Semi and fully automated immunostaining sample preparation platforms improve live leukocyte recovery, reproducibility, and flow cytometry data quality

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ABSTRACT

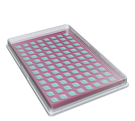
BACKGROUND: Limited innovation in automated cell and organelle sample preparation methodology limits the effectiveness of modern analytical methods, such as single-cell 'omics, flow and mass cytometry. These techniques traditionally rely on manual centrifugation-based protocols for cell washing and suspension preparation, hampering researchers' access to the reproducibility and scalability benefits of automation.

METHODS: We have developed a suite of cell suspension preparation systems that enable semi and full automation of cell washing protocols. These Laminar Wash™ technologies robustly, gently, and efficiently remove debris, dead cells, and unbound reagent using laminar flow and liquid handling robotics, rather than turbulent and harsh pelleting-plus-pipetting methods. Murine and humanized mouse peripheral blood mononuclear cells (PBMCs) and tumor infiltrating lymphocytes (TILs) were prepared and immunostained for flow cytometry analysis. Workflow improvements were assessed, as well as data quality by flow cytometry gating strategies isolating live cells and various lymphocyte subpopulations.

RESULTS: Adaptation of standard protocols to Laminar Wash automation typically improves repetitive immunostaining processes and workflows, in terms of reduced hands-on time and inter- and intra-operator variability. We demonstrate the superior live cell retention and reproducibility of Laminar Wash over centrifugation in processing murine and humanized mouse PBMCs and TILs for flow cytometry. Furthermore, we show how Laminar Wash improves flow cytometry data quality, in terms of debris removal and separation of immune cell subsets for both PBMCs and TILs.

CONCLUSIONS: Overall, these results show how Laminar Wash methodology assists in standardizing sample preparation for cytometric analysis, an important and unmet need in cancer immunotherapy discovery and manufacturing workflows.

LAMINAR WASH SYSTEMS



LW96 plate
Wall-less hydrophobic (red) and hydrophilic (blue) surfaces

HT2000
Semi-automated 96 wells

AUTO1000
Fully automated HT2000 within Hamilton NIMBUS User interface

SEMI-AUTOMATED IMMNOSTAINING WORKFOW

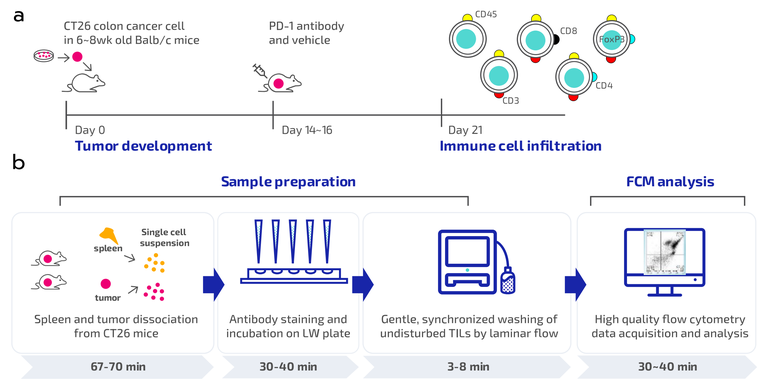


Figure 1. Overview of CT26 syngeneic mouse model and Laminar Wash workflow. **(a)** CT26 colon tumor cells were transplanted subcutaneously to 6~8-week-old mice and established for approximately 2 to 3 weeks followed by the i.v. injection of PD-1 antibody or vehicle, respectively. Spleen and tumor samples were processed into single cell suspensions and analyzed for immune cell subsets by flow cytometry. **(b)** Overview of the sample preparation procedure using the Laminar Wash system. Dissociated tumor cells and splenocytes were transferred to a LW96 plate and washed on HT1000 during the staining procedure prior to flow cytometry.

LAMINAR WASH IMPROVES RESOLUTION OF TUMOR INFILTRATING LYMPHOCYTES (TILs)

