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**Detection of Modified Nucleotides  
Using Nanopore Sequencing and  
Deep Learning Methods**

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*Umjesto ove stranice umetnite izvornik Vašeg rada.  
Da bi ste uklonili ovu stranicu obrišite naredbu \izvornik.*

*Foremost, I am profoundly grateful to my mentor, Professor Mile Šikić, for his guidance, encouragement, and sharing his expertise with me over the past few years. Further on, I would like to thank my supervisor, Dominik Stanojević, for providing useful advice and thoughtful comments regarding this thesis. Finally, I am thankful to my family and friends, especially my parents, for their unconditional love and support throughout this entire process.*

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# **1. Introduction**

Uvod rada. Nakon uvoda dolaze poglavlja u kojima se obrađuje tema.

## 2. Background

Background.

### 2.1. Related work

### 2.2. Rockfish

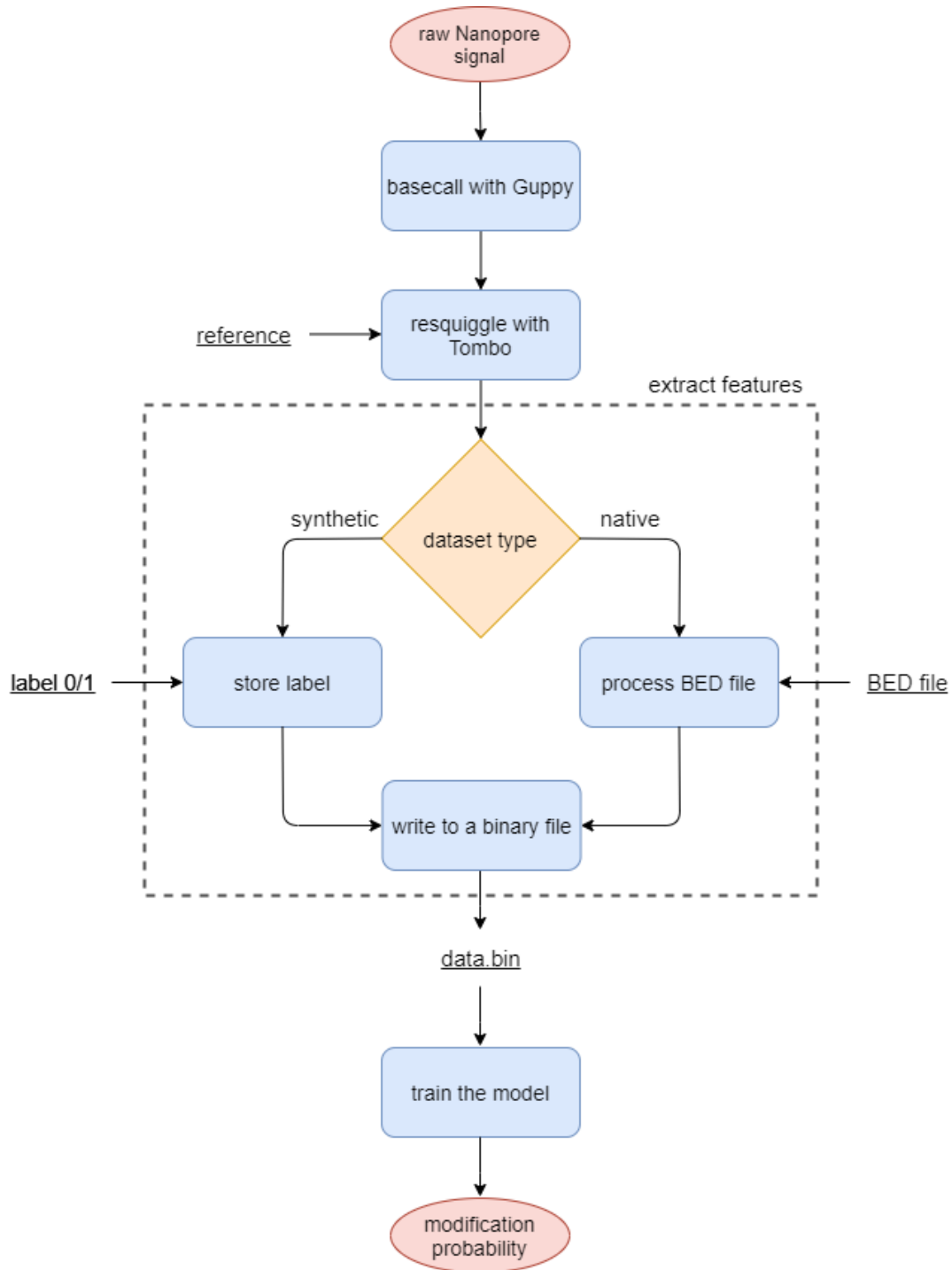
Rockfish<sup>1</sup> is a deep learning method for detecting DNA base modifications from Nanopore signal, developed by my supervisor Dominik Stanojević. The method can be used for several different tasks, such as read-level and genomic-level 5mC modification detection, cross-dataset generalization, and bisulfite sequencing. Rockfish has been tested on sequenced *Escherichia coli* Repli-G/M.SssI data and NA12878 human data, described in more details in chapters 3.1 and 3.2, respectively. The testing shows that Rockfish achieves state-of-the-art results, or at least comparable performance, as the methods described in section 2.1.

The Rockfish code consists of the following three parts:

1. extract features
2. train
3. inference

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<sup>1</sup><https://github.com/lbcb-sci/Rockfish>



**Figure 2.1:** Rockfish pipeline

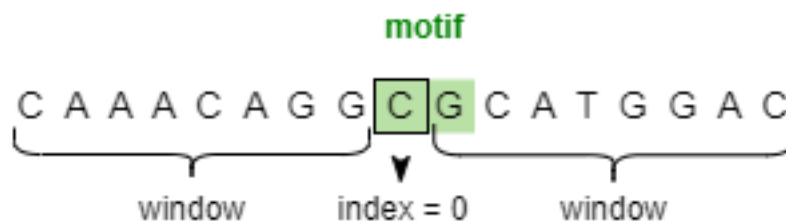
Figure 2.1 illustrates Rockfish pipeline which is composed of four parts. First, Nanopore reads are basecalled using Guppy basecaller, in order to infer nucleobase sequence from the raw signal.

Next, Tombo’s re-squiggle algorithm is used to map basecalled reads to the given reference using Minimapp2 (Li, 2018), and to map signal points to the reference, thus



correcting possible basecalling errors.

Third step is feature extraction using event table which is the output of re-squiggle algorithm. For every read, CpG motifs must be found, whilst taking care of alignment strand as described in 4.3, thus obtaining CpG regions of 17 nucleobases, because the window parameter is set to 8 by default, as shown in Figure 2.2. It is also possible to change the motif, which is "CG" by default, and central position index, which defaults to zero, thus representing the nucleobase "C". For example, motif "AATG", index 2, and window 7 might be provided, meaning that the nucleobase "T" is in the center, and length of the region equals 15. Nucleobases in the said regions get 20 signal points sampled from the corresponding event. If an event is longer than 20 points, some of the signal points are removed, and if it is shorter than 20 points, some of the points are repeated. The resulting signal vectors have exactly 340 elements, and they are stored in a binary file, together with event lengths, sequences of 17 nucleobases, and labels. Labels are provided for synthetic datasets, but need to be determined from bed file for native datasets, as depicted in Figure 2.1.



**Figure 2.2:** An example of CpG region, window = 8

Finally, the model is trained on the aforementioned binary file, in addition to encoded nucleobase vector, which is attained by assigning different integers to different nucleobases in the relevant region. The length of these vector should be 340 such as the signal vector, which is achieved by repeating each label 20 times, hence mapping 20 signal points to every nucleobase. The Rockfish model consists of encoder network and transformer module, and it outputs modification probability for the given CpG region. The encoder network is used to build latent representations of the input region, and to increase latent dimension for every timestamp. The encoder has three convolutional blocks, comprised of one-dimensional convolutional layer, GELU activation function (Hendrycks i Gimpel, 2016) and instance normalization (Ulyanov et al., 2016). Further on, data is processed using transformer module, i.e transformer encoder and decoder. The transformer encoder is equivalent to the encoder defined in

(Vaswani et al., 2017). Furthermore, the transformer decoder consists of global average pooling operation which reduces sequence dimension and a linear layer which outputs logit value, i.e. the wanted modification probability.

Inference takes trained model checkpoint file and re-segmented fast5 files as input, and outputs the modification probability for relevant CpG regions, alongside with a few other important information, such as contig, read name, alignment strand, and position of the central nucleobase (C) in the reference. The last output value is the predicted modification label, which equals 1 if the logit value is greater than 0, meaning that the modification occurred, and zero otherwise, which means there is no modification.

### **2.3. Problem formulation**

Rockfish model described in previous chapter achieves state-of-the-art results. However, Tombo's re-squiggle algorithm is a bottleneck in Rockfish pipeline (Figure 2.1), because it is quite slow. The main goal of this thesis is to replace Tombo with another tool whose task is to remap signal points at indels (insertions and deletions), hence correcting basecalling errors.

