Droplet-Based Combinatorial Assay for Cell Cytotoxicity and Cytokine Release Evaluation

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Balancing the maximal efficacy of interferon gamma (IFN-γ)-based therapies with its side effects is a great challenge for future cytokine treatments. To achieve this, the development of single-cell technologies that study IFN-γ release in correlation with antitumor activity would represent a huge step forward. To this end, droplet-based microfluidics is employed to quantitatively investigate IFN-γ secretion from single natural killer (NK) cells in correlation with their cytotoxic activity against a specific target. The method relies on co-encapsulation of NK-92 cells, target cancer cells, polystyrene beads conjugated with specific IFN-γ capture antibodies, and fluorescently labeled detection antibodies inside water-in-oil compartments. The secreted cytokines are captured and detected by localized fluorescence at the periphery of the beads. NK-92’s cytotoxicity is evaluated simultaneously by means of a fluorescent DNA intercalating agent, which penetrates the membranes of dead target cells. To deepen the understanding of the role of the cytokine in antitumor immunomodulation, the impact of different doses of human recombinant IFN-γ on the cytolytic activity of NK-92 cells shows a trend that the higher the dose the lower the cytolytic activity of NK cells. The developed method represents a simple quantitative approach to unravel the complex heterogeneity of NK cells toward IFN-γ secretion and cytolytic activity.

1. Introduction

Interferon gamma (IFN-γ) is a cytokine predominantly produced by natural killer (NK) cells and effector T cells, which is intensively involved in both innate and adaptive immunity. While IFN-γ was originally considered to only immunomodulate responses against bacterial and viral infections, its fundamental role in regulating tumor-related mechanisms was discovered and investigated more recently. IFN-γ has been found to influence both immune cells and tumor cells. For example, it enhances NK cell activation and also shows specific antitumor effects by inhibiting proliferation and inducing cell death (apoptosis- or necrosis-like). Although the employment of IFN-γ is positively associated with antitumor immunity, recent IFN-γ-based anticancer therapy studies have exhibited only limited success. This has mainly been explained by the recently discovered controversial effects of IFN-γ on promoting tumor growth and immunosurveillance escape. In order to maximize the IFN-γ antitumor efficacy and minimize the side effect burden, it is important to determine the optimal dosage for treatment with cytokines. Therefore, quantitative investigations that determine single-cell IFN-γ release and look at how recombinant IFN-γ addition affects the cytolytic activity of immune cells are necessary.

So far, most of the standardized techniques utilized for the measurement of immune cell cytokine release (e.g., enzyme-linked immunosorbent assay (ELISA) or enzyme-linked immunospot (ELISPOT) assays) or immune cell cytotoxic activity (e.g., chromium-release assay or flow cytometry) were employed independently of each other and mainly in bulk environmental conditions. These approaches generally provided...
information on universal and average cell-to-cell behavior outcomes, such as the amount of IFN-γ per volume or the percentage of IFN-γ positive cells, thus disregarding immune cell heterogeneity and possible overlapping effects. Moreover, these methodologies do not allow dynamic data collection from individual cells, thus lacking spatio-temporal resolution.

High-throughput, droplet-based microfluidics allows for single-cell, multiplexed analysis of subsets of cells. Recent advances in the field of microfluidics also make it possible to overcome challenges correlated with standardized assay techniques. The microfluidics platform grants for fast production (kHz) of highly monodisperse surfactant-stabilized water-in-oil droplets, hence generating picoliter compartments functioning as efficient micro-laboratory for manipulation and investigation of cellular behavior at a single-cell level. In the past decade, microfluidic technology has been used to establish separate platforms for measuring single-cell cytokine secretion and single-cell cytotoxicity. Very recently, Yuan et al. utilized droplet-based microfluidics to improve the accuracy of bulk-based cytokine capture assays. They co-encapsulated effector and target cells in picoliter droplets and evaluated IFN-γ release by fluorescence readout on the surface of the effector cells. Sequentially, by means of fluorescence-activated cell sorting (FACS), they determined and separated those effector cells releasing the cytokine of interest. Despite the potential of this approach, the researchers failed to provide quantitative information regarding IFN-γ secretion on the single-cell level.

In a recent study, Zhou and colleagues developed a sophisticated on-chip analysis platform to evaluate single-cell cytokine release and cell–cell interaction in a high-throughput manner. Such a platform would definitely benefit from the possibility of high-throughput manipulation of cells with defined properties. To achieve that, a droplet-based microfluidic approach for the combinatorial investigation of NK cell cytokotoxicity and IFN-γ release on a single cell level has to be developed.

