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EDITORIAL SUMMARY This protocol describes a microfluidic platform for dynamic high-throughput analysis the phenotype of single cells. Cell-surface markers and secreted proteins are quantified and characterized by fluorescence detection using tailored immunoassays, simultaneously with measurement of other cellular characteristics, including endocytosis activity and viability.

TWEET A new protocol describes a microfluidics-based assay for high-throughput interrogation of protein secretion kinetics in single cells. @BIOASTER @EyerFira @ETH_DCHAB

COVER TEASER Microfluidics-based analysis of protein secretion

Up to three primary research articles where the protocol has been used and/or developed.

1. Eyer, K., Doineau, R. C. L., Castrillon, C. E., Briseño-Roa, L., Menrath, V., Mottet, G., et al. (2017). Single-cell deep phenotyping of IgG-secreting cells for high-resolution immune monitoring. Nature Biotechnology, 35(10), 977–982. http://doi.org/10.1038/nbt.3964

ABSTRACT

Characterization of immune responses is currently hampered by the lack of systems allowing quantitative and dynamic phenotypic characterization of individual cells, and especially analysis of secreted proteins such as cytokines and antibodies. We recently developed a simple and robust microfluidic platform, DropMap, to measure simultaneously the kinetics of secretion and other cellular characteristics, including endocytosis activity, viability and expression of cell-surface markers, from tens of thousands of single immune cells. Single cells are compartmentalized in 50 pL droplets and analyzed using fluorescence microscopy combined with an immunoassay based on fluorescence relocation to paramagnetic nanoparticles aligned to form beadlines in a magnetic field. The protocol typically takes 8-10 hours after preparations of microfluidic chips and chambers, which can be prepared in advance. In contrast, enzyme-linked immunospot (ELISPOT), flow cytometry, time of flight mass cytometry (CyTOF), and single-cell sequencing only allow end-point measurements, and do not allow direct, quantitative measurement of secreted proteins. We illustrate how this system can be used to profile downregulation of tumor necrosis factor-α (TNF-α) secretion by single monocytes in septic shock patients, and to study immune responses by measuring rates of cytokine secretion from single T cells and to measure affinity of antibodies secreted by single B cells.

Introduction

The last few years have seen rapid progress of single-cell sequencing technologies in both fundamental and clinical research[1-3]. Single-cell RNA sequencing (scRNA-seq), in particular, is proving to be a powerful tool to study gene expression at the single-cell level and to characterize cell-type diversity[4]. Although methods such as droplet-based scRNA-seq[5] enable high-throughput analysis of tens-of-thousands of individual cells, measuring transcriptional profiles is not sufficient to fully understand cellular activity, as protein concentration and subcellular localization cannot be reliably inferred from mRNA expression levels[6, 7]. As proteins are implicated in most biological functions, numerous approaches have been developed to assess protein expression at the single-cell level. In particular, fluorescence-based flow cytometry[8] and time of flight mass cytometry (CyTOF)[9], which allow medium to high-throughput single-cell phenotyping based on the detection of intracellular and cell-surface proteins, but not secreted proteins. The inability to directly detect secreted proteins can be partially alleviated using experimental strategies such as blockage of secretion pathways[10, 11], but it is neither possible to tell whether the protein is secreted or stored intracellularly, nor to measure secretion rates. Fluorescence microscopy and flow cytometry allow reliable simultaneous analysis of 6-10 distinct markers, while CyTOF allows simultaneous detection of as many as 40 markers[9, 12,13]. Recently, droplet-based single-cell sequencing techniques have also been developed to quantify cell-surface proteins using antibodies labeled with oligonucleotides[7, 14, 15] (rather than fluorescence or mass tags), which allows simultaneous analysis of the transcriptome of the same cell[7, 14]. In contrast, enzyme-linked immunospot (ELISPOT)[16] has been used extensively to detect proteins secreted from individual cells. Although simple to implement, this technique is poorly quantitative. ELISPOT assays can be used to determine the frequency of secreting cells but, even then, discrimination between signals due to secretion and background staining can be difficult[17, 18]. Importantly, ELISPOT, flow cytometry, CyTOF, and single-cell sequencing are all only based on end-point measurements, which do not reflect the dynamic nature of cellular behavior.

There is, hence, a need for quantitative high-throughput systems that allow dynamic single-cell phenotyping, including detection of secretion rates and binding activities of both cell-surface and secreted proteins. This information is crucial for the accurate analysis of immune responses that are mediated by both cell-membrane receptors and secretion of soluble factors, notably antibodies, chemokines and cytokines[19]. Deciphering dynamic processes at the single-cell level is critical to describe and understand the fundamental mechanisms underlying immunity, to develop new and improved strategies for vaccination and cancer immunotherapy, and to diagnose and treat inflammatory, autoimmune and infectious diseases.

Recently, novel technologies, based on microfabrication and microfluidics, have been developed to analyze the secretome of individual cells[20-31], some of which have been used to measure the secretion of multiple cytokines and to detect antibody secretion and antigen binding[30, 31]. Compartmentalization of individual cells in picoliter volume droplets in microfluidic systems[32, 33] has also been used for high-throughput screening and sorting of antibody-secreting cells based on the binding or inhibitory activity of secreted antibodies[34-36] and to analyze cellular heterogeneity in cytokine-secreting immune cells[37]. Although promising, these assays are only based on endpoint data and do not reflect the dynamic expression of immune response.

To overcome these limitations, we have recently described a simple and robust droplet microfluidic method, DropMap (Fig. 1), which we used to study the humoral immune response in mice immunized with tetanus toxoid[38]. With DropMap, single IgG-secreting cells are compartmentalized in tens of thousands of 50 pL droplets to form a stationary two-dimensional droplet array allowing kinetic analysis by optical microscopy. A fast and ultrasensitive in-droplet immunoassay, based on fluorescence relocation to paramagnetic nanoparticles that are aligned to form beadlines in a magnetic field, allows simultaneous measurement of the IgG secretion rate and affinity for thousands of single cells in parallel. In our study, we discovered that immunization results in dramatic increases in the range of both single-cell secretion rates and IgG affinities, which spanned at maximum 3 and 4 logs, respectively[38]. We also showed differences in dynamics of secretion rates and affinities of IgGs within and between spleen and bone marrow[38].

Here we present a detailed step-by-step protocol for the DropMap system, and describe how the system can be extended to study a range of different immune cells, including B cells, T cells and monocytes, and to analyze the secretion of a variety of different proteins, such as different antibody isotypes, and a variety of murine and human cytokines (including TNF-α, IFN-γ; IL-2 and IL-6). We also describe how the measurement of secretion can be combined with the measurement of other parameters, including affinities of secreted antibodies, expression of cell-surface markers, endocytosis and cell viability. The method is rapid and sensitive, enabling, for example, the detection of secreted proteins from a single cells in as little as 30 min, compared to generally more than 6 hours for conventional single-cell assays such as ELISPOT[16]. It is a flexible system to analyze humoral, cellular and innate immune responses. To illustrate the performance of DropMap, we describe its applications to immune responses analyses in a murine immunization model by measuring secretion rate and affinity of antibodies secreted by single B cells. Importantly, from a clinical standpoint, we also reveal the downregulation profiles of TNF-α secretion by single monocytes obtained from septic shock patients.

Comparison with other methods

ELISPOT, flow cytometry, CyTOF, and scRNA-seq are all based on endpoint measurements, which is advantageous for throughput but does not reflect the dynamic nature of cellular behavior. They do not allow the quantitative characterization of secreted proteins by living cells. Recently, commercially available technologies based on compartmentalizing cells in microfabricated chambers have been developed to perform a range of assays, including real-time IgG and cytokine secretion, apoptosis and cell-cell interaction[39-41]. For example, the Berkeley Lights system allows optofluidic handling and analysis of single cells, including recovery of individual cells for sequence analysis, but can analyze ~10,000 cells as the system currently has 14115 chambers ("nanopens"), whereas the DropMap system can currently analyze up to 300,000 cells. Furthermore, when used to analyze antibody secretion, the Berkeley Lights system only provide a binary (yes/no) result[41], whereas, DropMap enable simultaneous determination of both antibody secretion rate and affinity.

Other technologies using droplet microfluidics have been developed for high-throughput, single-cell, protein secretion measurement [35, 37, 42, 43]. However, as previously mentioned, these are endpoint approaches, and do not assess the dynamic nature of single-cell secretome. Droplet microfluidics has also been used for real-time measurement of T cell activation upon recognition of target tumor cells Segaliny *et al*. However, the number of droplets containing T cells and target cancer cells was low (due to Poisson statistics of cell encapsulation)[44].

DropMap, in contrast, allows the direct measurement of protein secretion by individual living cells and the quantitative assessment of secreted proteins. Hence, using DropMap, the dynamic behavior of single-cells can be measured over time, allowing, for example, the direct and quantitative assessment of secretion rates of proteins such as antibodies, cytokines and chemokines, and, in the case of antibodies, DropMap enables simultaneous determination of both antibody secretion rate and affinity. A DropMap chamber surface of 1 cm^2 allows the observation and analysis of 60,000 droplets: i.e. around 20,000 cells over up to 12 hours. Currently, the maximum number of cells we have detected simultaneously with DropMap is around 300,000 cells with a time resolution around 21 minutes. The number of eukaryotic cells that can be analyzed is therefore similar to the number that can be analyzed by scRNA-seq (~ 10^5), ELISPOT (~10^5 per well) and using microfabricated nanowells (~10^5), but less than can be analyzed using flow cytometry (~10^8) CyTOF (~10^7) or droplet microfluidics with in-flow detection, where, using a similar magnetic beadline-based immunoassay, as many as 2.5 million eukaryotic cells can be analyzed per experiment[45]. The maximum number of markers that can currently be detected simultaneously with DropMap is 4, as described herein, but it should be possible to extend this to 6-10 markers, as for the other systems based on epifluorescence microscopy. The use, in DropMap, of an assay based on fluorescence relocation also negates the need for washing within the protocol.

Protocol limitations

Our protocol inherits the generic limitations of closed homogenous fluorescence-based assays. Once immobilized, nothing can be added or extracted from the droplets without losing their identity (as defined by their position). Therefore, the immunoassay cannot be washed, no enzymes can be used for signal amplification, and multi-step assays are not possible. The immunoassays sensitivity and range is defined by the number of added nanoparticles and detection reagent, therefore care should be taken when adapting the sensitivity and range of the assay to prevent excess false-positive or negative results (see also Box 1 and 2).

The biggest drawback of the protocol is the need for a known analyte of interest, since in the sandwich immunoassays the antibody pair is chosen according to the analyte, and the performance of the assay depends critically on the affinity and specificity of the antibodies. Also, when assaying secreted antibodies, since purified antigens are used in the assays, cross-reactivities or unspecific off-target binding of the antibodies are not taken into account and need to be characterized using other methods.

Due to the need for imaging in different fluorescent channels, care should be taken regarding optical phenomena such as pixel saturation, photo-bleaching or spectral overlap. This overlap also limits the number of readouts that could be used to probe several cellular functions within the droplets, thus reducing the multiplexing capability when compared to CYTOF or single-cell sequencing.

Long-term studies over days are also limited due to the inability to add fresh nutrients to the cells without losing their identity. Therefore, the longest experimental time is defined by the disappearance of a crucial nutrient, or the accumulation of waste products in the droplets. This depends on the nature of the cells analyzed, but using mammalian cells (Chinese hamster ovary, CHO cells), the longest assay we have performed is 12 hours. The frequency of dead cells (stained with propidium iodine) encapsulated in droplets was not significantly different from the reference culture in a standard CO_2 incubator over 12 hours of incubation time (3.2 ± 1.6 % and 2.6 ± 1.6 %, for droplets and the reference culture respectively. N = 3, two-tailed unpaired t test p = 0.67).

Expertise needed to perform the protocol

A competent graduate student or post-doc can perform the protocol. There are two crucial parts of the protocol. First, the extraction and preparation of the biological sample, i.e. the cells, is of great importance. These should be extracted and prepared with the least stress possible since even minor changes in the protocol might result in lower secretion rates, and therefore variations in the results.

The work with human and animal samples needs to be performed by properly trained and skilled experimenters, with appropriate safety precautions, with ethical approval where necessary, and in accordance with local legislation. Second, the proper preparation of the chamber and droplets is of high importance to eliminate or restrict droplet movement over time and improve data quality and reliability of the process, but a researcher from any discipline easily masters the procedure.

Experimental design

The DropMap protocol is based on compartmentalizing single cells in tens of thousands of 50 pL droplets that are immobilized in two-dimensional droplet arrays and analysed by fluorescence microscopy[38] (Fig.1). The droplets can be therefore monitored over time and cellular dynamics are captured by taking images at regular intervals. Compartmentalization of single cells in droplets allows the measurement of secreted molecules which remain in the droplets, in combination with measurements of cell-surface markers and other phenotypic characteristics. Secreted molecules are quantified using a fluorescence relocation-based, sandwich immunoassay. The analytes are captured on antibody-coated paramagnetic nanoparticles in the droplets using a capture antibody. These nanoparticles are aligned to form a beadline by applying a magnetic field, and the captured analytes are quantified by measuring relocation of a fluorescently-labelled detection antibody to the beadline. The protocol consists of six major stages:

Microfabrication of the droplet production chip (Steps 1-22). Microfluidic chips for droplet production are fabricated in poly(dimethysiloxane) (PDMS) using soft-lithography[46] as described previously[35]. Droplet-makers are also commercially available from a variety of commercial suppliers for laboratories without the necessary equipment and facilities to perform these steps. The droplet production chip is designed to produce monodisperse 50 pL droplets using hydrodynamic flow focusing[47] and contains three inlets to introduce, respectively, (i) the fluorinated carrier oil containing fluorosurfactant, (ii) the suspension of cells and (iii) the assay reagents (functionalized nanobeads, labelled detection probes, a live/dead cell probe, and others), as well as one outlet for collecting the produced droplets[35] (Fig. 1 and see Supplementary Data 1 for the complete chip design - CAD file).

Microfabrication of the droplet incubation chamber (Steps 23-30). The immobilization and storage of aqueous droplets in a 2D array is the core of the DropMap approach. Immobilization of the droplets allows for time-course measurements of droplet-based biological assays[48-53]. Due to the height of the chamber being less than the droplet diameter, the observation chamber is able to immobilize 50 pL droplets. Incubations and observations over time are therefore made possible for short to medium time ranges (5 min to < 24h) at temperatures between 4°C and 37 °C.

We use a simple, cost-effective and reproducible method based on double-sided tape and two standard microscope slides (75 mm x 25 mm x 1 mm) to fabricate the droplet incubation device (Fig. 2, see Supplementary Data 2 and 3 for the mask for the double-sided tape to prepare the observation chamber and the mask for laser ablation). The fabrication method is flexible and customized microfluidic chambers can be fabricated depending on the droplet volume and the number of droplets to be analyzed: several double-sided adhesive tapes with different thicknesses are commercially available (for example from 3M) and any observation chamber shape and size can be made by cutting the tape using a plotter. The use of glass results in an optically transparent system, whilst the double-sided tapes used adheres strongly and stably to the glass surfaces preventing fluid leakage and evaporation. Lastly, after the experiments the chamber can be cleaned and reused several times. To do so, the outer surfaces are cleaned with ethanol or isopropanol and the chamber flushed with pure fluorinated oil to remove droplets.

Functionalization of nanoparticles (Steps 31A-31B). Another important key feature of our droplet-based immunoassay is the use of many paramagnetic nanoparticles per droplet (see also Box 3). Magnetic nanoparticles are well-suited to immunodetection technologies owing to their large surface-to-volume ratio, monodispersity, stability and easy manipulation in a magnetic field. Compared to approaches relying on the co-encapsulation of a single cell and a single microparticle[35], the use of nanoparticles increases the binding capacity and lowers the number of droplets containing no particles, which improved the performance of the assay. The protocol was optimized so that, once a strong magnetic field is applied, each droplet contains a single, homogeneous, elongated aggregate of hundreds of nanoparticles, which appears in the bright-field images as a horizontal dark beadline located in the droplet (Fig. 1, 3). The number of nanoparticles per droplet defines the capacity of the immunoassay, and can be adjusted to the expected secretion rates to allow optimal results. However, a certain minimum (100-200 beads/droplet) is needed to form a measurable beadline.

Secreted molecules are captured onto paramagnetic nanoparticles for detection, typically *via* capture antibodies. The immobilization of capture antibodies onto the nanoparticles is a crucial step for the success of the protocol[54-59]. The performance of antibody-based biosensors is directly related to the orientation of antibodies on the nanoparticles and accessibility of the paratope that binds the targeted analyte. In this protocol, we describe the use of two targeted coupling strategies. First, we describe a boronic acid (BA)-based coupling strategy for precise orientation of capture antibodies on the surface of magnetic nanoparticles[54, 60-62] (Fig. 3a-c) and second, a strategy based on biotinylated capture antibodies coupled to streptavidin-coated nanoparticles (Fig. 3d-f).

Automated image acquisition (Steps 38-45). To perform imaging, the microfluidic chamber is placed on a motorized stage of an inverted epifluorescence microscope. In our case, 10X magnification was used as a compromise between scanning time and resolution for object detection within each droplet. We image between 75-225 fields of view in multiple channels (up to five, depending on the integrated assays): 75 fields of view (135 mm^2) results in around 60,000 imaged droplets; i.e. 10-20,000 cells. Throughput can be improved by increasing the imaged area. However, when scanning large areas of a sample with high resolution, scanning time as well as processing time (of large amount of imaging data) can become limiting steps for the throughput of the procedure. Here, binning of pixels during acquisition may help to solve some of these issues.

As both automated object detection and fluorescence intensity measurements rely on focus quality, control of focus drift throughout the imaging procedure (of a large area over long times) is crucial. Mechanical effects, vibrations, thermal changes and droplets movement can cause the focus to drift. We addressed this limitation by using the Perfect Focus System (PFS) of the Nikon microscope, but other microscopes might offer similar solutions.

Cell preparation, encapsulation and incubation in droplets (Steps 46A-46B). This analytical system is suitable for many different cells from various tissues and species. Primary cells are sensitive and fragile, therefore requiring gentle handling during extraction and manipulation. Due to the quantitative nature of the measurements described in this protocol, even small deviations might result in significant differences. Gentle handling and rapid extraction of cells is important, and for optimal results, the cells should be present as a singularized suspension. Before encapsulation, the cell density needs to be adjusted so that the frequency of droplets containing a single cell is maximized, while minimizing the frequency of droplets containing multiple cells (see Box 3)[35].

Compartmentalization of cells in droplets will further affect cell survival. The surfactant is a key reagent for the droplet assays[35, 63]: it must be non-cytotoxic, and must stabilize droplet interfaces and prevent the coalescence of droplets during incubation. The use of the nonionic perfluoro-polyether fluorosurfactant (600 g/mol PEPE tails and 2000 g/mol heads)[35] in combination with fluorinated carrier oils such as HFE-7500 (3M) is recommended. At some point, nutrient depletion and pH changes will be a limiting factor for long-term studies of the encapsulated cells. The in-droplet pH is maintained by the addition of HEPES[64], and the fluorinated oil, which can dissolve ~20 times more oxygen than water[65], used as a continuous phase, additionally serves as an, albeit limited, oxygen reservoir[66, 67]. In addition, as fluorinated oils are very poor solvents for non-fluorinated molecules[68, 69] they are well suited for cell-based and biochemical assays. Various biocompatible fluorinated oils and

fluorosurfactants can be ordered from a large number of suppliers such as 3M, RAN Biotechnologies, Dolomite, Sphere Fluidics and Bio-Rad. We confirmed the survival of primary monocytes over a 12-hour time span, and the survival of primary murine splenocytes over the same time-period in 50 pL droplets. Furthermore, we added in our protocol a simple and sensitive cytotoxicity assay to discriminate dead and live cells to exclude any signals that might stem from dead or dying cells.

Image processing (Steps 47A-47B). We developed a robust, custom-made Matlab-based software to detect and, if necessary, track droplets in time-lapse image sequences and monitor the status of their content over time. The software analyses the images over time in different fluorescence and bright field channels. First, the droplets are detected in bright field images using an algorithm that detects circles of a given diameter. In a next step, a binary mask of the detected droplets is generated, and this mask is overlaid onto the following fluorescence images to analyze each droplet separately. Within each droplet, the software measures the fluorescence of droplets, cells and beadlines (Fig. 4).

The software output is a spreadsheet containing morphological and fluorescence intensity parameters for different intra-droplet objects or regions over time. All measured fluorescence intensities on objects (i.e. magnetic beadline, cell or whole droplet) are normalized by the background fluorescence of the droplet in order to compensate locally for potential heterogeneity of illumination of the field, increase the sensitivity (signal-to-noise ratio is more differentiating than the raw beadline signal), and reduce the impact of photobleaching on results.

