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Single cell genome sequencing

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Whole genome amplification and next-generation sequencing of single cells have become a powerful approach for studying uncultivated microorganisms that represent 90–99% of all environmental microbes. Single cell sequencing enables not only the identification of microbes but also linking of functions to species, a feat not achievable by metagenomic techniques. Moreover, it allows the analysis of low abundance species that may be missed in community-based analyses. It has also proved very useful in complementing metagenomics in the assembly and binning of single genomes. With the advent of drastically cheaper and higher throughput sequencing technologies, it is expected that single cell sequencing will become a standard tool in studying the genome and transcriptome of microbial communities.

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Introduction

Microbes, the most abundant species on earth, play an important role in ecological processes in making, breaking down, and recycling the essential chemicals of life. Microbes are also related to our health and are abundant in our bodies — we have 10 times as many microbes living on and inside us as human cells. Despite their importance, it is estimated that 90–99% of microbes have not been characterized because they cannot be cultured in a laboratory. Hence, culture-independent techniques such as fluorescence *in situ* hybridization (FISH), PCR, microarrays, and sequencing of the 16S rRNA gene are relied upon to detect and analyze microbes. More recently, the advent of next-generation sequencing has allowed large-scale shotgun sequencing of collective genomes in a microbial community. This has allowed an unprecedented access to uncultured microbial communities and their activities and has been applied to a wide variety of habitats ranging from termite gut to marine environments [1,2]. However, a major drawback with the shotgun sequencing of the metagenome is that while it provides information on the species present and the function(s) of the community, it cannot link the function back to the species. Moreover, in the majority of cases, metagenomic sequencing does not allow assembly of individual genomes in the community.

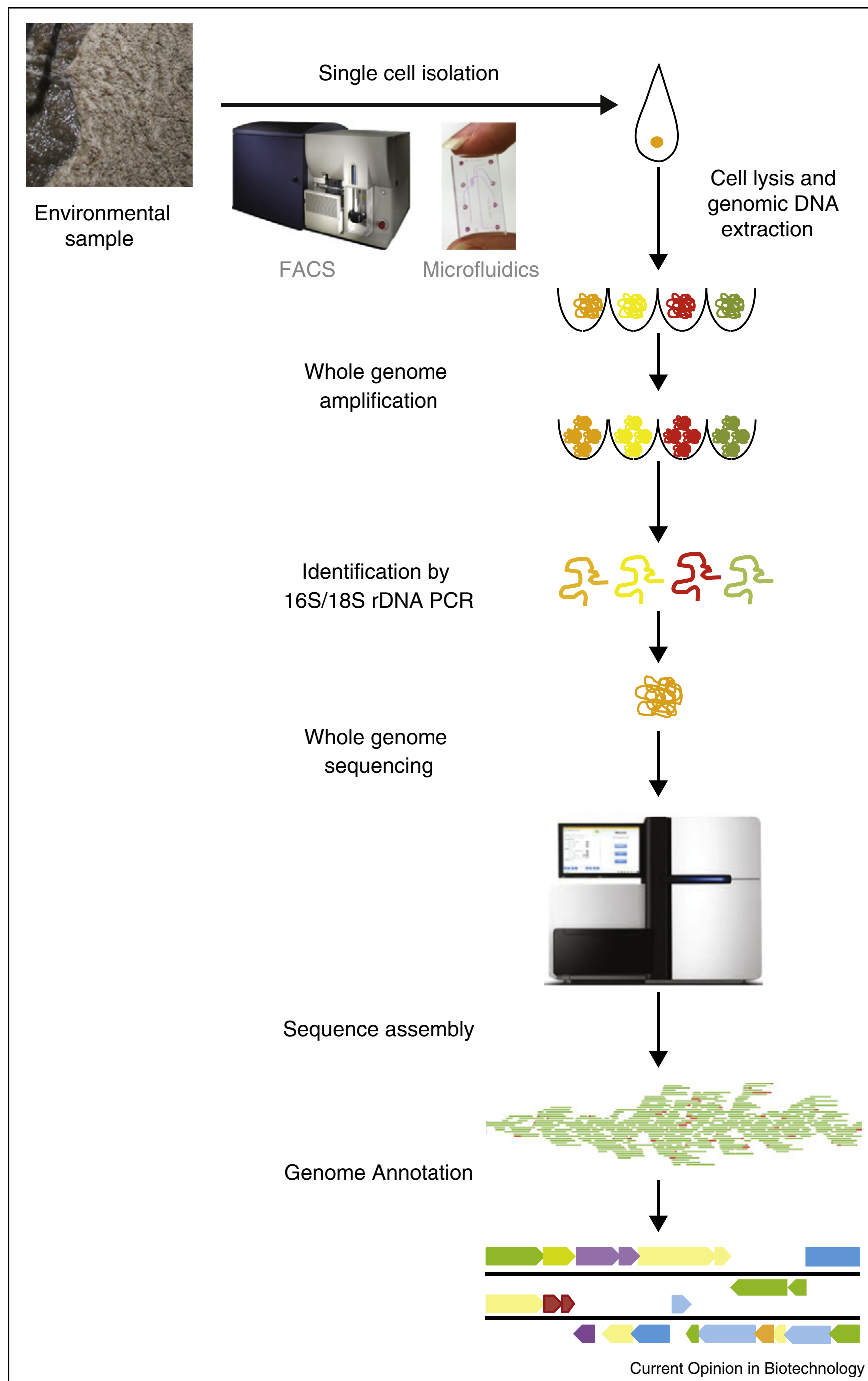
To overcome the problems associated with metagenomics, research efforts have generally focused on the sequencing of individual cells via the employment of whole genome amplification strategies [3]. Single cell sequencing has been applied to numerous environmental microbes (prokaryotic and eukaryotic microorganisms as well as environmental viruses) including T7 from human mouth and soil, Flavobacteria and heterotrophic protists from ocean, Termite Group 1 and *Desulfovibrio* from termite gut, *Leptothrix* from iron mats, *Sulcia* from a green sharpshooter, ammonia oxidizing archaea from a low-salinity estuary, and Poribacteria from marine sponge [3,4••,5–9,10•,11,12••]. Single cell genome sequencing involves the isolation of single cells from the environmental sample, cell lysis and multiple displacement amplification (MDA) followed by whole genome sequencing and analysis as depicted in Figure 1 and described below.