Therefore, in this study, we describe a droplet-based microfluidic approach that enables the combinatorial investigation of IFN-γ release and NK cell cytotoxicity in a sensitive (− fg cell−1), quantitative, and multiplexed way. Toward this end, we co-encapsulated NK-92 cells and KS62 cancer cells with polystyrene beads functionalized with specific IFN-γ capture antibodies and free-floating fluorescently labeled detection antibodies inside surfactant-stabilized water-in-oil droplets. Each droplet, containing these ingredients, represents an individual screening chamber in which cell cytotoxicity and IFN-γ amount can be investigated and correlated in a time-dependent manner. Antibody-functionalized polystyrene beads were employed for the quantitative analysis of IFN-γ secretion and DNA intercalating agents for NK cell cytotoxicity analysis within picoliter droplets. We expect the developed droplet-based assay to provide not only insights into the role of IFN-γ for tumor immunomodulation, but overall to represent a broadly applicable technological tool with the potential to shed light on the intrinsic heterogeneity of NK cells. Here, we chose cell cytotoxicity and IFN-γ secretion as an example for the development of our platform. However, the tunability of the multipurpose technology leaves space for the investigation of other biological scenarios such as ligand repertoire, neoantigen diversity in the case of NK cell–tumor cell interactions, and production of perforin and granzyme B in the case of NK cell cytotoxic activity.

2. Results and Discussion

2.1. Droplet Production and Bead Calibration

To overcome the drawbacks related to state-of-the-art bulk methods for cytokine detection, such as limited spatiotemporal resolution and the necessity of intracellular staining, we set out to establish an assay for the detection of IFN-γ release from NK cells within the picoliter compartment. Moreover, to correlate the secretion of cytokines with the cytokytic capabilities of NK cells, we combined the IFN-γ detection assay with a cytotoxicity assay. Toward this end, we used droplet-based microfluidics to co-encapsulate NK-92 cells (IFN-γ releasing cells), K562 cells as target cancer cells, IFN-γ sensing beads, and a suitable detection antibody inside compartments of roughly 110 pl volume. By means of confocal fluorescence microscopy, we evaluated the percentage of IFN-γ releasing cells over a period of 12 h, estimated the amount of released IFN-γ (down to the detection limit of a few femtograms per cell), and analyzed the NK-92 cells’ cytolytic activity (Figure 1B). The design of the IFN-γ sensing beads (see Experimental Section) was readapted from procedures reported earlier. Briefly, we utilized polystyrene beads and two antibodies with high specificity for IFN-γ: 1) the capture antibody, which was used to immobilize the released IFN-γ on the periphery of the bead; and 2) the detection antibody, a fluorescently labeled antibody that interacted with the immobilized IFN-γ. Notably, capture and detection antibodies have been chosen to interact with two different epitopes of the same antigen (IFN-γ). Upon titration, the capture antibody was immobilized on the surface of the polystyrene beads (Figures 2A,B). Likewise, after titration, fluorescently labeled detection antibodies were used to detect the immobilized cytokines at the periphery of the beads (Figure 2C).

For the calibration of IFN-γ sensing beads, different amounts of IFN-γ (0 to 70 fg droplet−1) were co-encapsulated with the detection antibody and the coated polystyrene beads inside droplets of roughly 110 pl volume. To correlate the fluorescence intensity at the beads’ periphery with the specific IFN-γ amount, 20–54 beads per condition were evaluated via confocal fluorescence microscopy (Figure 2D) and analyzed with Fiji macros by defining a circular region of interest (ROI) at the periphery of each bead, hence quantifying the peripheral fluorescence intensity (for further information see Data Analysis under Experimental Section). Figure 2E shows the calibration curve for the IFN-γ sensing beads. Note, the concentration range of IFN-γ employed for calibration was chosen based on values obtained in previous studies as well as an additional ELISA that we performed (see Figure S1, Supporting Information). Usually, ELISA measurements obtained from half a million of activated NK cells mL−1 showed an IFN-γ release of 1–3 ng mL−1 or higher. Assuming homogeneous cytokine release within the cell population, the average amount released would be in the range of 2 to 6 fg cell−1. To map the heterogeneity of NK cell populations regarding IFN-γ release, we developed a sensor capable of detecting between only a few fg cell−1 up to 70 fg cell−1, covering values equivalent to a tenfold increase of cytokine concentration.
2.2. Detection of NK-92 Cells’ IFN-γ Release Inside Droplets

