Nanopore direct RNA sequencing reveals modification in full-length coronavirus genomes

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ABSTRACT

Background

Sequence analyses of RNA virus genomes remain challenging due to the exceptional genetic plasticity of these viruses. Because of high mutation and recombination rates, genome replication by viral RNA-dependent RNA polymerases leads to populations of closely related viruses that are generally referred to as 'quasispecies'. Although standard (short-read) sequencing technologies allow to readily determine consensus sequences for these 'quasispecies', it is far more difficult to reconstruct large numbers of full-length haplotypes of (i) RNA virus genomes and (ii) subgenome-length (sg) RNAs comprised of noncontiguous genome regions that may be present in these virus populations. Here, we used a full-length, direct RNA sequencing (DRS) approach without any amplification step to characterize viral RNAs produced in cells infected with a human coronavirus representing one of the largest RNA virus genomes known to date.

Results

Using DRS, we were able to map the longest (∼26 kb) contiguous read to the viral reference genome. By combining Illumina and nanopore sequencing, a highly accurate consensus sequence of the human coronavirus (HCoV) 229E genome (27.3 kb) was reconstructed. Furthermore, using long reads that did not require an assembly step, we were able to identify, in infected cells, diverse and novel HCoV-229E sg RNAs that remain to be characterized. Also, the DRS approach, which does not require reverse transcription and amplification of RNA, allowed us to detect methylation sites in viral RNAs. Our work paves the way for haplotype-based analyses of viral quasispecies by demonstrating the feasibility of intra-sample haplotype separation. We also show how supplementary short-read sequencing (Illumina) can be used to reduce the error rate of nanopore sequencing.

Conclusions

Even though a number of technical challenges remain to be addressed to fully exploit the potential of the nanopore technology, our work illustrates that direct RNA sequencing may significantly advance genomic studies of complex virus populations, including predictions on long-range interactions in individual full-length viral RNA haplotypes.

Key words: direct RNA sequencing, Coronavirus, nanopore, haplotype, subgenomic

Background

Coronaviruses (subfamily *Coronavirinae*, family *Coronaviridae*, order *Nidovirales*) are enveloped positive-sense (+) single-stranded (ss) RNA viruses that infect a variety of mammalian and avian hosts and are of significant medical and economic importance, as illustrated by recent zoonotic transmissions from diverse animal hosts to humans [1,2]. The genome sizes of coronaviruses (∼30 kb) exceed those of most other RNA viruses. Coronaviruses use a special mechanism called

discountinuous extension of minus strands[3,4] to produce a nested set of 5'- and 3'-coterminal subgenomic (sg) mRNAs that carry a common 5' leader sequence that is identical to the 5' end of the viral genome[5,6]. These sg mRNAs contain a different number of open reading frames (ORFs) that encode the viral structural proteins and several accessory proteins. With very few exceptions, only the 5'-located ORF (which is absent from the next smaller sg mRNA) is translated into protein (Fig. 1).

In HCoV-229E-infected cells, at total of 7 major viral RNAs are produced. The viral genome (RNA 1) is occasionally referred to as mRNA 1 because it (also) has an mRNA function. In its 5'-terminal region, the genome RNA contains two large ORFs, 1a and 1b, that encode the viral replicase polyproteins 1a and 1ab. mRNAs 2, 4, 5, 6, and 7 are used to produce the S protein, accessory protein 4, E protein, M protein and N protein, respectively. The 5'-ORF present in RNA 3 starts contains the central and 3' regions of the S gene. Although this sg RNA has been consistently identified in HCoV-229E-infected cells, its mRNA function has been disputed and there is currently no evidence that this RNA is translated into protein[7–9].

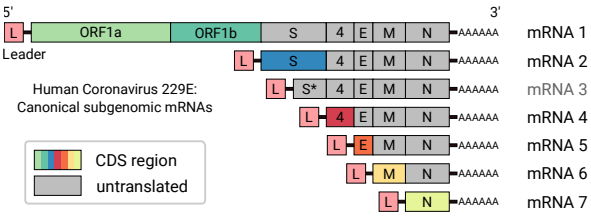

Figure 1: Scheme of genomic and subgenomic (sg) RNAs produced in HCoV-229E-infected cells[7,8]. Translation of the 5'-terminal ORF(s) of the respective RNA gives rise to the various viral structural and nonstructural proteins (indicated by different colors). RNA 3 is considered defective and unlikely to be translated into protein. Each RNA has a 3'-polyA tail and carries a 5'-leader sequence that is identical to the 5'-end of the genome. In a process called discontinuous extension of negative strands, negative-strand RNA synthesis is attenuated at specific genomic positions. After transfer of the nascent strand to an upstream position on the template, RNA synthesis is continued by copying the 5'-leader sequence. As a result, the 3'-ends of coronavirus minus-strand RNAs are equipped with the complement of the 5'-leader. The latter process is guided by base-pairing interactions between the transcription-regulating sequence (TRS) located immediately downstream of the leader sequence (TRS-L) at the 5'-end of the genome and the sequence complement of a TRS located upstream of one of the ORFs present in the 3'-proximal genome region (TRS-B).

Like many other +RNA viruses, coronaviruses show high rates of recombination[10–12]. In fact, the mechanism to produce 5' leader-containing sg mRNAs represents a prime example for copy-choice RNA recombination that, in this particular case, is guided by complex RNA-RNA interactions involving the transcription-regulating sequence (TRS) core sequences and likely requires additional interactions of viral proteins with specific RNA signals. In other virus systems, RNA recombination has been shown to generate 'transcriptional units' that control the expression of individual components of the genome[13]. The mechanisms involved in viral RNA recombination are diverse and may even extend to non-replicating systems[14]. In the vast majority of cases, recombination results in defective RNA (dRNA) copies that lack essential cis-active elements and thus cannot be replicated. In other cases, functional recombinant RNA with new properties, such as the ability to replicate in a new host, may emerge[15–18]. In yet other cases, defective interfering RNAs (DI-RNAs) may be produced. These defective (subgenome-length) RNAs contain all the cis-acting elements required for efficient replication by a helper virus polymerase and, therefore, represent parasitic RNAs that compete for components of the viral replication/transcription complex with non-defective viral RNAs[19].

To elucidate the many facets of recombination and to determine full-length haplotypes of, for example, virus mutants/variants in complex viral populations (quasispecies), long-read sequencing has become the method of choice. Short-read Next-Generation Sequencing technologies (NGS) – such as IonTorrent and Illumina – are restricted by read length (200-400 nucleotides[20]). For example, the use of inevitably highly fragmented viral RNAs considerably complicates the investigation of haplotypes[21,22]. Since the nested coronavirus mRNAs are almost identical to the original genome sequence, short-read data can usually not be unambiguously assigned to specific sg RNA species.

