Accelerating **cancer research** through comprehensive genomic analysis

Oxford Nanopore Technologies

Contents

Introduction

The rapid evolution of sequencing technologies has revolutionised human genome analysis, often replacing traditional low-resolution methods, such as karyotyping and microarrays. High-throughput Oxford Nanopore sequencing allows researchers to investigate complex genomic variants at nucleotide-level resolution, enabling deeper insights into the genetic underpinnings of cancer. Novel biomarkers identified through nanopore sequencing have the potential to support and improve diagnostics, prognostics, and treatment decisions.

While sequencing technologies have advanced novel discoveries, cancer remains a highly complex disease, often influenced by factors beyond the simple point mutations that are detectable by short-read sequencing methods. Structural variants (SVs), DNA methylation, and transcript isoforms play crucial roles in tumour biology, yet legacy sequencing techniques struggle to capture these elements efficiently and comprehensively. Additionally, phasing mutations to maternal or paternal chromosomes is essential for understanding their biological impact — another limitation of legacy methods.

Addressing these challenges is key to accelerating cancer research and advancing precision oncology. Oxford Nanopore sequencing offers a unique solution by enabling the real-time analysis of native DNA and RNA fragments of any length, capturing previously hidden variants and modifications in a single, comprehensive assay. This approach eliminates the need for multiple, often imprecise techniques and streamlines workflows while enhancing resolution and accuracy.

This white paper explores how Oxford Nanopore sequencing is transforming cancer genomics by delivering an unprecedented, comprehensive view of cancer biology with multiomic insights.

Addressing the challenges of cancer research

Access complex genomic regions with reads of any length

Read the following case studies for more on the benefits of full-length nanopore reads:

Case study 1 Page 18

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Sequencing technologies have traditionally relied on the fragmentation and subsequent amplification of short lengths of DNA or RNA, typically between 150 to 300 bp in length. During this process, the relative positional information of each fragment is lost and, for most applications, must be aligned to a reference genome and collapsed into an imperfect consensus sequence — adding significant time and complexity to analyses. While this may present less of a challenge when examining individual single nucleotide variants (SNVs) or mutation hotspots, it confounds the analysis of many other common forms of genomic variation, including SVs, repeat regions, phasing, and transcript isoforms (**Figure 1**). Furthermore, the requirement for amplification removes base modification information (see **The importance of methylation to reveal novel oncological insights**).

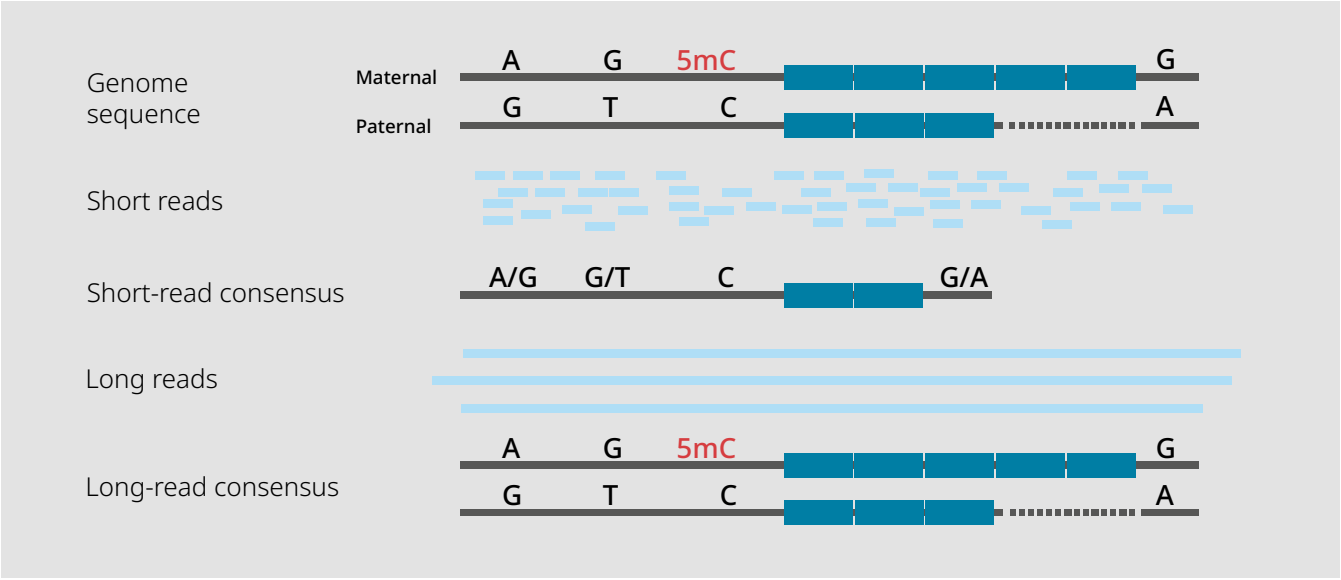

Figure 1. Schematic highlighting the advantages of long, direct sequencing reads in *de novo* assembly and characterisation of a wide variety of genomic variants, including SNVs (black nucleotides), repetitive regions (blue boxes), and base modifications (red nucleotide depicting 5-methylcytosine [5mC]). Long, native DNA sequencing reads allow complete resolution of the genomic region, including base modifications, for both maternal and paternal alleles, while short-read-only sequencing struggles to resolve genomic repeats and phasing and requires a separate sequencing run to identify base modifications. The dotted line indicates the alignment gap between both alleles due to the different number of repeats.

With Oxford Nanopore sequencing, read length is equal to DNA or RNA fragment length, enabling the analysis of any length of DNA or RNA molecule — from short fragments, which support applications such as cell-free DNA analysis[1], to ultra-long molecules, for which read lengths of >4 Mb have been demonstrated[2]. Clearly, such long reads are more likely to span and resolve large genomic aberrations intractable to legacy short-read-only sequencing technologies.

Read the case studies featured at the end of this white paper for detailed examples of the benefits of reads of unrestricted length.

Structural variants

SVs, which are defined as deletions, duplications, inversions, translocations, and insertions of 50 bp or greater in size (**Figure 2**), account for a greater number of variable bases than single nucleotide variants (SNVs)[3]. According to van Belzen *et al.* 'At least 30% of cancers have a known pathogenic SV used in diagnosis or treatment stratification. However, research into the role of SVs in cancer has been limited due to difficulties in detection'[4].

'nanopore sequencing can improve structural-variant resolution, while providing phasing and methylation information'

O'Neil, K.[5]

It has been estimated that at least 48% of deletions and 83% of insertions are routinely missed by short-read sequencing technologies[3]. Primarily because SVs predominantly occur in repetitive genomic regions, which short-read-only technologies struggle to resolve due to ambiguous multimapping, whereby reads map to multiple locations[3,5].

Conversely, the reads of unrestricted length generated by Oxford Nanopore technology have been shown to allow reliable and accurate detection of SVs, as demonstrated in **Case study 2**. In research samples, Geyer *et al.* found that nanopore reads easily resolved SVs and complex structural rearrangements associated with acute leukaemias that are known to be difficult to characterise and typically missed with conventional diagnostic methods[6].

'nanopore-generated WGS data provided additional information about structural variation that was not detected through clinical assays'

Geyer, J. *et al.*[6]

Learn more about characterising SVs with Oxford Nanopore reads at: nanoporetech.com/structural-variation

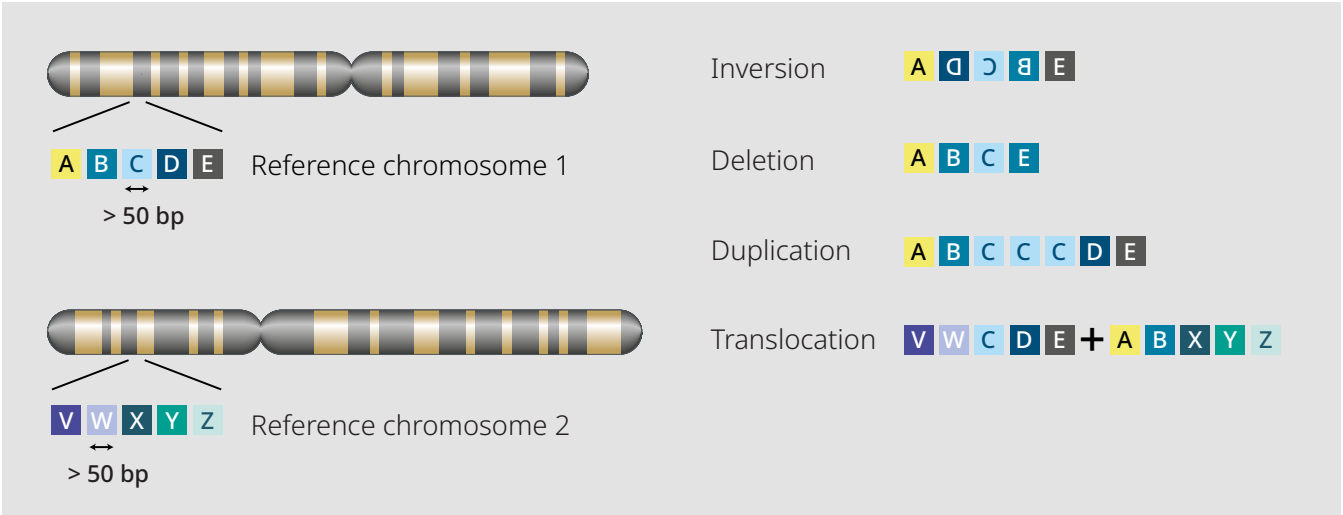

Figure 2. Schematic of common structural variants.

