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ORIGINAL ARTICLE

CLINICAL CYTOMETRY WILEY

Synthetic abnormal mast cell particles successfully mimic neoplastic mast cells by flow cytometry

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Abstract

Clinical flow cytometry laboratories require quality control materials for assay development, validation, and performance monitoring, including new reagent lot qualification. However, finding suitable controls for populations with uncommonly expressed antigens or for rare populations, such as mast cells, can be difficult. To that end, we evaluated synthetic abnormal mast cell particles (SAMCP), developed together with, and manufactured by, Slingshot Biosciences. The SAMCP's were designed to phenotypically mimic abnormal neoplastic mast cells: they were customized to have the same light scatter and autofluorescence properties of mast cells, along with surface antigen levels of CD45, CD33, CD117, CD2, CD25, and CD30 consistent with that seen in mast cell disease. We evaluated several performance characteristics of these particles using ARUP's high sensitivity clinical mast cell assay, including limit of detection, off-target activity and FMO controls, precision, scatter properties of the particles utilizing several different cytometer platforms, and particle antigen stability. The phenotype of the SAMCP mimicked abnormal mast cells, and they could be distinguished from normal native mast cells. FMO controls demonstrated specificity of each of the markers, and no off-target binding was detected. The limit of detection of the particles spiked into normal bone marrow was found to be ≤0.003% in a limiting dilution assay. The mast cell particles were found to perform similarly on Becton Dickinson Lyric, Cytek Aurora, and Beckman Coulter Navios and CytoFLEX platforms. Within run and between run precision were less than 10% CV. SAMCP were stable up to 13 days with minimal loss of antigen fluorescence intensity. The SAMCP's were able to successfully mimic neoplastic mast cells based on the results of our high sensitivity mast cell flow cytometry panel. These synthetic cell particles represent an exciting and innovative technology, which can fulfill vital needs in clinical flow cytometry

such as serving as standardized control materials for assay development and performance monitoring.

KEYWORDS
flow cytometry, mast cell, synthetic particle, systemic mastocytosis

1 | INTRODUCTION

Devising quality control material for flow cytometric assays is a laborious and difficult task. Control material is necessary to both determine if an assay is in control, as well as for developing and validating new assays. While Fc (Fragment crystallizable) controls are ubiquitous and well characterized, Fab (Fragment antigen-binding) controls pose a distinct challenge. These Fab controls provide antigen targets to test the sensitivity and specificity of antibody-fluorophore conjugates both singly and in combination (cocktails). Classically, three methods exist: (1) internal positive controls, (2) cell line external controls, and (3) preserved specimens. In the clinical laboratory, the first method suffices for most antigens as most markers are expressed on relatively common cell types found in most specimens. For example, normal internal positive and negative controls can be found for T cell markers such as CD2, CD3, CD5, CD7, and CD8 among lymphoid cell populations. On the other hand, this method often fails when the population of interest is in a rare specimen type (e.g. CD103+ intraepithelial T lymphocytes of the GI tract) or is in a small subset of total cells in common specimens (e.g. CD30 in immunoblasts of a lymph node). In such situations, the second and third methods are commonly used to maintain confidence in assay performance. For cell line controls, cells are purchased and/or grown requiring cell culture facilities and increased technologist labor and training to maintain these cell lines. Alternatively, cryopreservation (or other chemical methods (Eidhof et al., 2021)) of neoplastic cells can be used to maintain a bank of control material for future use (e.g., freezing down a sample of hairy cell leukemia with known expression of CD103).

These latter methods pose significant challenges for most clinical flow cytometry laboratories including the lack of cell culture facilities or (more pressingly) lack of skilled personnel to maintain cell culture lines. Likewise, preservation of the appropriate neoplastic material is dependent on having adequate access to often rare patient disease specimens. While statistics work in the favor of large clinical reference laboratories, for smaller laboratories, months, if not years, may pass between suitable specimens resulting in an absence of critically important control material.

In rare diseases such as systemic mastocytosis (SM), the incidence of the disease is very low (1:10,000) (Brockow, 2014) and therefore the frequency of SM specimens tested in any given lab is exceedingly low. Cell lines exist, but do not bear the full panoply of phenotypic abnormalities required to satisfy the minor diagnostic criteria for the disease (i.e., CD2, CD25 and CD30 expression on neoplastic mast cells) (Valent et al., 2021) and often have physical light scatter properties and abnormal antigen expression, which do not mimic the neoplastic mast cells seen in SM. In this setting, all three methods would be required to both validate and QC an SM assay with all the attendant problems associated with finding rare specimens, storing, or preserving them, and hoping for the correct phenotypic abnormalities of interest. To that end, we evaluated a new technology that allows for conjugation of multiple protein epitopes to a synthetic particle, which was designed to mimic the light scatter and autofluorescence properties of the abnormal mast cells seen in SM.

2 | MATERIALS AND METHODS

2.1 | Synthetic abnormal mast cell particles

The customized synthetic mast cell mimic particles were made using Slingshot Bioscience's proprietary technologies. These cell

TABLE 1 ARUP's clinically validated mast cell panel.

Marker	Fluor	Vendor	Catalog #
CD123	PE	Becton Dickinson	555644
CD34	PE-CF594	Becton Dickinson	562383
CD25	PE Cy5.5	Beckman Coulter	A79386
CD33	PE Cy7	Beckman Coulter	A54824
CD117	APC	Beckman Coulter	IM3638
CD30	APC A700	Beckman Coulter	B42017
CD2	APC/Fire 750	Biolegend	300226
DAPI	DAPI	Thermo Fisher	62248
CD45	Krome Orange	Beckman Coulter	A96416

TABLE 2 Alternative mast cell panel for FACSLyric cytometer.

