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Circulating leukocytes are indispensable components of the immune system, and rapid analysis of their native state or functionalities can help to unravel their pathophysiological roles and identify novel prognostics biomarkers in health and diseases. Herein we report a novel high throughput “sample-in-answer-out” integrated platform for continuous leukocyte sorting and single-cell electrical profiling in a label-free manner. The multi-staged platform enables isolation of neutrophils and monocytes from diluted or lysed blood samples directly within minutes based on Dean Flow Fractionation (DFF) (stage 1). DFF-purified leukocytes are next inertially focused in serpentine channels into single stream (stage 2) prior impedance detection (stage 3). As a proof-of-concept for neutrophil functional characterization towards diabetes testing, we characterised the formation of neutrophil extracellular traps (NETosis) of healthy and glucose-treated neutrophils and observed significant changes in dielectric properties (size and opacity) between both groups. Interestingly, the NETosis profile induced by calcium ionophore (Cal) and phorbol 12-myristate 13-acetate (PMA) were also electrically different, which could be attributed to the differential rates of cell enlargement and attenuated membrane permeability. Taken together, these results clearly demonstrated the potential of the developed platform for rapid (~mins) and label-free leukocyte profiling, and the use of impedance signatures as novel functional biomarkers for point-of-care testing in diabetes.

Introduction

Circulating leukocytes are known to orchestrate various biological processes either as a part of host defense mechanisms or in pathogenesis of major diseases such as cancer, HIV, diabetes mellitus or cardiovascular diseases¹. Neutrophils are the key effector cells of the innate immunity, and formation of neutrophil extracellular traps (NETosis) is a new defense function to resolve an inflammation by releasing DNA web-like structures comprising of decondensed chromatin, histones and granule proteins to trap and kill pathogens²⁻⁴. NETosis can also contribute to disease development (e.g. cancer) and host tissue damage in diabetes mellitus⁵⁻⁷, and have been proposed as novel prognostics markers or drug targets^{8, 9}. As most NETosis assays are based on ELISA¹⁰ or immunofluorescence¹¹ with a bulk-measurement readout, a better understanding of the NETosis-induced biophysical and dielectric changes at single-cell resolution can potentially be exploited for development of novel diagnostics or immune risk stratification strategies. Conventional fluorescence- and magnetic-activated cell sorting (FACS, MACS) are commonly

used for leukocyte separation in research settings, but they require antibodies labeling which is expensive and time consuming. Our group has previously reported a spiral microfluidic device (Dean Flow Fractionation, DFF) for size-based leukocyte fractionation^{12, 13}. By exploiting the presence of inertial coupled Dean flow in spiral channel, particles/cells experience size-dependent hydrodynamics forces and migrate to different lateral positions based on their size. This enables continuous size-based cell separation in a high throughput manner ($\sim 10^4$ – 10^6 cells/min)¹⁴.

For label-free cell measurements, electrorotation or impedance spectroscopy are attractive techniques which characterize intrinsic dielectric properties of single cells¹⁵. These techniques have been widely used for leukocyte studies including cell identification¹⁶⁻¹⁸, leukocyte activation^{19, 20}, and study of immune functions (NETosis)^{21, 22}. However, impedance cytometry till date has limited clinical utilities due to low flow rate operation and the inability to handle complex biosamples (e.g. blood) directly^{15, 23, 24}. One key concern is the presence of RBCs and platelets which can co-flow with leukocytes through the detection region simultaneously, thereby causing error in impedance readout and complicating signal analysis. Hence, there exists a critical need to develop impedance biosensing platforms with leukocyte sorting capabilities to facilitate assay automation and point-of-care testing. A few notable examples include recent work from Bashir et al^{16, 25} and Han et al^{26, 27} for CD4⁺ cell counting and circulating tumor cells (CTCs) analysis, respectively. A major limitation remains in the use of antibodies

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to isolate specific target cells which can complicate device operation and increase assay cost.

