Obtaining genomes from uncultivated environmental microorganisms using FACS–based single-cell genomics

Christian Rinke[1], Janey Lee[1], Nandita Nath[1], Danielle Goudeau[1], Brian Thompson[2], Nicole Poulton[2], Elizabeth Dmitrieff[2], Rex Malmstrom[1], Ramunas Stepanauskas[2] & Tanja Woyke[1]

[1]Department of Energy (DOE) Joint Genome Institute, Walnut Creek, California, USA. [2]Bigelow Laboratory for Ocean Sciences, East Boothbay, Maine, USA. Correspondence should be addressed to T.W. (twoyke@lbl.gov).

Published online 10 April 2014; doi:10.1038/nprot.2014.067

Single-cell genomics is a powerful tool for exploring the genetic makeup of environmental microorganisms, the vast majority of which are difficult, if not impossible, to cultivate with current approaches. Here we present a comprehensive protocol for obtaining genomes from uncultivated environmental microbes via high-throughput single-cell isolation by FACS. The protocol encompasses the preservation and pretreatment of differing environmental samples, followed by the physical separation, lysis, whole-genome amplification and 16S rRNA–based identification of individual bacterial and archaeal cells. The described procedure can be performed with standard molecular biology equipment and a FACS machine. It takes <12 h of bench time over a 4-d time period, and it generates up to 1 μg of genomic DNA from an individual microbial cell, which is suitable for downstream applications such as PCR amplification and shotgun sequencing. The completeness of the recovered genomes varies, with an average of ~50%.

© 2014 Nature America, Inc. All rights reserved.

INTRODUCTION

The majority of all sequenced bacterial and archaeal genomes belong to only four bacterial phyla, severely skewing our view of microbial genetic diversity[1]. This bias partly results from our inability to cultivate most microbes[2], which is a necessary step for traditional whole-genome sequencing. Through cultivation-independent approaches, namely the assembly and binning of metagenome shotgun sequencing data[3,4] and single-cell genomics (SCG)[5,6], one can now access the genetic makeup of uncultivated microbes. Both methods bypass the conventional cultivation step, and they can be applied directly to environmental samples. Natural populations that have a high degree of genomic heterogeneity will be more accessible through SCG than through metagenomics, as cross-assembly of multiple strains is avoided. The ability to assign genome fragments to a particular single cell makes SCG a powerful tool to explore the genetic diversity and metabolic potential of uncultivated environmental microorganisms.

By applying the protocol described here, we successfully amplified genomes from 201 single cells belonging to 29 mostly uncharted branches of the tree of life[1]. This enabled us to explore their phylogenetic relationships and to discover novel metabolic features of microbial dark matter, groups with no cultivated representatives. The reference genomes generated by SCG also facilitate the interpretation of metagenomic data sets. For example, we demonstrated that single-cell genomes from uncultivated phyla substantially improve the phylogenetic anchoring of metagenomic reads[1]. Mapping of metagenome sequences to single-cell genomes has not only been applied to validating metagenome assembly and binning[7] but also to subsequently improving the single-cell assemblies, as has been shown for SR1 bacteria, atribacteria and ammonia-oxidizing archaea[8–10]. Other studies successfully used fragment recruitment by single-cell genomes to investigate biogeographic distribution of uncultivated, marine taxa[11,12]. Although the bulk of the studies using FACS-enabled SCG have focused on bacteria and archaea, this protocol can also be adapted to microbial eukaryotes[13] and mammalian cells[14].

Several different methods for the isolation of single cells for SCG exist (for a review, see ref. 5), including serial dilution[15], micromanipulation[16], optofluidics (optical tweezing in conjunction with microfluidics[17]) and laser-capture microdissection of tissue samples[18]. Key advantages of optofluidics include the ability to capture the cell morphology and the reduced reaction volumes within the microfluidic chambers[17]. The main disadvantage is the lower throughput compared with FACS. Owing to its high speed and throughput and its ability to separate individual environmental cells on the basis of various cellular properties (e.g., size, fluorescence, granularity), FACS has become the preferred method for single-cell isolation in the context of SCG. The major limitation is the inability to visually inspect a cell and to minimize reaction volumes to nanogram range. FACS has also been successfully applied to populations. A recent protocol describes the use of FACS to enrich uncultivated symbiont populations[19]. The enriched pool of symbiont cells can undergo whole-genome amplification (WGA) followed by sequencing, yielding a population genome assembly or a homogeneous draft assembly in the case of a clonal population.

Here we describe a protocol for obtaining genomes from individual, uncultivated environmental microorganisms by using random isolation of single cells by FACS. The protocol outlines the pretreatment of environmental samples, cryopreservation and the physical separation of individual cells, followed by lysis, WGA of the genomic DNA and 16S rRNA gene–based identification (**Fig. 1**).

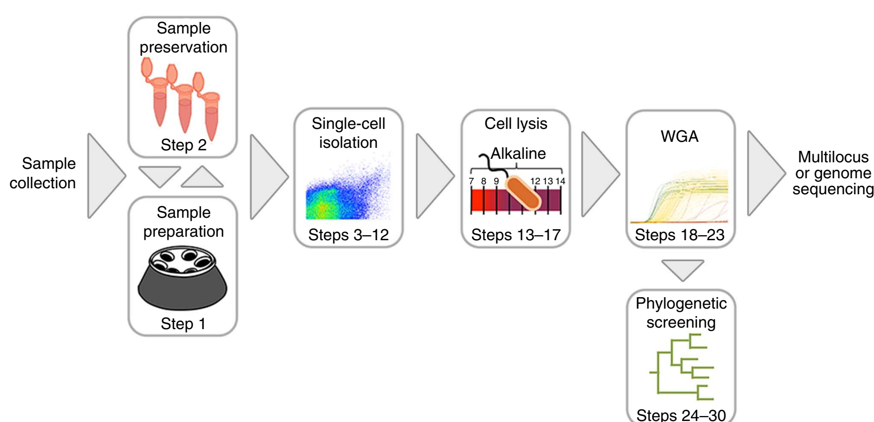

Figure 1 | Single-cell whole-genome amplification workflow. The workflow includes sample preparation (Step 1), sample preservation (betaine, glycerol; Step 2), single-cell isolation (flow cytometry; Steps 3–12), cell lysis (alkaline solution; Steps 13–17), WGA (MDA; Steps 18–23) and phylogenetic screening (16S rRNA or other marker genes; Steps 24–30). Specific amplified genomes then undergo multilocus or shotgun genome sequencing. Please note that only a small aliquot of DNA generated in the WGA is used for the phylogenetic screening and that phylogenetic screening is optional.

Experimental design

Sample preservation and preparation. If the environmental samples are not used immediately after collection, storage is required to preserve the integrity of the cell and its DNA. We recommend flash-freezing the samples in liquid nitrogen with a cryopreservant such as betaine or glycerol, which does not interfere with the downstream cell separation, lysis and DNA amplification steps[1,11,20]. Precipitant-free, aquatic samples can be directly mixed with the cryopreservant. Nonaquatic samples, such as sediments, soil, fecal samples, biofilms and sludge, may require additional sample preparation to disaggregate the cells and remove noncellular particles. It is generally advisable to minimize the length of sample handling before cryopreservation and to limit changes in ionic strength, pH and temperature. For sediments, soil and fecal samples, we recommend vortexing with a sterile, isotonic buffer, followed by brief centrifugation (Step 1A). Repeated pipetting through a syringe may be used to disperse cells comprising some microbial mats. Dense biofilm samples may require a more extensive mechanical disaggregation to make the sample suitable for flow cytometry (Step 1B). Sludge samples benefit from a centrifugation step and subsequent manual grinding to make the biomass accessible (Step 1C). The volumes given below can be scaled up or down according to the amount of biomass present in the sample. Ideally, preparation should be performed on fresh samples, followed by immediate cryopreservation; however, postprocessing of cryopreserved samples is also possible.

