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From the Bench

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An Improved Method for Bacterial Immunofluorescence Staining To Eliminate Antibody Exclusion from the Fixed Nucleoid

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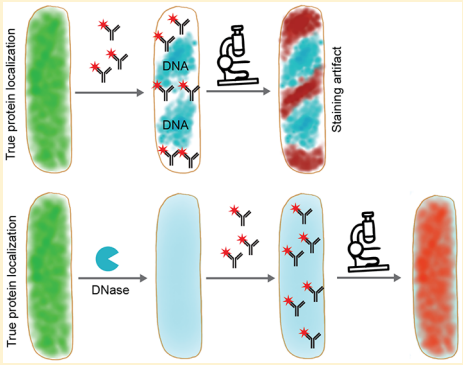
Supporting Information

ABSTRACT: Immunofluorescence (IF) is widely used to study the cellular localization and organization of proteins. However, steps such as fixation and permeabilization may affect cell morphology and/or introduce artifacts. For bacterial cells, commonly used permeabilization methods for IF include treatment with lysozyme. Here, we demonstrate two potential pitfalls in IF due to specific permeabilization methods: flattening or disruption of the cells caused by lysozyme treatment and inaccessibility of the antibody to the fixed nucleoid region. To solve these issues, we propose an improved IF method for bacterial cells, which includes the combined treatment with 70% ethanol, lysozyme, and DNase I. Treatment with 70% ethanol before the lysozyme permeabilization can better preserve the three-dimensional shape of the cell, and treatment with DNase I after the lysozyme permeabilization can eliminate the inaccessibility of the antibody to the nucleoid region. We further demonstrate that the DNase I treatment does not affect the preservation of the DNA-associated structure or organization of proteins. Finally, the method is also compatible with applications in which IF needs to be combined with RNA fluorescence *in situ* hybridization.



Immunofluorescence (IF) has been used for decades as one of the vital methods to detect the presence, abundance, localization, and organization of biomolecules with high specificity in many cell types, tissues, and organisms. Multiple targets can be imaged together to study their interactions under various conditions. However, it was illustrated in mammalian cell lines that fixation and permeabilization steps can cause IF artifacts due to the inaccessibility of antibodies.[1−3] Therefore, fixation and permeabilization methods need to be carefully evaluated and selected with important controls when applying IF. Bacterial cells, due to the presence of the cell wall, commonly require lysozyme treatment for permeabilization, as lysozyme hydrolyzes peptidoglycan, the major component of the bacterial cell wall.[4] Various concentrations of lysozyme have been used in immunofluorescence, ranging widely from 1 μg/mL to 2 mg/mL.[5−9] In addition, 70% ethanol is commonly used for permeabilization in RNA fluorescence *in situ* hybridization (FISH) for both bacterial cells and eukaryotic cell lines[10−12] and is also used in combination with lysozyme permeabilization in bacterial IF preparation.[13] Whether and how different permeabilization methods affect the IF studies of bacterial protein localization have not been evaluated. It is not uncommon that the same bacterial proteins are observed to be in different cellular localizations in IF imaging with different permeabilization methods. For example, Hfq proteins were observed to occupy the cytoplasmic space avoiding the nucleoid, localize to the cell poles or localize throughout the cell, forming a helical pattern using different IF preparation methods.[14−16]

In this report, we mainly use RecA protein in *Escherichia coli* cells as a test case and examined two commonly used permeabilization methods in IF: (1) lysozyme permeabilization and (2) a combination of ethanol and lysozyme treatment. RecA protein is fused with GFP[17] so that the localization of the protein can be independently reported by GFP. Our results show that 70% ethanol treatment alone is not sufficient for permeabilization of the bulky antibody, whereas lysozyme treatment alone causes flattening of the cells. Combination of 70% ethanol and lysozyme treatment best preserves the three-dimensional (3D) morphology of the bacterial cells, indicating that ethanol served as an additional fixative to preserve the cell structure. In addition, depending on the permeabilization methods, we observe different degrees of antibody exclusion effect from the fixed nucleoid, which generates bias toward the cytoplasmic localization. We propose to include an additional step, DNase I treatment, during the sample preparation and demonstrate that the modified protocol can best reveal the localization of the protein and can also be applied to the combined IF and FISH imaging.

■ MATERIALS AND METHODS

Bacterial Strains and Growth. The chromosomally expressing RecA-GFP strain (SS3085, denoted as "*recA-gfp*"

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here) and the RecA knockout strain [SS5912, a Δ*recA*::kan (Keio collection) derivative of the JC12509 strain, denoted as "Δ*recA*" here] are generous gifts from S. J. Sandler.[17,18] The chromosomally expressing RNase E-GFP strain is a derivative of MG1655, with the endogenous RNase E tagged by GFP (Δ*lacX*74 *rne/rne-gfp-kan*, denoted as "*rne-gfp*" here), and was generously shared by É. Massé. Strains were grown in LB medium with appropriate antibiotics at 37 °C. Overnight cultures were diluted by 100-fold in LB medium and incubated to an OD_{600} of ∼0.3. For cells with mitomycin C (MMC, Sigma-Aldrich M0503) treatment, MMC was added to the cell culture to a final concentration of 5 μg/mL, when the OD_{600} reached ∼0.2. Cell cultures were treated with MMC for 15−25 min. For cells expressing FtsZ-GFP, DH5α containing FtsZ-GFP-expressing plasmid (Addgene pXY027, shared by J. Xiao) was grown in MOPS EZ Rich medium (Teknova M2105) with 0.2% fructose. The Δ*ptsG* strain (CS196, generated using lambda red recombination, as reported previously[12]) was grown in LB and used as a negative control for FISH imaging.

