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Scanning Electron Microscopy to characterize cell shape in *Sulfolobus acidocaldarius*

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Abstract

This protocol describes the preparation of *Sulfolobus acidocaldarius* cells for imaging in Scanning Electron Microscopy (SEM). *Sulfolobus acidocaldarius* is a species of thermoacidophilic archaea. Due to their small cell size (~1µm), morphological studies rely on high-resolution imaging methods like super-resolution light microscopy or electron microscopy. Electron microscopy is the method of choice when imaging fine ultrastructural details. SEM has the advantage of imaging surface features of whole cells, as opposed to ultrathin sections used in transmission electron microscopy, providing a three-dimension-like view of cells.

Background

Cell shape is a fundamental aspect of cell biology. In bacteria with a rigid peptidoglycan-based cell wall, cell shape in each species is stereotypical (rod, coccus, etc), very little variation exists in given experimental conditions, and cells can typically withstand harsh preparation steps required for SEM imaging (strong chemical fixation, dehydration). On the contrary, most archaea do not possess a rigid peptidoglycan-based cell wall. Instead, the cell membrane is topped by a thin layer of proteins called the S-layer (surface-layer). Cell shape in archaea is often less stereotypical, exhibits larger variations under the same experimental conditions, and is typically more sensitive to harsh preparation steps. *Sulfolobus acidocaldarius* is a good example. Cells in the family Sulfolobus are described as irregular cocci of ~0.8-1.3 µm, and conventional transmission and scanning electron microscopy assays typically reveal large-scale surface features (Brock et al., 1972; Zhang et al., 2019).

In comparison, imaging methods that typically include less sample dehydration, such as immunofluorescence or cryo-electron microscopy show cells that are round, with a smooth surface (Samson et al., 2008; Dobro et al., 2013), consistent with live-cell imaging data where cells are imaged under physiological conditions (Pulschen et al., 2020; Charles-Orszag et al., 2021; Cezanne et al., 2023). Therefore, Sulfolobus cell shape appears sensitive to dehydration. Here we describe a protocol for SEM with fewer dehydration artifacts. We also compare two primary chemical fixation methods commonly used in Sulfolobus labs (alcohol fixation and aldehyde fixation) that result in similar overall cell shapes, but different levels of details at the cell surface.

Materials

Product name	Brand	Manufacturer	Catalogue number	Notes
German Glass Coverslips #1.5, 12 mm	Electron Microscopy Sciences		72290-04	
Poly-L-lysine solution	Sigma-Aldrich		P4707-50ML	Stock solution is 0.01%.
Harrick Plasma Expanded Plasma Cleaner 115V	VWR	Harrick Plasma	PDC001	Any plasma cleaner/glow discharger.
32% Paraformaldehyde Aqueous Solution, EM Grade	Electron Microscopy Sciences		15714	After opening glass ampules, transfer solution to a 15-mL conical tube and store in a cold room. Avoid storing in a fridge used to keep antibodies or other proteins. Use within 1 month of opening. Only handle in a fume hood, and dispose of as toxic liquid.
Ethanol, 200 proof	Thomas Scientific	Decon Labs V1016G	C745K43	
0.1 M HEPES pH 7.4				Used to prepare OsO_4 solutions. Other buffers like PBS tend to precipitate.
EMS Osmium Tetroxide, 2% Aqueous Solution	Electron Microscopy Sciences		19152	OsO_4 is extremely dangerous and should only be handled in a fume hood. Only use fresh (discard any remaining solution).

Product name	Brand	Manufacturer	Catalogue number	Notes
				Dispose of solution and wash steps containing osmium in a dedicated liquid waste container in the fume hood. Dispose of pipettes, tips, gloves, in a dedicated solid waste bag in the fume hood.

Equipment

Equipment name	Brand	Manufacturer	Catalogue number	Notes
Critical Point Drier Leica EM CPD300	Leica Microsystems		16771301	Similar results are expected with a different critical point drier.
Sputter coater Leica EM ACE600	Leica Microsystems		16771525	Similar results are expected with a different sputter coater.
Phenom Pharos G2 Desktop FEG-SEM	Thermo Fisher	Nanoscience Instruments		Similar results are expected on other SEM equipped with a Field Emission Gun, and backscattered and secondary electron detectors.

