A NOVEL SAMPLE PREPARATION METHOD FOR IMPROVED TUMOR-INFILTRATING LEUKOCYTE RECOVERY

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ABSTRACT

Murine syngeneic tumor models have significantly contributed to our understanding of cancer biology. Elucidating the roles of tumor-infiltrating lymphocytes (TILs) in solid tumors have become increasingly important in anti-cancer immunotherapies, yet a robust sample preparation method for consistent isolation of well-defined TIL populations is lacking. Here we introduce an innovative sample preparation platform using gentle Laminar Wash™ technology for isolating and characterizing TILs from dissociated CT26 syngeneic tumor samples. We demonstrate that the cell washing approach consistently improved overall TIL recovery with enhanced identification of immune subset populations, as compared to the conventional centrifugation-based method. This novel system simplifies workflows by requiring less hands-on time and limiting inter-operator variability, and it is applicable to a broad range of downstream analyses for dissociated tumors that present with challenging TIL recovery.

INTRODUCTION

Cancer immunotherapy has emerged as the revolutionary frontier in oncology. Since the original success in recalcitrant metastatic cancers[1], significant advances in immuno-oncology have led to novel therapeutic approaches and combinations to treat diverse cancer types in various stages of clinical development. The successful anti-tumor immune response is largely driven by tumor infiltrating lymphocytes (TILs), which display prognostic and predictive value in controlling cancer growth[2,3].

Naturally occurring TILs cohabitate the complex tumor microenvironment (TME) comprised of the extracellular matrix, blood vessels, and stromal and endothelial components, in addition to tumor cells. Such cellular heterogeneity within a solid tumor poses considerable technical challenges in isolating and characterizing the TILs for downstream analysis using multi-color flow cytometry[4] or single-cell sequencing[5]. A common problem with TIL preparation occurs during solid tumor dissociation, whereby TILs occupy a mixture with tissue debris and dead cells in suspension. Consequently, autologous TILs preparation often requires additional costly and laborious processing, such as density gradient centrifugation, immune cell sorting and enrichment, and dead cell and debris removal. Typically, such processing is accomplished with multiple centrifugation steps that introduce additional cellular stress, reducing TIL recovery[6]. Furthermore, such challenges in sample preparation can be further exacerbated in animal models, including the highly immunogenic murine CT26 colon carcinoma line, which has become a pre-clinical model for evaluating experimental anti-cancer drugs and their pathways in vivo[7].

Laminar Wash systems effectively remove floating debris in suspension by relying on gravity to settle live cells, followed by a gentle and continuous flow of buffer across the wells[8]. Avoiding repetitive centrifugation steps can also significantly reduce manual handling time. We surmised that Laminar Wash technology may be applied to improve TIL recovery and workflow. Using anti-PD1-challenged, murine CT26 dissociated tumors as a proof-of-concept, we report that this novel washing system results in significantly higher recovery of TILs and reduced labor-intensive washing time, while enhancing overall data quality.

MATERIALS AND METHODS

Mice

Balb/c mice were supplied from Taconic Biosciences (Rensselaer, New York, USA). Mice were 6–8 weeks old unless otherwise indicated; all animal protocols were executed in compliance with the Institutional Animal Care and Use Committee (IACUC) at Charles River Laboratories (IACUC No. I033).

Tumor challenge and isolation

Mice were challenged subcutaneously with 1×10^5 CT26 colon carcinoma tumor cells and sacrificed two weeks post inoculation. Spleens and the tumors from the CT26 tumor-bearing mice were removed, and the tissues were mechanically dissociated on a gentleMACS™ Octo Dissociator (Miltenyi Biotec). Enzymatic digestion of tumors was accomplished with the mouse tumor dissociation kit from Miltenyi Biotec (no. 130-096-730). Cells were washed, resuspended, counted, and transferred to either a round-bottom deep 96-well plate or on a Laminar Wash 96 (LW96) plate at a density of 1×10^6, 2×10^6, and 5×10^6 cells per well for antibody staining and flow cytometry analysis[9,10].

