Improving Charge Variant Analysis with Maurice Native Fluorescence

Introduction

Most post-translational and degradation events affect the biological activity of therapeutic proteins, making charge heterogeneity analysis a critical quality attribute for molecule characterization. Both iCE280 and iCE3 use protein absorption at 280 nm to monitor charge heterogeneity, and now Maurice adds native fluorescence detection to greatly increase cIEF capabilities.

Maurice's native fluorescence detection for cIEF works by measuring the fluorescence emission of tryptophan's aromatic group. It's label-free so you're not wasting time optimizing protein labeling or dealing with the background noise when label unconjugates from your protein. Baselines are significantly cleaner and less sensitive to ampholyte interference, giving you more options when optimizing your pH gradient.

You'll also get 3-5X more sensitivity compared to UV absorption. That means you can nix concentrating or desalting your samples, decreasing your sample preparation time. And, because proteins tend to aggregate less at lower concentrations, you'll be able to reduce or even remove urea completely in some of your methods.

Meet Maurice and Maurice C.

Maurice and Maurice C. use the cIEF cartridge (**Figure 1**) to analyze protein charge heterogeneity and identity. Each cartridge lets you run a total of 100 injections in a max of 20 batches and has an RFID tag that tracks how many injections you have left. The set up is very easy (**Figure 2**), simply prepare your samples, batch reagents, and cIEF cartridge, then put everything in Maurice. After you set up your batch parameters in Compass for iCE, hit **Start** and watch as Maurice simultaneously delivers high resolution absorbance and fluorescence data for every injection.

FIGURE 1. cIEF Cartridge.

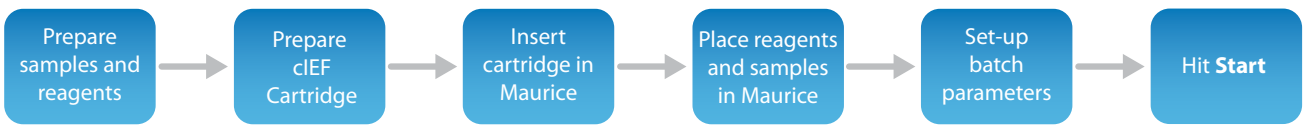

FIGURE 2. Maurice simplifies your cIEF workflow.

cIEF fluorescence mode

SAMPLE AND CARTRIDGE PREPARATION

Preparing samples for iCE3 or iCE280 has always been simple, and nothing's changed with Maurice. You'll still prep samples with methyl cellulose, carrier ampholytes, pI markers, and additives like arginine or urea. The only difference is you'll need to use Maurice pI markers instead, since they are specifically formulated for both absorbance and fluorescence detection modes. Batch reagents, like 0.5% Methyl Cellulose, will also be familiar for those of you who routinely run iCE methods. Maurice just has an extra fluorescent calibration standard that you'll run with each batch to make sure you get the cleanest baselines.

STARTING YOUR RUN

To prepare your cartridge, pipet 2 mL of Anolyte into the H+ electrolyte tank (red) and 2 mL of Catholyte into the OH- electrolyte tank (white). Seal the electrolyte tanks by firmly inserting the rubber stoppers in the tanks and you're ready to go.

Place your reagents, samples, and cIEF cartridge in Maurice, set up your batch parameters in Compass for iCE, hit **Start** and Maurice takes over. Compass for iCE monitors your runs in real-time and analyzes the injection data for you automatically.

Maurice's cIEF cartridge uses a 100 µm ID x 5 cm FC-coated capillary. The capillary is pressure-filled with 0.5% Methyl Cellulose at the beginning of each injection before sample is vacuum loaded. Voltage is then applied so proteins will migrate to their isoelectric point. Separation is monitored in absorbance in real-time with Compass for iCE and at the end of the separation an absorbance reading and multiple fluorescence readings are taken so you'll have data for both modes of detection for each injection. Fluorescence exposure times can be edited when you set up your batch so you can increase your exposure time whenever you need just a little bit more sensitivity. Added bonus - you can start analyzing completed injections with Compass for iCE while the batch is still running. And it's only a one-click operation to switch between viewing your data in absorbance or fluorescence mode.

