APPLICATION NOTE

From Variability to Consistency: Standardizing Scatter Signals with Slingshot Biosciences ScatterBridge™ Controls

Introduction

Forward scatter (FSC) and side scatter (SSC) are parameters in flow cytometry that reflect cell morphology, including light-scattering properties related to size and granularity. Scatter signals are highly instrument-dependent and influenced by optical configuration, making cross-platform standardization inherently challenging.

Despite their importance, FSC and SSC profiles lack widely adopted standardized controls for instrument harmonization, resulting in variability across instruments, operators, and sites.

This variability presents a major challenge for assay consistency and regulatory compliance—particularly in multi-site studies, manufacturing workflows, and other regulated environments where consistent, reproducible data are critical.

Traditional controls, including fluorescence calibration beads, can monitor detector sensitivity but do not correct for variability in forward and side scatter detection of experimental samples. Biological controls, including healthy donor peripheral blood mononuclear cells (PBMCs), can more closely standardize scatter detection but present new challenges related to biohazard risk, instability, and lot-to-lot variability.

ScatterBridge™, developed by Slingshot Biosciences, is a non-biohazardous, hydrogel-based reference control designed to standardize FSC and SSC. It provides a stable and reproducible solution for aligning scatter signals across instruments, operators and over time. ScatterBridge™ contains nine distinct populations that resolve into a 3×3 grid on FSC vs SSC plots, spanning a broad dynamic range to support instrument lifecycle management, cross-platform comparability, and longitudinal tracking for regulatory environments.

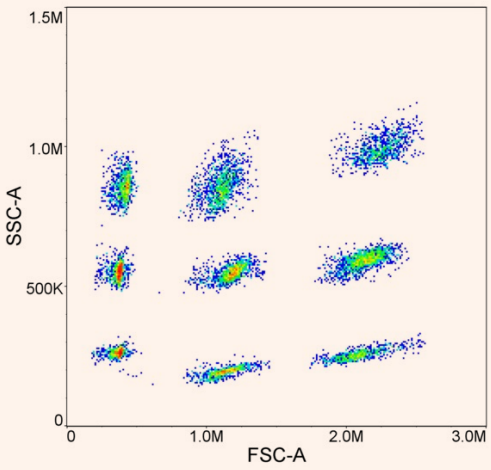


Figure 1: ScatterBridge™ FSC vs SSC Dot Plot – ScatterBridge™ contains nine distinct hydrogel particle populations that form a 3×3 grid when plotted on forward scatter (FSC) versus side scatter (SSC). This plot demonstrates how ScatterBridge™ provides a reproducible reference for instrument setup, cross-platform harmonization, and long-term monitoring of scatter.

Methods

Reagent Preparation

- ScatterBridge™ was stored at 2–8°C and equilibrated to room temperature prior to use.
- ScatterBridge was vortexed on high for 2 seconds to mix thoroughly.
- One drop of Scatterbridge was added to 250uL of Stain Buffer in a 5mL FACS tube.
- The sample was vortexed for 5–10 seconds to ensure uniform suspension.

Data Acquisition

- Samples were acquired on a flow cytometer at low to medium flow rate.
- A minimum of 10,000 events were collected per run.
- FSC and SSC gains or voltages were adjusted to resolve all nine populations simultaneously.

Experimental Design

Scatter Standardization Across Instruments and Acquisition Dates

The application of ScatterBridge controls for comprehensive scatter calibration was evaluated across multiple use cases and instruments, including two 5–laser cytometers (“A” and “B”) and one 3–laser cytometer (“C”). For each experimental condition, a single baseline cytometer (5–laser instrument “A”) was designated, enabling quantification of variability between instruments and over time.

