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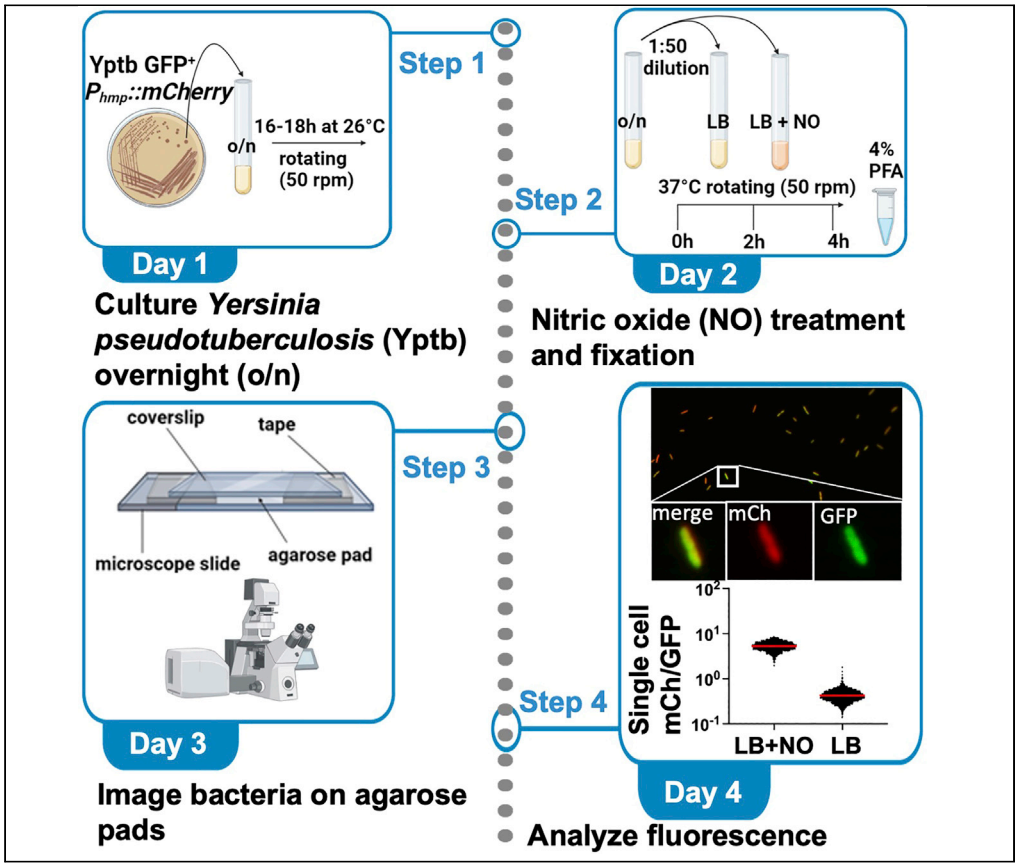
Protocol for detecting *Yersinia pseudotuberculosis* nitric oxide exposure during *in vitro* growth



Yersinia pseudotuberculosis (Yptb) is a bacterial pathogen that causes foodborne illness. Defense against the host antimicrobial gas, nitric oxide (NO), by the bacterial NO-detoxifying gene, *hmp*, promotes Yptb replication in mouse models of infection. Here, we detail the use of fluorescent signals as readouts for NO exposure within individual cells and subsequent detection of heterogeneity within a population, using single-cell imaging and analysis. This protocol quantifies NO exposure in culture, without capturing the full complexity of the host environment.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Quantify nitric oxide (NO) exposure during *in vitro* growth of bacteria

Use fluorescent signals as readouts for NO exposure within individual cells

Detect heterogeneity within a population using single-cell imaging and analysis

Approach can be easily applied to other bacterial species

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Protocol for detecting *Yersinia pseudotuberculosis* nitric oxide exposure during *in vitro* growth

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SUMMARY

***Yersinia pseudotuberculosis* (Yptb) is a bacterial pathogen that causes food-borne illness. Defense against the host antimicrobial gas, nitric oxide (NO), by the bacterial NO-detoxifying gene, *hmp*, promotes Yptb replication in mouse models of infection. Here, we detail the use of fluorescent signals as readouts for NO exposure within individual cells and subsequent detection of heterogeneity within a population, using single-cell imaging and analysis. This protocol quantifies NO exposure in culture, without capturing the full complexity of the host environment.**

For complete details on the use and execution of this protocol, please refer to Patel et al. (2021).

BEFORE YOU BEGIN

The protocol below describes the specific steps for quantifying nitric oxide (NO) exposure during *in vitro* growth of *Yersinia pseudotuberculosis*. We use our wild type (WT) strain IP2666 containing our GFP$^+$ plasmid and fluorescent reporter for *hmp* expression (P_{hmp}*::mCherry*) plasmid (see key resources table), which respectively confer constitutive GFP expression (GFP$^+$) and mCherry reporter expression driven by the promoter for *hmp* (P*hmp*), a nitric oxide detoxifying gene (Davis et al., 2015). Although we focus on NO exposure in culture in the presence of a chemical NO donor (DETA NONOate) in this protocol, we have also used this specific strain to quantify exposure of *Y. pseudotuberculosis* cells to extracellular NO stress during *in vivo* murine infection (Davis et al., 2015; Raneses et al., 2020).

