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

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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 and 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 a 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. Still, such systems require either a commercial microfluidics system or a custom-built 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

In order to conduct live-fluorescence co-culture experiments it is necessary to purify nanohaloarchaeal cells from the enrichment culture they are maintained in. This accomplishes two main things, firstly it allows staining of *Ca.* Nha. antarcticus separately to *Hrr. lacusprofundi* which enables the identification of the two based on the dye used for staining (assuming different dyes are used for each species). Secondly, by purifying nanohaloarchaeal cells and then re-introducing them to pure host cultures the stage of interactions can be approximately synchronised and tracked from start to finish as opposed to the enrichment culture where it is not possible to infer the stage of any given interaction at the time of imaging. Filter sizes used (0.45 µm and 0.2 µm) are based on a cell size range of 0.15 - 0.6 µm for the nanohaloarchaeon. Rod-shaped host cells have widths of ~0.5 µm which necessitates the 0.2 µm filtration step. Other systems may not require such stringent filtration or may require different pore sizes based on relative cell sizes of the host and symbiont.

a. Remove the plunger from the syringe and attach a 0.45 μm syringe filter to the end
b. Pour ~10 mL of Nanohaloarchaeal Enrichment Culture (Late-exponential stage, ~3x10^8 cells/mL) into the syringe
c. Filter into a fresh Falcon tube (Replace filter if it becomes too clogged with cells to filter effectively)
d. Repeat steps b and c in a new Falcon tube
e. Discard the used syringe and filters
f. Repeat step a with a 0.2 μm filter and pour the filtrate into the syringe
g. Filter into a new Falcon tube (Replace the filter if it becomes too clogged with cells to filter effectively)
h. Aliquot filtered cells into 1.5 mL Eppendorf tubes (1.5 mL of filtered cells per tube)
i. Centrifuge cells at 20,000 G in a 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

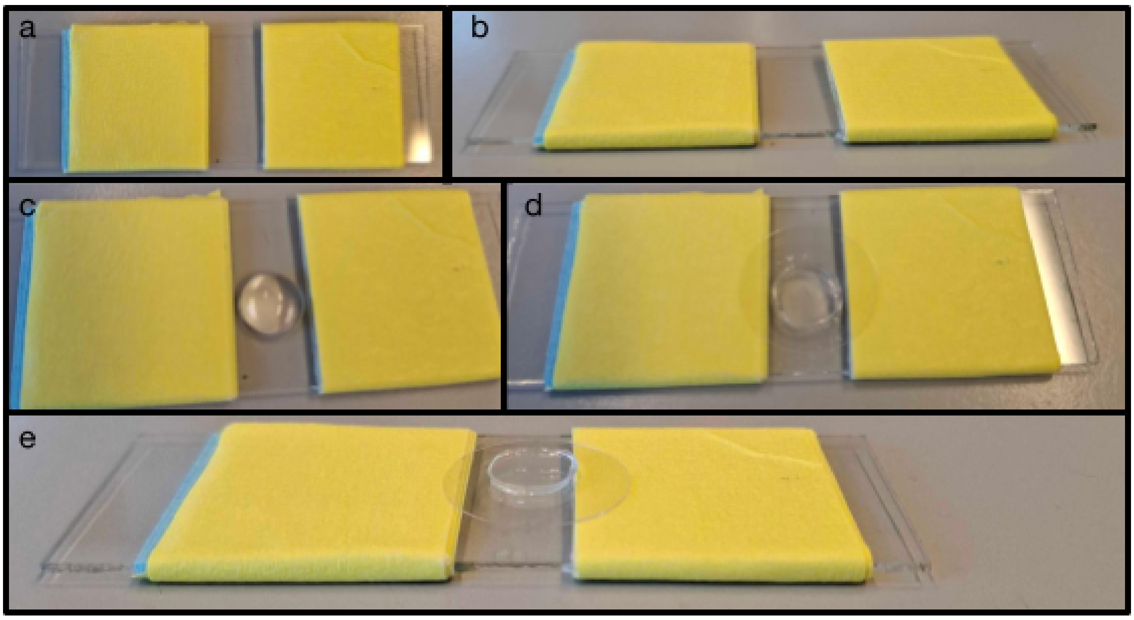
In order to stain both *Ca.* Nha. antarcticus and *Hrr. lacusprofundi* with non-cytotoxic dyes for visualisation of interactions MitoTracker dyes are used. Originally developed for staining of mitochondria, MitoTracker dyes are mildly lipophilic and at lower concentrations can be used for membrane staining. At moderate concentrations (such as used in this protocol) the dyes also accumulate in the cytoplasm, providing whole cell staining. Growth experiments show no impact on cell viability in batch cultures. Staining of each species with a different MitoTracker allows differentiation of the two in fluorescence micrographs.

