RNA sequencing: current and prospective uses in metabolic research

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Abstract

Previous global RNA analysis was restricted to known transcripts in species with a defined transcriptome. Next generation sequencing has transformed transcriptomics by making it possible to analyse expressed genes with an exon level resolution from any tissue in any species without any a priori knowledge of which genes that are being expressed, splice patterns or their nucleotide sequence. In addition, RNA sequencing is a more sensitive technique compared with microarrays with a larger dynamic range, and it also allows for investigation of imprinting and allele-specific expression. This can be done for a cost that is able to compete with that of a microarray, making RNA sequencing a technique available to most researchers. Therefore RNA sequencing has recently become the state of the art with regards to large-scale RNA investigations and has to a large extent replaced microarrays. The only drawback is the large data amounts produced, which together with the complexity of the data can make a researcher spend far more time on analysis than performing the actual experiment.

Introduction

The transcriptome is defined as all the RNAs in a cell at a given time and researchers have for a long time been interested in its composition. It has been reported that as many as 90% of eukaryotic genes can undergo alternative splicing (Pan et al. 2008) and that this is utilized to increase the complexity of the transcriptome (Modrek & Lee 2002, Pan et al. 2008, Wang et al. 2009). Further variation is in part done both in a tissue-specific manner (Le et al. 2004, Sugnet et al. 2006, Cooper et al. 2009) and by cellular signals (Ortis et al. 2010) and much of this is variation is not seen with the conventional techniques. In the early days, the amount of a given transcript was semi-quantitatively measured using hybridization techniques such as northern blotting analysis (Kevil et al. 1997) with the subsequent development allowing for the use of macroarrays (Rast et al. 2000) and then microarrays (Schena et al. 1995) and finally a fully quantitative technique, real-time PCR also known as qPCR (Heid et al. 1996). All of these techniques were limited to the investigation of previously defined transcripts with a known sequence and the choice had to be made between a global or a quantitative analysis, producing little splice-specific information. As the work to define transcripts was both time consuming and cumbersome (Agulnik et al. 1998), the definition of new transcripts took time. These limitations meant that if you worked outside of the more common model organisms you would have to use the sequence from other species as a base for your work, and...
when working with well-studied transcriptomes you would still have to choose the splice variant(s) of interest with the risk of other splice variants affecting your end result (Table 1).

RNA sequencing on the other hand allows for a hypothesis-free investigation of all the RNAs in a sample. It investigates the entire transcript in comparison with the other methods that focuses on a targeted part of the transcript and is able to discover novel exons, splice sites, and gene fusions (Mortazavi et al. 2008, Sultan et al. 2008, Asmann et al. 2009, Pickrell et al. 2010, Richard et al. 2010).

It also gives the total expression level of a gene, as well as the ratio between different isoforms, splice variation (Fig. 1) and expression levels of exon resolution with a better correlation with the subsequent protein levels as compared with microarrays and a higher sensitivity (Degner et al. 2009, Wang et al. 2009).

**Technical overview of RNA sequencing**

There are currently three major suppliers of sequencing equipment: Illumina, Pacific Bioscience, and Life Technologies. All three technologies have their pros and cons and it is beyond the scope of this review to properly address them. An overview is presented in Table 2. Briefly Pacific Bioscience produces by far the longest reads, but carries a hefty price tag. Life Technologies have the lowest entry price, but have a higher running cost and a somewhat lower base quality. Illumina sports the highest throughput and lowest cost per base, although on their most expensive equipment with their less expensive equipment having a lower throughput and somewhat higher cost per base.

For the last few years, Illumina’s chemistry and technology has been by far the most commonly used sequencing equipment for RNA-sequencing studies.

