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

Melvin Lye[1], Christoph Eberle[2], Nadiezda Fernandez Oropeza[1], Ann Wang[1], Chyan Ying Ke[1] & Namyong Kim[1]
[1]Curiox Biosystems Inc., Woburn, MA; [2]Charles River Laboratories, Worcester, MA

CURIOX BIOLOGY AT ITS BEST

www.curiox.com

charles river | every step of the way.

ABSTRACT

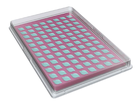
BACKGROUND: Limited innovation in automated cell and organelle sample preparation methodology limits the effectiveness of modern analytical methods, such as single-cell 'omics, flow and mass cytometry. These techniques traditionally rely on manual centrifugation-based protocols for cell washing and suspension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

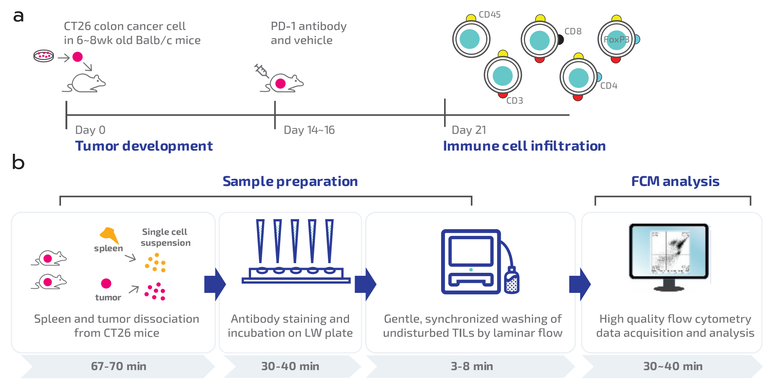


Figure 1. Overview of CT26 syngeneic mouse model and Laminar Wash workflow. (a) CT26 colon tumor cells were transplanted subcutaneously to 6~8-week-old mice and established for approximately 2 to 3 weeks followed by the i.v. injection of PD-1 antibody or vehicle, respectively. Spleen and tumor samples 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)

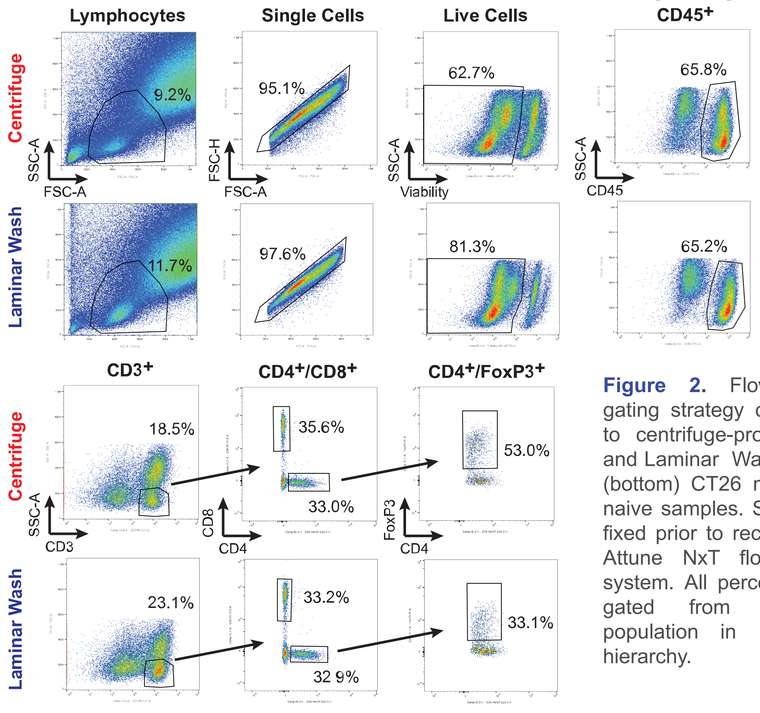
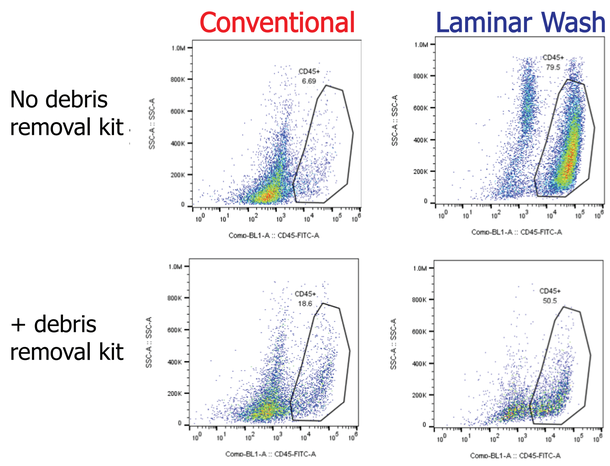
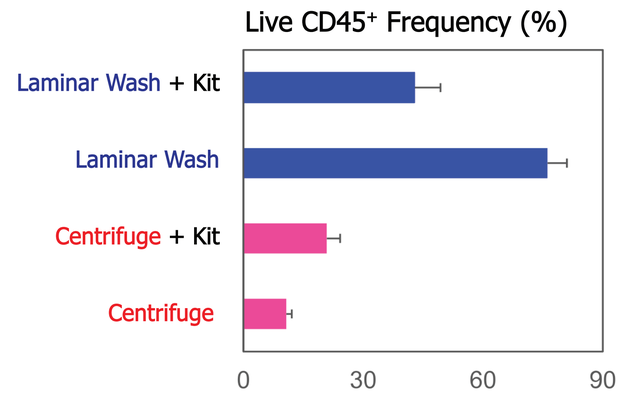


Figure 2. Flow cytometry gating strategy corresponding to centrifuge-processed (top) and Laminar Wash-processed (bottom) CT26 mouse tumor-naive samples. Samples were fixed prior to recording at an Attune NxT flow cytometry system. All percentages were gated from the parent population in the gating hierarchy.

