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Running Head: Super-resolution imaging of archaeal DNA replication

Key Words
Haloferax volcanii
Archaea
Replication
BrdU
Nascent DNA labelling
Super-resolution imaging
Stochastic Optical Reconstruction Microscopy (STORM)
3D Structured Illuminated Microscopy (3D-SIM)

Summary/Abstract

The labelling and specific detection of nascent DNA by the incorporation of thymidine analogues provide crucial information about DNA replication dynamics without requiring the intracellular expression of fluorescent proteins. After cell fixation and permeabilization, specific detection of thymidine analogues by antibodies can be performed using super-resolution imaging techniques. Here we describe a protocol to label nascent DNA using 5'-bromo-2'-deoxyuridine (BrdU) in *Haloferax volcanii* cells and generate super-resolved images of neo-synthesized DNA foci either by 3D Structured illumination microscopy (3D-SIM) or Stochastic Optical Reconstruction Microscopy (STORM).

1. Introduction

DNA is replicated by dynamic multiprotein machines called replisomes. These cellular machineries couple several activities required to efficiently and faithfully copy genetic information. In Bacteria and Eukarya, key understandings of replication dynamics have been provided by studies of the spatiotemporal localization of replisomes using functional fluorescent derivatives of replisomes components [1-6]. Classical fluorescence microscopy imaging (~200-250 nm resolution) enables non-invasive observation of protein organisation and localization in live cells with high specificity. Moreover, in recent years, the development of super-resolution techniques has provided sub-diffraction resolution and a higher level of detail, making these techniques essential for a better understanding of fundamental processes such as replication. A growing number of studies of archaeal cells took advantage of live-cell imaging techniques to investigate their biology using fluorescently-labelled proteins [7-10]. But they are restricted to few archaeal species as they require both genetic tools and intracellular conditions compatible with the functional expression of fluorescent proteins (FP) (high temperatures and/or anaerobic conditions being the two main limiting factors).

In vivo labelling of nascent DNA offers an alternative to provide crucial information about DNA replication dynamics without requiring the intracellular expression of FP proteins. It relies on the ability of thymidine salvage pathways to direct the incorporation of thymidine analogues into newly synthesized DNA. The key enzyme in this process is the thymidine kinase (TK), which converts 2'-deoxythymidine (dT) to 5'-thymidylate (dTMP). Thymidilate is then phosphorylated to yield 5'-deoxythymidine triphosphates (dTTP), which is incorporated into replicating DNA. This pathway allows extracellular thymidine analogues added to the medium of proliferating cells to be incorporated into nascent DNA. Various analogues of thymidine can be used and specific detection methods have been developed according to their distinct properties. Among them, 5'-bromo-2'-deoxyuridine (BrdU) has been widely used to investigate cell proliferation in eukaryotes, from plants to mammalian cells [11, 12]. In BrdU, the 5'-methyl group of thymidine is substituted by bromine (*see* **Fig. 1**). Alternatively, the

5’-methyl group of thymidine can be replaced by other halogens such as chlorine (5’-chloro-2’-deoxyuridine, CldU) and iodine (5’-iodo-2’-deoxyuridine, ldU), which allows the specific detection of incorporated BrdU, or other halogenated thymidine analogues, using specific antibodies. However, a significant drawback of their detection is the need to denature DNA to allow targeting by antibodies. Antibody-based detection of BrdU demands the permeabilization of cellular membranes and DNA denaturation, leading to harsh labelling conditions that may alter the sample. It is also important to highlight the importance of minimizing exposure to exogenous sources of DNA damage while culturing cells in BrdU because BrdU incorporation into DNA increases the sensitivity of cells to a wide range of chemicals, including the DNA-damaging agent mitomycin C [13] and the intercalating agent Hoechst [14], as well as the DNA-damaging UV radiations [15]. A more recent thymidine analogue, 5’-ethynyl-2’-deoxyuridine (EdU), can be detected by “Click Chemistry”, a reaction between azide and alkyne groups yielding covalent product. Because there are no azides and alkynes in native biomolecules, this Click Chemistry reaction is very selective. In EdU, the 5’-methyl group of thymidine is substituted by an alkyne group (*see* **Fig. 1**) [16] and can be targeted by azide-containing biomolecules like standard organic dyes. The click chemistry reaction allows efficient EdU detection at high efficiency in a range of solvent and pH conditions. Moreover, the molecules targeting EdU are small and permeable. They readily penetrate the cell to target EdU incorporated in double-stranded DNA. Thus, providing efficient and well-tolerated EdU incorporation during DNA replication, EdU labelling offers greater sensibility and reliability [16-18].