Washing of the Primary Antibody. To reduce the level of nonspecific binding, the RecA primary antibody was washed against Δ*recA* cells. Overnight cultures of Δ*recA* cells were diluted 100-fold in 4 mL of LB and grown until the OD_{600} reached ∼0.6. Cells were collected by centrifugation (4000*g* for 5 min; this centrifugation condition was used throughout unless stated otherwise). Cells were fixed by 4% formaldehyde in 1× phosphate-buffered saline (PBS) for 30 min at room temperature (RT) and then washed twice with 1× PBS. Fixed cells were permeabilized with 1 mg/mL lysozyme in TEG buffer [25 mM Tris-HCl, 10 mM EDTA, and 50 mM glucose (pH adjusted to 8.0)] for 30 min on a nutator at RT (1 mL final volume), followed by five washes with 1× PBS (1000*g* for 5 min). Then cells were resuspended in 1 mL of 1× PBS containing 2 μL of the RecA antibody (Abcam ab63797, stock concentration of 1 mg/mL) and incubated on a nutator for 1 h at RT. The antibody/cell mixture was centrifuged at 1000*g* for 5 min. The supernatant was collected and centrifuged again at 20000*g* for 2 min. The supernatant containing the washed antibody was collected. The GFP antibody (Rockland 600-101-215S, stock concentration of 1 mg/mL) was washed in the same way, using MG1655 cells (not expressing any GFP proteins). The washed antibodies can be kept at 4 °C for later use for up to 1 week. The exact storage conditions may depend on the specific antibodies and should be experimentally tested. In general, we tested the functionality and specificity of antibodies by comparing positive samples against negative samples where the proteins of interest were absent.

Antibody Labeling. Twenty-four microliters of the donkey anti-rabbit secondary antibody (Rockland 611-701-127, stock concentration of 1 mg/mL) or donkey anti-goat secondary antibody (Jackson Immunoresearch 705-005-147, stock concentration of 1 mg/mL) was mixed with 3 μL of 1 M sodium bicarbonate, 2.5 μL of 10× PBS, and 1 μL of Alexa Fluor 647 NHS Ester dye (A647, Invitrogen A20006, an aliquot at a concentration of 1 mg/mL in DMSO), Cy3B NHS Ester dye (GE Healthcare PA63101, aliquots made in the same way as A647), or Atto 655 NHS Ester (Atto-tec, aliquots made in the same way as A647) and incubated for 30 min in the dark. The reaction mixture was purified with a Micro Bio-Spin P6 Gel column (Bio-Rad, 7326221) to remove the free fluorophores. In this report, each antibody was labeled with 1−1.9 dyes.

Immunofluorescence Staining. *Fixation.* Five to ten milliliters of cells was collected by centrifugation at 4000*g* for 5 min. For fixation, cells were resuspended in 1 mL of 4% formaldehyde in 1× PBS and incubated at RT for 30 min. Then, cells were washed twice with 1 mL of 1× PBS, with centrifugation at 1500*g* for 5 min followed by resuspension.

Ethanol Treatment. For permeabilization conditions requiring 70% ethanol treatment, fixed cells were resuspended in 70% ethanol (mixed with water first, and then 100% ethanol was added to reach a final concentration of 70%) and incubated on a nutator for 1 h at RT. The volume of 70% ethanol was 10 μL per 10^8 cells.

Cell Immobilization. After fixation and ethanol treatment, cells were immobilized on an eight-well chambered cover glass (Cellvis C8-1.5H-N) for the following sample preparation steps. Chambered cover glass was precoated with poly-L-lysine (Sigma-Aldrich P8920) for 1 h followed by three washes with water and air drying. For cells without 70% ethanol treatment, 10 μL of cells in 1× PBS was mixed with 120 μL of 1× PBS and added to a single well. For cells with 70% ethanol treatment, 5 μL of cells was mixed with 5 μL of water and then centrifuged at 600*g* for 5 min. The cell pellet was resuspended in 130 μL of 1× PBS and added to a single well. The eight-well cover glass was then placed in 4 °C for 1 h. The immobilized cell density was monitored under a microscope until a desired cell density was achieved. The unattached cells were removed by replacement with fresh 1× PBS.

Lysozyme Treatment. For permeabilization conditions requiring lysozyme treatment, cells were incubated with the desired concentration of lysozyme (Sigma-Aldrich 62971) in TEG buffer for 30 min at RT and then washed three times with 1× PBS. One micrograms per milliliter corresponds to 70 units/mL.

DNase I Treatment. For permeabilization conditions requiring DNase I treatment, cells were incubated with DNase I (Roche 0471678001) at the desired concentration for 1 h at 37 °C and washed three times with 1× PBS.

Immunostaining. Cells were incubated in blocking buffer [0.1% ultrapure BSA (Invitrogen AM2616) and 0.05% Tween 20 (Fisher BP337) in 1× PBS] for 1 h at RT. In the primary antibody staining, cells were incubated with the washed primary antibody for 1.5 h followed by three washes with 1× PBS with a 5 min incubation for each wash. In the secondary antibody staining, cells were incubated with 2 μg/mL labeled secondary antibody in 1× PBS for 1 h, followed by three washes with 1× PBS with a 5 min incubation for each wash.

