Published in final edited form as:

Methods Mol Biol. 2018 ; 1749: 351–360. doi:10.1007/978-1-4939-7701-7_25.

Neutrophil Chemotaxis in One Droplet of Blood Using Microfluidic Assays

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Abstract

Neutrophils are the most abundant leukocytes in blood serving as the first line of host defense in tissue damage and infections. Upon activation by chemokines released from pathogens or injured tissues, neutrophils migrate through tissues toward sites of infections along the chemokine gradients, in a process named chemotaxis. Studying neutrophil chemotaxis using conventional tools, such as a transwell assay, often requires isolation of neutrophils from whole blood. This process requires milliliters of blood, trained personnel, and can easily alter the ability of chemotaxis. Microfluidics is an enabling technology for studying chemotaxis of neutrophils in vitro with high temporal and spatial resolution. In this chapter, we describe a procedure for probing human neutrophil chemotaxis directly in one droplet of whole blood, without neutrophil isolation, using microfluidic devices. The same devices can be applied to the study the chemotaxis of neutrophils from small animals, e.g., mice and rats.

Keywords

Chemotaxis; Microfluidics; Neutrophils; Speed; Persistence; Blood

1 Introduction

Neutrophils are the first line of host defense in tissue injury and infections [1]. Upon activation by chemokines released from pathogens or injured tissues, neutrophils transmigrate from peripheral blood and then migrate toward sites of infection along gradients of chemokines. This process is named chemotaxis. Impaired neutrophil chemotaxis, due to genetic defects [2] or to pathological conditions such as major burn [3] or drug treatment such as chemotherapy [4], weakens the host defense against pathogens and may further lead to severe infections. On the opposite side, excessive activation and recruitment of neutrophils in an uncontrolled manner may lead to chronic inflammation [5] and organ failure [6]. Thus, understanding neutrophil chemotaxis in homeostasis and pathology can provide important insights in clinical diagnosis, treatment, and prognosis of diseases.

During the past four decades, a variety of assays including Boyden chamber [7], Zigmond chamber [8], Dunn chamber [9], and micropipette [10] have been developed to study chemotaxis in vitro. Although these techniques enabled numerous discoveries and elevated our understanding of chemotaxis, they have several limitations that hinder them from being

used in clinical research. These assays lack the ability to control the gradient with precision over time and only use isolated neutrophils. Therefore, they require large volume of blood (>1 mL) and significant amount of work and time to isolate homogenous subpopulations of cells from the blood. This sample preparation process has the potential to alter neutrophil phenotype, interfering with the chemotaxis measurements in subsequent experiments.

Microfluidics is a technology using microfabricated valves, channels, and chambers to manipulate microscale fluids and organisms with high spatial and temporal resolution [11]. The application of microfluidics to the study of leukocyte biology has facilitated several insights into neutrophil chemotaxis [12]. Compared to the conventional chemotaxis assays, microfluidic assays enable the use of stable gradients, of sophisticated spatial profiles [13]. Microfluidic assays also enable precise mechanical confinement of the migrating cells, which resemble the interstitial spaces present in vivo in tissues [14]. Moreover, multifunctional microfluidic assays allow for seamless integration of sample preparation with downstream chemotaxis assay on a single chip [15–22]. A majority of these chips accomplish the on-chip blood purification first, and then probe neutrophil chemotaxis [15, 19–21]. Although these devices significantly simplify and reduce the time for sample preparation, they rely on P-selectin functionalized surfaces, which could activate the neutrophils and interfere with the chemotaxis measurements [15].

In this chapter, we present the complete work flow (Fig. 1) used in our group to measure neutrophil chemotaxis directly in a droplet of whole blood [16–18]. The device allows investigation of neutrophil chemotaxis, with high temporal and spatial resolutions, in microchannels that mimic the interstitial spaces. The moving neutrophils emerge from the droplet of blood at the time when they enter the channels, in a self-sorting process that does not interfere with their original activation status. The assay also preserves the serum components which are well known to be important for the functional status of the neutrophils [23].