To localize DNA replication in archaea, both EdU and BrdU have been used, and their efficient incorporation has required genetic modifications [7, 19]. DNA replication by nascent DNA labelling was first investigated in the crenarchea *Saccharalobus solfataricus* (formerly *Sulfolobus solfataricus*) and *Sulfolobus acidocaldarius* [19]. Both species lack genes for thymidine kinase (TK), which have a very restricted phylogenetic distribution in archaea. Thus, strains of *S. solfataricus* and *S. acidocaldarius* expressing the *tk* gene from the hyperthermophilic archaea *Pyrobaculum aerophilum* were engineered. The thymidine salvage pathway hence generated allowed efficient incorporation of both EdU and BrdU [19]. Obvious growth retardation was observed after extended growth in BrdU/EdU, with a dose-dependent effect more pronounced in the presence of EdU than BrdU, suggesting higher toxicity of EdU incorporation. Nevertheless, EdU was used for pulse-labelling DNA because cell treatment required for BrdU detection altered the structure of *Sulfolobus* nucleoid [19]. EdU pulse-labelling of nascent DNA revealed that synchronized *Sulfolobus* cells contain one, two or three replication foci preferentially located at the periphery of the cell. Moreover, replication foci revealed by EdU pulse-labelling were in good agreement with replication foci revealed by immunodetection of the key replication protein PCNA [19]. Localization of replication foci was also investigated by nascent DNA labelling in the euryarchaea *Haloferax volcanii* using BrdU incorporation and immunolocalization [7]. *H. volcanii* genome encodes a thymidine kinase gene, but efficient BrdU incorporation was achieved by inactivating the *de novo* pathway of thymidine synthesis. For that purpose, a thymidine auxotrophic strain was engineered by

deletion of both *hts* and *hdrB* genes encoding thymidylate synthase and dihydrofolate reductase, respectively [20]. Growth of Δ*hts-hdrB* cells in rich media containing BrdU resulted in efficient BrdU incorporation. The intracellular localization of BrdU-labelled nascent DNA using conventional microscopy imaging (~200 nm resolution) revealed discrete intracellular fluorescence foci, with an increasing number of replication foci over BrdU-incorporation time. Replication foci revealed by BrdU-labelling of nascent DNA were in good agreement with replication foci revealed by localisation of the key replication protein RPA2 fused to the GFP in living cells. Thus, the use of EdU/BrdU labelling and localization of nascent DNA has contributed to a better understanding of archaeal replication dynamics. Coupled to super-resolution microscopy imaging, BrdU labelling and detection of nascent DNA also offers the unique possibility to go further in our understanding of DNA replication processes. This chapter aims at providing a detailed protocol for nascent DNA labelling of *Haloferax volcanii* and BrdU localization in fixed cells compatible with super-resolution imaging techniques, paving the way for further studies of archaeal replication foci at higher resolution to reveal details of the structure and dynamics that were not previously accessible by conventional microscopy imaging methods. Two different super-resolution microscopy imaging techniques will be presented: 3D Structured illumination microscopy (3D-SIM), and Stochastic Optical Reconstruction Microscopy (STORM). 3D-SIM is based on the excitation of the sample with a known spatially structured pattern of light and relies on the generation of interference patterns known as Moiré effect [21]. Different images are acquired, and by mathematically deconvolving the interference signal, a super-resolution image is obtained. Using this technique, a twofold improvement in resolution compared to confocal microscopy can be obtained in the x/y and z axes (~ 100 nm in x/y using a green emitter). STORM is a type of super-resolution microscopy technique based on stochastic switching of single-molecule fluorescence signal. STORM utilizes fluorescent probes that can switch between fluorescent and dark states so that in every snapshot, only a small, optically resolvable fraction of the fluorophores is detected. This enables determining their positions with high precision from the center positions of the fluorescent spots. With multiple snapshots of the sample, each capturing a random subset of the fluorophores, a final super-resolution image can be reconstructed from the accumulated positions (~ 10-30 nm in 2D) [22]. The protocol provided in this chapter allows to obtain high-resolution images of nascent-DNA foci by 3D-SIM or STORM in *H. volcanii* fixed-cells (*see* **Fig. 2**). We also included a quantification of the mean intensity of BrdU signal per pixel measured by wide-field imaging prior to 3D-SIM acquisition (*see* **Fig. 2B**).

2. Materials

Prepare all solutions using ultrapure water and analytical grade reagents when possible.

2.1. *Haloferax volcanii* strain

Efficient BrdU incorporation is achieved in Δ*hts* Δ*hdrB* background. HvYC0 strain, published in [7], was used for the development of this method.