- **Instrument Harmonization:** Initial instrument variability was assessed by recording ScatterBridge controls prior to adjusting gain settings on the two experimental instruments to match the FSC and SSC median scatter intensity (MSI) of each ScatterBridge population on the baseline cytometer. After this calibration, the ScatterBridge control was re-acquired to evaluate post-calibration consistency across all instruments.
- **Biological Sample–Guided Scatter Calibration:** Representative biological samples (PBMCs or cultured cells) were used to initially adjust scatter voltages across multiple instruments by matching the MSI of target cell populations. ScatterBridge was then used as an independent reference control to monitor instrument stability for up to one month post-calibration. During this time, the instruments underwent regularly scheduled preventative maintenance (PM).

Instrument Lifecycle Management

To support comprehensive instrument lifecycle management, ScatterBridge was utilized during critical transition events. This included

- **Lab Move Evaluation:** ScatterBridge controls were acquired before and after instrument relocation to ensure optical alignment was maintained during transit.
- **Preventative Maintenance Intervention (PMI):** ScatterBridge was acquired pre-, mid- and post-maintenance to quantify shifts and validate recalibration.

Data Analysis

1. FSC vs SSC plots were evaluated for:
   a. Population resolution
   b. Positional alignment
2. MSI values, percent changes from baseline (baseline instrument or pre-maintenance timepoint) and the coefficient of variation (CV) across conditions were calculated.

Results and Discussion

Scatter Standardization Across Instruments

Instrument Harmonization

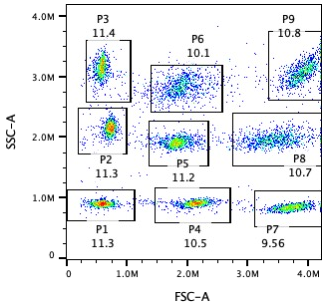
Prior to gain adjustment, population positions varied noticeably across the cytometers - indicating differences in detector sensitivity and signal scaling. Following adjustment of FSC and SSC voltages using ScatterBridge, population distributions across all instruments were aligned to the reference cytometer, resulting in close agreement with the defined standard.

This harmonization resulted in:

- Consistent scatter measurements across instruments
- Reduced inter-instrument variability
- Improved comparability across operators and sites

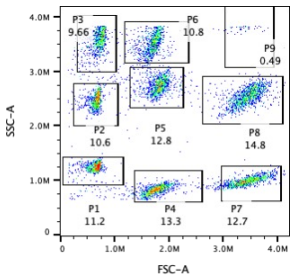
Such standardization is particularly critical for laboratories undergoing regulatory or quality audits, as it provides quantitative evidence of instrument equivalence beyond what fluorescence calibration beads can demonstrate alone.

Instrument A (Baseline)

