51 GeoChips for Analysis of Microbial Functional Communities

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1 Introduction 4040

2 Experimental Approach 4041
2.1 Target Preparation 4041
2.1.1 Amplification 4042
2.1.2 Labeling 4043
2.2 Hybridization 4044
2.2.1 Slide Preparation 4044
2.2.2 Preparation of Microarray Assembly 4044
2.2.3 Sample Preparation 4044
2.2.4 Posthybridization 4045
2.2.5 Data Acquisition and Preprocessing 4046
2.2.6 Solutions and Materials 4046
2.2.7 Time Considerations 4047
2.2.8 Troubleshooting 4048

3 Research Needs 4048

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Abstract: Functional gene arrays (FGA) are microarrays that contain probes for genes encoding proteins or enzymes involved in functions of interest and allow for the study of thousands of genes at one time. The most comprehensive FGA to date is the GeoChip, which contains ~24,000 probes for ~10,000 genes involved in the geochemical cycling of C, N, P, and S, as well as genes involved in metal resistance and reduction and contaminant degradation. This chapter details the methods necessary for GeoChip analysis. Methods covered include preparation of DNA (whole community genome amplification and labeling), array setup (prehybridization steps), hybridization (sample and hybridization buffers), and post hybridization steps (slide washing and array scanning).

1 Introduction

Microarrays are a powerful, high throughput tool that allows for the study of thousands of genes at one time. While microarrays were first designed to study individual organisms (Schena et al., 1995), their potential to study microorganisms in the environment was later proposed (Guschin et al., 1997). Several types of microarrays have since been developed to examine microbial communities. These include phylogenetic arrays composed of 16S rRNA gene probes or other conserved functional genes for examining species diversity or community composition (Loy et al., 2002; Small et al., 2001; Wilson et al., 2002), community genome arrays comprised of whole genomic DNA from microbial isolates that can be used to determine microbial identity or to screen for species of interest (Wu et al., 2004, 2008; Zhang et al., 2004), and functional gene arrays (FGA), which contain probes for genes encoding proteins or enzymes involved in functions of interest (Gentry et al., 2006; He et al., 2007; Tiquia et al., 2004; Wu et al., 2001). Because probes are designed for sequences with known functions, FGAs, unlike phylogenetic and community genome arrays, can provide information regarding the potential functional capabilities of microbial communities and are ideally suited for linking microbial functional structure with geochemical and other environmental processes. In addition, unlike traditional polymerase chain reaction (PCR)-based approaches, FGAs allow hundreds or thousands of genes or gene groups to be examined simultaneously without the need to design specific primers (Gentry et al., 2006; He et al., 2007; Wagner et al., 2007; Wu et al., 2001, 2006; Zhou et al., 2008; Zhou and Thompson, 2002). FGAs have been designed to examine specific microbial populations or processes of interest, such as N cycling (Tiquia et al., 2004; Wu et al., 2001), *nifH* diversity (Steward et al., 2004), contaminant degradation and metal resistance (Rhee et al., 2004), and acid mine drainage sites (Yin et al., 2007), among others (Gentry et al., 2006). Evaluation of FGAs have shown these arrays to be both sensitive and specific (Cho and Tiedje, 2002; He et al., 2007; Leigh et al., 2007; Rhee et al., 2004; Tiquia et al., 2004; Wu et al., 2001, 2006, 2008; Yergeau et al., 2007).

The most comprehensive FGA available to date is the GeoChip 2.0, which contains ~24,000 probes for genes involved in the geochemical cycling of N, C, and S, metal reduction and resistance, and in organic contaminant degradation (He et al., 2007). The GeoChip was designed to address two of the major challenges in the use of FGAs for microbial community analysis: (1) the need for adequate oligonucleotide probe specificity for genes that have high homologies and (2) the lack of a truly comprehensive FGA probe set (He et al., 2007). This array has been used in a variety of studies to examine the functional potential of microbial communities (He et al., 2007; Rodríguez-Martínez et al., 2006; Wu et al., 2006, Wu et al., 2008; Yergeau et al., 2007; Zhou et al., 2008), to probe pure culture isolates for specific gene

functions (Van Nostrand et al., 2007), in stable isotope probing experiments (Leigh et al., 2007), and for the detection of microbial community RNA (Gao et al., 2007). The next generation of GeoChip (GeoChip 3.0), which has ~47,000 probes, representing 292 gene families, has been developed and is currently being tested and validated (Deng et al., unpublished data).