Antibodies, staining reagents, and flow cytometry

Purified anti-mouse CD16/32 antibody for Fc receptor blocking was purchased from BioLegend. Mouse antibodies specific for CD3 (17A2), CD4 (GK1.5) and CD8 (53-6.7) were purchased from BioLegend, and those specific for CD45 (30-F11), FoxP3 (FJK-16s) and the viability marker (APC-eFluor 780 dye) were purchased from ThermoFisher Scientific for immunophenotyping by flow cytometry. Buffer for fixation and intracellular staining were freshly prepared from the Invitrogen™ eBioscience™ FoxP3 / Transcription Factor Staining Buffer Set (ThermoFisher Scientific, no. 00-5523-00)

according to the manufacturer's instructions. Samples washed by either centrifugation or the Laminar Wash method were collected into flat round-bottom 96-well plates and recorded at 100 µL/min on an Invitrogen™ Attune™ NxT flow cytometer (ThermoFisher Scientific, no. A24858). Immune cell populations were gated using FlowJo v10, and data were analyzed and graphically represented with PRISM software.

Antibody staining

Following washing by centrifugation or Laminar Wash, cells were resuspended in their residual buffer in either a round-bottom deep 96-well plate or a LW96 plate, respectively. Cells were first incubated with viability dye in 1X DPBS for 20 minutes on ice, protected from light. Subsequently, cells were washed, immersed with Fc receptor blocking reagent for 5 minutes on ice, then surface staining antibody mastermix was added, and cells were incubated for 40 minutes on ice, protected from light. After antibody incubation, samples in the centrifuge-processed group were diluted with 2 mL BD Stain buffer and centrifuged at 350 g for 5 minutes. Samples processed by Laminar Wash were washed for 9 rounds at 5 µL/s on a LW96 plate in an HT1000 instrument. Prior to intracellular FoxP3 staining, cells were fixed for 20 minutes on ice, protected from light. Cells were then washed and incubated on ice for 50-60 minutes, protected from light. Cells were then washed and resuspended in a final volume of 300 µL of BD Stain buffer for acquisition on the flow cytometer.

RESULTS AND DISCUSSION

Splenocytes or tumor samples were isolated from either naïve or anti-PD-1-challenged CT26 Balb/c mice, respectively (**Figure 1a**). Dissociated tumor cells and splenocytes in suspension were seeded in the density of 1, 2, and 5×10^6 cells per well and washed by either conventional centrifugation (i.e., manual method) or the Laminar Wash method prior to flow cytometric analysis (**Figure 1b**). Comparisons in the two washing methods in terms of the overall TIL isolation efficiency from the CT26 tumor-bearing mouse model are presented below.

Samples processed with Laminar Wash show higher viability and increased cell recovery

To compare the relative percentages of viable TILs from the dissociated tumors, the cell mixtures were stained with a live/dead marker. In further assessing operator-to-operator variability, samples were washed in
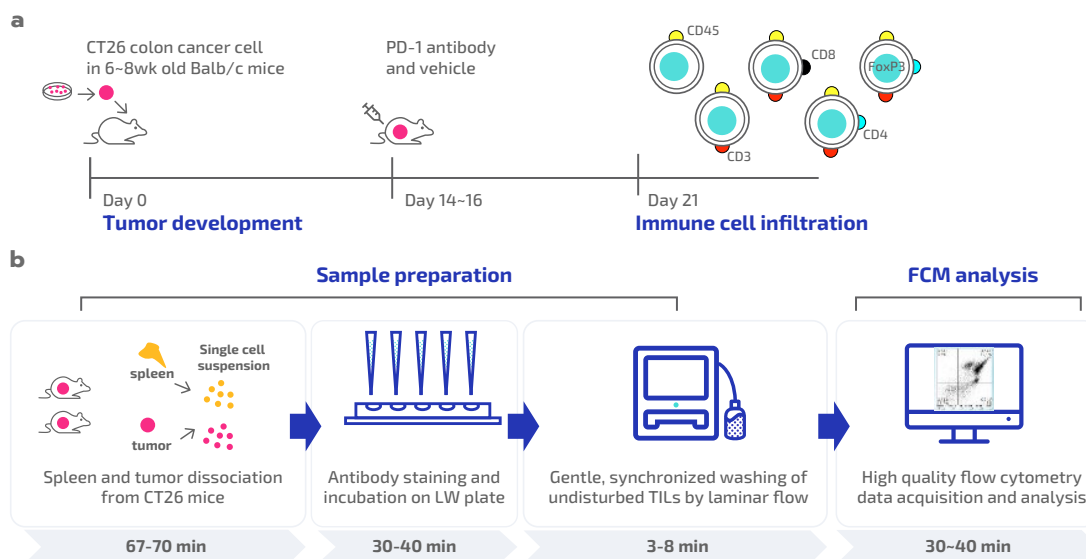
Figure 1. Schematics of CT26 syngeneic mouse model generation and overview of the Laminar Wash workflow. (a) *CT26 syngeneic mouse model and dose regimen. CT26 colon tumor cells were transplanted subcutaneously to 6~8-week-old mice and established for approximately 2 to 3 weeks followed by the i.v. injection 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.*

