Video Article

An All-on-chip Method for Rapid Neutrophil Chemotaxis Analysis Directly from a Drop of Blood

Ke Yang*[1,2,3], Jiandong Wu*[3,4], Ling Zhu[1], Yong Liu[1], Michael Zhang[5], Francis Lin[3,4,6,7]

[1]Institute of Applied Technology, Hefei Institutes of Physical Science, Chinese Academy of Sciences

[2]University of Science and Technology of China

[3]Department of Physics and Astronomy, University of Manitoba

[4]Department of Biosystems Engineering, University of Manitoba

[5]Seven Oaks General Hospital

[6]Department of Immunology, University of Manitoba

[7]Department of Biological Sciences, University of Manitoba

*These authors contributed equally

Correspondence to: Francis Lin at Francis.Lin@umanitoba.ca

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Abstract

Neutrophil migration and chemotaxis are critical for our body's immune system. Microfluidic devices are increasingly used for investigating neutrophil migration and chemotaxis owing to their advantages in real-time visualization, precise control of chemical concentration gradient generation, and reduced reagent and sample consumption. Recently, a growing effort has been made by the microfluidic researchers toward developing integrated and easily operated microfluidic chemotaxis analysis systems, directly from whole blood. In this direction, the first all-on-chip method was developed for integrating the magnetic negative purification of neutrophils and the chemotaxis assay from small blood volume samples. This new method permits a rapid sample-to-result neutrophil chemotaxis test in 25 min. In this paper, we provide detailed construction, operation and data analysis method for this all-on-chip chemotaxis assay with a discussion on troubleshooting strategies, limitations and future directions. Representative results of the neutrophil chemotaxis assay testing a defined chemoattractant, *N*-Formyl-Met-Leu-Phe (fMLP), and sputum from a chronic obstructive pulmonary disease (COPD) patient, using this all-on-chip method are shown. This method is applicable to many cell migration-related investigations and clinical applications.

Video Link

The video component of this article can be found at https://www.jove.com/video/55615/

Introduction

Chemotaxis, a process of directed cell migration to soluble chemical concentration gradient, is critically involved in many biological processes including immune response[1,2,3], tissue development[4] and cancer metastasis[5]. Neutrophils are the most abundant white blood cell subset and play crucial roles in enabling the body's innate host defense functions, as well as in mediating adaptive immune responses[6,7]. Neutrophils are equipped with highly-regulated chemotactic machinery allowing these motile immune cells to respond to both pathogen-derived chemoattractants (*e.g.* fMLP) and host-derived chemoattractants (*e.g.* interleukin-8) throughchemotaxis[8]. Neutrophil migration and chemotaxis mediate various physiological problems and diseases such as inflammation and cancers[1,9]. Thus, the accurate assessment of neutrophil chemotaxis provides an important functional readout for studying the neutrophil biology and the associated diseases.

Compared to the widely-used conventional chemotaxis assays (*e.g.* transwell assay[10]), the microfluidic devices show great promise for quantitative evaluation of cell migration and chemotaxis owing to the precisely controlled chemical gradient generation and miniaturization[11,12,13]. Over the last two decades or so, various microfluidic devices have been developed to study the chemotaxis of different biological cell types, especially neutrophils[11]. Significant effort was devoted to characterizing neutrophil migration in spatiotemporally complex chemical gradients that were configured in the microfluidic devices[14,15]. Interesting strategies were also developed to study directional decision making by neutrophils using the microfluidic devices[16].Aside from biologically-oriented research, the applications of microfluidic devices have been extended to test clinical samples for disease evaluation[17,18,19]. However, the use of many microfluidic devices is limited to specialized research laboratories and requires lengthy neutrophil isolation from large volume of blood samples. Therefore, there has been a growing trend of developing integrated microfluidic devices for rapid neutrophil chemotaxis analysis directly from a drop of whole blood[20,21,22,23,24].

