Automated Tumor Infiltrating Lymphocyte (TIL) Enrichment for Improved Single Cell Next Generation Sequencing

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INTRODUCTION

Immunotherapy with immune check point blockage (ICB) has revolutionized cancer treatment. A hypothesis for the mechanism of immunotherapy is that certain tumor infiltrating lymphocytes (TILs) in the tumor microenvironment are activated by ICB to react to the neoantigens expressed on the cancer cells. To better understand tumor microenvironment and the function of TILs in immunotherapy and further better utilize TILs in cancer cell therapy, high throughput single cell mRNA sequencing has been applied to study TILs and tumor microenvironment.

At Applied Cells, we developed a proprietary sample prep/cell separation instrument, MARS®. MARS stands for multi-physics automated reconfigurable separation. This instrument enables automation of entire cell sample washing and preparation from complex biological samples. It is an integrated system based on multiple physical principles with built-in novel technologies to achieve cell processing and target cell isolation (see Figure 1). On the Cell Wash Module, sample and the sample wash buffer are passing through the cell processing chip (CPC) simultaneously. In the flow stream on the CPC, the cells are forced into the center by the pressure generated by acoustic wave and the debris and small particles remain at the periphery. The cells and debris are separated and collected separately. The washed cells are ready for analysis or further separation or processing. On the Immuno-Magnetic Module, the cells are separated by in-flow magnetic force to capture the target cells and deplete the unwanted cells. MARS can positively or negatively separation the target cells. The Cell Concentration Module can decrease the sample volume.

Using MARS, we developed protocol to wash dissociated single cells from solid tumor tissue and enrich TILs from dissociated tumor samples. The MARS-washed dissociated tumor cells and the magnetically enriched TILs will give better results for high throughput single cells sequencing.

METHODS

Tissue dissociation Patient consented, surgically removed patient cancer tissue samples were freshly transferred to a research lab in RPMI (Roswell Park Memorial Institute) medium and processed within 24 hours of the tissue removal. The tissues samples were sliced into small pieces and incubated in enzymatic Tissue Dissociation Solution from Singeron (Nanjing, China) following Manufacture's protocol. After enzyme digestion, cells were passed through a 40 µm strainer and collected by centrifugation.

Flow cytometry The dissociated tissue single cells samples were resuspended in 1x PBS, stained with PE EpCAM for epithelial cells, Alexa Fluor® 488 CD45 for white blood cells and 7-AAD for live dead discrimination. The stained samples were analyzed on a 3-laser CytoFlex™ cytometer (Beckman Coulter, Miami, FL). The MARS washed samples and magnetically separated samples were also stained and analyzed by flow cytometry.

Automated sample wash The dissociated tumor single cell samples were loaded onto MARS Cell Wash Module and run CPC Wash Program. The samples were run at 1 mL/min speed.

Immune cell magnetic separation TILs were isolated on MARS by running the MAG Separation Program on the automated Immuno-Magnetic Module. Both positive and negative separation protocols were developed and applied. Both protocols used two-step indirect labeling procedures.

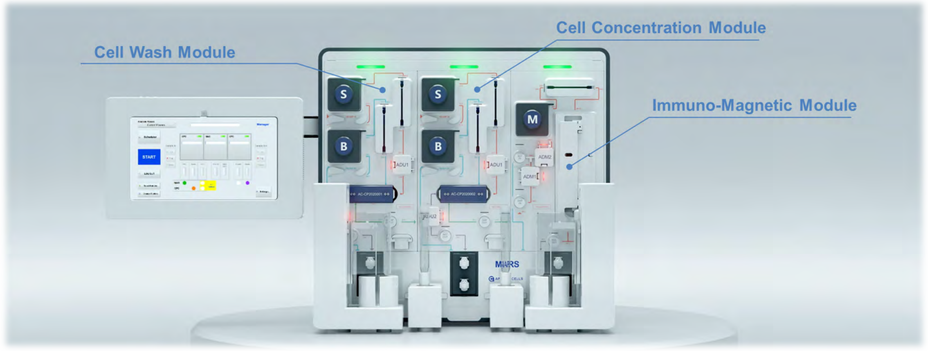
For the positive separation, the samples were labeled with CD45 antibody and magnetic particles.

The CD45-expressing TILs were labeled with the magnetic particles, which were pulled into the positive fraction. For the negative separation, the samples were labelled with EpCAM antibody and magnetic particles. The EpCAM-expressing epithelial cells were labeled with magnetic particles were depleted during magnetic separation. And the TILs were recovered in the negative fraction.