In this study, we performed direct RNA sequencing (DRS) on an array of nanopores, as developed by Oxford Nanopore Technologies (ONT)[23]. Nanopore sequencing does not have a limited reading length but is limited only by fragmentation of the input material[24–26]. Further, by using DRS, we avoid several drawbacks of previous sequencing methods, in particular cDNA synthesis and amplification of the input material. Thus, for example, cDNA synthesis can create artificial RNA-RNA chimerae[27] that are difficult to discriminate from naturally occurring chimerae (such as spliced RNAs). Also, amplification prior to

sequencing would remove all RNA modifications from the input material, whereas the nanopore sequencing technology preserves these modifications[23,28].

Recently, nanopore sequencing has been used for metagenomic forays into the virosphere[29] and studies focusing on transmission routes[30,31]. Furthermore, viral transcriptomes have been investigated using nanopore sequencing of cDNA[32–35], being subject to bias from reverse transcription and amplification. Other studies used DRS to study the human poly(A) transcriptome[36] and the transcriptome of DNA viruses such as HSV[37]. Furthermore, the genome of Influenza A virus has been completely sequenced in its original form using direct RNA sequencing[38].

In the present study, we sequenced one of the largest known RNA genomes, that of HCoV-229E, a member of the genus Alphacoronavirus, with a genome size of about 27,300 nt and determined complex architectural details for viral sg RNAs produced in cells infected with recombinant HCoV-229E. Using DRS, the longest read that was mapped to the viral reference genome had a contiguous length of over 26 kb, covering nearly the entire coronavirus genome. Sequence analysis of thousands of full-length sg RNAs allowed us to determine the architectures (including leader-body junction sites) of the major viral mRNAs. In addition, our study revealed an enormous diversity of additional HCoV-229E sg RNAs, probably including DI-RNAs. It remains to be studied if (some of) the newly identified sg RNAs have specific functions in the viral replication cycle.
This is also the first work to present data on RNA methylation without prior *in vitro* treatment of the sample. The RNA modifications were called directly from the raw nanopore signal, as has been shown for DNA[39,40].

Results

Full genome sequencing without amplification

We sequenced total RNA samples obtained from Huh-7 cells infected with serially passaged recombinant human coronaviruses: wild-type (WT) HCoV-229E, HCoV-229E_SL2-SARS, and HCoV-229E_SL2-BCoV, respectively. In the latter two viruses, a conserved stem-loop structure (SL2) residing in the HCoV-229E 5'-UTR was replaced with the equivalent SL2 element from SARS-CoV and BCoV, respectively[41]. Total RNA samples obtained for the latter two (chimeric) viruses were pooled prior to sequence analysis. Hereafter, we refer to the first sample as WT RNA and to the second (pooled) sample as SL2 RNA (see Methods and Materials).

We performed two direct RNA sequencing runs on a MinION nanopore sequencer. As shown in Table 1, we achieved a throughput of about 0.25 gigabases with 200 k reads per sample. See SFig. 1 A for an overview of the read length distribution. About one third of the reads mapped to the reference human and HCoV-229E sequences, respectively. About 16 % mapped to the yeast enolase 2 gene, a calibration standard added during the library preparation. The remaining 16 % are of unknown source.

The visualized raw voltage signal of a nanopore read is commonly called "squiggle" (see Fig. 2). Different from all previous sequencing technologies, nanopore sequencing preserves the information about base modifications in the raw signal[23]. However, one of the biggest challenges is the accurate mapping of the raw voltage signal to bases ("base calling").

As expected for nanopore DRS[23,38], reads had a median uncorrected error rate of about 15.5 % (see Fig. 3). This included gaps but omitted dis-continuous sites longer than six nucleotides since they indicated recombination. Half of all errors were deletions, which occur because the signal from homopolymer sequences is often misinterpreted by the basecaller. A quarter of the errors were substitutions, which we argue are largely due to modified bases that impede the basecaller's ability to assign bases correctly.

The HCoV-229E genome was 99.86 % covered, with a large coverage bias towards both ends (see Fig. 4 and Fig. 1). The high coverage of the 3'-end reflects the higher abundance of mRNAs produced from the 3'-terminal genome regions and is a result of the discontinuous transcription mechanism employed by coronaviruses and several other nidoviruses[5,42,43]. The 3'-coverage is further increased by the directional sequencing that starts from the mRNA 3'-terminal poly(A) tail. Also, the observed coverage bias for the very 5'-end results from the coronavirus-specific transcription mechanism because all viral mRNAs are equipped with the 65-nt 5'-leader sequence derived from the 5'-end of the genome. The remainder of the high 5'-coverage bias likely reflects the presence of high numbers of DI-RNAs in which 5'- and 3'-proximal genomic sequences were fused, probably resulting from illegitimate recombination events as shown previously for other coronaviruses[10,12,44].

An alignment of the longest reads from both samples to the HCoV-229E reference indicates that they represent near complete virus genomes (SFig 1 B). The observed peaks in the aligned reads length distribution (see Fig. 6) corresponded very well with the abundances of the known

Table 1: Sequencing and error statistics. Both samples mainly contain HCoV-229E reads, but also host (*Homo sapiens*) transcripts and *S. cerevisiae* enolase 2 (ENO2) mRNA (which was used as a calibration standard added during library preparation). Half of the sequencing errors were deletion errors, probably resulting to a large extent from basecalling at homopolymer stretches. The *S. cerevisiae* enolase 2 mRNA reads display an overall reduced error rate because the `Albacore` basecaller was trained on this calibration strand. Note that all error rates report differences to the reference genome and thus include actual genetic variation.

