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Single-Cell Techniques in Environmental Microbiology

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Abstract: Environmental microbiology has been an essential part of environmental research because it provides effective solutions to most pollutants. Hence, there is an interest in investigating microorganism behavior, such as observation, identification, isolation of pollutant degraders, and interactions between microbial species. To comprehensively understand cell heterogeneity, diverse approaches at the single-cell level are demanded. Thus far, the traditional bulk biological tools such as petri dishes are technically challenging for single cells, which could mask the heterogeneity. Single-cell technologies can reveal complex and rare cell populations by detecting heterogeneity among individual cells, which offers advantages of higher resolution, higher throughput, more accurate analysis, etc. Here, we overviewed several single-cell techniques on observation, isolation, and identification from aspects of methods and applications. Microscopic observation, sequencing identification, flow cytometric identification and isolation, Raman spectroscopy-based identification and isolation, and their applications are mainly discussed. Further development on multi-technique integrations at the single-cell level may highly advance the research progress of environmental microbiology, thereby giving more indication in the environmental microbial ecology.

Keywords: single-cell techniques; flow cytometry; observation; isolation; identification

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1. Introduction

Microorganisms are ubiquitous in the environment; they interact with the soil-, water-, and gas-phase environments, and they play essential roles in the environment. Environmental microbiology uses microbes to clean up contaminants, so the observation, identification, and isolation of high-efficiency contaminant degraders are essential. Bulk analysis techniques give average results for the population/community, but (i) fail to capture the contribution of small subpopulations ("phenotypic or genotypic heterogeneity") to the functionality of the community, or (ii) make up a non-existing averaged population, resulting in limited/false exploitation of these differences to steer microbial response (Figure 1). Therefore, more detailed analysis techniques at the single-cell level are highly demanded.

Research on single-cell technology is not new. Dating back to the 17th century, Antonie van Leeuwenhoek, known as "the Father of Microbiology", was the first person to observe and describe single microbes using his handcrafted microscopes [1]. In the 19th century, the first isolation of single bacteria was achieved by Julius Richard Petri and Robert Koch; individual bacterial cells were isolated on the petri dish and cultivated into single "clonal" bacterial colonies [2]. Microorganisms, which represent most of the earth's biodiversity, are primary drivers and play important roles in the environment, ecosystem functioning, and nutrient cycling [3–5]. While the vast majority of microorganisms (>99%) in the natural environment are currently unculturable in laboratory research [6]. These "unculturable" microorganisms represent a large, untouched pool of species with novel biological and chemical properties [7]. The characterization of microbial communities

and their management are becoming increasingly appealing in both natural and industrial processes. Identification of "unculturable" microorganisms from nature started to emerge in the 1990s by sequencing [8,9], followed the development of high-throughput next-generation sequencing or 16S rRNA sequencing, which remains dominant nowadays. Yet, the state-of-the-art molecular techniques are still semi-quantitative at best and at bulk levels [10]. Single-cell techniques can help to understand how individual phenotypic and genotypic traits determine functionality and link heterogeneity between individuals and performance. Effective single-cell techniques without the need for a time-consuming cultural step have been an issue for several years, and several methods already exist that address this problem.