Single-cell separation. FACS is the most commonly used high-throughput approach to separate the sample into single cells[6]. Microbial cells can be identified and recovered by FACS on the basis of their DNA content (fluorescence) and scatter signals, which are related to cell size and granularity. Only a few picoliters of the original sample are sorted with the cell, minimizing the risk of contamination from extracellular DNA. The wide variety of cell sorters, operational parameters and environmental samples precludes a single detailed protocol for cell sorting. The protocol below outlines general tips for sorting cells from environmental samples, and it includes details of a few suitable automated liquid-handling systems.

Automation. Approximately half of the single cells isolated for the study reported in ref. 1 were generated by the manual process described in the protocol, which is scalable to higher throughput because of the plate format and is more straightforward to set up than an automated liquid-handling system. Automated liquid-handling systems are, however, suitable for consistent high-throughput SCG applications, and they allow a streamlined and more reliable workflow. We have included a selection of three systems (see Equipment). As scripts will be very specific to each model and configuration and will require customized solutions, we do not include them here.

Box 1 | Volume reduction

Reducing the reaction volumes lowers the risk of reagent-based contamination, as the ratio of single-cell DNA to contaminating DNA is increased. Reduced 2-µl MDA reactions can be performed with the Echo 550 Omnics2 (Labcyte) liquid transfer system in a high-throughput manner, as shown here. This tipless instrument allows acoustic dispensing of minute volumes and rapid setup of low-volume MDA reactions, while limiting the opportunity for introduction of DNA contamination and reducing tip waste. Reducing the reaction volumes also decreases the costs of MDA reagents. At the DOE Joint Genome Institute, we apply the REPLI-g Single Cell reagents (Qiagen, cat. no. 150345) for our low-volume MDA reactions performed using the Echo, as they do not require additional UV decontamination, thus simplifying the workflow.

© 2014 Nature America, Inc. All rights reserved.
npg

© 2014 Nature America, Inc. All rights reserved.

Cell lysis. The purpose of the lysis step is to degrade the microbial cell envelope without damaging the chromosomal DNA, introducing contamination or inhibiting subsequent amplification reactions. The majority of single-cell studies rely on an alkaline lysis procedure first described by Raghunathan *et al.*[21], although alternative or supplementary treatments such as heat, freeze-thaw, detergents and treatment with hydrolytic enzymes have been used[6]. However, these lysis techniques are not universally effective on all microbes, and thus a substantial fraction of genomes may not be recovered from some environmental samples. We apply the alkaline lysis method in the subsequent procedure.

WGA. The most commonly used approach to amplify a whole genome is multiple displacement amplification (MDA). This method was first introduced by Dean *et al.*[22], and it relies on the Phi29 polymerase from *Bacillus* bacteriophage to amplify femtogram levels of DNA. This isothermal, strand-displacing amplification yields, on average, >10-kb-long overlapping amplicons, which are suitable for whole-genome sequencing and *de novo* assembly, similarly to sequence data from DNA extracts of pure cultures. However, MDA results in uneven genome coverage that can be partially mitigated by wet-bench and bioinformatic normalization methods[1,20,23,24]. Genomic rearrangements, or chimeras, are formed during MDA and can complicate genome assembly by linking noncontiguous chromosomal regions[15,25]. The effect of these rearrangements can be limited by high sequence coverage and by avoiding long mate-pair libraries[24]. The remaining challenges are the limited genome recovery—on average, approximately 40–55% of a single-cell genome is recovered[1,11]—and the overall low efficiency of accessing the genomic DNA within single cells, which is in part linked to the inability to successfully lyse cells. The lysis and MDA success rates vary widely among environmental samples, ranging from <10 to 100% (refs. 1,11,16).

Contamination. MDA reactions are sensitive to DNA contamination present in the sample, reagents or contamination introduced through sample handling. The effect of DNA contamination can be controlled by establishing clean cell-sorting procedures[12,20,24,26], decontaminating commercially available MDA reagents[1,20,23], expressing a custom ultrapure Phi29 enzyme[27] or by using commercial precleaned MDA kits that are specifically designed for single-cell templates. Volume reduction can also limit the impact of reagent contamination (**Box 1**). To minimize lab-introduced contamination, sorted single-cell plates should never be opened outside a clean PCR hood. To assess the degree of potential contamination, the inclusion of several plate rows of negative controls is highly recommended (Step 11).

Phylogenetic screening. Target genomes are typically identified after MDA by PCR amplification, sequencing and phylogenetic analysis of marker genes, e.g., 16S rRNA genes. We successfully used the following 'universal pyrotag' primer set—forward 926wF (5′-AAACTYAAAKGAATTGRCGG3′) and reverse 1392R (5′-ACGGGCGGTGTGTRC3′)—for archaea and bacteria (**Table 1**).

TABLE 1 | Optional primer sets for phylogenetic screening of single-cell MDA products.

Description	Name	Sequence (5′→3′)	Refs.
Bacterial full length	27F-YM	AGAGTTTGATYMTGGCTCAG	33,34
	1492R	TACGGYTACCTTGTTACGACTT	35
Universal i-tag	515F	GTGCCAGCMGCCGCGGTAA	36
(targeting V4 region)	806R	GGACTACHVGGGTWTCTAAT	36
Universal pyrotag	926wF	AAACTYAAAKGAATTGRCGG	37,38
(targeting V6-8 regions)	1392R	ACGGGCGGTGTGTRC	39,40
Bacterial	27F	AGRGTTYGATYMTGGCTCAG	41
	907R	CCGTCAATTCMTTTRAGTTT	35,39
Archaeal	Arc344F	ACGGGGYGCAGCAGGCGCGA	42,43
	Arc915R	GTGCTCCCCCGCCAATTCCT	44
Eukarya	Euk528F	CCGCGGTAATTCCAGCTC	45
	EukB	GATCCTTCTGCAGGTTCACCTAC	46

'Universal' refers to primers targeting bacteria and archaea; for all nonuniversal primers the domain bacteria, archaea or eukarya is specified.

Short 16S rRNA gene amplicons, resulting from the primer set mentioned above or from 'universal i-tag' primers listed in **Table 1**, provide, in our experience, sufficient resolution to assign a taxonomic classification at the phylum level. For taxonomic assignments below the phylum level, full-length 16S rRNA gene amplicons (generated, for example, by the primer pair 27F-YM and 1492R (**Table 1**)) are more suitable. However, the high rRNA gene diversity among microbial genomes makes it challenging to design truly universal primers that are suitable for the entire range of diverse microbial taxa. Please note that the purpose of the phylogenetic screening is to determine the taxonomic classification of the single amplified genomes (SAGs). This will allow single cells of interest to be selected for further downstream processing, such as multilocus or shotgun sequencing. Only a small aliquot of DNA generated in the WGA is used for the phylogenetic screening, and phylogenetic screening is optional.

