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Applications of Time-Resolved Flow Cytometry

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BACKGROUND

We have pursued a unified approach of using time-resolved flow cytometry to address a number of long-standing, broadly felt problems and unmet needs in flow cytometry, including spectral spillover, limits on multiplexing, and cellular autofluorescence. Previous attempts at solving these, such as mass cytometry and spectral cytometry, create additional problems (such as obliteration of analyzed cells and increased spreading of population clusters). We have adapted the concept of the long-standing field of measuring fluorescence lifetime under static conditions (fluorescence lifetime imaging microscopy, or FLIM) to the high-throughput arena of flow cytometry. This method requires fast pulsed laser-cell interactions (**Figure 1**) to excite the fluorophore and the measurement of its characteristic decay time. This occurs hundreds of times during the microsecond time scale that the cell is resident in the beam path.

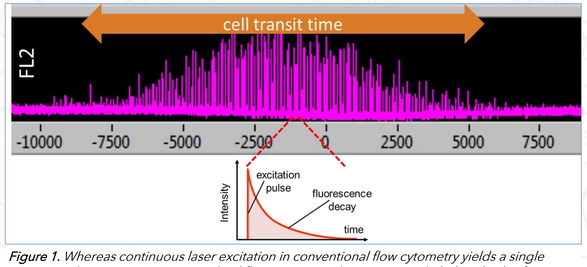

Figure 1. *Whereas continuous laser excitation in conventional flow cytometry yields a single intensity value per event, time-resolved fluorescence pulses excitation light hundreds of times per event, each pulse yielding a decay curve measured during the off period. Each fluorophore has its own characteristic decay lifetime.*

METHODS

We have developed and patented a suite of related technologies (*Arno*, *Colorado*, *Tiber*, *Danube*) that rely on direct time-domain measurements of fluorescence decay. Nanosecond-scale excitation pulses and fast electronic sampling allow resolution of fluorescence decays from about 500 psec to hundreds of nsec or longer. One unique advantage of this approach is the ability to simultaneously detect multiple fluorophore emissions, completely overlapping spectrally, using the same detector, and separating the different contributions based on fluorescence lifetime (**Figure 2**). Our time-resolved approach relies on proprietary hardware design and signal processing algorithms and has been tailored to address the key unmet needs in flow cytometry.

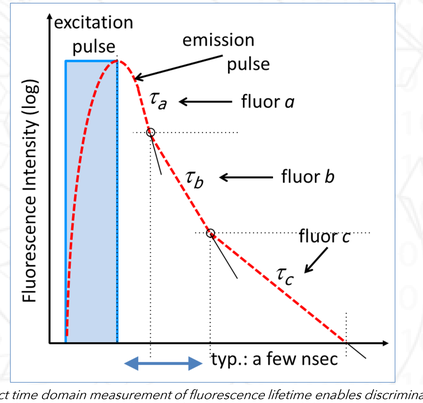

Figure 2. *Direct time domain measurement of fluorescence lifetime enables discrimination of multiple fluorophores simultaneously. Note the multi-exponential decay curve.*

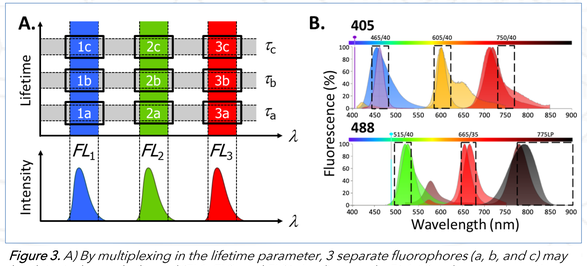

Figure 3. *A) By multiplexing in the lifetime parameter, 3 separate fluorophores (a, b, and c) may be detected in each channel (FL1, FL2, and FL3) simultaneously. B) A 12-color, compensation-free* Arno *design uses two lasers (405 and 488 nm) and six detectors, with two spectrally overlapping fluorophores detected in each channel.*