Marker	Fluor	Vendor	Catalog #
CD34	FITC	Becton Dickinson	555821
CD123	PE	Becton Dickinson	555644
CD25	PE Cy5.5	Beckman Coulter	A79386
CD33	PE Cy7	Beckman Coulter	A54824
CD117	APC	Beckman Coulter	IM3638
CD30	APC A700	Beckman Coulter	B42017
CD2	APC/Fire 750	Biolegend	300226
DAPI	DAPI	Thermo Fisher	62248
CD45	Krome Orange	Beckman Coulter	A96416

mimics are polymer particles that are customized with respect to their forward and side scatter properties and exhibit autofluorescent properties similar to mast cells. The base particles are also designed to mimic the biophysical properties of a target cell, including, but not limited to, its elastic modulus, proportional protein display (e.g. immunological synapse), and internal features. In

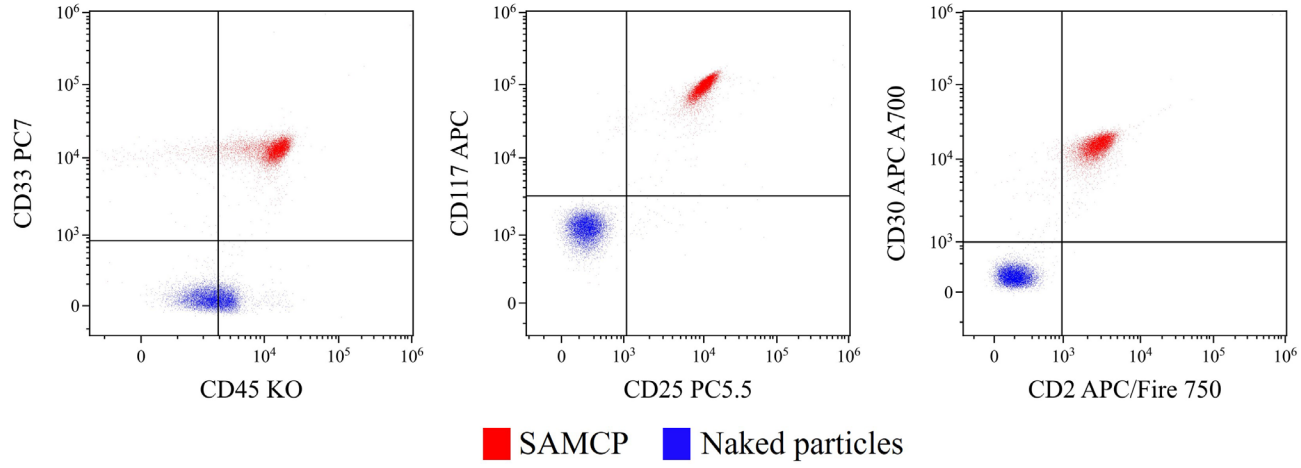

FIGURE 1 Phenotype of SAMCP. SAMCP or naked particles were stained with the mast cell panel in Table 1, run on a Navios flow cytometer and analyzed in Kaluza software. Data are shown as dot plot overlays of SAMCP (in red) with naked particles (in blue). [Color figure can be viewed at wileyonlinelibrary.com]