Sample	Subset	#reads	longest	median	%subst.	%insert.	%deletions	%errors
WT	complete sample	224,724	26,210	826	-	-	-	-
	HCoV-229E reference	74,815	26,210	1,415	4.292	2.564	8.264	15.119
mapped to	*H. sapiens*	60,773	9,562	778	4.370	2.653	8.720	15.743
	S. cerevisiae ENO2	35,490	6,604	636	3.752	2.381	6.360	**12.492**
SL2	complete sample	180,906	25,885	1342	-	-	-	-
	HCoV-229E w/ SARS SL2	65,044	25,885	1,626	4.396	2.681	8.508	15.585
mapped to	*H. sapiens*	54,599	11,723	960	4.557	2.763	8.929	16.248
	S. cerevisiae ENO2	18,597	7,905	860	4.021	2.464	6.890	**13.374**

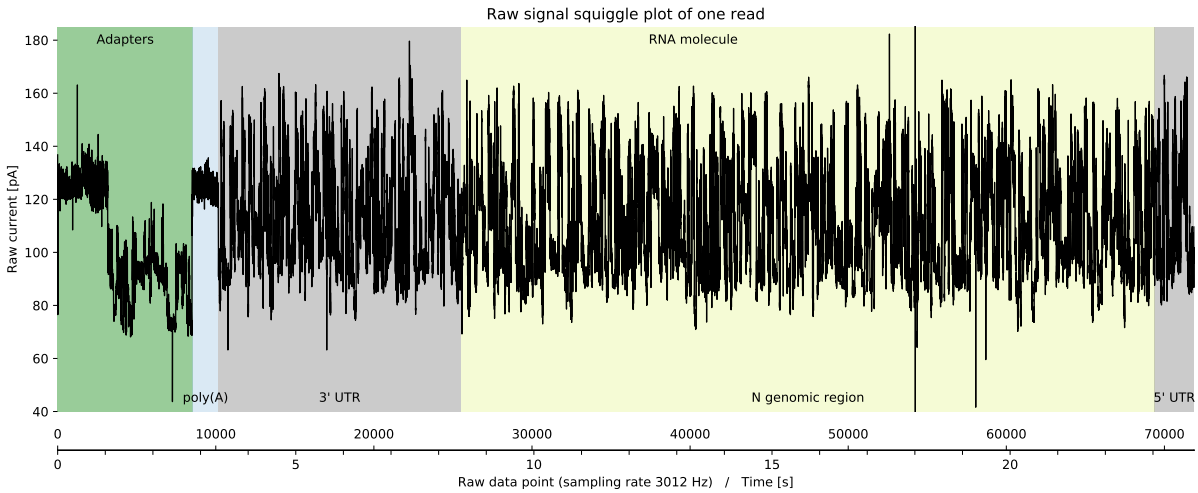

Figure 2: Raw signal "squiggle" plot of one example read from the WT sample. Of note, sequencing starts from the 3'-polyadenylated end of the mRNA. The MinION measures the current inside the nanopore with a fixed sampling rate while a molecule passes through. Basecalling algorithms work directly on this raw signal data to infer the nucleotide sequence[23]. Unlike all other current sequencing technologies, nanopore sequencing conserves the information about base modifications in the raw signal[23]. SFig. 2 exemplarily shows a 5mC methylation pattern detected on raw signal data.

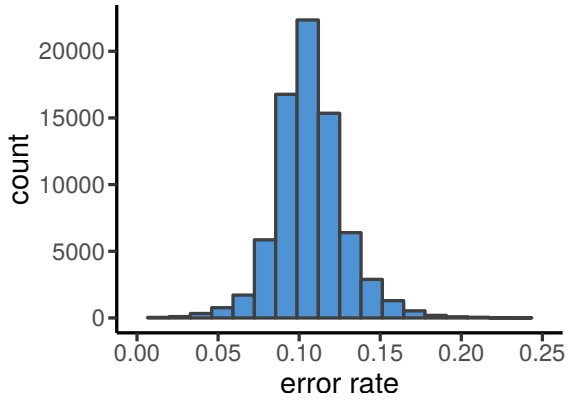

Figure 3: The distribution of correctly sequenced bases for direct RNA sequencing using nanopores has a median of about 90 %, as illustrated here for the WT HCoV-229E sample.

mRNAs produced in HCoV-229E-infected cells[7–9] (see Fig. 1).

The median read length for the combined set of reads from both samples was 826 nt, with a maximum of 26,210 nt, covering 99.86 % of the 27,276-nt-long virus genome, missing only 21 nt at the 5'-end, 15 nt at the 3'-end and those nucleotides that correspond to the skewed error distribution, with 5.7 percentage points more deletions than insertions (see Tab. 1). The median read length might sound short, however most of the viral RNAs (including many DI-RNAs) identified in HCoV-229E-infected cells were below 2,000 nt in length. Furthermore, this number nearly doubles the longest read length that can be obtained with short-read sequencing methods. We observed an abundance of very short reads due to RNA degradation during the extraction and library preparation. Innovations in these fields will directly translate into larger median read lengths.
We corrected the reads and obtained 99 % identity in both samples (WT, SL2) with the help of the consensus caller `ococo`[45] using the reference genome and all reads mapping to it. In addition, we assembled WT and SL2 consensus sequences using Nanopore and Illumina data with `HG-CoLoR`[46] in an approach that uses long nanopore reads to traverse an assembly graph constructed from short Illumina reads. We thereby recovered 99.57 % of the reference genome at 99.90 % sequence identity from an assembly graph traversal with the longest read in our dataset. This hybrid approach illustrates how short- and long-read technologies can be combined to reconstruct long transcripts accurately, which will greatly facilitate studies of haplotypes.

Uncharacterized subgenomic and defective interfering RNAs

In addition to the leader-to-body junctions expected for the canonical sg mRNAs 2, 4, 5, 6, and 7, we observed a surprisingly high number of recombination sites (Fig. 5) which were consistently found in our samples but have not been described previously (Fig. 5). In this study, we defined a recombination site as any site that flanks more than 100 consecutive gaps, as determined in a discontinuous mapping ("spliced" mapping). While there is currently no consensus on how to define such sites, we believe this to be a conservative definition, as this type of pattern is unlikely to result from e.g. miscalled homopolymer runs which, in our experience, typically affect less than 10 consecutive bases. We observe all known canonical HCoV-229E mRNAs at their expected lengths, including the (presumably) non-coding mRNA 3 (Fig. 6).

The aligned reads distribution revealed clusters for all known mRNAs which closely fit the expected molecule lengths (Fig. 6). The cluster positions show double peaks with a consistent distance of ∼65 nt, i.e. the length of the leader sequence. We observed that the 5'-end of reads has larger-than-average error rates and is often missing nucleotides. This might be due to a bias of the basecaller towards the end of reads. This is plausible, because the underlying classification algorithm is a bidirectional (i.e. forward and backward looking) long-short-term memory neural network (LSTM). The mapping algorithm was often unable to align these erroneous 5'-ends, leading to soft-clipped bases. Thus, for many reads representing canonical mRNAs, the included leader sequence was not aligned, which gives rise to the secondary peak at each cluster position. Many leader sequences can be recovered with a hidden markov model on these soft-clipped 5'-ends (data not shown). We also observed additional clusters which likely correspond to highly abundant dRNAs (Fig. 6).

