More Robust Fluorescence Lifetime Measurements for Cell Biology and Multiplexing

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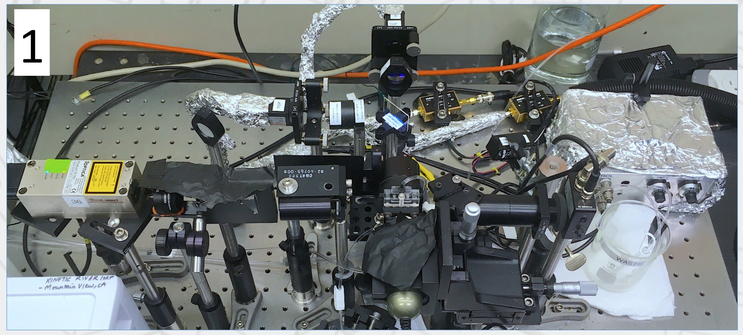
BACKGROUND

Fluorescence lifetime, long established in microscopy, is emerging as a valuable independent flow cytometry measurement parameter for use in studies of protein-protein interactions, FRET assays, and for expansion of multiplexing. While techniques based on frequency modulation have been successfully put to use, they have limitations in assessing lifetime heterogeneity. To detect multiple lifetimes using frequency-based approaches, one can employ multi-frequency systems, or computationally intensive phasor plots, however, neither of these methods are well suited for high-throughput analysis of data.



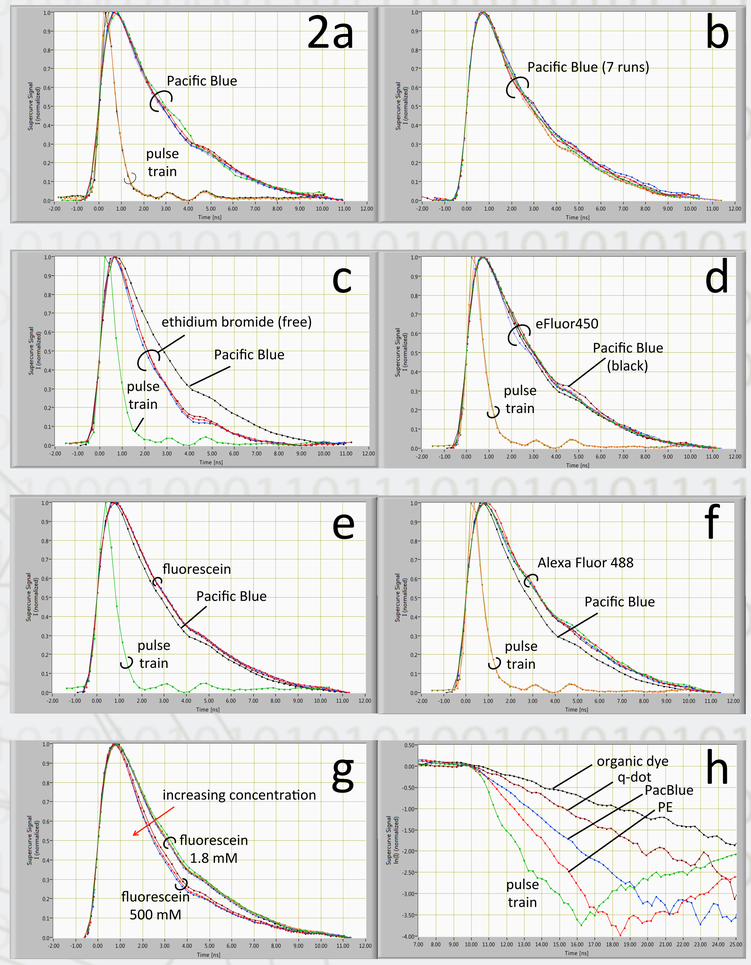
METHODS

Direct time-domain techniques can produce highly accurate multiexponential lifetime measurements. The traditional approach used in imaging, time-correlated single-photon counting, is too slow for use in flow cytometry. We have developed a faster method based instead on direct capture of decays following pulsed excitation at high peak power. The use of high-bandwidth electronics in the excitation path (1-GHz modulation source, 250-MHz 405-nm laser) and in the detection path (2-GHz detector, 1.5-GHz amplifier, 2-GHz digitizer, **Fig. 1**) allows fast turn-on/turn-off pulses in the nanosecond range and enables the resolution of fast decay transients with high fidelity. An alternate light source, a frequency-doubled laser oscillator producing ultrashort (200-fs) 400-nm pulses at a 80 MHz repetition rate, allows even sharper resolution of exponential decay behavior.

RESULTS

We have used our dual excitation measurement setup to perform flow-based fluorescence lifetime analysis of two dozen common fluorophores in bulk, bound to microparticles, and conjugated to antibodies bound to human cells. Measured fluorophores include workhorses such as FITC, PE, and Pacific Blue, as well as nanocrystals and Brilliant Violet polymer dyes. For several materials, our measurements constitute the first known determination of fluorescence lifetime. Measurements were carried out on single-fluorophore solutions as well as on mixtures. Single- and multi-exponential decay lifetime measurements ranged from 1 to 30 ns; with the laser oscillator source, the lifetime reproducibility was 0.1 ns and the smallest measurable lifetime difference was 0.2 ns. Fluorophore mixtures were measured in ratios ranging over 3 orders of magnitude.

Figs. 2(a)–(h) show direct time-domain lifetime measurements for selected fluorophores. The lifetime decays in Figs. 2(a)–(g) were collected on our short-pulse system, with sub-nanosecond pulses and an Instrument Response Function (IRF) of 0.8 ns; the decays in Fig. 2(h) were collected on our 250-MHz bandwidth modulated laser system with 10-ns pulses. (a) Pulse train showing the IRF, and several decays from Pacific Blue. (b) 7 Pacific Blue runs showing system reproducibility. (c) Ethidium bromide (free dye) in comparison to Pacific Blue. (d) eFluor 450 and Pacific Blue. (e) Fluorescein and Pacific Blue. (f) Alexa Fluor 488 and Pacific Blue. (g) The effect of increasing dye concentration showing quenching and lifetime shortening in fluorescein. (h) Comparison of four fluorophores—a commercial long-lifetime organic dye, a commercial quantum dot, Pacific Blue, and R-phycoerythrin.



CONCLUSION

Direct time-domain capture of fluorescence lifetime is a viable and highly accurate approach to reliably quantify multiexponential decays in flow. Based on our Danube instrument platform, we are currently designing a next-generation instrument architecture that will be capable of high-throughput analysis with real-time lifetime extraction, ultimately suitable for sorting applications.

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