Application note

Samplix

Revealing granzyme B secretion and cell killing dynamics in a single-cell format

David Leander Petersen, Mette Juul Jacobsen, Bárbara Schlicht, Marie Just Mikkelsen, and Peter Mouritzen
Samplix ApS, Birkerød, Denmark

Summary

- This Xdrop® DE50 droplet-based workflow enables simultaneous detection of granzyme B secretion and cell killing with single-cell resolution.
- The results are a biologically relevant demonstration of granzyme B secretion preceding cell killing.
- The workflow is designed to reveal the functional heterogeneity of cell populations.

Introduction

Immunotherapy development requires an effective cell characterization strategy to guide process design.[1, 2] Currently, most functional assays for cell characterization are bulk assays, which offer limited insights into cell population heterogeneity.[3] Determining the percentage of cells with the desired functionality within a sample population continues to be challenging.

Here, Xdrop is used to prepare cells for simultaneous measurement of granzyme B (GzmB) secretion and cell-killing activity. It offers mechanistic insights into cytotoxic events at the single-cell level and enhances the understanding of cell population heterogeneity. The results confirm that natural killer (NK) cell secretion of GzmB precedes cell killing, aligning with the well-established understanding of this protease's pivotal role in apoptosis-induced target cell death.[4]

Our approach involves the co-encapsulation of human NK cells (effector) and K562 human lymphoblast cells (target) in double-emulsion droplets with a volume of ~100 pl (DE50 droplets). This allows the analysis of individual effector cell behavior: their GzmB secretion activity and their ability to kill target cells. The small droplet volume ensures the swift accumulation of GzmB, so the signal is detectable sooner than in a larger reaction volume.

Co-encapsulation of target and effector cells

The workflow is illustrated in Figure 1A. Before encapsulation, the human NK cells were divided into two cultures. One was incubated with stimulation by 50 ng/ml interleukin 15 (IL15) for 24 h. The other (the control) was incubated for 24 h without stimulation.

To facilitate tracking of the cell content within the DE50 droplets, the NK cells were stained with CellTrace™ Violet (Conc. 1:5,000) and the K562 cells with CellTrace Far Red (Conc. 1:5,000).

The NK and K562 cells were separately resuspended in complete MEM α medium supplemented with 10% OptiPrep™, 1 µg/ml propidium iodide (PI) and 4 µM GzmB FITC-labelled peptide substrate. The peptide substrate fluoresces when it is cleaved by secreted GzmB, resulting in the accumulation of an FITC signal inside the droplets (Figure 1B). PI is used to monitor cell death.

