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A fluorescence-based method to study bacterial gene regulation in infected tissues --Manuscript Draft--

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Corresponding Author:	Shaun Brinsmade Georgetown University Washington, DC UNITED STATES
Corresponding Author's Institution:	Georgetown University
Corresponding Author E-Mail:	Shaun.Brinsmade@georgetown.edu
Order of Authors:	Ranjan Behera Kevin Mlynek Matthew Linz Shaun Brinsmade
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Department of Biology

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Dear Dr. Steindel,

Thank you and the reviewers for the generally positive response to our manuscript, JOVE59055, entitled “A fluorescence-based method to study bacterial gene regulation in infected tissues” by R.K. Behera, K.D. Mlynek, M.S. Linz, and S.R. Brinsmade, and for the useful comments.

Our responses to your comments and those of the reviewers are attached. We addressed all concerns and followed suggestions for modifying the text to improve the overall clarity of the manuscript as well as to better align the manuscript with JoVE’s mission to advance scientific research and education through visual learning solutions.

Once again, thank you very much for the opportunity to contribute to *Journal of Visualized Experiments*. We hope that with these changes the manuscript can now be considered for publication.

Sincerely yours,

A handwritten signature in black ink, reading "Shaun R. Brinsmade".

Shaun R. Brinsmade, Ph.D.
Assistant Professor
Tel. (202) 687-6549

406 Reiss Science Building
37th and O Streets, NW
Washington, DC 20057-1229
202-687-6247

TITLE:**A Fluorescence-based Method to Study Bacterial Gene Regulation in Infected Tissues.****AUTHORS AND AFFILIATIONS:**Ranjan K. Behera¹, Kevin D. Mlynek¹, Matthew S. Linz¹, Shaun R. Brinsmade¹¹Department of Biology, Georgetown University, Washington DC**CORRESPONDING AUTHOR:**

Shaun R. Brinsmade, Ph.D. (sb1344@georgetown.edu)

Tel: (202) 687-6549

EMAIL ADDRESSES OF CO-AUTHORS:

Ranjan K. Behera (rkb44@georgetown.edu)

Kevin D. Mlynek (kdm60@georgetown.edu)

Matthew S. Linz (msl110@georgetown.edu)

KEYWORDS:

Gene expression, virulence, fluorescence, confocal microscopy, histopathology, bacteria, pathogen, spatial regulation, Sae Two Component System, nuclease, kidney, abscess

SUMMARY:

Described here is a method for analyzing bacterial gene expression in animal tissues at a cellular level. This method provides a resource for studying the phenotypic diversity occurring within a bacterial population in response to the tissue environment during an infection.

ABSTRACT:

Bacterial virulence genes are often regulated at the transcriptional level by multiple factors that respond to different environmental signals. Some factors act directly on virulence genes; others control pathogenesis by adjusting the expression of downstream regulators or the accumulation of signals that affect regulator activity. While regulation has been studied extensively during *in vitro* growth, relatively little is known about how gene expression is adjusted during infection. Such information is important when a particular gene product is a candidate for therapeutic intervention. Transcriptional approaches like quantitative, real-time RT-PCR and RNA-Seq are powerful ways to examine gene expression on a global level but suffer from many technical challenges including low abundance of bacterial RNA compared to host RNA, and sample degradation by RNases. Evaluating regulation using fluorescent reporters is relatively easy and can be multiplexed with fluorescent proteins with unique spectral properties. The method allows for single-cell, spatiotemporal analysis of gene expression in tissues that exhibit complex three-dimensional architecture and physiochemical gradients that affect bacterial regulatory networks. Such information is lost when data are averaged over the bulk population. Herein, we describe a method for quantifying gene expression in bacterial pathogens *in situ*. The method is based on simple tissue processing and direct observation of fluorescence from reporter proteins. We demonstrate the utility of this system by examining the expression of *Staphylococcus aureus*

thermonuclease (*nuc*), whose gene product is required for immune evasion and full virulence *ex vivo* and *in vivo*. We show that *nuc-gfp* is strongly expressed in renal abscesses and reveal heterogeneous gene expression due in part to apparent spatial regulation of *nuc* promoter activity in abscesses fully engaged with the immune response. The method can be applied to any bacterium with a manipulatable genetic system and any infection model, providing valuable information for preclinical studies and drug development.

INTRODUCTION:

Bacteria respond to changing physiological conditions and alterations in the nutritional state of their environment by differentially expressing genes required for adaptation and survival. For instance, opportunistic pathogens colonize body surfaces at relatively low densities, and are often harmless. However, once the bacterium has penetrated physical and chemical barriers, it must contend with host immune cell counter-defenses and restricted nutrient availability¹. As an example, *Staphylococcus aureus* colonizes approximately one third of the population asymptomatically but is also the cause of devastating skin and soft tissue infections, osteomyelitis, endocarditis, and bacteremia². The success of *S. aureus* as a pathogen is often attributed to its metabolic flexibility as well as an arsenal of surface-associated and secreted virulence factors that enable the bacterium to escape the bloodstream and replicate in peripheral tissues³⁻⁵. Because host death due to staphylococcal disease is an evolutionary dead end and limits transmission to new hosts⁶, the commitment to virulence factor production must be carefully controlled.

A complex regulatory network of proteins and non-coding RNAs responds to a variety of environmental stimuli, including cell density, growth phase, neutrophil-associated factors, and nutrient availability, to ensure that virulence genes are expressed at the precise time and location within host tissues^{7,8-13}. For instance, the SaeR/S two component system (TCS) regulates expression of several virulence factors via the sensor kinase (SaeS) and the response regulator (SaeR)¹⁴. SaeS is autophosphorylated on a conserved histidine residue in response to host signals (*e.g.*, human neutrophil peptides [HNPs], calprotectin)^{8,15,16}. The phosphoryl group is then transferred to an aspartate residue on SaeR, activating it as a DNA-binding protein (SaeR~P)¹⁷. The SaeR/S TCS regulates over 20 genes that contribute to pathogenesis including fibronectin binding proteins (FnBPs), leukocidins, and coagulase^{14,18-20}. Targets can be classified into high-affinity and low-affinity gene targets, which are likely induced as the level of SaeR~P rises when exposed to its cues²¹. The SaeR/S activity is controlled by other regulators of gene expression such as the Agr quorum sensing system, repressor of toxins protein (Rot), and the alternative sigma factor B (SigB)²²⁻²⁴.

nuc is an Sae-dependent virulence gene in *Staphylococcus aureus* and encodes thermonuclease (Nuc), which is essential for escaping from neutrophilic extracellular traps (NETs) and for dissemination during the course of infection^{25,26}. The expression of *nuc* is also strongly indirectly repressed by CodY in the presence of branched-chain amino acids and GTP²⁷, and directly repressed by the staphylococcal accessory regulator protein SarA^{28,29}, whose activity is influenced by oxygen (redox state) and pH³⁰. Given that *sae* and *nuc* mutants are attenuated in mouse models of infection, there is interest in developing chemical interventions that inhibit

their corresponding activities^{26,31}. Despite this, there is no information regarding their regulation during infection.

Fluorescent reporters have been used to monitor and quantify gene expression on the single cell level. Herein, we present a method for quantifying *S. aureus* gene expression during infection that, when paired with *in vitro* transcriptome analysis and powerful imaging techniques like magnetic resonance imaging (MRI) and magnetic resonance spectroscopy (MRS), can reveal how bacterial physiology is regulated *in vivo* and the relative abundances of nutrients in certain niches. The method can be applied to any bacterial pathogen with a tractable genetic system.

Overview of the genome integrative vector.

The genome integrative vector pRB4 contains 500 base pairs each from the upstream and downstream regions of the *S. aureus* USA300 SAUSA300_0087 pseudogene to facilitate homologous recombination. pRB4 is derived from the temperature-sensitive pMAD vector backbone containing the erythromycin resistance cassette (*ermC*) and thermostable beta-galactosidase gene *bgaB* for blue/white screening of recombinants³². The engineered reporter construct also contains a chloramphenicol resistance marker (*cat*) for selection after genome integration and plasmid elimination, as well as EcoRI and SmaI sites to fuse the regulatory region of interest to superfolder green fluorescent protein (sGFP) (**Figure 1**). It is known that the choice of ribosome binding site (RBS) influences the activity of the reporter, and often requires empirical optimization³³. Thus, an RBS is not supplied. Here, the native ribosome binding site is used to provide for a more natural pattern of gene expression, but other sites may be used.

PROTOCOL:

All methods described here have been approved by the Institutional Animal Care and Use Committee (IACUC) of Georgetown University.

1. Generation of the Fluorescent Reporter Strain

1.1. Digest the genome integrative pRB4 vector sequentially with EcoRI and SmaI restriction enzymes. Following the manufacturer's protocol, set up an overnight digestion with SmaI using 1 µg of pRB4 at 25 °C, and then proceed with the second digestion for 1h at 37 °C by adding EcoRI to the reaction mixture. Inactivate the enzymes by incubating at 65 °C for 20 min.