a. Aliquot 999 µL of Hrr. lacusprofundi culture grown to stationary phase in an Eppendorf tube
b. Thaw two different MitoTracker dye stock solutions (1 mM) on ice e.g. MitoTracker Orange and MitoTracker DeepRed
c. Add 1 μL of one MitoTracker dye stock solution to Nanohaloarchaeal cells from step A.j. to achieve the desired final concentration (for halophilic archaea: 1 μM)
d. Add 1 μL of the other MitoTracker dye stock solution to *Hrr. lacusprofundi* cells from step B.a. to achieve the desired final concentration (for halophilic archaea: 1 μM)
e. Mix tubes thoroughly by shaking
f. Incubate in the dark for 1 h
g. Pellet cells by centrifugation at ~20, 000 G in a tabletop centrifuge for 10 minutes
h. Resuspend cells in fresh DBCM2 media and transfer to a new Eppendorf tube
i. Repeat steps g and h
j. Repeat step g 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

To immobilise cells in place for time-lapse imaging we use agarose pads prepared with the same media components as found in the cultivation media used for growing *Ca.* Nha. antarcticus and *Hrr. lacusprofundi*. Cells are mounted between the pads and the imaging surface resulting in a solid mount which reduces movement of the cells during imaging.

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 (Nippon Genetics, Catalog Number: AG01) to make a 1% w/v mixture in the desired volume (e.g., 0.1 g of agarose for 10 mL of volume) and add to a glass bottle
c. Aliquot the desired volume of DBCM2 media into the bottle with agarose
d. Melt agarose in the microwave for ~30 – 45 seconds
e. Once agarose has cooled slightly, use a pipette 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 solidify (Fig. 1d)



D. Preparation for Imaging

In order to perform time-lapse imaging over longer periods of time it is necessary to mount samples in a set up that maintains conditions necessary for healthy growth of cells. For this purpose 35 mm imaging dishes are used so that fresh media can be aliquoted to provide a constant source of nutrients and prevent the agarose pad from drying during imaging. Additionally, temperature control to maintain a constant growth temperature is required and handled by an Okolab stage top chamber (if necessary this can also regulate gas flow for maintaining anaerobic conditions).

a. Remove the lid from a 35 mm imaging dish (Ibidi, Catalog Number: 81156)
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 coverslip 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 an agarose pad, do not allow sample to dry before performing next step (see Notes: Sample Loading)
f. Place agarose pad and coverslip into the 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 the sample onto the microscope and turn on the Okolab stage top chamber for temperature control (30 °C for *Ca.* Nha. antarcticus and *Hrr. lacusprofundi*)

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. If this is a problem, either switch to an alternative brand of agarose or try washing the agarose before using it to clean any detergent from the mix. To wash agarose, 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 the 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 the effects of pad dehydration during the imaging runs. The agarose percentage should be optimised as part of the initial testing of the protocol.

Pad Size:

Pad dimensions are important factors in the success of the imaging. If the cells appear to be compressed and lyse, it may be that 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 too 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, so this should be done as part of the initial experiment testing phase.

Sample Loading:

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

If the sample is allowed to dry before being loaded into the imaging dish then two issues can occur. Firstly, the drying will result in formation of salt crystals which are highly autofluorescent and will interfere with the imaging of the samples. Secondly, dehydration of the pad can result in the media that sits between the pad and imaging surface being drawn into the gel matrix during imaging. If this happens then the haloarchaeal cells may be subjected to pressure differentials that result in morphological defects or, if strong enough, lysis of the cells. Ideally there should be a consistent, thin layer of liquid media between the pad surface and imaging surface in regions selected for time-lapse images so that cells being visualised are not subjected to excess pressure from the pad. The same effect on morphology can result from excess pressure being placed on the sample when loading is done i.e. by applying pressure to the pad after placing it in the dish.

Dye Selection:

In the original optimisation of the protocol, we tested 3 MitoTracker Dyes: Green (490/516 nm Ex/Em), Orange (554/576 nm, Ex/Em), and DeepRed (644/665 nm, Ex/Em). We found that all three 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 mainly 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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