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Single-cell, Time-lapse Reactive Oxygen Species Detection in *E. coli*

Zhilin Yang¹ and Heejun Choi²

¹Department of Chemistry, University of Wisconsin-Madison

²Janelia Research Campus, Howard Hughes Medical Institute

Abstract

We have developed single-cell, time-lapse assays to detect reactive oxygen species (ROS) in live *E. coli*. The assay utilizes flow systems on a fluorescence microscopy to correlate symptoms aroused from biological or chemical perturbations with the in situ detection of ROS. ROS is detected by fluorogenic dyes that accumulate inside the cell, allowing detection of ROS in single cells in both homogeneous and heterogeneous samples using CellROX Green and Amplex Red/APEX2.

Keywords

Reactive Oxygen Species; *E. coli*; fluorescence microscopy; single-cell; time-lapse

Introduction

Aerobically growing organisms constantly face challenges imposed by opportunistic reduction of oxygen molecules. Reduced oxygen molecules such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical ($\cdot OH$), are collectively called reactive oxygen species (ROS). To prevent damage to cellular processes, cells produce ROS scavengers like superoxide dismutase (SOD) and catalases/peroxidases to fully reduce ROS into water (H_2O). In *E. coli*, this process efficiently reduces the steady-state levels of superoxide and hydrogen peroxide to low nanomolar concentrations (Imlay, 2008). ROS production that exceeds the level at which SOD/catalase can neutralize ROS will lead to damage to nucleic acids and proteins (D. J. Dwyer, Kohanski, & Collins, 2009; Imlay, 2013).

Many conventional bulk biochemical assays are developed to quantitatively measure the amount of ROS formation in cells. These assays monitor changes in cytochrome c absorption upon superoxide formation (Huycke et al., 2001; Korshunov & Imlay, 2006) or the fluorescence increase from ROS sensitive dyes such as dihydroxyfluorescein (DCFH) (Caldefie-Chzet et al., 2002) and Amplex Red/Horse Radish Peroxidase (HRP) (Zhou, Diwu, Panchuk-Voloshina, & Haugland, 1997). These assays provide ensemble measurements and assume a homogeneous population within a sample. However, the recent development of single-cell techniques reveals that clonal populations of *E. coli* exhibit high

cell-to-cell variability in gene expression and other phenotypic traits (Newman et al., 2006; Peter Openshaw, Vernon Maino, & O'Garra*, 1995; Wheeler et al., 2003).

This unit describes a simple way to circumvent population averaging by using flow systems on a fluorescence microscope to measure the ROS in individual cells with high spatial and temporal resolution. Moreover, this method allows visualization of multiple symptoms or phenotypes that can be correlated with the onset of ROS formation (Choi, Yang, & Weisshaar, 2015, 2017). Our approach can provide quantitative measurements of ROS ($O_2^{\cdot-}/\bullet OH$ or H_2O_2) in single *E. coli* cells in real time using fluorescence microscopy and commercially available dyes. The Basic Protocols provide instructions for measuring ROS in single live *E. coli* cells; in the Support Protocols, we describe the procedure for constructing microfluidic chambers and simple anaerobic chambers.

Basic Protocol 1

Time-Lapse Detection of $O_2^{\cdot-}$ and $\bullet OH$ in Single Live *E. coli* Cells—This assay utilizes the fluorogenic, ROS-sensitive dye CellROX Green to measure superoxide ($O_2^{\cdot-}$) and hydroxyl radical ($\bullet OH$) in live cells. The dye is readily permeable to *E. coli* cells and becomes fluorescent upon binding to DNA after being oxidized by $O_2^{\cdot-}$ and/or $\bullet OH$ (Manina & McKinney, 2013). Tight binding of CellROX Green to DNA allows quantification of ROS in individual cells by measuring the resulting intensity of the oxidized dye on *E. coli* nucleoids in real time.

Materials: Microfluidic device (See Support Protocol 1)

Escherichia coli (MG1655 k12)

EZ rich defined medium (Teknova M2105) (See **REAGENTS AND SOLUTIONS**)

Orbital shaker with incubation

Epifluorescence microscope equipped with EM-CCD or sCMOS camera and 488nm laser

2.5 mM CellROX Green (Invitrogen C10444)

Sets of dichroic and emission filters for CellROX Green (EX525/50m)

Falcon Round-Bottom Polystyrene Tubes (Fisher 352057)

Millipore Sterile disposable vacuum Filter Units (Fisher SCGP00525)

Syringe pump (New Era pump Inc.)