1.2. PCR amplify the regulatory region of interest from genomic DNA (in this case, a ~350 base pair DNA fragment containing the thermonuclease [*nuc*] promoter region), incorporating a 5' EcoRI restriction site and a 3' SmaI restriction site. The SmaI recognition sequence must be in-frame with the translational start codon. In this study, the PCR was performed using a high-fidelity DNA Polymerase according to the manufacturer's recommendations with forward primer oRB015 (5'—ATCATTGAATTCTCCAAAGTAAATTATAAGTTATAC—3') and reverse primer oRB016 (5'—GGGCATAACTAACACCTCTTTCTTTTATAG—3'). The annealing temperature was 53 °C. Purify the resulting fragment using a PCR clean-up kit following the manufacturer's instructions.

1.3. Digest the resulting PCR product with EcoRI and SmaI as performed in step 1.1 and ligate the digested DNA fragment to the same sites of pRB4 using T4 DNA ligase as recommended by the manufacturer to generate the integrative construct. Introduce the ligation mixture into *E. coli* and confirm construct sequence.

1.4. Electroporate the construct into *S. aureus* strain RN4220 as previously described³⁴. In brief, use 1 µg of plasmid with 70 µL of electro-competent cells (100 Ω, 25 µF, and 2.3 kV) in B2 broth (1% [w/v] Casein hydrolysate, 2.5% [w/v] Yeast extract, 2.5% [w/v] NaCl, 0.1% [w/v] K₂PO₄, 0.5% [w/v] Glucose). Select for erythromycin resistance (Em^r) at the permissive temperature (30 °C) on tryptic soy agar (TSA) supplemented with erythromycin (5 µg mL⁻¹) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; 80 µg mL⁻¹). The resulting transformants are blue due to plasmid-encoded β-galactosidase activity.

NOTE: Transfer of DNA into clinical isolates is extremely difficult due to a strong restriction-modification barrier³⁵. Thus, *S. aureus* RN4220 was used as an intermediate recipient of the fusion construct because it is restriction deficient and highly transformable.

1.5. Transfer plasmid to *S. aureus* USA300 strain of interest using electroporation or phage-mediated transduction³⁶ at the permissive temperature. In brief, propagate Φ₁₁ bacteriophage particles on the donor strain using TSA plates containing 5 mM CaCl₂ overnight at 30°C, and then sterilize by filtration through a 0.45 µm cellulose acetate syringe filter. Use lysates to transduce *S. aureus* strain LAC to Em^r.

NOTE: LAC is a widely used clinical isolate that was previously made Em sensitive by serial passage in TSB medium to cure the strain of the native plasmid pUSA03 that confers Em^r^{37,38}.

1.6. Integrate the construct by two, successive homologous recombination events into the chromosome as described previously³². The recombinants are chloramphenicol-resistant (Cm^r) on TSA plates containing 5 µg mL⁻¹ of chloramphenicol, erythromycin-susceptible (Erm^s), and no longer blue.

NOTE: When possible, re-introduce the marked fusion into USA300 via phage-mediated transduction to avoid the accumulation of mutations associated with *in vitro* strain passage³⁹ and temperature shifts.

2. Animal Infection: Preparation of the Inoculum, Systemic Infection, and Tissue Processing

2.1. Preparation of bacterial inoculum

2.1.1. Streak *S. aureus* strains of interest for isolation on blood agar plates from a frozen glycerol stock. Incubate at 37 °C for 16-24 h to confirm hemolytic phenotypes based on their ability to lyse red blood cells (RBCs) and form clear transparent zones.

2.1.2. Initiate overnight cultures by inoculating single colonies of each strain to 4 mL of tryptic soy broth (TSB) in sterile glass tubes. Rotate the tubes at 37 °C for 16-24 h in a tube roller set at approximately a 70-degree angle and 70 rotations per minute [RPM].

2.1.3. Use a spectrophotometer to measure the optical density at 600 nm (OD_{600}) of the cultures from step 2.1.2. Use sterile medium as an optical reference (blank). Dilute these cells to an OD_{600} of 0.05 in 25 mL of sterile Tryptic Soy Broth (TSB) in separate, 125 mL DeLong flasks (5:1 flask to volume ratio) and incubate cultures in a water bath (for proper heat transfer to the cultures), shaking at 280 RPM and set to 37 °C.

NOTE: Growth of the inoculum can be performed in various ways; one method for growing cells to exponential phase is presented. Regardless, it is important to consistently use the same method for preparing the cells for inoculation.

2.1.4. At an OD_{600} of ~ 1 , re-dilute the cells into fresh medium to a starting OD_{600} of 0.05 to ensure the cells achieve exponential phase and that factors that accumulate during overnight incubation have been reduced to exponential phase levels.

2.1.5. Harvest the cells during exponential phase ($OD_{600} \sim 0.6-0.8$) by centrifuging at $3000 \times g$ for 10 min at room temperature.

2.1.6. Wash the pellets twice in equivalent volume of sterile 1x phosphate-buffered saline (PBS; pH 7.4).

2.1.7. Suspend the cells in 1X PBS (pH 7.4) to a concentration of 1×10^8 colony forming units (CFUs) mL^{-1} or as appropriate for the desired experiment.

NOTE: it is important to determine the relationship between OD_{600} and CFU mL^{-1} for each strain of interest because strain-dependent alterations of cell size and shape can affect light scattering properties and ultimately, the infection dose.

2.2. Preparation of the animals and bacterial infection

2.2.1. Acclimate mice for 7 days on purified diet AIN-93. The diet is formulated to provide adequate nutrition while reducing tissue auto-fluorescence associated with the consumption of plant-based ingredients used in standard mouse chow⁴⁰.

NOTE: Female C57/BL6 mice (6-8 weeks old) are used here, but the mouse strain and sex will depend on the study.

2.2.2. Before infection, dilate mouse tail veins with lukewarm water.

2.2.3. Infect animals by injecting 100 μ L of the bacterial inoculum via tail-veins to produce a systemic infection. Save an aliquot of the initial inoculum if performing flow cytometry (see Section 4).

2.2.4. Monitor animals daily and evaluate their health status using a monitoring system reviewed and approved by one's Institutional Animal Care and Use Committee.

2.2.5. Allow the infection to progress for the desired duration. Here, the experiments are terminated 3 days post-infection.

NOTE: Under these conditions, abscesses consist of a staphylococcal abscess community of bacteria, enclosed by fibrin deposits, and surrounded by concentric layers of immune cells^{5,41}.

2.3. Harvesting the organs and tissue processing

2.3.1. Euthanize the animals by CO₂ inhalation and cervical dislocation as a secondary method and perform necropsy.

2.3.2. Harvest kidney (right) and other vital organs (heart, liver, lungs, spleen) and transfer into 50 mL polypropylene tubes containing 10% [v/v] buffered formalin. Proceed with these organs to step 2.3.4 below.

2.3.3. Transfer the left kidney to a sterile 2 mL impact-resistant tube containing ~500 μ L of 2 mm silica beads and 1 mL sterile 1x PBS (pH 7.4). Proceed with this organ to step 4.2.

2.3.4. Allow organs from step 2.3.2 to fix in the dark at room temperature with gentle shaking or rotation for at least 24 hours but no more than 48 hours.

2.3.5. Embed organs in clear tissue freezing medium and store the tissues at -80 °C.

2.3.6. Using a cryostat, section tissue into slices of 10 μ m thickness.

2.3.7. Dry the sections on a pre-cleaned, charged glass slide for 20 min in darkness, apply hard mounting medium with 4',6-diamidino-2-phenylindole (DAPI) stain, and apply coverslip. Cure mounted slides at room temperature overnight, and transfer to 4 °C for long-term storage.

3. Laser Scanning Confocal Microscopy and Image Processing

3.1. Examine mounted slides to locate lesions using lasers appropriate for the fluorescent reporter being used. In this case, the green (GFP), red (tdTomato), and blue (DAPI) fluorescence signals are used.

3.2. Acquire the image using the appropriate objective for visualizing individual cells (*e.g.*, typically 20X or 40X objectives are used).

3.3. Measure the fluorescence intensities in the confocal images.

3.3.1. Open the confocal image in Image J and adjust brightness/contrast to properly visualize the fluorescence signal of the lesion.

3.3.2. Define the region of interest, and by using the **Thresholding** option, set the lower and upper fluorescence limits as necessary.

3.3.3. Define the centroid by selecting **Area** → **Mean gray value** → **Centroid** → **Limit to Threshold** under the **Analyze** tab in Image J.

3.3.4. Extract centroid fluorescence intensity value or measure the mean fluorescence intensity in a given lesion per unit area (mean fluorescence intensity, MFI) for GFP and tdTomato. Here, areas of one μm^2 were used.

NOTE: A blinded second analysis minimizes bias when the identity of the sample is known.

3.3.5. Plot the data and perform the appropriate statistical analyses for each comparison.

4. Flow Cytometry Analysis

4.1. (Optional) Determine fusion activity of the inoculum on the day of the infection (from section 2.2.3)

4.1.1. To 899 μL of 1X sterile PBS (pH 7.4) add 100 μL of each inoculum and 1 μL of membrane permeant nucleic acid stain (see **Table of Materials**). As a control, be sure to include a sample lacking nucleic acid stain.

4.1.2. Perform flow cytometry on all samples.

NOTE: For the very first experiment, it is useful to include a vector only control for the fusion of interest to determine the proper voltage to use. A clear separation between positive and negative samples is essential for data analysis, indicating the reporter is well above background signal.

4.1.3. To identify the bacterial population, plot events using nucleic acid stain (y-axis) and forward scatter (x-axis).

4.1.4. Draw an inclusion gate around events that are both nucleic acid stain positive and the correct size of the bacterium based on the forward scatter (between 0.5 to 2.0 μm for *S. aureus*).

