGN for final *in silico* method optimization.

If not known *a priori*, particle size estimates may be obtained with batch DLS, NTA or TEM. With VISION's

capabilities, method development becomes more efficient and less experiments may be required.

The FFF control center

VISION RUN is the control center of the system. Its dashboard view shows all the connected instruments and provides direct access to their configuration.

Sample sequences are started from a single table. The parameters for all components are entered in the table, and the run is started with one mouse click. The methods and sequence tables can be stored, retrieved and appended, which helps to create complex sequences efficiently and with minimal effort.

- Automated functions are available for system purge, flushing of a new membrane and equilibration after a solvent change. The number of injections is logged, and the user can configure a reminder to change the membrane.
- Intelligent solvent management monitors solvent consumption and prevents the system from running dry. The user can choose to recycle the cross flow which is generally pure solvent, enabling longer sample sequences and reducing the effort of solvent preparation.

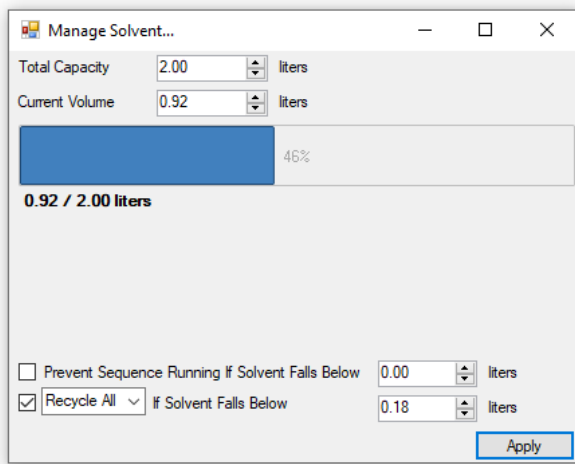


Figure 3. Solvent Management functionality. The software keeps track of solvent consumption; if the reservoir is low, it can either prevent sequences from starting, or recycle eluting solvent back to the bottle.

Diagnosis and advice

VISION RUN receives and processes live input from the sensors and system parameters, diagnosing system status and actively informing the user of any deviations.

- The Coriolis flow devices deliver information on flow rates and density at 3 different points in the flow path. Software analyzes this information and conveys essential messages regarding the system status. For example, the system knows when a solvent exchange is completed since the solvent density becomes uniform in all measurements.
- The position and operation of each and every switching valve is monitored; inlet, channel and cross flow pressures are measured and compared to the expected values. Discrepancies are run through smart algorithms to figure out when things are not as they should be and the user is informed.
- Leak and pressure sensors in the Eclipse are monitored continuously and analyzed to immediately pinpoint the type and specific location of problems, should they arise.

Eclipse channels

The FFF channel, where separation takes place, is a crucial element in a successful FFF-MALS experiment. With the release of Eclipse NEON, Eclipse channels have been redesigned for major performance improvements.

The new channel blocks are machined from 316 stainless steel which are more rigid and resist internal volume expansion from fluid pressure.

The channel cavity is formed by the trapezoidal shape cut into the spacer. The spacer needs to be perfectly sealed to the top and bottom blocks to prevent sample loss and pressure fluctuations. In the new Eclipse channels, two O-rings seal against the PTFE-coated spacer from above and below. This arrangement guarantees robust, reliable sealing every time the channel is assembled.

Variable vs. fixed height

Traditional FFF channels offer spacers with different thicknesses as a flexible means of adapting to varied applications. In addition to these 'variable-height channels', Wyatt also offers a novel channel design with a fixed channel height. 'Fixed-height channels' offer simplicity of assembly with better run-to-run reproducibility of the retention time, even after changing the membrane. The channel cavity is milled directly into the top plate, which forms a metal-to-metal seal with the bottom plate. Unlike variable-height channels, no torque wrench is needed during assembly, and the channel height is guaranteed to be highly reproducible.



