

Probing cellular heterogeneity in cytokine-secreting immune cells using droplet-based microfluidics†

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Here, we present a platform to detect cytokine (IL-2, IFN- γ , TNF- α) secretion of single, activated T-cells in droplets over time. We use a novel droplet-based microfluidic approach to encapsulate cells in monodisperse agarose droplets together with functionalized cytokine-capture beads for subsequent binding and detection of secreted cytokines from single cells. This method allows high-throughput detection of cellular heterogeneity and maps subsets within cell populations with specific functions.

Our current understanding of cellular responses to external stimuli has generally been obtained using bulk assays on populations of cells. These studies hide the response of individual cells, which do not necessarily reflect the bulk behavior.^{1,2} This lack of insight into cellular behavior at the single-cell level is particularly important in immunology. This notion was recently supported by Shalek *et al.* who demonstrated a widespread bimodality in the individual transcriptional response of murine dendritic cells to LPS stimulation.³ Another study demonstrated that the activation of NF- κ B is heterogeneous and is a digital process at the single-cell level,⁴ in contrast to what was found in bulk assays.⁵ Our immune system comprises a wide variety of cells all with their specific function; among these are the CD8⁺ cytotoxic T cells, regarded as the ‘soldiers’ of our immune system that lead the charge against tumors and viruses. It has been shown that the killer T cell responses and disease control depend on the quality rather than on the quantity of these killer T cells.⁶

The quality of killer T cells is defined by multiple functions: killing, proliferation, migration and cytokine release. This implies that out of the complete population of activated killer T cells, only designated subsets may actually be important for immunity. Understanding how these multifunctional responses are induced and exerted by individual cells will provide pivotal information on how vaccines based on killer T cells can be tuned and improved to facilitate superior immune protection. Therefore, we need new technologies to identify, quantify and probe these cellular sub-populations.

In recent years, microfluidic tools have emerged that allow the study of hundreds to a few thousands of individual cells under precisely defined conditions.^{7–9} These techniques have contributed greatly to our quantitative understanding of cellular heterogeneity and temporal variations in cellular behaviour. However, the study of individual cells in fluidic devices also presents a number of challenges, as i) many cells cannot be cultured easily on-chip,¹⁰ ii) the total number of cells is limited by the size of the chip,¹¹ iii) cellular behaviour might be different from other culture platforms,¹² and iv) the technology often requires a large investment in infrastructure. For our studies in immunology, the most important drawback is the difficulty of studying the secretion of molecules from individual cells using these advanced fluidic tools.¹³ Flow cytometry is the standard tool in immunology for high-throughput detection, sorting and collection of cells, but it is less well suited to studying secreted molecules. Cytokine secretion is inferred from intracellular staining of fixed individual cells, which merely indicates intracellular pools of cytokines but not actual secretion. Furthermore, the technique does not allow data collection over time at the individual cell level, precluding dynamic monitoring of individual cell functions. Droplet microfluidics enables the manipulation of discrete fluidic packets in the form of picolitre droplets and addresses the need for lower costs, higher throughput and higher sensitivities at which the assays can be performed. The technique is well adapted to perform

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operations and manipulations in series, like encapsulation and screening.^{14,15} Water-in-oil droplets have been used to compartmentalize individual cells and to study antibody production by single cells dating back to the 1950s,¹⁶ and recent advances in droplet microfluidics have greatly expanded their potential for single cell studies.^{7,14,17–21} Indeed, these picolitre droplets can be considered ‘nanolabs’ that accommodate both single cells and all factors secreted by that cell.^{7,22–24} In this paper, we present a droplet-based microfluidic strategy to encapsulate cells and cytokine-capture beads in monodisperse agarose droplets for detecting cytokine secretion, phenotypical maturation and proliferation of single, activated Jurkat T cells in droplets over time.