Interestingly, we also observed several unexpected recombination sites, e.g. at positions 3,000 to 4,000 (within ORF1a, see Fig. 5). These sites were confirmed by both nanopore and Illumina sequencing. They had a high read support and defined margins, suggesting a specific synthesis/amplification of these sg RNAs which, most likely, represent DI-RNAs. Since DI-RNAs are byproducts of viral replication and transcription, they present a larger diversity than the canonical viral mRNAs[47–51].

Nanopore sequencing captures recombination events far better than Illumina, which allowed us to study even complex sg RNAs (composed

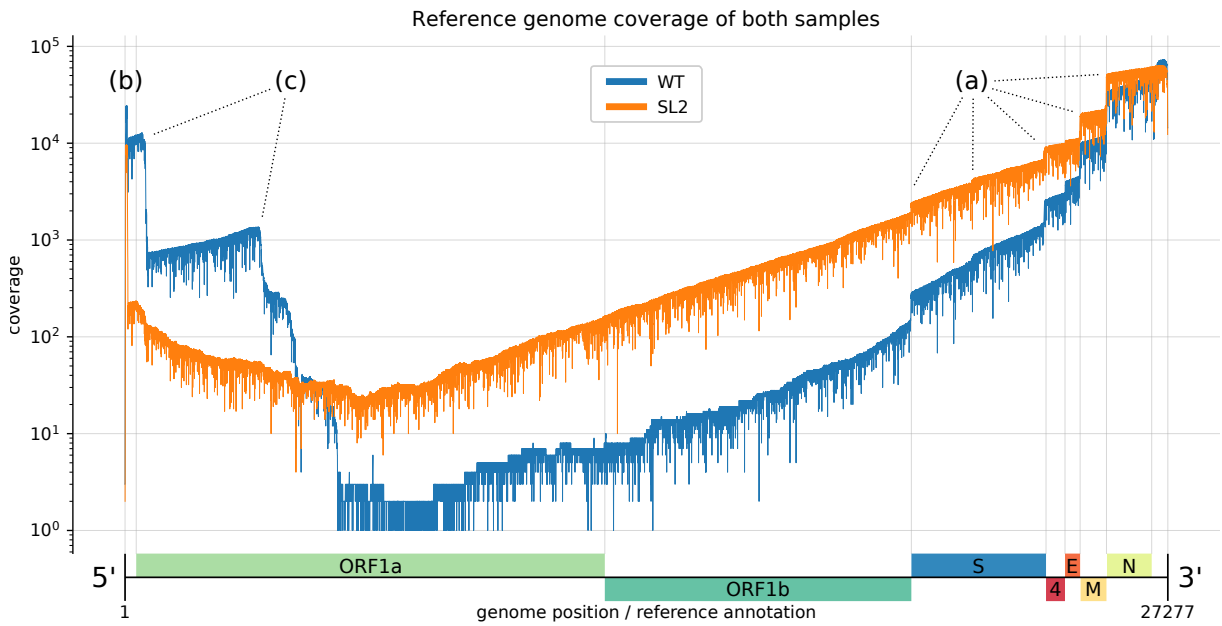

Figure 4: Reference genome coverage of the HCoV-229E WT sample (blue) and the HCoV-229E SL2 sample (orange) based on alignments with `minimap2`. There is an inverse correlation between sg RNA abundance and length. **(a)** Notable vertical 'steps' in the coverage correspond to borders expected for the canonical sg RNAs (see Fig. 1). **(b)** The presence of the leader sequence (ca. 65 nt) in canonical sg RNAs gives rise to the sharp coverage peak at the 5'-end. **(c)** We also observed unexpected 'steps', especially in the WT sample (blue). We hypothesize that the sequences correspond to DI-RNA molecules that may arise by recombination at TRS-like sequence motifs as well as other sites displaying sequence similarities that are sufficient to support illegitimate recombination events (see Fig. 5). We attribute the difference in the observed (non-canonical) recombination sites between the two samples to biological factors that we either did not control for or do not know (see also legend to Fig. 5).

of sequences derived from more than 2 non-contiguous genome regions) at much higher resolution: For example, we found sg RNAs with up to four recombination sites in the 5'- and 3'-terminal genome regions (Fig. 5).

Many sg RNAs do not represent canonical mRNAs

As pointed out above, a specific form of copy-choice RNA recombination (called discontinous extension of negative strands) has been shown to generate appropriate minus-strand templates for the production of 5' leader-containing mRNAs[3–5]. A critical step in this process involves base-pairing interactions of TRS elements located at distant positions in the viral genome[43,52]. Coronavirus TRS elements share a virus-specific conserved octanucleotide core sequence that, in the case of HCoV-229E, conforms to `UCU(C/A)AACU`. Only one TRS (which precedes ORF4) carries a replacement at position 1 of the TRS core sequence pairing[52], meaning that mismatches are tolerated as long as the energetic stability of the base-pairing interaction remains above a critical threshold.

Using our nanopore reads, we were able to provide further support for this model. Using about 3,000 candidate mRNAs, the nanopore reads were mapped to these 'mock' transcripts in a non-discontinuous manner (i.e. all reads had to map consecutively without large gaps). The majority of these '*in silico* transcripts' were found to represent known mRNAs containing the canonical leader-body junction sites, for which a high read support was obtained (see Fig. 5). However, we also identified sg RNAs that had recombined at other sites at low frequencies. We hypothesize that most of these RNAs represent DI-RNAs.

Using this procedure, about 12 % of the reads were confirmed to map to mRNAs produced according to the standard discontinuous transcription model. This likely represents an underestimate, as we omitted truncated sg RNAs probably resulting from RNA degradation and transcripts with more than one recombination site. Nevertheless, the proportion of canonical mRNAs in the total population of sg RNAs identified in these cells was surprisingly low.

