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Applications of functional gene microarrays for profiling microbial communities

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Functional gene arrays (FGAs) have been considered as a specific, sensitive, quantitative, and high throughput metagenomic tool to detect, monitor and characterize microbial communities. Especially GeoChips, the most comprehensive FGAs have been applied to analyze the functional diversity, composition, structure, and metabolic potential or activity of a variety of microbial communities from different habitats, such as aquatic ecosystems, soils, contaminated sites, extreme environments, and bioreactors. FGAs are able to address fundamental questions related to global change, bioremediation, land use, human health, and ecological theories, and link the microbial community structure to environmental properties and ecosystem functioning. This review focuses on applications of FGA technology for profiling microbial communities, including target preparation, hybridization and data processing, and data analysis. We also discuss challenges and future directions of FGA applications.

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Introduction

Microorganisms can live in almost all imaginable environments and play integral roles in the biosphere. Owing to their highly diversity, nonculturability, and metabolic versatility, characterization, identification, and quantitative analysis of microbial communities in natural or controlled environments are challenging. The recent development of metagenomic technologies such as high throughput sequencing [1–3] and microarrays [4,5,6••,7••,8••] provides powerful tools for characterizing microbial communities.

Functional gene arrays (FGAs) are comprised of probes targeting key genes involved in microbial functional processes, such as biogeochemical cycling of carbon (C), nitrogen (N), sulfur (S), phosphorus (P), and metals, biodegradation of environmental contaminants, stress responses, and virulence and antibiotic resistance. In the past decade, a variety of FGAs have been developed and applied for analyzing the functional diversity, composition, structure, function, activities, and dynamics of microbial communities from different habitats [5,6••,7••,8••,9,10,11••,12]. All results demonstrate that FGAs are a robust, powerful, high-throughput tool to specifically, sensitively and quantitatively profile microbial communities and link their composition and structure with environmental factors and ecosystem functioning.

This review is focused on applications of FGA technology for profiling microbial communities, including target preparation, hybridization and data processing, and data analysis to complement another review for FGA development [13••]. We will also discuss several challenges and future directions in FGA applications.

FGA analysis

Generally, FGA analysis includes three major steps: target preparation, FGA hybridization and data processing, and data analysis (Figure 1).

Target preparation

The key step toward successful FGA hybridizations is to obtain high quality nucleic acids from environmental samples. Environmental DNA is generally extracted using a well established method [14,15], which produces large fragments of DNA, especially important for subsequent DNA amplification. DNA can be purified using various methods, but for samples with large amounts of contaminants (e.g. humic compounds), agarose gel purification followed by phenol–chloroform–butanol extraction is preferred [16•]. The purified DNA and RNA should have a ratio of A_{260} to A_{280} around 1.80 and >1.90, respectively, and a ratio of A_{260} to A_{230} >1.70. Generally, 1–5 μg DNA or cDNA (reversely transcribed from mRNA) is required, otherwise amplification may be necessary. For DNA, whole-community genome amplification (WCGA) [17] may be used with 1–100 ng of DNA. It is more challenging to use mRNA but several methods are available for extraction of microbial community RNA from environmental samples [15,18,19•]. To overcome the low quantity of mRNA obtained, whole community RNA amplification (WCRA) may be used with 50–100 ng of total RNA [20]. RNA with or without amplification is reversely transcribed to cDNA. Finally,

