

*Red text is to be completed by The Society after review *Blue text is to be replaced with protocol details *Green text is general advice

Live Cell Imaging of Ca. Nha. antarcticus and Hrr. lacusprofundi using agarose pads

| Corresponding author: | Joshua N Hamm |
|-----------------------|---|
| List of authors: | Joshua N Hamm, Anja Spang |
| Group leader: | Anja Spang |
| Institution: | Netherlands Institute for Sea Research (NIOZ) |

Zenodo DOI: [To be added after scientific review by the Society for Archaeal Biology]

| Protocol Category: | [Restate the category provided in the submission form] |
|--------------------|--|
| Model organism(s): | Ca. Nha. antarcticus, Hrr. lacusprofundi |
| Tags: | DPANN, Haloarchaea, Microscopy, Live-cell imaging |

Abstract

This protocol is an adapted form of the protocol developed for imaging haloarchaea (Liao *et al.*, 2021) and subsequently applied to co-cultures containing nanohaloarchaea and haloarchaea (Hamm *et al.*, 2024). The purpose is to provide a cost-effective reliable protocol for immobilising cells in imaging chambers for conducting live cell imaging experiments.

Related publication(s): Hamm et al, 2024 and Liao et al, 2021

Background

DPANN archaea are a diverse group primarily composed of symbionts with small cells and reduced genomes that rely on direct cell-cell interactions with a host species (Dombrowski *et al.*, 2019). Within this, the Nanohaloarchaeota are an approximately phylum level lineage which have multiple cultivated representatives all of whom require host from the *Halobacteriales* (Hamm *et al.*, 2019; La Cono *et al.*, 2020; Reva *et al.*, 2023). Little is understood regarding the dynamics of these inter-species interactions and so techniques for tracking interactions in real-time are necessary. This protocol describes a workflow with which cells of the nanohaloarchaeon *Ca.* Nha. antarcticus and those of its host *Hrr. lacusprofundi* can be stained with non-cytotoxic dyes and imaged over periods of several days using agarose pads. The major alternative to agarose pad based imaging is microfluidics systems but such systems require either a commercial microfluidics

system or a custom build system both of which would require optimisation for working with halophiles. Agarose pads provide a cost-effective alternative that when executed properly can provide data of comparable quality at a fraction of the cost.

Materials.

| Product name | Brand | Manufacturer | Catalogue number | Notes |
|---------------------------------------|-----------------|--------------|------------------|--|
| Agarose | Nippon Genetics | | AG01 | |
| Imaging Dishes, µ-Dish 35 mm, high | lbidi | | 81156 | |
| Cover Slips, 18x18mm | Roth | Epredia | P233.1 | Smaller diameter coverslips can also be used |
| MitoTracker Dyes | ThermoFisher | Invitrogen | M7512 | |
| DBCM2 Media | Made in-house | | | See Dyall-Smith, 2015 for recipe |
| Microscope Slides | | | | |

Equipment

| Equipment name | Brand | Manufacturer | Catalogue number | Notes |
|-----------------------------------|---------------|--------------|-------------------------------|-------|
| 0.45 µm syringe filter | Sigma-Aldrich | Millex | SLHPX13 | |
| 0.2 µm syringe filter | Sigma-Aldrich | Millex | SLGP033RK | |
| Okolab stage top chamber | Okolab | | CO2-O2 Unit-BL [0-10;1-18] | |
| Zeiss Axio Observer Microscope | Zeiss | | | |

Protocol

- A. Purification of Nanohaloarchaeal Cells
 - a. Remove plunger from syringe and attach 0.45 µm syringe filter to end
 - b. Pour ~10 mL of Nanohaloarchaeal Enrichment Culture into syringe
 - c. Filter into fresh falcon tube (Replace filter if it becomes too clogged with cells to filter effectively)
 - d. Repeat steps b and c into a new falcon tube
 - e. Discard used syringe and filters
 - f. Repeat step a with 0.2 µm filter and pour filtrate into syringe
 - g. Filter into new falcon tube (Replace filter if it becomes too clogged with cells to filter effectively)
 - h. Aliquot filtered cells into Eppendorf tubes
 - i. Centrifuge cells at 20,000 G in tabletop centrifuge for 10 minutes

- j. Resuspend all pellets in the same 999 μ L of fresh DBCM2 media and aliquot into a new Eppendorf tube
- B. Staining of Cells with MitoTracker Dyes
 - a. Aliquot 999 µL of Hrr. lacusprofundi culture grown to stationary phase into an Eppendorf tube
 - b. Thaw MitoTracker dyes and add to cells as per manufacturer's instructions to achieve desired concentration (typically 1 : 999 µL)
 - c. Mix thoroughly by shaking tubes
 - d. Incubate in the dark for 1 h
 - e. Pellet cells by centrifugation at ~20, 000 G in tabletop centrifuge for 10 minutes
 - f. Resuspend cells in fresh DBCM2 media and transfer to a new Eppendorf tube
 - g. Repeat steps e and f
 - h. Repeat step e one more time and following this resuspend the *Hrr. lacusprofundi* cells in ~500 μ L of fresh DBCM2 media and the *Ca.* Nha. antarcticus cells in ~50 μ l of fresh DBCM2 media
- C. Making of Agarose Pads
 - a. If needed, prepare microscope slides for making pads by wrapping multiple layers of tape around the slide to create two raised surfaces ~1 mm thick (Fig. 1a and b)
 - Weigh out enough agarose to make a 1% w/v mixture in the desired volume (e.g. 0.1g of agarose for 10 mL of volume) and add to glass bottle
 - c. Aliquot the desired volume of DBCM2 media into bottle with agarose
 - d. Melt agarose in the microwave for ~30 45 seconds
 - e. Once agarose has cooled slightly use a pipet to aliquot \sim 50 100 µL of molten agarose onto a microscope slide between the raised surfaces of tape such that the top of the agarose droplet reaches the height of the tape (Fig. 1c)
 - f. Place a coverslip directly over the agarose so that the coverslip is supported by the layers of tape but contacts the agarose creating a flat surface (Fig. 1d)
 - g. Allow agarose to dry (Fig. 1d)



