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# Comparative analysis of single-cell parallel sequencing approaches in oocyte application

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ABSTRACT

Single-cell parallel sequencing allows us to explore how genetic and epigenetic variations correlate of gene expression in the same cell. Beads-based approach and non-beads-based approach are the two present methods to separate DNA and RNA from the same cell. However, systematic difference between the two methods are lacking. In our study, we compared the performances of the two methods using transcriptome and methylome profiles generated simultaneously from single mouse oocytes. Our results showed that the beads-based approach could capture maximum quantity of mRNA but loss of DNA was inevitable, while the non-beads-based approach could obtain more DNA due to the undamaged nucleus obtained but at a cost of partial loss of mRNA. As the sequencing coverage of methylome sequencing in a single cell was relatively low, single-cell whole genome bisulfite sequencing (scWGBS) was preferable to generate the methylome map in single-cell parallel sequencing in comparison to single-cell reduced representation bisulfite sequencing (scRRBS). To the best of our knowledge, this is the first study to compare the two methods of single-cell parallel sequencing which offers a basic idea for deciding between the two methods and a direction of single-cell parallel sequencing development.

## 1. Introduction

Single-cell high throughout sequencing technology has been developed at an amazing speed in recent ten years. The first single-cell whole transcriptome sequencing was performed by Tang et al. in 2009 (Tang et al., 2009). Subsequently, single-cell whole genome sequencing and whole exome sequencing technologies were successively established in 2011 and 2012 (Hou et al., 2012; Navin et al., 2011). Besides genetics, epigenetics profiles in single cells could also be generated through single-cell reduced representation bisulfite sequencing (scRRBS) or single cell whole genome bisulfite sequencing (scWGBS) (Nagano et al., 2013; Smallwood et al., 2014). Nowadays, such single cell sequencing technologies have been widely applied to explore cell heterogeneity, new cell types, cell differentiation, embryonic development and so on. However, the above approaches only allow us to study DNA and RNA separately in the same cell. Since cell-to-cell variability has been demonstrated in genomics, mRNA expression and also epigenetics and the complexity of gene expression regulation has been recognized, the conventional single cell sequencing methods could not actually uncover the causal relationship between epigenetic variations and mRNA transcription profiles. Besides, in the study of several cell types, like female gametes, the collection of samples is limited. Thus, mulitomic assays can provide a better understanding of the same cell, particularly in the case of oocyte study.

In 2016, single cell parallel sequencing technology was firstly reported to link transcriptome and genome simultaneously through applying biotinylated oligo-dT primer coated magnetic beads to separate DNA and RNA in one cell (Angermueller et al., 2016). Similarly, in the same year, Youjin Hu, et al. reported another separation method. It gently isolated cytosolic RNA to resemble soma RNA, so DNA in the

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remaining nucleus of the cell was used for further genome sequencing (Hu et al., 2016). However, comparison between the two different parallel sequencing methods is still lacking. To address this issue, we generated parallel transcriptome and methylome profiles from single mouse oocytes using the above methods, and finally compared their performance.

## 2. Material and methods

### 2.1. Animals and mouse oocyte isolation

C57BL/J female mice aged 6 weeks old were used in this study. The study was approved by the Animal Experimentation Ethics Committee of the Chinese University of Hong Kong. Female mice underwent superovulation treatment as previously described (Ledent et al., 2005). Briefly, female mice were injected intraperitoneally with 10 unit of pregnant mare's serum gonadotropin (PMSG, Sigma). 48 h after PMSG injection, mice were then injected intraperitoneally with 10 unit of human chorionic gonadotropin (hCG, Sigma). 14–16 h after superovulation treatment, mice were sacrificed and tissues containing oviduct and connected ovary were dissected out. In the dish containing M2 medium (Sigma), the ampulla of oviduct was chopped by a needle, and oocyte-cumulus-complexes (COC) were moved out and were transferred to M2 medium containing 10% hyaluronidase (Sigma) to detach cumulus cells from oocytes. After that, cumulus-free metaphase II (MII) oocytes were collected and washed with M2 medium and 1x PBS respectively by month pipette. One MII oocyte was finally transferred into one PCR tube with less than 1 μl of PBS and stored in -80 °C freezer.