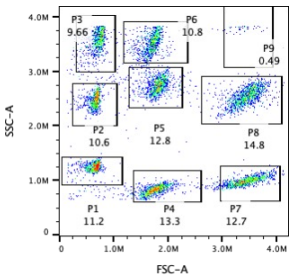


Instrument B

Pre-Calibration

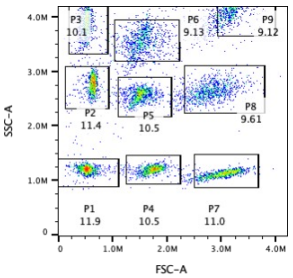


Post-Calibration

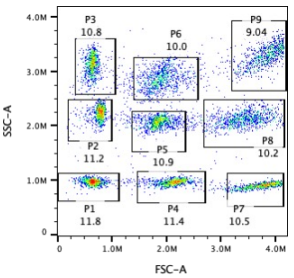


Instrument C

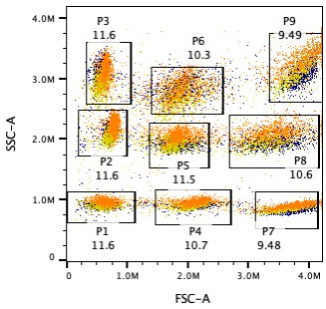
Pre-Calibration



Post-Calibration



Post-Calibration Overlay of All Instruments



Populations	Instrument A (Baseline)		Instrument B		Instrument C		%CV Across Instruments	
	FSC MSI	SSC MSI	FSC MSI	SSC MSI	FSC MSI	SSC MSI	FSC MSI	SSC MSI
P1	593525	899691	674709	1246277	526177	1203885	12.43	16.93
P2	726811	2140031	675692	2476247	640531	2764097	6.37	12.70
P3	586759	3130802	737128	3595589	521738	3866508	17.96	10.54
P4	2124086	909053	1753798	830796	1731719	1199885	11.79	19.85
P5	1815607	1905725	1816051	2730343	1496001	2560696	10.80	18.15
P6	1875313	2815478	1716851	3525309	1544034	3550350	9.68	12.65
P7	3705922	841139	3431922	986049	3038964	1123125	9.88	14.34
P8	3393114	1949552	3449166	2545480	2767816	2613133	11.81	15.41
P9	3858300	3049027	3602442	4194304	3058340	3991682	11.65	16.32

Table 1: Pre-calibration median scatter intensity (MSI) of ScatterBridge populations in forward scatter (FSC) and side scatter (SSC) across three instruments.

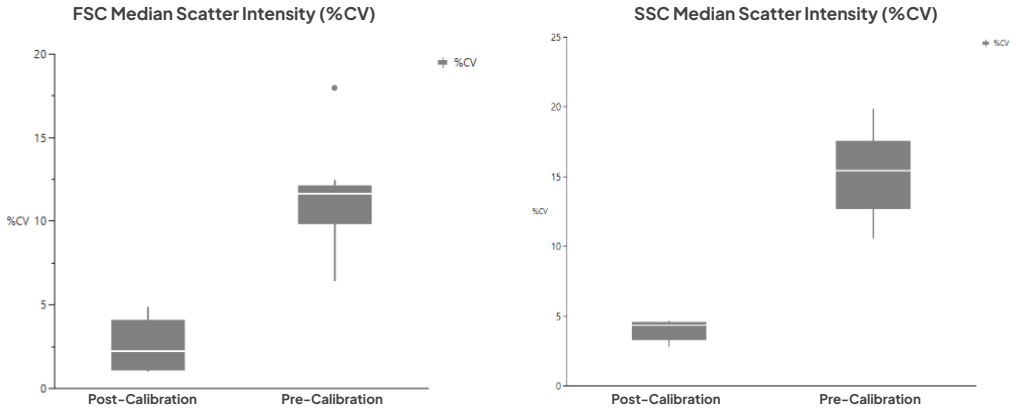
Populations	Instrument A (Baseline)		Instrument B		Instrument C		%CV Across Instruments	
	FSC MSI	SSC MSI	FSC MSI	SSC MSI	FSC MSI	SSC MSI	FSC MSI	SSC MSI
P1	593525	899691	614668	911067	645304	977372	4.21	4.51
P2	726811	2140031	744817	2099884	783953	2241535	3.89	3.38
P3	586759	3130802	600528	2967565	643502	3135826	4.85	3.11
P4	2124086	909053	2052788	915660	2120969	969533	1.92	3.56
P5	1815607	1905725	1782869	1944686	1814367	2070179	1.03	4.35
P6	1875313	2815478	1853295	2706121	1889987	2858537	0.99	2.81
P7	3705922	841139	3597398	844334	3766765	907240	2.33	4.31
P8	3393114	1949552	3282601	1961615	3423748	2117134	2.21	4.65
P9	3858300	3049027	3778224	3053785	3841974	3304741	1.11	4.66

Table 2: Post-calibration median scatter intensity (MSI) of ScatterBridge populations in forward scatter (FSC) and side scatter (SSC) across three instruments.

Pre- vs Post-Adjustment Comparison

Comparison of Table 1 and Table 2 highlight a marked reduction in inter-instrument variability after utilizing ScatterBridge controls for scatter detector calibration. The decrease in %CV values across all nine populations confirms successful harmonization of scatter signals, with populations consistently aligned to the reference instrument. This improvement demonstrates the effectiveness of ScatterBridge in standardizing instrument performance and ensuring reproducible, comparable data across multiple platforms.