EdU Treatment and Detection. Labeling the DNA with the Click-iT EdU kit (Invitrogen C10337) was performed according to the protocol from the manufacturer. Specifically, 12 μL of the EdU stock solution (2.5 mg/mL in DMSO) was added to 1 mL of the cell culture and incubated for 15 min before fixation. Cells were then fixed and treated with 70% ethanol and 25 μg/mL lysozyme (described above). Cells were washed three times in 100 μL of 1× PBS (4000*g* for 5 min). Ten microliters of water was added to the Alexa Fluor dye azide in the kit to make 10 aliquots of 1 μL each, and water was evaporated by a vacuum concentrator. 100 μL of Click-iT reaction cocktail was prepared by mixing the following items from the kit, i.e., 10 μL of reaction buffer, 80 μL of $CuSO_4$ (5 mM), 10 μL of 1× buffer additive, and one dye azide aliquot. This cocktail was added immediately to the cell pellet, mixed, and incubated for 1 h in the dark. Cells were washed five times in 100 μL of 1× PBS (4000*g* for 5 min) and immobilized onto

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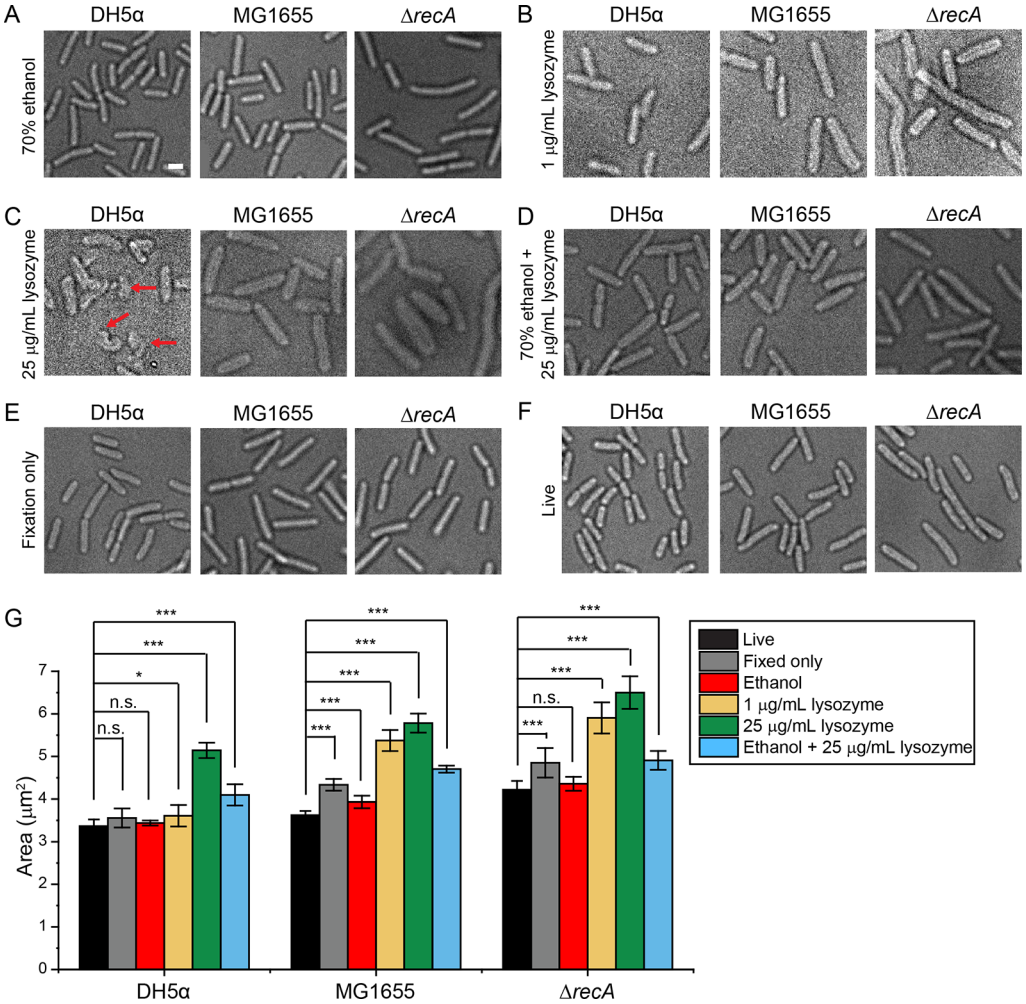

Figure 1. DIC images of *E. coli* cells with different permeabilization conditions: (A) 70% ethanol, (B) 1 μg/mL lysozyme, (C) 25 μg/mL lysozyme (red arrows indicate severely disrupted cells), (D) 70% ethanol with 25 μg/mL lysozyme, (E) fixed only, and (F) live cells. The scale bar represents 2 μm. (G) Areas of cells under different permeabilization conditions, quantified by cell segmentation based on the DIC images.[25] Error bars represent means and standard deviations of 5−10 images, with each image containing 70−300 cells. *p* values from the *t* test are added to indicate significance. n.s. stands for "not significant".

the chamber for the following sample preparation steps. The remaining dye azide aliquots can be kept in −20 °C for later use.

Fluorescence *in Situ* Hybridization. In each well of the eight-well chambered cover glass, cells were preincubated with 130 μL of 10% FISH wash buffer (10% formamide in 2× SSC) and then incubated overnight in 130 μL of hybridization buffer [10% dextran sulfate (Sigma D8906) and 10% formamide in 2× SSC] containing 105 nM FISH probes labeled with Alexa Fluor 568 NHS Ester dye (A568, Invitrogen A20003) at 30 °C.[12] Cells were washed three times with 10% FISH wash buffer, with incubation at 30 °C for 30 min each time.