Herein, we report a novel integrated platform combining spiral inertial microfluidics (DFF) and impedance cytometry for continuous, label-free leukocyte sorting and single-cell electrical profiling (Fig 1). Neutrophils or monocytes are first isolated directly from blood samples using DFF (stage 1), and the DFF-purified leukocytes are subsequently inertially focused in asymmetric serpentine channels into single stream (stage 2) prior impedance detection (stage 3). By accounting for flow rate mismatch between each stage using circuit network analysis, the developed “sample in-answer out” platform facilitates single-step user operation with rapid readout (<5 min). We first demonstrated neutrophil and monocyte isolation from various

minimally processed blood samples including lysed blood, diluted blood or peripheral blood mononuclear cells (PBMCs), and impedance-based leukocyte enumeration using our platform. As a proof-of-concept for diabetes testing, we measured NETosis profiles of healthy and glucose-treated neutrophils and observed significant changes in dielectric properties (size and opacity) between both groups. A comparative study of NETosis induced by calcium ionophore (Cal) and phorbol 12-myristate 13-acetate (PMA) using immunostaining, fluorescence spectroscopy and our impedance biosensor further revealed that the dielectric changes are dependent on the NETosis stimulus and could be attributed to the differential rates of cell enlargement and attenuated membrane permeability. Taken together, our

Fig. 1 Integrated platform for leukocyte subset sorting and impedance phenotyping (A) Workflow of integrated device. Different colored boxes highlight different stages namely the particle sorting (DFF) (stage 1, red), flow rate reduction (FRR) (stage 2, green), and impedance detection (stage 3, blue) (B) Schematic illustrations depicting target cells flow behavior at different stages.

results clearly demonstrated the potential of the developed platform for high throughput ($\sim 10^4$ cells/min), single-step and label-free leukocyte profiling in clinical settings, and the use of impedance signatures as novel functional biomarkers for point-of-care testing in diabetes and other inflammatory diseases.

Experimental section

Device design and operation

The two-layer integrated platform was fabricated in polydimethylsiloxane (PDMS) using standard soft lithography techniques (See supplementary for more detail). For cell separation, a spiral microchannel ($500\ \mu\text{m}$ (w) $\times$ $115\ \mu\text{m}$ (h)) has a radius of 0.9–1 cm with a total length of 7–8 cm. For the stage 2, the flow rate reduction (FRR) consists of small curved channels (60 – $150\ \mu\text{m}$ (w) $\times$ $115\ \mu\text{m}$ (h)) and big curved channels (100 – $300\ \mu\text{m}$ (w) $\times$ $115\ \mu\text{m}$ (h)). The height for both stages was chosen to comply inertial focusing criteria ($a_p/h > 0.07$ where a_p is particle size) for leukocytes (~ 10 – $15\ \mu\text{m}$) but not the smaller RBCs (~ 6 – $8\ \mu\text{m}$) and platelets ($2\ \mu\text{m}$). Lastly, the impedance detector (stage 3) with a stepped decrease in channel height ($30\ \mu\text{m}$ (w) $\times$ $20\ \mu\text{m}$ (h)) is comparable to target cell size to maximize signal sensitivity for optimal single-cell impedance measurement with coplanar electrodes ($30\ \mu\text{m}$ (w) and $20\ \mu\text{m}$ (gap)).

Prior to any experiment, the device was primed with 1% bovine serum albumin (BSA, Biowest) in phosphate buffered saline (PBS, Lonza) for at least 1 h to prevent non-specific adhesion to the device. The sample and sheath were loaded to outer wall inlet and inlet wall inlet respectively, and perfused at a flow rate ratio of 1:10 by syringe pumps (PHD ULTRA™, Harvard Apparatus). Before any measurement or imaging, the platform was allowed to run for 1 min to reach stabilized state. During experiments, the platform was mounted on an inverted microscope (Nikon Eclipse Ti). Fluorescent images and high-speed images were taken using Hamamatsu ORCA-Flash4.0 and Phantom V9.1, respectively.

Sample preparation

Depending on the leukocyte of interest, the whole blood samples were pre-processed differently. For neutrophil isolation from lysed blood, RBC lysis buffer (eBioscience) was added into whole blood (10:1 v/v) for 3 min and quenched with 0.5% BSA in PBS. For neutrophil isolation from diluted blood, whole blood was diluted in 0.1% BSA in PBS (1:500). To acquire peripheral blood mononuclear cells (PBMCs) for monocyte isolation, whole blood was subjected to Ficoll-Paque™ Plus (GE Healthcare) density centrifugation according to manufacturer's protocol. For impedance measurement, $10\ \mu\text{m}$ fluorescein isothiocyanate (FITC) beads (Polysciences) were added to every sample at 1 – 2.5×10^4 beads/mL. Since the beads have consistent dielectric properties, they will serve as a reference for comparisons across different experiments and devices.

