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Peer Review File

Quantifying neutrophil phagocytes by droplet digital cell PCR for diagnosis of bacteremia

Corresponding Author: Professor Naoki Uno

This file contains all editorial decision letters in order by version, followed by all author rebuttals in order by version.

Version 0:

Decision Letter:

Dear Professor Uno,

Thank you for submitting your manuscript, "Quantifying neutrophil phagocytosis by digital cell PCR for diagnosis of bacteremia", to Communications Materials. It has now been seen by 2 referees, whose comments are appended below. In the light of their advice I regret to inform you that we cannot publish your manuscript in Communications Materials.

You will see that, while the reviewers find your work of some interest, they raise substantive concerns that cast doubt on the advance your findings represent over related work and the strength of the novel conclusions that can be drawn at this stage. Unfortunately, these reservations are sufficiently important to preclude publication of this study in Communications Materials.

Although we cannot publish your paper, it may be appropriate for another journal in the Nature Portfolio. If you wish to explore the journals and transfer your manuscript please use our manuscript transfer portal. You will not have to re-supply manuscript metadata and files, unless you wish to make modifications. For more information, please see our manuscript transfer FAQ page.

I am sorry that we cannot be more positive on this occasion and thank you for the opportunity to consider your work.

Best regards,

Rona Chandrawati, PhD
Editorial Board Member
Communications Materials
orcid.org/0000-0002-9780-8844

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

In this manuscript by Uno et al., an approach termed “droplet digital cell PCR” (ddcPCR) to detect neutrophils that have phagocytosed bacteria was developed, aiming to offer a culture-independent diagnostic method for bacteremia. The authors encapsulate single neutrophils in droplets and perform ddPCR, enabling detection of bacterial DNA within individual cells. The method is tested using S. aureus as the target and compared with flow cytometry and blood culture data. The authors report that ddcPCR can detect phagocytosed S. aureus in some culture-negative clinical samples, potentially reflecting antibiotic-suppressed bacteremia.
After careful evaluation of the manuscript, the reviewer cannot recommend it for publication based on the following limitations:
1. The manuscript lacks a comprehensive overview of the current state-of-the-art in bloodstream infection diagnostics. While in situ hybridization (ISH) is mentioned, other more widely adopted molecular approaches, such as nucleic acid amplification tests, are missing. Without a detailed comparison of current methods and their limitations, the novelty and clinical value of the proposed ddcPCR approach are not clearly established.

2. The manuscript focuses on phagocytosis in neutrophils, but does not justify why this is preferable to assessing phagocytosis across a broader population of immune cells (e.g., macrophages or monocytes in PBMCs). Expanding to PBMCs could theoretically increase sensitivity, especially in cases with low neutrophil counts.

3. The authors cite their previous development of in situ PCR-based flow cytometry (reference 18). It is unclear what advantages ddcPCR offers over that method.

4. In determining optimal cell input to maintain single-cell occupancy per droplet (Section on Page 5), the authors rely on empirical testing but do not reference Poisson distribution principles, which are standard in digital assays. Additionally, their empirical determination of 5000 cells per 20 µL reaction appears inconsistent with expected single-cell loading rates based on Poisson statistics. This discrepancy is confusing and undermines confidence in their experimental design.

5. In Fig. 3d, the presence of significant numbers of S. aureus-only positive droplets raises concern about specificity. The authors should investigate and clarify the source of these single-positive signals.
6. The data show that ddcPCR underestimates phagocyte counts compared to flow cytometry, especially at shorter incubation times. This raises concerns about the sensitivity of the approach and suggests that ddcPCR may miss significant phagocytosis events, limiting its clinical utility.

7. Given the thick peptidoglycan layer of S. aureus, which makes it more resistant to heat-induced lysis, the efficiency of intracellular bacterial DNA release and amplification is critical. However, the manuscript lacks data validating that S. aureus DNA is reliably amplified under the ddcPCR conditions. This is a significant methodological gap.

8. On Page 10, the authors claim that only 1 mL of blood is needed for the assay, yet this conclusion is based solely on the detection of human HBB DNA, not on bacterial detection, which is misleading.

Reviewer #2 (Remarks to the Author):

In this study, authors have developed a culture-free molecular diagnostic method called droplet digital PCR (ddPCR) platform to detect bacteremia based on the measurement of neutrophil phagocytosis in blood derived from patients with suspected bacteremia. Overall, study is performed comprehensivly and well characterized. I also appreciate the clinical study done on 254 patients. However, study and manuscript has certain limitations which are decribed below for authors' consideration:

1. The novelty of the ddPCR platform compared to existing flpw cytomtery an dlfourecnece miscropy approaches is unclear. Even in this manuscript and current study, similar manual sample processing techniques were employed inlcuding neutrophils extraction, incubation, permeabalization etc. The superiority of this method vs. flow and microscopy employed as controls here needs to be clearly addressed.
2. In addition, recently several have developed microfluidic devices for on-chip phagocytosis monitoring based on GMR, impednace, magnetic sensing. A comparative analysis of ddPCR with these recent advances can further help make the case.
3. Further, several technolaogies are being developed on detect bacterimia or perform rapid ~ a few hours blood culture both in research and commercial space. A rapid blood culture may out compete the need for rapid phagocytosis monitoring. Discussion on this would be hellpful for the readers.
4. Significance of Fig. 3i is unlcear i.e., phagocytes detected at less than 0.5% by flow cytometry.
5. Scale bars in all microscopy images in main manscript and extended data are missing.
6. Why the 2 hr. of incubation time is used as a standard for most experiments? Neutrophils can ingest bacterial within a few min as also seen in Extended Data Flg. 3.