Toward this direction, an all-on-chip method was developed that integrates the magnetic negative neutrophil purification and the subsequent chemotaxis assay on a single microfluidic device[25]. This all-on-chip method has the following novel features: 1) in contrast to previous on-

chip strategies that isolate neutrophils from the blood by adhesion-based cell capture or cell size-based filtering[20,22], this new method permits high purity, on-chip magnetic separation of the neutrophils from small volumes of whole blood, as well as chemotaxis measurement upon chemoattractant stimulation; 2) the cell docking structure helps align the initial positions of the neutrophils close to the chemical gradient channel and permits simple chemotaxis analysis without single cell tracking; 3) the integration of the neutrophil isolation and chemotaxis assay on a single microfluidic device permits rapid sample-to-result chemotaxis analysis in 25 min when there is no interruption between experimental steps.

This paper provides a detailed protocol for the construction, operation and data analysis method of this all-on-chip chemotaxis assay. The paper demonstrates the effective use of the developed method for performing neutrophil chemotaxis by testing a known recombinant chemoattractant and complex chemotactic samples from patients, followed by a discussion on troubleshooting strategies, limitations and future directions.

Protocol

All human sample collection protocols were approved by the Joint-Faculty Research Ethics Board at the University of Manitoba, Winnipeg.

1. Microfluidic Device Fabrication (Figure 1A)

1. **Design and print transparency mask.**
    1. Design the device as detailed previously[25]. See **Figure 1A**.
    NOTE: The device includes two layers. The first layer (4 µm high) defines the cell docking barrier channel to trap the cells beside the gradient channel. The second layer (60 µm high) defines the gradient generating channel, the port and channel for cell loading, the chemical inlet reservoirs and waste outlet. The alignment marks are designed for the two layers. For the second layer, the length and width of the upstream serpentine input channel is 60 mm and 200 µm, respectively; the length and width of the downstream serpentine input channel is 6 mm and 280 µm, respectively.
    2. Print the first and second layer features to a transparency mask using a high-resolution printer.
    NOTE: The printing resolution depends on the minimum features in the design. In the current design, 32,000 dpi was chosen for 10 µm minimum feature.

2. **Clean the silicon wafer.**
    1. Place a 3 in silicon wafer into the plasma cleaner. Apply vacuum for 3 min.
    2. Turn on the plasma power and set the level to HIGH. Use the oxygen plasma to treat the silicon wafer for 30 min.
    3. Turn off the plasma cleaner and take out the silicon wafer; the silicon wafer is ready for fabricating the device mold.

3. **Fabricate the first layer by photolithography in a cleanroom facility.**
NOTE: The exact fabrication parameters may vary depending on the fabrication facility.
    1. Dilute 10 mL SU-8 2025 with 10 mL SU-8 2000 in a glass breaker to prepare the designed photoresist. Leave the mixture in the fume hood for 10 min until the bubbles disappear.
    2. Carefully place the cleaned silicon wafer on the spinner with the suitable chuck and apply the vacuum to immobilize the silicon wafer.
    3. Carefully pour 3 mL of the photoresist mixture onto the center of the silicon wafer. Spin at 500 rpm for 5 s. Then spin at 3,000 rpm for 30 s to obtain a final 4 µm thickness photoresist coating on the silicon wafer.
    4. Carefully remove the silicon wafer from the spinner and bake the silicon wafer on a hotplate for 4 min at 95 °C.
    5. Carefully place the silicon wafer on a mask aligner and set the UV exposure time to 6 s. Carefully attach the transparency mask of the first layer on a transparent glass plate using adhesive tape.
    6. Gently place the transparent glass plate with the attached mask to the aligner and align the mask with the silicon wafer. Expose the silicon wafer to the UV to pattern the cell docking structure.
    7. Carefully remove the glass plate and take out the exposed silicon wafer. Bake the silicon wafer on a hotplate for 4 min at 95 °C.
    8. Transfer the silicon wafer to a fume hood and place it in a glass pan containing the SU-8 developer. Gently shake this for 30 s.
    9. Clean the silicon wafer using fresh SU-8 developer followed by isopropyl alcohol (IPA) inside the fume hood. Dry the silicon wafer by nitrogen gas inside the fume hood; the first layer is ready.

