

Bacterial single-cell RNA sequencing captures biofilm transcriptional heterogeneity and differential responses to immune pressure

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This manuscript has been previously reviewed at another journal. This document only contains information relating to versions considered at Nature Communications.

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #2

(Remarks to the Author)

The authors have demonstrated a commendable level of diligence in addressing the reviewers' comments. Upon transfer of the manuscript to Nature Communications, the editors decided that additional functional assays or validation experiments are not required; therefore, the authors have focused on improving the technical description of the BaSSSh-seq protocol. The revision clarifies the distinctive features of their method compared to previously published single-cell RNA sequencing techniques in bacteria and describes the implemented optimizations and improvements in a transparent manner. This addresses our overall concerns, but we suggest a few additional clarifications that would strengthen the impact of the manuscript (detailed below):

Remaining comments:

1. The authors state in their response to reviewers:

"We agree that more information was needed to highlight how BaSSSh-seq differs from these existing methods. We initially attempted both microSPLiT and PETRI-seq protocols for our studies; however, neither were successful for biofilm."

We strongly encourage the authors to include evidence of these unsuccessful attempts with the microSPLiT and PETRI-seq protocols. This addition would enhance the perceived value of the BaSSSh-seq method, clearly demonstrating its advantages over existing techniques.

2. There is an inaccuracy in Extended Figure 1. The MATQ-seq protocol does not omit rRNA depletion; it employs a Cas9-based approach (DASH). Please correct this to ensure accuracy in the comparison of methods.

3. The authors have provided more details on gene detection, stating on p.6 "BaSSSh-seq captured an average of 12-60 mRNA reads per cell across all samples (covering a range of 7 to >2,000 reads per cell, and a range of 1 to >1,000 detected genes)". Nevertheless, we request the following changes:

a. Instead of using a range for average mRNA reads per cell, please provide a single, precise average number.

b. For the number of genes detected, the range of 1 to 1,000 is too broad to be informative. Please include specific average numbers of genes detected for both biofilm and planktonic growth. For example: "An average of X genes per cell were detected in biofilm samples, while Y genes per cell were detected in planktonic samples."
The data should also be included as a violin plot in Extended Figure 9.

4. In Extended Data Figure 8A-C, please clarify what normalized counts refers to. Are these counts per gene, per transcript, ...?

5. In the methods section the authors mention that "Cells were counted with a hemocytometer"

We recommend expanding this description to include:

- a. The specific type of hemocytometer used.
- b. Whether cell staining was required for accurate counting.
- c. Any other relevant details of the cell counting procedure, or a suitable reference will suffice.

These additions will enhance the reproducibility of the method and the transparency of the experimental procedures.

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We thank the reviewer for their remaining comments, which are addressed on a point-by-point basis below. All changes in the revised manuscript are indicated by yellow highlighting.

Reviewer #2

Remaining comments:

1. The authors state in their response to reviewers:

“We agree that more information was needed to highlight how BaSSSh-seq differs from these existing methods. We initially attempted both microSPLiT and PETRI-seq protocols for our studies; however, neither were successful for biofilm.”

We strongly encourage the authors to include evidence of these unsuccessful attempts with the microSPLiT and PETRI-seq protocols. This addition would enhance the perceived value of the BaSSSh-seq method, clearly demonstrating its advantages over existing techniques.

We agree with the reviewer that including this information is important to support the utility of our approach compared to existing protocols and for transparency. We have added details to the second paragraph in the Results section “BaSSSh-seq enables bacterial scRNA-seq of biofilm and incorporates rRNA depletion” to more clearly describe our failed attempts at these other two protocols. First, we note how the microSPLiT method used template switching for generating double-stranded cDNA, and that we obtained erroneous concatamerization during our attempts with template switching (as illustrated in Supplementary Figure 3). Next, we discuss how the PETRI-seq method used second strand synthesis without subsequent amplification, and that we were unable to generate any measurable, quality cDNA libraries while attempting this approach. Each of the respective methods is cited in the text where we describe these failed efforts. Since we could not proceed with the respective protocols with double-stranded cDNA, there is no downstream evidence to show.

2. There is an inaccuracy in Extended Figure 1. The MATQ-seq protocol does not omit rRNA depletion; it employs a Cas9-based approach (DASH). Please correct this to ensure accuracy in the comparison of methods.

We apologize for this oversight, which has been corrected in the noted figure (now Supplementary Figure 1).

3. The authors have provided more details on gene detection, stating on p.6 “BaSSSh-seq captured an average of 12-60 mRNA reads per cell across all samples (covering a range of 7 to >2,000 reads per cell, and a range of 1 to >1,000 detected genes)”. Nevertheless, we request the following changes:

a. Instead of using a range for average mRNA reads per cell, please provide a single, precise average number.

A range was reported because there is a notable difference between mRNA reads per cell under biofilm and planktonic growth conditions (as shown in Supplementary Figures 9 and 12). However, we have now adjusted our description of mRNA reads per cell in the revised manuscript to include a single average number separate for biofilm and planktonic growth.

b. For the number of genes detected, the range of 1 to 1,000 is too broad to be informative. Please include specific average numbers of genes detected for both biofilm and planktonic growth. For example: “An average of X genes per cell were detected in biofilm samples, while Y genes per

cell were detected in planktonic samples." The data should also be included as a violin plot in Extended Figure 9.

As we describe in both the Discussion and Methods sections, there is an inherent noise level resulting from a combination of factors related to the low metabolic activity of cells within biofilm including random concentrations used, fragmentation conditions, and alignment parameters. This noise artificially inflates the total gene counts when calculating the number of detected genes. We do not wish to report a value that is falsely high and could potentially mislead readers. Therefore, we have elected to maintain the broader range of detected genes, and as we note, future efforts will look to address the noise in the methodology.

4. In Extended Data Figure 8A-C, please clarify what normalized counts refers to. Are these counts per gene, per transcript, ...?

Normalized counts refer to counts per gene. We have included this clarification in the legend of what is now Supplementary Figure 8 when describing panels A-C.

5. In the methods section the authors mention that "Cells were counted with a hemocytometer" We recommend expanding this description to include:

a. The specific type of hemocytometer used.

We have provided additional details in the Methods section "Cell fixation, permeabilization, and counting". We specify that a Reichert Bright-Line, Hausser Scientific, #1492 hemocytometer was used.

b. Whether cell staining was required for accurate counting.

We specify that cells were diluted in 0.2 μm -filtered trypan blue. This was merely for contrast to aid in visualizing the cells but is not necessary.

c. Any other relevant details of the cell counting procedure, or a suitable reference will suffice.

We also added that cells were allowed to settle for 10 min following loading onto the hemocytometer before visualization under 40X magnification for counting.