WHITEPAPER

# The Cell Analysis Conundrum- Garbage In/Garbage Out

High quality data begins with high quality sample preparation: centrifuge-free, automated, and non-disruptive approaches lead to reproducible and higher resolution biological datasets

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# Introduction

The value of mammalian single-cell analyses lies in the high-resolution data generated to address critical questions in biological discovery, assay development, and generation of advanced therapies. The challenge has been ensuring the quality of upstream protocols, namely high-quality processing of samples to guarantee experimental consistency, reliability, and data quality. Even though centrifugation has damaging effects on cells, it remains a standard sample processing technique (Lu et al., 2019). Implementing a novel centrifuge-free automated approach presents significant advantages in cell sample processing, including (but not limited to) increased laboratory efficiency and reproducibility, as well as cell viability and debris removal, thereby improving the integrity of downstream data.

This methodology can improve reproducibility, quality and consistency between datasets generated using high-content techniques, such as single cell analysis, flow cytometry and immunoassays. For immunotherapy discovery, infectious diseases or tumour microenvironments, such technologies are highly warranted, when working with small precious samples and rare cells of interest for pre-clinical and clinical research.

# Content

1. **Cells are a precious research tool, and a considerable amount of information can be extracted when each cell is analysed in isolation.**
   There is an ever-increasing demand for novel scientific tools to advance healthcare and drug development. Demand is skyrocketing for single cell technologies, whereby millions of cells can be isolated and lysed using emulsification microfluidics and their genetic material subsequently analysed. Advances in single cell technology allow for the acquisition of higher resolution data, and the assembly of large biological datasets that can be used to answer important scientific questions (Armand et al., 2021). However, the imperative for these technologies to succeed is that the upstream cell sample prep is fast, effective, and gentle to maintain the native cellular expression profiles. When done well, these technologies have enabled numerous break-through discoveries: unravelling mechanisms for colorectal cancer (Zhang et al., 2018), immunophenotyping (Papalexi et al., 2018) and diversity of cell types in the brain (Zhong et al., 2018), thereby contributing to the understanding of plethora of molecular mechanisms and cellular development, hence, the possibility of future treatment for terminal diseases (Tang et al., 2019).

2. **Automating and miniaturizing sample preparation to deliver higher downstream results**
   Multiple technologies have been developed to improve the throughput and analytical dimensions of single-cell analyses. As the first and ubiquitous step in any bioassay, sample preparation is paramount to delivering reliable and meaningful downstream results. Systematic protocols to automate and miniaturize sample preparation can help to overcome deviations in sample quality, that may arise from human error and other preanalytical factors.

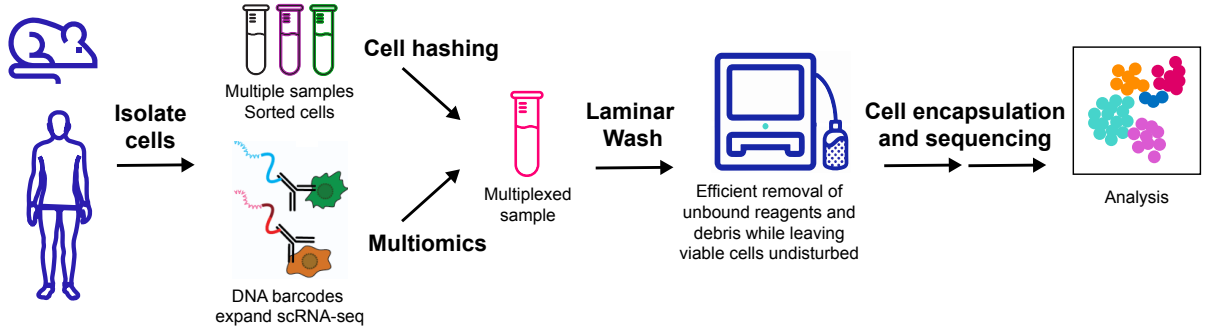
Various multiplexing techniques have been developed to acquire samples with different cell types or origins at the same time, limiting costs and maximising statistical power. Multiplex immunoassays capture ligands of interest in parallel arrays (Ellington et al., 2010). Flow cytometry machines measure the optical and fluorescent characteristics of individual cells and sort them according to these parameters. The fluidic system, light-scattering and signal detection and processing components of flow cytometry are all programmed functions of the apparatus (Adan et al., 2017). Mass cytometry utilizes both flow cytometry and mass spectrometry to increase the number of cellular parameters that can be sampled simultaneously (Spitzer et al., 2016).