Fig. 1 shows the schematic overview of our high-throughput microfluidic single-cell cytokine detection method. Two streams of dispersed aqueous phases of 1.8 w/v% ultra-low temperature gelling agarose (type IX-A, Sigma Aldrich) are mixed prior to the droplet formation junction using a PDMS chip (see the ESI† for device fabrication). One stream contains Jurkat T cells ($4 \times 10^6 \text{ mL}^{-1}$) in culture medium and stimulated with ionomycin ($0.2 \mu\text{g mL}^{-1}$) and phorbol myristate acetate (PMA; $1 \mu\text{g mL}^{-1}$). The other stream delivers polystyrene capture beads (500 nm, 2.422×10^{10} microspheres per mL, Bang Labs) functionalized with biotinylated mAbs specific for IL-2, TNF- α , or IFN- γ . Automated syringe pumps (Cetoni GmbH) controlled the flow rates inside the microfluidic device which was maintained above room temperature for a steady and uniform droplet production at the flow-focusing region of the device²⁵ (69 pL; variance < 1%) at a production frequency of 450 droplets per s (Fig. 1 and S1, ESI†). Droplets are stabilized using a biocompatible Krytox®-based tri-block copolymer surfactant.²⁶ Thereafter, the emulsion

was collected at the outlet of the channel and incubated for 18–24 h at 37 °C in a humidified atmosphere (Fig. 1). During incubation, single activated Jurkat T cells secreted cytokines that bind to functionalized capture beads confined within the same droplet. After incubation, the emulsion was cooled to 4 °C. Cooling the ultra-low gelling agarose containing droplets induces the formation of strong spherical gel beads which were stable and remained solid at room temperature. They were separated from oil by breaking the emulsion and subsequently carefully washed (see the ESI† for details). The mechanical strength of the agarose beads allows centrifugation during the washing process which helped flexible downstream processes. After solidification and removal of the oil phase, the beads were incubated with fluorescently labelled antibodies against IL-2, TNF- α , and IFN- γ , and the intensity of staining of the beads was measured by flow cytometry.

In accordance with previous studies, the process of encapsulating cells inside droplets is random and follows a Poisson distribution.^{7,9,27} To obtain the highest single-cell encapsulation efficiency, we used 2×10^6 and 4×10^6 cells per mL at the droplet production junction. Analysis revealed an encapsulation efficiency of approximately 11 and 19%, respectively (Poisson: $\lambda = 0.11$ and 0.23) (Fig. S2A, ESI†). The fraction of double- and triple-cell encapsulation depends on starting cell concentration and was tolerable and generally less than 1.5% and 0.5%. Analysis of all steps from production to analysis revealed that there is no specific loss of cell-containing droplets (Fig. S2B, ESI†). Next, we assessed the ability of Jurkat T cells to proliferate in picolitre droplets up to 48 hours after production. After production, the large majority of cell-containing droplets contained one cell (80%) and only less

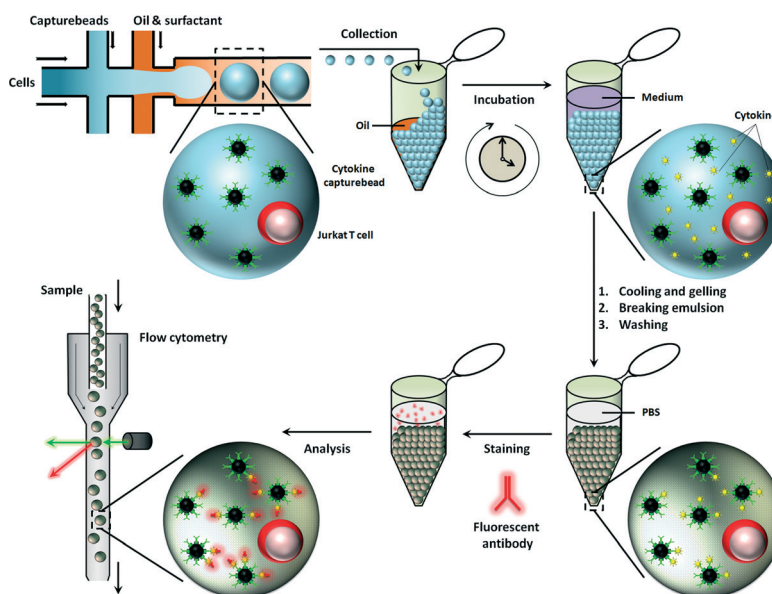


Fig. 1 Schematic overview of the work flow of the droplet-based microfluidic method for single-cell encapsulation and detection of secreted cytokines. Single Jurkat T cells are encapsulated in agarose-gel droplets together with functionalized capture beads. After collection and during incubation, Jurkat cells secrete cytokines that bind to the capture beads confined within the same droplet. The droplets are gelled and washed to break the emulsion. The agarose beads are incubated with fluorescently labeled detection antibodies that bind to the cytokines. Subsequently, the secretion of cytokines by single cells and the signal intensity in the beads are quantified by flow cytometry.