NOTE: Be rigorous when identifying this population as it is critical to include events that are clearly within these parameters. Be sure this gate excludes all events for the negative controls

under other conditions. In this study, roughly 70% the cell population met these requirements in the analysis.

4.2. Analysis of tissue samples after sacrifice:

4.2.1. Euthanize the animals, harvest the left kidneys (see step 2.3), and transfer into bead-beating tubes containing 1 mL of 1x sterile PBS (pH 7.4) and 250 μ L of 2 mm borosilicate beads.

4.2.2. Disrupt tissues to release bacterial cells. For kidneys, use a homogenizer to disrupt cells. Here, three 30 s bursts at 6800 rpm with 1 min cooling periods on wet ice between cycles were used to minimize heating samples.

4.2.3. Pellet the larger tissue debris by centrifuging at 250 x g for 3 min in a tabletop microcentrifuge cooled to 4 $^{\circ}$ C.

NOTE: Typically, there is a cell pellet at the bottom of the tube and a layer of floating debris on the top. The middle aqueous layer contains the bacterial cells.

4.2.4. Transfer 10 μ L from the aqueous layer into a clean 1.5 mL microcentrifuge tube containing 1 μ L of nucleic acid stain in 989 μ L of PBS (total volume 1 mL).

4.2.5. Perform flow cytometry using the same data acquisition parameters and gating as for the initial inocula.

NOTE: Bacterial cell counts will be very low because the sample mainly contains tissue debris. The “Live gate” option can also be used if the cells are nucleic acid stain positive and size-gated when data are loaded into the application. This will also help to analyze more of the target events and reduce file size. Nearly one million events per sample are counted, but more event counts may be necessary depending on the severity of the infection and size of tissue analyzed.

4.2.6. Data Analysis: Determine mean fluorescent intensity (MFI) for each fluorophore in a given sample using the inclusion gates determined in section 4.1.4. Normalize samples in flow analysis software to event counts. 10,000 counts are usually sufficient in the analysis.

NOTE: It is not uncommon to find samples that contain less than this number of events since this is dependent on abscess formation and infection severity. Using this approach, 500-40,000 events are typically found in infected tissue.

REPRESENTATIVE RESULTS:

We developed a plasmid derived from pMAD³² that can deliver any reporter fusion construct into the chromosome by double crossover homologous recombination (**Figure 1**). This construct allows for quantitative analysis of any regulatory region that supports the production of GFP protein and fluorescent signal above background. The plasmid confers ampicillin resistance (Ap^r)

for maintenance and propagation in *E. coli* and confers erythromycin resistance (Em^r) in *S. aureus*. The construct also confers chloramphenicol resistance (Cm^r), which allows for easy transfer of the integrated reporter fusion between various mutant strains for sophisticated genetic analysis in *S. aureus*.

As proof of principle, we fused the *nuc* regulatory region to *gfp*. *nuc* was chosen because its gene product is required for full virulence and is required for restricting macrophages from *S. aureus*-induced abscesses^{42,43}. The construct was integrated into the chromosome as described in steps 1.4-1.6 of the protocol. The integrated fusion was then transferred to AH3926 that contains a P_{sarAP1}-tdTomato fusion. The *sarA* P1 promoter has been shown previously to be constitutively active⁴⁴ and thus, labels all cells.

We first verified that the reporter fusions were active during *in vitro* shake flask growth in Tryptic Soy Broth (TSB; a rich, complex medium) and that the levels of fluorescence were above the cellular auto-fluorescent background (**Figure 2**). To determine how the fluorescent reporters behave *in vivo*, a modified renal abscess model was used⁵. Groups of female C57BL/6 mice were challenged intravenously with 1x10⁷ CFU each of *S. aureus* LAC cells lacking reporter fusions or LAC cells carrying both the *nuc-sGFP* and *sarAp1-tdTomato* fusions. Animals were sacrificed three days post infection. Then, harvested organs were fixed in 10% [v/v] buffered formalin, cryo-sectioned, and imaged by confocal microscopy after DAPI staining as described in steps 2 and 3 (**Figure 3A,B**). The images were analyzed using Image J and fluorescence per unit area in the renal lesions was measured. As shown in **Figure 3C**, *nuc-gfp* fusion fluorescence was on average nearly 9-fold higher in cells carrying the fusion than in cells not carrying the reporter fusion; the latter signal constitutes the limit of detection (auto-fluorescence) (compare *nuc-sGFP* to LAC). Similarly, P_{sarAP1}-tdtomato fusion fluorescence was ~6-fold higher than the no reporter control (**Figure 3E**, compare *sarAP1*-tdT vs LAC). Using flow cytometry, the pattern of reporter activities was confirmed using kidney homogenates and flow cytometry as described in step 4, though the fold differences in fluorescence were lower (**Figure 3D,F**).

Interestingly, the fluorescence data from lesions formed by cells carrying fluorescent reporter fusions appeared to show higher variability than those lacking the reporters. We wondered whether the variation in the fluorescence measurements observed was due to heterogeneous expression of the reporters (that is, of biological origins). Indeed, within ~100 μm distance it was found that some lesions expressed either one or both reporters (**Figure 4**). Importantly, examining reporter activity with single cell resolution in staphylococcal abscess communities (SACs) revealed spatial regulation of *nuc* expression in abscesses circumscribed with strong DAPI staining, likely associated with the formation and release of neutrophil extracellular traps (**Figure 5A-C**)⁴⁵. For instance, we measured significantly higher *nuc-sGFP* fusion fluorescence in the interior core of the SAC compared with that on the periphery (**Figure 5B,E**). In the same abscess, the pattern for *sarAp1-tdTomato* fusion fluorescence appeared to be inverted (**Figure 5D**). However, the pattern was not statistically significant using the same number of animals (**Figure 5F**).

FIGURE LEGENDS:

Figure 1: Schematic representation of the genome integrative reporter plasmid pRB4. Plasmid pRB4 is a derivative of pMAD with a temperature sensitive origin of replication in *S. aureus* (Ori pE194^{ts}). There are three drug resistance markers: (i) *bla*, conferring resistance to ampicillin in *Escherichia coli*; (ii) *ermC*, conferring erythromycin resistance in *S. aureus*; and (iii) *cat*, conferring resistance to chloramphenicol in *S. aureus*. The reporter construct is flanked by ~500 bp each from the upstream and downstream sequences of the pseudogene SAUSA300_0087 (reference genome: FPR3757) for double crossover homologous recombination and genome integration. sGFP, green fluorescent protein gene; CS, cloning site; TT, strong bidirectional transcriptional terminator. Figure is not to scale.

Figure 2: Integrated *nuc-sGFP* and *sarAp1-tdTomato* reporters are expressed during *in vitro* growth. *S. aureus* LAC cells (wild-type [WT]), with and without the **A) *nuc-sGFP*** or **B) *sarAp1-tdTomato*** reporters) were grown to exponential phase in TSB and re-diluted into the same medium. Optical density (OD) and fluorescence were measured at the indicated optical density values. Background-subtracted fluorescence intensity values (excitation/emission: sGFP, 485/535 nm; tdTomato [tdT], 535/590 nm) were divided by the optical density values to generate Relative Fluorescence Units. The data are the means +/- SEM from two independent experiments.

Figure 3: Fluorescent reporters are visible in renal tissue. Groups of 13 C57BL/6 mice were challenged intravenously with LAC cells or LAC cells carrying both *nuc-sGFP* and *sarAp1-tdTomato* (*tdT*), and the infection was allowed to proceed for three days. Mice were euthanized, and organs were processed as described in steps 2 and 3 of the protocol. Representative kidney section showing multiple lesions and the associated fluorescence for **A) *nuc-sGFP*** and **B) *sarAp1-tdTomato***. Scale bars = 250 μ m (10X objective). The Relative Fluorescence Units per unit area (RFU [μ m²]⁻¹) associated with renal lesions were measured using image analysis as described in step 3.3 and were plotted for **C) *nuc-sGFP*** and **E) *sarAp1-tdTomato***; each dot represents one lesion and bars indicate the median; 3-5 lesions analyzed per mouse. Flow cytometry analysis of **D) *nuc-sGFP*** and **F) *sarAp1-tdTomato*** fusion fluorescence in bacterial populations isolated from infected kidney homogenates three days post infection. The Mean Fluorescence Intensity (MFI) is indicated by the solid bar, and each dot is one kidney. LAC, reporterless wild-type strain. Statistics: Mann-Whitney Test; ****p < 0.05. The data are representative of two independent experiments. The excitation wavelengths for the fluorescence channels are as follows: DAPI, 405 nm; GFP, 488 nm; tdTomato, 561 nm. Emitted fluorescence data were collected over a range of wavelengths: DAPI, 419-481 nm; sGFP, 505-551 nm; tdTomato, 575-630 nm.