MATERIALS

REAGENTS

Sample preparation and preservation

- Sample (for example, sediment, soil, fecal material, biofilm or granular sludge)
- (Optional) Sterile, filtered buffer solution, such as 1× PBS (Calbiochem, OmniPur, cat. no. 6505)
- Betaine: betaine anhydrous (Fisher, cat. no. AC20424-1000; see Reagent Setup)
- TE, 100×, pH 8.0 (e.g., Fluka, cat. no. 86377; see Reagent Setup)
- Milli-Q water (Millipore)
- Molecular-grade glycerol (Acros, cat. no. 15892-0010; see Reagent Setup)
- Sterile UV-treated seawater (sterile seawater; Sigma, cat. no. S9148-1L)

Cell separation with a FACS

- Ultrapure water, such as Milli-Q water (Millipore), or filtered molecular biology–grade water (Fisher, cat. no. BP2810)
- Household bleach, a 3–8% (wt/vol) solution of sodium hypochlorite (Clorox; see Reagent Setup)
- PBS liquid concentrate, 10×, 4 liters, sterile (OmniPur, cat. no. 6505-4L; see Reagent Setup)
- NaCl (Sigma-Aldrich, cat. no. 71386; see Reagent Setup)
- SYBR Green fluorescent nucleic acid stain (e.g., Invitrogen SYBR Green nucleic acid gel stain; Invitrogen, cat. no. S-7585)

Single-cell lysis and whole-genome amplification by MDA

- RepliPHI Phi29 reagent set 0.1 µg/µl (Epicentre, cat. no. RH040210): it contains Phi29 DNA polymerase (100 U/µl), Phi29 10× reaction buffer, dNTP solution (25 mM each dATP, dCTP, dGTP and dTTP) and DTT, 100 mM (not used in this protocol) ▲ CRITICAL Phi29 enzyme performance can vary markedly between vendors and even between lots of the same vendor. Thus, we recommend testing the performance of each new lot.
- Buffer DLB (Qiagen, cat. no. 1031206; see Reagent Setup) ! CAUTION It contains potassium hydroxide, and it is corrosive and harmful. Avoid skin contact, eye contact and ingestion.
- STOP solution (Qiagen, cat. no. 1032393)
- Nuclease-free water (Fisher Scientific, cat. no. BP2484-100)
- Random hexamers, 50-µM (Integrated DNA Technologies (IDT); see Reagent Setup)
- DMSO (Sigma-Aldrich, cat. no. D8418-100ML)
- SYTO 13, 5 mM (Invitrogen, cat. no. S7575)
- DTT, 1 M (Sigma-Aldrich, cat. no. 646563-10X.5ML; see Reagent Setup)
- Household bleach (3–8% (wt/vol) solution of sodium hypochlorite)

Phylogenetic screening

- Ultrapure water, such as Milli-Q water (Millipore) or other filtered and deionized water (DIW)
- SsoAdvanced SYBR Green Supermix (Bio-Rad, cat. no. 172-5264)
- 926wF primer, 10 µM (5′-GAAACTYAAAKGAATTGRCGG-3′; IDT)
- 1392R primer, 10 µM (5′-ACGGGCGGTGTGTRC-3′; IDT)
- ExoSAP-IT (Affymetrix, cat. no. 78201)

EQUIPMENT

Sample preparation and preservation

- Microcentrifuge tubes, 2 ml (e.g., Eppendorf Safe-Lock tubes 2.0 ml, clear; Eppendorf, cat. no. 0030 120.094)
- Microcentrifuge (e.g., Eppendorf 5424 ventilated microcentrifuge; Eppendorf, cat. no. 5424 000.410)
- Vortex (VWR Scientific, Vortex Genie 2)
- Centrifuge (Eppendorf, cat. no. 5810R)
- Standard light microscope (Zeiss)
- Sterile cotton tip (Fisher Scientific, Fisherbrand cotton-tipped applicators, cat. no. 23-400-114)
- Ultrasonic water bath (e.g., Spectralab ultrasonic cleaning bath; Spectralab Instruments, cat. no. UCB-30D)
- Falcon tube, 50 ml (Thermo Scientific, cat. no. 362697)
- Glass beads, 2 mm diameter (e.g., solid-glass beads, borosilicate, diameter 2 mm; Sigma-Aldrich, cat. no. Z273627-1EA)
- Filter with a 40-µm pore size (e.g., BD Falcon cell strainer 40 µm; BD Biosciences, cat. no. 352340)
- Filter, 0.2 µm (e.g., Millipore Millex-FG syringe filter unit, 0.2 µm; Millipore, cat. no. SLFG025LS)
- Flask, 250 ml (Pyrex, cat. no. 4980)

Single-cell collection via FACS

- Cell sorter: we use the Influx (BD Biosciences) or MoFlo (Beckman Coulter) with a 70-nm nozzle and 488-nm excitation laser to detect and sort prokaryotic cells labeled with the DNA stain SYBR green. However, cells could be sorted by using a variety of stains and cell sorters
- Clean the PCR hood with UV light for decontaminating sheath fluid, sheath tanks and collection tubes (e.g., Labconco, cat. no. 3970302) ! CAUTION Avoid direct UV exposure to skin or eyes. Always wear appropriate personal protective equipment when operating.
- Two 2-liter quartz flasks for UV treatment of sheath fluid
- Two stir plates and stir bars for sheath fluid UV treatment
- BD Falcon 40-µm nylon cell strainer (BD Biosciences, cat. no. 352340)
- Polypropylene round-bottom tubes, 5 ml: BD Falcon 12 × 75-mm style, disposable tubes (BD Biosciences, cat. no. 352063)
- Pall Acrodisc, 32-mm syringe filter with 0.1-µm Supor membrane (Pall, cat. no. 4651)
- BD Luer-Lok tip disposable syringe, 10 ml (BD Biosciences, cat. no. 309604)
- Optical micro-well plates to receive sorted single cells (e.g., LightCycler multiwell plate 384; Roche, cat. no. 05102430001)

Single-cell lysis and whole-genome amplification by MDA

- Spectraline XL-1500 UV cross-linker (Fisher Scientific, cat. no. 11-992-90) ! CAUTION Avoid direct UV exposure to skin or eyes. Always wear appropriate personal protective equipment.
- Clean the PCR hood with UV light for decontaminating work surfaces (e.g., Labconco, cat. no. 3970302) ! CAUTION Avoid direct UV exposure to skin or eyes. Always wear appropriate personal protective equipment.
- Plate reader with temperature control (e.g., BMG Labtech FLUOstar Omega) or a real-time thermocycler (e.g., Roche LightCycler 480; Roche, cat. no. 05015243001)
- (Optional) Robotic liquid handler, such as Bravo (Agilent Technologies), Freedom EVO (Tecan), Echo (Labcyte) or similar ▲ CRITICAL Every step can be performed with single-channel manual pipettes; however, multichannel pipettes or robotic liquid handlers are recommended especially when working with 96- or 384-well plates.
- Eppendorf Safe-Lock tubes, 1.5 ml (Eppendorf, cat. no. 0030 120.086) ▲ CRITICAL UV treatment must always occur with the reagent in the Eppendorf Safe-Lock tubes.
- Gamma-irradiated 5-ml conical tubes (Daigger, cat. no. EF3159F) ▲ CRITICAL If you are working with large volumes owing to a large number of reactions, these sterile 5-ml tubes may be used instead of the Eppendorf Safe-Lock 1.5-ml tubes for handling reagents.
- EMD colorpHast pH strips (Fisher Scientific, cat. no. M95903)
- Quartz plate (e.g., 210-mm round dishes; Quartz Scientific)
- Aluminum foil
- Ice box, ice packs, foil-lined container (e.g., pipette-tip box lid)

Phylogenetic screening

- Standard thermocycler or a real-time thermocycler (e.g., Roche LightCycler 480; Roche, cat. no. 05015243001)
- Plate shaker (e.g., MTS 2/4 digital shaker; IKA, cat. no. 0003208001)
- Optical microtiter plate (e.g., LightCycler Multiwell Plate 384; Roche, cat. no. 05102430001)

REAGENT SETUP

Betaine stock, ~38% (wt/vol) Dissolve 48 g of betaine in 80 ml of DIW. Bring the volume up to 125 ml with DIW. Pass the solution through a 0.1-µm filter. Store it refrigerated at 4 °C for up to 1 year and re-filter it every month.