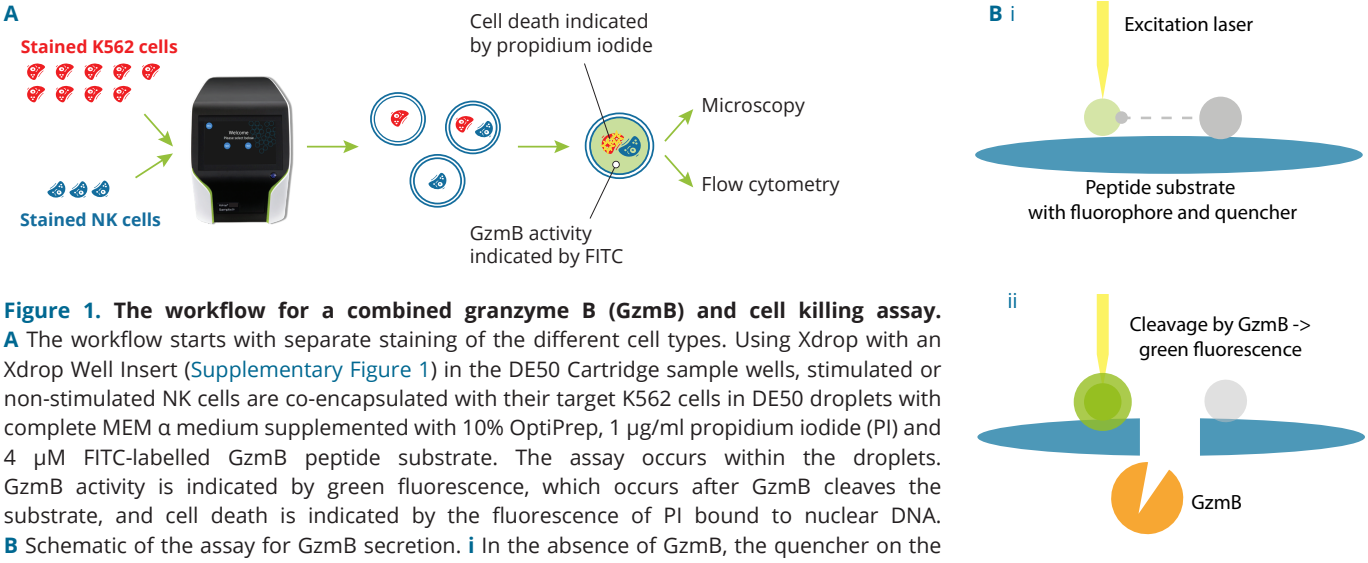


Figure 1. The workflow for a combined granzyme B (GzmB) and cell killing assay. **A** The workflow starts with separate staining of the different cell types. Using Xdrop with an Xdrop Well Insert (Supplementary Figure 1) in the DE50 Cartridge sample wells, stimulated or non-stimulated NK cells are co-encapsulated with their target K562 cells in DE50 droplets with complete MEM α medium supplemented with 10% OptiPrep, 1 µg/ml propidium iodide (PI) and 4 µM FITC-labelled GzmB peptide substrate. The assay occurs within the droplets. GzmB activity is indicated by green fluorescence, which occurs after GzmB cleaves the substrate, and cell death is indicated by the fluorescence of PI bound to nuclear DNA. **B** Schematic of the assay for GzmB secretion. **i** In the absence of GzmB, the quencher on the peptide substrate prevents the fluorophore from fluorescing. **ii** GzmB cleaves the peptide substrate. The quencher no longer prevents fluorescence.

The media for the stimulated NK cells and their co-encapsulated K562 cells were supplemented with IL15 to continue NK stimulation (NK:K562 +IL15). The media for the control NK cells and their co-encapsulated K562 cells were not supplemented with IL15 (NK:K562 -IL15).

An Xdrop Well Insert (Supplementary Figure 1) was placed into each of the sample wells of an Xdrop DE50 Cartridge. The NK cells in their media (+IL15 or -IL15) were pipetted into the left side of the Xdrop Well Insert and the K562 cells in their media (+IL15 or -IL15) were pippetted into the right. We used an NK:K562 cell ratio of 1:3 for this experiment, with 0.5 x 10^6 NK cells and 1.5 x10^6 K562 cells per sample well.

A standard Xdrop protocol for DE50 droplet generation was used to co-encapsulate the cells in DE50 droplets. Following encapsulation, each DE50 droplet production (NK:K562 +IL15 or NK:K562 -IL15) was divided into two incubation tubes and supplemented with complete MEM α, 33.3% Stabilizing solution for cells, and 10% OptiPrep.

The cells were incubated within the droplets for either 30 min or 4 h at 37°C in 5% CO_2, and then analyzed using microscopy and flow cytometry.