Following the calibration of IFN-γ sensing beads, we set out to assess their efficiency in detecting IFN-γ released from individual NK cells. Toward this end, we co-encapsulated the beads and the detection antibody with NK-92 and K562 cells inside droplet-based confinements of roughly 60 µm in diameter (see Figure S2, Supporting Information). Following co-encapsulation, confocal fluorescence microscopy was performed at different time points (0.5 h, and after 3, 6, and 12 h) to measure IFN-γ release. The investigated conditions included droplets containing only NK-92 cells (either in an activated or a not activated state) and activated NK-92 cells co-encapsulated with K562 target cancer cells. Droplets containing only beads and K562 cells were used as a control. For each condition, 28–50 beads were characterized. The droplets contained one cell of interest (or two in the case of NK-92 co-encapsulated with K562 cells) and one IFN-γ sensing bead (for details please see Statistical Analysis, Supporting Information). Figure 3A presents a summary of the fluorescence intensity measured at the beads’ peripheries in each droplet and its correlation to the amount of IFN-γ detected per cell. It is important to mention that the sensitivity of the detection is limited to IFN-γ amounts above 7.5 fg cell⁻¹. Fluorescence intensities at the periphery of the beads corresponding to values below 7.5 fg cell⁻¹ are not significantly distinguishable from the background. Notably, in some cases non-homogeneous distribution of IFN-γ over the beads’ periphery was observed.

As expected, soon after co-encapsulation (t = 0.5 h), no detectable amount of IFN-γ was observed for all analyzed conditions. Following 3 h of incubation, IFN-γ release was in the range of 75 to 18 fg cell⁻¹ for the activated NK-92 cells alone and for the activated NK-92 co-encapsulated with K562 cells, with higher IFN-γ values attributed to co-encapsulated cells. The secreted amount of IFN-γ further increased following 6 h of incubation, reaching values ranging from 75 to 85 fg cell⁻¹. Importantly, at this time point, 27% of the single NK-92 cells and 29% of the co-encapsulated NK-92 cells showed detectable amounts of IFN-γ (see Figure 3A, Supporting Information). These observations underline the heterogeneity of the NK cell population in terms of IFN-γ release. After 12 h of incubation, almost half of the screened population of activated NK-92 cells (alone or co-encapsulated with K562 cells) secreted detectable amounts of IFN-γ (see Figure S3A, Supporting Information). Moreover, a tendency to higher values (up to 90 fg cell⁻¹) of secreted IFN-γ can be extrapolated from the data. As expected, only 8% of non-activated NK-92 cells secreted a detectable amount of IFN-γ after 12 h of incubation (see Figure S3A, Supporting Information). Note, for the quantification of the percentages of NK-92 cells secreting detectable amounts of IFN-γ, only droplets containing one NK-92 cell and one bead were considered. Figure 3B shows representative confocal fluorescence images of activated NK-92 cell co-encapsulated with the IFN-γ detection bead at 0.5 h (Figure 3B-i) and after 12 h of incubation (Figure 3B-ii). Due to IFN-γ release, a fluorescent ring at the periphery of the detection bead is visible after 12 h of incubation (Figure 3B-ii). After 12 h of incubation, the number of NK-92 cells secreting cytokine amounts greater than 75 fg cell⁻¹ had increased even further to 49% of activated NK-92 cells and 50% of activated NK-92 cells co-encapsulated with a target cell. Moreover, a tendency to higher values of released IFN-γ can be extrapolated from the data (see Figure S3A, Supporting Information). As expected, very low percentages of a detectable amount of IFN-γ were observed in the droplets containing not activated NK-92 cells (from 6% up to 8% from 6 to 12 h) or only K562 cells (from 2% up to 14% from 6 to 12 h) over time. Notably, up to 6 h, the percentage of NK-92 cells releasing a detectable amount of IFN-γ is lower than the percentage of...
2.3. Combined Evaluation of NK-92 Cells’ IFN-γ Release and Cytolytic Activity

