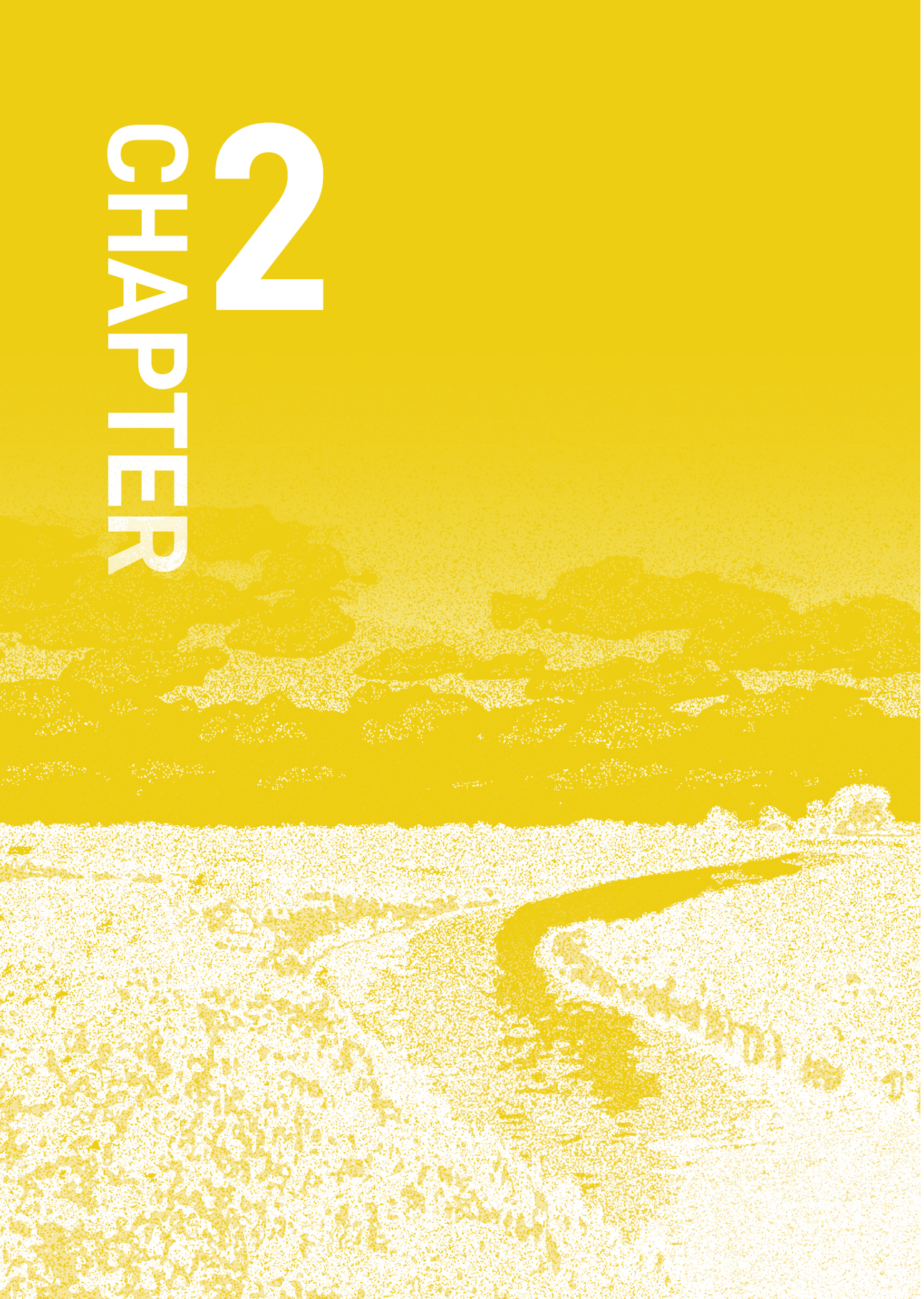
Systemic immune dynamics in cancer

Bakker, E.A.M.

CHAPTER 2



Ex vivo assessment of Human Neutrophil Motility and Migration

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Abstract

Neutrophils are pivotal in orchestrating tumor-induced systemic inflammation and are increasingly recognized for their critical involvement in both the initiation and progression of cancer. A fundamental facet of neutrophil biology is their migratory capacity, which enables them to extravasate and infiltrate tumors other tissues, where they carry out essential effector functions. Unraveling the intricate mechanisms of neutrophil motility and migration is crucial for comprehending immune responses and inflammatory processes, shedding light on their substantial contribution to cancer progression. Here, we provide a comprehensive protocol to assess direct *ex vivo* motility and migration of freshly isolated human neutrophils, offering valuable insights into their behavior.

