Responses to Reviewers

Editorial comments:

General:

Q1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

R1. We thank the editor for this comment. We have reviewed the manuscript thoroughly and edited any issues we found.

Q2. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please limit the use of commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: Zetag®, SylgardTM, HetaSep, Nikon Ti2, RayBiotech, etc.

R2. We removed the instances of commercial language. We changed Zetag® to the generic "cationic polyelectrolyte" (CP) and HetaSep to "erythrocyte aggregation agent."

Keywords:

Q1. Please provide at least 6 key words or phrases.

R1. The key words were edited as follows: "Microarray, microstamping, cell migration, neutrophil, swarming, proteins"

Protocol:

Q1. Please include an ethics statement before the numbered protocol steps, indicating that the protocol follows the guidelines of your institution's human research ethics committee.

R1. We added the following ethics statement to the beginning of the protocol section: "**Ethics statement:** The authors acknowledge our healthy volunteers who kindly donated their blood. Blood specimens were obtained after informed volunteer consent according to institutional review board (IRB) protocol #2018H0268 reviewed by the Biomedical Sciences Committee at The Ohio State University."

Q2. For each protocol step, please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. If revisions cause a step to have more than 2-3 actions and 4 sentences per step, please split into separate steps or substeps.

R2. We have expanded many of the protocol steps to more effectively explain how to perform the steps.

Specific Protocol steps:
Q1. 4.1: Are separate microarrays used for each time point? Please clarify.

R1. Yes, the supernatant of each time point came from a different microarray. This is necessary because the concentration of the proteins is low and the volume of each well is required to achive the minimum level of detection of the protein array assay. The following text has been added to step 4.1: "To overcome the limit of detection of the protein array assay, the entire volume of supernatant of a single well (200 µL) was used for each time point."

Figures:
Q1. Figures 2 and 3: Please use 'µm' and 'µL' instead of 'um' and 'uL'.

R1. Figures 2 and 3 were corrected to use 'µm' and 'µL' instead of 'um' and 'uL'.

Q2. Figure 3: What are the fitted curved here?

R2. The curves fitted here are smoothing splines with $\lambda = 0.05$. The caption for Fig. 3 was edited for clarification: "The data points were fitted with smoothing splines ($\lambda = 0.05$)."

Discussion:
Q1. Please also discuss critical steps of the protocol and modifications/troubleshooting in the Discussion section.

R1. The following text has been added to the discussion section: "During the development of our microstamping protocol, several challenges arose that required careful troubleshooting. For instance, the CP used for the microstamping is highly hydrophilic. The PDMS stamps are hydrophobic. Since the CP does not have a high affinity for PDMS, our procedure was carefully designed to avoid the formation of bubbles and promote wetting. By first priming the stamp with CP while face-up (step 1.8), we minimize the formation of bubbles between the CP and the stamp. The stamp is then inverted onto a layer of CP and incubated for 1 h. This long incubation time ensures every section of the stamp is wetted. Second, the process of removing excess CP before stamping on a clean glass slide (step 1.11) can be inconsistent. While performing step 1.11, look closely at the stamp. When the pattern begins to become visible, the stamp is ready for step 1.12. Additionally, the required vacuum time to dry the stamps (step 1.12) can vary. This is primarily dependent on the weather. On a warm, humid day, 2 min of vacuum time is required. On a cool, dry day, 1 min of vacuum time is sufficient."

Table of Materials:
Q1. Please ensure the Table of Materials has information on all materials and equipment used, especially those mentioned in the Protocol.

R1. The Table of Materials was updated to include all materials and equipment used as mentioned in the protocol.

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
The manuscript describe a method to fabricate a bioparticle microarray to study human neutrophil swarming. Neutrophil behavior as well as neutrophil derived cytokines can be studies in detail with this technique. Overall this protocol will be very useful for researchers studying neutrophil behavior and inflammation.

R. We thank the reviewer for the comments that will improve the quality of our manuscript. We have addressed all his/her comments.

Major Concerns:
none.

Minor Concerns:
Q1. It will be helpful to specify the volumes taken at step 4.1 at each time point.

R1. Step 4.1 has been edited as follows: "To overcome the limit of detection of the protein array assay, the entire volume of supernatant of a single well (200 µL) was used for each time point."

