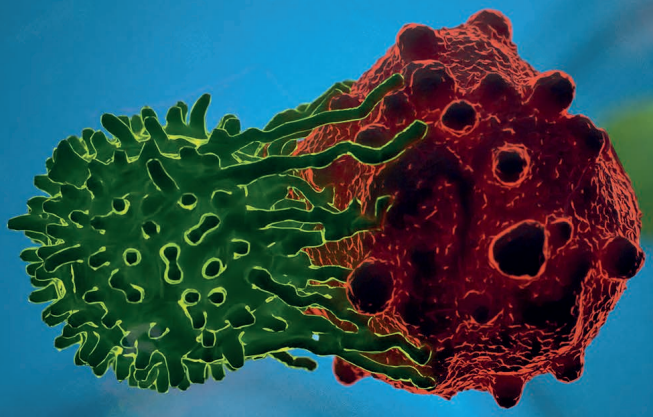
Reveal cell function with single-cell resolution



How the double-emulsion droplet could revolutionize immunotherapy research

Samplix

CONTENTS

INTRODUCTION

How droplet microfluidics will change immunotherapy

The heroes of this eBook are double-emulsion droplets called Xdrop® DE50 droplets. With a volume of around 100 picoliters, these tiny water-in-oil-in-water spheres are the right size to hold a single living mammalian cell, ready for incubation, flow cytometry, sorting, and other assays. In just eight minutes, Samplix's benchtop microfluidics instrument, Xdrop, can produce millions of DE50 droplets containing cells. They have other applications — as do their siblings, the even smaller DE20 droplets — but here, we're focusing on DE50 droplets and mammalian immune cells, because together, they're going to help realize the potential of immunotherapy.

That's a bold claim: after all, unlocking the full potential of immunotherapy is a major goal in medical research. One of the big obstacles is that the traditional methods for cell function analysis cannot truly reveal the extent of the heterogeneity of cell function within a population.

A single-cell view of immune cell functions, such as cytokine secretion, antibody secretion or cell-killing activity, is critical to determine the potency of an immune cell population intended for therapeutic use. It's the only way to find the cells with the right activity (e.g., that secrete the desired cytokine or killing agent) and weed out the ones that aren't right for the patient (e.g., that don't show the right functional response).

Bulk functional assays mask the view of individual cell activity. They can only deliver average readouts for cell function within the heterogeneous population. There is nothing to prevent cross-talk between immune cells with different activities in the bulk solution. Bulk assays also won't reveal whether a natural killer cell population consists mostly of moderately active cells or a minority of highly active cells.

Transcriptomics can give this single-cell insight, but the cells are killed in the workflow. That precludes the recover and expansion of the cells with the right functional properties.

Enter DE50 droplets. As mentioned, these are picoliter-sized reaction chambers for living cells. They are stable for days and compatible with key cell research instruments, such as incubators, flow cytometers, and cell sorters. The oil shell is permeable to gases and small molecules, but not to large proteins. That means that cells stay alive within the droplets, with simple nutrients, gases, and water passing between the inner and outer media. Cytokines, antibodies, and enzymes produced by the encapsulated cell cannot pass out of the droplet. Therefore, they build up quickly in the small volume, enabling rapid differentiation of the active secretors from the inactive ones.

Assays for the detection of secreted IFN-γ, TNF-α, and granzyme B have already been tested. Furthermore, we have successfully separated the cells with the desired activity from the rest of the population and recovered them alive. Experiments on the co-encapsulation of natural killer cells and their target lymphoblasts have also been tested, with similar success.

The distinguishing feature of our Xdrop droplet technology lies in its capacity to co-encapsulate immune cells and their target cells. Both engineered and native immune cells can be evaluated for their cytotoxic potential and simultaneously assayed for the release of cytokines, granzyme B and other agents.

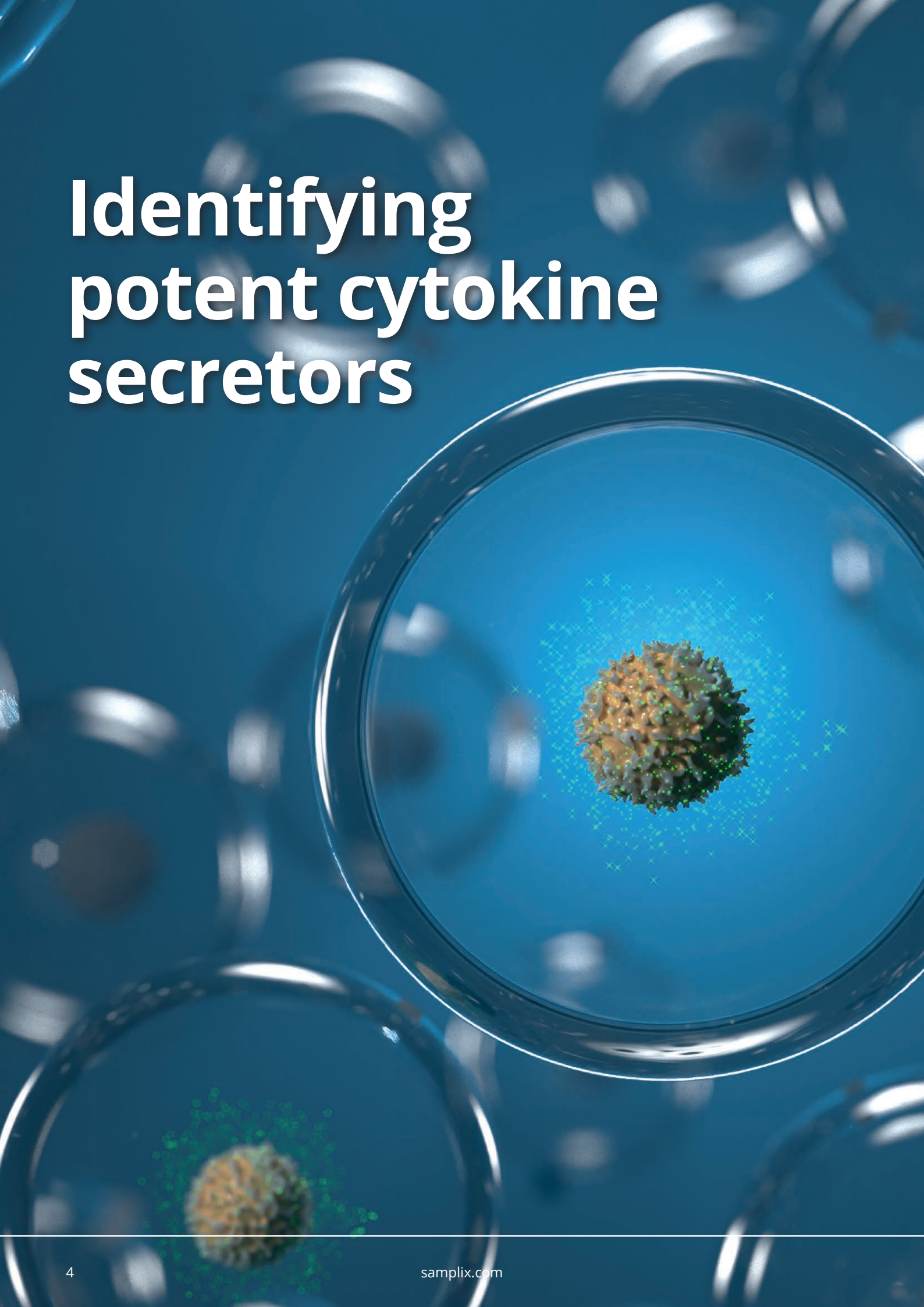
The compatibility of Xdrop DE50 droplets with flow cytometers and cell sorters significantly broadens the range of assays that can be performed on these instruments.