### 2.2. DNA and RNA separation from single cell

In beads-based (BB) approach, we applied streptabidin-coupled magnetic beads (ThermoFisher Scientific) to capture mRNA from single cell lysate according to the published protocol (Angermueller et al., 2016). In brief, 2.5 μl of RLT Plus buffer (Qiagen) and 1 μl of a 1:250,000 dilution of ERCC spike-in mixture A (Life Technologies) were added into the tubes containing single oocytes. Then, 10 μl of streptabidin-coupled magnetic beads conjugated with a modified oligo-dT primer (5'-biotin-triethyleneglyco-AAGCAGTGGTATCAACGCAGAGT $ACT_{30}$VN-3', where V is either A, C or G, and N is any base; IDT) in advance were added into the cell lysate and the mixture was incubated at room temperature for 20 min to capture polyadenylated mRNA. Subsequently, the tube was placed on a magnet and the supernatant, containing gDNA, was transferred into a new tube. To obtain as much gDNA as possible, we washed the beads with 10 μl of wash buffer (50 mM Tris-HCl (pH = 8.3), 75 mM KCl, 3 mM MgCl2, 10 mM DTT, 0.5% Tween-20, 0.2x RNAse inhibitor (SUPERasin, Life Technologies)) for four times. The wash buffer was pooled with the original supernatant. gDNA in the mixture of the supernatant and wash buffer was precipitated on Ampure Beads (1:0.6 ratio, Beckman Coulter) and eluted into 10 μl of $H_2O$ for further methylome sequencing. mRNA captured by beads was directly used for scRNA-seq.

In the non-beads-based (NBB) approach, for isolating nucleus and cytoplasma from a single oocyte, we followed the previously established protocol with some modifications (Hu et al., 2016). 7 μl of soft buffer (500 mM KCl, 100 mM Tris-HCl (pH = 8.3), 1.35 mM $MgCl_2$, 4.5 mM DTT, 0.45% Nonidet P-40, 0.18U SUPERase-In, and 0.36U RNase-inhibitor) were added into one PCR tube containing a single oocyte and the mixture was incubated at 4 °C for 30 min. Then, the lysate was vortexed at room temperature for 1 min and then centrifuged at 1000 x *g* for 5 min to leave the nucleus at the bottom of the tube. In order to minimize RNA loss, 6.5 μl of lysis product supernatant was carefully transferred to another new PCR tube containing 1 μl of 10μM oligo-dT30VN primer, 1 μl of dNTP mix and 1 μl of a 1:250,000 dilution of ERCC spike-in mixture A and proceeding to scRNA-seq, and the remaining 1.5 μl of lysis solution (containing the nucleus) was subjected for whole genome bisulfite sequencing.