Reviewer #2:
Manuscript Summary:
Reategui and Walters describe in their manuscript an experimental method to study swarming of human neutrophils on slides with patterns of inert bioparticles in vitro. The great potential of this method was already proven by their earlier work (Reategui et al., Nat Biomed Eng. 2017) with the identification of novel regulators of neutrophil swarming. Now, the authors present an optimized protocol of their microarray assay. Here, they describe in detail how their technical platform is fabricated. In addition, they explain how the assay can

be used to obtain reproducible data about migration dynamics and mediator release of swarming neutrophils. In conclusion, the standardization and high-throughput design makes their in vitro neutrophil swarming platform an unprecedented tool and powerful alternative to in vivo swarming assays. Before considering for publication, more detailed information should be included in some parts of the protocol.

R. We thank the reviewer for the comments that will improve the quality of our manuscript. We have addressed all his/her comments.

Minor Concerns:
My main questions and suggestions for the authors are:

Introduction:
Q1. Line 62-64: The authors refer to differing neutrophil migratory behavior between species. However, human neutrophil swarming has been shown to follow the same dynamics as in mice (Refs. 11, 15) and use similar chemotactic mediators (Refs. 5, 15). Please adapt this passage. This also refers to the discussion (Lines 255-257).

R1. The passages in the introduction and the discussion were updated to reflect this. In the introduction, the passage was modified as follows: "Additionally, research describing human neutrophil coordinated migration is limited.[16] On an *in vitro* swarming platform, human neutrophils can be directly analyzed." The discussion now says: "Additionally, in vivo models are typically performed in rodents[11–13, 15, 22, 23] or zebrafish.[11, 12, 15, 23] Our platform uses human neutrophils, which enables us to more directly interpret our results in the context of human disease, though certain similarities between mouse and human neutrophils have been observed.[5, 11]".

Q2. Line 72: What is the advantage and feature of Zetag? This is nowhere explained throughout the text. Please comment on this is any part of this protocol.

R2. We removed the commercial name Zetag and replaced it with the generic "cationic polyelectrolyte" (abbreviated CP). The following explanation was added to the introduction: "By first patterning the CP layer, we are able to selectively pattern negatively charged bioparticles to generate the desired neutrophil swarming pattern. The positively charged layer holds the negatively charged bioparticles through the vigorous washing step that removes the bioparticles from the areas on the glass slide that do not have the CP. Additionally, the CP used here, a copolymer of acrylamide and quaternized cationic monomer, is biocompatible, so it does not induce a response from the neutrophils. It has a very high surface charge, which immobilizes the micron-sized bioparticles to the glass slide,

thus inhibiting neutrophils from removing the particles from the patterned position on the glass slide." (Lines 71 – 79)

Protocol:

Q1. General statement to Point 1 (Microfabrication of Bioparticle Microarray): For biologists this protocol part requires a complete visualization in the video, from start to end. Please also adapt Figure 1 with better explanations in the figure, starting with steps from 1.1.

R1. Figure one has been updated to include the microfabrication process, and in the video we will include the microfabrication of the microstamps in detail. Additionally, the following has been added to the Fig. 1 caption: "A silicon wafer coated with negative photoresist is exposed to UV light through a chrome photomask (**A**). After the silicon wafer is baked and developed, a photoresist pattern remains on the surface of the silicon wafer. This is the master wafer (**B**). In a petri dish, a PDMS mixture is added to the top of the master wafer. The PDMS is cured overnight at 65 °C to form the PDMS mold (**C**). The PDMS mold is cut from the master wafer and a biopsy punch is used to punch out individual PDMS stamps (**D**)."

Q2. Please explain the generation of the master mold silicon waver including the design of the patterns (pattern size, distance between patterns etc.) and the used equipment in more detail or refer to a protocol with a detailed description [1.1, 87].

R2. Step 1.1. was expanded as follows:

"1.1. Using standard photolithography procedures, generate the master silicon wafer.

1.1.1. Generate a proof of the desired design using a computer-aided design (CAD) software. This design can be sent to a photomask manufacturer to produce a chrome photomask. The design used here is 4 mm x 4 mm rectangular arrays of 30-µm diameter filled in circles with a 150-µm center-to-center spacing. This design can be modified as desired for different applications.

1.1.2. Spincoat a 40-µm thick layer of a negative photoresist onto a silicon wafer. Bake wafer at 65 °C for 5 min and 95 °C for 10 min.

1.1.3. Expose wafer to UV light through a chrome photomask with 150 – 160 mJ/cm^2 (**Fig. 1A**).

1.1.4. Bake wafer at 65 °C for 5 min and 95 °C for 10 min. Submerge wafer in photoresist developer for 10 min and rinse with isopropyl alcohol. At this stage, the pattern should be visible on the wafer (**Fig. 1B**)."