At the end of the batch, simply remove your samples and reagents. Maurice automatically cleans your cartridge at the end of the batch so all you need to do is rinse the electrolyte tanks three times with deionized water using an electrolyte pipet or low vacuum. Then just store the cartridge in its original packaging until you're ready for your next batch. All of the waste is retained in the cartridge, so you don't need to worry about handling hazardous waste at the end of the run. Once the cartridge reaches it's injection limit, discard as per your institute's safey guidelines.

Super quiet baselines

Baselines in native fluorescence mode on Maurice are quieter than ever since carrier ampholytes don't fluoresce at the 320-450 nm emission wavelength used to detect proteins. Samples containing just 2 to 8% 3-10 Pharmalyte had significantly quieter baselines in fluorescence mode compared to absorbance mode (**Figure 3**). Low baseline

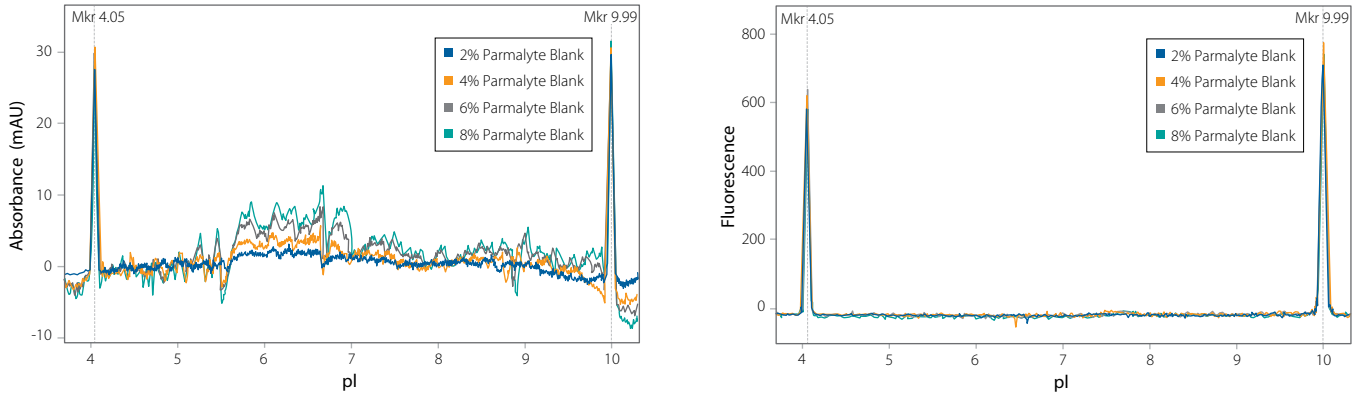

FIGURE 3. Comparison of Pharmalyte backgrounds observed in Maurice's absorbance mode (left) and fluorescence mode (right). Samples containing 2-8% of 3-10 Pharmalyte were loaded into the cartridge and pre-focused for 1 min at 1500 V followed by focusing for 4.5 min at 3000 V. The fluorescent image was taken with a 10 sec exposure and baseline uncorrected data is shown. Baselines were significantly lower in fluorescence, even in the presence of as much as 8% carrier ampholyte.

noise means more options when it comes to optimizing a pH gradient that will gives you the best resolution for your molecule. Carrier ampholyte noise can also be lower in fluorescence mode compared to absorbance mode when using 1% Servalyts. So you can get quieter baselines and better signal-to-noise if you're using Servalyts to analyze your fusion proteins.

Quieter backgrounds also make method transfer easier. Because you won't need to worry about minimizing background noise during method development, you can just focus on optimizing the signal and resolution for your molecule.

