Lab on a Chip

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Clockwork PCR Including Sample Preparation**

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With a few exceptions, the micro total analysis systems (μTASs) currently available have failed to live up to the ideal of the miniaturization of multiple laboratory operations onto a single chip.[1] These systems perform sample preparation off chip and only pursue a single function.[2] Hence, the definitive challenge is to interface the processing of real-world biological samples with downstream applications.[3] To this end, the manipulation of individual droplets on a planar surface offers an attractive option for a μTAS.[4] Herein, we transform a free droplet containing surface-functionalized superparamagnetic particles into a virtual μTAS with a (sub)microliter (μL) volume. Aside from being force mediators for actuating the droplet in a magnetic field, the superparamagnetic particles serve as a solid support for the sequential performance of laboratory or (bio)chemical processes. Depending on its particular task, the droplet temporarily becomes a pump, valve, mixer, extractor, or thermocycler. In an automated experiment, 30 green-fluorescent protein (GFP) transfected THP-1 cells are isolated from 25 μL of blood, 100-fold preconcentrated, purified, lysed, and subjected to a real-time PCR (RT-PCR) targeting the transfection vector, all within 17 min. Fast thermocycles of 8 s take place on a disposable substrate under time–space conversion by rotating the droplet clockwise over different temperature zones. Other PCR-based (bio)assay formats are easily adaptable, which makes this μTAS an attractive candidate for decentralized diagnostics.

Most bench-scale thermocyclers rely on a thermoelectrically heated metal block holding plastic tubes containing up to 50 μL of PCR mixture. This setup results in a high thermal mass and the PCR run time—typically hours—is limited by the low heating and cooling rates. Downscaling and/or the utilization of highly heat-conductive materials can overcome these limitations and a chip-based (sub)microscale PCR can perform the job within minutes.[5] There are two ways of conducting an on-chip PCR.[6–9] In the time domain, a stationary PCR mixture is thermocycled between three different temperatures. Under time–space conversion, a PCR mixture is driven through a microchannel that is constantly held at three different temperatures. This is why the PCR mixture reaches its thermal equilibrium quickly and a PCR in the space domain allows for fast thermocycling.

With a few exceptions,[3,6,10,11] the on-chip PCR is based on template DNA that has already been preprocessed off chip by using established bench-scale procedures. Complex samples like blood, saliva, or cell-culture medium contain (bio)chemicals, air bubbles, particulates, food residues, cell debris, etc. that have to be removed because they hamper the microfluidic operations and/or the subsequent PCR. This front-end sample workup is highly specific and cannot always be done on the (sub)microscale.[1] Clearly, the ability to integrate a basic set of laboratory operations into a single device and to directly handle real-world biological samples will be key in defining the commercial success of any μTAS.[2,3]

Almost all bench-scale (bio)chemical protocols depend on handling fluid boluses by using a pipette. A droplet-based (bio)chemical protocol is functionally equivalent to its bench-scale version: its reconfiguration or scale-down simply requires the rearrangement or volume variation of the droplets.[4] This flexibility cannot be matched by conventional microfluidic architectures that rely on a continuous flow of liquids in rigid microchannels permanently micromachined into silicon, glass, or polymer substrates.[12]

Electrowetting-on-dielectric,[13] dielectrophoresis,[14] surface-acoustic waves,[15] and (electro)magnetic forces[16] are popular techniques that are used to actuate droplets either sandwiched between two plates or positioned on an open surface. Among these, (electro)magnetic actuation is unique in that it is unaffected by surface charges, pH values, or ionic strength. Thus, it is compatible with a wide range of substrate materials and (bio)chemical processes. Furthermore, external permanent or electromagnets that remotely control the superparamagnetic particles make the running of a dedicated test on a low-cost disposable possible. Notably, the most important tasks within a bioassay—sample isolation/preconcentration, labeling, and detection—can be assigned to superparamagnetic particles.[17]

In our approach, a free droplet spontaneously self-organizes on a Teflon-coated glass substrate by emulsifying an aqueous suspension of superparamagnetic particles in an immiscible liquid (Figure 1 a). Sealing of the droplet in mineral oil prevents the aqueous phase from evaporating and renders a complicated chip design for the perpetuation of a humidifying atmosphere unnecessary.

