Video Article

Bromodeoxyuridine (BrdU) Labeling and Subsequent Fluorescence Activated Cell Sorting for Culture-independent Identification of Dissolved Organic Carbon-degrading Bacterioplankton

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Abstract

Microbes are major agents mediating the degradation of numerous dissolved organic carbon (DOC) substrates in aquatic environments. However, identification of bacterial taxa that transform specific pools of DOC in nature poses a technical challenge.

Here we describe an approach that couples bromodeoxyuridine (BrdU) incorporation, fluorescence activated cell sorting (FACS), and 16S rRNA gene-based molecular analysis that allows culture-independent identification of bacterioplankton capable of degrading a specific DOC compound in aquatic environments. Triplicate bacterioplankton microcosms are set up to receive both BrdU and a model DOC compound (DOC amendments), or only BrdU (no-addition control). BrdU substitutes the positions of thymidine in newly synthesized bacterial DNA and BrdU-labeled DNA can be readily immunodetected [1,2]. Through a 24-hr incubation, bacterioplankton that are able to use the added DOC compound are expected to be selectively activated, and therefore have higher levels of BrdU incorporation (HI cells) than non-responsive cells in the DOC amendments and cells in no-addition controls (low BrdU incorporation cells, LI cells). After fluorescence immunodetection, HI cells are distinguished and physically separated from the LI cells by fluorescence activated cell sorting (FACS) [3]. Sorted DOC-responsive cells (HI cells) are extracted for DNA and taxonomically identified through subsequent 16S rRNA gene-based analyses including PCR, clone library construction and sequencing.

Video Link

The video component of this article can be found at http://www.jove.com/details.php?id=2855

Protocol

1. Water sample processing

1. Filter 10L environmental water through 1 µm-pore-size membrane filters to remove large particles and bacteriovores. Collect the water filtrate in a carboy.
2. Transfer 36 ml filtrate each into 3 sterile Eppendorf tubes (50 ml) containing 4 ml freshly prepared paraformaldehyde solution (PFA; 10%). Incubate for 2 hrs at room temperature to preserve cells. Collect cells onto 0.22-µm-pore-size white membrane filters by vacuum filtration. Wash the filter by passing 10 ml phosphate-buffered saline (PBS) through the filter by vacuum filtration. Label the filters as negative controls and store them at -20°C.

Steps 1.3-1.4 are optional for establishing DOC-limited conditions.

3. Add a mixture of inorganic nitrogen and phosphorus (5 µM NH_4Cl, 5 µM $NaNO_3$, and 1µM NaH_2PO_4 final concentration) into the carboy.
4. Incubate in the dark at *in situ* temperature for 48 hours with occasional agitations.

2. Microcosm establishment and incubation

1. Fill each of six 1-L glass flasks with 800 ml water sample from the carboy of step 1.4 to establish microcosms. Add BrdU (10 µM, final concentration) to each microcosm. Mix well.
2. Add 1 ml model DOC compound solution into three of the microcosms, these will serve as DOC amendments. Add 1 ml sterile PBS into the three remaining microcosms, these will serve as no-addition controls.
3. Incubate all microcosms in an incubator shaker and incubate in the dark at *in situ* temperature while shaking at 100 rpm.

4. Collect 36 ml of water sample from each microcosm and transfer into 50 ml sterile Eppendorf tubes at time points of 0, 8, 16, and 24 hours. Immediately add 4 ml of fresh PFA (10%) to collection tubes and incubate for 2 hrs at room temperature to preserve the cells.
5. Filter preserved cells through 0.22-µm-pore-size polycarbonate membrane filters. Wash the filters with 10 ml PBS. Proceed immediately to the next step or store the filters at -20°C.

3. *In situ* immunodetection for BrdU Incorporation

1. Thaw the filters (DOC amended samples, no-addition controls and negative controls) at room temperature.
2. Apply 1 ml of lysozyme solution [10 mg/ml lysozyme egg white in 100 mM Tris, 50 nM EDTA (pH = 8)] [4] to cover bacterial cells on the filter. Incubate at room temperature for 30 minutes. Wash the filter by passing 10 ml PBS through it under suction.
3. Add 1 ml of proteinase K solution [2 mg/ml proteinase K in 100 mM Tris, 50 nM EDTA (pH = 8)] [4] to cover bacterial cells on the filter. Incubate at room temperature for 30 minutes. Wash the filter by passing 10 ml PBS through it under suction.
4. Add 1 ml exonuclease solution [exonuclease III (50 U/ml) in 5 mM $MgCl_2$ and 50 mM Tris-HCl] [5] to cover bacterial cells on the filter. Incubate at 37 °C for 30 minutes. Wash the filter by passing 10 ml PBS through it under suction.
5. Assemble frame-seal incubation chambers (Bio-Rad) according to the manufacturer's instructions.
6. Slice the filter into eighths using a sterile blade on an alcohol-cleaned dry surface.
7. Using forceps, place an eighth section of a filter into one assembled frame-seal incubation chamber (eight frame-seal chambers are needed for each filter sample). The side of the filter section that contains cells should face upwards. The moisture at the back of the filter will allow the filter stick to the slide. If filter becomes dry, apply a tiny drop (2 µl) of diH_2O in the center of the chamber before placing the filter section on the slide.