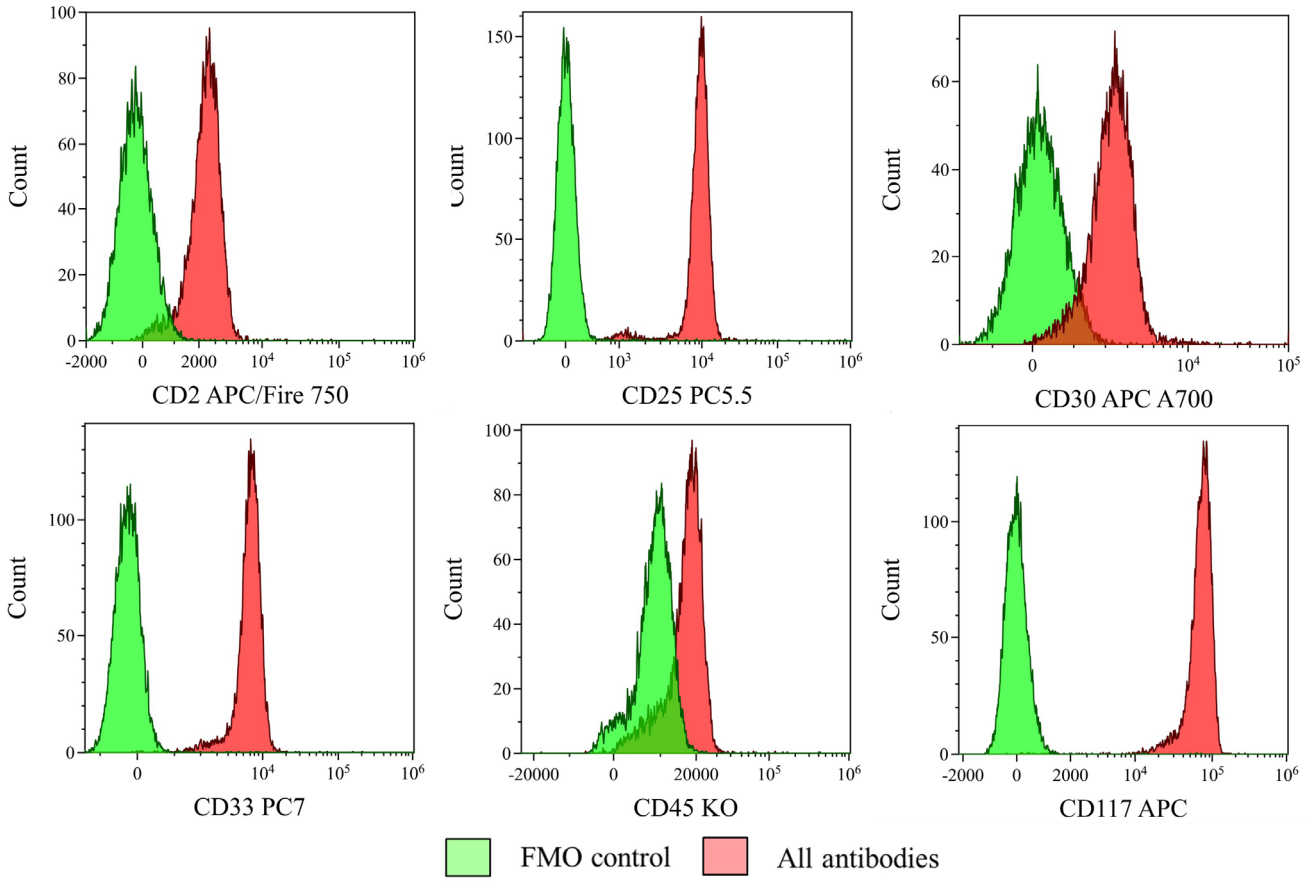

FIGURE 2 Fluorescence minus one (FMO) controls. SAMCP were stained with the full mast cell panel in Table 1, or with the panel missing either CD2 APC Fire/A750, CD25 PE Cy5.5, CD30 APC A700, CD33 PE Cy7, CD45 Krome Orange, or CD117 APC. Samples were run on a Navios flow cytometer and analyzed in Kaluza. Data are shown as histogram overlays of the full panel (in red) and with the panel missing the indicated marker (in green). [Color figure can be viewed at wileyonlinelibrary.com]

addition, the synthetic cells are designed to offer a range of benefits typically not found in biologically derived cells, including resistance to multiple freeze–thaw cycles, lyophilization and reconstitution, and consistent manufacturing quality. Finally, the synthetic cells are designed to be fully compatible with traditional flow cytometry workflows, including fixation, and can be directly spiked into whole blood or bone marrow samples to mimic clinical process workflows. The surface of these particles was modified with protein antigens to mimic the abnormal mast cell phenotype seen in SM: CD45, CD33, CD117, CD25, CD2, and CD30. The

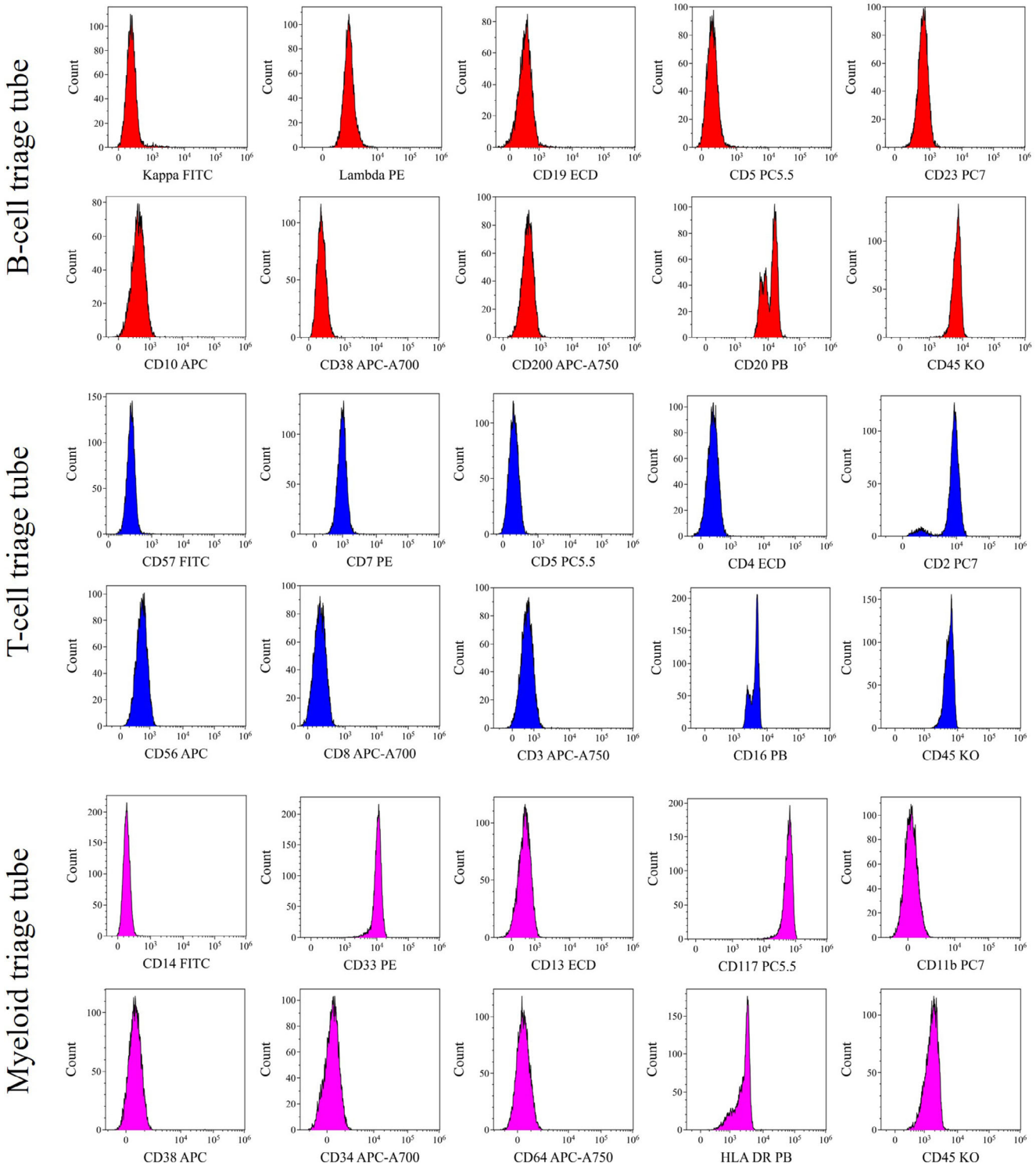

FIGURE 3 Off-target binding. SAMCP were stained with ARUP's validated three-tube triage panel encompassing an array of B-cell, T-cell, and myeloid markers, run on a Navios flow cytometer, and analyzed in Kaluza software. Data are shown as histograms of each the markers the three triage tubes. [Color figure can be viewed at wileyonlinelibrary.com]

particles were shipped lyophilized in vials and reconstituted prior to use in phosphate buffered saline.