Additionally, the *hmp* gene and its homologs are highly conserved throughout different bacterial species and can be found in both Gram-negative and Gram-positive bacteria, from *Escherichia coli* (Membrillo-Hernández et al., 1998; Stevanin et al., 2007) to *Bacillus subtilis* (LaCelle et al., 1996), and *Mycobacterium tuberculosis* (Hu et al., 1999), and even in the fungi *Saccharomyces cerevisiae* (Zhao et al., 1996). Thus, use of this P_{hmp} system and our NO exposure protocol could be broadly applicable to other microbial pathogens. As such, bacterial strains of interest must already be acquired or transformed with the GFP$^+$ and P_{hmp}*::mCherry* plasmids (see key resources table and materials availability) according to your specific pathogen's transformation protocols, prior to beginning the protocol outlined here.

To assess bacterial responses to NO treatment, the mCherry reporter signal intensity will be divided by the constitutive GFP signal intensity to generate a mCherry/GFP value that represents relative reporter expression. The mCherry/GFP values in response to NO treatment will then be normalized to

the values for untreated samples to serve as the readout for NO exposure during *in vitro* growth. Constitutive GFP detection is needed to assess the overall transcriptional and translational activity of each bacterial cell. Fluctuations in these activities would result in global changes to fluorescent protein expression, and reporter signal alterations could be misinterpreted to have biological significance in the absence of this important control. Staining methods, such as DAPI, or whole-cell labeling approaches, would lack this control for levels of transcription and translation. For example, fluorescent signals can appear quenched in bacterial cells with reduced metabolic activity, or appear higher in more metabolically-active cells. A constitutive GFP signal allows for another layer of control, so that mCherry signals can be attributed to P_{hmp} expression, and not a question of viability or changes in global metabolic activity.

In addition, a control strain that lacks the P*hmp* reporter (e.g., IP2666 GFP$^+$) can be used as a baseline control for background mCherry fluorescence (see troubleshooting, problem 2 section). Please refer to the materials availability section below for information on these reporter strains and plasmids.

To prepare for the **Major Steps** of the NO exposure experiment outlined below, overnight (o/n) cultures of the strains of interest must be prepared (see preparing the bacterial culture). On the day of the experiment, working concentrations of both the slow-releasing NO donor (DETA NONOate) and 4% paraformaldehyde (PFA) for cell fixation must be prepared fresh (see preparing the working stocks).

Institutional permissions

This protocol involves working with a Biosafety Level-2 (BSL2) pathogen, *Yersinia pseudotuberculosis*. You will need to obtain the proper approval from your Institutional Biosafety Committee to work with biohazards prior to experiments with *Y. pseudotuberculosis* and other BSL2 pathogens.

Preparing the bacterial culture

Timing: 10–15 min (16–18 h before experiment)

1. Prepare the bacterial strains of interest for an overnight (o/n) culture (16–18 h). Here, we will prepare a culture of the NO-exposure reporter strain (IP2666 GFP$^+$ P_{hmp}*::mCherry*). Our NO-exposure reporter strain *Yersinia pseudotuberculosis* (Yptb) has constitutive GFP expression (GFP$^+$), and mCherry reporter expression driven by the *Phmp* promoter (Davis et al., 2015).
   a. Streak the strain of interest from a glycerol freezer stock for single colonies on selective LB agar plates. Our strain requires LB agar plate with chloramphenicol (Cm, 25 μg/mL) and carbenicillin (Cb, 100 μg/mL).
      i. For Yptb strains, this requires 2 days of growth at 26°C, as previously described (Davidson and Davis, 2020).
      ii. Alternatively, if no 26°C incubator is available, Yptb plates can be grown on the benchtop at room temperature for 2 days.
   b. On the second day, use the plate to start an o/n culture. Culturing Yptb does not differ from traditional microbiological techniques, however, for complete details on how to streak and culture this bacterium, please refer to: (Davidson and Davis, 2020).
      i. Briefly, the IP2666 GFP$^+$ *Phmp::mCherry* reporter strain should be cultured in 2 mL LB broth + chloramphenicol (Cm, 25 μg/mL) + carbenicillin (Cb, 100 μg/mL) to maintain the GFP$^+$ and *Phmp::mCherry* containing plasmids, respectively.
      ii. For each o/n culture, pick one colony from the agar plate using a sterilized inoculating loop or a sterile wooden stick, and swirl in a glass culture tube with 2 mL LB media (+ antibiotics). All colonies on the plate should have similar, uniform morphology if Yptb is growing normally. We typically culture bacteria in glass tubes, but plastic tubes could be used as well.

c. Prepare one tube for each biological replicate. We recommend preparing 3 biological replicates (one separate colony into an o/n culture per biological replicate) for each strain.

2. Incubate the culture tubes with rotation at 50 rpm in a rotor (or in an orbital shaker at 200 rpm) o/n (16–18 h) at 26°C.
   a. Growth at 26°C is specific to Yptb. The culture temperature can be altered if different bacterial species are used.

Preparing the working stocks

Timing: ~5–10 min (on the day of the experiment)

3. Weigh and dissolve DETA NONOate, a slow-release NO donor, into LB at a final concentration of 2.5 mM.
   a. Use 4.0795 mg of DETA NONOate for every 10 mL of LB (see key resources table).
      i. Calculate how much total LB you will need if you use 3 mL LB per sample.
      ii. Our LB recipe: 10 g/L NaCl, 10 g/L tryptone, 5 g/L yeast extract dissolved in distilled water (pH7.0), and autoclaved. This should be prepared ahead of time, and will be used for the overnight (o/n) cultures as well.
4. Prepare LB without DETA NONOate to use for the control, untreated group.
5. Prepare fresh 4% paraformaldehyde (PFA) diluted in 1× PBS to use for cell fixation.
   a. Our main stock is 16% PFA (see key resources table). You will need 200 µL/sample for fixation. Calculate how much total 4% PFA you will need for all your samples.

