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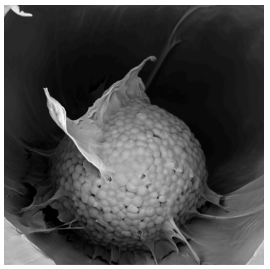
Scanning electron microscopy (SEM) protocol for imaging living materials



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We use this protocol and it's working

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Abstract

Scanning electron microscopy (SEM) can be used to image cells and colonies immobilized inside hydrogels after supercritical carbon dioxide (CO_2) extraction. Supercritical CO_2 extraction can also be used on suspension cells after filtering the sample onto a 0.2 µM filter attached into the extractors carriers. This protocol gives an overview on how different techniques can be used to characterize triblock copolymer hydrogels.

Materials

Reagents:

- formaldehyde
- Ethanol, 99.5 - 100 %
- MilliQ water, sterile
- 0.2 M phosphate buffer (20.44 g of Na2HPO4 and 6.72 g of NaH2PO4 per litre)
- liquid N_2

Supplies:

- falcon tubes/ependorfs/glass vials
- scalpels
- 12.5 mm aluminum SEM pin stubs
- conductive double sided carbon tabs/tape
- sharpie marker

Safety warnings

Formaldehyde (FA) is toxic and should handled accordingly. Wear protective gear!

N_2 cooled scalpels can break during sample cutting. Wear protective eyewear!

Supercritical CO_2 extraction involves high pressure. Do not leave extractor unattended while chamber temperature is rising.

Sample fixation

1 Prepare fixation solution ([M] 3.7 % volume formaldehyde in [M] 0.1 Molarity (M) phosphate buffer) 30m

2 Submerge sample into the fixation solution and incubate at Room temperature for 24:00:00 1d

3 Replace fixation solution and incubate at Room temperature for 24:00:00 1d

Sample Dehydration 1d 0h 30m

4 Prepare ethanol (EtOH) dilutions in milli-Q water as indicated below. 30m

5 Samples are dehydrated at Room temperature in an ascending EtOH series (40 – 90 %, 10 % steps; 96 %, 99.5 %).
Submerge sample into EtOH solution, let it incubate (minimum 02:00:00 per step), change the EtOH solution. 1d

EtOH steps:

1. [M] 40 % volume EtOH
2. [M] 50 % volume EtOH
3. [M] 60 % volume EtOH
4. [M] 70 % volume EtOH
5. [M] 80 % volume EtOH
6. [M] 90 % volume EtOH
7. [M] 96 % volume EtOH
8. [M] 99.5 % volume EtOH (Overnight)
9. [M] 99.5 % volume (for storage)

Supercritical CO_2 extraction

7h 25m

6 Cool the critical point dryer (E3100, Quorum Technologies) to 15 °C with a thermostat (Proline RP 1845, LAUDA) using thermostat external temperature probe. 1h

7 Connect the critical point dryer outlet to a bottle containing EtOH (half full) under fume hood (it is used to capture residues during extraction and to estimate the gas realese speed). 5m

8 Open the critical point dryer and mount the samples. Close the critical point dryer according to producers instructions. 10m

9 Open CO_2 inlet and fill the chamber with liquid CO_2. 2m

10 Slightly open the outlet and purge the chamber for 00:05:00 (bubbling inside the external EtOH bottle should not be too intensive). 10m

After purging close the outlet first then the inlet (to avoid pressure drop inside the chamber).

11 The chamber should be purged with fresh CO_2 6 – 8 times in 30 – 60 min intervals (let the EtOH diffuse out of the structure and purge it out of the chamber, use shorter intervals at the beginning of this process). 6h

- open the CO_2 inlet, then slightly open the outlet
- purge for 00:05:00
- close the outlet, then close the CO_2 inlet
- repeat 6-8 times in 30 – 60 min intervals

12 Increase the thermostat temperature to 37 °C (inlet and outlet of the chamber should be closed at this point). 1h 30m

Do not leave the critical point dryer unattended while the temperature is rising as the pressure can exceed the safety limit of the chamber.