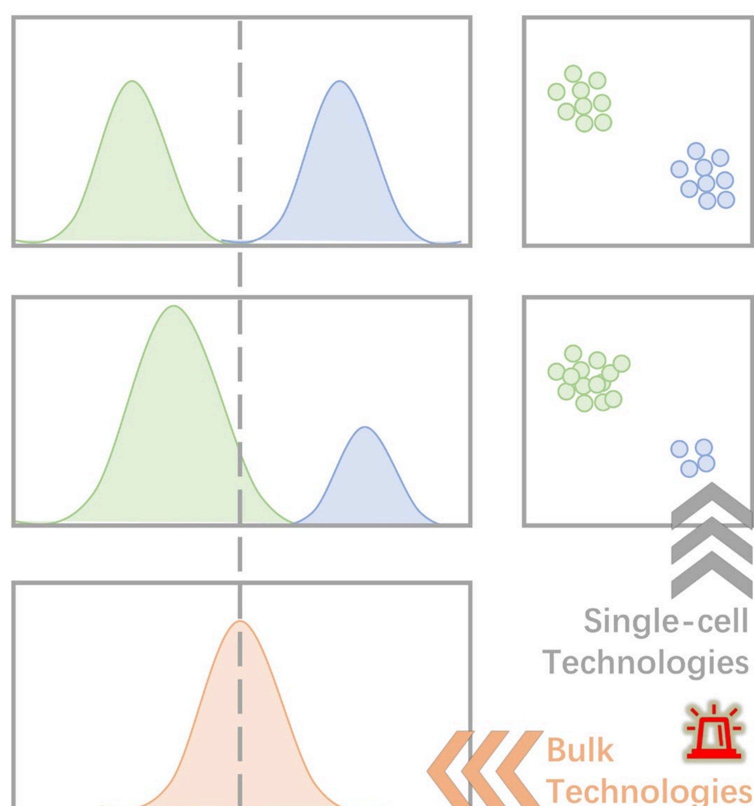

Figure 1. Comparison between bulk and single-cell techniques. Bulk analysis techniques provide average microbial information, while single-cell techniques provide more accurate microbial phenotypic heterogeneity.

The single-cell technique for diversified and efficient observation, isolation, and identification analyses of single biological cells has been a research hotspot in the respected field. This review highlighted the relevant single-cell techniques from the aspects of methods and applications. The advantages and disadvantages are discussed. The state-of-art techniques in single-cell analysis are reviewed, including microscopy, sequencing, flow cytometry, and Raman spectroscopy, in terms of their advantages and multi-technique application, with the intention of indicating the approaches to overcome the bottlenecks in environmental single-cell analysis.

2. Single-Cell Techniques

2.1. Microscopic Observation

Fluorescence microscopy is one of the most common tools in molecular biology/biochemistry laboratories. Reliable and strong fluorescent signals are necessary to monitor individual cell physiology through staining or using antibodies against proteins of interest. High-resolution single-cell microscopy, such as confocal microscopy, allows imaging of

three-dimensional structure distributions of a thick specimen by optical sectioning (a theoretical lateral resolution of 0.1–0.2 μm and a vertical resolution of 0.2–0.4 μm, depending on the numerical aperture of the objective and the wavelength of the emitted light [11,12]), and electron microscopy (e.g., scanning electron microscopy—SEM, transmission electron microscopy—TEM) observe the surface or interior of cells through sectioning and negative staining. An Energy Dispersive X-ray Analysis (EDX) system is an attachment to SEM or TEM that identifies the elemental composition of a sample. In situations where combined microscopy and EDX data are insufficient to identify a specimen, complementary techniques such as Raman microscopy are available. Furthermore, the combination of different techniques allows for more detailed information about a sample; for example, confocal Raman microscopy, which combines Raman spectroscopy with a confocal microscope, reveals the spatial distribution of the compounds within a sample [13].

With microscopic observation alone, it can be extremely hard to achieve a reasonable functional resolution for the diversity of microbes typically found in an environmental sample [2]. To solve this problem, image processing and analysis of the microscopic images are applied in order to observe sufficient sample numbers to allow meaningful statistical analyses, as well as rapid characterization of cell populations. This has been used in monitoring filamentous bacteria in active sludges of wastewater treatment plants [14,15]. While to give accurate quantification of flocs and filaments, a good performance of the image segmentation algorithms is very important. Furthermore, microscopy of biological samples in microfluidic devices may serve the pressing need for microscopy of cells and other biological systems and materials in their native liquid state, e.g., with a resolution of a few nanometers—the dimension of proteins—by combining electron microscopy and microfluidic devices [16]. Additionally, the development of new fluorescent dyes may further enhance the resolution of the microscopy image (e.g., the benzene ring in the rhodamine core is replaced with a permanently charged 1,3-disubstituted imidazolium), which boosts photoswitching behavior and overall performance of (direct) stochastic optical reconstruction microscopy ((d)STORM) [17].