⚠ CRITICAL: 2.5 mM of DETA NONOate inhibits Yptb growth in the first 4 h of treatment, after which bacteria can recover and start growing in the presence of NO. If using another pathogen, growth curves with dose responses in different DETA NONOate concentrations would first be needed to determine the desired concentration specific to your pathogen.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
IP2666 GFP$^+$ *Phmp::mCherry* (species: *Yersinia pseudotuberculosis*)	(Davis et al., 2015)	N/A
Chemicals, peptides, and recombinant proteins		
DETA NONOate	Cayman Chemical	CAS: 146724-94-9
Recombinant DNA		
P_{hmp}::*mCherry* plasmid (pMMB67EH)	(Davis et al., 2015)	N/A
GFP$^+$ plasmid (pACYC184)	(Crimmins et al., 2012; Davis et al., 2015)	N/A
Software and algorithms		
Zen2 image acquisition software	ZEISS	N/A
Volocity image analysis software	N/A	https://www.volocity4d.com/
ImageJ software	N/A	https://imagej.nih.gov/ij/
MicrobeJ software	(Ducret et al., 2016)	https://www.microbej.com/download-2/
Other		
16% Paraformaldehyde (w/v aq. soln.)	Alfa Aesar	CAS: 50-00-0
Agarose RA	VWR	N605-500G
LB (for liquid media)	Sigma	L3522-1KG
LB agar (for plates)	Sigma	L3147-1KG
1× PBS	Gibco	Ref: 10010-023

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chloramphenicol	Sigma	C0857-25G
Carbenicillin	Sigma	C1389-10G
96-well cell culture plate (sterile, flatbottom with lid)	Greiner Bio-One	Cat: 655-180
Synergy H1M microplate reader	BioTek	N/A
Microscope slides	VWR	Cat: 48300-026
Cover slips	VWR	Cat: 48366-045
16 × 150 mm Glass culture tubes	VWR	Cat: 10545-944
Zeiss Axio Observer.Z1 fluorescent microscope	ZEISS	N/A
Immersion oil, Immersol 518F	ZEISS	Cat: 444970-9010-000
26°C incubator	VWR	Cat: 89508-420
37°C incubator	VWR	Cat: 89511-430
Digital Dual Heat Block	VWR	Cat: 12621-088

MATERIALS AND EQUIPMENT

LB + DETA NONOate		
Reagent	Final concentration	Amount
DETA NONOate	2.5 mM	4.0795 mg
LB	25 g/L	10 mL

DETA NONOate powder should be stored at temperatures between −20°C to −70°C in a dessication jar; only prepare the needed amount per experiment. DETA NONOate powder can be stored for long periods of time at this temperature, please see the expiration date on each particular bottle for recommended time of storage. Once dissolved in LB liquid, the DETA-NONOate compound has a half-life of 24 h at room temperature, and so this cannot be stored for future experiments. Sterile liquid LB should be stored at room temperature, and can be stored un-opened for one year or longer. We typically use sterile opened LB within 6 months of opening.

⚠ CRITICAL: DETA NONOate was not formulated for human or veterinary use. Although it is non- hazardous, it is best to avoid inhalation and consumption of this product. We recommend working with this product in a chemical hood.

IP2666 strains are BSL-2 pathogens. Apply BSL-2 sterile techniques and precautions.

Alternatives: A fast-release NO donor is also available: Diethylamine NONOate from Sigma (CAS: 56329-27-2). Depending on the pathogen and purposes, this may be used instead of the slow-release NO donor (DETA NONOate) referenced in this protocol. We have found that our Yptb IP2666 strains are strongly growth-arrested in the presence of this fast-release NO donor.

For issues with leaky *Phmp::mCherry* expression in the absence of the NO donor, our IP2666 GFP$^+$ strain with the GFP$^+$ plasmid (pACYC184) from (Davis et al., 2015) may be used as a control for true background fluorescence (see troubleshooting, problem 2 for details).

STEP-BY-STEP METHOD DETAILS

Part one: Growth during NO exposure

Timing: 4–6 h

This is the main NO-exposure experiment. Bacterial cells are grown in the presence or absence of the NO donor. The GFP$^+$ *Phmp::mCherry* strain will identify bacteria that are responding to the NO treatment, since *Phmp* should drive mCherry expression in the presence of NO, while the untreated control will serve as the baseline for background mCherry expression. Every two hours after treatment, aliquots of samples are measured for absorbance (A600 nm) to assess bacterial growth or growth inhibition by NO treatment. Additionally, aliquots are taken for fixation. These fixed samples will be used for microscopy imaging in **Major Steps Two** and **Three**.