Phasing

Long and ultra-long nanopore reads further allow the phasing of variants (e.g. SNVs, SVs, methylated bases) to haplotypes, enabling researchers to more comprehensively understand cancer biology. Additionally, the long phase blocks and direct DNA sequencing delivered by Oxford Nanopore technology open the possibility of using maternal and paternal methylation markers to trace inherited variants of interest to the parent of origin, without the use and expense of trio (mother/father/child) sequencing[7,8], as demonstrated in **Case study 1**.

'By exploiting the long reads produced by MinION sequencing, it is possible to evaluate the phasing of mutations, clarifying the allelic context of mutations affecting the same gene, but too geographically distant to be detected with short-read sequencing'

Orsini, P. *et al*.[9]

Find out more about SNV and phasing at:
nanoporetech.com/snvs-phasing

Fusion genes

Nanopore reads of any length are also advantageous for the rapid and accurate resolution of fusion genes. Fusion genes occur when the DNA or RNA of two different genes are joined together and translated to produce a fusion protein. These types of variants are associated with many forms of cancer, particularly haematological cancers. Using Oxford Nanopore sequencing, researchers have investigated gene fusion events indicative of different forms of leukaemia, with one team reporting confident identification of a *PML-RARA* fusion in less than 50 minutes of sequencing on the MinION™ device, with the first *PML-RARA* read being detected in just over one minute[10]. Importantly, unlike the traditional techniques of fluorescence *in situ* hybridisation (FISH) and quantitative RT-PCR, Oxford Nanopore sequencing only requires information from one, rather than both, fusion partners, enabling the detection of novel gene fusions and reducing the time, cost, and number of assays required for analyses of known fusion partners. Read **Case study 2** to find out how full-length nanopore reads resolved an SV in a research sample that led to the identification of a *DUX4:IGH* gene fusion that was missed by standard clinical tests[6].

For more information about fusion transcripts, see the application page:
nanoporetech.com/fusion-transcripts

Methylation

Oxford Nanopore Technologies provides comprehensive, PCR-free, direct sequencing of DNA and RNA that preserves base modifications to reveal novel insights about cancer biology; results that are unattainable with short-read methods. Just one example of where Oxford Nanopore technology provides valuable insights for cancer research is the methylation profiling of circulating tumour DNA (ctDNA). Nanopore reads enable the detection of distinct methylation patterns that can be used to predict the tissue of origin for ctDNA[11].

Furthermore, in **Case study 1** the authors illustrate how methylation detection via nanopore sequencing can be used to determine the parent-of-origin effect and characterise altered gene expression of imprinted genes[12].

See **The importance of methylation to reveal novel oncological insights** for further examples of how methylation profiling with nanopore reads can be leveraged for cancer studies.

Find out more about methylation with nanopore sequencing at:
nanoporetech.com/applications/epigenetics

Enhancing targeted oncology sequencing capabilities

Read the following case studies for more on the benefits of targeted nanopore sequencing:

Case study 1
Page 18

Case study 2
Page 20

The advantages of Oxford Nanopore sequencing are not confined to whole-genome or whole-transcriptome analysis. Nanopore sequencing is compatible with all common targeted and enrichment strategies, including PCR amplification (amplicon) and hybridisation-based capture. In addition, unique to Oxford Nanopore sequencing, is adaptive sampling — an on-device target enrichment methodology that requires no additional upfront cumbersome sample preparation (**Figure 3**). With any-length, real-time reads generated by nanopore technology, the capabilities of targeted sequencing are expanded, allowing the detection of all the variants possible with non-targeted nanopore sequencing — such as repeats, SVs, base modifications[7,13]*, and SNVs — in a single sequencing run.

'we leverage the ability of targeted long-read sequencing to simultaneously assay genetic and epigenetic base level changes to provide haplotype-phased long-read genome and CpG methylome data... in a single sequencing run'

Stacey, A. *et al*.[12]

Oxford Nanopore provides fast and flexible workflows for both PCR-based and PCR-free target enrichment options, providing additional unique advantages over conventional targeted sequencing methods. Oxford Nanopore technology and long-range PCR can be used to enrich and sequence targets spanning several kilobases to a high depth of coverage. Additionally, hybridisation-based capture and nanopore sequencing can also be used to target large kilobase-sized regions of interest. Researchers have successfully used this approach to improve targeted gene coverage during single-cell sequencing to enhance transcriptome analysis. They generated a 14-fold enrichment in *SF3B1* and *BTK* genes and detected gene-specific mutations and splicing events at the single-cell level[14].

For sequences that are not amenable to PCR, such as repetitive or GC-rich regions or for studies that require rapid results, adaptive sampling can be utilised. This PCR-free method requires a BED file with target regions and then leverages real-time basecalling to selectively sequence or reject DNA molecules as they pass through the nanopore. Rejection of molecules that fall outside of the chosen criteria (e.g. non-homology to target sequence) is achieved through reversing the current applied to the individual nanopore, thereby making it available to sequence an alternative DNA fragment. As amplification is not required, long read lengths of native DNA that retain base modification information can be generated. A significant advantage of adaptive sampling is the facility to easily incorporate new targets into panel designs without the need for new panels and primer or probe synthesis. The removal of long and often cumbersome lab-based enrichment steps further increases the speed of the assay.

As part of the DNA selection process, the first 400–500 bp of each read is sequenced prior to being either accepted or rejected, the millions of short, rejected reads can also be used to generate low-pass whole-genome coverage, allowing genome-wide analysis of copy number changes alongside more in-depth genomic characterisation of the target regions[15].

Researchers used targeted nanopore sequencing with adaptive sampling in **Case study 1**, providing 'enhanced depth of coverage of the RB1 locus to identify the variant

*Requires an amplification-free targeted sequencing approach such as adaptive sampling.

at a VAF [variant allele frequency] of 16%'[12]. **Case study 2** specifically demonstrates the flexibility and speed of adaptive sampling as it enabled the authors to easily alter the regions of interest and perform targeted sequencing to provide results in three to six hours[6].

Many researchers are now assessing the utility of adaptive sampling to provide rapid, comprehensive, and cost-effective genomic characterisation of cancer-related genes and promoter regions[7,15,16–18].

Find out more about applying adaptive sampling to cancer research:
nanoporetech.com/cancer-associated-variants