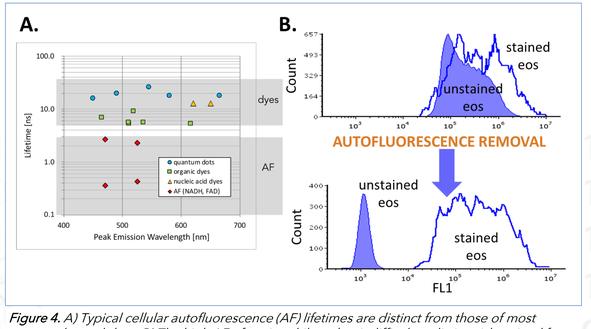

Figure 4. *A) Typical cellular autofluorescence (AF) lifetimes are distinct from those of most commonly used dyes. B) The high AF of eosinophils makes it difficult to distinguish stained from unstained. The* Colorado *can remove this AF contribution, making the two populations distinct.*

RESULTS

In the realm of spectral spillover and limits on multiplexing, we have developed and demonstrated a compensation-free analyzer designed to use only 2 lasers and 6 detectors for assays of up to 12 colors (the *Arno*; see **Figure 3** and Poster #292 – "Compensation-Free Flow Cytometry"). The same architecture will be used for a system that doubles or triples the number of simultaneous colors measurable on a traditional flow cytometer. Our *Colorado* proof-of-concept platform demonstrated the ability to isolate and eliminate interference from cellular autofluorescence excited at 375 nm (see **Figure 4** and Poster #269 – "Automated Elimination of Autofluorescence Interference in Flow Cytometry"). The *Tiber* system showed that the lifetime of cellular autofluorescence can be used to distinguish free and bound NADH, a ubiquitous metabolic cofactor, which will ultimately be used for label-free detection of cancer cells (see **Figure 5** and Poster #264 – "Label-Free Flow Cytometric Measurements of Cellular Metabolic Factors for Detection of Cancer Cells"). Finally, our *Danube* system is capable of precise measurements of lifetime, which can be used to detect changes in the fluorophore's cellular microenvironment for a variety of applications (**Figure 6**).

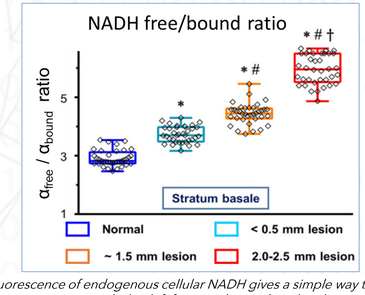

Figure 5. *Autofluorescence of endogenous cellular NADH gives a simple way to discriminate cancer cells. Cancer causes a metabolic shift from oxidative phosphorylation to aerobic glycolysis, which in turn changes in the free versus protein-bound levels of NADH in the cell. This change in bound state of NADH yields a measurable difference in lifetime, which can be used as an indicator of cancer. From Pastore* et al. *(2017) Exp. Derm. 26, 607-614.*

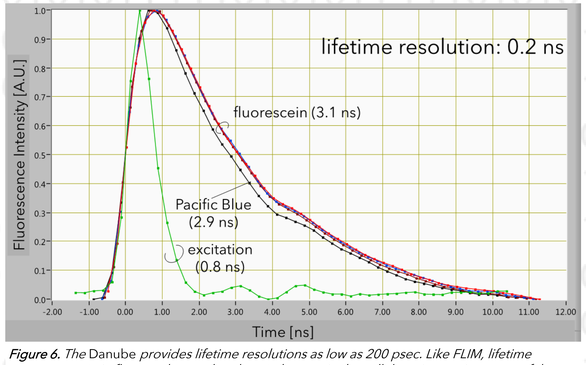

Figure 6. *The* Danube *provides lifetime resolutions as low as 200 psec. Like FLIM, lifetime measurements in flow can be used to detect changes in the cellular microenvironment of the fluorophore, which can alter lifetimes. This enables assays of changes in protein conformation, protease activity, receptor ligand interactions, pH and calcium concentrations, and more.*

CONCLUSION

Our direct time-resolved measurements of fluorescence have ushered in solutions to previously intractable or poorly handled problems, such as spectral compensation and autofluorescence interference. In addition, they are paving the way for long-desired applications, like two- or three-fold expansion in the number of measurable parameters, and label-free identification of cancer cells.

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The Arno, Colorado, Tiber, *and* Danube, *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.*