Materials

Biological materials

- Human blood ! **CAUTION** Experiments using human blood samples must conform to the relevant Institutional and National regulations and requires informed consent from all participants. See just below for the permissions obtained for the use of human blood for this Protocol:
  - Samples from septic shock patients (Table 1) were obtained from the prospective cohort MICROFLU-SEPSIS which has been approved by the French Ethical Committee CPP Sud Ouest et Outre Mer 4 (registration number 2017-A01134-49). Patients were included in the medical intensive care unit of Cochin hospital and informed consent was obtained from all the participants or their legally authorized representatives.
  - For sepsis application, blood sample collection from healthy donors was performed according to the Ethical Committee of the Investigation Clinique et Acces aux Resources Biologiques (ICAReB) platform (Dr Marie-Noelle Ungeheuer, Centre de Recherche Translationnelle, Institut Pasteur, Paris, France) as part of the DIAGMICOLL or CoSImmGEn (N° CORC: 2008-16 and 201-06) protocol, which has been approved by the French Ethical Committee (CPP), Ile-de-France. Informed consent was obtained from all donors (Table 1).
  - For cytokine-secreting T-cells application, Peripheral Human blood was also collected from healthy patients at the "Etablissement Français du Sang". Donors were recruited after a medical selection process complying with French regulations, and they gave their informed consent to participation in the study.

- Mouse spleen and bone marrow. In this protocol, we used Mus musculus, BALB/cJRj, females, age 8-10 weeks at the start of the immunization, supplied by Janvier Laboratories. ! **CAUTION** Experiments using animal samples must conform to the relevant Institutional and National regulations. In this work, the mice experiments have been validated by the CETEA ethics committee number 89 (Institute Pasteur, Paris, France) under #2013-0103, and by the French Ministery of Research under agreement #00513.02, and were part of larger scientific study.

	Gender	**Age**	**Sepsis source**	**SOFA**[#]
Septic Patient 1	Male	61	Cutaneous	8
Septic Patient 2	Female	88	Urinary tract	6

Septic Patient 3	Female	63	Osteo-articular	8
Healthy Donor 1	Male	47	ND	ND
Healthy Donor 2	Female	42	ND	ND
Healthy Donor 3	Female	35	ND	ND

- **Table 1. Characteristics of healthy donors and septic patients at admission in the intensive care unit.** Gender, age, sepsis source and SOFA score. The SOFA (Sequential Organ Failure Assessment) score is a bedside applicable score designed to score organ dysfunction with less focus on mortality prediction.

Reagents -

CAUTION Wear appropriate protective clothing and equipment when manipulating chemical and biological reagents.

- Ultrapure DNase/RNase-free distilled water (Invitrogen, cat. no.10977049).
- Isopropanol (VWR 208224321). **! CAUTION** Isopropanol liquid and vapor is highly flammable. Handle away from ignition sources and fire.
- Acetone (VWR 322201). **! CAUTION** Acetone liquid and vapor is highly flammable. Handle away from ignition sources and fire.
- Ethanol (VWR 208224321**). ! CAUTION** Ethanol liquid and vapor is highly flammable. Handle away from ignition sources and fire.
- 1H, 1H, 2H, 2H-Perfluorododecyltrichlorosilane (Sigma-Aldrich, cat. no. 729965)**! CAUTION** This reagent is corrosive and moisture sensitive. Work in a fume hood to avoid inhalation of silane.
- HFE-7500 (3M, cat. no. 98-0212-2928-5)**! CAUTION** Avoid direct contact with this liquid, as it may cause respiratory, skin and eye irritation.
- Fluorinated surfactant (Ran Biotechnologies, cat. no 008).
- Poly(dimethyl siloxane) (PDMS) and curing agent (Dow Corning, Sylgard 184).

Application sepsis

- Bovine Serum Albumin (BSA) (Sigma-Aldrich, cat. no A7960).
- Roswell Park Memorial Institute (RPMI) 1640 Medium (PanBiotech, cat. no. P04-17500).
- Phosphate Buffered Saline (PBS) (Dominique Dutscher, cat. no. L0612-500).
- Penicillin-streptomycin (Life Technologies, cat. no. 15140-122).
- HEPES (Dominique Dutscher, cat. no. L0180-100).
- Fetal Bovine Serum (FBS) (HyClone cat. no. SH30070.03).
- Anti-CD14, blue violet conjugate (Clone TÜK4) (Miltenyi Biotec Cat# 130-094-364, RRID:AB_10831023), Lot# 5171130409.

- Capture anti-TNF-α antibody (Antibody TNF5) (MABTECH Cat# 3510-6-1000, RRID:AB_907379), Lot#16, non-biotinylated).
- Detection PE-anti-TNF-α antibody Clone: cA2 (Miltenyi Biotec Cat# 130-120-489 without azide Cat#120-014-229, RRID:AB_2752115), Lot#5160222264.
- Recombinant TNF-α protein (Biolegend, cat. no. 570108).
- NucView488 (Biotium, cat. no. 99949).
- MitoView633 (Biotium, cat. no. 99950).
- LPS (Sigma-Aldrich, cat. no. L2637, 3012 and 3137).
- Dextran 20 % (wt/wt), 500 kDa (Sigma-Aldrich, cat. no. D8802).
- 300 nm dimeter Carboxyl Adembeads (Ademtech, cat. no 2131).
- EDC (Thermo Fisher Scientific, cat. no. 77149). **! CRITICAL** This material is moisture sensitive. Upon receipt, make aliquots and store them at -20°C.
- SulfoNHS (Thermo Fisher Scientific, cat. no 24510). **! CRITICAL** This material is moisture sensitive. Upon receipt, store at 4°C.
- Ethanolamine (Sigma-Aldrich, cat. no E9508). **! CAUTION** This material is irritant. Work in a fume hood.
- 10 X Activation buffer 10X for Carboxyl-Adembeads Coupling (Ademtech, cat. no 10101)
- 10 X Storage Buffer 10X (Ademtech, cat. no 10201)
- 3-aminophenylboronic Acid (Sigma, cat. no A71751)
- EasySep Direct Human Monocyte isolation Kit (STEMCELL Technology, cat. no 19669).

Application murine IgG-secreting cells

- Knockout Serum Replacement (Thermo Fisher Scientific, cat. no. 1082808).
- Human Serum Albumin (rHSA), recombinant (SigmaAldrich, cat. no. A9731).
- HEPES (ThermoFisher, cat. no. 15630080).
- DPBS (ThermoFisher, cat. no. 14190250).
- Penicillin-streptomycin (Life Technologies, cat. no. 15140-122).
- Pluronic F-68, 10% (wt/vol) sterile filtered solution (Thermo Fisher Scientific, cat. no. 24040032).
- Pluronic F-127, 10% (wt/vol) sterile filtered solution (Thermo Fisher Scientific, cat. no. P6866).
- RPMI 1640 Medium without phenol red (ThermoFisher, cat. no. 11835030).
- 300 nm diameter Bio-Adembeads Strepatividin plus (Ademtech, cat. no. 03132).
- CaptureSelect™ Biotin Anti-LC-kappa (Murine) Conjugate (ThermoFisher Scientific, cat. no. 7103152100), Lot#160707-02.
- AffiniPure F(ab')$_2$ Fragment Rabbit Anti-Mouse IgG, Fcγ fragment specific (Jackson ImmunoResearch Labs Cat# 315-606-047, RRID:AB_2340252), Lot#103179.
- CellTrace™ Violet Cell Proliferation Kit (ThermoFisher Scientific, cat. no. C34557).
- Zenon™ Alexa Fluor™ 488 Mouse IgG2b Labeling Kit (Thermo Fisher Scientific Cat# Z25202, RRID:AB_2736943), Lot#1450052 .

Application cytokine-secreting T-cells

- IFN gamma Monoclonal Antibody (MD-1) (Thermo Fisher Scientific Cat# 14-7317-85, RRID:AB_468474), Lot#4295084, biotinylated in house.
- IFN gamma Monoclonal Antibody (clone 4S.B3), APC conjugate (Thermo Fisher Scientific Cat# 17-7319-82, RRID:AB_469506), Lot#4281150.
- TNF alpha Monoclonal Antibody (MAb1) (Thermo Fisher Scientific Cat# 14-7348-85, RRID:AB_468488), Lot#E05529-432, biotinylated in house.
- TNF alpha Monoclonal Antibody (MAb11), PE conjugate (Thermo Fisher Scientific Cat# 12-7349-81, RRID:AB_466207), Lot#4290466.
- IL-2 Polyclonal Antibody, Biotin (Thermo Fisher Scientific Cat# 13-7028-81, RRID:AB_466900), Lot4307122 and 2049448.
- IL-2 rabbit anti-human (PeproTech Cat# 500-P22-100ug, RRID:AB_147898), Lot#0204CY12RB, labelled in house with Alexa 555
- IL-4 Monoclonal Antibody (MP4-25D2), Biotin conjugate (Thermo Fisher Scientific Cat# 13-7048-81, RRID:AB_466904), Lot#1963900.
- IL-4 Monoclonal Antibody (8D4-8), APC conjugate, (Thermo Fisher Scientific Cat# 17-7049-81, RRID:AB_469497), Lot#4275080.
- Anti-human CD3, clone HIT3a, Alexa Fluor® 647 conjugate (BioLegend Cat# 300322, RRID:AB_493693), Lot#RT2101610.

EQUIPMENT –

General lab equipment

- Gloves (Crolex powder-free nitrile).
- Microscope slides, frosted ground-edges (VWR, cat. no. 63115553).
- Ultraviolet curing adhesive (Nurland optical adhesive, cat. no.68 P/N 6801).
- Disposable slide for cell counting: Fast Reader 102 (Dominique Dutscher, cat. no. 390498).
- Reusable biopsy punch diameter 0.75 mm and 6 mm (WPI, cat. no. 504529 and 504532).
- Sterican needles 23 G for 0.56 mm diameter microtubing (Braun, cat no; 921021).
- Sterican needles 27 G for 0.30mm diameter microtubing (Braun, cat no. 921018).
- PTFE microtubing (0.56 × 1.07 mm; Fisher Scientific, cat. no. 1192-9445).
- PTFE microtubing (0.30 × 0.76 mm; Fisher Scientific, cat. no. 1191-9445).
- Sterile 1 ml syringes (Braun Omnifix, cat. no. 9204512).
- Sterile low binding microcentrifuge tubes (Dominique Dutscher, cat.no 27210).
- Petri dishes (100 mm diameter × 15 mm; BD Falcon, cat. no. 351029).
- Nanoport 6-32 (Cil , cat no N-126-01).
- Cap (Cil, cat no P-555).
- MicroTight fittings (Cil, cat no P126S).
- Three low pressure syringe pumps neMESYS 290N, and control module Base 120 (both Cetoni).
- Vortex LMS (Dominique Dutscher, cat. no 079030).

- Adjustable 2.5, 10, 20, 200 and 1,000 µl pipettes and sterile pipette tips (Eppendorf, cat no.: 3123000012; 3123000020; 3123000039; 3123000055; 3123000063).
- 2.5, 10, 20, 200 and 1,000 µl sterile pipette tips ART (Thermo Fisher Scientific).
- Carousel holder for 6 micropipettes Research or 6 Multipettes (Dominique Dutscher, cat. no 33076).
- Smart Spatula (Dominique Dutscher, cat.no. 059362).
- Centrifuge (Eppendorf 5810R and 5418R).
- Magnet holder for 1.5 microtube (Ademtech, cat. no 20101).
- EasySep Magnet (STEMCELL Technology, cat.no. 180001). **! CAUTION** Strong magnetic field. Handle it carefully and keep it away from objects that respond to magnetic field.
- Falcon® Round-Bottom Polystyrene Tubes, 5 and 15 mL (Dominique Dutscher, cat. no. 352051).
- Rapid-flow filter unit 0.2 µm-PES membrane 0.75 mm (Thermo Fisher Scientific, cat. no. 566-0020).
- Sonicator: Bransonic Model B200 ultrasonic cleaner (Dominique Dutscher, cat. no. 910586).
- Mini shaker vortex Lab Dancer (Dominique Dutscher, cat. no 250097).
- Rotating shaker SB3 Stuart (Dominique Dutscher, cat. no 250097).
- Drum 40 microtubes 1.5 mL (Dominique Dutscher, cat. no 90435).
- Inverted microscope for droplet generation (Olympus IX83).
- 96-well microtiter microplate (Greiner, cat.no. 650185).
- 96-well dark microtiter plate (Corning, cat.no 3991).
- Magnet holder for 96-well plate (Ademtech, cat. no 20106).
- Plate reader infinite M200 Pro (TECAN).

Chip fabrication

- Disposable mixing cups (Dominique Dutscher, cat no 045108).
- Aluminum foil (Alupro, cat. no. A0626).
- Oven (Binder 300°C, Dominique Dutscher cat. no. 9010-0190).
- Vacuum desiccator (Sigma-Aldrich, cat. no D2672).
- Scalpel (Becton Dickinson, No. 11, cat. no. 371611).
- Oxygen plasma cleaner (Femto Science, Inc. CUTE.MRP).

Chamber fabrication

- UV light source 5 (Opticure LED, Tech Optics Led, cat. no. 5090-200).
- Tweezers Dark blue forceps 111mm (Dutscher, cat no. 037876).
- CO_2 Laser (AxysLaser, C180 ii-CO2), or alternative method for drilling 1 mm holes in glass.
- Graphtec Cutting plotter CE6000-40 (Graphtec) or CAMEO plotter (Silhouette).
-

Chamber fabrication for sepsis application

- Double-sided adhesive tape (Adhesive Research, cat. no. 92712).
- Binder clips (safe tool, cat no.: 8140.19; 8140.32).
- Magnet (FIRST4MAGNETS, cat. no. F75103). **! CAUTION** These magnets are powerful. They can be dangerous, handle them carefully. Keep it away from objects that respond to magnetic field.
- Oven (Binder 300°C, Dominique Dutscher cat. no. 9010-0190).

Chamber fabrication for IgG application

- Orafol Tape Series 1375 (Orafol, SDAG Adhésifs).
- Silicone paper (135 g/m^2, SDAG Adhésifs). **! NOTE** The Orafol 1375 is only covered on one side with paper. Use this tape to cover the other side, and cut through this tape in the assembly to generate optimal results for chamber fabrication.
- 2 Magnets (K&J Magnetics, cat. no. BZX082). **! CAUTION** These magnets are powerful. They can be dangerous, handle them carefully. Keep it away from objects that respond to magnetic field.
- 3D printed holder for magnets (according to the Supplementary File 4)
- Thermopress capable of a pressing 7 bar and heating to 150 °C (Scamex).

Microscope and image acquisition

- Inverted microscope for time-lapse imaging (Nikon, Eclipse Ti-2E):
- Digital CMOS camera (ORCA FLASH 4 LT, Hamamatsu); 2048(V) x 2048(H) pixel array with 6.5 x 6.5 µm^2 pixel size.
- Transmission light source (powerful white LED, pE-100wht, CoolLED). It replaces typical 100W halogen lamp.
- LED-based excitation light engine (Lumencor, SOLA SE II,).It replaces mercury arc lamp.
- 10x CFI Plan Apochromat Objective (Nikon, cat. no. MDR00105).
- Microscope enclosure and temperature controller (Oko-lab, cat. no. H201-T-UNIT-BL).
- Motorized *xy* stage (Nikon, cat no MECS MEC56110).
- Software for stage control and image acquisition (Nikon, cat no NIS-AR Log MQS3 1000).
- Perfect Focusing System 2 (PFS2, Nikon, cat. no. MEP59390). **! CRITICAL**: Needed to retain focus over large measurement areas.
- DAPI fluorescence cube (Nikon, cat. no. DAPI-5060C: Exc. 377/50 nm, Em. 447/60 nm, Cutoff 409 nm)
- GFP fluorescence cube (Nikon, cat. no. GFP-1828A: Exc. 482/18 nm, Em. 520/28 nm, Cutoff 495 nm)
- CY3 fluorescence cube (Nikon, cat. no. CY3-4040C: Exc. 531/40 nm, Em. 593/40 nm, Cutoff 562 nm)

- CY5 fluorescence cube (Nikon, cat. no. CY5-4040C: Exc. 628/40 nm, Em. 692/40 nm, Cutoff 660 nm)

Software and analysis workstation

- MATLAB (MathWorks, version 2018a, Image processing toolbox, VLFeat open source library, https://www.vlfeat.org/install-matlab.html).
- MATLAB Image analysis workstation: 8 Virtual processors, 2.6GHz, RAM 64 GB.
- DropCell.exe (https://doi.org/10.6084/m9.figshare.11336663.v1). **CRITICAL** Before first use of the Matlab application, it will have to be installed on your computer. This is done automatically by running the provided MyAppInstaller_web.exe. The same installer file will offer to download and install the required version 9.4 (R2018a) of the MATLAB Runtime, if not already installed on your machine. An example of input_parameters.txt file as well as a readme_dropcell.txt file will be automatically copied to the folder where you will choose to install the application. **CRITICAL** Read carefully the readme_dropcell.txt before first use of DropCell.exe. It describes the installation procedure as well as the input images naming rules.

REAGENTS SETUP–

SILANE Solution

To prepare 1% (vol/vol) silane solution, dilute 10 µl 1H, 1H, 2H, 2H-Perfluorododecyltrichlorosilane in 990 µl filtered HFE-7500 oil. This solution should be prepared immediately before use.

! CAUTION This solution is irritant and corrosive, work in a fume hood with appropriate materials.

CRITICAL Use this reagent immediately after preparation.

Coupling buffer for boronic-based functionalization

Dilute activation buffer 10-fold in pure distilled water. This buffer should be freshly prepared before use.

Storage buffer for boronic-based functionalization

Dilute storage buffer 10-fold in pure distilled water. This buffer should be freshly prepared before use.

Quenching buffer for boronic-based functionalization

100 mM ethanolamine in PBS. This buffer should be freshly prepared before use

! CAUTION This solution is corrosive and irritating; work in a fume hood.

CRITICAL Use this reagent immediately after preparation. Do not store.

Blocking buffer for boronic-based functionalization

Blocking buffer is 1% (vol/vol) Dextran. Dilute dextran 20% (wt/wt) 20-fold in pure distilled water to obtain 1% (vol/vol) blocking buffer. This solution can be stored at 4°C for up to 6 months.

! CAUTION: Dextran 20% (wt/wt) is viscous pipette this solution very slowly.

Cell buffer for sepsis application

Heat the FBS at 56°C in a water bath for 35 minutes. Cool at room temperature (20-25°C) and filter the serum using rapid-flow filter unit of 0.2 µm-PES membrane. Make 50-ml aliquots and store at −20°C. After thawing, centrifuge the serum at 3000g for 10 minutes at room temperature to remove large debris. Supplement RPMI 1640 Glutamax with 10% heat-inactivated FBS (vol/vol), 5% Penicillin-streptomycin (vol/vol), and 20 mM HEPES. Store culture medium at 4 °C and use it within 2 weeks after preparation.

Blocking buffer murine IgG application

5% (vol/vol) Pluronic F-68 in PBS, sterile. Can be stored at -20°C in small aliquots ready to use. For short term storage, store at 4°C for up to 4 weeks.

Cell buffer 1 IgG application

RPMI 1640 with Glutamax (w/o phenol red) supplemented with 10% KnockOut Serum Replacement (vol/vol), 1x Penicillin-streptomycin, 0.1% Pluronic F-127 (vol/vol), rHSA 0.5% (wt/vol) and 25 mM HEPES. Aliquot cell buffer and freeze at -20°C. For short term storage, store at 4°C for up to 1 week. Up to 4 weeks are possible when working in a sterile environment.

! CRITICAL: The use of rHSA and the removal of all products from animal origin reduces assay background and increases sensitivity.

Immobilization reagent IgG application

CaptureSelect™ Biotin Anti-LC-kappa (Murine) Conjugate (stock concentration 1 mg/ml), diluted 1 to 5 (v/v) in PBS. Aliquot in 50 µl and store at -20°C. For short term storage, store at 4°C for up to 4 weeks.

PROCEDURE

Microfluidic device fabrication from existing molds. Timing 4 h

CRITICAL: Follow the instructions reported by Mazutis and colleagues[35] to design microfluidic chips and fabricate the wafer mold (see Supplementary Data 1 for our wafer design). Ready-to-use wafer

molds can be obtained from various commercially suppliers, thus avoiding photo-lithography steps (not described here, see reference[70]). Furthermore, finished droplet makers can be obtained directly from a variety of commercial suppliers such as Dolomite, Elveflow and others, avoiding steps 1-22. If purchased, droplet makers should be capable of forming 50 pL droplets. S

1. Place the wafer on a circular piece of aluminum foil (around 2 cm larger diameter than wafer). Form a cup around the mold.