Maurice detects different protein types

We tested three different types of proteins with different pIs, isoform profiles, and molecular weight ranges to show how versatile Maurice fluorescent mode is. Separation conditions for a 70 kDa therapeutic protein, 200 kDa fusion protein, and 150 kDa monoclonal antibody separation conditions were optimized for resolution and signal for each molecule (**Figure 4**).

All proteins had intrinsic fluorescent properties that generated high resolution profiles, confirming there is no bias when it comes to the type of protein you can run on Maurice. Any protein that contains tryptophan residues can be analyzed on Maurice in fluorescence mode with an ampholyte mixture that delivers the required resolution.

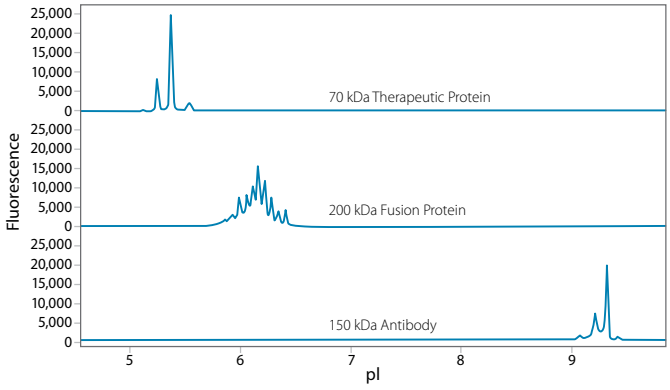

FIGURE 4. A therapeutic protein, fusion protein, and a monoclonal antibody were run on Maurice and detected in fluorescence mode. 200 µg/mL of therapeutic protein and 250 µg/mL of mAb were run with 6% 3-10 Pharmalyte, 10 mM arginine, 10 mM iminodiacetic acid, and 2M urea, then separated for 1 minute at 1500 V followed by 7 minutes at 3000 V. 400 µg/mL of fusion protein was run with 4% 5-8 Pharmalyte/2% 3-10 Pharmalyte, 10 mM arginine, 10 mM iminodiacetic acid and focused for 1 minute at 1500 V followed by 8 min at 3000 V. The fluorescence exposure time was 10 seconds for the fusion protein and mAb, and 5 seconds for the therapeutic protein.

Maurice linear dynamic range

The linear dynamic range of Maurice's cIEF fluorescence mode was determined by serially diluting a monoclonal antibody. Sample was serially diluted 2-fold from 125 µg/mL down to 0.5 µg/mL in 1X Sample Buffer to determine the linear dynamic range you'll get with Maurice in fluorescence mode (**Figure 5**). The total peak area was extremely linear across 2-logs with an R^2 of 0.999.