Day 1

1. Prepare a 2 mL overnight culture of *E. coli* in EZ rich defined medium (EZRDM) from a glycerol stock at appropriate temperature

In EZRDM at 30 °C, the doubling time of K12 MG1655 *E. coli* strain is roughly 50 minutes. Because EZRDM is buffered with MOPS, it is not advised to autoclave this solution. Rather,

it is recommended to sterilize via sterile filtration. Cell growth and ROS formation rely on proper aeration, which depends on many factors including the size of tubes, medium volume, and rotation speeds.

Day 2

1. Inoculate *E. coli* (1:100 dilution from the overnight culture) in 2 mL of pre-warmed EZRDM at appropriate temperature until O.D reaches 0.2-0.6.
2. Warm EZRDM to appropriate temperature for dilution of CellROX Green (5 nM) and/or desired drugs, and for washing away unbound cells.

Remember that the dissolved oxygen concentration in the medium is inversely proportional to the temperature.

3. Turn on the computer that controls the microscope, necessary laser lines, and camera(s).

It is important to pre-cool the camera as the sensitivity of camera depends on the temperature of the detector. The laser power is tuned such that the intensity measured at the sample is roughly 5 W/cm². Be sure to maintain a consistent laser intensity for the CellROX intensity comparison.

4. Flow the culture of *E. coli* into the pre-warmed microfluidic chamber on the inverted microscope staged equipped with a heating element.

In general, the surface of microfluidic chamber (glass coverslip with thickness depending on the objective of choice) is coated with poly-lysine. Detailed instructions for making microfluidic devices are in the Support Protocols. We have observed that lower molecular weight poly-lysine (<1500 Da) kills *E. coli* cells. However, higher molecular weight poly-lysine (~30,000 Da) does not affect cell growth and has no visible phenotypic change on *E. coli*.

5. Wash out cells that are not attached to the coverslip by flowing pre-warmed EZRDM.

We avoid using Luria-Bertani (LB) broth because flowing LB detaches surface-bound *E. coli*. The exact cause is not known but we suspect that tryptone (or peptone) that contains negatively charged peptides may wash out the poly-lysine from the glass surface.

6. Setup a flow system with 2.5 µM CellROX Green dissolved in EZRDM. Drugs/inducer/inhibitor can be incubated with this solution as necessary.

We have noticed that H₂O₂ alone quenches CellROX fluorescence in vitro. So caution is needed when using other inhibitors to see whether quenching of fluorescence can occur. The concentration of CellROX Green may need to be tuned based on your system.

7. Monitor CellROX Green fluorescence using the proper emission filter for 488 nm excitation and alternate with phase contrast to monitor cell growth during continuous flow of EZRDM.

If using a commercial microscope, a filter set for GFP fluorescence should suffice.

8. Clean up and turn off the system.

Support Protocol 1 (Optional)

Construction of Microfluidic Chamber—In this Support Protocol, the procedure for microfluidic chamber construction is described in detail. Although a microfluidic device is not essential for the Basic Protocol, it is crucial to use a continuous flow system to replenish oxygen, as *E. coli* exhausts oxygen at a fast rate. Any commercial flow chamber can be used. However, a microfluidic device is recommended as it provides the additional benefits of utilizing an oxygen-permeable substrate *and* reducing the amount of reagents used for flow experiments.

Materials: Silanized silicon wafer master

Sylgard 184 silicone elastomer kit (Dow Corning, Inc.)

23G Blunt-end hypodermic needles

23G PE tubing

Aluminum foil

Vacuum desiccator

Vacuum pump

Sterile syringe

Plasma cleaner (Harrick Plasma)

Coverslips (Fisher 12-548-5C)

Poly-lysine (0.01% m/v, Sigma Aldrich P1524)

1. Wash the silanized master with ethanol and dry thoroughly with nitrogen.

Any residual water may affect the curing time and plasma bonding process. The design for the master that was used for the study can be requested. Gloves are required.

2. Place the silanized master on a dish made of aluminum foil.

Be sure to make a flat bottom, as any curvature on the microfluidics surface may distort the phase contrast images

3. Measure and mix the SYLGARD 184 Silicone Elastomer kit thoroughly at 1:10 ratio as described in the kit.

This will affect the hardness of the resulting Polydimethylsiloxane (PDMS).