Version 1:

Decision Letter:

** Please ensure you delete the link to your author homepage in this email if you wish to forward it to your coauthors **

Dear Professor Uno,

Thank you again for submitting your manuscript "Quantifying neutrophil phagocytes by digital cell PCR for diagnosis of bacteremia" to Communications Materials. Please also accept our apologies for the long duration of this round of review, as we had to contact new referees.

We have now received reports from 3 reviewers and, based on their comments, we have decided to invite another minor revision of your work. You will find the reviewers' reports below. While they find your work of interest, they have raised additional important points which must be addressed in a revised manuscript.

To allow us to move forward with your work, we also ask that you edit your manuscript according to the table linked below. **Please read this document carefully as we will be unable to further assess your revised paper until these important points are addressed.** Please outline all revisions made in the right-hand column of the table.

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- A clean version of your revised manuscript with no mark-ups
- A marked-up version of your paper with all changes highlighted in a different colour

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** This url links to your confidential home page and associated information about manuscripts you may have submitted or that your are reviewing for us. If you wish to forward this email to co-authors, please delete the link to your homepage first **

We hope to receive your revised paper within six weeks, but we understand that the revisions may take longer. Please let us know if you find that the revision process will take substantially more time.

We are committed to providing a fair and constructive peer-review process. Please do not hesitate to contact me if you have any questions or would like to discuss these revisions further. We look forward to seeing the revised manuscript and thank you for the opportunity to review your work.

Best regards,

Rona Chandrawati, PhD
Editorial Board Member
Communications Materials
orcid.org/0000-0002-9780-8844

Reviewers' comments:
Reviewer #2 (Remarks to the Author):

Authors have addressed my concerns and I recommend the publication of this article.

Reviewer #3 (Remarks to the Author):

The authors have sufficiently replied to most of the comments of reviewer one. While reading this manuscript a couple of additional remarks came up.

Introduction: the method of cell encapsulation is not new and has been described almost a decade ago, also in the case of intracellular pathogen detection. The authors should sufficiently refer to that literature.

Reviewer one pointed out that the input cell number of 5000 cells does not add up to what would be expected in the effective observed encapsulation of cells. The authors rightfully respond that a rather large fraction gets lost. This point is interesting to discuss, also in the discussion section. Apart from cells not becoming encapsultated, some cells may also be lysed before encapsulation, which may lead to an underestimation of the encapsulated cell equivalents. If that is the case, the mix would also contain 'environmental' DNA.

Concerning the false positives, the universal probe may be binding to e-coli derived DNA that is often used for the generation of PCR mixtures. Could this be a reason for the low levels of false positives?

Concerning the sensitivity of dPCR versus flow cytometry: did the authors take the input volumes into account. If dPCR is performed on 5000 cells, but flow cytometry assesses many more cells, then the increased sensitivity is not methodological in nature, but a discrepancy between different sample sizes. This is an important point to discuss.

A Limit of detection is mentioned. Usually the limit of detection is the level at wich 95% of replicates contain the target. I have the impression that the authors use another definition, i.e. the smallest amount of detectable target. This is not wrong, but the definition of the LOD should be made explicit.

The authors performed in vitro experiments to encapsulate bacteria in neutrophils. Do they know, or can they monitor whether the level of phagocytized bacteria in vitro is similar to the level expected in neutrophils in vivo. This is an important point, as the sensitivity may be overestimated due to a (probably) higher level of bacteria per neutrophil in the experiments.

Finally, I would strongly encourage the authors to read the dMIQE guidelenes and make sure that their reportation of the methods is in line with these guidelines. On that note, ddPCR should be replaced by dPCR (the generic term) and droplets should be termed 'partitions'.

A small discussion on the transferrability of this method to other dPCR systems (droplet or plate based) could be of interest to the readers. On that note, it would be good to provide some references of succesfull cell encapsulation papers with other systems.

Reviewer #4 (Remarks to the Author):

Uno et al describe the development of an innovative diagnostic ddcPCR approach for bacteremia diagnostics. I'm enthousiastic about their approach. The methodology is technically sound and all conserderations and steps are clearly presented in the manuscript. I also believe that the authors have adequately addressed the previous reviewers' comments and suggestions, resulting in a theoretically and technically robust revision.