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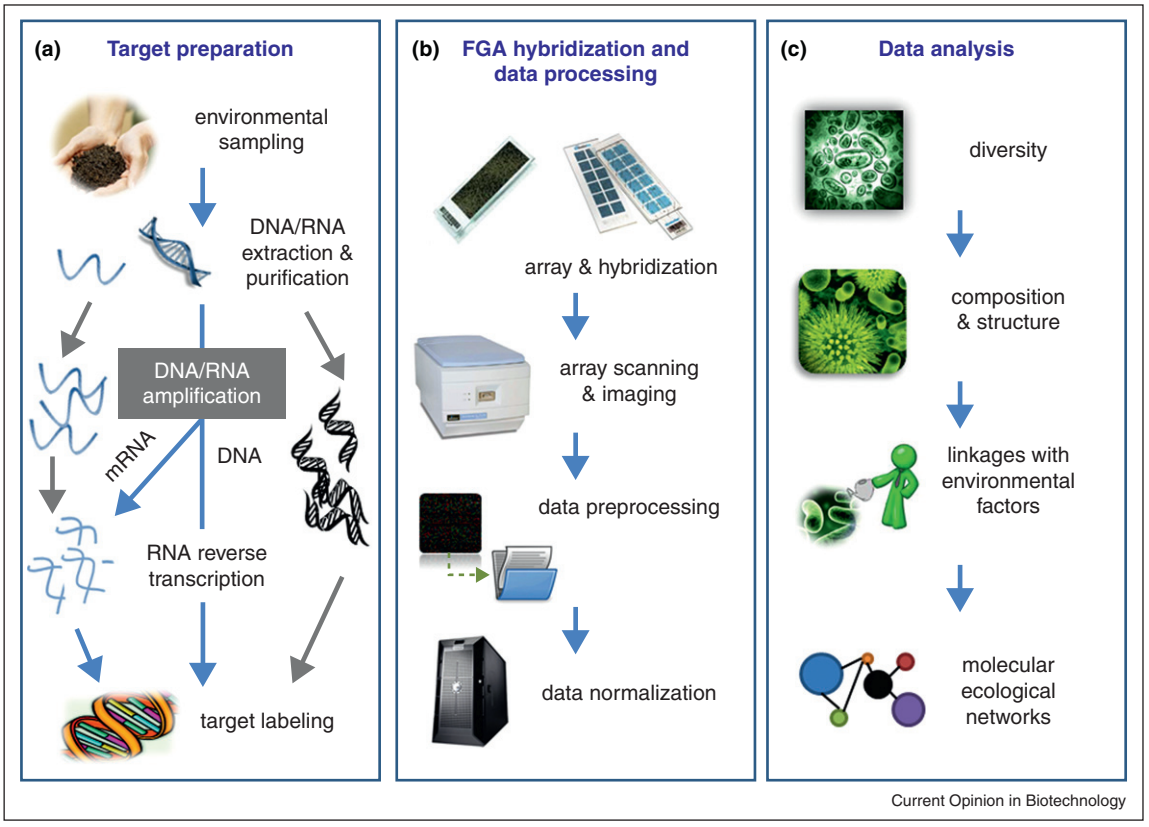
Figure 1



Schematic presentation of FGA applications in analysis of microbial communities from a variety of habitats. **(a)** Target preparation; **(b)** hybridization and data processing; **(c)** data analysis. The DNA/RNA amplification steps highlighted in gray are optional but often used if the amount of RNA or DNA is not enough for GeoChip analysis.

DNA or cDNA is labeled with fluorescent dyes and then purified for hybridization [17,21] (Figure 1a).

Hybridization, imaging, and data processing

Labeled nucleic acid target is hybridized to an FGA at 42–50°C with 40–50% formamide [5,6••,7••,11••,16•,22••]. After hybridization, FGAs are imaged with a microarray scanner with an appropriate resolution (e.g. 5 μm for home-made arrays, 2 μm for NimbleGen arrays). Microarray analysis software is then used to obtain quantitative information based on pixel density for each spot, and spot quality is evaluated. Positive spots are identified, usually using a threshold based on signal-to-noise ratio (SNR) [23] or signal-to-both-standard-deviations ratio (SDDR) [24]. Raw FGA data are further evaluated. For example, GeoChip data are processed via a pipeline [6••], including the quality of individual spots, evenness of control spots, background levels, overall array quality, overlap of replicates, and removal of outliers [6••,24,25]. The signal intensities are then normalized for further statistical analysis (Figure 1b).

Data analysis

FGA data analysis is challenging, and a variety of statistical methods are available. Generally, to eliminate or minimize potential cross-hybridization, relative comparisons are recommended [5,6••]. First, various diversity indices (e.g. richness, evenness, diversity) are calculated based on the number of genes detected and their abundances, and unique and shared genes can be identified among different samples or treatments [6••,17,22••,26]. Second, to discern overall differences in the microbial community composition and structure under different treatments, ANOVA, ANOISM, adonis, clustering analysis (CA), detrended correspondence analysis (DCA), MRPP, and response ratio can be used [5,6••,7••,11••,16•,17,22••,26]. Third, to correlate the microbial community structure with environmental properties, canonical correspondence analysis (CCA), partial CCA, Mantel test, and partial Mantel test are generally used [26–29]. Similarly, the relative influence of environmental properties on the microbial community can be determined using variance partitioning analysis (VPA) [27,30,31]. Finally, FGA data can be used to construct

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Table 1

Summary of representative applications of FGAs for microbial community analyses.