In this eBook, we'll look at some of these experiments more closely, showing the setup and the results. I believe you'll find the results compelling and see why we believe DE50 droplets can answer these key questions in immunotherapy research.

Lars Kongsbak,
CEO of Samplix

Learn more about Xdrop and DE50 droplets at samplix.com

Identifying potent cytokine secretors



Revealing and retrieving highly potent IFN-γ secretors using an Xdrop® single-cell format workflow based on double-emulsion droplets

Mette Juul Jacobsen, Mary Amasia, Christian Hussing, Rikke Stick Højmark, Thomas Mørch Frøsig, Peter Mouritzen, and Marie Just Mikkelsen

Samplix ApS, Birkerød, Denmark

Summary

- Bulk functional assays of immune cells miss highly potent cells within the population.
- This Xdrop single-cell format workflow for IFN-γ secretion assessment reveals these highly potent individual cells, enabling their retrieval and expansion.

Introduction

Bulk functional assays of mammalian cells can only deliver average readouts for the heterogeneous population, masking the view of individual cell activity. This is due to the cross-talk between cells with different activities in the bulk solution.

In immunotherapy research and clinical development, it is critical to have a single-cell view of immune cell activities, e.g., cytokine secretion. Transcriptomics can give this insight, but the cells are killed in the workflow, meaning no possibility for cell recovery or expansion.

Samplix has developed Xdrop and the Xdrop DE50 Cartridge to encapsulate living mammalian cells in highly stable double-emulsion droplets (DE50 droplets) for incubation, flow cytometry, and sorting. Here, we show the application for cytokine secretion analysis to reveal highly potent cells that were missed in the bulk assay.

IFN-γ secretion assay

Natural killer (NK) cells were washed and labelled with IFN-γ catch reagent according to the Miltenyi Biotec® IFN-γ Secretion Assay protocol. The cells were then resuspended in MEM α with 100 ng/ml IL-2 and FITC-IFN-γ antibody. Half of this activated population was incubated in bulk. We encapsulated the cells of the other half for incubation in double-emulsion droplets using an Xdrop DE50 Cartridge on an Xdrop.

In parallel, non-activated NK cells were prepared: they received the same treatment without exposure to IL-2.

Cells from all four incubations (bulk activated, bulk non-activated, single-cell activated, single-cell non-activated) were analyzed using a SONY® SH800S cell sorter. Figure 1 shows the Xdrop-based workflow for activated cells. Figure 2 shows the results.

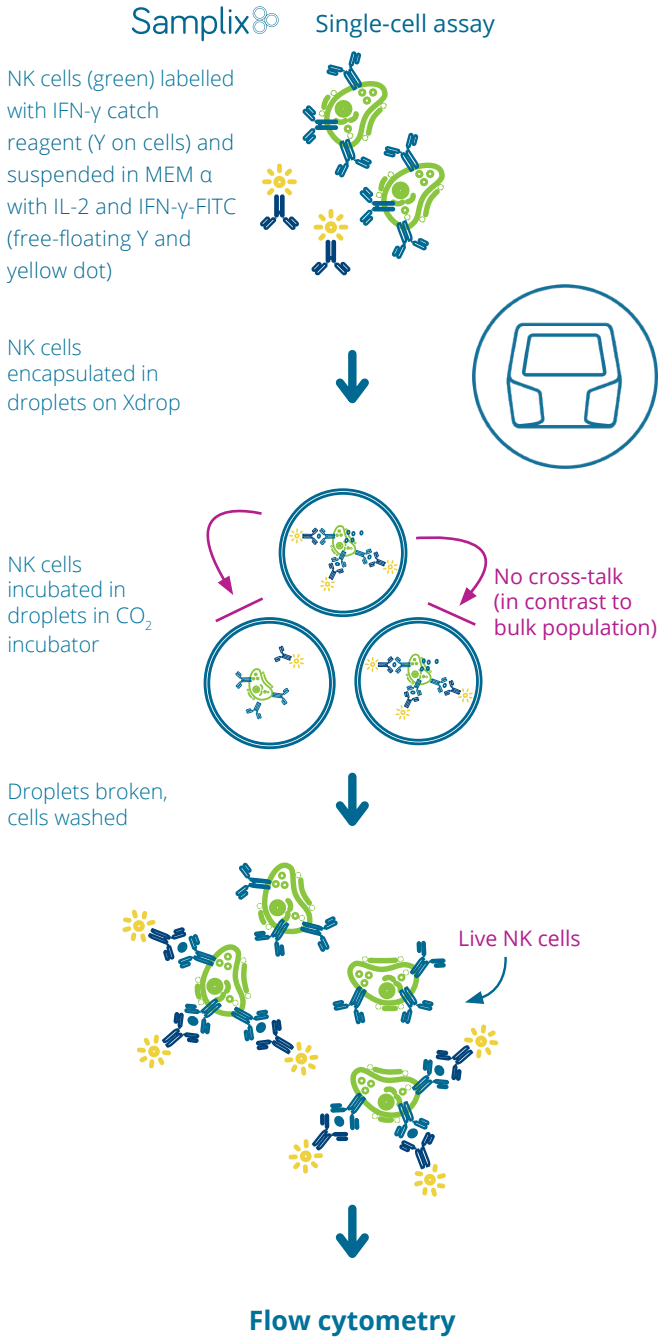


Figure 1. The Xdrop-based workflow for a single-cell format IFN-γ secretion assay

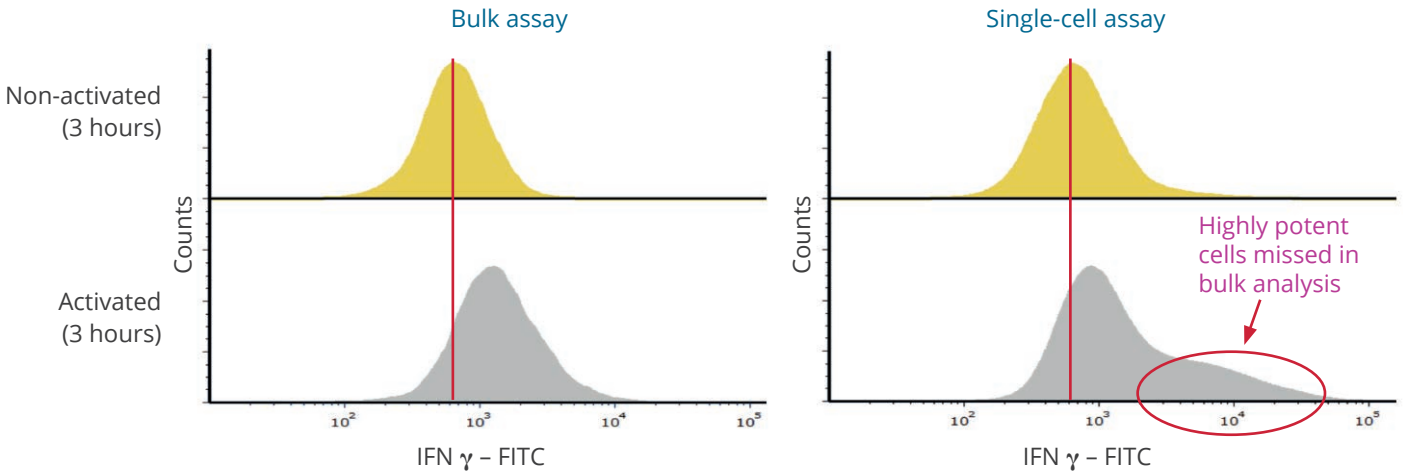


Figure 2. The results for IFN-γ secretion from non-activated and IL-2-activated NK cells incubated for 3 hours in MEM α in bulk (bulk assay) or within double-emulsion droplets generated with an Xdrop DE50 Cartridge on an Xdrop (single-cell assay). The Xdrop-based single-cell format workflow reveals highly potent individual NK cells that are hidden in the averaged readout for the bulk assay.

