HiCcompare: an R-package for joint normalization of Hi-C datasets and differential chromatin interaction detection

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# Abstract

**Motivation:** Changes in spatial chromatin interactions are now emerging as a unifying mechanism orchestrating regulation of gene expression. Hi-C sequencing technology allows insight into chromatin interactions on a genome-wide scale. However, Hi-C data contains many DNA sequence- and technology-driven biases. These biases prevent effective comparison of chromatin interactions aimed at identifying genomic regions differentially interacting between, e.g., disease-normal states or different cell types. Several methods have been developed for normalizing individual Hi-C datasets. However, they fail to account for biases *between two or more Hi-C datasets*, hindering comparative analysis of chromatin interactions.  
**Results:** We developed a simple and effective method, HiCcompare, for the joint normalization and differential analysis of multiple Hi-C datasets. The method introduces a distance-centric analysis of Hi-C data analysis by explicitly considering the distance between interacting chromatin regions. The distance-centric visualization of the differences between two Hi-C datasets on a single MD plot allows for a data-driven normalization of biases using locally weighted linear regression (loess). HiCcompare outperforms methods for normalizing individual Hi-C datasets in detecting *a priori* known chromatin interaction differences while preserving the detection of genomic structures, such as A/B compartments.  
**Availability:** HiCcompare is freely available as a Bioconductor R package <https://bioconductor.org/packages/HiCcompare/>.  
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**Supplementary information:** Supplementary data are available at *Bioinformatics* online.

# 1 Introduction

The 3D chromatin structure of the genome is emerging as a unifying regulatory framework orchestrating gene expression by bringing transcription factors, enhancers and co-activators in spatial proximity to the promoters of genes (Mifsud *et al.*, 2015; Sexton and Cavalli, 2015; Li *et al.*, 2012; Papantonis and Cook, 2013). Together with epigenomic profiles, changes in chromatin interactions shape cell type-specific gene expression (Jin *et al.*, 2013; Lieberman-Aiden *et al.*, 2009; Schmitt *et al.*, 2016; Nora *et al.*, 2012), as well as misregulation of oncogenes and tumor suppressors in cancer (Taberlay *et al.*, 2016; Hnisz *et al.*, 2016; Franke *et al.*, 2016) and other diseases (Li *et al.*, 2012). Identifying changes in chromatin interactions is the next logical step in understanding genomic regulation.

Evolution of Chromatin Conformation Capture (3C) technologies into Hi-C sequencing now allows the detection of “all vs. all” long-distance chromatin interactions across the whole genome (Lieberman-Aiden *et al.*, 2009; Sanborn *et al.*, 2015). Soon after public Hi-C datasets became available, it was clear that technology- and DNA sequence-driven biases substantially affect chromatin interactions (Yaffe and Tanay, 2011). The technology-specific biases include cutting length of a restriction enzyme (HindIII, MboI, or NcoI), cross-linking conditions, circularization length, etc. The DNA sequence-driven biases include GC content, mappability, nucleotide composition. Discovery of these biases led to the development of methods for normalizing individual datasets (Cournac *et al.*, 2012; Lieberman-Aiden *et al.*, 2009; Imakaev *et al.*, 2012; Yaffe and Tanay, 2011; Knight and Ruiz, 2012). Although normalization of individual datasets improves reproducibility within replicates of Hi-C data (Imakaev *et al.*, 2012; Yaffe and Tanay, 2011), these methods do not consider biases between multiple Hi-C datasets.

Accounting for the between-dataset biases is critical for the correct identification of chromatin interaction changes between, e.g., disease-normal states, or cell types. Left unchecked, biases can be mistaken for biologically relevant differential interactions. While DNA sequence-driven biases affect two datasets similarly (e.g., CG content of genomic regions tested for interaction differences is the same), technology-driven biases are poorly characterized and affect chromatin interactions unpredictably. Importantly, another source of chromatin interaction differences stems from large-scale genomic rearrangements, such as copy number variations (Servant *et al.*, 2015), a frequent event in cancer genomes (Rickman *et al.*, 2012). Accounting for such biases is needed for the detection of differential chromatin interactions between Hi-C datasets.