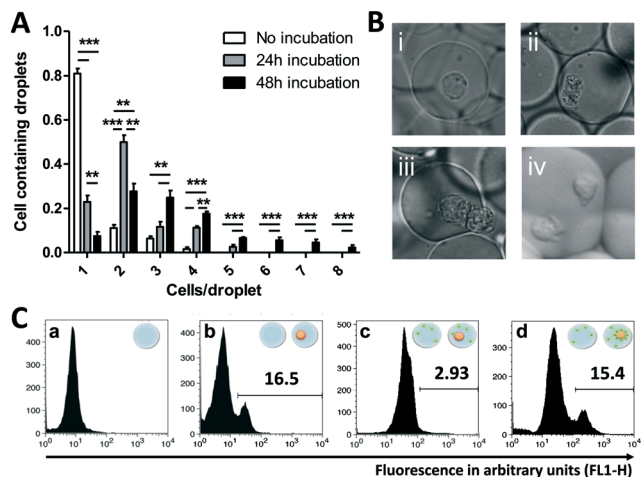


Fig. 2 A) Cell survival during incubation in droplets: an increase in fraction of droplets containing two cells or more can be measured after 24 hours. Up to 48 hours of incubation, further expansion of this fraction can be found. B) Different stages in the proliferation of Jurkat cells were observed with the droplets: i) single cells, ii) late anaphase, iii) telophase, iv) two cells. C) Detection of T cell co-receptor CD3 molecules expressed on the cell membrane: a) Empty agarose beads are not fluorescent. b) Encapsulated Jurkat cells show auto fluorescence at low intensity (16.5%). c) Incubation of agarose beads with isotype antibodies leads to weak staining of the empty and occupied agarose beads (2.93%). d) Treatment with anti-CD3 antibodies leads to specific staining of Jurkat cells expressing TCR co-receptor CD3 (15.4%).

than 20% contained two or more cells (Fig. 2A). The percentage of one-cell containing droplets significantly decreased after 24 hours of incubation, and at the same time, we observed a significant increase in the number of droplets containing

two cells (Fig. 2A). These data demonstrate that picolitre droplets contain sufficient nutrients and space for Jurkat T cells to divide in. This notion is underscored by the observation that various stages of cell division of Jurkat T cells are present in droplets (Fig. 2B). Bio-compatibility was achieved using fluorinated oil (HFE 7500, 3M) that has good solubility for gases^{28,29} together with a custom-made biocompatible surfactant^{26,30} (further described in the ESI†). The proliferation of Jurkat T cells provides evidence for cell viability and biocompatibility of the surfactant and shows that the encapsulated cells are not negatively impacted by compartmentalization-specific factors such as the oil and surfactant.

The gelation of agarose droplets into agarose gel beads upon cooling to 4 °C allowed us to study both the phenotype and the secretome of individual cells. An agarose droplet gelation method was developed for single-molecule emulsion PCR methods.^{9,31} In our case, gel beads were first incubated with fluorescently labeled anti-CD3 or control antibodies to detect molecules expressed on the plasma membrane of cells. Control samples demonstrated that cell-containing droplets can readily be detected based on their auto-fluorescence (Fig. 2C). In accordance with bulk experiments, we found that all single-encapsulated Jurkat T cells expressed the T cell co-receptor CD3 (Fig. 2C). Next, cytokine secretion was studied in gel beads containing stimulated Jurkat T cells and cytokine-capture beads and compared to gel beads containing non-stimulated Jurkat T cells. Although Jurkat T cells are known to constitutively produce low levels of IL-2, the secretion can be largely enhanced upon stimulation.³²

