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REVIEW

Recent advances and application in whole-genome multiple displacement amplification

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***Background*: The extremely small amount of DNA in a cell makes it difficult to study the whole genome of single cells, so whole-genome amplification (WGA) is necessary to increase the DNA amount and enable downstream analyses. Multiple displacement amplification (MDA) is the most widely used WGA technique.**
***Results*: Compared with amplification methods based on PCR and other methods, MDA renders high-quality DNA products and better genome coverage by using phi29 DNA polymerase. Moreover, recently developed advanced MDA technologies such as microreactor MDA, emulsion MDA, and micro-channel MDA have improved amplification uniformity. Additionally, the development of other novel methods such as TruePrime WGA allows for amplification without primers.**
***Conclusion*: Here, we reviewed a selection of recently developed MDA methods, their advantages over other WGA methods, and improved MDA-based technologies, followed by a discussion of future perspectives. With the continuous development of MDA and the successive update of detection technologies, MDA will be applied in increasingly more fields and provide a solid foundation for scientific research.**

Keywords: whole genome amplification; multiple displacement amplification; improved MDA-based approaches

Author summary: The extremely small amount of DNA in a cell makes it difficult to study the whole genome of single cells, multiple displacement amplification (MDA), as the most widely used whole-genome amplification (WGA) technique, is necessary to increase the DNA amount and enable downstream analyses. In this review, we focus on the principles and characteristics of MDA and summarize the advantages and disadvantages of MDA compared with other WGA methods. We also discuss a selection of recently developed MDA methods, their advantages over other WGA methods, and improved MDA-based technologies, followed by a discussion of future perspectives.

INTRODUCTION

Cells are the fundamental units of life and every important life activity is closely related to individual differences between cells. Cells are heterogeneous and have unique genomes, which is true not only for tumor cells but also somatic cells [1,2]. Some important issues in life sciences and medicine rely on very few cells such as human oocytes [3] and circulating tumor cells (CTCs) [4]. However, the extremely small amount of DNA in a cell makes it difficult to study the whole genome of single cells. In these cases, whole-genome amplification (WGA) is necessary to increase the DNA amount and enable downstream analyses.

A plethora of different WGA methods have been reported in the past decades, and some traditional methods have also undergone continuous optimization and innovation. Current WGA methods can be divided into three categories: (i) methods based on polymerase chain reaction (PCR), (ii) methods based on multiple displacement amplification (MDA), and (iii) other novel methods. Zhang *et al.* [5] implemented primer extension pre-amplification polymerase chain reaction (PEP-PCR)

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technology to achieve the amplification of single haploid cells in 1992. PEP-PCR was among the first single-cell WGA methods used, followed by the more widely adopted degenerate oligonucleotide-primed PCR (DOP-PCR) [6]. The concept of MDA based on strand displacement and rolling circular amplification was first proposed in 1998 [7] and was developed into a mature WGA method by Dean *et al.* [8]. Other novel methods, such as multiple annealing and looping based amplification cycles (MALBAC) [9] and linear amplification via transposon insertion (LIANTI) [10] were reported in recent years, and provide alternatives for WGA. Nevertheless, MDA continues to be the most popular WGA method. MDA mainly depends on a mild isothermal reaction, and therefore a thermal cycler is not needed. High-quality DNA products are conveniently obtained using phi29 DNA polymerase and random hexamer primers. Amplification bias was once the main disadvantage of this method, which prevented MDA from being more widely adopted. Some innovative MDA methods using microfluidic chips, micro-channels, and droplets have been developed to further improve amplification uniformity. This review focuses on the principles, advantages, and innovations of MDA. Further discussion of other WGA methods is beyond the scope of this article, as there are several detailed reviews on this topic [11,12].

In this review, we focus on the principles and characteristics of MDA and summarize the advantages and disadvantages of MDA compared with other WGA methods. We also discuss how recent studies have solved the drawbacks of non-uniform MDA, including some approaches that implement modified enzymes or primers in the reaction conditions. Additionally, this review also addresses the applications of MDA in specific biomedical fields such as cancer research, DNA identification, preimplantation genetic diagnosis (PGD), and metagenome research.

MDA CHARACTERISTICS AND PROCESS

Currently, WGA technology can effectively amplify single-cell or even single-molecular-weight nucleic acids to a certain extent. Due to the effectiveness of WGA, single-cell whole-genome sequencing has become an important branch of molecular biology studies. MDA is the most commonly used approach out of the current WGA methods [12] due to its high DNA yield, high fidelity, and low amplification bias [8].

phi29 DNA polymerase amplification process

MDA has been widely adopted since it was first described. The process is catalyzed by phi29 DNA polymerase, which has a strong strand displacement and exonuclease capacity under isothermal conditions (typically 30°C) using circular or linear DNA as a template. The random hexamer primers employed in this method become endonuclease-resistant after being modified with phosphorothioate [13]. Annealing begins when the random hexamer primers are linked to multiple sites on the DNA template sequences. Then, the phi29 DNA polymerase begins synthesizing DNA strands along the template from the multiple positions where the primers have adhered. The newly synthesized DNA strand ultimately replaces the complementary strand of the template in the extension reaction, and the replaced DNA strand is used as the new template to start a new replication [14]. Finally, a network of hyperbranched DNA structures is generated (see Fig. 1). Therefore, DNA is amplified through MDA until enough sample is accumulated to meet the research needs. Furthermore, molecular crowding can be achieved by adding polyethylene glycol (PEG) to the reaction to increase MDA efficiency [15], which enables the detection of a large number of alleles in multiplex short tandem repeat (STR) genotyping. However, heating, temperature control, and detection are separate modules of the MDA reaction, which reduces the portability of the method. Combining sensors for different physical parameters into one system (*e.g.*, light and temperature sensors [16]) can improve the level of integration. In 2019, Bruijns *et al.* [17] constructed a lab-on-a-chip (LOC) system for real-time monitoring of MDA, which integrated all sensors (temperature and photosensor) and actuators (heater). DNA production is monitored by a fluorescent dye $[Ru(phen)_2(DPPZ)]^{2+}$ that has strong photoluminescence in the presence of DNA, which allows us to observe the amount of DNA or the reaction process of MDA in real-time through fluorescence.

