Extracellular protein monitoring in the ResolveOME™ genomic and transcriptomic dual workflow to uncover cancer pathology mechanisms in single cells.

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Abstract

Cancer is a disease of remarkable cell heterogeneity and is driven by complex, interconnected omic tiers. Single cell research has become an instrumental method in interrogating these multiple levels, as the bulk sequencing does not have enough resolution to reveal the true heterogeneity of the samples. The ResolveOME workflow aids understanding of cell-to-cell heterogeneity by providing unified genomics and transcriptomic information—including assessment of genome-wide single nucleotide variation, copy number changes, regulatory variants, splice isoform variation and cell state changes from the same single cell.

Here, we have adapted the existing ResolveOME genomic/transcriptomic workflow to include the detection of cell-surface protein expression using the TotalSeq™-A Human T-cell, B-cell, Natural Killer (TBNK) panel (BioLegend) of antibody-conjugated oligonucleotides. Primary peripheral blood mononuclear cells (PBMCs) were processed using the ResolveOME workflow which unifies template-switching single-cell RNAseq chemistry and Primary Template-directed Amplification (PTA) for whole genome amplification (WGA). We incorporated the TotalSeq™-A antibody-oligo cocktail to this workflow, whereby both cellular mRNAs and antibody-derived oligos specifically bound to PBMC antigens anneal to oligo dT primers, followed by template switch-based reverse transcription. This resulted in the creation of first-strand cDNA molecules and antibody-derived tag molecules that could be affinity purified and pre-amplified following whole genome amplification by PTA. Separate protein+RNA and DNA fractions are processed as distinct library preparations at the end of the workflow. Sequencing was performed with the G4™ Sequencing Platform from Singular Genomics.

Using this strategy with PBMC cells, we detected all nine barcode IDs corresponding to the specific antibodies in the TotalSeq™-A panel. The total number of detected barcodes varied between antibodies as well as between single cells, whereby CD19, CD3, CD4, CD16 and CD56 showed the most significant variation between individual single cells. We are extending these analyses to other cellular systems as well as to primary cancer cells, using antibody-conjugated antibody panels tailored to the application. Our study has devised a method for simultaneous detection of three omic tiers: whole genomic and transcriptomic signatures coupled to assessment of defined protein panels at the individual cell level, empowering insights into the interplay between the three tiers not possible in isolation.