1. From the o/n cultures, dilute bacterial cells 1:50 into fresh LB or fresh LB + DETA NONOate prepared earlier (See preparing the working stocks).
   a. For Yptb growth, we typically start with 3 mL LB per tube.
      i. Add 60 μL of overnight (o/n) culture to 3 mL LB or LB + DETA NONOate.
2. Collect cells for the t = 0 timepoint.
   a. Aliquot 500 μL from each culture tube.
      i. For each sample, put 200 μL into a 96-well plate and 300 μL into a microcentrifuge tube. Place the microcentrifuge tube on ice.
      ii. Place the culture tubes from step 1 at 37°C with rotation at 50 rpm in a rotor (or in an orbital shaker at 200 rpm), to begin incubation of the samples.
      iii. Add LB only (without bacterial cells) into one well of the 96-well plate to use as a blank.
   b. Take the 96-well plate from step 2ai. Check the OD600 of the t = 0 samples in a plate reader, using a well containing LB without bacteria as a blank. Save and record these values.
      i. Any resulting OD_{600} value from the blank well should be deducted from the sample values in order to get the true absorbance value.
      ii. A typical OD600 of the t = 0 sample is ~0.02.
   c. Take the entire 200 μL out of each well and transfer into the corresponding microcentrifuge tube with the other 300 μL saved on ice (this ensures there are enough bacterial cells in the samples to analyze for microscopy, especially at t = 0 and since samples will be growth arrested in the presence of NO).
      i. Based on the typical growth of Yptb, we estimate there to be approximately 1–5 × 10^7 total CFUs (colony forming units) in this 500 μL sample. 1 × 10^7 CFUs being on the low end for a growth arrested or 0 h timepoint sample and 5 × 10^7 CFUs being on the high end for a 4 h timepoint sample that is growing very well.
3. Fix the collected samples with 4% PFA (See preparing the working stocks).
   a. Centrifuge samples in the microcentrifuge tubes at in a benchtop microcentrifuge at 15,000 rpm (20,627 × *g*) for 1 min at room temperature. Discard the supernatant by aspirating.
   b. Resuspend each pellet with 200 μL of 4% PFA.
   c. Allow fixation 12–16 h at 4°C in the dark (or by covering tubes with aluminum foil).
4. Every 2 h, repeat steps 2 and 3 above, until 4 h.
   a. Check the plate reader raw data to make sure the cells incubated in LB + DETA NONOate are growth-arrested compared to the samples grown in LB only (lower A600 nm, no change in values between t = 0 and t = 4 h). For problems, refer to the troubleshooting, problem 1 section below.
5. Incubate the culture tubes from step 1 with rotation (50 rpm in a rotor, or in an orbital shaker at 200 rpm) at 37°C for a total of 4 h.

Summary comment: At the end of this experiment, you should have samples collected and fixed at t = 0, 2, and 4 h for both treated (LB + DETA NONOate) and untreated (LB only) groups.

⚠ CRITICAL: Yptb starts to recover from *in vitro* NO exposure around 4 h and tends to start normal growth at this timepoint. If working with other pathogens, prior growth curves with the preferred NO concentration may be needed in order to determine the length of time it takes for your pathogen of choice to recover and start normal growth after NO exposure.

Pause point: Collected cells can be fixed and incubated at 4°C for a maximum of 7 days. Prior to day 7, proceed to **Major Step Two**. All samples from a single biological replicate should be processed on the same day, together.

Note: A plate reader is not necessary to measure A600. Any other means of measuring A600 will suffice, as long as an appropriate volume of sample is taken. Avoid taking large amounts of samples (>500 μL) as this will decrease the total volume left in the culture and can affect the growth of the bacteria.

Part two: Agarose pad preparation

Timing: ~3 h, for three biological replicates; approximately 1 h/biological replicate/6 samples (1–7 days after NO experiment)

In this step, cells that were fixed from the NO exposure experiment will be processed on microscope slides with agarose pads in preparation for the microscopy in **Major Step Three**. Agarose pads are used here to immobilize bacterial cells for imaging, to ensure bacterial cells have not moved between capturing images in different fluorescent channels. Otherwise, bacterial cells in liquid suspension can move (e.g., with Brownian motion), thus preparation of the agarose pads is an important step prior to imaging (**Major Step Three**). Please keep in mind that the agarose pad preparation is time sensitive; agarose pads will begin to evaporate and need to be used within 4 h of preparing.

6. Prepare the agarose.
   a. Dissolve 1% agarose in PBS (typically 10 mL PBS + 100 mg agarose) using a microwave.
   b. Place the beaker containing the agarose solution on a heat block set at 70°C. This prevents the agarose from solidifying.
      i. Use the smallest volume of beaker possible, to ensure that more of the agarose solution is being heated on the heat block.
7. Prepare microscope slides by adding two strips of labeling tape on top.
   a. The distance between the two strips of tape should not be bigger than the width of the cover slip being utilized (see Graphical Abstract).
   b. Firmly press down on the strips of tape to ensure they are fully adhered to the slide.
   c. Place the slides on the 70°C heat block (see key resources table for an example heat block). This ensures that the slide is warm enough for the agarose to spread on the surface.
8. Cut the end off of a 20–200 μL pipet tip to make a beveled edge (Figure 1A).
   a. Set the pipet volume to 50 μL (with tip cut off, this will pipet approximately 25 μL).
   b. Pipet the 1% agarose from the beaker.
9. Immediately add the agarose in between the tape strips, and immediately add the coverslip (Figure 1B).
   a. Allow agarose to spread on the slide surface (~3–5 s) before removing the slide from the heat block.
   b. Let the agarose solidify at room temperature on a bench for ~20 min.
10. Use a razor blade to peel off the coverslip (Figure 1C), then carefully pull off the tape strips. For problems, check out the troubleshooting, problem 3 section below.
    a. Clean any excess agarose off of the slide.
    b. It may be necessary to cut off the edges of the agarose pad to even out the sides. It is best to have the agarose pad as flat and even as possible.
11. Prepare the fixed bacterial cells.
    a. Take out the fixed cells from the 4°C fridge.
    b. Centrifuge samples in a tabletop microcentrifuge at 15,000 rpm (20,627 × *g*) for 1 min at room temperature. Discard the supernatant by aspirating.