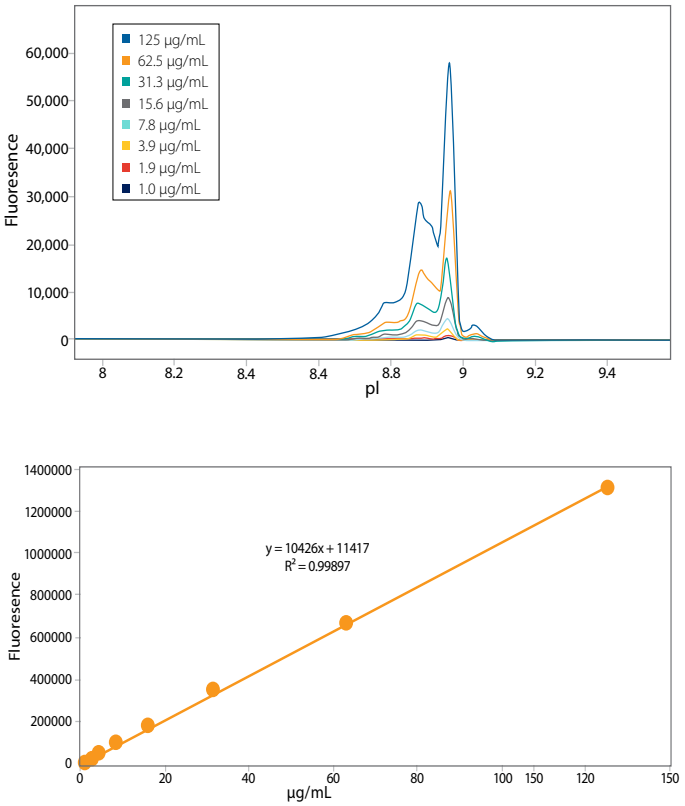

FIGURE 5. A monoclonal antibody was serially diluted from 125 µg/mL down to 0.5 µg/mL to demonstrate the linearity of fluorescence on Maurice (top). Linear regression of the total peak area demonstrates 2-log linearity with an R^2 of 0.999 (bottom). The fluorescence image was taken with a 40 second exposure time.

Increased sensitivity

A dilution series of mouse IgG was run on Maurice to demonstrate the increased sensitivity fluorescence mode delivers over absorbance. Samples were serially diluted in 1X Sample Buffer and the limit of detection (LOD) of the main peak (Peak 4) was calculated by dividing three times the standard deviation of the noise by the dilution curve slope.

The LOD for Peak 4 of the IgG was 3 µg/mL in absorbance mode, but fluorescence was able to detect the IgG with as little as 0.7 µg/mL (**Figure 6**). That's more than a 4X gain in sensitivity! The best part? Maurice detected an additional peak (Peak 6) in fluorescence mode because of the improved signal to noise.

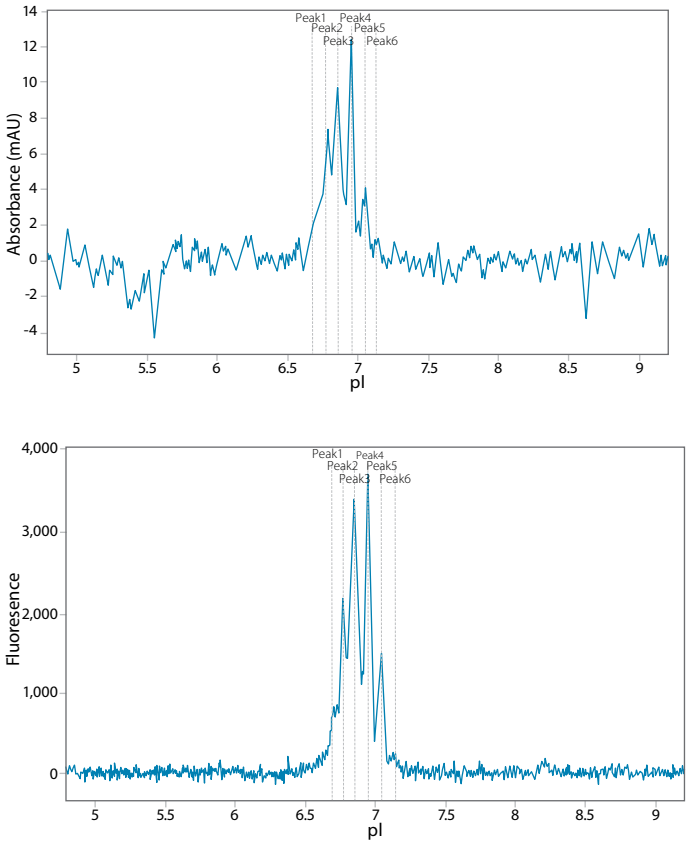

FIGURE 6. Fluorescence detection (bottom) is 4X more sensitive compared to absorbance (top). A serial dilution was used to calculate the LOD of Peak 4, and data from the 15.6 µg/mL sample shown. Samples were prepared in 4% 3-10, 10 mM arginine, 10 mM iminodiacetic acid and focused for 1 min at 1500 V followed by 6 min at 3000 V. The fluorescence exposure time was 30 seconds.

