Imaging of gene expression in single cells by visualizing mRNA using HCR-FISH v3.0 on filtered cells from marine sediment

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ABSTRACT

The purpose of this protocol is to visualize gene expression in single cells by targeting the mRNA of genes of interest using HCR-FISH v3.0. This protocol is specific to imaging mRNA within multicellular aggregates of anaerobic methanotrophic archaea (ANME) and sulfate reducing bacteria (SRB), separated from marine sediment samples. It can be used directly for imaging large cells in marine sediment cells or for smaller cells by changing the pore size of the polycarbonate filter.

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Protocol status: Working
We use this protocol and it's working but it is very dependent on the targeted gene. Some genes, especially longer genes just make better targets.

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Density separation of ANME-SRB aggregates from sediment

26m 20s

1 Take 50 µL of sediment slurry in a 2mL Eppendorf tube. Add 850 µL TE Buffer (1M Tris, 0.1 M EDTA, pH 8) and 100 µL 100 µM pyrophosphate.

2 Incubate at 60 °C for 00:03:00, in a heating block. 3m

3 Allow tube to cool on ice, and sonicate using the sonication wand for 00:00:10, three times. Allow 00:00:10 rest between each cycle of sonication. Rinse the wand with nanopure water and wipe clean with a Kim Wipe before using for the next sample. (Sonication disrupts sediment-attached cells from sediment particles.) 20s

4 Add 1 mL of Percoll using a pipette. Insert the tip all the way to the bottom of the tube and allow the Percoll to settle below the sediment layer.

5 Centrifuge at 16000 rpm, 4°C for 00:20:00 20m

6 After this spin, you will notice that the sample has separated into several layers, with a sediment pellet at the bottom, followed by Percoll and the aqueous layer on top. At the interface of the Percoll and the aqueous layer, lies the brown organic material, which includes the aggregates and other cells.

6.1 Remove the aqueous layer and the brown material and move to a separate 2 mL tube.

7 Centrifuge the aqueous layer at 16000 rpm, 4°C for 00:03:00 3m

8 Remove the supernatant to a separate 2 mL tube. You can preserve this if you feel that cells are present in the supernatant. You can filter the cells in the supernatant onto a 0.22 µm pore size filter and image that.

9 Re-suspend the brown pellet in 200 µL of 1X PBS. Centrifuge at 16000 rpm, 4°C for 00:03:00 again, to wash the pellet in PBS. 3m

10 Discard the supernatant of the PBS-washed cells and re-suspend the pellet in 200 µL of 1:1:: 1X PBS: Ethanol.

11 Preserve the sample in PBS:Ethanol at -20 °C or proceed with microscopy.

Preparation of filters containing density-separated ANME-SRB...

12 Preparation of glass filtration setup: Fit the fritted glass top with rubber septum onto an Erlenmeyer flask. Rinse the frit with DI water first. Attach the rubber tubing on the flask to the vacuum pump. Add the measuring glass filter tower on top of the frit and clamp down with the silver clamp.

12.1 Wash the entire set up with atleast 10 mL of milliQ water and 5 mL of PBS. Set aside the measuring glass filter tower and clamp.

13 Use tweezers to take a 5 µm PVDF backing filter (EMD Millipore), dip it in nanopure water and place on the glass frit. Add a 3 µm (use a 0.2 µm filter if you want cells smaller than ANME-SRB aggregates) white polycarbonate filter (EMD Millipore) on top. Add the measuring glass filter tower on top of the polycarbonate filter and clamp down with the silver clamp. Filter down 200 µL of density-separated cells from sediment in PBS:ethanol from Step 10.

14 Wash twice with 3 mL of PBS each. This can take from 00:02:00 to 00:15:00 depending on the density of the sample. 17m

15 Allow the polycarbonate filter to air-dry. Cut the filter into quarters. Use one quarter filter for each reaction with a different set of probes and for a control reaction.

