CHAPTER SIX

In vivo fluorescent TUNEL detection of single stranded DNA gaps and breaks induced by *dnaB* helicase mutants in *Escherichia coli*

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Abstract

The genome of prokaryotes can be damaged by a variety of endogenous and exogenous factors, including reactive oxygen species, UV exposure, and antibiotics. To better understand these repair processes and the impact they may have on DNA replication, normal genome maintenance processes can be perturbed by removing or editing associated genes and monitoring DNA repair outcomes. In particular, the replisome activities of DNA unwinding by the helicase and DNA synthesis by the polymerase must be tightly coupled to prevent any appreciable single strand DNA (ssDNA) from accumulating and amplifying genomic stress. If decoupled, vulnerable ssDNA would persist, likely leading to double strand breaks (DSBs) or requiring replication restart mechanisms

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downstream of a stall. In either case, free 3′-OH strands would exist, resulting from ssDNA gaps in the leading strand or complete DSBs. Terminal deoxyribonucleotide transferase (TdT)-mediated dUTP nick end labeling (TUNEL) can enzymatically label ssDNA ends with bromo-deoxy uridine triphosphate (BrdU) to detect free 3′-OH DNA ends in the *E. coli* genome. Labeled DNA ends can be detected and quantified using fluorescence microscopy or flow cytometry. This methodology is useful in applications where *in situ* investigation of DNA damage and repair are of interest, including effects from enzyme mutations or deletions and exposure to various environmental conditions.

1. Introduction

Terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling (TUNEL) assay was originally designed to detect eukaryotic apoptotic cells and tissues, which undergo extensive DNA degradation and fragmentation during the late stages of apoptosis (Gavrieli, Sherman, & Ben-Sasson, 1992). Since then, the technique has been adapted and honed for more fine-tuned identification of DNA breaks, nicks, and gaps, and utilized in a variety of organisms and systems (Dwyer, Camacho, Kohanski, Callura, & Collins, 2012; Li, Melamed, & Darzynkiewicz, 1996; Rohwer & Azam, 2000; Sarac & Hollenstein, 2019). The linchpin of the TUNEL method is the TdT enzyme, which adds nucleotides to the 3'-OH ends of DNA breaks, including blunt ends, and is independent of a template (Michelson & Orkin, 1982; Motea & Berdis, 2010). This enzyme is an X-family DNA polymerase and biologically catalyzes the addition of random nucleotides to 3′-overhangs during V(*D*)J recombination (Sarac & Hollenstein, 2019). The unusual biological function and biochemical properties intrinsic to the TdT polymerase have motivated development of various oligonucleotide-based tools, notably synthetic tagging when combined with non-canonical nucleotides.

Several variants of TUNEL methodology exist utilizing direct or indirect fluorochrome-labeling of 3′-OH termini of DNA strand breaks *in situ* with the use of exogenous TdT and different synthetic nucleotides. Here, we discuss strand gap or break labeling with 5-bromo-2′-deoxyuridine triphosphate (BrdU), which is subsequently detected by antibody labeling with an anti-BrdU antibody (Fig. 1). TUNEL with BrdU detects free DNA ends with greater sensitivities compared to other DNA break labeling techniques (Shee et al., 2013) or different modified nucleotide additions (Darzynkiewicz, Galkowski, & Zhao, 2008). Simultaneous staining of DNA

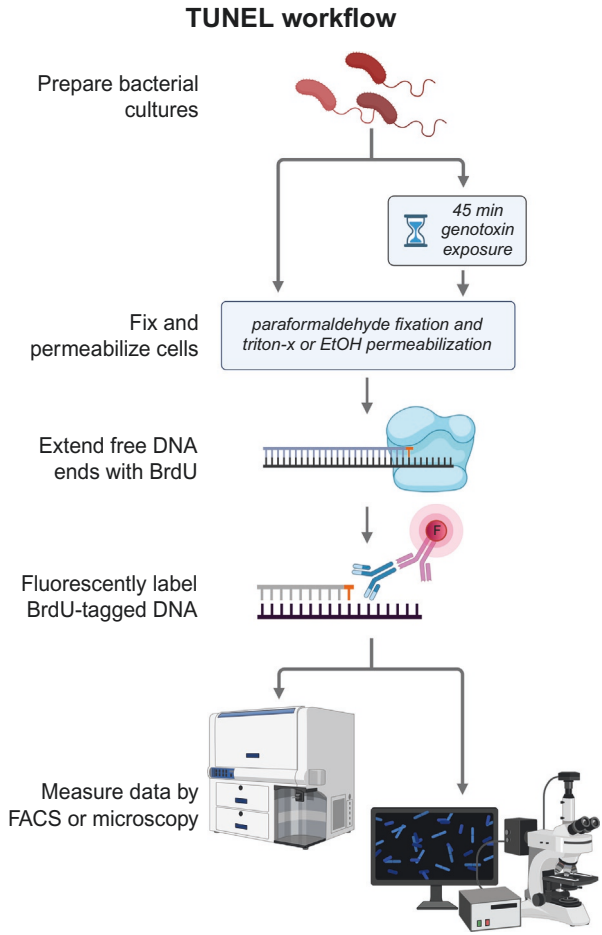

Fig. 1 TUNEL assay flowchart. A diagram showing method progression from overnight sample strains to final analysis. Cultures of the test and control strains are grown to log phase, then introduced to a genotoxic or blank solution of DMSO for 45 min. Cells are fixed with paraformaldehyde, permeabilized, and subsequently labeled with TUNEL incorporated BrdU. The labeled DNA is then tagged with a fluorescent antibody and analyzed by a combination of fluorescence microscopy and flow cytometry. Figure created with the help of BioRender.

with propidium iodide, Sytox Green, or 4′,6-diamidino-2-phenylindole (DAPI) and quantification of signal by flow cytometry enables correlation of cell growth or phase with the frequency of nicks, gaps, and breaks.

