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Sequence analysis

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Discovering chimeric transcripts in paired-end RNA-seq data by using EricScript

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ABSTRACT

Motivation: The discovery of novel gene fusions can lead to a better comprehension of cancer progression and development. The emergence of deep sequencing of trancriptome, known as RNA-seq, has opened many opportunities for the identification of this class of genomic alterations, leading to the discovery of novel chimeric transcripts in melanomas, breast cancers and lymphomas. Nowadays, few computational approaches have been developed for the detection of chimeric transcripts. Although all of these computational methods show good sensitivity, much work remains to reduce the huge number of false-positive calls that arises from this analysis.

Results: We proposed a novel computational framework, named chimEric tranScript detection algorithm (EricScript), for the identification of gene fusion products in paired-end RNA-seq data. Our simulation study on synthetic data demonstrates that EricScript enables to achieve higher sensitivity and specificity than existing methods with noticeably lower running times. We also applied our method to publicly available RNA-seq tumour datasets, and we showed its capability in rediscovering known gene fusions.

Availability: The EricScript package is freely available under GPL v3 license at http://ericscript.sourceforge.net.

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1 INTRODUCTION

The identification of genomic rearrangements in cancer research plays a main role to investigate causes and development of the disease. Gene fusions are common alterations in which two genes are fused, leading to the production of a chimeric transcript that may have a new or altered activity. Gene fusions are well-known mechanisms for oncogene activation in leukaemias, lymphomas and sarcomas (Mitelman *et al.*, 2007), but recent studies found novel chimeric transcripts also in common epithelial cancers, such as prostate cancers (Tomlins *et al.*, 2005) and non–small-cell lung cancer (Soda *et al.*, 2007). The past few years have seen the emergence of several high-throughput sequencing platforms that enable to sequence hundreds of millions of short sequences (reads) simultaneously and have routinely being applied to genome, epigenome and transcriptome studies.

The sequencing of trancriptome (Mortazavi *et al.*, 2008; Nagalakshmi *et al.*, 2008), known as RNA-seq, has been widely used for the study of abundance estimation (Jiang and Wong, 2009), RNA editing (Picardi *et al.*, 2010), identification of novel transcripts (Robertson *et al.*, 2010) and splicing variants detection (Trapnell *et al.*, 2010; Wang *et al.*, 2010). In 2009, Maher *et al.* (2009) proposed a new methodology to comprehensively catalogue functional gene fusions in cancer by using paired-end (PE) transcriptome sequencing data. The basic idea behind their approach was the identification of paired reads mapping against different genes (discordant alignments). Following this original approach, several studies for the detection of gene fusions have been carried out, Pflueger *et al.* (2011) found non-ETS gene fusions in human prostate cancer, Berger *et al.* (2010) identified 11 novel melanoma gene fusions produced by underlying genomic rearrangements and 12 novel read-through transcripts, Edgren *et al.* (2011) detected 24 novel and 3 previously known fusion genes in breast cancer cells and Steidl *et al.* (2011) found highly expressed gene fusion involving the major histocompatibility complex class II transactivator CIITA in KM-H2 cells.

At present, several computational approaches have been developed for the detection of chimeric transcripts in RNA-seq data. FusionSeq (Sboner *et al.*, 2010) identifies gene fusions by means of a two step analysis, identification of potential fusions based on PE mapping and the application of a sophisticated filtration cascade to filter out analysis artifacts. DeFuse (McPherson *et al.*, 2011) is a software package that uses clusters of discordant paired-end alignments to perform a split read alignment analysis for finding fusion boundaries. ChimeraScan (Iyer *et al.*, 2011) is a tool that implements the original computational methodology followed by Maher *et al.* (2009). FusionMap is able to search for gene fusion products in both single-end and paired-end sequencing by using 'seed reads' (Ge *et al.*, 2011). TopHat fusion implements several changes to the TopHat aligner, all designed to enable the discovery of fusion transcripts (Kim and Salzberg, 2011). ShortFuse (Kinsella *et al.*, 2011) detects chimeric transcripts RNA-seq data by using both

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unique and ambiguously mapping read pairs. Although all of these computational methods show good sensitivity in discovering chimeric transcript events, much work remains to reduce the huge number of false-positive results that arises from this kind of analysis.

Here, we present a novel computational method, named EricScript (chimERIC tranSCRIPT detection algorithm), for the detection of chimeric transcripts in PE RNA-seq data. The novelty of our approach consists in an efficient recalibration process of the exon junction reference that enables to increase sensitivity and specificity and to reduce running times. Moreover, we introduce a set of scores that enable to distinguish with high precision between true chimeric transcripts and false-positive events and reduce the large amount of calls generated from data analyses. To evaluate the performance of EricScript, we generated synthetic datasets of different read length simulating different levels of coverage, and we compared it with other four state-of-the-art algorithms. The synthetic datasets were also used to train an adaptive boosting (AdaBoost) classifier to rank analysis results. We implemented the procedure that we used to simulate gene fusions in the EricScript package. Our simulation study demonstrates that EricScript enables to achieve higher sensitivity and specificity than existing methods with noticeably lower running times. We also applied our method to publicly available PE RNA-seq tumour dataset, and we showed its capability in rediscovering known gene fusions.