GlyTE Make the GlyTE stock in a 250-ml flask by adding 20 ml of 100× TE (pH 8.0), 60 ml of DIW and 100 ml of molecular-grade glycerol (glycerol is best transferred with a syringe). Pass it through a 0.1-µm filter. Store the stock at −20 °C for up to 1 year.

PBS buffer, 1× Dilute 10× sterile PBS solution to a 1:10 ratio in ultrapure water, such as Milli-Q water. Prepare 4 liters of 1× PBS, 2 liters each in 2-liter flasks. Freshly prepare the buffer before every use.

NaCl solution, 15 p.p.t. Dissolve 105 g of combusted NaCl in 500 ml of UV-treated ultrapure water, and then filter the hypersaline solution through a 0.1-µm filter and dilute it to 7 liters with UV-treated ultrapure water. Freshly prepare the solution before every use. ▲ CRITICAL PBS buffer (1×) and 15 p.p.t. NaCl solution are used with freshwater and marine samples, respectively.

npg © 2014 Nature America, Inc. All rights reserved.

Bleach solution, 10% (wt/vol) Dilute household bleach at a 1:10 ratio in ultrapure water, such as Milli-Q water (0.3–0.8% (wt/vol) sodium hypochlorite, final concentration). Freshly make 1 liter of bleach solution before every use.

SCG-grade water Pour 75 ml of nuclease-free water into a quartz dish bottom. Replace the quartz dish cover and place the dish into a tray lined with aluminum foil (to increase reflectivity). UV-irradiate it in a Spectraline XL-1500 UV cross-linker for 16 h, and then aliquot it into UV-treated 1.5-ml Eppendorf Safe-Lock tubes for single use only. This SCG-grade water can be stored at −20 °C indefinitely.

Lysis buffer D2 To lyophilized buffer DLB (Qiagen), add 500 µl of SCG-grade water and 45.5 µl of 1 M DTT. Mix it well by vortexing. With pH strips, check for a correct pH of 14. Lysis buffer D2 can be stored at −20 °C for 6 months.

Random hexamers, 0.5 mM Use the IDT website to order 10 µmol of 'random hexamers'. Select the 'standard desalting' and 'hand-mix randomization' parameters. Hexamers should have phosphorothioate bonds between the last two nucleotides at the 3′ end (5′-NNNN*N*N-3′). Resuspend them in SCG-grade water to 500 µM and aliquot into UV-treated 1.5-ml tubes for single use only. Aliquots can be stored at −20 °C for 1 year.
▲ **CRITICAL** Hexamer quality can vary markedly between vendors and even between lots of the same vendor. Thus, we recommend testing the performance of each new lot.

DTT solution, 1 M Owing to the negative effects of oxidation over time, 1 M DTT should be transferred into UV-treated 1.5-ml tubes for single use only. If you are making dilutions of 1 M DTT, make them with SCG-grade water. DTT solution can be stored at −20 °C for 6 months.

PROCEDURE

Sample collection, preparation and preservation ● TIMING up to 1 h 10 min

1| Sample-processing procedures vary depending on the type of the sample. Process the samples as follows for a sediment, soil or fecal sample (option A), a biofilm sample (option B)[1] or a granular sludge sample (option C)[1].

(A) Sediment, soil or fecal sample ● TIMING 10 min

(i) Mix ~5 g of sample with 10–30 ml of sterile buffer in a 50-ml tube. For soil samples, fecal samples and freshwater sediments, use 1× PBS as the buffer. For marine sediments, use sterile-filtered UV-treated seawater.
(ii) Vortex the sample for 30 s at 16,000*g* or highest setting.
(iii) Centrifuge the sample for 30 s at 2,500*g* at room temperature (25 °C) to remove large particles.
(iv) Collect the supernatant.

(B) Biofilm sample ● TIMING 30 min

(i) Scratch the biomass from the solid medium by using a cotton stick, and collect it into microcentrifuge tubes containing a sterile-filtered, isotonic buffer solution, such as 1× PBS or seawater.
(ii) Sonicate the tube with the collected biofilm in an ultrasonic water bath by floating the tube for 10 min at the default setting at room temperature.
(iii) Shake the tube by hand for another 5 min.
(iv) Examine the sample under a microscope to observe the effectiveness of dispersion. If necessary, repeat Step 1B(ii,iii).

(C) Granular sludge sample ● TIMING 1 h

(i) Sample about 5–40 ml of liquid sludge. Transfer it into a 50-ml Falcon tube, and fill it up to a 50-ml volume with a sterile-filtered, isotonic buffer solution, such as 1× PBS or seawater.
(ii) Centrifuge the sample to form a pellet (e.g., 15 min at 16,000*g*).
(iii) Remove most of the supernatant to reduce the total liquid volume to 15 ml in the 50-ml Falcon tube.
(iv) Add 0.1 g of 2-mm-diameter glass beads into the tube and shear the pellet by hand mixing for 10 min.
(v) Let the tube stand for 3 min, and then collect the upper half of the suspension and transfer it into a new tube.
(vi) Remove the glass beads by filtration through a 40-µm gauze filter.
(vii) Examine the sample under a microscope to observe the effectiveness of dispersion. If necessary, repeat Step 1C(ii–v). An alternative to manual grinding is the dispersal of the sludge samples by ultrasonic treatment, for example, with the Sonifier II model 150 (Branson), as shown in Miyauchi *et al.*[28].

2| Cryopreserve the processed samples containing cells in betaine (option A) or glycerol (option B).

(A) Betaine stock ● TIMING 10 min

(i) Transfer 200 µl of betaine stock and 1 ml of unfiltered sample to a sterile cryovial (resulting in an ~6% (wt/vol) betaine solution).
(ii) Mix the vial gently and incubate it for 1 min at room temperature.
(iii) Store the sample mixture in liquid nitrogen or at −80 °C.
(iv) Prepare several replicate vials for each sample. This method was found to work well on most samples; however, hypersaline samples yielded a low number of preserved cells.

(B) GlyTE stock ● TIMING 10 min

(i) Transfer 100 µl of GlyTE stock and 1 ml of sample to a sterile cryovial.
(ii) Mix the vial gently and incubate it for 1 min at room temperature.
(iii) Store the sample mixture in liquid nitrogen or at −80 °C.

© 2014 Nature America, Inc. All rights reserved.

npg

(iv) Prepare several replicate vials for each sample. This method was found to work well for marine and freshwater samples.
■ **PAUSE POINT** Betaine and GlyTE stocks can be stored at –80 °C for several months.

Preparation of FACS for sterile sort ● TIMING 1 d

3| Prepare 4 liters of 1× PBS or 15 p.p.t. NaCl solution in two 2-liter quartz flasks and begin stirring with magnetic stir bars and plates within the clean hood. Position the empty sheath fluid tank and inverted lid inside the hood so that UV will shine on the inner surfaces. Close the hood and start the overnight UV exposure. We suggest dedicating one tank to clean sheath fluid. After UV exposure, carefully add sheath fluid to the tank while it is still inside the clean hood. Save at least 10 ml of clean sheath fluid for later use while sorting.
▲ **CRITICAL STEP** Clean cell-sorter and sheath fluid are essential for successful single-cell sorting and MDA. Fluidic lines on the cell sorter can be decontaminated by running bleach through the instrument, whereas sheath fluid can be decontaminated by UV treatment. We have found that the instrument remains clean for at least 3 d after decontamination, as long as clean sheath fluid is used. We also find that this procedure is suitable for decontaminating the same fluidic lines repeatedly for more than one use, and it is not necessary to replace fluidic lines between clean sorting runs.