2.2. Sequencing Identification

The sequencing technologies continued to evolve with the advent of next generation sequencing (NGS) in the early 2000s, providing more comprehensive data with efficient, rapid, reduced-cost, and accurate DNA sequencing [18,19]. Over the past decade, there has been a surge of interest in obtaining high-resolution views of single-cell heterogeneity on a global scale. Genome sequencing of single microbial cells directly isolated from environmental samples ranging from deep-sea hydrothermal vents to insect guts has become almost routine, providing a powerful complement to shotgun metagenomics in microbial community studies [20]. Further, third-generation sequencing allows for long-read sequencing in real-time with low alignment and mapping errors during library construction, which is much faster than traditional first- or second-generation technologies. It can provide results in a matter of minutes. Therefore, speed is one of the most important advantages of third-generation sequencing, allowing, for example, efficient pathogen identification [21]. Furthermore, the relatively long reads make it possible to completely sequence a viral or bacterial genome with high accuracy [22,23]. Third-generation sequencing is widely used in microbiology to sequence genomes from individual cells isolated from environmental samples, e.g., its application in a large-scale wastewater treatment plant to understand the mechanism of pollutants removal in a comprehensive way [24].

2.3. Flow Cytometric Identification and Isolation

Although microscopy and sequencing made us aware of "who is there" in the microbial world, it was not until the advent of flow cytometry (FCM) in the late 20th century that complex and high-throughput studies were first performed on the microbial single-cell level, such as the cellular DNA/protein content and viability of bacteria, algae, fungi, and yeasts [25]. Nowadays, FCM has been developed and recognized as the gold standard for

fast and reliable single microorganism analysis [26–30]. It is (i) a high-throughput technique where cells are analyzed by passing through a beam of light in a fluid stream and measuring their fluorescence or scatter properties; (ii) capable of multiple detections through the multitude of available stains; and (iii) efficient in single-cell sorting for downstream analysis, such as physical-chemical, biological, or molecular analysis [31,32].

Flow cytometry is now commonly used in aquatic microbiology, either at the lab scale to gain deep insights into the heterogeneity of populations and the functioning of microbial communities [33–36], in large-scale wastewater treatment plants for long-term investigations of dynamic community assembly to discover perturbation-associated symptoms for community control [37], or for automatic online monitoring, where the community data obtained is used as an early-warning tool to reflect/control drinking water process operation [38–41]. These applications rely heavily on maximizing the use of FCM data stored in single-cell phenotypic characteristics. Several novel computational FCM analysis tools that differ in their approach and purpose have been developed and reviewed [42]. Image-processing-based approaches are represented by the Dalmatian Plot [43] and Cytometric Histogram Image Comparison (CHIC) [44], which give a general comparison at the sample level. Further, gating-based approaches (CyBar) allow extra identification of individual subpopulation/subcommunity dynamics/responses of each sample in addition to the general trend interpreted by image processing [45], which has been proven to be of a similar resolution as a 16S rRNA gene analysis [32]. However, the gating is performed manually, which requires expertise and may vary from person to person. So automated approaches are on demand and established for phenotypic diversification, such as (i) binning strategies for the flow cytometry-derived alpha diversity metrics of "phenoflow" [46] and flow FP [47], and (ii) the Gaussian mixture model-based gating tools—"PhenoGMM" [48] enables efficient predictions of biodiversity, and "flowEMMi" [49] provides fast and accurate identification of cell clusters in FCM data. The integration of artificial intelligence and machine learning algorithms is the advancement in flow cytometry application. Additionally, there have been recent developments in the use of microfluidic devices for flow cytometry, which allows for the miniaturization of the technique and the potential for high-throughput analysis of large numbers of cells [50].