To shed light on the remarkable heterogeneity of NK cell populations regarding IFN-γ release and to better understand the relationship between the amount of released cytokines and the cytotoxic antitumor activity of NK-92 cells, we developed a combinatorial droplet-based assay linking NK-92 IFN-γ secretion with the efficiency of the respective cell to kill a cancer target cell. Toward this end, we performed confocal fluorescence microscopy analysis of droplets containing a combination of NK-92 and K562 cells, and beads for up to 12 h of incubation. At each time point, 28–50 beads were analyzed. Note, the majority of droplets contained one NK-92 cell, one K562 cell, and at least one IFN-γ sensing bead (for details please see Statistical Analysis, Supporting Information). Figure 3C depicts the fluorescence intensity measured on the beads’ periphery inside droplets at different time points as it correlates to the amount of IFN-γ released from cells. To investigate NK-92 cells heterogeneity in terms of IFN-γ secretion and cytotoxicity, we further quantify the percentages of: 1) NK-92 cells that secreted a detectable amount of IFN-γ; 2) cytotoxic NK-92 cells; and 3) cytotoxic NK-92 cells that secrete a detectable amount of IFN-γ (see Figure S3B,C, Supporting Information). As expected, at the beginning of the assay (t = 0.5 h), NK-92 cells produced only small amounts of IFN-γ (≤7.5 fg cell⁻¹), and 33% of the NK-92 cells showed cytolgetic activity against their targets. After 3 h of incubation, about 6% of the NK-92 cells secreted detectable amounts of IFN-γ, ranging between 7.5 to 18 fg cell⁻¹. Nevertheless, out of roughly 47% of the cytotoxic NK-92 cells, only 4% released a detectable amount of IFN-γ. The percentage of NK-92 cells simultaneously exhibiting IFN-γ release and cytolytic activity increased with time, reaching 29% and 53% after 6 and 12 h of incubation, respectively. Note, for the quantification of the percentages of NK-92 cells secreting detectable amount of IFN-γ, only droplets containing one activated NK-92 cell, one K562 cell, and one bead were considered.

Figure 3D shows representative confocal fluorescence images of a NK-92 cell encapsulated with one K562 target cell and one bead, at 0.5 h (Figure 3D-i) and after 6 h of incubation (Figure 3D-ii). Due to IFN-γ release, a fluorescence signal at the periphery of the detection bead can be observed after 5 h of incubation (Figure 3D-ii). All in all, the amount of NK-92 cells killing their target increased from 33% up to 67% over time—results that align with previously performed cytotoxicity assays (data not shown). At the same time, the number of cytotoxic NK-92 cells that also released quantifiable IFN-γ amounts increased from 0 up to roughly 54% (see Figure S3B,C, Supporting Information). Therefore, even though we did not detect a clear-cut IFN-γ threshold concentration that outlines cellular cytotoxicity, we indeed detected a general correlation between an increase of cytotoxicity and higher IFN-γ release during the 12 h of the assay. The viability of NK-92 cells and K562 cells encapsulated within the droplets considered for the evaluation of IFN-γ release was monitored (Figure S4, Supporting Information). Importantly, the mortality of K562 cells solitary confined in droplets remained significantly lower throughout the whole assay in comparison with K562 cells co-encapsulated...
Figure 3. Correlation between NK-92 cells’ IFN-γ release and their cytolytic activity. A) IFN-γ release in droplets at different incubation times (0.5 h and after 3, 6, and 12 h). The fluorescence intensity measured at the periphery of each bead (left Y axis) correlated with the respective amount of IFN-γ released from cells (fg cell⁻¹, right Y axis) is depicted. Activated or not activated NK-92 cells solitarily confined or activated NK-92 cells co-encapsulated
with activated NK-92 cells (see Figure S4B, Supporting Information).

Due to the various effects of IFN-γ on tumor-regulated mechanisms, the effectiveness of future IFN-γ-based therapies may require an optimization of the amount of cytokines used. Toward this end, we utilized our droplet-based microfluidic approach for a quantitative evaluation of NK cell cytotoxicity at different concentrations of added human recombinant IFN-γ (hrIFN-γ). We produced cell-laden droplets containing NK-92 and K562 cells in assay working media containing different amounts of hrIFN-γ: 0, 250, and 480 ng mL\(^{-1}\), which respectively corresponds to roughly 40 or 80 fg droplet\(^{-1}\) considering droplets of \(\approx 60 \mu\)m diameter. Next, we evaluated NK-92 cell cytotoxicity through PI staining and confocal fluorescence microscopy (at 0.5 h, and after 3, 6, and 12 h). For every condition (i.e., \(0, 40, \text{ or } 80 \mu\)g droplet\(^{-1}\) of added hrIFN-γ), \(27\)–\(75\) different droplets containing one NK-92 and one target cell were analyzed. Note, droplet diameters ranged from \(50\)–\(65 \mu\)m. Figure 3E, which shows NK-92 cytotoxicity behavior, reveals an interesting trend. The higher the initial concentration of hrIFN-γ inside the droplets, the lower the overall NK-92 cytolytic activity. In droplets with 480 ng mL\(^{-1}\) (\(\approx 80 \)fg droplet\(^{-1}\)) of added hrIFN-γ, only 42% of NK-92 cells had killed the target cell after 6 h of incubation (down from 61% when no further cytokine had been added), and after 12 h, instead of 66% (without additional cytokine), only 47% had killed their target. From these results, we observed a trend that higher doses of exogenous IFN-γ might trigger a negative feedback loop toward NK cell cytotoxicity, in line with past literature findings. This trend is, however, not statistically significant and would have to be confirmed in follow-up studies before drawing any conclusions.