2.2 | Biological samples

Where indicated, cells from healthy adult donors with no history of mast cell disease (normal donors) or from SM patients were obtained from bone marrow aspirates and used after ammonium chloride lysis to remove erythrocytes. All human specimens were obtained under IRB protocol #77285_19.

2.3 | Mast cell antibody panel

The mast cell antibody panel used for these studies was developed and validated at ARUP Laboratories for the detection of abnormal mast cells in SM patients. The panel combines CD45, CD33, CD117, and CD34 to identify mast cells, includes the mast cell aberrancy markers of CD2, CD25, CD30, and CD123, and utilizes DAPI as a viability marker (Table 1). When the panel is used clinically, 1.7×10^6 events are collected, and the assay has a limit of detection of 0.003%.

2.4 | Flow cytometric analysis

Synthetic abnormal mast cell particles (SAMCP), stained with the mast cell antibody panel described above, were assessed by flow cytometry either alone or in combination with bone marrow cells from normal or SM patients. Naked particles, not modified with any added antigen markers, were evaluated in some experiments. Most assays were performed on Navios EX cytometers (Beckman Coulter, Brea, CA, USA), but the particles' performance was also evaluated on a CytoFLEX cytometer (Beckman Coulter), an Aurora 5-laser spectral cytometer (Cytek Biosciences, Fremont, CA, USA) and a FACSLyric cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). For the FACSLyric cytometer, an alternative mast cell antibody panel was used because of the different configuration of the lasers (Table 2). Experimental data was analyzed using Kaluza software (Beckman Coulter).

3 | RESULTS

3.1 | Phenotype of mast cell particles

We began by examining the SAMCP expression of the specific markers added to the particles (Figure 1). By design, the particles express high levels of CD33, CD117 and CD25, and have more modest expression of CD2 and CD30; similar to levels expressed by neoplastic mast cells (Orfao et al., 1996; Teodosio et al., 2015). CD45 is also expressed at modest levels. The naked mast cell particles exhibit low background expression of all those markers. However, as described above, SAMCP are designed with autofluorescent properties in the violet and blue channels, similar to normal mast cells, and thus the naked particles appear to express low levels of CD45 Krome Orange as well.

3.2 | Fluorescence minus one controls

Further evidence of the specificity of the surface marker expression on the SAMCP was supported by fluorescence minus one experiments (Figure 2). Again, the particles exhibited high levels of CD33, CD117, and CD25, and more modest expression of CD2 and CD30. CD45 was modestly expressed and the fluorescence minus one control for CD45 Krome Orange exhibited background expression of CD45 due to the autofluorescent properties of the particles.

TABLE 3 Calculation of limit of detection of SAMCP.

% particles predicted	# of particle events	# of viable events	% particles observed
1.0000%	15,553	1,518,373	1.0243%
0.3162%	5034	1,513,986	0.3325%
0.1000%	1863	1,512,022	0.1232%
0.0316%	599	1,521,942	0.0394%
0.0100%	251	1,510,482	0.0166%
0.0032%	88	1,514,422	0.0058%
0.0010%	43	1,518,124	0.0028%
0.0003%	17	1,510,181	0.0011%
none	2	1,505,536	0.0001%

Note: SAMCP were spiked into normal bone marrow at an initial concentration of 1%. Serial half-log dilutions of this initial concentration were performed into normal bone marrow down to a predicted particle concentration of 0.0003%. Predicted SAMCP concentrations versus observed particle concentrations are shown for each of the serial dilutions.

3.3 | Off-target binding

SAMCP were stained with ARUP's validated three-tube triage panel encompassing an array of B-cell, T-cell, and myeloid markers (Figure 3). CD33 and CD117 in the myeloid tube, as well as CD45 in all three tubes, appear strongly positive as expected since the particles express these markers. The other markers in the violet channel, including CD20, CD16, and HLA-DR, exhibit some background staining due to simulated autofluorescence in that channel. All other markers exhibited negative or dim staining.

