Analysis of methanotroph community composition using a *pmoA*-based microbial diagnostic microarray

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Microbial diagnostic microarrays (MDMs) are highly parallel hybridization platforms containing multiple sets of immobilized oligonucleotide probes used for parallel detection and identification of many different microorganisms in environmental and clinical samples. Each probe is approximately specific to a given group of organisms. Here we describe the protocol used to develop and validate an MDM method for the semiquantification of a range of functional genes—in this case, particulate methane monooxygenase (pmoA)—and we give an example of its application to the study of the community structure of methanotrophs and functionally related bacteria in the environment. The development and validation of an MDM, following this protocol, takes ~6 months. The pmoA MDM described in detail comprises 199 probes and addresses ~50 different species-level clades. An experiment comprising 24 samples can be completed, from DNA extraction to data acquisition, within 3 d (12–13 h bench work).

INTRODUCTION

The analysis of functional diversity in the environment is an important step toward understanding microbial ecology. The application of molecular techniques, including nucleic-acid fingerprinting tools and genetic markers[1–3], allowed detailed studies of microorganisms, their interaction with the surrounding environment and their role in ecosystem functioning. These methods have been extended recently by next-generation sequencing[4,5] and microarray-based analyses[6], including MDMs. MDMs have provided the basis for a greater sample throughput and highly parallel detection of complex microbial communities in a wide range of samples[7] (e.g., soil[8], wastewater[9] and blood[10]) and for a wide variety of applications (e.g., food safety[11], environmental[12], medical[13,14] and metagenomic applications[15,16]). MDMs allow semiquantitative characterization of target genes by specific hybridization of labeled target gene sequences (amplified from the environment) to the corresponding oligonucleotide probes on a small solid surface (glass slide). Functional gene arrays (FGAs) are a subset of MDMs that contain probes targeting genes involved in ecological and environmental processes of interest, such as nitrogen or methane metabolism or antibiotic resistance[17–22]. FGAs are therefore useful for monitoring the physiological status and functional activities of microbial communities in natural environments.

Here we provide a protocol for the validation and application of a comprehensive FGA consisting of oligonucleotide probes complementary to *pmoA* and closely related ammonia-monooxygenase (amoA) genes, encoding for one of the subunits of the particulate methane monooxygenase (pMMO) and the ammonia monooxygenase (AMO), based on our previous publications[17,18]. The pMMO catalyzes methane oxidation in methanotrophs, whereas the AMO catalyzes the oxidation of ammonia in nitrifiers. Both methane and ammonia oxidation are important environmental processes that are crucial to biogeochemical transformations of nitrogen and methane; as such they are linked to environmentally important processes such as productivity, global warming, soil fertility and eutrophication. The FGA has subsequently been updated several times as new sequences were discovered[17,18,23], but the methodology used to design, validate and apply the microarray has not changed. The current probe set is shown in **Supplementary Table 1**.

The first step in the development of this FGA was the design of oligonucleotide probes; these were based on a comprehensive sequence database consisting of published and unpublished (obtained through correspondence, from groups working on methanotrophs) *pmoA* sequences. *In silico* designed probes were validated with a comprehensive reference set of pure cultures and environmental clones covering almost the entire known diversity of methanotrophs and bacteria carrying *pmoA* (**Fig. 1**). The *pmoA/amoA* genes were PCR-amplified from environmental samples or reference strains/clones (for validation) and used as template for a subsequent *in vitro* transcription reaction to generate labeled single-stranded RNA transcripts. Labeled targets were then fragmented and hybridized to complementary oligonucleotide probes on the array. Validation and data analysis have been previously described in detail by Bodrossy *et al.*[17] and Stralis-Pavese *et al.*[18]. The current array comprises 199 probes targeting different phylogenetic levels and has been validated using 81 reference targets[17,18].

The advantage of the *pmoA*-based microarray is that it enables the high-resolution (species) analysis of currently uncultivated organisms; it has the potential, when coupled with environmental RNA (cDNA), to also deliver information on the active populations[24]. With some modifications, the technique described here is suitable for the development of new FGAs targeting other genes and groups of organisms (see EXPERIMENTAL DESIGN).

A number of alternative methodologies for the analysis of *pmoA* diversity have been previously described including denaturing gradient gel electrophoresis (DGGE)[25], terminal restriction fragment length polymorphism (T-RFLP)[26] and direct sequencing from PCR product[27]. The *pmoA* microarray described here provides a depth of coverage and sensitivity comparable with all of these methods, while providing information about the composition of the community not provided by DGGE or T-RFLP and at a lower cost than comparable sequencing-based studies. Although the method provides substantial information on the composition of the *pmoA* gene

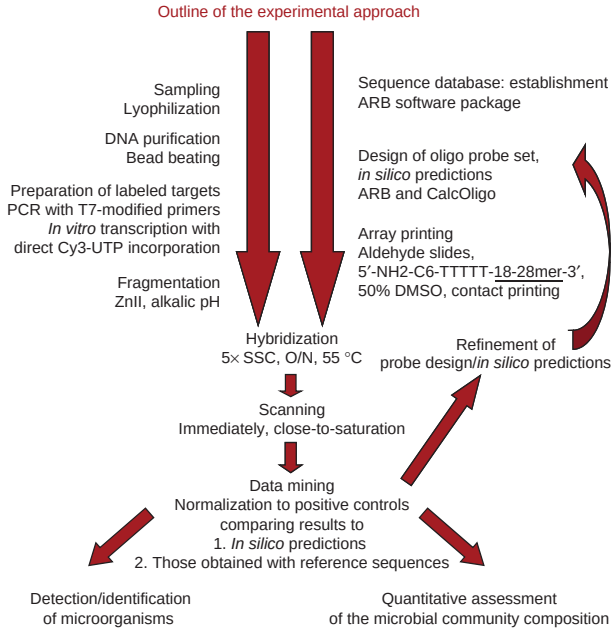

Figure 1 | Experimental scheme of microbial diagnostic microarray development.

within a given sample, it does not give the same resolution as direct sequencing. For this reason, a comprehensive study of a novel environment may involve both approaches to best account for any new diversity. The method has been used for the study of a number of environments including landfill cover soil[17], alpine meadow soil[28], coal mine soil[29], estuarine sediment[30], peat[31] and peat moss[32].

The sensitivity of a given MDM is limited by the relative abundance of the microbial population within the community amplified. Detection limits for environmental MDMs are in the range of 1–5% (ref. 17). The *FGA* described in this protocol has proven to be an efficient method for providing reliable information for functional microbial community analysis. However, quantification of the relative abundance of organisms is biased because of differences in the number of cellular *pmoA*/*amoA* genes[33]. The technique is well-suited to semiquantitative comparison of different samples (e.g., different treatments, environments or time points).

Experimental design

The protocol described here outlines the validation and application of an FGA for the high-throughput detection, identification and analysis of community structure of methanotrophs and functionally related bacteria using the *pmoA* gene. The methodology is suitable for application to other such functional genes. The resolution of the technique is limited only by the degree of conservation of the target gene. A flowchart in **Figure 1** outlines the steps in the development and application of the technique for study of a functional gene in environmental samples. A number of points should be considered when applying this method to the study of a new functional gene; these are outlined below.

PCR primer and microarray probe design. PCR primers targeting the gene of interest (in this case, *pmoA*) should be designed to cover the maximum diversity of the gene possible. Primers should be designed to cover the largest region of the gene in order to maximize the regions that can be used to design probes. Primers should include the T7 tag when being validated.

For the design of oligonucleotide probes, the ARB (http://www.arb-home.de/) and CalcOligo (http://www.calcoligo.info/) software packages are used. Probes are typically 18–28 nucleotides in length, preceded by the 5′ end thymidine residues and the spacer detailed below. Diagnostic positions within the probes are ideally positioned centrally. Probes with two or more central mismatches to non-target sequences were found to be specific (i.e., they did not hybridize nonspecifically with non-target sequences). As there is no available method to predict probe specificity with a certainty of 100%, probe sets must be thoroughly validated with a representative set of reference targets. More details about probe design and validation is included in **Box 1**; the reader is also referred to other publications[17,18,34].

Microarray printing considerations. Steric effects (electrostatic and actual physical hindrance) have been shown to seriously hinder the accessibility of immobilized oligonucleotide probes[35–37]. Steric hindrance can be mitigated by the application of spacer molecules[38]. The linker used in this protocol comprises C_6 spacer and an extra five thymidine (T) residues at the 5′ end of the probe. This linker provided optimal spacing, whereas the addition of further T residues did not show a significant effect on the accessibility of the probes tested.

The protocol uses 50% (vol/vol) DMSO in distilled water as a printing buffer. The advantage of DMSO-based printing buffers is that they do not dry out during long spotting rounds (unlike aqueous printing buffers). Furthermore, DMSO ensures that more uniform spots are printed on the slides.