2 METHODS

EricScript is a computational framework (see Fig. 1) that uses a combination of four alignment processes to identify fusion transcript signatures. It comprises the following steps:

- Mapping of the reads against the transcriptome.
- Identification of discordant alignments and building of the exon junction reference.
- Recalibration of the exon junction reference.
- Scoring and filtering the candidate gene fusions.

The first alignment [performed by Burrows–Wheeler alignment (BWA) (Li and Durbin, 2009)] is used to identify discordant alignments and to build an exon junction reference. This step is followed by the mapping of all the reads against this novel reference to detect reads that are not properly mapped (i.e. partially mapped and unmapped reads). To precisely estimate the exact borders of the junctions, our method performs a further local realignment [performed by BLAT (Kent, 2002)] of the not properly mapped reads against the exon junction reference. The last mapping procedure allows for the identification of the spanning reads, that is, the reads that span across the junctions and produce a list of candidate fusions. Finally, EricScript estimates a probability score for each predicted fusion and uses several heuristic filters to remove analysis artifacts.

2.1 Transcriptome reference

EricScript includes a pre-built transcriptome reference for the alignment process and the retrieval of information about genes. The set of genes we consider for the analysis of chimeric transcripts is created by including the Ensembl genes with the HUGO Gene Nomenclature Committee (HGNC) identifier (Seal *et al.*, 2011). The sequences of each gene are built by joining the sequences of the exons from the different transcript isoforms (exon-union model). We distribute EricScript with the latest version of Ensembl Genes (http://www.ensembl.org); therefore, no other reference is required to perform the analysis.

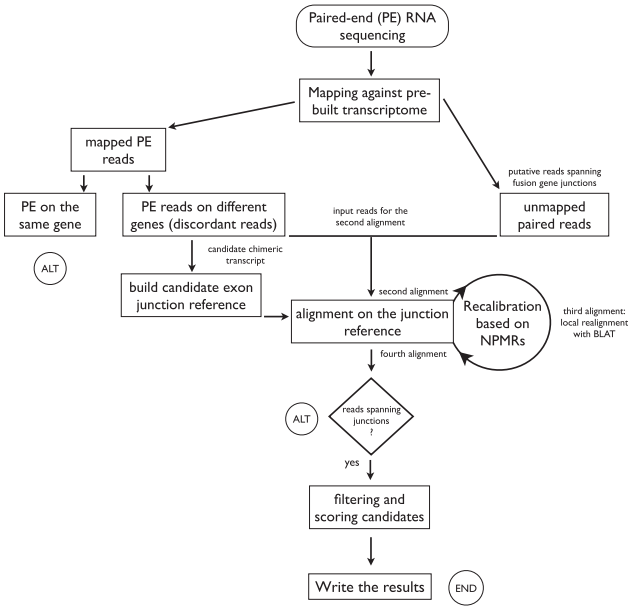

Fig. 1. The computational pipeline of EricScript

2.2 Identification of discordant reads

The first essential step in chimeric transcripts identification from PE RNA-seq data is to select reads for which each mate of the fragment aligns against different genes with opposite orientation. Although our approach is independent by the short-read aligner, we decided to use the BWA tool, as it reaches the best balance between sensitivity, specificity and computational time (Ruffalo *et al.*, 2011). The BWA mapping of all the reads against the pre-built Ensembl transcriptome is used to identify discordant alignments. To increase the sensitivity of BWA in discovering discordant alignments (especially when the length of the reads is $\geq$75 nt), the parameter *ntrim* allows EricScript to trim PE reads to a selected value only for this alignment (see Supplementary Material). EricScript enables to choose discordant alignments supported by a minimum number of the reads (*minreads*) and a minimum mapping quality of the supporting reads (*MAPQ*). The set of reads that map to the same pairs of genes in the same orientation are considered by EricScript to build a reference of putative gene fusion events (Fig. 2a and Supplementary Material). Discordant alignments between paralogous genes (Ensembl Paralogous Human Genes) are filtered out and not considered for the downstream analysis.

The usage of exon-union model together with BWA allows us to exploit the MAPQ's BWA estimation. In fact, the quality of the mapping assigned by BWA to each alignment is an excellent way to reduce false-positive results in the mapping process (Ruffalo *et al.*, 2011). Moreover, exon-union model reports all the exons once, allowing us to completely exploit the MAPQ information, mainly by excluding discordant alignments with MAPQ lower than a selectable threshold value.