Figure 4: Abscesses exhibit variable expression of reporters in renal tissues. Groups of 13 C57BL/6 mice were challenged with 1 x 10⁷ CFU of *S. aureus* cells via the tail-vein. Animals were euthanized three days post infection. Kidneys were harvested, fixed, and sectioned for fluorescence microscopy (40X objective) as described in step 2 of the protocol. Shown are three lesions in close proximity with variable levels of **(A) *nuc-sGFP*** and **(B) *sarAp1-tdTomato*** fluorescence. **(C)** Nucleic acid from staphylococci and host cells is indicated by the DAPI staining. The green and red channels are merged in **(D)**. Similar results were seen in other sections. The images were acquired using a 40X objective and the scale bar indicates 25 μ m. The excitation

wavelengths for the fluorescence channels are as follows: DAPI, 405 nm; GFP, 488 nm; tdTomato, 561 nm. Emitted fluorescence data were collected over a range of wavelengths: DAPI, 419-481 nm; sGFP, 505-551 nm; tdTomato, 575-630 nm.

Figure 5: *nuc-sGFP* is spatially regulated in the staphylococcal abscess micro-environment.

Representative confocal laser scanning microscopy (CLSM) images of a staphylococcal abscess community (SAC) lesion produced by *S. aureus* strain LAC carrying *nuc-sGFP* and *sarAp1-tdTomato* reporter fusions. Shown are the (A) merged channels for *nuc-sGFP* and *sarAp1-tdtomato*, (B) *nuc-sGFP* fluorescence (green), (C) DAPI staining of nucleic acids (blue), and (D) *sarAp1-tdTomato* fluorescence (red). Asterisks indicate cells in the core (centroid) and arrows indicate cells on the periphery. The fluorescence intensities for (E) *nuc-sGFP* and (F) *sarAp1-tdTomato* are shown for cells in the core and periphery of the SAC. The excitation wavelengths for the fluorescence channels are as follows: DAPI, 405 nm; GFP, 488 nm; tdTomato, 561 nm. Emitted fluorescence data were collected over a range of wavelengths: DAPI, 419-481 nm; sGFP, 505-551 nm; tdTomato, 575-630 nm. The data are derived from 8 kidneys (one per mouse), and 1-2 lesions were imaged from each kidney. Bars indicate median. Dashed line, limit of detection. Statistics: Normal distribution of data, Student's t-test (unpaired), **** $p < 0.05$. (Scale bar: 20 μ m; applies to all images.)

DISCUSSION:

Bacterial infectious diseases are an increasing health problem worldwide due to the acquisition of antibiotic resistance determinants⁴⁶. Because adaptation to host environments is essential for growth and survival during infection, strategies targeting gene expression programs that increase pathogen fitness may prove useful therapeutically. One such program is the set of genes controlled by the SaeR/S two component system (TCS), shown previously to play an essential role in immune evasion⁴⁷. SaeR/S is induced by a variety of factors, most notably those associated with neutrophils^{8,20}. During infection, *S. aureus* elicits a strong inflammatory response in which neutrophils and other phagocytes are recruited to the site of infection². Liquefaction necrosis and fibrin deposition follow, forming an abscess to prevent further tissue damage⁴⁸. Within these immune privileged sites, *S. aureus* cells use a number of Sae-dependent gene products to reprogram the abscess to facilitate bacterial multiplication^{5,48}. One Sae-dependent gene, *nuc*, codes for nuclease and metabolizes NETs to produce 2'-deoxyadenosine to kill macrophages⁴³. Thus, Nuc is an important secreted enzyme that is essential for full virulence⁴² and is expressed *in vivo* (Figure 3, Figure 4, and Figure 5).

Although the virulence factors of *S. aureus* have been studied extensively, how the bacterium grows in the host is an understudied area, as is understanding how its physiology is regulated during infection. Here we describe a method for probing bacterial gene expression and behavior on the single cell level using a modified integrative vector that mitigates concern for plasmid loss in the absence of selection. Our methodology allows for direct visualization of gene expression in abscesses without having to permeabilize cell walls, and without the need for antibodies and labeling. We detected strong expression of the *nuc-sGFP* fusion as well as the *sarAp1-tdTomato* fusion in renal abscess SACs formed by wild-type cells three days post infection in an acute

systemic infection model. However, the experimental design can be modified to answer questions regarding gene expression in other sites. Indeed, because C57BL/6 mice are unable to clear *S. aureus* from their tissues, the bacteria invade diverse anatomical sites including the skeletal system (bones and joints) and the brain, heart, spleen, and liver, all of which have different physiology and nutritive properties^{5,49}. Thus, much can be learned by using *S. aureus* to probe the nature of host tissues. It is important to note that it is known that certain host niches are hypoxic in nature or otherwise exhibit a strong oxygen gradient⁵⁰. Fluorescent proteins require molecular oxygen for activity, and some are more sensitive to oxygen partial pressures than others⁵¹. While we see fluorescence signal deep within the abscess, the magnitudes may be an underestimate. Using codon-optimized fluorescent proteins developed for use in *Clostridium difficile* could be used to mitigate this concern⁵². A second point to note is that the fluorescent proteins (sGFP and tdTomato) produced by the reporter strains used in this study are stable. Therefore, the fluorescent data reflect accumulation over the course of experiment rather than a recent response. Generating a construct containing an unstable sGFP or tdTomato gene would greatly increase the utility of the system for dynamic experiments.

The reporter system described here provides a powerful tool to quantitatively study gene regulation *in vitro* and *in vivo*. Because the reporter is maintained in single copy on the chromosome, the system is well-suited for strongly expressed genes (that is, having high promoter activity). Biosynthetic genes or other lowly expressed genes may not be visible because the level of fluorescence expression could fall below the limit of detection or background autofluorescence. It is known that the ribosome binding site (RBS) influences the activity of reporter fusions³³. A potential solution to this problem is to use a stronger RBS such as the translation initiation region (TIR)⁵³ or to integrate tandem copies of promoters of interest into the chromosome. Alternatively, stable multi-copy plasmids could be used⁵⁴.

A limitation of the method described is that, unlike a cDNA preparation from RNA extracted from the tissues, a relatively small number of genes can be interrogated simultaneously (limited by available fluorescent channels without spectral overlap). However, what is gained can be potentially more informative. qRT-PCR and other readouts that average the bulk population are unable to capture population heterogeneity at the site of infection. Indeed, we observed heterogeneity in the level of *nuc-sGFP* and *sarAp1-tdTomato* expression between abscess lesions in close proximity (**Figure 4**). This is similar to what was recently reported by Cassat *et al.*⁵⁵ At this time, the origin of this heterogeneity is not known. However, nutrient availability, variable induction of nutrient acquisition systems, or other host factors could possibly explain the phenomenon. Moreover, the host-pathogen interaction occurs within microenvironments of organs or abscesses that have complex three-dimensional structure and various cell types. Within these structures, tissues can vary considerably with respect to pH, osmolarity, oxygenation, and nutrient availability, a phenomenon known as metabolic zonation^{56,57}. We serendipitously discovered that a pattern of spatial regulation arises in wild-type cells residing in the abscess (**Figure 5**). This is to our knowledge the first observation of its kind in a Gram-positive pathogen during infection. The spatial regulation reported here is similar to that obtained by Davis *et al.* in splenic tissue containing microcolonies of the Gram-negative pathogen *Yersinia pseudotuberculosis*⁵⁸. In that instance, host produced nitric oxide (NO*) stimulated the

production of nitric oxide dioxygenase (Hmp) in cells on the periphery of the microcolony closest to the diffusing NO*, sparing interior cells the need to induce expression of *hmp*. In staphylococcal abscesses, *nuc* expression is strongest at the core of the SAC, and weakest along the periphery. Because abscesses are surrounded by a cuff of neutrophils, it is tempting to speculate that neutrophil-derived cues are guiding the behavior of the staphylococci, polarizing the cells into two phenotypically distinct populations reminiscent of morphogenic regulation of differentiation during development in higher life⁵⁹. The mechanistic underpinning of this pattern is unknown and is a subject of intense study in our laboratory.

We believe our method provides an effective tool for studying the fine detail of gene expression in individual cells during infection. The method provides a unique opportunity to observe and to eventually understand the physiological origins of heterogeneity and spatial patterning of gene expression in tissues. This heterogeneous behavior must be taken into account when developing new treatment modalities, as only a subpopulation of cells (*i.e.*, those expressing the genes) may be targeted by the therapeutic.

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The authors declare that they have no competing financial interests.

REFERENCES:

- 1 Hood, M. I. & Skaar, E. P. Nutritional immunity: transition metals at the pathogen-host interface. *Nature Reviews Microbiology*. **10** (8), 525-537, (2012).
- 2 Lowy, F. *Staphylococcus aureus* infections. *The New England Journal of Medicine*. **339** (8), 520-532, (1998).
- 3 Grosser, M. R. *et al.* Genetic requirements for *Staphylococcus aureus* nitric oxide resistance and virulence. *PLoS Pathogen*. **14** (3), e1006907, (2018).
- 4 Valentino, M. *et al.* Genes contributing to *Staphylococcus aureus* fitness in abscess- and infection-related ecologies. *MBio*. **5** (5), e01729-01714, (2014).
- 5 Cheng, A. *et al.* Genetic requirements for *Staphylococcus aureus* abscess formation and persistence in host tissues. *FASEB Journal*. **23** (10), 3393-3404, (2009).
- 6 Meyers, L. A., Levin, B. R., Richardson, A. R. & Stojiljkovic, I. Epidemiology, hypermutation, within-host evolution and the virulence of *Neisseria meningitidis*. *Proceedings of the Royal Society B: Biological Sciences*. **270** (1525), 1667-1677, (2003).
- 7 Balasubramanian, D. *et al.* *Staphylococcus aureus* Coordinates Leukocidin Expression and Pathogenesis by Sensing Metabolic Fluxes via RpiRc. *MBio*. **7** (3), e00818-00816, (2016).