Retrieval and expansion of cells with desired IFN-γ activity

NK-92 cells were labelled and activated with IL-2 as described for the IFN-γ secretion assay. Using an Xdrop DE50 Cartridge on an Xdrop, the cells were encapsulated along with FITC-IFN-γ antibody and prodidium iodide (PI) in MEM α. The IFN-γ secretion was assessed using a a SONY SH800S cell sorter. Cells with a high IFN-γ secretion profile were enriched via sorting on the SONY instrument and cultured for 2 weeks. In parallel, non–activated cells were cultured for 2 weeks in the same medium.

A second droplet-based analysis of the NK cells' IFN-γ secretion profiles after exposure to IL-2 indicates that those selected for high activation in the first round maintain this high potential over time (Figure 3). Notably, the background signal for the selected but not activated NK cells (B) is slightly higher than that for the not selected, not activated ones (D). This will be investigated further.

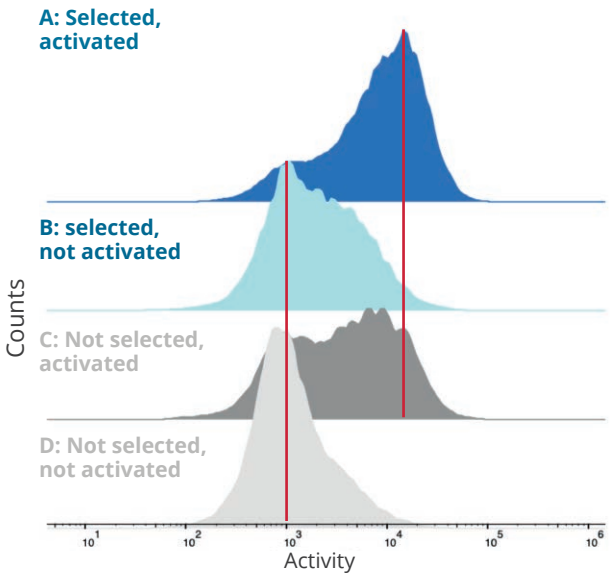


Figure 3. The results for IFN-γ secretion from NK cell populations that were: **A and B –** previously selected for high IFN-γ secretion, incubated for two weeks, then activated with IL-2 (A) or not activated (B); **C and D –** incubated for two weeks without any prior activity-based selection, then activated with IL-2 (C) or not activated (D).

Conclusion

The Xdrop workflows described here enable the identification of highly potent IFN-γ-secreting NK cells and the successful selection and expansion of those cells. This is a unique approach for cytokine secretion assays, delivering single-cell results for living cells within a short time frame.

How Xdrop supports functional assays of mammalian cells in a single-cell format

Using the Xdrop DE50 Cartridge, Xdrop encapsulates living mammalian cells in highly stable, ~100-picoliter, double-emulsion droplets. This can accelerate assays thanks to the picoliter reaction spaces, which force faster cell–cell interactions or cell secretion buildup. Xdrop processes up to 8 samples in parallel, with ~150,000 single-cell assays generated per sample in just 8 minutes. It is possible to incubate cells within droplets in a CO_2 incubator, analyze single cells or droplets on a flow cytometer, and sort and recover cells for expansion and molecular profiling.

Learn more about Xdrop at samplix.com or contact us at samplix.com/contact.

Notes

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

Identifying highly potent TNF-α-secreting T cells from blood samples in 7 hours using the Xdrop® single-cell format, droplet-based workflow

Mette Juul Jacobsen, Christian Hussing, Rikke Stick Højmark, Thomas Mørch Frøsig, Peter Mouritzen, and Marie Just Mikkelsen
Samplix ApS, Birkerød, Denmark

Summary

- Bulk functional assays of immune cells miss highly potent cells within the population.
- The Xdrop droplet-based assay reveals individual highly potent TNF-α-secreting T cells in blood samples.

Introduction

A single-cell view of immune cell activities, such as cytokine secretion, is critical to immunotherapy research. However, most common secretion analyses use a bulk workflow, where cross-talk between secreting and non-secreting cells creates a readout bias (Figure 1, lower workflow).

Samplix has developed Xdrop and the Xdrop DE50 Cartridge to encapsulate living mammalian cells in highly stable double-emulsion droplets (DE50 droplets) for incubation, flow cytometry, and sorting. The workflows provide the required single-cell view of activity levels.

Here, we use the Xdrop workflow for TNF-α secretion analysis (Figure 1, upper workflow), identifying and isolating highly potent T cells that would be missed in a bulk assay. What's more, the workflow does not require an overnight incubation, allowing completion within a working day.

TNF-α secretion assay

Human PBMCs (peripheral blood mononuclear cells) from a healthy donor were used for a TNF-α secretion assay based on the Xdrop workflow and a bulk assay.

The PBMCs were labeled with TNF-α capture reagents according to the Miltenyi Biotec® TNF-α Secretion Assay protocol. Then, the population was split into four groups. The cells of one group were encapsulated in DE50 droplets together with TNF-α-PE antibody and cell stimulation cocktail (PMA/Ionomycin). The cells of the second were also encapsulated with TNF-α-PE antibody but without cell stimulation cocktail. The third and fourth groups were non-encapsulated cells respectively with or without cell stimulation cocktail.

All cells were then incubated (in droplets or in bulk) at 37°C in 5% CO_2 for 1, 2, or 4 hours. TNF-α-PE antibody was added to the bulk groups after incubation and breakage of the droplets.

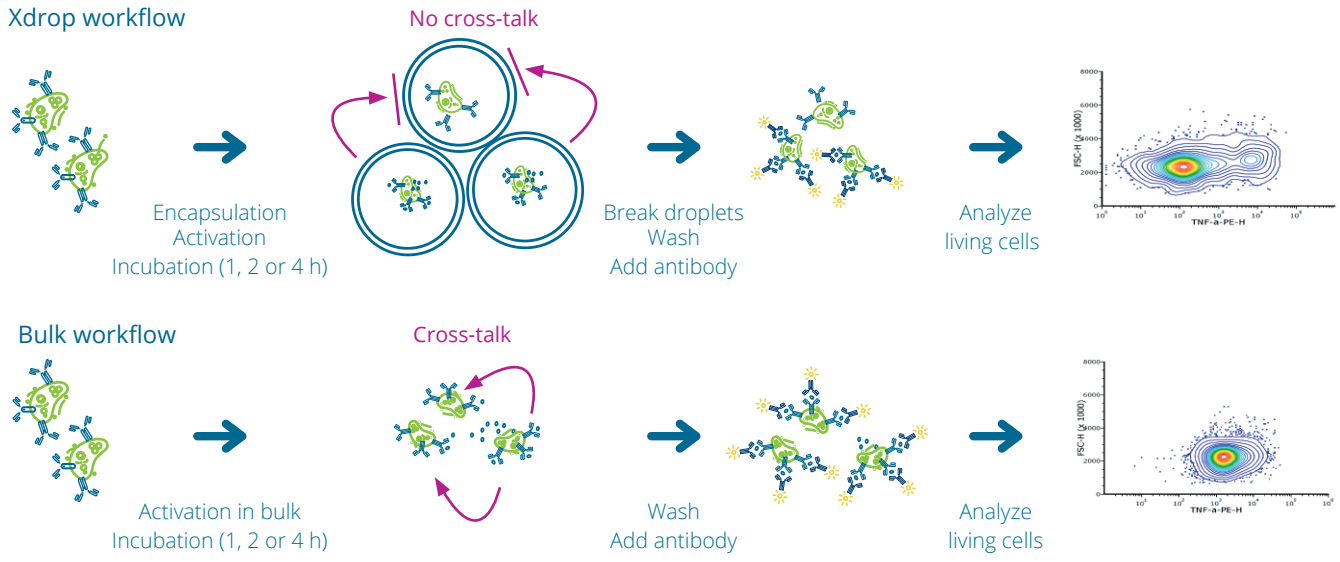


Figure 1. The Xdrop workflow for a single-cell TNF-α secretion assay with 1, 2, or 4 hours of incubation (top) and the equivalent bulk assay with the same incubation times (bottom). The whole Xdrop workflow takes 5 to 7 hours depending on the incubation time. Set up: 1 h 20 min. Droplet generation: 8 min. Incubation: 1–4 h. Droplet breaking and staining: 1 h. Analysis on flow cytometer: 5 min/per sample. While this version of the bulk workflow takes the same amount of time, it does not deliver equivalent results.