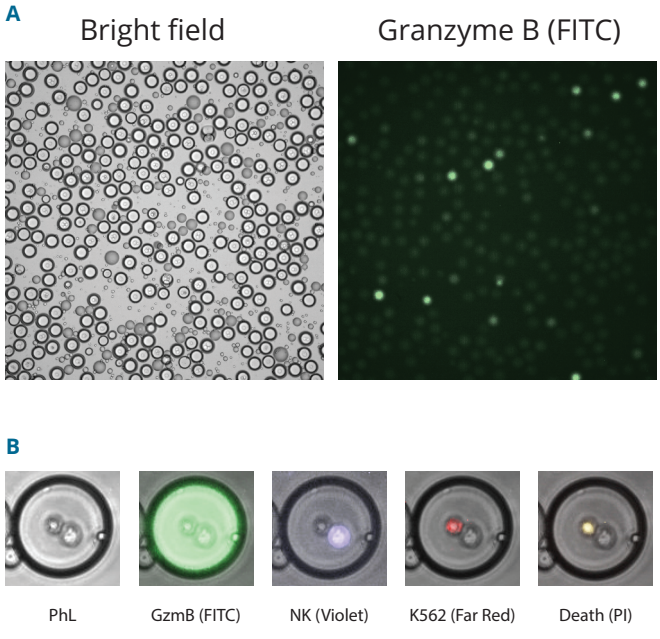


Figure 2. Microscopy images of encapsulated cells after 4 h incubation. A Microscopy images of DE50 droplets with IL15-stimulated NK and K562 cells. A bright FITC signal (green) is observed for droplets with GzmB activity. **B** Microscopy images of a single DE50 droplet containing a GzmB-positive, IL15-stimulated NK cell and a K562 cell. From left to right: phase contrast image; and fluorescence microcopy images overlaid on the phase contrast image to show the signal from GzmB activity (green); CellTrace Violet-stained NK cells (blue); CellTrace Far Red-stained K562 cells (red); and PI detection of cell death (yellow).

Figure 2A shows bright field and fluorescence microscopy images, with the intensity of the green fluorescence showing the level of GzmB activity of IL15-stimulated NK cells in DE50 droplets after 4 h incubation. Figure 2B shows phase contrast and fluorescence microscopy images of a single GzmB-positive droplet containing an IL-15-stimulated live NK cell and a dead K562 target cell, also after 4 h incubation.

Double-emulsion droplet flow cytometry reveals the dynamics of NK cell killing

To quantify the GzmB activity and cell killing activity, DE50 droplets from the NK:K562 +IL15 and NK:K562 -IL15 productions were analyzed using a NovoCyte® Quanteon flow cytometer (Agilent® Technologies). Specific staining of K562 cells with CellTrace Far Red and NK cells with CellTrace Violet facilitated the identification of four distinct droplet populations (Figure 3A). The droplets containing both K562 and NK cells were gated and analyzed for signals indicating GzmB activity (FITC) and cell death (PI; Figure 3B).

The number of NK+K562 droplets analyzed per data point ranged from 1.2–3.3 x10^4 (data not shown). After only 30 min incubation, 27% of the NK+K562 +IL15 droplets were positive for GzmB (Figure 3D), showing how rapidly the accumulated GzmB can be detected. After 4 h incubation, the number of GzmB-positive droplets had increased to 42%. NK+K562 -IL15 droplets showed limited GzmB activity at both time points.

After 30 min incubation, 17% of the NK+K562 +IL15 droplets showed GzmB without traces of cell death while 10% showed both GzmB and cell death. After 4 h, the frequency of droplets with both GzmB and cell death had doubled to 20%, while that of droplets with GzmB alone only increased from 17% to 21% (Figure 3D). Notably, the percentage of droplets with dead cells but no GzmB remained stable from the start until the end of the experiment (Figure 3C), i.e., there was no observed increase in the frequency of cell death without concomitant presence of GzmB. Thus, GzmB secretion is a valid and functional early indicator of active cell killing potential.

Droplets containing only NK cells or only K562 cells were also analyzed for GzmB activity and cell death (data not shown). Only 2.3% of droplets containing IL15-stimulated NK cells alone and 0.6% containing K562 alone were positive for GzmB secretion after 30 min incubation. The incidence of droplets with cell death remained the same throughout the experiment for both droplet types (data not shown).

These results confirm that GzmB secretion is prompted by NK cell recognition of target cells, and this effect is notably enhanced when NK cells are stimulated with IL15. GzmB secretion is succeeded by cell death, which is a consequence of GzmB-induced apoptosis. These results conform to the widely accepted sequence of events in cell killing. The Xdrop system provides the required single-cell resolution for this combined GzmB–cell killing assay.