For those proteins with fewer tryptophan residues, you can always increase the concentration of sample to increase your signal. You can also compensate for lower intrinsic fluorescence by taking a longer exposure when you need even higher sensitivity (**Figure 7**). Just make sure your profile stays in the method linear dynamic range and keep an eye out for signal quenching at the high end of the profile to make sure you data stays quantitive.

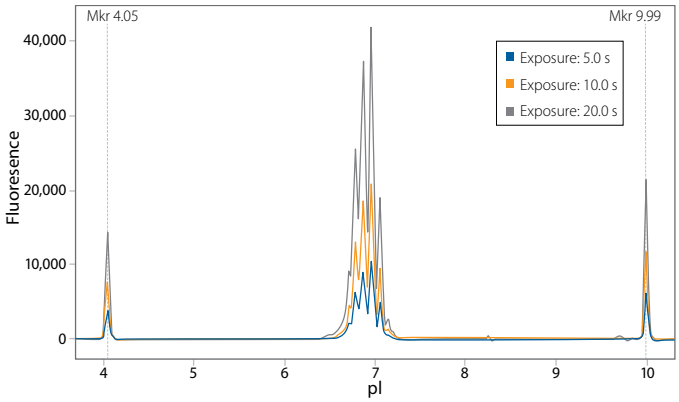

FIGURE 7. Increasing the fluorescence exposure time can increase signal. A mouse mAb was run on Maurice with 3-10% Pharmalyte, 10 mM arginine, and 10 mM iminiodiacetic acid and pre-focused for 1 minute at 1500 V followed by focusing for 6 minutes at 3000 V. Data for a 5 second, 10 second, and 20 second fluorescence exposures time shown.

Reproducible data with Both Modes

When you look at post-translation modifications of a single molecule, charge profiles between absorbance and fluorescence modes will be comparable since the tryptophan content will be the same for each isoform. To confirm this, we compared absorbance and fluorescence profiles for the therapeutic protein, fusion protein, and monoclonal antibody and found them to be equivalent (**Figure 8**). But, in cases when you're looking at multiple molecules in one injection, just keep in mind that the tryptophan content between the two molecules might be different. So you may see different ratios of molecule 1 to molecule 2 when comparing between the two detection modes.