Single cell isolation

The first step in single cell genomics is the isolation of individual cells from microbial communities. Several single cell isolation methods have been developed including serial dilution, micromanipulation, laser capture microdissection, Raman tweezers, fluorescence activated cell sorting (FACS), and microfluidics. Serial dilution, a simple and inexpensive technique mostly used for culturing studies, has been used to isolate single cells of *Escherichia coli* and *Prochlorococcus marinus* for genome sequencing [13]. However, serial dilution is prone to error and loss of cells and does not allow targeted isolation of cells. Hence, it is not a preferred method for isolation of cells from complex microbial samples.

Micromanipulation has been used to successfully isolate single cells of a number of uncultivated organisms from environmental samples - for example, Chrenarchaeota from soil [14], endosymbionts from termite gut [6,9], and a symbiont from green sharpshooter gut [3]. Although micromanipulation permits the visual evaluation of single cells during isolation, its extremely low throughput limits widespread use [15]. Another drawback is the mechanical shearing of cells during extraction. In the case of laser capture microdissection, this technique is

Figure 1



Single cell genomics: a flow diagram of the steps involved in single cell genomics.

almost exclusively utilized in human health-related single cell genomics studies [16,17] to isolate cells from tissues. However, it has been used recently to isolate single cells of *Burkholderia thailandensis* [18] in a single cell transcriptomics study. The real power of this technique comes from the integration of labeling with dissection [17]. Despite having a lower throughput, laser capture microdissection may prove useful in the isolation of single cells from a complex matrix (e.g. biofilm) where isolation is guided by fluorescent probes. A drawback of this method is the possibility of loss of genetic material or addition of impurities due to imprecise slicing. Raman tweezers is another isolation technique that combines Raman microspectroscopy with optical trapping. Raman

microspectroscopy enables the differentiation of cell types by biochemically profiling cells without external labeling [19]. After the cells of interest have been identified by Raman microspectroscopy, they are subsequently trapped via a laser. Raman tweezers was recently used by Huang *et al.* to separate single yeast and bacterial cells from an artificial cell mixture [20]. A limitation of this technique is that it can only isolate cells that are physiologically distinct.

FACS has become the preferred method for single cell separation since it is high throughput and has the ability to sort based on multiple cellular properties (e.g. size, granularity, intrinsic or extrinsic fluorescence). Advances in high-speed cell sorters capable of multiparametric measurements have facilitated the simultaneous assessment of physiological and taxonomic properties to detect and separate specific cells [21]. Its high sensitivity of detection has made it possible to sort small cells and particles such as viruses [22••] and cells trapped in microdroplets [23].

