

SMURF-seq: efficient short-read sequencing on long-read sequencers

Rishvanth K. Prabakar

Quantitative and Computational Biology Section,
Department of Biological Sciences,
University of Southern California,
1050 Childs Way,
Los Angeles 90089, USA

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Abstract

Somatic copy number alterations (CNA) play a significant role cancer, and can be leveraged for diagnostic and personalized approaches to treatment. High-throughput short-read sequencing has been extremely efficient in copy number profiling; however, its applicability depends on the availability of instrument, and time to obtain profiles can vary from a few days to weeks. We present SMURF-seq, a protocol to efficiently sequence short DNA molecules on a long-read sequencer by randomly ligating them to form long molecules. Applying SMURF-seq using the highly portable and inexpensive Oxford Nanopore MinION yields up to 30 fragments per read, providing an average of 6.2 and up to 7.5 million mappable fragments per run, increasing information throughput for read-counting applications. We apply SMURF-seq on the MinION to generate copy number profiles, demonstrate that multiple samples can be multiplexed and sequenced in a single sequencing run, and show that concordant profiles are obtained with reads sequenced in the first 45 minutes of a run. A comparison with profiles from Illumina sequencing reveals that SMURF-seq attains similar accuracy. More broadly, SMURF-seq expands the utility of long-read sequencers for read-counting applications.

Individuals are not stable things, they are fleeting. Chromosomes too are shuffled into oblivion, like hands of cards soon after they are dealt. But the cards themselves survive the shuffling. The cards are the genes. The genes are not destroyed by crossing-over, they merely change partners and march on. Of course they march on. That is their business. They are the replicators and we are their survival machines. When we have served our purpose we are cast aside. But genes are denizens of geological time: genes are forever.

Richard Dawkins, The selfish gene

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Chapter 1

Introduction

In the last decade, massively parallel high-throughput short-read sequencing has revolutionized the efficiency and breadth of applications for DNA sequencing (Kircher and Kelso, 2010). These high-throughput sequencing methods produce millions to billions of short reads in a single run, and have led to the development of many applications that depend on “read-counting” to measure the abundance of specific sequences in a sample. Examples include RNA-seq, ChIP-seq, and whole genome copy number profiling.

Recently, long-read technologies have been developed that are filling the gap left by short-read sequencers in applications such as genome assembly (Jain et al., 2018a; Loman et al., 2015), which benefit from connecting more distant sequences within a contiguous molecule. Among these the MinION instrument, from Oxford Nanopore Technologies, is highly portable and inexpensive and has shown its unique value for analysis outside of central sequencing facilities (Quick et al., 2016). Long-read sequencers such as the MinION typically produce vastly fewer reads from a sequencing run, and are therefore less efficient in applications that use sequenced reads purely as a means to count molecules. However, these technologies have the enormous advantage of operating in near real-time, with a turnaround time that can be measured in hours for some applications, rather than days or weeks.

Copy number variation (CNV) has been used successfully to understand a variety of diseases (Sebat et al., 2007) – notably cancers, which exhibit both extreme variation and recurrent trends that can be used for diagnostics and personalized approaches to treatment. For example, the amplification and loss of certain genes, such as *RB1* deletion and *MYCN* amplification in retinoblastoma, can be prognostic or even predictive for treatment (Berry et al., 2017). High-throughput short-read sequencing has been extremely effective in copy number profiling of cancers (Chiang et al., 2009), including profiling single tumor cells (Navin et al., 2011). However, for many potential users, the efficiency of high-throughput short-read sequencing in CNV analysis is determined by the availability of instruments and need for heavy multiplexing to hit reasonable cost per profile. A sequencing core is typically involved and an individual profile must wait for a “full” run before it can be processed. The MinION sequencer has an accessible buy-in and is easy to use. Unfortunately the MinION has optimal nucleotide throughput when producing reads that are orders of magnitude longer than needed for CNV profiling.

To make full use of the advantages offered by the MinION sequencer, we introduce sampling molecules using re-ligated fragments (SMURF)-seq, a protocol to efficiently sequence short DNA molecules on a long-read sequencer. The strategy of SMURF-seq is to concatenate short fragments into very long molecules (~8 kb) prior to sequencing. After (or possibly concurrent with) sequencing, the SMURF-seq reads are mapped to the reference genome by splitting them into their constituent fragments, each aligning to a distinct location in the genome. We demonstrate the utility of SMURF-seq with the low-cost MinION sequencer to obtain data similar to that expected from typical short-read sequencing, and generated high-quality copy number profiles from this output.

More broadly, SMURF-seq is an approach for efficient short-read sequencing, as required for read-counting, on long-read sequencing machines. Here, we describe the details of the SMURF-seq approach; both the SMURF-seq protocol prior to sequencing and mapping the sequenced SMURF-seq reads. This study is organized as follows:

In the second chapter, we review the relevant background. First, we discuss the concept of nanopore sequencing and summarize its history, the MinION sequencing instrument and its utility, library construction methods, and properties of reads sequenced on these machines. Then, we discuss the copy number profiling and its implications in diversity and disease; especially its involvement in cancer, and the utility of copy number analysis in understanding the biology of cancer and diagnostic evaluation of tumors. We summarize protocols for copy number profiling and computational methods to for generating profiles. Finally, we discuss prior protocols that are similar in spirit to SMURF-seq; these protocols include SAGE and its variants, SMASH, and Concat-seq.

In the third chapter, we describe the details of the SMURF-seq approach and demonstrate the accuracy of this approach for CNV profiling. We start with a discussion of sequencing long-reads or short-reads directly for read-counting on nanopore machines, and the merits and limitations of these methods. Then, we introduce the SMURF-seq protocol for efficient short-read sequencing on long-read machines; SMURF-seq combines the merits of sequencing long or short reads directly, while alleviating the limitations. We demonstrate that SMURF-seq generates higher read-counts from a sequencing run in comparison to these other method, the copy number profiles generated with SMURF-seq are as accurate as profiles generated on an Illumina platform, multiple samples can be multiplexed and sequencing in the same sequencing run, and the reads generated in the first few minutes of sequencing are sufficient to generate accurate profiles. Finally, we provide future directions for further improving the efficiency and for expanding the utility of SMURF-seq.

The fourth chapter is dedicated to algorithmic and statistical aspects of mapping SMURF-seq reads. We discuss the challenges associated with mapping SMURF-seq reads as the fragments get shorter. We introduce the fragment identification problem as a way of identifying fragment boundaries and estimating the optimal number of fragments on a SMURF-seq read. Next, we define a score function for aligning SMURF-seq reads and describe algorithms to find fragment boundaries on a read such that the score is maximized. Then, we determine the null distribution of aligning a SMURF-seq read generated at random in order to calculate a p-value for a particular

fragmentation of a read. We use these p-values to determine the optimal number of fragments on a read. Finally, we suggest future directions of aligning SMURF-seq reads with short fragments to large reference genomes.

We conclude this study by highlighting the merits of using the SMURF-seq approach for short-read sequencing on long-read sequencers. We envision a broadening of the applications of SMURF-seq as the underlying sequencing technology evolves and as SMURF-seq itself improves by continual decrease in fragment lengths, increase in sequenced read length, and data analysis methods optimized for SMURF-seq resulting in an increase in information yield per nucleotide sequenced.

Chapter 2

Background

2.1 Nanopore sequencing

A brief history of nanopore sequencing The concept of nanopore sequencing is based on the idea that as a single-stranded DNA (or RNA) translocates through a nanometer sized pore, a nanopore, in the presence of an electric field, the change in current level measured across the nanopore would be dependent on the nucleotide passing through the nanopore; Thus, measuring the current over time could be leveraged to determine the sequence of nucleotides (Fig. 2.1b). This idea of using transmembrane proteins an nanopores for sensing and sequencing nucleic acids was independently thought of by several researches including David Deamer, Hagan Bayley, and George Church (Bayley, 2015; Branton et al., 2009; Deamer et al., 2016).

Initial experiments showed that as a single-stranded DNA or RNA molecules could be driven through a *Staphylococcus aureus* α -hemolysin in the presence of an electric field (Kasianowicz et al., 1996). The current through the pore remained constant in the absence of oligomers; and the presence of oligomers caused transient decreases in current, with the duration of the decrease proportional to the length of the oligomer. Further research demonstrated that decrease in amplitude of current could be used to differentiate between poly-purine and poly-pyrimidine sequences

of RNA (Akeson et al., 1999) and DNA (Meller et al., 2000). It was also observed that the DNA molecules translocate through the nanopore at few microseconds per base (Meller et al., 2000).

Although these experiments demonstrated the potential for nanopores to distinguish nucleic acid polymers, several challenges remained to be addressed to use this approach for reading individual bases on a DNA or RNA molecule. The most important of these were detecting the bases on a molecule at single nucleotide resolution and slowing the rate of translocation through the nanopore so that a readout can be obtained (Bayley, 2015; Branton et al., 2009). These challenges were resolved in the forthcoming years. A few notable milestones are described below.

In regard to identifying individual nucleobases, all four bases were identified in single-stranded DNA that have a terminal hairpin structure (Ashkenasy et al., 2005) and single-stranded DNA attached to streptavidin with a biotin linker (Purnell and Schmidt, 2009; Stoddart et al., 2009). These structures immobilized the DNA in the nanopore (either a wildtype α -hemolysin or an engineered

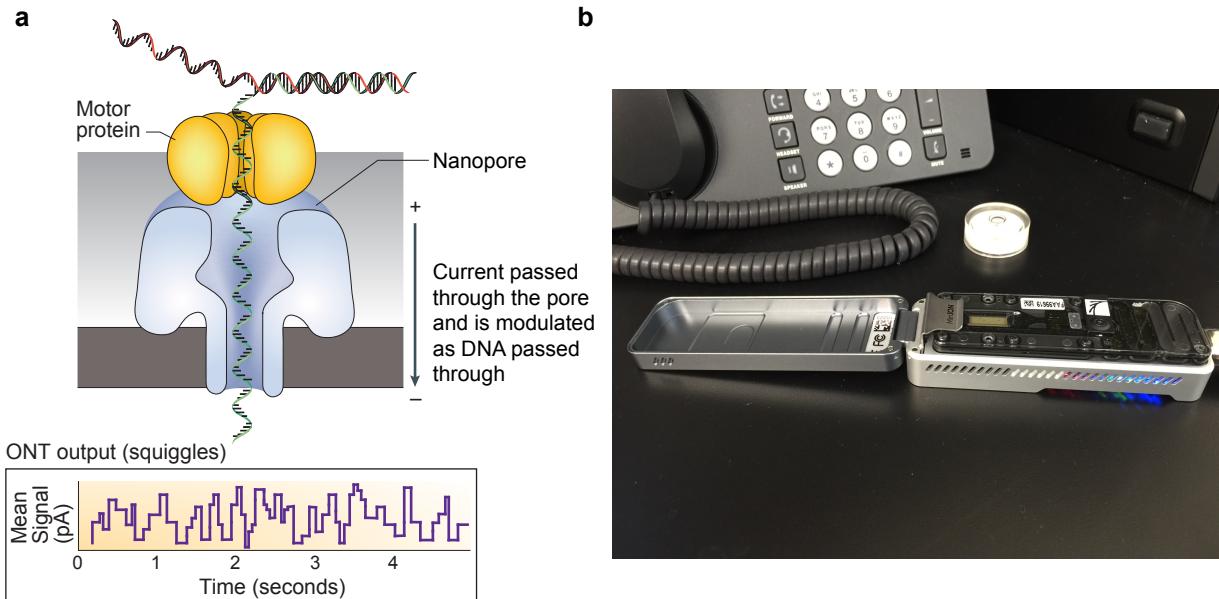


Figure 2.1: Nanopore sequencing. (a) Current across a nanopore is measured as a DNA molecule translocates through a nanopore. This figure is adapted from Figure 5Ab in Goodwin et al. (2016). (b) Oxford Nanopore Technologies MinION instrument.

form of it) allowing sufficient time for detection. Thus, with the appropriate sequence, each base could be resolved, and further, the location within a nanopore that is most sensitive to detect bases was also determined.

The frequency of translocation of DNA molecules through a α -hemolysin pore was increased and the voltage threshold for translocation was decreased by engineering the pore to have positive charged groups in the lining of the lumen (Maglia et al., 2008). However, a disadvantage of the α -hemolysin pores is that the region that is sensitive to the nucleotides in the pore is too wide, and so the current differences are small between nucleotides, making single nucleotide detection difficult. Pores derived from *Mycobacterium smegmatis* porin A has a narrower sensitive region, and could detect nucleotides at a higher resolution (Butler et al., 2008; Manrao et al., 2011).

In regard to controlling the rate of translocation through a nanopore, there were two approaches, exo-sequencing and strand-sequencing. In the exo-sequencing approach, individual bases from a DNA are cleaved into a nanopore with an exonuclease and unidentified one at a time (Astier et al., 2006; Clarke et al., 2009). Alternatively, in the strand sequencing approach a DNA molecule is threaded though a nanopore at a controlled rate and the bases are identified from the continuous change in current levels. Initial approaches in strand sequencing used a DNA polymerase (derived from *Escherichia coli* Kleenow fragment or bacteriophage T7) and recorded the current levels as the DNA translocates through a pore with the incorporation of each nucleotide (Benner et al., 2007; Chu et al., 2010; Cockroft et al., 2008; Gyarfas et al., 2009). Subsequently it was shown that ϕ 29 polymerase could be used to control ratcheting in both the forward and the reverse direction (Cherf et al., 2012; Lieberman et al., 2010; Manrao et al., 2012). ϕ 29 was used to sequence reads up to 4.5 kb from the ϕ X174 genome using nanopores (Laszlo et al., 2014).

Oxford Nanopore Technologies Oxford Nanopore Technologies was founded in 2005 by Hagan Bayley and colleagues (Deamer et al., 2016). Oxford Nanopore Technologies announced the MinION instrument at the Advances in Genome Biology and Technology meeting in 2012 and

made it available to early access researchers in 2014 (Bayley, 2015; Deamer et al., 2016).

The MinION sequencer (Fig. 2.1b) is a portable instrument requiring just a modest computer for control and data acquisition. A MinION flowcell of 2048 nanopores (at present, a derivative of *Escherichia coli* CsgG protein (Brown and Clarke, 2016)) embedded on a membrane, of which 512 pores can sequence molecules in parallel.

Whole genomes of several organisms including humans have been sequenced using the MinION instrument (Bowden et al., 2019; Jain et al., 2018a; Loman et al., 2015; Moss et al., 2020; Stancu et al., 2017). It has also been used in several other applications such as disease surveillance (Faria et al., 2016; Quick et al., 2016), metagenomics (Charalampous et al., 2019; Goordial et al., 2017; Leggett et al., 2020), direct RNA sequencing (Depledge et al., 2019; Garalde et al., 2018; Workman et al., 2019), and detecting methylated bases (Liu et al., 2019; Rand et al., 2017; Simpson et al., 2017) among others (Jain et al., 2016).

In addition to the MinION instrument, Oxford Nanopore Technologies currently offers various nanopore devices including the bench-top GridION and PromethION which allows parallel sequencing with up to 5 and 48 flowcells, respectively.

Nanopore library preparation and sequencing Sequencing nucleic acids requires prepossessing the sample DNA for compatibility with the underlying sequencing technology, a process traditionally referred to as library preparation. Sequencing on a nanopore machine usually requires fragmenting DNA molecules to the appropriate length and attaching sequencing adapters. Oxford nanopore technologies offers several commercially library preparation kits for both DNA and RNA samples. The most frequently used of these kits are the Ligation Sequencing Kit family and the Rapid Sequencing Kit family.

In theory, there is no limit on the length of a molecule that can be sequenced with a nanopore, and thus, the length is determined by the downstream application or the limitations of handling high molecular weight DNA. For the ligation sequencing kit (SQK-LSK108 1D DNA by ligation),

the recommended length is ~8 kb when starting with 1ug of sample to ensure appropriate molar concentration in the subsequent steps. DNA molecules can be fragmented to the appropriate length using a variety of methods including the Covaris g-TUBE. These molecules are then optionally repaired to remove any nicks, and then the DNA ends are prepared to have a dA tail. Finally, sequencing adapters (that have a dT tail) are ligated to the end-prepared DNA. These adapters contain specific DNA sequenced with attached enzymes that regulate translocation of the DNA molecule into a nanopore. Library preparation with the ligation kit takes approximately 60 minutes.

The rapid library preparation kit (SQK-RAD003 Rapid sequencing) offers a faster method, by simultaneously fragmenting and tagging the ends of high molecular weight DNA (recommended > 30 kb). Adapters are then attached to these tags. Library preparation with the rapid kit takes approximately 10 minutes.

Both of these kits offer barcoding capabilities for multiplexing several samples in a single sequencing run. For example, the Native Barcode Kit (EXP-NBD103) is used in addition to the ligation sequencing kit, and adds a barcode sequence to the end-prepared DNA molecules prior to ligating sequencing adapters. After library preparation with a unique barcode for each sample, they can pooled in appropriate molar concentrations before sequencing.

Oxford Nanopore Technologies offers severs other preparation kits, such as the 1D² kit for higher accuracy reads and PCR based kits when starting with nanogram or picogram amounts of DNA.

After library construction the sample is ready to be sequenced. The flowcell is loaded on the sequencing machine, primed with the appropriate buffers, and the sample is loaded. After which the sequencing can be started and the reads are available as they are sequenced in real-time. The sequencing process is controlled by the MinKNOW tool, and can continue for up to 48 hours. The sequenced reads can either be base-called in real time using the MinKNOW or at a later time using the Guppy tool.

Properties of nanopore reads

2.2 Copy number variation and profiling

Copy number variation Sources of genomic variation in humans include single-nucleotide polymorphisms (SNPs), short insertions and deletions, and repeats (). Another source of variation, copy number variation (CNV), is the change in number of copies of a region of the genome larger than 1 kb with respect to a reference genome (Feuk et al., 2006; Redon et al., 2006). The number of copies of a region of the genome could increase resulting in a copy number “gain” (also referred to as amplification or duplication), or decrease resulting in a copy number “loss” (also referred to as deletion). Changes in copy number can occur due to mechanisms such as homologous recombination and non-homologous DNA repair mechanisms (Hastings et al., 2009; Van Binsbergen, 2011).

Copy number variations contribute both to diversity in the human population and to disease (). Variations that contribute to diversity are present in the germline, whereas those that contribute to diseases could in the germline or somatic. (Somatic copy number variations are generally referred to as somatic copy number alterations, or CNA in short.)

CNV in diversity CNVs as a significant source of diversity in humans was established by Sebat et al. (2004) and Iafrate et al. (2004). Sebat et al. (2004) identified 221 copy number differences in 20 individuals with an average of 11 differences between individuals; these variations had an average length of 465 kb (median length of 222 kb). Iafrate et al. (2004) identified 225 regions in 55 individuals with an average of 12.4 differences between individuals; these variations ranged from 150 kb to 425 kb. These studies were extended to larger populations and to populations of different ancestry (Li et al., 2009; Redon et al., 2006). CNVs in the human genome and that those contribute to diversity are reviewed in (Feuk et al., 2006; Freeman et al., 2006; Zarrei et al., 2015).

Copy number variation in diseases Changes in copy number of genes or whole chromosomes are implicated in several diseases. The most notable example is the trisomy of chromosome 21 in Down's syndrome ()�.

Although, most copy number alterations are implicated in diseases, they can sometimes prevent disease.

Copy number alterations in cancer Evolution of a “normal” single cell into a “tumor” cell, a tumor cell into a mass of cells, into sub-clones of cells, and into metastatic tumor cells all involve genomic changes to the cells (Stratton et al., 2009). Of the possible genomic changes, somatic copy number alterations play a significant role (Beroukhim et al., 2010; Zack et al., 2013).

Applications of CNA profiling of cancer can be broadly classified into two categories, understanding the biology of cancer and diagnostic evaluation of tumors. These categories are not mutually exclusive and the advance in one drives advances in the other.

CNA profiling for understanding the biology of cancer typically involve profiling and analyzing a collection of tumor samples within or across cancer types. CNA profiles exhibit several recurrent alterations that occur in a significant fraction of samples within a population. These recurrent alterations are considered to be “driver” alterations and play an active role in tumor progression; Whereas alterations that are unique to few samples are considered to be “passenger” alterations which are neutral to the evolution of tumor (Beroukhim et al., 2010; Bignell et al., 2010). These frequently altered driver alterations are identified from regions on the genome that are frequently gained or lost above a significance threshold in a population of cancer samples (Mermel et al., 2011). Driver alterations play significant roles in tumor progression (Beroukhim et al., 2010; Bignell et al., 2010). Several studies have identified recurrent alteration in different tumor types (Beroukhim et al., 2007; Etemadmoghadam et al., 2009; Lin et al., 2008; Weir et al., 2007).