569 8 Geiger, T., Goerke, C., Mainiero, M., Kraus, D. & Wolz, C. The virulence regulator Sae of
570 *Staphylococcus aureus*: promoter activities and response to phagocytosis-related signals.
571 *Journal of Bacteriology*. **190** (10), 3419-3428, (2008).

572 9 Weiss, A., Broach, W. H. & Shaw, L. N. Characterizing the transcriptional adaptation of
573 *Staphylococcus aureus* to stationary phase growth. *Pathogens and Disease*. **74** (5),
574 pii:ftw046, (2016).

575 10 Balaban, N. & Novick, R. P. Autocrine regulation of toxin synthesis by *Staphylococcus*
576 *aureus*. *Proceedings of the National Academy of Sciences of the United States of America*.
577 **92** (5), 1619-1623, (1995).

578 11 Pohl, K. *et al.* CodY in *Staphylococcus aureus*: a regulatory link between metabolism and
579 virulence gene expression. *Journal of Bacteriology*. **191** (9), 2953-2963, (2009).

580 12 Majerczyk, C. D. *et al.* *Staphylococcus aureus* CodY negatively regulates virulence gene
581 expression. *Journal of Bacteriology*. **190** (7), 2257-2265, (2008).

582 13 McNamara, P. J., Milligan-Monroe, K. C., Khalili, S. & Proctor, R. A. Identification, cloning,
583 and initial characterization of rot, a locus encoding a regulator of virulence factor
584 expression in *Staphylococcus aureus*. *Journal of Bacteriology*. **182** (11), 3197-3203, (2000).

585 14 Liu, Q., Yeo, W. S. & Bae, T. The SaeRS Two-Component System of *Staphylococcus aureus*.
586 *Genes (Basel)*. **7** (10), E81, (2016).

587 15 Giraudo, A., Calzolari, A., Cataldi, A., Bogni, C. & Nagel, R. The *sae* locus of *Staphylococcus*
588 *aureus* encodes a two-component regulatory system. *FEMS Microbiology Letters*. **177** (1),
589 15-22, (1999).

590 16 Cho, H. *et al.* Calprotectin Increases the Activity of the SaeRS Two Component System and
591 Murine Mortality during *Staphylococcus aureus* Infections. *PLoS Pathogen*. **11** (7),
592 e1005026, (2015).

593 17 Sun, F. *et al.* In the *Staphylococcus aureus* two-component system *sae*, the response
594 regulator SaeR binds to a direct repeat sequence and DNA binding requires
595 phosphorylation by the sensor kinase SaeS. *Journal of Bacteriology*. **192** (8), 2111-2127,
596 (2010).

597 18 Liang, X. *et al.* Inactivation of a two-component signal transduction system, SaeRS,
598 eliminates adherence and attenuates virulence of *Staphylococcus aureus*. *Infection and*
599 *Immunity*. **74** (8), 4655-4665, (2006).

600 19 Giraudo, A. T., Cheung, A. L. & Nagel, R. The *sae* locus of *Staphylococcus aureus* controls
601 exoprotein synthesis at the transcriptional level. *Archives of Microbiology*. **168** (1), 53-58,
602 (1997).

603 20 Flack, C. E. *et al.* Differential regulation of staphylococcal virulence by the sensor kinase
604 SaeS in response to neutrophil-derived stimuli. *Proceedings of the National Academy of*
605 *Sciences of the United States of America*. **111** (19), E2037-2045, (2014).

606 21 Mainiero, M. *et al.* Differential target gene activation by the *Staphylococcus aureus* two-
607 component system *saeRS*. *Journal of Bacteriology*. **192** (3), 613-623, (2010).

608 22 Li, D. & Cheung, A. Repression of *hla* by *rot* is dependent on *sae* in *Staphylococcus aureus*.
609 *Infection and Immunity*. **76** (3), 1068-1075, (2008).

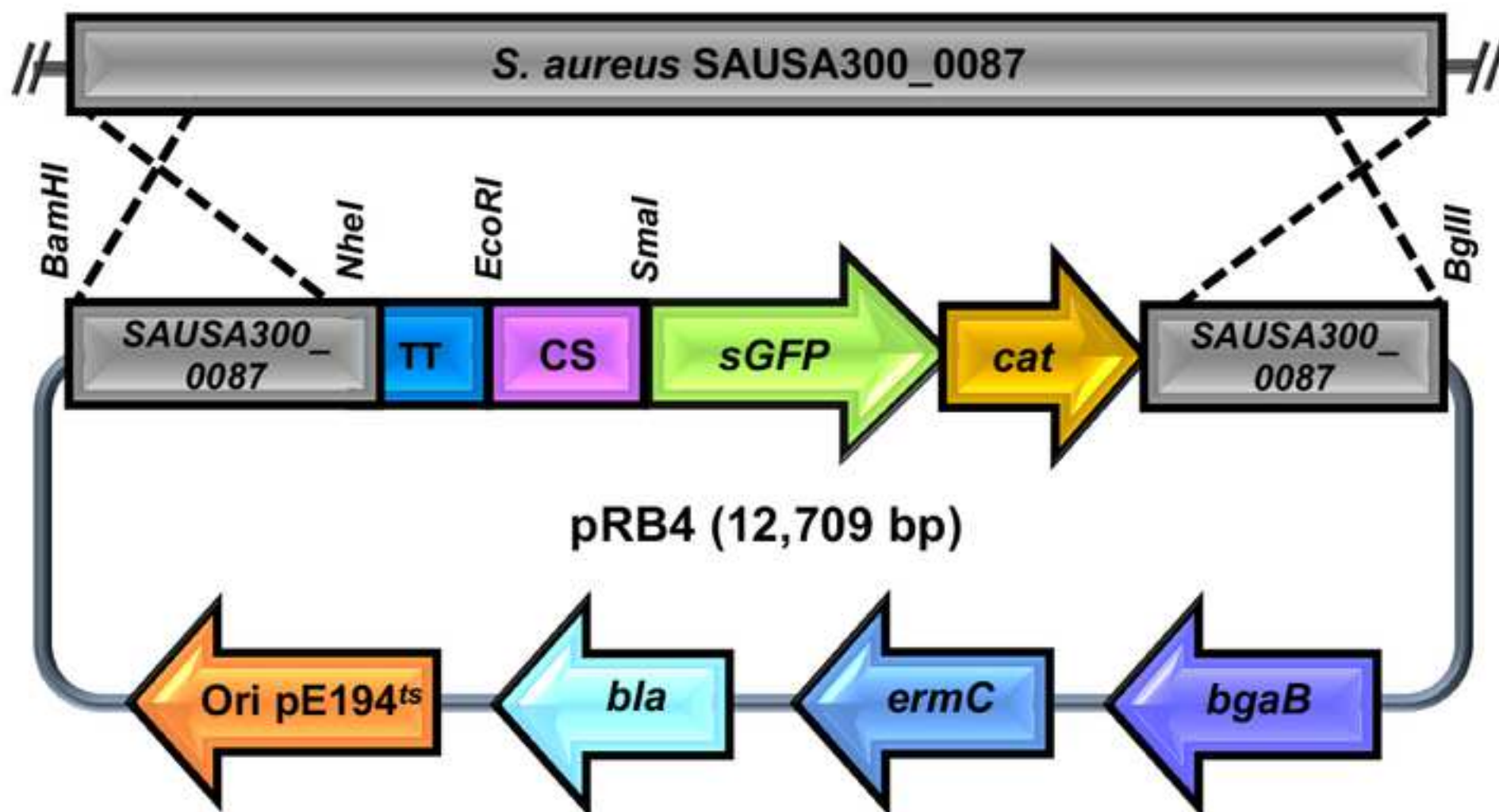
610 23 Bischoff, M. *et al.* Microarray-based analysis of the *Staphylococcus aureus* sigmaB
611 regulon. *Journal of Bacteriology*. **186** (13), 4085-4099, (2004).