Steps 3.8-3.20 use the reagents from the ROCHE In Situ Cell Proliferation Kit, FLUOS following procedures modified from the manufacturer's instructions. Except for PBS, all reagents are provided within the kit.

8. Apply enough incubation buffer (0.5% bovine serum albumen, 0.1% Tween20 in PBS,) to evenly cover the entire filter section in the incubation chamber without causing overflow once the seal is applied.
9. Place a polyester frame cover over the incubation chamber frame. Press down to tightly seal the incubation chamber. Avoid air bubbles above the filter. Incubate the sealed chambers for 10 min at room temperature in the dark.
10. Remove the polyester seal and open the incubation chambers. (Note: opening the chambers sometimes will pull up the frame seal from the slide. In that case, prepare a new chamber for the following steps.)
11. Pipet out the incubation buffer from one corner of the chamber. Avoid contact with the filter.
12. Wash the filter by gently pipetting 100 µl PBS in and out from the chamber 3 times.
13. Prepare anti-BrdU-FLUOS working solution following the steps recommended by the manufacturer immediately before use.
14. Pipet 120 µl anti-BrdU-FLUOS working solution onto the filter, taking care to evenly cover the entire surface.
15. Reseal the chamber with a new polyester cover. Avoid air bubbles on top of the filter. Incubate the chamber in the dark at 37 °C for 3 hours. This step will label BrdU incorporated DNA *in situ* with fluorescein isothiocyanate (FITC).
16. Remove the polyester cover and open the incubation chamber. Pipet out the anti-BrdU-FLUOS working solution. Wash the filter 3 times with PBS.
17. Transfer the filter from the incubation chamber to a sterile surface. Slice the filter section into small pieces using a sterile blade.
18. Transfer the filter pieces into 2 ml microcentrifuge tubes, each containing 1 ml PBS. Tightly cap the tube and seal with parafilm. Incubate at 37 °C and 200 rpm for 10 min.
19. Secure the tubes onto a vortexer and vortex at the maximum speed for 5 minutes. Pipet the supernatant into a sterile 15 ml Eppendorf tube. Repeat incubation and vortex steps for 5 more times. Combine the supernatant in the same 15 ml Eppendorf tube for each sample. Typically, 80% cells can be recovered in the suspension.
20. Store the supernatant with resuspended cells at 4 °C. Sort within 2 days.

4. FACS analysis

A procedure for a BD FACSAria flow cytometer and corresponding software is described here.

1. Optimize the setting of the flow cytometer following steps recommended by the manufacturer. This entails: adjusting the amplification control to set the required breakoff parameters and the sweet spot; optimizing the laser delay and area scaling factors for the experiment sheath pressure and optimizing settings for FSC and SSC voltages, FSC threshold, FSC fluorescence scaling, fluorescence PMT voltages, etc.
2. Run negative control samples on the flow cytometer (FCM) based on fluorescence intensity of FITC and side scatter (SSC). Increase the FITC threshold until no cells in the negative controls can be visualized by the FITC-SSC acquisition display.
3. Run no-addition control samples and examine the distribution pattern of 10,000 cells based on FITC and SSC acquisition. Define a gate to enclose all the cells and designate them as "low intensity cells" (LIs).
4. Run DOC-amended samples and examine the distribution pattern of 10,000 cells based on FITC and SSC. Some cells will appear in the preset LI gate. Define another gate to enclose the cells that have higher fluorescence intensity (HIs) than LIs. Acquire statistics to view the relative abundance of gated cells.
5. Sort HI cells into collection tubes containing 500 µl PBS at "purify 1 drop" mode. Terminate sorting when the number of HIs reaches 500,000 counts.