2.2. Cell cultures

1. 30% Salt-Water (SW): 240 g NaCl, 30 g Hexahydrate $MgCl_2$, 35 g Heptahydrate $MgSO_4$, 7 g KCl, 20 mL of 1 M Tris-HCl solution pH 7.5, bring volume to 1 L with water (30% SW). Store at room temperature.
2. 18% SW: dilute 30% SW in water to reach 18% SW. Autoclave (*see* **Note 1**). Store at room temperature.
3. 18% HEPES-SW: Replace the 1 M Tris-HCl solution pH 7.5 in 30% SW by a 1 M HEPES solution pH 7.5.
4. 10x YPC: add 10.2 g Yeast extract, 2.04 g peptone (from Oxoid supplier, *see* **Note 2**) and 2.04 g Casamino acids in 150 mL of water. While stirring, slowly add 3.6 mL 1 M KOH solution. Adjust the final volume to 204 mL with water. Use extemporaneously.
5. YPC liquid media: 300 mL 30% SW, 50 mL 10x YPC, 1.5 mL 1 M $CaCl_2$ solution, bring volume to 500 mL with water. Sterilize by filtration (*see* **Note 3**).
6. 3x Agar solution: 7.5 g Agar in 150 mL water. Autoclave. Store at room temperature until used.
7. YPC plates:
   - Mix 300 mL 30% SW, 50 mL 10x YPC and 1.5 mL 1 M $CaCl_2$ solution. Filter-sterilise.
   - Melt the 150 mL 3x agar solution by microwaving the bottle. Heat in 30 seconds intervals on low power, gently swirling between intervals to make sure the agar is melting evenly.
   - Add the melted-agar solution to the sterilized YPC solution and immediately poor about 25-30 mL of liquid in sterile Petri dish.
   - Allow plates to cool before use. Unused plates can be stored at 4 °C for 2-4 months (*see* **Note 4**).
8. 4 mg/mL thymidine stock solution: 40 mg of thymidine dissolved in 8 mL water, adjust the final volume to 10 mL after complete dissolution. Sterilise by filtration through polyethersulfone (PES) filter with a pore size of 0.22 µm in a sterile 15 mL tube and store at 4 °C.
9. YPC + thy liquid media: add 4 mg/mL thymidine stock solution (made in water and filter-sterilised) to reach a 40 µg/mL final solution before use.
10. YPC + thy plates: follow step 7 adding to the mix 5 mL of 4 mg/mL thymidine solution prior to sterilisation.
11. 10 mM 5'-bromo-2'-deoxyuridine stock solution: dissolve BrdU powder in sterile water to reach 10 mM final concentration. 1 mL-aliquots stored at -20 °C.

2.3. Cells chemical fixation

1. 37% Formaldehyde (25 mL bottle).

2. Ethanol absolute.
3. 100% Formamide.
4. 1x PBS solution: 8 g NaCl, 0.2 g KCl, 1.44 g $Na_2HPO_4 \cdot 2\ H_2O$ and 0.24 g KH_2PO_4 dissolved in 800 mL water. Adjust pH to 7.4. Complete with water to reach 1 L total volume. Autoclave. We recommend preparing a 10x stock solution.

2.4. BrdU revelation

1. 20% Tween-20 solution.
2. Bovine Serum Albumin (BSA), in powder.
3. Blocking solution: 1x PBS, 5% (w/v %) BSA, 0.1% Tween.
4. Primary and Secondary antibodies solution: 1x PBS, 1% (w/v %) BSA, 0.1% Tween in which the antibodies are diluted.
5. Anti-BrdU antibody (*see* **Note 5**).
6. Secondary antibody to target anti-BrdU antibodies (*see* **Note 6**).

2.5. Sample mounting (for SIM and/or STORM imaging)

1. Coverslips: 22 x 22 mm (for 3D-SIM) or 25 mm diameter (for STORM) No 1.5H high precision 170 ± 5 μm (*see* **Note 7**).
2. Attofluor imaging chamber (Thermofisher Scientific – A7816).
3. 1 M KOH solution for coverslip cleaning
4. Poly-L-lysine solution, 0.1 % (w/v) in H_2O, diluted in water to reach a 0.01% final concentration.
5. Mounting solution: 25% Vectashield - 75% glycerol with 2% w/w n-Propyl Galate (*see* **Note 8**).
6. Microscope slide.
7. 18mm diameter coverglass Size 1.
8. Nail varnish for sealing.

2.6. Microscopy components and setup for STORM imaging (*see* **Note 9**)

1. IX83 fully motorized inverted microscope.
2. 100x 1.49 TIRF objective.
3. Filter set in UFF filter cube.
4. 638nm laser 140mW (*see* **Note 10**).
5. Orca Fusion sCMOS camera (*see* **Note 11**).