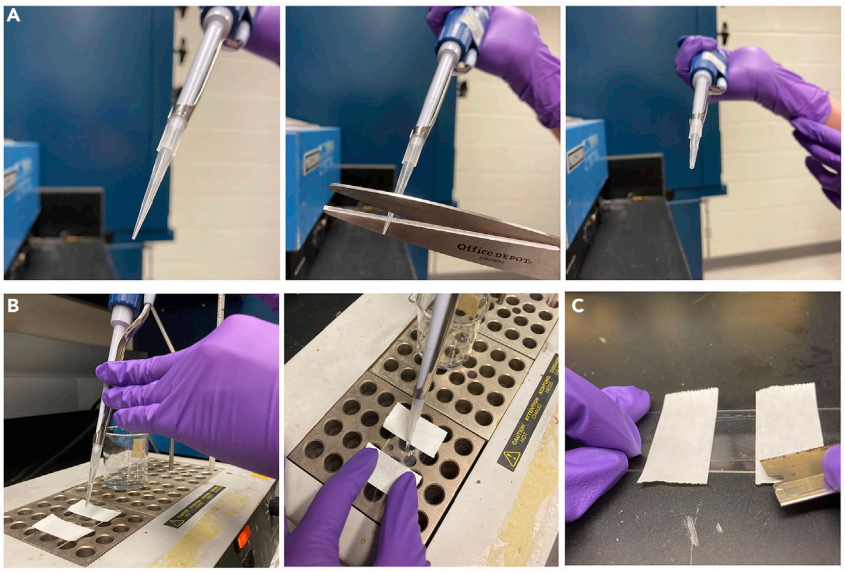

Figure 1. Agarose pad preparation

(A) The end of the pipet tip is cut off to make a beveled edge. The pipet is set to 50 μL so that approximately 20 μL will be pipetted with the beveled edge pipet tip.

(B) Glass microscope slides are prepared with pieces of tape evenly distanced apart (such that a coverslip can lay evenly on top of the pieces of tape) and set on a hot plate. Approximately 20 μL of agarose is pipetted onto the glass slide in between the pieces of tape. A coverslip is immediately placed on top so that the agarose spreads evenly underneath the coverslip between the pieces of tape and allowed to cool for 20 min at room temperature.

(C) After the agarose solidifies, the first coverslip can be taken off with a razor blade, in preparation for adding the fixed bacterial cells on top of the agarose pad with a fresh coverslip.

c. Resuspend cells in 50 μL of 1× PBS (depending on the growth of your particular bacterium, the volume used to resuspend cells can vary in order to have an optimal density of bacterial cells when imaging; see troubleshooting, problem 4 below).
d. Add 5–8 μL fixed bacteria (from steps 3–4) to the center of the agarose. We estimate the concentration of the fixed bacterial sample to be approximately 1–5 × 10^6 CFUs total.
e. Add a fresh coverslip on top.
   i. The cells are now ready to image. Proceed immediately to **Major Step Three.**

⚠ CRITICAL: After adding the bacterial cells on top (after step 11e), the agarose pads can dry out after a couple of hours (~4 h). Once prepared, microscope imaging must be done within that window. Otherwise, the agarose pad preparation steps will have to be redone. It is best to have the agarose pad as flat and even as possible. Unevenness will make it difficult to focus on the bacteria when imaging as some bacteria may be in a different plane of view.

Optional: Fixed cells resuspended in PBS (step 11) can be kept at 4°C for a few days, in case samples need to be re-imaged.

Part three: Microscopy and single cell analysis

Timing: ~2 h, depending on number of samples (imaging must be performed immediately after agarose pad preparation)

In this step, samples from the NO exposure experiment will be imaged via fluorescence microscopy. Here, we will describe single cell analyses of these images to determine individual cells' fluorescent

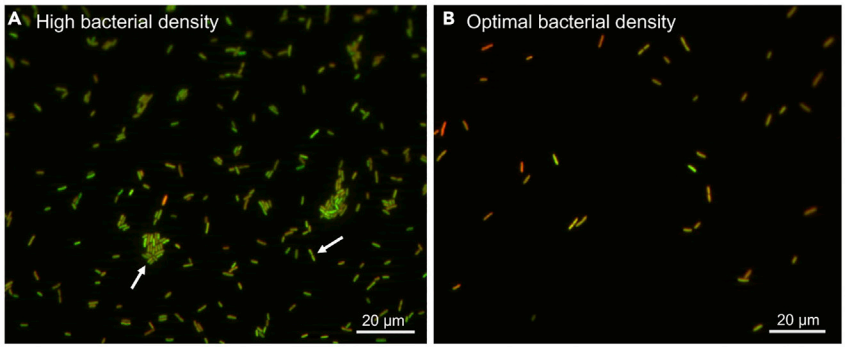

Figure 2. Representative images

(A) Representative image of a dense area of bacterial cells. Using image analysis software, bacterial cells will be identified by their constitutive GFP signal and size. A size selection criterion of 1 μm^2–6 μm^2 is typically used to select for single cells. White arrows point to an aggregate of multiple bacteria (left) and two bacteria in a doublet (right), indicating bacteria that would be excluded from analysis since they are not single cells.

(B) Representative image of an optimal density of bacterial cells. Almost all cells in this field of view, other than a few doublets, will be included in the analysis. Scale bars: 20 μm.

expression; GFP expression will be constitutive and consistent across cells, while mCherry expression is dependent on the levels and length of NO exposure.