4| Fill a second sheath tank with 1 liter of 10% (wt/vol) bleach (0.3–0.8% (wt/vol) sodium hypochlorite, final concentration) and run it through the cell sorter for 2 h to decontaminate fluidic tubing.

5| Dispose of any remaining bleach and rinse the sheath tank with sterile water. Run 1 liter of sterile water through the cell sorter for 30 min to rinse the fluidic tubing.

Cell separation by flow cytometry ● TIMING 1 d

6| Install the tank with clean sheath fluid and begin running the sort. Follow the procedure specified in your FACS manual to center the stream, to adjust the laser and detectors and to adjust and calculate drop delay.

7| Before sorting, UV-treat the microtiter plates openly (without a cover) for 10 min. To each well, add 1 µl of UV-treated, 1× TE buffer per well. UV-treat the plates again, this time with a cover, for another 10 min.
▲ **CRITICAL STEP** We recently found that the TE buffer can be omitted and that sorting in dry plates does not affect the downstream application. When you are performing a 'dry sort', the volume of sterile water in the WGA master mix needs to be increased by 1 µl per well to keep the overall volume constant.

8| To avoid clogging the nozzle with any kind of aggregates, filter each sample through an appropriate filter (e.g., 40-µm nylon mesh when using a 70-µm nozzle).

9| Stain the cells with 1× SYBR Green fluorescent nucleic acid stain for 15 min in the dark at 4 °C. SYBR Green is supplied at 10,000× concentration, and we use it at a 1× final concentration, which amounts to a 10,000-fold dilution.
▲ **CRITICAL STEP** A wide variety of nucleic acid stains could be used at this step, and some work better than others for particular samples.

10| Run the stained sample and target your desired microbial population with a sort gate (**Fig. 2**).
▲ **CRITICAL STEP** It can be challenging to find the target population in some environmental samples. For example, samples with high cell density (>10^6 cells per ml) and low background signal show a distinct microbial population (**Fig. 2b**), whereas sediment samples generally display a less-well-defined target (**Fig. 2d**,**e**). It may be helpful to compare stained and unstained samples when you are identifying target populations. Sample dilution may also be required (ensure that the diluent, such as clean sheath fluid, is UV-treated).
? TROUBLESHOOTING

11| Sort the target cells into the UV-treated microtiter plates containing 1 µl of 1× TE buffer per well. We recommend sorting the cells according to **Figure 3**, which includes four columns of negative controls (no template). To avoid co-sorting of >1 cell, use stringent sort mask and a low event rate.
▲ **CRITICAL STEP** For samples with an expected high concentration of extracellular DNA or nontarget events, a two-step sort may be used to dilute this extracellular DNA. First, sort at least 10,000 target cells into 1 ml of UV-treated 1× PBS. Next, back-flush the sample line for 1 min with sheath fluid, run a 10% (wt/vol) bleach solution through the sample line for 5 min and back-flush it again for 5 min. After this, re-stain the sorted cells with 1× SYTO and use as a source sample for FACS deposition into microtiter plates.

© 2014 Nature America, Inc. All rights reserved.

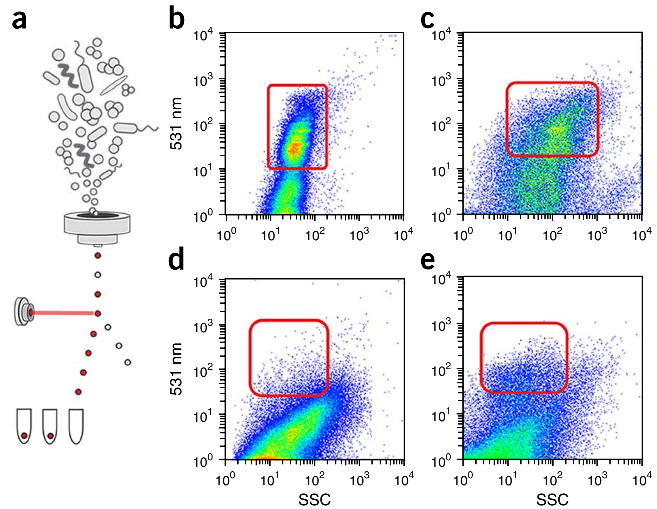

Figure 2 | High-throughput single-cell sorting. (**a**) Schematic drawing of the fluorescence-activated cell sorter. The environmental sample is arranged in a thin stream that moves one single cell at a time in front of the laser and detector, and the cells of interest are subsequently deflected into wells of a microtiter plate in a random manner, whereas the unwanted portion of the sample goes to waste. (**b**–**e**) Flow cytometry signals and sort windows (red outlines). (**b**) Granular sludge from a bioreactor, prepared as described in Step 1C. (**c**) Biofilm sample, prepared as described in Step 1B. (**d**,**e**) Marine sediment sample (**d**) and freshwater sediment sample (**e**), both prepared as described in Step 1A. The *x* axis shows the side scatter (SSC), and the *y* axis shows the relative green fluorescence of SYBR Green–stained samples at a 531-nm wavelength.

12| Cover the plates with sterile foil and store them at −80 °C. The plates should never be opened outside of a clean PCR hood before completing MDA.
■ **PAUSE POINT** Sorted cells can be stored at −80 °C for several months.

Single-cell lysis ● TIMING 2 h

13| Before performing any work, wipe down all clean hood surfaces, pipettes and equipment with 10% (wt/vol) bleach. UV-treat the clean hood for 60 min with equipment inside.
▲ **CRITICAL STEP** It is essential to prepare as sterile an environment as possible when you are performing single-cell amplification. UV treatment of all equipment and disposables are recommended, followed by wiping with 10% (wt/vol) bleach, if applicable. It is recommended to have a dedicated area reserved for single-cell work only, in a separate location free of DNA, with separate equipment.

14| Thaw lysis buffer D2 and STOP buffer (as prepared in Reagent Setup). The amount of lysis buffer added for each reaction should be calculated as follows: the cell in TE and the lysis buffer should be at a 1:1 ratio. At the same time, the final concentration of lysis buffer in the whole MDA reaction should be approximately 6–7% (vol/vol). The same amount of STOP buffer should also be added. For this example, a single cell was sorted into 1 µl of TE. A volume of 1 µl of lysis buffer should be added to obtain a final concentration of 6.7% in an MDA reaction of 15 µl. Calculate how much is needed based on the number of reactions, and then transfer the appropriate volume of both buffers to separate 1.5-ml Eppendorf Safe-Lock microcentrifuge tubes.
▲ **CRITICAL STEP** The following protocol describes performing MDA in 15-µl reactions. These reaction volumes may be adjusted up or down according to the parameters or requirements of individual experiments.
▲ **CRITICAL STEP** Another important consideration is that the larger the well that the single cell has been sorted into, the larger the reaction volume must be for the lysis buffer to completely cover the bottom of the well and to ensure a complete lysis of the cell.

15| Place the tubes of lysis and STOP buffers in a small, foil-lined container (e.g., a pipette tip-box lid). UV-treat them in the Spectraline XL-1500 UV cross-linker for 60 min.

16| To each well (of the plates from Step 12) containing a sorted single cell, add 1 µl of lysis buffer D2. Spin down the plate at 1,000*g* for 1 min and incubate them for 5 min at room temperature.

17| Add 1 µl of STOP buffer to each well. Spin down the plate at 1,000*g* for 1 min. If you are not adding MDA master mix immediately, store the lysed cells at 4 °C for no more than 1 h.