In vitro isothermal methods, which work at a temperature range of 30–65°C, rely on the strand-displacement activity of mesophilic DNA polymerases such as phi29 [18–20]. The first article on the use of phi29 DNA polymerase on single-cell MDA was published in 2004 [21], which was followed by more detailed studies. Moreover, phi29 DNA polymerase is now known to possess great advantages over others, for instance, this polymerase binds strongly to templates, allowing for continuous amplification of 100 kb DNA products without separation from the template [22,23]. The average fragment length is > 10 kb after amplification [24]. Because of its strong template-binding force, the amplification process is not affected by the base composition of the template sequence, short tandem repeats (STR), or secondary structures, which reduces the slippage rate of enzymes from microsatellite loci. The enzyme also has 3′–5′ exonuclease activity, and therefore the mismatch rate during DNA synthesis is only between

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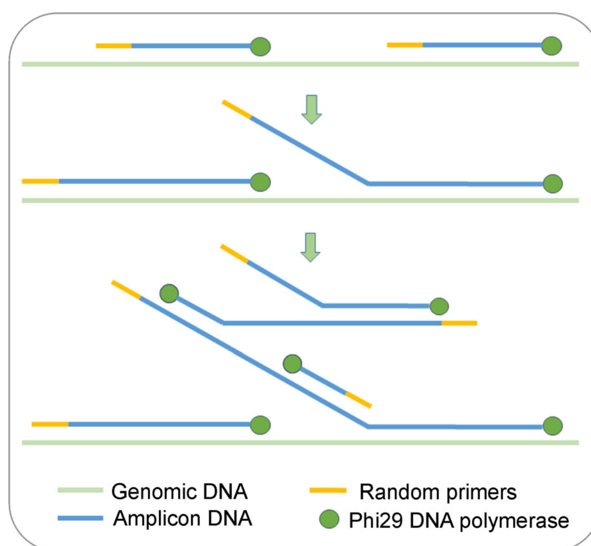

Figure 1. Multiple displacement amplification (MDA) process diagram.

$10^{-6}-10^{-7}$, which is 100 times lower than that of Taq DNA polymerase [25,26]. This feature provides high replication fidelity and also promotes its processivity. Furthermore, phi29 has an inherently high processivity that allows it to replicate the whole genome from a single binding event without the support of processivity or unwinding factors. Therefore, MDA can exponentially amplify trace DNA templates (from 1–10 copies of genomic DNA) to produce 20–30 µg of highly-stabilized DNA product. A Haplox-method single-cell whole genome amplification kit (HaploX) was thought to amplify from 0.1 copies of the genome (approximately 3 pg) with higher coverage and specificity than traditional MDA [27]. The yield of each MDA reaction was largely similar regardless of the amount of template, which is ideal for high-throughput experiments [8]. Finally, the most convenient aspect of MDA with phi29 DNA polymerase is that it eliminates the need to design target-specific primers. However, the MDA process becomes rather chaotic due to the presence of random primers. Therefore, developing an MDA process simulation software [28] and monitoring the reaction process through pH variations [29] and fluorescent dyes [17] is of great significance.

MDA characteristics and advantages

Compared with PEP-PCR and DOP-PCR, MDA provides higher uniformity and complete genome sequences, which minimizes site amplification error and maintains the genetic information of the product consistent with the template. Traditional samples often require many processing steps, which makes WGA labor-intensive and limits its throughput to some extent. In an MDA reaction, the sample can be used as a template directly without purification [30]. Additionally, if 0.05 µL of blood is added to 100 µL of the MDA reaction system, the blood is diluted 2000 times. However, some factors that inhibit the reaction in the blood, such as hemoglobin and EDTA, are also diluted 2000 times and therefore their effect becomes negligible [31]. As stated above, the whole genome must be amplified with minimum site errors to provide the highest genome coverage possible so that the product maintains its complete genetic information. Each WGA method produces a certain sequence bias depending on primer efficiency, template binding force, GC content, and distance between telomeres and centromeres [32]. MDA can achieve similar amplification efficiency for each site distributed in different positions of the genome, with an average amplification bias of only 2.5, whereas the range of DOP-PCR is 10^3-10^4 and that of PEP-PCR is 10^2-10^4 [33]. This indicates that MDA can completely amplify each locus, thus ensuring the accuracy of downstream genetic analyses.

The Illustra GenomiPhi V2 DNA Amplification Kit (GE Healthcare) utilizes the traditional MDA protocol of random hexamer primers. However, similar to the random octamer primer mentioned above [27], the REPLI-g kit (Qiagen) was found to improve the performance of unbiased MDA by using specific primers for common genome sequences instead of random primers [34]. For example, highly complex nucleic acid samples can be efficiently amplified using only one or more specifically selected or designed primers with specific nucleic acid

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sequences. The REPLI-g series of WGA kits is developing rapidly, and even an UltraFast version (REPLI-g UltraFast Mini Kit) has been released, which can effectively and uniformly amplify the target genome within 1.5–2 h. One study compared the performance of different WGA kits in allele recovery, allele dropout (ADO), and allele drop-in (ADI). Although this study included GEH-MDA (Illustra GenomiPhi V2 DNA Amplification, GE Healthcare), the results indicated that RG-MDA (REPLI-g Single Cell kit, Qiagen) was the only method to increase the sensitivity of DNA profiling [35].

Lasken pointed out that MALBAC showed higher and more uniform genomic coverage than MDA under the same conditions, but also higher false positives, which may be due to the low fidelity of the Bst and Taq polymerases [36]. De Bourcy *et al*. [37] evaluated the strengths and weaknesses of three WGA methods (PEP-PCR, MDA, and MALBAC) by accounting for the effect of reaction gain on uniformity, error rate, and the level of background contamination. Some studies have concluded that the product of MDA is directly proportional to the amplification bias, with higher yields leading to greater bias. However, there was no significant correlation between the amplification uniformity and DNA product in MALBAC and PEP, which is a problem that remains to be solved.