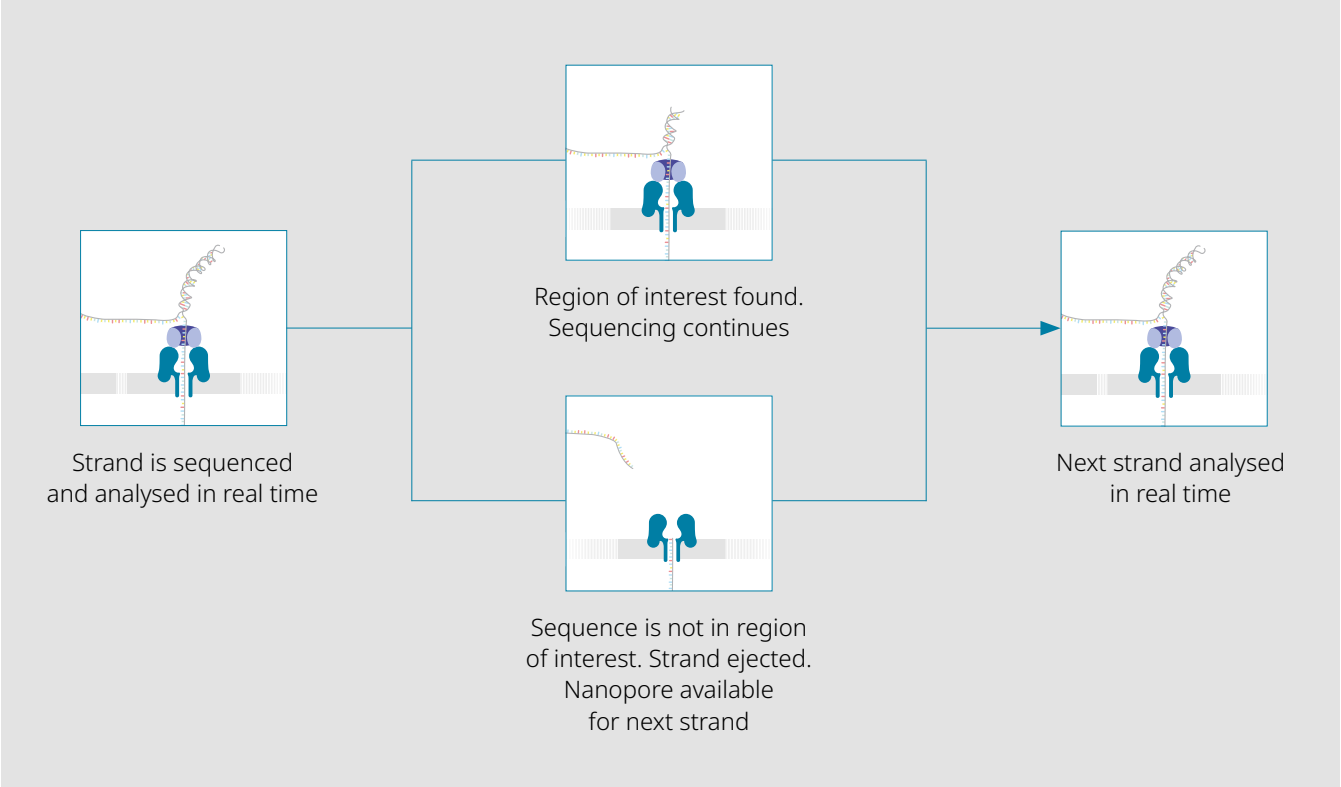

Figure 3. Adaptive sampling is a unique, on-device approach to targeted sequencing that requires no wet lab library enrichment steps, primers, or probes. Using real-time basecalling, DNA fragments can be accepted or rejected for further sequencing based on their initial sequence composition. A flexible list of regions to be enriched or rejected is provided as a BED file. Adaptive sampling can be implemented in advance of, or even during, a run to increase coverage of specific targets.

Harnessing the power of full-length transcripts and single-cell sequencing in cancer research

Read the following case study for more on the benefits of full-length transcript sequencing:

Case study 3
Page 22

It is estimated that protein-coding genes in the human genome generate, on average, seven different transcript isoforms[19]. However, due to the sequence similarity of related isoforms and the read-length limitations of legacy sequencing technologies, routine, in-depth analysis of the role of individual transcripts has been widely overlooked.

'The human genome contains more than 200,000 gene isoforms. However, different isoforms can be highly similar, and with an average length of 1.5 kb remain difficult to study with short-read sequencing'

Chen, Y. *et al*.[19]

Alternative splicing and isoform switching are involved in the development of many cancers, and these mechanisms can also contribute to treatment resistance[20]. As such, the ability of Oxford Nanopore sequencing to deliver long, full-length reads spanning whole transcripts provides researchers with a unique opportunity to quantify and fully characterise gene expression at the isoform level[7,19].

For example, researchers at the University of Athens used a targeted nanopore sequencing approach to characterise the transcript isoforms of the *RAS* genes, a family of proteins that are known to play a crucial role in many cancers[21]. Using a single MinION Flow Cell, they profiled the RAS transcripts across cell lines representing 13 different cancer types (and a non-cancerous control). In total, they identified 39 novel transcript isoforms, which were shown to be differentially expressed in the different cancer types.

Read **Case study 3** for a detailed example demonstrating cDNA characterisation via nanopore sequencing. In this study, the researchers successfully identified rare deep intronic variants, which contributed to inherited cancer risk, that were previously missed by conventional sequencing methods[22].

While most gene expression analysis to date has been performed on bulk cell populations, it is now also possible to interrogate isoform usage at the single-cell level (**Figure 4**), delivering new insights into previously hidden intricacies of cancer development[23,24].

'Understanding the relationship between the patterns of alternative splicing and cancer could help to gain insights into the origins of cancer formation and elicit potential therapies targeting cancer-specific protein isoforms'

Karakulak, T. *et al*.[25]

Find out more about single-cell nanopore sequencing at: nanoporetech.com/single-cell.

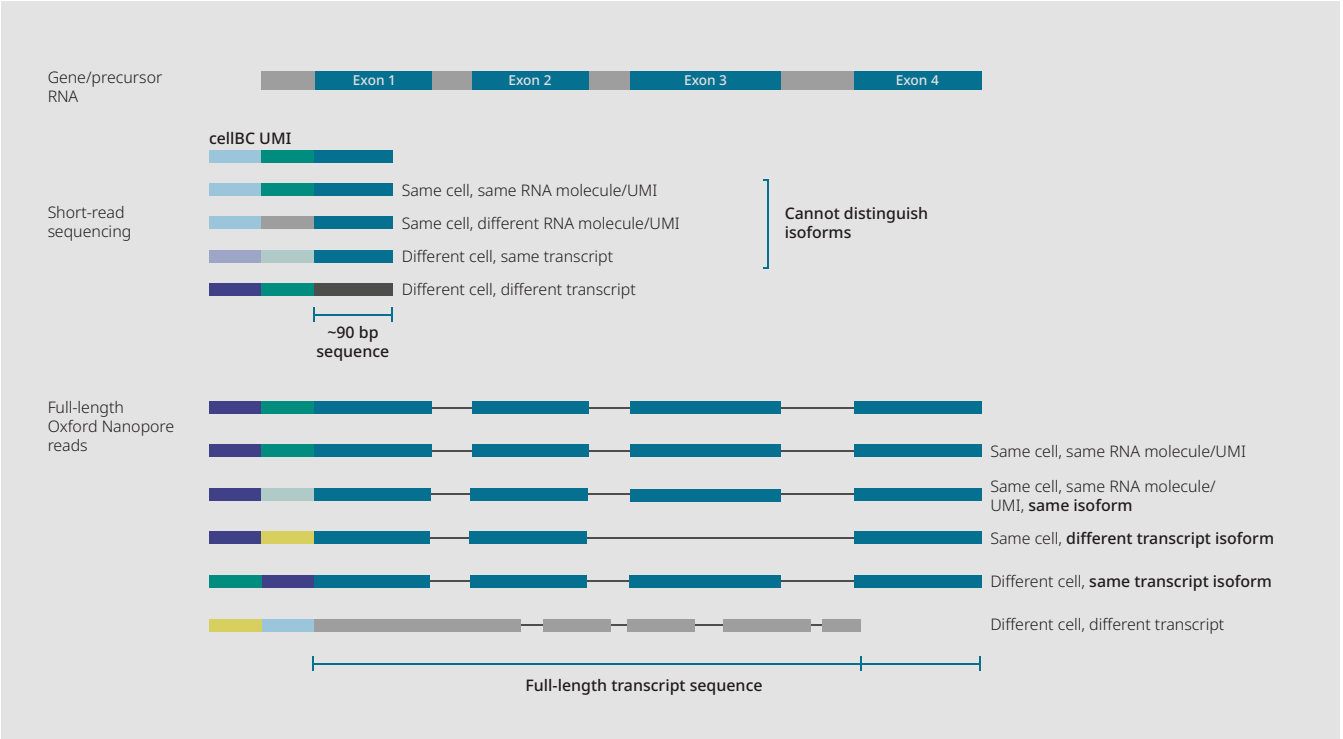

Figure 4. The power of single-cell sequencing comes from the facility to pool, sequence, and subsequently identify transcripts from hundreds of individual cells. All transcripts derived from the same cell can be identified using a unique cell barcode (cellBC). A unique molecular identifier (UMI), added to the original transcripts prior to amplification, further enables the identification of PCR duplicates that could impact transcript quantification and can be used to generate more accurate consensus sequences. Short-read sequencing technologies only read approximately 90 bp of transcript sequence, precluding the identification of transcript isoforms. In contrast, long Oxford Nanopore reads can span complete transcripts, enabling in-depth, isoform-level gene expression analysis from single cells.