Methods

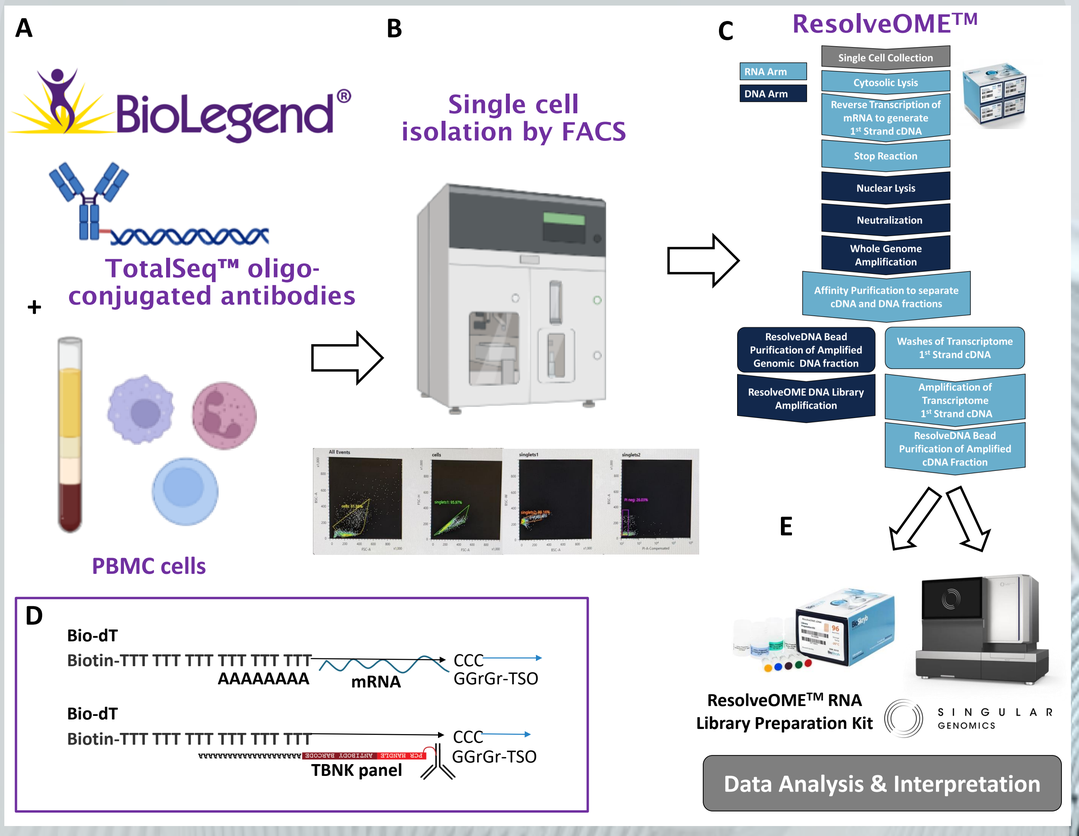


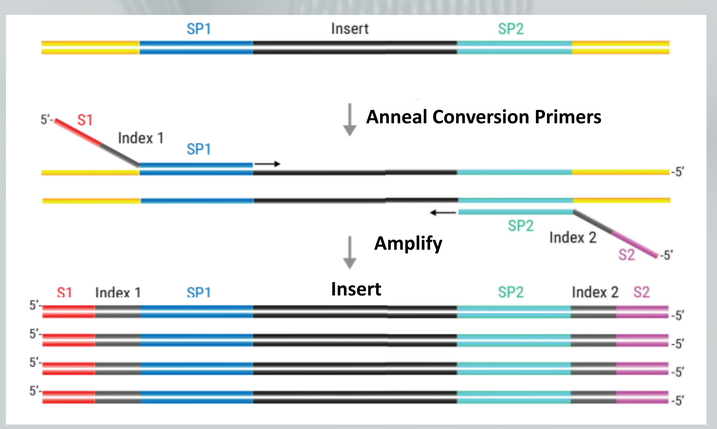
Figure 1. Schematic diagram of the expanded ResolveOME™ workflow with detection of cell-surface proteins.

A) We followed the BioLegend protocol to prepare Human TotalSeq™-A T-cell, B-cell, Natural Killer (TBNK) panel of nine antibody-conjugated oligonucleotides. Next, we prepared 40ul of ~1.0E6 fresh or frozen primary Peripheral Blood Mononuclear Cells (PBMCs) and incubated them with the TotalSeq™ antibody panel for 30 minutes, followed by washes with the Cell Staining Buffer.

B) Subsequently, flow cytometry was performed. Following Calcein AM and propidium iodide staining, singlet and live cell (PI negative, top 70% Calcein-AM positive) gating was established and ~1.0E6 PBMC single cells were sorted for the ResolveOME™ workflow.

C-D) We incorporated the TotalSeq™-A antibody-oligo cocktail into the ResolveOME™ workflow, whereby both cellular mRNAs and antibody-derived oligos specifically bound to PBMC antigens anneal to oligo dT primers, followed by template switch-based reverse transcription **(D).** We created first-strand cDNA molecules and antibody-derived tag (ADT) molecules that could be affinity purified and pre-amplified following whole genome amplification with ResolveDNA™ chemistry.

E) The resulting ADT+RNA and DNA fractions are processed separately for library preparation, sequencing and data analysis by BaseJumper™ at the end of the workflow. DNA and RNA libraries have been processed using ResolveOME™ DNA and RNA Library preparation kits. We collaborated with Singular Genomics and converted ResolveOME™ RNA libraries to the libraries compatible with G4 sequencing (Figure 2).



Insert – existing customer library

SP1 and SP2 – legacy sequencing primers

S1 and S2 – Singular Genomics Clustering Tags

Index 1 and Index 2 – unique 12 bp indices for Singular Genomics

Figure 2. Schematic diagram of converting existing libraries to Singular Genomics libraries.
Existing RNA libraries were converted to the compatible Singular Genomics libraries. Using the PCR approach, legacy clustering tags (yellow) have been replaced with Singular Genomics S1 and S2 sequences. Subsequently, the library product was purified, clustered, and sequenced (indexed 2X75 bp) on the Singular Genomics G4 Sequencing Platform generating 181 M PE reads.