From the perspective of laboratory professionals who will ultimately be responsible for performing such assays in clinical practice, several points merit further consideration. In Figure 4c, only a single Staphylococcus aureus–positive droplet per patient is observed. In a diagnostic laboratory setting, it is questionable whether this constitutes a sufficiently robust result, as the risk of missing a single positive droplet is a legitimate concern.

Furthermore, while this study focuses primarily on S. aureus, bacteremia involves a broad range of clinically relevant pathogens. With currently available ddPCR platforms, a maximum of six channels can be analyzed simultaneously. It remains unclear whether this level of multiplexing is sufficient to address the complexity of real-world bacteremia diagnostics. Furthermore, AMR detection is relevant as well and has not been discussed. I would like to suggest that the authors take this into consideration for at least the discussion.

Overall, I consider this manuscript a valuable first technical step to new diagnostic approaches for the complex clinical cases of bacteremia. Advantages of this new method are clearly described in the discussion. However, similar to multiplex PCR performed on whole blood, clinical implementation is likely to be highly challenging.

Version 2:

Decision Letter:

Dear Professor Uno,

Thank you again for submitting your revised manuscript "Quantifying neutrophil phagocytes by droplet digital cell PCR for diagnosis of bacteremia" to Communications Materials. We have now received reports from Reviewer 3 and 4 and, based on their comments, we have decided to invite a final revision of your work, to address the remaining comment of Reviewer 3. You will find the reviewers' reports below.

When resubmitting, please also include:

- A point-by-point response to the reviewers' comments. If you are unable to address specific reviewer requests or find any points invalid, please explain

- A clean version of your revised manuscript with no mark-ups
- A marked-up version of your paper with all changes highlighted in a different colour

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** This url links to your confidential home page and associated information about manuscripts you may have submitted or that your are reviewing for us. If you wish to forward this email to co-authors, please delete the link to your homepage first **

We hope to receive your revised paper within six weeks, but we understand that the revisions may take longer. Please let us know if you find that the revision process will take substantially more time.

We are committed to providing a fair and constructive peer-review process. Please do not hesitate to contact me if you have any questions or would like to discuss these revisions further. We look forward to seeing the revised manuscript and thank you for the opportunity to review your work.

Best regards,

Rona Chandrawati, PhD
Editorial Board Member
Communications Materials
orcid.org/0000-0002-9780-8844

Reviewers' comments:
Reviewer #3 (Remarks to the Author):

The authors have sufficiently adressed my comments

The only point that I still wish to make, is that I do not agree that only droplet based systems are compatible with single cell encapsulation. I do agree that droplet based methods are very suitable, but there are different 'chamber based' dPCR methods and depending on the partition generation some of these systems may be compatible with single cell encapsulation. Hence, I would still propose the authors to use the more generic dPCR form as they now dismiss all types of plate-based methods without having tested these.

Reviewer #4 (Remarks to the Author):

After carefully rereading the manuscript, I find that all comments raised by myself and the other reviewers have been satisfactorily addressed.

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Version 3:

Decision Letter:

Dear Professor Uno,

We are delighted to accept your manuscript titled "Quantifying neutrophil phagocytes by droplet digital cell PCR for diagnosis of bacteremia" for publication in Communications Materials. Thank you for choosing to publish your interesting work with us.

Acceptance of your manuscript is conditional on all authors' agreement with our publication policies. In particular, your manuscript must

not be published elsewhere and there must be no announcement of the work in the media until the publication date.

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Providing great service is very important to us. We would greatly appreciate any comments you have about your experience at Communications Materials. We look forward to publishing your paper, and we hope to work with you again in the future.

Best regards,

Rona Chandrawati, PhD
Editorial Board Member
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orcid.org/0000-0002-9780-8844

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Reviewers' comments:

Reviewer #1 (Remarks to the Author): In this manuscript by Uno et al., an approach termed “droplet digital cell PCR” (ddcPCR) to detect neutrophils that have phagocytosed bacteria was developed, aiming to offer a culture-independent diagnostic method for bacteremia. The authors encapsulate single neutrophils in droplets and perform ddPCR, enabling detection of bacterial DNA within individual cells. The method is tested using S. aureus as the target and compared with flow cytometry and blood culture data. The authors report that ddcPCR can detect phagocytosed S. aureus in some culture-negative clinical samples, potentially reflecting antibiotic-suppressed bacteremia. After careful evaluation of the manuscript, the reviewer cannot recommend it for publication based on the following limitations:

1. The manuscript lacks a comprehensive overview of the current state-of-the-art in bloodstream infection diagnostics. While in situ hybridization (ISH) is mentioned, other more widely adopted molecular approaches, such as nucleic acid amplification tests, are missing. Without a detailed comparison of current methods and their limitations, the novelty and clinical value of the proposed ddcPCR approach are not clearly established.