The importance of methylation to reveal novel oncological insights

Read the following case study for more on the benefits of characterising methylation:

Case study 1
Page 18

Nucleotide variation does not fully explain disease susceptibility; an increasing number of human diseases are now known to be associated with base modifications (e.g. the addition of a methyl group to cytosine to form 5-methylcytosine [5mC], an increasingly recognised biomarker in cancer progression). Methylation plays a key role in gene expression, and methylation patterns have been linked to many forms of cancer, including glioblastoma[5], colorectal cancer[5], cervical cancer[26], and myeloid leukaemia[27], and may offer significant potential as a diagnostic and prognostic indicator. It has also been shown that methylation profiles can be used to classify cancer samples[28,29] and identify cell of origin for cfDNA[11,30] (**Figure 5**).

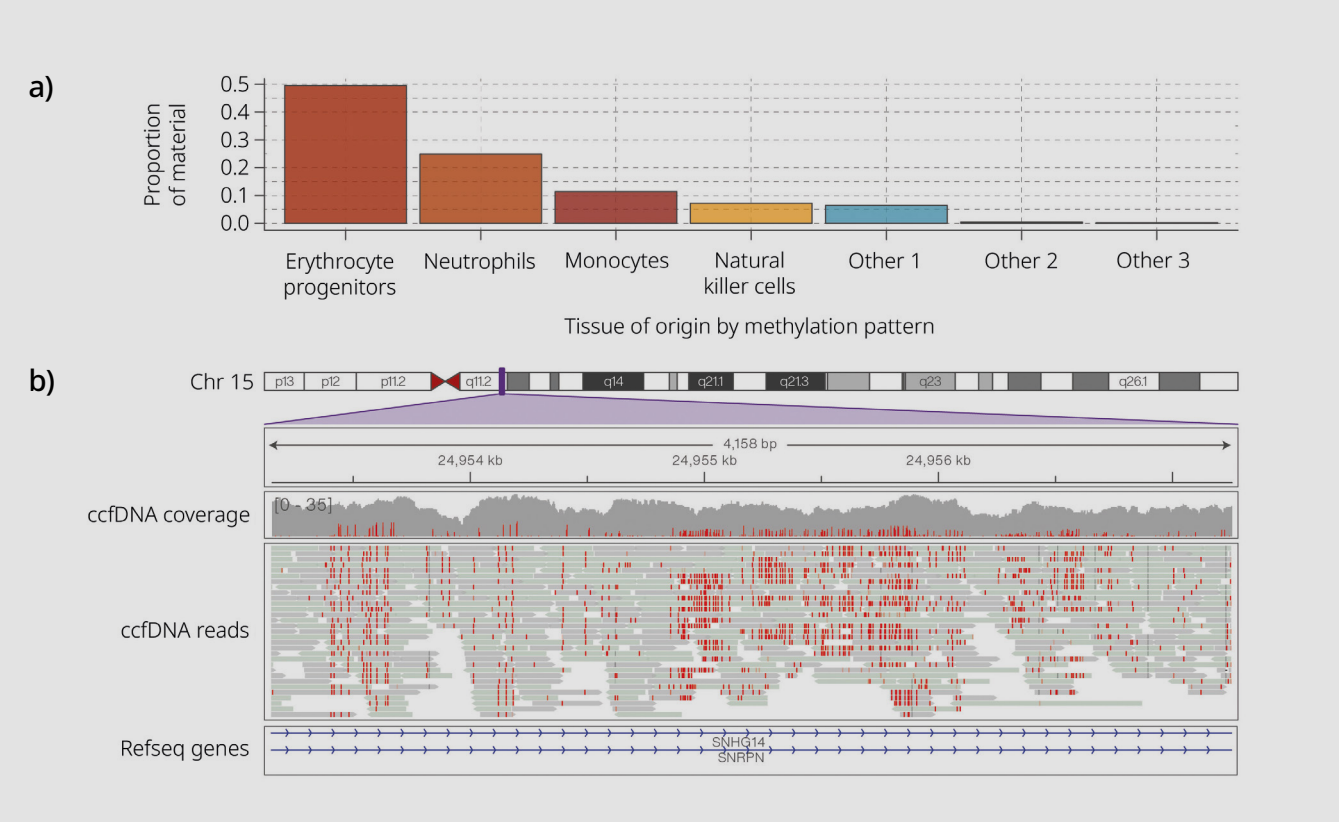

Figure 5. Nanopore-based methylation calling of cfDNA enables cell-of-origin identification. a) Tissue of origin by methylation pattern, and b) methylation signatures shown in IGV[13].

The amplification of nucleic acids — a prerequisite for legacy short-read sequencing technologies — erases base modifications, meaning they cannot be detected without additional time-consuming and often inefficient sample processing methods, such as bisulfite conversion. As mentioned previously, the Oxford Nanopore platform can directly sequence native DNA, meaning base modifications are retained and can be called alongside the canonical nucleotide sequence in the same sequencing run — without additional sample prep. Furthermore, when using reads of unrestricted length, it is possible to phase methylation to haplotype, allowing easy identification of genetically imprinted regions (**Figure 6**). Unlike array-based methylation analysis technologies, which typically survey up to 850,000 CpG sites in the human genome, nanopore sequencing can access all 28 million CpG sites.

This makes nanopore sequencing ideal for the discovery of novel differential methylation patterns between cancer and normal cells or different cancer subtypes that may be missed when using array-based approaches. Furthermore, Oxford Nanopore technology provides a cost-effective and reproducible method that generates comprehensive genomic and epigenomic sequencing data in a single run without the need for multiple assays. The use of adaptive sampling (see **Enhancing targeted oncology sequencing capabilities**) also offers a streamlined, lower-cost alternative for targeted analysis of specific CpGs than reduced representation bisulphite sequencing (RRBS) (**Figure 7**).

'Nanopore sequencing was able to cover the highest number of CpGs not covered by other assays'

Foox, J. *et al.*[31]

Researchers have used Oxford Nanopore sequencing as part of the personalised oncogenomics (POG) dataset, demonstrating the additional information native nanopore reads provide for cancer research. They found nanopore sequencing provides 'a significant advantage' because they generated methylation data within standard whole-genome sequencing in a single run and discovered allelically differentially methylated regions in cancer genes *RET* and *CDKN2A*[32].

Oxford Nanopore sequencing has also been successfully used to simplify the acute leukaemia genomic subtyping workflow based on genome-wide DNA methylation profiling. By using the free epigenomic data from Oxford Nanopore native sequencing, the researchers demonstrate a rapid and cost-effective single-assay workflow, overcoming the limitations of conventional methods that take several days to provide results[33].

'low-pass whole-genome nanopore sequencing can be used to generate DNA methylation profiles of a tumor biopsy that is sufficient for accurate classification in less than 2 h'

Djirackor, L. *et al.*[29]

The power of sequencing native DNA to provide valuable base modification information for cancer research has been further demonstrated by Mathilde Filser and her colleagues at the Institut Curie, France. They utilised Oxford Nanopore sequencing to rapidly and reliably classify medulloblastoma research samples into genomic subtypes based on methylome profiles[34].

Filser *et al.* demonstrated nanopore sequencing is a rapid and cost-effective method for precise subtyping, overcoming the limitations of current diagnostic methods that are laborious, costly, and not widely available. The researchers concluded that 'this approach could emerge as a valuable alternative for [medulloblastoma] subtyping' in the future[34].