Over the last few years, microfluidic devices, owing to their microscale dimensions (similar to the size of a cell) and potential for integration of multiple processes, are widely being used for the analysis of single cells [4••,24–26]. They provide a sealed environment for isolation and amplification, which reduces the risk of contamination from the environment. Recently, a microfluidic chip was described that automates and integrates the steps of sample preparation and analysis by flow cytometry and requires less than thousand cells for analysis of environmental samples [27]. It is expected that chips will be developed that integrate and automate the entire process of single cell isolation, preparation, and amplification (Figure 2). Another advantage of microfluidics is that it enables pico-to-nano liter reactions permitting high template concentrations hence minimizing the emergence of chimeras [25,28]. A key disadvantage of current microfluidic chips is the lower throughput compared to commercial FACS.

For many environmental samples, it is not always possible or desirable to isolate individual cells. Examples include cells from a biofilm where two or more species of cells could adhere to each other for physiological and metabolic considerations. In those situations, the best practice would be to isolate the smallest possible group of cells to reduce complexity and maximize the probability of assembly and genome reconstruction.

Cell lysis and gDNA extraction

After isolating single cells, the next step is to lyse them to extract genomic DNA (gDNA). This is perhaps the most critical step as the success of subsequent whole genome amplification depends on the availability and quality of gDNA, especially for small prokaryotic cells that contain only a few femtograms of DNA. The lysis method should be harsh enough to lyse the cells while gentle enough to preserve the integrity of gDNA. Of the several lysis methods available, none exists that can handle every cell type. The cell type (i.e. cell wall characteristics), downstream application (whole genome amplification), and the platform where single cell amplification will be performed are the critical factors in the selection of the proper lysis technique. A combination of more than one method may also be used for more effective cell lysis when required. Cell lysis procedures can be classified into three broad categories: physical, chemical, and enzymatic.

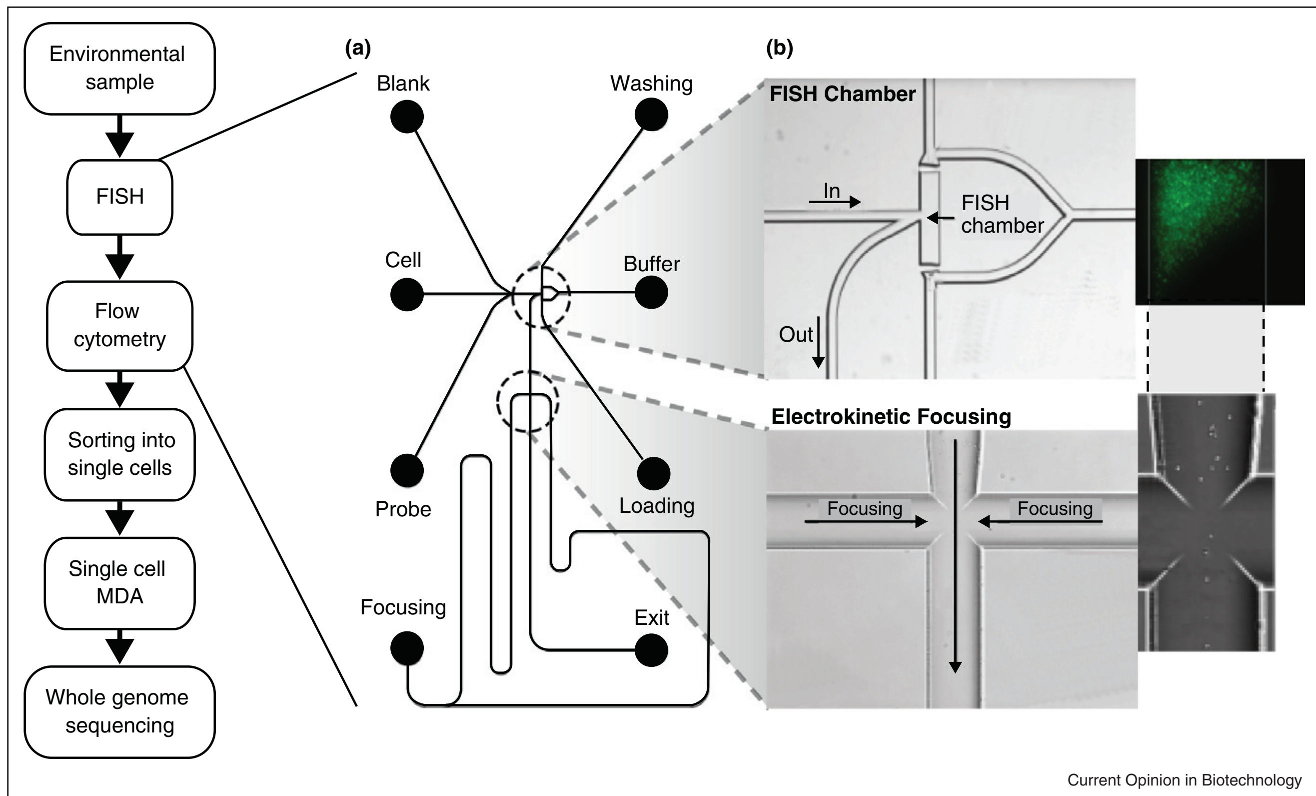
Physical methods involve techniques such as sonication and freeze/thawing. Availability of commercial high throughput sonication systems has the potential for lysing a large number of single cells simultaneously and with minimal contamination. Freeze/thawing is a gentle method of lysis and is typically combined with another method such as enzymatic digestion for complete lysis [10•,14,29]. Chemical lysis is fast and is typically done using a strong base such as NaOH or KOH, which is accompanied by a neutralization step. Another commonly used method is enyzmatic lysis using enzymes such as lysozyme and proteinase K [23]. Enzymatic cell lysis is the most gentle lysis method as no mechanical forces or extremes of pH are required that can potentially degrade the DNA. While the majority of Gram-negative bacteria can easily be lysed by lysozyme, there are a few lysozyme-resistant Gram-positive bacteria, which would require other enzymes such as achromopeptidases or muramidases with broader specificity. Therefore, for a complex community, a cocktail of enzymes is frequently used to achieve complete lysis [5]. A few other specialized lysis techniques such as pulsed laser microbeam-induced cell lysis, nanoknives, and electroporation have also been suggested for single cell applications [30].