Another great advantage of GeoChip, and other FGAs, is the ability to customize the array to focus on functions or processes of interest to the researcher and the ability to continuously update the sequences covered on the array. Sequences that are optimal for inclusion are those that encode a key step or protein in the process of interest, are conserved but still provide enough sequence variance for probe design, and have sufficient sequences available in public databases (Gentry et al., 2006). The sequences chosen for inclusion should be carefully evaluated and tested so that only sequences with confirmed functions (either experimentally or via sequence similarity) are included.

Several software programs are available for probe design which can provide probe sequences appropriate for FGAs. However, a study by our lab suggested that there could be some issues regarding specificity of these designed probes among similar sequences (Li et al., 2005). As such, to provide the greatest specificity, we recommend the use of the CommOligo software for probe design (Gentry et al., 2006; Li et al., 2005). Criteria for probe design and selection has been discussed in detail previously (Gentry et al., 2006; He et al., 2007). GeoChip 2.0 is comprised of 24,243 50-mer oligonucleotide probes which are contact printed onto UltraGAPS (gamma amino propyl silane) coated slides (Corning; Lowell, MA). These probes cover >10,000 genes in >150 functional groups. More information regarding the development of FGAs and the GeoChip is in Chapter 20, Vol. 4, Part 3 (Van Nostrand et al., 2009). In addition, several recent publications are available that provide more detailed information regarding the design and preparation of FGAs, including sequence selection, probe design criteria, and array printing (Bodrossy et al., 2003; Gentry et al., 2006, 2007; He et al., 2005a, b, 2007; Li et al., 2005; Liebich et al., 2006; Rhee et al., 2004). The following protocols are specific to the GeoChip, although other array platforms are available which would require use of different or modified methods.

2 Experimental Approach

2.1 Target Preparation

A detailed description of DNA extraction and purification protocols will not be described here, but as these are critical first steps in sample preparation, we felt it important to address several important issues. While many methods for DNA extraction are available, the method chosen should result in large fragments of genomic DNA as this is crucial for the whole community genome amplification step. Our lab routinely uses a freeze-grind method followed by alkaline lysis (Hurt et al., 2001; Zhou et al., 1996). A full description of this method is available at our lab's website (http://ieg.ou.edu/). The extracted DNA should be purified as soon as possible after extraction to prevent degradation. We have observed degradation of raw DNA extracts after as little as one month, even at −20°C. Ideally, DNA should have a 260/280 ratio ≥1.8 and 260/230 ratio ≥1.7. Gel purification of soil and sediment DNA has worked very well for our lab. A column purification kit can also be used, especially for samples with low DNA yields. While these steps can be tedious, it is critical that DNA used for

hybridizations be as pure as possible; therefore, it is beneficial (both in terms of time management and cost) to spend the time necessary on DNA preparation before proceeding with subsequent steps. Any impurities remaining in the DNA can interfere with amplification, labeling, and hybridization.

2.1.1 Amplification

The GeoChip requires 2–5 μg of genomic DNA for hybridization. Depending on the amount of DNA available, whole genome amplification (WGA) may be required. WGA can be performed using either phage Φ29 (Dean et al., 2001; Wu et al., 2006) or *Bacillus stearothermophilus* DNA polymerases (Aviel-Ronen et al., 2006; Lage et al., 2003). Amplification using Φ29 (Templiphi, GE Healthcare; Piscataway, NJ) for WGA of microbial community DNA has been systematically evaluated and shown to provide sensitive (10 fg detection limit) and representative amplification (<0.5% of amplified genes showed >2-fold different from unamplified) (Wu et al., 2006). The following protocol uses the Templiphi amplification kit (GE Healthcare) and is based on a previously published protocol (Wu et al., 2006). All steps should be carried out in a laminar flow hood or PCR workstation hood.