2. Dust-off the wafer mold with compressed nitrogen or air.

! CRITICAL STEP Care should be taken to remove any dust that might form clumps in the channels.

3. Place the aluminum foil cup containing the mold in a petri dish.

4. Per wafer, pour 45 g of PDMS and 5 g of curing agent (9:1 PDMS/curing (wt/wt) agent ratio) in a disposable beaker, then vigorously mix with 1000 µl pipette tip or plastic fork.

CRITICAL STEP Do not forget to add the curing agent; and mix generously. It is difficult to remove non- or semi-cured PDMS from the wafer mold.

5. Degas the PDMS-curing agent mixture using a vacuum chamber until all air bubbles are removed.

6. Slowly poor the degassed PDMS-curing agent mixture on top of the wafer from Step 3.

7. Put the Petri dish under vacuum again and degas the PDMS until no bubbles are visible. If bubbles persist, gently remove remaining bubbles with an air blower then cover the petri dish with lid to protect the PDMS from dust.

8. Bake the wafer mold-containing Petri dish in a drying oven at 65 °C for 90 to 120 min to harden the mixture.

9. Cool down to room temperature and carefully peel off the PDMS from the wafer using a scalpel. Clean the PDMS and wafer with clean nitrogen or air.

CRITICAL STEP The wafer is very fragile, any excessive pressure or bending force will break it. Wear protective glasses. Remove any under-flown cured PDMS first, and slowly remove PDMS by starting carefully, lifting the PDMS from the edges. The wafer can be re-used many times when handled properly.

PAUSE POINT The cured PDMS can be stored in a dust-free environment (petri dish) at room temperature for several months.

10. Place the PDMS on a cutting mat with the patterned side facing upward. Cut PDMS into separate microfluidic chips using a scalpel.

11. Punch with biopsy puncher (diameter 0.75 mm) into the dedicated places (inlet and outlet holes, 4 per chip. See Supplementary Data 2 for the mask for the double-sided tape to prepare the observation chamber) to make outside connections.

? TROUBLESHOOTING

CRITICAL STEP The diameter of the biopsy puncher is critical. We use a 0.75 mm-diameter biopsy puncher to make inlet holes that fits tightly a 200 µl pipette tip (for aqueous phases, Supplementary Fig. 1) and 0.3 mm PTFE microtubing (for oil phase).

12. Cover both sides of the PDMS slab with frosted Scotch tape, and then peel off the tape from both sides to remove PDMS debris that may be formed during punching. Alternatively, dust-off the PDMS slaps with compressed air or nitrogen to remove any remaining PDMS debris.

CRITICAL STEP! Repeat step 12 if necessary. Care should be taken to remove any resulting PDMS debris or dust that can otherwise clog the microfluidic chip.

13. Place a clean glass slide and the PDMS slab in the plasma chamber with the patterned side facing upward.

14. Run the oxygen plasma program following the manufactory's recommendation. We use 30s and 35 Watts.

? TROUBLESHOOTING

15. Carefully remove the glass slide and PDMS slab from the plasma chamber; then bring the treated surfaces into contact. Press the PDMS slab gently onto the glass slide to ensure uniform bonding.

? TROUBLESHOOTING

CRITICAL STEP To avoid microfluidic channel collapse and damage, only press gently on the PDMS slab. If plasma activation is successful, the chip will attach almost by itself.

CRITICAL STEP Contact between PDMS slab and glass slide should be made quickly after plasma exposition to avoid the hydrophobic reconstitution of PDMS (< 5minutes). Be careful when placing the PDMS, once contact is established, PDMS parts cannot be moved or removed.

16. Place the microfluidic device in the oven at 90°C for 5-20 min.

CRITICAL STEP The chip needs surface treatment for the formation of droplets. Perform this step directly after plasma treatment and bonding.

17. Remove the device from the oven, and cool down at room temperature.

18. Connect a 27-gauge needle to a 0.30 mm diameter PTFE microtubing using tweezers. Fill a 1 ml volume syringe with silane solution, afterwards attach a 0.22-µm Millipore filter to the syringe. Connect the needle-microtubing to the syringe-filter and fill the whole assembly with silane solution. Prepare a second syringe filled with filtered HFE-7500 only.

! CAUTION Use tweezers to hold the tubing. Take care to not pierce your skin.

! CAUTION 1H, 1H, 2H, 2H-Perfluorododecyltrichlorosilane is corrosive and highly irritant upon inhalation. Work in a fume hood. Store the product under argon; or aliquot and freeze since the silane will react with humid air rapidly and form crystals.

19. Put the microtubing into the oil inlet and flush the microfluidic channels with silane solution for about 10-30 sec.

20. After 1-minute incubation, flush the device with compressed clean air or nitrogen.

! CAUTION Stay clear of aerosol; work under fume hood. We attach a 200 µl pipette tip to the gas outlet to facilitate the connection.

21. Repeat steps 19 to 21 twice with pure HFE7500 oil to wash the microfluidic device.

22. Check for dust inclusion of microfluidic device using light microscopy, especially at the orifice for droplet generation. If dust is present, mark the chips and do not use them. Cover the PDMS chip with Scotch tape.

? TROUBLESHOOTING

PAUSE POINT The microfluidic chip can be stored in a close box at room temperature for several months. Care should be taken to protect the devices from dust.

Production of chamber mask in double-sided tape for the observation chamber. Timing 5 min

23. Load the provided mask for the double-sided tape (see Supplementary Data 2 for the mask for the double-sided tape to prepare the observation chamber) into the cutting plotter.

24. Load the double-sided tape into the plotter and initiate the machine according to the user handbook.

25. Use the according plotter software (Graphtec or Silhouette Cameo) to send the design to the plotter. Use the following conditions for plotting using the Graphtec Cutting plotter:

Parameter	**Setting**
Offset	0
Speed	25
Acceleration	1
Cut force	21
Blade offset	2.1

And using Silhouette Cameo:

Parameter	**Setting**
Blade setting	3
Speed	5 cm/sec
Thickness	33

Plot the chamber design into the double-sided tape; cut with scissors into rectangles.

CRITICAL STEP The above settings will result in a plot that cuts the top protective layer and the double-sided tape, but not the bottom protective layer.

PAUSE POINT The cut tape can be stored in a close box at room temperature for several months.

Production of glass slides with access holes. TIMING 5 min per slide

26. Produce two holes (diameter around 1 mm, see Supplementary Data 3 for the mask for laser ablation) in a 76x26x1 mm microscopy slide using laser ablation. The parameters for laser ablation will depend on the type of the machine used. We use the AxysLaser with 95% energy, 20 repetitions, and cluster 10 mm to speed up the process.

CRITICAL STEP If laser ablation is not available, good alternatives are drilling, or sand-blasting. Take care to wear appropriate safety equipment when working with the glass.

27. Rinse and clean the glass slides with acetone. Repeat this process with isopropanol then clean water. Dry with compressed air or nitrogen. Clean also a glass slide without holes (for the bottom of the chamber).

PAUSE POINT The glass slides can be stored in a close box at room temperature for several months.

Microfluidic chamber assembly

28. We use two variations of the same procedure to fabricate the droplet observation chamber. When using low numbers of beads (<500 beads per droplets ; such as the sepsis application) follow Option A, when using high numbers of beads (> 500 beads per droplet ; such as for the murine IgG application) follow Option B. Both are based on the use of double-sided tape and glass slides. The principle difference between the procedures is the equipment needed to press the chamber (binder clips in Option A; thermos-press in Option B).

A) Sepsis application. TIMING 3-5 h

i) Take a nanoport and place it on one of the outlet holes of the top microcopy slide (from steps 26-27). Use a 23-gauge needle to align the nanoport with the access holes on the glass slide (Fig. 2c).

ii) Add a drop of a UV-curable glue at the base of the nanoport and cure with UV light.

CRITICAL STEP This step must be performed carefully to avoid the clogging of the holes. Deposit a drop of viscous glue carefully which should be introduced between the slide and nanoport by capillary forces (Fig. 2c). Make sure not to move the nanoport during this process.

! CAUTION Wear appropriate eye and skin protection when using UV-light to cure the glue.

iii) Repeat steps i and ii for the second hole, and then expose the glass to oxygen plasma.

iv) Cover the top slide with the patterned double-sided adhesive tape (Fig. 2c, from step 25) then bring the bottom slide (from step 27) into contact to form 2D incubation chamber. Gently press the glass the glass chamber to ensure uniform sealing.

v) Clamp the slides together using binder clips to seal the chamber (Fig. 2c)

vi) Incubate the clamped device for at least 2 hours at 90°C.

vii) Cool the chamber to room temperature; and remove the clamps.

? TROUBLESHOOTING

CRITICAL STEP Inspect the sealing quality with an optical microscope: well-sealed device should not contain Saffman-Taylor fingers (Fig. 2d).

viii) Attach a magnet onto the bottom slide of the microfluidic chamber using double-sided adhesive tape (Fig. 2e). Proceed to step 28.

CRTICAL STEP The magnet is 3 mm thick, which is convenient for images acquisition using the inverted microscope. Do not heat the magnet since heating may affect the magnetization.

! CAUTION This magnet is very powerful and generate a strong magnetic flied (0.22T). Care should be taken during handling and keep the magnet away from objects that respond to magnetic field.

PAUSE POINT

B) Murine IgG application. TIMING 1.5 h

i) Clean the two glass slides (top slide from steps 26-27 and bottom microscopy slide from step 27) in air plasma for 10 min. If plasma is not available, this cleaning step can be replaced with frosted scotch tape or compressed air or nitrogen.

ii) Remove the protective layer from the shaped double-sided tape, and put the top slide (with holes) onto the adhesive tape. Flip, remove carefully the second layer and bring the bottom slide into contact to form 2D incubation chamber. Gently press the glass chamber to ensure uniform sealing.

CRITICAL STEP Make sure to work clean and dust-free. In our experience, the tape tends to slightly adhere to the protective layers. To reduce this problem, the tape can be put at -20°C before application. Remove carefully not to rupture or form deposits of tape. Deposits of tape will result in a chamber of incorrect height and the user will observe droplet movement during measurements.

iii) Transfer the assembly to the thermo-press, start and heat up the thermos-press to 150°C.

iv) Put the assembly for 5 minutes at 150°C, and press with 7 bars to achieve a chamber with a height of 33±2 µm.

! CAUTION Use appropriate safety equipment. The glass will be hot and might crack under pressure if deposits of dust, tape or other impurities are present.

v) Perform Step 28(A).i-ii to attach the two nanoports to the assembly.

vi) Attach magnets either on the side of the chamber with UV glue, or directly at the microscope using an appropriate support (see Supplementary Data 4 for the mask for the magnet holder).

! CAUTION This magnet is very powerful and generate a strong magnetic field. Care should be taken during handling and keep the magnet away from objects that respond to magnetic field. If beadlines appear to be points, the magnetic field is vertical instead of horizontal.

PAUSE POINT

Surface treatment of the chamber. TIMING 10 min

29. Repeat step 18 and then connect the tubing to the nanoport using microtight fitting adapter.

30. Flush the chamber with 0.5-1 ml silane solution, incubate for 1 to 3 minutes, completely flush with nitrogen and then fill with 1 ml HFE7500 oil. Close the assembly with two caps (Fig. 2e).

! CAUTION 1H, 1H, 2H, 2H-Perfluorododecyltrichlorosilane is corrosive and highly irritant upon inhalation. Work in a fume hood.

PAUSE POINT The chamber can be stored at room temperature for several months.

Nanoparticle functionalization

31. Use one of the two following approaches to functionalize the nanoparticles. One approach uses boronic acid coupling via glycosyl groups on the antibody to immobilize the antibody on the

nanoparticles to measure TNF-α secretion by single monocytes (Option A). The second approach uses the interaction between biotin and streptavidin to functionalize the beads to measure antibody secretion rate and affinity from single B cells (Option B).

A) Boronic acid coupling for sepsis application. TIMING 4 h and overnight incubation

i) Re-suspend the Carboxyl-Adembeads by pipetting and vortexing.

? TROUBLESHOOTING

! CRITICAL STEP Care should be taken to avoid foaming and bubble formation.

ii) Transfer 1 mg (33.33 µl) of beads into a clean 1.5 ml microtube.

! CAUTION Use low binding 1.5 ml tube to perform bead functionalization.

iii) Add 100 µl of activation buffer and vortex gently.

iv) Place the tube in a magnet holder for about 1 minute at room temperature to pellet the beads.

v) Carefully discard the supernatant using a 200 µl pipette.

! CRITICAL STEP Do not disturb the bead pellet. A clear supernatant indicates a complete bead collection.

vi) Remove the tube from the magnet holder, and then vigorously resuspend the beads in 100 µl activation buffer by vortexing.

CRITICAL STEP Care should be taken during washing steps. Do not allow beads to dry out. Drying reduces performances. After removing the supernatant, add buffer very quickly.

vii) Repeat steps iv – vi twice to complete the washing.

viii) Sonicate the beads for 3 minutes at room temperature at 30 W and 46 kHz. The beads are ready for EDC-sulfoNHS-based activation.

CRITICAL STEP Sonication breaks nanoparticle aggregates. The presence of aggregates reduces the efficiency of antibody coupling.

! CAUTION Wear ear protection when sonicating beads.

ix) Put aliquots of EDC and sulfoNHS (see solution setup) to room temperature for 5 to 10 minutes and prepare 10 mg/ml solution for each.

x) Mix 0.5 ml of EDC solution with 0.5 ml of sulfoNHS solution to form a solution of 5 mg/ml of activated EDC-sulfo-NHS.

xi) Place the tube containing the beads in a magnet holder for about 1 minute; and carefully discard the supernatant.

xii) Re-suspend the beads in 1 ml of activated EDC-sulfoNHS solution. Sonicate the beads for 3 minutes at room temperature.

xiii) Incubate for 20 minutes on a rotating wheel at room temperature.

xiv) Place the tube in a magnet holder for 1 min to pellet the beads.

xv) Carefully discard the supernatant.

xvi) Add 100 µl of activation buffer to the beads (see solution setup).

xvii) Remove the tube from the magnet holder and mix well by vortexing.

xviii) Repeat steps xiv – xvii twice. The beads are now activated and ready to react with boronic acid.

xix) Weigh out 50 mg of 3-Aminophenylboronic acid hemisulfate and dissolve it in 1 ml coupling buffer (later referred to as BA solution).

xx) Place the tube in a magnet holder for 1 min to pellet the beads.

xxi) Carefully discard the supernatant.

xxii) Re-suspend the beads in 1 ml BA solution, and then sonicate them for 3 minutes at room temperature.

xxiii) Incubate for 2 hours on a rotating wheel at room temperature.

xxiv) Place the tube in a magnet holder for about 3 minutes at room temperature.

xxv) Carefully discard the supernatant. Do not disturb the bead pellet.

xxvi) Add 100 µl of PBS to beads.

xxvii) Remove the tube from the magnet holder and mix well by vortexing.

xxviii) Repeat steps xxiv - xxvii twice.

xxix) Pellet the beads by placing the microtube in a magnet holder for 1 min.

xxx) Carefully discard the supernatant. Do not disturb the bead pellet.

xxxi) Re-suspend the beads in 500 µl of 100 mM ethanolamine to quench the reaction.

! CAUTION Ethanolamine is an irritant substance; handle it carefully under a chemical hood.

xxxii) Sonicate for 3 minutes, and then incubate 30 min at room temperature on a rotating wheel.

xxxiii) Place the tube in a magnet holder for about 1 minute at room temperature.

xxxiv) Carefully discard the supernatant. Do not disturb the bead pellet.

xxxv) Add 100 µl of PBS to beads.

xxxvi) Remove the tube from the magnet holder and mix well by vortexing.

xxxvii) Repeat steps xxxiii - xxxvi twice.

xxxviii) Re-suspend the beads in 50 µl PBS by vortexing, then sonicate for 3 minutes at room temperature.

xxxix) Add 50 µg of antibody (50 µl of 1mg/ml) to 1 mg of boronic-coated beads.

! CAUTION The anti-TNF-α antibody is provided in PBS buffer without sodium azide and users should work under a biological safety cabinet and aseptic conditions to avoid any contamination.

CRITICAL STEP For optimal coupling we recommend testing 10 to 50 µg of antibody per mg of boronic-coated beads. If other antibodies are used, this optimum might be different.

CRITICAL STEP Antibody coupling will only be successful if the antibody is glycosylated.

CRITICAL STEP If other antibodies are used, these antibody solutions must be free from sodium azide, storage proteins or amine-based buffers (such as HEPES, MOPS, TRIS etc.) that could interfere with the coupling procedure. If present, the buffer needs to be exchanged using a desalting spin column or similar.

xl) Mix gently; then incubate overnight at 4 °C whilst gentle shaking.

xli) After overnight incubation, place the tube in a magnet holder for 1 min to pellet the beads.

xlii) Carefully discard the supernatant whilst not disturbing the bead pellet.

xliii) Add 100 µl of PBS to beads.

xliv) Remove the tube from the magnet holder and mix well by vortexing.

xlv) Repeat steps xli – xliv twice.

xlvi) After washing, re-suspend the beads in 200 µl storage buffer. The concentration of beads is 5 mg/ml (1 mg in 0.2 ml).

xlvii) Sonicate the beads for 1 to 2 minutes at room temperature and store the Ab-coated beads at 4°C.

CRITICAL STEP Keep the beads at 4 °C and do not freeze. Freezing will cause the beads to aggregate and reduces the sensitivity of the bioassay.

PAUSE POINT The antibody-coated nanoparticles are stable for at least 2 months at 4°C without affecting the performance of the bioassay. Longer storage time will need to be determined by users.

xlviii) For experiments, vortex the beads vigorously for 1 minute to re-suspend the formed pellet.

xlix) Re-suspend 10 µL of antibody-coated beads in 100 µl blocking buffer.

CRITICAL STEP Prior to using the beads in a binding assay, treat the beads with 1% dextran blocking solution (vol/vol) to reduce non-specific binding.

l) Incubate for 45 minutes at room temperature, shaking at 700 rpm.

li) Place the tube in a magnet holder for about 1 minute to pellet the beads.

lii) Carefully discard the supernatant. Do not disturb the bead pellet.

liii) Add 100 µl of PBS buffer to beads.

liv) Remove the tube from the magnet holder and mix well by vortexing.

lv) Repeat steps li – liv twice.

lvi) After the washing step, sonicate the beads for 1 min at room temperature. Use immediately for the assay.

B) Biotin-streptavidin coupling for the murine IgG application. TIMING 2 h

i) Re-suspend the Streptavidin plus-Adembeads by vortexing for 10s.

? TROUBLESHOOTING

ii) Per experiment, transfer 25 µl of beads into a clean low-binding 1.5 ml microtube. Add 25 µl of PBS per 25 µl of nanoparticles.

! CRTICAL STEP Adapt accordingly if more or less nanoparticles are wanted in a droplet (see also Box 1 and 2).

iii) Add 2.5 µl of Ig immobilization reagent stock to each 50 µl bead solution. Deposit the drop at the top of the Eppendorf, and wash down with beads. Mix by pipetting, and incubate for 20-30 min at room temperature.

! CRITICAL STEP For other biotinylated Antibodies, add 5 µl of a 0.5 mg/ml stock to 100 µl nanoparticles volume. This might need optimization.

iv) Pellet the beads by placing the microtube in a magnet holder for about 1 minute.

v) Carefully discard the supernatant.

! CRITICAL STEP Do not disturb the bead pellet. A clear supernatant indicates a complete bead collection.

vi) Remove the tube from the magnet holder, and then vigorously re-suspend the beads in Blocking Buffer by using the respective volume (same total volume as used in step ii). Incubate for 20-30 min.

CRITICAL STEP Care should be taken during washing steps to not loose particles due to inefficient re-suspension or collection. Do not allow beads to dry out as drying reduces performance.

vii) Afterwards, repeat steps iv and v.

viii) Add Cell Buffer 1 (the same volume as used in step ii). Re-suspend the beads, and incubate for 20-30 minutes to block the bead surfaces.

PAUSE STEP: The beads can be stored overnight at 4°C after this step; and used on the next day.

ix) Shortly before experiments, repeat steps iv and v.

Quality control of functionalized nanoparticles

32. Assess the binding capacity of immobilized antibody using a fluorescent-based immune-sandwich assay in microtiter plates (bulk assay; Box 4) or within droplets. For the droplet microfluidic assay for the sepsis application, follow Option A. For the droplet microfluidic assay for the murine IgG application, follow Option B.