2.7. Microscopy components and setup for SIM imaging (*see* **Note 12**)

1. Ethanol 100% for lens and coverslip cleaning.
2. Immersion oil with the appropriate refractive index (*see* **Note 13**).

3. Microscope setup used: OMX-SR (Cytiva – formerly GE Healthcare) with 60X/1.42-numerical aperture PlanApo oil immersion objective and pco Edge 4.2 sCMOS cameras.

3. Methods

3.1. 5'-bromo-2'-deoxyuridine incorporation in replicating cells

H. volcanii strains are stored in [18% SW – 80% glycerol] at -80°C.

Otherwise stated, *H. volcanii* manipulation are done using sterile material on the bench.

1. Streak the *H. volcanii* strain on YPC plates complemented with 40 µg/mL thymidine (YPC + thy) plates.
2. Incubate the plate for 5-7 days at 45 °C in a plastic container to avoid drying.
3. Start a culture by suspending a single colony in 3 mL YPC + thy into 14 mL round-bottom sterile tubes. Grow overnight at 45 °C with agitation at 150 rpm.
4. The next morning, dilute to OD_{600nm} ~0.025 (*see* **Note 14**) in YPC + thy (50 mL final volume in 250 mL flasks for optimal aeration) and incubate at 45 °C with agitation at 150 rpm.
5. When reaching OD_{600nm} ~0.05 (*see* **Note 15**), centrifuge the cells for 10 minutes at 4,120 RCF (Relative Centrifugal Force, or g force) using 50 mL sterile tubes and remove the media.
6. Resuspend the cell pellet in 50 mL prewarmed YPC media and measure OD_{600nm} (*see* **Note 16**).
7. Add 500 µL of 10 mM BrdU solution to reach 100 µM final concentration, and incubate the culture at 45 °C with agitation at 150 rpm.
8. From addition of BrdU, corresponding to the time-point zero (T0), for each time-point of interest:
   - Measure OD_{600nm}, to monitor the growth (*see* **Note 17**).
   - Collect 10 mL from the culture in a 15 mL Sterile tube, and store them on ice.

3.2. Cell fixation

1. Centrifuge the cells for 10 minutes at 4,120 RCF (at room temperature) and discard the supernatant.
2. Resuspend the cells in 4,460 µL of 18% HEPES-SW (*see* **Note 18**) and add 540 µL of 37% formaldehyde (FA) to obtain a 4% final concentration.
3. Incubate 1 hour on a rotating wheel (at room temperature), then incubate at 4 °C overnight (no agitation required) (*see* **Note 19**).

3.3. Dehydration and Formamide DNA denaturation

2 mL-rounded sterile tubes should be used. Removing of the supernatant after centrifugation steps should be performed by inversing the tube (avoid pipetting). Unless stated otherwise, all steps are performed at room temperature.

1. Collect 2 mL of cells in 4% FA in a 2 mL-rounded sterile tube (*see* **Note 20**).
2. Centrifuge the cells for 5 minutes at 2,410 RCF and discard the supernatant.

3. Resuspend the cells in 300 µL 18% SW and add 1,5 mL 70% Ethanol solution (freshly prepared from ethanol absolute) to reach a 60% final concentration.
4. Centrifuge the cells for 5 minutes at 2,410 RCF and discard the supernatant.
5. Resuspend the cells in 2 mL 18% SW.
6. Centrifuge the cells for 5 minutes at 2,410 RCF and discard the supernatant.
7. Resuspend the cells in 2 mL 18% SW.
8. Centrifuge the cells for 5 minutes at 2,410 RCF and discard the supernatant.
9. Resuspend the cells in 1,300 µL 18% SW and add 700 µL of 100% formamide solution (35% final concentration).
10. Incubate 1 hour at 45 °C (Reverse the tube every ~10 minutes for agitation).
11. Centrifuge the cells for 5 minutes at 2,410 RCF and discard the supernatant.
12. Resuspend the cells in 2 mL 1x PBS.
13. Centrifuge the cells for 5 minutes at 2,410 RCF and discard the supernatant.
14. Resuspend the cells in 1 mL 1x PBS (*see* **Note 21**).

3.4. 5'-bromo-2'-deoxyuridine revelation

2 mL-rounded sterile tubes should be used. Removing of the supernatant after centrifugation steps should be performed by inversing the tube (avoid pipetting). Unless stated otherwise, all steps are performed at room temperature.