Figure 4. Exploded view of the Eclipse channel. The top and bottom plates are machined from 316 stainless steel for stability. A temperature regulation block added to the bottom plate keeps the temperature stable to improve reproducibility of the retention time.

Built-in temperature regulation

All Eclipse NEON channels come with a temperature regulation block. It is screwed directly on to the bottom plate and keeps the channel at a constant temperature, which may be set from ambient to 50°C. This simple and efficient device plays an important role in improving reproducibility of the retention time, as shown in Figure 5.

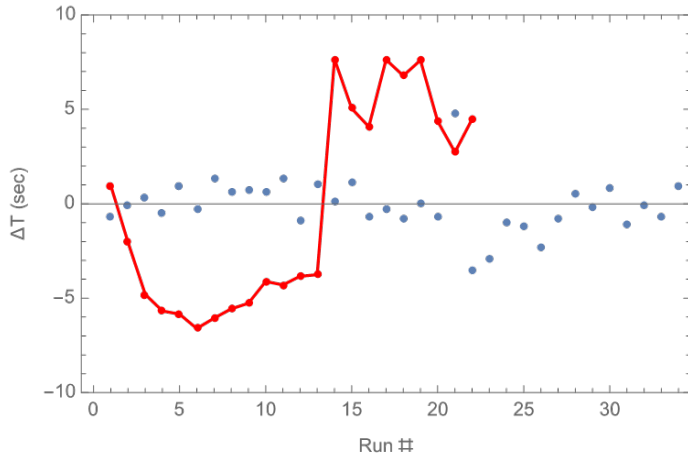


Figure 5. Effect of temperature regulation on the monomer elution time ΔT across multiple BSA runs. Without regulation (red line), the elution time fluctuates by ± 8 seconds with diurnal changes of the ambient temperature. Upon activating the temperature regulation (blue dots), a much tighter run-to-run variation, ± 2.5 seconds, is achieved.

Selecting the optimal channel for the job

A range of channel geometries is available to accommodate different applications. The benefits of having different channels are:

- **Get optimal results for a specific sample load.** From SC (Short Channel) to LC (Long Channel) to SP (Semi-Preparative Channel) sample load can be increased, starting from less than a microgram on SC to milligrams per injection on the SP channel. The flow program can be recalculated by VISION DESIGN to effectively transfer separation conditions from one geometry to another.
- **Purify fractions.** The SP channel is most suitable for use with a fraction collector, which is also controlled by VISION RUN. For SP applications, the Dilution Control Module (detailed in the next section) is indispensable; it prevents the higher dilution prevalent with the thick spacers usually used with the SP channel, and concentrated fractions can be generated.

- **Optimize efficiency versus concentration stress on the sample.** Depending on the application, maximum resolution and efficiency (narrow peaks) may be preferred, or the gentlest possible conditions may be more important. Higher resolution and narrow peaks are the result of high cross flow density. At the same volumetric flowrate SC will have superior resolution compared to LC or even SP. On the other hand, the concentration of the sample at the membrane is reduced with bigger channel area. Proteins and most virus particles will tolerate higher cross flow density compared to most liposomes or lipid nanoparticles.

For very sensitive samples, the Dispersion Inlet Channel is preferred, because it works without a focusing/relaxation step that concentrates the sample against the membrane. Instead, the sample is driven towards the membrane on the fly by a cross flow entering the channel from the top plate downstream of the channel inlet port.

- **Specialized channel: Mobility.** This channel, together with the Mobility module, is useful for the determination of sample charge, as explained below.