CNA profiling for diagnostic applications would involve profiling a tumor from a patient, and making treatment decisions based on prior knowledge of copy number alterations. These include

deciding the effectiveness of treatment and predicting the survival of breast cancer patients (Hicks et al., 2006; Stuart and Sellers, 2009). Amplification of 19q12 and 20q11.22-q13.12 is associated with poor response to platinum-based chemotherapy and survival in ovarian carcinomas (Etemadmoghadam et al., 2009). Recurrent alterations are associated with gains of oncogenes and loss of tumor suppressor genes. For example, gain of *MYC*, and loss of *PTEN* and *RBI* play important roles in prostate cancer (Alexander et al., 2018). The expression of several genes in these altered regions are correlated with the gain or loss of genomic region harboring the gene (Chitale et al., 2009; Lu et al., 2011; Pollack et al., 2002). Few studies have directly linked the alteration in CNA profiles with diagnostic outcomes (Bardelli et al., 2013; Berry et al., 2018; Etemadmoghadam et al., 2009), and several others have shown that the expression level of these genes are associated with tumor progression and response to treatment (Gorre et al., 2001; Shattuck et al., 2008; Villanueva et al., 2013).

CNA profiling methods

2.3 Prior protocols based on concatenating DNA molecules

Serial analysis of gene expression (SAGE) The concept of ligating short DNA molecules to improve the efficiency of sequencing was introduced in serial analysis of gene expression (SAGE) (), and subsequently its variants such as LongSAGE and SuperSAGE (). SAGE

Variants of SAGE

Digital karyotyping

SMASH

Concat-seq

Chapter 3

Sampling molecules using re-ligated fragments (SMURF)-seq

3.1 Naive approaches to read-counting on nanopore machines

Copy number profiling, and read-counting in general, can be done on nanopore sequencers with long-reads ($\sim 8\text{kb}$) following the standard sequencing procedure (Fig. 3.1). Since nanopore machines are optimized for long-read sequencing, this method has the advantage of using any standard library preparation protocol that are commercially available. Sequencing long molecules using a nanopore keeps a pore occupied for a longer duration once a pore is loaded followed by an open pore waiting for a molecule to be reload. Further, technical nucleotides, such as sequencing adapters and barcodes, are sequenced one (or twice) every $\sim 8\text{k}$ bases, and thus, the fraction of time a nanopore spends sequencing technical nucleotides is low. However, read-counting applications do not benefit from longer reads beyond what is necessary for unique mapping to the reference genome. In these applications, for any fixed number of nucleotides sequenced, more information would be obtained if those nucleotides are organized as more DNA molecules, rather than longer contiguous fragments.

An alternate approach for read-counting is to sequence short reads (~ 150 bp) directly on a nanopore sequencer (Fig. 3.1). In general, for a given sample of DNA, a nanopore instrument will generate more reads if the corresponding molecules are shorter. Once a molecule is loaded into a pore, the time spent sequencing is less for shorter reads. In addition, for a fixed amount of DNA, shorter molecules result in higher molar concentration when loaded onto the machine, increasing the rate at which each pore captures molecules (Muthukumar, 2010; Wanunu et al., 2008). Therefore, sequencing short reads on a nanopore machine would generate more reads from a sequencing run than sequencing long reads. However, sequencing short reads requires ad-hoc modifications to the library preparation protocol as these are optimized for longer molecules. Sequencing these shorter molecules keeps a pore occupied for a shorter duration once a pore is loaded followed by waiting for a pore to be reloaded (but the reload time is usually shorter due to the higher molar concentration). Moreover, technical nucleotides are sequenced every ~ 150 bp, increasing the fraction of time a nanopore sequences the technical bases.

SMURF-seq approach combines the advantages of both of these methods and alleviates the

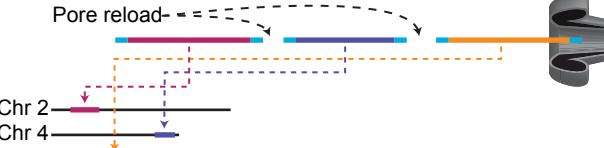
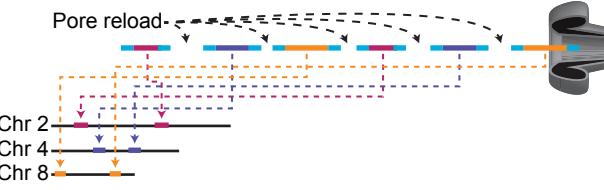
Method	Advantages	Disadvantages
Long-read sequencing (~8k bp)	<ul style="list-style-type: none"> Standard lib. prep. Adapter and barcode every ~8k bp Pore reload every ~8k bp Optimal sequencing speed (nucs./sec) 	<ul style="list-style-type: none"> Longer than required for read-counting Low read count per run
Short-read sequencing (~150 bp)	<ul style="list-style-type: none"> Optimal read length for read-counting Higher read count per run 	<ul style="list-style-type: none"> Modified lib. prep. Adapter and barcode every ~150 bp Pore reload every ~150 bp Reduced sequencing speed (nucs./sec)

Figure 3.1: Naive approaches to read-counting on nanopore machines. Sequencing long-reads directly is optimized for nanopore machines but not for read-counting applications. Sequencing short-read is optimized for read-counting applications but not for nanopore sequencing.

drawbacks by using a nanopore instrument as intended for long-read sequencing, while generating the desired short fragments. Using the SMURF-seq approach, we generate higher read counts per run than sequencing long or short molecules directly.

3.2 SMURF-seq approach to read counting

The SMURF-seq protocol involves cleaving genomic DNA into short fragments, with length just sufficient for an acceptable rate of uniquely mapping fragments in the reference genome. These fragmented molecules are then randomly ligated back together to form artificial long DNA molecules, as required for long-read sequencing. The long re-ligated molecules are sequenced following the standard MinION library preparation protocol. After (or possibly concurrent with)

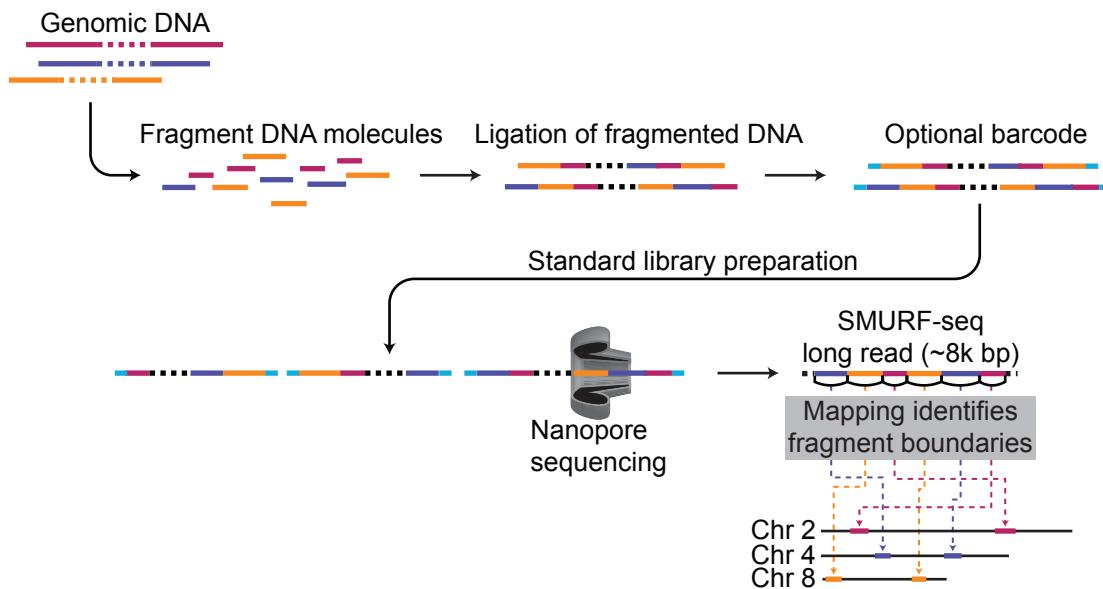


Figure 3.2: SMURF-seq approach to sequencing short fragments. SMURF-seq efficiently sequences short fragments of DNA for read-counting applications with a reference genome on long-read sequencers, and yields up to 30 countable fragments per sequenced read. SMURF-seq sequences short DNA molecules by generating long concatenated molecules from these. SMURF-seq reads are aligned by splitting them into multiple fragments, each aligning to a distinct region in the genome.

sequencing, the SMURF-seq reads are mapped to the reference genome in a way that simultaneously splits them into their constituent fragments, each aligning to a distinct location in the genome (Fig. 3.2).

More specifically, genomic DNA was fragmented using restriction enzymes and ligated with T4 DNA ligase, with clean-up steps in between. SMURF-seq protocol is completely enzymatic and takes less than 90 minutes to complete (Fig. 3.3). The details of these steps are given below:

1. Restriction enzyme digestion: restriction enzymes recognize and cleave specific DNA sequences,

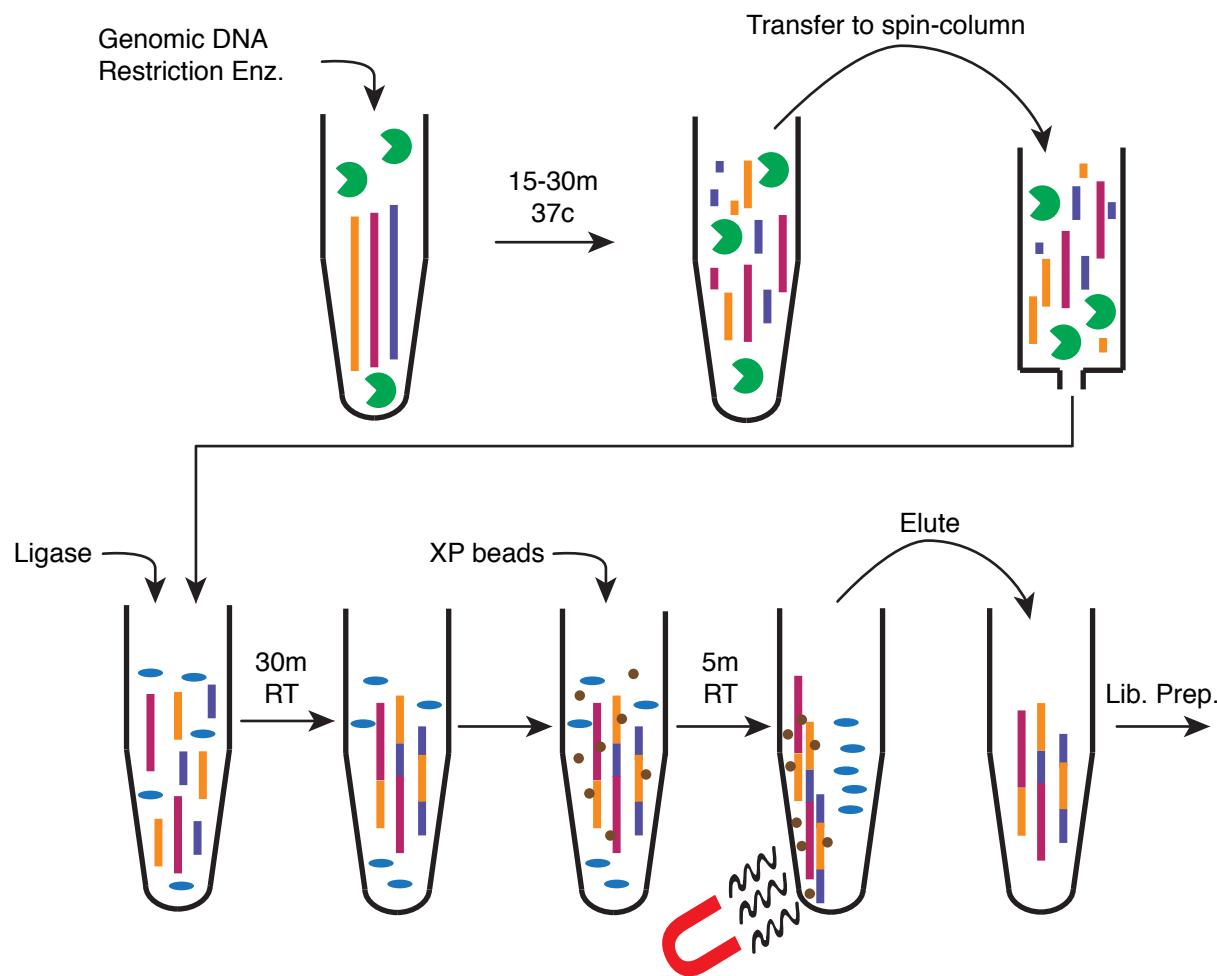


Figure 3.3: Schematic of SMURF-seq protocol. SMURF-seq consists of four steps: restriction enzyme digestion, spin-column clean-up, re-ligation of fragmented DNA, and Ampure XP beads clean-up.

typically producing sticky-ended DNA molecules. The choice of restriction enzyme used is primarily dependent on the size of the fragmented molecules produced. Based on the downstream application, this could also be influenced by other factors such as any sequence-specific biases restriction enzymes could introduce. An advantage of using restriction enzymes to fragment DNA molecules, over other fragmentation techniques, is that the fragmented molecules have a uniform ends (either overhangs with the same sequence or blunt-ends) and are thus compatible for ligation without an end-repair step in between.

2. Clean-up: the reaction containing the restriction enzymes and the fragmented DNA molecules is cleaned to wash out the enzymes and retain the fragmented DNA molecules. The choice of clean-up kit used determines the length of the retained DNA molecules. We used a spin-column based clean-up that typically retains molecules that are over ~70 bp. However, other clean-up kits, such as bead-based kits, could also be used at this step.
3. Re-ligation: fragmented DNA molecules with uniform ends are ligated at random with T4 DNA ligase enzymes. The most important factor in a ligation reaction is the concentration of compatible DNA ends (Dugaiczyk et al., 1975). At high concentrations, the chances are higher for ligation between two molecules than a molecule self-ligating. At low concentrations, the chances are higher for self-ligation. Thus, the main consideration during the ligation step is the duration of the ligation reaction, as the molar concentration of DNA molecules decrease with time. Too little time would lead to insufficient ligation, resulting in molecules of length that do not achieve optimal SMURF-seq efficiency. On the other extreme, too much time would result in circular molecules that are incompatible with the most downstream library preparation process. A typical ligation reaction would contain both short and circularized molecules, and achieving a balance between these determines the efficiency of SMURF-seq. Other factors such as the temperature and buffer contents also affect the ligation process. In our experiments, the ligation reaction was performed at a DNA concentration of 25 ng/ μ l (500 ng of DNA in 10 μ l nuclease-free water and 10 μ l DNA ligase) for 30 min.

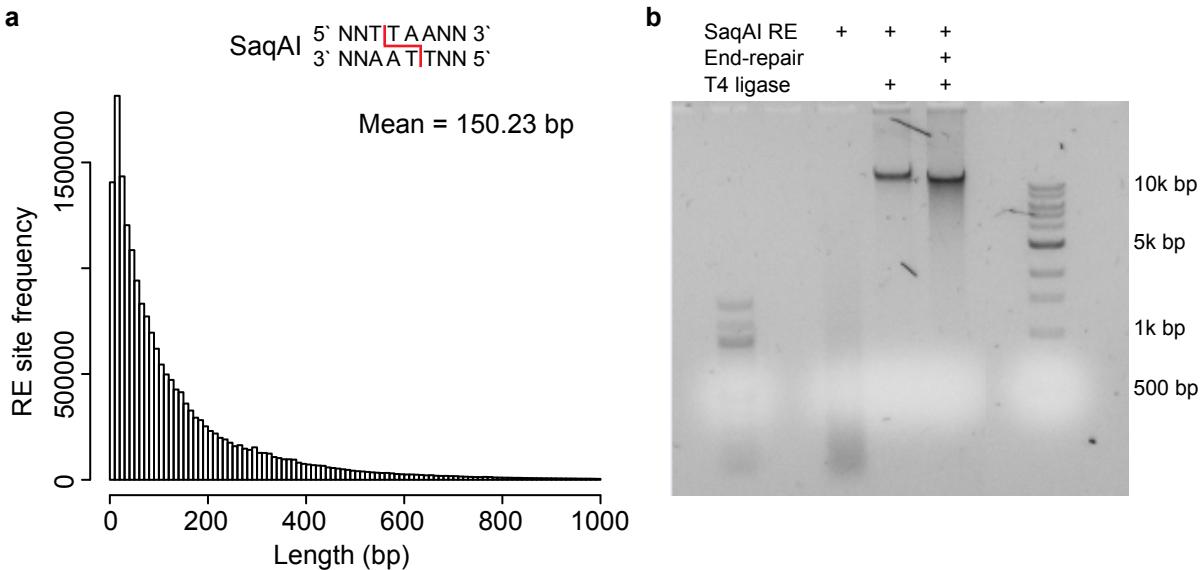


Figure 3.4: Restriction digestion and ligation of DNA molecules. (a) Distribution of length between restriction sites computed by measuring the distance between the recognition sites on the human reference genome. SaqAI recognizes the sequence TTAA and leaves a 2 bp overhang. (b) Negative gel image of fragmented and ligated normal diploid DNA using SaqAI restriction enzyme and T4 DNA ligase. Sticky-end and blunt-end ligation (by end-repair) of fragmented DNA are shown, and both yield ligated molecules of approximately the same length.

4. Bead-based clean-up: the reaction containing the ligase enzymes and ligated DNA molecules is cleaned to retain only the ligated molecules. We used a bead-based clean-up to avoid damage to long DNA molecules that are typical of spin-column based methods.

DNA molecules that are resultant of the SMURF-seq protocol are long DNA molecules that are typically several kilo-bases, and therefore, any standard library preparation kits that are available for nanopore machines can be used with SMURF-seq molecules. These molecules can also be barcoded with one (or two) barcode sequences per molecule. Thus, the SMURF-seq approach overcomes the disadvantages of sequencing long or short DNA molecules directly on a nanopore machine for read-counting applications, and improves its efficiency of read-counting applications.

We also tested dsDNA Fragmentase enzymes (New England Biolabs) and acoustic shearing (Covaris) to fragment DNA. However, these methods require an additional end-repair step after fragmentation and the ligated molecules failed to reach the lengths we obtained by using restriction

fragmentation.

In our applications, we used SaqAI restriction enzyme, which recognizes the sequence TTAA and produces molecules with mean lengths of 150.2 bp (Fig. 3.4a). The fragmented DNA molecules are then ligated randomly to form longer molecules using T4 DNA ligase enzyme (Fig. 3.4b). In our experiments, the resulting long DNA molecules were prepared for sequencing using the Oxford Nanopore Technologies 1D DNA by ligation kit (SQK-LSK108) or the rapid sequencing kit (SQK-RAD003) following the standard manufacturers protocol. We also multiplexed samples using the 1D native barcoding genomic DNA kit (EXP-NBD103) followed by library preparation using the 1D DNA by ligation kit. The sequencing was performed on a MinION instrument. The detailed SMURF-seq protocol is given in Appendix A.

3.3 Mapping SMURF-seq reads

The reads sequenced using SMURF-seq can be mapped to a reference genome by first identifying short matches within the reads, corresponding to parts of the individual fragments, and then extending those to locate fragment boundaries. As currently implemented, SMURF-seq fragments are longer than \sim 150bp, and mapping these reads is handled nicely using the seed-and-extend paradigm implemented in many existing long-read mapping tools. Although none of these tools were designed to align SMURF-seq reads, several long-read aligners such as BWA-MEM (Li, 2013), Minimap2 (Li, 2018), and LAST (Kiebasa et al., 2011) include steps designed for split-read alignment, which can be leveraged for aligning SMURF-seq reads.

Aligning SMURF-seq reads with long-read mapping tools typically involve variations of the following steps:

- Identifying seeds: Mapping tools have a step of identifying seeds, which are short exactly matching parts of the read with parts of the reference genome. Choices in how seeds are defined and used are often made for mapping speed. The total size of SMURF-seq data sets is currently

(relatively) small, so speed is not our primary concern. We favor the most sensitive seed strategy, but depending on implementation too many seed hits could lead to ambiguity later in the mapping process.

- Chaining seeds: The identified seeds that are close to one another on the read and the reference, and have the same orientations could be merged. These are further extended into proto alignments, and filtered to avoid aligning potentially false-positive seed hits.
- Aligning within the chains: In this stage a Smith-Waterman alignment is performed, typically allowing users to specify a mismatch penalty along with penalties for both gap-open and gap-extend.
- Selecting best alignments: When high-scoring alignments overlap within a read, one of them (or both) could be trimmed or one is selected and the other discarded. The choices made here could lead to discarding entire fragments.

Mapping tools have several parameter options, in general, these are related to: (1) the seeding and chaining algorithm used by the individual tool. (2) The Smith-Waterman alignment scores, i.e. the match score, and the mismatch and indel penalty. The seeding and chaining parameters control the number of proto alignments that are further refined by aligning parts of the read to the reference genome using the specified alignment scores.

The Smith-Waterman alignment score used to align fragments to the reference genome is crucial for determining the optimal fragment length. On one extreme, a match score of 1 with a mismatch and indel penalty of 0 will result in one identified fragment covering the entire read and mapping perfectly, but will always map ambiguously. On the other extreme, a match score of 1 with a mismatch and indel penalty of $-\infty$ will result in any mismatch or indel on the read to be considered as a fragment boundary. Therefore, it is crucial to determine the optimal alignment score for mapping a SMURF-seq read.