4. **Fabricate the second layer on the first layer.**
    1. Use adhesive tape to cover the alignment marks on the first layer. Carefully place the silicon wafer with the first layer on the vacuum chuck of the spinner and apply vacuum to immobilize the silicon wafer.
    2. Pour 3 mL of SU-8 2025 photoresist on the silicon wafer. Spin the silicon wafer at 500 rpm for 5 s. Then spin at 2000 rpm for 30 s to obtain a final 60 µm thickness photoresist coating on the silicon wafer.
    3. Carefully remove the silicon wafer from the spinner and transfer it to a hotplate; bake at 65 °C for 2 min.
    4. Gently remove the adhesive tape to expose the alignment marks on the first layer. Place the silicon wafer on a hotplate and bake at 95 °C for 6 min.
    5. Carefully place the silicon wafer on a mask aligner and set the UV exposure time to be 18 s.
    6. Carefully attach the transparency mask of the second layer on a transparent glass plate using adhesive tape.
    7. Carefully place the glass plate with the attached mask to the aligner, and align the mask and the first layer on the silicon wafer by the crossing alignment marks using the inspection microscope of the aligner.
    8. Expose the photoresist coated silicon wafer to UV to pattern the cell loading and gradient channels.
    9. Carefully remove the glass plate and take out the silicon wafer. Bake the silicon wafer on a hotplate at 65 °C for 2 min and then transfer the silicon wafer to another 95 °C hot plate and bake for 6 min.
    10. Transfer the silicon wafer to the fume hood and place it in a glass pan containing the SU-8 developer. Gently shake this for 6 min.
    11. Clean the silicon wafer using fresh SU-8 developer followed by IPA inside the fume hood.
    12. Dry the silicon wafer using nitrogen gas inside the fume hood. Place the silicon wafer on a hotplate and hard bake the mold at 150 °C for 30 min; the second layer is ready.

5. **Master mold surface modification.**
NOTE: A silanization surface modification step is applied to the SU-8 mold to facilitate polydimethylsiloxane (PDMS) release from the mold in soft-lithography.
    1. Take 10 µL of tridecafluoro-1,1,2,2-tetrahydrooctyl (trichlorosilane) solution in a micropipette tip. Put the micropipette tip into a 15 mL plastic tube and loosen the cap of the tube.
    2. Place the tube and the SU-8 patterned silicon wafer inside a desiccator and apply the vacuum for 1 h; the mask mold is ready for fabricating the PDMS device.

6. **Fabricate the PDMS device.**
    1. Prepare the PDMS solution by mixing 40 g PDMS base and 4 g curing agent in a plastic beaker. Place the prepared SU-8 master mold in a Petri dish and carefully pour 44 g PDMS solution onto the mold.
    2. Place the Petri dish in a desiccator and apply vacuum to degas the PDMS solution for 20 min. Then place the Petri dish in an oven and cure the PDMS at 80 °C for 2 h.
    3. After baking, take out the Petri dish and place it on a clean bench. Carefully cut and peel off the PDMS slab from the SU-8 mold.
    4. Punch out the cell loading port using a 3 mm diameter puncher. Punch out the chemical inlet reservoirs and the waste outlet using a 6 mm diameter puncher.
    5. Remove the dust on the surface of the PDMS slab using adhesive tape. Place the PDMS slab and a clean glass slide into the plasma machine. Apply the vacuum for 3 min.
    6. Turn on the plasma power and set the level to HIGH. Gently adjust the air valve and plasmatreat the PDMS slab and the glass slide for 3 min.
    7. Turn off the plasma power and release the vacuum. Carefully take out the PDMS slab and the glass slide using tweezers.
    8. Immediately place the PDMS slab (with channel structures face-down) on top of the glass slide; gently press the PDMS slab to bond it to the glass. Fill the microfluidic channel with deionized water immediately; the microfluidic device fabrication and assembly are completed.