Thank you for your instructive comment. I have added a comprehensive overview of the current state-of-the-art in bloodstream infection diagnostics in introduction of the revised manuscript. I have described clinically available nucleic acid tests using positive blood cultures and culture-free PCR-based technologies. To clarify the novelty of our method, I have explained that current molecular diagnostic strategies are designed to detect bacteria circulating in bloodstream, whereas our method do not detect circulating bacteria but detect bacteria in circulating neutrophils. This approach helps identify causative agents rather than contaminants, which cannot be distinguished from true infection by current molecular approaches, and also allows to understand the extent of phagocytosis induced to prevent host from bacteremia. The latter information, which is an important host response to the infection, cannot be obtained from current molecular strategies but help assess bacteremia because bacteremia develops as a result of imbalances in the complex interplay between the invading microorganism and the host defense mechanisms. The novelty of the ddcPCR method should be clear in introduction of the revised manuscript. The clinical values of ddcPCR are explained by comparison with blood culture in discussion with Supplementary Table 2, because blood culture is the only standard test in clinical practice.

2. The manuscript focuses on phagocytosis in neutrophils, but does not justify why this is preferable to assessing phagocytosis across a broader population of immune cells (e.g., macrophages or monocytes in PBMCs). Expanding to PBMCs could theoretically increase sensitivity, especially in cases with low neutrophil counts.

Thank you for your comment. As written in introduction of the initial manuscript (Line 44), neutrophils are most abundant leukocytes in blood. Although phagocytes include macrophages, macrophages are rarely found in peripheral blood. Therefore, it would be reasonable to use neutrophils to assess phagocytes when using blood samples. We measured the fraction of phagocytes in neutrophils. If PBMCs were used, non-phagocytic leukocytes such as lymphocytes would lead to a significant background and reduce the sensitivity.

3. The authors cite their previous development of in situ PCR-based flow cytometry (reference 18). It is unclear what advantages ddcPCR offers over that method.

Thank you for your constructive comment. The in situ PCR-based flowcytometry technology was not sufficiently quantitative and difficult to detect a rare cell population. We attributed the limitation to the release of PCR products from cells. Since cells are permeabilized to be accessible to PCR reagents, PCR products synthesized intracellularly can move out from the cells and move in other cells. To overcome the limitation, we employed the droplet technology. If we encapsulate single cells in droplets and performed in situ PCR, the PCR products which

are synthesized and fluorescently labelled in a cell can move out from the cell but cannot move out from the droplet because each droplet is separated by oil. In addition, the extracellular PCR products are further amplified and accumulate in the droplet, thereby enhancing signals. We have described the rationale behind the development of the assay in introduction of the revised manuscript (Line 68-73 in the revised manuscript).

4. In determining optimal cell input to maintain single-cell occupancy per droplet (Section on Page 5), the authors rely on empirical testing but do not reference Poisson distribution principles, which are standard in digital assays. Additionally, their empirical determination of 5000 cells per 20 µL reaction appears inconsistent with expected single-cell loading rates based on Poisson statistics. This discrepancy is confusing and undermines confidence in their experimental design.

Thank you for your comment. Yes, I agree that Poisson distribution principles are useful in theory. However, in practice, it isn't because cells that can be analyzed are not able to be estimated by the theory. When I put 5000 cells in a reaction, cells that can be analyzed are approximately 2000 cells (Line 150 in the initial manuscript). This discrepancy is due to the loss of cells during the process from cell counting to the droplet generation. We separate neutrophils, count cells, and take the appropriate number of cells. However, many cells are lost during the subsequent process including fixation, proteinase digestion, and resuspension with PCR reaction mixture. In addition, some cells are not encapsulated in droplets. In consequence, although 5000 cells are used, 2000 cells are encapsulated and analyzed. When the wide range of cell input are tested, the actual number of cells to be analyzed cannot be estimated by the theory and therefore should be tested experimentally. Unlike conventional digital PCR that uses nucleic acids, the input number of cells are inevitably discrepant from those are actually analyzed. Therefore, we determined the optimal cell input experimentally.

5. In Fig. 3d, the presence of significant numbers of S. aureus-only positive droplets raises concern about specificity. The authors should investigate and clarify the source of these single-positive signals.

Fig. 3d is the result of phagocytosis ex vivo. Neutrophils freshly separated from whole blood were mixed and incubated with S. aureus for 2 hours to allow phagocytosis ex vivo. After incubation cells were washed twice with PBS and then fixed. The bacteria that were not eaten by neutrophils were residual even after two washes because relatively many bacteria were used. The residual S. aureus are no longer eaten by neutrophils after fixation and therefore led to S. aureus-single positive droplets. We did not explain the S. aureus-single positive droplets in Fig. 3d, because they do not affect the assessment of phagocytosis. However, we found S. aureus-single positive droplets in several clinical samples. Therefore, we explained their source in the discussion section of the initial manuscript (Line 302-307 in page 11). We separated neutrophils from patients' blood and fixed them in a clean bench but carried out following experiments out the hood. Therefore, S. aureus can be contaminated during the experiments after fixation because S. aureus is normally present on skin. The contaminated S. aureus results in S. aureus-single positive droplets but not double positive droplets, because contaminated S. aureus cannot be eaten by neutrophils after fixation. Therefore, in clinical validation, S. aureus-single positive droplets indicate the contamination of S. aureus after fixation. Please note that S. aureus-single positive droplets do not affect the phagocyte assessment, because the phagocyte fraction is the proportion of double positive droplets out of total HBB-positive droplets.