We evaluated mapping tools on simulated SMURF-seq data generated by concatenating random fragments from real Oxford Nanopore reads. This emulates idealized SMURF-seq reads.

Within the simulated reads, the boundaries of each fragment are known *a priori*, as are their mapping locations when in the context of their original long reads. We used this information to evaluate mapping tools in terms of (1) how well they identify fragments purely for the purpose of counting molecules, which is the primary information used in CNV analysis, and (2) how well they identify individual mapping bases within reads. After mapping these reads, we calculated precision and recall for identifying both the correct fragment locations, and the individual mapping bases within the fragments (i.e. the correct fragment boundaries). Using this simulation setup, we determined the optimal Smith-Waterman alignment score for mapping SMURF-seq reads (detailed procedure is given in Appendix B).

Based on these results BWA-MEM outperformed other tools, and thus, we used BWA-MEM to align SMURF-seq reads. Briefly, BWA-MEM uses short seed hits originating from different parts of the long reads (and therefore, in our application, different fragments within those long reads), to form clusters of seed hits in the reference genome. Nearby clusters are joined, and then extended, eventually resulting in (for most fragments) one alignment per fragment. In our analysis, we employed BWA-MEM without any modifications to optimize identification of fragment boundaries. According to our simulations, this mode of operation may not perfectly identify fragment boundaries, but performs well in identifying mapping locations of the individual fragments, which is the information passed to subsequent steps in our analysis.

3.4 Generating higher fragment counts with SMURF-seq

A typical Oxford MinION sequencing run generates approximately 500k reads (length \sim 8 kb) (Jain et al., 2018a; Tyson et al., 2018) using the standard library preparation and sequencing protocols. Several studies have used these long reads for copy number profiling (Euskirchen et al., 2017; Magi et al., 2019). We tested the ability of the Oxford MinION instrument to sequence short DNA molecules by sequencing restriction enzyme (SaqAI) digested normal diploid genome. The

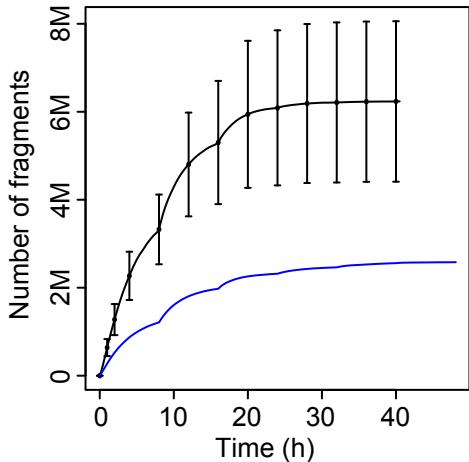


Figure 3.5: SMURF-seq generates fragments at a faster rate than sequencing short molecules directly. Number of fragments obtained from reads plotted as a function of sequencing time. For SMURF-seq, the average number of fragments from runs using the 1D sequencing by ligation kits are plotted (Error bars indicate one standard deviation). For short-read sequencing run, each read is considered as one fragment.

sequencing run produced 2.58 million reads with a mean read length of 630.93 bp. Using the same instrument, with SMURF-seq, we report here an average of 6.2 million mapped fragments per run, which is substantially more fragments than directly sequencing long or short reads directly. Further, the SMURF-seq approach generated fragments at a substantially faster rate than sequencing short molecules directly (Fig. 3.5).

The most important factor in the performance of SMURF-seq over sequencing short molecules directly is that sequencing concatenated fragments effectively eliminates the pore reload time for all but the first fragment in each read. However, there are a variety of additional factors that favor further optimization of the approach employed by SMURF-seq. First, reduction of resources spent on technical nucleotides: SMURF-seq uses a single barcode and sequencing adapter per read consisting of multiple fragments; sequencing short reads uses one barcode and adapter per fragment, adding approximately 50 bases to each fragment. This increases the time to sequence each short read. In sequencing short reads, as the reads get shorter the time consumed by these

technical bases increases. In SMURF-seq, sequencing either shorter fragments in fixed length reads, or longer reads containing fragments of fixed average length, both reduce the time consumed sequencing these technical bases. In the limit, assuming 100bp DNA fragments, sequencing those fragments as short-reads corresponds to 33% technical nucleotides; for SMURF-seq, the portion of technical nucleotides remains low. Second, more nucleotides sequenced at full speed: We observed that the speed of sequencing was lower when sequencing short molecules. For example, the average sequencing speed was 315.54 bases per second for sequencing the diploid genome without SMURF-seq, and 400.29 bases per second when sequencing using SMURF-seq on the MinION sequencer. Third, leveraging optimizations to long-read protocols: The rapidly evolving nanopore library construction kits are continually optimized for long-read sequencing, and would likely require significant ad-hoc modifications to optimize sequencing of short molecules of length optimal for read-counting applications.

3.5 Efficient CNV profiling using SMURF-seq

To demonstrate the utility of SMURF-seq, we generated CNV profiles of normal diploid and highly rearranged cancer genomes. The mapped fragments were grouped into variable length “bins” across the genome and bin counts were used to generate CNV profiles as described in (Baslan et al., 2012; Kendall and Krasnitz, 2014).

3.5.1 Accurate CNV profiles using SMURF-seq

We sequenced a normal diploid female genome with SMURF-seq, resulting in 270.8k reads (mean read length of 6.75 kb) in a single run. These reads were split into 7.28 million fragments (26.87 mean fragments per read; Fig. 3.6). A CNV profile for this normal diploid genome, with the expected (approximately flat) appearance can be seen in Fig. 3.7a. A replicate of this experiment resulted in 497.9k reads (mean read length of 3.7 kb), which were split into 7.55 million fragments

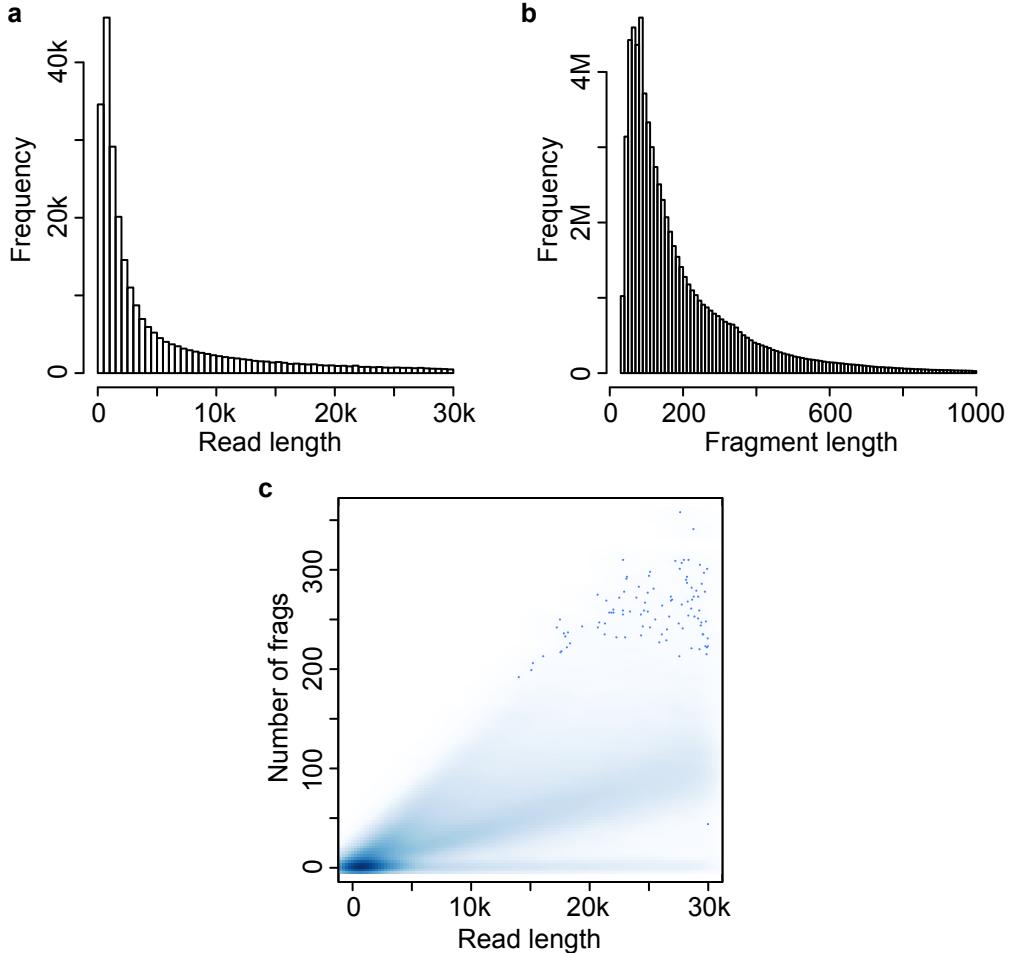


Figure 3.6: Read and fragment lengths from a SMURF-seq sequencing run. (a) Sequenced read length distribution. (b) Mapped fragment length distribution. (c) Scatter plot of read length and the number of fragments contained in the read.

(15.16 mean fragments per read).

The Rapid sequencing kit from Oxford Nanopore Technologies offers an extremely fast (10 minute) and simple (2 step) protocol for library preparation. We verified that the SMURF-seq procedure behaves similarly using the Rapid Sequencing Kit. The 213.38k sequenced reads had a mean read length of 3.9 kb, and were split into 2.81 million fragments. The copy number profile of the diploid genome was flat, as expected.

Next, we applied SMURF-seq to the breast cancer line SK-BR-3, generating 147.0k reads with

mean length of 7.62 kb, which were split into 4.52 million fragments (30.78 mean fragments per read). We then obtained a CNV profile using 5,000 bins, corresponding to an average bin size of approximately 600 kb (Fig. 3.7b).

To provide a quantification of accuracy in terms of individual CNV events we conducted whole-genome sequencing (WGS) on the same SK-BR-3 using Illumina (5.56 million reads; 130 bp, single-end). We used this to define a ground truth by calling CNV events for each of the pre-

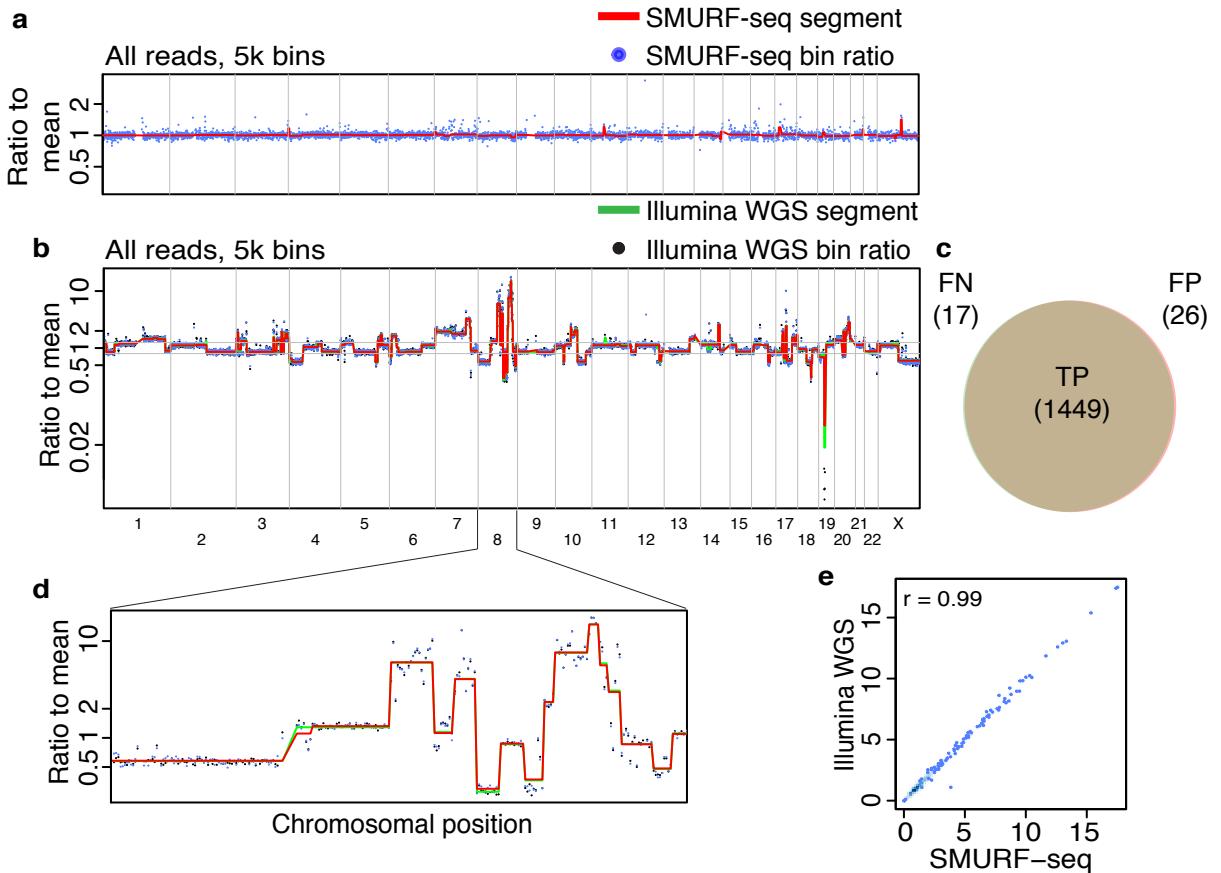


Figure 3.7: Accurate copy number profiles with SMURF-seq. (a) CNV profile of a normal diploid genome. Each blue point is a bin ratio and the red line is the segmented bin ratio. (b) Superimposed CNV profiles of SK-BR-3 genome generated using SMURF-seq and Illumina WGS reads. (c) Venn diagram illustrating the accuracy of event calls using SMURF-seq compared with Illumina WGS. (d) Zoom-in of copy number changes on chromosome 8. (e) Scatter plot of bin ratio of SK-BR-3 genome using SMURF-seq and Illumina WGS reads. Pearson correlation of the data is shown.

defined bins (both amplifications and deletions) based on the segmented signal with a cutoff of 1.25/0.8 (Fig. 3.7b) (Berry et al., 2017; Dago et al., 2014). This resulted in 1,466 events (886 amplifications, 580 deletions) from 4,953 bins. We then called events using the identical procedure with SMURF-seq data from the same SK-BR-3 sample. The precision and recall for SMURF-seq relative to the Illumina calls were 0.982 and 0.988, respectively (Fig. 3.7c). Fig. 3.7d shows a zoom-in of a region with extreme copy number alterations. The bin ratios for the Illumina WGS and the SMURF-seq profiles are highly correlated (Pearson $r = 0.99$; Fig. 3.7e). A replicate of this experiment resulted in 132.64k reads (mean read length of 7.3 kb), which were split into 4.02 million fragments (30.31 mean fragments per read).

We also generated higher-resolution CNV profiles with 20,000 and 50,000 bins, corresponding to an average of approximately 150 kb and 60 kb in length respectively (Fig. 3.8a, b). The profiles obtained at these resolutions have a high correlation with the profiles obtained using Illumina WGS (Pearson $r > 0.97$; Fig. 3.8c, d).

3.5.2 Concordant profiles from fewer countable fragments

Several cancer-related studies have employed CNV profiling based on low-coverage WGS (Kader et al., 2016; Macintyre et al., 2018). It has previously been demonstrated that 250k reads are sufficient for accurate genome-wide CNV profiling of single cells (Baslan et al., 2015). At the same time, the CNV profiles from a population of cells has been shown to have a high correlation with single-cell profiles (Baslan et al., 2015; Navin et al., 2011). We reasoned that using 250k fragments for CNV profiling using a population of cells would give useful profiles if they remained sufficiently accurate. By down-sampling our SMURF-seq data, we verified that 10k reads, approximately 250k fragments, result in highly-correlated CNV profiles (Pearson $r = 0.98$; Fig. 3.9a, b).

Given the total capacity of the MinION instrument, this indicates that multiple samples can effectively be barcoded and multiplexed in a single sequencing run. To verify this, we sequenced

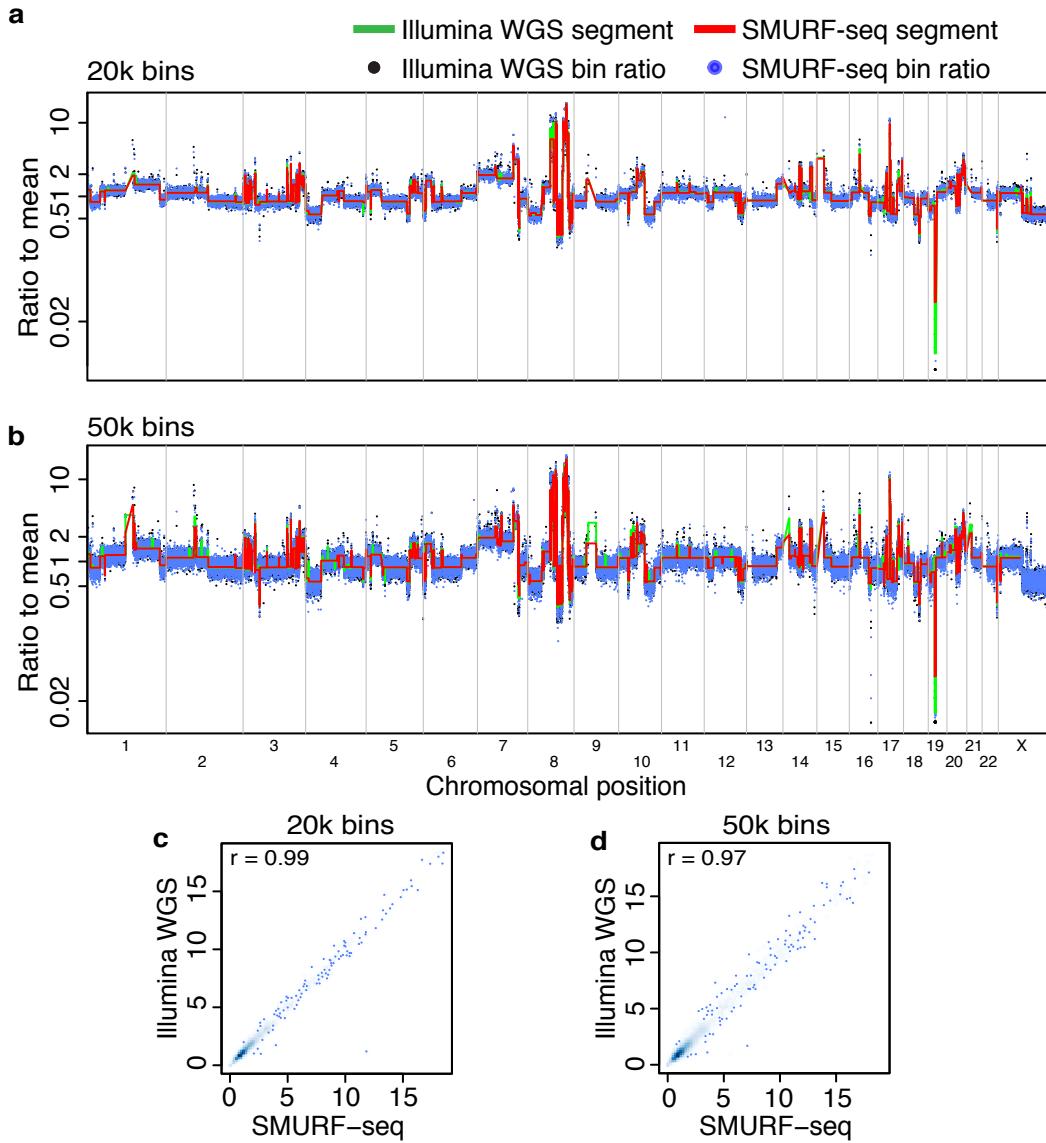


Figure 3.8: High resolution CNV profile generated using SMURF-seq is highly concordant with the profile generated with Illumina WGS. (a, b) Superimposed CNV profiles of SK-BR-3 genome generated using SMURF-seq and Illumina WGS at 20,000 and 50,000 bin resolutions. (c, d) Scatter plot of bin ratios of SK-BR-3 genome using SMURF-seq and Illumina WGS reads at 20,000 and 50,000 bin resolutions.

two DNA samples (normal diploid female and SK-BR-3) in a single run. These samples were processed with SMURF-seq protocol and then barcoded following the standard library construction. After demultiplexing and mapping the reads, the diploid genome had a CNV profile as expected