Q3. Please state the required volumes of polymers that need to be prepared to cover a master wafer [1.2, 91]

R3. Step 1.2 has been modified as follows: "1.2. Thoroughly mix a 10:1 ratio of polydimethylsiloxane prepolymer and its curing agent (i.e., 20 g prepolymer and 2 g curing agent) and pour the uncured polydimethylsiloxane (PDMS) mixture over the master wafer in a petri dish (**Fig. 1C**)."

Q4. Please briefly explain why the balanced weight is required [1.9, 111].

R4. Step 1.15 has been modified to explain the balanced weight. "1.14. Place a 5.6 ± 0.1 g balanced weight on top of the stamp and allow 10 min for stamping (**Figure 1D**). A balanced weight is required to ensure the stamp is pressed evenly onto the glass slide and promote even transfer of the CP to the glass slide."

Q5. Please make clear that you used 8 individual stamps per slide and not one (e.g. 1.15, 125). Be more careful with the phrasing here.

R5. Steps 1.5 – 1.15 were modified to more clearly explain that 8 stamps are used per slide. Some of the major changes were in step 1.5, "For one glass slide, 8 stamps will be needed," and steps 1.13-1.14, "1.13. Adhere an imaging spacer (8 well, 9-mm diameter) on the top of a clean glass slide as a guide for stamp placement. With this spacer, each glass slide can have 8 microarrays. 1.14 Place a stamp face down on the glass slide in the center of each well of the imaging spacer (i.e., use 8 stamps total)."

Q6. At what temperature does the drying of the Zetag® layer occur? [1.15, 125].

R6. The Zetag (cationic polyelctrolyte) dries at room temperature. Step 1.15 has been modified as follows: "1.16. Remove the weight and stamp from the glass slide (**Figure 1I**). Allow the CP layer to dry at room temperature for 24 h before adding the bioparticles (continuing with step 1.17)."

Q7. Please explain in more detail what you mean with "adding the second layer" [1.15, 125]. Do you continue with step 1.7? Please clarify this.

R7. The second layer refers to the bioparticle layer added onto the Zetag. Step 1.16 has been modified as follows: "1.16. Remove the weight and stamp from the glass slide (**Figure 1I**). Allow 1 day for the CP layer to dry at room temperature before adding the bioparticles (continuing with step 1.17)."

Q8. The addition of a blank PDMS slab to the glass slide is not visible in Figure 1f [1.16, 128-130]. The slides look the same in Fig1e and 1f. The authors should consider illustrating the whole fabrication process of the slides including the blank PDMS slab and the SecureSealTM spacer.

R8. We modified the schematic to show the addition of the PDMS slab (**Fig. 1J-L**). In addition, the following was added to the figure caption: "A PDMS slab with pre-cut wells is

adhered to the glass slide (**J**).". The following reference was added to the protocol section of the text: "1.17. Cut a blank PDMS slab to the size of the imaging spacer and use the 8-mm biopsy punch to create wells in the PDMS that align with the wells of an imaging spacer. Adhere the PDMS slab to the glass slide with the imaging spacer (**Fig. 1J**)."

Q9. In which buffer/medium should bioparticles be diluted [1.17, 132]. Do they need to be opsonized? Is this the same for both zymosan and E. coli particles (as mentioned in the Material Table)? Please clarify this.

R9. The bioparticles should be diluted in pure water. Opsonization of the particles is not necessary. Neutrophils contain receptors that are able to directly recognize molecules present on the bioparticles.[1] For example, TLR4 can recognize lipopolysaccharide (LPS), which is present on the cell membrane of gram negative bacteria like *E. coli*.[2] TLR2/TLR6 heterodimer recognizes zymosan, which is present on the cell wall of fungi.[2, 3] Here, neutrophil swarming occurs without artificial opsonization. Step 1.18 was modified as follows: "1.18. Thaw a solution of bioparticles (e.g. *E. coli* or zymosan) and dilute to 500 µg/mL in water for injection (WFI). Note: The bioparticles do not need to be opsonized. Neutrophil surface receptors directly recognize molecules on these bioparticles.[17–19]"

Q10. Which well are the authors referring to in 1.18 (line 132)?

R10. Step 1.19 was modified as follows: "1.19. Add 100 µL of bioparticle solution to each PDMS well on the glass slide (**Figure 1K**)."

Q11. Please give an approximate volume of blood / number of isolated neutrophils that should be used with the described assay [2.1, 143].