The secondary structure of the target molecule may significantly influence the hybridization signal, thus it is necessary to fragment the target before hybridization. As RNA can be chemically fragmented in a random manner[39], we use RNA targets for hybridization to the array (hybridization on microarrays is reverse to more traditional techniques, the target being the labeled sample hybridized to the immobilized probes). The T7 RNA polymerase is very efficient in incorporating the cyanine (Cy)-labeled nucleotides into the synthesized target molecule. RNA yield decreased only to 75% when replacing 50% of UTP (uridine triphsophate) with Cy3-UTP during *in vitro* transcription. Target yields were in the range of 50 ng μl^{-1} (in 50 µl final volume, starting from 8 µl 50 ng μl^{-1} template). Labeling efficiency was high; on average, every 10th–12th nucleotide was labeled. Hybridization efficiency was considerably enhanced by the fragmentation of the target RNA to an average fragment size of 50 nt[39]. Although Cy dyes are sensitive to nucleophilic attack (such as alkaline treatment with divalent cations used to fragment RNA), Cy3 signals increased by over an order of magnitude upon target fragmentation. In many cases fragmentation also decreased the differences in hybridization capacities between probes.

Amplification of target gene. Amplification of the target gene (in this case *pmoA*/*amoA*) was performed using modified primers based on the forward pmoA189 and either one of the reverse primers mb661 (ref. 40) (specific for methanotrophs; product size 508 bp) or A682 (ref. 41) (specific for methanotrophs, ammonia-oxidizing bacteria and homologous genes from environmental libraries; product size 531 bp). The modification comprised the

BOX 1 | DESIGNING PROBES FOR AN FGA ● TIMING 2–6 WEEKS, DEPENDING ON PROBE SET AND DATABASE SIZE

1. Create a database in ARB comprising all available sequences for the gene of interest. It is important to maximize probe specificity, to have as many sequences for the group of microorganisms to be investigated as possible. A detailed explanation of the use of the ARB package, including the functions utilized in the following steps, can be found at http://www.arb-home.de/documentation.html.
2. Align the sequences to each other using ClustalW in ARB. Following this, the alignment will need to be manually edited to ensure that it is robust and that the subsequent clustering is accurate. Once the alignment is complete, use it to create a PT-server (a special ARB database: ARB-Probes-PT_Server Admin-Build Server) from which probes can be designed.
3. Create phylogenetic trees. Use bootstrap values to check the validity of the indicated phylogenetic branches. Phylogenetically coherent groups are generally easier to design probes against. Creating trees using alternative calculations is also helpful to check the validity of the phylogenetic branches.
4. On the basis of the best tree (with relatively high branching pattern stability), select different groups (depending on the resolution required, these can be single strains, subspecies, species or higher groups) and design probes using the Probe_Design function of ARB. The optimal parameter settings for the Probe_Design function vary depending on the targeted group (monophyletic or not; level of relatedness; closely related non-target groups; and so on). The following parameters provide a good starting point:
   - Length of output, 50;
   - Maximum non-group hits, 0–5% of the total number of sequences targeted;
   - Maximum hairpin bonds, 4;
   - Minimum group hits, 80–100%;
   - Length of probe, 18–20;
   - Temperature, 50–70 °C; G + C content (35–75%);
   - ECOLI position, 0–10,000 (this way no regions will be excluded during design).
5. Use the Probe_Design output window as input for the Probe_Match function. Probe_Match is used to visualize the predicted specificity of the potential probes identified by Probe_Design.
6. With the nearest neighbor model, check the predicted melting temperature of the selected probes. This function is available on many web sites (i.e., http://www.idtdna.com/analyzer/applications/oligoanalyzer/) as well as in most molecular biology software. If necessary, repeat Probe_Design with longer or shorter settings for probe length to match the range of 58–62 °C for predicted melting point (T_m).
If possible, select 3–4 alternative probes for each targeted group, run Probe_Match allowing for 5 mismatches. Print results to ASCII file (this saves results as a text file).
7. Run CalcOligo.exe (http://www.calcoligo.info/), using the Probe_Match output files as input. CalcOligo creates a table (tab-delimited text file format), displaying the weighed mismatches of each of the probes in the probe set against the entire sequence database used for Probe_Design.
8. Using Conditional Formatting in Excel, you should visualize weighed mismatch categories (i.e., <0.5, <1.5, <2.5 and 3.5). Organize the Excel table according to phylogeny. Sort rows (sequences) according to the order of sequences in the phylogenetic tree used during Probe_Design. Sort columns (probes) into a matching order.
9. Select a panel of reference sequences representing the entire known diversity of the targeted group of microorganisms. Obtain strains/clones with the reference sequences for validating the probe set.
10. Order oligonucleotide probes. The probe sequence designed should be preceded by a 5′ NH_2 end modification, a C_6 spacer and five extra thymidine residues, in order to minimize steric hindrance. Ordering probe sets at a 0.2 μM scale is usually enough for at least ten spotting plates (with 30 μl 50 μM oligonucleotide solution per well). Each spotting plate in turn is sufficient to spot up to 3,000 arrays in triplicate.

addition of two tags, T3c and T7c, based on the T3- and T7-promoter sites, to the forward and reverse primers, respectively. Using these tags, a two-stage PCR was carried out. Amplification in the first 15 cycles used the target gene (*pmoA*)-specific primer regions; the subsequent 25 cycles were based only on the T3c and T7c primers targeting the introduced tag regions. This procedure allowed amplification of the target gene from difficult samples in which traditional PCR using the target gene-specific primers only did not yield products. The T7 promoter also enabled *in vitro* transcription of DNA templates into RNA using T7 RNA polymerase.

Experimental controls. A number of experimental controls are included in the protocol. First, positive and negative controls should be used in PCR steps to ensure specificity of target amplification. An internal control (*hyaB* PCR product, **Box 2**) is included during the *in vitro* transcription step; this control has a dedicated probe on the array and shows that hybridization has worked as expected. A number of positive control probes on the array (perfect matches to the forward and reverse primers) are also included; these probes serve as controls for the PCR amplification, labeling and hybridization. The combination of these control probes allows troubleshooting of the array (see TROUBLESHOOTING section).

Statistical analysis of final array data. The final data output from the microarray can be combined with multivariate statistical techniques to define the difference between a number

BOX 2 | *HYAB* PCR AMPLIFICATION ● TIMING ~5 H

hyaB is a hydrogenase gene in *Escherichia coli*, used as an external spike control. This spike is used as quality control for target labeling and hybridization and as a landing light (positive control spots used for orientation) during microarray analysis. Amplify *hyaB* as a large batch and use aliquots of the purified PCR product.

1. Set up ten 50-µl reactions as tabulated below.

Component	Amount (µl)	Final
PCR buffer (10×)	50	1×
dNTP mixture (2.5 mM for each dNTP)	40	0.2 µM
$MgCl_2$ (50 mM)	15	1.5 µM
Forward primer (1.5 µM)	10	1.5 pmol
Reverse primer (1.5 µM)	10	1.5 pmol
Taq polymerase (1 U $µl^{-1}$)	10	1 U
E. coli genomic DNA (1 ng $µl^{-1}$)	10	30 ng/1 ng/0.1 ng
Ultrapure water	Up to 500; split into ten 50-µl aliquots for PCR amplification	

2. Run the PCR using the following cycling conditions.

Cycle number	Denature	Anneal	Extend
1	95 °C, 5 min		
2–35	95 °C, 1 min	56 °C, 1 min	72 °C, 1 min
36			72 °C, 10 min

3. Check PCR products by running 5 µl of PCR product on a 1.5% (wt/vol) agarose gel.

■ **PAUSE POINT** PCR product may be stored for up to 1 year at −20 °C at this point.

4. Purify PCR product with a commercial PCR purification kit according to the manufacturer's instructions. Dissolve or elute purified DNA in ultrapure water.

5. Check PCR products by running 5 µl of PCR product on a 1.5% (wt/vol) agarose gel.

■ **PAUSE POINT** PCR product can be stored long term at −20 °C.

of samples. A good review of applicable statistical methods is given by Ramette[42]. In this way the effect of a sample treatment, spatial or temporal factor on the methanotroph community can be determined. **Figure 2** shows the application of multivariate statistical analysis to determine the difference between a number of array analyses from the same data set as **Figure 3**.