Whole-genome amplification ● TIMING 20 h (overnight)

18| Under a clean PCR hood, thaw the following reagents for making the MDA master mix: Phi29 10× reaction buffer, 25 mM dNTP solution, 0.5 mM hexamers, 1 M DTT, DMSO, SYTO 13 and SCG-grade water. Do not thaw Phi29 DNA polymerase.

19| Gently mix all reagents by vortexing and spinning them down in a microcentrifuge. Each 15 µl of MDA reaction contains 0.4 µl of Phi29 enzyme, 1.5 µl of Phi29 10× reaction buffer, 0.24 µl of dNTP solution (25 mM), 1.5 µl of hexamers (0.5 mM), 0.15 µl of DTT (1 M), 0.75 µl of DMSO, 0.0015 µl of SYTO 13 and 7.46 µl of SCG-grade water. Prepare enough master mix for all reactions, adding Phi29 DNA polymerase at the end. Mix by vortexing, and prepare aliquots of the master mix in 1.5-ml

© 2014 Nature America, Inc. All rights reserved.
npg

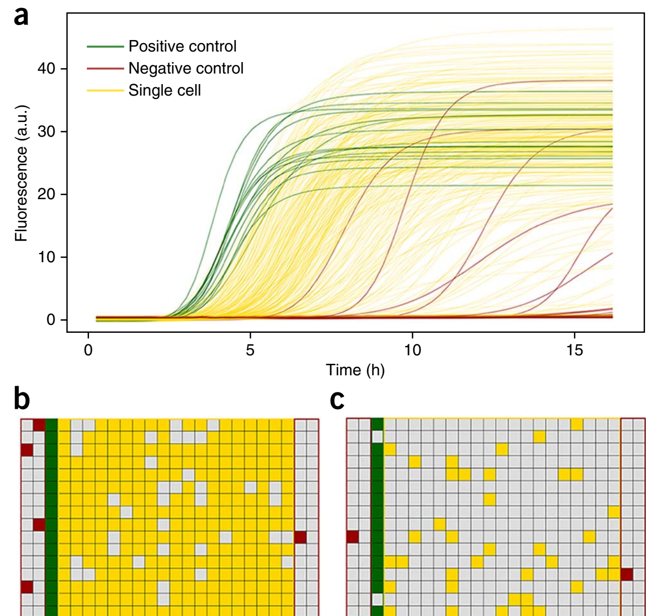

Figure 3 | Real-time whole-genome amplification and 16S rRNA gene PCR. (**a**) MDA kinetics for a 384-well plate of sorted single cells with positive (100 cells per well) and negative (no-template) controls. (**b**) Plate layout for MDA showing wells with successful amplification (success is measured by a fluorescence value above 10 arbitrary units (a.u.) in this case). (**c**) Plate layout for real-time quantitative PCR (qPCR) showing wells with successful 16S rRNA gene amplification. Gray wells show no amplification. Green, positive controls (100 cells); red, no-template controls; yellow, single cells.

Eppendorf Safe-Lock microcentrifuge tubes (with a maximum aliquot volume of 1 ml). See **Box 1** for information about how to carry out lower-volume MDA reactions.
▲ **CRITICAL STEP** Do not add SYTO 13 to the master mix at this point, as UV irradiation will degrade the dye.
▲ **CRITICAL STEP** Do not let the MDA master mix warm to a temperature above 30 °C. The Phi29 enzyme becomes inactivated at temperatures above 30 °C.

20| UV-treat the tubes of master mix in a reflective container on ice for 30–90 min in the Spectraline UV cross-linker, as described previously[23]. Arrange the tubes to rest ~8 cm below the UV bulb.
▲ **CRITICAL STEP** Because UV irradiation will raise the temperature within the UV cross-linker, it is important to UV-treat the MDA master mix on ice to avoid Phi29 enzyme deactivation.

21| After UV treatment, add SYTO 13 (0.0015 µl per reaction) to the master mix to obtain a final concentration of 0.5 µM. Vortex and spin down the mixture.

22| To the lysed cells from Step 17, add the 12 µl of MDA master mix to each well. Cover the plate with an optical seal and spin it down at 1,000*g* for 1 min. Incubate the plate at 30 °C for 16 h in the Roche LightCycler 480 or similar real-time thermocycler or plate reader. **Figure 3a**,**b** exemplifies the MDA kinetics and plate layout for a 384-well plate.
? TROUBLESHOOTING

23| Heat-inactivate the Phi29 enzyme by incubating the completed MDA reaction at 65 °C for 10 min.
▲ **CRITICAL STEP** Although previous reports suggest debranching of the MDA products with S1 nuclease before sequencing[15], we do not recommend this step. Our previous results suggest that S1 debranching has no effect on the chimera rate[12], and we routinely sequence and phylogenetically screen single-cell MDA products without debranching.
■ **PAUSE POINT** The amplified DNA is ready for phylogenetic screening and sequencing (Steps 24–30), and it can be stored for several months at −80 °C. However, freezing the MDA product results in a nonhomogenized DNA product upon thawing, and thus we suggest moving directly to phylogenetic screening when possible. Please note that the purpose of the phylogenetic screening is to determine the taxonomic classification of the SAGs. This will allow single cells of interest to be selected for further downstream processing, such as multilocus or shotgun sequencing. Only a small aliquot of the DNA generated in the WGA is used for phylogenetic screening, and phylogenetic screening is optional.

Phylogenetic screening ● TIMING 3 h

24| Make a 1:20 dilution of the MDA product in nuclease-free water. Mix it thoroughly by hand-pipetting up and down. If a large number of samples precludes hand-pipetting, mix the samples in a plate shaker for 15 min at the highest setting.
▲ **CRITICAL STEP** The MDA product is highly viscous. It is crucial to ensure that it is mixed thoroughly. Robotic instrumentation may also be helpful with pipette mixing.

25| Transfer 1 µl of diluted MDA product as template to an optical microtiter plate (e.g., LightCycler multiwell plate 384).

26| Thaw the following reagents on ice for the 16S rRNA gene PCR: SsoAdvanced SYBR Green Supermix, 10-µM 926wF primer and 10-µM 1392R primer. Alternate primers are listed in **Table 1**.
▲ **CRITICAL STEP** Minimize the exposure of SsoAdvance SYBR Green Supermix to direct light.

© 2014 Nature America, Inc. All rights reserved.
npg

27| Gently mix all reagents by vortexing and spinning down. Each 10-μl PCR contains 3.6 μl of nuclease-free water, 5 μl of SsoAdvance SYBR Green Supermix (2×), 0.2 μl of 926wF primer (10 μM), 0.2 μl of 1392R primer (10 μM) and 1 μl of diluted MDA product as template. Prepare a sufficient amount of master mix for all reactions. Mix the reactions by vortexing and spinning down.

28| To each well of 1 μl of diluted MDA product template, add 9 μl of master mix. Seal the plate with optical seal and spin down at 1,000*g* for 1 min.

29| In a real-time thermocycling instrument, PCR-amplify the samples by using the cycling program recommended by the PCR kit manufacturer's instructions (**Fig. 3c**). Incorporating a melt curve step into the cycling program will aid in the analysis of PCR products.
? TROUBLESHOOTING

30| Purify and sequence PCR products to identify the single-cell genomes. We clean up PCR products by using ExoSAP-IT according to the manufacturer's instructions, and we then Sanger-sequence them via an outside service. We amplify and sequence the 16S rRNA gene for the taxonomic classification of our single cells, as the 16S (or SSU) rRNA is considered the gold standard in bacterial and archaeal classification[29], and databases and online tools facilitating the taxonomic identification are readily available[30,31].

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 2**.

TABLE 2 | Troubleshooting table.