Single-cell multi-omics is a streamlined process beginning with cell sorting (using cytometry, droplet-based microfluidics or droplet-based methods). 'Cell-hashing' or CITE-seq uses oligotagged antibodies against widely expressed surface proteins that have barcodes to exclusively label cells from different samples, which can then be pooled and run in one parallel scRNA-seq run (Stoeckius, 2017). (Figure 1) Both mass cytometry (Bendall et al., 2014) and single-cell transcriptomics have been used to detect novel cell populations (Stewart et al., 2021). Unlike cytometry however, gating out debris or sub-populations of cells is not possible in single-cell sequencing, so the cell preparation must be as clean as possible prior to analysis. Otherwise, single-cell isolation applications can result in transcriptome-wide alterations and/or useless expensive data arising from sequencing these cells (Van den Brink et al., 2017).

The Laminar Wash system is currently being used as a model for standardisation of sample preparation. It was included in the Flow Cytometry Standards Consortium, in partnership with the National institute for standards and technology (NIST), as a contribution to the adoption of a universal flow cytometry method.

Fig. 1

Conceptual for single cell utility.



Single-cell technologies remain exceptionally expensive, from the device usage to the sequencing power required. Maximising efficiency and the ratio of data attained per sample is vital to saving costs. Moreover, challenges arise in the interpretation of complex datasets, so the minimization of confounding variation is crucial. Automating and miniaturizing sample preparations can greatly limit artefacts, reducing the signal to noise ratio and reduce costs.

Automating sample preparation can improve workflows by enabling reproducible implementation of protocols while limiting human intervention in sample handling, therefore reducing variability and error. Study costs are also reduced by miniaturizing sample preparations, which allows for a lower input of rare and valuable cells and removing the need to repeat experiments due to user variability. In addition, this allows for wider accessibility, and adoption, of various protocols from research groups around the world constrained by limited financial and spatial resources. This is invaluable to scientists, allowing for more time and freedom to innovate (Holland and Davies, 2020). Finally, cell fractionation prior to downstream analysis can lead to increased resolution in specific studies, such as proteomic profiling (Stewart et al., 2021; Larance and Lamond, 2015).

### 3. Traditional methods of sample preparation disrupt valuable cell types

The future of sample preparation determines the future characterisation standards of biological samples, as well as the launching of cell-based products (Lu et al., 2019). A major requirement of cellular processing is that cells are transferred from their native environment to a suitable solution for measurement. Centrifugation is the most common method for isolating cells, whereby the cells are repeatedly pelleted and subsequently re-suspended in a clean, specified medium. While limiting the time and centrifugal force of these so-called wash steps can reduce some stress (Katkov and Mazur, 1999), many cells will remain in the supernatant and are subsequently not recovered. This leads to a material loss in the number of viable cells, as well as increased variability in the purity of the resulting nucleic acid or protein preparations (Lu et al., 2019).

BioLegend®, a pioneer in scRNA-seq multiomics analysis, evaluated Laminar Wash methodology in their R&D lab to demonstrate how it performed for TotalSeq, a popular and leading multi-omics enabling product. They found it improved workflows and an optimized method is now recommended and described in TotalSeq protocols.

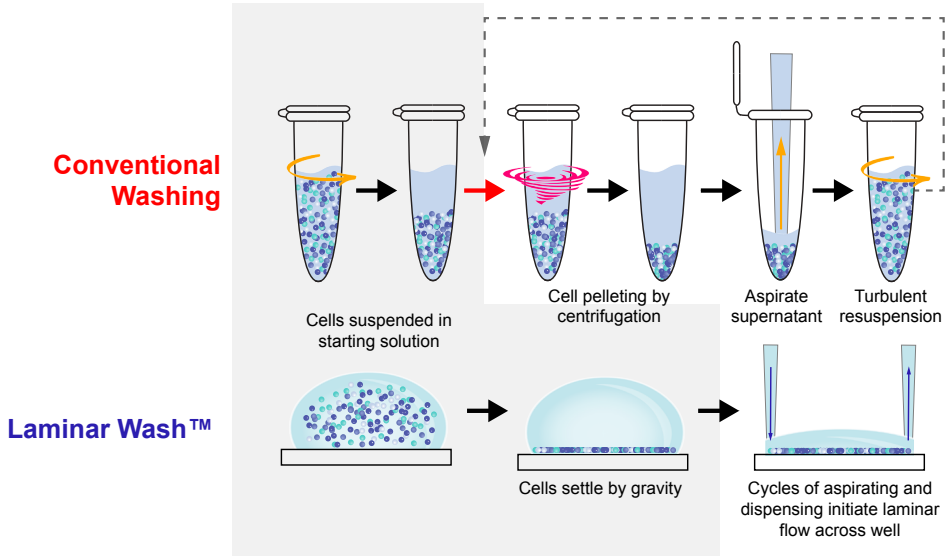
The variability among centrifugation techniques and skill of researchers handling the samples also contributes to discrepancies in cell quality. For example, if not performed properly, the 'plate flick' can leave inconsistent volumes in a 96-well plate. Centrifugation can dramatically alter cellular signalling and cell cycle dynamics (Soto et al., 2007) which will generate a heterogeneous cell population, confounding data analysis. Moreover, pellet resuspension involves mechanical agitation, leading to a reduction in cellular integrity (Katov and Mazur, 1998), including membrane leakage (Barbee et al., 2006), metabolic and physiological alterations (Al-Rubeai et al., 1995) – in other words, altered surface marker content (Dhondalay et al., 2014)) and apoptosis (Mollet et al., 2007).