An external permanent magnet is used for droplet actuation. The magnetic-field gradient exerts a translational force on the superparamagnetic particles suspended in the aqueous phase, a force that is transferred onto the inner aqueous phase/mineral oil interface. To maximize the mag-

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Supporting information for this article is available on the WWW under http://www.angewandte.org or from the author.

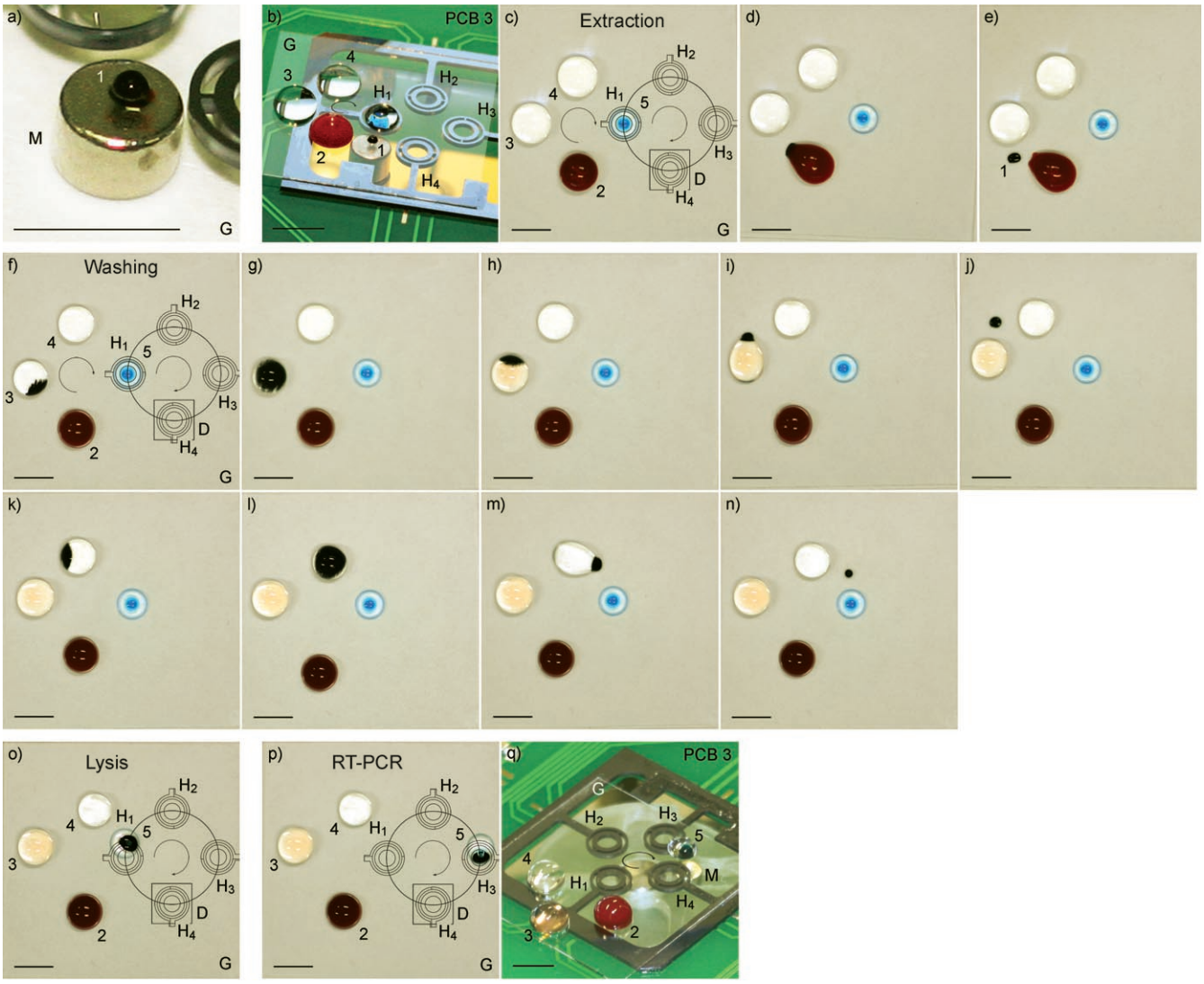

Figure 1. Sample preparation and RT-PCR. a) A droplet (250 nL) of Dynabeads CD15 sealed with mineral oil (100 nL) on a Teflonized surface. Moving, merging, mixing, and splitting of droplets is moderated by a permanent magnet. b) Droplet arrangement. c–n) After immunocapture through their CD15 cell-surface marker (c), the CD15-bound GFP-transfected THP-1 cells were extracted/preconcentrated from the blood droplet (d, e) and purified twice in washing-solution droplets (f–n). Potential PCR inhibitors enclosed in the slurry of Dynabeads CD15, such as erythrocytes, remained in the washing solution. o) Thereafter, the purified GFP-transfected THP-1 cells were merged with the RT-PCR mixture droplet and thermally lysed in temperature zone 1. p, q) Finally, the PCR took place in the space domain by clockwise rotation/pausing of the droplet over the four different temperature zones. Interrogation of the droplet at every turn in the extension zone (zone 4) by a fluorescence detector enabled real-time monitoring. D: fluorescence detector; G: Teflon-coated glass substrate; H_1–H_4: heaters/temperature sensors; PCB 3: printed circuit board 3; M: permanent magnet fixed to a stepper motor; 1: Dynabeads CD15 (250 nL) with immunocaptured GFP-transfected THP-1 cells; 2: blood droplet (25 μL) spiked with 30 GFP-transfected THP-1 cells; 3 and 4: washing-solution droplets (25 μL); 5 (blue): RT-PCR mixture droplet (1.5 μL) overlaid with mineral oil (4.5 μL). The arrows indicate the start/end point and direction of the sample preparation and RT-PCR, respectively. Scale bar: 5 mm.

netic force, the concentration of superparamagnetic particles is raised 40-fold. Whether the droplet containing the superparamagnetic particles moves or splits depends on a subtle balance between the magnetic force acting on the superparamagnetic particles, the interfacial tension of the droplet, and the frictional force between the droplet and the Teflon-coated substrate surface (see Movie 1 in the Supporting Information). The droplet moves as long as the interfacial tension overcomes the frictional force. The anti-CD15-coated superparamagnetic particles usually remain trapped inside the droplet because their hydrophilic surface makes them possess a greater affinity towards the aqueous phase than the surrounding mineral oil. The droplet splits asymmetrically into a big and small droplet if the frictional force overrides the interfacial tension. After splitting, the small droplet enclosing the aqueous suspension of superparamagnetic particles is still emulsified by a thin film of mineral oil. Experimentally, the outcome—moving or splitting—can easily be controlled by variation in the volumes of the interacting droplets: if we combine a 250 nL droplet comprising the superparamagnetic particles with another droplet and their combined volume is less than 10 μL, the resulting droplet moves (Figure 1p and

q); if the combined volume is more than 10 μL, the 250-nL droplet holding the superparamagnetic particles splits (Figure 1 d, i, and m).