Introduction

Neutrophils are the most abundant white blood cell type in human circulation, comprising 40-70% of all white blood cells. They play a key role in the innate immune response, especially as first line of defense against infections. Recently, neutrophils have been intensively studied in the context of cancer immunology due to growing evidence of their involvement in various aspects of cancer onset and progression (1). Crosstalk between cancer cells and immune cells can lead to systemic accumulation and activation of neutrophils, resulting in a chronic inflammatory state (2-5). Tumor-induced systemic inflammation is clinically scored as the Neutrophil-to-Lymphocyte Ratio (NLR), indicative of the relative abundance of neutrophils versus lymphocytes in the peripheral circulation. Clinical investigations revealed a correlation between a high NLR and unfavorable disease outcomes as well as suboptimal therapy responses across various cancer types (6-9). Furthermore, preclinical research has shown that neutrophils contribute to the formation of metastases through various mechanisms. These include inducing systemic immune suppression, assisting circulating cancer cells and promoting the creation of the (pre-)metastatic environment (10-15). Additionally, pre-clinical investigations demonstrated that during the initial phases of tumor development, bone marrow neutrophils display a pronounced inherent capacity for spontaneous migration. This capability allows them to effectively navigate to distant organs, thereby promoting the infiltration of cancer cells into remote tissues and consequently facilitating the progression of tumor metastasis (16, 17). Collectively, these studies have revealed the pivotal role of neutrophils in metastasis formation, sparking a growing interest in studying these cells within the context of cancer (18).

Despite the growing recognition of the importance of neutrophils in cancer, they have often been overlooked in scientific investigations. While neutrophils constitute the most abundant white blood cell in human blood, their representation is notably absent in archival specimens such as frozen peripheral blood mononuclear cells (PBMCs). This absence can

be attributed to the inherent vulnerability of neutrophils; they do not withstand a freeze-thaw cycle, leading to their virtual elimination in preserved specimens. Additionally, their short lifespan, typically lasting from a few hours to a few days, poses a significant challenge for researchers, making them difficult to work with. Hence, possessing the necessary technical knowledge about human neutrophil isolation and *ex vivo* handling is crucial for the successful execution of functional experiments.

This methodology chapter provides detailed protocols to study the *ex vivo* motility and migration of human neutrophils. Motility refers to the ability of cells to move actively and undirected and is indispensable for neutrophils to execute their effector functions, such as patrolling the bloodstream and tissues, actively seeking out and destroying pathogens. Migration is a critical property that allows neutrophils to exit the bloodstream and actively navigate into tissues or sites of infection (or tumor, in the case of cancer), attracted by a gradient of chemical stimuli called chemoattractants. Important chemoattractants for neutrophils include interleukin-8 (IL-8, CXCL8), leukotriene B4 (LTB4), growth-regulated oncogene-1 (GRO-1, GROα, CXCL1), complement component C5a, N-Formylmethionyl-leucyl-phenylalanine (fMLF or fMLP) and stromal cell-derived factor-1 (SDF-1, CXCL12)(19, 20). In the migration assay described in this protocol, IL-8 and LTB-4 are used as chemoattractants because they are well characterized and commonly used moderately potent chemoattractants that exhibit minimal interference with adhesive properties and activation status of the neutrophils.

Understanding the intricacies of neutrophil motility and migration in different organs and disease contexts is essential for unraveling the dynamics of immune responses and inflammatory processes. *Ex vivo* motility and migration assays can be helpful tools to compare neutrophil functionality in homeostasis and disease or across different tissue sites. Furthermore, these assays can serve as a platform for fundamental research aimed at investigating mechanisms of neutrophil migration, and offer the potential to explore strategies targeting neutrophil migration. By comprehending the complexities of neutrophil motility and migration across various organs and disease scenarios, researchers can unravel the dynamics of immune responses and inflammatory processes, thereby illuminating the crucial role neutrophils play in cancer progression.

Materials

Common disposables

- Pipet tips
- 15 mL Falcon® Tubes (Thermo Fischer Scientific) (see Note 1)
- 50 mL Falcon® Tubes (Thermo Fischer Scientific) (see Note 1)
- Falcon® 24-well Clear Flat Bottom TC-treated Multiwell Cell Culture Plate, with Lid,

Individually Wrapped, Sterile (Corning) (see Note 1)
- 96-transwell plates with 3.0 µm pore polycarbonate permeable membranes (Sigma Aldrich) (see Note 1)
- Black 96-well flat bottom OptiPlates; low-binding surface (Perkin Elmer)
- Aluminum foil to keep stained cells in the dark

Cells and reagents
- Fresh human blood sample (2-4 mL), collected in EDTA tube (see Notes 2, 3 and 4)
- MACSxpress® Whole Blood Neutrophil Isolation Kit, human (Miltenyi)
- Medium: 20/80 mixed medium (20% Roswell Park Memorial Institute (RPMI)/ 80% AIM- V medium) (Gibco) (see Note 5), supplemented with 1% Human Serum (Sigma Aldrich)
- Red Blood Cell Lysis Buffer (dH2O, 8.02 g/L NH4Cl, 0.84 g/L NaHCCO3, 0.37g/L EDTA)
- Calcein acetoxymethyl, cell-permeant dye (Thermo Fischer Scientific)
- N-Formylmethionine-leucyl-phenylalanine (also known as fMLF or fMLP) (Sigma-Aldrich), make 10 µM stock
- HTAB buffer (1g/L Tween20, 2g/L CTAB, 2g/L BSA, 7.44 g/L EDTA)
- Recombinant Human IL-8 (Peprotech), make 10 µg/mL stock (= 100x)
- Recombinant LTB-4 (Sigma-Aldrich), make 1 µg/mL stock (=100x)

