# Identification, retrieval, and RNA sequencing of single rare antigen-specific T cells from circulation using the RareCyte® platform

Nolan Ericson, Eric Kaldjian, Tad George, Lance U'Ren
RareCyte, Inc.

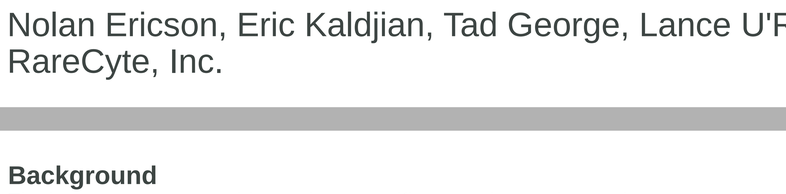
## Background

The immune system provides antigen-specific protection against pathogens as well as malignancies, both of which evolve strategies to evade immune surveillance and containment. Effective immune response often depends on activation of rare antigen-specific immune cell sub-types. The RareCyte platform provides integrated multi-parameter imaging and retrieval capabilities that allow phenotypic identification and isolation of rare cells for sequence and transcript level analyses in order to study the complexity of host defense.

## Methods

Antigen-specific T cells were identified by immunofluorescent staining of live purified T cells from normal donors with the following panel: CD3; CD8; exclusionary cocktail (CD4, CD14, CD15, CD20, and DAPI); and HLA-A2 restricted tetramer (MBL). Tetramers had specificity against influenza-M1 (GILGFVFTL) and the melanoma antigen Mart-1 (ELAGIGILTV). For differential expression experiments, cells were stimulated overnight with specific target peptide or control irrelevant peptide, stained with the multiparameter panel containing the relevant tetramer, then imaged using the CyteFinder® Imaging System. Automated image analysis identified candidate cells with positive signal in the tetramer and CD8 channels and negative signal in the exclusion channel. Single cells displaying membrane ring tetramer distributions were retrieved using the CytePicker® Retrieval Module, followed by single cell RNA sequencing using the SMART-Seq® v4 kit (Takara Bio) and the MiSeq® instrument (Illumina). Differential gene expression of stimulated versus unstimulated cells was performed using DESeq2 software (BioConductor). Single-cell RNA seq FASTQ files were analyzed with the TraCeR computational method to identify TCR alpha and beta chains in both stimulated and unstimulated cells.

## RareCyte Platform Technology



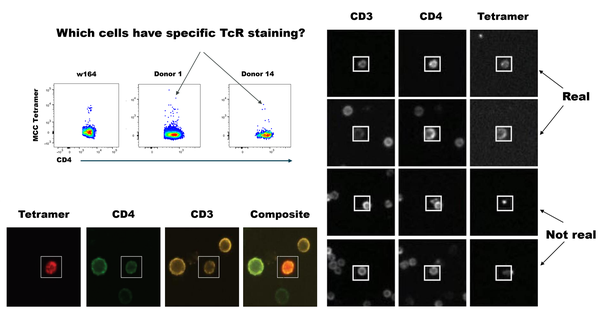

Visualize by CyteFinder® Imaging System with CyteMapper® analysis tools

Pick cells with CytePicker® Module

Transfer cell to PCR tube and re-visualize

**Figure 1. The RareCyte platform enables rare live cell detection and retrieval.** Cells are stained for multiparameter imaging by the CyteFinder System to detect rare cells. Identified rare cells of interest are retrieved and transferred to PCR tubes with the integrated CytePicker module for molecular analysis.

**Figure 2. Identification of rare T cells by tetramer.** RareCyte-enabled visualization offers improved specificity. In this example, tetramer against Merkel cell carcinoma polyoma virus-specific T cell receptors was used to identify CD4+ T cells. Flow cytometry cannot distinguish between specific membrane staining (“real”) and non-specific staining (“not real”) by the tetramer.




Paul Ngheim, University of Washington

## Experimental Design

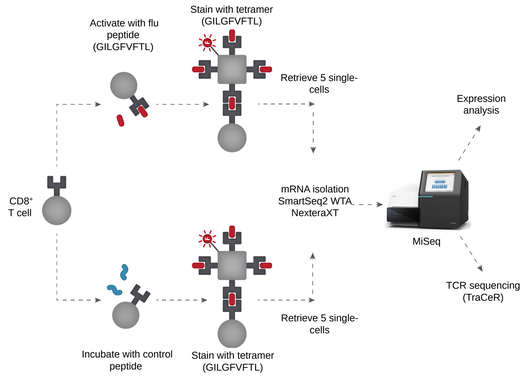



**Figure 3. Strategy for validation of T cell specificity.** CD8-purified T cells are incubated with activating peptide or control peptide. Cells are stained with tetramer specific to the activating antigen, as well as a cocktail to confirm viability and CD8+ T cell identity. Cells are retrieved, followed by RNA isolation, whole transcriptome amplification, and RNA-seq for differential expression and TCR sequence analysis.