Habitat or system	Ecosystem/sample type	FGA description	Ref
Aquatic systems	Choptank River sediment	N cycling gene array	[10]
	Choptank River water	*nifH* gene array	[33]
	Tap/river water, and tertiary effluent	VMG Biochip with >30 genes	[12]
	Marine microbial mats	*nifH* gene arrays	[36]
	Marine sediment	GeoChip 1.0	[29]
	Ebro and Elbe river sediment	GeoChip 2.0	[35]
	Coral-associated marine water	GeoChip 2.0	[34•]
Soils	Landfill soil	Methanotroph array (*pmoA*)	[9]
	Antarctic latitudinal transect soil	GeoChip 2.0	[28]
	Deciduous forest soil	GeoChip 2.0	[26]
	Native grassland soil	GeoChip 2.0	[39]
	Strawberry farmland soil	GeoChip 2.0	[37]
	Grassland soil	GeoChip 2.0	[40]
	Agricultural soil	E-FGA	[8••]
	Grassland soil	GeoChip 3.0	[22••]
	Citrus rhizosphere soil with	GeoChip 3.0	[38•]
Contaminated sites	U-contaminated underground water	GeoChip 1.0/2.0	[17,27,41]
	U-contaminated sediment	GeoChip 2.0	[31]
	PCB-contaminated soil	GeoChip 2.0	[42]
	Oil-contaminated soil	GeoChip 2.0	[16•]
	Arsenic-contaminated soil	GeoChip 3.0	[43]
	Oil-spill sea water	GeoChip 4.0	[7••,11••]
Extreme environments	Bioleaching system (water)	Bioleaching array	[44]
	Deep sea hydrothermal vent (chimney)	GeoChip 2.0	[45]
	Deep sea basalt samples	GeoChip 2.0	[46]
	GSL hypersaline water	GeoChip 2.0	[47]
	Acid mine drainage (water)	GeoChip 2.0	[48•]
Bioreactors	Fluidized bed reactor for bioremediation	GeoChip 2.0	[49]
	Microbial electrolysis cell for hydrogen production	GeoChip 3.0	[50•]

functional molecular ecological networks for revealing the interaction of microbial functional genes and their associated populations. A recent study showed that elevated CO_2 dramatically altered the network interactions, and the shift in network structure is significantly correlated with soil properties [32••] (Figure 1c).

FGA applications

FGAs have been used to analyze microbial communities from different habitats to address fundamental scientific questions related to global change, bioremediation, land-use change, and ecological theories (Table 1). Some representative studies with GeoChips, the most comprehensive FGAs, are highlighted below.

Aquatic ecosystems

Different FGAs have been used to study water [12,33,34•] and sediment [10,29,35] microbial communities as well as microbial mats [36] in such ecosystems. For example, sediment microbial communities from two pesticide-impacted European rivers were examined with GeoChip 2.0. The results showed that spatial and temporal fluctuations were reflected not only in the abundance of *Dehalococcoides* spp. but also in the diversity and composition of reductive dehalogenase genes, and that the strongest drivers of the functional structure were the C/N ratio, depth, total N, and location [35]. GeoChip 2.0 was also used to analyze the functional diversity, composition and structure of microbial communities associated with healthy and yellow-band diseased (YBD) coral *Montastraea faveolata*, suggesting a general shift in functional potential involving major nutrient cycles, metal transformations, and contaminant degradation [34•].

Soils

Soil may harbor the most complex microbial communities among known habitats, and FGAs have been used to address fundamental ecological questions, such as global change [22••,28], agriculture [8••,37,38•], land use [9,39], plant invasion [40], and ecology theories [26]. GeoChip 2.0 data were used to address ecological theories related to the gene–area relationship for better understanding of spatial scaling in forest soil microbial communities [26]. GeoChip 3.0 was used to examine how elevated CO_2 affected soil microbial communities. The results showed that the functional composition, structure, and metabolic potential of soil microbial communities shifted, which is significantly correlated with soil C and N contents and plant productivity [22••]. GeoChip 3.0 was also applied to profile rhizosphere microbial communities of *Candidatus Liberibacter asiaticus* infected citrus trees. The results showed that the communities shifted away from using

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more easily degraded sources of carbon to more recalcitrant forms, suggesting that the change in plant physiology mediated by '*Ca. L. asiaticus*' infection could elicit shifts in the composition and functional potential of rhizosphere microbial communities [38•].

Contaminated sites

To explore the potential of bioremediation by indigenous microbial communities, FGAs have been used to characterize microbial communities from uranium-contaminated ground waters [17,27,41] and sediments [31], PCB-contaminated and arsenic-contaminated sites [42,43], and oil-contaminated waters [7••,11••] and soils [16•]. GeoChip 3.0 was used to profile microbial communities in response to arsenic contamination in the rhizosphere, and the results showed that rhizosphere microorganisms might play an important role during soil arsenic uptake and hyperaccumulation processes by *Pteris vittata* [43]. Recently, the latest version GeoChip 4.0 was used to analyze oil-contaminated water microbial communities from the oil spill in the Gulf of Mexico, and the results showed that the dispersed hydrocarbon plume stimulated deep-sea indigenous microorganisms, and that the functional composition and structure of oil-contaminated microbial communities were dramatically altered [7••,11••]. Those studies suggest that indigenous microbial communities appeared to be undergoing rapid adaptation and succession in response to environmental contaminants, indicating the potential of bioremediation of contaminated sites.