Cell fixation and permeabilization is critical to successfully label DNA strand breaks. Cells are first fixed with a crosslinking agent, *i.e.* paraformaldehyde, which prevents the extraction or loss of low molecular weight

DNA fragments during the frequent centrifugation and washing steps (Darzynkiewicz et al., 2008). Fixed cells are then permeabilized by suspension in ethanol or by using detergents to allow TdT and BrdU access to the chromosome. The 3′-termini of any DNA nick, break, or single-stranded DNA (ssDNA) gap serves as a primer and becomes labeled with BrdU by TdT (Li et al., 1996; Li & Darzynkiewicz, 1995). The incorporated BrdU is then immunochemically detected by a BrdU antibody conjugated with a fluorescent dye for easy detection (Dolbeare & Selden, 1994).

In *E. coli*, replication initiates from a single origin (called *oriC*) and migrates bi-directionally around the circular chromosome with the two replication forks meeting opposite the origin at the replication terminus. During replication, the replisome frequently encounters replication blocks such as DNA crosslinks or lesions, bound or crosslinked proteins, alkylation, or complex structures like G-quadruplexes (Perera, Behrmann, Hoang, Griffin, & Trakselis, 2019; Romero et al., 2020). When lesions or blocks are encountered by the replisome, resolution and repair can take many forms, and most involve the generation of free DNA ends as an intermediate. In conditions with high levels of genomic stress, these broken DNA lesions or repair intermediates can persist and even be left unrepaired entirely (Erental, Sharon, & Engelberg-Kulka, 2012). TUNEL can be utilized to detect the presence of these ignored or unrepaired DNA damage intermediates.

Daughter ssDNA gaps can be generated on either the leading or lagging strand by damage-tolerant lesion bypass. While translesion synthesis (TLS) is a well-documented DNA damage tolerant repair pathway (Cranford, Kaszubowski, & Trakselis, 2020; Kath et al., 2016), some lesions can be bypassed by lesion skipping and repriming mechanisms. This involves the replisome 'skipping over' the replication block on either the leading or lagging strand and reinitiating with helicase-dependent downstream repriming, similar to regular Okazaki fragment synthesis (Laureti, Demol, Fuchs, & Pages, 2015; McInerney & O'Donnell, 2004; Mezzina, Menck, Courtin, & Sarasin, 1988). This type of damage tolerant lesion bypass leaves a gap behind the replisome in one of the "daughter strands," which is then repaired post-replicatively by TLS, RecA-dependent pathways (Henrikus et al., 2018), RecFOR (Morimatsu & Kowalczykowski, 2003), or a specific subset of the PriA/B/C pathways (Sandler, 2000). Downstream repriming is used to couple lagging strand synthesis to unwinding and may also be involved in maintaining coordination between leading strand

synthesis and unwinding during polymerase switching or helicase-polymerase decoupling events (Beattie et al., 2017; Kim, Dallmann, McHenry, & Marians, 1996; Lewis et al., 2017).

Replicative helicases unwind DNA, interact with the replisome dynamically, and are critically involved in replisome function, coordination, and genome conservation. In bacteria, the replicative hexameric helicase (DnaB) serves as a stable anchor for dynamic and transient interactions of other fork-associated proteins. Replisome-coupled helicase-polymerase activity is strongly favored for efficient replication. If a helicase decouples from the DNA polymerase, a molecular mechanism engages to slow the unwinding rate by over threefold in order to protect the DNA duplex and to encourage recoupling (Graham, Marians, & Kowalczykowski, 2017; Strycharska et al., 2013). Recently, we investigated the genomic impact of four *in vivo* DnaB mutations that disrupted this molecular break, promoting in a more constricted hexameric structure that correlates with faster unwinding, promoting replisome decoupling (Behrmann et al., 2021). For those *dnaB* mutants, there was evidence of increased frequencies of ssDNA gaps and DNA breaks detected by this TUNEL method. The TUNEL method is useful for specifically investigating the impact of helicase mutations on genome maintenance, coupled unwinding and synthesis activity, and other diverse DNA damage events but can also be utilized to probe the impact of many other enzymes involved in replisome coupling, restart, or repair in both prokaryotes and eukaryotes.

2. Materials and equipment

2.1 Materials

- Overnight cultures of bacterial strains to be tested (Table 1) (Behrmann et al., 2021; Costantino & Court, 2003)
- LB media: 10 g tryptone, 10 g NaCl, 5 g yeast extract per L, pH 7.0
- Genotoxins for inducing damage, such as:
  Mitomycin C (MMC): FisherScientific, BP25312
  Nitrofurazone (NFZ): TCI America, N020025G
- Sterile 1× PBS: 0.137 M NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4
- Sterile ddH_2O water
- Triton-X: ThermoScientific, A16046AP
- Tween-20: (Fisher BioReagents, BP151-100)

Table 1 Strains.