1. Collect 200 µL of formamide-treated cells in a 2 mL-rounded sterile tube.
2. Centrifuge the cells for 5 minutes at 2,410 RCF and discard the supernatant.
3. Resuspend the cells in 200 µL blocking solution.
4. Incubate at least 45 minutes on a rotating wheel.
5. Centrifuge the cells for 5 minutes at 2,410 RCF and discard the supernatant.
6. Resuspend the cells in 200 µL primary antibody solution.
7. Incubate overnight at 4 °C on a rotating wheel (*see* **Note 22**).
8. Centrifuge the cells for 5 minutes at 2,410 RCF and discard the supernatant.
9. Resuspend the cells in 2 mL [PBS - 0.1% Tween].
10. Incubate 5 minutes on a rotating wheel.
11. Repeat twice from steps 8 to 10 to perform 3 washes.
12. Centrifuge the cells for 5 minutes at 2,410 RCF and discard the supernatant.
13. Resuspend the cells in 200 µL secondary antibody solution.

The following steps should be performed in the dark to avoid light-exposure of the cells.

14. Incubate 1 hour on a rotating wheel.
15. Centrifuge the cells for 5 minutes at 2,410 RCF and discard the supernatant.
16. Resuspend the cells in 2 mL [1x PBS - 0.1% Tween].
17. Incubate 5 minutes on a rotating wheel.

18. Repeat twice from steps 15 to 17 to perform 3 washes.
19. Centrifuge the cells for 5 minutes at 2,410 RCF and discard the supernatant (*see* **Note 23**).
20. Resuspend the cells in 20 µL 1x PBS for imaging.

3.6. Sample mounting (STORM and/or 3D-SIM)

1. Wash the coverslips in 96% Ethanol for 20 minutes.
2. Wash three times with deionised water.
3. Wash the coverslips in 1 M KOH solution for 20 minutes.
4. Wash three times with deionised water.
5. Cover each coverslip with 1 mL of 0.01% poly-L-lysine solution (*see* **Note 24**).
6. Incubate at least 30 minutes.
7. Wash three times with deionised water.
8. Air-dry.
9. Spot the 20 µL of BrdU-labelled cells on the center of the poly-L-lysine coverslip.
10. Incubate at least 20 minutes.
11. Wash three times with 1x PBS.
12. Wash one time with deionised water.
13. For 3D-SIM imaging:
    - Clean the microscope slide and place a drop of ~15µL of mountant at the centre.
    - Slowly put the coverslip down on the mounting solution, avoiding air bubbles.
    - Carefully wipe excess mounting solution.
    - Seal with nail-polish.
14. For STORM imaging:
    - Place the coverslip on the Atto chamber, cells-covered surface up.
    - Add 30 µL of mounting solution on the cells-covered surface.
    - Cover with a clean 18 mm coverslip, spreading the mounting solution in between the two coverslips, avoiding air bubbles.

3.7. STORM imaging

1. Place the sample holder onto the microscope stage.
2. Adjust the focal plane and identify a region of interest using the fluorescence lamp at low intensity.
3. Set camera settings to 2x2 pixel binning (1 pixel = 130 nm) and integration time = 50 ms.
4. Acquire STORM images under continuous illumination with 638 nm laser (power on the sample of ~1.5 kW/cm²). Collect between 10,000 and 20,000 images.
5. For samples with a low density of molecules, illumination with a 405 nm laser (power of ~10W/cm²) for photo-reactivation can be added.

3.8. Widefield imaging (*see* **Note 12**)

For each image, a widefield image was also acquired with the OMX-SR prior to 3D-SIM acquisition.

1. Oil immersion drop of the appropriate RI (1.518) was placed in the middle of the coverslip and the sample placed on the sample holder of the OMX-SR.
2. Illumination settings were optimized on T120 sample so as to cover the entire dynamic range of the sCMOS camera without saturating it (14bit). These settings were kept constant for all widefield images to allow quantitative analysis of BrdU incorporation at different times.
3. 3D image stacks (z-step 125 nm) of 512 x 512-pixel field of view were acquired, taking care to capture the entire volume.
4. 3D-SIM images were acquired without changing the stage position but adjusting the illumination settings for optimal intensity range (see below).

3.9. 3D-SIM imaging (*see* **Note 12**)

1. Oil immersion drop of the appropriate RI (1.518) was placed in the middle of the coverslip and the sample placed on the sample holder of the OMX-SR.
2. Illumination settings were optimized for each image so as to cover the entire dynamic range of the sCMOS camera without saturating it (14bit) and ensure adequate signal for reconstruction.
3. 3D image stacks (z-step 125 nm) of 512 x 512 pixel field of view were acquired in 3D-SIM mode, taking care to capture the entire volume (unchanged from the widefield image).
4. The raw data was reconstructed using SoftWoRx version 7.2.0 (Cytiva) using the 647 channel-specific OTF recorded using immersion oil with RI 1.518 and automatic Wiener filter settings.
5. All 3D-SIM data (raw and reconstructed) was quality-controlled using SIMCheck [23], an open-source ImageJ/Fiji plugin.