A few fundamental decisions on design need to be made before running the experiment. As can be seen in the summary in Table 2, all instruments produce a finite amount of data. Due to this one of the first decisions that needs to be made is how to focus the sequencing power toward the transcripts of interest. The two most common approaches being the investigation of either rRNA-depleted or polyA-enriched samples, this is because otherwise ~99% of the sequencing power will go toward the rRNAs. The quality of the sample can affect this decision with poly(A) selection, working on high-quality samples and rRNA depletion being the recommended approach for lower quality RNA such as from formalin-fixed paraffin-embedded samples. The RNAs are then fragmented (optional step) and made into cDNA that is subsequently sequenced. This can either be done as a paired end (PE), meaning that the fragment is sequenced from both ends or single read (SR), meaning that it is only sequenced from one side. This will then produce either one stretch of sequence, one read, of a given length or two bi-directional reads per fragment sequenced. This is done on several million fragments simultaneously creating several million reads per sample. These reads are subsequently compared and aligned to the genome and/or the transcriptome of the species investigated and the expression levels of both known and novel transcripts are defined (Fig. 2).

If your aims are to investigate both known and unknown transcripts and splice variants, a longer read length together with PE sequencing is recommended for use. Longer reads will give more information with regards to the actual splice events, i.e., data from exon/exon a boundary that not only indicate the expression levels of the exon but also show which exons are linked. There is also the possibility to perform a so-called stranded sequencing, meaning that the data produced are defined as being from either of the two chromosomal strands. This gives more stringent information and can help in discerning between reads from genomic regions which are shared by genes on different strands. In the case of genes sharing exons and genes on the same strand, such as insulin (INS) and insulin-like growth factor 2 (INS-IGF2), the strand specific data do not help, instead longer reads and paired-end sequencing increases the possibility to clearly define which gene a read comes from.

**Table 1** A summary of the most common techniques to investigate RNA. Previously there were a tradeoff between doing a global or a quantitative analysis, RNA sequencing can however be used to combine a global and quantitative analysis

<table>
<thead>
<tr>
<th>A priori knowledge</th>
<th>Northern blotting</th>
<th>Arrays, macro and micro</th>
<th>Real-time PCR</th>
<th>Sequencing</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quantitative</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Number of RNAs</td>
<td>Semi</td>
<td>Semi</td>
<td>Semi</td>
<td>Yes</td>
</tr>
<tr>
<td>detected</td>
<td>1–5</td>
<td>Up to 30–40,000</td>
<td>1–4 at a time</td>
<td>All</td>
</tr>
<tr>
<td>Dynamic range</td>
<td>$10^3$–$10^6$</td>
<td>$10^1$–$10^4$</td>
<td>$10^9$ copies</td>
<td>1–infinity</td>
</tr>
</tbody>
</table>

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If you only are interested in the gene expression then the read length is of little value except for the alignment precision. Here, however, the number of reads are very important because a higher number of reads will give a statistically more solid investigation, especially of transcripts with a lower expression. Hence, in this study, the focus need not be on the total amount of data produced, 100 million clusters worth of data from a single-end run with 76 bp long reads will be more valuable than 40 million 100 bp long paired-end reads despite the fact that the data amount produced is basically the same.

**Statistical analysis**

Data analysis can broadly be divided into four main steps: alignment, count summarization, normalization, and identification of differentially expressed genes. The alignment process is carried out by comparing the data produced with the genome and/or the transcriptome to see where there is a high degree of sequence similarity. The output after alignment will be genomic positions where the sequence reads match the genome. If a read comes from more than one exon, i.e. exon-exon spanning, the genomic region where they map is specified and a junction where there is a presumed intronic region. Paired-end data will increase the mapping quality because the information from both reads is taken into account increasing the alignment specificity. There is the option to perform the analysis and create transcripts using only the sequencing data by aligning the reads together without the help of a scaffold. However, this is very time-consuming and tend to be artifact-prone, especially with short-read data such as that produced with Illumina and Life Technologies equipment. Unless the organism investigated completely lacks genomic and transcriptomic data, something that is becoming increasingly rare, the best option is to use previously produced data as a scaffold for the alignment. The two main aligners used are Tophat and STAR, and in our experience they produce similar results. STAR is much faster but requires more RAM to run (Engstrom et al. 2013). The recommended pipeline from BROAD (http://gatkforums.broadinstitute.org/) to enable variant calling in the data uses STAR as an aligner; however, the output from Tophat works for the said pipeline. This process has been compared with taking all papers in a news stand, shredding them and then trying to piece them back together (Korf 2013).