Neutrophil treatment for NETosis studies

To study NETosis, neutrophils from lysed blood were prepared from 1 mL of whole blood. After washing twice, the sample were treated with Cal (Sigma-Aldrich, $20\ \mu\text{M}$) or PMA (Sigma-Aldrich, $2\ \mu\text{M}$) and incubated at 37°C . The neutrophils undergoing NETosis were sampled for impedance measurement at various time points: 30 min, 60 min and 120 min. For glucose treated samples, whole blood was treated with 30 mM of D-glucose (Sigma-Aldrich) for 4 h at room temperature prior RBC lysis.

Imaging of NETosis progression

For nucleus visualization, DFF-isolated neutrophils were stained with Hoechst 33342 dye (Life Technologies) for 15 min at 4°C . Following washing, cells ($\sim 10^4$ cells/50 μL) were seeded in a 5 mm PDMS well coated with poly-L-lysine (Sigma-Aldrich, 0.01% w/v) and SYTOX green nucleic acid stain (ThermoFisher Scientific) was added into the well. The fluorescent images were captured as unstimulated at 0 min. After that, NETs stimulus ($20\ \mu\text{M}$ Cal or $2\ \mu\text{M}$ PMA) was added and images were taken at different time points (30, 60 and 120 min). During the course of imaging, the setup was mounted on an on-stage incubator at 37°C .

Quantification of NETosis using microplate reader

Neutrophils were seeded on a 96-well clear bottom black plate (BD Biosciences) at a concentration of $\sim 10^5$ cells per well in 200 μL of media (0.1% BSA in PBS), and stimulated with $20\ \mu\text{M}$ Cal or $1\ \mu\text{M}$ PMA. Unstimulated neutrophils served as negative controls. For each condition, either 500 nM SYTOX green were added to detect DNA release. The progress of NETosis was assessed by measuring the fluorescence of SYTOX Green-DNA interactions (excitation = 485 nm, emission = 528 nm) using a microplate reader (BioTek Synergy H1 Hybride multi-mode reader) every 30 min for 120 min after stimulation. Duplicates for each condition were performed.

Flow cytometry analysis

The staining protocols for leukocyte identification are similar as previously described¹⁹. To characterize neutrophil activation before and after sorting. The samples were stained for 30 min at 4°C with FITC-labelled CD11b and allophycocyanin (APC)-labelled CD66b antibodies. Non-specific antibody binding was examined using corresponding isotype negative control antibodies. The cells were washed once prior to flow cytometry analysis. All antibodies were purchased from eBioscience (1:20 dilution) and analyzed using a BD LSR Fortessa flow cytometer (BD Biosciences).

Statistical analysis

Paired t-tests were used to determine the statistical significance of the difference between two sets of data. $P < 0.05$ was regarded as statistically significant. All analysis was performed using GraphPad Prism V7.0 (GraphPad software).

Study approval

Written informed consent was obtained for all subjects during recruitment. All protocols were approved by the institutional review board of Nanyang Technological University (IRB-2014-04/27 and IRB-2018-10-015-02), Tan Tock Seng Hospital (2014/00416) in compliance with the Human Biomedical Research Act (Ministry of Health, Singapore). Blood samples were collected from venipuncture into a 4 mL K2 EDTA tube (BD vacutainer) of healthy donors for experiments.

For more information on device fabrication, measurement setup and data presentation, refer to supplementary information.