The capabilities of flow cytometers have improved dramatically over the years by integrating more lasers and detectors, allowing the detection of more markers per individual cell. Due to the compensation, conventional flow cytometry based on fluorescence has limitations in detecting channels ranging up to 15–20 colors. With the development of mass cytometry in 2009, the number of detection channels evaluated using metal-conjugated antibodies expanded by more than 40 [51] due to the low background value of metals in cells. Yet, mass cytometry is a cell-destructive technique (e.g., atomized and ionized) with no possibility of recovery of cells for sorting. The up-to-date full-spectrum flow cytometry breaks through the bottleneck of conventional flow cytometry with limited detecting channels and mass cytometry with destructive cell analysis, which measures the entire emission spectrum of every fluorochrome [52,53] across all laser lines. Up to 40 fluorescently labeled antibodies are possible for individual cell analysis and sorting. Full-spectrum flow cytometry allows for high-dimensional parameter space and high subcellular resolution.

2.4. Raman Spectroscopy-Based Identification and Isolation

Raman spectroscopy is another powerful and non-destructive spectroscopic technique for single-cell phenotypic/genotypic characterization. The incident light (normally monochromatic light close to the infrared or ultraviolet range) illuminating the sample results in the photons undergoing elastic scattering (energy/wavelength unchanged from incident light) and inelastic scattering (energy/wavelength changed—Raman spectrum). Each peak in the Raman spectrum corresponds to a specific molecular vibration mode, for example, the individual bonds of C-C, C-H, C=C, and N-O, and groups of bonds such as the breathing mode of the aromatic carbon ring, polymer chain vibrations, lattice modes, etc. In general, it is capable of acquiring more than 1000 Raman bands from a single cell within

30 s in the Raman shift range of 500–2000 cm^{-1}, which provides comprehensive and intrinsic biochemical information and single-cell identification as a molecular fingerprint [54]. Raman spectroscopy is now a remarkable label-free technique for single particle characterization, and researchers have used this technique to identify and quantify microplastics [55]. Normally, spontaneous Raman spectroscopy has a weak scattering effect because only 1 in 106–108 photons will undergo Raman scattering, which results in high laser power and a long acquisition time. While coherent Raman spectroscopy provides much stronger signals compared to spontaneous Raman spectroscopy. Raman signal can be enhanced through nonlinear optical effects, such as stimulated Raman spectroscopy (SRS) and coherent anti-Stokes Raman spectroscopy (CARS), providing higher spatial resolution and allowing rapid data collection. The sensitivity of Raman spectroscopy can also be enhanced through other techniques, such as resonance Raman spectroscopy, surface-enhanced Raman spectroscopy (SERS), or tip-enhanced Raman scattering (TERS) [54]. TERS combines scanning probe microscopy (SPM) with Raman spectroscopy to achieve high spatial resolution spectroscopy, down to the nanometer level, which can investigate biological processes such as protein folding and DNA replication.

Biological samples are complex and diverse, which results in varied Raman spectra data. Biological molecules such as lipids, proteins, nucleic acids, and carbohydrates share some bonds (e.g., C-H, C=O, etc.), which make the Raman spectra of different biological molecules overlap, thereby presenting difficulties in data interpretation. Therefore, effective raw data pretreatment (spectral axis alignment, spike removal, background correction, smoothing, normalization, and outlier removal [56]) is essential for the precise qualitative and quantitative analysis of biological samples. It is believed that an accurate peak definition can have a significant influence on the reliability of the results. A wide range of the most frequently seen peaks in biological Raman studies are presented [57]. Further, the multivariate analysis methods of Raman data can be broadly classified into two categories: unsupervised (unlabeled) and supervised classifications (labeled for discrimination), where principal component analysis (PCA) is a commonly used unsupervised procedure for complex datasets to find significant spectral variance and linear discriminant classifiers (LDCs), partial least squares regression (PLSR), and support vector regression (SVR) are represented methods often used in supervised classification [58].