2. Microfluidic Cell Migration Assay Preparation

1. **Microfluidic device preparation.**
    1. Prepare 50 µg/mL fibronectin solution by diluting 50 µL of stock fibronectin solution (1 mg/mL) to 950 µL Dulbecco's phosphate-buffered saline (DPBS) inside a biosafety cabinet.
    2. Prepare the migration medium by mixing 9 mL Roswell Park Memorial Institute medium (RPMI-1640) and 1 mL of RPMI-1640 with 4% bovine serum albumin (BSA).
    3. Remove the deionized water from the device.
    4. Add 100 µL fibronectin solution to the device from the outlet. Wait 3 min to ensure that all the channels are filled with fibronectin solution. Place the microfluidic device in a covered Petri dish for 1 h at room temperature.
    5. Remove the fibronectin solution from the device. Add 100 µL migration medium from the outlet. Wait 3 min to ensure that all the channels are filled with migration medium.
    6. Incubate the device for another 1 h at room temperature; the device is then ready for the chemotaxis experiment.

2. **Chemoattractant solution preparation for chemotaxis experiment.**
    1. Prepare 100 nM fMLP solution in total 1 mL migration medium. Mix 5 µL of stock FITC-Dextran (10 kDa, 1 mM) with the fMLP solution in a 1.5 mL tube.
    NOTE: FITC-Dextran is used for gradient measurement. Alternatively, use Rhodamine as the gradient indicator. The fMLP chemoattractant solution is then ready for chemotaxis experiment.

3. **Sputum sample preparation.**
NOTE: Neutrophil chemotaxis induced by a gradient of sputum sample from COPD patients was tested as a clinical diagnostic application of this all-on-chip method.
    1. Obtain a human ethics protocol to collect sputum samples from COPD patients.
    NOTE: We obtained approvals to collect samples at the Seven Oaks General Hospital in Winnipeg (approved by the University of Manitoba).
    2. Obtain the informed written consent forms from all subjects.
    3. Collect COPD patients' spontaneous sputum samples. Place 500 µL sputum sample in a 1.5 mL tube.
    4. Add 500 µL 0.1% dithiothreitol in the 1.5 mL tube and gently mix. Place the tube in a water bath at 37 °C for 15 min.
    5. Centrifuge the sample at 753 x g for 10 min and then collect the supernatant. Centrifuge the supernatant at 865 x g for 5 min and then collect the final supernatant. Store the collected supernatant inside a -80 °C freezer before use.
    6. When ready for chemotaxis experiment, thaw the sputum solution; transfer 900 µL migration medium to a 1.5 mL tube and mix with 100 µL sputum solution inside a biosafety cabinet; the sputum solution is then ready for chemotaxis experiment.

4. **Blood sample collection.**
    1. Obtain a human ethics protocol to collect blood samples from healthy donors. Obtain the informed written consent forms from all blood donors.
    NOTE: Here samples were obtained at the Victoria General Hospital in Winnipeg (approved by the Joint-Faculty Research Ethics Board at the University of Manitoba).
    2. Collect the blood sample by venipuncture and put the sample into an EDTA-coated tube. Keep the tube in a biosafety cabinet before the experiment.

3. All-on-chip Chemotaxis Assay Operation

1. **On-chip cell isolation (Figure 1B).**
    1. Place 10 µL whole blood in a 1.5 mL tube inside a biosafety cabinet.
    NOTE: Details of blood sample collection are in section 2.4.
    2. Add 2 µL antibody cocktail (Ab) and 2 µL magnetic particles (MP) from the neutrophil isolation kit (see the table of materials) into the 1.5 mL tube and gently mix; this will label cells in the blood except the neutrophils.
    3. Incubate the blood-Ab-MP mixture for 5 min at room temperature.
    NOTE: This will magnetically tag the antibody labeled cells in blood.
    4. Attach two small magnetic disks to the two sides of the cell loading port of the device. Aspirate the medium from all ports of the device.
    5. Slowly pipette 2 µL blood-Ab-MP mixture into the microfluidic device from the cell loading port.
    NOTE: The magnetically labeled cells are trapped to the side walls of the cell loading port while neutrophils will flow into the device and become trapped at the cell docking structure.
    6. Wait a few minutes until enough neutrophils are trapped at the cell docking area.

2. **Chemotaxis assay (Figure 1C).**
    1. Place the microfluidic device on the temperature controlled microscope stage at 37 °C.
    2. Add 100 µL chemoattractant solution (fMLP or sputum solution) and 100 µL migration medium to their designated inlet reservoirs using two pipettors; this will generate a chemoattractant gradient in the gradient channel by continuous laminar flow-based chemical mixing assisted by a pressure balancing structure.
    NOTE: Details of the sputum collection from COPD patients are in section 2.3.
        1. For the medium control experiment, only add migration medium to both of the inlet reservoirs.
    3. Acquire the fluorescence image of FITC-Dextran in the gradient channel.
    4. Import the image to ImageJ software using the command "File|Open".
    5. Measure the fluorescence intensity profile across the gradient channel using the command "Analyze|Plot Profile".
    6. Export the measurement data to a spreadsheet for further plotting.
    7. Incubate the device on the temperature controlled microscope stage or in a conventional cell culture incubator for 15 min.
    8. Image the gradient channel using a 10X objective to record the cells' final positions for data analysis.
    9. If needed, record the cell migration in the device by time-lapse microscopy.

4. Cell Migration Data Analysis (Figure 1C)

1. Analyze the chemotaxis assay by calculating the cell migration distance from the docking structure as described below. See **Figure 1C**.
2. Import the image into NIH ImageJ software (ver. 1.45).
3. Select the center of each cell that moved into the gradient channel.
4. Measure the coordinates of the selected cells for their final positions. Measure the coordinate of a point at the edge of the docking structure as the initial reference position.
5. Export the measured coordinate data to a spreadsheet software (*e.g.* Excel). Calculate the migration distance of the cells as the difference between a cell's final position and the initial reference position along the gradient direction.
6. Calibrate the distance to a micrometer. Calculate the average and deviation of the migration distance of all cells as a measure of chemotaxis.
7. Compare the migration distance in the presence of a chemoattractant gradient to the medium control experiment using the Student's *t*-test.
8. If the time-lapse images of the cell migration are recorded, the cell migration and chemotaxis can be further analyzed by cell tracking analysis[15].
NOTE: The materials required to construct and perform the all-on-chip chemotaxis assay are detailed in the table of materials.

Representative Results

Neutrophils are negatively selected from a drop of whole blood directly in the microfluidic device. The purity of the isolated neutrophils was verified by on-chip Giemsa staining and the results showed the typical ring-shaped and lobe-shaped nuclei of neutrophils (**Figure 2A**)[25]. This indicates an effective on-chip neutrophil isolation at high purity from a small volume of whole blood. Furthermore, the docking structure can effectively align cells next to the gradient channel before applying the chemical gradient (**Figure 2B**)[25].

Gradient generation is based on the continuous laminar flow chemical mixing, and the flows are driven by the pressure difference from the different levels of the inlet and outlet solutions. No external pumps are required. The chemical gradient is established within a few minutes in the microfluidic device, which is characterized by the fluorescence intensity profile of FITC-Dextran across the gradient channel. The gradient is stable for at least 1 h, which is enough time for the current neutrophil chemotaxis experiment (**Figure 1C**).

To demonstrate the use of the all-on-chip method for cell migration research, the neutrophil chemotaxis in medium alone or in a fMLP gradient were compared. The test results showed that few cells crawled through the barrier channel in the medium control experiment. By contrast, many neutrophils rapidly moved through the barrier channel and migrated toward the 100 nM fMLP gradient (**Figure 2B**)[25]. The cell migration test is quantitatively measured by the migration distance, which is significantly higher for the fMLP gradient than the medium control (**Figure 2C**)[25].

Furthermore, the all-on-chip method was demonstrated for potential clinical applications by comparing the neutrophil migration in medium alone to a gradient of sputum sample from COPD patients. The results showed a strong cell migration to the COPD sputum gradient, which is quantitatively indicated by the significantly higher migration distance compared to the medium control (**Figure 2B-C**)[25].

