TCR sequencing from tissue micro-regions and single cells utilizing RareCyte CytePicker® and Archer® Immunoverse™ technologies

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Introduction

T-cell receptor (TCR) based immunotherapies are becoming an important cornerstone of immuno-oncology. Complete TCR sequencing requires single-cell resolution to capture both α and β chains. There is great interest in obtaining single-cell TCR sequences from archived tumor tissue. This task requires technology that can not only retrieve single cells but also sequence degraded RNA from archived tissues samples at the single cell level. The RareCyte CyteFinder® platform provides integrated multi-parameter imaging and retrieval capabilities for identification and isolation of rare cells and microscopic regions of interest (ROI) for molecular analyses. Archer® has developed the Immunoverse™ platform of targeted Next-Generation Sequencing (NGS) assays to characterize the human immune repertoire from partially degraded RNA inputs. Combining these two technologies affords the unique potential to accelerate engineered cell-based therapeutic by sequencing TCR from individual cells.

Methods

ROIs (~40 µm) containing 1 to 10 T-cells were isolated from fresh/frozen (OCT) melanoma or OCT and FFPE tonsil using the CyteFinder® system with CytePicker® module. In addition, single flu antigen-specific T-cells were retrieved from live cell preparations. OCT tonsil sections were stained with a 3-color panel to discriminate T-cell and B-cell zones, and OCT tumor sections were stained with a 6-color panel to identify immune infiltrate. FFPE sections were stained with a 6 color panel which guided picking from a serial section stained with only a nuclear marker. The Archer® Immunoverse™ TCR All Chains library preparation kit was used to generate libraries which were then sequenced on the Illumina NextSeq platform. The resultant library sequences were de-duplicated, error corrected, aligned to reference V, (D), J and C regions of TCRs, and assembled to identify clonotypes from α and β chains using the Archer® Analysis tool.

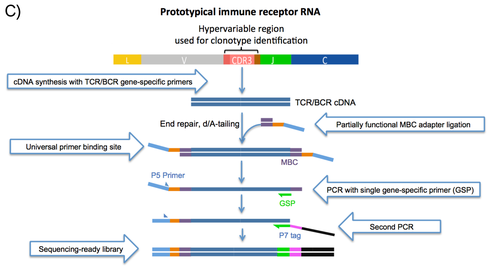
Figure 1. Rare cell isolation, RNA extraction and multiplexed TCR sequencing assay.
(A) Conditions tested in each experiment. (B) T-cells of interest were isolated using the CyteFinder® system with CytePicker® module. (C) RNA from T-cells were analyzed with the Immunoverse™ TCR assay for simultaneous interrogation of TCR α, β, γ and δ chains. The assay uses ligation-based molecular barcoding of reverse transcribed cDNA and subsequent multiplexed PCR. Universal primer binding sites are ligated to the variable TCR gene segment and opposing gene specific primers (GSP) are used to generate a sequencing library containing the CDR3. (D) Archer® Analysis was used for de-multiplexing, PCR de-duplication, and identification of CDR3, V, D, J and C regions on a per-molecule basis.

A)