4. Pour the mixture into the aluminum foil dish containing the silanized master.
5. Place the aluminum foil containing the mixture and the master into a vacuum desiccator.
6. Degas the PDMS by turning on the vacuum pump.

The duration of degassing will depend on the strength of vacuum pump. The goal here is to remove bubbles around structures to avoid the deformation of the microfluidic device.
7. Upon complete degassing, carefully remove the dish and place in the oven.

The curing time will depend on the temperature of the oven. Please refer to manufacturer's guideline on the curing time. From our experience, it takes approximately 1.5 hr at 60°C and 30 min at 110°C.
8. Remove the PDMS mixture from the oven and cool at room temperature.

Handle with care! It is hot!
9. Carefully remove the PDMS from the master by removing the aluminum foil.
10. Cut PDMS to the size of the microfluidic device.
11. Punch a hole with a 0.75 mm hole puncher to create an insert for a 23G hypodermic needle that is burred and cut at 1 inch.
12. Clean both surfaces thoroughly with a clear tape three times both sides.
13. Clean coverslip with water and then acetone. Sonicate coverslip in the acetone for 30 min. After sonication, dry coverslip completely with flowing nitrogen.
14. Place the PDMS and coverslip in the plasma cleaner and treat for 1 min.

The plasma cleaning duration may need to be adjusted based on the plasma cleaning system for the good binding of PDMS to coverslip. Remember to have the PDMS side with flow channels facing up.
15. Remove the PDMS and coverslip from the plasma cleaner.
16. Bond the PDMS and the coverslip together by placing PDMS with features facing down on the coverslip and gently pressing on the PDMS.
17. Inject poly-lysine solution into the chamber and incubate for at least 1 hr.
18. Insert 23G hypodermic needle and connect it with 23G PE tubing and a burred 23G needle as an adapter.
19. Wash out poly-lysine solution with sterile water.

This step also helps determine whether there is any leakage in the microfluidic device.
20. Dry out all liquid from the microfluidic device and store until needed.

We recommend making fresh devices and using the devices within one week for optimal bacterial binding.

Basic Protocol 2

Time-Lapse Detection H₂O₂ in Single Live *E. coli* Cells—This assay utilizes the fluorogenic, ROS-sensitive dye Amplex Red upon interacting with mutated ascorbate peroxidase (APEX2) and hydrogen peroxide (Daniel J. Dwyer et al., 2014; Lam et al., 2015). APEX2 can be expressed under an inducible promoter without hampering the growth of *E. coli* cells. Additionally, the transition state of APEX2 upon interacting with hydrogen peroxide catalyzes a reaction that turns non-fluorescent Amplex Red into fluorescent resorufin. Hence, the fluorescence from resorufin can be monitored to assess the level of hydrogen peroxide formation inside the cell.

Materials: Microfluidic device (See Support Protocol 1)

Escherichia coli (MG1655 K12) with plasmids expressing APEX2 (available upon request)

EZ rich defined medium (Teknova)

Orbital shaker with incubation

Epifluorescence microscope equipped with EM-CCD or sCMOS camera and laser excitation

10 mM Amplex Red (Invitrogen A22188)

561 nm laser and a set of dichroic and emission filters for Amplex Red (EX 617/73)

0.05 mg/ml Anhydrotetracycline (Sigma Aldrich 37919)

Falcon round-bottom polystyrene tubes (Fisher 352057)

Day 1

1. Prepare a 2 mL overnight culture of *E. coli* in EZ rich defined medium (EZRDM) with ampicillin (100 µg/mL) from a glycerol stock at 30°C.

Ampicillin is added so that the cells carrying the plasmid encoding APEX2 are selected. We have engineered our APEX2 plasmid into the pASK-IBA3+ vector. APEX2 expression is controlled by tetracycline repressor element.

Day 2

1. Inoculate *E. coli* (1:100 dilution from the overnight culture) in 2 mL of pre-warmed EZRDM with ampicillin (100 µg/mL) at appropriate temperature.
2. Warm EZRDM for Amplex Red dilution (10 µM) in step 11 below and EZRDM with ampicillin for washing steps to appropriate temperature.
3. Until O.D reaches ~0.1, induce APEX2 expression by adding 1.5 µL of 50 µg/mL anhydrotetracycline into the culture for 5 min.
4. Transfer cultures to two sterile Eppendorf 1.7 mL tubes and centrifuge in a mini-centrifuge for 2 min at 10,000×g.

