



# Connecting cytotoxic function and TCR identity in tumor-infiltrating lymphocytes (TILs) using droplet microfluidics

## Summary

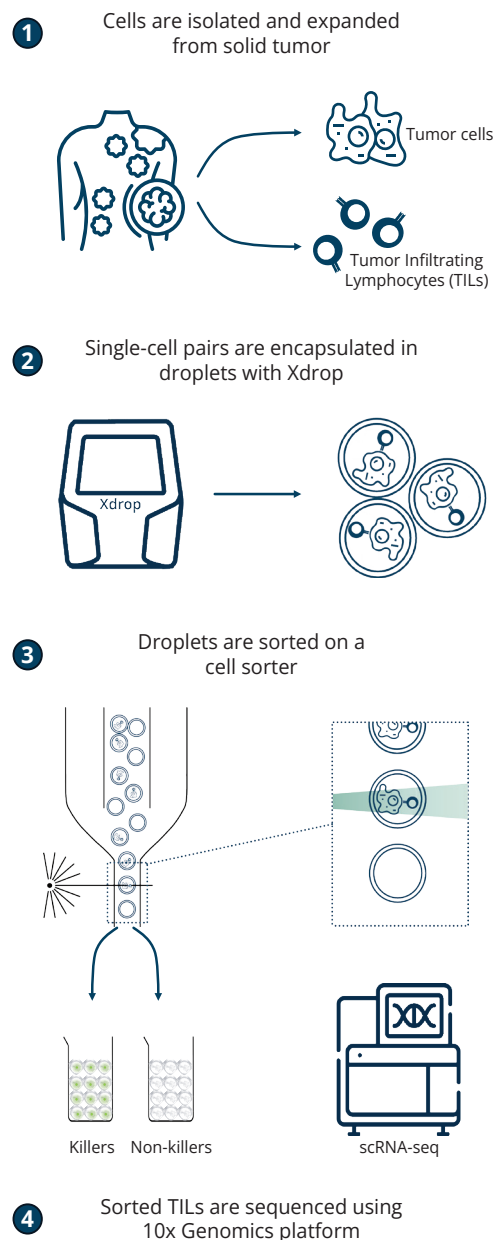
- High-throughput droplet-based co-encapsulation of tumor-infiltrating lymphocytes (TILs) and tumor cells enables functional profiling and sequencing.
- Based on cytotoxic activity, we functionally characterize and enrich TILs from solid tumors.
- Subsequent single-cell sequencing yields thousands of paired functional T-cell receptor (TCR)  $\alpha$ - and  $\beta$ -chain sequences, potentially revealing novel tumor-specific TCRs.

## Introduction

Adoptive T cell therapy is a powerful cancer immunotherapy approach but challenges remain in predicting T cell potency and variable treatment responses. A major limitation is the pronounced heterogeneity in T cell cytotoxicity, which current methods fail to resolve due to lack of single-cell functional resolution<sup>1</sup>.

Single-cell technologies directly address this gap by resolving T cell heterogeneity and enabling identification of therapeutically relevant clones<sup>2</sup>. Droplet microfluidics enables precise single-cell encapsulation for combined functional and molecular profiling of immune cells.

Kiel-Rasmussen *et al.* demonstrated the power of double-emulsion droplet technology for analyzing key cell types used in adoptive cell therapy, including natural killer cells, tumor-infiltrating lymphocytes (TILs), and CAR T cells<sup>3</sup>. Here, we extend this approach to demonstrate combined single-cell functional analysis and single cell transcriptome profiling of active and non-active TILs co-encapsulated with matched patient tumor cells.



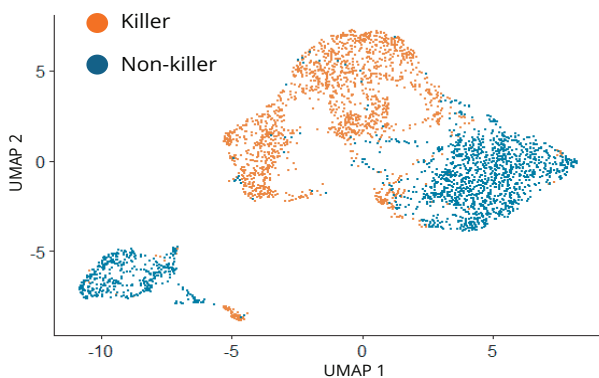
**Figure 1.** In this study, tumor cells and TILs were isolated from a solid tumor and expanded in vitro. These cells were co-encapsulated using Samplix's Xdrop platform to assess cytotoxic activity at the single-cell level. The double-emulsion droplets were sorted on a standard cell sorter and the sorted populations were then sequenced using the 10x Genomics platform.