MATERIALS

REAGENTS

- Oligonucleotide probes (VBC Genomics) synthesized with a 5′ NH_2-C_6 spacer, followed by and five thymidines preceding the probe sequence. See **Supplementary Table 1** for a list of probes used in our current *pmoA* MDM.
- NaCl (Carl Roth, cat. no. 3957.1)
- KCl (Carl Roth, cat. no. 6781.3)
- Na_2HPO_4 (Carl Roth, cat. no. PO30.1)
- KH_2PO_4 (Carl Roth, cat. no. 3904.2)
- DMSO (Sigma-Aldrich, cat. no. D8418) ! **CAUTION** DMSO is toxic; work in a fume hood, wear gloves and protective clothing.
- Sodium borohydride ($NaBH_4$; Sigma-Aldrich, cat. no. 452173, granular or cat. no. 452882, powder) ! **CAUTION** Borohydride is very toxic and harmful if inhaled or in direct contact with skin. Handle using appropriate gloves, wear protective clothing and work under a fume hood.
- Phenol (Sigma-Aldrich, cat. no. P4557) ! **CAUTION** Phenol is highly toxic; wear gloves and protective clothing and work in a fume hood.
- $CHCl_3$ (Carl Roth, cat. no. 3313) ! **CAUTION** Chloroform is highly toxic; wear gloves and protective clothing and work in a fume hood.
- Isoamyl alcohol (Carl Roth, cat. no. T870)
- Proteinase K (Roche Diagnostics, cat. no. 3115852)
- Potassium acetate (Sigma-Aldrich, P1190)
- NaH_2PO_4 (Carl Roth, cat. no. T878.1)
- $Na_2HPO_4.7H_2O$ (Carl Roth, cat. no. X987.1)
- NaCl (Carl Roth, cat. no. 3957.1)
- Hexadecyltrimethylammonium bromide (CTAB; Sigma-Aldrich, cat. no. H6269)
- MasterAmp 2× PCR PreMix F (Epicentre Biotechnologies, cat. no. MO7205F)

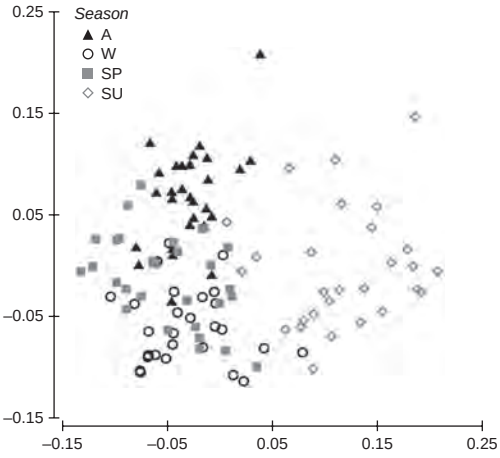

Figure 2 | An example of multivariate statistical analysis of square root-transformed microarray data. Microarray data is the same as that used in **Figure 3**, and highlights the difference in methane-oxidizing bacterial community structure between alpine meadow samples taken during different seasons: autumn (A), winter (W), spring (SP) and summer (SU). Adapted from Abell *et al.*[28].

- Milli-Q ultrapurified water (Millipore)
- Taq DNA Polymerase, Recombinant (Invitrogen, cat. no. 10342-020)
- Primer T3c-pmoA189 5′-CAGAGATGCAAATTAACCCTCACTAAAGGG NGACTGGGACTTCTGG-3′
- Primer T7c-mb661 5′-CCAAGCCTTCTAATACGACTCACTATAGCCGG MGCAACGTCYTTACC-3′
- Primer T7c-A682 5′-CCAAGCCTTCTAATACGACTCACTATAGGAASG CNGAGAAGAASGC-3′
- Primer T3c—5′-CAGAGATGCAAATTAACCCTCACTAAAG-3′
- Primer T7c—5′-CCAAGCCTTCTAATACGACTCACTATAG-3′
- Primer hyaB1-5′-GACCCGATTACGCGCATCGAAGG-3′
- Primer T7-hyaB2—5′-TAATACGACTCACTATAGCCAGTAGCCATTGC GGAAGATCC-3′
- PCR buffer (Supplied with Taq DNA Polymerase, Recombinant, Invitrogen)
- $MgCl_2$ (50 mM, Supplied with Taq DNA Polymerase, Recombinant, Invitrogen)
- dNTP set, PCR grade (100 mM, Invitrogen, cat. no. 10297-117)
- HighPure PCR purification kit (Roche Diagnostics GmbH, cat. no. 11732676001), 250 preps
- RNAsin (40 U μl^{-1}; Promega, cat. no. N251B)
- ATP (10 mM; Invitrogen, cat. no. 18330-019)
- CTP (10 mM; Invitrogen, cat. no. 18331-017)
- GTP (10 mM; Invitrogen, cat. no. 18332-015)
- UTP (10 mM; Invitrogen, cat. no. 18333-013)
- T7 RNA polymerase (2 × 2,500 U, 50 U μl^{-1}; Invitrogen, cat. no. 18033-100)
- T7 RNA polymerase buffer (5×; supplied with T7 RNA polymerase; Invitrogen)
- DTT (100 mM; supplied with T7 RNA polymerase; Invitrogen)
- Cy3 UTP (25 nmol; GE Healthcare Bio-Sciences, cat. no. PA 53026)
- Cy5-UTP (25 nmol; GE Healthcare Bio-Sciences, cat. no. PA 55026)
- Qiagen RNeasy kit (Quiagen, cat. no. 74104, 50 preps)
- $ZnSO_4 \cdot H_2O$ (Sigma-Aldrich, cat. no. 96495)
- Tris-Cl pH 7.4 (1 M; Sigma-Aldrich, cat. no. 93313)
- Agarose (Helena Biosciences, cat. no. 8201-07)
- Ethanol absolute (VWR, cat. no. 1.00983.2500
- Diethylpyrocarbonate (DEPC; Carl Roth, cat. no. K028.1) **! CAUTION** DEPC is a toxic carcinogen; wear gloves and protective clothing and work in a fume hood.
- Denhardt's reagent (50× lyophilized powder; Sigma-Aldrich, cat. no. D9905-5 ml)
- Sodium chloride (Carl Roth, cat. no. 3957.1)
- Sodium citrate (Sigma-Aldrich, cat. no. S1804)
- SDS (Sigma-Aldrich, cat. no. L4390)
- EDTA (Sigma-Aldrich, cat. no E5134)
- EDTA (0.5 M and pH 8.0; Fisher Scientific, cat. no. BP2482-100)
- Tris (Carl Roth, cat. no. 4855)
- PBS (see REAGENT SETUP)
- Sodium borohydride solution (see REAGENT SETUP) **! CAUTION** Borohydride is very toxic and harmful if inhaled or in direct contact with skin. Handle using appropriate gloves, wear protective clothing and work under a fume hood.
- Tris-HCl (1 M, pH 8.0) (see REAGENT SETUP)
- TE Buffer (pH 8.0; Applied Biosystems, Ambion, cat. no. AM9849)
- Proteinase K (10 mg ml^{-1}; see REAGENT SETUP)
- Potassium acetate (7.5 M; see REAGENT SETUP)

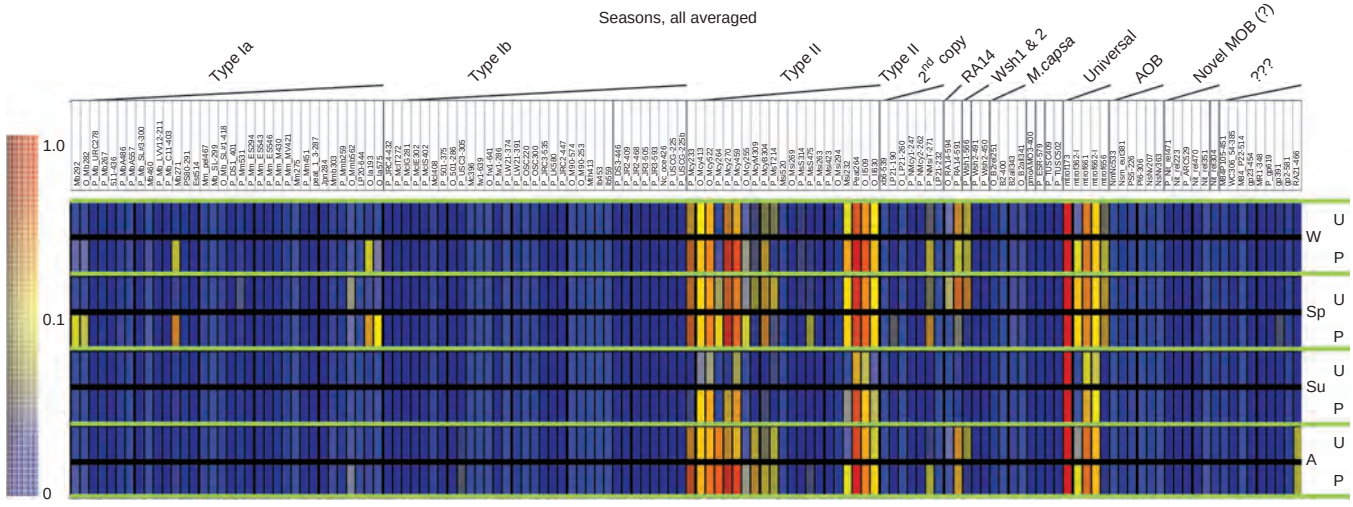

Figure 3 | An example of the application of the microarray for the study of methane oxidizing bacteria in environmental samples. The figure indicates the relative abundance of targets corresponding to each of the probes in each of the different samples analyzed. The figure allows visual comparison of a number of different samples. As such, the figure shows the difference in composition of the methane oxidizing bacterial community according to season (W, winter; Sp, spring; Su, summer; A, autumn) and land usage (unperturbed, U and perturbed, P sites). Adapted from Abell *et al*[28]. 'Type I/II', 'Wsh 1& 2' and 'RA14' refer to different major clades of methane oxidizing bacteria (MOB); AOB refers to ammonia oxidizing bacteria; 'Novel MOB' refers to a novel clade of methane oxidizing bacteria; '???' denotes a clade of sequences related to *pmoA* with uncertain physiology (i.e., it is not known whether the microbes carrying these genes are MOB or AOB or something different from both).