Equipment
- Pipets
- Magnet: MACSxpress Separator (Miltenyi)
- Cell counting equipment (manual/automated)
- Humidified cell culture incubator (37°C and 5% CO_2)
- Laboratory biosafety cabinet
- High-quality, inverted, wide field microscope system with motorized stage control (for time lapse), autofocus and temperature and CO_2 control for live cell imaging, (like the Zeiss Axio Observer Z1 Live) with standard filter cubes for GFP (Figure 1). The system needs to be equipped with a camera (like a sensitive Hamamatsu Orca Flash 4 monochrome camera for bright field, DIC, phase contrast and fluorescence imaging (LED or HXP light source) (see Note 1)
- Plate reader with excitation 485 and emission 520 such as PHERAstar FS (BMG labtech) (see Note 1)

Software
- PHERAstar FS plate reader software (BGM labtech)
- ZEN lite (Zeiss groep)
- TrackMate plugin Fiji (ImageJ)

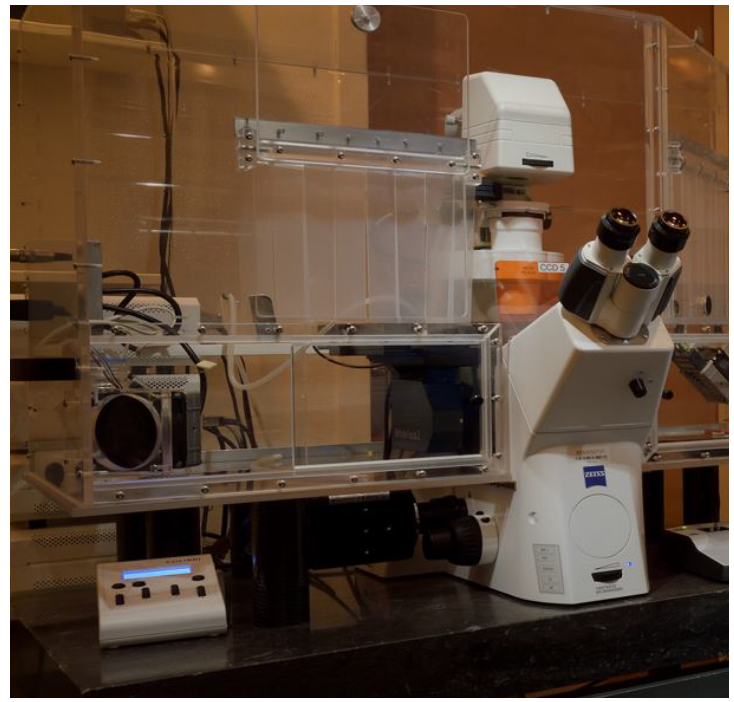

Figure 1: *Picture of the microscope, including the climate control chamber surrounding the microscope, maintaining the levels of CO_2 constantly at 5% and temperature at 37°C.*

Methods

Neutrophil isolation and staining

1. A ~5 mL peripheral blood sample is drawn and collected in an EDTA blood tube (see Notes 2-4). Keep the blood at RT and proceed to the next steps immediately.
2. Neutrophils are isolated according to the MACSxpress® Whole Blood Neutrophil Isolation Kit manual (see Note 6), which is a negative selection kit (see Note 7). In short:
   a. Beads are dissolved within 2 mL of Buffer A, which is included in the Kit (see Note 8).
   b. For each mL of whole blood that is used, 0.125 µL dissolved beads are mixed with 0.125 µL of Buffer B, which is included in the Kit (see Note 9).
   c. The appropriate amount of blood (for example 4 mL) is transferred to a 15 mL tube (see Note 10), after which the bead mix is added and incubated for 5 min. at RT. After 2-3 min., cell-bead suspension is mixed very gently by slowly pipetting up and down, without creating any bubbles.
   d. The 15 mL tube is placed in the magnet for 15 min.
   e. After 15 min., the fraction that does not stick to the magnet is harvested.

3. Cells are spun down for 5 min. at 250 *g*.
4. Red blood cells are lysed in 10 mL Red Blood Cell Lysis Buffer for 5 min. at RT.
5. 10 mL of medium is added and cells are spun down for 5 min. at 250 *g*.
6. Supernatant is discarded, the pellet is dissolved in 10 mL of mL of medium by gently pipetting up and down without creating any bubbles and cells are spun down again for 5 min., 250 *g*.
7. Cells are counted and diluted in medium to a final concentration of $4*10^6$/mL. It is strongly advised to perform a flow cytometry-based purity check on part of the isolated neutrophils (see Note 11).
8. The neutrophil suspension is transferred to a new 15 mL tube for staining (see Note 12).
9. Calcein is added to stain the neutrophils (final concentration: 1 µM).
10. Neutrophils are incubated for 30 min. at 37°C, and kept dark.
11. 10 mL of medium is added and cells are spun for 5 min. at 250*g*.
12. Supernatant is discarded, another 10 mL of medium is added and cells are spun at 5 min. at 250*g*.
13. Pellet is suspended in medium to a final concentration of $1*10^6$/mL.
14. Stained neutrophils can now be used for the motility (See 3.2) and migration assays (See 3.3).