6. The data show that ddcPCR underestimates phagocyte counts compared to flow cytometry, especially at shorter incubation times. This raises concerns about the sensitivity of the approach and suggests that ddcPCR may miss significant phagocytosis events, limiting its clinical utility.

Yes, the percentage of phagocytes measured by ddcPCR was lower than that measured by flow cytometry when the incubation time was short. We described this in the initial manuscript (Line 195-196). We also stated that

ddcPCR may underestimate the number of phagocytes if they contain a few bacteria (Line 202 in the initial manuscript) and that possibly because ddcPCR did not detect phagocytes that contained few bacteria. (Line 215 in the initial manuscript). Therefore, we had the same concerns about the sensitivity of the approach and thought that ddcPCR could miss phagocytosis events. However, the ddcPCR results using clinical samples were fairly consistent with clinical diagnosis and ddcPCR identified patients who were compatible with bacteremia but tested negative by blood cultures. The results demonstrate its clinical utility. We understand your comment and do not deny your concern. However, the clinical utility of the assay was verified in a clinical setting.

7. Given the thick peptidoglycan layer of S. aureus, which makes it more resistant to heat-induced lysis, the efficiency of intracellular bacterial DNA release and amplification is critical. However, the manuscript lacks data validating that S. aureus DNA is reliably amplified under the ddcPCR conditions. This is a significant methodological gap.

I agree that S. aureus can be difficult to detect owing to the thick peptidoglycan layer. However, Fig. 3 demonstrates that S. aureus DNA is reliably amplified under the ddcPCR conditions. We attempted to detect S. aureus, not only because it is a leading cause of bacteremia but because the assay that can detect S. aureus will detect other bacteria. We determined optimal conditions in which human genes including single-copy gene were amplified efficiently without cell lysis as shown in Fig. 1. We thought S. aureus DNA could be harder to be amplified than human genomic DNA. However, we successfully detected S. aureus DNA without lysing neutrophils as demonstrated in Fig. 3. However, we found that phagocytes containing a few S. aureus were difficult to detect as you pointed out in the previous comment. This could be attributed to the thick cell wall of S. aureus.

8. On Page 10, the authors claim that only 1 mL of blood is needed for the assay, yet this conclusion is based solely on the detection of human HBB DNA, not on bacterial detection, which is misleading.

I'm sorry I made the confusion. This conclusion is based on bacterial detection, because we performed the ddcPCR assay using 1 mL of patient blood as described in line 342 in the initial manuscript and obtained data shown in Fig. 4. The subheading 'ddcPCR can be conducted using only 1 mL of blood' was not appropriate for the subsection. I described in the section whether 1 mL of blood was sufficient for ddcPCR even if there were a few neutrophils in the blood. The number of neutrophils varies with patient. A few patients with bacteremia have neutropenia. I wanted to know whether ddcPCR was feasible even in patients with neutropenia. The ddcPCR assay works as far as HBB signals are detected sufficiently. However, we found no or little HBB signals generated in some patients. We investigated and found that ddcPCR could be performed using 1 mL blood as long as > 1000 leukocytes/μL were present, but might not be feasible when blood was derived from patients with neutropenia associated with hematologic malignancy. We have revised the subheading to avoid misunderstanding.

Reviewer #2 (Remarks to the Author): In this study, authors have developed a culture-free molecular diagnostic method called droplet digital PCR (ddPCR) platform to detect bacteremia based on the measurement of neutrophil phagocytosis in blood derived from patients with suspected bacteremia. Overall, study is performed comprehensivly and well characterized. I also appreciate the clinical study done on 254 patients. However, study and manuscript has certain limitations which are decribed below for authors' consideration:

1. The novelty of the ddPCR platform compared to existing flpw cytomtery an dlfourecnece miscropy approaches is unclear. Even in this manuscript and current study, similar manual sample processing techniques were employed inlcuding neutrophils extraction, incubation, permeabalization etc. The superiority of this method vs. flow and microscopy employed as controls here needs to be clearly addressed.

Thank you for your comment. Firstly, the flow cytometry approach to detection of phagocytes does not exist for clinical use, because bacteria circulating in patient blood are not labelled with fluorescence. I made phagocytosis occur ex vivo by adding fluorescently labelled bacteria to neutrophils and measured phagocytes by flow cytometry in order to assess whether ddcPCR could measure phagocytes quantitatively. Secondly, the culture-free microscopy approach does not exist for clinical use neither, except for Japan. Although bacterial nucleic acids in neutrophils can be detected by FISH without culture, the FISH-based assessment is not fully objective, because it relies on microscopy and depends on the proficiency of the personal as described in Line 54-57 of the initial manuscript. The culture-free FISH-based method is available for diagnostic use in only Japan but rarely used in clinical practice and poorly supported by clinical evidence. Therefore, its clinical utility has never been established. I agree that ddcPCR requires manual handling such as neutrophil separation and fixation but is more objective and less technically demanding than the FISH-based method. Please note that I used flow cytometry and microscopy in order to show that ddcPCR quantified phagocytes. I have described the overview of technologies developed for diagnosis of bacteremia in the introduction section of the revised manuscript.