three major groups: two groups by independent manual operators and the third by the Laminar Wash method. Overall, Laminar Wash resulted in increased viability across all sets of samples, regardless of the treatment status or the initial cell density (**Figure 2**). Notably, viability differences between the conventional and Laminar Wash methods were more pronounced among the tumor samples (**Figure 2c–d**) than the splenocytes (**Figure 2a–b**), suggesting Laminar Wash more effec-

tively removed debris from the tumor-bearing tissues.

Taken together, these data demonstrate that the Laminar Wash method resulted in higher cell retention and viability, leading to an enhanced recovery of TILs from freshly dissociated tumor samples. The increased enrichment of a viable TIL population with Laminar Wash is likely attributed to more efficient removal of the floating debris and dead cells in suspension, while keeping the live cells settled at the bottom of the LW96
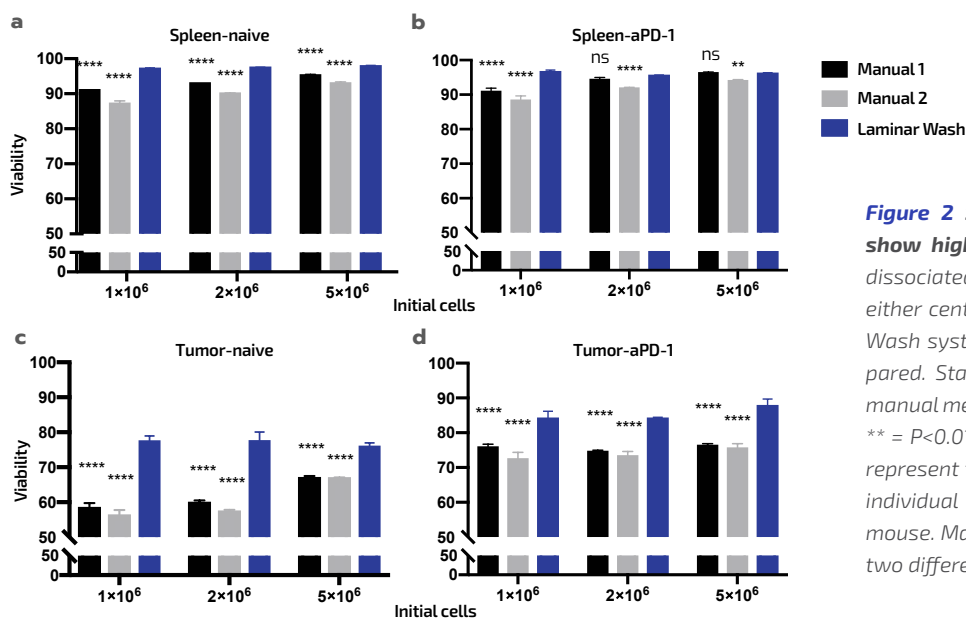
Figure 2 Samples processed with Laminar Wash show higher viability. *Splenocytes* (a) *and* (b) *and dissociated tumor cells* (c) *and* (d) *were washed with either centrifugation (manual 1 and 2) or the Laminar Wash system and viability measurements were compared. Statistical significance is reported 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.*
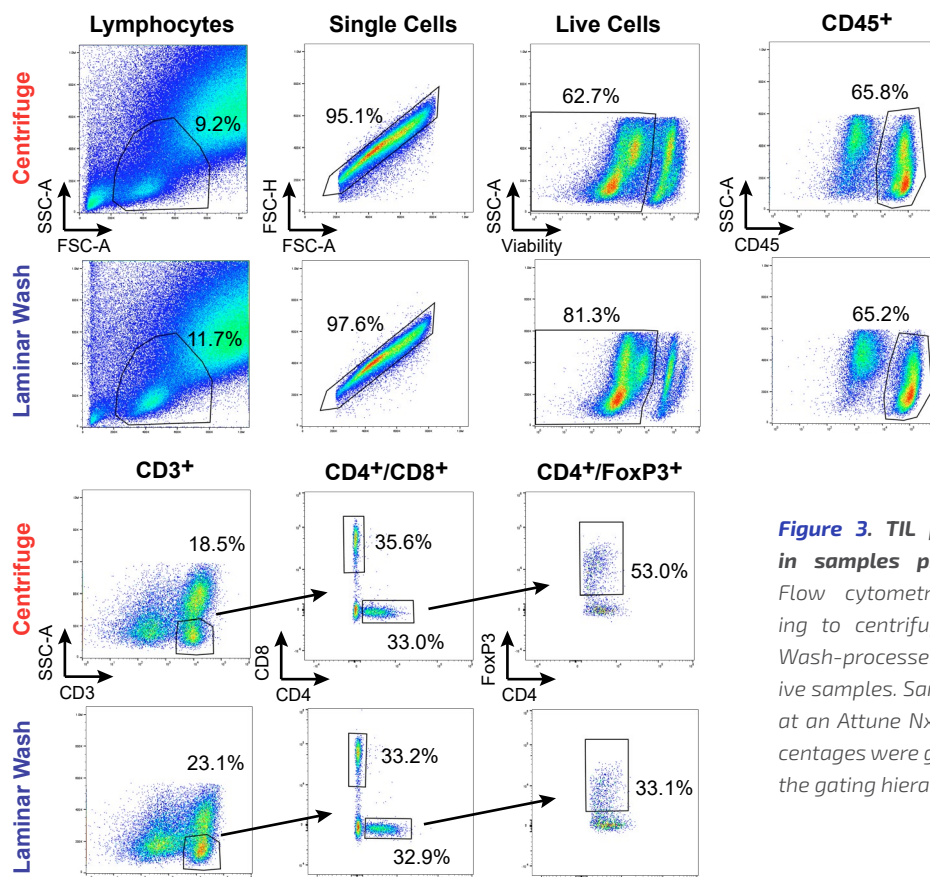
Figure 3. *TIL populations are better defined in samples processed with Laminar Wash. Flow cytometry gating strategy corresponding to centrifuge-processed (top) and Laminar Wash-processed (bottom) CT26 mouse tumor-naive samples. Samples were fixed prior to recording at an Attune NxT flow cytometry system. All percentages were gated from the parent population in the gating hierarchy.*