A. Detection of TNF-α using a droplet microfluidic assay. Timing 2 h

CRITICAL: By following this protocol and the respective dilutions, this will lead to a beadline capacity of around 3 nM within each droplet; and a number of nanoparticles of around 170 per droplet.

i. Prepare the syringes for the protein solutions by connecting a 0.56 mm diameter PTFE microtubing to 200 µl pipette tips using PDMS slabs (6 mm diameter, with a hole in the middle of 0.75 mm diameter, see also Supplementary Fig. 1), and UV light NOA curing. Connect the tip-tubing assembly to a 27-gauge needle using tweezers, and then screw the needle to the syringe. Fill the whole system with HFE-7500.

ii. Prepare a second syringe for the reagents assay sample as described in step i.

iii. Prepare a syringe for the continuous phase by putting a 23-gauge needle on the syringe, then connect it to a 0.3 mm diameter PTFE microtubing using tweezers and fill the whole assembly with HFE-7500 oil containing 2% (wt/wt) fluorosurfactant.

! CRITICAL STEP Care should be taken to remove any air bubbles. If air bubbles are present, hold the syringe in a vertical position with the opening facing upwards and push the plunger until the air is completely removed.

iv. Mount the syringes on the syringe pumps and prime the assembly by applying a flow rate of 1000 µl/h for the reagent syringes to remove air bubbles from the assembly (syringes from step i and ii, reagent syringes) for about 30 seconds. The station is ready to load the samples.

v. Take antibody-coated beads from step 31(A).lvi, pipette 10 µl of antibody-coated beads into new 1.5-ml tube and then sonicate for 3 minutes at room temperature.

? TROUBLESHOOTING

vi. Prepare the assay reagents for syringe 1 as follows: Add PE anti TNF-α antibody to a final concentration of 0.4 µg/ml (from 20 µg/ml stock) and antibody-coated beads at a concentration of 0.2 mg/ml (from 5mg/ml stock) in cell buffer 2. For each calibration point, prepare around 25 µl.

CRITICAL STEP Centrifuge the PE anti TNF-α antibody at 10,000 g for 5 minutes at 4 °C to remove aggregates.

vii. For syringe 2, prepare dilutions of recombinant TNF-α to desired concentration in cell culture media (0-100 nM).

B. Detection of murine IgG using a droplet microfluidic assay. Timing 2 h

CRITICAL: By following this protocol and the respective dilutions, this will lead to a beadline capacity of around 60 nM within each droplet, and around 1,200 nanoparticles per droplet. Each concentration counts as an experiment, so prepare a sufficient volume of beads (Step 31(B)i-ix).

i) Follow Step 32(A).i-iv.

ii) Take the functionalized nanoparticles (25 µl of nanoparticles per experiment) from step ix (Nanoparticle functionalization, option B, Biotin-streptavidin coupling for IgG application). Collect nanoparticles, discard supernatant and re-suspend the beads in the same volume of fresh cell buffer 1. Add to the following reagents(all final concentrations): 150 nM AffiniPure $F(ab')_2$

Fragment Rabbit Anti-Mouse IgG, Fcγ fragment specific, 60 nM Zenon™ Alexa Fluor™ 488 Mouse IgG2b Labeling Kit; and 60 nM fluorescent antigen of interest.

CRITICAL STEP Centrifuge the fluorescent proteins at 10,000 g for 5 minutes at 4 °C to remove big aggregates before taking the solution. As for the preparation of fluorescent molecules, refer to Box 1 and 2.

CRITICAL STEP Over prolonged storage time, the beads will slowly sediment. Re-suspended every hour by using a pipette.

? TROUBLESHOOTING

iii) For calibration, prepare a dilution series of murine calibration Ig in cell buffer 1. Start with 200 nM, and divide concentration by a factor of two down to 6.25 nM. Do not forget to prepare IgG cell buffer 1 with no added IgG as a blank measure.

CRITICAL STEP Before calibrating your own antibodies, we recommend to use murine control isotypes (such as supplied by ThermoFisher) for a general calibration of the system.

33. Generation of droplets. Place the pipette tips of the sample syringes in a sample tube (Supplementary Fig. 1), one in the desired concentration of calibration solution and the other in the final bead solution, and aspirate 25 µl at 2000 µl/h rate of the beads and the calibration solution.

34. Connect the syringes to the corresponding inlet holes of the microfluidic device and connect the device outlet to waste with 0.30 mm diameter PTFE microtubing.

35. Set the flow rates of aqueous solutions and oil phase at 100 µl/h, then start the pumps to inject fluids into the chip. Once the droplet production is stabilized and the fluid leaves the outlet (around 8-10 µl volume used), increase the flow rate of oil to 300 µl/h to generate droplets of 50 pL volume.

36. In order to collect the droplets, connect the microfluidic device outlet to the incubation chamber (from step 28(A) or 28(B) according the application) using a new piece of 0.3 mm diameter PTFE microtubing. Attach the tubing to the nanoport of the observation chamber using microtight fitting adapter. Fill the chamber with droplets (visible as a grey, homogenous and turbid layer). Once filled, remove the tubing and fluid remaining in the nanoports, and close the chamber with the caps.

? TROUBLESHOOTING

CRITICAL STEP The loading of the cells in a pipette tips (rather than a syringe), the encapsulation process and the chamber filling should take less than 5 min, and is typically performed at room temperature. If longer operating time is required, density-matching agents (such as OptiPrep, Percoll, Methyl Cellulose) can be added to the cell buffer. However, cells can also be cooled to 4°C to reduce

secretion before encapsulation in droplets, if required, though this may slow secretion rate, at least initially, after encapsulating and changing to the desired temperature for secretion measurements. To cool, a small hole is drilled or punched into the bottom of a 2 ml Eppendorf tube. The pipette tip is then introduced into the chip through the Eppendorf tube and glued in place. The volume between the pipette tip and the tube is filled with iced water during droplet loading.

CRITICAL STEP Clean the outside surfaces of the incubation chamber with isopropanol and water before filling. Apply Scotch tape to the glass chamber to remove residual dust particles.

37. Transfer the filled chamber to the microscope for imaging acquisition (Steps 38-45) and repeat steps 33-45 for all other calibration solutions.

? TROUBLESHOOTING

Image acquisition. Timing 1 h

38. Start the epifluorescence microscope and set the heating at 37°C at least 30 minutes before starting acquisition.

CRITICAL STEP Care should be taken so that the experiment is performed at 37°C as the biological responses are optimal at this temperature. Imaging at room–temperature will result in a reduced cellular secretion rate and may also interfere with cellular secretion *per se*.

39. Open the acquisition software and set the image acquisition program. We typically perform quality controls of the droplets by acquiring manually a few images using a 10X objective (BF (20 ms), DAPI (300 ms - 1 sec), GFP (200 - 500 ms), CY3 (200ms - 1 sec), and CY5 (200 - 500 ms). Once acquisition exposure times have been defined, they should not be modified for a defined assay if a calibration curve is performed.

CRITICAL STEP Clean the objective before imaging.

CRITICAL STEP Exposure times will depend on the exact setup used, and may be adapted depending on the assay and reagents used. Fix exposure time to ensure absence of saturation and optimal fluorescence quantification.

? TROUBLESHOOTING

40. Position the chamber on the microscope stage insert (Supplementary Fig. 2).

- *Chamber for sepsis application:* When one magnet is attached to the chamber, place the chamber so that the magnet is located on the left when facing the microscope; the beadline is located in the left part of each droplet on the acquired bright-field image.
- *Chamber for IgG application:* Position the chamber on the microscope stage insert; and place the magnet holder above it so that the magnet is located in the front and back of the stage. Check with bright-field: The beadline should be located in the top or bottom part of each droplet and span over 2/3 of the droplet diameters; and should be vertical or horizontal in orientation.

41. Start the Perfect Focus System (PFS).

CRITICAL STEP This step is very critical as it allows maintaining the focus at different locations of the incubation chamber over time (corrects for potential drift of focus over time and scan).

42. Use the bright-field channel and adjust the focus manually so that the contours of droplets and beadlines appear as dark objects on the bright-field image (to facilitate automated detection).

CRITICAL STEP This step is very critical since sharp focus is needed for optimal signals as well as automated analysis.

43. Position the objective in the middle of the incubation chamber and then set up the imaging matrix to be acquired using the microscope software.

CRITICAL STEP Do not acquire images close to the magnet. Before imaging and after installation of the chamber, wait for 2-5 min for the temperature to equilibrate to reduce droplet movement due to this difference.

44. After the completion of the experiment, save the images as ND2 files and switch off the microscope and the heating system.

? TROUBLESHOOTING

45. At this step of the protocol, quality control of the images is needed. Potential issues are undesired movement of droplets during acquisitions, aggregation of nanobeads, irregular distribution of beads within the droplets, presence of clusters, more than one layer of droplet with the imaging microfluidic chamber (see Table 2 in the Troubleshooting section).
Quality control movement: Open the images in the software, choose the bright field image and focus on a single field of view. Activate the time-lapse function, the software will show you the images over time. If no movement is visible (<1/10 of the diameter can be tolerated), repeat this step for two other fields of view. A small fraction of droplets (<2%) might rearrange their position, these need to be removed manually or tracked.
Quality control droplets and layers: Similarly, the presence of droplets with varying diameter (>95% should be similar in diameter) can be easily assessed by looking at the bright field images, as well as the presence of multiple layers of droplets. In the former case, some data might be useable, in the latter there is no other way than to repeat the experiment. We recommend repeating the experiment in both cases.
Aggregation of nanobeads: Aggregates of nanoparticles can also be seen in the bright field image. Usually, these are potato-shaped clusters that show bright fluorescence. Therefore, also check in the empty droplets where no secreting cell is present. These can lead to false-positive events (see also Troubleshooting Table 2).
Irregular distribution of beads within the droplets: This phenomenon can be checked in bright field; and the fluorescence channels. Often, this issue is accompanied by droplets showing different levels of fluorescence (brighter, darker droplets), and speaks for problems during encapsulation. We recommend repeating the experiment in this case.
Presence of clusters: The presence of cell clusters can also be checked in bright field. A relatively easy control is to calculate the cells/droplet ratio, and compare the distribution of individual cells/droplets with the expected distribution following Poisson. If no other issues occur, this data

might still be usable, but is usually followed by an uneven distribution of dyes and antibodies as well /see above); making its analysis difficult.

Cell measurements.

46. The protocol can be used for a variety of different cells. The first application uses human monocytes from healthy donors and septic shock patients (Option A), whereas the second application uses murine IgG-secreting cells from immunized animals that were extracted from spleen and bone marrow (Option B). Other tissues or cell types can also be analyzed. However, cell preparation should be rapid and gentle, generating a singularized suspension of un-clumped cells.

A) Sepsis application. TIMING 5.5 h

i) Start the laminar flow hood for cell culture 15 minutes before starting cell extraction.

ii) Use EasySep Direct human monocyte isolation kit from STEMCELLS technologies to isolate highly pure CD14+ monocytes from human whole blood.

CRITICAL STEP This protocol allows for the isolation of live cells without using density gradient centrifugation or cell lysis buffers. For optimal recovery of monocyte isolation, use EDTA as an anticoagulant to collect blood samples. Follow EasySep Kit instructions to isolate monocytes from 3 ml of human blood.

iii) After the end of the EasySep protocol, pipette 7 µl to count the cells using FastRead 102 slides.

iv) Add 20 µl of the fluorescently labelled anti-CD14 antibody stock to the cells to stain monocytes.

v) Incubate for 12 minutes at room temperature in dark.

vi) Centrifuge the cells at 2800g for 7 minutes at room temperature or 22-25 yy°C and carefully remove the plasma from the cell pellet.

CRITICAL STEP Carefully discard the supernatant. Do not disturb the cell pellet.

CRITICAL STEP If residual red blood cells are present in the isolated cells, re-suspend the pellet in PBS for one additional separation step. The red blood cells can be also removed by lysis using Ammonium Chloride Solution from STEMCELL technologies (cat.no 07800).

vii) Take antibody-coated beads from step 31(A)lvi, pipette 10 µl of antibody-coated beads into new 1.5 ml tube and then sonicate for 3 minutes at room temperature.

? TROUBLESHOOTING

viii) Re-suspend at 10^6 monocytes from step vi per 100 µl of cell culture buffer containing 20 mM HEPES. This is the final cell sample.

ix) To a separate volume of cell buffer 2, add the following assay reagents (all final concentrations): 0.5 µg/ml LPS, 0.4 µg/ml PE anti TNF-α, 0.2 mg/ml antibody-coated nanoparticles, Nucview and Mitoview probes at 2X.

? TROUBLESHOOTING

CRITICAL STEP LPS is omitted for control experiment. Centrifuge the PE anti TNF-α antibody at 10,000 g for 5 minutes at 4 °C to remove aggregates.

x) Prepare the syringes for cell sample and reagent assay samples as described in Step 32(A)i.

xi) Prepare a syringe for the continuous phase as described in Step 32(A)iii.

! CRITICAL STEP Care should be taken to avoid air bubble formation. If air bubbles are formed, hold the syringe in a vertical position with the opening facing upwards and push the plunger until air bubbles are completely removed.

xii) Mount the syringes on the Nemesys pump and prime the assembly by applying a flow rate of 1000 µl/h for about 50 µl. Discard fluid. The station is ready to load the samples.

xiii) Place the pipette tips in the sample tubes containing cell sample and assay reagents separately (Supplementary Fig. 1) and aspirate 50 µl at 2000 µl/h rate.

xiv) Connect pipette tips to the corresponding inlet holes of the microfluidic device and connect the device outlet to waste with 0.30 mm diameter PTFE microtubing.

xv) Set the flow rates of aqueous solutions and oil phase at 100 µl/h then start the pumps to inject fluids into the chip. Once droplet production is stabilized (1-2 min), increase the flow rate of oil to 300 µL/h to generate droplets of 50 pL volume.

? TROUBLESHOOTING

xvi) In order to collect the droplets, connect the microfluidic device outlet to the incubation chamber using new PTFE microtubing. Fill the chamber with droplets (visible as a gray, homogenous and turbid layer). Once filled, remove the tubing and fluid remaining in the nanoports, and close the chamber with the caps.

xvii) Immediately proceed to image acquisition as described in steps 38-45. In Step 39, use the following image acquisition settings: BF (20 ms), DAPI (1 sec), GFP (500 ms), CY3 (1 sec), and CY5 (500 ms) images using a 10X objective. 75 fields (25x3) every 30 minutes during 3 hours (7 time points). If a calibration curve is used for secretion, the same acquisition exposure times defined at step 39 need to be used.

? TROUBLESHOOTING

CRITICAL STEP If the chamber is not clean, wipe the surfaces with ethanol, or dust off using compressed air or scotch.

B) Murine Ig-measurements application. TIMING 2 h

i) Prepare spleen, bone marrow or other organs of interest according to the established protocol of your laboratory, or follow reference[38]. Note that the cells should be well-suspended, and present as individual cells to proceed and give optimal results.

CRITICAL STEP In our experience, different protocols will result in slightly different secretion rates that are measured. Harsh conditions and protocols, i.e. strong shear forces or chemical stress, may result in decreased secretion rates.

ii) Count cells in the suspension, and centrifuge the volume equivalent of 2 million of cells. Re-suspend the cells in PBS.

iii) Add 1 µl/ml of CellTrace Violet (5 mM stock in DMSO); mix by pipetting and incubate the cells for 15-30 min at 37°C. Dilute the cells afterwards in cell buffer 1 to quench the reaction.

iv) Take the beads from the assay from Step 32(B).ii

v) Centrifuge the labelled cells at 400g for 5 min and carefully remove the supernatant from the cell pellet. Re-suspend gently but fully at 150 µl of Cell buffer 1. Proceed immediately to cell assay and encapsulation.

CRITICAL STEP The concentration of cells is optimized to result in the optimum amount of droplets containing an individual cell. Work fast, and proceed directly to the encapsulation since the cells will continue to secrete in bulk.

CRITICAL STEP The cellular density is quite high in this volume. For short term (< 1-2 h), the cells can be stored on ice. Longer storage will result in a significant decrease in Ig secretion and cellular response.

? TROUBLESHOOTING

vi) Prepare the syringes for cell sample, bead solution as well as continuous phase as described in Step 32(A)i and Step 32(A)iii.

vii) Mount the syringes on the Nemesys pumps and prime the assembly by flowing 50 µl of solutions with a flow rate of 1000 µl/h. Clean the pipette tips from oil by wiping them with tissue. No air bubbles should be visible now in the pipette tips.

viii) Place the syringes with added pipette tips in the sample tubes (Supplementary Fig. 1), one in the prepared cell suspension and the other in the final bead solution, and aspirate 50 µl at 2000 µl/h rate of the beads and the calibration dilution.

ix) Connect the syringes to the corresponding inlet holes of the microfluidic device.

x) Set the flow rates of aqueous solutions and oil phase at 100 µl/h then start the pumps to inject fluids into the chip. Once the droplet production is stabilized, increase the flow rate of oil to 300 µl/h to generate droplets of 50 pL volume.

? TROUBLESHOOTING

xi) In order to collect the droplets, connect the microfluidic device outlet to the incubation chamber using a 0.30mm diameter PTFE microtubing.

xii) Immediately proceed to image the droplets as described in steps 38-45. In Step 39, use the following image acquisition settings: DAPI (300 ms), GFP (200 ms), CY3 (200 ms), CY5 (200 ms) and BF (20 ms) images using a 10X objective. 100 fields of view (10x10) every 10 minutes during 60 min (7 time points). If a calibration curve is used for secretion, the same acquisition exposure times defined at step 39 need to be used.

Automated image processing.

47. For image processing of data obtained for the sepsis application, follow Option A. For image processing of data obtained for the murine IgG measurements, follow Option B.

A) Sepsis application. TIMING 4 h

i) Extract images from ND2 files (Nikon file format) and save them in a TIFF format: 5250 images are extracted per experiment, including both the kinetics acquired with and without LPS.

ii) Open the input_parameter.txt file and set the required parameters (path to the folder containing the input images, vector of field numbers, vector of time points, various display and saving parameters, etc).

iii) Run the standalone Matlab application DropCell.exe (https://doi.org/10.6084/m9.figshare.11336663.v1).

CRITICAL STEP Image data without LPS and with LPS must be located in different folders and be analyzed separately.

CRITICAL STEP Make sure of the presence of five TIFF images per field in the input data folder, named according to the required naming rules.

CRITICAL STEP Make sure (by checking a few bright-field images) that the beadline is always located in the left-hand-part of the droplets, and that its length is lower than the third of the droplets diameter.

CRITICAL STEP Modify the input_parameters.txt carefully, observing format instructions.

CRITICAL STEP Do not open Excel while running DropCell.exe.

CRITICAL STEP Check the suitability of the analysis parameters on a few fields and time points before running the analysis of an entire experiment.

iv) Process the output STATdrops_sorting_YYYY-M-D_H-M-S.xlsx file obtained after the execution of DropCell.exe is completed. The Single-cell sheet focuses on the single-cell-containing droplet. CY3 relocalization fluorescence signal (signal-to-noise ratio) per droplet and time point is given in the columns “t**_Signal_CY3”. “trackingfail_time”, “rodfail_time”, “death_time” and

"phagocytosis_time" columns are used, when setting appropriate filters, to select the droplets of interest (single, live, and non-endocytic cell through the entire kinetics)

B) Murine Ig-measurements. TIMING 3-4 h

i) Extract images from ND2 files (Nikon file format) and save them in a TIFF format (Nikon file format; 1 TIFF file per time point) and transfer the images to the workstation for calculation.

ii) Open the Matlab script and run the provided script (Analyzer.m, https://github.com/LCMD-ESPCI/dropmap-analyzer).

iii) A window will pop up. Within the first line, define the channels where you want to measure the beadline (as channel index within the file, see also readme file on the GitHub). Next, define the bright field channel; this will be used to detect the droplets. Define the number of time points and the average droplet radius in pixels. If you follow the protocol described herein, 25 pixels is a good a starting point for 50 pL droplets.

CRITICAL STEP The algorithm is sensitive to the droplet diameter you define. You might need to increase or decrease the diameter if you change certain variables in the protocol.

iv) The output files are a Microsoft Excel file wherein every channel is a separate sheet, sorted by columns for beadline signal over time, average droplet signal over time and ratio of the two values over time. In addition, Matlab file where the images of the recognized droplets are saved by index.

v) Use the calibration curve to transfer ratios to concentration of analyte.

CRITICAL STEP Be sure to take the right curve for the proper channel.

vi) Please refer to Box 2 for the extraction of parameters. Sort and select droplets of interest in a separate sheet and combine the information.

vii) After analysis, check the selected droplets with the verify function (verify.m) to exclude false-positive events. The verif program will show the droplets to verify their content. It will ask for a list of indices to check.

CRITICAL STEP Control the selected events for false-positive events (dead cells or other); and exclude those from further analysis.

Chamber cleaning after experiments. TIMING 5 min

CRITICAL If desired, the chambers can be used over 4-8 weeks for multiple experiments. After the measurements, the droplets should be removed from the chamber before storage.

48. Repeat step 18 but fill the syringe with HFE7500 oil. Connect the tubing to the nanoport using microtight fitting adaptors.