'Nanopore sequencing demonstrates a robust capacity for precise subtyping of [medulloblastoma], a critical advancement'

Filser, M. *et al.*[34]

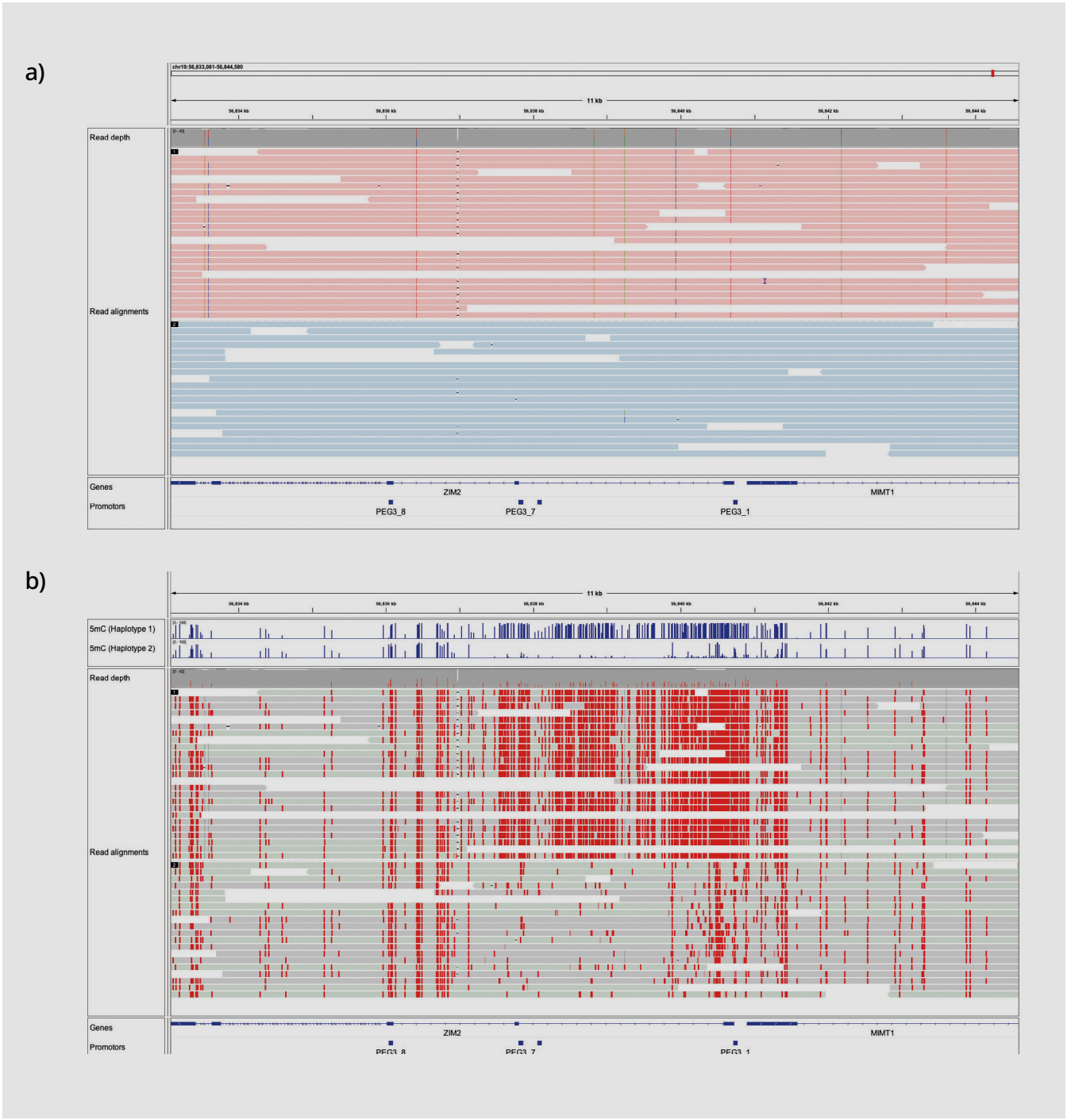

Figure 6. Phasing methylation using nanopore reads. The MinKNOW™ operating system, which powers Oxford Nanopore devices, provides integrated, simultaneous calling of canonical bases and modified bases (using Dorado). Dorado has minimal impact on basecalling speed and is easy to train for the detection of different base modifications and methylation motifs. a) The long nanopore reads can be phased to haplotype using SNV information (pink: haplotype 1; blue haplotype 2). b) The same region of the same dataset visualised using 5mC modification information, showing differentially methylated haplotypes.

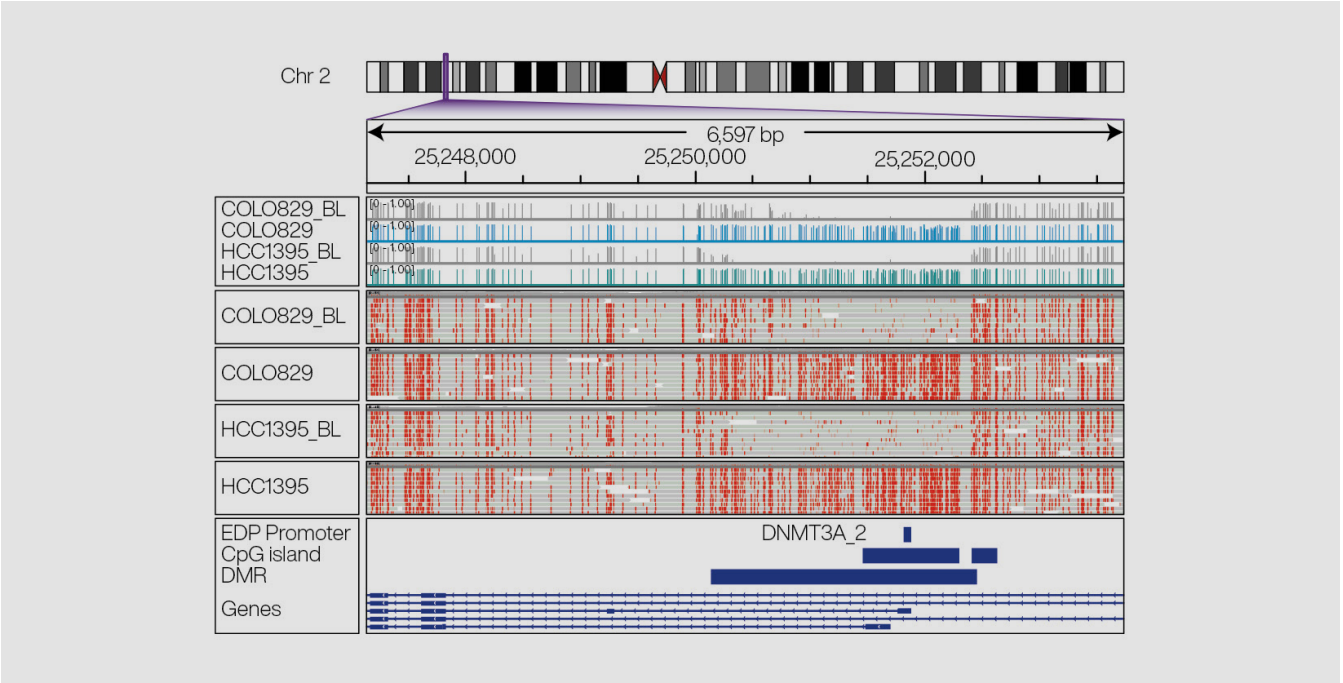

Figure 7. Adaptive sampling enables cost-effective, genome-wide characterisation of methylation patterns. Differential 5mC methylation is identified between a metastatic melanoma cell line/normal pair (COLO829/COLO829_BL) and also for a triple negative breast cancer cell-line/normal pair (HCC1395/HCC1935_BL) on Chromosome 2 overlapping *de novo* methyltransferase *DNMT3A*[13]. This region is clearly methylated in both tumour samples. Methyltransferases are essential for establishing and maintaining normal levels of methylation, so their dysregulation can contribute to cancer development. This type of analysis, therefore, has potential as a method of monitoring tumour progression.

With the latest Oxford Nanopore sequencing chemistry and software, the calling of 5mC in CpG contexts has been shown to be more accurate than that achieved using the previous gold standard of bisulfite sequencing[35]. Additionally, Oxford Nanopore sequencing can differentiate between modifications such as 5mC and 5hmC, and the identification of novel modifications can be trained, ensuring a complete methylation picture from a single experiment.

Learn more about modified base detection using Oxford Nanopore sequencing:
nanoporetech.com/epigenetics

Case study 1 provides a detailed example of how Oxford Nanopore sequencing provides comprehensive epigenomic data rapidly to reveal novel insights previously missed by conventional methods. Read the case study to find out how nanopore sequencing solves the parent of origin of *de novo* mutations in hereditary retinoblastoma in a single assay[12].

Democratising sequencing access to characterise cancers accurately worldwide

Read the following case study for more on the benefits of accessible sequencing:

Case study 2
Page 20

Rapid, real-time results

Unlike other sequencing technologies, which deliver all data at the end of a fixed and usually lengthy run time, the Oxford Nanopore platform provides data in real time during a sequencing run, enabling immediate access to results. As previously discussed, cancer researchers are utilising this capability to accelerate their work, including the genomic subtyping of acute leukaemias in six to nine hours using a single-assay workflow[6] and methylation-based brain tumour classification in under two hours[36].

In addition to offering rapid sample characterisation, real-time sequencing allows researchers to stop sequencing once a result has been obtained, wash, and then reuse the flow cell — providing additional time and cost savings to alternative sequencing-based approaches. The Oxford Nanopore sequencing software MinKNOW™ controls the nanopore sequencing devices and provides access to control sequencing during a run with access to data in real time.