Inter-Instrument Variability Pre-and Post-Calibration



Biological Sample-Guided Scatter Calibration

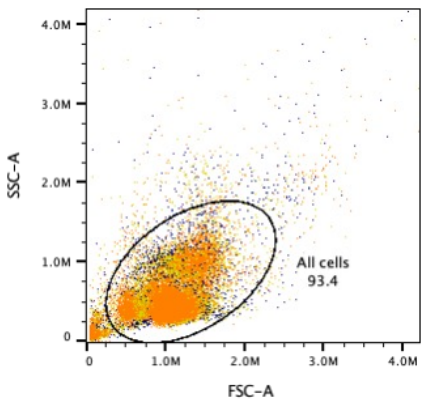
Overlays of the biological sample across all three instruments confirmed strong alignment of key populations, demonstrating successful normalization of detector settings under biologically relevant conditions.

Because gains were optimized for the biological sample, not all nine ScatterBridge populations appeared on the plots. This was expected, as instrument settings were optimized based on display of the biological sample rather than the full dynamic range of ScatterBridge.

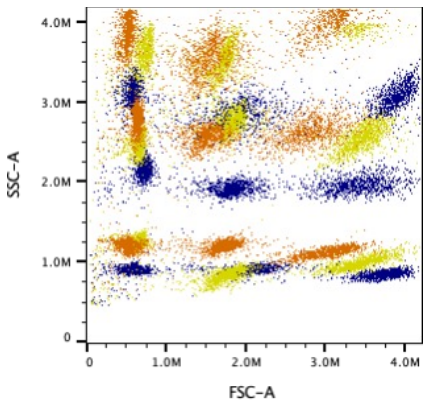
The goal of using ScatterBridge in this context is not to capture all nine populations, but to monitor instrument performance. Populations that appear within the scatterplot axes are sufficient to track changes in sensitivity, detect shifts, and ensure consistency across instruments over time. Differences in how populations appear on each instrument are expected, as each cytometer has slightly different detector sensitivity, laser power, and optics. Despite this variance, the visible ScatterBridge populations provide a reliable reference for longitudinal performance monitoring.

Overlays of ScatterBridge controls acquired from each instrument one month post-calibration demonstrate how instrument performance can be monitored longitudinally using the visible reference populations.

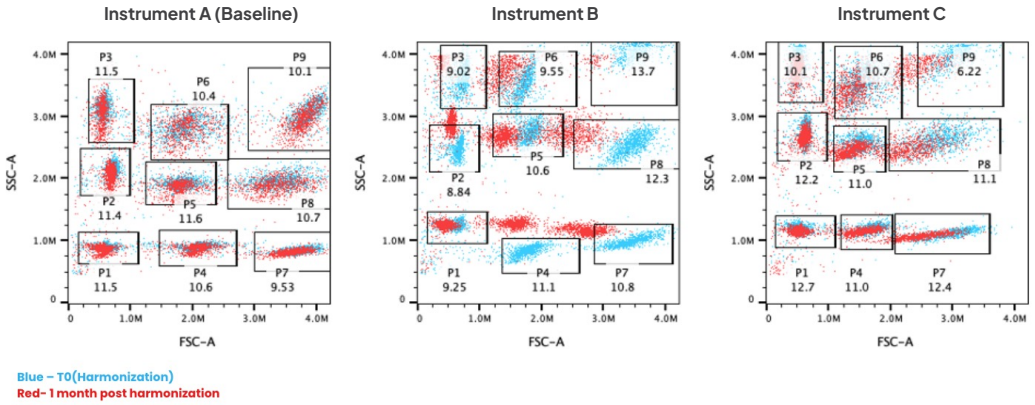
Overlay of Biological Sample FSC/SSC Profiles Across Three Instruments After Biological Sample Scatter Calibration