49. Flush the chamber with 1 ml HFE7500 oil to remove droplets.

50. Close the nanoport chamber with caps and keep it in a dust-free environment.

TROUBLESHOOTING

Troubleshooting guidance can be found in **Table 2.**

Table 2. Troubleshooting Table.

Step	Problem	Possible reason	Solution
11	Cracking in the inlets or outlets during punching.	Blunt or clogged puncher.	Use a new puncher.
		Insufficient mixing of PDMS and curing agent.	Vigorously mix PDMS and curing agent to achieve a homogenous mixture of the two components. Do not cure for prolonged times (> 24 h).
14	Microfluidic device delaminates during surface treatment.	Plasma protocol was not optimal.	Check and adapt the parameters of your plasma procedure.
		Not sufficiently cleaned glass slides.	Use clean glass slides: wash the glass slides with acetone, isopropanol, milli-Q water. Repeat if necessary.
15, 18-22	No droplet formation during experiments.	Collapsed microfluidic channels.	Reduce force when sealing PDMS slab to glass slide.
		Aqueous solutions wet the microfluidics channels.	Repeat the surface treatment of the device.
		Dirt present within the microfluidic assembly.	Observe microfluidic chips after fabrication for crystal formation, or the presence of dust particles.
		Absence of sufficient concentration of surfactant.	Use HFE7500 with added 2.0% fluorosurfactant-008.

28 (A) i-vii.	Microfluidic chamber leakage.	Inefficient sealing.	Use clean glass slides and work clean. Increase the duration of incubation during step 28 (A) vi. Make sure that the nanoports are well sealed with UV glue.
36, 46 (A) xvi, 45	Observed movement of droplets during acquisitions.	Droplet coalescence occurs.	Increase the concentration of surfactant.
		Chamber height is bigger than droplet diameter.	Fabricate a chamber with proper height (< 90% of droplet diameter). Make sure that the height of the chamber is optimal for your droplet volume.
		Presence of air within the chamber.	Remove air bubbles before acquiring images.
31 (A) i and 31 (B) i		Microfluidic chamber is leaking.	Check steps 27 and 28 (A) i – vii.
	Aggregation of nanobeads.	Inherent to the beads. Long term storage of beads.	Increase sonication time. It should be noted that extended sonication time, can heat the nanoparticles denaturing the functionalized antibodies. Ice can be added to the bath to limit nanoparticle overheating.
32 (A) i, 32 (B) i, 37, 46 A) vii	Irregular distribution of beads within the droplets, presence of clusters.	Beads aggregates during coupling.	COOH beads: increase sonication time. Streptavidin-beads: Bead-crosslinking due to over-biotinylated immobilization reagent. After step 31 (B) iii, add 10 µM biotin before

		During storage of coupled beads.	collecting the beads. Incubate for 5-10 min before collection. Reversible formation of clusters is normal, but should be reversible by pipetting or sonication.
BOX4 step18, 44	Failure to detect the target molecule.	Insufficient coupling efficiency.	Adapt concentration or incubation times. Use a fluorescent antibody to establish optimal labelling of beads. Additionally, in our experience, fluorescent analyte (TNF-A488, or Ig-A488) helps greatly to analyze problems.
		Positive calibration samples did not result in fluorescence relocation.	Make sure that calibrate is not degraded. Make sure of calculations of concentrations. Make sure that no free dye is present in the fluorescent detection antibody solution. Remove free dye by centrifugation with molecular cut-off spin columns.
		In cell experiments.	Use alternative method to confirm cellular secretion of the analyte (ELISA; ELISPOT; flow cytometry with brefeldin A or similar).
36, 46 (A) xvi	More than one layer of droplet is formed.	The thickness of chamber is bigger than droplet diameter.	Reduce the thickness of the chamber or increase the volume of droplets.

46 (A) xv 46 (B) i	Cell aggregates and clumps are present in the microfluidic chip during encapsulation.	Nucleic acids are released from dead cells.	Add Benzonase to cell suspension for DNA and RNA removal.
46 (A) ix 45	Cells are not fluorescent.	Cells are necrotic. Live and dead cells staining protocol is not optimal.	Discard these cells from the analyses. Use a probe that can detect necrotic cells. Optimize the concentration of live/dead probes.
39-45	Rapid decrease of droplet and beadline fluorescence	Photobleaching	Optimally, reduce illumination intensity and/or exposure time. Otherwise, reduce the total number of time points.

1243

1244

1245

TIMING

Steps 1-22	Droplet maker fabrication	4 h
Steps 23-30	Observation chamber fabrication	2-5 h
	Microfluidic chamber assembly	
	28(A) Sepsis application.	3-5 h
	28(B) Murine IgG application.	1.5h
Steps 31(A)- 31(B)	Nanoparticle functionalization	
	31(A) Boronic acid coupling	4h, ON incubation
	31(B) Biotin-streptavidin coupling	2h
Steps 32-45	Quality control of functionalized nanoparticles	
	BOX 4 Microtiter plate assay	3h
	32(A) Detection of TNF-α droplet assay	2h
	32(B) Detection of murine IgG droplet assay	2h
	Steps 33-45 Droplet generation and image acquisition	1h
Steps 46(A) – 46(B)	Cell measurements.	
	46A) Sepsis Application	5.5h
	46B) Murine Ig-measurements application	2h
Steps 47(A) – 47(B)	Automated Image processing.	
	47(A) Sepsis application	4h
	47(B) Murine Ig-measurements	3-4h

Anticipated results

The DropMap system is a flexible platform for high-throughput single-cell phenotypic analysis that is particularly well adapted to the characterization of immune responses. The protocol can be modified to allow the single cell quantitative analysis of a wide range of cell-surface markers and secreted molecules expressed by a range of different cells types, simultaneously with other cellular characteristics such as endocytosis and viability. The development described here expands its potential from the analysis of humoral to cell-mediated and innate immune responses.

The ability of DropMap to simultaneously measure secretion rates and affinities of antibodies from thousands of single B cells makes it a powerful tool to study humoral immune responses[38], for example following vaccination[71, 72] or infection. Furthermore, the large data sets from these single-cell experiments are well adapted as training sets for deep learning/artificial intelligence strategies, and allow *in silico* modeling and simulation of the immune responses following vaccination. Such strategies could ultimately contribute to the reduction or replacement of animal experiments.

The system is also well adapted to study the role of T cells in both humoral and cell-mediated immune responses. Given their constitutive role in immune-surveillance, aberrant T cell functions are linked to autoimmune diseases as well as to carcinogenesis and to defective protection against pathogen infections [73, 74]. In this protocol, we demonstrate our approach by integrating the measurement of secretion of a range of murine and human cytokines produced by T cells identified using a fluorescent anti-CD3 antibody. For example, we show here the measurement of secretion rates of TNF-α, IFN-γ, IL-2 and IL-4 from human T cells (Fig. 5, LODs of 10.4 nM, 0.6 nM, 0.5 nM and 0.2 nM for human IL-2, IL-4, TNF-α and IFN-γ, respectively). Distribution of secretion rates are shown for *ex vivo* stimulated and unstimulated peripheral blood mononuclear cells (PBMCs; stimulated with PMA/Ionomycin).

Interestingly, stimulation with PMA/ionomycin did not significantly affect the median secretion rates for TNF-α (N=4 independent experiments, unstimulated 25 ± 13 molecules/s; stimulated 28 ± 15 molecules/s, p-value 0.77, mean ± standard deviation of the median values for the independent experiments, two-tailed unpaired t-test), but significantly increased the percentage of secreting T cells by around 8-fold (from 2.5 ± 0.6 % to 17 ± 11 %, mean ± standard deviation, p-value 0.05, two-tailed unpaired t-test, Fig. 5a). In contrast, upon stimulation, there were significant increases in the frequency and secretion rates of T cells secreting INF-γ (N=4 independent experiments, 1 ± 1 % vs. 19 ± 5 %, p-value 0.004; and 4.7 ± 2.8 molecules/s vs. 22.5 ± 12.8 molecules/s, p-value 0.03, both mean ± standard deviation of the median values for the independent experiments, two-tailed unpaired t-test, Fig. 5b), a pro-inflammatory cytokine signature for Th1 T cells subset that is critical for fighting

viral infections[75-77]. The average median rate of IL-2 secretion by T cells (N=3 independent experiments, 0 ± 1.1 % vs. 24 ± 10 %, p-value 0.01, mean ± standard deviation; and 43 ± 5 molecules/s vs. 73 ± 18 molecules/s, p-value 0.05, mean ± standard deviation of the median values for the independent experiments, two-tailed unpaired t-test, Fig. 5c) was equivalent with or without stimulation, while a 10-fold increase of the frequency of IL2 secreting cells was found in the stimulated sample. A low frequency (<2%) and low (but measurable) secretion rates (N=3 independent experiments, 0.6 ± 0.7 % vs. 1.7 ± 0.7 %, non-significant in a two-tailed unpaired t-test, mean ± standard deviation; and 4.4 ± 3.2 molecules/s vs. 4.2 ± 2.7 molecules/s, non-significant in a two-tailed unpaired t-test, mean ± standard deviation of the median values for the independent experiments, Fig. 5d) were observed for IL-4 secreting cells in both samples. The detection of IL-4 at low expression level in a small subset of cells nicely illustrates the advantages of DropMap, and its use for the analysis of cytokine secretion by T cells at the single-cell level. This opens up new opportunities to improve our fundamental understanding of immune responses, for example to study T cell plasticity[78], which may lead to the development of novel therapeutic strategies. It is also a promising approach for clinical single-cell immuno-monitoring for patient stratification and personalized medicine.

We also extend the DropMap system to analyse the innate immune responses and to monitor the dysfunction of monocytes from septic shock patients (Table 1). Septic shock is the most severe form of sepsis[79, 80]. Sepsis is a complex clinical syndrome characterized by an early dysregulated inflammatory response. This response, triggered by the recognition of microbial patterns by receptors such as Toll-like receptors (TLR), is often followed by a post-infective complex immune dysfunction combining innate and adaptive immune defects, such as monocyte dysfunction, characterized by decreased TNF-α production and release[81].

To evaluate the potential of DropMap in this context, we first controlled its ability to maintain viable primary human monocyte population in a confined environment over the course of an experiment with and without pro-inflammatory lipopolysaccharide (LPS). For all experiments, only a small fraction of cells showed apoptotic activity (0.49 ± 0.09 % for N=3 healthy donors and 1.94 ± 1.75 % for N=3 septic patients, mean ± standard deviation for the independent experiments, Table 3), demonstrating that the encapsulation process allows functional analysis.

Deactivation of circulating monocytes is currently viewed as the soundest indicator of sepsis-induced immunosuppression[79, 80] and reduced production rate of TNF-α by monocytes in bulk, upon *in vitro* stimulation by LPS, is used a biomarker to monitor sepsis-induced immunosuppression in intensive care unit patients[81]. However, no standardized tool is currently available in routine practice to assess

the functionality of septic patients' immune system. Here, we show that the DropMap system can be used to assess TNF-α secretion rate of single monocytes from septic shock patients in response to LPS stimulation.

In agreement with previous studies[22], single-cell DropMap measurements revealed lower rates of TNF-α secretion by monocytes upon LPS activation in septic shock patients (N=3 patients, 1.3 ± 0.5 molecules/s, mean ± standard deviation of the median values for the independent experiments) compared to healthy donors (N=3 donors, 2.5 ± 1.1 molecules/s, mean ± standard deviation of the median values for the independent experiments, Fig. 6a and Table 3). Without LPS, only a small fraction (3%) of monocytes secrete TNF-α (N=1 donor, n=32 monocytes, 0.9 ± 0.4 molecules/s, mean ± standard deviation) demonstrating that cell activation is specifically trigged by LPS stimulation (Fig. 6b). Interestingly, the frequency of monocytes, having a TNF-α secretion rate below 0.5 molecules per second (i.e. below the limit of detection of the bioassay), was higher in septic shock patients (N=3 patients, 29 ± 8%, mean ± standard deviation for the independent experiments), than in healthy donors (N=3 donors, 6 ± 6%, mean ± standard deviation for the independent experiments). These differences were at the limit of statistical significance (p=0.05 with one-sided Mann-Whitney test) (Fig. 6c).

Furthermore, monocytes from sepsis patients have been reported to exhibit higher endocytic activity compared to healthy donors[82]. The uptake of nanobeads is usually used as a readout for functional phenotyping of cell endocytosis[83-85]. We thus used the DropMap platform to monitor over time the uptake of nanobeads (endocytic activity) by monocytes. As expected, we observed differences in nanobead uptake between cells of healthy donors (N=3 donors, 29 ± 3 %, mean ± standard deviation for the independent experiments) and septic shock patients (N=3 patients, 56 ± 15 %, mean ± standard deviation for the independent experiments, Table 3). These differences were at the limit of statistical significance (p=0.05 with one-sided Mann-Whitney test). This result should be taken cautiously given the small number of samples. Overall, these results are exemplifying the potential application and added value of the DropMap technology in clinical studies.

The DropMap system is a valuable tool for monitoring cell responses and probing heterogeneity with single-cell resolution that can be applied in the field of immuno-monitoring, and beyond, to address a large panel of fundamental research or clinical questions. It is robust and simple, and it can be easily adapted to measure other cell types, in healthy and diseased states, and to measure other secreted molecules (such as microRNAs, other cytokines, chemokines and enzymes).

Table 3. **Analysis of single monocytes from healthy donors and septic shock patients using DropMap.**

Number of LPS stimulated cells per experiment, percentage of dead cells, secretion rate (molecules/sec), percentage of low secreting cells, and the percentage of endocytic cells of 3 healthy donors and 3 septic shock patients. Dead monocytes were determined by measuring caspase-3 activity in droplets. Endocytic activity was assessed by counting the number of single monocytes that bind to or internalize the magnetic beadline.

	Cell number	Dead cells (%)	Secretion rate (molecules/s)	Low secreting cells (%)	Endocytic cells (%)
Healthy Donor 1	2215	0.6	3.6	1.8	31
Healthy Donor 2	3008	0.5	2.5	5.3	31
Healthy Donor 3	4495	0.4	1.4	13.3	26
Septic Patient 1	2473	1.6	0.9	34.1	61
Septic Patient 2	1122	3.8	1.9	20.0	69
Septic Patient 3	3077	0.4	1.0	31.9	39

Box 1 Assay set-up

Choosing a pair of antibodies for the immunoassay:

When choosing a new pair of antibodies to set up another immunoassay, we use consider the following, ranked approaches:

1) Study literature and/or suppliers for antibody pairs that are tested for sandwich immunoassays.
2) A neutralizing and a non-neutralizing clone will recognize different epitopes, and binding interference is less likely.
3) The combination of polyclonal IgG for immobilization and monoclonal IgG for detection can also show good results, although recalibration is necessary when different batches of antibody are used.
4) Use a variety of different monoclonal antibodies and test the various combinations.

Labelled antigens and antibodies:

Important points to consider when preparing the reagents for the in-droplet assay:

- Purify antibodies and antigens as much as possible. Pure reagents will allow for better results and more standardized and repeatable results.
- Be sure to remove any free dye or biotin before using the proteins in the assays. Any non-bound molecules will increase the background and reduce the sensitivity of the assay. We use several rounds centrifugation and 10K MWCO Protein Concentrators (Thermo Scientific™ Pierce™) to remove free dyes.
- When labelling the antigen with biotin or fluorescent dye, use a random method to conserve all available epitopes on the antigen.
- Do not over-label proteins. We aim for a degree of labelling (molecules/protein) of around 2. Over-labelled antigens will not allow to set-up sensitive immunoassays.
- After labelling and purification, determine the exact concentration of the protein since the bioassay performance it highly variable with concentration.
- Test the labelled proteins in an ELISA format to determine the absence of interference. For antigens, we usually assay this by ELISA. Coat the labelled and un-labelled antigen on the ELISA plate (separately), and add the antibody in varying concentration. Compare both binding curves.

Setup concentration range:

The in-droplet assay allows for a wide range of measurements. The assay can be set up to be very sensitive (LOD of 500 molecules); or to span a wider concentration range. Use bulk measurements that help set up single-cell measurements. Look for literature values of bulk measurements that indicate the number of cells, time, volume and concentrations of the experiment to estimate the secretion rate. This crude estimation can serve as a starting point for assay set up.

There are four determining factors to set up the assay: concentration of beads, immobilization reagent, detection reagent and the apparent dissociation constant of the pair of antibodies for capture and detection. For starting values, please refer to the table below.

Parameter	**Number of nanoparticles**	**Immobilization antibody**	**Labelled detection antibody**	**Apparent dissociation constant of capture and detection antibodies**
Influence on capacity of the beads[#]	+++	+++	-	-
Influence on sensitivity[#]	+	+++	+++	++
Influence on measurement range[#]	++	++	++	+
Use of too much will result in…	Plateau in the calibration curve. Great for Yes/No answers, less suitable to quantify secretion rates	Increased waste	Decreased sensitivity; increased LOD	N.A.
Use of too little…	Absence of beadline; no reliable measurement of relocation	Plateau in the calibration curve. Great for Yes/No answers, less suitable to quantify secretion rates	Lower relocation values	N.A.
Ideally	Binding capacity matches the quantity of labelled detection reagent	Calculated as 1-2x capacity of the beads (minimized waste)	Matches capacity of beadline	As high-affinity as possible
Starting point for	170 beads	34 µM per mg of	0.8nM	As high-affinity as

0.1-10 molecules/s (30 min assay time)	(in-droplet dilution of factor 50 from stock)	beads		possible
Starting point for 10-1000 molecules/s (0.5 hour assay time)	1,200 beads (in-droplet dilution of factor 4 from stock)	75 nM per droplet	75 nM (30 nM for antigen)	As high-affinity as possible

[#]The magnitude of the effect is indicated: +++, strong; ++, medium; +, weak; -, no effect.

- END OF BOX 1 –

Box 2 Extraction of parameters

Limit of detection: This requires a calibration curve, a negative sample (e.g. cells not producing the analyte, and a positive sample (e.g. cells producing the analyte).

First, you will have to define a limit of detection (LOD) for the extracted secretion rate. Below this value, you will not be able to determine whether a cell secretes the analyte. To set-up the LOD, we recommend that you have a calibration curve, a negative sample as well as a sample (such as a cell line) with positive events. Use the data from the calibration curve to set various thresholds, and use them on the negative and positive samples to quantify the number of false-positives and negatives given a certain threshold. As a starting point, we use the definition of the limit of detection (LOD) by Armbuster and Pry[86] that states:

$$Limit\ of\ detection\ (LOD) = \overline{x_{blank}} + 1.645 * \sigma_{blank} + 1.645 * \sigma_{low}$$

where x_{blank} is the mean fluorescence relocation in a negative sample, σ_{blank} its standard deviation and σ_{low} the standard deviation of a sample with low concentration. If this threshold is too low, you will include mostly false-positives events (visible by an increased number of events in the negative sample), whereas a too high threshold increases false-negatives (a decreased number of events in the positive sample). If the analytical resolution is too low, set-up the assay anew with different antibodies or concentration ranges (Box 1) and repeat the experiments.

CRITICAL: A good labelling and high signals will decrease the limit of detection and decrease the frequency of false-positives and negatives.

Number of cells: Droplets with living cells are automatically detected in the analytical pathway. It is important to have a low mean number of cells per droplet ($\lambda < 0.2$) and the absence of cell clusters.

Secretion rates: This requires a calibration curve and data measured over time.

Your calibration curve will consist of an increasing phase of fluorescence relocation, a plateau and an ensuing decreasing phase, due the Hook effect (see Fig. 3b, e).Present in one-step immunoassays, the Hook effect leads to a decrease in signal at very high analyte concentrations. In our case, at analyte concentrations higher than beadline capacity, the non-bound analyte competes with the bound analyte for the detection reagent; and the measured fluorescence relocation decreases. Therefore, take care to use only the increasing phase for calculations of secretion rates in the cell measurements. Use the fitting parameters from the calibration curve to calculate the measured in-droplet fluorescence relocation into concentration. The secretion rate is then simply calculated by the change of concentration over time. For each droplet followed over time, we can therefore define:

1. $$Secretion\ rate\ \left[\frac{molecules}{s}\right] = \left(\frac{1}{n-1}\left(\sum_{1}^{n-1}\frac{c_{i+1}-c_i}{\Delta t}\right)\right) * N_A * V$$

with n as the number of time points, c as the concentration present in the droplet at each time (in pM; according to the calibration curve), Δt as the time difference between two measurement points, N_A as the Avogadro constant and V as the volume of the droplet (in pL).