- Phosphate Buffer (100 mM; see REAGENT SETUP)
- DMSO (50%, vol/vol; see REAGENT SETUP) ! CAUTION DMSO is toxic; work in a fume hood, wear gloves and protective clothing.
- $ZnSO_4$ (100 mM; see REAGENT SETUP)
- EDTA (500 mM, pH 8; see REAGENT SETUP)
- SDS (10%, wt/vol, RNase free; see REAGENT SETUP)
- SDS (10%, wt/vol; see REAGENT SETUP)
- SSC (20×; see REAGENT SETUP)
- SSC (2×), SDS 0.1% (vol/vol) (see REAGENT SETUP)
- SSC (0.2×, vol/vol; see REAGENT SETUP)
- SSC (0.1×, vol/vol; see REAGENT SETUP)
- DEPC-treated water (see REAGENT SETUP) ! CAUTION DEPC is a toxic carcinogen; wear gloves and protective clothing and work in a fume hood.

EQUIPMENT

- OmniGrid 100 spotter (GeneMachines)
- TeleChem SMP3 pin (TeleChem International, cat. no. SMP3)
- Scanner GenePix 4000B laser scanner (Axon Instrument).
- VSS-25 silylated (aldehyde) slides, 25 slides/box (TeleChem International, cat. no. VSS-25)
- Software:
  - ARB (including ClustalW),
  - CalcOligo (Shareware; http://www.calcoligo.info/),
  - Excel (Microsoft; http://www.microsoft.com/),
  - Primer 6 (Primer-e; http://www.primer-e.com/),
- Thermocycler (Peqlab, cat. no. 95-4002)
- Spectrophotometer NanoDrop (Peqlab, cat. no. 91-ND-1000)
- Belly Dancer (Stovall, Sigma-Aldrich, cat. no. Z367648-1EA)
- Aluminum block (custom-made with slots to fit slides). For detailed specifications, see **Figure 4**
- Heating block (Peqlab, cat. no. 91-D1100)
- Incubator (Thermo Fisher Scientific, cat. no. 51015264)
- FastPrep24 (MP Biomedicals, cat. no. 6002-500)
- Eppendorf shaker (Sigma-Aldrich, cat. no. Z404160)
- Hard Shell 384-well microtiter plate (Biozym, cat. no. 621541)
- Staining system (staining jar, rack and handle) (VWR, cat. no. 631-9328)
- HybriWell chambers (triple, custom designed, 100 µl; Grace BioLabs, cat. no. 46170)
- Seal spots (Grace Bio-Labs, supplied with HybriWell)
- Air gun fitted with a cotton-wool filter inside or oil-free compressed air or nitrogen
- Multipipette (5–50 µl 8-channel; Thermo Fisher Scientific, cat. no. 320 71 71)
- Centrifuge 5424 (Eppendorf, cat. no. 5424 000)
- Concentrator 5301 (Eppendorf, cat. no. 5301 000)
- FastDNA spin kit for soil (QBiogene, cat. no. 6560-200, 50 preps).
- Gloves, non-powder, nitrile (Carl Roth, cat. no. P777.1)
- Microcentrifuge tubes (2.0 ml; Eppendorf, cat. no. 0030 123.344)
- Microcentrifuge tubes (1.5 ml; Eppendorf, cat. no. 0030 123.328)
- PCR tubes (0.2 ml; Eppendorf, cat. no. 0030 124.332)
- Corning filter tips (0.2–10 µl; Szabo-Scandic, cat. no. COS 4807)
- Corning filter tips (1–30 µl; Szabo-Scandic, cat. no. COS 4821)
- Corning filter tips (1–200 µl; Szabo-Scandic, cat. no. COS 4823)
- Corning filter tips (100–1,000 µl; Szabo-Scandic, cat. no. COS 4809)
- Vacuplus (Petra-Electric, cat. no. 91106800)
- SpeedVac centrifuge
- Capillary sequencer (Applied Biosystems)

REAGENT SETUP

▲ CRITICAL Diethylpyrocarbonate (DEPC) is frequently used to remove RNase activity from solutions and glassware. To treat solutions or glassware, add 0.1% (wt/vol) DEPC, shake and incubate overnight at 37 °C. Following overnight incubation, autoclave at 121 °C for 60 min to inactivate DEPC. DEPC treatment is not compatible with Tris and other amine-containing buffers. These buffers are best bought RNase-free from major suppliers.

▲ CRITICAL Follow basic guidelines for working under RNase-free conditions. When working with RNA, always use clean gloves; work with filter tips specific to RNA work; work with RNA-free solutions and plasticware autoclaved at 121 °C for 60 min. When preparing solutions, use chemicals specific to RNA work and, if possible, measure them directly into RNase-free glassware without using spatulas. If spatulas have to be used, sterilize first by ethanol flaming.

PBS Dissolve 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4 and 0.24 g KH_2PO_4 in 800 ml of Milli-Q water. Adjust to pH 7.4 with 1 M HCl and add Milli-Q water to prepare a volume of 1,000 ml. Autoclave at 121 °C for 20 min. Store at room temperature (22 °C) for a maximum of 12 months.

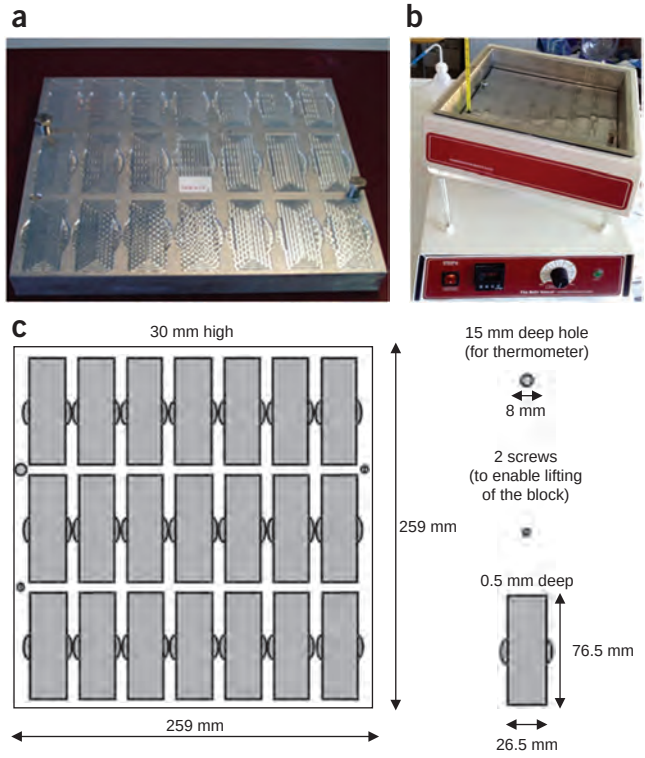

Figure 4 | The Belly Dancer and its components. (**a**) Aluminum block, (**b**) assembled Belly Dancer (aluminum block into the hybridization water bath), (**c**) plan of the aluminum block.

Sodium borohydride solution Dissolve 0.5 g of $NaBH_4$ in 150 ml PBS. Add 44 ml of 100% ethanol to reduce bubbling. Prepare fresh. ! CAUTION Sodium borohydride is toxic; work in a fume hood and wear gloves and protective clothing.

Tris-HCl (1 M, pH 8.0) Dissolve 121.1 g of Tris base in 800 ml of Milli-Q water. Adjust to pH 8.0 with 1 M HCl and add Milli-Q water to adjust the volume to 1.0 liter. Store at room temperature for up to 12 months.

TE Buffer, pH 8.0 Purchase from Applied Biosystems, Ambion (cat. no. AM9849) or prepare stock solution: 1 M Tris and 0.5 M EDTA. Prepare a solution of 10 mM Tris-HCl, 1 mM EDTA in Milli-Q water, and adjust to pH 8.0. Autoclave at 121 °C for 20 min. Store at room temperature for up to 12 months.

Proteinase K, 10 mg ml^{-1} Dissolve 10 mg of proteinase K in 1 ml of Milli-Q water. Aliquot and autoclave at 121 °C for 20 min. Store at −20 °C for up to 2 months.

Potassium acetate, 7.5 M Dissolve 73.6 g of potassium acetate in 80 ml of Milli-Q water. Adjust to pH 7.5 and add Milli-Q water to adjust the volume to 100 ml. Autoclave at 121 °C for 20 min. Store at room temperature for up to 12 months.