Results

NA12878 QC metrics	>90%	>65%	<4%	<10%	>2000
	Reads Aligned % Total	% Exonic Reads	% Intergenic Reads	% MT	Number Genes Detected
PBMC 5C	90	54	3	5	3,586
Frozen PBMC SC	86	48	3	5	2,180
Fresh PBMC SC	91	60	2	5	1,503
NA12878 Bulk	85	78	2	8	5,553
NA12878 SC	91	67	2	7	3,963

Table 1. QC metrics for RNA fraction of ResolveOME™.
ResolveOME™ RNA metrics for NA12878 cells, and fresh or frozen PBMC cells, indicating that addition of the TBNK antibody panel upstream of the protocol did not significantly affect the performance of the ResolveOME™ protocol. Green boxes indicate the samples that passed DNA QC metrics.

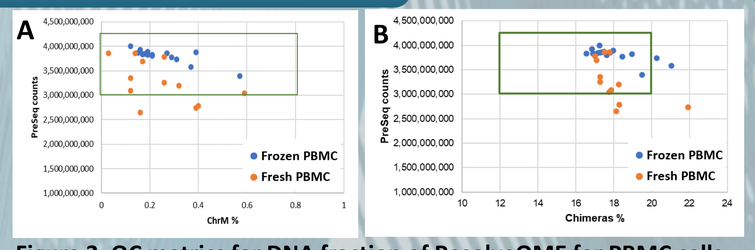


Figure 3. QC metrics for DNA fraction of ResolveOME for PBMC cells.
PreSeq counts as a measure of library complexity (A), % Mitochondrial reads and % chimeras (B) were within specification for ResolveOME™ samples, indicating that addition of the TBNK antibody panel upstream of the protocol did not significantly affect the performance of the ResolveOME™ . Green boxes indicate samples that passed DNA QC metrics.

Cells	Antibody	CD19 HB19	CD3 UCHT1	CD16 3G8	CD4 RPA-T4	CD11c S-HCL-3	CD56 5.1H11	CD14 M5E2	CD8 SK1	CD45 2D1
SC1	No	1	1	5	1	1	4	1	1	1
SC2	No	1	1	1	1	1	7	1	1	1
SC3	No	2	1	36	1	1	3	1	1	1
SC4	No	1	1	2	1	1	18	1	1	1
SC5	No	1	1	1	1	1	8	1	1	1
SC6	No	1	3	5	1	5	1	2	1	5
SC7	No	1	1	1	1	1	11	11	1	1
SC8	No	1	27	2	1	1	1	1	1	1
SC9	No	11	1	1	1	1	1	5	1	1
SC10	No	2	1	1	2	1	1	1	1	1
SC11	No	1	1	1	1	1	1	1	1	1
SC12	No	1	1	1	1	1	1	8	1	1
SC1	YES	1687	123	778	110	1076	92	86	56	387
SC2	YES	29	1417	275	1508	41	16	26	13	99
SC3	YES	35	590	279	43	48	20	23	776	96
SC4	YES	18	217	49	198	9	3	8	2	29
SC5	YES	3023	233	1680	244	413	216	123	74	1218
SC6	YES	21	538	241	50	40	11	22	850	114
SC7	YES	17	436	201	1343	36	15	18	7	85
SC8	YES	11	334	65	551	19	6	7	1	48
SC9	YES	8	408	115	1100	32	11	6	10	101
SC10	YES	4	599	49	22	11	2	1	10	33
SC11	YES	15	1542	124	1206	27	14	5	8	52
SC12	YES	5713	115	835	136	201	83	60	20	567
SC13	YES	14	312	155	17	31	14	6	304	77
SC14	YES	26	987	132	1018	31	6	14	9	72
SC15	YES	6	344	43	456	5	1	3	1	29

Table 2. TBNK antibody barcodes detected in fresh PBMC cells.
As shown in the Table 2, we observed significant differences in the number of barcodes detected in fresh PBMC cells with the TBNK antibody panel and without antibody control. The background signal without antibody staining was less than 50 reads. The total number of detected barcodes in the cells with antibodies staining varied between antibodies as well as between single cells. For example, CD19, CD3, CD4, CD8 and CD16 showed the most significant variation between individual single cells, while CD11c, CD14, CD45 were detected less frequently and were less variable between single cells. Interestingly, CD3 and CD4 barcodes reads were associated more often in the same single cells then any other barcodes (Figure 5).