5. Filter PCR amplification of 16S rRNA genes

Filter PCR procedures are modified from Kirchman et al. [6]

1. Filter sorted cells onto a white, 0.22 µm-pore-size, 25 mm-diameter, polycarbonate membrane filter. Trim off the edge of the filter that contains no bacterial cells using a sterile blade. Slice the filter into 8 equal sized pieces.
2. Place a single filter piece into a PCR reaction tube, with the cells facing inward of the tube. Add 45 µl PCR grade water into the PCR reaction tube. Submerge the filter entirely in the water.
3. Add 2 ROCHE illustra PuReTaq Read-To-Go PCR Beads into the PCR reaction tube, briefly vortex. Add 2 µl each of the forward and reverse 16S rRNA gene primers (0.4 µM final concentration for each primer), such as 27F and 1492R [7], into each of the PCR reaction tubes.

4. (Optional) Add 1µl bovine serum albumin solution (BSA, final concentration is 30µg/100 µl) to the PCR reaction mixture to help adsorb amplification inhibitors. If one chooses not to add BSA, add 1µl of water instead.
5. Perform PCR amplification on a thermal cycler. A touch down PCR program is recommended, which has the annealing temperature sequentially decreasing from 62 to 52°C by 1°C per cycle for 11 cycles, followed by 15 cycles with annealing temperature of 52°C. All cycles include denaturing (at 95°C), annealing (at 62 to 52°C), and extension (at 72°C) steps of 50s duration. An initial 3-min denaturation and final 10-min extension step is also included in the PCR program.
6. Confirm PCR amplification by electrophoresis on an ethidium bromide-stained 1% agarose gel. Excise the PCR amplicons from the gel and clean with the QIAGEN QIAquick gel extraction kit.
7. Perform two additional PCR amplifications for each sample, each time use a new filter section. After PCR gel purification, pool the amplicons of the same sample together. Purified 16S rDNA amplicons are now ready for a number of molecular analyses that allow taxonomic identification, such as clone library construction and sequencing.

6. Representative results:

Representative results are described using a study of putrescine-degrading bacteria as an example. Water samples were collected from a coastal site of Georgia and processed following the procedures described above. FACS analysis revealed that putrescine addition induced development of a group of bacteria with high FITC fluorescence intensity, indicating high BrdU incorporation rate (Figure 1). These cells were designated as high-BrdU-incorporation cells (HIs) and were expected to contain mostly putrescine-degrading bacteria. HIs were missing in the no-addition controls, which only contained cells with lower levels of BrdU incorporation (LIs). LIs were expected to mainly contain bacterioplankton that were unable to use added putrescine. HIs were sorted into separated tubes and then collected onto membrane filters. 16S rRNA gene amplicons were obtained for high HI cells using filter PCR.

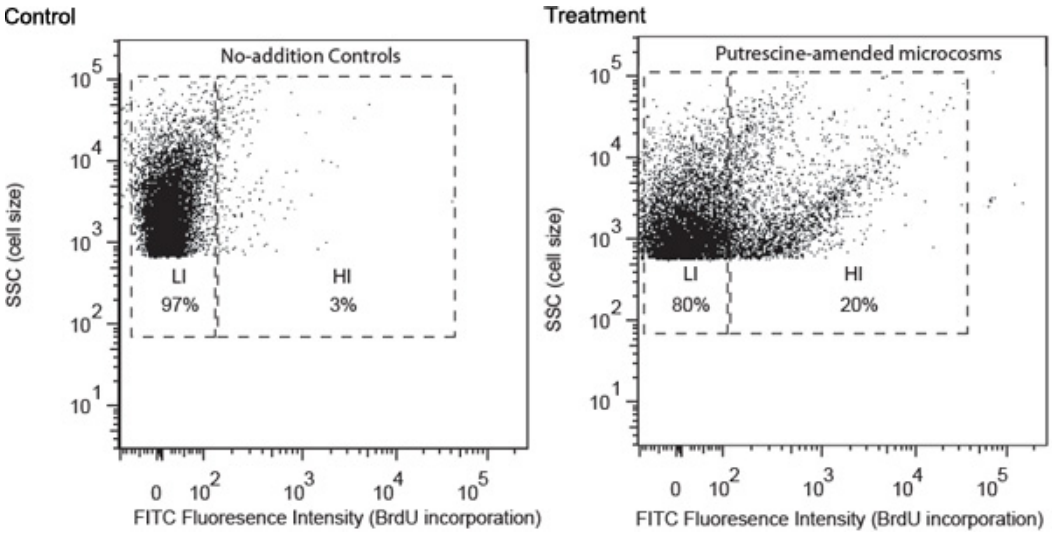

Figure 1. Flow cytometric analysis of no-addition control and model-compound-amended (putrescine as an example here) samples collected after 24 h of incubation. Cell distribution analysis was based on (1) fluorescence intensity of FITC labeling (x-axis), which is positively related to level of BrdU incorporation, and (2) side scatter (SSC, y-axis), which is positively related to cell size. Gate notation is based on level of BrdU incorporation, (HI, high-BrdU-incorporation; LI, low-BrdU-incorporation). The relative percentage of HI and LI cells are shown in corresponding gates.