The coefficient of variation (CV) is typically used as the key parameter to evaluate amplification uniformity in CNV analysis results based on next-generation sequencing (NGS). Lower CV values are indicative of a higher CNV analysis accuracy. Fibroblast samples were subjected to low-depth, high-throughput sequencing at the single-cell level. Afterward, the detection rate of CNV and the coefficient of ADO were compared between MALBAC and MDA [38]. It was observed that the CV of CNV detection in the MALBAC group was significantly better than that of MDA. Moreover, the total ADO rate of the MALBAC group was 4.55%, which was significantly lower than 22.5% in the MDA group. Nonetheless, when the amount of initial templates increased, the ADO of MDA decreased significantly and there was no visible distinction between the two methods. Furthermore, some comprehensive technologies serving PGT-M such as single-nucleotide polymorphism arrays (SNP-arrays), metaphase comparative genomic hybridization (mCGH), and array-CGH (aCGH), require DNA amplification, and therefore several WGA technologies have been implemented. Del Rey *et al*. [39] tested four different WGA kits (DOP-PCR, MALBAC, GEH-MDA, and RG-MDA) using the TruSight One (TSO) Sequencing Panel (Illumina). Compared with DOP-PCR and MALBAC, both MDA-based methods were suitable for the TSO-NGS platform; however, RG-MDA performed better. This study also reported that the Nexus software can be used to analyze the TSO sequencing data from RG-MDA products for CNV with decent performance.

However, other studies have indicated that MDA is less suitable for CNV analysis than several other WGA methods. In a study by De Bourcy *et al*. [37], high coverage rates and inherently low error rates made MDA more suitable for the study of single-nucleotide variants (SNVs), in which the amplification-induced error rate of MALBAC and PicoPLEX was approximately 10 times that of MDA. Similarly, He *et al.* [40] conducted β-thalassemia genotyping and SNP/CNV through MALBAC and MDA and concluded that the amplification efficiency of MDA was higher than that of MALBAC, with superior SNP detection using NGS. However, when CNVs were detected at the single-cell level, MALBAC displayed higher stability than MDA. The authors also concluded that MALBAC is more well-suited for CNV detection, whereas MDA is more appropriate for SNV detection. To optimize the application of MDA in CNV, multifraction amplification (mfA) technology based on digital counting coupled with single-cell whole-genome sequencing may achieve a higher CNV detection resolution, in addition to higher uniformity and lower noise [41]. To address the fact that not every DNA fragment can be successfully amplified, mfA utilizes the segment-merge maximum likelihood algorithm to estimate copy numbers by accounting for the probability of amplification.

In addition to the above-described methods, many other WGA methods have been reported. In 2017, Xie *et al*. proposed the linear amplification via transposon insertion (LIANTI) method, a novel WGA approach. This entire process relies exclusively on linear amplification, which greatly enhances amplification stability and reduces PCR interference. Furthermore, this technology enables micro-CNV detection at kilobase resolutions and with genome coverages as high as 97% [10]. Nonetheless, this technology was proposed relatively late and had few applications. Different WGA methods have different advantages and disadvantages. Table 1 summarizes the differences between the above-described methods to enable researchers to select the appropriate methods based on their desired amplification results.

IMPROVED MDA-BASED APPROACHES

The MDA reaction is known to have three main defects: non-specificity of amplification due to random primers or DNA contamination or primer dimers, non-uniformity due to amplification bias, and generally long reaction times. The third can be addressed with more quantitative templates, such as the UltraFast version of REPLI-g. Non-specific or template-independent amplification (TIA) caused false-positive results. Blainey *et al*. [45] reported

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Table 1 Comparison of mainstream WGA methods

WGA methods	Enzyme	Product length	Coverage	Advantage	Disadvantage	Refs.
PEP	DNA polymerase	< 2 kb	~50%	Low template quality requirements, simple operation, and easy improvement	Low yield and fidelity; easy to cause amplification bias and fragment loss	[5,42]
DOP-PCR	DNA polymerase	< 2 kb	~40%	Simple operation, the minimum template amount can reach 50 pg, and the product fragment is 0.5~10 kb	Large amplification bias at low template amounts	[6,43]
MDA	Phi29 DNA polymerase	< 100 kb	~70%	High product, low DNA template amount, great fidelity	Non-uniformity, may have non-specific products	[8,37]
MALBAC	Bst enzyme and Taq DNA polymerase	< 2 kb	~90%	Simple operation, high yield, minimum template amount is several pg, uniform amplification	Amplification is difficult when the amount of initial template is low, and non-specific amplification may occur	[9,44]
LIANTI	Transcriptase and T7 RNA polymerase	~400 bp	~97%	Linear amplification enhances amplification stability and high coverage	Multiple amplification steps and few applications	[10]

that high molecular weight contaminants in the polymerase mixture and not the MDA reagents resulted in background amplification. To solve this defect, Wang [46] developed a reliable MDA protocol using a 5′end-blocked random pentamer primer. Random pentamer primer MDA reactions rely on the template for amplification, and therefore this reaction is referred to as template-dependent MDA (tdMDA). The potential of tdMDA has also been demonstrated using a protocol that effectively amplifies circular RNAs (circRNAs) and almost completely inhibits TIA [47]. A selection of innovative and widely-adopted approaches will be discussed below.

TruePrime

In addition to improving MDA system primers, Picher *et al*. [48] innovatively applied a *Thermus thermophilus* primase-polymerase (*Tth*PrimPol) enzyme, which had strong primase activity and preferentially utilized dNTPs as substrates, unlike conventional primases. *Tth*PrimPol can bind denatured DNA templates at multiple sites to produce short primers. Afterward, the phi29 DNA polymerase can take over the extension of these primers and perform strand displacement (Fig. 2). TruePrime, a method that combines *Tth*PrimPol with phi29 DNA polymerase, is certified primer-free and delivers even genome coverage and preeminent SNP detection with low ADO and chimera formation as exemplified by sequencing HEK293 cells. Furthermore, better CNV calling results were obtained with this approach compared with MDA methods based on random primers, which were expected to accelerate single-cell genome analysis. Based on these observations, the TruePrime single-cell WGA kit (Sygnis GmbH, Germany) represents a sizeable proportion of the market.