Neutrophil motility assay

Plate preparation

1. 10.000 neutrophils are plated per well of a 24-well plate (10 µl of the stained neutrophil suspension in 1mL of medium per well) (see Note 13).
2. The remaining cells are kept at RT for use in the migration assay described in 3.3.
3. Neutrophils are allowed to settle in the plate for 30 min.
4. In the meantime, the microscope is prepared for the experiment (temperature is set at 37°C and CO_2 at 5%, the instrument is calibrated for a 24-well plate, correct focus and auto focus are established).

Data acquisition

1. Neutrophils are imaged using a 20x 0,4 objective and two channels:

a. Phase contrast, 30 ms exposure time.

b. Fluorescence contrast EGFP (Excitation wavelength 488, Emission wavelength 509), 50 ms exposure time.

2. To allow time lapse analysis, one frame every 2.5 min. is taken. Acquisition duration is typically between 30 min. and 4 hours, and can be chosen according to your experimental question (see Note 14).

Data analysis

1. After data acquisition, the pictures of one condition are digitally linked using the stitch function.
2. A fixed area of interest is chosen (see Note 15), a timespan is defined that is equal for all samples (typically 2-4 hours) and this homogenized data is exported.
3. For the quantification and visualization of neutrophil motility, TrackMate software is used (21, 22) (see Note 16).
4. To start, cells are segmented by overlaying each cell with a purple spot (see enlargement in Figure 2, Frame 1). It is important to choose a spot-size that fits your data (10-12 µm for neutrophils). This can be done by choosing 10-12 micron as 'Estimated object diameter'. Press 'Preview' to see whether the settings of choice correctly identify the cells in one sample frame. All cells will be automatically recognized in all frames.
5. Next, a check for software mistakes in cell fragmentation and a manual adjustment of the spot selection needs to be performed. In Figure 2, three consecutive frames are shown, illustrating the importance of this step. In some cases, multiple neutrophils are moving in close proximity to each other and they are recognized as one single cell by the software. Consequently, a spot can be lost during acquisition, resulting in fragmented tracks later on in the analysis. In this case, an extra spot should be added manually. On the other hand, the software sometimes considers a cell protrusion as a new cell and it incorrectly adds an extra spot. In this case, this extra spot must be removed manually (see Figure 2 Frame 3). If the software frequently misidentifies cell protrusions and adds inappropriate spots, it is recommended to increase the spot size to enhance accuracy.

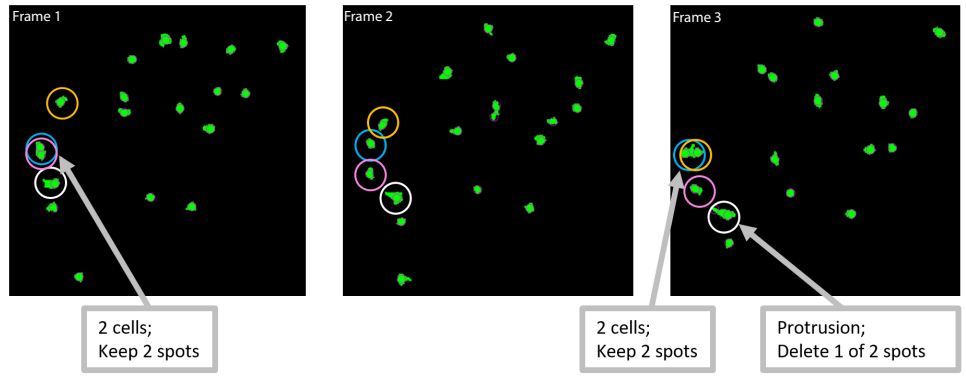

Figure 2: *Segmentation process of three consecutive frames of the same area in TrackMate. In green, the neutrophils are visible. The software annotates each cell with a purple circle that will eventually be tracked (see 2x enlargement in Frames 1 and 3). The four squares in yellow, blue, red and white are highlighting the same individual cells overtime for illustration purposes.*

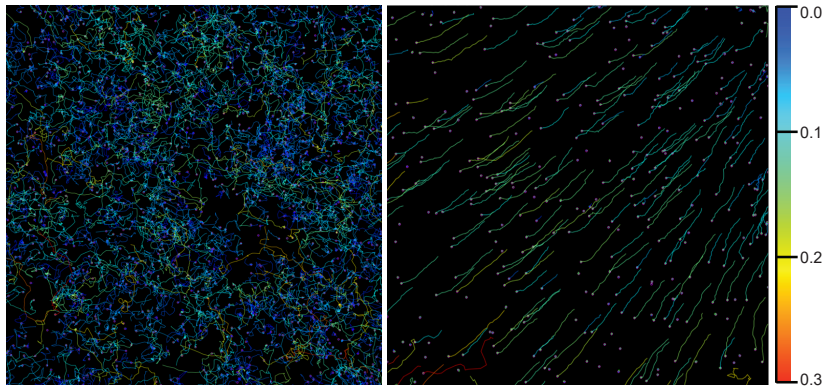

Figure 3: *Tracking live cell motility of human neutrophils for three hours: a good (left) and a bad (right) example (10x magnification plus 2x digital zoom). Tracks are colored for mean track velocity (micron/sec) and values are indicated by the bar on the right.*