3.10. STORM image reconstruction

1. The raw data is opened with FIJI [24].
2. We used Detection of Molecules (FIJI plugin https://github.com/ekatrukha/DoM_Utrecht. There are 3 steps , we give the relevant parameters as FIJI macro code (*see* **Note 25**):

   - w=getWidth();
   - h=getHeight();

3. Detection and fitting of molecules:

   run("Detect Molecules", "task=[Detect molecules and fit] psf=1.2 intensity=4 pixel 130 parallel=1000 fitting=5 ignore");

4. Drift correction:

   run("Drift Correction", "pixel=20 batch=1500 method=[Direct CC first batch and all others] apply");

5. Image reconstruction:

run("Reconstruct Image", "for=[Only true positives] pixel=10 width="+w+" height="+h+" sd=[Constant value] value=10 cut=25 x_offset=0 y_offset=0 range=1-99999 average update render=Z-stack z-distance=100 lut=Fire"); (*see* **Note 26**).

4. Notes

1. Sterile filtration can also be performed (see Note 3).
2. Only peptone from Oxoid supplier allows growth of *H. volcanii*.
3. Do not autoclave as salts precipitation may occur. The use of complete single-use sterile vacuum filtration units (retention< 10 µg/cm² IgG), with reservoir and receiving flask, is recommended. The sterile YPC media can be stored directly in the receiving flask at room temperature, protected from light, for weeks.
4. After storage at 4 °C, warm-up the petri dish prior to use. In this purpose, place the bottom portion containing the media up-side-down, half-opened on its lid, for 1 hour at 45 °C.
5. We have been using 1/100 dilution (v/v) of purified mouse Anti-BrdU antibodies, clone 3D4 (RUO), 0.5 mg/mL.
6. We have been using 1/100 dilution (v/v) of affinity purified Anti-Mouse IgG (H+L) antibodies, DyLight649-conjugated, 1.5 mg/mL. AlexaFluor-647 and CF-647 are equally valid options for STORM. Better resolution in 3D-SIM can be obtained by using blue or green fluorophores such as CF405M or AlexaFluor-488, respectively.
7. Super-resolution imaging requires coverslips with controlled and uniform thickness and high precision to avoid aberrations. Coverslips were cleaned in demineralized H_2O to remove dust and stored in ethanol 100% prior to use. Before plating, coverslips were air-dried to remove traces of ethanol.
8. STORM imaging buffer vary depending on secondary dyes and microscope used. For AlexaFluor-647, Dylight-649 and Cf-647, Vectashield is an easy option that yields high quality images. Vectashield can also be diluted in Glycerol or 1x PBS. done when the laser power of the STORM microscope is limited. A dilution of 25% Vectashield in glycerol works readily on multiple optical setups. See (25).
9. We used an Olympus IX83 inverted microscope, equipped with an Olympus 100x 1.49NA objective, a Chroma filter set (49006), a Hamamatsu sCMOS camera and a Vortran laser but everything we describe should be generally applicable to an inverted microscope with laser excitation and a sensitive camera. One important parameter that will change is the pixel size (here 6.5um/100=65nm) that should be kept in the 100-160 nm range which is why we use a 2x2 pixel binning (*see* **Note 11**).
10. Anything between 630 and 650 nm will work.