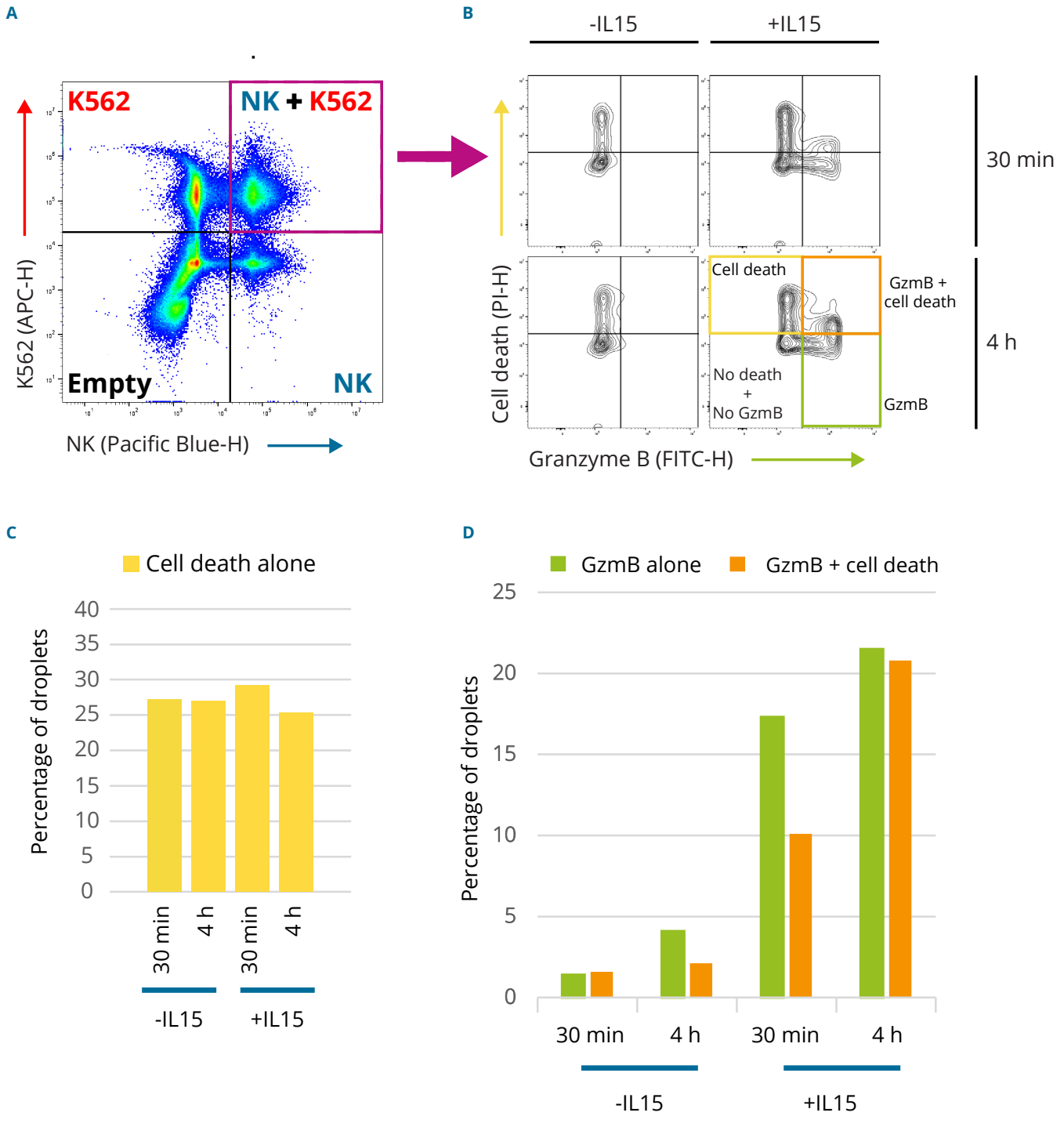


Figure 3. Quantitative double-emulsion flow cytometry analysis of the DE50 droplet-based, combined GzmB and cell killing assay. **A** Density diagram with gated DE50 droplets plotted based on the signal from the two applied cell stains: NK — CellTrace Violet (Pacific Blue-H) and K562 — CellTrace Far Red (APC-H). Four droplet populations are visible: droplets containing both NK and K562 cells; droplets with K562 cells alone; droplets with NK cells alone; and empty droplets. **B** Contour plots with the gated DE50 droplets containing both NK and K562 cells plotted based on cell death (PI-H) and GzmB (FITC-H) signals. The time points are 30 min and 4 h for NK cells stimulated with IL15 or non-stimulated NK cells. **C** Percentage of droplets with cell death only from **B**, illustrating that cell death without secretion of GzmB did not increase over the course of the experiment. **D** Percentage of droplets in quadrants from **B** with GzmB activity only or with simultaneous GzmB activity and cell death.