Control the internal pressure so it does not exceed 110 bar by opening the chamber outlet (should be done slowly as too fast gas release can cool the reactor and turn supercritical state back to liquid state).

Adjust the outlet so that the pressure gauge stays stable around 105 bar as the temperature is rising

Leave the outlet open as it is, when 37 °C is achieved (do not open the outlet more, as the faster gas release can cool the reactor).

STEP CASE

Slow pressure release 9 steps

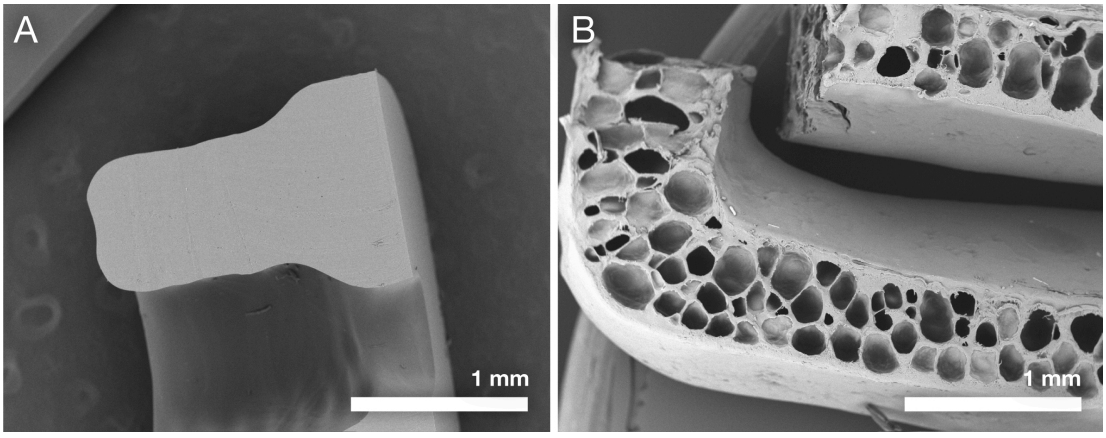

Figure 1 Slow pressure release (A) vs. fast pressure release (B)

13 Leave the outlet open until the chamber is ready to be opened (Overnight). 12h

As the pressure drops so does the bubbling.
Adjust the outlet so that there is always slight bubbling (do not over do it as it can result in pore formation **figure 1B**)

14 Before opening the chamber remove outlet tube from EtOH bottle that is situated under the hood (to avoid sucking EtOH into the chamber while opening it). 10m

Remove samples from the chamber and store in a sealable container (ependorf, glass vial, falcon tube)

Sample cutting and mounting

15 Attache conductive double sided carbon tabs/tape on aluminum SEM pin stubs and then lable them with a sharpie marker. 5m