Imaging and Data Analysis. Imaging was performed on a custom inverted microscope (Nikon Ti-E with 100× NA 1.49 CFI HP TIRF oil immersion objective).[19] Molecules labeled with A647 or Atto 655 were imaged with a 647 nm laser (Cobolt 0647-06-01-0120-100). Molecules labeled with A568 or Cy3B were imaged with a 561 nm laser (Coherent Obis LS). GFP was imaged with a 488 nm later (Cobolt 0488-06-01-0060-100). The DAPI signal was imaged with the LED lamp (X-Cite 120 LED) with a DAPI filter cube (Chroma ET49000). SMLM imaging and analysis were conducted as described in the previous report.[12]

■ RESULTS AND DISCUSSION

We first tested the effect of permeabilization by DIC (differential interference contrast) imaging on three bacterial strains, DH5α, MG1655, and a Δ*recA* strain, under four permeabilization conditions after formaldehyde fixation: (1) 70% ethanol only (Figure 1A), (2) 1 μg/mL lysozyme only (Figure 1B), (3) 25 μg/mL lysozyme only (Figure 1C), and (4) 70% ethanol followed by 25 μg/mL lysozyme (Figure 1D). These three strains showed different levels of morphology change, as revealed by an increase in the cell size in two dimensions compared to the cells that were fixed but not permeabilized and the live cells (Figure 1E,F), which we interpret as a flattening effect after membrane disruption. Specifically, 70% ethanol treatment gave minimal changes in the cell size for all tested strains (Figure 1A,G). The low concentration of lysozyme preserved the morphology slightly better than the high concentration (Figure 1B,C,G). In addition, the degree of the flattening effect of the lysozyme treatment was strain-dependent. At high concentrations of

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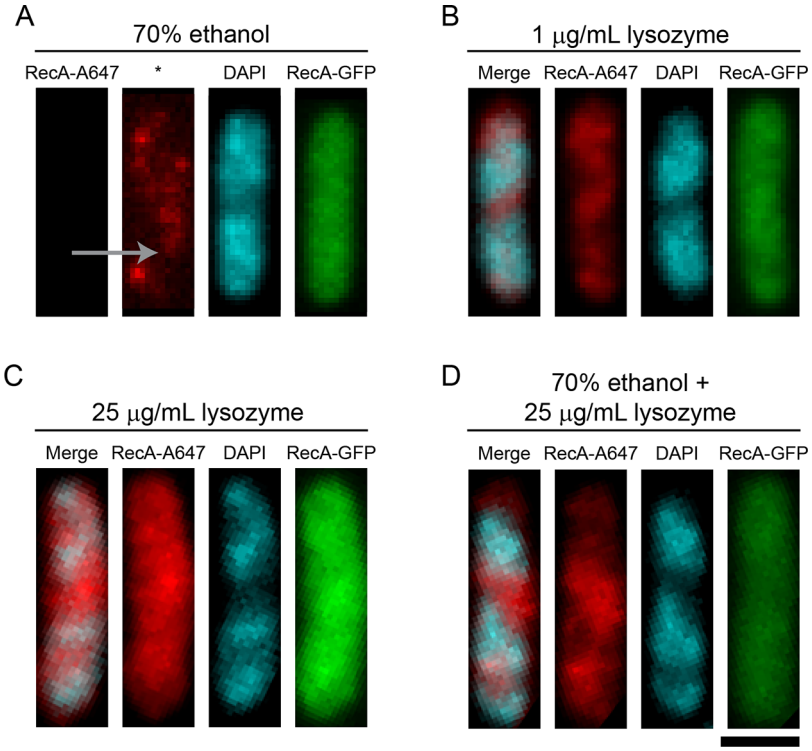

Figure 2. Immunofluorescence images of RecA-GFP with different permeabilization conditions. (A) With 70% ethanol. The panel labeled with "RecA-A647" is shown with a similar contrast as in all other A647 panels. The panel marked with an asterisk is the same image as in the "RecA-A647" panel but shown at an ∼5−10-fold lower intensity contrast due to inefficient IF labeling with this permeabilization method. (B) With 1 μg/mL lysozyme. (C) With 25 μg/mL lysozyme. (D) With 70% ethanol and 25 μg/mL lysozyme. The scale bar represents 2 μm.

lysozyme treatment, some cells were completely damaged (indicated by the red arrows as in Figure 1C); 70% ethanol followed by 25 μg/mL lysozyme preserved the cell morphology and showed a minimal flattening effect (Figure 1D,G). The exact cause of the strain-dependent sensitivity to lysosome treatment is unclear but is likely due to the changes in the cell mechanical properties under a different genetic background.[20]

We next tested the effect of different permeabilization methods on the IF signal. We used RecA-GFP as an example and stained *recA-gfp* cells with the washed primary antibody against RecA and the secondary antibody labeled with Alexa Fluor 647 NHS Ester dye (A647). The A647 signal could be compared with the GFP signal to examine whether IF reveals the true localization of the protein (Figure 2). With 70% ethanol only, IF labeling was deficient, suggesting that permeabilization by ethanol is not sufficient for the bulky antibody (Figure 2A). With the 70% ethanol followed by the lysozyme treatment, the IF signal on RecA-GFP demonstrated a patterned distribution that was anticorrelated with DAPI staining on DNA, whereas the GFP signal from the RecA-GFP fusion showed a relatively uniform distribution rather than the avoidance of the DNA (Figure 2D and Figure S1A). These observations suggest that the permeabilization with ethanol followed by lysozyme treatment can lead to a staining artifact, and this staining artifact is likely due to the inaccessibility of the antibody to the more condensed nucleoid region after fixation rather than changes of protein localization itself upon fixation. The same antibody exclusion effect was also observed under treatment with a low lysozyme concentration without 70% ethanol pretreatment (Figure 2B) but improved by treatment with a high concentration of lysozyme (Figure 2C). To better reveal the effect of permeabilization methods on the IF signal, we quantified the correlation between the A647 and DAPI signals (Figure S2). Permeabilization with 1 μg/mL lysozyme (Figure 2B) or 70% ethanol followed by 25 μg/mL lysozyme (Figure 2C) resulted in an average negative correlation coefficient between the A647 and DAPI signals, compared to permeabilization with 25 μg/mL lysozyme without pretreatment with ethanol, which had an average correlation coefficient of ∼0 (Figure S2B).