6. Once all cells are correctly segmented throughout all frames, the next step is to connect the spots to study the tracks. Choose 'Simple LAP tracker' in the dropdown menu and set appropriate parameters (see Note 17).
7. When pressing "Next", the software creates 'links' connecting two spots in consecutive frames and 'tracks' that are the sum of all the links.
8. Make sure to verify that the plugin connected the correct dots, and adjust manually if necessary. Figure 3 shows two examples of cell tracking results. On the left, cells are actively moving across the well's surface, creating intricate patterns in their tracks. The left picture is therefore a good example of what actively moving neutrophils look like. On the right is an illustrative example of tracking caused by cells drifting within the well, as all the cells are following the same direction at the same speed; this does not represent actual active cell movement (see Note 18). The picture on the right is therefore an example of what passive/artefact movements look like.
9. After the tracks have been manually curated frame by frame, the resulting track data can be exported in a .txt or .xlsx format for statistical analysis and graphing purposes.
10. To obtain information about the percentage of moving neutrophils, you can set a very low threshold (e.g. 10 µm) to identify all cells that (hardly) moved, and calculate this as a fraction of total measured tracks. Alternatively, you can divide the number of tracks over the number of spots.
11. Additional informative parameters to investigate are:
    a. Mean track velocity: velocity values are defined as the distance between two spots (the link length) divided by the time difference for a single link. Mean velocity, is the average of the link velocities over all the links of the track.

    b. Track length: total distance travelled; sum of all link lengths for each track.
    c. Track displacement: net distance from start point to end point.
    d. Track displacement in combination with track length provides information about to which degree the neutrophils move linearly through space.

In Figure 4, an example is presented of the final data analysis results, in which multiple experiments are combined: 11 donors from Group A to 13 donors in Group B (see Note 19).

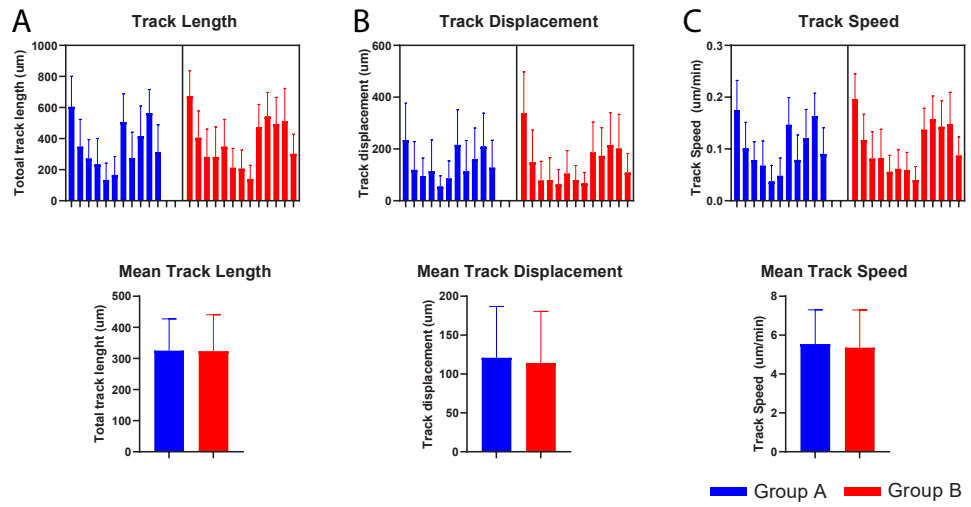

Figure 4: *Combined results of multiple experiments showing* ***(A)*** *Track Length,* ***(B)*** *Track Displacement and* ***(C)*** *Track Speed of individual donors (top row) and mean values per group (bottom row).*

Neutrophil migration assay

Plate preparation

1. After staining/washing the neutrophils as described in 3.1, allow them to rest for 30 min. in the dark at RT.
2. In the meantime, prepare the lower wells of a 96-well Transwell plate with a medium control and the chemoattractants (see Figure 5 for IL-8 and LTB-4 titrations).
3. Fill the bottom wells with 200 µL of the following conditions as described in Table 1 and illustrated in Figure 6 (the use of triplicates is recommended):
    a. Positive control: 100.000 cells (use 100 µL of cell suspension and add 100 µl of medium)
    b. Medium
    c. Medium +IL8 (final concentration of 0.1 µg/mL) (see Notes 20 and 21)
    d. Medium +LTB4 (final concentration of 0.01 µg/mL) (see Notes 20 and 21)

4. After the 30 min rest, the top wells are placed on the lower wells and 100 µL calcein stained neutrophils (100.000 cells) are added to the upper wells, according to the table 1.
5. The plate is incubated for 40 min. at 37°C.
6. After incubation, upper wells are removed and neutrophils are harvested from the lower wells by gently pipetting up and down without creating bubbles, and transferred to a V- 96-well plate (see Notes 22 and 23).
7. The V-bottom plate is spun down for 5 min. at 250 *g,* and supernatant is removed.
8. Cells are resuspended in 50 µL of medium + 1% HS and transferred to a black flat bottom plate.
9. 50 µL of HTAB buffer is added to all samples. Pipet up and down to lyse the cells.
10. Samples are kept dark until data acquisition, preferably on the same day.