2.3 Candidate exon junction reference

The identification of discordant alignments enables to build the candidate exon junction reference that will be used to search for split-read signatures. For each set of discordant alignments (i.e all the discordant reads that map against the same couple of genes), 5′-gene is identified by the signature of forward reads (first reads of the pairs) mapping to it, whereas

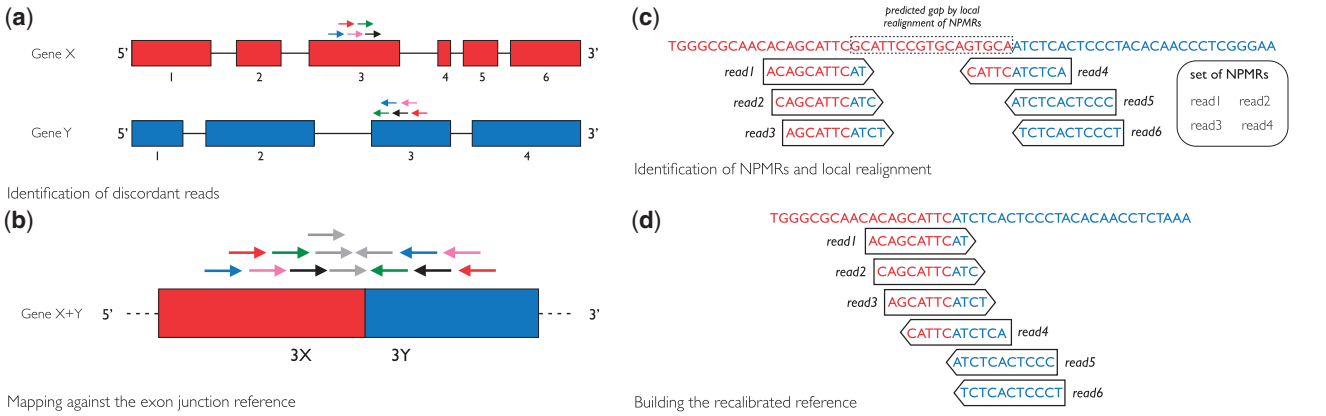

Fig. 2. A simplified scheme for illustrating the recalibration procedure of EricScript. (**a**) Identification of discordant alignments and construction of the exon junction reference. The reads with the same colour belong to the same cDNA fragment, that is, they are mates. (**b**) Mapping of all the short reads against the exon junction reference by means of the BWA aligner. The NPMRs are reported in grey colour. (**c**) Identification of NPMRs and local realignment of them against the exon junction reference by using BLAT. The local realignment reports a gap (**d**) that allows EricScript to recalibrate the exon junction reference. The mapping of all the reads against the augmented recalibrated reference (see text) enables the identification of the junction spanning reads

the reverse reads are exploited to identify 3′-gene. Let R_5 (R_3) be the genomic region encompassing all the alignments of crossing reads to 5′-gene (3′-gene). Let the m (n) candidate exons for 5′-gene (3′-gene) be all exons overlapping R_5 (R_3). As schematically illustrated in Figure 2b and c, exon junction reference is generated by joining together the sequences of the m exons of 5′-gene with the n exons of 3′-gene, according to the strand of transcription of both the genes. Bearing this in mind, the candidate exon junction will result from the union of the last nucleotide of the m-th exon with the first nucleotide of the n-th exon, according to the strand of transcription of both the genes.

2.4 Recalibration of the exon junction reference

Gene fusions can involve both fusions between genomic boundary of exons and fusions between any genomic positions of the two exons. Moreover, as we build the set of putative fusions by joining the exon boundaries of the candidate 5′–3′ fused genes, our junction reference strictly depends on the Ensembl transcriptome. Bearing this in mind, all the reads are mapped against the exon junction reference, and the reads that are not properly mapped (not properly mapped reads, NPMRs) are identified. We define NPMRs as the reads that map for a fraction of their length against the reference or are unmapped (Fig. 2b). NPMRs represent the candidate set of reads that span a fusion boundary and allow us to find fusions that involve middle of exons. Our pipeline classifies reads as NPMRs if they are unmapped or their string for mismatching positions (i.e. MD:Z tag of SAM file) reports a mismatch, and its mapping quality is >0. Each NPMR is then locally realigned against its corresponding junction by means of the BLAT aligner to predict the existence of gaps >3 bp (see Supplementary Material for more details). As schematically illustrated in Figure 2b and c, this step allows us to investigate whether the majority of the NPMRs predict a gap. This means that when two or more gene fusion isoforms are expressed in a sample, EricScript is able to only detect the transcript with the highest expression level. The exon junction reference is then recalibrated by taking into account the predicted gap (Fig. 2d).

2.5 Mapping against the recalibrated reference

After the recalibration step, we build a novel reference that comprises both the pre-built Ensembl transcriptome and the previously recalibrated junction reference. All the reads are mapped against this augmented reference by means of the BWA aligner. Putative gene fusion junction is selected for downstream analysis, if there exist at least one read that spans the junction.

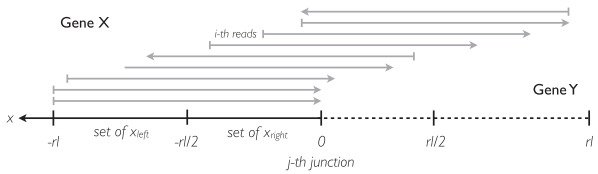

Fig. 3. Coordinate system and variables for evaluating the scores of EricScript

2.6 Scoring the candidate fusions

As already reported by Edgren *et al.* (2011), we expect genuine fusion junctions to be characterized by a ladder-like pattern of short reads alignment across the junctions. On the other hand, a typical false-positive event because of a 'wrong' pattern is represented by short reads aligning around to the same position (shifted at maximum of 2–3 bp). To distinguish genuine from wrong patterns, we introduced three novel scores, named genuine junction score (*GJS*), Edge Score (*ES*) and Uniformity Score (*US*). To produce a single score, we trained an AdaBoost classifier on the aforementioned scores that allows EricScript to rank predicted gene fusions. To comprehend the parameters used to define these score, refer to Figure 3.