To test whether the antibody exclusion effect is specifically associated with formaldehyde fixation, we performed the IF on methanol-fixed cells. Following the published methods,[21,22] we permeabilized methanol-fixed cell with three different lysozyme concentrations, 1 μg/mL, 25 μg/mL, and 2 mg/mL. All preparation conditions generated different degrees of the antibody exclusion effect (Figure S3A). Severe cell disruption was very frequently observed with high-lysozyme concentration treatment (Figure S3B). In addition, we also tested whether the antibody exclusion effect is specifically associated with the fluorophore A647, due to its negative charge. We performed the IF with the relatively neutrally charged Atto 655-labeled secondary antibody on 70% ethanol- and 25 μg/mL lysozyme-treated cells and observed the same behavior visually as well as by the correlation analysis (Figures S2B and S4). These observations indicate that the antibody exclusion effect is not specific to the formaldehyde fixation method or the charged dye.

To overcome the antibody exclusion effect, we treated cells with DNase I after lysozyme permeabilization. For both *recA-gfp* and MG1655 cells, the RecA distribution became mostly uniform and is consistent with the GFP distribution (Figure 3A and Figure S1B), demonstrating that DNase I successfully

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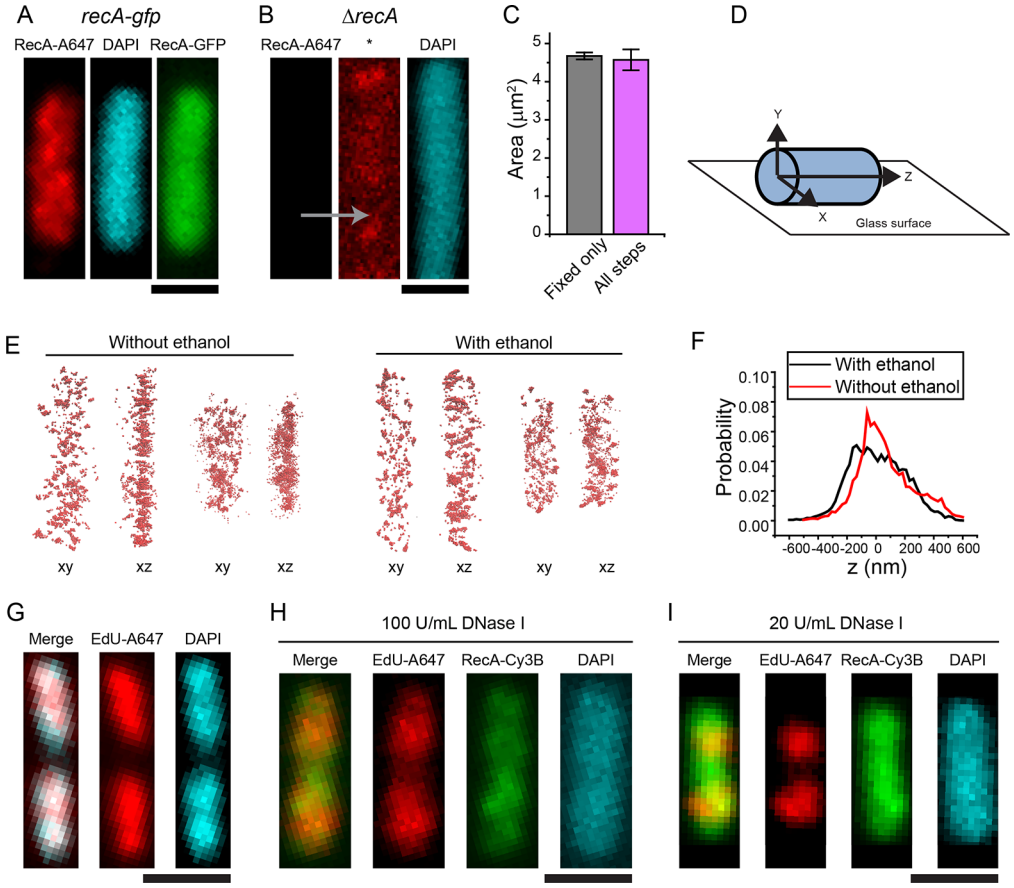