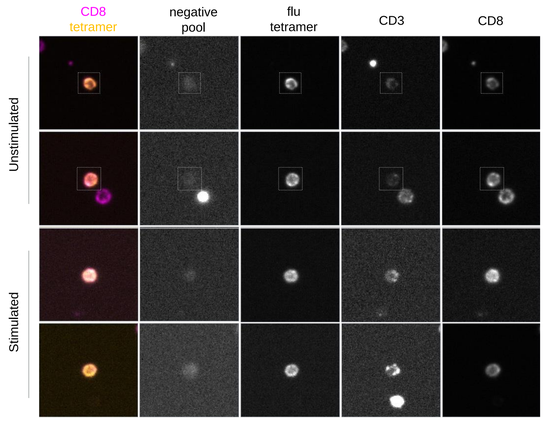



**Figure 4. Multiparameter imaging of flu tetramer-stained T cells.** Representative images of flu tetramer-positive T cells identified in unstimulated (control peptide) and stimulated (flu peptide) populations. Identified cell also display staining for T cell markers CD3 and CD8.

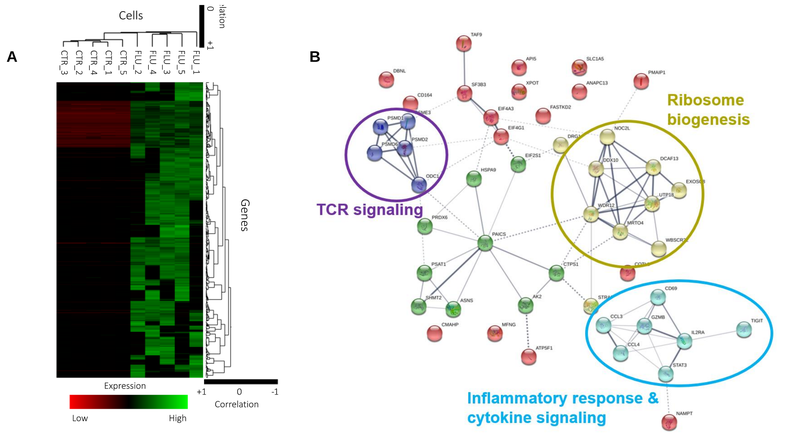



**Figure 5. RNA-seq analysis of tetramer-stained CD8+ T cells.** RNA sequencing was performed on retrieved tetramer-positive live T cells. **(A)** DESeq2 and hierarchical clustering analysis revealed 760 differentially expressed genes. (**B)** Protein STRING analysis of the top 50 differentially expressed genes demonstrated the involvement of TCR signaling and inflammatory response/cytokine signaling pathways, further validating the specificity of the method.

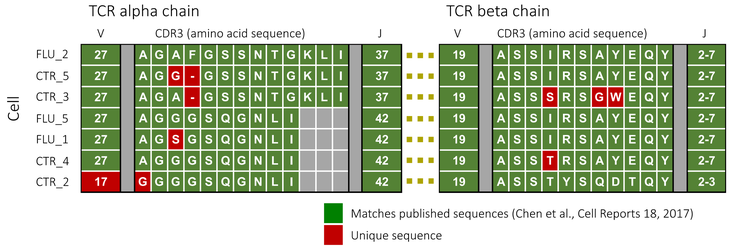



**Figure 6. TCR sequence analysis in flu tetramer-positive T cells.** RNA-seq data from flu tetramer-positive T cells was mined by TraCeR analysis to identify TCR sequences for each cell. 10 of the 10 cells retrieved had productive TCR α/β pairs, and 7 of 10 had exact or near-exact matches against published flu-specific TCR sequences (shown).

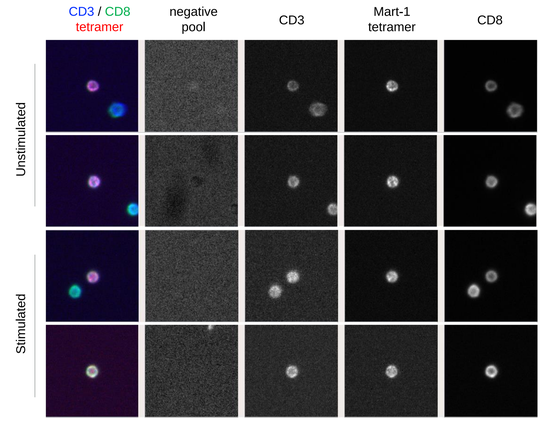



**Figure 7. Melanoma antigen MART-1 tetramer-staining in CD8⁺ T cells.** Representative images of MART-1 tetramer-positive T cells identified in unstimulated (control peptide) and stimulated (MART-1 peptide) populations.

## Results

Single influenza and Mart-1 antigen-specific T cells in donor blood samples were detected and retrieved using the CyteFinder System. Anti-influenza specificity was confirmed by RNA sequencing, which revealed a majority of alpha/beta TCR pairings with identical match to literature reports. Comparative gene expression analysis of activated and control flu tetramer-positive T cells revealed 73 significantly down-regulated and 687 significantly up-regulated genes. Pathway analysis of differential expression revealed T cell activation-associated pathways, including TCR signaling and inflammatory response/cytokine signaling.

## Conclusions

The RareCyte platform can be used to visually identify and retrieve rare antigen-specific T cells from a bulk population by using tetramers against influenza-specific T cell receptors. T cell receptor sequencing confirmed the flu-specific identity of the tetramer-positive cells. RNA signatures of activation were identified at the single T cell level after peptide stimulation.