2.6.1 GJS The aim of introducing GJS is to assign higher scores to those gene fusions characterized by the presence of reads spanning a comparable number of bases in both the genes. For each junction j, duplicated spanning reads are considered only once. This set of $n_{j,\,\mathrm{unique}}$ reads is used to calculate the *GJS* as follows:

$$GJS_j = \frac{\sum_{i=1}^{n_{j,\,\mathrm{unique}}} N(x_i|\mu,\sigma)}{\sum_{i=1}^{n_{j,\,\mathrm{unique}}} N(z_i|\mu,\sigma)}, \quad (1)$$

where x_i is the relative position of each read with respect to the junction, N is the normal distribution with mean $\mu = -rl/2$ and SD $\sigma = rl/4$ (rl is the read length of the reads) and z is a vector made up of $n_{j,\,unique}$ 'sham' positions that would have minimum distance of $-rl/2$, that is, the position for which reads span both the genes with the maximum number of bases. The choice of using z_i enables to constrain GJS_j between 0 and 1. To be clear, we report the following example: let's assume we have two cases (i) $rl = 50$, $x = -1$, -5, -40, -5 and (ii) $rl = 50$, $x = (-10, -25, -30)$. For both the cases, the vector $z = (-25, -24, -26)$. z has 3 elements as the number of unique positions for both the examples we reported ($n_{j,\,unique} = 3$), and its elements are the positions with minimum distance for $-rl/2$. In case (i) we obtain a GJS = 0.31, whereas in case (ii) GJS = 0.81.

2.6.2 ES For each junction j, we parted reads in two sets, reads with relative position $x > -rl/2$ with respect to the junction (we marked them by the *right* subscript) and reads with $x \leq -rl/2$ (we marked them by the *left* subscript). We defined *ES* with the following formula:

$$ES_j = 1 - 1.1^{\left(\frac{-\overline{x_{j,\,\mathrm{left}}} + rl + \overline{x_{j,\,\mathrm{right}}}}{2}\right)}, \quad (2)$$

where the overlines represent the mean, and the 1.1 base is arbitrarily chosen to soften variations in the exponent. The *ES* score allows us to give lower score values to events with the majority of reads that fall in proximity of the fusion junction or in proximity of $-rl$.

2.6.3 US The *US* score was conceived to assign higher scores to events in which the number of spanning and crossing reads are comparable. For each junction j, *US* is defined as the follows:

$$US_j = \frac{\min(n_{j,\,cross}, n_{j,\,span})}{\max(n_{j,\,cross}, n_{j,\,span})}, \quad (3)$$

where $n_{j,\,cross}$ and $n_{j,\,span}$ are the number of crossing and spanning reads, respectively.

2.6.4 AdaBoost classifier To better rank predicted gene fusions, we used an AdaBoost classifier trained with synthetic data (see 'Results' section and Supplementary Material). As already reported by McPherson *et al.* (2011), AdaBoost was selected because it enables to improve the predictive power of each individual score and summarize the aforementioned measures into a single score (we indicate it as 'EricScore').

2.7 Filtering the results

Filtering is an essential procedure when dealing with the detection of chimeric transcripts, as several types of noise of both sequencing and analysis process can lead to the detection of a large amount of gene fusion artifacts (Edgren *et al.*, 2011; Sboner *et al.*, 2010). To this end, we designed a set of heuristic filters with the aim of discarding these false-positive events.

2.7.1 Duplicate reads We discard all the PE reads that exactly map to the same position, as they may derive from polymerase chain reaction or optical artifacts. We use the command *rmdup* of SAMtools to remove these events.

2.7.2 Pattern of short reads Scoring the candidate fusions by means of *EricScore* allows us to assign to each candidate a probability score of 'well' pattern, and thus classify all the fusions for discriminating between real transcripts and false-positive events.

2.7.3 Transcript similarity Reads mapping on homologue regions of different genes can lead to chimeric transcript artifacts. To minimize these events, we use BLAT to map the 100 bp sequence region around the wild-type junction against the Ensembl transcriptome. If BLAT finds that the 100 bp window sequence map $\geq 80\%$ of its length against one of the two candidate fused genes, we remove the candidate fusion.

2.7.4 Junction homology The junction coming from fusion process can be a homologue with other regions of the transcriptome. To take into consideration these events, we map the 100 bp sequence region around the predicted junction against the Ensembl transcriptome with BLAT. If BLAT finds a homology with other genes, EricScript reports the percentage of homology, that is, the percentage of the bases of the homologue gene(s) that overlaps the 100 bp junction sequence.