Figure 3. DNase I treatment removes the antibody exclusion effect. IF images of RecA-GFP in (A) a *recA-gfp* cell and (B) a Δ*recA* cell. IF was performed with 70% ethanol, 25 μg/mL lysozyme, and 100 units/mL DNase I treatments. The panel marked with an asterisk is the same image as in "RecA-A647" panel in panel B but shown at an ∼5−10-fold lower intensity contrast to show minimal nonspecific binding of the antibody. (C) Average cell area quantified by segmentation of DIC images of the *recA-gfp* cells that were fixed only and after all of the steps of IF preparation, including ethanol, lysozyme, and DNase I treatments. Error bars represent means and standard deviations of six or seven images, with each image containing 70−300 cells. (D) Illustration of coordinates of a cell from SMLM imaging. (E) Representative SMLM images of the A647 signal from RecA IF in individual DH5α cells, without and with 70% ethanol treatment. The 3D visualization of RecA SMLM data is presented by Visual Molecular Dynamics (VMD) software and its custom plugin.[26] (F) Distribution of localizations of RecA IF signals from the SMLM images. Cells are aligned with the middle plane set to $z = 0$ nm. Curves represent the average distribution from ∼10 cells for each case. (G) One MG1655 cell image right after A647 labeling of the DNA using the Click-iT EdU kit, in the comparison with DAPI staining. (H) One MG1655 cell treated with the EdU kit and all IF steps performed as in panel A. (I) The same treatment as in panel H except that DNase I was at 20 units/mL. The scale bars represent 2 μm.

eliminated the antibody exclusion effect. It is worth pointing out that a positive correlation between the A647 and DAPI signals with this permeabilization method was largely contributed by the loss of the DNA and a uniform DAPI signal rather than a true positive correlation between RecA distribution and DNA under our growth condition (Figure S2B). The minimal signal was detected in the Δ*recA* cells (Figure 3B), suggesting that introducing DNase treatment did not cause any nonspecific staining. In addition, DNase I treatment did not cause additional flattening effects, as revealed by similar cell sizes after fixation and after all steps in IF preparation, measured in DIC imaging (Figure 3C). To estimate the cell height in the z direction (Figure 3D), we also performed super-resolution imaging on A647-labeled RecA in the DH5α strain by a 3D single-molecule localization microscope (SMLM).[19] Cells without the pretreatment of 70% ethanol demonstrated a reduced height in the z direction (Figure 3E,F) compared to cells with the 70% ethanol treatment, consistent with the increase in the two-dimensional cell area quantified from the DIC images (Figure 1C,D,G). These results demonstrate that the modified IF method can eliminate the antibody exclusion effect while better maintaining the 3D shape of the cell.

To exclude the possibility that the DNase I treatment artificially leads to the uniform IF staining because of the clearance of the DNA, we performed two additional experiments. In the first case, we labeled the DNA with the Click-iT EdU Imaging Kit. Briefly, 5-ethynyl-2′-deoxyuridine (EdU) was added to the cell culture and incorporated into the replicated DNA. Cells were then fixed with formaldehyde, permeabilized with 70% ethanol and lysozyme, and labeled with A647 azide. Images of EdU-A647-labeled DNA were taken before and after DNase I treatment. Before the DNase I treatment, DAPI staining verified that the EdU-A647 signal correlates well with DNA (Figure 3G). After the DNase I treatment, the intensity of the EdU-A647 signal was reduced, as digested small DNA fragments were very likely washed out from the permeabilized cells. However, the remaining EdU-A647 signal could still reveal the original nucleoid pattern (Figure 3H). We also decreased the DNase I concentration

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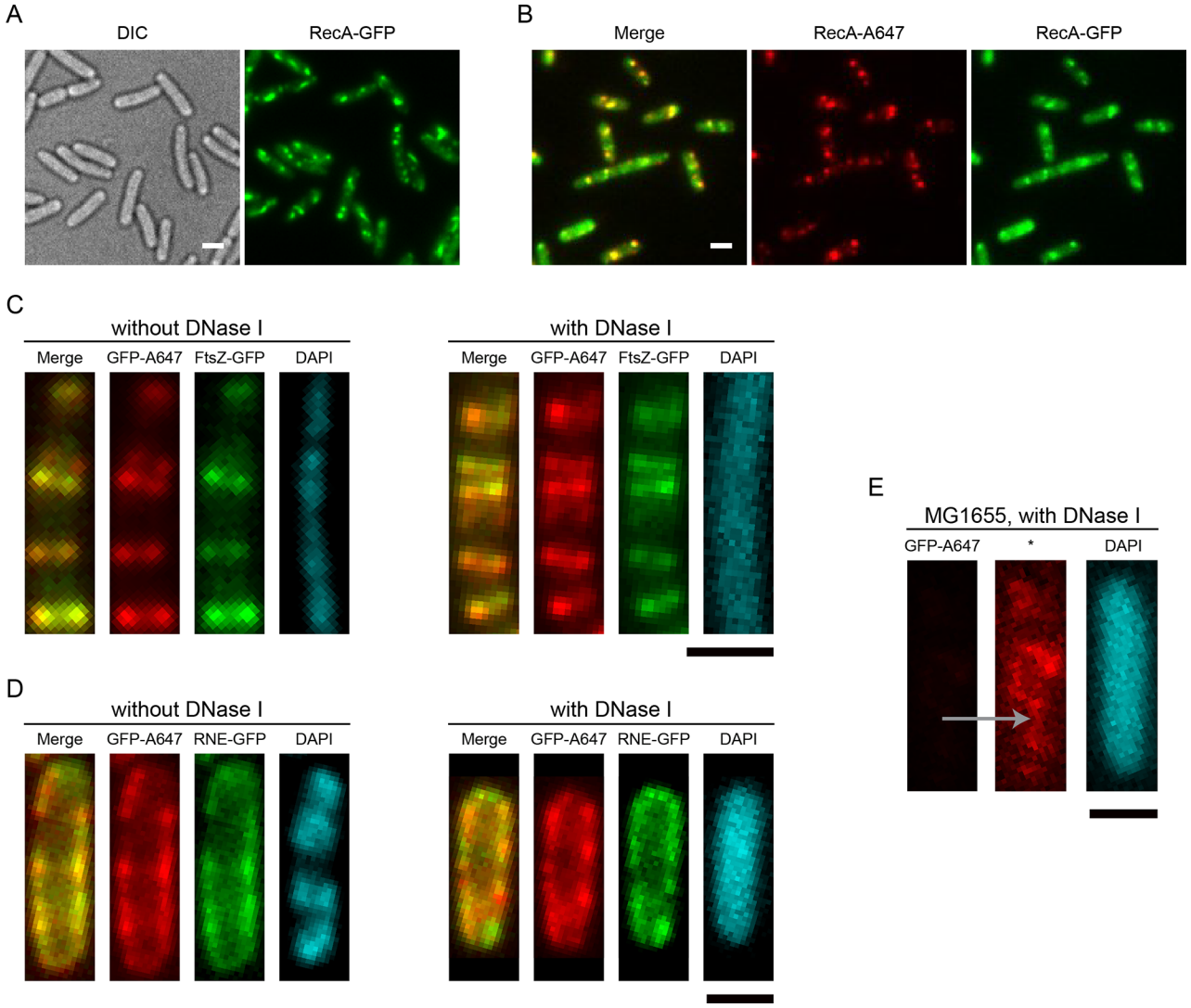