**Figure 1**

Different splice variations that has been observed with RNA sequencing. This includes but is not limited to differential exon usage, inclusion and exclusion, partial differential exon usage both on the 5’ and 3’ ends as well as intron inclusion and mutually exclusive exons. This kind of variation can be seen, thanks to the exon level resolution from RNA sequencing data and it is especially clear with data from exon-exon spanning reads.

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Following the alignment, the gene expression is calculated. The most straightforward way to do this is to count the number of reads that is aligned to a given gene. This is done by comparing the reads genomic coordinates with those of genes genomic, and the sum of the number of reads that map to a certain gene is summarized defining its expression level. Here, longer reads and PE data helps. The longer reads can be mapped more accurately and they contain more exon–exon junction information due to their length. The PE data help because the mapping information from both reads is used together to define the optimal alignment. By dividing the number of counts with the gene length and the total number of reads sequenced from the sample, it is possible to get a value that is comparable between genes as well as between samples and runs. This is usually defined either as fragments per kilobase of exon per million fragments mapped (FPKM) or read per kilobase of exon per million fragments mapped (RPKM). So for a SR experiment, the FPKM value would be analogous to the RPKM while for a PE experiment the RPKM value would be two times the FPKM value. This is not always true because during the data production the reads can be produced without a pair and sometimes only one read from a pair maps. However, for most applications this simplification works. This calculation can also be done on an exon level. If the aim is to identify genes that differ between conditions the normalization for gene length is usually omitted as it introduces problems on its own (Cai et al. 2012).

Often the aim of an RNA experiment is to investigate whether the expression levels are different between tissues...

Figure 2
An overview of RNA sequencing as performed using Illumina's Technology here depicted as a paired-end sequencing set-up. The two most common RNA sequencing approaches are to either sequence mRNA selected with an poly(A)n tail selection step or a negative selection step where the rRNAs are removed (A). rRNA depletion is common when working with RNA samples where the RNA quality is lower such as for RNA from formalin-fixed paraffin-embedded samples. The RNAs are then fragmented and turned to cDNA and adaptors ligated to the ends (B, C, and D). Following this, the fragments are sequenced from both end creating two reads from the same fragment (E and F). The reads are then aligned to the genome and/or the transcriptome and the transcripts expression levels are measured (G and H). It is possible to extract information regarding the expression levels of individual exons as well as how the different exons are linked together from exon–exon junction spanning reads.
and/or treatment groups. Following the development of RNA sequencing, softwares have been developed to aid in this analysis. The group sizes and expression levels of the genes of interest will be pivotal in deciding which software works for a given experiment and there are reviews of the most commonly used softwares (Soneson & Delorenzi 2013). These softwares works for the analysis of both entire genes and transcripts or exons.

An alternative way to analyse the data is to create transcripts from each gene found expressed by combining the junction and exon level data. The most common way of doing this is through Cufflinks (Trapnell et al. 2012). This software package contains tools to create the transcripts and to subsequently compare the transcript levels between groups. The creation of complete transcripts is hard; however, as there are linked events such as mutually exclusive exons which Cufflinks can have issues handling.

There are several software packages that can help in the visualization of RNA sequence data, IGV with the Sashimi plot add on being one. In Fig. 3, both the exon levels and the number of reads that link different exons together are visualized with both an exon skipping event and a 3’ splice site being evident. This kind of data is where the strength of junction reads, reads spanning exon–exon junctions, comes in. These unequivocally shows exon skipping and exon–exon linkage, something that is only indirectly seen in the levels of an exon.

Figure 3
The use of a sahshimi plot can show differential exon usage (A). Here it shows the exclusion/inclusion of an exon in the blue and the green samples, an event that is not seen in the red and the brown samples. The numbers indicate the number of reads that spans from the 4th to the 6th exon in the figure (A). In (B) it shows complete or partial usage of an exon, an event present in all four of the samples. This here is a partial usage of the second exon in the figure and it indicates that there are two 3’ splice sites.
Imprinting and allele-specific expression are events when an allele is predominately expressed due to preference of, for example, DNA methylation or when a genomic variant acts in cis and affect a gene's expression. Several articles have been published on this subject (Nag et al. 2013, Savova et al. 2013). The option to use RNA sequencing data to call SNPs (Fig. 4) in genes expressed makes it possible to investigate whether there is an allelic imbalance in the gene expression, from imprinting or from another source. In (A) there is clear evidence of a C/G SNP with an even distribution, with 57 C vs 60 G carrying reads, between the alleles. In (B) however there is an uneven distribution, with 20 C vs 10 T carrying reads, between the two alleles. This uneven distribution indicates an allelic imbalance with one allele expressed with twice the level.