First, the data analysis is conducted and its results are examined (see Section 3.3), in order to implement new methods in the best possible way. Next, the Remapper model is implemented and thoroughly explained in chapters 4 and 5. Besides the sole implementation, Remapper needs to be successfully integrated into Rockfish pipeline. The biggest difference from the original Rockfish code is the fact that signal vectors now have variable lengths, instead of their length being fixated at exactly 340 signal points. Lastly, Remapper is tested, and results between implemented remapping methods and the original Rockfish code are compared (see Chapter 6).

## 3. Dataset

This chapter gives a brief description of the two datasets used in this thesis, *Escherichia coli* and *Homo sapiens* data. Moreover, data analysis, crucial for the subsequent chapters, is provided. The detailed data analysis is crucial, since it helps whilst making decisions on what algorithms to implement, what parameters to use, and what thresholds to put.

### 3.1. *Escherichia coli* data

*Escherichia coli* strain K12 MG1655 has been kindly gifted to us by Dr. Swaine Chen's laboratory in Genome Institute of Singapore, A\*STAR, Singapore. The modifications on the genomic DNA obtained from the grown *E. coli* were eliminated using REPLI-g Mini Kit. Afterwards, the resulting whole genome amplified sample was treated with M.SssI methyltransferase. The obtained synthetic *E. coli* data is primarily used for the data analysis, since it is known which reads are modified. For that purpose, 1,000 modified and 1,000 unmodified reads are examined.

The reference genome has been downloaded from NCBI (National Center for Biotechnology Information) GenBank under accession number NC\_000913.3. There is a total of 346,793 CpG sites in the reference genome.

### 3.2. *Homo sapiens* data

NA12878 *Homo sapiens* native dataset (Jain et al., 2018) has been obtained from European Nucleotide Archive under accession number PRJEB23027. Human genome assembly GRCh38 (Schneider et al., 2017) was used as the input reference for data extraction. Bisulfite sequencing used as a ground truth for NA12878 data was acquired from ENCODE Project (Consortium et al., 2012) under accession number ENCFF835NTC. The described human dataset consists of 406,821 reads in total, mapped to the chromosome 21 and 22. The data is partitioned into training, validation, and test set by

80%, 10%, and 10%, respectively, as can be seen in the Table 3.1.

**Table 3.1:** Distribution of Homo sapiens data

<b>Chromosome</b>	<b>Training</b>	<b>Validation</b>	<b>Test</b>
chr21	176,040	22,005	22,006
chr22	149,416	18,677	18,677
<b>Total</b>	<b>325,456</b>	<b>40,682</b>	<b>18,677</b>

For training the model only the high-confidence CpG positions, i.e. the positions which have at least 10 mapped reads, are included. This definition is in the accordance with DeepSignal (Ni et al., 2019). Furthermore, only the positions with unambiguous methylation are considered, meaning that the position is labeled as unmethylated if the methylation frequency is 0%, whereas it is labeled as methylated if the frequency is 100%. The final outcome are 5,084,927 unmethylated and 6,211,372 methylated high-confidence CpG positions.

### 3.3. Data analysis

The mentioned synthetic Escherichia coli dataset was thoroughly examined and analysed, taking into consideration if the reads are modified or unmodified. The native NA12878 human dataset is very large, and is not clearly separated according read modification, thus it is not analysed in this section.

A large amount of different analyses has been made, however, only the most important ones are presented in this thesis. In Subsection signal lengths are plotted and commented. Subsequently, in Subsection alignment between reference and queries, i.e. basecalled reads, is explored and the most important findings written down. At last, start index of raw signals is analysed, compared against the end of the signal, and plotted in Subsection .

The data analysis is important because it points us in the right direction regarding the selection of methods to implement, what exactly to keep in mind while coding them, on which values to set the parameters, etc.

### 3.3.1. Signal length

This subsection briefly gives raw signal lengths of 1,000 modified and 1,000 unmodified E.coli reads, presented in Figure 3.1. It can be observed that a lot of unmodified reads have shorter signal lengths, and then also a few of them have extremely long signal lengths, around 500,000 to 600,000 signal points. On the contrary, modified reads' signal lengths are distributed more evenly, mostly below 150,000, and with a maximum value below 400,000.

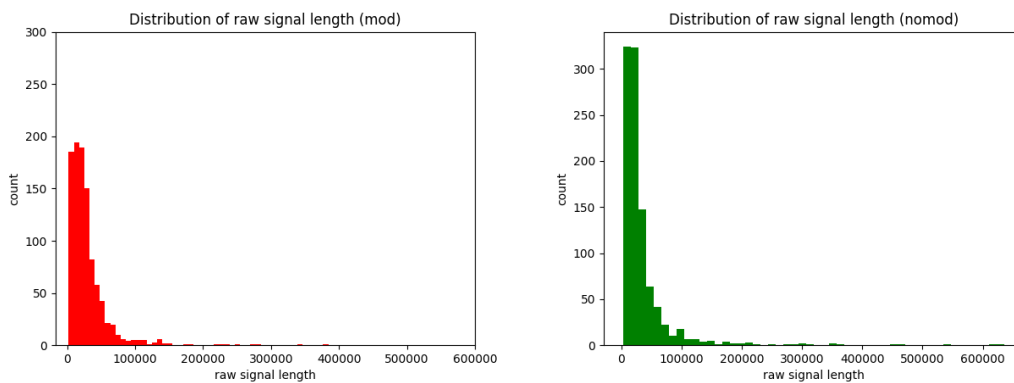



Figure 3.1: Distribution of raw signal lengths for modified vs. unmodified reads 

### 3.3.2. Alignment

Alignment is obtained by mapping basecalled reads to the reference, and it is consisted of the four following CIGAR operations: match, mismatch, deletion, and insertion. The values in Table 3.2 are calculated as the amount of certain operation in alignment divided by the length of alignment.

First two rows show the distribution of operations on all bases, from which it can be concluded that alignments are pretty accurate, considering they have more than 90% of matches. It can also be seen that modified reads have less matches, and more mismatches, deletions, and insertions. In conclusion, modified reads are harder to map correctly, because, as their name states, they contain modifications.

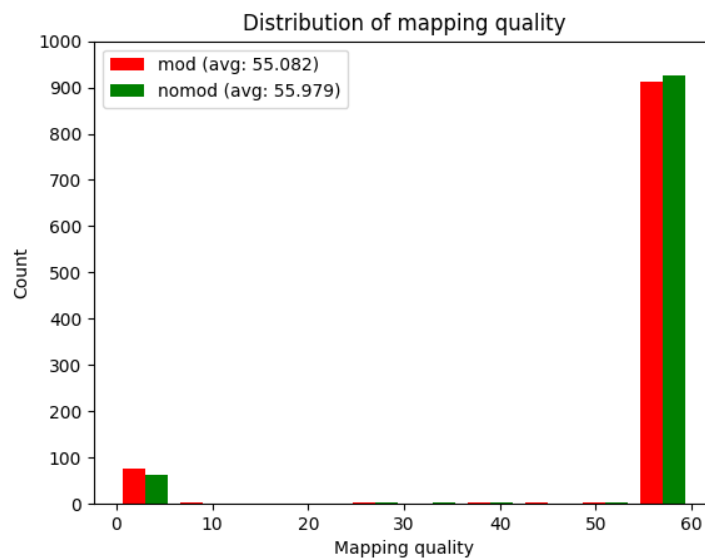
Last two rows show the comparison of operations at CpG positions, i.e. if "C" is matched, mismatched, deleted, or inserted. We can conclude that modified reads once again have less matches, more mismatches and deletions, but, surprisingly, less insertions. Therefore, we are not going to consider inserted "C" as a modification at CpG context. We will implement reference anchoring, and only look for the CpG positions on the reference, as it is considered to be the ground truth.

**Table 3.2:** Average amount of CIGAR operations across the alignments [%]

Position	Modification	Match	Mismatch	Deletion	Insertion
Any	mod	91.177	3.217	3.295	2.311
	nomod	93.867	2.260	2.403	1.470
CpG	mod	5.606	0.294	0.142	0.021
	nomod	6.275	0.170	0.056	0.055

After aligning basecalled reads to the reference using Mappy, we have noticed that some reads do not yield any alignment. The further investigation was conducted, and the findings were that 97.794% of reads with no alignments have mapping quality of zero. In general, reads with lower mapping quality have either no alignment, or short and quite incorrect one.


Based on the distribution of mapping qualities, shown in Figure 3.2, it is decided to put the mapping quality threshold to 10, meaning that all alignments that have the mapping quality below said threshold are discarded. It can be observed that Mappy has done a pretty good job, because a vast majority of alignments have the maximum mapping quality of 60. Lastly, the average mapping quality is, as can be expected, higher for unmodified reads, because they have less mistakes as has been previously shown in Table 3.2.