11. An EMCCD would be an equally valid option, in which case choose 1x1 binning is typically used due to bigger pixels.
12. The sample preparation we describe should be applicable to any 3D-SIM microscope, but the data acquisition and analysis steps are quite manufacturer-dependent.
13. 3D-SIM reconstruction is sensitive to artefacts due to refractive index (RI) mismatch between the system's optical transfer function (OTF = Fourier transform of the point spread function, PSF) that reflects the optical properties at a given wavelengths and that of the biological sample. For imaging experiments on Deltavision OMX-SR system (Cytiva), we recommend the use of channel-specific measured OTFs. The RI should be selected to provide a symmetric PSF for the wavelength used (e.g 1.518 RI for Alexa 647-coated beads). As *H. volcanii* is close to the coverslip, we used 1.518 oil to image Alexa647-labelled BrdU-foci. See also [26].
14. In order to perform efficient BrdU incorporation in proliferating cells, we recommend not to exceed OD_{600nm} 0.1-0.2 (8). Thus, we dilute the culture to OD_{600nm} ~0.025.
15. Should take ~3 hours (~120 minutes generation time).
16. 10 mL of cells can be collected prior to BrdU addition to establish background level of the method.
17. OD_{600nm} should increase over time for at least 360 minutes (~3 generations).
18. It is important not to use Tris buffers in formaldehyde cross-linking because the amine groups will cross-link to the lysines of proteins.
19. Fixed-cells can be stored 2-3 weeks at 4 °C prior to use.
20. Dehydration and DNA denaturation of 2 mL of fixed cells will allow 5 BrdU-labelled samples to be prepared.
21. Cells can be stored in 1x PBS 2-3 weeks at 4°C prior to use.
22. Primary antibodies incubation can be performed at room temperature for 1 hour on a rotating wheel.
23. At this step, Hoechst labelling of DNA can be performed if needed. To do so, resuspend the cells in 200 µL of Hoechst 33342 solution at 5 µg/mL, incubate 10 minutes at room temperature, centrifuge the cells for 5 minutes at 2,410 RCF, discard the supernatant and resuspend the cells in 20 µL 1x PBS for imaging.
24. 0.01% poly-L-lysine solution can be stored at 4 °C and use several times (~5-10 times) for coating.
25. For a better description of the range of parameters that can be used, see the documentation at https://github.com/ekatrukha/DoM_Utrecht.
26. If the density of fluorescent emitters is too high (for example in some T120 cells), a high-density fitting software such as UNLOC [27] is recommended.

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6. Acknowledgements

We thank the Morphoscope Bioimaging Facility partly supported by Agence Nationale de la Recherche (ANR-11-EQPX-0029 Morphoscope2, ANR-10-INBS-04 France BioImaging). We also thank Emmanuel Beaurepaire, Pierre Mahou and Marie-Claire Schanne-Klein for helpful discussions.

Figures Legends

Figure 1. Chemical structures of the thymidine and its analogues BrdU and EdU. (A) 2'-deoxythymidine (dT). (B) 5'-bromo-2'-deoxyuridine (BrdU). (C) 5'-ethynyl-2'-deoxyuridine (EdU).

Figure 2. BrdU-labelled nascent DNA localization in Δ*hts-hdrB* cells of *Halofeax volcanii* using superesolution imaging. Cells were analysed before BrdU addition in the medium, at the starting point of BrdU incorporation (T0), after 30 minutes, 60 minutes and 120 minutes. Anti-BrdU antibodies conjugated to DyLight649 were used. (A) SIM and STORM images (scale bar is 2 µm) including inset of STORM images (scale bar is 500 nm) whose area is represented by a dotted white square. Yellow cell outlines were drawn manually. (B) Mean intensity of the cellular fluorescent signal (in arbitrary units) measured from conventional wide-field images. Error bars represent standard deviation of at least three fields.