Results and discussion

Device principle

Fig 1A shows the integrated platform workflow. The platform consists of three stages namely (1) Dean flow fractionation (DFF), (2) flow rate reduction (FRR), and (3) impedance detection using coplanar electrodes. For cell separation using DFF (stage 1), sample and sheath buffer were introduced at the outer and inner wall of the channel, respectively at a sample to sheath flow rate ratio of 1:10. As the cells flow through the spiral channel, they undergo lateral migration towards the inner wall due to the presence of the Dean drag force (F_D). Near the inner wall, the larger cells (neutrophils/monocytes) experience additional inertial lift forces (F_L) and equilibrate near the inner wall, while smaller components (lymphocytes, RBCs and platelets) recirculate back to the outer wall to achieve

separation (**Fig 1B**). The larger leukocytes then continue to the FRR module (stage 2) where they experience alternating Dean vortices in a serpentine channel and inertially focus into single stream at the channel centre²⁸. This is important as it facilitates removal of excess fluid volume from the side channels to reduce overall volume flow rate entering the impedance detector (**Fig 1B**). Lastly, the target cells will enter the impedance detector and two dielectric characteristics are acquired for each cell; (1) opacity (ratio of impedance magnitude at 1.7×10^6 Hz ($|Z_{HF}|$)) to impedance magnitude at 0.3×10^6 Hz ($|Z_{LF}|$) reflecting electrical properties of cell membrane, and (2) $|Z_{LF}|$ which reflects cell size.

Device characterization with different sized beads

To determine the optimal flow rate condition for leukocytes sorting, 10 μm , 7 μm and 5 μm beads which represent monocytes/neutrophils ($\sim 10\text{--}12$ μm), lymphocytes ($\sim 7\text{--}8$ μm) and RBCs ($\sim 5\text{--}7$ μm), respectively, were used for flow rate characterization. At the optimized sample inlet flow rate range of 150–170 $\mu\text{L}/\text{min}$, 10 μm beads experienced strong inertial forces ($a_p/h > 0.07$ where a_p is particle size, and h is height of the channel) and focused near the inner wall. As expected, smaller 7 μm and 5 μm beads ($a_p/h < 0.07$) recirculated towards the outer wall due to dominant Dean force, which resulted in their separation into the waste outlet (**Fig 2A**). Flow rate characterization was also performed for the FRR design, and we showed that a similar flow working rate range of 140–170 $\mu\text{L}/\text{min}$ could successfully focus the 10 μm beads to the center

Fig. 2 Device characterization with microbeads at different device stages. **(A)** Composite fluorescence images of 10 μm (left), 7 μm (center) and 5 μm (right) bead trajectory at particle sorting stage (DFF). **(B)** Composite fluorescence images of 10 μm at the 1st FRR, and **(C)** 2nd FRR (prior impedance detection). Scale bar = 100 μm . **(D)** Sorting purity of 10 μm from a mixture of different sized beads (5 μm , 7 μm and 10 μm) Data are presented as mean $\pm$ s.e.m (n=4).

of the channel for efficient fluid removal via the side outlets (**Fig S2A, B**).

We next determined the sorting efficiency of the developed platform with a bead mixture (5 μm , 7 μm and 10 μm) using high speed imaging and flow cytometry analysis. The high-speed microscopic images were taken at different sections of the device to show particle focusing streams (**Fig S3A**). Eluent was collected from the impedance outlet for flow cytometry analysis. $\sim 98.9\%$ sorting purity of 10 μm beads was achieved at the impedance outlet at the optimal flow rate of 150-160 $\mu\text{L}/\text{min}$ (**Fig 2D**). Sorting efficiency decreased slightly at 170 $\mu\text{L}/\text{min}$ to $\sim 90.3\%$ due to higher 5 μm beads contamination as they recirculated back to the inner wall. We next evaluated the enrichment ratio (ratio of sorted target cell concentration to initial target cell concentration), and found that the 10 μm bead concentration was increased by ~ 3 -fold (**Fig S3B**). Taken together, our results clearly indicate that the platform can effectively separate and concentrate the 10 μm beads to the impedance interrogation region (stage 3) while eliminating smaller beads to the waste during DFF (stage 1).

We also examined the effect of particle concentration ranging from 10^5 to 10^6 beads/mL on 10 μm bead trajectory at different stages and observed negligible differences in particle focusing at DFF and FRR1 (**Fig S4**). However, at bead concentration of 106 beads/mL, the sorting efficiency was slightly deteriorated in FRR2 as evident by shifting of the bead trajectory towards the bottom waste outlet. This would possibly occur as the beads become too concentrated after two stages of FRR ($\sim 3\times$ enrichment, thus $\sim 3\times 10^6$ beads/mL).

Device characterization with different blood samples

We next characterized the leukocyte isolation efficacy from diluted whole blood. Whole blood samples (stained with CD66b, CD14 and CD3/19, to identify neutrophils, monocytes and lymphocytes, respectively) were diluted 1:500 and pumped into the integrated platform at 150-170 $\mu\text{L}/\text{min}$. The eluent was collected from the impedance outlet for flow cytometric analysis. **Fig 3A** shows the stacked high-speed microscopic images of the integrated platform at different stages. Due to the

Fig. 3 Device characterization of different blood samples. Composite high speed images of blood cells from (A) diluted blood (1:500) (DB) and (B) peripheral blood mononuclear cells (PBMCs) at different device stages. Scale bar = 100 μm . Sorting purity of neutrophils from (C) diluted blood (500x) and (D) lysed blood and (E) monocytes from PBMCs. Data are presented as mean $\pm$ s.e.m (n=4).

high RBC concentration in the inlet sample, a thick RBC band was observed near the outer wall which was similar to the 5 μm beads flow profile. In the FRR stage, DFF-purified leukocytes also focused efficiently to the channel center towards the impedance detector. Based on flow cytometry analysis, the integrated platform sorting achieved $\sim 80\%$ neutrophil purity with significant lymphocyte and RBC depletion (Fig 3C). This resulted in an enrichment ratio of ~ 177 -fold due to significant RBCs depletion (Fig S5B, C). Similar neutrophil sorting performance was also demonstrated using lysed blood samples with neutrophil purity of $>80\%$ (Fig 3D), and an enrichment ratio of 2.86-fold at the impedance outlet (Fig S6B).

Besides neutrophil sorting, we further showed that the device can be used for efficient monocyte isolation from PBMCs sample obtained from Ficoll density gradient centrifugation. PBMCs ("buffy coat") primarily consists of lymphocytes, monocytes and platelets as the denser RBCs and granulocytes (neutrophils) would be sedimented at the bottom. PBMCs samples were processed at similar flow rate conditions and eluent from the impedance outlet was collected for high speed imaging and flow cytometric analysis. As expected, monocytes equilibrated near the inner wall as they were similar in size with neutrophils, while lymphocytes and platelets were removed into the waste outlet (Fig 3B). Fig 3E shows $>80\%$ monocyte sorting purity was achieved for all flow rate conditions tested with comparable enrichment ratio (~ 10 -fold) at the impedance outlet (Fig S7B).

To determine if the developed technology will result in non-specific neutrophil activation during operation, we quantified neutrophil CD66b and CD11b expressions which are reported as activation markers²⁹. Negligible differences in both cell markers were observed for healthy (control) and activated (PMA-treated) neutrophils before and after sorting, which was consistent with our previous work¹². (Fig 4A). Although cells encounter high shear stresses during flow, the total transit time of the cells inside the device is ~ 450 ms, which leads to minimal shear-induced effects. Cell viability also remained high ($>95\%$) in post-sorted neutrophils based on trypan blue staining (Fig S6E). Taken together, these results clearly indicated the platform versatility in processing different blood samples for gentle and label-free neutrophil and monocyte isolation at high throughput ($\sim 10^4$ cells/min).

Leukocyte identification and counting using integrated platform

After optimizing the DFF and FRR stages, we performed impedance measurements of sorted neutrophils and monocytes from lysed blood and PBMCs, respectively. Impedance profile of single cells were plotted as a 2D scatter distribution plot based on cell size (μm) and opacity ($|Z_{\text{HF}}|/|Z_{\text{LF}}|$) which corresponds to cell membrane property^{30, 31}. See supplementary information for data presentation. Fig 4B (left) shows a representative impedance profile of sorted neutrophils from lysed blood with dotted lines representing average or centroid of the population distribution. We then compared the electrical counting using the developed platform

Fig. 4 Leukocyte activation and neutrophil impedance enumeration. Representative flow cytometric analysis of (A) CD66b (left) and CD11b (center) expression of healthy and PMA-treated neutrophils before and after sorting and neutrophil activation (%) quantification based on CD11b (n=3) (right). *P ≤ 0.05 (B) Impedance profiling of neutrophils sorted from lysed blood. Dotted lines represent the centroid of the population (left). Comparison of neutrophil enumeration between the integrated platform and hemacytometer (right).