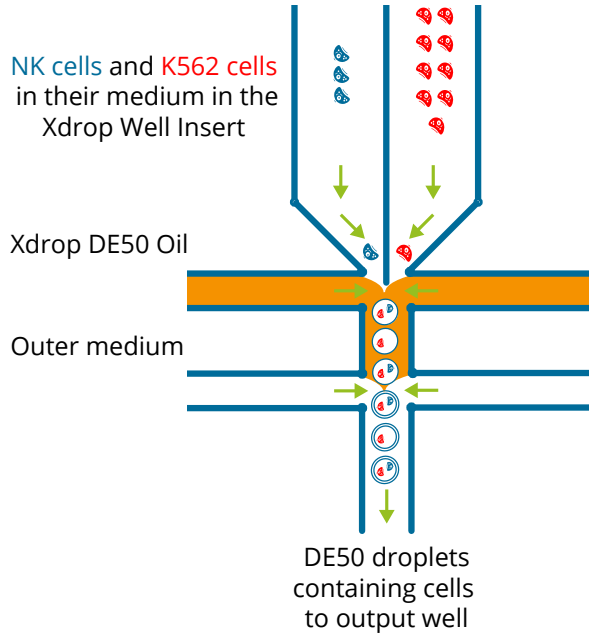
Conclusion

Xdrop reveals the heterogeneity in cell function within cell populations, offering rapid, biologically relevant, single-cell resolution for flow cytometry assays. Our findings here support the widely accepted dynamics of GzmB secretion and cell killing, showing the utility of Xdrop in advancing our understanding of vital cell biology processes and as a powerful tool for immune cell therapy development.

For more information about Xdrop products and applications, visit samplix.com.

A

B



Supplementary Figure 1. Cell co-encapsulation using the Xdrop Well Insert. **A** Photo of an Xdrop Well Insert in the sample input well of an Xdrop DE50 Cartridge. **B** Illustration of the process of co-encapsulation of two cell types (in this case, stained natural killer cells and stained human lymphoblast cells, K562 in a 1:3 ratio). The cells in their medium enter the channels of the Xdrop DE50 Cartridge separately through the two sides of the Xdrop Well Insert. They meet with the Xdrop DE50 Oil at the first junction and are co-encapsulated in the oil. When the droplets containing the cells meet the outer medium at the second junction, the double emulsion shell forms. This highly stable double-emulsion droplet acts as a picoliter-sized incubation or assay chamber for the cell interaction.

Notes and references

Xdrop and the Xdrop DE50 Cartridge are for research use only, not for use in any diagnostic procedures.

1. U.S. Department of Health and Human Services, Food and Drug Administration. 2022. Considerations for the development of chimeric antigen receptor (CAR) T cell products. Draft Guidance for Industry. https://www.fda.gov/media/156896/download, accessed December 2023.
2. European Medicines Agency. 2008. Guideline on human cell-based medicinal products. https://www.ema.europa.eu/en/documents/scientific-guideline/guideline-human-cell-based-medicinal-products_en.pdf, accessed December 2023.
3. Antona, S., et al. 2020. Droplet-based combinatorial assay for cell cytotoxicity and cytokine release evaluation. Adv. Funct. Mater. 30(46), 2003479.
4. Boivin, W.A., Cooper, D.M., Hiebert, P.R., and Granville, D.J. 2009. Intracellular versus extracellular granzyme B in immunity and disease: challenging the dogma. Laboratory Investigation, 89, 1195–1220.

SX-000649-AN, ver. 1