Figure 1

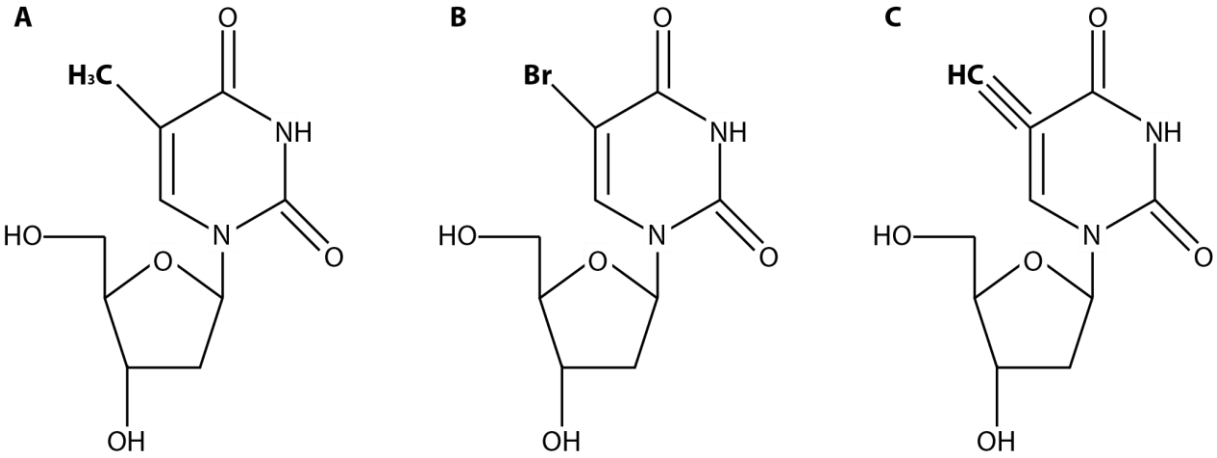
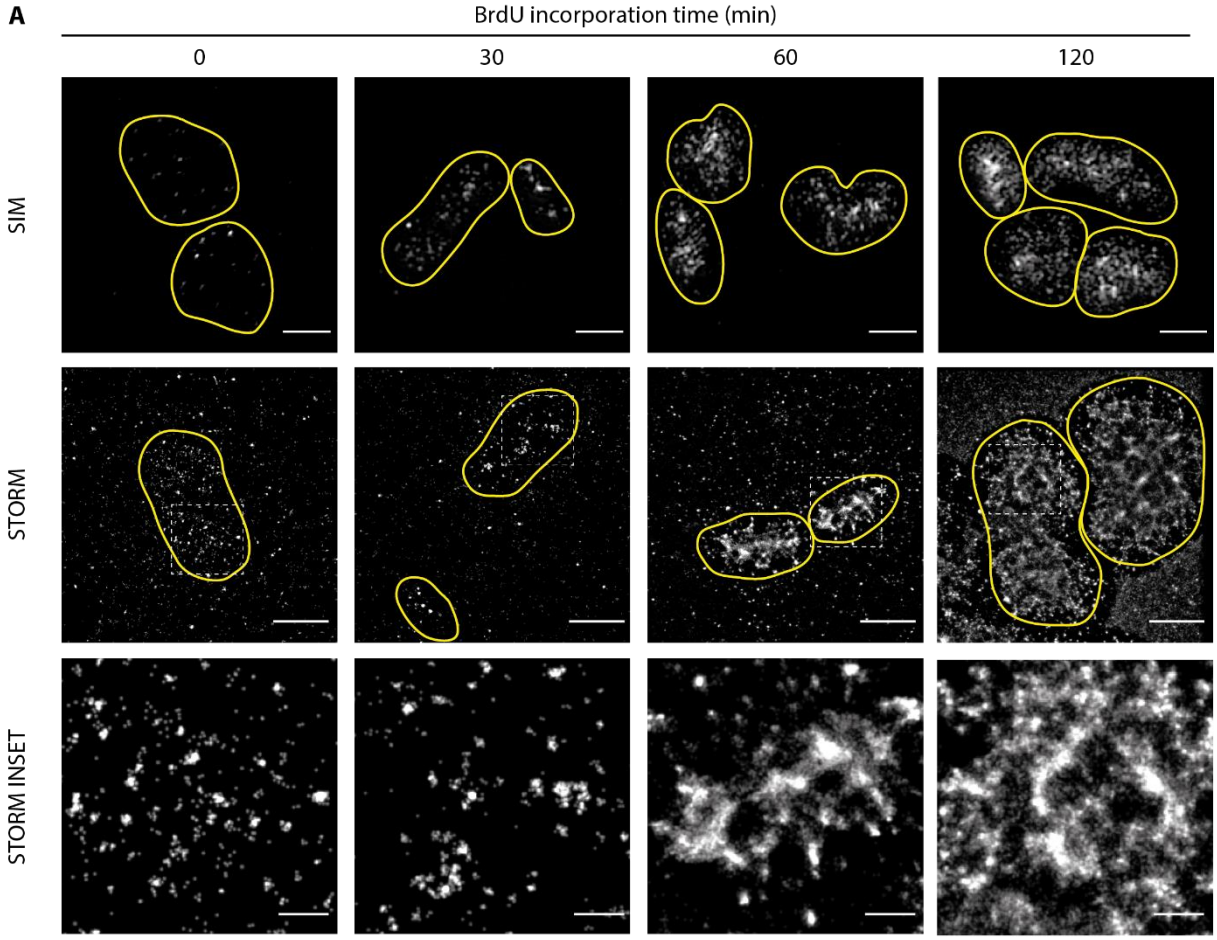
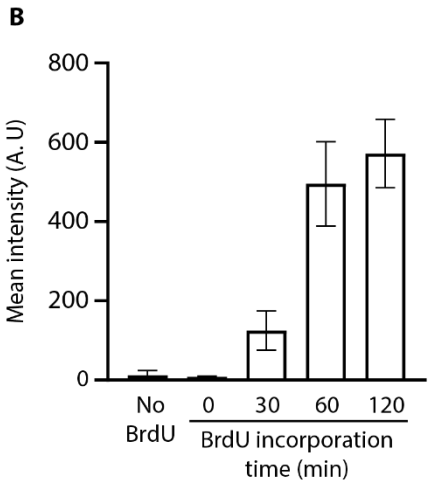


Figure 2


A
BrdU incorporation time (min)
0
30
60
120
SIM
STORM
STORM INSET


B
Mean intensity (A. U)
800
600
400
200
0
No BrdU
0
30
60
120
BrdU incorporation time (min)