REALTIME UNCERTAINTY QUANTIFICATION VIA ULTRA-PRECISE PARTICLE MATCHING FOR HIGH-THROUGHPUT SERIAL CYTOMETRY

Matthew DiSalvo[1,2], Paul N. Patrone[2], and Gregory A. Cooksey[2]
[1]*Johns Hopkins University, USA and*
[2]*National Institute of Standards and Technology, USA*

ABSTRACT

A central paradigm in flow cytometry is the one-time optical interrogation of cells, a practice that has limited the ability to address important questions associated with reproducibility and repeatability of measurements. Serial flow cytometry has pioneered the quantification of measurement uncertainties by optically interrogating each object more than once along a flow path. Here, we address the throughput limitations of serial cytometry with an algorithm to match signals across different interrogation regions. The algorithm operated real-time in an automated microfluidic serial cytometer and matched 99.96 % (95 % confidence interval [99.91 %, 99.98 %]) of particles at 94 Hz.

KEYWORDS: Flow Cytometry, Microfluidics, Optofluidics, Reproducibility

INTRODUCTION

Previously, we reported on a microfluidic serial cytometer that used integrated waveguides and a novel inertial and 3-D hydrodynamic flow focusing strategy to demonstrate the feasibility of quantifying measurement uncertainties in individual events [1, 2]. The cytometer achieved particle velocity variations of ≈ 0.3 % and median fluorescence area measurement precisions of ≈ 2 % from calibration microspheres. However, operation of the device was restricted to a throughput of 1 Hz to avoid challenges associated with matching, thus limiting its utility.

THEORY

In a strict approach to particle matching, unambiguous matching proceeds if and only if the time-of-flight (TOF) is less than interparticle latency (Figure 1). In contrast, for forward-projection time subdivision (FPTS), a particle was matched if, for the k^{th} non-reference signal channel, there existed exactly 1 index m_k where the peak time $t_{m_k,k}$ landed between two sequential time boundaries. The boundaries $b_{n,k}$, dividing the time for $n = 1 \ldots N$ particles shifted by estimated TOFs $\delta_{n,k}$ and peak times $t_{n,ref}$ from the reference channel, are given by Equation (1). Otherwise, series of particles whose signals did not uniquely occupy an equally-sized series of time windows were matched in order.

$$b_{n,k} = \begin{cases} t_{n,ref} + \frac{t_{n+1,ref} - t_{n,ref}}{2} + \delta_{n,k}, & n < N \\ \infty, & n = N \end{cases} \tag{1}$$

EXPERIMENTAL

An average of 10 000 green fluorescent polystyrene microspheres (15.3 µm nominal diameter) were measured under each of 19 different flow-focusing conditions by a microfluidic serial flow cytometer configured with six detectors (two fluorescence and one transmission for each of two laser excitation regions). The conditions included particle-based Reynold's numbers ranging from 2.7 to 3.7, sheath-to-core ratios (SCRs) from 1 to 130, and event rates from 2 Hz to 760 Hz. Simulated events were timed according to a Poisson process with invariant event order. Confidence intervals (CIs) for matching proportions represent Clopper-Pearson binomial proportion intervals; CIs for velocities represent bias-corrected and accelerated bootstrap intervals.

RESULTS AND DISCUSSION

The performances of the strict and FPTS algorithms were evaluated on experimental and synthetic signals from particles at various velocities, velocity variations, and event rates (Figure 2). The tracking yield of both algorithms were limited by event rates; however, while the strict approach improved at higher velocities, the FPTS algorithm improved at lower velocity variations. At ideal operating conditions, experimental and synthetic data indicate that the failure point (loss > 0.1 %) occurs at ≈ 1 Hz for the strict approach and ≈ 100 Hz for FPTS. The FPTS algorithm was incorporated into automation routines and particles flowing at 0.771 ± 0.020 m/s (mean ± standard deviation of 27 461 particles at 762 Hz) were successfully analyzed, displayed, and logged in real-time (4 s buffer and 500 ns sampling interval). Novel metrics afforded by serial nature of the measurements, such as particle velocity and measurement precision, were used to support on-the-fly performance tracking of the instrument under various flow focusing conditions (Figure 3). When challenged with a 556 Hz event rate and low hydrodynamic flow focusing

(SCR = 3), the matching yield was 98.96 % (95 % CI [98.82 %, 99.10 %]) and the precision of integrated fluorescence area was 0.7 % to 1.8 % (25th to 75th percentile of individual particle replication coefficient of variations).

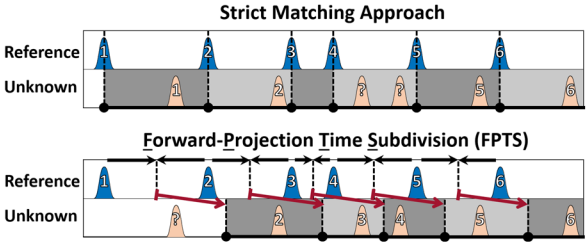

Figure 1: Particle matching strategies. Top: Signals in a reference channel (blue) are matched to downstream channel (tan) with unknown peak identities only if one unknown peak uniquely occupied the time windows (grey) spanning reference signals. Bottom: In FPTS, time window boundaries are extrapolated away from halfway (black arrows) between reference peaks using an estimated time-of-flight (red arrows).

