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# Compensation-Free Flow Cytometry

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## BACKGROUND

In flow cytometry, compensation is required to account for and remove the contribution of signal spillover between spectral channels. The problem worsens as the level of multiplexing increases and more wavelength bands are crowded into a limited spectral window (**Figure 1**). While the technique of spectral flow cytometry can resolve some overlapping fluorophores, it requires spectral unmixing, which is simply another term for compensation; and it does not reduce the population spread associated with the overlaps. To solve these problems, we have developed time-resolved flow cytometry technology that distinguishes completely overlapping fluors based on differences in their fluorescence decay lifetimes (**Figure 2**). In doing so, we can increase the multiplexing capability of our instrument while avoiding spectral spillover.

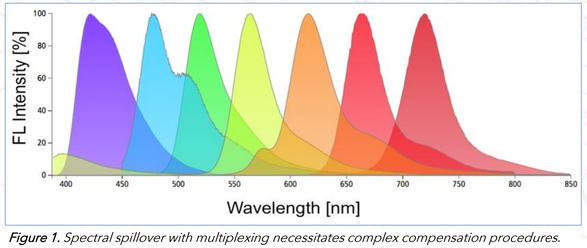


*Figure 1. Spectral spillover with multiplexing necessitates complex compensation procedures.*

## METHODS

We reported previously on our compensation-free feasibility efforts (CYTO 2016–2019). The new 15-parameter *Arno* platform has two spatially separated, pulsed excitation sources (Toptica iBeam 405 and 488); one FSC and two SSC channels; and 12 fluorescence detection channels (6 per laser), from violet to infrared. The 12 fiber-coupled fluorescence signals are sensed by only 6 physical detectors (Hamamatsu PMTs). **Each detector collects light from two spectrally overlapping fluors**, and the contribution from each fluor is resolved using our proprietary time-domain multiplexing technology (**Figure 3**). Ultrastable sheath flow is established with our custom-built *Shasta* fluidic control system (see Poster #261 – "A Highly Customizable Fluidics Control Module for Flow Cytometry"). Data acquisition is performed using 8-channel 1.25-GHz sampling on a National Instrument PXI platform and custom-written LabVIEW code. Signal processing is performed on a dedicated computing platform running custom algorithms.

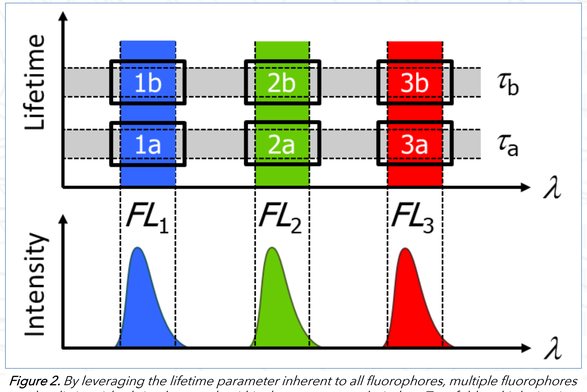


*Figure 2. By leveraging the lifetime parameter inherent to all fluorophores, multiple fluorophores can be distinguished simultaneously within the same spectral window. Two-fold multiplexing is shown here, but 3 or more per channel is possible.*

## RESULTS

The *Arno* system has been fully characterized with respect to sensitivity, dynamic range, CVs, and time resolution in each of the 12 fluorescence 'channels' (six spectral bands, three from each laser, each with both a short- and a long-lifetime component). **Figure 4** shows a typical example of dynamic range measurements from the short-lifetime component of the blue channel.

By having only three detectors per laser, each wavelength band can be spectrally well separated, leading to less than 5% spillover in all channels, with most below 1%. We are in the process of full system validation with sequentially more complex cell-based assays using 4-, 8-, and 12-color panels. **Figure 5** shows a typical example of a multiparameter assay run without compensation on the *Arno*. CD3 and CD4 are detected simultaneously in the **same spectral band** and lymphocyte populations can be easily distinguished. Importantly, although the *Arno* relies on lifetime measurements, data presented to the end-user is no different than that from a conventional flow cytometer. Final validation will include benchmarking our compensation-free results with *Arno* against a traditional flow cytometry system with compensation.