Extraction of affinity (IgG assay): Additionally, when a fluorescent variant of the antigen of interest is introduced into the assay, affinity of the secreted antibodies can be assayed (for more information, see reference[38]). The ratio of relocated antigen fluorescence onto the beadline and free antigen within the droplet can be used to calculate the strength of the interaction (K_D) once the concentration of antibody is known. This concentration is accessible using calibration curves (see Secretion rate above). If the secreted IgG recognizes the antigen, the ratio of fluorescent antigen bound to IgG on the beadline and mean fluorescence within the droplet can be used to calculate the strength of the interaction (K_D). The K_D is determined from the slope of the line defined by plotting the relocation of fluorescent anti-IgG F(ab')2 (concentration dependent) against the relocation of fluorescent antigen to the beadline at different concentrations of IgG below the maximum capacity of the beadline (≤50 nM, concentration and affinity dependent).

[PRODUCTION : Please place Figure for Box 2 here].

Measurement of murine IgG affinity. Relocation of fluorescent antigen and fluorescent anti-IgG detection antibody are determined by dividing the fluorescence intensity measured on the magnetic beadline (I_B) by the background fluorescence of the droplet (I_D). To calculate K_D, antigen relocation (I_B/I_D antigen) is plotted against anti-IgG detection antibody relocation (I_B/I_D anti-IgG). The slope of this plot increases with increasing affinity, i.e. decreasing K_D. The use of several antibodies with

different, but known affinities (measured, for example, using surface plasmon resonance) allows calibration of the slope to determine K_D.

Slopes of several antibodies with known affinity against the antigen are needed to calibrate the assay. We determined the slopes for a panel of 7 purified anti-TT IgGs with affinities ranging from 300 to 0.02 nM in our initial study[38], and have since reproduced this curve using a variety of different antigens. If the antigen is multimeric, or epitopes are present more than once, an apparent dissociation constant will be measured instead of K_D.

Be aware that the slope for different isotypes (e.g. for IgG1 and IgG2) will need different calibration curves due to their difference in fluorescence relocation[38]. If the slope is below 0.03; no affinity can be detected. If the slope is above 1; K_D is at or below 0.1 nM but cannot be resolved anymore. In order to validate the antigen and antibodies, and to make sure that the calibration curve can be used for the system of interest, we assay 2-3 antibodies with known but different affinity; and use the slope to calculate the K_D. The K_D should be compared with the K_D measured using an independent method, such as Surface Plasmon Resonance.

- END OF BOX 2 –

Box 3 Distribution of cells and nanoparticles per droplet

Cells. In the absence of cell clusters (i.e. the cells are well individualized and suspended), the cellular loading in this protocol follows a Poisson distribution, which is a special case of the binomial distribution. The mean number of cells per droplet, λ, is determined by the number of cells in the initial solution and the volume of an individual droplet (see the table below, 50 pL droplets). While a higher λ will increase the throughput and might be advantageous to increase the number of low frequency events, lower λ is preferable for single-cell measurements. Higher λ will increase the frequency of droplets containing multiple cells, and data analysis needs to be adapted to cope with these events. We usually aim for a λ of around 0.2-0.3, which is a compromise between maximizing the frequency of droplets with one cell and minimizing the frequency of with multiple cells (see the table below). We recommend validating the λ for each experiment by counting the number of cells in a subset of droplets (e.g. 50 droplets) since it will also be needed to calculate the total number of cells, and the frequency of positive events (see Box 2).

Cells per ml [Mio/ml]	λ	Empty droplet	Droplets with a single cell	Droplets with multiple cells

5	0.1	90%	9.5%	0.5%
10	0.2	78%	19%	3%
20	0.4	67%	27%	6%

Nanoparticles. An advantage of the protocol described here is the co-encapsulation of single cells with multiple nanoparticles instead of the co-encapsulation of a single cell and a single microparticle[35, 36]. With a homogenous suspension of nanoparticles and a mean, λ, of hundreds to thousands of nanoparticles per droplet, the distribution is binomial. For the two conditions presented here (170 and 1,200 nanoparticles per droplet), the standard deviation is 7 and 17, respectively, and the coefficient of variance (CV) below 0.05. Therefore, every droplet contains approximately the same number of nanoparticles and the low CV assures that drop-to-drop variation has a minimal impact on the results. If droplets with no beads are present during analysis, these likely stem from errors in the flow ratios during encapsulation, due, for example, to the presence of clusters of beads or cells, dust in the microfluidic chips or operating errors.

- END OF BOX 3 –

BOX 4. Detection of protein of interest using a microtiter plate (bulk) assay. TIMING 3 h As an alternative to the droplet-based quality control assays of the functionalized nanoparticles, one can also perform a fluorescent-based immune-sandwich assay in microtiter plates. It should be noted that bulk assays use more reagents and a calibration curve can also be performed directly within droplets to allow for the quantitative measurement of secretion rates (Box 2, Fig. 3b and e).

Procedure

1. Prepare a serial dilution of recombinant protein of interest (typically in the range 0-100 nM) in PBS containing 1% (wt/vol) BSA in a 96-well microtiter microplate. Final volumes should be 90 µl each.
2. Add 10 µl of beads to each well containing the protein of interest, and then mix well by pipetting.
3. Incubate, shaking at 700 rpm, for 1 hour at room temperature.
4. Place the microtiter plate on a magnet holder for about 1 minute at room temperature.
5. Carefully discard the supernatant. Do not disturb the bead pellet.
6. Add 100 µl of PBS buffer to beads.
7. Remove the microtiter plate from the magnet holder and mix well by vortexing.
8. Repeat steps iv-vii once.

9. Collect the beads, carefully discard the supernatant then add 100 µl of solution containing 0.8 µg/ml of fluorescent-labeled detection antibody to each well.
10. Incubate, shaking at 700 rpm, for 1 hour at room temperature.
11. Place the microtiter plate on a magnet holder for about 1 minute at room temperature.
12. Carefully discard the supernatant. Do not disturb the bead pellet.
13. Add 100 µl of PBS buffer to beads.
14. Remove the microtiter plate from the magnet holder and mix well by vortexing.
15. Repeat steps xi-xiv once.
16. Pellet the beads using the magnet and carefully discard the supernatant. Re-suspend the beads directly in 50 µL PBS, mix gently by pipetting.
17. Transfer the samples to a black 96-well microtiter microplate and measure fluorescence at the wavelength optimal for the fluorophore on the detection antibody using the microplate reader.
18. Plot the curve of fluorescence intensity against the concentration of added protein of interest.

? TROUBLESHOOTING

- End of BOX 4-

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Competing interests

Some of the authors (Jean Baudry, Jérôme Bibette, Andrew Griffiths, Yacine Bounab, Christophe Védrine) are inventors on patent applications based on certain ideas described in this manuscript and may receive financial compensation via their employer’s rewards to inventors scheme.

Author Contributions

Y.B., K.E., M.R. and N.A. performed and optimized the experiments described in this protocol, S.D. and G.C. provided the respective Matlab Scripts for data analysis, C.C and T.T. provided the statistical tools. M.M. optimized the boronic acid nanobead functionalization protocol. CV and J.Ba. managed

the optical bench set-up. J-F.L supplied the septic clinical samples. K.E, J.Bi., J.Ba and A.D.G. developed the DropMap technology[38] and contributed to the early stage definition of the new technology. Y.B. and C.V. extended DropMap technology to measure low cytokine secretion profile and to overcome limitations of cell endocytic activity. G.M., A.P., J.T. designed and setup the clinical study involving sepsis and matched-control patients. A.T., C.G., P.L., V.M., F.V., P.C., I.G. supervised the work, participated to the design of technical experiments and of the clinical study and actively contributed in writing different sections of the manuscript. Y.B., S.D. and C.V. analyzed the data for the sepsis application, and Y.B., K.E., J.Ba., A.D.G., C.V. wrote the manuscript. All authors edited and proof-read the paper.

Code availability statement

DropMap Matlab script is available from GitHub repository, https://github.com/LCMD-ESPCI/dropmap-analyzer. Matlab scripts illustrating the main functions performed by the DropCell.exe Matlab application are available from GitHub repository, https://github.com/bioaster/dropcell.git. Installation file for DropCell.exe Matlab application as well as an image data set that support/illustrate the findings of this study are available in figshare repository, with the identifiers https://doi.org/10.6084/m9.figshare.11336663.v1 and https://figshare.com/articles/dropcell_image_data_set/11342426, respectively.

Data availability statement

The datasets generated during and/or analysed during the current study are not publicly available due to confidentiality and contractual obligations towards industrial partners of the project in which the data have been generated, but are available from the corresponding author on reasonable request.

References

1. Lawson, D.A., Kessenbrock, K., Davis, R.T., Pervolarakis, N. & Werb, Z. Tumour heterogeneity and metastasis at single-cell resolution. *Nat Cell Biol* **20**, 1349-1360 (2018).
2. Potter, S.S. Single-cell RNA sequencing for the study of development, physiology and disease. *Nat Rev Nephrol* **14**, 479-492 (2018).
3. Stuart, T. & Satija, R. Integrative single-cell analysis. *Nat Rev Genet* **20**, 257-272 (2019).
4. Svensson, V., Vento-Tormo, R. & Teichmann, S.A. Exponential scaling of single-cell RNA-seq in the past decade. *Nat Protoc* **13**, 599-604 (2018).
5. Zhang, X. et al. Comparative Analysis of Droplet-Based Ultra-High- Throughput Single-Cell RNA-Seq Systems. *Molecular Cell*, 1-19 (2018).
6. Battle, A. et al. Genomic variation. Impact of regulatory variation from RNA to protein. *Science (New York, N.Y.)* **347**, 664-667 (2015).
7. Peterson, V.M. et al. Multiplexed quantification of proteins and transcripts in single cells. *Nature Biotechnology* **35**, 936-939 (2017).
8. Shapiro, H.M. Practical Flow Cytometry. (Wiley-Liss, New York; 2003).
9. Bendall, S.C. et al. Single-cell mass cytometry of differential immune and drug responses across a human hematopoietic continuum. *Science (New York, NY)* **332**, 687-696 (2011).
10. Becattini, S. et al. Functional heterogeneity of human memory CD4(+) T cell clones primed by pathogens or vaccines. *Science (New York, N.Y.)* **347**, 400-406 (2015).
11. Betts, M.R. et al. Sensitive and viable identification of antigen-specific CD8+ T cells by a flow cytometric assay for degranulation. *J Immunol Methods* **281**, 65-78 (2003).
12. Han, G., Spitzer, M.H., Bendall, S.C., Fantl, W.J. & Nolan, G.P. Metal-isotope-tagged monoclonal antibodies for high-dimensional mass cytometry. *Nature protocols* **13**, 2121-2148 (2018).
13. Korin, B., Dubovik, T. & Rolls, A. Mass cytometry analysis of immune cells in the brain. *Nature protocols* **13**, 377-391 (2018).
14. Stoeckius, M. et al. Simultaneous epitope and transcriptome measurement in single cells. *Nature methods* **14**, 865-868 (2017).
15. Shahi, P., Kim, S.C., Haliburton, J.R., Gartner, Z.J. & Abate, A.R. Abseq: Ultrahigh-throughput single cell protein profiling with droplet microfluidic barcoding. *Scientific reports* **7**, 44447 (2017).
16. Czerkinsky, C.C., Nilsson, L.A., Nygren, H., Ouchterlony, O. & Tarkowski, A. A solid-phase enzyme-linked immunospot (ELISPOT) assay for enumeration of specific antibody-secreting cells. *Journal of immunological methods* **65**, 109-121 (1983).
17. Kouwenhoven, M. et al. Enzyme-linked immunospot assays provide a sensitive tool for detection of cytokine secretion by monocytes. *Clin Diagn Lab Immunol* **8**, 1248-1257 (2001).
18. Schultes, B.C. & Whiteside, T.L. Monitoring of immune responses to CA125 with an IFN-gamma ELISPOT assay. *J Immunol Methods* **279**, 1-15 (2003).
19. Mogensen, T.H. Pathogen recognition and inflammatory signaling in innate immune defenses. *Clinical microbiology reviews* **22**, 240-273, Table of Contents (2009).
20. Garcia-Cordero, J.L., Nembrini, C., Stano, A., Hubbell, J.A. & Maerkl, S.J. A high-throughput nanoimmunoassay chip applied to large-scale vaccine adjuvant screening. *Integrative biology : quantitative biosciences from nano to macro* **5**, 650-658 (2013).
21. Han, Q. et al. Polyfunctional responses by human T cells result from sequential release of cytokines. *Proceedings of the National Academy of Sciences of the United States of America* **109**, 1607-1612 (2012).
22. Han, Q., Bradshaw, E.M., Nilsson, B., Hafler, D.A. & Love, J.C. Multidimensional analysis of the frequencies and rates of cytokine secretion from single cells by quantitative microengraving. *Lab on a chip* **10**, 1391-1400 (2010).
23. Shirasaki, Y. et al. Real-time single-cell imaging of protein secretion. *Sci Rep* **4**, 4736 (2014).

24. Son, K.J. et al. Microfluidic compartments with sensing microbeads for dynamic monitoring of cytokine and exosome release from single cells. *The Analyst* **141**, 679-688 (2016).
25. Varadarajan, N. et al. A high-throughput single-cell analysis of human CD8(+) T cell functions reveals discordance for cytokine secretion and cytolysis. *The Journal of clinical investigation* **121**, 4322-4331 (2011).
26. Xue, Q. et al. Single-cell multiplexed cytokine profiling of CD19 CAR-T cells reveals a diverse landscape of polyfunctional antigen-specific response. *Journal for immunotherapy of cancer* **5**, 85 (2017).
27. Xue, Q. et al. Analysis of single-cell cytokine secretion reveals a role for paracrine signaling in coordinating macrophage responses to TLR4 stimulation. *Science signaling* **8**, ra59 (2015).
28. Yamanaka, Y.J. et al. Cellular barcodes for efficiently profiling single-cell secretory responses by microengraving. *Analytical chemistry* **84**, 10531-10536 (2012).
29. Seah, Y.F.S., Hu, H. & Merten, C.A. Microfluidic single-cell technology in immunology and antibody screening. *Molecular aspects of medicine* **59**, 47-61 (2018).
30. Love, J., Ronan, J., Grotenbreg, G., Van Der Veen, A. & Ploegh, H. A microengraving method for rapid selection of single cells producing antigen-specific antibodies. *Nat Biotechnol* **24**, 703-707 (2006).
31. Jin, A. et al. Rapid isolation of antigen-specific antibody-secreting cells using a chip-based immunospot array. *Nature protocols* **6**, 668-676 (2011).
32. Köster, S. et al. Drop-based microfluidic devices for encapsulation of single cells. *Lab on a chip* **8**, 1110-1115 (2008).
33. Clausell-Tormos, J. et al. Droplet-based microfluidic platforms for the encapsulation and screening of Mammalian cells and multicellular organisms. *Chemistry & biology* **15**, 427-437 (2008).
34. El Debs, B., Utharala, R., Balyasnikova, I.V., Griffiths, A.D. & Merten, C.A. Functional single-cell hybridoma screening using droplet-based microfluidics. *Proceedings of the National Academy of Sciences* **109**, 11570-11575 (2012).
35. Mazutis, L. et al. Single-cell analysis and sorting using droplet-based microfluidics. *Nature protocols* **8**, 870-891 (2013).
36. Shembekar, N., Hu, H., Eustace, D. & Merten, C.A. Single-Cell Droplet Microfluidic Screening for Antibodies Specifically Binding to Target Cells. *CellReports* **22**, 2206-2215 (2018).
37. Chokkalingam, V. et al. Probing cellular heterogeneity in cytokine-secreting immune cells using droplet-based microfluidics. *Lab Chip* **13**, 4740-4744 (2013).
38. Eyer, K. et al. Single-cell deep phenotyping of IgG-secreting cells for high-resolution immune monitoring. *Nat Biotechnol* **35**, 977-982 (2017).
39. Jorgolli, M. et al. Nanoscale integration of single cell biologics discovery processes using optofluidic manipulation and monitoring. *Biotechnol Bioeng* **116**, 2393-2411 (2019).
40. Mocciaro, A. et al. Light-activated cell identification and sorting (LACIS) for selection of edited clones on a nanofluidic device. *Commun Biol* **1**, 41 (2018).
41. Winters, A. et al. Rapid single B cell antibody discovery using nanopens and structured light. *mAbs* **11**, 1025-1035 (2019).
42. Konry, T., Dominguez-Villar, M., Baecher-Allan, C., Hafler, D.A. & Yarmush, M.L. Droplet-based microfluidic platforms for single T cell secretion analysis of IL-10 cytokine. *Biosens Bioelectron* **26**, 2707-2710 (2011).
43. Qiu, L. et al. A membrane-anchored aptamer sensor for probing IFNgamma secretion by single cells. *Chem Commun (Camb)* **53**, 8066-8069 (2017).
44. Segaliny, A.I. et al. Functional TCR T cell screening using single-cell droplet microfluidics. *Lab on a chip* **18**, 3733-3749 (2018).
45. Gérard, A., Woolfe, A. & Mottet, G., Reichen, M., Castrillon, C., Menrath, V., Ellouze, S., Poitou, A., Doineau, R., Briseno-Roa, L., Canales-Herrerias, P., Mary, P., Rose, G., Ortega, C., Delincé, M., Essono, S., Jia, B., Iannascoli, B., Richard-LeGoff, O., Kumar, R., Stewart, S.N., Pousse, Y., Shen, B., Grosselin, K., Saudemont, B., Sautel-Caillé, A., Godina, A., McNamara, S.,

Eyer, K., Millot, G.A., Baudry, J., England, P., Nizak, C., Jensen, A., Griffiths, A.D., Bruhns, P., and Brenan. C. High-throughput single-cell activity-based screening and sequencing of antibodies using droplet microfluidics. . *Nature Biotechnology* (In press).
46. Duffy, D.C., McDonald, J.C., Schueller, O.J.A. & G.M., W. Rapid prototyping of microfluidic systems in poly(dimethylsiloxane). *Analytical chemistry* **70**, 4974-4984 (1998).
47. Anna, S.L., Bontoux, N. & Stone, H.A. Formation of dispersions using "flow focusing" in microchannels. *Appl Phys Lett* **82**, 364-366 (2003).
48. Akhtar, M., Driesche, S. & Vellekoop, M.J. On-chip storage of droplets in parylene-AF4 coated PDMS channels. (2014).
49. Di Carlo, D., Aghdam, N. & Lee, L.P. Single-cell enzyme concentrations, kinetics, and inhibition analysis using high-density hydrodynamic cell isolation arrays. *Analytical chemistry* **78**, 4925-4930 (2006).
50. Jin, S.H., Jeong, H.H., Lee, B., Lee, S.S. & Lee, C.S. A programmable microfluidic static droplet array for droplet generation, transportation, fusion, storage, and retrieval. *Lab on a chip* **15**, 3677-3686 (2015).
51. Liu, C., Liu, J., Gao, D., Ding, M. & Lin, J.M. Fabrication of microwell arrays based on two-dimensional ordered polystyrene microspheres for high-throughput single-cell analysis. *Analytical chemistry* **82**, 9418-9424 (2010).
52. Ochsner, M. et al. Micro-well arrays for 3D shape control and high resolution analysis of single cells. *Lab on a chip* **7**, 1074-1077 (2007).
53. Schmitz, C.H., Rowat, A.C., Koster, S. & Weitz, D.A. Dropspots: a picoliter array in a microfluidic device. *Lab on a chip* **9**, 44-49 (2009).
54. Duval, F., van Beek, T.A. & Zuilhof, H. Key steps towards the oriented immobilization of antibodies using boronic acids. *The Analyst* **140**, 6467-6472 (2015).
55. Kumar, S., Aaron, J. & Sokolov, K. Directional conjugation of antibodies to nanoparticles for synthesis of multiplexed optical contrast agents with both delivery and targeting moieties. *Nature protocols* **3**, 314-320 (2008).
56. Saha, B., Evers, T.H. & Prins, M.W. How antibody surface coverage on nanoparticles determines the activity and kinetics of antigen capturing for biosensing. *Anal Chem* **86**, 8158-8166 (2014).
57. Saha, B., Songe, P., Evers, T.H. & Prins, M.W.J. The influence of covalent immobilization conditions on antibody accessibility on nanoparticles. *The Analyst* **142**, 4247-4256 (2017).
58. Sivaram, A.J., Wardiana, A., Howard, C.B., Mahler, S.M. & Thurecht, K.J. Recent Advances in the Generation of Antibody-Nanomaterial Conjugates. *Advanced healthcare materials* **7** (2018).
59. Welch, N.G., Scoble, J.A., Muir, B.W. & Pigram, P.J. Orientation and characterization of immobilized antibodies for improved immunoassays (Review). *Biointerphases* **12**, 02d301 (2017).
60. Dhadge, V.L., Hussain, A., Azevedo, A.M., Aires-Barros, R. & Roque, A.C. Boronic acid-modified magnetic materials for antibody purification. *Journal of the Royal Society, Interface* **11**, 20130875 (2014).
61. Lin, P.C. et al. Fabrication of oriented antibody-conjugated magnetic nanoprobes and their immunoaffinity application. *Anal Chem* **81**, 8774-8782 (2009).
62. Wang, X., Xia, N. & Liu, L. Boronic Acid-based approach for separation and immobilization of glycoproteins and its application in sensing. *International journal of molecular sciences* **14**, 20890-20912 (2013).
63. Wagner, O. et al. Biocompatible fluorinated polyglycerols for droplet microfluidics as an alternative to PEG-based copolymer surfactants. *Lab on a chip* **16**, 65-69 (2016).
64. Williamson, J.D. & Cox, P. Use of a new buffer in the culture of animal cells. *The Journal of general virology* **2**, 309-312 (1968).
65. Lowe, K.C. Perfluorochemical respiratory gas carriers: benefits to cell culture systems. *Journal of Fluorine Chemistry* **118**, 19-26 (2002).

