# Protocol for Bacteria observation (Heavy metal stain)

The reagents required

1) 0.01% poly-L-lysine, 2) PBS (phosphate buffered saline), 3) 4% paraformaldehyde/1% glutaraldehyde in PBS, 4) DDW (double distilled water), 5) 3% platinum blue[1] (Nisshin EM #335, TI blue, the stock solution density is 6%) in DDW, 6) 5% PTA (phosphotungstic acid) in DDW (The pH of PTA is adjusted with NaOH to pH 7.0), and 7) water solution of an ascorbic acid (10 mg/ml) or dextrose (10 mg/ml).

1. Coat the ASEM dish membrane with 0.01% poly-L-lysine (drop 2 µL of poly-L-lysine on the membrane and dried). Wash the membrane twice with DDW, and dried.
2. Wash bacterial cell with PBS (3000 rpm, 10 minutes, twice) in a centrifuge tube.
3. Suspend the bacterial cell in PBS.
4. Apply the bacterial cell suspension on the ASEM dish membrane (30 - 60 minutes).
5. Fixation: Remove the suspension followed by adding 4% paraformaldehyde/1% glutaraldehyde for 10 minutes.
   (do not change the order of the 4 and 5 process)
6. Wash three times with DDW.
7. Stain for ASEM: Remove DDW followed by adding 3% platinum blue for 30 - 60 minutes.
8. Wash three times with DDW.
9. Remove DDW followed by adding 5% PTA for 15 - 30 minutes.
10. Wash three times with DDW.
11. Exchange DDW for ascorbic acid (10 mg/ml) or dextrose (10 mg/ml) prior to ASEM observation.
12. Place dish on ClairScope.
13. Start the observation under proper conditions (for example, spot size = 30 - 40, acceleration voltage = 30 kV) with ASEM (Fig. 1).

[1] S. Inaga *et al., Arch. Histol. Cytol.* 70, 43-49 (2007).

Note 1: For staining, the general reagents ($OsO_4$, Uranyl acetate etc.) may be applicable.

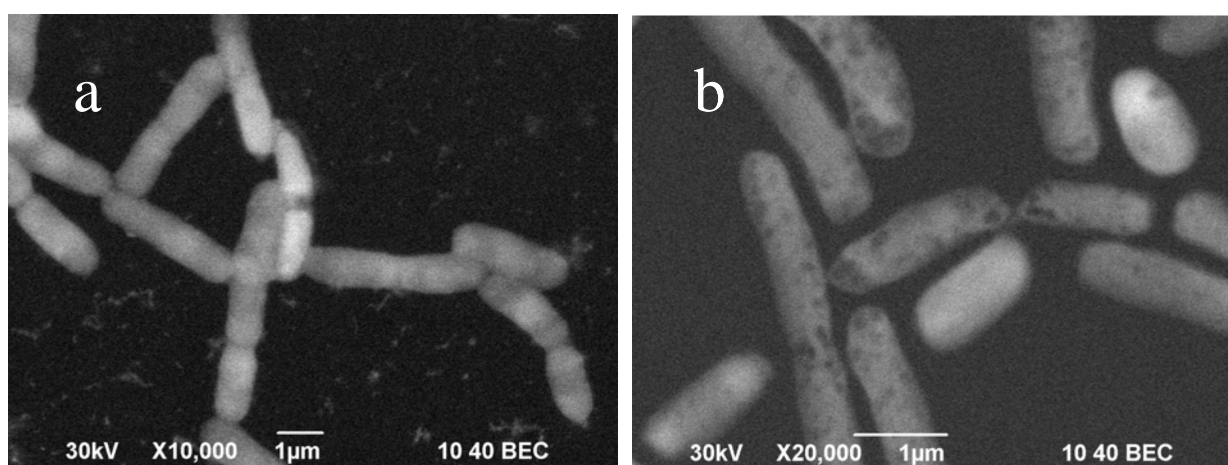


Fig. 1. ASEM images of *Escherichia coli* cultured for 2 hours (a) and for 48 hours (b).



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