MG1655	*E. coli* K12	One of the two parent strain derivatives of *E. coli* K12
W3110	*E. coli* K12	One of the two parent strain derivatives of *E. coli* K12
HME6	W3110 $galK_{tyr145UAG}$ Δ*lacU169* [λ *cI857* Δ(*cro-bioA*)]	This strain contains the *galK* assay system for oligo recombination.
HME63	HME6 *mutS<>amp*	Defective for MMR thus gives high frequency oligo recombination.
MSB5	HME63 *dnaB:R328/9A*	Contains a *dnaB* point mutation

- Paraformaldehyde (PFA): Acros, 41678-5000
- Cold 77% ethanol at −20 °C
- 5-Bromo-2'-Deoxyuridine (BrdU): FisherScientific, B23151
- Terminal Deoxynucleotidyl Transferase (TdT) enzyme and buffer: NEB, M0315S
- Cobalt chloride (CoCl2) (*optional*)
- 1× TBST: 50 mM Tris–Cl, pH 7.5, 150 mM NaCl, 1% Tween-20
- Bovine Serum Albumin (BSA): FisherScientific, BP1600-100
- 0.45 μm syringe filters or other filtration sterilization method
- Mouse-α-BrdU: BD Biosciences, 347,580 at 1:100 in 2% BSA in TBST
- α-mouse IgG-Alexa647: Thermo Fisher, A-21235 at 1:500 in 2% BSA in TBST
- Sodium citrate ($Na_3C_6H_5O_7$)
- 1,4-Diazabicyclo[2.2.2]octane (DABCO): ThermoFisher, 112471000
- DABCO mounting medium: (25 mg/mL DABCO in 90% glycerol/10% 1xPBS, pH 8.6)
- DAPI fluorescent DNA stain for microscopy: Invitrogen, D1306
- Glass slides, coverslips, and clear nail polish or other sealant
- Sytox Green DNA stain for FACS: Invitrogen, S7020
- Sterilized 250 mL glass Erlenmeyer flasks (one for each strain)
- 50 mL sterile conical tubes, such as falcon tubes, or equivalent sterile container

2.2 Equipment

- Temperature controlled shaker (*able to maintain 225 RPM and 37 °C*)
- 37 °C water bath or equivalent benchtop incubator
- Olympus brightfield microscope IX-81 with a 60× oil immersion lens (*or equivalent*)
- BD Biosciences FACSverse (*or equivalent*)

3. Culture preparation

3.1 Growth and treatment

This assay is used to investigate the presence of genomic single-stranded DNA ends or double -strand breaks, and so, it is important to include both positive and negative controls to eliminate any background signals of native repair in the strains. When testing strains with genomic mutations, deletions or insertions, it is important to also test the parental strain, such as W3110, MG1655, or others containing any secondary mutations. Based on our experimentation, the presence of additional genomic material, such as plasmids, does not appear to have a significant effect on either the amount or location of BrdU foci signal (as seen in Fig. 2), and so, it is not absolutely necessary to remove vectors before performing the TUNEL assay. However, if vectors are utilized, we do recommend that they be present in both the control and test strains.

3.1.1 Plasmid curing

1. To cure an *E. coli* strain of plasmid(s), first check to see if the plasmid has an internal curing mechanic, such as a heat sensitive origin or inducible expression of targeted restriction enzymes. Many modern plasmid systems have these "self-curing" mechanisms to increase the efficiency of removal. If no in-built curing mechanism exists, plasmids can be cured by repeated growth in the absence of the selective agent(s) (*i.e. without selective antibiotics*).
2. Begin by streaking out a glycerol stock onto LB-agar that lacks the plasmid selective agent. From this plate, pick a single colony to grow overnight in LB media absent of plasmid-specific selective agent(s).
3. Once the culture is turbid, streak out once again onto agar lacking the selective agent(s) and place in a 37 °C incubator overnight.
4. Pick a minimum of eight individual colonies and streak dually on to two separate agar plates marked into eight sections, one with and one without the selective agent.
5. Select a plasmid-free colony based on sensitivity to the vector's resistance marker. Grow overnight in liquid culture before adding 15% glycerol and flash freezing in liquid nitrogen.
6. If no sensitivity is detected, repeat the passaging process (*steps 2–4*) until sensitive colonies are identified.

