Giraffe: a tool for comprehensive processing and visualization of multiple long-read sequencing data

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Abstract

Motivation

Third-generation sequencing techniques have gained popularity due to the ability to generate super-long reads with high quality. Utilizing datasets obtained from various samples and multiple sequencing platforms for comparative and comprehensive analysis is crucial for exploring biological mechanisms and establishing benchmark baselines. However, existing tools for long reads primarily focus on quality control (QC) and read processing for individual samples, which complicates the profiling and comparison of multiple datasets. The lack of tools for data comparison and results visualization poses challenges for researchers with limited experience in bioinformatics. Furthermore, benchmark baselines, crucial for choosing appropriate sequencing platforms, necessitate the development of a comprehensive long-read QC method that facilitates comparative analysis and visualization across multi-samples and multi-platforms.

Results

The Giraffe, a Python3-based command line tool, was designed for comparative analysis and visualization across multiple samples and platforms. The tool stands out by offering features that allow for the assessment of read quality, sequencing bias, and genomic regional methylation proportions of DNA reads and direct RNA sequencing reads. The usability of the Giraffe has been demonstrated through its applications in various scenarios, including comparisons between different biological processing methods (whole genome amplification and shotgun), sequencing platforms (Oxford Nanopore Technology and Pacific Biosciences), tissues (Kidney marrow with and without blood), and biological replicates (Kidney marrows). Additionally, we found that Oxford Nanopore duplex reads outperformed PacBio HiFi reads in homopolymer identification and GC evenness, with an overall comparable read quality.

Availability and implementation

The source code is available at https://github.com/lrslab/Giraffe_View and PyPI (https://pypi.org/project/Giraffe-View).

1 Introduction

Comparative analysis of samples collected from different individuals or generated from different sequencing platforms is crucial for exploring biological mechanisms and establishing benchmark baselines. Third-generation sequencing platforms, such as Oxford Nanopore Technologies (ONT) and Pacific Biosciences (PacBio), have gained significant popularity in biological research due to their ability to generate long and high-quality reads (Branton, et al., 2008; Karst, et al., 2021; Liu, et al., 2023; Ni, et al., 2023; Rhoads and Au, 2015; Wang, et al., 2021). However, current tools for quality control and long-read assessment, such as pycoQC (Leger and Leonardi, 2019) and minion_qc (Lanfear, et al., 2019), are primarily designed to analyze in-house sequencing data. They can only handle individual samples, lacking the capability for comparative analysis across multiple samples and platforms. To address this gap, we developed Giraffe, a set of Python3-based scripts specifically designed for multiple long-read data comparisons in read quality, sequencing bias, and distribution of genomic regional methylation proportion. Compared to alternative tools like NanoComp (De Coster and Rademakers, 2023), Giraffe provides greater features and options such as estimated and observed read accuracy, facilitating users in understanding their data quality better and judging whether the quality is reliable enough for downstream analysis.

2 Software description

Giraffe has four functions, including “estimate” for estimated read quality assessment, “observe” for observed read accuracy evaluation, “gcbias” for sequencing bias detection, and “modbin” for genomic regional methylation proportion comparison, with the requirement of FASTQ reads or alignment SAM/ BAM file (**Fig. 1**).

2.1 Installation and dependencies

Giraffe and the associated Python scripts are conveniently accessible through the public software repositories PyPI, which can be installed using pip. To support the read processing functionalities, Giraffe relies on dependencies including minimap2 (Li, 2018; Li, 2021), samtools (Li, et al., 2009), and bedtools (Quinlan and Hall,

2010), which can be obtained through conda (https://conda.org). The scripts are developed utilizing several third-party Python modules, including pysam (Bonfield, et al., 2021; Danecek, et al., 2021; Li, et al., 2009), plotnine (https://github.com/has2k1/plotnine), numpy (Harris, et al., 2020), pandas (https://github.com/pandas-dev/pandas), tqdm (da Costa-Luis, 2019), Biopython (https://biopython.org), and termcolor (https://github.com/termcolor/termcolor).

2.2 Estimated read quality

The "estimate" function provides the user with basic information, generating a table that includes estimated accuracy, estimated error proportion, Q score, read length, and GC content (**Table S1**). The function requires input from a table containing sample ID, sequencing platform (ONT/ Pacbio), and the path of the data in FASTQ format. To facilitate simpler and more intuitive comparisons of data quality, we have transformed the Q score into estimated accuracy, providing values ranging from 0 to 100 % (**Equation S1**).