16 5m

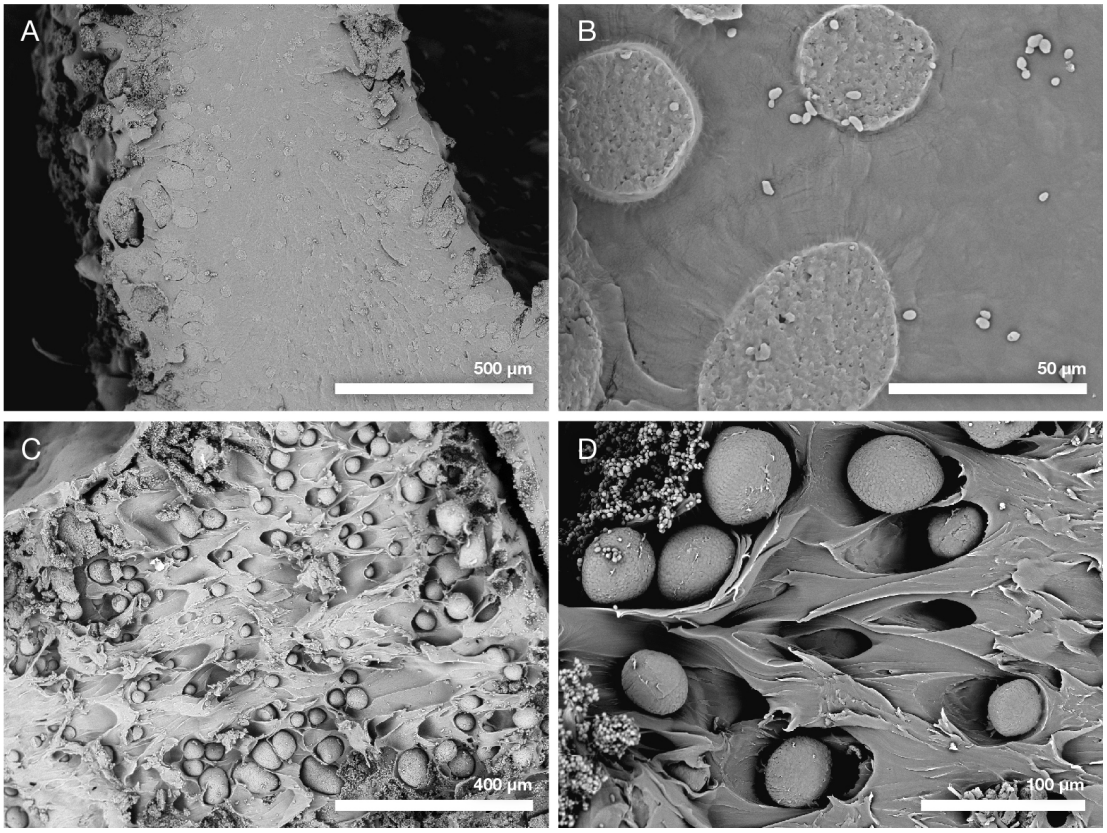

Figure 3: Sample cutting - exposing cell-material interactions in hydrogels. Varying the sample and the blade temperature together with the speed of cutting can be used to demonstrate various aspects of LMs. A combination of sample and scalpel cooling (~20 s) together with fast incisions results in the most accurate SEM images in terms of polymeric material and colony localization (A, B), but with this technique it is impossible to evaluate the colony size and shape because of the unknown location of the obtained cross-section in respect to the colony. A shorter duration of sample and scalpel cooling (~10 s) together with slow incision highlights biologically relevant information such as cell-polymer encapsulations (Figure 5 A - C) and colony size and shape (C, D) but results in cutting marks across the polymer (D). Different sample cuttings and resulting images: samples prepared with longer cooling of sample and scalpel showing relatively smooth cuts (A, B). Samples prepared with short sample and scalpel cooling showing clear colonies (C, D).

16.1 **Fast incision (Figure 3: A, B) - for acquiring artifact-free cross-sections** 20s

Immerse the sample with forceps and scalpel into liquid N_2 for 00:00:20 and instantly cut with fast incision (N_2 cooled scalpels can break during sample cutting. Wear protective eyewear!).

16.2 **Slow incision (Figure 3: C, D) - for acquiring information of colony-material interactions, colony size and shape** 15s

Immerse the sample with forceps and scalpel in liquid N_2 for 00:00:10 and cut after 00:00:03 at room temperature with slow incision.

17 Using forceps, pick up the cut sample and gently press it onto the two-sided carbon tape. 1m

Sputter Coating

18 Coat the sample with a 7.5 nm gold layer using a high vacuum sputter coater (EM ACE600, Leica Microsystems). 1h

SEM imaging 1d

19 Gold-coated samples were imaged with a tabletop scanning electron microscope (TM3000, Hitachi).
The imaging was done under a high vacuum and 15 kV accelerating voltage. 1d