WGA-X

Microbial single-cell genomics is a tool that is often used to characterize metabolic potential, interactions, and evolution of uncultured microorganisms. Although MDA is currently the most widely used method in microbial genomics, as mentioned earlier, single-cell MDA has an inherent and potential bias against high GC templates. Additionally, this approach recovers only an average of < 50% of the genome from individual microbial cells, even at a depth of 1,000× sequencing [49]. Stepanauskas *et al*. [50] developed WGA-X, a novel gDNA amplification method, by utilizing a thermostable mutant of the phi29 polymerase, which exhibited a better genome recovery from individual microbial cells and viral particles than conventional MDA. According to the authors, genome recovery improvements may derive from the higher reaction temperature of WGA-X (45°C), which promoted initiation, DNA denaturation, and dissociation of high GC regions from other cellular components. The combination of WGA-X, high-throughput genome sequencing, and fluorescence-activated cell sorting (FACS) enables the analysis of genome sequences from limited cell numbers (*i.e*., hundreds of individuals), uncultured bacteria, archaea, protozoa, and viruses that involve natural microbiomes.

Primary template-directed amplification

In particular, one of the factors that result in MDA non-uniformity is error re-copying. Gawad *et al*. [51] of St. Jude Children's Research Hospital developed the primary template-directed amplification (PTA) approach, which is a new MDA-based WGA method that incorporates an extension terminator to achieve a

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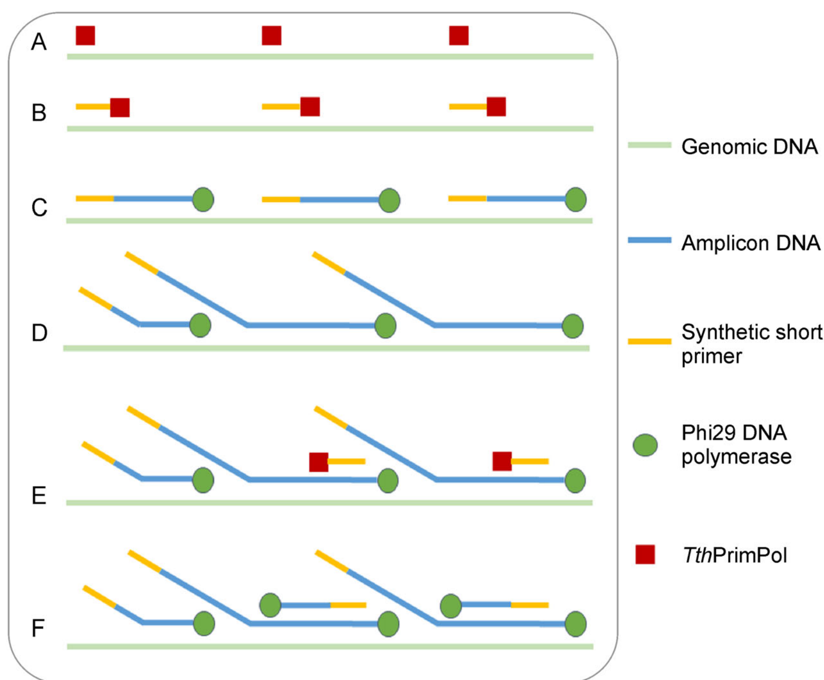

Figure 2. Schematic diagram of the TruePrime reaction process. (A) T*th*PrimPol binds to denatured DNA templates at multiple sites. (B) Synthesis of short DNA primers. (C) The phi29 DNA polymerase takes over the extension of these primers. (D) The strand-displacement capacity of phi29 DNA polymerase leads to the exposure of new single-stranded template regions. (E) Synthesis of new primers on the displaced ssDNA. (F) The new primers are extended by phi29 DNA polymerase resulting in exponential amplification.

quasi-linear amplification process. Exonuclease-resistant random primers (6–9-mers) were added to the reaction to randomly combine with DNA templates. Unlike previous MDA methods, they used an amplification mix containing alpha-thio-ddNTPs to prevent recopying of the amplification products, which reduced errors and genome coverage was dramatically increased as the amplification reaction became significantly more uniform (Fig. 3). This study conducted a comprehensive comparison between PTA and common single-cell WGA methods. Surprisingly, PTA demonstrated excellent genome uniformity performance, SNV sensitivity, and SNV specificity, as well as greater cell-to-cell reproducibility. This approach is currently the core technology of BioSkryb corporation (USA), which provides new solutions for cell heterogeneity and clinical research. The characteristics and advantages of representative improved approaches based on MDA discussed in this section are summarized in Table 2.

NOVEL MDA METHODS BASED ON MICROFLUIDIC TECHNOLOGY

Due to inherent biases in the MDA reaction mechanism, the main source of non-uniform amplification is the continuous amplification of what was preferentially amplified early in the reaction and is further determined by the reaction conditions. The maturity of MDA as a WGA technology depends on overcoming or greatly reducing these biases. To achieve this, microfluidics offers new possibilities for MDA development.

MDA microreactor

Non-uniform amplification is reflected as highly uneven sequencing depth distribution in the final sequencing data, which greatly limits the application of MDA for CNV detection. To address the problem of uneven single-cell MDA amplification, Quake *et al*. [52] used a microfluidic chip to isolate single *E. coli* cells into microfluidic cavities for nanoliter-level MDA reactions in 2007. Compared with the microliter volume of conventional MDA experiments in centrifuge tubes, the proposed procedure inhibited non-specific amplification and rendered higher genome coverage. In 2013, Gole *et al*. [53] reported a microwell displacement amplification system (MIDAS), in which single cells were randomly distributed into microwells, after which MDA was performed in the physically isolated nanoscale wells. Based on the same

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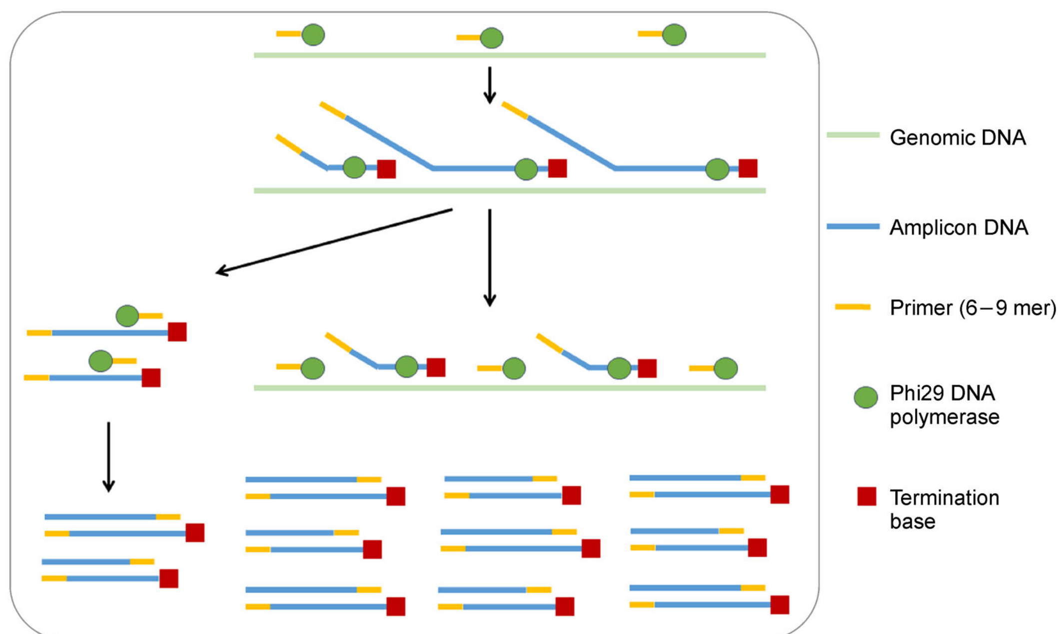