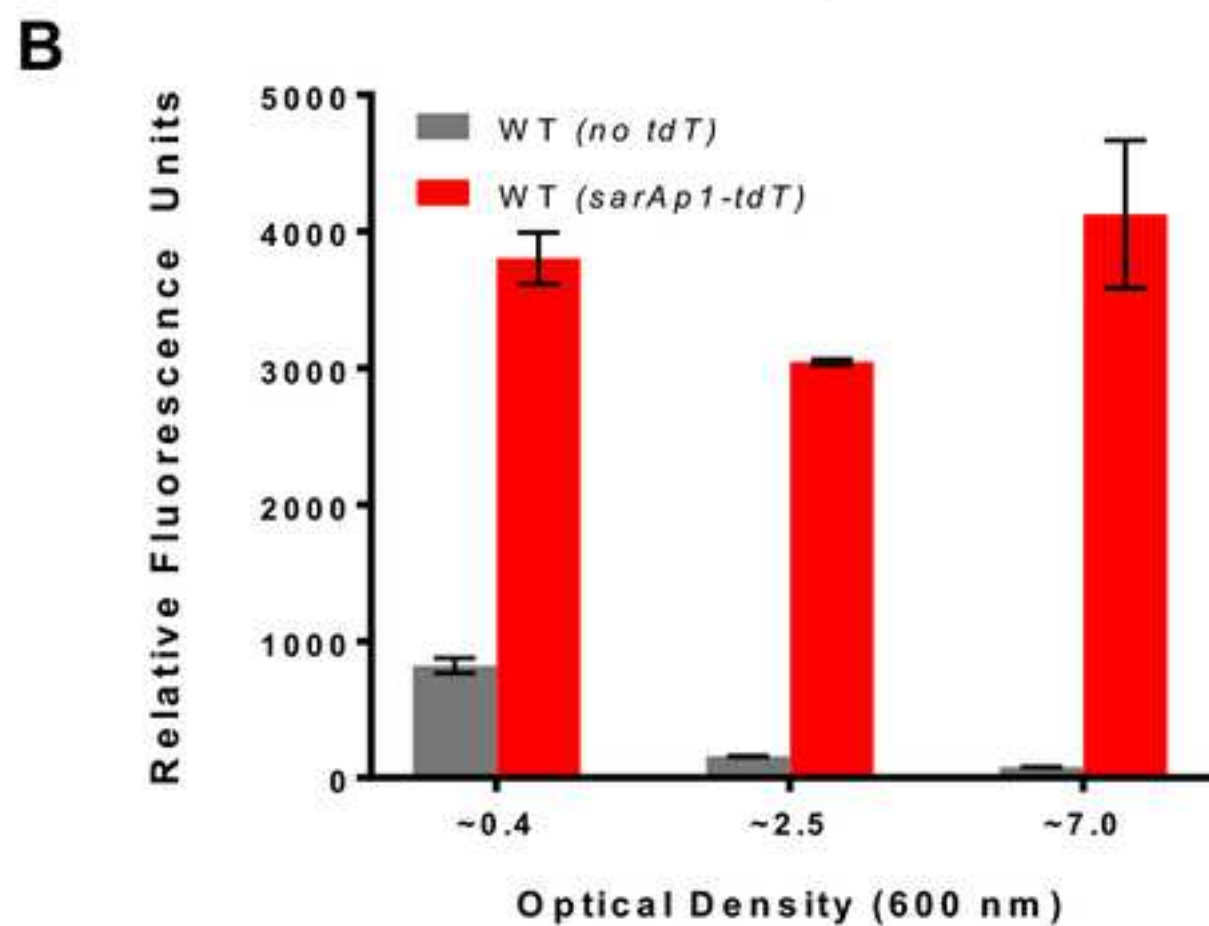
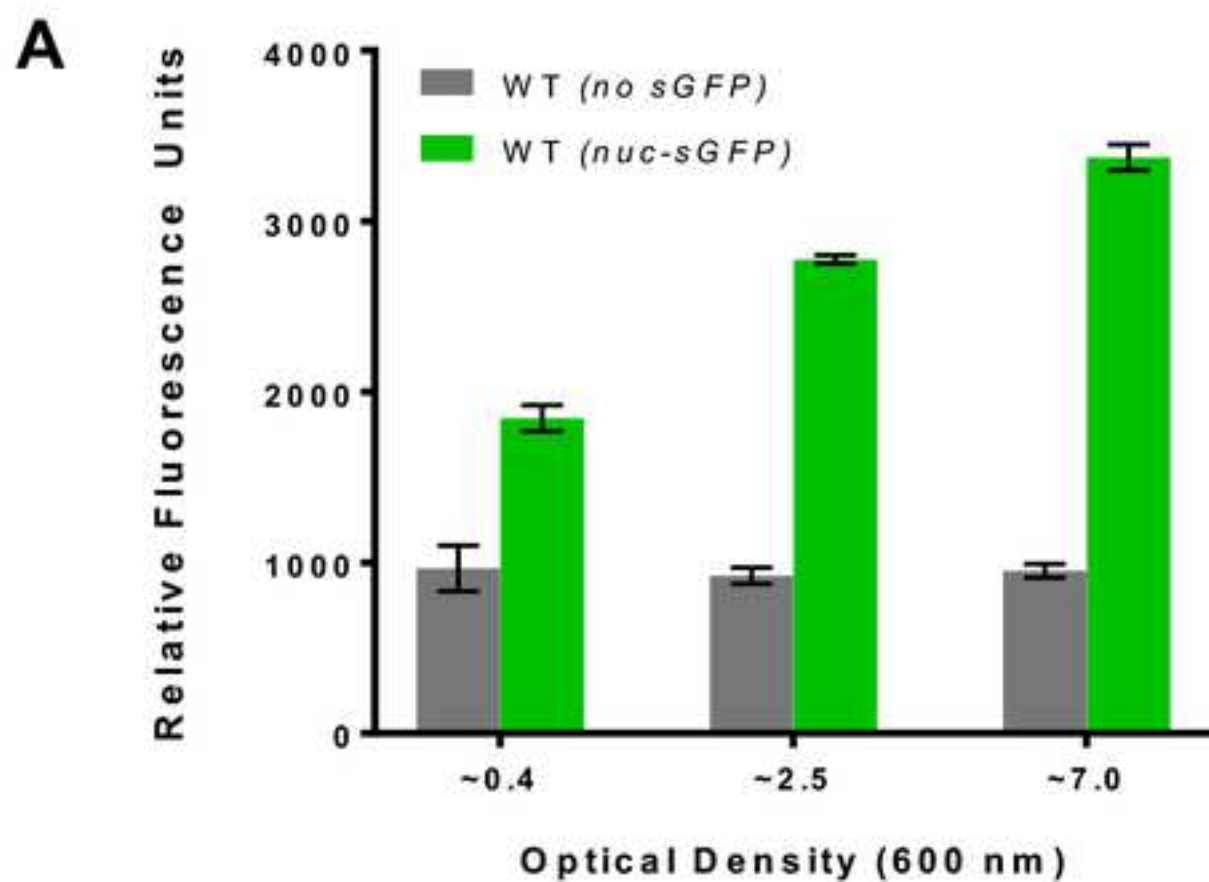
- 24 Novick, R. & Jiang, D. The staphylococcal *saeRS* system coordinates environmental signals with *agr* quorum sensing. *Microbiology*. **149** (Pt 10), 2709-2717, (2003).
- 25 Olson, M. E. *et al.* *Staphylococcus aureus* nuclease is an SaeRS-dependent virulence factor. *Infection and Immunity*. **81** (4), 1316-1324, (2013).
- 26 Berends, E. *et al.* Nuclease expression by *Staphylococcus aureus* facilitates escape from neutrophil extracellular traps. *Journal of Innate Immunity*. **2** (6), 576-587, (2010).
- 27 Waters, N. R. *et al.* A spectrum of CodY activities drives metabolic reorganization and virulence gene expression in *Staphylococcus aureus*. *Molecular Microbiology*. **101** (3), 495-514, (2016).
- 28 Cassat, J. *et al.* Transcriptional profiling of a *Staphylococcus aureus* clinical isolate and its isogenic *agr* and *sarA* mutants reveals global differences in comparison to the laboratory strain RN6390. *Microbiology*. **152** (Pt 10), 3075-3090, (2006).
- 29 Kiedrowski, M. *et al.* Nuclease modulates biofilm formation in community-associated methicillin-resistant *Staphylococcus aureus*. *PLoS One*. **6** (11), e26714, (2011).
- 30 Fujimoto, D. F. *et al.* *Staphylococcus aureus* SarA is a regulatory protein responsive to redox and pH that can support bacteriophage lambda integrase-mediated excision/recombination. *Molecular Microbiology*. **74** (6), 1445-1458, (2009).
- 31 Yeo, W. S. *et al.* The FDA-approved anti-cancer drugs, streptozotocin and floxuridine, reduce the virulence of *Staphylococcus aureus*. *Scientific Reports*. **8** (1), 2521, (2018).
- 32 Arnaud, M., Chastanet, A. & Débarbouillé, M. New vector for efficient allelic replacement in naturally nontransformable, low-GC-content, Gram-positive bacteria. *Applied and Environmental Microbiology*. **70** (11), 6887-6891, (2004).
- 33 Malone, C. L. *et al.* Fluorescent reporters for *Staphylococcus aureus*. *Journal of Microbiological Methods*. **77** (3), 251-260, (2009).
- 34 Schenk, S. & Laddaga, R. A. Improved method for electroporation of *Staphylococcus aureus*. *FEMS Microbiology Letters*. **73** (1-2), 133-138, (1992).
- 35 Monk, I. R. & Foster, T. J. Genetic manipulation of Staphylococci—breaking through the barrier. *Frontiers in Cellular and Infection Microbiology*. **2**, 49, (2012).
- 36 Novick, R. P. Genetic systems in staphylococci. *Methods in Enzymology*. **204**, 587-636, (1991).
- 37 Diep, B. A. *et al.* Complete genome sequence of USA300, an epidemic clone of community-acquired methicillin-resistant *Staphylococcus aureus*. *Lancet*. **367** (9512), 731-739, (2006).
- 38 Boles, B. R., Thoendel, M., Roth, A. J. & Horswill, A. R. Identification of genes involved in polysaccharide-independent *Staphylococcus aureus* biofilm formation. *PLoS One*. **5** (4), e10146, (2010).
- 39 Villaruz, A. E. *et al.* A point mutation in the *agr* locus rather than expression of the Pantone-Valentine leukocidin caused previously reported phenotypes in *Staphylococcus aureus* pneumonia and gene regulation. *Journal of Infect Diseases*. **200** (5), 724-734, (2009).
- 40 Reeves, P. G., Nielsen, F. H. & Fahey, G. C. J. AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition *ad hoc* writing committee on the reformulation of the AIN-76A rodent diet. *Journal of Nutrition*. **123** (11), 1939-1951, (1993).