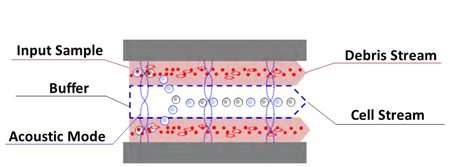
The samples were run at 1 mL/min speed.

High throughput single cell mRNAseq The magnetically enriched TILs and washed, dissociated cancer single cell sample without magnetic enrichment will be subjected to high throughput single cell mRNA sequencing respectively using Singleron GEXSCOPE™ single cell RNAseq kit.

SAMPLE WASH and CELL SEPARATION on MARS

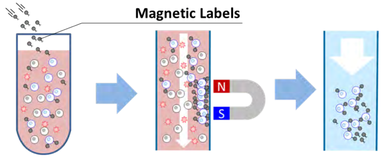


Acoustic Cell Manipulation



- Acoustic force separates particles based on the difference in physical parameters (size, etc.) with high speed within MARS CPC (cell processing chip) component

In-Flow Immuno-Magnetic Cell Separation



- MARS immuno-magnetic cell separation targets specific cell surface markers
- In-flow magnetic cell separation of MARS uses no column

Figure 1 Overview of MARS Instrument and Technology

DISCUSSIONS and FUTURE DIRECTIONS

Good understanding of solid tumor cancer cells and tumor microenvironment is important for cancer research and drug discovery. However, the sample processing methods don't always meet the needs of producing good quality samples for good sample analysis results.

Here, we developed a protocol to automate washing of dissociated solid tumor single cells and TIL separation and enrichment using Applied Cell proprietary MARS system.

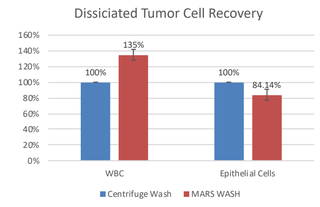
MARS WASH module isolates the dissociated single cells with high recovery and viability. MARS Immuno-Magnetic Separation module enriches TILs up to greater than 90 folds.

The enriched TILs will be subjected to high throughput single cell sequencing. It was shown MARS WASH results in cleaner samples. Therefore we expect to see improved single cell RNA sequencing sensitivity. This could help reach better understanding of the lymphocyte clonal evolution and transcriptional profiling in solid tumor microenvironment.

The viable TILs separated by MARS may also be further cultured, which could be used for TIL mediated immuno-therapy drug development.

RESULTS

High cell recovery with MARS Wash without compromising cell viability



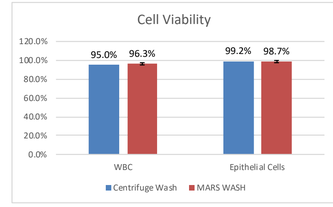


Figure 2. In this experiment, MARS Wash was compared with centrifuge wash. High cell viability and recovery were achieved with MARS WASH.

MARS Immuno-Magnetically enriches TILs

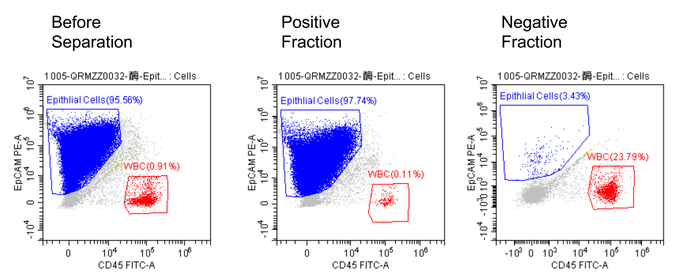


Figure 3. In this experiment, EpCAM antibody was used for TIL negative separation. Before the magnetic separation, greater than 95% of the dissociated tissue sample cells were epithelial cells. After the magnetic separation, most of the epithelial cells were pulled into the EpCAM positive fraction, while the white blood cells remained in the negative fraction.

	Sample 1	Sample 2	Sample 3	
Enrichment Method	EpCAM Negative Selection	EpCAM Negative Selection	EpCAM Negative Selection	CD45 Positive Selection
WBC Percentage before Separation	0.94%	10.48%	80.20%	80.20%
WBC Percentage after Separation	87.4%	63.9%	97.6%	98.4%
Fold Enrichment	92.7	6.1	1.2	1.2

Table 1. The TIL enrichments of a few representative samples are shown with high (Sample 1), medium (Sample 2) and low (Sample 3) levels of enrichment. Sample 1 is the same sample as shown in Figure 3. In our system, negative separation with EpCAM antibody appeared to be a better method to enrich TILs.