The facility of real-time basecalling further underpins the unique, on-device target enrichment methodology of adaptive sampling, which enables the streamlined development of cancer panels to support the cost-effective interrogation of SNVs, SVs, repeats, and methylation in specific regions of interest (see **Enhancing targeted oncology sequencing capabilities**).

Find out more about real-time sequencing at:
nanoporetech.com/how-it-works/advantages-of-real-time-sequencing

Cost-effective, scalable, and on-demand analysis

A range of Oxford Nanopore sequencing devices and flow cells are available, which utilise the same sequencing chemistry and kits, allowing researchers to discover new biology at the scale and flexibility required (**Figure 8**).

Julie Geyer and her colleagues, in collaboration with St. Jude Children's Research Hospital, USA, demonstrated that nanopore technology is an accessible sequencing method for low- and middle-income countries. Read **Case study 2** for more details about how Geyer *et al.* used Oxford Nanopore sequencing as a simple and cost-effective approach to classify paediatric acute leukaemias in six to nine hours from the lab bench[6].

Figure 8. Oxford Nanopore sequencing platforms (top row, from left to right): the portable MinION, PromethION 2 Solo; PromethION 2 Integrated and (bottom row, from left to right): GridION, with capacity for five MinION Flow Cells; and the high-output PromethION 24 device.

MinION packs (including MinION device, a flow cell, and sequencing reagents) can deliver up to 15–35 Gb* of data per flow cell, ideal for targeted sequencing applications, low-coverage whole-genome analysis, and isoform-level gene expression studies.

The GridION™ device can run up to five MinION Flow Cells independently or in parallel, offering a flexible, on-demand platform for busy cancer research labs.

The PromethION™ family of devices offer the highest sequencing outputs, ideal for the analysis of whole cancer genomes at high depth and the characterisation of low-abundance transcript isoforms from whole transcriptomes. Running up to two or 24 high-output flow cells, respectively, the PromethION 2 Integrated, PromethION 2 Solo, and PromethION 24 devices provide the ultimate sequencing power and flexibility. Each PromethION Flow Cell can deliver up to 100–200 Gb* of sequencing data and can be run independently or in parallel.

A range of streamlined library preparation kits is available, including options for sample multiplexing to increase throughput and reduce costs.

Find out more about Oxford Nanopore devices:
nanoporetech.com/products

*Flow cell output will vary according to library type, run conditions, and other sample-specific factors. For more information on data output, visit: nanoporetech.com/products/specifications.

Accessible to any laboratory

Combining high performance with low cost and small size, nanopore devices ensure that the advantages of high-throughput sequencing are no longer limited to well-funded, centralised service laboratories. The uniquely portable MinION is ideal for local implementation and resource-limited settings that ordinarily might not have access to such powerful and versatile sequencing technology.

The small footprint of the high-output GridION and PromethION devices make them suitable for any laboratory, while the high-performance compute modules allow real-time data analysis — even at full sequencing capacity — and reduce the potential burden of installing additional data analysis hardware[†].

The preliminary data from Dr Carika Weldon showcased how she has harnessed nanopore sequencing to analyse genetic differences in breast cancer in Caribbean women in Bermuda.

This study was a first of its kind where the entire genomic workflow was conducted and analysed locally in Bermuda without shipping samples to centralised labs in the USA[37]. Oxford Nanopore offers EPI2ME™, a fully supported analysis platform that provides easy access to best-practice analysis workflows. This platform offers real-time data analysis tools covering a range of routine applications, such as SNV calling, SV calling, differential gene expression analysis, and isoform annotation. The wf-single-cell is one of many pipelines available. It analyses nanopore data, including mapping reads to references, assigns genes and transcripts, identifies cell barcodes, and calls cell-level SNVs. Sequencing data can also be exported in standard FASTQ, FAST5, and POD5 file formats suitable for further downstream analysis using a wide range of community-developed tools.

Find out more about analysing Oxford Nanopore sequencing data at: nanoporetech.com/analyse

[†]PromethION 2 Solo does not include integrated compute and requires external compute resource (e.g. GridION device or suitably specified computer).

Summary

High-throughput genomic technologies, such as microarrays and short-read sequencing, have afforded significant breakthroughs in cancer research; however, many regions of the human genome remain challenging to access or are even completely hidden from view due to the limitations of these techniques. To truly understand cancer biology and fulfil the promise of precision medicine, researchers are now exploring Oxford Nanopore sequencing to overcome these challenges.

'Multi-modality of nanopore is a huge asset — methylation, SV, gene fusions, single mutations and phasing — it's amazing to have all that in the single assay'

Hamza, A.[38]

Delivering unrestricted read lengths, accurate variant calling with simultaneous methylation analysis, real-time results, and a range of flexible and affordable kits and devices, Oxford Nanopore sequencing provides the most comprehensive insights into cancer genomes available from a single technology.

Case study 1

Solving the parent-of-origin effect in retinoblastoma to determine disease severity

Retinoblastoma is a common paediatric eye cancer in children under five years of age[39,12]. It is most often caused by biallelic mutations of the retinoblastoma gene (*RB1*), resulting in a loss of function and tumour formation[40].

This cancer has high survival rates[39], but heritable retinoblastoma — caused by a germline mutation, then a secondary somatic mutation[12] — has variable clinical outcomes depending on which parental allele the mutation resides in, known as the parent-of-origin effect[41].

What is the parent-of-origin effect?

The parent-of-origin effect is an epigenetic event caused by genomic imprinting that impacts methylation and gene expression[41]. This causes the phenotypic effects of a gene to vary depending on whether a genetic mutation is inherited maternally or paternally[12]. This effect is best characterised in imprinting disorders, such as Prader-Willi and Angelman syndromes, where the parent-of-origin effect on chromosome 15 results in different syndromes and phenotypes[42].

In the case of heritable retinoblastoma, the parent-of-origin effect alters clinical outcomes[12]. For example, paternally inherited *RB1* variants are associated with a higher likelihood of developing bilateral tumours and secondary cancers in later life than maternally inherited variants[43–45]. A study by Eloy *et al*. suggests that this is due to a differentially methylated region (DMR) in intron 2 of *RB1* that impacts the tumour-suppressor activity of the gene. They proposed that maternally inherited mutations have a weaker effect on tumour-suppressor activity than paternally inherited mutations, which cause minimal suppressor activity, leading to more severe tumour progression[46]. Therefore, it is beneficial to identify the parent of origin to inform clinical treatment plans early on.

However, it is estimated that more than 90% of germline cases are caused by *de novo* mutations, meaning that the parent of origin cannot be assigned because conventional testing requires trio sequencing of familial DNA samples, which would not harbour the mutation[12]. Yet, despite these difficulties in assigning *de novo* mutations, they are less likely to predispose to secondary cancers[45].

The difficulties of assigning the parent of origin

Studies have determined that the DMR in *RB1* is methylated on the maternal allele and unmethylated on the paternal allele[43], meaning it can be used to identify the parent of origin of a mutation without the need for trio sequencing[12].

To assign the parent of origin of an *RB1* mutation by analysing methylation in this region, the variant must therefore, be phased with the DMR. However, legacy short-read sequencing methods cannot sequence the entire *RB1* locus containing the mutation and DMR because short reads cannot provide chromosomal phase information due to their limited read length. Furthermore, secondary assays are required to characterise epigenetic changes, 'limiting their ability to provide parent-of-origin diagnostics' in a comprehensive or rapid manner[12].

Stacey *et al*. based at the University of Washington, USA, investigated the potential use of targeted Oxford Nanopore sequencing, as the multiomic method provides epigenetic and genetic data in a single assay. Furthermore, nanopore sequencing generates reads of any length, allowing long fragments of native DNA to be sequenced without losing base modifications. This means that a variant in *RB1* can be analysed and phased with the DMR using the same nanopore reads in a single sequencing run, demonstrating the potential to enable confirmation of the parent of origin from just the affected individual.

To target nanopore sequencing, Stacey *et al*. utilised adaptive sampling, a unique feature of Oxford Nanopore sequencing that provides real-time enrichment of selected regions of interest during sequencing without additional library preparation. The team sequenced seven research samples from individuals diagnosed with hereditary retinoblastoma and nine retinoblastoma research samples with *de novo* germline mutations. Peripheral blood samples were prepared using the Ligation Sequencing Kit before sequencing on a MinION for 72 hours. Adaptive sampling was employed during sequencing to target the region of the *RB1* gene containing both the DMR in intron 2 and the pathogenic variant, with an additional 50 kb flanking region on either side of the targeted locus.