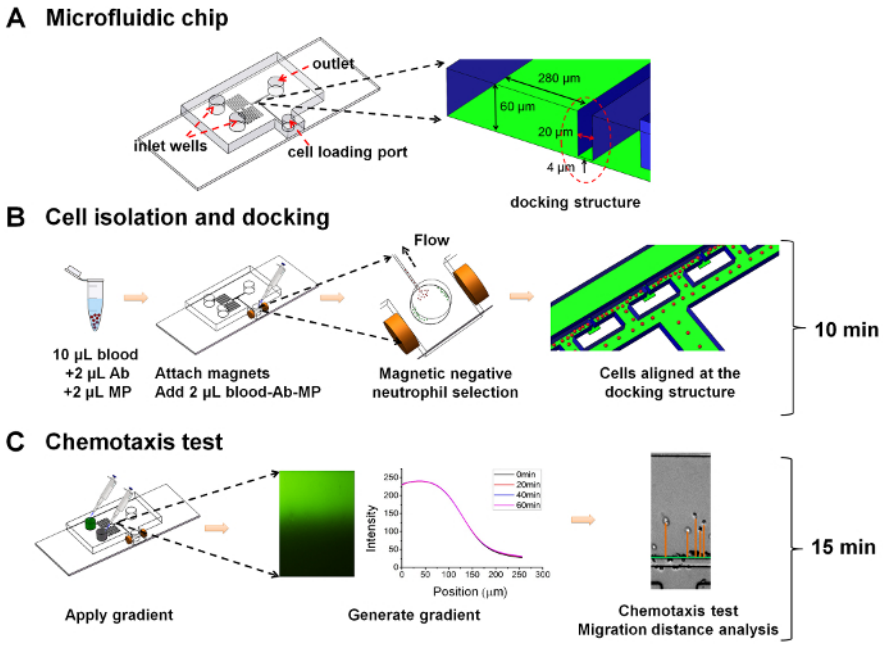

Figure 1: Illustration of the all-on-chip method for neutrophil chemotaxis analysis. (**A**) Illustration of the microfluidic device. The device includes two layers. The first layer (4 μm high) defines the cell docking barrier channel to trap the cells beside the gradient channel. The second layer (60 μm high) defines the gradient generating channel, the port and channel for cell loading, the chemical inlet reservoirs and the waste outlet. Alignment marks are designed for the two layers. For the second layer, the length and width of the upstream serpentine input channel is 60 mm and 200 μm, respectively; the length and width of the downstream serpentine input channel is 6 mm and 280 μm, respectively; (**B**) Illustration of the all-on-chip cell isolation method; (**C**) Illustration of the chemotaxis test. Please click here to view a larger version of this figure.

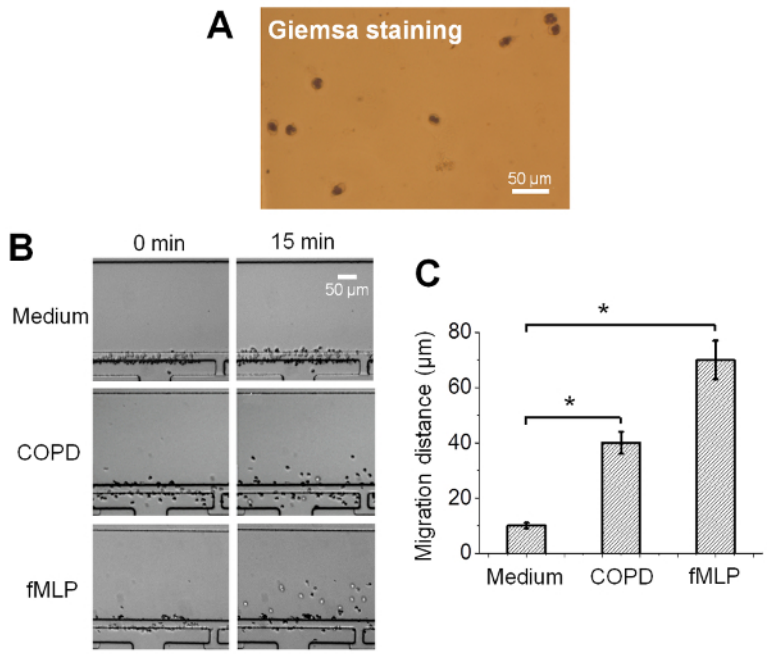

Figure 2: Representative results of the all-on-chip neutrophil chemotaxis analysis[25]. (**A**) Giemsa staining image (using a 60X objective) of the all-on-chip isolated cells in the microfluidic channel; (**B**) Comparison of the cell distribution in the medium control, a 100 nM fMLP gradient and a COPD sputum gradient; (**C**) The averaged cell migration distance in the gradient channel in the medium control, a fMLP gradient and a COPD sputum gradient. The error bars indicate the standard error of the mean (SEM). *indicates p <0.05 from the Student's t-test.The figures were adapted from reference[25] with permission from World Scientific Publishing. Please click here to view a larger version of this figure.