with hemocytometer counting and found that our electrical-based leukocyte enumeration was in good agreement with cell counting results over a wide range of cell concentrations (10^{2-5} cells/mL) (**Fig 4B** (right)). Monocytes isolated from PBMC sample were also electrically measured which showed good correlations with hemocytometer counting (**Fig S7D**). Consistent with our previous work¹⁹, we found that monocytes had lower opacity than neutrophils due to subtle size difference which affects the membrane capacitance ($\text{Opacity} \propto C_{\text{mem}}^{-1}$, $C_{\text{mem}} \propto A$).

Additionally, an investigation of the effect of particle concentration on impedance profile was performed using 10 μm beads. As shown in **Fig S9A**, the cell-cell interspacing below 100 μm indicated that two cells reside within detection region (100 μm width) and two signals merged together as evident in a distorted signal (**Fig S9A** red). It was observed that if distance between two cells were more than 100 μm , signals become separable without any magnitude distortion. Therefore, the quantification of the occurrence of cell-cell interspacing less than 100 μm at different concentrations (**Fig S9B**) was performed. At high sample concentration (10^6 beads/mL), the impedance distribution revealed higher particles larger than 11 μm (**Fig S9C**, right), which could be due to the presence of two particles in between electrode pairs resulting in a superposition of two signals. Hence, the target cell concentration was around 10^5 – 5×10^5 cells/mL to minimize measurement error.

For impedance throughput, as >80% of beads/cells will enter the impedance detector, the throughput for particle detection will be ~ 200 beads/sec (at 10^6 beads/mL). For a 500 $\times$ diluted blood sample (whole blood $\sim 5 \times 10^6$ WBCs/mL), the diluted cell concentration is 10^4 WBCs/mL. Assuming $\sim 60\%$ WBCs are neutrophils/monocytes and a separation efficiency of $\sim 80\%$, the impedance measurement throughput will be ~ 13 neutrophil/sec. A 5 min sample processing of 500 $\times$ diluted blood will give us ~ 4000 single cell impedance measurements. Noteworthy, the high sample flow rate (~ 140 – 160 $\mu\text{L}/\text{min}$) of our device is significantly faster than other impedance devices (< 50 $\mu\text{L}/\text{min}$)^{17, 30}. This minimizes cell sedimentation inside the sample syringe and tubing, which is beneficial for cell counting applications as well as processing of rare target cells.

NETosis electrical profiling for functional assessment

To determine if the integrated platform can be used for rapid functional phenotyping, we characterized neutrophils undergoing NETosis induced by calcium ionophore (Cal). Neutrophils were stained with Hoechst for nucleus identification and SYTOX green, a cell-impermeant DNA dye. Time-lapse fluorescent imaging was then performed to observe morphological changes during NETosis progression for 120 minutes. As shown in **Fig 5B**, neutrophil size (red dotted line) and its nucleus became larger with time. The leakage of nuclear content, due to membrane permeability changes during NETosis, was visualized by SYTOX green dye after 60 min. We next evaluated the NETosis process using a plate reader and observed a significant increase in fluorescence signal over time. Glucose-treated neutrophils (30 mM) also exhibited a higher

increase in fluorescence intensity as compared to untreated (control) neutrophils, which indicated an upregulation of NETosis (**Fig S10**). We further quantified NETosis process using our developed impedance cytometer and showed that the opacity and size distribution was higher for neutrophils undergoing NETosis (NET 30 min, 60min and 120 min) as compared to neutrophils without stimulation (control, 0 min) obtained from the same individual (**Fig 5C**). The mean opacity and cell size based on the centroid of the impedance profiles from different donors ($n = 5$) were quantified and normalized with their respective control (control 0 min) to obtain relative changes. Both relative opacity and cell size were higher for NETosis neutrophils as compared to unstimulated neutrophils (**Fig 5D**). These dielectric changes-increase in opacity (increase in impedance response at high frequency) and cell size (increase in impedance response at low frequency) for neutrophils undergoing NETosis were similar with previous findings by Schröter et al. in which they observed elevated impedance spectra²². Interestingly, while a larger cell size typically corresponds to a larger surface area and decreased opacity ($\text{Opacity} \propto C_{\text{mem}}^{-1}$, $C_{\text{mem}} \propto \text{surface area}$), we observed an increase in opacity for neutrophils undergoing NETosis which could be attributed to changes in cell membrane or cytoplasm conductivity as Cal induces a massive calcium influx³². Griffith et al. have also reported that changes in electrorotation spectra of activated neutrophils was due to the expulsion of ions which led to an increase in cell size and reduction of cytoplasm conductivity³³. Additionally, these dielectric changes could also be associated with nucleus degradation which can be modelled as a structural transformation from a double shelled particle to a single shelled particle. Further studies will be warranted to test these hypotheses.