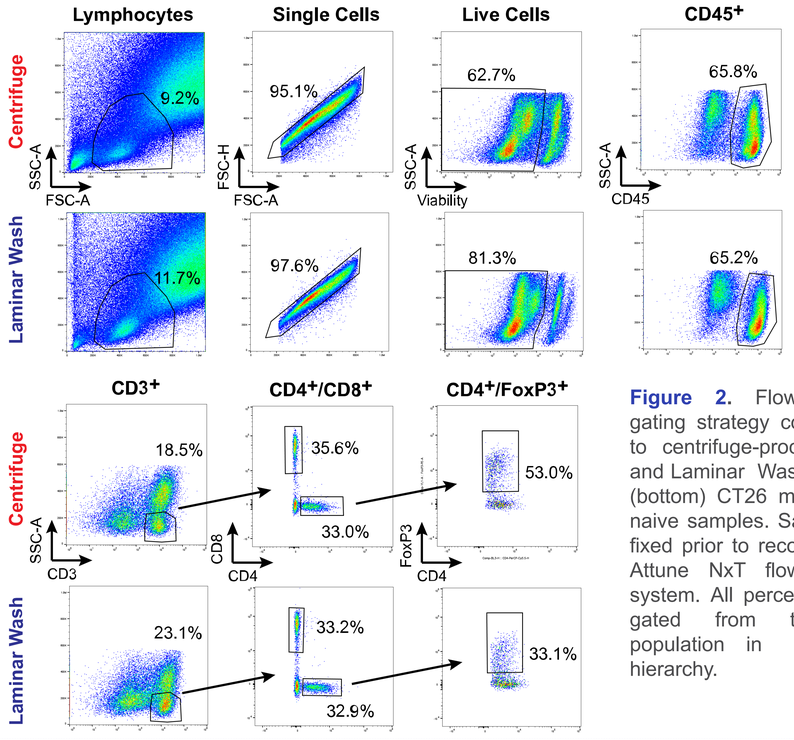


Figure 2. Flow cytometry gating strategy corresponding to centrifuge-processed (top) and Laminar Wash-processed (bottom) CT26 mouse tumor-naive samples. Samples were fixed prior to recording at an Attune NxT flow cytometry system. All percentages were gated from the parent population in the gating hierarchy.

LAMINAR WASH IMPROVES RECOVERY OF VIABLE TILs

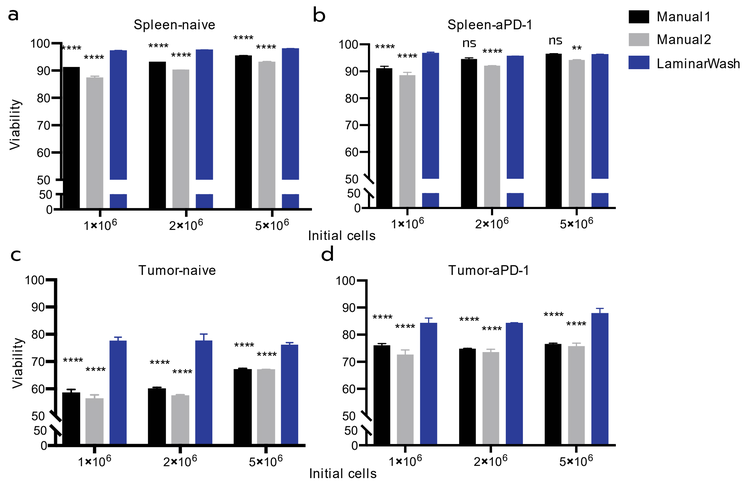


Figure 3. Splenocytes **(a)** and **(b)** and dissociated tumor cells **(c)** and **(d)** were washed with either centrifugation (manual 1 and 2) or the Laminar Wash system and viability measurements were compared. Statistical significance is reported among the manual methods vs Laminar Wash: ns = not significant,** = P<0.01, *** = P<0.001, **** = P<0.0001. The values represent technical triplicates of the samples from an individual naïve and an individual challenged Balb/c mouse. Manually processed samples were handled by two different analysts.

LAMINAR WASH REDUCES HANDS-ON TIME

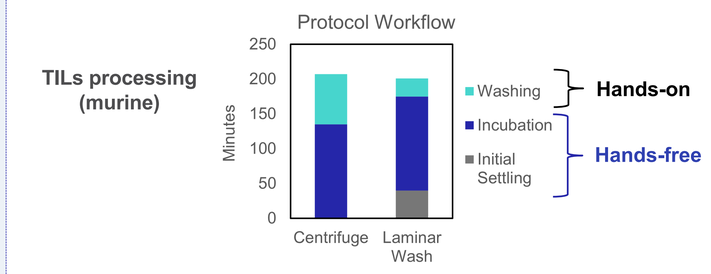


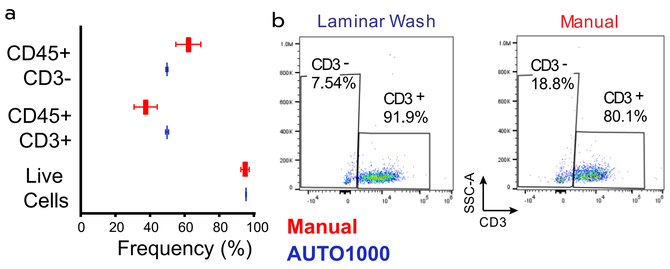
Figure 4. Procedural workflows were considered either incubation (blue) or washing (cyan) steps and were subsequently timed accordingly. Laminar Wash has an additional initial settling time that is counted as a washing step in the conventional method. Incubation and settling are hands-free steps.