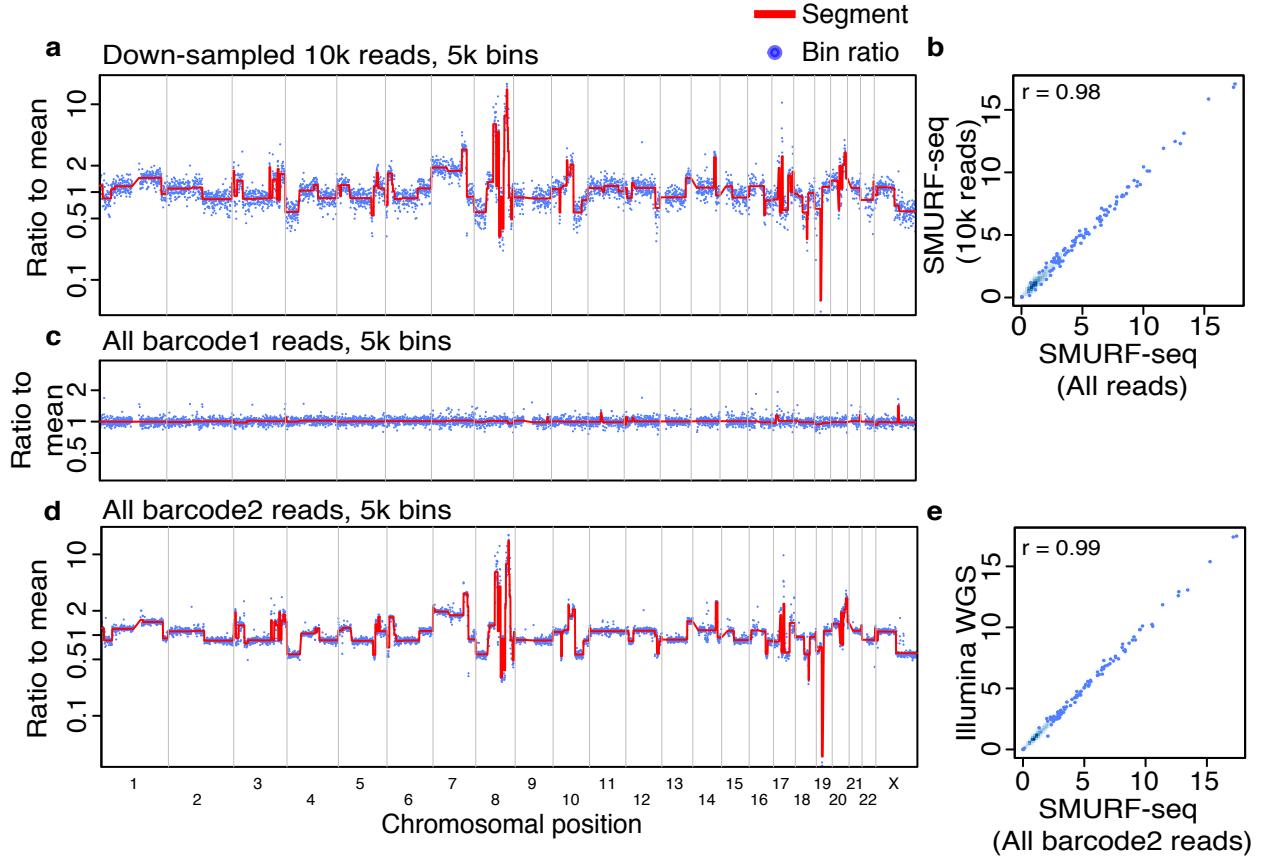


Figure 3.9: Multiple SMURF-seq CNV profiles by multiplexing in a single run. (a) CNV profile of SK-BR-3 genome with down-sampled 10k SMURF-seq reads. (b) Scatter plot of normalized bin counts of the original SMURF-seq data and data down-sampled to 10k SMURF-seq reads. Pearson correlation of the data is shown. (c) CNV profile of barcode01 (Normal diploid genome) reads. (d) CNV profile of barcode02 (SK-BR-3 cancer genome) reads. (e) Scatter plot of bin ratios of SK-BR-3 genome using multiplexed SMURF-seq and Illumina WGS reads.

(Fig. 3.9c) and the SK-BR-3 CNV profile was nearly identical to the profile obtained using Illumina WGS (Pearson $r = 0.99$; Fig. 3.9d, e). All the sequencing runs and the availability of sequence data are summarized in Appendix C.

Further, we verified that the CNV profile with reads generated in the first 45, 90, and 180 minutes of starting a sequencing run had a high correlation to the profile with reads from the complete run (Pearson $r > 0.98$; Fig. 3.10).

In summary, our results demonstrate that SMURF-seq can generate more information for CNV

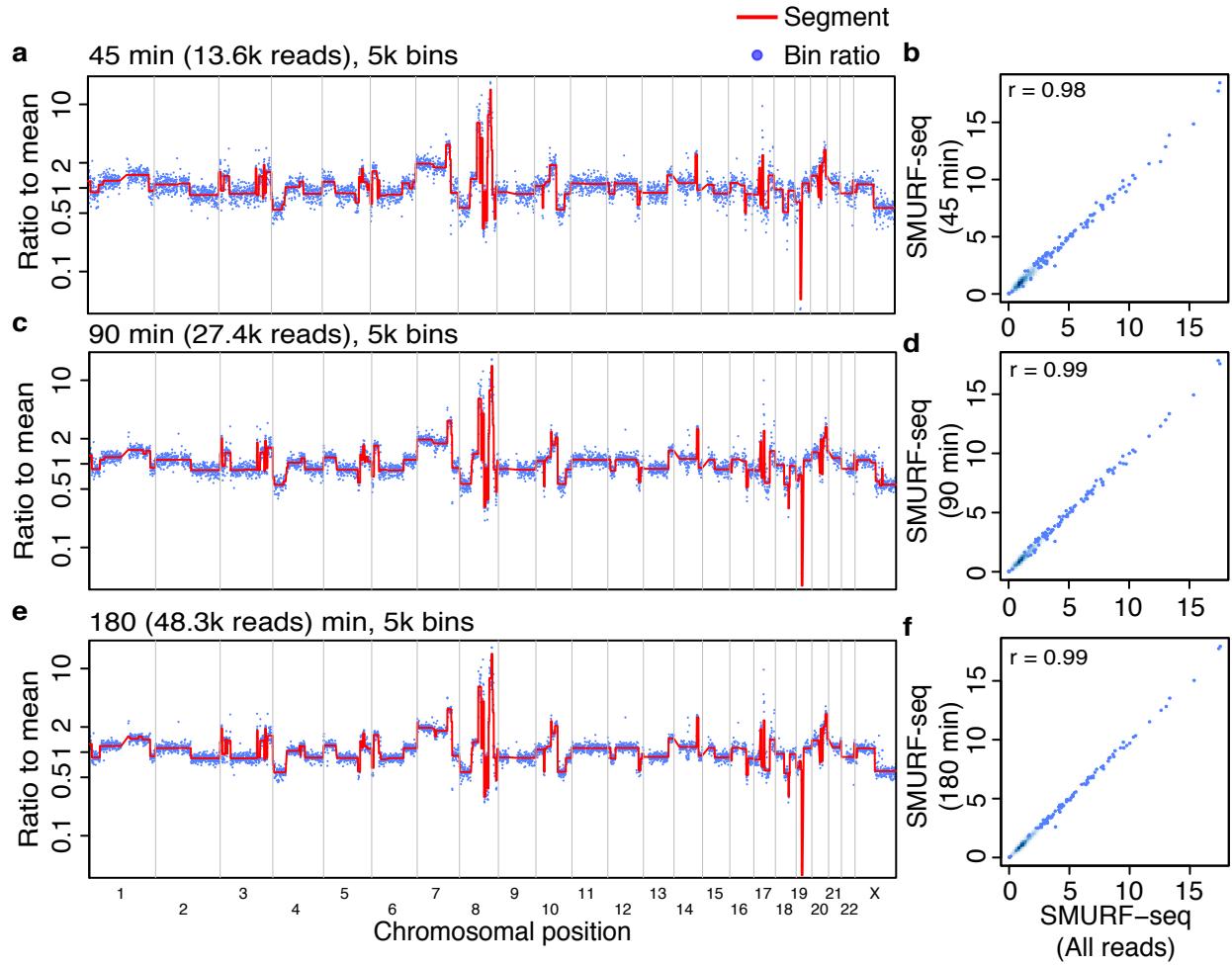


Figure 3.10: CNV profile with reads obtained in first few minutes of sequencing. (a, c, e) CNV profile with reads obtained in the first 45, 90, and 180 minutes of sequencing. (b, d, f) Scatter plot of bin ratios of the original SMURF-seq data and data obtained in first 45, 90, and 180 minutes of sequencing.

analysis in a single run of the Oxford MinION sequencer, compared with either sequencing long reads in the usual way or direct short-read sequencing on the same instrument. This increased information is in the form of increased numbers of distinct DNA fragments sequenced, and can be leveraged in multiple ways. Applying SMURF-seq on a single sample for a full run corresponds to higher counts for downstream analysis. In CNV analysis, increased counts either add confidence for a fixed resolution, or can allow higher resolution analysis (i.e. smaller bins) at the same level

of confidence. Alternatively, the increased information throughput can effectively reduce the time required to produce the same number of counts for CNV analysis by terminating the sequencing earlier. Finally, the increased information yield can be directed towards reducing the cost of generating CNV profiles by allowing a greater degree of multiplexing. For CNV analysis at resolutions permitted by 250k mapped fragments, our results show SMURF-seq allows roughly 20 and up to 30 samples in a single run, compared with 10 per run directly using short-read sequencing.

3.6 Future of SMURF-seq

SMURF-seq with shorter fragments

For CNV profiling with SMURF-seq, we used restriction enzyme SaqAI which fragments the human genome generating molecules with a mean length of 150 bp. For CNV profiling and other read-counting applications, the length of the fragmented molecules needs to be just long enough to ensure unique mappability to a sufficient fraction of the genome. Thus, reducing the fragment lengths could increase the number of fragments in a SMURF-seq read, and would increase the number of read-counts obtained per sequencing run.

The length of the fragmented DNA molecules can be reduced by digestion with a combination of restriction enzymes, as these would recognize more sites on the genome. Depending on the choice of enzymes used, the fragmented molecules could have blunts ends, sticky ends that are not compatible with one another for ligation, or a combination of blunt and sticky ends. These fragmented DNA could be end-repaired to have blunt ends on all molecules prior to ligation. However, this step adds an end-repair and a clean-up step (after end-repair) to the SMURF-seq protocol. Another option is to directly ligate the fragmented molecules even though they do not have compatible ends. As an example, after digestion with two restriction enzymes that generate different ends, the fragmented molecules can be of three types: The fragmented molecule could have both ends from

the first enzyme, both ends from the second enzyme, or one end from each of the enzymes. Ligation these molecules directly (i.e. without end-repair) would concatenate the two types of molecules that have the same ends within each type; the type of molecules that have two different ends would act as bridges between the molecules that have the same ends. Although, this protocol would have fewer steps, the efficiency of ligation could be reduced and would need to be evaluated.

The use of restriction enzymes or a combination of enzymes for fragmentation could lead to a bias in read counts for the downstream application. For example, in terms of copy number proofing, the number of restriction sites in a bin could vary, causing variation in bin counts that does not reflect the sequenced sample. The effect of the biases needs to be assessed based on the enzymes used and the downstream application.

Sequencing fragmented molecules with SMURF-seq

Some read-counting applications, such as Ribo-seq (Ingolia et al., 2009), involve extensive steps to produce the molecules of interest. These resultant DNA molecules from these protocols could already fragmented to length optimal for SMURF-seq. Other applications such as targeted-capture methods and whole-exome sequencing protocols typically produce molecules that are ~150-200 bp. Further, cell-free DNA molecules obtained from blood samples are of length ~150 bp, and are increasingly used in low-coverage copy number profiling (Adalsteinsson et al., 2017; Underhill et al., 2016).

Sequencing these molecules using SMURF-seq would require that these molecules have compatible ends for ligation, and the quantity of DNA is sufficient for nanopore sequencing. To meet these requirements, DNA molecules from these protocols could be PCR amplified by first dA-tailing and ligating adapters to bind the PCR primers. If these adapters are designed to have an appropriate restriction site in them, after amplification they could be removed with restriction digestion, leaving behind DNA molecules with sticky-ends as required for SMURF-seq (Fig 3.11).

Designing PCR adapters with restriction sites also provides an opportunity to insert an addi-

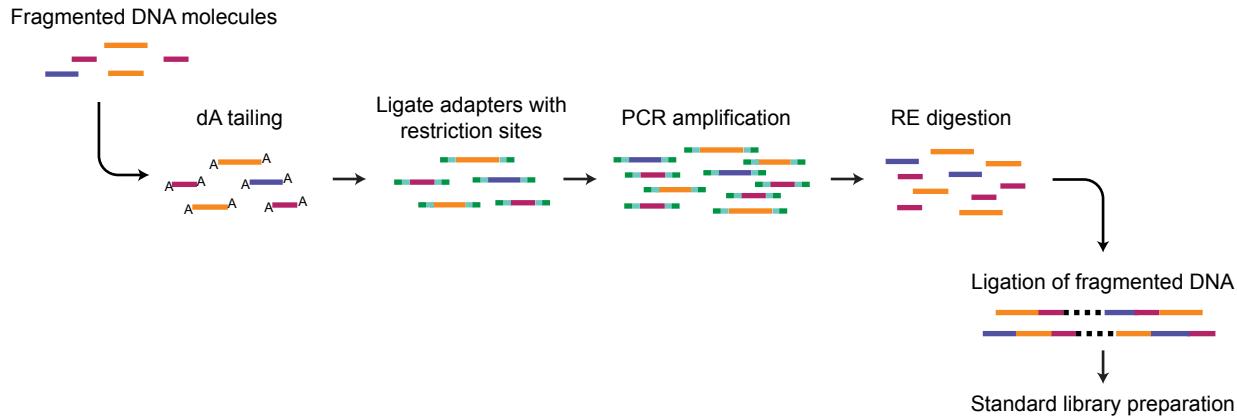


Figure 3.11: SMURF-seq protocol to sequence DNA molecules that are already fragmented. The light green bands on the adapters represent restriction sites. These molecules are amplified with an adapter containing a restriction site, followed by restriction digestion, and re-ligation for sequencing on long-read machines.

tional sequence in an adapter prior to restriction site. This sequence could be modified depending on the downstream application. For example, it could be a barcode sequence or a unique molecular identifier sequence.

However, SMURF-seq is still limited by the number of fragments produced from a run on the sequencing instrument. We believe that the evolution of SMURF-seq and the improvements to the underlying sequencing technology would expand the utility of SMURF-seq, enabling applications on nanopore instruments that are currently only possible with short-read high-throughput machines.

Chapter 4

Identifying fragment boundaries on a SMURF-seq read

4.1 Motivation

New sequencing methods motivate development of new algorithms for mapping and analysis of sequences generated using these methods. A few significant developments include BLAST (Altschul et al., 1990) and FASTA (Pearson and Lipman, 1988) motivated by database searches with the advent of Sanger sequencing, BWA (Li and Durbin, 2009) and Bowtie (Langmead et al., 2009) inspired by high-throughput short-read sequencing, and BLASR (Chaisson and Tesler, 2012) by single-molecule long-read sequencing. SMURF-seq has enabled efficient short-read sequencing for read-counting applications on portable long-read machines. However, efficient methods tailored for mapping SMURF-seq reads are still lacking; Especially as SMURF-seq protocol evolves and the fragments become shorter, and thus, making the mapping process challenging in terms of identifying accurate fragment locations and boundaries.

As currently implemented, SMURF-seq protocol uses a single restriction enzyme (SaqAI) to fragment DNA molecules to \sim 150 bp. However, depending on the downstream application, the

fragment lengths need to be just long enough to ensure unique mappability to a sufficient fraction of the genome. Fragments could be made shorter using methods discussed in section 3.6. As an example, for copy-number profiling (at low resolutions, as used for tumor samples) the fragment lengths could be as short as 40 bp.

We used BWA-MEM (Li, 2013) to align SMURF-seq reads generated with the current protocol, which consists of fragments that are typically over 100 bp. Though not designed to align SMURF-seq reads, BWA-MEM is designed for split read alignment, and it works sufficiently well at these fragment lengths. SMURF-seq reads can also be aligned with other mapping tools capable of split-read alignment such as Minimap2 (Li, 2018) and LAST (Kiełbasa et al., 2011). However, all of these tools are either designed for aligning short reads with low sequencing error or long reads with high sequencing error.

Aligning SMURF-seq reads, especially as the fragments get shorter, would differ significantly from these tools in the following aspects: (1) A seeding approach designed for short fragments sequenced with a high error-rate. (2) An approach to estimate the number of fragments on a SMURF-seq read and determine the optimal fragment boundaries.

The initial step of a typical alignment tool, called seeding, is to find candidate locations of a read on the reference genome, and limit the downstream steps to these locations. This is usually accomplished using a hash table based data structure to find exact matches (Altschul et al., 1990, 1997; Kent, 2002) or non-contiguous exact matches (Chen et al., 2009; Ma et al., 2002), or by using a suffix tree based data structure (Kurtz et al., 2004; Langmead et al., 2009; Li, 2013; Li and Durbin, 2009, 2010). The choice of data structure and parameters such as the length of the match, number of matches, etc. are determined by factors such as the read length and the error profile of the underlying sequencing technology. For example, the optimal seeds parameters for expressed sequence tags and whole genome sequencing (prior to short-read high-throughput sequencing) was determined in BLAT (Kent, 2002), similarly for low-error rate short-reads in RazerS (Weese et al., 2009), and high-error rate long-reads in BLASR (Chaisson and Tesler, 2012). The fragments could

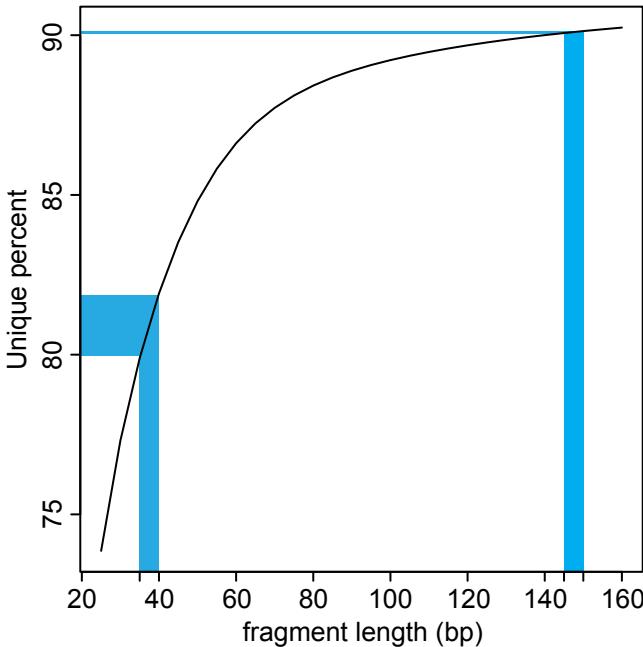


Figure 4.1: The fraction of genome that is uniquely mappable decreases with fragment length.

be as short as 40 bp in a SMURF-seq read, and aligning these reads requires identifying candidate locations for these fragments in the presence of the characteristic error profile of nanopore machines.

The other aspect of aligning a SMURF-seq read to split the read into its constituent fragments. As the fragments become shorter it becomes crucial challenging to determine the number of fragments and fragment boundaries on a read. As the fragments become shorter, the number of the fraction of the reference genome that is uniquely mappable would decrease. For the human reference genome (hg19), when allowing mismatches and indels are not allowed, the fraction of the genome that is uniquely mappable reduces by 0.06% when going from 150 to 145 bp, whereas it reduces by 2.02% when going from 40bp to 35bp (Fig. 4.1). The unique fraction of the genome decreases further when mismatches are allowed (Derrien et al., 2012). Due to shorter fragment, the probability of a fragment that originated from a unique location on the genome to misalign to an ambiguous location (or vice-versa) due to having incorrect fragment boundaries increases.

Thus, as the fragments become shorter, the odds of inaccurate fragment boundaries leading to misalignment of fragments or missing fragments entirely increases.

Although both seeding and determining the accurate fragmentation is crucial to align a SMURF-seq read, in this study we focus only on the second. To this end, we define the fragment identification problem for identifying the number of fragments and the fragment boundaries on a SMURF-seq read. We approach the fragment identification problem by defining a score function for aligning a SMURF-seq read, provide algorithms to align a read maximizing this score function, study the null score distribution of aligning reads and reference generated at random, and estimate the number of fragments in a SMURF-seq read by comparing its alignment score with the null distribution. Finally, we discuss the challenges and future directions to utilize this method for aligning real SMURF-seq reads to large reference genomes.

4.2 Background

In the early days of DNA sequencing, as the number of nucleotides sequenced grew, comparison of DNA sequences became an indispensable tool to a biologist. DNA sequence comparison can be broadly classified into global alignment (Needleman and Wunsch, 1970) and local alignment (Smith et al., 1981). A global alignment seeks an optimal alignment between two sequences such that each base of one sequence is aligned to each base of the other sequences. On the other hand, a local alignment seeks an optimal alignment between any subsequences of the sequences being compared.

Comparison of two sequences, even unrelated or random sequences, always produces an optimal alignment. This motivated the development of approaches to differentiate a “meaningful” alignment from alignment of unrelated sequences. These methods determine the significance of an alignment between two sequences by comparing the alignment score with a null distribution of alignment scores of unrelated sequences. Determining the appropriate null distribution was the

subject of an enormous amount of research, some of which are summarized below.