Discussion

In this paper, a detailed protocol to directly isolate neutrophils from whole blood followed by the chemotaxis test, all on a single microfluidic chip, was described. This method offers useful features in its easy operation, negative selection of high purity neutrophils, rapid sample-to-result

chemotaxis test, reduced reagents and sample consumption, and accurate cell migration data analysis. As a rough estimate, at least 25% of the neutrophils from the input whole blood sample effectively entered the docking structure in the device and we found the neutrophil purity is high by on-chip Giemsa staining.

This developed all-on-chip chemotaxis analysis method has great potential in various cell migration research and clinical applications. A research application of this method was demonstrated by comparing neutrophil chemotaxis in medium alone to a fMLP gradient. Similarly, this method can be used to test neutrophil chemotaxis in COPD sputum as an example of developing a cell functional biomarker for clinical diagnosis. With this method, a researcher can easily test neutrophil chemotaxis to different chemoattractants individually or in combinations. Researchers can also test neutrophil chemotaxis to complex chemotactic factors from patients or test neutrophils from diseased patients for the potentially altered chemotaxis response using this method. This integrated all-on-chip method is particularly useful for performing the test in research or clinical labs that do not have specialized cell culture and live cell imaging facilities. The test can be easily operated by researchers or clinicians following this protocol. For more advanced research applications, this method allows time-lapse microscopy to track individual cell movement.

In general, this all-on-chip method is easy to operate and the result is robust. Several technical reminders will further ensure a successful experiment. First, the PDMS replica should be gently pressed onto the class substrate during plasma bonding to avoid damaging the very thin barrier channel. Second, the evaporation of the medium in the cell loading port can disturb the chemical gradient. It is recommended to cover the cell loading port with a sealing tab during the chemotaxis test. Third, the blood sample should be gently loaded onto the device to avoid high pressure that can push the cells over the barrier channel before the chemotaxis experiment. Fourth, in the current setting, we recommend keeping the magnets attached to the cell loading port during the chemotaxis assay to prevent unwanted cells from entering the channel. Alternatively, a separate piece of PDMS with a through-hole and the magnetic disks attached can be aligned to the cell loading port of the device. In this case, the top PDMS part with the magnetic disks and the trapped cells can be removed from the device after cell isolation.

This all-on-chip method can be further developed to overcome its current limitations and to improve and expand its functionalities. First, the current device only allows a single assay at a time thus limiting the throughput. Further development of the device with multiple parallel test units will improve the experimental throughput requirement. Second, the current flow-based chemical gradient generator limits gradient generation in 1D. Further development of 2D or 3D flow-free gradient generators will better mimic the physiological gradient conditions. Third, in addition to neutrophils, this all-on-chip method in principle can be used to test other white blood cell types such as T cells, B cells and NK cells using similar magnetic cell isolation kits. It will be important to study if this method can be effectively used to test blood cell populations at lower frequency and those cells that require on-chip activation and culture before the chemotaxis test. Then the all-on-chip cell isolation method can be further extended for some other applications. Different barrier channel thickness were tested and the results showed that 3-4 μm is most suitable for the neutrophil migration experiment; that is, it sufficiently trapped the un-stimulated cells and allowed the cells to crawl through the barrier channel upon stimulation. The barrier channel dimension should be optimized for different cell types. Finally, this integrated and rapid chemotaxis test will allow researchers to explore relevant clinical applications. To enable practical testing in clinics, a portable system has been developed that integrates the microfluidic device, temperature and stage control, as well as smartphone-based optical imaging and data analysis modules. In addition to the COPD-related study as demonstrated in this paper, the cell migration for other relevant diseases such as chronic kidney disease is being tested using this all-on-chip method.

Disclosures

There are no conflicts of interest to disclose.

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