Whole genome amplification

Next-generation sequencing technologies require micrograms of DNA and hence, amplification is a crucial step for sequencing of cells typically containing femtograms of DNA. MDA has become the method of choice for whole genome amplification from single cells. It is an isothermal amplification method that uses random primers and Phi29 DNA polymerase for generating fairly large fragments (10–20 kb) with high fidelity [31]. Although it is proven to be the best method for whole genome amplification (compared to PCR-based techniques), it is not completely unbiased and error-free [32]. The key drawbacks include uneven genome coverage, chimeric sequences, and contamination issues discussed below.

Uneven genome coverage by MDA: Because of the stochastic priming and amplification at the early stages of the MDA reaction, substantial variations in the amplifications of different regions occur in single amplified genomes

Figure 2



Integrated microfluidic chip for isolation and analysis of single cells. Left panel shows the steps that can be integrated into a microfluidic chip. **(a)** is a schematic of a microchip for fluorescence *in situ* hybridization (FISH) and flow cytometry (μFlowFISH). **(b)** is an image of the FISH chamber formed by two photopolymerized membranes in the channel and the cross-channel structure for electrokinetically focusing microbial cells into a single streamline along the center of the vertical channel for flow cytometry. The top panel shows bacteria labeled with a green FISH probe and the bottom panel shows the enlarged image of the cross-channel showing single cells being focused in the center [27].

(SAGs) [32,33,34••]. To overcome uneven genome coverage, the pooling of MDA reactions from different individuals of the same species has been suggested [1,4••,33,35]. Although this strategy may work fine for clonal populations, the resulting genome should be treated as a pangenome for analyzing microbial communities due to cellular heterogeneities in natural populations. Another approach used to minimize bias is to use smaller reaction volumes or crowding agents to increase effective template concentration. For example, Marcy *et al.* demonstrated that reducing the total reaction volume using microfluidic devices increases the specific template concentration, thus reducing the amplification bias [25]. Similarly, while it has yet to be applied to single cell amplification, molecular crowding agents such as trehalose or PEG400 can provide more homogenous amplifications via the volume reduction effect [36,37]. Another strategy tested to even out the genome coverage is the normalization of DNA libraries by a duplex-specific nuclease to remove high-abundant dsDNA [34••,38]. While postamplification normalization has provided substantial improvement for sequence coverages <100×, no significant improvement was observed at higher coverages [34••]. Bioinformatic normalization via removal of reads from highly overrepresented regions has been found to be very effective for the *de novo* assembly of SAGs [34••].

Chimeric sequences: MDA can cause genomic rearrangements due to the chimeric sequences formed during amplification, further complicating genome assembly, where the average chimera formation rate is approximately 1 per 10 kb [34,39]. As single stranded MDA reaction intermediates are believed to lead to chimeras, postamplification treatment with S1-nuclease has previously been reported to reduce chimeras by up to 80% [13]; however, when Woyke *et al.* compared small insert clone libraries prepared from branched and unbranched MDA of a single cell, they were not able to detect any notable chimera reduction in the S1-treated samples [11]. A last resort is to identify and remove chimeras computationally during analysis if sufficient sequence coverage is provided.