Experiment	Input Type	RNA QC (tissue)	Lysis buffer	RNA isolation	WTA	Tissue	# Cells	Carrier RNA	PCR 1&2 Cycling #	PCR 1&2 Extension Times (min.)
2	Live single T-cell	N/A	SmartSeq (no ProK)	Agencourt Formapure	none	Flu or Jurkat	1	No	16/20	3/3
3	Live single T-cell	N/A	SmartSeq (no ProK)	Agencourt Formapure	none	Flu or Jurkat	1	Yes or No	16/20	3/3
3	Live single T-cell	N/A	Qiagen WTA kit buffer (no ProK)	none	Qiagen Repli-G	Flu or Jurkat	1	Yes or No	16/20	3/3
1	OCT tissue ROI	none	Qiagen PKD (ProK)	Agencourt Formapure	none	Tonsil	4-1000	No	16/20	3/3
1	OCT tissue ROI	none	Qiagen PKD (ProK)	Agencourt Formapure	none	Tonsil	4-1000	No	16/20	3/3
2	OCT tissue ROI	none	Qiagen PKD (ProK)	Agencourt Formapure	none	Melanoma	1-10	No	16/20	3/3
4	OCT tissue ROI	yes	Qiagen PKD (ProK)	Agencourt Formapure	none	lung	1-9	No	16/20	15/3
4	OCT tissue ROI	yes	Qiagen PKD (ProK)	Agencourt Formapure	Qiagen Repli-G	lung	1-9	No	8/20	15/3
4	OCT tissue ROI	yes	Qiagen PKD (ProK)	Oligo(dT) beads	SmartSeq v4	breast	1-9	No	8/20	15/3
1	FFPE tissue ROI	none	Qiagen PKD (ProK)	Agencourt Formapure	none	Tonsil	~4-1000	No	16/20	3/3
1	FFPE tissue ROI	none	Qiagen PKD (ProK)	Oligo(dT) beads	none	Tonsil	~4-1000	No	16/20	3/3
4	FFPE tissue ROI	yes	Qiagen PKD (ProK)	Agencourt Formapure	none	lung	~5-10	No	16/20	15/3
4	FFPE tissue ROI	yes	Qiagen PKD (ProK)	Agencourt Formapure	Qiagen Repli-G	lung	~5-10	No	8/20	15/3

B) Image on CyteFinder → Retrieve with CytePicker → Confirm → RNA isolation

C)



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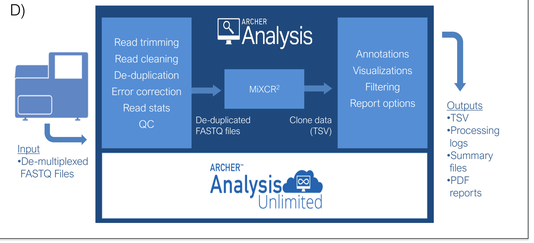
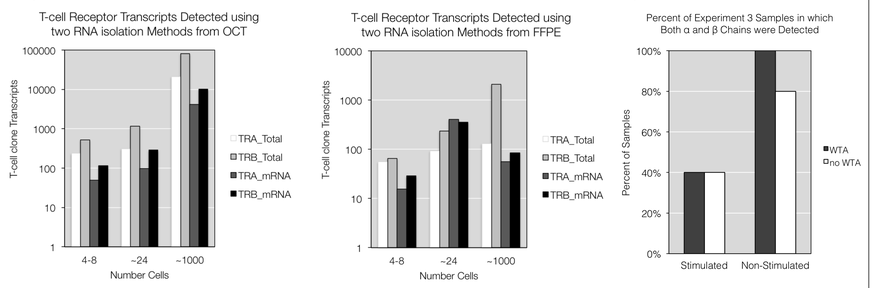


Results

Figure 2. Transcript Detection Trends with Input Mass and RNA Preparation Method.
(A) TCR transcripts were detected from RNA isolated from ROI retrieved from OCT and FFPE tonsil when processed with the Immunoverse™ TCR assay. The numbers of transcripts observed correlated with the number of isolated cells analyzed. The sample source also affected the number of observed clonotypes with more being observed in samples of RNA isolated from OCT tissue compared to FFPE. (B) Data from live T-Cell input show WTA increased the number of samples in which both α and β chains are observed, and stimulated T-cells had a lower rate of detection of both α and β chains from the same sample. This reduced number of observed chains in stimulated cells may be due to decreased expression levels (Gallegos et al., 2016 *Nat. Immunol.)*

A)



B)

Figure 3. α/β Chain Transcripts Detected in Optimal Cutting Temperature and FFPE Samples
(A) Heat map of per-sample clonotype detection on a per-chain level. (B) Percent of samples in which both α and β chain transcripts were detected.