66. Holtze, C. et al. Biocompatible surfactants for water-in-fluorocarbon emulsions. *Lab on a chip* **8**, 1632-1639 (2008).
67. Mazutis, L. & Griffiths, A.D. Selective droplet coalescence using microfluidic systems. *Lab on a chip* **12**, 1800-1806 (2012).
68. Scott, R.L. The solubility of fluorocarbons. *J Am Chem Soc* **70**, 4090-4093 (1948).
69. Simons, J.H. & Linevsky, M.J. The Solubility of Organic Solids in Fluorocarbon Derivatives. *Journal of the American Chemical Society* **74**, 4750-4751 (1952).
70. Qin, D., Xia, Y. & Whitesides, G.M. Soft lithography for micro- and nanoscale patterning. *Nature protocols* **5**, 491-502 (2010).
71. Heo, M. et al. Deep-phenotypic characterization of the immunization induced anti-bacterial immunoglobulin-G repertoire. (submitted).
72. Eyer, K. et al. Recall analysis as an integrative study tool of immunizations. (submitted).
73. Raphael, I., Nalawade, S., Eagar, T.N. & Forsthuber, T.G. T cell subsets and their signature cytokines in autoimmune and inflammatory diseases. *Cytokine* **74**, 5-17 (2015).
74. Tanaka, A. & Sakaguchi, S. Regulatory T cells in cancer immunotherapy. *Cell Res* **27**, 109-118 (2017).
75. Kang, S., Brown, H.M. & Hwang, S. Direct Antiviral Mechanisms of Interferon-Gamma. *Immune Netw* **18**, e33 (2018).
76. Mosmann, T.R., Cherwinski, H., Bond, M.W., Giedlin, M.A. & Coffman, R.L. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J Immunol* **136**, 2348-2357 (1986).
77. Wheelock, E.F. Interferon-Like Virus-Inhibitor Induced in Human Leukocytes by Phytohemagglutinin. *Science (New York, N.Y.)* **149**, 310-311 (1965).
78. DuPage, M. & Bluestone, J.A. Harnessing the plasticity of CD4(+) T cells to treat immune-mediated disease. *Nat Rev Immunol* **16**, 149-163 (2016).
79. Cecconi, M., Evans, L., Levy, M. & Rhodes, A. Sepsis and septic shock. *Lancet* **392**, 75-87 (2018).
80. Gyawali, B., Ramakrishna, K. & Dhamoon, A.S. Sepsis: The evolution in definition, pathophysiology, and management. *SAGE Open Med* **7**, 2050312119835043 (2019).
81. Monneret, G. et al. Novel Approach in Monocyte Intracellular TNF Measurement: Application to Sepsis-Induced Immune Alterations. *Shock (Augusta, Ga.)* **47**, 318-322 (2017).
82. Shalova, I.N. et al. Human monocytes undergo functional re-programming during sepsis mediated by hypoxia-inducible factor-1alpha. *Immunity* **42**, 484-498 (2015).
83. Kumar, P., Pai, K., Pandey, H.P. & Sundar, S. Study on pinocytosis by monocytes from visceral leishmaniasis patients, Vol. 83. (2002).
84. Luciani, N., Gazeau, F. & Wilhelm, C. Reactivity of the monocyte/macrophage system to superparamagnetic anionic nanoparticles. *Journal of Materials Chemistry* **19**, 6373-6380 (2009).
85. Robert, D. et al. Cell sorting by endocytotic capacity in a microfluidic magnetophoresis device. *Lab on a chip* **11**, 1902-1910 (2011).
86. Armbruster, D.A. & Pry, T. Limit of blank, limit of detection and limit of quantitation. *Clin Biochem Rev* **29 Suppl 1**, S49-52 (2008).

FIGURE LEGENDS

Figure 1. Single cell secretion assay in microfluidic droplets. (**a**) Co-encapsulation of cells and assay reagents into picoliter droplets. Red arrows show individual cells in droplets. Scale bars 50 µm (**b, c**) Principle of the single-cell secretion assay in droplets. The bioassay comprises magnetic nanoparticles

(300 nm diameter) functionalized with capture antibodies, and fluorescently-labeled detection antibodies. In the absence of secreted analyte (**b**), no binding occurs on the magnetic beadline and the fluorescent signal remains homogenous within the droplet. In the presence of secreted analyte (**c**), antibody-coated beads capture the secreted analyte and the fluorescent signal becomes relocalized to the beadline, due to the fluorescently-labelled detection antibody binding the captured analyte. Black arrows (B->) below the droplet indicate the direction of the magnetic field that induces beadline formation in the droplets. Both scale bars 25 µm. (**d**) A brightfield image of beadlines and monocytes in a compact array of 50 pL droplets. Scale bar 50 µm. (**e**) Complete chip design used to co-encapsulate cells and reagents. Scale bar 750 µm.

Figure 2. Fabrication of the microfluidic glass chamber using double-sided adhesive tape. (**a**) Dimensions of the lower and upper glass slides enclosing the chamber. The upper slide contains two 1 mm wide holes. Droplets are injected through one hole (the other one acting as a vent). (**b**) Dimensions of the adhesive tape and pattern of the chamber. (**c**) Key steps for droplet chamber fabrication (step numbers refer to the steps described in the Procedure section of the manuscript). (**d**) Brightfield images of the glass/tap composite: Saffman-Taylor fingers are formed at room temperature (RT); they are not formed after a 90°C incubation (28(A).vi) . (**e-f**) Device composition: two glass slides (at bottom and top) sealed with the double-sided adhesive tape, two inlet/outlet nanoports (in the top glass slide, sealed with two caps), and a magnet glued to the bottom glass slide (alternative chamber fabrication 1).

Figure 3. Characterization of functionalized nanoparticles in droplets. (**a**) Schematic representation of nanoparticle coating for TNF-α detection. The bioassay comprises paramagnetic nanobeads functionalized with anti-TNF-α antibodies using boronic acid coupling to glycosyl groups. **(b)** Detection of human recombinant TNF-α in 50 pL droplets. The relocation of the fluorescently-labeled detection antibodies in the presence of different concentrations of TNF-α. The signal was determined by dividing the fluorescence intensity measured on the magnetic beadline (I_B) by the background fluorescence of the droplet (I_D). Top left, brightfield image. Bottom left, fluorescent image. Right, signal (I_B/I_D) as a function of TNF-α concentration. (**c**) Time courses of TNF-α secretion by individual monocytes. Each curve represents the kinetic signal of a single cell**.** Top left, brightfield image. Bottom left, fluorescent image. Right, signal (I_B/I_D) as a function of time, each curve represents the signal of a single cell. Black lines indicate the relocation within droplets with non-secreting cells. (**d**) Schematic representation of nanoparticle coating for IgG detection. The bioassay comprises paramagnetic nanobeads functionalized with anti-mouse kappa light chain V_HH that capture IgG onto

the bead surface. (**e**) Detection of murine recombinant IgG1 (circles) and IgG2 (squares) in 50 pL droplets. Top left, brightfield image. Bottom left, fluorescent image. Right, signal (I_B/I_D) as a function of IgG concentration (IgG1 detection reagent, red lines; IgG2 detection reagent, green lines). (**f**) Time course analyses of IgG secretion of individual B cells. Top left, brightfield image. Bottom left, fluorescent image. Right, signal (I_B/I_D) as a function of time, each curve represents the mean signal of a single cell (IgG1, red lines; IgG2, green lines). Black lines indicate the relocation within droplets with non-secreting cells. All scale bars are 25 μm.

Figure 4. Analysis pipeline. (**a**) Acquisition of images of the monolayer of droplets, illustrated for a single field of view in 5 imaging channels. Measurements were repeated at regular time intervals. (**b**) Detection of droplet contours in the field, and of the intra-droplet beadline (bright-field image) (**c**). Tracking of droplets allows continued analysis in spite of potential small droplet movements (supplementary Fig. 3 and supplementary method 1)**.** (**d**) Images illustrating multiplexed and dynamic analyses of single monocytes. The droplet sub-image is shown in the five different imaging channels. Bright-field is used to detect droplets and magnetic beadlines, a Cy3 cube (orange fluorescence) is used to detect the secreted TNF-α on the beadline, a Cy5 cube (far red fluorescence) is used to detect living cells, a GFP cube (far-red fluorescence) is used to detect dead cells, and a DAPI cube (blue violet conjugate fluorescence) is used to detect cells expressing the cell surface marker CD14. The CD14 positive monocytes remained alive over 180 min and TNF-α relocalization on the beadline is detected 60 min after stimulation in droplets at 37°C. At 180 min, the cell shows an apoptotic activity indicating the death of the cell. Live and dead monocytes were determined by staining with MitoView and NucView to measuring mitochondrial membrane potential and caspase-3 activity, respectively. Scale bar 50 μm.

Figure 5. Analysis of cytokine secretion from human single T cells. (**a**) Distribution of the frequencies of TNF-α (17±, (**b**) IFN-γ, (**c**) IL-2 and (**d**) IL-4 secreting T cells (identified using a fluorescent FITC-conjugated anti-CD3 antibody) in PMA/ionomycin stimulated human PBMCs (orange) and non-stimulated PBMCs (black). Panels a and b depict 4,515 cells for stimulated, and 7'525 cells for non-stimulated PBMCs. Panels c and d depict 8,640 and 8,606 individually assayed cells for stimulated and non-stimulated cells respectively. The data represents a single experiment. No or only little secretion was found in the population of CD3 negative cells. Gray bars represent the part of the distribution that could not be analyzed due to the LOD of the assays (see also Box 1 and 2).

Figure 6. Analysis of TNF-α secretion from human single monocytes of healthy donor and septic shock patient. (**a**) Distribution of the frequencies of TNF-α secreting monocytes upon LPS stimulation

of a healthy donor (black line, 3008 cells) and septic shock patient (orange line, 2473 cells). (**b**) Distribution of the frequencies of TNF-α secreting monocytes upon LPS stimulation (black line, 3008 cells) and non-stimulated cells (dashed line, 1099 cells) of a healthy donor. Low secreting cells exhibit TNF-α secretion rate below 0.5 molecules per second. The data represents a single experiment. Gray bars (a and b) represent the part of the distribution that could not be analyzed due to the LOD (TNF-α secretion rate below 0.5 molecules per second) of the assay (see also Box 1 and 2). (**c**) Percentage of low secreting monocytes of septic patients (N=3 patients, black squares, 29 ± 8%, mean ± standard deviation for the independent experiments) and healthy donors (N=3 donors, black circles, 6 ± 6%, mean ± standard deviation for the independent experiments). Error bars show standard deviation.

List of supplementary information

Supplementary Figure 1. Explanatory figure for steps 33-35 to assemble the tip connectors and the syringes for the aqueous phases.

Supplementary Figure 2. Explanatory figure for step 40 to position the chamber on the microscope stage insert.

Supplementary Figure 3. Explanatory figure describing the pipeline analysis of droplet detection and tracking.

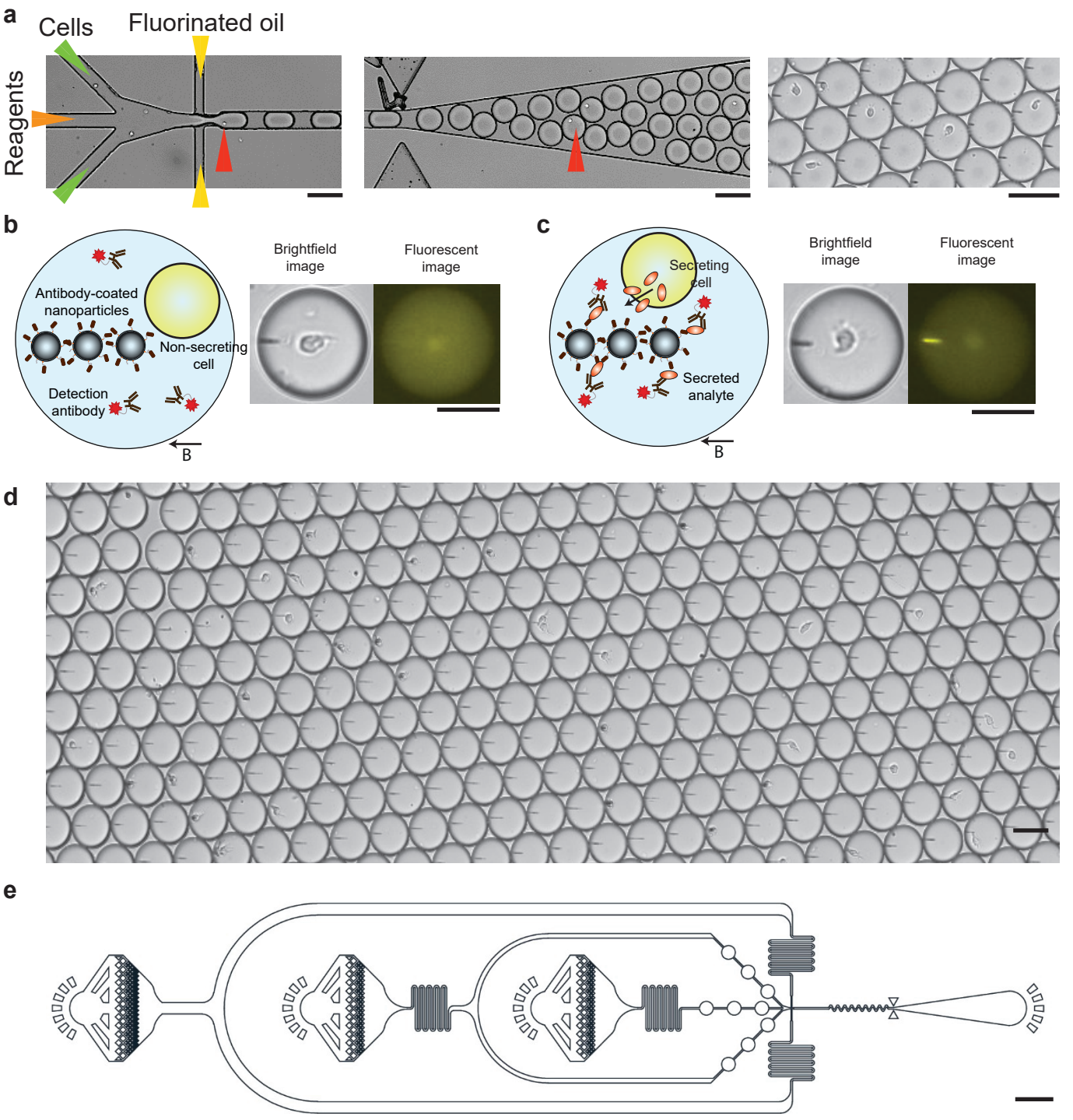
Supplementary Method 1. Pipeline analysis of droplet detection and tracking.

Supplementary Data 1. Complete chip design (CAD file).

Supplementary Data 2. Mask for the double-sided tape to prepare the observation chamber.

Supplementary Data 3. Mask for laser ablation.