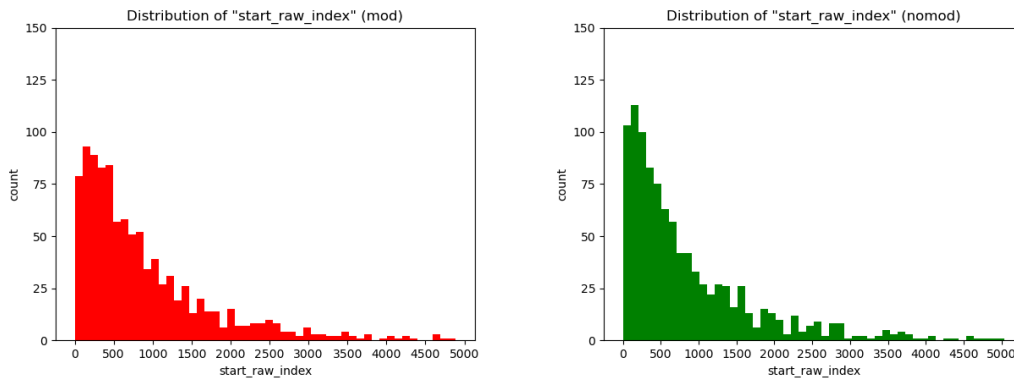


**Figure 3.2:** Mapping quality of alignments for modified vs. unmodified reads

### 3.3.3. Start row index

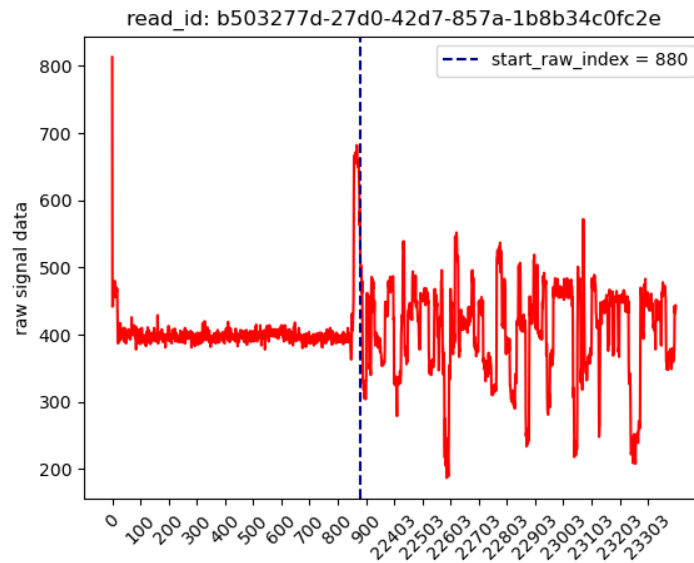
Start index of raw signals is the index at which the signal actually starts, that can be noticed by a sudden peak in the signal amplitude. The assumption is that the first N signal points before the mentioned peak have a small amplitude, and thus a small standard deviation.

First, distribution of start row indices may be seen in Figure 3.3 and it can be concluded that those indices are generally lower for unmodified reads. The reasoning behind that might lie in the fact that unmodified reads are easier to basecall, therefore they have smaller starting area with low amplitude, i.e. the real signal values start before than in modified reads. 

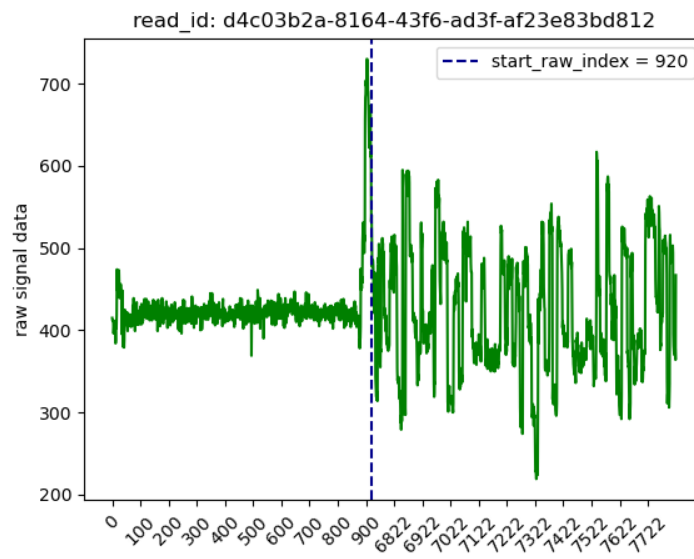


**Figure 3.3:** Distribution of start index of raw signals for modified vs. unmodified reads

In order to further explain the idea around the start row index, the first and the last 1,000 signal points are drawn for one representative modified read, and one unmodified, as shown in Figure 3.4 and 3.5. It can be noticed that signal remains still until the sudden start row index peak, and then continues to deviate around the center value. Furthermore, it can be concluded that there exists no such thing as an end row index, since the signal has larger amplitude until the very end of the read.



**Figure 3.4:** The first and last 1,000 signal points for a representative modified read

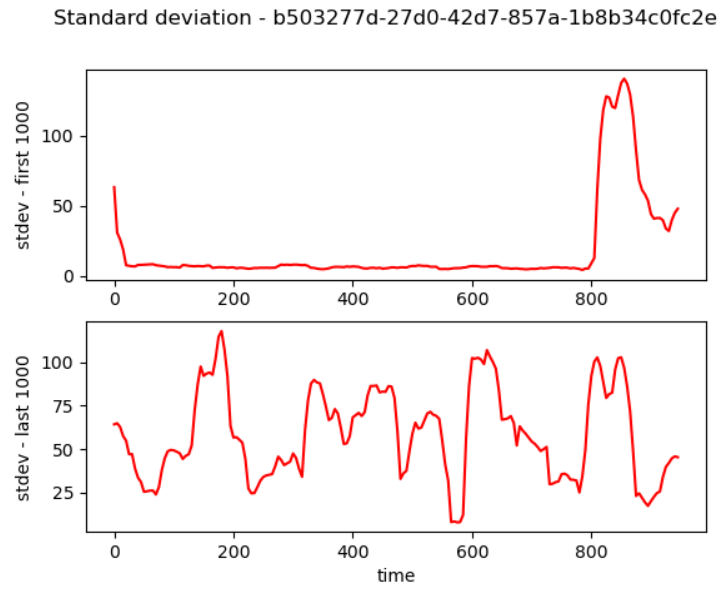


**Figure 3.5:** The first and last 1,000 signal points for a representative unmodified read

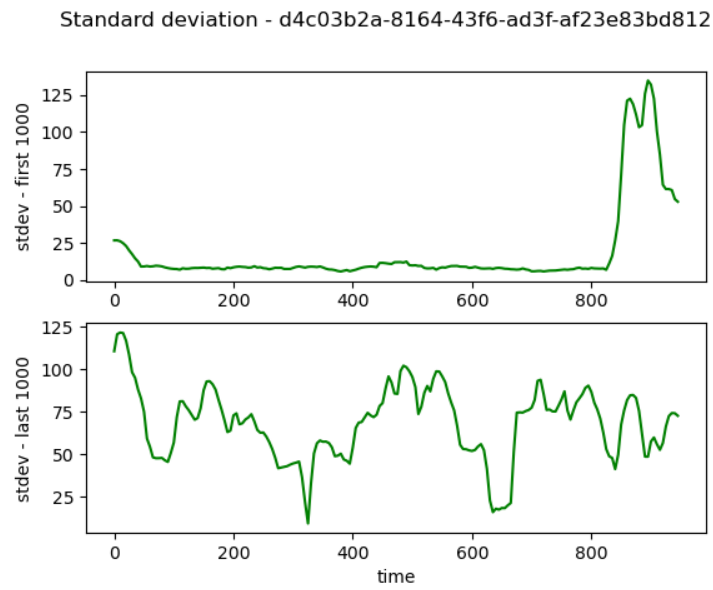
We have also decided to look at the standard deviation of signal points before jumping to any conclusions. As can be concluded from Figure 3.5 and 3.6, standard deviation is close to zero until the abrupt appearance of start row index for both modified and unmodified representative read. Further on, the standard deviation diverges until the very end of the signal, which confirms that there does not exist an end row index. Based on the findings in this subsection, it is decided to trim the signal, so it begins from the start row index until the end of the signal, thus obtaining only the relevant



signal points.



**Figure 3.6:** Standard deviation of the first and last 1,000 signal points for a modified read



**Figure 3.7:** Standard deviation of the first and last 1,000 signal points for an unmodified read

## 4. Methods

This chapter explains underlying concepts and algorithms necessary for the final Remapper implementation described in Chapter 5. Remapper is a tool developed for the sake of replacing Tombo framework used in the original Rockfish code described in 2.2, and hopefully to lower the overall execution time. After aligning basecalled reads to the reference using Mappy, signal points at indels should be remapped, hence the name Remapper. First, insertions are resolved as depicted in Section 4.1 using the Half-half method (see Subsection 4.1.1). Next, as explained in Section 4.2, the deletions are dealt with in one of the two possible ways, Concatenate and divide method (see Subsection 4.1.1) or Greater neighbour method (see Subsection 4.2.1). Thirdly, Section 4.3 provides a short overview of dealing with two different types of alignment strand. At last, Section 4.4 describes Binary writer, used for storing the extracted features into binary file, later on used for training.

### 4.1. Resolving insertions

Insertions occur when a basecalled read contains a nucleobase, or several consecutive nucleobases, which are not present in the reference at the same position in the alignment. The reference is considered to be the ground truth, and insertions to mainly be mistakes made during the basecalling process. Therefore, it is necessary to remove insertions and remap their signal points to the neighbouring bases. In order to do so, the Half-half method, described in the succeeding subsection, has been developed. As shown in Algorithm 1 the method takes alignment obtained using Mappy - "a" and signal points intervals for the observed read - "raw", as inputs. The outputs are signal points intervals mapped to the reference, and intervals of indices at which deletions have taken place, that facilitate future handling of deletions.