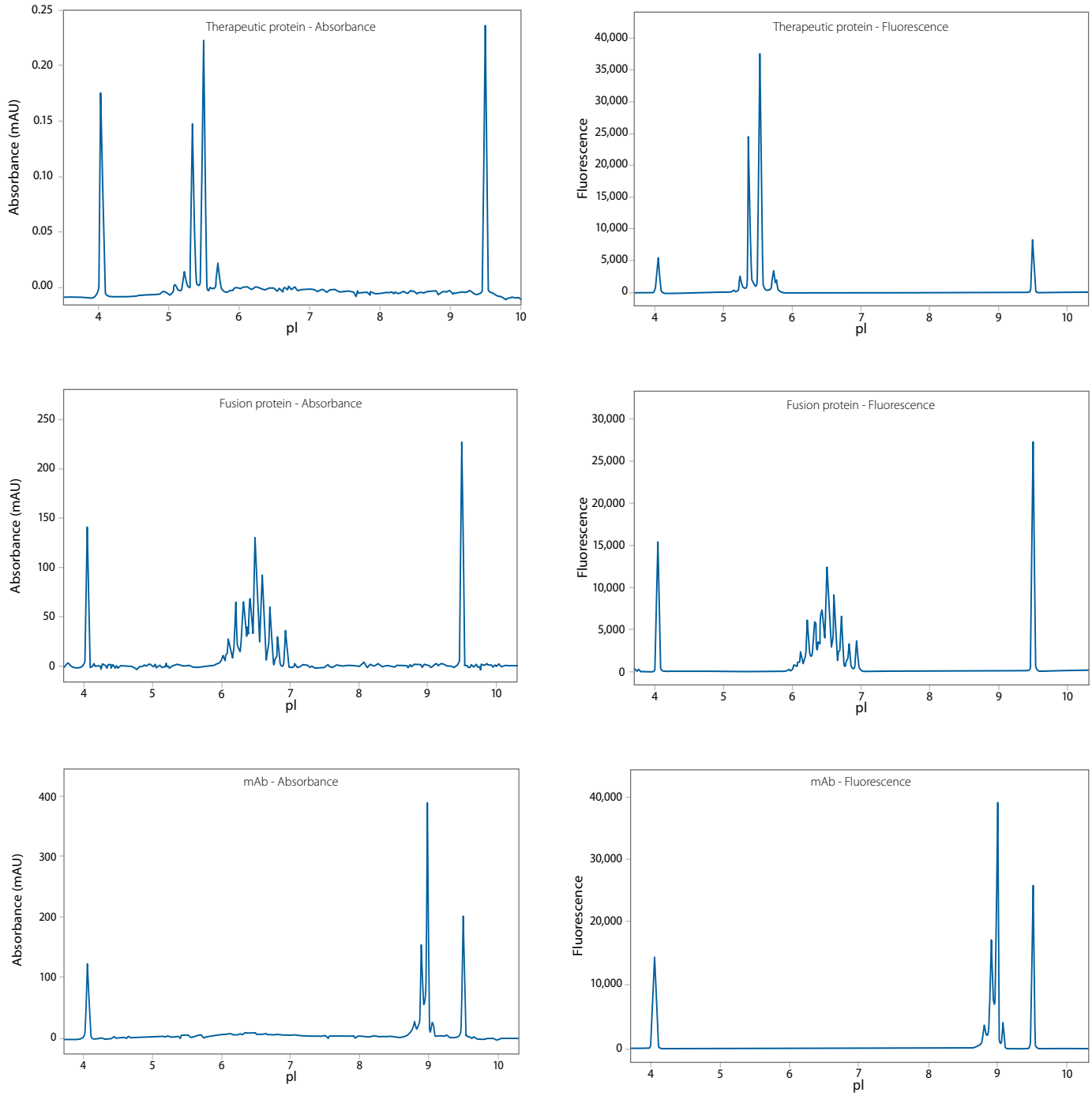

FIGURE 8. Comparison of charge profiles in both absorbance and fluorescence mode. The therapeutic protein (top), fusion protein (middle), and mAb (bottom) were run on Maurice using the same the methods described in Figure 4 to demonstrate equivalence between absorbance (left) and fluorescence (right) detection. Profiles between all three molecules were all comparable.

We also separated the mouse IgG across 100 injections and found equivalent profiles at the beginning and at the end of the batch, demonstrating Maurice's robustness in both detection modes (**Figure 9**). Quantitation comparison between absorbance and fluorescence detection confirmed equivalency of data collected with the two modes across the board with % compositions between absorbance and fluorescence all within 1% (**Table 1**). Data was also robust in both modes with RSDs all under 6%, and RSDs for peaks with greater than 10% composition all under 3%.

Simpler sample preparation

The extra sensitivity you get with Maurice's fluorescence mode has a few other benefits besides better detection. The added sensitivity can save the time it takes to concentrate your sample and also enables you to dilute the salt concentration of your samples instead of desalting them. In both cases, you'll save time and remove the chance that your sample might change during additional prep procedures.