12. Under 63× oil immersion objective (1.4 numerical aperture), take 5 images at distinctly different fields of view.
    a. For single cell analysis specifically, it is useful to choose fields of view that already have single cells or cells that are more spread apart (Figure 2). For problems, check out the troubleshooting, problem 4 section below.
    b. We use a Zeiss Axio Observer.Z1 fluorescent microscope with Colibri.2 LED light source and an ORCA-R2 digital CCD camera, but alternative fluorescent microscopes with the appropriate channels could be used.
    c. With the strain used here, the DIC, GFP, and mCherry channels need to be utilized.
        i. Use the DIC to focus on bacterial cells. Avoid scanning and focusing using one of the fluorescent channels, since this could potentially lead to photobleaching.
        ii. When capturing images, keep exposure times of the fluorescent channels consistent between samples and replicates. Exposure times we have used in the past include: 75 ms for GFP and 240 ms for mCherry. However, users need to keep in mind this is only a starting point, and they may need to use different settings.
        iii. Look through both $mCherry^+$ and $mCherry^-$ samples beforehand to determine optimal exposure times and minimize pixel saturation or background signal. The same mCherry and GFP exposure times need to be used for all imaging. GFP signal should be consistent throughout the samples, unless a sample has very high cell density. Higher cell density correlates with higher fluorescent signals, and is not optimal for single cell analyses (see Figure 2).

Pause point: Images can be saved and stored at this point, with the next step of single cell analysis completed at another time.

13 .Perform single cell analysis using Volocity software.
    a. In order to open images on Volocity software, CVI images from Zeiss software must first be converted into ZVI images.
    b. To identify bacteria specifically as objects (Find Objects), use the constitutive GFP signal as a reference, and specify size range of the objects from 1 μm^2 to 6 μm^2, anything larger or

smaller must be excluded. This highlights single cell bacteria only and excludes doublets or cells in groups (Figure 2).

i. DIC cannot identify bacteria as objects well, and using mCherry as the reference for identifying bacterial cells as objects prevents identification of samples grown without the NO donor (no mCherry expression), or cells that had low NO exposure.

ii. It is important to have all bacteria within the field of view appropriately focused on, otherwise fluorescence values will be impacted. If the agarose pad is uneven and causing bacteria to be in different planes of view, the image can be cropped to select for only bacteria that are appropriately focused and in the same plane of view.

c. Record the mean single cell GFP and mCherry signals for each image. If you are using Volocity software (https://www.volocity4d.com/), this can be compiled using the Measurement item function, and exported into a single excel spreadsheet.

d. Graph and perform further statistical analyses as needed (see quantification and statistical analysis).

Optional: Analyses can also be performed differently, using other software such as Zen, ImageJ, or MicrobeJ. Please see an alternative method of analyzing the captured images using the free software program, MicrobeJ, below.

14. The following is an alternative method of analyzing the captured images using MicrobeJ (Ducret et al., 2016).

a. Open images in ImageJ/Fiji in their raw format (our images taken with Zeiss are in CZI format). This will open the 'Bio-Formats Import Options' tab where you can select how the image will be imported. We recommend selecting 'split channels' and keeping all other default options. This will split the image into the individual channels (for our images, 'C=0' corresponds to GFP, 'C=1' corresponds to mCherry, and 'C=2' corresponds to DIC).

b. In MicrobeJ under the 'Images' tab, change 'Image' to 'Channel(s)'. This allows MicrobeJ to recognize each channel appropriately. We recommend assigning 'C=1' to 'Ch. 1' for mCherry and 'C=0' to 'Ch.2' for GFP. To the right of these dropdown menus, click the camera icon. This merges the images into a hyperstack and allows you to analyze all channels at once.

c. Identify individual bacterial cells under the 'Bacteria' tab. In the leftmost dropdown menu, select the channel associated with GFP (Channel 2). This makes the identification of bacteria more consistent across samples since all samples will have constitutive GFP.

d. Immediately to the right, in the next dropdown menu, select "Dark" instead of "Bright" to indicate that the background is darker than the objects ("Bright" is used for phase contrast images, which have a lighter background in comparison to fluorescence images).

e. In the first dropdown menu below, change 'Medial Axis' to 'Fit Shape', this should automatically switch the adjacent dropdown (fit mode) to 'Rod-Shaped' which is suitable for detecting Yptb cells, but other "fit modes" are available for other bacterial shapes.

f. Set area (μm^2) to 1–6 and click the blue play button at the bottom of this dialog box to test the current settings. Collected cells will be outlined in green and excluded cells will be outlined in red. This should select for single cell bacteria, but the area can be set differently depending on the size of your particular bacterium.

g. If the area parameters are unable to exclude some bacteria that are clumped together or in doublets, there is an 'Experiment Editor' function (utilized by clicking on the pencil icon located in the bottom right) that will allow you to select individual objects and exclude them from analysis. However, this function is best used only after you are ready to run all of the parameters (see step h) i. below).

h. Under the 'Options' box, immediately to the right, select "Intensity" to measure the mean signal intensity for each channel.

i. Click the blue play button in the very bottom right corner to run all of the settings, then display the results table using the icon in the far right bottom corner.

j. If some bacteria need to be excluded from analysis use the 'Experiment Editor' function and select bacteria that are in clumps or doublets and remove them using the trash can icon in the bottom right. Once finished, click the results icon at the top within the 'Experiment Editor' tab. This will generate a results table with only the bacteria that you want to include in the analysis. If you click the results icon within the 'Bacteria' tab, the bacteria you selected for exclusion with the 'Experiment Editor' will still be included.

k. In the 'Result' tab, 'Bacteria' should be listed and a table showing the measurements for each single bacterium should be displayed.
   i. To measure the mean mCherry intensity for each cell, type the following formula into the boxes above the table "mCh int mean = bacteria.INTENSITY.ch1.mean". This will generate a new column in the table named "mCh int mean" with the mean fluorescence intensity of mCherry for each individual bacterial cell.
   ii. To measure the mean GFP intensity for each cell, type the following formula into the boxes above the table "GFP int mean = bacteria.INTENSITY.ch2.mean". This will generate a new column in the table named "GFP int mean" with the mean fluorescence intensity of GFP for each individual bacterial cell.
   iii. Record these values and perform further analyses as needed (see quantification and statistical analysis).