'This work demonstrates parent-of-origin in disease can be resolved by phasing a disease variant with a differentially methylated signal in the genome, either local or distant, with a targeted long-read sequencing approach'

Stacey *et al*. 2024[12]

Capturing *de novo* germline mutations

Using this method, the *RB1* variant was sequenced and phased with the DMR, identifying the parent of origin in all research samples from the affected individual for both inherited and *de novo* variants — without familial DNA samples. Oxford Nanopore sequencing also identified the parent of origin for complex *de novo* mutations caused by structural variants and somatic mosaicisms, which typically require multiple secondary assays with legacy short-read methods. Furthermore, all 'previously unknown cases with *de novo* pathogenic variants in *RB1*, which account for the majority of heritable cases of [retinoblastoma]', were resolved with this method.

The results also demonstrated that low-frequency variants can be identified at a variant allele fraction (VAF) of 16% from peripheral blood cells. This was made possible with adaptive sampling, which focused sequencing depth on a targeted region. Stacey *et al*. found 'a distinct advantage [with targeted adaptive sequencing' because it 'provides the adequate read depth and ability to phase variants to identify pathogenic variants rapidly'[12], and provides methylation information.

Accessing novel prognostic biomarkers

The team found that Oxford Nanopore sequencing solves the parent-of-origin question for all cases of retinoblastoma from just the proband, providing access to a potentially important prognostic biomarker for this disease. This is particularly important for identifying variants on the paternal allele, which typically demonstrate worse clinical outcomes with more severe ocular disease than maternally inherited mutations.

In conclusion, targeted Oxford Nanopore sequencing provides 'the ability to phase a germline variant with an imprinting signal in a single sequencing run', potentially providing prognostic information for all retinoblastoma cases. Stacey *et al*. proposed that implementing this multiomic platform in clinical settings has the potential to provide 'a more complete diagnostic outlook for children and their families afflicted with [retinoblastoma] to better guide management and therapy in a more personalised manner'[12].

Case study 2

Improving the characterisation of acute paediatric leukaemia worldwide

There are many different types of blood cancers that impact various stages of the blood production cycle, with the most common type in children being leukaemia[47,48]. B cell acute lymphoblastic leukaemia (B-ALL) is one of the most common types of leukaemia, which causes uncontrolled growth of non-functional B cell lymphocytes[48,6]. Acute myeloid leukaemia (AML) is another type of leukaemia, leading to the fast growth of monocytes and granulocytes[49]. Both lead to the build-up of non-functional cells in the blood and bone marrow, preventing normal blood cells from developing[47,49].

Not only is B-ALL the most common type of leukaemia, but it is also the most common type of paediatric cancer, with AML making up 15–20% of paediatric cancers[6]. Due to both being acute leukaemias, disease progression is fast[49] and requires timely classification into genomic subtypes to determine prognosis and treatment plans[6].

The arsenal of genetic tests used in the standard-of-care pipeline

Currently, B-ALL and AML are classified into genomic subtypes through a range of complicated and expensive genetic tests known as the 'standard-of-care' diagnostic pipeline. Tests include karyotyping, fluorescence *in situ* hybridisation (FISH), PCR, targeted panels, and whole-genome sequencing[6]. However, the specific genetic tests used in the diagnostic odyssey vary between different clinical facilities, especially in low- and middle-income countries (LMICs) where resources are limited[6,50]. Despite these limitations, most cancer treatment centres across the globe are in LMICs[6] with significantly lower incidence rates[50], highlighting the need for accessible sequencing.

To improve access to testing, Geyer *et al*. investigated how to create a single, sequencing-based classification workflow to simplify the standard-of-care pipeline, with the aim of reducing testing costs and time. Typically, short-read-only sequencing methods are used to perform genomic subtyping of AML and B-ALL to resolve structural variants (SVs). However, Geyer *et al*. investigated Oxford Nanopore sequencing — a platform capable of generating reads of any length, from short to ultra long — because it has the 'potential to generate faster and more cost-effective results compared with short-read sequencing methods' on a single consolidated platform[6].

Moving the standard-of-care pipeline to a single platform

Oxford Nanopore sequencing can generate reads of unrestricted length, providing more comprehensive data than short reads, because the long nanopore reads can span complex and repetitive genomic regions. Geyer *et al*. noted that nanopore data is streamed in real time, enabling rapid access to results, and that adaptive sampling — a unique targeted sequencing method without additional sample preparation — provides 'comprehensive genomic sequencing, offering a more efficient use of resources and time'[6].

Geyer and colleagues performed Oxford Nanopore whole-genome sequencing on 57 acute leukaemia research samples. The samples were sequenced in singleplex and multiplex for up to 72 hours on MinION and PromethION 2 Solo devices; however, they found results could be inferred from as little as 15 minutes and up to six hours of sequencing. During sequencing, adaptive

sampling was employed to enrich 59 genes commonly involved in translocations or fusions in B-ALL and AML.

For six samples, 152 genes were targeted with adaptive sampling to enrich for additional genes and regions associated with AML, B-ALL, and T cell ALL, another type of acute lymphoblastic leukaemia.

'The results presented suggest that, as a single-assay classification tool, nanopore-based adaptive whole-genome sequencing accurately classifies B-ALL into genomic subtypes, with the potential to identify clinically relevant AML genomic subtypes, as well as clinically actionable pharmacogenetic subtypes'

Geyer *et al.* 2024[6]

Generating more comprehensive data than traditional genetic tests

From sample receipt to data analysis, the Oxford Nanopore sequencing-based pipeline took between six to nine hours and provided data that was '100% consistent with clinically derived genomic subtype classification'[6]. Using a novel bioinformatics pipeline, Geyer *et al.* analysed nanopore data to identify genetic variants from chromosome-level abnormalities and large-scale SVs down to copy number and single nucleotide variants in a single rapid assay.

Geyer *et al.* achieved good depth of sequencing coverage across the genome (average of 12x), and an average of 86x coverage over targeted genes. This provided enough comprehensive data to identify genomic variants that typically require multiple traditional molecular tests to characterise. Using both whole-genome and targeted sequencing data, the team accurately identified clinically relevant karyotype profiles and gene fusions in acute leukaemias. Additionally, targeted data led to the identification of small-scale structural variants, copy number variants, and single nucleotide variants. Taken together, these results demonstrate that the Oxford Nanopore assay reliably detects clinically relevant genomic variation and tumorigenic drivers, which have the potential to improve future diagnostic outcomes.

Furthermore, long Oxford Nanopore reads provided additional information inaccessible to legacy sequencing methods, such as data to identify complex genomic variants. For example, *DUX4* rearrangements are the cause of 14% of B-ALL cases and are not well-characterised using traditional methods[51]. Geyer *et al.* detected an SV and corresponding *DUX4:IGH* gene fusion using nanopore sequencing from a single run, demonstrating the potential of Oxford Nanopore sequencing for advanced diagnostic benefits in the future.

'We have demonstrated proof of principle that nanopore long-read WGS can provide all clinically relevant genomic information currently offered by traditional diagnostic testing (karyotype, FISH, and occasional microarray) for pediatric acute leukemia'

Geyer *et al.* 2024[6]

Democratising access to sequencing

Geyer *et al.* highlighted that the nanopore sequencing-based workflow is more cost-effective than the traditional standard-of-care pipeline comprising multiple molecular tests, due to simple nanopore workflows that require few reagents. The authors concluded that further 'optimization and automation of the sequencing pipeline are essential to democratizing this approach'[6]. However, this study demonstrates that Oxford Nanopore Technologies has a role in potential improvements in the current variable complex standard-of-care assays for classifying paediatric acute leukaemias by providing a cost-effective and rapid single-platform assay.

Case study 3

Accurate identification of cancer-predisposing deep intronic variants in tumour-suppressor genes with Oxford Nanopore sequencing

Detecting deep intronic variants is challenging but important

Deep intronic variants are genetic mutations located within introns. Whilst most variants of this type are thought to be benign, some rare variants can alter RNA splicing[52]. This alteration in splicing can lead to exonification of intronic sequence, creating pseudoexons and causing abnormal transcriptional consequences such as premature stop codons and loss of gene function[53]. Damaging deep intronic variants are likely to be understudied and underreported causes of disease because they are missed — most clinical genetic tests do not sequence full intronic regions and many of these variants are within complex repetitive areas of the genome that cannot be aligned with legacy short-read sequencing techniques.