As a proof-of-concept for diabetes testing, we investigated the influence of glucose treatment on NETosis electrical profiles. Similar trends were observed in the impedance profile of glucose-treated neutrophils undergoing NETosis (**Fig 5D**). For both cell size and opacity, there were negligible differences between untreated and glucose-treated neutrophils at the early stage of NETosis (30min). However, the difference in cell size (glucose > untreated) became significant ($P < 0.05$) after 60 min and at 120 min. We also observed a slight increase in opacity (untreated > glucose) after 120 min, which could be associated with the larger number neutrophils undergoing NETosis^{6, 34}. These impedance results were also consistent with our SYTOX green fluorescence spectrometry which showed higher fluorescence intensities for glucose-treated neutrophils after 60 min (**Fig 5B**).

Previous literatures have reported electrorotation³³ and impedance spectroscopy²² for NETosis characterization and quantification. However, electrorotation suffers from low throughput (< 50 cells/s), and impedance spectroscopy measures the bulk or averaged impedance spectra of neutrophil population undergoing NETosis on interdigitated electrodes. This is a major shortcoming since it is difficult to pinpoint whether the changes in electrical signal are caused by the released NETs or the structural cellular changes of neutrophils.

Fig. 5 NETosis profiling using impedance cytometry. (A) Experimental workflow (B) Overlaid fluorescent (Hoechst and SYTOX Green) and brightfield images showing NETosis progression (using Cal) over the course of 2 hour. Red dotted lines highlight cell boundary determined from brightfield images. Scalebar = 10 μm (C) Representative impedance profiling of neutrophils sorted from lysed blood undergoing NETosis at different time points. Dotted lines indicate means of each cluster. (D) Average relative neutrophil cell size (left) and opacity (right) with respect to unstimulated neutrophil (0 min) for control and glucose-treated data are presented as mean ± s.e.m (n = 5). ***P ≤ 0.001.

Additionally, laborious and time-consuming sample preparation processes (Dextran-Ficoll-Lysis, >60 min) were employed for neutrophil isolation²². Our developed platform addressed aforementioned issues by integrating high throughput neutrophil purification and impedance-based NETosis at single-cell resolution within minutes (<5 min).

As NETosis is time-sensitive cellular function, we used lysed blood samples (containing more neutrophils) to perform rapid impedance measurement (3 min) to avoid the neutrophils from undergoing physical changes during the microfluidic processing. Nevertheless, it is also possible to use diluted blood for NETosis characterization (Fig S11). We performed NETosis experiments with diluted blood and longer measurement time (5 mins) to get comparable data points as lysed blood samples. The results

revealed similar neutrophil impedance profiles and NETosis-induced changes and suggests the feasibility of using diluted blood samples for point-of-care impedance-based NETosis assay.

In future clinical use, both untreated (as negative control), and a sample treated with a specific NETosis stimulus will be measured to study patient-specific NETosis-induced impedance changes. Future work includes increasing sample size to study NETosis profile in healthy and T2DM individuals to design a proper gating strategy to determine NETosis events in an unknown sample.