A)

Input Type	TRA Clonotypes	TRB Clonotypes
OCT Lung - Single Cell	0	3
OCT Lung - Single Cell	1	1
OCT Lung - Single Cell	1	5
OCT Lung - Multi Cell	1	0
OCT Lung - Multi Cell	0	2
OCT Lung - Multi Cell	0	4
WTA - OCT Lung - Single Cell *	0	8
WTA - OCT Lung - Single Cell	0	9
WTA - OCT Lung - Single Cell	0	13
WTA - OCT Lung - Single Cell	0	14
WTA - OCT Lung - Single Cell	0	20
WTA - OCT Lung - Single Cell	1	13
WTA - OCT Lung - Single Cell	1	5
WTA - OCT Lung - Single Cell	2	24
WTA - OCT Lung - Multi Cell	0	15
WTA - OCT Lung - Multi Cell	0	21
WTA - OCT Lung - Multi Cell	1	6
WTA - OCT Lung - Multi Cell	1	7
WTA - OCT Lung - Multi Cell	1	11
WTA - OCT Lung - Multi Cell	2	18
WTA - OCT Lung - Multi Cell	4	29
WTA - OCT Lung - Multi Cell	5	12
FFPE Lung - Cell Rich	0	1
FFPE Lung - Cell Rich	0	5
FFPE Lung - Cell Rich	1	8
WTA - FFPE Lung - Cell Rich	0	0
WTA - FFPE Lung - Cell Rich	0	0
WTA - FFPE Lung - Cell Rich	0	0
WTA - FFPE Lung - Cell Rich	0	0
WTA - FFPE Lung - Cell Rich	0	7
WTA - FFPE Lung - Cell Rich	0	8
WTA - FFPE Lung - Cell Rich	0	24
WTA - FFPE Lung - Cell Rich	1	5
WTA - OCT Lung - Pre-Pick Control	1	3
WTA - FFPE Lung - Pre-Pick Control	1	3
WTA - OCT Lung - Post-Pick Control	0	1
WTA - NTC	0	0

* This sample also showed a γ and δ chain pair.

B) % Samples with both α and β Chains Detected

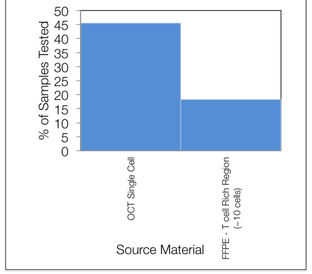


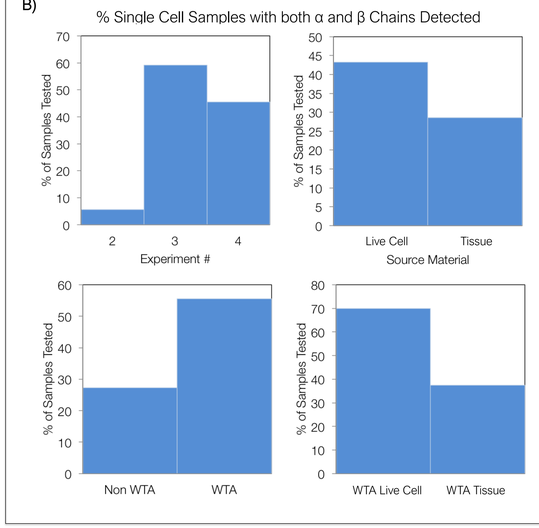
Figure 4. % Single Cell Samples with both α and β Chains Detected Varies by Source Material and Condition.
Three experiments were performed in which RNA from a single cell was studied. Each experiment used different input sources, extraction methods, and RNA pre-treatments (see Figure 1A). The different conditions affected the percent of samples in which both α and β chain transcripts were detected. (A) Heat map of single-cell clonotype detection on a per-chain level. (B) The optimal method for ensuring detection of both α and β chain transcripts within 1 sample is when using whole transcriptome amplified (WTA) RNA obtained from a single live T cell. In addition to the data presented here, both a γ and a δ chain were identified in one sample.