2.3 Observe read accuracy

The Q score is calculated by the estimated probability of the base being wrong, which is commonly used to quantify the quality of baseballs in sequencing data. However, there are situations, such as overfitting of the basecalling model, where the Q score may not accurately reflect the read quality, which leads to the development of the "observe" function based on the read alignment (**Equation S2-S7**). Considering that detecting the homopolymer in long-read sequencing accurately is still challenging, homopolymer identification is also included in the "observe" function as a comparative metric. "Observe" requires a table identical to that of "estimate" and a reference as input, generating two summary tables, one specifically for homopolymer identification accuracy (**Tables S2**) and another for observation information (**Tables S3**) with detailed information such as length of insertions, deletions, substitutions, and matches, as well as the observed read accuracy.

2.4 Sequencing bias

In wet lab experiments, some processes can introduce sequencing bias such as polymerase chain reaction (PCR). More specifically, during DNA application, the use of non-random primers or different affinities of polymerase enzyme to DNA sequences can result in uneven coverage and increased depth of specific regions. The results generated by analyzing reads with a sequencing bias are unreliable, especially for methods based on counting. The “gcbias” function was designed to assess the relationship between GC content and sequencing depth, reflecting the level of sequencing bias.

2.5 Genomic regional methylation profiling

The methylation proportion in eukaryotes is associated with the strength of promoter activity and downstream protein expression, which has significant implications for exploring the mechanisms underlying various diseases. Nanopore sequencing for native DNA can now give accurate 5mC methylation profiling for every CpG site, especially for human samples. The methylation level for each functional region would be required for downstream analysis. The “modbin” function was designed to facilitate the comparison of methylation profiling files at the genomic regional level and detect the difference between samples.

2.6 Additional scripts and options

Considering that the scales of normalization may not be reasonable or suitable for all users in the sequencing bias detection part, additional scripts, including “renormalization_sequencing_bias” and “replot_sequencing_bias”, are provided to allow users to manually define the normalized scale based on the distribution of bins and replot figure. Additionally, another script, named “homopolymer_count.py”, is offered to count the number, position, and type of homopolymers in the reference genome. To support direct RNA sequencing on the ONT platform, we have included the RNA alignment strategy in Giraffe. The user can indicate “ONT_RNA” as the sequencing platform in the input table to assess the quality of RNA data.

3 Usage examples and discussion

Here, we showcased four applications of Giraffe: exploring the influence of amplification, benchmarking the read quality between different sequencing platforms, identifying the influence of sample purity on sequencing data, and investigating the consistency of biological repetitions. The details of all datasets are available in **Table S4**.

DNA amplification is an essential step before obtaining genome information from limited sample availability. To evaluate the impact of amplification on sequencing reads, DNA samples with and without whole genome amplification derived from the human HCC78 cell line were sequenced using ONT platforms with R9.4.1 flow cells. Upon observation, it became apparent that the amplification process had minimal impact on the sequencing quality and GC content (**Fig. 2A**, **2B**, and **2D-2F**). However, a noticeable bias was identified in the amplified data (**Fig. 2H**). In terms of methylation proportion at the 1M bin level, a significant majority of the bins derived from amplified reads clustered near 0 percent methylation (**Fig. 2I**).

Different long-read sequencing platforms offer distinct advantages depending on the specific requirements. To assess the read accuracy of long-read sequencing platforms, the ONT duplex reads and PacBio HiFi reads derived from human HG002 datasets were employed. Remarkably, both datasets achieved exceptional accuracy levels exceeding 99% and showcased similar GC content of approximately 40% (**Fig. S1A**, **S1B**, and **S1D**). Notably, the PacBio reads exhibited an enrichment of around 20 Kb in read length, whereas the ONT reads displayed a more evenly distributed range (**Fig. S1C**). Furthermore, in comparison to HiFi reads, duplex reads demonstrated superior performance in homopolymer identification and less bias in terms of read coverage across the reference genome (**Fig. S1F** and **S1H**).