We developed an R package, HiCcompare, for the joint normalization and comparative analysis of multiple Hi-C datasets, summarized as chromatin interaction matrices. Our method is based on the observation that chromatin interactions are highly stable (Dixon *et al.*, 2012; Fudenberg *et al.*, 2016; Rao *et al.*, 2014; Schmitt *et al.*, 2016), suggesting that the majority of them, excluding large genomic rearrangement of regions, can serve as a reference to build a rescaling model. We present the novel concept of the MD plot (difference vs. distance plot), a modification of the MA plot (Dudoit *et al.*, 2002). The MD plot allows for visualizing the differences between interacting chromatin regions in two Hi-C datasets while explicitly accounting for the linear distance between interacting regions. The MD plot concept naturally allows for fitting the local regression model, a procedure termed loess, and jointly normalizing the two datasets by balancing biases between them. The distance-centric view of chromatin interaction differences allows for detecting statistically significant differential chromatin interactions between two Hi-C datasets using a simple but robust permutation method. We show improved performance of differential chromatin interaction detection when using the jointly vs. individually normalized simulated and real Hi-C datasets. Our method is broadly applicable to a range of biological problems, such as identifying differential chromatin interactions between tumor and normal cells, immune cell types, and normal tissues.

# 2 Methods

## 2.1 Visualization of the differences between two Hi-C datasets using an MD plot

A chromosome-specific Hi-C matrix is a square matrix of size , where is the number of genomic regions of size on a chromosome. The size of the genomic regions defines the resolution of the Hi-C data. Each cell in the matrix contains an interaction frequency , where and are the indices of the interacting regions. For this study, data in the sparse upper triangular format from the GM12878 and RWPE1 cell lines were used (Supplementary Section 1).

The first step of the HiCcompare procedure is to convert the data into what we refer to as an MD plot. The MD plot is similar to the MA plot (Bland-Altman plot) commonly used to visualize gene expression differences (Dudoit *et al.*, 2002). is defined as the log difference between the two data sets , where and are interaction frequencies of the first and the second Hi-C datasets, respectively. is defined as the distance between two interacting regions, expressed in unit-length of the resolution of the Hi-C data. In terms of chromatin interaction matrices, corresponds to the off-diagonal traces of interaction frequencies (Supplementary Section 2). Because chromatin interaction matrices are sparse, i.e. contain an excess of zero interaction frequencies, by default only the non-zero pairwise interaction are used for the construction of the MD plot with an option to use partial interactions, i.e. with a zero value in one of the matrices and a non-zero IF in the other.

## 2.2 Joint normalization of multiple Hi-C data using loess regression

After the transformation of the data into an MD plot, loess regression (Cleveland, 1979) is performed with as the predictor for . The fitted values are then used to normalize the original IFs:

where is the predicted value from the loess regression at a distance . The values are then anti-logged to obtain the normalized IFs. Note that for both Hi-C datasets the average interaction frequency remains unchanged, as the one set is increased by the factor of while the other is decreased by the same amount. Any normalized IFs with values less than one are not considered in further analyses. The joint normalization was tested against five methods for normalizing individual Hi-C matrices, ChromoR (Shavit and Lio’, 2014), ICE (Imakaev *et al.*, 2012), KR (Knight and Ruiz, 2012), SCN (Cournac *et al.*, 2012), MA (Lun and Smyth, 2015) (Supplementary Section 1).