Figure 3. Schematic diagram of PTA. Primers bind to DNA templates at multiple sites and begin to extend under the action of the phi29 DNA polymerase. Afterward, a proprietary set of nucleotides is used to prevent the recopying of the amplification products. The reaction conditions are then modified to drive the primers back to the original template, preventing re-copying of daughter amplicons. The whole process is quasi-linear.

Table 2 Characteristics and advantages of representative improved approaches

Methods	Characteristics	Advantages	Refs.
TruePrime	*Tth*PrimPol enzyme	Primer-free, evenness of genome coverage, preeminent SNP detection with low ADO, better CNV calling. TruePrime single-cell WGA kit (Sygnis GmbH, Germany) is currently in the market	[48]
WGA-X	A thermostable mutant of phi29 polymerase	Better genome recovery from individual microbial cells and viral particles, the higher reaction temperature (45°C), may address the potential bias of high GC templates	[50]
PTA	Extension terminator	A quasi-linear amplification process, excellent genome uniformity performance, SNV sensitivity, SNV specificity, and greater cell-to-cell reproducibility	[51]

principle, the DNA template is then wrapped into picoliter-level droplets, and subjected to the MDA reaction. The emulsion WGA (eWGA) method [14] is an example of this approach.

Conventional WGA methods such as DOP-PCR, MDA, and MALBAC require extensive amplification of the cell genome with DNA polymerase *in vitro*, after which a library is constructed for high-throughput sequencing. However, DNA polymerase amplification errors can lead to false positives and high-throughput sequencing reads are unlikely to contain any haplotype information due to their relatively short lengths. Single-stranded sequencing using microfluidic reactors (SIS-SOR) can solve the above problems [44]. First, the positive and negative double strands of chromosomal DNA of a single cell were separated using a microfluidic processor. The DNA was then randomly divided into a large number of nano-liter components, after which the separated DNA fragments were randomly distributed using a rotary pump and MDA was performed. Finally, all amplified DNA was collected and converted into a barcoded sequencing library so that the complementary double strands of homologous chromosomes could be sequenced independently. Potential applications of this technology include accurate detection of CTCs in blood, screening of healthy embryonic cells for *in vitro* fertilization, and detection of gene-edited human cells.

Emulsion MDA

eWGA utilizes microfluidic technology to encapsulate DNA fragments in a picoliter of emulsion

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for amplification and to include other materials for isothermal MDA. Each fragment is amplified in a limited system, which is more uniform than a conventional MDA reaction. eWGA is superior to the existing single-cell amplification methods in many aspects. For example, MDA is more susceptible to environmental contamination, and eWGA prevents over-amplification, thereby effectively reducing DNA contamination. eWGA was proposed as a solution to the relatively poor performance of MDA for CNV detection. The results show that, compared with MDA, MALBAC, and Dopo-PCR, eWGA not only improves genome coverage but also detects SNV and CNV simultaneously with higher accuracy and resolution [14,54]. Abate *et al*. [55] also adopted the droplet MDA method and named it ddMDA. The authors investigated the improvement in coverage and uniformity of MDA with monodisperse droplets generated by a microfluidic chip and droplets of various sizes obtained by vortexing. Rhee *et al*. [56] comprehensively elaborated on the advantages of ddMDA, and further explained the potential relationship between inhibition preference amplification, GC content, and amplification ratio. Figure 4 illustrates the differences between traditional MDA and improved MDA technology using microfluidics.

Innovative droplet generation methods

It is worth mentioning that although microfluidic devices can be created with relative ease, Chen *et al.* [57] proposed a new microfluidic generation device (SiMPLE) to simplify the procedure and overcome some limitations. This device takes ddMDA as the application object, thereby further reducing the difficulty of obtaining monodisperse microdroplets. Uniform monodisperse droplets were generated using shear force by inserting a glass pipette with a diameter of 15 μm into the oil phase and exerting appropriate pressure to liquid in the pipette while rotating the pipette horizontally (Fig. 5A). The method is simple to operate and prevents the possibility of contamination in the process of frequent liquid transportation. Moreover, in 2019 the authors combined microcapillary array (MiCA) droplet generation [60] with eMDA to achieve high-throughput sequencing experiments with zero sample loss, which can be carried out easily in traditional biology labs with common equipment with excellent results [58] (Fig. 5B). Kim *et al.* [59] also developed a method of rapidly emulsifying ddMDA (re-ddMDA), which used a manual microfluidic emulsifier to produce a monodisperse emulsion. The emulsified sample was loaded into a syringe and manually injected into the device. Millions of monodisperse droplets can be generated in second (Fig. 5C). Although this method requires a microfluidic device consisting of a network of bifurcated channels, the device is easy to manufacture and can be purchased from existing commercial suppliers. Furthermore, by applying this method to detect CNVs in single cancer cells, the authors confirmed that similar measurements could be obtained from either sequencing data or unamplified cancer genomes from millions of cells.