Software and bioinformatics tools

Software/tool/script	Company/Developer	Version	Web address	Github repository
Phenom Pharos G2 Desktop FEG-SEM	Thermo Fisher	Nanoscience Instruments		

Protocol

A. Cell culture and synchronization

1. Grow cells of interest (new species, cell shape or S-layer mutant strain) in appropriate media.

Here we grew *Sulfolobus acidocaldarius* strain MW001 (*pyrEF*) described in (Wagner et al., 2012) in Brock medium at pH 2.5 supplemented with 0.2% sucrose, 0.1% tryptone and 20 µg.mL^{-1} uracil (Sigma Aldrich),

at 76°C with shaking (140 rpm).

2. If necessary, synchronize the culture. Here we synchronized *Sulfolobus acidocaldarius* cultures using the acetic acid method (link to protocol by Gabriel Tarrason Risa) to minimize potential cell shape differences associated to differences in cell cycle phases. Cells were fixed at 120 min after release from the acetic acid.

A. PLL-coating of glass coverslips

1. Add the desired number of 12 mm glass coverslips to a 24-well plate.
   Note: we recommend preparing two technical replicates for each condition, as coverslips break easily.
2. Activate the glass surface by plasma treating for 1 min on high RF settings.
   Note: make sure to remove the lid of the 24-well plate so the glass is exposed to the plasma.
3. Add 300 µL 0.01% poly-L-lysine (PLL) solution to each coverslip and incubate for 30 min at room temperature.
4. Discard PLL, rinse three times with double distilled water, and allow to air dry.
   Note: PLL-coated coverslips can be kept dry at 4°C for several days prior to use.

B. Fixation Methods

i. PFA fixation

a) Add 0.5-1 mL of cells in Brock medium to each well and incubate without shaking at 76°C for 30-60 min. Fill the empty wells with the same volume of water to prevent evaporation.
   Note: the volume of cells should be determined empirically to reach an optimal cell density on the glass coverslip.
b) In the fume hood, add the required volume of 32% PFA so the final concentration in each well is ~4% (e.g.: 125 µL in 1 mL)
c) Incubate at room temperature for 1 hour.
d) Discard the media containing the PFA, and rinse three times with 500 µL PBS 1X.

ii. Stepwise ethanol fixation

a) Add 3 mL of cells in Brock medium directly to 1.5 mL ice-cold ethanol and incubate at RT for 10 min.
b) Add a further 1.5 ml ice-cold ethanol and incubate at RT for 10 min.
c) Add ice-cold ethanol up to a final volume of 10ml. The final sample should be in 77% ethanol and can be stored at 4℃ for up to 2 months.
d) Wash 1ml of fixed cells twice with 1 mL PBS 1X by centrifugation at 5,000 x*g* for 5 min to remove ethanol.
e) Add cells to the 24-well plate containing the PLL-coated coverslips and incubate for 30-60 min.
   Note: Alternatively, cell adhesion can be enhanced by centrifugation in the plate.

C. Post-fixation with osmium tetroxide (everything in the fume hood)

1. Transfer 2% OsO_4 solution to a conical (this avoids having to pipet from the glass ampule in the next step).

   Note: Be *extremely* careful when handling OsO_4 ampules, have solid and liquid waste disposal containers ready. OsO_4 are light-sensitive and not very stable and should be used right away or discarded.
2. Add one volume of 2% OsO_4 to each well. Cover the plate in foil (lid on) and incubate at room temperature for 1 hour.

   Note: pipette tips should go to osmium solid waste.
3. Remove foil and discard liquid in the wells.

   Note: liquid should go to osmium liquid waste.
4. Wash with 0.1 M HEPES pH 7.4 three times.

   Note: work fast enough so the cells are never allowed to fully dry.

D. Dehydration in graded series of ethanol

   Note: have 25%, 50%, 75% and 95% EtOH solutions ready. Work fast enough so the cells are never allowed to fully dry.

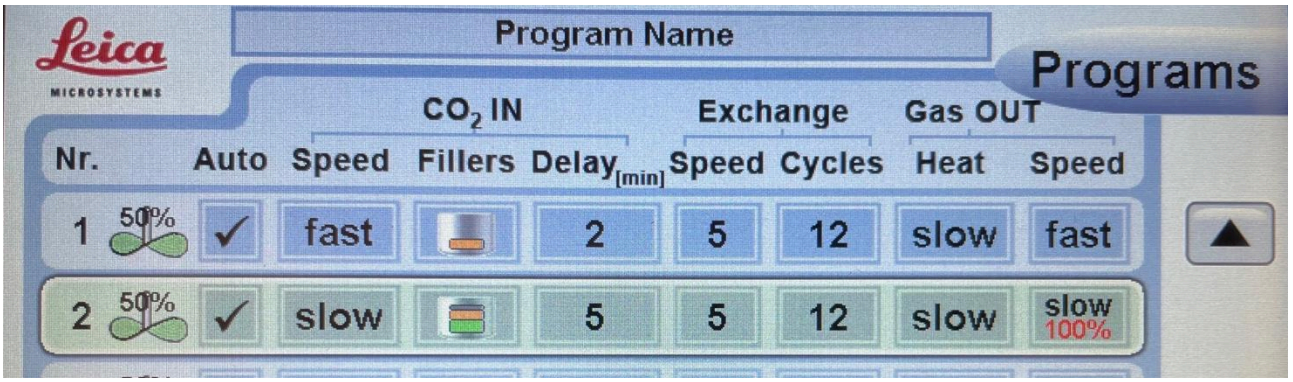
   Note: ethanol between each step is contaminated with OsO_4 and should be discarded accordingly (EtOH and OsO_4 are chemically compatible).