Discussion

Our approach couples BrdU incorporation, FACS and 16S rDNA analysis to allow species-level identification of bacterioplankton that metabolize individual DOC components in aquatic environments. The BrdU incorporation assay labels bacterial cells based on metabolic activities, which allows analysis only on active bacteria and thus does not include dormant cells. In our approach, BrdU incorporation is *in situ* immunodetected and bacteria that have different levels of BrdU incorporation are subsequently visualized, grouped and sorted using FACS. Analysis of BrdU incorporated cells has also been reported using magnetic bead immunocapture of BrdU-labeled DNA followed by molecular analyses [2,8,9]. One limitation of the latter methodology is its inability to distinguish cells of different activity levels. In that case, the analysis will include cells that remain only slightly active by using indigenous DOC in the water samples (Figure 1, LIs).

The coupled approach has been used to identify the taxonomic structures of bacteria that are able to degrade DOC compounds such as dimethylsulfoniopropionate (DMSP), vanillate, glycine betaine in coastal seawater [3]. Its application can be broadly extrapolated to study taxonomy of bacteria that use other single or mixed dissolved substrates in aquatic environments. In addition to 16S rRNA genes, functional gene markers can also be similarly analyzed. Molecular analysis on sorted cells is challenging due to the small abundance of sorted cells, which is typically less than 1 million cells. Therefore, DNA template amplification is typically required for further molecular analysis. Although limited in cell quantity, sufficient resolution of community structure is usually obtained. Studies have demonstrated that community finger prints generated by terminal restriction fragment length polymorphism (T-RFLP) from as low as 2,000 cells (~ 1 µl seawater) largely resemble those generated from $2x10^9$ cells (~1 L seawater) in coastal seawater[10]. In addition to the single gene amplification using PCR, metagenomic analysis of sorted cells is possible through a non-PCR based genomic amplification technique, namely multiple displacement amplification, MDA [11].

Critical steps

To maintain conditions close to nature, addition of excessive amounts of external DOC compounds to microcosms and prolonged incubation should be avoided. Preliminary experiments are highly recommended to determine the lowest amounts of model DOC compounds that are required to induce development of HI cells in a relatively short time (< 72 hours). Longer incubation times may raise the concern of development

of bottle-effect in microcosms. Pre-incubation with inorganic N and P is optional, but may help shorten the length of incubation time that is required for the development of HI cells in DOC amended microcosms.

Conditions for cell fixation before BrdU fluorescence immunodetection are critically important. Insufficient fixation will cause loss of cells during cell wall permeabilization steps (lysozyme and proteinase K treatments). On the other hand, over-preserved cells tend to be too rigid to lyse and release DNA template for the filter-PCR amplification. Selection of preservative, preservative concentration and preservation time length should be optimized for studied bacterial community. Preservation procedures listed in this protocol have been optimized for a coastal marine bacterioplankton community that is largely dominated by *Proteobacteria* in the alpha- and gamma- subdivisions [10].

Multiple steps of BrdU immunodetection are performed on membrane filters. Caution should be taken to avoid over drying the filters. Dehydrated cells on over-dried filters may strongly attach to the filter membranes and will then be difficult to resuspend for later FACS analysis. Cell counts of bacteria before and after BrdU immunodetection should be always measured to determine the recovery rate of bacterial cells.

When performing filter-PCR [6], the volume of PCR reagents should be enough to submerge the filter piece entirely. An optimized hot-start and touch-down PCR program in general will help to produce good gene amplification. Further PCR optimization can be performed by using a PCR optimization kit (such as the EPICENTRE FailSafe PCR System) or manipulating the concentrations of Mg^{2+}, BSA and other ingredients.

Method limitation

This coupled approach allows collection of bacteria that can potentially degrade a specific substrate and can culture-independently identify these functional cells to detailed taxonomic levels. However, several considerations should be taken into account when interpreting results. The approach initiates by incubation of natural bacterioplankton with specific compounds, typically at non-tracer levels in glass bottles. Cells identified as functional assemblages may differ from those that process the given compound(s) under *in situ* conditions. BrdU incorporation has been found to be undetectable for some bacteria [1,2]. These bacteria, therefore, are inaccessible using this method. Bacteria that are not directly involved in compound degradation but take up degradation products of added compound(s) may give false-positive signals that are indistinguishable from the true degraders.

Disclosures

No conflicts of interest declared.

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