Phosphate buffer, 100 mM Prepare stock solution:

(i) Stock solution A: 200 mM solution of monobasic sodium phosphate ($NaH_2PO_4 \cdot H_2O$). Dissolve 27.8 g in 1,000 ml Milli-Q water. Autoclave at 121 °C for 20 min. Store at room temperature for up to 12 months.

(ii) Stock solution B: 200 mM solution of dibasic sodium phosphate. Dissolve 52.65 g of $Na_2HPO_4 \cdot 7\ H_2O$ in 1,000 ml of distilled water. Autoclave at 121 °C for 20 min. Store at room temperature for up to 12 months.

Mix 39.0 ml of stock solution A (200 mM NaH_2PO_4) with 61.0 ml of stock solution B (200 mM Na_2HPO_4) to obtain 100 ml of 200 mM $NaHPO_4$, pH 7.0. Add 80 ml of Milli-Q water, 17.54 g of NaCl and 2 g of CTAB. Dissolve; check pH (7.0), dilute to a total of 200 ml with Milli-Q water to obtain 100 mM phosphate buffer. Autoclave at 121 °C for 20 min. Store at room

temperature for up to 12 months. ▲ CRITICAL Add lysozyme to an aliquot at 5 mg ml^{-1} concentration just before use (i.e., 5 mg to a 1 ml aliquot).
DMSO (50%, vol/vol) Add 50 ml of DMSO in 50 ml of Milli-Q water. Autoclave at 121 °C for 20 min. ! CAUTION DMSO is toxic; work in a fume hood, wear gloves and protective clothing.
$ZnSO_4$, 100 mM Dissolve 1.8 g of $ZnSO_4 \cdot H_2O$ in 100 ml of Milli-Q water; add 100 µl DEPC, shake intensively and incubate at 37 °C overnight. Autoclave at 121 °C for 30 min. Store at room temperature for up to 12 months. ! CAUTION DEPC is a toxic carcinogen; wear gloves and protective clothing and work in a fume hood.
EDTA, 500 mM, pH 8 Dissolve 18.6 g of EDTA (mw 372.24) in 80 ml of Milli-Q water. Adjust the pH to 8.0 with 1 M NaOH (EDTA does not completely dissolve until the pH reaches ~8) and add Milli-Q water to adjust the volume to 100 ml. Add 100 µl DEPC, shake thoroughly, incubate at 37 °C overnight and autoclave at 121 °C for 30 min. Store at room temperature for up to 12 months. ! CAUTION DEPC is a toxic carcinogen; wear gloves and protective clothing and work in a fume hood.
SDS (10%, wt/vol) RNase free Dissolve 1 g of SDS in DEPC water and adjust the volume to 10 ml. Use RNase-free tube. SDS cannot be autoclaved. Store at room temperature for up to 12 months.
SDS (10%, wt/vol) Dissolve 10 g of SDS in Milli-Q water and adjust the volume to 100 ml. SDS cannot be autoclaved. Store at room temperature for up to 12 months.
SSC (20×; 3 M sodium chloride, 0.3 M sodium citrate) Dissolve 175.3 g of NaCl and 88.2 g of sodium citrate dihydrate in 800 ml of Milli-Q water. Adjust to pH 7 with 1 M HCl and add Milli-Q water to prepare a volume of 1,000 ml. Autoclave at 121 °C for 20 min. Store at room temperature for up to 12 months.
SSC (2×), SDS (0.1%, vol/vol) Add 100 ml of 20× SSC and 10 ml of SDS (10%, wt/vol) to 890 ml Milli-Q water. Store at room temperature for up to 12 months.
SSC (0.2×, vol/vol) Add 10 ml of 20× SSC to 990 ml of Milli-Q water. Store at room temperature for up to 12 months.
SSC (0.1×, vol/vol) Add 5 ml of 20× SSC to 995 ml Milli-Q water. Store at room temperature for up to 12 months.
Lysis buffer, pH 7.0 (FastDNA spin kit) $NaPO_4$ (100 mM, pH 7.0); 1% (wt/vol) CTAB; 1.5 M NaCl; and 5 mg ml^{-1} of lysozyme (lysozyme added immediately before use).
DEPC-treated water Add 100 µl of DEPC to 100 ml of Milli-Q water. Shake thoroughly; incubate at 37 °C overnight and autoclave at 121 °C for 30 min. Store at room temperature for up to 12 months. ! CAUTION DEPC is a toxic carcinogen; wear gloves and protective clothing and work in a fume hood.

EQUIPMENT SETUP

Aluminum block for Belly Dancer The purpose of the aluminum block is to provide a constant, controlled temperature for the microarray slides throughout the hybridization process. To fulfill this, it has to meet two requirements: (i) it has to fit inside the water bath of the BellyDancer unit leaving only a minimal gap (~1 mm on each side) for water; and (ii) it needs to have slots carved to fit the microarray slides. **Figure 4a** shows the design of such a custom-made aluminum block. The inner size of the BellyDancer water baths may vary slightly; we recommend checking the particular unit's exact size before cutting the aluminum block.

PROCEDURE

Oligonucleotide probe design ● TIMING 30 min per probe

1| Design and order-specific oligonucleotide probes. If methanotrophs and nitrifiers are to be analyzed, a comprehensive list of validated probes is provided in **Supplementary Table 1**. If you are applying the approach for the first time to a new functional gene, probes can be designed as described in **Box 1**.

Microarray preparation ● TIMING 30 h

2| Prepare 50 µM oligonucleotide probe solutions in 50% (vol/vol) DMSO and add 30 µl of each probe to separate wells of a 384-well flat-bottom plate. Arrange the probes within the microtiter plate so that they correspond to the final layout of the printed microarray. **Figure 5** provides an example of corresponding layouts.
! CAUTION DMSO is toxic; work in a fume hood, wear gloves and protective clothing.

3| Spot arrays onto silylated slides using an OmniGrid spotter (1 TeleChem SMP3 pin). Set relative humidity to 50% and temperature to 22 °C. Spotting with single pins is recommended. This avoids variations inherent to spotting with multiple pins. The SMP3 pin used in our protocol takes 250 nl of sample per run and deposits 0.6 nl per spot. To reduce experimental error, each array should contain probes in triplicates. It is also possible to spot three arrays (in triplicate) on the same slide, allowing hybridization of three different samples per slide using triplicate chambers. Check the quality of printed spots by scanning the first and the last slide (if re-dipping during spotting, check the first and the last slide for every visit of the spotting head to the spotting plate) using the microarray scanner.

4| Place spotted slides carefully, with spotted sides in the same orientation, in a rack and incubate them overnight at room temperature (20–25 °C) and <30% relative humidity. For this purpose we use a 5-liter sandwich box, containing 0.5 liters of saturated NaCl solution.

5| Rinse slides twice in 0.2% (wt/vol) SDS for 2 min at room temperature with vigorous agitation to remove the unbound oligos.

6| Rinse slides twice in distilled water for 2 min at room temperature with vigorous agitation.

7| Transfer slides into distilled water at 95–100 °C for 2 min to denature DNA, then allow them to cool for 5 min at room temperature.

8| Treat slides in a freshly prepared sodium borohydride solution for ~5 min.
! CAUTION Borohydride is very toxic and harmful if inhaled or in direct contact with skin. Handle using appropriate gloves, wear protective clothing and work under a fume hood.

a

1	mtrof173	2	mtrof362-I	3	mtrof661	4	mtrof662-I	5	mtrof656	6	hyaBp	7	Blank	8	mtrof173
9	Blank	10	Blank	11	Blank	12	Blank	13	Blank	14	Blank	15	Blank	16	Blank
17	Mm275	18	PS80-291	19	peat_1_3-287	20	Mb460	21	Mb271	22	511-436	23	O_BB51-299	24	Mb292
25	O_Mb282	26	O_Mb_SI#1-41	27	Mb_SL-299	28	Jpn284	29	Mm_pel467	30	Est514	31	LP20-644	32	Mmb303
33	O_Mmb562	34	Blank	35	Blank	36	Blank	37	Blank	38	Blank	39	O_la193	40	O_la575
41	Nc_oce426	42	Mth413	43	Mc396	44	O_501-286	45	Mcl408	46	O_M90-253	47	O_M90-574	48	fw1-639
49	O_fw1-641	50	Blank	51	Blank	52	Blank	53	Blank	54	Blank	55	lb453	56	lb559
57	Blank	58	Blank	59	Blank	60	Blank	61	Blank	62	Blank	63	Blank	64	Blank
65	O_Mcy413	66	O_Mcy522	67	O_Mcy255	68	Peat264	69	Msi520	70	O_Msi269	71	O_Msi294	72	Msi232
73	Blank	74	Blank	75	Blank	76	Blank	77	Blank	78	Blank	79	O_Il509	80	O_Il630
81	O_B2rel251	82	B2-400	83	B2all343	84	O_B2all341	85	Blank	86	Blank	87	omoAMO3-406	88	O_RA14-594
89	Blank	90	Blank	91	xb6-539	92	LP21-190	93	LP21-232	94	O_LP21-260	95	Blank	96	Blank
97	NmNc533	98	Nsm_eut381	99	PS5-226	100	P16-306	101	NsNv207	102	NsNv363	103	Blank	104	Blank
105	Nrt_rel223	106	Nit_rel351	107	Nit_rel470	108	Nit_rel304	109	Blank	110	Blank	111	Blank	112	Blank
113	Blank	114	Blank	115	Blank	116	Blank	117	Blank	118	Blank	119	Blank	120	Blank
121	M84P105-451	122	VC306_54-38	123	M84_P22-514	124	gp23-454	125	MR1-348	126	gp391	127	gp2-581	128	RA21-466

