Application Note

Immunotherapy Research

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Trusted Answers

Rapidly Identifying Active Natural Killer Cells

Using an Xdrop single-cell format assay based on flow cytometry analysis of double-emulsion droplets

Authors

Mette Juul Jacobsen[1], David Leander Petersen[1], Mary Amasia[1], Thomas Mørch Frøsig[1], Garret Guenther[2], Robert Sleiman[2], Peter Mouritzen[1], and Marie Just Mikkelsen[1]

[1] Samplix ApS
Birkerød, Denmark

[2] Agilent Technologies, Inc.

Samplix

Abstract

Samplix Xdrop technology and the Agilent NovoCyte Quanteon flow cytometer were applied in a cell-killing assay to rapidly identify active natural killer (NK) cells. This workflow can enable fast quantification in personalized therapies.

Introduction

Conventional cytotoxicity assays for analyzing NK cell activity often rely on bulk, averaged end-point measurements.[1–3] However, these methods fail to reveal crucial insights into the heterogeneity of effector and target cell interactions, emphasizing the importance of dynamic single-cell analysis. Single-cell assays enable the study of cytotoxicity dynamics within cell populations. Besides, single-cell assays reveal the inherent heterogeneity within and between single cell types to uncover seemingly aberrant toxicity profiles.

Single-cell assays of individual NK cells reveal varying strengths in effector function, allowing targeted selection of the "best killers" within a population. This type of individual behavior is typically masked in conventional studies. In cell therapy research, a single-cell view of immune cell activities, particularly cell killing, is of utmost importance for tailoring personalized treatments.[4,5] While transcriptomics such as RNA-Seq offers useable insights, these cells are killed during execution of the workflow, thereby preventing cell recovery or expansion for downstream in-line workflows.

To address these limitations, Samplix developed Xdrop, a technology, which, in conjunction with the Xdrop DE50 cartridge, allows for encapsulation of single living cells (or two different single cells) within double-emulsion droplets called DE50 droplets. This technology not only facilitates single-cell encapsulation but also high-throughput multiparameter analysis with the NovoCyte Quanteon flow cytometer and cell deposition and recovery through fluorescence activated cell sorting.

This application note demonstrates the application of Xdrop in a cell-killing assay, enabling rapid identification of active NK cells.

Cell-killing assay

Human lymphoblasts (K562 cells) were stained with eFluor 670. Human NK cells were incubated for 24 hours in two ways: one with interleukin 5 (IL15) for stimulation, and the other without IL15 as a control. After incubation, NK cells were stained with CFSE for 20 minutes before co-encapsulation.

CFSE-stained cells were resuspended separately in complete MEM α and co-encapsulated in double-emulsion droplets using the Xdrop protocol with an Xdrop DE50 cartridge and Xdrop well insert (Figure 1A and B). Cells were encapsulated with propidium iodide (PI) for viability studies. The effector target ratio for the sample mix was 1:3, being one NK cell to three K562 cells.

Droplets containing cells were incubated for 0, 1, 2, 4, or 24 hours at 37 °C in 5% CO_2 and the analysis was performed using a NovoCyte Quanteon flow cytometer, using novel silicon photomultiplier (SiPM) detector technology (Figure 1C).

Before analysis, droplets were visually inspected on a microscope to verify correct labeling of cells. Figure 2 shows overlaid brightfield and fluorescence microscopy images of a droplet containing both cell types after 24 hours of incubation.

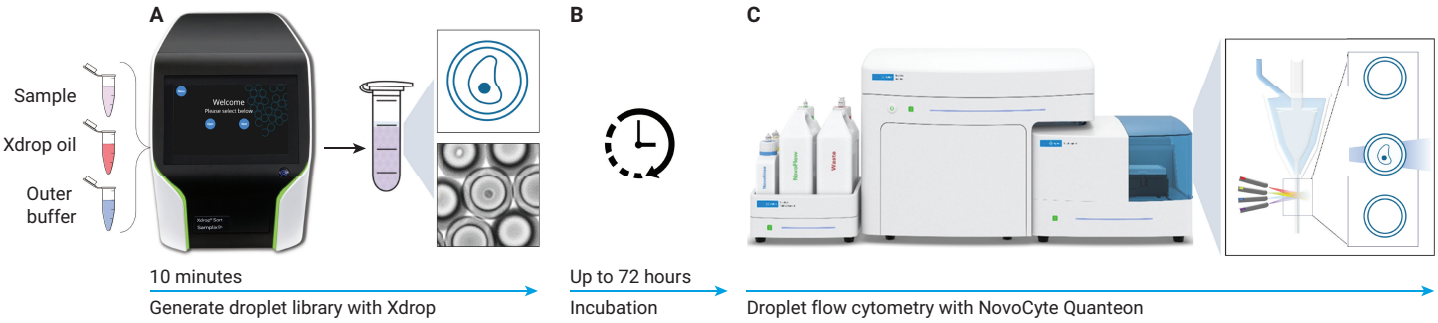

Figure 1. Schematic workflow for cell encapsulation using the Xdrop and flow cytometry analysis using an Agilent NovoCyte Quanteon analyzer. (A) Cells are co-encapsulated in double emulsion droplets from single-cell suspensions in an oil shell and surrounding outer buffer. The Xdrop generates 1 million droplets in 8 minutes. (B) Droplets containing single cells can be incubated in a standard tissue culture (CO_2) incubator for up to 72 hours to facilitate homeostasis. (C) Flow cytometry analysis was conducted using an Agilent NovoCyte Quanteon cytometer analyzer using protocols optimized for fluidic stability and cell characterization.