### 2.3. Single cell RNA-seq (scRNA-seq)

2 μl of cell lysis buffer (0.2% Triton-X and 2U/μl RNase inhibitor), 1 μl of 10 μM oligo-$dT_{30}$VN primer and 1 μl of dNTP mix (NEB) were added into the tube containing single oocyte. The cell lysate was vortexed and incubated at 72 °C for 3 min. SMART-seq2 was then employed for cDNA synthesis and amplification from cell lysate, captured RNA in BB approach or isolated RNA from NBB approach (Picelli et al., 2014). In brief, 7 μl of the first-strand reaction mix, containing 0.5 μl of SuperScript II reverse transcriptase (200 U/μl, Invitrogen), 0.25 μl of RNAse inhibitor (40 U/μl, Life Tech), 2 μl of Supercript II first-strand buffer (5x, Invitrogen), 0.25 μl of DTT (100 μM, Invitorgen), 2 μl of Betaine (5 M, Sigma), 0.3 μl of $MgCl_2$ (0.2 M, Sigma) and 1 μl of TSO (20μM) and 0.7 μl of $H_2O$, were added into each sample. Reverse transcription reaction was carried out by incubating at 42 °C for 90 min, followed by 10 cycles of 50 °C for 2 min and 42 °C for 2 min, and finally incubating at 70 °C for 15 min for inactivation. Next, 15 μl of PCR master mix, containing 12.5 μl of KAPA HiFi HotStart ReadyMix (2x, KAPA), 0.25 μl of IS PCR primer (10 μM, 5'-AAGCAGTGGTATCAACGC AGAGT-3') and 2.25 μl of $H_2O$, were added into RT product for PCR preamplification. The reaction was incubated at 95 °C for 1 min, then cycled 16 times with 95 °C 15 s, 65 °C 30 s and 68 °C 6 min, and finally incubated at 72 °C for 10 min for extension. PCR products were purified by AMPure XP beads (Beckman Coulter) with 1:1 ratio and eluted in 15 μl of $ddH_2O$. Then, 5 ng of amplified cDNA for each oocyte sample was subjected to construct scRNA-seq library using TruePrep DNA Library Prep Kit V2 for Illumina (Vazyme).

### 2.4. Single cell whole genome bisulfite sequencing (scWGBS) and reduced representation bisculfite sequencing (scRRBS)

Either single cell lysate in RLT plus buffer (QIGEN) or gDNA isolated from single cell by NBB approach was subjected for scWGBS. Bisulfite conversion, amplification and library construction were performed with Pico Methyl-Seq™ Library Prep Kit (Zymo Research) following the manufacturer's instruction. gDNA isolated by BB approach was applied to perform scRRBS according to the published protocol (Guo et al., 2015).

### 2.5. Sequencing data processing and data analysis

All sequencing libraries, including scRNA-seq, scRRBS and scWGBS, were assessed for quality using High-Sensitivity DNA chips on the Agilent Bioanalyzer and measured quantity with Qubit 3.0 fluorometer. Libraries were sequenced on Illumina HiSeq2500 platform for 150-bp pair-end sequencing.

Raw sequencing reads from scRNA-seq libraries were trimmed and mapped to mouse reference genome (mm10) with TopHat (v2.0.13) (Trapnell et al., 2009). Then, Cufflinks (v2.2.1) was employed to generate a transcriptome assembly for each sample and to normalize and calculate the expression level of each transcript or gene with FPKM (fragments per kilobase of transcript per million mapped fragments) (Trapnell et al., 2010). In Principal Component Analysis (PCA), published oocyte dataset was obtained under accession GEO: GSE94813 (Wang et al., 2018), while, the embryonic stem cell (ESC) dataset and cumulus cell (CC) dataset were downloaded from accession GEO: GSE86585 and GSE70605 respectively (Liu et al., 2016; von Meyenn et al., 2016).

For raw sequencing reads from scRRBS or scWGBS libraries and downloaded scWGBS-seq dataset from GEO: GSE56879, Trim_Galore (v0.4.2, http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/) was used to remove adaptors and poor-quality reads. The trimmed sequencing data was mapped to the reference genome (mm10) using Bismark (v0.18.1, https://www.bioinformatics.babraham.ac.uk/

projects/bismark/). The function of Deduplication in Bismark was applied to exclude duplicate sequences. Methylation sites were called using the function of Methylation Extractor in Bismark. A sliding window of 2 kb in size was used to subdivided the genome and estimate the methylation level of each sample, while those windows containing less than 5 CpG sites were removed in the analysis (Smallwood et al., 2014).

Data analysis were carried out using the program R (www.r-project.org) and data presentation were performed using R packages, such as ggplot2 (Wickham, 2009), reshape2 (Wickham, 2007), SeqPlots (Stempor and Ahringer, 2016) and so on.