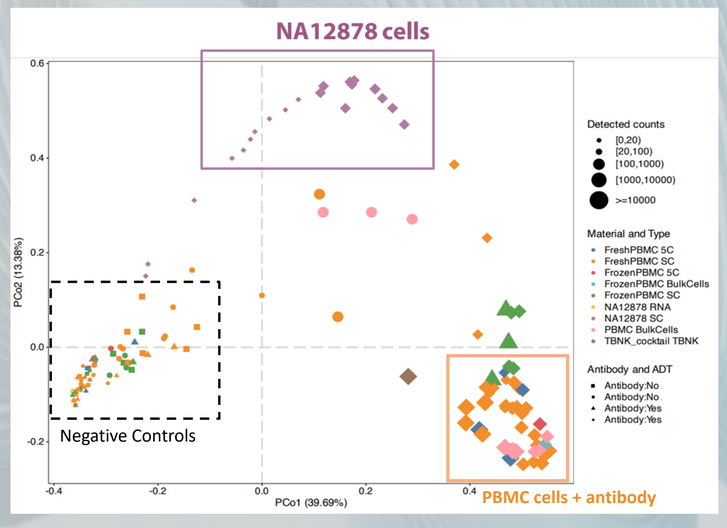


Figure 4. PC analysis of sequencing read counts.
PCA analysis showing clear separation between cells incubated with the TBNK antibody panel (shown with squares and triangles) and not (shown with circles and rhomboids). In addition, B-Lymphocyte NA12878 cells are clearly clustering separately from PBMC frozen or fresh cells.

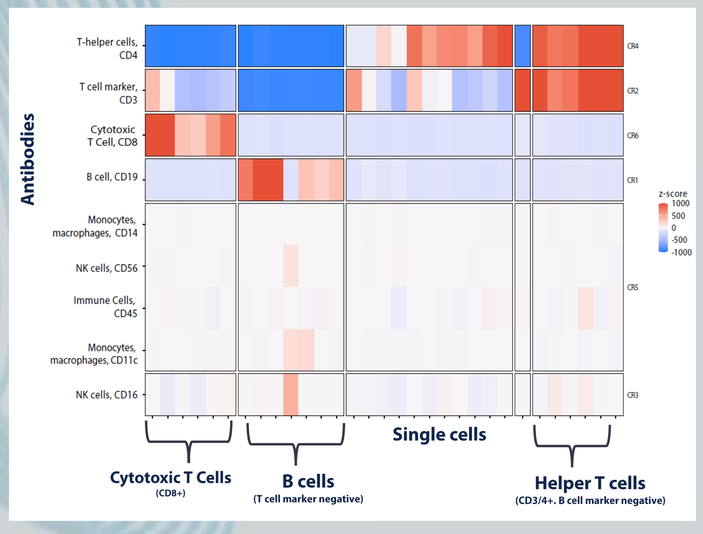


Figure 5. Biological insights in fresh PBMC cells.
TotalSeq™-A staining revealed signatures indicative of cytotoxic T cells, B cells, and helper T cells from the PBMC sample.

Summary

- We successfully incorporated the TotalSeq™-A TBNK panel into the ResolveOME™ workflow. The result is a tri-modal dataset originating from the same single cell: genomics, transcriptomics, and targeted cell-surface proteomics.
- We are extending these analyses to other cellular systems as well as to primary cancer cells, using antibody-conjugated antibody panels tailored to the applications.
- RNA libraries converted for compatibility with the Singular Genomics G4 Sequencing platform provides comparable data to the ResolveOME™ RNA libraries.

References

1. Gonzalez-Pena, V. *et al.* Accurate genomic variant detection in single cells with primary template-directed amplification. *Proc Natl Acad Sci U S A* **118**, e2024176118 (2021).
2. Zawistowski, J.S. *et al.*, Unifying genomics and transcriptomics in single cells with ResolveOME amplification chemistry to illuminate oncogenic and drug resistance mechanisms. BioRxiv, https://doi.org/10.1101/2022.04.29.489440
3. https://www.biolegend.com/
4. https://singulargenomics.com/

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