plate unperturbed. This contrasts with conventional centrifugation, which pellets both debris and dead cells along with live TILs, particularly with the heterogeneous tumor samples.

Laminar Wash yields better defined and identifiable TIL populations

To identify the various TIL populations from the dissociated cell mixture, we first pre-gated on live, single, CD45+-positive lymphocytes. For immunophenotyping, we employed a gating strategy specific to the various staining markers (e.g., single cells, CD45+, CD3+, CD3+CD4+, CD3+CD4+FoxP3+, and CD3+CD8+ cells, **Figure 3**). Remarkably, cells prepared with the Laminar Wash system showed a significant increase in the CD45+ populations compared to those washed conventionally with a centrifuge. In addition, signals corresponding to the CD4+ and CD8+ subpopulations of the CD3+ cells were better resolved with Laminar Wash, while the relative frequencies were unperturbed.

For each gating of subpopulations, the Laminar Wash

method appears to provide a cleaner, more appreciable separation. The overall sample clearly shows less debris, and the CD45+ and CD3+ subpopulations show discernable borders. These data suggest that the gentle washing associated with the Laminar Wash method maintains specific lymphocyte subpopulations unperturbed in their native states, leading to better definitions and clearer identifications, compared to the harsh pelleting imparted by centrifugation.

Laminar Wash generally reduces variation in lymphocyte recovery

To investigate the consistency of the two wash methods, three technical replicates were performed, and the resulting coefficients of variation (CVs) were calculated for each of the two centrifuge operators and Laminar Wash. The Laminar Wash method consistently yielded higher levels of viable cells over the three replicates compared to either manual operator (**Table 1**). Samples processed by the Laminar Wash method typically showed lower CV values for lymphocyte subtypes