Dilution Control Module

The Dilution Control Module (DCM) principle is shown in Figure 6. Briefly, excess solvent is removed from the channel in a carefully controlled fashion before it has the opportunity to mix with the separated sample exiting the channel. The DCM is a proprietary advancement which has several benefits:

- The sample concentration at the detector can be increased by a factor of up to 5 for analytical purposes (with negligible loss of resolution) and up to 10 for fraction collection or samples with a broad size distribution.
- The detector flow rate is measured and controlled with high precision—typically better than 0.1%—and the pressure in the channel is regulated at the same time. This increases the reproducibility of the retention time and improves the signal-to-noise ratio of the detector chain.
- The detector flow becomes independent of the channel flow rate. Separation methods using high channel-

flow and cross-flow rates are now possible, which increases efficiency while maintaining a moderate flow rate at the detector to avoid overpressure. In conjunction with the intelligent solvent management feature, DCM outlet flow consisting of pure solvent can be recycled to minimize solvent consumption.

- Dynamic light scattering is less sensitive than MALS, and its accuracy (especially for larger particle sizes) is impacted by high flow rates. The combination of reduced detector flow rate and increased sample concentration specifically improves DLS measurements. Meaningful results can be obtained at lower concentrations and larger particle sizes.

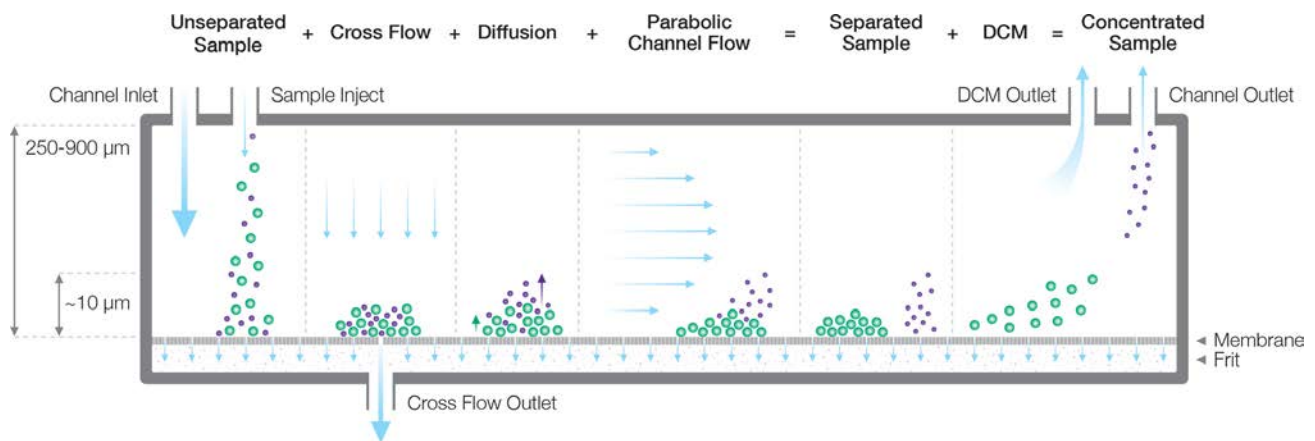


Figure 6. Principle of the Dilution Control Module. During the elution step, the DCM port is activated, and a programmable part of the channel flow is extracted upstream of the channel outlet. This reduces the dilution of the sample layer in proportion to the volume ratio at the two ports.

Eclipse Mobility

The Eclipse Mobility module realizes electrical/asymmetric-flow FFF (EAF4). The method, depicted in Figure 7, applies an electrical field parallel or anti-parallel to the cross flow during the elution process. This generates a force on particles that depends on their charge, causing a shift in

retention time compared to an experiment without an electrical field. The shift in retention time is determined as a function of the electrical field strength to calculate the electrophoretic mobility³. As the particle size may be