## 2.3 Excluding potentially problematic regions from the joint normalization

The between-dataset biases may occur due to large-scale genomic rearrangements and copy number variants (CNVs), a frequent case in tumor-normal comparisons (Rickman *et al.*, 2012). Similar to removing other biases, the joint loess normalization removes CNV-driven biases by design, allowing for the detection of chromatin interaction differences within CNV regions. However, CNVs introduce large changes in chromatin interactions (Servant *et al.*, 2015), which may be of interest to consider separately. Therefore, unless cells/tissues with normal karyotypes are compared, we provide functionality for the detection and removal of genomic regions containing CNVs from the joint normalization. The QDNAseq (Scheinin *et al.*, 2014) R package is used to detect and exclude CNVs from the HiCcompare analysis. Alternatively, CNV regions can be detected separately and provided to HiCcompare as a BED file. Additionally, the HiCcompare package includes the ENCODE blacklisted regions for hg19 and hg38 genome assemblies, which can be excluded from further analysis.

## 2.4 Detection of differential chromatin interactions

After joint normalization, the chromatin interaction matrices are ready to be compared for differences. Again, the MD plot is used to represent the differences between two normalized datasets at a distance . The jointly normalized values are centered around 0 and are approximately normally distributed across all distances (Supplementary Section 1). values can be converted to Z-scores using the standard approach:

where is the mean value of all ’s on the chromosome and is the standard deviation of all values on the chromosome and is the th interacting pair on the chromosome.

## 2.5 Filtering low-abundance interaction pairs

During Z-score conversion the average expression of each interacting pair is considered. Due to the nature of , a difference represented by an interacting pair with IFs 1 and 10 is equivalent to an interacting pair of IFs 10 and 100 with both differences producing an value of 3.32. However, the average expression of these two differences is 5.5 and 55, respectively. Differences with higher average expression are supported by the larger number of sequencing reads and are therefore more trustworthy than the low average expression differences. Thus, we filter out differences with low average expression by setting the Z-scores to 0 when average expression () is less than a user set quantile (typically 5-20%) or a user set specific value of (Supplementary Section 1). Filtering takes place such that the and are calculated using only the values remaining after filtering. The Z-scores can then be converted to p-values using the standard normal distribution.

## 2.6 Multiple testing correction

Analyzing Hi-C data for differences necessarily involves testing of multiple hypotheses. Multiple testing correction (False Discovery Rate (FDR) by default) is applied on a per-distance basis by default, with an option to apply it on a chromosomal basis.

## 2.7 Benchmarking the differential chromatin interaction detection

As there is no “gold standard” for differential chromatin interactions, we created such *a priori* known differences by introducing controlled changes to replicate Hi-C datasets (Dozmorov *et al.*, 2010). The benefit of using joint normalization vs. individually normalized datasets was quantified by the improvement in power of detecting the pre-defined chromatin interaction differences. Standard classifier performance measures (Supplementary Section 4), summarized in the Matthews Correlation Coefficient (MCC) metric, were assessed. The results of HiCcompare analysis were further compared with those obtained with diffHiC method (Lun and Smyth, 2015).

## 2.8 Concordance between A/B genomic compartments

To assess the effect of normalization of the detection of A/B compartments, they were defined using the Principal Components Analysis (PCA) method (Lieberman-Aiden *et al.*, 2009) using the raw, jointly and individually normalized Hi-C data. The concordance of compartment detection was evaluated using three metrics: 1) the Pearson correlation coefficient between the vectors of principal components (PCs) detected from raw and normalized data, 2) the overlap of signs of PCs defining A (positive) and B (negative) compartments, and 3) the Jaccard overlap statistics.

## Parallelization

The biggest advantage of loess - the ability to model any biases in the data without explicitly specifying them - comes at the cost of increased computation. We implemented a parallelization strategy for processing chromosome-specific chromatin interaction matrices on multiple cores, improving the total run time (Supplementary Section 1).

## Availability and Future Directions

HiCcompare is available on Bioconductor at <https://bioconductor.org/packages/HiCcompare/> or on GitHub at <https://github.com/dozmorovlab/HiCcompare>. The package includes vignettes with test data and documentation for all functions. The “manuscript\_bioinformatics” branch of the GitHub repository contains code to generate all results referenced in this manuscript. HiCcompare is released under the MIT open source software license.

