**Editorial comments:**

Changes to be made by the author(s) regarding the manuscript:  
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

*Done as requested.*

2. Please revise step 3.1.2 to avoid previously published text.

*Done as requested.*

3. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

*Done as requested.*

4. Please revise the protocol to contain only action items that direct the reader to do something (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible.

*Done as requested.*

5. Lines 118-130: The Protocol should contain only action items that direct the reader to do something. Please move the overview to the Introduction or Discussion.

*Done as requested.*

6. 2.1: Please describe how to digest the pRB4 vector. Alternatively, add references to published material specifying how to perform the protocol action.

*Done as requested – these are actions using commercial reagents; thus, we state that we performed the action as per the manufacturer’s instructions.*

7. 2.2: Please ensure that conditions and primers are listed all PCR procedures.

*Done as requested.*

8. 2.3: Please describe how to digest the PCR product and ligate the digested DNA fragment.

*Done as requested; see point 6 above.*

9. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Please note that some of the shorter Protocol steps can be combined so that individual steps contain 2-3 actions and maximum of 4 sentences per step.

*Done as requested.*

10. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense. Please do not highlight any steps describing anesthetization and euthanasia.

*Done as requested.*

11. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

*Done as requested.*

12. Figure 1: The figure has “CAT” while figure legend has “cat”. Please be consistent.

*Done as requested.*

13. Discussion: Please discuss critical steps within the protocol.

*Done as requested.*

14. For in-text references, the corresponding reference numbers should appear as superscripts after the appropriate statement(s) in the text (before punctuation but after closed parenthesis). The references should be numbered in order of appearance.

*Done as requested.*

15. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al. See the example below:  
Bedford, C.D., Harris, R.N., Howd, R.A., Goff, D.A., Koolpe, G.A. Quaternary salts of 2-[(hydroxyimino)methyl]imidazole. Journal of Medicinal Chemistry. 32 (2), 493-503 (1998).

16. References: Please do not abbreviate journal titles.  
*Done as requested.*

**Reviewers' comments:**  
  
  
  
Reviewer #1:  
  
Manuscript Summary:  
Behera et al. describe a novel fluorescence-based method to analyze gene regulation in bacterial pathogens in situ. The authors use the well-established Staphylococcus aureus renal abscess model along with a new reporter plasmid system to show expression of staphylococcal nuclease (nuc) in infected tissues. This method is simple, effective and applicable to other bacteria. The technique is reasonably well described. Below are some recommendations which the authors may find helpful.  
*We thank the reviewer for their helpful comments and suggestions.*

Major Concerns:  
  
Comments as they arise in the manuscript:  
Line 93: change leucocidans to leukocidins

*Done as requested. (Line 86)*

Line 121: The authors used USA300HOU\_0087 to facilitate homologous recombination of the integrative plasmid pRB4. What was the rationale for using this particular gene? Are the authors unconcerned about disruption of gene 0087? Did the author verify that this is indeed a pseudogene?

*The reviewer brings up a good point. The gene USA300HOU\_0087 is annotated as a pseudogene in the genome reference, and we indeed find multiple premature stop codons that are predicted to result in a truncated protein after ~270 amino acid residues (the gene codes for a polypeptide ~600 amino acids in length). Moreover, when constructs were integrated into the locus (and inactivating the putative gene) we did not observe a detectable in vitro growth defect in rich medium nor a virulence phenotype in vivo relative to WT. Therefore, we consider this a useful site in which to integrate our construct.*

Line 143-149: The authors should explain why the plasmid is transformed into strain RN4220 before transformation into USA300. Technical details for electroporation should be provided rather than citing references 38-39, especially if the electroporation procedure differs between the two strains. The names of phages that can be used for efficient transduction of the plasmid in strain USA300 should be indicated. The amount of antibiotics should be indicated for each strain. Strain USA300 is intrinsically resistant to erythromycin. The authors should explain how they are able to select for plasmid-encoded erythromycin-resistance.

*Done as requested.*

*The USA300 strain used in the study has been cured of the native plasmid conferring Emr and is susceptible to 5 g ml-1 of erythromycin. This is now stated in the manuscript (Lines 153-155).*

Line 153: The authors should describe how "back cross" is performed. They should also explain the meaning of "back cross".

*We have replaced “back cross” with “re-introduce” and have indicated why (Lines 159-161). We apologize for the confusion.*

Line 174: Is the use of a water bath absolutely necessary (this wasn't specified above)?

*No, this is not necessary. However, we found it is important to remain consistent with our cultivation conditions. We have added a note in step 2.1.3. (Now Lines 181-183)*

Line 176: Steps 3.1.2 and 3.1.4 describes how the bacterial culture is "refreshed" twice in fresh medium. What is the rationale for the second dilution of the culture?

*This ensures exponential phase and steady state growth. Fluorescent proteins are stable and must be diluted over several generations to get an accurate measurement of promoter activity during early growth. The step has been updated to make this point. (Lines 185-187)*

Lines 179, 182: Specify temperature for centrifugation.