b

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	1	9	17	25	33	41	49	57	65	73	81	89	97	105	113	121								
B																								
C	2	10	18	26	34	42	50	58	66	74	82	90	98	106	114	122								
D																								
E	3	11	19	27	35	43	51	59	67	75	83	91	99	107	115	123								
F																								
G	4	12	20	28	36	44	52	60	68	76	84	92	100	108	116	124								
H																								
I	5	13	21	29	37	45	53	61	69	77	85	93	101	109	117	125								
J																								
K	6	14	22	30	38	46	54	62	70	78	86	94	102	110	118	126								
L																								
M	7	15	23	31	39	47	55	63	71	79	87	95	103	111	119	127								
N																								
O	8	16	24	32	40	48	56	64	72	80	88	96	104	112	120	128								
P																								

c

	1	2	3	4	5	6	7	8	9	10	11	12
A	1	9	17	25	33	41	49	57	65	73	81	89
	mtrof173	Blank	Mm275	O_Mb282	O_Mmb562	Nc_oce426	O_fw1-641	Blank	O_Mcy413	Blank	O_B2rel251	Blank
B	2	10	18	26	34	42	50	58	66	74	82	90
	mtrof362-I	Blank	PS80-291	O_Mb_Stv1-41	Blank	Mth413	Blank	Blank	O_Mcy522	Blank	B2-400	Blank
C	3	11	19	27	35	43	51	59	67	75	83	91
	mtrof661	Blank	peat_1_3-287	Mb_SL-299	Blank	Mc396	Blank	Blank	O_Mcy255	Blank	B2all343	xb6-539
D	4	12	20	28	36	44	52	60	68	76	84	92
	mtrof662-I	Blank	Mb460	Jpn284	Blank	O_501-286	Blank	Blank	Peat264	Blank	O_B2all341	LP21-190
E	5	13	21	29	37	45	53	61	69	77	85	93
	mtrof656	Blank	Mb271	Mm_pel467	Blank	Mcl408	Blank	Blank	Msi520	Blank	Blank	LP21-232
F	6	14	22	30	38	46	54	62	70	78	86	94
	hyaBp	Blank	511-436	Est514	Blank	O_M90-253	Blank	Blank	O_Msi269	Blank	Blank	O_LP21-260
G	7	15	23	31	39	47	55	63	71	79	87	95
	Blank	Blank	O_BB51-299	LP20-644	O_la193	O_M90-574	lb453	Blank	O_Msi294	O_Il509	OmoAMO3-40	Blank
H	8	16	24	32	40	48	56	64	72	80	88	96
	mtrof173	Blank	Mb292	Mmb303	O_la575	fw1-639	lb559	Blank	Msi232	O_Il630	O_RA14-594	Blank

d

	1	2	3	4	5	6	7	8	9	10	11	12
A	97	105	113	121								
	NmNc533	Nit_rel223	Blank	M84P105-451								
B	98	106	114	122								
	Nsm_eut381	Nit_rel351	Blank	VC306_54-385								
C	99	107	115	123								
	PS5-226	Nit_rel470	Blank	M84_P22-514								
D	100	108	116	124								
	PI6-306	Nit_rel304	Blank	gp23-454								
E	101	109	117	125								
	NsNv207	Blank	Blank	MR1-348								
F	102	110	118	126								
	NsNv363	Blank	Blank	gp391								
G	103	111	119	127								
	Blank	Blank	Blank	gp2-581								
H	104	112	120	128								
	Blank	Blank	Blank	RA21-466								

Figure 5 | An example of microarray layout versus spotting plate layout. (**a**) Microarray layout—one subarray shown (this subarray is spotted in triplicate for one array; one slide normally contains three microarrrays, consisting of three subarrays each). (**b**) Layout of the 384-well spotting plate. (**c**,**d**) Layout of the 96-well (deep-well) plates containing the stock oligoprobe solutions.

9| Rinse slides three times in 0.2% (wt/vol) SDS for 1 min each at room temperature.

10| Rinse once in distilled water for 1 min each at room temperature.

11| Dry slides by centrifugation at 100*g* for 2 min. Alternatively, an air gun fitted with a cotton-wool filter (to filter out oil microdroplets) can be used to dry slides individually. If an air gun is used, a modest stream of air should first be applied to the field containing the spotted probes; the aim is to blow the drops down and off the slide rather than drying them onto it.
■ PAUSE POINT Dried slides can be stored for up to 3 months at room temperature, desiccated, in the dark.

Environmental DNA purification

● TIMING 2 h

12| Extract DNA from soil samples; the method described here is based on the FastDNA spin kit for soil. Add 0.3 g of soil and 780 µl of lysis buffer into a Multimix FastPrep tube and homogenize by vortexing. Incubate at 37 °C for 30 min.

13| Add 122 µl of MT buffer into the tubes and shake them in the FastPrep instrument for 30 s at 5.5 m s^{-1}.

14| Centrifuge samples for 15 min at 12,000*g* and collect the supernatant (~700 µl) into a microcentrifuge tube.

15| Add 5 µl of 10 mg ml^{-1} freshly prepared proteinase K to the tube and incubate at 65 °C for 30 min.

16| Add 300 µl of phenol; vortex. Add 300 µl of chloroform-isoamyl alcohol (24:1), vortex and spin for 5 min at 12,000*g*. Transfer the supernatant to a new tube.
! CAUTION Phenol and chloroform are both highly toxic; wear gloves and protective clothing and work in a fume hood.

17| Add 600 µl of chloroform-isoamyl alcohol (24:1), mix carefully and then spin for 5 min at 12,000*g* and transfer the supernatant into a new microcentrifuge tube.

18| Add 125 µl of 7.5 M potassium acetate (pH 7.5); incubate the tubes on ice for 5 min.

19| Centrifuge at 12,000*g* for 10 min. Transfer the supernatant (~600 µl) into a new tube.

20| Add 700 µl of binding matrix and mix tubes for 5 min in an Eppendorf shaker at 100 r.p.m.

21| Centrifuge binding matrix with bound DNA for 1 min at 12,000*g*. Discard supernatant.

22| Resuspend the pellet in 500 µl of wash buffer (included in the kit). Add the resulting suspension into a spin filter (included in the kit) and centrifuge for 1 min at 12,000*g*. Discard the eluate.

23| Wash the pellet again with 500 µl wash buffer. Discard the eluate.

24| Centrifuge the spin filter for a further 10 s to dry the pellet.

25| Place the filter into a new tube and add 150 µl of TE (pH 8.0) and incubate for 1 min at room temperature.

26| Centrifuge for 1 min at 12,000*g*. Collect eluate containing the purified DNA in the catch tube.
■ **PAUSE POINT** DNA can be stored, for up to 1 year, at −20 or −80 °C.

Target amplification by two-stage PCR ● TIMING 4 h

27| *First-stage PCR amplification*: For each target, set up three 50-µl PCR reactions using appropriate primer pairs; for *pmoA* and related genes we use forward primer T3c-pmoA189 and the reverse primer T7c-mb661 or T7c-A682. Running three parallel PCR reactions provides enough material for standardized target preparation; in addition, using three separate reactions instead of a single, larger-volume one also helps minimize the chance of random PCR drifts. Include a negative control (with water replacing DNA template) and a positive control (containing a *pmoA* plasmid, methanotroph genomic DNA or environmental DNA previously shown to give rise to *pmoA* PCR product). Prepare each 50-µl reaction as tabulated below.

Component	Amount (µl)	Final
PCR buffer (10×)	5	1×
dNTP mixture (2.5 mM for each dNTP)	4	0.2 µM
$MgCl_2$ (50 mM)	1.5	1.5 µM
Forward primer (1.5 µM)	1	1.5 pmol
Reverse primer (1.5 µM)	1	1.5 pmol
Taq polymerase (1 U μl^{-1})	1	1 U
Template DNA		
(30 ng μl^{-1} environmental DNA or 1 ng μl^{-1} genomic DNA or 0.1 ng μl^{-1} plasmid DNA)	1	30 ng/1 ng/0.1 ng
Ultrapure water	Up to 50	

▲ **CRITICAL STEP** We found that MasterAmp 2× Premix F substantially improved PCR yield from all soil environments tested. Buffer, Mg and dNTP are included in Premix F.