The cells from the first two groups were then released from the droplets and all cells were washed in 0.5% BSA in dPBS, stained with live/dead-FITC stain and CD3-PerCP, and analyzed using a BD Accuri™ flow cytometer. The T cells in the samples were specifically detected via gating based on the $CD3^+$-PerCP signal.

Figure 1 shows the Xdrop-based and bulk workflows for the activated cells. Figure 2 shows the TNF-α secretion profiles of the T cells from the blood samples.

Highly active TNF-α-secreting T cells are detected after 2 hours of incubation in droplets

After 1 hour of incubation, the TNF-α secretion profiles for the activated T cells from the bulk and Xdrop groups looked similar to each other (Figure 2A).

After 2 hours of incubation, the profile for the activated T cells from the two workflows were distinctly different, with a subpopulation of highly potent cells clearly present in the Xdrop group (Figure 2B). After 4 hours, this difference was even more pronounced (Figure 2C). At no point is such a subpopulation of highly potent T cells discernable from the bulk activated profile.

What's more, the peak value for the bulk activated group increases significantly over the four hours, while the peak value for the Xdrop activated group only increases by a relatively small amount.

These results indicate that there is cross-talk between the cells in the bulk activated group, and that only a minor fraction of the cells in the bulk group are activated, but they present with a highly increased signal.

Conclusion

The Xdrop workflow using the Xdrop DE50 Cartridge allows for precise TNF-α secretion analysis in a single-cell format for human T cells in a population of PBMCs within an 8-hour working day. The incubation time is just 2 to 4 hours. Overnight incubation is not necessary. The results clearly show how significant a population of highly potent secretors can be hidden in the results of a bulk assay.

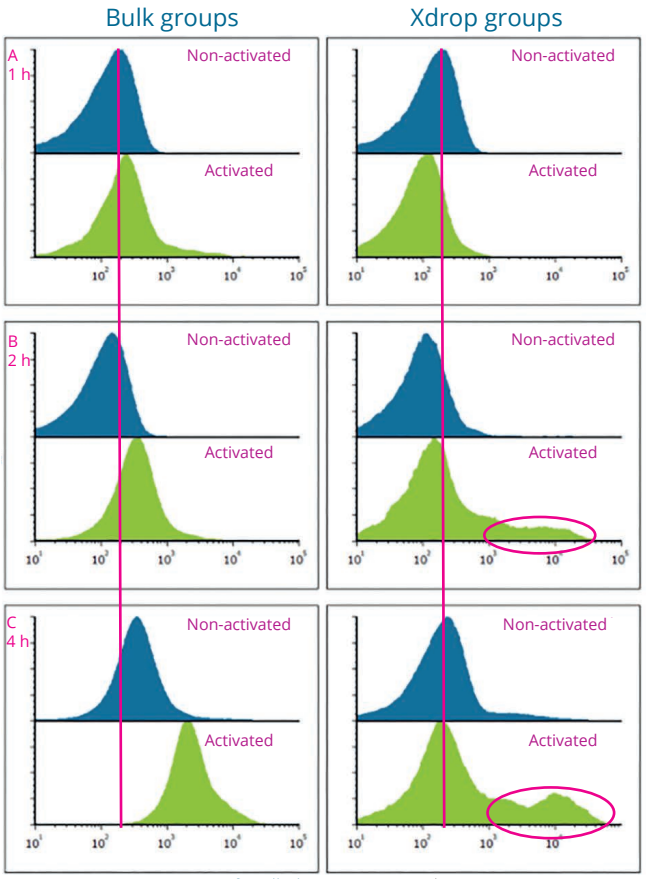


Figure 2. The TNF-α secretion profiles of the T cells from the blood samples. The cells were processed in four groups: activated and non-activated bulk groups; and activated and non-activated Xdrop groups (cells encapsulated in DE50 droplets). **A** The results after 1 hour of incubation. **B** The results after 2 hours of incubation. Note the circled area of the profile, indicating highly potent T cells. **C** The results after 4 hours of incubation. Note the circled area is larger and the peak for the bulk activated group is significantly different.

How Xdrop supports functional assays of mammalian cells with single-cell resolution

Using the Xdrop DE50 Cartridge, Xdrop encapsulates living mammalian cells in highly stable, ~100-picoliter, double-emulsion droplets. This can accelerate assays thanks to the picoliter reaction spaces, which force faster cell–cell interactions or cell secretion buildup. Xdrop processes up to 8 samples in parallel, with ~150,000 single-cell assays generated per sample in just 8 minutes. It is possible to incubate cells within droplets in a CO_2 incubator, analyze single cells or droplets on a flow cytometer, and sort and recover cells for expansion and molecular profiling.

Learn more about Xdrop at samplix.com or contact us at samplix.com/contact.

Notes

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

Quantifying individual T cells or natural killer cells secreting IFN-γ, TNF-α, or both cytokines in a multiplex, single-cell assay with Xdrop®

Mette Juul Jacobsen, Mary Amasia, Christian Hussing, Rikke Stick Højmark, Thomas Mørch Frøsig, and Marie Just Mikkelsen
Samplix ApS, Birkerød, Denmark

Summary

- This Xdrop workflow enables rapid, multiplex quantification of the individual immune cells in a population that are secreting one or more cytokines.
- The results for single-cell format multiplex and singleplex assay concurred and were reproducible.

Introduction

Bulk cell analyses can miss crucial information on the heterogeneity of immune cell populations and the diversity of individual immune cell responses. Within immunotherapy research, there is a clear need for rapid, sensitive, and reliable functional analyses of cells with single-cell resolution to gain these critical insights.[1]

Samplix has developed Xdrop and the Xdrop DE50 Cartridge to encapsulate living mammalian cells in highly stable double-emulsion droplets (DE50 droplets) for single-cell format incubation, flow cytometry, and sorting.

The Xdrop workflow provides the required single-cell view of cytokine levels, because the droplet encapsulation prevents crosstalk between the cells. It allows for the analysis of 150,000 single cells per sample with the option to recover immune cells with the desired functionality for later molecular profiling or further cell expansion.

Furthermore, results are obtained in less than eight hours. This is thanks to the small droplet volumes (~100 pl), which ensure secreted cytokines rapidly reach detectable levels.

Here, we use the Xdrop workflow to encapsulate immune cells in droplets for the quantification of TNF-α and IFN-γ secretion and digital counting of the number of actively secreting cells. We also show that the workflow results are highly reproducible.

Experimental setup

Human natural killer cells (NK cell line) and a population of human peripheral blood mononuclear cells (PBMCs) from a healthy donor were analyzed in parallel.