2. In addition, recently several have developed microfluidic devices for on-chip phagocytosis monitoring based on GMR, impednace, magnetic sensing. A comparative analysis of ddPCR with these recent advances can further help make the case.

Thank you for your comment. Yes, several microfluidic lab-on-a-chip technologies have been developed for the assessment of phagocytosis with GMR sensors. They allow real-time monitoring of magnetic particle uptake but neither quantify phagocytes nor identify bacteria. The objective of ddcPCR is quantification of phagocytes containing target bacteria out of thousands of neutrophils and is not monitoring of the magnetic particle uptake. We have revised the title of the article to clarify that.

3. Further, several technologies are being developed on detect bacterimia or perform rapid ~ a few hours blood culture both in research and commercial space. A rapid blood culture may out compete the need for rapid phagocytosis monitoring. Discussion on this would be hellpful for the readers.

Thank you for your comment. I have added a comprehensive overview of technical advances in molecular diagnostics of bacteremia in the introduction section of the revised manuscript. Molecular technologies detecting pathogen nucleic acids in positive blood cultures are clinically available and certainly faster than the conventional blood culture approach. However, the positive blood culture is typically obtained more than 8 h after incubation. Therefore, their turnaround times are comparable with that of ddcPCR. We have revised the introduction section to clarify the difference between ddcPCR and the blood culture-based approach.

4. Significance of Fig. 3i is unlcear i.e., phagocytes detected at less than 0.5% by flow cytometry.

In patients with bacteremia, phagocytes are a rare population in blood. Therefore, we assessed whether ddcPCR could detect and quantify phagocytes constituting less than 0.5 % of total neutrophils. Fig. 3i shows ddcPCR can detect phagocytes constituting down to 0.05%, suggesting that ddcPCR is clinically useful.

5. Scale bars in all microscopy images in main manscript and extended data are missing.

Thank you for your comment. We have added scale bars in Fig. 2c, Fig. 3b, and Extended Data Fig. 3.

6. Why the 2 hr. of incubation time is used as a standard for most experiments? Neutrophils can ingest bacterial within a few min as also seen in Extended Data FIg. 3.

Yes, neutrophils ingest bacteria within 5 mins as shown by flow cytometry results (Fig. 3e). However, as described in the initial manuscript (Line 192-203), the percentage of phagocytes measured by ddcPCR at 5 mins was lower than that measured by flow cytometry, but increased gradually and reached as same level as flow cytometry after 2 h (Fig. 3e, f). We used 2 h for the quantitative assessment of ddcPCR (Fig. 3h and i), because the quantitative assessment of ddcPCR should be investigated in the condition in which phagocytes are fully detectable by ddcPCR.

Reviewers' comments:

Reviewer #2 (Remarks to the Author):Authors have addressed my concerns and I recommend the publication of this article.

Thank you for reviewing our manuscript. Your comments greatly helped improve the manuscript.

Reviewer #3 (Remarks to the Author):The authors have sufficiently replied to most of the comments of reviewer one. While reading this manuscript a couple of additional remarks came up. Introduction: the method of cell encapsulation is not new and has been described almost a decade ago, also in the case of intracellular pathogen detection. The authors should sufficiently refer to that literature.

Thank you for your valuable comments. Yes, past studies have demonstrated single-cell encapsulation in droplets. We have referred to the literature in the introduction section (Line 86-87 in the re-revised manuscript). Our technology is however distinct from them, because cells are not lysed after single-cell encapsulation. We carried out *in situ* PCR instead. We described briefly the technical novelty and advantages of the lysis-free method in the introduction section (Line 87-98 in the re-revised manuscript). I agree that some studies reported intracellular viral detection following single-cell encapsulation in droplets. However, to the best of my knowledge, no study has achieved intracellular bacterial detection in droplets. Since bacterial cell walls are difficult to lyse particularly in Gram-positive bacteria such as *S. aureus*, enzymatic or chemical lysis is often insufficient to lyse bacterial walls. Therefore, mechanical lysis is generally used to extract bacterial nucleic acids. However, mechanical lysis is not possible when single cells are encapsulated in droplets. Strong enzymatic or chemical lysis may be considered, but harsh lysis buffer can lead to lysis of human cells before they are encapsulated in droplets, which makes single-cell analysis difficult. Thus, intracellular bacterial detection in droplets is a challenge. We addressed the issue by eliminating the cell lysis step using the *in situ* PCR technique that we developed previously[23]. We permeabilized both human cell membrane and bacterial cell walls, performed *in situ* PCR, and detected both human gene and bacterial DNA successfully. Bacterial DNA was detected successfully, presumably because bacterial cell walls had been already broken by phagocytosis to some extent. In other words, bacterial cell walls were damaged physiologically in phagosome, which could help detect bacterial nucleic acids.