1. Add 10 μL of sample buffer (supplied with kit) to a PCR tube or micro-well plate.
2. Transfer 10–100 ng DNA to the sample buffer. The total volume of DNA added should be no more than 5 μL and the sample volume should be the same for all samples. Use nuclease-free water to bring the volume up, if necessary.
3. Mix the DNA and buffer thoroughly and incubate 10 min at room temperature.
4. While DNA and buffer are incubating, prepare the Templiphi premix [for each reaction: 10 μL reaction buffer, 0.6 μL enzyme mixture (both supplied in the kit), and single stranded binding protein (USB; Cleveland, OH) and spermidine to a final concentration of 260 ng μL^{-1} and 0.1 μM, respectively.]
5. Transfer 12.85 μL of the Templiphi premix to the DNA/buffer mixture (or the equivalent volume for one sample).
6. Incubate the reaction at 30°C for 3 h and then heat-inactivate the enzyme at 65°C for 10 min.
7. To evaluate the amplification quality, run approximately 2 μL of amplified product on a gel. The product should produce a smear rather than a single band.
8. Quantify the amplified DNA using a dye-binding assay, such as PicoGreen (Quant-iT PicoGreen dsDNA kit; Invitrogen, Carlsbad, CA). The amplified product can not be measured using 260/280 ratios due to primers and dNTPs remaining in the sample. There should be at least 2 μg of amplified DNA. If there is less than this, the amplification should be repeated.

Notes: The best amplification results will be obtained using freshly extracted, high molecular weight DNA of the highest quality obtainable. However, DNA from samples with very low biomass, limited sample size, or that would be impossible to replace may not be of optimum quality yet are important to analyze. These samples can still be amplified, but may require some additional steps. If the DNA sample is very dilute, the DNA can be concentrated so that more DNA can be added to the reaction. If no or poor amplification occurs, try decreasing the amount of sample volume used to dilute out any inhibitors that may be present. Serial dilution (2–3 dilution steps) of the sample can be used to "wash" the DNA. This approach has been

used successfully in our lab for a variety of samples. Serial dilution can also be used if the DNA quality is above the recommended thresholds, but the amplification results are poor. Other options that can be tried include re-precipitating the DNA (using an ethanol or isopropanol protocol) to try and remove any inhibitors, increasing amplification time or performing multiple amplifications of low-product samples and combining the products to increase the total amount of amplified DNA.

This is a very sensitive reaction and any contaminating DNA will be amplified. Steps to limit the amount of background DNA have been outlined in Zhang et al. (2006) and include UV irradiation of the hood and all items to be used in the protocol, including tips, tubes, pipettors, tube racks, ice and ice bucket, etc. Due to the sensitivity of this reaction, negative controls should always be run alongside the samples.

2.1.2 Labeling

DNA for hybridization is generally labeled using fluorescent dyes, primarily Cy5 or Cy3. The DNA can be labeled directly (dyes are directly integrated into the target DNA) or indirectly (targets are labeled after hybridization). Our lab uses a direct labeling approach which is detailed below.

1. Combine 20 μL random primers (octamers, 750 μg mL^{-1}; BioPrime DNA Labeling System; Invitrogen) with 2–5 μg DNA (amplified or unamplified).
2. If necessary, bring volume up to 35 μL with water (if using amplified DNA, volume may already be larger than 35 μL).
3. Incubate 5 min at 99.9°C in a thermocycler and immediately chill tubes on ice.
4. In a separate tube create a master mix sufficient for all samples (per sample: 2.5 μL 5 mM dNTP mix [2.5 mM dTTP], 1 μL CyDye [25 nM, Cy5-dUTP; GE Healthcare], 2 μL Klenow [40 U μL^{-1}; BioPrime DNA Labeling System, Invitrogen]). The final reaction volume should be 50 μL, so adjust the master mix volume with nuclease free water to obtain this volume.
5. Add an appropriate volume of master mix to the primer DNA solution.
6. Incubate at 37°C for 3 h, followed by 3 min at 95°C. Labeled DNA can be kept at 4°C in the thermocycler if labeling is done overnight, or proceed to purification.
7. Purify the labeled DNA using a PCR clean-up kit as per the manufacturer's directions. Our lab uses the Qiagen QIAquick PCR purification kit (Qiagen; Valencia, CA). DNA can be eluted using water or the elution buffer supplied.
8. Check Cy-dye incorporation by absorbance at 550 nm for Cy3 or 650 nm for Cy5. The Nanodrop ND-1000 (Thermo Scientific; Wilmington, DE) or similar spectrophotometers require only 1 μL of sample. Several measures can be used to determine incorporation efficiency. The ND-1000 provides the pmol of dye μL^{-1} for each sample; a minimum dye incorporation required is 50 pmol (pmol μL^{-1} * total μL). Specific activity (SA) can be determined using two different formulas: (i) SA = (ng of target × 1000)/(pmol dye incorp × 324.5), where the SA should be >20. (ii) The SA can also be determined using a ratio. The A_{260}/A_{550} ratio for Cy3 should be between 8 and 15, while the A_{260}/A_{650} for Cy5 should be between 10 and 20 (Zhou and Thompson, 2004).
9. Dry the sample using a speedvac at 45°C for 45 min.
10. At this point, samples can be kept at −20°C until ready for hybridization.