5. Remove the supernatant and resuspend the pellets in 1 mL warm EZRDM with ampicillin (100 µg/mL) from step 2.
6. Repeat Step 4.
7. Repeat Step 5.
8. Move the liquid culture from Step 7 into a new culture tube.
9. Incubate the culture at appropriate temperature for 1.5 hr.
10. Turn on the computer that controls the microscope, necessary laser lines, and camera(s).
11. Use pre-warmed media to make solutions that contain 10 µM Amplex Red and desired drugs.

Flow *E. coli* culture into the microfluidic chamber on inverted microscope stage equipped with a heating element.
12. Wash out cells that are not attached to the coverslip by flowing pre-warmed EZRDM.
13. Set up a flow system with 10 µM Amplex Red dissolved in EZRDM. Any drugs/inducers/inhibitors can be incubated with this solution as necessary.

If ampicillin (or other penicillin binding protein inhibitors) is required to study the behavior, it is necessary to swap out the resistance gene from the vector.
14. Monitor Amplex Red fluorescence upon 561 nm excitation and alternate with phase contrast to monitor cell growth during continuous flow of media. Amplex Red can also be applied with other fluorescent indicators, enabling 2-color imaging plus phase contrast.

If using a commercial microscope with pre-installed filter sets, a filter set for mCherry fluorescence will work. However, this may not allow collecting maximum amount of photons. Emission profile of Amplex Red is available at the Life Technologies website.
15. Clean up and turn off the system.

Support Protocol 2

Anaerobic Assay for Control Experiments—This section describes a simple anaerobic assay for control experiments. This is crucial as dyes can be nonspecifically oxidized by an increased pool of oxidants inside the cell. This assay addresses the potential problem of nonspecific oxidation of dyes.

Materials: Anaerobic chamber

Protocatechuic acid (PCA, Sigma Aldrich 03930590)

Protocatechuate dioxygenase (PCD, Sigma Aldrich PB279)

Glass bottles with septa

Parafilm

23G Hypodermic needle

Prepare anaerobic EZRDM

1. Prepare 1 ml of fresh 50 mM PCA solution with 20 μ L of 1M KOH at 50°C.
2. Prepare 10 μ M PCD stock solution with 50 mM Tris•HCl at pH 7.6. The PCD stock solution can be stored at -20°C for up to 2 months. Aliquots are strongly recommended.
3. Prepare 2 mL of anaerobic EZRDM, by adding 1.89 mL EZRDM, 100 μ L 50mM PCA, and 10 μ L 10 μ M PCD to a clean 2 mL glass bottle.
4. Cap the glass bottle quickly with a septum and parafilm the cap for the better sealing.
5. Leave the glass bottle in the 30°C incubator for at least 1 hr before flowing cells into microfluidic device.

Anaerobic detection

1. Put microfluidic device in the anaerobic chamber and seal the anaerobic chamber. Connect N₂ tubing to anaerobic chamber and then turn on N₂ flow for at least 1.5 hr before flowing the cells.
2. Warm microfluidic device for at least 30 min before flowing the cells.
3. When cells reach O.D. of 0.4, add the cells to the microfluidic chamber.
4. Take out 1 mL of anaerobic EZRDM using a 1 mL sterile syringe and needle and flow onto the adsorbed cells.
5. Continue to flow the anaerobic media for >30min. Cover the needle hole with parafilm for the best seal and to prevent air dissolving in the solution.
6. Add drugs/inducers/inhibitors along with the appropriate amount of CellROX or Amplex Red in a separate anaerobic EZRDM bottle. Wrap the bottle with aluminum foil and incubate in the 30°C incubator for about 10 min prior to injection.
7. Wash away any unbound cells and then change to the anaerobic solution containing CellROX or Amplex Red and the drugs/inducers/inhibitors.
8. Perform the fluorescence detection according to the *BASIC PROTOCOL 1* and *BASIC PROTOCOL 2* above.
9. Clean up and turn off the system

Reagents and Solutions

Use Millipore water in all recipes and protocol steps

EZRDM, 1X

5 ml MOPS-buffered solution with supplemented metal ions (M2130; Teknova)

5 ml Supplemental amino acids and vitamins (M2104; Teknova)

10 ml Nitrogenous bases (M2103; Teknova)

Glucose (2 mg/ml)