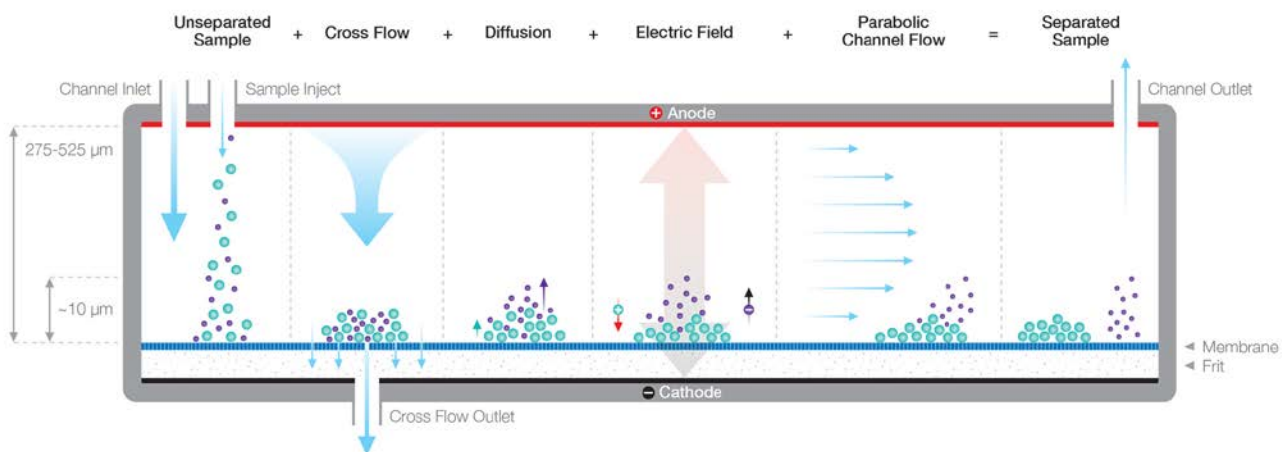
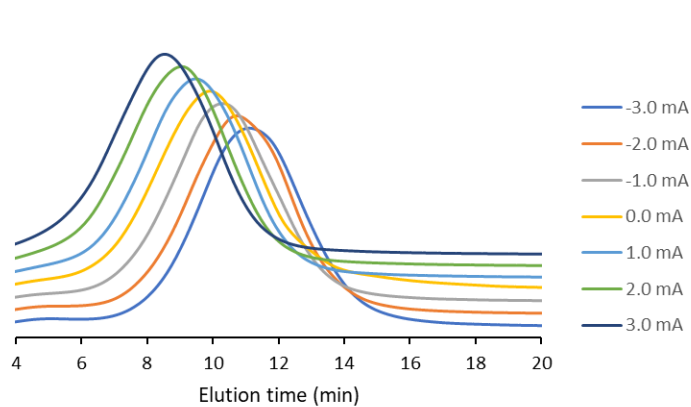


Figure 7. Principle of electrical/asymmetric-flow field-flow fractionation (EAF4). Two electrodes are added to the channel: one at the top plate and the counter electrode beneath the frit. Upon applying a current, an electrical field is generated which exerts a force on the sample components in proportion to their charge. This interaction results in a shift in retention time, from which the electrophoretic mobility can be calculated. Combined with the known size from light scattering or retention time, the zeta potential can also be determined.

determined from light scattering or estimated from the retention time using FFF theory, the zeta potential can be calculated, as shown in Figure 8.

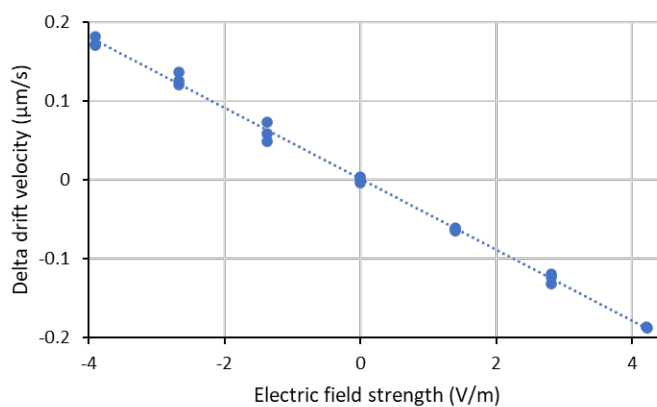
The unique capability of EAF4 is calculation of zeta potential for different populations in the fractogram. A peak containing equally sized particles with different charges will split under an applied field, while distinct peaks will shift to different degrees depending on the specific zeta potential of each. For a sample with a broad size distribution, the homogeneity of the charge distribution can be estimated. This is done by overlaying the shifted fracto-



grams. If the shape changes under influence of the electrical field, then different components have different charge.