Additionally, the anti-CD15-coated superparamagnetic particles temporarily serve as a solid support for (bio)chemical processes. Placing the Teflon-coated glass substrate on micromachined heaters with integrated optics allows the observation of temperature-controlled (bio)chemical processes in real time (Figure 1 b–q). In this context, every droplet and/or droplet manipulation is equivalent to an item/piece of equipment or task in a laboratory. For example, a droplet is like a test tube (Figure 1 c), splitting of the droplet enclosing the anti-CD15-coated superparamagnetic particles/CD15-expressing THP-1 cells from blood resembles an extraction (Figure 1 b–e), and a rotating droplet covered by mineral oil pausing on a microfabricated heater corresponds to a thermocycler (Figure 1 p and q). All essential components of the μTAS are virtual, which is why we can assemble a new prototype in minutes. It is possible to place a single-use chip, run a dedicated test, and dispose of the chip because the magnetic force acting on the superparamagnetic particles is remote from the surface.

Anticoagulants used for blood preservation, RNases/DNases present in blood plasma and some leucocytes subtypes, as well as hemoglobin incorporated in erythrocytes, significantly inhibit a PCR and have to be removed. The capability of the anti-CD15-coated superparamagnetic particles to specifically isolate, preconcentrate, and purify 30 CD15-expressing THP-1 cells spiked into 25 μL of blood[18] and to pass these cells on to the PCR is the key feature for performing sequential (bio)chemical processes (Figure 1 b–q; see Movie 1 in the Supporting Information). Consecutively, a 250 nL droplet containing the surface-bound THP-1 cells is split from a 25 μL blood droplet, purified in two 25 μL washing-solution droplets, and merged with a 1.5 μL cell-lysis/PCR mixture droplet. For optimization of the extraction and lysis of the THP-1 cells, we use GFP-transfected THP-1 cells (see the Supporting Information). No GFP-transfected THP-1 cells are subsequently observed in the blood and washing solutions. After the extraction, the GFP-transfected THP-1 cells are transferred from a 25 μL to a 250 nL volume, which corresponds to a 100-fold preconcentration. Potential PCR inhibitors largely stay behind in the blood or are diluted twice by using the washing solutions. As the volume ratio of the superparamagnetic-particle suspension and the washing solution is 1:100, the contaminants possibly carried off in the split droplet are diluted 10^4-fold ($(1/100)^2$). For example, on average, 1.2×10^4 [0] out of 1.3×10^8 erythrocytes present in a 25 μL blood volume are counted in the first [second] washing solution, which represents a $10^{4[8]}$-fold reduction. We quantitatively recover the GFP-transfected THP-1 cells in less than six minutes with a purity suited for a PCR.

After removal of the PCR inhibitors, the surface-bound GFP-transfected THP-1 cells are thermally lysed within the PCR mixture located in the temperature zone 1 (Figure 1 o). Lysis at 95 °C for 1 min is sufficient to release the DNA from the GFP-transfected THP-1 cells into the PCR mixture and to extinguish the fluorescence of the GFP.[19] Neither higher temperatures of up to 130 °C nor longer lysis times of up to 10 min affect the thermocycle threshold (C_T) in the subsequent RT-PCR targeting the GFP-transfection vector. The GFP-transfection vector was choosen as the template, because we plan use it as an internal control for multiplex RT-PCR experiments employing a *Taq*Man-based[20] two-step thermocycling format.[21]

From temperature zone 1, the droplet rotates clockwise and pauses on temperature zones 2–4 (Figure 1 p and q). For the two-step thermocycling protocol 1, we keep temperature zone 1 and temperature zones 2–4 constantly at the denaturation and annealing/extension temperatures, respectively. Stable temperatures for, homogeneous temperature distributions within, and a minimal thermal cross-talk between the four temperature zones are critical for the thermal layout of the PCR chip. The temperature variation measured by the temperature sensors is ±1 °C between 25 and 95 °C. Modeling by using finite-element analysis (FEA) (see the Supporting Information) and IR imaging of the Teflon-coated glass-substrate surface (Figure 2) show a temperature uniformity of

