CASSE STUDY

Cell-free DNA nanopore sequencing and methylation detection — promising potential for non-invasive cancer monitoring

The analysis of circulating tumour DNA (ctDNA) from liquid biopsies is an active area of research and has been shown to be a powerful method for minimally-invasive cancer detection and monitoring. Additionally, the epigenetic status of cell-free DNA (cfDNA) is emerging as a promising biomarker in personalised disease monitoring, and may also prove valuable in cancer screening. Nanopore sequencing technology has been shown to allow accurate and sensitive detection of low-abundance cfDNA and ctDNA, and with the ability to directly detect DNA methylation from nanopore sequencing data, without the need for conversion or amplification steps during sample preparation, the platform shows potential as a ‘powerful tool for liquid biopsy’ in the future.

Martignano and colleagues, based at ISPRO, Florence, Italy, have previously published their innovative work using shallow nanopore whole-genome sequencing (WGS) to detect copy number aberrations (CNAs) from liquid biopsy cancer research samples. Building on their knowledge, the group joined Katsman et al. at the Hebrew University in Jerusalem, Israel, and more recently demonstrated that shallow nanopore WGS of cfDNA and ctDNA from liquid biopsies could also be used to detect cell of origin, cancer-associated fragmentation signatures, and cancer-specific methylation features of cfDNA, with comparable results to short-read-based datasets.

Martignano and colleagues, based at ISPRO, Florence, Italy, have previously published their innovative work using shallow nanopore whole-genome sequencing (WGS) to detect copy number aberrations (CNAs) from liquid biopsy cancer research samples. Building on their knowledge, the group joined Katsman et al. at the Hebrew University in Jerusalem, Israel, and more recently demonstrated that shallow nanopore WGS of cfDNA and ctDNA from liquid biopsies could also be used to detect cell of origin, cancer-associated fragmentation signatures, and cancer-specific methylation features of cfDNA, with comparable results to short-read-based datasets. Summarising the platform, Katsman et al. stated that ‘the simplicity of native [Oxford Nanopore] sequencing and the number of features that can be extracted from a single run, combined with the low cost and portability of [the] sequencer, make it an interesting proposition’ for clinical research settings.

Using nanopore sequencing for methylation calling appealed to Katsman and colleagues as bisulfite WGS approaches for methylation detection can result in significant sample degradation, and loss of input material, and can obscure fragmentation patterns. Additionally, bisulfite sequencing cannot distinguish between 5mC and other modifications, such as 5hmC, 5fC, and 5CaC, which ‘are all in principle detectable by nanopore’.

Katsman et al. estimated the prevalence of cancer cells in their samples, based on cfDNA analysis alone, using nanopore sequencing and comparison with matched short-read WGS datasets. To better understand the impact of low sequencing depth, the team performed downsampling experiments, demonstrating that results were consistent when nanopore WGS data was downsampled to 0.2x average depth of coverage. While the short-read libraries were sequenced at significantly higher depth (median 1.3x), the tumour fraction estimates were highly similar between Oxford Nanopore and short-read sequencing.

They also showed that shallow nanopore sequencing reproduced the cell type composition of the normal, non-cancerous cells, which could only be determined from short-read bisulfite (WGBS) data with much higher sequencing depth.

Furthermore, Katsman et al. found that the high tumour fraction samples had shorter fragment sizes, and suggested, therefore, that fragment length differences could also potentially be used to classify different cancer types. With Oxford Nanopore technology, short fragments can be sequenced efficiently while maintaining the ability to capture much longer cfDNA fragments than possible with short-read sequencing (as shown recently by Yu et al.).

While noting that their sample size was small, the results obtained by Katsman et al. suggested that cancer-specific features of DNA methylation, fragmentation, and CNA were broadly concordant between their nanopore data and short-read WGS and WGBS datasets. Downsampling analysis showed that the genomic coverage targeted with nanopore sequencing (minimum of 0.2x) was sufficient to detect cancer-derived DNA in all samples based on DNA methylation profiles.

The authors noted other work in the field showing that fast sample preparation and rapid nanopore sequencing can allow methylation-based classification of tumour DNA in as little as 1–3 hours. While their study did not test this rapid sequencing paradigm, senior author Ben Berman of Hebrew University suggested that this is part of the ultimate goal of their nanopore-based liquid biopsy research – ‘the ability to perform a complete analysis within hours at the point of care’.

Products used

<table>
<thead>
<tr>
<th>Kit</th>
<th>Ligation Sequencing Kit</th>
<th>Native Barcoding Expansion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Device</td>
<td>MinION™</td>
<td></td>
</tr>
<tr>
<td>Tools</td>
<td>Guppy (v3.0.2)</td>
<td>Megalodon (v2.4.2)*</td>
</tr>
</tbody>
</table>

Find out more: nanoporetech.com/products
Cell-free DNA sequencing from <1 ng starting material, without PCR

Despite fast development in the field of cfDNA analysis, the fact that extracted cfDNA yields range from single nanograms, or less, per millilitre of plasma remains a bottleneck, as sequencing often requires hundreds of nanograms of DNA. To tackle this issue, Lau and colleagues developed a PCR- and bisulfite-free nanopore sequencing-based approach to efficiently characterise methylation profiles from cfDNA. Specifically, the team developed a series of steps to efficiently incorporate sample barcodes and nanopore sequencing adapters into cfDNA, and systematically optimised reaction conditions to maximise cfDNA library yield.

Their approach generated up to hundreds of millions of reads per cfDNA sample, which is an order of magnitude improvement over existing methods. For example, from 5 ng of input DNA, the yield was approximately six million aligned and filtered reads, and with 100 pg of input DNA, the yield was approximately 140,000 aligned reads.

They applied their method to the characterisation of cfDNA methylationomes of cancer research samples, and demonstrated the potential of using such profiles to perform longitudinal monitoring during treatment (Figure 1). The methylation profiles obtained revealed specific treatment responses and the emergence of treatment-resistant metastatic cancer.

Discussing their long-term vision for this research, the authors concluded that this approach has the potential to impact liquid biopsy diagnostics for cancer detection and characterisation.

This PCR-free process generates sequencing libraries from nanogram amounts or less of cfDNA per sample

This feasibility study suggests that Oxford Nanopore shallow WGS could be a powerful tool for liquid biopsy

---

**References**

7. Berman, B. Personal communication with Oxford Nanopore Technologies on 23 August 2022
8. Lau, BT. et al. bioRxiv. 497080 (2022)

---

**Nanopore Sequencing**

- Enabled direct detection of DNA methylation — with no need for amplification or conversion during sample prep
- Delivered fast access to results through rapid workflows, from sample prep to methylation analysis
- Enabled analysis of fragmentation, copy number, and methylation status, through efficient capture of short to long cfDNA fragments

Find out more about using nanopore sequencing for cancer research: nanoporetech.com/cancer-research

---

**Figure 1:** Longitudinal methylation profiles of cfDNA from colorectal cancer research samples. Total cfDNA reads (upper panel) and reads with methylation profiles matching those of the matched tumour (lower panel). After day 400, the fraction of reads with tumour-specific methylation changes dramatically increased, correlating with metastatic progression. (Figure from Lau et al. 2022).