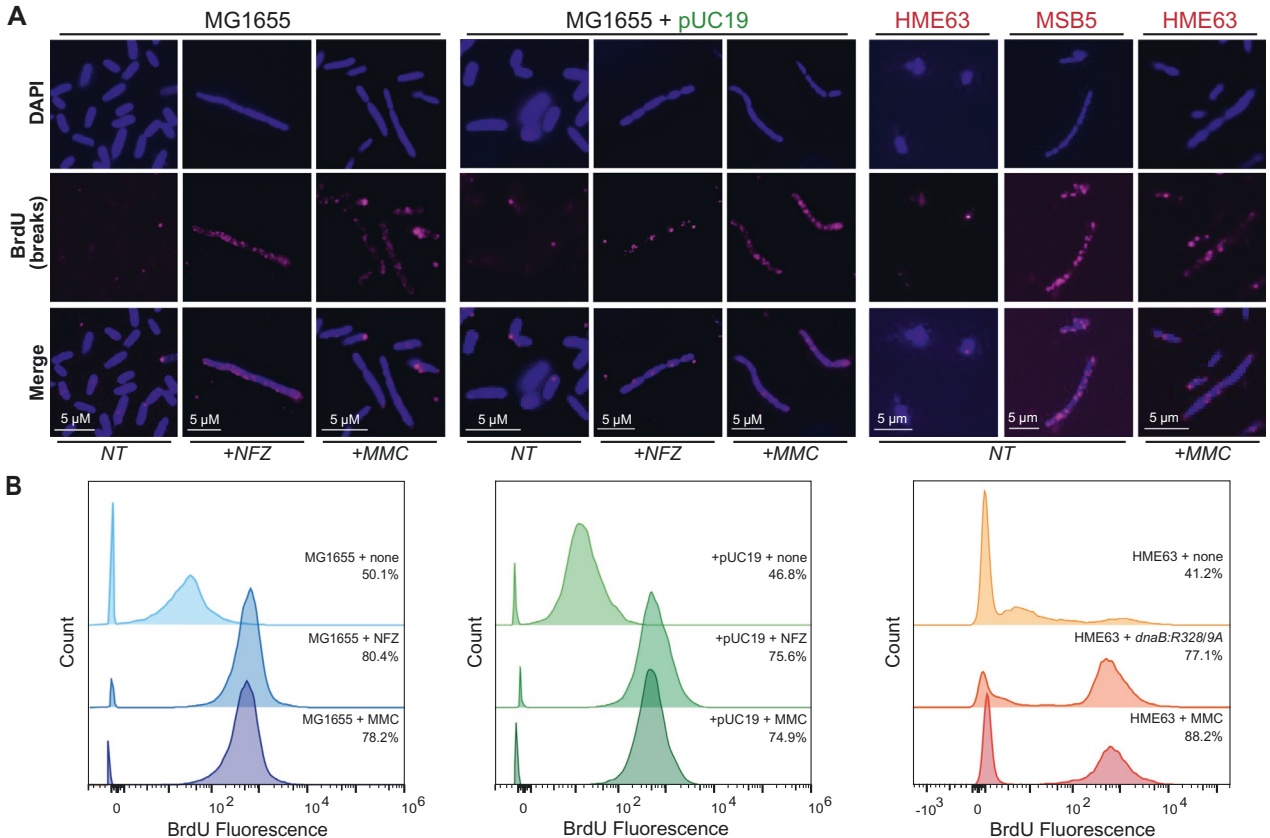

Fig. 2 BrdU foci increase with exogenous damage but not extragenomic DNA. (A) Microscopy images of MG1655 with and without the high-copy plasmid pUC19 (*middle* and *left*, respectively), HME63 (HME6 *mutS<>amp*), and the conformationally constricted helicase mutant strain MSB5 (HME63 *dnaB:R328/9A*). Cells were probed for DNA breaks using TUNEL; blue (DAPI) represents DNA staining, and pink (BrdU) represents tagged DNA breaks. NT—nontreated; +MMC—treated with 0.01 μg/mL MMC. Images shown are representative of the population observed. The fraction of BrdU labeled cells was (B) measured and quantified by flow cytometry (FACS), utilizing the same exponential growth cultures as the microscopy images. Cells were gated for +/-BrdU signal; BrdU positive populations are recorded on the right of each plot.

3.1.2 Growing up test and control strains

1. Start with overnight cultures of the test and control strains grown in appropriate media.
2. Dilute each strain 1:100 in LB media (with selective antibiotics if necessary) into 30 mL of media in a 250 mL sterile Erlenmeyer flask. This will grow enough sample for two test conditions.
3. Incubate at 37 °C and shake at 220 RPM until the optical density (OD_{600}) reads between 0.3 and 0.4, early log phase.
4. As each test culture reaches the appropriate OD_{600}, split the contents of the flask in to two ~10 mL aliquots in 50 mL tubes and discard any remaining culture according to appropriate biohazard and containment protocols. For the control strain, separate into three 10 mL aliquots, reserving one to be an "unstained" control for flow cytometry. *Do not expose this third control aliquot to genotoxins or TdT extension.*
5. Treat one tube with an appropriate test genotoxic agent and the other with mock control conditions before returning to shaking incubation. Examples include 0.01 μg/mL MMC or 5 μM NFZ for 45 min, although both the concentrations and exposure times can be varied.

   Note: While an appropriate control strain serves as an effective baseline, hydroxyurea can also be used for an additional or alternative negative control. For this method, once the strain reaches early log phase, add 10 mM HU and return to incubation with shaking for 4 h.
6. Harvest strains by spinning down at 4000 × *g* for 12–15 min until a pellet forms. Discard the media and begin washing by resuspending the cell pellet in 1 mL of filter sterilized PBS and transfer to a 1.5 mL microcentrifuge tube. Repeat this pelleting and resuspension procedure a total of three times. After the initial centrifugation, all subsequent spins listed in this method are performed in a microcentrifuge at 20,000 × *g* for 1 min. From this point forward, all solutions and samples are kept cold and on ice unless otherwise stated.

3.2 Fixation and permeabilization

After fixation, there are two different methods for cell permeabilization that can be used.

3.2.1 Paraformaldehyde fixation

Paraformaldehyde (PFA) fixation is necessary to crosslink and prevent the loss of small DNA fragments.

1. Pellet cells by microcentrifugation at 20,000 × *g* for 1 min, wash in PBS, and then resuspend in 1 mL PFA fixing solution *(4% paraformaldehyde in 1× PBS)*.
2. Let cells incubate in the fixing solution for 20 min at room temperature.
3. Spin down fixed cells, wash in cold PBS, then proceed immediately to the chosen permeabilization method.

   Note: Paraformaldehyde dissolves in water best when provided with mild heat, alkalinity, agitation, and time. This solution should be made ahead of time and chilled. Paraformaldehyde degrades within a few weeks when kept in the fridge but can last in aliquots in the freezer at −20 °C for several months. Avoid freeze-thaw cycles.

3.2.2 Detergent permeabilization

PFA fixation followed by Triton-X permeabilization allows for downstream utilization of the cells and has been shown to preserve cell surface ultrastructures (Chao & Zhang, 2011); however, it requires sample analysis within a short time frame from fixation as it can be reversible.

1. Resuspend fixed pelleted cells in 500 μL permeabilization solution (*0.1% Triton X-100 and 0.1% sodium citrate in ddH_2O*) and let sit for 2 min on ice.
2. Pellet permeabilized cells, wash with 1 mL cold PBS, and then keep on ice or at 4 °C until ready to proceed with DNA end labeling

   Note: An alternative detergent like Tween-20 can be substituted for Triton-X.