The NK cells and PBMCs were respectively labeled with IFN-γ and TNF-α capture reagents according to the Miltenyi Biotec® IFN-γ and TNF-α Secretion Assay protocols. The cell populations were divided in two: a non-activated control subpopulation that was not exposed to cytokine stimulants; and an activated population that was exposed to 25 ng/ml cell stimulation cocktail (PMA/Ionomycin), 100 ng/ml IL-2, and 2.5 µg/ml PHA-M. This was earlier shown to activate the NK cells and the T cells among the PMBCs (data not shown).

The control and activated subpopulations of each cell type were then divided into samples of ~460,000 cells (6,700 cells per µl medium) for encapsulation in DE50 droplets together with IFN-γ-APC and TNF-α-PE antibodies.

The cells within the DE droplets were subsequently incubated for 3 hours at 37°C in 5% CO_2. After incubation, all the cells were recovered from the droplets, washed, and stained with LIVE/DEAD Fixable Green Dead Stain (Thermo Fisher Scientific®) to identify living cells. The PBMCs were also stained with CD3-PerCP to identify the T cells within the population. All the cells were then analyzed using a BD Accuri™ flow cytometer.

To verify that the multiplex assay yields comparable results to a singleplex assay, the experiment was repeated for each cell type and each cytokine singly, i.e., with one capture reagent bound to the cells instead of two.

The reproducibility of this Xdrop workflow for cytokine secretion analysis was determined by repeating the same experiments 5 times on 3 different days (Figure 2).

Figure 1 shows the workflow for a single-cell secretion assay.

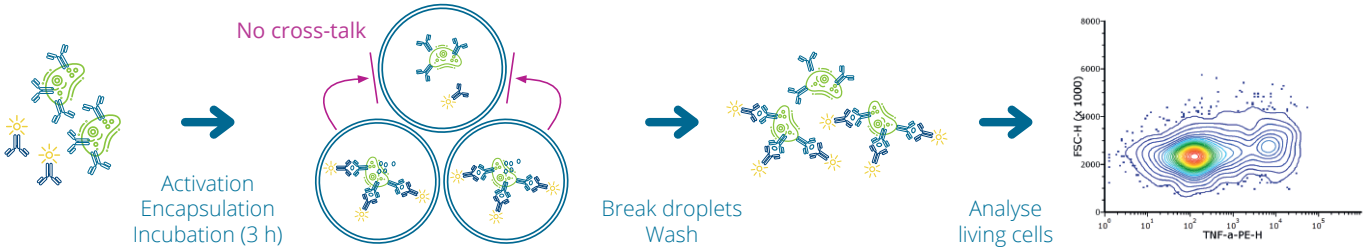


Figure 1. The Xdrop workflow for a single-cell cytokine secretion assay. The whole workflow takes ~6 hours based on the 3 hours of incubation used here. Two to four hours of incubation has been found to be sufficient in other experiments (data not shown).

Highly active TNF-α and IFN-γ secretors are reproducibly detected after 3 hours of incubation in droplets

We retrieved 5 replicates of the TNF-α and IFN-γ secretion profiles from non-activated (control) and activated NK cells and T cells. Figure 2 shows the box plot for the 5 replicates, discriminating between cells secreting TNF-α, IFN-γ, or both of these cytokines.

As expected, non-activated cells display a very low secretion profile, while activated cells show a high profile. The activated T cells mainly secreted TNF-α (45%) and the activated NK cells IFN-γ (82%). Interestingly, almost all the T cells that secreted IFN-γ also secreted TNF-α, and almost all the NK cells that secreted TNF-α also secreted IFN-γ. The standard deviation of <5% illustrates the very high reproducibility.

Figure 3 shows the consistent results for the singleplex and multiplex versions of the droplet-based single-cell assay.

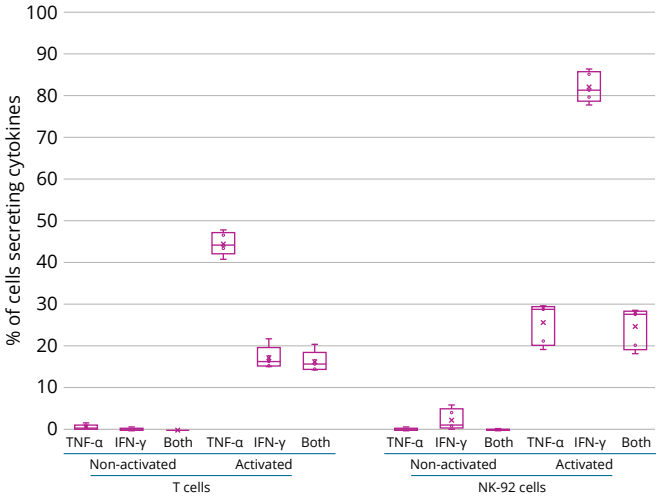


Figure 2. Box plot showing the TNF-α and IFN-γ secretion profiles for non-activated (control) and activated T cells within a PMBC population from a human blood sample and for non-activated (control) and activated NK cells. Data derived from 5 replicates performed on 3 different days.

Conclusion

The Xdrop workflow allows for cytokine secretion profiling with precise measurements at the single-cell level. The results are highly consistent and reproducible, and it is possible to investigate one or more cytokine secretion profiles simultaneously and within one working day.

How Xdrop supports functional assays of mammalian cells in a single-cell format

Using the Xdrop DE50 Cartridge, Xdrop encapsulates living mammalian cells in highly stable, ~100-picoliter, double-emulsion droplets. The picoliter reaction spaces force faster cell secretion buildup, accelerating workflows. Xdrop processes up to 8 samples in parallel, with ~150,000 single-cell assays generated per sample in just 8 minutes. It is possible to incubate cells within droplets in a CO_2 incubator, analyze single cells or droplets on a flow cytometer, and sort and recover cells for expansion and molecular profiling.

Learn more about Xdrop at samplix.com.