## 3. Results and discussion

Mouse MII oocytes were harvested from six-weeks-old C57/BL female mice under superovulation treatment. In this study, we performed two different separation methods (the beads-based and the non-beads-based approaches, separately) on single mouse MII oocytes with two replicates for each approach. After obtaining simultaneously DNA and RNA from single oocytes, they were subjected to further generation of RNA-seq libraries by SMART-seq2 and methylation sequencing libraries by published scRRBS or scWGBS protocol. Meanwhile, single cell transcriptome and methylome profiles were generated using regular single cell SMART-seq2 and scRRBS as comparison reference. Then all transcriptome or methylome libraries were pooled together for subsequent sequencing.

For each transcriptome library, average 2.92 M (0.18 M–7.41 M) reads were generated. After filtering out reads with low quality, around 40.24% (13.6%–62.0%) reads were unambiguously aligned to mm10 mouse reference genome (Table 1). The gene coverage over the entire gene body was detected. The non-beads-based approach showed even coverage despite the lower reads and relatively higher multiple alignment ratio, but the beads-based approach demonstrated 3' mapping bias (Fig. 1A). Though TSO used in both two approaches was the same, RNA degradation might reflect in sequencing data because just RNA containing 3' end would be collected in the standard poly-A selection (Sigurgeirsson et al., 2014). During this study, oocytes subjected to the beads-based approach were stored in -80 °C freezer for relatively long term, which might increase the chance of RNA degradation. Different from library construction of bulk samples, it is unavailable to check RNA quality before cDNA synthesis for single cell. Thus, fresh or short-term frozen samples should be used for scRNA-seq library construction to reduce the impact of RNA degradation. We pooled sequencing data of two replicates per approach, and detected 10,555 genes for the beads based approach and 8038 genes for the non-beads-based approach. Meanwhile, we detected 8586 genes from regular scRNA-seq data. Around 2000 genes were shared in three different approaches and nearly 7500 genes were detected by both the bead-based approach and regular scRNA-seq. While around 5500 genes were specific to the non-beads-based approach, around 3000 genes were specific to the beads-based approach (Fig. 1B). To detect the similarity of two different approach, Person's correlation analysis was performed across the above samples. Two replicates of the beads-based approach showed relatively high correlation ($R = 0.94$), but the correlation of two replicates of the non-beads-based approach was relatively low ($R = 0.79$, Fig. 1C). Uneven gene coverage of non-beads-based approach might contribute to it. Besides, during separation in the non-beads-based approach, limited volume of cell lysis containing RNA left to reserve intact nucleus, which would cause massive and uneven loss of RNA. Principle component analysis (PCA) was studied on our samples, published oocyte dataset GEO: GSE94813 (Wang et al., 2018), published embryonic stem cell (ESC) dataset GEO: GSE86586 (von Meyenn et al., 2016) and published cumulus cell (CC) dataset GEO: GSE70605 (Liu et al., 2016), and demonstrated that all oocyte samples were clustered together (Fig. 1D). Furthermore, high expression of oocyte specific genes, like *Gdf9* and *Nobox,* was observed in all oocyte samples (Fig. 1E). These observations demonstrate that both approaches can generate transcriptome profiles successfully from single cells in which the beads-based approach was more consistent and could provide more comprehensive information of transcriptome.

**Table 1**
Comparison of scRNA-seq preference between the beads-based approach and the non-beads-based approach.

| Sample ID | Clean reads | Mapped reads | Mapping efficiency | Multiple alignment | No. of detected genes |
|---|---|---|---|---|---|
| BB-1 | 5,654,832 | 1,535,027 | 27.3% | 3.4% | 9823 |
| BB-2 | 183,775 | 108,530 | 59.1% | 5.8% | 7747 |
| NBB-1 | 868,873 | 366,621 | 39.2% | 34.2% | 4348 |
| NBB-2 | 517,012 | 326,968 | 62.0% | 4.8% | 7372 |
| Regular[a] | 7,415,974 | 2,012,387 | 13.6% | 11.0% | 8586 |

[a] Regular represents regular scRNA-seq.