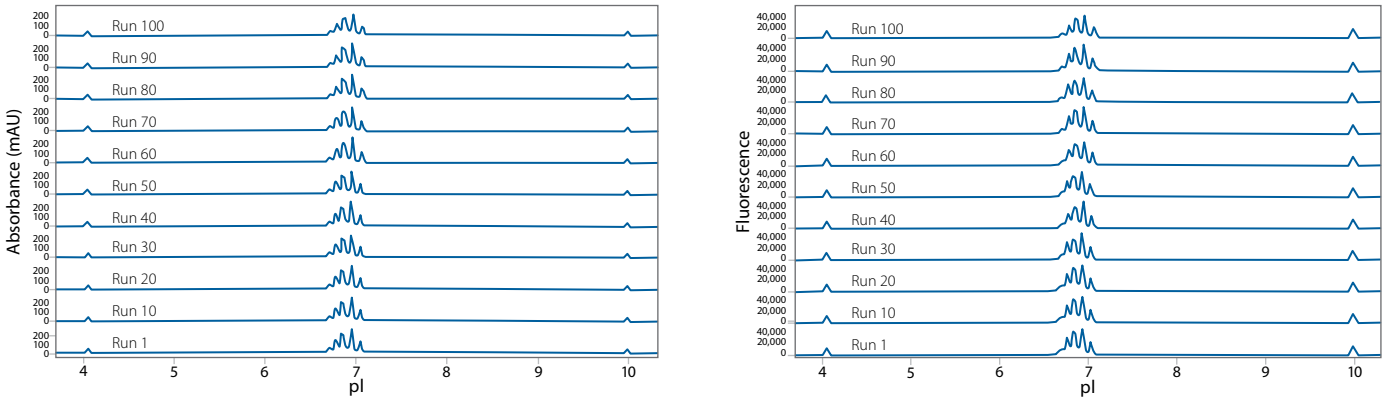

FIGURE 9. Mouse IgG was run across 100 injections on Maurice. Data for 11 injections across the batch shows how robust the data is from the beginning to the end of the batch. Additionally, absorbance data (left) and fluorescence data (right) demonstrates equivalency between the two detection modes. The fluorescence exposure time was 20 seconds.

	% COMPOSITION (ABSORBANCE)						% COMPOSITION (FLUORESCENCE)					
Injection	Peak1	Peak2	Peak3	Peak4	Peak5	Peak6	Peak1	Peak2	Peak3	Peak4	Peak5	Peak6
1	6.90	17.50	32.90	28.90	11.00	2.80	6.70	18.90	31.20	29.10	12.10	2.00
10	7.50	17.70	31.80	29.10	11.30	2.70	6.90	19.60	31.90	27.40	12.20	2.00
20	6.80	18.40	32.40	28.40	11.30	2.70	6.90	19.20	31.80	27.70	12.20	2.00
30	7.20	18.10	32.00	28.90	11.30	2.50	6.50	19.10	31.90	28.40	12.20	2.00
40	6.30	18.80	32.90	27.80	11.40	2.70	6.80	19.40	31.90	27.70	12.10	2.10
50	6.40	18.30	32.60	28.80	11.30	2.70	7.00	18.50	31.80	28.40	12.30	2.00
60	6.40	18.60	32.50	28.70	11.20	2.60	6.70	19.40	32.00	27.80	12.20	1.90
70	6.70	18.40	31.80	28.90	11.40	2.70	7.10	19.60	31.20	27.80	12.30	2.10
80	6.20	19.20	31.50	29.00	11.60	2.60	6.50	19.10	31.00	29.10	12.30	2.00
90	6.80	18.00	32.20	28.80	11.60	2.60	6.50	19.20	31.80	28.30	12.20	2.00
100	6.80	18.10	31.70	29.20	11.50	2.70	6.70	19.20	31.70	28.10	12.10	2.10
Average	6.73	18.28	32.21	28.77	11.35	2.66	6.75	19.20	31.65	28.16	12.20	2.02
Std. Dev.	0.39	0.48	0.49	0.38	0.18	0.08	0.21	0.32	0.35	0.56	0.08	0.06
% RSD	5.84	2.64	1.51	1.34	1.54	3.04	3.06	1.65	1.10	2.00	0.63	2.99

TABLE 1. % peak compositions between absorbance and fluorescence. Quantitation and summary statists for the % composition of a mouse IgG demonstrate comparable data between the two detection modes and instrument precision across 100 injections.