Note: Intensity values range from 0-16383 for a 16-bit image. If the image is overexposed, all cells will have a maximum intensity value of 16383. When taking images, choose exposure times carefully to prevent pixel saturation (see step 12. c. iii).

⚠ CRITICAL: Unevenness in the agarose pad will impact analysis. MicrobeJ is sensitive to bumps in the agarose pad and will attempt to identify curvature in the agarose pad as bacteria. Additionally, MicrobeJ will be unable to identify bacteria adequately if cells are in different planes of view. To circumvent this, use ImageJ to draw a box around only the bacteria that are appropriately focused on and in the same plane of view. Only bacteria within this box will be identified and measured for fluorescence intensity.

EXPECTED OUTCOMES

For this NO exposure experiment using a GFP$^+$ P_{hmp}::*mCherry* reporter strain, mCherry signal should increase in response to the NO treatment. Mean single cell mCherry signal for the NO treated sample can be graphed side by side with the untreated sample (Figure 3A) to show how the *Phmp::mCherry* reporter signal compares to background mCherry levels in the untreated sample. Additionally, mean mCherry signal of the NO treated cells can be normalized to the average mCherry signal of the untreated sample to represent fold change in mCherry signal in response to NO treatment (Figure 3B).

QUANTIFICATION AND STATISTICAL ANALYSIS

Volocity analyses will yield raw fluorescence values of mean mCherry and GFP signals for each cell (Table 1). The resulting mCherry signals should be normalized to the corresponding constitutive GFP signal per cell. To do this, simply divide the mean single cell mCherry value by the mean single cell GFP value for every cell (Table 2, Figure 3A).

To further normalize single cell values to the untreated samples (-NO), first take the average fluorescence signal of each timepoint for the untreated samples (Table 2). Then, divide each single cell value of the treated samples (+NO) by the average fluorescence value of the untreated samples for each timepoint. This results in values of 1 (fluorescence of the treated sample is equal to the baseline fluorescence of the untreated samples) or above 1 (mCherry signal is higher than baseline) to represent fold change in mCherry signal in response to NO treatment (Table 2, Figure 3B). For statistical analyses of the finished graphs (Figure 3), we recommend using Kruskal-Wallis one-way

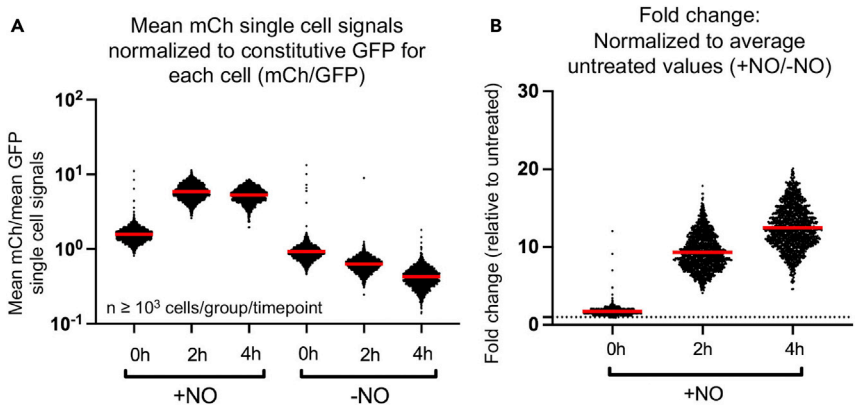

Figure 3. Expected outcomes; mCherry levels increase upon NO exposure

(A) The mean single cell mCherry fluorescence signal is normalized to the mean single cell GFP fluorescence signal for each cell analyzed (mean single cell mCh/mean single cell GFP). NO treated cells (+NO) for each time point are graphed side by side with the untreated controls (-NO).

(B) Data from (A) can also be normalized to the average signal of the untreated samples (+NO/-NO) to represent fold change in mCherry/GFP ratio in response to NO treatment. Dashed line at 1 represents fluorescence value that is equal to the baseline fluorescence of the untreated sample. (A and B) Solid bar lines represent median values.

ANOVA with Dunn's posttest to assess significant differences between the different groups or timepoints.

We typically collect between 20 to as many as 100 cells/image. With 5 fields of view, this would equate to 500 cells/biological replicate, and across three biological replicates equate to 1,500 cells. The data shown in Figure 3 are close to these values. However, with 20 cells/image, we would be collecting closer to 300 cells for the 3 replicates. We ensure the collection and analysis is equivalent in terms of numbers for each experimental dataset.

LIMITATIONS

This protocol has been optimized for single cell analyses of the bacterial population via fluorescence microscopy after growth in the presence or absence of NO, not for fluorescent reads with a plate reader. Fluorescence from a plate reader could be used as a raw readout and quick check for fluorescent signal, but plate readers measure the overall fluorescence of the population and will not detect how individual cells within the population are responding to NO stress.

TROUBLESHOOTING

Problem 1

Unexpected growth kinetics in the presence of the NO donor.

Samples are not growth-arrested in the presence of the NO donor, DETA NONOate, or are only growth-arrested for a short time frame then continue normal growth kinetics (see Protocol step 4A).