For generating methylome profiles, we first performed scRRBS on DNA collected by the beads-based approach. However, a very low mapping efficiency (0.3% and 1.5%, Table 2) and limited number of detected CpG sites (14,163 and 98,852) were observed. However, 46.8% of mapping efficiency and nearly 0.6 M CpG sites were detected in regular scRRBS based on the similar sequencing throughout. It indicated that there was huge DNA loss during beads-based separation step. In this case, we then performed scWGBS on nucleus retained from the non-beads-based approach and much higher mapping efficiency (1.8% and 36.5%) was observed. In two replicates, 59,256 and 1,695,593 CpG sites were detected respectively. In total four samples from two methods, two of them (BB-2 and NBB-1) had aberrant CpG methylation level (92.54% and 93.59%), which showed the poor data quality and were filtered out for further analysis. Almost all the CpG sites detected in two samples were full, half or empty methylated (Fig. 2A), which was consistent with two DNA copies in one cell. The sample (NBB-2), which underwent the non-bead-based approach combined with scWGBS, showed 60.52% methylation level of all detected CpG sites, while the other sample (BB-1), which underwent the beads-based approach combined with scRRBS, showed 65.59% methylation level (Table 2). The methylation level measured was consistent with previous study on bulk oocyte samples (Smallwood et al., 2011). To analyze the correlation between two different approaches, average methylation level of 2 kb windows (> 5 CpG sites involved) was calculated in three samples with good data quality. Moreover, the conventional scWGBS oocyte data was obtained from public accession GEO: GSE56879 and included in the further analysis. The correlation coefficient between scRRBS sample (BB-1) from the beads-based approach and scWGBS sample (NBB-2) from the non-beads-based approach was more than 0.6, while regular scRRBS sample (scRRBS) showed relatively higher correlation with BB-1 ($R = 0.7139$) than NBB-2 ($R = 0.6954$). Besides, NBB-2 showed relatively high correlation with conventional scWGBS data ($R = 0.8164$, Fig. 2B). These results suggested that both two separation approaches and two methylome profiling methods could generate relatively consistent methylome profiles from single cells. But the non-beads-based approach allows a more comprehensive methylome map due to the retainability of intact gDNA. Besides MII oocytes, germinal vesicle (GV) oocytes are often involved in the study of female gametes. MII oocytes contain condensed chromosomes at metaphase II, while GV oocytes contain the intact nuclear membrane. Thus, it will be better to apply the non-beads-based approach on GV oocytes for retaining the intact nucleus to minimize gDNA loss and the genome coverage could be further improved when compared with MII oocytes.

Since RRBS method enriches for high-CpG regions of the genome via restriction digestion (Stevens et al., 2013), scWGBS could show a relatively lower methylation level in oocyte than scRRBS due to the involvement of more low-CpG regions. The distribution of detected CpG