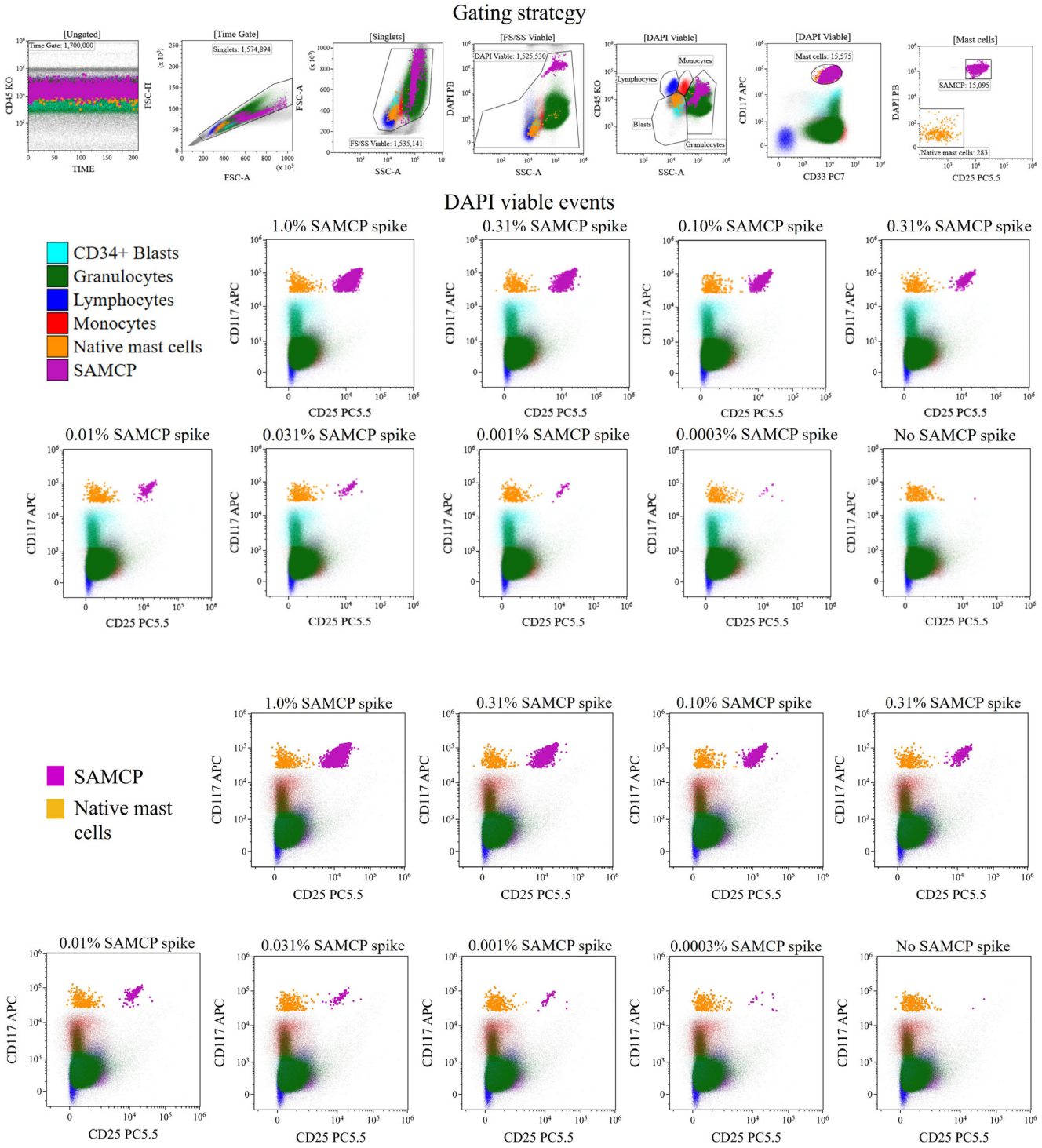

FIGURE 4 Dot plots of limit of detection study. Dot plots of each of the serial dilutions performed in the limit of detection analysis (Table 3) are shown with SAMCP in purple and native mast cells in orange. [Color figure can be viewed at wileyonlinelibrary.com]

3.4 | Limit of detection and linearity

ARUP's clinical mast cell assay is validated for a limit of detection of 0.003% when 1.7×10^6 events are collected. We sought to replicate this limit of detection using a serial dilution of mast cell particles spiked into normal bone marrow. We detected the mast cell particles down to a level of 0.0028% (Table 3). The particles were easily distinguishable from the native normal mast cells in the bone marrow sample (Figure 4). A plot of predicted versus observed SAMCP in normal bone marrow in the serial dilution analysis demonstrated an R^2 value of 0.9995 (Figure 5).

3.5 | Scatter properties of SAMCP

We analyzed how the SAMCP performed on various cytometer platforms. The side scatter of the particles looked similar across Navios, CytoFLEX, Aurora and FACSLyric cytometers (Figure 6). Forward scatter looked similar on Navios and CytoFLEX cytometers, but the particles fell higher on the forward scatter scale on the Aurora and particularly the FACSLyric cytometers. The SAMCP antigen/antibody performance appeared similar across all four flow cytometry platforms.

3.6 | Precision

Within run (Table 4), and between run (Table 5), precision of SAMCP spiked into normal bone marrow revealed coefficients of variability (CVs) of replicates of 7.32% and 3.32%, respectively, indicating excellent assay precision when using the SAMCP.

3.7 | SAMCP stability

While the lyophilized shelf life of the synthetic particles can reach 18 months or greater based on the manufacturer's information, we specifically evaluated SAMCP on days 4, 8, 13, and 21 after reconstitution in phosphate buffered saline and compared them to freshly reconstituted particles (Figure 7). Forward scatter and side scatter were unchanged at all-time points (Table 6). The antigen markers experienced some minor losses of median fluorescence intensity (MFI) by days 4, 8, and 13 of up to 30%. However, by day 21, CD45, CD117, CD2, CD25, and CD30 experienced more significant losses of MFI of greater than 30%, while CD33 remained unchanged compared to freshly reconstituted particles.