1.32 mM K_2HPO_4

76 mM NaCl

H₂O to 50 ml

Filter through a 0.2 μ m pore filter

Store up to 2 month at -20°C, and 3 days at 4°C

CellROX Green (up to ~6 months at -20°C)

Amplex Red (up to 12 months when stored at -20°C)

PCA (up to 12 months when stored at 4°C)

PCD (up to 2 months when stored at -20°C)

Commentary

Background Information

Molecular oxygen (O₂) serves as an excellent electron acceptor for cellular respiration (Borisov & Verkhovsky, 2009). Unfortunately, adventitious reduction of oxygen poses great danger to cellular growth and survival. O₂ by itself does not cause much damage to cells. Partially reduced oxygen species by the opportunistic reduction in living cells, however, provide much damage associated with reactive oxygen species (ROS). ROS generally refers to superoxide (O₂⁻), hydrogen peroxide (H₂O₂), and hydroxyl radical ($\cdot$ OH). The accumulation of ROS inside a cell can be detrimental, as exemplified by the multiple growth defects in aerobically grown *E. coli* mutants that lack ROS scavenging proteins (Jang & Imlay, 2007; Park, You, & Imlay, 2005). It has been known that perturbation of redox cycling antibiotics and possibly other bactericidal antibiotics induce ROS formation (Daniel J. Dwyer et al., 2014; D. J. Dwyer, Kohanski, & Collins, 2009).

Conventional ROS monitoring in cells either directly utilizes fluorogenic dyes or measure the perfused ROS in the medium. Fluorogenic dyes such as reduced fluorescein or attaching ROS-mediated leaving groups on fluorescent molecules have been incubated directly with

cells and substances of interest. As for the measuring perfused ROS in the medium, Hpx⁻ (*katG katE ahpCF*) strain of *E. coli* has been used to carefully quantify the production of hydrogen peroxide by using horseradish peroxidase (HRP) and Amplex red (Seaver & Imlay, 2001). The perfused hydrogen peroxide from *E. coli* reacts with HRP. The transition state of HRP with hydrogen peroxide oxidizes nonfluorescent Amplex red to fluorescent resorufin.

Although pre-existing techniques can lead to quantification of ROS in live cells, these assays lack the spatiotemporal requirement for the correlation of multiple symptoms arising from specific perturbation studies, such as antibiotic addition, transcription/translation responses, among many. This is more challenging when the appearance of symptoms is temporally heterogeneous. The protocol described here provides a way to temporally correlate the production of ROS and visible symptoms due to a given perturbation in single *E. coli* cells.

Critical Parameters

Reactive oxygen sensitive dyes, while requiring a strong oxidant, can also be oxidized by non-ROS, highly reactive metal oxidants. Therefore, it is critical to test whether the resultant fluorescence is due to the formation of reactive oxygen species under the given treatment. Imlay and coworkers have demonstrated that ferrocyanide and horse radish peroxidase can directly oxidize DCFH without producing ROS (Liu & Imlay, 2013). Although we provide a simple way to test via anaerobic conditions, it is recommended to perform tests with conventional biochemical assays to validate ROS production.

Additionally, we have observed that it is important to use a fresh aliquot to perform the experiment, as the materials are sensitive to oxygen and light. We suggest making fresh aliquots of these dyes to avoid multiple cycles of freezing and thaw. In anaerobic conditions, it is extremely important to equilibrate the oxygen level prior to the injection, as any reintroduction of air requires time for the oxygen to be quenched.

Anticipated Results

These protocols show the fluorescence signal inside the cell upon ROS formation. In the Basic Protocol 1, the nucleoid shaped fluorescence signal forms upon ROS production (Fig. 2A). In the Basic Protocol 2, the uniform fluorescence signal in the cytoplasm should occur when ROS is produced (Fig. 2B).

Time Considerations—Transformation of APEX2 plasmid into a working bacterial strain takes 24 hrs. The transformed cells can be stored in 40% glycerol in -80 °C. The set-up of aerobic experiments will take ~ 1 hr to warm up the stage and microfluidics device. The preparation of anaerobic chamber takes extra ~1 hr for creating the anaerobic environment around the chamber and removing dissolved oxygen in the medium. The microfluidics device will take about 3 hr to prepare. It is recommended not to prepare more than 2 days prior to the experiments. The expression of APEX2 takes about 1.5 hr. The actual experiment time will vary depending on the time scale of onset of a perturbed phenotype. For example, ampicillin takes ~60 min to act on bacterial cells compared to 5 min for chloramphenicol or kanamycin.