**Imprinting and allele-specific expression**

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Main problems and future of RNA sequencing

All techniques have their Achilles heel and the major problem in RNA sequencings is with sampling. When performing RNA sequencing experiments, more abundant transcripts are sampled more than lowly expressed transcripts and as much as 70% of the gene expression can come from very few genes (Shin et al. 2014). While it still is relatively easy to investigate gene expression levels of transcripts that are highly as well as lowly expressed at the same time more complex analysis such as allelic imbalance and splice variation can be harder. This is because only a fraction of the data from a given gene is going to be from the SNP investigated or the exon/exon boundaries of interest, increasing the risk of not enough data for a statistically stable investigation. Therefore, when investigating specific splice variants from a gene, the total amount of data needed is higher. However, it might not be practically or economically feasible to increases the read depth enough, especially when the gene(s) of interest is very lowly expressed.

There are ways to alleviate the problem. In part, this is built into the sample preparations themselves because the poly(A) selection or rRNA depletion focuses the sequencing power vs the type of RNA of interest. If this is not sufficient, there is a possibility to either remove more transcripts or to select for the transcripts of interest. When investigating the expression levels in blood, it is common to remove globin RNAs because these transcripts can make up to 70% of the total amount of transcripts in the samples (Shin et al. 2014). If you have a specific set of transcripts that you are interested in, then it is possible to perform a pull down of the said transcripts using probes (Mercer et al. 2011), thereby ensuring that the sequencing is predominantly performed on transcripts of interest. Of these two methods, globin depletion still allows for a set up without any prior assumptions, while the active selection is somewhat dependent on the targets being known. A targeted pull down followed by sequencing, in the future if possible, could even replace qPCR as the main quantitative method to measure even only a few transcripts when the transcriptome in a model system is clearly defined. By using a smart probe placement one could target all the transcripts of interest and ensure an in-depth analysis of all splice variants, with potentially different functions (Small & Pickering 2009, Kahkhaie et al. 2014, Michel et al. 2014), simultaneously. However, for it to be feasible to replace qPCR for one or a few genes, the cost of targeting needs to drop drastically.

One other drawback or limitation is the read length. The fact that the read length is shorter than the transcripts investigated means that there is the need for alignments
and transcript reassembly, two processes that comes with inherent issues. The sequencing technology is under constant development and longer reads lengths are possible all the time. Notably, the read length from Pacific Bioscience have been reported to produce reads of up to 30–40 kb in length with an average read length per run close to 10 kb. This would largely solve the problem that the data are uncoupled, i.e., that the exon usage in the beginning and the end of a transcript is ‘only’ statistically perceived as prejudicing the impartiality of this review. By sequencing the entire transcript in one long read, the exact sequence of a transcript would be defined helping in the analysis of allele-specific expression and allowing for a definite link between exon usage, clearly showing when there are mutually exclusive exons in transcripts (Stephan et al. 2007). There are however other limitations to the Pacific Bioscience set-up and one major factor limiting in its use is the cost. Oxford nanopore is one company that promises long read lengths without the costly initial investigation. They are currently performing a worldwide test to show their capabilities and this will tell whether their technology works.

Summary

The development of NGS sequencing has been a mile stone in the investigation of RNAs. For the first time, we are able to investigate the expression in a given sample without priori knowledge in a quantitative manner. Based on this, we can detect what happened during the development of disease, after treatment, or during development without filtering the investigation on previously known transcripts for the first time. Considering the complexity of the transcriptome with tissue-specific splicing creating functionally different transcripts from the same a tools such as RNA sequencing is sorely needed.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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