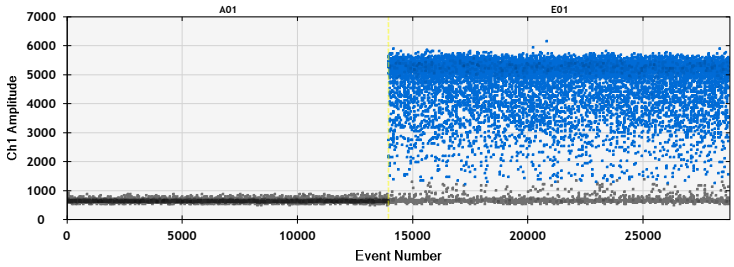
Reviewer one pointed out that the input cell number of 5000 cells does not add up to what would be expected in the effective observed encapsulation of cells. The authors rightfully respond that a rather large fraction gets lost. This point is interesting to discuss, also in the discussion section. Apart from cells not becoming encapsulated, some cells may also be lysed before encapsulation, which may lead to

an underestimation of the encapsulated cell equivalents. If that is the case, the mix would also contain 'environmental' DNA.

Thank you for your important comment. I have written the considerable loss of cells in the discussion section (Line 344-353 in the re-revised manuscript) and added Supplementary Fig. 7. In terms of the possibility of cell lysis, cells are not lysed before encapsulation, because we do not use lysis buffer throughout the experiment. If lysis buffer was used, some cells could be lysed before encapsulation, which could cause a serious issue of single-cell analysis. We addressed the issue by removing the lysis step. We performed *in situ* PCR to amplify both intracellular bacterial DNA and a human gene without cell lysis. Target DNA can be amplified without cell lysis, because bacterial cell walls as well as neutrophil cell membrane are fixed and permeabilized prior to PCR. We worried that thick bacterial cell walls could be difficult to permeabilize, but bacterial DNA was detected readily, partially because bacterial cell walls were lysed in phagosome as I explained previously.

Concerning the false positives, the universal probe may be binding to e-coli derived DNA that is often used for the generation of PCR mixtures. Could this be a reason for the low levels of false positives?

Thank you for your point. Our coauthor also pointed out it. If DNA derived from *E. coli* was present in the reaction, it could be amplified and bind to the Universal probe. However, it was unlikely, because we used another probe that was specific to *E. coli* and observed no background noise in the absence of bacteria as shown below.



E. coli	-	+
FAM-labeled probe	*E. coli*	

Concerning the sensitivity of dPCR versus flow cytometry: did the authors take the input volumes into account. If dPCR is performed on 5000 cells, but flow cytometry assesses many more cells, then the increased sensitivity is not methodological in nature, but a discrepancy between different sample sizes. This is an important point to discuss.

Thank you for your comment. Yes, we took the input cell number into consideration. We used 5000 cells per reaction but performed multiple reactions per sample (8 reactions can be performed simultaneously by Bio-Rad QX100). We obtain approximately 2000 *HBB*-positive droplets when 5000 cells are used in a reaction. Therefore, we prepared 2-5 reactions per sample so that 4,000-10,000 cells could be analyzed per sample. We analyzed 4,000-10,000 cells by flow cytometry in the same day to make assessment fair. We repeated this experiment on 5 different days and obtained results shown in Figure 3i.

A Limit of detection is mentioned. Usually the limit of detection is the level at which 95% of replicates contain the target. I have the impression that the authors use another definition, i.e. the smallest amount of detectable target. This is not wrong, but the definition of the LOD should be made explicit.

Yes, you are right. To avoid the misunderstanding, we have deleted the term limit of detection. Instead, we have simply described that ddcPCR detected male cells comprising 0.05% in the re-revised manuscript.

The authors performed in vitro experiments to encapsulate bacteria in neutrophils. Do they know, or can they monitor whether the level of phagocytized bacteria in vitro is similar to the level expected in neutrophils in vivo. This is an important point, as the sensitivity may be overestimated due to a (probably) higher level of bacteria per neutrophil in the experiments.

No, we don't know whether the level of phagocytosed bacteria *in vitro* is similar to the level expected in neutrophils *in vivo*. I totally agree with you. Bacteria that were ingested in neutrophils *in vivo* could be significantly fewer than those ingested in neutrophils *in vitro*. We found that ddcPCR could miss neutrophils that contained a few bacteria (Figure 3f and g). Therefore, we wrote in the previous manuscript "ddcPCR may underestimate the number of phagocytes if they contain a few bacteria" on line 217 and "possibly because ddcPCR did not detect phagocytes that contained few bacteria" on line 230-231. We have further added a sentence "ddcPCR may underestimate phagocytes when the phagocytes constitute less than 0.5% of total neutrophils" on line 333-334 in the re-revised manuscript. Thus, phagocytes containing a few bacteria are not always detected by ddcPCR. Nevertheless, clinical results were promising. Table 1 shows that one phagocyte out of 3000-7000 bloodstream neutrophils contains bacteria enough to be detected by ddcPCR. Future studies will have to elucidate how many bacteria are phagocytosed by bloodstream neutrophils in patients with bacteremia.