Figure 4. Subcellular localization and higher-order structures are preserved after the DNase I treatment. (A) Live *recA-gfp* cells after MMC treatment. (B) IF images of RecA-GFP in *recA-gfp* cells with MMC treatment. IF was performed with 70% ethanol, 25 μg/mL lysozyme, and 100 units/mL DNase I. (C) Single cells expressing FtsZ-GFP, without and with the DNase I treatment. (D) Single cells expressing RNase E-GFP, without and with the DNase I treatment. (E) A MG1655 cell as a control for the GFP primary antibody. The panel denoted with an asterisk is the same image as in the "GFP-A647" panel but shown at an ∼5−10-fold lower intensity contrast to show minimal nonspecific binding of the antibody. Experiments depicted in panels C−E were conducted via the same protocol as those depicted in panel B with the primary antibody against GFP. The scale bars represent 2 μm.

and could preserve a stronger EdU-A647 signal and a more defined nucleoid pattern (Figure 3I). In contrast, the DAPI staining signal was completely lost after the DNase I treatment with both concentrations (Figure 3H,I). In addition, a lower concentration of DNase I was also sufficient to eliminate the antibody exclusion effect, as revealed by the RecA-Cy3B signal (Figure 3H,I). In the second case, we utilized the osmotic stress condition induced by treating the cells with a high salt concentration, under which the DNA became highly compact (Figure S5). Due to DNA compaction, the ribosomes, labeled by FISH staining of 16S rRNA, were highly demixed from the nucleoid, as reflected by "holes" in the 16S FISH signal that correspond to the nucleoid regions (Figure S5, left). With the DNase I treatment, the distribution of the 16S FISH signal was maintained, while the DAPI signal was lost after clearance of the DNA (Figure S5, right). These results collectively suggest that the fixation can well preserve the cellular organization, specifically the original nucleoid region, even though the DNA is largely digested by DNase I.

To test whether the modified IF method, particularly the involvement of DNase I, will disrupt any structure formed in the cell, we applied the method to proteins that have characteristic subcellular localization or form higher-order structures. DNA damage by mitomycin C (MMC) is known to cause the formation of RecA foci.[17] We observed that the distinct RecA foci revealed by RecA-GFP from the live cells (Figure 4A), were well captured by IF after the DNase I treatment and that the IF signal on RecA-GFP was co-localized well with the GFP signal (Figure 4B). We also observed a similar pattern of RecA foci in MG1655 cells (Figure S1C), with the same sample preparation protocol. The FtsZ protein, which is known to form the z-ring structure in the midplane of the bacterial cell for cell division,[23] and RNase E, which is known to be localized on the inner membrane of the bacterial cells,[24] both showed the expected localization and structure

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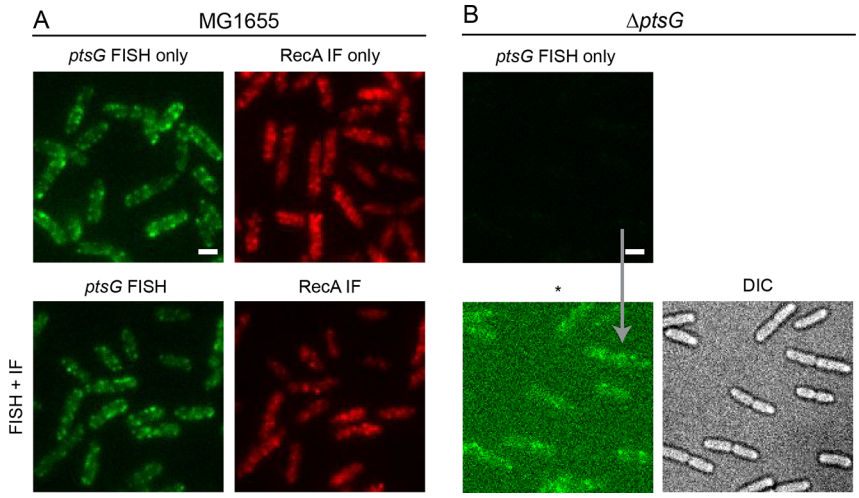

Figure 5. Combination of IF with FISH. (A) MG1655 cells were permeabilized with 70% ethanol, 25 μg/mL lysozyme, and 100 units/mL DNase I. RecA proteins were stained with the RecA primary antibody and the A647-labeled secondary antibody, and *ptsG* mRNAs were detected by A568-labeled FISH probes. As a comparison, the FISH-only sample was prepared with permeabilization by 70% ethanol. The IF-only signal was captured by imaging the IF+FISH sample before the FISH labeling step. (B) As a control for FISH staining, Δ*ptsG* cells were treated under the same condition for FISH staining as in panel A. The panel denoted with an asterisk is the same image as in the "*ptsG* FISH-only" panel in part B but shown at an ∼5−10-fold lower-intensity contrast to show minimal nonspecific binding of the FISH probe. The scale bar represents 2 μm.