In the context of local alignment, at the time of the initial studies on the score distribution of unrelated sequences, mathematical tools to understand the null distributions were still lacking, and these studies relied on empirical distributions generated from aligning unrelated sequences. In (Smith et al., 1985), it is shown that the similarity score is proportional to the logarithm of the length of the sequences being compared, and the standard deviation is independent of the sequence length. The significance of an alignment was determined from the number of standard deviations over mean of the alignment score. These studies (Lipman et al., 1984) also highlight that the statistical properties (Smith et al., 1983), such as nucleotide frequencies or codon usage, of the sequences affect the distribution of the alignment scores. Generating a null distribution from an incorrect model could lead to an alignment of unrelated sequences being dubiously declared significant. Several methods are available to generate random sequences preserving these statistical properties (Altschul and Erickson, 1985; Fitch, 1983).

Erdos and Renyi (Erdös and Révész, 1975) presented results for the length of the longest headrun in a the first n tosses of a biased coin. The length of the longest headrun in coin tosses is equivalent to the number of matches between two DNA sequences when shifts in the starting and ending positions of the sequences are not allowed, with the probability of head equal to the probability of match between letters of the DNA alphabet. In (Arratia and Waterman, 1985), this is generalized to matches between DNA sequences, while allowing shifts. These results indicate that allowing shifts doubles the length of the longest headrun. Results for the longest headrun allowing for up to k mismatches and sequences generated from a Markov chain are also considered. In (Arratia et al., 1986; Gordon et al., 1986) the distribution of the longest matches is shown to have an extreme value distribution with mean that is proportional to the logarithm of the sequences lengths and variance independent of sequence length. Here, when considering only matches, the asymptotic extreme value distribution is shown by considering a maximum of geometric distributions, and when mismatches are allowed, it is shown by considering a maximum of negative binomial

distributions. An alternate approach is a Poisson approximation for the distribution of the longest match (Arratia et al., 1989).

A crucial aspect of aligning nucleic acid and protein sequences is using an appropriate score function. For example, PAM (Dayhoff et al., 1978) and BLOSUM (Henikoff and Henikoff, 1992) matrices are commonly used for protein sequences. The score function used alters the score of the aligned sequences and thus the alignment score distribution of unaligned sequences. However, the approach based on the length of the headruns does not consider the score function used for an alignment. In (Karlin and Altschul, 1990; Karlin et al., 1990), it is shown that the maximal score of aligning unrelated sequences using any score function (that has at least one positive score and the expected score is negative) takes the form of an extreme value distribution, and explicit formulas for its parameters are provided. Further, the number of high-scoring alignments is closely approximated by the Poisson distribution. Thus, enabling the calculation of the probability that an alignment of random sequences having a score greater than any given value (usually the score of aligning two sequences of interest).

These analytical formulas enable the calculation of parameters for ungapped alignment. However, these formulas do not hold for gapped alignments. Empirical studies showed that similar theory holds for gapped alignments as well (Altschul and Gish, 1996; Pearson, 1998; Smith et al., 1985). For gapped alignments, the parameters for the distribution are estimated empirically from the comparison of random sequences (Altschul and Gish, 1996; Waterman and Vingron, 1994a,b). These approaches are employed in database search tools such as BLAST (Altschul et al., 1990, 1997) and FASTA (Pearson, 1995; Pearson and Lipman, 1988) to determine the significance of matches of a query to a database of sequences.

Multiple sequence alignments of families of similar proteins can be summarized in position specific scoring table called a profile (Gribskov et al., 1987). It has been shown that the maximum alignment score of aligning a profile to a genome generated at random is well approximated by an extreme value distribution (Goldstein and Waterman, 1994).

4.3 Fragment Identification problem

A SMURF-seq molecule consists of short fragments of DNA from distinct regions of the genome concatenated into a longer molecule. After sequencing, aligning these reads requires splitting them into their constituent fragments, each aligning to a distinct region on the genome. Here, we define the fragment identification problem for identifying the number of fragments and the fragment boundaries on a SMURF-seq read.

Let Σ be an alphabet. A string X is a sequence of letters $a_0a_1\dots a_{n-1}$, where $a_i \in \Sigma$; $|X|$ denotes the length of the string X ; and $X[i\dots j] = a_i\dots a_{j-1}$ is a substring of X .

The reference string T is generated from the DNA alphabet $\Sigma = \{A, T, G, C\}$, with $|T| = n$. A SMURF-seq read S is generated by concatenating substrings (called fragments) of T , with no information available *a priori* about the number, length, orientation (forward or reverse-complement), and the position on T of these fragments. Further, S contains sequencing errors with a rate ρ . Let $|S| = m$ and $m \ll n$.

A fragment set P is an set of start locations of fragments on S . $P \subset \{0\dots m-1\}$ and $|P| = k$, with the rule that 0 is in P always. By convention we consider the set P to be ordered such that if $i < j$ then $P_i < P_j$. For a fragment set P , $\sum_{i=1}^k P_{i+1} - P_i = m$ and we say that the i^{th} fragment of S is the substring $S[P_i\dots P_{i+1}]$, with $P_{k+1} = m$.

For a given T and S , the fragment identification problem is to determine the elements of the fragment set P such that it corresponds to the start locations of fragments contained in S .

4.3.1 Approach to the fragment identification problem

We approach the fragment identification problem by defining a score function as follows: For a given fragment set P , we define the score of aligning S to T as:

$$score_T(S, P) = \sum_{i=1}^k \max\{score(T[u\dots v], S[P_i\dots P_{i+1}]) : 0 \leq u < v \leq n\}.$$

This allows us to consider the fragment identification problem as two inter-related problems: (1) Determining k , the size of the fragment set, and (2) given k , determining the elements of P such that $score_T(S, P)$ is maximized.

By the score function defined above, determining the elements of the fragment set P requires the knowledge of the number of fragments k , and this is not known *a priori*. Further, the k that maximizes the score function would almost never correspond to the optimal fragment set. As an example, taking $k = m - 1$ which corresponds to taking each base as a fragment would maximize the score, however, this is a non-sensical alignment.

We propose to estimate the number of fragments k by aligning a SMURF-seq read to the reference genome with different k . For each of these fragmentations, we determine the p-value by comparing the alignment score with the null distribution generated from aligning reads generated at random to a reference genome generated at random. Finally, we choose the fragmentation with lowest p-value as the optimal fragmentation. The following sections describe each of these steps in detail.

The fragment identification problem differs from the alignment problems described in section 4.2 in a crucial manner. For the fragment identification problem we have the reference genome, and it is assumed that the reads always arise from this genome; the score distribution of sequences generated at random is used to determine the optimal number of fragments on a SMURF-seq read. Whereas, in the context of local alignment the score distribution of aligning random reads are used to determine a “meaningful” alignment by comparing the alignment score of sequences with the random null distribution.

4.4 Identifying fragment boundaries on a SMURF-seq read

A SMURF-seq read of length m can be fragmented into $k = 1$ to $k = m - 1$ fragments. For each of these fragmentations, the $k - 1$ fragment boundaries on the read can be at any of the $O(m/k)$

positions. Aligning a SMURF-seq read requires identifying both the number of fragments k and the fragment boundaries such that the alignment score is maximized.

In this section we present algorithms for identifying the optimal fragment boundaries for any given value of k . First, an algorithm that does not allow for mismatches or indels is described, and then an algorithm for a generalized score functions. Limitations of these algorithms and how the optimal fragment boundaries can be identified on a SMURF-seq read in practice are then presented.

4.4.1 Fragment boundary identification under exact matching

We first examine the fragment boundary identification problem assuming the score function requires exact matching

$$score(a, b) = \begin{cases} 1 & \text{if } a = b \\ -\infty & \text{otherwise.} \end{cases}$$

The fragment identification problem then becomes an exact matching problem where the goal is to minimize the number of fragments such that $score_T(P, S)$ is maximized.

A simple linear time solution to this problem can be obtained as follows. First, we assume some data structure for T has been constructed in linear time and allows for longest prefix matches to be computed in time proportional to the length of the query string. The data structure could be a suffix tree (McCreight, 1976), or a more space efficient and a modern structure like an FM-index (Ferragina and Manzini, 2000). The principle of the algorithm can be seen by starting at the beginning of S , and identifying the longest prefix match of S in T . Then retain j as the first position of where this longest prefix matches in T , and denote the first mismatching position on S as i . Repeat the procedure solving the subproblem of fragment identification for $S[i \dots m]$. Repeating these steps, the algorithm iteratively solves the longest prefix match problems, retaining as P_{i+1} the position of mismatch that terminates matching during iteration i . The following pseudocode describes the procedure.

Algorithm 1 ExactFragmentMatching(T, S):

```
1:  $i \leftarrow 0$ 
2: while  $i < m$  do
3:    $P \leftarrow P \cup \{i\}$ 
4:    $i \leftarrow \text{longest-match-length}(S, i, T)$ 
5: return  $P$ 
```

Proof: Consider an optimal solution to this problem, where the identified fragment set P_{opt} has minimal size. To prove the optimality of our algorithm we need to show that it finds the same number of fragments as the optimal solution, i.e. $|P| = |P_{\text{opt}}|$.

The first iteration of the greedy algorithm will find the longest prefix match. If the optimal solution has its first fragmentation ending before P_1 , i.e. $P_{\text{opt}1} < P_1$. Then the longest match starting at $P_{\text{opt}1}$ will end at or before P_2 , the end of the second fragment found by the greedy algorithm. If it ends at P_2 then the greedy algorithm has the same number of fragments as the optimal solution so far. And it cannot end before P_2 , because then the optimal solution will have more fragments than found by the greedy algorithm. Moreover, we cannot have $P_{\text{opt}1} > P_1$ as this would imply a longer prefix than found by the longest prefix match exists. With this reasoning we can say that this greedy approach will find just as little fragments as the optimal solution.

4.4.2 Fragment boundary identification allowing mismatches and indels

Now we examine fragment boundary identification for a generalized score function allowing mismatches and indels. A dynamic programming approach is presented. This algorithm is based on and is inspired by the local alignment algorithm (Smith et al., 1981) and the local alignment with inversions problem (Schöniger and Waterman, 1992).

Let M denote a table with $m+1$ rows, $n+1$ columns and $k+1$ dimensions, where k is the maximum number of fragments ($1 \leq k \leq m$). $\max_{0 \leq j \leq n} M(i, j, l)$ represents the best fragmentation of $S[1 \dots i]$ with l fragments. The entries of M are computed as follows:

Algorithm 2 FragBoundaryIdentification(T, S, k)

```

1:  $M(i, j, 0) \leftarrow -\infty$  for all  $0 \leq i \leq m, 0 \leq j \leq n$ 
2:  $M(0, j, 1) \leftarrow 0$  for all  $0 \leq j \leq n$ 
3:  $M(i, 0, 1) \leftarrow M(i - 1, 0, 1) + score(S[i], -)$  for all  $0 \leq i \leq m$ 
4:  $M(l - 1, j, l) \leftarrow -\infty$  for all  $2 \leq l \leq k, 0 \leq j \leq n$ 
5:  $M(i, 0, l) \leftarrow -\infty$  for all  $2 \leq l \leq k, l \leq i \leq m$ 
6: for  $l \leftarrow 1$  to  $k$  do
7:   for  $i \leftarrow l$  to  $m$  do
8:     for  $j \leftarrow 1$  to  $n$  do
9:       
$$M(i, j, l) \leftarrow \max \begin{cases} M(i - 1, j - 1, l) + score(S[i], T[j]) \\ M(i - 1, j, l) + score(S[i], -) \\ M(i, j - 1, l) + score(-, T[j]) \\ \max_{0 \leq h \leq n} M(i - 1, h, l - 1) + score(S[i], T[j]). \end{cases}$$


```

Time and space complexity: Each entry of M is computed in constant time by storing the value of $\max_{0 \leq j \leq n} M(i - 1, j, l - 1)$ for every row of M in a separate array. The algorithm runs in $O(knm)$ time and uses $O(knm + km)$ space, where the additional $O(km)$ is used to store the values of $\max_{0 \leq j \leq n} M(i - 1, j, l - 1)$.

The optimal alignment and fragment boundaries are determined from the usual traceback procedure starting from $\max_{1 \leq j \leq n} M(m, j, k)$ and ending in $M_{1 \leq j \leq n}(1, j, 1)$, with the exception of storing if a new fragment maximized the score at a cell.

Intuitively, this algorithm is similar to the local alignment algorithm but instead of picking an empty alignment when the score of an extension is negative, this algorithm starts a new fragment when the score of extending a fragment is less than score of starting a new fragment. In terms of an alignment graph, each node has a zero-weight incoming edge from the node corresponding to $\max_{0 \leq j \leq n} M(i - 1, j, l - 1)$, in addition to the weighted match/mismatch and indel edges (Fig. 4.2).

Although this algorithm provides the exact solution to determine optimal fragment boundaries for each k , it has several limitations for use with real SMURF-seq reads in practice: (1) This algorithm always starts at $k = 1$, and for each k builds an alignment based on the best fragmentation of $k - 1$. However, in practice the approximate number of fragments and the fragment boundaries

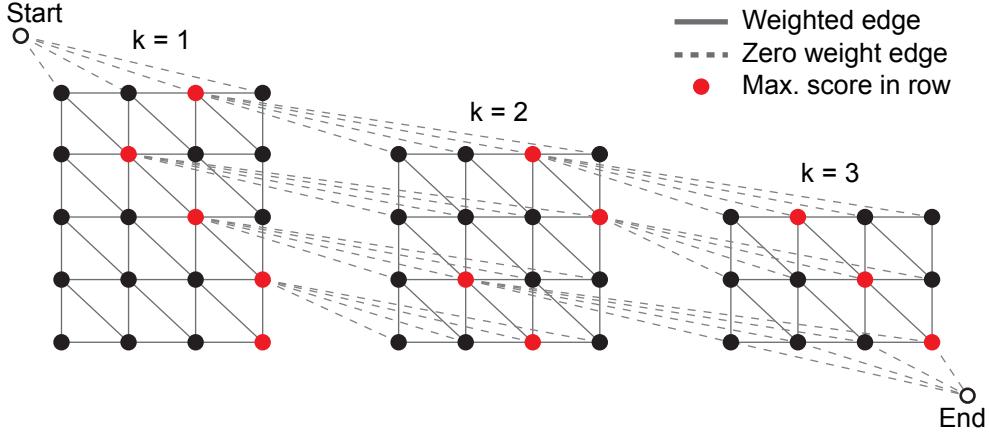


Figure 4.2: Alignment graph for fragment boundary identification algorithm with an arbitrary score function. The direction of arrows are omitted for clarity. The horizontal edges are directed from left to right and all other edges are directed from top to bottom.

are expected to be known after the initial seeding step, and starting at $k = 1$ is inefficient. (2) The time and space complexity is much too large to be used with any reference genome. Further, after initial seeding, the approximate locations of fragments on the genome would also be known, which is not taken into consideration here to reduce the time or space. (3) This algorithm does not allow for a separate gap open cost for affine gap alignments. (4) Not all bases of a SMURF-seq read can be expected to align to a reference genome, or fragments on a read could align equally well to multiple locations.

4.4.3 Identifying fragment boundaries in practice

The initial step in any traditional mapping algorithm is to determine the approximate mapping locations of the read on the reference genome using a seeding approach. Some algorithms further generate proto-alignments using the initial seeds. For example, BWA-MEM joins seeds that are close to on the read and reference coordinates and have the same orientation into a “chain” (Li, 2013). Typically, the final step is to perform a Smith-Waterman alignment between the read and a small region on the reference genome.

Any algorithm to aligning SMURF-seq reads can be expected to have similar steps. After seeding a SMURF-seq read, seeds from different fragments on a read would cluster at different locations on the genome; likely several different location for each fragment due to repeats or seeding parameters (such as seed length). These seed hits could be further processed to generate preliminary alignments between parts of the read and the reference genome. Thus, after these steps it is reasonable to assume that approximate number of fragments and the boundaries of these fragments are known.

The final step of the algorithm would be finalize the number of fragments and fragment boundaries on a read. For a given number of fragments, the fragment boundaries can be easily determined such that the alignment score is maximized (this problem is equivalent to finding a longest path in a single-source directed acyclic graph). For finding the number of fragments on a SMURF-seq read, for each fragmentation, we use the alignment score distribution of random sequences with the same fragment set to determine the p-value for a fragmentation using the procedure described in the next section.

4.5 Alignment score of a SMURF-seq read

The alignment score of a SMURF-seq read is defined as the sum of the alignment score of each of individual fragment for a particular fragmentation with k fragments, and algorithms given in section 4.4 determine the best fragmentation so that the alignment score is maximized.

The alignment score of a SMURF-seq read is a non-decreasing function with the number of fragments, as the score function does not penalize for increasing the number of fragments on a read. Thus, a SMURF-seq read aligned with k fragments can always be aligned with $k + 1$ fragments with a score at least as high as with k fragments.

As an example of how the alignment score of a SMURF-seq read grows as a function of k , consider a read consisting of f fragments (typically > 20 for a SMURF-seq read), however, f is

not known *a priori*. If the read is aligned to the reference genome as one fragment, it is likely going to align to a random location on the reference genome; Alternatively, one of the fragments in the read could align close to its true location, and the flanking fragments would align to flanking regions on the genome (essentially aligning these flanking fragments to a random location on the genome). Similarly, if the read is aligned as two fragments, it is likely going to align to two random location on the genome, or at most two fragments could align to regions close to their true location. With the alignment score for two fragments at least as high as with one fragment. As the read is aligned with increasing k up to $f - 1$, the number of the number of bases on a read that are aligned to its true location on the genome would increase, and number of based aligned to random location would decrease. Although there will always bases that are not mapped to its true location. At $k = f$ all the fragments on the read would be aligned to their true locations with no bases aligned to random locations. As k increased beyond f , each fragment will be split into shorter fragments, the fragmentation location likely being at locations of sequencing errors (with a small increase in the alignment score). And if there are no more sequencing errors, the split can be anywhere on the read, with the alignment score remaining the same. At $k = m$, i.e. each base on the read aligns as one fragment, the alignment would have the highest possible score.

Consider a simulated SMURF-seq read from a genome of length 50 kb, the read has 20 fragments each of length 40 bp, and the read does not have any sequencing errors. Fig. 4.3a shows the alignment score as a function of the number of fragments k , with scoring a match as 1, a mismatch as 0, and not allowing indels. At $k = 1$, the score is at the lowest, and increases with k . At $k = 20$ every fragment is mapped to it true location, and since the read does not have any sequencing errors, the score is equal to the read length. For $k > 20$, the score remains at the maximum.

When a SMURF-seq read has sequencing errors, the alignment score increases with the number of fragments, but would not reach the peak at $k = 20$ (Fig 4.3b; the SMURF-seq read is similar to 4.3a, but with 10% mismatch errors). The alignment score continues to increase beyond $k = 20$ but at a lower rate, and would eventually equal the read length as the number of fragments increase.

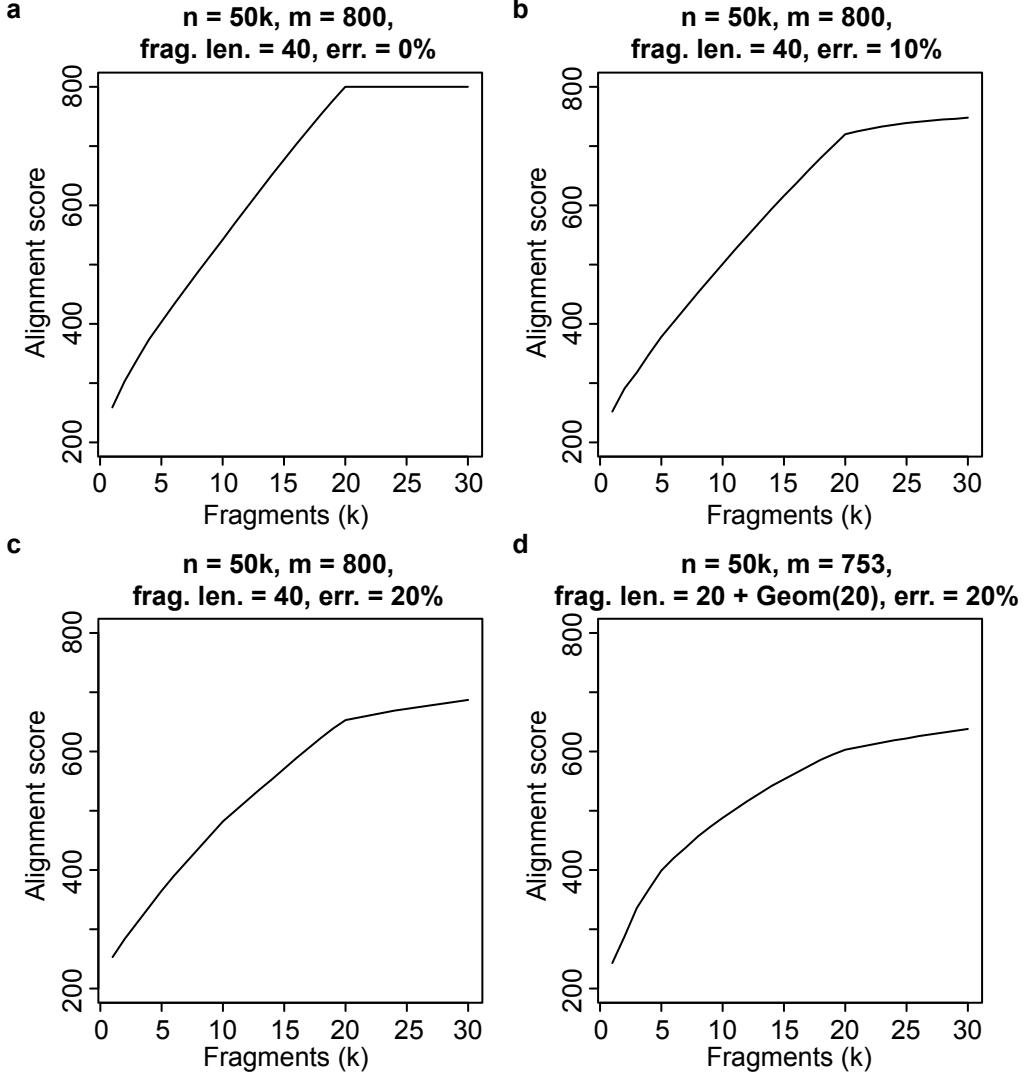


Figure 4.3: Alignment score of SMURF-seq read as a function of number of fragments. (a) Alignment score of a SMURF-seq read with 20 fragment (40 bp each) that does not have any errors. (b) Alignment score with 10% errors. (c) Alignment score with 20% errors. (d) Alignment score of a read with 20 fragments generated from $20 + \text{Geom}(20)$ distribution and with 20% errors.