Extreme environments

FGAs have been applied for analyzing microbial communities from extreme environments, including bioleaching systems [44], deep sea hydrothermal vents [45], deep sea basalts [46], hypersaline lake [47], and acid mine drainage (AMD) [48•]. GeoChip 2.0 analysis of a mature chimney and the inner and outer portions of a five-day-old chimney suggests those microbial communities appear to be undergoing dynamic succession in response to rapid changes in temperature and chemical gradients across the chimney [45]. GeoChip 2.0 was also used to analyze microbial communities from AMD ecosystems, and the results revealed that the functional diversity of AMD microbial communities may be much higher than it was previously thought [48•].

Bioreactors

FGAs have also been used for analysis of microbial communities from controlled environments like bioreactor systems. For example, one system used fluidized bed reactors to perform bioremediation of hydrocarbon-contaminated water [49], and another system used microbial electrolysis cells (MECs) for hydrogen production from wastewater [50•]. GeoChip analyses indicate that genes associated with hydrocarbon degradation, denitrification, and sulfate reduction were detected or stimulated in bioremediation reactors [49], and that the microbial community composition largely affected MEC performance [50•].

Challenges and future directions

Although FGAs have been applied to profile microbial communities from different habitats as shown above, some challenges remain, including nucleic acid quality, call for positive signals, and the detection of active microbial communities. Future directions of FGA applications should focus on addressing those challenges and improving data processing, data analysis, and data interpretation.

Nucleic acid quality

Successful FGA hybridizations require high quality nucleic acids, including: firstly, large DNA fragments or nondegraded RNA as nucleic acid fragmentation decreases amplification efficiency; secondly, fresh DNA or RNA since even short-term storage may result in degradation or fragmentation; and thirdly, no or little inhibitors and contaminants are present in DNA or RNA solution since they may inhibit amplification and labeling enzymes [51] or increase degradation. Although gel purification plus phenol–chloroform–butanol extraction may obtain high quality DNA, relatively large amounts of DNA are necessary due to low recovery. Also, using microbial community mRNA for FGA hybridization is even more challenging due to their low abundance, rapid turnover, instability, and a small proportion (~5%) of the total RNA. Therefore, new DNA/RNA extraction and purification approaches need to be further developed.

Detection of functional activity

Although microbial activity can be reflected in population changes, this may not be accurate [9,20]. To monitor microbial activity, mRNA or other functional markers (e.g. stable isotope probing) should be used [9,20,42]. As discussed above, obtaining enough environmental mRNA is still difficult, resulting in only a limited number of studies for FGA analyses of environmental mRNA [8••,15,20,52]. Also, for FGA analysis, mRNA amplification is generally required. Since current mRNA amplification protocols are tedious, time consuming and potentially biased, more efficient mRNA amplification methods are needed.

Call for positive signals

Distinguishing positive signals from background noise is challenging. On the basis of image processing, SNR is used to call a positive signal, but no thresholds have been established although SNR > 2.0 has been used for microarrays with known targets [23]. For environmental samples, the composition of sequences in the environment is largely unknown. Recently, a new calculation, SDDR has been proposed and evaluated, which shows the lowest percentage of false positives and false negatives, suggesting that it may be a better calculation for

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more accurate determination of thresholds, especially for environmental samples [24]. Various mathematic algorithms [53,54] can be used to denoise images by decreasing both signal and background standard deviations. Therefore, more robust methods that will improve images and distinction of positive spots are needed.

Future directions

FGA-based analysis has revolutionized our understanding of microbial community diversity, composition, structure, functional activity/potential, and population dynamics as well as their linkages with environmental properties and ecosystem functioning. However, to fulfill the potential of FGA applications, challenges in technology, experiment, and data analysis still remain, especially for complex microbial communities. Future directions should focus on the following key issues: first, improving experimental design to minimize inherent variations among array hybridizations; second, increasing replicates to enhance statistical power; third, developing novel algorithms, bioinformatic tools, and knowledge-based databases for imaging, data processing, data analysis, and data interpretation; fourth, integrating FGA data with environmental properties and ecosystem functioning; and fifth, expanding FGA data to molecular ecological network analysis and environmental modeling. Recently, next generation sequencing technologies [1–3] challenge microarray-based technology for microbial community analyses. Metatranscriptomics is able to measure gene expression in microbial communities [55,56]. For complex microbial communities, however, the sequencing-based technology suffers from random sampling, under-sampling, and rRNA interference [26,57,58]. Therefore, it is believed that FGAs will be irreplaceable due to their unique features, such as quick sample preparation, rapid output, quick data analysis, robustness for community comparisons, and resistance to contaminants. It is preferred that both technologies are complementarily used to better address fundamental questions in microbial ecology and environmental microbiology.

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