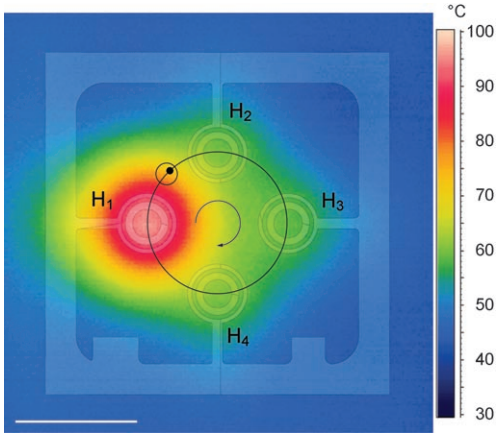

Figure 2. IR image of the temperature distribution on the Teflon-coated glass substrate, based on thermocycling protocol 1. Illustrations of the four heaters/temperature sensors, the traveling path of the droplet, and the droplet containing the Dynabeads CD15/PCR mixture (●) emulsified in mineral oil (○) are superpositioned. The mean temperatures within the inner rings were 94.5, 60.6, 60.4, and 60.6 °C for temperature zones 1–4, respectively. Scale bar: 10 mm.

±1 °C between 25 and 95 °C on the surface areas occupied by the droplet during the PCR. Large air gaps thermally isolating the four heaters/temperature sensors from one another eliminate thermal cross-talk between adjacent temperature zones on the glass-substrate surface and result in a smooth temperature transition between them (see the Supporting Information). Typically, the angular velocity during a rotation is 90° s^{-1}, which correlates with heating and cooling rates of ±35 °C s^{-1}.

We monitor the PCR in real time (Figure 3) by periodically recording the fluorescence of the paused droplet in temperature zone 4 by using a fluorescence microscope combined with a photomultiplier tube (see the Supporting Information). Inclusive of the transition times of 1 s each between adjacent temperature zones, it just takes 8 s for one thermocycle (see Movie 2 in the Supporting Information).

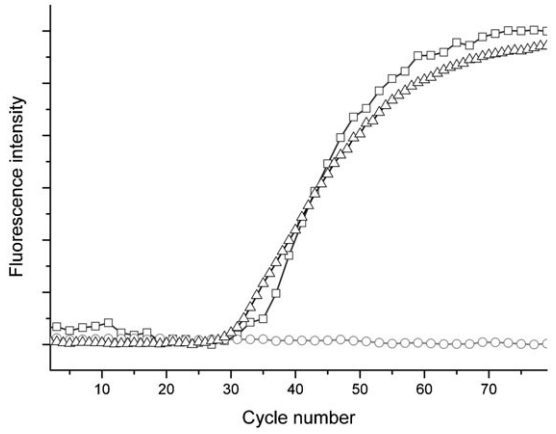

Figure 3. Results of the RT-PCR with thermocycling protocol 1 (△) and thermocycling protocol 2 (□). The C_T values were 29.4 and 30.6, respectively. A negative template control (NTC) was also performed (○).

One thermocycle relates to one revolution and results in an overall PCR run time of 10 min and 40 s for 80 thermocycles. For the two-step thermocycling protocol 2, the temperatures of successive temperature zones are altered between 95 and 60 °C, which further reduces the time for a thermocycle to 7 s (see Movie 3 in the Supporting Information). However, with 2 thermocycles per revolution, the resolution of the C_T value has an uncertainty of ±1 (Figure 3). To verify the PCR-product specificity and yield, capillary electrophoresis (CE) is used (Figure 4). Even after 80 thermocycles, no nonspecifically amplified PCR products are observable, a result indicating an optimized PCR. A droplet volume of 1.5 μL yields 31–34 ng of PCR product per μL.

With the exception of increasing the relative concentration of the superparamagnetic particles, we use established bench-scale protocols throughout the experiment. The overall reaction time of only 17 min is due to the short diffusion distances between the anti-CD15-coated surface of the superparamagnetic particles and the GFP-transfected THP-1 cells during the sample preparation, as well as the fast heat transfer that is intrinsic in a microscale PCR.