Micro-channel MDA

The above three methods greatly reduce the difficulty of obtaining droplets for experiments. However, they still essentially use droplets as the MDA reaction system. Therefore, micro-channel (μcMDA) was also discussed

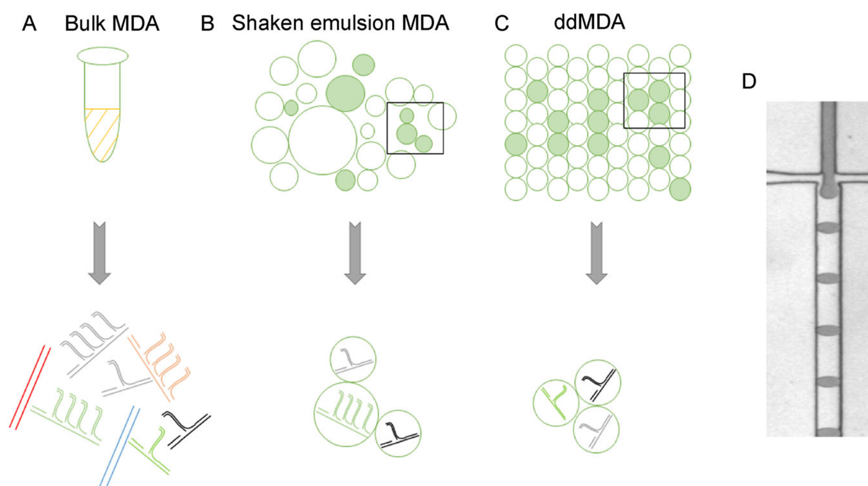

Figure 4. Reaction mode of MDA in different systems. (A) Uncompartmentalized amplification results in uneven amplification. (B) Emulsion MDA greatly improves homogeneity and sequencing coverage. (C) A microfluidic device is used to generate picoliter-level droplets with high uniformity, which can provide complete coverage. (D) Droplets generation process.

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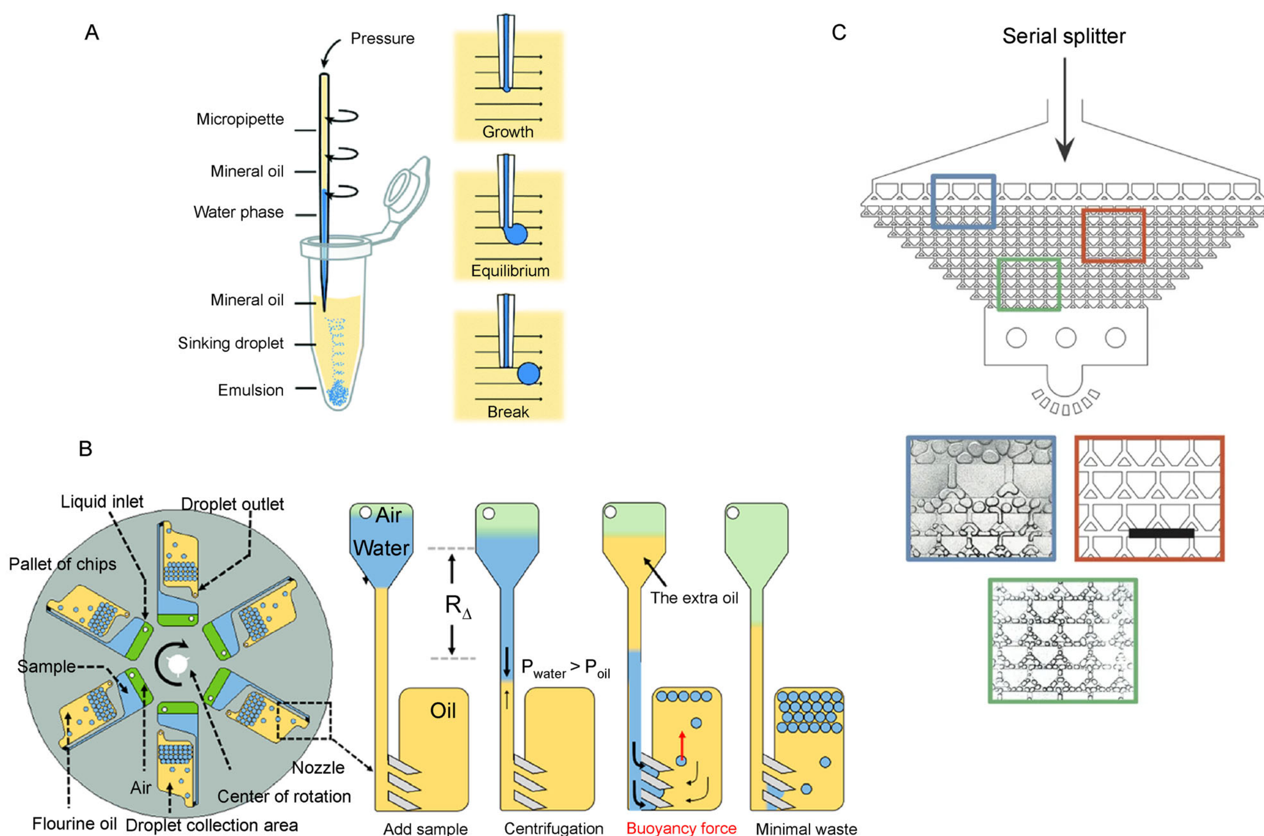

Figure 5. Some innovative droplet generation methods. (A) Schematic diagram of a SiMPLE generator [57]. When the pipette is rotated horizontally, the appropriate pressure is exerted to the liquid in the pipette, and uniform monodisperse droplets will be generated by shear force. (B) Schematic diagram of MiCA [58]. This is a centrifuge-driven droplet generation process based on a micro-capillary array. (C) Schematic diagram of re-ddMDA [59]. Millions of monodisperse droplets can be generated in seconds by manual injection.

herein as a technology that improves uniformity. Li *et al.* [61] optimized the uniformity of MDA by adopting an elongated polytetrafluoroethylene (PTFE/Teflon) capillary as a reaction vessel for MDA, and the conventional three-dimensional reaction space was replaced with an approximately one-dimensional linear reaction space (Fig. 6). The MDA reaction units were evenly distributed in the narrow flow channel, which greatly improved the uniformity of MDA amplification. Furthermore, they also detected CNVs at the single-cell-level through μcMDA, which confirmed that this method can enable subsequent CNV analysis with appropriate resolution and sensitivity [62]. Another benefit of μcMDA is its high GC correction reliability, thereby greatly improving upon the limitations of other microfluidic equipment.