28| Run PCR using the following cycling conditions.

Cycle number	Denature	Anneal	Extend
1	95 °C, 5 min		
2–16	95 °C, 1 min	56 °C, 1 min	72 °C, 1 min
17			72 °C, 10 min

29| Second-stage PCR amplification. To each PCR reaction from Step 28, add 15 pmol of each primer T3c and T7c and an additional 1 U of Taq DNA polymerase. Carry out a further 25 cycles using an annealing temperature of 58 °C as detailed in the table below.

Cycle number	Denature	Anneal	Extend
1	95 °C, 5 min		
2–26	95 °C, 1 min	58 °C, 1 min	72 °C, 1 min
27			72 °C, 10 min

30| Check PCR products by running 5 µl of PCR product on 1.5% (wt/vol) agarose gel.
■ **PAUSE POINT** PCR products may be stored for up to 1 year at this point.
? TROUBLESHOOTING

PCR purification ● TIMING 0.5 h
31| Pool parallel PCR products (3 × 50 µl) and purify with a commercial PCR purification kit according to manufacturer's instructions. Dissolve or elute purified DNA in ultrapure water. Avoid using excessive amounts for elution; the concentration of the PCR product has to be adjusted to 50 ng μl^{-1}.
▲ **CRITICAL STEP** Self-made filtration columns may leave significant amounts of unincorporated nucleotides in the purified product, interfering with the spectrophotometric concentration measurement.

32| Measure the concentration of purified DNA by spectrophotometry (e.g., with NanoDrop spectrophotometer). Adjust concentration to 50 ng μl^{-1} with ultrapure water (if DNA concentration is below 50 ng μl^{-1}, concentrate DNA by evaporation in a SpeedVac centrifuge (without heating) and quantify DNA again). Store at −20 °C until use.
■ **PAUSE POINT** PCR product can be stored long term, at −20 °C.

***In vitro* transcription ● TIMING 5 h**
33| Working under standard RNase-free conditions, set up an *in vitro* transcription reaction for each purified PCR product from Step 32 as tabulated below. Incubate at 37 °C for 4 h. *hyaB* PCR product is prepared as described in **Box 2**.

Component	Amount (µl)	Final
Purified *pmoA* PCR product (50 ng μl^{-1})	7	350 ng
Purified *hyaB* PCR product (50 ng μl^{-1})	1	50 ng
T7 RNA polymerase buffer (5×)	4	1×
DTT (100 mM)	2	10 mM
RNAsin (40 U μl^{-1})	0.5	20 U
ATP (10 mM)	1	0.5 mM
CTP (10 mM)	1	0.5 mM
GTP (10 mM)	1	0.5 mM
UTP (10 mM)	0.5	0.25 mM
T7 RNA polymerase (50 U μl^{-1})	1	50 U
Cy3-UTP (5 mM)	1	0.25 mM

34| Purify labeled RNA immediately. This step removes unincorporated nucleotides, DNA template, T7 polymerase and salts. We use the Qiagen RNeasy kit with minor modifications. To the tube from Step 33, containing the IVT (*in vitro* transcription) mix, add 80 µl of DEPC-treated water and 350 µl of RLT solution (provided with the kit), mix thoroughly and then add 250 µl of ethanol.
! CAUTION Ethanol is flammable.

35| After mixing thoroughly, transfer sample (700 µl) into an RNeasy mini column and centrifuge for 15 s at >12,000*g*.

36| Transfer the column to a new 1.5-ml collection tube, add 500 µl RPE solution (provided with the kit) and centrifuge for 15 s at >12,000*g*.

37| Add 500 µl RPE and centrifuge for 2 min at >12,000*g*.

38| Transfer the column to a new 1.5-ml collection tube, add 50 µl RNase-free water and centrifuge for 1 min at >12,000*g*. Eluted RNA should have a pink color (intensity may vary, but at least a faint color is usually visible to the eye). If not, check labeling by spectrophotometry at wavelength corresponding to Cy3 (550 nm). Here the *hyaB* PCR product is used as an internal positive control for labeling.
▲ **CRITICAL STEP** We recommend measuring RNA yields and dye incorporation rates by spectrophotometry.
? TROUBLESHOOTING

Fragmentation of RNA with Zn^{2+} ● TIMING 1 h
39| Work under standard RNase-free conditions. To 50 µl of purified RNA (in a 1.5 ml microcentrifuge tube, from Step 38), add 1.43 µl of 1 M Tris.Cl (pH 7.4), 5.71 µl of 100 mM $ZnSO_4$ and mix; incubate at 60 °C for 30 min.
▲ **CRITICAL STEP** Fragmentation has been calibrated to yield the ideal length of RNA. Too short fragments would negatively influence hybridization between target and probe; too long fragments would allow strong secondary structures to be formed within target RNA, outcompeting hybridization between target and probe. This protocol is optimized for a dry block without mixing. The condensation on the inside of the tube lid, which causes a gradual concentration of the reaction components over the 30 min incubation, is included in optimization of the protocol. In our experience, following this protocol yields reproducible results between laboratories.
▲ **CRITICAL STEP** RNA work is demanding because of the chemical instability of RNA and the ubiquitous presence of RNases. Use RNase-free labware and chemicals, and wear RNase-free gloves when working with RNA. Use RNAsin to inhibit RNase activity in RNA solutions and reaction mixtures containing RNA. RNAsin can be inactivated by denaturing reagents (i.e., SDS, urea) and elevated temperatures. RNases, on the other hand, do generally survive such treatments. If exposed to such conditions, replenish RNAsin.

40| Add 1.43 µl of 500-mM EDTA (pH 8.0) to stop the reaction (EDTA chelate Zn^{2+}). Place on ice for 1 min then add 1-µl 40 U μl^{-1} RNasin. For quality assessment, run the fragmented RNA target on an ABI capillary sequencer with a standard size marker (e.g., 500 bp LIZ).
■ **PAUSE POINT** Fragmented, labeled RNA target can be stored at −20 °C for several months.

Hybridization ● TIMING 19 h
41| Pour ~300 ml of water into the hybridization water bath (Belly Dancer) and then place the aluminum block into it. Cap the Belly Dancer, start rotation at low speed (30–40 r.p.m.) and preheat the hybridization block to 55 °C for at least 30 min to allow the temperature to stabilize; always check the temperature with a thermometer before proceeding. Also preheat an Eppendorf incubator (dry block) to 65 °C.
▲ **CRITICAL STEP** Hybridization should be carried out in the dark, whenever possible, to minimize photobleaching of the fluorescent dyes. Carry out hybridization in a custom-tailored aluminum block (see EQUIPMENT SETUP) used as an insert for a temperature-controlled Belly Dancer set at maximum bending (~10°).
▲ **CRITICAL STEP** Ensure that no air bubbles are trapped below the block. After 55 °C is reached, remove excess water using a sponge. At the end, the block surface must be dry, but there must be water visible around the block (see **Fig. 4**).

42| Place the slides containing the arrays (face up) on a flat surface. Peel HybriWell chamber off the release liner (HybriWells are RNase-free, therefore, take care not to contaminate exposed working surface). Apply HybriWell onto the slides (adhesive side down) aligning the edges of the slide with the edges of the HybriWell. Ensure a secure seal by pressing the edge of the HybriWell (black stick border) with the flat edge of the smoothing tool (provided with HybriWells).

43| For each hybridization using triplicate chambers, set up a hybridization mixture in a 1.5-ml microcentrifuge tube as tabulated below. If chambers of different volume are used, the concentration of labeled target should be maintained for consistent results. Mix the hybridization mixture by vortexing gently, centrifuge at room temperature at 12,000*g* for 2 min and incubate at 65 °C for 1–10 min.

Component	Amount (µl)	Final
DEPC-treated water	62	—
SDS (10%)	1	0.1%
Denhardt's reagent (50×)	2	1×
SSC (20×)	30	6×
Target RNA	5	200 ng

44| Remove any water from the upper surface of the aluminum block with a tissue and arrange assembled slide on it. Ensure that the slides do not get wet when the Belly Dancer is rotating. Preheat the slides for ~2 min; the Belly Dancer should be set to maximum bending.

45| Apply preheated hybridization mixtures to the assembled slides via the port in the lower position, allowing air to escape through the other port in the upper position (to minimize risk of air bubbles being trapped within the chamber). Seal chamber (the two ports) using adhesive seal tabs (provided with HybriWells).
▲ **CRITICAL STEP** Ensure that both the microarray slide and the hybridization mixture are preheated to the hybridization temperature. Ensuring good control over hybridization conditions throughout the entire procedure maximizes specificity.
▲ **CRITICAL STEP** Ensure that the hybridization chamber is completely filled with the hybridization mixture and no bubbles are formed in the array area. Light pressure applied to the sealed port in the lower position (while port in the upper position is still not sealed) will help to fill the chamber completely (take care not to "wick" reagents from the port) and dislodge air bubbles if they appear, then seal the port in the upper position.

