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Automated Elimination of Autofluorescence Interference in Flow Cytometry

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BACKGROUND

Most assays in flow cytometry rely on signals from exogenously added fluorescent labels. However, often an unwanted background to the signal arises due to cellular autofluorescence (AF). Common sources of AF are the metabolic cofactors nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FAD). These two compounds are excited strongly at commonly used UV and violet wavelengths, and their emission spectrum is broad (**Figure 1**), contributing unwanted background to most spectral channels. Kinetic River is leveraging our proprietary time-resolved flow cytometry technology to address this problem. Time-resolved flow cytometry uses a modulated laser source that can excite the sample and monitor fluorescence decay (fluorescence lifetime) hundreds of times per event (**Figure 2**).

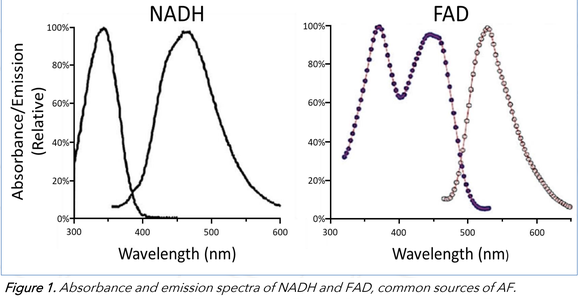

Figure 1. *Absorbance and emission spectra of NADH and FAD, common sources of AF.*

METHODS

We have developed the *Colorado* time-resolved multiparametric analyzer to address the problem of AF interference in cell assays. The system uses pulsed laser excitation at 375 nm (QuixX 375, Omicron Laserage) and detection at 460/36, 510/42, 593LP (channels which overlap with AF from NADH and/or FAD), FSC, and SSC. Each channel is capable of discriminating multiple fluorophores based on their lifetime decays and analysis of a multi-exponential decay curve (**Figure 3**). Ultrastable sheath flow is established with our custom-built *Shasta* fluidic control system for both sample and sheath (see Poster #261 – "A Highly Customizable Fluidics Control Module for Flow Cytometry"). Data acquisition was performed using 4-channel 1.25-GHz sampling on an NI PXI platform and custom-written LabVIEW code. Signal processing was performed on a dedicated computing platform running custom-written algorithms.

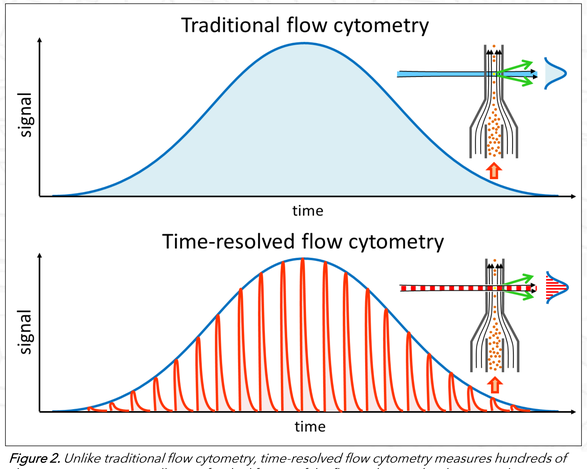

Figure 2. *Unlike traditional flow cytometry, time-resolved flow cytometry measures hundreds of decay curves per event, allowing for the lifetime of the fluorophore to be determined.*

RESULTS

Using 6-Peak Rainbow Calibration Beads (Spherotech), we characterized the FL1 (blue) and FL2 (green) channels (**Figure 4**). In both channels we were able to resolve 5 ½ peaks, corresponding to a minimum of 2.5 decades of dynamic range. The sensitivity of each channel was measured using silica microspheres