# 3 Results

## 3.1 The off-diagonal concept of distance between regions in chromatin interaction matrices

Our study focuses on the joint analysis of multiple Hi-C datasets represented by chromatin interaction matrices, where rows and columns represent genomic regions (bins), and cells contain interaction counts (frequencies). The values on the diagonal trace represent interaction frequencies of self-interacting regions. Each off-diagonal trace of values represents interaction frequencies for a pair of regions at a given unit-length distance. The unit-length distance is expressed in terms of resolution of the data (the size of genomic regions). The concept of considering interaction frequencies at each off-diagonal trace is central for the joint normalization and differential chromatin interaction detection (Figure 1).

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| --- |
| **Figure 1. Distance-centric (off-diagonal) view of chromatin interaction matrices.** Each off-diagonal vector of interaction frequencies represents interactions at a given distance between pairs of regions. Triangles mark pairs of genomic regions interacting at the same distance. Data for chromosome 1, K562 cell line, 50KB resolution, spanning 0 - 7.5Mb is shown. |

The interaction frequency drops as the distance between interacting regions increases. Numerous attempts have been made to parametrically model the inverse relationship between chromatin interaction frequency and the distance between interacting regions (Supplementary Section 2). However, Hi-C data are affected by technology- and DNA sequence-driven biases (Yaffe and Tanay, 2011; Cournac *et al.*, 2012; Imakaev *et al.*, 2012), unpredictably altering chromatin interaction frequencies. Consequently, parametric approaches fail to model interaction frequencies across the full range of distances (Sanborn *et al.*, 2015), confirmed by our observations (Supplementary Section 2).

## 3.2 Elimination of biases in jointly, but not individually, normalized Hi-C data

Discovery of biases in Hi-C data led to the development of numerous methods for normalizing *individual* datasets (Lieberman-Aiden *et al.*, 2009; Imakaev *et al.*, 2012; Knight and Ruiz, 2012; Cournac *et al.*, 2012). Although normalization of individual datasets improves reproducibility of replicated Hi-C data (Imakaev *et al.*, 2012; Yaffe and Tanay, 2011), these methods do not explicitly account for biases between *multiple* Hi-C data. The between-datasets biases are particularly problematic when comparing Hi-C datasets between biological conditions (Supplementary Section 3). When the detection of chromatin interaction differences due to biology, not biases, is important, normalization that removes the between-datasets biases is critical.

To assess the between-dataset biases, we visualize two Hi-C datasets on a single MD plot (see Methods). Briefly, differences in chromatin interaction frequencies (**M**inus) are visualized on a per-unit-length distance basis. Chromatin interactions are highly conserved (Dixon *et al.*, 2012; Fudenberg *et al.*, 2016; Rao *et al.*, 2014); thus, the majority of the **M** differences should be centered around zero. The MD plot visualization allows us to identify systematic biases appearing as the offset of the cloud of **M** differences from zero. Visualizing replicates of Hi-C data (Gm12878 cell line) showed the presence of biases in the individually normalized datasets (Figure 2, Supplementary Section 3), suggesting that the performance of individual normalization methods may be sub-optimal when comparing multiple Hi-C datasets.

To account for the between-datasets biases, we developed a non-parametric joint normalization method that makes no assumptions about the theoretical distribution of the chromatin interaction frequencies. It utilizes the well-known loess (locally weighted polynomial regression) smoothing algorithm - a regression-based method for fitting simple models to segments of data (Cleveland, 1979). The main advantage of loess is that it accounts for any local irregularities *between* the datasets that cannot be modeled by parametric methods. Thus, loess is particularly appealing when normalizing two Hi-C datasets, as the internal biases in Hi-C data are poorly understood (Figure 2).