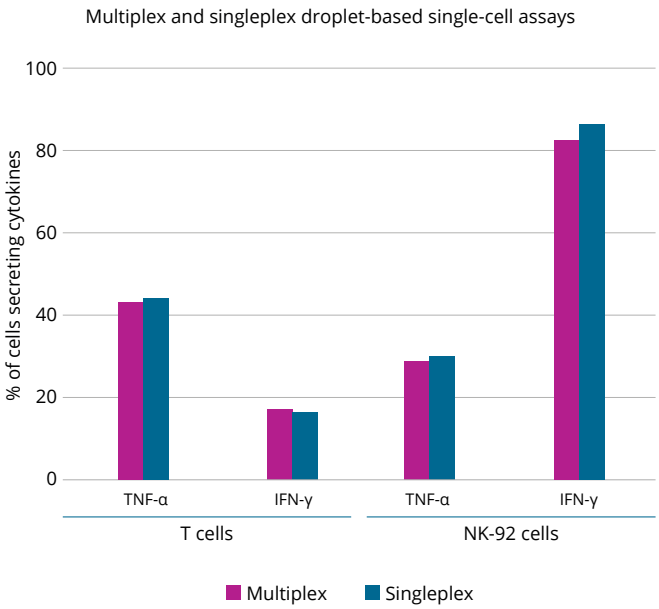


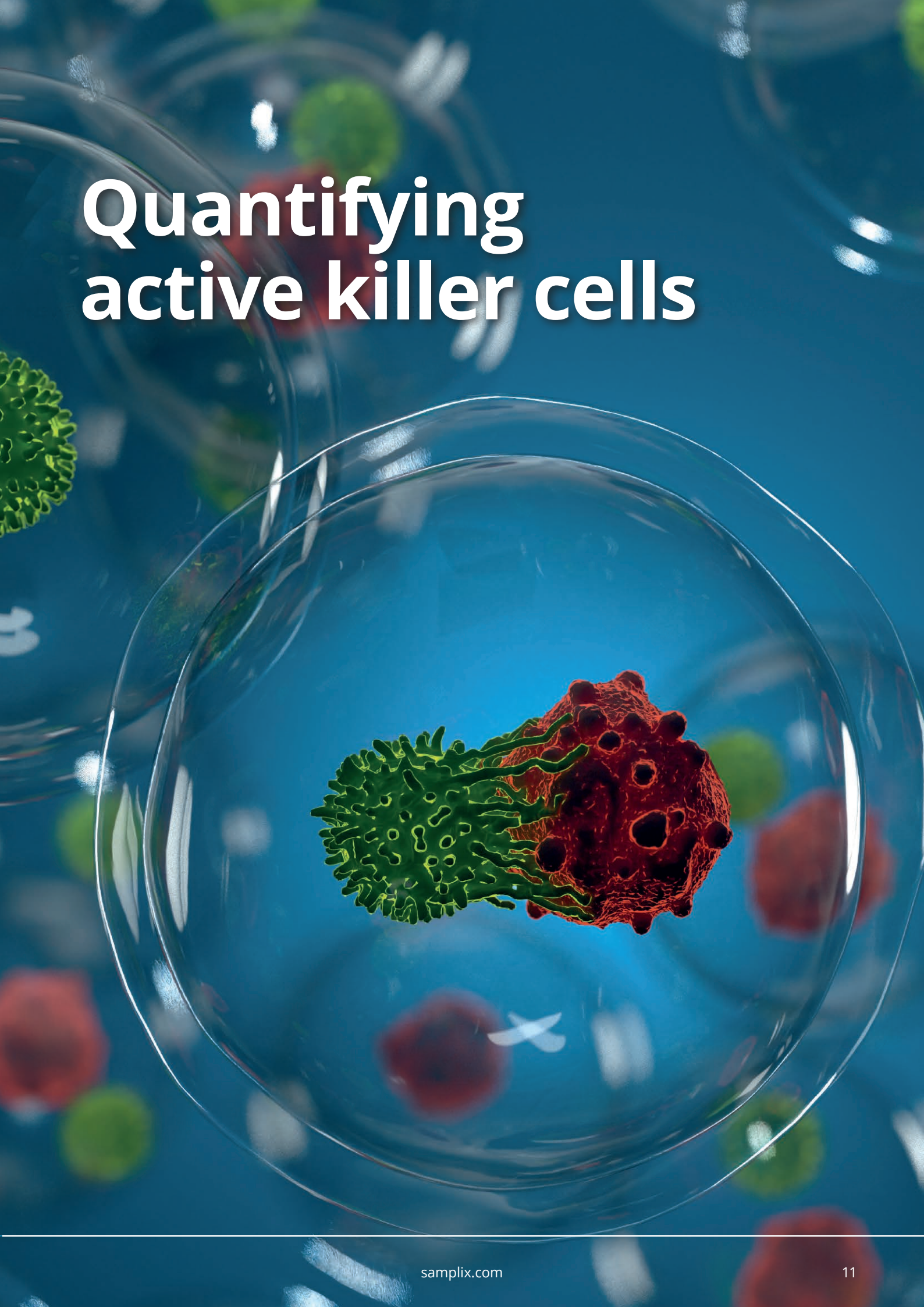
Figure 3. Clustered histogram showing the percentages of NK cells and T cells secreting TNF-α and IFN-γ in multiplex and singleplex versions of the Xdrop workflow.

Notes and references

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

1. European Medicines Agency. 2016. Guideline on potency testing of cell-based immunotherapy medicinal products for the treatment of cancer. Technical update downloaded from www.ema.europa.eu in August 2022

Quantifying active killer cells



Application Note

Immunotherapy Research

Agilent 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.

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.

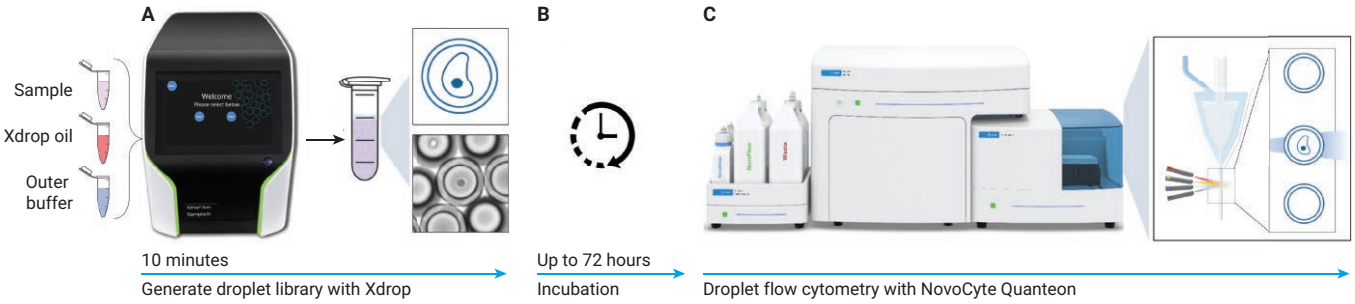


Figure 1. Schematic workflow for cell encapsulation using the Xdrop and flow cytometry analysis using an Agilent NovoCyte Quanteon analyzer. (A) Cells are co-encapsulated in double emulsion droplets from single-cell suspensions in an oil shell and surrounding outer buffer. The Xdrop generates 1 million 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.

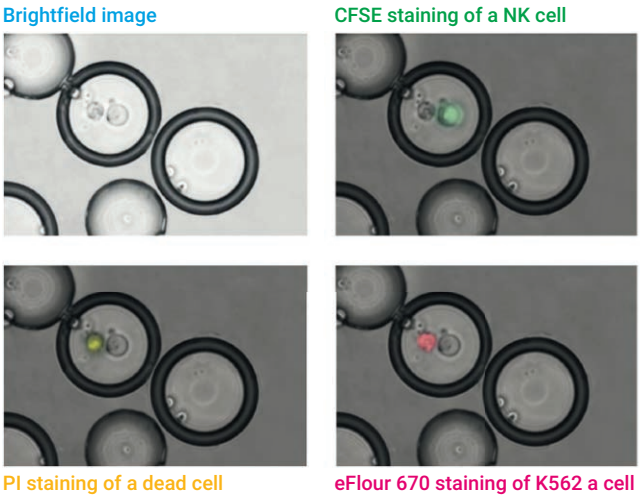


Figure 2. Overlaid brightfield and fluorescence microscopy images showing cells co-encapsulated in a double-emulsion droplet. Green FL represents an NK cell stained with CFSE, and Red FL represents a K562 lymphoblast stained with eFluor 670. Yellow FL indicates a positive PI signal, indicating loss of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

