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Extraction of microbial cells from methane-seep carbonate rocks for single-cell analyses (e.g. BONCAT-FISH) using “combination buffer” and percoll density centrifugation

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We use this protocol and it's working

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Abstract

Protocol to separate cells from carbonate rock to be able to image cells on a filter or slide with a microscope, using e.g. DAPI staining, BONCAT-FISH to see translationally active cells or for other subsequent single-cell techniques. In this protocol we use a combination buffer (Eichorst et al 2015) originally developed for soil and combine it with a percoll density centrifugation to separate the cells from rock material. The steps included in the protocol are: Cell fixation, cell extraction, density separation, preparing filter for DAPI staining or slides for BONCAT-FISH.

Materials

Syringe
Syringe filter (0.2 µm)
1X PBS (phosphate-buffered saline)
3X PBS
2% PFA in 3X PBS e.g. from 20% ampules
Ethanol
Water ice
Sonication wand (with thin probe to fit 2 ml tube)
50 ml tubes
Mortar and pestle
Optional: diamond saw, rotary tool with diamond wheel
RNAse away
70% Ethanol
Kimwipes
Transparent 2 ml tubes for centrifuge
MOBIO vortex adapter for 2 ml tubes or similar
Percoll (P4937, Sigma)
1 ml pipette and tips
balance
Nanopure water or similar
centrifuge up to 18000 g cooled to 4 °C, or paced in fridge
Tween 20
sodium pyrophosphate
PVP 40000 or PVPP

Making filter/slide
filtration unit
vacuum pump
polycarbonate filter membrane white or black 0.2 µm pore size (Millipore)
cover slips
poly-L-lysine coated, teflon printed slide (e.g. Tekdon)
big cover slips
pencil
small petri dishes
DAPI-Citifluor mounting mix
fluorescence microscope

Carbonate fixation

1 Make sure all solutions are filtered 0.2 µm the same day, e.g. using a syringe filter.

2 Fix small piece or crushed rock with 2% PFA in 3xPBS (marine samples) over night at 4 °C.
(PFA is toxic dispose properly and use PPE accordingly).

3 Wash at least 2x with 1x or 3xPBS and store in 30:70 1xPBS:Ethanol. Keep over night at 4 °C (to allow the ethanol mix to go inside the rock prior to freezing). Move to -20 °C for long term storage.

4 Run a positive and a negative control for the extraction, especially for a new sample type.
Positive control: e.g. a rock you know has lots of cells. Negative control: e.g. protocol without sample, or piece of cell-free calcite.

Carbonate cleaning and crushing

5 Take out sample from freezer and place on ice.

6 Wash rocks - to remove contamination and/or remove outside cells.
Option 1: Place carbonate into freshly filtered 1xPBS and sonicate 2x15 sec (sonication wand). Take out place into fresh buffer in fresh tube. Wash three times. After last time crush with mortar and pestle.
Option 2: If rock is too crumbly for sonication, shake or vortex 3 times in freshly filtered 1xPBS in fresh tube.
Option 3: remove surface of rock with a diamond saw e.g. if a lot of cells need to be removed or use a rotary tool with diamond wheel then wash with Opt.1 or Opt.2 for interior cells.

7 Wash agate mortar and pestle: 1) remove all visible dirt and rinse thoroughly with DI water 2) wipe with RNAse away 3) wipe with 70% Ethanol use clean Kimwipes. Then crush the clean rock piece with clean mortar and pestle to a fine paste.

Cell extraction with combination buffer

8 Add approx. 0.1 g crushed carbonate to transparent pre-weighed 2 ml snap cap tube. Do not overload the percoll centrifugation (add more or less depending on cell numbers, for more rock larger tubes and upscaling might be necessary).
Weigh after adding rock for an estimate of amount added.

9 Add 400 µl "combination buffer" (Eichorst et al. 2015), freshly filtered day of. (see recipe below, can be prepared in advance and stored sterile filtered at 4 °C in the dark for weeks to months).

(To the remaining rock not used for extraction add 1xPBS:Ethanol 30:70 and store at -20 °C.)
Place 2ml tubes with rock and comb. buffer on ice.
Sonicate 3x10 sec. with sonication probe, pause for 10 sec between to avoid heating of sample. Clean sonicator thoroughly between samples (Qsonica, Q55, lowest setting 10).
Shake tubes gently on e.g. MOBIO/Qiagen vortex adapter for 2 ml tubes for 30 min.

Percoll separation

10 Place 2ml tubes on ice. Place ethanol on ice. Place percoll on ice. All reagents need to be cold. Do not skip this step.

11 Add 450 µl pure 4 °C ethanol to tube. Place on ice until cold for 5 minutes. Do not skip this step.
(For rock powder the gradient formation worked reliable only with cold reagents which may depend on density, viscosity or other factors, do not skip for reliable gradient formation.)

12 Shake tube briefly and slowly pipette 950 µl of 4 °C percoll to the bottom of tube. Percoll should form a layer underneath the layer of combination buffer+ethanol+rock+cells.

13 Centrifuge at 4 °C 20 min 18 000g (use refrigerated centrifuge, alternatively place centrifuge into fridge). Take out and place carefully on water ice.

14 There should be a pellet of the rock material at the bottom, a percoll phase and the separated cells in the middle "fluffy interface" and some supernatant above. Pipette supernatant and fluffy interface to a fresh tube. Avoid percoll. Keep tube with rock pellet to measure weight later (see below).