3.2.3 Ethanol permeabilization/fixation

Ethanol is a quick and effective permeabilization method that results in permanent fixation but can cause significant cell lysis in some genera (Zhu, Rajendram, & Huang, 2021). Alcohol preservation methods have been shown to reduce damage and impact to DNA because of rapid entrance into the cell, condensing DNA into a stable form that readily resumes native structure when rehydrated (Srinivasan, Sedmak, & Jewell, 2002). Because it causes permeant fixation, it allows for long term storage with minimal to no change in sample overtime, and for these reasons, it is the preferred method. While the presence of ethanol can quench fluorophores, this can be nullified by thorough washing before introducing dyes and antibodies.

1. Pellet cells by centrifugation, wash once in PBS, and then resuspend in ice cold 70% ethanol, immediately pipetting up and down several times to avoid aggregation and clumping.

2. This fixation method is permanent, and cells have an extremely good shelf life when stored in ethanol in the freezer or fridge. We have kept cells in the fridge for up to 4 months and the freezer for over a year with no detectable change in sample integrity.

At this point, the fixed cells can be stored at 4 °C until ready to proceed to the DNA end labeling. It is not recommended to store PFA and Triton-X treated cells for more than 7–10 days in total (Lanier & Warner, 1981; Sedek et al., 2020), and so, it is recommended to label and image samples within a few days when using this method. For longer storage, use PFA fixation and ethanol permeabilization.

4. TUNEL labeling

4.1 TdT extension assay

Terminal deoxynucleotidyl transferase (TdT) enzyme adds a single nucleotide to free 3'-OH DNA ends, including synthetic nucleotides such as BrdU (Brouwer et al., 2001).

1. To make TdT reaction buffer, combine 100–300 μM BrdU (or other synthetic nucleotide) with 5 U of TdT in 1 × commercial TdT buffer with optional 2.5 mM cobalt chloride.

   Note: the optional addition of cobalt chloride enhances the tailing reaction.
2. Spin down fixed and permeabilized cells, wash two-three times in PBS, and then resuspend in 100 μL of TdT reaction buffer.
3. Incubate cells in a water bath or water-filled heat block at 37 °C for 60 min to allow 3′ extension to occur.
4. After elongation, pellet cells, wash with 1 mL PBS, and then resuspend in 1 mL PBS.

   Note: At this point cells can again be stored at 4 °C, but it is recommended to proceed directly to antibody probing.

   Note: from this point forward samples are kept in PBS or TBST buffers and thus are not suitable for long term storage. Samples should be imaged within 10–14 days of performing TUNEL extension, or sooner if treated with PFA and Triton-X.

4.2 Antibody probing

TdT tagged DNA nicks, breaks, or gaps with BrdU can be detected with appropriate fluorescent secondary anti-BrdU antibodies. Permeabilized cells

allow antibodies to freely flow in and interact with internal components; however, over-fixation in PFA can create a cross-linking network that prevents effective diffusion within the cell. If antibody probing is weak on the positive control, we recommend experimenting with shorter fixation times.

1. Spin down cells and resuspend in an antibody blocking solution of 4% BSA in TBST. Let cells block by rocking at room temperature for 60 min.
2. Pellet cells and resuspend in 100 μL of 1:100 anti-BrdU primary antibody in 2% BSA in TBST. Incubate while rocking at room temperature for 60 min.
3. Wash three times by pelleting and resuspending in TBST. Then, resuspend in 100 μL of 1:500 fluorescent secondary antibody in TBST and incubate for 60 min at room temperature.

   Note: At this point, remember to protect everything from light for minimal signal loss.
4. Pellet the cells and wash 3 × with TBST to remove any unbound antibody. Resuspend cells in PBS and keep on ice or in the fridge

   Note: BSA can take a while to dissolve in water, and even after extended mixing there are often a few remaining small chunks and clumps. Prepare the BSA solution ahead of time and filter sterilize using a 0.45 μM syringe filter or equivalent. Aliquot and store in the −20 °C freezer indefinitely.

   Note: Any of the blocking and antibody incubation steps can also be performed at 4 °C overnight.

5. Analyzing your TUNEL samples

Fluorescent microscopic imaging and flow cytometry allow for both quantitative and qualitative analysis of a bacterial TUNEL assay. While neither method is dependent on the other, the data pairs nicely and provides two independent datasets for comparisons. To minimize variation from environmental conditions and instrument variation during flow cytometry, all samples from one data set must be run and analyzed during one session. Therefore, if you do plan to do both methods, perform microscopy first to screen the samples and re-prepare any that were not harvested or treated correctly before moving on to flow cytometry.

5.1 Microscopy

1. Spin down fixed cells, wash once, and then resuspend in 1 mL PBS. Add 10 μL of this solution to 90 μL of sterile PBS.

2. Working with clean labeled microscope slides, spot 2 μL each of the concentrated and 1:10 diluted solutions onto different areas of the slide. Cover and leave on the benchtop for ~10 min until dry.
3. Top each spot with 2–3 μL of DABCO with DAPI mounting solution and let rest for ~15 min. Cover each spot with a coverslip and seal with clear nail polish.
4. Let the sealant dry, and then store the slides protected from light at 4 °C overnight.
5. Image on an epifluorescent light microscope using a 60 × or 100 × oil immersion lens using DAPI and Cy5 preset filters. Bacteria exposed to MMC or NFZ should have a subpopulation of cells with evident foci, as seen in Fig. 2.
6. Collect and store images from multiple fields of view. Overlay DAPI and Cy5 channels according to microscope software, adjusting the signal intensity as needed to reduce background.
7. The total amount of Cy5 fluorescence or the number of Cy5 foci per cell can be quantified using commercial microscopy software or using Image J (Rasband, 1997–2016, 17 October 2015).