Different tissues within a biological organism generally exhibit distinct patterns of protein expression and methylation proportion due to their specialized functions. However, isolating a specific tissue without any contamination from other tissues can be challenging in practice. To investigate the impact of other tissue contamination on downstream analysis results, the ONT R9.4.1 reads obtained from the kidney marrow of zebrafish, with and without the presence of blood, were employed. A clear

observation was made regarding the decreased quality of kidney marrow with blood (KMB) reads (**Fig. S2A** and **S2D**). The decrease in quality was evidenced by higher mismatch proportion and low homopolymer identification accuracy (**Fig. S2E** and **S2F**).

Biological systems are complex and can be influenced by numerous variables, making it difficult to isolate and control a single changing factor in experiments. The biological repetitions are necessary to evaluate the consistency and reproducibility of their results. Here, two sets of zebrafish kidney marrow duplicate samples sequenced by ONT platforms equipped with R10.4.1 flow cells were used for evaluation. The similar read quality, GC content, and methylation pattern of the two samples can be regarded as important metrics of being a qualified replication (**Fig. S3**).

Considering the large amount of data in the aforementioned datasets, we provided users with selected subsets of datasets to facilitate the operation of Giraffe. These datasets include ONT R10.4.1 and R9.4.1 reads collected from *Escherichia coli* with a reference and 5mC methylation files collected from zebrafish blood and kidney samples. The downloading and running commands are available in the supplementary materials. The size of the demo datasets is approximately 246 Mb, and it takes about 7 minutes to conduct four functions with 4 threads.

4 Conclusion

Giraffe is a tool designed for comparing multiple long-read sequencing samples. It offers four main functions: “estimate”, “observe”, “gcbias”, and “modbin” involving read quality, sequencing bias, and genomic regional methylation proportion. The practicability and applicability of the Giraffe are well-validated in several research backgrounds.

Data availability

All the datasets used in this work can be found in **Table S4**. The PacBio HiFi and ONT duplex reads are published, which are available at https://humanpangenome.org/data. The other three datasets can be found at the National Centre for Biotechnology Information (NCBI) with project ID

PRJNA1095401. The testing demo datasets are available in Figshare (https://figshare.com/articles/dataset/demo_datasets/25378693).

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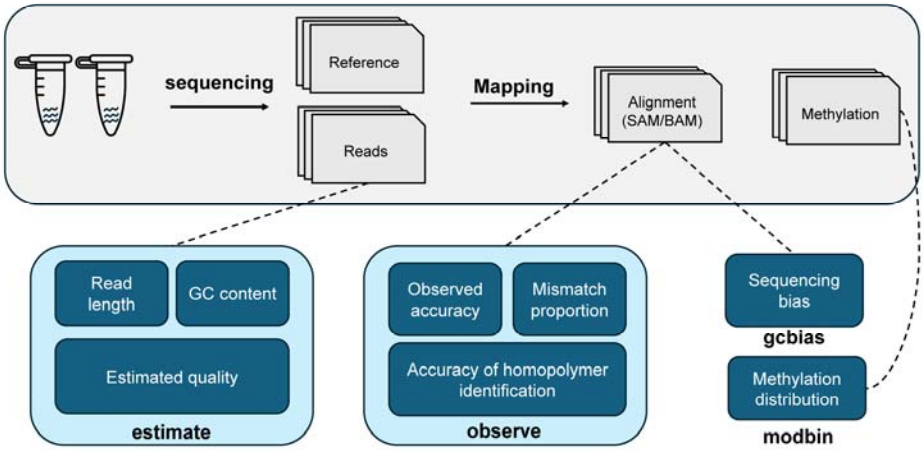

Fig. 1. The workflow of the Giraffe. The basic information included in length, GC content, and estimated accuracy for each read can be profiled by the "estimate" function. After aligning reads against the reference, observed accuracy, mismatch proportion, and accuracy of homopolymer identification are calculated by the "observe" function. The alignment files can be also used by the "gcbias" function to detect the sequencing bias. As for the genomic regional methylation proportion, it can be summarized by the "modbin" function.