Potentially, an overall similar conclusion could be drawn from conventional bulk co-culture system. However, droplet-compartmentalization offers the additional potential to evaluate and unravel single-cell information that would have been lost in a bulk assay. Single-cell information contained within droplets could be further exploited by detailed sequential analysis, droplet sorting, and finally cell release toward standard assessments. Therefore, the developed approach provides a more flexible platform toward custom investigation of the particular impact of a single molecule on specific cell–cell interactions. We, therefore, envision that the developed microfluidic method can serve as an efficient analytical tool for quantitative evaluations of IFN-γ-based therapies.

3. Summary and Conclusion

In this study, we developed a microfluidic droplet-based assay for the quantitative combinatorial analysis of NK-92 cells concerning their IFN-γ release and cytotoxic activity against a specific target. Toward this end, antibody-functionalized polystyrene beads were developed as sensors for IFN-γ release, allowing for IFN-γ detection down to a few femtograms per bead. The developed beads and the detection antibodies were co-encapsulated with NK-92 cells and K562 cancer cells inside surfactant-stabilized water-in-oil droplets, thus using each cell-laden droplet as an individual screening chamber for a time-dependent IFN-γ secretion and cytotoxicity investigation. We observed that, at the early stage of the assay (up to 6 h), the number of IFN-γ releasing NK-92 cells is lower (\(\leq 29\%)\) than those cells that do not release detectable amounts of cytokines, hence underlying the heterogeneity of the NK cell population with regard to IFN-γ release. However, at the end of the assay, almost half of the analyzed population of activated NK-92 cells (alone or in combination with K562 cells) released a detectable amount of IFN-γ. In order to gain deeper insights into the role of IFN-γ in anti-tumor modulation, we cross-correlated the cytolytic efficiency of NK cells with their ability to release the cytokine. Although no particular threshold amount of released IFN-γ that triggers NK cell cytotoxicity has been determined, nevertheless, we did detect a correlation between...
increased NK-92 cytotoxicity and greater IFN-γ release after 12 h assay runtime. Moreover, due to possible diverse effects of IFN-γ on tumor-regulated mechanisms, we also investigated the effect of the exogenous addition of hrIFN-γ on the NK-92 cells’ cytolytic behavior. Interestingly, we observed a trend of lower NK-92 cell cytotoxicity in conjunction with exposure to a higher concentration of exogenous IFN-γ. This observation correlates with reports of recent clinical studies on IFN-γ-based therapies that have been of limited success, mainly due to adverse effects of IFN-γ on the tumor-regulated mechanisms, such as the promotion of tumor-growth and immunosurveillance evasion.[1a,3a,5] The outcomes of our study highlighted the importance of balancing the dose of hrIFN-γ for maximizing the efficiency of future IFN-γ-based therapies.

We expect the developed droplet-based assay to represent a broadly applicable technological tool with the potential to shed light on the intrinsic heterogeneity of NK cells under different perspectives. Furthermore, we envision this combinatorial platform to pave the way for hitherto challenging approaches toward cell screening for fundamental studies and clinical applications. These include cytokine- and cell-based immunotherapy as well as all cell analyses where collective cell phenomena are critical for a macroscopic read-out, such as in cancer, embryo development, and wound healing research. However, to advance this assay further, the developed approach must be integrated into state-of-the-art microfluidic manipulation and sorting techniques.