- 41 Thomer, L., Schneewind, O. & Missiakas, D. Pathogenesis of *Staphylococcus aureus* Bloodstream Infections. *Annual Review of Pathology*. **11**, 343-364, (2016).
- 42 Olson, M. *et al.* *Staphylococcus aureus* nuclease is an SaeRS-dependent virulence factor. *Infection and Immunity*. **81** (4), 1316-1324, (2013).
- 43 Thammavongsa, V., Missiakas, D. & Schneewind, O. *Staphylococcus aureus* degrades neutrophil extracellular traps to promote immune cell death. *Science*. **342** (6160), 863-866, (2013).
- 44 Cheung, A. L., Nast, C. C. & Bayer, A. S. Selective activation of *sar* promoters with the use of green fluorescent protein transcriptional fusions as the detection system in the rabbit endocarditis model. *Infection and Immunity*. **66** (12), 5988-5993, (1998).
- 45 Pilsczek, F. H. *et al.* A novel mechanism of rapid nuclear neutrophil extracellular trap formation in response to *Staphylococcus aureus*. *Journal of Immunology*. **185** (12), 7413-7425, (2010).
- 46 Uhlemann, A. C., Otto, M., Lowy, F. D. & DeLeo, F. R. Evolution of community- and healthcare-associated methicillin-resistant *Staphylococcus aureus*. *Infection, Genetics and Evolution*. **21**, 563-574, (2014).
- 47 Voyich, J. M. *et al.* The SaeR/S gene regulatory system is essential for innate immune evasion by *Staphylococcus aureus*. *Journal of Infectious Diseases*. **199** (11), 1698-1706, (2009).
- 48 Cheng, A., DeDent, A., Schneewind, O. & Missiakas, D. A play in four acts: *Staphylococcus aureus* abscess formation. *Trends in Microbiology*. **19** (5), 225-232, (2011).
- 49 Balasubramanian, D., Harper, L., Shopsin, B. & Torres, V. J. *Staphylococcus aureus* pathogenesis in diverse host environments. *Pathogens and Disease*. **75** (1), (2017).
- 50 Vitko, N. P., Grosser, M. R., Khatri, D., Thurlow, L. R. & Richardson, A. R. Expanded Glucose Import Capability Affords *Staphylococcus aureus* Optimized Glycolytic Flux during Infection. *MBio*. **7** (3), e00296-00216, (2016).
- 51 Landete, J. M. *et al.* Use of anaerobic green fluorescent protein versus green fluorescent protein as reporter in lactic acid bacteria. *Applied Microbiology and Biotechnol.* **99** (16), 6865-6877, (2015).
- 52 Buckley, A. M. *et al.* Lighting Up Clostridium Difficile: Reporting Gene Expression Using Fluorescent Lov Domains. *Scientific Reports*. **6** 23463, (2016).
- 53 Bose, J. L. Genetic manipulation of staphylococci. *Methods in Molecular Biology*. **1106** 101-111, (2014).
- 54 Krute, C. N. *et al.* Generation of a stable plasmid for *in vitro* and *in vivo* studies of *Staphylococcus*. *Applied and Environmental Microbiology*. 10.1128/AEM.02370-16, (2016).
- 55 Cassat, J. E. *et al.* Integrated molecular imaging reveals tissue heterogeneity driving host-pathogen interactions. *Science Translational Medicine*. **10** (432), pii: eaan6361, (2018).
- 56 Handlogten, M. E., Hong, S. P., Westhoff, C. M. & Weiner, I. D. Apical ammonia transport by the mouse inner medullary collecting duct cell (mIMCD-3). *American Journal of Physiology-Renal Physiology*. **289** (2), F347-358, (2005).
- 57 Hijmans, B. S., Grefhorst, A., Oosterveer, M. H. & Groen, A. K. Zonation of glucose and fatty acid metabolism in the liver: mechanism and metabolic consequences. *Biochimie Journal*. **96** 121-129, (2014).

699 58 Davis, K. M., Mohammadi, S. & Isberg, R. R. Community behavior and spatial regulation
700 within a bacterial microcolony in deep tissue sites serves to protect against host attack.
701 *Cell Host & Microbe*. **17** (1), 21-31, (2015).
702 59 Stenman, J. M. *et al.* Canonical Wnt signaling regulates organ-specific assembly and
703 differentiation of CNS vasculature. *Science*. **322** (5905), 1247-1250, (2008).
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Figure 1