   1. Discard HEPES and add 500 µL 25% EtOH. Incubate for 20 min.
   2. Discard 25% EtOH and add 500 µL 50% EtOH. Incubate for 20 min.
   3. Discard 50% EtOH and add 500 µL 75% EtOH. Incubate for 20 min.
   4. Discard 75% EtOH and add 500 µL 95% EtOH. Incubate for 20 min.
   5. Discard 95% EtOH and add 500 µL 100% EtOH. Incubate for 20 min.
   6. Repeat this step once.

      Note: steps can be as short as 5 min if necessary for small cells, but better results are obtained with longer steps like 20 min.

E. Critical point drying and sputter coating

   1. Place the coverslips in the critical point dryer sample chamber and cover in 100% EtOH, making sure to never allow the cells to dry.
   2. Dry using a soft and slow automated program. An example program is given below:



3. Place the dried coverslips in the sputter coater and coat with 6-8 nm iridium.

   Note: Platinum or gold-palladium can also be used. Iridium tends to have a finer grain and give a more stable film.

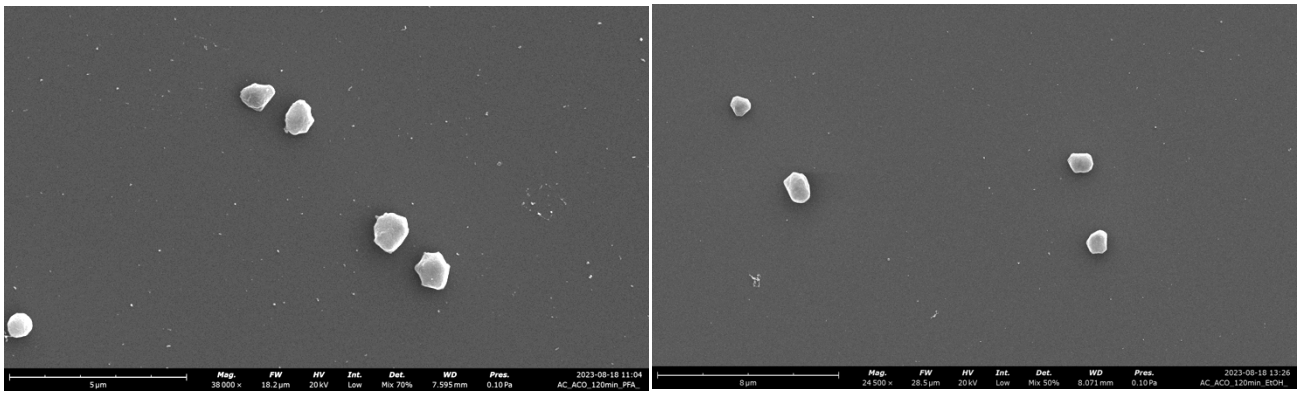
   Note: Do not allow the samples to rehydrate in ambient air between drying and sputter coating.

4. Image or store in a dry location.

   Note: some prefer to store SEM samples in a desiccation chamber containing solid desiccant to avoid rehydration.

F. Image in the Scanning Electron Microscope

Image using your favorite SEM. We recommend a SEM that uses a Field Emission Gun (brighter source) as well as backscattered and secondary electron detectors.



Comparison of *Sulfolobus acidocaldarius* morphology in PFA- (left) versus ethanol-fixed cells (right) at low magnification. In both cases, cells have an overall coccoid morphology. They look like irregular rocks of <1 µm. They look hydrated and their surface is smooth, with some cells exhibiting sharp angles. In both cases cell morphology is comparable with that of live cells (Pulschen et al., 2020; Charles-Orszag et al., 2021; Cezanne et al., 2023), suggesting that it is not a result of dehydration.