Visualization of gene expression in single cells using HCR-FIS... 1h

16 The rest of this protocol is adapted from the HCR-FISH v3.0 protocol for "bacteria in suspension" from Molecular Technologies.
https://www.moleculartechnologies.org/supp/HCRv3_protocol_bacteria.pdf
Reagents and probes were purchased from Molecular Technologies.

16.1 For details on the principles behind HCR-FISH v3.0, refer the Molecular Technologies website. https://www.moleculartechnologies.org/info/protocol and Choi *et al.* Development (2018).

16.2 Target selection: Choose target genes of interest and design probes using the default settings available on the Molecular Technologies website. (From my experience, probes targeting longer genes typically have higher signal and are more successful.)

16.3 Fluorophore selection: Combine the probe design with an appropriate initiator and fluorescent amplification hairpin set. (We typically use hairpins in the cy5 or FITC channel since the ANME probes are brightest in the cy3 channel while they are sometimes autofluorescent in the TRITC channel.)

HCR-FISH v3.0 - Hybridization stage 1h

17 Move the filter with density-separated cells into a 1.5 mL Eppendorf tube. Add 200 µL of 30% LMW probe hybridization buffer to the tube and immerse the filter for 01:00:00 at 37 °C .

18 **Preparation of the probe solution:** While the filter is under pre-hybridization, prepare probe solution by adding 2 pmol of each probe mixture (odd & even: 1 µL of 2 µM stock per probe mixture) to 100 µL of 30% LMW probe hybridization buffer at 37 °C . Final concentration of probes is set to be 4 nM.

19 Remove the 30% LMW probe hybridization buffer from the tube containing the filter. Add the probe solution directly to the filter.

20 Incubate the filter at 37 °C overnight for 16:00:00. 16h

21 Remove the 30% LMW probe hybridization buffer.

22 **Wash step:** Add 1mL of pre-heated (at 37 °C) probe wash buffer to the sample. Incubate for 00:05:00 at 37 °C . Remove the probe wash buffer. 5m

23 Repeat Step 22 two more times, only incubate for 00:10:00 at 37 °C . Remove the probe wash buffer. 10m

24 Proceed to amplification step.

HCR-FISH v3.0 - Amplification stage

1h 1m 30s

25 Incubate the filter in 150 µL amplification buffer in the same 1.5 mL Eppendorf tube for 00:30:00 at Room temperature for pre-amplification. 30m

26 **Preparation of hairpins:** While the filters are in the pre-amplification stage, prepare 15 pmol of hairpin H1 and 15 pmol of hairpin H2 by snap cooling 5 µL of 3 µM stock (heat at 95 °C for 00:01:30 and cool to Room temperature in a dark drawer for 00:30:00. 31m 30s

26.1 **Preparation of hairpins:** Add hairpins to 100 µL LMW amplification buffer.

27 Add 100 µL of hairpin mixture directly to the tube with filter containing 150 µL amplification buffer to reach a final hairpin concentration of 60 nM.

28 Allow amplification to occur at Room temperature in the dark for 12:00:00 (up to 16 hours). (Sometimes, I only do this for 6 hours. This only works if the signal is really bright for a given target probe-amplifier combination). 12h

HCR-FISH v3.0 - Final washing stage 15m

29 Add 1 mL of 5X SSCT at room temperature to the 1.5 mL Eppendorf tube containing the filter to dilute the solution.

30 Remove the 5X SSCT solution. Add 500 µL of fresh 5X SSCT to the tube and incubate at Room temperature for 00:05:00. 5m

31 Remove the SSCT solution. Repeat Step 30 twice, only incubate the filter for 00:10:00 each time. 10m

32 Allow the filter to air-dry in the dark and mount on a glass slide with 25 µL of DAPI:citifluor and cover with a 25x25 mm cover slip.

33 Proceed with imaging the slide using a fluorescent microscope.