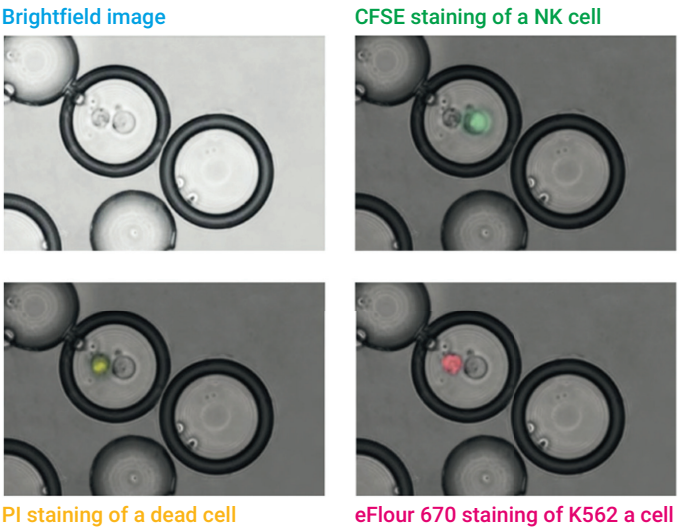

Figure 2. Overlaid brightfield and fluorescence microscopy images showing cells co-encapsulated in a double-emulsion droplet. Green FL represents an NK cell stained with CFSE, and Red FL represents a K562 lymphoblast stained with eFluor 670. Yellow FL indicates a positive PI signal, indicating loss of K562 cell viability consequent upon viable NK cell killing.

Flow cytometry analysis

The eFluor 670 FL (APC-H), representing double emulsions containing K562 target cells, were plotted against the CFSE FL (FITC-H), representing droplets containing NK effectors.

The analysis enabled the identification of four distinct droplet populations: empty droplets (64.2%), droplets with only K562 cells (28.3%), droplets with only NK cells (3.9%), and co-encapsulated droplets with both K562 and NK cells (3.6%) arising from the total droplet count generated by Xdrop and analyzed on the Quanteon (Figure 3A). The count of droplets with PI-negative (living cells) or PI-positive (dead cells) signals was plotted (Figure 3B) for each distinct droplet population. The highest count of dead cells (43.4%) was observed in the droplets where both K562 and NK cells were co-encapsulated.