D. Preparation for Imaging

- a. Remove the lid from an imaging dish
- b. Carefully move a coverslip mounted agarose pad to the edge of the microscope slide placing slight downward pressure to ensure the pad moves with the coverslip
- c. Slide the coverslip and agarose pad off the slide together so that the pad remains attached to the cover slip and place the combination with the pad facing upwards on the microscope slide (Fig. 1e)
- d. Mix 10 µl of each of the cell types (final volume 20 µL) together in a fresh tube
- e. Load 6 μ L of co-culture onto agarose pad
- f. Place agarose pad and coverslip into imaging dish with the pad facing downwards
- g. Aliquot 4 mL of fresh DBCM2 media onto the coverslip-pad combination taking care not to disturb the pad
- h. Mount sample onto microscope and turn on climate control
- E. Image sample

Recipes

The recipe for DBCM2 media can be found in the Halohandbook (Dyall-Smith, 2015).

Additional Notes

• This protocol should work for live imaging of other haloarchaeal species as well

Trial and error

Agarose Supplier:

Some brands of agarose (and agar) contain too much residual detergent used during the manufacturing process. This detergent can disrupt the cell walls of haloarchaea and cause them to lyse. The brand used here (Nippon Genetics) does not appear to have this issue but other brands may. In case this is a problem either switch to an alternative brand of agarose or try washing the agarose before use to clean any detergent from the mix. To wash agararose, suspend the agarose in milliQ water and mix thoroughly, wait for the agarose to settle at the bottom of the container and then pour off the excess water, repeat this 3 times and the agarose is washed.

Agarose Percentage:

In this protocol we use an agarose percentage of 1%. Some other protocols suggest use of pads as low as 0.1%. In our experience lower percentage agarose gels resulted in higher frequencies of lysis in control samples which may be related to effects of pad dehydration during the imaging runs. Optimisation of agarose percentage should be performed as part of the initial testing of the protocol.

Pad Size:

Pad dimensions are important factors in success of the imaging. If the cells appear to be compressed and lyse it may be the agarose pad is too thick, if the pad-coverslip combination does not remain stationary it may be that the pad is too thin. Similarly, a pad that is too large or small in area may cause issues with compression or movement. Optimisation of pad dimensions is necessary and will take several attempts to get right. The optimal dimensions will vary between setups and so this is something that should be done as part of the initial experiment testing phase.

Sample Loading:

Volume of sample loaded onto the pad will influence stability of the pad in the imaging chamber as the liquid volume applied to the pad will remain between the pad and imaging surface. The volume used above (6 μ L) is what we have found to work best for our set up but it's possible that this may not work on alternative set ups so along with pad dimensions the sample volume should be optimised during initial testing.

Dye Selection:

In the original optimisation of the protocol we tested 3 MitoTracker Dyes: Green, Orange, and DeepRed. We found that all 3 work similarly well for *Hrr. lacusprofundi* but MitoTracker Orange works less well for *Ca*. Nha. antarcticus than the others. If the staining protocol above is followed i.e. 1 hr staining, then this dye provides sufficient staining for imaging but it seems uptake of the Green and DeepRed dyes is faster than Orange in *Ca*. Nha. antarcticus. For this reason we mostly use DeepRed for *Ca*. Nha. antarcticus and Orange for *Hrr. lacusprofundi*.

Competing interests

The authors declare that they have no conflict of interest

Acknowledgments

We would like to thank Yan Liao for development of the original protocol on which this was based. We would also like to thank Alexandre Bisson for providing valuable feedback and advice in optimising the protocol.

References

Dombrowski, N. *et al.* (2019) 'Genomic diversity, lifestyles and evolutionary origins of DPANN archaea', *FEMS Microbiol Lett*, 366(2). Available at: https://doi.org/10.1093/femsle/fnz008.

Dyall-Smith, M. (2015) The Halohandbook v7.3.

Hamm, J.N. *et al.* (2019) 'Unexpected host dependency of Antarctic Nanohaloarchaeota', *Proc Natl Acad Sci U S A*, 116(29), pp. 14661–14670. Available at: https://doi.org/10.1073/pnas.1905179116.

Hamm, J.N. *et al.* (2024) 'The parasitic lifestyle of an archaeal symbiont', *Nature Communications*, 15(1), p. 6449. Available at: https://doi.org/10.1038/s41467-024-49962-y.

La Cono, V. *et al.* (2020) 'Symbiosis between nanohaloarchaeon and haloarchaeon is based on utilization of different polysaccharides', *Proc Natl Acad Sci U S A*, 117(33), pp. 20223–20234. Available at: https://doi.org/10.1073/pnas.2007232117.

Liao, Y. *et al.* (2021) 'Cell division in the archaeon Haloferax volcanii relies on two FtsZ proteins with distinct functions in division ring assembly and constriction', *Nature Microbiology*, 6(5), pp. 594–605. Available at: https://doi.org/10.1038/s41564-021-00894-z.

Reva, O. *et al.* (2023) 'Functional diversity of nanohaloarchaea within xylan-degrading consortia', *Frontiers in Microbiology*, 14. Available at: https://doi.org/10.3389/fmicb.2023.1182464.

This protocol template was inspired by the <u>Bio-protocol Manuscript template</u>