Consistent 5mC methylation signatures of coronavirus RNA

Nanopore sequencing preserves information on nucleotide modifications. Using a trained model, DNA and RNA modifications such as 5mC methy-

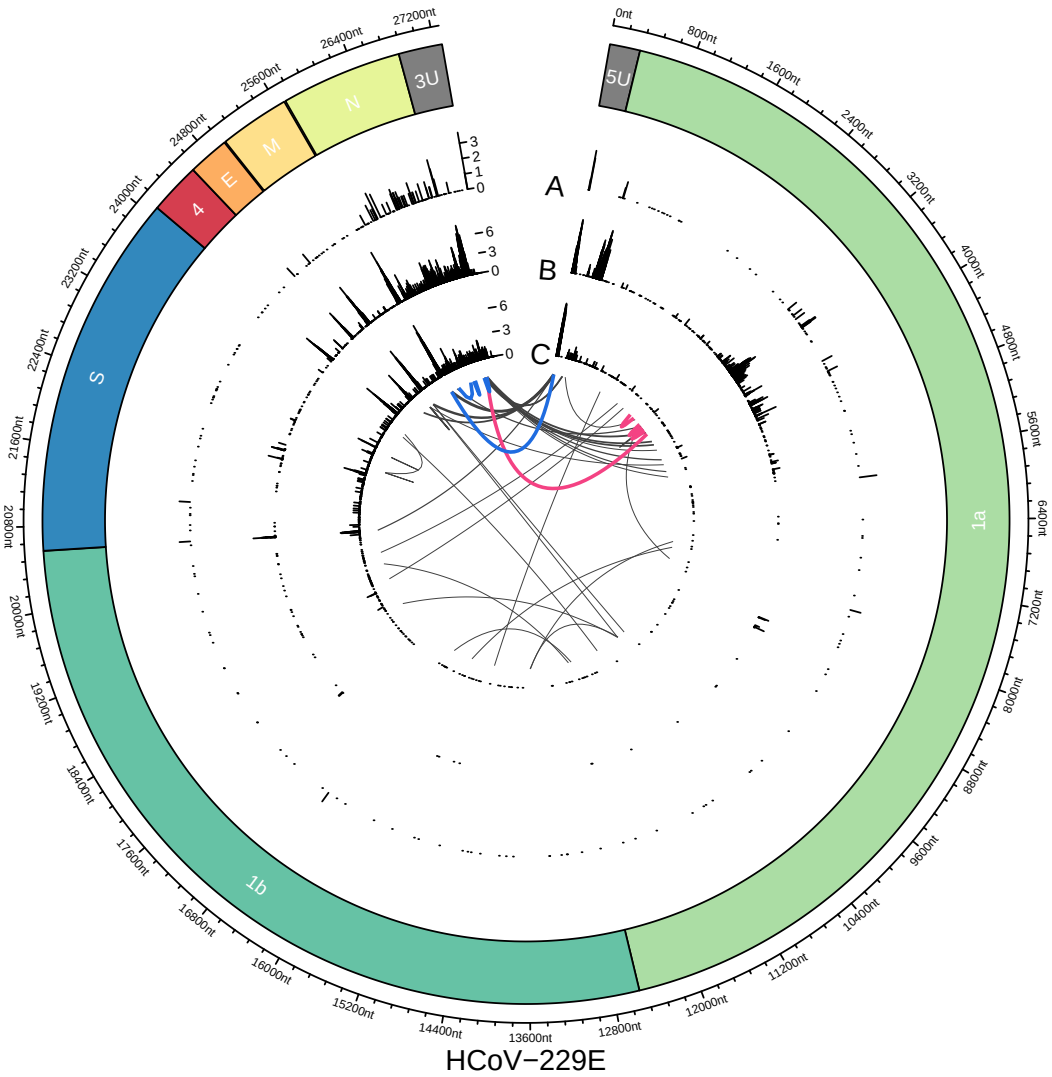

Figure 5: Joining of non-contiguous genome sequences in sg RNAs identified in HCoV-229E-infected cells. On the **circular axis** the annotations of the reference genome (including 5'-UTR (5U) and 3'-UTR (3U)) are shown. Genomic positions of 'discontinuous sites' identified in Illumina reads **(A, outer track)**, nanopore reads of sample HCoV-229E WT **(B, middle track)** and nanopore reads of sample HCoV-229E SL2 **(C, inner track)** reveal multiple recombination sites across the whole genome. An aggregation of recombination sites can be observed in the region that encodes the viral N protein. Furthermore, clear recombination sites can be seen at intergenic boundaries and at the 5'- and 3'-UTRs, with the former corresponding to the boundary between the leader sequence and the rest of the genome. Another prominent cluster can be observed in ORF1a in the WT nanopore sample, but not in SL2. This cluster is supported by the WT Illumina data, excluding sequencing bias as a potential source of error. We hypothesize that since samples WT and SL2 were obtained from non-plaque-purified serially passaged virus populations derived from *in vitro*-transcribed genome RNAs transfected into Huh-7 cells, differences in the proportion of full-length transcripts versus abortive transcripts could translate into different patterns of recombination. Generally, nanopore-based sequencing allows more detailed analysis of recombination events due to the long read length. Even complex isoforms such as two exemplary reads, each with four discontinuous segments, can be observed (blue and pink). Simulated *in silico* transcripts (black lines) are shown in the center. These were generated by recombining *in silico* 5' and 3' genome segments using pairs of putative TRS motifs in the genome. The aim was to explore the space of putative isoforms given the model assumption of TRS-based, similarity-assisted copy-choice recombination. We considered a putative isoform as plausible if it had a read support of 5. Only plausible isoforms are displayed.