2.8 Writing results

After the filtering process, EricScript reports the candidate fusions in two tab-delimited files, one file contains all the predicted fusions, whereas the other reports the fusions with *EricScore* >0.5. For each predicted gene fusion, EricScript outputs several information that include the names of 5′ and 3′ genes and their corresponding biological descriptions, the breakpoint positions for both the genes, the sequence that arises from the fusion process and the type of fusion (inter-chromosomal, intra-chromosomal, read-through or *cis*-acting transcripts). Moreover, we report the four scores (*GJS*, *ES*, *US*, *EricScore*) and the estimation of gene expression of wild-type genes and of the gene fusion product by using a read count approach (see Supplementary Material for more details).

2.9 Implementation, requirements and availability

EricScript is written in perl, R and bash scripts. It requires the BWA aligner to perform the mapping of the PE RNA-seq short reads against the transcriptome, the SAMtools software package (Li *et al.*, 2009) to handle with the SAM/BAM files created during the analysis and the BLAT tool to perform the local realignment of the NPMRs against the exon junction reference. EricScript is freely available under GPL v3 license at http://ericscript.sourceforge.net.

3 RESULTS

3.1 Synthetic data

To assess a reliable estimation of the performance of EricScript, we simulated PE RNA-seq data with synthetic gene fusions, and we compared our method with ChimeraScan (Iyer *et al.*, 2011), DeFuse (McPherson *et al.*, 2011), FusionMap (Ge *et al.*, 2011) and ShortFuse (Kinsella *et al.*, 2011). We generated each synthetic dataset with the following recipe: we randomly extracted 2 million short reads from the RNA-seq data of untreated human pulmonary microvascular endothelial cells generated by Zhang *et al.* (2012) (SRA accession code: SRX099065). This dataset is made of 10.3G PE 100 bp reads sequenced by the Illumina HiScan SQ (Illumina Inc., San Diego, CA, USA). By aligning all the reads against the Ensembl transcriptome database version 65 with BWA (version 0.6.2-r126), we estimated that cDNA fragments were generated from cDNA fragments of length ~164 and SD ~48. The reads were also trimmed to 50 and 75 bp to evaluate the performance of each algorithm for different read lengths. The purpose of introducing these reads in our study is to simulate a background of 'synthetic' gene activity. To simulate synthetic gene fusion products, we sampled 50 5′-transcripts and 50 3′-transcripts

from the Ensembl transcriptome database version 65. We created two distinct datasets as follows:

- Intact exons (IE): Each sampled 5′-transcript was joined with the corresponding 3′-transcript, and the breakpoints for both transcripts were randomly chosen among all the known splicing sites of synthetically fused genes.
- Broken exons (BE): Each sampled 5′-transcript was joined with the corresponding 3′-transcript, and the breakpoints for both transcripts were randomly chosen without exploiting information of the known splicing sites of synthetically fused genes.

From these novel references, we simulated 50, 75 and 100 bp PE reads by means of wgsim (http://github.com/lh3/wgsim) (with -d 164 -r 0.0001 -R 0.001 -s 48). We varied the number of reads generated by wgsim to simulate different levels of coverage (from 1 to 50). The final synthetic PE RNA-seq dataset is built by merging, for each read length data, the background dataset and the simulated gene fusions (for both IE and BE data). Although such a synthetic dataset is an ideal and simplistic case for simulating gene fusion processes, the use of it allows us to objectively assess chimeric transcripts discovery algorithms. To this end, we generated 50 synthetic PE RNA-seq datasets for each read length data and for both BE and IE events (for synthetic datasets and 15 000 synthetic gene fusions), and we analysed them by using EricScript (with $minreads = 2$, $MAPQ = 1$ and $ntrim = 50$), ChimeraScan, DeFuse, FusionMap and ShortFuse (see Supplementary Material and Supplementary Table S1 for more details). We compared the performance of these algorithms by using the following statistical indices:

- True-positive rate (TPR) or *detection sensitivity*. We defined TPR as the number of gene fusions correctly predicted by the algorithm divided by the number of simulated fusions (50).
- False-positive rate (FPR) or *detection specificity*. We defined FPR as the number of predicted gene fusions that are not in the list of simulated fusions divided by the number of detected events.
- False-negative rate (FNR). FNR corresponds to the number of undetected gene fusions divided by the number of simulated fusions (i.e. 1- *detection sensitivity*).
- True-positive sequence rate (TPSR). TPSR is the number of correctly determined junction sequences divided by the number of correctly predicted gene fusions.
- Area under the curve (AUC). AUC is a measure of the accuracy of each algorithm in discriminating between true- and false-positive results. This parameter is estimated by means of the receiver operating characteristic (ROC) curve. Details on how ROC curves were built are available in Supplementary Material.

The TPR, FPR and FNR statistical indices are useful to estimate 'detection accuracy' of each algorithm. In fact, these measures considered all the calls, irrespective of scores assigned to the identified fusion events. On the other hand, AUC and the ROC curves reported in this manuscript enable us to evaluate 'scoring accuracy' of each algorithm, which means the ability of such an algorithm in discriminating between true- and false-positive events.