2 Materials and Equipment

Soft lithography is the most widely used method for prototyping microfluidic devices. It includes two major steps: fabrication of a master mold in SU-8 and replication of devices in Polydimethylsiloxane (PDMS) from the mold. The fabrication of the SU-8 mold is conducted in class 100 clean room using standard microfabrication equipment. Basic tools and containers such as tweezers and beakers are broadly available in research labs, thus they are not mentioned in the section.

2.1 Soft Lithography

2.1.1 Fabrication of the SU-8 Master Mold

1. Photomasks with microfluidic designs (Front Range Photo Mask, Palmer Lake, CO).
2. 10 cm silicon wafers (Desert Silicon, Grandale, AZ).
3. SU-8 5 negative photoresist (SU-8 5, Microchem, Newton, MA) for patterning the first layer of the mold, which contains the migration channels.

4. SU-8 100 negative photoresist (SU-8100, Microchem) for patterning the second layer of the mold, which contains the chemokine chambers.
5. SU-8 developer (BTS-220, J.T. Baker, Center Valley, PA).
6. Dehydration oven at 200 °C.
7. Plasma treatment system (March PX-2527 Plasma System, Nordson March, Concord, CA).
8. Spin coater for uniformly coating layers of SU-8 with desired thickness.
9. Hot plates for baking SU-8 before and after exposure.
10. Mask aligner for multilayer alignment and exposure.
11. Profilometer (Contour Elite K, Bruker BioSpin Corporation, Billerica, MA) for precision metrology and quality control of the fabricated mold.

2.1.2 PDMS Casting and Bonding

1. SU-8 master mold fabricated from the SU-8 process.
2. PDMS base and curing agent (PDMS, Sylgard, 184, Elsworth Adhesives, Wilmington, MA).
3. Digital weighing balance with >0.1 g resolution for weighing PDMS base and curing agent.
4. Disposable cup and stirring stick for mixing PDMS.
5. Oven or hotplate for curing of PDMS at 65–80 °C.
6. Surgical scalpel with #11 blade.
7. 1.5 and 5 mm punchers (Harris Uni-Core, Ted Pella Inc., Reading, CA).
8. Clean-room adhesive tape.
9. Glass-bottom well plates (MatTek Co., Ashland, MA).
10. Plasma treatment system.

2.2 Preparation of Chemoattractant

1. Chemokine stock purchased from vendors, such as Sigma-Aldrich, Cayman Chemicals, R&D Systems Inc. (*see* Note 1).
2. Medium: Iscove's Modified Dulbecco's Medium (IMDM) containing 20% FBS. Mix 400 mL (IMDM) with 100 mL FBS. Filter the mixture and store at 4 °C. Warm up to 37 °C in a bead or water bath before use.
3. Human fibronectin (R&D systems, Inc., Minneapolis, MN): the stock is aliquoted into small vials and stored at 4 °C.

¹We recommend thawing chemokine solutions right before priming the device. Repetitive thawing and freezing should be avoided.

4. 500 μ L snap vials.

2.3 Preparation of Human Whole Blood Sample

1. Lancing devices and disposable lancets (BD Contact-Activated Lancet 30G, Becton Dickinson, Franklin Lakes, NJ).
2. Media: IMDM containing 20% FBS.
3. Nucleus staining solution: Hoechst 33342 (10 mg/mL solution in water). Store at 4 °C.
4. 500 μ L snap vials.

2.4 Time-Lapse Imaging

1. Nikon Eclipse TiE microscope equipped with Nikon perfect focus system.
2. Biochamber compatible with the microscope stage, set at 37 °C temperature and 5% CO₂ content.

3 Methods

3.1 Design and Fabrication of the Microfluidic Device

3.1.1 Designing the Microfluidic Device

1. Draw the microfluidic design in AutoCAD software (*see* Note 2).
2. Send the AutoCAD file to a vendor to fabricate the photomasks with desired resolutions (*see* Note 3).