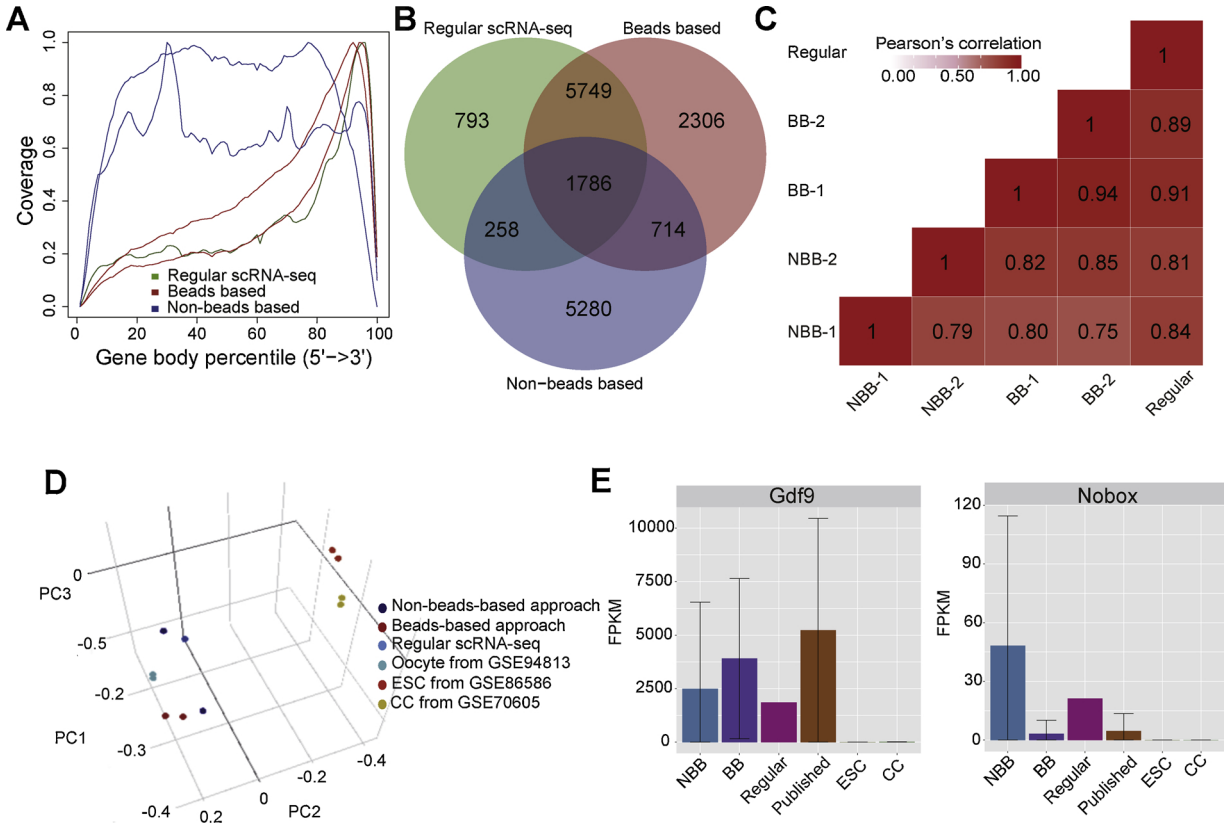


**Fig. 1.** XXX.

sites in two approaches showed more enrichment of CpG sites in promoters in scRRBS and wider coverage of CpG sites in scWGBS (Fig. 2C). RRBS is a powerful tool to assess a large fraction of promoters but is limited to measure other important regulatory regions, like enhances, due to lower coverage, which is not a true whole genome approach in fact (Clark et al., 2016). However, scWGBS is a post-bisulfite adapter-tagging approach which defends adaptor-tagged fragments against degradation, and allows detecting up to 50% of CpG sites and measuring distal enhancer methylation. Thus, generation of methylome profile by scRRBS is an effective and costless method if the aim or design of the study is focused on the relationship between methylation in promoters and gene expression. Otherwise, more information for a greater number of CpG sites would be obtained from scWGBS. In bulk samples, methylome libraries are often sequenced in very high depth to get reliable data, which limits the wide application of WGBS (Doherty and Couldrey, 2014). Since one cell contains only two copies of the genome, scWGBS library could be sequenced at much lower coverage, which reduces the cost of study, but would not affect further bioinformatics analysis. Thus, the choice of scWGBS or scRRBS will no longer be affected by study budget and only influenced by the study design.

In summary, we systematically compared sequencing performance and assessed the reliability and consistency of two single cell parallel sequencing technologies. Since these two approaches utilize different principles of separating DNA and RNA from individual cells, the beads-based approach shows the disadvantage of DNA loss while more RNA loss is observed in the non-beads-based approach. Given that each cell has two copies of the genome but contains more copies of mRNA, we tend to apply the non-beads-based approach to separate DNA and RNA in single cells, but it calls for higher level of experimental techniques to reduce inter-experimental variation as much as possible. SMART-Seq2 is recommended as a preferable method for quantifying transcriptomes of fewer cells (Ziegenhain et al., 2017), and both two separation approaches showed a high consistency in transcriptome. As the requirement of sequencing of coverage in single cell methylome is relatively low, scWGBS is a preferable method of profiling single cell methylome with more comprehensive sequencing information.