Notes: High quality, fresh reagents should be used to insure the best possible results (Wu et al., 2001). The higher the labeling efficiency, the better the hybridization results will be. Cy-dyes are light sensitive and should be protected from the light as much as possible. We have stored dried, labeled products for months with no apparent loss of efficacy.

2.2 Hybridization

2.2.1 Slide Preparation

Microarray slides must be pretreated prior to hybridization. The exact protocol will vary depending upon the slide chemistry used. The following protocol is used for UltraGAPS coated slides (Corning; Lowell, MA).

1. Prepare enough prehybridization solution (5x SSC, 0.1% SDS, 0.1 mg mL^{-1} BSA) for the number of slides being prepared and warm to 42°C (or the temperature used for hybridization). A coplin jar (50 mL volume, 5 slide capacity) can be used for this step.
2. Immerse array slides in the warmed prehybridization solution and incubate for 45–60 min at 42°C.
3. Transfer slides to 0.1x SSC and incubate at room temperature 5 min.
4. Repeat step 3.
5. Transfer slides to room temperature DI water for 30 s.
6. Dry slides with purified canned air.
7. Examine slides for cleanliness. If the slides do not look clean, repeat steps 3 through 6.

2.2.2 Preparation of Microarray Assembly

Because the GeoChip is sensitive to temperature variations, especially to decreases in temperature below the hybridization temperature, all samples and microarray items are heated to several degrees above the hybridization temperature prior to sample loading.

1. Preheat coverslips (Lifter slips, 22 × 60 mm), array slides, tips, hybridization chambers, and an aliquot of 3x SSC at 60°C for at least 20 min in a hybridization oven. For ease of use in subsequent steps, the microarrays, coverslips, and hybridization chambers can be preassembled prior to placement in the hybridization oven.
2. Heat a heatblock to 60°C.
3. Set a waterbath to the appropriate hybridization temperature.

2.2.3 Sample Preparation

1. Prepare sufficient hybridization buffer for all of the samples (40 μL per array; 50% formamide, 3x SSC, 0.3% SDS, 0.7 μg μL^{-1} herring sperm DNA, 0.85 mM DTT).
2. Rehydrate the labeled DNA with 40 μL hybridization buffer.
3. Denature hydridization solution at 95°C for 3–5 min in a thermocycler and then maintain the temperature at 60°C.

4. At this point all solutions, slides, tips, and chambers should be maintained at 60°C, until the arrays are placed into the waterbath.
5. Remove a hybridization chamber, slide, and cover slip from the heating oven and place on the 60°C heat block.
6. Using the tips from the heating oven, add 17.5 μL of hot 3x SSC to the two wells in the hybridization chamber.
7. Apply sample to the arrays using one of two methods:
   a. Pipette the hybridization buffer onto the slide approximately 1/3 the way down the microarray. Add the Lifter slip by gently leaning it onto the slide and slowly lowering it until it touches the droplet on the slide, then release. Try to avoid air bubbles when assembling the array.
   b. Preassemble the array with the Lifter slip in place. Slowly pipette the entire sample solution onto the surface of the slide beginning at the edge of the Lifter slip. The solution should fill all the space between the slide and the Lifter slip by capillary action.
8. Seal the hybridization chamber and immediately immerse it in the pre-warmed waterbath.
9. Incubate over night at 42°C (~12–16 h).

Notes: While this protocol used a non-mixing hybridization, mixing during hybridization has been shown to increase sensitivity. A three fold increase in sensitivity was obtained for low abundance DNA using the MAUI mixer compared to non-mixed hybridization reactions (Adey et al., 2002). Several automated or semi-automated hybridization stations are available which provide mixing. In addition, there are hybridization ovens and special gaskets which allow mixing during hybridization. The use of smaller hybridization volumes has also been shown to increase sensitivity (Shalon et al., 1996).

When the GeoChip was first designed, optimal hybridization conditions were determined to be 50°C and 50% formamide (He et al., 2007). However, subsequent experiments by our lab have shown that hybridization conditions of 42°C and 50% formamide provided results consistent with other PCR-based methods.