3.1.2 Fabrication of the SU-8 Master Mold

1. Dehydrate two 4" wafers at 200 °C in the dehydration oven for 90 min (*see* Notes 4 and 5).
2. Spin-coat SU-8 5 on the wafers with a thickness of 5 μ m (*see* Notes 6 and 7).
3. Bake the wafers on a 75 °C hotplate and then on a separate 100 °C hotplate.
4. Load the first photomask and the wafer on the mask aligner and expose the wafer under UV light.
5. Bake the wafers on a 75 °C hotplate and then on a separate 100 °C hotplate.

²The presented microfluidic design has two layers: a 5 μ m thick layer containing channels for cell migration and a 50 μ m thick layer containing chemokine chambers. Fabrication of the two-layer SU-8 master mold requires two photomasks with proper alignment marks.

³When ordering a mask from a company, the resolution of the photo mask needs to be smaller than the smallest features of the design. The mask for the first layer of our design has a resolution of 3 μ m. The mask for the second layer has a resolution of 20 μ m.

⁴SU-8 process is a standard process; thus, it is only briefly described in this chapter.

⁵We recommend fabricating multiple master molds in parallel.

⁶The ramping rate, spinning rate, and time for specific thickness of SU-8 film can be found from standard SU-8 protocol (<http://www.microchem.com/Prod-SU8.htm>).

⁷The actual thickness of SU-8 after spinning may vary with temperature and humidity of the clean-room environment. These parameters are specific to each clean room and should be optimized accordingly.

6. Fill a 15 cm-diameter glass container with ~30 mL SU-8 developer (the level of the developer should be ~1.5 cm above the bottom). Develop the SU-8 until unexposed SU-8 is completely removed (*see* Note 8).
7. Dry the wafer under nitrogen flow (*see* Note 9).
8. Repeat the similar process to fabricate the second layer, 50 μ m thick, on top of the first layer (*see* Note 10).
9. Place the SU-8 master mold in a 15 cm diameter petri dish and tape the edge of the wafer to fix it.

3.1.3 PDMS Casting and Bonding

1. Weigh PDMS base and curing agent at a ratio of 10:1 (*see* Note 11).
2. Mix the base and curing agent homogeneously with a disposable stirring stick or a fork and pour the mixture on the mold.
3. Place the master mold into a vacuum chamber and degas until the PDMS is bubble-free.
4. Cure the PDMS in an oven or on a hot plate at 75 °C overnight.
5. After curing, cut PDMS along the outline of the wafer with a scalpel and peel out the entire PDMS layer.
6. Punch the inlet of the device with a 1.25 mm puncher and then remove the device from the PDMS layer with a 5 mm puncher.
7. Clean the surface of the device multiple times with clean-room adhesive tape.
8. Treat a glass bottom dish or multi-well plate and the device with O₂ plasma for 35 s (*see* Note 12).
9. Bond the PDMS device on top of the plate. Press the device gently with tweezers to ensure the PDMS and glass contact seamlessly.
10. Place the dish or plate on a hotplate for 20 min at 65 °C to enhance the bonding. Figure 2a is a photo of the device that is ready to use. Figure 3a shows a microscopic image of the microchannels and chambers.

⁸The baking time, exposure time and development time may vary. We recommend using the parameters in the standard SU-8 protocol as a reference and then modify them.

⁹The wafer should be directly dried with N₂ flow after development. Do not rinse the wafer with water or other reagent.

¹⁰The proper alignment of the two SU-8 layers is critical. This is achieved by aligning the alignment marks in the second mask with the alignment marks on the wafer using the mask aligner.

¹¹We use 40 g of PDMS base and 4 g of PDMS curing agent for a 15 cm diameter Petri dish. This will yield a ~3 mm thick PDMS layer.

¹²Single dishes and 6, 12, 24, and 96 glass bottom multi-well plates are available. Using a multi-well plates enables simultaneous study of neutrophil chemotaxis from multiple samples, in various experimental conditions.