			Viable cells	CD45^{+}	CD3^{+}	CD4^{+}	CD8^{+}	FoxP3^{+}
Manual 1	Naïve	Mean	58.23%	76.20%	29.47%	34.53%	30.63%	48.07%
		%CV	2.62	2.75	13.13	15.47	3.20	3.52
	a-PD1	Mean	75.87%	91.27%	36.60%	22.50%	58.67%	63.87%
		%CV	1.12	0.38	11.82	6.27	3.06	3.21
Manual 2	Naïve	Mean	56.07%	79.87%	34.23%	35.33%	29.57%	45.13%
		%CV	3.07	1.25	1.76	1.88	1.74	3.55
	a-PD-1	Mean	72.50%	93.07%	38.50%	22.90%	56.53%	61.20%
		%CV	2.58	0.48	1.35	0.76	1.18	1.61
Laminar Wash	Naïve	Mean	77.43%	66.30%	38.07%	24.73%	31.47%	46.83%
		%CV	1.94	4.58	1.71	1.02	3.68	4.92
	a-PD-1	Mean	84.10%	91.60%	45.43%	16.23%	57.37%	60.70%
		%CV	2.46	1.22	1.79	4.78	1.91	3.99

*Table 1. Comparison of the means and %CVs between manual and LW methods. The percentages of the viable cells and the lymphocyte subsets for tumor samples challenged with or without anti-PD-1 antibody are presented for each of the two manual operators and the Laminar Wash method. The values represent the variability among technical triplicates for samples containing 1x10⁶ cells per well.*

with fewer outliers, compared to those processed by centrifugation. This trend was particularly true for the CD3^{+} and CD4^{+} TIL subtypes, for which manual inter-operator variability was high. Furthermore, the trend was observed regardless of the starting cell density for both splenocytes and dissociated tumors (data not shown). For intracellular staining of CD4^{+}FoxP3^{+} cells, Laminar Wash generated higher CV values that were still <5%. It should be noted that this method was not optimized prior to adaptation to Laminar Wash and subsequent analysis.

The Laminar Wash methodology has fewer hands-on steps that depend on operator experience and technique, which here appears to positively impact consistency in cell recovery. Notably, the percentage of the CD45^{+}-expressing lymphocyte populations remained relatively unchanged among the methods and analysts, even though Laminar Wash yielded a higher percentage of viable cells, suggesting that the Laminar Wash method maintained the relative proportions of stained lymphocyte subpopulations. Furthermore, the expected PD-1 antibody-dependent shift in TIL population density from CD4^{+} to CD8^{+} cells was well-maintained among all the methods, providing further evidence the underlying biology was unaffected. Hence, the Laminar Wash system dually addresses both operator operator-dependent variability and cell loss by bypassing the handling steps associated with centrifugation.

The Laminar Wash method simplifies sample preparation workflow

In order to assess whether the Laminar Wash system can streamline the sample preparation workflow, we evaluated a time course of the entire procedure comparing the two washing methods, from initial sample transfer to CT26 tumor isolation (**Figure 4, Supplementary Table 1**). Laminar Wash requires an initial 40-minute step whereby cells settle to the
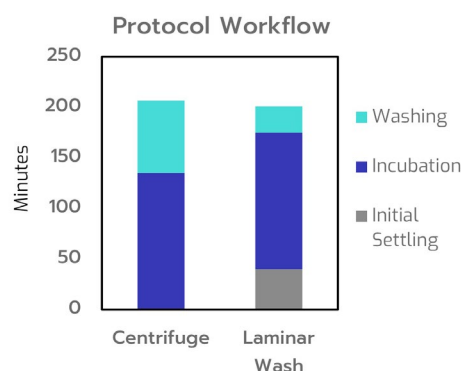
Figure 4. Laminar Wash reduces the time to complete labor-intensive wash steps. Procedural workflows were considered either incubation (cyan) or washing (blue) steps and were subsequently timed accordingly. Laminar Wash has an additional initial settling time that is counted as a washing step in the conventional method (See Supplementary Table 1).

bottom of the LW96 plate by gravity, which the conventional method accomplishes at the first wash step. Fixation and permeabilization times were counted together with viability and immunostaining as incubation steps. Overall, incubation workflows were comparable among the two methods. However, the washing steps were performed in 26 minutes with the Laminar Wash system, compared to 72 minutes with a conventional centrifuge, a savings of 46 minutes in favor of Laminar Wash. Taking into account the unique initial cell settling period, the Laminar Wash method completed the entire procedure 6 minutes faster than the conventional method.