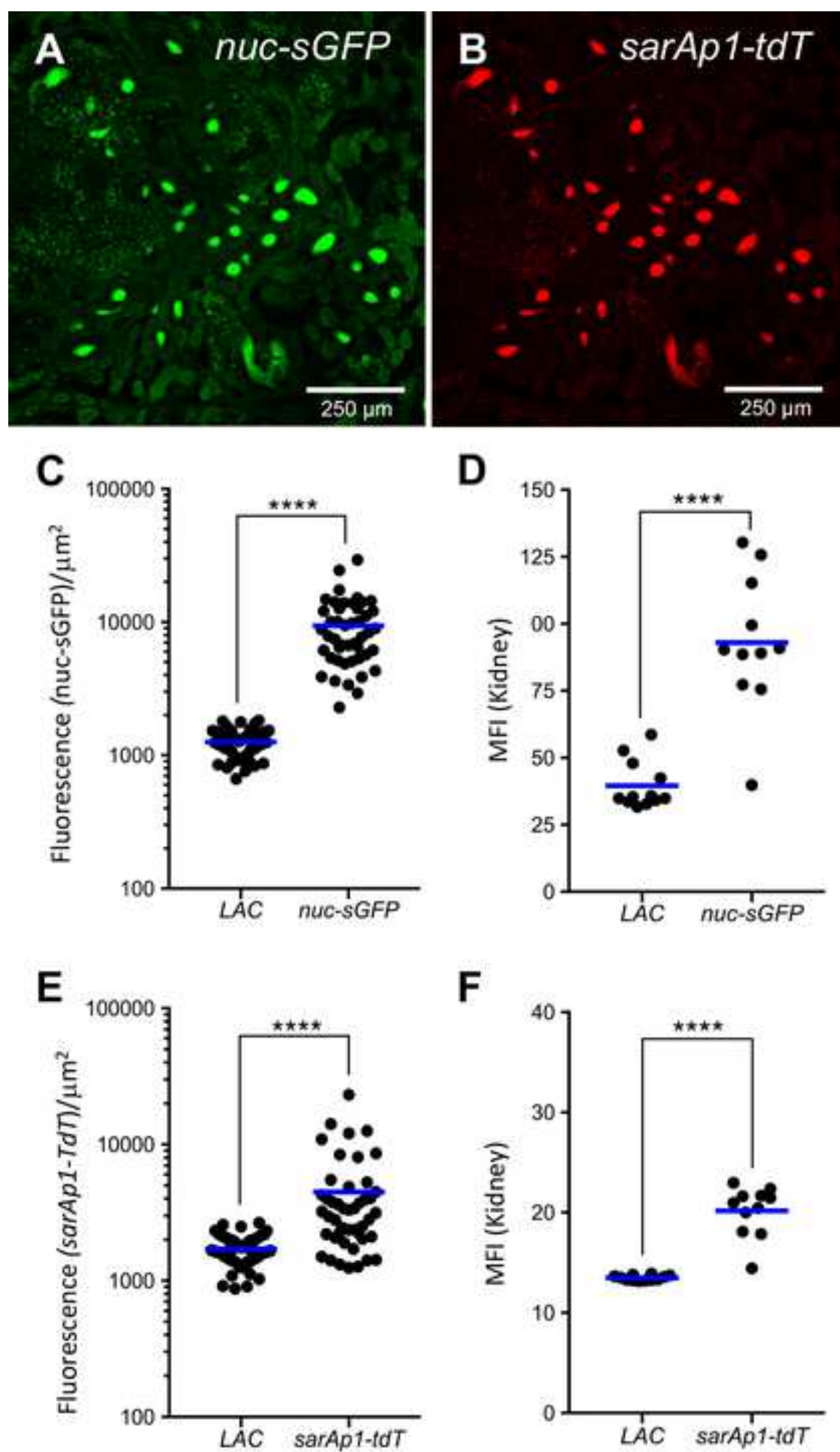
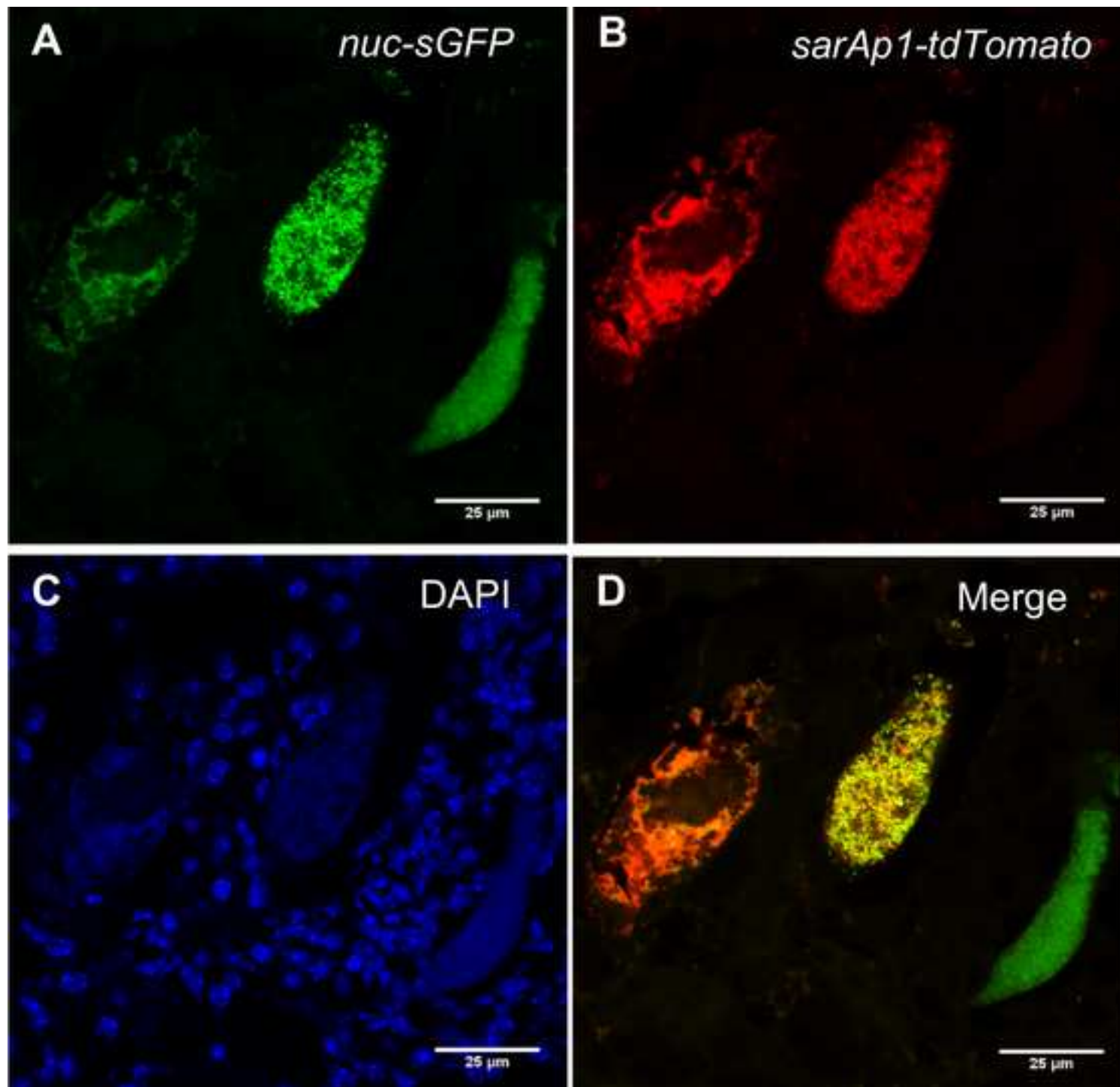
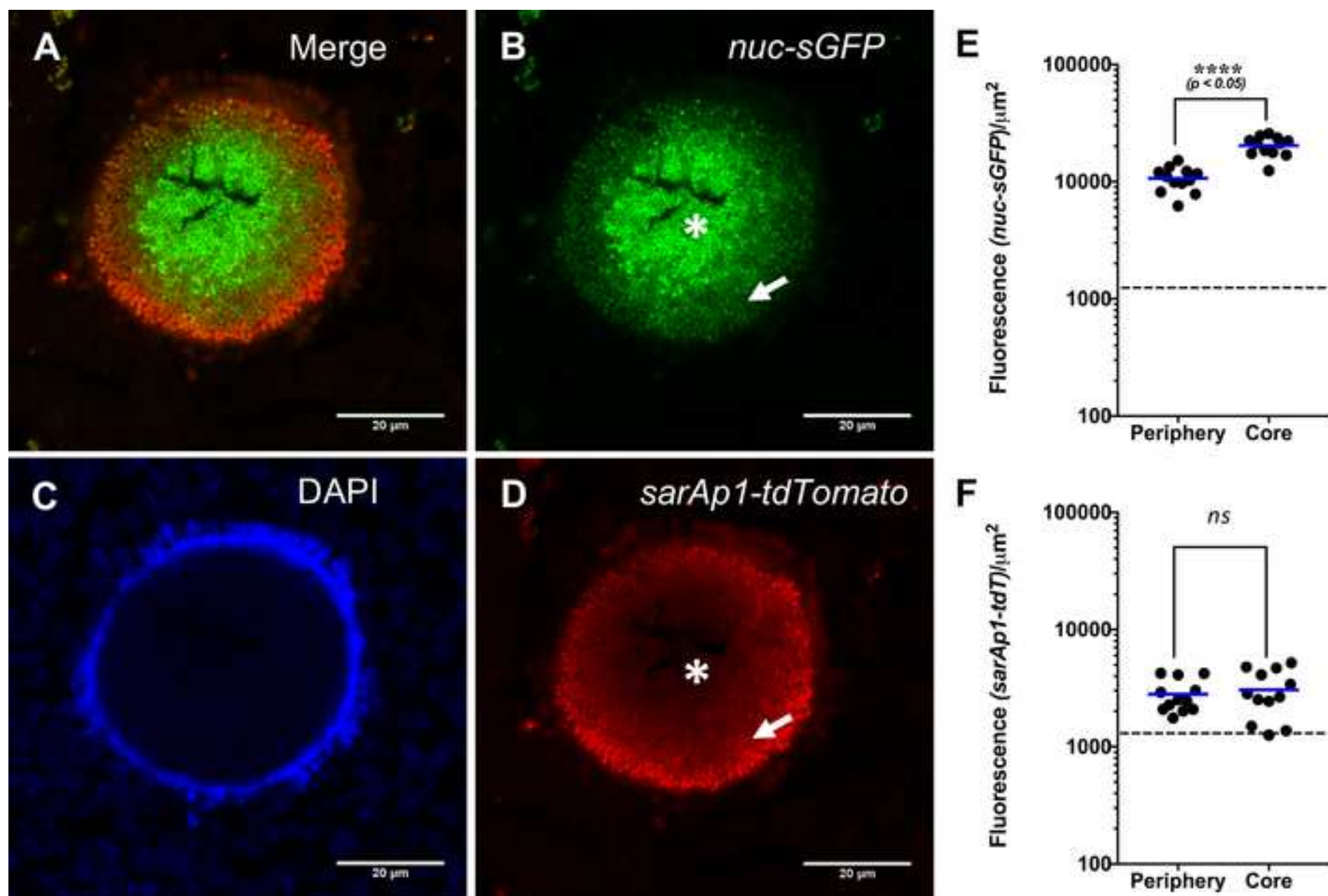


Figure 4





Name of Material/ Equipment	Company	Catalog Number
5% sheep blood	Hardy Diagnostics (Santa Maria, CA)	A10
5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal)	ThermoScientific	R0402
Ampicillin	Fisher Scientific	BP1760-25
C57Bl/6 Mice	Charles River	NA
Chloramphenicol	Sigma-Aldrich	C-0378
confocal laser scanning microscope	Zeiss	NA
cryostat microtome	Thermo Scientific	NA
Culture, Cap	VWR International	2005-02512
D+2:25NA Oligo	Integrated DNA Technologies (Coralville, Iowa)	NA
DNA Ligase	New England Biolabs	M0202S
Erythromycin	Sigma-Aldrich	E5389
Flow Analyzer	Becton Dickinson	NA
glass beads	Sigma	Z273627
Miniprep, plasmid	Promega	A1220
orbital shaking water bath	New Brunswick Innova	NA
PCR purification	QIAGEN	28106
Phosphate Buffer Saline (PBS)	Cellgrow	46-013-CM
Plate reader	Tecan	NA
Precellys 24 homogenizer	Bertin Laboratories	NA
pUC57-Kan	GenScript (Piscataway, NJ)	NA
Q5 Taq DNA Polymerase	New England Biolabs (Ipswich, MA)	M0491S R0150S (PvuI), R3136S (BamHI), R0144S (BglII), R3131S (NheI), R0101S (EcoRI), R0141S (SmaI).
Restriction Enzymes	New England Biolabs (Ipswich, MA)	
Reverse transcriptase	New England BioLabs	E6300L
Sanger Sequencing	Genewiz (Germantown, MD)	NA
Sub Xero clear tissue freezing medium	Mercedes Medical	MER5000
Superfrost Plus microscope slides	Fisher Scientific	12-550-15

superloop	GE Lifesciences	18111382
Syringe, Filter	VWR International	28145-481
Syto 40	Thermo Fisher Scientific	S11351
Tetracycline	Sigma-Aldrich	T7660
Tryptic Soy Broth	VWR	90000-376
UV-visible spectrophotometer	Beckman Coulter-DU350	NA
Vectashield Antifade Mounting medium with DAPI	Vector Laboratories	H-1500

Comments/Description

Membrane permeant nucleic acid stain



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CORRESPONDING AUTHOR:

Name:	Shaun Brinsmade	
Department:	Biology	
Institution:	Georgetown University	
Article Title:	A fluorescence-based method to study bacterial gene regulation in infected tissues.	
Signature:		Date: <div>Text</div>

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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 Em^r and is susceptible to 5 µg 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 1×10^7 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 P_{sarA} -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.