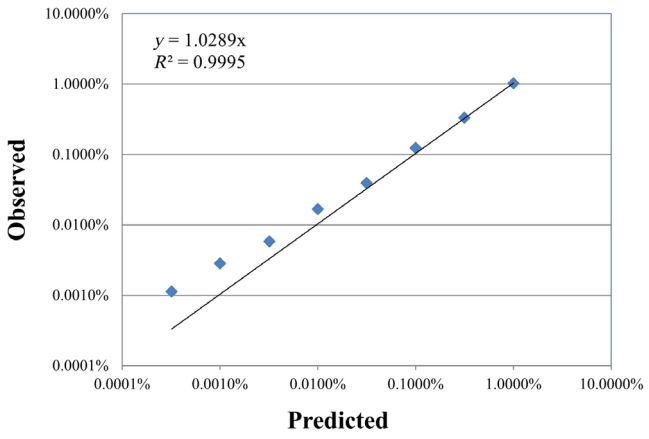

FIGURE 5 Plot of predicted versus observed SAMCP serial dilutions. The values from the SAMCP serial dilution study were plotted, and an R^2 value was calculated. [Color figure can be viewed at wileyonlinelibrary.com]

4 | DISCUSSION

The SAMCP were found to perform similarly on BC Navios and CytoFLEX, BD FACSLyric, and Cytek Aurora platforms. FMO controls demonstrated specificity of each of the coupled markers. Furthermore, when tested against our extensive library of antibodies, the particles exhibited no evidence of off target binding and inter- and intra-assay precision were less than 10% CV.

These synthetic particles provide several benefits including the ability to add multiple protein markers (up to 6 in this example and potentially more), as well as tunable light scatter and autofluorescence characteristics. The open vial stability and recognition by different antibody clones make these particles ideal for internal quality control processes across laboratories and opens the possibility for a reference particle that can be used to harmonize assays between laboratories. Indeed, harmonization (Miller & Greenberg, 2021; Rawstron et al., 2013) (i.e. ensuring equivalent performance without worrying about the intervening process) is logistically and politically easier compared with prior pushes for standardization (Kalina et al., 2012) (i.e. ensuring a single 'standard' process resulting in the same result). Fundamentally, both strategies result in assays that can be readily compared across laboratories, which offers significant benefits to patients and the scientific community.

One issue seen when testing these synthetic particles on multiple flow cytometry platforms is that the light scatter characteristics of the particles, when tuned for a particular instrument type, can differ from that of clinical samples when run on a different platform. The synthetic particles used in this study were specifically developed for use on Navios cytometers. In this respect, the use of these synthetic particles in blinded proficiency material may be limited, although further work by both Slingshot and instrument manufacturers may result in a more generalized solution. Nevertheless, outside of this limitation, these synthetic particles can be a useful reference and in cases of rare diseases, allow for the development and validation of assays even in smaller laboratories, particularly if these particles can themselves be validated in multiple laboratories against rare clinical cases. Using this specific study as an example, the SAMCP we tested could then become a translatable/transferable reference standard used by other laboratories to develop and harmonize their assays for SM.

To our knowledge, this is the first peer reviewed publication of Slingshot's synthetic particles tested in a clinical flow cytometry setting. These synthetic cell mimics show great promise in addressing a