Conventional wash methods are typically labor-intensive, involving complicated movements that introduce variability among users, sites, and time. In contrast, Laminar Wash technology automates many of these steps, providing a more consistent workflow, which may explain some of the reduced CVs presented in **Table 1**. For example, the instrument fluidics accomplish continuous buffer exchange, rather than user "flicking" or aspirating of supernatant post-centrifugation. These results indicate that the Laminar Wash protocol not only improved the staining index, but it also simplified the workflow and shortened the hands-on processing time required to prepare samples for flow cytometric analysis. Additionally, we have observed similar trends in data quality improvement with other syngeneic models, as well as humanized mice, demonstrating that this finding is applicable to a wide range of animal tumor models (data not shown).

CONCLUSION

Reliable methods for analyzing TILs from tumor samples are critical for understanding the roles of these precious cells in immuno-oncology and evaluating therapies. Here, we demonstrate that sample processing with Laminar Wash is superior to conventional centrifugation in recovering TILs from murine CT26 syngeneic tumors. The gentle and automated Laminar Wash method effectively removes debris and dead cells from dissociated tumors, leading to better and more consistent identification of TILs for more accurate analysis without altering the subpopulation composition. Finally, the method simplified the workflow, yielding considerable time savings in laborious washing steps, while reducing manual manipulation and inter-operator variability. In conclusion, the novel Laminar Wash system represents an elegant, reproducible, and partially automated approach to recovering viable and well-defined TIL subpopulations for quantification and analysis by multicolor flow cytometry.

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Time (min)	Conventional SOP	Laminar Wash method	Time (min)
Viability staining			
---	Aliquot cells at 1x10^6 in azide-free and protein-free PBS.	Aliquot 1x10^6 cells in azide-free and protein-free PBS and allow to settle by gravity.	40
8	Wash cells with 2 mL of PBS, centrifuge at 350 x g for 5 min. Discard supernatant.	Wash on HT1000 12 rounds with PBS.	5
---	Add 0.5 µL of viability dye per 100 µL cell suspension and mix immediately.	Add 0.25 µL of viability dye per 50 µL cell suspension and mix immediately.	---
20	Incubate for 20 min on ice in dark.	Incubate for 20 min on ice in dark.	20
16	Wash cells with 1 mL of Stain Buffer, centrifuge at 350 x g for 5 min twice. Discard supernatant.	Wash on HT1000 12 rounds with Stain Buffer.	5
Extracellular staining			
---	Resuspend cells in 100 µL of Stain Buffer.	Cells are currently in 25 µL of Stain Buffer	---
5	Add 2 µL of mouse Fc receptor blocking reagent per 100 µL of cell suspension, mix and incubate on ice for 5 min.	Add 1 µL of mouse Fc receptor blocking reagent per 50 µL of cell suspension, mix and incubate on ice for 5 min.	5
40	Add freshly prepared antibody mix and incubate on ice for 40 min protected from light.	Add freshly prepared antibody mix and incubate on ice for 40 min protected from light.	40
16	Centrifuge at 350 x g for 5 min. Discard supernatant. Wash cells with 2 mL of Stain Buffer, centrifuge at 350 x g for 5 min again. Discard supernatant.	Wash on HT1000 12 rounds with Stain Buffer.	5
Cell fixation and permeabilization			
20	Fix cells in 0.5 mL/well using Fix/Perm Buffer in the dark for 20 min on ice.	Add 50 µL Fix/Perm Buffer to each well and incubate in the dark for 20 min on ice.	20
16	Add 1 mL of Perm/Wash Buffer, centrifuge at 350 x g for 5 min twice. Discard supernatant.	Wash on HT1000 12 rounds with Perm/Wash Buffer.	5
Intracellular staining			
50	Add 100 µL of freshly prepared staining mix and incubate on ice for 50 min protected from light.	Add 50 µL of freshly prepared staining mix and incubate on ice for 50 min protected from light.	50
16	Wash cells with 2 mL of Perm/Wash Buffer, centrifuge cells 350 x g for 5 min twice. Discard supernatant after each wash step.	Wash on HT1000 5 rounds with Perm/Wash Buffer.	3
---	Resuspend cells in 300 µL of Stain Buffer and store cells at 4°C.	Wash on HT1000 5 rounds with Stain Buffer. Store cells at 4°C.	3
Total procedure: 207min **Wash steps:** 72 min **Incubation steps:** 135 min		**Total procedure**: 201 min **Wash steps:** 26 min **Incubation steps:** 135 min **Settling step:** 40 min	

Supplementary Table 1. *Step-by-step comparison of the overall processing procedure between the conventional and the Laminar Wash method. Colored boxes correspond to wash (cyan) or Laminar Wash settling (gray) steps.*