Overlay of ScatterBridge™ FSC/SSC Profiles Acquired Using Biological Sample Calibration Across Three Instruments



Overlay of ScatterBridge™ FSC/SSC profiles on three instruments, one month after Biological Sample Scatter Calibration



Populations	Instrument A (Baseline)		Instrument B		Instrument C		%CV Across Instruments	
	FSC	SSC	FSC	SSC	FSC	SSC	FSC	SSC
P1	6.9	2.1	28.1	0.1	1.0	3.2	12.43	16.93
P2	6.2	0.8	14.5	10.5	2.6	2.9	6.37	12.70
P3	7.0	2.4	35.4	9.3	2.6	1.8	17.96	10.54
P4	7.0	1.3	7.2	29.3	7.4	3.3	11.79	19.85
P5	5.3	2.6	19.4	1.6	5.3	3.2	10.80	18.15
P6	6.1	1.6	13.0	4.7	6.7	2.3	9.68	12.65
P7	5.5	1.3	15.7	11.1	12.7	3.1	9.88	14.34
P8	7.2	1.0	23.2	4.3	8.4	3.2	11.81	15.41
P9	7.8	1.4	16.1	0.00	12.4	1.9	11.65	16.32

Table 3: %CV of FSC and SSC Median Values Across Cytometers One Month Post-Harmonization and PM

Instrument performance was tracked one month post-harmonization (Table 3), during which routine preventive maintenance (PM) was also performed on all three instruments. Overall, both the 5-Laser Cytometer-A (reference) and the 3-Laser Cytometer maintained stable performance, with %CV for FSC and SSC remaining low across most populations. For Cytometer-A, FSC %CV ranged from 5.3–7.8% and SSC %CV from 0.8–2.6%, while the 3-Laser cytometer showed FSC %CV of 1.0–12.7% and SSC %CV of 1.8–3.3%, indicating minimal inter-instrument variability.

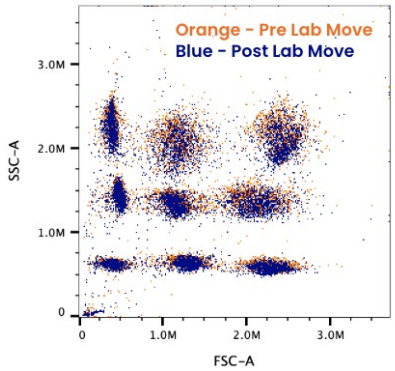
In contrast, the 5-Laser Cytometer-B exhibited marked variability in several populations following PM. FSC %CV ranged from 7.2–35.4% and SSC %CV from 0–29.3%, with particularly large shifts observed in P1 (FSC 28.1%), P3 (FSC 35.4%), and P4 (SSC 29.3%). These deviations corresponded to measurable shifts in FSC and SSC population positions, highlighting localized changes in instrument response. ScatterBridge™ effectively detected these early, instrument-specific gain drifts.

These observations demonstrate that ScatterBridge™ can sensitively monitor instrument-specific performance changes after maintenance events while showing that the other cytometers remained stable, all without the need for additional biological sample acquisition.

Instrument Lifecycle Management

Scatter Stability During Lab Move

ScatterBridge™ showed consistent scatter profiles before and after instrument relocation, with minimal deviation in population positioning. This confirms that scatter performance remained stable and that any deviations could be readily identified and corrected. This use case highlights its utility in maintaining data continuity during lab transitions.