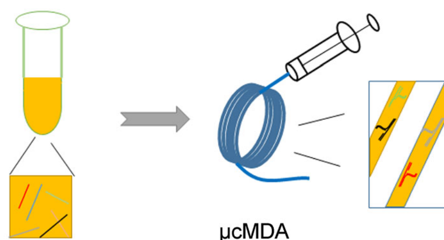

Figure 6. Schematic diagram of μcMDA.

In summary, innovative MDA amplification uniformity technology can be roughly divided into three categories. The first comprises MDA microreactors implemented with a microfluidic device, which appeared earlier. Emulsion MDA was then developed to confine the MDA system to microdroplets. In this stage, droplets of different sizes are generated by the shaken MDA emulsion, and continuous monodisperse droplets are generated by the microfluidic chip. It is worth mentioning that μcMDA not only does not rely on microfluidic technology but also greatly improves amplification uniformity. Therefore, the simple operation and low

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equipment requirements of μcMDA make this a promising technology for whole genome amplification. Table 3 provides a comparison between the three method categories.

MDA APPLICATIONS

As mentioned earlier, the MDA reaction is carried out in a water bath instead of a thermal cycler, and therefore nonspecific amplification products brought by the PCR reaction are avoided. Moreover, the reaction system can be specifically designed to meet the experiment requirements. The MDA technique using phi29 DNA polymerase has been effectively applied in many fields, and may even replace the traditional Taq-PCR and be used in clinical applications. For example, Zhu *et al.* [63] constructed a DNA-nanoparticle composite probe to detect salmonella infections with phi29 DNA polymerase and gold nanoparticles, highlighting the potential of MDA in clinical detection, food safety, and environmental monitoring. Moreover, this technology has also been widely used in single-cell sequencing research [64], clinical diagnosis [65], DNA identification analysis [66], forensic medicine [67], PGD, and metagenomics.

Cancer research

Genomic aberrations are different in cancers of the same histological type. Regardless of the characteristics of somatic genetic aberrations in any two types of tumors, malignant tumor cells can adapt to the complex microenvironment of the host. Tumor cells also exhibit differences, variations, and morphological imparities [68]. Single-cell genome analysis will contribute to the development of more effective cell-targeted therapies, as well as the study of tumor development trends and metastatic mechanisms [69]. In 2012, the BGI institute developed an MDA-based single-cell sequencing technology, which combined sequencing technology with MDA to achieve breakthrough progress in sequencing approaches (Fig. 7). This research group performed whole-exome sequencing of 90 cells from one patient with essential thrombocythemia (ET) using MDA, and confirmed that mutations in SESN2 and NTRK1 were closely related to the occurrence and evolution of ET [70]. MDA-based single-cell sequencing technology has been applied in many studies and cell types including myeloproliferative tumors, colon cancer, and renal clear cell carcinoma, and the findings of these studies have offered insights into previously unknown mechanisms of disease onset.

Table 3 Comparison between microfluidics-based improved approaches

Evaluation parameter	MDA	Microreactor MDA	Emulsion MDA	Micro-channel MDA
Uniformity	Low	Intermediate	High	High
Efficiency	High	Intermediate	High	High
Equipment requirements	Low	High	High or intermediate	Low
Experimental difficulty	Low	High	High or intermediate	Intermediate

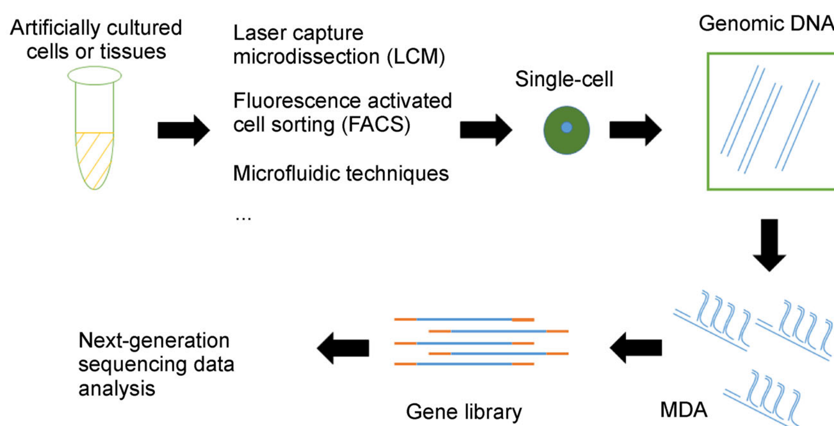

Figure 7. MDA single-cell genome sequencing process. Single cells are isolated from the cell population (cultured cells or tissues) through various single cell separation techniques. The single cell is then lysed to extract its genome and amplified via MDA to obtain a genomic library. The sequencing data is then analyzed for SNP and CNV detection or cell type identification.

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In 2014, Wang *et al*. [71] developed an MDA-based whole-genome and exome single-cell sequencing approach called nuc-seq for breast cancer research. The method is mainly used to analyze single-cell nuclei, monitor the diversity of cancer clones, assess the development and evolution of different clonal subgroups, characterize tumor development, and establish potential treatments. Moreover, MDA was found to be more effective in the unbiased amplification of fresh rare cell DNA than DOP-PCR in metastatic colorectal cancer (CRC) research, which resulted in precise variant calling using the targeted NGS [72]. Additionally, the development of eMDA and μcMDA not only improved the coverage of conventional MDA, but also possess better amplification uniformity. This approach can also accurately detect smaller CNVs in single cells without correction and simultaneously achieves high-precision SNV detection. Several studies have linked CNVs to tumor formation and metastasis, and therefore the development of these technologies has broadened the applications of MDA in tumor research.

DNA identification analysis

Short tandem repeats (STRs), also known as microsatellite DNA, is a short tandem repeat structure with a core sequence of 2–6 bases. In 1991, STR loci were first used as important genetic markers for human paternity tests [73]. Given that there are so many STRs in the human genome, individuals can be clearly distinguished and parent-child relationships can be established by characterizing these polymorphisms. Deleye *et al*. [74] employed four different WGA kits to amplify the input of one or three micromanipulated cells to assess their performance for downstream human STR profiling. After MDA, all selected STR markers could be detected in some samples with a low dropout rate, which highlighted the suitability of MDA products for STR analysis.