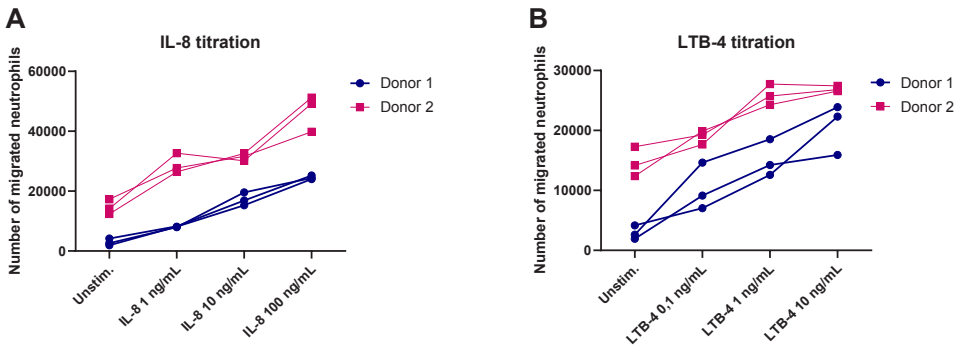

Figure 5: *Titration of chemoattractants (A) IL-8 and (B) LTB-4. Chemoattractant titrations were performed in medium supplemented with 1% human serum. Triplicates of two donors are shown here.*

Table 1: *Schematic overview of the experimental conditions of the neutrophil migration assay.*

	Bottom well	**Upper well**
Positive control	100,000 cells (100 µL) + 100 µL medium	Empty
Medium	Medium (200 µL)	100,000 cells (100 µL)
IL8	Medium + IL-8 (200 µL)	100,000 cells (100 µL)
LTB4	Medium + LTB4 (200 µL)	100,000 cells (100 µL)

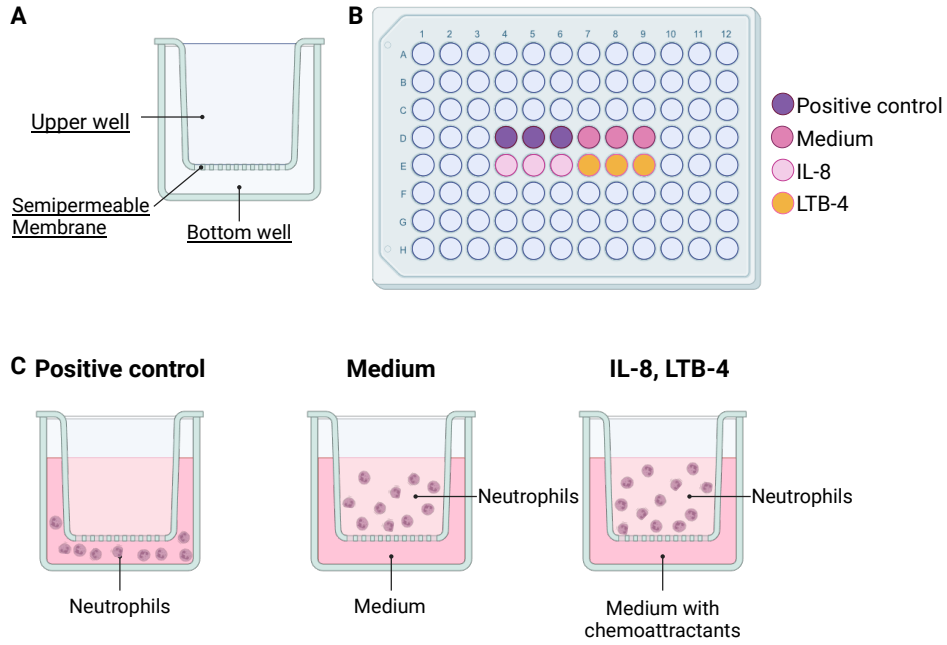

Figure 6*: Schematic overview of the experimental setup for the neutrophil migration assay, illustrating* ***(A)*** *the transwell components,* ***(B)*** *plate layout and* ***(C)*** *starting experimental conditions. Created with BioRender.com.*

Data acquisition and analysis

1. The plate reader is set up as follows: the focal height is determined and the gain is adjusted to prevent signal saturation in any of the wells.
2. 20 laser beam flashes per well are used.
3. The fluorescent signal is measured and quantified with the plate reader (excitation 485/ emission 520).
4. The average of the triplicates is used for each condition.
5. The positive control condition serves as reference (=100%) and migration rates of the other conditions are calculated relative to this signal.

Notes

1. Suppliers are given for reference, but equivalent products can be purchased from different providers or manufacturers.
2. Blood sampling is a reserved medical procedure, and may only be performed by certified

personnel. Subjects must be counseled and sign informed consent. Be sure to follow applicable regulations within your country and research institution.