Step	Problem	Possible reason	Solution
10	No sortable population is visible on the FACS instrument	High level of non-cell particle staining	Apply different sample preparation methods before sorting; alternatively, use different DNA stains
		Cell concentration is too low	Concentrate your cell population, for example, by centrifugation and discharging of part of the supernatant
		Mismatched refractive index between the sample and the sheath	Attempt to match the refractive index (i.e., density or salt content) of your sample with your sheath fluid
22	No MDA amplification across the plate	Too much UV irradiation affected Phi29 activity	Perform a quality control (QC) test with different time points of UV exposure (e.g., 0, 10, 20, 40, 60 min). Include positive controls (genomic DNA) and negative controls (single-cell genomics–grade water). Select the time point with the least amount of amplification in the negative controls, but sufficient activity in the positive controls and single cell wells
		Plate temperature exceeded 30 °C	Keep reagents and/or the plate on ice when handling. If you are using a real-time instrument, ensure that the interior is cooled to <30 °C before loading the plate (for example, open the instrument lid for a few minutes before loading)
		Cell was not sorted into the well	Spike a few of the wells with 1 ng of genomic DNA. If these wells amplify, but the positive control wells (100 cells) do not, review the sorting procedure used for possible errors
		Incomplete cell lysis	Alkaline lysis is not effective on all taxa. Alternate methods include freeze-thawing and heat lysis (for example, at 60 °C), although harsher lysis methods may damage the genomic DNA. Note that when using alternative lysis procedures, we still recommend the addition of lysis buffer to denature the DNA

(continued)

© 2014 Nature America, Inc. All rights reserved.
npg

TABLE 2 | Troubleshooting table (continued).

Step	Problem	Possible reason	Solution
22	MDA contamination (entire plate amplifies)	MDA kit or reagents are severely contaminated	Perform a QC test with different time points of UV exposure, e.g., 0, 10, 20, 40, 60 min. Include positive controls (genomic DNA) and negative controls (single-cell genomics–grade water). Ensure that the time point selected contains little or no background amplification in the negative controls. This QC is recommended when introducing a new lot of reagents, such as Phi29 or random hexamers. Alternatively, use the REPLI-g single cell kit, which is already pretreated to remove contamination from reagents
		Water is not of single-cell genomics–grade quality	It is best practice to always pretreat water before use. Refer to the Reagent Setup for the protocol. Note: water can be UV-irradiated for a longer period than what we state in this protocol, if extra 'cleaning' is still required. Alternatively, certified DNA-free water can be used (e.g., Mo-Bio, cat. no. 17000-11)
		Equipment and/or consumables carry contaminating DNA	Ensure that all equipment has been wiped thoroughly with a 10% (wt/vol) bleach solution and that all single-cell genomics work is performed within a UV-treated hood. Ideally, the hood should be stationed within a designated room with positive-pressure airflow. Contaminated objects may include pipettes, tips and tubes used to hold MDA reagents. These may be bleached and also UV treated in the Spectraline UV cross-linker, if necessary. If you use multichannel electronic pipettes, it may be helpful to take apart and thoroughly bleach all lines and channels. All personnel should wear designated and disposable lab coats and gloves
29	No PCR amplification	Too much or too little MDA DNA template	If there is a high background baseline, dilute the MDA DNA product by 1:50 or 1:100 (Step 23)
		MDA DNA template is not mixed sufficiently	Because MDA DNA is a branched product, the MDA product does not homogenize into solution easily. Mixing by vortexing is not as effective as using a plate shaker at the highest setting for 15 min. Even better results can be achieved by pipetting the MDA product up and down 15 times (either by hand or by using robotics; Step 23)
		Primers do not match the target organism	Full-length 16S rRNA gene primers can be used (for example, bacterial 27F and 1391R), but the shorter universal primers (926wF and 1392RV) generally result in more SAGs with PCR products

© 2014 Nature America, Inc. All rights reserved.

● **TIMING**

Steps 1 and 2, day 1, sample collection, preparation and preservation: up to 1 h 10 min
Steps 3–5, day 1, preparation of FACS for sterile sort: 1 d (overnight)
Steps 6–12, day 2, cell separation by flow cytometry: 1 d
Steps 13–17, day 3, single-cell lysis: 2 h
Steps 18–22, day 3, whole-genome amplification: 20 h (overnight)
Step 23, day 4, Phi29 enzyme deactivation: 10 min
Steps 24–29, day 4, phylogenetic screening: 3 h
Step 30, day 4, PCR product purification for sequencing: 1 h

ANTICIPATED RESULTS

This protocol enables recovery of amplified genomes from single cells found in a wide variety of environmental samples. The number of successfully amplified single cells can vary substantially between samples, and sample-specific preparation may be necessary for best results. In our experience, freshwater and marine samples yield the highest percentages of successfully amplified genomes (up to 40%), whereas success rates for soil samples tend to be low (<10%). Possible reasons for the high variability in genome amplification success between different sample types include resistance to lysis by some taxa and the presence of inhibitors of the MDA reaction. The successful single-cell amplification generally yields 100–200 ng/µl DNA, which can be directly used for multilocus or whole-genome sequencing without the need for re-amplification. Amplified single-cell genomes can also be used for the fabrication of DNA microarrays of select organisms of interest, which allows microarray-based monitoring of uncultivated microbes in various ecosystems and/or laboratory

enrichments[32]. For sequenced single cells, the genomes recovered are, on average, 40–55% complete, ranging from a few percent to greater than 90%. This variation may be attributed to incomplete cell lysis restricting the access of the Phi29 enzyme to the DNA, partial degradation of the template DNA before MDA and/or bias of the amplification reaction. Performing combined assemblies of closely related single cells allows estimated genome recoveries approaching completeness[1,8,10].

ACKNOWLEDGMENTS The work conducted by the US DOE Joint Genome Institute is supported by the Office of Science of the US Department of Energy under contract no. DE-AC02-05CH11231. Work conducted by Bigelow Laboratory for Ocean Sciences is supported by National Science Foundation grants OCE-1232982, OCE-821374, EF-0633142, EF-826924 and MCB-738232.

AUTHOR CONTRIBUTIONS C.R., T.W., R.M. and R.S. conceived the strategies. C.R., J.L., N.N., D.G., B.T., N.P. and E.D. performed the experiments and analyzed the data. C.R. wrote the paper with significant input from T.W., R.M. and R.S.

COMPETING FINANCIAL INTERESTS The authors declare no competing financial interests.

Reprints and permissions information is available online at http://www.nature.com/reprints/index.html.

© 2014 Nature America, Inc. All rights reserved.