2.2.4 Posthybridization

Once hybridization is completed, the microarray must be washed to remove unbound labeled DNA and hybridization buffer components that may increase background signal. The post-hybridization wash protocol may differ depending on the slide chemistry used. The following protocol is based on Corning UltraGAPS slides.

1. Immediately disassemble the chamber and remove the coverslip by immersion of the slide in 42°C (or the temperature of hybridization) Wash Buffer I (1x SSC, 0.1% SDS). Do not agitate the slides.
2. Once the coverslip falls off, transfer the slide to fresh 42°C Wash Buffer I and incubate at 42°C for 5 min.
3. Transfer slides to room temperature Wash Buffer II (0.1x SSC, 0.1% SDS) and incubate with gentle shaking for 5 min.
4. Repeat step 3.
5. Transfer slides to Wash Buffer III (0.1x SSC) and incubate with gentle shaking for 1 min.
6. Repeat step 5 five times, for a total of 6, 1 min washes.
7. Transfer slides to Wash Buffer IV (0.01x SSC) for 10 s.
8. Dry slides using purified canned air.

Notes: To reduce handling time of the slides at room temperature after hybridization, take out only one hybridization chamber at a time from the water bath.

Completed arrays should be protected from the light until imaged. A black or foil wrapped slide box works well. Imaging should be done within a few hours of removing the arrays from the hybridization chamber to minimize loss of signal.

2.2.5 Data Acquisition and Preprocessing

Scanner settings will depend upon the fluorescent dye used. This protocol will detail settings for cyanine dyes.

1. The arrays should be imaged with a laser scanner using wavelengths appropriate to the dyes used, for example 630 nm for Cy5 and 543 nm for Cy3.
2. Use a prescan setting with a low resolution (30–50 μm) to determine the appropriate photomultiplier tube (PMT) gain (%) and the laser power (%) settings. The settings should provide maximum signal intensity without saturating the spots.
3. Once appropriate settings have been determined, scan the array using a higher resolution (5–10 μm).
4. The scanned image can then be analyzed using a variety of array analysis software, including ArrayStar (DNAStar, Madison, WI), GenePix Pro (Molecular Devices, Sunnyvale, CA), GeneSpotter (MicroDiscovery, San Diego, CA), GeneChip (Affymetrix, Santa Clara, CA) and ImaGene (BioDiscovery, El Segundo, CA).

Notes: While increasing the PMT increases signal intensity, this also increases background. The hybridization signal can be photobleached, so the number of scans should be limited. The hybridization quality should be evaluated both during scanning (e.g., presence of positive control spots, even hybridization signals across the array, minimal background intensity) and after image analysis (e.g., presence of weak or poor spots). Several sources are available that provide more information regarding image preprocessing and analysis (Gentry et al., 2007; He et al., 2007; Zhou and Thompson, 2004).

2.2.6 Solutions and Materials

2.2.6.1 General Laboratory Supplies and Equipment Required

Pipettes (with appropriate tips), 0.2 mL PCR tubes, 1.8 mL microcentrifuge tubes, thermocycler, water bath, heatblock, gel electrophoresis unit (with appropriate agarose, buffers, and staining supplies), ice bucket, nuclease-free or PCR quality water, Coplin jar or similar container

2.2.6.2 Specific/Special Equipment Required

- Laminar flow hood/PCR workstation (e.g., AirClean 600 PCR Workstation; AirClean Systems, Raleigh, NC)
- Fluorescence reader (e.g., FLUOstar OPTIMA; BMG Labtech, Durham, NC)
- Spectrophotometer (e.g., NanoDrop-1000; NanoDrop Technologies, Wilmington, DE)
- SpeedVac (e.g., SPD 1010 SpeedVac system; Thermo Savant, Waltham, MA)
- Hybridization oven (e.g., PersonalHyb Hybridization Oven; Strategene, La Jolla, CA)

- Hybridization chambers (product number 2551; Corning, Lowell, MA)
- Lifter slips (22 × 60 mm; product number 100499–610; VWR, West Chester, PA)
- Purified canned air (e.g., Whoosh Duster; product number 16650–027; VWR)
- Microarray scanner (e.g., ProScan Array; Perkin Elmer, Waltham, Massachusetts)
- Microarray analysis software (e.g., Imagene 6.1.0; BioDiscovery, El Secundo, CA)