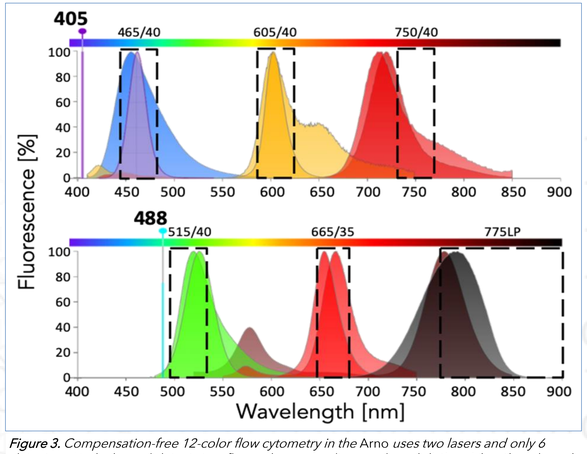


*Figure 3. Compensation-free 12-color flow cytometry in the* Arno *uses two lasers and only 6 detectors. Each channel detects two fluorophores simultaneously and distinguishes them based on their lifetime characteristics. Each channel is spectrally well-separated, eliminating spillover.*

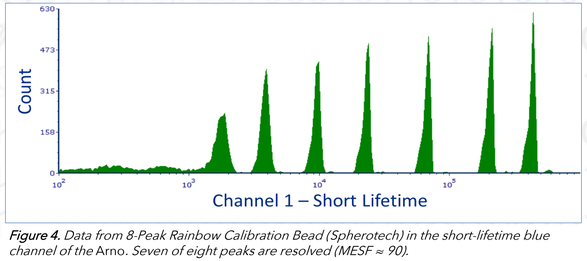


*Figure 4. Data from 8-Peak Rainbow Calibration Bead (Spherotech) in the short-lifetime blue channel of the* Arno. *Seven of eight peaks are resolved (MESF ≈ 90).*

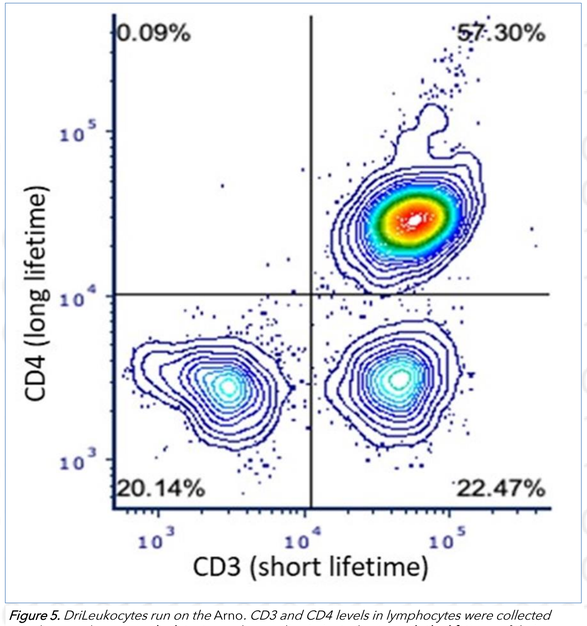


*Figure 5. DriLeukocytes run on the* Arno. *CD3 and CD4 levels in lymphocytes were collected simultaneously on a* ***single detector*** *and were discriminated* ***using only the lifetimes*** *of their respective fluorophores.*

## CONCLUSION

We have expanded on our previous proof-of-concept *Arno* system by designing and building a 15-parameter, compensation-free cell analyzer. Our *Arno* platform leverages Kinetic River's proprietary time-resolved flow cytometry technology to achieve compensation-free 12-color (plus one forward and two side scattering channels) using only two lasers and six spectral detectors.

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*The* Arno *and* Shasta, *or use thereof, may be covered in whole or in part by patents in the U.S. and other jurisdictions. A current list of applicable patents can be found at https://www.kineticriver.com/kinetic-river-corp-patents.*