14.1 See example image for the density gradient. The image shows an upscaled gradient for better visibility of layers. For microscopy much less material should be used (see above). Hold against light or different wall textures if phases are difficult to see. Even if no "fluffy interface" is visible, or only a few flakes, there might be enough cells for microscopy. The gradient can be upscaled easily but a fast refrigerated centrifuge for larger volumes and suitable tubes are necessary. If no density gradient is visible make sure to keep everything cold throughout, ethanol was added, and check densities of solutions (using a balance or calculate).

15 a. *Optional*: second percoll. If necessary for different reasons e.g. testing.
b. Fill tube to 1 ml with cold ethanol. Place on ice.
c. Vortex briefly and add 950 µl 4 °C percoll to bottom of tube.
d. Centrifuge at 4°C 20 min approx. 18 000 g. Take out and place on ice.
e. Pipette supernatant and fluffy interface to fresh tube. Avoid any percoll.

16 Proceed to putting cells on a filter or a slide. *Optional*: keep extracted cells in 50:50 1xPBS:Ethanol. Note that keeping cells in 50:50 1xPBS:Ethanol does not always work well for rock cells, but will depend a lot on the sample.

Put cells on a filter for counting DAPI cells (or FISH)

17 Add 1 ml Nanopure and spin down cells in extract for 3 min, 20000 g. (How to prepare a glass slide see further down)

18 Remove supernatant carefully, leave some liquid behind to not loose cells (approx. 100 µl).

19 Add 1900 µl 1xPBS and vortex gently. Spin down again.

20 Repeat last two steps then go to next step.

21 Resuspend in 1xPBS e.g. 25-400 µl. Pipette up and down to resuspend with 1 ml pipette if necessary.

22 Clean filter unit. Use either a glass funnel or 12-well Millipore vacuum filtration unit for more samples.

23 Add supporting filter, e.g. 5 µm pore size and on top polycarbonate white (for FISH) or black (for DAPI) 0.2 µm pore size filter with 25 mm diameter.

24 Add 5 ml 1XPBS, 0.2 µm prefiltered, to each well.

25 Measure the total volume of your cell extract if you do not use all of it (for cell nr calculation).

26 Add sufficient amount of cell extract to each well e.g. 100-1000 µl. Note down volume. Use 200 mbar pressure.

27 Wash with approx. 2x5 ml 1XPBS and 1x5 ml Nanopure or more if necessary. This can take some time.
(If a layer forms on the filter there might have been percoll left - avoid percoll when pipetting cell fraction or wash cells more before mounting them on the filter)

28 Label filters with pencil, place into small petri dish and dry 10 min at 40 °C.

29 Always check cell extraction success with a DAPI slide/filter before doing BONCAT or FISH. For 1/8th filter use 10-15 µl DAPI-Citifluor mix.

30 Proceed to microscopy or further FISH reaction and/or store filter at 4 °C short term or -20 °C longer term.

Put cells on a slide for BONCAT-FISH

31 Spin down cells 3 min 20 000 g. Orient all tubes same way. Remove liquid but 100-200 µl. Add 1 ml Nanopure. Wash 3 times.

32 Last time keep e.g. 20-300 µl depending on cell numbers. If pellet does not resuspend use e.g. sonication bath briefly.

33 Spot e.g. 5-8 µl on poly-L-lysine coated slide “10-well” and let dry at 40 °C for 10-15 min until dry (Only keep slide at 40 °C until dry, store at 4 °C until further steps e.g. BONCAT and/or FISH reaction).

Always check cell extraction success with a DAPI filter/slide before doing BONCAT or FISH. To check cell extraction success and cell density add approx. 3.5 µl DAPI- Citifluor mix to each well and place a big cover slip on top.

Continue with BONCAT and FISH reaction. As a recommendation do not put more than one sample on a slide, and use only one FISH probe combination per slide to avoid any cross-contamination.

Rock dry weight

34 Centrifuge the pellet with percoll layer again 3 min 20 000g. Remove percoll and wash rock pellet 1x in 1 ml Nanopure, centrifuge, remove Nanopure. Evaporate water at 40 °C (27-55 °C) over night or until dry and record weight. (Pre-weigh the 2 ml tube prior to adding rock and subtract tube weight).

Combination buffer recipe

35 0.5% v/v Tween 20, 3 mM sodium pyrophosphate, and 0.35% wt/v PVP 40000 or PVPP not specified (Eichorst et al 2015). I dissolved everything in 1xPBS. I filtered the buffer 0.2 µm and it worked very well.
The combination buffer was developed for soil and works well for rocks too. Store sterile filtered combination buffer at 4 °C and sterile filter an aliquot prior to use. Can be prepared in advance and stored sterile filtered at 4 °C in the dark for weeks to months, wrap lid with parafilm to avoid evaporation.

Percoll notes

36 Percoll should be aliquoted under sterile conditions in a laminar flow and stored at 4 °C. Can be filtered 0.2 µm. The density is not that high therefore decreasing the density of the supernat. seems to help to improve success rate (meaning the gradient forms nicely). Acidic samples will make a gel out of percoll. (there are other options for density centrifugation e.g. Nycodenz but in our case percoll worked well).

DAPI-Citifluor anti fade mounting mix

37 Mix:
50 ul DAPI working stock,1 mg/ml (Middle Rm -20 °C freezer, DAPI Box)
10 ml Citiflour AF1 (EMS cat#17970)
1 ml 1X PBS, pH 7 to 7.5 (filter sterilized)

Final concentration DAPI: 4.5 µg/ml=4.5ng/µl

(Dapi working stock: Made from parent stock of 10 mg DAPI per ml H2O, which is made by adding 1 ml 0.2 µm filtered Nanopure water to vial of e.g. Sigma# D9542-10MG, e.g. McGlynn et al. 2015)

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