Mock (time-motion study)

	Total Run Time, min:sec	Hands-On Time (% of total)	Walk-Away Time (% of total)
Centrifuge	25:06	10:06 (40.2%)	15:00 (59.8%)
HT2000	5:04	0:32 (10.5%)	4:32 (89.5%)

Table 1. A time-motion study comparing manual and Laminar Wash methodology was carried out, beginning after the antibody incubation step. Equivalent washes were performed: 3 runs of 5 min 1500 rpm spins on the centrifuge, 12 wash cycles with the Laminar Wash HT2000.

FULLY AUTOMATED WORKFLOW WITH AUTO1000 IMPROVES CONSISTENCY OF MOUSE PBMC IMMUNOSTAINING

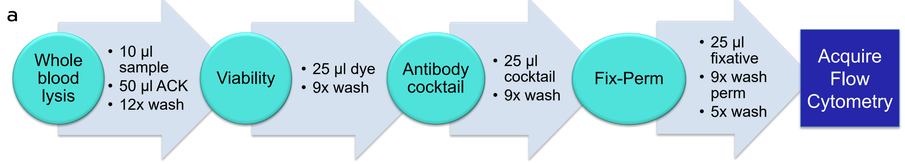


c

Mouse	Method		Live cells	hCD45+	CD3+	CD3-
1	Manual	Mean	97.67%	94.23%	76.47%	21.00%
		%CV	0.46	3.16	5.43	19.21
	AUTO1000	Mean	97.97%	97.37%	87.73%	11.73%
		%CV	0.50	0.31	0.43	2.14
2	Manual	Mean	95.50%	91.97%	32.67%	66.73%
		%CV	0.65	0.66	25.43	12.55
	AUTO1000	Mean	94.37%	86.67%	33.13%	66.53%
		%CV	0.53	1.22	7.42	3.86
3	Manual	Mean	94.80%	87.97%	37.20	62.23%
		%CV	2.59	1.61	18.62	11.37
	AUTO1000	Mean	95.37%	80.17%	49.87%	49.63%
		%CV	0.26	2.57	1.61	1.42

Figure 5. Humanized mouse PBMCs from naïve humanized mice (hNCG, n=5) comparing manual and AUTO1000 preparations. (a) Overall post ACK-lysed and recovery of parent CD45+CD3- and CD45+CD3+ lymphocytes were comparable with the AUTO generating tighter overall CVs (6.2% vs 20.8%). Error bars represent s.d. Technical replicates: manual (n=3) and AUTO (n=4). **(b)** Scatterplot analysis reveals improved resolution and auto-gating strategies. **(c)** Case studies between selected animals reveal triplicate intra-reproducibility improvements via automated handling and washing when analyzing CD45+CD3- and CD45+CD3+ subpopulations. All percentages were gated from the parent population.

HUMAN WHOLE BLOOD CAN BE REPRODUCIBLY LYSED AND IMMUNOSTAINED HANDS-FREE ON AUTO1000



b

		CD45+	CD19+	NK cells
7.5 µl whole blood (n=4)	Mean	97.10%	20.43%	13.95%
	%CV	0.98	7.32	10.61
10 µl whole blood (n=4)	Mean	97.90%	24.93%	18.38%
	%CV	0.64	5.67	5.19

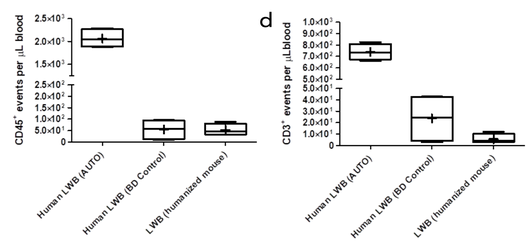


Figure 6. Proof-of-concept: automated lysis and immunostaining of 10 µL human whole blood on the AUTO1000 system. (a) schematic of AUTO1000 workflow. Settling and incubation occurs prior to wash cycles; post-wash volumes are at 25 µl. **(b)** Table depicting CV values for immune cell subtypes. All percentages were gated from the parent population. Representative total live events for **(c)** CD45+ and **(d)** CD3+ cells measured in the 10 µl AUTO1000 experiment compared to 100 µl manual controls (run on separate days). Data are normalized to volume of blood. Mean indicated as +.