The results of these analyses are reported in Figure 4, Table 1 and Supplementary Material. The ROC curves of Figure 4a and Supplementary Figure S2 clearly show that our algorithm obtains better performance than the other state-of-the-art methods in distinguishing between true- and false-positive events. EricScript outperforms the other methods in all the simulations we performed with different read lengths for both BE and IE datasets, with the exception of data with read length of 75 and 100 bp in which FusionMap reaches similar results. Figure 4b shows the capability of the five algorithms in identifying the correct fusion genes versus the simulated coverage for all the IE datasets, whereas Supplementary Figure S2b is related to BE simulations. For both the datasets, when coverage is $<10\times$, all the algorithms are not able to reliably discover fused

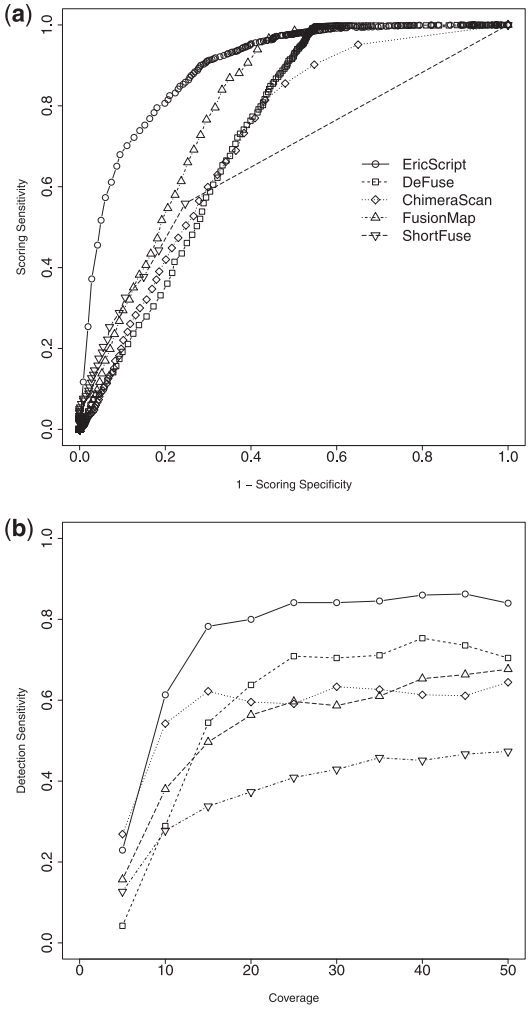

Fig. 4. Results of the simulation study among 150 synthetic IE datasets. (**a**) Comparison between ROC curves obtained for EricScript and the other state-of-the-art gene fusion detection methods. (**b**) TPR of EricScript and the other fusion discovery methods versus coverage (each point of the plot represents a bin of five values of coverage). The legend of (a) is relative to both the plots

Table 1. Comparison of statistical indices between EricScript and the other gene fusion detection methods among the 150 synthetic IE datasets[a]

Method	TPR	FPR	FNR	TPSR[b]	AUC	Time[c]
EricScript	0.75	0.15	0.25	>0.99	0.90	0.53
ChimeraScan	0.58	0.40	0.42	—	0.72	0.76
DeFuse	0.58	0.39	0.42	>0.99	0.76	1.3
FusionMap	0.54	0.63	0.46	>0.99	0.80	1.6
ShortFuse	0.38	0.13	0.62	—	0.67	0.33

[a]All the values are obtained by averaging across all the simulations we performed.
[b]ChimeraScan and ShortFuse do not output fusion junction sequence.
[c]Expressed as CPU hours.

transcripts. On the other hand, for IE data and for coverage >10×, our method detects gene fusions with TPR >0.8 followed by DeFuse that is able to discover almost 70% of the fusions we simulated. ChimeraScan and FusionMap detect ∼60% of gene fusion events. For BE data (Supplementary Fig. S2b), ChimeraScan and ShortFuse lose their prediction capability, whereas EricScript, DeFuse and FusionMap do not. The strong performance of EricScript and DeFuse in BE data is because of the fact that both the algorithms detect fused transcripts by a split-read approach, allowing them to identify fusions involving middle of exons. FusionMap performs well on these datasets, as we run it with $G = 0$ to not penalize non-canonical splice patterns (Supplementary Material). Conversely, ChimeraScan and ShortFuse are computationally designed to privilege fusions involving known splicing sites. The results corresponding to different read lengths are reported in Supplementary Figures S3–S8. In these plots, we observe that the overall performance of Defuse and ShortFuse decreases, whereas the length of the reads increases. This is because of two main reasons (i) these algorithms have been calibrated on reads of 50 nt in size and (ii) at fixed coverage, the longer are the reads, the smaller is the number of discordant reads. Increasing read length does not affect ChimeraScan, EricScript and FusionMap performance. The results reported in Table 1 and Supplementary Table S6 are obtained by averaging across the 150 synthetic IE datasets and 150 synthetic BE datasets, respectively. The FPRs reported in these Tables highlight that our algorithm outperforms the majority of the other methods; the probability of EricScript to make a wrong call is ∼0.15, whereas other algorithms obtain FPR values that range between 0.15 (ShortFuse) and 0.63 (FusionMap). However, it is important to note that FPR may be misleading, as a tool could predict thousands of low scoring false-positive results. This would affect FPR, even though these events are easily discernable from true-positive results based on their low score (see the comments aforementioned on ROC curves analysis) or on the fact that these events are supported by a few number of supporting reads. In fact, when we consider calls with predicted number of supporting reads >5, all the algorithms (especially FusionMap) show a strong increase in specificity (see values in parentheses of Supplementary Tables S7–S12 and Supplementary Fig. S9) to the detriment of a small decrease in terms of sensitivity. The simulation study we performed also shows that EricScript, DeFuse and FusionMap obtain excellent results in reconstructing the correct fusion gene junction sequences, whereas ChimeraScan and ShortFuse do not output this information (TPSR score). Table 1 also reports the average computational time taken by each algorithm to complete the analysis; ShortFuse obtains the best performance and requires ∼80% less time than FusionMap. Although EricScript uses a four-step alignment pipeline, it takes only 0.53 h per CPUs to perform the analysis. This is because of the fact that we use a transcriptome instead of a genome reference to map the reads; this feature does not allow EricScript to detect fusions involving unannotated transcripts. Supplementary Tables S7–S12 report the results relative to different read lengths and make clear the aforementioned coverage effect. If we set $ntrim = 0$, EricScript is also affected by coverage effect mainly for read length of 100 bp and coverage <10× (Supplementary Tables S2 and S3). This is the reason why we performed these analyses with $ntrim = 50$ for read length of 75 and 100 bp (see Supplementary Material for more details).