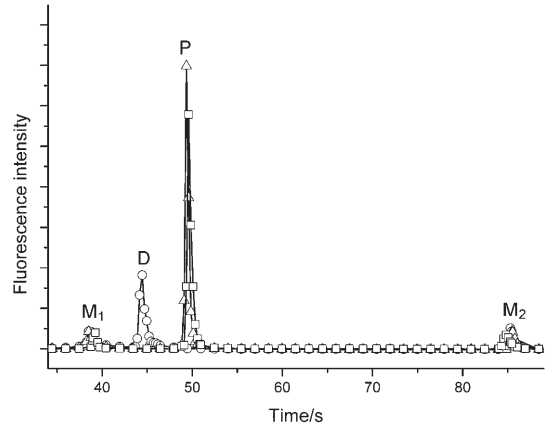

Figure 4. Results of the CE of the PCR products with thermocycling protocol 1 (△) and thermocycling protocol 2 (□). An NTC was also performed (○). For all experiments, 30 GFP-transfected THP-1 cells were used as input. The yields of the PCR products were 32.1 and 34.2 ng μL^{-1}, respectively. M_1: marker 1 (15 bp); M_2: marker 2 (600 bp); D: primer dimer; P: PCR product (99 bp).

Handheld units for point-of-care diagnostic tests in low-resource settings call for low-cost instruments and/or disposables. Besides the thermal management, all components of our prototype instrument originate from a CD-ROM drive, such as the power supply, the stepper motor for the circular microfluidic actuation, and the optics for the fluorescence detection (see the Supporting Information). At present, we are working on optical multiplexing by interrogating the rotating droplet at different locations among temperature zones 2–4 by using a miniaturized detector array working at different wavelengths.

Experimental Section

(Heptadecafluoro-1,1,1,1-tetrahydrodecyl)trimethoxysilane (Gelest)-modified no. 1 BB022022A1-glass microscope cover slips (Menzel) were spin coated with a 1% solution of Teflon AF 1600 (DuPont) in FC-75 Fluorinert (3M).[22] The 0.1–0.3 μm thick Teflon-like films had static contact angles with water (Millipore) and M5904 mineral oil (Sigma) of 115 ± 2 and 85 ± 2°, respectively.

For rotation of the droplet containing the superparamagnetic particles, a stack of permanent N30H neodynium iron boron disc magnets (Assemtech) was attached to a 0.9° size-17 Super Slim Line stepper motor (NetMotion) controlled by a K179 stepper driver (Ozitronics). The distance between the top magnet and the droplet was around 0.8 mm; the superparamagnetic particles were therefore exposed to a magnetic-field strength of around 0.4 T. To linearly move the droplet, the stepper motor was mounted on a ProScanII motorized-stage system (Prior Scientific). A program written in LabVIEW 8 software (National Instruments) served as the user interface for droplet manipulation. Unless stated otherwise, all reactions were carried out at 25 °C.

The thermal management of our silicon-micromachined PCR chip is described in detail elsewhere.[23] However, the printed circuit board comprising the thermal management was numbered up for accommodating individual heaters/temperature sensors 1–4 (see the Supporting Information).

For fixing the Teflon-coated glass substrate on top of the four heaters/temperature sensors and improving the heat transfer into the corresponding temperature zones, M5904 mineral oil (100 nL) was applied. The temperature distribution on the Teflon-coated glass substrate was measured by using a PM200-IR camera (MTech Imaging) calibrated with a precision of ±0.2 °C.

The fluorescence signal was continuously recorded into a text file using a BX-51-fluorescence microscope (Olympus) equipped with a 20× M Plan Apo objective (Mitutoyo), a 49002 filter set (Chroma), an X-Cite 120 PC-fluorescence illumination system (EXFO Life Sciences), a H5784-20 photomultiplier tube (Hamamatsu), and a TDS5054B digital phosphor oscilloscope (Tektronix). The raw fluorescence signal was smoothed by three-point-averaging by using Origin Pro 7 software (OriginLab).