3. Blood samples are considered primary human tissues and should be handled as biohazardous material under Biosafety Level 2: work in a Class II bio-safety cabinet while wearing appropriate protective PPE certified gloves and clothing.
4. Neutrophils follow the circadian rhythm in exiting the bone marrow. It is best to collect blood samples in the morning and to avoid large variation in time of blood draw. Since neutrophils do not survive a freeze-thaw procedure, the use of fresh blood samples is essential.
5. If no serum free T cell mixed medium is available, experiments can also be conducted with RPMI 1640 Medium without Phenol red (Gibco).
6. Depending on the number of magnets you have, you can process multiple samples at the same time.
7. To avoid neutrophil activation as much as possible, it is important to use a negative selection kit during neutrophil isolation.
8. Neutrophil isolation mix must be prepared freshly before each cell separation procedure, and reagents should be placed at RT before use for 15min.
9. Half the amount of Miltenyi Neutrophil Isolation beads was tested and was found to be equally effective.
10. When pipetting neutrophils, make sure to handle them very gently (e.g. avoid flicking the pellet after centrifugation and do not create bubbles).
11. For the purity check after neutrophil isolation, use forward scatter and side scatter to identify your single cells, life/dead staining in combination with a dump lineage channel containing markers like anti-CD3, anti-CD19 and anti-CD56, and antibodies against CD11b, CD66b and CD16 to identify neutrophils (see Figure 7).
12. The remaining cells can be used for other purposes, e.g. proteomics, secretomics, NET-formation assay.
13. Only the central wells of the 24 well plate are used and water is added to the wells that are not used to prevent evaporation of medium in the wells containing the neutrophils.
14. It is advisable to choose the acquisition duration generously. After acquisition, the user can choose the window of time of interest to export, for instance excluding the end if cells die.
15. To avoid (unintentional) bias in selecting the region of analysis, it is advised to work with fixed coordinates within the well.
16. TrackMate is a plugin within FIJI, with a user friendly interface. It follows a classical approach in which the cell segmentation step is distinct from the particle-linking steps. There is other tracking software available (like e.g. Spottracker), but TrackMate is most commonly used.

17. The parameters used in this experiment are: Linking max distance: 50,0 micron, Gap-closing max distance: 100,0 micron, Gap-closing max frame gap: 3. It is advisable to adjust these values to determine the parameters that best fit your data. In which the "Linking max distance" dictates the maximal distance between two spots. "Gap-closing max distance" dictates how far two spots can be apart, in case the spot was missed, and "Gap-closing max frame gap" describes the maximum allowed number of consecutive missed frames.

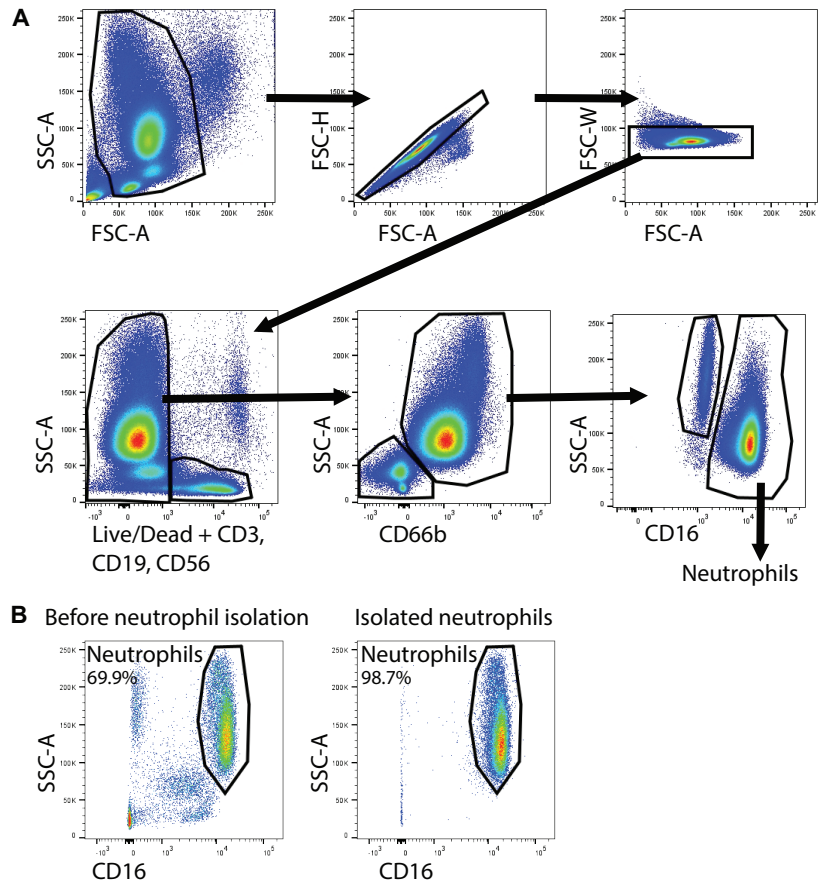

Figure 7: *Neutrophil isolation purity check based on flow cytometry.* ***A)*** *Representative flow cytometry dot plots illustrating strategy to gate on neutrophils (gating on singlets, live, lineage-, high side scatter, CD66b+ CD16+).* ***B)*** *Percentage of neutrophils from single live cells before and after neutrophil isolation.*