As seen in Fig. 2A (*left* panels), exogenous damage of MG1655 by either NFZ or MMC shows cellular elongation and prevalent BrdU foci throughout the cell. Transfection of a high copy number plasmid does not appear to change the distribution of foci through the cell (Fig. 2A, *middle* panels) Interestingly, genomic mutation of *dnaB* to create DnaB(R328/329) contained within the MSB5 strain (Table 1) shows prevalent BrdU TUNEL foci and cellular lengthening, even in the absence of any exogenous damage (Fig. 2A, *right* panels). This *dnaB* mutation along with several others has been thoroughly characterized (Behrmann et al., 2021).

5.2 Flow cytometry

1. After removing a few microliters of sample for microscopy, spin down the remaining sample and wash three times in PBS to remove any lingering ethanol. Resuspend test strains in 1 mL sterile 1 × PBS with 1.5 μM Sytox Green (SG).
2. Split both the BrdU-tagged control and the reserved unstained sample into two separate 1.5 mL tubes. Resuspend one tube in 1 mL sterile 1 × PBS with 1.5 μM SG, and the other in an equal volume of PBS only. There are now four controls: unstained, BrdU+, SG+, and a regular BrdU+/SG+ control strain sample.
3. Incubate samples for 30 min at room temperature while protecting from light.

4. Depending on cell density and the instrument specifications, dilute samples with sheath fluid or sterile 1 × PBS *(usually ~3–5 mL)* immediately prior to analysis.
5. Set the forward scatter (FSC) and side scatter (SSC) gain by running the reserved sample of unstained cells. Ensure that the entire cell population is visible on the scatter plot.
6. Using the unstained control, SG + only control, and BrdU + only control, set the gain for the FITC *(Sytox Green)* and Cy5.5 *(AlexaFluor 647)* laser presets using histograms (Fig. 3). Signal intensity should be optimized to make best use of the static X-axis range, as shown for the FITC laser preset in Fig. 3A
7. Once parameters are set, collect data from 100,000 events for all samples.
8. Using an analysis software for .fcs files, such as FloJo, begin by gating all samples for single cells. To do this, plot a scatter plot of FSC-H by FSC-A and gate for the population of cells that fall linearly on the diagonal, as shown in Fig. 3B.
9. Next, use the SG + and unstained controls to gate all single cell events for +/− SG staining to exclude any data from impermeabilized cells or cell contaminants (Fig. 3C). As with FACS signal gain optimization, correct gating should be visually intuitive and clearly differentiate between controls.
10. Selecting the subpopulation of SG-stained single cell events, set a universal gate for +/− BrdU signal using the BrdU + and SG + *(which is also BrdU-)* controls as reference (Fig. 3D). The +/− gated populations *(in this case, the fraction of cells with DNA gaps)* are listed in the correlating top corners of each graph and can be exported to excel directly from the FloJo workspace.
11. Check BrdU gating against the control strain (*MG1655*) and control strain exposed to genotoxin (*MG1655 + MMC*); ensure that the location of the gate falls naturally between peaks for cells with and without BrdU staining. Examples histograms are shown in Fig. 2B, with MG1655 +/− genotoxins (*left*) and the parental test strain HME63 +/− a genomic *dnaB* helicase mutation (*right*).

As seen in Fig. 2B, the addition of exogenous DNA damage agents, NFZ or MMC, shows a increase in BrdU fluorescence indicated by a large shift to the right for MG1655 and HME63. Interestingly, when *dnaB* is mutated on the genome, there is also a significant increase in BrdU even in the absence of any exogenous damage. The similarity in BrdU staining and