Electrical profiling of NETosis by different stimulus

NETosis can be induced by a variety of stimulus such as PMA, Cal and bacteria^{35, 36}. We next explored whether impedance cytometry can be used to distinguish NETosis induced by different stimulus (Cal and PMA). We first compared time-lapse fluorescent images of PMA and Cal and observed that Cal induced NETosis more rapidly than PMA, as evident in the faster nuclear degradation and an earlier presence of intracellular SYTOX green expression due to compromised membrane at 60 min (**Fig 6A**). Fluorescent spectrometry data also showed that Cal-induced NETosis led to a more rapid and higher SYTOX green expression as compared to PMA (**Fig 6C**). The impedance profiles of unstimulated neutrophils and neutrophils

undergoing NETosis stimulated by Cal and PMA (120 min) were then characterized using our device (**Fig 6B**). Interestingly, the impedance profile of PMA-stimulated neutrophils was opposite to Cal-stimulated neutrophils as it shifted towards a lower opacity with a larger cell size differences as compared to control (unstimulated). The mean opacity and cell size based on the centroid of the impedance distribution were further quantified, and we showed that the relative opacity change was significantly different between both stimulus ($P \leq 0.01$) (**Fig. 6D**). The change in cell size was also much larger for PMA ($P \leq 0.05$) (**Fig. 6E**). Since calcium is required for NETosis³², it is likely that Cal and PMA trigger a cell membrane dielectric attenuation

Fig. 6 NETosis impedance profiling with different stimulus. **(A)** Fluorescent (Hoechst and SYTOX Green) and brightfield images showing NETosis progression via Cal (left) and PMA (right) over the course of 2 h. White dotted lines highlight cell boundaries. Scalebar = 10 μm **(B)** Representative impedance profiling of unstimulated neutrophils (left) and neutrophils undergoing NETosis via Cal (center) and PMA (right). Dotted lines indicate centroid of each cluster. **(C)** Fluorescent intensity profiles of SYTOX green expression for neutrophils undergoing NETosis via Cal and PMA for 2 h. Data are presented as mean $\pm$ s.e.m. * $P \leq 0.05$, ** $P \leq 0.01$. **(D)** Average relative neutrophil opacity and **(E)** cell size with respect to unstimulated control (0 min). Data are presented as mean $\pm$ s.e.m (n = 4). * $P \leq 0.05$, ** $P \leq 0.01$.

(and opacity) through calcium ion transport. Cytoplasm conductivity could also be affected as it was reported that Cal resulted in a higher calcium flux and intracellular calcium concentration as compared to PMA³⁷. Further studies will be performed to evaluate the observed differences in dielectric properties between PMA and Cal. Collectively, these results demonstrate that the developed impedance cytometry can be a novel and high throughput single-cell electrical and biophysical characterization tool for NETosis.

This work solely presents the development of the integrated microfluidic device with a single-step sample processing to purify and electrically detect neutrophils. This greatly minimizes user sample handling and sample preparation (e.g. centrifugation) prior detection. To facilitate future clinical uses, it is necessary to incorporate microfluidic chip, fluidic pumps and detection apparatus into a single machine. We envision the developed microfluidic device as a small cartridge which the user will load into the machine prior sample processing. For data processing, it is currently done offline and generally takes about 2-5 min with simple computational tasks such as digital filtering and peak detection. By implementing data processing on embedded systems such as digital signal processor (DSP) chip or field programmable gate arrays (FPGA), parallel computing can be exploited to shorten analysis time tremendously, hence enabling near real time data interpretation.

Conclusion

In summary, we have developed a novel integrated platform for inertial-based cell sorting and electrical phenotyping using impedance cytometry. The “sample in-answer out” platform offers great versatility in blood processing and it also significantly minimizes sample handling and user operation. Critically, since both cell sorting and impedance measurement are label-free, the “untouched” sorted cells after impedance measurements can be continuously collected off-chip for downstream assays. Besides leukocyte sorting, identification and counting, the developed platform was used for functional characterization of NETosis in which we observed significant differences in dielectric properties (opacity and size) between healthy and glucose treated neutrophils, as well as between different NETosis stimulus (Cal and PMA). We envision our developed platform can be further developed and translated into a point-of-care testing technology for rapid and multi-parametric impedance-based functional immunophenotyping to identify novel biomarkers for disease diagnostics and risk stratification.

Conflicts of interest

There are no conflicts of interest to declare.

Author contributions

C.P, K.H.H.L and H.W.H designed research. C.P and H.M.T performed experiments and analyzed the data. C.P, H.M.T and

H.W.H wrote the manuscript. All authors reviewed the manuscript.

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