A)

Input Type	TRA Clonotypes	TRB Clonotypes
Flu - Single Cell	0	1
Flu - Single Cell	0	1
Flu - Single Cell	0	1
Flu - Single Cell	0	1
Flu - Single Cell	0	1
Flu - Single Cell	0	2
Flu - Single Cell	0	6
Flu - Single Cell	0	7
Flu - Single Cell	0	54
Flu - Single Cell	1	0
Flu - Single Cell	1	2
Flu - Single Cell	1	3
Flu - Single Cell	1	4
Flu - Single Cell	1	13
Flu - Single Cell	1	13
Flu - Single Cell	1	13
Flu - Single Cell	2	2
Flu - Single Cell	2	1
Flu - Single Cell	3	4
Flu - Single Cell	3	69
Flu - Single Cell	4	8
Flu - Single Cell	13	0
Flu - Single Cell	19	11
Flu - Single Cell	19	4
Jurkat - Single Cell	5	76
Jurkat - Single Cell	119	114
OCT Lung - Single Cell	0	3
OCT Lung - Single Cell	1	1
OCT Lung - Single Cell	1	5
OCT Melanoma - Single Cell	1	0
OCT Melanoma - Single Cell	1	0
WTA - OCT Lung - Single Cell *	0	8
WTA - OCT Lung - Single Cell	0	9
WTA - OCT Lung - Single Cell	0	13
WTA - OCT Lung - Single Cell	0	14
WTA - OCT Lung - Single Cell	0	20
WTA - OCT Lung - Single Cell	1	5
WTA - OCT Lung - Single Cell	1	13
WTA - OCT Lung - Single Cell	2	24
WTA - OCT Breast - Single Cell	0	0
WTA - OCT Breast - Single Cell	0	0
WTA - OCT Breast - Single Cell	1	15

* This sample also showed a γ and δ chain pair.

B) % Single Cell Samples with both α and β Chains Detected



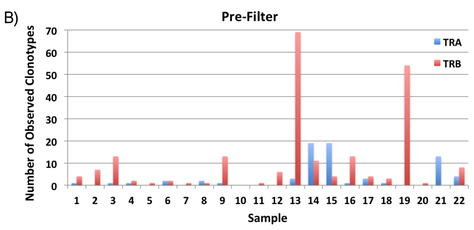
Results, Continued

Figure 5. Archer® Analysis was used to identify T-cell receptor clonotypes from the sequencing data.
(A) Many single cell samples have more than 1 α and 1 β chains identified, likely due to PCR and or sequencing errors. The most abundant clonotype identified per sample often only differed from the other clonotypes by a few insertions, deletions or mismatches. After applying an empirically derived frequency filter of 0.45, the number of single cell samples in which exactly 1 α and 1 β clonotype was identified increased from 0/22 (B) to 20/22 (C) .

A)

Clonotype Sequence	Clone Abundance	Raw Fragment Abundance	Clone Frequency
TGTGCTCTGAGTCCAAGGAGGCAACACAGGCAAACTAATCTTT	12067	300496	0.995955761
TGTGCTCTGAGTCCAAGGAGGCAACACAGGCAAACT-ATCTTT	13	13	0.001072961
TGTGCTCTGAGTC-AAGGAGGCAACACAGGCAAACTAATCTTT	12	12	0.000990426
TGTGCTCTGAGTCCAAG-AGGCAACACAGGCAAACTAATCTTT	4	4	0.000330142
TGTGCTCTGAGTCCAAGGAGGCAACACAGGCAAACTAATCTTTT	2	96	0.000165071
TGTGCTCTGAGTCCAAGGAGGCAACACAGGCAAACTAATCTTT	2	7	0.000165071
TGTGCTCTGAGTCCAAGGAGGCAACACAGGCAA-CTAATCTTT	2	2	0.000165071
TGCGCTGTGAGAGATGGTCCGGCAGGAACTGCTCTGATCTTT	2	4	0.000165071
TGTGCTCTGAGTCCAAGGAGGCAACA--GGCAAACTAATCTTT	2	2	0.000165071
TGTGCTCTGAGTCCAAGGAGGCAACACAGGCAAACTAATCTTTG	1	1	8.25E-05
TGCGCTCTGAGTCCAAGGAGGCAACACAGGCAAACTAATCTTT	1	1	8.25E-05
TGGGCTCTGAGTCCAAGGAGGCAGGACAGGCAAACGAGGCTTT	1	1	8.25E-05
TGTGCTCTGAGTCCAAGGAGGCAGCACAGGCAAACTA-TCTTT	1	1	8.25E-05
TGTGCTCTGAGTCCAAGGAGGCAACACAGGCAAA-TAATCTTT	1	1	8.25E-05
TGTGCTCTGAGTCCAAGGAGGCAACACAGGCAAACTAATCTT-	1	1	8.25E-05
TGTGCTCTGAGTCCAAGGAGGGA-CACAGGCAAACTAATCTTT	1	1	8.25E-05
TGTGCTCTGAGTC-AAGGAGGCAACACAAGCAAACTAATCTTT	1	1	8.25E-05
TGTGCTCTG--TCCAAGGAGGCAACACAGGCAAACTAATCTTT	1	1	8.25E-05
TG---CTCTGAGTCCAAGGAGGCAACACAGGCAAACTAATCTTT	1	1	8.25E-05