3.2 Application to previously reported gene fusions

We applied EricScript to publicly available PE RNA-seq datasets (Table 2) with the aim of evaluating its capability in discovering previously characterized gene fusion products. We analysed the NCI-H660 prostate cell line dataset for the TMPRSS2–ERG and FOXP1–RYBP fusions (Pflueger *et al*., 2011; Sboner *et al*., 2010) (see Supplementary Material for a comparison between EricScript and DeFuse on these data), and we searched for the 23 validated gene fusions in the four breast cancer cell lines of Edgren *et al*. (2011) (SRA accession: SRP003186). We run EricScript on these datasets with two different sets of input parameters, setting *a* with $minreads = 2$ and $MAPQ = 1$ and setting *b* with $minreads = 3$ and $MAPQ = 20$. In both cases, EricScript took ∼14 CPU h to complete the whole analysis on ∼60 million reads. The results of the analyses are reported in Table 3.

With parameter setting *a*, our method predicted 489 fusions (Supplementary File S1). It was able to detect 22 of the 25 known fusions, and for all of them, EricScript is able to assemble the correct sequence of the junction. In the BT-474 library, we predicted 9/10 validated gene fusion, whereas we missed the fusion CPNE1–PI3. This fusion was filtered out by EricScript, as BWA found three discordant alignments with $MAPQ = 0$ between CPNE1 and both PI3 and RBM12, potentially indicating a read-through between CPNE1 and RBM12. This situation also happens for the ANKHD1–PCDH1 fusion in the SK-BR-3 sample. BWA found six discordant alignments with $MAPQ = 0$ between ANKHD1 and both PCDH1 and ANKHD1–EIF4EBP3 (ENSG00000254996). EricScript identified all the other validated fusions of SK-BR-3 dataset, including the DHX35–ITCH fusion that, as reported by Kim and Salzberg (2011), neither DeFuse nor TopHat fusion are able to detect. In the KPL-4 cell line, we were able to detect all the known fusions, whereas in the MCF-7 sample, our method was not able to rediscover the RPS6KB1–TMEM49 fusion, as BWA found no discordant read.

With setting *b*, EricScript predicted 20 of the 25 validated fusions (see Supplementary File S2) and missed the events supported by less than three supporting reads (WDR67–ZNF704

Table 2. RNA-seq datasets used for EricScript validation[a]

Reference	Library	Number of reads	Read length	Time (CPU hours)[b]	Predicted fusions (setting *a*)	Predicted fusions (setting *b*)
Sboner *et al.* (2010)	NCIH660	6 512 688	51	1.2	31 (7)	12 (5)
Edgren *et al.* (2011)	BT-474	21 423 697	50	3.8	193 (53)	84 (43)
Edgren *et al.* (2011)	SK-BR-3	18 240 246	50	3.4	180 (35)	61 (22)
Edgren *et al.* (2011)	KPL-4	6 796 443	50	1.1	39 (8)	15 (4)
Edgren *et al.* (2011)	MCF-7	8 409 785	50	1.4	46 (9)	21 (10)

[a]The number of gene fusions identified by EricScript with *EricScore* >0.5 is reported between parentheses.
[b]The reported run time is for EricScript with setting *a*.