R11. Step 2.1 was modified as follows: "2.1. Collect at least 2 mL fresh blood from the desired donor in K2-EDTA tubes. The expected yield of neutrophils is 1-2 million cells / mL whole blood. The imaging assay requires approximately 0.15 million neutrophils, and the analysis of the supernatant requires 1 million neutrophils. Use the blood within 4 hours."

Q12. At which temperature should the centrifugation take place [2.4, 150]. Also [2.10, 171]. Also [4.4, 212].

R12. All centrifugation steps take place at room temperature (20 °C). Steps 2.4, 2.7, 2.10, and 4.4 were edited to reflect this.

Q13. Please refer to the concentration and not dilution factor of Hoechst 33342 [2.9, 168].

R13. Step 2.9 was modified as follows: "2.9. Stain nuclei with 20 µg/mL Hoechst 33342 for 10 min at 37 °C."

Q14. Please illustrate the sealing with a coverslip in more detail [2.12, 175].

R14. Step 2.13 was added to explain in further detail: "2.13. Seal with a 12-mm diameter coverslip. Cover the opening of the PDMS well with a 12-mm diameter coverslip. Press down gently onto the coverslip with tweezers so the excess cell suspension escapes to the edge of the well. Use a tissue to remove the excess cell suspension."

Q15. Please specify the microscope and stage incubator type in the material & methods section only or explain why only this system can be used [3.1, 180-181]. Is O2 necessary for this assay to work?

R15. The commercial names of the microscope and incubator were removed from the protocol. Step 3.1 now reads, "3.1. Load microparticle array with cells on the live cell imaging station with a microscope equipped with a cage incubator set to 37 °C, 5 % CO_2, and 90 % relative humidity."

Q16. Please explain in more detail how you generate individual tracks of neutrophils using Imaris software. Do you track them manually or do you use automated tracking, and if so, which parameters/settings do you use for automated tracking? [3.2, 186-187].

R16. Step 3.3 was modified as follows:

"3.3. Use an automated cell tracking software to track the migration of individual neutrophils toward the bioparticle cluster.

3.3.1. Use the autoregression mode of a spot detection cell tracking software. Set the spot radius to 5 µm (the approximate size of a neutrophil nucleus). Set the minimum track length to 120 s and a maximum gap size of one frame.

3.3.2. From the data generated by the cell tracking software, extract the files that contain the neutrophil position and speed. These files can be used with a graphing software to generate neutrophil migration tracks (**Fig. 2C**) and a heat map of speed vs. time (**Fig. 2D**), respectively."

Q17. The generation of heat maps of speed vs. time are generated with software different to Imaris, right? [3.3, 189].

R17. We used Matlab and JMP to generate the plots in Figure 2. However, it is not important which graphing software you use to generate the plots from the Imaris data. We clarified that a separate graphing software was used to generate the plots in step 3.3.2. as follows: "3.3.2. From the data generated by the cell tracking software, extract the files that contain the neutrophil position and speed. These files can be used with a graphing software to generate neutrophil migration tracks (**Fig. 2C**) and a heat map of speed vs. time (**Fig. 2D**), respectively."

Q18. Please show in detail the steps in 3.4 to analyze the bioparticle cluster, either in video or Figure.

R18. The following analysis steps are described for the analysis of the swarms:

"3.4. Use the 405 nm fluorescent images to track swarm size over time on an image analysis software of your choice.
3.4.1. Define regions of interest (ROIs) around each bioparticle cluster where neutrophils will swarm. Keep the same size ROI to analyze each bioparticle cluster.
3.4.2. Analyze the mean fluorescent intensity of the 405-nm images within each ROI over time.
3.4.3. Generate a calibration curve of mean fluorescent intensity to swarm size by taking manual measurements at various swarm sizes from 0 μm^2 to the maximum swarm size. Use this calibration to calculate the swarm size over time."

Q19. Do you track neutrophils inside the cluster or only their recruitment to the cluster? Fig2c only shows tracks of recruited neutrophils, but not tracks of neutrophils in the swarm. It is not clear in Fig2c, if there are tracks underneath the red circle that indicates the micropattern.

R19. We only track the recruitment of neutrophils to the cluster. When neutrophils reach the cluster, their nuclei overlap with other nuclei in the cluster. Thus, we cannot accurately track a neutrophil within the cluster. The following text was added to the Results section: "We track the migration of individual neutrophil nuclei as they migrate toward the bioparticle cluster. When neutrophils reach the bioparticle cluster, their nuclei overlap with other nuclei in the cluster. Thus, we cannot accurately track a neutrophil within the cluster."

Q20. Did you use 30-μm sized E. coli bioparticle patterns for all your experiments? Please indicate at beginning of the result section. The Table of Materials has zymosan and E. coli particles.