---

**Algorithm 1** Resolve insertions

---

```
1: function RESOLVE_INSERTIONS(al, raw)
2:   cigar  $\leftarrow$  al.cigar if al.strand == 1 else reversed(al.cigar)
3:   r_pos, q_pos  $\leftarrow$  0, al.q_st
4:   r_len  $\leftarrow$  al.r_en - al.r_st
5:   intervals  $\leftarrow$  [None] * r_len
6:   insertion  $\leftarrow$  False
7:   deletion_idx  $\leftarrow$  []


8:   for length, operation in cigar do
9:     if operation in {0, 7, 8} then
10:      if insertion then
11:        intervals[r_pos]  $\leftarrow$  (center, raw[q_pos].end)
12:        insertion, length  $\leftarrow$  False, length - 1
13:        r_pos, q_pos  $\leftarrow$  r_pos + 1, q_pos + 1
14:        for i = 0 to length do
15:          intervals[r_pos + i]  $\leftarrow$  raw[q_pos + i]
16:          r_pos, q_pos  $\leftarrow$  r_pos + length, q_pos + length

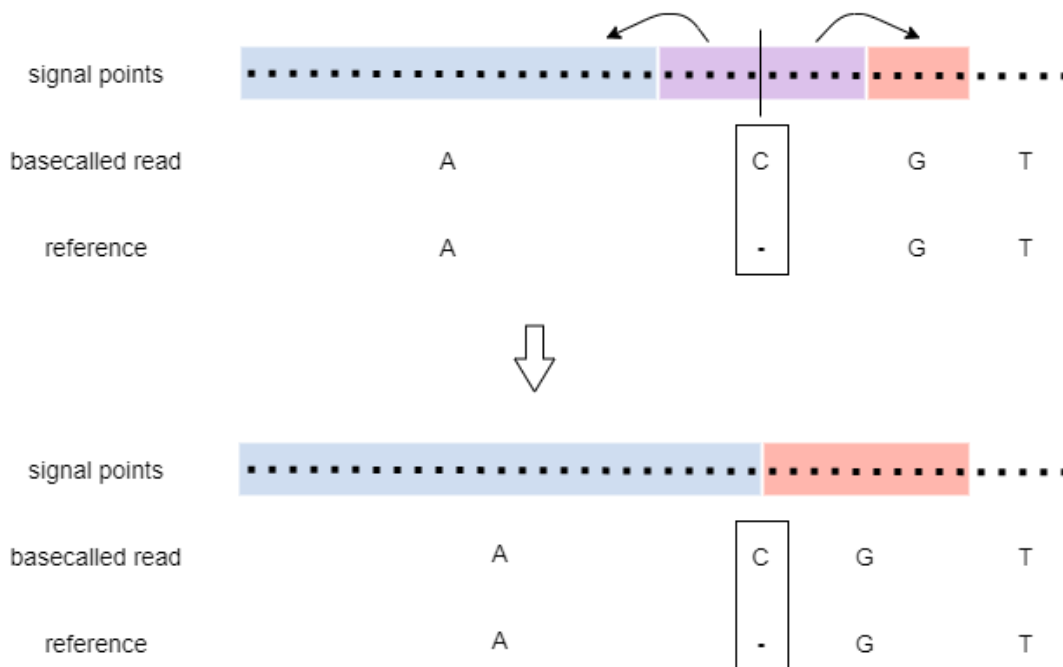
17:      else if operation == 1 then
18:        ins_interval  $\leftarrow$  (raw[q_pos].start, raw[q_pos] + length.start)
19:        center  $\leftarrow$  int(np.mean(ins_interval))
20:        intervals[r_pos - 1]  $\leftarrow$  (intervals[r_pos - 1].start, center)
21:        insertion  $\leftarrow$  True
22:        q_pos  $\leftarrow$  q_pos + length

23:      else if operation in {2, 3} then
24:        deletion_idx.append((r_pos, r_pos + length))
25:        if insertion then
26:          intervals[r_pos]  $\leftarrow$  (center, raw[q_pos].start)
27:          insertion, length  $\leftarrow$  False, length - 1
28:          r_pos  $\leftarrow$  r_pos + 1
29:          for i = 0 to length do
30:            intervals[r_pos + i]  $\leftarrow$  (raw[q_pos].start, raw[q_pos].start)
31:            r_pos  $\leftarrow$  r_pos + length
32:   return intervals, deletion_idx
```

---

### 4.1.1. Half-half method

The thinking process behind the occurrence of inserted bases is that they should not be present, and that they contain signal points which in reality belong to their neighbouring bases. For that reason, the Half-half method deals with insertions applying a simple heuristic of assigning half of their signal points to the left neighbour, and half of their points to the right neighbour, as shown in Figure 4.1. If there is an odd number of signal points, then the right neighbour gets one point more. For example, a total of 5 signal points would be divided into 2 and 3 points, and assigned to the left and right neighbour, respectively. 



**Figure 4.1:** Half-half method for resolving insertions

Algorithm 1 demonstrates pseudocode for resolving insertions using the Half-half method. Input arguments are alignment obtained by aligning basecalled read to the reference using Mappy, and raw signal points that are written as intervals, from which the exact signal point values are easily attainable. Start of mentioned intervals is inclusive, whilst the end is exclusive.

Firstly, CIGAR operations and corresponding lengths are extracted from alignment, depending on the alignment strand (for further explanation on strands see 4.3). A few other variables are initialized, amongst which the starting reference and query positions that are going to be crucial in the continuation.

Subsequently, we iterate through CIGAR, and check if an operation is match (or mismatch), insertion, or deletion, according to SAM format specification<sup>1</sup>. If the operation is insertion (consult line 17 in Algorithm 1), insertion interval is found, taking into account number of consecutive insertions, then the center index of mentioned interval is remembered. The end index of left neighbour is set to the center index, insertion flag is marked as True, and query position is updated depending on number of insertions. Only the query position is updated because insertions are the type of operation which consume query, i.e. they appear on the query, and are missing on the reference.

Next, if the operation is match or mismatch (see line 9 in Algorithm 1), and the insertion flag is set, i.e. insertion occurred in the last iteration, then the start index of the current base is set to the remembered center index. Then, the insertion flag is set to False, length of match operation is decreased by one, because the current base is "resolved", and positions on both query and reference are incremented, which concludes adjustment of the right neighbour. Then, remaining matches are resolved by simply mapping the signal points intervals from query to reference. Matches and mismatches consume both query and reference, thus both of the positions must be increased by the length of the matches.

Moreover, handling of deletions can be seen at line 23 in Algorithm 1, which is done in a similar fashion as the previously described matches, with the main difference lying in remembering deletion intervals essential for the following portion of the Remapper implementation. Again, if the insertion happened in the last iteration, then half of the insertion's signal points are given to the deletion, i.e. the right neighbour. The variables are adjusted similarly as for the matches, with the difference that only the position on the reference is incremented, because deletions consume reference. Afterwards, or immediately if there were no previous insertions detected, the signal intervals are assigned to deletions, but in a way that they get zero signal points, e.g. interval (350, 350). If they have not gotten any signal points from possible insertions, deletions enter the next phase with having assigned intervals, but containing zero points. Lastly, the reference position is increased by the number of deletions.

Finally, signal intervals assigned to the reference with resolved insertions and deletions written as empty intervals, together with a list of indices where the deletions occurred, are returned as the output.

---

<sup>1</sup><https://samtools.github.io/hts-specs/SAMv1.pdf>

## 4.2. Resolving deletions

Deletions appear when reference contains certain nucleobases which are "deleted", i.e. not present, in the basecalled read at the same positions in the alignment. This event is considered to be the result of mistakes whilst basacalling the reads, because bases, which should be a part of the sequence, are wrongly omitted and "contain zero signal points". Those deleted bases should have certain amount of signal points, mistakenly attributed to their neighbours. For this purpose, two methods for resolving deletions are implemented and compared. On the one hand, there is Concatenate and divide method, described in Section 4.2.1, which attempts to remap signal points from neighbours to deletions uniformly. On the other hand, Greatest neighbour method is created and explained in Section 4.2.2, giving deletions signal points from the neighbour that holds more of them. The Algorithm 2 presents the resolve deletions algorithm, which takes outputs from the resolve insertions algorithm, and the type of deletion method as the input. At the end, the algorithm outputs the resulting signal points intervals remapped at indels, and mapped to the reference.