**4. Laminar Wash™ technology addresses the limitations of conventional cell preparation methodology**

Laminar Wash technology is a novel approach to eliminating the centrifugation bottleneck in between sample preparation and analysis. Depending on the Laminar Wash instrument or system being employed, the cell washing step and/or the staining steps can be automated and customized with gentle and reproducible workflows, drastically reducing human intervention, variability, and stress on cells. Wells on the unique Laminar Wash plate maintain each sample as an individual droplet via surface tension and hydrophobic interactions with the surrounding environment. Samples from a single cell up to 5 million cells in a buffer can be dispensed onto the plate using multichannel or automated pipetting systems. The cells fall by gravity to the bottom of the wells where they settle on the hydrophilic coating without physical attachment. This is crucial as it permits continuous binding of cells to staining reagents, while cell debris, contaminants, and unbound proteins are retained in the bulk buffer solution. Robotic fluidics are utilised to dispense and aspirate the solution in the desired number of cycles. The userfriendly touch-screen interface enables researchers to customize the thoroughness of the wash (e.g., number of washes and flow rate) tailored to individual application. By limiting hands on time, resources can be saved and there are fewer instances where human intervention can obscure the data.

Moreover, by isolating the samples in each well, cells on the surface of the plate are maintained at a negligible loss and antigen-antibody surface and cytoplasmic interactions are undisturbed. This key feature preserves cells in their native state, increasing viability and reducing cellular stress, as well as removing debris and unbound antibodies for cell-hashing and multi-omics purposes. Laminar Wash technology is currently the only solution on the market that addresses these challenges.

Fig. 2
Laminar Wash™ technology addresses the limitations of conventional cell preparation methodology.




## 5. Standardization and Unification Of scRNA-seq Methodology

The Laminar Wash system is currently being used as a model for standardisation of sample preparation. It was included in the Flow Cytometry Standards Consortium, in partnership with the National institute for standards and technology (NIST), as a contribution to the adoption of a universal flow cytometry method.

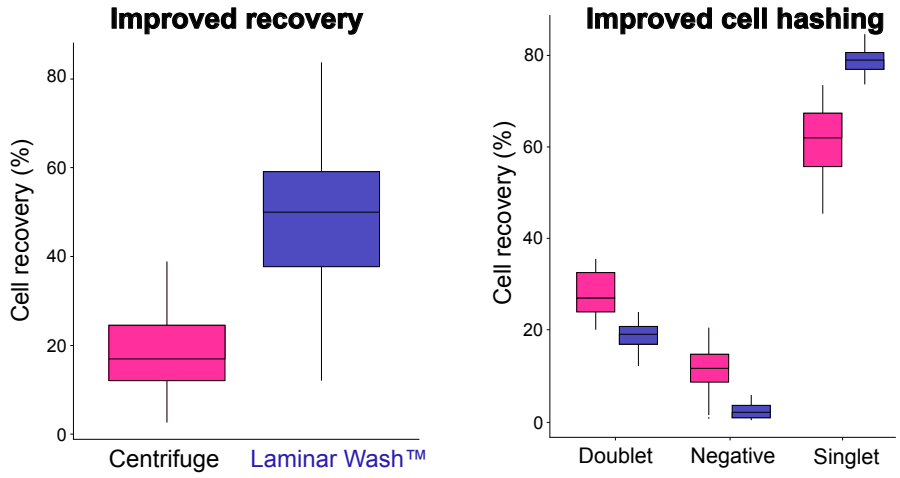
Moreover, BioLegend, a pioneer in scRNA-seq multiomics analysis, evaluated Laminar Wash methodology in their R&D lab to demonstrate how it performed for TotalSeq, a popular and leading multi-omics enabling product. They found it improved workflows and an optimized method is now recommended and described in TotalSeq protocols. In a 2020 webinar, BioLegend reported that Laminar Wash technology improves processing time while yielding comparable data to traditional centrifugation. Furthermore, Laminar Wash is the preferred choice for high throughput sample processing at therapeutics company ImmunAI. With multiplexing many samples using TotalSeq hashing, Laminar Wash prepared samples had elevated retention of single cells and fewer doublets and cells unstained with antibody, compared to conventional centrifugation preparation (Figure 3).