3.2 Device Priming

1. Dilute chemokine to desired concentration in IMDM containing 20% FBS with 100 nM fibronectin. In this experiment, we use 100 nM LTB4 as the chemoattractant.
2. Pipetting 5 μ L chemoattractant from the inlet into the device and degas the device in a vacuum chamber for 10 min.
3. Remove the device from the vacuum chamber and wait for 20 min until the microchannels and chambers are completely filled with chemoattractant.
4. To establish the gradient, flush the central cell-loading chamber with chemoattractant-free media using a 1 mL a pipette.
5. Immerse the device under 4 mL media (Fig. 2b).
6. Wait for 30 min to allow the gradient to stabilize (*see* Note 13) (Fig. 3b).

3.3 Loading Human Whole Blood Sample

1. Acquire 5 μ L human whole blood by finger prick and transfer it into a 0.5 mL tube containing 5 μ L of media, heparin anticoagulant, and $\sim$ 4 μ M of Hoechst stain.
2. After gentle mixing with pipette, incubate the sample at 37 °C and 5% CO₂ for 15 min to allow proper staining of the nuclei.
3. Dilute the sample further with media in a ratio of 1:3.
4. Load 1 μ L diluted human blood per device, gently, from the inlet using a gel loading tip (Fig. 2c).

3.4 Time-Lapse Imaging

1. Place the dish or multi-well-plate on the microscope stage.
2. Cover the plate with the biochamber which maintains the temperature and CO₂ content at 37 °C and 5%.
3. Set up imaging positions, filter cubes, time duration of each cycle and total imaging time in the software (*see* Note 14) and start the imaging process.
4. After the experiment, import the time-lapse images into ImageJ or other imaging processing software for further analysis (*see* Note 15). For example, cell migration trajectories can be tracked and analyzed using Trackmate module in ImageJ. Figure 4 shows a neutrophil migrating up the LTB4 gradient in the microchannel.

¹³To visualize the chemoattractant gradient, we use a fluorescent dye with similar molecular weight as the chemokine. Figure 3b demonstrates a chemoattractant gradient established between the chemoattractant chamber and central cell-loading chamber.

¹⁴It is critical to make sure that the microscope takes images at the same focal plane during the entire imaging period. We use the Nikon perfect focus system (PFS) to automatically maintain the focal plane during the time-lapse imaging process.

¹⁵Using the microfluidic assay, chemotaxis of single leukocytes can be investigated with high temporal and spatial resolution. The characteristic parameters of chemotaxis we investigate are the number of recruited cells, migration velocity, directionality, persistence, and trajectory.

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Fig. 1.

The work flow of developing microfluidic devices for chemotaxis studies is an iterative process that may include several cycles of design, fabrication, and testing. A microfluidic design is first captured as an AutoCAD drawing. The design is implemented in SU-8 photoresist on silicon wafers using photolithography masks printed from the AutoCAD drawings. Devices are then fabricated in PDMS that is cast on the SU-8-silicon mold. The devices are then tested with whole blood samples. Depending on the initial results, the design may need to be optimized further

Fig. 2.

The microfluidic device or neutrophil chemotaxis in a droplet of whole blood. **(a)** A macrophotography of the microfluidic device fabricated in a single well plate. **(b)** The device immersed in IMDM media after priming. **(c)** Loading of 1 μ L whole blood in the device using a fine pipette tip. The scale bars are 5 mm

Fig. 3. Geometrical details of the microfluidic device for neutrophil chemotaxis. **(a)** Bright-field microscopic image showing the microfluidic unit for characterizing neutrophil trafficking from a drop of whole blood. **(b)** Fluorescence microscopic image showing the chemogradient established in the unit. The scale bars are 40 μm

Fig. 4. Human neutrophil chemotaxis from whole blood. A montage of time-lapse images shows one neutrophil (nucleus stained in blue), which migrates from the whole blood in the central chamber (bottom) toward the LTB₄-filled chemoattractant chamber (top). The scale bar is 40 μ m. Time interval between successive frames is 2 min