R20. *E. coli* and zymosan particles were used for different experiments. The following explanation was added to the results section: "Zymosan and *E. coli* bioparticle clusters both result in the generation of neutrophil swarms. For our results, **Fig. 2B** uses data from neutrophil swarms generated by *E. coli* particles. **Fig. 3** and the other panels of **Fig. 2** use neutrophil swarms generated by zymosan particles."

Q21. Please clarify the number of replicates you used in Fig2. Is n the number of different neutrophil donors, and N the number of clusters you analyzed from a total of n donors?

R21. The figure caption was modified for clarity. For **Fig. 2B**, the data comes from 32 neutrophil swarms (n) from one donor (N). The caption for Fig. 2B was modified as follows:

"ANOVA, $p < 0.0001$, n = 32 neutrophil swarms, N = 1 donor, error bars = standard deviation."

Q22. What does ‚count' mean in Fig1D? Is this plot a representative of one experiment or does it show data from different replicates?

R22. The caption for **Fig. 2D** was edited as follows: "Each count on the heat map represents a neutrophil with the given instantaneous speed at the given time point. These heat maps are representative of 1 experiment (n = 1 swarm, N = 1 donor). (ANOVA, 6114 neutrophils (swarming), 32116 neutrophils (control), $p < 0.0001$)"

Q23. Please double-check if you used a cytokine microarray or a microarray that detects a range of human proteins. For example, some of the differentially regulated proteins are receptors (TLR2, IL-6R) and not cytokines [4, 203-216 and discussion].

R23. It is a microarray that detects a range of human proteins. Step 4.6. was edited as follows: "4.6. Use a microarray kit that detects a range of human proteins to process samples."

Q24. How reproducible is the identification of the proteins that you found in the supernatant of swarming neutrophils? Please include a measure of variability in Fig3.

R24. The supernatant experiment was done in triplicates for the same donor. We have added the errors bars to Figure 3. The following text was added to the **Fig. 3** caption: "(Error bars = standard deviation, n = 3 replicates, N = 1 donor)" The goal of this manuscript is to highlight the method, and how the method can enable quantification of different cytokines or proteins. In this manuscript we are not making any claims about what protein has or has not been identified.

Q25. Please refer to the cytokines that were already identified in Ref. 5 before.

R25. The following text was added to the results section. "Of the 16 proteins we identified, 12 proteins were identified by our previous publication.[5] Adipsin, galectin-3, nidogen-1, pentraxin 3, TIMP-1, and TLR2 were shown to be swarming specific, while clusterin, IL-6R, MMP-8, and MMP-9, RANK, and trappin-2 were not.[5]" The following was added to the discussion section, "Some of the proteins we identified were previously shown to be swarming specific, while others were expressed differentially by activated non-swarming neutrophils.[5]"

Q26. The authors attribute functions to proteins differentially expressed during swarming (e.g. protein is "unnecessary" if downregulated during swarming). This part is pure speculation without any proof and should be phrased more carefully (e.g. further studies are

needed to understand role of differentially regulated proteins during neutrophil swarming) [discussion, 281-287].

R26. The discussion section has been edited. The section in question now reads: "The relationship between other proteins and inflammation is less well known. The proteins that spike or decrease during swarming may be involved in the regulation of inflammation. However, further research is necessary to understand the role in inflammation of many of the proteins that are differentially expressed during swarming."

Reviewer #3:

Manuscript Summary:

Authors describe a protocol for using microarray to asses neutrophil swarming.

R. We thank the reviewer for the comments that will improve the quality of our manuscript. We have addressed all his/her comments.

Major Concerns:

The manuscript has mert; however, four major concerns should be addressed.

Q1. The introduction fails to set the stage for the need and application of the assay. A paragraph describing the research or clinical use of the assay is needed to round the paper out.

R1. The following text was added to the introduction: "In our previous publication, we demonstrated that patients with certain medical conditions (trauma, autoimmune disease, and sepsis), had neutrophils that functioned differently than healthy donors.[5] In future research studies, our platform could be used to analyze neutrophil function among a variety of patient populations. This platform can quantitively analyze the complex coordination involved in neutrophil swarming. Additional studies can be done to provide insight on the neutrophil function of a specific patient population or neutrophil response to a pathogen of interest."

Q2. An in vivo comparison with the microarray to determine the effectiveness of the assay is warranted.