Typically two samples are taken along: a. before isolation sample; b. isolated neutrophils

18. To avoid cell drifting, lower the speed of the stage while moving to the next well to 50% with acceleration 30%.
19. In addition TrackMate plugin offers the opportunity to plot several other features of interest regarding the Spots, Links and Tracks.
20. IL-8 and LTB-4 concentrations were optimized and optimal conditions were chosen (Figure 8).
21. It is desirable to test multiple concentrations of the chemoattractants while setting up the experiments. In our lab, these were the optimal conditions with the biggest difference between the case and control group, but this might vary depending on the experiment and the lab. Additionally, researchers may opt to utilize alternative chemoattractants. In that case too, it is imperative to optimize the protocol accordingly.
22. V-bottom plates are preferred when spinning down only a small amount of cells because the pellet is more firm. Alternatively, a U-bottom plate can be used as well.
23. In some cases, the volume in the lower well might exceed the maximum of what can be plated in a V-bottom well (~220 µL). In this case, you need to separate the sample over two wells and combine them after the first spin. Wash wells to minimize cell loss.

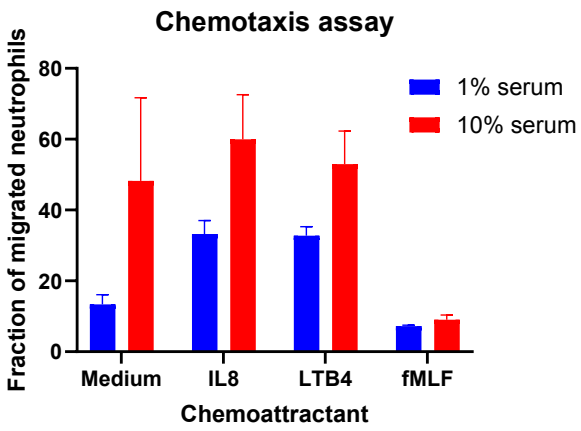

Figure 8: *Fraction of migrated neutrophils at different concentrations of human serum. For testing the best serum concentration, the chemoattractants were used in the following concentration: 100 ng/mL IL-8, 10 ng/mL LTB-4 and 100 nM fMLF.*

Concluding remarks

While descriptive analyses can provide correlative and static snapshots of cellular capabilities, functional assays give dynamic insights into cellular activity, and provide opportunities for mechanistic analyses of biological processes. Investigating the functional behavior of neutrophils takes on considerable importance within the context of tumor-induced systemic inflammation and tumor progression (16, 17). In this chapter we have described two distinct *ex vivo* assays tailored to quantify human neutrophil motility and migratory capacity. Taking into account the difficulties of working with unpredictable human samples, especially when dealing with cancer patients, our protocol offers a feasible and reproducible approach that allows a standardized sample analysis over extended periods of time. The motility and migration assays described in this chapter have a wide range of potential applications. These include, but are not limited to, comparing the migration capacity of neutrophils from healthy individuals with those from patients affected by a particular disease. Furthermore, these assays can function as a screening tool to evaluate the impact of specific drugs on neutrophil migration.

When a preference for more physiologically relevant conditions arises, the migration assay can be modified with the addition of a HUVEC (Human umbilical vein endothelial cells) monolayer in the transwell. This will better resemble the transmigration process across the blood vasculature. However, the execution of these assays poses significant logistical challenges, primarily due to the concomitant requirement for fresh blood samples and an already formed HUVEC cell monolayer in the transwell.

We advocate combining these functional assays with complementary techniques such as RNA sequencing and proteomics. This integrative approach not only enhances analytical depth but could also facilitate the elucidation of the underlying molecular mechanisms responsible for potential differences in migration between experimental and control groups.

When stimulating neutrophil migration and motility with chemoattractants, it is important to carefully consider the choice of the stimulus. IL-8 and LTB4 are acknowledged as “intermediary” signals, directing neutrophils from the bloodstream to the general vicinity of their target. These intermediary signals are disregarded once neutrophils perceive “end-target” signals in close proximity to their final destination (23). To draw a comparative analysis between “low-priority input” denoted by IL-8 and LTB4, and “high-priority input”, chemoattractants such as fMLF and C5a can be examined. However, previous studies have outlined that fMLF and complement C5a result in swift alterations in neutrophil morphology and adherence properties (24, 25), which can impact the outcomes of migration experiments. In our hands, fMLF persistently resulted in less migration than in the medium control condition (Figure 5), because the neutrophils adhere to the transwells. Therefore, when alternative chemoattractants like fMLF, C5a or others are intended to be used, additional refinement of this protocol is required.

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Conflicts of interest

N.A.M.B. and C.B. have no conflicts of interest to declare. K.E.d.V. reports research funding from Roche/Genentech and is consultant for Macomics, outside the scope of this work.

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