**Table 2**
Comparison of methylation sequencing preference between the beads-based approach and the non-beads-based approach.

| Sample ID | Raw reads (M) | Mapping efficiency | Bisulfite conversion efficiency[a] | No. of detected CpG sites (> 1x) | Methylation of detected CpG sites |
|---|---|---|---|---|---|
| BB-1 | 23.2 | 0.3% | 99.2% | 14,163 | 65.59% |
| BB-2 | 14.1 | 1.5% | 99.2% | 98,852 | 93.59% |
| NBB-1 | 13.1 | 1.8% | 86.7% | 59,256 | 92.54% |
| NBB-2 | 14.9 | 36.5% | 96.7% | 1,695,593 | 60.52% |
| scRRBS | 10.1 | 46.8% | 99.4% | 667,986 | 97.88% |
| scWGBS (GSE56879) | 11.3 | 33.2% | 96.3% | 4,059,728 | 41.35% |

[a] Bisulfite conversion efficiency of BB-1, BB-2 and scRRBS was calculated on the methylation level of lambda-DNA spike-in, while it of NBB-1, NBB-2 and scWGBS was calculated on the methylation level of CHH.

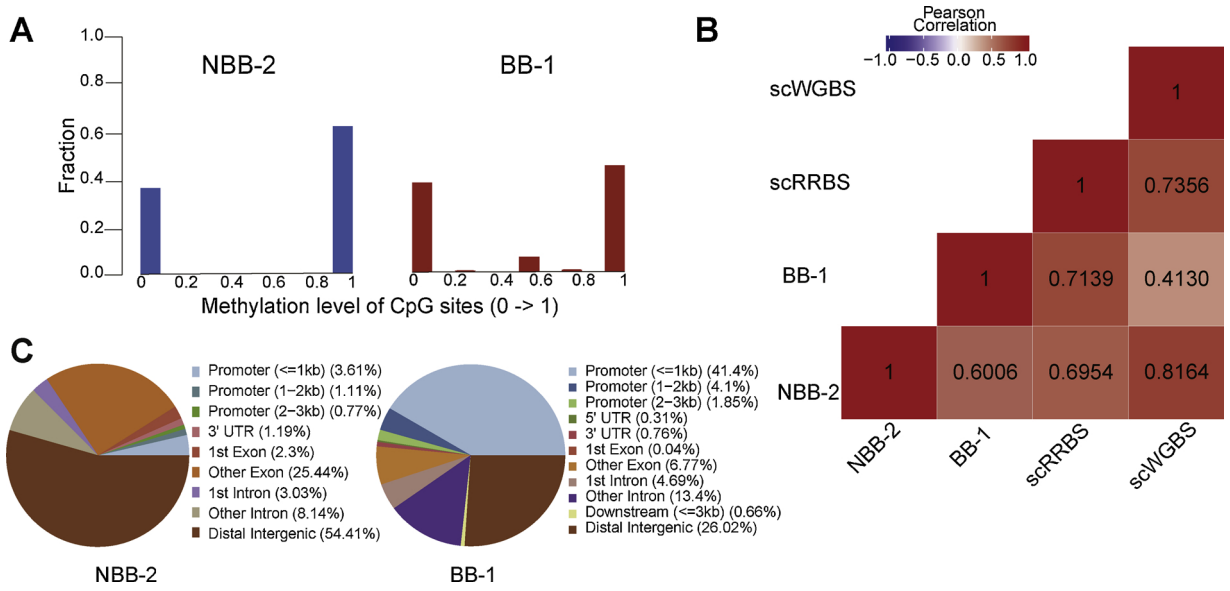


**Fig. 2.** XXX.

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