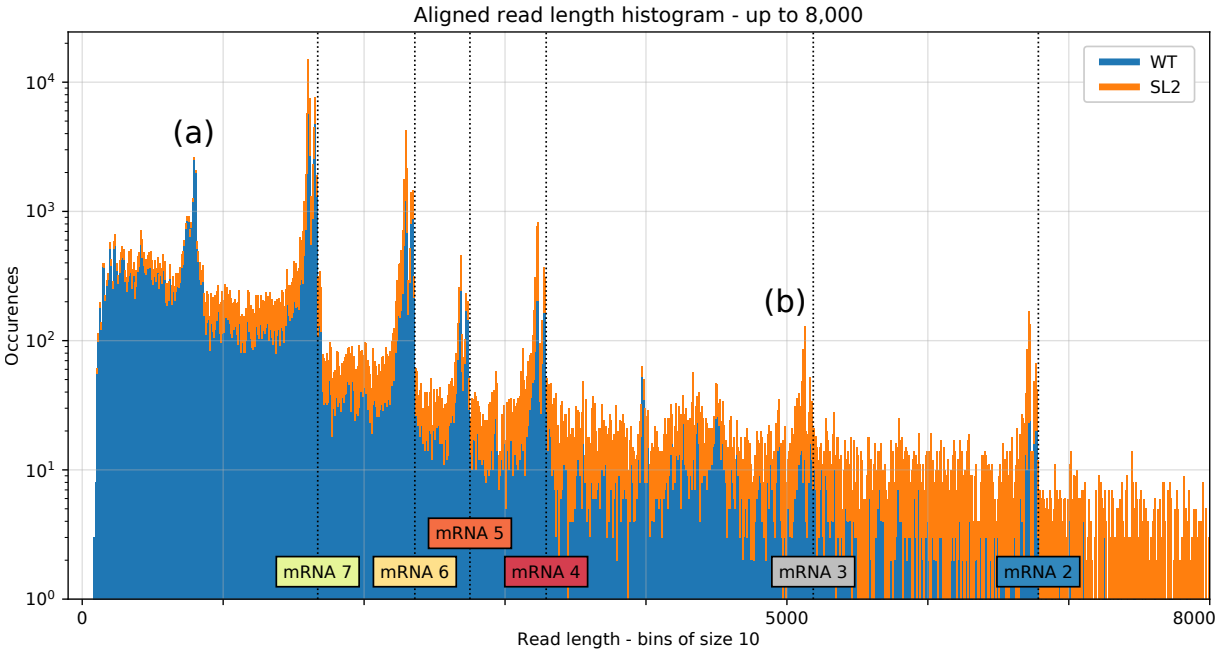

Figure 6: Distribution of aligned read lengths up to 8,000 nt for both samples (stacked) based on alignments with `minimap2`. We observed clusters that correspond well with the transcript lengths expected for canonical mRNAs (vertical dotted lines). The distribution shows double peaks at the cluster positions because reads corresponding to mRNAs often miss the leader sequence, possibly due to basecalling or mapping errors. We also observed additional clusters that likely correspond to highly abundant DI-RNAs. **(a)** indicates a cluster in the WT sample that respresents chimeric ∼820-nt sg RNAs comprised of both 5'- and 3'-terminal genome regions (∼540 nt and ∼280 nt, respectively). We propose the template switch for this transcript occurs around position 27,000 and RNA synthesis resumes at around position 540 (see corresponding peaks in Fig. 5 track B). **(b)** indicates a cluster from the SL2 sample that contains transcripts with an approximate length of 5,150 nt which represents mRNA 3 (see Fig. 1). These transcripts are probably formed due to a transcription stop at a TRS motif around position 22,150 (see corresponding peak in Fig. 5 track C).

lation could be identified (Fig. 7). To assess the false positive rate (FPR) of the methylation calling, we used an unmethylated RNA calibration standard (RCS) as a negative control which was added in the standard library preparation protocol for DRS. We considered a position to be methylated if at least 90 % of the reads showed a methylation signal for this particular position. Using this threshold, the estimated FPR was calculated to be below 5 %. Our experimental setup did not include a positive methylation control.

When analyzing 5mC methylation across various RNAs, we observed consistent patterns (Figure 7) that were reproducible for the corresponding genomic positions in different RNAs, suggesting that the methylation of coronavirus RNAs is sequence-specific and/or controlled by RNA structural elements. Methylated nucleotides could be identified across the genome, both in the leader sequence and in the body regions of viral mRNAs.

Discussion

We found the diversity of sg RNAs identified in coronavirus-infected cells to be surprisingly high, with many sg RNAs not corresponding to the known canonical mRNAs. These 'non-canonical' sg RNAs had abundant read support and full-length sequences could be obtained for most of these RNAs.

As indicated above, only 12 % of the sg RNAs were found to conform to our current understanding of discontinuous mRNA transcription in coronaviruses, resulting in mRNAs that (all) carry an identical 5'-leader sequence that is fused to the 3'-coding ('body') sequence of the respective mRNA. We however believe that 12 % represents an underestimate because a large number of sg RNAs were probably omitted from the analysis: (1) RNA molecules degrade rapidly under laboratory conditions, even when handled carefully. The resulting fragments will only be sequenced if they contain a poly(A) tail. (2) The high sequencing error may introduce mismappings, especially for low-quality reads. These reads would not be assigned to the canonical model under our assumptions because of the high number of mismatches. However, we think the associated bias is low, because `minimap2` is very robust against high error rates and because the reads are very long, thus ensuring that the mapper has sufficient aggregate information on a given read to position it very reliably on a reference. (3) The library preparation protocol for DRS includes the ligation of adapters via a T4 ligase. Any ligase will introduce artificial chimera. Again, we think that this does not affect our results substantially: First, this bias is random and it seems unlikely that we would observe the very same RNA 'isoform' many times if it was created by random ligation. Second, many 'isoforms' that we observed only once (e.g. those colored pink and blue in Figure 5) were structured plausibly: They contained a leader sequence and had recombined at expected (self-similar) sites corresponding to putative or validated TRSs, with downstream sequences being arranged in a linear 5'-3'-order. (4) Finally, it is important to note that the RNA used for DRS was isolated from cells infected with a serially passaged pool of recombinant viruses rescued after transfection of *in vitro*-transcribed genome-length (27.3 kb) RNAs. Transfection of preparations of *in vitro*-transcribed RNA of this large size likely included a significant proportion of abortive transcripts that lacked varying parts of the 3' genome regions, rendering them dysfunctional. It is reasonable to suggest that the presence of replication-incompetent RNAs lacking essential 3'-terminal genome regions may have triggered recombination events resulting in the emergence of DI-RNAs that contained all the 5'- and 3'-terminal cis-active elements required for RNA replication, but lacked non-essential internal genome regions. Upon serial passaging of the cell culture supernants for 21 times, DI-RNAs may have been enriched, especially in the HCoV-229E (wild-type) sample (Figure 5). Comparative DRS analyses of RNA obtained from cells infected with (i) plaque-purified HCoV-229E and (ii) newly rescued recombinant HCoV-229E (without prior plaque purification), respectively, would help to address the possible role of prematurely terminated *in vitro* transcripts produced from full-length cDNA in triggering the large number of DI-RNAs observed in our study.

Although, for the above reasons, the low percentage of canonical mRNAs (12 %) in our samples likely represents an underestimate, our study may stimulate additional studies, for example to revisit the production of mRNAs from non-canonical templates[52,53]. Also, it is worth mentioning in this context that, for several other nidoviruses, such as murine hepatitis virus (MHV), bovine coronavirus (BCoV), and arteriviruses, evidence has been obtained that sg RNA transcription may also involve non-canonical TRS motifs [54–58].
The majority of sg RNAs (other than mRNAs) we found in our samples likely represent DI-RNAs, which are a common occurrence in coronavirus *in vitro* studies[19].