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| **Figure 2.** MD plot data visualization and the effects of different normalization techniques. MD plots of the differences M between two replicated Hi-C datasets (GM12878 cell line, chromosome 11, 1MB resolution, DpnII and MboI restriction enzymes) plotted vs. distance D between interacting regions. (A) Before normalization, (B) after loess joint normalization, (C) ChromoR, (D) Iterative Correction and Eigenvector decomposition (ICE), (E) Knight-Ruiz (KR), (F) Sequential Component Normalization (SCN). |

Existing Hi-C data at high resolutions still suffer from a limited dynamic range of chromatin interaction frequencies, with the majority of them being small or zero, especially at large distances between interacting regions. This sparsity places limits on loess joint normalization, as it builds a rescaling model from many non-zero pairwise comparisons. A way to alleviate this limitation is to consider interactions only within a range of short interaction distances, where genomic regions interact more frequently, and the proportion of zero interaction frequencies is the lowest. Our evaluation of the loess joint normalization showed it performs best at resolutions between 1MB and 100KB (Supplementary Section 3).

## 3.3 Detecting differential chromatin interactions

To detect significant chromatin interaction differences, we introduced the *a priori* known chromatin interaction differences in replicate data from the GM12878 cell line. The benefits of the joint normalization vs. individually normalized datasets were evaluated in detecting the known differences. HiCcompare is able to detect most of the added differences with a relatively low number of false positives across the range of fold changes (Table 1, Supplementary Section 4).

**Table 1. Evaluation of the effect of normalization on differential chromatin interaction detection.** Matthews Correlation Coefficient of detecting 200 controlled differences in jointly (HiCcompare) vs individually normalized Gm12878 datasets, chromosome 1, 1Mb resolution.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Fold change | HiCcompare | MA | ICE | SCN | KR |
| 2 | 0.847 | 0.823 | 0.835 | 0.768 | 0.748 |
| 3 | 0.973 | 0.934 | 0.802 | 0.721 | 0.764 |
| 4 | 0.995 | 0.98 | 0.953 | 0.881 | 0.868 |
| 5 | 0.972 | 0.967 | 0.748 | 0.722 | 0.755 |

## 3.4 Comparison with diffHiC

The diffHiC pipeline was designed to process two raw Hi-C sequencing datasets and detect chromatin interaction differences using the generalized linear framework developed in the edgeR package (Lun and Smyth, 2015). We compared the results of Hi-C data analyzed in the diffHiC paper (human prostate epithelial cells RWPE1 overexpressing the EGR protein or GFP (Rickman *et al.*, 2012)) with the results obtained with HiCcompare.

diffHic detected a total of 5,737 significant differences (FDR < 0.05), while HiCcompare tended to be more conservative, detecting 314 differences (FDR < 0.05) and 7,620 differences when multiple testing correction was not applied (p-value < 0.05). Of the 314 differences, 121 overlapped with the regions detected by diffHic and of the 7,620 differences, 1,654 overlapped with the regions detected by diffHic. Surprisingly, although diffHiC used CNV correction in their analysis, 2,567 (44.7%) of the detected differentially interacting regions overlapped with CNV regions detected in our analysis, and/or blacklisted regions. diffHic tended to detect differentially interacting regions with smaller fold changes as compared to HiCcompare, and at shorter distances between interacting regions, while HiCcompare can detect differences across the full range of distances (Supplementary Section 5). These results suggest that detecting chromatin interaction differences represented in the MD coordinates, as implemented in HiCcompare, may be useful in detecting large chromatin interaction differences across the full range of distances, potentially having a more significant biological effect.

## 3.5 Preservation of A/B compartments

A/B compartments are the best known genomic structures that can be detected from Hi-C data (Lieberman-Aiden *et al.*, 2009). To understand the consequences of the joint vs. individual normalization methods on the detection of A/B compartments we compared principal components defining compartments in raw vs. normalized data. A/B compartments detected following the joint normalization were the most similar to those detected in the raw data (Table 2). These results suggest that the joint HiCcompare normalization preserves properties of Hi-C data needed for the accurate detection of A/B compartments.