---

**Algorithm 2** Resolve deletions

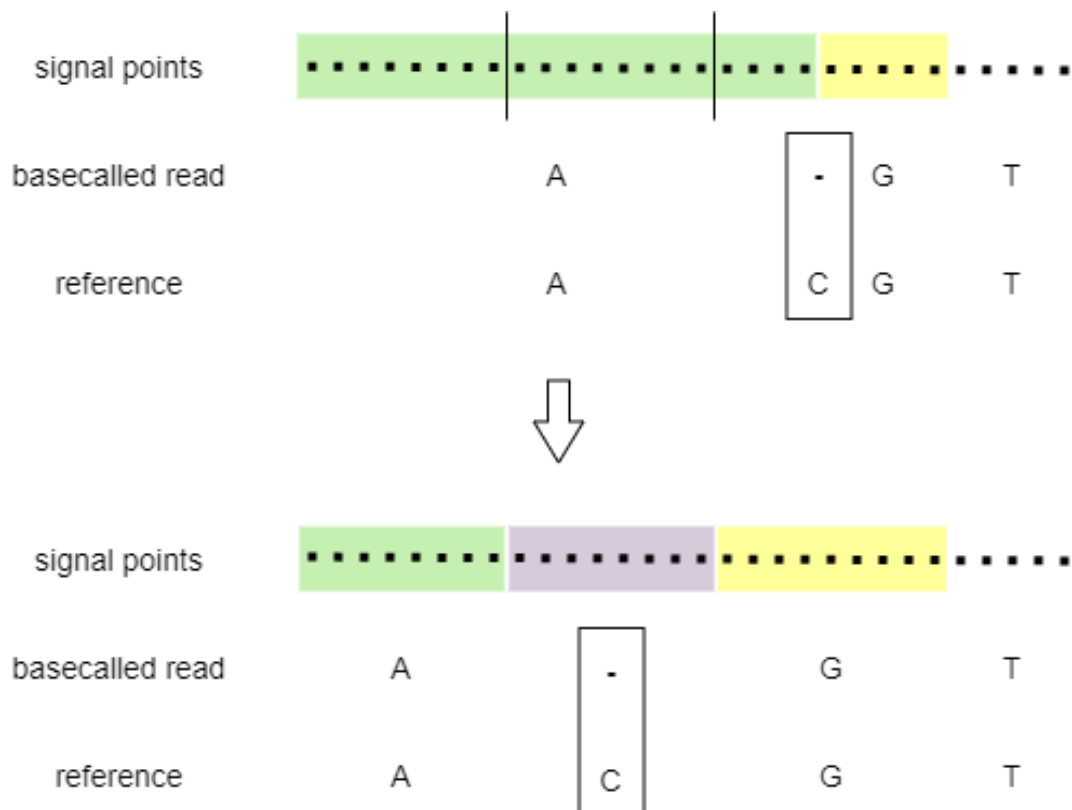
---

```
1: function RESOLVE_DELETIONS(intervals, deletion_idx, del_method)
2:   for  $del\_st, del\_en$  in  $deletion\_idx$  do
3:      $left \leftarrow intervals[del\_st - 1]$ 
4:      $right \leftarrow intervals[del\_en]$ 
5:     if  $del\_method == 'concatenate\_and\_divide'$  then
6:        $sig\_st, sig\_en \leftarrow left.start, right.end$ 
7:     else if  $del\_method == 'greatest\_neighbour'$  then
8:        $left\_len \leftarrow left.end - left.start$ 
9:        $right\_len \leftarrow right.end - right.start$ 
10:       $del\_len \leftarrow del\_en - del\_st$ 
11:      if  $left\_len > right\_len$  and  $left\_len > del\_len$  then
12:         $sig\_st, sig\_en \leftarrow left.start, left.end$ 
13:      else if  $right\_len > left\_len$  and  $right\_len > del\_len$  then
14:         $sig\_st, sig\_en \leftarrow right.start, right.end$ 
15:      else
16:         $sig\_st, sig\_en \leftarrow left.start, right.end$ 
17:       $points \leftarrow np.array\_split(range(sig\_st, sig\_en), del\_en - del\_st + 2)$ 
18:      if  $len(points[-1]) == 0$  then
19:        while  $len(points[-1]) == 0$  do
20:           $points.pop(-1)$ 
21:           $interval \leftarrow points.pop(-1)$ 
22:           $intervals[del\_en] \leftarrow (interval[0], interval[-1] + 1)$ 
23:      for  $i = del\_st - 1$  to  $del\_en + 1$  do
24:        if  $len(points) == 0$  then
25:          if  $i < del\_en$  then
26:             $intervals[i] \leftarrow intervals[del\_en].start, intervals[del\_en].start)$ 
27:          continue
28:           $interval \leftarrow points.pop(0)$ 
29:           $intervals[i] = (interval[0], interval[-1] + 1)$ 
30:      return  $intervals$ 
```

---

### 4.2.1. Concatenate and divide method

Deletions are bases which exist on the reference, but are deleted on the basecalled read, as shown in Figure 4.2, where "C" in the CpG context is modified, or more precisely deleted, on the read. One of the possible heuristics to deal with that issue is Concatenate and divide method, whose goal is to divide signal points between deletions and their first neighbours uniformly. The signal points from left and right neighbour are concatenated and divided into equal parts. If there exists a remainder whilst dividing the points, quite the opposite from the aforementioned insertion resolving process, the bases to the left get more points. For example, in Figure 4.2, 25 points shall be divided to three bases, which is achieved in the following way: left neighbour gets 9 points, deleted base gets 8 points, and, finally, 8 points are assigned to the right neighbour.



**Figure 4.2:** Concatenate and divide method for resolving deletions

The pseudocode for the Concatenate and divide method is given in Algorithm 2. The inputs are signal intervals mapped to the reference already modified by the resolve insertions method, intervals at which deletions occurred, and wanted deletion method. The resolve deletions method begins with a for loop going through deletion intervals,



and finding an interval for the left and right neighbour.

Next, signal start is defined as the start position of left neighbour, and the signal end as the end position of right neighbour, thus concatenating necessary signal points. At line 17 in Algorithm 2 points are divided uniformly between neighbours and deletions, depending on the number of deletions.

The case where there is not enough signal points to divide amongst bases is covered from line 18 to 22. This part of code makes sure that the right neighbour keeps at least one signal point. It can be observed that the left neighbour always has at least one point, at least one point is explicitly given to the right one, and deletions might have zero or more points. For example, if left neighbour has one point, the right one two points, and there are two deletions, then the points are given as follows: one to the left neighbour, one to the first deletion, zero to the second deletion, and, lastly, one to the right neighbour.

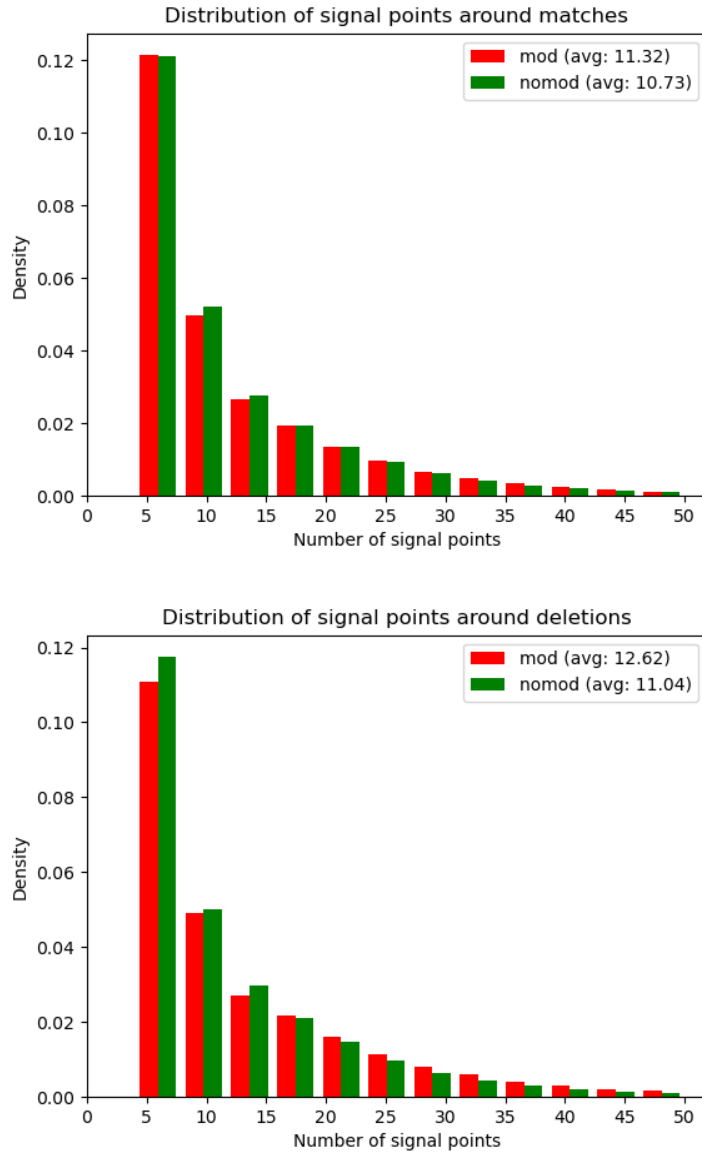
Finally, the program loops through the relevant positions on the reference and maps points to them, more precisely it adjusts the signal interval start and end. If there are zero signal points, then an empty interval is made, e.g. (350, 350). After the adjustment of all deletion intervals, new signal point intervals is returned as the output.

#### 4.2.2. Greater neighbour method

Concatenate and divide method might seem a bit unfair, in sense that it divides signal points in an uniform way, disregarding the cases in which one neighbour has a lot more points than the other. For instance, if there is one deletion, and one neighbour has 5 points, whilst the other has 85 points, is it fair to divide points as 30-30-30, taking away a vast amount of points from the latter neighbour? We have decided to analyse the direct neighbours on the E. coli dataset (see Subsection 3.1), and based on the results think about an implementation of an alternative method.