4. Experimental Section

Materials: SPHERO Protein G Coated Particles (6.8 µm) were purchased from SpheroTech (SpheroTech, Inc., USA). Purified anti-human IFN-γ antibody (NIB42) and Alexa Fluor 488 anti-human IFN-γ antibody (45.B3) were bought from BioLegend (BioLegend, Inc., USA). Human recombinant IFN-γ protein was purchased from Invitrogen (Invitrogen Corporation, USA). TWEEN20 was purchased from Sigma-Aldrich (Merck KGaA, Germany), and 008-FluoroSurfactant from RAN Biotechnologies (RAN Biotechnologies, Inc., USA). 3M Novex 7500 engineered fluid was acquired from 3M (3M, USA). Heat inactivated fetal bovine serum (h.f. FBS), heat-inactivated horse serum (h.i. HS), Gibco GlutaMAX (100x), Gibco 2-mercaptoethanol, sodium pyruvate (100 mM), Molecular Probes CellTracker Blue CMAC Dye, RPMI 1640 Medium, propidium iodide (PI), CO2 independent media, and SwitchingBlock (TBS) Blocking Buffer were purchased from Thermo Fisher Scientific (TFS, Germany). Interleukin-2, human recombinant (hrIL-2), Minimum Essential Medium Eagle—Alpha Modification (xMEM) and Phorbol-12-myristate-13-acetate (PMA) were purchased from Merck (Merck KGaA, Germany). Iscove’s Modified Dulbecco’s Medium (IMDM) was purchased from ATCC (ATCC, Germany). Ionomycin was bought from STEMCELL Technologies.

Cell Lines: NK-92 cells were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Germany) and cultured in xMEM medium enriched with 10% v/v h.f. FBS, 10% v/v h.i. HS, 2 mM L-glutamate, 1 mM sodium pyruvate, 100 µM 2-mercaptoethanol, and 10 ng mL−1 IL-2. Chronic myelogenous leukemia cells K562 were purchased from ATCC and cultured in IMDM supplemented with 10% v/v FBS. All cell lines were cultured in suspension at 37 °C and 5% CO2 following the manufacturer’s recommendations. When necessary (in the condition of “NK-92–activated” and “NK-92 + K562”), NK-92 cells were activated by adding in the encapsulation media 50 ng mL−1 of phorbol-12-myristate-13-acetate (PMA) and 5 µg mL−1 ionomycin. For recognition purposes, K562 cells were stained with CellTracker Blue (Thermo Fisher Scientific, Germany) following the manufacturer’s protocol.

Preparation of Polystyrene Bead-Based IFN-γ Sensors: For bead preparation, previously published procedures were adapted.[10] Briefly, for IFN-γ detection, Protein G-coated polystyrene beads, purified anti-human IFN-γ antibody (NIB42) as the capture antibody, and Alexa Fluor 488 anti-human IFN-γ antibody (45.B3) as the detection antibody were employed. Following the manufacturer’s (BioLegend) recommendation, antibody clones NIB42 and 45.B3 were chosen in conjunction. Due to their high affinity toward two separate epitopes on native human IFN-γ, these antibodies have previously been used in ELISA sandwich assays.[16] Immobilization of the antibodies on the bead surface is based on the interaction between Protein G and the antibodies’Fc region.[17]

Capture and detection antibodies were titrated on the beads’ surface, followed by bead calibration with defined amounts of IFN-γ. In order to quantify the fluorescence-based sensitivity of the sensor, investigating the saturation of the beads’ surface by titrating the fluorescently labeled detection antibody on the surface of the beads was commenced (Figure 2A). Toward this end, 7.25 × 106 beads mL−1 were resuspended in PBS containing 0.1% Tween 20 and different concentrations of fluorescently labeled detection antibody ranging from 0 to 6 µg mL−1. To allow sufficient time for the detection antibody to bind to the bead surface, the solutions were incubated for 20 min at room temperature (RT) while shaking. After washing with PBS (to remove unspecific binding of antibodies), the coated particles were resuspended in the assay-working medium (enriched with 2 mM L-glutamate, 1 mM sodium pyruvate, 100 µM 2-mercaptoethanol, and 10 ng mL−1 IL-2) and 48–103 beads per condition were imaged in order to evaluate the fluorescence intensity at their periphery (Figure S5A, Supporting Information). Based on these experiments, a concentration of 4 µg mL−1 detection antibody was chosen for further experiments. To optimize the sensitivity capacity of the beads, the proper amount of capture antibody required for full coverage of the bead periphery was defined (Figure 2B). 7.25 × 106 beads mL−1 were resuspended and incubated in PBS containing 0.1% Tween 20 and different concentrations of capture antibody ranging from 0 to 50 µg mL−1. Next, PBS was replaced by cell medium and the beads were further incubated for 60 min at RT in cell medium containing 4 µg mL−1 of detection antibody. Please note that the incubation with the detection antibody was done as a control step in order to exclude the possibility of incomplete coverage with a capture antibody. Based on this analysis (77–117 beads per condition, Figure S5B, Supporting Information), a concentration of 35 µg mL−1 of capture antibody was chosen for further experiments.