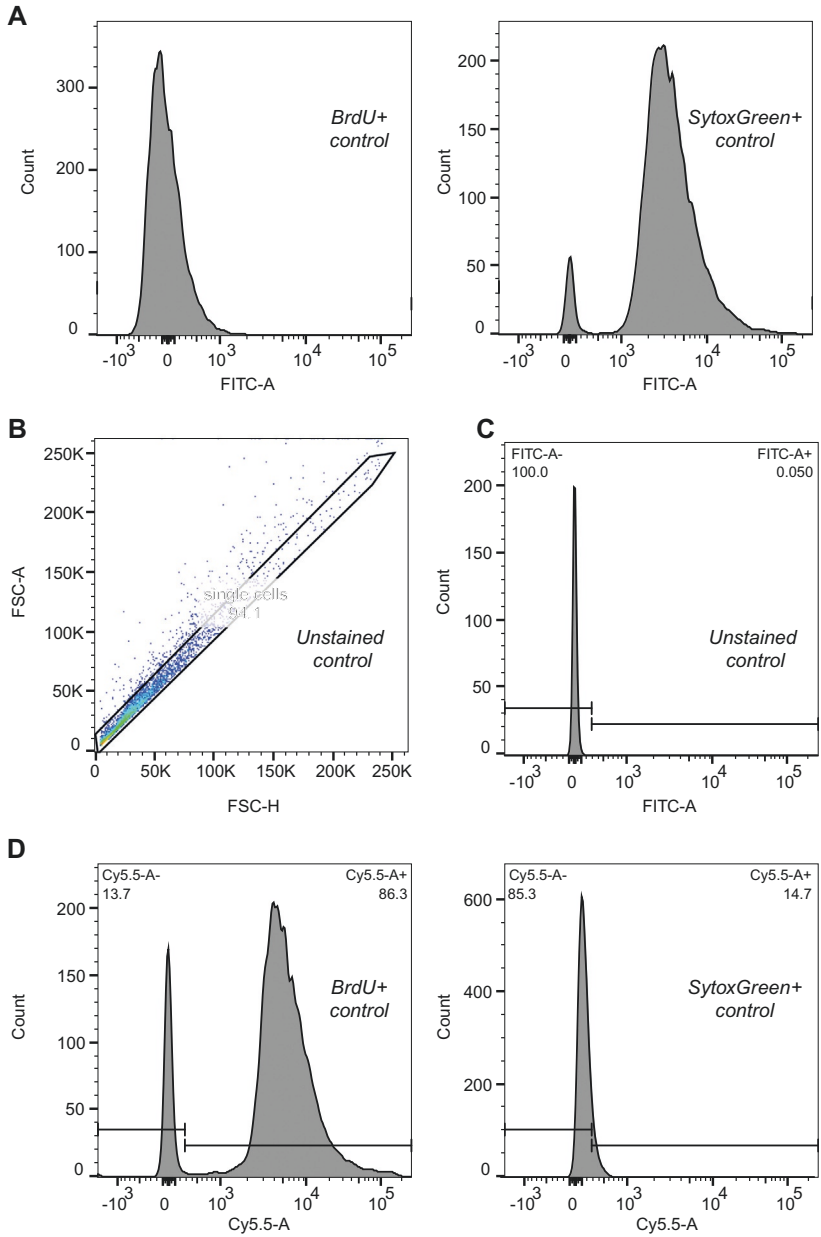

Fig. 3 Flow cytometry data collection and gating. Examples showing how to gate flow cytometry data in FloJo. (A) Prior to data collection, adjust the signal voltage so that the controls show up appropriately as high or low total signal based on their respective lasers, as shown for the green FITC laser. (B) The first gate applied to all samples is along the FSC-A/FSC-H diagonal for single cell events, followed by (C) gating for cells positive for SG DNA staining based on the unstained and SG+ controls. (D) To quantify the fraction of BrdU-containing cells, gate for +/− BrdU signal using the BrdU+ and unstained control, adjusting so that the SG+ control shows up as negative for BrdU fluorescence to avoid signal overlap.

intensities for either exogenously damaged cells or those of MSB5 suggest that this helicase mutant increases the frequency of decoupling, contributing to prevalent ssDNA gaps, nicks, or breaks.

6. Summary

DNA end labeling by TUNEL, an assay which has previously been utilized primarily for the detection of eukaryotic apoptosis, is a powerful technique for detecting and visualizing single stranded gaps and DNA ends in bacteria *in vivo*. Fluorescently tagging single strand DNA ends allows for detection of single strand nicks and gaps, which are important repair intermediates and fragile sites for replication or stress induced damage. While TUNEL does not specifically monitor ssDNA gaps, this method can be combined with an SOS induction assay to determine the likelihood of damage-induced foci as we did previously (Behrmann et al., 2021), or a secondary damage labeling technique like the DSB-specific binding of fluorescent Gam (Shee et al., 2013). This technique proves informative when generating a landscape of global replication and repair processes, especially when paired with targeted DNA damage assays and enzyme manipulation, such as DnaB helicase point mutations to test the consequences of altering the conformation to induce decoupling of unwinding synthesis.

References

Beattie, T. R., Kapadia, N., Nicolas, E., Uphoff, S., Wollman, A. J., Leake, M. C., et al. (2017). Frequent exchange of the DNA polymerase during bacterial chromosome replication. *eLife*, *6*, e21763.

Behrmann, M. S., Perera, H. M., Hoang, J. M., Venkat, T. A., Visser, B. J., Bates, D., et al. (2021). Targeted chromosomal Escherichia coli:dnaB exterior surface residues regulate DNA helicase behavior to maintain genomic stability and organismal fitness. *PLoS Genetics*, *17*, e1009886.

Brouwer, A. M., Frochot, C., Gatti, F. G., Leigh, D. A., Mottier, L., Paolucci, F., et al. (2001). Photoinduction of fast, reversible translational motion in a hydrogen-bonded molecular shuttle. *Science*, *291*, 2124–2128.

Chao, Y., & Zhang, T. (2011). Optimization of fixation methods for observation of bacterial cell morphology and surface ultrastructures by atomic force microscopy. *Applied Microbiology and Biotechnology*, *92*(2), 381–392. https://doi.org/10.1007/s00253-011-3551-5.

Costantino, N., & Court, D. L. (2003). Enhanced levels of lambda red-mediated recombinants in mismatch repair mutants. *Proceedings of the National Academy of Sciences of the United States of America*, *100*, 15748–15753.

Cranford, M. T., Kaszubowski, J. D., & Trakselis, M. A. (2020). A hand-off of DNA between archaeal polymerases allows high-fidelity replication to resume at a discrete intermediate three bases past 8-oxoguanine. *Nucleic Acids Research*, *48*, 10986–10997.