Table 1. Example raw data for four single cells from each timepoint and treatment condition

Raw data (mean single cell mCherry signal)						Raw data (mean single cell GFP signal)					
+NO			-NO			+NO			-NO		
0 h	2 h	4 h	0 h	2 h	4 h	0 h	2 h	4 h	0 h	2 h	4 h
1643.73	15845.19	16380.64	1689.35	2070.02	1499.7	1454.5	2917.48	2289.42	1512.88	2302.67	4295.64
2078.45	15434.61	16077.28	1535.03	1389.64	2297.99	1723.44	2956.28	2072.41	1600.71	2443.3	3633.75
2127.5	14084.64	16383	1314.63	1483.64	1941.47	1523.06	2795.78	2942.98	1848.04	1991.08	6417.79
1683.21	16120.57	16383	1534.11	1662.04	1412.23	1291.72	2670.46	3439.68	1684.96	2454.4	6153.31

Table 2. Example of normalized data from four single cells

Normalized to GFP (mCherry/GFP)						Normalized to untreated (+NO/-NO)		
+NO			-NO			+NO		
0 h	2 h	4 h	0 h	2 h	4 h	0 h	2 h	4 h
1.13010	5.43112	7.15493	1.11665	0.89897	0.34912	1.22258	7.49110	18.9089
1.20599	5.22096	7.75777	0.95897	0.56876	0.63240	1.30468	7.20122	20.5020
1.39686	5.03782	5.56681	0.71136	0.75514	0.30251	1.51112	6.94862	14.7118
1.30308	6.03663	4.76294	0.91047	0.67717	0.22951	1.40971	8.32527	12.5873
Average			**0.92436**	**0.72501**	**0.37839**			

Potential solution

2.5 mM of DETA NONOate inhibits growth of Yptb for about 4 h, then recovery occurs through detoxification, and cells start growing in the presence of NO.

- If using Yptb, some growth is expected towards the end of the experiment (Liu et al., 2022).
- If using another pathogen, growth curves with dose responses in different DETA NONOate concentrations would first be needed to determine the desired concentration specific to your pathogen.
- Ensure that the strain used has normal growth kinetics without DETA NONOate (e.g., not slow-growing or fast-growing relative to the parental WT strain). Different normal growth kinetics of the strain, irrespective of the NO donor, may affect this experiment.

Problem 2

Leaky NO reporter expression.

If the NO reporter strain (in this protocol, we use *Phmp::mCherry*) has high detectable mCherry expression in the absence of the NO donor (LB only), the NO reporter may be "leaky." We have not observed this in our IP2666 GFP$^+$ *Phmp::mCherry* strain, but this may occur if the *Phmp::mCherry* plasmid is transformed into other strains or pathogens. See notes in before you begin and materials and equipment, Alternatives.

Potential solution

- A constitutive fluorescent strain without the NO reporter plasmid can be used, such as our IP2666 GFP$^+$ strain to determine the amount of background fluorescence.
  - Any mCherry readouts from this strain is true background fluorescence, and can be deducted from all mCherry signals in the NO reporter strain. See materials availability below.

Problem 3

Agarose pad difficulties.

When preparing the agarose pads, there could be difficulties in ensuring the agarose stays on the surface of the slide, and isn't pulled off the slide with the cover slip. Other problems with the agarose pad preparation may include uneven surfaces or bubbles on the agarose pad (see Protocol step 10).

Potential solution

- For agarose pads staying on the slide surface:
  - Ensure that the solution being used is 1% agarose in 1× PBS
    - TAE and similar buffers will be more difficult to work with, as they do not solidify properly on the slide surface.

- If problem persists with the correct agarose solution, try waiting for the agarose pad to dry out/solidify for 30 min, instead of 20 min.

- For other agarose pad preparation problems:
  - Ensure the razor blade is only lifting the cover slip off of the slide, and not the agarose pad. Partial lifting of the pad could cause uneven surfaces.
  - Ensure no bubbles are pipetted out when pipetting the agarose onto the slide to minimize bubbles on the agarose pad.
  - When needed, use the razor blade to cut away small sections of uneven or bubbled agarose pads.

Problem 4

High cell density/clumping on images.

When imaging the cells, some samples may contain high cell density and/or clumping of too many cells (Figure 2A), which hinders the single cell analyses. We estimate we add approximately 1×10^5 CFU to each agarose pad (see Protocol steps 11c and 12a).

Potential solution

- Ensure that the cells are resuspended in 1× PBS thoroughly.
- Add more volume of 1× PBS to decrease the overall concentration of cells in the sample.
- For bacterial strains that naturally aggregate or clump more easily, vortex each sample for at least 10 s after resuspension in 1× PBS, and before pipetting cells onto the agarose pad.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Kimberly M. Davis (kdavi140@jhu.edu).

Materials availability

This study did not generate new unique reagents. The laboratory will provide the plasmid constructs described here per request. Please reach out to the lead contact (kdavi140@jhu.edu) with plasmid requests.

Data and code availability

No codes were generated; all datasets generated and analyzed with this protocol are included in the referenced published study (Liu et al., 2022).

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AUTHOR CONTRIBUTIONS

Conceptualization: R.E.B., K.L.C., K.M.D.; formal analysis: R.E.B., K.L.C.; funding acquisition and supervision: K.M.D.; investigation: R.E.B.; methodology: R.E.B., K.L.C., K.M.D.; writing – original draft: R.E.B., K.L.C.; writing – review and editing: R.E.B., K.L.C., K.M.D.; visualization: R.E.B., K.L.C.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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