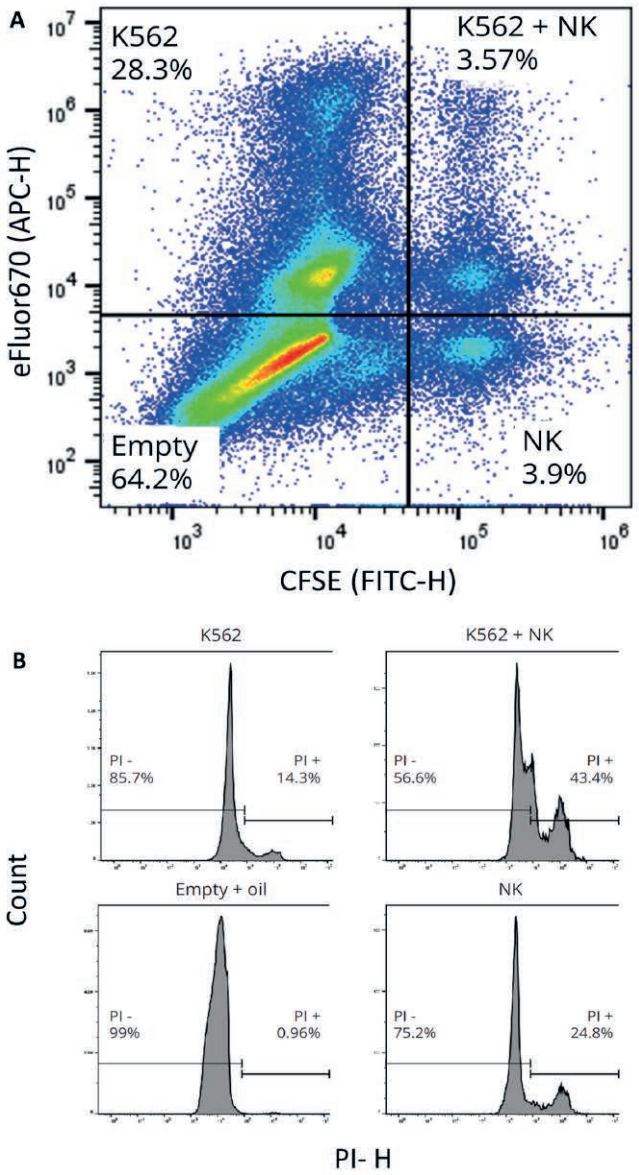


Figure 3. Flow cytometry analysis. (A) Bivariate dot-plot showing eFluor 670 (APC-H) FL plotted against CFSE (FITC-H) FL. From these data four distinct droplet populations were determined: empty droplets (low eFluor 670, low CFSE), droplets with only K562 cells (high eFluor 670, low CFSE), droplets with 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).

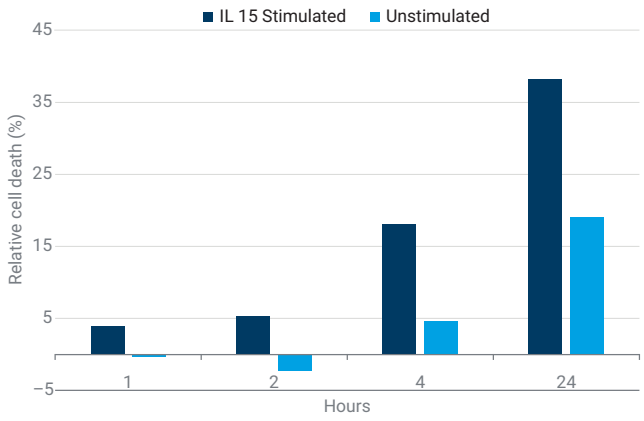


Figure 4. Relative cell death from three experiments ± SEM. K562 cells were co-encapsulated in double-emulsion droplets with IL15-stimulated or unstimulated NK cells. After flow cytometry analysis, relative cell death was determined by subtracting the background death (PI+ signal for droplets with co-encapsulated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

www.agilent.com

RA45205.6532986111

This information is subject to change without notice.

Printed in the USA, November 3, 2023
5994-6863EN

Agilent
Trusted Answers

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.

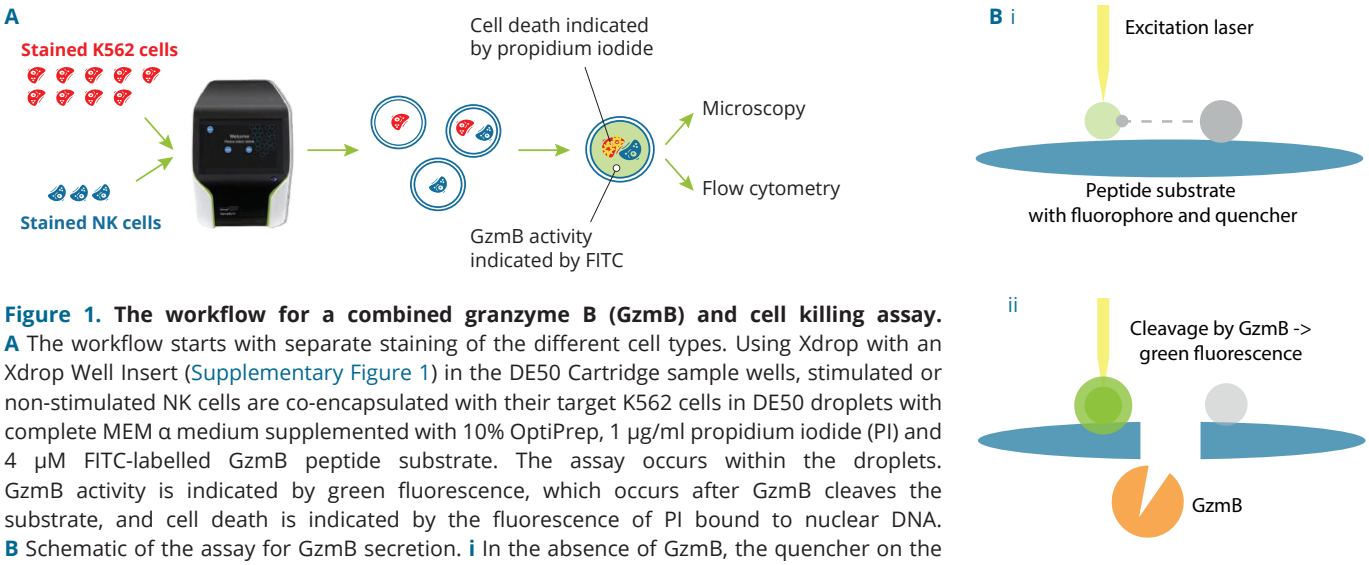


Figure 1. The workflow for a combined granzyme B (GzmB) and cell killing assay. **A** The workflow starts with separate staining of the different cell types. Using Xdrop with an Xdrop Well Insert (Supplementary Figure 1) in the DE50 Cartridge sample wells, stimulated or non-stimulated NK cells are co-encapsulated 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.

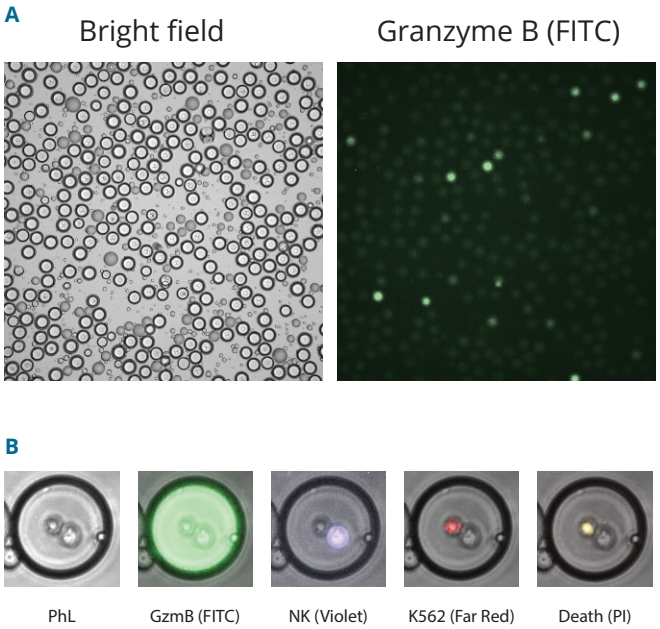


Figure 2. Microscopy images of encapsulated cells after 4 h incubation. A Microscopy images of DE50 droplets with IL15-stimulated NK and K562 cells. A bright FITC signal (green) is observed for droplets with GzmB activity. **B** Microscopy images of a single DE50 droplet containing a GzmB-positive, IL15-stimulated 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.