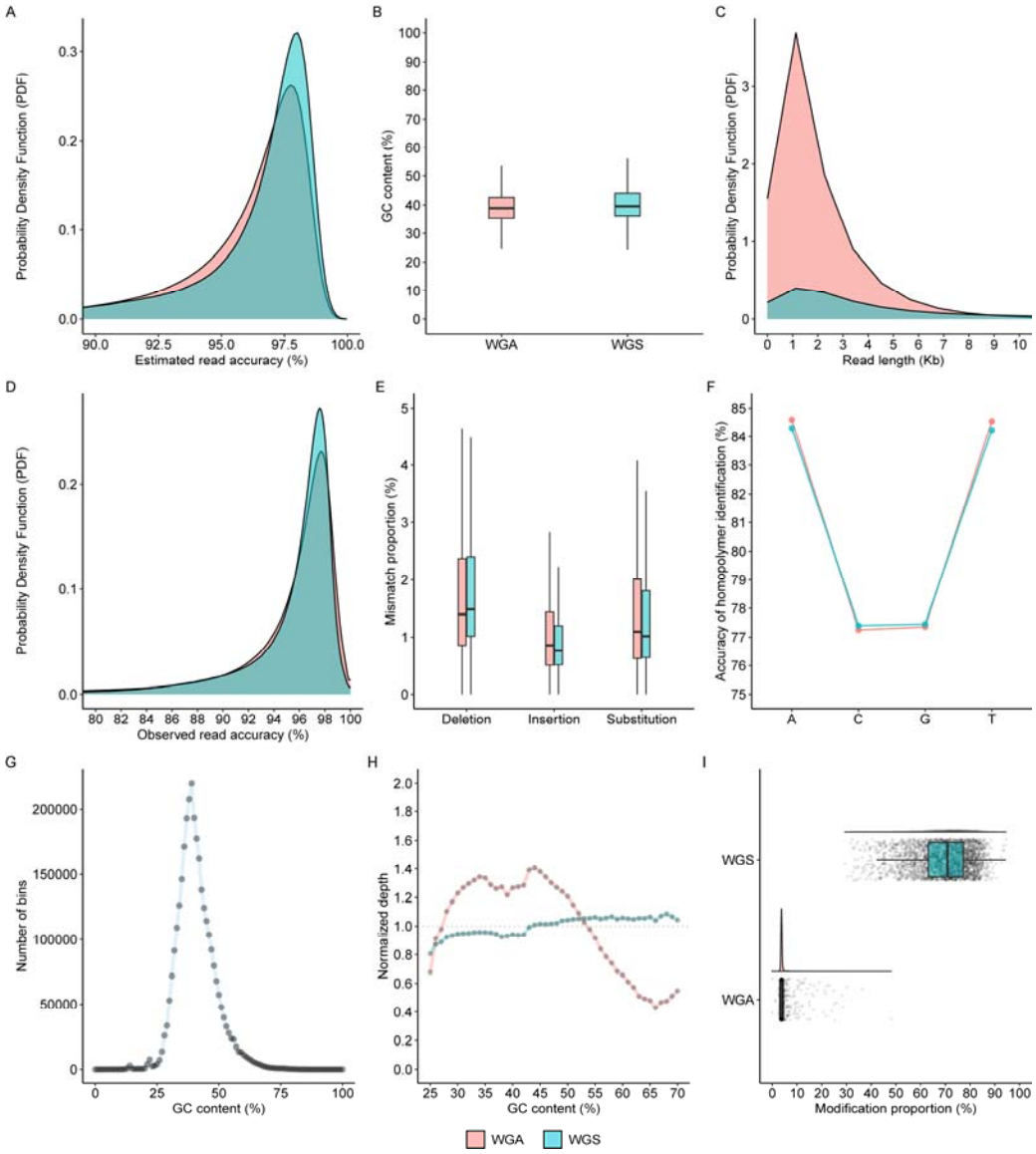

Fig. 2. Comparison of human HCC78 cell line between WGS and WGA reads. (**A**), (**B**), and (**C**) the distribution of estimated accuracy, GC content, and length. (**D**) and (**E**) the distribution of observed accuracy and mismatch proportion. (**F**) The accuracy of homopolymer identification for each base type. (**G**) The distribution of 1kp bin numbers within 0 to 100% GC content. (**H**) Relationship between sequencing depth and GC content at 1k bin level. (**I**) The distribution of 5mC methylation proportion at 1M bin level. WGS: whole genome sequencing; WGA: whole-genome amplification.