Darzynkiewicz, Z., Galkowski, D., & Zhao, H. (2008). Analysis of apoptosis by cytometry using TUNEL assay. *Methods*, *44*, 250–254.
Dolbeare, F., & Selden, J. R. (1994). Immunochemical quantitation of bromodeoxyuridine: Application to cell-cycle kinetics. *Methods in Cell Biology*, *41*, 297–316.
Dwyer, D. J., Camacho, D. M., Kohanski, M. A., Callura, J. M., & Collins, J. J. (2012). Antibiotic-induced bacterial cell death exhibits physiological and biochemical hallmarks of apoptosis. *Molecular Cell*, *46*, 561–572.
Erental, A., Sharon, I., & Engelberg-Kulka, H. (2012). Two programmed cell death systems in *Escherichia coli*: An apoptotic-like death is inhibited by the *mazEF*-mediated death pathway. *PLOS Biology*, *10*(3), e1001281.
Gavrieli, Y., Sherman, Y., & Ben-Sasson, S. A. (1992). Identification of programmed cell death *in situ* via specific labeling of nuclear DNA fragmentation. *The Journal of Cell Biology*, *119*, 493–501.
Graham, J. E., Marians, K. J., & Kowalczykowski, S. C. (2017). Independent and stochastic action of DNA polymerases in the replisome. *Cell*, *169*, 1201–1213.e1217.
Henrikus, S. S., Wood, E. A., McDonald, J. P., Cox, M. M., Woodgate, R., Goodman, M. F., et al. (2018). DNA polymerase IV primarily operates outside of DNA replication forks in *Escherichia coli*. *PLoS Genetics*, *14*, e1007161.
Kath, J. E., Chang, S., Scotland, M. K., Wilbertz, J. H., Jergic, S., Dixon, N. E., et al. (2016). Exchange between *Escherichia coli* polymerases II and III on a processivity clamp. *Nucleic Acids Research*, *44*, 1681–1690.
Kim, S., Dallmann, H. G., McHenry, C. S., & Marians, K. J. (1996). Coupling of a replicative polymerase and helicase: A tau-DnaB interaction mediates rapid replication fork movement. *Cell*, *84*, 643–650.
Lanier, L. L., & Warner, N. L. (1981). Paraformaldehyde fixation of hematopoietic cells for quantitative flow cytometry (FACS) analysis. *Journal of Immunological Methods*, *47*, 25–30.
Laureti, L., Demol, J., Fuchs, R. P., & Pages, V. (2015). Bacterial proliferation: Keep dividing and don't mind the gap. *PLoS Genetics*, *11*, e1005757.
Lewis, J. S., Spenkelink, L. M., Jergic, S., Wood, E. A., Monachino, E., Horan, N. P., et al. (2017). Single-molecule visualization of fast polymerase turnover in the bacterial replisome. *eLife*, *6*, e23932.
Li, X., & Darzynkiewicz, Z. (1995). Labelling DNA strand breaks with BrdUTP. Detection of apoptosis and cell proliferation. *Cell Proliferation*, *28*, 571–579.
Li, X., Melamed, M. R., & Darzynkiewicz, Z. (1996). Detection of apoptosis and DNA replication by differential labeling of DNA strand breaks with fluorochromes of different color. *Experimental Cell Research*, *222*, 28–37.
McInerney, P., & O'Donnell, M. (2004). Functional uncoupling of twin polymerases: Mechanism of polymerase dissociation from a lagging-strand block. *The Journal of Biological Chemistry*, *279*, 21543–21551.
Mezzina, M., Menck, C. F., Courtin, P., & Sarasin, A. (1988). Replication of simian virus 40 DNA after UV irradiation: Evidence of growing fork blockage and single-stranded gaps in daughter strands. *Journal of Virology*, *62*, 4249–4258.
Michelson, A. M., & Orkin, S. H. (1982). Characterization of the homopolymer tailing reaction catalyzed by terminal deoxynucleotidyl transferase. Implications for the cloning of cDNA. *The Journal of Biological Chemistry*, *257*, 14773–14782.
Morimatsu, K., & Kowalczykowski, S. C. (2003). RecFOR proteins load RecA protein onto gapped DNA to accelerate DNA strand exchange: A universal step of recombinational repair. *Molecular Cell*, *11*, 1337–1347.
Motea, E. A., & Berdis, A. J. (2010). Terminal deoxynucleotidyl transferase: The story of a misguided DNA polymerase. *Biochimica et Biophysica Acta*, *1804*, 1151–1166.
Perera, H. M., Behrmann, M. S., Hoang, J. M., Griffin, W. C., & Trakselis, M. A. (2019). Contacts and context that regulate DNA helicase unwinding and replisome progression. *Enzyme*, *45*, 183–223.

Rasband, W. S. (1997–2016, 17 October 2015). *ImageJ*. Bethesda, Maryland, USA: U. S. National Institutes of Health.

Rohwer, F., & Azam, F. (2000). Detection of DNA damage in prokaryotes by terminal deoxyribonucleotide transferase-mediated dUTP nick end labeling. *Applied and Environmental Microbiology*, *66*, 1001–1006.

Romero, Z. J., Chen, S. H., Armstrong, T., Wood, E. A., van Oijen, A., Robinson, A., et al. (2020). Resolving toxic DNA repair intermediates in every *E. coli* replication cycle: Critical roles for RecG, Uup and RadD. *Nucleic Acids Research*, *48*, 8445–8460.

Sandler, S. J. (2000). Multiple genetic pathways for restarting DNA replication forks in *Escherichia coli* K-12. *Genetics*, *155*, 487–497.

Sarac, I., & Hollenstein, M. (2019). Terminal deoxynucleotidyl transferase in the synthesis and modification of nucleic acids. *Chembiochem*, *20*, 860–871.

Sedek, L., Kulis, J., Slota, L., Twardoch, M., Pierzyna-SwitaLa, M., Perkowski, B., et al. (2020). The influence of fixation of biological samples on cell count and marker expression stability in flow cytometric analyses. *Central European Journal of Immunology*, *45*, 206–213.

Shee, C., Cox, B. D., Gu, F., Luengas, E. M., Joshi, M. C., Chiu, L. Y., et al. (2013). Engineered proteins detect spontaneous DNA breakage in human and bacterial cells. *eLife*, *2*, e01222.

Srinivasan, M., Sedmak, D., & Jewell, S. (2002). Effect of fixatives and tissue processing on the content and integrity of nucleic acids. *The American Journal of Pathology*, *161*, 1961–1971.

Strycharska, M. S., Arias-Palomo, E., Lyubimov, A. Y., Erzberger, J. P., O'Shea, V. L., Bustamante, C. J., et al. (2013). Nucleotide and partner-protein control of bacterial replicative helicase structure and function. *Molecular Cell*, *52*, 844–854.

Zhu, L., Rajendram, M., & Huang, K. C. (2021). Effects of fixation on bacterial cellular dimensions and integrity. *iScience*, *24*, 102348.