Supplementary Data 4. Mask for the magnet holder.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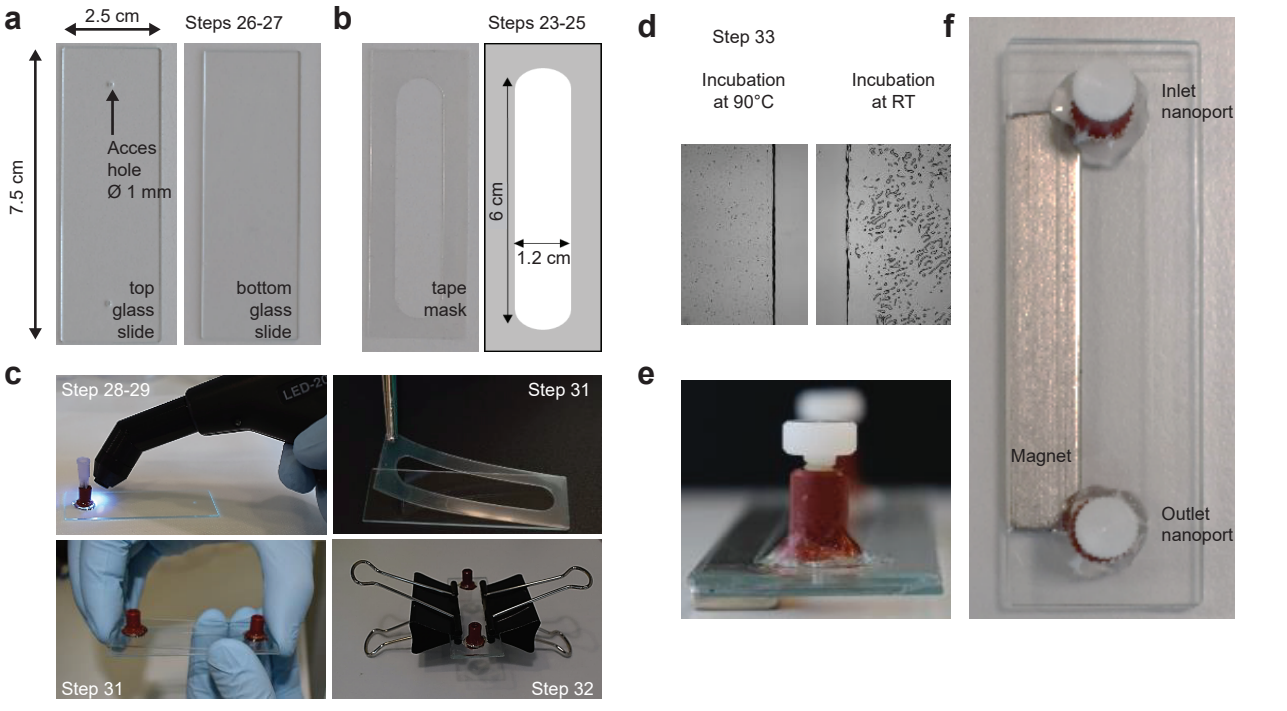
Fluorinated oil
Reagents
b
Antibody-coated nanoparticles
Non-secreting cell
Detection antibody
B
Brightfield image
Fluorescent image
c
Secreting cell
Secreted analyte
B
Brightfield image
Fluorescent image
d
e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Steps 26-27
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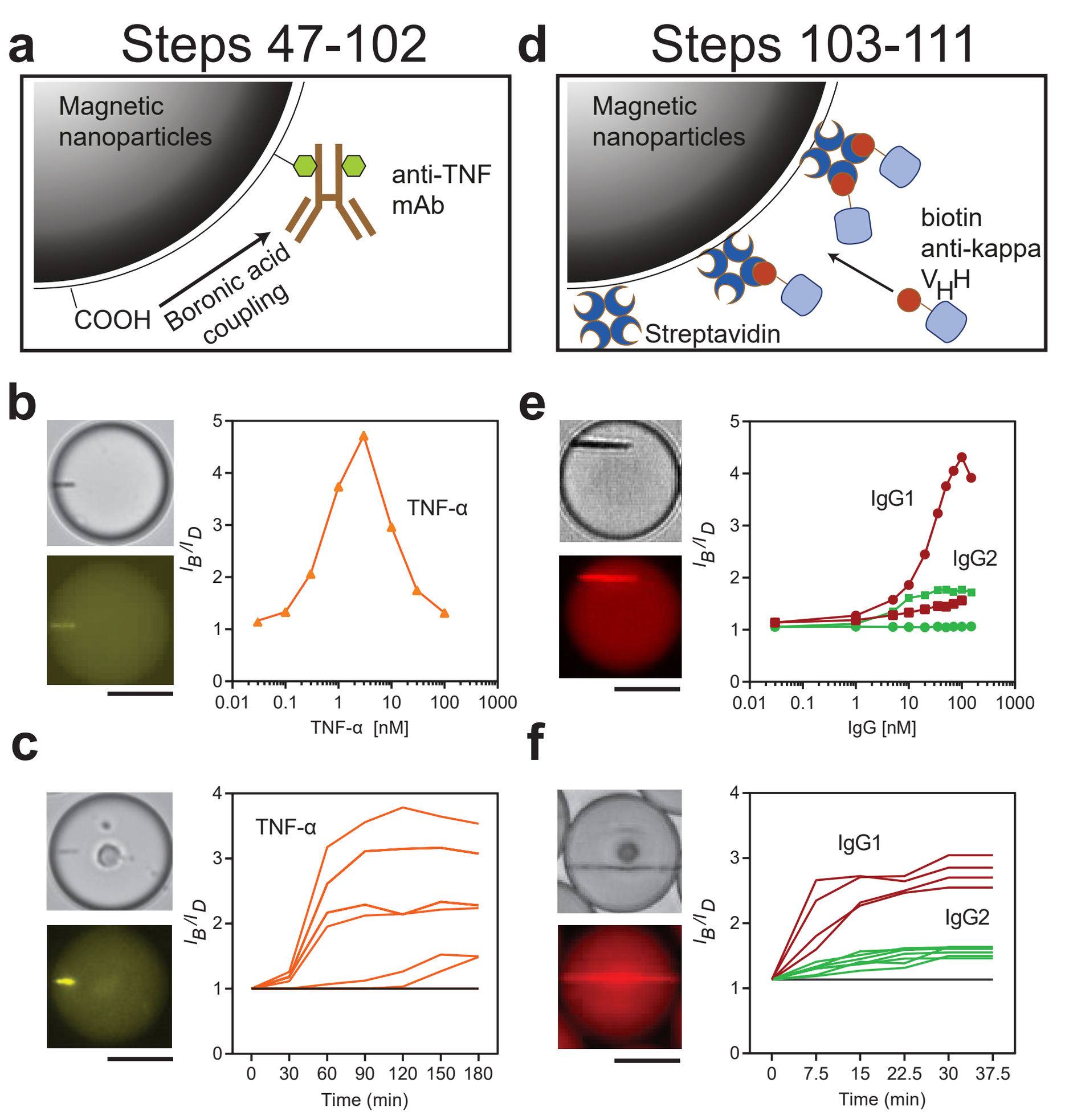
Acces
hole
Ø 1 mm
top
glass
slide
bottom
glass
slide
b
Steps 23-25
6 cm
1.2 cm
tape
mask
c
Step 28-29
LED-2
Step 31
Step 31
Step 32
d
Step 33
Incubation
at 90°C
Incubation
at RT
e
f
Inlet
nanoport
Magnet
Outlet
nanoport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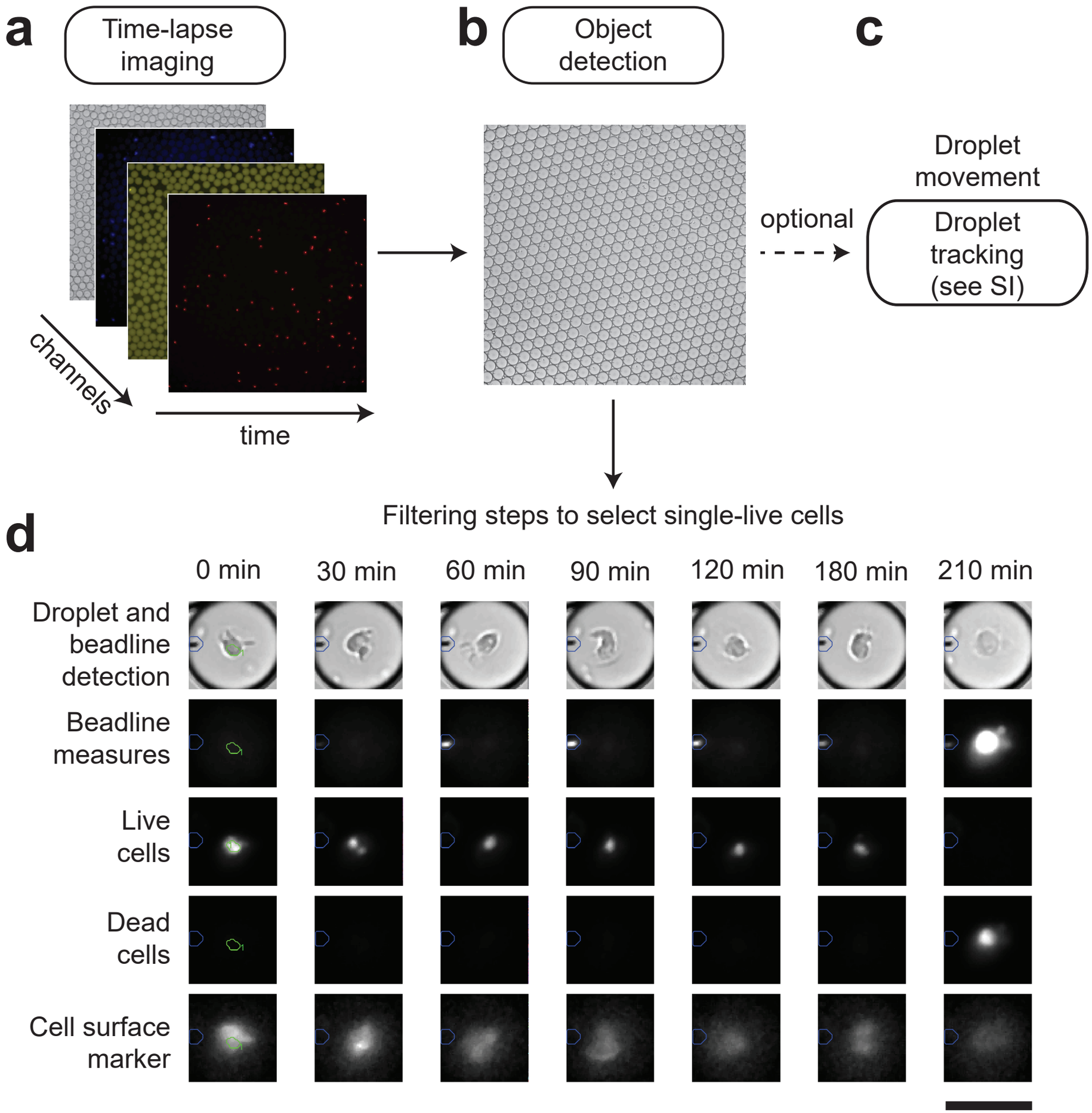
Streptavidin
b
TNF-α
I_B/I_D
TNF-α [nM]
e
IgG1
IgG2
I_B/I_D
IgG [nM]
c
TNF-α
I_B/I_D
Time (min)
f
IgG1
IgG2
I_B/I_D
Time (min)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Time-lapse imaging
b
Object detection
c
Droplet movement
optional
Droplet tracking (see SI)
channels
time
Filtering steps to select single-live cells
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90 min
120 min
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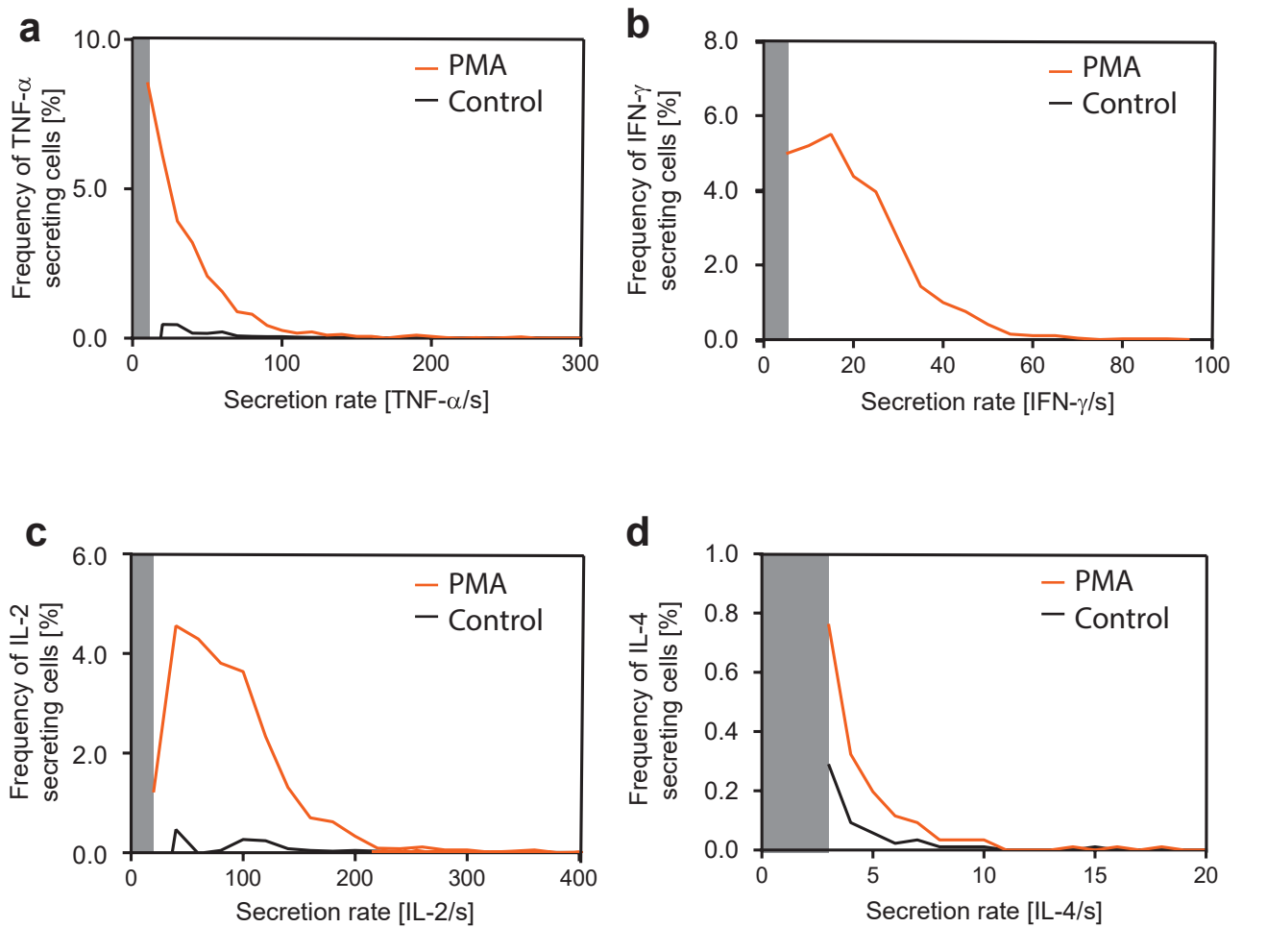
210 min
Droplet and beadline detection
Beadline measures
Live cells
Dead cells
Cell surface marker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0
100
200
300
Secretion rate [TNF-α/s]
PMA
Control
b
Frequency of IFN-γ secreting cells [%]
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6.0
4.0
2.0
0.0
0
20
40
60
80
100
Secretion rate [IFN-γ/s]
PMA
Control
c
Frequency of IL-2 secreting cells [%]
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2.0
0.0
0
100
200
300
400
Secretion rate [IL-2/s]
PMA
Control
d
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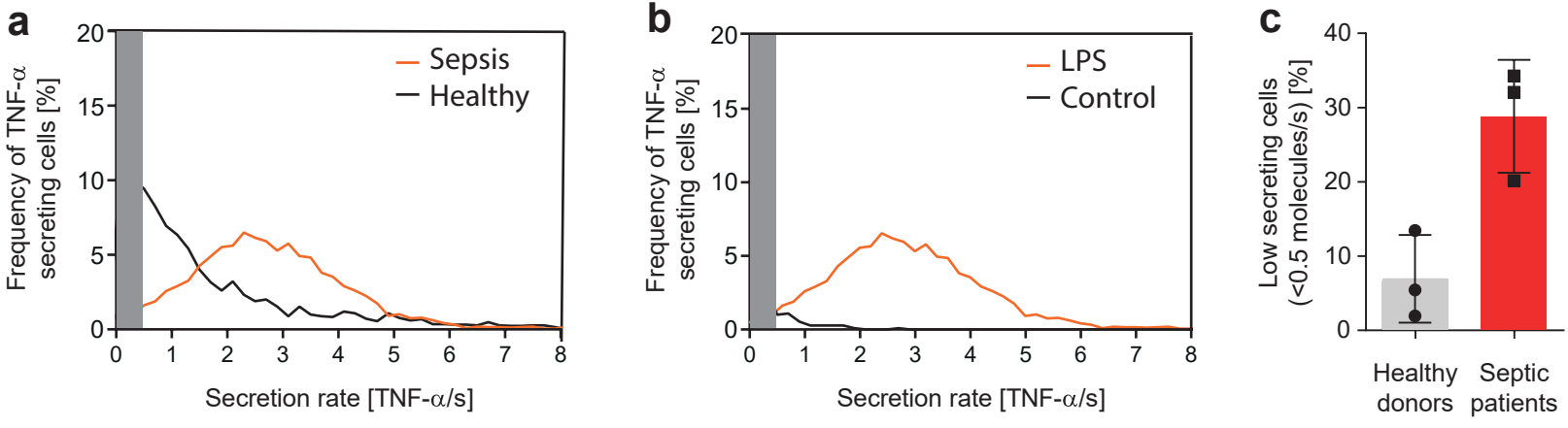
Control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Sepsis
Healthy
Secretion rate [TNF-α/s]
b
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Control
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c
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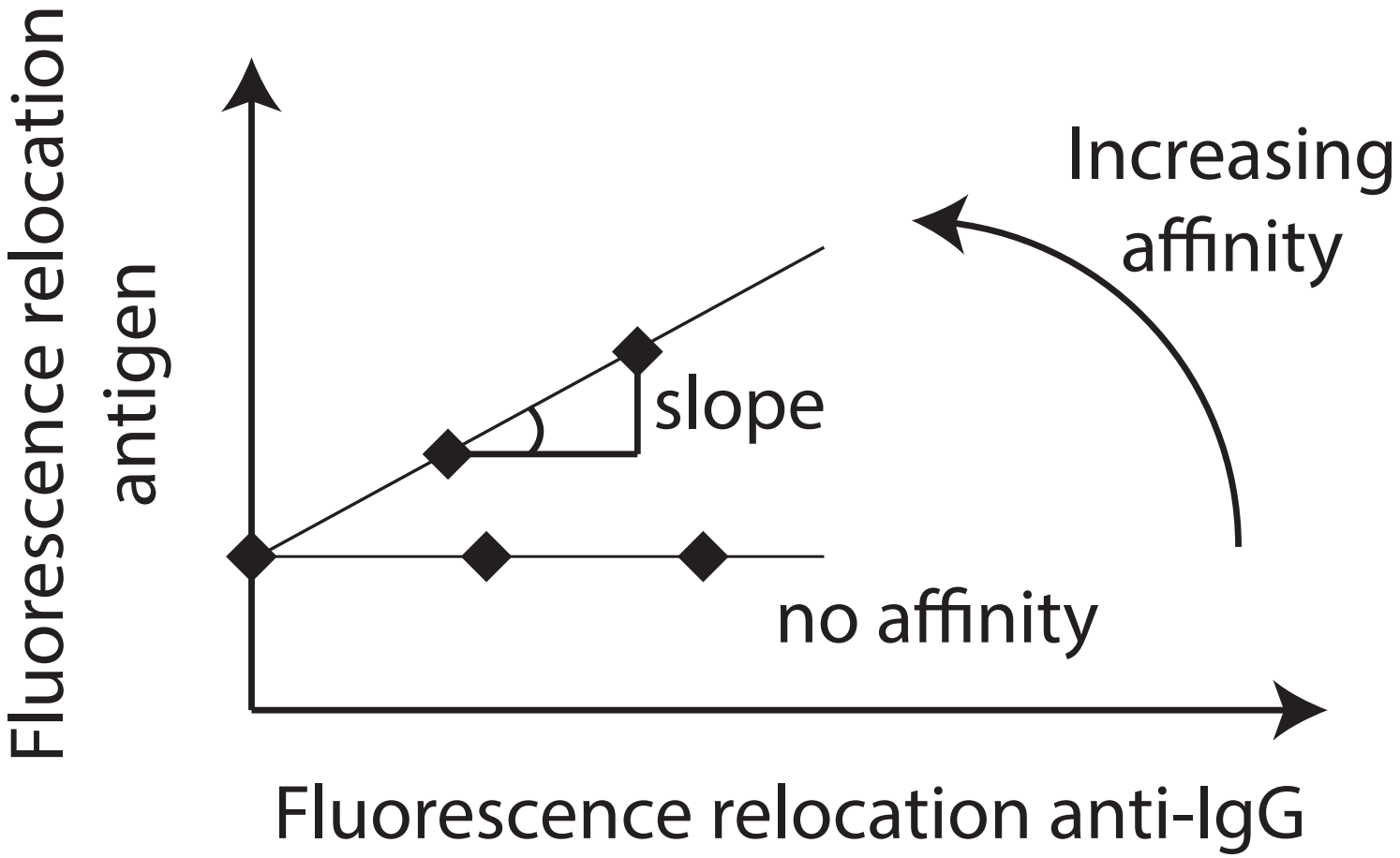
Healthy donors
Septic patients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Increasing affinity
slope
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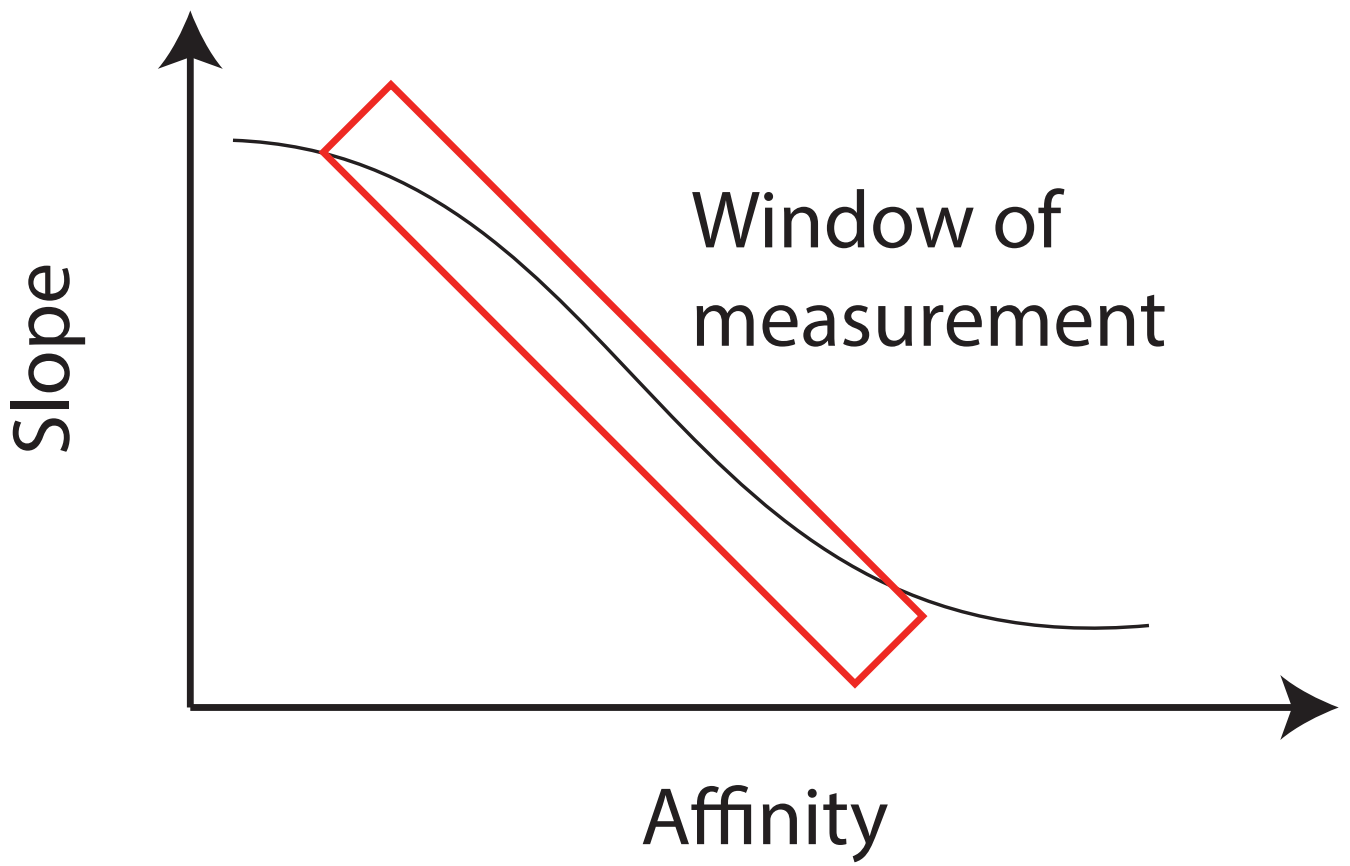
Fluorescence relocation anti-IgG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Window of measurement
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