R2. This manuscript describes an *in vitro* method that mimics neutrophil swarming. In the past, our method was highlighted for its accuracy by one of the world experts in the field of *in vivo* leucocyte migration imaging. The author mentions that while other *in vitro* methods "do not provide any insight into how neutrophils coordinate their dynamics and effector processes," this microarray swarming platform is a "solution for monitoring the swarming

response of human neutrophils, enabling the detailed analysis of neutrophil-derived factors released during swarming."[5] Additionally, he writes, "These microarrays in combination with live-cell imaging represent a high-throughput, standardized and precisely controlled experimental system to study neutrophil swarming."[5] Here we are describing a refined method to our original publication.[4] A comparison with an *in vivo* experiment is out of the scope of this manuscript.

Q3. The statistics fail miserably on this paper. Traditional microarray numbers are necessary including CV, standard deviation, etc. A Z' score may also be warranted.

R3. We have corrected the statistics presented in the manuscript. The following text was added to the **Fig. 3** caption: "(Error bars = standard deviation, n = 3 replicates, N = 1 donor)"

Q4. The study needs biological and technical replicates.

R4. The purpose of this manuscript is to present a refined version of our previous publication on neutrophil swarming.[4] Extensive experiments testing different conditions (e.g., biological and technical replicates) were conducted on that study.[4] We do not consider necessary the experiments suggested by the reviewer.

Minor Concerns:
None noted

Reviewer #4:
Manuscript Summary:
Neutrophils, the most abundant white blood cell in the bloodstream, are gaining attention as potential diagnostic and therapeutic targets because neutrophil activity has been attributed to a variety of medical conditions including sepsis, trauma, and cancer. This protocol describes a multistep microstamping process to generate a bioparticle microarray that stimulates precisely controlled swarming by mimicking an in vivo infection. On the microarray, neutrophils increase in speed and form stable swarms around bioparticle clusters. Significantly, they also demonstrate that secreted cytokines are differentially expressed and "spike" over the course of swarming. This in vitro swarming platform facilitates direct analysis of neutrophil migration and cytokine release in 43 a reproducible, spatially controlled manner. Neutrophil swarming is extremely important in many inflammatory diseases and plays a primary role in controlling larger pathogens such as fungi. This method is an interesting way to precisely control and quantify neutrophil swarming in vitro. This may be used to quantify "swarming" ability as a biomarker for

patient immune function in the future or as a tool to understand neutrophil communication mechanisms that facilitate efficient swarming.

R. We thank the reviewer for the comments that will improve the quality of our manuscript. We have addressed all his/her comments.

Major Concerns:

Q1. Explanation of procedure for preparing the microarray is not well explained. No description of the Zetag solution and how to prepare it.

R1. Step 1.7. and its sub steps were added to explain the preparation of the Zetag (cationic polyelectrolyte, CP) solution as follows:

"1.7. In advance, prepare a 1.6 mg/mL solution of CP in water.

1.7.1. Add the proper amount of the CP powder to water (e.g., 0.8 g to 500 mL).

1.7.2. Mix on a stir plate at room temperature overnight, or until all of the solid is dissolved into the water. The CP solution can be stored at room temperature for 6 months.

1.7.3. If desired, the CP solution can be made fluorescent by adding poly-L-lysine labelled with fluorescein isothiocyanate (PLL-FITC).

1.7.3.1. Aliquot about 10 mL of the CP solution. Add a small amount of PLL-FITC (0.05 mg, the amount can be altered to adjust the brightness of fluorescence as desired) to the aliquoted volume.

1.7.3.2. Vortex the CP solution labeled with FITC for 20 s, or until the solution is a uniform color. The solution should be a pale yellow. Protect from light and store at 4 °C for up to 1 month."

Q2. Figures 1H and 1I are not referenced in the text.

R2. A reference to **Fig. 1M** (**Fig. 1H** in the previous version) was added to step 1.16. as follows: "If the CP is tagged with FITC, the effectiveness of the stamping can be checked at this point with a fluorescent microscope at 488 nm before proceeding to step 1.17 (**Fig. 1M**)." A reference to **Fig. 1N** (**Fig. 1I** in the previous version) was added to step 1.21. as follows: "At this point, the pattern should be checked with a fluorescent microscope at 594 nm before proceeding to step 2.1 (**Fig. 1N**)."

Q3. Did not sufficiently explain bioparticles.