Raman spectroscopy has recently been reported to have higher resolution than FCM at the single-cell level [59,60], which can reflect more bacterial genotypes than phenotypes because more biochemical information can be recorded. Additionally, combinations such as Raman isotope probing and Raman-FISH can be used to characterize metabolic activity at the single-cell level [61]. Furthermore, to actively link the function with their genotypes, Raman-activated cell sorting techniques, such as Raman-activated cell ejection (RACE, [62]), Raman-activated microfluidic sorting (RAMS, [63]), and optical tweezers [64], are available to offer a culture-independent approach to isolate individual cells for downstream sequencing.

2.5. Integrated Microfluidic Single-Cell Techniques

Microfluidics is a technique for manipulating and controlling fluids in the range of micro- to pico-liters in networks of channels with dimensions from tens to hundreds of microns [65]. Microfluidic chips can be flexibly designed to have desired size, shape, and geometry to fulfill the demands of diverse single-cell manipulation and analysis tasks. There are different microfluidic methods to capture or isolate single cells, such as the hydrodynamic, electrical, optical, acoustic, and magnetic methods, among which the electrical methods, especially dielectrophoresis (DEP), have high accuracy and great flexibility owing to the various parameters covered (e.g., the size of the cells, the dielectric properties of the cells and the surrounding solution, the gradient of the electric field, and the frequency of the electric field), but they have shortcomings such as lower throughput than the hydrodynamic methods. Optical tweezers combined with microfluidic chips could manipulate particles within the size range of nanometers to tens of microns under

continuous flow for cell sorting [66]. Further, there are various detection methods, such as fluorescence microscopy, fluorometry, and mass spectroscopy, that can be combined with microfluidic systems for single-cell analysis, from cell morphology to secreted proteins.

Automated and integrated microfluidic chips allow for real-time observations and investigations of the environmental signals' influence on cellular response dynamics by delivering input signals to single and isolated suspensions or adherent cells in a precisely controlled manner [67]. Furthermore, microfluidic devices can be used to isolate and analyze individual cells from environmental samples, such as soil or water, allowing for environmental metagenomics analysis at the single-cell level. This can provide a statistically rigorous approach to extracting novel microbial genomes while preserving single-cell resolution [68]. Further, microfluidic devices can mimic environmental stress conditions, such as changes in temperature, pH, or nutrient availability, allowing for the study of stress response phenotypes at the single-cell level [69].

3. Applications in Environmental Microbiology

Single-cell analysis may highly accelerate the research on environmental microbiology for the observation, isolation, and identification (Figure 2) of the functionally tolerating/sensitive microbial species, such as the bio-degraders of emerging contaminants. These approaches may shorten the time it takes to derive contaminant degraders and their synergetic species, thereby supporting contamination remediation. In order to provide an overview of the differences among single-cell technologies, a comparison table was created to highlight the achievements and variations in the measurement and analysis of different methods (Table 1). This allows for an easy and comprehensive evaluation of the technologies available.