To our knowledge, this study is the first to perform RNA modification calling without prior treatment of the input sample. It only relies on the raw nanopore signal. While DNA modifications such as 5mC methylation have been explored exten-

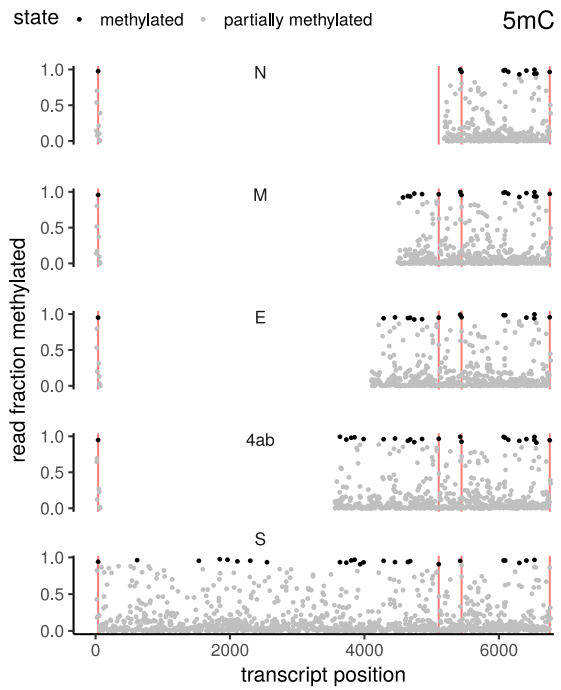

Figure 7: 5mC methylation of various annotated coronavirus sg RNAs. The transcripts have been aligned such that corresponding genomic positions can be found in the same vertical column across facets. Both the leader sequence (including the TRS) as well as the nested subgenomic sequences show consistent patterns of methylation across transcripts. Exemplary positions that display consistent methylation across all investigated transcripts are marked as red vertical lines. Note that while coronavirus recombination uses two TRS, the resulting transcript has only one TRS, because of self-similarity based pairing of the TRS. Positions have been labelled 'methylated' if at least 90 % of the reads show a methylation signal. Using this threshold, the estimated FPR is below 5 %.

sively[59], less is known about RNA modifications[60], the importance of which is debated[61]. We found consistent 5mC methylation patterns across viral RNAs when tested at a FPR below 5 %. We were not able to assess the sensitivity and specificity of the methylation calling due to the absence of a positive control group, which was beyond the scope of this study. In addition, we observed that the software used[39] will likely present high error rates in regions of low coverage or where the underlying reference assembly is erroneous. This is because the resquiggle algorithm – upon which this method is based – has to align the raw nanopore read signal to the basecalled read sequence. This is necessary to test the raw signal against learned modification models, of which at the time of manuscript preparation (November 2018) only 5mC was implemented for RNA. Nevertheless, new options to call these modifications at an acceptable error rate without any RNA pretreatment is a powerful method.

We were able to reconstruct accurate consensus sequences, both for the Illumina and nanopore data. We also demonstrated that individual transcripts can be characterized. More problematic was the resolution of quasispecies in our experimental setup. Although DRS allowed us to confirm the presence of each of the two heterologous SL2 structures present in the SL2 sample, this was only possible for subgenome-length (DI-) RNAs. It appears that the high error rate of more than 10 % was a critical limitation when analyzing the SL2 region located at the extreme 5'-terminal end of the 27.3 kb genome RNA. This high error rate made variant calling difficult, particularly under low-coverage conditions, as was the case in our analyses of the 5'-UTR of genome-length RNA (results not included).

We used a hybrid error correction method (`HG-CoLoR`[46]) that uses Illumina data to correct read-level errors. However, it remains questionable whether the corrected read sequence is truly representative of the ground truth read sequence. Signal-based correction methods such as `Nanopolish`[62] may be more promising, however, at the time of manuscript writing (November 2018) correction on direct RNA data has not been implemented. We expect this to become available in the near future. Combined with the ever-increasing accuracy of the nanopore technology, we think this method might be able to study quasispecies soon.

Conclusions

In this work, we demonstrated the potential of long-read data as produced by nanopore sequencing. We were able to directly sequence the RNA molecules of two different samples of one of the largest RNA virus genomes known to date. We showed how very large RNA genomes and a diverse set of sg RNAs with complex structures can be investigated at high resolution without the need for a prior assembly step and without the bias introduced by cDNA synthesis that is typically required for transcriptome studies.

The detail and quality of the available data still require significant bioinformatic expertise as the available tooling is still at an early development stage. However, the technological potential of nanopore sequencing for new insights into different aspects of viral replication and evolution is very promising.

Future studies should investigate both strands of the coronavirus transcriptome. Studies focusing on RNA modifications need to employ well-defined positive and negative controls to assess the error rate of the current software alternatives. Also, the

DRS method will be extremely powerful if it comes to analyzing the nature and dynamics of specific haplotypes in coronavirus populations under specific selection pressures, for example mutations and/or drugs affecting replication efficiency and others.

Our work also serves as a proof-of-concept demonstrating that consistent RNA modifications can be detected using nanopore DRS.

To fully exploit the potential of DRS, several improvements are needed: First and foremost, a significant reduction of the currently very high per-read error rate is crucial. This is especially problematic in studies focusing on intra-sample heterogeneity and haplotypes. Secondly, protocols that limit RNA degradation during library preparation would be of great value. This could be achieved by shortening the library protocol. To limit the cost of DRS, barcoded adapters would be desirable. On the bioinformatics side, the basecaller for DRS data is still at an early stage and, for example, cannot accurately call the poly(A)-regions as well as the RNA-DNA-hybrid adapter sequences. Further basecalling errors likely result from RNA modifications, which need to be modelled more accurately. However, once these limitations will be fixed, the use of nanopore-based DRS can be expected to greatly advance our understanding of the genomics of virus populations and their multiple haplotypes.

Methods

RNA virus samples

The two total RNA samples used in this study for DRS (ONT MinION) and Illumina sequencing were prepared at 24 h post infection from Huh-7 cells infected at an MOI of 3 with recombinant HCoV-229E WT, HCoV-229E_SL2-SARS and HCoV-229E_SL2-BCoV, respectively[41]. Prior to sequence analysis, the two RNA samples obtained from HCoV-229E_SL2-SARS- and HCoV-229E_SL2-BCoV-infected cells were pooled (SL2 sample, see SFig. 3).