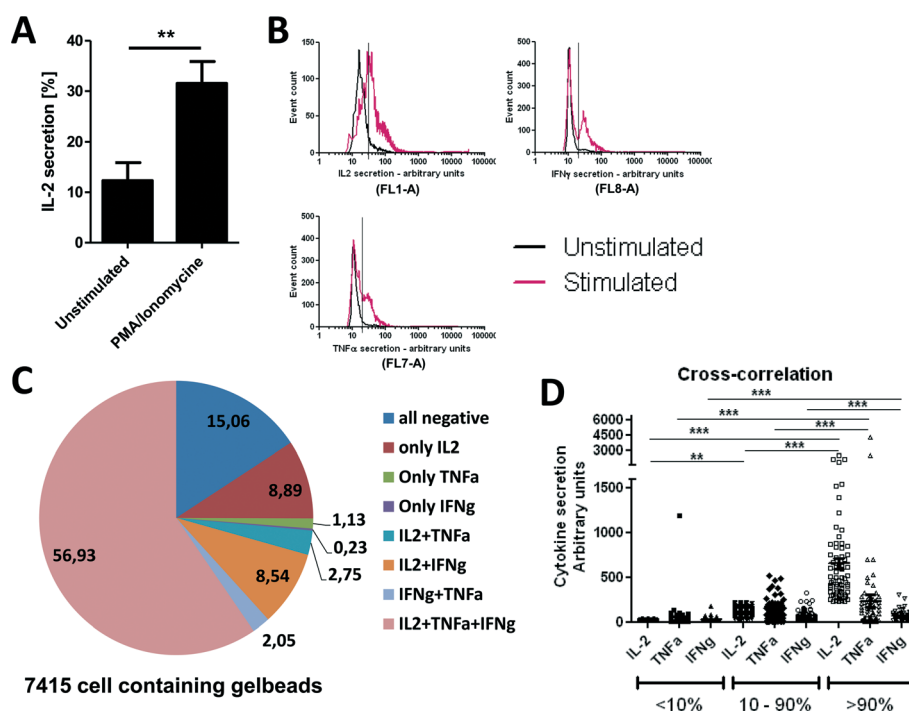


Fig. 3 A) Secretion of IL-2: stimulated Jurkat cells in droplets showed more secretion than the unstimulated cells. B) Fluorescence flow cytometry analysis of agarose beads resulting from single-cell secretion of cytokines IL-2, TNFα, IFNγ by stimulated cells compared to the unstimulated cells. C) Heterogeneity present in the secretion of different tested cytokines in the Jurkat cell population considered. Only 15% of the cells did not secrete any of the tested cytokines. D) Cross-correlation plot revealing the heterogeneity within the cell population.

Our data show that stimulated Jurkat T cells secreted significantly higher levels of IL-2 compared to unstimulated Jurkat T cells (Fig. 3A). The concurrent detection of multiple cytokines is essential for understanding the different functions exerted by activated T cells. Therefore, the number of tested cytokines was extended with TNF α and IFN γ , and multidimensional analysis allowed us to perform cross-correlation on these secreted cytokines. Analysis of more than 60 000 gel beads, of which 7415 are cell-containing, revealed the presence of 8 different cellular subpopulations, and that 85% of all individual Jurkat T cells secreted one or more cytokines upon overnight in-droplet stimulation with PMA and ionomycin (Fig. 3B). In the present study, we analyzed up to 8000 cell-containing gel beads; however, the throughput of our system can easily be scaled up by adjusting the collection time during droplet production (now 3 minutes). Furthermore, we show that almost 57% of stimulated Jurkat T cells secreted all 3 tested cytokines (Fig. 3C). These findings are in line with recent studies, exploiting nanowells, that demonstrated that the production and secretion of cytokines by T-cells occurred in a dynamic and heterogeneous manner.^{33,34} Furthermore, the observed cellular heterogeneity is in accordance with intracellular cytokine staining performed in bulk assays (data not shown) and might be attributed to various factors, *e.g.* differences in cell cycle stage, genetic perturbations or the transcriptional activity.³³ More in-depth analysis revealed that cells that secreted large amounts of IL-2 (>10 percentile) also secreted significantly higher levels of TNF α and IFN γ (Fig. 3D). Moreover, we also observed a population of cells that secreted intermediate levels of IL-2, but significantly higher levels of TNF α . Generally, these populations and individual responses would have been masked by the average response of the total cell population. Therefore, we believe that these findings underscore the strength of our developed system to probe cellular heterogeneity and unravel undefined cell populations with specific functions. Obviously, the secretion of soluble proteins is only one parameter that cells can exploit when activated. Cells can also communicate with other cells in a contact dependent manner, highlighting the importance of concurrently understanding the cells' phenotype. Furthermore, cells often only secrete proteins when activated; therefore, it is highly interesting to see whether certain populations of cells can also be defined based on the phenotype of a cell, which would allow isolation of cells with specific features prior to activation and use in cellular vaccines. The ability to detect the phenotype of immune cells together with the secretome of a cell provides a high-throughput systems immunology platform to unravel the functional properties of individual cells which is virtually impossible using current conventional methods.