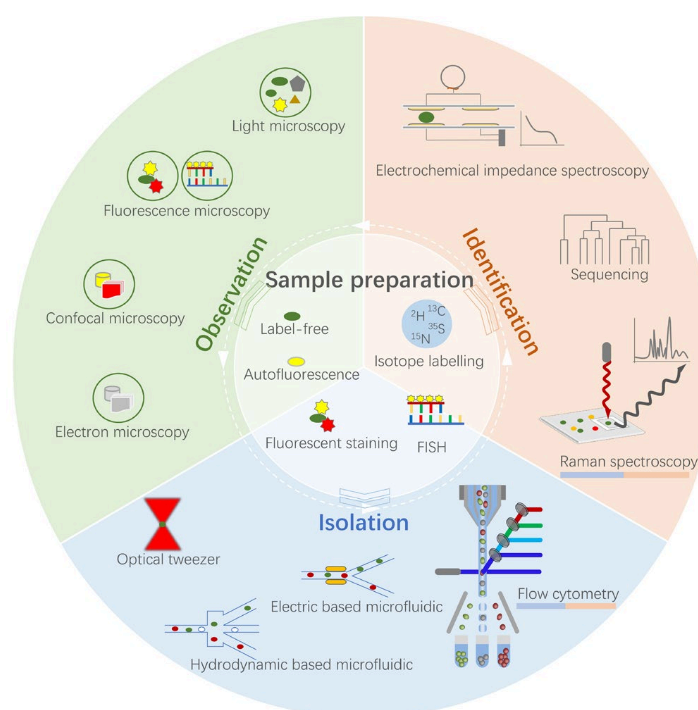

Figure 2. Workflow for single-cell analysis in terms of observation, identification, and isolation of cells at the single-cell level. Sample preparation mainly includes label-free, autofluorescent, and fluorescently stained cells, isotope-labeled cells, and FISH-labeled cells. Observation of single cells mainly uses light microscopy, fluorescent microscopy, confocal microscopy, and electron microscopy. Identification of single cells can be achieved by sequencing, electrochemical impedance spectroscopy, and Raman spectroscopy. Isolation of targeted single cells can be performed through optical tweezers, microfluidic chips, and flow cytometry.

Table 1. A comparison table in terms of achievements and variations in the measurement and analysis of different methods.

Technologies	Examples in Achievements	Regression Analysis and Outlook
Microscope	Energy Dispersive X-ray Analysis (EDX) system combines with SEM or TEM to identify the elemental composition in a sample [35].	Microscopic images are subject to a variety of factors, such as instrument limitations, signal intensity or image contrast, sample preparation, and experimental conditions. Regression analysis can be employed to examine the correlation variables to eliminate or control the discrepancies, leading to more precise and reliable measurements. The future of microscopy will focus on the application of super-resolution microscopy, multiphoton microscopy, and CRISPR-based microscopy to visualize specific DNA or RNA sequences within cells.
	Confocal microscopy combined with Raman spectroscopy reveals the spatial distribution of the compounds within a sample [13].	
Flow cytometry	Investigate the dynamic community assembly in wastewater treatment plants to discover perturbation-associated symptoms for community control [37].	Flow cytometry measurements are susceptible to several sources of variability, including instrument noise, variations in sample preparation, and different experimental conditions. Statistical regression analysis can be applied to flow cytometry data to account for these sources of errors. Therefore, regression analysis is required to correlate the fluorescence signal from the cell population with various independent variables, such as cell size, granularity, instrument gain, and protein expression levels. The future of flow cytometry looks promising, with advancements moving towards high-throughput analysis, imaging flow cytometry, and AI-powered data analysis. With these developments, the potential for this technology to revolutionize biological research is enormous.
	Automatic online monitoring of the community changes as an early-warning tool to reflect/control drinking water processing operation [38–40].	
	Automated approaches have been established for flow cytometric phenotypic diversification, including phenoflow [46], flow FP [47], PhenoGMM [48], and flowEMMi [49].	
Raman spectroscopy	By analyzing the Raman spectra of small particles in water samples, researchers have been able to identify and quantify microplastics, which pose a threat to marine life and ecosystems.	There are various sources of errors in Raman spectroscopy, including instrumental noise, sample heterogeneity, fluorescence, and solvent effects. To account for the error in Raman spectroscopy, regression analysis can be used to quantify the amount of a particular chemical in a sample based on the signal intensity of a Raman peak that is associated with the chemical, or to correct for interferences or background signals that may be present in the Raman spectrum to improve the accuracy of the quantitation method. The outlook for the development of Raman spectroscopy is to develop portable systems for in-field applications. And combining Raman with other techniques, such as infrared spectroscopy, surface-enhanced Raman spectroscopy (SERS), and fluorescence spectroscopy, can obtain more comprehensive information about samples.
	Confocal Raman microscopy allows for three-dimensional imaging of samples with high spatial resolution [13].	
	Tip-enhanced Raman spectroscopy combines scanning probe microscopy (SPM) with Raman spectroscopy to achieve high spatial resolution spectroscopy down to the nanometer level, which can investigate biological processes such as protein folding and DNA replication [54].	
Microfluidic single-cell techniques	The microfluidic device allows for real-time observations of apoptosis in intracellular signaling pathways in single cells [67].	In microfluidic chips, the regression of error refers to the process of analyzing and quantifying the accuracy and precision of the device's performance. This is typically achieved by comparing the results obtained from the chip to a known value or established standard. To accomplish regression of error in microfluidic chips, various statistical methods are used, such as linear regression and least-squares analysis. These methods allow researchers to determine the relationship between different variables and identify any sources of error in the system. The future of microfluidic chips is exciting and full of potential. Major advancements are expected in the development of miniaturized, easy-to-use, inexpensive, and highly integrated microfluidic systems.
	Microfluidic devices can be used to isolate and analyze individual cells from environmental samples, such as soil or water, allowing for environmental metagenomics analysis at the single-cell level. This can provide a more accurate understanding of microbial diversity and function in complex environments [68].	
	Microfluidic devices can mimic environmental stress conditions, such as changes in temperature, pH, or nutrient availability, allowing for the study of stress response phenotypes at the single-cell level [69].	