The first assumption is that the bases around deletions have more signal points than those around matches. The reasoning behind that lies in the previously explored thought that the basecaller has made a mistake in not detecting the deleted base, and that it assigned its signal points to the one of the neighbours. Therefore, the deletion should have probably been in the place where there is more signal points than the average. The Figure 4.3 confirms the assumption that there are more signal points in average around deletions than matches. It can be observed that matches have larger values for 5 signal points, for 10 signal points values are similar, and for larger values, deletions start to take over. Lastly, it can also be seen in the right figure that unmodified

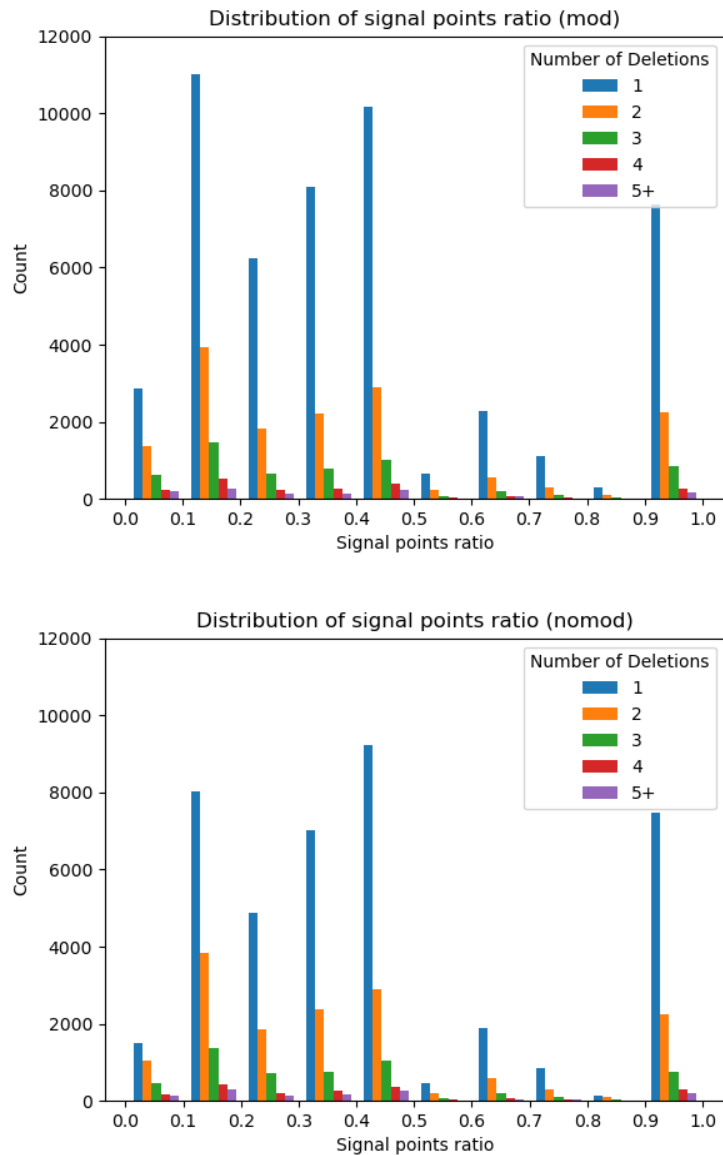
reads have larger values for smaller number of signal points, and modified ones take over after 15 points. This can be interpreted in a way that modified reads have more deletions at CpG positions.



**Figure 4.3:** Distribution of signal points around matches vs. deletions

Now that we have established that there are indeed more points around deletions than usual, we can proceed to formulate the second assumption. It looks at the signal ratio, i.e. the neighbour with less signal points divided by the neighbour with more signal points, which is a number between zero and 1. So, the second assumption, which is the foundation of the Greatest neighbour method, claims that the signal points from the supposed deleted base have been assigned to the neighbour who now has more points.

We wonder how often the case that one neighbour has more points than the other happens. Also we are interested in signal ratios, i.e. how many more signal points does the larger of two neighbours have. Ratios below 0.5 prove a large difference between neighbours, for instance, ratio for opening example of 5 and 85 points is 0.0625. On the contrary, ratios above 0.5 show a smaller difference between neighbours, whilst the ratio equal to 1 means that the two neighbours have the same amount of signal points. Figure 4.4 shows distribution of signal points ratios for *E. coli* reads, and interestingly strongly confirms the aforementioned statement that modified reads have more deletions than unmodified. Moreover, there are indeed a vast amount of deletions with ratios below 0.5 which arises the need for the alternative method which will take this discovered fact into consideration. There is also quite a lot cases with ratio equal to 1, which can be covered with the previously implemented Concatenate and divide method. Finally, the interesting fact is that one or two consecutive deletions prevail, confirming that the basecaller works good, i.e. that it has not basecalled a lot of consecutive deletions.

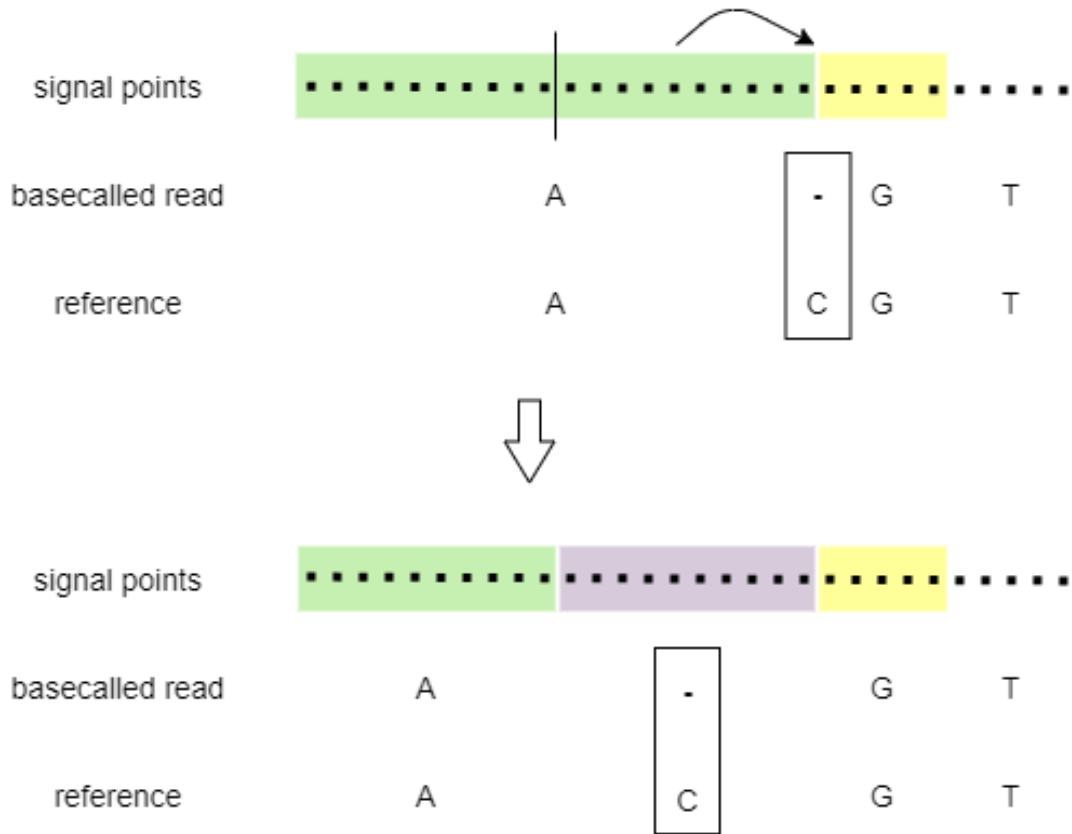


**Figure 4.4:** Distribution of signal points ratio for modified vs. unmodified reads


Greatest neighbour method first compares the number of signal points of left and right neighbour and decides which one is greater. Then, the signal points of the greater neighbour are divided between the neighbour and the deletions in a uniform way, same as in the Concatenate and divide method. For further explanation consult Figure 4.5 where the same starting example from Figure 4.2 is now resolved using the alternative method.

There exist two edge cases, left and right neighbour having the same amount of signal points, and not having enough signal points to divide between all the deletions. The latter case is quite rare because there are mostly one or two consecutive deletions as

proved above, and there are usually enough signal points to assign to them. Nevertheless, both cases are solved with Concatenate and divide approach.



**Figure 4.5:** Greater neighbour method for resolving deletions

The lines 7 to 16 in Algorithm 2 are key if we choose the Greatest Neighbour approach. The number of signal points of left and right neighbour are compared. If one neighbour is larger than the other, additionally we need to check if its number of signal points is larger than the number of deletions, i.e. if it has enough points to cover all of them. If both conditions are met, signal start and signal end are adjusted according to start and end of that neighbour. Otherwise, signal start and signal end are set as before and classic Concatenate and divide method is applied. 