Single-cell applications are highly dependent on every cell being processed and analysed individually. This necessitates the use of technologies that focuses on improving retention, resolution and recovery of viability and physiological state for every cell. While the growing list of available single-cell RNA sequencing (scRNA-seq) multi-omics assays enables researchers to ask more targeted questions, objective assessments of their utility, strengths, and weaknesses are lacking. In a benchmarking study of multi-omics methods, Zu and colleagues exploited Laminar Wash technology's intrinsic standardization properties to compare single-cell trimodal DOGMA-Seq (Mimitou, 2021) with bimodal

CITE-seq (Stoeckius, 2017) assays under different conditions, leading to optimization recommendations (Zu et al., 2021). This evaluation provides researchers with a valuable resource to confidently deploy the scRNA-seq method suited to their research question.

Fig. 3:

ImmunAI data from webinar shows higher recovery and cell hashing efficiency.




## 6. Enabling High-Content Analysis of Rare And Low Abundance Cells

Peripheral blood mononuclear cells (PBMCs), are found in peripheral blood, defined as any blood cell with a round nucleus (Kleiveland, 2015). B cells are inherently low in abundance, comprising 5-10% of all PBMCs and B cell sub-populations (evolved in a particular immune response) are even fewer in proportion and are typically poorly defined.

Stewart and colleagues (2021) made use of Laminar Wash to process samples in a single-cell omics study of B cells. The study utilised Fluorescence-activated cell sorting (FACS) sorting to isolate and separate five peripheral B cell subsets which were then subjected to NGS to generate single-cell transcriptomic data. Study validation samples were isolated and pooled using CITE-seq and then run on one lane of the 10x Genomics -instrument to cut down on research costs. The additional samples were prepared with Laminar Wash methodology instead of classic centrifugation.

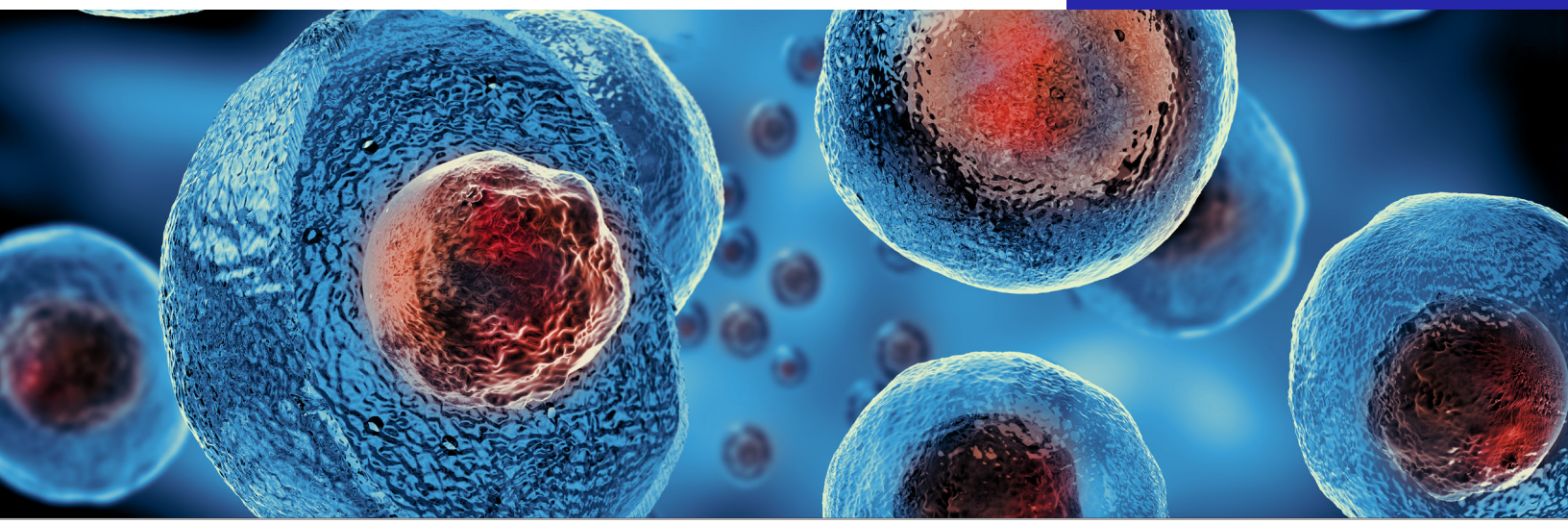
This study revealed developmental courses for 10 subclusters of B cells and pinpointed a unique population of B cells expressing IgE antibodies. Significantly, the Laminar-Wash prepared samples that re-generated the initial map, despite having ten times fewer starting donor cells per isolated subpopulation. In the future, variations of this B cell landscape can be used to inform new therapy and diagnostic modalities, whereby monitoring of shifts in B cell subsets during infection or chronic disease challenge,