Mobility channel design

The Mobility channel has been engineered to be robust and durable. The frame is made of titanium and the electrodes are platinum, preventing corrosion even under high-salt buffers. The channel can be operated at pressures of 20 bars, eliminating bubbles which may be formed by electrolysis at the upper electrode at higher field strength, thus extending the range of operation.



Hydrodynamic radius (nm)	Diffusion coefficient (10^{-12} m ² /s)	Electrophoretic mobility (10^8 m ² /V·s)
50.00 ± 0.15	5.572 ± 0.017	-4.50 ± 0.06

Figure 8. Determination of mobility and zeta potential and net charge from the shift of retention time for a polystyrene latex particle. The slope of a linear regression of drift velocity versus the electrical field strength gives the electrophoretic mobility.

FFF-SEC switching

The optional FFF-SEC switching function, which may be incorporated into the Eclipse instrument, provides the flexibility of using either SEC-MALS or FFF-MALS without manually switching fluidic pathways – the user need only select the separation mode in the VISION sequence. This capability doubles the return on investment in the FFF-MALS system, since it utilizes the same fluidic components (degasser, pump, autosampler) and the same detector modules (UV, MALS, DLS, dRI) for both characterization methods. One use case is to compare results between both separation modes for a specific sample, using the same instrumentation and the exact same sample solution, as illustrated in Figure 9.

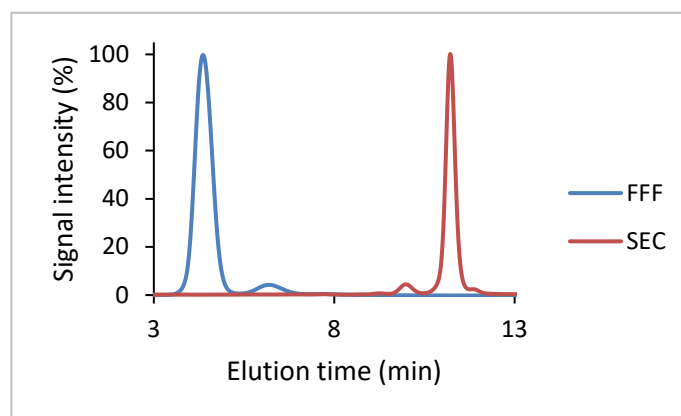


Figure 9. Comparison of BSA separations via FFF and SEC done with the FFF-SEC switching module. Both experiments drew BSA from the same vial. This is a useful configuration to compare these orthogonal methods.

Summary

Wyatt Technology pioneered the use of MALS to measure absolute molar mass and size distributions downstream of FFF, and the Eclipse NEON system brings to bear three decades of experience of FFF coupled to light scattering. Top-notch engineering and cutting-edge technological advances have provided solutions to pressing issues raised by customers, leading to a system that is suitable for demanding pharmaceutical quality control applications as well as research and development.

A complete FFF-MALS system (Figure 10), controlled by VISION software and running ASTRA software for detector data acquisition and analysis, raises FFF-MALS to a whole new level of performance, robustness, productivity, versatility, and usability.

To learn more about Eclipse and FFF-MALS, visit www.wyatt.com/Eclipse or www.wyatt.com/FFF-MALS. To schedule an application review and/or demo, please contact us via the Product Information Request form on the Wyatt web site: <https://www.wyatt.com/products/eclipse-product-info-request.html>.



Figure 10. A complete Wyatt FFF-MALS system includes an Eclipse FFF controller and channel, Agilent 1260 fluid handling modules (degasser, pump, autosampler) and a full contingent of online detectors: DAWN (MALS), Optilab (dRI), and Agilent 1260 UV (VWD/MWD/DAD).

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