Finally, I would strongly encourage the authors to read the dMIQE guidelines and make sure that their reportation of the methods is in line with these guidelines. On that note, ddPCR should be replaced by dPCR (the generic term) and droplets should be termed 'partitions'.

Thank you for your comment. I have added a dMIQE checklist in Supplementary Information. I understand the terminology defined in dMIQE. However, we would like to maintain the terms, both ddPCR and droplets, because our method is not generalizable beyond droplet-based dPCR platforms as written on line 354-360 in the re-revised manuscript.

A small discussion on the transferability of this method to other dPCR systems (droplet or plate based) could be of interest to the readers. On that note, it would be good to provide some references of successful cell encapsulation papers with other systems.

Thank you for your comment. Your point is of great interest to me too. I actually carried out ddcPCR using the other droplet-based dPCR platform, a naica system (Stilla). It worked but less than satisfactory and therefore requires further research for optimization. I have discussed the potential compatibility with other dPCR platforms in the discussion section and also provided references of single-cell trapping methods based on droplet and microplate technologies (Line 354-360 in the re-revised manuscript).

Reviewer #4 (Remarks to the Author):Uno et al describe the development of an innovative diagnostic ddcPCR approach for bacteremia diagnostics. I'm enthusiastic about their approach. The methodology is technically sound and all considerations and steps are clearly presented in the manuscript. I also believe that the authors have adequately addressed the previous reviewers' comments and suggestions, resulting in a theoretically and technically robust revision.From the perspective of laboratory professionals who will ultimately be responsible for performing such assays in clinical practice, several points merit further consideration. In Figure 4c, only a single Staphylococcus aureus–positive droplet per patient is observed. In a diagnostic laboratory setting, it is questionable whether this constitutes a sufficiently robust result, as the risk of missing a single positive droplet is a legitimate concern.

Thank you for your comment. There might be a risk of missing a single positive droplet if you saw only scatterplots. However, we also see tables that automatically show the number of positive and negative droplets for each color. We always see the table in an Excel file and pay careful attention to the number of double-positive droplets. In addition, if you see the top of a scatterplot shown in Fig. 4c, you see the number of droplets of each category. You can see the number of double positive droplets at Ch1+Ch2+. Thus, we can avoid missing a single positive droplet.

Furthermore, while this study focuses primarily on S. aureus, bacteremia involves a broad range of clinically relevant pathogens. With currently available ddPCR platforms, a maximum of six channels can

be analyzed simultaneously. It remains unclear whether this level of multiplexing is sufficient to address the complexity of real-world bacteremia diagnostics. Furthermore, AMR detection is relevant as well and has not been discussed. I would like to suggest that the authors take this into consideration for at least the discussion.

Thank you for your valuable comment. I think six-plex is not sufficient to address the complexity of real-world bacteremia diagnostics. However, more pathogens can be detected if more reactions are prepared for a sample. For example, 12-plex is possible, if two reactions are prepared for one sample. We isolated 400,000 neutrophils from 1 mL of blood in most cases and used 5,000 neutrophils per reaction. Therefore, you can prepare multiple reactions per sample to detect multiple pathogens. AMR detection is indeed important. I have added it in the discussion section (Line 374-377 in the re-revised manuscript).

Overall, I consider this manuscript a valuable first technical step to new diagnostic approaches for the complex clinical cases of bacteremia. Advantages of this new method are clearly described in the discussion. However, similar to multiplex PCR performed on whole blood, clinical implementation is likely to be highly challenging.

Thank you for your comment. Yes, I totally agree with you. Clinical implementation is a challenge. I will continue to improve ddcPCR technology to make it implementable in clinical practice.

Reviewers' comments:
Reviewer #3 (Remarks to the Author):

The authors have sufficiently adressed my comments

The only point that I still wish to make, is that I do not agree that only droplet based systems are compatible with single cell encapsulation. I do agree that droplet based methods are very suitable, but there are different 'chamber based' dPCR methods and depending on the partition generation some of these systems may be compatible with single cell encapsulation. Hence, I would still propose the authors to use the more generic dPCR form as they now dismiss all types of plate-based methods without having tested these.

Thank you for your coment. We have replaced the term ‘ddPCR’ by ‘dPCR’ as you proposed. We agree that some of dPCR systems may be compatible with single cell encapsulation. We have revised the text accordingly (Line 357-358 in the revised manuscript).
Once again, thank you for reviewing our manuscript thoroughly and giving constructive comments. Your comments helped improve our manuscript substantially.

Reviewer #4 (Remarks to the Author):

After carefully rereading the manuscript, I find that all comments raised by myself and the other reviewers have been satisfactorily addressed.

Thank you for reviewing our manuscript in detail. We are grateful to you for all your feedback.