Similarly, Fig. 4.3c shows the alignment score for a read with 20% mismatch errors. However, the due to higher errors, the score is lower at $k = 20$, and the curve “flattens”.

For a real SMURF-seq read, the fragment lengths cannot be expected to be constant. Fig. 4.3d shows the increase in alignment score of read with 20 fragments, with each fragment length sampled from a $20 + \text{Geom}(20)$ (mean fragment length of 40 bp) distribution. The alignment score has

increase, but the change in slope at $k = 20$ is not prominent.

4.6 Score distribution under a random model

Calculation of p-value for aligning a SMURF-seq read with a given fragmentation requires the null distribution of aligning reads generated at random with the same with the same fragmentation. The problem of finding the null distribution is defined as follows: consider strings T and S are generated by drawing letters independently from the same distribution from an alphabet $a \in \Sigma$ with probability p_a such that $\sum_{a \in \Sigma} p_a = 1$. For a given fragment set P containing k elements, we need to determine the distribution of $score_T(S, P)$. We use the following score function to obtain the distribution of $score_T(S, k)$:

$$score(a, b) = \begin{cases} 1 & \text{if } a = b \\ 0 & \text{if } a \neq b \\ -\infty & \text{otherwise.} \end{cases}$$

To determine the distribution of $score_T(S, P)$, we first consider the score distribution when $k = 1$, i.e. the entire read aligns as one fragment. Then, we consider the score distribution when $k > 1$ as the sum of $k = 1$ distributions.

4.6.1 Score distribution of one fragment

The score distribution of $score_T(S, 1)$ has similarities to the score distribution of local alignment scores (Altschul and Gish, 1996; Smith et al., 1983) and profile alignment scores (Goldstein and Waterman, 1994), but also differs from these. The distribution of $score_T(S, 1)$ differs from the local alignment as we require an end-to-end alignment of S to a substring of T , and also differs from the profile score distribution since the letters of S are generated at random. Based on these

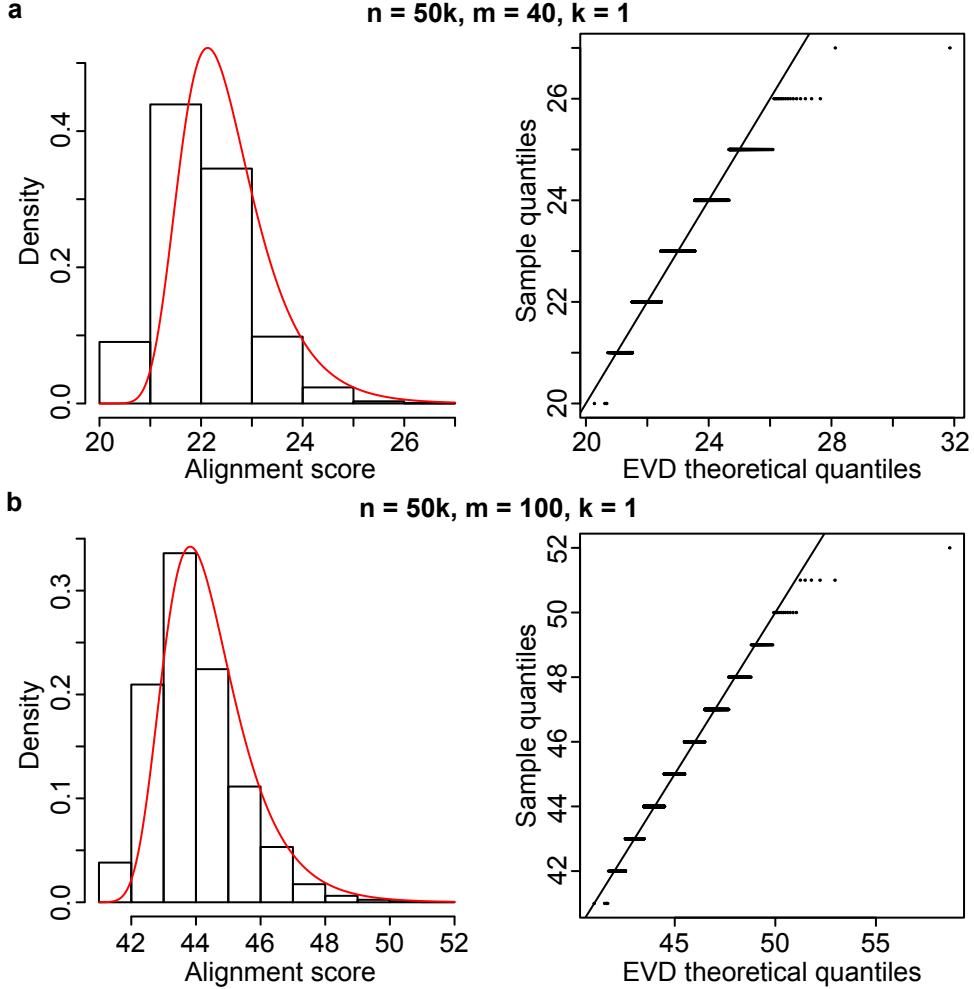


Figure 4.4: Extreme value approximation for $score_T(S, 1)$. Empirical score distribution of $score_T(S, 1)$ with a fitted EVD using the method of moments estimator. Q-Q plot comparing the theoretical and empirical distributions are shown. (a) $m = 40$. (b) $m = 100$.

results, we hypothesized that the distribution of $score_T(S, 1)$ is likely to follow an extreme value distribution.

Let X_j denote the score of aligning S with $T[j \dots j + m - 1]$, a substring of T starting at j , then

$$X_j = \sum_{i=0}^{m-1} score(S[i], T[j+i]), j = 0, \dots, n - m + 1.$$

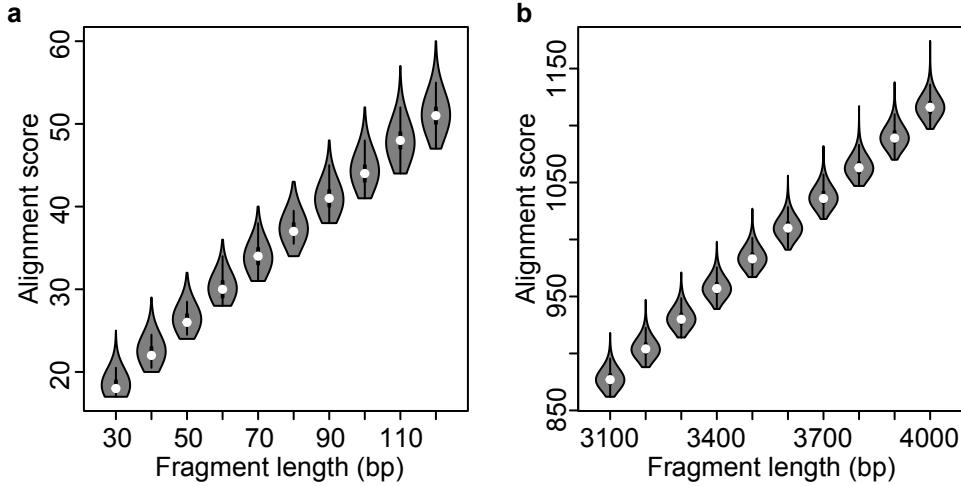


Figure 4.5: Empirical score distribution approximation for $score_T(S, 1)$. (a) Empirical score distribution for m corresponding to shorter fragments. (b) Empirical score distribution form m corresponding to longer fragments.

Since the letters of T and S are iid, we have

$$X_j \sim binom(m, p)$$

where $p = \sum_{a \in \Sigma} p_a^2$. For a large enough m , X_j can be approximated by a normal distribution as

$$X_j \sim N(mp, mp(1 - p)).$$

$score_T(S, 1)$ is the maximum score over all positions in T ,

$$score_T(S, 1) = \max_{0 \leq j \leq n-m+1} X_j.$$

$score_T(S, 1)$ is a maximum of normal distributions, which follows an extreme value distribution (EVD) (Kotz and Nadarajah, 2000).

We verified that score distribution of $score_T(S, 1)$ is well approximated by a extreme value

distribution by generating a random genome of length 50kb from the DNA alphabet with equal probabilities, and reads of length 40 bp and 100 bp. For each read length, we determined the score distribution by aligning 5,000 reads generated at random (Fig. 4.4a, b). The parameters for the EVD was estimated using the method of moments. Further, the score distribution for increasing read lengths shows an increasing trend in the mean and standard deviation of the distribution (Fig. 4.4c, d).

4.6.2 Score distribution for a given fragment set

The distribution of $score_T(S, k)$ for $k > 1$ and a given P is the sum of k independent distributions of $score_T(S, 1)$, i.e the distribution of $score_T(S, k)$ is the sum of k independent extreme value distributions

$$score_T(S, k) = \sum_{i=1}^k score_T(S[P_i \dots P_{i+1}], 1).$$

The independence of the distributions for each fragment is justified because it is required that $n >> m$, and the probability of two fragments to aligning to overlapping location on T is extremely small.

The distribution of the sum and linear combination of extreme value distributions has been studied (Cetinkaya et al., 2001; Loaiciga and Leipnik, 1999; Marques et al., 2015; Nadarajah, 2008). In (Loaiciga and Leipnik, 1999) the exact distribution of two independent Gumbel distributions is given and in (Nadarajah, 2008) the exact distribution of the linear combination of Gumbel distributions is given. However, these distributions do not follow a “standard” distribution.

Since each the distribution of score of each fragment is independent, when the fragments are of equal lengths, the distribution of $score_T(S, k)$ is a sum of i.i.d. random variables. Thus, we can apply the central limit theorem to approximate the score distribution to a normal distribution as $k \rightarrow \infty$. To test the convergence to $score_T(S, k)$ to normal, we compared the empirical score distribution to the normal distribution for $k = 20$ and 40 , values that are typical for a SMURF-seq read (Fig. 4.6). The fragments lengths for all the comparisons were kept constant at 40 bp, and the

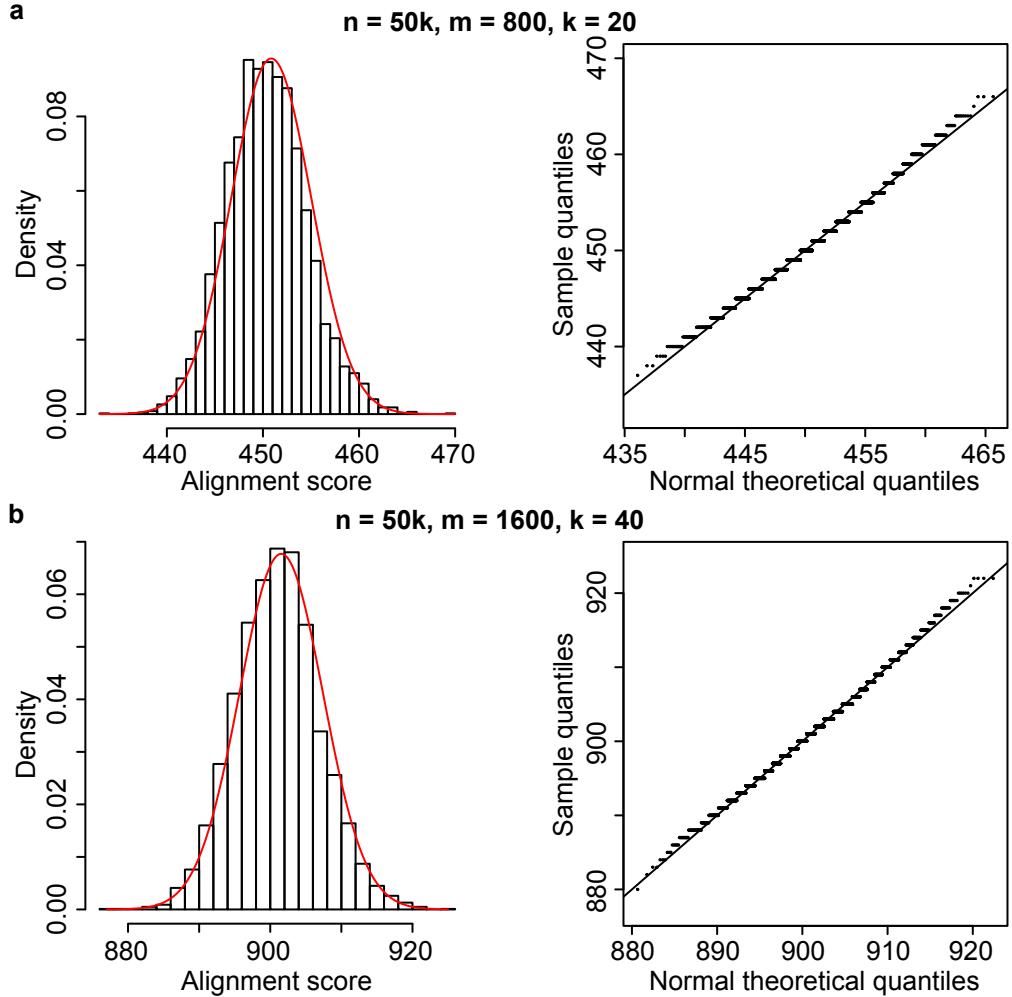


Figure 4.6: Normal approximation for $score_T(S, k)$ with equal fragment lengths. Empirical score distribution of $score_T(S, k)$ with a fitted normal using the method of moments estimator. All fragments are 40 bp. Q-Q plot comparing the theoretical and empirical distributions are shown. (a) $m = 800, k = 20$. (b) $m = 1600, k = 40$.

parameters for the normal distribution was determined using the method of moments estimator.

In aligning a SMURF-seq read, we cannot expect the fragment lengths to be equal. The distribution of $score_T(S, k)$, when the fragment lengths differ, is a sum of independent, but not identical, random variables. We empirically verified that this distribution converges to normal (Fig. 4.7). For each k , the fragment lengths were generated from at random from a geometric distribution.

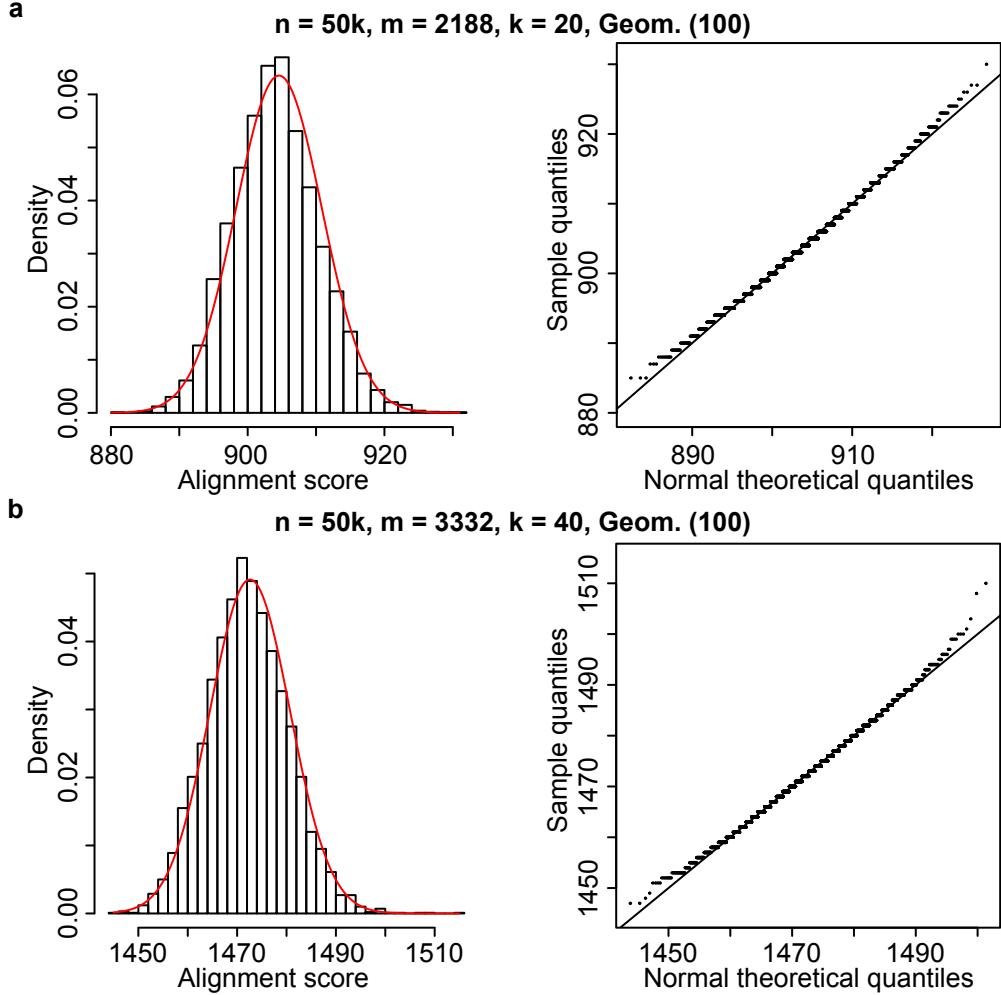


Figure 4.7: Normal approximation for $score_T(S, k)$ with random fragment lengths. Empirical score distribution of $score_T(S, k)$ with a fitted normal using the method of moments estimator. The fragment lengths are generated from a geometric distribution with mean 100. Q-Q plot comparing the theoretical and empirical distributions are shown. (a) $m = 2188, k = 20$. (b) $m = 3332, k = 40$.

4.7 Estimating the optimal fragment set

The score distribution of aligning a random read S_{rand} to a random genome T_{rand} can be used to estimate the optimal k for aligning a SMURF-seq read S_{SMURF} to a reference genome T_{ref} . For a SMURF-seq read, find the best alignment score k_{score} and the fragment set k_P for all k from 1 to m using algorithms given in section 4.4. In practice, k can be restricted to a much smaller subset

of possible values, and the goal is to determine a value of k that best represents the fragments that were ligated to generate the read.

As described in the previous section, the null score distribution of aligning a read generated at random for $k = 1$ follows an extreme value distribution, and a normal distribution for sufficiently large k . For each k , the parameters for the null distributions can be estimated from the empirical distribution by aligning random reads with the fragment set k_P . The p-value for each k can be determined by finding the probability of a score greater than k_{score} from the null distribution. Finally, the optimal k for a read is the one with the lowest p-value (Algorithm 3).

Algorithm 3 OptimalK (T, S)

```

1:  $k_{\text{opt}} \leftarrow 1$ 
2:  $\Pr_{\text{opt}} \leftarrow 1$ 
3: for  $k \leftarrow 1$  to  $m - 1$  do
4:    $k_{\text{score}}, k_P \leftarrow \text{FragMatch}(T_{\text{ref}}, S_{\text{SMURF}}, k)$ 
5:    $k_{\Pr} \leftarrow \Pr(score_{T_{\text{rand}}}(S_{\text{rand}}, k_P) > k_{\text{score}})$ 
6:   if  $k_{\Pr} < \Pr_{\text{opt}}$  then
7:      $\Pr_{\text{opt}} \leftarrow k_{\Pr}$ 
8:      $k_{\text{opt}} \leftarrow k$ 
9: return  $k_{\text{opt}}$ 
```

As discussed in section 4.5, as k increases up to k_{opt} the number of bases on a SMURF-seq that are aligned to its true location on the reference genome would increase and the bases aligned to random locations would decrease. Thus, getting further away from a random alignment with an expected decrease in p-value. At $k = k_{\text{opt}}$, all bases are aligned to their true locations, and would have the lowest p-value. As k gets farther away from k_{opt} , fragments on a read are further split into smaller fragments with a small increase in alignment score; eventually, with fragment aligning to random locations on the reference, and thus, getting closer to a random alignment with an expected increase in p-value.