B)



C)

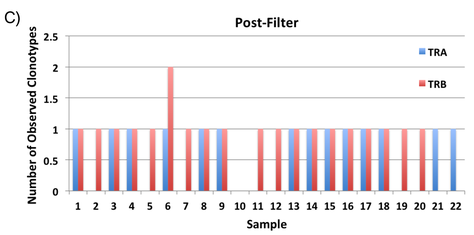


Figure 6. Previously published sequences identified in sequencing results from single T-cell trained against Flu antigen.
α-β chain pairs were identified in several live T cells trained against a flu antigen and in live Jurkat cell line cells. CDR3 sequences from single flu-targeting T-cells (*Blue*) were found to match previously published data (Chen et al., 2017, *Cell Reports*). Other, un-published, CDR3 sequences were also found in single flu-targeting T-Cells (*Black*). Furthermore, single Jurkat cell line CDR3 sequences were obtained (*Red*) and found to match previously identified sequences (IMGT/LIGM-DB: K02777 and K02779 for α and β, respectively).

α-β Pair	TRA	TRB
Flu #1	**CAGGGSQGNLIF**	**CASSVRSSYEQYF**
Flu #2	*Not Detected*	CASSGRSTDTQYF
Flu #3	**CAGAIGSSNTGKLIF**	CASSQYVPGRRRNIQYF
Flu #4	CAMSGGGGSQGNLIF	CASSIRSTDTQYF
Flu #5	**CAAGGSQGNLIF**	**CASSIRSSYEQYF**
Flu #6	**CAGAGGGSQGNLIF**	CASSTRSSETQYF
Flu #7	CAVRDGTGANNLFF	CASSHGLSSYEQYF
Flu #8	CALSPRRQHRQTIF	CSARSGGILNEQFF
Flu #9	CAVRWGGFGNVLHC	CASSSILKQYF
Jurkat	**CAVSDLEPNSSASKIIF**	**CASSFSTCSANYGYTF**

Conclusions and Discussion

These results support the utility of the RareCyte CyteFinder® platform with CytePicker® module combined with Archer® Immunoverse™ chemistry and Archer® Analysis software for profiling RNA derived from single cell to low numbers of cells in either fresh/frozen tissue, FFPE tissue, or live cells. Using a workflow that combines RareCyte and Archer® technologies, we have identified α and β chain pairs from single T-cells isolated from OCT, live cell culture, and the Jurkat cell line.

These combined methodologies are a potentially promising lead for the future of T-cell therapies that require characterization of specific T-cell receptor sequences that target specific antigens.

References:

1. Sequence and Structural Analyses Reveal Distinct and Highly Diverse Human CD8+ TCR Repertoires to Immunodominant Viral Antigens. Chen et al., 2017, Cell Reports 19, 569–583.
2. Bolotin et al., Nat. Methods. 5, 380-381 (2015).
3. Control of T cell antigen reactivity via programmed TCR downregulation. Gallegos et al., 2016, Nat. Immunol. 17(4), 379-386.
4. IMGT/LIGM-DB. Giudicelli, V. et al., Nucleic Acids Res., 34, D781-D784 (2006)