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Literature Cited

- Borisov VB, Verkhovsky MI. Oxygen as Acceptor. *EcoSal Plus*. 2009; 3(2):doi: 10.1128/ecosalplus.3.2.7
- Caldefie-Chézet F, Walrand S, Moinard C, Tridon A, Chassagne J, Vasson MP. Is the neutrophil reactive oxygen species production measured by luminol and lucigenin chemiluminescence intra or extracellular? Comparison with DCFH-DA flow cytometry and cytochrome c reduction. *Clinica Chimica Acta*. 2002; 319(1):9–17.
- Choi H, Yang Z, Weisshaar JC. Single-cell, real-time detection of oxidative stress induced in *Escherichia coli* by the antimicrobial peptide CM15. *Proc Natl Acad Sci*. 2015; 112(3):E303–310. DOI: 10.1073/pnas.1417703112 [PubMed: 25561551]
- Choi H, Yang Z, Weisshaar JC. Oxidative stress induced in *E. coli* by the human antimicrobial peptide LL-37. *PLoS Pathog*. 2017; 13(6):e1006481.doi: 10.1371/journal.ppat.1006481 [PubMed: 28665988]
- Dwyer DJ, Belenky PA, Yang JH, MacDonald IC, Martell JD, Takahashi N, et al. Collins JJ. Antibiotics induce redox-related physiological alterations as part of their lethality. *Proc Natl Acad Sci*. 2014; 111(20):E2100–E2109. DOI: 10.1073/pnas.1401876111 [PubMed: 24803433]
- Dwyer DJ, Kohanski MA, Collins JJ. Role of reactive oxygen species in antibiotic action and resistance. *Curr Opin Microbiol*. 2009; 12(5):482–489. DOI: 10.1016/j.mib.2009.06.018 [PubMed: 19647477]
- Huycke MM, Moore D, Joyce W, Wise P, Shepard L, Kotake Y, Gilmore MS. Extracellular superoxide production by *Enterococcus faecalis* requires demethylmenaquinone and is attenuated by functional terminal quinol oxidases. *Molecular Microbiology*. 2001; 42(3):729–740. DOI: 10.1046/j.1365-2958.2001.02638.x [PubMed: 11722738]
- Imlay JA. Cellular defenses against superoxide and hydrogen peroxide. *Annu Rev Biochem*. 2008; 77:755–776. DOI: 10.1146/annurev.biochem.77.061606.161055 [PubMed: 18173371]
- Jang S, Imlay JA. Micromolar intracellular hydrogen peroxide disrupts metabolism by damaging iron-sulfur enzymes. *J Biol Chem*. 2007; 282(2):929–937. DOI: 10.1074/jbc.M607646200 [PubMed: 17102132]
- Korshunov S, Imlay JA. Detection and quantification of superoxide formed within the periplasm of *Escherichia coli*. *J Bacteriol*. 2006; 188(17):6326–6334. DOI: 10.1128/JB.00554-06 [PubMed: 16923900]
- Lam SS, Martell JD, Kamer KJ, Deerinck TJ, Ellisman MH, Mootha VK, Ting AY. Directed evolution of APEX2 for electron microscopy and proximity labeling. *Nat Meth*. 2015; 12(1):51–54. DOI: 10.1038/nmeth.3179
- Liu Y, Imlay JA. Cell Death from Antibiotics Without the Involvement of Reactive Oxygen Species. *Science*. 2013; 339:1210–1213. DOI: 10.1126/science.1232751 [PubMed: 23471409]
- Manina G, McKinney JD. A single-cell perspective on non-growing but metabolically active (NGMA) bacteria. *Curr Top Microbiol Immunol*. 2013; 374:135–161. DOI: 10.1007/82_2013_333 [PubMed: 23793585]
- Newman JRS, Ghaemmaghami S, Ihmels J, Breslow DK, Noble M, DeRisi JL, Weissman JS. Single-cell proteomic analysis of *S. cerevisiae* reveals the architecture of biological noise. *Nature*. 2006; 441(7095):840–846. DOI: 10.1038/nature04785 [PubMed: 16699522]
- Park S, You X, Imlay JA. Substantial DNA damage from submicromolar intracellular hydrogen peroxide detected in Hpx- mutants of *Escherichia coli*. *Proc Natl Acad Sci*. 2005; 102(26):9317–9322. DOI: 10.1073/pnas.0502051102 [PubMed: 15967999]