1. Rinke, C. *et al.* Insights into the phylogeny and coding potential of microbial dark matter. *Nature* **499**, 431–437 (2013).
2. Amann, R.I., Ludwig, W. & Schleifer, K.H. Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. *Microbiol. Rev.* **59**, 143–169 (1995).
3. Albertsen, M. *et al.* Genome sequences of rare, uncultured bacteria obtained by differential coverage binning of multiple metagenomes. *Nat. Biotechnol.* **31**, 533–538 (2013).
4. Wrighton, K.C. *et al.* Fermentation, hydrogen, and sulfur metabolism in multiple uncultivated bacterial phyla. *Science* **337**, 1661–1665 (2012).
5. Blainey, P.C. The future is now: single-cell genomics of bacteria and archaea. *FEMS Microbiol. Rev.* **37**, 407–427 (2013).
6. Stepanauskas, R. Single cell genomics: an individual look at microbes. *Curr. Opin. Microbiol.* **15**, 613–620 (2012).
7. Hess, M. *et al.* Metagenomic discovery of biomass-degrading genes and genomes from cow rumen. *Science* **331**, 463–467 (2011).
8. Blainey, P.C., Mosier, A.C., Potanina, A., Francis, C.A. & Quake, S.R. Genome of a low-salinity ammonia-oxidizing archaeon determined by single-cell and metagenomic analysis. *PLoS ONE* **6**, e16626 (2011).
9. Campbell, J.H. *et al.* UGA is an additional glycine codon in uncultured SR1 bacteria from the human microbiota. *Proc. Natl. Acad. Sci. USA* **110**, 5540–5545 (2013).
10. Dodsworth, J.A. *et al.* Single-cell and metagenomic analyses indicate a fermentative and saccharolytic lifestyle for members of the OP9 lineage. *Nat. Commun.* **4**, 1854 (2013).
11. Swan, B.K. *et al.* Prevalent genome streamlining and latitudinal divergence of planktonic bacteria in the surface ocean. *Proc. Natl. Acad. Sci. USA* **110**, 11463–11468 (2013).
12. Woyke, T. *et al.* Assembling the marine metagenome, one cell at a time. *PLoS ONE* **4**, e5299 (2009).
13. Yoon, H.S. *et al.* Single-cell genomics reveals organismal interactions in uncultivated marine protists. *Science* **332**, 714–717 (2011).
14. McConnell, M.J. *et al.* Mosaic copy number variation in human neurons. *Science* **342**, 632–637 (2013).
15. Zhang, K. *et al.* Sequencing genomes from single cells by polymerase cloning. *Nat. Biotechnol.* **24**, 680–686 (2006).
16. Woyke, T. *et al.* One bacterial cell, one complete genome. *PLoS ONE* **5**, e10314 (2010).
17. Landry, Z.C., Giovanonni, S.J., Quake, S.R. & Blainey, P.C. Optofluidic cell selection from complex microbial communities for single-genome analysis. *Methods Enzymol.* **531**, 61–90 (2013).
18. Frumkin, D. *et al.* Amplification of multiple genomic loci from single cells isolated by laser micro-dissection of tissues. *BMC Biotechnol.* **8**, 17 (2008).
19. Thompson, A., Bench, S., Carter, B. & Zehr, J. Coupling FACS and genomic methods for the characterization of uncultivated symbionts. *Methods Enzymol.* **531**, 45–60 (2013).
20. Swan, B.K. *et al.* Potential for chemolithoautotrophy among ubiquitous bacteria lineages in the dark ocean. *Science* **333**, 1296–1300 (2011).
21. Raghunathan, A. *et al.* Genomic DNA amplification from a single bacterium. *Appl. Environ. Microbiol.* **71**, 3342–3347 (2005).
22. Dean, F.B. *et al.* Comprehensive human genome amplification using multiple displacement amplification. *Proc. Natl. Acad. Sci. USA* **99**, 5261–5266 (2002).
23. Woyke, T. *et al.* Decontamination of MDA reagents for single cell whole genome amplification. *PLoS ONE* **6**, e26161 (2011).
24. Rodrigue, S. *et al.* Whole genome amplification and *de novo* assembly of single bacterial cells. *PLoS ONE* **4**, e6864 (2009).
25. Lasken, R.S. & Stockwell, T.B. Mechanism of chimera formation during the multiple displacement amplification reaction. *BMC Biotechnol.* **7**, 19 (2007).
26. Stepanauskas, R. & Sieracki, M.E. Matching phylogeny and metabolism in the uncultured marine bacteria, one cell at a time. *Proc. Natl. Acad. Sci. USA* **104**, 9052–9057 (2007).
27. Blainey, P.C. & Quake, S.R. Digital MDA for enumeration of total nucleic acid contamination. *Nucleic Acids Res.* **39**, e19 (2011).
28. Miyauchi, R., Oki, K., Aoi, Y. & Tsuneda, S. Diversity of nitrite reductase genes in '*Candidatus Accumulibacter phosphatis*'-dominated cultures enriched by flow-cytometric sorting. *Appl. Environ. Microbiol.* **73**, 5331–5337 (2007).
29. Stackebrandt, E. & Ebers, J. Taxonomic parameters revisited: tarnished gold standard. *Microbiology Today* **33**, 152–155 (2006).
30. Yarza, P. *et al.* Update of the All-Species Living Tree Project based on 16S and 23S rRNA sequence analyses. *Syst. Appl. Microbiol.* **33**, 291–299 (2010).
31. McDonald, D. *et al.* An improved Greengenes taxonomy with explicit ranks for ecological and evolutionary analyses of bacteria and archaea. *ISME J.* **6**, 610–618 (2012).
32. Chang, H.W. *et al.* Development of microbial genome-probing microarrays using digital multiple displacement amplification of uncultivated microbial single cells. *Environ. Sci. Technol.* **42**, 6058–6064 (2008).
33. Frank, J.A. *et al.* Critical evaluation of two primers commonly used for amplification of bacterial 16S rRNA genes. *Appl. Environ. Microbiol.* **74**, 2461–2470 (2008).
34. Mao, D.P., Zhou, Q., Chen, C.Y. & Quan, Z.X. Coverage evaluation of universal bacterial primers using the metagenomic datasets. *BMC Microbiol.* **12**, 66 (2012).
35. Lane, D.J. in *Nucleic Acid Techniques in Bacterial Systematics* (eds. Stackebrandt, E. & Goodfellow, M.) 115–175 (John Wiley and Sons, 1991).
36. Caporaso, J.G. *et al.* Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *Proc. Natl. Acad. Sci. USA* **108** (suppl. 1), 4516–4522 (2011).
37. An, D. *et al.* Metagenomics of hydrocarbon resource environments indicates aerobic taxa and genes to be unexpectedly common. *Environ. Sci. Technol.* **47**, 10708–10717 (2013).
38. Cole, J.K. *et al.* Sediment microbial communities in Great Boiling Spring are controlled by temperature and distinct from water communities. *ISME J.* **7**, 718–729 (2013).
39. Lane, D.J. *et al.* Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses. *Proc. Natl. Acad. Sci. USA* **82**, 6955–6959 (1985).
40. Pace, N.R.S., Stahl, D.A., Lane, D.J. & Olsen, C.J. The use of rRNA sequences to characterize natural microbial populations. *Adv. Microb. Ecol.* **9**, 1–55 (1986).
41. Page, K.A., Connon, S.A. & Giovannoni, S.J. Representative freshwater bacterioplankton isolated from Crater Lake, Oregon. *Appl. Environ. Microbiol.* **70**, 6542–6550 (2004).
42. Casamayor, E.O., Schafer, H., Baneras, L., Pedros-Alio, C. & Muyzer, G. Identification of and spatio-temporal differences between microbial assemblages from two neighboring sulfurous lakes: comparison by microscopy and denaturing gradient gel electrophoresis. *Appl. Environ. Microbiol.* **66**, 499–508 (2000).
43. Raskin, L., Stromley, J.M., Rittmann, B.E. & Stahl, D.A. Group-specific 16S rRNA hybridization probes to describe natural communities of methanogens. *Appl. Environ. Microbiol.* **60**, 1232–1240 (1994).
44. Stahl, D.A.A. & Amann, R. in *Nucleic Acid Techniques in Bacterial Systematics* (eds. Stackebrandt, E. & Goodfellow, M.) 205–248 (John Wiley and Sons, 1991).
45. Zhu, F., Massana, R., Not, F., Marie, D. & Vaulot, D. Mapping of picoeucaryotes in marine ecosystems with quantitative PCR of the 18S rRNA gene. *FEMS Microbiol. Ecol.* **52**, 79–92 (2005).
46. Medlin, L., Elwood, H.J., Stickel, S. & Sogin, M.L. The characterization of enzymatically amplified eukaryotic 16S-like rRNA-coding regions. *Gene* **71**, 491–499 (1988).