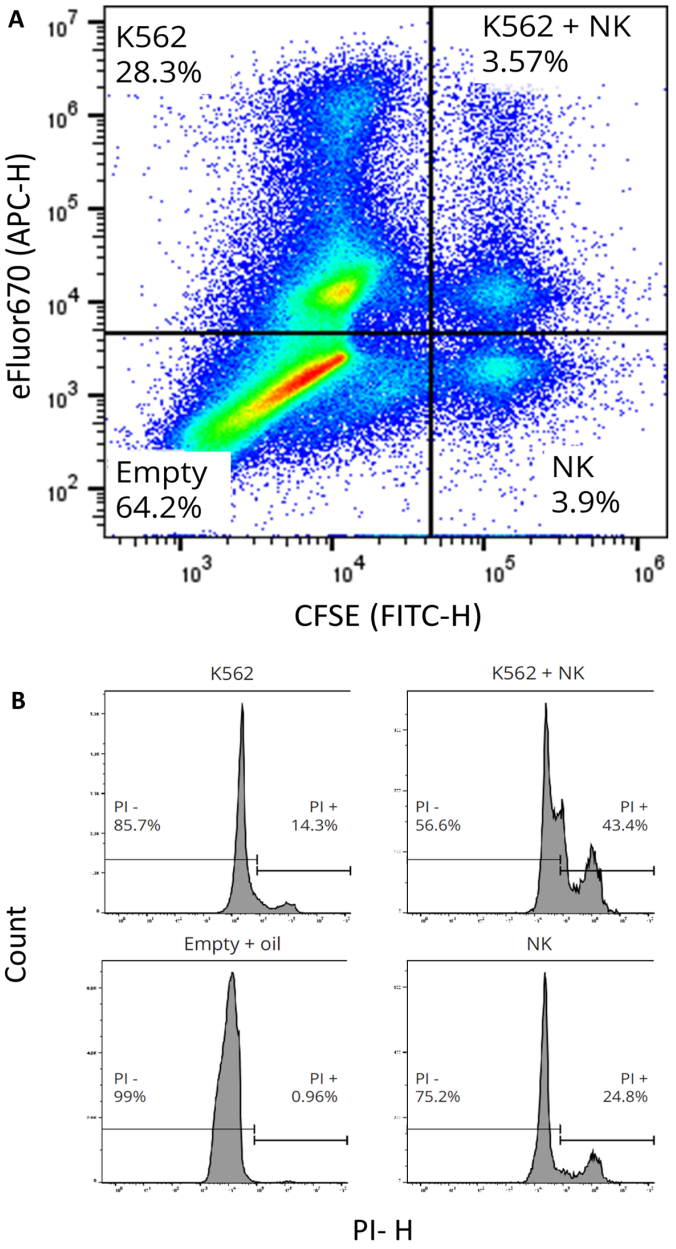

Figure 3. Flow cytometry analysis. (A) Bivariate dot-plot showing eFluor 670 (APC-H) FL plotted against CFSE (FITC-H) FL. From these data four distinct droplet populations were determined: empty droplets (low eFluor 670, low CFSE), droplets with only K562 cells (high eFluor 670, low CFSE), droplets with only NK cells (low eFluor 670, high CFSE), and droplets containing both K562 and NK cells (high eFluor 670, high CFSE). (B) Histograms showing the PI signal for each one of the four quadrant gates. A PI+ signal indicates dead cells. This was used to determine the level of late apoptotic cell death. All data were FL-compensated with single-FL stained samples.

Confirmation of cell-killing activity

Percentage of dead cells was determined based on PI FL intensity measured after 0, 1, 2, 4, or 24 hours. Cell killing was calculated by subtracting the background death at 0 hours and normalizing to the number of live cells at the same time point. The droplets co-encapsulated with both K562 and IL15-stimulated NK cells, the percentage of dead cells progressed from 3.9% after 1 hour to 38.1% after 24 hours. In the unstimulated cultures, cell killing was delayed and less pronounced even after 24 hours (Figure 4).

In droplets containing only NK cells, there was no increase in the percentage of dead cells during the experiment. In droplets containing only K562 cells, an increase in the percentage of dead cells was not observed for the first 4 hours of the experiment. After 24 hours, a slight increase (7 to 8%) in dead cells was observed (data not shown).

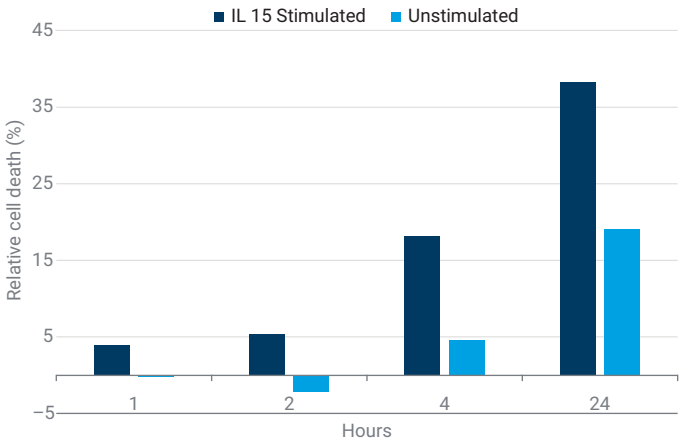

Figure 4. Relative cell death from three experiments ± SEM. K562 cells were co-encapsulated in double-emulsion droplets with IL15-stimulated or unstimulated NK cells. After flow cytometry analysis, relative cell death was determined by subtracting the background death (PI+ signal for droplets with co-encapsulated NK and K562 cells at 0 hours) and normalizing to the number of live cells at 0 hours.

Conclusion

The Xdrop workflow for co-encapsulation of immune cells and their targets offers a unique opportunity, in conjunction with Agilent NovoCyte Quanteon flow cytometry, to rapidly and reproducibly quantify the behavior of NK cells exhibiting different cytotoxicity levels in a population. Target cell killing can be observed within one hour of incubation, allowing the identification of the "fastest killers" within the heterogeneous population. This workflow represents a significant advancement in the study and analysis of intrinsic and engineered immune cells in a single-cell format.

Reference

1. Wang, D.; Bodovitz, S. Single Cell Analysis: The New Frontier in 'Omics'. *Trends Biotechnol.* **2010**, *28(6)*, 281–290.
2. Chattopadhyay, P. K.; Gierahn, T. M.; Roederer, M.; Love, J. C. Single-Cell Technologies for Monitoring Immune Systems. *Nat. Immunol.* **2014**, *15(2)*, 128–135.
3. Toriello, N. M. *et al.* Integrated Microfluidic Bioprocessor for Single-Cell Gene Expression Analysis. *Proc. Natl. Acad. Sci. USA* **2008**, *105(51)*, 20173–20178.
4. Santra, T. S.; Tseng, F. G. Single-Cell Analysis. *Cells* 2020, *9(9)*, **1993**.
5. Bode, D.; Cull, A. H.; Rubio-Lara, J. A.; Kent, D. G. Exploiting Single-Cell Tools in Gene and Cell Therapy. *Front. Immunol.* **2021**, 12, 702636.

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