- Peter Openshaw EEM, Hosken Nancy A, Vernon Maino wKD, Kenneth Murphy WI, O'Garra A. Heterogeneity of intracellular cytokine synthesis at the single-cell level in polarized T helper 1 and T helper 2 populations. *The Journal of Experimental Medicine*. 1995; 182(5):1357–1367. [PubMed: 7595206]
- Seaver LC, Imlay JA. Alkyl hydroperoxide reductase is the primary scavenger of endogenous hydrogen peroxide in *Escherichia coli*. *J Bacteriol*. 2001; 183(24):7173–7181. DOI: 10.1128/JB.183.24.7173-7181.2001 [PubMed: 11717276]
- Wheeler AR, Thronset WR, Whelan RJ, Leach AM, Zare RN, Liao YH, et al. Daridon A. Microfluidic Device for Single-Cell Analysis. *Analytical Chemistry*. 2003; 75(14):3581–3586. DOI: 10.1021/ac0340758 [PubMed: 14570213]
- Zhou M, Diwu Z, Panchuk-Voloshina N, Haugland RP. A Stable Nonfluorescent Derivative of Resorufin for the Fluorometric Determination of Trace Hydrogen Peroxide: Applications in Detecting the Activity of Phagocyte NADPH Oxidase and Other Oxidases. *Analytical Biochemistry*. 1997; 253(2):162–168. DOI: 10.1006/abio.1997.2391 [PubMed: 9367498]

Significance Statement

Detection of reactive oxygen species (ROS) in bacteria has been limited to bulk biochemical assays. Although they are powerful and quantitative tools to understand the overall production of ROS in *E. coli*, such assays provide limited spatial and temporal information when correlating cellular phenotype with perturbations such as antibiotics or other treatments. This unit describes a method for detecting in situ ROS in individual *E. coli* cells via quantitative fluorescence microscopy, enabling detection of ROS in both homogeneous and heterogeneous samples at high spatiotemporal resolutions.

Figure 1.

A typical set-up for flow experiments using the microfluidic device with a syringe pump. **(a)** *E. coli* cells are injected into the pre-warmed microfluidic chamber that is coated with 0.1% poly-L-lysine. **(b)** Residual cells are washed off and **(c)** solutions can be injected or flowed for any perturbation needed for the study.

Figure 2.

Schematic of (A) CellROX Green and (B) Amplex Red/APEX II system. (A) Upon oxidation of CellROX Green by superoxide or hydroxyl radical, the dye binds to DNA and becomes fluorescent. A representative image of oxidized CellROX Green (denoted as CellROX*) is shown in the fluorescent image (left) with the phase contrast image of the cells (right). (B) Amplex Red in the solution is oxidized by APEX II after APEXII interacts with hydrogen peroxide. The signal can be interpreted as an increased formation of hydrogen peroxide inside a cell. A representative image of resorufin (oxidized Amplex Red by APEX II) is shown in the fluorescent image (left) with the phase contrast image of the cells (right).

Table 1
Troubleshooting Guide

Problem	Possible Cause	Possible Solution
Bright CellROX Green signal prior injection without any perturbation	CellROX Green is oxidized	Make a fresh aliquot. It is advised to store CellROX Green under desiccation and avoid light for less than 6 month
	CellROX Green concentration is too high	Reduce the concentration to avoid oxidation from basal ROS level
Bright Resorufin Signal upon Amplex Red without any perturbation	Amplex Red is oxidized	Same as above
No Resorufin Signal upon ROS treatment	APEX2 is not expressed well inside the cell	Control the expression level through different range of anhydrotetracycline. Recommend to make a fresh aliquot of anhydrotetracycline
Cells are washed away from the microfluidics chamber upon injection	Introduction of air bubbles	Make sure there is no bubble in the syringe prior to the injection and while putting the syringe to the adapter
	Flow rate is too fast	Decrease the flow rate
	Insufficient poly-lysine coating	Make a fresh aliquot of polylysine and the microfluidics device should be used within 2 days post-coating
Bright CellROX or Amplex signal under anaerobic condition	Failure to achieve anaerobic condition	Make fresh PCA and PCD aliquot on the day of the experiment.
		Lower the exposure of the solvent to air to decrease the accumulation of oxygen into the medium