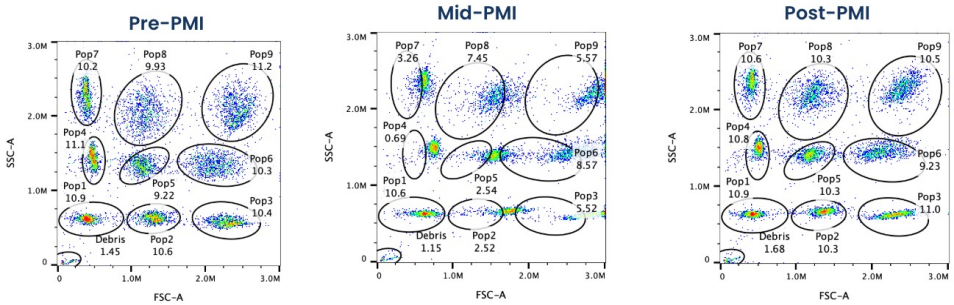


Monitoring and Optimizing PMI Impact

During PMI, mid-maintenance data revealed temporary shifts in FSC values. Utilizing ScatterBridge™ as part of the instrument lifecycle management workflow allowed the laboratory to:

- Quantify the magnitude of change introduced
- Adjust settings more precisely
- Restore original performance specifications with high fidelity
- Ensure regulatory compliance by documenting that the instrument returned to its validated state post-servicing

Post-PMI results showed strong alignment with pre-PMI baseline, confirming effective recalibration.



Observed variation between pre- and post-PMI:

- FSC variation: –2.2% to +4.5%
- SSC variation: –1.5% to +3.6%

These results indicate high reproducibility, minimal drift and reliable recovery following maintenance.

Advantages Over Existing Controls:

- Audit Readiness: Synthetic controls offer the stability required for long-term studies, where biological drift could invalidate the audit trail.
- Fluorescent beads differ substantially from most biological samples in light scatter properties, while ScatterBridge controls offer a wide range of populations to represent biological samples.
- Unlike biological controls, ScatterBridge helps enable biologically relevant scatter standardization without the biohazardous risk, short shelf life and donor or culture variability.

By integrating ScatterBridge™ into workflows, laboratories can establish consistent scatter, improving reproducibility across studies and systems.

Conclusion

ScatterBridge™ provides a robust and reproducible solution for standardizing and harmonizing forward and side scatter (FSC and SSC) in flow cytometry. By enabling alignment across instruments, including platforms with different laser configurations, it ensures that scatter measurements are consistent and comparable across instruments, sites, and operators.

Key benefits demonstrated in this study include:

- **Instrument-to-instrument alignment:** Harmonization of FSC and SSC using ScatterBridge allows consistent population positioning across cytometers with varying detector sensitivities and laser configurations.
- **Cross-site reproducibility:** Biological sample-guided calibration confirms that median scatter values could be compared across instruments, supporting comparable measurements across laboratories.
- **Long-term performance monitoring:** Visible ScatterBridge populations provided a reliable reference to track instrument sensitivity, detect drift, and confirm stability over time, including one month post-calibration.
- **Lifecycle management:** ScatterBridge effectively monitored instrument performance during critical events such as preventive maintenance interventions (PMI) and laboratory relocations, to ensure rapid identification and correction of deviations.
- **Regulatory compliance and audit readiness:** As a non-biological, stable control, ScatterBridge provides reproducible scatter data suitable for documentation and validation in regulated environments.

By filling a critical gap in scatter standardization, ScatterBridge complements existing fluorescence-based controls, establishes reproducible FSC/SSC baselines, and provides a framework for harmonized, audit-ready flow cytometry workflows from installation through routine operation.

References

1 Slingshot Biosciences — ScatterBridge™ Product Documentation

2 CLSI H62. *Validation of Assay Performance for Multivariate Flow Cytometry; Approved Guideline.* (Verification of regulatory compliance and standardization protocols).

3 BD Biosciences. *Introduction to BD Cytometer Setup and Tracking (CS&T) Technology.* (Reference for CS&T baseline and performance monitoring).

4 Shapiro, H. M. (2003). *Practical Flow Cytometry,* 4th Edition, Wiley-Liss.

5 Kalina, T. (2020). Reproducibility of flow cytometry through standardization: Opportunities and challenges. *Cytometry Part A,* 97, 137–147.