2.2.6.3 Reagents Required for Amplification

- Illustra Templiphi amplification kit (product number 25-6400-50; GE Healthcare, Piscataway, NJ)
- Single-stranded binding protein (SSB, product number 70032Z; USB, Cleveland, Ohio)
- Ultrapure spermidine (product number 21760; USB)
- Quant-iT PicoGreen dsDNA reagent (product number P11495; Invitrogen, Carlsbad, CA)

2.2.6.4 Reagents Required for Labeling

- Cy5-dUTP (product number PA55022 or PA55322; GE Healthcare)
- BioPrime DNA labeling system (for Klenow and Random primers; product number 18094–011; Invitrogen)
- dNTPs (product number 10297–018; Invitrogen)
- QIAquick PCR purification kit (product number 28106; Qiagen, Valencia, CA)

2.2.6.5 Reagents Required for Hybridizations

For Buffers

- 20x SSC buffer (3 M NaCl, 0.3 M sodium citrate; product number 100514–344; VWR)
- 10% sodium dodecyl sulfate (SDS; product number 12001–392; VWR)
- Omnipure bovine albumin (BSA) (product number EM-2930; VWR)
- Ultrapure formamide (product number 15515–026; Invitrogen)
- Ultrapure herring sperm DNA (product number 15634–017; Invitrogen)
- Dithiothreitol (DTT; product number PI20291; VWR)

Buffer Recipes

- Prehybridization buffer (5x SSC, 0.1% SDS, 1 mg mL^{-1} BSA)
- Hybridization buffer (40 μL per sample; 50% formamide, 3x SSC, 0.3% SDS; 0.7 mg mL^{-1} herring sperm DNA, 0.85 mM DTT)
- Wash buffer I (1x SSC, 0.1% SDS)
- Wash buffer II (0.1x SSC, 0.1% SDS)
- Wash buffer III (0.1x SSC)
- Wash buffer IV (0.01x SSC)

2.2.7 Time Considerations

The amplification reaction can be completed in one day (5–6 h total). Reaction set-up, including UV irradiation and preparation of hood, takes from 30 min to 1 h. Post-amplification quality checks (gel electrophoresis and PicoGreen assay) take approximately 1 h. Once

amplification has been confirmed, the labeling reaction can be done overnight (~3 h total). Approximately 30 min will be required for labeling reaction set up. An additional 2 h will be required afterwards for purification, quality control, and drying of samples. Preparations for hybridization take approximately 2–3 h, including preheating solutions and equipment, pre-hybridization incubations, and preheating of arrays and chambers. It is convenient to prepare the microarrays at the end of the day so that hybridizations occur overnight (12–16 h). Buffers for rinsing can be pre-heated overnight so that they are ready to use immediately once hybridizations are completed. Post-hybridization washes take 30 min to 1 h, depending on the number of arrays. Scanning generally takes 5–10 min per array, depending on the resolution and the number of dyes used.

2.2.8 Troubleshooting

The most important step for successful hybridizations is to make sure the starting DNA quality is optimal. In addition, the sample quality should be evaluated at each step to determine whether to proceed. Poor amplifications are often due to inhibitors present in the sample so reducing the amount of DNA added or performing a serial dilution can often solve this problem. Low labeling efficiency may be due to poor amplification results or the use of older reagents.

Hybridization temperature or the concentration of formamide can be varied to modify stringency and specificity. Each percent of formamide is equivalent to an increase of 0.6°C. Our lab has observed that a hybridization range of 42–50°C and 50% formamide provides accurate and specific hybridization results.

Making sure the arrays are clean after prehybridization, preventing bubble formation when setting up arrays, and thorough rinsing of arrays after hybridization will minimize background intensities, although the use of community DNA tends to result in a "dirtier" array than pure culture DNA. The presence of a high amount of atmospheric ozone can interfere with Cy-dye fluorescence (Branham et al., 2007).

3 Research Needs

Several challenges still exist for microarray analysis including quality of nucleic acids, specificity, sensitivity, and quantitative ability of microarrays. DNA quality and purity is critical to subsequent steps, but currently the most effective methods are also the most time consuming so more efficient, higher throughput purification methods are needed. Advances in probe design criteria, slide chemistry, and printing technology could help improve the specificity of arrays. More sensitive signal detection methods and new labeling methods (more sensitive dyes) would increase array sensitivity. Finally, while several studies have shown that FGAs can be quantitatively accurate, methods to distinguish between signal intensity changes owing to gene abundance or from sequence divergence need to be developed.

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