The results from this study validate droplet microfluidics as a strong and innovative tool to study and manipulate single-cell behaviour. The impact of rapid expansion of high-throughput single-cell analysis is evident in its great potential for numerous applications, including drug discovery, diagnostics,

cancer research, regenerative medicine, system and synthetic biology, and many others. A better understanding of the immune response, including the heterogeneity of individual subpopulations of cells and the activation of killer T cells by other immune cells, is of particular interest because it may facilitate the development of new vaccines and immunotherapies.³⁵

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References

- 1 S. J. Altschuler and L. F. Wu, *Cell*, 2010, **141**, 559–563.
- 2 Y. Lu, J. J. Chen, L. Mu, Q. Xue, Y. Wu, P. H. Wu, J. Li, A. O. Vortmeyer, K. Miller-Jensen, D. Wirtz and R. Fan, *Anal. Chem.*, 2013, **85**, 2548–2556.
- 3 A. K. Shalek, R. Satija, X. Adiconis, R. S. Gertner, J. T. Gaublomme, R. Raychowdhury, S. Schwartz, N. Yosef, C. Malboeuf, D. Lu, J. J. Trombetta, D. Gennert, A. Gnirke, A. Goren, N. Hacohen, J. Z. Levin, H. Park and A. Regev, *Nature*, 2013, **498**, 236–240.
- 4 S. Tay, J. J. Hughey, T. K. Lee, T. Lipniacki, S. R. Quake and M. W. Covert, *Nature*, 2010, **466**, 267–271.
- 5 R. Cheong, A. Bergmann, S. L. Werner, J. Regal, A. Hoffmann and A. Levchenko, *J. Biol. Chem.*, 2006, **281**, 2945–2950.
- 6 M. R. Betts, M. C. Nason, S. M. West, S. C. De Rosa, S. A. Migueles, J. Abraham, M. M. Lederman, J. M. Benito, P. A. Goepfert, M. Connors, M. Roederer and R. A. Koup, *Blood*, 2006, **107**, 4781–4789.
- 7 B. El Debs, R. Utharala, I. V. Balyasnikova, A. D. Griffiths and C. A. Merten, *Proc. Natl. Acad. Sci. U. S. A.*, 2012, **109**, 11570–11575.
- 8 P. Mary, A. Chen, I. Chen, A. R. Abate and D. A. Weitz, *Lab Chip*, 2011, **11**, 2066–2070.
- 9 R. Novak, Y. Zeng, J. Shuga, G. Venugopalan, D. A. Fletcher, M. T. Smith and R. A. Mathies, *Angew. Chem., Int. Ed.*, 2011, **50**, 390–395.
- 10 S. Srigunapalan, I. A. Eydelnant, C. A. Simmons and A. R. Wheeler, *Lab Chip*, 2012, **12**, 369–375.
- 11 I. Barbulovic-Nad, S. H. Au and A. R. Wheeler, *Lab Chip*, 2010, **10**, 1536–1542.
- 12 J. Shao, L. Wu, J. Wu, Y. Zheng, H. Zhao, Q. Jin and J. Zhao, *Lab Chip*, 2009, **9**, 3118–3125.
- 13 H. N. Joensson and H. Andersson Svahn, *Angew. Chem., Int. Ed.*, 2012, **51**, 12176–12192.
- 14 M. T. Guo, A. Rotem, J. A. Heyman and D. A. Weitz, *Lab Chip*, 2012, **12**, 2146–2155.
- 15 A. M. Streets and Y. Huang, *Biomicrofluidics*, 2013, **7**, 11302.
- 16 G. J. Nossal and J. Lederberg, *Nature*, 1958, **181**, 1419–1420.

