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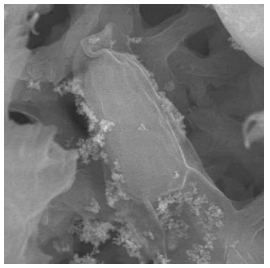
Magnetic Labeling, Filtration, Immobilization, and Processing of Bacillus cereus Spores for SEM Imaging V.3



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We use this protocol and it's working

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Abstract

This protocol describes a novel method for labeling *Bacillus cereus* spores with magnetic nanoparticles, filtering excess particles, immobilizing spores onto membranes, and preparing samples for scanning electron microscopy (SEM) imaging. It combines immunomagnetic labeling, filtration, chemical fixation, dehydration using tertiary butyl alcohol, osmium tetroxide vapor post-fixation, and sputter coating, offering a robust strategy for high-quality SEM imaging of bacterial spores.

Guidelines

- Always maintain cold chain and protect samples from drying out during filtration and labeling steps.
- Perform all fixation and dehydration steps with appropriate ventilation.
- Tertiary butyl alcohol must be completely melted before use.
- Osmium tetroxide handling requires special care: always work inside a fume hood.
- Ensure complete evaporation of tertiary butyl alcohol in the vacuum chamber to avoid sample loss.

Materials

- *Bacillus cereus* spores (ATCC 14579)
- 100 nm superparamagnetic nanoparticles conjugated to Protein A (nanomag®-D protein A, Micromod, product code: 84-20-102)
- 250 nm superparamagnetic nanoparticles conjugated to Protein A (nanomag®-D protein A, Micromod, product code: 09-20-252)
- Rabbit polyclonal anti-*B. cereus* antibody (ab20556, Abcam)
- Phosphate Buffered Saline (PBS) pH 7.4
- PBS with 0.5% Triton-X
- Ultrapure sterile water
- 2.5% Glutaraldehyde + 1% Formaldehyde in 0.1 M Cacodylate Buffer
- Ethanol series (30%, 50%, 70%, 90%, absolute ethanol)
- Tertiary butyl alcohol (≥99.5%)
- Osmium tetroxide solution (for vapor fixation)
- 0.45 µm PES membrane filters (Supor® Membrane, PALL Corporation)
- Water filtration system (funnels, manifolds, vacuum pump)
- Orbital shaker
- Glass petri dishes
- SEM metal stubs
- Copper adhesive tape
- Sputter coater (Cressington 108auto or equivalent)
- Scanning Electron Microscope (SEM)

Safety warnings

- Superparamagnetic nanoparticles must be resuspended homogeneously before use.
- Never pipette osmium tetroxide directly; use closed containers.
- Dehydration steps must be performed swiftly to prevent sample collapse.

Use appropriate PPE (lab coat, gloves, eye protection) throughout the protocol.

Before start

- Prepare fresh solutions of PBS 0.5% Triton-X, glutaraldehyde/formaldehyde fixative, and cacodylate buffer.
- Preheat tertiary butyl alcohol to 40°C.
- Turn on sputter coater and SEM equipment to allow warming up.
- Prepare vacuum filtration apparatus and test for leaks.
- Cool down a freezer to -20°C for freezing steps.
- Pre-cut copper adhesive tapes and mount them in metal SEM stubs.

Preparation of 100 nm Nanoparticles

1 Transfer 995 µL of PBS (pH 8.0) into a separate 1.5 mL Eppendorf tube.

2 Add 5 µL of 100 nm nanoparticle stock solution and mix gently.

3 Place the tube in a magnetic separator for 3 min.

4 Carefully discard the supernatant.

5 Remove from magnetic separator and ressuspend in 1000 µL of PBS (pH 8.0);

6 Repeat steps 3 - 5 two more times.

7 Resuspend the pellet in 100 µL of PBS (pH 8.0).

Note: This ensures a ratio of approximately 300 nanoparticles per spore when combined

Preparation of 250 nm Nanoparticles

8 Transfer 938.8 µL of PBS (pH 8.0) into a separate 1.5 mL Eppendorf tube.

9 Add 61.2 µL 100 nm nanoparticle stock solution and mix gently.

10 Place the tube in a magnetic separator for 3 min.

11 Carefully discard the supernatant.

12 Remove from magnetic separator and ressuspend in 1000 µL of PBS (pH 8.0);

13 Repeat steps 3 - 5 two more times.

14 Resuspend the pellet in 100 µL of PBS (pH 8.0).

Note: This ensures a ratio of approximately 300 nanoparticles per spore when combined

Preparation of Bacillus cereus Spores

15 Grow *B. cereus* spores following the "High-Yield Sporulation and Purification of Bacillus cereus
and Bacillus thuringiensis Spores" protocol from Antunes & Fonseca (dx.doi.org/10.17504/protocols.io.14egnwxwqg5d/v1).

Labeling of Bacillus cereus Spores

16 Harvest spores by centrifugation at 4000 × g, 10 min, wash three times with sterile deionized (DI) water.

17 Adjust final spore suspension to OD600 = 0.8 (~10^8 CFU/mL).

18 Centrifuge spores and resuspend pellet in 100 µL of a 1:50 dilution of anti-*B. cereus* antibody in PBS 0.5% Triton-X.

19 Incubate 30 minutes at room temperature (22–25°C) in a rotator at ~60 RPM.

20 Centrifuge and resuspend pellet in 100 µL of superparamagnetic nanoparticle suspension (~300 nanoparticles per spore) in PBS 0.5% Triton-X.

21 Incubate for 30 minutes at room temperature (22–25°C) in a rotator at ~60 RPM.

Filtration and Immobilization

22 Set up the filtration system with 0.45 µm polyethersulfone (PES) membrane filter.

Note: The filtration setup consisted of 250 mL funnels, 0.45 µm PES filters, suction manifolds, and a vacuum pump - components commonly used in water sample processing for microbiological analysis.

23 Turn on vacuum pump and add 250 mL PBS to pre-wet the system.

24 Mix labeled spore suspension with 20 mL PBS 0.5% Triton-X.

25 Pour the mixture into the funnel containing remaining PBS.

26 After full filtration, rinse the membrane with an additional 250 mL PBS.

27 Allow membrane to dry under suction for 2 minutes before turning off the pump.

Fixation and Dehydration

28 Cut a 10 mm × 10 mm section of the PES membrane filter.

29 Immerse in fixative solution (2.5% glutaraldehyde + 1% formaldehyde in 0.1 M cacodylate buffer) for 30 minutes with moderate orbital agitation.

30 Wash in sterile DI water.

31 Dehydrate sequentially in ethanol series (1 minute per step in a petri dish with moderate orbital agitation: 30%, 50%, 70%, 90%, absolute ethanol).

32 Transfer to melted tertiary butyl alcohol at 40°C in an eppendorf tube for homogenization.

Freezing and Drying

33 Mount the sample onto SEM stubs with copper adhesive tape.

34 Freeze the mounted samples at -20°C for 30 minutes.

35 Quickly place samples in the sputter coater vacuum chamber; evaporate tertiary butyl alcohol under vacuum.

Post-fixation and Sputter Coating

36 Expose samples to osmium tetroxide vapor for 5 minutes inside a closed Petri dish.

37 Sputter coat samples with an 8 nm gold layer (10 mA for 15 seconds).

38 Samples are ready for SEM visualization.