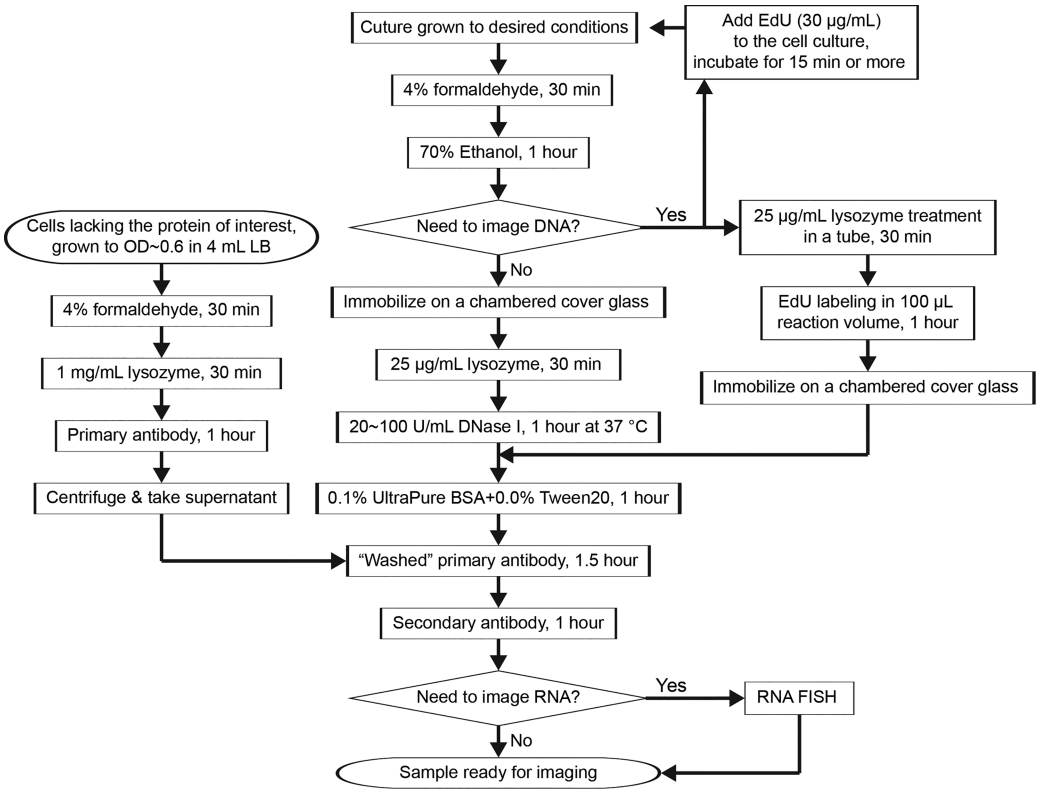

Figure 6. Flowchart of the modified IF method. Unless specified otherwise, all of the steps are conducted at room temperature.

with IF staining against GFP on the GFP-fused proteins (Figure 4C,D). These results collectively demonstrate that the modified IF method can well preserve the protein localization and formation of higher-order structures, including structures associated with DNA.

There is often a desire to image proteins with RNAs together in cells by performing IF with FISH on the same sample. To test if our protocol could be combined with FISH, we used *ptsG* mRNA as an example and imaged it together with RecA.[12] FISH probe hybridization has to follow IF due to the use of DNase I. Our results showed that the RecA IF signal did not change before and after FISH probe hybridization, and FISH signals with and without IF sample preparation were identical, demonstrating that our revised IF method is quite compatible with FISH (Figure 5). Finally, for the cases in which co-staining of DNA is desired with IF, we found that prelabeling the DNA with the Click-iT EdU kit could partially tolerate the DNase I treatment, allowing preservation of the

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nucleoid region while eliminating the antibody exclusion effect (Figure 3H,I). Alternatively, carefully titrating the DNase I concentration could achieve IF together with DNA staining (Figures S2B and S6).

In conclusion, we demonstrate two potential pitfalls in IF due to specific permeabilization methods: flattening of the cells caused by the lysozyme treatment and inaccessibility of the antibody to the fixed nucleoid region. These issues may affect the interpretation of data in addressing specific questions. Flattening of the cells needs to be considered when studying 3D localization of the biomolecules of interest, and DNA exclusion can lead to overestimated cytoplasmic localization of the protein of interest. We therefore improve the IF method by including the 70% ethanol treatment before lysozyme permeabilization and the DNase I treatment after permeabilization (Figure 6). We find that the treatment with 70% ethanol before lysozyme permeabilization can better preserve the 3D shape of the cell, likely because ethanol can provide additional fixation to make the cells stiffer after the digestion of the cell wall, and that treatment with DNase I after permeabilization can eliminate the inaccessibility of the antibody into the fixed nucleoid region. The modified IF protocol can preserve the subcellular localization and higher-order structure or organization of proteins and is compatible with RNA FISH.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.9b00724.

Figures S1−S6 (PDF)

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Notes

The authors declare no competing financial interest.

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