The THP-1 cells (American Type Culture Collection) were transiently transfected with the pmaxGFP vector encoding the GFP by using the Cell Line Nucleofector Kit V (Amaxa).[24] After 24 h, the transfection efficiency was around 80%.

Capillary whole blood was taken from one of the authors by finger pricking and stored at 4 °C for 0–24 h in ethylenediaminetetraacetic acid coated Microtainer blood-collection tubes (BD). Either reverse pipetting or wetting of the inner and outer surfaces of the pipette tip with a thin film of M5904 mineral oil was used for metering the blood and suspension of the superparamagnetic particles.

For the extraction, a suspension (250 nL) of 400 μg μL^{-1} Dynabeads CD15 (Dynal Biotech) in 0.01M phosphate-buffered saline (PBS; pH 7.4; Sigma)/0.1% bovine serum albumin (BSA; Carl Roth) was added into a blood droplet (25 μL) spiked with 30 GFP-transfected THP-1 cells. This was further mixed by pipetting up and down 10 times and was then incubated for 5 min. Thereafter, a droplet (250 nL) containing the THP-1 cells immunocaptured onto the Dynabeads CD15 was split from the blood droplet and washed successively in two droplets (25 μL) of 0.01M PBS/0.1% BSA. No fluorescent GFP-transfected THP-1 cells were visible any longer in the droplets containing the blood and the washing solutions. To manually count the number of residual erythrocytes carried off into the washing solutions, a Bright Line hemacyctometer (Sigma–Aldrich) was used.

The droplet containing the purified surface-immobilized THP-1 cells was then merged with a droplet containing PCR mixture (1.5 μL) in temperature zone 1 and the cells were thermally lysed at 95°C for 1 min to make their DNA accessible.

The forward primer 5′-ATG ACC AAC AAG ATG AAG AGC A-3′ and reverse primer 5′-GTA GGT GCC GAA GTG GTA GAA G-3′ amplified a 99-bp fragment of the transfected pmaxGFP vector,[25] which was monitored in real time by employing the *Taq*Man probe 5′-carboxyfluorescein(5′-FAM)-AAG GCG CCC TGA CCT T-CA GCC CTA-3′–Eclipse Dark Quencher (all from Research Biolabs). The PCR cocktail was based on the *Taq* PCR Core Kit (Qiagen) and had the following composition: water (28.0 μL), Q solution (10.0 μL), Qiagen PCR buffer (5.0 μL), deoxynucleotide phosphates (1.0 μL in total), 10 μM *Taq*Man probe (0.5 μL), 10 μM forward and reverse primers (2.5 μL each), and *Taq* polymerase (0.5 μL). Of that mixture, 1.5 μL are used for the miniaturized PCR and the remainder for a bench-scale PCR on a DNA Engine Opticon 2 thermocycler (MJ Research). Nontransfected THP-1 cells served as an NTC.

For the PCR, the droplet rotated 40–80 times clockwise over temperature zones 1–4 (Table 1). The angular velocity of the rotating droplet was 90° s^{-1}, which translated to 7–8 s for one thermocycle. We centered the long-distance objective of the fluorescence microscope above temperature zone 4 to acquire the real-time data during the last second of the extension step. Alternatively, a miniaturized fluorescence detector was used (see the Supporting Information).[26] The PCR-product specificity and yield were verified by CE by using a Bioanalyzer 2100 instrument (Agilent).

Table 1: Thermoycling protocols 1 and 2.

Heater	Protocol 1[a]		Protocol 2[a,b]	
	T [°C]	*t* [s]	*T* [°C]	*t* [s]
1	95	1	95	1
2	60	1	60	4
3	95	1	95	1
4	60	1	60	4

[a] For the trajectory of the droplet in the first thermocycle, see the Supporting Information. [b] One revolution of the droplet corresponded to two thermocycles.

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