R3. More information regarding the bioparticles was added. In the protocol section, step 1.18. was updated as follows: "1.18. Thaw a solution of bioparticles (e.g. *E. coli* or zymosan) and dilute to 500 µg/mL in water for injection (WFI). Note: The bioparticles do not need to

be opsonized. Neutrophil surface receptors directly recognize molecules on these bioparticles.[19-21]" Additionally, the following text was added to the results section: "When neutrophils are added to the bioparticle microarray, neutrophils that come into contact with the bioparticle clusters become activated and initiate the swarming response," and "Zymosan and *E. coli* bioparticle clusters both result in the generation of neutrophil swarms. For our results, **Fig. 2B** uses data from neutrophil swarms generated by *E. coli* particles. **Fig. 3** and the other panels of **Fig. 2** use neutrophil swarms generated by zymosan particles."

Q4. Did not define quantification parameters (chemotactic index, radial velocity, speed, and total distance traveled).

R4. The results section was updated as follows: "We measured the speed (distance travelled / time) of swarming and non-activated neutrophils" The discussion was updated as follows:, "For example, we have previously shown that this platform can be used to calculate the chemotactic index (CI, the cosine of the angle between the neutrophil velocity vector and the position vector between the neutrophil and the nearest bioparticle cluster), speed (distance neutrophil travels divided by time), radial velocity (speed multiplied by CI), and total distance traveled (difference between initial and final neutrophil position) of individual migrating neutrophils.[5]"

Q5. Data generated by Imaris (step 3.3) not well explained.

R5. Step 3.3 was expanded as follows:

"3.3. Use an automated cell tracking software to track the migration of individual neutrophils toward the bioparticle cluster.

3.3.1. Use the autoregression mode of a spot detection cell tracking software. Set the spot radius to 5 µm (the approximate size of a neutrophil nucleus). Set the minimum track length to 120 s and a maximum gap size of one frame.

3.3.2. From the data generated by the cell tracking software, extract the files that contain the neutrophil position and speed. These files can be used with a graphing software to generate neutrophil migration tracks (**Fig. 2C**) and a heat map of speed vs. time (**Fig. 2D**), respectively."

Minor Concerns:
Q6. Microscopy images and protocol do not mention what magnification to use.

R6. The magnification was added to step 3.2. of the protocol as follows: "Use time-lapse fluorescent and brightfield microscopy to record images at 10x magnification every 10 seconds at 405 nm, 594 nm, and brightfield."

Q7. Videos do not have captions or scale bars.

R7. Scale bars have been added to the videos. Captions have been added to the videos as follows:

"**Video S1. Neutrophil swarming toward zymosan bioparticle clusters.** Neutrophil nuclei are shown in blue. Zymosan targets are marked with red circles. Scale bar: 50 µm. Original acquisition time: 60 min.

Video S2. Non-activated neutrophil random migration. Neutrophil nuclei are shown in blue. Scale bar: 50 µm. Original acquisition time: 60 min.

Q8. Some typos/copy editing issues.

R8. We thank the reviewer for this comment. We have reread the manuscript and made several edits.

Reviewer #5:

Manuscript Summary:

The manuscript describes the steps for manufacturing an assay for neutrophil swarming and the protocols for testing human neutrophil swarming function.

R. We thank the reviewer for the comments that will improve the quality of our manuscript. We have addressed all his/her comments.

Major Concerns:

Q1. A procedure for verifying the accuracy of stamping after step 1.15 should be included in the protocol. Similarly, after step 1.20, a procedure should be described for verifying that the beads are just on the spots and not on the rest of the glass surface.

R1. The following was added to step 1.16: "If the CP is tagged with FITC, the effectiveness of the stamping can be checked at this point with a fluorescent microscope at 488 nm before proceeding to step 1.17 (**Fig. 1M**)." The following was added to step 1.21: "At this point, the pattern should be checked with a fluorescent microscope at 594 nm before proceeding to step 2.1 (**Fig. 1N**)."

Q2. The procedure for Image analysis is overly simplified and all steps should be described in detail. Screen captures should be used to document the steps and their effect on the analysis.

R2. The procedure for image analysis was expanded as follows:

"3.3. Use an automated cell tracking software to track the migration of individual neutrophils toward the bioparticle cluster.

3.3.1. Use the autoregression mode of a spot detection cell tracking software. Set the spot radius to 5 μm (the approximate size of a neutrophil nucleus). Set the minimum track length to 120 s and a maximum gap size of one frame.

3.3.2. From the data generated by the cell tracking software, extract the files that contain the neutrophil position and speed. These files can be used with a graphing software to generate neutrophil migration tracks (**Fig. 2C**) and a heat map of speed vs. time (**Fig. 2D**), respectively.