**Table 2. Similarity between A/B compartments detected following various normalization methods.** “Correlation” - Pearson correlation coefficient between principal components defining A/B compartments in raw vs. normalized Hi-C data; “Prop. Match Sign” - the proportion of regions with matching sign defining A/B compartments; “Jaccard A/B” - Jaccard overlap statistics between A/B compartments, respectively. All values represent averages over all chromosomes.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Comparison | Correlation | Prop. Match Sign | Jaccard A | Jaccard B |
| HiCcompare vs. Raw | 0.9954 | 0.8537 | 0.8719 | 0.8284 |
| SCN vs. Raw | 0.9309 | 0.8083 | 0.6641 | 0.5737 |
| ICE vs. Raw | 0.9795 | 0.785 | 0.6969 | 0.6141 |

# Discussion

This work introduces three novel concepts for the joint normalization and differential analysis of Hi-C data, implemented in the HiCcompare R package. First, we introduce the representation of the differences between two Hi-C datasets on an MD plot, a modification of the MA plot (Dudoit *et al.*, 2002). Importantly, we consider the data on a per-distance basis, allowing the data-driven normalization of global biases without distorting the relative distribution of interaction frequencies of the interacting regions. Second, we implement a non-parametric loess normalization method that minimizes biases-driven differences between the datasets. There is compelling evidence that non-parametric normalization methods, such as quantile- and loess normalization, are particularly suitable for removing the between-dataset biases (Shao *et al.*, 2012; Bolstad *et al.*, 2003), confirmed by our application of loess to the joint normalization of Hi-C data. Third, we develop and benchmark a simple but rigorous statistical method for the differential analysis of Hi-C datasets.

The importance of joint normalization in removing the between-dataset biases has been demonstrated using the MA normalization introduced in the diffHiC R package (Lun and Smyth, 2015). The MA normalization uses a similar concept of representing measures from two datasets on a single plot (Lun and Smyth, 2015), except it uses the **A**verage chromatin interaction frequency as an X-axis instead of the **D**istance used by us. The MA normalization performed second to HiCcompare (Table 1, Supplementary Section 4). This may be due to the power-law decay of interaction measures leading to the limited dynamic range of average chromatin interaction frequencies and making fitting a loess curve difficult. Instead, the more balanced representation of chromatin interaction differences **M** (Y-axis) as a function of distance **D** (X-axis) improves the performance of loess fit for the joint normalization and the subsequent detection of chromatin interaction differences.

The discrepancy of differential chromatin interaction detection between diffHiC and HiCcompare (Supplementary Section 5) could arise from multiple factors. diffHiC’s implementation of the MA normalization favors differences at shorter distances and small fold changes while HiCcompare’s loess fitting through the MD plot allows for the detection of large chromatin interaction differences across the full range of interaction frequencies (Supplementary Section 5). diffHiC operates on logCPM counts while HiCcompare uses log interaction frequency counts. diffHiC uses enzyme cut sites to define bins when partitioning the genome while HiCcompare uses fixed bin size. diffHiC uses median inter-chromosomal interaction frequency cutoff to filters low-abundance bin pairs while HiCcompare filters based on average IFs of the chromosome being considered. Finally, the RWPE1 data analyzed by diffHiC is relatively sparse even at 1Mb resolution, potentially interfering with HiCcompare statistical analyses. In summary, diffHiC and HiCcompare may provide complementary views on chromatin interaction differences, with HiCcompare being better suited for removing the between-datasets biases and the detection of biology-driven chromatin interaction differences.

Despite the ability of Hi-C technology to simultaneously capture all genomic interactions, current resolution of Hi-C data (1Mb - 1kb) remains insufficient to resolve individual *cis*-regulatory elements (~100b-1kb). Alternative techniques, such as ChiA-PET (Fullwood *et al.*, 2009), Capture Hi-C (Mifsud *et al.*, 2015) have been designed to identify targeted 3D interactions, e.g., between promoters and distant regions. These data require specialized normalization methods (Cairns *et al.*, 2016). Our future goals include extending the loess joint normalization method for chromosome conformation capture data other than Hi-C.

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