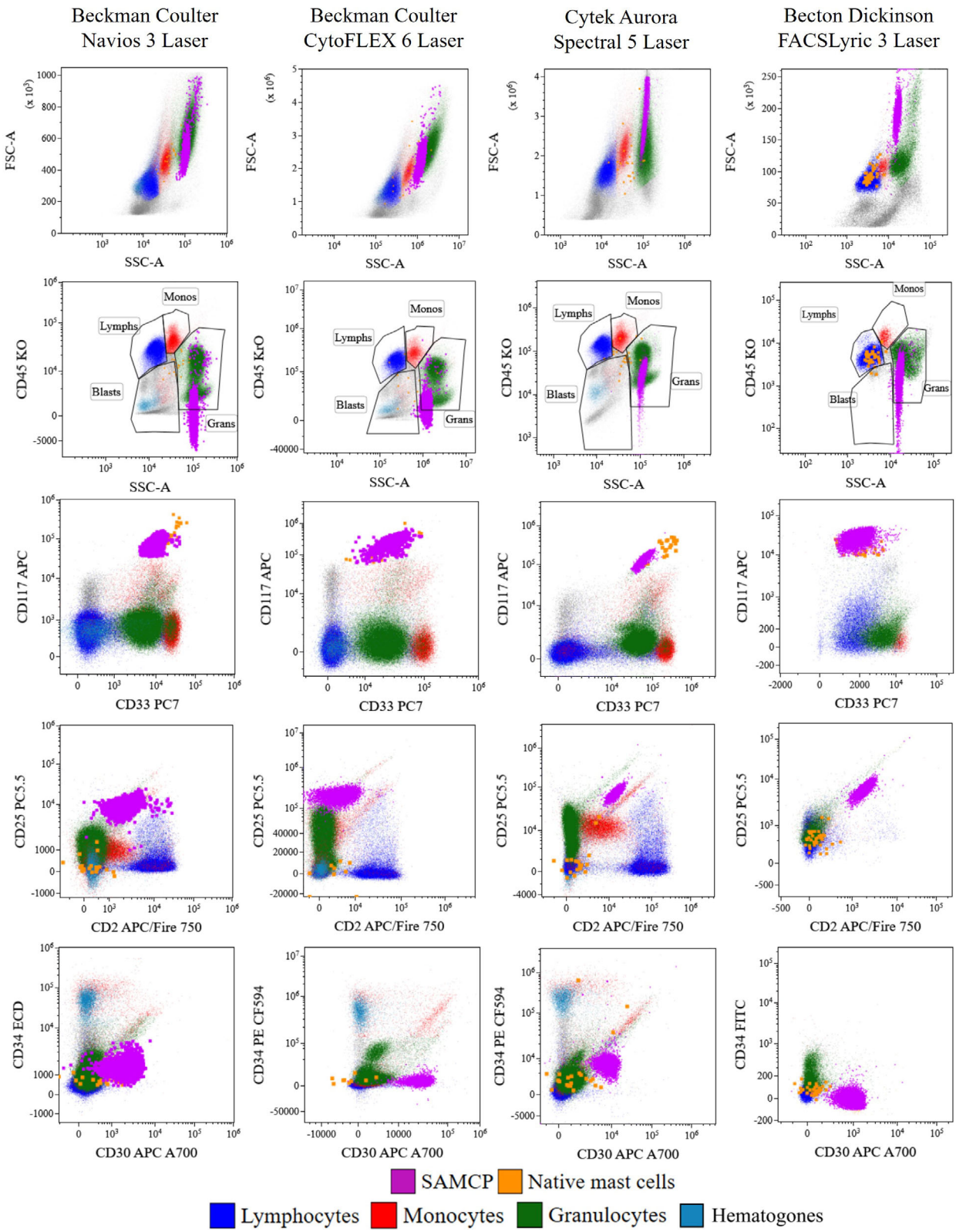

FIGURE 6 Scatter properties of SAMCP on various cytometer platforms. SAMCP were spiked in normal bone marrow at a concentration of 1%, stained with either the clinical mast cell panel (Table 1, for the Navios, CytoFLEX or Aurora platforms) or the alternative mast cell panel (Table 2, for the FACSLyric platform), run on the indicated platforms and analyzed on Kaluza software. Dot plots of side scatter versus forward scatter (top row), side scatter versus CD45 (middle row) or CD33 versus CD117 (bottom row) are shown for each of the cytometer platforms. SAMCP are shown in purple and native mast cells are shown in gold. [Color figure can be viewed at wileyonlinelibrary.com]

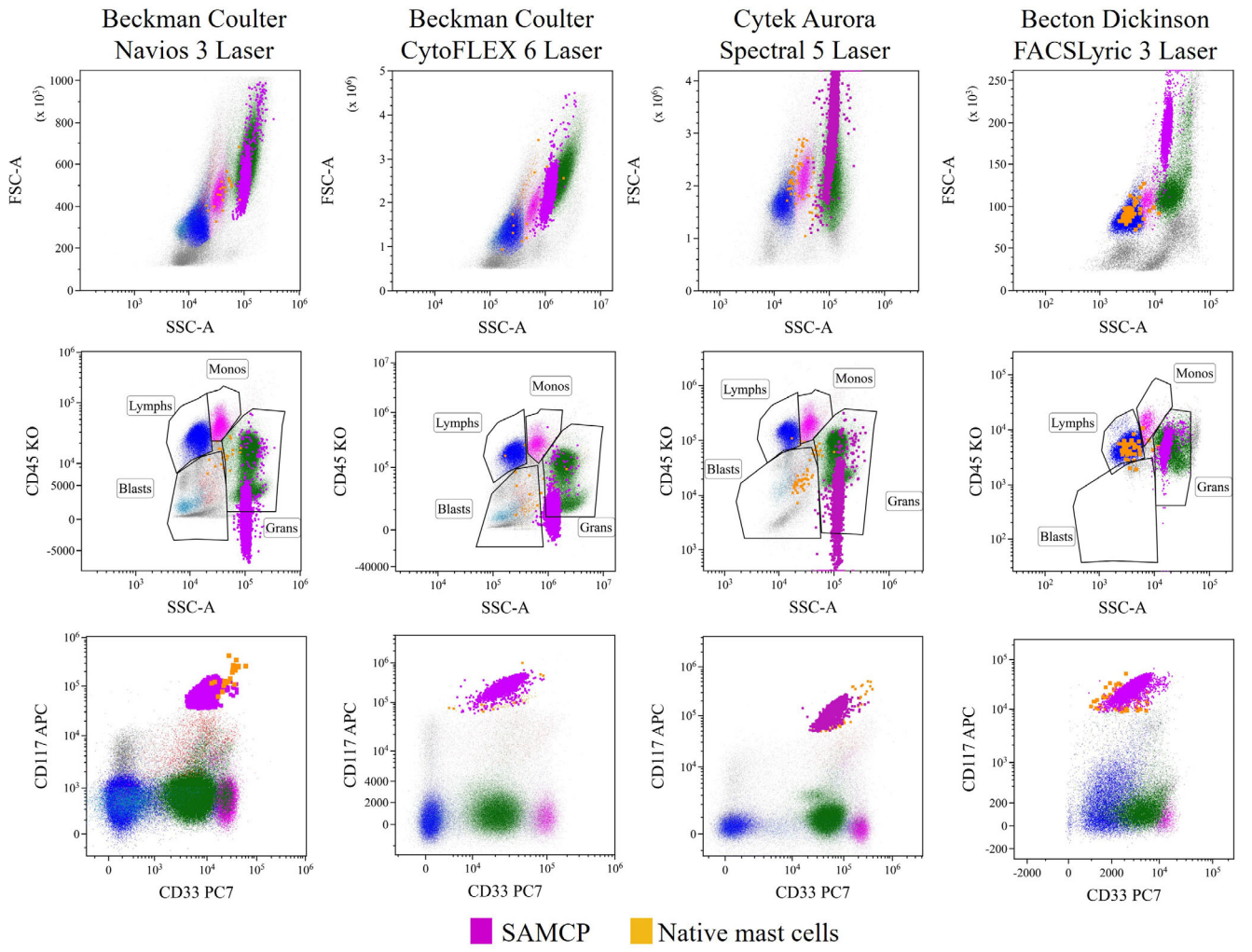

FIGURE 6 (Continued)

TABLE 4 Within run precision of SAMCP.

	# FS/SS total events	# particle events	% particles
Run 1	239,562	2647	1.10%
Run 2	243,144	2528	1.04%
Run 3	240,698	2613	1.09%
Run 4	238,112	2559	1.07%
Run 5	240,156	2444	1.02%
Average			1.06%
Standard deviation			0.04%
% CV			3.32%

Note: SAMCP were spiked into normal bone marrow at a concentration of about 1%. The sample was run five times on a Navios cytometer and analyzed in Kaluza.

TABLE 5 Between run precision of SAMCP.