Single nucleotide polymorphisms (SNPs) comprise a new generation of genetic markers. However, amplifying enough SNP sites from trace DNA for personal identification remains a challenge. The combination of MDA and microsequence analysis technology is an effective way to solve this problem. To address the difficulty of SNP genotyping from long DNA fragments, Michikawa [75] placed diluted DNA samples in alkaline agarose gels to conduct MDA reactions with a MassARRY system. Upon comparing two WGA technologies (MDA and MALBAC), the authors concluded that the amplification efficiency of MDA was higher than that of MALBAC, and was therefore more suitable for SNP detection [40]. In 2018, Lieselot *et al*. [76] tested the amplification products of the input of three micromanipulated cells by four different WGA kits via the TSO sequencing panel. The excellent performance of MDA in SNP genotyping makes it a promising technology in the fields of preimplantation genetic testing (PGT), CTC liquid biopsy, and tumor cell profiling.

Preimplantation genetic testing

The concept of PGD was proposed in 1967, when Edwords and Gardner successfully carried out gender identification of rabbit embryos [77]. Afterward, Hellani *et al*. [78,79] reported two MDA applications for single-cell WGA in the PGD field. The validity and accuracy of the method were verified by the successful amplification of cystic fibrosis- and thalassemia-related genes, as well as multiple STR sequences using single-cell MDA products. The authors predicted that MDA could replace conventional PCR-based approaches and MDA would become the first step in the PGD standard procedure. MDA has been the mainstream amplification method in preimplantation genetic testing for monogenic disease (PGT-M) since its invention, and successful implementations of this technique have been reported in various monogenic disease studies [80–82].

Currently, the phenomenon of allele dropout (ADO) is inevitable in both PCR and single-cell genome amplification, which limits the sensitivity and efficiency of PGT-M to detect single-gene diseases. Therefore, polymorphic loci should be monitored during the PGT process for linkage analysis to avoid misdiagnosis. The effective use of MDA in STR and SNP genotyping has promoted its application in PGD. For a long time, STRs have been the preferred linkage marker for PGT. Compared with STRs, the number of SNPs is larger, which is conducive to the automated detection and analysis of high-throughput genomic data. The use of the SNP-NGS platform in PGT-M renders more accurate and effective results because SNPs are more frequent [83]. Moreover, preimplantation genetic testing for aneuploidies (PGT-A) can also be carried out effectively with this platform, which enables the selection of euploid embryos to improve pregnancy success rates. In recent years, karyomapping with SNP arrays and NGS based on SNP markers have been successfully implemented in the field of PGT and its clinical application was first reported in 2015 [84]. This technology scans more than 300,000 SNP markers across the entire genome for chromosomal screening. Moreover, haplotypes are established by SNP markers located in the target gene region and linkage analysis is used to infer whether the embryos carry pathogenic mutations. Therefore, karyomapping is considered a new landmark approach in the study of monogenic genetic diseases [85].

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Metagenomic research

The diversity of most of the world's microbes remains unknown. However, the rise in high-throughput sequencing capabilities and the reduction in costs has enabled the acquisition of high-quality sequencing libraries from microbial trace DNA, which has greatly facilitated metagenomic research. Therefore, MDA is commonly used to amplify small amounts of microbial genomic DNA. One example is the frequent application of MDA in the study of cyanobacteria diversity. Davison *et al*. [86] obtained data from CRISPR spacers after amplification of single-cell genomes of cyanobacteria with different growth temperatures and combined the reference sequences of the host and viruses to study the mechanisms of host/virus co-evolution. Similarly, in 2019, Tu *et al*. proposed a pervasive metagenome construction and analysis pipeline to provide insights into the reciprocal relationships within *Microcystis* colonies and cyanobacterial bloom mechanisms [87]. Despite the inherent bias in traditional MDA protocols, some researchers have reported that MDA bias has little impact on the beta diversity of human salivary viruses [88] or can be effectively alleviated by sample dilution [89]. As mentioned earlier, there are many ways to ameliorate this bias, which expands the application of MDA in metagenomics. For example, WGA-X was used to analyze the genome sequences and cell sizes of hundreds of individuals, uncultured bacteria, archaea, protozoa, and viruses that comprise natural microbiomes [50]. Moreover, monodisperse droplets can be easily created via microfluidic technology to make MDA reactions more uniform [90].

DISCUSSION AND FUTURE PERSPECTIVES

Each WGA method has its unique advantages. Strand-displacement amplification results in a hyper-branched product with a high molecular weight ($>$ 10 kb), which is ideal for library construction in NGS. However, problems such as non-specific amplification of MDA, abnormal amplification of microsatellites, and loss of amplification fragments near the telomere and centromere will invariably exist. Preferential amplification (PA) and ADO are common problems in single-cell PCR and also exist in the process of MDA, albeit not as prevalently as in single-cell PCR, which is a key issue that may lead to misdiagnosis. Fortunately, many innovative MDA-based technologies such as PTA, WGA-X, and TruePrime have emerged, all of which possess excellent amplification ability and even solve the non-specificity and ADO problems of MDA to some extent.

Furthermore, the combination of MDA and microfluidics enables the high-throughput and accurate detection of CNVs and SNVs from a single cell, and is therefore currently considered the best single-cell WGA technology. However, microfluidic device limitations and experimental repeatability must be further optimized. Therefore, μcMDA is suitable for laboratories lacking microfluidic devices and therefore has great potential for WGA applications. In the future, the improvement of MDA technologies will likely be focused on solving the above-mentioned challenges. Moreover, the development of "precision medicine" will likely promote the adoption of WGA-based single-cell sequencing technology in clinical settings. MDA undoubtedly occupies an important position in WGA approaches. Therefore, future MDA research will focus on meeting both convenience and accuracy requirements for amplification. For example, the creation of a disposable MDA-based DNA amplification chip with an integrated heater has proven to be a promising approach for fast point-of-use detection despite requiring further research and optimizations [91]. With the continuous development of MDA and the successive update of detection technologies, we believe that MDA will be applied in increasingly more fields and provide a solid foundation for scientific research.

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COMPLIANCE WITH ETHICS GUIDELINES

The authors Naiyun Long, Yi Qiao, Zheyun Xu, Jing Tu and Zuhong Lu declare that they have no conflict of interests.

This article is a review article and does not contain any studies with human or animal subjects performed by any of the authors.

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