- 17 A. B. Theberge, F. Courtois, Y. Schaerli, M. Fischlechner, C. Abell, F. Hollfelder and W. T. Huck, *Angew. Chem., Int. Ed.*, 2010, **49**, 5846–5868.
- 18 A. Huebner, M. Srisa-Art, D. Holt, C. Abell, F. Hollfelder, A. J. deMello and J. B. Edel, *Chem. Commun.*, 2007, 1218–1220.
- 19 J. C. Baret, O. J. Miller, V. Taly, M. Ryckelynck, A. El-Harrak, L. Frenz, C. Rick, M. L. Samuels, J. B. Hutchison, J. J. Agresti, D. R. Link, D. A. Weitz and A. D. Griffiths, *Lab Chip*, 2009, **9**, 1850–1858.
- 20 A. Huebner, L. F. Olguin, D. Bratton, G. Whyte, W. T. Huck, A. J. de Mello, J. B. Edel, C. Abell and F. Hollfelder, *Anal. Chem.*, 2008, **80**, 3890–3896.
- 21 F. Courtois, L. F. Olguin, G. Whyte, A. B. Theberge, W. T. Huck, F. Hollfelder and C. Abell, *Anal. Chem.*, 2009, **81**, 3008–3016.
- 22 L. Mazutis, J. Gilbert, W. L. Ung, D. A. Weitz, A. D. Griffiths and J. A. Heyman, *Nat. Protoc.*, 2013, **8**, 870–891.
- 23 O. J. Miller, A. El Harrak, T. Mangeat, J. C. Baret, L. Frenz, B. El Debs, E. Mayot, M. L. Samuels, E. K. Rooney, P. Dieu, M. Galvan, D. R. Link and A. D. Griffiths, *Proc. Natl. Acad. Sci. U. S. A.*, 2012, **109**, 378–383.
- 24 C. J. Easley, J. V. Rocheleau, W. S. Head and D. W. Piston, *Anal. Chem.*, 2009, **81**, 9086–9095.
- 25 M. A. Unger, H. P. Chou, T. Thorsen, A. Scherer and S. R. Quake, *Science*, 2000, **288**, 113–116.
- 26 C. Holtze, A. C. Rowat, J. J. Agresti, J. B. Hutchison, F. E. Angile, C. H. Schmitz, S. Koster, H. Duan, K. J. Humphry, R. A. Scanga, J. S. Johnson, D. Pisignano and D. A. Weitz, *Lab Chip*, 2008, **8**, 1632–1639.
- 27 E. M. Bradshaw, S. C. Kent, V. Tripuraneni, T. Orban, H. L. Ploegh, D. A. Hafler and J. C. Love, *Clin. Immunol.*, 2008, **129**, 10–18.
- 28 L. S. Roach, H. Song and R. F. Ismagilov, *Anal. Chem.*, 2005, **77**, 785–796.
- 29 H. Song, D. L. Chen and R. F. Ismagilov, *Angew. Chem., Int. Ed.*, 2006, **45**, 7336–7356.
- 30 J. C. Baret, *Lab Chip*, 2012, **12**, 422–433.
- 31 X. Leng, W. Zhang, C. Wang, L. Cui and C. J. Yang, *Lab Chip*, 2010, **10**, 2841–2843.
- 32 G. Pawelec, A. Borowitz, P. H. Krammer and P. Wernet, *Eur. J. Immunol.*, 1982, **12**, 387–392.
- 33 Q. Han, N. Bagheri, E. M. Bradshaw, D. A. Hafler, D. A. Lauffenburger and J. C. Love, *Proc. Natl. Acad. Sci. U. S. A.*, 2012, **109**, 1607–1612.
- 34 Q. Han, E. M. Bradshaw, B. Nilsson, D. A. Hafler and J. C. Love, *Lab Chip*, 2010, **10**, 1391–1400.
- 35 L. Bird, *Nat. Rev. Immunol.*, 2012, **12**, 230–231.