It is important to mention here that commercially available IFN-γ sensing beads[18] were not used in research due to several reasons. Most of the commercially available beads were designed and calibrated for bulk cell culture assays with an IFN-γ sensitivity in the range of pg mL−1. Moreover, only partial information was provided by the company concerning antibody capture and detection. Last, but not least, some of the commercial beads rely on detection methods that are either based on luminescence or on a more complex strategy, such as biotinylated detection antibodies visualized by a streptavidin-conjugated fluorophore.

Calibration of IFN-γ Sensing Beads: Based on the above-described titration experiments, 35 µg mL−1 of capture antibody and 4 µg mL−1 of detection antibody were chosen for further experiments. Beads were prepared for calibration as follows (Figure 2C): 7.25 × 106 beads mL−1 were resuspended in 0.1% Tween 20 in PBS containing 35 µg mL−1 capture antibody and incubated for 20 min at room temperature (RT) under shaking. After washing with PBS, the coated beads were centrifugated for 2 min at 6000 rpm (Labnet C1301-B), and uncoated, free surface areas (i.e., the areas between Protein G spots) were blocked with TBS Blocking Buffer for 30 min at RT. In order to calibrate the IFN-γ sensing beads, fluorescently labeled detection antibodies (4 µg mL−1), 3 × 10−6 M−1 of polystyrene beads coated with capture antibody, and defined amounts of IFN-γ (ranging from 0 to 70 fg droplet−1) were co-encapsulated in droplets of roughly 60 µm in diameter (~113 pl volume). After 3 h of incubation, the fluorescence

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intensity at the periphery of the beads (20–54 beads measured for every IFN-γ amount tested) was detected by confocal fluorescence microscopy (Figure 2D) and analyzed using Fiji macros (see Data Analysis under Experimental Section) in order to correlate the beads’ periphery fluorescence intensity with the corresponding amount of IFN-γ (Figure 2E). To this end, linear regression analysis (see Data Analysis under Experimental Section) was performed commencing from the data point at which the fluorescence at the bead periphery became distinguishable from the background.

Microfluidic Chip Design and Fabrication: The polydimethylsiloxane (PDMS-Sylgard 184 Elastomer Kit, Dow Corning) microfluidic device employed for droplet production and cell/bead encapsulation was produced following previously described photo and soft lithography protocols.[20,21] The water inlet of the chip was connected to a spiral channel used to separate suspended beads/cells in the aqueous phase prior to droplet generation at the T-junction (the channel width is 80 μm) (Figure S5, Supporting Information). The device was used to produce droplets with a diameter of roughly 50–70 μm.

Encapsulation of IFN-γ Sensing Beads and Cells in Droplet-Based Confinements: Stable droplets of roughly 60 μm in diameter were produced using 3% 008-FluoroSurfactant in 3M Novec7500 fluorinated fluid as the oil phase and NK-92 cells (2.5 × 10^6 mL^−1), fluorescently labeled K562 cells (2.5 × 10^6 mL^−1), IFN-γ sensing beads (3 × 10^6 mL^−1), fluorescently labeled detection antibody, and PI (1 μg mL^−1) suspended in CO2 independent medium (enriched with 2 mM l-glutamate, 1 mM sodium pyruvate, 100 μM 2-mercaptoethanol, 10 ng mL^−1 IL-2, and PMA/Iono when required) as the aqueous phase. Aqueous and oil phases were injected into the microfluidic PDMS-based device through polytetrafluoroethylene (PTFE) tubes (0.4–0.9 mm, Bola, Germany), using a flow controller system (ELVEFLOW Pressure Controller OBI MK3). A high-speed camera (Phantom 7.2, Vision Research, USA) was used to follow droplet production quality.

Droplet-Based IFN-γ Release Evaluation: In order to evaluate IFN-γ release from NK-92 cells within droplets compartment, 3 × 10^6 mL^−1 of IFN-γ sensing beads coated with capture antibody were co-encapsulated together with fluorescently labeled detection antibody and 5 × 10^6 mL^−1 of the cells of interest (either activated or not activated NK-92 cells, a combination of activated NK-92 cells with K562 target cancer cells, or only K562 cells as a negative control) inside droplets of roughly 60 μm in diameter. Cell-laden droplets were produced, collected within a microtube (Eppendorf), and stored in a cell incubator (5% CO2, 37 °C) in CO2 independent medium (enriched with 2 mM l-glutamate, 1 mM sodium pyruvate, 100 μM 2-mercaptoethanol, 10 ng mL^−1 IL-2, and PMA/Iono when required) as the aqueous phase. Aqueous and oil phases were injected into the microfluidic PDMS-based device through polytetrafluoroethylene (PTFE) tubes (0.4–0.9 mm, Bola, Germany), using a flow controller system (ELVEFLOW Pressure Controller OBI MK3). A high-speed camera (Phantom 7.2, Vision Research, USA) was used to follow droplet production quality.