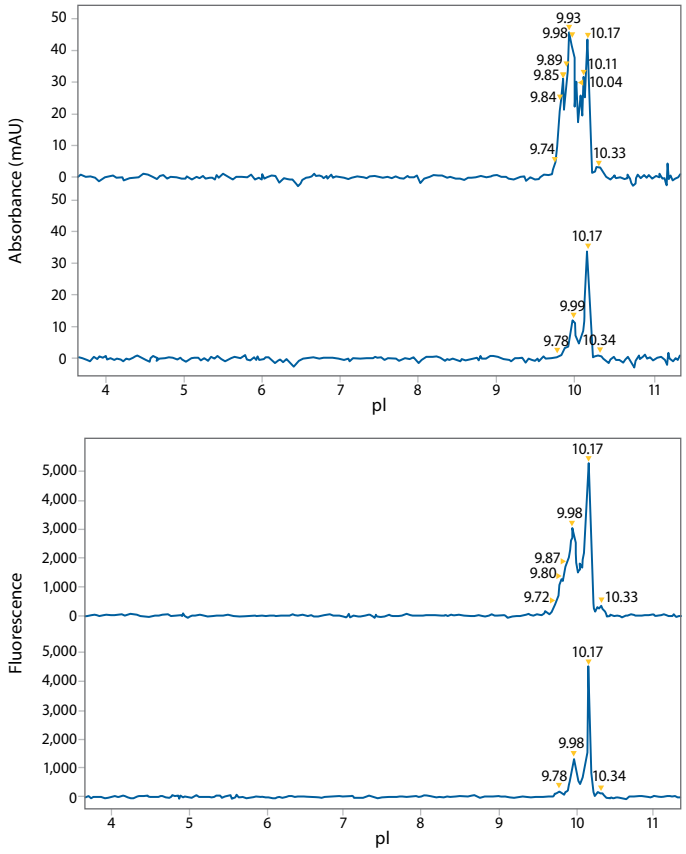

FIGURE 10. Samples normally run with 4 M urea still exhibit protein aggregation at 0.4 mg/mL (top electropherogram). Protein aggregation is no longer observed at 0.1 mg/mL (bottom electropherogram). However, minor peaks at 9.78 and 10.34 are more easily detected in fluorescence mode (bottom panel) compared to absorbance mode (top panel) due to the signal-to-noise improvement. The fluorescence exposure time was 10 seconds.

Some proteins also tend to aggregate at higher concentrations. So you may be able to remove urea from your method by analyzing less protein in fluorescence mode. This is a huge advantage since urea needs to be made fresh every day to avoid isocyanic acid, a degradation product that increases the % composition of your molecule's acidic species. To demonstrate this, a model IgG that requires 4 M urea to prevent aggregation at 1 mg/mL in absorbance mode was run on Maurice at 0.4 mg/mL and 0.1 mg/mL (**Figure 10**) without urea. At 0.1 mg/mL, the IgG no longer shows the signs of aggregation still present at 0.4 mg/mL. However, the signal-to-noise with absorbance detection made it difficult to discern some of the minor peaks. The cleaner baselines and higher sensitivity with fluorescence detection allowed detection of the minor peaks with only a 10-second exposure time in the 0.1 mg/mL sample. You can improve that even further if you use longer exposure times.

Conclusion

Maurice's cIEF application gives you the same great absorbance data you've always gotten with the gold-standard iCE3/iCE280, and now you'll also get native fluorescence detection for every injection. Experiments showed equivalent data between absorbance or fluorescence mode with precision RSDs below 6% for all six peaks across 100 injections. And when we ran three different types of proteins there wasn't any bias between detection modes.

Baselines with fluorescence detection are also much quieter, simplifying method development and transfer. Fluorescence detection also gives you 4X more sensitivity than absorbance. So you can potentially save time since you won't have to concentrate your sample to get within the linear detection range or desalt them to maximize resolution. And last but not least, the improved sensitivity means you may be able to remove urea from your method if you can run sample concentrations that are low enough where the molecule no longer aggregates. So cIEF methods on Maurice gives you the same great data you've come to rely on but takes it up a notch with native fluorescence mode.

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