Generation of recombinant viruses and total RNA isolation were carried out as described previously[41]. Briefly, full-length cDNA copies of the genomes of HCoV-229E (GenBank accession number NC_002645), HCoV-229E_SL2-SARS and HCoV-229E_SL2-BCoV, respectively, were engineered into recombinant vaccinia viruses using previously described methods[63–65]. Next, full-length genomic RNAs of HCoV-229E, HCoV-229E_SL2-SARS and HCoV-229E_SL2-BCoV, respectively, were transcribed in vitro using purified ClaI-digested genomic DNA of the corresponding recombinant vaccinia virus as a template. 1.5 µg of full-length viral genome RNA, along with 0.75 µg of in vitro-transcribed HCoV-229E nucleocapsid protein mRNA, were used to transfect 1×10^6 Huh-7 cells using the TransIT® mRNA transfection kit according to the manufacturer's instructions (Mirus Bio LLC). At 72 h post transfection (p.t.), cell culture supernatants were collected and serially passaged in Huh-7 cells for 21 (WT) or 12 times (HCoV-229E_SL2-SARS and HCoV-229E_SL2-BCoV), respectively.

Nanopore sequencing and long-read assessment

For nanopore sequencing, 1 µg of RNA in 9 µl was carried into the library preparation with the Oxford nanopore direct RNA sequencing protocol (SQK-RNA001). All steps were followed according to the manufacturer's specifications. The library was then loaded on an R9.4 flow cell and sequenced on a MinION device (Oxford Nanopore Technologies). The sequencing run was terminated after 48 h.

The raw signal data was basecalled using `Albacore` (v2.2.7, available through the Oxford Nanopore community forum). Due to the size of the raw signal files, only the basecalled data were deposited at the Open Science Framework (OSF; doi.org/10.17605/OSF.IO/UP7B4).

While it is customary to remove adapters after DNA sequencing experiments, we did not perform this preprocessing step. The reason is that the sequenced RNA is attached to the adapter molecule via a DNA linker, effectively creating a DNA-RNA chimera. The current basecaller – being trained on RNA – is not able to reliably translate the DNA part of the sequence into base space, which makes adapter trimming based on sequence distance unreliable. However, we found that the subsequent mapping is very robust against these adapter sequences. All mappings were performed with `minimap2`[66] (v2.8-r672) using the 'spliced' preset without observing the canonical `GU...AG` splicing motif (parameter `-u n`), and k-mer size set to 14 (`-k 14`).

Raw reads coverage, sequence identity to the HCoV-229E reference genome (Genbank, NC_002645.1) and sequencing error statistics were determined from the mappings to the references produced by `minimap2`. Identity and error rates are the number of matching nucleotides (or number of nucleotide substitutions, insertions or deletions) divided by the total length of the alignment including gaps from indels. Reads were also mapped against the human reference genome (GRCh37/hg19) and

the yeast phosphopyruvate hydratase ENO2 gene (Genbank, NP_012044.1).
Consensus calling of nanopore reads was performed with `ococo`[45] (v0.1.2.6). The minimum required base quality was set to 0 in order to avoid gaps in low coverage domains.
We used the hybrid error correction tool `HG-CoLoR`[46] in conjunction with the Illumina HiSeq short-read data sets of both samples to reduce errors in all reads that exceed 20k nt in length. The program builds a *de Bruijn* graph from the near noise-free short-read data and then substitutes fragments of the noisy long reads with paths found in the graph which correspond to that same fragment of the sequence. `HG-CoLoR` was run with default parameters except for the maximum order of the *de Bruijn* graph, which was set to 50 in order to fit the length of the short reads.

Illumina HiSeq sequencing and assembly

Illumina short-read sequencing was performed using the TruSeq RNA v2 kit to obtain RNA from species with polyA-tails and without any strand information. The three samples (WT, SL2_SARS, SL2_BCoV) selected for this study were prepared on a HiSeq 2500 lane and sequenced with 51 cycles. After demultiplexing, 23.2, 22.0, and 23.8 million single-end reads were obtained for the WT and the two SL2 samples, respectively. The raw sequencing data was deposited at the OSF (doi.org/10.17605/OSF.IO/UP7B4).

Characterization of transcript isoforms and subgenomic RNAs

We first defined TRS as a 8-mer with a maximum Hamming distance of 2 from the motif `UCUCAACU`. We then searched the HCoV-229E reference genome (Genbank, NC_002645.1) for all matching 8-mers. We then synthesized sg RNAs *in silico* as follows: For each pair of complementary 8-mers (5'-TRS, 3'-TRS) we accepted at most 1 mismatch to simulate base pairing under a stable energy state. We then joined two reference subsequences for each pair or TRS: First, the 5'-end up to but not including the 5'-TRS. Second, the 3'-end of the reference genome including the 3'-TRS and excluding the poly(A)-tail.
This way we obtained about 5,000 candidate sg RNAs. To validate them, we mapped the nanopore reads to these 'mock' sg RNAs in a non-discontinuous manner, i.e. all reads had to map consecutively without large gaps. To count as a putative hit, 95 % of the read length had to uniquely map to a given mock transcript, and the mock transcript could not be more than 5 % longer than the read.
We only considered putative hits as plausible if they had a read support of at least 5. With this threshold, we aim to balance the sensitivity of finding plausible novel transcripts with a need to control the number of false positives.

Identification of 5mC methylation

We used `Tombo` (v1.3, default parameters)[39] to identify signal level changes corresponding to 5mC methylation.
To assess the false positive rate (FPR) of the methylation calling, we used an RNA calibration standard (RCS) as a negative control. It is added in the standard library preparation protocol for direct RNA sequencing. This mRNA standard is derived from the yeast enolase II (YHR174W) gene[67] and is produced using an *in vitro* transcription system. As a consequence, the mRNA standard is not methylated.
For a conservative resquiggle match score of 1.3 (part of the `Tombo` algorithm, default setting for RNA) and a methylation threshold of 0.9, the FPR was 4.67 %, which met our requirement that the FPR be smaller than 5 %.
Our experimental setup did not include a positive methylation control.

We used `Inkscape` version 0.92.1 (available from inkscape.org) to finalize our figures for publication.

Availability of data and material Both the short-read cDNA (Illumina) and the basecalled long-read RNA (ONT) data were archived and stored at the Open Science Framework, under accession doi.org/10.17605/OSF.IO/UP7B4.

Competing interests The authors declare that they have no competing interests.

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Authors' contributions AV developed the experimental design for sequencing with nanopores. AV, SK and KL analyzed and interpreted the data. JZ, RM, MH and MM were major contributors for discussion and in writing the manuscript. All authors wrote, commented, edited and approved the final manuscript.

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