	# FS/SS total events	# particle events	% particles
Replicate 1	239,562	2647	1.10%
Replicate 2	238,333	2395	1.00%
Replicate 3	243,562	2777	1.14%
Replicate 4	247,689	2963	1.20%
Replicate 5	244,568	2963	1.21%
Average			1.13%
Standard deviation			0.08%
% CV			7.32%

Note: SAMCP were spiked into normal bone marrow at a concentration of about 1% with five replicates. The samples were run on a Navios cytometer and analyzed in Kaluza.

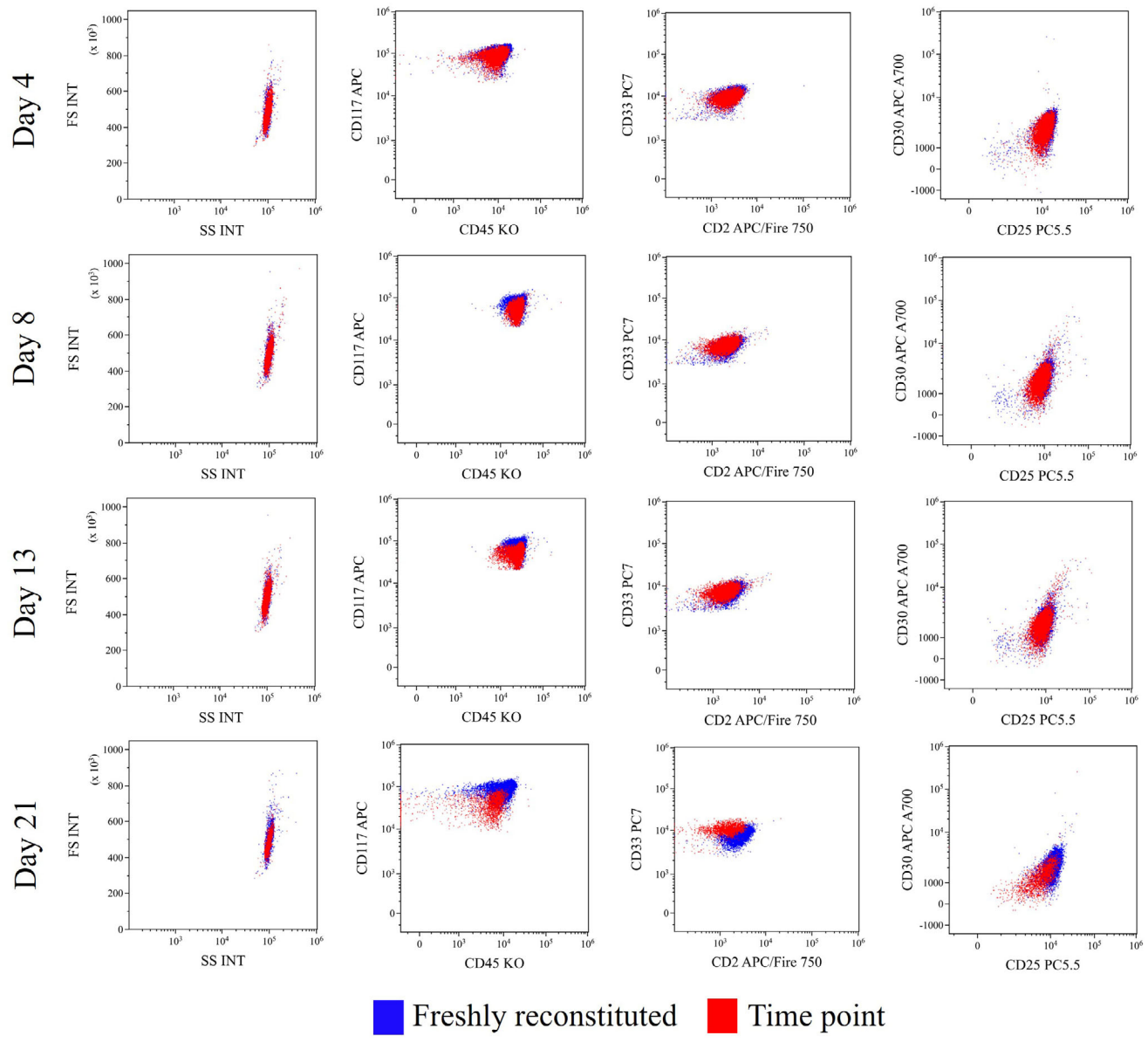

FIGURE 7 Dot plots of SAMCP stability. SAMCP were reconstituted from lyophilized vials, stored at 4°C. for the indicated length of time, and each time point were compared to freshly reconstituted SAMCP after staining with the mast cell panel and analysis in Kaluza. Overlay dot plots of particles at each time point (in red), versus freshly reconstituted particles (in blue), are shown for side scatter versus forward scatter and each of the expressed mast cell markers. [Color figure can be viewed at wileyonlinelibrary.com]

TABLE 6 SAMCP stability changes to MFI at time points.

	Change in MFI							
	FS	SS	CD45	CD33	CD117	CD2	CD25	CD30
Day 4	0.0%	0.2%	−17.1%	−12.8%	−14.9%	−10.7%	−11.6%	−11.7%
Day 8	0.6%	0.8%	−10.1%	0.6%	−23.1%	−17.6%	−12.0%	−15.7%
Day 13	0.2%	0.2%	−8.7%	−0.2%	−29.5%	−22.2%	−10.5%	−15.6%
Day 21	0.4%	0.0%	−44.9%	−1.2%	−47.7%	−36.7%	−30.1%	−35.4%

critical need for many clinical flow cytometry laboratories, particularly for rare disease testing.

CONFLICT OF INTEREST STATEMENT

Artificial mast cell particles were provided free of charge to ARUP by Slingshot Biosciences for testing purposes.

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