Development of Enhanced Reduced Representation Bisulfite Sequencing Method for Single-cell Methylome Analysis

Junko Yamane¹, Tomoya Mori¹, Nobuko Taniyama¹, Kenta Kobayashi¹, Wataru Fujibuchi¹∗

¹Center for iPS Cell Research and Application (CiRA), Kyoto University, 53 Kawahara-cho, Shogoin, Sakyo-ku, Kyoto, 606-8507 Japan

∗Correspondence: Tel: +81 75 366 7012; Fax: +81 75 366 7013; Email: fujibuchi-g@ciira.kyoto-u.ac.jp

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SUMMARY

Single-cell analysis provides molecular signatures to define cell identity. To characterize cell types, DNA methylation patterns are often used as a flag of internal molecular status. There are a few reports of single-cell methylome techniques that involves bisulfite conversion. However, the step often causes DNA fragmentation, which leads to severe PCR substrate reduction. Here we developed a new version of single-cell reduced representation bisulfite sequencing (scRRBS) method to recover more CpG sites to be analysed. Our method succeeded to increase of 4.1 times in sample yield and 1.6 times in CpG site coverage. Importantly, our results indicate that the obtained single-cell DNA methylation sites are homogeneous among 12 cells, thus it may provide molecular barcodes for cell types. In summary, we succeed to develop enhanced scRRBS method and it will be more useful tool to define cell identity in the near future.

KEYWORDS

Single-cell; Methylome; RRBS

INTRODUCTION

An organism consists of a large number of cells with extracellular matrix in well-structured manner. In a normal tissue and organism, cells communicate with each other and localize in defined locations. It is important to know how these organized positioning of cells is configured. Recently some papers reported on cellular interaction events using an organoid, which mimics tissue formation [1].

To investigate these cellular functions and morphogenesis phenomenon, various kinds of molecular features have been investigated. Although the analysis of cellular epigenetic status, which regulates gene expression, has made remarkable progress, most of the analysis is still at tissue level. Therefore, the differences of individual cells have to be averaged [2, 3]. As each cell in organism has information to develop higher structure of tissues, single-cell analysis is important to analyze how the information in individual cells is designed and expressed in a coordinated manner. Since disease tissues also contain heterogeneous population, single-cell analysis become more important in cancer treatment for investigating drug effects or making decision for treatment [4].

Recently, many researches using single-cell transcriptome and methylome have been reported [5–9]. Among these, we focused on DNA methylation. DNA methylation plays important roles in development, tumor formation and other processes [10]. It becomes more stable after differentiation than in early embryo and primordial germ cells. In these early stages, cells are mainly demethylated except at internal retrovirus regions [11]. DNA methylation is deeply associated with cellular identity and fate determination.

There are several DNA methylome techniques that can be applied to single-cells [12, 13]. As shown in Table 1, major DNA methylome techniques at single-cell level are PBAT (Post Bisulfite Adapter Tagging) and RRBS method [14, 15]. PBAT method uses whole genome sequencing, while RRBS method analyzes only methylated regions. Different DNA methylation mainly occurs at CpG rich regions [5].

Both methods involve bisulfite treatment to convert unmethylated cytosines to uracils. The most severe effect of the bisulfite conversion is to reduce sample yields. As the reaction treats DNA in a severe condition, such as low pH and high temperature, DNA becomes fragmented and only 55% of samples remain after bisulfite conversion [16]. If the condition is relaxed, conversion rate becomes poor, leading to the detection of larger number of methylated cytosines than the actual count [17].

Table 1: List of single-cell DNA methylome assays. Among various DNA methylome assays, these three assays can be applied to single-cell analysis.

We developed a new method to recover fragmented DNA caused by bisulfite conversion. From the viewpoints of sequence depth, costs, and analysis region, we simply modified the original scRRBS method by inserting the rescue protocol. Our method consists of three enzymatic processes.
Figure 1: An overview of enhanced scRRBS protocol. Rescue steps were inserted between bisulfite conversion and PCR enrichment steps.

Figure 2: Comparison of detected CpG sites. Red lines are detected cytosine’s positions in each chromosome. There is no bias observed among all chromosomes. Gray regions are "N" sequences.
RESULTS

Developing enhanced scRRBS method

Figure 1 shows our enhanced scRRBS protocol. The differences from the original scRRBS method are as follows: 1) biotinylated of 5' end of universal adapters and 3' end of index adapters; and 2) insertion of rescue steps.

The method successfully increases PCR amplifiable DNA sequences. In the case of 100 single-cells of human iPS cells, the yield in control was 1.26E-04 pM, while the yield decreased to 9.12E-07 pM upon the bisulfite treatment. After the rescue step, the yield increased to 3.77E-06 pM. So the rescue efficiency was 4.1 times. In the stricter experimental condition such as 12 single-cells of human iPS cells, rescue efficiency was 4.8 times.

Increase of detected CpG sites by enhanced scRRBS protocol

We compared detected CpG sites without rescue (-) and with rescue (+) samples. The number of detected CpGs of rescue (+) was higher than rescue (-) in the case of non-redundant counting. And there were no bias of detected CpG sites observed over all chromosomes (Figure 2). This data’s sequence depth was more than 1.

The number of CpG sites were 15,181 in rescue (-), and 24,413 in rescue (+). Thus, the rescue ratio was 1.6 times.

Conformation of methylation status at some sequence depth

Next, we checked the variance for methylation levels in mixed single-cells. At the threshold of depth 10 or more, the methylation patterns are largely common (i.e., the variance is 0) among 12 single-cells. This means that there may be little variance of methylation status in the same cell type (Figure 3). When we checked the methylome data produced by the original scRRBS method, we confirmed that this conserved DNA methylation are observed.

Interestingly, when we draw a histogram of the methylation rate to check the statistical distribution, it was either 0, or 100% in most cases and almost no partially methylated CpG sites are observed. (Figure 4).

Taken together, these results indicate that the DNA methylation patterns are highly conserved among single-cells of the same cell type, and the patterns are all-or-none in the four strands of diploid chromosomes in most cases of iPS cells.

DISCUSSION

In our enhanced scRRBS method, we could obtain more CpG sites than the original method and there is little bias among all chromosomes. We have succeeded to rescue fragmented DNAs and convert them PCR amplifiable. However, in this version of our method, we cannot guarantee that rescued DNAs were ligated with the original counterparts. Currently, we are trying to develop new version of the protocol.

Figure 3 and Figure 4 show that the methylation rate patterns are so distinct and may be used as epigenetic cell barcodes (ECBs) for cell identification. Currently, we are examining the ECBs of other cell types using tissue samples, for example, mouse early blastocysts, to see if they can be used to identify cell types as strict markers.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES


