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

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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	Ibidi		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			

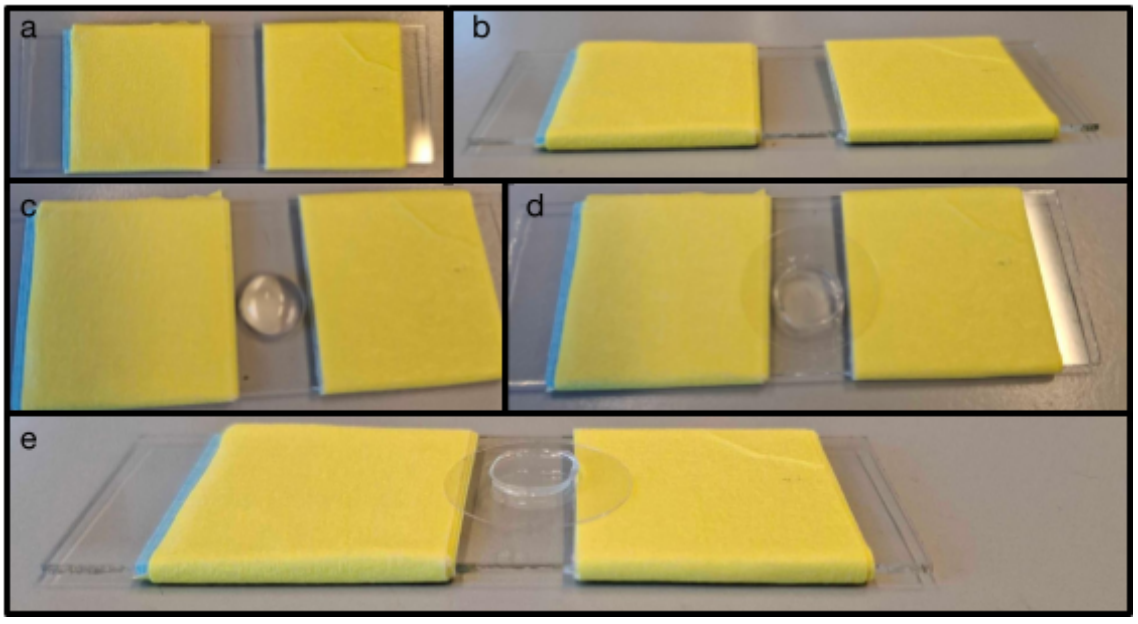
Protocol

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 µL 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 µL of fresh DBCM2 media and the *Ca.* Nha. antarcticus cells in ~50 µl 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)
   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 ~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 µL 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 µL) 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

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