46| Cap the Belly Dancer incubator unit to stop evaporation of water from the unit itself. Incubate overnight (14–18 h to ensure complete hybridization) in the Belly Dancer (30–40 r.p.m. circulation at maximum bending to ensure uniform hybridization across the whole microarray) in the dark.

47| Following hybridization, grasp the tab end firmly and peel the HybriWell slowly away from the slides and immerse the slide immediately into 2× SSC and 0.1% (wt/vol) SDS at room temperature (22 °C).
▲ **CRITICAL STEP** Place one slide at a time into the wash solution to minimize the risk of drying.
▲ **CRITICAL STEP** It is important to minimize handling time in this step. Once the slide is removed from the hybridization platform, temperature quickly drops, allowing nonspecific hybridization to build up. Once the HybriWell is opened, free, Cy3-labeled RNA target may dry onto the slide surface, giving rise to nonspecific signal that cannot be removed by the wash procedure described below.

48| Wash the slides by shaking at room temperature for 5 min in 2× SSC, 0.1% (wt/vol) SDS.

49| Wash twice for 5 min in 0.2× SSC at room temperature.

50| Wash for 5 min in 0.1× SSC at room temperature.

51| Dry the slides individually using an oil-free air gun or with a gentle stream of compressed nitrogen (approximately 20–30 psi).
■ **PAUSE POINT** Store the slides at room temperature in the dark, scan the same day.
▲ **CRITICAL STEP** As a precaution against, e.g., light- or ozone-mediated bleaching of fluorescent signal, it is generally recommended to scan hybridized slides as soon as possible. Although it should be noted that we found Cy3 signals on slides stored in the dark for 2 months to show comparable signals.

Scanning and data analysis ● TIMING 2 h (for 24 arrays)
52| Perform a preview scan to adjust the photomultiplier tube settings (to avoid saturated pixels) at wavelengths of 532 nm and 635 nm for Cy3 and Cy5, respectively. We use a GenePix 4000A laser scanner.

53| Change to an average of three lines and perform a full resolution scan.
? TROUBLESHOOTING

54| Normalize results to a positive control: express hybridization signal for each probe as percentage of the signal of the positive control probe mtrof173 (universal to all amplified genes) on the same array. As signal, use the median minus local background values.
▲ **CRITICAL STEP** Steps 54–57 are carried out in Excel using custom-recorded macros (see **Supplementary Data 1**).

55| Determine the average of the normalized intensities and standard deviations on the triplicate spots for each probe.

56| As several probes produce nonspecific background signal up to 3% of their maximum signal, consider hybridization between a probe and a target positive only if the signal is at least 5% of the reference value. The reference value is the strongest signal obtained for that given probe during validation using the reference strains/clones.
? TROUBLESHOOTING

57| For probes in which no reference target perfectly matching the probe is available, and in which the strongest signal is below 60, set this reference value arbitrarily to 60. In our experience this minimizes false-positive calls while not creating false negative calls.
? TROUBLESHOOTING

Statistical analysis of array data ● TIMING 1 h
58| Combine data from all arrays to be compared in a spreadsheet with sample name as a header and including probe names.

59| Import the data into a standard multivariate statistical analysis package such as Primer 6.

60| Remove the control probes (*hyaB* and mtrof probes) and standardize the probe intensities within each sample and then transform as required. Use the factors function to define the different groups within the experiment (Treatments, spatial zones and so on).

61| Use ordination to generate a two-dimensional plot of all the samples within the study. This plot can be used to illustrate the relationship between methanotroph community compositions across different samples (as demonstrated in **Fig. 2**).

62| Test significant differences between groups using ANOSIM[43] or PERMANOVA[44] routines.

63| Use similarity percentage analysis to determine the probes that best describe any significant differences between groups of samples.

? TROUBLESHOOTING
Troubleshooting advice is provided in **Table 1**.

TABLE 1 | Troubleshooting table.

Step	Problem	Possible reason	Solution
30	No PCR product	Low-purity environmental DNA	Repeat DNA purification, try different purification method, try using more or less environmental sample Try to repeat PCR with less environmental DNA as template
		PCR reagents expired	Try to replace PCR reagents with fresh stocks and working solutions
		Low abundance of methanotrophs in the environmental sample	Try to repeat PCR with more environmental DNA as template
30	Negative PCR control is positive	PCR reagents contaminated	Try to replace PCR reagents with fresh stocks and working solutions
38	Purified RNA target is colorless	*In vitro* transcription did not work	Repeat with fresh reagents
		No fluorophore incorporated into the synthesized RNA	Repeat with fresh Cy3-UTP
53	Irregular spot morphology	Deformed or contaminated spotting pin	Replace or clean spotting pin
		Poor printing	Check if the pin takes the correct amount of probe
		Improperly spotted or processed array	Repeat printing and processing Check humidity and mixing of air inside the spotter
53	Low signal	Target was not labeled efficiently or has been exposed to light too long	Check the sample for labeling efficiency. If poor label incorporation is observed, repeat *in vitro* transcription. Protect labeled sample from exposure to light and store at low temperature

(continued)

TABLE 1 | Troubleshooting table (continued).

Step	Problem	Possible reason	Solution
56	Hybridization: false negative or positive results	Probe melting temperature can cause loss of signal if too high, and nonspecific signal if too low	Confirm temperature placing a calibrated thermometer in the small whole of the aluminum block
53	Local differences in signal and background intensity	Bubbles	Make sure that the ports in the HybriWell are properly sealed and the Belly Dancer is at maximum bending with circulation between 30 and 40 r.p.m. Repeat hybridization Avoid drawing air into pipette tip; good loading technique is essential to prevent bubble formation
		Array was improperly processed	Repeat hybridization using arrays from a different printing batch
53	High background following hybridization	Unincorporated fluorochrome molecules are a common source of background signal	Prepare a new target
56	Poor reproducibility	Array inconsistencies	Array spotting and processing are prominent sources of reproducibility problems. Always use, in any one experiment, successive arrays from the same printing batch. Running replicate arrays is a common method of overcoming array inconsistencies
57	No reference target available	Probe targets sequences, from a public database, for which no reference target is available	Set reference value arbitrarily to 60

● TIMING

Microarray development (Step 1 and **Box 1).** The development and validation of an MDM following this protocol takes ~6 months, subject to availability of reference clones used in validation.

Microarray spotting (Steps 2–11). Preparing the spotting plate and starting the microarray spotter takes ~6 h, depending on the complexity of the array design and the spotter used. The time for spotting itself depends on the instrumentation, the number of arrays and probes to be spotted. Spotting an array of 100 probes in triplicate onto 100 slides takes ~6 h using the OmniGrid 100 spotter. Including an overnight incubation and subsequent processing, the total time is ~30 h.

Timing for a typical experiment on 24 samples by an experienced operator:

Day 1 (Steps 12–29). Isolation of DNA from 24 environmental samples, gel analysis of purified DNA, quantification and PCR (overnight): ~5 h bench work.

Day 2 (Steps 30–46 and Box 2). Gel analysis of PCR products, purification, quantification, concentration adjustment; *in vitro* transcription, purification, fragmentation and hybridization: ~5 h bench work.

Day 3 (Steps 47–63). Washing and scanning of slides; analysis of data: approximately 4–5 h bench work.

Total hands-on time: 13–14 h.

ANTICIPATED RESULTS

In our laboratory, the *pmoA*-based microarray technology has been evaluated and applied successfully for the analysis of methanotroph communities in a range of environmental studies[24,28–31,45–51]. The microarray provides a high-resolution fingerprint of the methanotroph community structure. Results are typically presented as a heatmap, indicating the presence and relative abundance of the different methanotroph clades targeted. Further analysis of the data using multivariate statistics can provide more in-depth information about the experimental system being studied. The detection limit of the technique is ~5% of the total population analyzed (i.e., of the total *pmoA*/*amoA* pool amplified). An example is shown in **Figure 3**, depicting the effect of land use on methane oxidizing bacteria in an Alpine meadow over four seasons. **Figure 2** provides an example of the type of analysis that can be carried out on such a data set and highlights the use of multivariate analysis of array data to differentiate different groups of samples (seasonal samples). Together these two figures demonstrate the relative abundance of groups of methanotrophs present in each of the samples analyzed (**Fig. 3**) and the relation of each sample to all the others (**Fig. 2**). From these two figures it is possible to see that there is an effect of a particular factor

(season) on the structure of the targeted methanotroph community, whereby the community in the summer samples is significantly different from the other seasons. However, the experimental questions being posed will ultimately determine the best method of analysis. Results obtained with the microarray have frequently been validated by sequencing of a small clone library (50–100 clones) from one of the samples analyzed within a study. These clone library analyses reproducibly confirmed microarray findings, except for rare cases where an understudied environment harbored major unknown groups of methanotrophs. The microarray described here has been updated several times since its first publication, taking into consideration such results as well as new sequences appearing in public databases[18,28,23].

Note: Supplementary information is available via the HTML version of this article.

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