*Done as requested. (Line 189)*

Line 197: Please provide the age of animals.

*Done as requested. (Line 204)*

Line 205: What is an "authorized animal welfare rubric"? Are the authors referring to a monitoring system reviewed and approved by veterinarian and IACUC?

*Yes, we have clarified this statement (Line 212-213). We apologize for any confusion.*

Lines 202 and 207-209: both low and high dose inocula lead to systemic infections whereby bacteria disseminate via the vasculature to organ tissues. At lower dose, S. aureus will form abscesses in organ tissues and will persist in these lesions. The bacteria are not found in the bloodstream. At the higher dose, animal succumb presumably of bacteremia (i.e. bacteria in the bloodstream). The authors should provide some references for these invasive disease models.

*The reviewer is indeed correct that relatively low doses lead to abscess formation in tissues, whereas relatively high doses lead to acute infection, bacteremia, and death. We used 1x107 CFU as the inoculum for systemic infection because it has been shown previously to lead to abscess formation. Our desire was to set up a host-pathogen interaction in the tissues to examine bacterial gene expression; thus, we sacrificed mice after 3-4 days post infection. Under these conditions, abscesses consist of a staphylococcal abscess community of bacteria, enclosed by fibrin deposits, and surrounded by concentric layers of immune cells (Thomer et al., 2016). We have now reference the primary literature revealing the kinetics of abscess formation as well as the Thomer* et al. *review article. (Lines 217-218)*

Line 341: The authors state that the reporter fusions were verified in in vitro assays but do not show the data. It would seem important to show such data so that other may use the data as a reference point.

*We completely agree with the reviewer. We now add new data to the manuscript revealing promoter activity during in vitro growth (Figure 2).*

Figure 2: Panels D and F (unlike panels C and E) lack the vector control for the sarA plasmid construct.  
*(Now Figure 3). We have removed the vector control data for GFP to make the figure and data symmetrical. We hope that this will be satisfactory.*

Minor Concerns:  
None  
  
  
  
  
Reviewer #2:  
  
The author reported a fused fluorescent protein method to image the heterogeneous expression of POI in infected tissues. To facilitate the repeatability of this manuscript, some issues should be addressed.

*We thank the reviewer for their valuable comments and suggestions.*

1. The source (brand, catalog number) or the sequence of the vector, POI and fluorescent protein should be provided.

*Done as requested.*

2. Electroporation and phage mediated transduction should be more detailed.

*Done as requested.*

3. How to confirm the hemolytic phenotype.

*Now described in the text (Lines 171-172)*

4. Source of pre-cleaned, charged glass slide should be provide.

*Provided as requested in ToM.*

5. In figure legends, specify the excitation, emission filter and objective used in figure 2, 3 and 4.

*Done as requested in the figures.*

6. Figure 4. Specify the roi selected for identify the periphery and core in the typical images.

*Now Figure 5. We indicate core and periphery using asterisks and arrows, respectively.*

7. ToM section. Specify the model of some instrument such as plate reader, confocal microscope.  
*Done as requested.*

Reviewer #3:  
  
Manuscript Summary:  
Overall, the manuscript provides detailed instructions for a protocol that is useful for the scientific community. Overall, the data matches the expected outcome of the procedure. Only a couple of concerns are listed.  
*We thank the reviewer for their careful reading of the manuscript and their suggestions.*

Major Concerns:  
1. The choice of tdT seems poor as ~50% of the signal is below the limit of detection, based on the top of the LAC data.

*We were in fact surprised that the PsarA-tdTomato fusion showed such variability in vivo, as the in vitro data lack this variability. Moreover, the promoter used in this construct is believed to be constitutive. At this time, we don’t know the nature of the variability we observed, and we believe that this is beyond the scope of this manuscript. It is indeed an interesting question for follow-up.*

2. If tdT is under the sarA promoter, and that promoter is constitutively active (to show where all cells are), then how can there be regions expressing just green and not red (Figure 3). The opposite could be possible.  
*The reviewer is quite right to point this out, and we wondered the same thing. As we mention above, we don’t yet understand the basis for the differences we see. Nevertheless, while surprising, we thought it was notable and should be communicated.*

Minor Concerns:

Line 93. spelling of leukocidins is wrong

*Corrected.*

Line 99: reword "directs the expression" this sounds like a regulatory phenomenon, not that nuc is translated into Nuc

*We absolutely agree and have rephrased the statement. (Line 92)*

Line 197: unless otherwise suggested by Jove, most guidelines prefer the term "sex" over "gender"

*Done as requested. (Line 204)*

Line 256: needs indenting

*Done as requested.*

Regarding pRB4. There is no real reason to have the blue/white screen along with an antibiotic selection. Regardless, the bgaB is not on the map nor in the figure legend. Is it really part of the plasmid?  
*We found it was useful to use the blue/white screening during the course of strain construction. Thus, we kept the* bgaB gene on pRB4. Figure 1 and its legend has been updated to reflect this. We apologize for any confusion.