Despite advances in the discovery and detection of the genes and variants responsible for inherited predisposition to cancer, a considerable portion of the hereditary risk associated with breast, ovarian, pancreatic, and metastatic prostate among other cancers remains unidentified. Recent research has pointed towards deep intronic variants as an explanation for this missing heritability. James *et al*. found significant enrichment for rare deep intronic variants in *BRCA1*, *BRCA2*, and *PALB2* — all tumour-suppressor genes — among patients with familial breast cancer versus older women who are cancer free[54]. Additionally, a study by Ambry Genetics, which used short-read RNA sequencing, identified likely pathogenic deep intronic variants that were previously classified as variants of uncertain significance[55].

Investigating cancer predisposition with targeted Oxford Nanopore sequencing

To systematically evaluate deep intronic variants, Gulsuner and AbuRayyan *et al*. used targeted nanopore sequencing to analyse DNA and cDNA from patient research samples[22]. These samples were taken from families severely affected with breast, ovarian, pancreatic, and/or metastatic prostate cancer, but with no causal variant identified by multiple conventional methods, including exome sequencing and short-read whole-genome sequencing. Oxford Nanopore technology generates reads of any length — from short to ultra long — meaning that, for DNA sequencing, entire intronic regions can be captured within a single read, overcoming the challenges associated with highly repetitive regions; and for cDNA sequencing, single nanopore reads can span multiple exons, revealing the precise locations of splice sites for all transcripts.

The authors carried out two nanopore sequencing workflows in tandem. First, they performed targeted DNA sequencing to identify deep intronic variants in 10 tumour-suppressor genes — *BRCA1*, *BRCA2*, *PALB2*, *ATM*, *CHEK2*, *BARD1*, *BRIP1*, *RAD51C*, *RAD51D*, and *TP53*. Loss-of-function variants in these genes are widely thought to be responsible for predisposition to breast and ovarian cancer. Then, the researchers used targeted cDNA sequencing to investigate whether the deep intronic variants identified within these genes had an impact on the transcripts.

> ‘Long-read genomic and cDNA sequencing, carried out in tandem, are effective in detecting this class of variation’
>
> Gulsuner, S. and AbuRayyan, A. *et al*.[22]

For DNA sequencing, the authors prepared patient-derived DNA from 240 affected relatives from 120 unsolved families using the Native Barcoding Kit and sequenced the libraries on PromethION Flow Cells for 72 hours. The researchers employed adaptive sampling to enrich for and sequence 1 Mb regions around the 10 tumour-suppressor genes. Adaptive sampling is a unique on-device target enrichment methodology from Oxford Nanopore that allows specific genomic regions to be targeted without upfront wet

laboratory sample enrichment, providing high depth of coverage for targets whilst 'simplifying library preparation'. This simple targeted nanopore workflow revealed 92 rare deep intronic variants in 88 (73%) of the families.

The team then used the *in silico* tools SpliceAI[56] and Pangolin[57] to predict which variants would have a functional consequence. This analysis prioritised seven deep intronic variants present in eight families for further evaluation — three in *BRCA1*, one in *PALB2*, and three in *ATM*. To perform targeted cDNA nanopore sequencing, the authors reverse transcribed patient-derived RNA with a pool of gene-specific primers, enriching the candidate genes. Individual cDNA libraries were sequenced on PromethION Flow Cells for 72 hours, resulting in >100x depth of sequencing coverage at the critical gene for each of the samples. All seven variants caused exonification and yielded transcripts with pseudoexons, introducing premature stop codons and ultimately leading to loss of gene function.

The future of deep intronic variant identification

Using targeted DNA and cDNA nanopore sequencing, Gulsuner and AbuRayyan *et al*. successfully identified rare deep intronic variants contributing to inherited cancer risk in eight of the 120 (6%) previously unsolved families. Nanopore sequencing effectively detected these variants that had been missed by legacy short-read sequencing methods and other conventional approaches.

Therefore, the integration of Oxford Nanopore sequencing into variant discovery workflows can better characterise previously undetected pathogenic variants. This approach has the potential to enhance genetic diagnostics and personalised medicine in the future, ensuring patients with rare deep intronic variants receive precise risk predictions, leading to tailored therapeutic strategies, such as adjusted cancer screening schedules.

About Oxford Nanopore Technologies

The goal of Oxford Nanopore Technologies is to enable the analysis of anything, by anyone, anywhere. The company offers the only sequencing technology to combine scalability — from portable to ultra-high-throughput formats — with real-time data delivery and the ability to elucidate accurate, rich biological data through the analysis of short to ultra-long fragments of native DNA or RNA (**Figure 9**).

A range of sequencing devices is available, suitable for all cancer research applications and sample throughput requirements (**Figure 9**).

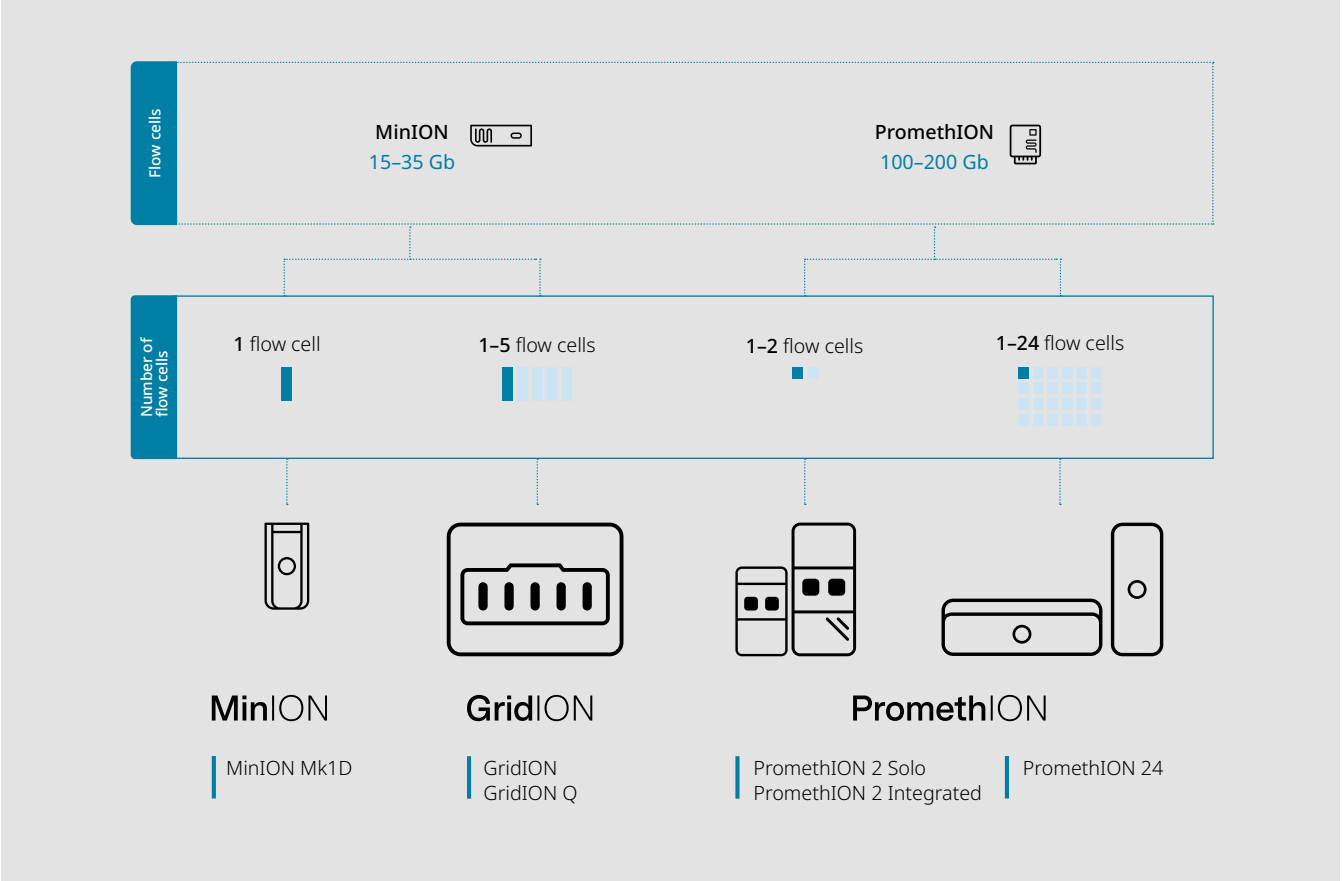

Figure 9. A range of Oxford Nanopore sequencing devices are available, providing high-output and scalable throughput to suit all requirements. The continual development of nanopore technology delivers increased output for even more cost-effective analyses. Information correct at time of publication, for the latest information, visit nanoporetech.com.

For more information about utilising nanopore sequencing for cancer research, including getting started guides, best practice workflows, and customer presentations, visit: nanoporetech.com/cancer-research.

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