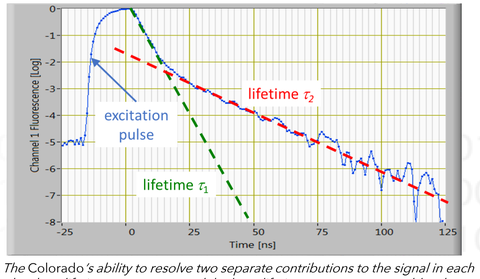

Figure 3. *The* Colorado *'s ability to resolve two separate contributions to the signal in each channel - the short-lifetime component and the long-lifetime component - enables the automated removal of AF contribution from each channel.*

(Spherotech) which exhibit essentially no AF. To test the *Colorado*'s ability to remove the contribution of AF, we used eosinophils, which are highly autofluorescent, stained for CD45 with either AlexaFluor 405 or QDot525 (ThermoFisher) in FL1 and FL2 respectively. Cellular autofluorescence, with a lifetime in the range of roughly 0.5 to 3 nsec, comprises the short lifetime component contaminating both the FL1 and FL2 channels. Both exogenous fluorophores exhibit detectably longer lifetimes and comprise the long-lifetime decay component in FL1 or FL2.

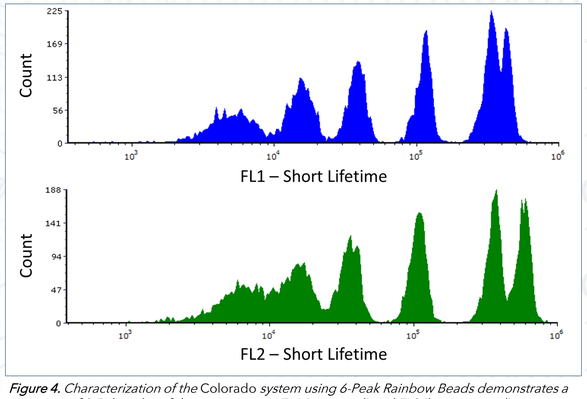

Figure 4. *Characterization of the* Colorado *system using 6-Peak Rainbow Beads demonstrates a minimum of 2.5 decades of dynamic range in FL1 (top panel) and FL2 (bottom panel)*

Whereas conventional cytometry cannot distinguish AF signal from that of the fluorophore, the *Colorado* can do so easily (**Figure 5**). Stained and unstained eosinophils (eos) have an overlapping fluorescence component in both FL1 (**Figure 5A**) and FL2 (**Figure 5B**). When using lifetime to distinguish between the AF and the exogenous fluorescence (**Figures 5C** and **5D**), stained cells are easily distinguished from unstained cells.

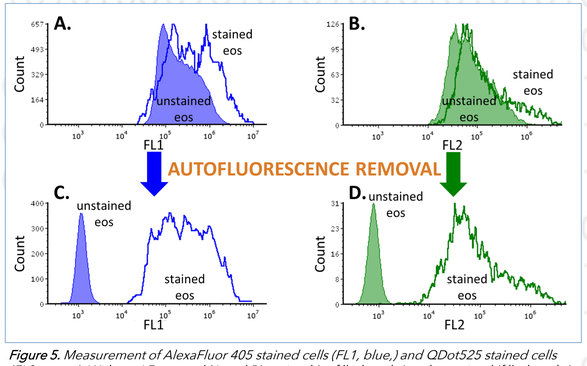

Figure 5. *Measurement of AlexaFluor 405 stained cells (FL1, blue,) and QDot525 stained cells (FL2, green). Without AF removal (A and B), stained (unfilled peaks) and unstained (filled peaks) eosinophil populations overlap in intensity. They are easily distinguished after automated AF removal (C and D).*

CONCLUSION

We designed and built the *Colorado*, a novel flow cytometry system capable of distinguishing AF from that of exogenous labels by exploiting a typically unused property of fluorophores (lifetime decay). The *Colorado* incorporates Kinetic River's proprietary time-resolved flow cytometry technology. We have demonstrated that this system is capable of distinguishing the signal derived from exogenously added fluorophores from AF background (e.g., from NADH and FAD). Ultimately, we will expand the system's multiplexing capabilities by increasing the number of lasers and detection channels and creating a cell analyzer platform that can automatically eliminate interference from AF.

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