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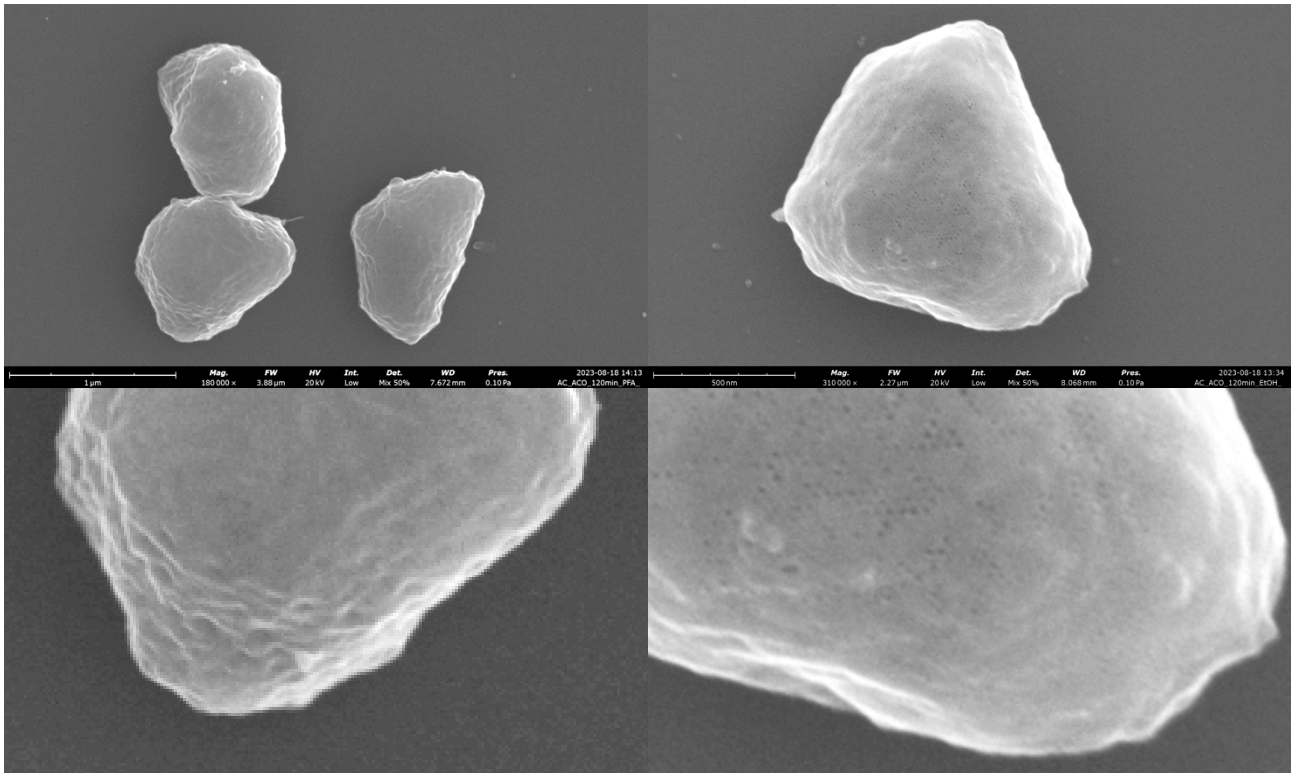

Comparison of *Sulfolobus acidocaldarius* surface features in PFA- (left) versus ethanol-fixed cells (right).

While PFA-fixed cells exhibit rough surface at high magnifications, ethanol-fixed cells reveal an overall smooth surface with regularly spaced holes that may correspond to the S-layer porous structure. Of note, a similar porous surface was observed in SEM images of *Sulfolobus islandicus* (Zhang et al., 2019).

Data analysis

Cell size measurements and image enhancement can be done in Fiji (Schindelin et al., 2012).

Recipes

The recipe for Brock medium can be found here (link to protocol by Fredrik Hurtig).

Additional Notes

- While the preparation of the glass coverslips (Step B) is optional and should be adapted in a species-specific way, we found it to be the best method for enhancing adhesion of *Sulfolobus acidocaldarius* to glass.
- Alternatively to step D, 1% OsO_4 can be added to highly adherent cells as follows: in the fume hood,

prepare a 1% solution of osmium tetroxide by mixing 2% OsO_4 with an equal volume of 0.1 M HEPES pH 7.4. Discard media/supernatant and add 500 µL 1% OsO_4 to each well.

- For dehydration in graded series ethanol, a gold standard is to use solutions made from anhydrous ethanol (prepared and stored with molecular sieves).

Competing interests

The authors declare that they have no conflict of interest.

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