provides an avenue to evaluate the resulting consequences on immune homeostasis. The primary author of this study, Alex Stewart, Ph.D., commented that the Laminar Wash system was “better at cleaning unbound antibodies when you do cell hashing, so you get less background when you run the samples through NGS”. Moreover, he described how the Curiox system “made life easier” by saving time and resources.

B cells are not the only low abundance cell type of interest; Professor Mats Bemark, University of Gottenberg, delivered a webinar on how Laminar Wash technology has enabled his research on antigen-specific immune responses using cell-hashing, CITE-seq and transcriptomics, with rare, small cell populations. Antigen-specific cells are extremely rare and according to Bemark, only the Laminar Wash system enabled to collect these cells, which would otherwise have been lost to the centrifuge. Mice infected with cholera-toxin were orally immunized and subsequently monitored for antigen-specific B cell immune response using CITE-seq and transcriptomics, with Laminar Wash for sample preparation of over 48 separate organs. The analysis clustered cells from distinctive organs at different timepoints (Komban et al., 2019).

Another study using similar methods, stratified severe and mild COVID-19 cases based on IgG antibody levels (Marklund et al., 2020), demonstrating that Laminar Wash technology has already been tried and tested for its healthcare potential in times of a global pandemic.

In a benchmarking study of multi-omic methods, Zu and colleagues exploited Laminar Wash technology’s intrinsic standardization properties to compare single-cell trimodal DOGMASeq (Mimitou, 2021) with bimodal CITE-Seq (Stoeckius, 2017) assays under different conditions, leading to optimization recommendations (Zu et al., 2021). This evaluation provides researchers with a valuable resource to confidently deploy the scRNA-seq method suited to their research question.



## 7. Conclusion

Back to the basics approach dictates the reason that advanced technologies like single cell multiomics and mass cytometry are enabling scientists to ask fundamental questions at a single cell level. This underlines the value of generating clean and consistent cell samples. The lingering challenge however, has been ensuring the quality of upstream protocols, to ensure reliably clean and biologically relevant sample preparation. Here we describe Laminar Wash: a novel technological approach that delivers high retention, debris-free, centrifuge free automated cell preparation system that can be used for a variety of cell types and cell numbers. Laminar Wash increases resolution of downstream data, whilst allowing for miniaturization and automation, leading to reliable and robust data for numerous and in vogue research applications such as single-cell multiomics, cell-based immunoassays and so forth. Additionally, increased adoption of Laminar Wash methodology can help to standardize and unify methods in scRNA-seq and multi-omics, flow cytometry, and mass cytometry: leading to faster and more efficient research, medical discovery, biotherapeutic development, and manufacturing. Considering the scalability of Laminar Wash arising from automation, these trends will almost certainly benefit leading biopharmaceutical, CROs, biomanufacturing companies, as well as regulatory bodies in the coming years.

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Spun out of the Agency for Science, Technology, and Research (A*STAR) in Singapore, Curiox brings together decades of scientific and engineering expertise in surface chemistry and life science instrumentation with the goal of overcoming critical challenges that slow the pace of life science research. By focusing on common assay steps where miniaturization and automation are currently underutilized, we've developed innovative technologies that simultaneously improve both productivity and data quality. We look forward to ushering in the next generation of innovations that accelerate the pace of life science research, diagnostics, and therapeutics discovery and development.

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