Table 3. EricScript results in the publicly available PE RNA-seq datasets of Edgren *et al.* (2011) and Sboner *et al.* (2010)[a]

Library	5′ gene	3′ gene	Crossing reads[b]	Spanning reads[c]	EricScript (setting *a*)	EricScript (setting *b*)	EricScript (correct sequence)	EricScore
NCIH660	TMPRSS2	ERG	18	15	✓	✓	✓	0.97
NCIH660	FOXP1	RYBP	12	6	✓	✓	✓	0.57
BT-474	ACACA	STAC2	56	80	✓	✓	✓	0.89
BT-474	VAPB	IKZF3	41	32	✓	✓	✓	0.97
BT-474	ZMYND8	CEP250	36	25	✓	✓	✓	0.96
BT-474	RAB22A	MYO9B	10	21	✓	✓	✓	0.94
BT-474	SKA2	MYO19	8	9	✓	✓	✓	0.97
BT-474	STARD3	DOK5	6	5	✓	✓	✓	0.93
BT-474	LAMP1	MCF2L	5	2	✓	✓	✓	0.88
BT-474	GLB1	CMTM7	6	4	✓	✓	✓	0.68
BT-474	CPNE1	PI3	—	—	×	×	×	—
SK-BR-3	TATDN1	GSDMB	118	463	✓	✓	✓	0.29
SK-BR-3	RARA	PKIA	13	10	✓	✓	✓	0.78
SK-BR-3	ANKHD1	PCDH1	—	—	×	×	×	—
SK-BR-3	CCDC85C	SETD3	5	6	✓	✓	✓	0.92
SK-BR-3	WDR67	ZNF704	2	4	✓	×	✓	0.73
SK-BR-3	CYTH1	EIF3H	31	24	✓	✓	✓	0.95
SK-BR-3	DHX35	ITCH	3	4	✓	✓	✓	0.33
KPL-4	BSG	NFIX	20	18	✓	✓	✓	0.90
KPL-4	PPP1R12A	SEPT10	2	6	✓	×	✓	0.65
KPL-4	NOTCH1	NUP214	5	7	✓	✓	✓	0.97
MCF-7	BCAS4	BCAS3	133	212	✓	✓	✓	0.80
MCF-7	ARFGEF2	SULF2	16	40	✓	✓	✓	0.91
MCF-7	RPS6KB1	TMEM49	—	—	×	×	×	—

[a]The scores are relative to EricScript with setting *a*.
[b]Crossing reads are the EricScript estimation of the number of reads that supports the discordant alignment.
[c]Spanning reads are the EricScript estimation of number of reads that covers the junction.

and PPP1R12A–SEPT10). In this case, our method predicted 193 fusions.

Table 3 also shows that all the predicted known fusions with the exception of DHX35–ITCH and TATDN1–GSDMB have an *EricScore* of >0.5 (11/22 present *EricScore* ≥0.90). In particular, the low score of TATDN1–GSDMB is because of a low value of US (*US* ∼0.29, Supplementary Files S1 and S2); US was introduced to assign a higher score to candidate fusion genes in which the number of junction spanning single reads and paired-end reads connecting the genes are similar. Although this is a valid assumption for most fusion genes, it may not be true for fusion genes in which only a short stretch of the 5′ (or 3′) gene is present. Moreover, this measure is dependent on library specific factors, including the length of the cDNA fragments and lengths of the reads. Generating a specific dataset that simulates these features for training our classifier would be useful to improve the classification power of *EricScore*. Despite of that, these results indicate that *EricScore* is reliable for discriminating between true- and false-positive calls also in real data. If we consider only the predicted gene fusions with *EricScore* >0.5, we are able to significantly reduce the number of our set of calls; indeed, we found 112 fusions for setting *a* and 84 fusions for setting *b*.

4 CONCLUSION

In this work, we discussed a novel computational approach to use discordant alignments of paired-end RNA-seq data to identify chimeric transcripts. Our method, named EricScript, makes use of the local realignment of the sequence reads that align across a gene fusion boundary to search for evidence of gene fusion events. We introduced three novel scores for classifying the 'goodness' of the distribution of the reads that span the junctions. The results we obtained demonstrate that these approaches, joined with the application of a filtering step, perform better than existing methods in distinguishing between real fusions and false-positive events, resulting in a smaller but robust set of calls. In fact, the analyses we performed on the synthetic gene fusion datasets showed that EricScript obtains good results in terms of both specificity and sensitivity with low-computational times. Moreover, our synthetic study demonstrated that split read-based methods (EricScript and DeFuse) obtain better performance than the other algorithms, and this is increasingly true if gene fusions involving middle of exons occur. The large amount of synthetic gene fusions we generated was also used to train an AdaBoost classifier that allows us to assign a reliable probability score to each predicted gene fusion event. The synthetic data generator has been included in the EricScript package; the synthetic data will represent a good resource for new developers when testing their methods. We also applied our algorithm to five publicly available datasets, and we tested its capability in rediscovering previously characterized gene fusions. Our analyses on both synthetic and real data demonstrated that EricScript is reliable in assembling the correct sequence of fusion junctions, allowing for the detection of chimeric events with a resolution of 1 bp. The main limitation of our method is the use of a transcriptome instead of a genome reference for mapping reads. Although this option allows us to bring down computational times, it does not enable us to discover gene fusions involving unannotated transcribed regions. Recent reports (Cabili *et al*., 2011) suggest that there are an abundance of unannotated tissue-specific genes; in this case methods, such as DeFuse (McPherson *et al*., 2011), will be more appropriated to screen fusions involving these genes.

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