As an example, we simulated a reference genome of length 50 kb with the DNA alphabet having equal probabilities. A simulated SMURF-seq read with 20 fragments each of length 40 bp was generated from this genome, and 10% mismatch sequencing errors were introduced. This

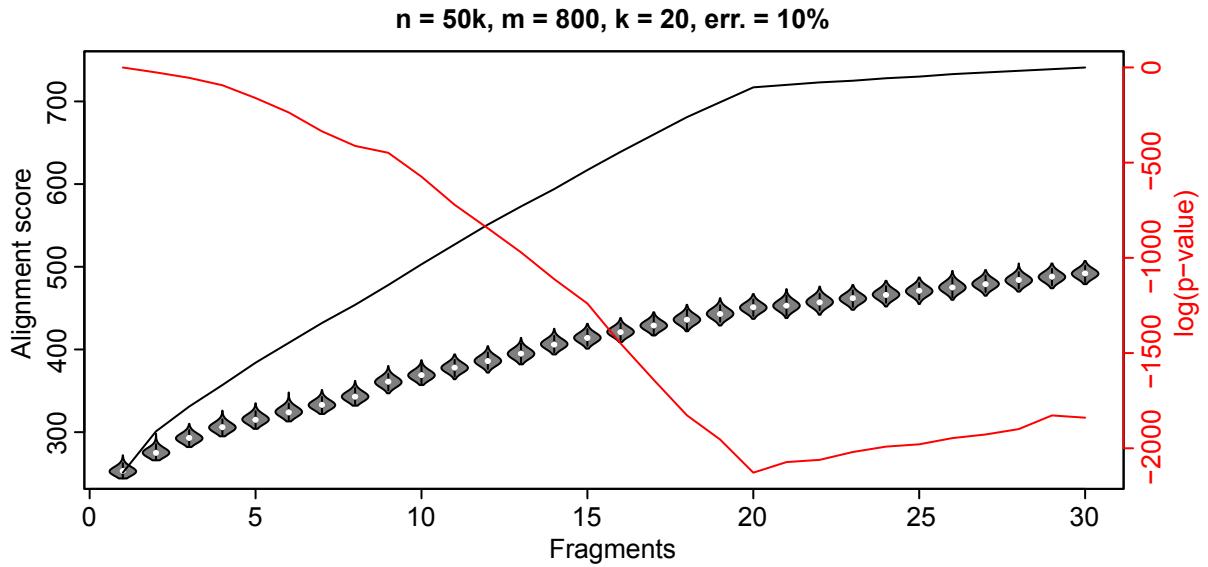


Figure 4.8: Determining the optimal fragmentation of a SMURF-seq read. The black line is the alignment score of a simulated SMURF-seq read with 20 fragments of 40 bp each, and with 10% mismatch errors. The violin plots are the empirical null distributions using fragment set corresponding the best alignment score for each k . The red line is the p-value for each k determined from the alignment score and the null distribution. The optimal fragmentation has the lowest p-value.

read was then mapped back to the reference genome for values of $k = 1$ to 30 using algorithm 2 (scoring a match as 1, a mismatch as 0, and not allowing indels), yielding the fragment set that maximizes the alignment score for each k . These fragment sets were then used to generate the null distribution by simulating a random reference genome with the same base probabilities, and aligning 10,000 random reads with fragment start locations based on the fragment set. The p-value for each fragmentation was determined using an EVD for $k = 1$ and normal distributions for $k > 1$ with parameters estimated using the method of moments from the simulated reads (although the normal approximation does not hold for small values of k , we have included it for clarity). The fragmentation with the smallest p-value was considered as the optimal fragmentation, and as expected, $k = 20$ has the lowest p-value; With the p-value increasing on either side of $k = 20$ (Fig. 4.8).

The example considered here is simplified to illustrate the procedure to determine the optimal fragmentation of a SMURF-seq read. In aligning real SMURF-seq reads: (1) The reference genome would be significantly larger (e.g. the human genome) with different base (or dinucleotide) probabilities. (2) The fragments lengths cannot expected to be constant. (3) The sequencing error model will depend on the properties of the sequencing technology used. (4) The alignment score used would depend on the sequencing error model and would allow indels (with possibly different penalties for gap open and gap extend). (5) Parameters for the null distribution were determined empirically, which cannot be done in practice. Some of these issues are considered in the following sections.

4.7.1 Fast computation of p-values

When aligning a SMURF-seq read, p-values need to calculated for a read to determine an optimal fragmentation among the potential fragmentations. It is thus important to be able to determine the p-value using an efficient procedure requiring the least computation. However, the procedure used above requires generating an empirical distribution for each fragmentation to determine the parameters for the null distribution. This process is computationally intensive and cannot be used in practice.

For any $k > 1$, the score distribution is the sum of k independent extreme value distributions. An approach for fast computation of p-values is to compute the mean and standard deviation for the $k = 1$ distribution for all possible values of fragment lengths (i.e. 1 to maximum possible read length); Then the mean and standard deviation for the normal distribution, for any fragmentation with $k > 1$ fragments, can be calculated from the sum of the mean and variance of the $k = 1$ distributions corresponding to the fragment set. As an example, for a fragmentation with three fragments of length 40, 100, 70 bp, the parameters for the normal distribution can be calculated as the sum of the mean and variance of the distribution of aligning 40 bp, 100 bp, 70 bp fragments individually to a random genome.

Thus, for a reference genome and a given score function, the parameters for the $k = 1$ distribution need to be determined just once. The parameters for the null distribution for any fragmentation can be computed by looking at the table and simply adding the values. We tested the effectiveness of this procedure by computing the parameters for a genome of length 50 kb generated at random. Figure 4.9 compares null distribution generated by aligning a 100,000 random reads and the null distribution computed as the sum of $k = 1$ distributions.

4.8 Limitations and future directions

As SMURF-seq evolves, the fragments can be expected to get shorter challenging the existing mapping tools. A crucial element of aligning a SMURF-seq read is to determine the number of fragments on a SMURF-seq read and to determine the optimal fragment boundaries on a SMURF-seq read. To this end, we defined a score function for a SMURF-seq read, suggested algorithms to find fragment boundaries, and provided a statistical procedure to estimate the number of fragments on a read. However, there are still several questions that remain to be answered in this regard, both in terms of using this procedure for real SMURF-seq reads and terms of understanding the score distribution of aligning SMURF-seq reads. Some of these are discussed in the sections below.

Aligning with a general score function

In the analysis of aligning a SMURF-seq read and the random distributions, we used a simple score function of scoring a match as 1, a mismatch as 0, and not allowing indels. However, such a score function is too simplified for use in practice. A score function for aligning a real SMURF-seq read would depend on several factors such as the error profile of the sequencing technology used and the algorithms used to align these reads.

When scoring a mismatch with a negative penalty and allowing indels X_j , which denotes the score of aligning a read S to a substring of the reference $T[j \dots j + m - 1]$, will not follow a

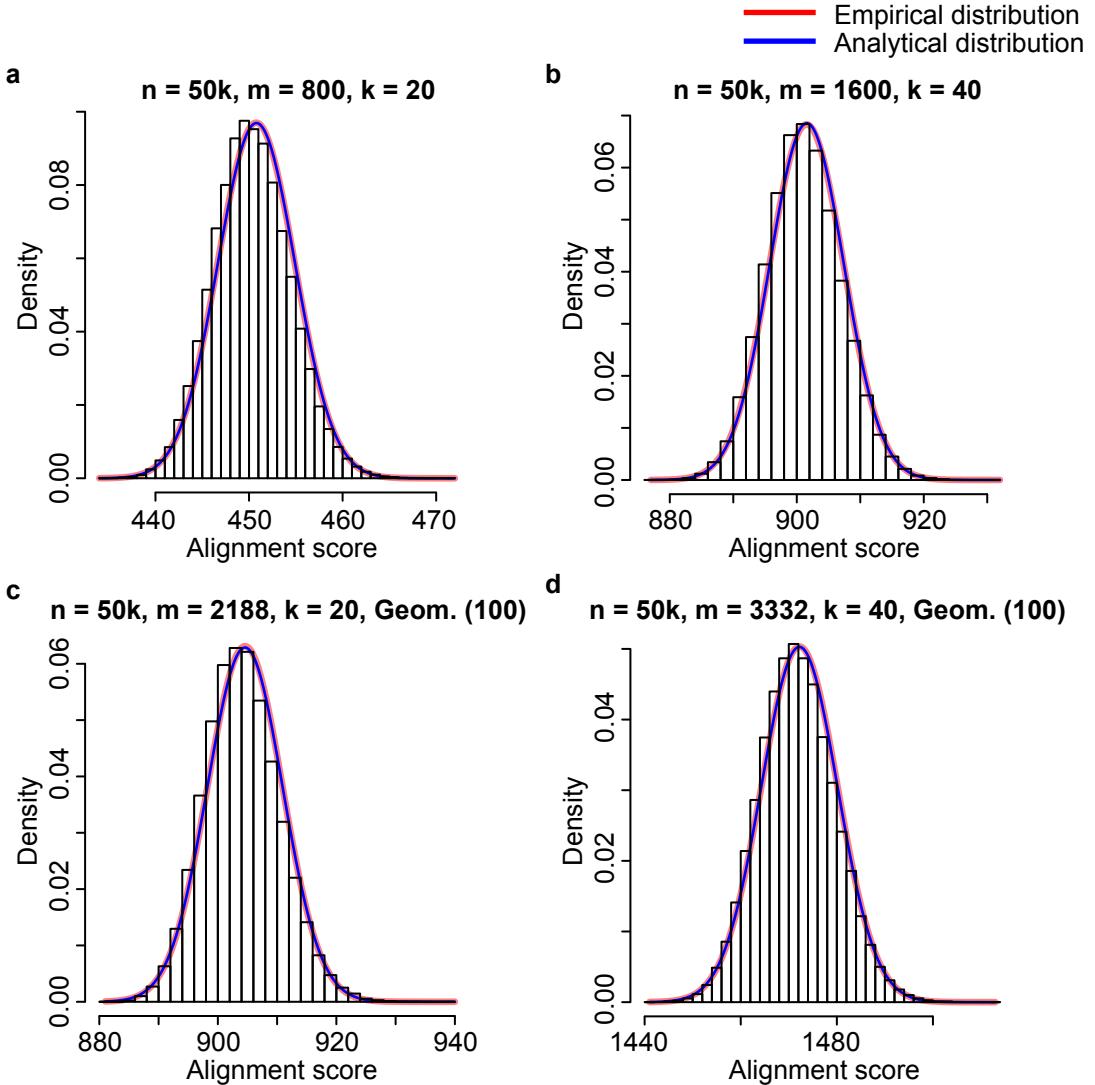


Figure 4.9: Fast computation of p-values as the sum of mean and variance of the k distributions corresponding to the fragment set. (a-b) Reads with $k = 20$ and $k = 40$ fragments of constant 40 bp length. (c-d) Reads with $k = 20$ and $k = 40$ fragments of length generated at random from a *Geom.(100)* distribution.

binomial distribution. The extreme value distribution can be used to approximate the maximum of any independent and identical distributions (i.e. not just the normal distribution) (Kotz and Nadarajah, 2000). Thus, we hypothesized that

$$score_T(S, 1) = \max_{0 \leq j \leq n-m+1} X_j$$

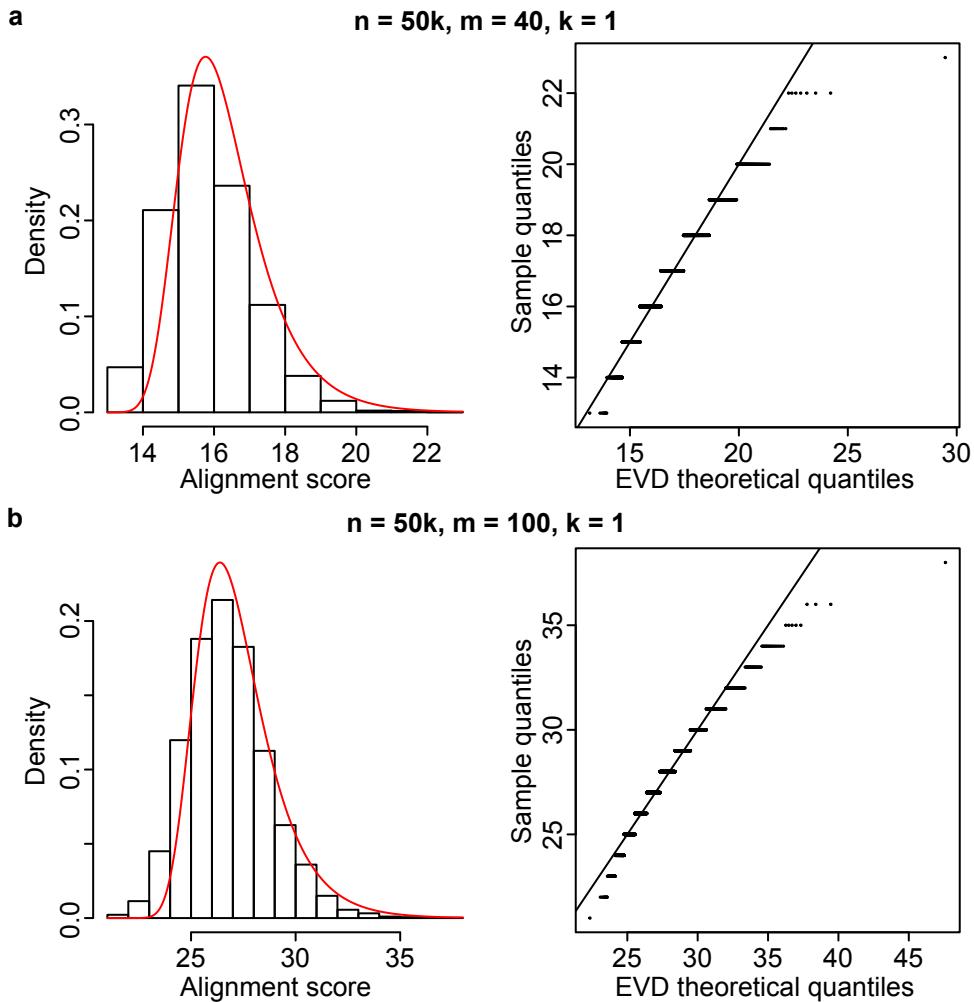


Figure 4.10: Extreme value approximation for $score_T(S, 1)$ with a general score function. A match is scored as 1, mismatch and indels as -1 . Empirical score distribution of $score_T(S, 1)$ with a fitted EVD using the method of moments estimator. Q-Q plot comparing the theoretical and empirical distributions are shown. (a) $m = 40$. (b) $m = 100$.

could be approximated with an EVD irrespective of the score function used.

In the context of local alignment, the theoretical background for the score distributions for a random model were derived when indels were not allowed (Karlin and Altschul, 1990; Karlin et al., 1990); And it was shown empirically that allowing indels follows similar distributions as allowing only mismatches, although a theoretical proof does not exist (Altschul and Gish, 1996; Pearson, 1998; Smith et al., 1985).

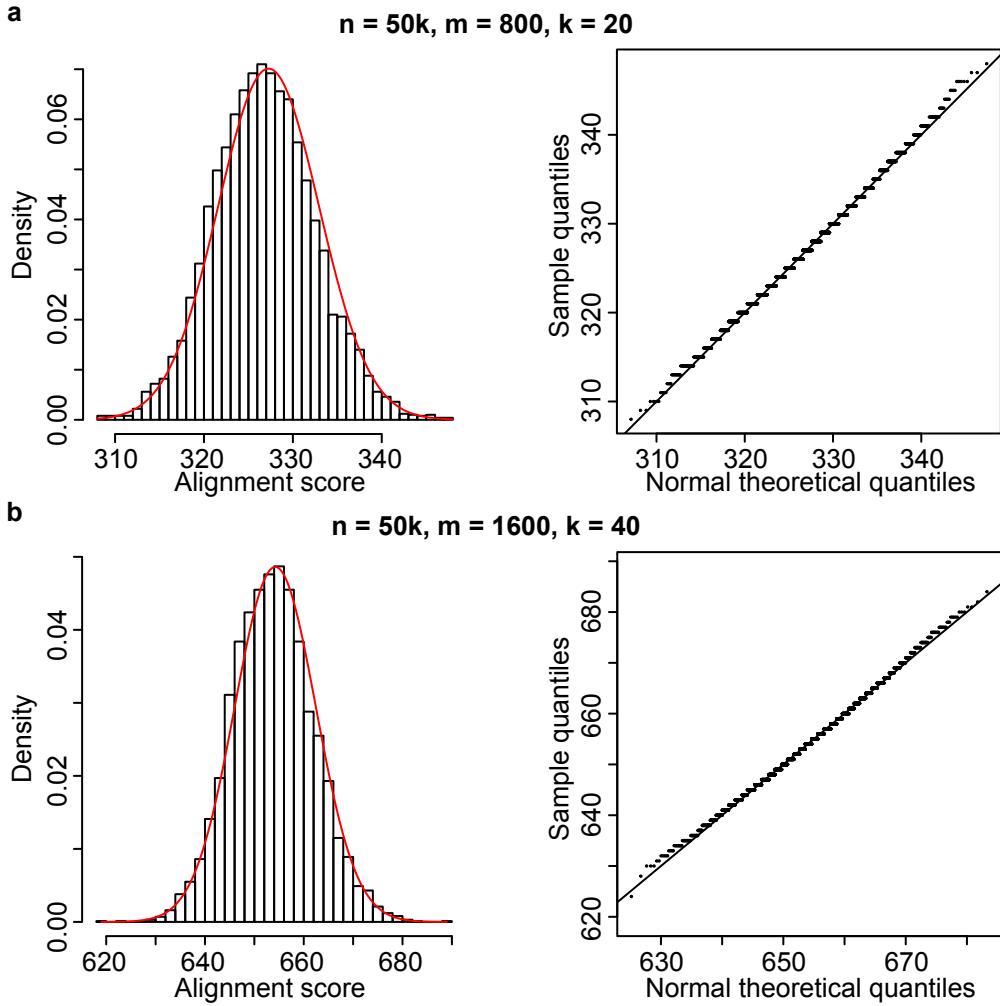


Figure 4.11: Normal approximation for $score_T(S, k)$ with a general score function. A match is scored as 1, mismatch and indels as -1 . Empirical score distribution of $score_T(S, k)$ with a fitted normal using the method of moments estimator. All fragments are 40 bp. Q-Q plot comparing the theoretical and empirical distributions are shown. (a) $m = 800, k = 20$. (b) $m = 1600, k = 40$.

We verified empirically that the score distribution of for $k = 1$, $score_T(S, 1)$, can still be approximated using an EVD when scoring a match as 1, and mismatch and indels scored as -1 . We generated a random genome of length 50kb from the DNA alphabet with equal probabilities, and reads of length 40 bp and 100 bp. For each read length, we determined the score distribution by aligning 5,000 reads generated at random (Fig. 4.10a, b). The parameters for the EVD was estimated using the method of moments.

Then, we also verified empirically that the score distribution for $k > 1$, which is still the sum of k independent extreme value distributions, is well approximated with a normal distribution. The fragments are 40 bp with 20 and 40 fragments per read (Fig. 4.11a, b).

Although the extreme value and normal distributions are good approximations for the score function tested above, we have not tested other score functions. These approximations need to be tested for the score function that is used to align SMURF-seq reads. Further, we have also not verified the validity of these approximation when gap open penalties are used in addition to gap extend and mismatch penalties.

Errors bounds extreme value and normal approximations

Any approximation for the score distributions are just approximations, and would have associated limitations. We have not assessed the limitations and error bounds in making these approximations.

Aligning a SMURF-seq read as one fragment is approximated with an extreme value distribution. Convergence to an EVD depends on the length of the genome, n . The minimum length of the genome with for convergence, the error in making this approximation, and its dependence on fragment length needs to be determined.

Aligning a SMURF-seq read with more than one fragment ($k > 1$) is approximated as a normal distribution, and the natural question is how large does k have to be for a good approximation. Further, understanding the dependence of the number of fragments and the fragment lengths are also crucial for determining the effective less of calculation the p-value for a fragmentation. For example, aligning two reads with a same k but one with shorter fragments and the other with longer could have to different error bounds for the normal approximation.

In aligning a real SMURF-seq read, the dependence of these approximation on the choice of score function used also needs to evaluated.

Effectiveness of p-value procedure

We determined the optimal fragmentation of a SMURF-seq read as the one with the lowest p-value. The effectiveness of this procedure for predicting the optimal fragmentation need to be evaluated. This could be done with simulated SMURF-seq reads for which the number of fragments and fragment boundaries are known. (Simulated SMURF-seq reads can be generated by sampling substrings from a reference genome and then adding sequencing errors. They can also be generated by concatenating substrings from a long read sequenced on a nanopore machine without SMURF-seq, and thus, preserving the error profile.)