Nonspecific amplification: MDA may lead to the amplification of even small quantities of contaminating DNA as well as dimerized primer pairs since random primers are

used to initiate polymerization [13,40]. Major sources of contamination result from laboratory environment, amplification reagents, and exogenous DNA in the environmental sample. Stringent cleaning measures must be applied to eliminate possible contaminations. As a common practice in the field of single cell genomics, all tubes, plates, and buffers are UV-treated before use, and cell isolation instruments are cleaned with bleach and rinsed with UV-treated water to remove any DNA contamination. Rodrigue *et al.* employed a two-step sorting strategy to reduce DNA contamination [34••]. They introduced exogenous DNA to their sample and found no contaminating DNA in two replicate SAGs after two cycles of FACS leading to a billion-fold dilution. This approach was successfully adapted to a microfluidic platform as well [24]. Arguably the most common contaminant from commercial MDA reagents is *Delftia acidovorans* [33]. Blainey and Quake devised a method to circumvent the problem of bacterial DNA found in commercial MDA reagents by expressing and affinity-purifying recombinant Phi29 DNA polymerase with dual affinity tags [41].

To evaluate the quality of MDA products before whole genome sequencing, amplification of some marker genes and Sanger sequencing are commonly used. However, quality controls relying on SSU rRNA gene were shown to be inadequate, as 90% of 57 Mb sequence identified as contaminants after shotgun sequencing; although no non-target DNA contamination was detected with SSU rRNA PCR [3]. Despite taking extensive precautions, it is possible or, even likely that contaminating DNA is still present in the sequenced data and the last resort is computational identification and removal [3,33].

Analysis of single cell sequence data

The earlier studies utilizing Sanger sequencing [8,13] or short-read length 454 pyrosequencing [7] resulted in partial recovery of SAGs. While Zhang *et al.* were able to span 66% of Prochlorococcus genome with 7.2 Mb of high-quality Sanger reads [13], Marcy *et al.* recovered an undetermined % of the genome in a fragmented assembly of 1825 scaffolds (~2.86 Mb) by pyrosequencing of three individual cells of uncultured TM7 from the human mouth [7].

The higher throughput and longer reads of next-generation sequencing have made it possible to finish SAGs. Woyke *et al.* were able to recover 80% of a marine Flavobacterium genome using a combination of Sanger and 454 pyrosequencing [11]. However, when combined with metagenomics, they were able to improve recovery to 91% leading to a high-quality draft *de novo* reconstruction of an uncultured bacterium from a complex microbial community.

With the increasing number of single cell genome sequences, algorithms are being developed to tackle problems intrinsic to single cell genomics. For example, SmashCell automates genome assembly, gene prediction and annotation from SAGs [42]. It utilizes sequence similarity and kmer-based tools to identify contaminants; uses custom scripts to downsample overrepresented regions of the SAG; and employs STRING database [43] to count single-copy orthologous groups, which then can be used to estimate genome completeness.

Conclusions

Despite tremendous sequencing efforts, it has not been possible to achieve complete assembly and metabolic reconstruction of individual genomes using metagenomics, except in very simple communities such as in acid mine drainage [44]. With the recent advances in whole genome amplification strategies and sequencing technologies, single cell genomics has complemented metagenomics in unraveling the individual genomes and making it possible to complete genome assembly of novel uncultivated organisms. However, single cell sequencing has many challenges remaining. Some of these can be attributed to multiple displacement amplification discussed earlier. A growing challenge in single cell genomics will be providing sufficient computational resources and expertise to analyze genomes as vast amounts of data are generated from single cell genome sequencing. Despite these limitations, the single cell genomics approach has enabled researchers to determine population level microheterogeneities [4••], study cell–cell interactions [12••], improve phylogenetic resolution of microbial diversity [45•], reclassify an organism [5], and even study single viral genomes [22••,46]. Another area where single cell sequencing will be important is in the optimization of the expression of novel genes revealed by sequencing. While, in theory, metagenomics allows us to find novel genes for functions of interest (e.g. cellulase), we frequently fail to express those genes in heterologous systems (or, even if we can express them, the activities are less than their native hosts). Single cell sequencing not only identifies the genes, it also provides contextual information on the gene such as transcriptional regulation, which can be very useful in designing successful expression platforms.

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