In the microbial environment, persistent organic pollutants are highly concerning due to their recalcitrance, toxicity, mutagenicity, and carcinogenicity. Flow cytometry has been used to detect the absorption of polycyclic aromatic hydrocarbons (PAHs) to cell surfaces and the toxicity to cell growth, as well as to study the microbial responses to the PAH degradation [70]. These relative studies mainly depend on the single-cell analysis (e.g., cell abundance changes over time) rather than single-cell isolation and enrichment. Currently,

the isolation and enrichment of target synergistic degraders mainly rely on cultivation approaches at the lab scale, with the majority of microorganisms remaining uncultured and therefore poorly characterized. Therefore, cultivation-independent technologies have been greatly expanded to reveal novel functional microorganisms. However, a single technique cannot provide a comprehensive understanding of the biology of these microorganisms and their functions; thus, the integration of cultivation-independent multi-techniques may broaden the view on understanding the microbial ecosystem. For example, microscopy combined with microfluidic chips could enable direct, high-resolution observation of the cell morphology of the natural bio-degrader biofilm surrounding the contaminants. Flow cytometry analysis and cell sorting combined with sequencing enable monitoring of the real-time-dependent microbial community succession under contaminant exposure with comprehensive information on identifying and isolating the synergetic microbial species. Raman spectroscopy combined with microfluidic chips gives deeper insight into individual cell differences at the genotype level.

Furthermore, for contaminants (e.g., PAHs) with known efficient degraders, single-cell techniques may help investigate the surviving mechanisms of degraders in natural soil communities. Real-time tracking fluorescence microscopy combines fluorescence microscopy with microfluidic chips to observe and quantify the phenotypic changes of individual pollutant-degrading cells and the dynamic changes of typical proteins within a degrader. This enables full understanding of the colonization and survival of invading contaminant degraders and their interactions with native environmental communities. The combination of flow cytometry and fluorescence staining (e.g., PI, SYBR Green, DAPI, etc.) further enables the observation and isolation of cells with special functionalities for deeper downstream analysis (e.g., sequencing to identify "who" is there or cultivating to enrich the abundance of one or multiple target strains as effective microbial inoculum for environmental microbiology applications).

Overall, microorganisms play a crucial role in the biogeochemical cycles of natural ecosystems. However, traditional methods of studying microbial communities often result in inaccurate results due to averaging across the whole population. Single-cell technologies have emerged as critical tools that help researchers study individual microbial cells accurately, providing a more comprehensive understanding of microbial diversity and their impact on the environment. In addition, single-cell technologies are crucial in identifying and studying unknown microorganisms for developing biotechnological applications such as bioremediation or biofuel production. Furthermore, environmental factors like pollutants, temperature change, and pH significantly impact microbial communities. Single-cell technologies are essential in studying the impact of these stressors on individual cells, providing valuable insights into the effects of environmental changes. Single-cell technologies provide vital information on the number and activities of microorganisms that supports environmental monitoring programs in evaluating the health of ecosystems. In summary, developing single-cell technologies is essential in the environmental field for understanding microbial diversity, identifying new microorganisms, assessing ecosystem health, and studying environmental stressors.

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