3.4. Use the 405 nm fluorescent images to track swarm size over time on an image analysis software of your choice.

3.4.1. Define regions of interest (ROIs) around each bioparticle cluster that encompasses the largest swarm size. Keep the same size ROI to analyze each bioparticle cluster.

3.4.2. Analyze the mean fluorescent intensity of the 405-nm images within each ROI over time.

3.4.3. Generate a calibration curve of mean fluorescent intensity to swarm size by taking manual measurements at various swarm sizes from 0 μm^2 to the maximum swarm size. Use this calibration to calculate the swarm size over time."

Q3. The procedure for using the cytokine array panel from Raybiotech is missing from the manuscript. This is important and the steps should be described in detail.

R3. Step 4.6. was expanded in the protocol section as follows:

"4.6. Use a microarray kit that detects a range of human proteins to process samples.

4.6.1. Add 200 μL of each sample to a separate dialysis tube (provided with kit).

4.6.2. Place dialysis tubes in a beaker containing at least 500 mL phosphate buffered saline (PBS, pH = 8.0). Stir gently on a stir plate for at least 3 h at 4 °C. Exchange PBS in beaker and repeat this step.

4.6.3. Transfer each sample to a clean centrifuge tube and centrifuge at 9,000 x g for 5 min to remove any precipitates. Transfer each supernatant to a clean tube.

4.6.4. Biotinylate each sample by adding 36 μL of 1X labeling reagent kit (provided with kit) per mg of total protein in dialyzed sample to 180 μL of dialyzed sample. Incubate at 20 °C for 30 min. Mix gently every 5 min.

4.6.5. Add 3 µL of Stop Solution (provided with kit) into each sample tube. Transfer each sample to a fresh dialysis tube and repeat steps 4.6.2 – 4.6.3. At this stage, the sample can be stored at -20 °C or -80 °C until you are ready to proceed.

4.6.6. Allow glass slide (provided with kit, stored at -20 °C) to come to room temperature. Place assembled glass slide in a laminar flow hood for 1 – 2 h at room temperature.

4.6.7. Add 400 µL of Blocking Buffer (provided with kit) into each well of assembled glass slide. Incubate at room temperature for 30 min.

4.6.8. Centrifuge prepared samples for 5 min at 9,000 x g to remove precipitates or particulates. Dilute 5x with Blocking Buffer.

4.6.9. Remove Blocking Buffer from each well. Add 400 µL of diluted samples into appropriate wells. Incubate for 2 h at room temperature while rocking.

4.6.10. Decant samples from each well. Wash 3x with 800 µL 1X Wash Buffer I (provided with kit) at room temperature for 5 min each while rocking.

4.6.11. In a clean container, submerge assembled glass slide in 1X Wash Buffer I. Wash 2x at room temperature for 5 min each while rocking.

4.6.12. Add 400 µL of 1X Cy3-Conjugated Streptavidin to each sub-array. Cover with plastic adhesive strips. Protect from light for the remainder of the protocol.

4.6.13. Incubate for 2 h at room temperature while rocking.

4.6.14. Decant solution and disassemble glass slide from the sample chambers.

4.6.15. In a 30 mL centrifuge tube (provided with kit), carefully add glass slide and enough 1X Wash Buffer I to cover the glass slide. Wash 3x for 10 min each at room temperature while rocking.

4.6.16. In the 30 mL centrifuge tube, wash 2x with 1X Wash Buffer II for 5 min each at room temperature while rocking.

4.6.17. Wash glass slide with 30 mL ddH_2O for 5 min. Remove the glass slide from the centrifuge tube and allow to dry for 20 min in a laminar flow hood. The prepared glass slide may be stored at -20 °C until ready to scan.

4.6.18. Scan the glass slide with a microarray scanner at a fluorescence emission of 555 nm."

Minor Concerns:

Q4. The manuscript needs careful editing. Several sentences are difficult to understand. On page 2 "neutrophil activity has been attributed to a variety of medical conditions" does not

make sense. The "two-layer process" in the second paragraph is confusing. I suppose a "two-step process" was more appropriate.

R4. We thank the reviewer for this comment. We have reread the manuscript and made several edits. For example, the first sentence in the introduction now reads, "Neutrophils, the most abundant white blood cell in the bloodstream,[1] are gaining attention as potential diagnostic and therapeutic targets[2, 3] because neutrophils may be involved in a variety of medical conditions including gout,[4] sepsis,[3] trauma,[5, 6] cancer,[1, 7, 8] and various autoimmune diseases.[5, 9]" Additionally, the noted sentence in the second paragraph has been changed to, "We generate bioparticle microarrays on glass slides in a two-step process."

References

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