Shorter fragments on a SMURF-seq read would lower the rate of increase of the alignment score with increasing the number of fragments on the read. This makes predicting the optimal number of fragments on a read challenging. Similarly, an increase in sequencing errors on a read would also make the prediction challenging. Thus, the mispredictions of the optimal fragmentation of a read needs to evaluated based on these factors.

A random model used to determine the null distribution would have a significant effect on the p-value, and so the effectiveness of the procedure to determine the optimal number of fragments on a read. Random models preserving nucleotide frequencies, dinucleotide frequencies, or local base composition can be used. The effect of the these random models for aligning SMURF-seq reads needs to assessed.

Extending to large genomes

Here, we used a genome of length 50 kb generated with letters of the DNA alphabet. However, a reference genome for use with SMURF-seq can be expected to be significantly larger (e.g. the human genome).

After determining the appropriate random model and the score function use use, the parameters for the null distribution need to be estimated. For example, if the method described in section 4.7.1

is used, the mean and variance of aligning fragment of different lengths need to be estimated. However, this requires aligning reads generated at random to a large genome to determine the maximum alignment score for a read. An alternate approach is to determine the dependence of the null distribution parameters on the length of the reads and length of the reference genome. Then the parameters for the required genome length and fragment length could be extrapolated from aligning reads to smaller genomes.

Theoretical proof for score distributions

We have shown empirically that the alignment score distribution of one fragment and multiple fragments are closely approximated by the extreme value and normal distributions, respectively. However, we have not theoretically proved the convergence to these distributions.

A better theoretic understanding of these approximations would enable, among others, determining the error bounds in making these approximations. Similar to BLAST statistics (Karlin and Altschul, 1990; Karlin et al., 1990), it could also provide an efficient analytical procedure for determining the p-value of aligning a SMURF-seq read.

SMURF-seq read mapping tool

Finally, as SMURF-seq evolves and the fragments get shorter, accuracy in identifying fragment boundaries will begin to impact the ability of aligners to recover fragments, and algorithms designed specifically to map SMURF-seq reads will become essential.

Mapping tools, in general, typically consists of a “search” step and an “alignment” step. The search step finds the candidate mapping locations of a read on the reference genome. The algorithm and the parameters used in this step determines the number of candidate locations that are passed on to the downstream steps. A “lenient” algorithm would pass too many candidates leading to an increase in the time spent in the subsequent steps. This could also lead to ambiguity in the

downstream steps by obscuring a true alignment. On the other extreme, a too “stringent” algorithm would lead to a decrease in the sensitivity of the alignments found. The candidate regions found in the search step could be further refined, typically produces an intermediate alignment to make the final align step efficient. Some tools use this step to further refine the candidate locations passed to the align step, and some tools skip this step entirely. The align step performs a more detailed evaluation of similar regions on the read and the reference found in the previous steps, typically using a some form of modified Smith-Waterman algorithm.

A mapping tool designed for SMURF-seq reads would vary substantially for both of these steps. The search step would require finding the candidate location of short fragments in the presence of high sequencing errors that is typical of nanopore sequencers. The align step for SMURF-seq reads requires incorporating a procedure to find accurate fragment boundaries and to estimate the optimal number of fragments on a read.

A SMURF-seq read could have fragments that do not or originate from the reference genome (due to DNA molecules from contaminants or regions of high-sequencing errors on a read), or a SMURF-seq read could have too few fragments for the normal approximation to hold. Developing a mapping tool for SMURF-seq reads would require taking into consideration these and several other factors, such as fragments aligning to multiple locations due to repeats on the genome, that we have not considered here.

With shorter fragments on a SMURF-seq read and with a dedicated mapping tool for these reads, we expect the number of fragments in a read to increase. Thus, improving the efficiency of short-read sequencing on long-read machines, and potentially expanding the utility of nanopore machines beyond read-counting applications.

Chapter 5

Conclusions

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Appendix A

Supplemental methods

DNA samples

The normal diploid female DNA was purchased from Promega (Cat. no. G1521). Breast cancer cell line SK-BR-3 (American Type of Culture Collection (ATCC), Cat. no. HTB-30) was cultured in RPMI-1640 medium (Thermo Fisher Scientific, Cat. no. 11875093) supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific, Cat. no. 35011CV) and was maintained at 37° in a humidified chamber supplied with 5% CO₂ and was regularly tested for mycoplasma infection.

Cell lysis and DNA purification

The DNA from SK-BR-3 cells was extracted and purified with the QIAamp DNA Blood Mini Kit (Qiagen, Cat. no. 51104) following the protocol for cultured cells given by the manufacturer. RNA and proteins in the cells were degraded using RNase A stock solution (100 mg/ml) (Qiagen, Cat. no. 19101) and Protease-K (Qiagen, Cat. no. 19133) respectively. Both purchased female diploid DNA and extracted SK-BR-3 DNA were treated with the same downstream processes.

Fragmenting genomic DNA

2-3 µg of genomic DNA was fragmented with restriction enzyme Anza 64 SaqAI (Thermo Fisher Scientific, Cat. no. IVGN0644) for 30 min at 37°. The fragmented DNA was cleaned with the QIAquick PCR purification kit (Qiagen, Cat. no. 8106) and eluted with 34 µl nuclease-free water. The concentration of DNA was quantified on a Qubit Fluorometer v3 (Thermo Fisher Scientific, cat. no. Q33216) with the Qubit dsDNA HS assay kit (Thermo Fisher Scientific, cat. no. Q32854).

Ligation of fragmented DNA

500 ng of fragmented DNA in 10 µl nuclease-free water was mixed with 10 µl Anza T4 DNA Ligase Master Mix (Thermo Fisher Scientific, Cat. no. IVGN210-4) and incubated for 30 min at room temperature. The ligated DNA was cleaned with 2× volume Ampure XP beads (Beckman Coulter, Cat. no. A63881) and eluted in nuclease-free water. This step was done in multiple tubes if more than 500 ng of fragmented DNA was needed to be ligated. The concentration of DNA was quantified on a Qubit Fluorometer v3 with the Qubit dsDNA HS assay kit to ensure $\geq 1 \mu\text{g}$ ($\geq 400 \text{ ng}$, if the Rapid kit was used for library preparation) remained. The size of the ligated DNA molecules were assessed with 1% agarose gel electrophoresis run at 90 V for 30 min.

Library preparation (SQK-LSK108 1D DNA by ligation)

1 µg of re-ligated DNA in 45 µl of nuclease-free water was end-repaired and dA-tailed (New England Biolabs (NEB), Cat. no. E7546), followed by elution in nuclease-free water after 1.5× volume Ampure XP beads clean-up. Sequencing adapters (AMX1D) were ligated with Blunt/TA Ligase Master Mix (NEB, Cat.no. M0367) and cleaned with 0.4× volume Ampure XP beads and eluted using 15 µl Elution Buffer (ELB) following the manufacturer's protocol (Oxford Nanopore Technologies (ONT), 1D genomic DNA by ligation protocol).

Multiplexed library preparation (EXP-NBD103 and SQK-LSK108)

700 ng of each re-ligated sample in 45 µl of nuclease-free water was end-repaired, dA-tailed (NEB, Cat. no. E7546), cleaned with 1.5× volume Ampure XP beads and eluted in nuclease-free water. Different Native Barcodes (NB-x) for each sample was ligated with Blunt/TA Ligase Master Mix (NEB, Cat.no. M0367), cleaned with 2× volume Ampure XP beads and eluted in nuclease-free water. Equimolar amounts of each sample was pooled to have 700 ng of DNA in 50 µl water. Barcode adapters (BAM) were ligated with Quick T4 DNA Ligase (NEB, Cat. no. E6056), cleaned with 0.4× volume Ampure XP beads and eluted using 15 µl Elution Buffer (ELB) following the manufacturer's protocol (ONT, 1D native barcoding genomic DNA).

Library preparation (SQK-RAD003 Rapid sequencing)

400 ng of re-ligated DNA was concentrated with 2× volume Ampure XP beads to 7.5 µl nuclease-free water. DNA was fragmented with Fragmentation Mix (FRA), and Rapid 1D Adapter (RPD) was attached following the manufacturer's protocol (ONT, rapid sequencing).

MinION sequencing and base-calling

All the prepared libraries were loaded on R9.5 Flowcells following the manufacturer's protocol (ONT) and sequenced for up to 48 hours using the script specific to library preparation protocol. Base-calling and de-multiplexing barcoded reads were performed using ONT Guppy (2.3.5) with the appropriate parameters based on the library preparation kit.

Sequencing RE digested normal diploid genome

1 µg of genomic DNA was fragmented with restriction enzyme Anza 64 SaqAI (Thermo Fisher Scientific, Cat. no. IVGN0644) for 30 min at 37°. The fragmented DNA was cleaned with the QIAquick PCR purification kit (Qiagen, Cat. no. 8106) and eluted with 31 µl nuclease-free water. The concentration of DNA was quantified on a Qubit Fluorometer v3 (Thermo Fisher Scientific, cat. no. Q33216) with the Qubit dsDNA HS assay kit (Thermo Fisher Scientific, cat. no. Q32854).

0.5 µg of restriction enzyme digested DNA in 45 µl of nuclease-free water was end-repaired and dA-tailed (New England Biolabs (NEB), Cat. no. E7546), followed by elution in nuclease-free water after 1.5× volume Ampure XP beads clean-up. Sequencing adapters (AMX1D) were ligated with Blunt/TA Ligase Master Mix (NEB, Cat.no. M0367) and cleaned with 1.0× volume Ampure XP beads (manufacturer's protocol uses 0.4× volume XP beads, we increased to 1.0× to get as many short molecules as possible) and eluted using 15 µl Elution Buffer (ELB) following the manufacturer's protocol (Oxford Nanopore Technologies (ONT), 1D genomic DNA by ligation protocol).

The prepared library was loaded on R9.4 Flowcell following the manufacturer's protocol (ONT) and sequenced for 48 hours. Base-calling was performed using ONT Guppy (2.3.5).

Estimation of copy number variations

CNV profiles were generated using the procedure described in (Baslan et al., 2012; Kendall and Krasnitz, 2014) with the modification employed in (Gerdsson et al., 2018; Malihi et al., 2018). Briefly, the human reference genome (hg19) was split into 5,000 (20,000 or 50,000) bins containing an equal number of uniquely mappable locations and the bin counts were determined using uniquely mapped fragments. Bins with spuriously high counts ('bad bins', typically around centromeric and telomeric regions) were masked for downstream analysis (Kendall and Krasnitz, 2014). This procedure normalizes bin counts for biases correlated with GC content by fitting a

LOWESS curve to the GC content by bin count, and subtracting the LOWESS estimate from each bin (Kendall and Krasnitz, 2014). Circular binary segmentation (CBS) (Olshen et al., 2004), implemented in DNAcopy (Seshan et al., 2010) package, then identifies breakpoints in the normalized bin counts. Following (Gerdtssohn et al., 2018; Malihi et al., 2018), after CBS, spurious segmentation calls were removed. The influence of the GC content correction can be seen in Additional file 1: Figure S14.

Comparison with Illumina WGS of SK-BR-3 genome.

DNA from SK-BR-3 cells was used to construct WGS library with the NEBNext UltraII FS DNA Library Prep Kit (NEB, Cat. no. E7805) following the manufacturer's instructions. After library quality and quantity assessment with Qubit 3.0 HS dsDNA assay and BioAnalyzer HS dsDNA assay (Agilent), libraries were sequenced on HiSeq 2500 (Illumina) with single-end 130 cycles mode.

The reads were mapped with BWA-MEM using the default parameters, PCR duplicates were removed, and CNV profiles were generated using exactly the same method as used for SMURF-seq reads. The scatter plots and Pearson correlations comparing the CNV profiles were produced using R.

Appendix B

Mapping SMURF-seq reads with long-read aligners

Simulating SMURF-seq reads to evaluate mapping programs

To test these mapping tools, we chose to create simulated reads with the technical characteristics we expect in idealized SMURF-seq data. We first selected a fragment length ℓ and a number k of fragments per read. Then, for a given WGS nanopore data set, we took the set of mapped long reads as determined by BWA-MEM (with `-x ont2d` option). Each of the mapped reads was split into fragments of length ℓ (with a random offset of 0 to $\ell - 1$ at the start of the long read). Each fragment was validated by requiring that it did not overlap a deadzone in the genome (as determined by the deadzone program available from <https://github.com/smithlabcode/utils> for 40 bp). The reason for excluding deadzones is that even when a short fragment has a “known” mapping location when it is part of a longer read, we cannot compare its reported mapping location as a short fragment with that known location, since we expect any good mapping algorithm to identify that the fragment maps ambiguously. Among these validated fragments, subsets of k were sampled uniformly at random and concatenated (in random order and orientation) to form simulated SMURF-seq reads.

The first and last fragments in a read should be slightly easier to identify and map than the rest, since one of their boundaries is known. Using the above procedure, we select $k = 20$ so that the simulated reads have a sufficient number of fragments to eliminate the influence of the first and last fragments in each read on the results. There is no need to have large k otherwise.

By lowering ℓ and making the fragments shorter, the task of mapping the fragments becomes more challenging. Real SMURF-seq reads have fragment lengths determined by restriction site density, size selection and other aspects of the experiments. But in testing mapping algorithms and optimizing parameters, there is no disadvantage to making the task more challenging. We only need to be able to distinguish the relative performance of different mapping tools and parameter combinations. Real SMURF-seq reads have varying fragment lengths, but in evaluating mapping tools, there is no need to randomize fragment lengths. None of the algorithms we evaluated are capable of either deducing or leveraging the fact that all simulated fragments have the same length. We selected $\ell = 100$, which begins to challenge the various mapping strategies. These values of ℓ are slightly lower than the average in real SMURF-seq data.

Evaluating performance using simulated SMURF-seq reads

Within the simulated reads, the boundaries of each fragment are known *a priori*, as are their mapping locations. We used this information to evaluate mapping tools in terms of (1) how well they identify fragments purely for the purpose of counting molecules, which is the primary information used in CNV analysis, and (2) how well they identify individual mapping bases within reads. The latter criteria becomes important in challenging cases and will be increasingly important as fragment sizes are reduced.

Performance on identifying fragments: After mapping these simulated reads, each mapping result is called a predicted fragment. Each predicted fragment is considered a positive prediction, and we assume an arbitrary order over positive predictions. A positive prediction is a true positive

if:

- The predicted fragment maps uniquely.
- The mapping locations of at least half the bases in the predicted fragment are equal to the original mapping locations for those bases, and those bases are all part of the same original fragment (we assume that it is unlikely for two fragments on a simulated read to have the same mapping location but opposite orientation, and thus do not check for the orientation of a fragment). In this case, we say the predicted fragment is associated with that original fragment.
- The predicted fragment is the first among predicted fragments associated the same original fragment.

False positives are predicted fragments that are not true positives. Any original fragment with no associated predicted fragment is a false negative. These criteria penalize splitting one original fragment or merging two original fragments. By defining true positives, false positives and false negatives we are able to calculate precision, recall, and F-score for a particular mapping strategy.

Performance on identifying individual mapping bases: After mapping simulated reads, each mapping result is decomposed into individual nucleotides and associated with a location in the genome. Those locations are retained. We keep multiplicities, so when two mapped fragments overlap in the genome we count certain nucleotides twice. These are the predicted positive bases in the reference. The condition positive bases are those known *a priori* from the simulation. The original fragment mapping locations may overlap in the reference genome, leading to multiplicities in the condition positive bases, but with low probability. The true positives are the intersection of the condition positive and the predicted positive bases. When there are multiplicities of mapped fragments and simulated fragments overlapping the same bases in the reference genome, this is determined by taking the smaller of the two values. After removing the true positives bases, the remaining predicted positive bases are false positives, and the remaining condition positive bases are false negatives. These criteria penalize mapping approaches that do not cover the entire simulated SMURF-seq reads, and also penalize approaches that predict fragments that overlap within

the read. The true positives, false positives, and false negatives here allow us to assign precision and recall in terms of individual bases and corresponding F-scores. Although the reference bases for both predicted positive and condition positive could involve multisets, since our simulations used relatively low coverage this almost never happened.

To generate simulated reads we used the standard long reads from four sequencing runs (Flow-cell ID: FAB42704, FAB42810, FAB49914, and FAF01253) in the public dataset available at <https://github.com/nanopore-wgs-consortium/NA12878/blob/master/Genome.md> (Jain et al., 2018a,b). We downloaded the raw data from EBI (Run accession: ERR2184696, ERR2184704, ERR2184712, and ERR2184722) and base-called these with Guppy (version: 2.3.5).

Initial selection of mapping tools

We tested the following mapping tools: BWA-MEM(Li, 2013), Minimap2(Li, 2018), LAST(Kiełbasa et al., 2011), GraphMap(Sović et al., 2016), BLASR(Chaisson and Tesler, 2012), rHAT(Liu et al., 2015), and LAMSA(Liu et al., 2017). These were selected either because they are known to perform well on certain mapping tasks or have unique properties that plausibly could help in mapping SMURF-seq reads. We tested each of these using default parameters on simulated reads and downsampled real SMURF-seq reads (data not shown). Among these BWA-MEM, Minimap2, and LAST had higher accuracy on simulated data, and the other tools identified at most 15 fragments per read on real data. Thus, we explored performance of BWA-MEM (0.7.17), LAST (963), and Minimap2 (2.15) in more detail, varying parameters to improve performance.

We remark that none of these tools were designed to map SMURF-seq reads; results we report here do not reflect the overall performance of the various mapping tools, only that the three aforementioned tools happened to perform relatively well on a task for which they were not directly designed for.

Determining the optimal Smith-Waterman score for SMURF-seq reads

In order to determine the optimal alignment score, we kept the seeding related parameters constant, and varied the alignment score combinations to perform a grid search. We varied the mismatch penalty from 1 to 6, gap open penalty from 0 to 4, and gap extend penalty from 1 to 4. The match score was fixed at 1. Thus for each tool we tested 120 ($6 \times 5 \times 4$) combinations of alignment scores.

The seeding and chaining related parameters for each tool was set as follows (along with the four alignment scores):

- BWA-MEM: `-x ont2d -k 12 -W 12 -T 30`
- Minimap2: `-w 1 -m 10 -s 30`
- LAST (NEAR): `lastal -Q0 -e 20` and `last-split -m 1 -s 30`

We set the seeding and chaining parameters in a liberal manner to allow for higher sensitivity than the default parameter of each tool, and the minimum alignment score to output was set at 30.

After aligning the simulated reads, we calculated the average precision and recall, each for the mapped fragment locations and nucleotides, for the four datasets. The F-score was computed for each, and the mean of the F-scores was used to determine the optimal alignment parameter for each tool. Based on these results BWA-MEM outperformed other tools for aligning SMURF-seq reads. BWA-MEM performed best with a mismatch, open, and extension penalty of 2, 1, 1 respectively.

To further refine the optimal alignment parameter for BWA-MEM, we aligned the simulated reads with parameter values around the value described above with a higher resolution. We varied the mismatch penalty from 1.5 to 2.5, and open and extend penalties from 0.5 to 1.5 in increments of 0.25. However, BWA-MEM does not accept floating point values for alignment score parameters. To overcome this, we scaled the alignment score proportionately to have integer values, i.e we varied the mismatch penalty from 6 to 10, open and extend penalties from 2 to 6, and fixed the match score at 4 (125 combinations). Based on these results, the highest accuracy was obtained

with the mismatch, open, and extension penalty of 2.5, 1.5, 0.75 respectively (corresponding scaled values are 10, 6 and 3). We used these optimal alignment scores for mapping real SMURF-seq read, and all the CNV profiles presented are based on these.

Appendix C

Data availability and summary of sequencing runs

Sample	Kit	Reads	Mean length	Fragments	Accession
Diploid	SQK-LSK108	270.82k	6.8 kb	7.28M	SRX5893474
Diploid	SQK-LSK108	497.92k	3.7 kb	7.55M	SRX5893475
SK-BR-3	SQK-LSK108	146.98k	7.6 kb	4.52M	SRX5893478
SK-BR-3	SQK-LSK108	132.64k	7.3 kb	4.02M	SRX5893479
Diploid	SQK-RAD003	213.38k	3.9 kb	2.81M	SRX5893473
Multiplexed run	EXP-NBD103 +	442.9k			
Diploid (BC01)	SQK-LSK108	138.19k	4.8 kb	2.95M	SRX5893472
SK-BR-3 (BC02)		144.57k	7.7 kb	4.97M	SRX5893476
Diploid (short-read)	SQK-LSK108	2.58M	630.9 bp		SRX5893480
SK-BR-3 (WGS)	Illumina WGS	5.56M	130 bp		SRX5893477

Table C.1: Summary of sequencing run. Samples are processed with the SMURF-seq protocol, unless indicated otherwise. Sequence data generated during the study are available in SRA with the accession number PRJNA454059.