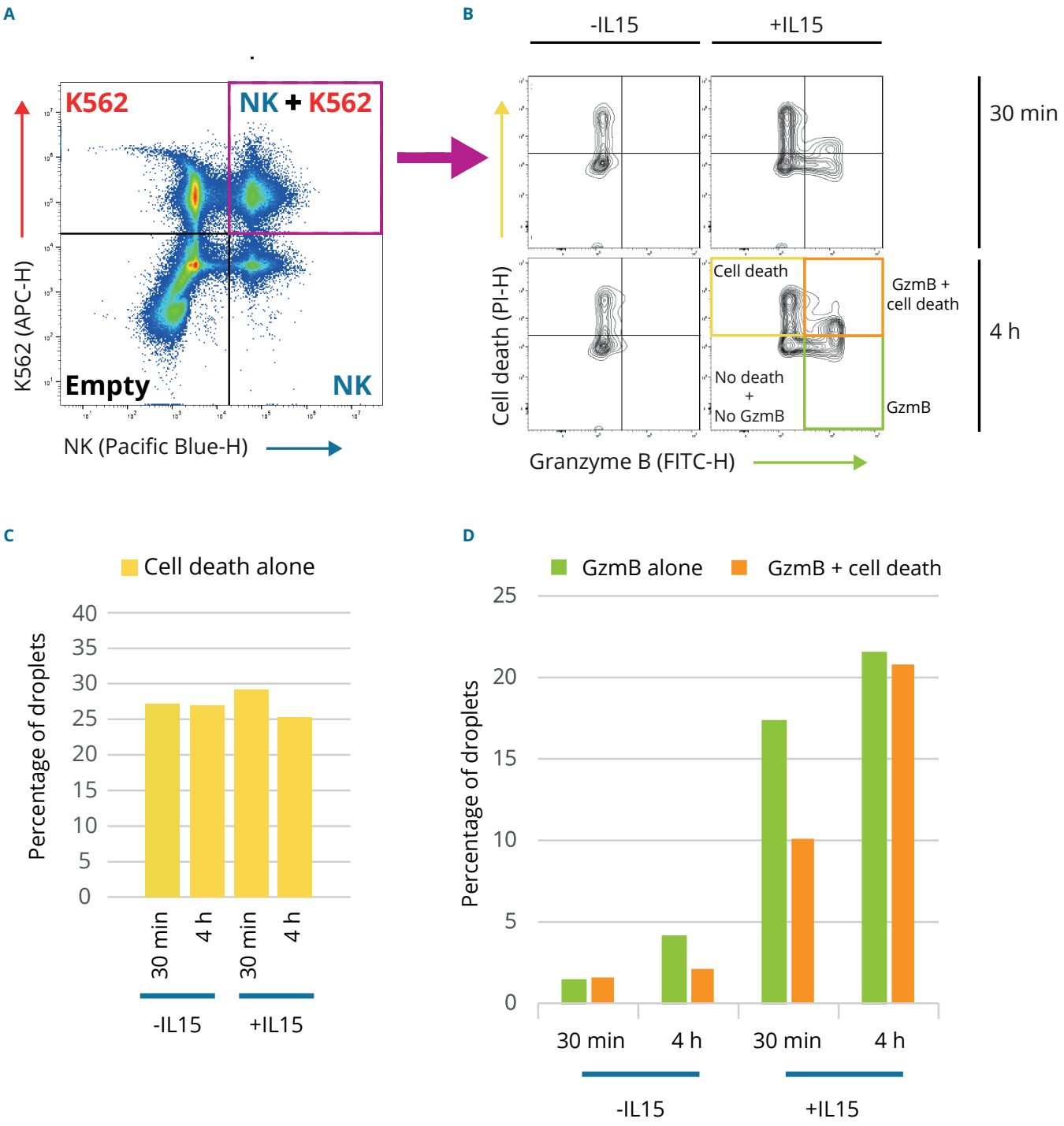


Figure 3. Quantitative double-emulsion flow cytometry analysis of the DE50 droplet-based, combined GzmB and cell killing assay. **A** Density diagram with gated DE50 droplets plotted based on the signal from the two applied cell stains: NK — CellTrace Violet (Pacific Blue-H) and K562 — CellTrace Far 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.

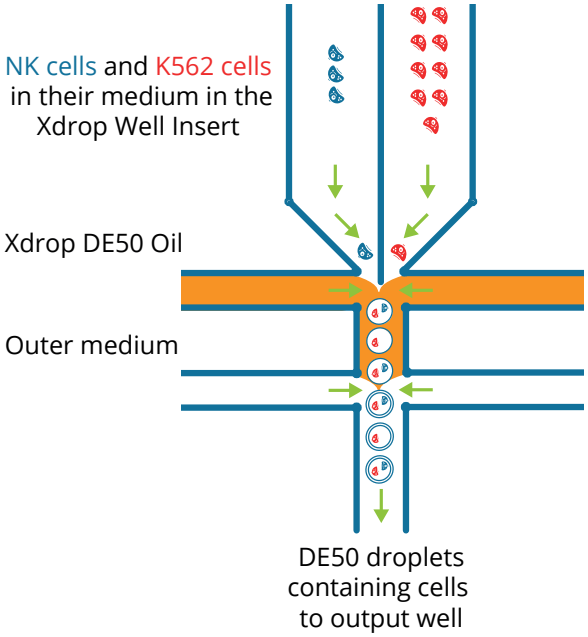
Conclusion

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



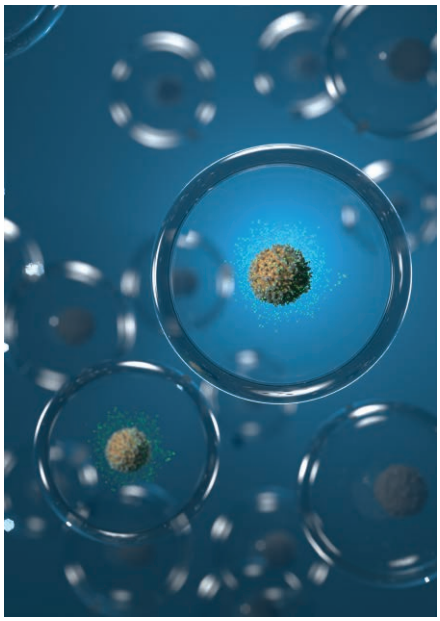
Supplementary Figure 1. Cell co-encapsulation using the Xdrop Well Insert. **A** Photo of an Xdrop Well Insert in the sample input well of an Xdrop DE50 Cartridge. **B** Illustration of the process of co-encapsulation of two cell types (in this case, stained natural killer cells and stained human lymphoblast 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.

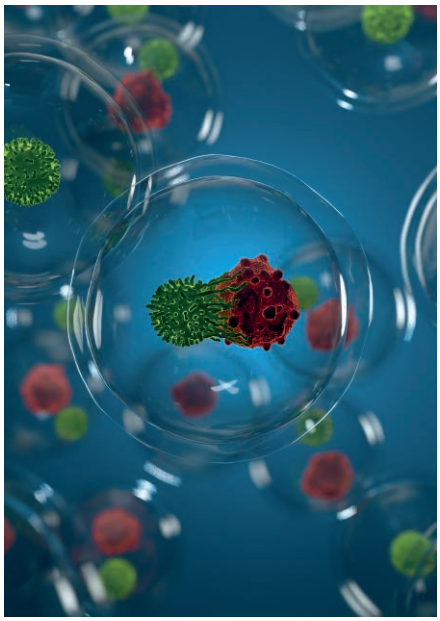
Notes and references

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.

Xdrop prepares immune cells for highly accurate functional analysis





Samplix

Discover more about double-emulsion droplets and book your remote demonstration of Xdrop products at **samplix.com**