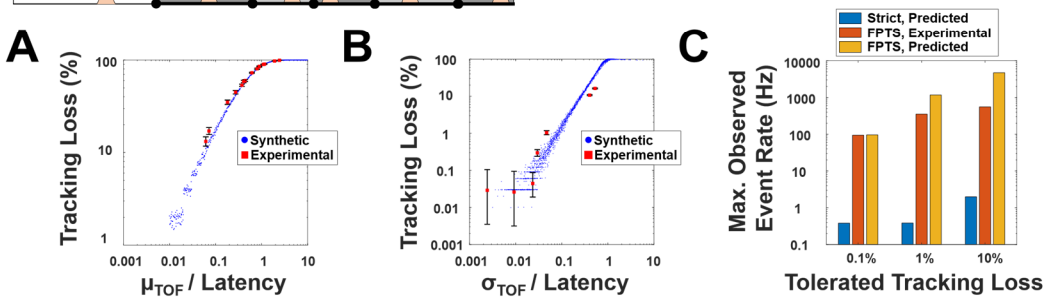

Figure 2: Characterization of particle matching limits. A) Strict matching and B) FPTS algorithm. Error bars represent 95 % binomial proportion confidence intervals. μ_{TOF} : mean TOF; σ_{TOF} : sample standard deviation of TOF; latency : time between sequential particles; tracking loss : proportion of particles detected but not matched across replicate measurement channels . Each synthetic data point is the result from N = 10 000 particles. C) Maximum observed event rates across synthetic and experimental datasets with tracking loss (95 % binomial proportion confidence interval) below tolerances. Predictions assumed ideal operating conditions of $\mu_{TOF} \approx 26$ ms and $\sigma_{TOF}/\mu_{TOF} \approx 0.2$ %. No experimental data had less than 10 % tracking loss using the strict matching method; the lowest observed loss using strict matching was 13 % at 2.4 Hz.

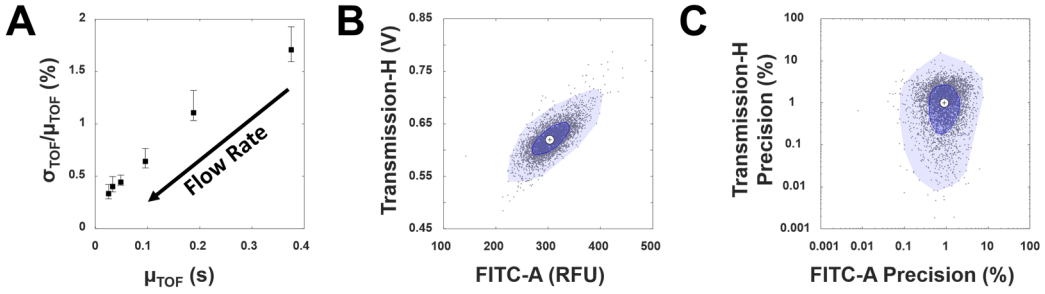

Figure 3: Serial cytometry results. A) Monitoring of particle velocity and velocity variability with changing flow rates. Error bars represent 95 % bootstrap confidence intervals. B) Traditional flow cytometry scatterplot of forward scatter versus fluorescence for N = 4305 particles with 100 % matching yield acquired at 35 Hz. C) Scatterplot of the same particles with the novel axes of measurement precisions. B,C) Dark blue: envelope containing central 50 % of data points; Light blue : envelope containing inliers; White crosshair : Tukey median. Transmission-H : height of the signal representing loss of transmitted light due to particle crossing the laser path (analogous to forward scatter); FITC-A : integrated area of the signal representing green fluorescence intensity emitted by the particles.

CONCLUSION

Automated particle matching enabled on-the-fly uncertainty quantification of flow cytometry measurement reproducibility *on a per-event basis*, alongside continual and simultaneous measurement and data logging. The approach allows us to characterize when the measurement system becomes unstable with respect to measurement reproducibility. We anticipate that serial cytometry will reveal and quantify additional sources of uncertainty arising from the instrumentation, sample, and analyses and provide better tools to compare rare events within a population.

REFERENCES

[1] M. DiSalvo, P. N. Patrone, G. A. Cooksey, in *24th Int. Conf. Miniaturized Syst. Chem. Life Sci*, 835, Oct. 2020.
[2] G. A. Cooksey, P. N. Patrone, J. R. Hands, *et al.*, *Anal. Chem.*, 91, 16, 10713–10722, Aug. 2019

CONTACT: G.A. Cooksey; phone: +1-301-975-5529; gregory.cooksey@nist.gov