### 4.3. Alignment strand

#### 4.3.1. Forward

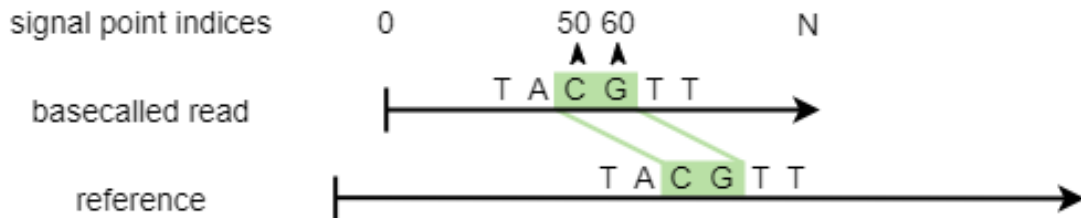


Figure 4.6: Forward alignment strand

#### 4.3.2. Reverse

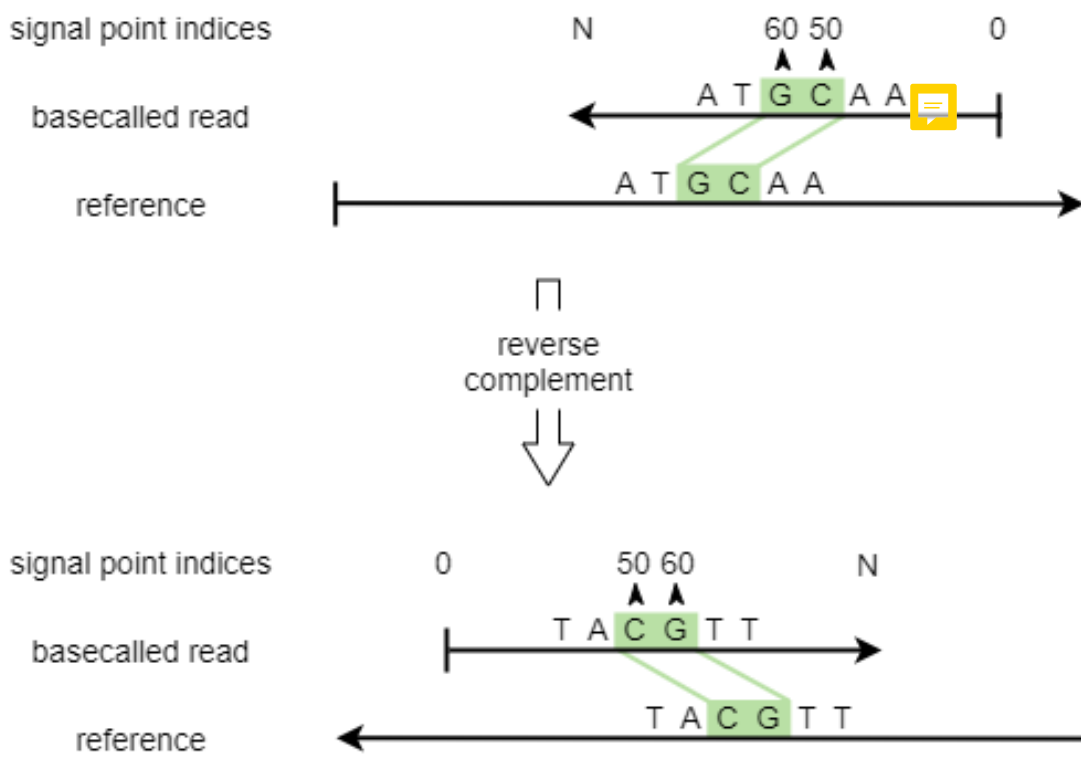


Figure 4.7: Reverse alignment strand

# 5. Implementation

This chapter provides an overview of external dependencies used for the implementation presented in Section 5.1. Moreover, detailed code structure and implementation details are given in Section 5.2. At last, Section 5.3 describes training procedure of the deep model.

## 5.1. Dependencies

This sections deals with external dependencies used for the final implementation. First, it gives a short introduction to Guppy, method used for basecalling the reads (see Subsection 5.1.1. Next, Mappy, the method used for aligning reads to the reference is outlined in Subsection 5.1.2. Furthermore, in Subsection 5.1.3 PyTorch and PyTorch Lightning, libraries commonly used for deep learning, are described. Lastly, Subsection 5.1.4 provides a brief overview of other libraries and tools used in this implementation.

### 5.1.1. Guppy

**Guppy**<sup>1</sup> is a basecaller based on the RNN architecture that transforms raw FAST5 data into canonical bases of DNA or RNA. The Guppy toolkit also performs modified basecalling (5mC, 6mA, and CpG) from the raw signal data, returning an additional FAST5 file of modified base probabilities as the output.

In this implementation, Guppy basecall server is run on a certain port, using basecalling of high accuracy and GPU mode, in order to obtain accurate basecalling at an acceptable speed. Furthermore, **ont-pyguppy-client-lib**<sup>2</sup> is a Python Guppy API, used as a client which connects to the said server and basecalls given FAST5 reads.

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<sup>1</sup><https://community.nanoporetech.com/protocols/Guppy-protocol/>

<sup>2</sup><https://pypi.org/project/ont-pyguppy-client-lib/>

Both Guppy basecall server and client shall have compatible versions, and in this implementation version 4.4.2 is used.

### 5.1.2. Mappy

**Mappy**<sup>3</sup> is a Python API built on top of the Minimap2 (Li, 2018) implementation. Minimap2 is a sequence alignment program which aligns DNA or mRNA sequences against a large reference genome. In this implementation it is used for mapping Oxford Nanopore genomic reads to the human genome, as well as E. coli reference.

### 5.1.3. PyTorch and PyTorch Lightning

**PyTorch**<sup>4</sup> (Paszke et al., 2019) is an open source deep learning Python library used for tensor computation with strong GPU acceleration, as well as building different deep neural network architectures.

**PyTorch Lightning**<sup>5</sup> is built on top of PyTorch and its main usage is organising the training code, making it more readable, easier to reproduce, less prone to errors, scalable to any hardware without changing the model, etc.

In this implementation, PyTorch is used to implement the deep model and perform certain tensor calculations, whilst PyTorch Lightning serves as a tool for organising the training code.

### 5.1.4. Other dependencies

**h5py**<sup>6</sup> is a Python interface to the HDF5 binary data format used to store huge amounts of numerical data, and later easily manipulate that data. In the implementation FAST5 reads are stored in HDF5 files. Furthermore, **ont\_fast5\_api**<sup>7</sup>, a simple Python interface to HDF5 files of the Oxford Nanopore .FAST5 file format, is used to handle those types of files directly from Python code.

Other dependencies are listed here briefly, due to their familiarity and widespread usage:

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<sup>3</sup><https://pypi.org/project/mappy/>

<sup>4</sup><https://pytorch.org/>

<sup>5</sup><https://www.pytorchlightning.ai/>

<sup>6</sup><https://www.h5py.org/>

<sup>7</sup>[https://github.com/nanoporetech/ont\\_fast5\\_api](https://github.com/nanoporetech/ont_fast5_api)



- **NumPy**<sup>8</sup> - fundamental package for scientific computing in Python
- **Biopython**<sup>9</sup> - set of tools for biological computation in Python
- **Matplotlib**<sup>10</sup> - Python library used for visualisation
- **tqdm**<sup>11</sup> - a fast, extensible progress bar for Python and CLI

## 5.2. Code structure

The whole implementation has been written in Python 3.7.10, with the help of the dependencies described in the previous section. The code is written in an object oriented matter, and using multiprocessing to achieve comparable speed with the original implementation. The code is publicly available under the MIT licence at the following link: <https://github.com/sanjadeur/Rockfish/tree/basecall>.

The code pipeline is shown in Figure 5.1 where it can be observed that the Tombo framework present in the original Rockfish implementation (see Figure 2.1) is replaced with the new Remapper tool. Unlike the original implementation which takes previously basecalled and re-segmented reads as inputs, this implementation simply takes raw FAST5 reads.

Next, feature extraction begins with basecalling, continues with the Remapper tool, and ends in the same way as before. The basecalling process is written completely in Python using tools mentioned in 5.1.1. In order to avoid basecalling to become a bottleneck in the pipeline, it is written using multiprocessing, using the producer-consumer pattern. The number of producers and consumers, called processors in this implementation, can be set as a parameter of the program. Processors are used to basecall the input raw data, and put processed reads into the queue. Then, processors take basecalled reads from the queue, and further process them using Remapper, and finally write them in a binary format. The program ends when the queue is empty, meaning there are no more basecalled reads to further process.