### Tumor cells and TILs co-encapsulated in droplets

Matched expanded TILs and tumor cells from the same patient were obtained from the National Center for Cancer Immune Therapy (CCIT-DK) at Herlev Hospital. Dead cells were removed by magnetic cell isolation (MACS, Milteney). TILs were stained with CellTrace Violet (Thermo Fisher Scientific) (1:5000), while tumor cells were stained with CellTrace Far Red (Thermo Fisher Scientific) (1:5000). Cells were mixed at a 1:2.25, TIL:tumor cell ratio in RPMI 1640 media + 10% FBS + 1% Pen/Strep with Incucyte Caspase-3/7 Green Dye (Sartorius) (1:1000) to monitor cytotoxicity. The cells and their media were immediately encapsulated in Xdrop DE50 double-emulsion droplets. After three hours of incubation, droplets were sorted on a Sony MA900 cell sorter.

### Functional and molecular profiling of T cells

Droplets triple-positive for TILs, tumor cells, and caspase-3/7 activity were classified as “Killers,” while those positive for TILs and tumor cells but not caspase-3/7 were “Non-killers.” Cells were recovered from sorted droplets using Droplet Break Solution (Samplix) and processed using the 10X Chromium platform for both gene expression and T cell Receptor (TCR) sequencing, followed by sequencing on Illumina NovaSeq 6000.

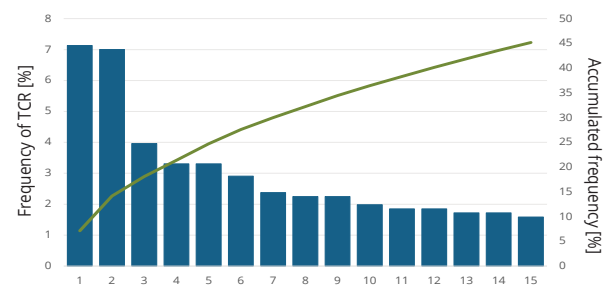


**Figure 2.** UMAP visualization of single-cell transcriptomic data overlaid with Xdrop-generated single-cell functional assessment of cytotoxicity. The figure includes data from TILs only, excluding cancer cells. Each dot represents a single cell.

This study demonstrates how Xdrop single-cell functional analysis can be combined with single-cell transcriptomics and TCR sequencing. In this particular patient sample, TILs were separated based on their ability to kill or not kill a cancer cell. In total, gene expression profiles were

acquired from 5,548 “Killer” and 11,391 “Non-killer” cells (mix of TILs and tumor cells). After selecting for CD3 expressing cells (T cells), >2,700 T cells remained in each group (Figure 2).

Interestingly, when the expression data is combined with Xdrop-derived single-cell functional data on TIL cytotoxicity, the unsupervised clustering of TILs aligns with their annotated functions as either “Killer” or “Non-killer”.



**Figure 3.** Frequency of the most abundant T-cell receptor (TCR) clonotypes in the “Killer” subpopulation identified via Xdrop functional single-cell screening and subsequent single-cell sequencing.

TCR sequencing identified >240 unique TCRs in the “Killer” TIL population. The 15 most frequent clonotypes overall accounted for >45% of the identified TCRs indicating a robust enrichment for these clonotypes (Figure 3). Future studies will define shared and distinct TCR repertoires between cytotoxic and non-cytotoxic T cells.

### Conclusion

Co-encapsulation of immune and cancer cells enables high-throughput functional analysis and sorting based on cytotoxicity-associated readouts, including caspase activation, granzyme B, and cytokine release. Here, we show how this information is expanded through downstream molecular profiling, such as RNA-sequencing.

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### References

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2. Liu, Y., Yan, X., Zhang, F. et al. TCR-T Immunotherapy: The Challenges and Solutions. *Frontiers in Oncology* vol. 11 (2022).
3. Kiel Rasmussen, AC., Hulen, T. M., Petersen, D. L. et al. Analyzing functional heterogeneity of effector cells for enhanced adoptive cell therapy applications. *Immuno-Oncology and Technology*, 24, 100738 (2024).