QUALITATIVE MEASUREMENT OF DEBRIS REMOVAL RATE WITH FRESHLY DISSOCIATED TILS AND LAMINAR WASH





Samples: MC38 Tumor Infiltrating Lymphocytes (murine)

Kit: Miltenyi Biotec Debris Removal Solution

Figure 3. After mechanical and enzymatic dissociation using GentleMACS system, the single tumor cell suspension yielded 6.89% viable CD45+ lymphocytes. The toughness of the tumor and presence of fibrosis or necrotic parts can be challenging for the dissociation and lead to low yields and cell viability. With the application of a ready-to-use density gradient reagent, Debris Removal Solution (Miltenyi Biotec) improved the recovery to 18.6% viable CD45+ cells. In comparison, the standalone application of Laminar Wash revealed a higher recovery of 79.5% viable CD45+ cells, indicating the important variables of debris removal and gentleness of the wash to recover more TME-resident CD45+ cells.

LAMINAR WASH DEBRIS REMOVAL METHOD REVEALS MORE TME EFFECTOR CELLS AS A FACTOR OF BREAST CANCER PROGRESSION

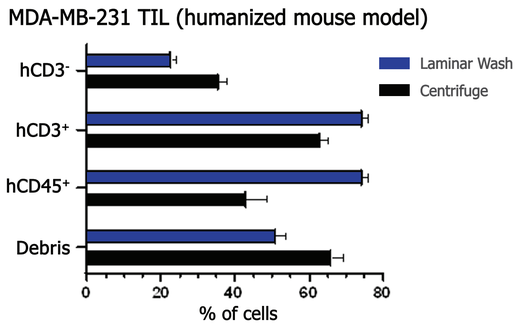


Figure 4. Comparison between the relative distribution of distinct cell populations evaluated on identifiable tumor cell dissociates in centrifuge method *(n=3, technical replicates)* and Laminar Wash *(n=3, technical replicates)* samples. Targeting MDA-MB-231 cells as a tumor-infiltrating leukocytes model for more aggressive breast cancer, the overall recovery of CD45+ cells was statistically improved with Laminar Wash. With 2 million cells at starting input, debris cleanup was improved down to 50% (from 66%) and CD45+ cell frequency to 76% (from 43%). Recovery of more tumor-associated CD3 lymphocyte frequencies assists in a more accurate CD3+ effector profile.

HIGH REPRODUCIBILITY AND TECH-TRANSFERABILITY DEMONSTRATED BY LAMINAR WASH WITH SPLENOCYTES AND TILS DERIVED FROM MICE TREATED WITH IMMUNOTHERAPY

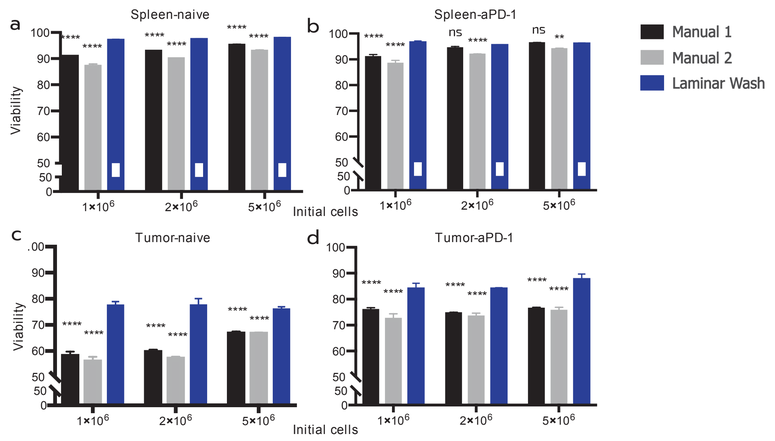


Figure 5. Splenocytes **(a)** and **(b)** and dissociated tumor cells **(c)** and **(d)** were washed with either centrifugation (manual 1 and 2) or the Laminar Wash system and viability measurements were compared. Statistical significance is reported among the manual methods vs. Laminar Wash: ns = not 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.

ENHANCED DEBRIS CLEANUP AND CELL RECOVERY SEEN BY LAMINAR WASH WITH FROZEN-THAWED PERIPHERAL BLOOD CELLS

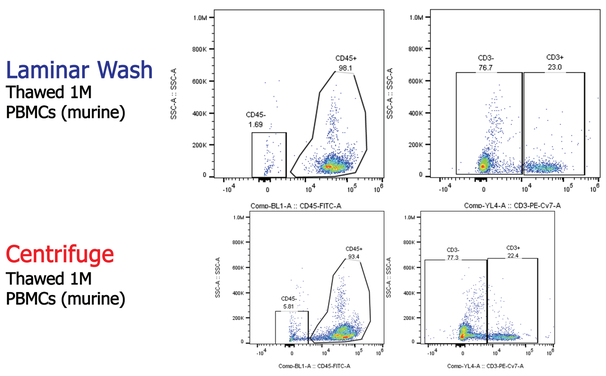


Figure 6. Investigation of differential loss of PBMC subtypes and phenotypic changes during thawing and incubation was performed with post-thawed washing and staining on Laminar Wash. Any harshness in post-thawing procedure significantly impacted PBMC viability and live cell recovery. Evaluating both viability and live PBMC recovery resolved more CD4+ cells (98.1% vs. 93.4%) and the cleanliness of thawed mouse PBMC in parallel processing showed distinctly better CD3- and CD3+ resolution.