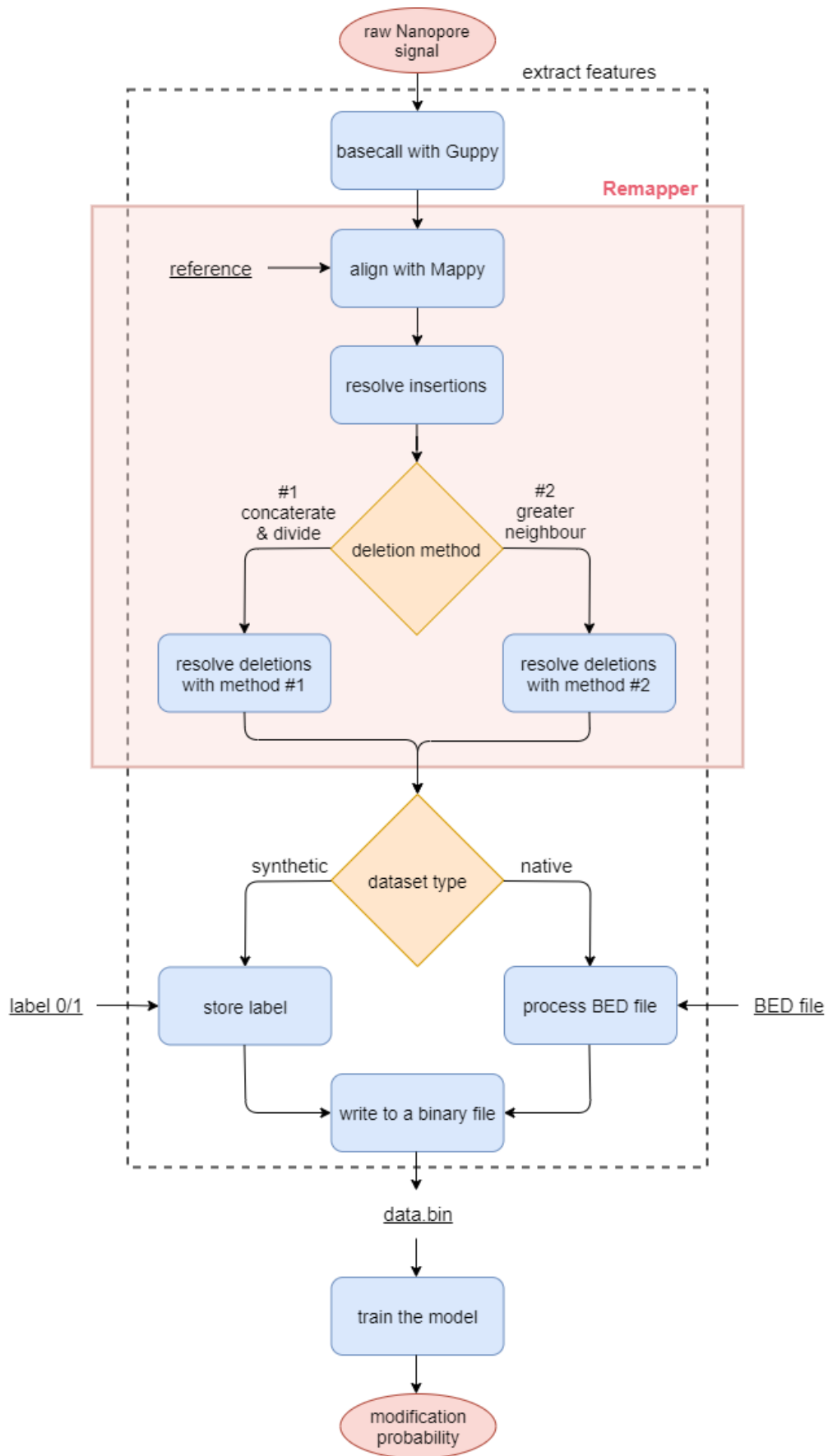
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<sup>8</sup><https://numpy.org/>

<sup>9</sup><https://biopython.org/>

<sup>10</sup><https://matplotlib.org/>

<sup>11</sup><https://github.com/tqdm/tqdm>



**Figure 5.1:** Rockfish with Remapper pipeline

Subsequently, an object of a class Remapper is instantiated and given as an argument to all of the processors. Remapper consists of seven most important steps, as follows:

1. find motif positions on the reference
2. align read to the reference using Mappy
3. convert sequence to raw signal
4. find relevant motif positions
5. resolve insertions
6. resolve deletions
7. extract relevant data

In the Figure 5.1 some of the steps above are omitted for the clarity sake, but are going to be thoroughly explained in the following text.

In the Remapper initialisation part Mappy aligner is instantiated, used for aligning the read to the reference later on. Also, the positions of central base in the motif, for instance "C" for the CpG context, are found on the reference, both for forward and reverse alignment strand. If it is dealt with native dataset, then BED positions shall be extracted, together with the alignment strand information. It is also possible to filter the BED positions, leaving only the high confidence ones. The intersection between found motif positions and allowed BED positions shall be made. Afterwards, the final motif positions are stored into a variable in a dictionary format, keys being different chromosomes, and values being a pair of two sets, one for forward strand, and one for the reverse one.

Next step is aligning read to the reference, and getting the relevant motif positions, in a way that it is observed which of the previously found motif positions are covered by the alignment, i.e. also match position on the query.

Then, raw signal intervals must be obtained using the basecalled data, and given to the resolve insertions method as an input. 

Steps 5. and 6. are not going to be explained into further details, because they have already been examined in sections 4.1 and 4.2, respectively.

Finally, the program loops through the relevant positions and extracts important information for each of them. The resegmentation data is structured in the following way:

- **position** - central base position on the reference
- **event\_intervals** - raw signal intervals mapped to the bases on the reference
- **event\_lens** - lengths of the event intervals
- **bases** - bases in the relevant region extracted from the reference (e.g. 17-mer for motif "CG" and window equal to 8 shown previously in Figure 2.2)

Even though, the remaining part of the pipeline looks the same as in the original implementation, actually quite a lot of changes needed to be done in order for whole pipeline to work correctly. Binary writer in the original implementation wrote signals of exactly 340 points in a binary file. Now, due to manual remapping, and removing the sampling of 20 signals per base, signals of variable lengths must be successfully written. In order to do so, a certain overhead shall be introduced, a header containing number of examples and lengths of every example.

Training portion of the code can now easily read mentioned binary data file, because offsets are given in the binary header file. Additional changes must be made in the training as well, because signals differ in length. First, bases in the 17-mer should be repeated based on event lengths, and not constantly 20 times as before. Secondly, padding containing zeros must be added to the beginning and the end of the signal in order to obtain same tensors. Signals which are too long are cut in a way that we keep the middle of the signal. At last, convolutional layers should be adjusted based on the new lengths (they are not always 340). After all of that successfully implemented, modification probabilities can be again obtained as the final output of the pipeline.

### 5.3. Training procedure

The model is trained for 30 epochs in mini-batches of 256 examples, using the back-propagation algorithm. Binary cross-entropy loss is used in order to measure the performance of the model. For training, AdamW optimizer (Loshchilov i Hutter, 2018), which implements weight decay regularization for Adam algorithm (Kingma i Ba, 2014), and cyclic learning rate scheduler (Smith, 2015) are utilised. Learning rate is cyclically changed between lower and upper boundary, which equal to  $10^{-5}$  and  $10^{-4}$ , respectively. After each cycle, upper bound is changed to half of the value of previous upper bound. One step size represents half cycle and is equal to  $4 \times iterations\_in\_epoch$ .

## 6. Results

This chapter offers an overview of the obtained results divided into two parts, measuring and comparing execution time for different methods, and comparing the accuracy of methods. Later on, a brief commentary on the obtained results is given, as well as the propositions for the future work in the field.

The assumption, and the very goal of replacing the Tombo framework, is that the runtime will be shorter than before. Tombo uses a more complex heuristic than those developed in this thesis, hence the execution time should be greater. On the other hand, it is expected that the accuracy whilst using Tombo is higher than with the Remapper tool. Nevertheless, we hope that heuristics developed in this thesis will yield good enough accuracy, while at the same time lowering the runtime.

### 6.1. Runtime

Runtime is first measured for the original Rockfish code, including basecalling using Guppy, re-squiggling using Tombo, and finally feature extraction. Secondly, runtime is measured for the Rockfish code including Remapper which only has feature extraction step. The measurements are conducted three times and the average value is taken, because there have been small differences across the measurements.


The results for E. coli dataset are presented in Table 6.1. Original Rockfish with Tombo, Remapper with the first deletion method (Concatenate and divide), and Remapper with the second deletion method (Greatest neighbour) are compared. Guppy runs in the GPU mode and basecalling is done with four basecallers, called producers in the Remapper, for all of the methods. Furthermore, 24 processors have been used to process the basecalled reads in all of the cases. All of the measurements are conducted on the same machine, and with the same setting, thus achieving comparable results.

**Table 6.1:** Comparison of runtimes for Escherichia coli dataset [sec]

<b>Model</b>	<b>Modification</b>	<b>Guppy</b>	<b>Tombo</b>	<b>Feature extraction</b>	<b>Total</b>
<u>Rockfish</u>	mod	31.29	52.55	83.17	167.01
	nomod	32.97	58.17	86.26	177.4
<u>Remapper</u>	mod	-	-	138.24	136.56
	<u>del #1</u>	-	-	146.43	<b>137.42</b>
<u>Remapper</u>	mod	-	-	133.66	<b>133.66</b>
	<u>del #2</u>	-	-	142.93	142.93

## 6.2. Accuracy

## 6.3. Discussion

The main goal of this thesis has been to find a heuristic which would replace Tombo and whose performance is similar to the original model, but the speed is improved. From taking a glance at Table 6.1 it can be concluded that we have succeeded in doing so. Remapper is faster than Rockfish in the both of its implementations, regarding the way of resolving deletions. All of the methods are slower for unmodified reads which can maybe be attributed to them having longer signals in average (see Subsection 3.3.1). The difference in runtimes might not seem so drastic on 2000 E. coli reads, but when the program is scaled for a large amount of data, the difference in the speed is indeed noticeable. 

## **7. Conclusion**

Zaključak.

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# **Detection of Modified Nucleotides Using Nanopore Sequencing and Deep Learning Methods**

## **Abstract**

Abstract.

**Keywords:** Keywords.

# **Određivanje modificiranih nukleotida koristeći sekvenciranje nanoporama i duboko učenje**

## **Sažetak**

Sažetak na hrvatskom jeziku.

**Ključne riječi:** Ključne riječi, odvojene zarezima.