Confocal Fluorescence Microscopy: In order to evaluate the dimension and monodispersity of the droplets, an LSM 800 confocal microscope (Carl Zeiss AG, Germany) with a 20x objective (Plan-Apochromat 20×/0.8 M27, Carl Zeiss AG, Germany) was used. To investigate IFN-γ release and cytotoxicity, droplets were sealed in a self-made glass-based observation chamber.[21] Unless stated otherwise, fluorescence and bright-field microscopy images of beads- and cell-laden droplets were acquired using a 63× oil-immersion objective (Plan-Apochromat 63×/1.4 Oil M27, Carl Zeiss AG, Germany) at different time points over 12 h (at 0.5 h and after 3, 6, and 12 h) with a resolution of 1024 × 1024 pixels. The time point 0.5 h indicates the beginning of the microscopy image acquisition, coinciding roughly with 30 min after production of cell/bead laden droplets. For the evaluation of NK-92 cells cytotoxicity in the case of different concentrations of hIFN-γ (Figure 3E, for gray and white bars), a 20x objective (Plan-Apochromat 20×/0.8 M27, Carl Zeiss AG, Germany) was used. Fluorophores were excited with a diode laser (λex = 405 nm for Cell Tracker Blue and λex = 488 nm for PI and detection antibody) and detected at intervals between 490 and 525 nm, 535 and 617 nm, and 353 and 466 nm for Alexa Fluor 488 (IFN-γ), PI (dead cells), and Cell Tracker Blue (K562 cells), respectively. The pinhole was set to 1 Airy unit for Alexa Fluor 488 emission and 200 Airy units for PI and CTB emission.

Data Analysis: Image processing and analysis (e.g., fluorescence intensity of the beads, target cell death, and droplet monodispersity) were performed using the open-source platform ImageJ. Briefly, fluorescence intensities at the periphery of beads were quantified with two Fiji macros. First off, a Fiji macro was used to recognize every bead’s periphery as a circular ROI. Toward this end, a Fiji’s built-in function for automated particle recognition was employed, requiring binarization (thresholding) of the image. As a control step, an additional user-manual optimization of the ROI selection was possible. Afterward, the ROI was stored as a backup for the accomplished data. At this point, a second macro was used to quantify every bead’s peripheral fluorescence intensity for further statistical processing. First, the macro opened the saved ROIs and 100 evenly spaced lines, orthogonal to the bead’s periphery, were drawn for statistical purposes (see Figure S7, Supporting Information). Next, Gaussian curves were fitted to the fluorescence intensity along every single line using a built-in curve fitting function. Every curve’s maximum value was saved externally as a backup for possible retracement and statistical processing and/or proof of significance. Gaussian curve fitting was used to not only consider the airy disks of confocal images but, in combination with the orthogonally drawn lines, it solved the problem of finding the exact position of the periphery as well. If the peak intensities of the fitted curves matched certain intervals, they were stored in a separate file. Afterward, all the saved peak values per bead were averaged. Further correlation between fluorescence intensity and IFN-γ concentration required linear regression performed using a custom-written Python script via non-linear least-square fit. Averaging the peaks’ values was done based on the assumption that a single bead is uniformly covered with detection antibody and thus emits the same fluorescence intensity at every point. A custom-written Python script was also used for the statistical analysis of the other experiments. For the titation curves (Figure S5, Supporting Information) and the calibration curve (Figure 2E), for each condition, all mean values per bead were averaged. As an error estimate, the standard deviation of the mean value was used. Furthermore, linear regression was performed using a non-linear least-square minimization inside the curve fit function of Python’s scipy package. Because statistical independence of the cells and the corresponding signal from the sensing beads can be assumed based on the heterogeneity of the cells, in all cell experiments, averaged results for 8 beads from a single droplet per bead were depicted without any further averaging (Figure 3A,C). Detailed information for experiments depicted in Figures 2E and 3A,C,E and Figure S4B, Supporting Information, concerning: 1) number of beads/droplets/cells analyzed per condition, 2) error bars (when applicable), and 3) statistical analysis performed (where possible) can be found in Statistical Analysis, Supporting Information. GraphPad Prism software (GraphPad Software Inc, USA) was used for graph depictions and calculation of statistical analysis.
Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

cytotoxicity assay, droplet-based microfluidics, interferon-gamma, natural killer cells, single-cell analysis

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